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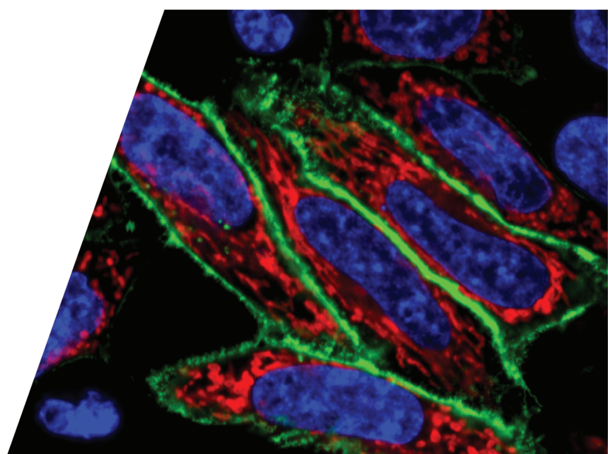

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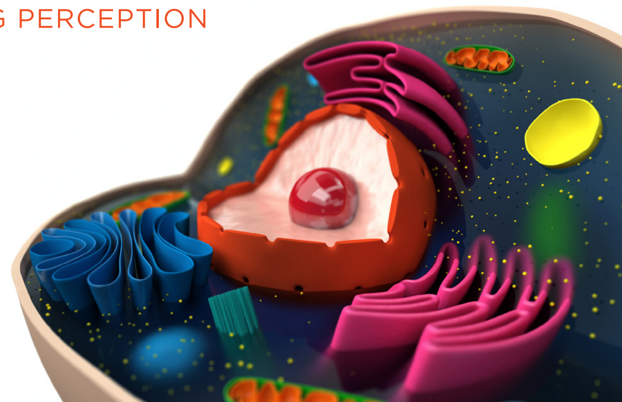


Living Proof: Live-Cell Analysis

Standard cell imaging methods require fixation, which is impractical for monitoring cellular dynamics and often results in experimental artifacts. Live-cell imaging, on the other hand, needs no fixation and is able to track cellular behaviors and interactions in real time. Live-cell analysis is considered the gold standard for probing complex and dynamic cellular events. However, traditional microscopy and stains weren't designed for use with live cells. More recently, various instruments, reagents, and probes have been developed for live-cell techniques.

Real Time, Over Time: Applications of Live-Cell Imaging

From simply studying cell structures and substructures to the dynamic localization of molecules, live-cell imaging has many uses. Common live-cell analyses include examining cell integrity during injury and repair mechanisms,[1] endocytosis and exocytosis,[2,3] signal transduction,[4] enzyme activity mapping,[5] and protein tracking,[6] as well as measuring responses to environmental assaults or perturbations.[7]

Why Live Cells?

If you had to tell a story using a single picture or a feature length film, which would you choose? Although fixed-cell imaging has its uses, in terms of storytelling, it doesn't come close to live-cell imaging. Most live cells, however, aren't meant to be exposed to light, which makes staining and imaging them somewhat more involved than fixed-cell analysis. It also means that the microscopy techniques that were developed for use with fixed cells have to be modified to ensure they can capture movement, focus on a dynamic object, and record multiple images in a short timeframe.

Instrumentation Involved in Live-Cell Imaging

Live-cell imaging is essentially high-rate time-lapse photography performed using a microscope. Various microscopy techniques can be employed for live-cell analyses, and each is useful for answering different biological questions. While simple forms of transmission microscopy such as bright field, dark field, phase contrast, and differential interference contrast microscopy are useful for studying cell shape, classic wide-field fluorescence microscopy or various forms of confocal microscopy using fluorescent markers are commonly used for experiments monitoring movement of molecules and proteins within, into, and out of the cell.

