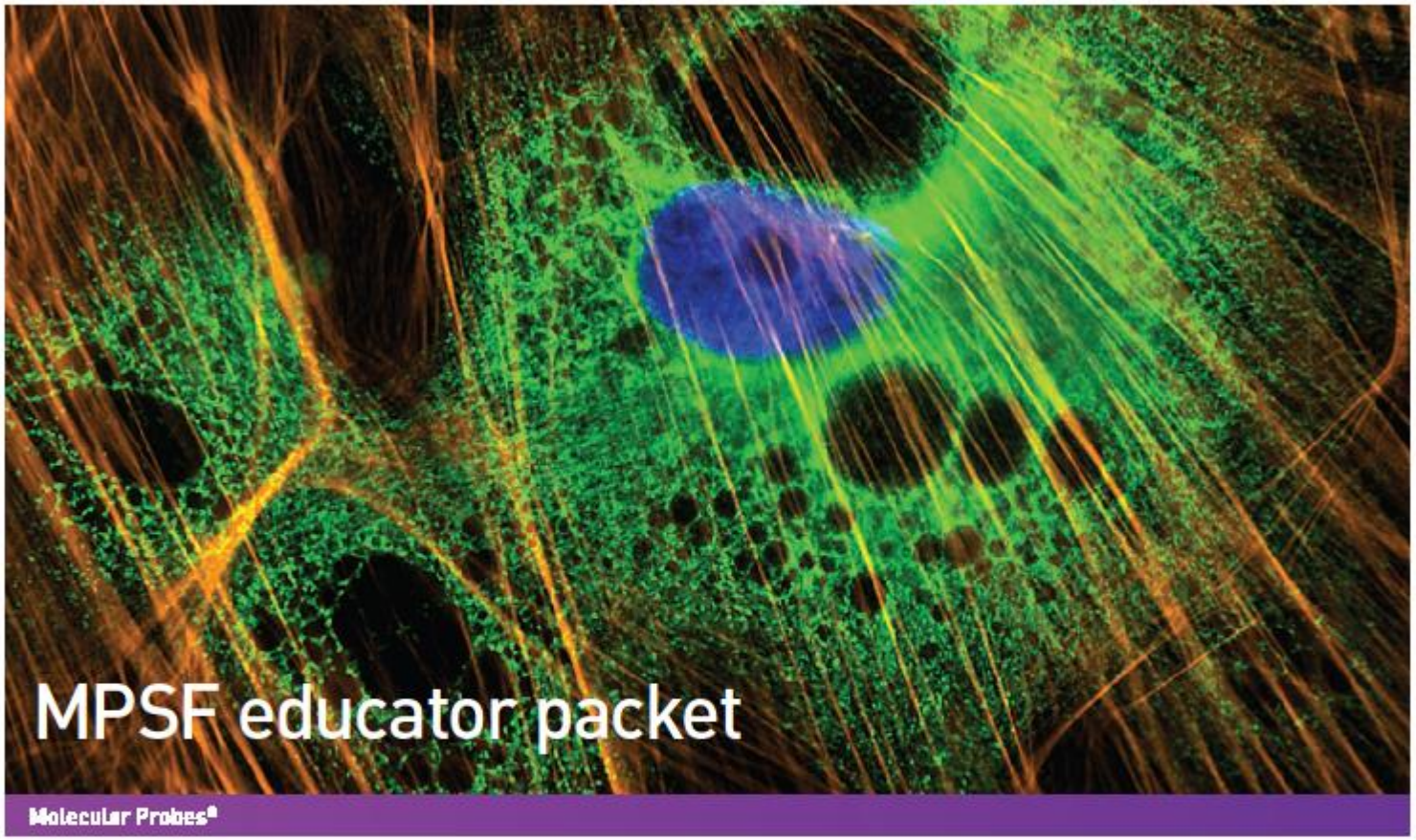
Microscopy & fluorescence

Prof. Dr. Andrei Leitão

Microscopy



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.

life technologies
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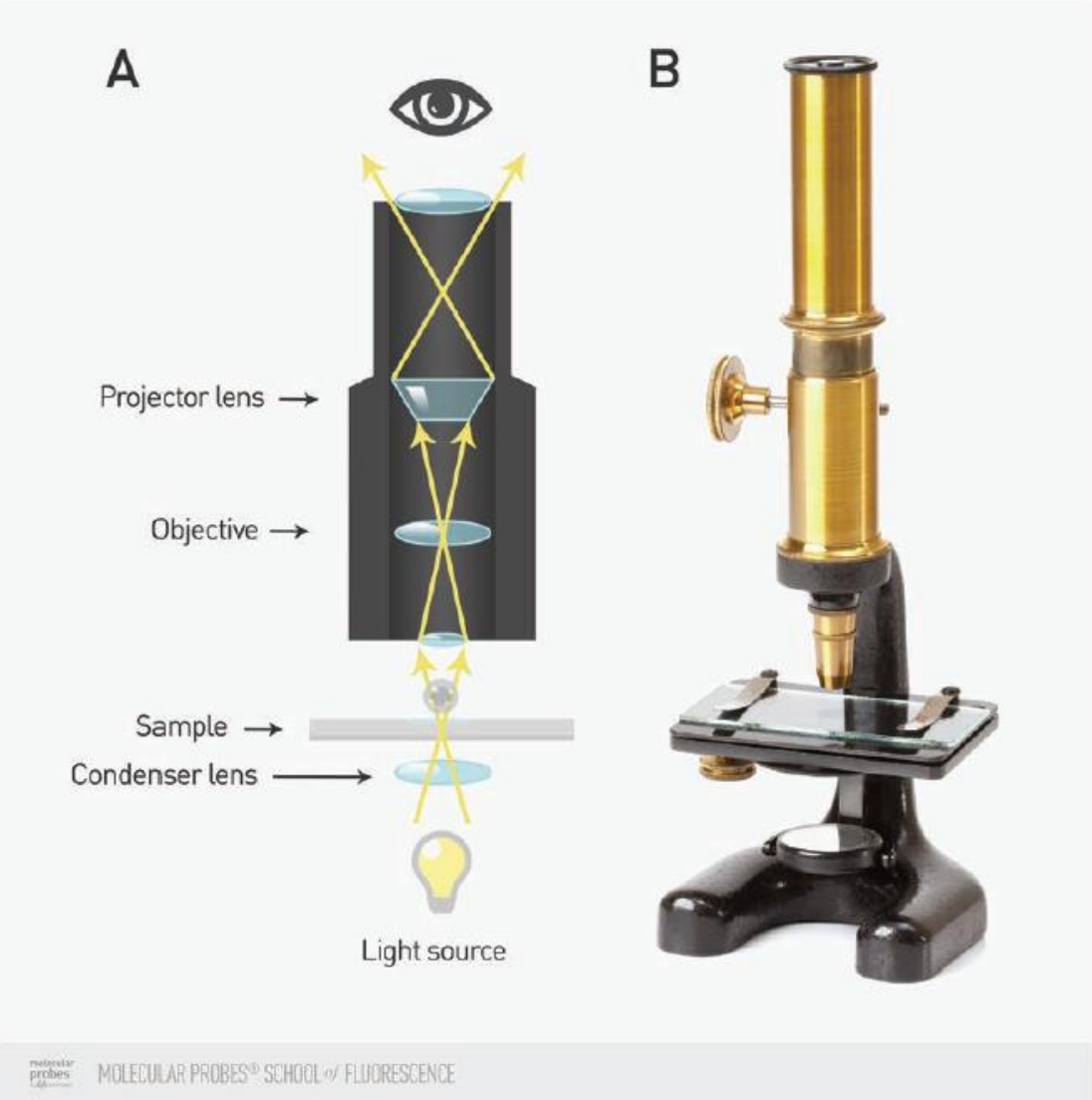
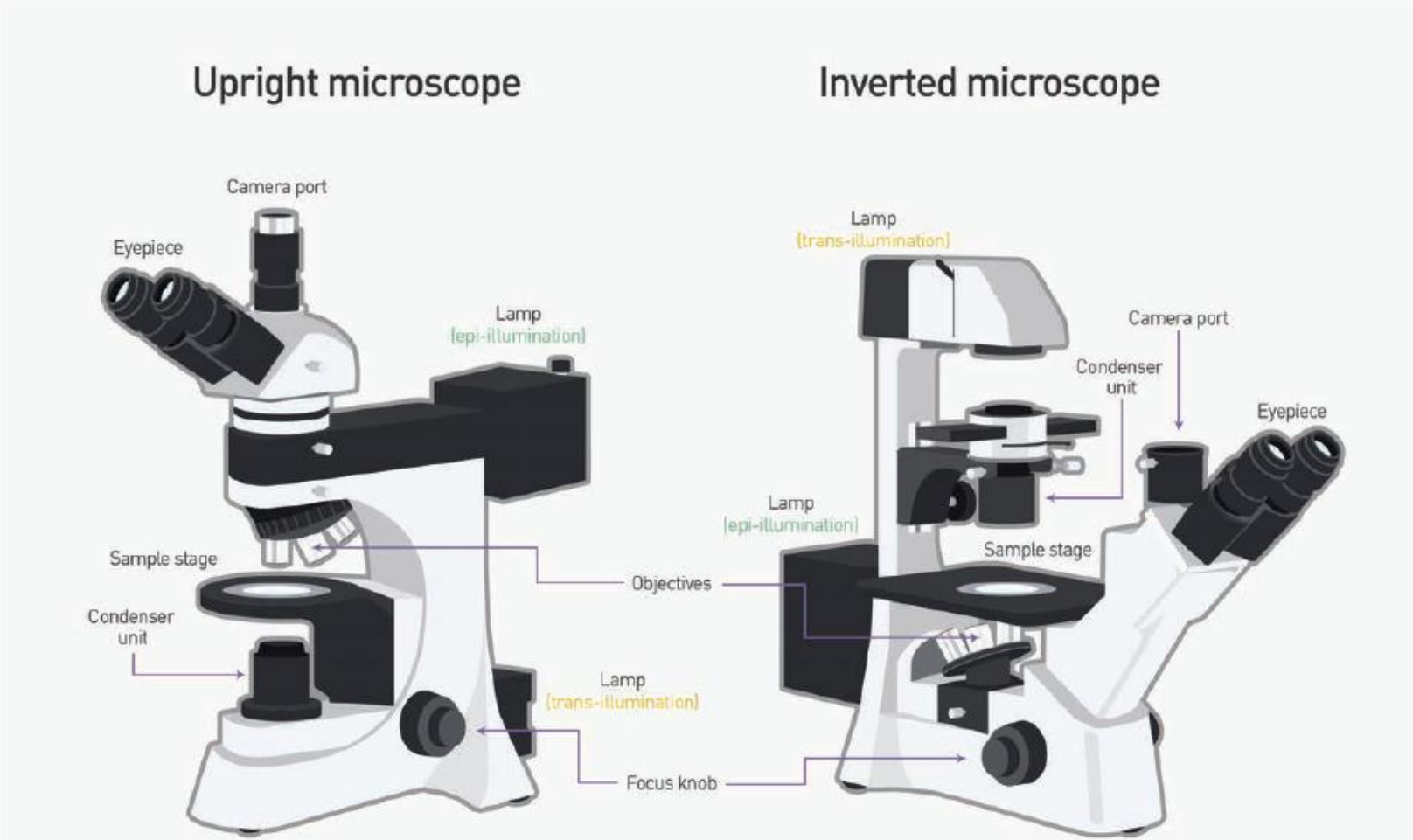
Microscopy

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



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.



Resolution

Figure 3.4A. Two 6 µm beads taken at 3 different magnifications, 4x, 10x, and 40x.

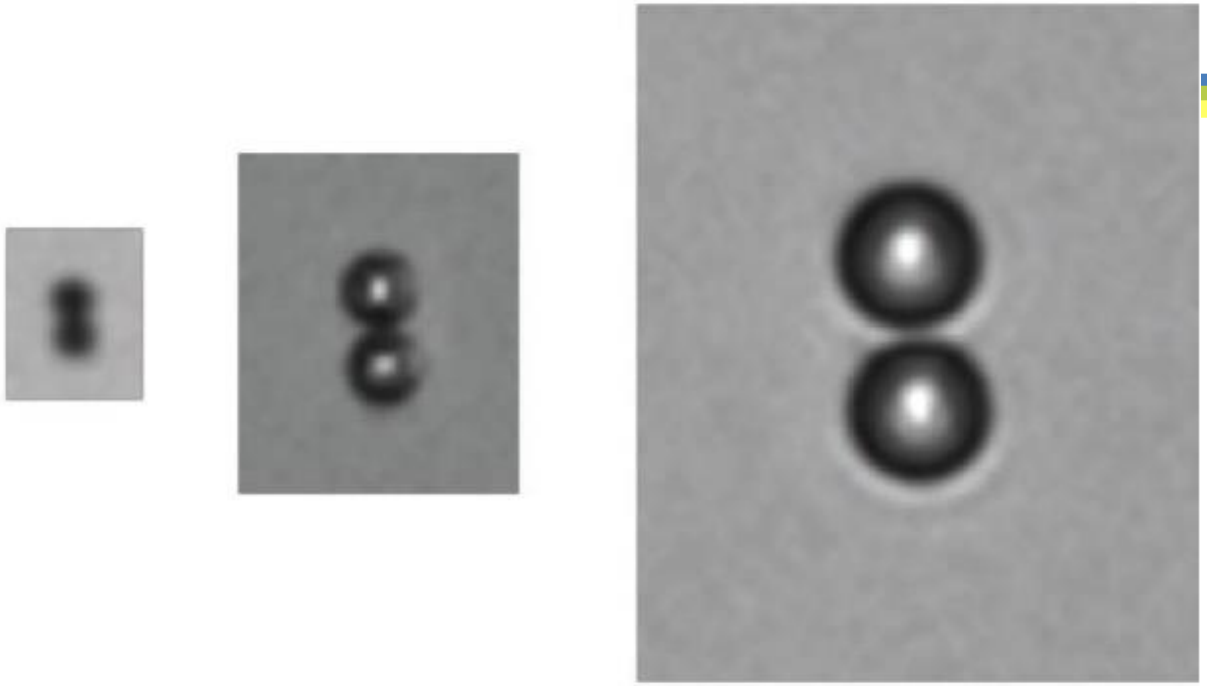
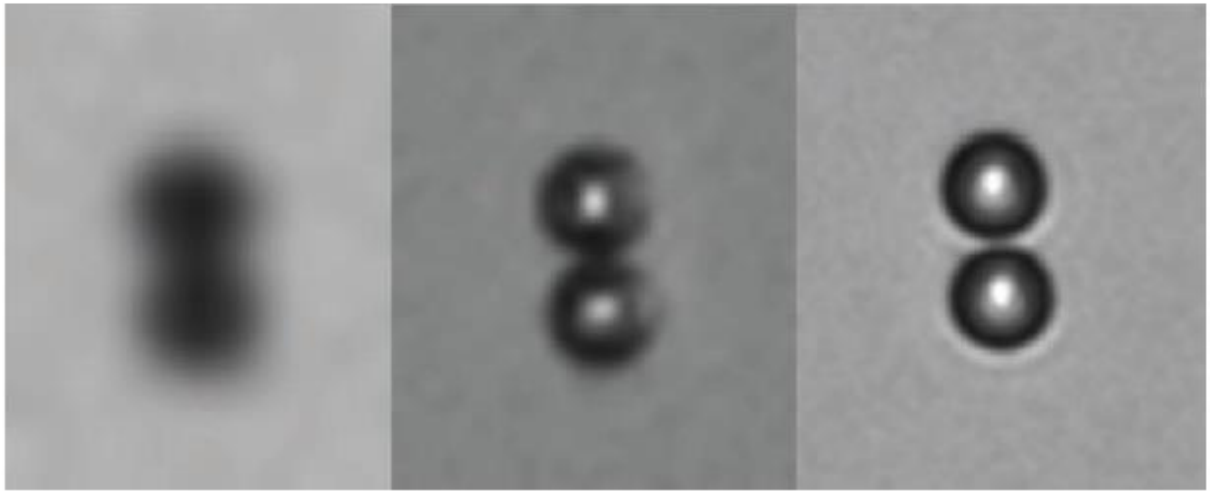


Figure 3.4B.



