Fluorescence Microscopy: A Field Guide for Biologists

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Chapter 1

Fluorescence Microscopy: A Field Guide for Biologists

Lucy H. Swift and Pina Colarusso

Abstract

Optical microscopy is a tool for observing objects, and features within objects, that are not visible to the unaided eye. In the life sciences, fluorescence microscopy has been widely adopted because it allows us to selectively observe molecules, organelles, and cells at multiple levels of organization. Fluorescence microscopy encompasses numerous techniques and applications that share a specialized technical language and concepts that can create barriers for researchers who are new to this area. Our goal is to meet the needs of researchers new to fluorescence microscopy, by introducing the essential concepts and mindset required to navigate and apply this powerful technology to the laboratory.

Key words Optical microscopy, Fluorescence microscopy, Magnification, Resolution, Numerical aperture, Digital image, Imaging workflow, Image acquisition, Lateral resolution, Nyquist sampling, Data management plan

1 Introduction

The optical microscope is a symbol of scientific discovery. Ask a child to draw a scientist and chances are the picture will include a microscope. The optical microscope is a standard fixture in education and research and its intuitive design allows us to explore samples from onion skins to human cells with relative ease. Although capturing images can be straightforward, it is difficult to make informed decisions at every step of an experiment without training in this discipline. Perhaps you want to examine the fate of a particular cell during development or decode why a novel virus readily infects the lungs. To achieve rigorous and reproducible results, it is vital to develop the required mind set and skills. To do so takes time and patience, just like learning to fix an engine, skate backward, or knit a sock. Yet you will be rewarded by systematic progress as you uncover new insights about the miniature worlds that surround us.

At its heart, microscopy is a tool that helps researchers observe objects, and features within objects, that cannot be seen with the

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unaided eye. Along with resolving minute features in a specimen, microscopes must also provide contrast to make these features apparent against the background. That is, a microscopic image must include variations in color and/or brightness or it will be invisible to the eye. Microscope techniques are grouped according to the way they implement contrast. Diverse techniques have been developed such as phase contrast [1], darkfield [1], polarization [2], differential interference contrast (DIC) microscopy [3] as well as fluorescence microscopy [4].

Fluorescence microscopy generates contrast from a type of luminescence that occurs when certain molecules, denoted as fluorophores, emit photons while being irradiated with light of characteristic frequencies. Fluorescence is rapid, occurring on the order of a few nanoseconds after photon excitation. The fluorescence is generally shifted to a lower frequency compared to the excitation light, and this difference allows us to isolate the weak fluorescence signal from the intense excitation light by using filters that can separate the different colors of light. This leads to the characteristic high contrast appearance of a fluorescence image, with bright features against a dark background, much like stars illuminating the night sky. The dark background means that fluorescence microscopy is highly sensitive; indeed, the technique can detect emission from a single fluorophore [5]. Fluorescence is also selective at multiple scales of organization; individual molecules, organelles, and cells can be labeled with a variety of extrinsic and genetically encoded fluorophores.

Here we present a practical introduction to fluorescence microscopy for the biologist. We start by defining and describing several foundational concepts, and then illustrating how to transfer these concepts into practice. We emphasize how to make informed choices at every step of the experimental workflow. Informed choices save time, precious samples, and reagents, and help you extract the maximum information and value from experiments. Throughout, we highlight information that is typically transferred informally in the laboratory but not always mentioned directly in the literature.

2 Foundational Concepts

2.1 The Photophysical Properties of Fluorescence Probes

Fluorescence is a photophysical process that occurs when molecules absorb and quickly reemit light within a few nanoseconds. Fluorescence is only exhibited by molecules with specific molecular symmetries [6], and we know these as fluorophores. When fluorophores absorb and reemit light, they do so between one or more defined electronic energy levels. These transitions involve changes within the fluorophore's electron cloud, as well as molecular vibrations and rotations. Thus, each energy state of the molecule

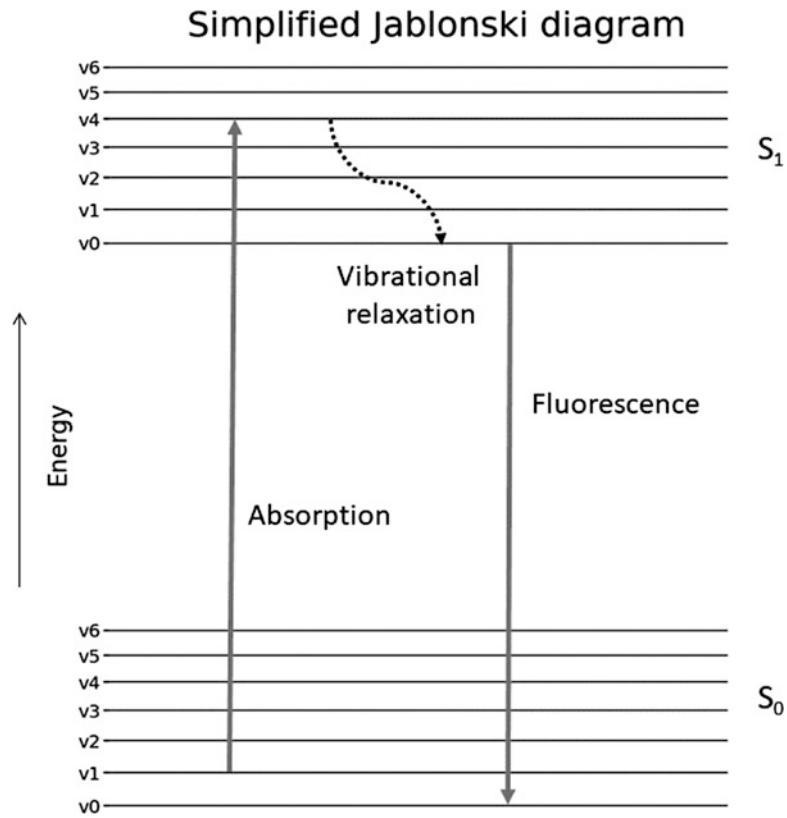

Fig. 1 A simplified Jablonski diagram illustrating the ground electronic state (S_0) and first excited state of a fluorophore (S_1). Each energy level is further subdivided into vibrational (v) and rotational levels, although only representative vibrational levels are depicted for clarity. Arrows indicate transitions among the different energy levels. Once a fluorophore absorbs a photon, it is excited to the upper electronic energy level. The fluorophore quickly relaxes to the lowest vibrational-rotational level of S_1. Next the fluorophore reemits the photon as it drops back down to the ground electronic state S_0. Usually the energy of the emitted photon is slightly less than the energy of the absorbed photon

corresponds to specific electronic, vibrational, and rotational levels. Figure 1 depicts the first two electronic energy states of a typical fluorophore, with the ground state labelled S_0 and the first excited state labelled S_1. Each electronic state is subdivided into discrete levels that correspond to molecular vibrations. They are not shown for clarity, but each vibrational level is further subdivided into rotational levels. The energy of a photon is directly proportional to its frequency, ν, and inversely proportional to its wavelength (λ).

$$E = h\nu = h\frac{c}{\lambda} \tag{1}$$

where h is Planck's constant, a fundamental physical constant [7], and c is the speed of light in the material. As the wavelength of

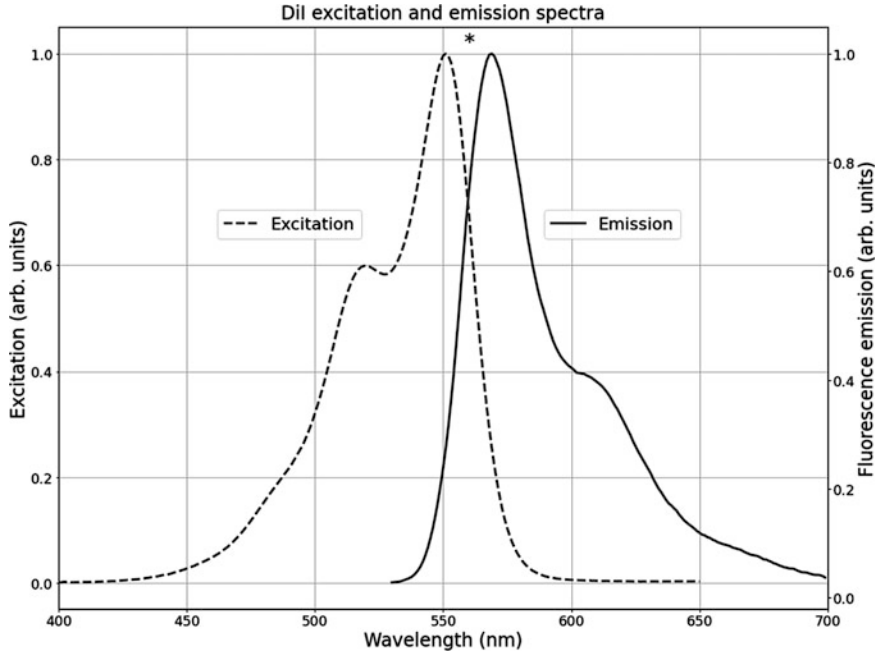

Fig. 2 The excitation and emission spectra of the fluorophore DiI (1,1′-dioctadecyl-3,3,3′,3-′-tetramethylindocarbocyanine perchlorate). Spectral data courtesy of Chroma Technologies. The Stokes shift (*) is the difference between the excitation and emission maxima. Note the emission spectrum is shifted to longer wavelengths (and lower energies) compared to the excitation spectrum

electromagnetic radiation decreases, the energy and frequency increase. Fluorescence microscopy involves energies in the visible range (with some overlap into the near-infrared and ultraviolet). As we move from red to violet in the visible spectrum, the energy and frequency increase, while the wavelength decreases.

Transitions between molecular energy states are governed by quantum mechanical selection rules, in which "allowed" transitions are likely, while "forbidden" transitions have a low probability of occurring [6]. When light of the appropriate energy shines on a fluorophore, it can be excited from the ground state to the first electronic excited state as long as the transitions between the states are allowed. The electronic transitions observed in fluorescence microscopy are excited from about 350–800 nm and involve simultaneous vibrational and rotational transitions. As shown in Fig. 1, a molecule can absorb light and undergo a transition from the ground state S_0 to the upper electronic state S_1. Next the fluorophore quickly relaxes to the lowest vibrational-rotational level of S_1 and then reemits the photon as it drops back down to the ground electronic state S_0. As the energy of the absorbed photon is slightly greater than the emitted energy, the energy difference allows us to use filters and related spectral devices to separate the weaker fluorescence emission from the intense light used to excite fluorescence.

