Cell Staining, step-by-step training

Introduction

Additional materials:

https://biotium.com/technology/cellular-stains/

https://www.abcam.com/secondary-antibodies/fluorescence-guide

https://www.labome.com/method/Live-Cell-Imaging.html

https://www.leica-microsystems.com/science-lab/introduction-to-live-cell-imaging/

Introduction

What is Cellular Staining?

Cell staining is a technique that can be used to better visualize cells and cell components under a microscope. By using different stains, one can stain certain cell components, such as a nucleus, a cell wall, or the entire cell. Most stains can be used on fixed, or non-living cells, while only some can be used on living cells; some stains can be used on either living or non-living cells.

Why Stain Cells?

The most basic reason that cells are stained is to enhance visualization of the cell or certain cellular components under a microscope. Cells may also be stained to highlight metabolic processes or to differentiate between live and dead cells in a sample. Cells may also be enumerated by staining cells to determine biomass in an environment of interest.

How Are Cells Stained and Slides Prepared?

Cell staining techniques and preparation depend on the type of stain and analysis used. One or more of the following procedures may be required to prepare a sample:

Fixation - serves to "fix" or preserve cell or tissue morphology through the preparation process. This process may involve several steps, but most fixation procedures involve adding a chemical fixative that creates chemical bonds between proteins to increase their rigidity. Common fixatives include formaldehyde, ethanol, methanol, and/or picric acid.

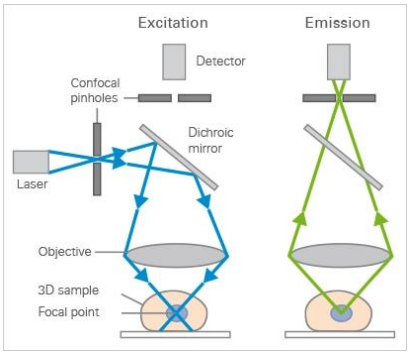
Permeabilization - treatment of cells, generally with a mild surfactant, which dissolves cell membranes in order to allow larger dye molecules to enter inside the cell.

Staining - application of stain to a sample to color cells, tissues, components, or metabolic processes. This process may involve immersing the sample (before or after fixation or mounting) in a dye solution and then rinsing and observing the sample under a microscope.

Mounting - involves attaching samples to a glass microscope slide for observation and analysis. Cells may either be grown directly to the slide or loose cells can be applied to a slide using a sterile technique. Thin sections (slices) of material such as tissue may also be applied to a microscope slide for observation.

Confocal Microscopy

Similar to the widefield microscope, the confocal microscope uses fluorescence optics. Instead of illuminating the whole sample at once, laser light is focused onto a defined spot at a specific depth within the sample. This leads to the emission of fluorescent light at exactly this point. A pinhole inside the optical pathway cuts off signals that are out of focus, thus allowing only the fluorescence signals from the illuminated spot to enter the light detector.



By scanning the specimen in a raster pattern, images of one single optical plane are created. 3D objects can be visualized by scanning several optical planes and stacking them using a suitable microscopy deconvolution software (z-stack). It is also possible to analyze multicolor immunofluorescence stainings using state-of-the-art confocal microscopes that include several lasers and emission/excitation filters.

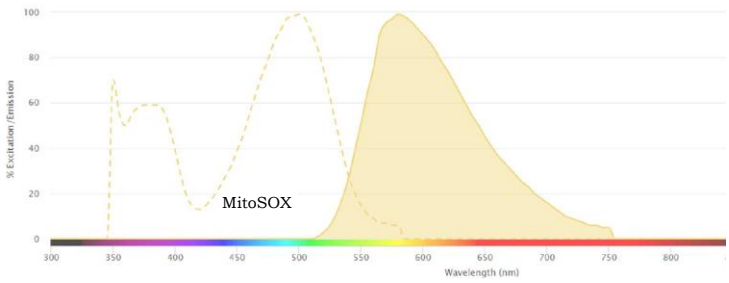
Part 1. Organelle staining

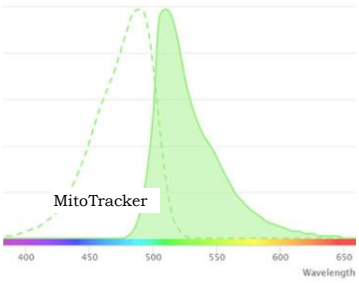
Eukaryotic cells contain subcellular structures responsible for carrying out essential functions for cell survival, including generating energy (ATP), synthesizing and trafficking proteins, and cell waste removal. Labeling key organelles, such as the cell membrane, nucleus, cytoplasm, mitochondria, lysosomes, endoplasmic reticulum (ER), Golgi apparatus, and cytoskeleton proteins (e.g., actin), can be used to monitor cell health, cell death, metabolic activity, autophagy, cell tracking, and cell migration.

