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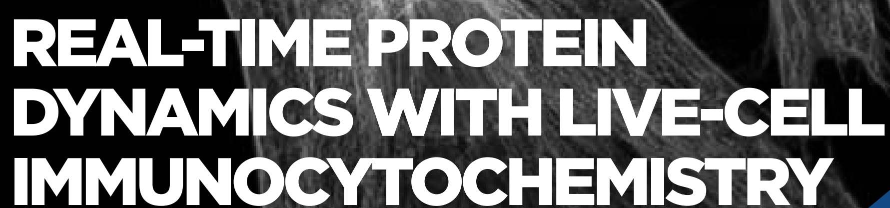


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# Expand immunological insights with real-time live-cell analysis

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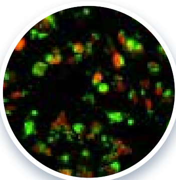

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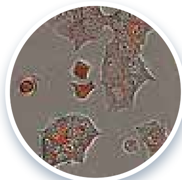

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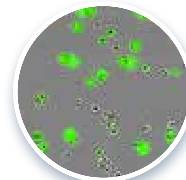

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Changes in protein-expression levels and locations are integral to cellular functions. Immunocytochemistry delivers excellent spatial resolution, and when coupled with real-time live-cell imaging techniques, also provides new insights into protein dynamics, allowing researchers to visualize a more complete picture of protein expression and protein-protein interactions.

# Immunocytochemistry: Essential and Evolving

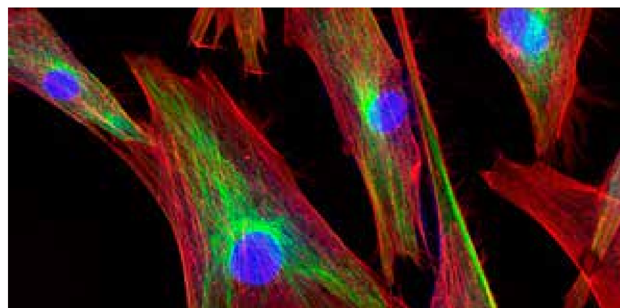
***"The ability to visualize cellular proteins [...] is instrumental to our capacity to identify cellular phenotypes and understand the mechanisms that govern cell behavior."***

The ability to visualize cellular proteins gives us the ability to obtain information on protein presence, location, and activity, and it is therefore instrumental to our capacity to identify cellular phenotypes and understand the mechanisms that govern cell behavior. Immunocytochemistry (ICC) uses selective labels to visualize proteins of interest in individual cells.[1] While radioimmunoassay antibodies were popular during ICC's infancy, today, standard ICC is most commonly performed using fluorophore-bound antibodies and fluorescence microscopy. Still, enzyme and particulate labels are still used in tandem with light and electron microscopy, respectively, and quantum dots present an interesting alternative to conventional antibodies.[1-3]

Conventional ICC is extremely versatile, as any cellular molecule can be theoretically detected if the appropriate label is available.[1] It also provides excellent spatial resolution, allowing researchers to not only delineate signals in close proximity to one another, but also to associate signal origin locations with cellular structures and organelles. These features have made ICC a *go-to* technique in many different fields, with scientists using ICC extensively to examine intracellular protein localization and protein-protein interactions/co-localization allowing them to associate protein profiles with cellular phenotypes and/or behaviors..

## Frozen in Time: The Limitations of Single Snapshots

Conventional ICC methodologies can only capture a single time point per cell.[4] Having only a single snapshot makes it difficult to place the observed phenomena into the context of a greater mechanism or series of mechanisms, and also hinders the decisive determination of the trigger for a given response. While increased sampling can mitigate this weakness, conventional ICC protocols require cellular fixation for structural and molecular preservation throughout the process. In order to obtain data from a different timepoint, a new sample must be prepared.[4] This inability to obtain multiple different images from the same cell increases data variability, particularly when studying cell populations (*e.g.,* immune cells, cancer cells) known for their plasticity and intra-population heterogeneity. There is no guarantee that multiple different cells will generate the same responses, despite the researchers' best efforts.



