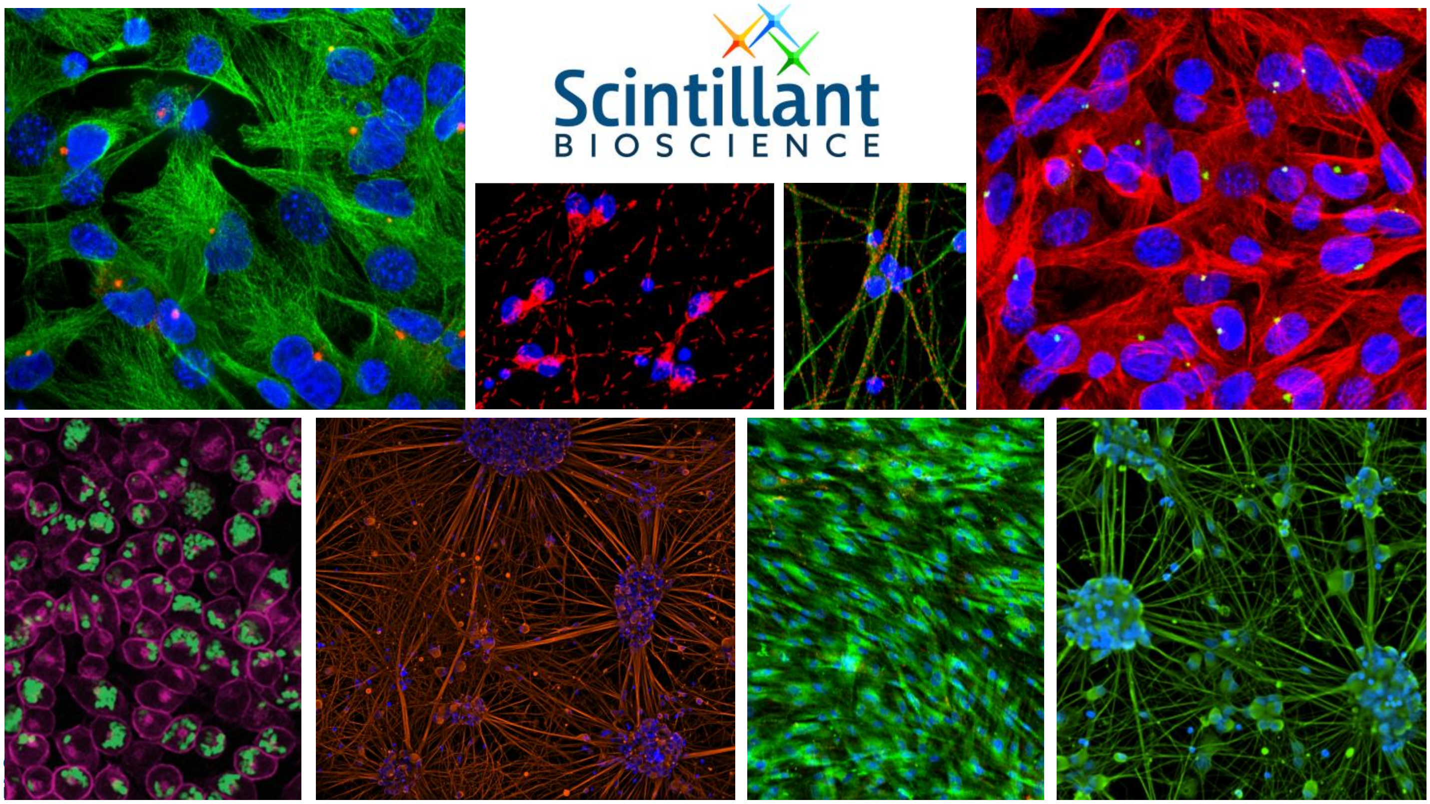
Scintillant
BIOSCIENCE

Fluorescence Microscopy Basics for Cell-Based Imaging Assays

Introduction

Step into a world where the hidden intricacies of cells are brought to light by the awe-inspiring power of fluorescence microscopy—a technology that has shaped our understanding of cell biology and transformed the landscape of drug discovery. Within this white paper, we embark on a journey to unravel the foundational principles of this technology, followed by an exploration of its practical applications. Like radiant beacons, fluorescent probes selectively latch onto cellular structures, granting us unprecedented access to the vibrant tapestry of living cells. Join us as we unveil the marvels of fluorescence microscopy and embark on an immersive exploration of its extraordinary contributions to cell-based imaging assays, propelling forward both fundamental biological research and the development of groundbreaking therapeutics.



All fluorescence images of cells shown in this white paper are from projects Scintillant Bioscience conducted for clients.

