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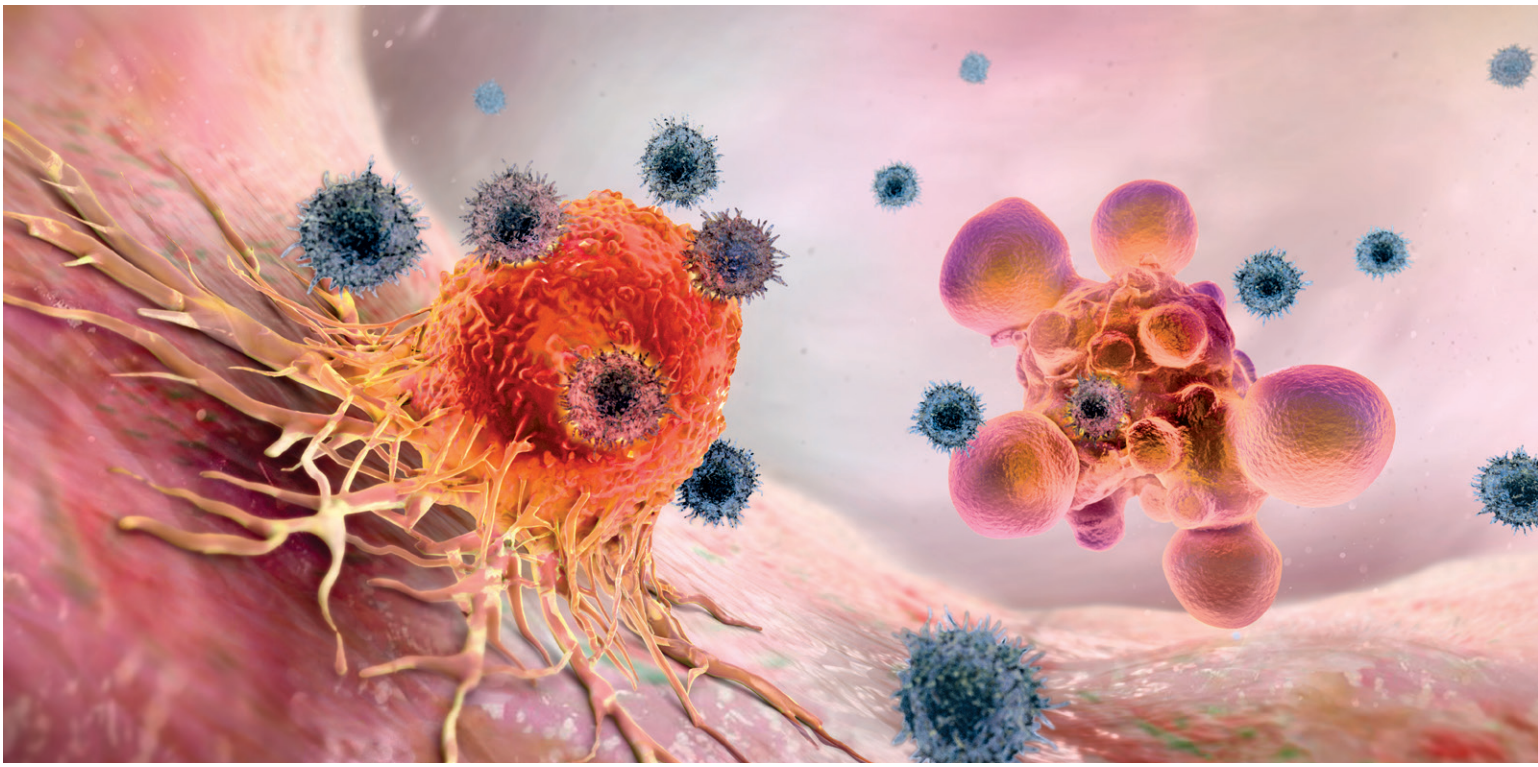
Live-Cell Analysis Handbook
A guide to real-time live-cell imaging & analysis

Third Edition



turning science into solutions

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Introducing Real-Time Live-Cell Analysis

The biomedical world has come a long way since Anton van Leeuwenhoek first observed living cells with a basic microscope in 1674. Using fluorescent probes and modern high resolution imaging techniques it is now possible to view labeled sub-cellular structures at the 10-50 nanometer scale. For researchers working with fixed (dead) cells, organelles can be studied at even higher resolution using electron microscopy. These methods provide tremendous insight into the structure and function of cells down to the molecular and atomic level.

The further development of cell imaging techniques has largely focused on resolving greater spatial detail within cells. Examples include higher magnification, three dimensional viewing and enhanced penetration into deep structures. Significant attention has also been paid to temporal resolution – time-lapse imaging has evolved for high-frame rate image capture from living cells to address "fast" biology such as synaptic transmission and muscle contractility. Any consideration for technology advances at lower spatial or temporal detail may initially seem mundane, or even unnecessary. However, this would fail to recognize some key unmet user needs.

First, there is an increasing realization that many important biological changes occur over far longer time periods than current imaging solutions enable. For example, maturation and differentiation of stem cells can take hours, days and sometime weeks, which is hard to track using existing methods. Second, imaging techniques are not readily accessible to all researchers nor on an everyday basis. This lack of accessibility is either due to virtue of instrumentation that is expensive and use-saturated or by complex software that renders image acquisition and analysis the sole domain of the expert user. Third, and particularly with regard to time-lapse measurement, the throughput of current solutions is typically too low for frontline use in industrial applications. Finally and most importantly, researchers are increasingly aware that any perturbance of the cells in the process of imaging (e.g. fixing, loss of environmental control) can introduce unwanted and misleading experimental artefacts. Together, these factors frame up the requirement for solutions that enable longer-term, non-perturbing analyses of cells at a throughput and ease of use commensurate with non-specialist users, and at industrial scale.

A new generation of specialized compact microscopes and live-cell imaging devices, are now emerging to meet this need. Designed to reside within the controlled, stable environment of a cell incubator, these systems gather cell images (phase contrast, bright-field and/or fluorescence) from assay micro-plates automatically, repeatedly and around the clock. Image acquisition is completely non-invasive and non-perturbing to cells, opening up the opportunity to capture the full, and as needed, long-term time course of the biology. Acquisition scheduling, analysis and data viewing can be conducted easily and remotely, without in-depth knowledge of image processing. Data is analyzed on the fly, image by image, to provide real-time insight into cell behavior. We refer to this paradigm, which is differentiated from straight live-cell imaging by the provision of analysed data at scale as opposed to simply images, as 'real-time live-cell analysis'.

In an ideal world, the images acquired from a live-cell imaging device would be collected only from photons produced by the sample of interest, and in perfect focus. However, this is not the usual case. There are multiple sources of confounding signal present in an image, each needing correction, removal, or cleaning in order to reveal information which has been generated by the sample elements of interest. Corrections are needed due to systematic aberrations in an imaging system stemming from multiple sources. For example, detector anomalies (e.g. detector bias, dark current variability, field flatness and thermal or gamma-ray noise), optical issues (non-flat optical components and illumination imperfections) or undesired signal introduced by the sample are common issues. Autofluorescence from cellular components or media, or non-biological signal sources such as shading, or patterns arising from sample matrices or non-uniform illumination due to meniscus effects in microwells must be removed before usable, replicable information can be extracted.

In order to perform these corrections, one must be aware of the effects of each process, and manipulations on the raw images must be repeatable, to ensure faithful capture of the measured biological signal across images, experiments, and devices. There are many tutorials and software toolkits available to process images, however systems that perform these corrections as a matter of course provide consistency and ease of use, particularly when coupled with standardized assays, reagents and consumables which normalize the experimental process (e.g. the IncuCyte Live-Cell Analysis System, and the assays and reagents available from Sartorius). The consistency with which images are acquired and processed strongly influences the ability to analyze the collected data. This can be a time-consuming task, and purpose-built software that presents only the tools necessary for a specific scientific question can remove what can be a significant hurdle in the image analysis workflow.

While traditional compact microscopes typically only image from a single micro-plate or flask at a time, new live-cell analysis devices such as IncuCyte can automatically capture and analyze images from multiple micro-plates in parallel, thereby significantly increasing throughput (e.g. IncuCyte = 6 x 384 well plates). With the IncuCyte® system, a unique moving optical path design means that the cells and cell plates remain stationary throughout the entire experiment. This further minimizes cell perturbance and enables imaging and analyses of both adherent and non-adherent cell types.

This combination of functionality, throughput and ease of use revolutionizes the way researchers can think about imaging assays in living cells. Real time live-cell analysis has now been applied to a wide range of phenotypic cellular assays including cell proliferation, cell death and apoptosis, immune-cell killing, migration, chemotaxis, angiogenesis, neurite outgrowth and phagocytosis. In each case, the full time-course data and 'mini-movies' of the assay provide greater biological insight than end point assays. Novel analyses such as area under curve, time to signal onset or threshold, and rate parameters (dx/dt) are at times highly value adding. Simply calculating the assay signal at its peak timepoint and/or at the optimal signal/background all helps in assembling robust and reproducible assays. Of course, transient effects of treatments can be detected by kinetic imaging that may otherwise be missed with end-point reads.

Due to its non-invasive nature, measurements from cells can be made not only during the assay itself but also during the cell preparation and 'pre-assay' stage. For example, the morphology and proliferation rates of cells can be monitored throughout the cell culture period and immediately post-seeding on the micro-titer assay plate. The parameter/phenotype of interest can be measured prior to the addition of treatments to provide a within well baseline measure. Quality control of cells and assay plates in this way helps improve assay performance and consistency by ensuring that experiments are only conducted on healthy, evenly plated cultures with the expected cell morphology.

The real-time live-cell analysis approach also provides the opportunity to make data driven decisions while the experiment is in progress. A researcher studying the biology of vascular or neuronal networks, for example, may wish to first establish a stable network before assessing the effects of compound treatments or genetic manipulations (e.g. siRNAs). With continuous live-cell analysis, it is straightforward to temporally track network parameters and use the real time data to judge when best to initiate the treatment regimes. The timing of adjunct studies such as analysis of metabolites or secreted proteins in supernatants can also be guided. Drug washout studies may be performed using the real time data to identify when an equilibrium response occurs and to trigger the timing of the washout regime. If for any reason it transpires that the experiment is not performing as expected then treatments could be withheld to save expensive reagents and follow-on experiments can be initiated more quickly to make up time.

Real-time live-cell analysis is extremely helpful when developing, validating and troubleshooting phenotypic assays. Within a small number of assay plates it's usually possible to obtain a clear understanding of the relationship over time between assay signal and treatments, cell plating densities, plate coatings and other protocol parameters. Scrutiny of the kinetic data and 'mini-movies' from each well help to rapidly pinpoint sources of within- and across-plate variance and to validate the biology of interest. This is particularly true for more advanced cell systems such as co-cultures where far more permutations and combinations of protocol parameters exist (e.g. cell plating ratios) and the biology is more complex.

In summary, real-time live-cell analysis is re-defining the possibilities and workflows of cell biology. The combination of ease of use, throughput, long term stability and non-invasive measurement enables researchers to monitor and measure cell behaviors at a scale and in ways that were previously not possible, or at the least, highly impractical. In the following chapters of this handbook, we illustrate this with a range of different application examples.

From Images to Answers

Introduction

The nature of cell biology research typically requires that image-based methods are used to capture moments in time to enable comparisons between treatment groups and across imaging modalities. Sample information is typically acquired using a microscope and a digital camera, and those moments in time are processed and analyzed. Images captured with a typical microscope camera are digital representations of the analog information contained in the sample, providing a means to automatically analyze the information in the sample. Once these digital snapshots are acquired, image processing is used to clean up the data, and image analysis is used to extract usable information for analysis.

At the core of all of these manipulations are numbers – images are comprised of pixels (picture elements), and each pixel in an image has a digital value representing the brightness or intensity of that portion of the sample, at a specific moment in time. By operating on these values, either in isolation, or while considering nearby values, the information in the images can be cleaned of aberrant information, and data relevant to the imaged sample can be extracted and measured.

The Image Processing Workflow

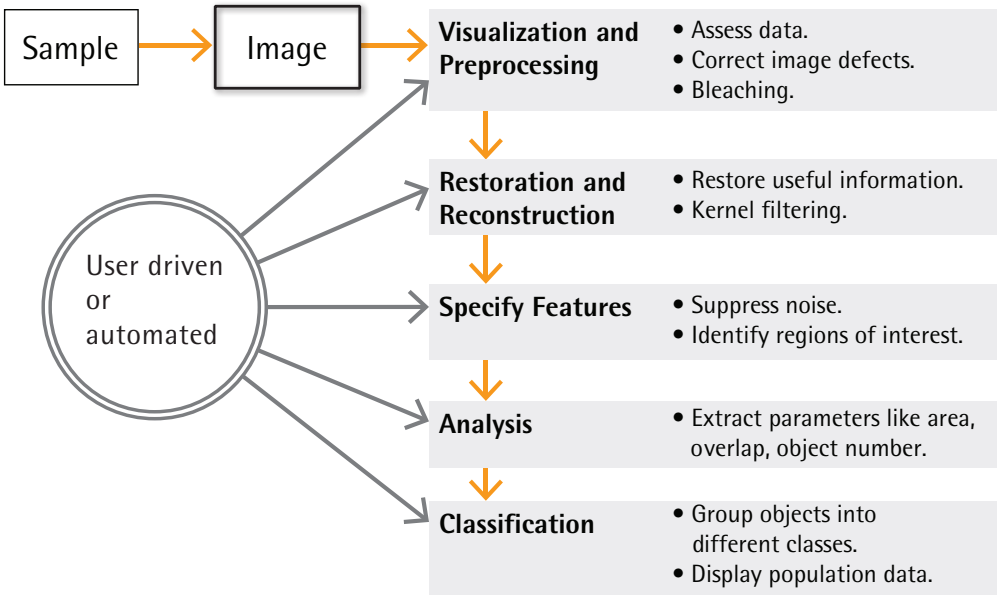


Image processing and analysis is accomplished using a number of techniques, guided by expert knowledge and software guidance. To ensure processing consistency across static and kinetic data, it is important to establish a set of image processing parameters which enable operation on all images in an identical manner. This contextually derived data processing workflow will seamlessly and automatically perform all of the necessary pre- and post- image processing steps, up to and including object analysis and graphical representation of the experimental result. Properly designed image analysis workflows are intended to require no human intervention and processes image archives, generating consistent and actionable results either in real-time, or post-acquisition.

Performing these steps on individual images to generate sufficient statistical power to support a hypothesis can be a tedious process. However, when operating on large numbers of images which have been collected in a substantially similar manner, the series of operations performed to clean up the data, extract desired information, and compare images may be recorded and automatically applied to many images in a single experiment. Once this data has been extracted, treatment groups may be compared to assess differences, and hypotheses evaluated. Scaling this to the analysis of live-cell experiments allows for the evaluation of temporal data, and extending this to microplate microscopy means that population data may be studied with ease. This basic workflow is the subject of countless tutorials and books, and the domain of numerous software packages that offer a cornucopia of tools intended to answer a broad range of scientific questions.

Image Processing to Remove Systematic or Sample-Induced Artifacts

The image data we have described above is typically captured by detectors that convert analog information, specifically photons, into digital signals. This analog information is collected in a matrix fashion, spatially rendered according to location in the sample. Ideally, the signal undergoing analog to digital conversion would come only from photons produced by the sample of interest, and in perfect focus. However, this is not the usual case. There are multiple sources of confounding signal present in an image, each needing correction, removal, or cleaning in order to reveal information which has been generated by the sample elements of interest. Corrections are needed due to systematic aberrations in an imaging system stemming from multiple sources. For example, detector anomalies (e.g. detector bias, dark current variability, field flatness and thermal or gamma-ray noise), optical issues (non-flat optical components and illumination imperfections) or undesired signal introduced by the sample are common issues. Autofluorescence from cellular componenets or media, or non-biological signal sources (i.e. shading or patterns arising from sample matrices, micro-fluidic channels, or non-uniform illumination effects in microwells) must be removed before usable, replicable information can be extracted.

In order to perform these corrections, one must be aware of the effects of each process, and manipulations on the raw images must be repeatable to ensure faithful capture of the true biological signal across images. There are many tutorials and software toolkits available to process images, however systems that perform these corrections as a matter of course provide consistency and ease of use, particularly when coupled with standardized assays, reagents and consumables which normalize the experimental process (e.g. the IncuCyte Live-Cell Analysis System, and the assays and reagents available from Sartorius). The consistency with which images are acquired and processed will influence the ability to analyze the collected data.

Identifying Biology of Interest via Image Masking or "Segmentation"

Once an image has been appropriately processed to remove aberrant signal, the next step is to identify the biology of interest. Image segmentation is a binary process, meaning pixels are classified as either "in" and are included in any enumeration process, or "out" and not considered as part of the sample. The simplest method for determining which pixels are in or out is by thresholding, or setting a boundary above which all pixels are "in", and below which, all pixels are "out". More complex tools do exist, and more complex interactions can be performed with multiple masks, and Boolean operations (e.g., AND, OR, NOT) in order to hone in on the exact pixels of scientific interest. Again, this can be a time-consuming task, and purpose-built software that presents only the tools necessary for a specific scientific question can remove what can be a significant hurdle in the image analysis workflow.

Generating Actionable Data

After the pixels which satisfy all of the measurement criteria are identified in an image, it is possible to operate on this binary mask of pixels. The mask may be analyzed whole (for total area, or confluence measurements) or broken into multiple subparts, for example when defining or counting objects in the image. Depending upon the labeling of the sample, e.g. label-free or tagged with a specific marker such as a fluorescent reagent labeling a specific organelle or structure, a wide variety of statistics may be generated. In the case of fluorescent reagent-labeled images, these statistics may include the mean intensity value of all the pixels in the mask, the total additive intensity, the minimum, maximum, or standard deviation of the collective intensity, or the fluorescence mask may be used to count numbers of objects. Statistics may be global for the image as just described (e.g. total size of the mask, or mean intensity of the mask) or per object (e.g., area occupied by individual cells). Once again, the appropriate choice of labels, image processing, and object identification can require deep technical expertise, as the number of options available to differentiate objects is very broad. For example, if you are looking for all red-labeled nuclei that are also labeled with a green reagent (for example, apoptotic cells labelled with Caspase Green), it is possible to identify individual cells first using a transmitted light image [mask 1], breaking that mask into objects representing cells using image processing tools like watershed split, and then classifying those objects/cells based on the included red and green mean intensity of the included nuclei. This task is more easily performed when the scientific question is well-defined, the appropriate tools are utilized, and the images processed systematically, and without bias.

Analyzing Image Data at Throughput

Now that a specific set of operations has been constructed to process and analyze a representative image, this same set of operations may be applied to all images in an experiment in exactly the same manner. If this set of operations inadequately processes the population of images included in an experiment, it may be necessary to make adjustments to the set of processing operations based upon the population of images collected for the task. In a live-cell imaging experiment performed in a 96-well plate, a data set containing thousands of images is perfectly reasonable. Many data sets will be considerably larger when capturing multiple channels, e.g. red fluorescence/green fluorescence/transmitted light, so variability between images in different treatment groups and at different time points should be expected. In analyzing a large image set, one must be assured that the set of operations is suitable across the data set (e.g., on dead or living cells). Traditional image analysis software does not offer the ability to assess a variety of images in an efficient manner, and thus typical live-cell microplate assays can be unwieldy, at best. Software must address the needs of the researcher by performing all the steps required to convert images to data at the scale of long-term time-lapse experiments, and at the rate of acquisition, in order to best understand biological processes while they are happening.

Cell Culture Quality Control Assays

Real-time monitoring and analysis of cell culture conditions and techniques

A large number of variables exist that alter the growth and function of cells in culture. Many sources of variability are largely uncontrollable because they are inherent to the stochastic processes in biological systems. Other factors can be identified and controlled. Some key controllable factors include:

- Poor CO_2 incubator performance due to lack of calibration and stability of temperature, humidity and CO_2 over time.
- Non-quantitative and/or inconsistent procedures for feeding and splitting cell cultures prior to running cell-based assays including inconsistent limits of cell density and feeding schedules, inconsistent cell density at the time of assay and changes to cell morphology.
- Alterations in media components due to lot-to-lot differences and alteration of concentrations over time due to degradation.
- Differences in cell culture growth surfaces such as variability among suppliers, vessel surface treatments and lot-to-lot variability.
- Biological issues resulting from growing cells in continuous culture for extended periods of time which in turn increases the risk of phenotypic drift, contamination by infectious agents and cross contamination with other cell types.

Each of these controllable variables can adversely affect the results and interpretation of data obtained from downstream assays leading to lost time and a waste of valuable, and at times, costly reagents. A key element in controlling adverse variables is to standardize on objective metrics, thereby eliminating human subjectivity and interpretation.

Advantages of performing quality control of cell cultures via continuous monitoring

The IncuCyte® real-time live-cell analysis system provides a label-free, non-invasive method for monitoring cells directly in the incubator. In sharp contrast to manual monitoring of cell culture processes, quality control monitoring with real-time live-cell analysis automates data capture and cell assessment (Figure 1). Cells can be monitored around-the-clock and at precise, regularly scheduled sampling intervals. In addition to supporting decisions for current culture processes, historical information can be retrieved months or years later for comparison of cell lines and culture growth characteristics.

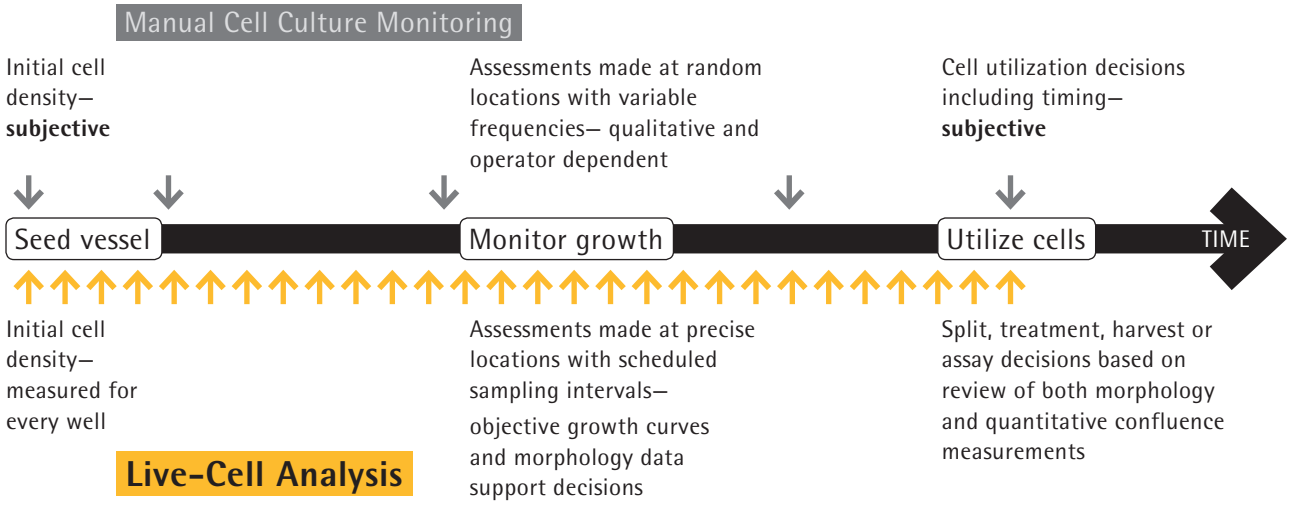


Figure 1. Comparison of cell culture monitoring methods. Vertical arrows represent interaction points with the cell culture using manual methods and real-time live-cell analysis. Subjective decisions made during manual monitoring result in variability for cell-based assays.

The IncuCyte system is compatible with multiple different types of cell culture vessels and can monitor multiple vessels at a time. Data consists of objective, image-based growth metrics to capture transient and time-dependent events.

In order to establish quality cell cultures and improve experimental outcomes in downstream assays, various aspects of cell culture need to be tightly controlled, optimized and documented.

- **Growth conditions**
  Prior to utilizing cells in quantitative assays, it is important to optimize and define cell culture regimens. Documenting variations in cell growth due to factors such as; lot-to-lot differences in cell culture media, changes in media component concentrations over time due to degradation, and inconsistent feeding schedules can all be identified using label-free phase image segmentation techniques that generate confluence measurements.
- **Documentation of cell morphology**
  Changes in cell morphology due to cell seeding density or phenotypic drift can be identified using a range of magnifications to capture fine details of cells and spatial coverage of cell populations.
- **Accurate assessment of overall monolayer**
  By imaging multiple areas of your vessel or using a whole-well imaging mode, spatial variations in cell distribution can be quantified and then reduced.
- **Cell seeding densities**
  To improve assay quality and consistency of kinetic assays, it is important to minimize variations in cell seeding densities due to pipetting errors during plate seeding which result in differential growth rates. Measuring confluence before utilizing assay plates eliminates assay variation, resulting in reproducible conditions.
- **Documenting time-dependent variables**
  Assessment of cell behavior and growth in association with cell treatment times can define experimental conditions to ensure consistent results.

Cell Culture QC Assays

Measuring proliferation to optimize growth conditions and seeding densities

The IncuCyte system can be used to track cell proliferation over time using label-free phase segmentation (confluence) metrics (Figure 2), allowing for the assessment of cell growth as well as monitoring cell culture and assay optimization.

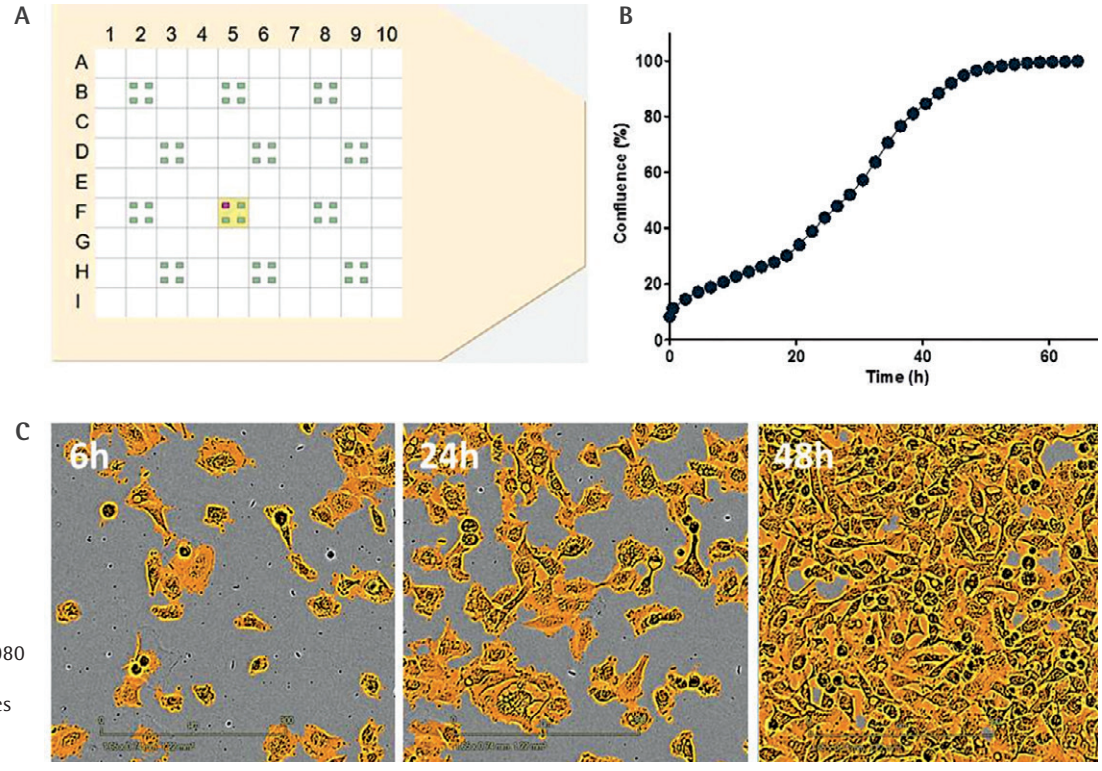


Figure 2. T-flask vessel view shows location of images acquired (A). Proliferation time-course for HT-1080 human fibrosarcoma cells is shown (B). (C) HD-phase time-lapse images of HT-1080 cells (confluence mask overlaid; right panels).

Figure 3 shows results of a study to assess optimal levels of fetal bovine serum (FBS) and cell density using the IncuCyte system.

Live-cell analysis can be used to accurately mask different morphologies and quantify label-free proliferation over time, as shown in Figure 4 below. In 4A and 4B, the variation in cell seeding density-dependent proliferation in two different cell lines, A172 human glioblastoma and LNCap human prostate carcinoma, is exemplified. Understanding cell seeding variation and its effects on proliferation is important for controlling assay variability. As shown in 4C, images reveal accurate segmentation of cell types irrespective of morphology.

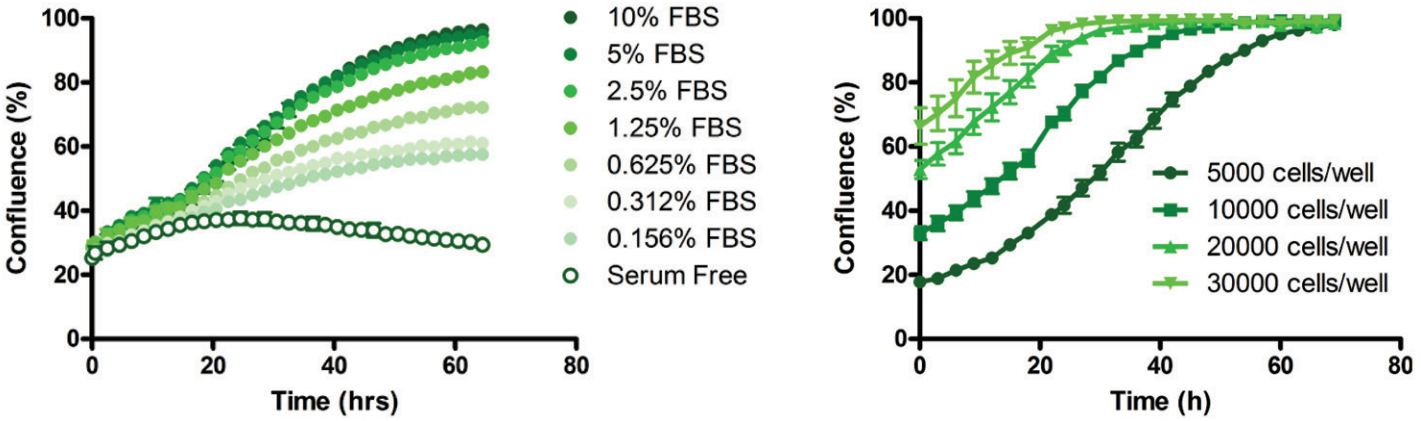


Figure 3. Human umbilical vein endothelial cells (HUVECs) cultured in basal cell media containing growth factors and supplemented with decreasing amounts of FBS. The optimal concentration of FBS for normal propagation was determined to be >2.5%.

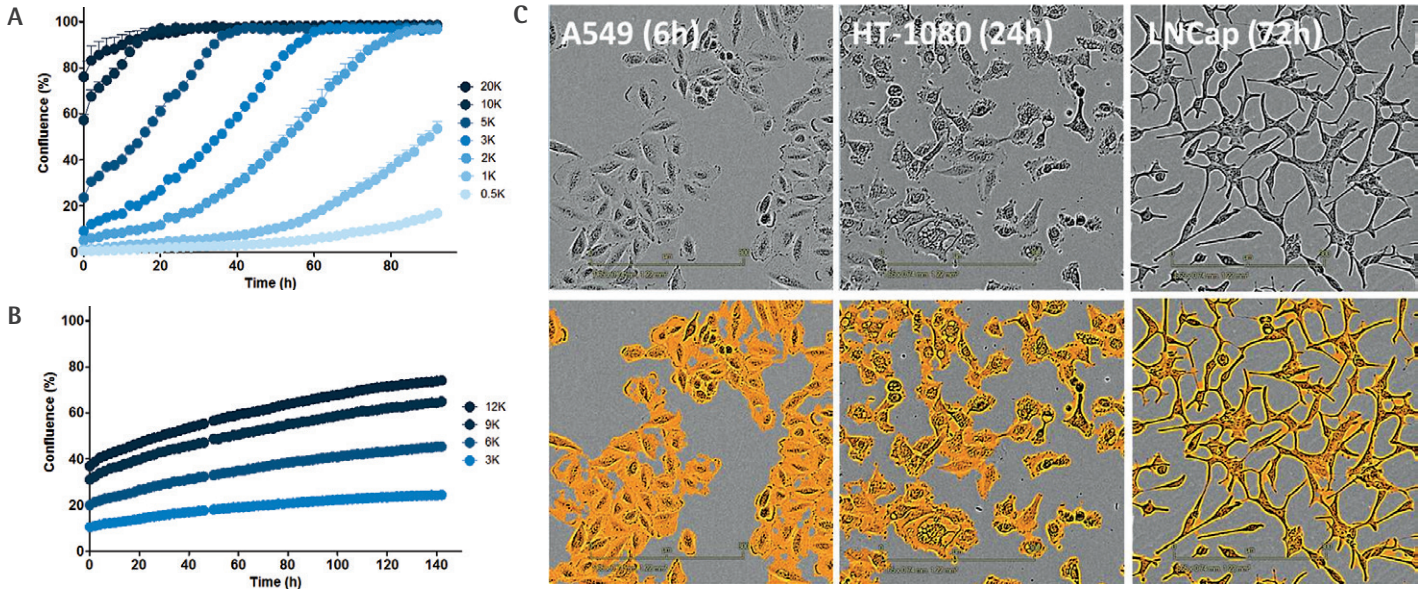
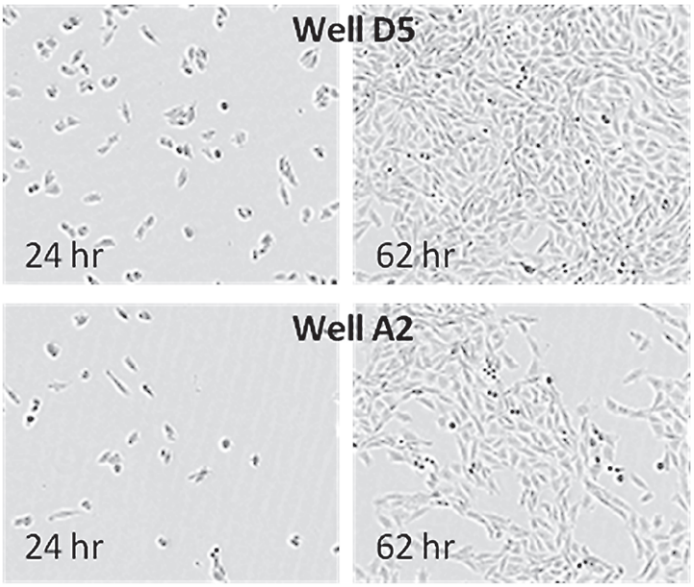


Figure 4. Quantification of A172 human glioblastoma (A) and LNCap human prostate carcinoma (B) growth curves using label-free confluence analysis. (C) HD-phase contrast images of A549 human lung carcinoma, HT-1080 human fibrosarcoma and LNCap cells shown without and with confluence mask (orange). Images reveal accurate confluence masking of all three cell types of varied morphology.

A



B

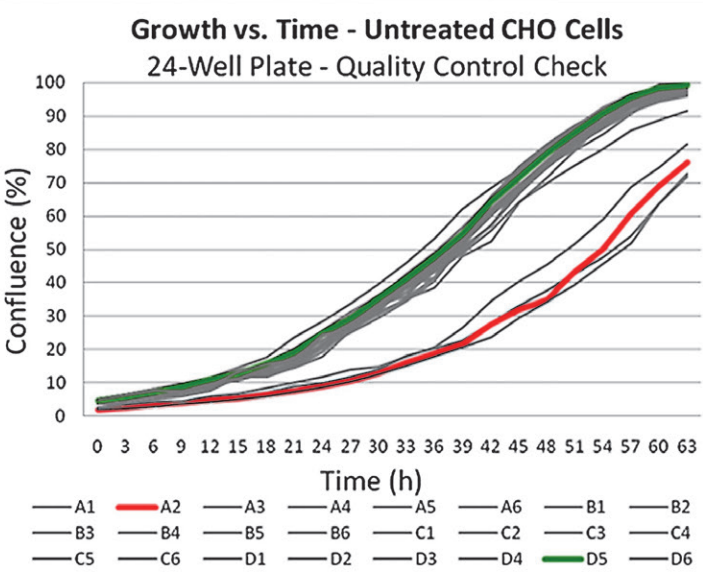


Figure 5. Images (A) and data (B) illustrating the effect of pipetting errors on growth. Wells were seeded at the same cell density and then checked prior to an endpoint assay for growth. Initial confluence for four wells were found to be ~50% that of the median initial confluence for the plate, a variable likely caused by pipetting errors.

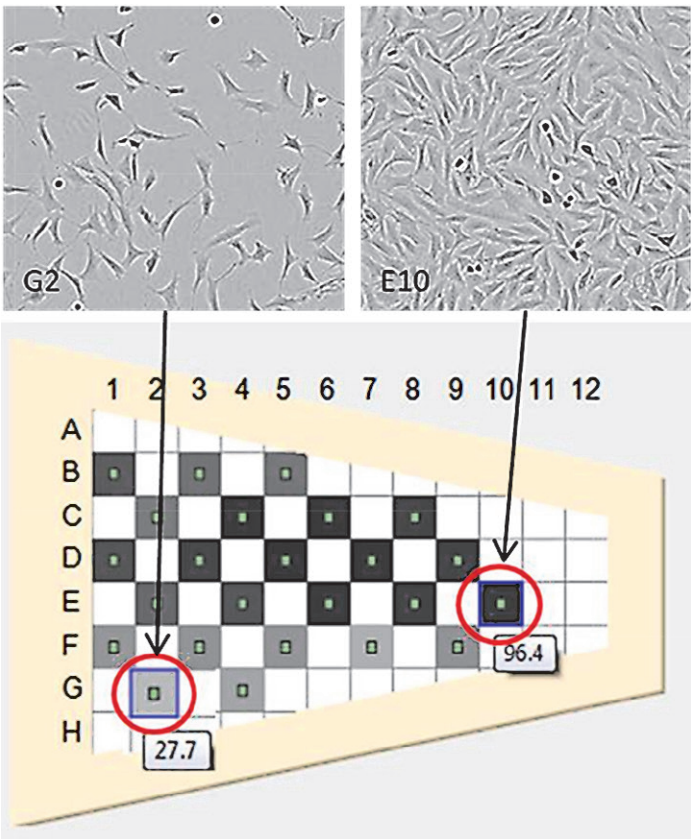


Figure 6. T-25 flask exhibiting uneven growth, often related to poor culture technique. Two representative images from a single flask show differences in confluence.

Conclusions

With real-time live-cell analysis it is possible to follow and quantify cell growth over time, effectively revealing both transient and time-dependent phenomena. This type of analysis is a powerful tool for cell culture quality control, providing a quantitative, objective, non-invasive and kinetic method of analyzing living cells, unperturbed in the incubator. Use of the IncuCyte system to document and monitor routine cell culture can improve cell-based assay quality and consistency.

Kinetic Cell Health and Viability Assays

Real-time automated measurements of cell health and viability

Measurements of cell health are essential for studying the effects of drugs, culture conditions or genetic modifications on cell growth or viability. Such studies are used to rank compounds in drug discovery screens, identifying off-target toxic compounds, as well as to investigate the cellular changes that underline disease pathologies.[1] In order to assess cell health and viability, a variety of end-point assays have been employed, such as ATP assays, LDH assays and vital dyes used in flow cytometry, but all fail to take into account the kinetics or have the capability to make simultaneous measurements in a single well. Understanding the biological time-dependence, along with the ability to evaluate multiple methods by which a cell dies, offers considerable advantages in the characterization of test compounds.[2]

IncuCyte® cell health and viability assays allow for the kinetic evaluation of effective concentration of the compound and the time needed for the compound to perturb the target, thereby discriminating if the test agent is fast acting or prolonged. Another advantage is the ability to perform multi-parametric analysis using non-perturbing reagents within a single well, enabling detection of the mechanism of cell death as well as monitoring cell viability utilizing reagents that label cell nuclei. These image-based detection methods can all be qualitatively validated with images to evaluate morphological changes associated with cell death.

Furthermore, these advantages of live-cell analysis are also ideally suited to meet the needs of more dynamic cellular models, such as co-culture immune cell killing assays, which require robust temporal and spatial quantitative measurements in physiologically-relevant conditions. Continuous live-cell imaging makes it possible to measure and visually validate the complex and dynamic interactions between target and effector cells in co-culture for the reliable analysis of new potential immunotherapies.

Live-cell imaging and analysis approaches

The IncuCyte cell health and viability assays enable the detection and analysis of cell proliferation, viability, apoptosis, and cytotoxicity. Images of cell cultures in 96- or 384-well formats are automatically acquired and analyzed to generate time courses and reveal concentration-dependent responses that can be used to calculate EC50 or IC50 values.

How Live-Cell Health and Viability Assays Work

Proliferation and viability assays

Measure growth or growth inhibition in real time over several cell divisions using label-free cell counts or confluence measurements in both adherent and non-adherent cell types. Additional assay strategies can be used to generate measurements of cell type-specific growth rates in co-cultures. IncuCyte® NucLight reagents can be used to fluorescently label nuclei and determine counts of viable cells.

Apoptosis assays

Detect apoptosis in living cells and in real time using mix-and-read reagents that measure caspase-3/7 activity or phosphatidyl serine externalization.

Cytotoxicity assays

Measure cell death upon loss of plasma membrane integrity over time using simple mix-and-read protocols.

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2. Abassi, Y.A., Xi, B, Zhang, W., Ye, P., Kirstein, S.L, Gayloard, M.R., Feinstein, S.C., Wang, X. and Xu, X. Kinetic Cell-Based Morphological Screening: Predisction of Mechanism of Compound Action and Off-target Effects. *Chemistry & Biology* 2009 16:712.

Recent IncuCyte Publications

Balvers, et. al., investigated the therapeutic benefit of combination therapy in patient-derived glioma stem-like cells (GSC). The current standard of care for Glioblastoma Multiforme consists of radiation along with temozolomide (TMZ) chemotherapy. A possible strategy to increase the efficacy of TMZ is through interference with the DNA damage repair machinery, by poly(ADP-ribose) polymerase protein inhibition(PARPi). Proliferation assays consisted of longitudinal imaging of cell confluency in the IncuCyte system. Phase contrast images were acquired at 1-3hr intervals and confluence per well was calculated.

ABT-888 enhances cytotoxic effects of temozolomide independent of MGMT status in serum free cultured glioma cells. Balvers, R. K., Lamfers, M., Kloezeman, J. J., Kleijn, A., Berghauser Pont, L., Dirven, C. and Leenstra, S. *Journal of Translational Medicine* 2015 13:74.

Ong, et. al., evaluated PAK1 dysregulation (copy number gain, mRNA and protein expression) in breast cancer tissues. A novel small molecule inhibitor, FRAX1036, and RNA interference were used to examine PAK1 loss of function and combination with docetaxel in vitro. The IncuCyte system was used for caspase 3/7 activation apoptosis assays. Cells were treated with DMSO, FRAX1036, and/or docetaxel. Caspase 3/7 reagent was added and cells imaged every 2 hours or 4 hours for 36 to 72 hours. Data was analyzed using IncuCyte analysis software to detect and quantify apoptotic cells.

Small molecule inhibition of group I p21-activated kinases in breast cancer induces apoptosis and potentiates the activity of microtubule stabilizing agents. Ong, C. C., Gierke, S., Pitt, C., Sagolla M., Cheng, C. K., Zhou, W., Jubb, A. M., Strickland, L., Schmidt, M., Duron, S. G., Campbell, D. A., Zheng, W., Dehdashti, S., Shen M., Yang, N., Behnke, M. L., Huang W., McKew, J. C., Chernoff J., Forrest, W.F., Haverty, P. M., Chin S., Rakha, E. A., Green, A. R., Ellis, I. O., Caldas, C., O'Brien, T., Friedman, L. S., Koeppen, H., Rudolph, J., Hoeflich, K. P. *Breast Cancer Research* 2015 17:59.

Barsyte-Lovejoy, et. al. provide a protocol for measuring early toxicity/apoptosis response to chemical probes using the IncuCyte platform.

Methods in Enzymology, 2016. Chapter 4: Chemical Biology Approaches for Characterization of Epigenetic Regulators. Barsyte-Lovejoy, D., Szewczyk, M.M., Prinos, P.

Novotny, et. al. evaluated the ability of reversible HER2 inhibitors to inhibit signaling and proliferation in cancer cell lines driven by HER2–HER3 heterodimers activated via various oncogenic mvechanisms. The IncuCyte platform was used for real-time cell proliferation assays. CHL-1 cells were plated in clear-bottom black 96-well plates (Corning; 3904) and allowed to adhere overnight. The following day, media was changed to fresh media that contained either DMSO or compound. Confluence was measured every 2 h for 96 h using two bright-field images per well.

Overcoming resistance to HER2 inhibitors through state-specific kinase binding. Novotny, C.J., Pollari, S., Park, J.H., Lemmon, M.A., Shen, W., Shokat, K.M. *Nature Chemical Biology* 2016 12:923.

Skolekova, et. al., measured caspase-3/7 activity corresponding to the induction of apoptosis in human mesenchymal stromal cells (MSCs) cultivated in the presence of cisplatin using the IncuCyte platform. Kinetic activation of caspase-3/7 was monitored and quantified using the IncuCyte® FLR object counting algorithm. The authors showed that the secretory phenotype and behavior of mesenchymal stromal cells influenced by cisplatin differed from naïve MSC. MSC were more resistant to cisplatin, which was cytotoxic for tumor cells.

Cisplatin-induced mesenchymal stromal cells-mediated mechanism contributing to decreased antitumor effect in breast cancer cells. Skolekova, S., Matuskova. M., Bohac, M., Toro, L., Durinikova, E., Tyciakova, S., Demkova, L., Gursky, J., and Kucerova, L. C*ell Communication and Signaling.* 2016 14:4.

Greenberg, et. al., studied whether Bcl-2–IP3R interaction is a potentially useful therapeutic target in small cell lung carcinoma (SCLC). The IncuCyte platform was used to detect caspase activation and nuclear condensation in SCLC cell lines.

Synergistic killing of human small cell lung cancer cells by the Bcl-2-inositol 1,4,5-trisphosphate receptor disruptor BIRD-2 and the BH3-mimetic ABT-263. Greenberg, E. F., McColl, K. S., Zhong, F., Wildey, G., Dowlati, A., Distelhorst, C. W. *Cell Death and Disease* 2015 6:e2034.

Yang, et. al., describe characterization of a quadruplex dye (G4-REP; quadruplex-specific red-edge probe) that provides fluorescence responses regardless of the excitation wavelength and modality thus allowing for diverse applications and most imaging facilities. Authors demonstrated use via cell images and associated quantifications collected through use of the IncuCyte real-time live-cell imaging system over an extended period, monitoring both non-cancerous and cancerous human cell lines.

Real-time and quantitative fluorescent live-cell imaging with quadruplex-specific red-edge probe (G4-REP). Yang, S. Y., Amor, S., Laguerre, A., Wong, J. M. Y., Monchaud, D. Biochimica et Biophysica Acta (BBA) - General Subjects http://dx.doi.org/10.1016/j.bbagen.2016.11.046.

Ano, et. al. sought to elucidate specific compounds that strongly suppress microglial inflammation by screening dairy products fermented with *Penicillium candidum*. Quantitative live cell imaging to assess apoptosis or neuronal cell-death was performed using an IncuCyte real-time imaging system. Images were obtained every 3 hour at 20x magnification in phase contrast mode. The number of cells that had undergone apoptosis or cell-death was determined using the object counting algorithm.

Identification of a novel dehydroergosterol enhancing microglial anti-inflammato ry activity in a dairy product Fermented with *Penicillium candidum***.** Ano, Y., Kutsukaka, T., Hoshi, A., Yoshida,, A., Nakayama, H. PLoS On 10, e0116598, 201.

Kinetic Proliferation Assays

Accurate and reproducible measurements in a variety of cell culture models

Introduction

Cell proliferation assays are a cornerstone of cancer therapeutic, developmental biology, and drug safety research. Analysis of the sustained signaling pathways that underlie the progression of tumors, for example, accounts for >12,000 manuscripts in PubMed, the majority of which use cell proliferation analysis to evaluate tumor cell growth. Despite this, there has not been a direct, straightforward, scalable method for quantifying cell proliferation as a continuous event. Rather, the traditional approaches are endpoints or at best a series of concatenated endpoints to measure the time-course.

Challenges in monitoring cell proliferation via traditional single end point, non-image based assays that utilize plate readers or flow cytometers include:

- A single data point may not provide enough information to effectively distinguish impacts of conditions or treatments, (e.g. when comparing early or late acting compounds, or discerning cell-type dependent proliferative effects of drugs).
- Use of concatenated endpoints utilizes samples from different wells that are measured at different points in time. This potentially introduces artifacts, primarily due to variations in cell seeding densities.
- Many biochemical detection measures (e.g., MTT, LDH, ATP detection) are indirect, destroy the sample, and do not represent the true cell number.
- Measurements cannot be readily verified visually, and morphology changes due to treatment effects cannot be discerned.
- Proliferation rates of cells grown in co-cultures cannot be distinguished.

Live-cell imaging alleviates many of these challenges by allowing for non-destructive, repeated scanning of the same sample over time using either transmitted or fluorescent imaging modalities. However, there are challenges associated with adopting a live-cell approach for measuring proliferation, such as:

- Many fluorescent detection reagents perturb cell growth and morphology and are therefore unsuitable for kinetic analysis.
- Imaging more than one sample location requires movement of the sample, which is particularly problematic for non-adherent cells that can easily move to the edge of the well, or dish, and cause artifacts.
- Imaging over several cell divisions requires leak-free, environmental control systems for temperature, oxygen, and carbon dioxide that can both maintain the environment and enable access for the operator.
- Most live-cell imaging platforms require deep operator expertise and are not easily scaled to microplate throughput, either due to difficulty with set-up of image acquisition or ineffective workflow when viewing and analyzing large numbers of images.

A successful strategy for adoption of a scalable, live-cell approach for proliferation measurements must address the challenges described above. In this chapter, we illustrate how proliferation assays using the IncuCyte® Live-Cell Analysis System in conjunction with fit-for-purpose software tools and non-perturbing reagents enable kinetic quantification of cell proliferation, at microplate scale, for both non-adherent and adherent cell cultures, both in mono- and co-culture. In addition, cell proliferation measurements can be multiplexed with cell health, morphology, or surface marker measurements.

IncuCyte® Proliferation Assays at a glance

A variety of strategies for kinetic measurement of proliferation are possible using the IncuCyte Live-Cell Analysis System. Selection of label-free or fluorescent assays depends on the specific scientific question being asked and cell models studied. Continuous live-cell assays for both adherent and non-adherent cells are possible, as cells stay stationary inside a standard tissue culture incubator while IncuCyte® optics move. There is no sample or stage movement that can cause non-adherent cells to migrate to the edges of microplate wells and negatively impact data accuracy.

IncuCyte® Live-Cell Imaging and Analyis enables non-invasive, label-free measurements of cell growth based on area (confluence) or cell number (count) metrics, both of which are generated via segmentation (masking) of high quality phase images. To resolve the challenge of quantifying low contrast cells that can be difficult to identify in phase contrast images, IncuCyte NucLight live-cell analysis reagents can be used to fluorescently label nuclei. Fluorescent IncuCyte images can then be acquired over time and analyzed to generate nuclear counts and derive doubling times in either mono- or co-cultures.

Additionally, IncuCyte Proliferation Assays can be multiplexed with IncuCyte Live-Cell Analysis fluorescence reagents for cell health assessments, including apoptosis (IncuCyte® Caspase 3/7, Annexin V), cytotoxicity (IncuCyte® Cytotox), or viability (IncuCyte® NucLight Lentivirus). Cell boundaries can be identified using the IncuCyte® Cell-by-Cell Analysis software tools, and simultaneous assessment of cell death or viability achieved by measuring the fluorescence intensity originating from within the individual cell boundary.

Shortcomings of Traditional Assays	Live-Cell Imaging and Analysis Approaches
• Data obtained from a single, **pre-defined time point** yields **minimal dynamic insight**.	• Continuous, real-time data can distinguish **temporal differences** in drug or treatment effects and enable decisions as experiments progress.
• **Concatenated end point** experiments are subject to **cell seeding artifacts**.	• Cells are measured continuously over time via repeated interrogation of the same well, without loss of environmental control.
• **Indirect** detection methods are subject to **artifacts** that cannot be readily verified by eye.	• True, **direct cell counts** are generated non-invasively and visually verified via image and movies.
• Co-cultures cannot be studied as the entire population is **analyzed indiscriminately**.	• Either **label-free** or **fluorescent** assays using non-perturbing reagents can be used for studying co-cultures.
• Complex and dynamic insight (e.g., drug mechanism of action, characteristics of activation, heterogeneous populations) are not easily achieved.	• Proliferation measurements of heterogeneous, adherent or non-adherent cells can be multiplexed with morphology, health, or functional readouts.

Sample Results

Validation data for label-free and fluorescent approaches

As mentioned, the choice of assay methodology depends on the scientific question and cell model at hand. Depending on the level of rigor required, confluence assays can be a high value and valid approach. They are simple to implement and can distinguish concentration dependent effects on proliferation (Figure 1).

If a true cell count is required, label-free cell counting can be accomplished with IncuCyte's Cell-by-Cell Analysis software tools that utilize proprietary image acquisition strategies and algorithms to identify individual cells in HD phase-contrast images. Figure 2 validates this approach, in both adherent and non-adherent cell modules, by comparing changes in cell number using phase-image segmentation versus fluorescent nuclei counting achieved with IncuCyte® NucLight Lentivirus reagents (described further below). Throughout the duration of the experiment, the label-free and fluorescent nuclei cell count values tracked very closely, indicative of a robust segmentation algorithm and cell counting method.

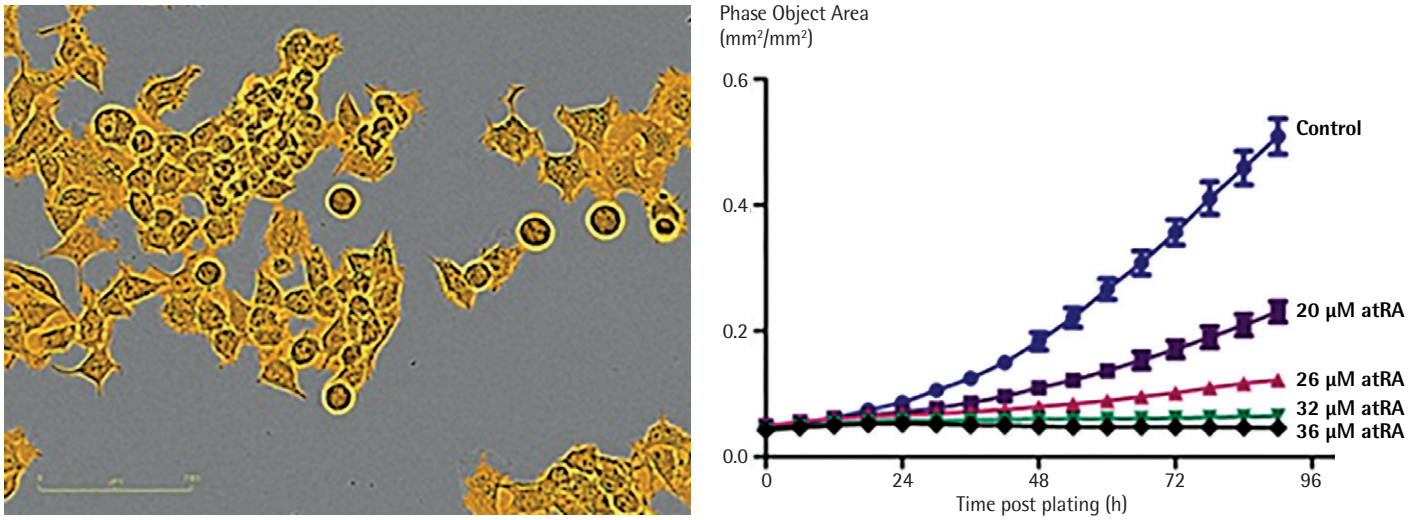


Figure 1. Proliferation is successfully measured by masking (segmenting) and analyzing IncuCyte® HD phase images. An IncuCyte mask (orange) identifies the area of the image containing neuro-2a cells. The proliferation of neuro-2a cells decreases under increasing concentrations of all-trans retinoic acid.

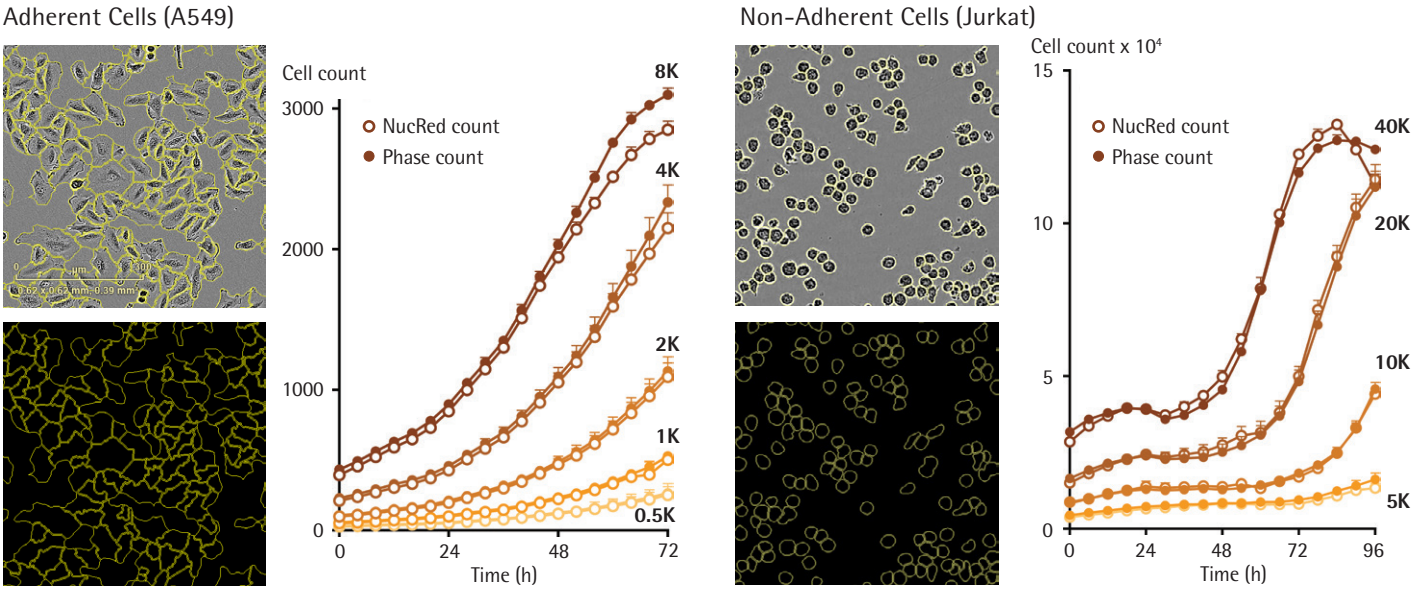


Figure 2. Label-free cell counting in adherent and non-adherent cell types using IncuCyte Cell-by-Cell analysis – Various densities of adherent A549 or non-adherent Jurkat NucLight Red cells were analyzed over time with IncuCyte Cell-by-Cell Analysis software tools and red object count to validate IncuCyte's label-free cell counting method. Images demonstrate individual cell masking using the Cell-by-Cell software. Time course of phase count and red count across densities shows overlay of label-free and fluorescent data. This validation has been repeated across a range of cell types (data not shown). Values shown are mean ± SEM of 4 wells.

If a label-free technique does not suffice, nuclear counts can be performed using IncuCyte NucLight Reagents. These reagents are available in either dye (IncuCyte® NucLight Rapid) or lentiviral (IncuCyte® NucLight Lentivirus) formats and are non-perturbing to cell health and morphology. IncuCyte NucLight Rapid is a cell permeable DNA stain that specifically stains nuclei in cells using a mix-and-read protocol. IncuCyte NucLight Lentivirus Reagents are compatible with convenient transduction protocols and provide homogenous expression of a nuclear-restricted fluorescent protein in your choice of primary, immortalized, dividing, or non-dividing cells without altering cell function and with minimal toxicity. These reagents are ideal for generating stable cell populations or clones using puromycin or bleomycin selection. Fluorescent techniques have particular value when morphology of cells are flat and/or thin, and therefore IncuCyte NucLight lentivirus reagent can be utilized not only to determine nuclear counts in living cells as described in Figure 3, but also in combination with label-free cell identification methods to measure viability (data not shown). Loss of viability is indicated by a loss in fluorescence, as fluorescent protein passes out of the nucleus as nuclear membrane integrity is lost.

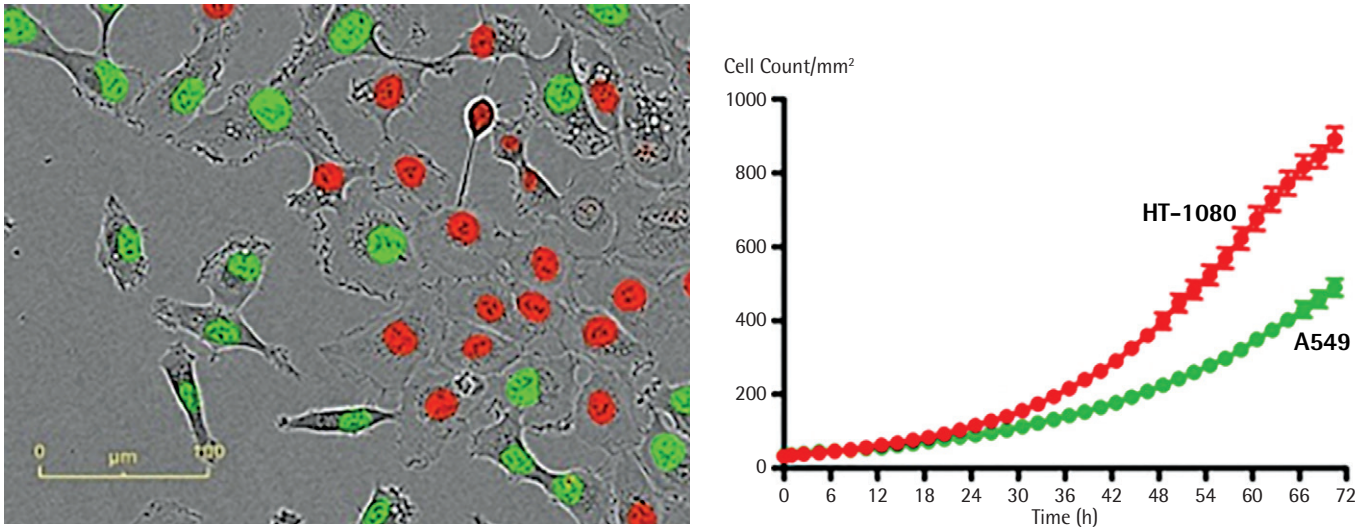


Figure 3. A wide range of co-culture-based assays are possible by fluorescently labeling the nuclei of one or more cell types (HT-1080 red; A549 green) with a non-perturbing nuclear-targeted fluorescent protein. IncuCyte fluorescent object counting software successfully quantifies real-time proliferation of viable cells.

Quantitative measurement of cancer cell proliferation in a co-culture model

Certain cancers are resistant to chemotherapy due to the biological activity of their neighboring cells, or within the context of the tumor microenvironment. For example, stromal cells have been observed to rescue tumors from drug-induced toxicity by secreting growth factors that impede apoptotic pathways.[1] Such cell interactions may be illuminated by juxtaposing monocultures and co-cultures through in vitro assays. In this study, we conducted kinetic experiments to further examine and confirm previous reports of culture-dependent drug sensitivity in a commonly used breast adenocarcinoma cell line, SK-BR-3, that overexpresses HER-2. Previously, Konecny et al demonstrated that monocultures of SK-BR-3 cells were sensitive to the drug lapatinib, which induces cell death by inhibiting tyrosine kinase activity of the HER-2 and EGFR pathways.[2] However, a second study showed that when SK-BR-3 cells were co-cultured with normal skin fibroblasts (CCD-1068Sk), these stromal cells were able to rescue the inhibitory effect of lapatinib. Interestingly, no such rescue effect was observed when SK-BR-3 cells grown in co-culture with normal human mammary fibroblasts (HMF).[1]

In our study, the proliferation of SK-BR-3 cells in monoculture and co-culture were continuously monitored for more than 8 days in the presence of increasing concentrations of lapatinib (Figure 4). SK-BR-3 cells expressing IncuCyte NucLight Red fluorescent protein were cultured in the presence or absence of fibroblasts, and then quantified using IncuCyte's nuclear counting algorithm. This method allowed real-time cell counting based on nuclear restricted fluorescent protein expression. Kinetic graphs of nuclear counts per mm² show SK-BR-3 cells grown with CCD-1068Sk fibroblasts grow at a significantly higher rate than those grown alone or with HMF's in the presence of 500 nM lapatinib (Figure 4A). In addition, IC50 values, calculated using the area under the curve (AUC) of nuclear counts per mm²/time, provide quantitative evidence for the differences in drug response between monoculture and co-cultures in the presence of lapatinib. Specifically, SK-BR-3 cells grown with CCD- 1068Sk fibroblasts are the least sensitive to lapatinib with an IC50 value of 1.162 µM, followed by SK-BR-3 cells grown with HMF's with an IC50 value of 0.581 µM (Figure 4B). Interestingly, SKBR-3 cells grown in mono-culture remain effectively sensitive to lapatinib with an IC50 value of 0.015 µM (Figure 4B) which is comparable to the published IC50 value, 0.037±0.031 µM.2 These striking data very clearly illustrate the difference inSK-BR-3 cell proliferation in the presence of stromal cells, thus highlighting the importance of considering the effect that the tumor microenvironment can have on drug resistance.

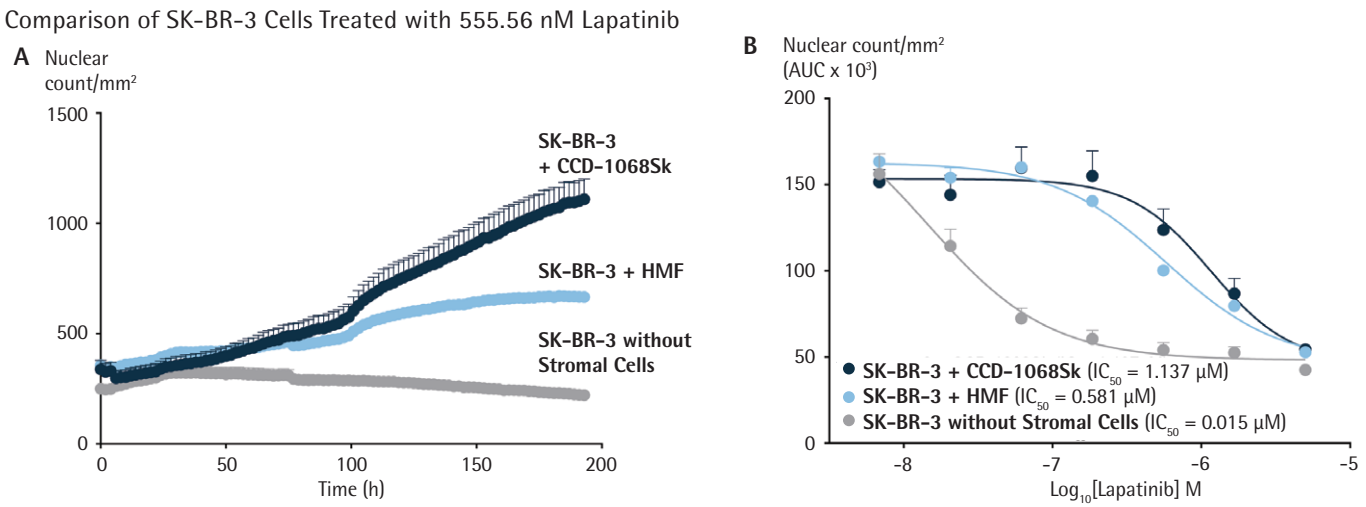


Figure 4. Proliferation of SK-BR-3 cells in co-culture and monoculture under lapatinib treatment. SK-BR-3 cells expressing a nuclear restricted red protein were grown with normal skin fibroblasts (CCD-1068Sk), human mammary fibroblasts (HMF), or in monoculture, and treated with varying concentrations of lapatinib for 8 days. (A) Nuclear counts per mm² of SK-BR-3 cells grown with or without stromal cells in the presence of 556 nM lapatinib illustrate the rescue effect of CCD-1068Sk fibroblasts compared to HMFs and mono-culture. (B) Area under the curve of nuclear counts per mm² over time for each concentration (n=4) was used to calculate and compare IC_{50} values of SK-BR-3 cells grown with or without stromal cells.

High-throughput compound testing using NucLight Green labeled HT-1080s

High-throughput compound testing is essential for efficiently advancing promising drugs through the drug discovery pipeline. To examine the ability of the IncuCyte system to meet this need, cell proliferation was measured over time in a higher-throughput format. To assess many pharmacological agents simultaneously, 16 literature-standard compounds (Table 1) were applied to HT-1080 tumor-derived fibrosarcoma in a 384-well format (Figure 5). An 11-point concentration response curve was constructed for each compound (Figure 6). Of the 16 compounds tested, the rank order of potency for inhibition of cell proliferation was: doxorubicin = staurosporine = camptothecin > mitomycin C >cycloheximide = RITA > PD-98059 > FAK inhibitor 14 = cisplatin > 10-DEBC = Chrysin = Compound 401. The compounds TAME, PAC1, KU0063794 and FPA-124 had little or no effect on cell proliferation under the conditions of the experiment.

Table 1. Drugs identified in literature as relevant to cell proliferation.

Drug	Description
Doxorubicin	chemotherapy drug, intercalates DNA[3]
Camptothesin	alkaloid inhibits topoisomerase, causing DNA damage[3]
Staurosporine	potent alkaloid inhibitor of protein kinase[4]
Mitomycin C	chemotherapy drug, alkylates DNA[5]
Cycloheximide	protein synthesis, inhibitor[6]
RITA	(reactivation of p53 and induction of tumor cell apoptosis) a small molecule, binds p53[7]
PD-98059	MAPK1/2 inhibitor[8]
Cisplatin	chemotherapy drug acts through crosslinking DNA[9]
FAK-inhibitor 14	selective inhibitor of focal adhension kinase[10]
10DEBC	selective inhibitor of Akt[11]
Chrysin	a flavonoid observed to inhibit growth in cancer cells[12]
TAME	tert-Amyl methyl ether; a gasoline additive with suspected toxic effects upon inhalation[13]
PAC1	(pro-caspase activating compound-1), a small-molecule activator of procaspase-3 to caspase-3[14]
KU0063794	specific inhibitor of mTORC1/2[15]
FPA-124	Akt inhibitor[16]
Compound 401	inhibitor of DNA-dependent kinase and mTOR[17]

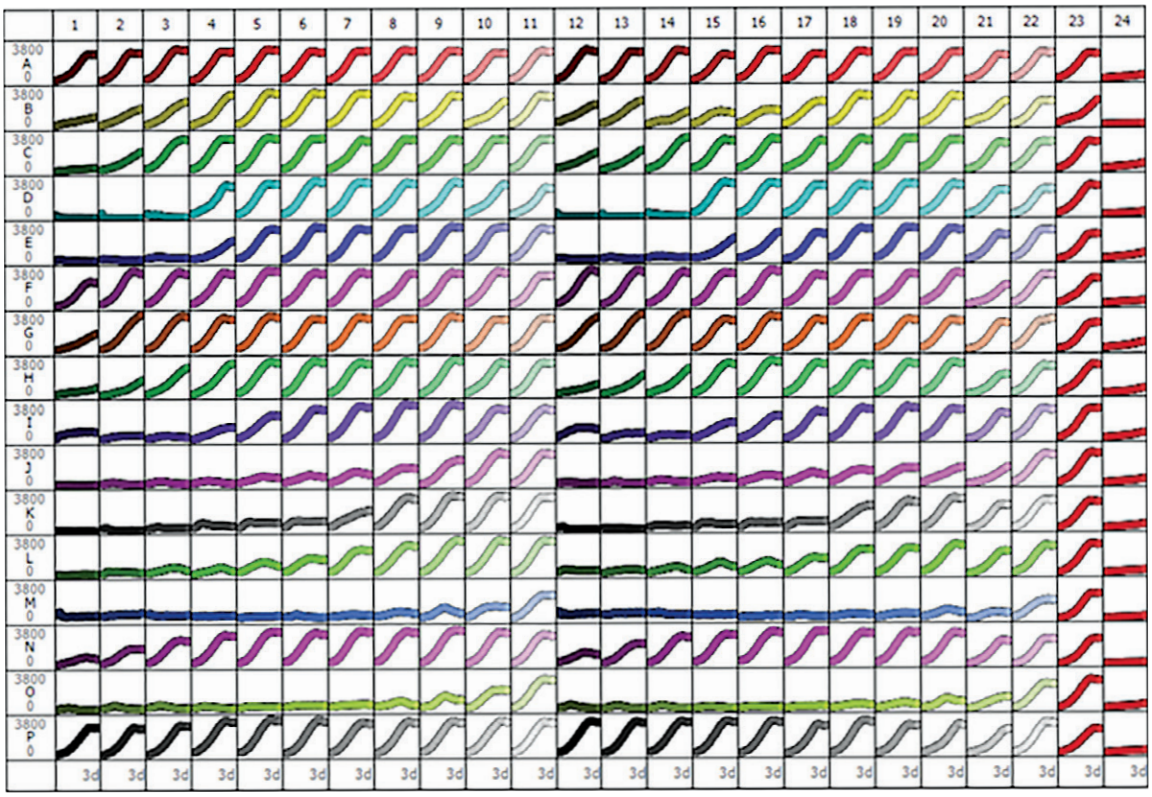


Figure 5. 384-well microplate view of HT-1080 NucLight Green cell proliferation with 16 different compounds, 11-point concentration-response curves in duplicate (different colors, high to low concentrations left to right). Columns 15 and 16 are vehicle (0.5% DMSO) and CHX (3 µM) controls, respectively. Note the potent concentration-dependent inhibition of cell proliferation for certain compounds (e.g. row J, row M, row O), and weaker effects/inactivity of others (e.g. row A, row P). Abscissa: time (0-72h), ordinate: fluorescent object count per well (0-3800).

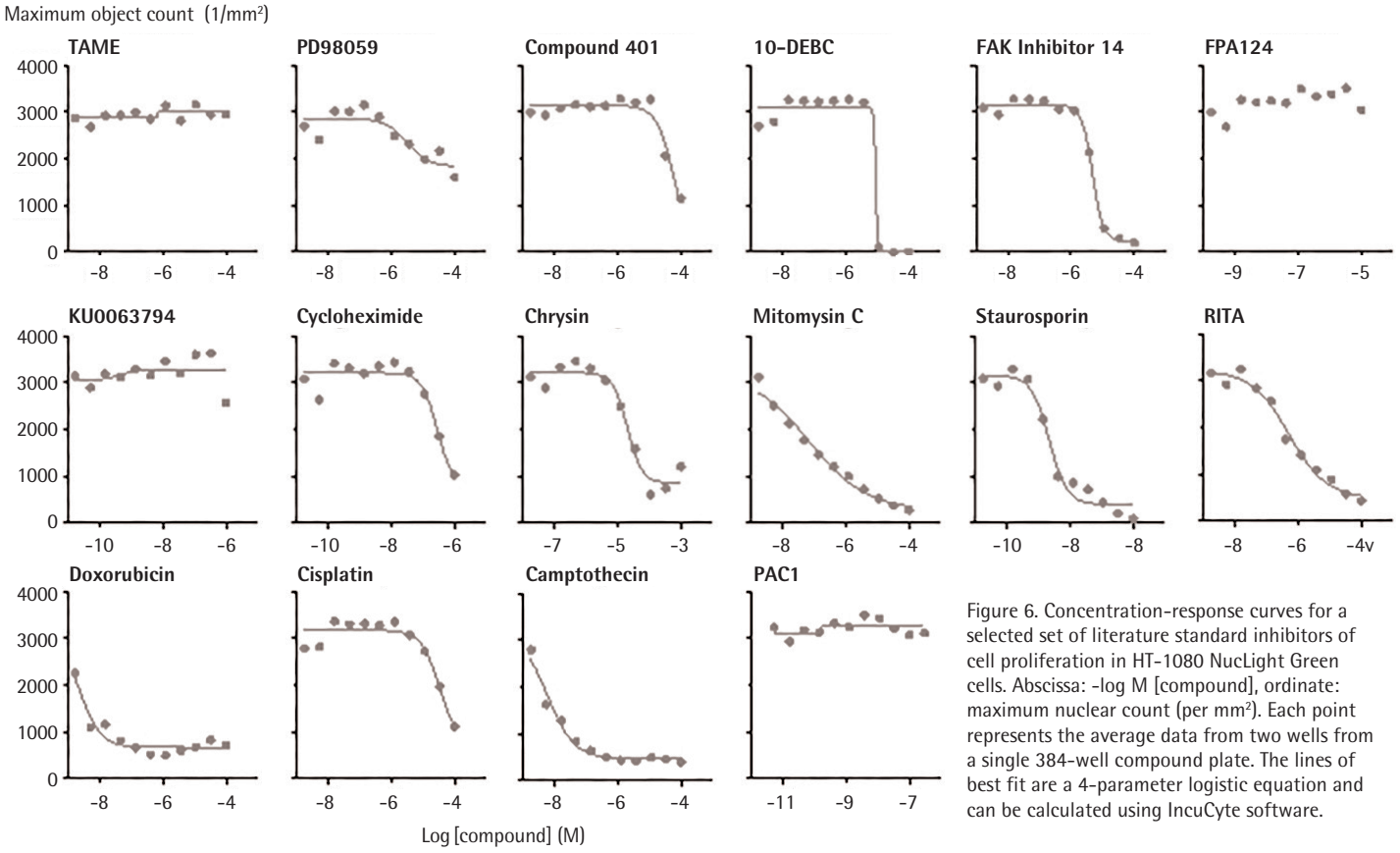


Figure 6. Concentration-response curves for a selected set of literature standard inhibitors of cell proliferation in HT-1080 NucLight Green cells. Abscissa: -log M [compound], ordinate: maximum nuclear count (per mm²). Each point represents the average data from two wells from a single 384-well compound plate. The lines of best fit are a 4-parameter logistic equation and can be calculated using IncuCyte software.

To verify and extend these findings, representative images of the cells exposed to test compounds at selected concentrations were inspected (Figure 7). Following exposure to an IC80 concentration for 24h, staurosporine produced profound changes in cell morphology, with extensive branching and condensation of the nucleus and cell body. The cells lost motility and there was clear evidence of cytotoxicity. In contrast, the inhibition of cell proliferation by RITA (also IC80, 24h) was not accompanied by any obvious morphological changes. At anti-proliferative concentrations, both camptothecin and doxorubicin treated cells appeared healthy with no evidence of cell death, suggesting that senescence had occurred. 10-DEBC (11 mM, 24h) produced overt cytotoxicity and complete cell lysis. These data show the potential of this kinetic and morphological approach to the screening, prioritization, and classification of compounds in drug discovery.

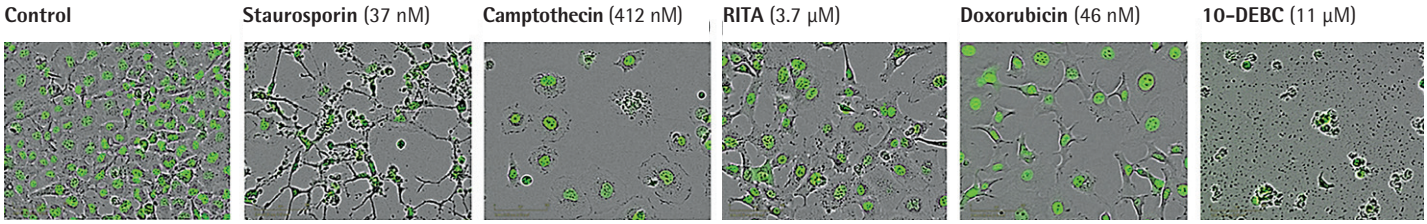


Figure 7. Representative 'blended' phase contrast/fluorescence images of HT- 1080 NucLight Green cells treated with different test compounds as labeled. Images shown were taken 24 h post compound treatment (10x). Note the reduction in total cell number for each treatment compared to the vehicle control, and the profound differences in cell morphology.

Label-free cell counting and immunophenotyping of heterogeneous, non-adherent cell models

The experiment (Figure 8) illustrates label-free counting of live cells in a heterogeneous population of a mixed B cell/T cell culture, as well as the characterization of the CD surface markers. Specific antibodies to the leucocyte common antigen, CD45 and the B-lymphocyte specific antigen CD20 were labeled with IncuCyte® FabFluor-488, a fluorescently labeled antibody fragment. The FabFluor/antibody conjugates were then directly added to mixed cultures of Jurkat and Ramos B cells in full cell culture media. Incuyte® Opti-Green, a background fluorescence suppressing reagent, was included to minimize non-specific fluorescence from unbound Fab/ antibody complexes. Images were analyzed using IncuCyte's Cell-by-Cell Analysis software module, then further classified based on CD45 positive and CD20 positive fluorescence (separate wells). In line with expectations, >95% of cells were labeled positive for CD45, irrespective of the proportion of Jurkat or Ramos cells added to the mix. CD20 positive cells were only observed in Ramos containing cultures at the proportions expected. In the continued presence of the Fab/antibody, an increase in the number of fluorescently-labeled cells was observed over 48h as the cells proliferated. This simple proof-of-concept experiment demonstrates the ability to specifically label, count and quantify subsets of cells in mixed cultures and to subsequently track long-term changes in these subsets over time.

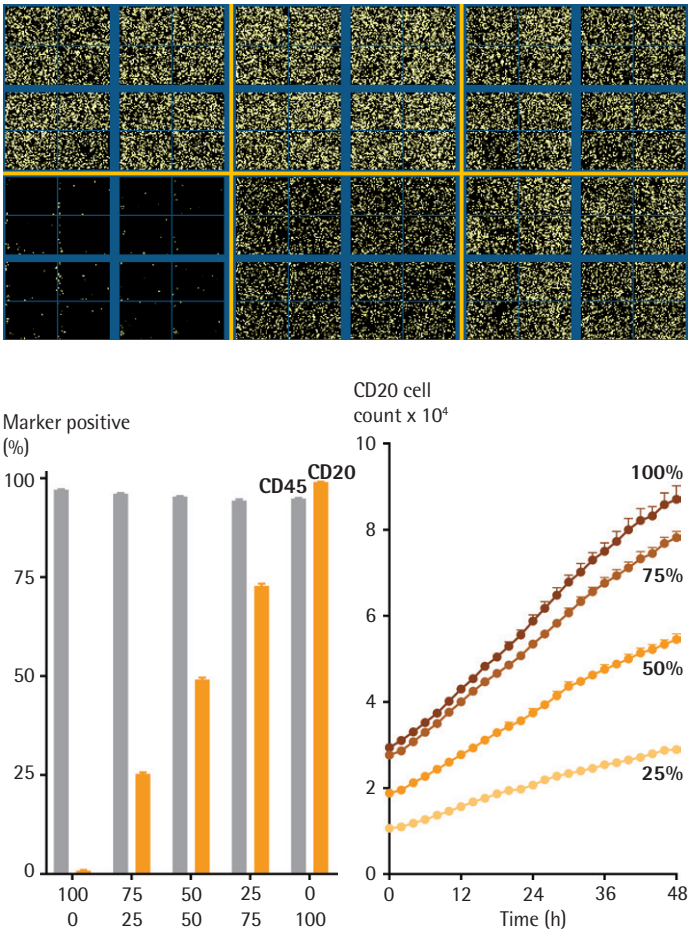


Figure 8. Label-free cell counting and characterization in heterogeneous non-adherent cultures. Characterization of a mixed B cell/T cell culture for the CD surface marker CD45 and CD20 using IncuCyte FabFluor-488 labeled Abs. (A) IncuCyte vessel view images (yellow = Ab-labeled cell) of mixed cell populations in the ratios shown. Note the greater proportion of CD20-labeled (Ramos) cells as the ratio of R:J increased in contrast to the CD45 that labels both cell types. (B) IncuCyte Cell-by-Cell quantification of the % expression in the mixed culture. (C) Time course of CD20+ cell count showing proliferation of CD20+ cells within the mixed culture. Values shown are mean ± SEM of 4 wells.

Continuous live-cell proliferation, clustering and viability assays for T-cells

To investigate the effect of cell density on T-cell proliferation and cluster formation, isolated human peripheral blood mononuclear cells (PBMCs) were seeded at various cell densities (20-50 K/well) on poly-l-ornithine (PLO) coated flat bottom 96-well plates. Cells were grown in the absence or presence of T-cell activators (α-CD-3 (100 ng/ml), hrIL-2 (10 ng/ml)) and HD phase contrast images were captured on an IncuCyte system over 144h. Images were analyzed for phase confluence (%) as a measure of cell proliferation and the number of phase objects above an area threshold (cluster count/well) to define cluster formation. The kinetic graph (Figure 9A) demonstrates little or no proliferation under basal conditions (grey lines) but rapid proliferation in the presence of activators (blue lines), which is seeding cell density dependent. Following activation, T-cells rapidly form clusters, which is displayed as a cluster count increase (Figure 9B) over time. Both data sets show an increase in the rate of proliferation or cluster formation with cell density (bar graphs, Figures 9A and 9B). The confluence in unstimulated PBMCs can be seen to drop over time due the possible presence of phagocytes. The IncuCyte system acquired phase-contrast images of IL-2/anti-CD3 activated PBMCs, confirming cluster formation as illustrated in Figure 9C.