## From Static to Kinetic: Live-Cell Immunocytochemistry

Live-cell analysis has been used extensively to study cellular behavior, mostly in terms of motility, morphology, and cell-cell interactions. However, since protein labeling has historically required cellular fixation and/or cell permeabilization, live-cell analysis has not been a popular option for studying protein expression dynamics. This is now changing, as technological advancements are facilitating modified ICC protocols that embrace live-cell analysis, adding a much valued temporal dimension to ICC-obtained data.

Live-cell ICC builds upon traditional ICC by expanding on the 'before and after' dichotomy. Instead of comparing two isolated timepoints in two separate cells, live-cell ICC imaging can be conducted in real time, allowing researchers not only to determine whether the expression levels of a given protein have changed, but also to identify the precise stimuli (*e.g.,* a cell-cell interaction, an environmental change, or a protein-protein interaction) involved in modifying expression levels and the temporal kinetics of these changes. Real-time ICC also augments existing ICC protocols by permitting repeated sampling of the same cell(s) over time, a feature that offers a particular advantage when studying the aforementioned highly plastic cell populations. Finally, live-cell ICC allows the real-time visualization of cellular behaviors in physiologically relevant environments, allowing researchers to observe interactions between the cells in culture and the culture media, extracellular matrix components, and other cells. In this way, data obtained using live-cell ICC can be more representative of cellular function in vivo.

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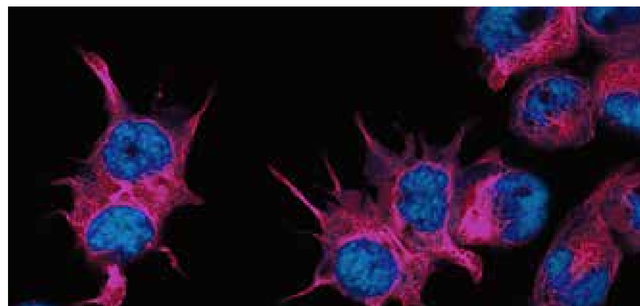
# Dynamic Data: Adapting Immunocytochemistry for Live-Cell Analysis



***"...a more in-depth and complete understanding of cellular function requires the ability to capture cellular processes as they happen."***

Static snapshots of cells have provided scientists with vast amounts of valuable information over the years, but a more in-depth and complete understanding of cellular function requires the ability to monitor cellular processes as they happen.[1] With this goal in mind, live-cell analysis has proven useful for examining cellular mechanisms such as wound healing, chemotaxis/migration, and tissue/vascular formation.

Adapting immunocytochemistry (ICC) for live-cell analysis has been somewhat more difficult.[2] Standard ICC protocols can involve manipulating cellular integrity (*e.g.*, permeabilization, antigen retrieval) for probes to access and label antigens.[3] This is not optimal for live-cell analysis, where cellular integrity needs to be preserved over extended periods of time in order to avoid triggering undesired stress-induced physiological responses. These prohibitions have left some researchers with the mistaken impression that live-cell ICC is only capable of analyzing cell-surface proteins. However, there are several options for researchers looking to visualize intracellular proteins. While membrane-permeable fluorophores are popular, there are other options. The cell's own endocytic pathway offers a direct route to intracellular labeling without altering cellular function; endocytic labeling has even been used to differentiate internalized vs. surface-expressed populations of the same protein.[4]

## Planning for Prosperity: Optimizing Live-Cell ICC

Whether investigating intracellular or cell-surface proteins, researchers need to be aware of certain factors and take certain precautions in order to optimize the quality of their data. This, of course, applies to every experiment, but is perhaps more important for live-cell ICC protocols because a single misstep can ruin many hours, or even days, of image/video capture.

