



# MPSF educator packet

Molecular Probes®

This packet contains illustrations and figures from the Molecular Probes® School of Fluorescence website. They illustrate concepts from the basic physical properties that underlie fluorescence through experiment planning and troubleshooting. The images and graphics on this page are copyrighted, but they are freely available for your use as long as the attribution “Molecular Probes®” remains intact.

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For more information, go to [lifetechnologies.com/imagingbasics](https://www.lifetechnologies.com/imagingbasics)

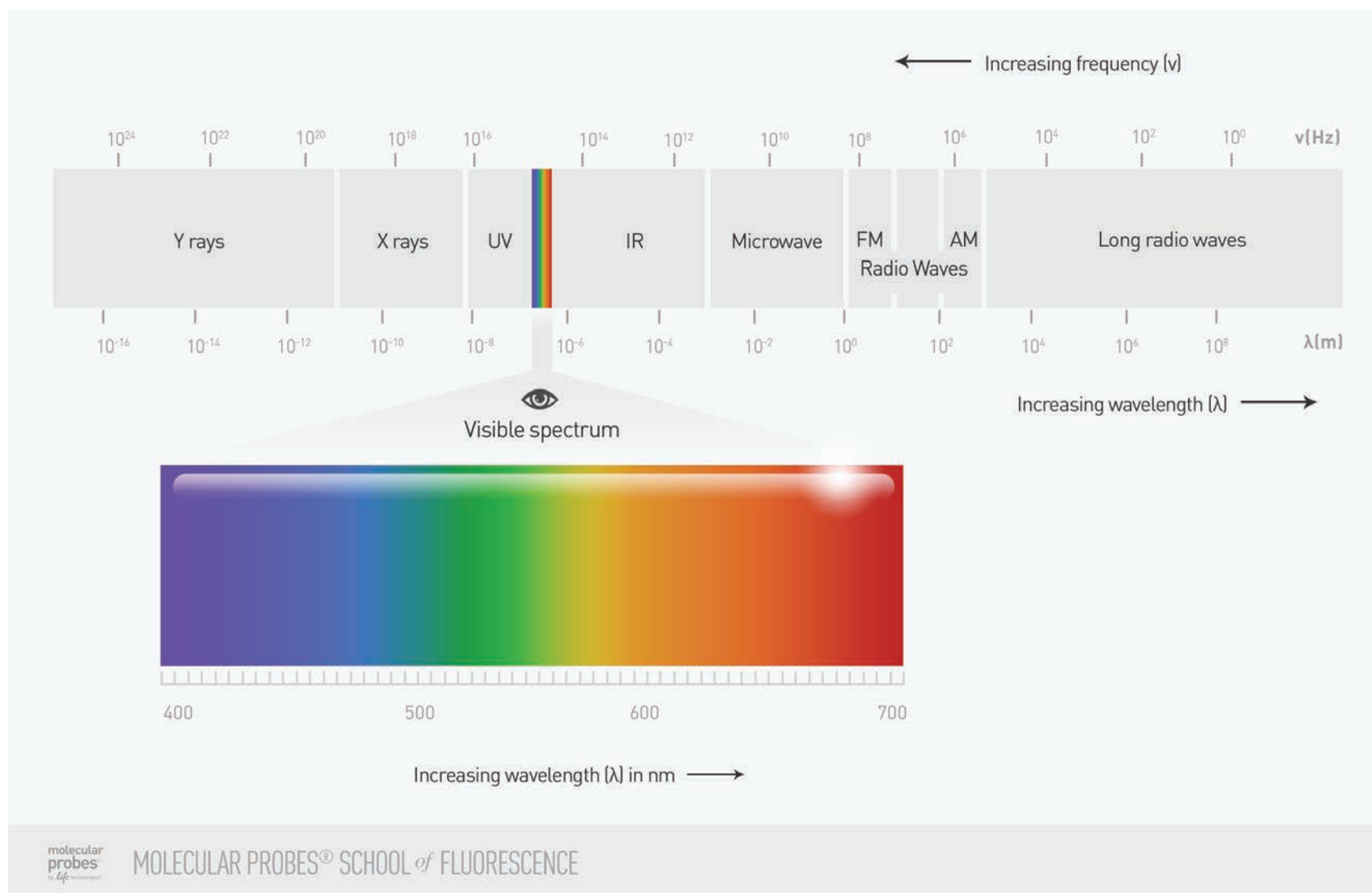


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# Part One: Fundamentals of Fluorescence Microscopy

## 1. Physical Properties That Define Fluorescence

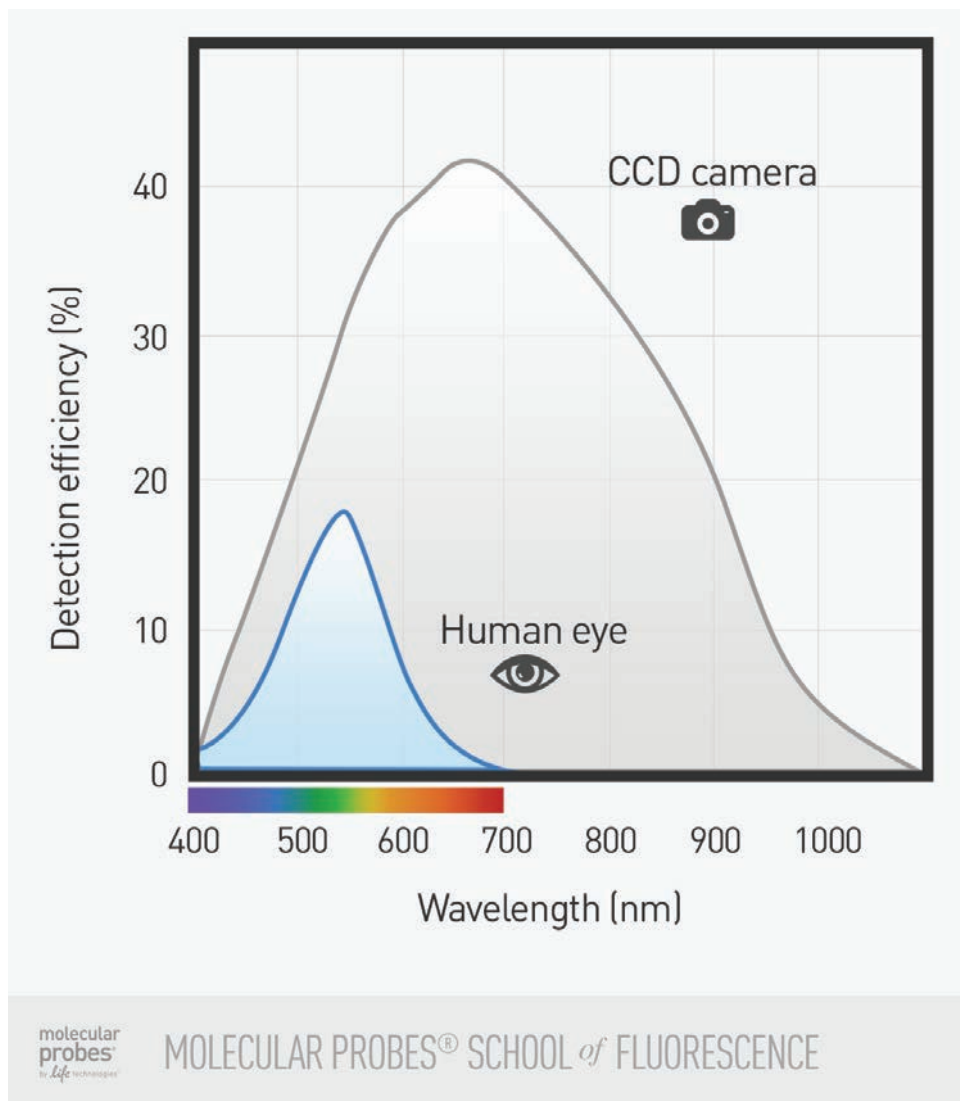
Figure 1.1. The electromagnetic spectrum, with visible wavelengths and their corresponding colors highlighted.





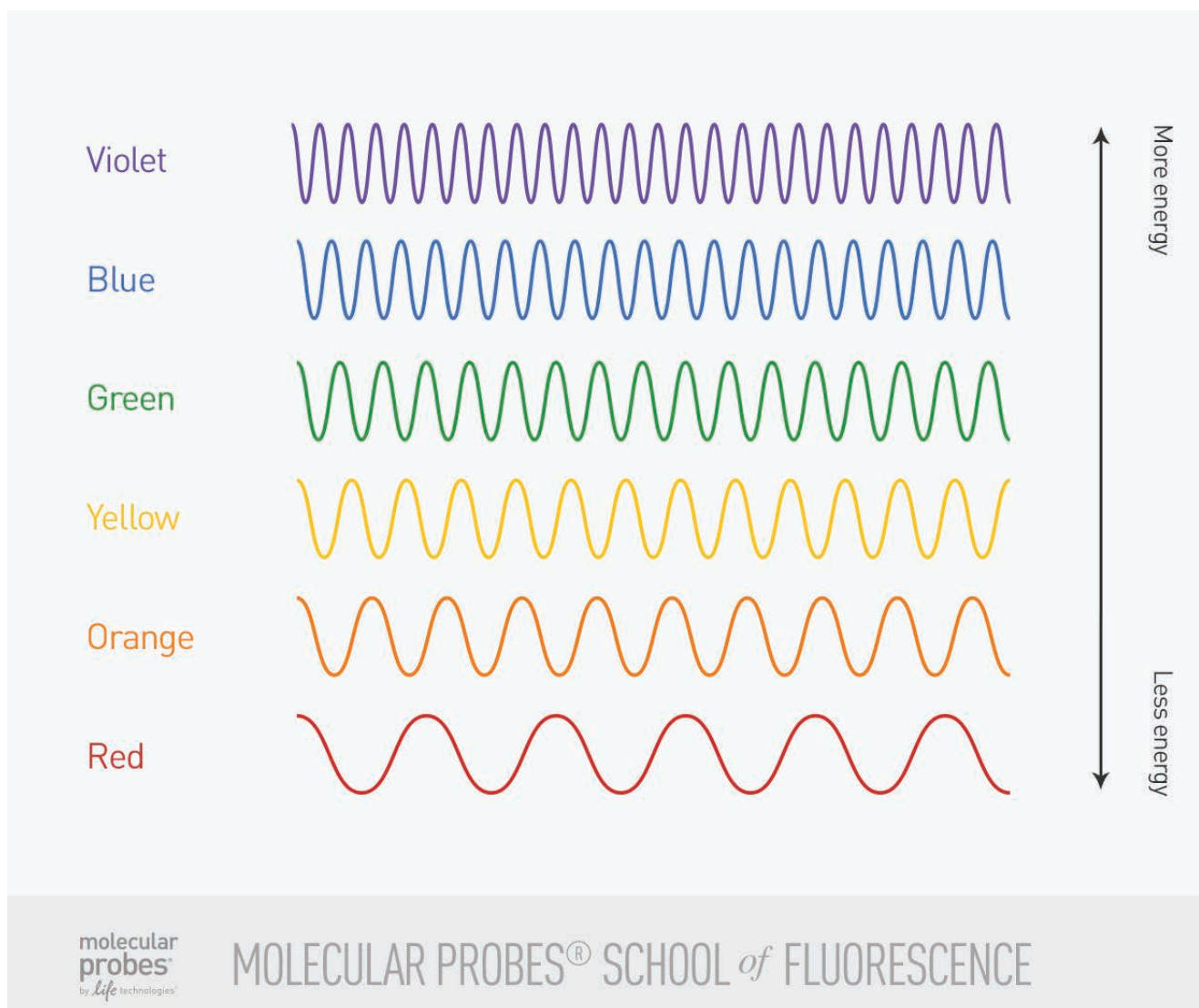
## Part One: Fundamentals of Fluorescence Microscopy

Figure 1.2. The range and efficiency of light detection for a CCD camera compared to that of the human eye.



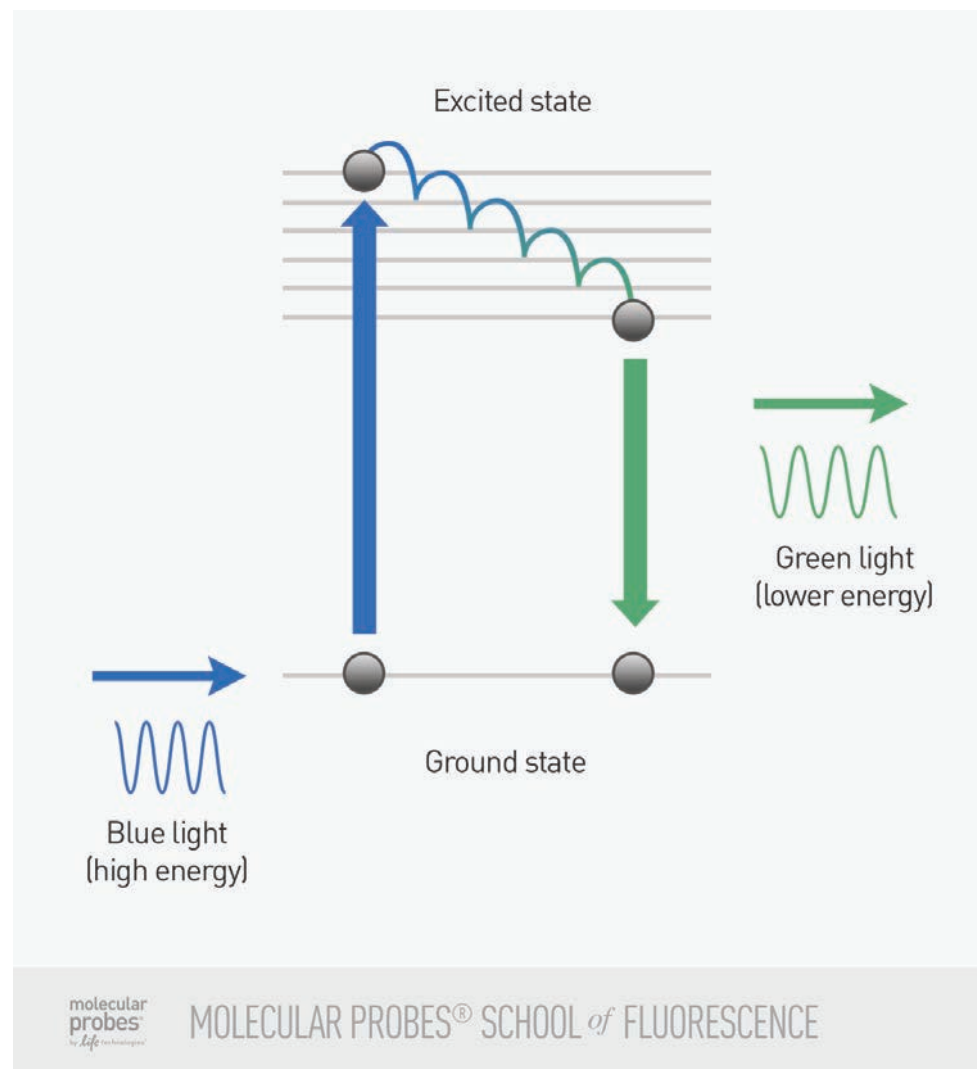
## Part One: Fundamentals of Fluorescence Microscopy

Figure 1.3. The inverse relationship between energy and wavelength in the visible spectrum.



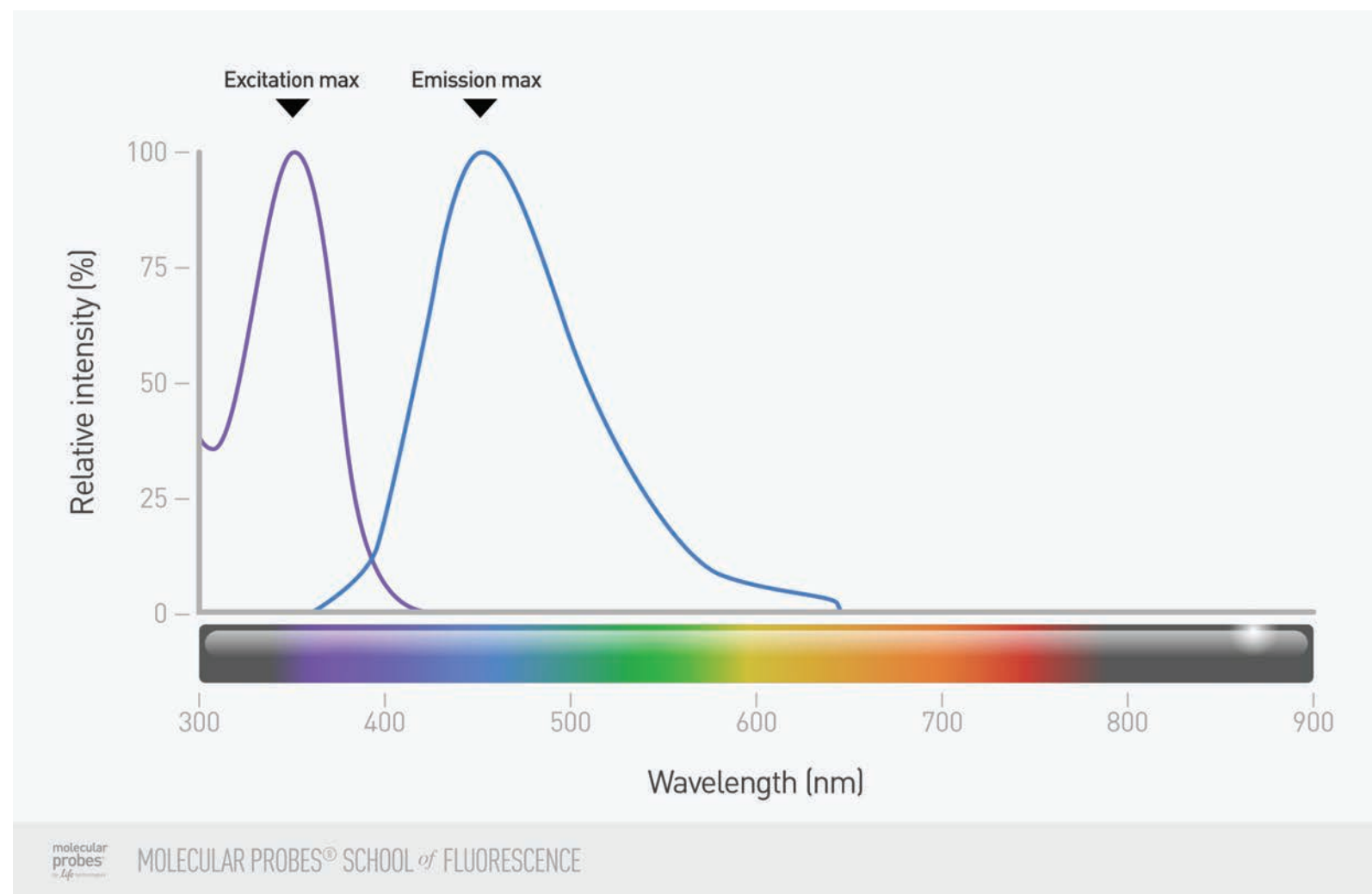
## Part One: Fundamentals of Fluorescence Microscopy

Figure 1.4. Simplified Jablonski diagram showing the energy state change of a fluorophore's electron as it undergoes fluorescence, with the corresponding change in the color of light.



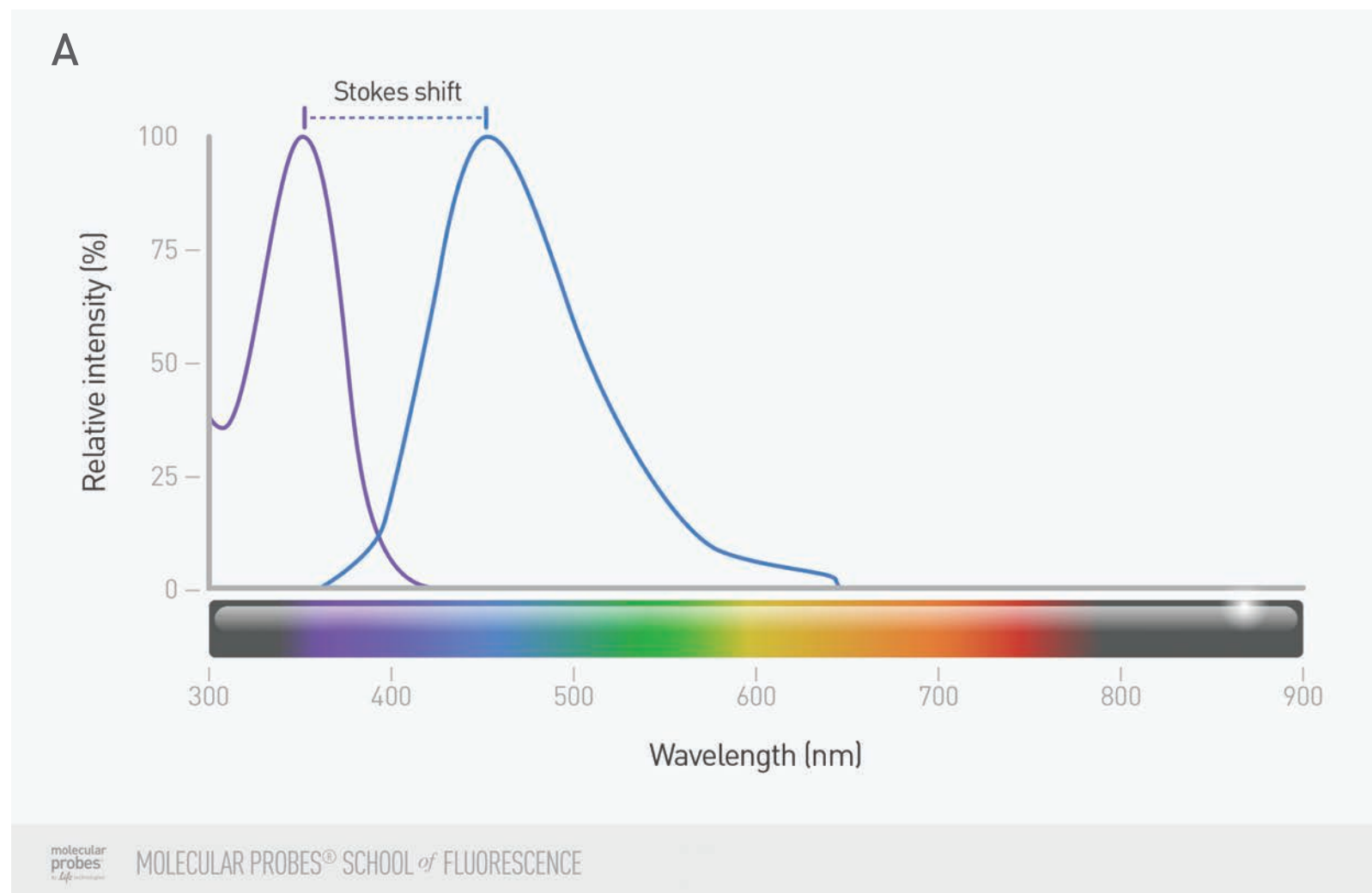
## Part One: Fundamentals of Fluorescence Microscopy

Figure 1.5. Excitation and emission spectra of a nuclear dye (DAPI). Shows both the fraction of light absorbed by the dye over a range of wavelengths (excitation, shown in purple) as well as the light emitted from the dye over a range of wavelengths (emission, shown in blue).



## Part One: Fundamentals of Fluorescence Microscopy

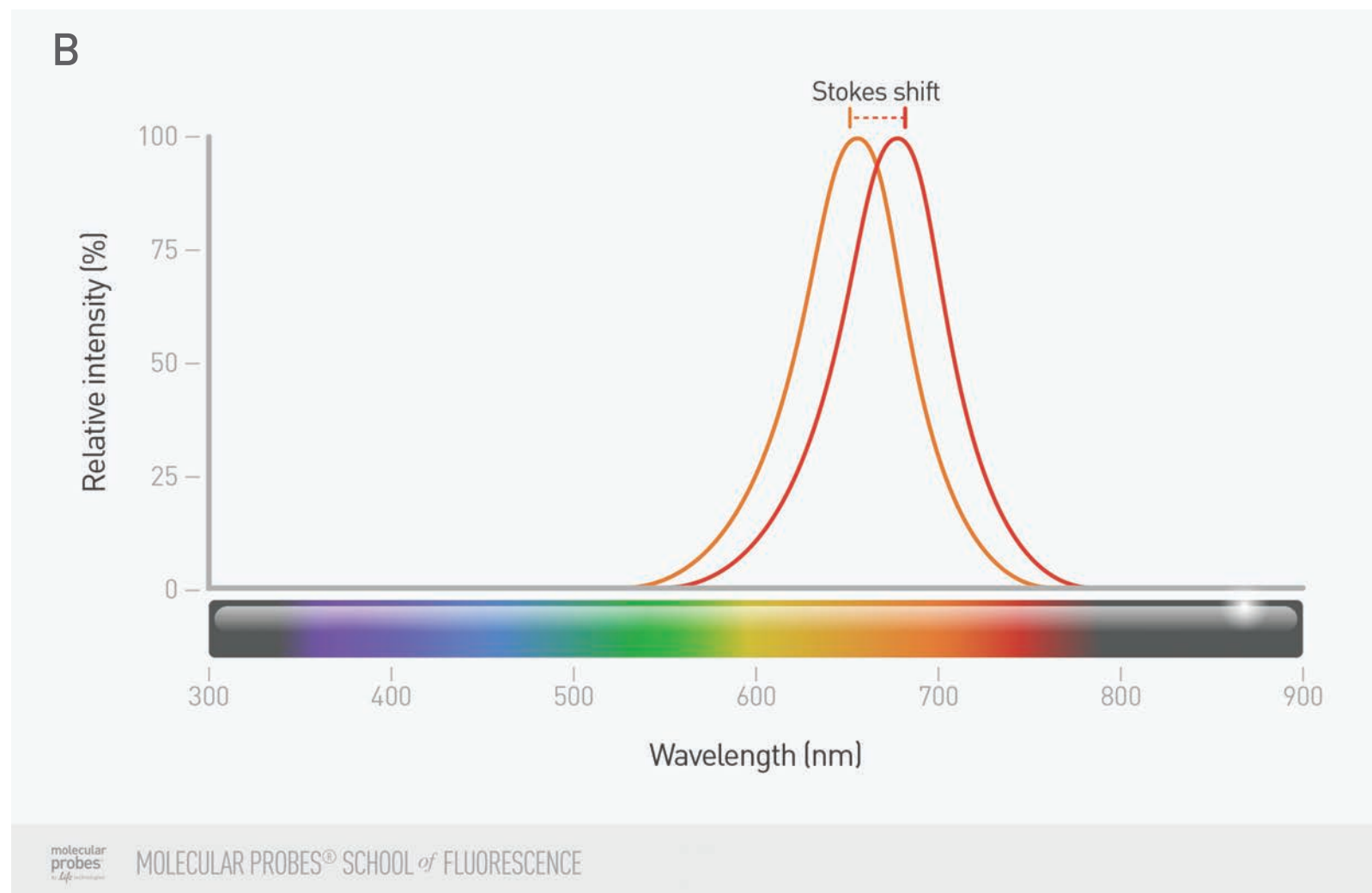
Figure 1.6A. A fluorophore with good separation between the excitation and emission maxima typically results in more reliable detection than a fluorophore with little separation. Compare the fluorophore with a large Stokes shift (A, purple and blue maximum peaks) to that of a fluorophore with a small Stokes shift (B, orange and red peaks).





## Part One: Fundamentals of Fluorescence Microscopy

Figure 1.6B. A fluorophore with good separation between the excitation and emission maxima typically results in more reliable detection than a fluorophore with little separation. Compare the fluorophore with a large Stokes shift (purple and blue maximum peaks) to that of a fluorophore with a small Stokes shift (orange and red peaks).