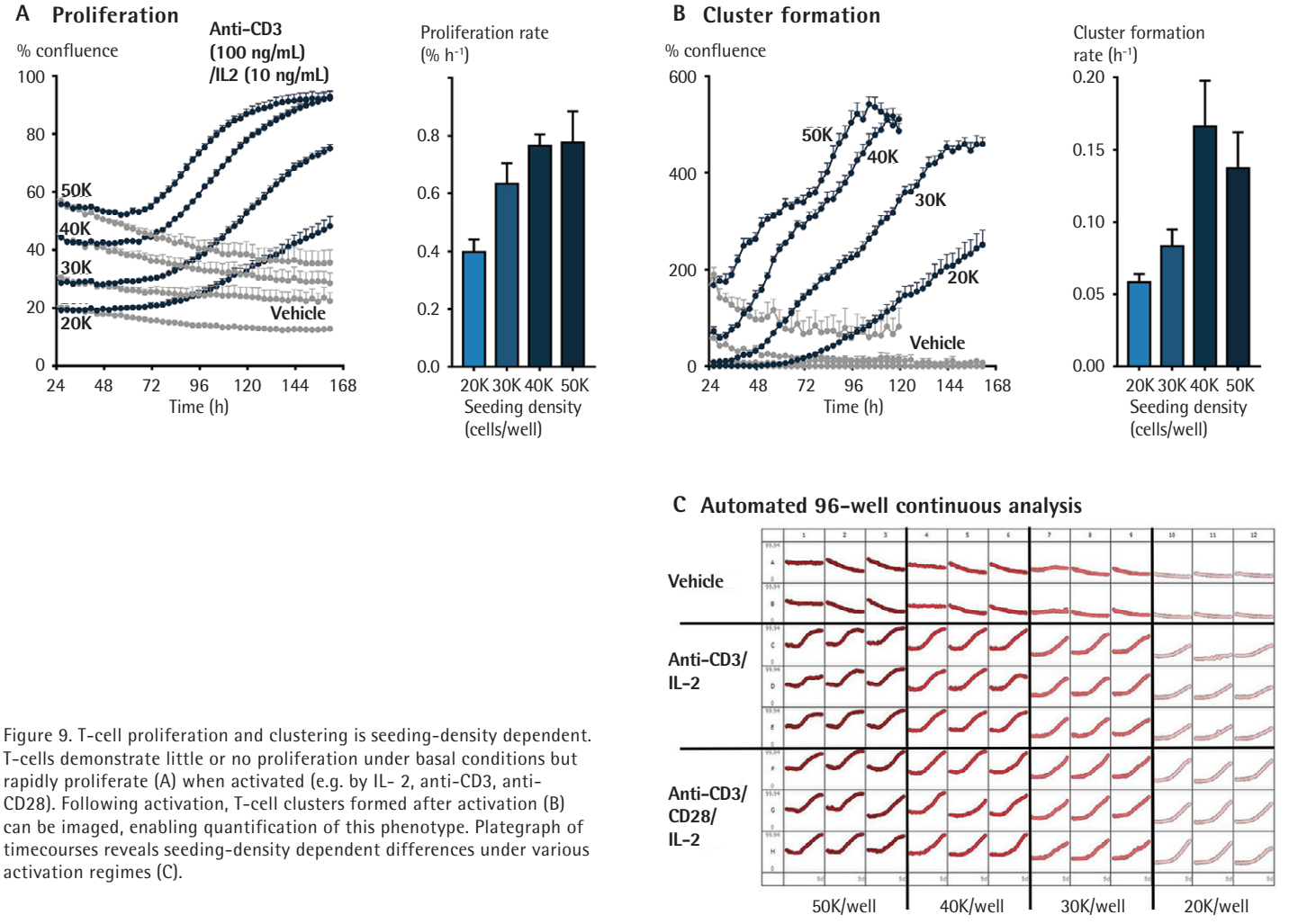


Figure 9. T-cell proliferation and clustering is seeding-density dependent. T-cells demonstrate little or no proliferation under basal conditions but rapidly proliferate (A) when activated (e.g. by IL- 2, anti-CD3, anti-CD28). Following activation, T-cell clusters formed after activation (B) can be imaged, enabling quantification of this phenotype. Plategraph of timecourses reveals seeding-density dependent differences under various activation regimes (C).

To capture the stimulus dependent effect on T-cell proliferation and cluster formation, isolated human PBMCs were seeded (20 K/well) on a PLO-coated flat bottom 96-well plate and grown in the presence of various T-cell activator combinations, hrIL-2 (10 ng/ml), a-CD-3 (0.1 ng/ml) and/or a-CD28 (1-100 ng/ml). HD phase contrast images were captured on the IncuCyte system over 240 hours and analyzed for phase confluence (%) as a measure of cell proliferation and the number of phase objects above an area threshold (cluster count/well) to define cluster formation. As illustrated in Figure 10A, little or no cell proliferation was observed in the presence of IL-2 alone, however, moderate proliferation was observed with the addition of a-CD-3. T-cell proliferation was further enhanced by the addition of a-CD-28, revealing a concentration dependent response (Figure 10A). A similar stimulus dependent cluster formation profile was also measured in the presence of various T-cell activator combinations (Figure 10B). These data show the potential of this kinetic and morphological approach to evaluating immune cell activation.

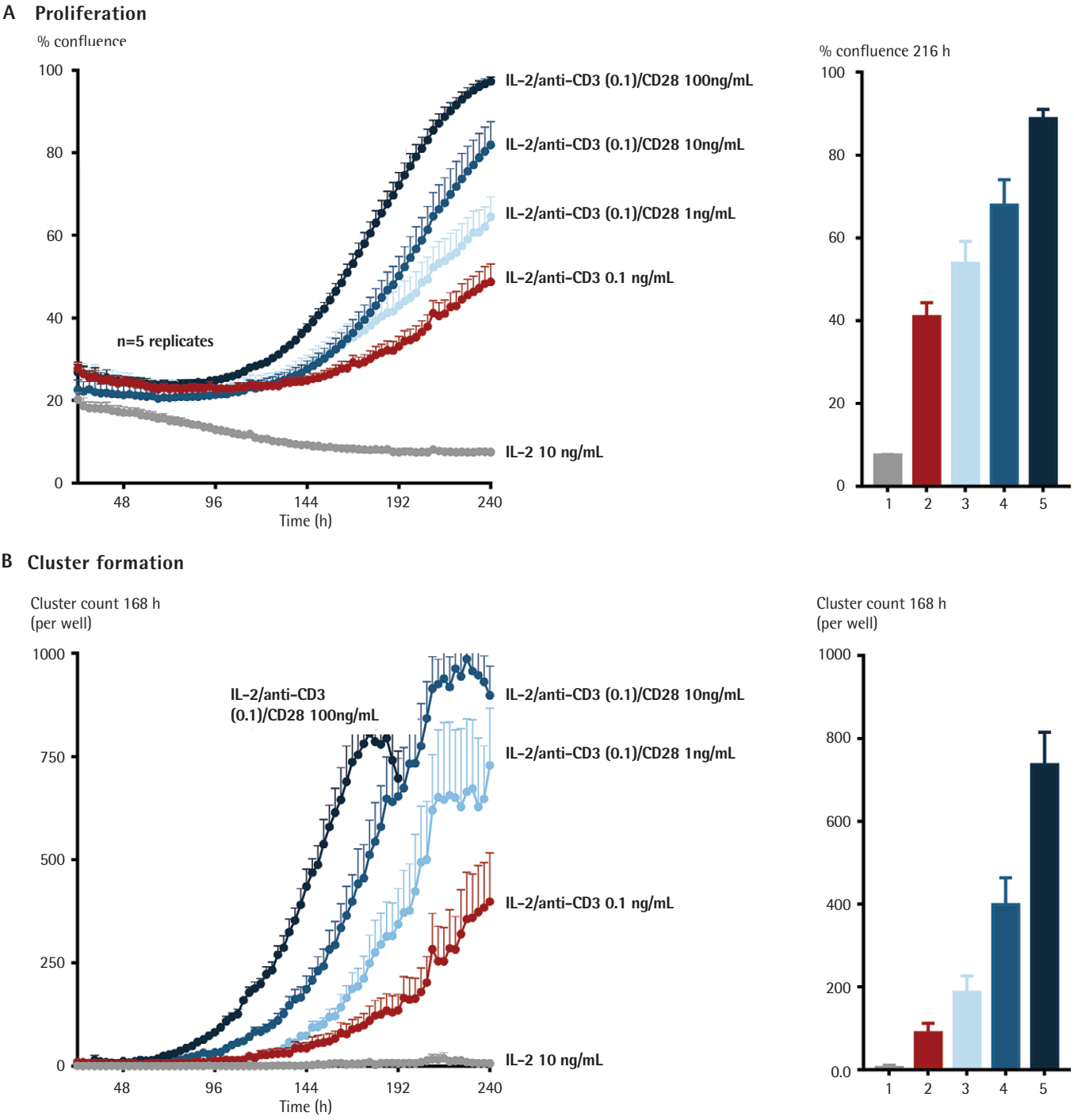
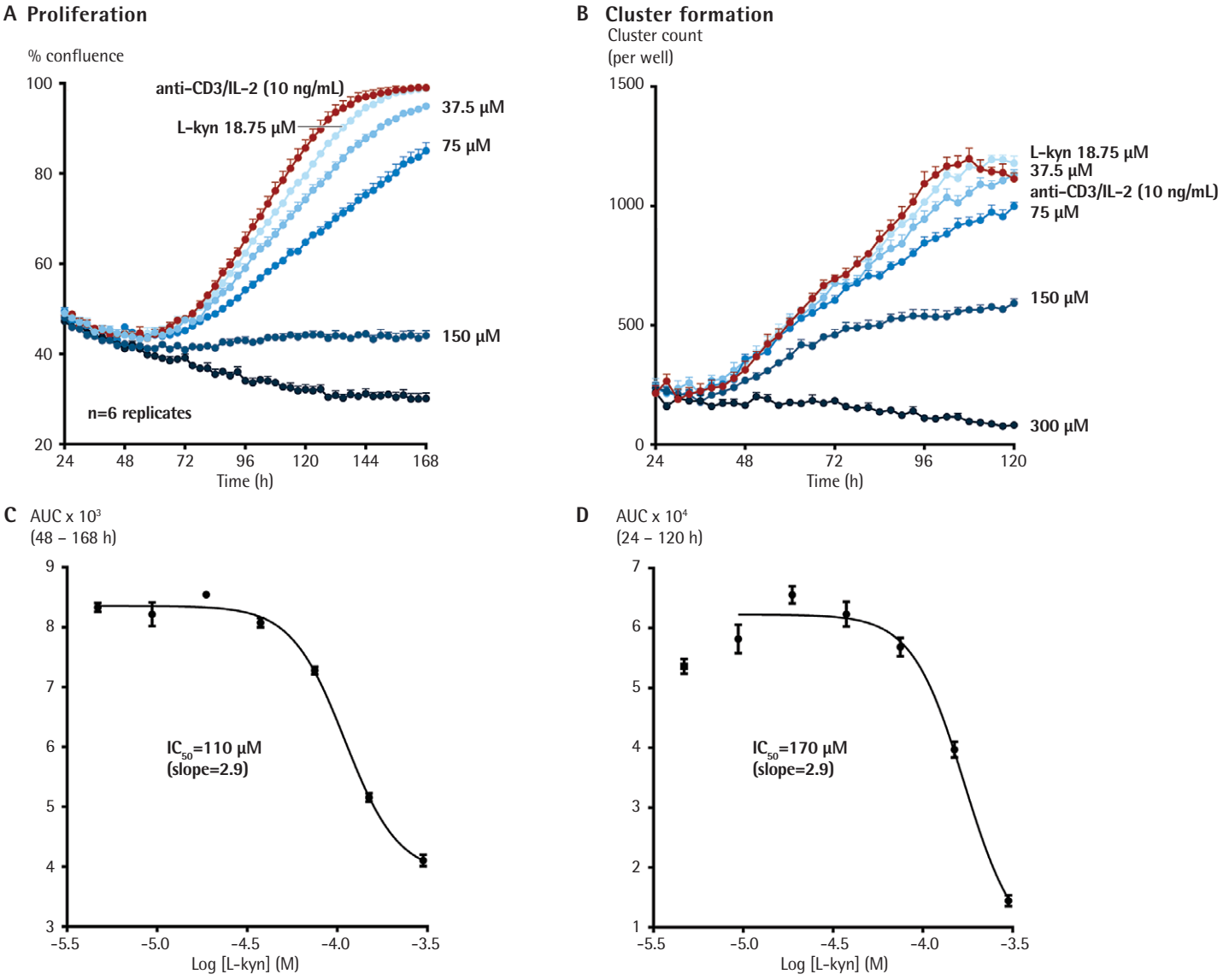


Figure 10. T-cell activation is stimulus and concentration-dependent. Data shown are for PBMCs treated with combinations of IL-2, anti-CD3, and anti-CD28.

Additionally, the IncuCyte system was used to study effects of L-kynurenine (L-kyn) on T-cell proliferation and clustering. L-kyn is a metabolite formed from the catabolism of L-tryptophan by the enzymes indoleamine 2,3-dioxygenase (IDO) and tryptophan 2,3-dioxygenase (TDO). Some cancers increase L-kyn production in a bid to block antigen-driven T-cell proliferation and induce T-cell death, thus allowing cancer cells to escape immune surveillance. Inhibitors of IDO and/or TDO are therefore promising therapeutic targets for the treatment of cancer. Figure 11 shows time-course results of L-kyn inhibition of T-cell proliferation and clustering.



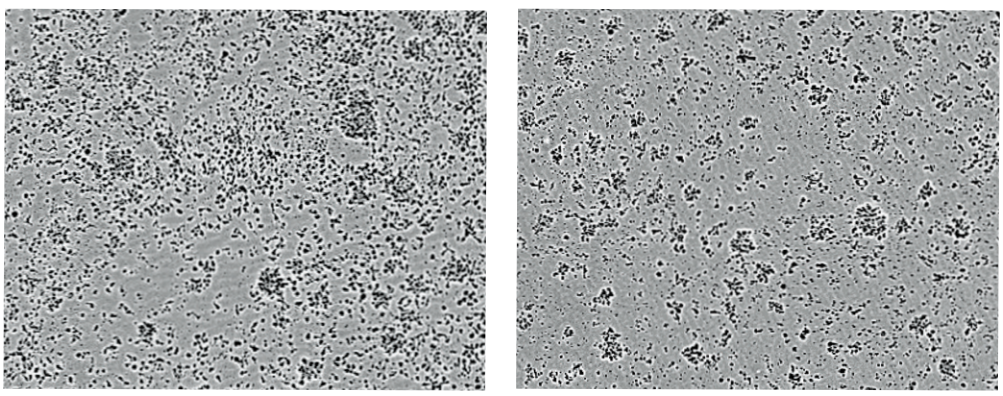


Figure 11. L-kynurenine inhibits T-cell proliferation and clustering. Data demonstrates a clear concentration-related inhibition of IL-2/anti-CD3 activated T-cell proliferation (A) and clustering (B) with the addition of exogenous L-kyn, over time. Time-course profiles enabled AUC analysis and generation of concentration-response curves from which IC50 values for inhibition of proliferation (C) and clustering (D) were determined. Images reveal reduction of clustered morphology with addition of L-kyunerenine (F) when compared to activated t-cells (E).

Conclusions

The IncuCyte Live-Cell Analysis System, in conjunction with proprietary software tools and IncuCyte's NucLight nuclear-labeling reagents, provide a flexible assay platform for kinetic measurements of proliferation. These assays achieve quantitative and reproducible analysis of both adherent and non-adherent cells, in monoculture and importantly, co-culture, and without removing cells from the physiologically-relevant environment of a tissue culture incubator. Image-based methodologies give the user the ability to monitor morphological changes in parallel with quantification, the combination of which is a powerful and unique tool for detecting pharmacological or genetic manipulations that alter cell viability or function.

- Kinetic, label-free confluence or cell count measurements over several cell divisions can be generated using phase image segmentation and without using fluorescent labels– an ideal strategy for mono-culture analysis.

- Fluorescent reagents that label nuclei (e.g., IncuCyte NucLight reagents) can be used to measure proliferation of mono-cultures when cells are of low contrast and therefore difficult to identify via segmentation of phase contrast images. Nuclear-labeling strategies can also be used to differentiate the effects of supporting cells on the proliferation of a labelled cell population.

- IncuCyte's kinetic label-free cell counting method can also differentiate between proliferation rates of subpopulations in co-cultures when individual cell types are of different size or shape (non-adherent cells only), or when the individual populations can be identified using cell surface markers (adherent or non-adherent cells), or when one population is fluorescently labelled with a nuclear marker (IncuCyte NucLight reagents).

- Proliferation assays can be run in microplates (96-well and 384-well) with high precision and reproducibility. In 384-well plates, a mix and read assay is exemplified whereby full concentration-response curves of 16 standard anti-proliferative agents were compared. In a single IncuCyte instrument, 6 x 384-well plates can be monitored providing >2000 wells of parallel data acquisition.

- All data and time points can be verified by inspecting individual images and/or time-lapse movies. Cell morphology observations provide additional validation and insight into mechanistic differences between treatments or conditions.

- Non-adherent cell proliferation and clustering can be visualized due to the non-perturbing nature of the IncuCyte's optical design. Non-adherent cells stay stationary; there is no stage or sample movement, rather the optics move.

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Kinetic Caspase-3/7 and Annexin Apoptosis Assays

Quantitative assays for apoptotic pathway analysis for both drug discovery and basic research

Introduction

Apoptosis, the biological process by which cells undergo programmed cell death, is required for normal tissue maintenance and development. However, aberrations in apoptotic signaling networks are implicated in numerous human diseases including neurodegeneration and cancer.[1] Apoptotic pathways are initiated by extrinsic factors that result in activation of pro-apoptotic receptors on the cell surface, or intrinsically by many different stimuli such as DNA damage, hypoxia, the absence of growth factors, defective cell cycle control, or other types of cellular stress that result in release of cytochrome C from mitochondria.

Stimulation of either the extrinsic or intrinsic apoptotic pathways triggers a signaling cascade that results in the activation of a family of proteins that play a major role in carrying out the apoptotic process called caspases.[2] Caspases (cysteinyl aspartate proteinases) cleave substrates following an Asp (D) amino acid residue. Effector targets of caspases include caspase family members themselves, proteins involved in fragmentation of cellular DNA (caspase-activated DNAses), nuclear lamins, as well as proteins that make up the cell cytoskeleton. Caspase proteins are traditionally separated into two groups, initiator caspases (caspase 2, 8, 9 and 10), and executioner or effector caspases (caspase 3, 6, and 7). As a primary executioner caspase in most systems, the activation of caspase-3 often results in the irreversible commitment of a cell to apoptosis. Therefore, the activation of caspase-3 is considered a reliable marker for cells undergoing apoptosis.

The regulated loss of plasma membrane phosphatidylserine (PS) symmetry is also a classical marker of apoptosis. Dying cells trigger the translocation of the normally inward-facing PS to the cellular surface, allowing for early phagocytic recognition of the dying cell by surrounding phagocytes.

Numerous enzymatic, plate-reader and flow-cytometric assays have been designed to measure caspase-3/7 activation or PS externalization. Most caspase-3/7 assays involve luciferase, colorimetric or fluorometric reagent substrates that incorporate a DEVD (Asp-Glu-Val-Asp) peptide motif[3] which is recognized by the enzyme. Annexin V is a recombinant protein with a high affinity and selectivity for PS residues, allowing it to be used for the detection of apoptosis. Apoptosis assays using annexin V conjugated to a fluoroprobe have been optimized for detection of PS externalization and are most commonly measured by flow-cytometry.

In this chapter, we will examine kinetic approaches for measuring apoptosis using IncuCyte® Caspase-3/7 and Annexin V fluorescent reagents. Unlike plate reader and flow-cytometric endpoint approaches, kinetic live-cell image-based analysis allows for the evaluation of time-dependent effects of treatments or cell-specific responses.

Live-cell Apoptosis Assays at a Glance

The IncuCyte® Caspsase-3/7 reagent is an inert, non-fluorescent substrate. When added to tissue culture growth medium, the substrate freely crosses the cell membrane where it is cleaved by activated caspase-3/7 resulting in the release of the DNA dye and green fluorescent labeling of DNA.[4, 5] IncuCyte® Annexin V reagents are specially formulated, highly-selective cyanine-based fluorescent dyes ideally suited to a simple mix-and-read, real-time quantification of apoptosis in living cells.

- The apoptotic signal relies on either the activation of Caspase-3/7, a primary and irreversible "executioner" pathway in most cell types, or using Annexin V conjugated to a fluoroprobe for detection of PS externalization.
- Multiplexing the Caspase-3/7 and Annexin V reagents detect and confirm apoptosis through two different pathways, verifying apoptosis as a mechanism of cell death.
- Multiplexing IncuCyte apoptosis reagents with a nuclear-labeling reagent such as IncuCyte NucLight enables differentiation between inhibition of cell growth and induction of cell death.
- High definition phase contrast images provide an additional qualitative validation of cell death based on morphological characteristics.
- Images are automatically acquired and analyzed to reveal concentration and time-dependent effects on biology.

Shortcomings of Traditional Assays	Live-Cell Imaging and Analysis Approaches
• Assays result in a **single, user-defined time point measurement** of caspase-3/7 activity.	• The assay provides a **full kinetic readout of apoptotic signaling over multiple days**, eliminating the need for determining a single, optimum, assay endpoint a-priori which can vary considerably for different cell types and for different compound treatment conditions.
• Techniques **require multiple wash steps or cell lifting** prior to data collection that may result in the loss of dying cells or lead to a loss in PS asymmetry.	• Cells can be simultaneously labeled with an IncuCyte apoptosis reagent and NucLight live-cell nuclear labeling reagent to **measure apoptotic cell death, cell proliferation and kinetically monitor anti-proliferative effects of compounds**.
• Assays are **not amenable to long-term measurements** due to increasing signal background over time.	• Addition of IncuCyte apoptosis reagents to normal, healthy cells are **non-perturbing to cell growth or morphology** and yield little to no intrinsic fluorescent signal.
• Manipulations can result in the **loss of cells or critical data** in experiments where cells undergo apoptosis at different rates according to treatment conditions.	• **IC50 and EC50 values can be calculated using kinetic area under the curve** values of nuclear counts and caspase-3/7 or annexin V counts, respectively.
	• 96- and 384-well assay using a homogeneous "mix and read" protocol which can be run over multiple days in full media. **No wash or lifting steps required**, negating the concern that cells are lost during the experiment or labeling process.
	• The assay has **high statistical reproducibility** and can be used both for **single-point screening or concentration response profiling.**
	• All data points and temporal data curves can be validated by individual images or time-lapse movies respectively. The kinetic readout of the IncuCyte® system provides both **high definition (HD) phase as well as quantitative fluorescent imaging**.

Sample Results

Quantitative measurement of caspase-3/7 kinetic activation

MDA-MB-231 cells, a human breast adenocarcinoma derived cell line, were treated with staurosporine (SSP) a well-known inducer of apoptosis. SSP was serially diluted in growth media containing 5 µM IncuCyte Caspase 3/7 reagent in a 96-well plate. Once treated, the cells were immediately placed inside the IncuCyte live-cell analysis system with a 10X objective in a standard cell culture incubator and both phase-contrast and fluorescent images were collected every 2-3 hours.

Alterations in cell morphology were evident within only a few hours of SSP treatment as illustrated in the phase image in Figure 1A. Using fluorescent images, we positively identified cells containing fluorescently stained DNA indicating activation of caspase-3/7, cleavage of the DEVD moiety in the kinetic apoptosis reagent, and fluorescent labeling of cellular DNA (green image in Figure 1A). Using the object counting algorithm, we successfully quantified the number of fluorescent objects , shown in Figure 1A. The object counting criteria were then applied to all images in the experiment at each time point. The data in Figure 1B indicate that caspase-3/7 activation is detectable within a few hours of SSP treatment, with a maximal response triggered in the presence of 333 nM SSP.

Increasing concentrations of SSP also significantly affected cell proliferation. To demonstrate this on the IncuCyte, we completed an end point analysis at the 48 hour time point. Vybrant® DyeCycle™ Green DNA dye was added directly (no wash required) to the wells at a final concentration of 1 µM in 50 µL of PBS. After a 30 minute incubation, the total number of DNA containing objects was enumerated using the object counting algorithm. As expected, our data indicate an inverse correlation between the total number of objects and the apoptotic index as a function of increasing concentrations of SSP (Figure 1C). The data clearly indicate the ability of the IncuCyte system to accurately identify the activation of Caspase-3/7, thus providing insight into the dynamics and timing of the apoptotic signaling pathway, thereby alleviating the need to pick an end-point for analysis prior to running the experiment.

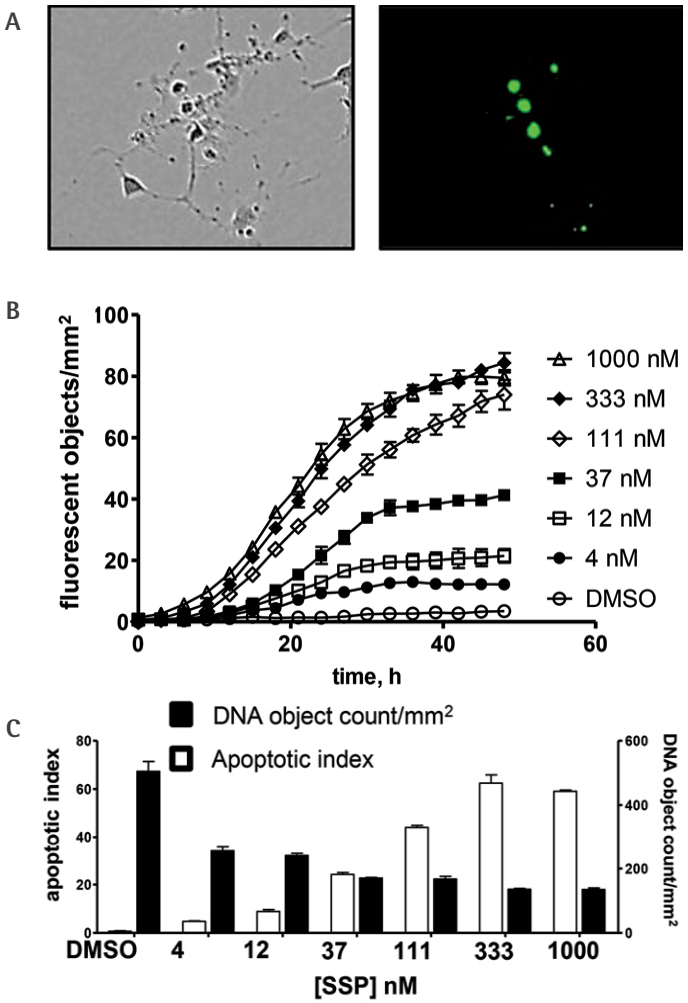

Figure 1: Staurosporine (SSP) induced caspase-3/7 activity in human breast adenocarcinoma cells (MDA-MB-231). (A) Representative phase contrast and fluorescent images reveal classical apoptotic cell morphologies and indicate activation of caspase-3/7, respectively. (B) Kinetic measures of the number of caspase-3/7 positive cells is recorded over time and plotted as fluorescent objects, n=3 wells per data point shown (C) At the 48 hour end point, the apoptotic index was calculated by dividing the number of caspase- 3/7 fluorescent objects by the total number of DNA containing objects following staining with Vybrant DyeCycle Green.

Multiplexed, kinetic measurements of proliferation and apoptosis

The fluorescent channels on the IncuCyte live-cell analysis system provide a way to kinetically measure caspase-3/7 activation in addition to proliferation (nuclear label) within the same well, eliminating the need for end-point analysis. In the next experiment, HeLa NucLight Red cells were treated with SSP in the presence of 5µM IncuCyte Caspase-3/7 reagent and phase-contrast, red, and green images were collected every 2 hours in the IncuCyte system using a 10x objective (Figure 2). These data illustrate typical results obtained using the IncuCyte Caspase-3/7 reagent multiplexed with IncuCyte NucLight Red cells to measure the kinetic induction of apoptosis and proliferative effects of drug treatment (Figure 2B and C, respectively). Using all of the kinetic data in Figure 2B and 2C, area under the curve (AUC) values were plotted and EC50 (apoptosis) and IC50 (proliferation) values were calculated. This 2-color kinetic assay provides a multiplex way to analyze the apoptotic and anti-proliferative effects of various treatments.

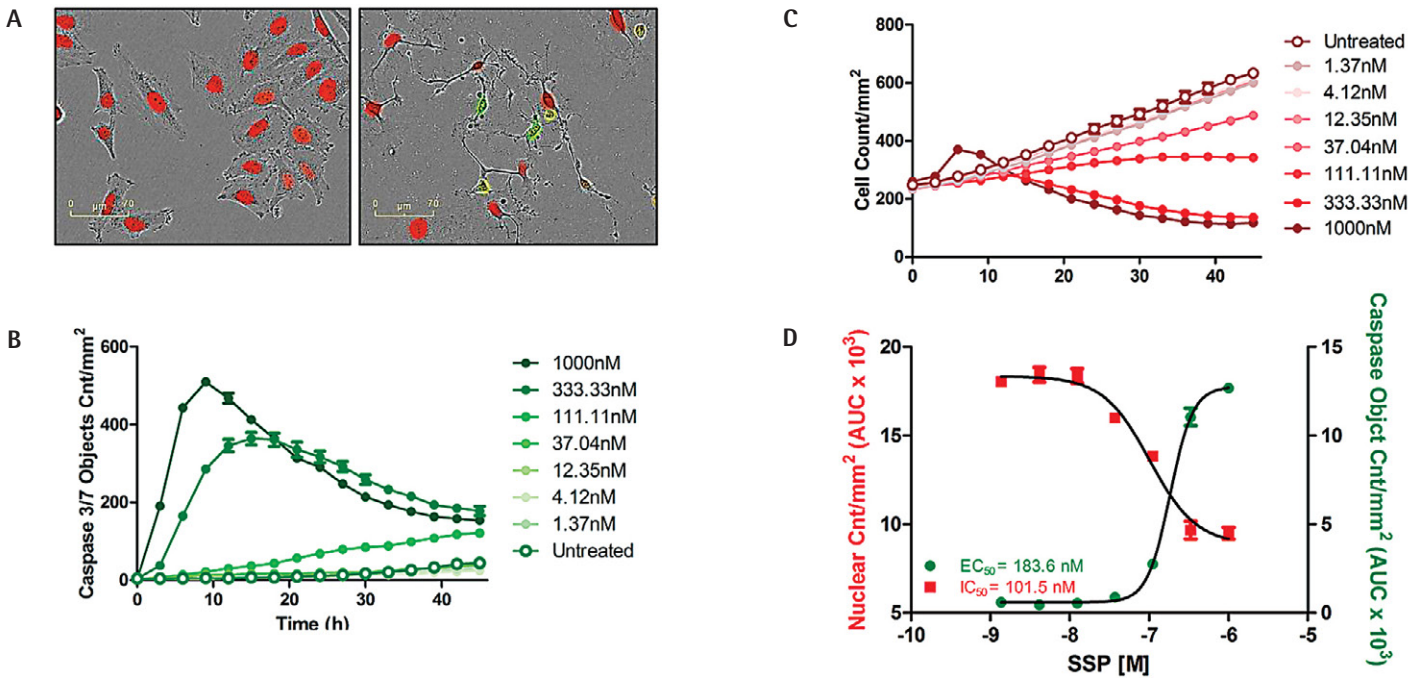

Figure 2: Pharmacological analysis of caspase-3/7 activation and nuclear counts in HeLa NucLight Red cells treated with SSP.
(A) Blended phase-contrast and red/green images taken at 20x show red nuclear signal and activation of caspase-3/7 as well as morphological differences in untreated cells (left) vs. cells treated with 300nM SSP (right). (B) Caspase-3/7 positive objects and (C) nuclear counts were measured over time in response to increasing concentrations of SSP. (D) Area under the curve (AUC) of nuclear counts/mm2 and caspase object counts/mm2 over time were measured and used to calculate IC50 and EC50 values, respectively.

Kinetic measurement of extrinsic activation of apoptosis

Depending on the cellular context, exposure of cells to tumor necrosis factor alpha (TNF-α) can induce either pro-survival, or cell death pathways. When used in isolation, TNF-α induces NFκB activity and subsequent expression of pro-survival signaling molecules (e.g. FLIP, XIAP, A20). Alternatively, when used in conjunction with cycloheximide (CHX), itself an inhibitor of translation, TNF-α is a potent inducer of apoptosis through caspase 3 mediated signaling pathways. To demonstrate this using the IncuCyte live-cell analysis system, A549 epithelial carcinoma cells were treated with increasing concentrations of TNF-α in the presence of 5µg/mL CHX. Caspase-3/7 was activated in a TNF-α concentration dependent manner (Figure 3A). The AUC values for caspase-3/7 positive objects over time were then used to calculate the EC50 value of 0.676 nM TNF-α (Figure 3B).

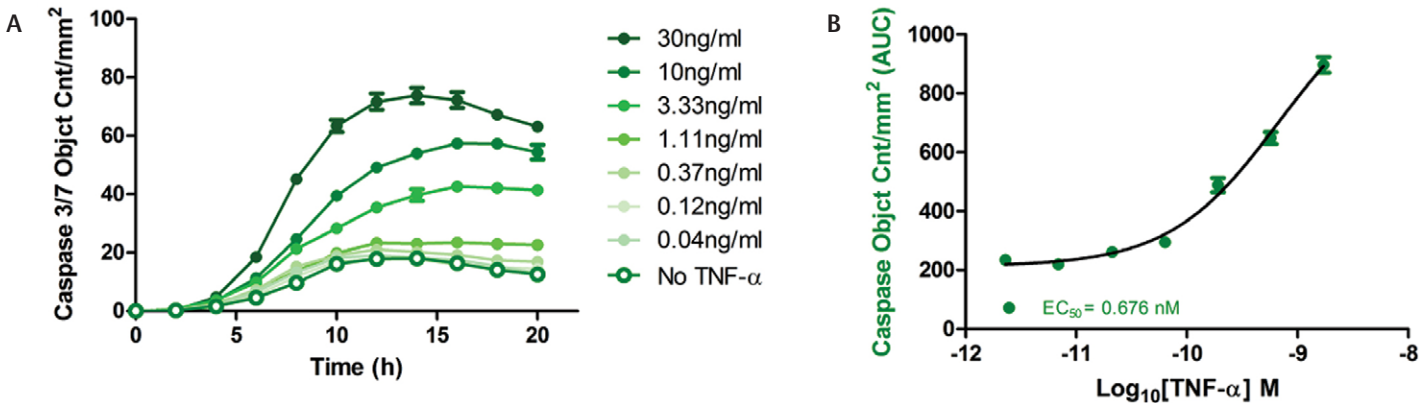

Figure 3: Extrinsic activation of caspase-3/7 in A549 lung epithelial cells. (A) Caspase-3/7 activation of A549 lung epithelial cells in response to varying concentrations of TNF-α in the presence of 5µg/mL cycloheximide (CHX). (B) The EC50 value of TNF-α was calculated by using the area under the curve (AUC) of caspase-3/7 objects/mm² over time.

Using images and movies to confirm signaling

One of the major advantages of live-cell analysis is the ability to verify the quantified kinetic data with both phase contrast and fluorescent images. Classical morphological changes associated with apoptosis include: cell shrinkage, membrane blebbing, nuclear condensation, and DNA fragmentation. The time lapse sequence presented in Figure 4 highlights this advantage, illustrating the ability to use phase contrast and fluorescent blended images to temporally correlate the activation of caspase-3/7 and the loss of red nuclei due to cell death with morphological changes in response to treatment with SSP. Using the IncuCyte system, the temporal responses in every well can be supplemented with a "movie" of either a phase contrast, fluorescence or blended time-lapse sequence. This ability significantly enhances the confidence in the measured response and any subsequent conclusions drawn from quantitative image analysis.

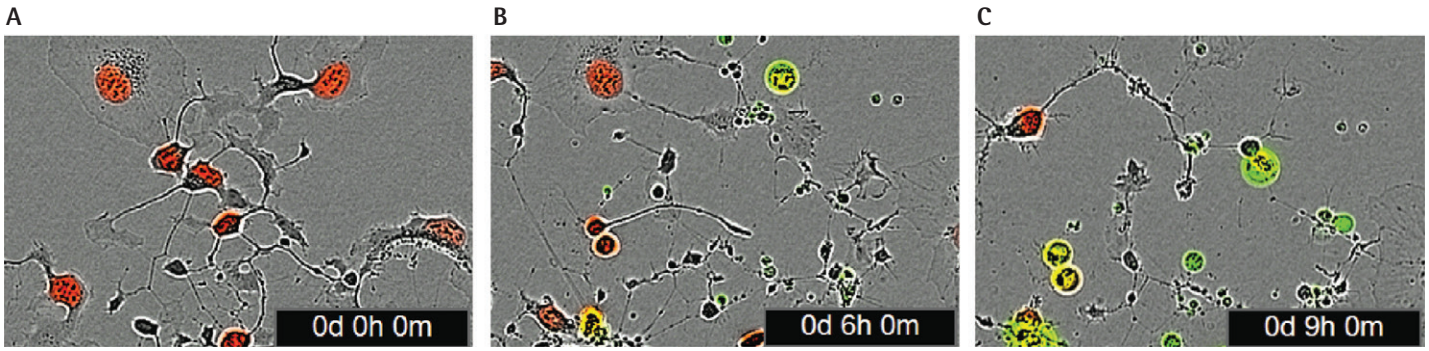

Figure 4: Time-lapse images and movies to detect SSP induced apoptosis in HT-1080 cells. HT-1080 NucLight Red cells were treated with 300nM SSP in the presence of 5µM Caspase-3/7 reagent and imaged in IncuCyte live-cell analysis system every 3 hours (A). Time-lapse images and movies monitor changes in morphology and confirm the activation of the green caspase-3/7 signal in (B) and the loss of the red nuclear signal (C).

Loss of phosphatidylserine asymmetry

Addition of the IncuCyte Annexin V reagent to normal healthy cells is non-perturbing to cell growth or morphology and yields little or no intrinsic fluorescent signal. Once cells become apoptotic, plasma membrane PS asymmetry is lost leading to exposure of PS to the extracellular surface and binding of the IncuCyte Annexin V reagent, yielding a bright and photostable fluorescent signal.

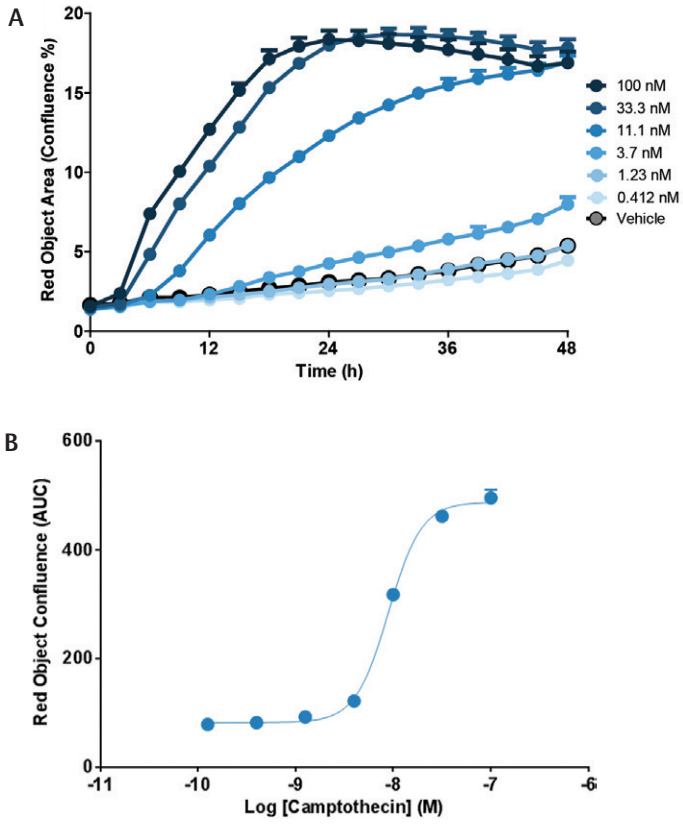

Figure 5: Concentration and time-dependent loss of PS asymmetry. Annexin V reagent was added to Jurkat human T-cell leukemia cells treated with the topoisomerase inhibitor camptothecin. (A) Time-course for the effects of camptothecin on Jurkat cell death (Red Object Confluence (%) presented as the mean ± SEM, n=3 wells). (B) Concentration response curve to camptothecin. Area under the curve (AUC) values have been determined from the time-course shown in panel A (0-36 hours) and are presented as the mean ± SEM, n=3 wells. Average AUC values were used to calculate pIC50 values (camptothecin pIC50 = 8.01).

Multiplex Assays to confirm apoptotic pathways

The IncuCyte system can be used to detect and confirm apoptosis through two different pathways by multiplexing caspase-3/7 and annexin V reagents to verify apoptosis as a mechanism of cell death.

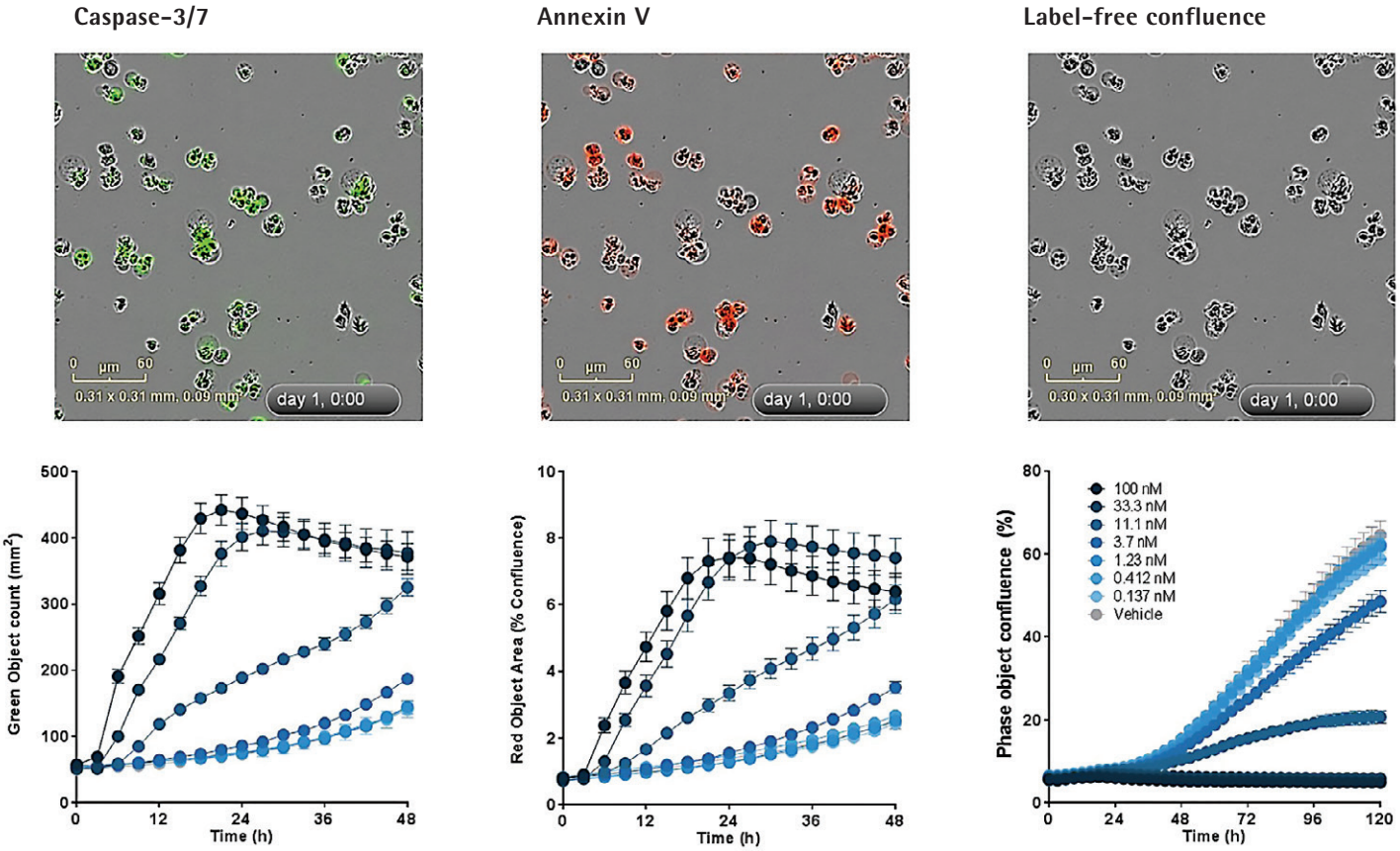

Figure 6: Detect both caspase-3/7 and annexin V signals in cultures. Automatically determine the time courses of apoptotic cell death and correlate with label free confluence measurements to provide an estimate of the proportion of apoptotic cells within the population (apoptotic index).

Assessment of immune cell killing of cancer cells using caspase signaling

Capsase-3/7 was used to evaluate targeted tumor cell death in a co-culture Antibody-Dependent Cell-mediated Cytotoxicity (ADCC) assay to measure tumor cell apoptosis and for visual validation of tumor-immune cell interactions. To demonstrate the utility of this approach, ADCC cell death was measured via Caspase-3/7 induction in Her2 positive SKOV3 or negative A549 NucLight Red cells (1.6K/well) co-cultured with with PBMCs (8K/well) in the presence of trastuzumab, a clinically used monoclonal antibody for Her-2 positive sold cancers.

A concentration-dependent decrease in proliferation (IC50 8.1 ng/mL) and increase in apoptosis (IC50 4.6 ng/mL) was measured in Her2-positive SKOV3 cells. No response was seen in Her2-negative A549 cells (data not shown). These data demonstrate that live cell imaging can be used to discern the full-time course and specificity of immune cell killing, which are traditionally conducted as flow cytometry or biochemical readouts, requiring selection of end points and lack visual confirmation of cellular interactions.

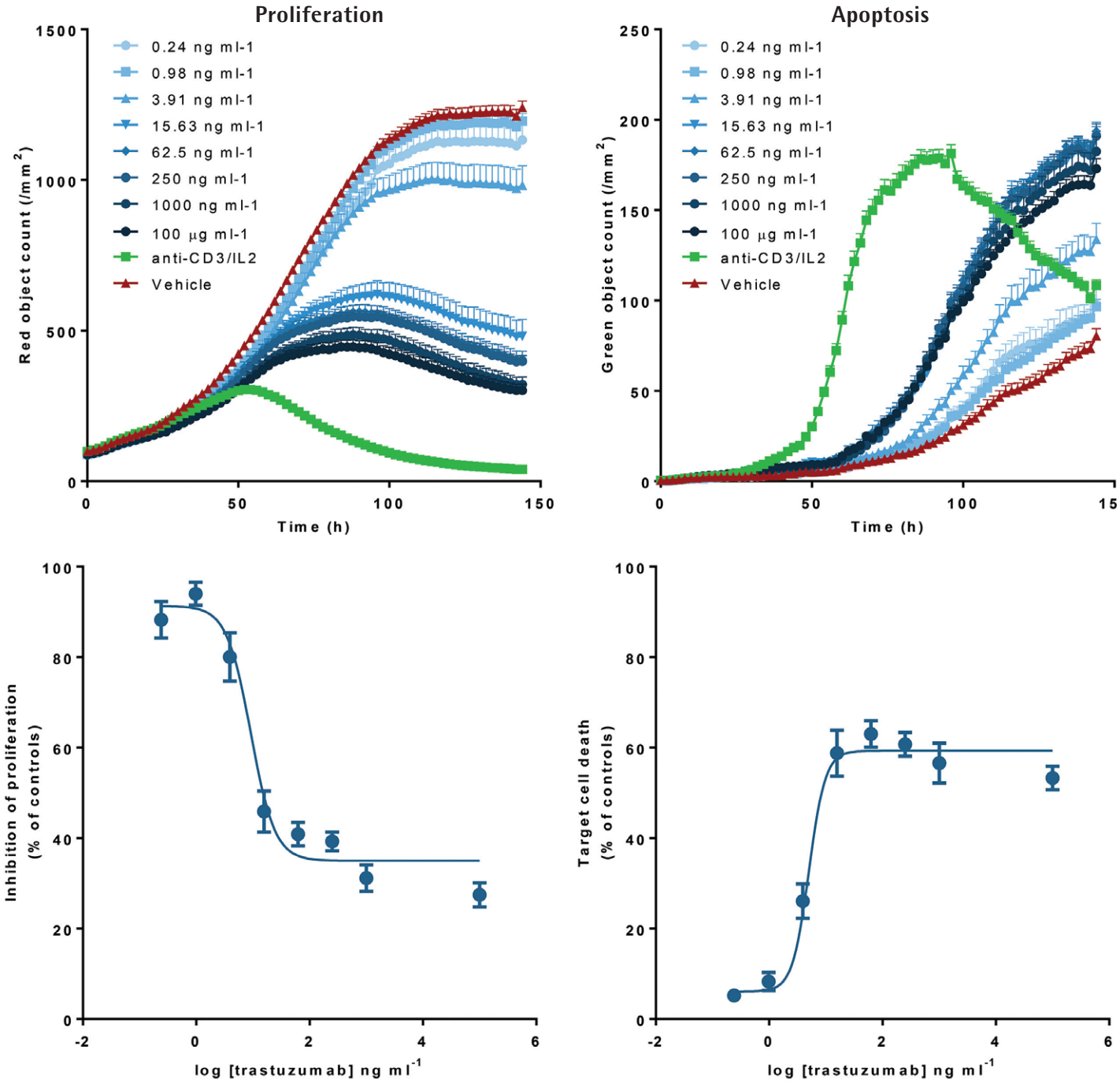

Figure 7. Trastuzumab induced ADCC in Her2 positive SKOV3 cells. SK-OV-3 cancer cells were seeded in combination with PBMCs in the presence of trastuzumab to induce ADCC. A concentration-dependent decrease in proliferation (IC50 8.1 ng/mL) and an increase in apoptosis (IC50 4.6 ng/mL) was seen.

Conclusions

Live-cell imaging and analysis is a powerful tool for the kinetic detection of apoptosis, demonstrating quantitative and reproducible data. This strategy of live-cell imaging and analysis gives the user the ability to monitor morphological changes in parallel with quantification, the combination of which is a powerful and unique tool for detecting pharmacological or genetic manipulations that alter cell health. In addition, NucLight reagents or cell lines, when used in conjunction with IncuCyte apoptosis reagents and the IncuCyte system, provide an additional parameter for measuring cytostatic (anti-proliferative) and apoptotic events. Together, these attributes provide new and unique assays for apoptotic pathway analysis for both drug discovery as well as basic cell biology research.

Key features of the apoptosis assays are:

- The irreversible "executioner" caspase-3/7 pathway or PS externalization can be kinetically monitored allowing for the detection of both short-term and long-term treatment effects.
- Non-perturbing IncuCyte Caspase-3/7 or Annexin V reagents are added as mix-and-read reagents directly to the cultured cells in complete growth media, removing the need for fluid aspiration steps, thus eliminating cell disruption or loss of impaired cells.
- All data points can be validated by individual images or time-lapse movies to confirm processing metrics, significantly enhancing the confidence in the measured response.
- Live-cell imaging of apoptosis can be used to discern the full-time course and specificity of co-culture models such as immune cell killing, with visual confirmation of cellular interactions.

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Kinetic Cytotoxicity Assays

Real-time, live-cell assay to quantify and visualize cytotoxic events

Introduction

The cellular response to cytotoxic exposure is controlled by complex biochemical pathways, such as necrosis or apoptosis, which results in cell death. In apoptosis, morphological changes include pseudopodia retraction, reduction of cellular volume (pyknosis), nuclear fragmentation (karyorrhexis) and eventually loss of plasma membrane integrity.[1] Morphological changes that characterize necrosis include cytoplasmic swelling and early rupture of plasma membrane.[2] Compounds that have cytotoxic effects often compromise cell membrane integrity regardless of the pathway.

Assays designed to measure cytotoxicity in vitro are used to predict tissue-specific toxicity or to identify and classify leads for anti-cancer therapies. Multiplexed, high-throughput screening (HTS) in vitro cytotoxicity assays measuring a variety of different readouts are being employed to assess the cytotoxicity of compounds in early drug development.[3] Commonly used cytotoxicity assays evaluate a range of end-point parameters, such as the release of lactate dehydrogenase (LDH) and glutathione (GSH) following membrane rupture, generation of reactive oxygen species (ROS), cell proliferation, and disruption of mitochondrial trans-membrane potential. Critical factors contributing to the predictive nature of these assays include compound concentration, and more importantly, the time allowed for the compound to elicit an effect.[4] Although these multiplexed assays are able to simultaneously measure multiple indicators of in vitro cytotoxicity, they typically assess a single time point and are unable to assess the biological activity over time.

In this chapter, we will examine kinetic approaches for measuring cytotoxicity using reagents that fluorescently stain the nuclear DNA of cells that have lost plasma membrane integrity. Unlike traditional endpoint approaches, kinetic live-cell imaging allows for the analysis of time-dependent variation in treatment response as well as the ability to differentiate between cytostatic and cytotoxic effects.

IncuCyte® Cytotoxicity Assay at a Glance

The IncuCyte Cytotoxicity assay uses highly sensitive cyanine nucelic acid dyes ideally suited for mix-and-read kinetic measurements of cell membrane integrity overtime. IncuCyte® CytoTox Red and Green Reagents are cell impermeant cyanine dimer nucleic acid stains that bind to dsDNA,5 and when added to the culture medium, these reagents fluorescently stain the nuclear DNA of cells that have lost plasma membrane integrity.

This cytotoxicity assay can be combined with IncuCyte® NucLight cell labeling reagents that incorporate a red or green nuclear label allowing for simultaneous measurement of proliferation and cytotoxicity in a single well. Non-perturbing NucLight reagents provide a means to kinetically quantify cell proliferation over time. The reagents allow for the expression of a nuclear-restricted GFP (green fluorescent protein) or mKate2 (red fluorescent protein) in mammalian cells without altering cell function and with minimal toxicity. NucLight lentivirus reagents allow for the creation of stable cell populations or clones.

Phase-contrast images can be used to qualitatively monitor associated morphological changes in the same cells over the same time course.

Shortcomings of Traditional Assays	Live-Cell Imaging and Analysis Approaches
• Assays result in a **single**, user-defined time point measurement.	• The assay provides a **full kinetic readout** of cytotoxicity over multiple days, eliminating the need for determining a single, optimum, assay endpoint a-priori which can vary considerably for different cell types and for different compound treatment conditions.
• **Lack of ability to assess biological activity over time** limits predictive nature.	• Addition of IncuCyte CytoTox reagents to normal, healthy cells are **nonperturbing to cell growth and morphology.**
• Manipulations can result in the **loss of cells or critical data in experiments** where cells undergo cell death at different rates according to treatment conditions.	• Cells can be **simultaneously labeled with an IncuCyte CytoTox reagent and NucLight nuclear labeling reagent to measure cytotoxicity and cell proliferation.**
	• 96- and 384-well assay format follows a homogeneous "mix and read" protocol which can be run over multiple days in full media. No wash or lifting steps required, negating the concern that cells are lost during the experiment or labeling process.
	• All data points and temporal data curves can be validated by individual images or time-lapse movies respectively. The kinetic readout of the IncuCyte® system provides both high definition (HD) phase as well as quantitative fluorescent imaging.

Sample Results

Quantitative measurement of cytotoxicity using IncuCyte CytoTox reagent

Staurosporine (SSP) and camptothecin (CMP) are compounds that are known to cause cell death due to cytotoxicity. SSP is a high affinity, non-selective, ATP-competitive kinase inhibitor and is classically used as a research tool to induce caspase-3 mediated apoptosis.[6, 7] CMP causes cell death by inhibition of the DNA enzyme, topoisomerase I (topo I), resulting in double strand breaks during S-phase and triggering the apoptotic program.[8] These two compounds were used to illustrate the ability of the cell impermeant DNA dye based cytotoxicity assay to measure cell death over-time using two different cell lines, HT 1080 (human tumor derived fibrosarcoma) and MDA-MB-231 (human tumor derived breast adenocarcinoma. In addition, we also measured the response of these same two cell types to the protein synthesis inhibitor, cycloheximide (CHX), a cytostatic compound which was predicted to inhibit cell proliferation while not affecting cell viability (Figure 1).

A 7-point concentration curve of each compound clearly illustrated that in both cell types, SSP and CMP induced a concentration-dependent cytotoxic response. Specifically, we observed a statistical induction of cytotoxicity in MDA-MB-231 cells at 16 and 26 hours for SSP and CMP treatments, respectively (Figure 1A). In identically treated HT 1080 cells, there was a more rapid induction of the cytotoxic responses correlating to 12 and 22 hours for SSP and CMP treatments, respectively, which illustrates a slight cell type dependent difference (Figure 1B).

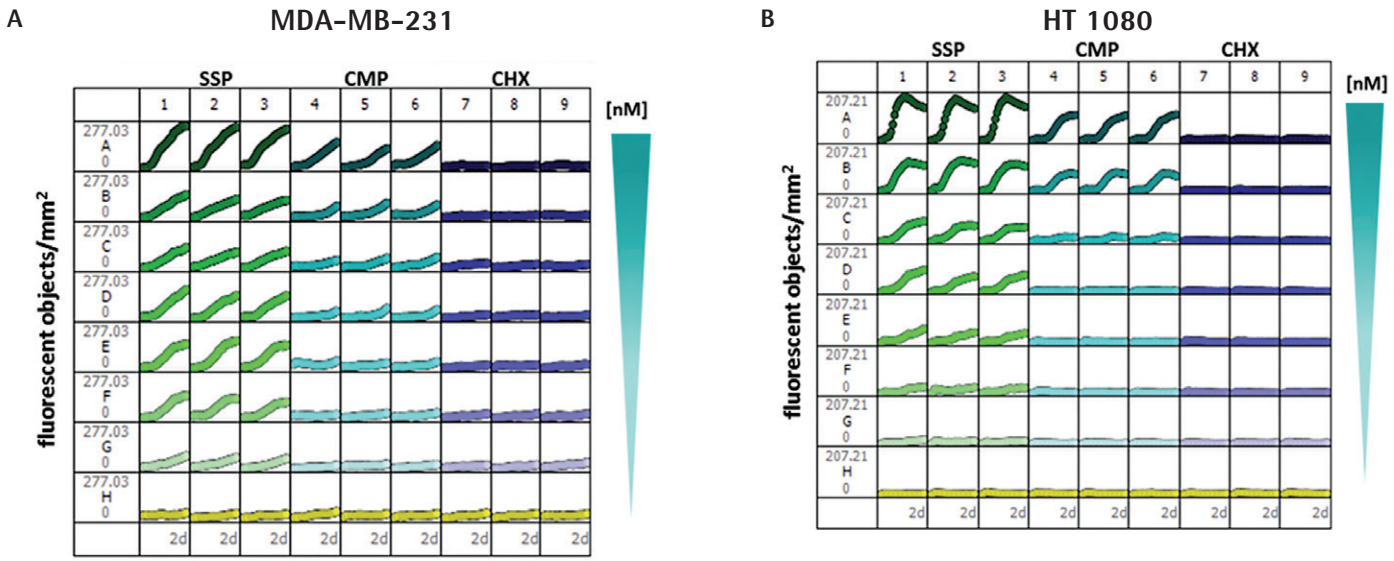


Figure 1. Discrimination of cytotoxic and cytostatic compounds. 96-well microplate graph showing the kinetic measurement of cell death as determined by CytoTox Green staining in response to several concentrations of SSP, CMP, and CHX in MDA-MB-231 cells (A) and HT 1080 cells (B).

In contrast, no statistical induction of cytotoxicity was observed when either cell type was treated with any of the tested concentrations of CHX (Figure 1A, B). However, a clear concentration-dependent inhibition of cell proliferation was observed as measured by the NucLight Red fluorescent signal (Figure 2). Moreover, end point normalization, which corrects for differences in proliferation within treatment groups, revealed a concentration-dependent cytotoxic index for both SSP and CMP in MDA-MB-231 and HT-1080 cells, whereas no cytotoxic responses was induced by treatment with CHX (Figure 3).

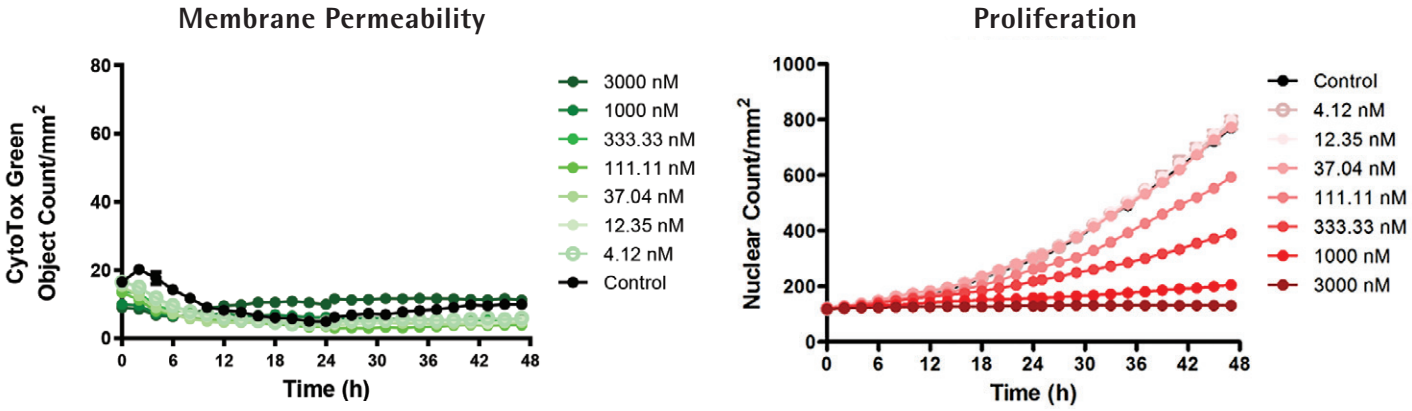


Figure 2. Cytostatic effect of cycloheximide (CHX). NucLight Red HT-1080 cells were treated with several concentrations of cycloheximide in the presence of CytoTox Green. Graphs illustrate no induction of cytotoxicity as measured by green fluorescence staining of DNA (A) however, inhibition of cell proliferation (B) as measured by fluorescent nuclear counts is observed. Cell morphology did not significantly differ from untreated cells as illustrated in Figure 5. Each data point represents the mean ± SE in N=3 wells.

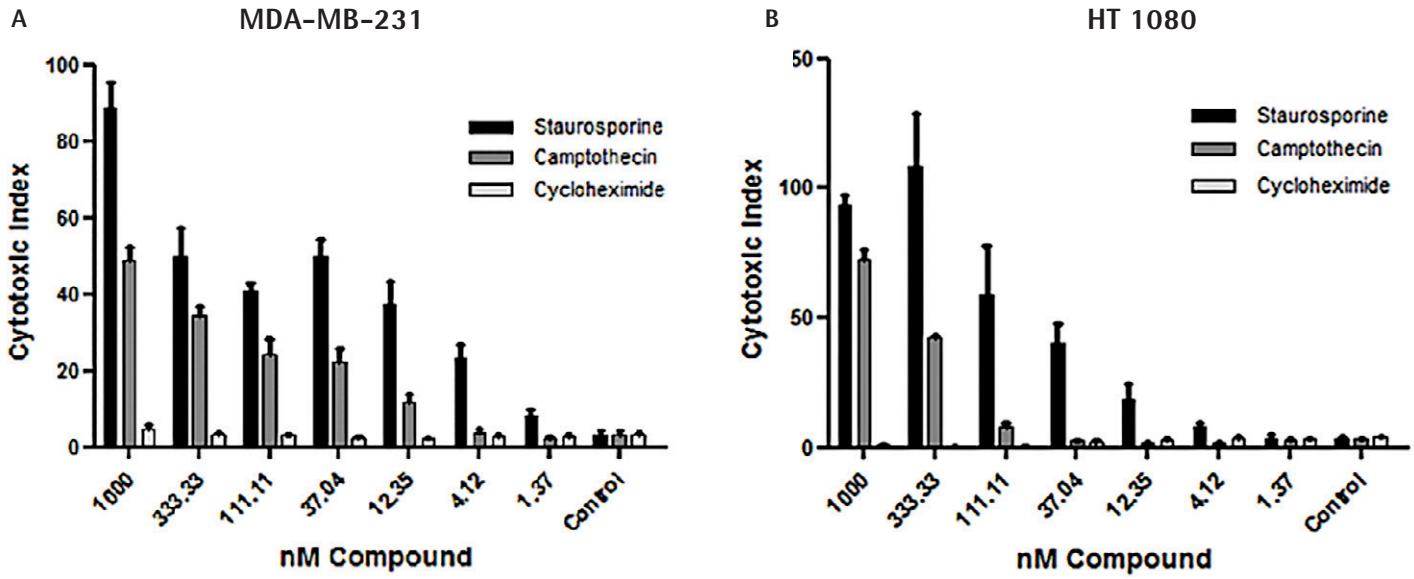


Figure 3. End point normalization of cytotoxic and cytostatic compounds. At the 72-hour end point, Triton X-100 at a final concentration of 0.0625% was added to allow nuclear dsDNA staining by CytoTox Green of all cells present/well. The cytotoxic index was calculated by dividing the number of CytoTox Green fluorescent objects by the total number of DNA containing objects (fluorescent objects counted post Triton X-100 treatment).

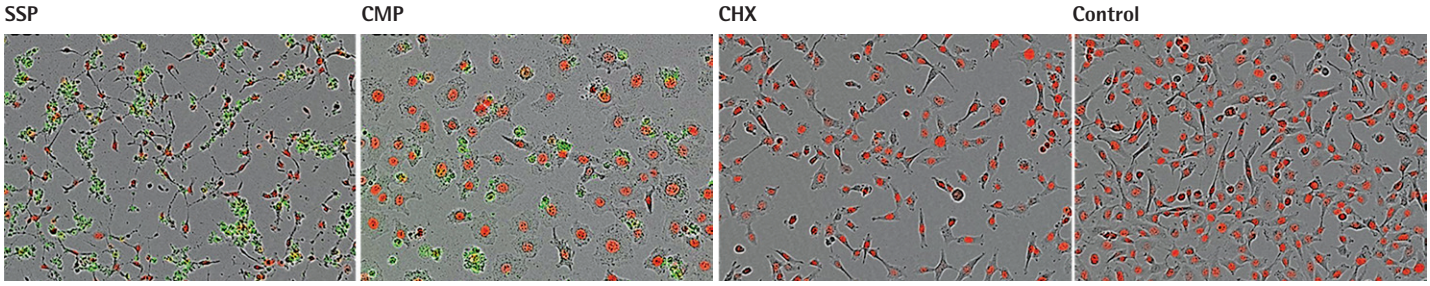


Figure 4. Morphological images. Phase-contrast and fluorescent images of NucLight Red HT-1080 cells 24hrs post-treatment, showing morphological changes in response to SSP, CMP and CHX.

The IncuCyte® system acquired phase-contrast and fluorescent images of HT-1080 cells, under all three compound treatments, confirmed fluorescent object count data as illustrated in Figure 4 (similar results were observed with MDA-MB-231 cells). These data show the potential of this kinetic and morphological approach to the screening, prioritization, and classification of compounds in drug discovery.

In order to evaluate the accuracy and reproducibility a series of experiments using MDA-MB-231 and HT-1080 cell lines and two known cytotoxic compounds (SSP and CMP) were used.

To assess intra-assay reproducibility, each well of a 96-well plate was seeded with 5,000 HT-1080 or MDA-MB-231 cells. Each row of cells was treated with 2-fold decreasing concentrations of CMP (2000 nM to 62.5 nM; n=12 wells per condition) in the presence of CytoTox Green and kinetic measurements of fluorescent objects were analyzed. As illustrated in the microplate graph view in Figure 5A, we obtained a highly reproducible kinetic measure of cell viability. In addition, using end point data, we were able to demonstrate an inverse relationship between total DNA object count and cytotoxic index (Figure 5B, C) in both cell lines analyzed.

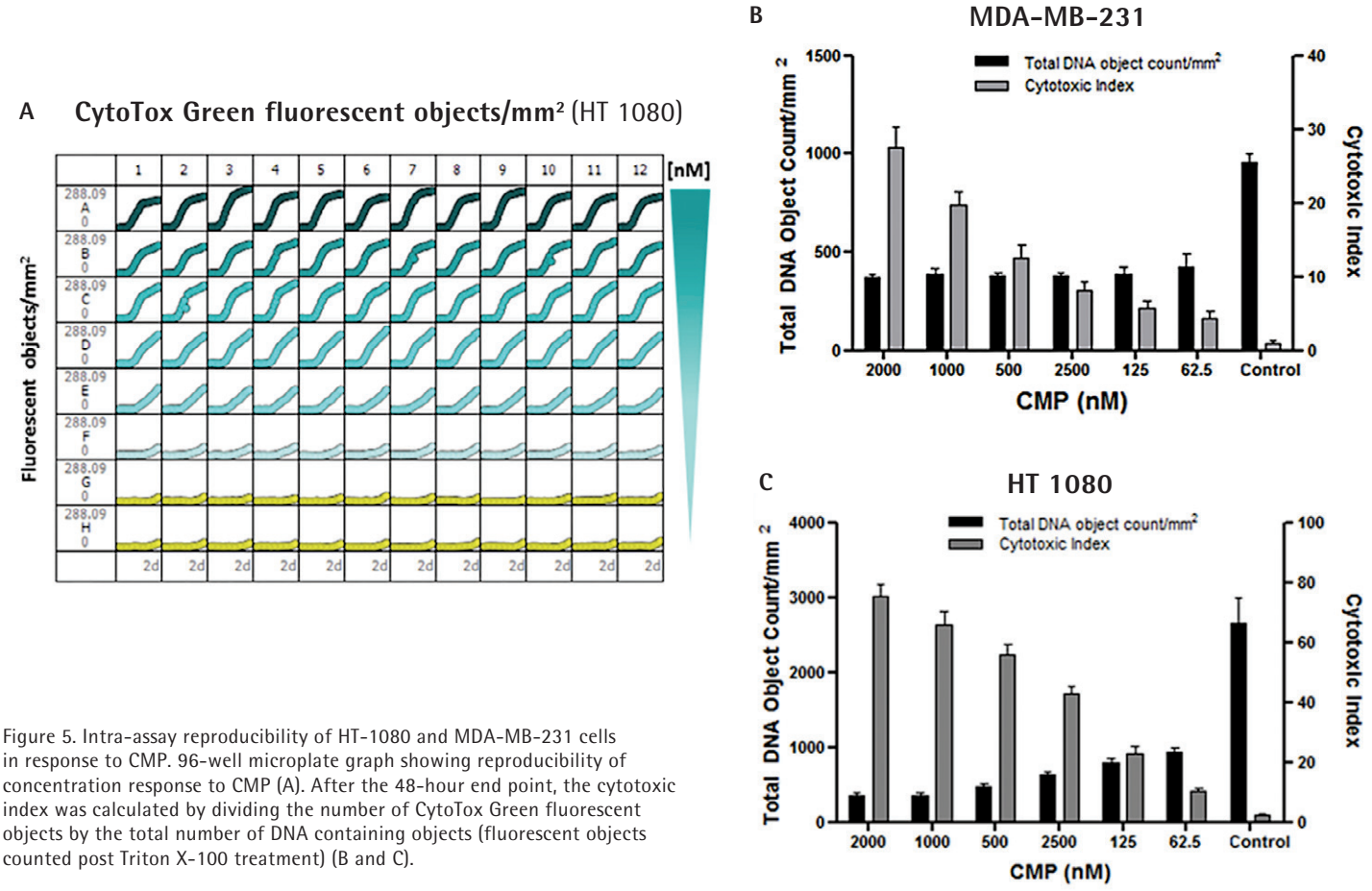


Figure 5. Intra-assay reproducibility of HT-1080 and MDA-MB-231 cells in response to CMP. 96-well microplate graph showing reproducibility of concentration response to CMP (A). After the 48-hour end point, the cytotoxic index was calculated by dividing the number of CytoTox Green fluorescent objects by the total number of DNA containing objects (fluorescent objects counted post Triton X-100 treatment) (B and C).

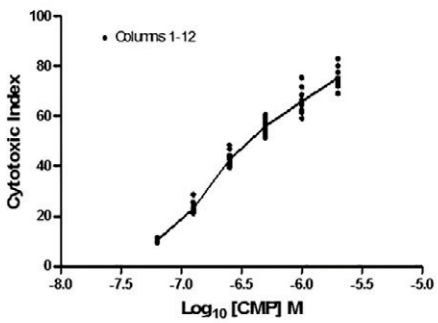
A

[CMP]	Cytotoxic Index (Mean)	SD	
2000 nm	75.280	3.805	z'=0.82
1000 nm	65.895	4.397	
500 nm	55.943	3.293	
250 nm	42.595	2.846	
125 nm	23.063	2.265	
62.5 nm	10.441	0.773	
Control	2.641	0.657	

B

[CMP]	Cytotoxic Index (Mean)	SD	
2000 nm	27.443	2.941	z'=0.64
1000 nm	19.829	1.768	
500 nm	12.636	1.826	
250 nm	8.080	1.234	
125 nm	5.690	1.102	
62.5 nm	4.338	1.078	
Control	0.984	0.264	

C



Column	pEC50
1	7.078
2	6.681
3	6.578
4	6.741
5	6.61
6	6.747
7	6.738
8	6.817
9	6.594
10	6.791
11	6.621
12	6.651
MEAN pEC50	6.721
MEAN 95% CI	6.501 to 6.933

Geometric Mean (antilog converted)

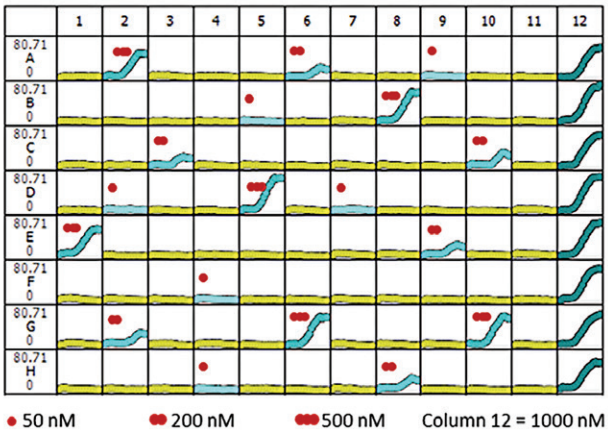
EC50	190.4 nM
95% CI	116.7 to 315.5 nM

Figure 6. Statistical analysis of intra-assay reproducibility. Calculated cytotoxic index of HT-1080 (A) and MD-MB-231 (B) cells to decreasing concentrations of CMP. (C) EC50 values determined from HT-1080 plate described in Figure 5A.

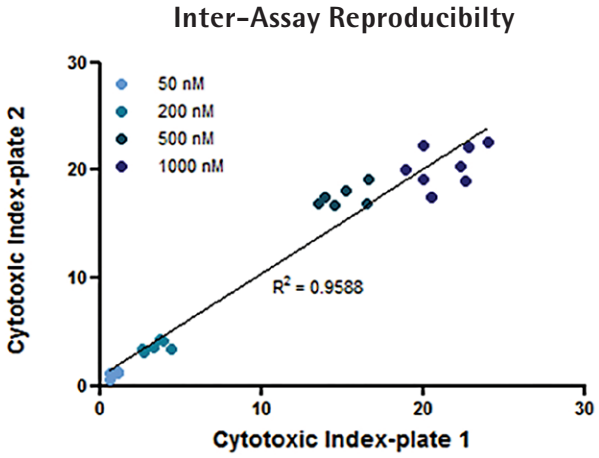
Assay Z' factors of 0.82 (HT 1080) and 0.64 (MD-MB-231) were calculated, indicating that this assay platform is amenable to screening protocols (Figure 6A, B). In addition, using the end point cytotoxic index from the HT-1080 data, we were able to calculate remarkably consistent pEC50 values from each column of the microplate with a total geometric mean of 198 nM (Figure 6C).

In order to determine inter-assay reproducibility and accuracy, HT-1080 cells were seeded in two 96-well plates at a density of 5,000 cells/well. Random wells of each plate were spiked with independently prepared stocks of CMP at 1000, 500, 200 and 50 nM (n=6 per concentration), as illustrated in Figure 7A. The data from both plates were then plotted on different axes and analyzed using linear regression (Figure 7B). The resulting R^2 value of 0.9588 demonstrates a strong correlation between identically treated wells on separate plates. Additionally, the Z' factors of 0.72 and 0.62 are again indicative of a high quality assay (Figure 7C).

A



B



C

Plate 1 : N = 6 per treatment

CMP	Cytotoxic Index (Mean)	SD	
50 nM	1.09	0.238	z' = 0.72
200 nM	3.65	0.514	
500 nM	17.57	0.936	
1000 nM	20.37	1.829	

Plate 2 : N = 6 per treatment

CMP	Cytotoxic Index (Mean)	SD	
50 nM	0.84	0.224	z' = 0.62
200 nM	3.48	0.703	
500 nM	15.06	1.309	
1000 nM	21.45	1.772	

Figure 7. Inter-assay reproducibility of HT-1080 response to CMP. At the 72-hour end point, Triton X-100 at a final concentration of 0.0625% was added to allow nuclear dsDNA staining by CytoTox Green of all cells present/well. The cytotoxic index was calculated by dividing the number of CytoTox Green fluorescent objects by the total number of DNA containing objects (fluorescent objects counted post Triton X-100 treatment). (A) A 96-well microplate graph showing reproducibility of singe-well responses to various concentrations of CMP on HT-1080 cells. (B) Replicate plates of HT-1080 were spiked with identical concentrations of CMP and results were graphed to show correlation. (C) Statistical measurements from the same plates showing Z' factors exceeding 0.60.

Differentiating cytotoxic and cytostatic treatments

A multiplexed assay capable of measuring cytotoxicity in addition to cell proliferation was created. HT-1080 NucLight Red cells were seeded at 5,000 cells/well and treated with serially diluted concentrations of SSP, CMP or CHX in the presence of 0.1 µM CytoTox Green (all data not shown). The IncuCyte system was used to mask the green fluorescent nuclear signal to quantitate cell death as well as the red fluorescent nuclear signal to monitor cell proliferation kinetic dose response curves for both CytoTox Green positive events as well as nuclear counts of NucLight Red HT-1080 cells were exported to GraphPad Prism.

Statistical analysis of the area under the curve (AUC) was calculated for time points within the kinetic curves. Replicate AUC values, at peak response, were used to calculate EC50 and IC50 values. Figure 8 shows the inverse relationship between cell proliferation (nuclear count) and membrane permeability (CytoTox Green positive objects) over time in the presence of SSP. The AUCs were then used to statistically determine at which concentration SSP exhibited purely cytostatic effects. The concentration at which the AUC of CytoTox Green objects/mm^2 over time was different from the control group was termed cytotoxic and/or cytostatic. Concentrations where the AUC of CytoTox Green objects/mm^2 over time was not different from that of the control, but the AUC of nuclear objects/mm^2 over time was significantly different from the control was termed purely cytostatic. These data show the potential of this kinetic and multi-parametric approach to the classification of compounds in drug discovery.

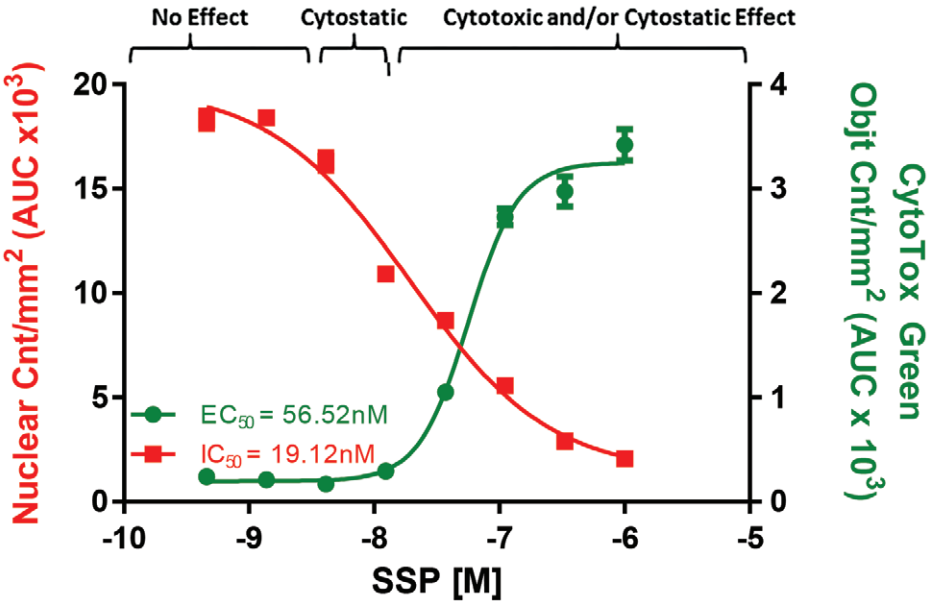


Figure 8. Inter-assay reproducibility of HT-1080 response to CMP. HT-1080 cells were treated with varying concentrations SSP. Dual fluorescent images were used to calculate the area under the curve (AUC) of cell death (CytoTox Green Object Cnt/mm2) and cell proliferation (Nuclear Cnt/mm2) over time. Average AUC values were then used to calculate EC50 and IC50 values, respectively. Dunnett's Multiple Comparison Test was used to compare the differences between AUCs at each concentration.

Conclusions

Using the IncuCyte system in conjunction with the IncuCyte CytoTox Green or Red reagents as a live-cell, kinetic assay for the measurement of cytotoxicity has demonstrated quantitative and reproducible detection of cell permeability, a hallmark of cell death. This strategy also gives the user the ability to monitor morphological changes in parallel with quantification, the combination of which is a powerful and unique tool for detecting pharmacological or genetic manipulations that alter cell viability. In addition, NucLight reagents or cells lines, when used with IncuCyte CytoTox Green or Red reagents and the IncuCyte live-cell analysis system, provide an additional parameter for measuring cytostatic (anti-proliferative) and cytotoxic events.

Key features of the cytotoxicity assay are:

- Non-perturbing cytotoxicity reagents are added directly to the cultured cells, removing the need for fluid aspiration steps, thus eliminating cell disruption or loss of impaired cells.
- Data generated using live-cell multi-parametric analysis of cytotoxicity and proliferation delivers more reliable data and allows for differentiation between cytosatic and cytotoxic effects of treatments as well as detection of both short-term and long-term alterations.
- All data points can be validated by individual images or time-lapse movies to confirm processing metrics, significantly enhancing the confidence in the measured response.

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Kinetic Cell Migration and Invasion Assays

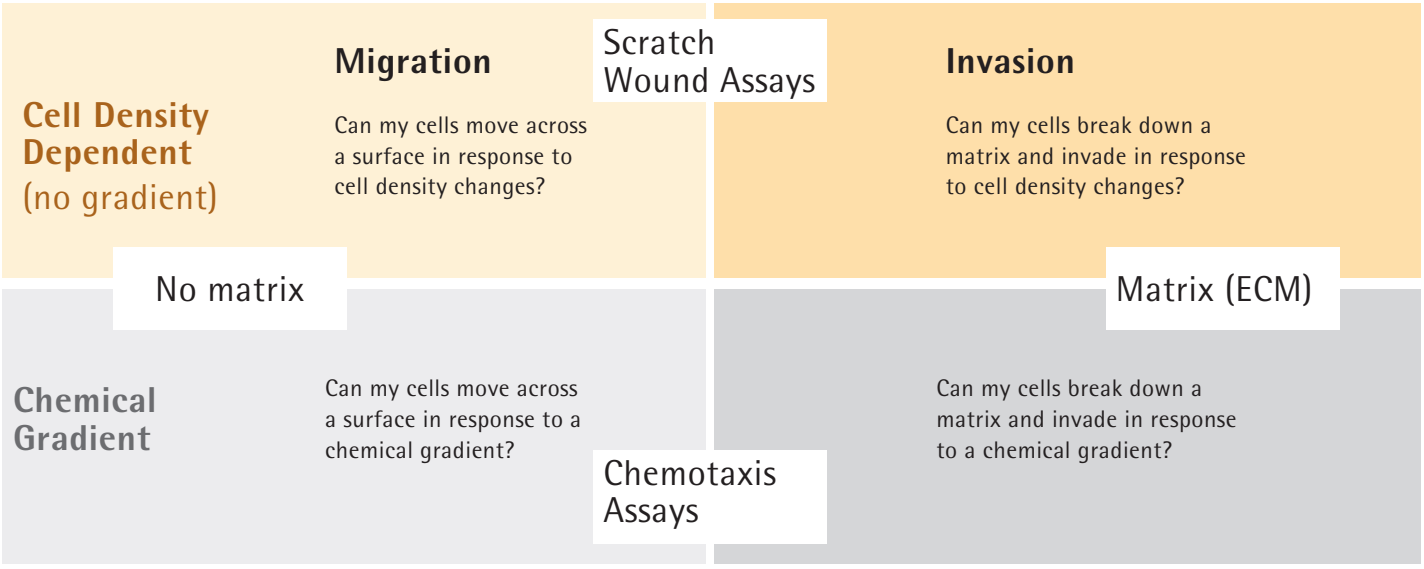
Real-time automated measurements of cell motility

Cell migration and invasion play a role in many normal and pathological processes including immune responses, embryonic development, angiogenesis, regeneration, tumor metastasis, and wound healing.[1] A wide variety of assays can be used to monitor and analyze cell migration and invasion *in vitro*, from very simple assays to very complex. The scratch assay permits velocity measurements of wound closure and is dependent upon a cell density gradient, whereas methods to study chemical factors that regulate migration and invasion of cells typically employ modifications of Boyden chamber assays. Scratch assays are typically performed by manually creating scratches in multiple well plates (e.g., 6-, 12-, 24-or 48-well) and then acquiring images using a microscope at defined time-points. This approach leads to variation in scratch uniformity, as well as disrupting the assay environmental control during image acquisition. Current chemotaxis assay approaches have inherent drawbacks such as non-linear gradients, inability to observe cell morphology and validate cell movement, are technically challenging and have cumbersome quantification steps. In order to drive development of novel therapeutics, a deeper understanding of these processes is required to gain valuable insights.

IncuCyte® live-cell migration and invasion assays allow continuous visualization and analysis of cell motility and kinetics. This approach addresses the inherent shortcomings of traditional assays that deliver a user-defined time points or a single end-point measurement and don't allow visualization of cells as they move in response to their environment. The IncuCyte® Scratch Wound Assay is a method that utilizes the IncuCyte® WoundMaker tool to create precise scratches within a cell monolayer, eliminating assay variability that is inherent in "home-brew" methods.[2] The IncuCyte® Chemotaxis Assay is a kinetic and visual chemotactic driven method, that alleviates hurdles such as laborious end point measurements, high cell usage, and non-linear gradient that is common in traditional transmembrane assays. These image-based detection methods offer simple and reliable assays which deliver new biological insights into cell migration and invasion.

Live-cell imaging and analysis approaches

The IncuCyte cell migration and invasion assays enable the detection and analysis of live-cell movement in 96-well formats. IncuCyte Scratch Wound and ClearView Chemotaxis assays allow you to kinetically monitor and analyze migration and invasion with or without a chemotactic gradient, and associated images allow for the ability to observe cell morphology.