To start, environmental stability needs to be maintained as much as possible. Temperature, humidity, and media composition can all affect cellular behavior, phenotype, and activity, and unplanned fluctuations in these variables can ruin an experimental run. Worse, if undetected, they can damage experimental integrity and reproducibility. Environmental stability is also important from an imaging perspective, as physical perturbations can bring specimens out of focus mid-experiment. From a stability standpoint, long-term dynamic studies present greater challenges than short-term ones, as they potentially face additional challenges such as the depletion of media nutrients and labeling agent potency. Reagents too need to be carefully examined, as some may contain cytotoxic compounds commonly used as preservatives such as sodium azide.

Second, the right labels with the right binding sites need to be selected. A binding site located on an intracellular domain of a surface protein could lead to diminished or no signal intensity unless the label was able to enter the cell. Maximizing label binding, and therefore signal intensity, is vital to limiting exposure time and minimizing harmful photobleaching.[1] Additionally, antibody signal longevity can pose a problem for extended longitudinal imaging. This can be overcome by a novel new method where antibodies specific for the protein of interest are further tagged with fluorescently-labeled Fc-targeting Fab fragments.

Third, the signal wavelengths involved in the experiment need to be carefully planned, especially if multiple probes are being used. Autofluorescence must be taken into consideration during this planning process. Organelles such as mitochondria and lysosomes naturally produce autofluorescence, as do certain molecules endogenously present in cells such as NAD(P)H.[1] Autofluorescence can result in false positives in the absence of probe-based signal or false negatives by eliminating the contrast between probe signals and the background.

Finally, incorporating the *Z*-plane for 4-dimensional (*x*, *y*, *z*, and time) imaging will allow researchers to distinguish between true signal co-localization and two physically distinct signals that happen to overlap when viewed from above.[1] This will allow them to determine whether two proteins are truly interacting with one another.

## Harnessing Time and Space: Using ICC for Protein Dynamics Analysis

Adapting ICC to real-time live cell analysis allows researchers to add the temporal dimension to their data, giving them the ability to not only discern *where*, but also *when* and *how*. Live-cell analysis brings with it additional considerations and potential challenges, but offers great potential when it comes to correlating protein dynamics with cellular morphologies, phenotypes, and behaviors.


**References:**

1. D.J. Stephens and V.J. Allan, "Light microscopy techniques for live cell imaging," *Science* 300(5616):82-86, 2003.
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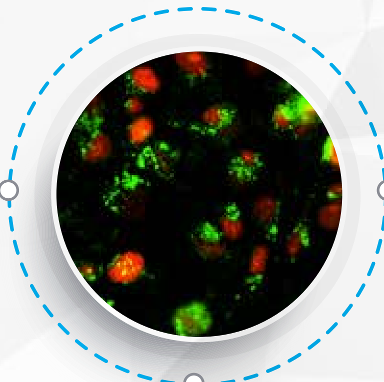
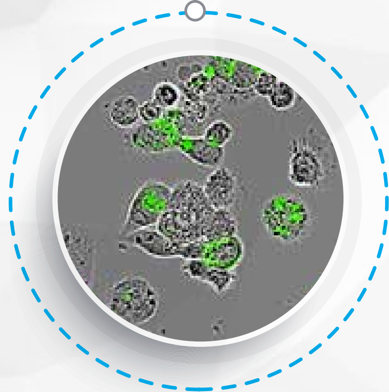
# EXPANDING YOUR PROTEIN ANALYSIS TOOLKIT:
## CONNECTING THE DOTS WITH REAL-TIME LIVE-CELL IMAGING

Immunocytochemistry is a fundamental tool used to identify specific proteins and their intracellular localization patterns. Learn how to expand this spatial data to reveal dynamic changes in protein expression over time and connect these temporal changes to cellular morphology and function.