## Part One: Fundamentals of Fluorescence Microscopy

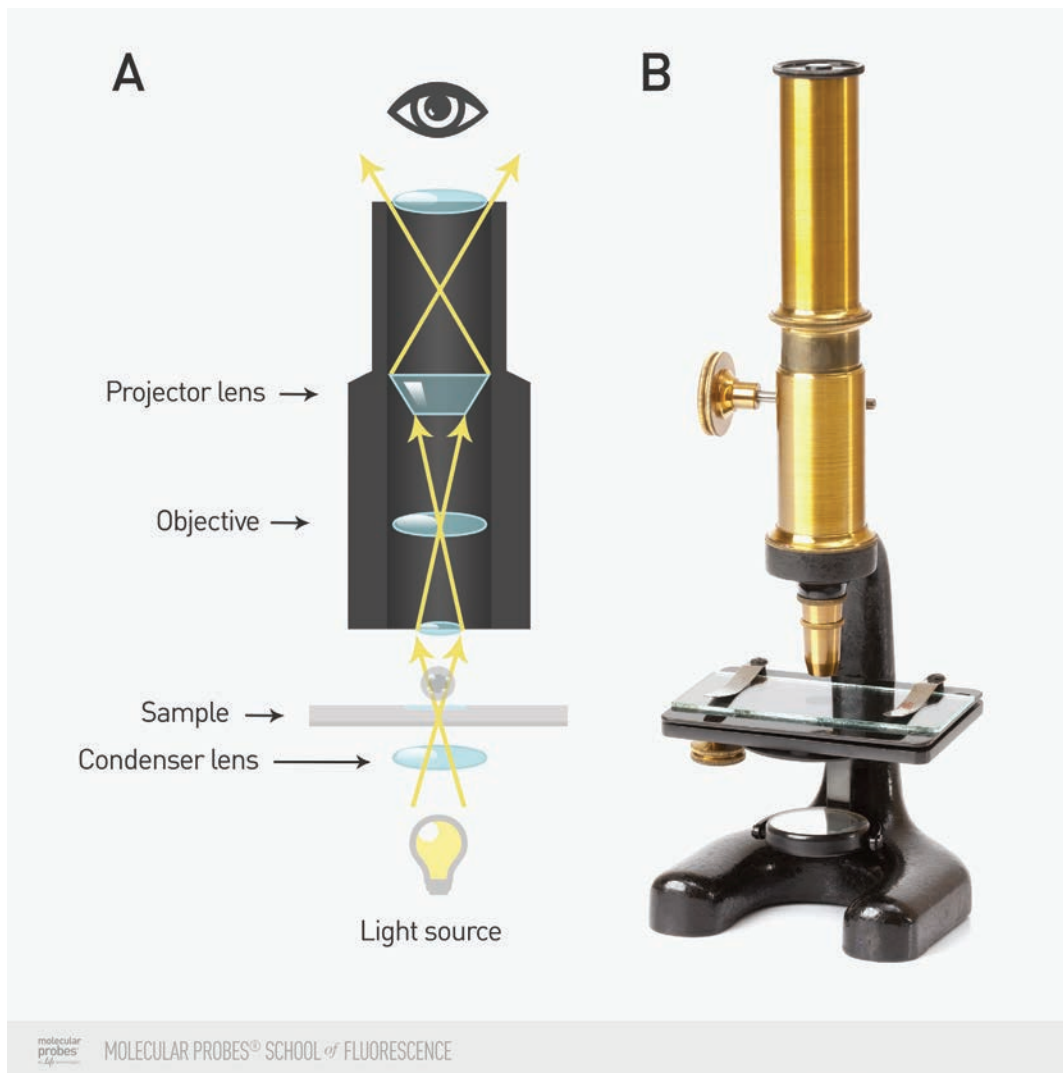
### 2. How Fluorescence Microscopy Works

Figure 2.1. A magnifying glass is the simplest form of lens used to view objects.



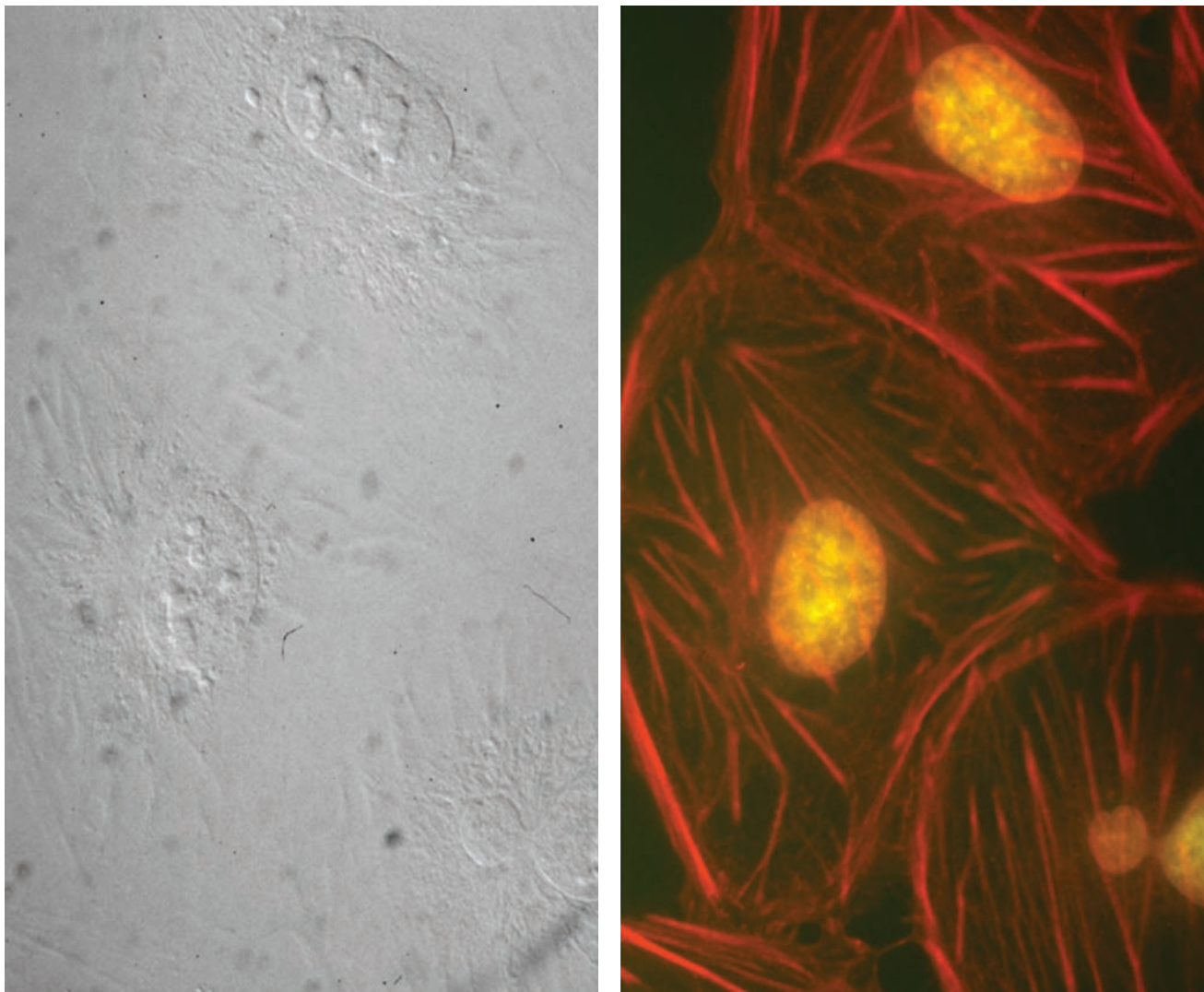
## Part One: Fundamentals of Fluorescence Microscopy

Figure 2.2. The light path through lenses and sample in basic brightfield microscopy (A). Antique 19th century drum-style compound microscope (B).



## Part One: Fundamentals of Fluorescence Microscopy

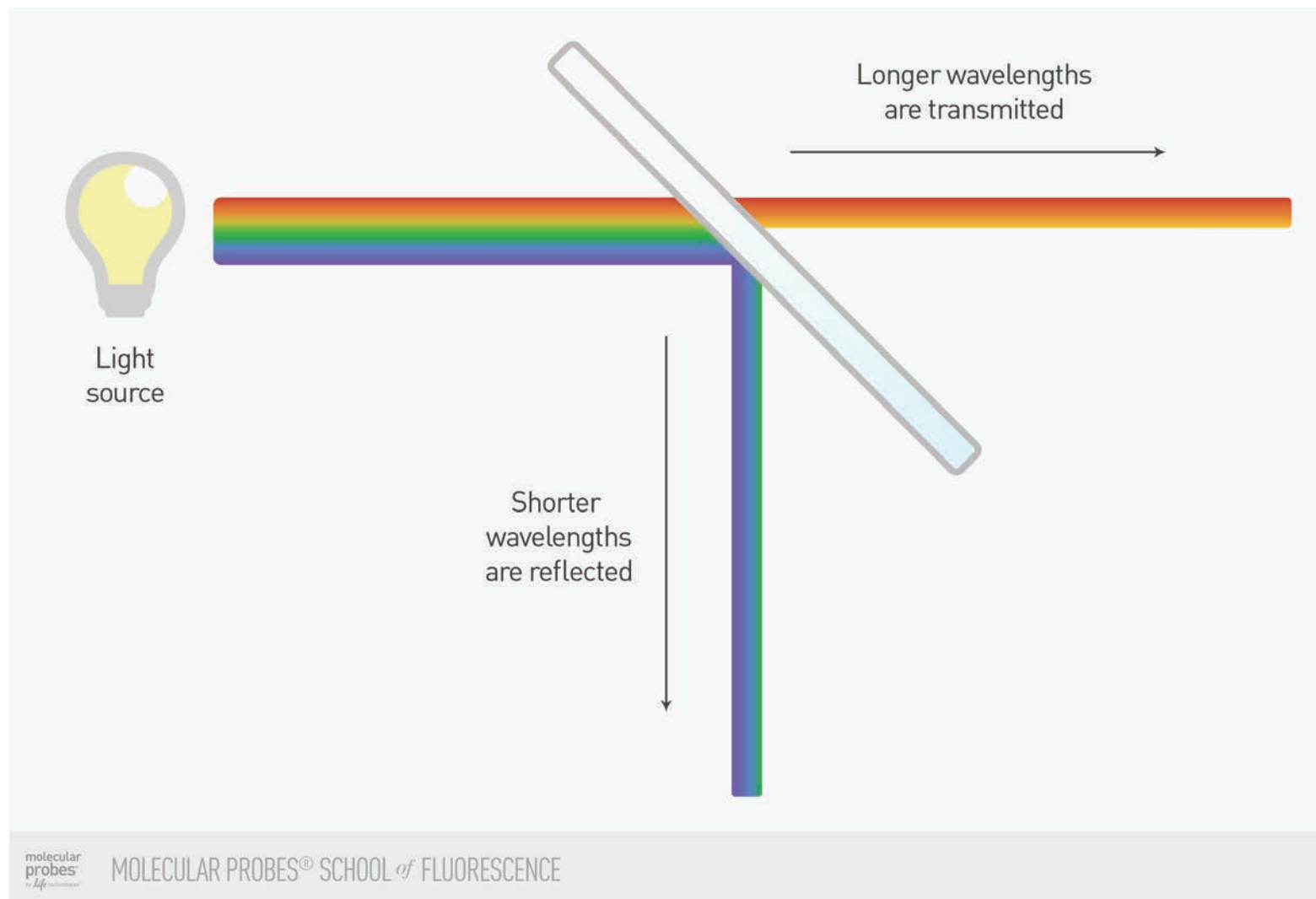
Figure 2.3. An image of the same field of BPAE cells captured using brightfield (left) and fluorescence (right) microscopy. Fluorescent labeling of the nucleus (yellow) and actin (red) makes it possible to see much more detailed cell structure.



## Part One: Fundamentals of Fluorescence Microscopy

### 3. Epifluorescence Microscope Basics

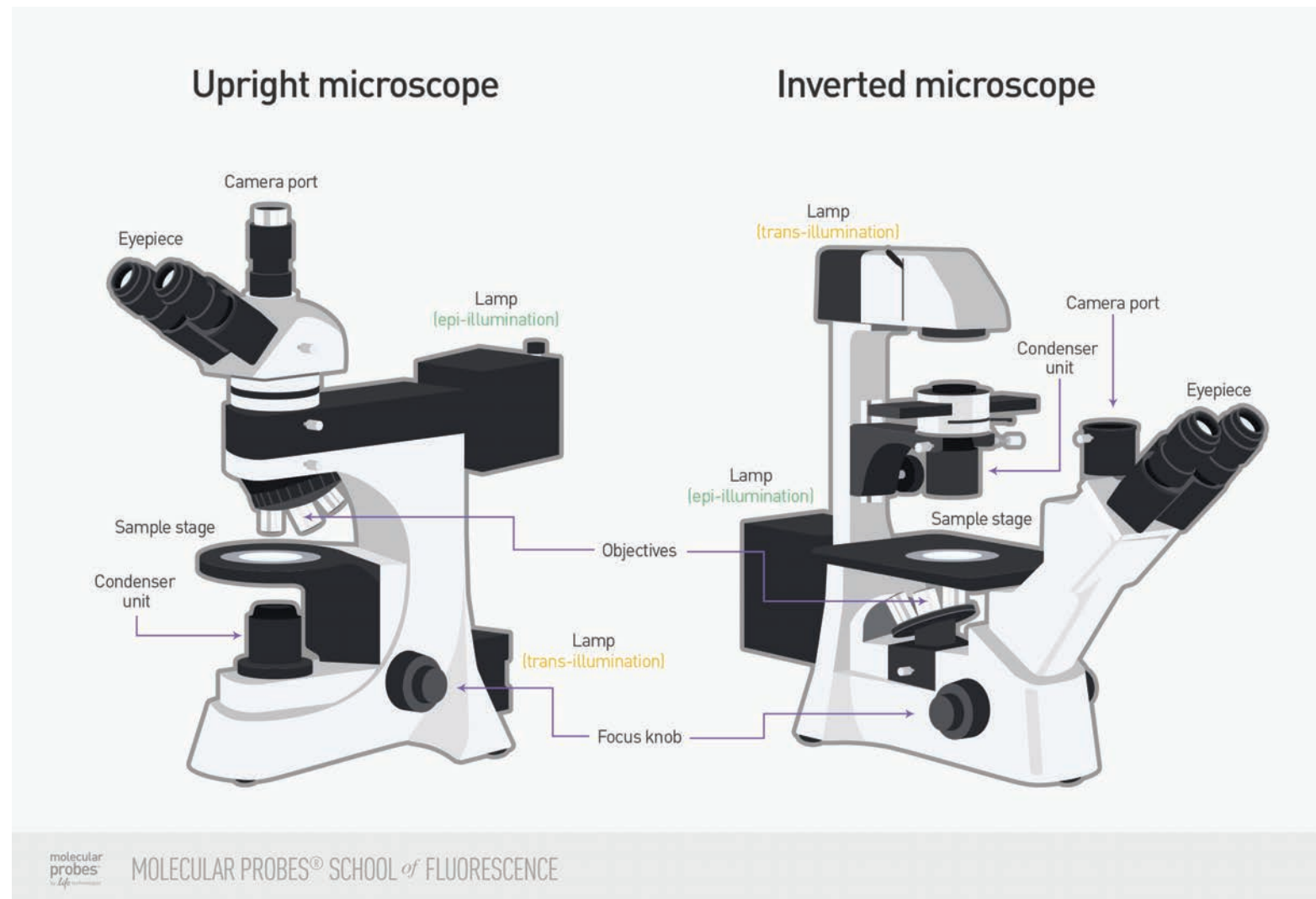
Figure 3.1 A dichroic beamsplitter allows longer wavelengths of light to pass through the filter while reflecting shorter wavelengths of light.





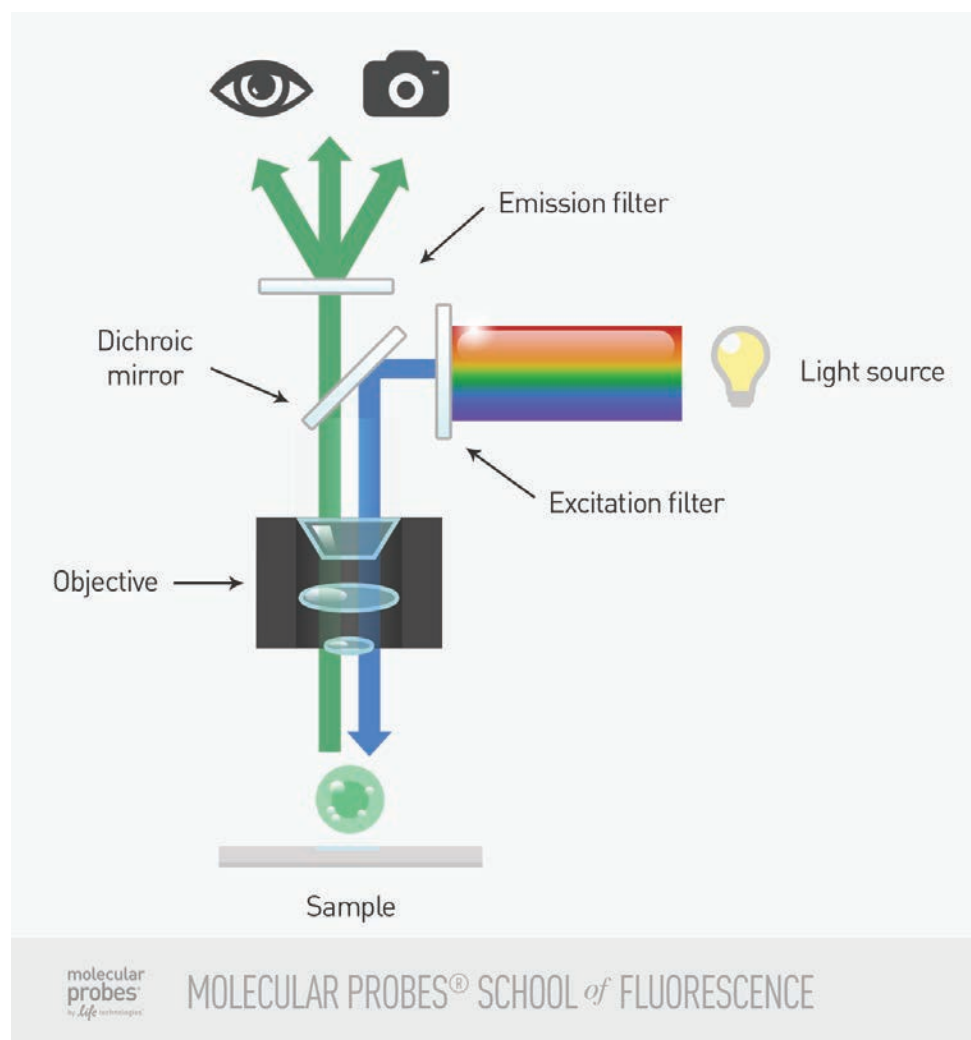
## Part One: Fundamentals of Fluorescence Microscopy

Figure 3.2. Inverted and upright microscopes both utilize epifluorescent illumination: the main difference is the location of the objectives relative to the stage where the sample is placed.



## Part One: Fundamentals of Fluorescence Microscopy

Figure 3.3 Typical light path in an epifluorescence microscope. Notice that the both excitation and emission are controlled by the dichroic, which reflects excitation light (shorter wavelengths) onto the sample and passes the resulting emission light (longer wavelengths) through the filter and on to the detector (the viewer or the camera).



## Part One: Fundamentals of Fluorescence Microscopy

Figure 3.4A. Two 6  $\mu\text{m}$  beads taken at 3 different magnifications, 4x, 10x, and 40x.

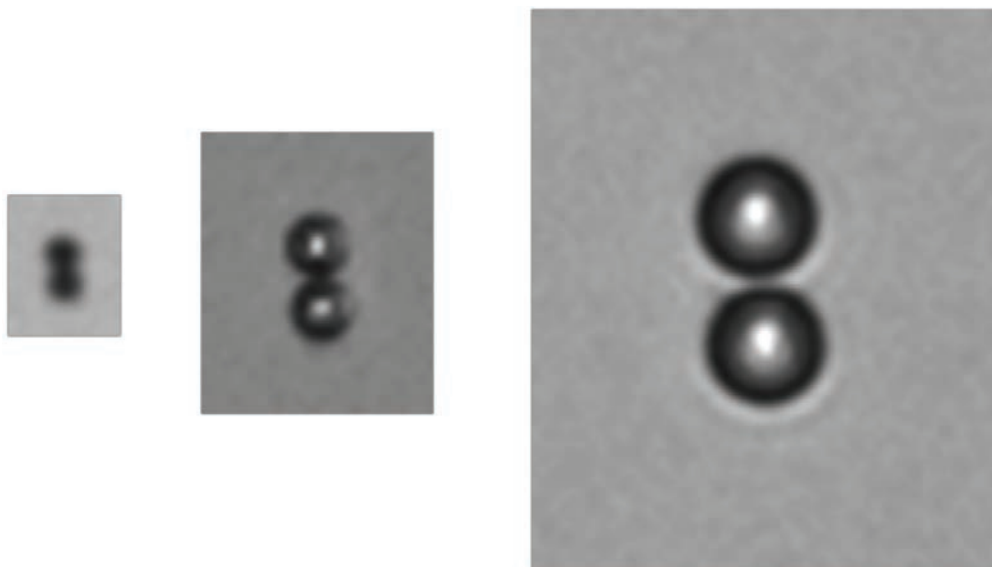
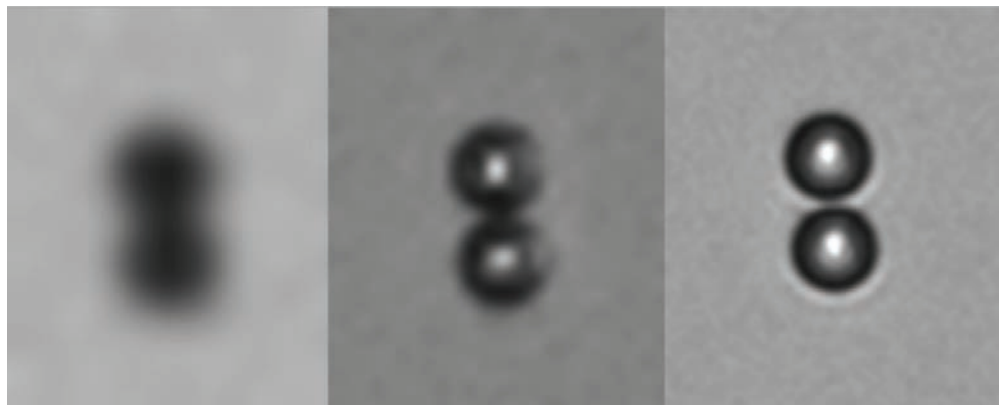
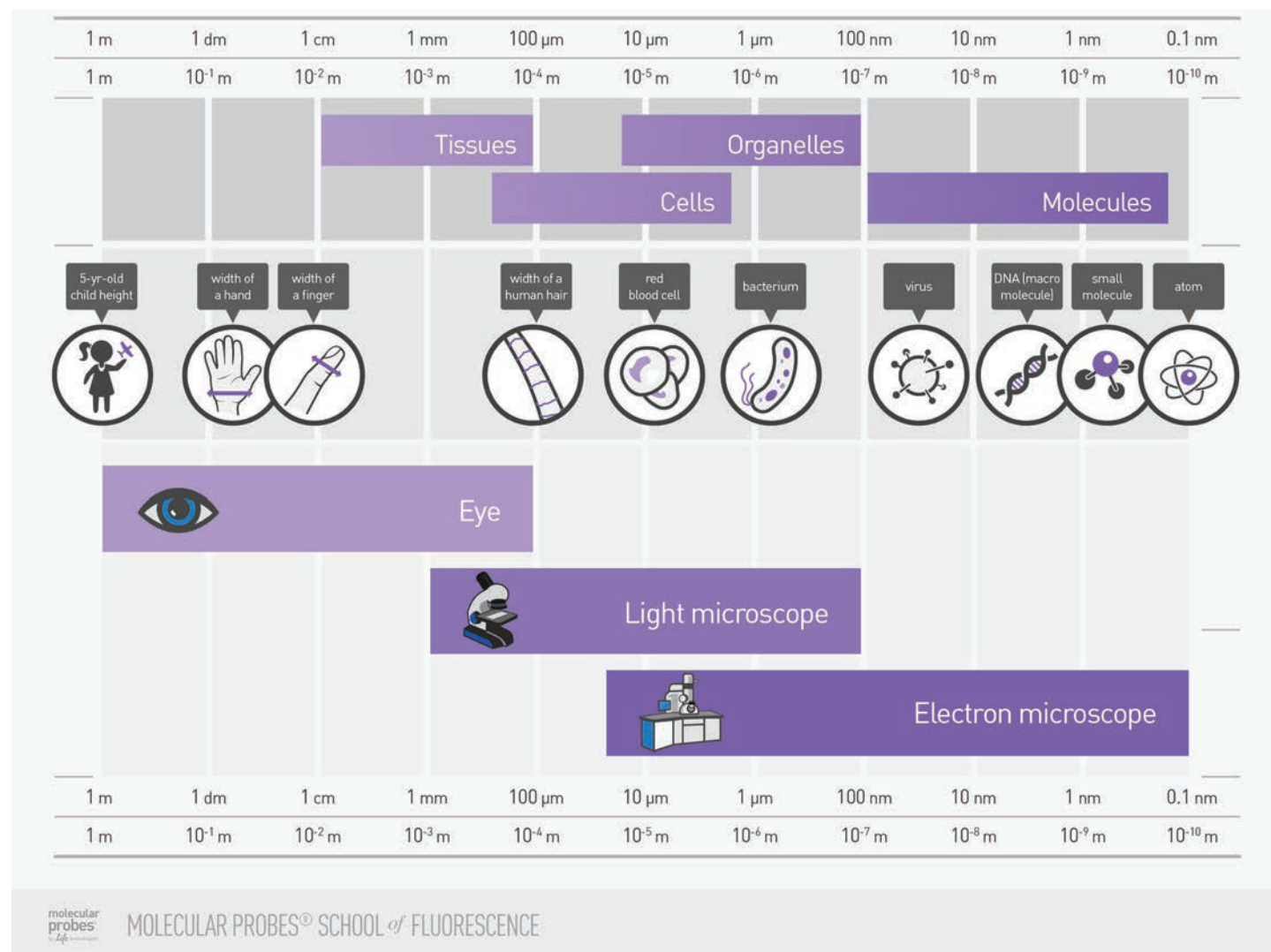


Figure 3.4B.



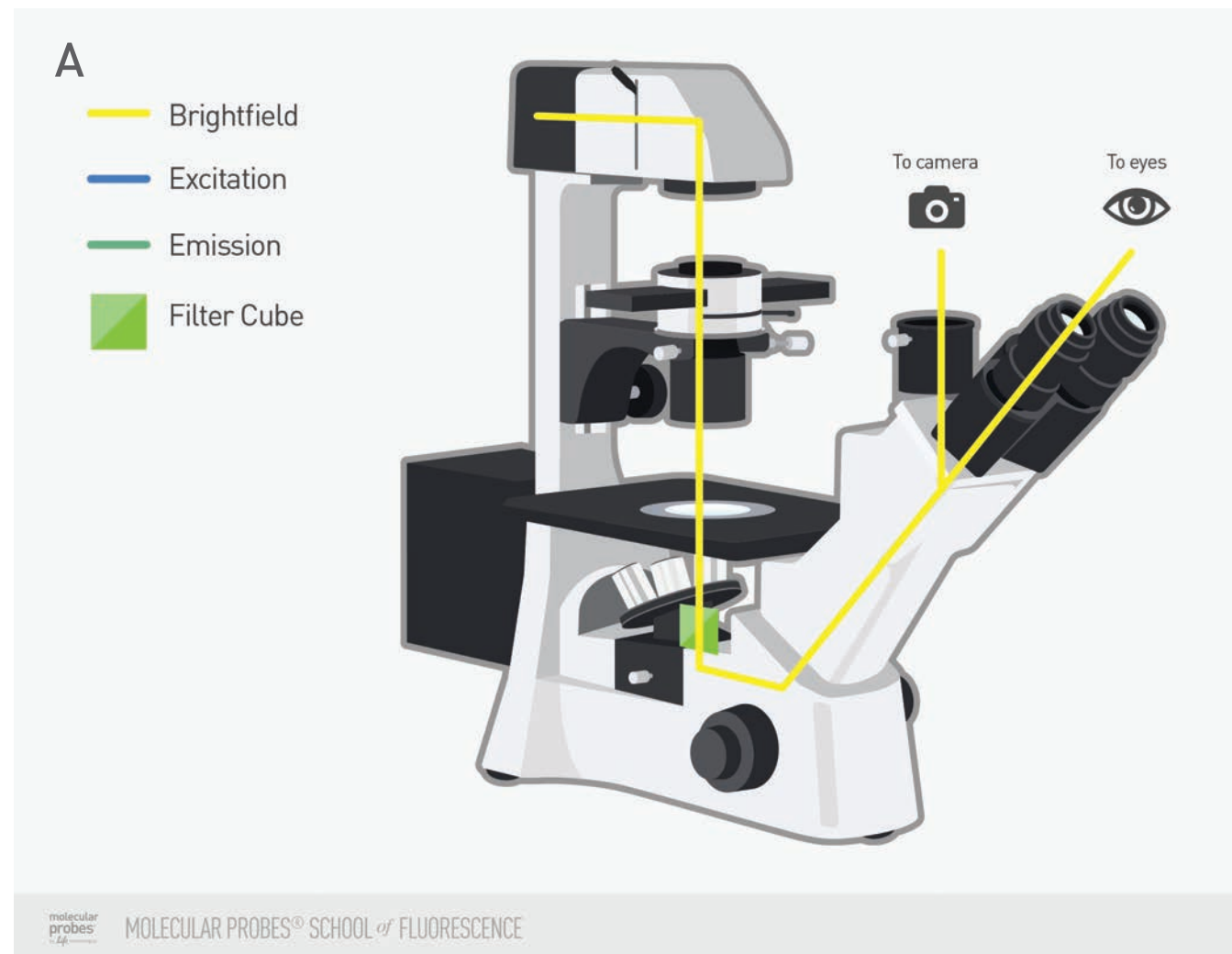
## Part One: Fundamentals of Fluorescence Microscopy

Figure 3.6. The resolving power of various microscopes, with representative objects within range for both light microscopes and electron microscopes.



