# Imaging, Staining, and Markers

*Vardan Avetisyan and Narine Sarvazyan*

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**What You will Learn in This Chapter and Associated Exercises**

The goal of this chapter is to cover the key concepts related to imaging, fluorescence, and sample labeling. Students will gain basic knowledge of viewing cells and tissues using a microscope as well as how to stain different structures using specific markers.

## 7.1 Visible Light

Humans observe the world using what is appropriately called the *visible light spectrum*. It corresponds to roughly 400–750 nm range of wavelengths. It is neighbored by the ultraviolet (UV, 10–400 nm) and infrared (IR, from 750 nm until about 1000 microns) light ranges. When photons of certain energy are absorbed by a specimen, it can be measured using spectrophotometer or seen as a darker area on microscopic images. This phenomenon is commonly referred to as *light absorption*.



## 7.2 Fluorescence

Fluorescence is the ability of molecules to emit light after absorbing photons of higher energy. The energy coming from a light source shifts an electron of an atom from a lower energy state to a higher (also called "excited") energy state (Fig. 7.1). When fluorophore returns to its ground state, energy is released in the form of another photon. This newly emitted photon has lower energy and therefore lower frequency. Having a lower frequency ($\nu$) means that the photon has a longer wavelength ($\lambda$). For example, if fluorophore emits red light, the sample needs to be illuminated using

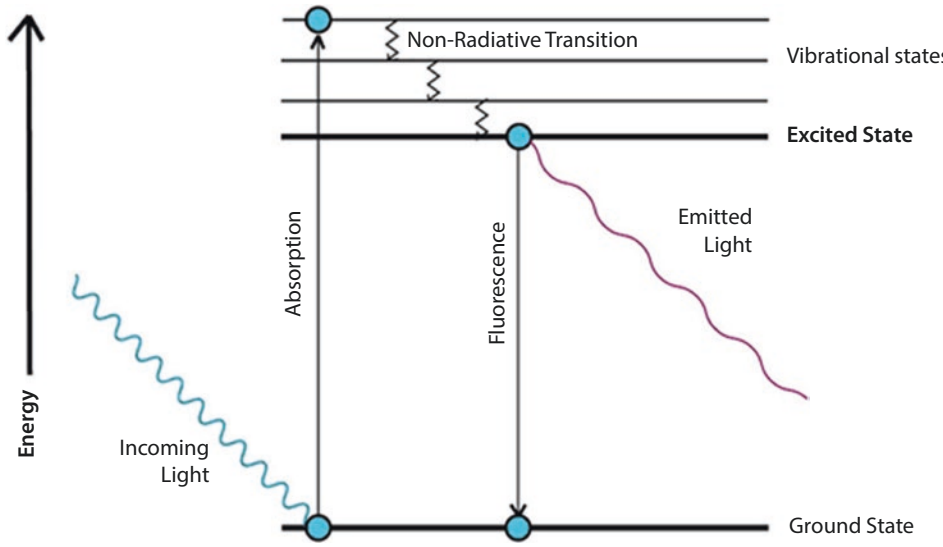



**Fig. 7.1** Jablonski diagram. This diagram illustrates the energy states of molecules and the transitions between them. Due to energy losses in an excited state, emitted photons always have lower energy than absorbed photons. Therefore, the emission wavelength is always longer than the excitation wavelength

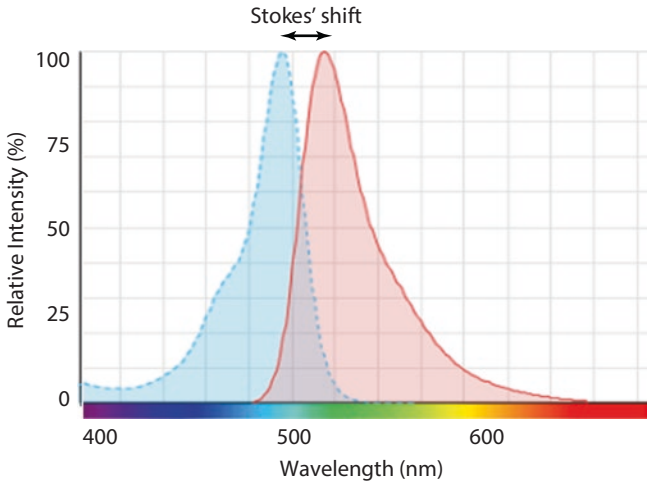



**Fig. 7.2** Example of excitation (shaded blue) and emission (shaded pink) spectra of Fluo-4, an indicator for intracellular calcium levels

higher energy wavelength (which will appear as green or blue light). The distance between optimal excitation wavelength and wavelength that has maximal emission intensity is called Stokes' shift. The larger the Stokes' shift is for a specific dye, the easier it is to select appropriate filters to image samples stained with it (Fig. 7.2).