The photophysical properties of fluorophores are important to consider when selecting the best probe or probes for an imaging experiment. When designing an experiment, it is important to reference and/or measure several properties of probes, including the excitation and emission spectra, brightness and photostability. These parameters influence the quality of the experimental results, including spatial and spectral resolution, the relative brightness of signal versus background, and sample viability when working with living specimens. Advanced techniques may require attention to additional properties, including fluorescence lifetime [8] and fluorescence anisotropy [9].

In the Jablonski diagram shown in Fig. 1, we only depict one of the millions of electronic transitions that occur when imaging with standard fluorescence microscopes. The excitation and emission spectra plot the relative probabilities of the multiple electronic transitions (and the accompanying vibrational and rotational transitions) as a function of wavelength. They are visual representations of the multiple photophysical events that occur when a population of fluorophores absorb and then reemit photons. It is important to refer to the detailed excitation and emission spectra when designing an experiment and choosing the appropriate filter settings required to image a given fluorophore. When designing experiments, it is important to look up the appropriate wavelength ranges for exciting and detecting the fluorescence of the selected probe(s). Sometimes product guides list a single excitation and emission wavelength; this information is incomplete and can be misleading because most fluorescence probes can be excited and emit over a broad range of wavelengths.

Often in fluorescence microscopy the terms “absorption spectrum” and “excitation spectrum” are used interchangeably, but they are not synonymous. An absorption spectrum measures the attenuation of light as a function of wavelength as it passes through a sample. An absorbed photon may or may not participate in generating fluorescence. Conversely, an excitation spectrum measures the wavelengths of light that can be used to generate fluorescence, with the height of the curve at each point illustrating the efficacy of each wavelength. Compared to an excitation spectrum, an absorption spectrum captures the effect of many different light-molecule interactions, including those that lead to fluorescence. In practice, either spectrum is a useful reference, but the absorption spectrum may not exactly match the excitation spectrum.

To illustrate excitation and emission spectra, let’s consider the fluorophore DiI (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate), a popular probe for studying lipid distribution in cells and tissues. Figure 2 illustrates the excitation and emission spectra of this molecule from 400–700 nm. The excitation spectrum reveals which wavelengths the molecule absorbs to produce fluorescence, while the emission spectrum depicts which

wavelengths are emitted. Note that the excitation spectrum is at a shorter wavelength and thus higher energy than the emission spectrum. This arises because the fluorophore loses energy as it drops to the lowest level of the excited electronic state, before releasing a photon (Fig. 1). Most often, the excitation and emission spectra are mirror images because the upper and lower electronic states have similar energy level distributions.

In Fig. 2, the spectral maxima for excitation (555 nm) and emission (569 nm) represent the most probable transitions for both photophysical events. The difference between the spectral maxima (here 14 nm) is known as the Stokes shift. The Stokes shift allows us to separate the emitted signal from the excitation light by using optical filters or related devices. The Stokes shift varies by fluorophore and is also influenced by the chemical environment. A large Stokes shift is generally favorable because it makes it easier to separate excitation light from emission light.

Spectra are available on numerous vendor websites and are often encoded in acquisition software on microscopes. When using more than one fluorophore, it is important to minimize spectral overlap between the excitation and emission spectra of the different probes, or you may obtain and interpret artifactual results [10–13]. For example, you may obtain a false positive if the emission from one probe is visible using the microscope filters and settings for a second probe. Additional caution is needed because most reference spectra have been recorded in solvents rather than in cells and tissues. As fluorescence spectra can shift in wavelength depending on their environment, the reference spectra may not be identical to the spectra of the probes within your sample. The emission spectrum of a fluorophore can be measured directly in the sample with a spectral imaging system [11].

To design fluorescence imaging experiments, it is important to evaluate probes for brightness. Fluorescence spectra, however, are reported in relative and arbitrary units and thus cannot be used to compare the brightness of different fluorophores. To check brightness, it is important to evaluate photophysical properties that yield a quantitative measure of the efficiency of light absorption and the subsequent reemission. The Beer–Lambert law tells us that the absorbance A of a molecule at a given wavelength λ is given by:

$$A(\lambda) = \varepsilon bc, \tag{2}$$

where ε is the molar absorption coefficient (also known as extinction coefficient), b is the physical path length (sample thickness), and c is the concentration of the molecular species. When comparing fluorophores, the greater the value of the extinction coefficient, the greater the number of photons absorbed. The second photophysical property that determines brightness is the quantum yield Φ, which is a measure of how many absorbed photons will be reemitted by the molecule. It is calculated by determining the

Table 1
Comparing relative brightness of fluorophores [14]. This table shows different photophysical properties of lipophilic dyes. Brightness is proportional to $\varepsilon\ \Phi$ where ε is the molar absorption coefficient ($M^{-1}\ cm^{-1}$) and Φ is the quantum yield. Note that these data are recorded in solvents, and results may vary for biological samples

Fluorophore (common name)	ε (λ in nm)	Φ_F	Brightness	Solvent used
DiR	240,000 (742)	0.28	67,200	Ethanol
Sulforhodamine B	99,000 (553)	0.7	69,300	Ethanol
Nile red	38,000 (519)	0.7	26,600	Dioxane

fraction of absorbed photons that are reemitted as fluorescent photons.

$$\Phi = \frac{\text{number of photons emitted}}{\text{number of photons absorbed}}. \tag{3}$$

The brightness of the probe is proportional to Φ, the product of the molar absorption extinction coefficient and quantum yield. As an example, Table 1 lists the relative brightness of three lipophilic probes.

Along with brightness, it is important to consider the photostability of a probe. For example, many probes used in flow cytometry are bright but are not suitable for fluorescence microscopy. The simplified Jablonski diagram in Fig. 1 does not depict the myriad photophysical processes that can accompany fluorescence. This includes photochemical reactions that can destroy the fluorophore so that it no longer emits light, a process known as photobleaching [15, 16]. Photobleaching occurs when the fluorophore decomposes at the molecular level, typically after undergoing repeated cycles of the fluorescence process [16]. Photobleaching leads to a decrease in signal over time. It can also induce phototoxicity in living samples, because the by-products of this process are often free radicals that induce damage to cells.

2.2 The Light Path of a Standard Fluorescence Microscope

To develop expertise in fluorescence microscopy, it is important to learn how to identify the various paths that light can travel from the source to the detector. A simple fluorescence microscope may have a single light path from the light source to the eyepiece, or two, if is also equipped with a camera. Advanced systems can have ten or more light paths. From the simplest to the most complex system, a light path in a fluorescence microscope contains a successive series of optical components that can be organized by function. These functions include:

- Exciting the fluorescence (light sources)
- Resolving spatial features (lenses)

- Separating the light by spectral range (filters)
- Detecting the fluorescence emission (cameras or other detectors)
- Converting the fluorescence signal to a digital image (computer and electronics)

When you begin working with a new microscope, start by tracing the light path from the light source to the detector. Try to identify the different components in the light path and describe how they work together to form the image that you view or acquire digitally. If you take the time to trace the light paths in a microscope when learning a new application, you will find it easier to troubleshoot problems and optimize your imaging.

To apply the light path to laboratory practice, let us consider how light propagates from the light source to the detector in a widefield fluorescence microscope. The term "widefield" refers to techniques where the image is captured simultaneously across the field of view using a camera. The advantages of widefield systems are their low cost, straightforward and modular design, and high sensitivity [17]. They are excellent choices for imaging thin preparations such as cultured cells and for live-cell work. A representative light path highlighting the components of a widefield microscope is shown in Fig. 3:

1. Light is emitted from one or more intense light sources, commonly arc lamps and light emitting diodes (LEDs) [18]. Arc lamps emit a broad range of wavelengths but require spectral filtering, generate heat and sometimes emit high UV levels. They are slowly being replaced by LEDs, solid state devices that consume less energy and require less maintenance.
2. The excitation filter transmits specific wavelengths while reflecting others; here it transmits the required excitation wavelengths while reflecting the undesired wavelengths back to the source. Ideally, the filter transmission curve matches the excitation spectrum of the fluorophore of interest.
3. The excitation light is reflected by a dichroic mirror, which is oriented at 45° relative to the sample. The excitation light passes through the objective and is delivered to the sample. As the sample is irradiated, fluorophores emit light of lower energy and longer wavelengths than the excitation light.
4. The fluorescence emission is captured by the objective, which provides an enlarged and spatially resolved image of the fluorescence emitted by the sample. The excitation light is reflected to the source.
5. The fluorescence emission passes through the dichroic filter and the emission filter. Both the dichroic filter and emission filter ideally transmit wavelength ranges that match the

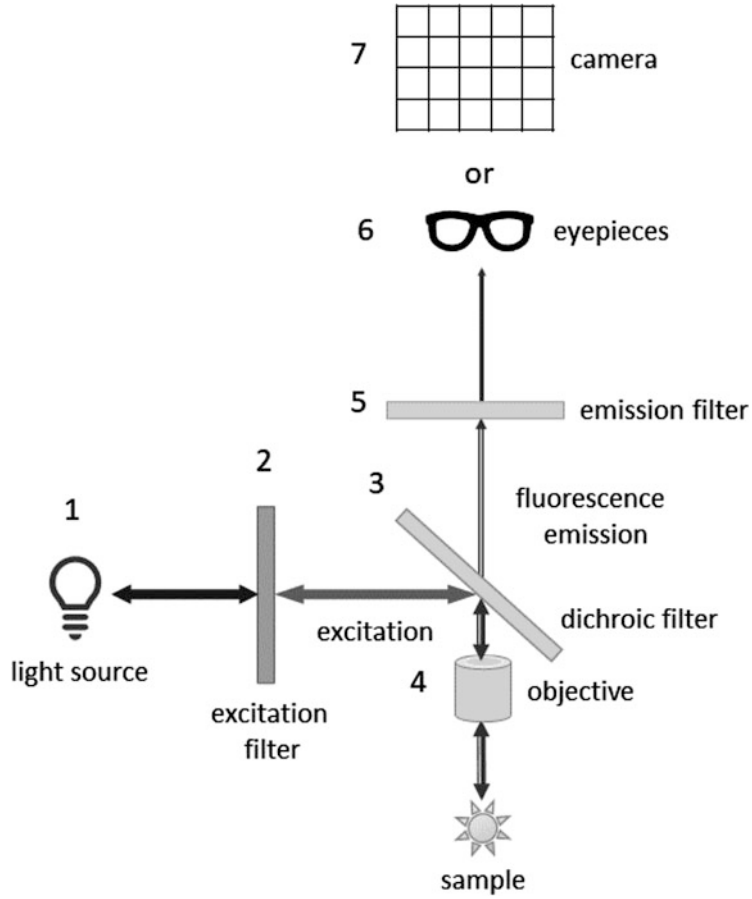

Fig. 3 Representative light path for a widefield microscope. Light is emitted from an intense light source (1). The excitation filter transmits light over a specific wavelength range (2). The excitation light is reflected by a dichroic mirror, which is oriented at 45° relative to the sample. The dichroic reflects light with shorter wavelengths and transmits light of longer wavelengths (3). Fluorophores emit light of lower energy and longer wavelength compared to the excitation light. The fluorescence emission is captured by the objective, which provides an enlarged and spatially resolved image of the fluorescence labelling on the sample (4). The signal passes through the dichroic mirror and then an emission filter matched to the fluorescence spectrum of the fluorophore of interest. The emission filter provides a layer of security to ensure that the much brighter excitation light is blocked, or it may overwhelm the fluorescence signal or create high background (5). The image is viewed through the eyepieces (6) OR the image is projected onto the camera (7)

fluorescence emission of the fluorophore of interest. The dichroic and emission filters are required to ensure that the much brighter excitation light is blocked. Otherwise, the fluorescence signal may be obscured by the much brighter excitation light.