## Part One: Fundamentals of Fluorescence Microscopy

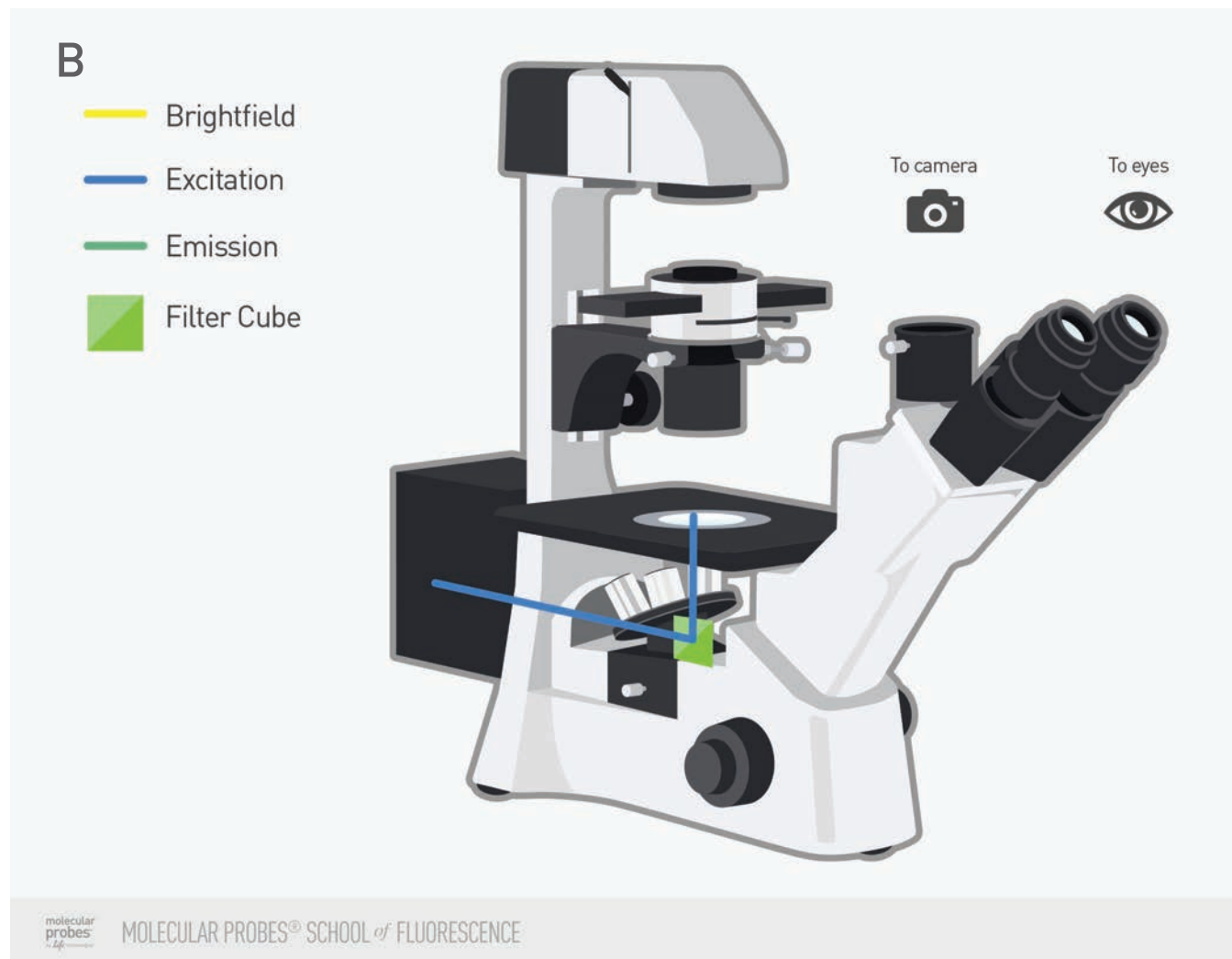
Figure 3.7A. The yellow line represents the light path for brightfield illumination. All of the illumination light does not travel through the objective, only the light that is transmitted through the sample. For this reason, images acquired using brightfield illumination are sometimes referred to as "transmitted" images.





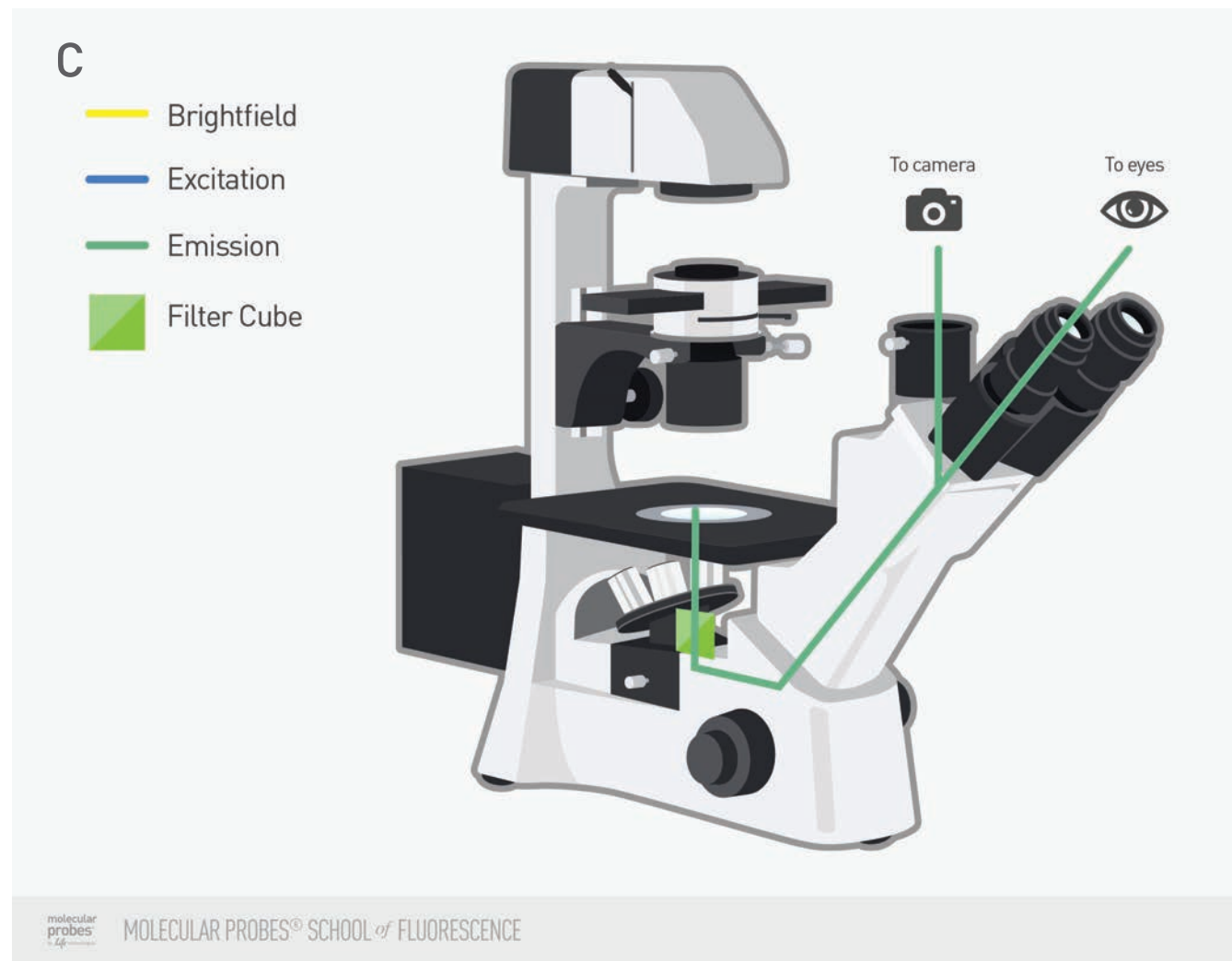
## Part One: Fundamentals of Fluorescence Microscopy

37.B The blue line illustrates the path of excitation light, which travels through the filter cube and the objective to the sample. In epifluorescence microscopy, both the excitation and emission light travel through the same objective.



## Part One: Fundamentals of Fluorescence Microscopy

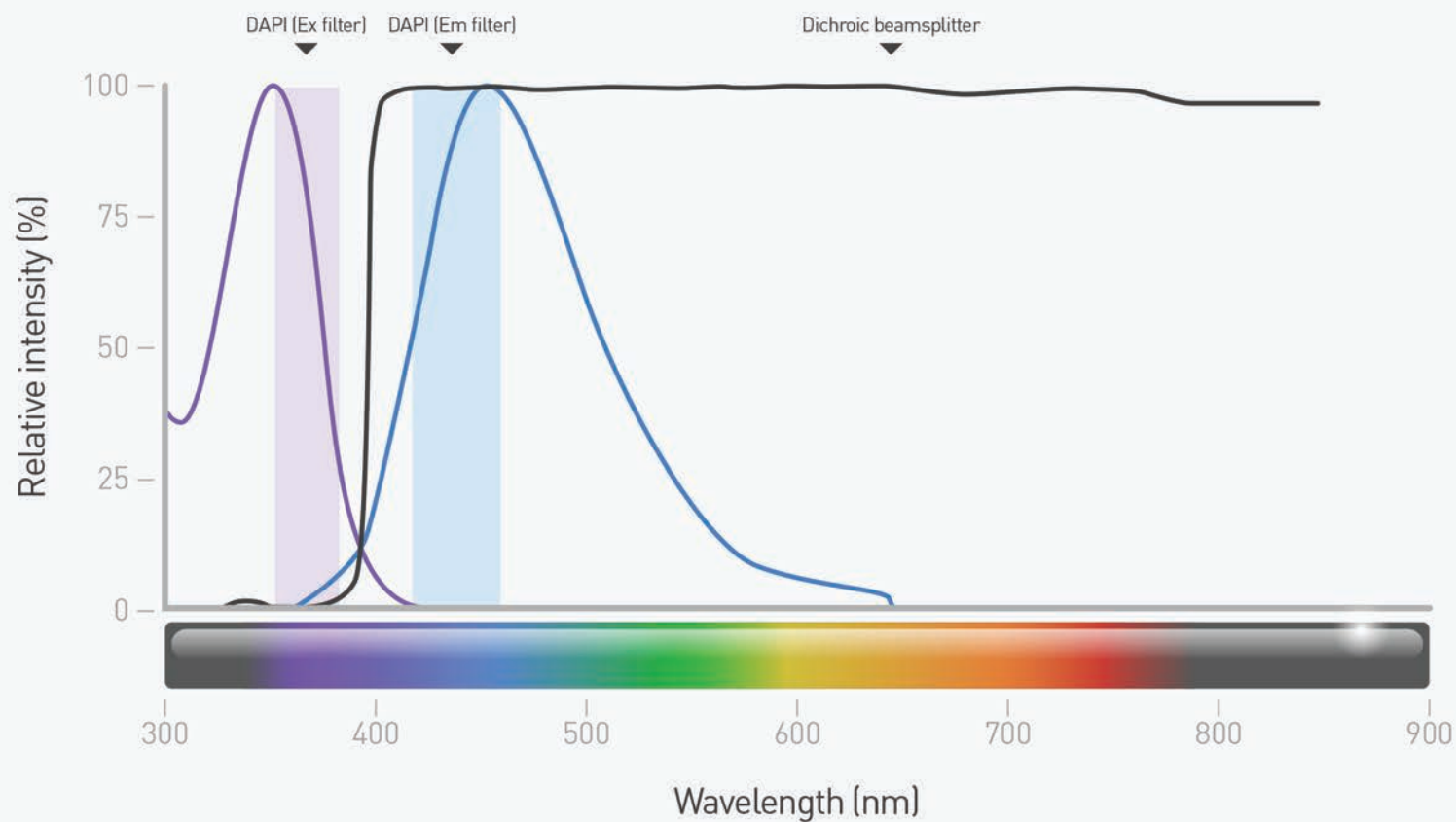
3.7C The green line illustrates the path for light emitted from the fluorescent sample upon excitation. It simultaneously travels through the objective and filter cube and onto the detectors. In epifluorescence microscopy, both the excitation and emission light travel through the same objective.



## Part One: Fundamentals of Fluorescence Microscopy

### 4. Using Filters to Capture Your Signal

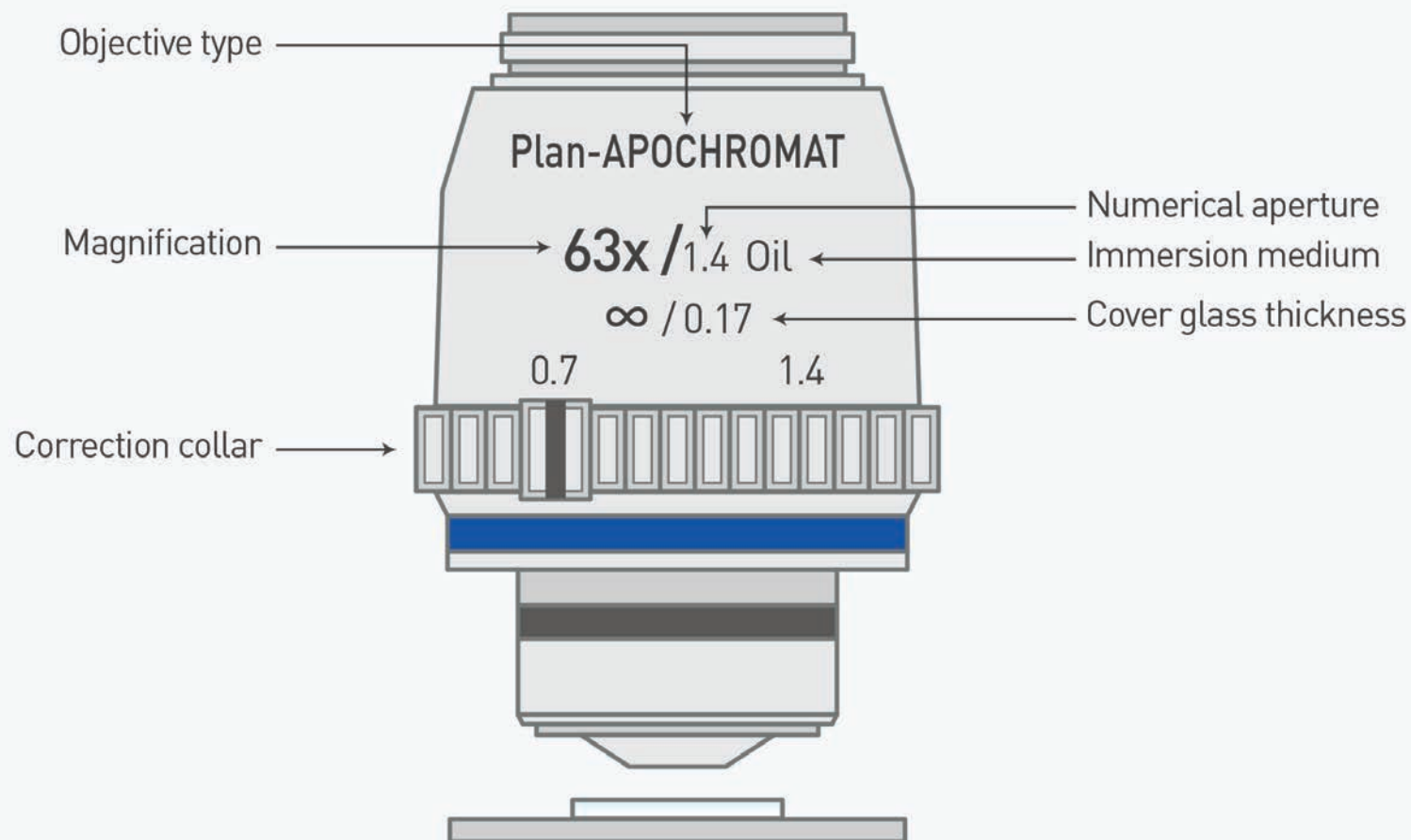
Figure 4.1. Excitation and emission spectra of a nuclear dye [DAPI] overlaid with the range of wavelengths passed through the filters for emission (purple box) and excitation (blue box). The black line depicts the transmission of the dichroic filter.



## Part One: Fundamentals of Fluorescence Microscopy

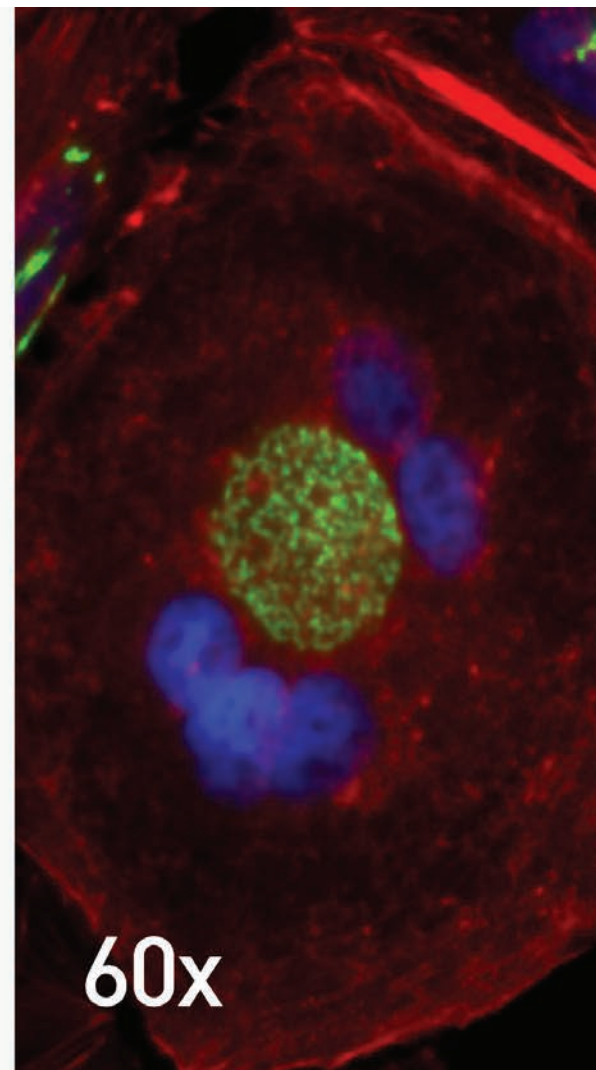
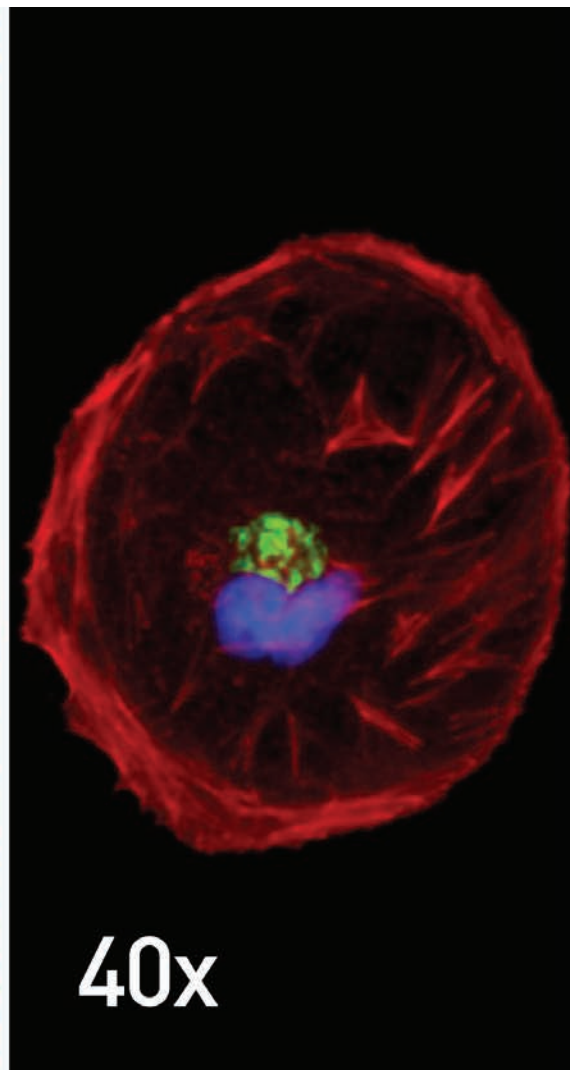
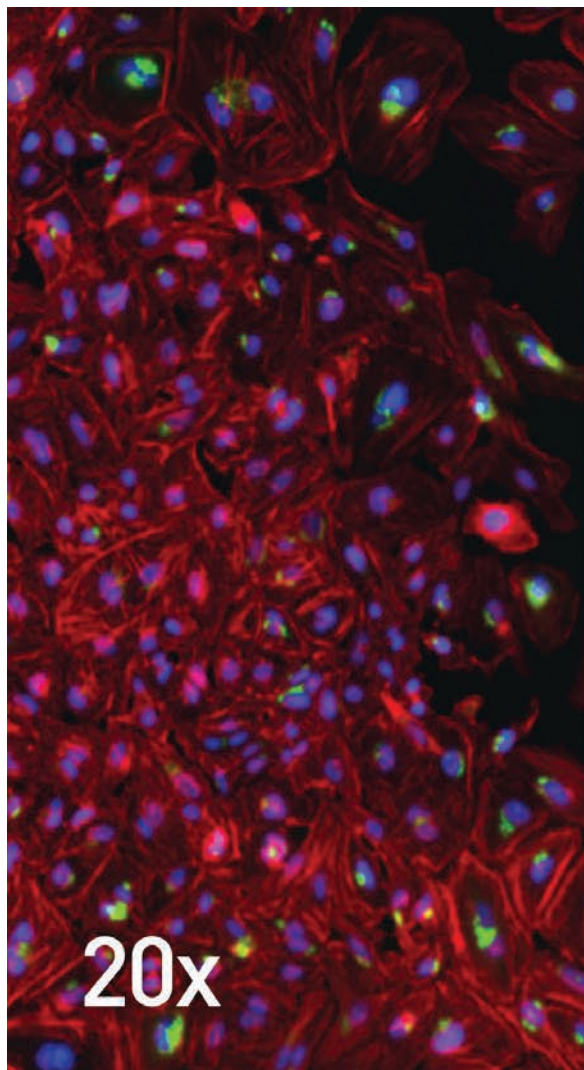
### 5. Understanding Your Objective

Figure 5.1. Common notations found on objectives and what they mean.



## Part One: Fundamentals of Fluorescence Microscopy

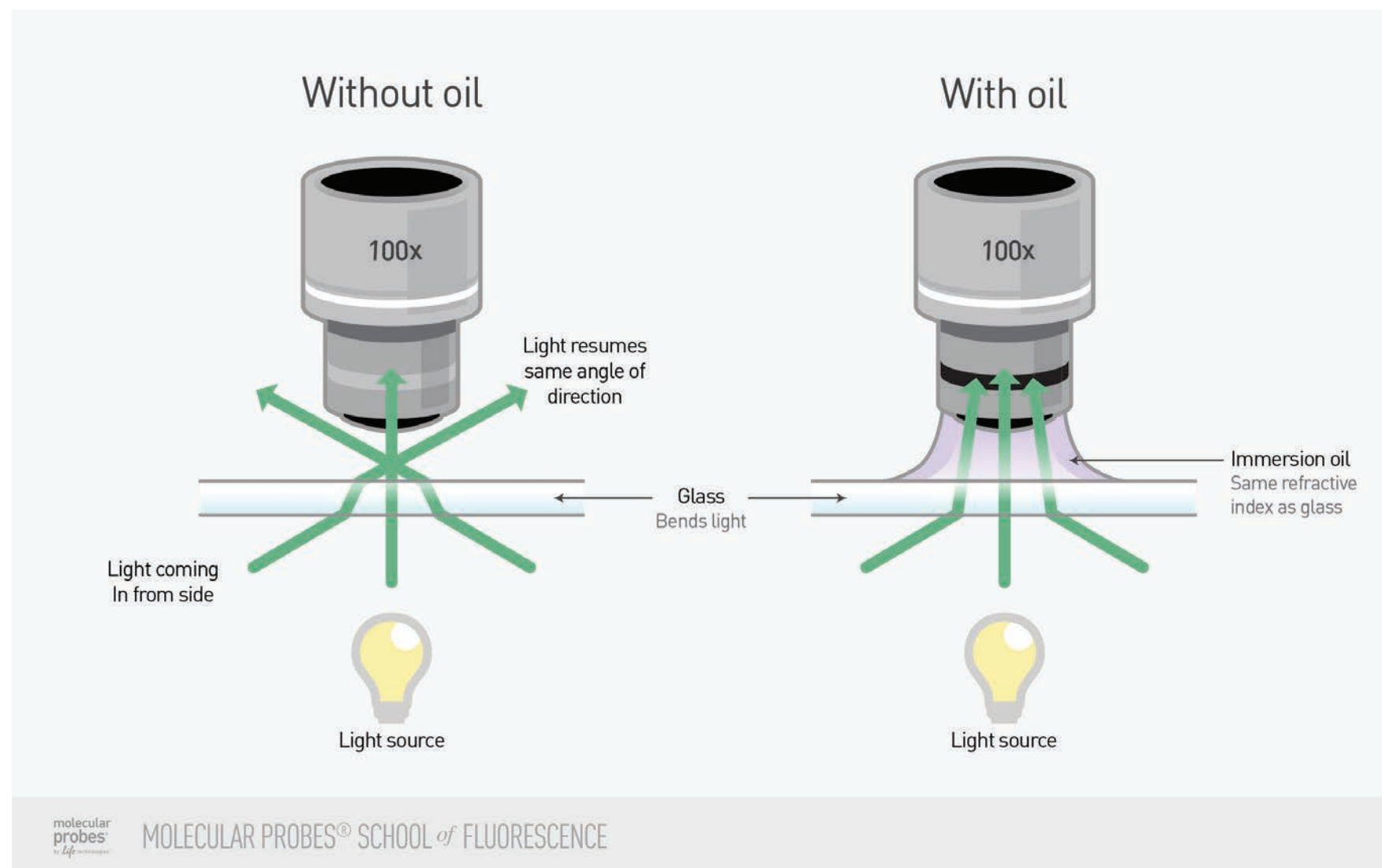
Figure 5.2. Same field of cells captured at different magnifications. Each magnification can offer different information, and the best choice for your experiment will vary depending on what you want to know.





## Part One: Fundamentals of Fluorescence Microscopy

Figure 5.3. Use of immersion media matched to the objective can minimize the refractive index differences between the objective and the sample.



## Part One: Fundamentals of Fluorescence Microscopy

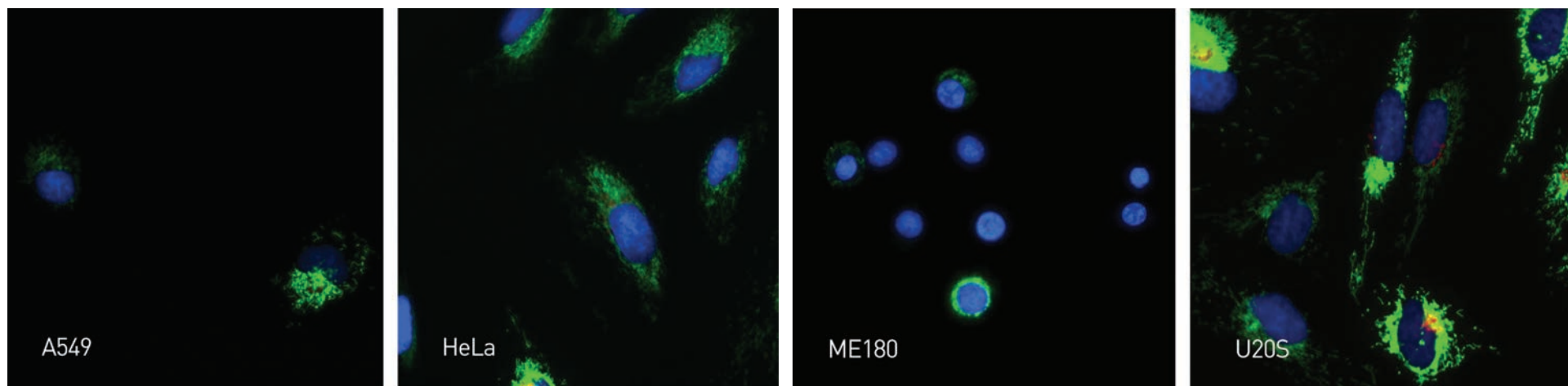
Figure 5.4. The pencil appears bent or broken because the refractive indexes of water and glass are different than that of air.



