A quick guide to light microscopy in cell biology

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ABSTRACT **Light microscopy is a key tool in modern cell biology. Light microscopy has several features that make it ideally suited for imaging biology in living cells: the resolution is well-matched to the sizes of subcellular structures, a diverse range of available fluorescent probes makes it possible to mark proteins, organelles, and other structures for imaging, and the relatively nonperturbing nature of light means that living cells can be imaged for long periods of time to follow their dynamics. Here I provide a brief introduction to using light microscopy in cell biology, with particular emphasis on factors to be considered when starting microscopy experiments.**

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INTRODUCTION

Most broadly, light microscopy techniques can be divided into two categories: brightfield and fluorescence. In brightfield microscopy, the light source and detection objective are placed on opposite sides of the sample, and the sample is imaged by its effect on the light passing through it as the sample absorbs, scatters, or deflects the light. Because most cells are thin and transparent, they do not absorb much light and so are difficult to see without adding optics that allows the phase shift of light induced by the cells to be seen. The two most commonly used techniques to visualize this phase shift are phase contrast, which causes cells to appear dark on a light background, and differential interference contrast (DIC), which gives a pseudo–three-dimensional (3D) shaded appearance to cells (Murphy and Davidson, 2012). Brightfield without phase contrast or DIC is usually sufficient to see the general outlines of cells, but phase contrast or DIC is necessary to achieve detailed, high-contrast brightfield images.

FLUORESCENCE MICROSCOPY

Fluorescence microscopy uses fluorescent dyes (fluorophores), which are molecules that absorb one wavelength of light (the excitation wavelength) and emit a second, longer wavelength of light (the emission wavelength). Most molecules in the cell are not very fluorescent, so fluorescent labels to be imaged are typically introduced by the experimenter. This allows the labels to be targeted to the molecule(s) of interest, either by genetically encoding a fluorescent protein or by binding a fluorescently labeled antibody. Multiple different fluorescent molecules can be distinguished simultaneously and can be detected at very low abundance (single molecules can be imaged), making this a very powerful technique. Fluorescence microscopy is typically done using epifluorescence, in which the fluorescence excitation light illuminates the sample through the same objective that is used to detect the emission from the sample. A fluorescence filter cube separates the light by wavelength so that the emitted light can be imaged without interference from the excitation light (Murphy and Davidson, 2012).

The two major techniques for introducing fluorescent labels into cells are immunofluorescence, in which fluorescently labeled antibodies that bind to specific proteins in cells are introduced, and genetic introduction of a fluorescent protein. In immunofluorescence, the cells are first fixed to cross-link proteins in the cell and then permeabilized to allow antibodies access to the cellular milieu. Typically, primary antibodies, which recognize the proteins of interest in the cell, are first introduced. After unbound antibodies are washed off, fluorescently labeled secondary antibodies, which bind to the primary antibodies, are added. This is known as indirect immunofluorescence and makes it easy to switch primary antibodies, as they do not need to be labeled, and the secondary antibodies typically have broad specificity (e.g., a goat anti-mouse secondary that recognizes all mouse immunoglobulin Gs).

Genetic introduction of a fluorescent protein involves fusing a fluorescent protein to a target of interest, which is then either introduced into the genome of the cell or expressed from a plasmid. This allows imaging of proteins in live cells and, if done by genomic introduction, means that the protein is expressed from its endogenous promoter at its endogenous level. For both immunofluorescence and fluorescent protein imaging, it is straightforward to image four colors in a cell. Most commonly, filter sets

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Abbreviations used: 3D, three dimensional; DIC, differential interference contrast; NA, numerical aperture; TIRF, total internal reflection fluorescence.