Today, a great number of fluorescent dyes have been developed. These dyes are capable of selectively labeling intracellular components of both live and fixed cells, to monitor the release of ions, to track the movement of cells or proteins, to examine the oxidative state of the cells, and the list goes on [1]. Many of these dyes are added to the cells or tissue in AM (*acetoxymethyl ester*) or DA (*diacetate*) form, which makes it easier for the dye to pass through cell membranes. Upon entering the cells, these added chemical groups are cleaved off by intracellular enzymes, making the active form of the dye trapped inside the cell.

In addition to low molecular weight dyes mentioned above, there is also a wide range of fluorescent proteins that were developed after the discovery of green fluorescent protein (GFP) [2]. Genes for GFP or its many analogs can be included in the DNA of an organism or individual cells. This results in cells being permanently autofluorescent, which is very helpful for tracing cells that are imbedded into scaffolds for extended periods. The same applies to tracing cells upon their implantation into live tissues.

## 7.3 Basics Light Microscope Types

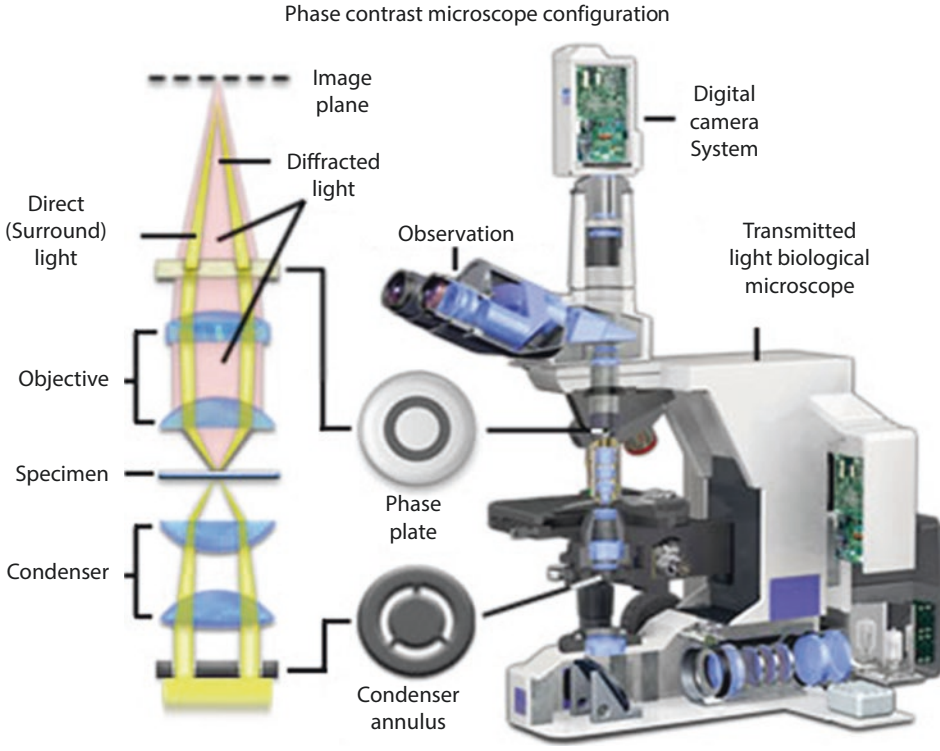
All microscopes have a goal of creating a magnified image of a specimen. They differ in the way the image is obtained, how the specimen is placed relative to the objective, the degree of magnification, spatial resolution, type of sensors, and other features. *Light Microscope* uses lenses and visible light to create a magnified image of the object [3]. There are two main types of light microscopes: upright and inverted. In case of an upright microscope, the object to be observed is positioned above the objective, while in case of an inverted microscope, objectives are placed beneath the object. The light from a light source on an opposite side passes through the object to the objective lens. Common magnification values include 4×, 10×, 20×, or 100×. The light then passes