## Part Two: Sample Considerations

### 6. Cell Morphology

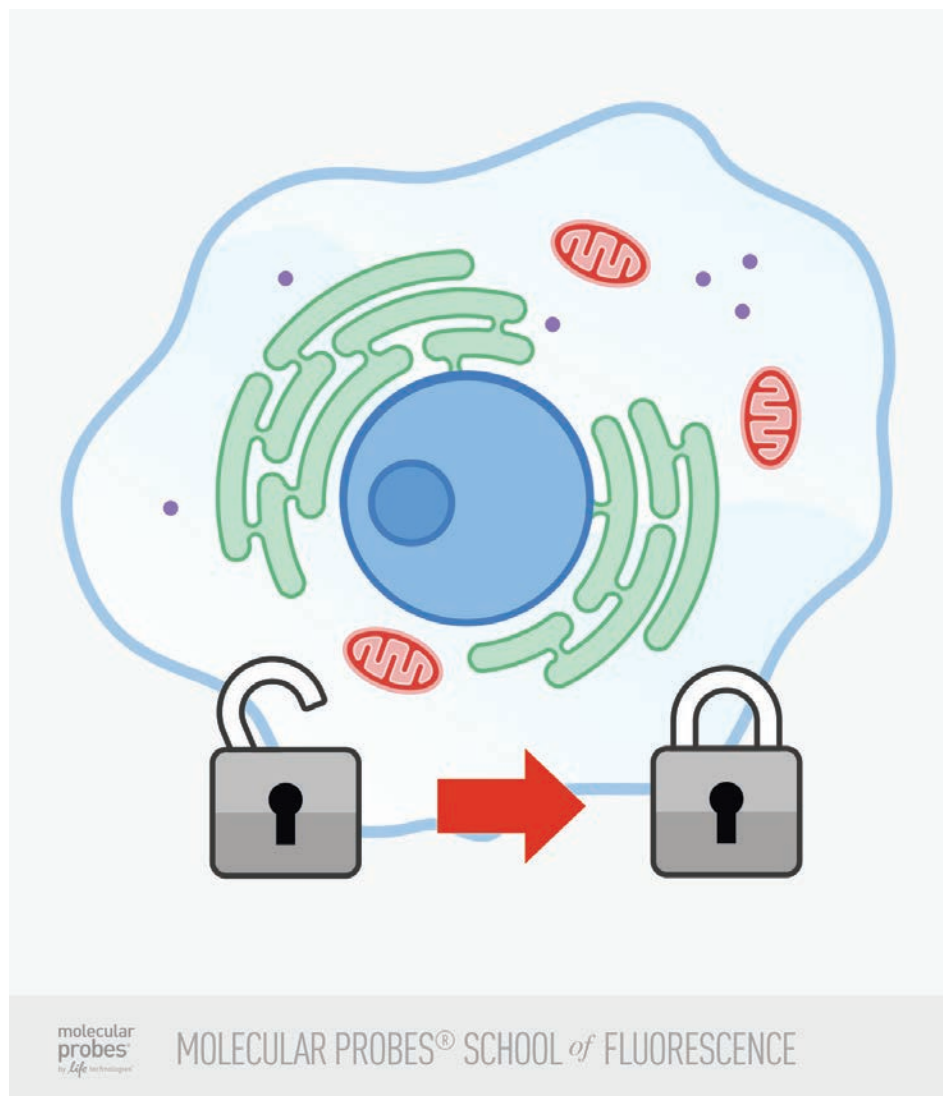
Figure 6.1. A549, HeLa, ME180, and U2OS cells all exhibit very different staining patterns for the same set of fluorescent reagents due to differences in their morphology and metabolic pathways. All four cell types were stained with NucBlue Live® reagent after transduction with CellLights® Golgi-RFP and Mito-GFP reagents.



## Part Two: Sample Considerations

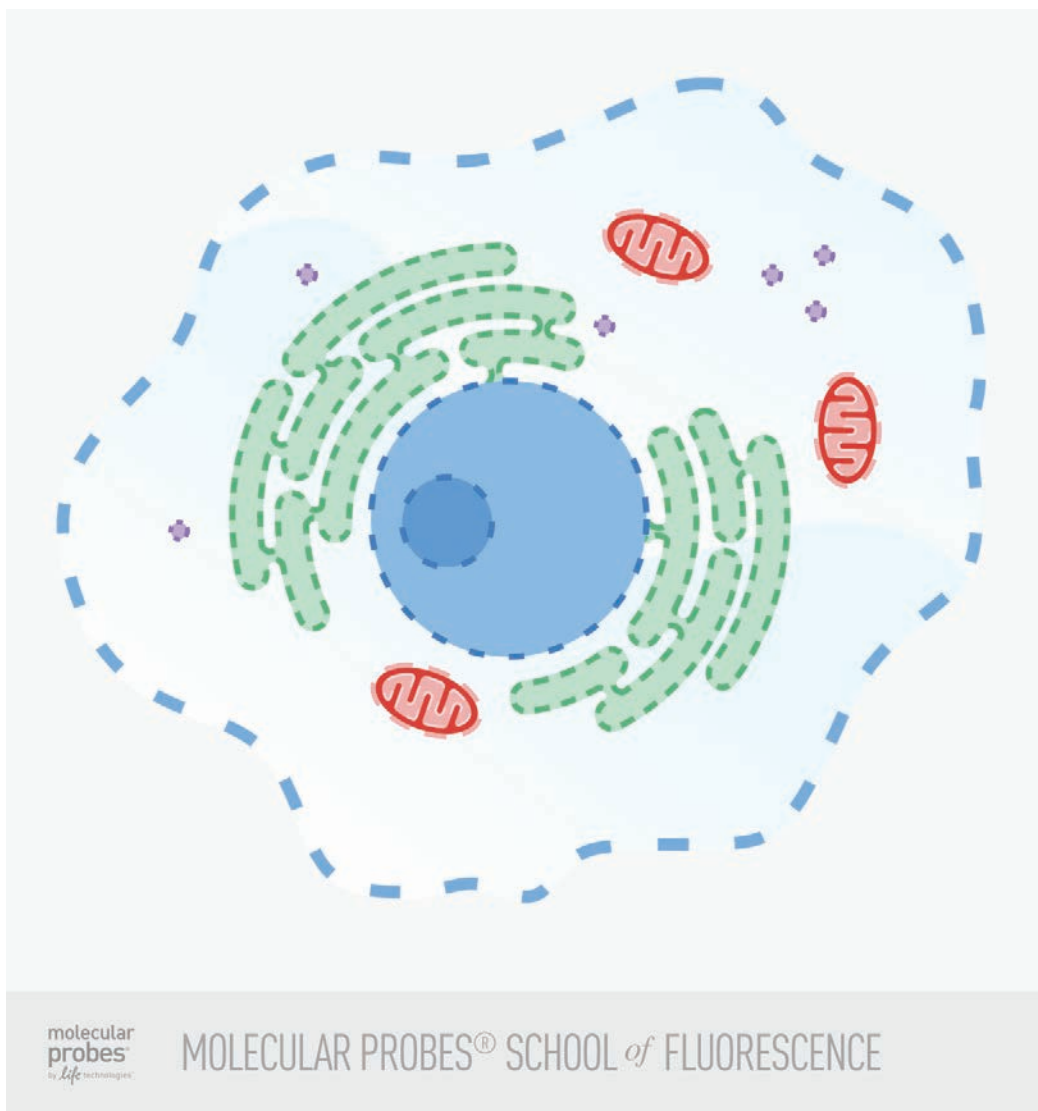
### 7. Preparing Fixed Cells for Labeling

Figure 7.1. Formaldehyde fixation essentially locks cellular structures in place.



## Part Two: Sample Considerations

Figure 7.2. The removal of cellular membrane lipids in the permeabilization step allows large molecules access to the interior of the cell.



## Part Two: Sample Considerations

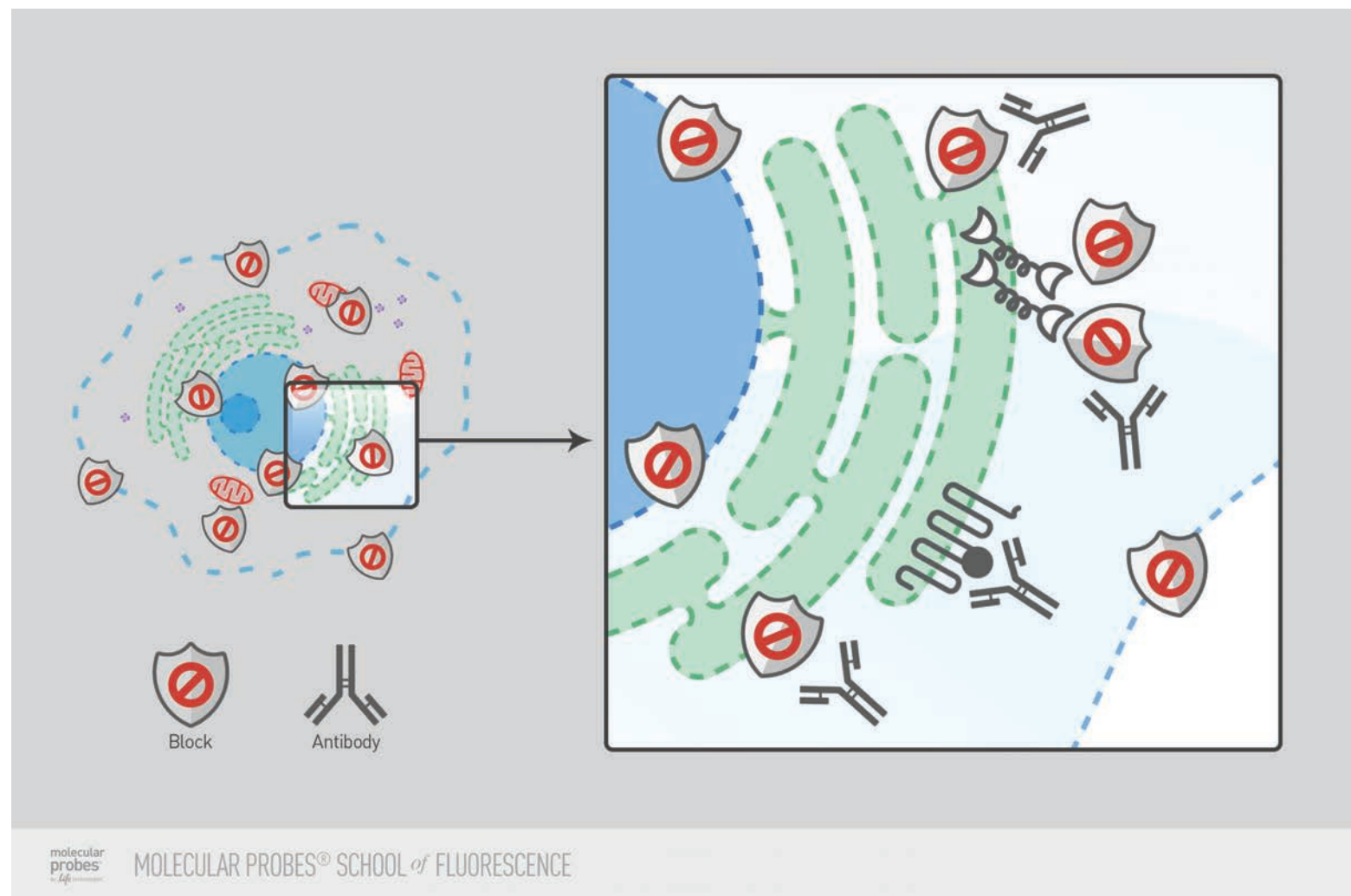
Figure 7.3. The use of protein-based blocking agents reduces nonspecific staining.





## Part Two: Sample Considerations

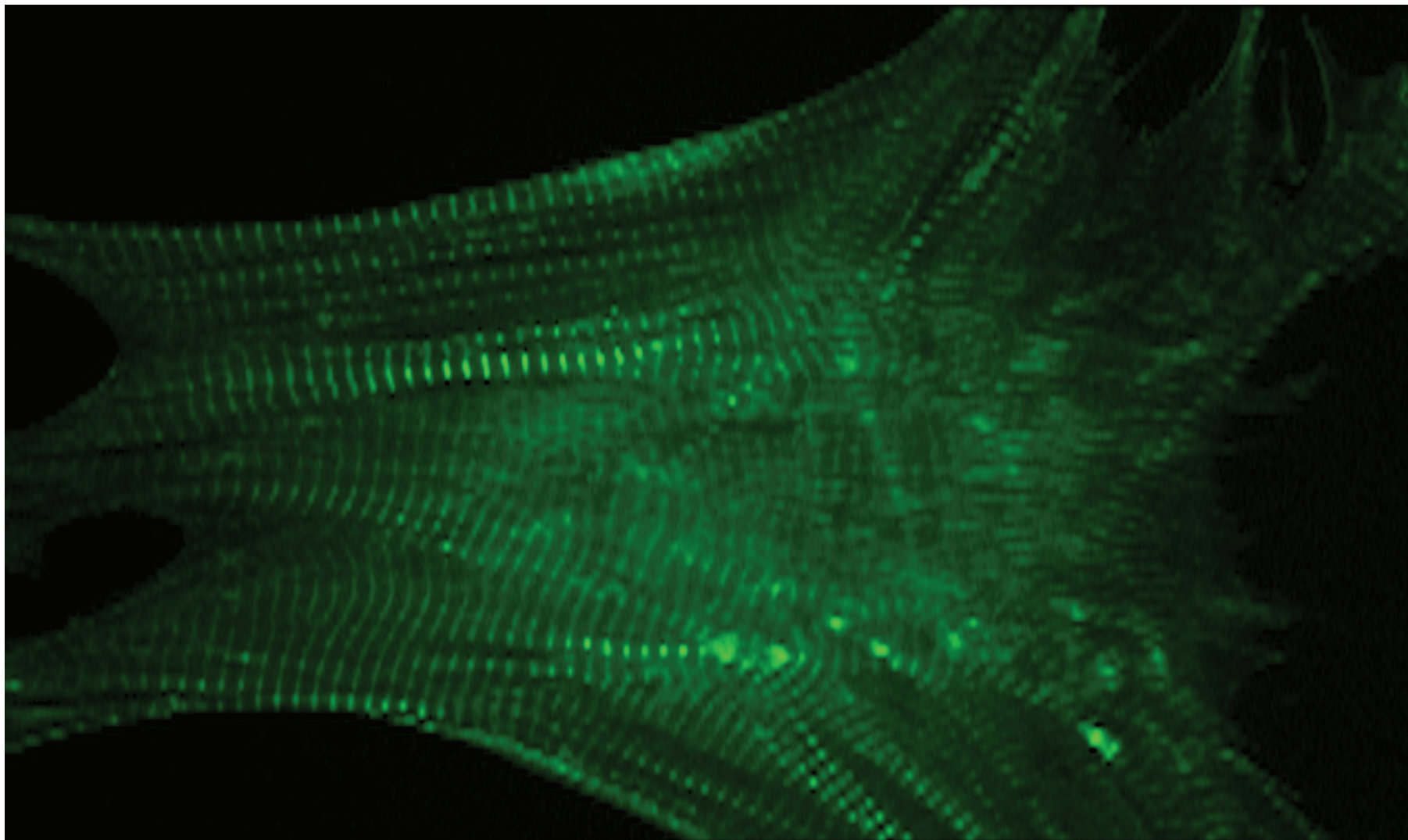
Figure 7.4. Protein-based blocking agents help reduce non-specific staining. Antibodies are able to displace the blocking proteins to form a high-affinity bond with their epitopes, while blocking proteins prevent low-affinity antibody interactions elsewhere in the sample.



## Part Two: Sample Considerations

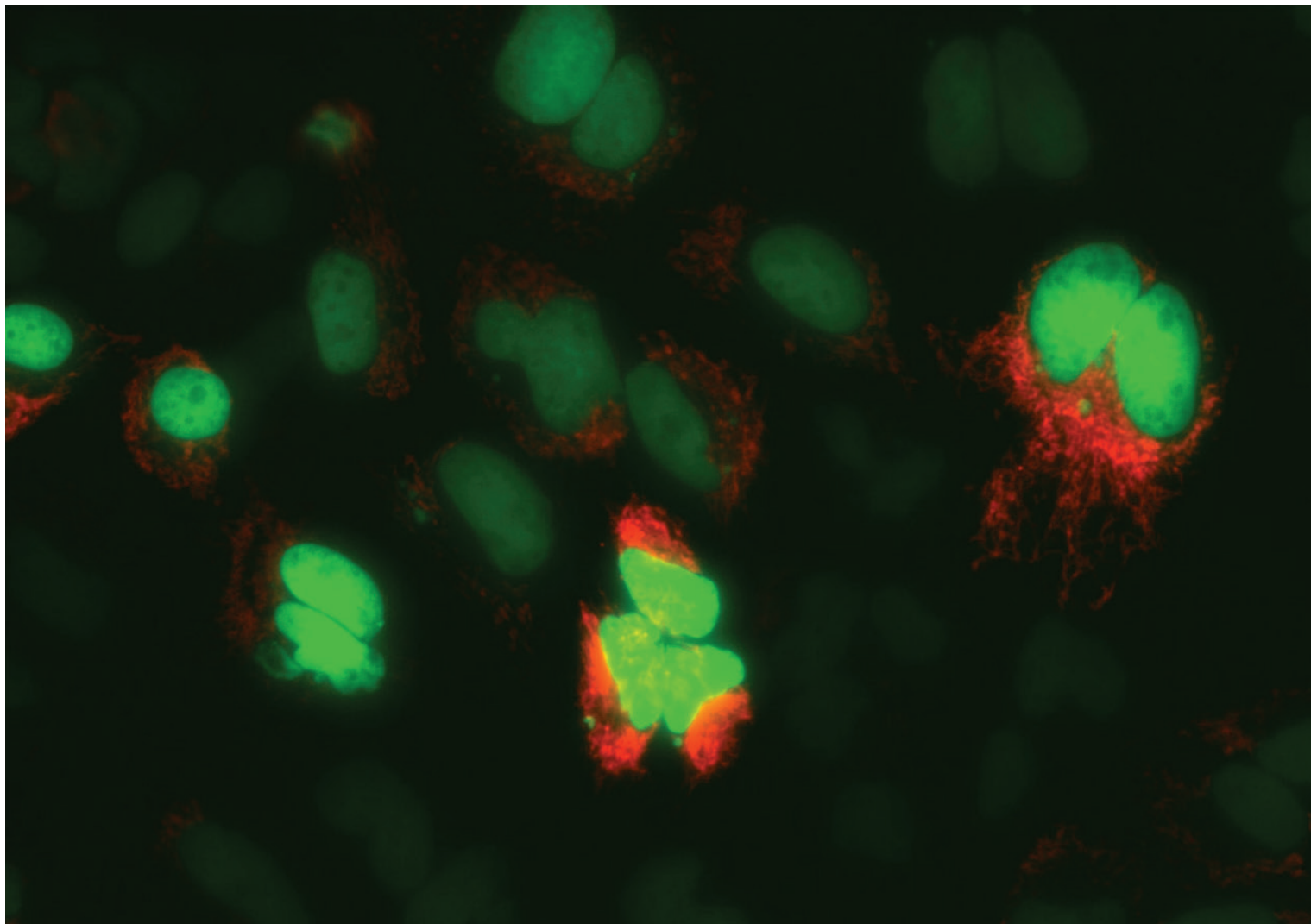
### 8. Preparing for Live-Cell Imaging

Figure 8.1. A still image captured from a movie of spontaneous contractions of mouse embryonic stem cell-derived cardiomyocytes, transduced with CellLights® Actin-GFP.



## Part Two: Sample Considerations

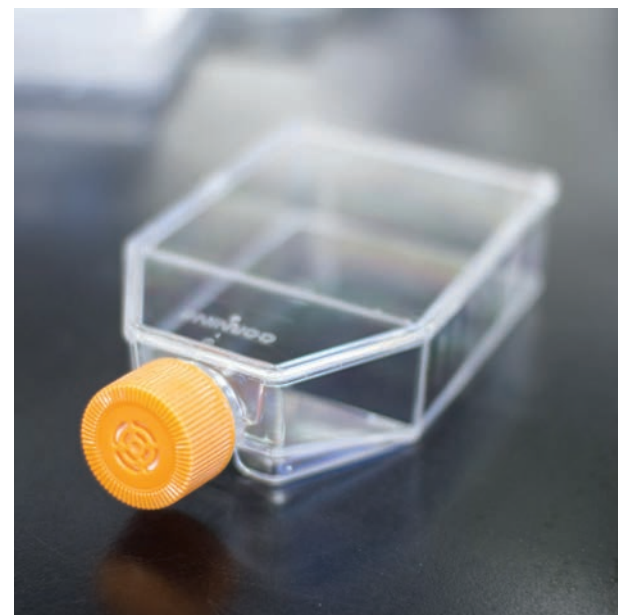
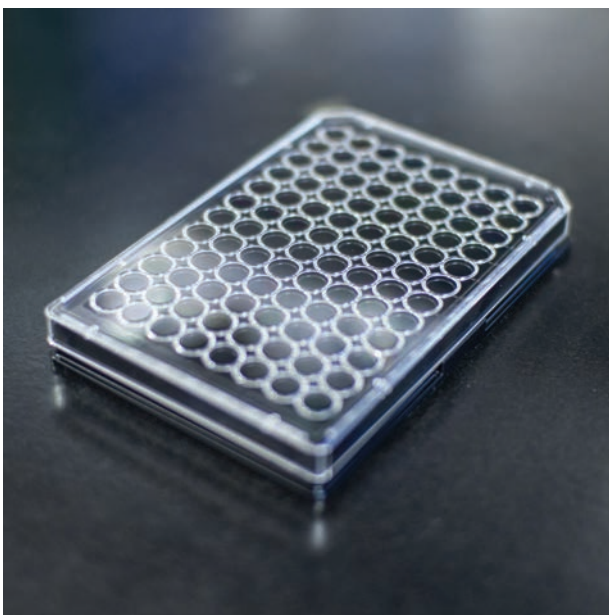
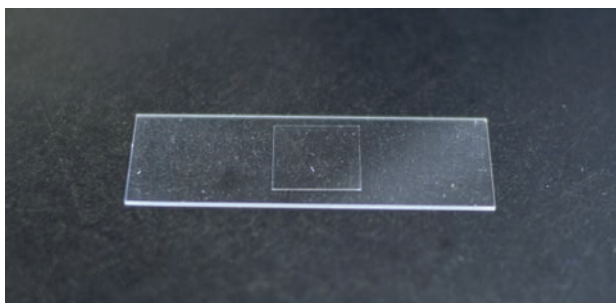
Figure 8.2. Live-cell fluorescence imaging (red and green channels) capturing mitotic division in HeLa cells. Cells were transduced with CellLight® Histone 2B-GFP and Mitochondria RFP.



## Part Two: Sample Considerations

### 9. Choosing a Vessel for Your Sample

Figure 9.1. There are several types of vessels through which you can image. Which one you choose depends on the type of experiment you are doing and the capabilities of your microscope. Vessels may have glass bottoms, or plastic, vary in volume, and may contain more than one well.

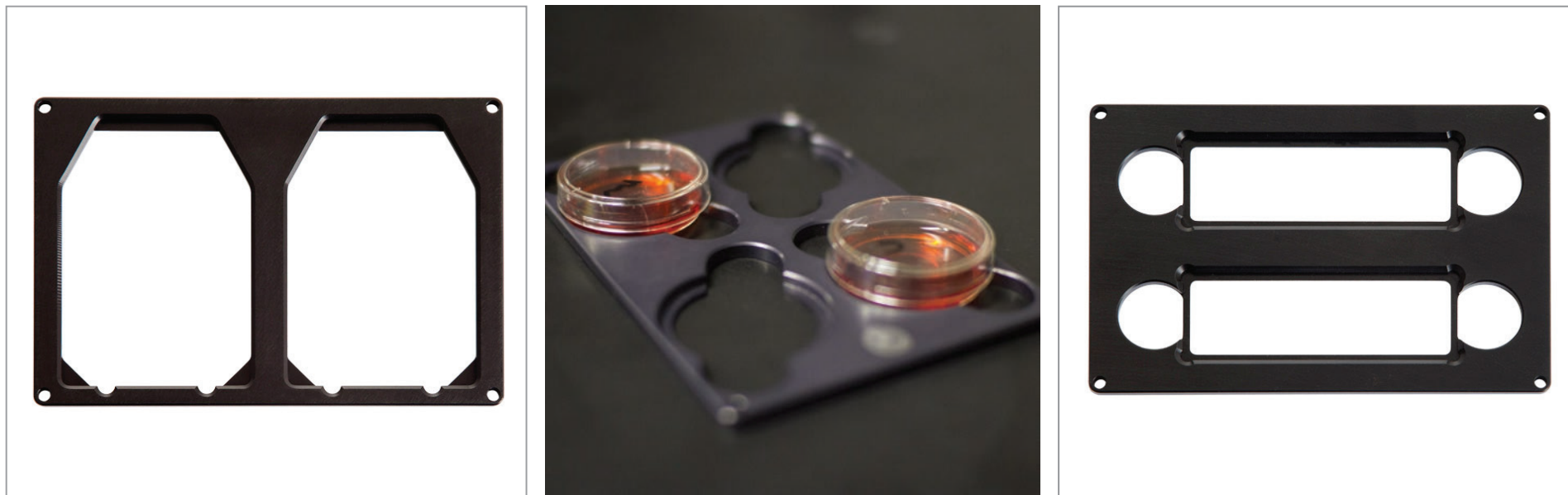




## Part Two: Sample Considerations

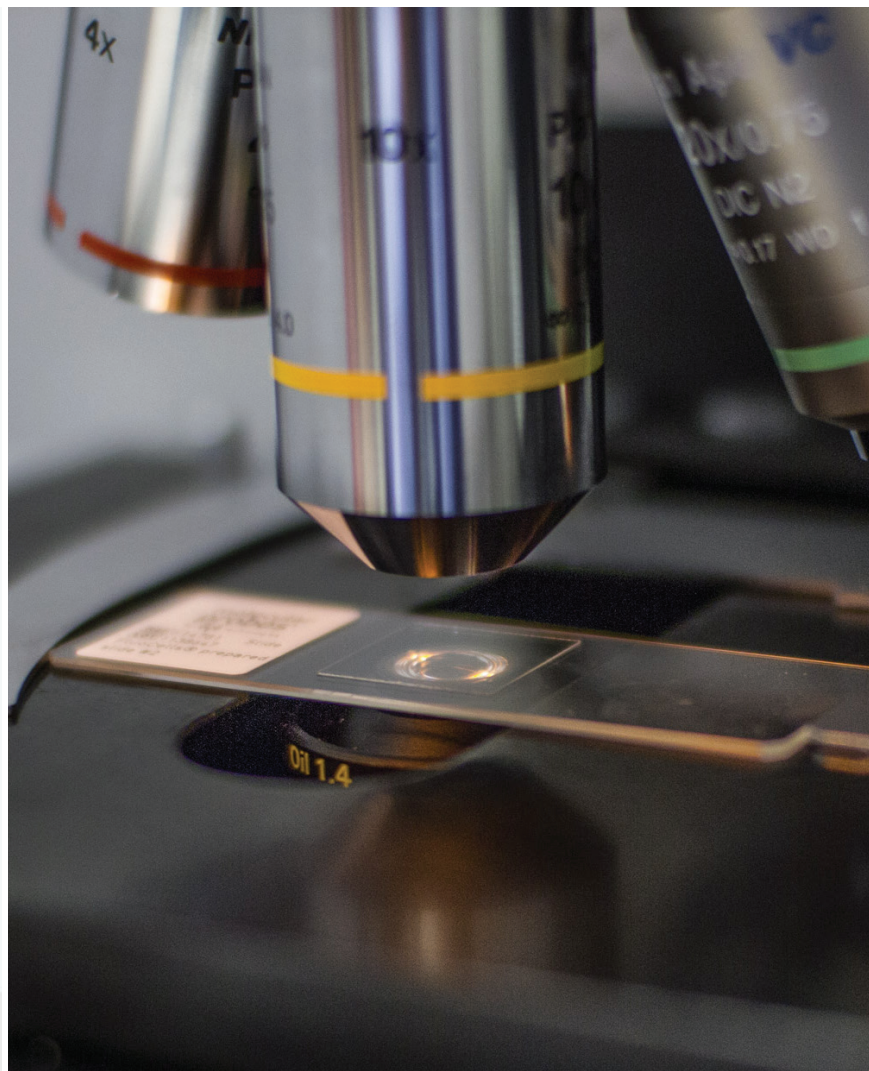
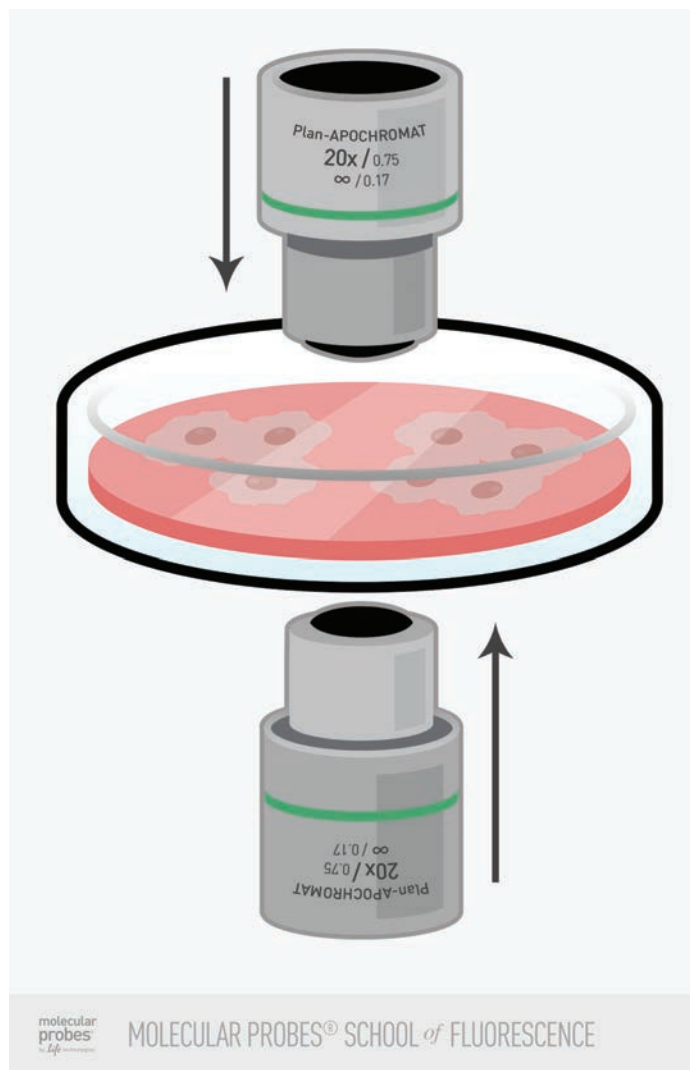
### Vessel Holders and Adaptors

Figure 9.2. Some stages require an adaptor to fit certain types of vessels.