6. The image is viewed through the eyepieces. Although having a second light path for visual inspection adds cost, and can lead to confusion, eyepieces allow for quick set up and provide a larger field of view compared to the camera.

   OR

7. The image is projected onto the camera, typically a charge-coupled device (CCD) or scientific complementary metal-oxide semiconductor (sCMOS) [19]. Cameras are also known as two-dimensional array detectors because they contain multiple photosensitive elements, also known as photosites, which allow the multiple points on an image to be detected at the same time. The camera converts the photons into electrical signals that are then converted into digital files for computational visualization and analysis.

Widefield fluorescence microscopes, like most fluorescence microscopes, are configured for reflection imaging. In fluorescence imaging, the objective serves a dual function by illuminating the sample as well as by collecting the fluorescence emission. This arrangement is known as epifluorescence, because the excitation and emission light both pass through the objective (from the Greek *epi* meaning "same"). The microscope is known as "upright" if the objective is above the sample and "inverted" if the objective is below the sample.

Tracing and systematically testing the components in a light path can help diagnose issues with imaging. For example, if you want to quickly inspect your sample but are unable to see any fluorescence emission through the eyepieces, try tracing the light path step by step. Is your light source switched off, turned to minimum intensity or is a shutter closed? Did you choose an incorrect filter set? Did you switch the light path to image with the camera rather than with the eyepiece? As shown here, the light path can help you systematically work through problems; otherwise, troubleshooting can be a frustrating exercise.

Although it requires more time initially, we recommend practicing with a standard sample before attempting your first imaging experiment. If you develop imaging skills ahead of time, when you start with experiments, you will be able to focus on the biological questions rather than the mechanics of operating a microscope. A standard sample need not be expensive, and you can use a discarded slide with robust fluorescence. Another option is to purchase standard slides from educational supply companies. Pollen or plant specimen slides are useful because they contain pigments that are naturally autofluorescent. Standard slides are also useful for troubleshooting when you start experiments. They allow you to check that you have set up the microscope properly

and reveal whether any issues encountered are with your experimental sample rather than the equipment.

Often widefield microscopes are also equipped for white-light imaging, including techniques such as brightfield, phase contrast or DIC. White-light techniques do not require any external labels and can be used alongside fluorescence to provide a view of the sample as a whole and with less photodamage. However, white-light imaging modes can create confusion when trying to identify the light paths in a microscope. Fluorescence imaging is configured for reflection, where the excitation and emission light both travel through the objective. By contrast, white-light imaging for life science research is typically set up in transmission, with a condenser illuminating the sample on one side and the objective forming the image on the other. New operators sometimes attempt to set up their microscope or diagnose problems with the epifluorescence light path by adjusting components in the white-light transmission path. To avoid this source of potential confusion, it is important to familiarize yourself with all the light paths of the microscope, not just the fluorescence ones.

A final note of advice on white-light imaging: while fluorescence light paths rarely need alignment, white-light paths take more time and effort to set up properly. If you have a white-light path, after bringing your sample into focus, ensure that you align the microscope for "Koehler illumination" as it is the required first step for white-light imaging [20, 21]. After you set up Koehler illumination, refer to the manufacturer instructions for your specific imaging mode.

Fluorescence microscopy allows us to label cells, organelles, proteins, or other cellular constituents and identify them using tags that emit over different wavelength ranges. However, the detectors, such as the cameras used in research grade fluorescence microscopes, are typically monochrome. This means they detect intensity but not colour and thus optical filters are required to distinguish among the fluorescent probes. It is important to compare the fluorophore excitation and emission spectra to the transmission curves for the excitation and emission filters available in your microscope so that the appropriate fluorophores can be selected. When looking up information about fluorophores, it is common to see the excitation and emission maxima listed. However, to fully understand how your fluorophore will perform with your microscope filter sets, it is important to consider the full spectral curves and compare them to the full filter transmission curves. This allows you to determine how well matched your filter set is to your fluorophore and determine if there will be any overlap.

In Fig. 4a, the excitation and emission spectra of GFP are illustrated, along with a filter set optimized for imaging this fluorophore. The percent transmission of the excitation, dichroic, and emission filters are also plotted. By inspection, the excitation and

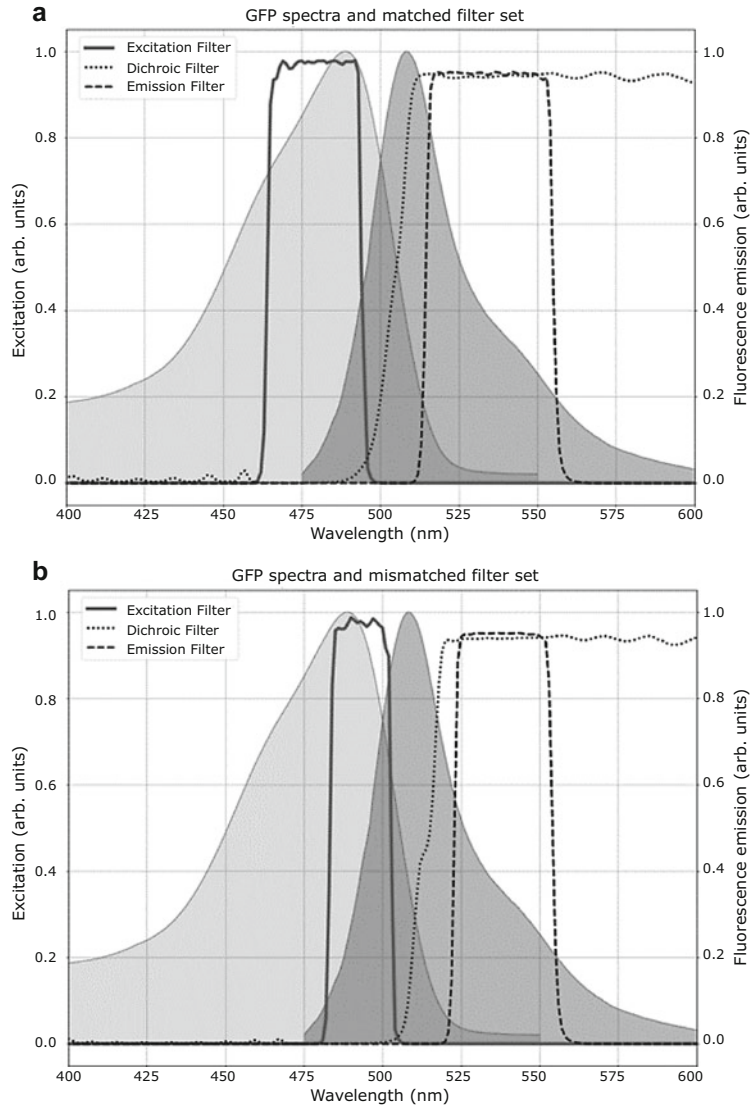

Fig. 4 Excitation and absorption spectra of a fluorophore should be matched to the channel, that is, the wavelengths for excitation and emission. The spectra and filter transmission curves should be carefully evaluated for optimal overlap. In (**a**), the filter set used was designed for imaging with GFP. The excitation and emission spectra of the fluorophore, here GFP, align with the transmission curves for the excitation, dichroic and emission filters. In (**b**), the filter set is designed for imaging with YFP and the GFP excitation and emission spectra imperfectly match the emission, dichroic and emission filter curves compared to the filter set in (**a**)

emission filters are seen to match the fluorophore spectra. The dichroic mirror reflects most of the excitation light (toward the sample) and transmits almost all of the fluorescence emission (toward the detector). We can conclude that this filter set is a

suitable choice for imaging GFP. In Fig. 4b, the excitation and emission spectra for GFP are shown with a nonoptimal filter set that is designed for imaging YFP. The excitation filter overlaps with the emission spectrum and only transmits a small range of the wavelengths that excite GFP. The dichroic reflects a large portion of the wavelengths of light emitted by GFP and the emission filter does not transmit the peak wavelengths emitted. Note that although the match is less than perfect, it may still be usable. As filters are expensive, it is always good to test whether the existing filters meet the demands of the imaging experiment rather than purchasing a new set for a single use case.

For multicolor imaging experiments, separate filter sets are used to differentiate among the two or more probes used to label the sample. Each filter set corresponds to a defined spectral range that is used for excitation and emission, corresponding to what we know as "channels." Carefully matching the probe spectra to the appropriate filters is essential because each channel should detect the emission from one intended target. If there is spectral contamination between the different channels (spectral cross talk and bleed-though are the technical terms), you may not be able to analyze your data. For more information, we direct you to several excellent reviews [10–13].

2.3 Magnification, Spatial Resolution, and Sampling

Like all microscopes, fluorescence microscopes spatially resolve features beyond the acuity of human vision. When working with optical microscopes, sometimes magnification is confused with the spatial resolving power of the system. Magnification is the difference in size of the image relative to the object. When imaged by a detector, we call this lateral magnification, while when observed by eye it is referred to as angular magnification [22]. Magnification, however, does not tell us how well the lens resolves individual points on an image. For example, a microscope may be equipped with two 20× objectives, but one objective may be better at resolving finer spatial features. This is because objectives and their resolving power are defined by the numerical aperture (NA), not magnification.

Numerical aperture refers to the ability of an objective to collect light and resolve objects. For an epifluorescent microscope, where the objective acts as the condenser, NA is calculated as follows:

$$\mathrm{NA} = n \sin \theta, \tag{4}$$

where n is the refractive index of the medium between the front lens and the sample, and θ is the acceptance angle of the objective (Fig. 5).