Magnification

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.

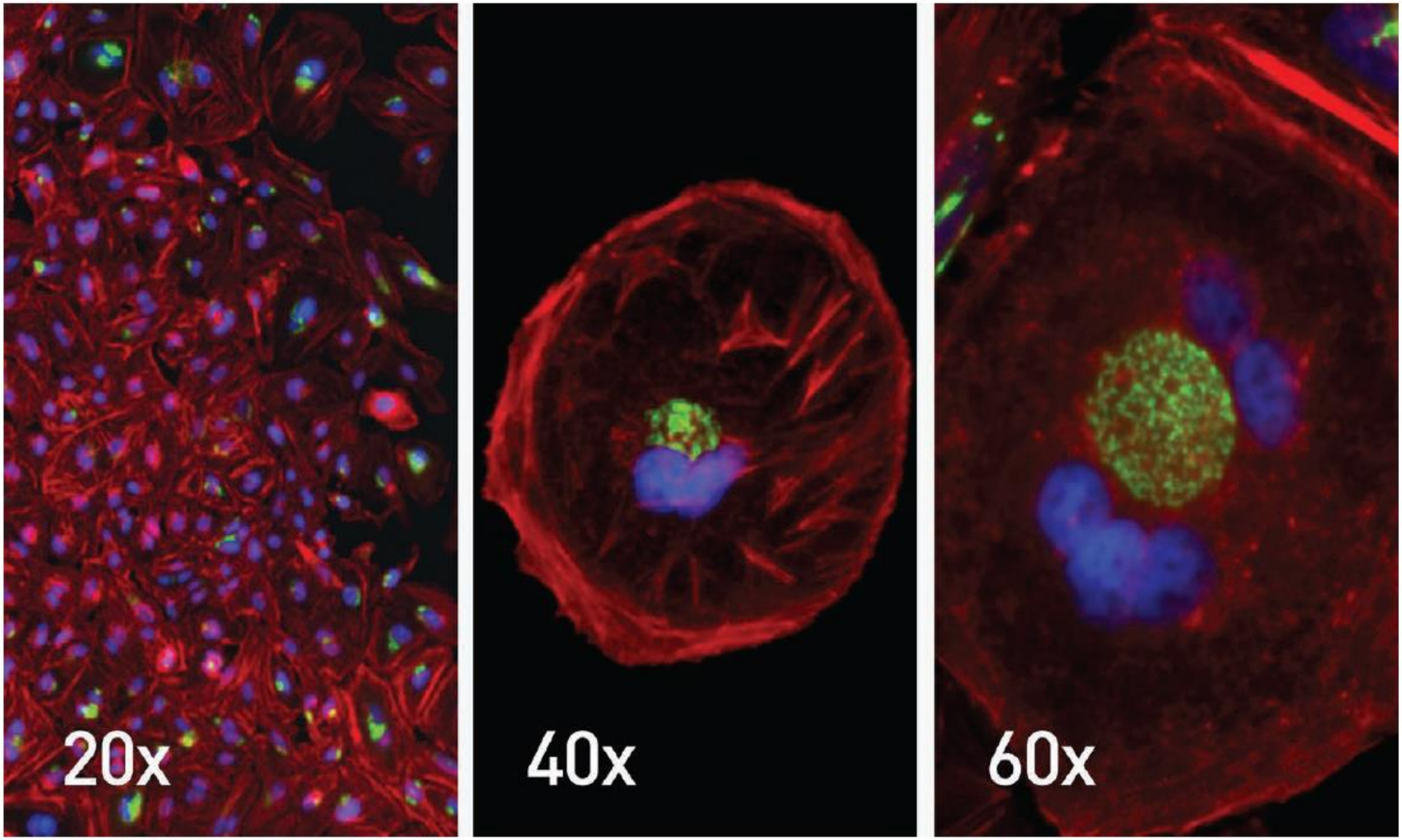
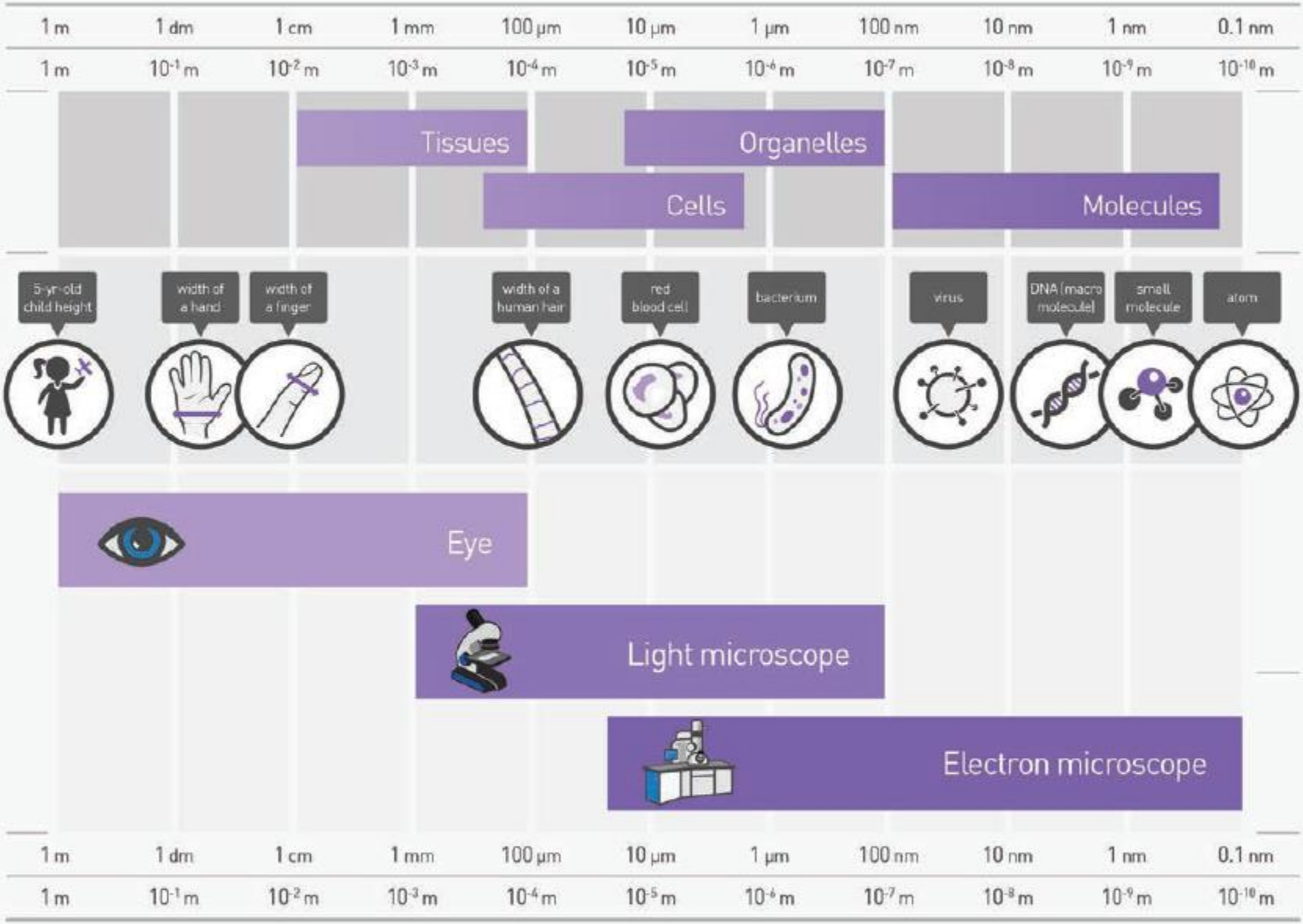


Figure 3.6. The resolving power of various microscopes, with representative objects within range for both light microscopes and electron microscopes.



Epifluorescence microscopy

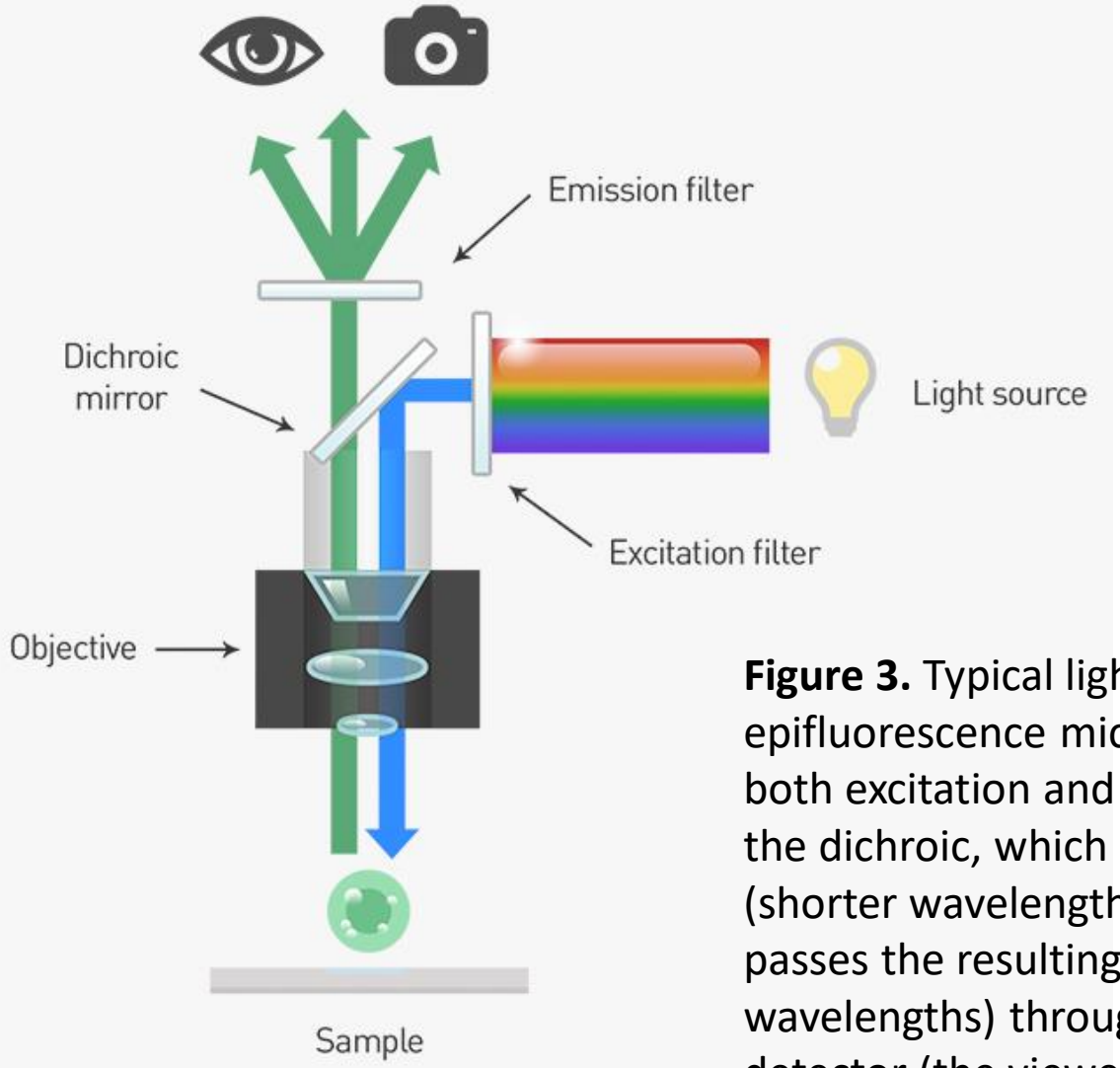


Figure 3. Typical light path in an epifluorescence microscope. Notice that the both excitation and emission are controlled by the dichroic, which reflects excitation light (shorter wavelengths) onto the sample and passes the resulting emission light (longer wavelengths) through the filter and on to the detector (the viewer or the camera).

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.