## Part Two: Sample Considerations

Figure 9.3. The position of your microscope's objective relative to the sample stage will determine which kinds of vessels will work the best for your imaging experiment.

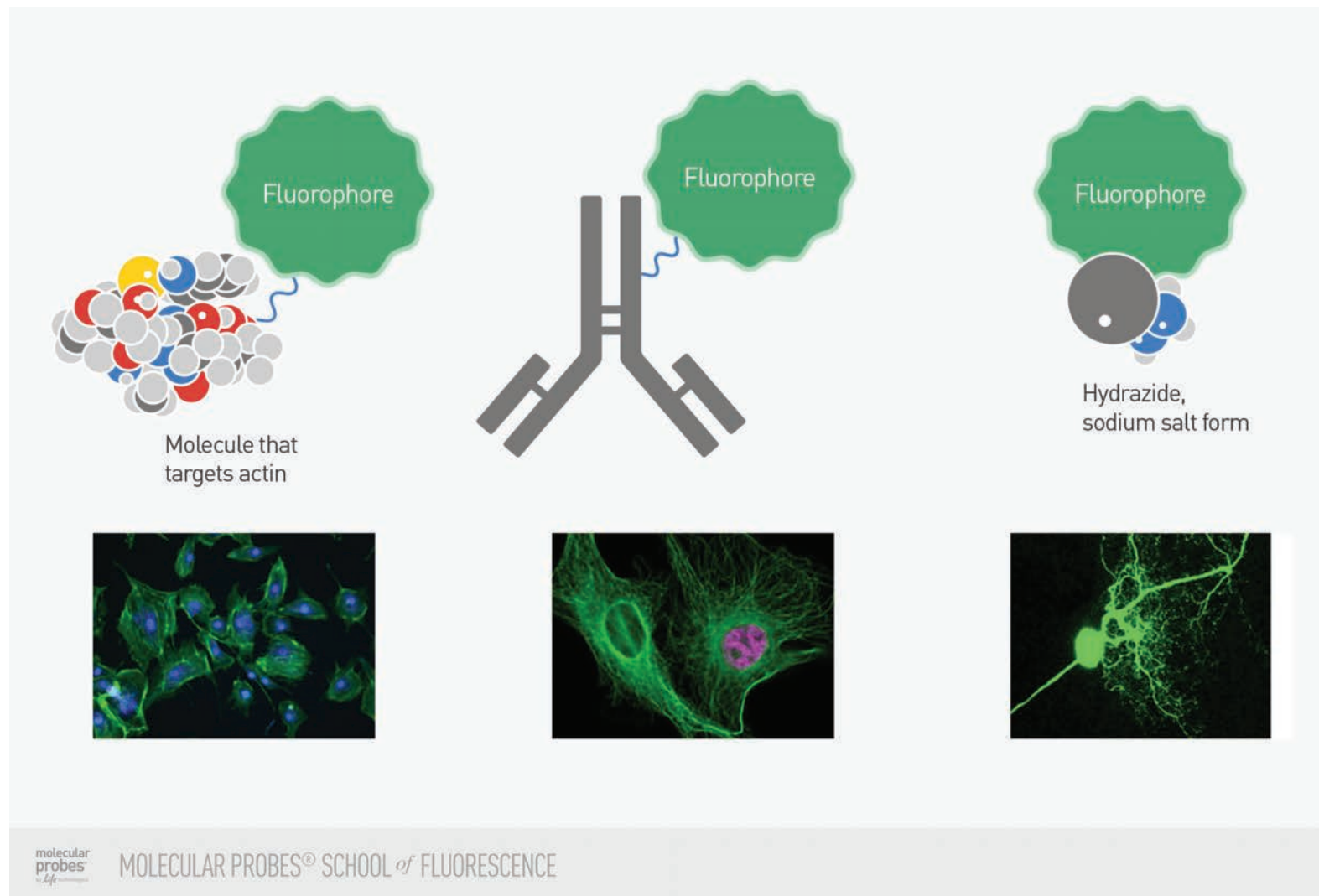




## Part Three: Labeling Your Samples

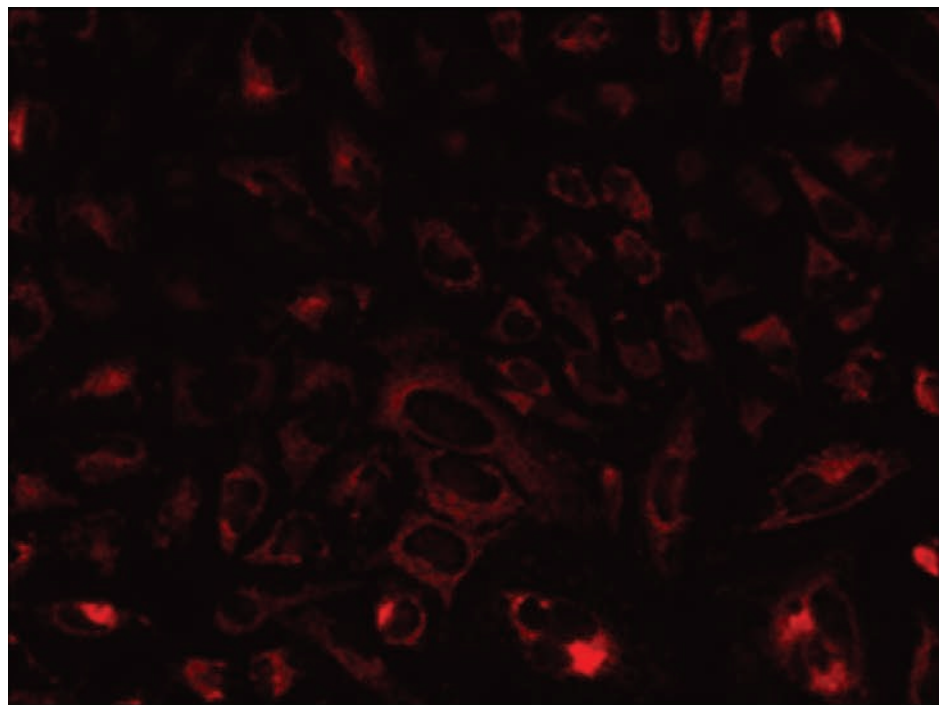
### 10. Different Ways to Add Fluorescent Labels

Figure 10.1. A single fluorophore can be modified to carry out any number of labeling jobs, including functionalized forms for labeling cell structure components such as actin (A) and tubulin (B) and salt (C) forms for whole-cell staining.

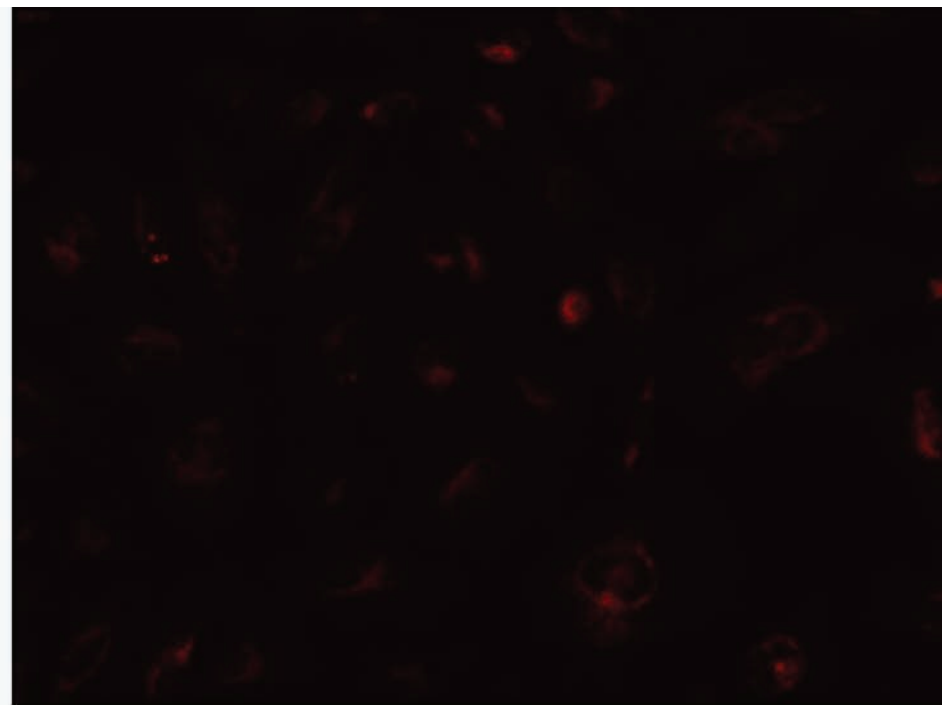


## Part Three: Labeling Your Samples

Figure 10.2. Panel A shows TMRM staining in healthy HeLa cells; panel B shows the loss of TMRM signal concurrent with treatment to destroy the mitochondrial membrane potential.



Control – bright TMRM signal

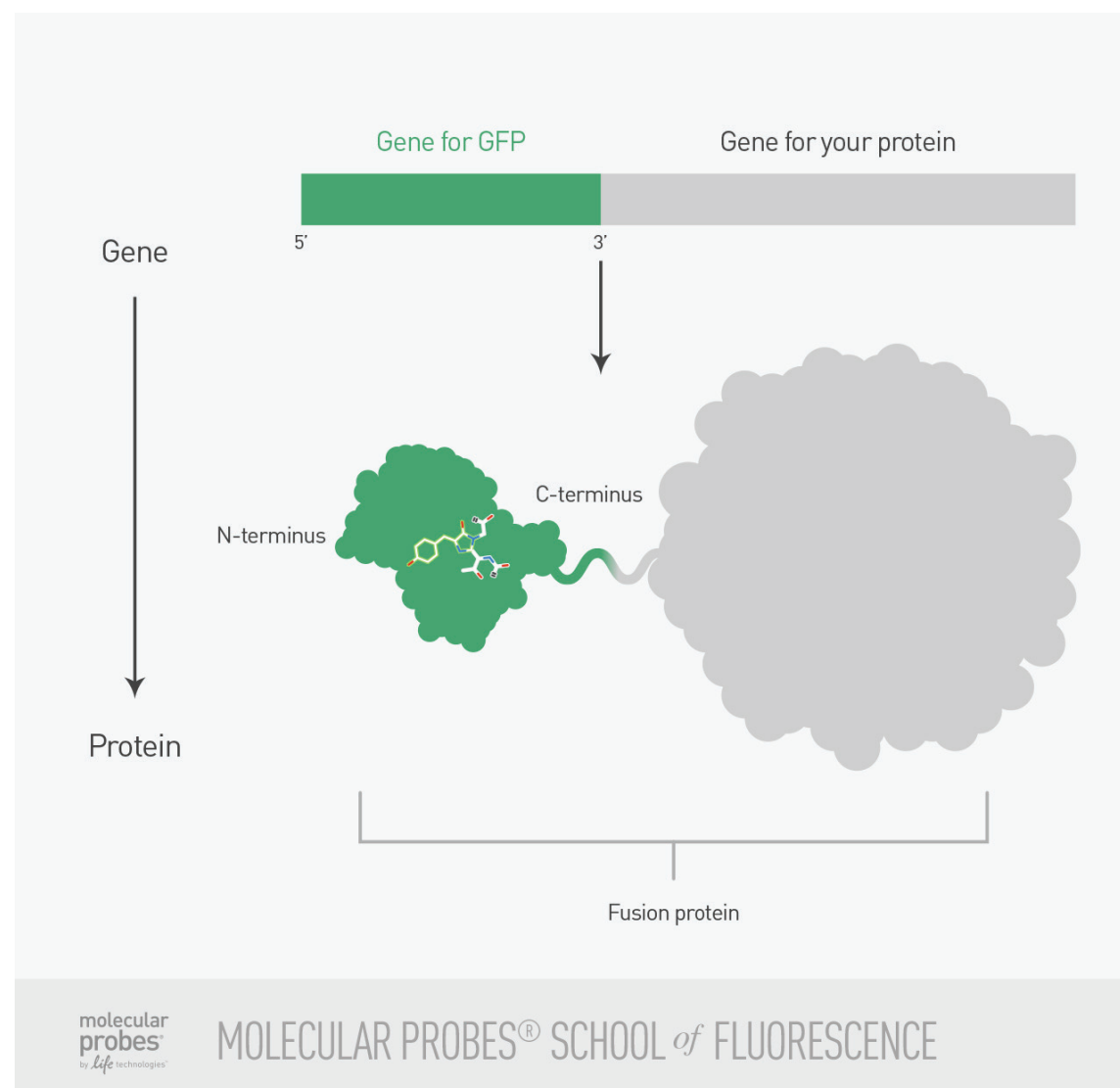


Treated – dim TMRM signal

## Part Three: Labeling Your Samples

### 11. Labeling Using Fluorescent Proteins

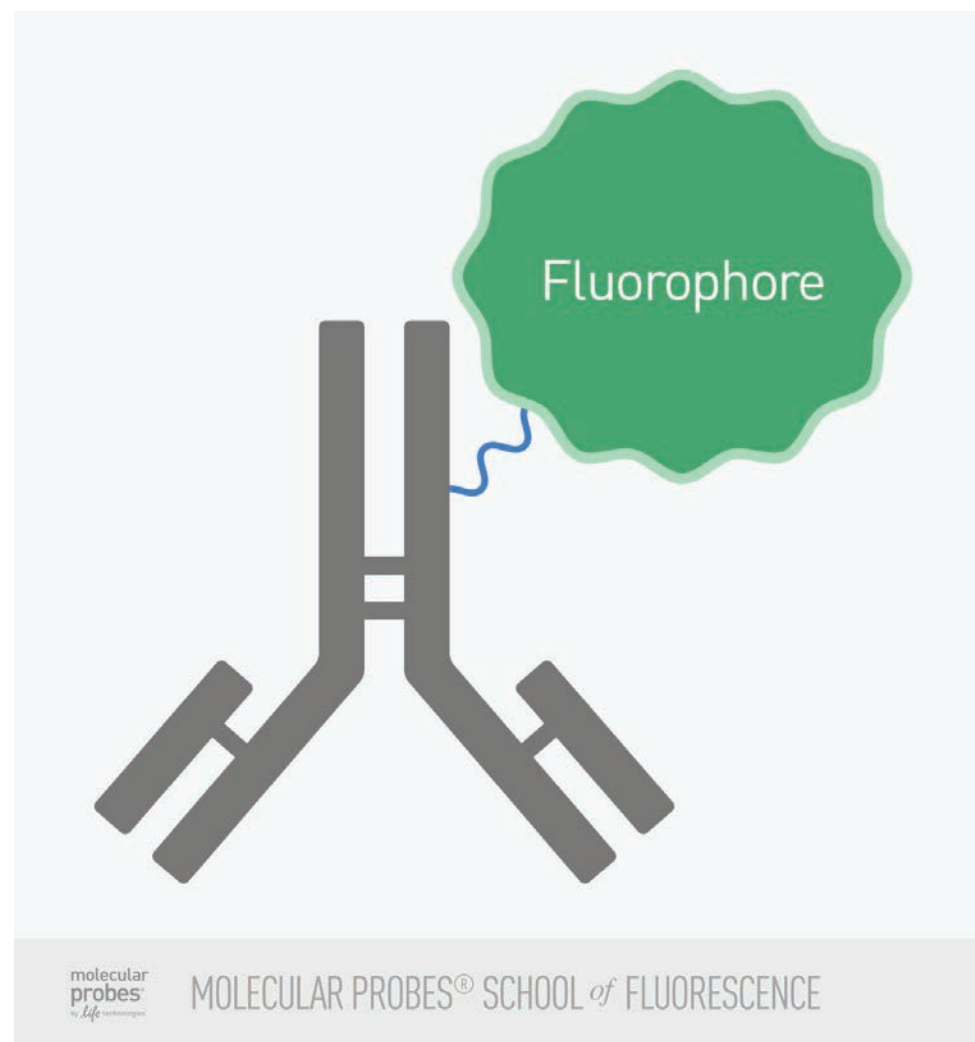
Figure 11.1. Optimal placement of your gene of interest is on the C-terminal end of the fluorescent protein gene.



## Part Three: Labeling Your Samples

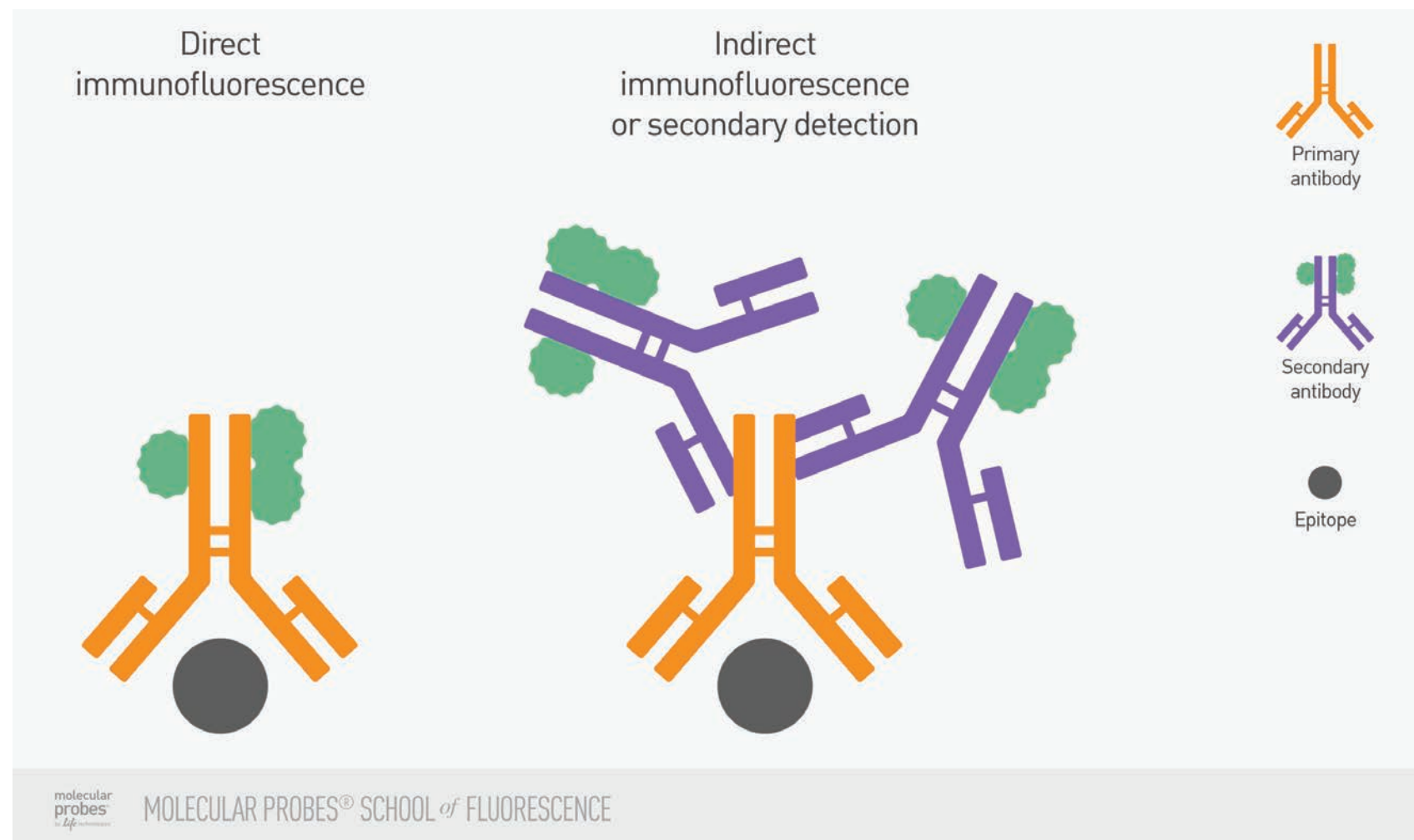
### 12. Immunolabeling

Figure 12.1. Immunofluorescence techniques rely on a fluorophore-conjugated antibody, which has specificity for a specific target. The illustration shows an antibody (grey) conjugated to a fluorophore (green). In reality, most commercially conjugated antibodies would be labeled with 2–7 of the same fluorophore molecules per antibody.



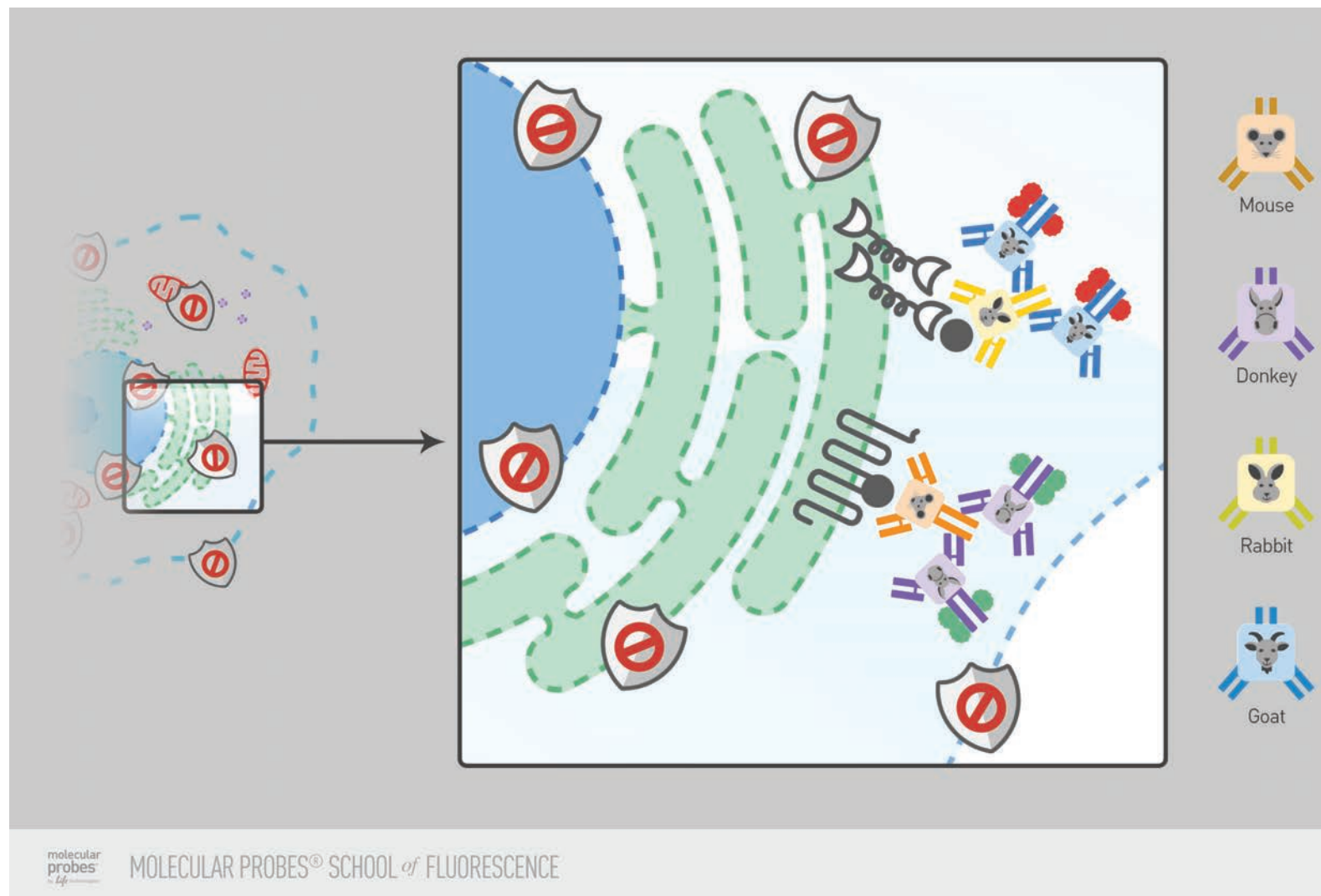
## Part Three: Labeling Your Samples

Figure 12.2. Immunolabeling can be accomplished in several ways. The antibody which binds a specific target or epitope is shown in orange. For direct immunofluorescence, the antibody binding the epitope is labeled with fluorophores (green). For indirect or secondary detection, the primary antibody binds the epitope and a fluorophore-labeled secondary antibody (purple) that has specificity for the primary antibody binds to it.



## Part Three: Labeling Your Samples

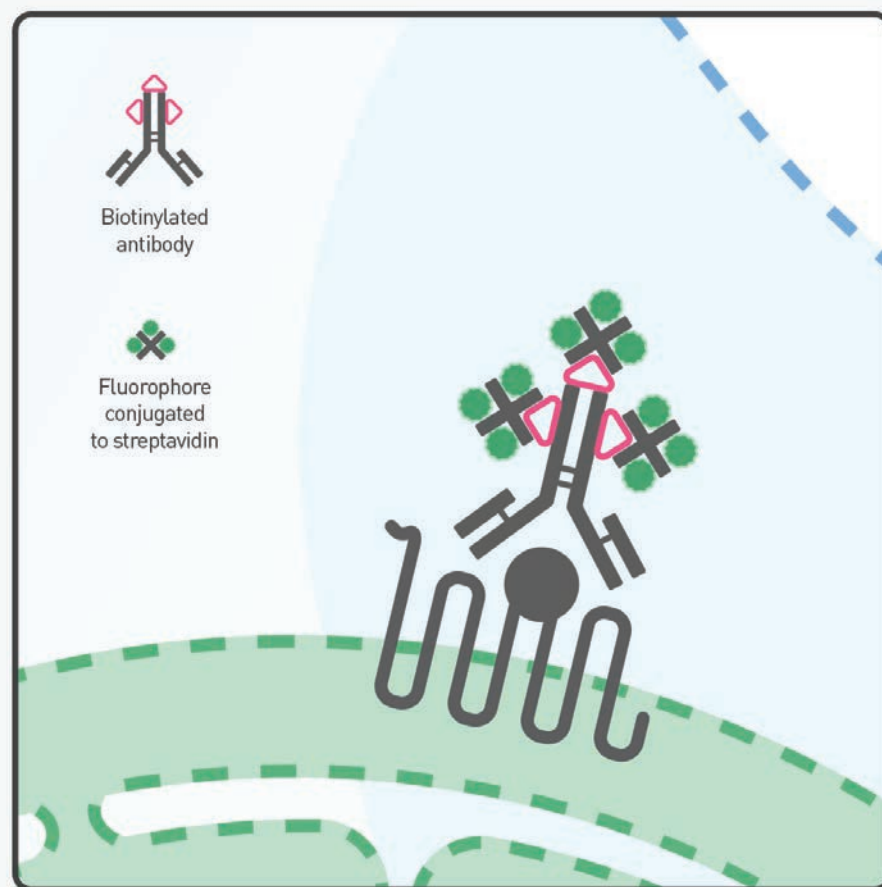
Figure 12.3. Secondary detection of two different targets in the same sample relies on the primary antibody for each target being raised in different host species to avoid cross-reactivity between the secondary antibodies.