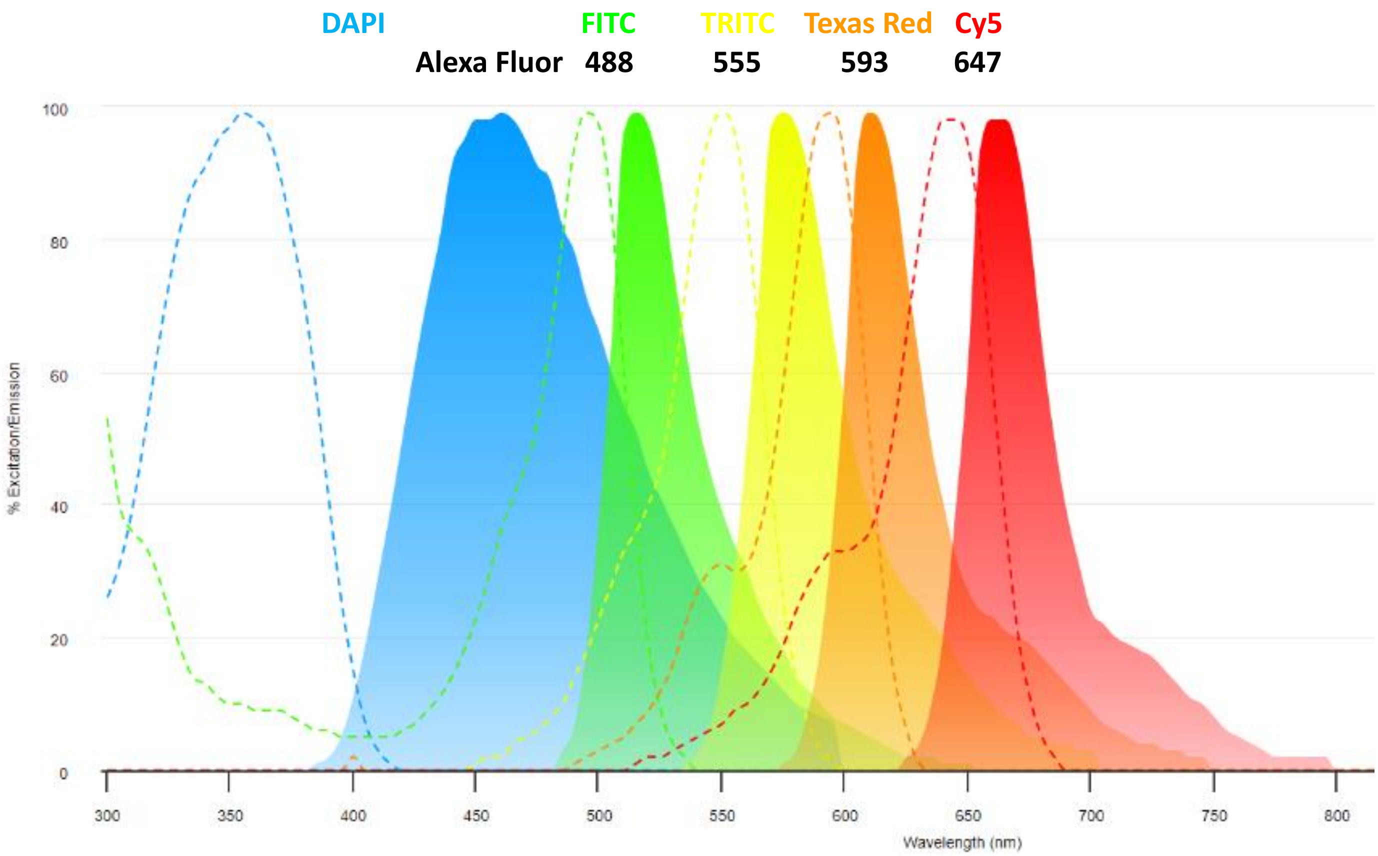
Reflected Light vs. Fluorescence

When it comes to understanding the behavior of light and its interaction with objects, two intriguing phenomena stand out: reflected light and fluorescence. These phenomena play crucial roles in shaping our perception of color and enable us to delve into the fascinating world of fluorescence microscopy. So, what sets them apart?

Let's start with reflected light. Picture a ray of light striking the surface of an object. Rather than being absorbed, certain wavelengths of this light bounce off the object, making their way to our eyes. It is this reflected light that bestows color upon the object, allowing us to appreciate its visual appearance. In essence, the colors we perceive are a result of the selective reflection of specific wavelengths.

On the other hand, fluorescence is a captivating phenomenon that occurs when a molecule absorbs light at a particular wavelength and subsequently emits light at a longer wavelength. This process involves a shift in the energy of the absorbed light, transitioning from higher energy (shorter wavelength) to lower energy (longer wavelength) in a phenomenon known as the Stokes shift. This unique property distinguishes fluorescence from reflected light.

Practical applications of fluorescence are enabled by the ability to selectively detect the emission of light from molecules called fluorophores or fluorochromes. By employing well-designed optical filters, scientists can separate the excitation light (the light used to trigger fluorescence) from the emitted fluorescence light, allowing them to exclusively capture the fluorescence emission from a sample of interest. This selective detection is made possible by leveraging the distinct Stokes shift exhibited by fluorescent molecules.



The accompanying figure above showcases the excitation and emission spectra of several commonly used fluorophores. These fluorophores, including DAPI (blue), FITC (green), TRITC (yellow), Texas Red (orange), and Cy5 (red), have been widely employed in fluorescence microscopy. Moreover, modern and highly photostable alternatives like the Alexa Fluor dyes from ThermoFisher Scientific have gained prominence in current fluorescence microscopy applications. We include the names of a few of the Alexa Fluor dyes that are comparable to traditional fluorophores in the figure (each named for its maximum excitation wavelength).