Förster (or fluorescence) resonance energy transfer (FRET) analysis, a process involving radiation-free transfer of energy between donor and acceptor fluorophores, is useful for monitoring protein-protein and protein-DNA interactions. FRET-based biosensors are available for monitoring cellular dynamics in heterogeneous populations and single cells.[8] Techniques including fluorescence recovery after photobleaching (FRAP), fluorescence correlation spectroscopy (FCS), and single particle tracking (SPT) are used to visualize protein diffusion. Epifluorescence, some types of confocal, and spinning disk microscopy are useful for protein colocalization experiments or those looking at the spatial organization of cells. The widespread use of these techniques for live-cell imaging is, in part, due to the enormous variety of fluorescent markers that are now available.[9]

Most live-cell microscopy techniques make use of an inverted microscope to study cells grown on glass coverslips for short-term studies. Environmentally controlled chambers or incubator-based imaging systems can be used for longer-term analysis.[4]

Choosing the Right Stain for Your Particular Biological Question

Fluorescence labeling has revolutionized live-cell imaging. To get the best live images, however, there are several factors to consider when choosing a cellular stain, label, or dye. These depend on the imaging technique being used as well as biological question being asked.

For example, green fluorescent protein (GFP) and its derivatives photobleach, or lose fluorescence under continuous illumination, and so aren't a good choice for longer-term live imaging. Probes must be nontoxic, and either cell permeant or introduced into cells by transfection or transduction. Some fluorescent probes aggregate or dimerize under certain temperatures or at a certain pH. Other probes take time to express or accumulate in cells, and so aren't a good choice when requiring immediate live imaging after stain addition. When imaging over long periods of time, to decrease phototoxicity, illumination with longer wavelengths is good practice, so a stain must be chosen accordingly.[10] Furthermore, some dyes migrate and localize in different areas over time, which may also affect live-cell imaging protocols. With the right tools, live-cell imaging promises to tell a multidimensional story. Learning how to select the right stains and techniques will set you on the right path to a happy ending.

For references, please see page 7.

STAINING SUBCELLULAR STRUCTURES

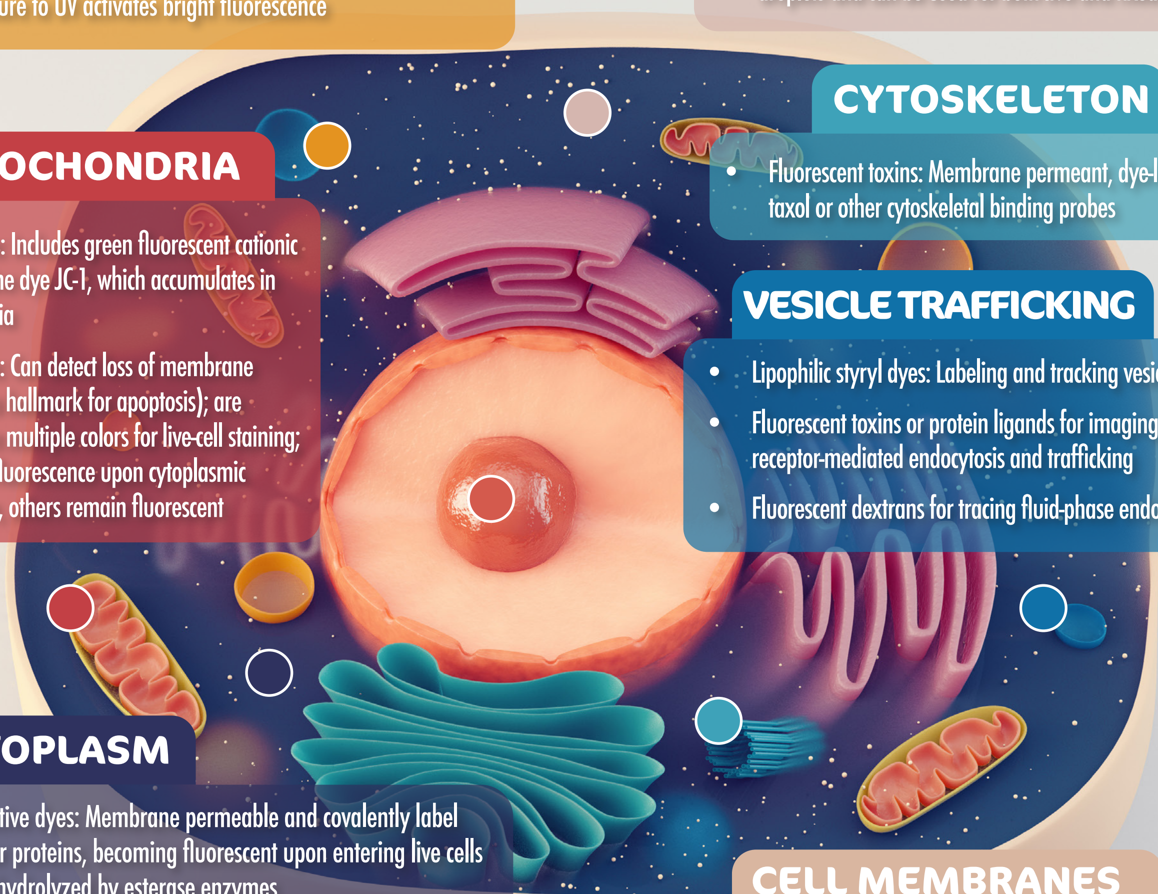
Various fluorescent probes are available for specifically staining certain subcellular structures. But which is the right choice? Our handy guide to some common stains will help guide you toward the right decision.