Examples:

a. **Mitochondria**. Double-membrane-bound organelles, responsible for cellular respiration and the production of energy as adenosine triphosphate (ATP). Mitochondria are also involved in other tasks, such as cell signaling, cellular differentiation, and cell death—as well as maintaining control of the cell cycle and growth. Dependent on membrane potential, fluorescent live cell dyes for mitochondria can be used to analyze overall cell health and vitality, in addition to mitochondrial activity.

Name	Excitation/Emission (nm)
MitoSOX Red	510/580
MitoTracker Green	490/516

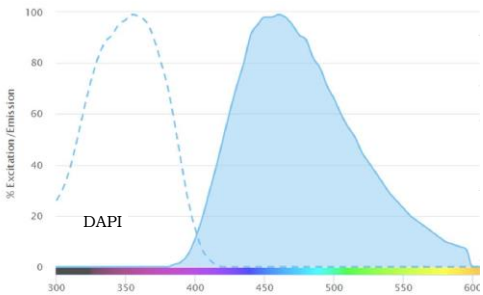


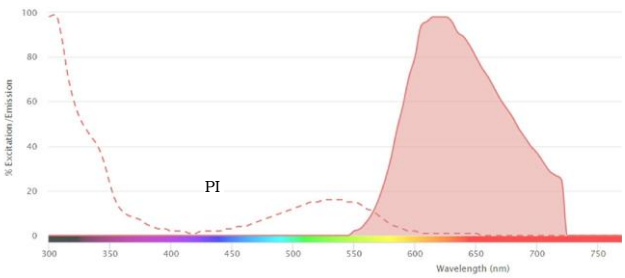


b. **Lysosome.** Membrane-bound organelles that act as the waste disposal system of the cell. As highly acidic (pH 4-5) organelles, lysosomes contain a variety of enzymes capable of breaking down various biomolecules including peptides, nucleic acids, carbohydrates, and lipids. In addition to biomolecule dissolution, lysosomes are also involved in autophagy and apoptosis. Depending on the internal acidic environment, live cell fluorescent dyes for lysosomes can be used to analyze lysosomal activity, autophagy events, vesicle trafficking, and overall cell health.

c. **Nucleus**. Containing nearly all of the cell's genetic material, the cell nucleus is organized in chromosomes, formed from the complexation of long linear DNA with proteins. Live cell nuclear stains can be used either for cell tracking, or as counterstains with other fluorescent dyes or cell reporters.

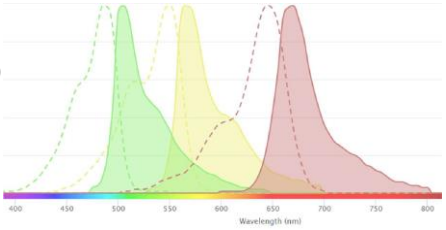
Name	Excitation/Emission (nm)
DAPI	360/460
Propidium Iodide	488/562-617





a. **Cell Membrane**. Protecting and sequestering the cell interior, the cell membrane is composed primarily of lipids and proteins. In addition to controlling movement across the bilayer, cell membranes are involved in a variety of cellular processes such as cell adhesion, ion conductivity, cell signaling, and extracellular structure attachment.

Name	Excitation/Emission (nm)
DiO/DiL/DiD	DiO 484/501 Dil 549/565 DiD 644/665



b. **Cytoskeleton**. As a complex network of interlinking filaments and microtubules extending throughout the cytoplasm, the cytoskeleton provides structural support to the cell and controls many processes, including endocytosis, cytokinesis, cell migration, invasion, and movement.

Example of staining protocol (MitoTracker Green, selectively targets mitochondria):

1. Wash coverslip twice with PBS.
2. Pippet 100 µL of the MitoTracker Green Working Solution (200 nM) onto the coverslip. Incubate the coverslip for 20 minutes at 37°C, protected from light.
3. Drain off the staining medium and wash the coverslips 2-3 times with PBS, dry.
4. Put a drop of mowiol 5 µL on a glass slide. Make sure that there aren't any bubbles.
5. Take coverslip with tweezers, hold it with the cell surface down and put on mowiol drop while tilted. Label the glass slide.
6. Image using the appropriate emission and excitation filters.