Most microscope objectives have NA values between 0.3 and 1.45. Air objectives have maximum NA of about 0.9 and are designed to work with no immersion medium between the front

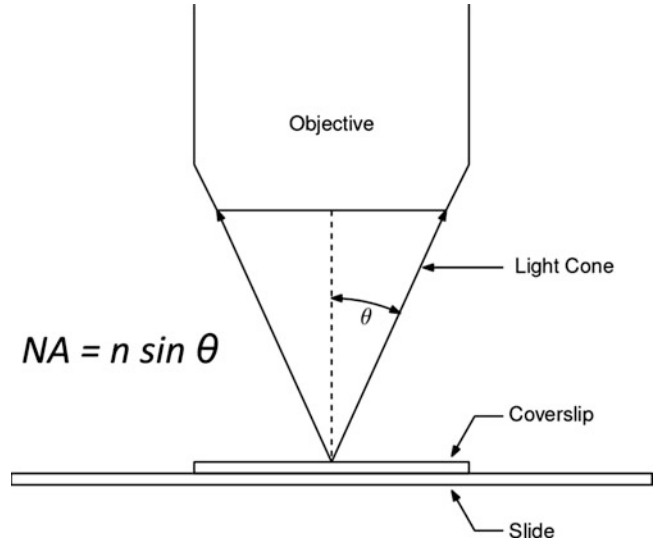

Fig. 5 A visual representation of the acceptance angle θ of an objective lens. It is defined as the largest half-cone of light that can propagate through the lens

lens and sample. To achieve an NA > 1, the immersion medium can be changed from air to water, oil, silicone, or some other substance. Objectives are commonly labelled by the name of the immersion medium and whether the objective is placed in it directly or separated from the medium by a coverslip or similar barrier. For example, water objectives can be used as "dipping" or "immersion" objectives, while oil objectives are almost always used as immersion objectives. Some specialized objectives can perform with multiple immersion media using both dipping and immersion configurations.

As NA increases, the spatial resolving power of an optical microscope improves, but only up to a point. Standard optical microscopes can, at best, resolve features on the order of 200–250 nm. This restriction arises from diffraction, a fundamental property of light-matter interactions [22]. Diffraction occurs whenever light interacts with an edge or aperture, such as a lens. Diffraction creates an interference pattern that is critical to image formation but is also associated with effects that degrade the quality of an image. In fluorescence microscopy, the heart of the microscope imaging system is the objective. When a point of light passes through the objective, the diffraction pattern that is formed consists of a central bright disk surrounded by concentric rings that fall off in brightness as they extend outward (Fig. 6a) [22]. This characteristic pattern is known as the Airy diffraction; it arises whenever light travels through a circular aperture such as an objective lens. In light microscopy, the central disk is denoted "the Airy disk" and the surrounding rings are known as "Airy rings."

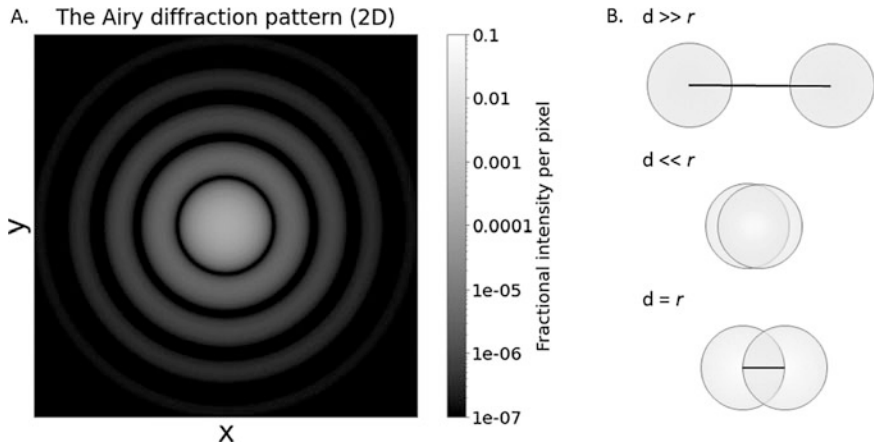

Fig. 6 Airy disk and resolution. (**a**) When imaging a subresolution feature such as a 100 nm bead, the resulting diffraction pattern (**a**) has a characteristic appearance that consists of a bright central disk surrounded by alternating bright and dark rings that gradually fall off in intensity. The intensities are displayed using a logarithmic scale, to highlight the diffraction rings. The radius of the Airy disk, *r,* is the lateral (*x,y*) resolution limit of the microscope (see Eq. 5). Here we show the diffraction pattern in a single image plane, but it also extends in 3D. Plot generated using POPPY, an open-source optical propagation Python package originally developed for the James Webb Space Telescope project [23]. (**b**) From top to bottom: two subresolution features are well-resolved; not resolved; just resolved when the centers of the Airy disks are separated by *r*. This is known as the resolution limit

By convention, we define the resolution limit of a standard widefield microscope by considering two bright subresolution features on the sample, represented as the central bright Airy disks in Fig. 6b. Three cases are shown, when the points are said to be "resolved," "not resolved," and "just resolved." The "just resolved" arises when the central disks in the Airy diffraction pattern are separated by a distance equal to the radius of each circle:

$$r(xy) = \frac{0.6\lambda}{\mathrm{NA}}, \tag{5}$$

where λ is the emission wavelength and NA is the numerical aperture of the objective. The value r (x,y) is known as the Rayleigh criterion for lateral (x,y) resolution [22]. Here we limit our description to a single imaging plane (x,y), and simplify $r(x,y)$ to r, but it is important to note that the diffraction pattern extends in three dimensions. How we define resolution in the z direction is covered in the following chapter. Note that the r value is also defined differently depending on the technique [24].

As r decreases, the resolution increases or improves. This is because r is a measure of how finely features can be resolved on a sample. An r value of 200 nm yields more spatial definition when compared to an r of 500 nm. Also note that as wavelength decreases, r also decreases and as NA increases, r decreases. If you want to improve resolution, you can use shorter wavelengths to image and/or increase the NA of your objective. However, be

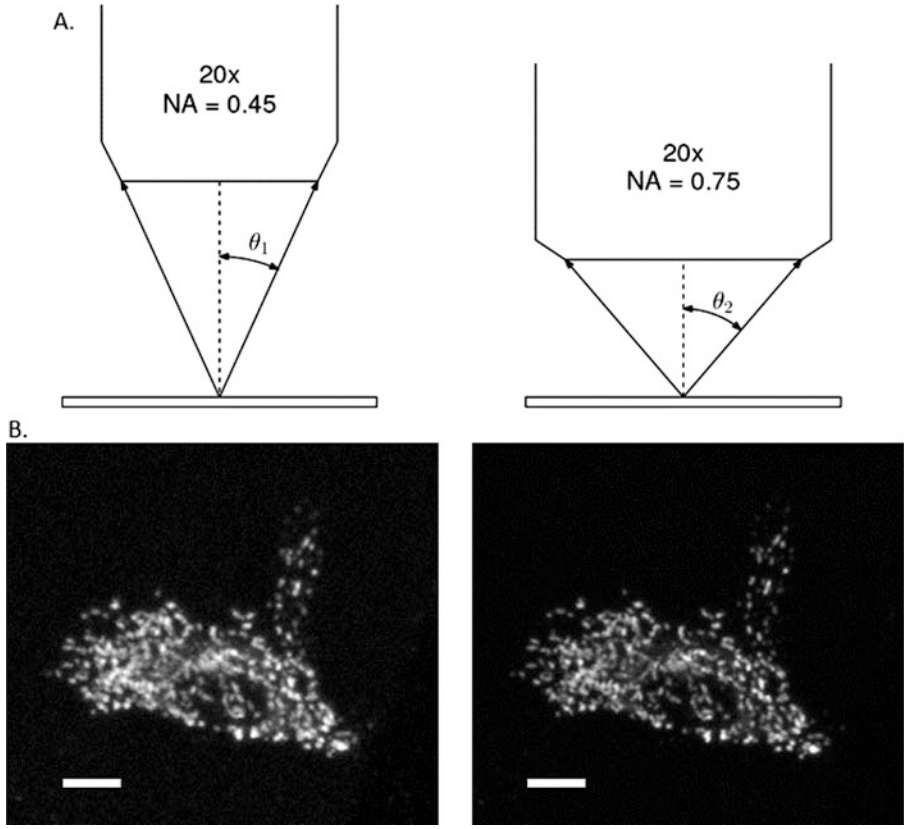

Fig. 7 Numerical aperture (NA), not magnification, sets the spatial resolution of a microscope objective. (**a**) The diagrams show the light collection and angle of acceptance of two different 20× objectives that have different NA values. The objective on the left has an NA of 0.45 and the objective on the right has an NA of 0.75. (**b**) The corresponding images obtained with the 20× objective depicted above each image. Although the same magnification (20×) was used, the features in the right image are more resolved than in the left image, because the NA is higher. Images record the fluorescence signal from a secondary antibody (STAR RED, Abberior, Göttingen, Germany) that targets a primary antibody used to label von Willebrand factor. Images were acquired using a laser scanning confocal microscope (Nikon A1R microscope, Ti2-E stand, NIS-Elements v 5.02 software; Nikon Canada, Mississauga, Ontario, Canada). Image acquisition settings: 639 nm laser excitation and an emission filter with transmission between approximately 660–735 nm. Scale bar = 10 μm

careful when imaging with wavelengths near 400 nm. Most microscope optics are tailored for visible light longer than 450 nm and perform poorly near 400 nm. It is better to work with wavelengths above 450 nm or choose an objective designed specifically for shorter wavelengths.

To illustrate the effect of NA on spatial resolution, Fig. 7 shows two images captured with two different 20× objectives that have different NA. The samples are dermal endothelial cells that have been stained using immunofluorescence to highlight von Willebrand factor, a large multimeric glycoprotein that is stored in Weibel-Palade bodies. Although both images are captured at the same magnification, the features in the image on the left (NA = 0.45,

$r = 0.89$ μm) are not as well resolved as in the image on the right (NA = 0.75, $r = 0.53$ μm). This highlights that NA, not magnification, sets the spatial resolution. When choosing an objective, consider the experimental aims. Here, if the aim of the experiment were to investigate the morphology and number of Weibel-Palade bodies following treatment with different drugs, the 20×/0.75 NA objective would be a better choice for the image acquisition than the 20×/0.45 NA objective. Unfortunately, this concept is often misapplied when magnification is prioritized without considering NA.