In the figure above, the excitation spectrum for each fluorophore is represented by a dotted line, indicating the range of wavelengths that can excite the fluorophore, with the highest point on the line indicating the wavelength that elicits the maximum excitation. Meanwhile, the emission spectrum for each fluorophore is displayed as a solid shape, with its peak indicating the wavelength at which the fluorescence emission reaches its maximum intensity.

We next will address the essential elements of fluorescence microscopy, where the true potential of cellular imaging reveals itself. Expect to be fascinated by the remarkable applications that have emerged from this transformative technology, including the precision with which cellular structures and molecules are visualized in real-time, allowing us to unlock the secrets of their interactions and responses to various stimuli. We will provide you with expert insights, illuminating images and practical tips that will empower you to harness the full potential of fluorescence microscopy.

The Basics of Fluorescence Microscopy

Fluorescence microscopy utilizes intense light sources like lasers, arc lamps, or LED lights to excite fluorescent molecules within cells or tissue samples. These molecules absorb the energy and emit light at longer wavelengths, generating a fluorescent signal that can be captured by a detector. This requires specialized optical components, including a filter cube that selectively transmits the excitation and emission wavelengths, a dichroic mirror that separates the excitation and emission light, and a detector, such as a charge-coupled device (CCD) or scientific CMOS (sCMOS) camera, that captures the emitted light, as shown in the figure below.

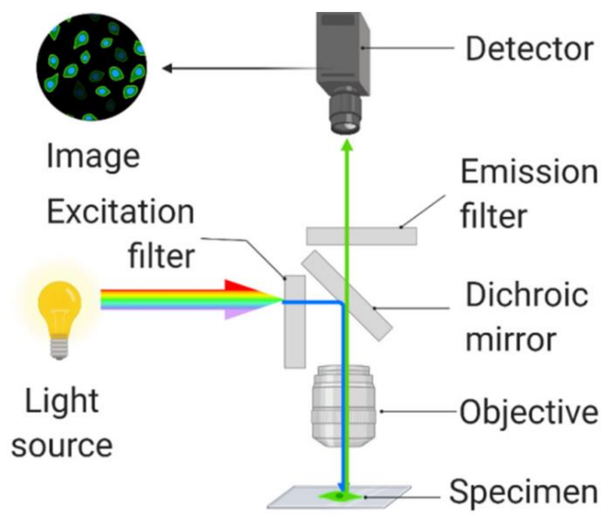


Image from: https://www.e3sconferences.org/articles/e3sconf/ pdf/2021/66/e3sconf_icgec2021_01031.pdf

While fluorescent molecules imaged within cells can be naturally occurring with fluorescent properties, they are typically introduced by researchers as fluorescent probes. Some probes passively enter cells, like lipophilic dyes, while others require fixation and permeabilization, such as antibodies, to reach their intracellular targets.

Different types of fluorescent probes share a common trait: a fluorophore or fluorochrome capable of absorbing specific wavelengths of light and emitting light at longer wavelengths.

Fluorescent dyes may exhibit unique properties, like DNA binding affinity (e.g., DAPI, Hoechst), the ability to penetrate highly negatively charged mitochondria, as seen in cationic lipophilic MitoTracker dyes, or increased fluorescence upon oxidation, as just a few examples.

Fluorophore-conjugated antibodies serve as versatile probes, leveraging their high binding affinity and specificity for single molecular targets, typically proteins. By attaching a fluorophore to an antibody, specific subcellular molecules or structures can be fluorescently labeled. Typically this involves first binding an unlabeled primary antibody to its protein target and subsequently utilizing a fluorophore-conjugated secondary antibody to bind the primary antibody. This approach offers flexibility in selecting fluorophores for labeling cellular structures without compromising the binding ability of the primary antibody.