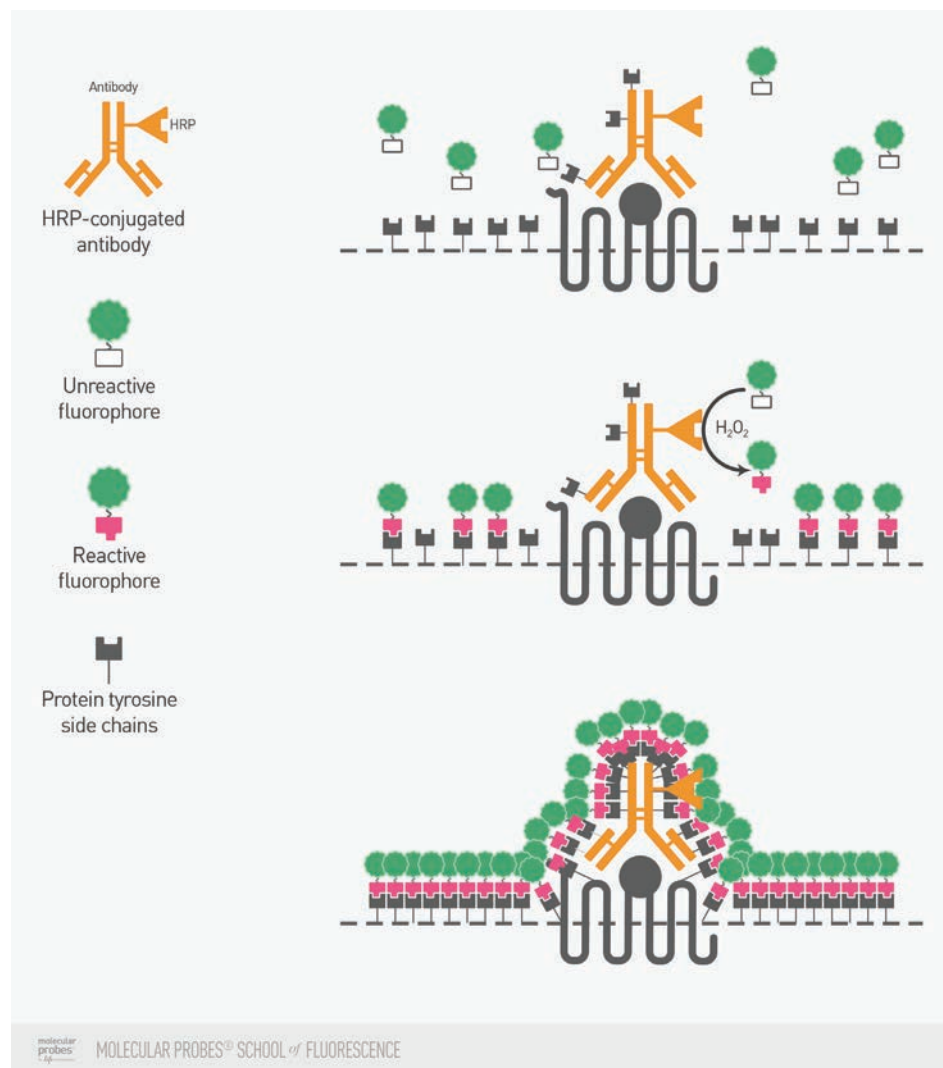
## Part Three: Labeling Your Samples

Figure 12.4. Biotinylated antibodies labeled using fluorophore-streptavidin conjugates can boost dim signals in your labeling experiments because more fluorophores can bind to each antibody molecule. Specificity for the epitope is retained.



## Part Three: Labeling Your Samples

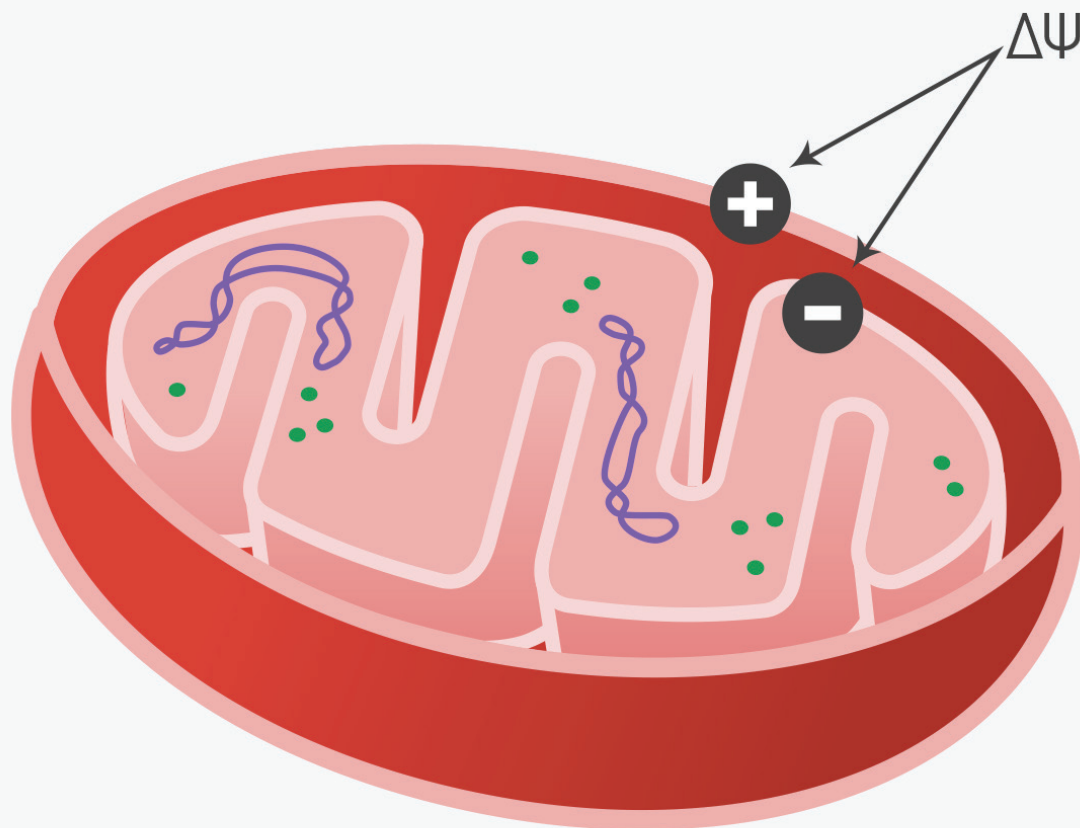
Figure 12.5. Tyrosine signal amplification can also amplify dim signals by adding many more fluorophores per epitope, but the labeling may be less specific due to reactive fluorophores binding to the immediate area surrounding the epitope.



## Part Three: Labeling Your Samples

### 13. Mitochondrial Labeling

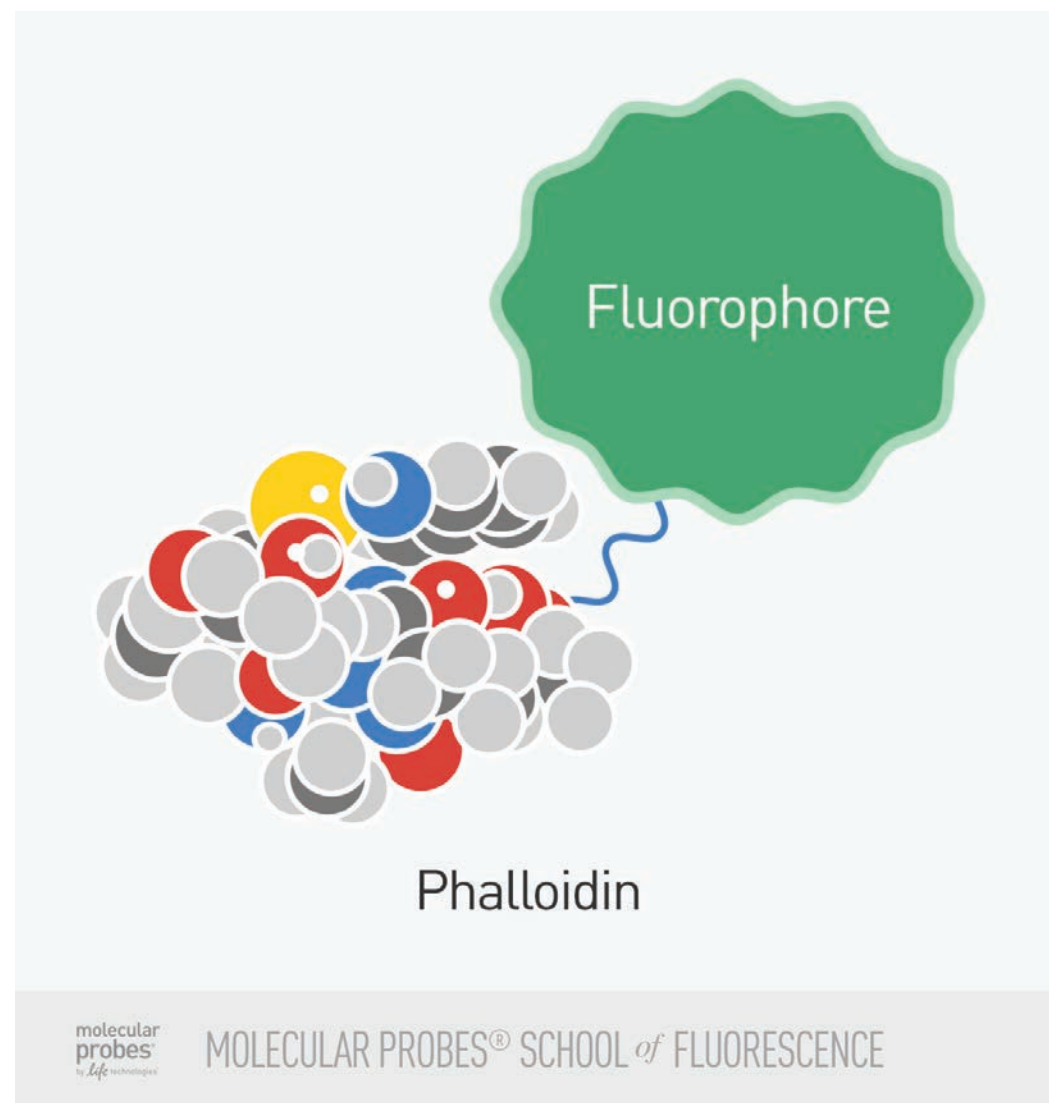
Figure 13.1. Illustration of a mitochondrion. The electric potential, noted as  $\Delta\Psi$ , is lost in non-functional mitochondria.



## Part Three: Labeling Your Samples

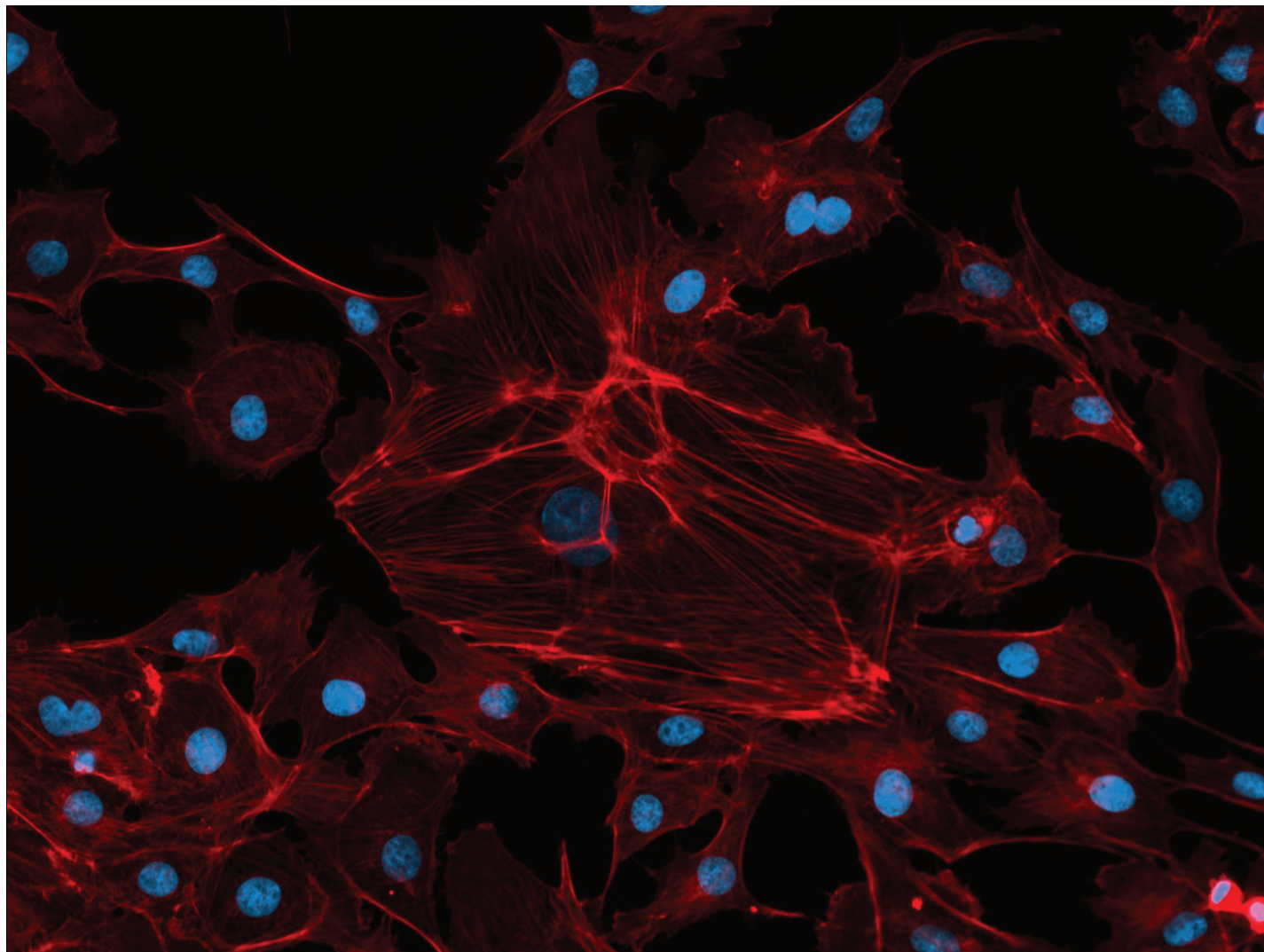
### 14. Actin Labeling

Figure 14.1. Space filling model of phalloidin conjugated to a fluorophore.



## Part Three: Labeling Your Samples

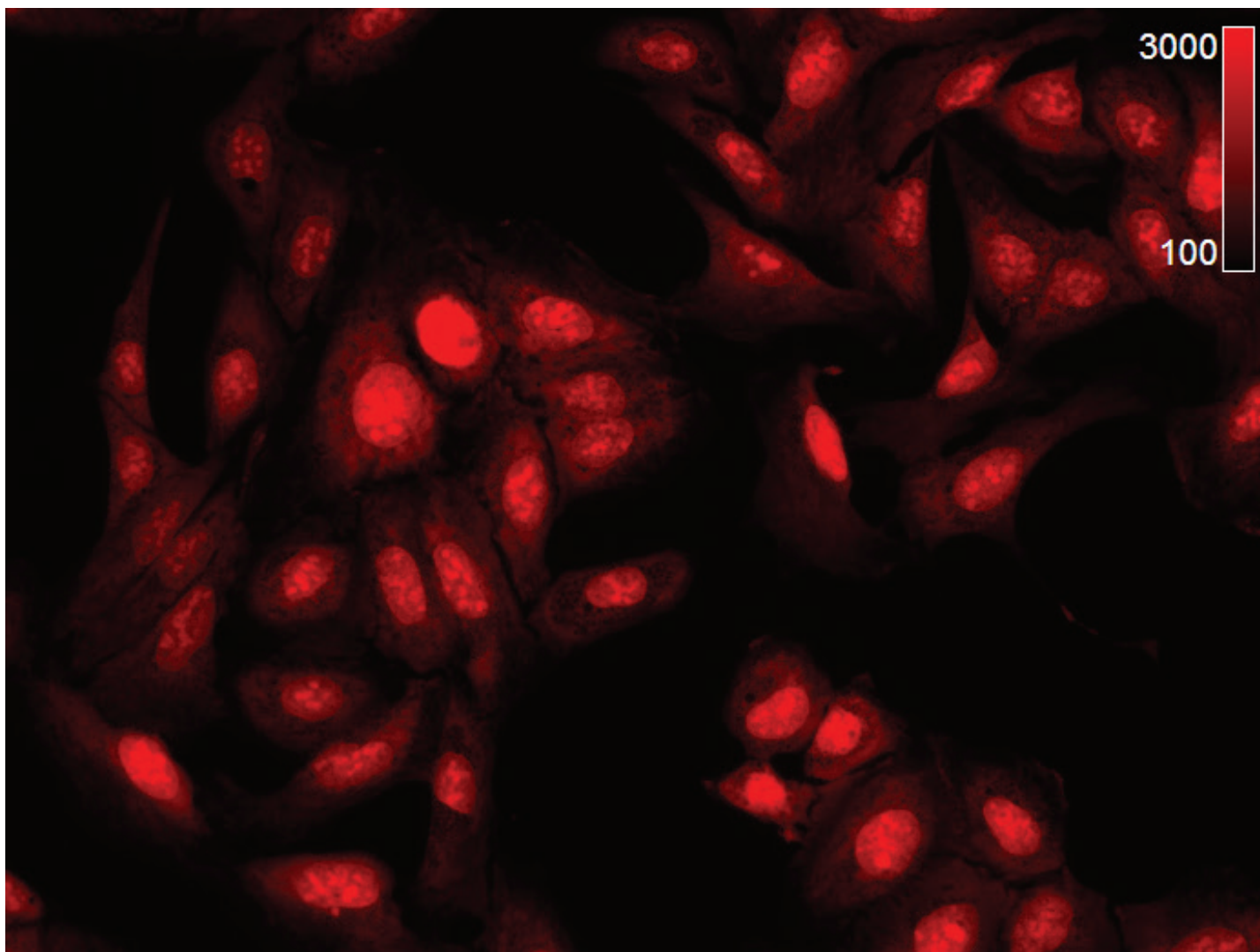
Figure 14.2. After fixing, permeabilizing, and blocking, BPAE cells were labeled with ActinRed™ 555 ReadyProbes® Reagent (TRITC-conjugated phalloidin that labels F-actin), and nuclei were labeled with NucBlue® Fixed Cell ReadyProbes® Reagent (a form of DAPI).



## Part Three: Labeling Your Samples

### 15. Nuclear Labeling

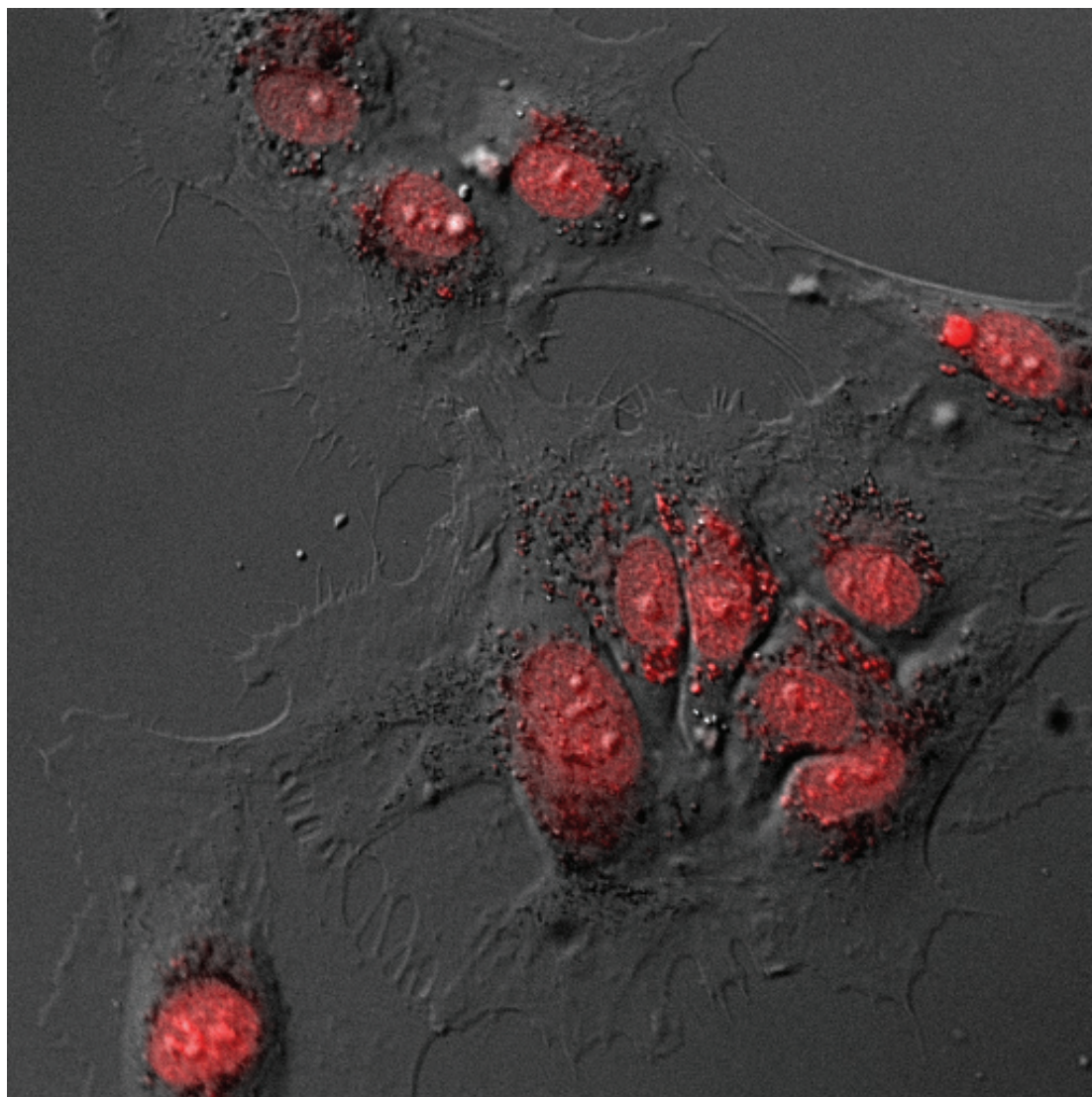
Figure 15.1. TO-PRO®-3 stain exhibits a bright signal in the nucleus and a dimmer signal in the cytoplasm (from RNA) in fixed and permeabilized U2OS cells.





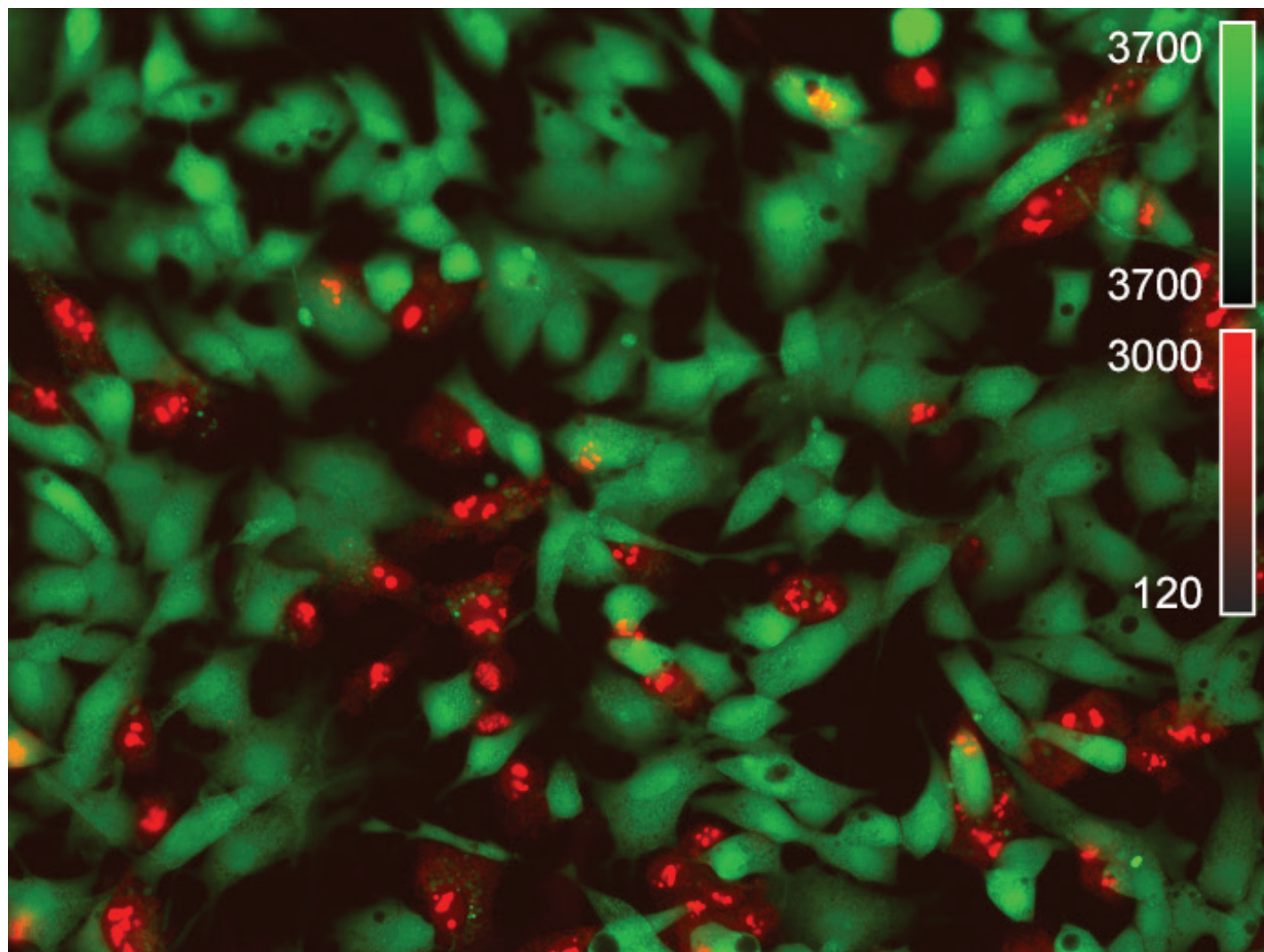
## Part Three: Labeling Your Samples

Figure 15.2. HUVECs labeled with NucRed® Live 647 ReadyProbes® Reagent, a cell-permeant nuclear stain.