When choosing an objective, NA is central as it sets the limit on the resolution of the imaging experiment. Besides NA, there are a number of other specifications to consider when choosing the best objective for the application [25]. As one example, the working distance of an objective can affect the imaging parameters. Oil immersion objectives may provide the highest resolution, but these objectives cannot be used to focus through standard plasticware. Rather, they require specialized thin dishes or cells grown on coverslips. In addition, when working with multicolor applications, it is critical to choose objectives with a high degree of chromatic correction, designated apochromats, that minimize mis-registration among different spectral channels. In some cases, applications like phase contrast or total internal reflection fluorescence (TIRF) [26] demand the use of a specific objective to perform any imaging at all. These and other key objective specifications are inscribed on the objective barrel as shown in Fig. 8, which depicts a stylized air objective as well as an objective with a correction collar. These are only two representative examples of the dozens of objectives available for any fluorescence microscope. Table 2 summarizes the key specifications of microscope objectives and their practical implications for fluorescence imaging of biological samples.

Diffraction not only limits spatial resolution, but also leads to blur and haze that degrades overall image quality. Techniques such as confocal, deconvolution, and multiphoton microscopy, collectively known as "optical sectioning" techniques, can improve the spatial resolution in x, y, and z, as described in the following chapters. To image with the highest resolution, superresolution techniques such as structured illumination microscopy (SIM), stimulated emission after depletion (STED) and point localization can be applied, as described in Chapters 14–18.

2.4 Capturing and Digitizing Images

The image formed by the microscope is a real object, just like your sample, though it is composed of photons rather than atoms and molecules. The focused image is projected onto a detector that coverts the photons into an electrical signal, which in turn, is converted into a digital file that we can visualize and analyze. In a widefield microscope, images are detected by CCD or sCMOS

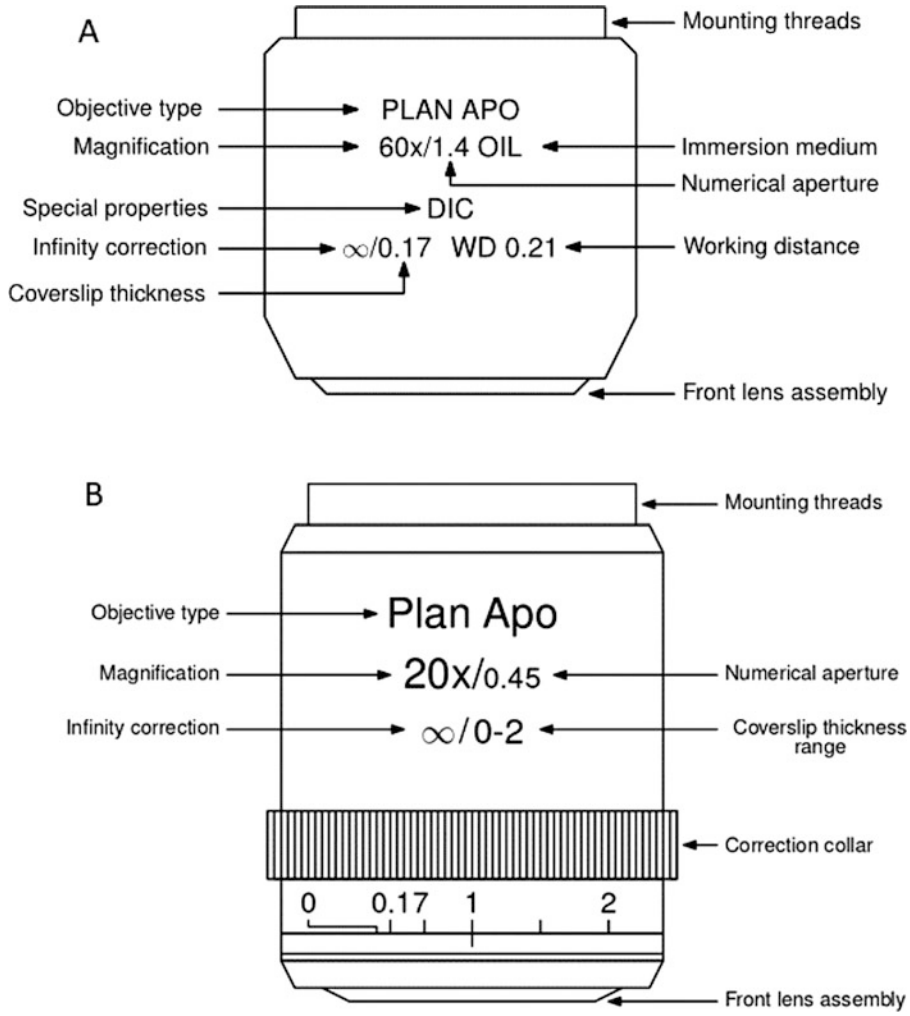

Fig. 8 Two representative objectives and typical specifications are depicted. The top schematic shows an oil immersion objective and the bottom schematic depicts an air objective with a correction collar. Table 2 summarizes the specifications and their relevance

cameras. These cameras contain photosites arranged in a two-dimensional array that can capture multiple points on the image at the same time. Each photosite generates an electrical signal that should be proportional to the number of photons captured. The electrical signal is a continuous signal that is digitized into discrete intensity readings. The resulting digital file contains a two-dimensional array of numbers that are organized into rows and columns that correspond to each photosensitive element on the camera (Fig. 9). These individual elements are known as "pixels." Each pixel encodes the emission intensity as well as the x and y positions, for each point on the original image. The digital files are then used to visualize and analyze the data computationally. When a

Table 2
Common specifications for objectives used in fluorescence microscopy. These specifications are often inscribed on the barrel of the objective

Specification	Definition	Practical notes
Manufacturer	Microscope objectives from different vendors are usually not interchangeable. They have threads, dimensions, and optical properties that are tailored for the vendor's systems	You can buy specialized adaptors to interchange objectives. If you interchange objectives, carefully assess performance such as colour registration among spectral channels and make sure that the objective has enough clearance if you mount it on a rotatable turret
Special properties	Optical designs customized for specific applications	Applications like phase contrast and total internal reflection (TIRF) microscopy require an objective designed for the application
Magnification	Lateral magnification provided relative to the sample size	Common objectives range from 4–100×. Color coding for magnification: Red: 4× or 5× Yellow: 10× Green: 16× or 20× Light blue: 40× or 50×, Bright blue: 60× or 63× White: 100×
Working distance (WD)	Working distance is the distance from the front lens of the objective to the specimen	As you increase the NA, WD typically decreases. Oil-immersion lenses have a short WD, typically very close to the specimen. If using an inverted microscope, you can image through cell culture dishes with most 4, 10, and 20× objectives. Standard plastic dishes are not compatible with high-NA objectives and specialized dishes or cells mounted on coverslips are needed
Numerical aperture (NA)	The greater the numerical aperture, the higher the spatial resolution in (x,y)	The NA appears after the magnification usually in the format mag/NA such as 10×/0.3 Often higher NA means that you have a shorter working distance.

(continued)

Table 2
(continued)

Specification	Definition	Practical notes
		We have not considered resolution in *z*; refer to Chapter 2 for more information
Immersion medium and/or dipping objective	Medium between the front lens and the sample surface or coverslip Some are dipping objectives and placed directly in the immersion medium without a coverslip or other barrier	Air, dipping or immersion medium. If using oil, research the different kinds because they have different optical properties that are suitable for different applications Dipping objectives have a second white ring below the magnification ring colour code
Tube length	Infinity for modern objectives 160 or 170 mm for older objectives	Modern microscope optics are infinity corrected to allow for the placement of optical components in the light path
Coverslip if applicable	"0" means no coverslip Standard coverslip thickness is 0.17 mm	Objectives are designed to work with no coverslip, a coverslip of fixed thickness (typically 0.17 mm) or a variable range of thicknesses. The appropriate condition is listed after the tube length, usually the infinity symbol for modern microscopes
Objective type	All lenses, including objectives, exhibit aberrations that can affect the quality of the image. The three major aberrations are spherical aberration, chromatic aberration, and field curvature Chromatic aberration poses the greatest risk to the integrity of data sets. Chromatic aberration is when images collected using different spectral channels do not register properly, so images are offset in x, y, or z, which can lead to erroneous interpretations Spherical aberration arises because rays at the edges of lenses do not focus at the same point as those that travel through the center A perfect image is perpendicular to the axis of the objective. Field curvature means the image formed is slightly curved at the edges and thus out of focus	Objective types and degree of correction for optical aberrations. Spherical aberration (Sph); chromatic aberration (Chr); field curvature (Fld). Numbers report how many wavelengths are corrected

Type	Sph	Chr	Fld
Achromat	1	2	No
Plan achromat	1	2	Yes
Fluorite	2–3	2–3	No
Plan fluorite	3–4	2–4	Yes
Plan apochromat	3–4	4–5	Yes

(continued)

Table 2 (continued)

Specification	Definition	Practical notes
Iris or collars	Collars are often used to correct for different properties. These can include correcting for chamber thickness and/or to improve optical quality when imaging into thick specimens and/or for imaging at different temperatures Irises limit the cone of light and thus NA. If you are imaging with fluorescence, the iris must be fully open to achieve the full NA and thus spatial resolution	Adjust for thickness of plate, coverslip thickness and temperature

camera or related detector captures an image on a fluorescence microscope, the image is digitized, meaning that it is converted from a continuous or analogue signal (the real image) to one consisting of discrete data points (the signal detected at each detector element).

Whenever we convert a continuous signal to a digital one, we must consider the sampling rate, that is, the extent to which we must sample to create an accurate representation of the original. The image formed by the microscope is continuous, but we convert it into discrete pixel readings so that we can visualize and analyze the signal computationally. To capture the image at high fidelity without losing information, we must sample the image with high enough spatial detail. This sampling rate is governed by a fundamental theorem on signal processing, known as the Nyquist Theorem. The Nyquist Theorem states that a continuous signal must be sampled at atleast twice the highest frequency in the original [27]. For a fluorescence image, the highest spatial frequency is the resolution lateral limit r. When imaging, if you do not want to lose spatial detail, you should configure your system so that

$$p \leq \frac{M_{\text{total}} r}{2}, \tag{6}$$

where p is the pixel length (assuming square pixels), M_{total} is the total magnification of the system, and r the smallest lateral (x,y) distance that is resolvable by the system, given by Eq. 5. If the smallest resolvable feature is 1 μm, then each image pixel should be about 500 nm. As long as the Nyquist Theorem is followed, then the spatial fidelity of the microscopic image is preserved, and sampling artifacts are avoided.