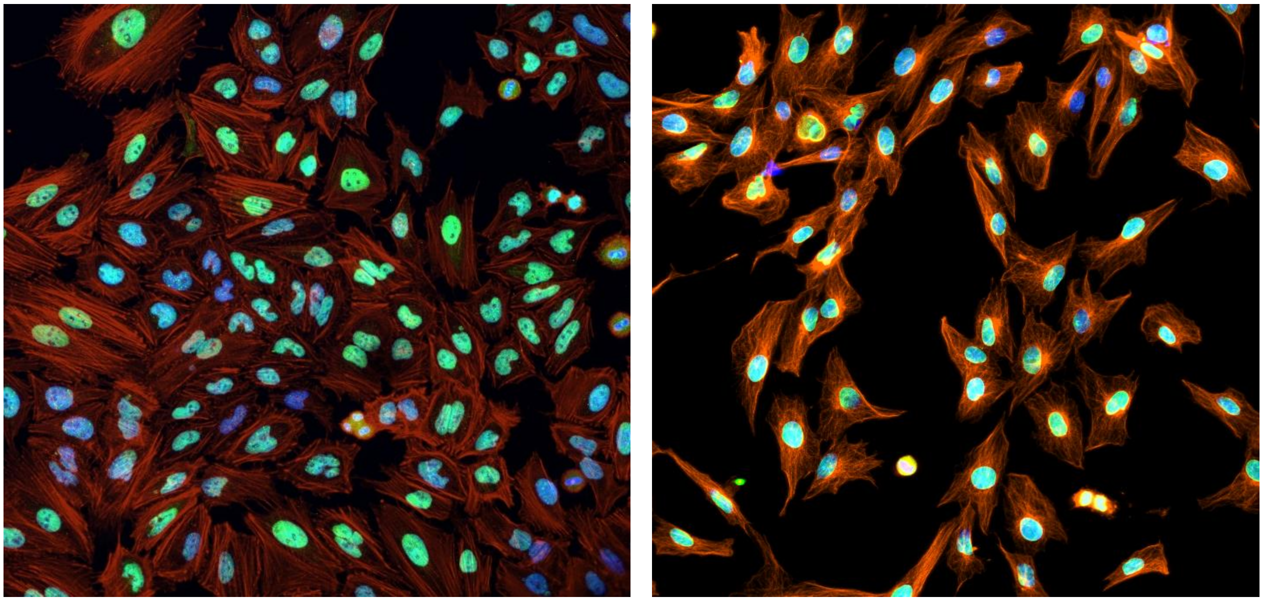
Another category of fluorescent probes includes genetically encoded fluorescent proteins that can be fused to other proteins through gene expression. These include green fluorescent protein (GFP), first isolated from the jellyfish *Aequorea Victoria*, followed by other fluorescent proteins discovered in nature and/or engineered with fluorescence emission across the color spectrum.

Applications of Fluorescence Microscopy in Cell-Based Imaging Assays

Fluorescence microscopy has transcended boundaries, finding its place in diverse domains, from the intricate realms of fundamental biological research to the frontiers of drug discovery and even clinical diagnostics. Now, let us delve into some of the applications that have harnessed the power of fluorescence microscopy for cell-based imaging assays.

Revealing the Intricacies: Visualizing Cellular Structures and Molecules

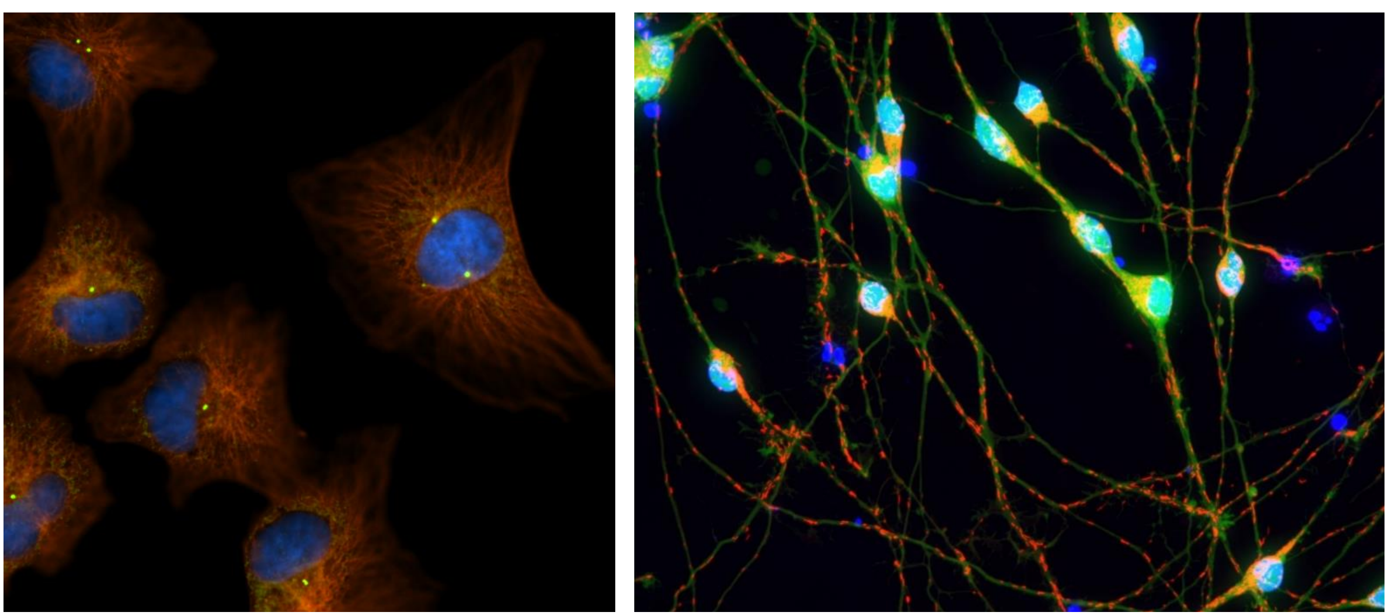
One of the most common applications of fluorescence microscopy is the visualization of cellular structures. For example, the proteins comprising the cytoskeleton give the cell its overall shape, as shown below:



Left Image: Cervical cancer cells: Blue, Hoechst 33342 (nuclei); Green, YAP1; Red, Actin Cytoskeleton.
Right Image: Glioblastoma cells: Blue, Hoechst 33342 (nuclei); Green, Lamin B1; Red, Microtubule Cytoskeleton

Fluorescence microscopy is also commonly used to visualize organelles. By utilizing fluorescent dyes, researchers can effectively label specific organelles, such as nuclei (as depicted in most cell images in this paper), mitochondria (as seen in the image below on the right), endoplasmic reticulum, and lysosomes. This labeling approach enables the examination of organelle morphology, distribution, and functions within living cells. Moreover, small-molecule fluorescent probes offer the ability to label cellular molecules like RNA and proteins, facilitating the investigation of their abundance, localization, and functions in cells.