### PROTEIN IDENTIFICATION

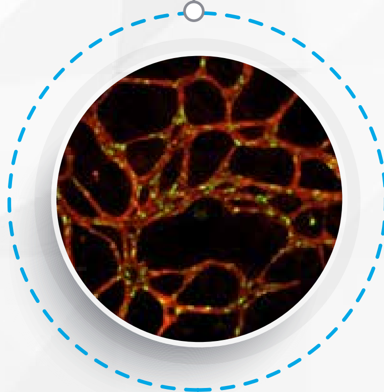
Track protein expression and distribution over time for new insights into intermediate mechanisms, transient expression patterns, and translocations





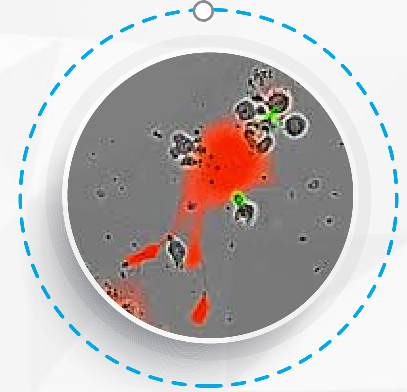
### MORPHOLOGY

Observe changes in cell morphology linked to dynamic changes in protein expression as they happen



### FUNCTION

Visualize and link changes in surface protein expression profile with cellular functions

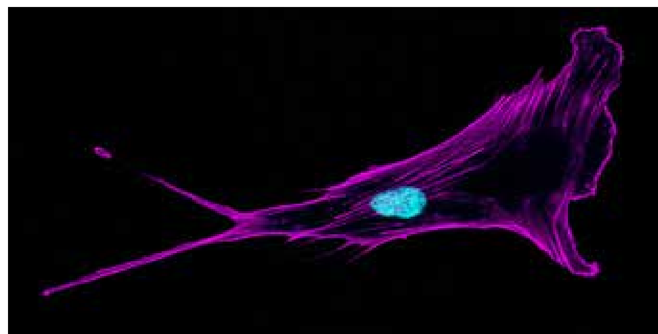


### CELL-CELL INTERACTIONS

Observe and quantify cell-cell interactions over time in complex co-culture models, revealing the intricacies of interplay between cells

# Applied Analysis: Live-Cell Immunocytochemistry for Disease Research

***"…live-cell analysis is capable of delineating specific protein expression profiles from individual cells, offering a window into […] cellular heterogeneity…"***



While it can be tempting to treat all cells of a given type in the same manner, the reality is that human cells are extremely heterogeneous and plastic. These properties allow cells to adapt to varying environmental conditions and respond appropriately to stimuli and stress. Understanding cellular heterogeneity and plasticity is critical for disease research, and live-cell immunocytochemistry (ICC) gives researchers a new window into the mechanisms and stimuli governing both the cellular changes instrumental to innate physiological defense mechanisms and pathogenic processes.

## Visualizing Cellular Individuality: Real-Time Live-Cell ICC

Cellular heterogeneity and plasticity can manifest in a number of ways, ranging from changes in intracellular mechanisms and protein expression profiles to differentiation into different cell types.[1] Increased data sampling is important for understanding the temporal and spatial dynamics governing the constantly shifting cellular properties and mechanisms responsible for health and disease. However, many common cell imaging techniques are unable to provide this depth of information – capturing data only at researcher-defined individual timepoints. These techniques are therefore unable to definitively link observed phenomena with cellular behaviors and/or activities.

Live-cell ICC monitors cellular protein expression in real-time, allowing researchers to visualize and observe protein regulation from start to finish. Instead of a single, final protein expression profile used to determine a phenotype, live-cell ICC analysis can provide information on mechanism triggers, any potential intermediate steps (including transient protein level fluctuations) and capture the possible role of environmental elements including other cells.

Single-timepoint imaging forces scientists to aggregate data from multiple cells to paint a composite picture, live-cell analysis is capable of delineating specific protein expression profiles over time from individual cells, offering a window into the cellular heterogeneity that is a defining hallmark of immune, stem, and cancer cells.