LYSOSOMES

- Lysosomotropic dyes: Contain weakly basic amines that accumulate in acidic organelles in mammalian cells and yeast
- UV-activatable lysosome stains: Initially show low fluorescence; brief exposure to UV activates bright fluorescence

LIPID DROPLETS

- Fluorogenic neutral lipid stains: Rapidly stain lipid droplets and can be used for both live and fixed cells



MITOCHONDRIA

- Classic dyes: Includes green fluorescent cationic carbocyanine dye JC-1, which accumulates in mitochondria
- Newer dyes: Can detect loss of membrane potential (a hallmark for apoptosis); are available in multiple colors for live-cell staining; some lose fluorescence upon cytoplasmic localization, others remain fluorescent

CYTOSKELETON

- Fluorescent toxins: Membrane permeant, dye-labeled taxol or other cytoskeletal binding probes

VESICLE TRAFFICKING

- Lipophilic styryl dyes: Labeling and tracking vesicles
- Fluorescent toxins or protein ligands for imaging receptor-mediated endocytosis and trafficking
- Fluorescent dextrans for tracing fluid-phase endocytosis

CYTOPLASM

- Amine-reactive dyes: Membrane permeable and covalently label intracellular proteins, becoming fluorescent upon entering live cells and being hydrolyzed by esterase enzymes
- Non-covalent dyes: Include calcein AM, which is a green fluorescent dye useful for determining cell viability or to monitor dye efflux by transporter proteins

CELL MEMBRANES AND CELL SURFACE

- Lipophilic carbocyanine dyes: Label membranes in a wide variety of live and fixed cell types; suitable for long-term cell labeling and tracking studies
- Lectin conjugates: Recognize specific sugar moieties on glycoproteins to selectively stain the cell surface of live cells, and the cell surface and organelles of fixed cells
- Newer covalent membrane and surface stains: Stains that react with plasma membrane proteins for fixable surface staining

NUCLEUS

- Classic nuclear stains: Include DAPI and Hoechst blue fluorescent stains that can be used for both live and fixed cells
- New stains: Green and red spectrum stains are now available with various properties for labeling fixed, dead, or live cells

Charting Change: Choosing a Stain

A suitable live-cell fluorescent stain should not damage the cells being analyzed, have good brightness and photostability, and possess a narrow emission spectrum so it can be used alongside other stains and probes with no overlap. Some stains are suitable for staining live or fixed cells, whereas others are suitable for live-cell imaging only. Cellular stains generally fall into four categories: fluorescent proteins, self-labeling tags, organic cellular stains and indicator dyes, and fluorescent bioconjugates.

GFP and Other Genetically Encoded Fluorescent Proteins

Fluorescent proteins include green fluorescent protein (GFP) and its derivatives such as blue (BFP), cyan (CFP), yellow (YFP), and DsRed fluorescent proteins. Recently, photoswitchable (PS) and photoactivatable (PA) fluorescent proteins have been developed, including PA-EGFP, which becomes 100 times brighter on illumination with UV, and Dronpa, which can be turned on or off with different wavelengths of light.[1]

- Pros: The fluorescent protein can be fused to a specific protein or isolated protein domain, allowing tracking and intracellular imaging of a specific functional protein in live cells.
- Cons: Recombinant expression vectors must be constructed for each probe, which is labor-intensive. The expression vectors must be exogenously introduced into cells by transfection or transduction, which can result in overexpression or heterogeneous expression in a cell population, and can be technically challenging for primary cells and other difficult-to-transfect cell types. In addition, fusion of a large fluorescent protein to a cellular protein may alter its function.