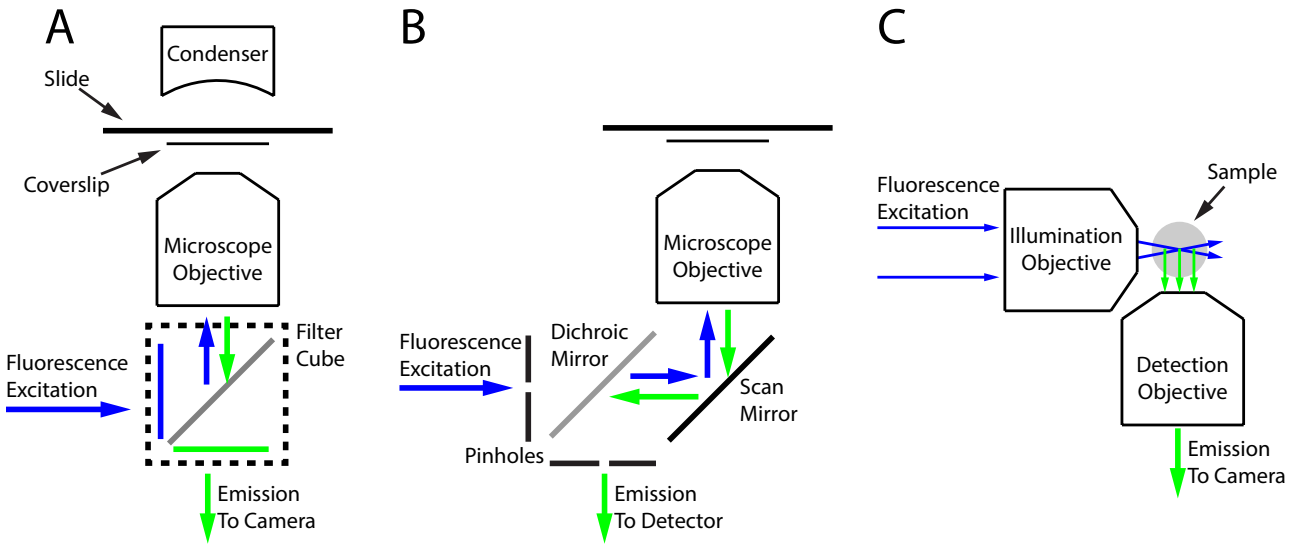

FIGURE 1: Schematic drawings of common microscopy techniques. (A) An inverted epifluorescence microscope. The sample sits between the slide and coverslip. The condenser lens delivers illumination for viewing light transmitted through the sample; the objective lens collects light from the sample and delivers excitation light for fluorescence microscopy. The filter cube consists of an excitation filter (blue), an emission filter (green), and a dichroic mirror (gray). The excitation and emission filters select the wavelengths that will illuminate the sample and be recorded on the camera, respectively, and the dichroic mirror reflects the excitation light to the sample while transmitting the emission light to the camera. An upright microscope has the same design, just rotated by 180°. (B) A confocal microscope. The excitation and emission pinholes are imaged onto the sample to define an illuminated point in the sample and to detect light from only that point. The lenses that image the pinholes onto the sample have been omitted for simplicity. The scan mirror scans the illuminated spot across the sample; because the scan mirror is in both the excitation and emission paths, the position of the spot detected from the sample is scanned in parallel with the excitation spot. (C) A light sheet microscope. An illumination objective, along with additional optics (not shown), is used to form a thin sheet of light that illuminates the sample. A detection objective images the light emitted from this sheet onto a camera.

with 405-, 488-, 561-, and 640-nm excitation wavelengths are used. For immunofluorescence, typical dyes used are the nuclear stain 4′,6-diamidino-2-phenylindole, green dyes such as Alexa 488 or fluorescein, red dyes such as Cy3, rhodamine, or Alexa 568, and far-red dyes such as Cy5 or Alexa 647. For fluorescent proteins, mTagBFP2, enhanced green fluorescent protein, mCherry/mRuby2/TagRFP-T, and monomeric infrared fluorescent protein can be used together. Particularly in the 640-nm-excitation channel, new fluorescent proteins are being developed rapidly, and better combinations may emerge in the near future; for reviews of fluorescent proteins see Day and Davidson (2009) and Dean and Palmer (2014).