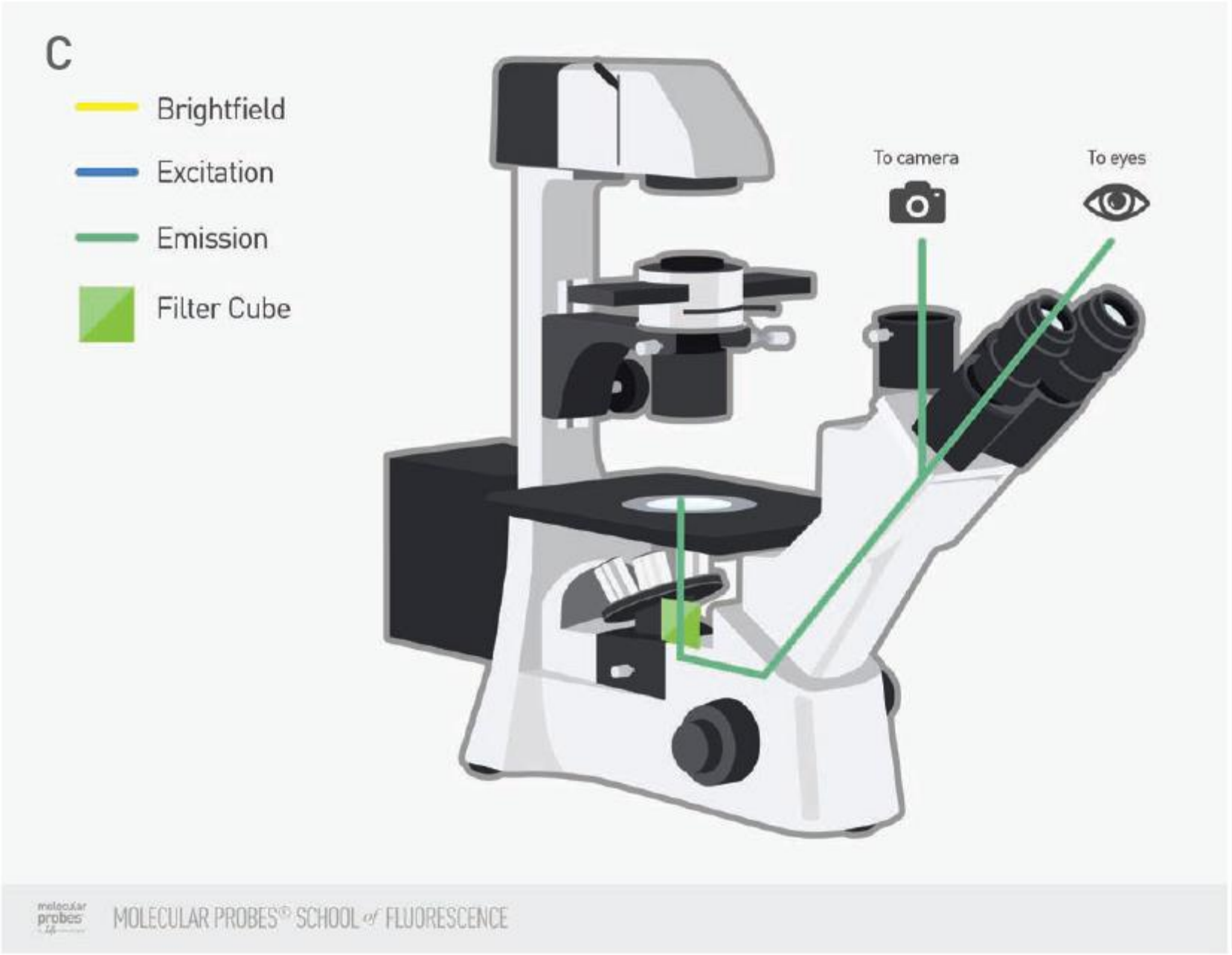
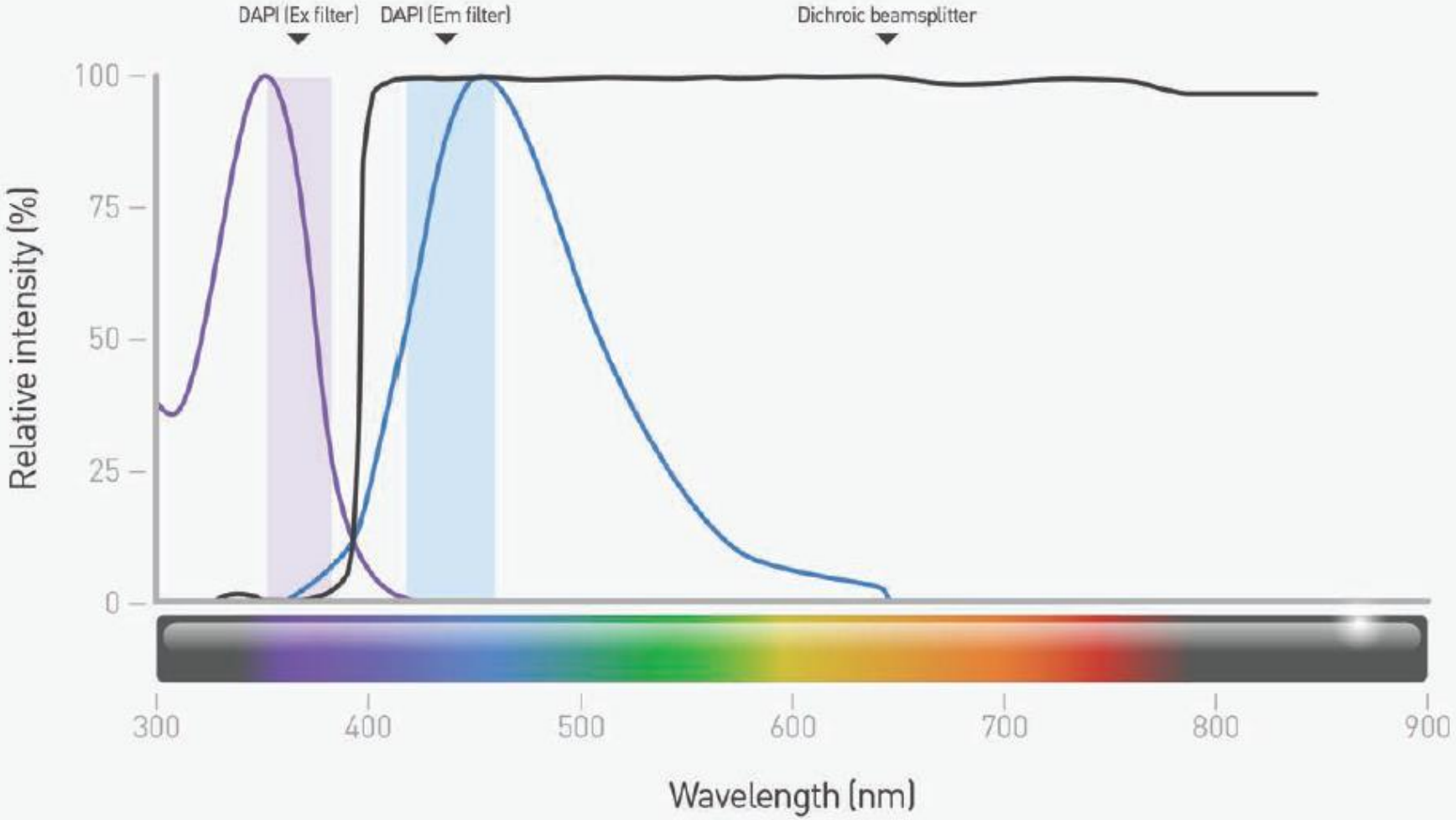
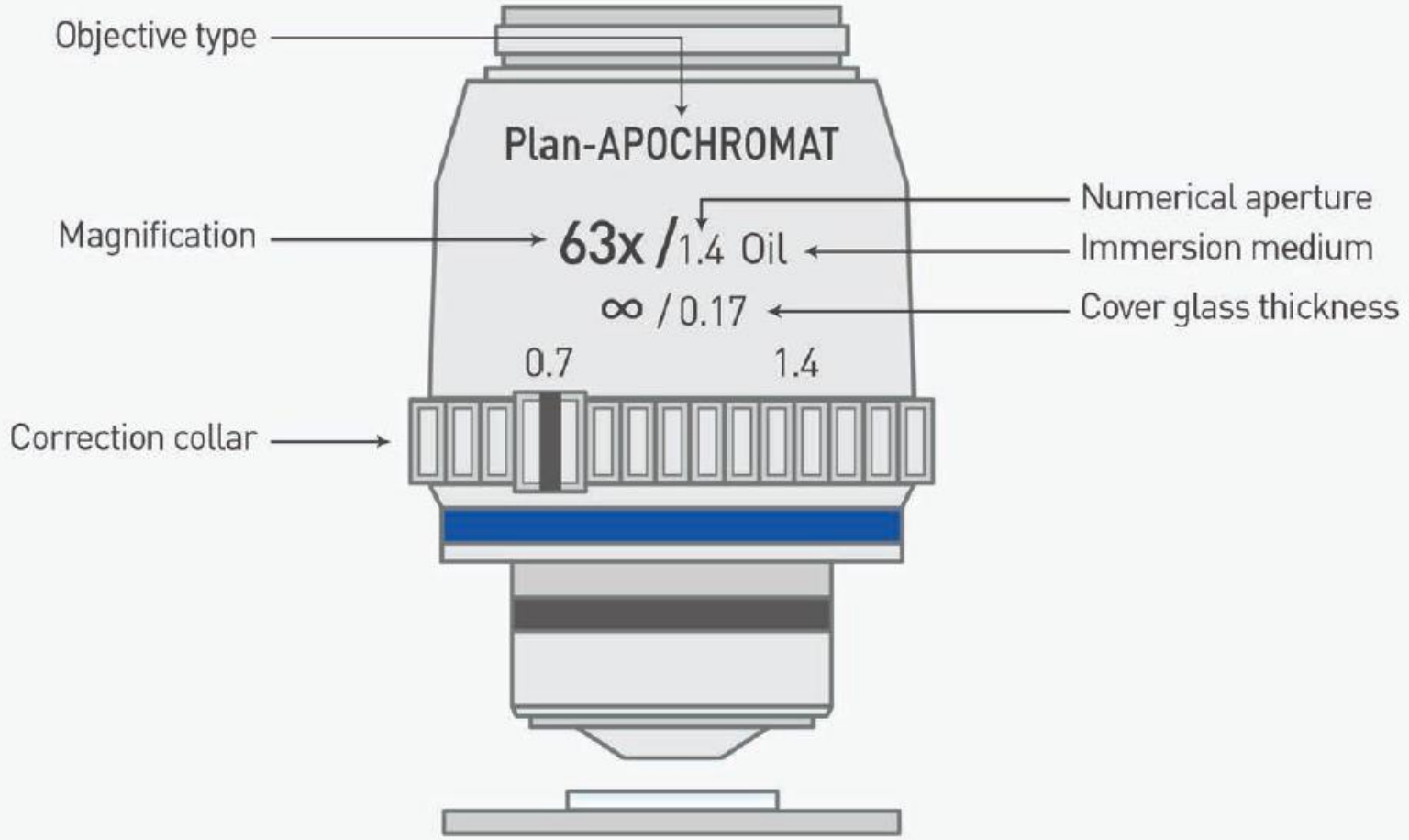


Figure 4.1. Excitation and emission spectra of a nuclear dye (DAPI) overlaid with the range of wavelengths passed through the filters for emission (purple box) and excitation (blue box). The black line depicts the transmission of the dichroic filter.