through the second pair of lenses located within eyepieces. The latter usually magnifies the image for an additional ten times. So, the user looking at let's say 20× objective using 10× eyepiece lenses ends up seeing 200 times larger object. *Stereo microscope* uses two separate optical paths from the objective lens to eyepieces. The different angles of these two pathways create a 3D image of an object. This type of microscope usually has low magnification. It is used to study the surfaces of solid structures, organ dissection, cannulation of small vessels, or microsurgery.

## 7.4 Microscopes Used for Cell Culture

In cases of cultured cells, it is more convenient to observe samples from beneath in order not to disturb the sterility of the cell environment. This is enabled by what is called an *inverted microscope* (■ Fig. 7.3). In the case of inverted microscopes, light is coming from the top, while the objective is beneath the stage. Another feature that is useful to observe cells is called phase contrast. It enables to enhance the contrast of transparent and colorless objects such as cells or thin tissue slices. To have this feature, the microscope needs to have a special plate that is inserted into its light path. The phase contrast plate must match with the objective. The difference in phase of the light wave is not noticeable to the human eye; however, when the change in phase is increased to half a wavelength by phase-plate, it causes a visible difference in brightness enabling to better see cell boundaries and other structures.






■ **Fig.7.3** A general appearance of an inverted, phase contrast cell culture microscope

## 7.5 Fluorescent Microscopes

Fluorescent microscopes employ a set of filters that enables separation of illuminating and collected photons based on their wavelength. To do so, the light path includes an excitation filter, a dichroic mirror, and an emission filter (Fig. 7.4). The filters and the dichroic mirror must be chosen to match the spectral properties of the dyes used to label the specimen. Several different fluorescent dyes can be imaged simultaneously by selecting the appropriate combination of filters so neither excitation nor emission ranges overlap. This enables the combination of several single-color images into one.

Let's consider an example. When selecting a dye to be used with a confocal microscope, the first step is to determine what lines of the available laser can excite the dye. In the case of Fluo-4 dye (spectra of which are shown in Fig. 7.2), 488 nm argon laser will be a good fit as far Fluo-4 excitation wavelength. The next step is to select a dichroic filter that can separate the two peaks (something around 500 nm will fit). Lastly, we need to select an appropriate emission filter (any filter that lets photons from 520 to 560 nm to pass through should work).

Filters that pass wavelengths *within a certain specified range* are called *bandpass filters*. Filters that allow light to pass with wavelengths *longer than the specified value* are called *long-pass filters*. Filters that let photons pass with a wavelength shorter than specified values are called *short-pass filters*. By changing multiple filters or excitation wavelengths, images of samples stained with several dyes can be obtained. In those multistained samples, it is critical to eliminate what is called "optical crosstalk." The latter occurs when filters are not optimized and there is insufficient separation between photons of different wavelengths. In those cases, a signal from one dye will "bleed" into a channel reserved for another dye.