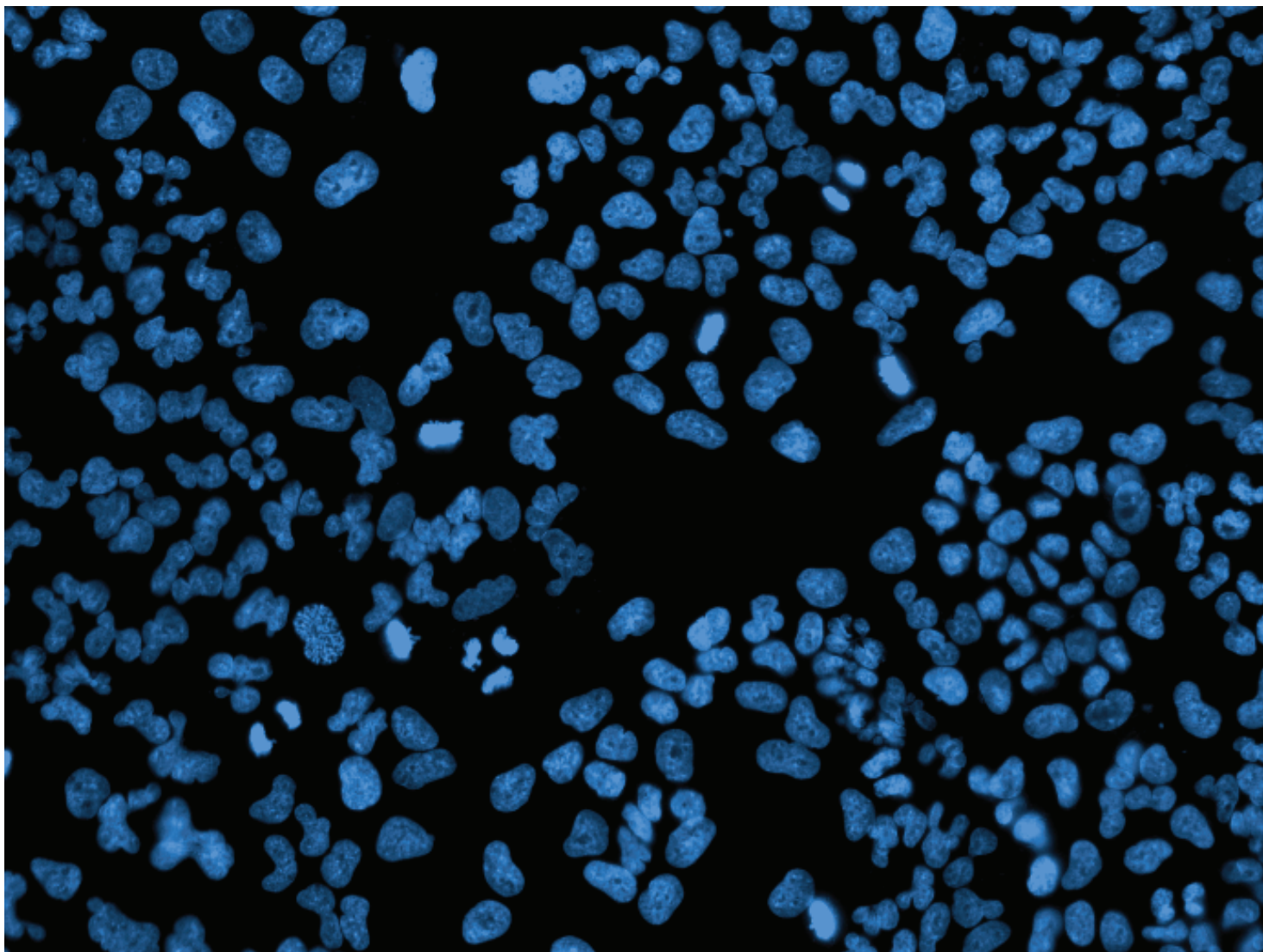
## Part Three: Labeling Your Samples

Figure 15.3. Live and dead HEPG2 cells stained using the LIVE/DEAD® Cell Imaging Kit. Dead cells (red) are labeled with a cell-impermeant dye (DeadRed™ reagent) and live cells (green) are stained with calcein.



## Part Three: Labeling Your Samples

Figure 15.4. Nuclear staining of fixed and permeabilized U2OS cells using NucBlue® Fixed Cell ReadyProbes® Reagent (a form of DAPI).

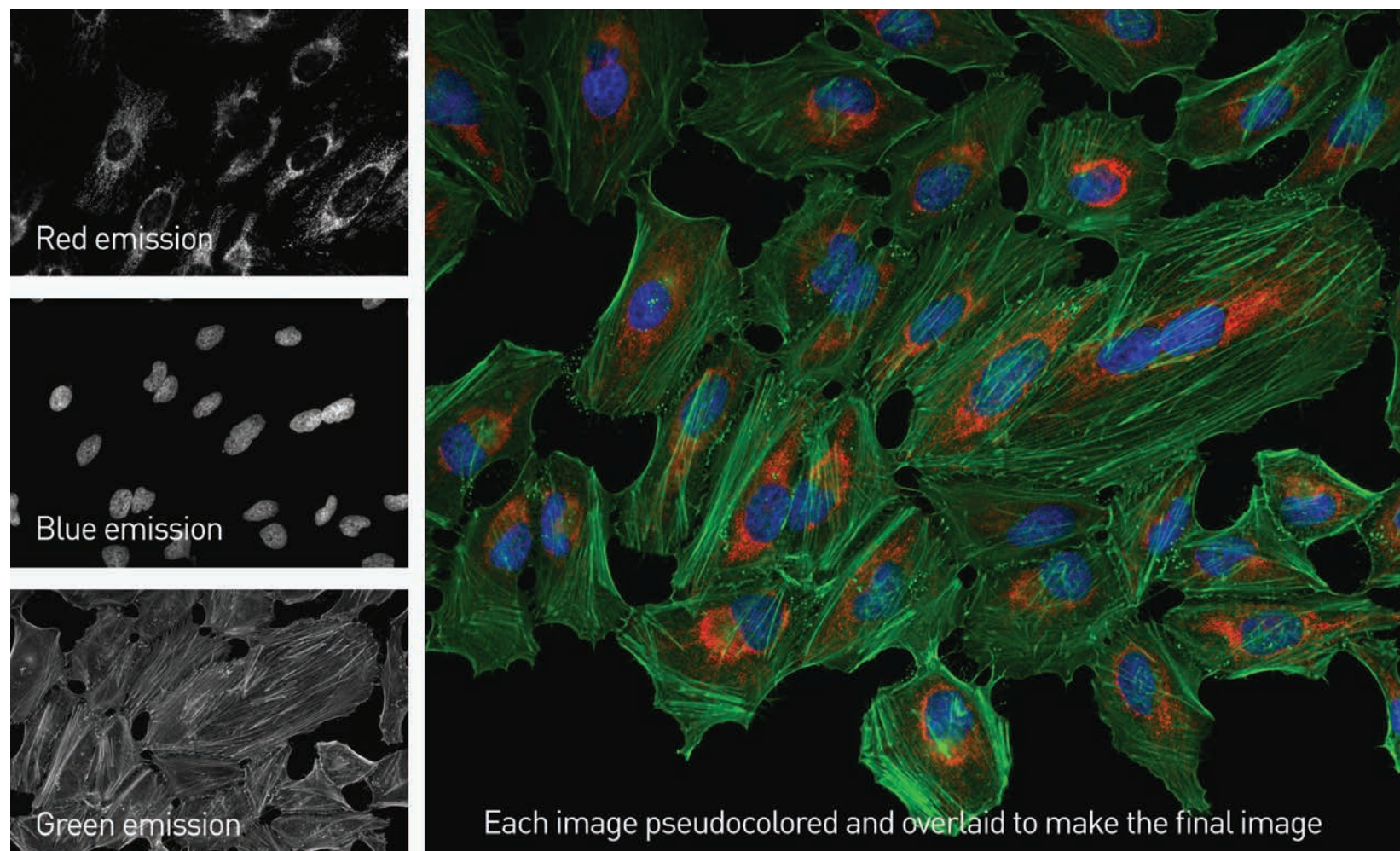




## Part Four: Capturing & Analyzing Your Samples

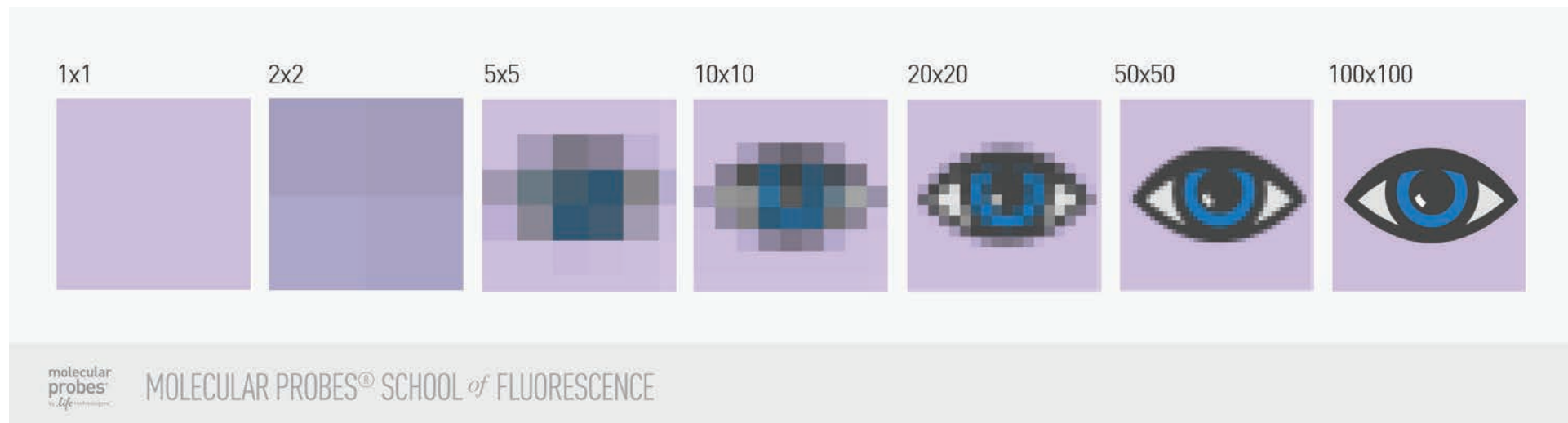
### 16. Pixels and Intensity

Figure 16.1. HeLa cells labeled with 3 different fluorescent labels: NucBlue® Fixed reagent (stains nuclei), ActinGreen™ ReadyProbes® reagent (stains actin filaments), and a primary antibody against mitochondria followed by a fluorescently labeled secondary antibody (stains mitochondria). Pseudocoloring allows you to show each channel (or fluorescent dye) in a different color. This makes it easier to differentiate multiple fluorescent dyes in the same sample (since each is a different color).



## Part Four: Capturing & Analyzing Your Samples

Figure 16.2. Each image in the series (from left to right) contains more pixels than the previous one. As the number of pixels increases, the image becomes clearer. Eventually, you can see the fine details of the image.

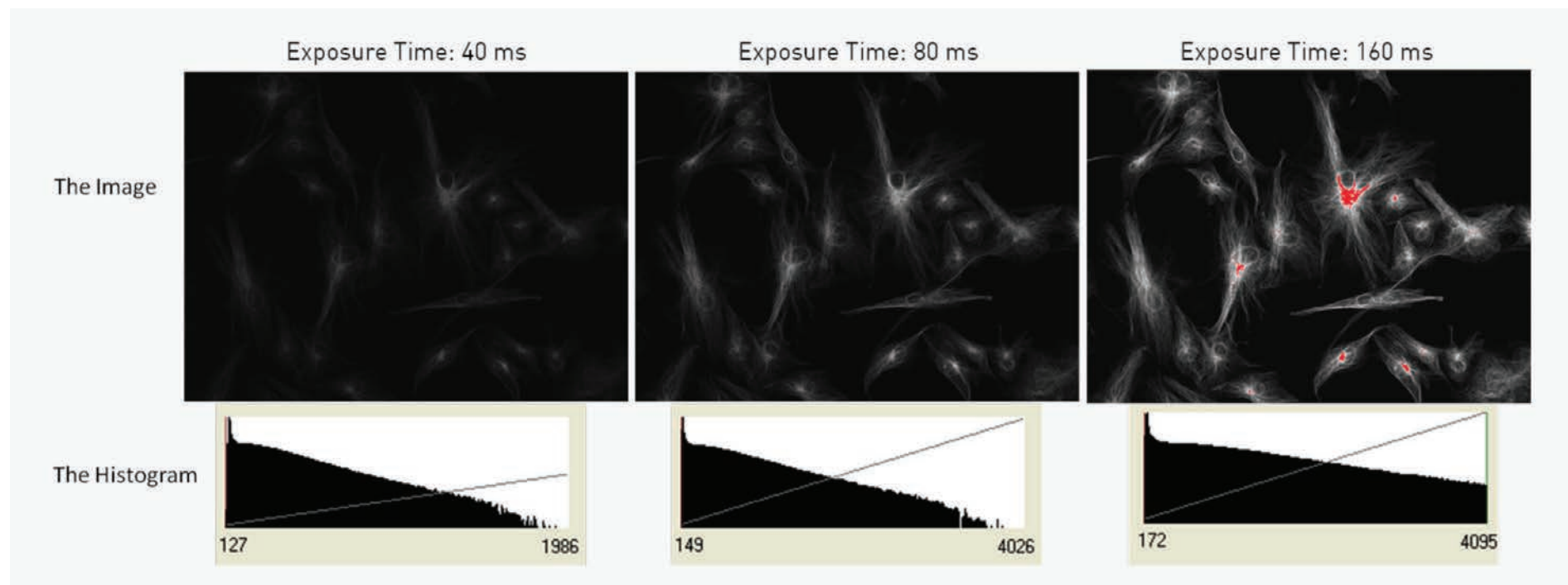




## Part Four: Capturing & Analyzing Your Samples

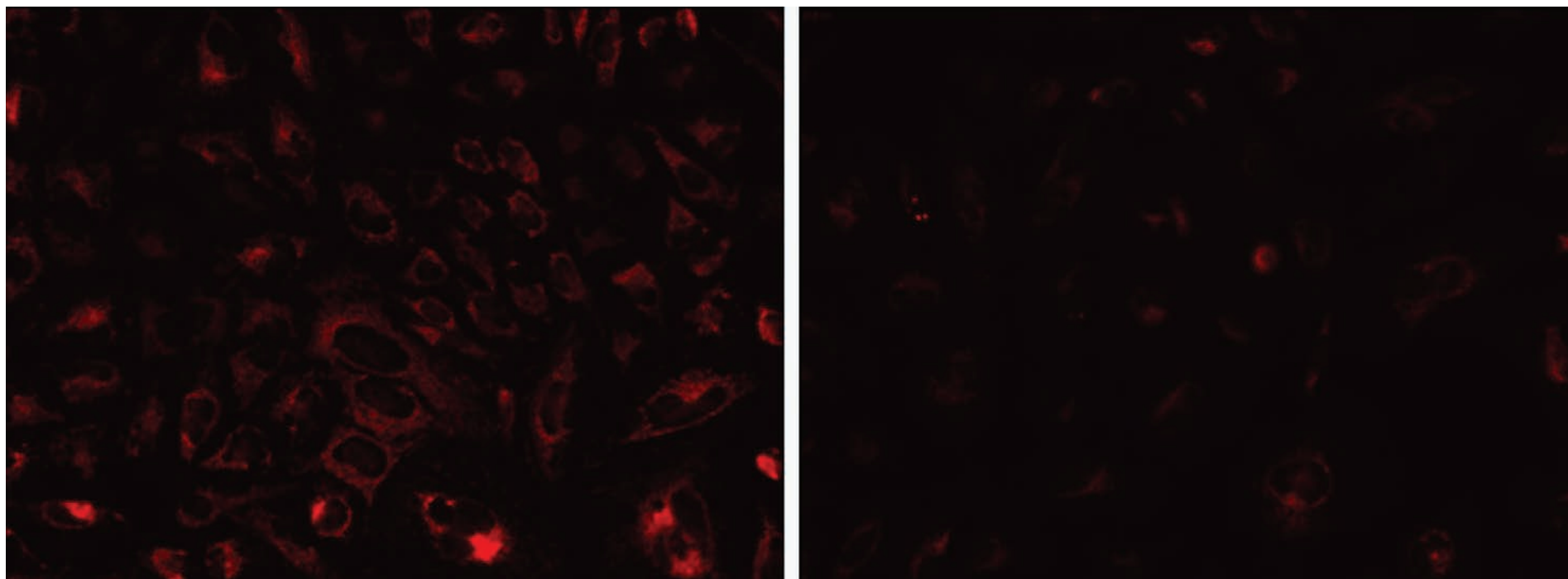
### 17. Exposure Times

Figure 17.1. Your imaging software program can help you choose a good exposure time by visually flagging saturated pixels and/or by presenting a histogram of intensity values for each pixel in the image. Ideally, you want to use the entire dynamic range of your camera without saturating any pixels.



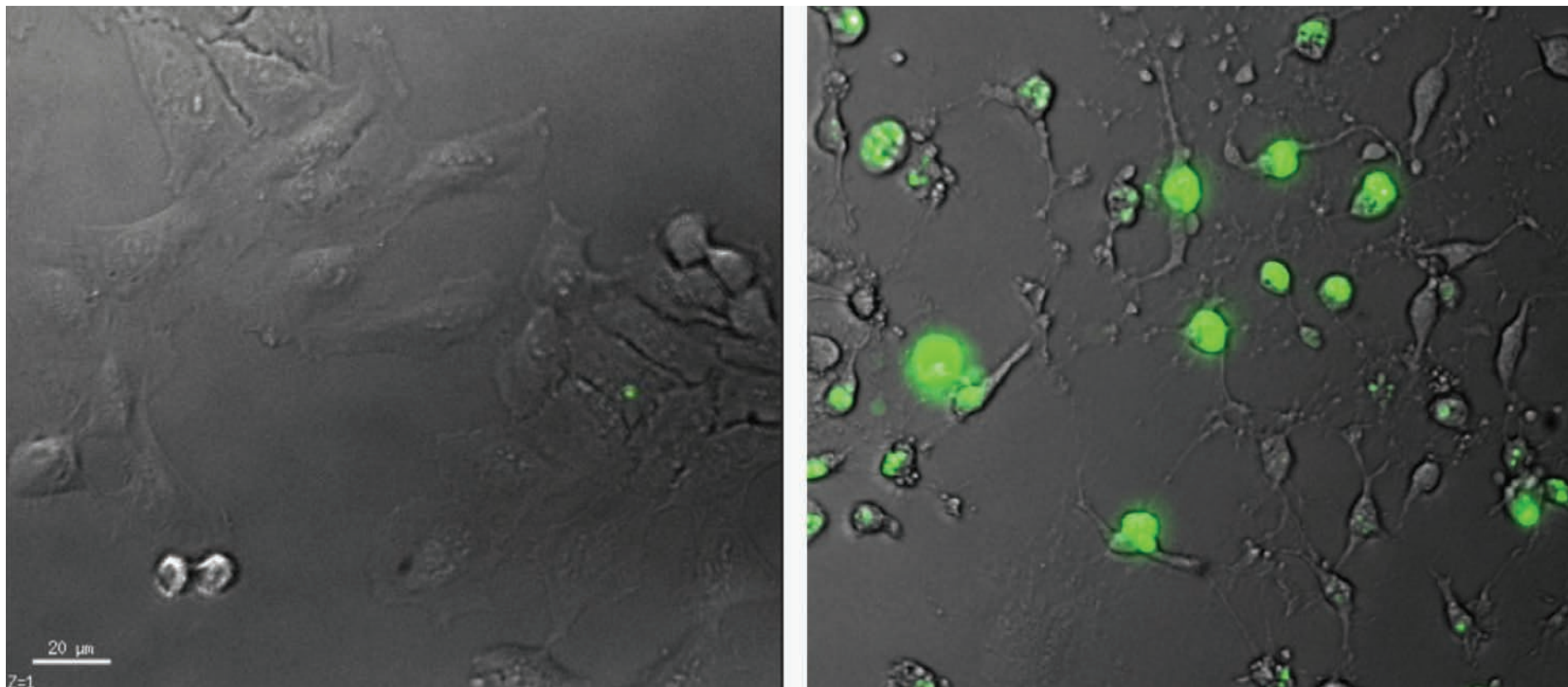
## Part Four: Capturing & Analyzing Your Samples

Figure 17.2. Example of loss of signal upon treatment. Untreated HeLa cells (left) exhibit bright fluorescence intensity when stained with mitochondrial dye TMRM. Cells treated with CCCP (carbonyl cyanide 3-chlorophenylhydrazone, which uncouples oxidative phosphorylation resulting in loss of mitochondrial membrane potential) exhibit dim fluorescence signal (right). In cases like these, use the untreated sample to set exposure time.



## Part Four: Capturing & Analyzing Your Samples

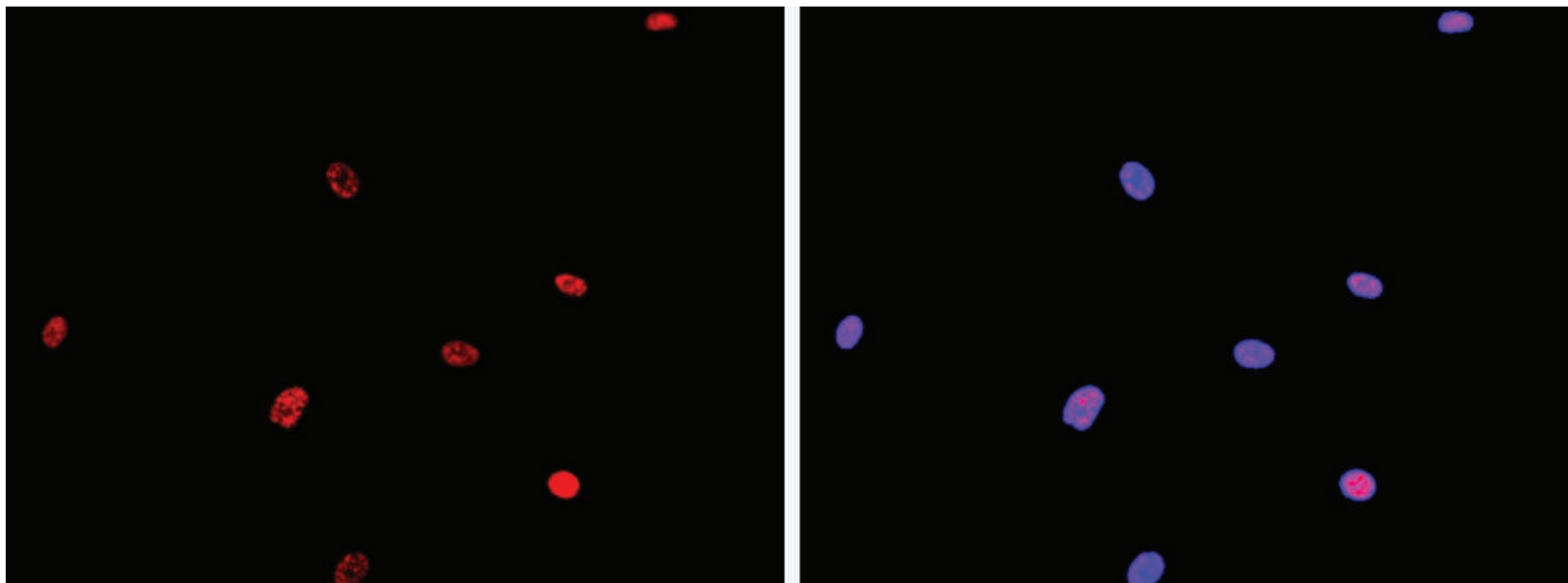
Figure 17.3. Example of increase in signal upon treatment. Untreated HeLa cells (left) stained with NucGreen® Dead reagent are dim. Cells treated with a drug to induce cell death, staurosporine (right) are bright green. In cases like these, use the treated sample to set exposure time.



## Part Four: Capturing & Analyzing Your Samples

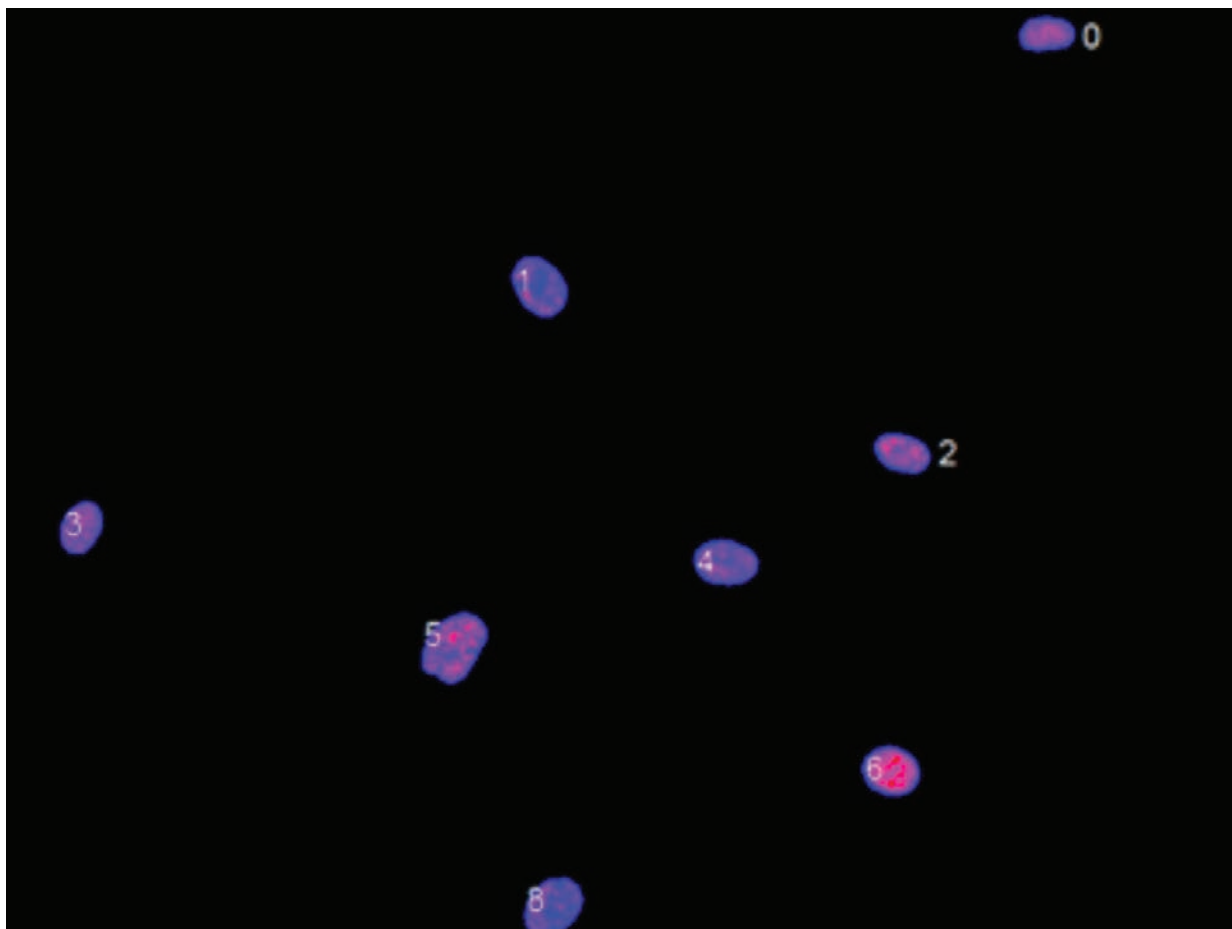
### 18. Analysis and Quantitation

Figure 18.1. Example of defining ROIs using an intensity threshold. The nucleus of each cell is labeled using a nuclear stain (red, left) and the user can then specify an intensity threshold. The software then overlays a mask (blue overlaid on red, right) on the regions that meet the specified threshold. Masking gives you a visual check that the threshold you've set includes all of the objects you're interested in, before you begin your quantitation and analysis..



## Part Four: Capturing & Analyzing Your Samples

Figure 18.2. Example of identifying individual objects. Each nuclei is counted as an individual object with its corresponding intensity value given, instead of an average intensity for all nuclei in the field.