Self-Labeling Protein Tags

Self-labeling protein tags offer a potentially less-disruptive alternative for live-cell imaging of recombinant proteins. These peptides, such as SNAP-tag®, CLIP-tag™, and HaloTag®, covalently react with small molecule ligands.[2] Like fluorescent proteins, self-labeling tags can be fused to target proteins of interest for recombinant expression, and then detected by incubating the cells with their fluorescent ligands. The ligands can be labeled with fluorescent dyes that have superior brightness, photostability, and other spectral properties compared to fluorescent proteins; in addition, dyes compatible with super-resolution imaging techniques can be used.[3] The use of exogenous ligands for imaging also allows temporal control of fluorescence. Fluorescent ligands can be labeled with membrane-permeant or impermeant dyes, for discrimination of cell surface and intracellular target.

- Pros: Genetic tagging of cellular proteins allows the use of superior fluorophores compared to using fluorescent proteins
- Cons: As with fluorescent proteins, the tagged proteins must be genetically engineered and exogenously expressed in the target cells

Organic Cellular Stains and Labeled Toxins

Several classes of small molecule live-cell probes have been developed to target specific organelles and other cellular targets. Cell-permean DNA-binding dyes are commonly used to image the cell nucleus or for cell counting. Lysosomal dyes, mitochondrial dyes, membrane dyes, and vesicle probes accumulate in their target organelles based on charge, pKa, and/or lipophilicity.[4] These organelle stains are available in a variety of fluorescent colors, allowing multiplex imaging in combination with each other or with fluorescent proteins. Many cellular stains are fluorogenic, meaning that they have minimal fluorescence in solution, but become brightly fluorescent inside of cells, allowing staining without a wash step. Many organelle stains have low toxicity to cells, but the stability of dye retention, as wells as cytotoxicity or other biological effects must be considered for long-term staining experiments. Depending on the specific probe, staining with organelle stains may or may not withstand fixation for subsequent immunofluorescence staining.

Fluorescently labeled toxins can be used for imaging of specific cellular structures, for example, membrane-permeant fluorescent paclitaxel (Taxol®) conjugates can be used for live cell imaging of microtubules. While conjugation usually greatly reduces the affinity, and therefore the potency, of toxins, potential disruption of cellular processes and cytotoxicity must be considered when using toxins for long-term imaging.

- Pros: Bright, wide spectral range, good photostability, small in size and therefore interfere minimally with cell function
- Cons: Prone to photobleaching, sometimes toxic, hydrophobic, low specificity compared to genetically integrated fluorescent proteins

Indicator Probes

Inorganic targets including ions, thiols, sulfides, and metals are commonly probed using spectrally sensitive indicator probes, which alter their fluorescent properties based on the surrounding cellular environment. An example is the Ca^{2+} group of sensors, which are useful for measuring cellular properties such as calcium flux into the cytoplasm.[4] Other common targets include cyclic AMP, zinc, potassium, magnesium, and sodium.

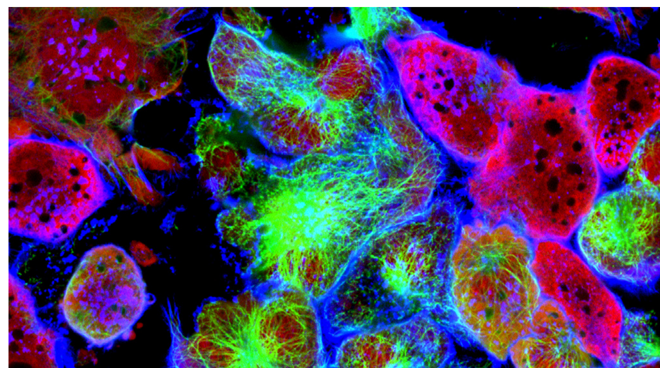
- Pros: Very specific for inorganic targets
- Cons: May require specialized equipment for detection of rapid ion flux