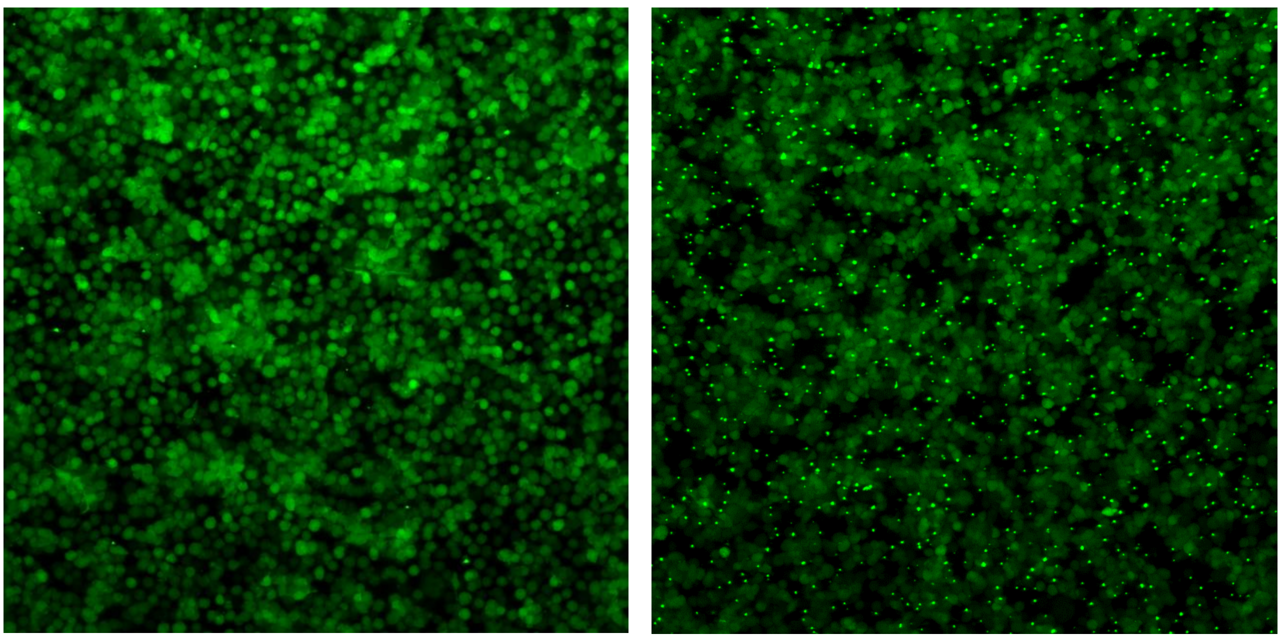
To visualize specific proteins within distinct cellular structures, fluorophore-conjugated antibodies are employed. This technique is referred to as immunostaining or immunocytochemistry or immunofluorescence microscopy. The microtubule staining demonstrated above was achieved using an antibody stain, while actin was stained using fluorophore-conjugated phalloidin—a toxin naturally occurring in the "death cap" mushroom. The left-hand image below showcases green dots representing centrosomes, which were identified through antibody binding to γ-tubulin, a protein component of centrosomes that serve as microtubule nucleating centers for cell division.



Left Image: Breast cancer cells: Blue, Nuclei; Red, Microtubules, Green, Centrosomes.
Right Image: Human iPSC-derived neurons: Blue, Nuclei; Red, Mitochondria; Green, Cytosol.

Fluorescent proteins play a pivotal role in fluorescence microscopy, offering a versatile tool for scientific investigations. Through genetic encoding, fluorescent proteins can be utilized to label specific proteins within cells using heterologous expression systems like transient or stable transfection. An excellent example is the fusion of green fluorescent protein (GFP) with a protein of interest, generating a fluorescent fusion protein that enables real-time visualization within live cells. In the images presented below, GFP is genetically encoded as a fusion protein with ASC, a vital component of the NLRP3 inflammasome.

In the left-hand image, the expression of the ASC-GFP fusion protein manifests as a diffuse presence throughout the cytosol after treating the cells with lipopolysaccharide (LPS), which induces expression of ASC. However, in the right-hand image, a fascinating phenomenon unfolds. Upon stimulation of inflammasome activation, triggered by nigericin, the ASC-GFP fusion protein undergoes an intriguing transformation. It aggregates into a singular speck per cell, signifying the assembly of inflammasome components and the ensuing activation process. This activation event leads to the secretion of the inflammatory cytokine IL-1β. Notably, ASC speck formation can be assessed as an assay endpoint or continuously monitored via time-lapse imaging, allowing for dynamic observations of the inflammasome assembly and its subsequent effects.