If we do not sample the image with enough pixels per resolution element r, we denote this as "undersampling." An undersampled image does not preserve all the spatial information that was resolved in the original image and can create artifacts. For example, in Fig. 10, the fluorescence image on the left has been

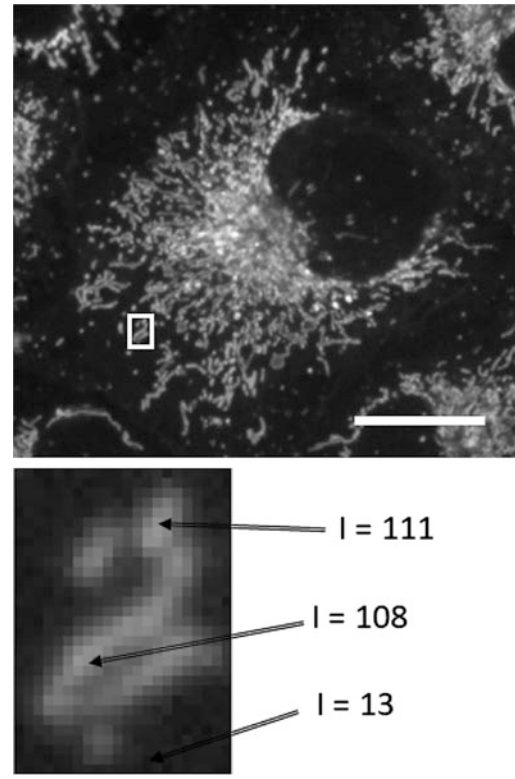

Fig. 9 Images are composed of individual pixel readings arranged in the two-dimensional (*x,y*) array. A fluorescent image of living cells labelled with a fluorescent probe that localizes to mitochondria is shown. The area of the cell bounded by the white box is shown below at higher zoom. The image is composed of pixels that encode intensity readings. The image is 8-bit, meaning that the possible intensity values range from 0 to 255. The representative intensity (I) pixel readings are indicated. The image was recorded using a spinning disk confocal microscope (Leica DMI6000 stand, Diskovery Flex system equipped with an Andor iXon Ultra 897 camera, MetaMorph software; Quorum Technologies, Guelph, Ontario, Canada). Image acquisition settings: 63×/1.35 NA objective, 488 nm laser excitation and an emission filter with transmission between approximately 500–575 nm to match the emission spectrum of the organelle probe MitoTracker Green FM (ThermoFisher Scientific, Canada). Scale bar = 20 μm

sampled with fewer pixels per micron than the one on the right. This means that the sampling is inadequate if we want to obtain the highest resolution possible from the experiment. Conversely, if we sample more than required, our images will contain more pixels and require more computer storage but will not lead to any more detail in the image. This is known as "oversampling" or "empty magnification." Another drawback of oversampling is that it can slow down image acquisition and even degrade the quality of imaging, because it can lead to photobleaching.

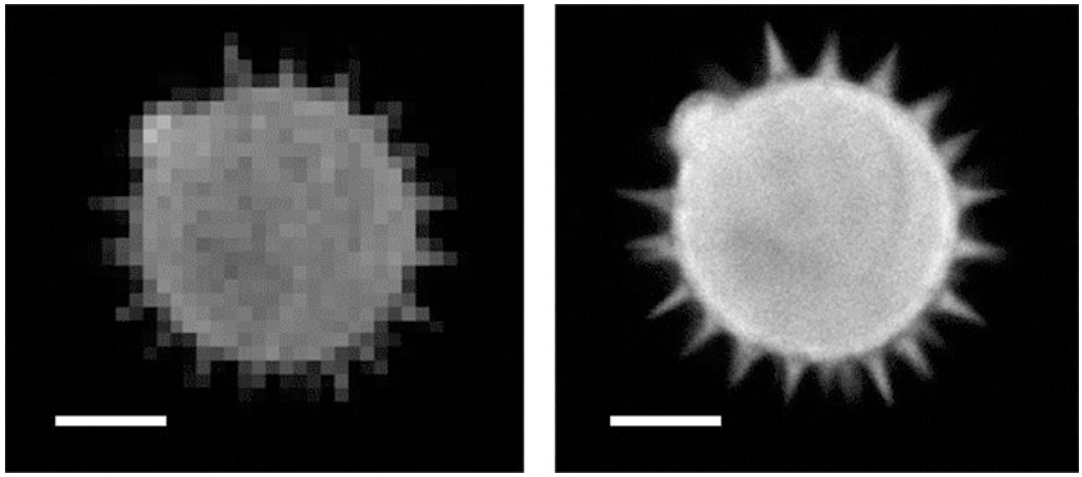

Fig. 10 The effect of sampling on spatial resolution. An autofluorescent pollen grain (#304264, Carolina Biological Supply Company, NC, USA) imaged using a laser scanning confocal microscope (Nikon A1R microscope, Ti2-E stand, NIS-Elements v 5.02 software; Nikon Canada, Mississauga, Ontario, Canada) and 20×/0.75 NA objective. The image on the left is undersampled and not all spatial resolution has been preserved. This image has been sampled with fewer pixels per micron (1.27 μm/pixel or 0.8 pixels/μm). The pollen on the right has been sampled using the Nyquist Theorem (0.18 μm/pixel or 5.5 pixels/μm). When the image is sampled using the Nyquist Theorem, features of the pollen are better resolved. For example, the spikes of the pollen are visible and can be counted and measured in the image on the right. Image acquisition settings: 561 nm laser excitation and an emission filter with transmission between approximately 570–625 nm. Scale bar = 10 μm

Widefield microscopes should typically be configured so that r obtained with the highest numerical aperture objective is sampled by at least two adjacent image pixels [28]. If you are not sure whether a system is sampling at Nyquist, you can calibrate your system by acquiring an image of a stage micrometer or other standard such as a USAF 1951 target, and then calculating the number of adjacent pixels per unit length in microns [29].

On systems such as laser scanning confocal or multiphoton microscopes, the sampling frequency is set by the operator rather than determined by a camera. Often, the default settings are not configured for optimal sampling but rather for speed. For this reason, it is important to check your sampling frequency on these systems. Sampling at a rate specified by the Nyquist theorem, however, is not an absolute requirement but rather depends on the research question and application. If you are not interested in imaging features close to the resolution limit then undersampling may be appropriate, especially if you need to acquire images quickly and with minimal photobleaching. Yet if you are exploring spatial relationships on the order of 200–500 nm, it is critical to ensure that you are sampling at the highest fidelity possible. Nyquist sampling is also relevant to imaging z-sections, described in

Chapter 2, and to sampling in the time domain, as in live cell imaging (Chapter 3).

For each pixel in a digital imaging file, the intensity readings and their ranges are set by the analogue to digital conversion (ADC) electronics. The ADC converts the analogue signal (the electrical signal generated by the photons hitting the photoelements) to a discrete integer value within specific ranges. These readings are expressed from 0 to 2^N-1 or 1 to 2^N, where N is the bit depth. The pixels in an 8-bit image will range from 0–255 (equivalently 1–256), while those in a 12-bit image will range from 0–4095 (or 1–4096). It is possible to record the same image using either bit depth. In each case, the 0 value represents the lowest possible value, while the top value (255 or 4095) represents the highest possible intensity, corresponding to the maximum number of photons that can be detected at each photosite. The number represents how many gradations are used to capture the original signal and a higher bit depth captures the signal with finer detail. This is like measuring a volume of liquid (73 mL) using a 100 mL graduated cylinder with markings every mL (high bit depth) versus a 100 mL beaker with markings every 50 mL (low bit depth).

When working with digital images, you may not see a difference between an 8-bit and 12-bit image because the human eye can, at best, distinguish about 700 shades of grey [30]. If you collect your image at 12-bit, but save it as an 8-bit image, important information about your signal will be irrevocably lost. It is critical to acquire and save your data at higher bit depths if you plan to carry out quantitative analysis of your images. Additionally, compressing data reduces the fidelity of an image and can introduce artifacts. When saving data, avoid file compression and save your data sets in the original proprietary format. When exporting files, use a lossless format such as PNG or TIF and avoid JPEG, as these can compress your data.

Another common misconception is that the detectors used in research-grade fluorescence microscopes detect images in colour. Except for cameras used for histology, most detectors are monochrome and only record the intensity of light, not colour. The images are acquired using filters, and then we assign a colour look up table (LUT) that by convention is often chosen to match the fluorescence emission peak. Although this is the convention, it is also appropriate and sometimes preferable to use a different colour scheme; however, to avoid confusion, it is good practice to report the spectral range used to detect the signal. When using colour to display images, it is important to use palettes that are accessible for people with colour deficiencies. The prevalence of inherited colour vision deficiencies varies, affecting 3–8% of males worldwide; rates for female sex are much lower [31, 32]. Colour vision deficiencies can also develop due to disease or environmental exposure. The

most common deficiency affects red-green perception. This means the common red-green palette used to depict fluorescence images is uninterpretable to a significant fraction of the population and can create unexpected issues when working with colleagues who may interpret the data in unexpected ways.

3 Transferring Concepts to Practice: The Fluorescence Imaging Experimental Workflow

Now let us apply the foundational concepts described above to the experimental workflow, as illustrated in Fig. 11. Ideally, consider these steps as an iterative cycle over the lifetime of a project. For example, you may have to carry out a few tests before finalizing your experimental design or selecting your preferred imaging mode. In addition, if you acquire all your imaging data before attempting to analyze it, you may find yourself repeating experiments. This is because image analysis often highlights issues in the workflow, and you may need to adjust your sample preparation and/or image acquisition settings to extract the information that you need from the images.

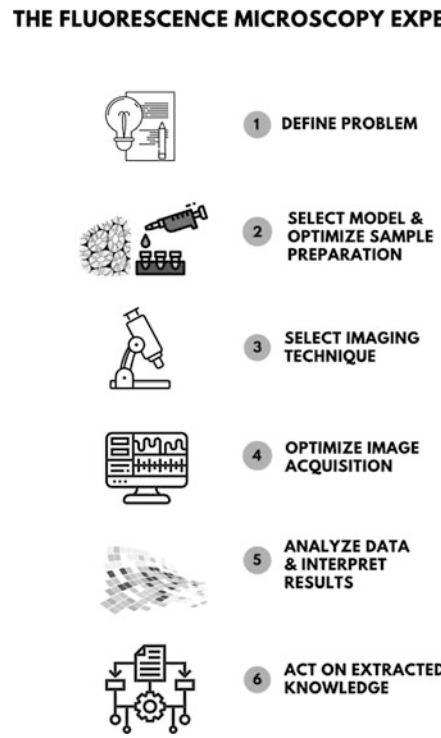

Fig. 11 The steps in a fluorescence imaging experiment. This workflow should not be considered linearly. You may need to revisit the different steps as your experiment progresses

3.1 Define Problem

Fluorescence microscopy requires an intensive investment in time, effort, and funding, and the goal is rigor and reproducibility [12, 33]. Before beginning an imaging experiment, make sure that you have well-defined aims with specific goals. Like with any research and development project, every step should be systematically mapped out, performed, and documented. It is important to define your project aims and how you will achieve them. Just because you are capturing images does not mean that you circumvent the need for controls or to document your work. Anecdotally, through working with hundreds of students and researchers, we note that there is a tendency for people new to the field to start off with a flurry of image acquisition rather than taking the time for experimental design, sample optimization and analysis. Even the most seasoned microscopists experience the siren call of the single representative image rather than taking the time to acquire systematic and statistically rigorous data.