## Closer to Home: Mimicking In Vivo Environments with Live-Cell ICC

The ability to observe cellular protein dynamics in environments which more closely mimic in vivo conditions is important, as cellular protein responses are chiefly dictated by external cues and stimuli. These can originate from changes in the extracellular environment (*e.g.*, pH, oxygenation, or ionic and electrophysiological properties), direct physical contact with other cells or non-cellular elements (*e.g.*, extracellular matrix components), and/or receptor activation through a variety of potential mechanisms (*e.g.*, ligands, neurotransmitters, receptor-receptor interactions, or cyto/chemokines).[1] The more closely an experimental set-up reflects in vivo conditions, the more applicable the data obtained is for disease research.

Live-cell ICC can be performed on cell lines or extracted primary cells in standard 2-D culture, but it really shines when used to visualize cells in 3-D interfaces, allowing researchers to view cellular behavior in real-time in a setting that more closely represents their native in vivo environments. For example, researchers have combined live-cell analysis with 3-D co-culture models to visualize proteolysis, identifying not only which proteases were implicated in this critical mechanism for cancer progression, but also when and where they were active.[2] Live-cell ICC analysis has also been used to examine cell-cycle transcription factor regulation,[3] cancer stem-cell migration,[4] and embryonic stem-cell differentiation.[5]

Cellular protein expression changes play a foundational role in modulating cellular function, morphology, and cell-cell interactions, thereby mediating the balance between homeostasis and pathology. This is perhaps most clear in immuno-oncology, where cancers may start as individual cellular mutations, but tumor formation and subsequent metastasis is heavily dependent on environmental conditions (the tumor microenvironment; TME) that manipulate cellular protein expression profiles for the abrogation and circumvention of the body's natural anti-cancer mechanisms.[1] Live-cell ICC provides researchers with informational depth, allowing them to move beyond a one-size-fits-all approach and uncover the temporal protein dynamics underlying cellular function, behavior, and interactions in inflammatory and infectious disease, cell therapeutics, and tissue regeneration/engineering.

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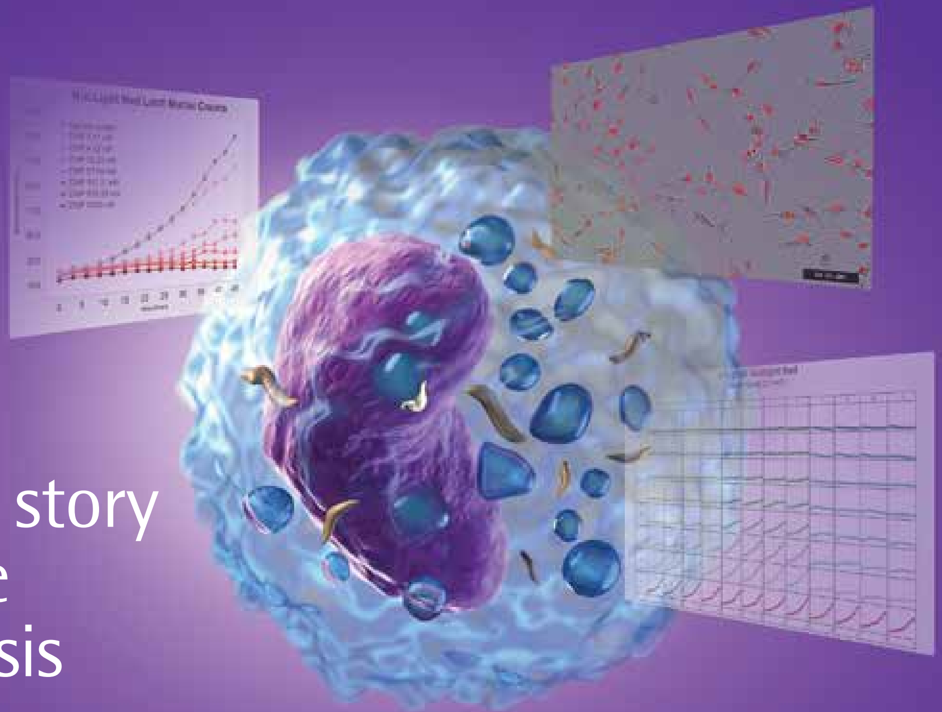

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