How Live-Cell Migration and Invasion Assays Work

IncuCyte® Scratch Wound Assay

The IncuCyte Scratch Wound Assay is a 96-well, high throughput wound assay that measures cell density-dependent migration and invasion. Utilizing the IncuCyte® WoundMaker, 96 precise, uniform, cell-free zones are created in cell monolayers and wound closure is visualized and analyzed in real-time with the IncuCyte® live-cell analysis system and software.

IncuCyte® Chemotaxis Assay

The IncuCyte Chemotaxis Assay enables real-time visualization and quantification of cell migration and invasion in response to a chemotactic gradient. Using an optically clear membrane insert that contains optimally spaced 8-micron pores, cell migration and invasion are automatically imaged and analyzed as the cells move through the pores, generating a chemotactic signal.

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Recent IncuCyte® Publications

Scratch wound assay

Drebert, et. al., used the IncuCyte scratch wound assay to assess HUVEC migration in response to conditioned medium from solvent- (CMS) and dexamethasone (Dex)-treated (CMD) colon cancer-derived myofibroblasts. In the HUVEC scratch assay CMS-induced acceleration of wound healing was blunted by CMD treatment.

Colon cancer-derived myofibroblasts increase endothelial cell migration by glucocorticoid-sensitive secretion of a pro-migratory factor. Drebert, Z., MacAskill, M., Doughty-Shenton, D., De Bosscher, K., Bracke, M., Hadoke, P. W., Beck, *I. M. Vascul. Pharmacol., Vascul Pharmacol.* 2016 Oct 4. pii: S1537-1891(16)30137-9. doi: 10.1016/j.vph.2016.10.004. [Epub ahead of print], 2017.

Härmä, et. al., cultured prostate cancer cells on ImageLock plates from Essen BioScience until fully confluent and scratched with the WoundMaker instrument. Wound closure was monitored and quantified with the IncuCyte® system. Study results suggest that some betulin-derivatives may specifically target cell motility and invasion by affecting the organization of filamentous actin fiber network at low nanomolar concentrations.

Optimization of invasion-specific effects of betulin derivatives on prostate cancer cells through lead development. Härmä, V., Haavikko, R., Virtanen, J., Ahonen, I., Schukov, H. P., Alakurtti, S., Purev, E., Rischer, H., Yli-Kauhaluoma, J., Moreira, V. M., Nees, M., Oksman-Caldentey, K. M. *PLoS ONE* 05/2015 10(5):e0126111.

Moody, et. al. conducted scratch wound assays using the IncuCyte platform to assess the functional consequence of growth arrest specific 6 (Gas6) neutralization in lung cancer cells. Gas6 is a vitamin-K dependent, 75 kDa growth factor-like protein involved in the regulation of a wide array of cellular activities, including adhesion, migration, mitogenesis, aggregation, stress-response, differentiation, immune activation, efferocytosis and apoptosis. A Gas6 neutralizing antibody was also tested for its ability to inhibit Gas6-induced proliferation using the same platform.

Antibody-mediated neutralization of autocrine Gas6 inhibits the growth of pancreatic ductal adenocarcinoma tumors in vivo. Moody, G., Belmontes, B., Masterman, S., Wang, W., King, C., Murawsky, C., Tsurda, T., Liu, S., Radinsky, R., Beltran, P. *Int J of Cancer.* 2016 139:1340.

This review article explores how advances in time-resolved imaging contributes to the characterization of distinct modes of invasion and molecular mechanisms. The author highlights the latest advances in kinetic imaging instrumentation applicable to in vitro and in vivo models of tumor invasion.

Profiling distinct mechanisms of tumor invasion for drug discovery: imaging adhesion, signalling and matrix turnover. Carragher, N. *Clinical & Exp Metastasis.* 2009 April 26(4).

Chemotaxis assay

Taylor, et.al., used the IncuCyte® chemotaxis assay to study netrin-1, a cellular guidance signal. The authors demonstrated that netrin-1 inhibits human monocyte and murine macrophage migration towards C5a, a complement peptide with potent chemotactic and anaphylatoxic properties that upregulate endothelial adhesion molecules, increase vascular permeability, and localize leukocytes and inflammatory molecules at sites of infection.

Netrin-1 reduces monocyte and macrophage chemotaxis towards the complement component C5a. Taylor, L., Brodermann, M. H.,McCaffary, D., Iqbal, A. J., Greaves, D. R. *PLoS ONE*, 2016 Aug 10; 11(8).

A study by Pasqualon, et. al., demonstrated that syndecan-1, a surface-expressed proteoglycan on epithelial tumor cells, promotes macrophage migration inhibitory factor (MIF) binding and MIF-mediated cell migration. Invasion studies showed that MIF induced the chemotactic migration of A549 tumor cells, wound closure and invasion into Matrigel without affecting cell proliferation. These MIF-induced responses were abrogated by silencing of syndecan-1. The authors conclude that this may represent a relevant mechanism through which MIF enhances tumor cell motility and metastasis.

Cell surface syndecan-1 contributes to binding and function of macrophage migration inhibitory factor (MIF) on epithelial tumor cells. Pasqualon, T., Lue, H., Groening, S., Pruessmeyer, J., Jahr, H., Denecke, B., Bernhagen, J., Ludwig,A. BBA-Mol Cell Res. 2016 Apr; 1864 (4).

Orphan G protein-coupled receptor GPRC5A modulates integrin β1-mediated epithelial cell adhesion. Bulanova, D; Akimov, Y; Rokka, A; Laajala, T; Aittokallio, T; Kouvonen, P; Pellinen, T; Kuznetsov, S. Cell Adhesion & Migration. 2016 Oct.

Potent EMT and CSC phenotypes are induced by oncostatin-M in pancreatic cancer. Smigiel, J; Parameswaran, N; Jackson, MMol Can Res. 2017 Jan.

Kinetic Scratch Wound Assays

Real-time assays to assess migration and invasion potential

Introduction

Cell migration is a multistep process that is a fundamental component of many biological and pathological processes such as embryonic development, tissue re-organization, angiogenesis, immune cell trafficking, chronic inflammation and tumor metastasis. Cell migration is initiated by a stimulus that activates a set of signaling pathways leading to cellular polarization and a rapid reorganization of actin filaments and microtubules. Cells advance by protruding their membrane at their leading cell border, which is followed by dynamic substrate adhesion via integrin adherence to the substrate. Membrane retraction at the lagging cell edge finishes the cycle, which is then repeated in rapid succession. The summation of this process results in cell migration.

In this chapter, we will review real-time visualization and analysis of cell movement that is driven by changes in cell density. Unlike traditional assays, which require repeatedly removing cells from an incubator to perform manual image acquisition and have variability in scratch width and uniformity, this live-cell assay approach provides continuous analysis and eliminates assay variability with the use of the WoundMaker tool to create precise scratches in the cell monolayer.

IncuCyte® Scratch Wound Assay at a Glance

The IncuCyte® scratch wound migration and invasion assay can be used to quantitatively assess both cellular migration and cell invasion potential in the presence of experimental agents. The 96-well IncuCyte® WoundMaker creates cell-free zones in cell monolayers cultured on IncuCyte® ImageLock 96-well plates with the use of precision engineered pins, without disrupting the underlying biomatrix or damaging the tissue culture treated plastic. After wounding, the scratch wound assay is initiated by simply adding media to the cells for the migration assay or by overlaying the cells with an optimized concentration of a biomatrix material for the invasion assay. IncuCyte® scratch wound analysis software enables real time, automated measurement of label-free or dual fluorescence of cell migration and invasion.

- Visualize cell migration and invasion, and assess morphological changes in real time with the scratch wound method.
- Assays are flexible, quantitative and easily reproducible with the IncuCyte WoundMaker tool.
- Monitor and quantify cell movements across a substrate (migration) or through a gel matrix (invasion) in the same plate, up to six 96-well plates at once.
- Validated with a wide range of adherent primary, immortalized and tumor cell types.

Shortcomings of Traditional Assays	Live-Cell Imaging and Analysis Approaches
• Assays result in a **single, user-defined time point measurement or requires manual intervention to generate multiple time points.**	• **Real-time, direct visualization** and automated analysis of migration and invasion.
• Lack of precision and reproducibility **in creating the wound and capturing data.**	• 96-well ImageLock plates **enable precise, scan to scan repeat imaging of the same field of view.**
	• Reproducible **kinetic data supported by images and time-lapse movies.**
	• **Spatio-temporal, label-free format** provides data on both the rate and the extent of migration and invasion for a given set of experimental variables.
	• Explore **time-dependent pharmacology**, in order to enhance assay sensitivity.
	• Measure migration **without fixing or staining steps.**

Sample Results

Assessing wound closure rates and morphology

The differences in wound closure rates and cell morphology between migrating and invading HT-1080 cells are shown in Figure 1. Migrating HT-1080 cells (odd numbered columns) closed the wound region at a significantly faster rate with complete wound closure detected by 10-12 hours post wounding. In contrast, HT-1080 cells invading 8 mg/ml Matrigel® reached 80% wound closure within 48 hours as measured by the Relative Wound Density metric. Morphological differences were also noted. Migrating cells maintained a fibroblastic morphology, had rounded lamellipodia, and advanced as a uniform population of cells. Invading HT-1080 cells adopted a mesenchymal phenotype displaying extended cell bodies and, in some cases, "spike-like" lamellipodia as the cells advanced into the Matrigel matrix in an irregular manner. The clear morphological differences between migrating and invading HT-1080 cells can be used to select the optimum condition for the invasion assay.

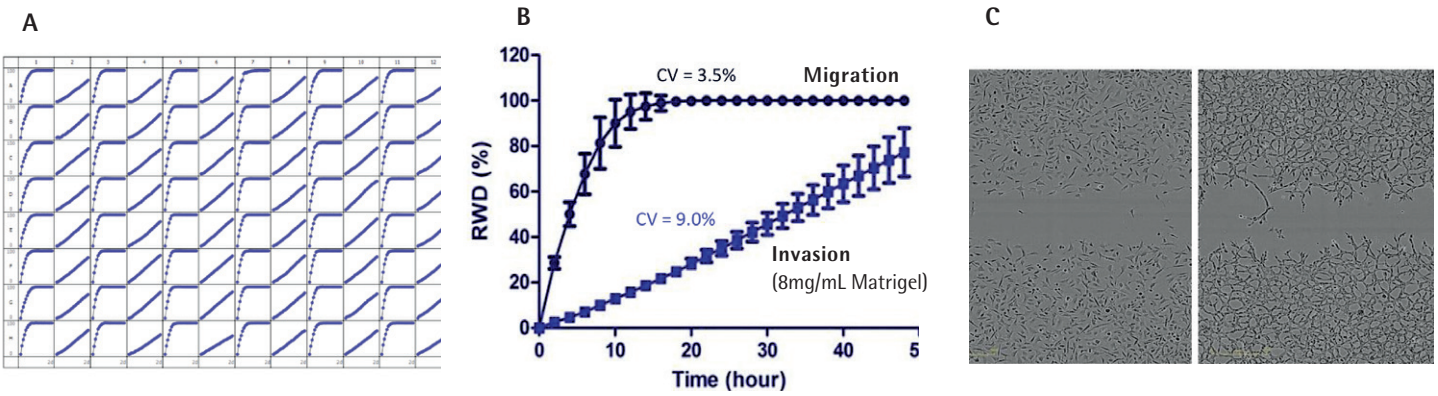

Figure 1. Measurement of the reproducibility the migration and invasion assay in the same microplate. HT-1080 cells were plated at 2 x 10^4 cells per well on 100 µg/ml Matrigel coated ImageLock plates. The cells in odd numbered columns had only media added after using the 96-well WoundMaker representing cell migration. The cells in even numbered columns were overlaid with 8 mg/mL Matrigel representing invasion. (A) Temporal progression of wound closure in each well with time using RWD as the metric to measure migration or invasion. (B) Time course of means of each condition. The respective coefficients of variation for each assay were averaged. (C) Representative images of HT-1080 cells migrating on Matrigel (left) and invading through 8mg/mL.

Differentiating between invasive and non-invasive cells

Both MDA-MB-231 and HT-1080 cells are highly invasive cell types. In contrast, MCF-7 cells are relatively non- invasive. All three cell types were tested in invasion and migration assays as indicated in Figure 2. As previously shown, HT-1080 cells migrated on collagen 1 coated plates and invaded collagen 1, with the rate of invasion slowing as the concentration of collagen 1 matrix was increased from 1-3 mg/ml. MDA-MB-231 cells also migrated on collagen 1-coated plates, but invaded collagen more slowly than HT-1080 cells. Interestingly, the rate of invasion of MDA-MB-231 cells was similar at all concentrations of collagen 1 tested.

MCF-7 cells, like the other two cells types, migrated on collagen 1-coated plates, but in contrast to the other two cells types, MCF-7 cells did not have the capacity to invade collagen 1 at the concentrations tested.

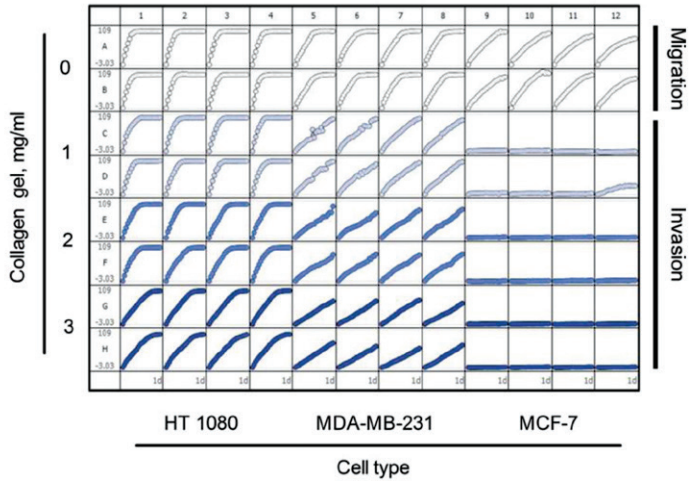

Figure 2. 96-well microplate graph of three cell types in the cell migration and invasion assay. All wells were coated with 300 µg/mL collagen 1. HT-1080 (2 x 10^4 cells per well) are in column 1-4, MDA-MB-231 (2.5 x 10^4 cells per well) are in column 5-8 and MCF-7 (5 x 10^4 cells per well) are in column 9-12. Rows A and B show the cell migration data for the three cell types. The same cells in the invasion assay are shown in rows C-D (1 mg/ml collagen 1), rows D-E (2 mg/ml collagen 1) and rows G-H (3 mg/ml collagen 1). The plate map graph shows the progression of each well with time using the RWD metric to measure migration or invasion.

Pharmacology applications

Blebbistatin is a pharmacological agent that is known to inhibit myosin by binding to the ATPase intermediate with ADP and phosphate bound at the active site, slowing the release of phosphate and inhibits locomotion of cells.[1] Previous studies have suggested that blebbistatin may be more effective at inhibiting cell invasion as compared to cell migration.[2] With the 96-well format, it is easy to set up an experiment to measure migration and invasion concurrently within the same microplate. Using this approach, the effect of blebbistatin on migration and invasion using HT-1080 cells was studied. The plate map in Figure 3A demonstrates a convenient way to set up this experiment. Three columns of cells were used for migration, and three were used for invasion. A 7-point concentration curve of blebbistatin was carried out as depicted in rows A-G. Row H was used a solvent control. The microplate graph in Figure 3B demonstrates the reproducibility of the assay and shows the effect of each concentration of drug in both assay formats. By inspection, it appeared that blebbistatin had a larger effect on invasion compared to migration. Plotting the data as the average of the treatment group for migration and invasion made it clear that blebbistatin had a much larger effect on invasion (Figure 3 C and D, respectively). Figure 3E shows the concentration response analysis at the 24-hour time point for both assays. From these data, the calculated IC50 of blebbistatin for migration and invasion is 92 and 5.2 µM, respectively. The Z' for this data set is 0.77.

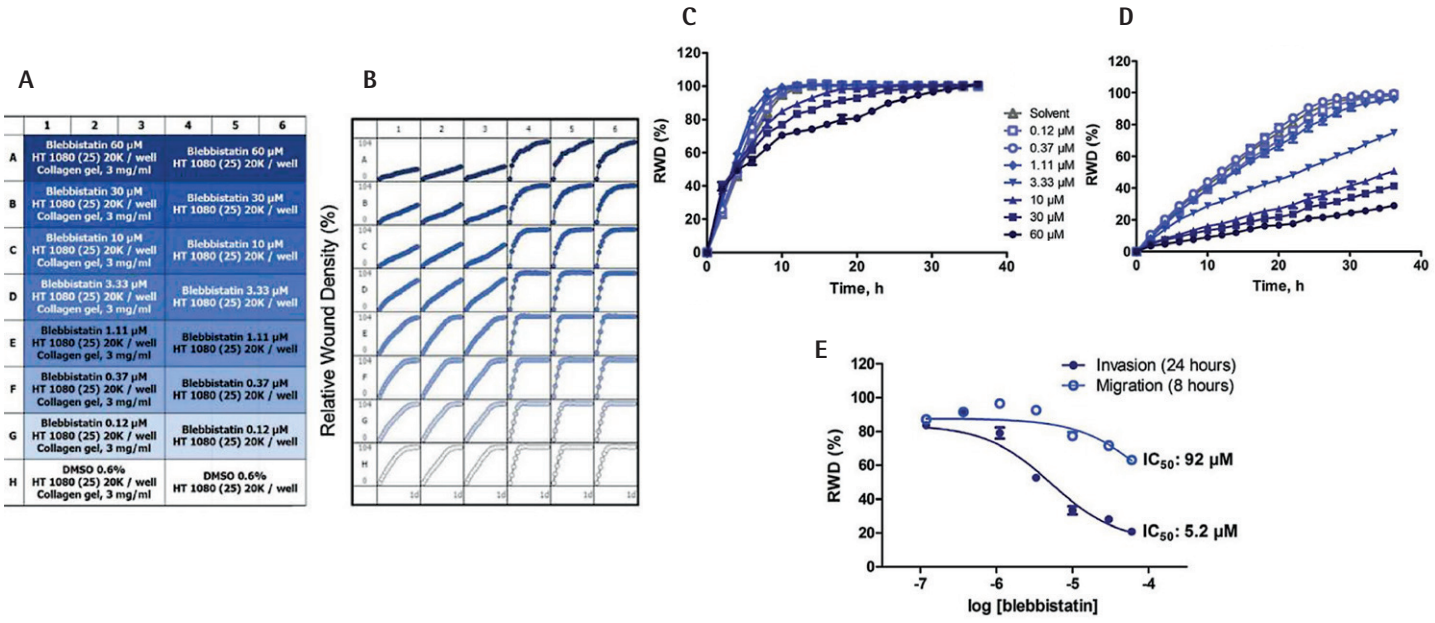

Figure 3. Effect of blebbistatin on the migration and invasion of HT-1080 cells. HT-1080 cells were plated at 2 x 104 cells per well on 300 µg/mL collagen 1 coated plates. Panel A shows the plate map for the experiment. The cells in columns 1-3 were overlaid with 3 mg/mL collagen 1 containing blebbistatin or solvent control. The cells in columns 4-6 were given complete growth media with blebbistatin or the solvent control. Panel B shows the microplate graph of the experiment. Panel C and D show the means of each treatment group for migration and invasion, respectively. Panel E shows the concentration response analysis of blebbistatin on cell migration and invasion. The IC50 calculation for each assay is included next to the concentration response curve.

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases that degrade collagen 1 and other basement membrane materials and are expressed at increased levels by many highly metastatic tumor derived cells. GM6001 is a broad MMP inhibitor that has been shown to inhibit the invasion of HT-1080 cells into collagen 1.[3] The 96-well Migration and Invasion Assay was configured in order to test the effect of this drug on invasion and migration on the same microplate. As shown in Figure 4A, GM6001 had no effect on the migration of HT-1080 cells. In contrast, as shown in Figure 4B, GM6001 inhibited invasion into collagen 1 in a concentration-dependent manner. Interestingly, the addition of a protease cocktail containing E-64 (25 µM) pepstatin A (100 µM), leupeptin (2 µM) and aportinin (2 µM) had no measurable effect on cell migration or cell invasion (data not shown).

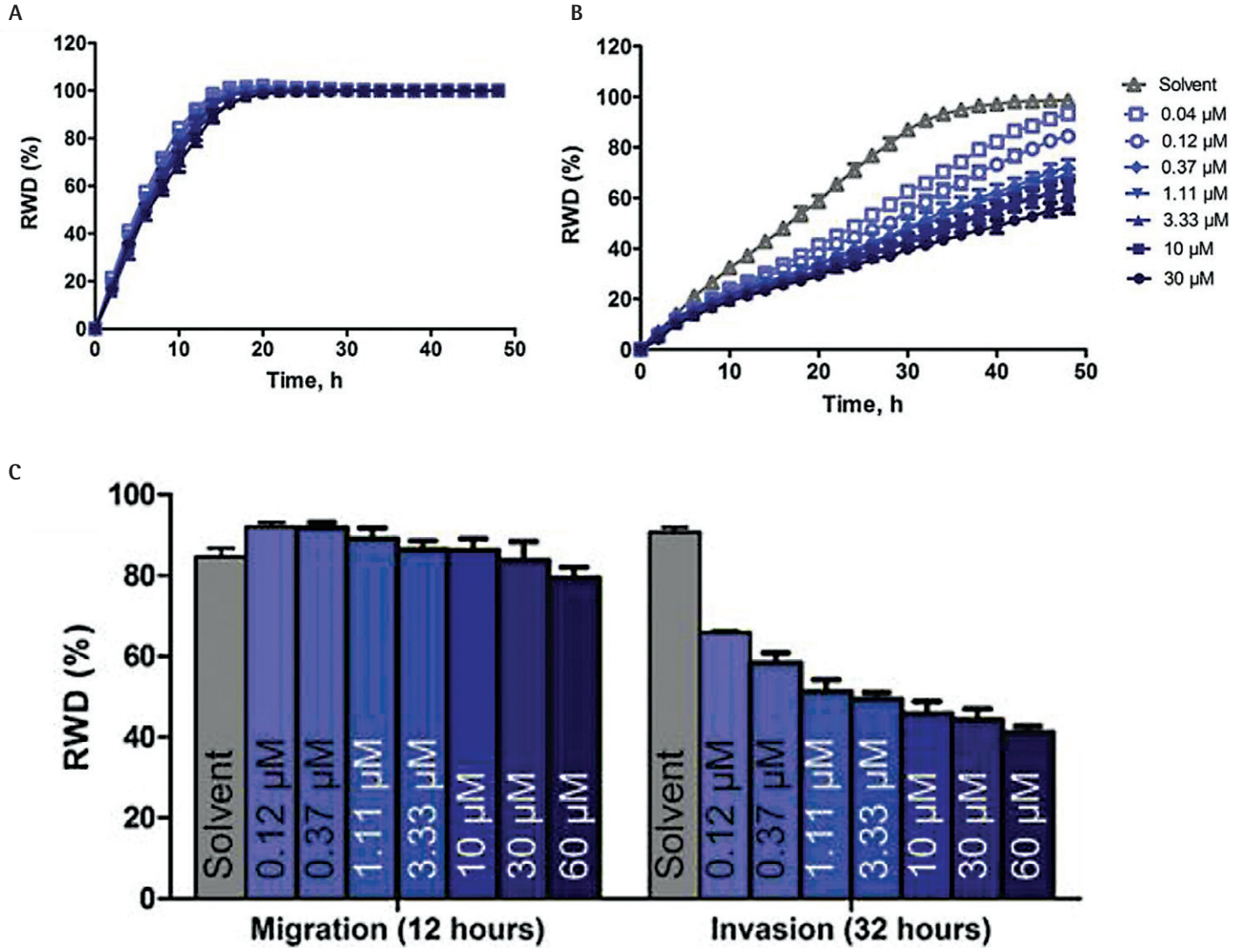

Figure 4. Effect of MMP inhibitor. HT-1080 cells were plated at 2 x 104 cells per well on 300 µg/mL collagen 1 coated plates. The GM6001 concentration response was carried out as described for blebbistatin experiment described in Figure 3. Panel A and B show the means of each treatment group for migration and invasion, respectively. Panel C shows the effect of GM6001 on cell migration and invasion in a bar chart format at the 32-hour time point.

Two-color fluorescent scratch wound experiments

In addition to phase contrast images, two-color images can be collected using IncuCyte® fluorescent labeling reagents. This allows study of the interactions between several cell types in a mixed culture, and how each affects migration, invasion, and proliferation of the other, all within one well of a 96-well plate. As shown in Figure 5, the non-invasive MCF- 7 cells were labeled with IncuCyte® NucLight Green Lentivirus Reagent, and the HT-1080 cells labeled with IncuCyte® NucLight Red Lentivirus Reagent were mixed in co-culture, and plated for an invasion assay through 8 mg/ml Matrigel®. Imaging in phase, red, and green channels revealed the HT- 1080 cells efficiently invaded the Matrigel® matrix whereas MCF-7 remained non-invasive.

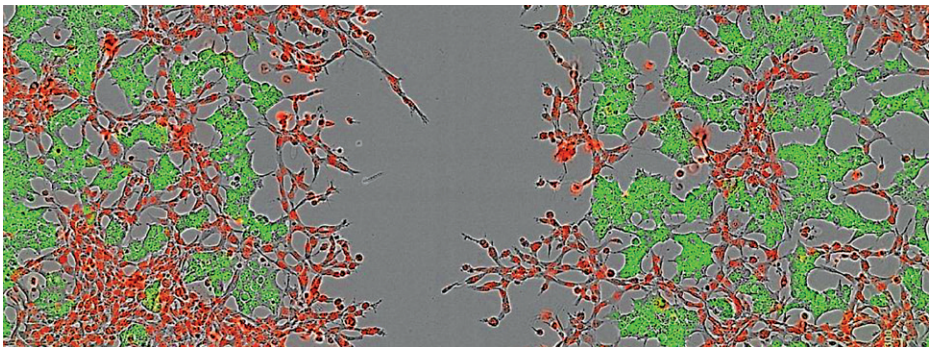

Figure 5. Two color fluorescent scratch wound. The ability to image cells in both wavelengths in addition to phase contrast allows users the ability to explore cell-cell interactions as it pertains to cell migration and invasion. In this example, HT-1080 NucLight Red cells were plated with MCF-7 NucLight Green cells and invasion through 8 mg/ml Matrigel® was monitored over time (Image shows the 24 hour time point).

Conclusions

IncuCyte scratch wound migration and invasion assays are flexible, quantitative, and reproducible. The unique tip design of the 96-well IncuCyte WoundMaker is a critical part of these assays, as it creates a cell-free zone from a confluent monolayer of cells making the biology consistent and reproducible. Both assays utilize IncuCyte's innovative high definition optics and integrative analysis algorithms to make quantitative measurements without the need for labeling cells. Having high-quality images at every time point gives an investigator access to both the kinetics and morphological changes occurring in migration and invasion experiments, enabling additional insight intothe effects test agents have on cells undergoing these processes. Key features and benefits include:

- Investigators can measure migration and invasion on the same microplate. This provides the best opportunity for determining the specificity of drugs and the utility of potential drug targets.
- The spatio-temporal, label-free format of both the Migration and Invasion Assays allows investigators to follow both the rate and the extent of migration and invasion for a given set of experimental variables. This feature can be used to explore time-dependent pharmacology, in order to enhance assay sensitivity.
- After the experiment is initiated, phase contrast and/ or fluorescence images are collected and processed automatically, yielding un-biased results.
- Precise wounding ensures that results are quantitative and reproducible.
- IncuCyte high definition optics eliminates the need to label the cells.
- HD images are acquired at every time point and can be automatically assembled into time-lapse movies for convenient visualization of morphology and wound closure.

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Kinetic Chemotaxis Assays

Powerful assays for quantification and visualization of chemotactic cell migration and invasion

Introduction

Chemotactic migration and invasion play an essential role in many normal and pathological processes including immune response, cell differentiation and tumor metastasis. Methods to study factors that regulate the directional migration of cells typically employ modifications of Boyden chamber assays. Boyden chamber assays consist of an upper and lower chamber separated by a porous filter, allowing for the diffusion of a chemotactic agent. Although these assays offer insight into the chemotactic response of cells, they have inherent drawbacks including the inability to visualize active cell migration and the requirement for a large number of cells, typically 50,000 to 100,000 cells per well. Most importantly, perhaps, Boyden chamber assays "*only afford the measurement of cell migration at a single time point, and as the kinetics of cell migration vary markedly depending on the cell type studied and the chemoattractant used, this limits the utility of this type of assay.*"[1]

In this chapter, we will review live-cell chemotatic cell migration and invasion assays, which accurately measure and visualize cell movement in response to a chemical gradient. This approach allows for the assay to be completed in a physiologically relevant environment, supports a stable gradient, and automatically analyzes images, alleviating technically challenging and cumbersome quantification steps in traditional approaches.

IncuCyte® Chemotaxis Assay at a Glance

The IncuCyte® ClearView chemotaxis cell migration assay is conducted in a 96-well migration plate consisting of an optically clear membrane insert and reservoir that allows for direct visualization of cell migration and morphological changes using phase contrast and/or fluorescent imaging. The plate is placed in the IncuCyte® instrument, a fully automated live-cell analysis system with integrated image analysis tools that is placed inside a standard tissue culture incubator. Integrated metrics precisely quantify the chemotactic response using 1,000 to 5,000 cells per well, a significant advantage when using rare primary hematopoietic cells from the blood. This assay method is sensitive to surface-integrin signaling, and sustains a linear gradient over several days. The result is 96 wells of reproducible kinetic data supported by images and movies, requiring very few cells and minimal hands-on time.

Shortcomings of Traditional Assays	Live-Cell Imaging and Analysis Approaches
• **Inability to visualize** active cell migration.	• **Real-time, direct visualization** and automated analysis of chemotactic migration, invasion in a 96-well well assay format.
• **Requirement for a large number of cells**. typically 50,000 to 100,000 cells per well.	• Reproducible **kinetic data supported by images and movies.**
• Measurement of cell migration **at a single time point.**	• Requires only **1,000 to 5,000 cells per well**, a significant advantage when using rare primary hematopoietic cells from the blood.
	• Assay is sensitive to surface-integrin signaling allowing **the study of migration on biologically-relevant surfaces.**
	• **Sustains a linear gradient** over several days.
	• Measure label-free or labeled cell migration **without fixing, staining or cell scraping steps.**
	• **Flexibility to study** adherent and non-adherent cell chemotaxis, in mono- or co-culture, with or without fluorescent labels.

Sample Results

Automated analysis and visualization

Whole-well images of cells on both the bottom and the top of the IncuCyte® ClearView plate membrane are captured at user-defined intervals. All images are processed using automated algorithms to quantify cell area on each side of the membrane (Figure 1). Directed cell migration can be reported as either an increase in area on the bottom side of the membrane for adherent cells, or a decrease in area on the top side of the membrane for non-adherent cells that migrate down the pore and fall off of the membrane.

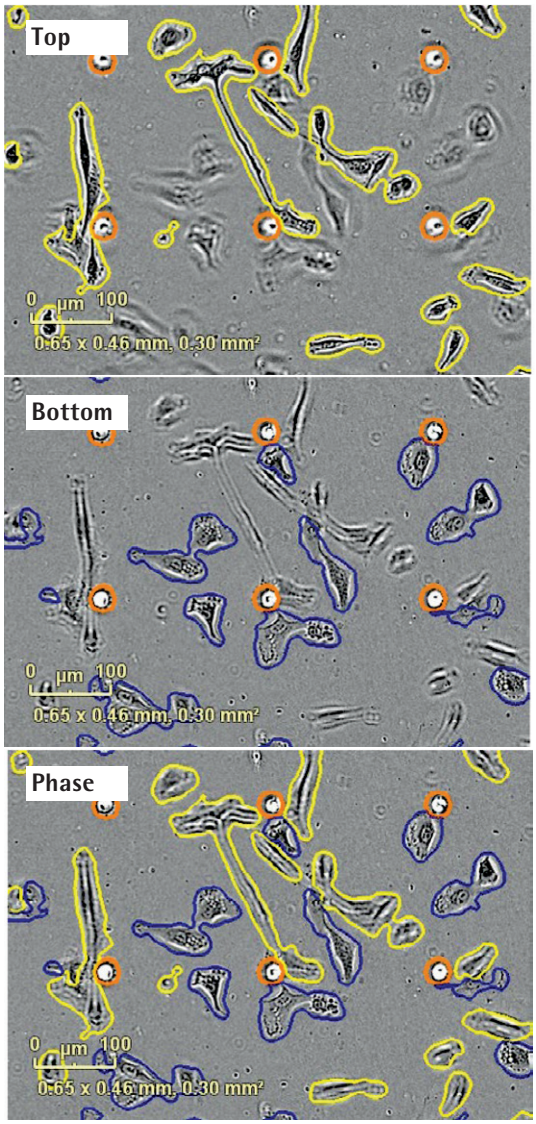

Figure 1. Chemotaxis quantification. HT-1080 fibrosarcoma cells were plated in the top chamber of the ClearView 96-well cell migration plate at a density of 1000 cells/well. 10% FBS was added to the bottom chamber as a chemoattractant. Images represent the top and bottom side of the membrane at the 36-hour time point. Automated image processing separates cells located on the top (outlined in yellow) and the bottom (outlined in blue) surface of the membrane. Pores are outlined in orange. Images are processed as they are acquired, and data can be plotted in real time.

Cancer cell chemotactic migration — adherent cells

The pharmacological effect of traditional signaling pathway inhibitors on the directed migration of HT-1080 fibrosarcoma cells toward fetal bovine serum (FBS) was investigated (Figure 2). We observed concentration-dependent inhibition of directed cell migration with each of the three inhibitors tested: KU0063794 (Akt pathway), U0126 (MEK/ERK MAPK pathway), and Wortmannin (PI3K pathway).

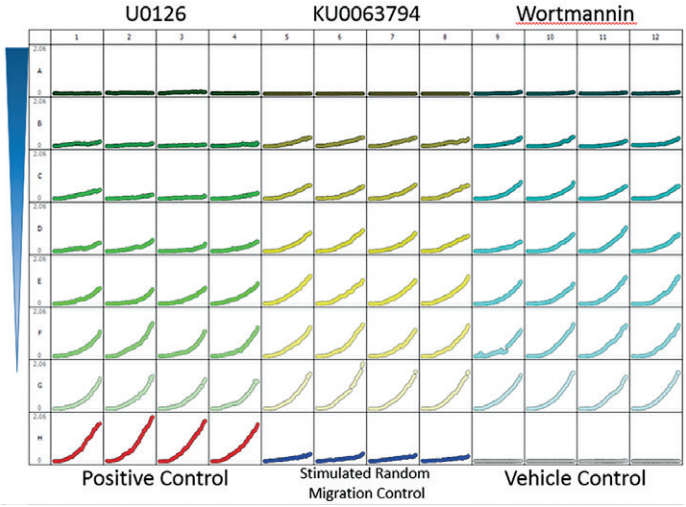

Figure 2. Inhibition of HT-1080 cancer cell migration. This 96-well microplate graph illustrates the kinetic measurement of HT-1080 cell migration toward 10% FBS in each well of the ClearView 96-well chemotaxis plate. Plotted in each well is the cell area on the bottom of the membrane (y-axis) over the course of a 48-hour assay (x-axis). U0126, KU0063974 or Wortmannin were added to 1,000 HT-1080 cells in the upper chamber and incubated at 37°C for approximately 30 minutes prior to exposing the cells to chemoattractant in the lower chamber. The concentration of inhibitors decreases from the top of the plate to the bottom. Positive (with chemoattractant) and negative (without chemoattractant) controls are located in the last row. These data were collected over a 48-hour period at 1-hour intervals.

Cancer cell chemotactic invasion

Directed cell invasion was studied by embedding HT-1080 cells in an extracellular biomatrix, and investigating the ability to specifically inhibit invasion using GM6001. Side-by side migration and invasion assays were performed (prepared by embedding 1,000 HT-1080 cells per well in 1 mg/mL Cultrex® Rat Tail Collagen 1) in ClearView plates (Figure 3) The biomatrix:cell layer was overlaid with three-fold serial dilutions of GM6001,a broad spectrum matrix metalloproteinase (MMP) inhibitor. After compound addition, 10% FBS was added to the reservoir wells and measurements of bottom-side nuclear counts were plotted over 72 hours. Data shows specific GM6001 concentration-dependent inhibition of HT-1080 invasion and no effect on the migratory response of HT-1080 cells toward 10% FBS.

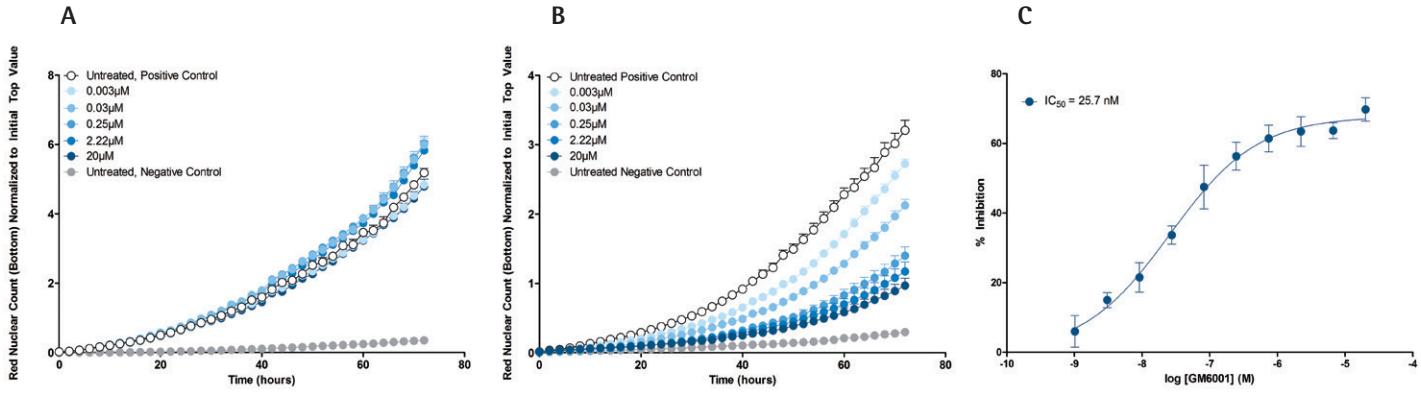

Figure 3. Effect of GM6001 on migration and invasion of HT-1080 cells in a collagen invasion assay. HT-1080 cells were plated at a density of 1,000 cells per well in assay medium (A, migration assay) or embedded in 1 mg/mL neutralized Cultrex® Rat Tail Collagen 1 (B, invasion assay). Serial dilutions of GM6001 were added to the cells in the migration assay or overlaid on the biomatrix:cell layer of the invasion assay at indicated concentrations prior to exposing them to 10% FBS. Analysis of pharmacological response was performed at t=72 hr. Inhibition curve for GM6001 indicating IC50 in the invasion assay (C). Data were collected over a 72-hour period at 2-hour intervals. Each data point represents mean ± SEM, n=4.

Immune cell chemotaxis – non-adherent cells

In a model of immune cell chemotaxis, the response of CD3/CD28 activated T cells toward two chemoattractants was investigated: CXCL11 and CXCL12 (aka SDF-1α), ligands for CXCR3 and CXCR4, respectively (Figure 4)[2]. By measuring the loss of cell area on the top of the membrane, we show that activated T cells migrate toward both CXCL11 and CXCL12. Results show the selective CXCR4 antagonist, AMD3100, inhibits chemotaxis toward CXCL12 (IC50 = 279 nM), with no effect on CXCL11-mediated chemotaxis. This experiment was completed on an ICAM-1–coated surface.

Interestingly, successful measurements of T cell chemotaxis were also made on fibronectin and Matrigel®/FBS–coated surfaces, while T cells did not migrate on an uncoated surface. This suggests that interactions between integrins and/or receptors on the cell surface and the substrate play a fundamental role in T cell chemotaxis in this assay. This was not the case when tested in a traditional Boyden chamber, where the surface coating was not required (data not shown).

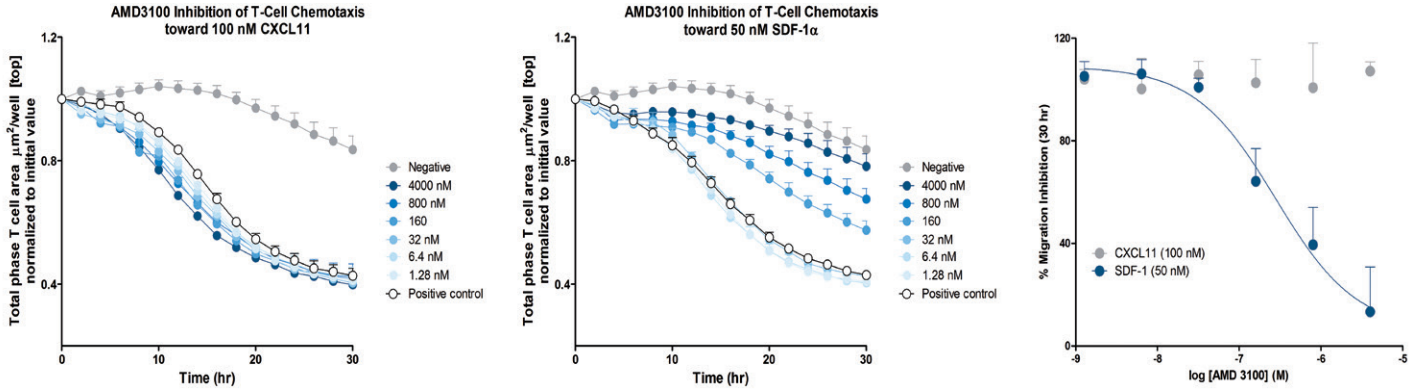

Figure 4. T cell chemotaxis toward CXCL11 and CXCL12 (SDF-1a). T-cells were plated at a density of 5,000 cells per well in the upper chamber of an ClearView 96-well chemotaxis plate coated with ICAM. AMD3100 was added to the cells at indicated concentrations and incubated at room temperature for approximately 1 hour prior to exposing the cells to chemoattractant gradient. CXC11 or SDF-1a was added to the reservoir plate at 100 nM or 50 nM, respectively, based on EC50 values obtained in agonist curve experiments (data not shown). Analysis of pharmacological response was performed at t=30 hr. Data were collected over a 30-hour period at 2-hour intervals. Each data point represents mean ± SEM, n=3.

Surface contact-mediated migration

The low pore density of the ClearView membrane ensures that cells must migrate across the biologically relevant surface towards the chemoattractant. Neutrophils seeded on an uncoated ClearView membrane were unable to migrate towards the chemoattractants IL-8 and fMLP; however, those on Matrigel®-coated membranes showed clear chemotactic profiles (data not shown). These data suggest that integrin and/or cell surface receptor interactions with the substrate play a key role in neutrophil chemotaxis in this model. In contrast, no coatings were required for neutrophil migration studies using a 96-well modified Boyden chamber assay (data not shown), suggesting that active migration of neutrophils in a Boyden chamber approach is absent.

Interestingly, the assay micro-environment is critical in supporting cell motility. Neutrophils suspended in RPMI supplemented with 0.5% BSA were unable to migrate toward C5a and IL-8 chemoattractant gradients. When media was supplemented with 0.5% HSA, however, neutrophils actively migrated toward both C5a and IL-8 (data not shown). Figure 5 shows that upon visual inspection of the wells, an observed difference in cell morphology of neutrophils isolated in RPMI + 0.5% BSA (rounded phenotype), compared to neutrophils isolated in RPMI + 0.5% HSA (activated phenotype). Furthermore, neutrophils assayed using a modified Boyden-chamber approach responded to both chemoattractants (data not shown), showing no sensitivity to the different albumins present in the assay media. Together, these quantitative and qualitative data suggest that the interaction of cell integrins with the substrate and the overall assay micro-environment are a crucial component in the IncuCyte chemotaxis cell migration assay.

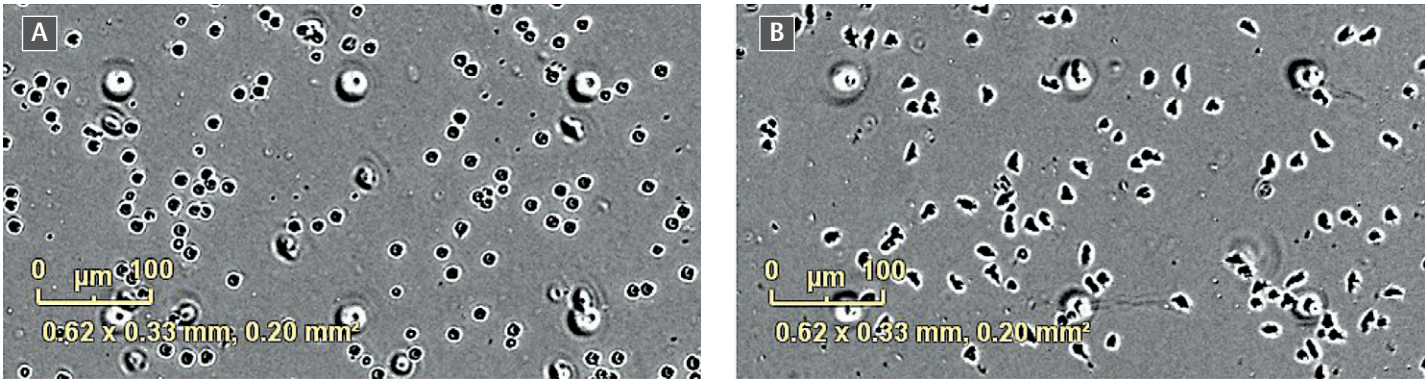

Figure 5. Morphological images. Phase-contrast images of neutrophils isolated in RPMI + 0.5% BSA (A) versus isolation in RPMI + 0.5% HSA (B), seeded on ClearView membranes coated with 50 µg/mL Matrigel + 10% FBS, showing morphological differences in response to 1 µM C5a (t=20 minutes). This difference in phenotype was also observed in neutrophils that were exposed to the chemoattractant IL-8.

Intra- and inter-plate reproducibility measurements

To assess assay reproducibility and precision of the IncuCyte chemotaxis assay, a series of four independent experiments using Jurkat (non-adherent) T cells was performed (Figure 6). Cells were plated at a density of 5,000 cells per well. Two-fold dilutions of CXCL12 were made across columns, and measurements of total phase object area normalized to the initial value were plotted over a 30-hour time course. Highly reproducible well-to-well kinetic measurements, with an average intra-assay CV of 6.3%, were observed. Similar results were obtained when measuring the directed migration of HT-1080 cells toward FBS and neutrophils toward IL-8, C5a and fMLP (data not shown).

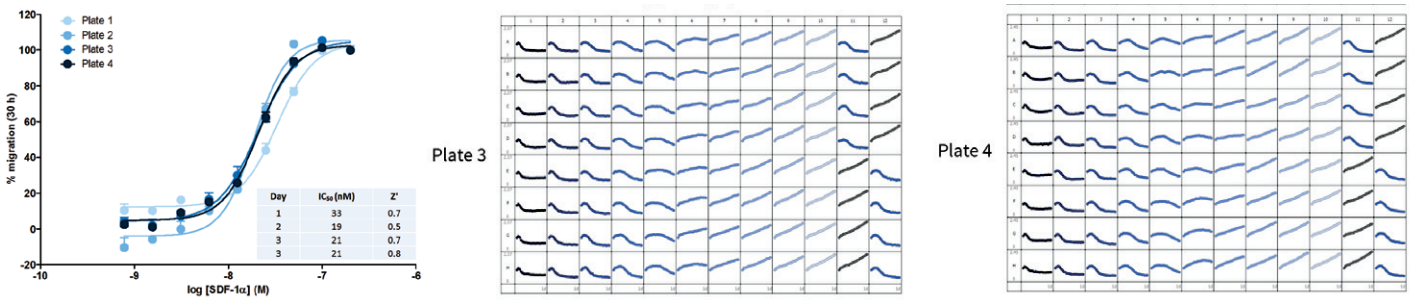

Figure 6. Precision and reproducibility of directed migration of Jurkat T cells toward CXCL12. Jurkat cells were plated at a density of 5,000 cells per well in the upper chamber of the ClearView chemotaxis cell migration plate. Two-fold dilutions of SDF-1a (n=8 per concentration) were added to the reservoir.

Stable gradient

A long-term, stable gradient is required to support chemotaxis over longer periods of time, and to increase cell participation rates. In an experiment designed to evaluate the stability of a chemoattractant gradient in the ClearView 96-Well chemotaxis plate and a 96-well modified Boyden-chamber assay, diffusion of a 10,000 kD dextran fluorescently labeled with Alexa Fluor® 594 was monitored over a 72-hour time course in each consumable. As shown in Figure 7, more than 50% of the gradient dissipates in the 96-well traditional Boyden-chamber plate within the first four hours. In contrast, >80% of the gradient remains intact in the ClearView plate at 72 hours. In a biological test of gradient stability, the HT-1080 cells migrated directionally toward FBS with equal rates in gradients that had been pre-established for 24, 48, and 72 hours, indicating a stable gradient across the membrane (Figure 7). These rates were slightly faster than those for cells migrating toward a gradient that had not been pre-established. This is likely a result of increased cell health when serum-starved cells are plated in established gradients.

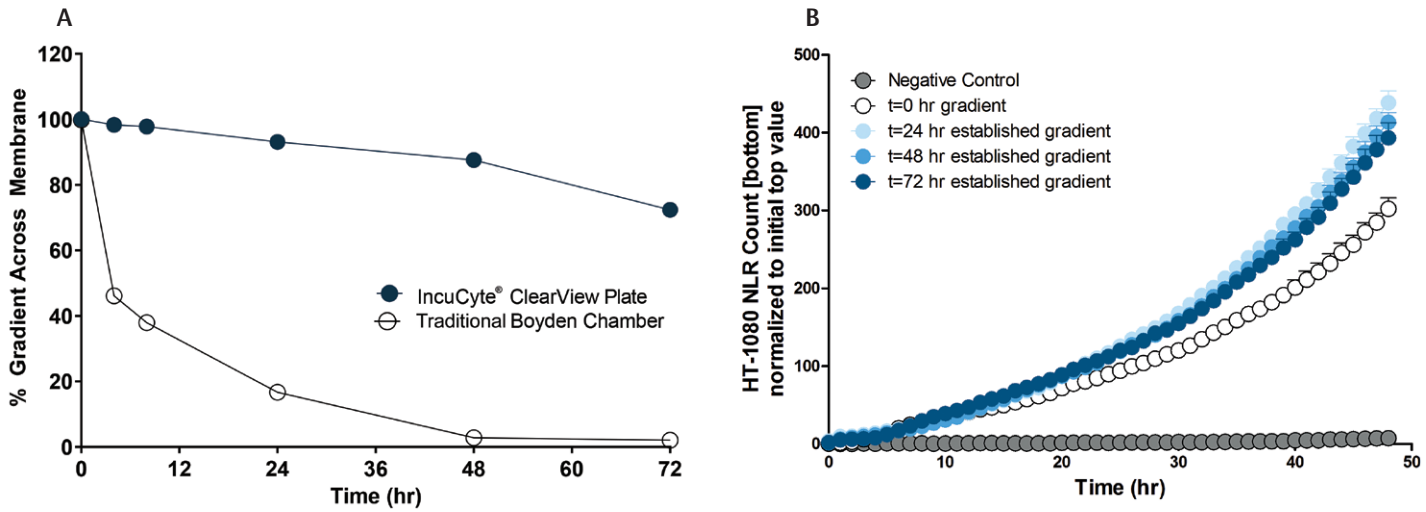

Figure 7. Evaluation of ClearView chemotaxis assay gradient. (A) 10,000 kDa dextran, labeled with Alexa Fluor® 594, was added to the reservoir plates for the ClearView and 96-well modified Boyden-chamber at a concentration of 10 µM to establish gradients over 72, 48, 24 and t=0 hours. Measurements of diffusion were made by sampling the insert wells on both plates and measuring fluorescent intensity on a microplate reader. Each data point represents mean ± SEM, N=3. (B). An FBS gradient was established over 72, 48, 24 and t=0 hours in separate wells. Serum starved NucLight Red HT-1080 cells were added at a density of 500 cells per insert well to the established gradients and FBS-induced chemotactic response was measured. Each data point represents mean ± SEM, N=3.

Conclusions

The IncuCyte chemotaxis assay is a quantitative and reproducible approach for measuring chemotaxis in both adherent and non-adherent cell types. This assay format allows for the kinetic detection of cell migration toward chemotactic gradients on a physiologically relevant substrate, with movies and images that support quantitative measurements and provide associated morphological and phenotypic insights.

Key features demonstrated in the IncuCyte chemotaxis assay are:

- Kinetic cell migration is automatically processed on both the top and bottom side of the optically clear membrane, allowing for real-time analysis of chemotactic driven migration of adherent and non-adherent cell types.
- Non-adherent cell migration is precisely quantified in a physiologically relevant environment, while the plate stays stationary, allowing for the evaluation of rare primary hematopoietic cells from the blood.
- Cells are required to migrate across a biologically relevant surface, demonstrating the need for cell-surface interaction, providing an opportunity for evaluation of integrin receptor signaling.
- All data points can be validated by individual images or time-lapse movies to confirm processing metrics, significantly enhancing the confidence in the measured response.

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2. Kawaguchi A, Orba Y et al: **Inhibition of the SDF-1a-CXCR4 axis by the CXCR4 antagonist AMD3100 suppreses the migration of cultured cells from ATL patients and murine lymphoblastoid cells from HTLV-U Tax transgenic mice.** *Blood* 2009, 114: 2961-2968.

Kinetic Transendothelial Migration Assays

Label-free, live cell imaging assay to visualize and quantify transendothelial migration

Introduction

The recruitment of leukocytes to sites of infection and their subsequent migration through the endothelium are critical steps of the immune response. This process of transendothelial migration (TEM) is essential in order for leukocytes to respond to foreign microorganisms, but if uncontrolled, can lead to autoimmune disorders such as inflammatory bowel disease and rheumatoid arthritis. Assays to evaluate the extravasation of leukocytes are also essential for the study of host-defense response and inflammatory disorders.

In this chapter, we will review live-cell transendothelial cell migration assay, which accurately quantifies directional leukocyte migration across an endothelial monolayer in real time. This approach allows for the assay to be completed in a physiologically relevant environment and automatically analyzes images, alleviating technically challenging and cumbersome quantification steps in traditional approaches.

IncuCyte® Transendothelial Migration Assay at a Glance

The IncuCyte® Chemotaxis transendothelial migration assay is a fully automated solution to quantify directional leukocyte migration across an endothelial monolayer using kinetic, image-based measurements. This label-free assay is suitable for testing multiple cell type pairings, evaluation of immunomodulation with blocking antibodies or inhibitors, along with the associated ability to assess monolayer integrity throughout the assay.

The transendothelial migration assay is conducted in a 96-well IncuCyte® ClearView Chemotaxis plate consisting of an optically clear membrane insert and reservoir that allows for direct visualization of leukocyte migration across an endothelial monolayer. The plate is placed in the IncuCyte® instrument, a fully automated live-cell analysis system with integrated image analysis tools, thus eliminating laborious endpoint analysis. Kinetic data of the 96-well plate is supported by images that allow for visualization of endothelial monolayer integrity and leukocyte diapedesis.

Shortcomings of Traditional Assays	Live-Cell Imaging and Analysis Approaches
• Standard Boyden chamber surfaces are **not easily amenable to imaging.**	• **Acquisition of high-definition, phase contrast images** enable verification of intact endothelium throughout the experiment.
• Require use of **intrinsically toxic dyes.**	• **Eliminates use** of intrinsically toxic dyes.
• Requires **fixing, staining and cell scraping steps.**	• Fixing, staining and cell scraping steps **not required.**
	• **Quantitate cells on top and bottom** of the membrane to confirm movement.

Sample Results

Assessment of monolayer integrity and visualization of TEM

Human umbilical vein endothelial cells (HUVECs) were grown to confluence on top of fibronectin, a physiologically relevant basement membrane. Integrity of the monolayer was evaluated both prior to leukocyte addition, using electrical resistance measurements as well as staining for E-Selectin, and after leukocyte addition by assessing phase contrast images.

HUVECs were seeded at 6,000 cells per ClearView insert and allowed to form a monolayer for approximately 24 hrs. Cells were permeabilized and fixed, and VE-cadherin adhesion junctions (green) were visualized from assembled 10x Z-stack confocal images; dashed circles mark membrane pore locations (Figure 1A).

Resistance measurements of HUVEC monolayers cultured on a supporting matrix of fibronectin was measured using a digital multimeter. HUVEC monolayers were grown for 24 hours in EGM-2, then growth medium was removed and 60µL of DPBS-/- was placed into the insert wells and 200 µL into the reservoir wells. The average resistance for inserts containing a monolayer was 31.9±1.7 compared to wells without cells14.9±0.9 (N=9 per condition), indicative of monolayer formation over the insert pores (Figure 1B).

Label-free CD3/CD28 Dynabead activated primary T cells were added to the endothelial monolayer cultured on fibronectin in the presence or absence of migration modulators. Images were acquired every minute (Figure 2) using the IncuCyte system's 10x objective. Yellow and orange arrows indicate leukocytes moving between HUVEC cells and eventually down the pores (blue circles) of the ClearView insert.

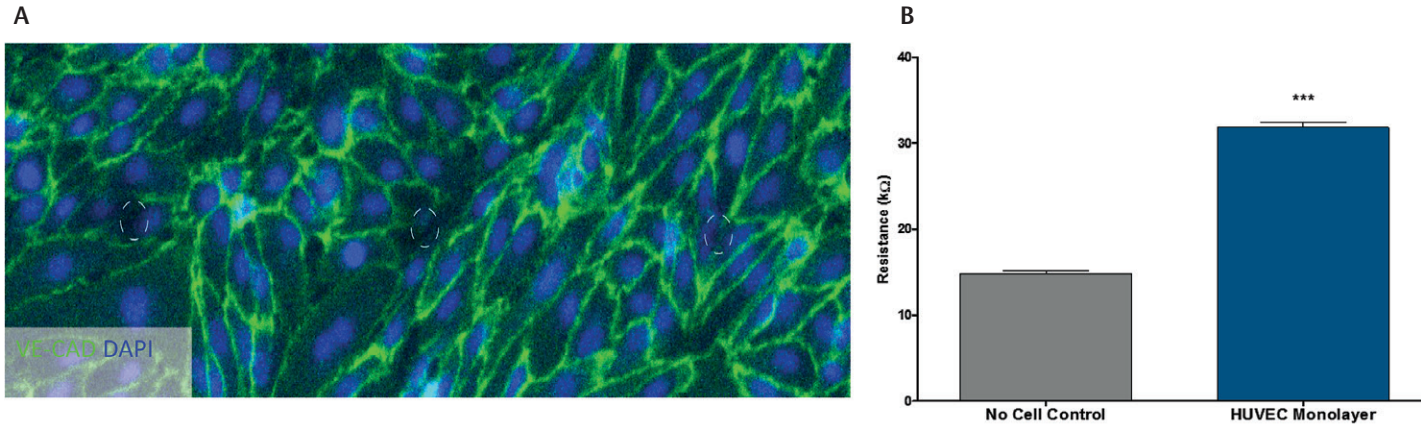

Figure 1. Assessment of monolayer integrity. VE-cadherin adhesion junctions (green) were visualized (A) and monolayer formation confirmed via measurement of resistance across the inserts (B).

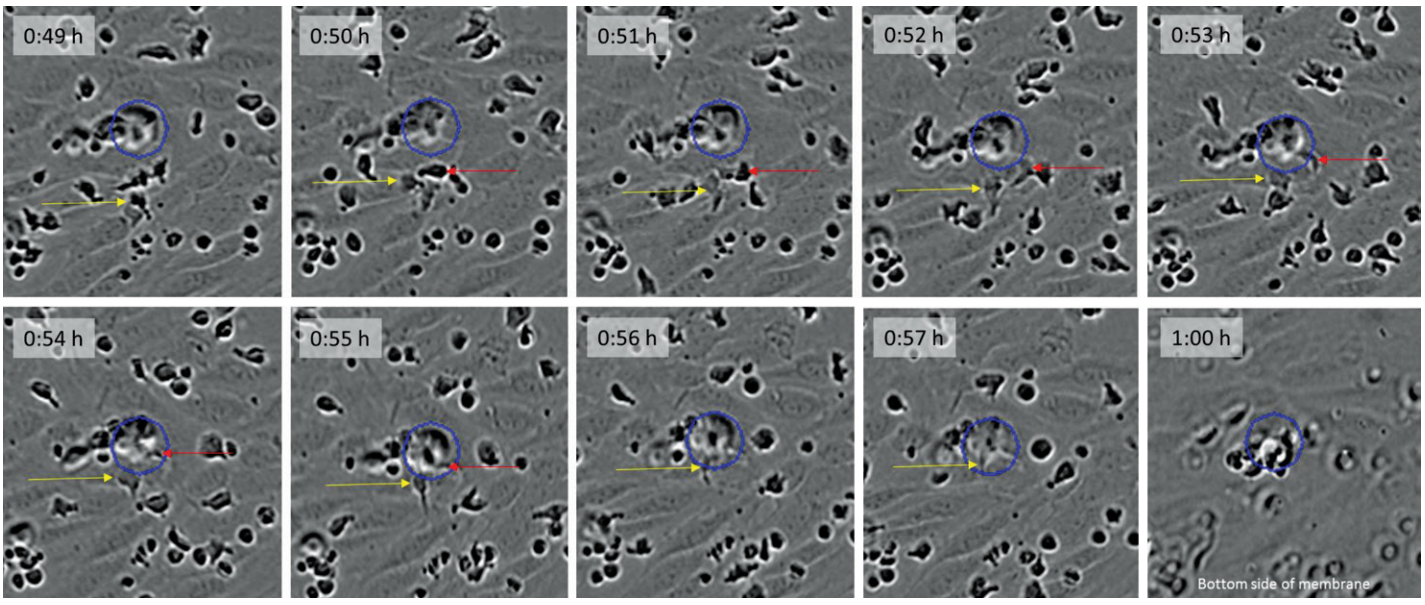

Figure 2. Visualization of leukocyte extravasation.

Real-time kinetic quantitation of label-free TEM

CD3/CD28 Dynabead activated primary T-cells were seeded on a HUVEC monolayer cultured on fibronectin in IncuCyte ClearView chemotaxis plates. Live cell images were captured at regular time intervals, and directed transendothelial migration toward CXCL12 (SDF-1α) was quantified using image analysis algorithms. Significant transendothelial migration, or diapedesis, was observed in response to increasing concentrations of SDF-1α, and could be completely inhibited using high concentrations of AMD3100, a selective inhibitor of CXCR4 and CXCL12 (SDF-1α) mediated chemotaxis.

Whole-well, phase-contrast images represent the top-side of the membrane at t=0 hour time point (Figure 3A). Automated image processing separates T cells (outlined in yellow) from the HUVEC monolayer (Figure 3B). Images are processed as they are acquired, and data can be plotted in real time as a decrease in area on the top side of the membrane for leukocytes that extravasate the endothelial monolayer and down the pore.

The insert containing T cell:HUVEC monolayer co-cultures was exposed to 3-fold decreasing concentrations of CXCL12 (SDF-1α) (Figure 4A). Images were acquired every 30 minutes and phase analysis was performed. Analysis of pharmacological response was performed at t=6hr; each data point represents mean ±SEM, N=4 (Figure 4B).

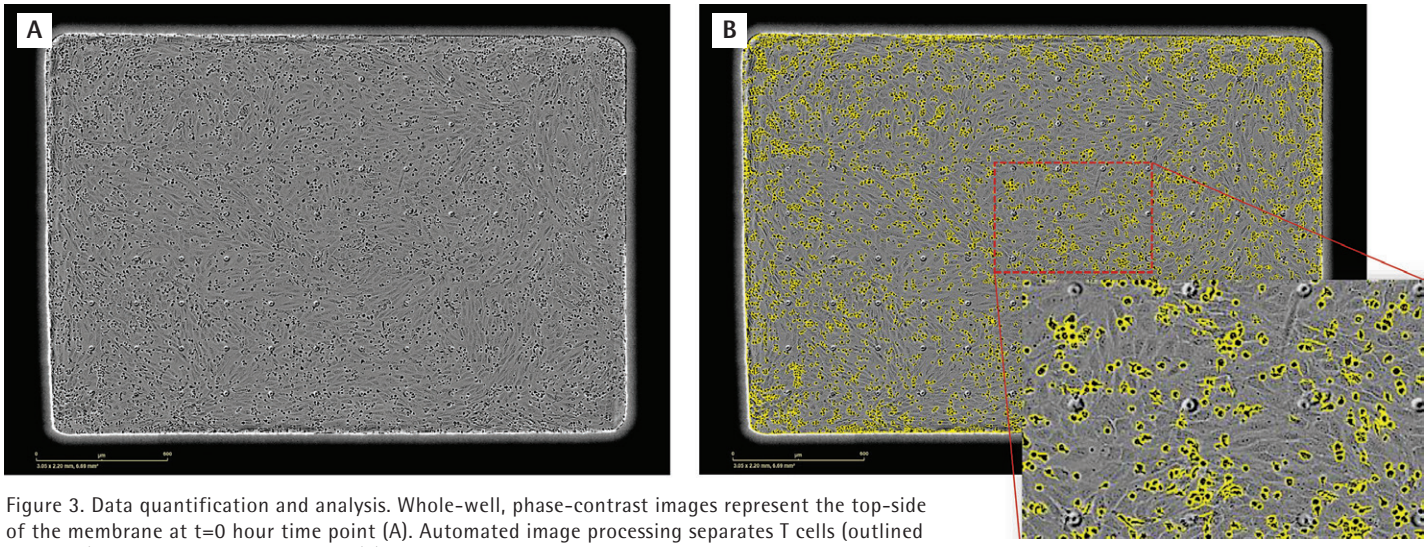

Figure 3. Data quantification and analysis. Whole-well, phase-contrast images represent the top-side of the membrane at t=0 hour time point (A). Automated image processing separates T cells (outlined in yellow) from the HUVEC monolayer (B).

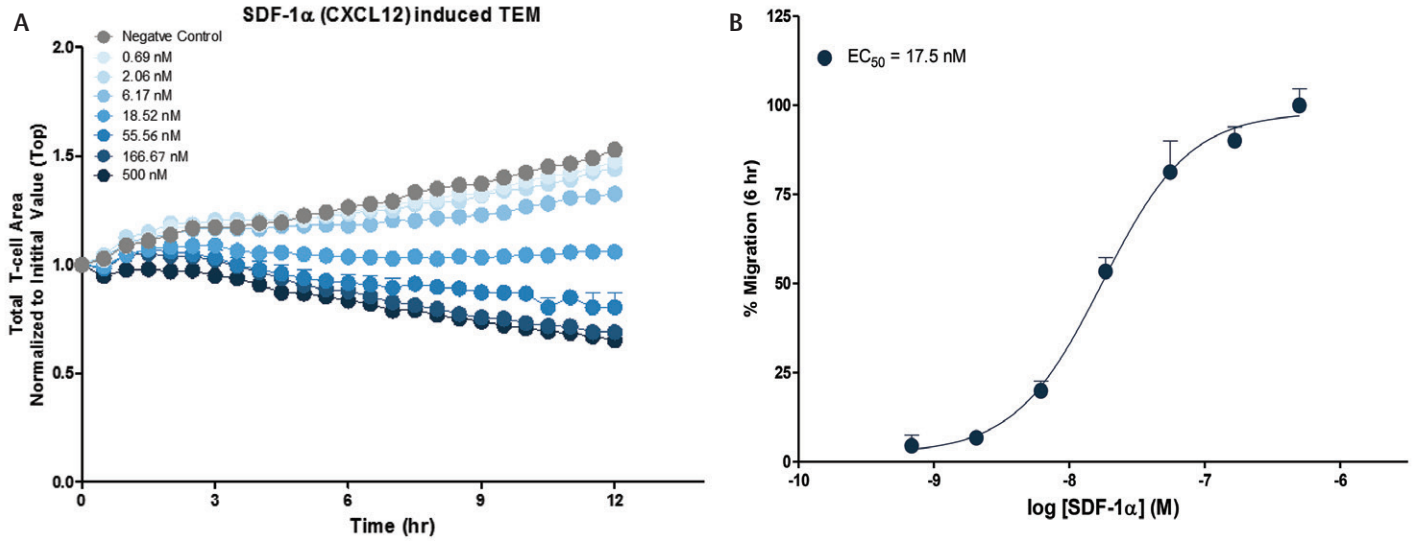

Figure 4. Primary T cells extravasation toward CXCL12. CXCL12 (SDF-1a) induced TEM (A). Pharmacological response of T cell migration (B).

Inhibition of leukocyte extravasation

For AMD3100 (CXCR4 receptor antagonist) and BIRT377 (allosteric modulator of LFA- 1) inhibition studies, CD3/CD28 activated T-cells were pre-treated for 1 hour at 37°C at indicated inhibitor concentrations, then seeded onto HUVEC monolayers and exposed to 100nM SDF-1α (Figure 5A and C).

Analysis of AMD3100 pharmacological response was performed at t=6 hr. Each data point represents mean ± SEM, N=4 (Figure 5B). For antibody inhibition, HUVEC monolayers cultured overnight on fibronectin were ± pre-treated with neutralizing ICAM-1 for 1 hour at 37°C (Figure 5D). CD3/CD28 Dynabead activated T cells were then plated at a density of 5,000 cells per well on to a HUVEC monolayer then exposed to 100nM SDF-1α (EC80concentration). Images were collected every hour over a 12 hour period and phase analysis performed.

The observed increase in total cell area is due to proliferation of C3/CD28 activated T cells. The inability to fully inhibit TEM via BIRT 377 and neutralizing ICAM-1 is believed to be caused by the centrifugation step, bringing the T cells to the HUVEC monolayer, thus bypassing the cell adhesion step.

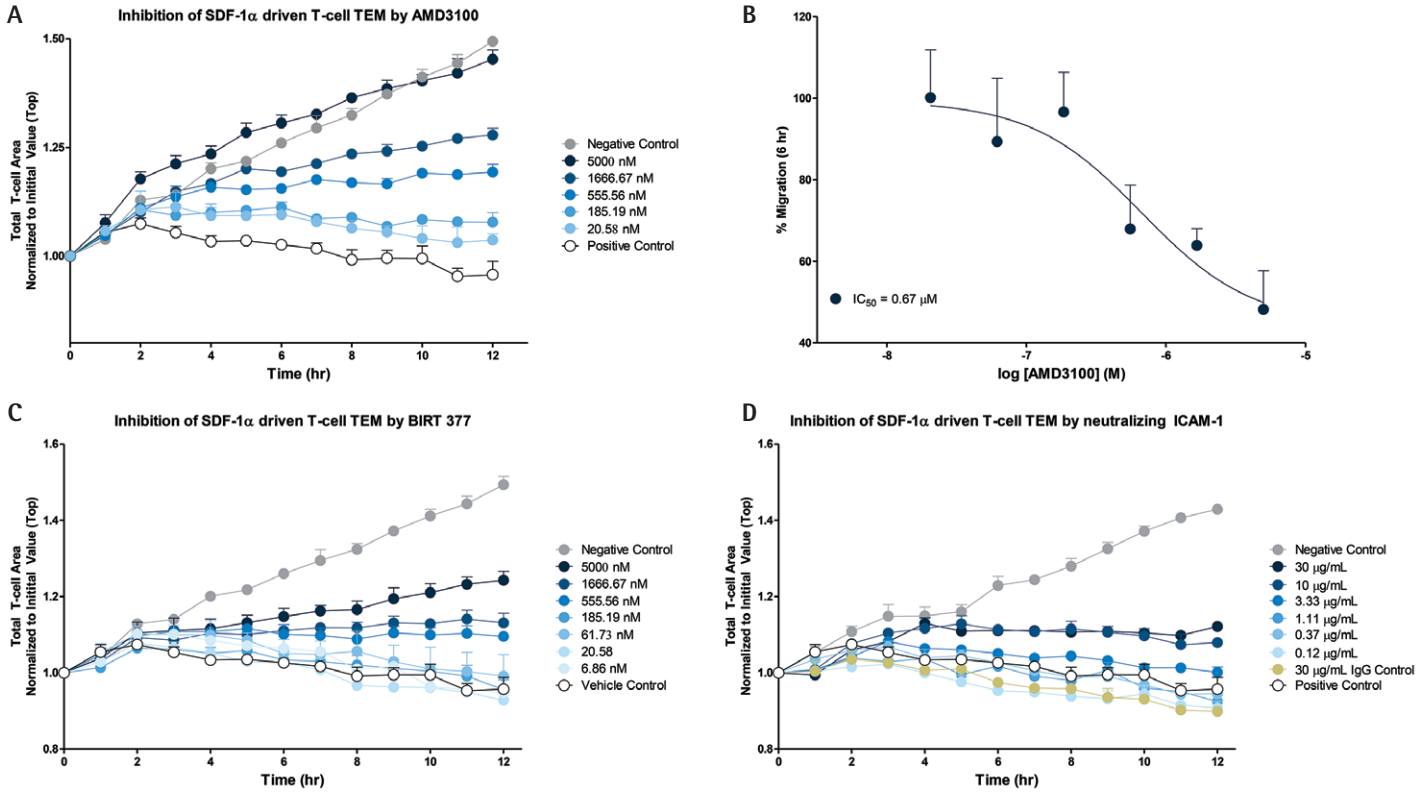

Figure 5. Treatment effects on leukocyte transendothelial migration. Inhibition of CXCL12 (SDF-1a) driven T cell migration by AMD3100 (A), BIRT 377 (C) and neutralizing ICAM-1. Analysis of AMD3100 pharmacological response (B). Migration across the endothelial layer was at least partially dependent on ICAM-1, as neutralizing ICAM-1 antibodies, and treatments with an allosteric inhibitor of LFA-1, BIRT377, significantly inhibited SDF-1a mediated diapedesis of T cells.

Conclusions

The IncuCyte® chemotaxis transendothelial assay can be used for real-time visualization and automated analysis of transendothelial migration in a 96-well format, in real-time.

- Acquisition of high-definition, phase contrast images ensure endothelium integrity throughout the experiment.
- Measurement of leukocyte extravasation is automatically processed without use of intrinsically toxic dyes, fixing, staining and cell scraping steps.
- All data points can be validated by individual images or time-lapse movies to confirm processing metrics, significantly enhancing the confidence in the measured response.

Kinetic Assays for Quantifying Protein Dynamics

Complementary, live-cell solutions for studying protein dynamics

Proteins – the building blocks of the cell – are required for virtually every biological process, from cell function, to structure and regulation. The study of proteins – how they move, interact and communicate with each other – is thus essential for our understanding of health and disease.

This is particularly true of proteins on the surface of cells involved in cell signaling and ligand binding, which requires the interaction of both extracellular proteins, such as cytokines and hormones, and membrane proteins. Such interactions are often the drivers of disease processes or the basis of drug mechanism of action. Antigen-mediated antibody internalization, for example, plays an important role in several antibody-based therapeutics and in differentiation-induced protein expression changes.

However, despite their importance, our ability to analyze these processes and link their properties to cell function has been hindered by the methodologies available to study them. Current methodologies for analysis of membrane proteins and their receptor function are limited to single time point analyses, not taking into account the dynamic changes in protein expression due to protein turnover or in response to biochemical cues. While traditional protein assays have provided insights into localization, binding and protein-protein interactions; these single snapshots in time cannot follow the dynamics of proteins in living cells under physiological conditions and therefore are lacking in physiological context.

IncuCyte's live-cell analysis system and antibody labeling reagents enable a new assay approach that delivers spatial and temporal information at scale in living cells. The unique antibody reagents can be mixed with any Fc-containing antibody, applied directly to cells and then monitored under physiological conditions for hours or days. The real-time image acquisition and analysis means it is possible to quantitatively link the dynamics of a target protein with cell morphology, proliferation and function. The protocol is simple despite the richness of data and insight generated, involving a single antibody-labeling step followed by direct addition to cells, thereby removing the need for laborious labeling and blocking procedures and wash steps that can result in unwanted cellular changes and misleading results.

IncuCyte® FabFluor antibody labeling reagents are highly specific and sensitive, amenable to both adherent and non-adherent cell models. The data below exemplifies how the assays can be used to study long-term protein dynamics and their relationship to cell function, morphology, as well as cell-cell interactions.

Live-cell imaging and analysis approaches to studying protein dynamics

Fluorescently labeled antibodies are widely used as markers for visualizing cell surface protein expression, receptor interactions and antibody internalization. To build on existing methodologies for studying protein expression, a novel strategy was developed based on fluorescently labeled antibody fragments (Fabs) that target the Fc region of any mouse, human or rat antibody. Two types of reagents have been developed, differing in the type of fluorescent molecule conjugated to the Fab and thereby enabling two different applications, antibody internalization and live-cell immunocytochemistry. These two reagents, when combined with the IncuCyte® Live-Cell Analysis System, provide an integrated approach to studying protein dynamics in real-time and under physiological conditions.

How Live-Cell Protein Assays Work

Antibody internalization assays

In the antibody internalization assay, a pH-sensitive antibody labeling reagent, IncuCyte® FabFluor-pH Red, allows the specific detection of antibodies as they are internalized into the acidic conditions of endosomes and lysosomes, enabling real-time, kinetic monitoring over the full time-course of internalization.

Live-cell immunocytochemistry

To detect cell surface markers, IncuCyte® FabFluor-488, a Fab conjugated to a continuously fluorescing molecule at physiological pH, is utilized for antibody labeling. Addition of the IncuCyte® FabFluor-antibody complex to living cells enables kinetic detection of cell surface markers, making it possible to track the movement and interaction of cell subsets, reveal the dynamics of their interactions, and link time- and concentration-dependent changes in cell surface protein expression to other morphological and phenotypic parameters.

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Kinetic Antibody Internalization Assays

Visualization and quantification of antibody internalization, in real time

Introduction

The growing focus on antibodies as therapeutics makes it essential to be able to study their dynamics in a realistic cell environment. Antibodies are used both as therapeutic agents that themselves block the activity of certain molecules, or for the targeted delivery of treatments to sites or cells of interest. This includes the delivery of highly toxic drugs to cancer cells via antibody drug conjugates (ADCs), removal or degradation of surface receptors from cancer cells (i.e. EGFR), and antibody immunotherapies used to identify tumor cells for immune cell killing (i.e. ADCC or ADCP).

Each of these therapeutic strategies hold the promise of increased efficacy and reduced side effects, and each requires a series of antibody features, for example, to enable maintenance on the cell surface for identification of tumor cells, or for rapid internalization when delivering ADCs. To realize their potential, it is important to understand the uptake profile and clearance of antibody candidates, and to be able to measure and optimize functional responses to antibodies for optimal antibody engineering and internalization characteristics. For example, pinocytosis, which is one of the main elimination routes of antibodies, requires antibody optimization for qualitative pharmacokinetic measurements during therapeutic antibody development.

Current methods for measuring antibody internalization have several limitations. These include the requirement to label each antibody, conduct multiple washing steps, and the ability to only do single-time point analysis. This can lead to unnecessary and labor-intensive processes, loss of cells through washing, and missed and incorrect measurements due to limitations of single time-point analysis.

There is an urgent need to have an efficient, affordable method that can be used throughout the entire antibody screening and functional characterization process to fully and rapidly quantify antibody internalization using real time live-cell analysis.

In this chapter, we will illustrate how novel pH-sensitive Fc-targeting antibody labeling reagents allow for real-time rapid, kinetic, high-throughput analysis of antibody internalization in living cells. This is an ideal tool for optimal antibody engineering early in the biologic discovery process.

IncuCyte® FabFluor antibody internalization assays at a glance

The IncuCyte® antibody internalization assay uses IncuCyte® FabFluor-pH Red reagent, a novel pH-sensitive Fc-targeting antibody fragment, isotype-matched to the antibody of interest. The IncuCyte® FabFluor-pH Red reagent and antibody of interest are mixed in a one-step, no wash, labeling protocol. At pH 7.0, the FabFluor-Ab complex has little or no fluorescence. When labeled antibodies are added to cells, a fluorogenic signal is seen as the antibody is internalized and processed in acidic (pH 4.5-5.5) lysosomes and endosomes. The full time-course of antibody internalization can then be followed with the IncuCyte Live-Cell Analysis System for real-time analysis of internalization rates. An overview of the workflow is shown on the next page. (Figure 1)

Shortcomings of Traditional Assays	Live-Cell Imaging and Analysis Approaches
• Requires **labor-intensive labeling** of each antibody.	• **Rapid, single-step labeling** allows efficient testing of panels of antibodies.
• Only provides **single-time point analysis**.	• **Real-time, kinetic measurements** of antibody responses.
• **Low throughput** due to combination of labeling approach and end-point analysis.	• **High-throughput, reproducible antibody screening** validated with individual images.

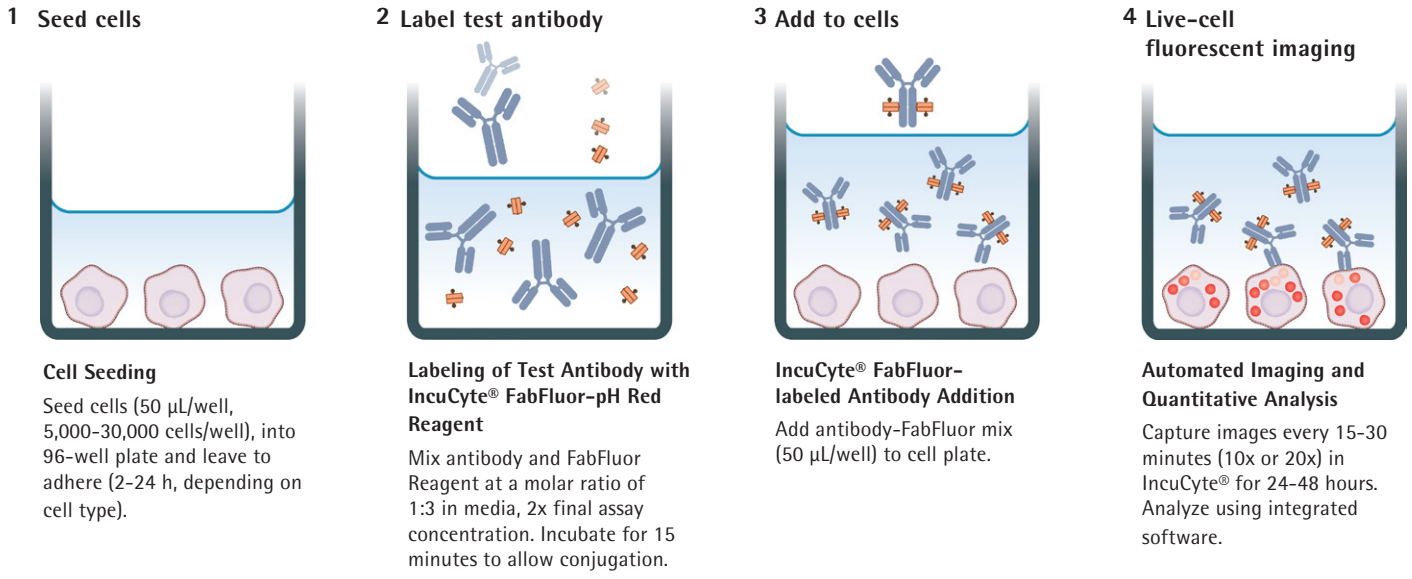


Figure 1. Overview of IncuCyte® antibody internalization assay

Sample Results

Antibody internalization with IncuCyte® FabFluor-pH Red reagent

Antibody internalization signal increases with cell number, but IncuCyte® antibody labels can distinguish the true internalization rate signal from that associated with cell proliferation by virtue of collecting both biochemical and morphological data simultaneously. Anti-CD71 internalization (red fluorescence area) was normalized to the total cell area (phase confluence), the internalization signals over time between different plates were highly similar. This normalization method helps to minimize the impact of variation in cell number between plates and reveals the true internalization rate of your antibody of interest.

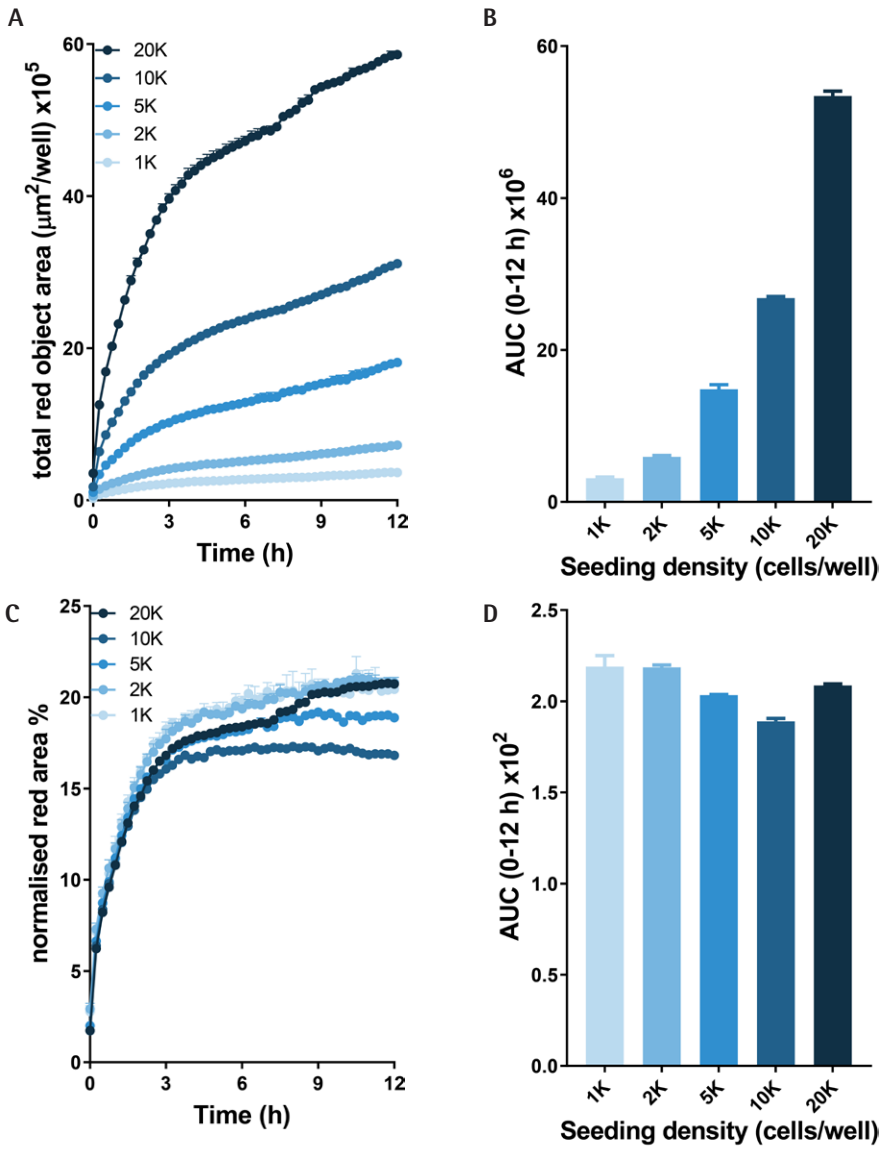


Figure 2. Antibody internalization response is cell number dependent. An increasing density of HT1080 cells were seeded (1-20K/well) and treated with IncuCyte® FabFluor-pH labeled α-CD71 (4 µg/mL). HD phase and red fluorescence mages (10X) were captured every 30 min over 12 h. The time-course of red object area data demonstrates an increasing internalization signal with increasing cell number (A and B). When the red object signal is normalized for phase area, it is clear the internalization response size is depending on cell number (C and D). All data shown as a mean of 3 wells ± SEM, bar graphs shown as area under the curve (AUC) calculated from time-course data.