If we want to use more than one dye at a time we have to make it properly. We have to select fluorochrome to avoid emission spectral overlap. To do that, you can use these spectrum viewers: ThermoFisher, BDbiosciences. and AAT.

Part 2. Immunostaining staining

Immunostaining is a technique that uses antibodies conjugated with fluorophore to detect a specific protein in a sample.

So, what is immunostaining? Immunostaining is the term for using antibodies to detect a specific protein in a sample. We will talk about immunofluorescence where antibodies conjugate with fluorophores. First of all, we have to know what antigen is. An antigen is any substance that causes your immune system to produce antibodies against it. So, it's something "not yours", strange and maybe dangerous. A specific region of this antigen is called epitope. But is it, antibody (or **immunoglobulin**)? It is a large, Y-shaped protein. There are five **antibody classes** known as IgA, IgD, IgE, IgG, and IgM. The classes differ in their biological properties, functional locations and ability to deal with different antigens. But for our training we need only IgG.

So, we need antibodies to bind specific proteins inside the cell. But why do we need this? We want to find out where a particular protein is expressed, or if it isn't expressed at all, and how many.

There are two types of antibodies available to scientists: polyclonal and monoclonal. **Polyclonal antibodies** contain a heterogeneous mixture of IgGs against the whole antigen, whereas **monoclonal antibodies** are composed of a single IgG against one epitope.

Polyclonal antibodies	**Monoclonal antibodies**
Refer to a mixture of immunoglobulin molecules that are secreted against a particular antigen.	Refer to a homogenous population of antibodies that are produced by a single clone of plasma B cells.
Produced by different clones of plasma B cells.	Produced by the same clone of plasma B cells.
Production does not require hybridoma cell lines.	Production requires hybridoma cell lines.
A heterogeneous antibody population.	A homogenous antibody population.
Interact with different epitopes on the same antigen.	Interact with a particular epitope on the antigen.

There are few steps before staining:

1. Fixation

1. Aspirate the culture medium from the dish or remove each coverslip as required with tweezers, and gently wash them with PBS at room temperature.
2. Incubate the coverslips in freshly prepared 2-4% formaldehyde – neutral PBS at room temperature for 10 minutes. Alternatively, the cells can be fixed for 10 minutes in chilled methanol (pre-equilibrated at -20°C) on ice.

   > Tip: Alternative fixation methods (ethanol, methanol or another) may be tested and compared to determine which is best at preserving the structure and epitope of the protein of interest.

3. Wash the coverslips off the fix buffer in PBS for 2 minutes.

2. Permeabilization

1. Incubate the coverslips in 0.5% Triton X-100 in PBS at room temperature for five minutes. Test different detergents (ex. digitonin, Tween-20) in a range of concentrations to find the optimal condition that best preserves cell structure and the target protein.

Tip: A permeabilization step is not required with methanol fixation because methanol acts as a fixative as well as cell permeabilization agent.

2. Wash the coverslips of the permeabilization buffer by incubating in PBS for 5 minutes.

3. Blocking

1. IgG from the secondary antibody may bind non-specifically to the sticky sites on the cells which often leads to non-specific background signals. To avoid this issue, block the coverslips in 1-5% BSA in PBS.

Multicolor labeling experiments are best carried out by sequentially incubating cells with primary and secondary antibodies, however it may be performed by employing one of the following three options:

a) Simultaneous incubation with unlabeled primary antibodies

This method is useful when the primary antibodies are from different hosts. For example, a mouse monoclonal antibody against antigen-X and rabbit polyclonal antibody against antigen Y.

1. After the blocking step, incubate the cells with unlabeled primary antibodies in the blocking buffer in a humidified chamber for 1h at RT or overnight at 4°C.
2. Decant the antibody solution and wash the cells three times in PBS (5 minutes for each wash).
3. Incubate the cells with both secondary antibodies in a blocking buffer for 1h at RT in the dark.

Tip: The secondary antibodies often come with a broad range of working dilutions. It is recommended to choose the dilutions very carefully and to employ additional optimization to see which dilution combination gives the best possible staining.

4. Decant the secondary antibody solution and wash three times with PBS for five minutes each in the dark.

b) Simultaneous incubation with directly labeled primary antibodies

This method is useful when the primary antibodies are from the same host. For example, a mouse monoclonal against antigen-X and a mouse monoclonal against antigen-Y.