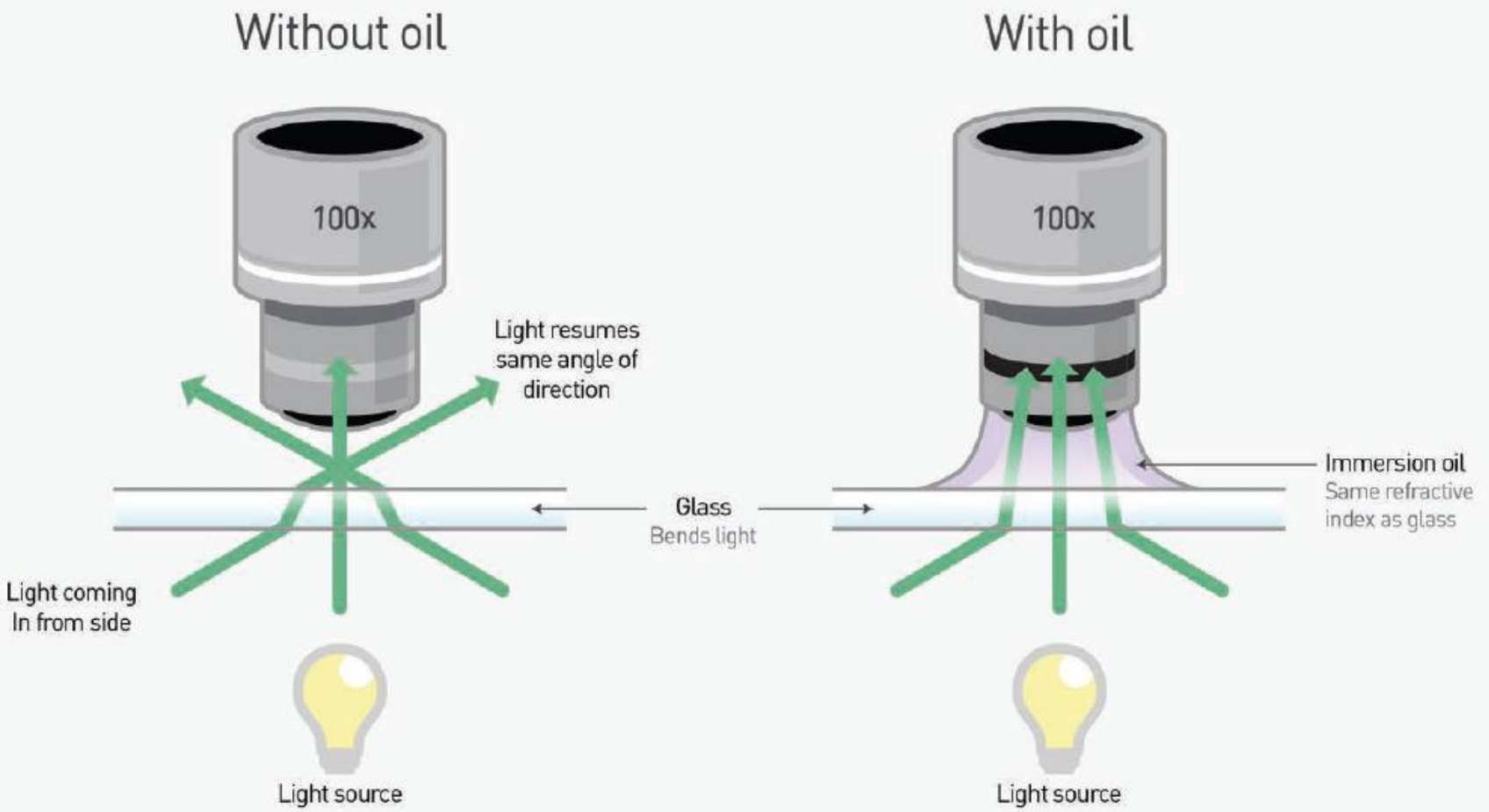
Lens

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



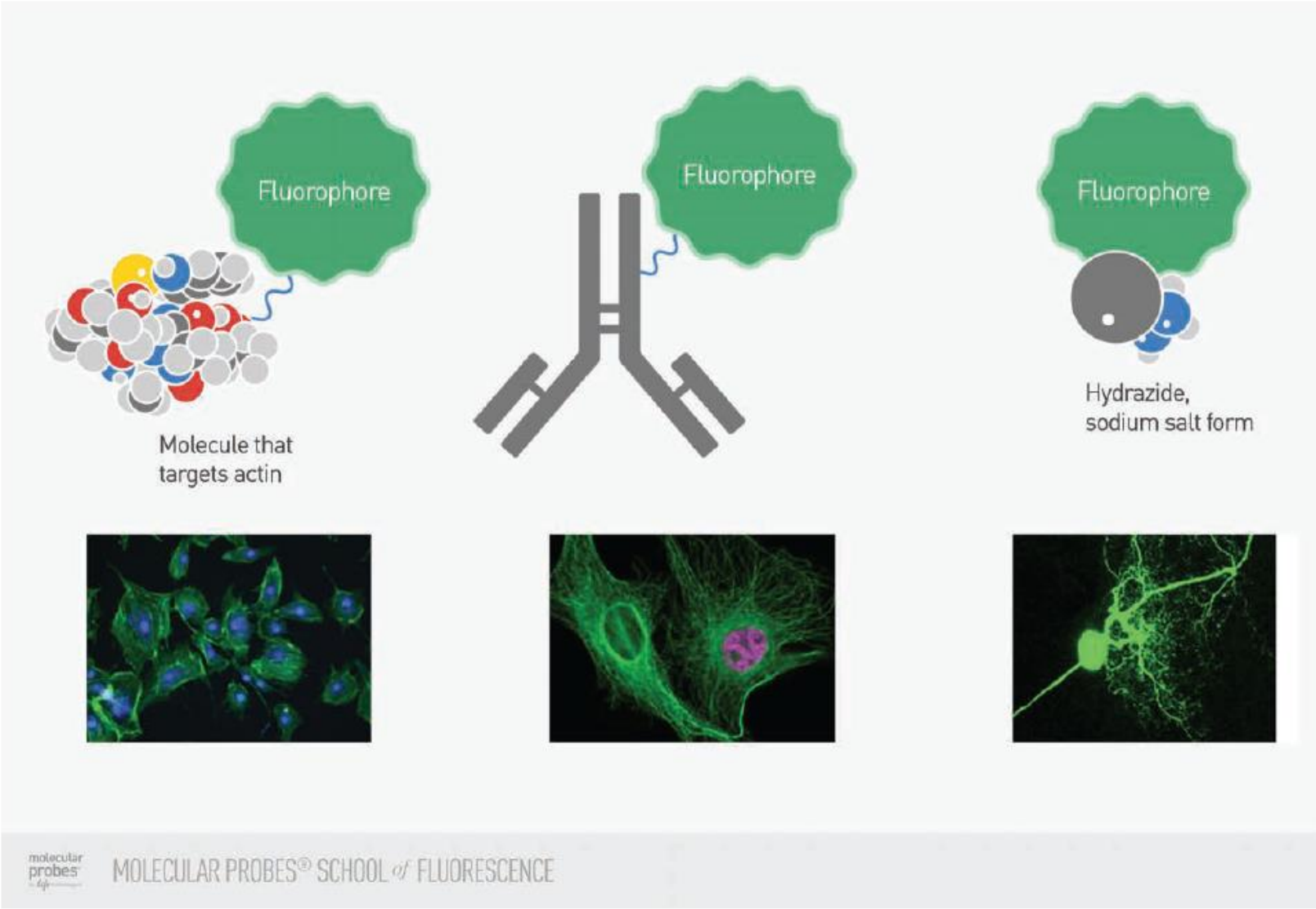
Lens

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



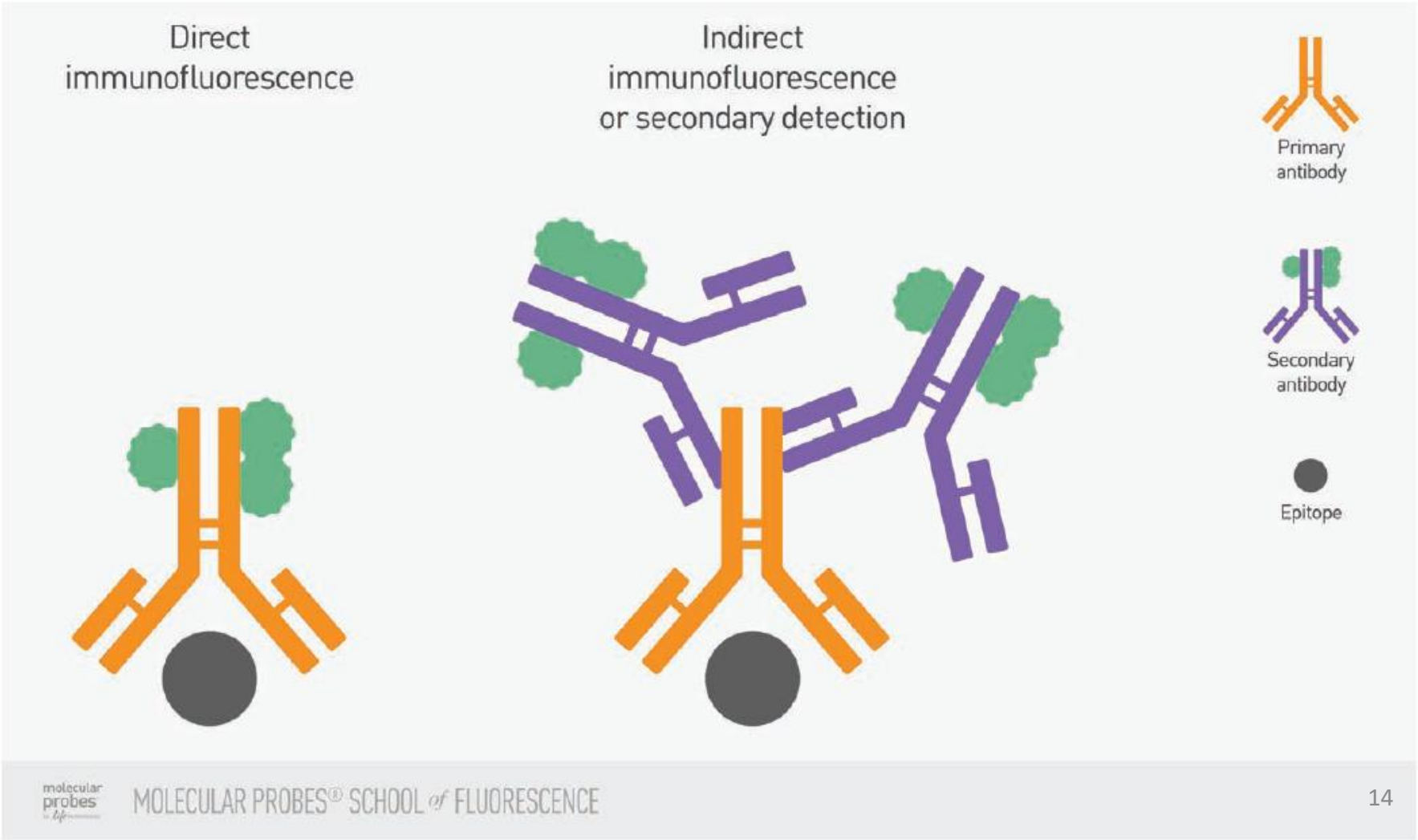
Ways to carry out the fluorophore

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.



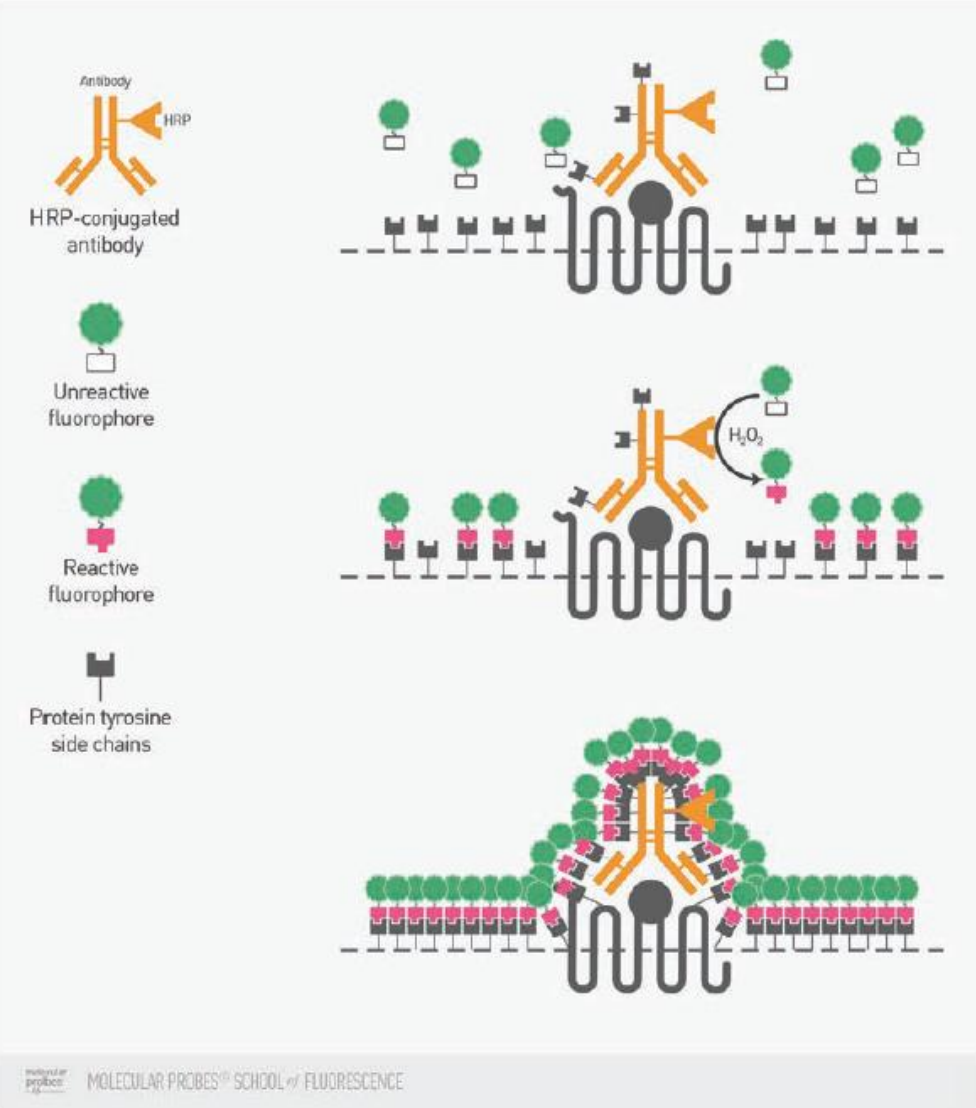
Labeling

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.



Labeling

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.



Direct immunofluorescence

(+) Pros	(-) Cons
•Direct labeling results in shorter sample staining time and a simpler workflow •Offers the best solution for specific targeting if multiple antibodies to multiple targets are raised in the same species •Avoids any cross-reactivity between secondary antibodies	•Fluorophore signal relies on the finite number of fluorophores that can be attached to a single antibody, and consequently, can limit detection to high-abundance targets •Workflow can be less flexible (fewer colors may be available), more expensive, and, if commercially labeled direct conjugates are unavailable, more difficult

Indirect immunofluorescence

(+) Pros		(+) Pros
•Offers greater sensitivity because more than one secondary antibody can bind to each primary antibody, resulting in signal amplification •Commercially produced secondary antibodies are relatively inexpensive, and are available in a wide spectrum of colors		•Potential for cross-reactivity; when performing multilabel experiments, indirect labeling requires the use of primary antibodies that are not raised in the same species •Secondary antibody may react with endogenous immunoglobulin in tissue samples, which can result in higher background fluorescence

Fixation of the sample

(+) Pros	(-) Cons
•Cells are preserved at a certain time point •Cells become permeable to large molecules •Gross cellular structures are protected and stabilized •Targets are easier to image when they are not moving	•Protein conformation may be changed by fixation •Most enzymes are inactivated •Soluble contents of the cell can be lost •Dynamic processes give more functional information

Fixation & permeabilization

Formaldehyde is the most commonly used fixative; it works by chemically bonding adjacent macromolecules, such as proteins, together. Most available formaldehyde preparations are actually paraformaldehyde (PFA, polymeric formaldehyde) dissolved in water or a buffer. The free methanediols in the resulting solution are reactive with amine groups on proteins and other cellular structures that contain nitrogen. PFA also solubilizes some lipids in cellular membranes. PFA is commonly diluted to 3.7–5% v/v and is applied to cells for 10–15 minutes.

The permeabilization step removes more cellular membrane lipids to allow large molecules like antibodies to get inside the cell. Triton® X-100 and NP-40 are detergents commonly used at 0.1–0.5% (v/v, in PBS) for permeabilization. A permeabilization time of 10–15 minutes is a good starting point, but if that isn't working well for your target you might need to try a shorter time or a different detergent. These detergents will also permeabilize the nuclear membrane, so they are suitable for a variety of target locations.

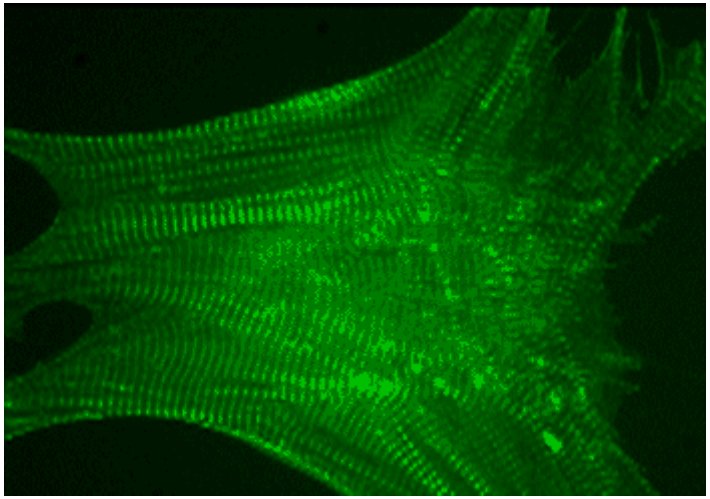
Blocking the antibody

The most common types of blocking solutions for ICC are 3% (w/v) bovine serum albumin in PBS and/or a 10% (v/v) solution of heat-inactivated species-specific serum in PBS, where the serum species matches the species of the secondary antibody. For example, if you are using a mouse anti–rabbit IgG secondary antibody, chose normal heat-inactivated mouse serum to make your blocking reagent.

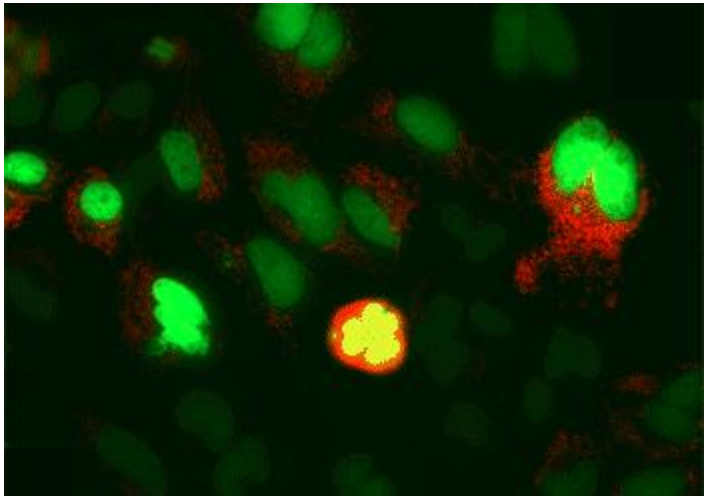
Live-cell imaging

(+) Pros	(-) Cons
•Cellular enzymes and other cytosolic biomolecules remain in the cell •Can observe dynamic cellular processes as they happen •Cellular structures can be studied in their native environment, so you get less experimental artifact •Cellular biomolecules and structures can be tracked over time •Interactions between cells can be observed	•Cells must be kept in their natural physiological ranges for pH, temperature, and osmolarity •Must have a specific way to label your target—whether it is a molecule, a cellular function, or a cellular state—and illuminate it with minimum toxicity •Living cells are not generally permeable to large molecules (i.e., antibodies) •Moving objects can be more difficult to keep in focus •Interrogation techniques can be harmful to living cells

Live-cell imaging



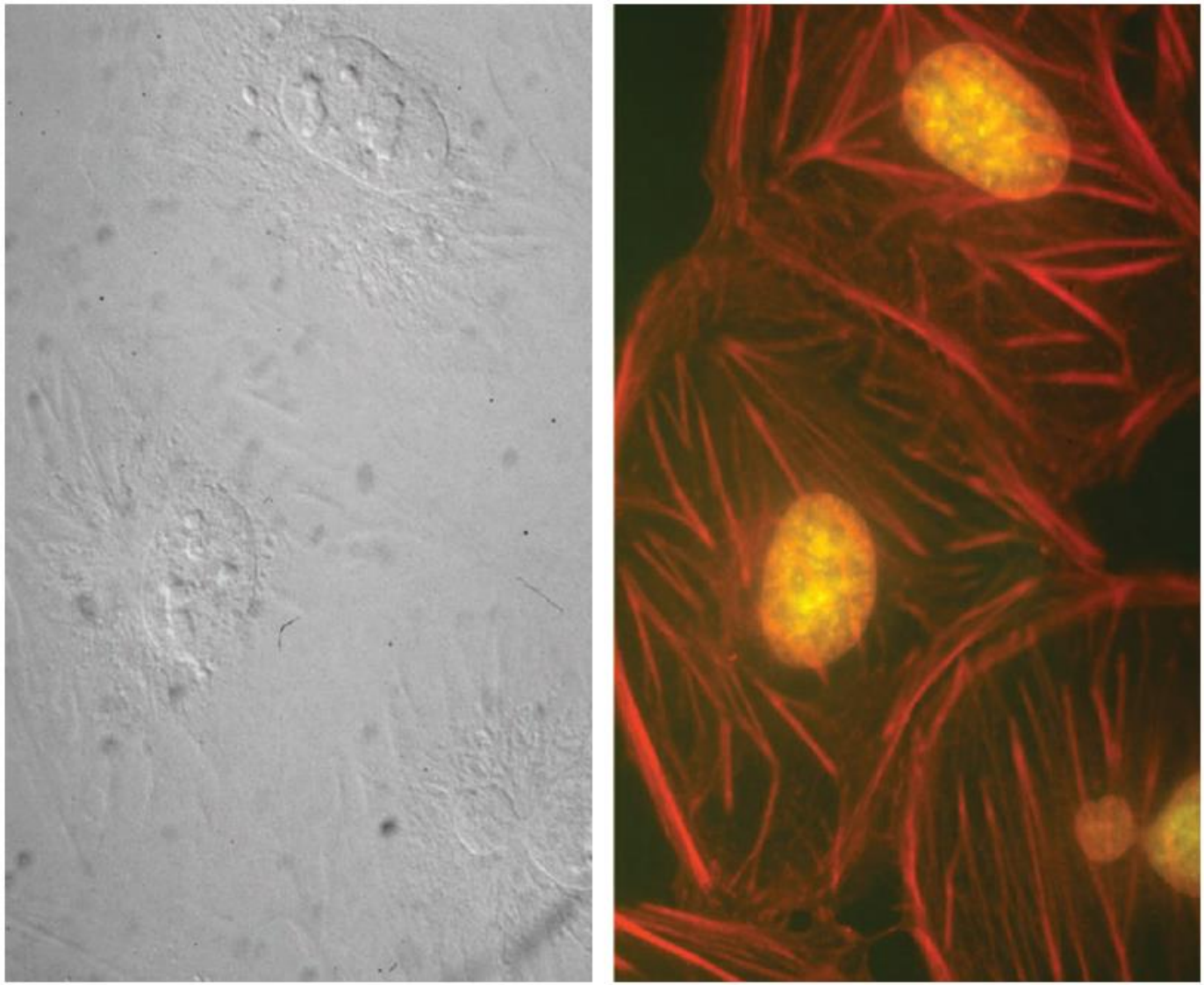
Spontaneous contractions in mouse embryonic stem cell–derived cardiomyocytes, transduced with CellLight® Actin-GFP.