3.2 Select Model and Optimize Sample Preparation

The models used in fluorescence imaging range from individual molecules to living organisms. Whether imaging an individual ion channel, root stem, or blood flow, the imaging tools and approaches developed for fluorescence imaging bridge these levels of organization. Careful sample preparation is essential for successful microscopy experiments. Nonfluorescent samples can be labelled by a number of methods, such as labelling organelles with specific dyes, expressing proteins tagged with fluorescent proteins [34], using fluorescently tagged DNA probes (such as DNA-PAINT) [35] and immunofluorescence [36]. Careful consideration should be given to how a sample will be stained and which fluorophores will be used. Always take the time to research suitable probes to address your research questions and check that they are compatible with your microscope filters. For immunofluorescence applications, it is also important to validate your antibody choice [37–39], to ensure you are labeling the intended structure or molecule.

When choosing probes it is important to compare the excitation and emission spectra to the spectral channels available on the microscope and to consider fluorophore brightness. It is also important to know the performance capability of the system itself. That is, a probe may be bright, but the light source on a microscope may be dim within that spectral range or the camera may exhibit lower sensitivity. The intensity of a light source versus wavelength and spectral sensitivity curves for detectors are available on vendor websites. It is a good idea to refer to these, along with probe information, as you plan an imaging experiment. When imaging only one target, it is preferable to choose a bright fluorophore for the most sensitive spectral channel on the microscope. When imaging more than one target, it is a good idea to assign the optimal channel to the one with the lowest concentration in the sample.

Another important consideration is the number of targets that can be imaged in one experiment. Finally, advanced techniques such as live cell imaging, optogenetics, multiphoton imaging and super-resolution techniques often require specialized probes that require more time and effort to identify if you are new to the field.

It is important to consider the possible sources of error and/or artifacts arising from the choice of model and labelling method in the planning stage. Proper controls are critical for the success of fluorescence microscopy experiments. In addition to experimental controls, you should also prepare positive and negative controls for imaging [12, 40]. For example, when carrying out multicolor experiments, it is important to test samples labeled with each fluorophore alone, as sometimes you may obtain false positives if there is overlap among the spectral channels. It is also important to check an unlabeled sample for autofluorescence arising from endogenous fluorophores. Autofluorescence often looks hazy and diffuse but can also appear as specific antibody labelling. Sometimes you can use this to simplify your experimental design by taking advantage of this inherent signal.

3.3 Select Imaging Technique

To choose a suitable microscope, consider your research aims and the availability of instrumentation. When reviewing techniques and systems, it is helpful to recognize that you will need to prioritize and balance different requirements. For example, if you need to capture images at high speed, you may not be able to acquire images with a high signal-to-noise ratio (S/N). In fluorescence microscopy, various approaches are used to quantitatively measure S/N [41] but here we use this term qualitatively to mean the extent of signal over the background. Other considerations are the number of probes and spectral ranges to be used, whether you will image single planes (2D) or volumes (3D), and the fraction of the sample area or volume you need to image for statistical rigor. For delicate samples, you also need to monitor photobleaching and, if working with living specimens, phototoxicity.

As a starting point, consider whether your sample is compatible with the optical setup of your microscope. Different microscopy techniques constrain the way you can mount and observe your sample, and you should check the requirements for your desired technique before planning any experiments. For guidance on which techniques are best suited to your research, we recommend the following chapters in this book, in addition to overviews in the literature [24, 42]. If you have the time and resources, sometimes the best way to find the optimal microscope is by testing applications on different systems to determine the advantages and drawbacks of each. When you work on your own research question, with your own samples, you will directly experience and recognize the choices and compromises involved with each technique and application.

If you do not have access to the optimal system for your experiment, this does not mean all is lost. You can go far with a widefield fluorescence microscope because it will help build the groundwork and help to identify whether a more sophisticated system is truly needed. A common misconception in the imaging field is that a more complex and more expensive system must out-perform a basic one, but this is not always the case. Sometimes a widefield microscope may be your best choice, when considering your research model and aims as well as other factors such as sensitivity, time, and cost. If you do need to access an advanced system, there are newly emerging national and international consortiums that are addressing the need for shared and open resources.

Although fluorescence microscopy is a powerful technique, it is limited to revealing a miniscule fraction of the numerous organelles and thousands of proteins, lipids, and other molecular constituents within a specimen. You may be pleased that the fluorescence images of your living cells look crisp and bright; yet if you don't take the time to examine them using white-light imaging, you might miss that they are stressed, exhibit condensed nuclei, swollen mitochondria, and blebbing. Alternatively, you may think that you are looking at individual isolated cells, but when you inspect your cells using white-light, you notice that your staining is highlighting a subpopulation of a monolayer. By using white-light imaging, you will expose your specimens to a much lower dose of light compared to fluorescence imaging so there is reduced risk of photobleaching. When you first inspect your sample through the eyepiece, use white-light imaging whenever possible to bring your sample into focus. This will minimize photobleaching and will reduce photodamage and phototoxicity if imaging live cells.

3.4 Optimize Image Acquisition

Once you have planned your imaging experiment and prepared your samples, the next step is to acquire images at the microscope. This can be daunting, especially as technologies advance and options at the microscope increase. However, there are several general principles that apply in fluorescence imaging. Applying these principles to experimental practice will help to ensure your imaging is optimal.

3.4.1 Know your Light Path(s)

When you sit down at a microscope for the first time, you might wonder where to start. We recommend always tracing the light path when working with a system that is new to you. Can you identify your light source(s)? Do you know what fluorescent filter sets are available and how to select them? Does the system have any add-ons or extras such as different stages? What objectives are available? How is the image detected and where? If you can identify some of the components on the microscope, then it will be easier to navigate the image acquisition software. Although commercial

packages have been slowly improving the user interface experience, the array of options and controls can be confusing or even overwhelming. In addition to tracing the light path, take time to learn how to operate the microscope using a standard slide, before moving to your experimental samples. Once you are confident on a system, you will find that your experiments will progress faster, because you will not be held back by basic operator errors.

3.4.2 Select Filter Sets

First, place the appropriate filters in the light path. You should have already matched your fluorophores to the microscope filter sets as part of your experimental design. If you did not do this in advance, you will need to check which filters best match the probes used in your sample. When the specifications for two filter sets have similar transmission curves, we recommend testing both with your sample to determine which gives the best imaging for your experimental needs.

3.4.3 Place Sample and Focus

Start your imaging session with an air objective that is positioned for maximum clearance from the slide. When switching between objectives, take care to use the electronic controls. If your system is not automated, switch the positions by handling the filter turret or holder. Do not press or pull on the objectives when switching between them, as unnecessary strain can misalign the lens assemblies within objectives. Objectives are the heart of the imaging system and costly and if you are not careful, wear and tear will affect the quality of the images acquired.

Next, you want to position your sample securely on the microscope stage. Microscopes are equipped with manual and/or motorized stages, each suited for different applications. For example, your microscope might be equipped with a piezo stage that will allow for fast and accurate collection of z-stacks and/or have a live cell imaging stage that allows you to control the local environment by modifying temperature, humidity and CO_2. When using an inverted microscope (objective below the sample), take care that the objective is centered within the insert that holds your sample and secure your sample in place with a pressure fitting or paper tape.

Now that the sample is secured, bring the sample into focus using the eyepiece or detector. It is a good idea to start with an air objective even if the experiment requires imaging with an oil immersion or other short working distance objective. Once you focus using the air objective, switch to the oil immersion objective, which may need further focusing to fine tune the image. Using the air objective to set up the imaging helps minimize the risk of driving the objective into the sample. Although the front lenses on objectives frequently have protective spring mechanisms, these cannot protect from extreme carelessness, such as rapidly crashing the objective into the sample. If you take the time to familiarize yourself

with focusing an objective, ideally with a standard slide, then you will have a much easier time when carrying out your experiments and minimize the risk of damaging the objective and related components.

When using an oil immersion objective, extra care is needed to prevent damage. Do not be tempted to add too much oil to the objective. Oil can creep into the spring mechanism of the objective, causing seizing, or can leak into the optics, which can mean a costly repair or worse. For optimal imaging, ensure that you clean an oil objective at regular intervals. Cleaning methods vary by laboratory and imaging center, and there is lively debate about how best to clean oil immersion objectives. Therefore, if you are not sure how to clean an objective, ask the person in charge of the microscope. If there is no one overseeing the microscope, you can ask the vendor.

Once your sample is in focus with the desired objective, you need to select regions of interest. When choosing where to image, consider possible bias. Ideally, the images captured represent your population of interest, without selectively choosing areas that support your hypothesis. To limit bias when choosing your fields of view, there are several options including: using white-light to focus your image and choose a field of view (this is also gentle on your cells); using the fluorescence channel of a nonexperimental marker (such as the nucleus) to focus and choose a field of view; using tiling to cover a larger area of the sample and labelling your samples with codes so that your imaging is blinded to the condition. Also consider how you will approach the statistical analysis of your data, as this may influence which and how many images are acquired.

3.4.4 Capture Images

After focusing with the eyepiece and choosing your field of view, switch to the camera to start acquiring data. Sometimes when you switch to the camera, the image on your camera is perfectly in focus. This optimal configuration, when both light paths have a common focal plane, is known as "parfocality." However, often you need to adjust the focus slightly because the light path to the eyepiece and the light path to the detector are not identical. If you see a large change in focus between the two light paths, it may be that your microscope needs to be adjusted by the person entrusted to its care or by the vendor.

When you acquire images, it is important to monitor the image and intensity readings at each pixel so that you ensure the image meets the needs of your experiment. For example, you may want a bright image with high S/N for immunofluorescence imaging of fixed cells, but you may sacrifice S/N for speed and/or viability when working with living specimens. To make these choices, you should monitor the intensity readings encoded by your images. You could rely on the brightness of the image itself, but images

displayed by the software can be artificially enhanced for maximum brightness and can be deceptive (beware the "Autoscale" button).

The image histogram is an invaluable tool for checking pixel intensity as you acquire images. Histograms plot the number of pixels versus the possible intensity values (Fig. 12). Different microscopes offer different ways to adjust the signal intensity. A widefield microscope with a camera will allow you to increase or decrease exposure time and may also let you modify camera gain and the intensity of the excitation light source. Histogram representations are a standard feature in software acquisition, and you should monitor them closely when setting up your light source intensity and exposure time. As with all aspects of microscopy, setting your signal can involve making compromises.