Specific detection and visualization of internalization

The IncuCyte® analysis software robustly detects when an IncuCyte® FabFluor-pH Red labeled antibody has been internalized. Dual-labeling experiments were conducted in HT1080 fibrosarcoma cells using a lysosomal marker (LysoSensor® Green, Thermo Scientific) and an IncuCyte® FabFluor-pH Red-labeled anti-CD71 antibody (Figure 3). After three hours, a strong co-incident signal was measured: 74% of the red anti-CD71 signal was colocalized with green, confirming that the labeled antibody was internalized in the lysosome.

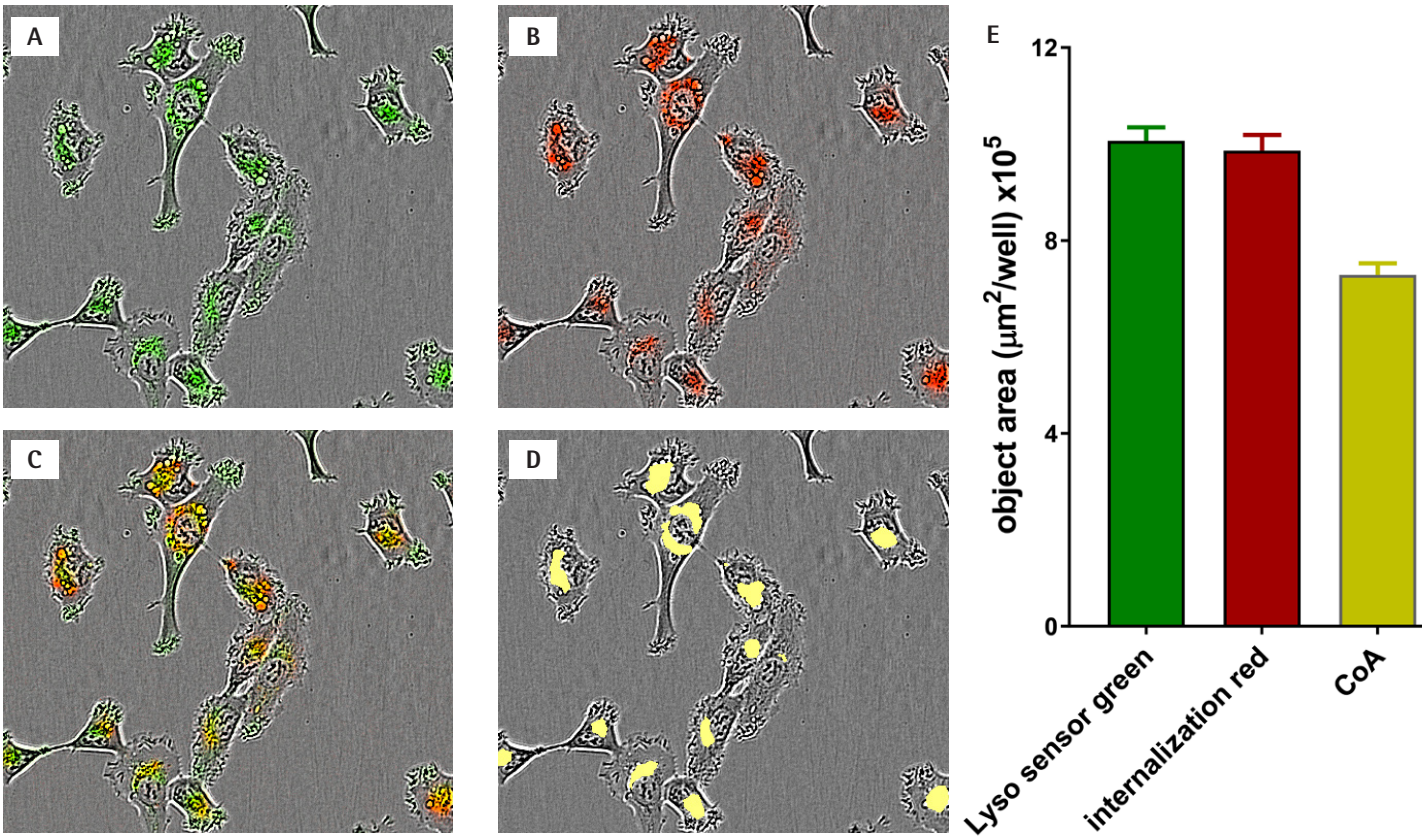


Figure 3. Colocalization of IncuCyte® FabFluor labeled α-CD71 and Lyso Sensor® Green in HT1080 cells.
Internalization of IncuCyte® FabFluor labeled α-CD71 (4 µg/mL) was established for 3 h in HT1080 cells before addition of LysoSensor® Green DND-189 (Thermo, 0.25 µM). Images show individual LysoSensor® Green and FabFluor labeled α-CD71 red signal (A and B), co-localization of red and green signals (C), and the co-localized analysis mask shown in yellow (D). (E) IncuCyte analysis of the coincidence of the red and green fluorescence confirms co-localization of 74% of the red signal with the green signal. Images captured at 20x magnification, 30 min post LysoSensor® addition, data shown as mean of 4 wells ± SEM.

Applicability across different cell types and antibodies

To demonstrate the broad application and specificity of the method, internalization was assessed for a range of test antibodies targeted against specific CD markers expressed in different cell lines (Figure 5). Anti-CD20 was internalized in the B-cell line Raji, but not Jurkat, a T-cell line. Conversely, anti-CD3 was internalized in Jurkat but not Raji. Antibodies to CD71 and CD45, general lymphocyte markers, were internalized in both cell types. Importantly, IgG was not internalized in either. These data are in alignment with the known CD surface marker expression of these cell lines, and provide strong confidence in the signal specificity and generic utility of the method.

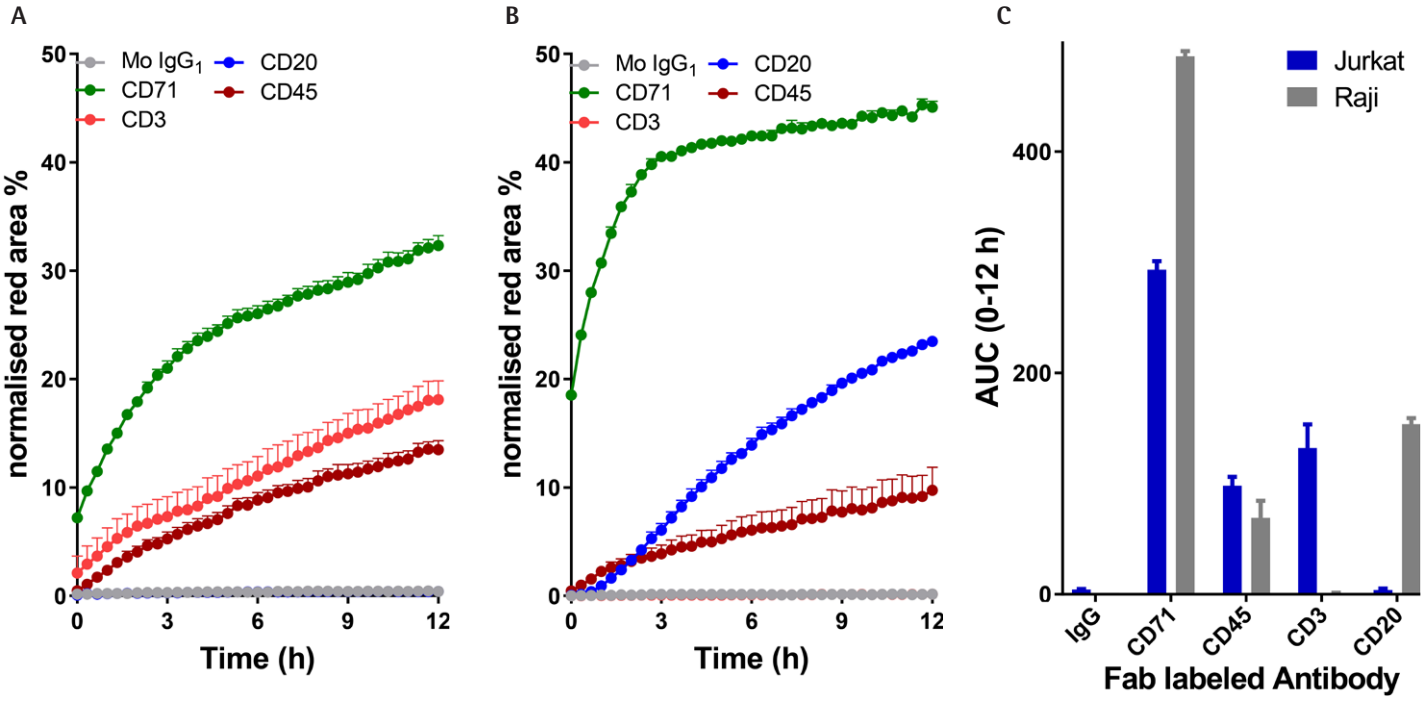


Figure 5. Internalization of CD surface marker targeted antibodies in lymphocytic cell lines.
Jurkat (T cell-like) and Raji (B cell-like) cells (30 K/well) were treated with different IncuCyte® FabFluor labeled antibodies (4 µg/mL). HD phase and red Images were captured every 30 minutes using a 20x objective over 12 h. Time course data (A and B) and area under the curve (AUC, C) analysis demonstrates the response profile in both cell lines. All data shown as a mean of at least 4 wells ± SEM, time course data shown as normalized red area.

Quantitative Pharmacological Analysis

Pharmacological, kinetic quantification of antibody internalization

To illustrate the quantitative nature of the method and suitability for analyzing therapeutic antibodies, we sought to determine EC50 values for the internalization of Herceptin (Trastuzumab) and Rituxan (Rituximab), two clinically used monoclonal antibodies. After labeling of each antibody with the FabFluor reagent, the antibody was serially diluted (1:2) prior to addition to the cells to enable construction of a concentration-response curve. Handling the labeled antibody this way is good practice to eliminate any variation in Fab labeling efficiency which could occur across the concentration range. In BT-474 Her2-positive breast carcinoma cells, clear time and concentration dependent internalization of Herceptin was observed over 48 h. From an area under the time-course (AUC) analysis, the EC_{50} value for internalization was 323 ng/mL ≡ 2.1 nM, Figure 6). In Raji cells, the EC_{50} value for Rituxan was 426 ng/mL ≡ 2.6 nM, Figure 7). These EC_{50} values are similar to the known KD values for Herceptin and Rituxan for their target receptors (both approximately 5 nM).

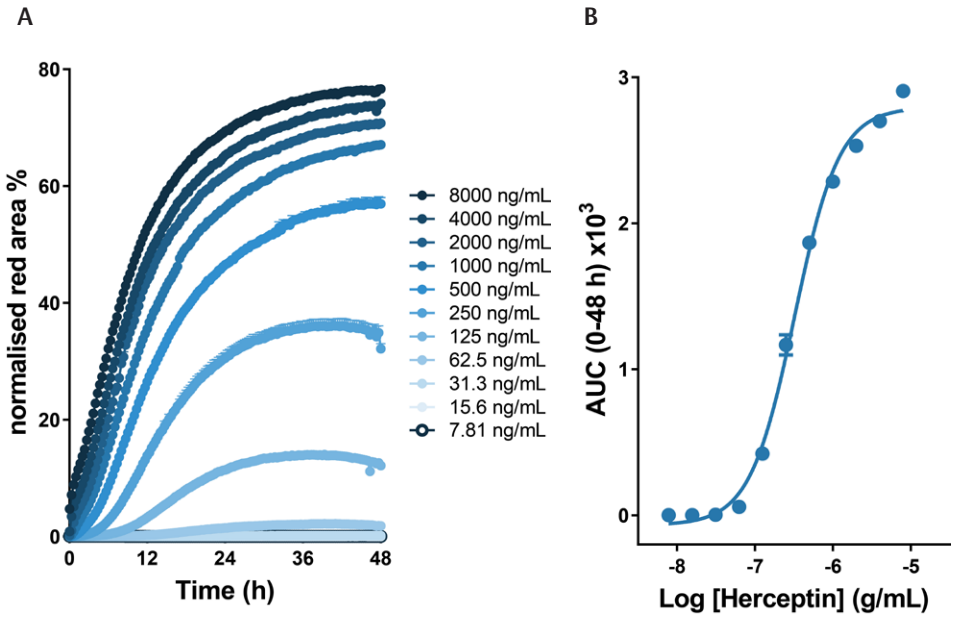


Figure 6. Quantitative pharmacological analysis of IncuCyte® FabFluor labeled Herceptin.
BT-474 Her2-positive cells were treated with increasing concentrations of FabFluor labeled Herceptin. The time course graph displays an increase normalized red area over time with increasing Herceptin concentrations (A). Area under the curve analysis of this response displays a clear concentration dependent response with an EC50 of 323 ng/mL (B). All data shown as a mean of 3 wells ± SEM, time course data shown as normalized red area.

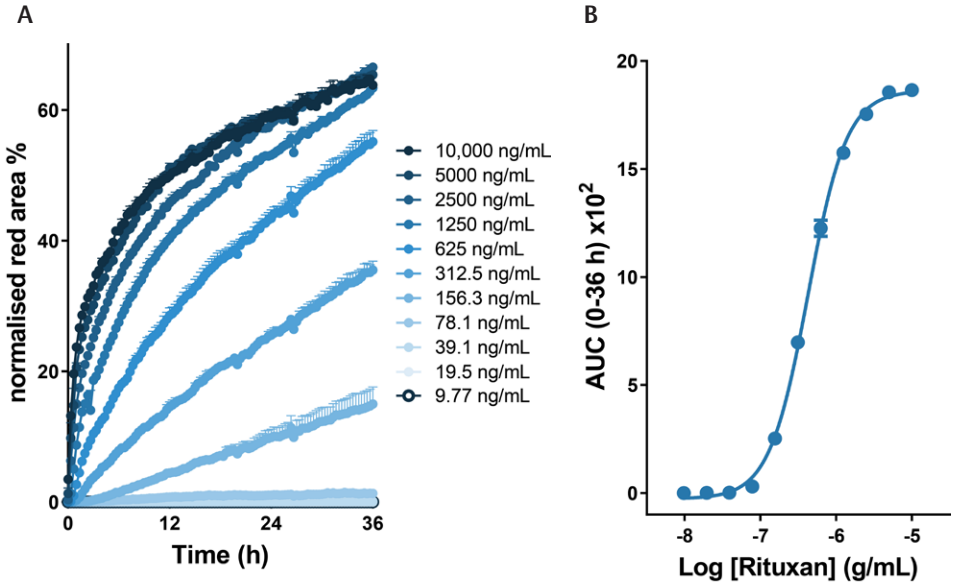


Figure 7. Quantitative pharmacological analysis of IncuCyte® FabFluor labeled Rituxan.
Raji cells were treated with increasing concentrations of FabFluor labeled Rituxan. The time course graph displays an increase normalized red area over time with increasing Rituxan concentrations (A). Area under the curve analysis of this response displays a clear concentration dependent response with an EC50 of 426 ng/mL (B). All data shown as a mean of 3 wells SEM, time course data shown as normalized red area.

Comparison of multiple test antibodies for high-throughput screening

The features of the IncuCyte and FabFluor solution are such that it should be facile to parallel label many antibodies and compare their internalization. To validate this we took 6 different commercially available anti-CD71 antibodies and compared their internalization properties head to head. The antibodies were plated in 96-well plates and labeled in full media with the IncuCyte® FabFluor labeling reagent. Serial dilutions were performed in full media (8 point, 1:2). Labeled IgG and FabFluor alone were added to control wells. Labeled antibodies were then added to pre-plated HT1080 cells and monitored for internalization for 12 h.

Of the 6 antibodies, 3 (Ab 1a, Ab2 and Ab 1b) produced large internalization signals and were detected at low concentrations (<0.05 ug ml-1). Reassuringly, Ab 1a and Ab 1b were the same antibody clone from different suppliers, and gave similar internalization responses. Abs 3, 4, and 5 were internalized more weakly and only at higher concentrations (Figure 8). From the control responses, a mean Z' value of 0.82 was determined (2 plates 0.75, 0.87) indicating a microplate assay with high robustness. These data confirm the suitability of the method for comparing the internalization of multiple antibodies at a single target, and illustrate that the internalization profile is a property of the antibody per se. Indeed, the assay precision and work flows are such that 100s of different antibodies could be compared at once and further throughput could be achieved through miniaturization to 384-well format.

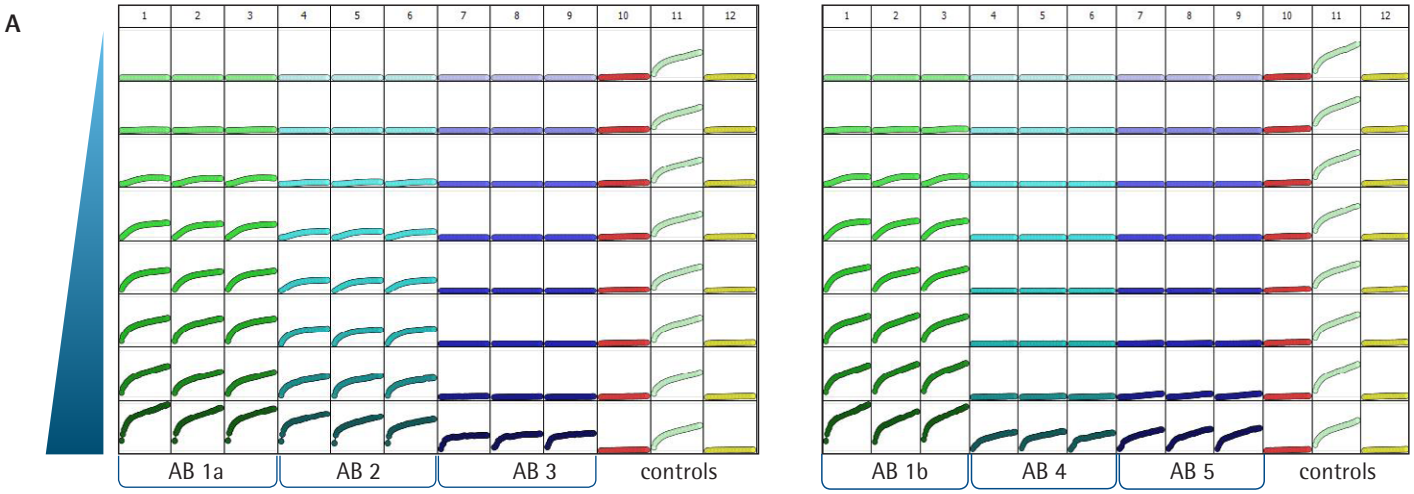


Figure 8. Screening test Abs for internalization. Six different CD71 antibodies including one clone from 2 different suppliers (clone 1a & 1b) were tested head to head in HT1080 cells. The antibodies were labeled with IncuCyte® FabFluor reagent prior to addition to cells and the internalization signal captured every 30 min over 12 h using a 10x magnification. Plate views taken from IncuCyte show clear positive and negative control responses in column 11 and 12 with concentration dependent responses for each antibody across two plates (A). Head to head analysis of antibody data shows a range of responses across these clones (B) control responses at 12 h display a clear positive response. All data shown as mean of 3 wells ± SEM, controls shown as mean of 8 wells.

Conclusions

The key features of the approach described in this application note are:

- A single step labeling protocol for easily tagging antibodies of interest with an Fc-targeted Fab coupled pH-sensitive dye (IncuCyte FabFluor). The labeling method is conducted in full media and is suitable for purified antibodies and antibody supernatants.
- An automated, image-based and real time analysis method (IncuCyte) for monitoring internalization in multiple 96-well microplates at once. The format is amenable to both adherent and non-adherent cells.
- An assay system that follows the full time course of the biology and reports internalization with high specificity, sensitivity and morphological information. The use of a pH-sensitive dye provides for low background signal and obviates the need to separate out fluorescence arising from antibody on the cell surface or in bulk solution.

Taken together, these attributes provide a simple, integrated and quantitative solution for directly studying internalization of antibodies into cells that can easily be scaled to compare many antibodies (10s-100's) in parallel. This method enables antibody internalization measurements to be implemented at earlier stages in the biologics discovery process, and will prove valuable in efficacy, safety and pharmacokinetic optimization of novel therapeutic antibodies. In addition, the method is suited to understanding basic mechanisms of endocytosis, pinocytosis and receptor turnover where antibodies can be employed.

Live-cell Immunocytochemistry

Long-term tracking and quantification of cell surface protein markers

Introduction

Immunocytochemistry (ICC) is a powerful laboratory technique for visualizing the cellular and subcellular location of proteins using fluorescent-labeled antibodies ('immunofluorescence'). The study of cell protein expression, and the modulation of it, is a crucial method used to understand gene expression, and ultimately the biology of the cell. With cell fixation protocols, labeled antibodies and specialized microscopes, spatial resolution down to the nanometer level can be achieved to evaluate the expression of proteins in the nucleus, specialized organelles and the cell membrane.

Where conventional ICC is less useful, however, is in studying dynamic changes in protein expression over time and under physiologically relevant conditions. The technique includes a number of lengthy and time-consuming steps – fixing, washes, and staining – which can take anything between six and 24 hours. These procedures can also lead to physical loss of cells, or result in unhealthy cells that are losing their viability because of fixation and are unlikely to give an accurate picture of the biology of interest.

ICC is a widely used technology for studying changes in protein surface molecules – the key communicators that dictate how a cell responds to external stimuli and changes its shape, protein expression and function in response. These cell surface molecules are the targets of many existing and future drugs and being able to monitor their activity in real-time and in their natural environment is paramount. Consequently, there is a strong unmet need for technical solutions that can allow the tracking of protein distribution and abundance over time in living cells, and link these vto cell morphology and function.

In this chapter, we illustrate how kinetic, live-cell analysis of surface protein expression using fluorescently-labeled antibodies opens up new possibilities for connecting long-term dynamic changes in protein abundance and distribution in cells with morphology and function.

IncuCyte® live-cell immunocytochemistry at a glance

In order to measure the dynamic changes in cell surface protein expression while simultaneously monitoring morphological changes, IncuCyte® FabFluor-488 Antibody labeling reagents are combined with Fc-containing antibodies. These conjugated FabFluor-Ab complexes are then added directly to live-cells in a single-step mix and read assay. Time-lapse images of cellular fluorescence can then be gathered over hours and days, and automatically analyzed to provide an index of the levels and pattern of expression over time. An overview of the assay workflow is shown below.

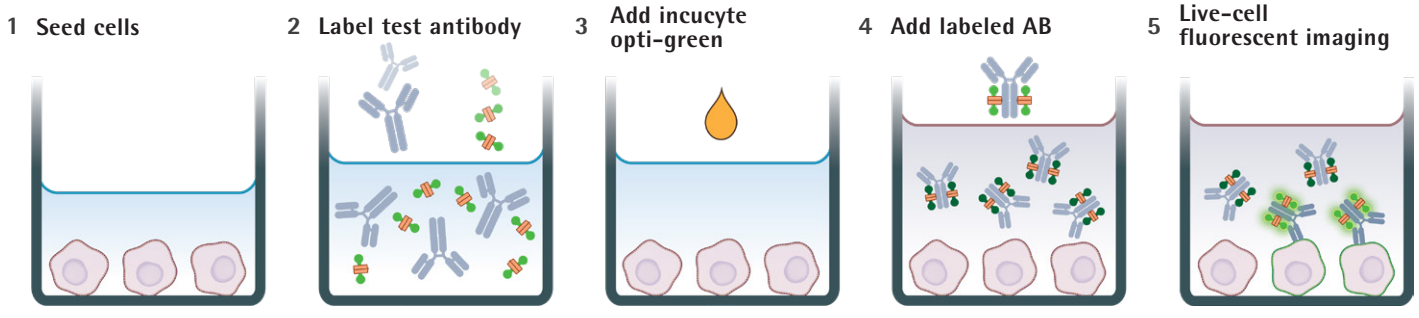


Figure 1. Overview of IncuCyte® live-cell immunocytochemistry workflow.

Key advantages:

- Measure surface protein expression and distribution over time using non-perturbing antibody labeling reagents in physiologically relevant conditions.
- Associate changes in surface protein expression with cell function and morphology to reveal informative, temporal changes in cell behavior.
- Visualize and quantify cell–cell interactions over time in complex co-culture models, revealing insight into the interplay of cells.
- Significantly increase productivity compared to conventional ICC by combining a rapid, single-step labeling protocol with automated acquisition and analysis. (Table 1).

	IncuCyte® Live-Cell ICC Approach	Traditional Fix and Stain ICC
Suitable for	Cell surface proteins	Both intracellular and cell surface proteins
Optimized for	Protein dynamics and linking changes to function and morphology. Cell identification and cell-cell interactions in motile systems	In-depth structural/morphological analysis, subcellular distribution, organelles, protein trafficking and redistribution
Reagents	Primary antibody + IncuCyte® FabFluor-488 + Opti-Green background suppressor	F-labeled primary or secondary Ab
Hardware	IncuCyte® S3 (and IncuCyte® ZOOM)	Fluorescent microscopes, high-content imagers (IncuCyte)
Protocols	Mix and read (one hour prep time)	Fix, wash and stain (6—24 hours?)
Resolution	Cellular, up to 20x magnification	Subcellular > 100x possible with oil immersion, etc.
Cell status	Living cells in media/serum	Dead/dying cells post fixation
Imaging paradigm	Repeated imaging over several days, time-lapse movies	Single time point (“end point”)

Table 1: Comparison of IncuCyte Live Cell ICC with conventional ‘fix and stain’ ICC.

Sample Results

Quantitative measurements of surface protein dynamics

Given the current explosion of checkpoint inhibitor cancer therapies, assays that expedite further studies on the regulation of immune-cell signaling pathways in tumors are an area of significant need. The below example illustrates how dynamic changes in cell surface checkpoint proteins can be quantified in living cells in response to an inflammatory stimulus, using Programmed Death Ligand-1 (PD-L1) as an archetype (Figure 2). The IncuCyte® FabFluor-488 antibody reagent was conjugated to anti-PD-L1 and applied the mixture to MDA-MB-231 breast cancer cells. Following treatment with IFN-γ, a time- and concentration-dependent increase in PD-L1 labeling was observed over 72 hours. IFN-γ had no effect on the growth rate of MDA-MB-231, indicating that the response was a specific upregulation of PD-L1.

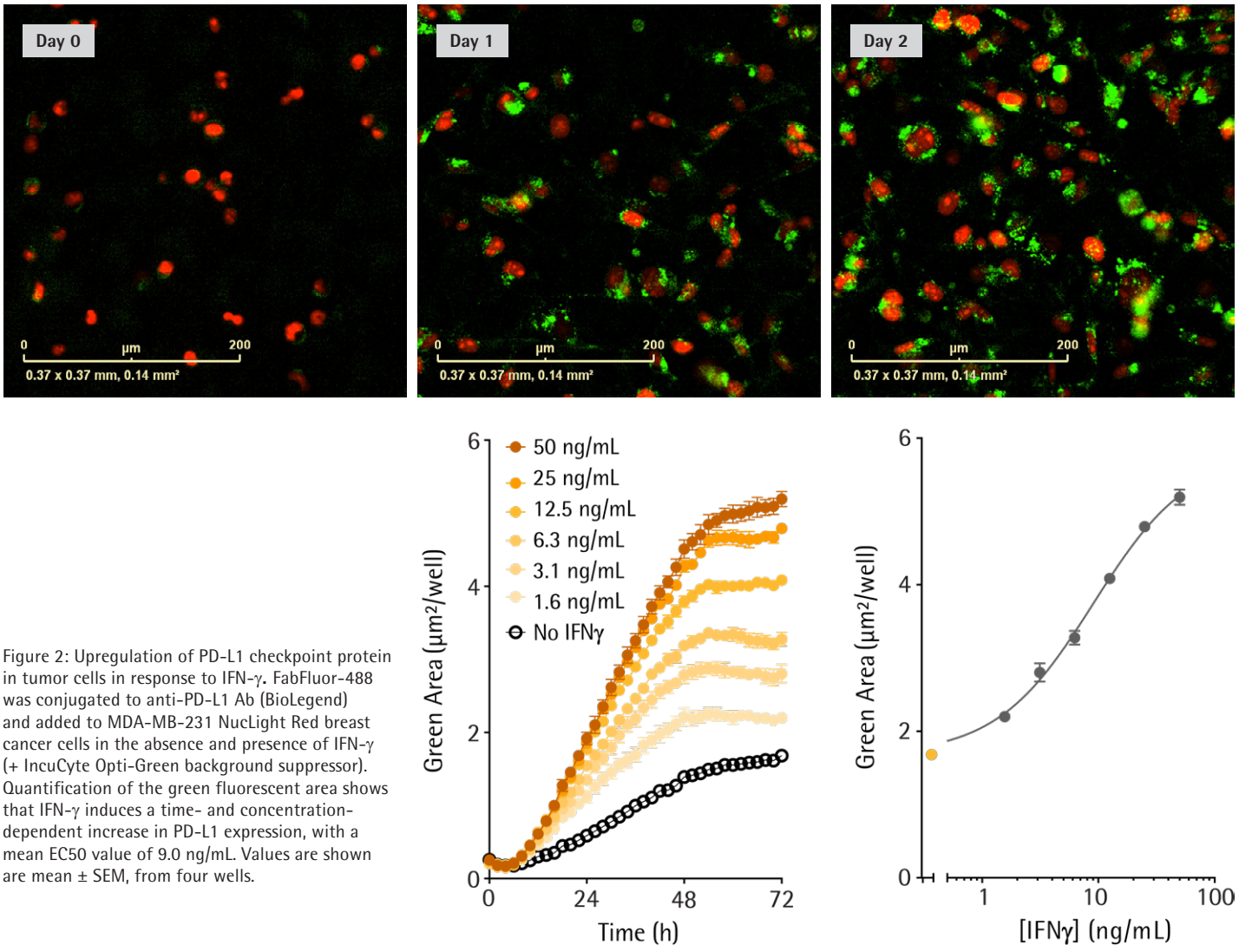


Figure 2: Upregulation of PD-L1 checkpoint protein in tumor cells in response to IFN-γ. FabFluor-488 was conjugated to anti-PD-L1 Ab (BioLegend) and added to MDA-MB-231 NucLight Red breast cancer cells in the absence and presence of IFN-γ (+ IncuCyte Opti-Green background suppressor). Quantification of the green fluorescent area shows that IFN-γ induces a time- and concentration-dependent increase in PD-L1 expression, with a mean EC50 value of 9.0 ng/mL. Values are shown are mean ± SEM, from four wells.

Coupling protein dynamics to morphological changes

Protein dynamics can also be coupled to morphological changes using IncuCyte® live-cell analysis. Here, measurements of cell surface markers were linked to morphological changes in human neuroblastoma cells (Figure 3). Here, once treated with a vitamin A derivative to induce differentiation, there was a clear association between the change in neural cell adhesion molecules (NCAMs) observed through live-cell ICC and the increase in neurite length over time.

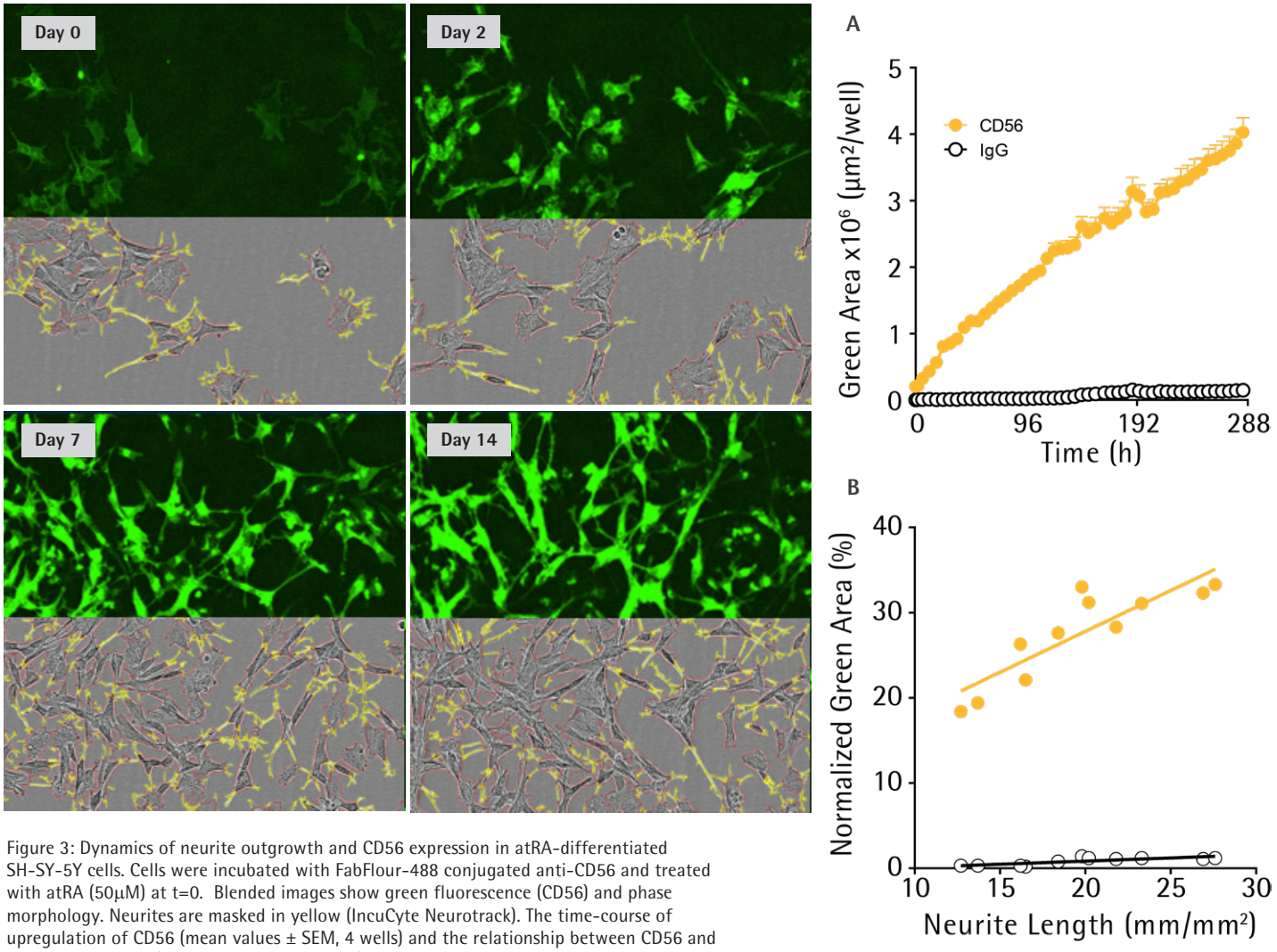
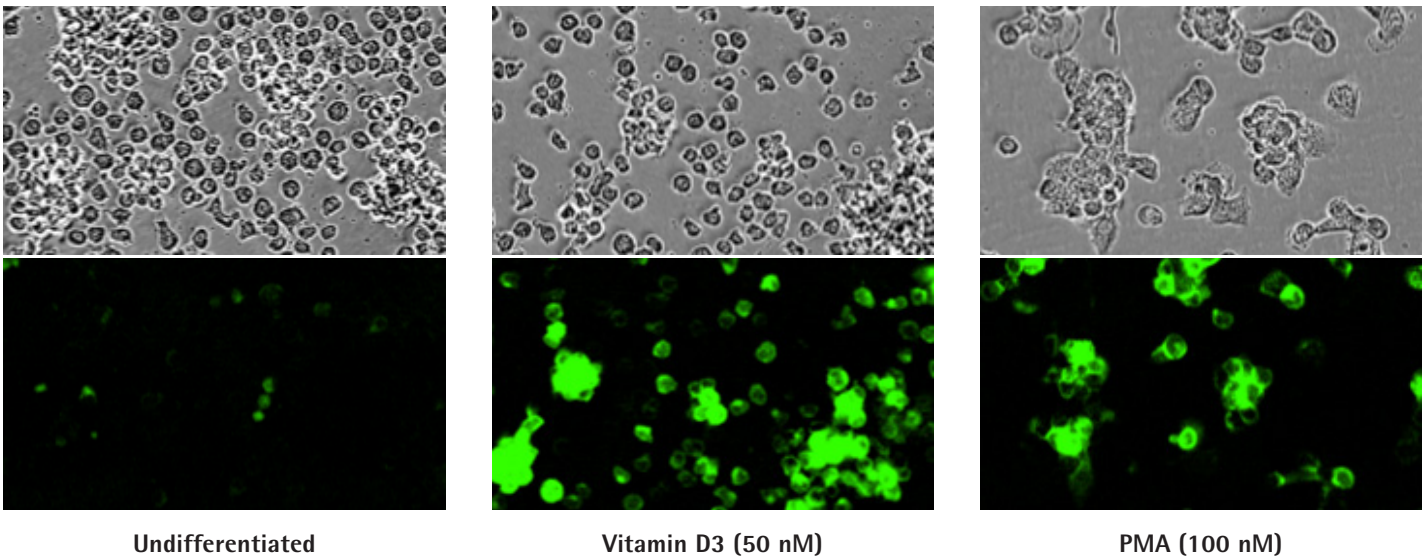
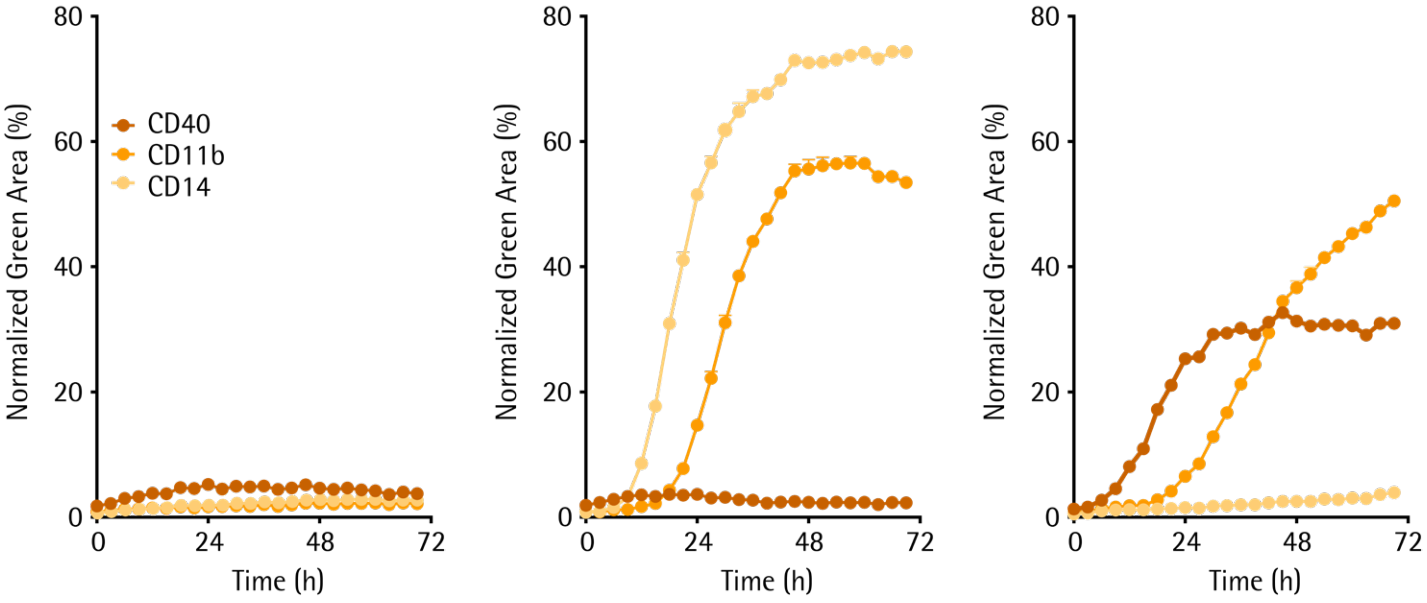


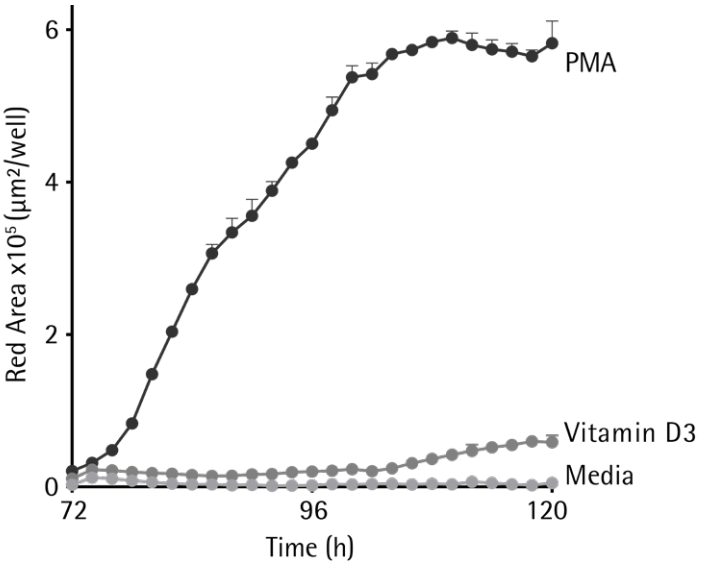
Figure 3: Dynamics of neurite outgrowth and CD56 expression in atRA-differentiated SH-SY-5Y cells. Cells were incubated with FabFlour-488 conjugated anti-CD56 and treated with atRA (50µM) at t=0. Blended images show green fluorescence (CD56) and phase morphology. Neurites are masked in yellow (IncuCyte Neurotrack). The time-course of upregulation of CD56 (mean values ± SEM, 4 wells) and the relationship between CD56 and neurite outgrowth (normalized to confluency) are shown in panels A and B, respectively. In panel B, each symbol represents data taken at different time-points throughout the experiment. Note the time-dependent increase in neurite length and associated CD56 signal.

Coupling protein dynamics to cell function

Live-cell ICC can also be used to correlate changes in morphology with function. In the below example, the differentiation of human monocytic cells into macrophage-like cells was tracked by IncuCyte® FabFluor-488 labeling of immune cell surface markers (Figure 4). It was found that only phorbol myristate acetate (PMA) produced a marked change in morphology, yielding large, flattened, adherent 'macrophage-like' cells. To correlate these observations with function, the ability of differentiated THP-cells to phagocytose IncuCyte® pHrodo-labeled apoptotic T cells was measured. Only those cells treated with PMA were phagocytic, illustrating that it is possible to associate protein measurements to the functional properties of cells.







	Undifferentiated	Vitamin D3	PMA
CD11b			
CD14			
CD40			
Growth inhibition			
Δ Morphology			
Phagocytosis			

Figure 4: Differentiation of hTHP-1 monocytes: surface marker and functional changes. hTHP-1 cells were exposed to media (undifferentiated), vitamin D3 (50 nM) or PMA (100 nM) in the presence of IncuCyte FabFluor-488 reagent complexed to CD11b, CD14 or CD40 Abs (+ IncuCyte Opti-Green). IncuCyte images (Top, 20x, every 3h) were analyzed for marker expression (green fluorescent area), normalized for confluency (Center: mean ± SEM, 4 wells). In the lower panel the phagocytic effects were determined by exposing the cells to pHrodo-labeled apoptotic Jurkat cells (1x10^6 per well) at 72h, and measuring red fluorescence. Note the association of the CD40 marker expression with the functional profiles (orange = +ve).

Monitoring cell-cell interactions

Finally, live-cell ICC was used to study the dynamic interactions of immune cells and tumor cells, in a co-culture model system (Figure 5). Close inspection of the IncuCyte® time-lapse movies revealed that individual and sometimes multiple immune cells associate with the target cell and remain attached even as the tumor cells move.

Using the CD8-labeling antibody it was possible to show that a subset of CD8+ cytotoxic T-lymphocytes specifically engaged with the tumor cells, and even detected a polarity to these cells, where the CD8+ region of the effector cell appeared to contact the target.

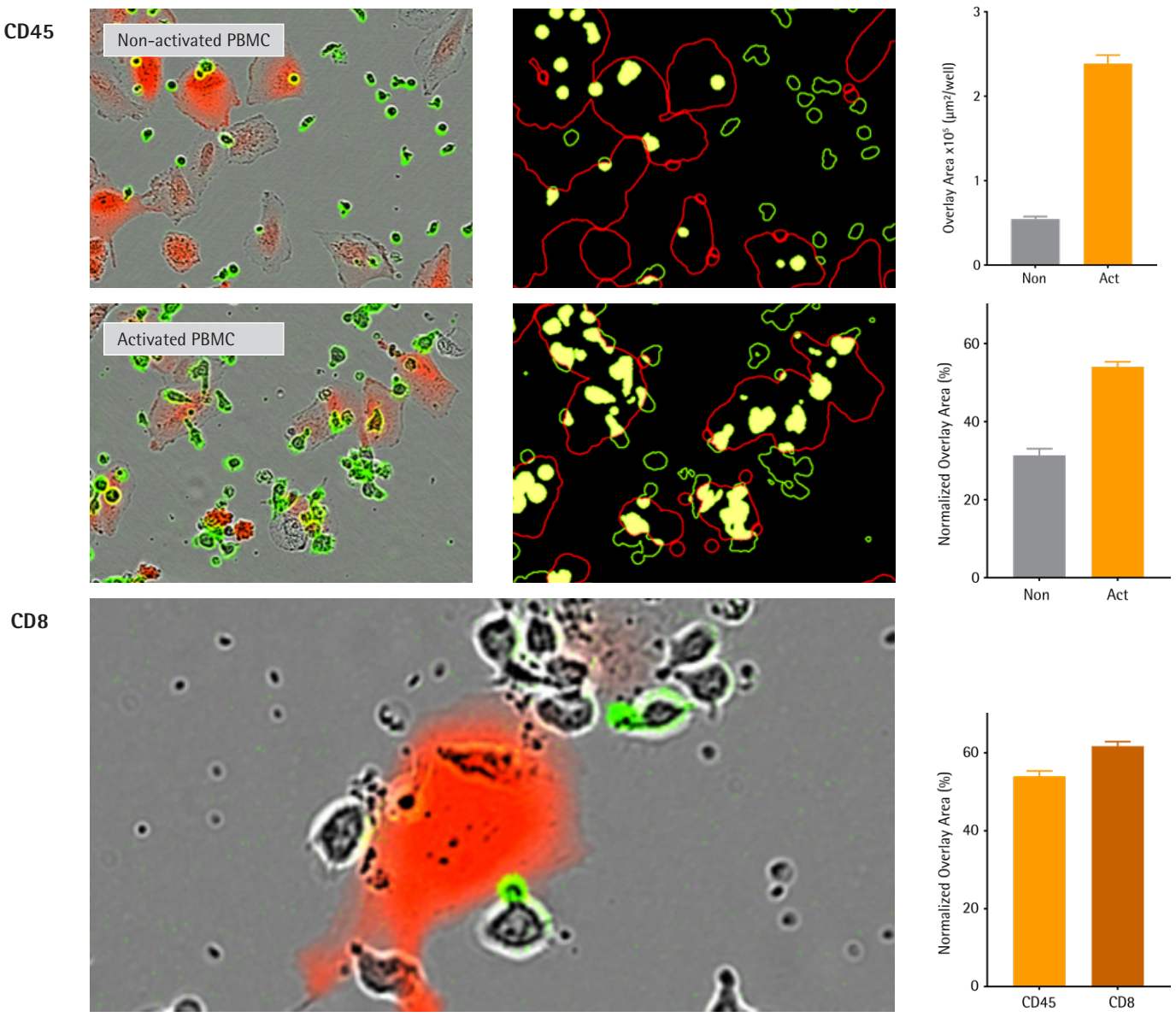


Figure 5: Visualization and quantification of cell-cell interactions. A549 CytoLight Red tumor cells were mixed with either pre-activated (anti-CD3/IL-2) or non-activated PBMC's in the presence of IncuCyte FabFluor-488-α-CD45 (upper) or CD8 (lower panel). The masked image (center) illustrates the overlay (yellow) of the immune cells (green) and tumor cells (red). The overlay area metric shows the increase in interaction between the two cell types upon activation, either without (upper) or with (middle) normalization to the green area. For CD8, note the clear polarity of the protein signal in the lymphocyte, and formation of the CD8/immune cell 'synapse'.

Conclusions

IncuCyte® live-cell Immunocytochemistry demonstrates how traditional ICC can be extended from a traditional 'fix and stain' approach to a method that allows for the dynamic monitoring of surface proteins in living cells over several days. Using a simple and robust labeling approach, IncuCyte® FabFluor-488 labeling reagents in conjunction with the IncuCyte® Live-Cell Analysis System, enables real-time quantification of dynamic changes in protein abundance and distribution with the ability to associate changes in cell morphology, function as well as study complex cell interactions.

- Live-cell ICC can reveal time-and concentration-dependent changes in cell surface, and can be combined with analyses of other morphological and phenotypic parameters to link surface protein expression changes with cell function over time proteins.
- By labeling different cell surface markers with IncuCyte® FabFluor-488 reagent it becomes possible to track the movement and interaction of subsets of living cells, revealing the dynamics of their interactions.
- Proximity relationships, such as those between the tumor and immune cells presented here, can be observed and quantified at throughput with unprecedented accessibility.
- IncuCyte® FabFluor-488 antibody labeling reagent is specific, stable under physiological conditions for long incubations, and allows image analysis without disturbing the biology, health or proliferation of cells.
- The reagent has been used successfully with a range of antibodies in a variety of cells displaying surface proteins – including immune cells, nerve cells and tumor cells.
- The reagent removes the need for researchers to source antibodies with appropriate fluorescent tags for their molecule of interest.
- The live-cell ICC method saves time – assays leverage automated acquisition and analysis of data and can be carried out simultaneously in up to six 384-well plates, without laborious fixation and washing protocols.

Kinetic Assays for Utilizing Complex Models

Live-cell microplate assays for studying growth and health of three-dimensional cultures

Introduction

It is well established that conventional monolayer cultures of tumor cells grown on plastic do not adequately reflect the in vivo situation.[1,2] Yet, in vitro culture models of cancer cells are still an integral component of clinical drug development and for the advancement of our understanding of cancer cell biology. 3D cell culture models, although still relatively new compared to traditional 2D monolayer cultures, are increasingly adopted as advanced tools to accelerate drug discovery efforts.[3] This shift is most notable in cancer biology, immuno-oncology and hepatotoxicity, where organoids and 3D microtissues can represent more relevant biological features such as hypoxic regions or tumor-immune cell interactions.[4,5] There are a multitude of approaches to generating these models and they can generally be described as scaffold-free (media-based) or scaffold-based.

Scaffold-free models are easily achieved using round-bottom ultra-low attachment (ULA) microplates to promote spheroid self-assembly. These models generally yield a single tumor spheroid per well and exhibit key features of solid tumors; larger spheroids consisting of proliferating, quiescent, and necrotic zones resulting from a radial gradient of nutrients, metabolites and oxygen.

Current scaffold-based models rely primarily on an extracellular matrix (ECM) such as Matrigel® or collagen and attempt to recapitulate both physical and biochemical characteristics of the tumor microenvironment. Generally, these models employ the use of flat bottom plates and result in multiple tumor spheroids per well.

Choosing a model depends on the scientific question or objective at hand. Single spheroid models may be more representative of large solid tumors and can be made with high reproducibility which is desirable for drug testing. Multi-spheroid models capture the inherent heterogeneity of tumor cells and are considered by many to be more physiologically relevant.

Employing these complex models is challenging due to inherent limitations for studying cell growth, kinetics, and other characteristics in this 3D setting. These include time-consuming, expensive or laborious workflows, labeling requirements that can affect cell biology, and single time-point or indirect read-outs that do not report the full experimental time-course and potentially miss valuable information about size or growth, or shifts in the characteristics of cell populations.

The IncuCyte® S3 Live-cell Analysis System, for both scaffold-free and scaffold-based 3D spheroid models, avoids such limitations by providing simple, cost-conscious protocols that result in 3D cultures amenable to imaging, employing either label-free techniques or use non-perturbing reagents, and enabling real-time monitoring of morphological and cell health measurements without removing precious samples from the incubator. The approach works both with single spheroids grown in round-bottomed ULA wells in 96- and 384-well formats, and multi-spheroid cultures grown on plates coated with Matrigel® to reproduce the ECM scaffold.

The IncuCyte® approach has many advantages over traditional cell analysis methods (summarized in Table 1). In the following chapter, the assays are described in detail, demonstrating their potential as a valuable tool for insightful scientific discovery and drug screening and development.

Shortcomings of Traditional Assays	Live-Cell Imaging and Analysis Approaches
• **Endpoint collection of data misses important information** between imaging intervals that is needed for complete insight into biological processes.	• Continuous long term **monitoring captures important kinetic information on cellular processes and morphology**, providing for additional mechanistic details.
• Repeated removal of cells from incubator for lengthy image acquisition results in **loss of control for O_2 and CO_2 and multiple environmental fluctuations.**	• Uninterrupted environmental control within incubator, coupled with automated image acquisition (DF Brightfield, HD Phase, and fluorescent) results in a **continuous physiologically-relevant environment.**
• Incorporation of labels that can **perturb cells** and may confound interpretation.	• **Label-free** analysis of growth and shrinkage.
• **Additional time required** for development and optimization of image acquisition parameters.	• **Non-perturbing**, optimized, validated cell health reagents for enhanced physiological relevance.
• Complex image processing **requires expert operation and training** for data generation and analysis.	• **Automated acquisition and analysis** of high quality, high contrast images, combined with lab-tested protocols, to quickly gather reproducible, quantitative data for pharmacological screening in 96 and 384-well formats.
	• Purpose built software tools and guided interface **enables non-expert operators** to perform image processing and generate publication-ready graphics.

Table 1. Traditional vs Live-cell Analysis.

How Live-Cell Spheroid Assays Work

Multi-spheroid assays

Scaffold-based, multi-spheroid assays allow for study of cell cultures in a more physiologically relevant, 3D format. These assays permit the tracking and quantification of spheroid formation, cell-cell, and cell-ECM interactions in real time inside a tissue culture incubator.

Single spheroid assays

These assays provide an integrated solution to automatically track and quantify single tumor spheroid formation, growth, and health in real time with minimal environmental disturbance inside a tissue culture incubator, providing additional physiological insights.

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1. Catchpole I, et al. **Engineering T-cells for adoptive cell therapy to overcome TGF-β-mediated immunosuppression in the tumour microenvironment.** *Annals of Oncology*;28 (S11) mdx711.081, 100P (2018)

Invading cells from 3D spheroid

2. Fujita, M, Imadome, K, and Imai, T. **Metabolic characterization of invaded cells of the pancreatic cancer cell line, PANC-1.** *Cancer Sci.*, 108(5); 961-971 (2018)

Tumorsphere Growth Kinetics and Cell Number

3. Cazet AS, et al. **Targeting stromal remodeling and cancer stem cell plasticity to overcome chemoresistance in triple negative breast cancer.** Pre-print under review Cold Spring Harbor Laboratory, *bioRxiv* preprint (2018) URL: https://www.biorxiv.org/content/early/2017/11/08/215954

4. Galbraith MD, et al. **CDK8 Kinase Activity Promotes Glycolysis.** *Cell Rep, Cell Rep*, 21(6); 1495-1506, (2018)

5. Cesi G, et al. R**OS production induced by BRAF inhibitor treatment rewires metabolic processes affecting cell growth of melanoma cells Cancer**, *Mol. Cancer* 16(1); 102, 42894 (2018).

Mammosphere Growth

6. Fettig LM et al. **Cross talk between progesterone receptors and retinoic acid receptors in regulation of cytokeratin 5-positive breast cancer cells.** *Oncogene*, 36(44);6074-6084 (2018)

Neurosphere Growth Kinetics

7. Griesinger AM et al. **Neuro NF-κB upregulation through epigenetic silencing of LDOC1 drives tumor biology and specific immunophenotype in Group A ependymoma .** *Neuro-Oncology*, Volume 19, Issue 10, 1 October;1350–1360 (2017)

Kinetic Multi-Spheroid Assays

Integrated turnkey solution for reproducible analysis of multi-spheroid cultures

Introduction

The use of multi-cell tumor spheroids as a model for oncology research has expanded rapidly in recent years. As 3D spheroid protocols become more accessible to researchers, the experimental models have become more complex with correspondingly greater translational potential.

Multi-spheroid models have a number of advantages over conventional cell culture. In 2D cell culture, tumor cells are grown in monolayers under conditions that are quite different from the physiological conditions of a tumor. They are grown on a rigid, non-biological surfaces, have an abundance of oxygen, and an excess of nutrients produces hyper-nourished cells with unrestricted and non-physiological proliferation characteristics. Using such cells for drug screening introduces unwanted bias, with a tendency to identify molecules that work only against a uniform population of proliferative cells, and overestimating efficacy.

A more realistic approach involves recreating more physiologic heterogeneity inherent to a 3D tumor structure and providing a microenvironment more closely recapitulating in vivo conditions, which includes key interactions between the tumor and the extracellular matrix (ECM).

Scaffold-based 3D spheroid models, in which tumor cell aggregates are grown in ECM scaffolds such as Matrigel®, more closely recapitulate physiological growth conditions of tumors, enabling the study of interactions between tumor cells and the microenvironment. Tumor cells grown in 3D scaffold-based culture can form cell-cell and cell-matrix interactions. The heterogeneous nature of the tumor microenvironment can also be studied through the use of co-culture models, such as tumor cells with fibroblasts or immune populations.

This chapter illustrates how IncuCyte's multi-spheroid assays can provide a rapid method for the pharmacological investigation of potential drug candidates. The approach allows the measurement of real-time viability and toxicity measurements in a meaningful, multicellular in vitro model that better reflects the heterogeneous nature of tumors and allows deeper study of their potential physiological interactions with the tumor microenvironment.

IncuCyte® S3 Multi-Spheroid assays at a glance

The IncuCyte® S3 Multi-Spheroid assays combine IncuCyte's proprietary DF Brightfield image acquisition mode with a 3D multi-spheroid model grown on a layer of extracellular matrix in a 96-well format. Multiple protocols have been developed to suit various experimental objectives and cost considerations.

After coating either flat-bottom or round-bottom culture plates[1] with Matrigel®, cells are added to the wells with or without IncuCyte® Cell Health Reagents. An optional layer of Matrigel can be added to "sandwich" the cultures and surround them with ECM. The cells are then automatically scanned in the IncuCyte® S3 Live-Cell Analysis System every six hours to monitor multi-spheroid formation.

After treatments are added, the spheroid growth and shrinkage assay is initiated and monitored by repeat scanning every six hours in the IncuCyte® S3 system for up to two weeks. Spheroid size is reported based on DF Brightfield image analysis and can be accessed in real time or on demand as needed. The simple protocol is shown below:

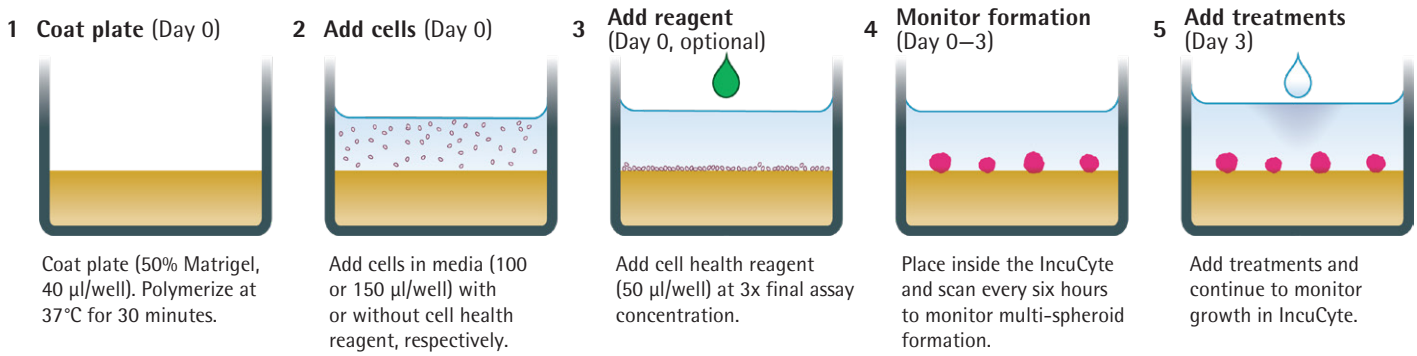


Figure 1. Overview of workflow for generation and analysis of multi-spheroid cultures in an IncuCyte® S3 Live-Cell Analysis System.

1. Protocols have been developed using either 96-well flat bottom or round bottom plates to address cost concerns when utilizing Matrigel. Round bottom plate protocols use four times less Matrigel than flat bottom plate protocols.

Key advantages:

- Quantify label-free growth and investigate morphology of multiple spheroids grown on ECM inside a standard tissue culture incubator, ensuring improved physiological relevance.
- Multiplex kinetic viability and toxicity measurements using IncuCyte® non-perturbing Cell Health Reagents to investigate drug mechanisms of action.
- Generate reproducible data suitable for pharmacological analysis with lab-tested and cost-conscious protocols, high quality images, and unbiased analysis.
- Rapidly create concentration response curves from thousands of images generated in 96-well plates.

Application of IncuCyte® S3 Multi-Spheroid live-cell assays

Combining the IncuCyte® DF Brightfield image acquisition with a 3D multi-spheroid cell model on ECM allows for monitoring and quantification of changes over time and under more physiological relevant conditions. As the spheroids remain undisturbed within the incubator while images are taken, it is possible to visualize and quantify spheroid growth, measure cell health, and better understand mechanisms of drug action.

Label-free image acquisition using novel DF-Brightfield reveals morphology and enables quantification of spheroid growth over time

Introduction of a new, enhanced depth of focus brightfield (DF Brightfield) image acquisition enables imaging of multiple tumor spheroids on a layer of extracellular matrix (Figure 2). This enhanced image acquisition results in high contrast brightfield images that can be reliably segmented and analyzed using IncuCyte's purpose-built processing algorithms.

The novel DF Brightfield image capture provides high-quality images of multi-spheroids formed from a range of tumor cells (Figure 3). Within just three days, different tumor cell lines begin to show distinctive morphology, demonstrating the physiological relevance of the models.

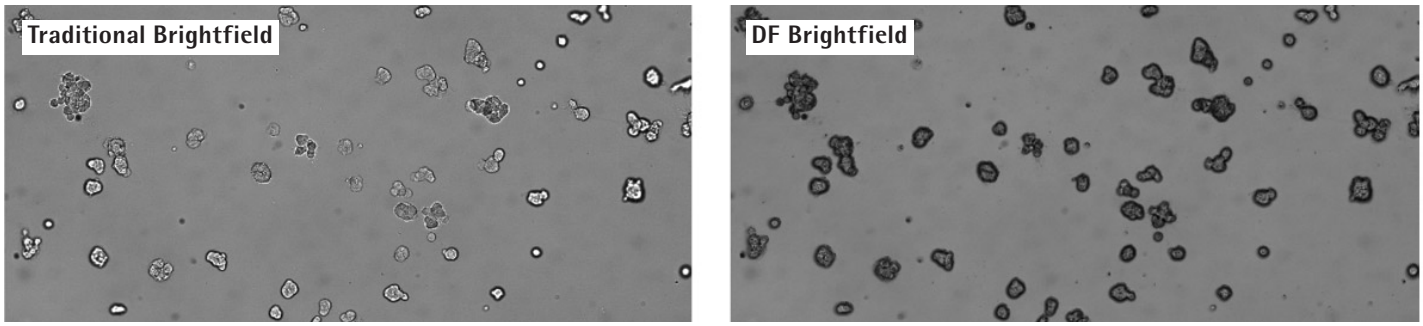


Figure 2. IncuCyte's proprietary image acquisition technique, DF Brightfield for 3D Cultures, generates high contrast, extended depth of focus images when used in conjunction with the IncuCyte S3 Multi-Tumor Spheroid protocol. Subsequent segmentation and analysis with IncuCyte S3's image processing algorithms results in reproducible data without operator bias.

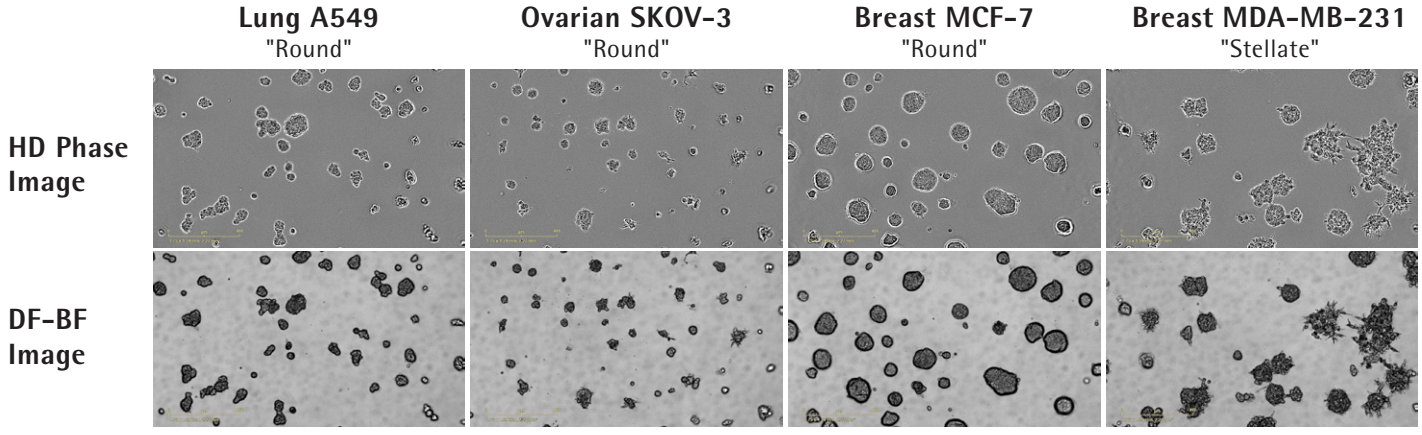


Figure 3. High quality HD Phase and DF Brightfield (DF BF) images of multi-spheroids (MS) formed from a range of tumor cell lines (5 d post seeding) on a Matrigel® base. Three day post seeding, A549, SKOV-3 and MCF-7 cells formed round aggregates, while MDA-MB-231 MS exhibited stellate branching distinctive of an invasive morphology.

IncuCyte S3 multi-spheroid automated image acquisition and analysis software tools enable label-free, kinetic quantification of growing and shrinking multi-spheroids over time. Figure 4 (below) illustrates the growth curves of three multi-spheroid tumor models over several days and demonstrates the inhibitory effects of the cytotoxic drug camptothecin (CMP) on their growth trajectories.

Segmented DF Brightfield images compare vehicle or CMP treated conditions at 168 h. Time courses show the individual well total Brightfield Object area (µm2) (y-axis) over 168 h and illustrate specific cell type-dependent kinetic profile of spheroid growth and shrinkage. Data were collected over 168 hour period at 6 hour intervals. All images captured at 10x magnification. Each data point represents mean ± SEM, n=6 wells.

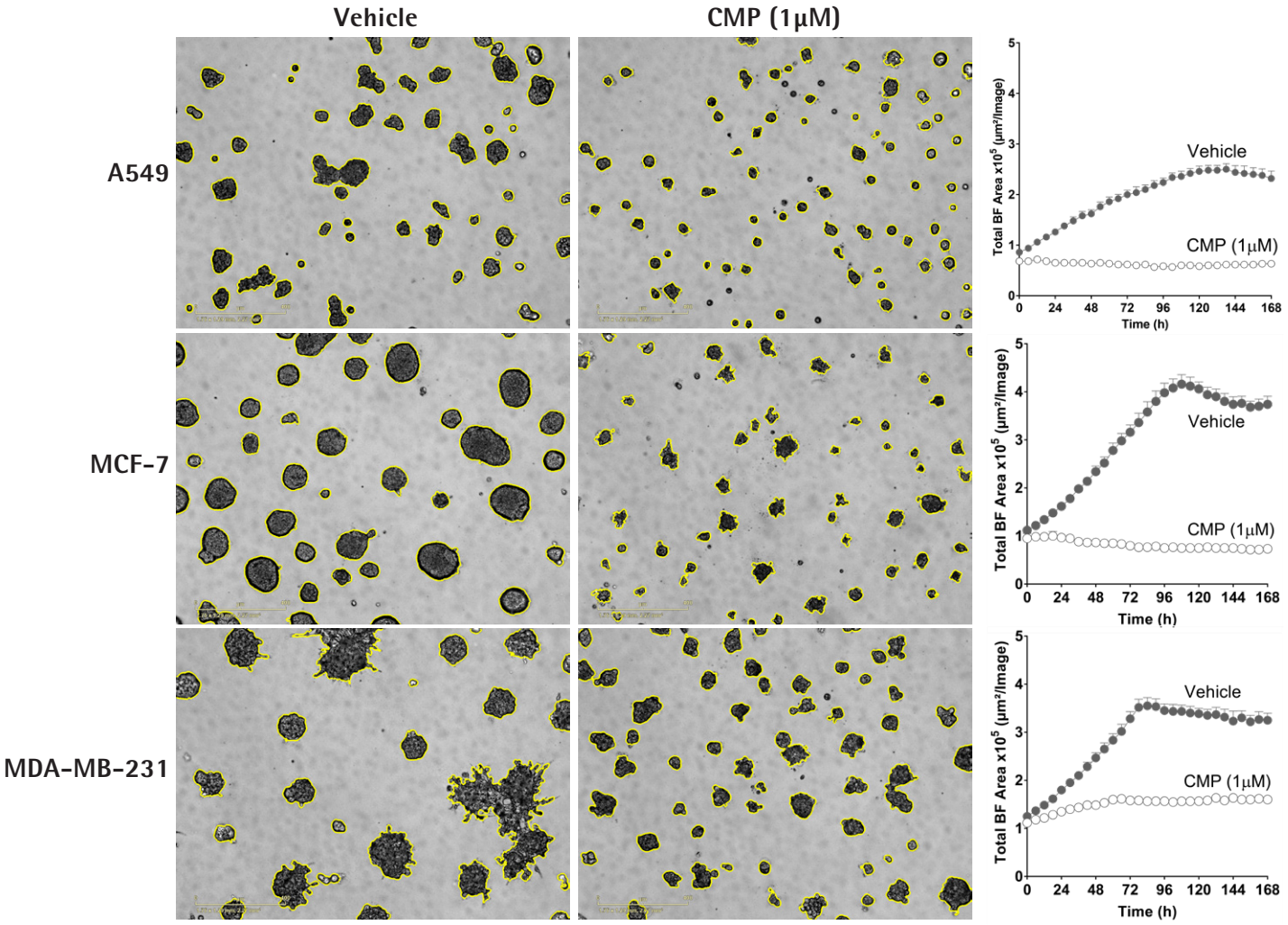


Figure 4. Real-time quantification of the growth of A549, MCF-7 and MDA-MB-231 spheroids. Cells were seeded in flat-bottom 96-well plates (2,000 cells per well) on a bed of Matrigel® and spheroids allowed to form (72 h). Spheroids were treated with either vehicle (0.1% DMSO) or CMP (10 µM).

Fluorescent readouts of stably expressing nuclear restricted fluorescent protein act as a surrogate for cell viability

When a range of antibodies were tested against different cell surface markers in various lymphocyte cell lines (Figure 4) it was found that the internalization patterns corresponded with the known surface marker expression of these cells. Labeling antibodies with IncuCyte® FabFluor-pH Red showed that anti-CD20 was internalized in a B-cell line, but not a T-cell line, whereas anti-CD3 was internalized in T-cells and not B-cells. Antibodies to CD71 and CD45, which are general lymphocyte markers, were internalized in both cell types. Crucially, the non-specific mouse IgG was not internalized in any of the cell lines. These data demonstrate the broad application and specificity across different cell types and molecules.

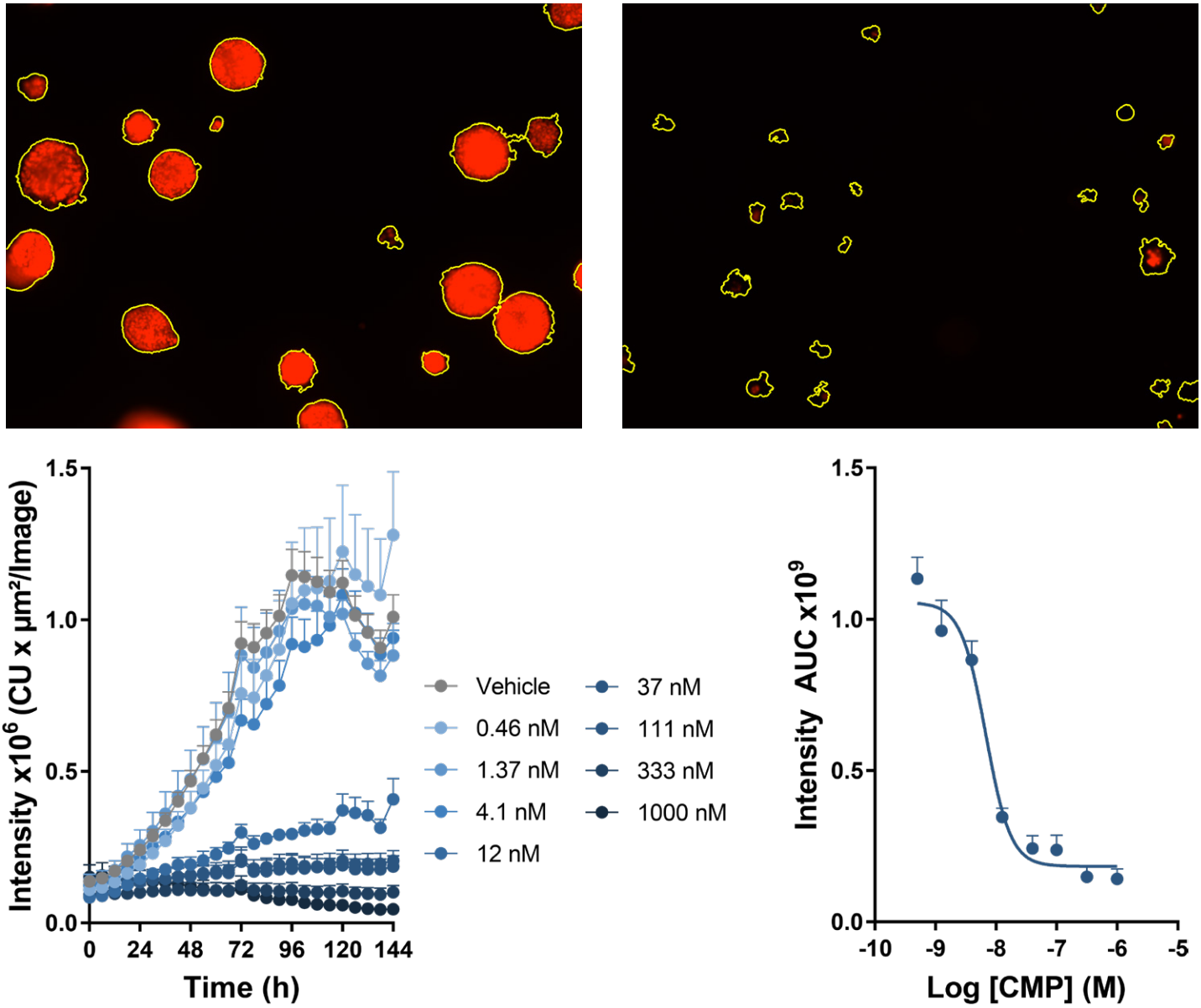


Figure 5. Analysis of spheroids expressing IncuCyte® NucLight Red fluorescent proteins enables determination of spheroid viability. Representative images taken at 144 h show a strong red fluorescent signal in a vehicle control spheroid, in contrast to a marked loss of red fluorescence in the CMP treated spheroid. The yellow boundary in the images represents the DF Brightfield mask outline. Monitoring the integrated intensity from within the DF Brightfield boundary highlights increasing fluorescence under vehicle control conditions corresponding to the growth of the spheroid. Upon treatment with CMP (0.4 nM - 1 µM), a concentration-dependent reduction in integrated fluorescence is observed, with complete loss of fluorescence at the highest concentration tested after 144 h.

Multiplexed viability and toxicity measurements enable investigation of mechanisms of action

In addition to screening for cytotoxicity, live-cell multi-spheroid assays can be used in conjunction with IncuCyte® Cell Health Reagents to determine the mechanism of action of drugs by combining data on size with levels of cytotoxicity and apoptosis (Figure 6). Here, the power of the assay is illustrated, distinguishing the cytotoxic effects of CMP from that of cycloheximide (CHX). The ability to multiplex reagents with fluorescent-expressing cell lines provides a depth of information on drug mechanism of action under realistic physiological conditions.

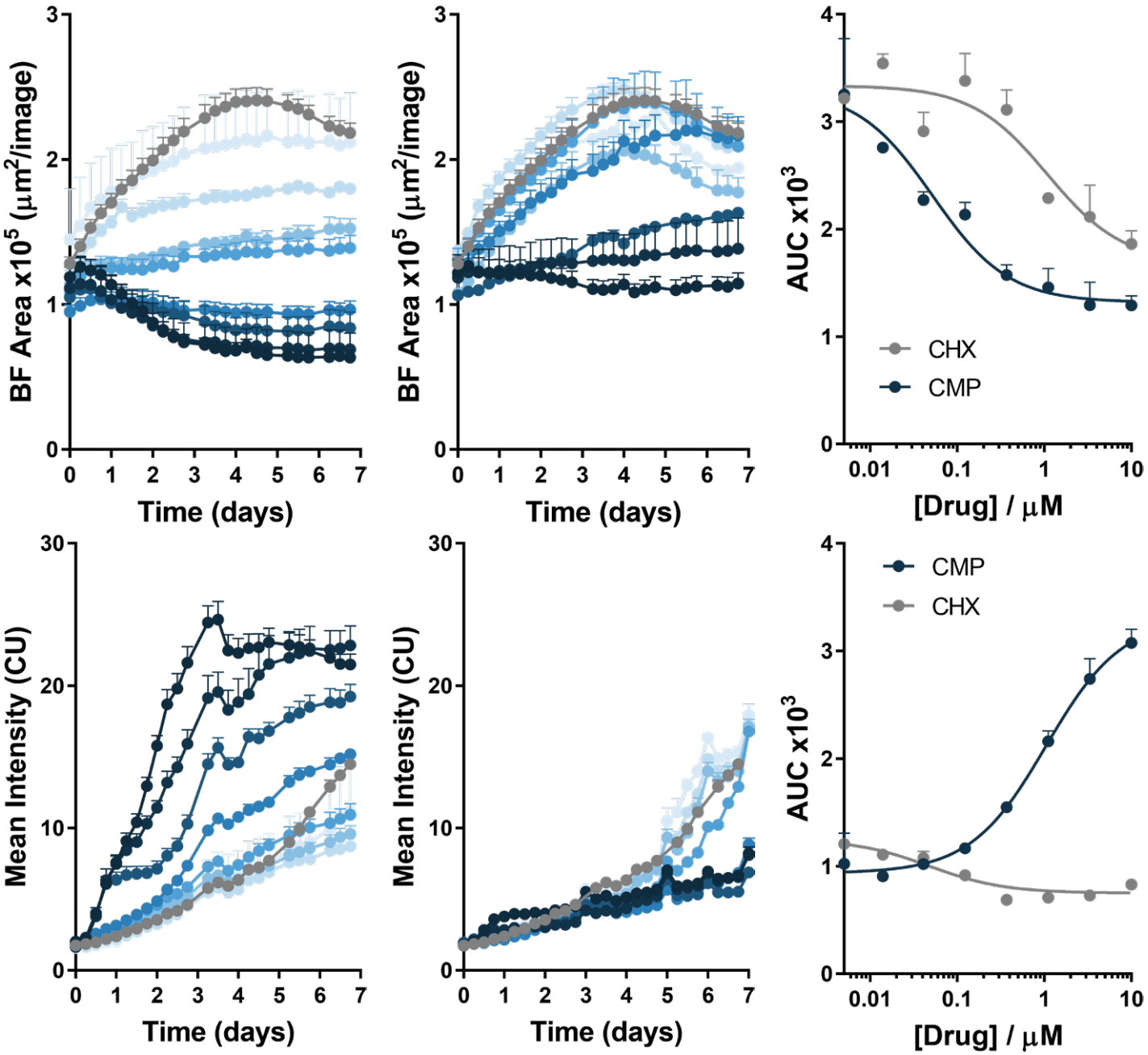


Figure 6. Cytotoxic and cytostatic mechanisms of action can be differentiated by measuring spheroid size and apoptosis. A549 cells were plated at a density of 2,000 cells per well and spheroid allowed to form (96 h). Spheroids were treated with increasing concentrations of CMP (top row, 4 nM – 10 µM) or CHX (bottom row, 4 nM – 10 µM) in the presence of IncuCyte® Annexin V Green reagent (1%). Images were taken every 6 h for 10 d. Time courses show change in size (Brightfield area) or apoptosis (IncuCyte Annexin V fluorescence intensity) over time. CRCs show the different profiles of cytotoxic and cytostatic mechanisms.

By enabling long term, continuous live-cell analysis under uninterrupted incubation, the IncuCyte® S3 multi-spheroid assay makes it possible to derive meaningful EC50 values for drugs by deriving concentration response curves over several days without disturbing cell biology. In the figure below (Figure 7) this is demonstrated for CMP and CHX, and this approach is used to further validate drug mechanism of action observed through earlier analysis.

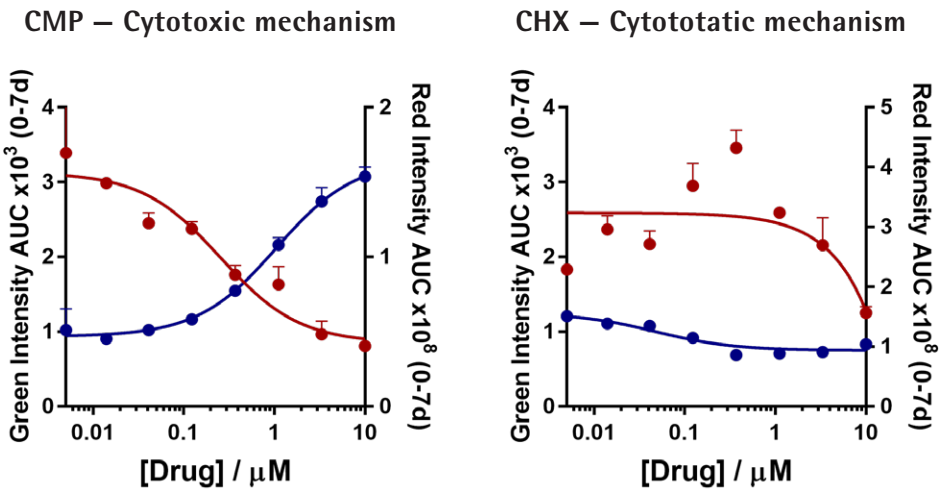


Figure 7. Determination of EC50s of CMP and CHX using the IncuCyte S3 Live-cell Analysis System to confirm mechanisms of action in response to cytotoxic (CMP) and cytostatic (CHX) drugs. IncuCyte A549 NucLight Red Cells (2k/well, nuclear-restricted FB indicating viability) were seeded in the present of IncuCyte V Annexin Green reagent (1%, apoptosis marker) and spheroids allowed to form for 3 days. Spheroids were then treated with a concentration range of either CMP, CHX, or vehicle control, and the cells were imaged every 6 h for 7 d. Both CMP (cytotoxic) and CHX (cytostatic) drugs caused a concentration-dependent inhibition of spheroid growth (total BF time courses, not shown). The CMP EC50, using AUC from 0-7 d after treatment, showed a concentration-dependent loss of viability and increase in apoptosis, while CHX EC50s showed little change in viability and no increase in apoptosis.

Reproducible, quantitative data at throughput suitable for pharmacological testing

A pharmacological study performed in MCF-7 breast cancer cells illustrates the potential of the IncuCyte's multi-spheroid assays for drug toxicity testing (Figure 8, below). The ability to view spheroids in 96-well plates over time provides a rapid method for comparing the potencies and toxicities of different compounds within the same assay.