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.

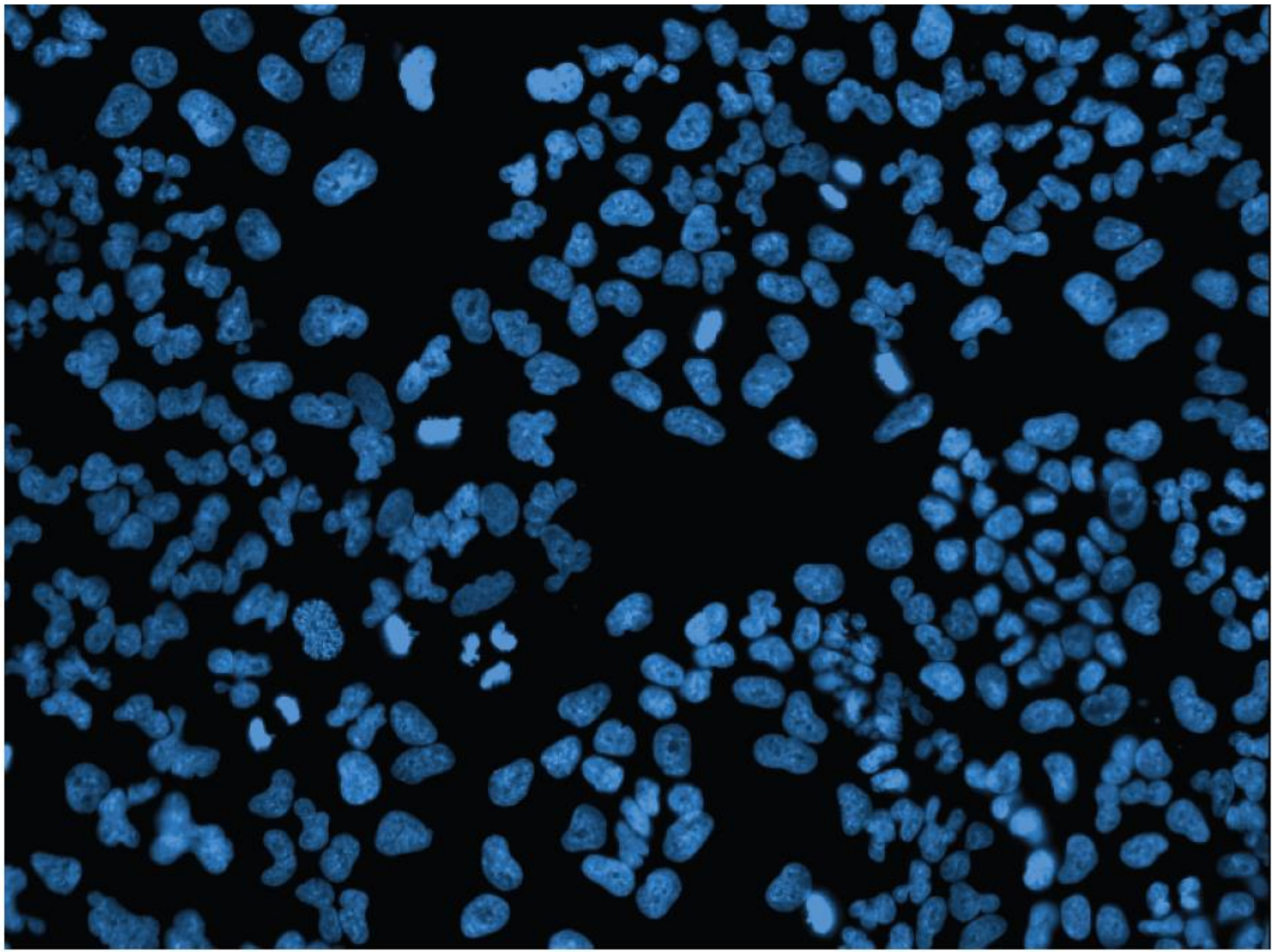
Bright field & fluorescence microscopy

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.



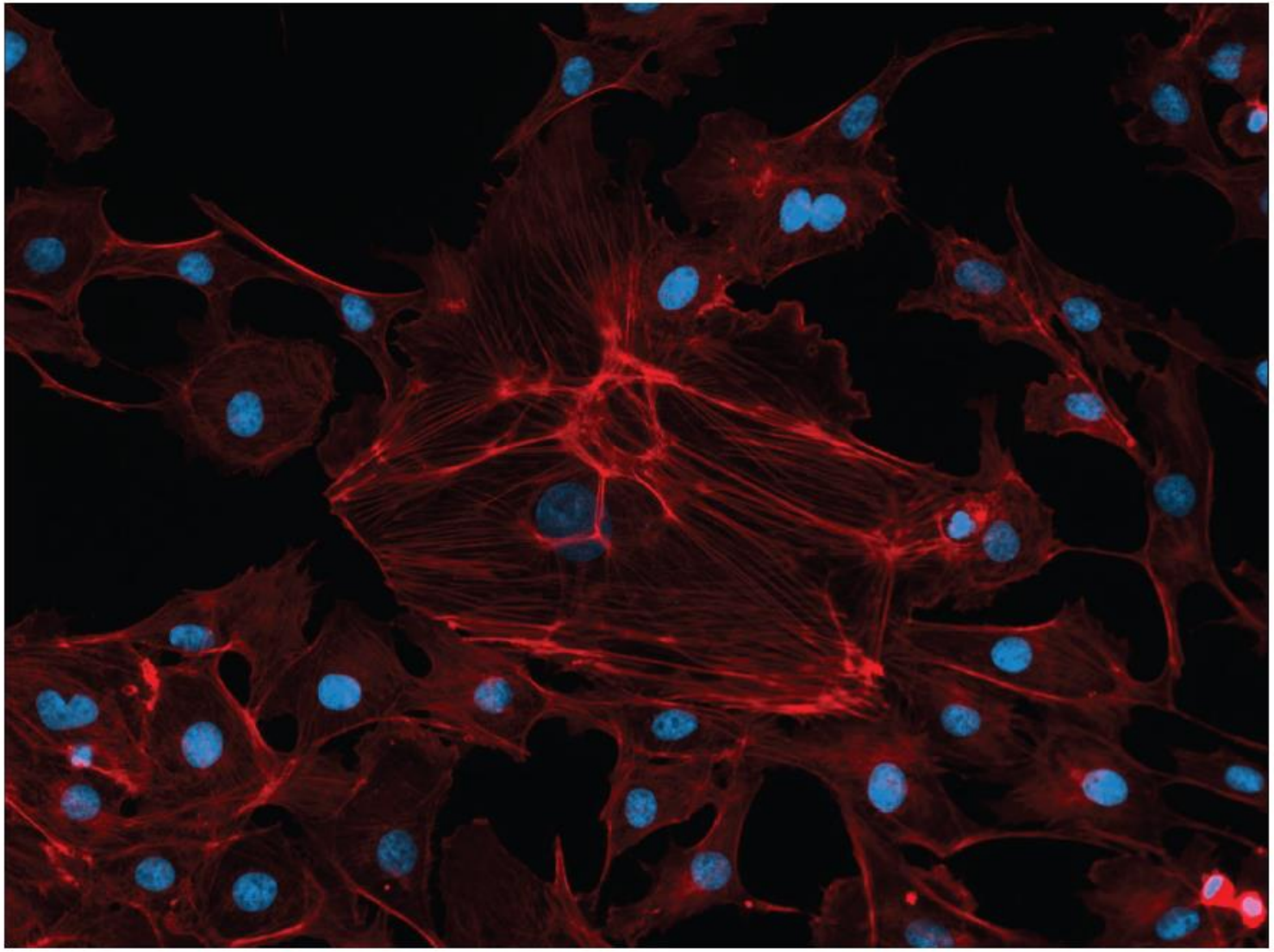
Application – nuclear staining

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



Application – cytoskeleton labeling

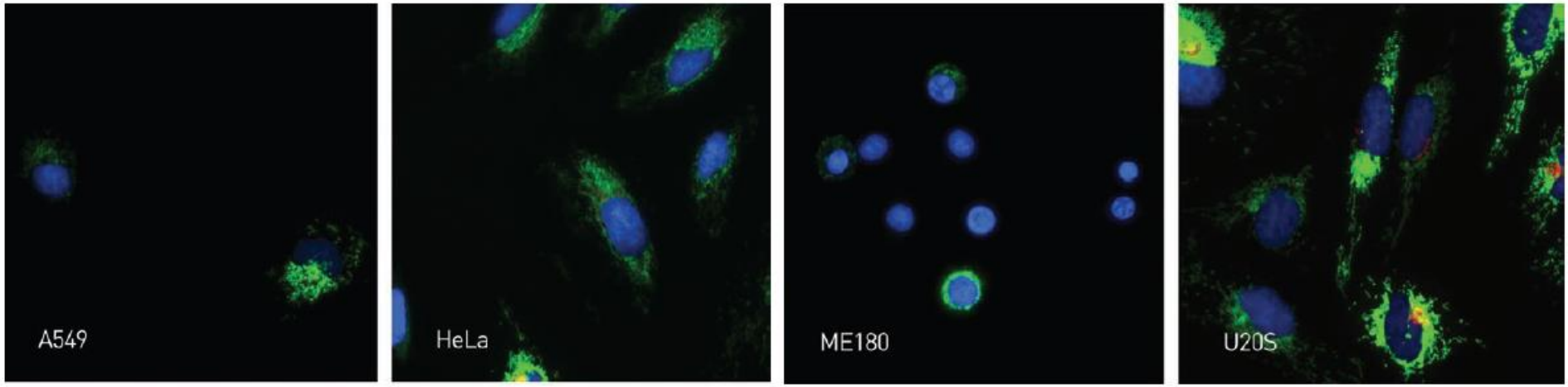
Figure 14.2. After fixing, permeabilizing, and blocking, BPAE cells were labeled with ActinRed™ 555 ReadyProbes® Reagent (TRITC-conjugated phalloidin that labels F-actin), and nuclei were labeled with NucBlue® Fixed Cell ReadyProbes® Reagent (a form of DAPI).



Application - morphology

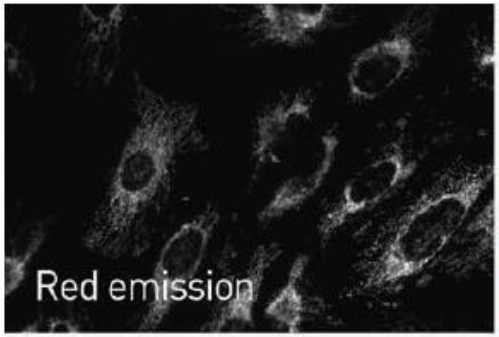
6. Cell Morphology

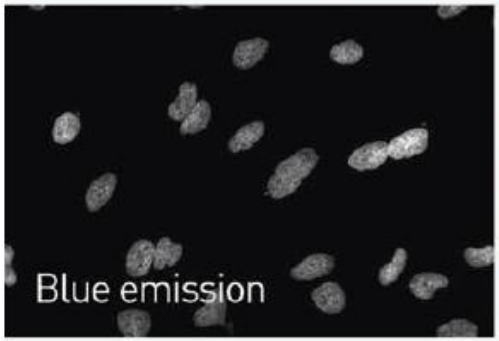
Figure 6.1. A549, HeLa, ME180, and U2OS cells all exhibit very different staining patterns for the same set of fluorescent reagents due to differences in their morphology and metabolic pathways. All four cell types were stained with NucBlue Live® reagent after transduction with CellLights® Golgi-RFP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

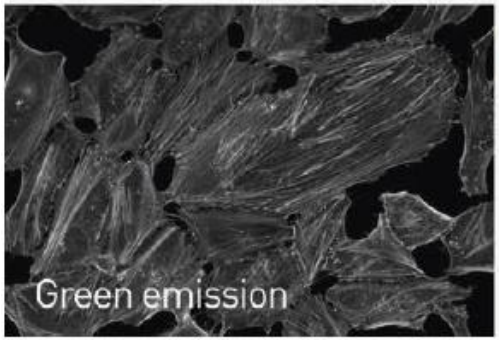


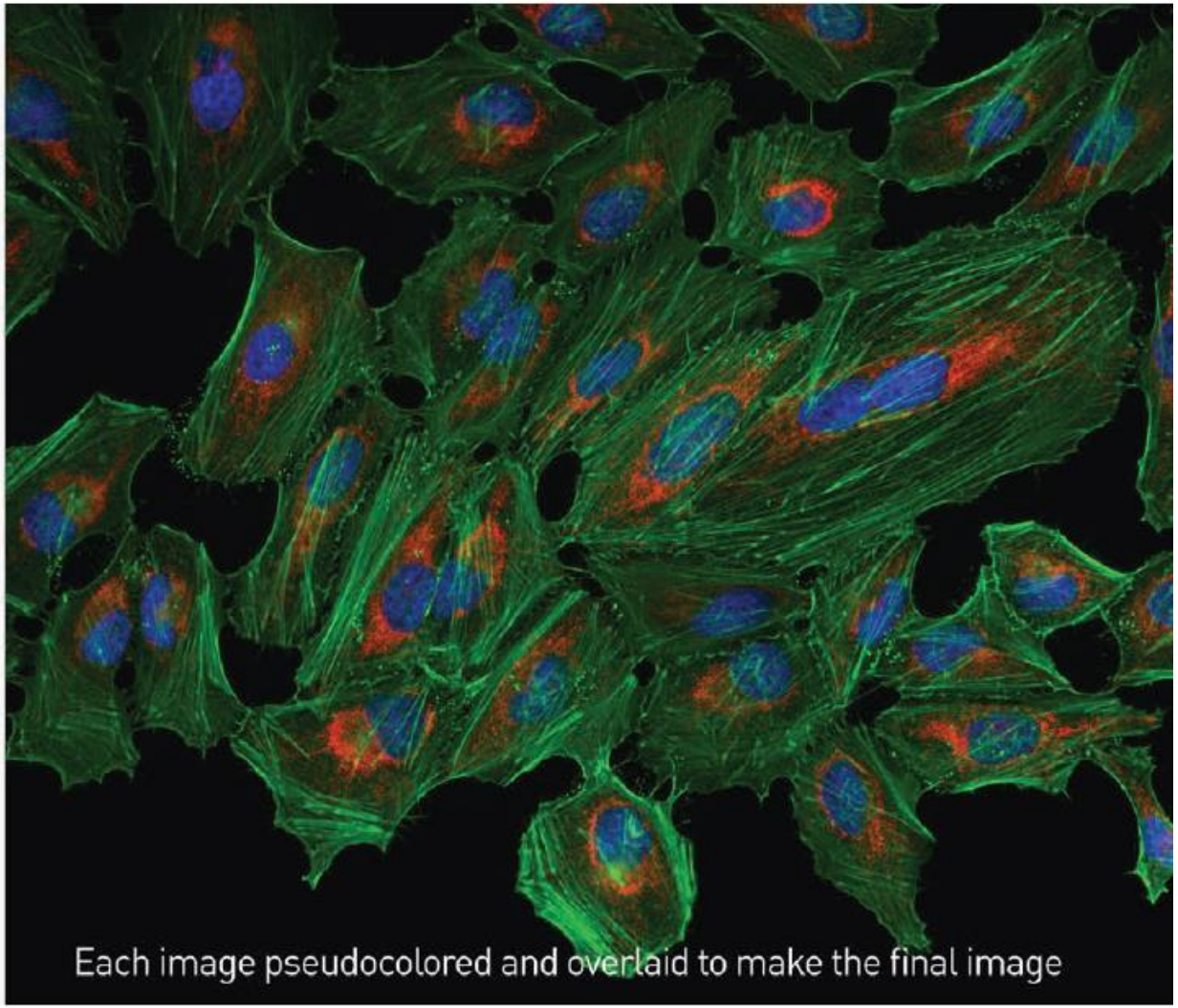
Application – multiple labeling

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).