Inorganic Probes

Designed to have reduced photobleaching and improved fluorescence intensity, inorganic fluorescent probes include semiconductor nanocrystals (quantum dots), as well as newer lanthanide-doped oxide nanoparticles, silicon nanoparticles, and fluorescent nanodiamonds. Quantum dots are brighter than their organic fluorophore cousins, and are very photostable, making them useful for longer term live cell analyses. However, as their core is generally composed of cytotoxic elements such as cadmium, they may not be compatible with long-term use in live cells.[5,6]

- Pros: Photostable, suitable for multiplex labeling, bright
- Cons: Coupling to other molecules is necessary

For references, please see page 7.



Fluorescence Staining Tips and Tricks

A good fluorescent live-cell staining protocol will enable reduced background noise and enhanced contrast for quantification of signal intensity without damaging the cell. But many factors can affect the outcome. Keep reading for common issues in sample preparation and how to resolve them, to help you get the most out of your live cell imaging.

Choose a Live-Cell-Specific Stain

While some stains can be used with either live or fixed cells, other stains are not suitable for live-cell imaging because they are toxic or cannot penetrate living cells. Get to know your stains and how they can be used before you start your staining protocol to ensure a good fit for your imaging to ensure a good fit for your experimental system.

Background Fluorescence of Culture Media

Most tissue culture media contains fluorescent compounds such as phenol red. Phenol red is highly fluorescent when excited at 440 nm, so phenol-red-free media should always be used for imaging of fluorescent proteins in the cyan spectrum.[1]

Matching Spectra to Filter Sets

Fluorescent tags are available to cover almost the entire visible spectrum. In reality, they are only applicable if the live-cell imaging apparatus being used has the correct excitation and emission filters. Emission filter sets should be optimized prior to live-cell imaging. When using less-flexible systems such as spinning disk confocal microscopes, ensure that the stain being used matches the available excitation and emission bands.[1]

Aggregation of Stains

Some fluorescent protein stains exhibit temperature-dependent dimerization or aggregation, so certain reducing agents or protocols must be used to prevent erroneous imaging results. Enhanced green fluorescent protein (EGFP) for example tends to fuse at a pH less than 7 and at high concentrations.[2] Work to carefully control your experiments when using fluorescent proteins prone to aggregation.

Understand the Workflow

While many staining protocols involve stain dilution, staining, and washing steps, these steps vary depending on the stain being used. Some stains require dilution in a particular buffer, some require staining to take place at a set temperature or for a certain period of time, and some require multiple washing steps. Get to know your workflow before you begin to avoid wasting your time, reagents, and cells.

Stain Buffers

Certain stains can be added directly to cell culture medium containing serum or buffers such as PBS, but many chemically reactive fluorescent stains have more stringent buffer requirements. Always check the buffer requirements of the stain before adding it to your cell culture.

Interference with Endogenous Structures

Size matters when it comes to live-cell fluorescent staining. Fluorescent protein fusions, such as endogenous proteins cloned with EGFP, can unsurprisingly interfere with cell function.[1] However, size and reactions with endogenous structures are factors when staining live cells with any dye. When choosing a live cell stain, choose wisely based on the dye's size, potential interactions, and propensity to stain structures outside of your target structure.

Photobleaching

Photobleaching is unavoidable in live-cell imaging, especially because antifade mounting media commonly used in fixed-cell imaging is not compatible with live-cell applications, although live-cell antifade media is now available. But photobleaching can be managed. Minimizing exposure time is the most straightforward approach to controlling photobleaching. Choosing the most photostable dye is another approach. A dye's core structure as well as outer groups contribute to its photostability. For example, xanthene-based fluorescent dyes containing a rhodamine core may be more photostable than other dyes in the same class.

For references, please see page 7.

Article 1 - Living Proof: Live-Cell Analysis

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Article 2 - Charting Change: Choosing a Stain

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Article 3 - Fluorescent Staining Tips and Tricks

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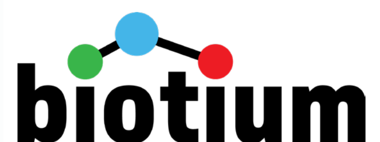

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