Figure 12 depicts three images of von Willebrand factor contained in Weibel Palade bodies in dermal microvascular endothelial cells. Each image was acquired using different exposure settings, with the exposure increasing in time from left to right. The camera is 12-bit, meaning each pixel intensity reading can range between 0 and 4095 (12 bit), where 0 is displayed as black, 4095 as white, with grayscale in between. In Fig. 12a, the readings range between 0 and 1000. Having a weak signal may be necessary for live cell or other photosensitive experiments, but if imaging fixed cells, you can improve image quality by increasing the brightness of your signal. In Fig. 12b, more of the intensity range is used, and the pixel intensity values range between 0 and 3000. Figure 12c illustrates saturation. This means that some pixels in the image have reached the maximum intensity value possible (4095) and the image no longer accurately represents the fluorescence signal because the photosites are being overfilled with photons. This is much like trying to accurately measure 105 mL when the 100 mL graduated cylinder is flowing over. If you were to continue to increase the signal intensity, more and more pixels would reach this maximum value and information contained in the image would be lost.

Sometimes operators will adjust the brightness and contrast of an image by manipulating the histogram settings, so the image looks brighter. The "Autoscale" function in software does this automatically, and conveniently brightens the image for you by adjusting the brightness and contrast. This is acceptable, if you recognize that adjusting the image histogram does not change acquisition settings, and by extension, the signal recorded by the camera. In short, by adjusting the histogram display settings, you have not done anything to change the pixel intensity values themselves, only how they are displayed. Therefore, you may get a false sense of the S/N of the data. To change the pixel intensity readings, you should modify the microscope settings by adjusting the light source intensity, camera exposure, or other instrumental settings.

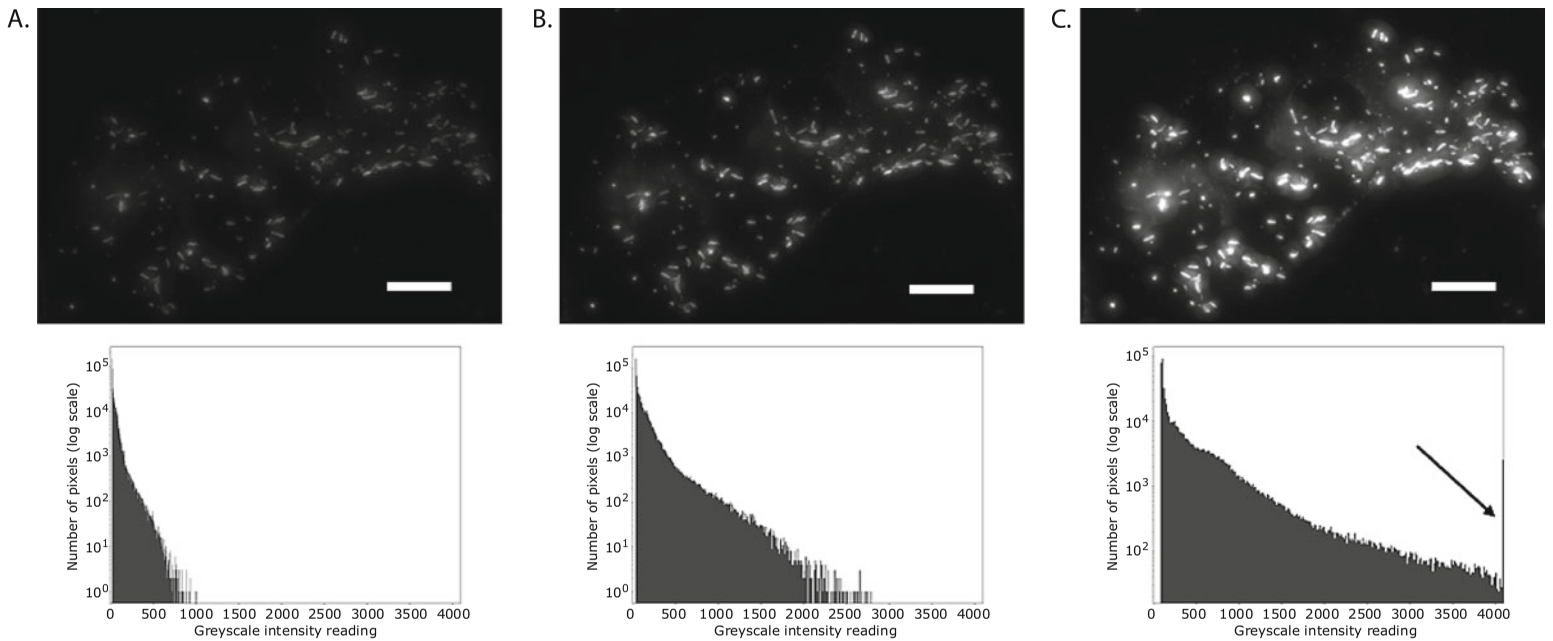

Fig. 12 Using histograms to optimize the intensity values within an image. The same region of a cell was imaged using different exposure settings. (**a**) This image has weak signal and it is difficult to observe the features in the image. The pixel intensities fall between 0 and 1000. (**b**) This image has optimal signal. The features are visible and easily distinguished. The pixel intensities fall between 0 and 3000. (**c**) This image is saturated. Although this may look pleasing to the eye, the saturation makes features appear larger and causes coalescing between features that should be resolved. Saturation can lead you to misinterpret your data sets. Sample: dermal microvascular endothelial cells labelled using indirect immunofluorescence to detect von Willebrand factor and STAR RED (Abberior, Göttingen, Germany). Images were acquired using a widefield microscope (Nikon Ti2 stand, NIS Elements software, ORCA Flash 2 sCMOS camera; Nikon Canada, Mississauga, Ontario, Canada). Image acquisition settings: 60×/1.4 NA objective, 640 nm LED excitation, and an emission filter with transmission between approximately 669–741 nm. Scale bars = 20 μm

When you acquire images, it is important to optimize your settings at the beginning of your experiment and then try to adhere to these throughout the course of your project.

In addition to optimizing your acquisition settings, consider the order of imaging samples. It may be tempting to acquire images of all control slides first, and then image slides of your different experimental conditions. However, as light sources and systems warm up, there can be slight changes in excitation light intensity and other inconsistencies. Therefore, we recommend alternating your samples so that you acquire images of one control, then one experimental condition. We also suggest that all images for a data set be acquired on the same day, because performance can be variable day to day. If the experiment cannot be completed in one imaging session, consider repeat imaging one or two samples when you return to the microscope, as an internal check for comparison between sessions.

Some microscopes are also equipped with additional features. It is worthwhile investigating the capabilities of your microscope as you learn to use the system so that you can optimize your image acquisition and be aware of any limitations. Common features include the following.

- A motorized stage that allows you to increase your field of view by taking multiple images to cover an entire area or around a set point (called "tiling"). These images can then be stitched together to create one large image.
- Autofocus capabilities that allow you to maintain focus when running time lapse imaging experiments, for example with live cells.
- Ability to acquire *z*-stacks, which allow you to visualize your sample in 3D.
- A live cell imaging stage that controls temperature, atmospheric gas composition, and humidity.
- Perfusion apparatus for imaging cells under flow conditions.
- Photoactivation apparatus for photobleaching or photoactivation experiments.

3.5 Analyze Data and Interpret Results

Image analysis should be considered from the start, when designing experiments. Do not treat the workflow in Fig. 11 linearly, as the analysis methodology may require further optimization of sample preparation and/or image acquisition. It is a good idea to check whether others in your group have developed approaches to address related questions, or to search the literature for publications that provide detailed protocols. Here, as in the other steps of the experimental workflow, you must make choices depending on your research question, background, and access to software

packages. There are many open source and commercial packages available for image analysis. If you have a choice, you should check which package works best for you.

If you are new to image analysis, it is important to develop image analysis skills in parallel with learning to operate the microscope so that you can review and analyze your data before carrying out your next experiments. This is because sometimes data that look acceptable by eye are not amenable to image analysis. If you wait to acquire all the images before starting to analyze the data, you may have to go back and repeat your experiments or invest time in *post-hoc* justification. As you develop your image analysis protocol, ask one or more colleagues to test your approach. There are numerous open source and commercial packages available for image analysis. A good starting point is Fiji (Fiji is just ImageJ) [43] as it is widely used for image analysis in the life sciences, and we also draw your attention to an excellent introductory textbook that is also open source [41].

Data management and storage are also critical. You should preserve all original data sets as these contain the metadata that encode the image acquisition settings required to reproduce the imaging. If using a manual system, take the time to note down the acquisition settings. As part of any project, you should save your data sets where they are secure. If you do not have access to a server with regular backups, keep copies of your data in different locations. Additionally, make sure that your supervisor or team leader has an organized and searchable archive of all your data sets, as well as a detailed protocol for the image analysis, similar to how you document all other experimental details in your logbooks. These are but a few of the considerations that should be part of a data management plan (DMP), which are now mandated by multiple granting agencies. A DMP is a document that clearly defines roles and responsibilities when it comes to documenting, acquiring, maintaining, storing and sharing data at all stages of the project [44–46]. A number of guides and checklists are available to the research community to aid in the development of DMPs, and these are useful tools for keeping the project on track at every stage of its life cycle [47–49].

3.6 Act on Extracted Knowledge

When you present, publish, or otherwise communicate your results and conclusions, it is important that you include information about every step in the experimental workflow, as described above. Reproducibility in the life sciences is an ongoing concern, and a recent paper highlights that most publications do not adhere to best practices for reporting imaging data [50]. Ensure that you keep up to date with best practices [12, 33, 51] and clearly report and document every step of your experiment, including image analysis. If you have developed custom software, follow guidelines for documenting and sharing code as well. If you can, consider depositing

the original data sets in centralized repositories. In this way, you will help improve standards and model good practices, as you create a body of work for you and the research community to build and act upon.

4 Next Steps

We have introduced concepts designed to develop the skills and mind set needed for successful microscopy experiments. Microscopy is a practice that demands continual learning and refinement. As you start applying microscopy to your research, we encourage you to read the literature, consult with experienced colleagues, and seek out learning opportunities through online collaboration and/or the numerous onsite workshops offered worldwide. Besides reinforcing concepts and skills, these opportunities facilitate the transfer of ideas and experiences. As Michael Polanyi stated, "we know more that we can tell" [52] and when you reach out to others, you will learn valuable informal information that does not always translate to text. Whether you are a newcomer or a seasoned expert, you can help deepen your expertise and that of others by creating vibrant communities that sustain and nurture good practice.

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