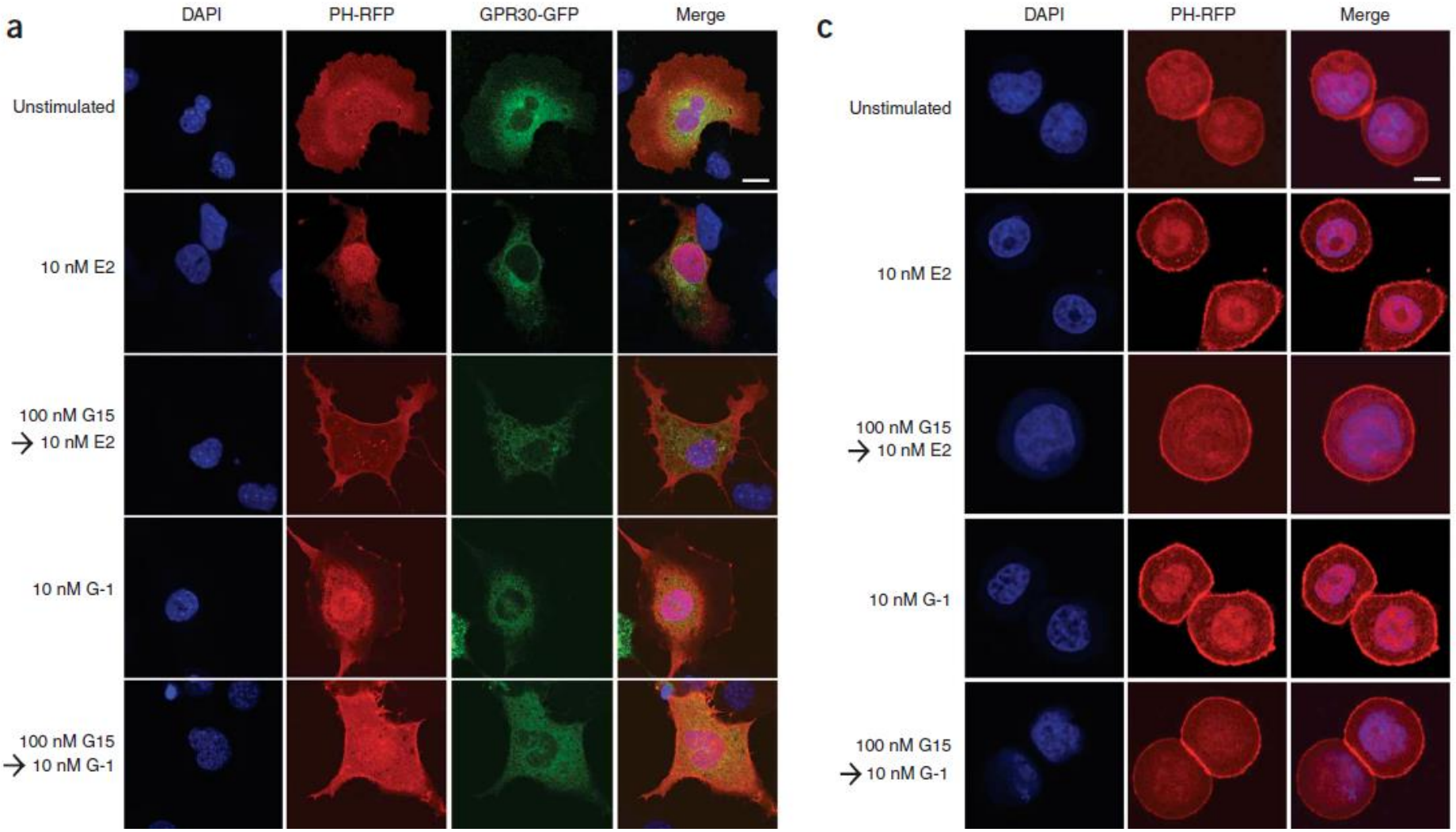








Application – cell signaling



Dennis, M.K.; et al. *Nat. Chem. Biol.* **2009**, *5*, 421-427.

Application – cell signaling

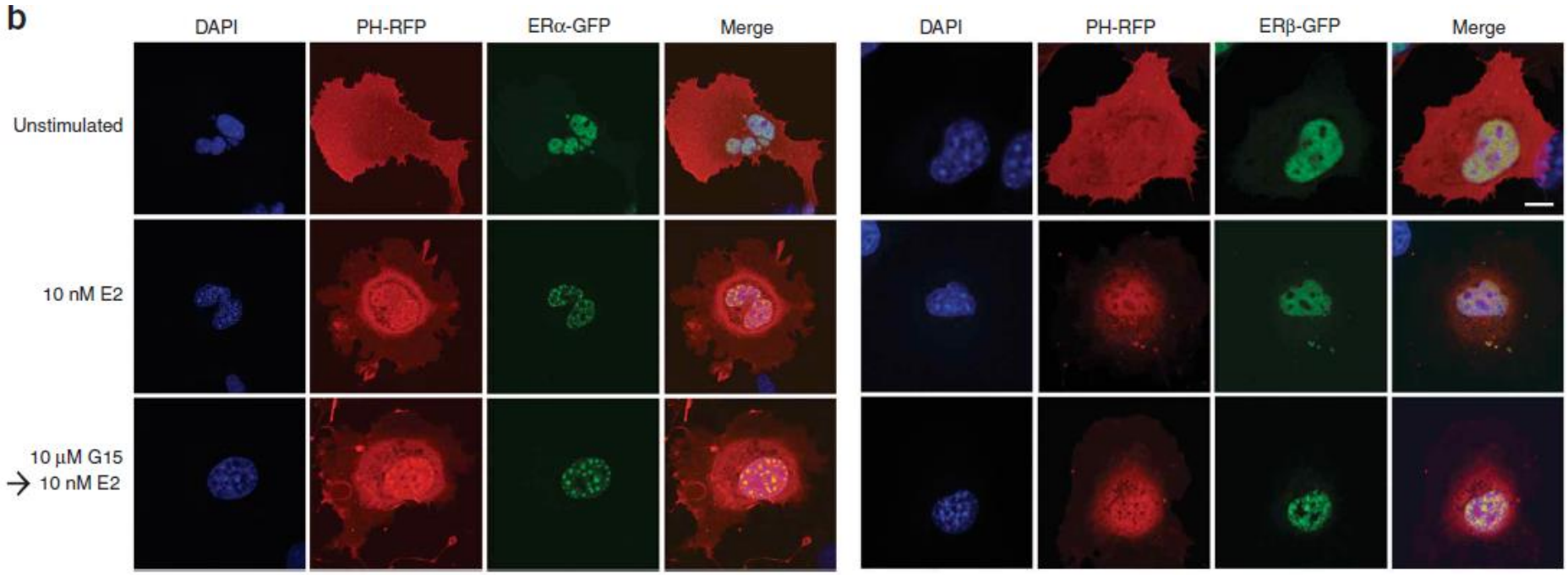
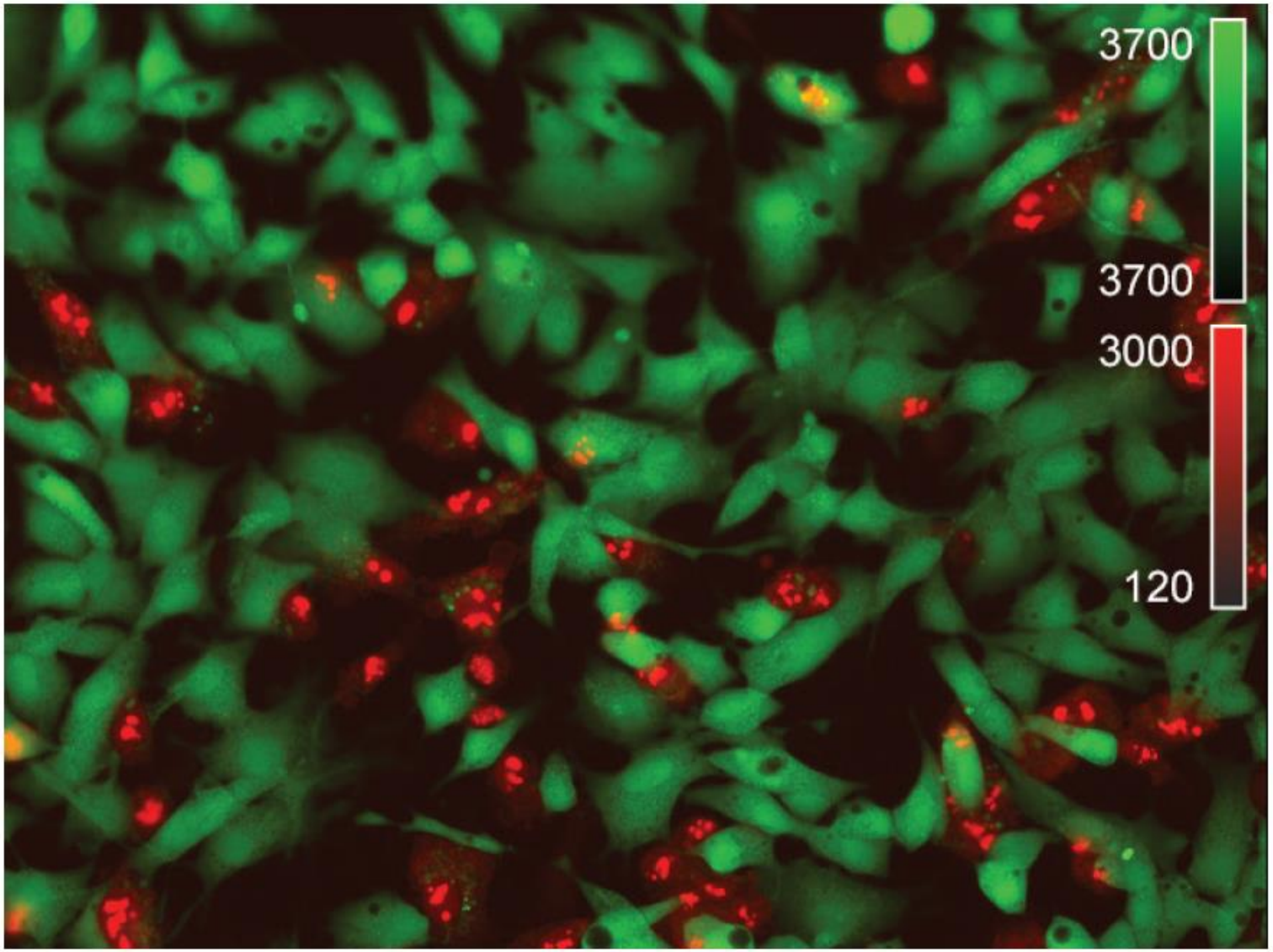


Figure 4 G15 antagonism of PI(3)K activation by GPR30. (**a**–**c**) The activity of G15 was evaluated using COS7 cells transfected with Akt-PH-mRFP1 and either GPR30-GFP (**a**), ERα-GFP or ERβ-GFP (**b**), or using SKBr3 cells transfected with Akt-PH-mRFP1 (**c**). 17β-estradiol, G-1 and G15 were used at the indicated concentrations. The white scale bar in the upper right panel of **a**–**c** denotes 10 μm for all images. Data are representative of three independent experiments.

Dennis, M.K.; et al. *Nat. Chem. Biol.* **2009**, *5*, 421-427.

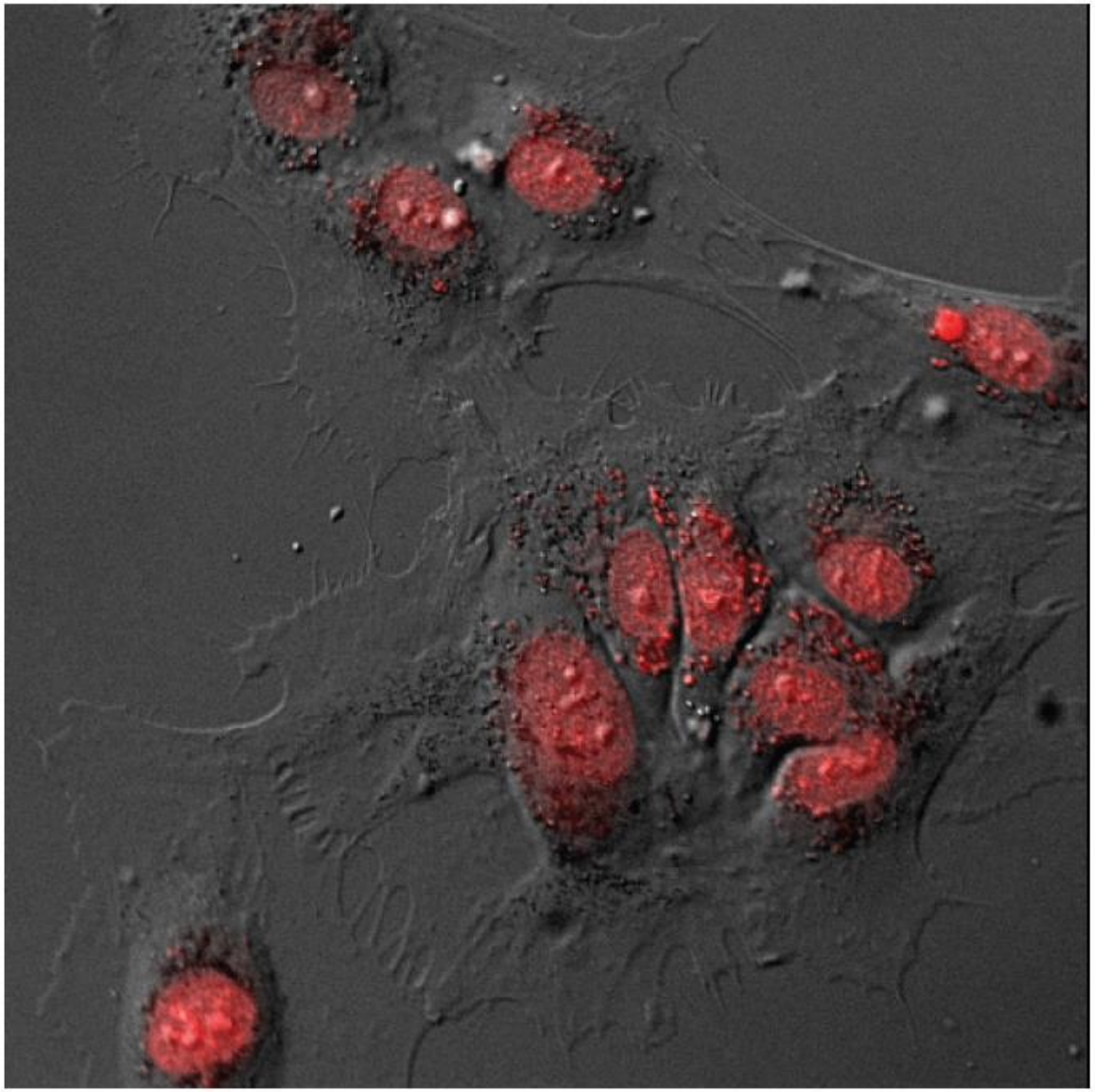
Application – cell death

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.