WIDEFIELD FLUORESCENCE MICROSCOPY AND RESOLUTION

Most cell biology imaging is done with widefield microscopy, in which the microscope simply forms an image of the sample on the camera, without any additional optical manipulation. Live cells are most commonly imaged on an inverted epifluorescence microscope (Figure 1). In such a microscope, the objective images the sample from below. Inverted microscopes are popular for cell biological imaging because they allow imaging through a glass coverslip to see cells grown above. This means that cells can be grown in coverslip-bottom Petri dishes or multiwell plates containing growth media, which can be left open at the top. Alternatively, an upright microscope can be used with a water-dipping objective, which is immersed into the medium in which the cells are grown, but this is less convenient and less common.

Most of the key properties of the microscope are dictated by the choice of objective lens. The objective determines the magnification and resolution of the image; it also determines how much light will be collected from the sample and hence the sensitivity of the microscope. The objective performance is largely determined by its magnification and numerical aperture (NA). In addition, objectives come in multiple classes according to how well aberrations have been corrected; these are referred to by terms such as Achromat and Plan Apochromat. The objective magnification specifies how large the image at the camera will be relative to the sample; a 60× objective produces an image of the sample at the camera that is 60-fold larger than the sample. The NA is defined as the sine of the largest angle of light emitted by the specimen that the objective can collect multiplied by the sample refractive index for which the objective is designed. This controls both the light-gathering power of the objective (collecting a larger range of angles collects more light; this scales as the square of the NA) and the resolution limit of the objective. In the *XY*-plane (the plane perpendicular to the focus axis), the theoretical resolution is given by $0.61\lambda/\mathrm{NA}$, and the theoretical *Z*-resolution is given by $2\lambda n/\mathrm{NA}^2$, where λ is the wavelength of light and n is the refractive index of the sample. Although the resolution of the objective lens is set by its NA, the objective magnification is important to ensure that the image is magnified sufficiently on the camera to capture that resolution (the rule is that there must be at least two camera pixels per resolvable element to capture the full resolution of the microscope objective; Jonkman *et al*., 2003; Inoué, 2006; Murphy and Davidson, 2012). Most objectives are designed to image through glass coverslips 0.17 mm in thickness. To acquire

the best images, it is important to grow cells on these coverslips. If cells must be grown on plastic, specialized objectives for imaging through plastic are available, although they typically have poorer performance than objectives designed for imaging through coverslips. Alternatively, specialized plastic dishes can be purchased that have optical properties similar to 0.17-mm glass coverslips.

CONFOCAL MICROSCOPY

A major limitation of conventional epifluorescence microscopy is that the illuminating light excites fluorophores in a cone throughout the sample, and the detection camera cannot distinguish this out-of-focus light from the light emitted by the focal plane of the sample. Hence the in-focus information that we seek to image is obscured by blurred images of the out-of-focus regions of the sample. For samples that are not too thick and not too densely labeled with fluorophores, this out of focus light is not a major problem. However, for thick, densely stained samples or in cases in which we wish to achieve well-resolved 3D images, this out-of-focus light can obscure valuable information. Many techniques have been developed to eliminate this out-of-focus light. The most commonly used is confocal microscopy, in which the sample is illuminated by a focused laser beam at a single point in the sample focal plane (Figure 1). Light from this point is detected after passage through a pinhole, such that only light emitted from the focal plane makes it through the pinhole and is recorded on the detector. Light from out-of-focus planes is blocked by the pinhole, and so the confocal only records light from the focal plane of the sample. Scanning mirrors are used to raster the laser spot across the sample, building up an image point by point (Inoué, 2006; Stelzer, 2006). A related technique is two-photon microscopy (Helmchen and Denk, 2005), which is primarily used for imaging very thick specimens (>200 µm), so it is not commonly used in cell biology.