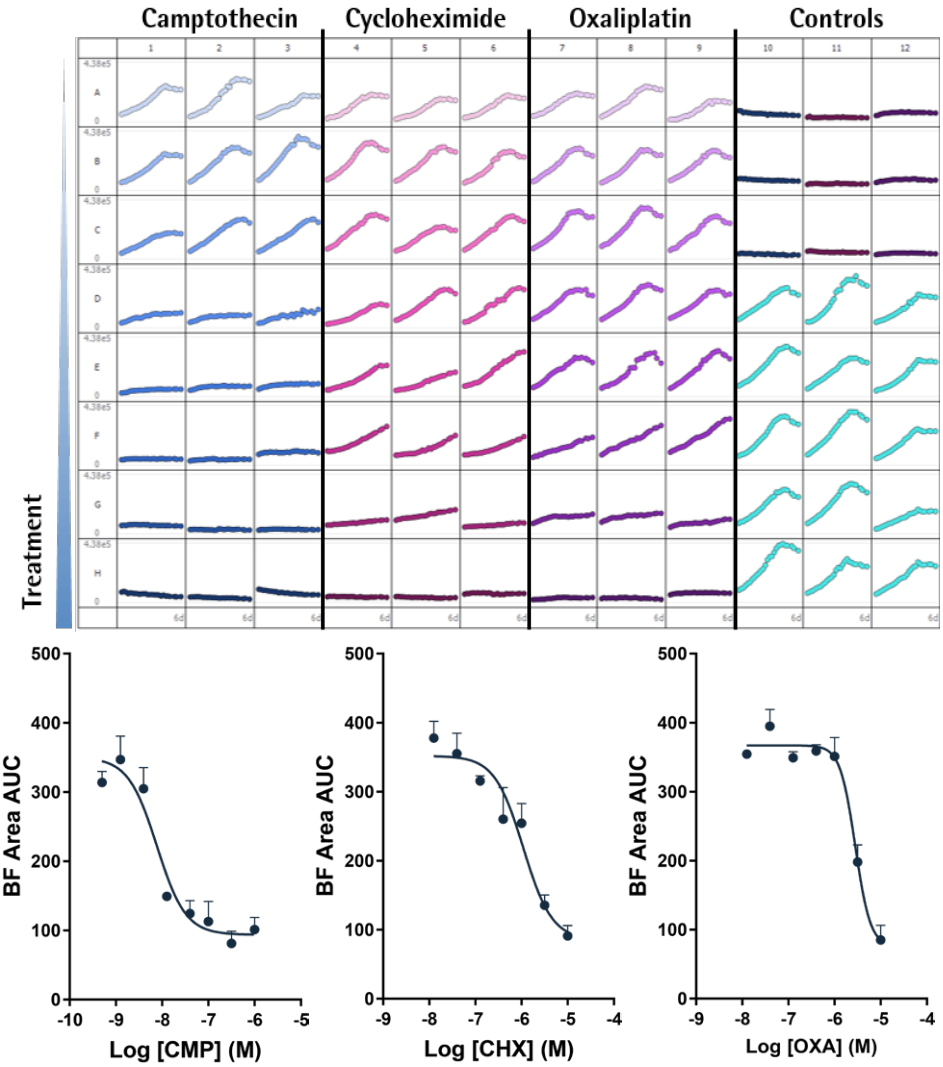


Figure 8. Effect of CMP, cisplatin (CIS) and oxaliplatinin (OXA) on growth of MCF-7 cells in a 3D spheroid assay. MCF-7 cells were plated at a density of 1,000 cells per well and spheroids allowed to form (72 h). Cells were then treated with serial dilutions of compounds and the kinetics of spheroid growth were obtained. Plate view shows the individual well Total Brightfield area (µm²) over time. Concentration response curves represent the area under curve of the Total Brightfield area time course (µm²) from 0 - 168 h post-treatment. Data were collected over 168 h period at 6 h intervals. Each data point represents mean ± SEM, n=3 wells.

Conclusions

The use of multi-spheroid assays as model systems for the evaluation of new treatments and study of disease has expanded in recent years. However, despite their ability to measure indicators of cell health, such as cytotoxicity and apoptosis, common assays rely on indirect, end-point measurements that are subject to artefacts and cannot be readily verified by morphological changes. The IncuCyte S3 Live-Cell Analysis System's Multi-Spheroid assay is a rapid method for the pharmacological investigation of potential drugs in a meaningful multicellular model, which allows researchers to:

- Study spheroid morphology, growth and shrinkage using a label-free, scaffold-based, kinetic 96-well plate assay without removing cultures from the incubator and without need for a predefined endpoint selection.
- Link morphology, growth, and toxicity via direct, kinetic measurements by combining novel DF Brightfield image acquisition and analysis with IncuCyte® Cell Health Reagents.
- Investigate mechanisms of action with multiplexed viability and toxicity measurements using non-perturbing reagents and cell lines that stably express fluorescent proteins.
- Produce reproducible well-to-well kinetic data in live spheroid cultures, in response to different compounds and conditions, using lab-tested protocols, high quality images, and unbiased analysis.
- Carry out automated analysis of multiple conditions or variables under more physiologically relevant conditions, minimizing interference with cell biology.

Kinetic Single Spheroid Assays

Reproducible quantitative analysis of single spheroid growth and health over time

Introduction

Single spheroids exhibit several physiological traits including relevant morphology, increased cell survival, and a hypoxic core that make them ideally suited to the study of larger solid tumors.

These scaffold-free (media-based) models are easily achieved using round-bottom ultra-low attachment (ULA) microplates to promote spheroid self-assembly. They generally yield a single tumor spheroid per well. By virtue of their size, they exhibit the key features of solid tumors: they are comprised of proliferating and quiescent cells, and contain necrotic zones resulting from a gradient of nutrients, metabolites and oxygen that recapitulates the authentic heterogeneity within a tumor.

Single spheroids formed in ultra-low attachment (ULA) plates also avoid the use of complex and poorly characterized biomatrices, and are ideal for studies that require high levels of well-to-well consistency.

A growing body of evidence suggests that more relevant and translational observations can be made in 3D multi-cell tumor models compared to 2D monolayer models. Most currently available 3D techniques for generating and quantifying spheroids are time consuming, laborious, costly and can lack reproducibility.

In this chapter we describe the validation and application of miniaturized IncuCyte live-cell single tumor spheroids formed in ULA plates and monitored up to two weeks. These assays are flexible, simple to run, and provide automated and direct measures of tumor size and health in real time.

IncuCyte® S3 Single Spheroid assays at a glance

Cells of interest are harvested, counted, and plated onto ULA round bottom 96- or 384-well plates and then centrifuged. Spheroid formation is monitored every six hours using DF Brightfield and HD phase contrast image acquisition until they reach the desired size. Test compounds are then added, and the spheroid growth and shrinkage assay is initiated. Automated monitoring continues every six hours for up to two weeks, with tumor spheroid size measurements made available in real time. The simple protocol is shown below:

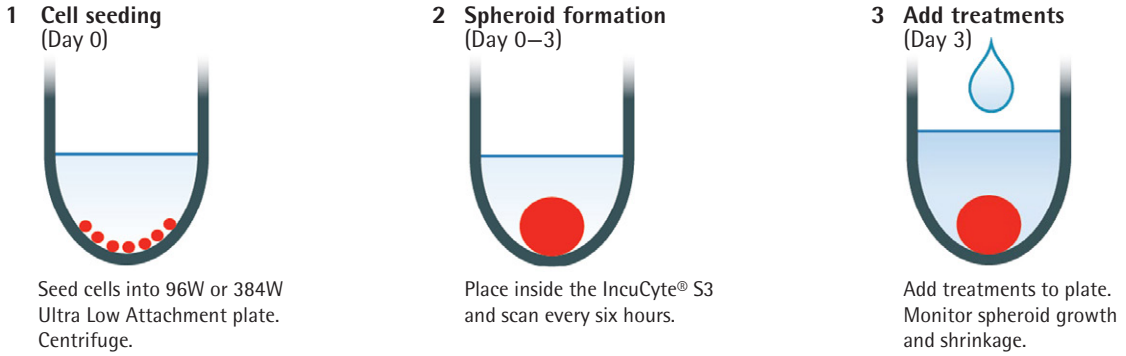


Figure 1. Overview of workflow for generation and analysis of single spheroid cultures in an IncuCyte® S3 Live-Cell Analysis System.

Key advantages:

- Quantify growth and investigate morphology of single spheroids grown in ultra-low attachment (ULA) round bottom plates without the need of labels, and leaving cells undisturbed inside a standard tissue culture incubator.
- Investigate mechanisms of action or immune modulation with kinetic viability and toxicity measurements using non-perturbing reagents.
- Lab-tested protocols, high quality images, and unbiased analysis delivers robust data suitable for pharmacological analysis.
- Automated acquisition and analysis tools enable rapid creation of concentration response curves from thousands of images generated from 96- or 384-well plates.

Application of IncuCyte® S3 Single Spheroid live-cell assays

IncuCyte® S3 DF Brightfield Image Acquisition (described in IncuCyte S3 Multi-Spheroid Assays, Figure 2) enables robust measurement of spheroid size, yielding information on spheroid growth rates and morphology without the need for labels. Following treatment with cytotoxic compounds, a strong Brightfield signature is obtained which, when combined with fluorescence analysis using IncuCyte® Cell Health Reagents, provides insight into the viability of tumor spheroids in response to different treatments.

Label-free monitoring of spheroid growth and morphology over time

The example below illustrates how the IncuCyte S3 DF Brightfield analysis can provide kinetic measurements of single spheroid size over time (Figure 2). The size of tumor spheroids was measured using an automated algorithm that masked the largest Brightfield object in the field of view. Changes in the size of spheroids derived from three types of tumor cell were monitored in response to camptothecin, allowing growth response curves to be generated in the absence and presence of the drug.

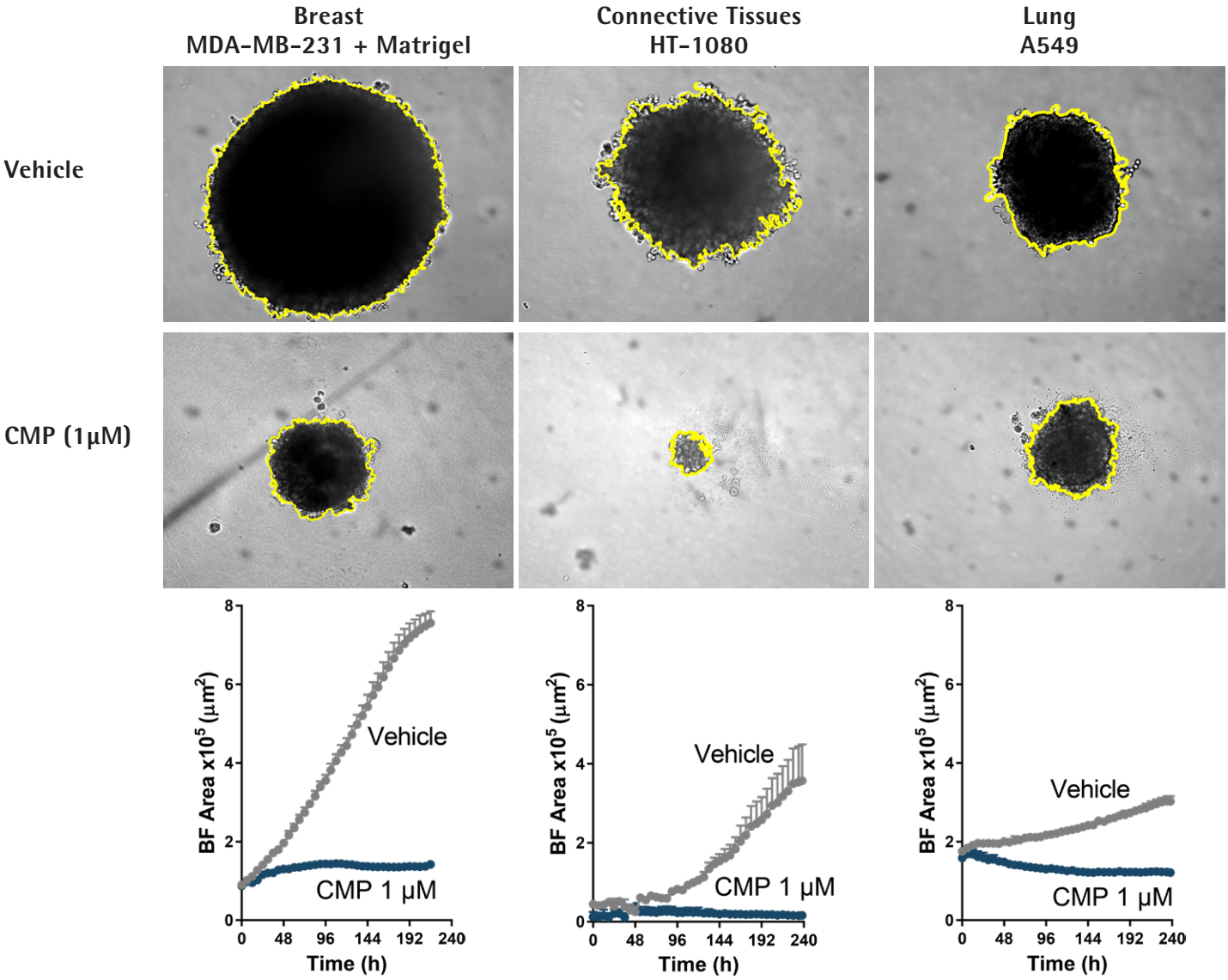


Figure 2. Brightfield analysis enables accurate kinetic quantification of spheroids. The differential pharmacological effect of 1 µM camptothecin (CMP) on growth of MDA-MB-231, HT-1080 and A549 cells in a 3D spheroid assay. Cells were grown in ULA round-bottom 96- well plates (2,500 cells per well) for 72 h and treatment with ± 1 µM CMP followed. Segmented DF Brightfield images compare treated vs. un-treated conditions at 240 h. Time courses illustrate the specific cell type-dependent kinetic profile of spheroid growth and shrinkage. The graphs display the Largest Brightfield Object area (µm²) (y-axis) over the course of a 240 h assay (x-axis) at 6 h intervals. All images captured at 10x magnification. Each data point represents mean ±SEM, n=4.

Reproducible, quantitative data to conduct pharmacological analysis

To demonstrate the pharmacological utility of the single spheroid assay, we performed a comparison of three different drugs using 3D spheroids derived from the SKOV-3 ovarian cancer line (Figure 3). The data shows a concentration-dependent inhibitory growth effect for all compounds, and illustrates how compound potencies can be directly compared within the same assay.

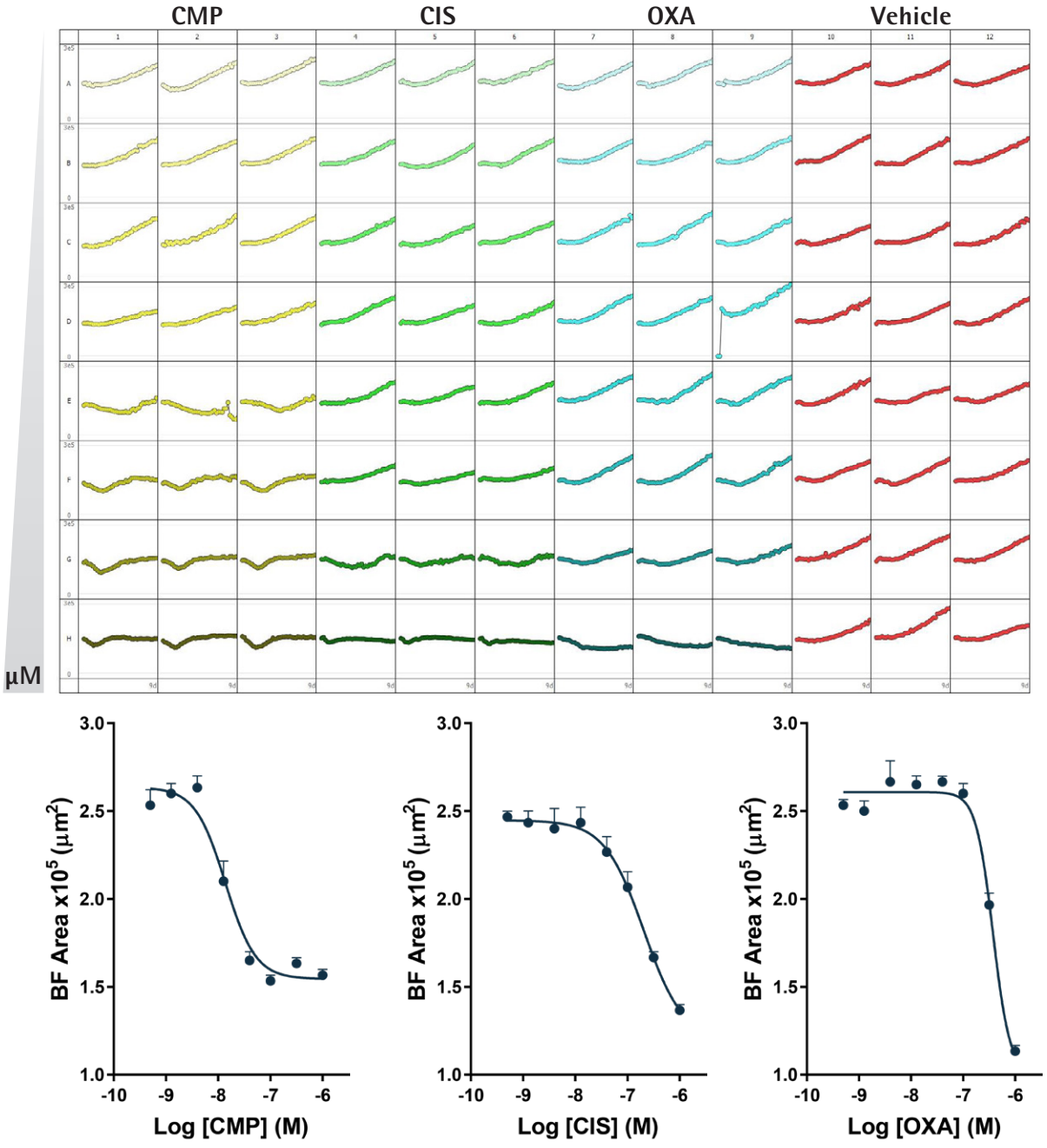


Figure 3. Effect of CMP, cisplatin (CIS) and oxaliplatin (OXA) on growth of SKOV-3 cells in a 3D spheroid assay. SKOV-3 cells were plated at a density of 5,000 cells per well and spheroid allowed to form (72 h). Cells were then treated with serial compound dilutions and kinetics of spheroid growth and shrinkage were obtained. Plate graph shows the individual well Largest Brightfield area (µm²) over time. IC50 curves represent the Largest Brightfield area (µm²) at 204 h post-treatment. Data were collected over 240 h period at 6 h intervals. Each data point represents mean ±SEM, n=8.

Miniaturizing the 3D spheroid cultures makes it possible to optimize the assay further by testing different seeding densities for multiple cell types in a single experiment. This is demonstrated below using three cell types plated at four different densities (Figure 4). The data demonstrates the cell area dependence with seeding density and differential growth profile across the cell types.

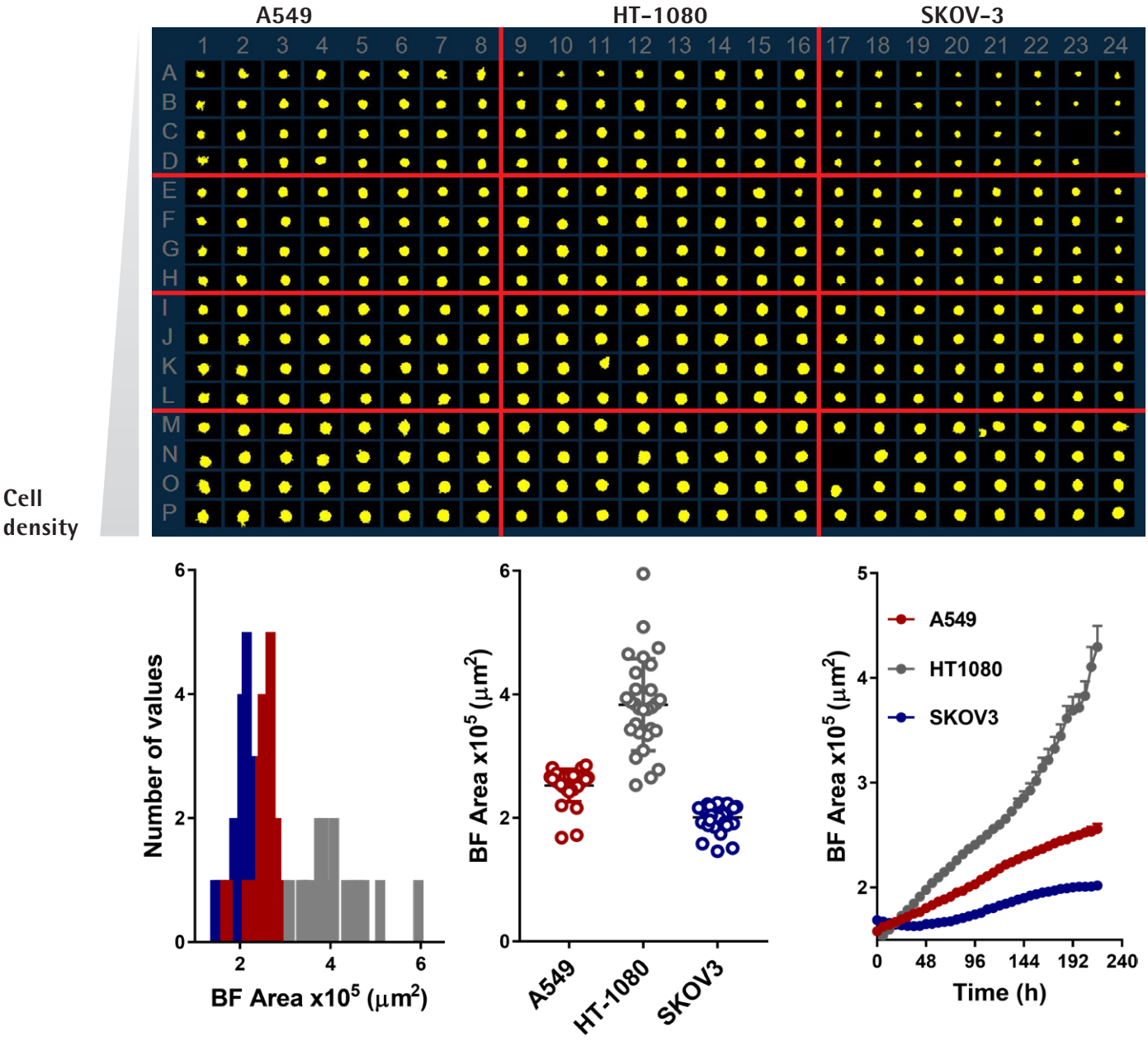
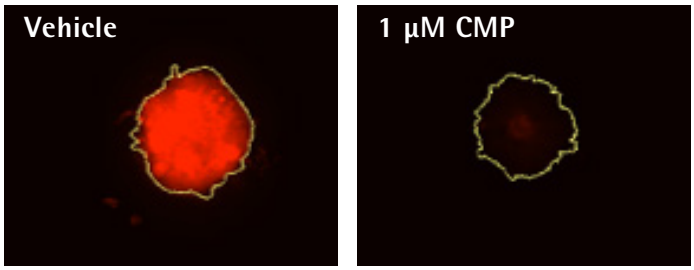


Figure 4. Miniaturizing spheroid growth and shrinkage assay for assay optimization. Comparison of temporal growth profiles of A549, HT-1080 and SKOV-3 cells in a miniaturized 3D spheroid assay. All cells seeded at a density ranging from 310 to 7,500 cells per well plated in a ULA round-bottom 384-well plate. Media was replenished 72 h post seeding. Microplate overview image shows DF Brightfield segmentation mask at 204 h post-media replenishment. Histogram compares the distribution frequency of the Brightfield area (µm²) across all cell types plated at 2,500 cells per well at this time point. Variability plot analysis shows the largest Brightfield area of individual wells at 204 h. Time-course plots represent the differential temporal profile of the Largest Brightfield Object Area metric (µm²) across the cell types. Data were collected over a 204 h period at 6 h intervals, all images captured at 10x magnification. Each data point represents mean ±SEM, n=32.

Viability and toxicity measurements using non-perturbing reagents enable investigation of drug mechanisms of action

Masking of the DF Brightfield channel allows measurement of other parameters of interest when non-perturbing reagents are employed. In Figure 5 (below) the effects of CMP on cell viability were studied by stably expressing a red fluorescent protein (using IncuCyte NucLight Red Lentiviral Reagent) in the spheroids.

Applying the 'fluorescence within the Brightfield boundary' feature in the IncuCyte® S3 spheroid software module negates the need to apply a fluorescence mask, and removes the impact of threshold settings.



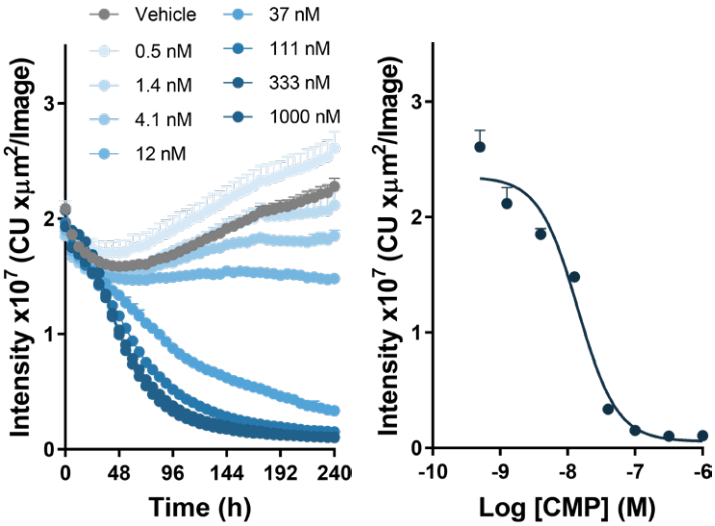
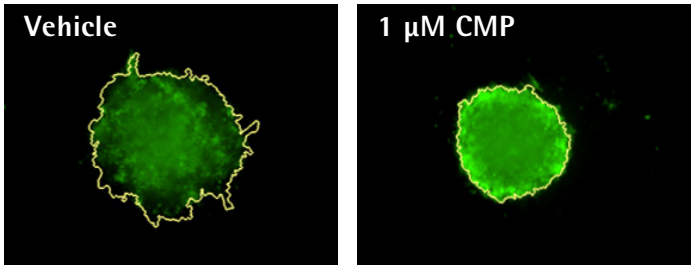


Figure 5. Analysis of spheroids expressing fluorescent proteins enables spheroid viability determination. Representative images taken at 240 h show a strong red fluorescent signal in a vehicle control spheroid, in contrast to a marked loss in red fluorescence in the CMP-treated spheroid. The yellow boundary in the images represents the Brightfield mask outline. Monitoring the integrated intensity from within the Brightfield boundary highlights a gradual increase in fluorescence under vehicle control conditions (red symbols) corresponding to the growth of the spheroid. Upon treatment with CMP, a concentration-dependent reduction in integrated fluorescence is observed, with abolishment of fluorescence with the highest concentration tested after 240 h.

A similar approach was used with IncuCyte® Cell Health Reagents to determine the mechanism of cell death after treatment with CMP (Figure 6). Little or no reduction in spheroid size was observed with the Brightfield phase contrast images, but fluorescence analysis revealed a concentration-dependent effect with increases in the mean intensity within the Brightfield boundary, suggesting induction of apoptosis.



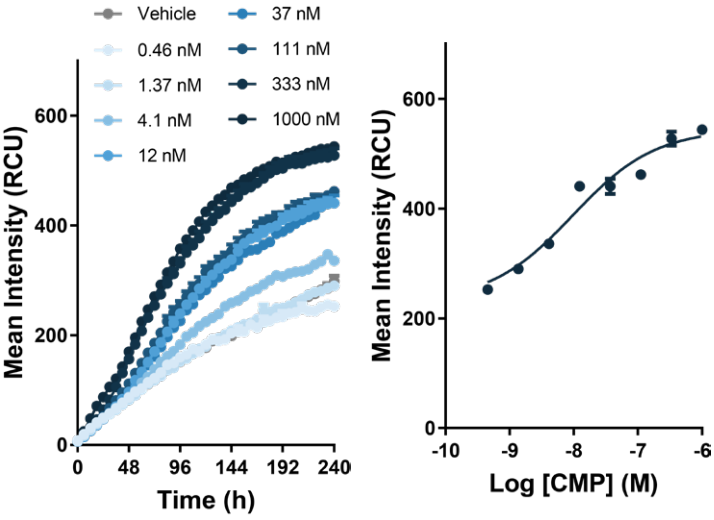


Figure 6. Effect of CMP on A549 cells reported by IncuCyte® Annexin V Green reagents in a 3D spheroid assay. A549 cells were seeded at a density of 2,500 cells per well in ULA round bottom plates and spheroids were formed for 96h. Spheroids were treated with CMP (0.4 nM - 1 µM) or vehicle (0.1% DMSO) and apoptosis was reported using IncuCyte Annexin V Green.

Finally, data is presented showing how combining the Brightfield and Cell Health readouts can be used in single spheroids to determine the mechanism of action of compounds (Figure 7). CMP caused a marked increase in fluorescence intensity, suggesting a cytotoxic mechanism, whereas CHX only caused a notable increase in fluorescence at the highest concentration tested. The clear separation between the size and cytotoxicity readouts supports the known cytostatic mechanism of CHX.

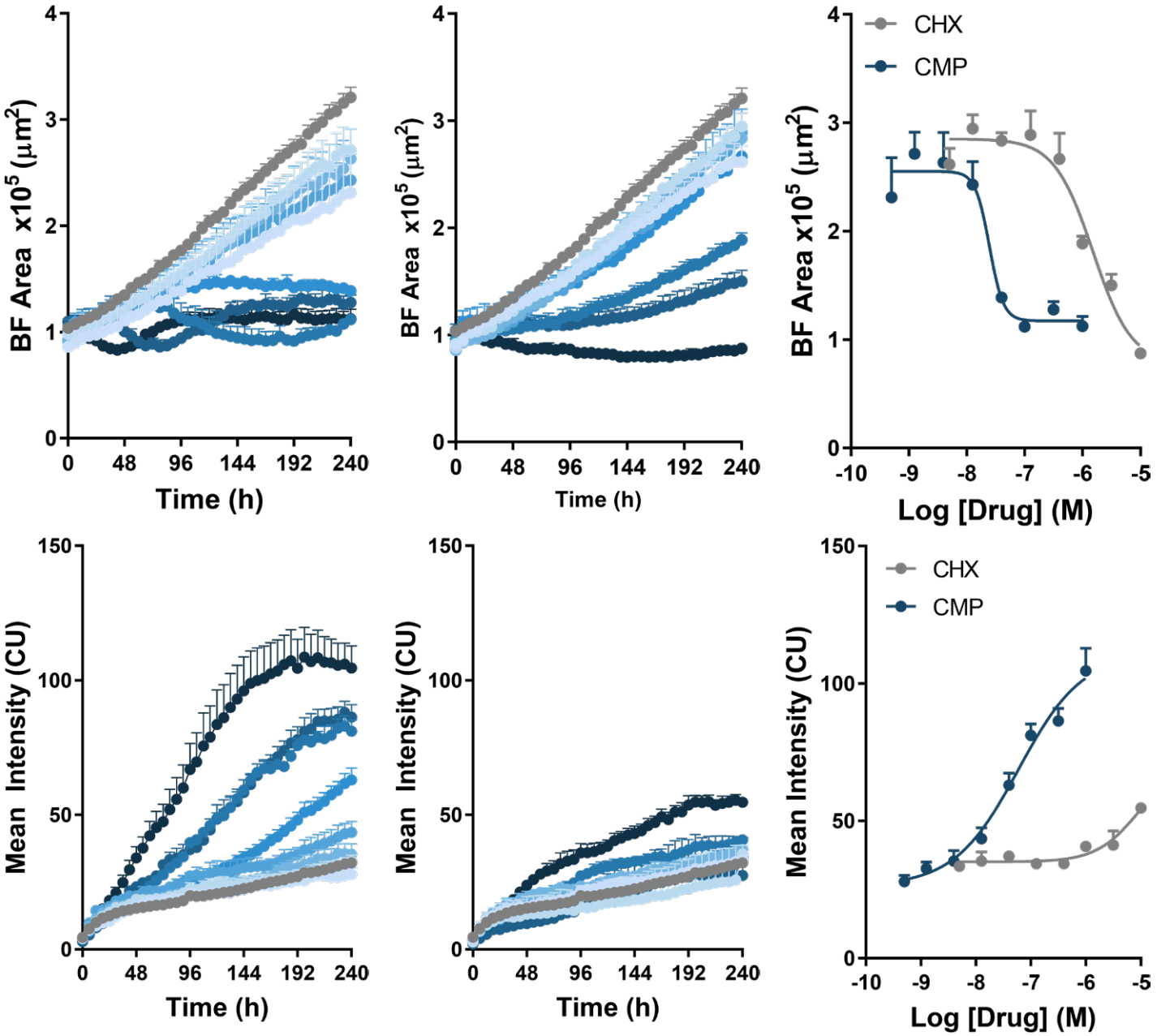


Figure 7. Cytotoxic and cytostatic mechanisms of action can be differentiated by measuring spheroid size and viability. SKOV-3 cells were plated at a density of 2,500 cells per well and spheroid allowed to form (96 h). Spheroids were treated with increasing concentrations CMP (0.5 nM – 1 µM) or CHX (1.4 nM – 10 µM) in the presence of IncuCyte® Cytotox Green reagent (25 nM). Images were taken every 6h for 10 days. Time courses show change in Brightfield area (top row) or fluorescent response (bottom row) over time. CRCs show the different profiles of cytotoxic and cytostatic mechanisms.

Flexible assay format can be adapted for co-culture studies using relevant immune-oncology cell models

In order to further demonstrate the utility of the IncuCyte® S3 Single Spheroid Assay as a translational cell-based assay, an immune cell killing model was optimized and employed to assess the efficacy of novel immune modulators. As illustrated below, new insights for greater translational relevance can be gained by utilizing 3D ADCC spheroid assays, as compared to 2D ADCC assays, for the examination of immune modulators, such as Herceptin. Such models may uncover important information regarding dose response kinetics.

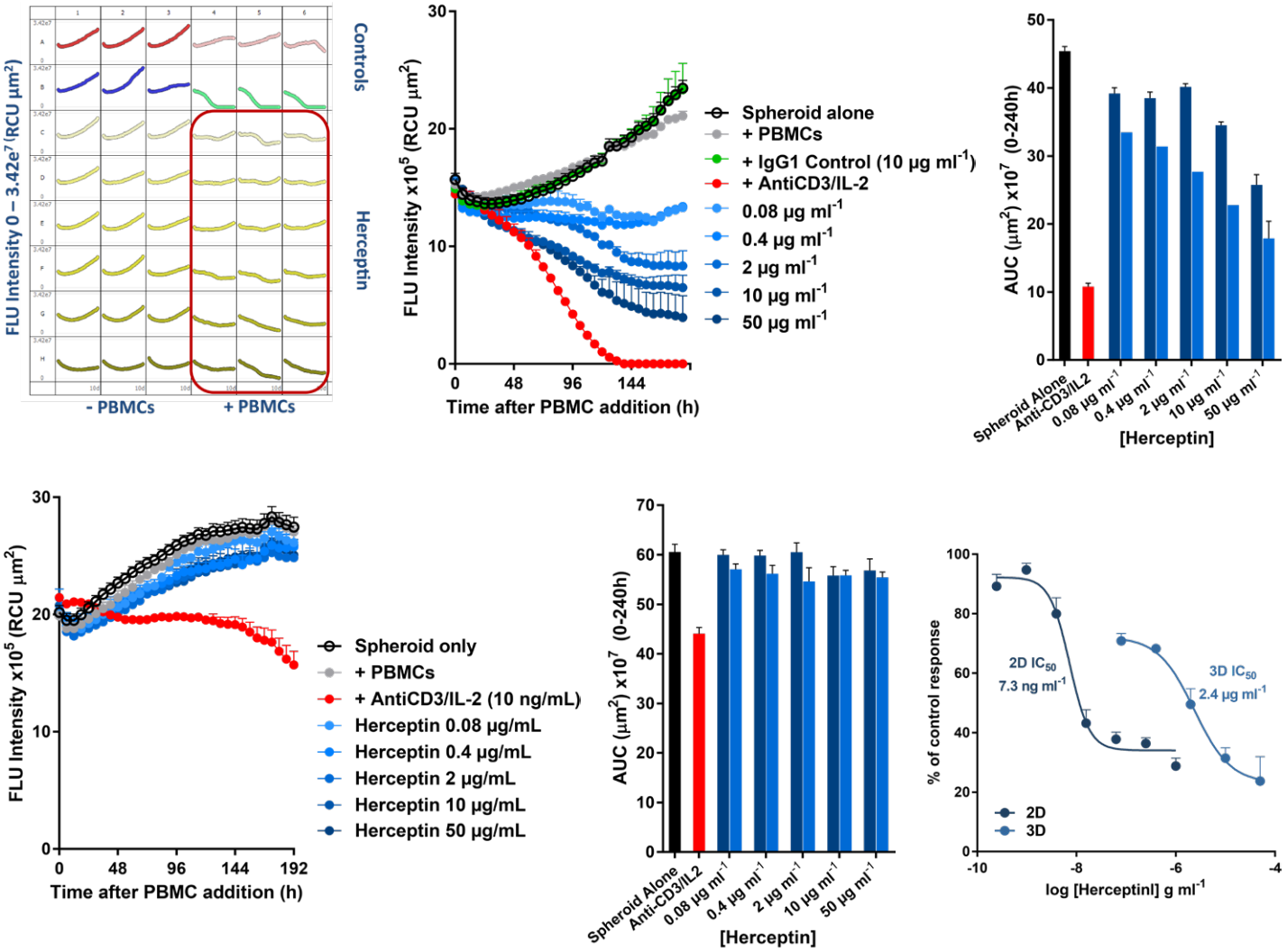


Figure 8. Herceptin-induced ADCC in HER2-positive SKOV-3 cells. HER2-positive SKOV-3 or HER2 negative A549 NucLight Red™ spheroids (2.5K/well) were seeded with PBMCs (6.25K/well) and treated with Herceptin (mAb targeting HER2 receptors). Herceptin-induced cytotoxicity was observed and measured in SKOV-3 but not A549 spheroids. A similar assay was conducted in a 2D culture model. SKOV-3 cells (1.6K/well) were seeded overnight prior to the addition of PBMCs (8K/well) and subsequent treatment with Herceptin. SKOV-3 tumor spheroids appear to exhibit ~300-fold lower Herceptin sensitivity in comparison to 2D. Note the apparent 34% inhibition of the 3D spheroid at the lowest test concentration (0.08 µg/ml), suggesting that a biphasic concentration response curve may exist; the outermost cells behave as in the 2D model, whereas the spheroid center has lower sensitivity.

Conclusions

3D micro-tissues and organoids are becoming increasingly popular for their more relevant morphology and cell survival, but current methods for assessing the growth and vitality of these models are limited. Most assays require cell labeling and only provide a single time point readout. By contrast, the IncuCyte® S3 Live-Cell Assay allows:

- Label-free study of spheroid morphology, growth and shrinkage in 96- and 384-well format.
- Generation of kinetic measurements and cell-dependent growth curves for spheroids derived from different types of cells.
- Monitoring of spheroid viability over time and in different treatment conditions using IncuCyte's Cell Health Reagents and/or stable expression of fluorescent reporter proteins and the ability to link this to morphological observations.
- Miniaturization of the assay to provide a high-throughput method of producing reproducible, quantitative pharmacological data in physiologically relevant cell models simultaneously for a variety of test compound.
- Flexibility in cell types and co-culture, enabling extension of the assay for study of immune modulators.
- IncuCyte® S3 Live-Cell Single Spheroid Assay provides a valuable and timely addition to the toolkit of those working in basic biology, medical research, and pharmacological drug development who seek to better understand cellular responses.

Kinetic Assays for Studying Neuronal Cell Models

Assays for studying neuronal cell health, function and morphology long-term

Effective modeling for neurological disease, injury, and development is critical for identifying novel and effective treatments. However, due to the complexity and plasticity of the nervous system, inaccessibility of human diseased tissue, sensitivity of neurons to perturbation, and lack oftranslational value of animal models, elucidating the function of the nervous system and identifying novel treatments is challenging. With advances in stem cell technologies, the promise to create differentiated neurons and support cells (e.g. microglia, astrocytes) that accurately represent human phenotypes in order to build translational and patient-specific human models now exists. To fulfill this promise, considerable work is required to optimize the reprogramming and differentiation methods, and to build and validate cellular bioassays that are representative of the native human pathophysiology.

To facilitate the evaluation and characterization of human induced pluripotent stem cells (hiPSCs), many technologies are employed to measure various aspects of cell health, morphology and function, such as immunocytochemistry methods, microelectrode electrophysiology techniques, and flow cytometry. Unfortunately, they are not amenable to monitoring long-term changes (days and weeks) and sample preparation can perturb fragile neuronal cells, thus compromising accurate analysis of dynamic biological changes and responses to treatment. The ability to evaluate long-term changes and characterize complex biological models in a non-invasive manner from a physiologically relevant environment offers considerable advantages in characterizing the function of the nervous system.

Live-cell analysis with the IncuCyte addresses the inherent shortcomings of traditional assays through non-invasive, automated time-lapse imaging and analysis from within a cell incubator. Phase-contrast, brightfield and fluorescence images are acquired under uninterrupted environmental control, and then analyzed and quantified in real time to report changes in morphology, movement, activity and function. Time-lapse videos can be created to verify the experimental outcomes. The critical attributes are (1) relevant, informative analyses based on the minimization of artifacts arising from cell perturbation, (2) long-term monitoring of biological events that unfold over days, weeks or even months, and (3) sufficient miniaturization, throughput and ease of use to enable replication, controls and overall experimental productivity. The attributes of live-cell analysis offer simple and reliable assays which offer new biological insights into basic and advanced neuronal cell models.In the subsequent chapters, the assays are described in detail, demonstrating their utility for characterizing and validating translational cell models for the discovery of novel neurotherapeutics.

Live-cell imaging and analysis approaches for studying neuronal cell dynamics

Building on existing methodologies employed to study cell health, morphology and function, novel strategies were developed in order to quantify long-term changes in neurite dynamics, neuronal activity, as well as readouts for neuroimmune functions, such as phagocytosis and chemotaxis of microglia. The IncuCyte Live-Cell Analysis methodology consists of instrumentation, software modules, lab-tested protocols and reagents that enable continuous interrogation of sensitive neuronal cell models. In order to accomplish this, instrumentation was designed to quantify cell behavior with uninterrupted incubation provided by a cell culture incubator and a mobile optical train the enables sample to stay stationary and reduce physical disturbance. Purpose-built software analysis modules automatically segment and analyze images to create full time course plots for each well in 96- and 384-well plates. Finally, combining novel, non-perturbing reagents that deploy longer wavelength fluorophores designed for neuronal-specific measurements along with lab-tested protocols ensures reproducible and unprecedented access to phenotypic information. The flexibility of this instrument, novel reagents, and standardized protocols, combined with higher throughput capabilities and capacity for long-term, unperturbed culture enables this system to overcome many of the challenges associated with traditional endpoint workflows.

How Live-Cell Assays for Neuroscience Work

IncuCyte® Neurite Analysis Assays

In the neurite analysis assay, image software analysis, IncuCyte® NeuroTrack Analysis Software module, is employed for automated quantification of neurite dynamics. This assay permits the analysis of neurons in monoculture (label-free) or in co-culture with astrocytes, using a non-perturbing IncuCyte® NeuroLight Orange Lentivirus reagent for continuous analysis of neurite length and branch points. Furthermore, the neurite analysis assay can be multiplexed with cell health reagents, IncuCyte® AnnexinV NIR or Orange, to determine the onset of apoptosis in real time.

Kinetic Neuronal Activity Assay

To detect changes in neuronal activity, an end-to-end solution consisting of instrumentation, software and reagent is employed. Addition of a genetically-encoded calcium indicator, IncuCyte® NeuroBurst Orange Lentiviral Reagent, allows for efficient, non-perturbing labeling of living neurons for the long-term detection of neuronal activity via calcium binding. Integrated software captures and analyzes short-term calcium flux kinetics for every active cell within each well to reveal the extent of connections in a network and automatically quantifies longitudinal changes of activity for the characterization of neuronal cell models.

Kinetic Neuroimmune Assays

To quantify neuroimmune function, applications include phagocytosis assays using non-perturbing cell-labeling reagents and chemotactic migration assays utilizing purpose-built consumables and software. The IncuCyte® pHrodo® Orange Cell labeling kit utilizes a pH-sensitive fluorescent probe to quantify cell clearance of diseased or dying cells or neuronal-associated proteins to efficiently study the full time course of phagocytosis in the model of your choice.

To evaluate microglial response, the IncuCyte® Chemotaxis Cell Migration Assay enables real-time visualization and quantification of cell migration in response to a chemical stimulus. Using an optically clear membrane, visual assessments of morphology can be linked to real-time measurements to gain deep phenotypic insights.

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Kinetic Neurite Analysis Assays

Quantification and visualization of neurite outgrowth, disruption and cell health

Introduction

Neurite dynamics are central to the study of neuropathological disorders, neuronal injury and regeneration, embryonic development and neuronal differentiation. During these processes, the integrity of neuronal networks is slowly altered, accompanied by changes in neurite outgrowth and disruption that may lead to impaired neuronal connectivity. Characterizing the dynamic changes of neurites *in vitro*, and how they interact with other cells or environmental stimuli, can be invaluable for optimizing *in vitro* models, identifying diseased cells from a heterogeneous population, studying phenotypic effects from genetic manipulation, or performing drug pharmacology studies.

Traditional in vitro approaches for analyzing neuronal structures rely on endpoint assays and imaging techniques that require immunochemical staining. Neuronal cultures are exposed to a treatment condition, fixed and stained at a pre-determined time post-treatment, and then imaged using either high content imaging or traditional fluorescent microscopes. Images are then analyzed to generate measurements of neurite length and branch points. Although this method has been an important tool in neurobiology, the process is labor-intensive, perturbing to fragile neurites, and produces only a single time point of data. In sum, the approach lacks temporal insight and is subject to artifact. Concatenated endpoints may be utilized to address the lack of temporal insight, but require large cell numbers and are subject to further artifacts caused by differences in cell plating or other variable culture conditions.

Continuous real-time imaging and analysis offers a significant advantage over end-point assays in that it provides a more physiologically relevant and dynamic approach to visualizing and analyzing neurite outgrowth and disruption. It is achieved using non-destructive, cell-sparing, repeated measurements of the same neuronal networks over extended periods of time (days or weeks), without perturbing cell health or delicate neurite structures. Conducting experiments at these time scales offers a significant advantage when investigating chronic toxicity, as toxic effects develop unpredictably over time.

In this chapter we will examine kinetic approaches for measuring changes in neurite outgrowth or disruption in both mono- and co-culture cell models. Cultures can be measured and visually validated for days, weeks, or even months, and at microplate throughput, making these approaches particularly well-suited for the optimization and analysis of neuronal cell models as well as for conducting pharmacological studies and investigations of drug mechanisms of action.

IncuCyte® Neurite Analysis Assays at a glance

In order to measure neurite dynamics, the IncuCyte® Live-Cell Analysis System automatically acquires and analyzes images over days or weeks in 96- or 384-well formats. Image acquisition is achieved without the motion of a motorized stage that can be disruptive to sensitive cells; instead, the optical train moves while cells stay stationary. In addition, cells are not subject to loss of environmental control at any point in the experiment. Post-image acquisition, the IncuCyte® NeuroTrack Analysis Software Module is used to segment neuronal cell bodies and neurites and quantify biologically relevant processes such as neurite initiation, neurite extension, branching, and loss of neurite length due to retraction. Either label-free or fluorescent methods may be employed depending on the cell culture model utilized and the scientific question at hand.

For studies of neurite outgrowth and branching in simple mono-culture systems, neurite parameters are derived 'label-free' using phase-contrast images. Cell bodies are segmented from background based on texture and/or brightness, and neurites (linear features) are segmented based on width and brightness. By normalizing the neurite length to the number of cell bodies, it is possible to directly compare the rates of outgrowth.

When studying neurite retraction, or in cases where co-cultures are required, neuronal fluorescent labeling methods can be employed. In these cases, phase-contrast images alone may not able to discriminate the neuronal projections from either neurite debris or support cells. In order to identify neuronal processes, neurons are transduced in a single step protocol using IncuCyte® NeuroLight Lentivirus Reagent. This VSV-G pseudotyped lentivirus encodes a non-perturbing fluorescent protein driven off of a synapsin promoter to strengthen neuronal expression and minimize non-neuronal crossover. Phototoxicity associated with repeat exposure to short wavelength lights must also be avoided when analyzing sensitive neuronal models, so longer wavelength fluorophores (e.g. orange, red) are preferred.[1] Once fluorescent images are acquired, NeuroTrack algorithms are employed to quantitatively assess neurite morphology and reveal temporal information.

Both label-free and fluorescent IncuCyte Neurite Analysis assays can be combined with apoptosis measurements to gain further insight into neurotoxic effects. Multiplexing apoptosis measured using IncuCyte® Annexin NIR and neurite measurements enables real-time tracking of neuronal morphology and cell health within the same population of cells in every single well.

Shortcomings of Traditional Assays	Live-Cell Imaging and Analysis Approaches
• Data obtained from a single, **pre-defined time point** yields **minimal dynamic insight**.	• Continuous, real-time data can be collected over days, weeks, or months without loss of environmental control or physical movement of cultures.
• **Concatenated end point** experiments are subject to **cell seeding artifacts** and require large cell numbers.	• The same network of cells is repeatedly interrogated over time, yielding maximum information from precious cells.
• Fixation and immunostaining steps are labor-intensive and destroy delicate neurites.	• Neurites are identified without fixation and multiple wash steps. Label-free and non-perturbing fluorescent approaches are available.
• Co-cultures cannot be studied as the entire population is **analyzed indiscriminately**.	• Purpose built software tools and guided interface enables non-expert operators to perform image processing and generate publication-ready graphics.

Sample results

Automated analysis and visualization in label-free monocultures

The IncuCyte Live-Cell Analysis System takes non-invasive phase-contrast images of neuronal cultures for a complete time course of neurite dynamics (Figure 1, top image). NeuroTrack software produces data on neurite dynamics by analyzing these images. The software analyzes a HD phase image of neurons in two steps: 1) Cell bodies are segmented from background based on texture and/or brightness, and are masked as cell body clusters (Figure 1, lower left). The number of clusters and the total area of clusters are normalized to image area. 2) Linear features are detected based on width and brightness, and are masked as neurites (Figure 1, lower right). The total neurite length and the total number of branch points are normalized to image area. The segmentation mask can be refined and filtered to tailor the mask to the specific cell type. Every time point of the assay generates metrics from the automated software analysis, which quantifies biologically relevant processes such as neurite initiation, neurite extension, branching, and loss of neurite length due to retraction or disintegration. Quantification of cell body cluster count and area provides methods to measure cell body size or proliferation, as well as providing two metrics for normalization.

To validate the data generated provided by the NeuroTrack software, data from a live-cell IncuCyte Neurite Analysis assay was compared to end-point data derived from fixed cells imaged on a high content screening system (Figure 2). At the three different time points, NeuroTrack's automated software quantified neurite length in unfixed cells over the course of a 6.5 day assay with a sensitivity statistically identical to the fixing/immunostaining method quantified using a high content screening system.

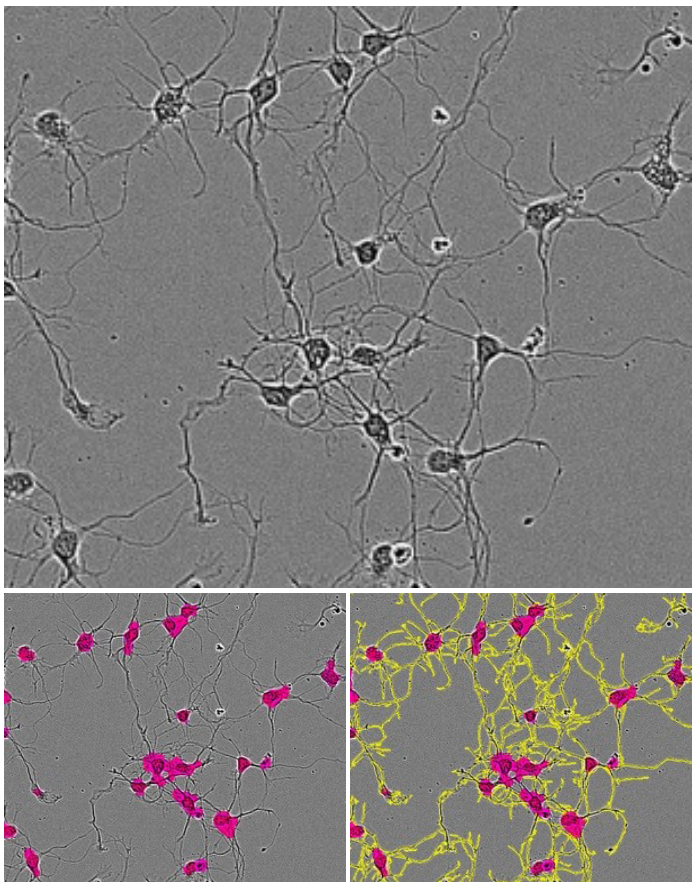


Figure 1. Analysis of rat E18 cortical neurons structures using label-free segmentation masking. Phase image of neurons (top) after 5 days in vitro (DIV). Cell body cluster mask applied to image (bottom left). Neurite mask and cell body cluster mask applied to image (bottom right).

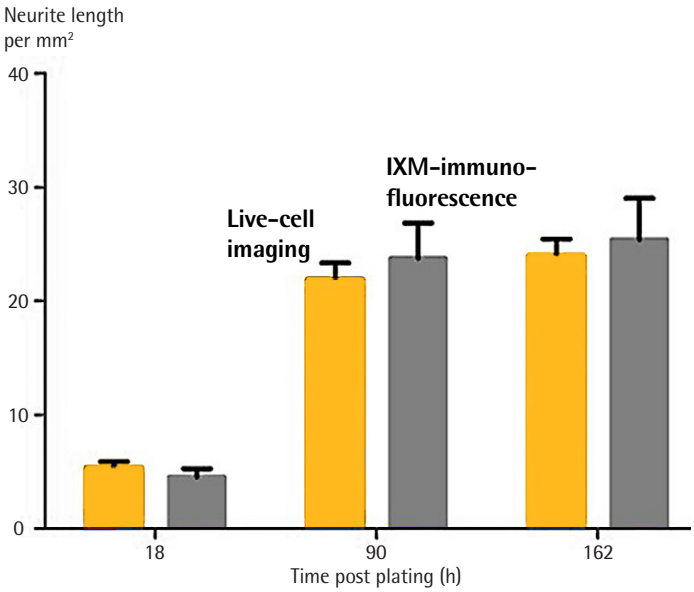
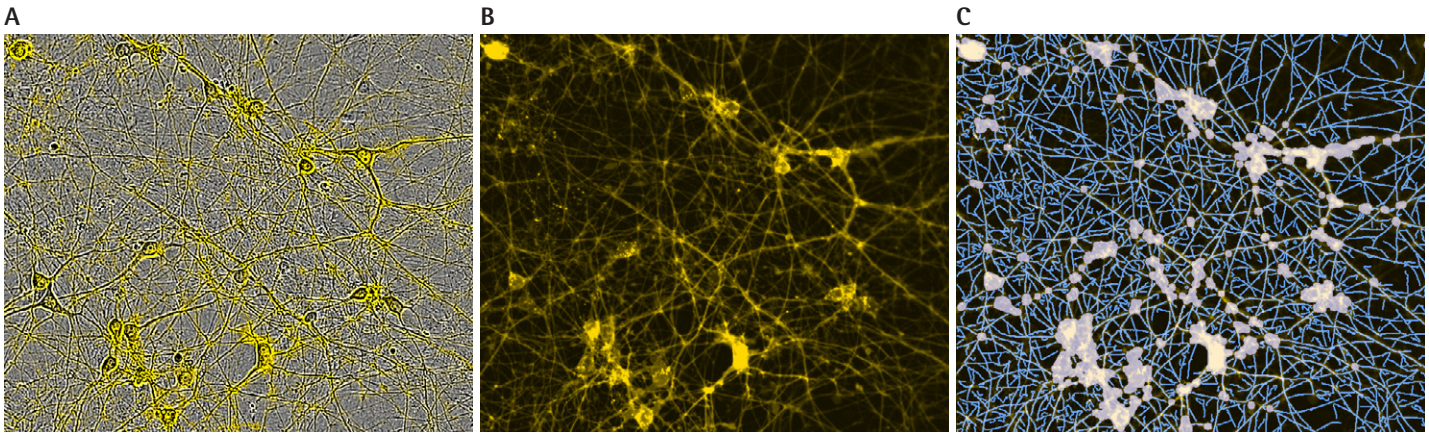


Figure 2: Comparison of automated IncuCyte NeuroTrack analysis to traditional fixed-point staining. Three 96- well plates from the same E18 rat cortical prep were seeded at 8,000 cells/well. One plate was imaged in the IncuCyte at each of three different time points post-plating (18, 90, and 162 hours). These images were quantified with IncuCyte NeuroTrack to measure neurite length/mm². Immediately after imaging in an IncuCyte®, plates were removed from the incubator, fixed, and immunostained for β-tubulin to mark the neurite structures. Cells were then imaged in an Image Xpress Micro high-content screening system and MetaXpress software was used to quantify neurite length/mm². The two data sets are statically indistinguishable at each time point. Error bars represent standard deviation, N=6.

Real-time quantification of neurite outgrowth in co-culture models

To identify and measure neuronal processes in co-cultures containing neurons and astrocytes, incorporation of a neuronal-specific fluorescent label is required. Figure 3 shows primary rat cortical neurons labeled with IncuCyte® NeuroLight Orange in co-culture with rat primary astrocytes to measure changes in neurite length over time. Rat cortical neurons were seeded in a 96-well poly-D-lysine coated micro-titer plate and allowed to adhere for 4 hours in an incubator. Neurons were then infected with IncuCyte NeuroLight Orange, exposing the cells to lentivirus (MOI 1) for 16-24 h. After the incubation period, the lentivirus was removed and media replaced followed by addition of astrocytes. The microtiter plate was placed in an IncuCyte live-cell analysis system, where phase and fluorescent images (4 images per well, 20x) were captured every 6 h for the entire assay duration and automatic analysis of fluorescent images was performed to identify both cell body clusters and neurite length.



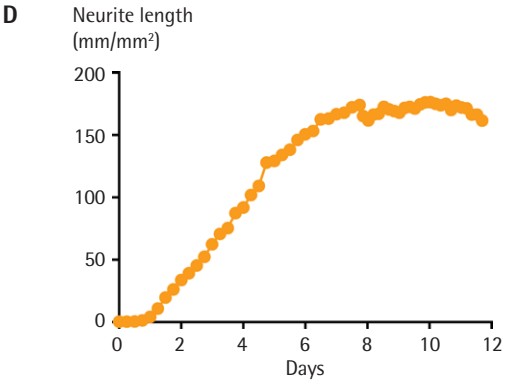


Figure 3. Quantitative assessment of neurite structures using IncuCyte NeuroLight Orange reagent in co-culture with astrocytes. (A) Merged HD phase and fluorescent image of rat cortical neurons in co-culture with rat astrocytes at 7 days post-infection. (B) Fluorescent only image is analyzed via automated image processing to identify (C) both cell body and neurite masks and (D) to quantify neurite length over 12 days.

Real-time quantification of neurite retraction in co-culture models

To illustrate the use of neurite analysis in a model of neurodegenerative disease and neurotoxicity, rat cortical neurons expressing IncuCyte NeuroLight Orange were co-cultured with rat astrocytes and exposed to glutamate. Figure 4 illustrates the use of the IncuCyte Live-Cell Analysis system and NeuroTrack software to analyze phenotypic retraction of neurites, providing quantitative pharmacology. As shown in figure 4A below, the addition of glutamate to co-cultures of primary neu rons and astrocytes at day 9 produced a concentration- and time-dependent decrease in neurite length. Measurements of neurite length at 286 hours after plating were fitted with a Hill equation, yielding a 29 µM IC50 for glutamate-induced decreases in neurite length with a 73% decline at maximal glutamate concentrations (Figure 4B). To investigate a potential mechanism mediating this toxicity, the neuron-astrocyte co-cultures were exposed on day 9 to MK-801, a specific NMDA receptor antagonist), 10 minutes prior to addition of a maximally effective glutamate concentration (250 µM). MK-801 afforded full protection from the effects of glutamate on neurite length (Figure 4C) with an IC50 value of 0.44 nM at 298 hours (Figure 4D), suggesting that the effects of glutamate on neurite length are mediated, at least in part, by NMDA receptors.

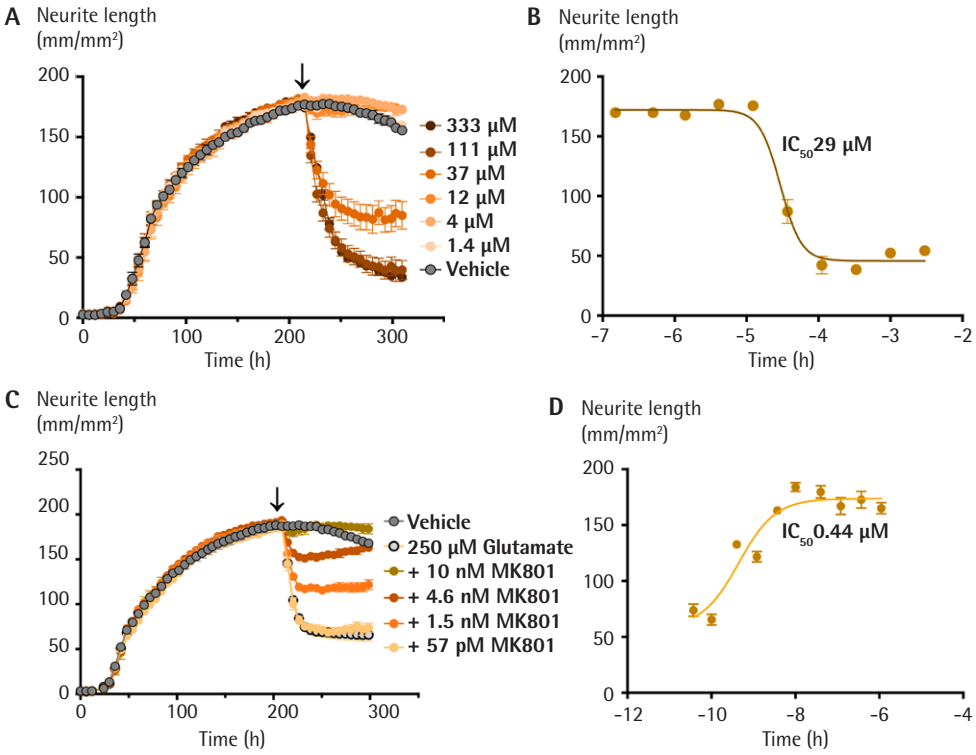


Figure 4. Glutamate-induced retraction of neurite projections. Time-course of effects of glutamate addition at day 9 (arrow) on neurite length is shown in A (mean + SEM, n=4). (B) Concentration-response analysis for data in A (mean + SEM, n=4) taken at 286 h time-point. (C) Addition of increasing concentrations of MK-801 10 minutes prior to addition of 250 µM glutamate (arrow) protects neurites from glutamate toxicity (mean + SEM, n=4). (D) Concentration-response data for MK-801 effects measured at 298 h (mean + SEM, n=4).

Live-cell analysis utility for characterization and development of iPSC-derived neuronal models

Neurite analysis can also be used to characterize and optimize basic culture conditions when plating and maintaining iPSC. An example of this is shown in Figure 5 where the culture of iCell® neurons (CDI) were tested in three common culture substrates (PDL, PLO, PEI) w/wo secondary laminin or Matrigel coating. iCell Neurons were seeded at 50,000 cells/well on PDL, PLO or PEI +/-Matrigel or +/-laminin, and images were capture at 10X magnification. Different morphologies were observed between the coatings, with dramatic cell clustering and radial neurite cabling demonstrated with PDL and PLO, while the morphology of the monolayer on PEI plates were more homogenous and representative of classical neurite outgrowth. Secondary coating with either laminin or Matrigel produced a neuronal monolayer with more pronounced neurite outgrowth, and laminin was chosen for further experimentation.

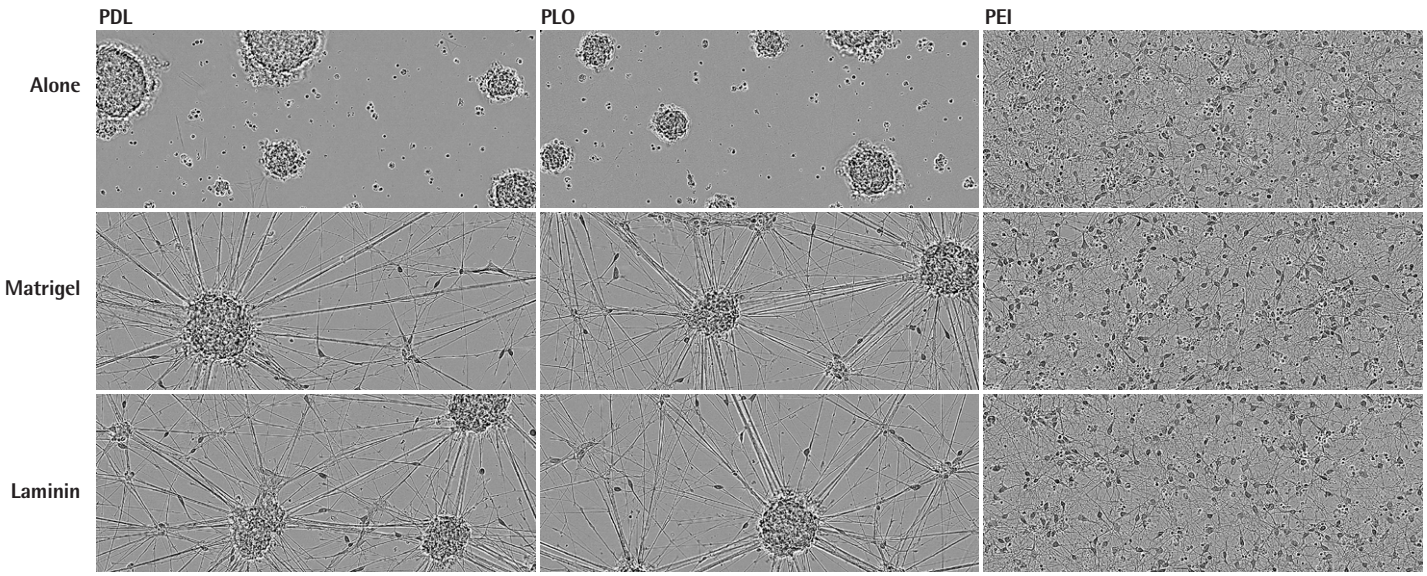
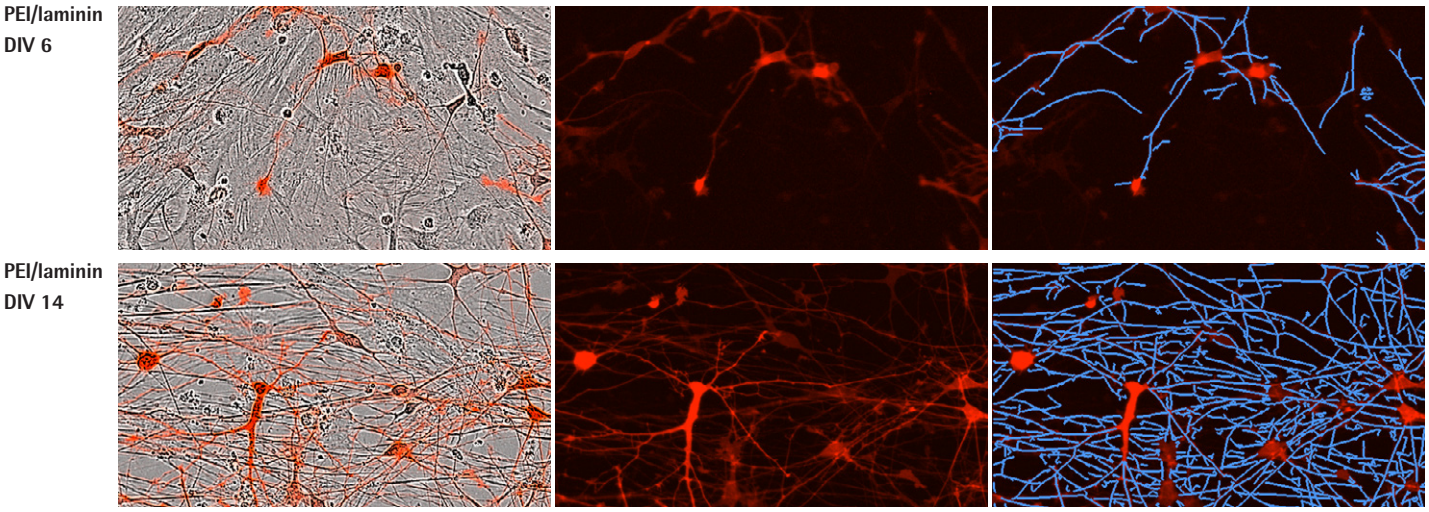


Figure 5. Determination of optimal adherence and morphology of iCell Neuron monocultures seeded on PEI + laminin iCell Neurons were seeded at 50,000 cells/well on PDL, PLO or PEI +/- Matrigel or +/- laminin. Cells plated on PDL or PLO formed large neurospheres by DIV14 in the presence or absence of additional laminin or Matrigel coating. Cells plated on PEI +/- laminin or Matrigel displayed a more homogenous monolayer. All images captured at 10x magnification.

Once culture conditions were optimized for iCell Neurons in monoculture on PEI + laminin, they were subsequently tested to evaluate if the conditions translated to iCell Neurons plated in co-culture with primary rat astrocytes. ICell Neurons and primary rat astrocytes were seeded at 10,000 and 15,000 cell/well respectively. The ICell neurons were then infected with IncuCyte® NeuroLight Red, images captured at 20X magnification, and neurite length was assessed with Fluorescent NeuroTrack software (Figure 6), which demonstrated robust neurite outgrowth. Although iCell Neurons in co-culture with astrocytes did not display a dramatic difference in morphology or neurite length with other coating conditions (data not shown), we recommend seeding on PEI + laminin to maintain consistency with monoculture experiments



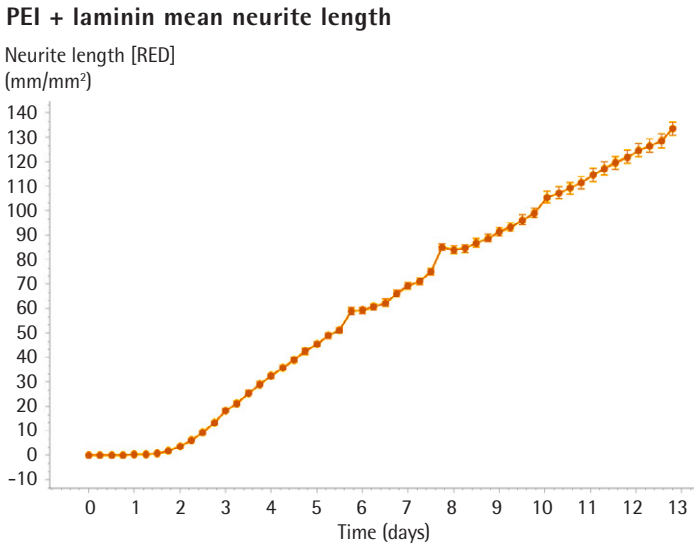
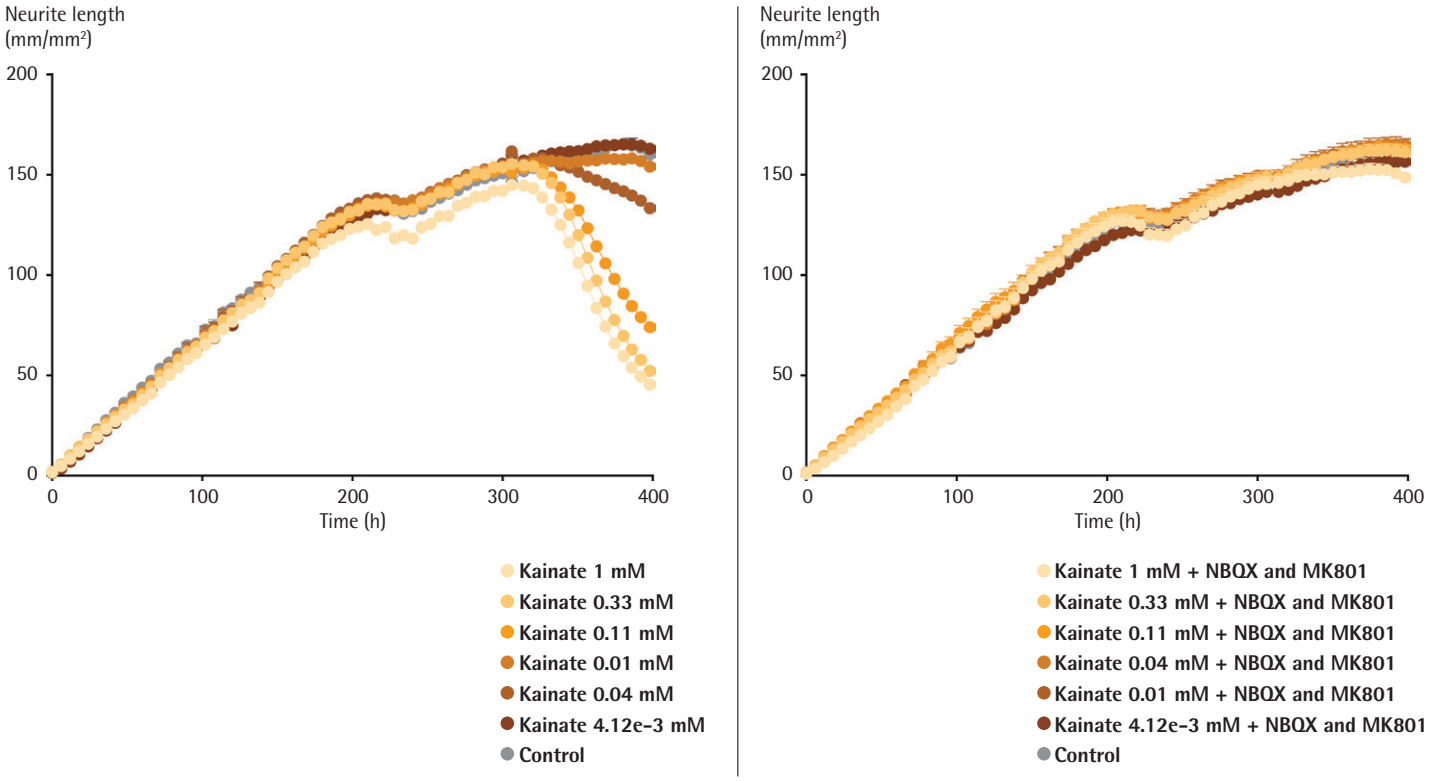


Figure 6. PEI + laminin coating enables robust neurite outgrowth in iCell Neuron co-culture with primary rat astrocytes. Cell culture plates were coated with PEI + laminin. All images captured at 20X magnification. Each data point represents mean =/-SEM, n=4.

Multiplexed, kinetic measurements of neurite dynamics and cell health

The flexibility of the IncuCyte system, integrated software, and associated non-perturbing reagents enables users to multiplex kinetic measurements of neurite length and branch points with cell health readouts in one assay platform, which spares precious sample, augments standardization, and enhances throughput for both mono-culture and co-culture workflows. Figure 7 shows the use of fluorescent (co-culture) NeuroTrack analysis software multiplexed with IncuCyte Annexin V NIR apoptosis reagent to quantitatively characterize potential long term neurotoxic effects of glutamate and kainate in hiPSC-derived neurons (IGluta Neuron). Addition of both glutamate and kainate at DIV14 produced a concentration- and time-dependent decrease in neurite length with concomitant increase in cell death in iGluta Neurons in co-culture with primary rat astrocytes. To investigate a potential mechanism mediating this toxicity, AMPA receptor and NMDA receptor antagonists (NBQX and MK801, respectively) were added at DIV14 along with glutamate or kainate. NBQX and MK801 reduced the neurotoxic effects of both glutamate and kainate, suggesting that these effects are, at least in part, glutamate receptor-mediated.

Neurite length–fluorescent neurotrack



Cell viability–IncuCyte® Annexin V NIR

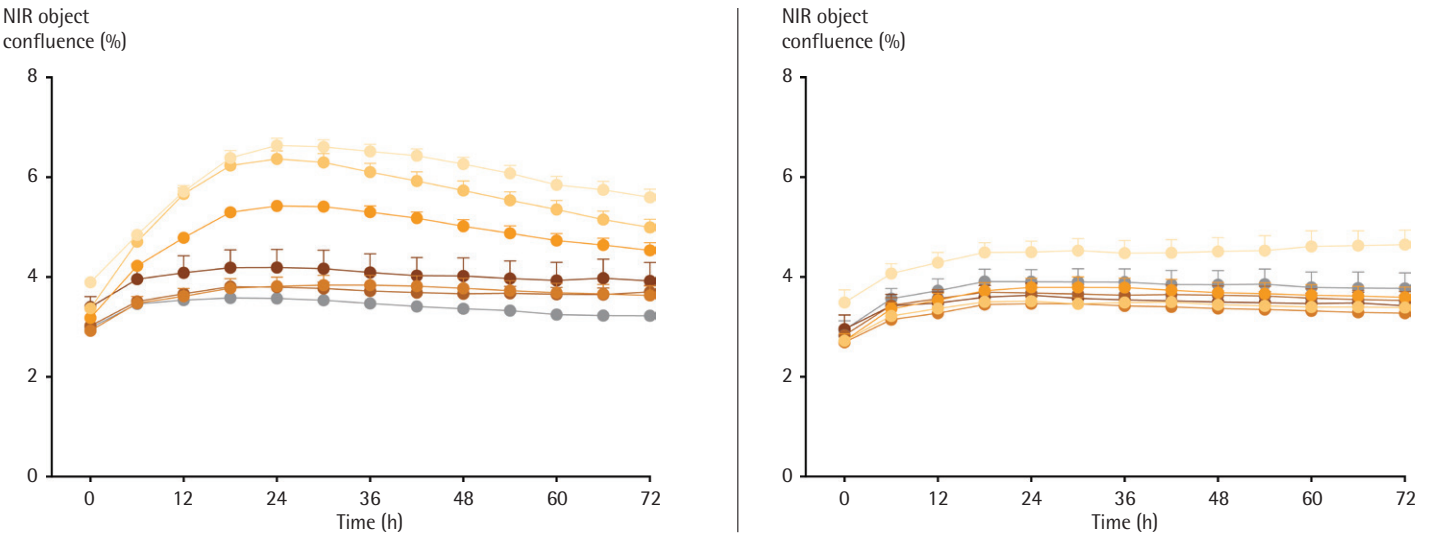


Figure 7. Kainate exposure concentration- and time-dependently decreases neurite length and increases cell death in iGluta Neuron co-culture. Time course of the effects of kainate addition at day 14 (arrow) on neurite length (top panels) and cell viability (bottom panels). Kainate causes a concentration-dependent decrease in neurite length with concomitant decrease in cell viability (increased IncuCyte Annexin V NIR fluorescence). Addition of NBQX (20 µM) and MK801 (10 µM) protects iGluta Neurons from kainate toxicity. Each data point represents mean +/- SEM, n=6.

Conclusions

IncuCyte live-cell neurite analysis assays enable long-term examination of neurite dynamics in primary, immortalized and iPSC-derived neuronal models. These assays are ideal for both optimizing in vitro models and performing drug pharmacology studies. By acquiring images in a physiologically relevant environment, and using robust, integrated software analysis, neurite analysis can be extended from a traditional 'fix-and-stain' approach, to kinetic, non-invasive evaluation of mono- or co-culture models to detect pharmacological and genetic manipulations that alter neurite formation, elongation, and disruption. This approach also allows users to optimize the culture conditions and maintenance of iPSC-derived neuronal models as well as providing a method to analyze cell health by deploying longer wavelength, non-perturbing fluorophores. The IncuCyte approach for real-time, long-term quantitative analysis of neuronal morphology and cell health fills a critical need in the study of human neurophysiological disorders and iPSC-derived neurons.

- The assay is flexible and can be utilized to study neurite outgrowth or retraction in a wide range of cell types, from large immortalized cell lines to primary neurons and iPSCs, in mono- or co-culture.
- Sensitive, kinetic measurements of neurite length, branch points, and cell body clusters capture a complete description of a highly dynamic process that cannot be provided by single time point data alone. Arbitrarily defined endpoints are not required.
- Assays are conducted in an optimal environment so that neurite dynamics can be studied over extended time periods (days or weeks), ideal for studying effects that may not present until later points in time. Cells do not experience loss of environmental control or physical movement as data is collected.
- All data and time points can be verified by inspecting individual images and/or time-lapse movies. Observation of cell morphology provides additional validation and insight into the biological effect of treatment groups.
- Assays are either label-free or employ non-perturbing IncuCyte reagents that can be multiplexed (e.g. to readout neurite length and cell death). Cell sparing, lab-tested protocols are provided to minimize troubleshooting.
- Images are automatically acquired and analyzed in 96- or 384-well format using an intuitive user interface. This allows for rapid assay optimization.
- Low well-to-well variability and minimal user effort make the assay amenable to medium throughput screening.
- The combination of long-term image-based analysis and microplate throughput in a physiologically relevant environment enables efficient optimization of culture conditions and maintenance of iPSC-derived neuronal models.

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Neuronal Activity Assay

Long-term quantification of synaptic activity and network connectivity

Introduction

The most fundamental function of the nervous system is the transmission and integration of information via electrical and chemical signals that pass from axon termini to receiving dendrites of neighboring neurons. In order to coordinate complex processes, this primary function of integration and propagation of cellular signals must be achieved. Activity measurements are therefore critical for characterizing the generation and maturation of neuronal networks to gain functional insights into relevant neuronal models and disease states. Model systems gaining the most traction in the field of neuroscience are human induced pluripotent stem cell (hiPSC)-derived neurons, which offer the opportunity to study basic neuronal development as well as representative disease conditions as they differentiate, mature and become functionally active. However, current technologies have limited ability to generate the information needed to develop these models and determine when they become functionally active.

Traditionally, neuronal activity measurements are made using sophisticated microelectrode electrophysiology techniques or microscopic analysis of calcium oscillations. Electrophysiology techniques, such as patch clamp measurements and multi-electrode arrays (MEA), may provide exquisite resolution of electrical changes, however these measurements are often derived at a single point in time without confirmation of cell morphology. Furthermore, MEA methods require neuronal cultures to be grown at high densities which sacrifices representation of *in vivo* conditions. Analysis of calcium oscillations offers the opportunity to analyze morphological changes associated with neuronal activity, however also fail to capture long-term changes of neuronal activity and cannot detect the formation of a mature, connected neuronal network.

Live-cell analysis alleviates many of these challenges by allowing for chronic evaluation (over days, weeks, or months) of neuronal activity and connectivity from thousands of cells per well, via non-destructive, repeated imaging of the same sample in a physiologically relevant environment. In this chapter, we illustrate how the neuronal activity assay using the IncuCyte® Live-Cell Analysis System in conjunction with fit-for-purpose software tools and a non-perturbing reagent enables kinetic quantification of activity and connectivity, at microplate scale.

Kinetic neuronal activity assays at a glance

To evaluate long-term neuronal activity measurements, IncuCyte® live-cell imaging and analysis employs an end-to-end solution consisting of instrumentation, software, and reagent utilized in a physiologically relevant environment. The IncuCyte® NeuroBurst Orange Reagent is a genetically encoded calcium indicator that can be expressed in a variety of neuronal cell types, including iPSC-derived models, to gain insight into the dynamic changes in activity via measurements of calcium oscillations. In order to acquire these neuronal activity changes, the reagent encodes a fluorescent protein that is excited at a higher wavelength to avoid phototoxic damage to the sensitive neurons that are being analyzed. The IncuCyte system and IncuCyte® Neuronal Activity Analysis Software Module captures these short-term calcium oscillations via high speed movie acquisition (3 fps for 30-180s) in every well of a 96-well microtiter plate. Acquired fluorescent IncuCyte movies are then analyzed to quantify the orange fluorescent signal to derive the active neuronal count and connectivity over the course of the experiment, enabling continuous characterization of developing networks as they become functional and mature. This approach provides researchers the opportunity to continuously analyze the same population of cells long-term, from days to weeks to months, in order to gain insight into how and when neurons become active and how their activity changes over time.

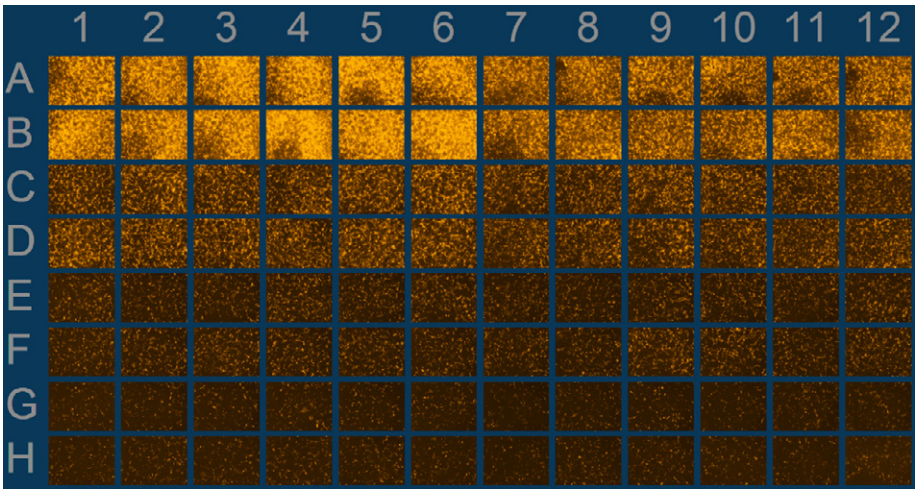
Shortcomings of Traditional Assays	Live-Cell Imaging and Analysis Approaches
• Data obtained from **at a single point in time** yields **minimal insight** as neuronal networks develop and mature.	• **Long-term, kinetic evaluation** shows longitudinal changes in network activity **to determine when neurons become active and how their activity changes over time**.
• Measurement of activity from a **limited number of cells** or by using **concatenated single time point** data causes **high variability in assay results**.	• Burst rate of **thousands of cells in every well of a 96-well** plate is measured with **high accuracy** via **continuous interrogation** in the **same population of cells** over days, weeks, or month.
• **Lack of environmental control** and **physical movement of plate** during analysis **disturbs sensitive neuronal structures** and the biology of interest.	• **Uninterrupted environmental control** provided by a tissue culture incubator, coupled with a non-perturbing reagent and automated image acquisition without physical movement of sample, **protects sensitive neuronal structures** and **maintains integrity of data**.
• **Poor/no visualization of cell morphology** and **limited spatial resolution** does not allow for evaluation of cell health or neuronal network formation.	• Qualitatively **monitor cell morphology** using HD phase image and **quantify functional connectivity** of entire network of active objects in each well of a 96-well plate.
• Complex instrumentation and image processing **requires expert operation and training** for data generation and analysis.	• **Purpose built software tools** and guided interface **enables scientific discovery** for even first time users.