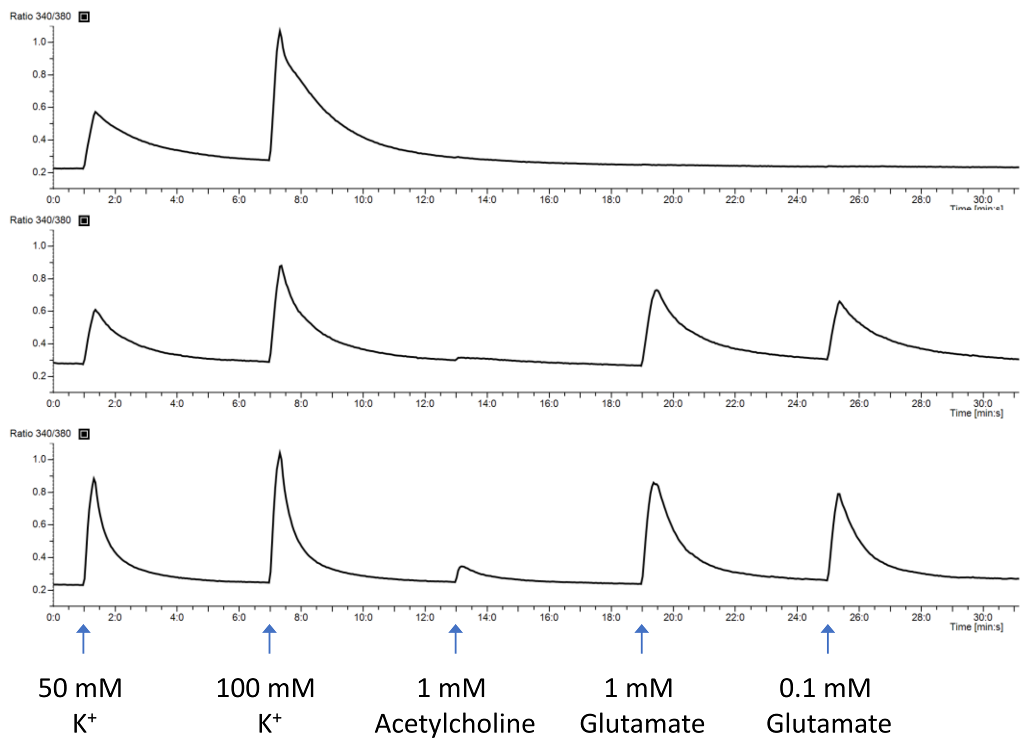
THP-1 monocyte cell line engineered to express ASC-GFP fusion protein.
Left Image: Diffuse expression of ASC-GFP after priming with LPS to induce expression of ASC-GFP.
Right Image: ASC-GFP speck formation after triggering inflammasome assembly and activation with nigericin.

Unraveling the Enigmatic: Illuminating Cellular Processes, Dynamics, and Signaling Pathways

Fluorescence microscopy is a powerful tool for quantifying cellular processes and unraveling the enigmatic functions that drive the living machinery. By peering into the captivating realm of live-cell time-lapse imaging, we gain the ability to witness the ever-changing landscape of cellular structures. Microtubules and actin filaments engage in graceful choreographies, constructing the tapestry of cytoskeletal structures. Meanwhile, organelles like mitochondria and lysosomes embark on mysterious journeys, their movements mapped before our eyes. This approach also unveils the transformative story of cellular morphology and dynamics, shedding light on cell migration, adhesion, division, and apoptosis. Moreover, it unravels the intricate interactions between cells themselves and their extracellular matrices, unmasking the secret dialogues that shape cellular destinies.

The wonders of fluorescence microscopy extend further with innovative techniques like fluorescence resonance energy transfer (FRET) and fluorescence lifetime imaging microscopy (FLIM). These methodologies enable us to probe the intricate realm of protein-protein interactions and capture real-time conformational changes, painting a vivid portrait of protein activity and signaling pathways. Like skilled detectives, we employ fluorescent probes designed to monitor the subtle shifts that occur in pathological processes, tracking changes in pH levels and reactive oxygen species, unveiling the hidden clues that may illuminate the path to understanding.

Within this luminescent landscape, fluorescent probes act as beacons, allowing us to measure the ebb and flow of intracellular signaling molecules. Calcium, cyclic AMP, and protein kinase activity can be continuously monitored, unveiling the symphony of signaling events in real-time. A valuable tool in our arsenal is the calcium indicator Fura-2, which elucidates our understanding of changes in intracellular calcium levels, a critical player in a multitude of signaling pathways. Through this technique, we can interrogate the very essence of numerous signaling cascades, uncovering the secrets that govern cellular behavior, as illustrated in the following figure depicting calcium-imaging traces of individual neurons, i.e., changes in intracellular calcium over time.



Calcium-imaging traces from human iPSC-derived neurons. Y-axis is the ratio of fluorescence emission of Fura-2 dye at 510 nM when excited alternatively with 340 nm and 380 nm light. X-axis is time in minutes. Each trace represents the response of a single neuron to various stimuli, as shown beneath the X-axis. Responses of hundreds of individual neurons can be monitored simultaneously.