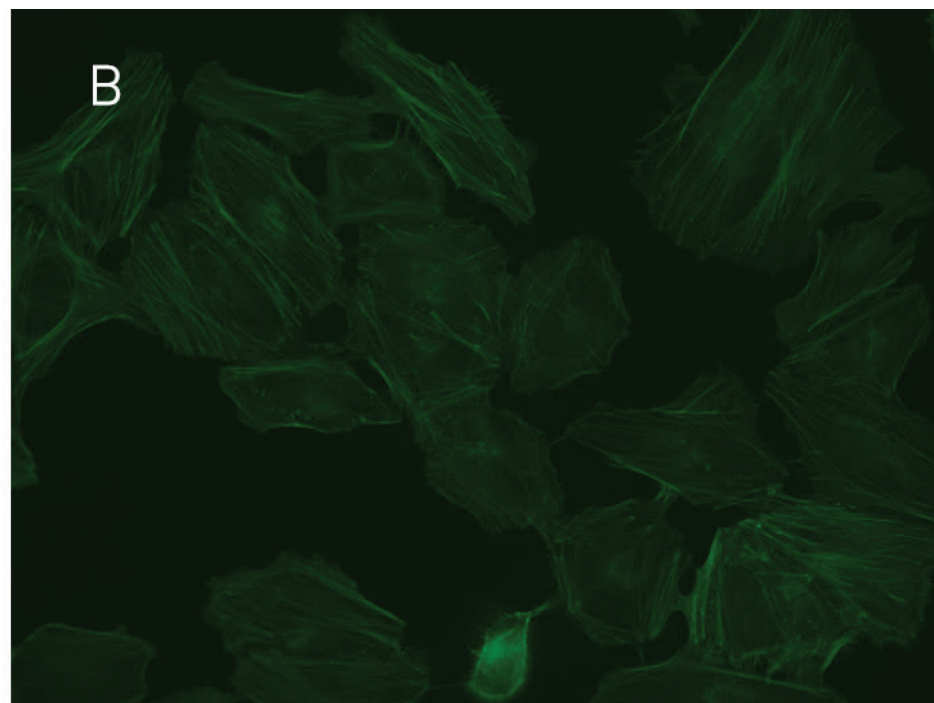
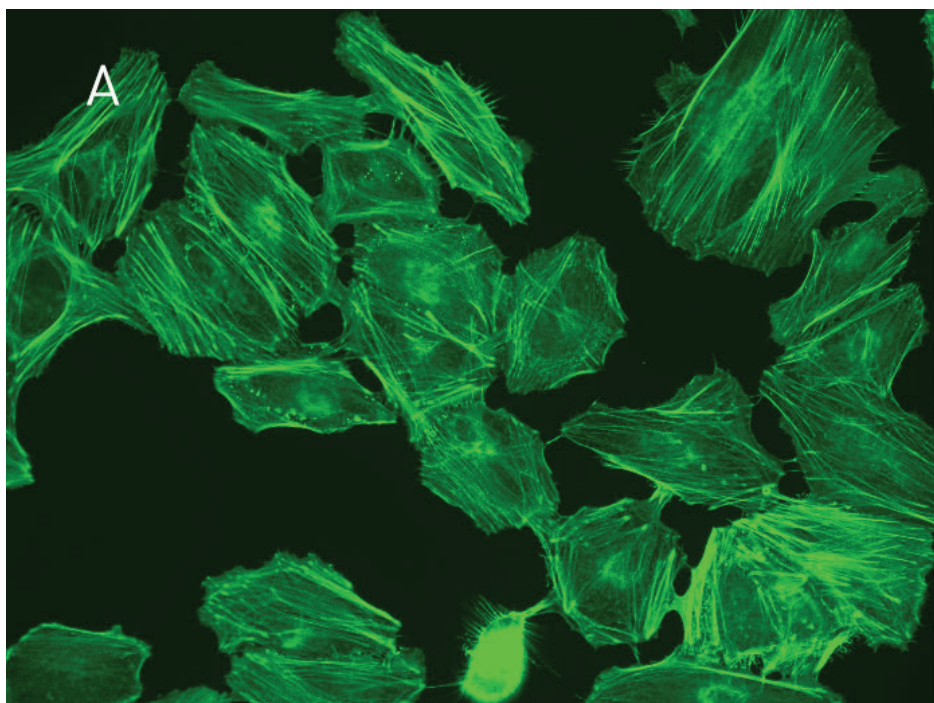




## Part Five: Troubleshooting

### 19. Photobleaching

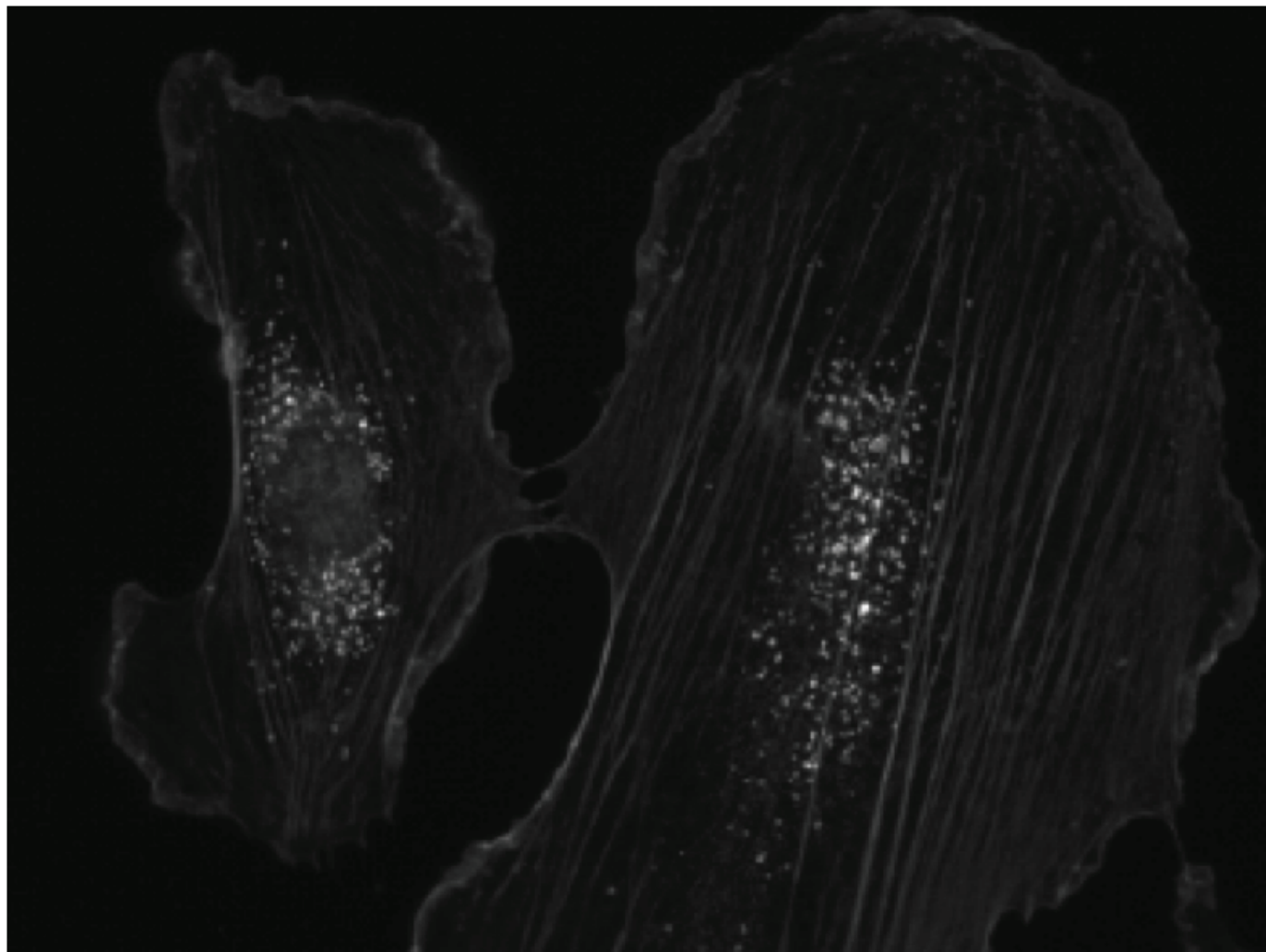
Figure 19.1. HeLa cells were fixed and labeled with FITC-conjugated phalloidin. Coverslips were mounted in 50% glycerol (in PBS). Panel (A) shows the initial intensity of the fluorophore, while panel (B) shows the photobleaching that occurs after 36 seconds of constant illumination



## Part Five: Troubleshooting

### 20. Bleed-Through

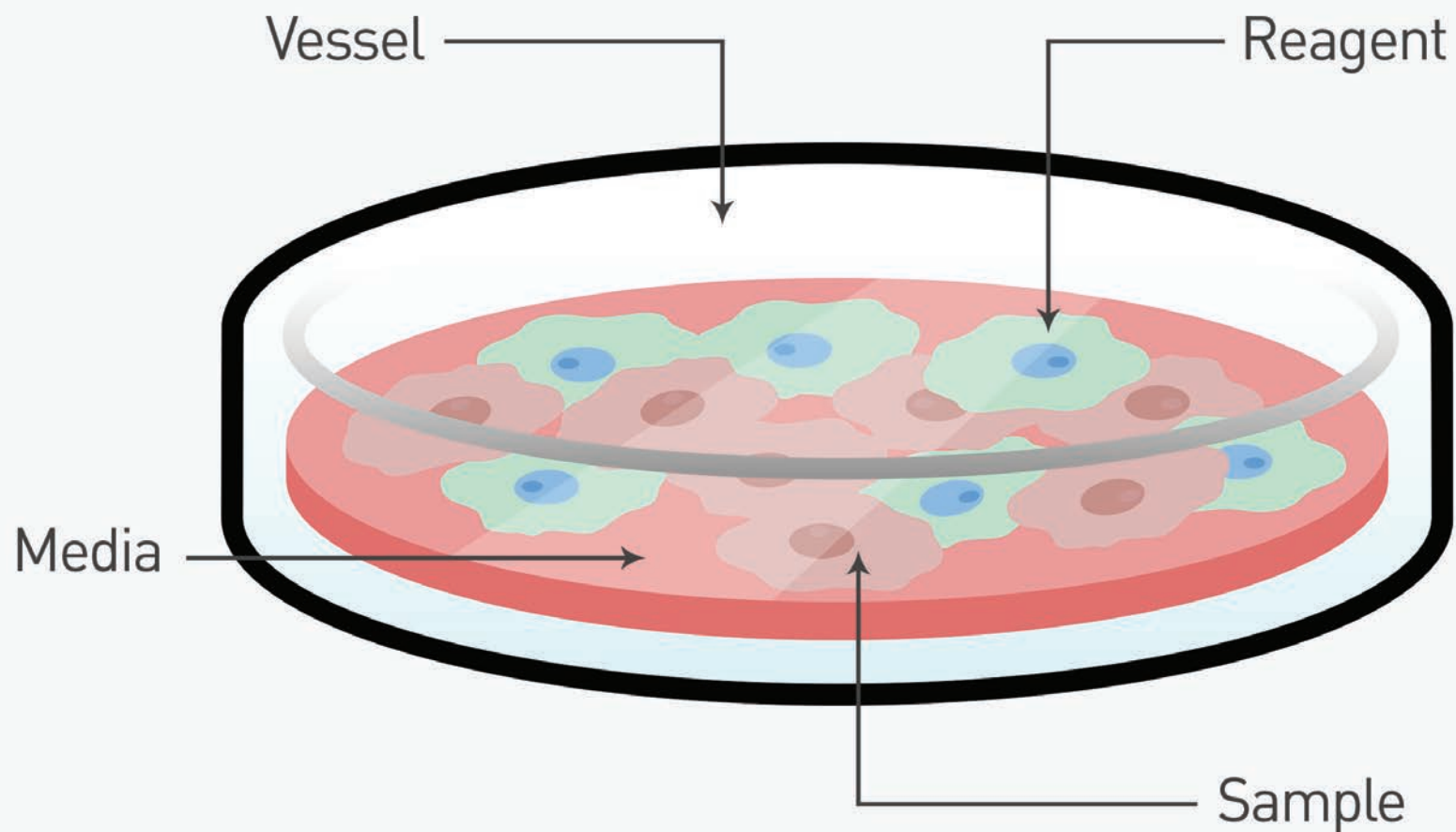
Figure 20.1. Signals from two different fluorophores can appear in the same channel if the emission/excitation of the fluorophores are not both carefully matched to filters. An example of bleed-through is shown, where the signal from a fluorophore detected in the TRITC filter also shows in the FITC filter.



## Part Five: Troubleshooting

### 21. Background Fluorescence

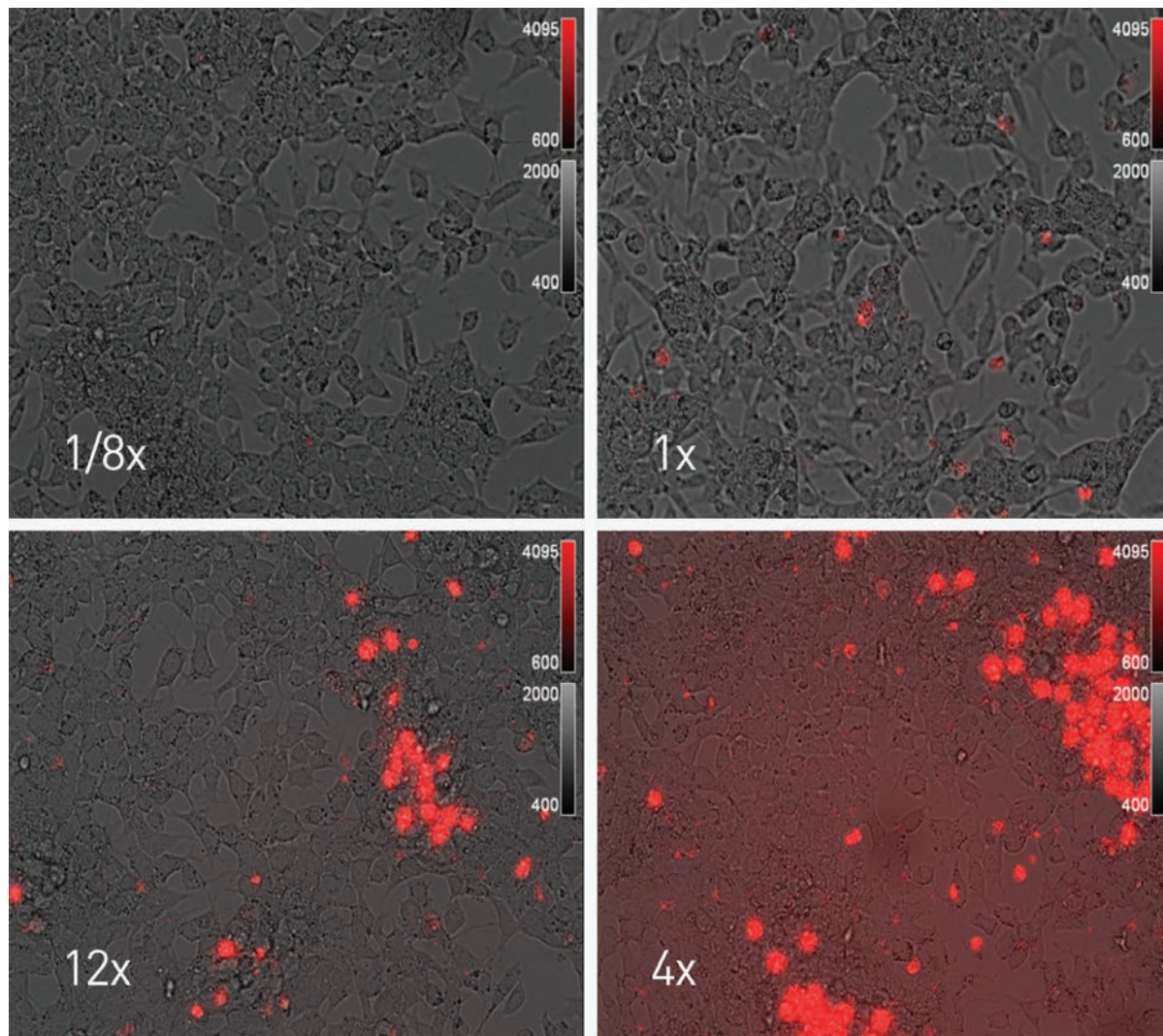
Figure 21.1. Background fluorescence can arise from many sources in your experiment.





## Part Five: Troubleshooting

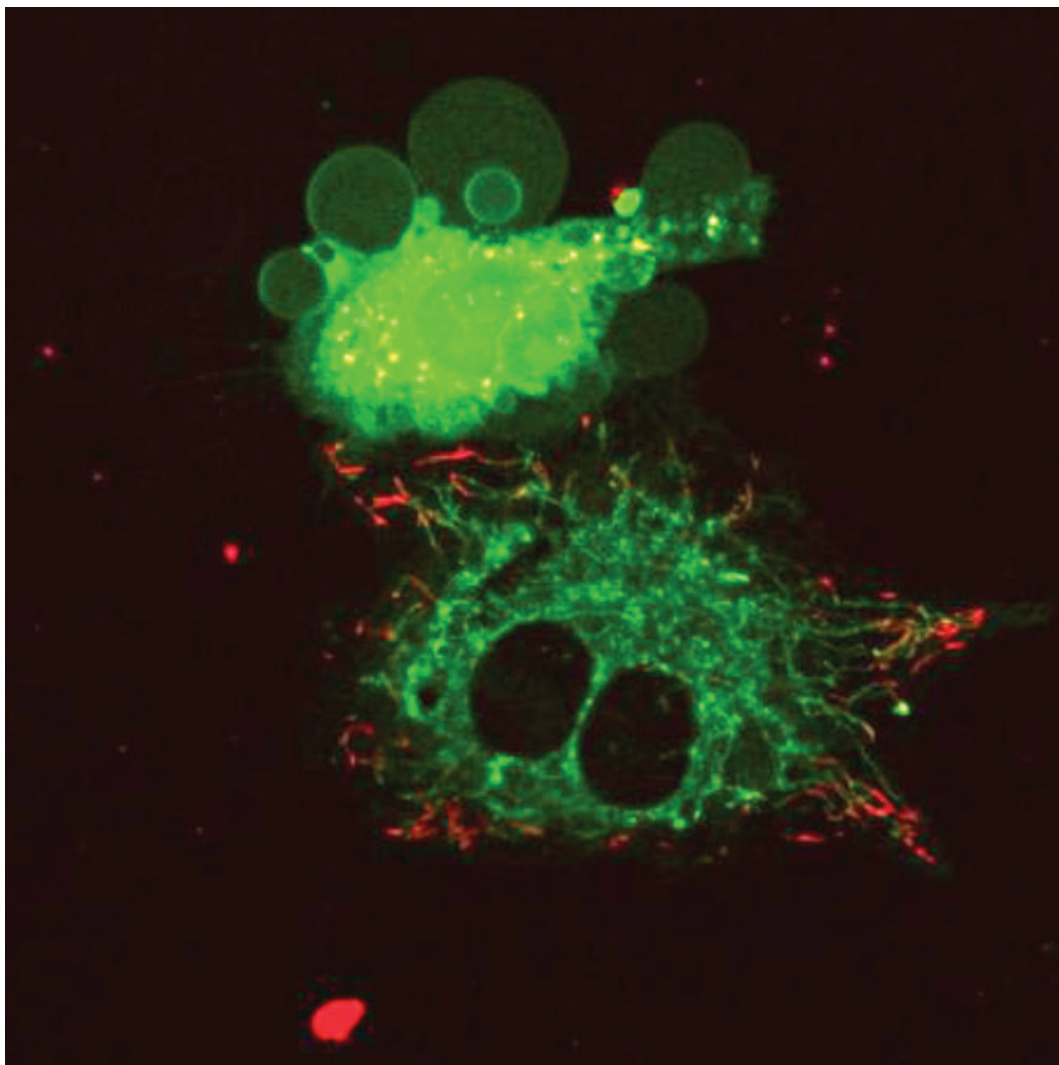
Figure 21.2. Different dye concentrations under identical experimental conditions can produce significantly different amounts of background.



## Part Five: Troubleshooting

### 22. Phototoxicity

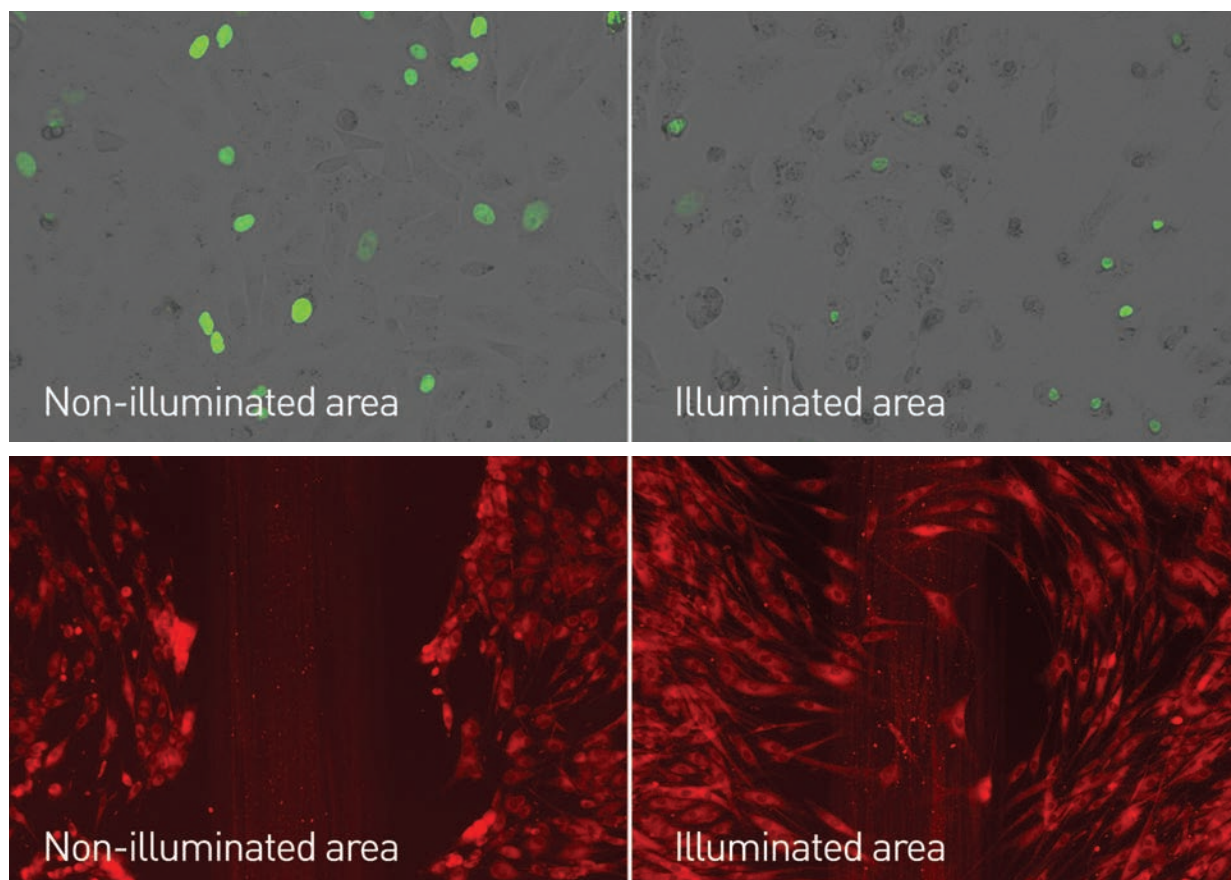
Figure 22.1. The cell in the top of the figure shows catastrophic blebbing of the cell membrane, while its neighbor remains relatively healthy.





## Part Five: Troubleshooting

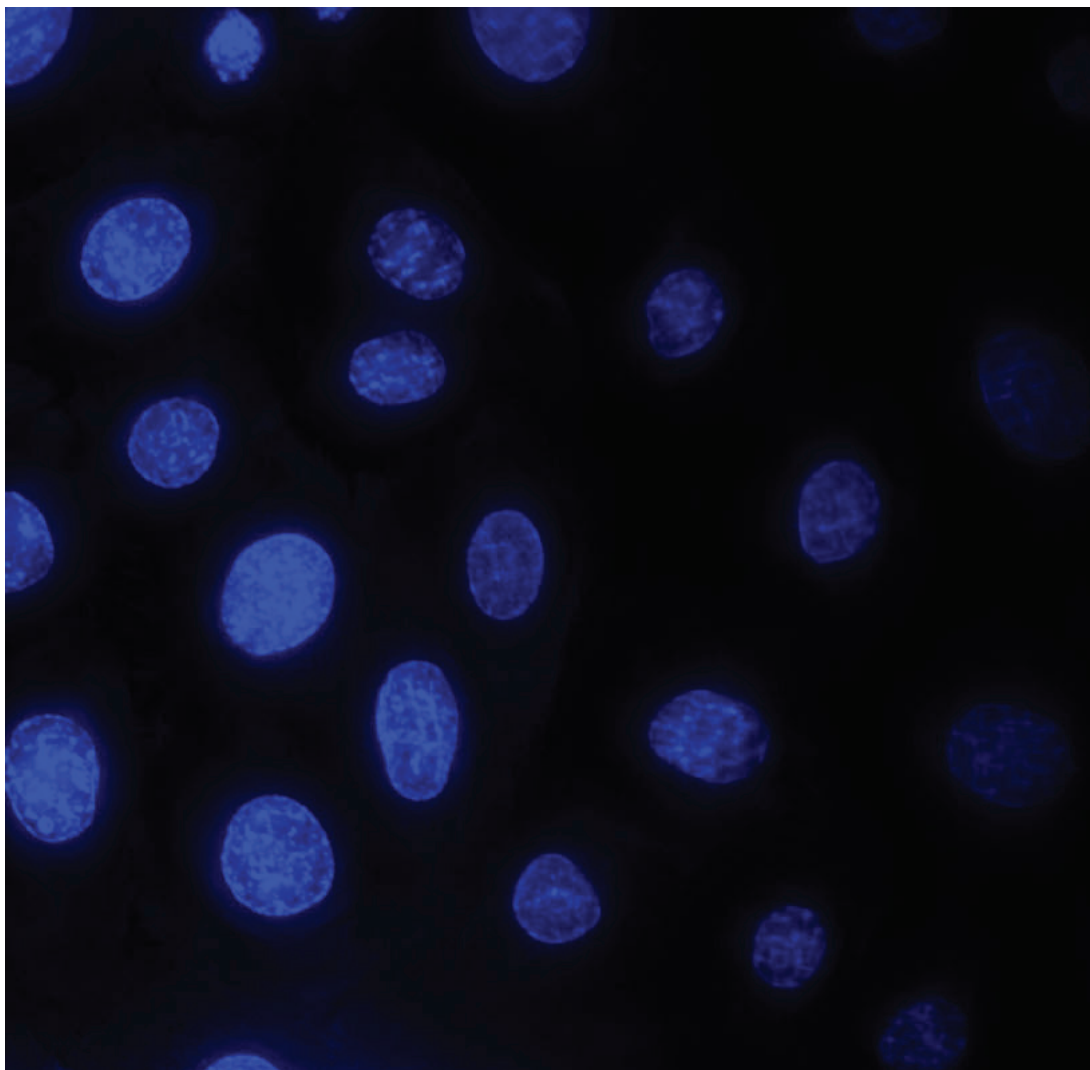
Figure 22.2. Top panel: brightfield and green channel overlay of a field of live HeLa cells transduced with CellLight® H2B-GFP reagent. Cells in the illuminated area have undergone repeated illumination for 10 hours prior to the capture of this image; cells in the non-illuminated area were illuminated only for the capture of this image. For the sample subjected to repeated illumination, we see dimming and loss of the GFP signal (green channel image), and we can also observe significant cell damage, including cell shrinking, cell rounding, and mitochondrial enlargement (brightfield channel image). Bottom panel: scratch wound in a culture of HDFn cells loaded with CellTracker® Deep Red reagent. The illuminated area was subjected to repeated illumination for 10 hr. Cells in this area shows a loss of viability were not able to grow into the wound, while cells in the non-illuminated area show viable cell growth into the wound.

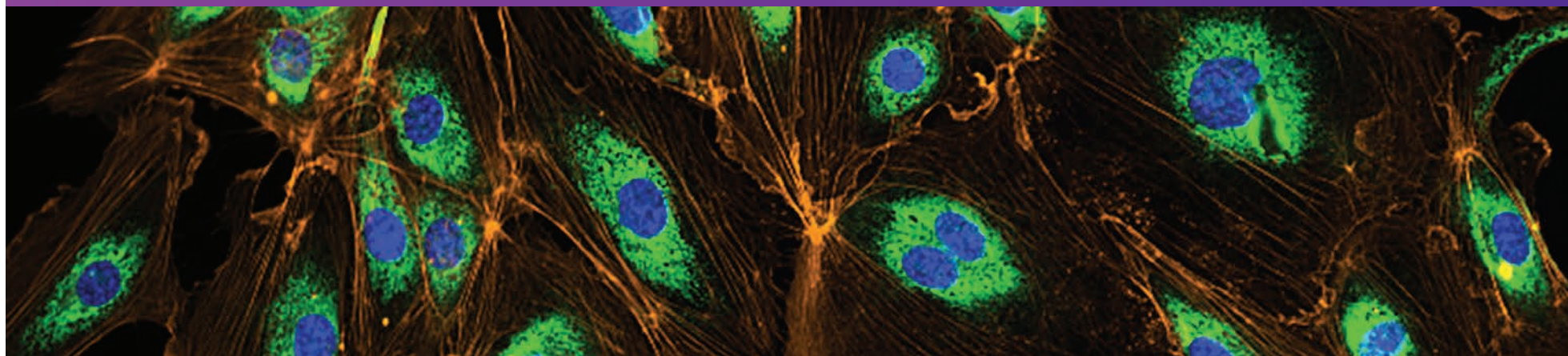


## Part Five: Troubleshooting

### 23. Uneven Illumination

Figure 23.1. Cells stained with a nucleic acid dye are not evenly illuminated, most likely due to a misaligned light path in the microscope.





## Tools for active learning in the biology classroom

One of the best ways to engage your students is with hands-on activities that reinforce the concepts presented in the lectures. A great way to learn about fluorescence imaging is to actually do it! We recommend the tools below for experiential learning in the biological sciences:



EVOS® FLoid® Cell Imaging Station, a self-contained instrument that captures high-quality, three-color fluorescent cell images right at the benchtop, with an interface that is so simple even novice users can collect data in just a few clicks of the mouse.



ReadyProbes® Ready-to-Use Imaging Reagents are designed to allow you to stain common structures in cells with:

- No calculations
- No dilutions



Cell Imaging Guide with Protocols allow you to find fluorescent dyes, reagents and protocols for cell biology related fluorescence microscopy applications.

For more information, go to [lifetechnologies.com/imagingbasics](http://lifetechnologies.com/imagingbasics)

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