Sample Results

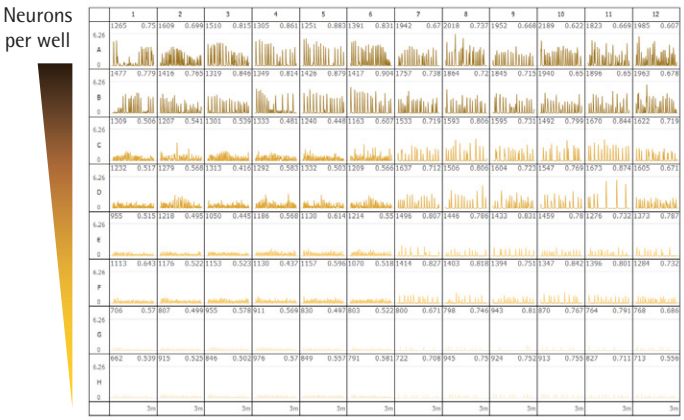
Monitoring neuronal activity via detection of calcium oscillations

IncuCyte NeuroBurst Orange is a neuronal specific, live-cell genetically-encoded calcium indicator (GECI) that, when used along with IncuCyte® Live-Cell Analysis System and IncuCyte® Neuronal Activity Analysis Software Module, enables automated quantification of transient calcium oscillations of thousands of functional neurons within a culture over long periods of time. Optimizing cell seeding densities for use with the IncuCyte Neuroburst Orange reagent for monitoring neuronal activity is illustrated below (Figure 1) using a primary model for neuronal activity; co-culture of primary rat neurons (E18) and primary rat astrocytes. In this experiment, E18 rat forebrain neurons were plated at decreasing cell densities (5-40K/well) in co-culture with a fixed number of rat astrocytes (15K per well). As visualized in Figure 1a, fluorescence intensity within the range image strongly correlates with cell density, with the highest amount of activity observed at 40K neurons/well. The range image also provides the researcher with a qualitative assessment of morphology, toxicity, and transduction efficiency. Summary traces of neuronal activity provide a quantitative assessment of activity within each well, and density of neurons tested was optimal for visualization of neuronal activity within each scan (Figure 1b) and detection of active objects over the full 12-day time course (Figure 1c).

A



B **Summary trace activity at Day 12**



C **Number of active objects over 12 days**

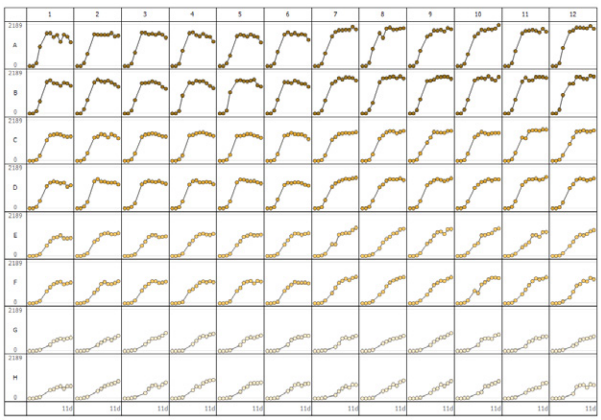


Figure 1. Optimizing cell seeding densities for use with IncuCyte NeuroBurst Orange for monitoring neuronal activity. Primary rat forebrain neurons were seeded at 40K (rows A and B), 20K (rows C and D), 10K (rows E and F), and 5K (rows G and H) cells/well. All densities of neurons were plated in a co-culture with primary rat astrocytes seeded at 15K cells/well and transduced with the NeuroBurst Orange Reagent. (A) 96-well vessel view of the range image over the course of the scan provides a snapshot of active wells at each time point. (B) Summary traces of fluorescence intensity across all active objects for the 96-well plate at day 12 provide an overview of activity and display metrics of bursting intensity, active object number and mean correlation. (C) 96-well throughput with high kinetic reproducibility over 12 days in culture.

The IncuCyte Live-Cell Analysis System automatically captures and analyzes short-term, calcium flux kinetics for every active cell within each well of a 96-well plate using IncuCyte® Stare Mode Movie acquisition. Each scan consists of a 30-180 second movie, captured at a rate of three frames per second, then distilled into a single range image to allow for simple viewing and image processing. This image represents the range of intensities that are detected from each cell within the culture over the specified scan time. Using this image, automated image segmentation tools are used to identify active objects (cells) within each well. Based on the changing fluorescent intensity of each individual cell, intensity traces are displayed for every active cell in the culture. Scanning is typically completed once every 24 hrs. Once these data are collected, several automated metrics are calculated for each well and at each scan time, allowing for simple visualization of changing metrics over the full time-course of the experiment (Figure 2).

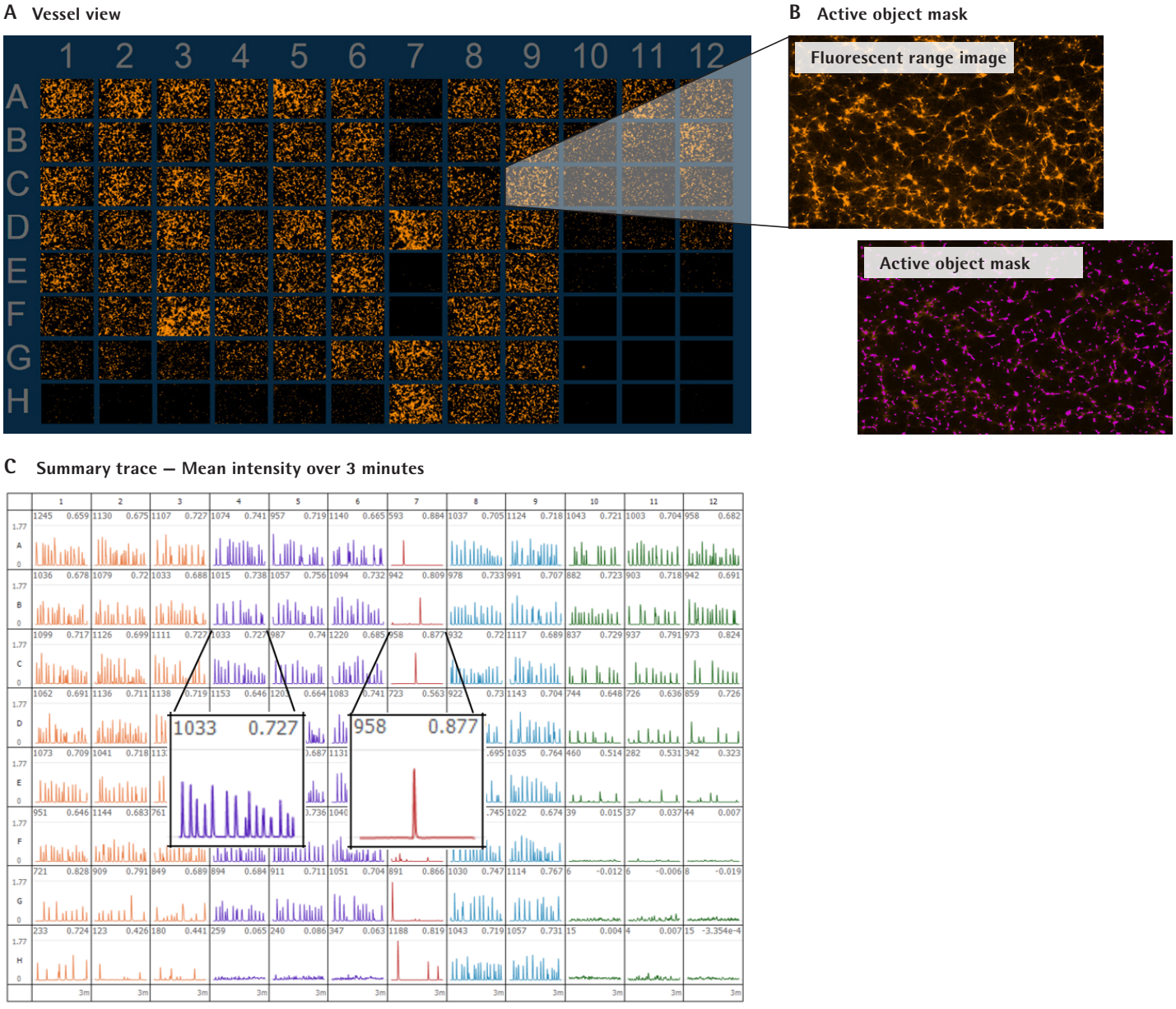


Figure 2. Identification and masking of active neurons using integrated IncuCyte Neuronal Activity Analysis Software Module. IncuCyte® Cortical Neurons seeded at 15,000 cells/well in a co-culture model with IncuCyte® rAstrocytes were subsequently infected with the IncuCyte® NeuroBurst Orange Reagent. Vessel View (A) displays a summary of active objects (range image) in each well of a 96-well plate acquired during movie acquisition on day 8, revealing differences in activity across the microplate. Identification of each active object via masking (shown in purple) of the range image (B) is performed using the integrated IncuCyte® Neuronal Activity Analysis Software Module. 96-well Summary Traces for movies acquired at a given timepoint (C) provide a full 96-well view of burst intensity, active object count (left inset value) and mean correlation (right inset value).

Characterizing changes in neuronal activity through chronic analysis

A key value of live-cell analysis is the ability to quantify longitudinal changes in activity for the characterization of neuronal cell models in physiologically relevant conditions. In Figure 3, iPSC-derived iCell® Gluta neurons in co-culture with rat astrocytes co-were transduced with IncuCyte NeuroBurst Orange Lentivirus reagent. After 24 hours, the lentivirus reagent was removed and live-cell analysis of calcium oscillations were captured every 24 hours for 20 days. Automated image processing identifies thousands of active neurons – many more than MEA - in each well of a 96-well plate and data is plotted in real time to display metrics such the mean burst rate and mean correlation of the samples being analyzed. Importantly, this analysis of the same population of cells is repeated over many days and weeks to build understanding of the development of the network and any long-term plastic changes.

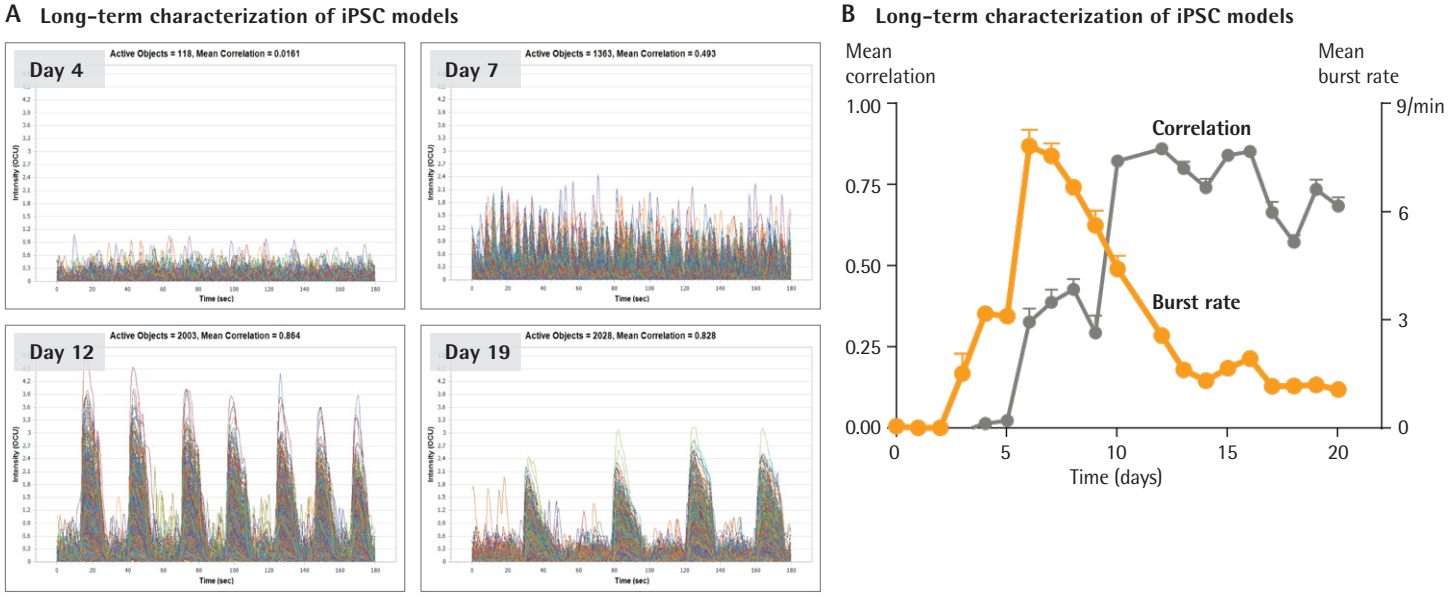


Figure 3. Long-term characterization of changes in spontaneous neuronal activity in iCell GlutaNeurons. iCell GlutaNeurons (Cellular Dynamics International) were seeded at 30K cells/well with a co-culture of rat astrocytes (15K cells/well) on PEI/laminin coated 96-well culture plates. Neurons were transduced with IncuCyte® NeuroBurst Orange Reagent at DIV 2, and spontaneous neuronal activity was analyzed over a period of 20 days. Active object traces (A) provide detailed insight into the dynamic changes in neuronal activity and connectivity for every acquired movie and are qualitatively confirmed with movie viewing tools. Kinetic quantification (B) of longitudinal, dynamic changes in neuronal activity of mean burst rate and mean correlation over time shows that during neuronal network maturation, an increase in burst rate occurs, peaking at day 5. Time course data also shows an increase in neuronal synaptic connections, as noted in an increase in correlation.

Kinetic profiles of different iPSC-derived neurons

Four different types of iPSC-derived neurons were evaluated over 30-50 days in culture to profile their functional activity. These included iCell GlutaNeurons (Figure 4a), iCell GABANeurons (Figure 4b), iCell DopaNeurons (Figure 4c) co-cultured with primary rat astrocytes, as well as CNS.4U neurons (Figure 4d). iCell GlutaNeurons, described as human glutamatergic-enriched cortical neurons derived from iPSCs, displayed a rapid induction of calcium burst activity in >1500 cells that became highly correlated within 10 days of co-culture. iCell GABANeurons, characterized as a culture of >95% pure population of GABAergic (inhibitory) neurons, also displayed a rapid increase in the number of cells with calcium burst activity within the first week of co-culture. However, iCell GABANeurons did not display significant correlation at any time-point tested, in line with their inhibitory phenotype. A closer examination of cellular activity at day 14, displayed as object traces over the full 3 min scan (Figure 5a and b), supports the observation of a significant number of active cells in both the iCell Gluta- and GABANeurons; the former displaying higher calcium burst intensity and synchronicity when compared to the latter. Interestingly, the kinetics of iCell DopaNeuron activity was strikingly similar to iCell GlutaNeurons, illustrating a very rapid induction of highly active, highly correlated networks within the first 10 days of culture. Ncardia's CNS.4U cells represent an *in vitro* co-culture model of hiPSC-derived neurons and astrocytes. These cells showed significant activity from nearly 1200 cells within the first week of culture and an increase in correlated activity (network connectivity) at approximately day 34 in culture, reaching a correlation of 0.7 at day 45 when the experiment was terminated.

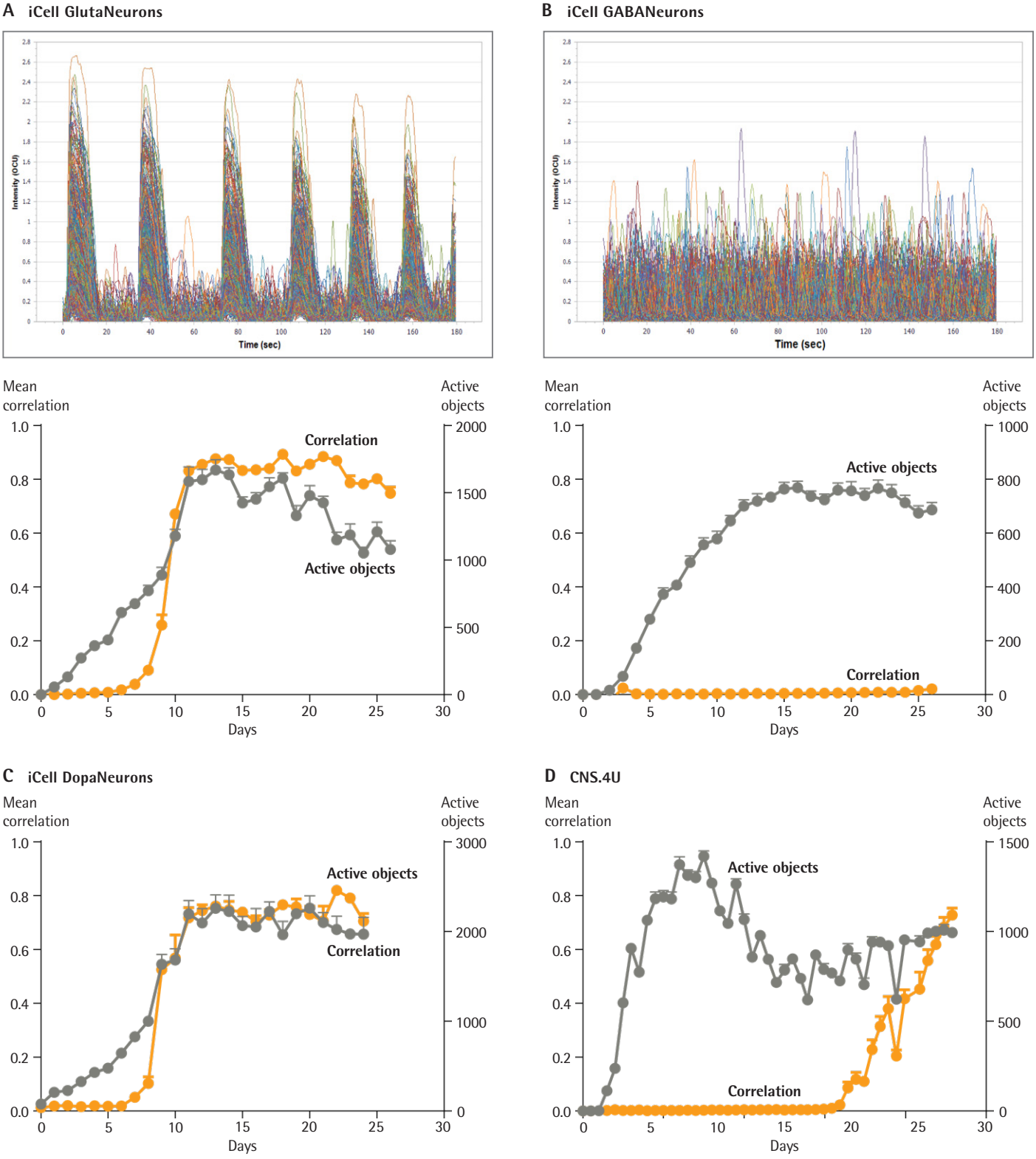


Figure 4. Comparison of differences in functional activity in iPSC-derived neurons. iCell GlutaNeurons, iCell GABANeurons, iCell DopaNeurons (Cellular Dynamics International) and CNS.4U neurons (Ncardia) were all seeded at 20K cells/well. iCell GlutaNeurons, iCell GABANeurons and iCell DopaNeurons were also plated with a co-culture of rat astrocytes seeded at 15K cells/well. Neurons were infected with NeuroBurst Orange reagent, and active object number and mean correlation were quantified for up to 45 days. Example calcium oscillation traces and kinetic graphs of activity metrics over time for iCell GlutaNeurons (A) and iCell GABANeurons (B). Mean correlation and active object number were quantified for iCell DopaNeurons (25 days) (C) and CNS.4U neurons (45 days) (D). Data points represent Mean ± SEM.

Measuring impact of culture conditions on neuronal activity in iPSC-derived neurons

To evaluate the impact of cell culture conditions on neuronal function, complete BrainPhys® Neuronal Medium, a serum-free, neurophysiological basal medium specifically developed for improved neuronal function, was evaluated to test whether the media could affect the function of iPSC-derived neurons, Peri.4Ucells, iPSC-derived peripheral neurons (Ncardia). Neurons were cultured in either BrainPhys medium or the media provided by the manufacturer (Neuro.4U; Figure 5). Qualitative inspection of cell morphology did not reveal obvious differences in cell health or neuronal network structure. Quantitative measurements of neuronal function indicate that both media types support neuronal activity with noticeably increased activity observed within the first three days of culture. However, the number of active objects observed in the Ncardia medium were higher than in complete BrainPhys medium. Interestingly, although the synchronicity of cells in both culture conditions remained low for the extent of the 25 day experiment, higher synchronicity was observed in co-cultures grown in BrainPhys medium compared to Ncardia medium. Although changes in environmental conditions (media and supplements) did not appear to affect qualitative observations of neuronal morphology and network complexity, these data illustrate that there were significant alterations in neuronal function and connectivity.

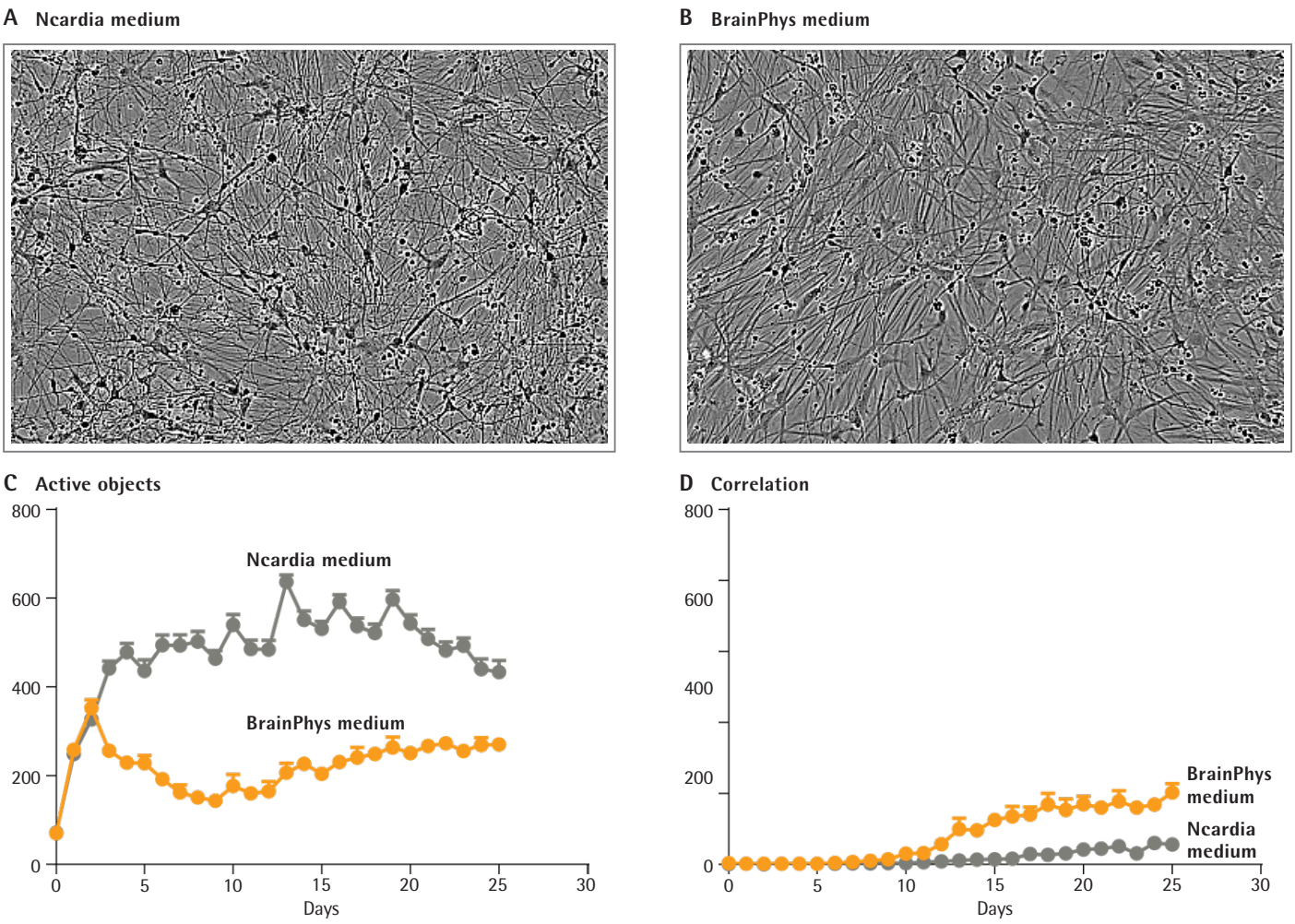


Figure 5. Impact of culture conditions on neuronal activity in iPSC-derived Peri/4Ucells. Peri.4U neurons were seeded at 25K cells/wells with a co-culture of primary rat astrocytes seeded at 15K cells/well on 96-well culture plates. Neurons were infected with NeuroBurst Orange reagent at day 2. Cells were then cultured in Complete BrainPhys Medium or Neuro.4U Medium Morphology of the neurons was not affected by media differences (A and B). Active object number (C) and mean correlation (D) were plotted over 25 days in culture. Data points represent Mean ± SEM.

Measurements of neuronal structure versus function to investigate neurotoxic effects

Additional insights can be obtained when measurements of neuronal structure and function are combined. Figure 6 shows how this was applied to study the potential neurotoxic effects of paclitaxel (Taxol®), which can sometimes be associated with neuropathic effects such as numbness and loss of sensory function. To study potential neurotoxic effects, primary rat cortical neurons were first co-cultured with primary rat astrocytes for 11 days, allowing the cultures to mature and stabilize. Baseline measurements of activity and morphology were made each day using IncuCyte NeuroBurst Orange and IncuCyte® Neurolight Orange respectively (Figure 6). At day 11, cultures were treated with a range of concentrations of Taxol. Activity and morphology were monitored for a further 11 days in culture. Figure 6 illustrates that by day 21, at sub-nanomolar (<10-9 M) concentrations of Taxol only small changes in neurite length were observed, while a reduction in neuronal activity occurred (Figure 6c). Individual well traces indicated both concentration- and time- dependent responses of neuronal activity following Taxol treatment as shown in Figure 6d.

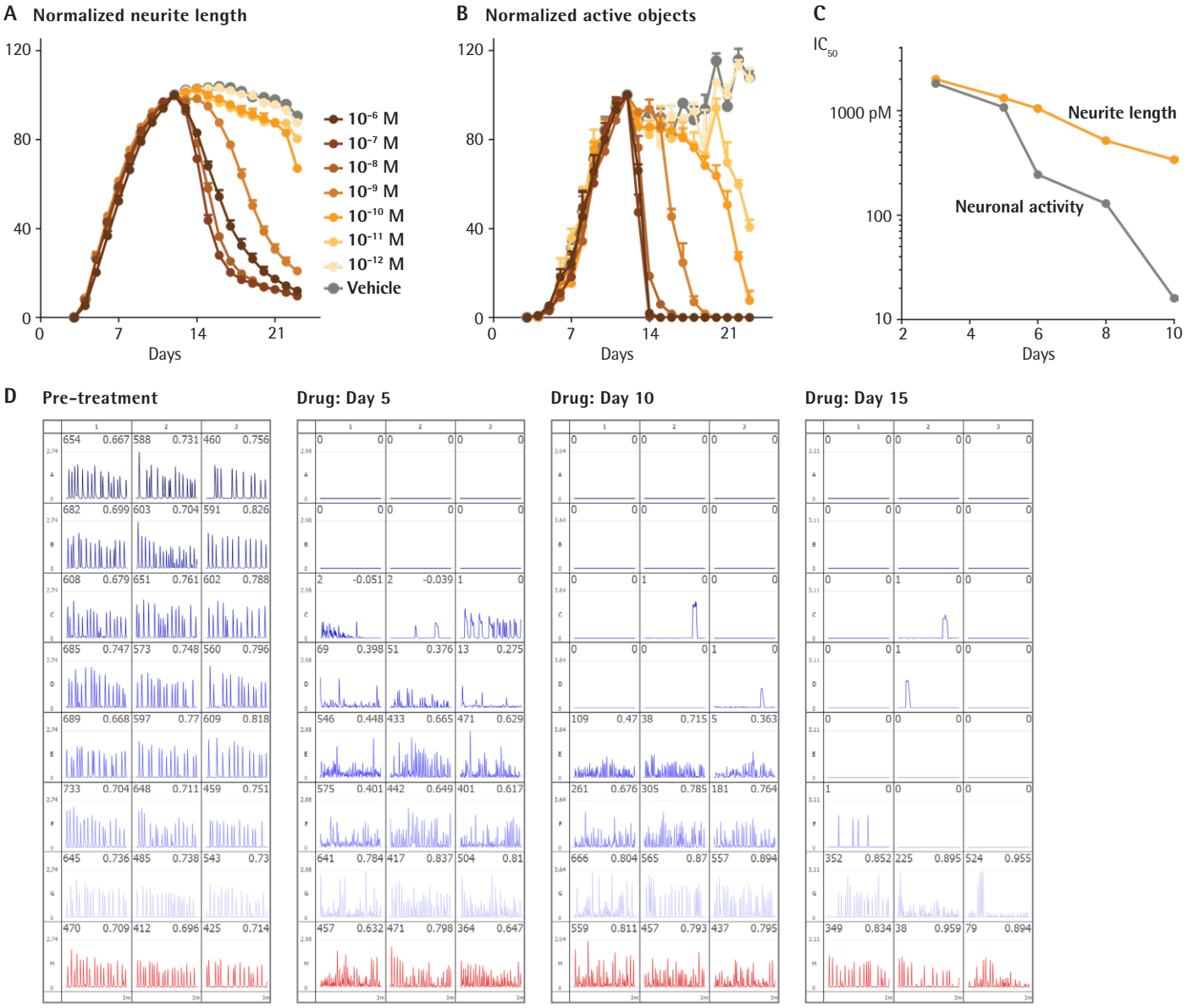


Figure 6. Taxol-induced changes in neurite length and neuronal activity in primary rat neurons. Rat cortical neurons seeded at 30K cells/well were co-cultured with rat astrocytes seeded at 15K cells/well and transduced with NeuroBurst Orange or NeuroLight Orange at day 3 in culture. Live-cell analysis measurements were made each day using IncuCyte for Neuroscience. After 11 days, neural networks had fully formed and stabilized. Taxol or vehicle control was then added and cultures were monitored for an additional 11 days. Time-courses of neurite development (A) and neuronal activity (B) prior to, and after the addition of, control or increasing concentrations of Taxol are shown. Potency (IC50 values) plotted against time post-treatment for neuronal activity (grey) and neurite length (orange) (C). Data is expressed as % neurite length or active object count, normalized to the pre-treatment value. Data points represent Mean ± SEM. Neuronal activity summary traces at pre-treatment and at 5, 10 and 15 days post-treatment display decreased activity levels over the course of the experiment (D).

Conclusions

The IncuCyte® Neuronal Activity Assay, consisting of instrumentation, software, and reagent, provides access to complex, neuronal activity and connectivity measurements as neurons and their networks mature and become functionally active through chronic evaluation of the same population of cells. This assay achieves long-term evaluation in a variety of neuronal cell models, such as primary cells and hiPSC-derived neurons, to evaluate when they become active and how this activity changes overtime without removing cells from the physiologically-relevant environment of a tissue culture incubator. Overall, live-cell analysis provides a valuable compliment to established techniques such as electrophysiology, for building and validating translational cell models for the discovery of novel neurotherapeutics.

- A non-invasive imaged-based approach allows quantitative monitoring of neuronal activity as neurons mature and networks develop; the need to select an arbitrary point in time to perform a single measurement is alleviated.

- Use of a genetically encoded calcium sensor (IncuCyte NeuroBurst Orange reagent) is non-perturbing and can be used with a variety of neuronal cell types (such as primary neurons and iPSC-derived models) to analyze short-term calcium flux kinetics.

- Acquisition of longer wavelength fluorophores (excitation > 500 nm) allows for chronic analysis of the same population of cells over weeks to months to detect functional changes and reveal differences among various iPSC-derived neuronal models or to measure impact of culture conditions on neuronal activity.

- Capture of spontaneous Ca2+ oscillations from thousands of neurons allows for highly reproducible kinetic profiling to determine both activity and connectivity using IncuCyte Neuronal Activity Analysis software tools.

- Additional insights can be obtained when measurements of neuronal structure and function are combined. As an example of utility when investigating potential neurotoxic effects, concentration response curves of paclitaxel were generated in an established co-culture of primary rat cortical neurons and astrocytes, revealing a substantial reduction in neuronal activity with only small changes in neurite length after 10 days of treatment.

Kinetic Neuroimmune Assays

Real-time quantification of chemotaxis and phagocytosis to evaluate neuroimmune function

Introduction

Several neurological disorders have been linked with the dysfunction of the immune system, including multiple sclerosis, Alzheimer's disease and brain cancers. Being the primary innate immune cells of the brain, microglia, play a role in both the etiology of such disease progression as well as in heathy brain development and maintenance. In their role as resident brain macrophages, microglia are responsible for immunosurveillance and neuroprotection, regulating brain development primarily through phagocytosis and the release of various immune proteins. However, with age, microglia become increasingly dysfunctional and lose their neuroprotective properties. Thus, studying the role of these innate immune cells is crucial in understanding the inflammatory response under both normal and degenerative conditions of the brain.

To evaluate microglia behavior and their impact on the regulation of functional neurons, detailed *in vitro* methodology is required. In particular, the cell models used, such as microglia cell lines, stem cell-derived microglia cultures and primary dissociated cell cultures, as well as their culture conditions is of pivotal importance to characterize the underlining functions of microglia. Assays designed to evaluate these models typically focus on the morphology of activated cells, their migratory response, and the ability to phagocytize diseased or dying cells as well as pathogens, employing technologies such as flow cytometry and Boyden chamber assays. However, the techniques used to study microglia behavior is typically endpoint, are performed in environments that are not representative of physiological conditions, include cumbersome assay preparation steps and do not offer insight into morphological changes associated with microglial activation.

Live-cell imaging and analysis addresses these inherent drawbacks via non-invasive, repeated monitoring of the same population of cells within a standard cell culture incubator. The includes incorporation of reagents and consumables that allow for real-time, quantification and visualization of microglial behavior, alleviating technically challenging preparation and quantification steps associated with traditional techniques. In this chapter, we will examine kinetic approaches that can shed new light on the function of microglia in order to characterize microglial response to neurodegeneration therapeutic interventions.

IncuCyte neuroimmune assays at a glance

In order to measure microglia function over time, various quantitative and qualitative assays are employed. IncuCyte® HD phase imaging allows for visual observation of activation-associated morphology changes, giving detailed insight into cellular changes associated with neurodegenerative diseases.

IncuCyte® Chemotaxis Assays are fully automated solutions to quantify directional migration in response to a stimulus. Utilizing a proprietary migration microplate, the IncuCyte® ClearView 96-Well Chemotaxis Plate, label-free analysis and precise quantification of the chemotactic response is generated throughout the experiment. In addition, visual assessment of morphological changes associated with stimuli response as the membrane of the plate is optically clear.

IncuCyte® Phagocytosis Assays enable real-time, automated analysis of both phagocytosis and efferocytosis within your cell culture incubator. Using non-perturbing, pH sensitive fluorescent probes, visualization and quantification of phagocytosis can be achieved in real-time over the entire assay time course.

Shortcomings of Traditional Assays	Live-Cell Imaging and Analysis Approaches
• Requires fixation and immunostaining steps.	• Real-time, label-free measurement of chemotaxis migration without fixing, staining or cell scraping steps.
• Measurements at user-defined time points.	• Real-time automated analysis in 96- or 384-well formats.
• Inability to visualize, confirm morphological changes.	• Direct visualization of morphological changes associated with biological response.
• Requirement for large number of cells for single-point measurements.	• Analyze sensitive and rare cells with a cell sparing, highly reproducible 96- or 384-well assays.

Sample Data

Differential morphology of microglia

Finding the optimal *in vitro* model for studying microglia in the pathogenesis of neurodegenerative disorders can be difficult. Current options for studying microglia, include immortalized cell lines, primary cell lines and human induced pluripotent stem cell (hiPSC)-derived microglia. In order to evaluate morphological characteristics of each of these models, we imaged rat primary microglia, two different sources of hiPSC-derived microglia, and three immortalized microglia cell lines. Dramatic differential morphology was observed depending on species and source (Figure 1). Evaluation of imaged-based changes in morphology gives insight into *in vitro* culture conditions, cell activation status and reprogramming events for the development of therapeutic targets for neurodegenerative disorders.

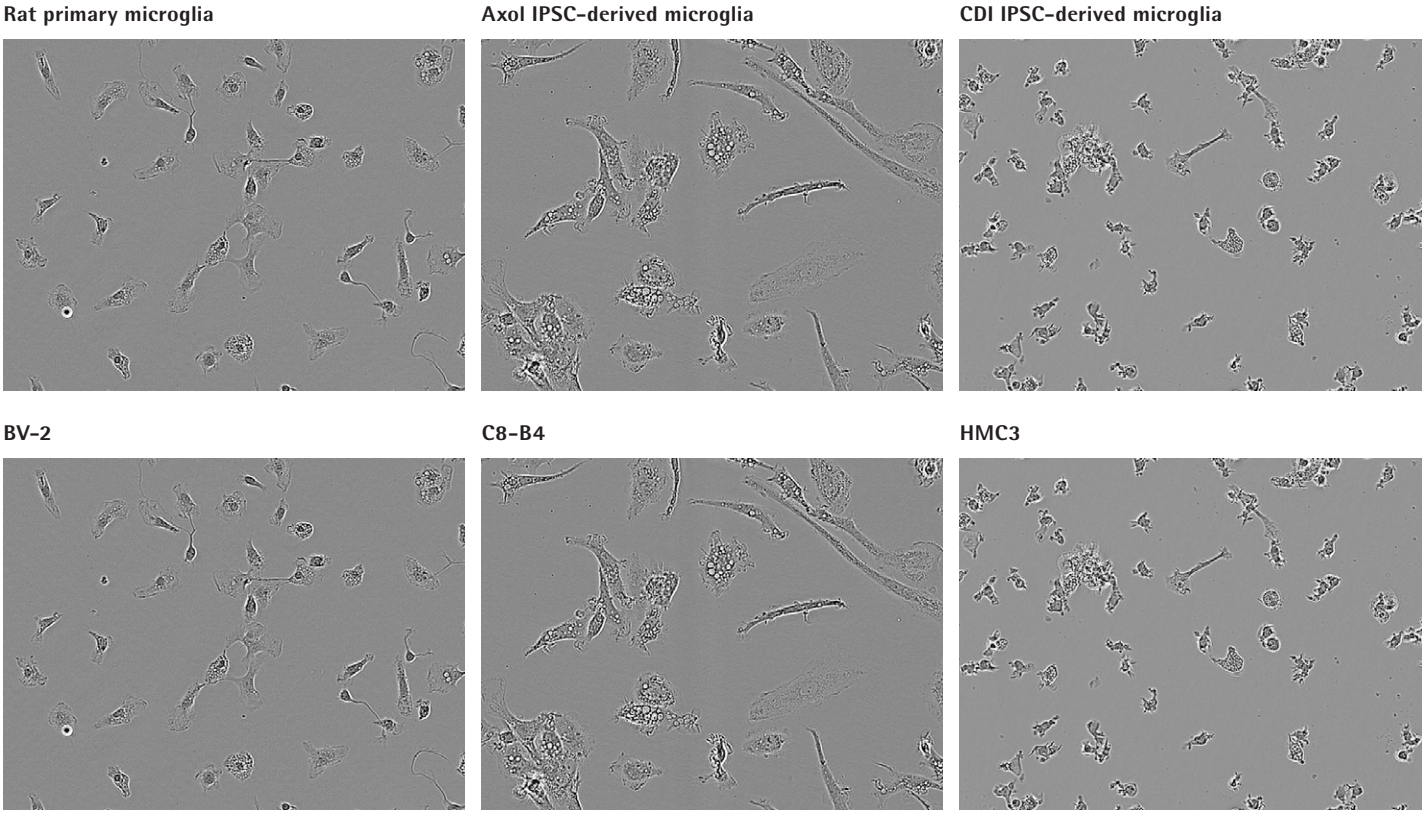
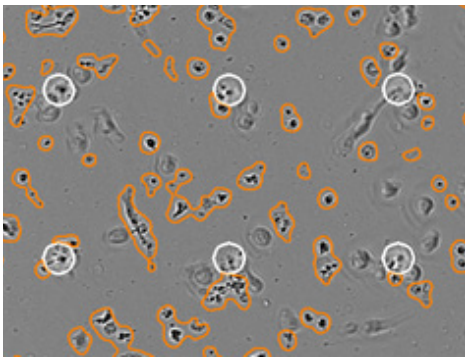


Figure 1. Visualization of morphological differences in microglia. Representative images from: Rat Primary microglia (Brain Bits – top left), iPSC-derived microglia (Axol BioSciences – top middle), iPSC-derived microgia (Cellular Dynamics – top right), immortalized muring microglia cell lines BV-2 (bottom left) and C8-B4 (bottom middle), and HMC3 human immortalized microglia (bottom right).

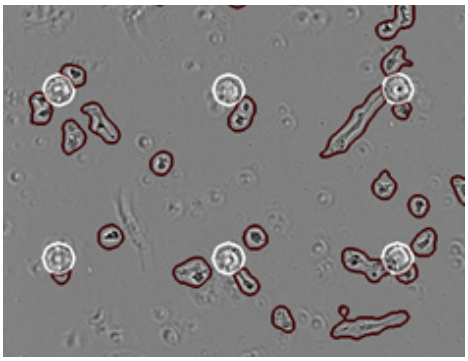
Automated quantification of microglia migration

In addition to providing insight into the morphology of microglia, the IncuCyte can also be utilized to quantify migratory capability of these cells via IncuCyte Chemotaxis Assays. Microglia are the resident immune cells of the brain, responding to extracellular signals and migrating towards the site of infection or neuronal damage. In a model of microglia migration response, iPSC-derived microglia (CDI) were seeded in an IncuCyte ClearView 96-well Chemotaxis Plate and exposed to C5a, a protein fragment involved in cell recruitment and activation of phagocytosis. After cell addition on the top membrane of the IncuCyte ClearView chemotaxis plate, three-fold dilutions of C5a were added to reservoir wells. Cells then migrated to the bottom of the membrane toward C5a. Label-free measurements of bottom-side confluence were analyzed in real time. Data shows concentration-dependent chemotactic migration of iPSC-derived microglia towards C5a over a period of 24 hours (Figure 2).

Top of membrane with mask



Bottom of membrane with mask



Concentration-dependent movement towards complement component 5a

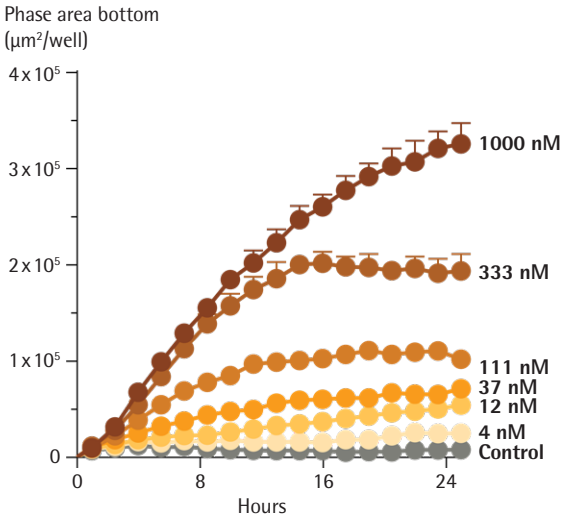


Figure 2. Concentration-dependent migration of iPSC-derived microglia (CDI) toward C5a. IPSC-derived microglia (CDI) were plated in the top chamber of the IncuCyte® ClearView 96-Well Cell Migration Plate at a density of 4,000 cells/well. Serial dilutions of C5a, starting at 1 nM, were added to the bottom reservoir wells and automated imaging and analysis was performed (data collected at 1-hour intervals). Images represent the top and bottom side of the membrane at the 30-hour time point. Automated image processing separates cells located on the top (outlined in yellow) and the bottom (outlined in black) surface of the membrane. Pores are outlined in white. Images are processed as they are acquired, and data can be plotted in real time.

Specific detection and visualization of cell engulfment by microglia

Another important function of microglia cells is the phagocytosis of cellular debris and dead neurons. To visualize and quantify the phagocytic potential of microglia, a non-perturbing pH sensitive pHrodo dye IncuCyte® pHrodo® Orange, was used. Neuro-2a target cells were treated with staurosporine to induce apoptosis and subsequently labelled with the pHrodo Orange Cell Labeling Kit. The target cells were added to wells containing iPSC-derived microglia and imaged in real time real time to visualize and quantify the engulfment of the labeled apoptotic N2A cells (Figure 3). As the engulfed cell enters the acidic phagosome of the microglia, the change in pH results in an orange fluorescent signal which is automatically segmented and quantified by integrated IncuCyte software. To verify sensitivity of the assay, increasing densities of pHrodo labelled apoptotic N2A cells were added to pre-plated rat primary microglia. Data reveals a N2A density-dependent response of orange intensity due to phagocytosis by microglia, and no fluorescent signal in wells containing only apoptotic N2A cells. (Figure 4).

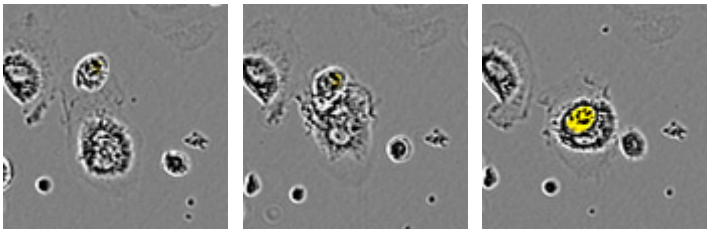


Figure 3. HD phase and fluorescent visualization of N2a engulfment by microglia. Time-lapse visualization of iPSC-derived microglia (Axol BioSciences) engulfing IncuCyte pHrodo Orange-labeled apoptotic N2A cells. Images verify the entry of apoptotic target cells into the phagosome of microglia.

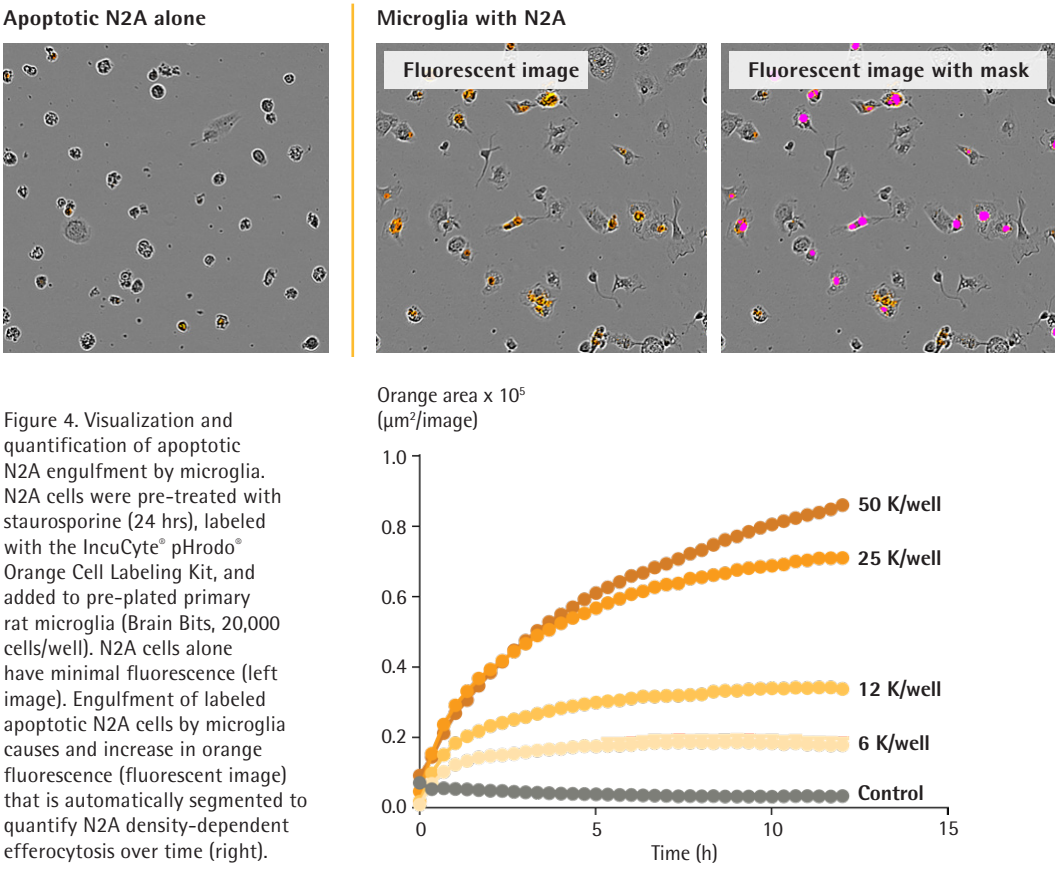


Figure 4. Visualization and quantification of apoptotic N2A engulfment by microglia. N2A cells were pre-treated with staurosporine (24 hrs), labeled with the IncuCyte® pHrodo® Orange Cell Labeling Kit, and added to pre-plated primary rat microglia (Brain Bits, 20,000 cells/well). N2A cells alone have minimal fluorescence (left image). Engulfment of labeled apoptotic N2A cells by microglia causes and increase in orange fluorescence (fluorescent image) that is automatically segmented to quantify N2A density-dependent efferocytosis over time (right).

Differential effects of inhibitors on efferocytosis and phagocytosis

The IncuCyte phagocytosis assays can also be used to evaluate pharmacological effects on microglial engulfment of dead or dying cells as well as bacterial particles. By looking at different types of target material, we can understand how treatments impact different phagocytic processes, as the receptors that mediate efferocytosis (uptake of dying/dead cells) are different from those that mediate phagocytosis of bacteria and other cellular debris. The BV2 microglia cell line was used to evaluate the effect of cytochalasin D and cilengitide on both the efferocytosis of apoptotic N2A cells and the phagocytosis of pHrodo labelled *E. coli* bioparticles. Cytochalasin D is an inhibitor of actin polymerization and elicited a concentration-dependent inhibition of both efferocytosis and phagocytosis. Cilengitide is an inhibitor of αVβ3 and αVβ5 integrins, and it selectively attenuated the efferocytosis of N2A cells with minimal effect on the phagocytosis of bioparticles. These data support the role of αVβ3 and αVβ5 integrins in the cell interactions required for efferocytosis, but not in the phagocytosis of bacteria-based bioparticles.

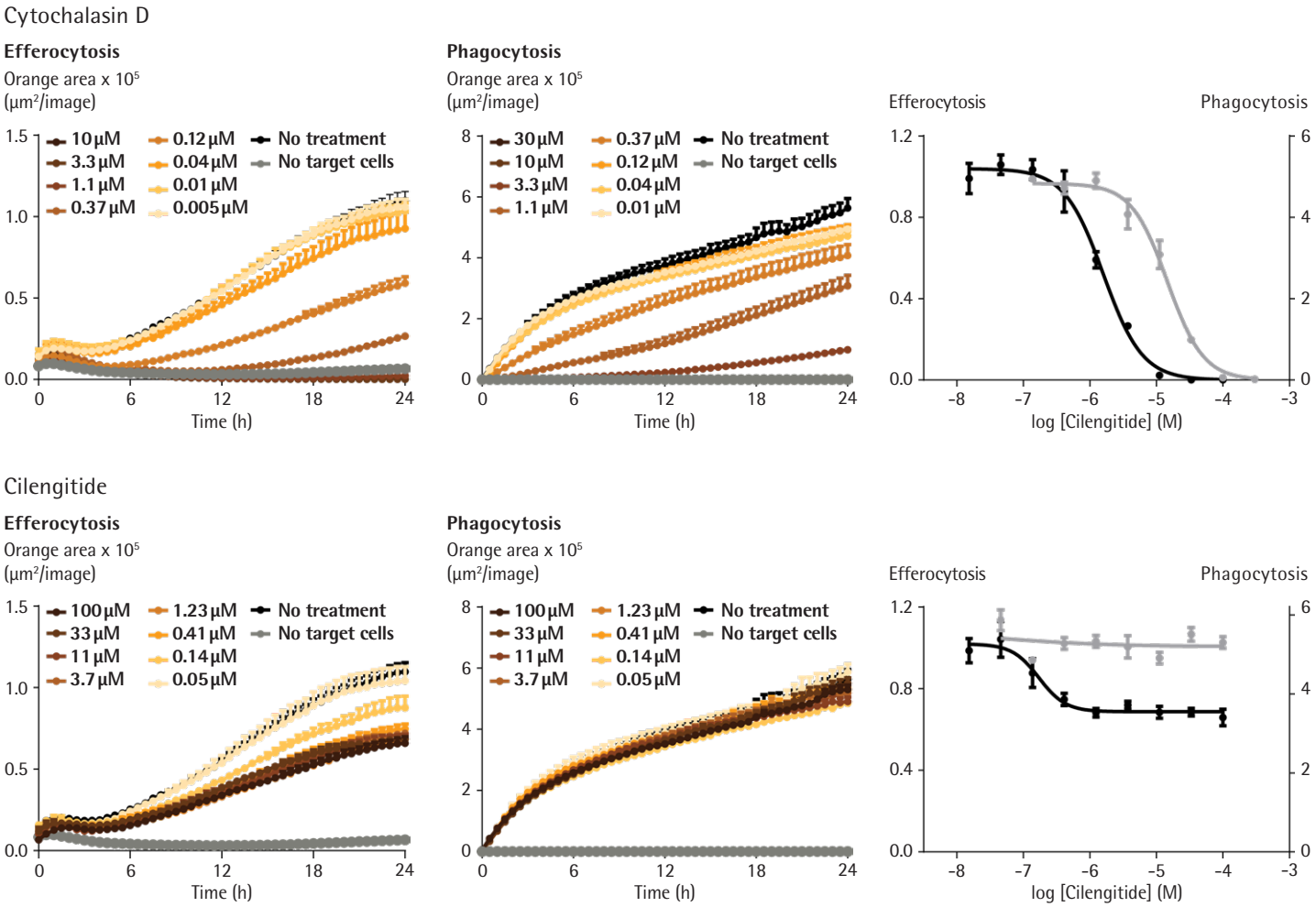


Figure 5. Quantitative pharmacological analysis of relevant target material. BV-2 effector cells (20,000 cells/well) efferocytose apoptotic N2A cells (left column) or *E. coli* bioparticles (middle column). Cytochalsin D (top row) elicits a concentration-dependent inhibition of both efferocytosis and phagocytosis, yielding IC50 values of 0.16 µM and 1.5 µM, respectively. Cilengitide, and inhibitor of αVβ3 and αVβ5 integrins, selectively attenuates efferocytosis (IC50 value of 0.16 µM), with minimal effect on phagocytosis at the highest concentration tested (100 µM).

Microglial phagocytosis of disease-related peptides

Microglia are also responsible for the clearance of other cellular debris and peptides associated with neurological disorders. In order to evaluate the engulfment of relevant disease-associated peptides, the IncuCyte® pHrodo labeling kit was used to label both apoptotic Neuro-2A, beta-amyloid, alpha-synuclein and myelin basic protein. Using iPSC-derived microglia, we observed a concentration-dependent phagocytic clearance of physiologically relevant material (Figure 6). These data support the use of pHrodo-labeled material to study microglia function in maintaining brain homeostasis to clear potential toxic factors.

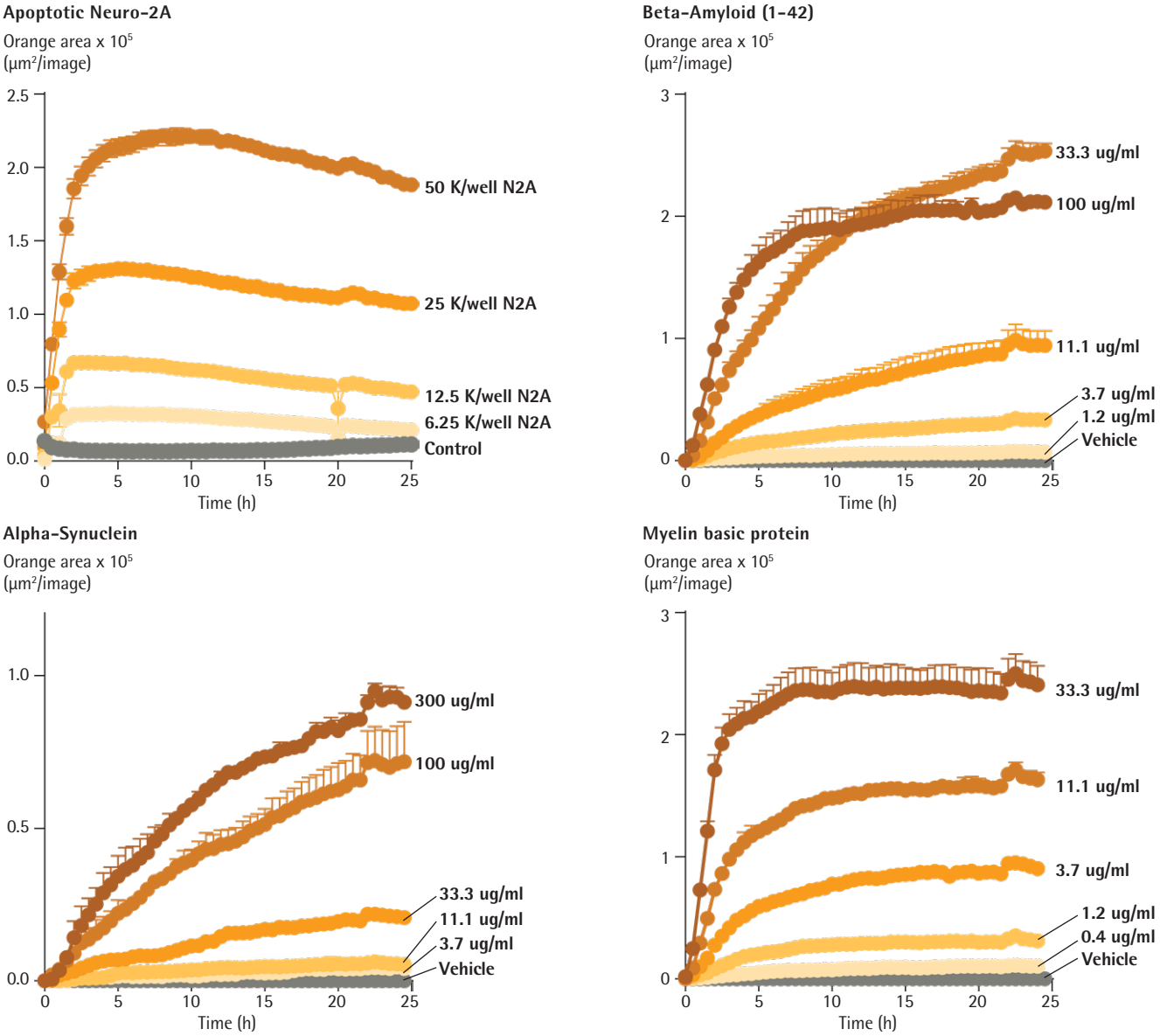


Figure 6. Comparison of phagocytic clearance of neuro-associated peptides in an iPSC-derived microglial cell model. iPSC-derived microglia (Axol BioSciences) seeded at 30,000 cells/well phagocytose physiologically relevant target material. Kinetic graphs display concentration-dependent response to pHrodo labeled apoptotic Neuro-2A, beta-amyloid, alpha-synuclein and myelin basic protein.

Conclusions

These results exemplify the IncuCyte Live-Cell Analysis system's capability for analysis of neuroimmune fucntion in live-cell models. Using non-perturbing reagents, a proprietary microplate, and purpose-built software analysis, microglia can be kinetically evaluated to monitor cell morphology, movement and function in a single, flexible platform. Specifically, this system allows for image-based evaluation of changes in microglia morphology during cell activation and reprogramming events as well as the kinetic quantification of microglia chemotaxis and phagocytosis in response to physiologically relevant material. The IncuCyte approach for real-time, long-term quantitative analysis of neuroimmune modulation is suited for development and characterization of therapeutic approaches for neurodegenerative disorders.

- System allows for users to optimize culture conditions and maintenance of different *in vitro* models by providing automated analysis of cell morphology, movement and function.
- Image-based acquisition enables qualitative assessment of morphological changes in different microglia *in vitro* models.
- Kinetic measurements of microglia chemotaxis and phagocytosis capture a complete account of dynamic cell function and movement.
- All data and time points can be verified by inspecting individual images and/or time-lapse movies.
- Low well-to-well variability and minimal user effort make the assay amenable to medium throughput screening in a 96- or 384-well format.
- This approach also provides a sensitive method to detect experimental manipulations that alter neuroimmune function to study microglia function in maintaining brain homeostasis.

IncuCyte User Publications

1. Balaban S, *et al.* **Adipocyte lipolysis links obesity to breast cancer growth: adipocyte-derived fatty acids drive breast cancer cell proliferation and migration.** *Cancer Metab*, 2017, Jan 5:1.
2. Bohlen C. J. *et al.* 2017. **Diverse requirements for microglial survival, specification, and function revealed by defined-medium cultures.** *Neuron*; 94 (4): 759-773.e8.
3. Brosius Lutz A. *et al.* 2017. **Schwann cells use TAM receptor-mediated phagocytosis in addition to autophagy to clear myelin in a mouse model of nerve injury.** *Proc. Natl. Acad. Sci. USA*; 114 (38): E8072-E8080.
4. Sellgren C. M. *et al.* 2017. **Increased microglial synapse elimination in patient-specific models of schizophrenia.** *https://www.biorxiv.org/content/early/2017/12/08/231290 Preprint bioRxiv*, 231290.
5. Zorina Y. *et al.* 2018. **Human IgM antibody rHIgM22 promotes phagocytic clearance of myelin debris by microglia.** *Sci. Rep.*; 8: 9392.

Appendix

IncuCyte® Cell Proliferation Assay Protocol

For label-free proliferation measurements of adherent or non-adherent cell lines

This protocol provides an overview of the IncuCyte® Cell Proliferation Assay methodology. It is compatible with the IncuCyte® live-cell analysis system using your choice of cells and treatments. The highly flexible assay format can be combined with our range of IncuCyte® cell health and viability reagents for multiplexed measurements of cytotoxicity and apoptosis alongside proliferation in the same well.

Required materials

- Flat bottom tissue culture plate (e.g., Corning 3595)
- Poly-L-ornithine (Sigma P4957)
  – optional, for non-adherent cells
- Fibronectin (Sigma F1141)
  – optional, for non-adherent cells

General guidelines

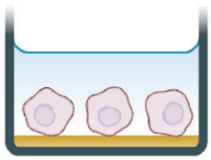
- Following cell seeding, place plates at ambient temperature (15 minutes for adherent cell lines and 45 minutes for non-adherent cell lines) to ensure homogenous cell settling.
- Remove bubbles from all wells by gently squeezing a wash bottle containing 70-100% ethanol with the inner straw removed, to blow vapor over the surface of each well.
- After placing the plate in the IncuCyte live-cell analysis system, allow the plate to warm to 37°C for 30 minutes prior to scanning.

Adherent cell line protocol

1 Coat wells

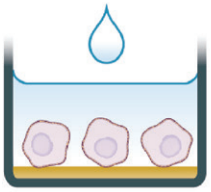
Coat wells of plate (50 µL/well) with appropriate matrix.
Optional for adherent cell lines.

2 Plate cells



Seed cells (100 µL/well, 1,000–10,000 for adherent and 5,000–50,000 for non-adherent) into a 96-well plate.

3 Add treatments



Add desired treatments (100 µL/well, 1x for adherent cell lines, 2x for non-adherent cell lines).

DAY 0:

1 Coat wells (optional)

1.1. Depending on cell line used, coat a 96-well flat bottom plate with relevant coating matrix according to manufacturer's recommendation.

2 Plate cells

2.1. Seed your choice of cells (100 µL per well) at an appropriate density into a 96-well plate, such that by day 1 the cell confluence is approximately 10-20%. The seeding density will need to be optimized for the cell line used; however, we have found that 1,000 to 2,500 cells per well (10,000 to 25,000 cells/mL seeding stock) are reasonable starting points.

a. Monitor cell growth using the IncuCyte® system to capture phase contrast images every 2 hours and analyze using the integrated confluence algorithm.

3 Add treatments

3.1. Prepare 1x concentrations of desired cell treatments in cell culture medium. The volumes may be varied; however, we recommend preparing enough volume of each desired treatment/dilution in order to achieve 100 µL per well.

3.2. Remove the cell plate from the incubator and aspirate medium from wells.

3.3. Add treatments and controls to appropriate wells of the 96-well plate.

3.4. Place the cell plate into the IncuCyte live-cell analysis system and allow the plate to warm to 37°C for 30 minutes prior to scanning.

a. Objective: 4x, 10x or 20x

b. Channel selection: Phase Contrast (+ "Green" or "Red" if fluorescent label or cell health reagents are used)

c. Scan type: Standard

d. Scan interval: Typically, every 1 to 2 hours, until your experiment is complete

Non-adherent cell line protocol

This protocol can also be used to evaluate immune cell clustering and proliferation following activation.

DAY 1:

1 Seed cells and add prepared treatments

1.1. Coat a 96-well flat bottom plate with relevant coating matrix. We recommend coating with 50 µL of either 0.01% poly-L-ornithine solution or 5 µg/mL fibronectin diluted in 0.1% BSA. Coat plates for 1 hour at ambient temperature, remove solution from wells, then allow plates to dry for 30-60 minutes prior to cell addition.

1.2. Prior to cell seeding, prepare cell treatments at 2x final assay concentration in enough cell culture medium to achieve a volume of 100 µL per well.

2 Plate cells

2.1. Seed your choice of cells (100 µL per well) at an appropriate density into a 96-well plate. The seeding density will need to be optimized for the cell line used; however, we have found that 5,000 to 50,000 cells per well (50,000–500,000 cells/mL seeding stock) are reasonable starting points.

NOTE: If studying immune cell clustering and proliferation, prepare cell activation treatments at 5x final concentration, and immediately add 50 µL per well containing cells. It is advised that some control wells containing only vehicle are included in the plate.

3 Add treatments

3.1. Immediately after cell seeding, add treatments and controls to appropriate wells of the 96-well plate containing cells. Triturate wells to appropriately mix the treatment to ensure cell exposure at 1x.

3.2. Place the cell plate into the IncuCyte live-cell analysis system and allow the plate to warm to 37°C for 30 minutes prior to scanning.

a. Objective: 4x (recommended 1 image per well or whole well) or 10x

b. Channel selection: Phase Contrast (+ "Green" or "Red" if fluorescent label or cell health reagents are used)

c. Scan type: Standard

d. Scan interval: Typically, every 1 to 2 hours, until your experiment is complete.

IncuCyte® Cell Transduction Protocol

For stable or transient fluorescent labelling of cell nuclei

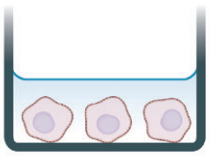
This protocol provides an overview of methodologies used for the transduction of cell lines with a range of IncuCyte® NucLight labeling reagents. The IncuCyte NucLight live-cell labelling reagents are used to fluorescently label the nuclei of living cells without perturbing cell function or biology, enabling real-time cell counting using your choice of cells and treatments. In addition, the highly flexible assay format can be combined with our range of IncuCyte® Cytotox Reagents, IncuCyte® Annexin V Apoptosis Reagents, or IncuCyte® Caspase-3/7 reagent for multiplexed measurements of cytotoxicity and apoptosis alongside proliferation in the same well.

Creating a cell population or clone that stably expresses a nuclear label

We recommend the use of IncuCyte NucLight Lentivirus Reagents to provide stable, homogenous expression of a nuclear-restricted green or red fluorescent protein (GFP or mKate2) in your choice of living mammalian cells without perturbing cell function and with minimal toxicity. These reagents are ideal for generating stable cell populations or clones using puromycin or bleomycin selection.

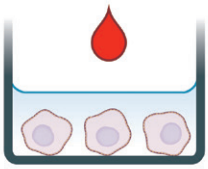
Protocol

1 Seed cells



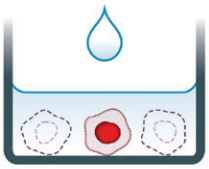
Seed cells in growth media and leave to adhere (4-24 hours). Cells should be 15-35% confluent at the time of transduction.

2 Transduce



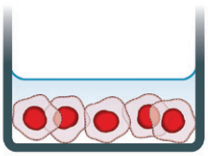
Add Green or Red NucLight Lentivirus Reagent (MOI 3 to 6) diluted in media ± Polybrene®. After 24 hours, replace the media with fresh growth media. Monitor expression using the IncuCyte system.

3 Apply selection



Apply anitobiotic selection to derive a stable, homogenous cell population or clone that expresses a nuclear restricted green or red fluorescent protein. Optional: Freeze cells and use for future assays.

4 Live cell fluorescent imaging



Capture images every 1 to 2 hours (4x, 10x or 20x) in an IncuCyte system. Analyze using integrated software.

DAY 0:

1 Seed cells

1.1. Seed cells in growth media of choice and at a density such that they are 15-35% confluent after 24 hours of incubation.

2 Transduce

2.1. Add the IncuCyte NucLight Lentivirus Reagent at desired multiplicity of infection (MOI = TU/cell). An MOI of 3 to 6 is recommended for most cell types, however, an optimized MOI should be determined for each cell type used. Polybrene® (1-8 µg/mL) may also be added to enhance transduction of some cell types (Note: Certain cell types can be sensitive to Polybrene® (e.g. neurons)).

2.2. Incubate at 37°C, 5% CO_2 for 24 hours, then remove and replace with fresh growth media.

2.3. Return to incubator for an additional 24-48 hours, monitoring expression using an IncuCyte system

3 Apply selection

3.1. Remove media and replace with fresh growth medium containing selection (i.e. puromycin or bleomycin).

Example: For HT-1080, A549, HeLa, and MDA-MB-231 cells, complete media containing 1 µg/mL puromycin is sufficient for efficient killing of non-transduced cells.

4 Live cell fluorescent imaging

4.1. Incubate for 72-96 hours, replacing media every 48 hours. Maintain stable population in a maintenance concentration of selection media.

Example: HT-1080, A549, HeLa, and MDA-MB-231 cells labeled with the IncuCyte NucLight Red Lentivirus Reagent (EF-1 µ, Puro) can be maintained in complete media containing 0.5 µg/mL puromycin.

IncuCyte® Apoptosis Assay Protocol

For the fluorescent detection of caspase-3/7 activation or phosphatidylserine externalization

This protocol provides an overview of the IncuCyte Apoptosis Assay methodology which uses mix-and read IncuCyte® Caspase 3/7 or Annexin V Reagents to detect apoptosis in real time. It is compatible with the IncuCyte® live-cell analysis system using your choice of cells and treatments. The highly flexible assay format can be combined with our range of IncuCyte® NucLight red nuclear labeling reagents or labeled cell lines for multiplexed measurements of proliferation and apoptosis in the same well.

Required materials

- IncuCyte® Caspase- 3/7 Apoptosis Reagent (EssenBioscience Cat #4440)

  or
- IncuCyte® Annexin V Red Reagent (EssenBioScience Cat #4641)

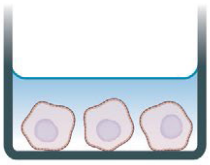
  or
- IncuCyte® Anexin V Green Reagent (EssenBioScience Cat #4642)
- Poly-L-ornithine (Sigma P4957) – optional, for non-adherent cells
- Fibronectin (Sigma F1141) - optional, for non-adherent cells
- Flat bottom tissue culture plate (e.g., Corning 3595)

General guidelines

- We recommend medium with low levels of riboflavin to reduce the green fluorescence background. EBM, F12-K, and Eagles MEM have low riboflavin (<0.2 mg/L). DMEM and RPMI have high riboflavin (>0.2 mg/L).
- Following cell seeding, place plates at ambient temperature (15 minutes for adherent cell lines and 45 minutes for nonadherent cell lines) to ensure homogenous cell settling.
- Remove bubbles from all wells by gently squeezing a wash bottle (containing 70-100% ethanol with the inner straw removed) to blow vapor over the surface of each well.
- After placing the plate in the IncuCyte® live-cell analysis system, allow the plate to warm to 37°C for 30 minutes prior to scanning.
- If monitoring apoptosis in primary neuronal cultures, we recommend use of the IncuCyte Annexin V Red reagent to eliminate risk of green channel excitation issues in these sensitive cell lines.

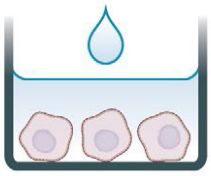
Adherent cell line protocol

1 Seed Cell



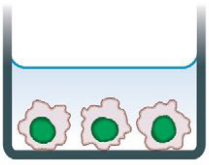
Seed cells (100 µL/well, 1,000-5,000) into a 96-well plate and incubate overnight.

2 Prepare apoptosis reagent and treat cells



Prepare the desired treatments at 1x in medium containing IncuCyte Caspase-3/7 or Annexin V Reagents. Aspirate media from wells and add treatment (100 µL/well).

3 Live cell fluorescent imaging



Capture images every 2-3 hours (20x or 10x) in the IncuCyte® System. Analyze using integrated software.

DAY 0:

1 Seed cell

1.1. Seed your choice of cells (100 µL per well) at an appropriate density into a 96-well plate, such that by day 1 the cell confluence is approximately 30%. The seeding density will need to be optimized for the cell line used; however, we have found that 1,000 to 5,000 cells per well (10,000–50,000 cells/mL seeding stock) are reasonable starting points.

a. Monitor cell growth using the IncuCyte system to capture phase contrast images every 2 hours and analyze using the integrated confluence algorithm.

DAY 1:

2 Apoptosis reagent preparation and cell treatment addition

2.1. Dilute apoptosis reagents in desired medium formulations

a. If using caspase-3/7, dilute reagent to a final concentration of 5 µM (1:1000 dilution)

b. If using Annexin V reagents, solubilize Annexin V by adding 100 µL of complete medium or PBS. The reagents may then be diluted in complete medium containing at least 1 mM $CaCl_2$ for a final dilution of 1:200.

NOTE: All test agents will be diluted in this reagent-containing medium, so make up a volume that will accommodate all treatment conditions. The volumes/ dilutions added to cells may be varied; however, a volume of 100 µL per well is generally sufficient for the duration of the assay.

2.2. Remove the cell plate from the incubator and aspirate off growth medium.

2.3. Add treatments and controls to appropriate wells of the96-well plate.

3 Live-Cell imaging of apoptosis

3.1. Place the cell plate into the IncuCyte Live-Cell Analysis System and allow the plate to warm to 37°C for 30 minutes prior to scanning.

a. Objective: 10x or 20x

b. Channel selection: Phase Contrast and Green (+ "Red" if using fluorescent label or an additional cell health reagent)

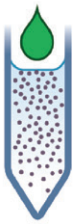
c. Scan type: Standard (2-4 images per well)

d. Scan interval: Typically, every 2 hours, until your experiment is complete.

1 Coat plate

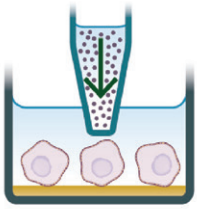
Coat plate with 0.01% poly-L-ornithine solution or 5 µL/mL fibronectin diluted in 0.1% BSA.

2 Prepare incucyte apoptosis reagent and treatment



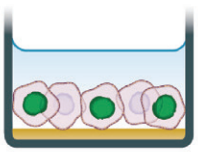
Dilute apoptosis reagent in medium and prepare cell treatments

3 Seed cells and add treatment



Seed cells (100 µL/well, 5,000–25,000 cells) into the coated 96-well plate. Immediately add apoptosis reagent ± treatments and triturate.

4 Live cell fluorescent analysis



Capture images every 2-3 hours (20x or 10x) in the IncuCyte® system.

Non-adherent cell line protocol

DAY 1:

1 Coat plate

1.1. Coat a 96-well flat bottom plate with appropriate coating matrix. We recommend coating with 50 µL of either 0.01% poly-L-ornithine solution or 5 µg/mL fibronectin diluted in 0.1% BSA. Coat plates for 1 hour at ambient temperature, remove solution from wells, then allow plates to dry for 30-60 minutes prior to cell addition.

2 Prepare apoptosis reagent and treatments

2.1. Prior to cell seeding, dilute apoptosis reagents in desired medium formulation.

a. If using caspase-3/7, dilute reagent to a final concentration of 5 µM (1:1000 dilution).

b. If using Annexin V reagents, solubilize Annexin V by adding 100 µL of complete medium or PBS. The reagents may then be diluted in complete medium containing at least 1 mM $CaCl_2$ for a final dilution of 1:200.

NOTE: All test agents will be diluted in this reagent-containing medium, so make up a volume that will accommodate all treatment conditions. The volumes/dilutions added to cells may be varied; however, a volume of 200 µL per well is generally sufficient for the duration of the assay.

2.2. Prepare cell treatments at 2x final assay concentration in enough cell culture medium containing caspase-3/7 or Annexin V to achieve a volume of 100 µL per well.

3 Seed cells and add prepared treatments

3.1. Seed your choice of cells (100 µL per well) at an appropriate density into a 96-well plate in medium containing Caspase 3/7 or Annexin V. The seeding density will need to be optimized for the cell line used; however, we have found that 5,000 to 25,000 cells per well (50,000–250,000 cells/mL seeding stock) are reasonable starting points.

3.2. Immediately add treatments and controls to appropriate wells of the 96-well plate containing cells. Triturate wells to appropriately mix the treatment to ensure cell exposure at 1x.

4 Live-Cell imaging of apoptosis

4.1. Place the cell plate into the IncuCyte® live-cell analysis system and allow the plate to warm to 37°C for 30 minutes prior to scanning.

a. Objective: 10x or 20x

b. Channel selection: Phase Contrast and Green (+ "Red" if using fluorescent label or an additional cell health reagent)

c. Scan type: Standard (2-4 images per well)

d. Scan interval: Typically, every 2 hours, until your experiment is complete.

IncuCyte® Cytotoxicity Assay

For the fluorescent quantification of cell death

This protocol provides an overview of the IncuCyte® Cytotoxicity Assay methodology which uses the mix-and-read IncuCyte® Green or Red Reagent to detect cell death in real time. The protocol is compatible with the IncuCyte® live-cell analysis system and your choice of cells (e.g., tumor, immune, neuronal) and treatments. Furthermore, this protocol can be used with cells labeled using the IncuCyte® NucLight nuclear labeling reagents to provide multiplexed measurements of proliferation alongside cell death in the same well.

Required materials

- IncuCyte® Red Cytotoxicity Reagent (EssenBioscience Cat #4632)

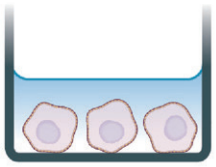
  or
- IncuCyte® Green Cytotoxicity Reagent (EssenBioscience Cat #4633)
- Poly-L-ornithine (Sigma P4957) – optional, for non-adherent cells
- Fibronectin (Sigma F1141) - optional, for non-adherent cells
- Flat bottom tissue culture plate (e.g., Corning 3595, TPP 92096 for neuronal cell health)

General guidelines

- We recommend medium with low levels of riboflavin to reduce the green fluorescence background. EBM, F12-K, and Eagles MEM have low riboflavin (<0.2 mg/L). DMEM and RPMI have high riboflavin (>0.2 mg/L).
- Following cell seeding, place plates at ambient temperature (15 minutes for adherent cell lines and 45 minutes for nonadherent cell lines) to ensure homogenous cell settling.
- Remove bubbles from all wells by gently squeezing a wash bottle (containing 70-100% ethanol with the inner straw removed) to blow vapor over the surface of each well.
- After placing the plate in the IncuCyte® live-cell analysis system, allow the plate to warm to 37°C for 30 minutes prior to scanning
- If monitoring cytotoxcity in primary neuronal cultures, we recommend use of the IncuCyte® Cytotox Red reagent to eliminate risk of green channel excitation issues in these sensitive cell types.

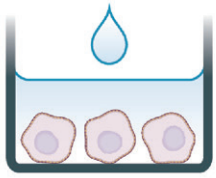
Adherent cell line protocol

1 Seed cells



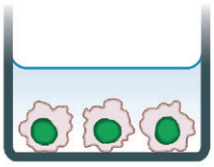
Seed cells (100 µL/well, 1,000–5,000) into a 96-well plate and incubate overnight.

2 Prepare cytotoxicity reagent and treat cells



Prepare the desired treatments at 1x in medium containing IncuCyte Cytotoxicity reagent. Aspirate media from wells and add treatment (100 µL/well).

3 Live cell fluorescent analysis



Capture images every 2-3 hours (20x or 10x) in the IncuCyte® System. Analyze using integrated software.

DAY 0:

1 Seed effector cells

1.1. Seed your choice of cells (100 µL per well) at an appropriate density into a 96-well plate, such that by day 1 the cell confluence is approximately 30%. The seeding density will need to be optimized for the cell line used; however, we have found that 1,000 to 5,000 cells per well (10,000–50,000 cells/mL seeding stock) are reasonable starting points.

NOTE: For non-proliferating cell lines (e.g., rate forebrain neurons) we recommend seeding at 15 x 10^3 to 30 x 10^3 cells per well, and culturing for 14 days for the neural network to establish, prior to evaluating cytotoxicity.

a. Monitor cell growth using the IncuCyte system to capture phase contrast images every 2 hours and analyze using the integrated confluence algorithm.

DAY 1:

2 Cytotoxicity reagent preparation and cell treatment addition

2.1. Dilute cytotoxicity reagent in desired medium formulation.

NOTE: All test agents will be diluted in this reagent-containing medium, so make up a volume that will accommodate all treatment conditions. The volumes/dilutions added to cells may be varied; however, a volume of 100 µL per well is generally sufficient for the duration of the assay.

2.2. Remove the cell plate from the incubator and aspirate off growth medium.

2.3. Add treatments and controls to appropriate wells of the 96-well plate.

3 Live-cell imaging of cytotoxicity

3.1. Place the cell plate into the IncuCyte Live-Cell Analysis System and allow the plate to warm to 37°C for 30 minutes prior to scanning.

a. Objective: 10x or 20x

b. Channel selection: Phase Contrast and Fluorescence

c. Scan type: Standard (2-4 images per well)

d. Scan interval: Typically, every 2 hours, until your experiment is complete.

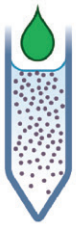
NOTE: For neuronal cultures we recommend scanning every 6 to 12 hours to minimize risk of phototoxicity.

Non-adherent cell line protocol

1 Coat Plate

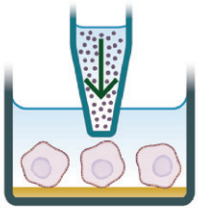
Coat plate with 0.01% poly- L-ornithine solution or 5 µL/mL fibronectin diluted in 0.1% BSA.

2 Prepare cytotoxicity reagent and treatment



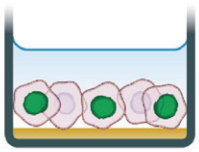
Dilute cytotoxicity reagent in medium at 1x and prepare cell treatments

3 Seed cells and add treatment



Seed cells (100 µL/well, 5,000–25,000 cells) into the coated 96-well plate. Immediately add cytotoxicity reagent ± treatments and triturate.

4 Live cell fluorescent analysis



Capture images every 2-3 hours (20x or 10x) in the IncuCyte® System.

DAY 1:

1 Coat plate

1.1. Coat a 96-well flat bottom plate with appropriate coating matrix. We recommend coating with 50 µL of either 0.01% poly-L-ornithine solution or 5 µg/mL fibronectin diluted in 0.1% BSA. Coat plates for 1 hour at ambient temperature, remove solution from wells, then allow plates to dry for 30-60 minutes prior to cell addition.

2 Prepare cytotoxity reagent and treatments

2.1.Prior to cell seeding, dilute cytotoxicity reagent to a final concentration of 250 nM (1:4000 dilution) in desired medium formulation.

NOTE: All test agents will be diluted in this reagent-containing medium, so make up a volume that will accommodate all treatment conditions. The volumes/ dilutions added to cells may be varied; however, a volume of 100 µL per well is generally sufficient for the duration of the assay.

2.2. Prepare cell treatments at 2x final assay concentration in enough cell culture medium the cytotoxicity reagent to achieve a volume of 100 µL per well.

3 Seed cells and add prepared treatments

3.1. Seed your choice of cells (100 µL per well) at an appropriate density into a 96-well plate in medium containing the cytotoxicity reagent. The seeding density will need to be optimized for the cell line used; however, we have found that 5,000 to 25,000 cells per well (50,000–250,000 cells/mL seeding stock) are reasonable starting points.

3.2. Immediately add treatments and controls to appropriate wells of the 96-well plate containing cells. Triturate wells to appropriately mix the treatment to ensure cell exposure at 1x.

4 Live-Cell Imaging of cytotoxicity

1.1. Place the cell plate into the IncuCyte® live-cell analysis system and allow the plate to warm to 37°C for 30 minutes prior to scanning.

a. Objective: 4x (recommended 1 image per well or whole well) or 10x

b. Channel selection: Phase Contrast and Fluorescence

c. Scan type: Standard

d. Scan interval: Typically, every 2 hours, until your experiment is complete.

IncuCyte® Immune Cell Killing Assay

For measurements of tumor cell death

This protocol provides an overview for the measurement of immune cell killing of adherent or non-adherent target tumor cells. The flexible assay format is suitable for cytotoxic T cell killing and antibody-dependent cell-mediated cytotoxicity (ADCC) assays using a co-culture methodology that combines direct measurements of tumor cell death with no-wash, mix-and-read protocols. This method utilizes either our IncuCyte® Caspase 3/7 reagent, a substrate that is cleaved during target cell apoptosis to release a green-fluorescent DNA dye that stains the nuclear DNA, or IncuCyte® Annexin V reagent which labels externalized phosphatidylserine (PS) moieties. IncuCyte® image analysis software enables automated detection and selective quantitation of tumor cell death in real time.