Using techniques together

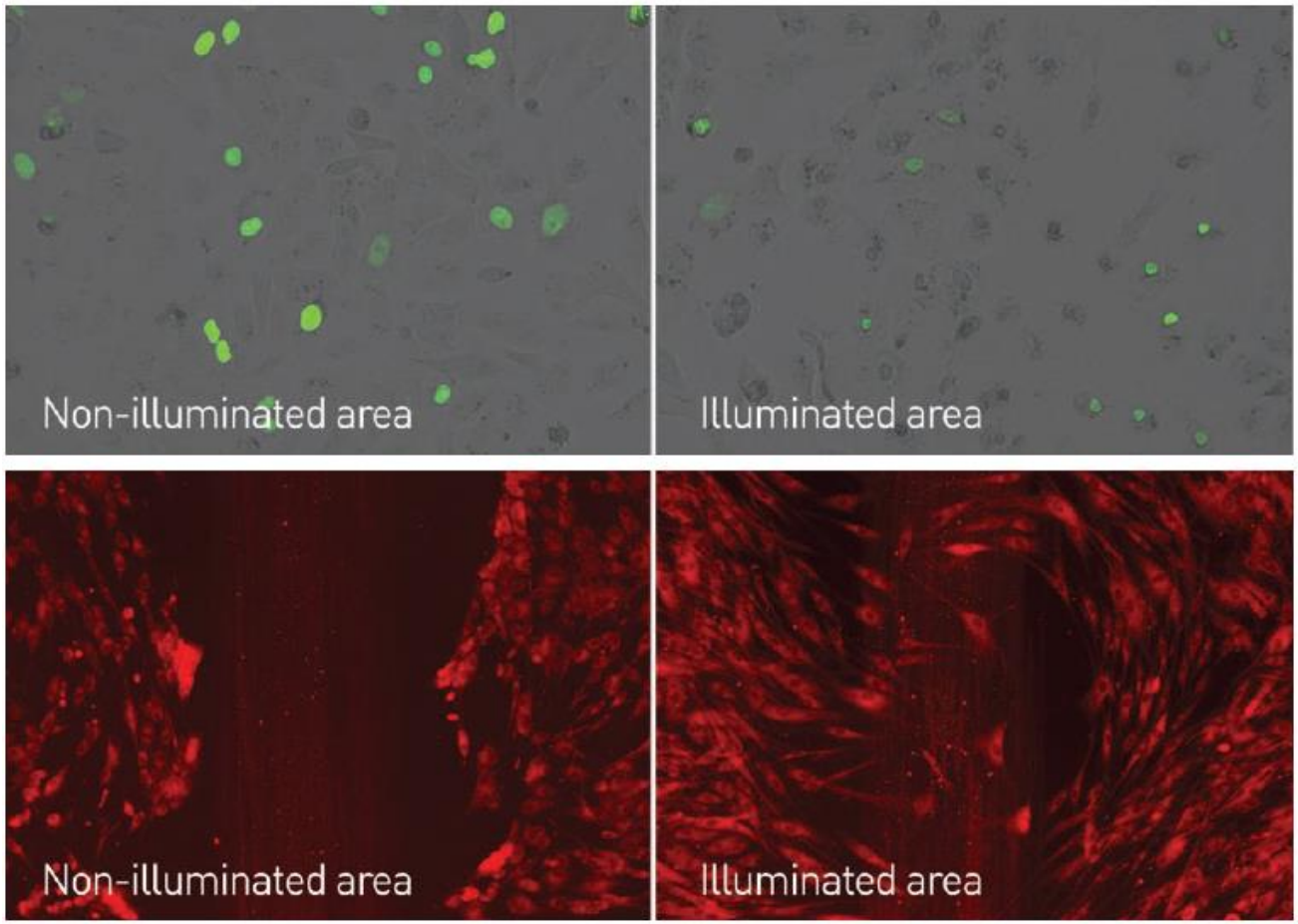
Figure 15.2. HUVECs labeled with NucRed® Live 647 ReadyProbes® Reagent, a cell-permeant nuclear stain



Fluorescence microscopy
Phase contrast microscopy

Wound healing

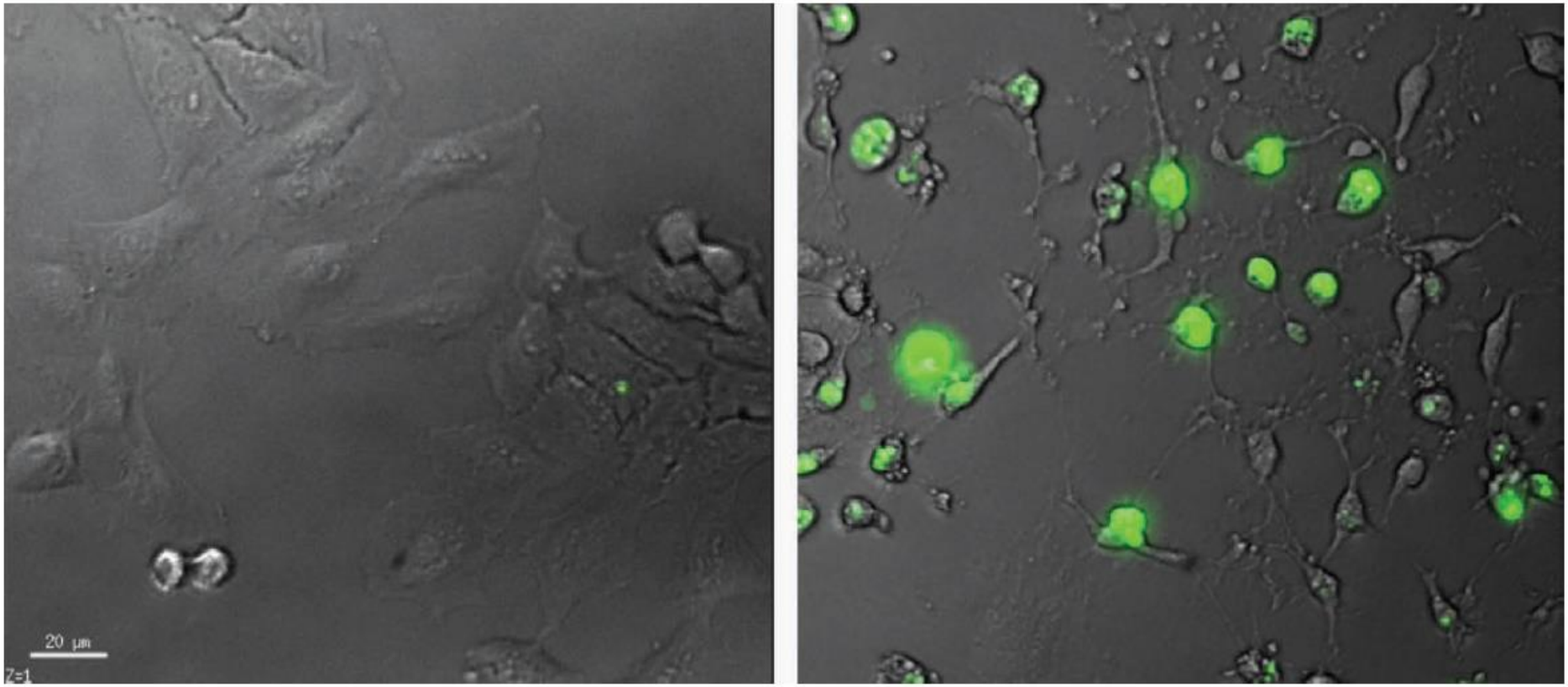
Figure 22.2. Top panel: brightfield and green channel overlay of a field of live HeLa cells transduced with CellLight® H2B-GFP reagent. Cells in the illuminated area have undergone repeated illumination for 10 hours prior to the capture of this image; cells in the non-illuminated area were illuminated 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.



Problems facing the obtention of the images in fluorescence microscopy

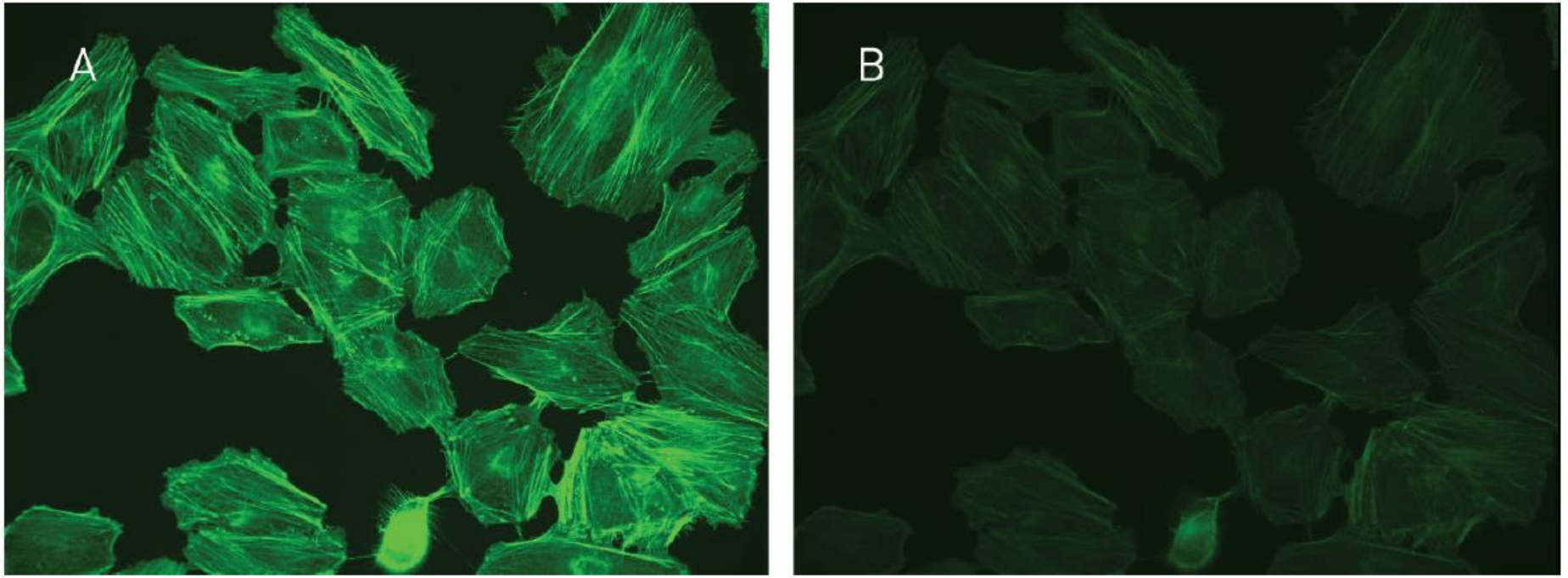
Treatment options

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.