Unlocking Therapeutic Potential: Fluorescence Microscopy in Drug Discovery and Development

Fluorescence microscopy has emerged as a powerful tool in drug discovery, particularly in the pharmaceutical industry's pursuit of novel compounds through cell-based phenotypic screening. This screening approach, commonly referred to as high-content screening (HCS) or high-content imaging (HCI), combines automated fluorescence microscopy with advanced image-analysis techniques in high-throughput formats such as 384- or 1536-well plates. By employing this technique, researchers can rapidly screen large compound libraries and identify substances that can restore diseased cells to a normal, healthy state or phenotype. Through the precise observation of cellular processes using fluorescence microscopy, HCS enables the exploration of drug effects at a microscopic level, unveiling previously unknown disease-associated phenotypes.

To manage the vast amount of image data generated by HCS, artificial intelligence (AI) algorithms are often employed for efficient image analysis. These algorithms utilize advanced machine learning techniques to identify and quantify various disease phenotypes, often measuring thousands of imaging parameters per cell or image. This enables a comprehensive evaluation of disease phenotypes and the efficacy of drug candidates in rescuing these phenotypes.

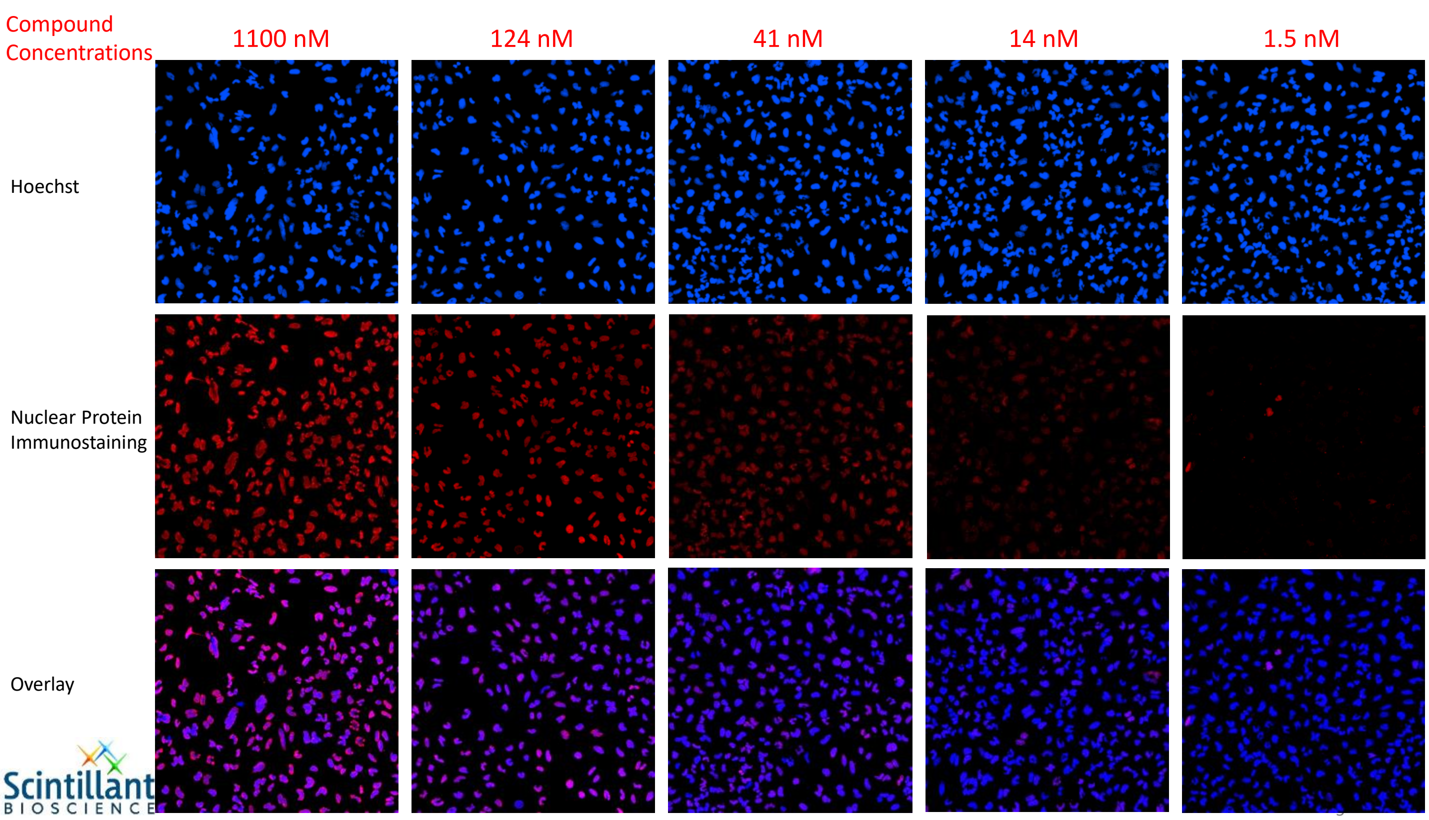
One popular variation of HCS is known as Cell Painting, where six fluorescent dyes are imaged in five fluorescent channels to label eight organelles or subcellular components. In the context of HCS, the analysis of these and other high-content images is commonly referred to as high-content analysis (HCA), reflecting the comprehensive examination of various cellular and subcellular features.