General guidelines

- We recommend medium with low levels of riboflavin to reduce the green fluorescence background. EBM, F12-K, and Eagles MEM have low riboflavin (<0.2 mg/L). DMEM and RPMI have high riboflavin (>0.2 mg/L).
- Following cell seeding, place plates at ambient temperature (15 minutes for adherent cell lines and 45 minutes for non-adherent cell lines) to ensure homogenous cell settling.
- Remove bubbles from all wells by gently squeezing a wash bottle (containing 70-100% ethanol with the inner straw removed) to blow vapor over the surface of each well.
- After placing the plate in the IncuCyte® live-cell analysis system, allow the plate to warm to 37°C for 30 minutes prior to scanning

REQUIRED MATERIALS

- IncuCyte® Caspase- 3/7 Apoptosis Reagent (Essen Bioscience Cat #4440)

or

- IncuCyte® Annexin V Green Reagent (Essen BioScience Cat #4642)

or

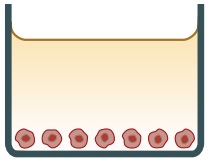
- IncuCyte® Annexin V Red Reagent (Essen BioScience Cat #4641)
- Poly-L-ornithine (Sigma P4957), for non-adherent cells

or

- Fibronectin (Sigma F1141), for non-adherent cells
- Flat bottom tissue culture plate (e.g., Corning 3595)
- Target cells of interest (non-adherent target cells are required to be labeled with NucLight live-cell labeling reagent to enable tumor cell counting).
- IncuCyte® NucLight Red Lentivirus Reagent (Essen BioScience Cat # 4476)
- IncuCyte® NucLight Green Lentivirus Reagent (Essen BioScience Cat # 4475)
- Immune (effector) cells of interest 96-well microplate (e.g., Corning® 3595)

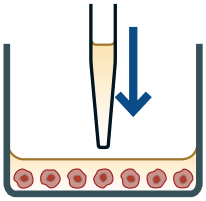
Immune cell killing of adher ent tumor cells protocol

1 Seed target cells



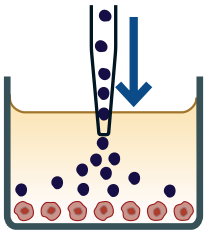
Seed tumor cells (100 µL/well, 1,000 to 3,000/well) into the 96-well plate. Optional: Target cells can be labeled with IncuCyte® NucLight Red live-cell labeling reagent (Essen BioScience 4476) to enable simultaneous tumor cell counting.

2 Treat cells



Aspirate the medium and add the Caspase 3/7 reagent or Annexin V reagent (50 µL/well) and desired treatments (50 µL/well) at 4x final assay concentrations.

3 Add immune cells



Add your choice of immune cells (100 µL/well, 10,000 to 30,000/well) to a 96-well plate.

DAY 0:

1 Seed target cells

1.1. Seed target cancer cells (100 µL per well) at an appropriate density into a 96-well flat-bottom plate such that by day 1 the cell confluency is approximately 20%. The seeding density will need to be optimized for each tumor cell line used; however, we have found that 1,000 to 3,000 cells per well are reasonable starting points.

a. Target cell growth can be monitored using the IncuCyte® live-cell analysis system and confluence algorithm.

b. Optional: Target cells can be labeled with NucLight Red live-cell labeling reagent (Catalog # 4475 or 4476) to enable simultaneous real-time counting of viable tumor cells.

DAY 1:

2 Prepare apoptosis reagent and treatments

2.1. Dilute apoptosis reagents, ensuring compatibility of target cell label and apoptotic marker, and treatments (e.g., T cell stimuli, antibodies, cytokines) at 4x final assay concentration in desired assay medium.

a. If using caspase-3/7, dilute reagent to a concentration of 20 µM (1:250 dilution), sufficient for 50 µL per well.

b. If using Annexin V reagents, solubilize Annexin V by adding 100 µL of complete medium or PBS. The reagents may then be diluted in complete medium containing at least 1 mM $CaCl_2$ for a dilution of 1:50, sufficient for 50 µL per well.

2.2. Remove the cell plate from the incubator and aspirate off growth medium.

2.3. Add 50 µL each of the prepared apoptosis reagent and treatments from step 2.1 above.

NOTE: For treatment controls, add 50 µL of assay medium.

3 Add treatments

3.1. Count chosen effector cells (e.g. T cells, PBMCs) and prepare a cell suspension at a density of 100,000 to 300,000 cells/mL (100 µL per well, 10,000 to 30,000 cells/ well). It is recommended that different target-to-effector cell ratios are tested (e.g., 1:5, 1:10).

NOTE: Assay duration may be reduced by pre-activating the effector cells before addition to assay plate, however, this may require a higher initial seeding density of target cells.

3.2. Seed 100 µL of effector cells into the appropriate wells of the cell plate to achieve a final assay volume of 200 µL. Allow plates to settle on level surface at ambient temperature for 30 minutes.

3.3. Place the assay plate into the IncuCyte live-cell analysis system and schedule 24 hour repeat scanning:

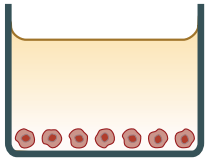
a. Objective: 10x

b. Channel selection: Phase Contrast + "Green" or "Red" depending on apoptosis reagent and target cell label used

c. Scan type: Standard (2 images per well)

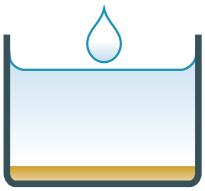
d. Scan interval: Every 3 hours

1 Coat plate



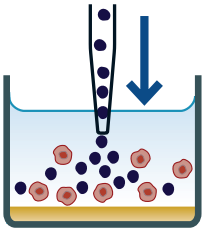
Coat plate surface to ensure even target cell coverage e.g. Poly-L-ornithine solution.

2 Prepare treatments



Prepare Annexin V reagent (50 µL/well) and desired treatments (50 µL/well) at 4x final assay concentrations.

3 Addition of target and effector cells



Add NucLight labeled target cells (50 µL/well, 10,000 to 20,000/ well) and immune cells (50 µL/ well, 100,000 to 200,000/well) to a 96-well plate.

Immune cell killing of adherent tumor cells protocol

DAY 1:

1 Coat plate

1.1. Coat a 96-well flat bottom plate with relevant coating matrix. We recommend coating with 50 µL of either 0.01% poly-L-ornithine solution or 5 µg/mL fibronectin diluted in 0.1% BSA. Coat plates for 1 hour at ambient temperature, remove solution from wells, then allow plates to dry for 30-60 minutes prior to cell addition. Choice of coating will need to be determined prior to the assay for target cells of interest.

2 Prepare apoptosis reagent and treatments

2.1. Prepare the following reagents in medium:

a. Test materials (e.g. T cell stimuli, antibodies, cytokines; 50 µL per well, prepared at 4x final assay concentration).

b. Apoptosis detection reagent (ensure compatibility of cell label and apoptotic marker), IncuCyte® Annexin V Reagent (Cat # 4641 or 4642): solubilize Annexin V by adding 100 µL of complete medium or PBS. The reagents may then be diluted in complete medium containing at least 1 mM $CaCl_2$ for a dilution of 1:50 (4x final assay concentration, 50 µL per well.

NOTE: Although either the IncuCyte® Annexin V or Caspase-3/7 reagents can be used to detect immune cell killing of target cells we recommend that the Annexin V reagent is used for non-adherent target cells. Non-adherent target and effector cell types can have very similar nuclear sizes negating the use of size filters to remove Caspase-3/7 labeled effector nuclei from the analysis. Additionally, we have observed raised levels of caspase 3/7 activity in some non-adherent cell types, particularly at higher confluency, which can interfere with the interpretation of immune cell driven target cell death. In our experience the Annexin V reagent labels fewer effector cells and provides lower non-specific background.

2.2. Add all prepared reagents to assay plate to achieve 100 µL per well.

3 Add immune cells

3.1. Count labeled target cells and prepare a cell suspension at a density of 40,000 – 80,000 cells/mL (seed 50 µL per well, 10,000 to 20,000 cells/well). Target cells can be labeled with NucLight Red or Green live-cell labeling reagent (Cat # 4476 or 4475) to enable simultaneous real-time counting of viable tumor cells.

3.2. Count chosen effector cells (e.g. T cells, PBMCs) and prepare a cell suspension at a density of 400,000 – 800,000 cells/mL (50 µL per well, 100,000 to 200,000 cells/well). It is recommended that different target-to-effector cell ratios are tested (e.g. 1:5, 1:10).

NOTE: Assay duration may be reduced by pre-activating the effector cells before addition to assay plate, however, this may require a higher initial seeding density of target cells.

3.3. Add target and effector cells to assay plate to achieve a final assay volume of 200 µL. Allow plates to settle on level surface at ambient temperature for 30 minutes.

3.4. Place the assay plate into the IncuCyte® instrument and schedule 24 hour repeat scanning:

a. Objective: 4x

b. Channel selection: Phase Contrast + “Green” and “Red”

c. Scan type: Standard (2 images per well)

d. Scan interval: Every 2–3 hours

IncuCyte® Scratch Wound Assay

For the measurement of invasion or migration into a wound region

This protocol provides an overview of our cell motility assay, which is suitable for the analysis of migration or invasion of adherent cell lines. This method utilizes our IncuCyte® WoundMaker tool to create 96 precise, uniform cell-free zones in cell monolayers cultured on IncuCyte® ImageLock 96-well plates. IncuCyte® Scratch Wound analysis software enables real time, automated measurement of label-free or dual fluorescence of cell migration and invasion *in vitro*.

Required materials

- Cell Migration/Invasion Kit (Essen BioScience Cat #4474), includes:
  - 96-well Cell Migration Software Application Module (Essen BioScience Cat #4400)
  - 96-pin IncuCyte WoundMaker Tool (Essen BioScience Cat #4563)
  - Two (2) Wound Maker Rinse Boats (Essen BioScience Cat # 5025-0191-A00)
  - Fifteen 96-well ImageLock plate (Essen BioScience Cat #4379)
- IncuCyte 96-well Scratch Wound Cell Invasion Accessories (Essen BioScience Cat #4444), for invasion assay includes:
  - 2 x CoolBox 96F System plus 2 x CoolSink 96F
  - 1 x extra CoolSink 96F
- Matrigel® (BD Cat#354234), for invasion assay

General guidelines

- Following cell seeding, place plates at ambient temperature for 15 minutes to ensure homogenous cell settling.
- Do not leave any empty (dry) wells– these will damage the WoundMaker pins when creating the scratch.
- Remove bubbles from all wells by gently squeezing a wash bottle (containing 70-100% ethanol with the inner straw removed) to blow vapor over the surface of each well.
- After placing the plate in the IncuCyte® live-cell analysis system, allow the plate to warm to 37°C for 30 minutes prior to scanning.

General guidelines

For optimal assay quality, for both IncuCyte Scratch Wound cell migration and cell invasion assays it is recommended that cell density, the timing of the scratch wound (post cell plating) and the density of biomatrix material (if required) be investigated and optimized for different cell types.

Cell Density: The most consistent wounds are generally made when the cell monolayer is at or very near to 100% confluence; typically seeding density will range from 10-50K cells per well.

Timing of Scratch: Plating cells at the end of the day and wounding cells in the morning of the following day works well for many cell types plated on tissue culture plastic. If a biomatrix material is being utilized, cells may adhere in just a few hours, and successful wounding may be possible on the same day of seeding. On occasion, cells will adhere too tightly, causing adhered cells debris after wounding, which blocks subsequent cell migration. Plating cells for shorter time periods, (e.g. 4 – 8h) can help improve the quality of the wounds.

Biomatrix: Coating the well with a biomatrix material (e.g. Collagen-I) or poly-D-Lysine will typically enhance the timing and strength of cell attachment. It can allow cells to adhere more tightly to the substrate as opposed to each other, avoiding cell sloughing or the removal of sheets of cells.

Migration Protocol

1 Coat Plate with ECM (optional)

Coat plate surface to ensure cell attachment (e.g., Collagen-1).

2 Coat wells

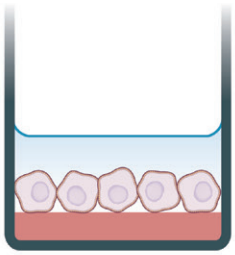
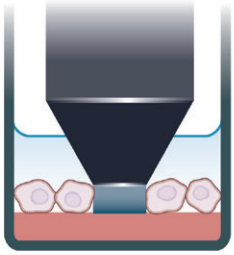


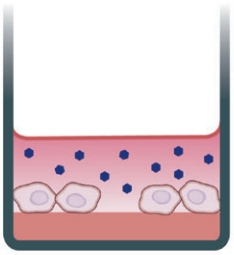
Plate cells (100 µL/well, 10,000-40,000 cells/well) and allow to adhere overnight.

3 Create wound area



Wound confluent cell monolayer using 96-well WoundMaker.

4 Add treatment



Add modulators of migration (100 µL/well).

DAY 0:

1 Coat plate with ECM (if required)

1.1. Coat a 96-well ImageLock plate with a thin layer (50 µL/well) of biomatrix. Gently rock the plate to ensure even coating of each well.

1.2. Depending on biomatrix used for coating, aspirate and wash coating from the wells prior to cell seeding.

2 Seed cells

2.1. Seed cells at a density of 10,000–40,000 cells/well (100 µL/well; 100,000–400,000 cells/mL stock) into each well of the coated 96-well ImageLock plate.

2.2. Allow the cells to settle at ambient temperature for 15 minutes, then place the plate into a 37°C incubator, 5% CO_2 overnight or as pre-determined in assay optimization.

DAY 1:

3 Create wound

3.1. Carefully remove the ImageLock plate from the incubator, and use the WoundMaker (refer to Appendix I, Creating wounds section) to simultaneously create wounds in all wells.

3.2. After wounding, immediately aspirate the media from each well and carefully wash the cells twice with culture media (100 µL/well; with our without serum) or Dulbecco's Phosphate Buffered Saline (dPBS), if desired.

4 Add treatments

4.1. After washing, add 100 µl of culture media ± test material (e.g. small molecules, antibodies) to each well.

4.2. Place the cell plate into the IncuCyte live-cell analysis system and allow the plate to warm to 37°C for 30 minutes prior to scanning.

a. Objective: 4x, 10x (recommended), or 20x

b. Channel selection: Phase Contrast (+ Fluorescence if analyzing cells with fluorescent labels)

c. Scan type: Scratch Wound (Wide Mode optional for 10x, required for 20x)

d. Scan interval: Every 1-3 hours

4.3. Wash and store the WoundMaker according to the wash protocol.

Invasion Protocol

1 Coat Plate with ECM (optional)

Coat plate surface with 100 mg/mL Matrigel (50 mL/well) to ensure cell attachment.

2 Seed cells

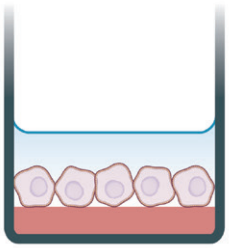
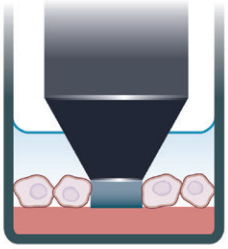


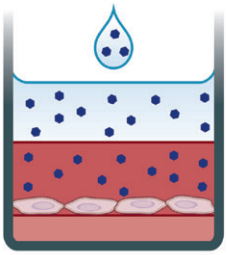
Plate cells (100 µL/well, 10,000-40,000 cells/well) and allow to adhere overnight.

3 Create wound area



Wound confluent cell monolayer using 96-well WoundMaker.

4 Add ECM + media containing treatment



Overlay cells with ECM (50 µL/well) ± treatment, polymerize, then overlay wells with media ± treatment (100 µL/well).

IMPORTANT:

In advance of invasion experiments it is important to have stored the Cool pack accessories at the correct temperatures for at least 4h:

- Coolbox x 2 (block with gelpack: -20°C), Coolsink 96F x2 (4°C),
- Coolsink 96F x 1 (37°C).
- CoolBox M30 System (block with gelpack: -20 °C) with CoolRack (4°C).

The Cool Packs are used to ensure close temperature control of Matrigel® in microplates. At 4-8°C, Matrigel is a viscous liquid. Polymerization will occur slowly at 4-8°C, and more rapidly when at room temperature or higher. For this reason, it is imperative to keep Matrigel solutions at 4-8°C during experimental set-up to avoid unwanted gelling. It is easier to handle low volume (<500µL) ECM solutions using pre-cooled (from a fridge), wide bore pipette tips or serological pipettes. We recommend sourcing a batch of Matrigel with a concentration of >9mg/mL and an endotoxin level of <1.5 (EU)/mL.

DAY 0:

1 Coat plate with ECM

1.1. 1.1. Using a CoolSink M30 System with CoolRack, dilute Matrigel® stock to 100 µg/mL in culture media.

Note: The day prior to coating the ImageLock plate, thaw a bottle of Matrigel®, packed in ice, overnight at + 4°C. When fully thawed, there should be no visible gel aggregates. If aggregates are present, replace the bottle on ice and thaw at + 4°C for a longer period of time. After thawing, chill ten 2 mL micro centrifuge tubes in the CoolSink M30 System (10min), and using a pre-cooled serological pipette, create 1 mL aliquots of Matrigel and store at -20°C.

1.3. Coat a 96-well well ImageLock plate with 50 µL/well of diluted Matrigel (100µg/mL). Gently rock the plate to ensure even coating of each well.

1.2. Place the plate in a 37°C incubator, 5% CO_2 and allow the biomatrix material to polymerize for 2 hours.

2 Seed cells

2.1. Remove plate from 37°C. Using a manual pipette, aspirate the Matrigel coating from the wells prior to cell seeding.

2.2. Seed cells at a density of 10,000–40,000 cells/well (100 µL/well, 100,000–400,000 cells/mL stock) into each well of the coated 96-well ImageLock plate.

2.3. Allow the cells to settle at ambient temperature for 15 minutes, then place the plate into a 37°C incubator, 5% CO_2 overnight.

DAY 1:

3 Prepare biomatrix top layer, then create wound

3.1. On ice, prepare the biomatrix top layer by diluting Matrigel, typically 4-8 mg/mL, in cold culture media ± treatments.

3.2. Carefully remove the ImageLock plate from the incubator, and use the WoundMaker (refer to Appendix I, Creating wounds section) to simultaneously create wounds in all wells.

3.3. After wounding, immediately aspirate the media from each well and carefully wash the cells twice with culture media (100 µL/well).

3.4. After washing, add 100 µl of culture media, then cool the cell plate to 4°C for 5 minutes using the CoolSink and CoolBox 96F.

4 Add ECM and media ± treatments

4.1. Aspirate media from wounded ImageLock plate, and carefully overlay cells with 50µL of the Matrigel top layer (prepared in step 3.1). Remove any bubbles from the assay plate.

4.2. To polymerize the biomatrix top layer, warm the cell plate to 37°C by placing the plate onto a pre-warmed CoolSink inside the incubator.

4.3. After 30 minutes, add additional media (100 µL/well ± treatments).

Note: Leaving the plate to polymerize longer than 60 minutes will cause the biomatrix top layer to dehydrate.

4.4. Place the cell plate into the IncuCyte live-cell analysis system and allow the plate to warm to 37°C for 30 minutes prior to scanning.

a. Objective: 4x, 10x (recommended), or 20x

b. Channel selection: Phase Contrast (+ Fluorescence if analyzing cells with fluorescent labels)

c. Scan type: Scratch Wound (Wide Mode optional for 10x, required for 20x)

d. Scan interval: Every 2-3 hours

4.5. Wash and store the WoundMaker according to the wash protocol.

Creating wounds: Six simple steps

The WoundMaker is a 96-pin mechanical device designed to create homogeneous, 700-800 µm wide wounds in cell monolayers on IncuCyte® ImageLock 96-well microplates. The device is simple to use and wounds can be created in seconds.

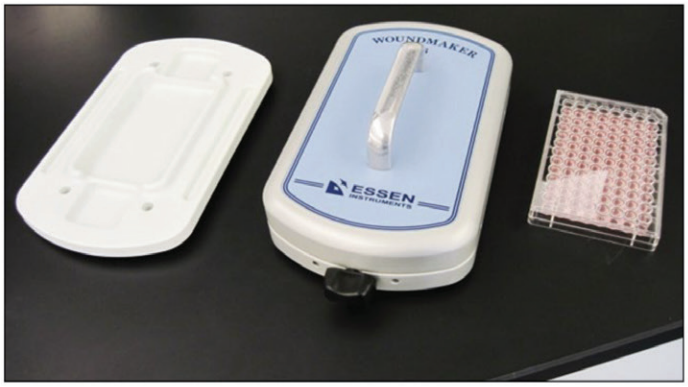
When used, stored and cleaned correctly, the WoundMaker should continue to provide clean, consistent wounds without damaging the cells or the underlying plastic or biomatrix. The WoundMaker is comprised of:

1 x 96 well pin block

1 x metal base

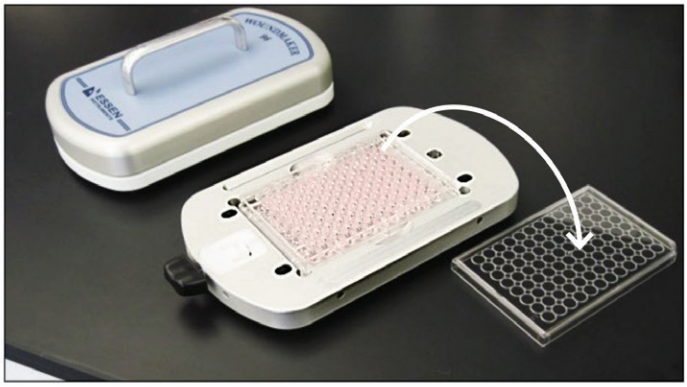
2 x 96 wash boats

The following steps should be performed in a biological safety cabinet:



STEP 1

- Remove top of WoundMaker.
- Set top in empty wash boat.



STEP 2

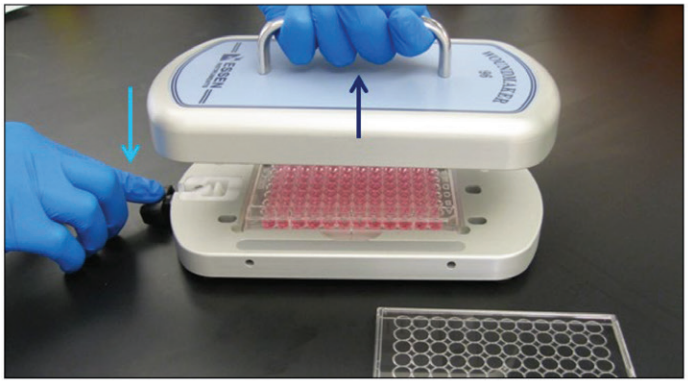
- Insert plate (containing cells & media) into base plate holder.
- Remove plate cover.

STEP 3

- Replace pin block by guiding the rear dowels of pin block into the rear holes of the base plate.
- Do not push down.

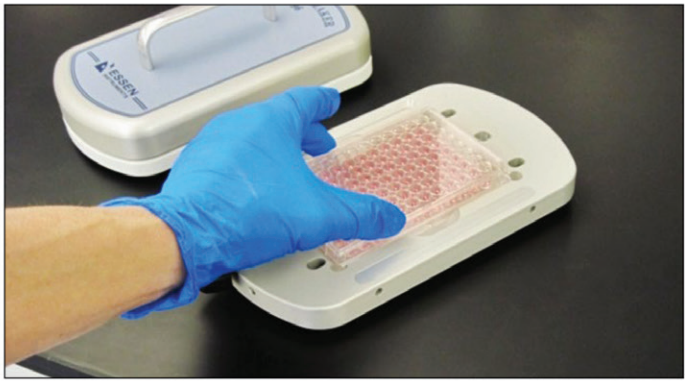
STEP 4

- Push and hold the black lever.



STEP 5

- Lift pin block while continuing to hold the black lever down.



STEP 6

- Replace plate cover.
- Wash wells (up to two washes).
- Add treatment conditions.
- Put into the IncuCyte and start acquiring data and images.

IncuCyte® Chemotaxis Cell Migration Assay

For the detection of chemotactic-induced cell migration

The IncuCyte® Chemotaxis Cell Migration Assay approach allows for automated imaging and analysis of cell migration using an optically clear membrane that allows for 96-well kinetic throughput. Using phase-contrast and/or fluorescent imaging in combination with IncuCyte® integrated metrics, we are able to precisely quantify the chemotactic response of adherent or non-adherent cell types. The assay method does not require labeling for quantification, is sensitive to surface integrin signaling, sustains a linear gradient over several days and allows for direct visualization of cell migration.

Required materials

- IncuCyte® ClearView 96-well Cell Migration Plate (EssenBioscience cat # 4582)
- IncuCyte® ClearView Reservoir Plate (EssenBioScience cat# 4600)
- IncuCyte® Chemotaxis Software Module (EssenBioScience cat# 9600-0015)

General guidelines

It is important to read the protocol in its entirety prior to initiating a chemotaxis assay. The IncuCyte® live-cell analysis system relies on images to process data; thus it is important to avoid bubbles and follow our protocol recommendations to achieve superior assay performance and imaging. We recommend the following techniques to eliminate bubbles from your experiment:

- Reverse-pipette at the coating step and when adding cells to the insert. Reverse pipetting reduces the risk of splashing or bubble formation. In reverse pipetting, the volume aspirated into the tip is larger than the volume delivered to the receiving vessel.
  - Press the plunger to the second stop.
  - Dip the pipette-tip into the solution.
  - Release the plunger until the starting position has been reached.
  - Move the pipette-tip to the receiving vessel.
  - Dispense the liquid by pressing the plunger to the first stop. SOME LIQUID WILL REMAIN IN THE TIP.
  - Repeat steps 2–5 until throughout the plate.
- Triturate with an additional cell volume or reduced volume setting (e.g., 60 µL cell volume added, mix by reverse-pipetting up and down with 30 µL) to dislodge bubbles that may have been trapped at the membrane-insert interface. Perform this immediately after cell addition.
- Remove bubbles at the liquid surface by gently squeezing a wash bottle containing 100% ethanol, with the inner straw removed, to blow vapor over the surface of each well.
- Gently place the insert into the pre-filled reservoir at a slight angle to allow air to move upwards across the membrane.

Optimization considerations

Cells in the IncuCyte® Chemotaxis Cell Migration Assay are required to move toward a chemoattractant gradient across the membrane surface and through a pore. As a result, cells must be maintained in a healthy state on a biologically relevant surface, in order to facilitate cell movement. Cell surface coatings, chemoattractant and assay medium formulations are parameters that should be optimized in order to achieve superior assay performance.

Prior to starting optimization assays, we recommend a thorough review of literature in order to become familiar with standard culturing techniques as well as Boyden chamber assay conditions for the cell type of interest. Although not all techniques will translate into the IncuCyte® Chemotaxis Cell Migration Assay, they will give guidance for an optimization strategy.

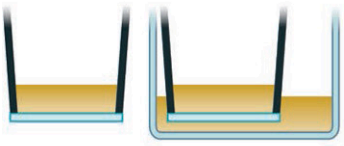
Coating: Migrating cells require interactions with the substrate in order to move. Collagen I, collagen IV, gelatin (Attachment Factor), fibronectin, Matrigel®, and protein G/ICAM surface coatings are commonly used to promote cell migration. Refer to "IncuCyte® ClearView Plate Coating Protocol" for recommended coating procedures.

Chemoattractant: The chemoattractant concentration required for an optimal assay should be determined through experimentation. Based on published literature and/or experience, we recommend testing the expected EC_{50} concentration as well as testing concentrations 1-2 logs above and below the EC_{50}.

Medium: We have found that including a reduced amount of serum (0.1%–2.5%) in the assay buffer allows the cells to attach to the surface and move, while not affecting their directional migration. If cells look unhealthy, an experiment should be designed to increase serum or growth factor levels until cells are healthy enough to attach and move. Adding Insulin-Transferrin-Selenium (ITS) to the assay medium is another way to make cells healthier if minimizing serum is required.

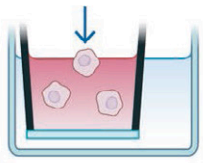
Protocol

1 Coat insert (optional)



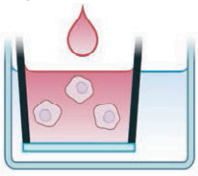
Coat membrane with extracellular matrix (ECM): 20 µL (insert side) and 150 µL (reservoir side).

2 Harvest and seed cells



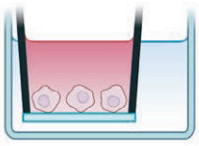
Seed cells (*60 µL/well, 1,000 or 5,000/well) into the IncuCyte® ClearView insert. *If assay requires addition of treatment, seeding volume should be reduced to 40 µL.

3 Treat cells (optional)



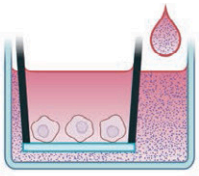
Prepare 3x concentrations of treatment and add 20 µL to appropriate wells. Mix by triturating a 30 µL volume.

4 Allow cells to settle



Place on level surface and allow cells to settle at ambient temperature for 15 to 60 minutes.

5 Add chemoattractant



Add 200 µL of chemoattractant or controls to reservoir plate. Place the insert containing cells into the pre-filled plate, and image in IncuCyte® system.

DAY 0:

1 Coat insert (optional)

Some cell lines may require the addition of an extracellular matrix protein (e.g., 5 µg/mL fibronectin) to promote light cell adherence and provide the necessary integrins for cell motility. Refer to Table 1 for cell-line specific coating recommendations.

1.1. Under sterile conditions prepare coating matrix at desired concentration.

1.2. Using reverse pipetting, aliquot 150 µL of the prepared matrix into the reservoir wells and 20 µL into the insert wells.

1.3. Incubate according to manufacturer's recommendations

1.4. If required, aspirate and wash coating from the reservoir and insert prior to cell seeding

2 Harvest and seed cells

2.1. Harvest cells using suitable dissociation solution.

2.2. Spin down cells and resuspend in appropriate assay medium (i.e. growth medium with reduced FBS, typically 0.5% FBS).

2.3. Determine cell concentration and prepare cell seeding stock to achieve 1,000 or 5,000 cells per well at a 60 µL volume, or, if using modulators of chemotaxis (refer to step 3 below), the seeding volume will be reduced to 40 µL.

NOTE: The seeding density will need to be optimized for each cell type used; however, we have found that 1,000 cells per well for adherent cell types and 5,000 cells per well for non-adherent cell types are reasonable starting points.

2.4. Using reverse pipetting, add cells (if not using inhibitors of chemotaxis, go directly to step 4).

SEEDING VOLUME	FINAL CELL DENSITY/WELL	CELL SEEDING STOCK (CELLS/mL)	RECOMMENED SEEDING STOCK VOLUME (mL)
40	1,000	25,000	6
	5,000	125,000	6
60	1,000	16,666.70	8
	5,000	83,333.30	8

3 Treat cells (optional)

Cell treatment should be prepared prior to cell seeding. It is critical to add modulators immediately following cell seeding in order to ensure appropriate cell exposure and homogenous cell settling.

3.1. Prepare 3x concentrations of treatment and add 20 µL to the insert wells containing cells immediately after cell seeding.

3.2. Using a 30 µL volume, triturate the cells to appropriately mix the treatment, so cell exposure during pre- treatment is at 1x.

4 Harvest and seed cells

4.1. Place the plate onto a level surface and allow the cells to settle at ambient temperature for 15 minutes (adherent cell types) to 45-60 minutes (non-adherent cell types).

NOTE: If treatments were added to an adherent cell type, we recommend a continued pre-incubation with inhibitors at 37°C for 30 minutes.

5 Chemoattractant addition and imaging

5.1. Add 200 µL of desired chemoattractant or control to the appropriate wells of the reservoir plate.

5.2. Carefully transfer the insert into the pre-loaded reservoir plate. Be careful not to introduce bubbles which can become trapped below the membrane when placing the insert into the pre-filled reservoir plate.

5.3. Place the ClearView Cell Migration plate into the IncuCyte® system and allow the plate to warm to 37°C for at least 15 minutes. After 15 minutes, carefully wipe away any condensation that may have accumulated on top of the plate lid or on the bottom of the reservoir plate. Schedule 24 hour repeat scanning:

a. Objective: 10x

b. Channel selection: Phase Contrast (+"Red" if fluorescent labeled cell line is used)

c. Scan type: Chemotaxis

d. Scan interval: Every 1 to 2 hours

Validated cell lines

NOTE: Detailed protocols are available for some of the validated cell lines by clicking on the cell type name.

	THERAPEUTIC AREA	CELL TYPE	ASSAY MEDIUM	COATING (20 µl TOP/ 150 µl BOTTOM	SEED DENSITY (CELLS/WELL)	CELL SETTING TIME (MIN)	CHEMOATTRACTANT (CONCENTRATION RANGE TESTED)	
							nM	ng/mL
Non-adherent	IMMUNOLOGY	Jurkat	RPMI + 0.5% FBS	5 µg/mL Fibronectin + 0.1% BSA in D-PBS / 50 µg/mL Matrigel + 10% FBS in assay medium (for clustered cell migration)	5,000	45-60	SDF-1α (12. 5 – 125)	SDF-1α (100– 1,000)
		T Cells	RPMI + 0.5% FBS	20 µg/mL Protein G followed by 5 µg/mL ICAM	5,000	45-60	SDF-1α (3.1 – 200) CXCL11 (12.5 – 800) RANTES (16)	SDF-1α (24.8 – 1,600) CXCL11 (103.8 – 6640) RANTES (125)
		Neutrophils	RPMI + 0.5% HSA	50 µg/mL Matrigel + 10% FBS in assay medium	5,000	45-60	C5a (2.6 – 1,900) fMLP (1.4 – 1,000) IL-8 (1.5 – 1120) LTB4 (1.4 – 1,000)	C5a (21.6 – 15,770) fMLP (0.6 – 437.6) IL-8 (13.4 – 10,000) LTB4 (0.5 – 336.5)
		CCRF-CEM	RPMI + 50% Human Serum	20 µg/mL Protein G followed by 5 µg/ mL ICAM OR 5 µg/mL Fibronectin	5,000	45-60	CCL22 (1 – 100) SDF-1α (1 – 100)	CCL22 (8.1 – 810) SDF-1α (8 – 800 nM)
		THP-1	RPMI + 0.5% dialyzed FBS	50 µg/mL Matrigel + 10% (dialyzed FBS in assay medium)	5,000	45-60	RANTES (08 – 780) MIP-1α (0.8 – 780) MCP-1 (12 – 8,600)	RANTES (0.1 – 100) MIP-1α (0.1 – 100) MCP-1 (1.4 – 1,000)
		Differentiated THP-1	RPMI + 0.5% FBS	5 µg/mL Fibronectin in D-PBS	2,500	**45-60	C5a (0.5 – 2,000)	C5a (4.2 – 16,600)
		Primary Macrophages	RPMI + 0.5% FBS	50 µg/mL Matrigel + 10% FBS in assay medium	2,000	15-30	C5a (10 – 1000)	C5a (83 – 8,300)
Adherent	VASCULAR	HUVEC	EBM-2 + ITS + 0.25% FBS With #supplements	5 µg/mL Fibronectin + 0.1% BSA in D-PB	1,000	15	VEGF (0.01 – 2.6)bFGF (0.02 – 17.4)FBS (0.4 – 10%)	VEGF (0.4 – 100) bFGF (0.4 – 300) FBS (0.4 – 10%)
		HMVEC	EBM-2 + 0.5% FBS + ITS with #supplements	10 µg/mL Fibronectin + 0.1% BSA in D-PB	1,000	15	bFGF (0.06 – 5.8) FBS (10%)	bFGF (0.01-0.1) FBS (10%)
	ONCOLOGY	HT 1080	F12 + ITS*	None	1,000	15	FBS (0.01 – 10)%	
		MDA-MB-231	DMEM + 2.5 FBS	None	1,000	15	EGF (0.2 – 1.6) FBS (10%) – slight response	EGF (1 – 10n) FBS (10%) – slight response
		MCF10a	DMEM/F12 + 10 µg/mL insulin hydrocortozone + 100ng/mL cholera toxin	5 µg/mL Collagen IV in 0.05M HCl	1,000	15	EGF (0.2 – 17,200)	EGF (0.1 – 1,000)

* Insulin-Transferrin-Selenium (Life Technologies Cat# 41400-045)
# Supplements: EGM-2 singlequot bullet kit (LONZA CC-4176) with growth factors removed and reduced FBS
** Cell settling time pre-differentiation

IncuCyte® Chemotaxis Cell Invasion Assay

For the detection of chemotactic-induced cell invasion

The IncuCyte® Chemotaxis Cell Invasion Assay approach allows for automated imaging and analysis of cell invasion using an optically clear membrane that allows for 96-well kinetic throughput. Measurements of chemotactic cell invasion can be made using nuclear labeled or unlabeled cells, however, we recommend labeling cells with a live-cell nuclear label (e.g. IncuCyte® NucLight reagents) to ensure optimal performance. Using IncuCyte® integrated metrics, we are able to precisely quantify the chemotactic response of adherent types, with direct visualization of cell invasion.

Required materials

- IncuCyte® ClearView 96-well Chemotaxis Plate (Essen Bioscience cat # 4582)
- IncuCyte® ClearView Reservoir Plate (Essen BioScience cat# 4600)
- IncuCyte® Cell Invasion Kit (Essen BioScience cat# 4444)
- Cultrex® 3-D Culture Matrix™ Reduced Growth Factor Basement Membrane Extract (Trevigen 3445-005-01) - optional
- Cultrex® Rat Collagen I (Trevigen 3440-100-01) - optional
- IncuCyte® Chemotaxis Software Module (Essen BioScience cat# 9600-0015)

General guidelines

Avoiding bubbles

The IncuCyte system relies on images to process data; thus, it is important to avoid bubbles and follow our protocol recommendations to achieve superior assay performance and imaging. We recommend the following techniques to eliminate bubbles from your experiment:

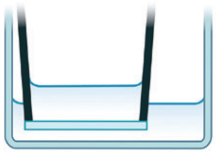
- Reverse-pipette at the coating step and when adding cells to the insert. Reverse pipetting reduces the risk of splashing or bubble formation. In reverse pipetting, the volume aspirated into the tip is larger than the volume delivered to the receiving vessel.
  - Press the plunger to the second stop.
  - Dip the pipette-tip into the solution.
  - Release the plunger until the starting has been reached.
  - Move the pipette-tip to the receiving vessel.
  - Dispense the liquid by pressing the plunger to the first stop. SOME LIQUID WILL REMAIN IN THE TIP.
  - Repeat steps 2–5 until throughout the plate.
- Triturate with an additional cell volume or reduced volume setting (e.g., 60 µL cell volume added, mix by reverse-pipetting up and down with 30 µL) to dislodge bubbles that may have been trapped at the membrane-insert interface. Perform this immediately after cell addition.
- Remove bubbles at the liquid surface by gently squeezing a wash bottle containing 100% ethanol, with the inner straw removed, to blow vapor over the surface of each well.

Reagent temperature control

- It is important to keep close temperature control of biomatrix materials such as Collagen-1 and basement membrane extract (BME) to prevent unwanted gelling.
- The IncuCyte® Cell Invasion Kit (Cat. No. 4444) includes a specialized CoolBox™ system to ensure the temperature of your assay plate and biomatrix materials are maintained between 4-8°C – preventing premature polymerization and eliminating edge effects. Crushed ice can be used as an alternative however non-uniform cooling can lead to assay variability.
- When handling cells that have been embedded in biomatrix material, ensure that all steps of cell handling is performed between 4-8°C, utilizing consumables that have been pre-chilled (e.g., a pre-cooled reservoir boat during cell seeding).

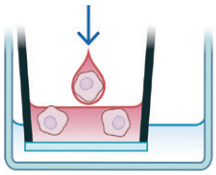
Protocol

1 Prime insert



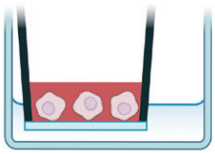
Prime membrane by adding 150 L of D-PBS to the reservoir of a pre-chilled ClearView Cell Migration plate. Incubate plate for 20 minutes at 4°C.

2 Harvest and seed cells



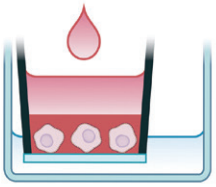
Prepare cells at 50,000 cells/mL in extracellular matrix. Dispense 20 µL/well of cell:matrix suspension (1,000 cells/well) into insert. Centrifuge for 3 minutes at 50x g.

3 Polymerize matrix



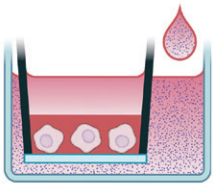
Place ClearView plate on a pre-warmed CoolSink at 37°C. Allow matrix with embedded cells to polymerize for 30-60 minutes.

4 Media addition



Add 40 µL assay medium on top of the polymerized matrix:cell layer.

5 Add chemoattractant



Add 200 µL of desired chemoattractant or controls to appropriate wells of the reservoir plate. Place the insert into the pre-filled and image in IncuCyte® system.

DAY 0:

1 Prime insert

1.1. Pre-cool a ClearVew Chemotaxis plate in a CoolBox system containing a frozen cold pack and CoolSink plate (4°C) for 5 minutes

1.2. Add 150 µL of D-PBS (4°C) to each reservoir well of the pre-chilled ClearView chemotaxis plate. Replace the ClearView insert and allow the membrane to

2 Harvest and seed cells

2.1. While the ClearView plate is priming, prepare your biomatrix reagent at the desired working concentration as per the manufacturer's instructions. Prepare a large dead volume to ensure available biomatrix for transfer to the assay plate (e.g., prepare 4 mL biomatrix solution to provide 20 µL per insert well).

NOTE: The required biomatrix density will be dependent on the matrix and cell types used. For HT-1080 cells we recommend BME (5 mg/mL) diluted in assay medium or Collagen I (1 mg/mL) diluted in neutralizing buffer (DMEM, Sigma D2429, + 7.5 g/L sodium bicarbonate + 0.004 g/L folic acid + 1% GlutaMax). When preparing collagen I it is important that it is properly neutralized to ensure cell health is maintained and gelling is uniform.

2.2. Harvest your cells and resuspend the pellet in the biomatrix solution. Cell density will need to be optimized for each cell type used; however, we have found that 50,000 cells/mL (1,000 cells per well) is a reasonable starting point. **Calculation:** *50,000 cells/mL x 0.02 mL/well = 1,000 cells/well*

NOTE: Some cell types may require reduced exposure to Fetal Bovine Serum (FBS) before initiating the transmembrane invasion assay (e.g., HT-1080s starved in F12 + Insulin-Transferrin-Selenium for ~20 hours).

2.3. Seed cells (20 µL per well, 1000 cells per well) into every well of the cooled ClearView insert plate.

2.4. Centrifuge the ClearView plate in a cooled centriguge for three minutes at 50 x g.

3 Polymerize matrix

3.1. Place the ClearVIew plate at 37°C on a pre-warmed CoolSink and allow the biomatrix to polymerize for 30 -60 minutes.

4 Media addition

4.1. Gently add 40 µL of assay medium ± modulators of invasion on top of the biomatrix:cell layer in the insert wells.

NOTE: If adding modulators of invasion, the working concentration should be 1.5x the desired final concentration to account for the volume of the biomatrix: cell layer

5 Chemoattractant addition and imaging

5.1. Add 200 µL of desired chemoattractant or control to the appropriate wells of a second reservoir plate.

5.2. Carefully transfer the insert into the pre-loaded reservoir plate. Be careful not to introduce bubbles which can become trapped below the membrane when placing the insert into the pre-filled reservoir plate.

5.3. Place the ClearView Cell Migration plate into the IncuCyte® system and allow the plate to warm to 37°C for at least 15 minutes. After 15 minutes, carefully wipe away any condensation that may have accumulated on top of the plate lid or on the bottom of the reservoir plate. Schedule 24 hour repeat scanning:

a. Objective: 10x

b. Channel selection: Phase Contrast +"Red" or "Green" channels selected (dependent on the fluorescent label used)

c. Scan type: Chemotaxis

d. Scan interval: Every 1 to 2 hours

Chemotatic Transendothelial Migration Assay

For quantification of leukocyte migration across an endothelial monolayers

This protocol is intended for the measurement of transendothelial migration by leukocytes. This method utilizes the IncuCyte® ClearView 96-well Chemotaxis plate and the IncuCyte live-cell analysis system for image-based measurements of diapedesis.

Required materials

- Leukocytes (e.g. activated primary T-cells or neutrophils)
- Endothelial cells (e.g. HUVEC or EA.hy9226)
- Endothelial growth medium
- Assay medium
- Collagen Type I Rat Tail (BD Biosciences 354236) or
- Fibronectin (Sigma Aldrich F1141)
- Acetic Acid
- D-PBS -/- (w/o Ca2+, Mg2+, Life Technologies 10010) – used in Fibronectin coating step
- D-PBS +/+ (with Ca2+, Mg2+, Life Technologies 14040) – used in monolayer wash step
- IncuCyte® ClearView 96-Well Chemotaxis Plate (Essen 4582 or 4599)

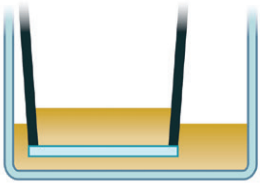
General guidelines

The IncuCyte system relies on images to process data; thus, it is important to avoid bubbles and follow our protocol recommendations to achieve superior assay performance and imaging. We recommend the following techniques to eliminate bubbles from your experiment:

- Reverse-pipette at the coating step and when adding cells to the insert. Reverse pipetting reduces the risk of splashing or bubble formation. In reverse pipetting, the volume aspirated into the tip is larger than the volume delivered to the receiving vessel.
  - Press the plunger to the second stop.
  - Dip the pipette-tip into the solution.
  - Release the plunger until the starting has been reached.
  - Move the pipette-tip to the receiving vessel.
  - Dispense the liquid by pressing the plunger to the first stop. SOME LIQUID WILL REMAIN IN THE TIP.
  - Repeat steps 2–5 until throughout the plate.
- Triturate with an additional cell volume or reduced volume setting (e.g., 60 µL cell volume added, mix by reverse-pipetting up and down with 30 µL) to dislodge bubbles that may have been trapped at the membrane-insert interface. Perform this immediately after cell addition.
- Remove bubbles at the liquid surface by gently squeezing a wash bottle containing 100% ethanol, with the inner straw removed, to blow vapor over the surface of each well.

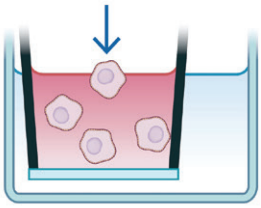
Protocol

1 Coat insert



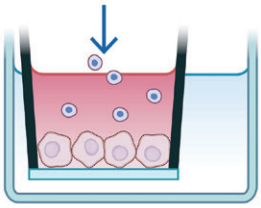
Coat membrane with extracellular matrix (ECM): 20 µL (insert side) and 150 µL (reservoir side).

2 Seed endothelial cells



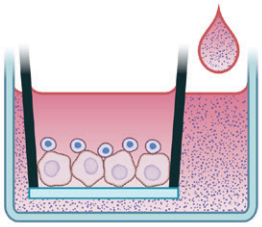
Seen endothelial cells (60 µL/well, 6,000 cells/mL) into the IncuCyte ClearView insert. Allow monolayer to form overnight.

3 Seed leukocytes



Seed leukocytes (60 µL/well, 5,000 cells/mL) onto endothelial monolayer.

4 Add chemoattractant



Add 200 µL of chemoattractant or controls to reservoir plate. Place the insert into the pre-filled reservoir plate and image in IncuCyte® system.

DAY 0:

1 Coat insert

1.1. Prepare extracellular matrix coating of either 50 µg/mL collagen diluted with 0.02N acetic acid or 5 µg/mL fibronectin diluted with D-PBS (-/-).

1.2. Aliquot 150 µL of coating solution into the reservoir. Gently place the insert into the reservoir and pipette 20 µL of the fibronectin, or collagen, solution into the insert.

1.3. Incubate for 1 hour at ambient temperature

2 Create endothelial cell monolayer

2.1. During incubation, harvest and count endothelial cells and prepare a cell seeding stock of 100,000 cells/mL in full growth medium.

2.2. Aspirate the coating from the reservoir plate and replace with 200 µL of D-PBS (-/-) and gently return the insert into to the reservoir plate.

2.3. To the insert, directly add 60 µL D-PBS (-/-) to the inserts containing coating, then aspirate the entire volume.

2.4. Immediately seed 60 µL of the endothelial seeding stock in growth medium using a multi-channel pipette into every well of the insert plate (60 µL per well, 6,000 cells per well).
Calculation: *100,000 cells/mL x 0.06 mL = 6,000 cells per insert well.*

2.5. Allow the cells to settle at ambient temperature on a level surface for 15 minutes.

2.6. Place the Chemotaxis plate containing cells at 37°C and incubate for 24 hours

DAY 1:

3 Seed leukocytes

3.1. After the endothelial monolayer has formed, gently wash the monolayer 2x with D-PBS (+/+), using partial washes

NOTE: It is important not to disrupt the monolayer. It is recommended to gently remove about half of the growth medium then add 60 µL D-PBS for both washes. At the final wash step, remove as much of the medium/D-PBS as possible without disrupting the monolayer.

3.2. Prepare leukocyte cell seeding stock at 83,333 cells/mL in appropriate assays medium.

3.3. Using a manual multi-channel pipette and reverse pipetting technique, seed 60 µL of the leukocytes seeding stock (5,000 cells per well) into every well of the insert plate, being careful not to disrupt the endothelial monolayer.
Calculation: *83,333 cells/mL x 0.06 mL = 5,000 cells per insert well.*

3.4. Centrifuge the chemotaxis plate for 3 minutes at 50 x g in order to quickly bring the leukocytes to the monolayer surface. Alternatively, if centrifugation is not possible, allow the leukocytes to settle on the endothelial monolayer at ambient temperature for 45-60 minutes.

4 Add chemoattractant

4.1. Using a manual multi-channel pipette, add 200 µL of the chemoattractant and control medium to the appropriate wells of the second reservoir plate.

4.1. Carefully transfer the insert plate containing the cells into the pre-filled second reservoir plate containing assay medium ± chemoattractant.

4.1. Place the IncuCyte® ClearView cell migration plate into the IncuCyte® live-cell analysis system and allow the plate to warm to 37°C for at least 15 minutes. After 15 minutes, wipe away any condensation that remains on the outside of the plate lid or bottom of the reservoir.

4.1. In the IncuCyte® software, schedule 24 hour repeat scanning (10x) for every 30 minutes.

a. Scan Type: Chemotaxis

b. Objective: 10x

c. Channel selection: Phase Contrast +"Red" or "Green" channel if leukocytes are fluorescently labeled

Leukocyte and endothelial cells that have been validated with the TEM and recommended growth and assay media

LEUKOCYTE	ENDOTHELIAL CELLS	ENDOTHELIAL GROWTH MEDIUM	ASSAY MEDIUM	COATING (20 µL TOP/150 µL BOTTOM)	SEEDING DENSITY ENDOTHELIAL: LEUKOCYTE (CELLS/WELL)
Jurkat with	HUVEC	EGM-2	EBM-2 + 2% FBS with #supplements	5 µg/mL fibronectin or 50 µg/mL collagen	6,000 : 5,000
Primary T-cells	HUVEC	EGM-2	EBM-2 + 2% FBS with #supplements	5 µg/mL fibronectin or 50 µg/mL collagen	
	EA.hy926	DMEM + 10% dialyzed FBS	RPMI + 0.5% dialyzed FBS	50 µg/mL collagen	
Neurophils with	HUVEC	EGM-2	EBM-2 + 2% FBS with #supplements	5 µg/mL fibronectin or 50 µg/mL collagen	

#supplement includes gentamycin, hydrocortisone, and ascorbic acid

IncuCyte® Antibody Internalization Assay

This protocol describes a solution for the measurement of antibody internalization by cells expressing antigen of interest. This method utilizes the IncuCyte® FabFluor-pH Red Antibody Labeling reagent and the IncuCyte® live-cell analysis system for image-based fluorescent measurements of antibody internalization.

Required materials

- IncuCyte® FabFluor-pH Red Antibody labeling reagent (Essen BioScience cat #4722 human, cat #4723 mouse IgG1, cat #4737 rat , cat #4750 mouse IgG2a, or cat #4751 mouse IgG2b).
- Test antibody of interest containing human, mouse, or rat Fc region (at known concentration).
- Target cells of interest.
- Target cell growth media.
- PBS (w/o Ca^{2+}/Mg^{2+}, Life Tech 14190).
- 96-well flat bottom microplate (e.g. Corning® 3595) for imaging.
- 96-well round black round bottom ULA plate (e.g. Corning® 45913799) or amber microtube (e.g. Cole parmer® MCT-150-X) for conjugation step.

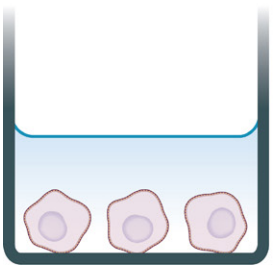
Recommended materials

It is strongly recommended that a positive and negative control is run alongside test antibodies and cell lines. CD71, which is an anti-human mouse antibody, is recommended as a positive control for the mouse Fab.

- Anti-CD71, clone MEM-189, IgG1 e.g. Sigma SAB4700520-100UG
- Anti-CD71, clone CYG4, IgG2a e.g. Biologend 334102
- Isotype controls, depending on isotype being studied
  - Mouse IgG1, e.g. R&D Systems, MAB002 or Biolegend 400124
  - Mouse IgG2a e.g. Biolegend 401501
  - Mouse IgG2b e.g. Biolegend 400322

Quick Guide

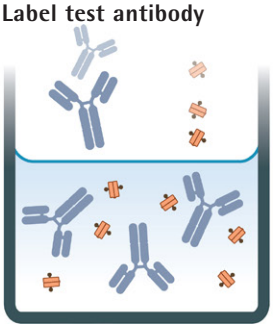
1 Seed cells



Cell Seeding

Seed cells (50 µL/well, 5,000-30,000 cells/well), into 96-well plate and leave to adhere (2-24 h, depending on cell type).

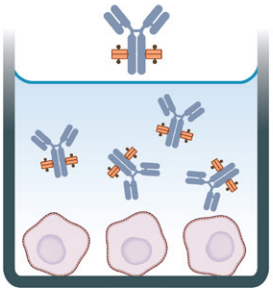
2 Label test antibody



Labeling of Test Antibody with IncuCyte® FabFluor-pH Red Reagent

Mix antibody and FabFluor Reagent at a molar ratio of 1:3 in media, 2x final assay concentration. Incubate for 15 minutes to allow conjugation.

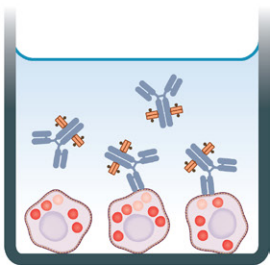
3 Add to cells



IncuCyte® FabFluor-labeled Antibody Addition

Add antibody-FabFluor mix (50 µL/well) to cell plate.

4 Live-cell Fluorescent imaging



Automated Imaging andQuantitative Analysis

Capture images every 15-30 minutes (10x or 20x) in IncuCyte® for 24-48 hours. Analyze using integrated software.

Preparation of IncuCyte® Antibody Internalization Assay

1 Seed Target target cells of interest

1.1 Harvest cells of interest and determine cell concentration (e.g. trypan blue + hemocytometer).

1.2 Prepare cell seeding stock in target cell growth media to achieve 40-50% confluence after 2-6 h. Suggested starting range 5,000-30,000 cells/well (depends on cell type used).

NOTE: The seeding density will need to be optimized for each cell type. For non-adherent cell types a well coating may be required e.g. Poly-L-ornithine (PLO, Sigma P4957) to maintain even cell coverage in well (see IncuCyte Cell Proliferation Assay protocol on www.Essenbioscience.com for details).

1.3 Using a multi-channel pipette, seed cells (50 µL per well) into a 96-well flat bottom microplate. Lightly tap plate side to ensure even liquid distribution in well.

1.4 Remove bubbles from all wells by gently squeezing a wash bottle (containing 70-100% ethanol with the inner straw removed) to blow vapor over the surface of each well.

1.5 Allow cells to settle on a level surface for 30 minutes at room temperature, then place in IncuCyte® live-cell analysis system to monitor cell confluence in well.

NOTE: Depending on cell type, plates can be used in assay once cells have adhered to plastic and achieved normal cell morphology e.g. 2-3 hr for HT1080 or 1-2 h for non-adherent cell types. Some cell types may require overnight incubation.

2 Labeling of test antibody

2.1 Rehydrate IncuCyte® FabFluor-pH Red Antibody Labeling reagent with 100 µL sterile water to result in a final concentration of 0.5 mg/mL.

NOTE: A 1:3 molar ratio of test antibody to IncuCyte® FabFluor-pH Red reagent is recommended. The labeling reagent is a third of the size of a standard antibody, so equal mg/ml quantities will produce a 1:3 molar ratio of test antibody to labeling Fab.

This reagent is light sensitive. It is advised to keep in amber tubes or foil wrapped tubes. Remaining re-hydrated reagent can be aliquoted and stored at -80°C (avoid freezing and thawing, stable for > year).

2.2 Mix test antibody with dilute IncuCyte®FabFluor-pH Red Antibody Labeling reagent and target cell growth media in a black round bottom microplate or amber tube to protect from light (50 ul/well).

a. Add test antibody at 2X the final antibody concentration. We suggest optimizing the assay by testing a final concentration of 4 ug/ml of test antibody (e.g., 2X working concentration=8 ug/ml).

b. Add IncuCyte®FabFluor-pH Red Antibody Labeling reagent at 2X the final concentration. We suggest optimizing the assay by testing a final concentration of 4 ug/ml of IncuCyte FabFluor-pH Red Antibody (e.g., 2X working concentration=8 ug/ml).

c. Add media to bring the total volume to 50 ul/well. Triturate to mix.

NOTES: If performing a range of concentrations of test antibody e.g. concentration response-curve, it is recommended to create dilution series post conjugation step in media to ensure consistent molar ratio labeling.

We strongly recommend the use of both a negative and positive control antibody (see Recommended Materials above).

3 Add IncuCyte FabFluor-pH Red reagent to cells

3.1 Remove cell plate from incubator.

3.2 Using a multi-channel pipette, add 50 µL of labeled antibody to required test wells, remove any bubbles and immediately place plate in IncuCyte® live-cell analysis system.

4 Acquire images and analyze

4.1 In the IncuCyte® software, schedule 24 hour repeat scanning for every 15-30 minute (depending on speed of internalization signal).

a. Scan on schedule, standard.

b. Channel selection: select “phase” and “red”

c. Objective: 10x or 20x depending on cell types used, generally 10x is recommended for adherent cells, and 20x for non-adherent or smaller cells.

NOTE: If trying to achieve rapid first image acquisition, scheduling can be set up on the instrument and no start time of scan attached prior to addition of reagents to plate. The scan time can then set once the plate is placed in the instrument.

By maintaining all reagents at 37°C prior to plate addition there is reduced risk of condensation formation on the lid and therefore no need for plate warming before first image acquisition.

4.2 To generate the metrics, user must create an Analysis Definition suited to the cell type, assay conditions and magnification selected.

4.3 Select images from a well containing a positive internalization signal and an isotype control well (negative signal) at a time point where internalization is visible.

4.4 In the Analysis Definition:

a. Set up the mask for the phase confluence measure with red channel turned off.

b. Red channel turned on: Exclude background fluorescence from the mask by using the background subtraction feature. The feature "Top-Hat" will subtract local background from brightly fluorescent objects within a given radius; this is a useful tool for analyzing objects which change in fluorescence intensity over time.

i. The radius chosen should reflect the size of the fluorescent object but contain enough background to reliably estimate background fluorescence in the image; 20-30 µm is often a useful starting point.

ii. The threshold chosen will ensure that objects below a fluorescence threshold will not be masked.

iii. Choose a threshold in which red objects are masked in the positive response image but low numbers in the isotype control, negative response well. For a very sensitive measurement, for example, if interested in early responses, we suggest a threshold of 0.2.

NOTE: The adaptive feature can be used for analysis but may not be as sensitive and may miss early responses. If interested in rate of response, Top Hat maybe preferable.

Example calculation for antibody labeling using positive control anti-CD71 at 1 mg/mL stock concentration

1. Determine final assay concentration of test antibody — 4 µg/mL for anti-CD71 is recommended for positive control wells. Working concentration will be 2X or 8 µg/mL.

2. Determine volume of labeled antibody required at 2X final assay concentration (dilution of 1:2 recommended upon addition to cells): [# wells] x 50µL (plus additional required to prepare dilution series if desired).

   For 8 replicates of highest concentration plus 8 replicates of 1:2 dilution of labeled test antibody:
   8 x 50 µL x 1.5 = 600 µL minimum (650µL used for this example)

3. Calculate volumes of test antibody, IncuCyte FabFluor-pH Red reagent, and media required to provide 2X final assay concentration of labeled test antibody.

   - Determine volume of test antibody:
     [total volume] µL x [working concentration test antibody] µg/mL / [stock concentration test antibody] mg/mL /1000
     650 µL x 8 µg/mL / 1 mg/mL / 1000 = 5.2 µL

   - volume of IncuCyte FabFluor:
     [volume of test antibody] µL X [stock concentration of test antibody] mg/mL / [stock concentration of test FabFluor] mg/mL
     5.2 µL x 1 mg/mL / 0.5 mg/mL = 10.4 µL

     NOTE: IncuCyte FabFluor-pH Red reagent is a third of the molecular weight of a standard antibody, so equal volumes of equal mg/mL quantities will produce a 1:3 molar ratio of test antibody to FabFluor as MW of a typical antibody is ~3x of FabFluor. In this case, the stock concentration in mg/mL of the test antibody is twice that of FabFluor, thus the FabFluor volume should be 2X the volume of the test antibody.

   - Determine volume of media:
     [total volume] – [test antibody volume] – [FabFluor volume]
     = 634.4 µL

Analysis Guidelines

As the labeled antibody is internalized into the acidic environment of the lysosome the area of fluorescence and intensity inside the cells increases. This can be reported in two ways:

i. An increase in fluorescence area ("total object area" or "red object confluence"). Suggested metric: Analyze using the "Total Red Object Area (µm²/well)".

ii. An increase in intensity, integrated over the area of detectable fluorescence ("Total Integrated Intensity"). Suggested metric: Analyze using "Total Red Object Integrated Intensity (RCU x µm²/well)" metrics.

IncuCyte® Live-Cell Immunocytochemistry Assay

For cell marker analysis

This protocol describes a solution for measuring immunocytochemistry in live cells expressing a surface antigen of interest. The method utilizes the IncuCyte® FabFluor-488 Antibody Labeling Reagent in combination with IncuCyte Opti-Green and the IncuCyte® S3 Live-Cell Analysis System for image-based fluorescent measurement enabling live-cell immunocytochemistry.

Required materials

- IncuCyte® FabFluor-488 Antibody Labeling Reagent plus IncuCyte® Opti-Green suppressor (Sartorius Cat #4745 mouse IgG1, Sartorius Cat# 4743 mouse IgG2a or Sartorius Cat#4744 mouse IgG2b).
- Test antibody of interest (at known concentration) containing mouse Fc region.
  - Recommend using Azide-free antibodies when available.
- Target cells of interest.
- Target cell growth media
- Effector cell culture media
- PBS (w/o Ca2+/Mg2+, Life Tech 14190).
- 96-well flat bottom microplate (e.g. Corning® 3595) for imaging
- 96-well round round bottom plate (e.g. Corning® 3799) or amber microtube (e.g. Cole parmer® MCT-150-X) for conjugation step.

Additional material for non-adherent cell types

- Poly-L-ornithine, PLO (Sigma P4957)

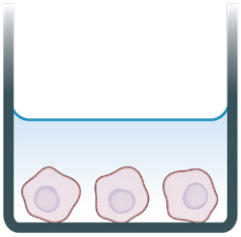
Recommended materials

It is strongly recommended to run both a positive and negative control alongside test antibodies and cell lines. CD71 (anti transferrin receptor) marker is recommended as a positive control for the mouse Fab IgG1 or 2a. Isotype matched IgG are recommended as negative controls.

- Anti-CD71, clone MEM-189, IgG1 e.g. Sigma SAB4700520-100UG
- Anti-CD71, clone CYG4, IgG2a e.g. Biologend 334102
- Isotype controls, depending on isotype being studied
  - Mouse IgG1, e.g. R&D Systems, MAB002 or Biolegend 400124
  - Mouse IgG2a e.g. Biolegend 401501
  - Mouse IgG2b e.g. Biolegend 400322

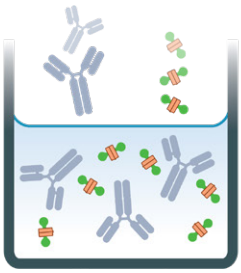
Quick Guide

1. Seed cells



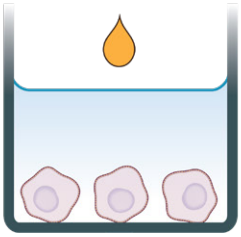
Cell seeding
Seed cells (50 µl/well, 5-30K/well) into 96-well plate.
NOTE: for non-adherent cell types, PLO coat plate prior to cell seeding.

2. Label test antibody



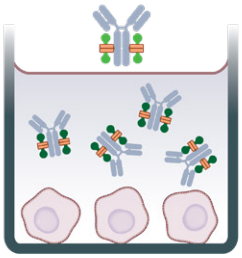
Labeling of test antibody with IncuCyte® FabFluor-488 reagent
Mix antibody and FabFluor-488 reagent at a molar ratio of 1:3 in media, 3x final concentration. Incubate for 15 minutes to allow conjugation.

3. Add Incucyte® opti-green



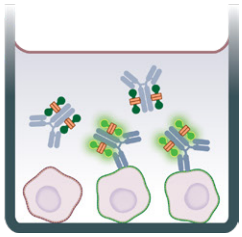
IncuCyte® Opti-Green background suppressor addition
Add 50 µl/well, (3x final concentration).

4. Add labeled AB



IncuCyte® FabFluor-488-labeled antibody addition
Add antibody-FabFluor mix (50 µl/well) to cell plate. Non-adherent cells – spin plate

5. Live-cell fluorescent imaging



Automated imaging and quantitative analysis
Capture images, (time span and objective depends on assay and cells type, 10x or 20x) in IncuCyte® S3 Live-Cell Analysis System.

IncuCyte® Live-cell Immunocytochemistry Assay Methodology

1a. Seed target cells of interest - Adherent cell

1.1. Harvest cells of interest and determine cell concentration (e.g. trypan blue + hemocytometer).

1.2. Prepare cell seeding stock in target cell growth media to achieve 40-50% confluence after 2-6 h. Suggested starting range 5,000-20,000 cells/well (depends on cell type used).

NOTE: Seeding density must be optimized for each cell type.

1.3. Using a multi-channel pipette, seed cells (50 µL per well) into a 96-well flat bottom microplate. Lightly tap plate side to ensure even liquid distribution in well.

1.4. Remove bubbles from all wells by gently squeezing a wash bottle (containing 70-100% ethanol with the inner straw removed) to blow vapor over the surface of each well.

1.5. Allow cells to settle on a level surface for 30 minutes at room temperature, then place in IncuCyte® S3 Live-Cell Analysis System to monitor cell confluence.

NOTE: Depending on cell type, plates can be used in assay once cells have adhered to plastic and achieved normal cell morphology e.g. 2-3 hr for HT1080. Some cell types may require overnight incubation.

1b. Non-adherent cells

NOTE: For this assay, non-adherent cells will be the last addition to the plate (prepare suspension during the antibody conjugation step).

1.1. Coat a 96-well flat bottom plate with relevant coating matrix. We recommend coating with 50 µL of either 0.01% poly-L-ornithine solution (Sigma P4957) or 5 µg/mL fibronectin (Sigma P4957) diluted in 0.1% BSA. Coat for 1 hour at ambient temperature, remove solution from wells, and then allow plates to dry for 30-60 minutes prior to cell addition.

NOTE: Some optimization of plate coatings may be required.

1.2. Count cells of interest and determine cell concentration (e.g. trypan blue + hemocytometer).

1.3. Prepare cell seeding stock in target cell growth media, suggest starting range of 20,000 – 40,000 cells/well in 50 µL (depends on cell type used).

1.4. Using a multi-channel pipette, seed cells (50 µL per well) into a 96-well flat bottom microplate.

1.5. Remove bubbles from all wells by gently squeezing a wash bottle (containing 70-100% ethanol with the inner straw removed) to blow vapor over the surface of each well.

1.6. Allow cells to settle on a level surface for 30 minutes at room temperature then place in IncuCyte® S3 Live-Cell Analysis System to monitor cell confluence.

NOTE: To reduce settling time cell plates can be centrifuged for 1 minute at 50 g.

2. Labeling of test antibody

NOTE: It is recommend to use low azide or azide-free antibodies (e.g. LEAF™ from Biolegend). Effects on cell growth from high concentrations of azide have been observed in some cell types. If this is of concern, the buffer can be exchanged using a simple desalting column (e.g. Zeba from Thermo Scientific).

2.1. Rehydrate IncuCyte® FabFluor-488 Antibody Labeling reagent with 100 µL sterile water (final concentration = 0.5 mg/mL).

NOTE: A 1:3 molar ratio of test antibody to IncuCyte® FabFluor-488 reagent is recommended. The labeling reagent is a third of the size of a standard antibody. Therefore, equal mg/mL quantities produce a 1:3 molar ratio of test antibody to labeling Fab.

NOTE: Reagent is light sensitive, keep in amber or foil wrapped tubes. Remaining re-hydrated reagent can be aliquoted and stored at -80°C (avoid freezing and thawing, stable for > year).

2.2. Mix test antibody with dilute IncuCyte® FabFluor-488 Antibody Labeling reagent and target cell growth media in a round bottom microplate or amber tube to protect from light. Prepare sufficient quantity to enable 50 µL/well at 3X final assay concentration.

NOTE: WE strongly recommend using both a negative and positive control antibody (see **Recommended Materials** above)

a. Add test antibody at 3X the final antibody concentration. Recommendation: A final concentration of <1.5 µg/mL of test antibody. A reasonable starting concentration is 1 µg/mL (e.g. 3X working concentration = 3 µg/mL).

b. Add IncuCyte® FabFluor-488 Antibody Labeling reagent at a 1:3 (test antibody:FabFluor) molar ratio. See **Example calculations** below.

c. Add media to dilute to 3X final assay concentration. Triturate to mix.

d. Incubate for 15 minutes at 37°C in the dark to allow for conjugation.

Example calculations of antibody labeling using positive control anti-CD71 at 1 mg/mL stock concentration

1. Required final assay concentration of test antibody — 1 µg/mL for anti-CD71 is recommended for positive control wells. Working concentration = 3X, or 3 µg/mL.
2. Determine volume of labeled antibody required at 3X final assay concentration (i.e. dilution of 1:3 recommended upon addition to cells): [# wells] x 50 µL (plus additional required to prepare dilution series if desired).

E.g. For 8 replicates of 1:3 dilution of labeled test antibody:

8 x 50 µL= 400 µL minimum (500µL used for this example)

3. Calculate volumes of test antibody, IncuCyte FabFluor reagent, and media required to provide 3X final assay concentration of labeled test antibody.

a. Determine volume of test antibody:

[Total volume] µL x [Working concentration test antibody] µg/mL / [Stock concentration test antibody] mg/mL /1000

500 µL x 3 µg/mL / 1 mg/mL / 1000 = 1.5 µL

b. Determine volume of IncuCyte FabFluor:

[Volume of test antibody] µL X [Stock concentration of test antibody] mg/mL / [Stock concentration of FabFluor] mg/mL

1.5 µL x 1 mg/mL / 0.5 mg/mL = 3.0 µL

NOTE: IncuCyte FabFluor reagent is a third of the molecular weight of a standard antibody. Therefore, equal volumes of equal mg/mL quantities produce a 1:3 molar ratio of test antibody to FabFluor as MW of a typical antibody is ~3x of FabFluor. In this case, the stock concentration in mg/mL of test antibody is twice that of FabFluor. Therefore, 2X volume of FabFluor is required.

c. Determine volume of media: [Total volume] – [Test antibody volume] – [FabFluor volume]

500 µL – 1.5 µL – 3.0 µL = 495.5 µL

3. Dilution of IncuCyte Opti-Green background suppressor

3.1. Dilute Opti-Green stock in complete growth media for a final assay concentration of 0.5 mM or 1:200 dilution of stock (see calculations below).

NOTE: A final assay concentration of 0.5 mM has proven to be suitable across a range of cell types, however some optimization may be required to assess cell proliferation in the presence of Opti-Green. For lower expressed markers, Opti-green may be increased in some cell types (1 mM or 1:100 dilution of stock final assay concentration), allowing for a higher concentration of test antibody to be added (<3 µg/mL). A full list of pre-assessed cell types is included at the end of this protocol.

Example calculations for Opti-Green background suppressor

1. Required final assay concentration of Opti-Green Background Suppressor — 0.5 mM. Working concentration = 3X, or 1.5 mM.

2. Determine volume of Opti-Green Background Suppressor required at 3X final assay concentration (i.e. dilution of 1:3 recommended upon addition to cells): [# wells] x 50 µL (plus additional required to prepare dilution series if desired).

   E.g. For 96 replicates of 1:3 dilution of Opti-Green Background Suppressor:

   96 x 50 µL= 4800 µL minimum (5000 µL used for this example)

3. Calculate volume of Opti-Green Background Suppressor required to provide 3X final assay concentration.

   a. Determine volume of Opti-Green Background Suppressor: [Total volume] µL x [Working concentration Opti-Green Background Suppressor] mM / [Stock concentration Opti-Green Background Suppressor] mM 5000 µL x 1.5 mM / 100 mM = 75 µL

   b. Determine volume of media:[Total volume] – [Opti-Green Background Suppressor]

   5000 µL – 75 µL = 4925 µL

4. Add IncuCyte FabFlour-488 test antibody and Opti-Green to Cells

Adherent cells

4.1 Remove cell plate from incubator.

4.2 Using a multi-channel pipette:

a. Add 50 µL of diluted Opti-Green to wells
b. Add 50 µL of labeled antibody to required test wells.
c. Remove any bubbles and place plate in IncuCyte® S3 Live-Cell Analysis System.

4.3 Place plate in IncuCyte® S3 Live-Cell Analysis System.

Non-adherent cells

4.1 Add reagents to matrix coated plate:

a. Add 50 µL of diluted Opti-green
b. Add 50 µL of labelled antibody to required test wells.
c. Add 50 µL of cell suspension to wells.
d. Remove any bubbles

4.2 Allow the plate to sit for 30 minutes at room temperature to allow even settling, or centrifuge at 50 g for 1 minute.

4.3 Place plate in IncuCyte® S3 Live-Cell Analysis System.

5. Aquire images and analyze

5.1 Using IncuCyte® Software, schedule 24-hour repeat scanning for every 2-3 h.

a. Scan on schedule, Standard.

b. Channel selection: select "phase" and "green"

c. Objective: 10x or 20x depending on cell types used. Generally, 10x is recommended for adherent cells, and 20x for non-adherent or smaller cells.

5.2 To generate the metrics, user must create an Analysis Definition suited to the cell type, assay conditions and magnification selected.

5.3 Select images from a well containing a positive signal and an isotype control well (negative signal) at a time point where staining is visible.

5.4 In the Analysis Definition:

a. Set mask for phase confluence measure with green channel turned off.

b. Turn green channel on and mask green objects. Exclude background fluorescence using the background subtraction feature. The feature "Top-Hat" will subtract local background from brightly fluorescent objects within a given radius; applicable for analyzing objects which change in fluorescence intensity over time.

i. The radius chosen should reflect the size of fluorescent objects but contain enough background to reliably estimate background fluorescence in the image; 20-30 µm is often a useful starting point.

ii. The threshold chosen will ensure that objects below a fluorescence threshold will not be masked.

iii. Choose a threshold in which green objects are masked in the positive response image but low numbers in the isotype control, negative response well.

NOTE: For both cell types, individual cell identification can be enabled with the use of the IncuCyte Cell-by-Cell Analysis Software Module (PN 9600 0031). This enables the subsequent classification into subpopulations based on properties including fluorescence intensity, size and shape. For further details of this analysis module and it's application see:

www.essenbioscience.com/cell-by-cell

Analysis Guidelines

Staining of surfaced expressed protein will appear as a green ring followed by intracellular green signal as there will be internalization of the signal over time (time depends on cell type studied). Suggested metrics for data analysis are shown below:

1. Quantification of fluorescence area ("total object area" or "green object confluence"). Suggested metric: Analyze using "Total green Object Area (µm^2/well)".
2. Quantification of intensity, integrated over the area of detectable fluorescence (i.e. "Total Integrated Intensity"). Suggested metric: Analyze using "Total green Object Integrated Intensity (GCU x µm^2/ well)" metrics.
3. To correct for cell proliferation, it is advisable to normalize the area measurement for cell coverage (e.g. "green object confluence"/"phase confluence").

NOTE: If using Cell-by-Cell Analysis, post classification the data can be displayed as either % of cells expressing red fluorescence or mean intensity of positive red objects.

IncuCyte® Multi-Spheroid Assay

This protocol describes a solution for creating multi-spheroids using 96-well flat bottom plates coated with Matrigel®. The method utilizes the IncuCyte® live-cell analysis system for image-based brightfield and fluorescence measurements of multi-spheroid size (area), number and health.

Required materials

- 96-well flat bottom TC-treated microplate (Corning Cat #3595)
- Matrigel®, protein concentration ≥ 8 mg/mL (Corning Cat #356234)
- Wet ice
- Serum-free cell culture media for Matrigel® dilutions
- Complete culture media for cell culture and assay
- Manual multi-channel pipette
- IncuCyte® S3 Spheroid Software Module, version 2018A (Essen Cat #9600-0019)

Recommended materials

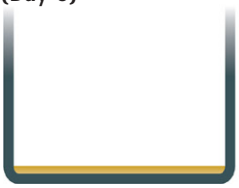
- IncuCyte® Cool Accessories (Cat # 1500-0078)
  - CoolBox 96F System (Includes x1 Block with gelpack and Coolsink 96F)
- IncuCyte® NucLight Red or Green Lentivirus Reagent (Essen Bioscience, Cat #4476 or #4475)
- IncuCyte® CytoLight Red or Green Lentivirus Reagent (Essen Bioscience, Cat #4481 or #4482)

General Guidelines

- All materials (e.g. culture-ware, reagents) that will come in contact with Matrigel® must be kept cold (on ice, stored at + 4°C).
- Follow manufacture's guidelines for thawing and storing of 100% Matrigel®. Thaw Corning® Matrigel® overnight by submerging the vial in ice cold water in the rear of a refrigerator (+ 4°C). Do not allow Matrigel® to warm to room temperature at any time as this will induce polymerization.
- We recommend sourcing a batch of Matrigel® with a concentration of ≥ 8 mg/mL.
- Following Matrigel® coating, cell seeding and after treatment addition, remove bubbles from all wells by gently squeezing a wash bottle containing 70 – 100% ethanol, with the inner straw removed, to blow vapor over the surface of each well.
- After placing the plate in the IncuCyte® live-cell analysis system, allow the plate to warm to 37°C for 30 minutes prior to scanning.

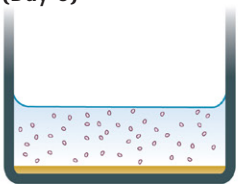
Quick guide

1 COAT PLATE (Day 0)



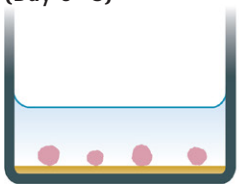
Coat plate (50% Matrigel®, 40 µL/well). Polymerize at 37°C for 30 minutes.

2 ADD CELLS (Day 0)



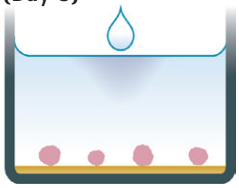
Add cells (150 µL/well) and allow multi-spheroids to form (3 days).

3 MONITOR FORMATION (Day 0–3)



Place inside the IncuCyte and scan every six hours to monitor multi-spheroid formation.

4 ADD TREATMENTS (Day 3)



Add appropriate treatments (50 µL/well) at 4x final assay concentration.

Protocol

IMPORTANT:

1. In advance of multi-spheroid experiments, it is important to have stored the Cool pack accessories at the correct temperatures for at least 4h:
   a. Coolbox x 1 (block with gelpack: -20°C),
   b. Coolsink 96F x1 (4°C)
2. Keep all culture-ware and reagents coming in contact with Matrigel® on ice during the entire process.
3. Store pipette tips used for dispensing diluted Matrigel® at + 4°C.

Day 0

1 Coat plate with Matrigel®

1.1 In a cell culture hood, chill plates (10 – 15 minutes) on a pre-chilled Coolsink 96F within a Coolbox 96F box.

1.2 In a cold polypropylene tube, dilute 100% Matrigel® 1:1 in cold serum-free culture media (keep all Matrigel® solutions on ice).

NOTE: To prevent incomplete gel formation, for coating we recommend using ≥ 4 mg/mL Matrigel®.

a. To coat a single 96-well plate, add 2.5 mL of cold serum-free culture media to a pre-chilled polypropylene tube.
b. Using a cold serological pipette, slowly pipette 2.5 mL of 100% Matrigel® into the serum-free media and, taking care to avoid bubbles, slowly mix by pipetting the solution up and down.

1.3 Pour prepared solution into a pre-chilled sterile reagent reservoir (keep on ice).

1.4 Using pre-chilled pipette tips and reverse pipetting technique, coat the pre-chilled 96-well plate by carefully adding 40 µL of diluted Matrigel® into the center of each well.

a. While the plate is cold and Matrigel® is still liquid, gently rock the plate once within the Coolbox to ensure even coating of each well.

NOTE: To avoid cell penetration to the base of the plate, coat wells with a minimum of 40 µL. Use of reverse pipetting technique is important to minimize bubbles.

1.5 Remove any bubbles using a wash bottle containing 70 – 100% ethanol, with the inner straw removed, to blow vapor over the surface of each well.

1.6 Place the plate in a 37°C incubator for 30 minutes to polymerize the Matrigel®.

2 Seed cells

2.1 Seed cells of interest (150 µL per well) at an appropriate density on top of polymerized Matrigel® base such that by day 3, multi-spheroids have formed with the desired size (e.g. 30 – 80 µm in diameter).

NOTE: Seeding density will need to be optimized for each cell type used. As a guide, we recommend seeding A549, MCF-7 and MDA-MB-231 at 1000 - 2000 cells per well or SKOV-3 at 2000 - 4000 cells per well.

2.2 Place plate in a 37°C incubator for 30 minutes prior to scanning.

Day 0–3

3 Monitor multi-spheroid formation

3.1 Place the cell plate into the IncuCyte® live-cell analysis System and schedule 24 hour repeat scanning:

a. Objective: 10x (96-well corning) 1 image per well
b. Channel selection: Phase Contrast + Brightfield and "Red" or "Green" if required
c. Scan type: Spheroid, Spheroid Type: Multi
d. Scan interval: Every 6 hours

Day 3

4 Add treatments

4.1 Once multi-spheroids have reached desired size, remove the plate from the IncuCyte and carefully add appropriate treatments at 4x final assay concentration (50 µL per well).

4.2. Continue to monitor multi-spheroid growth (e.g. every 6 hours for 7 days).

5 Re-feed cultures (optional)

5.1 Re-feed cultures every 3 – 4 days. Remove plate from IncuCyte. Carefully remove 100 µL of media per well and replace with 100 µL of media containing test agents (1x final assay concentration).

5.2 Return plate to the IncuCyte and continue to monitor multi-spheroid growth.

Analysis Guidelines

Result: Size, number and viability/health measurements

Suggested metric: Brightfield Object Area (Total, Average), Object Count

Secondary metrics: Fluorescent metrics within a Brightfield Object Boundary

IncuCyte® Single Spheroid Viability Assay

For the quantification of fluorescently labeled spheroid growth and shrinkage.

This protocol describes a solution for creating single spheroids using a 96- or 384- well round-bottom, ultra-low attachment plate. This method utilizes the IncuCyte® live-cell analysis system for image-based Brightfield and fluorescence within the Brightfield boundary of spheroid area measurements. Cell lines expressing fluorescent protein can be used to monitor spheroid health.

General Guidelines

- Remove bubbles from all wells by gently squeezing a wash bottle containing 70-100% ethanol, with the inner straw removed, to blow vapor over the surface of each well.
- After placing the plate in the IncuCyte® live-cell analysis system, allow the plate to warm to 37 °C for 30 minutes prior to scanning.

Required materials

- IncuCyte® S3 Spheroid Software Module (Essen Cat # 9600-0019)
- IncuCyte® S3 Spheroid software version 2017B

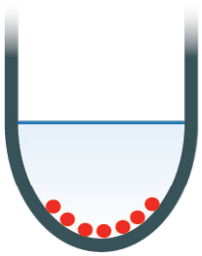
Cell fluorescent label reagents and consumables

- IncuCyte® NucLight Red or Green BacMam 3.0 Reagent for nuclear labeling (Essen Cat # 4621 or 4622)
- IncuCyte® NucLight Red or Green Lentivirus Reagent (EF-1 α, Puro) for nuclear labeling (Essen Cat # 4624 or 4625)
- IncuCyte® CytoLight Red or Green Lentivirus Reagent (EF-1 α, Puro) for cytoplasmic labeling (Essen Cat # 4481 or 4482)
- Matrigel® (Corning Cat#356234), optional
- 96-well round-bottom, ultra-low attachment plate (e.g., Corning® Cat#7007, S-BIO Cat#MS-9096UZ, BRANDplates® Cat#7816 60, 7819 00, 7819 60)
- 384-well round-bottom, ultra-low attachment plate (e.g., S-BIO Cat#MS-9384UZ)

NOTE: Combination of cells expressing fluorescent proteins with cell health reagents (Cytotox, Annexin V) is NOT recommended.

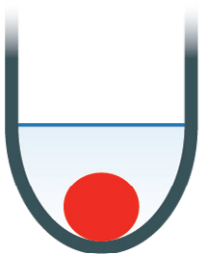
Protocol

1 CELL SEEDING (Day 0)



Seed cells into 96W or 384W Ultra Low Attachment plate. Centrifuge.

2 SPHEROID FORMATION (Day 0–3)



Place plate inside the IncuCyte and scan every six hours.

3 ADD TREATMENTS Day 3

Add treatments to plate. Monitor spheroid growth and shrinkage.

Day 0

1 Seed cells

1.1. Seed cells of interest (100 µL per well for 96-well, 50 µL for 384-well) at an appropriate density into a 96- or 384-well ULA plate such that by day 3, spheroids have formed with the desired size (e.g., 200 – 500 µm after 3 days). Seeding density will need to be optimized for each cell line used, however, we recommend a range of 1,000 – 5,000 cells per well (10,000 – 50,000 cells per mL seeding stock).

NOTE: Some cell lines may require the addition of a basement membrane extract, typically 2.5% v/v Matrigel®, to promote tight spheroid formation.