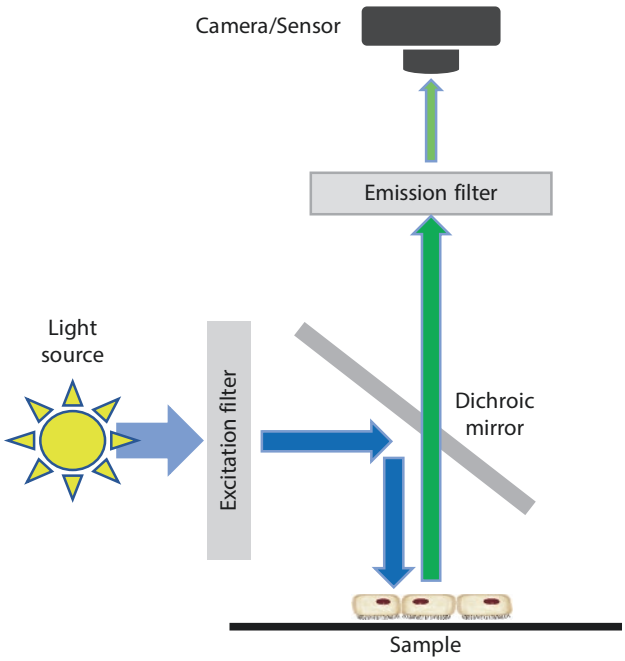



**Fig. 7.4** Basic principles behind fluorescence microscopy. The light source can be a lamp, a laser, or an LED source

## 7.6 Confocal Microscopy

In regular fluorescence microscopes, photons are coming from the full thickness of the specimen; thus, it is not possible to acquire crisp images from a single focal plane. In contrast, confocal microscopy eliminates this out-of-focus information by means of a confocal "pinhole" situated in front of the image plane. It allows only the in-focus portion of the light to be collected. Light from above and below the plane of focus of the object is eliminated from the final image. A diagram of the confocal principle is shown in Fig. 7.5.

*Laser scanning confocal microscopes* use lasers as the light source. The laser beam is then "scanned" through the object by using x-y scanning mirrors, while altering the focus allows the acquisition of different focal planes. Multiple planes are then reconstructed into 3D images using a computer. Confocal microscopy is also widely used to acquire crisp pictures of individual stains, which can be then combined into a pseudocolor image of a sample with each cellular target assigned a different color (Fig. 7.6).



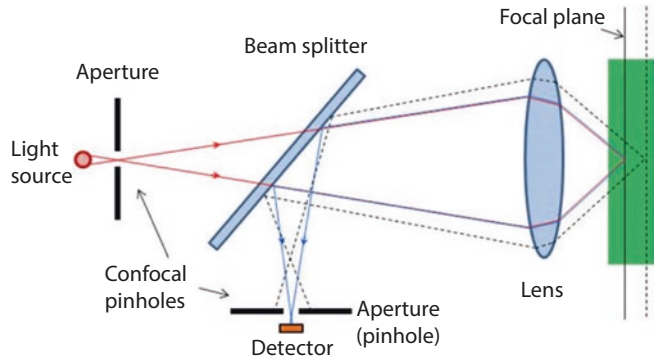



**Fig. 7.5** Diagram showing the main principles behind confocal microscopy. The presence of pinholes removes out-of-focus light enabling optical sectioning of the sample

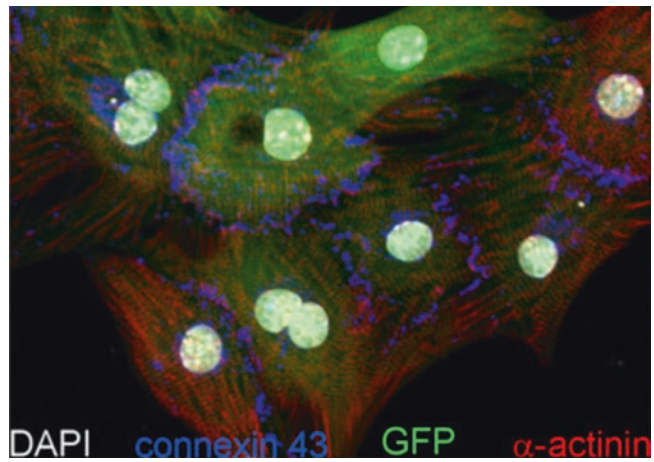



**Fig. 7.6** Confocal image of cardiac myocytes derived from GFP-expressing mouse embryonic stem cells. Cells were fixed and stained with DAPI (white), and with antibodies for alpha-actinin (Cy5, red) and connexin 43 (Cy3, blue)

## 7.7 Electron Microscopes

In the case of electron microscopy, the light source is replaced by a stream of electrons that pass through a set of electromagnets. The wavelength of an electron is much smaller than the visible light, so this approach allows one to create much more detailed images.

*Scanning electron microscope (SEM)* creates a 3D image of an object with high resolution and magnification by covering the source of an object with an electron reflecting film. So, the cells or tissue structures are essentially observed "from an outside."

*Transmission electron microscope (TEM)* creates highly detailed 2D images of cell infrastructure as electrons pass through a very thin specimen while being absorbed by its denser components. Other types of microscopes are currently available, and interested students are referred to numerous online resources on this subject.