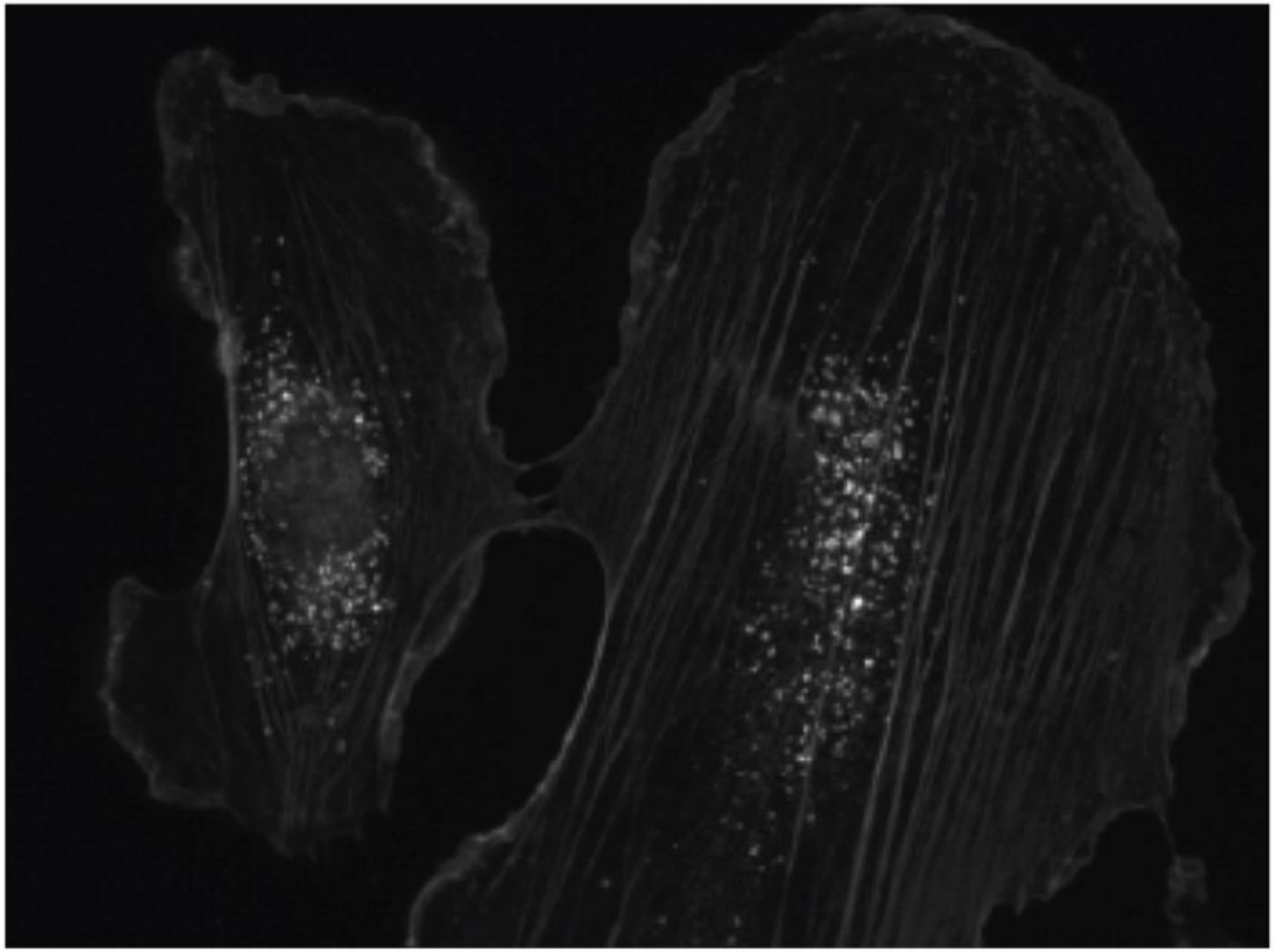
Photobleaching

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



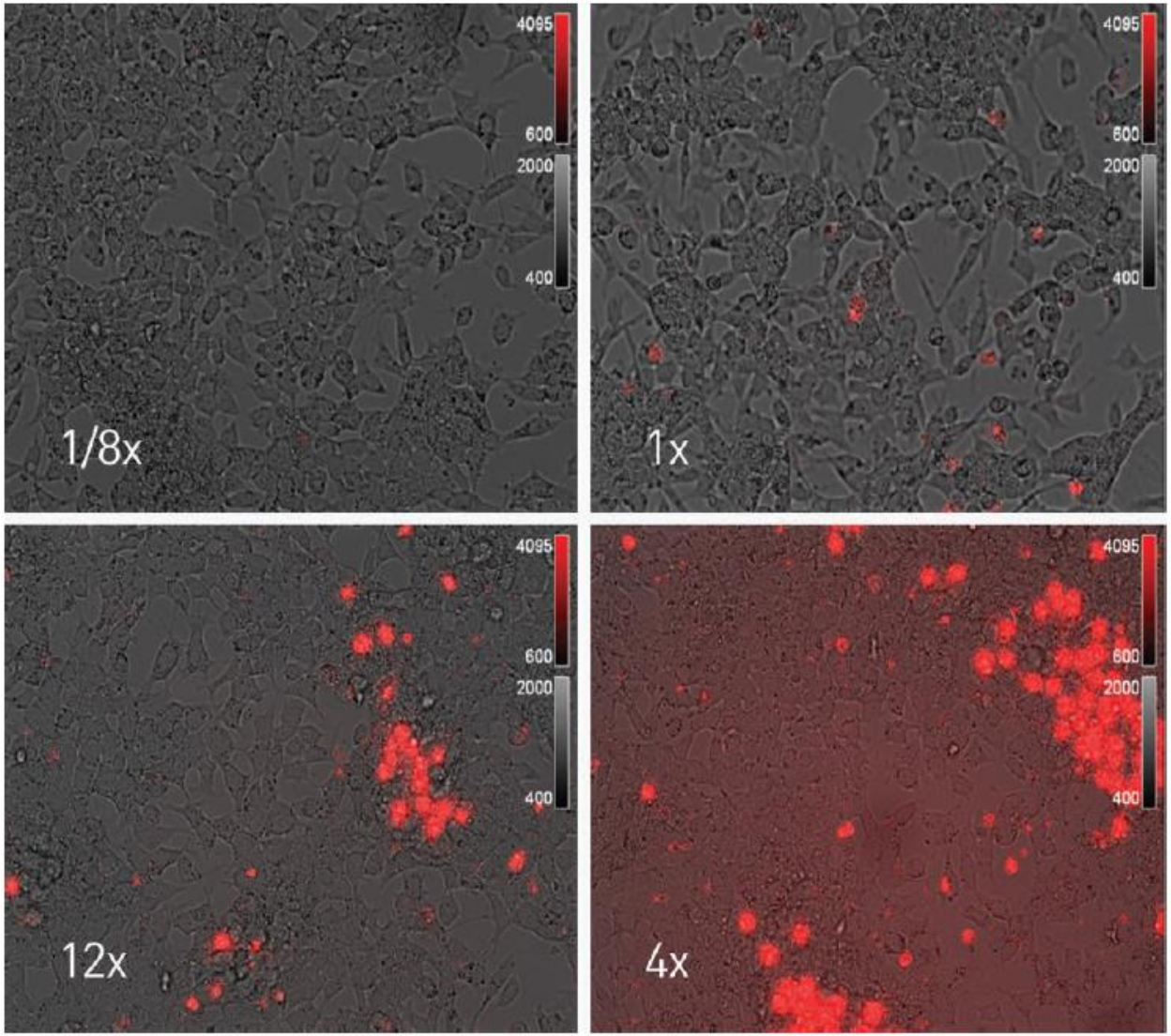
Bleed-Through

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.



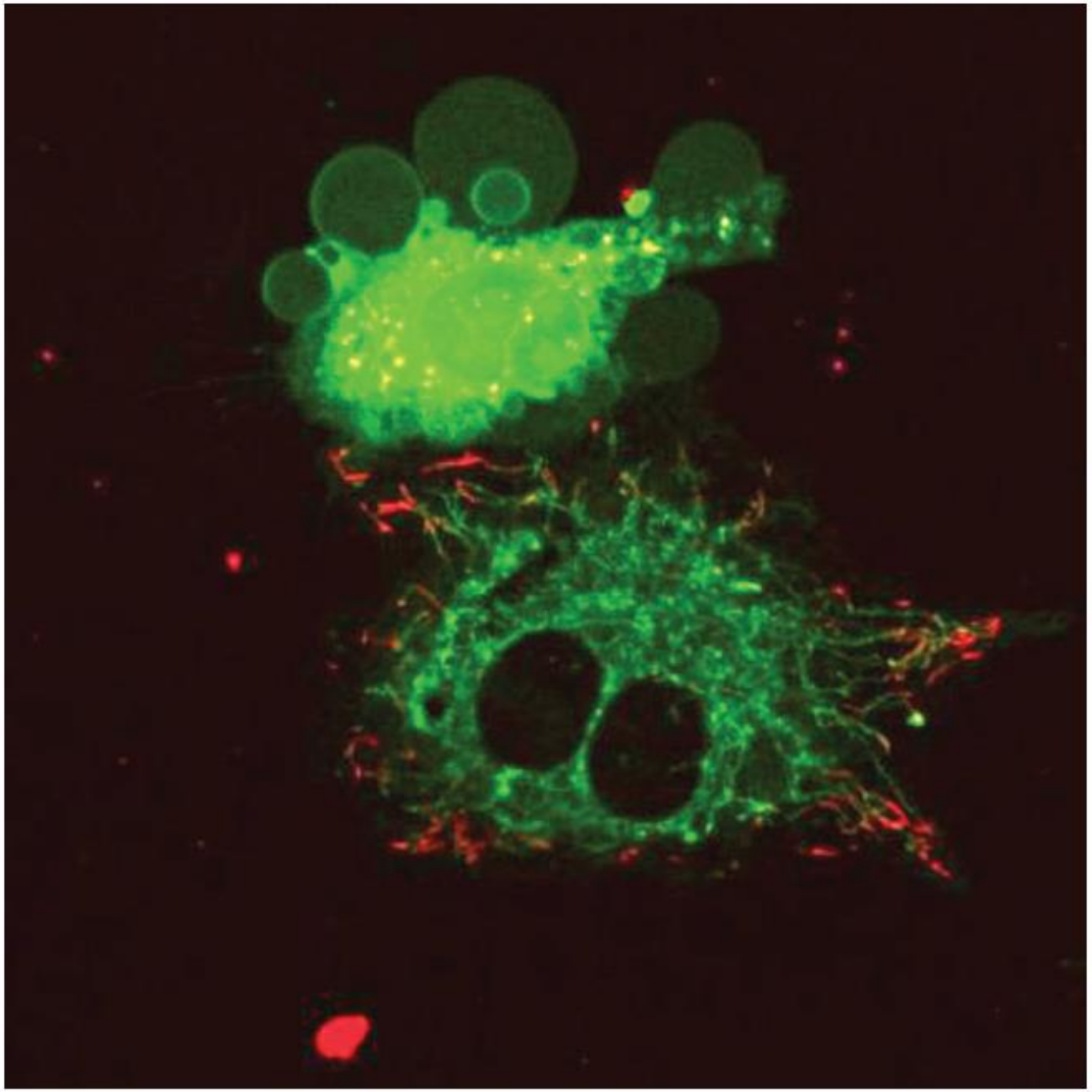
Background noise

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



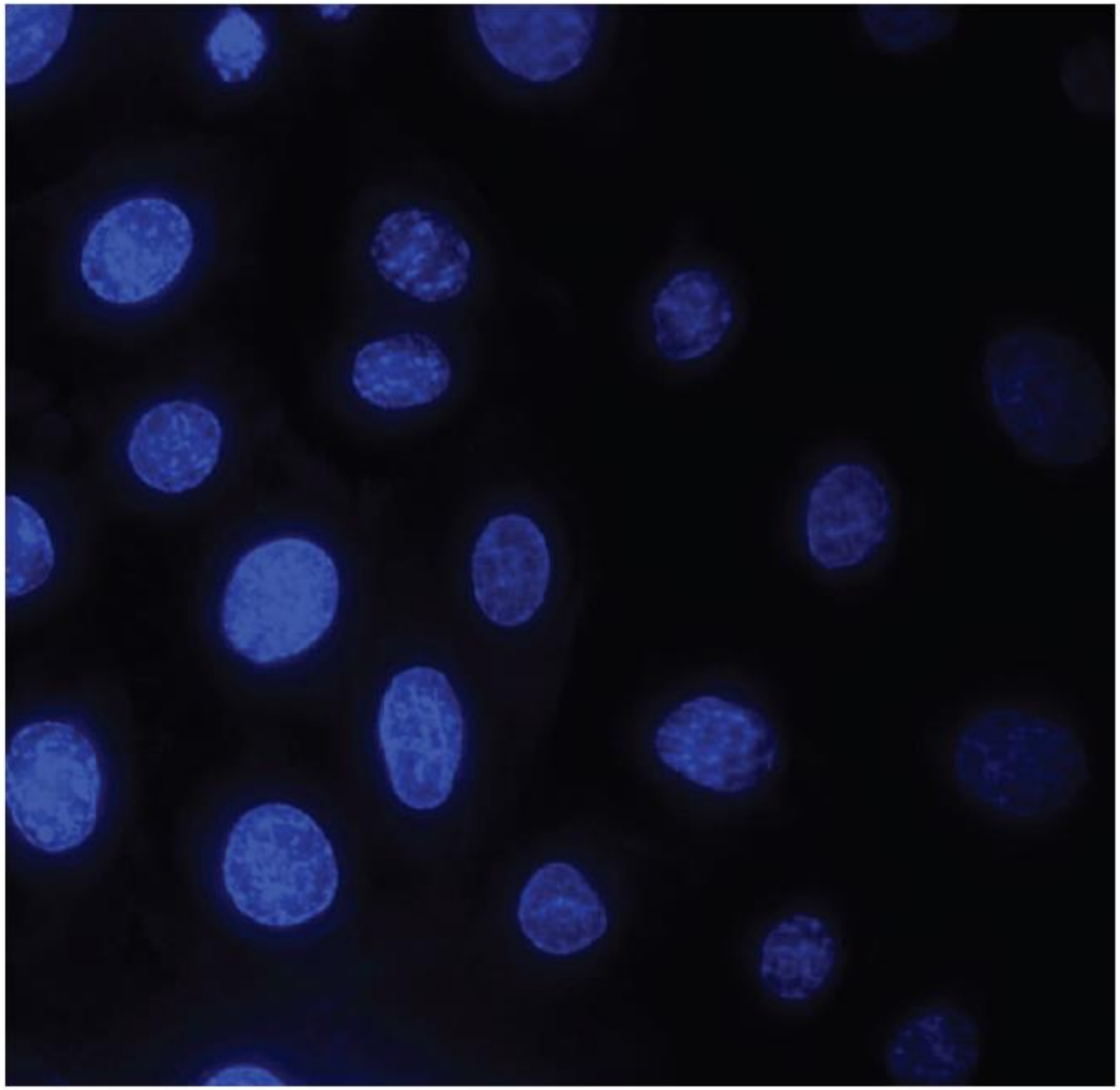
Photoxicity

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



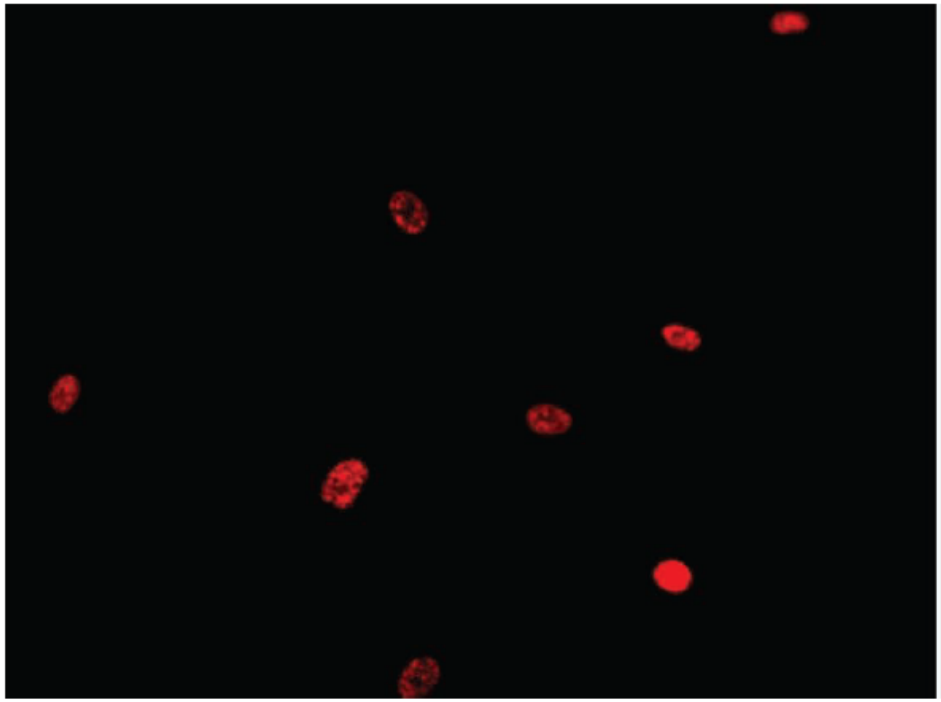
Uneven illumination

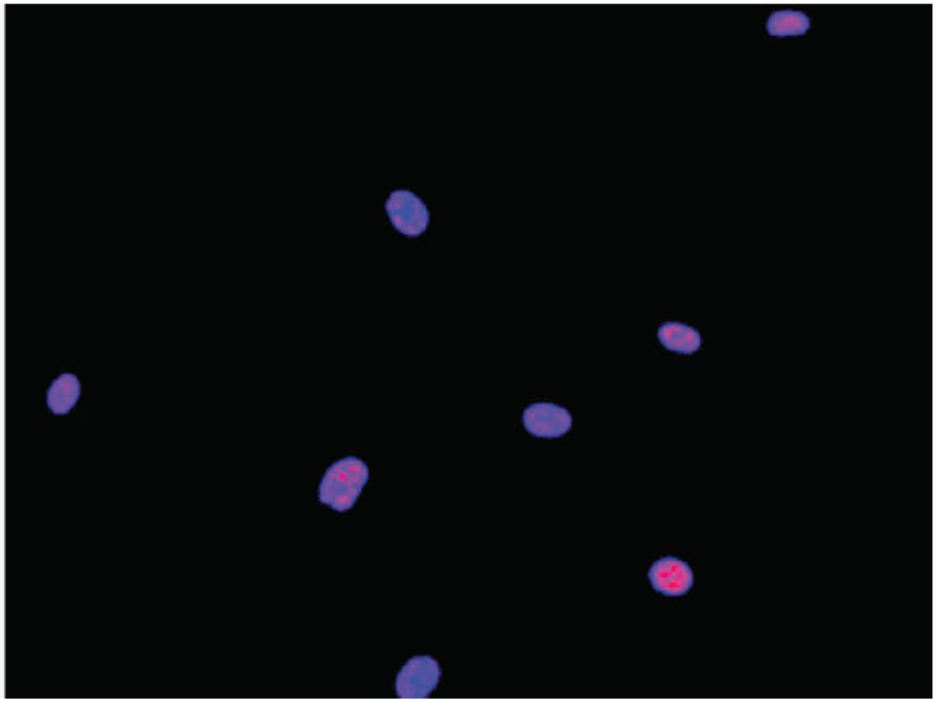
Figure 23.1. Cells stained with a nucleic acid dye are not evenly illuminated, most likely due to a misaligned light path in the microscope.



Masking the images

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..





Post-processing – analysing the results

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.