By leveraging fluorescence microscopy, automated image analysis, and AI-driven algorithms, HCS and HCA have revolutionized the drug discovery process. These techniques provide researchers with a deeper understanding of disease mechanisms, uncovering potential targets and facilitating the development of innovative therapeutics. With their capacity to reveal intricate cellular details and identify promising drug candidates, fluorescence microscopy and high-content analysis continue to be indispensable tools in modern pharmaceutical research.

In addition to the cell-based phenotypic screening described above, automated fluorescence microscopy is also used for target-based screening of potential new drug candidates. Such high-throughput screening assays capitalize on the use of fluorescently labeled cells to identify compounds that modulate specific targets or pathways.

As mentioned earlier, the employment of fluorescent calcium indicators enables researchers to screen for compounds that influence calcium signaling through time-lapse imaging. Similarly, the use of fluorescently labeled reporter cells, such as THP-1 monocytes expressing ASC-GFP showcased earlier, facilitates the screening of compounds that affect specific gene expression or protein dynamics. These are just two of the many ways that automated fluorescence microscopy can be used for target-based or pathway-based drug discovery.

Illustrating another exemplary scenario, the following figure demonstrates the effect of a drug that increases the abundance of a nuclear protein (kept confidential on behalf of our client). To support a client in their lead-optimization program, we conducted this assay in 384-well format, simultaneously testing 30-50 compounds, each in 10-point dose-response. We ran this assay iteratively across multiple medicinal-chemistry cycles, which helped the client to find progressively more potent compounds.



Fluorescence microscopy allows researchers to gain valuable insights into drug targets and their interactions. By introducing fluorescent labels into proteins, cells, or tissues, it becomes possible to visualize and analyze the localization of drug targets. Among the various techniques available, fluorescence recovery after photobleaching (FRAP) is useful for studying the dynamics of drug targets in the plasma membrane. By selectively bleaching a region of fluorescence and observing the recovery over time, researchers can examine the movement and turnover of drug targets within the membrane. Another technique, fluorescence correlation spectroscopy (FCS), provides a means to track the binding and distribution of drugs within cells. FCS enables the measurement of diffusion and binding kinetics, offering insights into how drugs interact with their targets in living cells. These approaches expand the possibilities for investigating drug mechanisms and provide a deeper understanding of the dynamics and interactions of drugs and their targets within the cellular context.

Another valuable application of fluorescence microscopy in drug discovery lies in the examination of drug resistance. Through cell-based imaging assays, subpopulations of cells with distinct drug-resistance characteristics can be identified. Such techniques have been applied to chemotherapy resistance in cancer and antibiotic resistance.

Fluorescence microscopy can be used in various ways to evaluate drug toxicity. It allows for the visualization of drug-induced morphological changes in cells, such as alterations in cell shape, membrane integrity, or organelle structure, which aids in identifying potential toxic effects. Fluorescence microscopy techniques can also assess drug-induced alterations in cellular processes and signaling pathways. For instance, fluorescent probes targeting specific cellular components or signaling molecules can be employed to monitor changes in cellular metabolism, oxidative stress, calcium levels, mitochondrial function and DNA damage. By using time-lapse microscopy, researchers can observe the temporal progression of drug toxicity, capturing cellular events such as apoptosis, cell-cycle arrest, or cellular senescence. This dynamic imaging approach provides a comprehensive understanding of the kinetics and temporal patterns of drug-induced toxicity, enhancing our knowledge of the underlying mechanisms.

Conclusion: The Future of Fluorescence Microscopy for Cell-Based Imaging Assays

In conclusion, the remarkable capabilities of fluorescence microscopy have positioned it as an indispensable tool for a broad spectrum of applications, encompassing fundamental cell-biology investigations and the frontiers of drug discovery. This versatile imaging technique offers invaluable insights into the intricate organization and dynamic functions of cells, enabling researchers to witness the vibrant processes that unfold within living cells and their complex molecular components. In the realm of drug discovery, automated fluorescence microscopy is used for cell-based phenotypic screening, as well as target-based and pathway-based screening. Fluorescence microscopy unveils a fascinating world of drug mechanisms by allowing researchers to visualize and comprehend the intricacies of drug targets and their interactions. It serves as a powerful tool to study crucial aspects such as drug efficacy, resistance, and toxicity, providing a comprehensive understanding of the complex interplay between drugs and biological systems.

The future of fluorescence microscopy is exceptionally promising, with ongoing advancements in fluorescent probes and imaging technologies. These innovations will undoubtedly propel the field forward, further enhancing our comprehension of cellular biology and contributing to the continuous improvement of human health. The enduring relevance of fluorescence microscopy in expanding our knowledge and capabilities in cellular research and drug development is undeniable, establishing it as an essential cornerstone in the scientific pursuit of unraveling the mysteries of life at the cellular level.

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