## 7.8 Histology

In addition to fluorescent dyes, many histological dyes can be very useful in TERM applications. In general, they are not as specific as fluorescent markers and are best used for tissue rather than for intracellular staining. Histology procedures are lengthy and involve sample fixation, mounting, slicing, staining, and finally imaging. Most of histology protocols can be found online [Ref: https://webpath.med.utah.edu/HISTHTML/MANUALS/MANUALS.html]. Due to the time it takes to perform all the steps, it is advised to use commercial histological facilities for better results.

## 7.9 Dye Aliquoting

Multiple cycles of freeze-thawing can affect the integrity of the samples, including fluorescent dyes. Therefore, the first step in any staining protocol is to divide the dye stock solution into small aliquots that can be individually defrosted for individual experiments. To perform aliquoting, one needs to estimate the most reasonable volume of individual aliquots based on the stock concentration and required dilution to achieve the volume of solution that is sufficient for one experiment. Most of the commercial dyes come as 10 mM stock solution in dimethyl sulfoxide (DMSO) or have to be diluted in DMSO per the product manual. For example, let's consider ThermoFisher nuclear stain 7-AAD (#A1310). It comes as a 1 mg solid. If diluted with either 78 μL methanol or DMSO, it will yield 10 mM concentration (7-AAD molecular weight is 1270). Considering the final dye concentration being about 5 μM, a 10 mM stock solution will require an additional 1:2000 dilution. Since 2 mL dye solution is usually enough to fully cover several glass coverslips, making 18 × 1 μL aliquots and 6 × 10 μL aliquots (for further aliquoting) makes a good sense. To make aliquots, multiple small size Eppendorf tubes have to be labeled, followed by the addition of a specified volume (1 μL or 10 μL in our case) of the dye to each of the Eppendorf tubes. The label needs to include the name of the dye, manufacturer and catalog number of the dye, the day of aliquoting, stock concentration, and aliquot volume. Aliquots are then frozen (unless product manual notes otherwise).

## 7.10 How to Observe Stained Samples

When imaging samples at low resolution using air objectives ranging from 2× to 40×, pretty much any clear dish can be used. Glass coverslips are preferable when looking at fluorescent samples, especially when a non-confocal setup is used to observe it. Higher resolutions usually require immersion oil (the latter is stated on the objective), in which case samples can be only imaged through 170 microns thick or what is called #1.5 size glass coverslip. When imaging fixed samples, this can be done by simply flipping samples with a coverslip surface facing the objective. Mounting media, such as Mowiol, are usually used to fix cells or thin tissue between the glass slide and the coverslip. Imaging of live cells requires medium presence; therefore, special chambers must be used. They can be bought or custom-made. Their main feature is 170-micron thick glass coverslip at the bottom of the dish that allows taking images of the sample from below (Fig. 7.7).

Alternatively, for live samples special water immersion objectives can be used. These objectives use water in place of the oil as the immersion medium. They enable approaching live sample from the top since these objectives can be in direct contact with the saline that surrounds living cells or tissues. Unfortunately, it is nearly impossible to maintain sterility of these samples afterward, unless the entire imaging setup is placed in a sterile environment.

## 7.11 Choosing the Dyes

Online resources such as the ThermoFisher Fluorescence SpectraViewer website can be used to find emission and excitation spectra for selected dyes. Multiple dyes can be added and removed from the graph, enabling to see overlaps in excitation and emission spectra. It is important to select dyes that can be excited with light sources available to a particular lab or the user. For example, if a user's confocal system does not have a UV laser, then he/she will not be able to use DAPI as its excitation lies in the 300–400 nm range.