1. After the blocking step, incubate the cells with directly labeled primary antibodies in the blocking buffer in a humidified chamber for 1h at RT or overnight at 4°C in the dark.
2. Decant the antibody solution and wash the cells three times in PBS (five minutes for each wash).

Part 3. Live cell staining

The acquisition of information about the "molecular state" of a sample already is a hard task in fixed cells or tissue. This becomes even harder, if the information has to be acquired in real-time, as the cells during an experiment have to be functioning as naturally as possible. Additionally, a high amount of information has to be sampled in a relatively short time, as many events only last seconds or even milliseconds (e.g. changes in cellular ion levels).

Live-cell imaging allows for investigation of dynamical physiological processes in living cells instead of giving a "snapshot" of a cell's current state. It turns Snapshots into movies. Live-cell imaging provides spatial and temporal information of dynamic molecular events in single cells.

1. It allows the determination of appropriate time points for endpoint studies: many biological processes take a long time (hours to weeks) and often choosing the most significant time points for analysis can be challenging, e.g. when to collect cells after treatment or differentiation, etc. Being able to monitor the cells' response in real-time enables more accurate identification of relevant endpoints
2. It provides additional and continuous data points of biological processes over time, giving more robust quantitative analysis
3. It enables quantification of transient phenotypic changes in cells that could be missed if only looking at given time points
4. It helps to improve the interpretation of conflicting results from endpoint studies that can be an outcome of reversible processes that are dynamic and controlled in space and time

Successful live-cell imaging experiments can be a major technical challenge. An important caution is to ensure that cells are in good condition and function normally while on the microscope stage with illumination in the presence of synthetic fluorophores or fluorescent proteins. The conditions under which cells are maintained on the microscope stage, although widely variable, often dictate the success or failure of an experiment.

Imaging Media

Various cell culture media are available based on the particular biochemical requirements of cells. Culture media contain various constituents, including amino acids, vitamins, inorganic salts (minerals), trace elements, nucleic acid constituents (bases and nucleosides), sugars, tricarboxylic acid cycle intermediates, lipids, and coenzymes. In tissue culture media, an important step is to control oxygen concentration, pH, buffering capacity, osmolarity, viscosity, and surface tension.

Maintenance of the Cellular Environment in Culture

A constant cellular environment is important to maintain. Cells should be grown in culture medium in a carbon dioxide incubator. For permanent equipment, a box can be constructed around the microscope and heated with warm air.

Choice of Imaging Chamber

Chambers need to keep the specimens viable, while also providing optical properties that are optimal for imaging. Long-term experiments can also be performed for imaging live cells on the microscope stage. The culture cells need to have optimum growth conditions for an extended period of time. In general, imaging chambers include a glass window, usually the thickness of a coverslip (approximately 170 μm), through which the cells are viewed with an objective lens. Short-term experiments can be performed by sandwiching two coverslips.

Phototoxicity

Cells are prone to photodamage, especially when fluorophores are present. When fluorescent molecules are in an excited state, they react with oxygen to produce free radicals that can damage subcellular components and adversely affect the cells. Even when fluorophores are absent, mammalian cells are sensitive to ultraviolet light.

For obtaining the maximum signal/noise ratio and resolution, cells need to be illuminated with very high light intensity. Many methods are used to reduce light-induced damage. An important protective step is to shut off the illuminating light when not. Optimized emission filters should be selected for a maximal

signal. Reducing oxygen levels can help limit photobleaching. Additionally, omitting phenol red and serum from the medium can help reduce background fluorescence.

Establishing Cell Morphology and Conditions in Culture

One of the most important aspects of live-cell imaging experiments is to establish criteria for determining the condition of the cells. These criteria will vary depending upon the type of experiment; however, one of the most important factors to determine is whether there is damage to the cells caused by the imaging process that might affect the results. One of the simplest ways for following an imaging experiment is to compare the morphology and condition of cells that were exposed to light during the study with neighboring cells in the same chamber that were not illuminated.

Example of staining protocol (MitoSOX Red selectively targets mitochondria):

1. Wash cells twice with Tyrode's solution.
2. Pipet 100 µL of the MitoSOX Working Solution (1 µM) to the dish. Incubate it for 20 minutes at 37°C, protected from light.
3. Drain off the staining medium and wash the coverslips 2-3 times with Tyrode's.
4. Wash twice with Tyrode's solution.
5. Add 300 µl Tyrode's solution to the dish.
6. After you set up confocal parameters (the appropriate emission and excitation filters, gain, offset etc), add 300 µl 0.5% H_2O_2 as an apoptosis inducer (but this concentration is high, so it induces necrosis).