1.2. Centrifuge the ULA plate (125 g, 10 minutes) at room temperature (20-25°C).

Day 0—3

2 Spheroid formation

2.1. Place the cell plate into the IncuCyte live-cell analysis System and schedule 24 hour repeat scanning:

a. Objective: 4x or 10x (96-well ULA) or 10x (384-well ULA), 1 image per well

b. Channel selection: Phase Contrast; Brightfield; "Green" or "Red" if fluorescent label OR if a cell health reagent will be added post spheroid formation.

c. Scan type: Spheroid.

d. Scan interval: Every 6 hours.

3 Add treatments

3.1. Once spheroids have reached desired size (e.g., 200 – 500 µm), remove the ULA plate from the incubator and carefully add culture media supplemented with cell heath reagent (100 µL per well for 96-well, 25 µL per well for 384-well) containing test material (e.g. small molecules, antibodies; prepared at 2x final assay concentration for 96-well, 3x final assay concentration for 384-well).

3.2. Continue to monitor spheroid growth (e.g. every 6 h for 10 days).

NOTE: It is not recommended to change media in this assay as it will disrupt spheroids containing necrosing or apoptotic cells.

Analysis Guidelines

NOTE: Utilize the IncuCyte® S3 Spheroid Software module in the Brightfield channel to identify spheroid boundaries and analyze fluorescence as needed. See "Guidelines for Analysis," which can be accessed from the IncuCyte® S3 Technical Notes folder as part of the GUI installer.

1. **For parental (non-transduced) cells:**

Brightfield Boundary Measurements

Result: Size of spheroid measurement

Suggested Metric: Largest Brightfield object (avoid segmentation of small fragments) IncuCyte. Carefully remove 100 µL of media per well and replace with 100 µL of media containing test agents (1x final assay concentration).

2. For cells expressing fluorescent protein:

Fluorescent and Brightfield Boundary Measurements

Result: Size and viability measurements

Suggested Metric: Integrated intensity

Secondary metric: Mean intensity

IncuCyte® Neurite Outgrowth and Cell Health Analysis Assay

For the demonstration of label-free neurite outgrowth and neuronal cell health

This protocol demonstrates IncuCyte® label-free measurements of neurite outgrowth multiplexed with real-time measures of neuronal cell health. Neuro-2a NucLight Red cells are treated with retinoic acid to induce neurite formation and are cultured in the presence of decreasing serum concentrations to generate treatment groups with varying populations of viable and apoptotic cells. Neurite length and cell health are quantified in real time using the IncuCyte® NeuroTrack software module and IncuCyte® Annexin V Green reagent.

Materials supplied by Essen

- IncuCyte® Neuro-2a NucLight Red Cells (Essen BioScience 4512)
- IncuCyte® Annexin V Green Reagent (Essen BioScience 4642)

Materials to be supplied by Customer:

- F-12K Nutrient Mixture (1x)
- Fetal Bovine Serum
- Puromycin
- TrypLE Express Enzyme
- D-PBS (w/o Ca2+, Mg2+)
- Retinoic Acid
- 96-well flat-bottom microplate

Protocol

Day -3

1) Prepare serum-free and complete media.
   a. Transfer 100 mL of F-12K media into two sterile bottles. Label one bottle "Serum free media". Do not add anything to this bottle as it will be used to prepare the compound plate.
   b. To the second bottle add 10 mL of FBS and 5.5 µL of 10 mg/mL puromycin and label "Neuro-2a complete media" (final media concentrations 10% FBS and 0.5 µg/mL puromycin).
2) Thaw the NucLight Red Neuro-2a cells.
   a. Warm the complete Neuro-2a media in a water bath prior to use.
   b. Add 20 mL of Neuro-2a complete media to one T75 flask.
   c. Remove one vial of Neuro-2a NucLight Red cells from liquid nitrogen and thaw in a 37 °C water bath for approximately 90 seconds, swirling gently. Remove vial when only a tiny ice crystal remains.
   d. Transfer the cells to the media in the T75 flask using a 5 mL serological pipette.
   e. Rinse the vial with 1 mL of Neuro-2a complete media and transfer to the T75 flask.

Day -2

3) Replace culture media with 20 mL fresh complete Neuro-2a media. Monitor cells in the IncuCyte and allow cells to grow until 70-80% confluent.

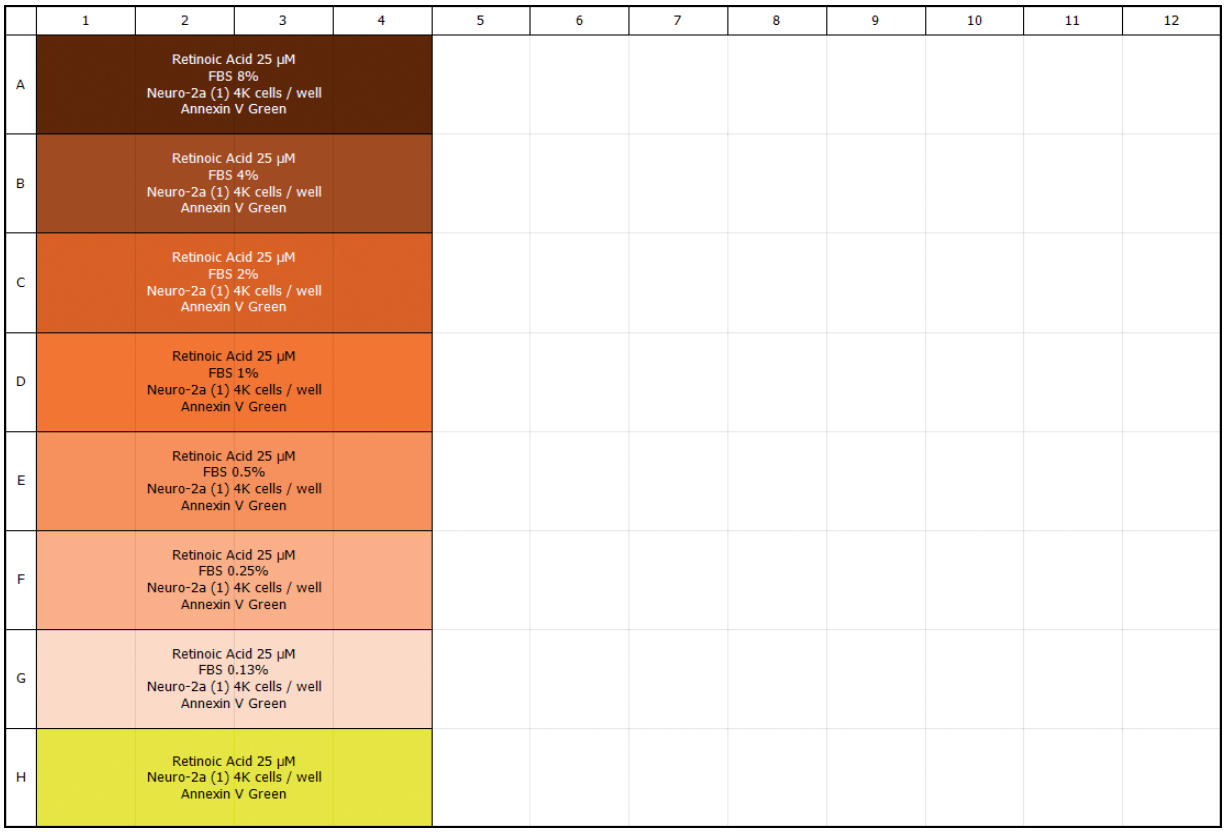
Day 0

1) Plate cells at 4,000 cells/well in complete media into a 96-well plate.
   a. Remove medium from the Neuro-2a NucLight Red cells and gently rinse twice with D-PBS.
   NOTE: Culture should be at 70–80% confluence in a T-75 flask.
   b. Harvest cells and perform a cell count (e.g., trypan bluestaining + hemacytometer).
   c. Dilute cells to 40,000 cells/mL and dispense 100 µL per well in columns 1-4 of a sterile 96-well Corning plate to obtain a density of 4,000 cells/well.
   d. Allow plate to sit at ambient temperature for 30 minutes and place in the incubator 3 hours prior to treatment.
2) Create a 10 mM stock of retinoic acid.
   a. Using a 50 mg ampule of retinoic acid (MW=300.44) gently tap the ampule to move all powder to the bottom and crack open the ampule. Add 1.664 mL DMSO to the ampule using a 1000 µL micropipette and triturate using a 200 µL micropipette until the retinoic acid is completely in solution (solution will be yellow and transparent), making a 100 mM retinoic acid solution.
   b. Transfer 200 µL of 100 mM retinoic acid from the ampule into a tube containing 1.8 mL DMSO to create a 10 mM retinoic acid stock solution.

3) Prepare assay media.
   a. Make 20 mL of a 25 µM retinoic acid by adding 50 µL of 10 mM retinoic acid stock solution to 19.95 mL serum-free F-12K Nutrient Mixture media in a 50 mL conical tube. This will be your Assay Media for setting up the compound plate and making up the Annexin V reagent.

   NOTE: Retinoic acid has poor aqueous solubility and should be vortexed thoroughly after adding it to the serum free media.
4. Make compound plate.
   a. Make up 2 mL of 12% FBS by adding 240 µL FBS to 1.760 mL Assay Media. This is 1.5x the final assay concentration of 8%. It will be used as the top concentration for the dilution series.
   b. Add 125 µL of Assay Media to rows B-H in columns 1-4 of a sterile 96-well plate.
   c. Add 250 µL of Assay Media containing 12% FBS to row A of columns 1-4.
   d. Serially dilute the FBS 1:2 by transferring 125 µL from row A into row B and mixing 10 times. Repeat until row G. Leave row H untouched, as that will contain 0% FBS.
5. Transfer compound plate to cell plate.
   a. Remove cell plate from incubator and place in hood
   b. Aspirate all media from the wells and gently wash with 100 µL per well of pre-warmed serum-free F12K Nutrient Mixture media. Ensure all media is removed from plate after this wash step.
   c. Using a multichannel pipette, gently transfer 100 µL from each well of the Compound Plate to the cell plate.
6. Addition of Annexin V reagent.
   a. To one vial of Annexin V Green reagent add 100 µL of Assay Media. Transfer the contents of the vial to 6.7mL of Assay Media.
   b. Using a multichannel pipette, add 50 µL of Assay Media containing Annexin V Green reagent to all wells in the cell plate (final assay Annexin V Reagent dilution 1:200).
7. Remove any bubbles from all wells by gently squeezing a wash bottle (containing 100% ethanol with inner straw removed) to blow vapor over the surface of each well. Keep the tip of the wash bottle approximately 5 cm from the surface of the medium.
8. Position the de-bubbled cell plate in the IncuCyte® Live-Cell Analysis System and allow to equilibrate for 20 min prior to the first scan. Schedule 24-hr repeat scanning (10x) for every 4 hours for 3 days.
   a. Objective: 10x.
   b. Vessel Type: Corning 3595.
   c. Scan Mode: Standard.
   d. Scan Pattern: 4 images per well.
   e. Channels: Phase + Green + Red.

Plate map set up



Analysis

Two separate IncuCyte® analysis jobs must be run to quantify both neurite morphology and neuronal cell health.

1. Neurite analysis
NeuroTrack: Run a NeuroTrack analysis job for the phase contrast channel to quantify Neurite Length.
NOTE: You will have the option to quantify nuclear counts using the NeuroTrack analysis job, however, we recommend that the Basic Analyzer is used to quantify both the nuclear counts Annexin V objects to enable easy cross comparison of cell viability data.

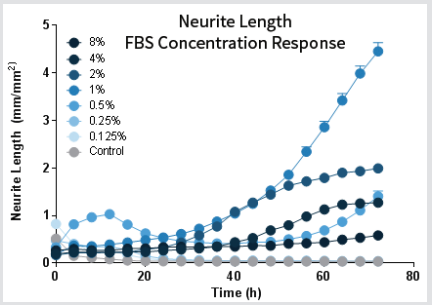
Suggested analysis parameters for Phase NeuroTrack:
- Segmentation Mode: Texture
- Min Cell Width: 15.0
- Neurite Sensitivity: 0.5
- Neurite Width: 2

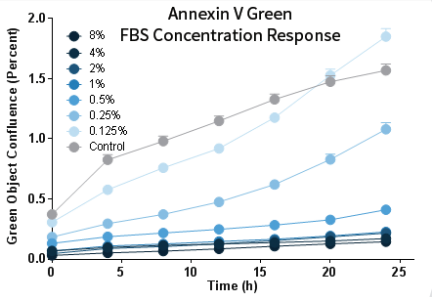
2. Quantification of apoptotic cells and viable cell count
Basic Analyzer: Run a Basic Analyzer job for both the red and green fluorescent channels. We recommend using red Object Count to quantify fluorescent nuclei (viable cells), and green Object Confluence to measure the Annexin V response (apoptotic cells).
NOTE: Retinoic acid may fall out of solution over the course of the assay forming small auto fluorescent green particles. These particles can be removed from the Annexin V green object analysis by setting a minimum green channel area filter threshold of around 50 µm².

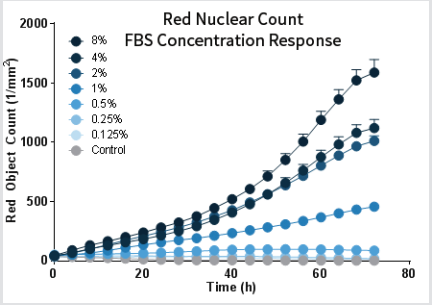
Suggested analysis parameters for Annexin V Green processing definition:
- Top-Hat analysis, radius 100 µm, threshold at 2.0 GCU
- Edge Split On, Sensitivity at -30
- Area Filter, min set at 50 µm²

Suggested analysis parameters for Red Nuclear Count processing Definition
- Top-Hat analysis, Radius 100 µm, threshold at 2.0 RCU

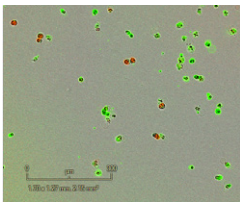
Example data

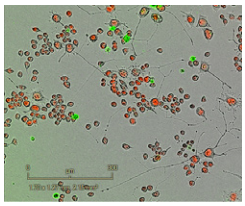


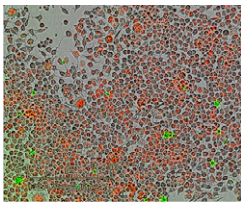




IncuCyte® images and metrics at 72 hours







	0.125% FBS	1% FBS	8% FBS
Neurite length	0.01 mm/mm²	6.01 mm/mm²	0.55 mm/mm²
Annexin V	2.97%	0.92%	0.67%
Nuclear count	14.4 nuclei/mm²	439 nuclei/mm²	2069 nuclei/mm²

Expected results and interpretation

Neuro-2a cells are a mouse neuroblastoma cell line and proliferate in growth media containing 8 to 10% serum. Neuro-2a cells can be differentiated into a neuronal-like morphology by reducing the serum concentration towards 2% and treating with 25 µM retinoic acid. Differentiated cells will form neurites and cease to proliferate. In the example data shown above maximal neurite length, reduced cell proliferation and minimal cell death were observed at 1% FBS.

Neurite outgrowth:

- Recommended metric: Neurite length (mm/mm2); the total neurite network length in mm expressed per mm2.

- Decreasing the media serum concentration from 8% to 2% promotes neurite outgrowth. Maximal neurite outgrowth is typically observed at FBS concentrations between 0.5 and 2% in the presence of 25 µM retinoic acid. At serum levels below 0.5% cell viability and neurite outgrowth is impaired.

Annexin V measurement (cell analysis):

- Recommended metric: Green object confluence (%); the percentage of the image area occupied by annexin V labeled objects.

- Serum concentrations below 1 to 0.5% will cause immediate cell death and illicit a concentration dependent Annexin V Green response in the first 24 hours. Please note it is normal to observe an Annexin V response for serum concentrations above 1% after approximately 3 days in culture as the cells become confluent and die. We recommend stopping the assay after 72 hours.

Nuclear Count (viable cell analysis):

- Recommended metric: Red object count (1/mm2); the number of NucLight red labeled nuclei per mm2.

- Serum concentrations above 1% will induce Neuro-2a cell proliferation in a concentration dependent manner. FBS levels below 1% will lead to a loss in cell viability and reduced nuclear count.

Combined Metrics:

It is possible to normalize neurite length to either Cell-Body Clusters or per Nucleus if a nuclear labeled cell line is used. This normalization would not be recommended for the FBS optimization experiment described here because the metric

can be difficult to interpret when significant cell death is occurring. This normalization would primarily be used to normalize for variations in seeding density when testing compounds that affect neurite outgrowth.

IncuCyte® Fluorescence Neurite Analysis Assay

This protocol provides an overview of the IncuCyte® Apoptosis Assay methodology combined with the IncuCyte® Neurite Analysis Assay.

The IncuCyte Apoptosis Assay uses mix-and-read IncuCyte® Annexin V NIR Reagent (Cat. No. 4768) to detect apoptosis in real time. The highly flexible apoptosis assay format can be combined with your choice of treatments. It is compatible with the IncuCyte® S3 Live-Cell Analysis System for Neuroscience (Cat. No. 4763) configured with the Orange/NIR Optical Module.

The IncuCyte® NeuroPrime Orange Kit (Cat. No. 4760) is a main component of the Neurite Analysis Assay. This kit contains cryopreserved vials of cortical neurons and astrocytes isolated from embryonic stage (E18) Sprague-Dawley rats as well as sufficient IncuCyte® NeuroLight Orange Reagent to perform one 96-well experiment. The lentivirus encodes an orange fluorescent protein regulated by a synapsin promoter that selectively drives expression in neurons and minimizes expression in non-neuronal cell types. After six days in co-culture the neurons form extensive neurite networks enabling the neurotoxic or neuroprotective effects of treatments to be assessed. Dynamic changes in network length and branching are measured using IncuCyte® S3 Live-Cell Analysis System for Neuroscience (Cat No. 4763) and the NeuroTrack Software Module (Cat. No. 9600-0010).

The NeuroPrime Orange Kit and Annexin NIR Reagent are not compatible with instruments configured with the Green/Red Optical Module, e.g. 4647.

Required materials

- IncuCyte® NeuroPrime Orange Kit (Sartorius Cat. No. 4760), containing:
  - 1X vial IncuCyte® rCortical Neurons (2 x 10^6 cells/vial)
  - 1X vial IncuCyte® rAstrocytes (2 x 10^6 cells/vial)
  - 1X vial IncuCyte® NeuroLight Orange Lentivirus-Synapsin promoter (0.45 mL/vial)
- IncuCyte® Annexin NIR Reagent (Sartorius Cat. No. 4768)

Materials required but not provided

Software:

- IncuCyte® S3 Live-Cell Analysis System for Neuroscience (Cat. No. 4763) with IncuCyte® NeuroTrack Software Module (Cat. No. 9600-0010)

Reagents:

- Poly-D-Lysine—Millipore (Cat. No. A-003-E)
- WFI water—Corning CellGro Mediatech (Cat. No. 25-055-CM)
- Neurobasal Media—Life Technologies (Cat. No. 21103049)
- B-27 Serum Free Supplement—Life Technologies (Cat. No. 17504044)
- GlutaMAX-I Supplement—Life Technologies (Cat. No. 35050061)
- DMEM—Life Technologies (Cat. No. 11965 or 41965)
- Fetal Bovine Serum—Sigma Aldrich (Cat. No. F2442) or Thermo Scientific (Cat. No. SH3007103)
- 5-Fluoro-2'-deoxyuridine—Sigma Aldrich (Cat. No. F0503)
- Uridine—Sigma Aldrich (Cat. No. U3003)
- 70% ethanol w/v

Material:

- Flat bottom tissue culture plate (e.g., Corning Cat. No. 3595)

IncuCyte® Annexin V NIR and IncuCyte® NeuroLight Orange Multiplex Protocol

Protocol Overview: IncuCyte NeuroPrime Orange Kit

1. Day 0

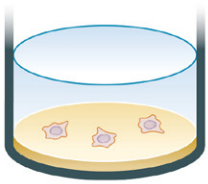
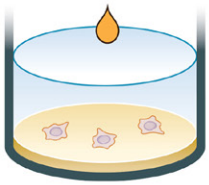


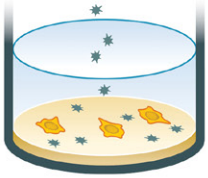
Plate rCortical neurons.

2. Day 0 (+ 4 hours)



Add NeuroLight Orange Reagent.

3. Day 1

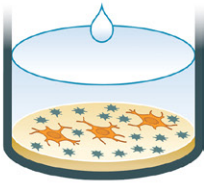


95% media replacement. Plate rAstrocytes. Begin IncuCyte® S3 scanning.

4. Day 3

50% media replacement. Add Uridine +5-Fluoro-2'-deoxyuridine.

5. Day 6, 9, 12...



50% media replacement. Treatments at Day 6 and beyond.

Solutions to prepare in advance

Day -1
Poly-D-Lysine

- 100 µg/mL in 12 mL of WFI quality water

Day 0
Neuronal Culture Media—for 50 mL. Recommended: make two batches (total 100 mL) to ensure sufficient volume for Day 1 media replacement

- 48.5 mL Neurobasal Media
- 0.5 mL GlutaMAX-I
- 1 mL B-27 Supplement

Day 1
Astrocyte Culture Media—for 50 mL

- 42.5 mL DMEM
- 7.5 mL FBS

Day 3

2X 5-Fluoro-2'-deoxyuridine and Uridine (FdU/U)

- Dissolve 8 mg of FdU and 28 mg of U in 100 mL Neurobasal to make 10X FdU/U stock solution.
- Dilute to 2X FdU/U in total volume of 12 mL in a 15 mL conical tube.
  - Add 2.4 mL 10X FdU/U
  - Add 9.6 mL NCM
- 10X FdU/U stock solution can be aliquoted and stored at -20° C for future use, at which point it can be thawed on ice.

CRITICAL: Use rigorous aseptic technique at all times. Only open the culture plate and medium bottles within a tissue culture hood.

Protocol

Day -1: Coat 96-well plate with Poly-D-Lysine

1. Coat one 96-well plate with Poly-D-Lysine. Prepare a 1X stock of Poly-D-Lysine (final concentration –100 µg/mL) in WFI quality water and add 100 µL to each well. Replace lid and incubate for 16-20 hours at room temperature in the tissue culture hood.
2. Aspirate and discard the Poly-D-Lysine and rinse the plate twice with 150 µL/well of WFI water. If excess Poly-D-Lysine is not washed away it can impair neurite outgrowth.
3. Leave the plate to dry for at least one hour with lid removed in the tissue culture hood.

Day 0: Thaw and plate neurons

1. Prepare the Neuronal Culture Media (NCM). For 50 mL of complete NCM, add 1 mL of B-27 Serum Free supplement and 0.5 mL GlutaMAX-I to 48.5 mL of Neurobasal media in a 50 mL conical tube. Recommended: make two batches (total 100 mL) to ensure sufficient volume for Day 1 media replacement.
   CRITICAL: Warm NCM to 37° C prior to thawing neurons.
2. Remove the vial of rCortical Neurons from liquid nitrogen storage and thaw in a 37° C water bath until only a tiny ice crystal remains (1 to 2 minutes).
   CRITICAL: Do not agitate the vial during this step.
3. Wipe outside of vial with 70% ethanol.
4. In tissue culture hood, use a P1000 pipettor to pre-wet a tip with 1 mL NCM.
5. Use the pre-wetted tip to transfer the 1 mL volume of thawed neuronal cells to a 50 mL conical tube.
6. Rinse the cryo-vial with 1 mL NCM and transfer the rinse media in a drop-wise fashion to the 50 mL conical tube containing neurons, while gently swirling the 50 mL conical tube.
   CRITICAL: Rapid addition of the media at this point can result in osmotic shock and cell death. The 1 mL addition should take about 30 seconds.
7. In drop-wise fashion, add a further 2 mL pre-warmed NCM to the 50 mL conical tube. The 2 mL addition should take about one minute.

8. Perform a cell count (e.g. Trypan Blue staining with hemocytometer) and dilute neurons to 150,000 cells/mL in pre-warmed NCM. The cell suspension can be transferred to a sterile trough in the tissue culture hood at this point in order to facilitate pipetting of cells in the next step.
9. Using a multichannel handheld pipette, dispense 100 µL of neuronal cell suspension into each well of the Poly-D-Lysine coated 96-well plate (15,000 neurons/well).
   CRITICAL: To ensure proper mixing and uniform seeding of the neurons, mix the cell suspension by gently pipetting up and down 1-2 times between seeding each row of the plate. Rocking the trough is also recommended to ensure equal cell distribution.
10. Let the plate stand at room temperature in the tissue culture hood for 30 minutes and then place inside the incubator.
    CRITICAL: This step ensures the uniform distribution of cells in each well.
11. Allow cells to settle on the plate for 2 to 3 hours before proceeding.

Infect neurons with NeuroLight Orange Reagent

1. Allow the NeuroLight Orange Reagent to thaw on ice (approximately 1 hour).
2. After neurons have adhered for 2-4 hours post-plating, add appropriately diluted NeuroLight Orange Reagent to achieve desired concentration. The final well volume should be 200 µL per well.
   Note: Quality control for the IncuCyte NeuroLight Orange Reagent is the ability to efficiently infect IncuCyte rCortical Neurons to express TagRFP, driven off of the synapsin promotor of the IncuCyte NeuroLight Orange Lentivirus, such that a volume of > 3.2 µL/20,000 neurons results in a neurite length of > 50 mm/mm^2 in a neurite outgrowth assay (rCortical Neurons/rAstrocytes co-culture experiment). We recommend performing a volumetric titration from 100-0.14 µL for each neuronal cell line evaluated. The lowest concentration that results in the highest neurite outgrowth measurement should be selected. Evaluation of neurite dynamics is to be performed on an IncuCyte S3 for Neuroscience.
   CRITICAL: Do not pipette up and down after adding the virus solution as this may result in damage to the plated neurons.

Day 1: Plate astrocytes

1. 16-24 hours after plating and infecting the neurons, warm NCM to 37° C.
2. Carefully remove 190 µL of medium per well using a multi-channel pipettor, and replace immediately with 140 µL of fresh, pre-warmed NCM. Volume should now be 150 µL per well.
3. Prepare 50 mL Astrocyte Culture Media (85% DMEM + 15% FBS; ACM) by adding 7.5 mL FBS to 42.5 mL DMEM and warm to 37° C.
4. Remove the vial of rAstrocytes from liquid nitrogen storage and thaw in a 37° C water bath until only a tiny ice crystal remains (1 to 2 minutes).
5. Wipe vial with 70% ethanol.
6. In tissue culture hood, use a P1000 pipettor to pre-wet a tip with 1 mL ACM.
7. Use the pre-wetted tip to transfer the 1 mL volume of thawed astrocytes to a 50 mL conical tube.
8. Rinse the cryo-vial with 1 mL ACM.
   CRITICAL: Rapid addition of the media to the cell suspension at this point can result in osmotic shock and cell death. Transfer the rinse media in a drop-wise fashion to the 50 mL conical tube containing astrocytes, while gently swirling the 50 mL conical tube.
9. Add 3 mL pre-warmed ACM to the 50 mL conical tube in a drop-wise fashion. The 3 mL addition should be performed slowly, taking at least 1 minute.
10. Centrifuge the astrocytes at 250 x g for 5 min. Carefully aspirate and discard the supernatant and resuspend the cell pellet in 5 mL of ACM. Using a P-1000 handheld pipettor set to 800 µL, triturate the cell suspension by gently aspirating and dispensing 10-15 times to ensure a single cell suspension.
11. Perform a cell count (e.g. Trypan Blue staining with hemocytometer) and dilute cells to 300,000 cells/mL in prewarmed ACM.
12. Using a multichannel handheld pipettor, plate 50 µL of astrocyte cell suspension into each well of the 96-well plate containing the cultured neurons (i.e. 15,000 astrocytes/well) CRITICAL: To ensure proper mixing and uniform seeding of the astrocytes, mix the cell suspension by gently pipetting up and down 1-2 times between seeding each row of the plate. Rocking the trough is also recommended to ensure equal cell distribution.
13. Place plate into the IncuCyte® S3 for Neuroscience and schedule to image every 2 to 12 hours in Phase and Orange image channels. (See IncuCyte User Manual for detailed instructions on setting up an imaging schedule.)

Day 3: Treat plate with 5-Fluoro-2'-deoxyuridine and Uridine
CRITICAL: Addition of 5-Fluoro-2'-deoxyuridine and Uridine (FdU/U) prevents proliferation of non-neuronal cell types.

1. Remove 100 µL of media from each well using a multi-channel pipette and replace with 100 µL fresh NCM containing 2X concentrations of FdU/U to a final assay concentration of 8 µg/mL and 28 µg/mL, respectively.

Feeding cultures

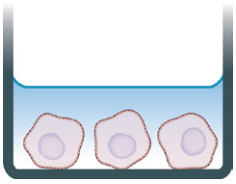
1. Feed cultures with fresh NCM by performing a 50% media change. To do this, remove 100 µL per well and replace with 100 µL of fresh media.
   CRITICAL: Only a single FdU/U treatment is required (Day 3, step 1). Addition of fresh FdU/U is not recommended on following days.
2. Cultures can be stopped at Day 11 or continued for desired length, with 50% media changes occurring every third day.

Days 6, 9, 12 and beyond: Solutions Required

1. Neuronal Culture Media—for 50 mL
   a. 48.5 mL Neurobasal Media
   b. 0.5 mL GlutaMAX I
   c. 1 mL B-27 Supplement

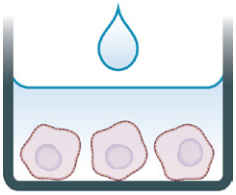
Protocol Overview: IncuCyte Annexin V NIR Reagent

1. Seed cells



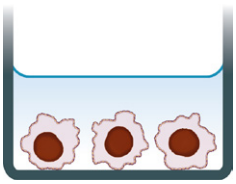
Seed cells (100 µL/well) into a 96-well plate.

2. Prepare apoptosis reagent and treat cells



Prepare the desired treatments at 2X in medium containing IncuCyte® Annexin V Reagent and add treatment.

3. Live-cell fluorescent analysis



Capture images every 2-3 hours (20X or 10X) in the IncuCyte® System. Analyze using integrated software.

Desired day: Annexin V NIR reagent preparation and cell treatment addition

1. Solubilize Annexin V NIR Reagent by adding 100 µL of complete medium or PBS. The reagent may then be diluted in complete medium containing at least 1 mM $CaCl_2$ for a final dilution of 1:100.
   Note: All test agents will be diluted in this reagent-containing medium to 2X concentration, so make up a volume that will accommodate all treatment conditions. The volumes/dilutions added to cells may be varied; however, a volume of 200 µL per well is generally sufficient for the duration of the assay.
2. Take the cell plate from the incubator and remove 100 µL media from wells (50%).
3. Add treatments and controls to appropriate wells of the 96-well plate to achieve a volume 200 µL per well. As solutions were made at 2X, we will have a 1X final dilution of treatments in individual wells (i.e., 1:200 final concentration of Annexin V NIR).
4. Place plate into the IncuCyte S3 for Neuroscience and allow the plate to warm to 37˚C for 30 minutes prior to scanning. Acquire images every 2-3 hours in Phase (optional), Orange, and NIR image channels, adjusting scan schedule if needed.

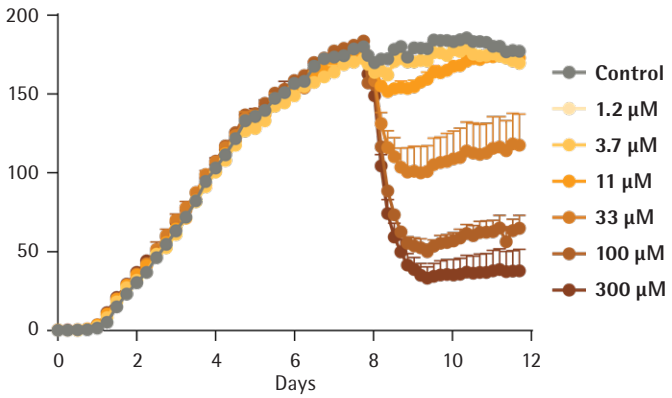
Analysis guidelines

1. Before starting your analysis, first set up the Spectral Unmixing parameters. We recommend removing 2-4% NIR channel from the orange channel.
   Note: Annexin V NIR signal bleeds into the orange channel. Using the Spectral Unmixing feature allows you to produce images that better represent the distribution of the two reagents.
2. Run 2 separate analysis jobs.
   a. For Neurite Analysis Assay:
      i. Analysis Type: NeuroTrack
      ii. Image Channels: Orange to quantify Neurite Length
      iii. Suggested analysis parameters:
         1. Min Cell Width: 15.0
         2. Neurite Fine Sensitivity: 0.5
         3. Neurite Width: 2
   b. For Apoptosis Assay:
      i. Analysis Type: Basic Analyzer
      ii. Image Channels: NIR to quantify apoptotic cells

Example Data

Measurements and images gathered using IncuCyte® S3 for Neuroscience. The figures below above show data and images obtained from a single 96-well plate assay over time.

A. Orange Neurite Length (mm/mm²)



B. NIR Total Fluorescent Area (10^5 mm²/image)

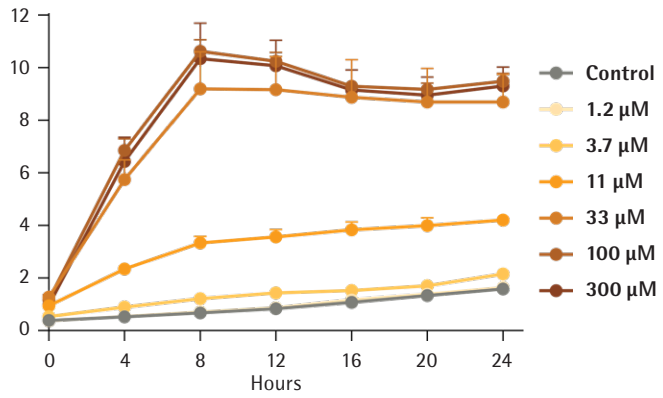
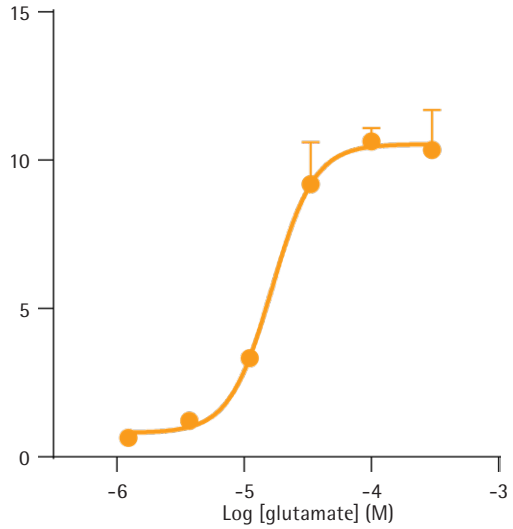
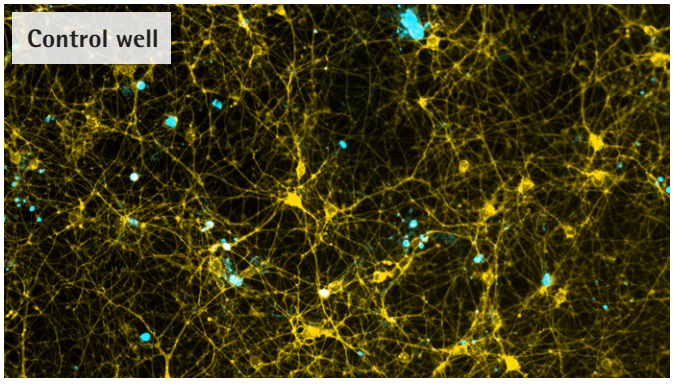
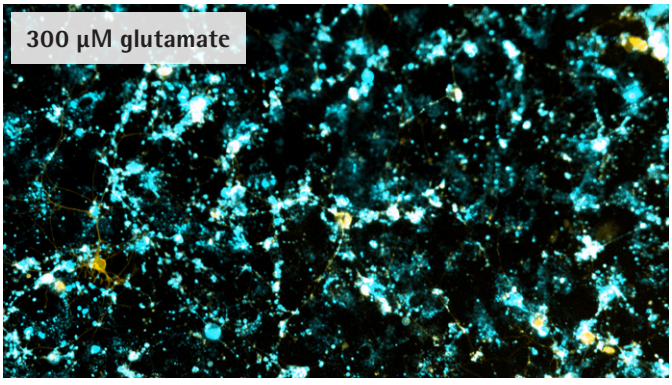


Figure A shows the disruption of Orange Neurite Length with the addition of various concentrations of glutamate at Day 8. Figure B shows the concentration-dependent response of Annexin V NIR Total Area to the glutamate treatment starting at time of addition, indicating neuronal cell death. Figure C is the concentration response curve of NIR Total Area generated with IncuCyte® Software providing an EC_{50} of 13.2 µM.

C. Concentration Response Curve







IncuCyte images showing NeuroLight Orange labeled rCortical Neurons in co-culture with rAstrocytes. The image on the left shows a control well treated with only Annexin V NIR, while the image on the right shows a well treated with Annexin V NIR and 300 µM glutamate. Annexin V NIR and treatments were added on Day 8 and images were taken 8 hours post addition. Cyan color depicts the Annexin V NIR response.

Safety considerations

The backbone of the Lentivirus particles in this system has been modified to improve their safety and minimize their relation to the wild-type, human HIV-1 virus. These modifications include:

1. The lentiviral particles are replication-incompetent and only carry the non-oncogenic gene of interest.
2. A deletion in the 3' LTR (ΔU3) resulting in "self-inactivation" (SIN) of the Lentivirus after transduction and genomic integration of the target cell (Yee et al., 1987; Yu et al., 1986; Zufferey et al., 1998). This alteration renders the lentiviral genome incapable of producing package able virus following host integration.
3. The envelope is psueudotyped with the VSV-G gene from Vesicular Stomatitis Virus of the HIV-1 envelope (Burns et al., 1993; Emi et al., 1991; Yee et al., 1994).

Replication-defective lentiviral vectors, such as the 3rd generation vector provided in this product, are not known to cause any diseases in humans or animals. However, lentivirus particles still pose some biohazardous risk because they can transduce primary human cells and can integrate into the host cell genome thus posing some risk of insertional mutagenesis. For this reason, **we highly recommend that you treat lentiviral stocks as Biosafety Level 2 (BSL-2, BL-2) organisms and strictly follow all published BL-2 guidelines with proper waste decontamination.**

For more information about the BL-2 guidelines and Lentivirus handling, we recommend referring to local documentation based on geography. The Essen BioScience 3rd generation HIV-based lentiviruses meet BL-2 requirements based on the criteria in the document, "Biosafety in Microbiological and Biomedical Laboratories", 5th Edition, published by the Centers for Disease Control (CDC). This document may be downloaded at **http://www.cdc.gov/biosafety/publications/bmbl5/index.htm**

Institutional Guidelines: Safety requirements for use and handling of lentiviruses may vary at individual institutions. We recommend consulting your institution's health and safety guidelines and/or officers prior to implementing the use of these reagents in your experiments.
A detailed discussion of lentiviral vectors is provided in Pauwels, K. et al (2009). **State-of-the-art lentiviral vectors for research use: Risk assessment and biosafety recommendations.** *Curr. Gene Ther.* 9: 459-474.

Biohazard note

The rCortical Neurons and rAstrocytes contain cells of rodent origin. Although the cells test negative for mycoplasma, bacteria and fungi, no test procedure can guarantee the absence of known and unknown infectious agents. Consequently, all products should always be considered potentially biohazardous and appropriate precautions should be taken. Use good laboratory practice and aseptic technique at all times.

IncuCyte® Neuronal Activity Assay

For the kinetic quantification of neuronal activity and functional connectivity

This protocol provides an overview of the IncuCyte® S3 Neuronal Activity Assay methodology which uses the lentiviral based genetically encoded orange fluorescent calcium indicator. The assay is compatible with the IncuCyte® S3 Live-Cell Analysis System for Neuroscience using your choice of cells and treatments.

Required materials

- IncuCyte® Neuronal Activity Analysis Software Module (Sartorius Cat# 9600-0032)
- IncuCyte® S3 Live-Cell Analysis System for Neuroscience (Sartorius Cat# 4763)
- IncuCyte® NeuroBurst Orange Lentivirus (Sartorius Cat# 4736)
- Flat bottom 96-well tissue culture plate (e.g., Corning Cat #3595)
- Water, cell culture grade
- Uridine (Sigma Cat# U3003)
- 5-Fluoro-2′-deoxyuridine (Sigma Cat#F0503)
- IncuCyte® rAstrocytes (Sartorius Cat #4586)
- BrainPhys™ Neuronal Medium (Stem Cell Technologies Cat# 05790)
- DMEM (Invitrogen Cat#11965)
- Fetal Bovine Serum (Sigma Aldrich Cat# F2442)
- For rat cortical neurons:
  - Poly-D-Lysine (PDL) (Millipore Cat#A-003-E)
  - IncuCyte® rCortical Neurons (Sartorius Cat #4753)
  - NeuroBasal® Medium (Thermo Fisher Cat #21103-049)
  - NeuroCult™ SM1 Neuronal Supplement (Stem Cell Technologies Cat# 05711)
  - GlutaMAX Supplement (Thermo Fisher Cat# 35050061)
- For iCell® GlutaNeurons:
  - Sodium borate solution (Thermo Fisher Cat#28341)
  - Polyethylenimine (PEI) (Sigma Cat#408727)
  - iCell® GlutaNeurons (Cellular Dynamics Cat # R1061)
  - Laminin (Sigma Cat# L2020)
  - iCell Neural Supplement B (Cellular Dynamics Cat# M1029)
  - N-2 Supplement (Thermo Fisher Cat#1750248)
  - iCell Nervous System Supplement (Cellular Dynamics Cat# M1031)

Recommended Materials for IncuCyte Neurite Analysis

- IncuCyte® NeuroTrack Software Module (Sartorius Cat# 9600-0010)
- IncuCyte® NeuroLight Orange Lentivirus Reagent (Sartorius Cat# 4758)

General guidelines

- Due to differences in neuronal sources, media and culture supplements may vary. We recommend using media/supplements supplied by the specific cell vendor.
- The protocol below outlines a coating of PEI/laminin for iCell® GlutaNeurons as an example of iPSC-derived neurons and PDL for primary rat cortical neurons. Depending on neuronal source, other preferred ECM plate coatings may be used.
- Thaw no more than 1 vial of neurons at one time. Maintain neurons in liquid nitrogen until immediately before thawing to ensure maximal performance of the cells. Complete the following steps of the thawing procedure in a time-efficient manner to facilitate optimal viability and performance.
- We recommend initiating activity experiments on a Monday in order to avoid weekend feedings.

Primary rat cortical and astrocyte cell culture media recipes

1. Complete Plating Medium (primary rat cortical neurons)

- 48.5 mL Neurobasal® Medium.
- 1 mL NeuroCult™ SM1 Neuronal Supplement.

  —Thaw one bottle of NeuroCult™ SM1 at room temperature (15 - 25°C) for 1 hour.

NOTE: If NeuroCult™ is not used immediately, aliquot and store at -20°C. Do not exceed the expiration date (EXP) as indicated on the label.

- 0.5 mL 100X GlutaMAX Supplement – stored at room temperature

2. Complete Maturation Medium (primary rat cortical neurons)

- 49 mL BrainPhys™ Neuronal Medium
- 1 mL NeuroCult™ SM1 Neuronal Supplement

— Thaw one bottle of NeuroCult™ SM1 at room temperature (15 - 25°C) for 1 hour.

NOTE: If NeuroCult™ is not used immediately, aliquot and store at -20°C. Do not exceed the expiration date (EXP) as indicated on the label.

NOTE: Complete Plating/Maturation Media can be stored at 2 - 8°C for up to 2 weeks. After 2 weeks, additional fresh complete maturation medium should be made to ensure neuronal cell health and function. **NOTE:** See recipe for culture of iCell® GlutaNeurons on page 6.

3. Rat Astrocyte Medium

- 425 mL DMEM
- 75 mL Characterized FBS NOTE: If not used immediately, medium can be stored at 4°C for 3 months.

Quick Guide: IncuCyte® NeuroBurst Orange Reagent

1. DIV: -1

Coat plate with matrix of choice and incubate at ambient temperature overnight.

2. DIV: 0

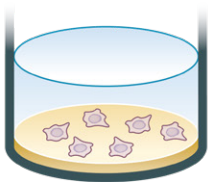


Plate rCortical neurons.

3. DIV: 0 + 2 hours

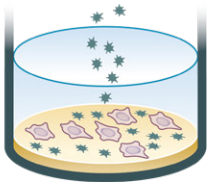


Plate rAstrocytes.

4. DIV: 2

Add NeuroBurst Orange Reagent.

5. DIV: 3

1. Remove NeuroBurst Orange Reagent.
2. Add Uridine + 5- Fluoro-2'-deoxyuridine.
3. Start Neuronal Activity scanning.

1. Plate Preparation DIV (day in vitro): -1

Primary Rat Cortical Neurons

1. Coat 1x 96-well plate with 100 µL/well of 0.1 mg/mL PDL in cell culture grade water.
2. Replace lid and incubate for 16-20 hrs at room temperature in tissue culture hood.

iCell® GlutaNeurons

We recommend plating iPSC-derived neuronal cultures on a dual coating of polyethylenimine (PEI) and laminin. *If other coatings are wanted for specific iPSC-derived neurons, customers can use what works for them.

1. Dilute 1M Borate stock buffer solution 10-fold in dH2O to achieve a concentration of 100 mM sodium borate solution.
2. Weigh out 0.11 g polyethylenimine and add 50 mL of 100 mM sodium borate solution.
3. Set on shaker or vortex until the PEI is completely dissolved.
4. Filter the solution through a 0.2 µM syringe filter (solution can be stored at 4°C for 7-10 days).
5. Coat 1x 96-well plate with 100 µL of PEI solution, replace lid, and incubate for 16-20 hrs at room temperature in tissue culture hood.

2. Plate Preparation DIV: 0

Primary Rat Cortical Neurons

1. Wash plate 3x with 200 µL cell culture grade water and let dry in tissue culture hood for approximately 1 hr (or until completely dry).

iCell GlutaNeurons

1. Using a vacuum manifold or multi-channel pipette, completely remove PEI from all wells.
2. Wash plate 3x with 200 µL sterile cell culture grade water and let dry in tissue culture hood for approximately 1 hr, ensuring no residual liquid remains in wells.
3. Laminin coating:
   a. Thaw 1 mg/mL laminin at 4°C to prevent premature gelling.
   b. When thawed, dilute 1 mg/mL laminin solution 1:300 in sterile culture grade water to a final concentration of 3.3. µg/mL immediately before use. Do not vortex the solution.
   c. Add 100 µL into each well and incubate at least 1 hr at 37°C.
   d. Remove laminin just before seeding cells. Seed each column separately to avoid drying of laminin coating.

3. Seeding of Neurons DIV: 0

Primary Rat Cortical Neurons

We recommend initial seeding in Complete Plating Medium and continued culture in Complete Maturation Medium (see included recipes).

NOTE: Equilibrate Complete Plating Medium to ambient temperature before thawing cells.

1. Thaw no more than 1 vial of neurons by immersing the cryovial in a 37°C water bath for approximately 2-3 min (avoid submerging the cap), holding the tube stationary (no swirling).
2. Immediately remove the cryovial from the water bath, spray with 70% ethanol, and place in tissue culture hood.
3. Pre-wet a sterile 50 mL centrifuge tube by addition and removal of 1 mL Complete Plating Medium.
4. Pre-wet P1000 tip with Complete Plating Medium and gently transfer the cryovial contents to the pre-wet 50 mL centrifuge tube.

NOTE: Use of a 50 mL centrifuge tube facilitates suitable mixing to minimize osmotic shock and increase neuron viability.

5. Rinse the empty cryovial with 1 mL of Complete Plating Medium to recover any residual cells from the vial.
6. Transfer the 1 mL of Complete Plating Medium from the cryovial drop-wise (~1 drop/sec) to the 50 mL centrifuge tube containing the neuronal suspension. Gently swirl the tube while adding the Complete Plating Medium to mix the solution completely and minimize osmotic shock on the thawed cells.
7. Slowly add an additional 3 mL of Complete Plating Medium to the 50 mL centrifuge tube drop-wise (~1-2 drops/sec). Gently swirl the centrifuge tube while adding the Complete Plating Medium.

NOTE: It is critical to add the Complete Plating Medium slowly to ensure maximum viability and attachment of the cells once plated. Avoid vigorous shaking or vortexing of the cell suspension.

8. Count live neurons with Trypan Blue (10 µL cell suspension + 10 µL Trypan Blue) using a hemocytometer. Adjust cell stock to 133,333 cells/mL to seed 20,000 neurons in 150 µL per well, using Complete Plating Medium.
9. Add 150 µL mixed cell suspension into each well (20,000 cells/well).

NOTE: While seeding neurons, gently mix cell suspension with a multichannel pipettor.

10. Let plate sit at room temperature in tissue culture hood for approximately 20 min to allow neurons to settle evenly in the wells.
11. Incubate plate(s) for approximately 2 hrs at 37°C before addition of astrocytes.

iCell GlutaNeurons

We recommend plating and keeping iCell GlutaNeurons in Complete BrainPhys Medium (see included recipe).

NOTE: Equilibrate Complete BrainPhys Medium to ambient temperature before thawing cells. We recommend a cell density of 20,000 cells per well, but this may need to be optimized for other iPSC-derived neuronal cell types.

1. Thaw no more than 1 vial of neurons by immersing the cryovial in a 37°C water bath for approximately 2-3 min (avoid submerging the cap), holding the tube stationary (no swirling).
2. Immediately remove the cryovial from the water bath, spray with 70% ethanol, and place in tissue culture hood.
3. Pre-wet a sterile 50 mL centrifuge tube by addition and removal of 1 mL Complete BrainPhys Medium.
4. Pre-wet P1000 tip with Complete BrainPhys Medium and gently transfer the cryovial contents to the pre-wet 50 mL centrifuge tube.

NOTE: Use of a 50 mL centrifuge tube facilitates suitable mixing to minimize osmotic shock and increase neuron viability.

5. Rinse the empty cryovial with 1 mL of Complete BrainPhys Medium to recover any residual cells from the vial.
6. Transfer the 1 mL of Complete BrainPhys Medium from the cryovial drop-wise (~1 drop/sec) to the 50 mL centrifuge tube containing the neuronal suspension. Gently swirl the tube while adding the Complete BrainPhys Medium to mix the solution completely and minimize osmotic shock on the thawed cells.
7. Slowly add an additional 8 mL of Complete BrainPhys Medium to the 50 mL centrifuge tube drop-wise (~1-2 drops/sec). Gently swirl the centrifuge tube while adding the Complete BrainPhys Medium.
8. Centrifuge the cell suspension at 400 x g at room temperature for 5 min.
9. Resuspend the cell pellet in 3 mL Complete BrainPhys Medium by gentle trituration (~ 30 sec).

NOTE: It is critical to add the Complete BrainPhys Medium slowly to ensure maximum viability and attachment of the cells once plated. Avoid vigorous shaking or vortexing of the cell suspension.
NOTE: Resuspension volume may need to be adjusted for other iPSC-derived neuronal cell types, depending on cell number.

10. Count live neurons with Trypan Blue (10 µL cell suspension + 10 µL Trypan Blue) using hemocytometer. Adjust cell stock to 133,333 cells/mL to seed 20,000 neurons in 150 µL per well, using Complete BrainPhys Medium.
11. Add 150 µL mixed cell suspension into each well (20,000 cells/well)

NOTE: While seeding neurons, gently mix cell suspension with a multichannel pipettor.

12. Let plate sit at room temperature in tissue culture hood for approximately 20 min to allow neurons to settle evenly in the wells.
13. Incubate plate(s) for approximately 2 hrs at 37°C before addition of astrocytes.

4. Seeding of Primary Rat Astrocytes for both Primary Rat Cortical Neurons and iCell GlutaNeurons DIV: 0 + 2 hours

NOTE: We recommend using rAstrocytes for both rCortical and iPSC co-cultures to maintain cell viability. Equilibrate astrocyte medium to ambient temperature before thawing cells.

1. Thaw 1 vial of astrocytes by immersing the cryovial in a 37°C water bath for approximately 2-3 min (avoid submerging the cap), holding the tube stationary (no swirling).
2. Immediately remove the cryovial from the water bath, spray with 70% ethanol, and place in tissue culture hood.
3. Pre-wet a sterile 50 mL centrifuge tube by addition and removal of 1 mL astrocyte medium.
4. Pre-wet P1000 tip with astrocyte medium and gently transfer the cryovial contents to the pre-wet 50 mL centrifuge tube.

NOTE: Use of a 50 mL centrifuge tube facilitates suitable mixing to minimize osmotic shock and increase astrocyte viability.

5. Rinse the empty cryovial with 1 mL of room temperature culture medium to recover any residual cells from the vial.
6. Transfer the 1 mL of astrocyte medium from the cryovial drop-wise (~1 drop/sec) to the 50 mL centrifuge tube containing the neuronal suspension. Gently swirl the tube while adding the astrocyte medium to mix the solution completely and minimize osmotic shock on the thawed cells.
7. Slowly add an additional 3 mL of astrocyte medium to the 50 mL centrifuge tube drop-wise (~1-2 drops/sec). Gently swirl the centrifuge tube while adding the astrocyte medium.
8. Centrifuge the cell suspension at 250 x g for 5 min at room temperature.
9. Carefully aspirate the supernatant leaving approximately 0.5 mL astrocyte medium in centrifuge tube. Resuspend cells in 5 mL astrocyte medium.
10. Count astrocytes with Trypan Blue (10 µL cell suspension + 10 µL Trypan Blue) using hemocytometer. Adjust cell stock to 300,000 cells/mL using astrocyte medium and gently seed 15,000 astrocytes in 50 µL per well (total volume per well should now be 200 µL) of 96-well plate containing neurons.

NOTE: While seeding astrocytes, gently mix cell suspension with a multichannel pipettor.

5. Infection of neurons with IncuCyte® NeuroBurst Orange Reagent DIV: 2

We recommend infection of neuronal cultures on DIV 2. However, later infections can be done without alterations in expression levels.

NOTE: IncuCyte® NeuroLight Orange Reagent can also be used on a separate plate for expression control.

1. Thaw NeuroBurst Orange Reagent on wet ice (approximately 1-2 hours).
2. Dilute NeuroBurst Orange Reagent in Complete Plating Medium for primary rat cortical neurons, Complete BrainPhys Medium for iCell GlutaNeurons or appropriate medium for other iPSC-derived neuronal cell types needed for a final addition volume of 100 uL/well.

NOTE: See optimization recommendations at end of protocol for determining the appropriate amount of virus to use for the neuronal cell type of interest.

3. Remove 100 µL Complete Plating Medium for primary rat cortical neurons, Complete BrainPhys Medium for iCell GlutaNeurons or appropriate medium for other iPSC-derived neuronal cell types from the assay plate containing the neuronal co-cultures.
4. Using a multichannel pipettor, gently add 100 µL/well of diluted NeuroBurst Orange Reagent prepared above to each well of your cell plate. Do not mix and return to incubator immediately.

CRITICAL: Do not pipette up and down after adding the virus solution as this may result in damage to the plated neurons.

6. Remove NeuroBurst Orange Reagent / 5-FDU/U Treatment DIV: 3

1. Prepare 90 mL of 5-FDUridine/Uridine (5-FDU/U).
   a. Weigh 7.2 mg 5-FDU (90 mL x 0.08 mg/mL = 7.2 mg).
   b. Weigh 25.2 mg U (90 mL x 0.28 mg/mL = 25.2 mg).
   c. Dissolve in 90 mL Complete Plating Medium for primary rat cortical neurons, Complete BrainPhys Medium for iCell GlutaNeurons or appropriate medium for other iPSC-derived neuronal cell types, and sterile filter.
   d. Make 4 mL aliquots and store at -20°C.
2. Remove 190 µL of medium from the assay plate containing neuronal/astrocyte co-cultures and replace with 90 µL of Complete Plating Medium for primary rat cortical neurons, Complete BrainPhys Medium for iCell GlutaNeurons or appropriate medium for other iPSC-derived neuronal cell types.

3. Add 100 µL/well of 2x 5-FDU/U for a final assay concentration of 8 µg/mL and 28 µg/mL, respectively, to inhibit astrocyte proliferation.

NOTE: If infection is performed at a later time point, add 5-FDU/U at DIV3 and remove NeuroBurst Orange Reagent (as described) 24 hrs after infection.

7. Scan Set-Up DIV: 3

Neuronal Activity Plate

1. Place the cell plate into the IncuCyte® S3 for Neuroscience and allow to warm to 37°C for 20-30 min prior to scanning (typical scan pattern is 3 min/well, 1 scan/day).
   a. Scan type: Neuronal Activity
   b. Channel selection: Phase and Orange
   c. Movie acquisition time: 30s-180s (default is set to 180s)
   d. Objective: 4x
   e. Scan interval: Every 24 hrs
2. Perform 50% media exchange (100 µL) with Complete Maturation Medium for primary rat cortical neurons, Complete BrainPhys Medium for iCell GlutaNeurons or appropriate medium for other iPSC-derived neuronal cell types 3x per week.

NeuroTrack Plate

1. Place the cell plate into the IncuCyte S3 for Neuroscience and allow to warm to 37°C for 20-30 min prior to scanning.
   a. Scan type: Standard
   b. Channel selection: Phase and Orange
   c. Objective: 20x
   d. 4 images per well
   e. Scan interval: Every 6 hrs
2. Perform 50% media exchange (100 µL) with Complete Maturation Medium for primary rat cortical neurons or Complete BrainPhys Medium for iCell GlutaNeurons 3x per week.

8. Feeding Cultures DIV: 6

Primary Rat Cortical Neurons

1. Make Complete Maturation Medium (see recipe above) and warm in a 37°C water batch.

NOTE: The Complete Maturation Medium will be used during the remainder of the assay.

2. Perform a 50% (100µL) media exchange with Complete Maturation Media 3x per week.

iCell GlutaNeurons

1. Use Complete BrainPhys Medium for iCell GlutaNeurons or optimized culture medium for other iPSC-derived neurons being used and perform 50% (100µL) medium changes 3x per week.

IncuCyte® NeuroBurst Orange Reagent Optimization

NOTE: We recommend optimizing NeuroBurst Orange Reagent volume per well for each uncharacterized cell type tested. Quality control for the IncuCyte NeuroBurst Orange Reagent is the ability to efficiently infect IncuCyte rCortical Neurons to express the mRuby-based IncuCyte NeuroBurst Orange Lentivirus driven off of the synapsin promoter, such that a concentration of > 3.7 µL/20,000 neurons results in an active object count > 500 at day 10 in a Neuronal Activity Assay (rCortical Neurons/rAstrocyte co-culture experiment). We recommend performing a volumetric titration from 100-0.14 µL for each neuronal cell line evaluated. The lowest concentration that results in the highest count of active objects should be selected. Evaluation of neuronal activity is to be performed on an IncuCyte S3 for Neuroscience.

1. Thaw reagent on wet ice: NeuroBurst Orange Reagent in DMEM (reagent is stored at -80°C).
2. To find the optimal reagent volume, we recommend testing a range of 100 µL to 0.14 µL per well from at least 4 wells for each plating density of neurons tested.

NOTE: We have found 20,000 neurons/well to be a good starting point for many cell types.

3. In a sterile 96-well culture plate, create serial dilutions of NeuroBurst Orange Reagent using the provided plate map (Figure 1).
   a. Add 180 µL of NeuroBurst Orange Reagent to wells A4-A7.
   b. Add 120 µL of appropriate medium to wells B4-B7 and continue down the entire plate to wells to wells H4-H7.
   c. Perform a 1:3 serial dilution by transferring 60 µL of NeuroBurst Orange Reagent from wells A4-A7 to wells B4-B7 and continue down the plate, stopping at row G. Row H is a no virus control and contains only medium.
4. We recommend using the IncuCyte NeuroLight Orange Reagent for expression control on a separate plate. The same dilution protocol should be used as NeuroBurst Orange Reagent optimization.

NOTE: NeuroLight Orange Reagent expression control optimization must be conducted on a separate plate, as a different scan type is used (Standard).

5. Once virus dilution plate is created, use a multichannel pipettor to gently add 100 µL/well of diluted NeuroBurst Orange Reagent prepared above to each well of your cell plate.

NOTE: Do not mix and return to incubator immediately.

IncuCyte® NeuroBurst Orange Reagent Optimization

NOTE: We recommend optimizing NeuroBurst Orange Reagent volume per well for each uncharacterized cell type tested. Quality control for the IncuCyte NeuroBurst Orange Reagent is the ability to efficiently infect IncuCyte rCortical Neurons to express the mRuby-based IncuCyte NeuroBurst Orange Lentivirus driven off of the synapsin promoter, such that a concentration of > 3.7 µL/20,000 neurons results in an active object count > 500 at day 10 in a Neuronal Activity Assay (rCortical Neurons/rAstrocyte co-culture experiment). We recommend performing a volumetric titration from 100-0.14 µL for each neuronal cell line evaluated. The lowest concentration that results in the highest count of active objects should be selected. Evaluation of neuronal activity is to be performed on an IncuCyte S3 for Neuroscience.

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4. We recommend using the IncuCyte NeuroLight Orange Reagent for expression control on a separate plate. The same dilution protocol should be used as NeuroBurst Orange Reagent optimization.

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5. Once virus dilution plate is created, use a multichannel pipettor to gently add 100 µL/well of diluted NeuroBurst Orange Reagent prepared above to each well of your cell plate.

NOTE: Do not mix and return to incubator immediately.

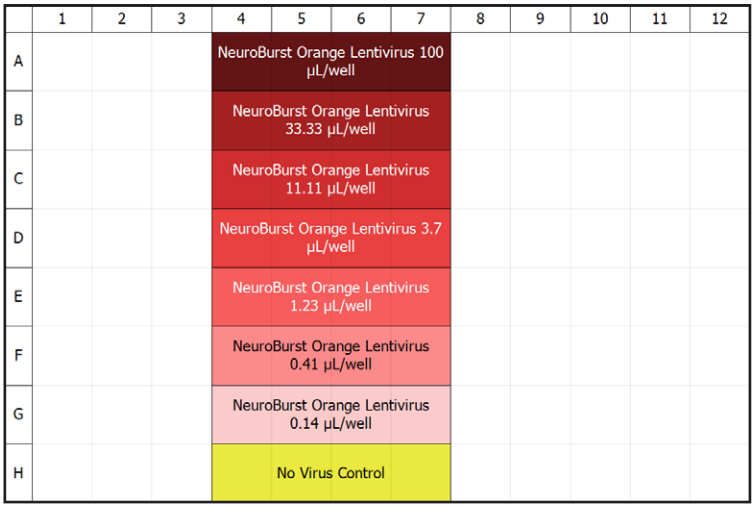


Figure 1

iCell GlutaNeurons Cell Culture Medium Recipe

iCell GlutaNeurons are cultured in Complete BrainPhys Medium comprised of BrainPhys Neuronal Medium, iCell® Neural Supplement B, iCell® Nervous System Supplement and N-2 supplement. The Complete BrainPhys Medium is serum-free and has been specially formulated to maintain the health and function of iCell GlutaNeurons while limiting the proliferation of progenitor or non-neuronal cells. iCell GlutaNeurons can be maintained in culture for at least 2 weeks in this medium without appreciable loss of viability or purity. For 200 mL:

- BrainPhys™ Neuronal Medium (192 mL)
- iCell Neural Supplement B (4 mL)
- iCell Nervous System Supplement (2 mL)
- N-2 Supplement (2 mL)

1. Thaw iCell Neural Supplement B, iCell Nervous System Supplement, and N-2 supplement at room temperature on the day of medium preparation.
2. Spray all medium components with 70% ethanol and place in a biological safety cabinet.
3. Using sterile technique, add the entire contents of the iCell Neural Supplement B vial (~2 mL), iCell Nervous System Supplement vial (~1 mL), and N-2 supplement (1 mL) to the BrainPhys Neuronal Medium (96 mL) to make the Complete BrainPhys Medium. Filter the Complete BrainPhys Medium through a 0.22 µm sterile filter unit.
4. Store the Complete BrainPhys Medium at 4°C, protected from light, for up to 2 weeks.

NOTE: We recommend using room temperature Complete BrainPhys Medium to thaw iCell GlutaNeurons.

NOTE: Freeze remaining N-2 supplement in 1 mL aliquots. Do not refreeze the other individual medium components or Complete BrainPhys Medium.

Determining Optimal NeuroBurst Orange Reagent Volume

- It is important to look at all metrics related to activity and connectivity when selecting the optimal volume of NeuroBurst Orange Reagent.
- The optimal amount will be the lowest volume producing the highest active object count while not affecting the other metrics of connectivity and activity.
- Figure 2 shows the results of a NeuroBurst Orange Reagent optimization experiment on iPSC-derived neurons. Active object count, mean correlation, mean burst rate, and mean burst strength are shown.
- The 100 and 33 µL per well concentrations show the highest number of active objects. These concentrations do not impact correlation measurements of network connectivity, and they show the highest level of burst strength. However, the burst rate is reduced when compared to lower concentrations in the time period between day 8 and 10.
- The 11 µL/well gives a sufficient active object count without significantly altering other metrics.
- This effect on burst rate can be observed by looking at the activity summary traces for the day 9 timepoint. Examples of these traces are shown in Figure 3.

Figure 2

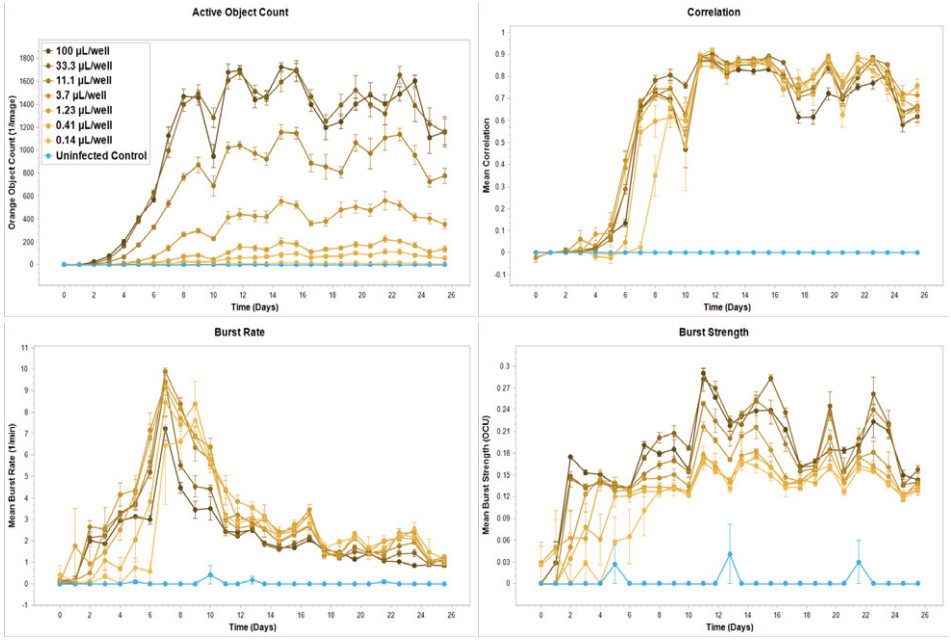
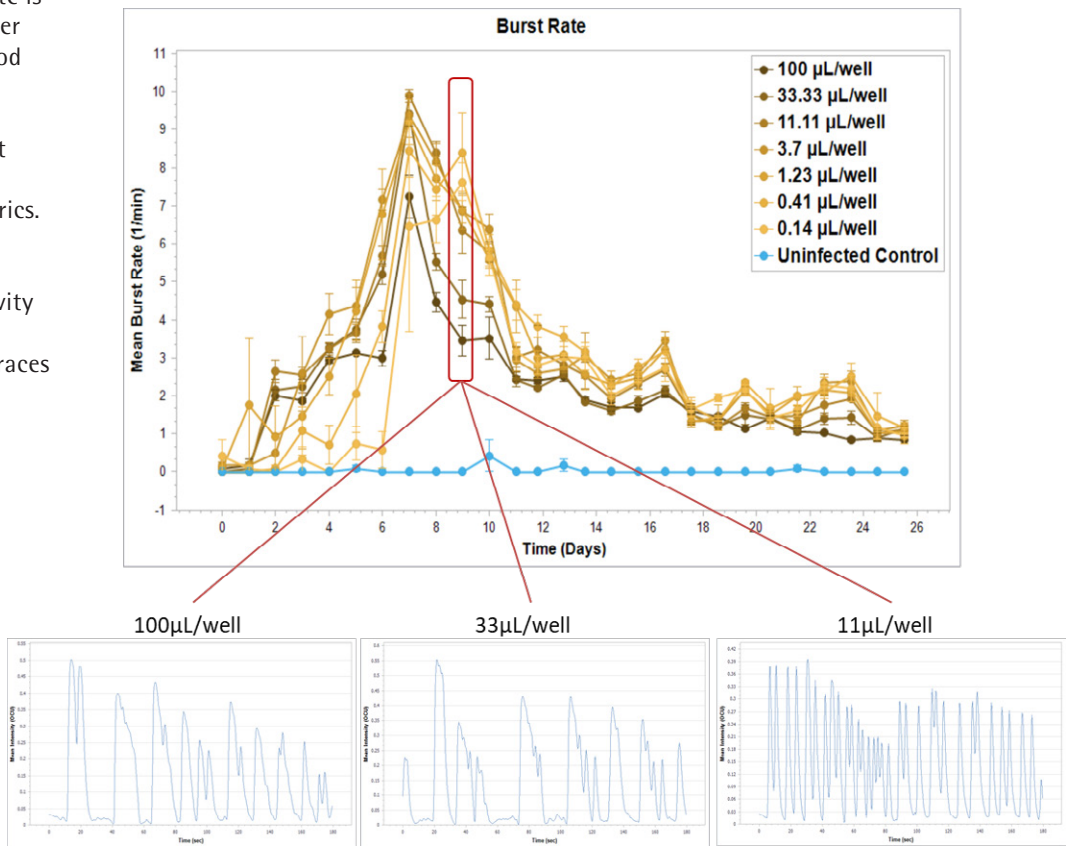


Figure 3



IncuCyte® pHrodo® Orange Phagocytosis Assay

For quantification of phagocytosis of apoptotic and non-apoptotic cell activity

This protocol is intended for the measurement of both apoptotic (efferocytosis) and non-apoptotic phagocytosis (antibody-dependent cellular phagocytosis) of cells by macrophages. This method utilizes the IncuCyte® pHrodo® Orange Cell Labeling Kit and the IncuCyte® S3 Live-Cell Analysis System for Neuroscience for image-based fluorescent measurements of phagocytosis.

Required materials

- IncuCyte® pHrodo® Orange Cell Labeling Kit for Phagocytosis (Sartorius Cat # 4766)
- Target cells of interest
- Target cell culture media
- Effector cells of interest
- Effector cell culture media
- 96-well microplate (e.g., Corning® Cat # 3595)

Initial Optimization Experiment

1. Assay Optimization

For optimal assay results, conduct preliminary experiment to determine the following assay parameters:

1.1 The seeding density of the effector cells which will result in 10-20% confluence 24 hours after plating. We have found that 10,000 effector cells per well is a reasonable starting point to reach ~20% cell confluency, and recommend optimizing above and below that density (e.g., 6, 7, 8, 9, 10, 11, 12 and 13k cells/well).

1.2 The lowest concentration of drug treatment (e.g., camptothecin or staurosporine) that will induce target cell apoptosis with limited cellular debris following a 24-hour exposure. Target cell apoptosis can be measured using the IncuCyte® Annexin V Reagent (Cat. No. 4759).

2. Target Cell Labeling Optimization

Target cells in the IncuCyte® pHrodo Orange Phagocytosis Assay must be efficiently labeled in order to detect phagocytic events. We recommend performing a serial dilution of the IncuCyte®pHrodo® Orange Cell Labeling Dye in DMSO and labeling your target cells per the optimization protocol below:

2.1 Suspend target cells at a density of 1 x106 cells/mL in IncuCyte® pHrodo® Labeling Buffer (component D). Separate the suspension into aliquots of 1mL.

2.2 Solubilize the IncuCyte pHrodo Orange Cell Labeling Dye (component A) by adding 100 µl of DMSO (component B) to create a stock concentration of 1 mg/mL.

2.3 Perform a serial dilution of the IncuCyte pHrodo Orange Cell Labeling Dye in DMSO.

a. For cells extracted from blood or tissue, generate a concentration range between 1 mg/mL (stock) and 100 µg/mL.

b. For cultured cell lines, generate a concentration range between 100 µg/mL and 10 µg/mL.

2.4 Add 10 µl of each concentration of dye to 1 mL cell suspension i.e., a 1:100 dilution, which will provide a final assay concentration range of

a. 0 µg/mL to 1 µg/mL,

b. 1 µg/mL to 100 ng/mL

2.5 Incubate for 1 hour at 37 °C. Harvest cells by centrifugation for 7 minutes at 1000 rpm.

2.6 Aspirate supernatant and wash cell pellet with 1 mL complete media (cell type appropriate). Harvest cells by centrifugation for 7 minutes at 1000 rpm, aspirate supernatant and resuspend in 1 mL complete media.

2.7 Prepare a citrate-based buffer solution at pH 4.0. For each dilution of IncuCyte pHrodo Orange Labeled Cells, prepare a micro-centrifuge tube containing 300 µL of buffer, and add 30 µL of labeled cells. Mix by trituration.

2.8 Per each buffered cell dilution, aliquot 100 µL to three wells of a 96-well plate and allow the cells to settle at ambient temperature. Scan the plate in phase and orange fluorescence. By counting the number of phase and fluorescent objects, a percentage of labeled cells may be obtained for each concentration of dye.

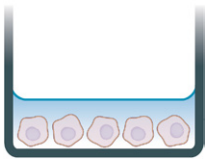
Apoptotic Phagocytosis Protocol (Efferocytosis)

This protocol provides an overview of the phagocytosis of dying cells by macrophage engulfment, known as efferocytosis. It combines the IncuCyte pHrodo Orange Cell Labeling Kit with the IncuCyte® S3 Live-Cell Analysis System for Neuroscience using your choice of target and phagocytic (effector) cells.

Prior to initiating the assay, it is important that your experimental design includes replicate wells of each condition being tested (e.g. effector cells alone, labeled apoptotic cells alone at each density, and target:effector co-cultures at each ratio) in order to determine the assay signal window.

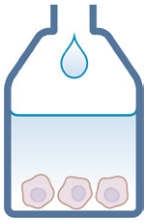
Quick Guide

1.Seed effector cells



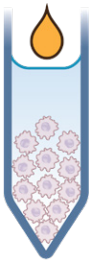
Seed phagocytotic effector cells (50µL/well). Culture overnight.

2. Treat target cells



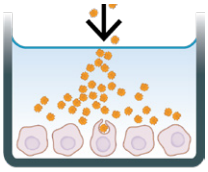
Treat target cells with apoptosis inducing reagent. Incubate for 24 hrs.

3.Label target cells



Label apoptotic target cells with IncuCyte pHrodo Labeling Dye.

4. Add Incucyte® pHrodo labeled target cells



Add IncuCyte pHrodo labeled target cells to treated wells (10 µg/well, 25 µL/well).

Day 0

1. Seed effector cells

1.1 Harvest effector cells and determine cell concentration (e.g., Trypan blue + hemocytometer).

NOTE: Grow enough effector cells in advance to accommodate the different cell densities required to set up the assay (e.g. 1 x 106 total cells for seeding 10,000 effector cells/well).

1.2 Prepare cell seeding stock in culture media to achieve 10-20% confluence after 24 hours.

NOTE: The seeding density will need to be optimized for each cell type used per the preliminary optimization protocol.

1.3 Using a multi-channel pipette, seed effector cells (50 µL per well) into a 96-well microplate.

1.4 Remove bubbles from all wells by gently squeezing a wash bottle (containing 70-100% ethanol with the inner straw removed) to blow vapor over the surface of each well.

1.5 Allow the cells to settle on a level surface for 30 minutes, then incubate overnight at 37 °C with 5% CO2.

2. Treat target cells with apoptotic agent

2.1 Harvest target cells and determine cell concentration (e.g. Trypan blue staining + hemocytometer).

NOTE: Grow enough target cells in advance to accommodate the different cell densities required to set up the assay. We recommend testing target-to-effector cell ratios by holding the effector cell constant, and creating a 7-point, two-fold serial dilution (500,000 cells/well to 7,812 cells/well) of the target cells.

2.2 Centrifuge the cell suspension for 7 minutes at 1000 rpm.

2.3 Aspirate supernatant and resuspend cell pellet in 50 mL fresh growth media at a final cell density of 1x106 cells/mL.

2.4 Add apoptosis inducing compound (e.g., camptothecin or staurosporine) at the optimal concentration identified in the preliminary optimization experiment to the target cells.

2.5 Dispense cells with apoptosis inducing treatment into a T175 flask and incubate for 24 hours at 37 °C with 5% CO_2.

Day 1

3. Label target cells with IncuCyte pHrodo Orange Cell Labeling Kit

3.1 Harvest apoptotic target cells and transfer into a 50 mL centrifuge tube. Centrifuge for 7 minutes at 1000 rpm.

3.2 Aspirate supernatant and resuspend cell pellet with 50 mL IncuCyte® pHrodo® Wash Buffer (component C). Gently mix cells by trituration and determine cell count using a hemocytometer (omit Trypan blue as cells are apoptotic).

3.3 Harvest cells by centrifugation for 7 minutes at 1000 rpm. Aspirate wash buffer and resuspend cell pellet in IncuCyte pHrodo Labeling Buffer (component D) to a density of 1x106 cells/mL.

3.4 Reconstitute IncuCyte pHrodo Orange Cell Labeling Dye (component A) in 100 µL of DMSO (component B) to create a stock concentration of 1 mg/mL.

3.5 Add the solubilized IncuCyte pHrodo Orange Cell Labeling Dye to the target cell suspension at the concentration determined during optimization (refer to Target Cell Labeling Optimization under General Guidelines). Incubate the centrifuge tube containing cells for 1 hour at 37 °C.

3.6. Remove excess pHrodo reagent from cells:

a. Centrifuge the cell:labeling dye suspension at 1000 rpm for 7 minutes. Aspirate off supernatant and resuspend apoptotic target cells in 50 mL of target cell media.

b. Harvest cells by centrifugation for 7 minutes at 1000 rpm. Aspirate supernatant and resuspend apoptotic target cells in effector cell media to yield a cell density of 1 x 10^7 cells/mL.

4. Add target cells to effector cells

4.1 Prepare dilutions of the labeled apoptotic target cells by creating a 7-point, two-fold serial dilution (500,000 cells/well to 7,812 cells/well).

4.2 Immediately following target cell resuspension, remove the effector cell plate from the incubator and add the target cell suspensions to the cell plate (50 µL per well) using a multichannel pipette.

4.3 Remove bubbles and immediately place the microplate in the IncuCyte S3 for Neuroscience (refer to Data Acquisition and Analysis section).

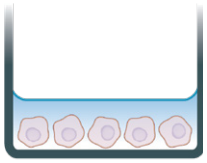
Non-apoptotic phagocytosis protocol (antibody-dependent cellular phagocytosis)

This protocol provides an overview of the phagocytosis of antibody-treated cells by macrophage engulfment, referred to as antibody-dependent cellular phagocytosis (ADCP). It combines the IncuCyte pHrodo Orange Cell Labeling Kit with the IncuCyte S3 for Neuroscience using your choice of target and phagocytic (effector) cells.

Prior to initiating the assay, it is important that your experimental design includes replicate wells of each condition being tested (e.g. labeled target cells alone as well as target:effector co-cultures at each ratio ± antibody, isotype, or vehicle controls).

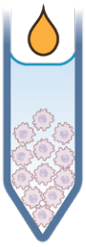
Quick Guide

1. Seed effector cells



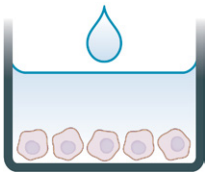
Seed phagocytotic effector cells

2. Treat target cells



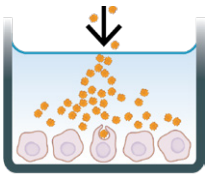
Label target cells with IncuCyte pHrodo Labeling Dye.

3. Treat effector cells



Treat effector cells with compounds prior to phagocytosis (0.5-24 h pretreatment, 25 µL/well).

4. Add Incucyte® pHrodo labeled target cells



Add IncuCyte pHrodo labeled target cells to treated wells (10 µg/well, 25 µL/well).

Day 0

1. Seed effector cells

1.1 Harvest effector cells and determine cell concentration (e.g., Trypan blue + hemocytometer).

NOTE: Grow enough effector cells in advance to accommodate the different cell densities required to set up the assay (e.g. 1 x 106 total cells for seeding 10,000 effector cells/well).

1.2 Prepare cell seeding stock in culture media to achieve 10-20% confluence after 24 hours.

NOTE: The seeding density will need to be optimized for each cell type used per the preliminary optimization protocol.

1.3 Using a multi-channel pipette, seed effector cells (50 µL per well) into a 96-well microplate.

1.4 Remove bubbles from all wells by gently squeezing a wash bottle (containing 70-100% ethanol with the inner straw removed) to blow vapor over the surface of each well.

1.5 Allow the cells to settle on a level surface for 30 minutes, then incubate overnight at 37 °C with 5% CO2.

Day 1

Prepare treatment plate

Using effector cell media, prepare 4x the final desired concentration of antibody, isotype control, and vehicle control in a separate 96-well plate (minimum volume per well should be 50 µL). Set plate aside.

2. Label target cells with IncuCyte pHrodo Orange Cell Labeling Kit

NOTE: Grow enough target cells in advance to accommodate the different cell densities required to set up the assay. We recommend testing target-to-effector cell ratios of 20:1, 10:1 and 5:1.

2.1 Harvest target cells and transfer into a 50 mL centrifuge tube. Centrifuge for 7 minutes at 1000 rpm.

2.2 Aspirate supernatant and resuspend cell pellet with 50 mL IncuCyte pHrodo Wash Buffer (component C). Gently mix cells by trituration and determine cell count using a hemocytometer.

2.3 Harvest cells by centrifugation for 7 minutes at 1000 rpm. Aspirate IncuCyte pHrodo Wash Buffer and resuspend cell pellet in IncuCyte pHrodo Labeling Buffer (component D) to a density of 1x106 cells/mL.

2.4 Reconstitute IncuCyte pHrodo Orange Cell Labeling Dye (component A) in 100 µL of DMSO (component B) to create a stock concentration of 1 mg/mL.

2.5 Add the solubilized IncuCyte pHrodo Orange Cell Labeling Dye to the target cell suspension at the concentration determined during optimization (refer to Target Cell Labeling Optimization under General Guidelines). Incubate the centrifuge tube containing cells for 1 h at 37 °C.

2.6 Remove excess IncuCyte pHrodo Orange Labeling Dye from cells:

a. Centrifuge the cell:labeling dye suspension at 1000 rpm for 7 minutes. Aspirate off supernatant and resuspend apoptotic target cells in 50 mL of target cell media.

b. Harvest cells by centrifugation for 7 minutes at 1000 rpm. Aspirate supernatant and resuspend apoptotic target cells in effector cell media to yield a cell density of 1 x 10^7 cells/mL.

3. Add treatment to effector cells

3.1 During target cell incubation, use a multichannel pipette to transfer 25 µL from the treatment plate containing the antibody, isotype control, and vehicle control to the effector cell plate. Incubate for 15-30 minutes at 37 °C.

4. Add IncuCyte® pHrodo® labeled target cells to effector cells

4.1 Create two-fold serial dilutions of the labeled target cells by creating a 7-point, two-fold serial dilution (500,000 cells/well to 7,812 cells/well).

Target Cell Seeding Density	Cell no. Per Well	Volume Per Well
1x10^7 cells/mL	250,000	25 µL
5x10^6 cells/mL	125,000	25 µL
2.5x10^6 cells/mL	62,500	25 µL

4.2 Immediately following target cell resuspension, remove the effector cell plate from the incubator and add the target cell suspensions to the cell plate (25 µL per well) using a multichannel pipette.

4.3 Remove bubbles and immediately place plate in the IncuCyte S3 for Neuroscience (refer to Data Acquisition and Analysis section).

Data acquisition and analysis

1. Acquisition

In the IncuCyte® software, schedule 24 hour repeat scanning for every 15-30 minutes, for up to 48 hours.

a. Objective: Ensure 10x or 20x objective is installed
b. Channel Selection: Select "Phase" and "Orange" (800 ms acquisition time)
c. Scan Mode: Select "Standard" scan type and Scan Pattern of 1 image per well if using 10x, and 4 images per well if using 20x.

2. Analysis

To generate the metrics, the user must create a Processing Definition suited to the cell type, assay conditions and magnification selected, then apply the Processing Definition to the data set as an Analysis Job.

2.1 Select an image from a well containing a high concentration of treated target cells (e.g., 250,000 cells/ well) but no effector cells during the peak assay response (e.g., 24 hours). Under Image Channels, expand the orange drop down and deselect the AutoScale option. Adjust the scale until no orange is observed in the image. Note the minimum and maximum values. Add the image to a New Image Collection by selecting "Create or Add to Image Collection."

2.2 Select an image from a well containing both effector cells and the same concentration of treated target cells selected in step 1 (e.g., 250,000 cell/well) during the peak assay response (e.g., 24 hours). Save the image to the Image Collection.

2.3 With this image collection, create a new Processing Definition:

a. Deselect the AutoScale option and set the minimum and maximum values as identified in step 1.
b. To exclude background fluorescence from the mask, use the background subtraction feature in the Parameters drop-down menu. The feature "Top-Hat" will subtract local background from brightly fluorescent objects within a given radius; this is a useful tool for analyzing objects which change in fluorescence intensity over time, as in this case.
c. The radius chosen should reflect the size of the cells; 20 µm is often a useful starting point.
d. The threshold chosen will ensure that objects below a fluorescence threshold will not be masked. Choose a threshold in which orange objects are masked in the image with effector cells but not masked in the image without effector cells.

2.4 Recommended Metrics for Phagocytosis of Cells: As effector cells engulf target cells, the area of fluorescence and intensity inside the effector cells increases. This can be reported in two ways: as an increase in fluorescence area ("Total Object Area") or increase in intensity, integrated over the area of detectable fluorescence ("Total Integrated Intensity"). Analyze using the "Total Orange Object Area (μm^2/image)" or "Total Orange Object Integrated Intensity (RCU x μm^2/image)" metrics.

Notes

Essen BioScience, Inc., a Sartorius Company, advances live-cell research world wide with the IncuCyte® Live-Cell Analysis System.

The IncuCyte system and suite of assays automatically collect and analyze images and data in real time—while cells remain undisturbed inside a standard incubator.

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