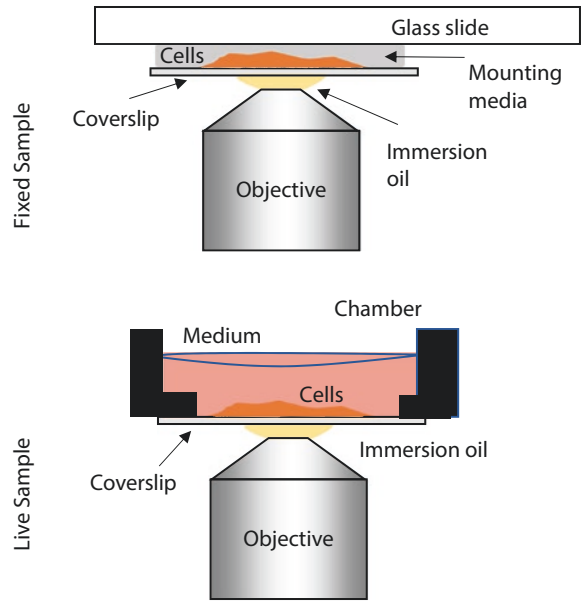



**Fig. 7.7** Sample placement for an inverted microscope with oil immersion objectives. Typical 60× or 100× oil objectives require 170-micron thick glass coverslip between the sample and the objective lens

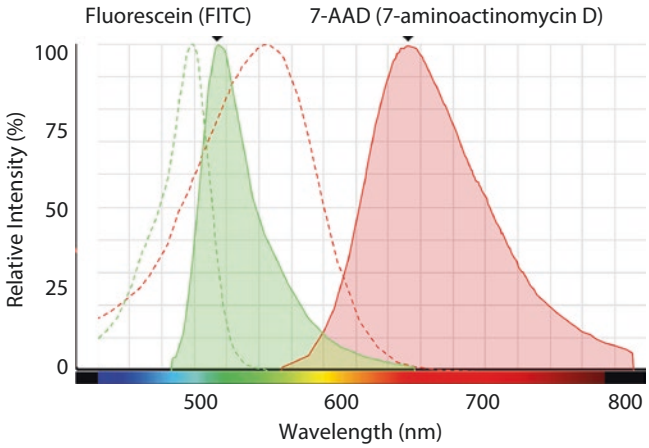



**Fig. 7.8** Principle of selecting suitable dyes for staining with multiple markers. Excitation spectra of FITC (dotted green line) indicate that it can be excited by blue light (about 490 nm) while collecting emitted green light at 510–530 nm range. Samples can be co-stained with 7-AAD, a red nuclear stain, that has excitation and emission peaks at 560 and 650 nm, respectively. Based on the spectra, the expected overlap between the two stainings should be negligible

It is recommended to choose dyes with well-separated emission or excitation spectra. For example, let's say the user wants to stain cells with 7-AAD (for nuclei) and TRITC-conjugated phalloidin (for actin filaments). Both can be excited by a 535 nm laser. Emission spectra are different, but there is a significant overlap in almost all areas of the spectrum. It is possible to use narrow emission bands to separate signals from these two fluorophores (i.e., 580–600 for TRITC and 660–740 nm for 7-AAD), but this will translate into low-intensity signals. Instead, the user will be better off by replacing 7-AAD with a different nuclear stain—let's say TO-PRO-3 or substitute TRITC-labeled phalloidin with FITC-conjugated phalloidin (Fig. 7.8). In both the cases, there will be a clear separation between peaks of both excitation and emission spectra.

### Session I

**Demonstration**

The instructor shows how to use different light paths on available fluorescent scopes to perform imaging of samples stained with different dyes. If a confocal setup is available, the use of Z-stacks to image tissue slices is also shown. Lastly, students are shown how to fix and stain cultured cells on a glass coverslip using multiple organelle-specific dyes. Previously stained samples with clearly delineated cell morphology (similar to the one shown in Fig. 7.6) can be used to demonstrate different optical paths and how specific filter can affect the intensity of the individual channels.

**Homework**

*Students are asked to familiarize themselves with the spectral properties of a given list of dyes to be used during Session II.*

### Session II

**Team Exercises**

Using known dye spectra and a list of available probes, each team selects the best dye combinations and filter settings to perform triple staining of given cell samples using DAPI, phalloidin, mitochondrial, or other organelle-specific markers. For this exercise, students need to match available dyes with hardware settings of the confocal system or any other lab-specific fluorescence imaging equipment. Cells isolated and plated by the teams during previous weeks can be used for these experiments.

**Homework**

*Students are tasked with taking images of their stained samples and making PowerPoint presentations that show respective cell structures.*

7

## Sample Protocols

**Cell preparation for team exercises**

1. Plate cells on gelatin-coated coverslips.
2. Culture to desired density/age.
3. Wash two times with PBS (phosphate-buffered saline).
4. Cover cells with 4% formaldehyde for 10 min at room temperature. Alternatively, cover cells with ice-cold 1:1 methanol-acetone solution and put the cell plate in the freezer for 10 min.
5. Wash two times with PBS.
6. Add 400 μL Wash Buffer/Blocking Buffer (PBS + 0.1% serum/1% albumin + 0.02–0.03% sodium azide).
7. Stain or store at 2–8 °C for up to 3 months.

**Multi-dye staining**

1. Choose dyes according to chosen organelles (e.g., nucleus, cell membrane, mitochondria, etc.). Make aliquots. Use one aliquot to make a dye solution with recommended final concentration.
2. Transfer fixed cells grown on 170-micron thick glass coverslips to a small, dry Petri dish. Coverslips of different sizes can be used depending on the final observation chamber.
3. Carefully add 10–20 μL of dye, diluted to a final concentration, on top of each coverslip. Cover Petri dish with a lid and wait 15 min at room temperature.
4. Wash coverslips two times with PBS (phosphate-buffered saline).
5. Check for positive cell staining using a fluorescent microscope with filters setting suitable for the dye. This can be done using low magnification objectives that do not require close contact with the sample.
6. Add second dye and then third dye as per steps 3–5.
7. Use high-resolution objectives to obtain good quality images illustrating different cell structures.

**Mounting slides with Mowiol**. Mowiol is a proprietary solution of polyvinyl alcohol. It fully hardens overnight and does not require sealing coverslips with nail polish. The latter is another way to mount samples. Opened aliquots of Mowiol can be stored in the fridge for about 1 month.

1. Wash coverslip with saline.
2. Put a small drop of Mowiol (5–10 μL) on a glass slide. Make sure that there aren't any bubbles.
3. Take coverslip with tweezers, hold it with cell surface down, and put on Mowiol drop while tilted.
4. Label the glass slide.
5. Wait at least 30 min for air-objective and 24 h if oil objectives are to be used for imaging. It is easy to smear Mowiol while it is not fully settled. Therefore, oil objectives, which come to very close contact with the surface of the coverslip, can shift the coverglass leading to irreversible sample damage.

**Take-Home Message/Lessons Learned**

After reading this chapter and performing the requested assignments and exercises, students should:

- Know the basic functions of different types of microscopes and objectives.
- Be able to select appropriate type of filters when viewing sample stained with a specified dye(s)
- Learn how to fix and stain cell or tissue samples with different fluorescent markers
- Be able to aliquot dyes for long-term storage
- Know how to use the online spectral libraries to select multiple dyes with appropriate emission/excitation properties.

## Self-Check Questions

Q.7.1. You stained cell samples with mitochondria-specific dye and want to observe individual organelles, which are approximately 1–2 micron in size. The most appropriate objectives will be

A. 2×
B. 10×
C. 20×
D. 100×

Q.7.2. You are trying to get a single image of a 2 × 2 mm piece of engineered tissue. The most appropriate objectives will be

A. 2×
B. 10×
C. 20×
D. 100×

Q.7.3. Use online spectra finder to determine if a single excitation source can be used to image sample co-stained with _____ antibody and a nuclear stain ___.

A. FITC & TO-PRO-3
B. FITC & 7-AAD
C. TRITC & TO-PRO-3
D. TRITC & 7-AAD

Q.7.4. Based on 7-AAD spectra, the following set of filters (excitation/dichroic/emission) should be suitable to image samples stained with it:

A. 480 nm short-pass, 500 nm, 600–650 nm band-pass
B. 540–570 nm, 600 nm, 620 nm long-pass
C. 480 nm long-pass, 650 nm, 600–650 nm band-pass
D. 500 nm long-pass, 550 nm, 600 nm short-pass

Q.7.5. Choose the correct statement.

A. A confocal microscope uses a beam of electrons to create crisp images of tissue at different depths.
B. Mowiol aliquots cannot be stored and must be prepared fresh.
C. The use of acetoxymethyl ester AM form of the dye enables to store stained cells for at least a month at room temperature.
D. The surface of a sample to be observed using a 63× oil objective with ∞/0.17 marking must be less than 170 microns away from the objective lens.



## References and Further Reading

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