Because laser-scanning confocal microscopes record an image point by point, they do not use cameras, but instead use a point detector, which tend to be less sensitive than cameras. To overcome this limitation, systems that scan multiple focus spots across the sample simultaneously and image the resulting emission on a camera have been designed. The most common of these is the spinning-disk confocal, which uses a disk of pinholes that sweep across the sample such that a revolution of the disk scans over every point in the sample during a single exposure (Toomre and Pawley, 2006). Spinning-disk confocal microscopes combine ease of use, high speed (up to hundreds of frames per second), and high sensitivity, so they have become widely used in cell biology. For samples thicker than ~30 µm, they are poorer at rejecting out-of-focus light than a laser-scanning confocal, but this is not a limitation for imaging most tissue culture cells. Spinning-disk confocal microscopy is believed to be more live-cell–friendly than widefield or laser-scanning confocal microscopy, but definitive evidence of this is lacking. Spinning-disk confocal microscopy is widely used for imaging protein and organelle dynamics in single cells—for example, imaging mitochondrial inheritance in yeast (Rafelski *et al.*, 2012) or imaging microtubule dynamics in mammalian cells (Wittmann and Waterman-Storer, 2005).

TOTAL INTERNAL REFLECTION FLUORESCENCE MICROSCOPY

Another microscopy technique widely used in cell biology is total internal reflection fluorescence (TIRF) microscopy (Axelrod, 2001). This technique relies on total internal reflection of a laser beam at the interface between the coverslip and the aqueous sample above it. The reflected laser beam sets up an evanescent light field at the coverslip interface; the evanescent field penetrates only a few hundred nanometers into the sample. This allows excitation of fluorophores within a few hundred nanometers of the coverslip but nowhere else in the sample. This highly localized excitation makes TIRF microscopy one of the most sensitive microscopy techniques, as there is essentially no background from the rest of the sample, but also limits its use to imaging samples that are immediately adjacent to the coverslip. This has made TIRF a very powerful technique for imaging membrane dynamics and trafficking (e.g., Yudowski *et al.*, 2009) and the cell cortex (e.g., Hu *et al.*, 2007), as the basal membrane of a cell grown on a coverslip lies very close to the coverslip.

LIGHT SHEET MICROSCOPY

Finally, a revolution in microscopy has been occurring with the development of light sheet microscopes (Weber and Huisken, 2011; Keller and Ahrens, 2015). These are microscopes that illuminate the sample from a plane orthogonal to the imaging plane (Figure 1). This eliminates the problem of out-of-focus light because only light from the focal plane or very close to it is excited. This selective illumination also reduces the total light exposure of the sample, which in turn reduces photobleaching and phototoxicity. If identical objectives are used to produce the light sheet and detect the fluorescence, their roles can be interchanged, with orthogonal views of the sample being captured through the two objectives. These images can then be fused to produce a 3D image of the sample with isotropic resolution, eliminating the poorer *Z*-resolution of other forms of microscopy (Wu *et al.*, 2013). Alternatively, sophisticated optical design can be used to produce microscopes that capture high-resolution 3D images at high speed (Chen *et al.*, 2014). Although these microscopes are being commercialized, they are not yet widely available. I expect that these and other light sheet designs will become widely available in the near future, heralding a revolution in 3D microscopy of live cells.

SUPERRESOLUTION MICROSCOPY

Although beyond the scope of this introduction, another recent revolution in light microscopy is the development of superresolution techniques that allow resolution beyond that of conventional light microscopy (Huang *et al.*, 2010). With these techniques, resolution as high as 20 nm in *XY* and 50 nm in *Z* can be achieved, although these often require the use of special sample preparation techniques, special microscopes, or special fluorophores and can be difficult to use in live cells. Although superresolution microscopes are commercially available, adoption of these techniques has been slow. However, it is likely that these techniques will be increasingly important for resolving cellular ultrastructure in the future.

CHOOSING THE RIGHT MICROSCOPE FOR YOUR EXPERIMENT

When planning a microscopy experiment, the first thing to do is to determine the systems to which you have access and how they are configured; check the systems both in your lab and in core facilities. Make sure that the microscopes have the filters and/or lasers to image the dyes you plan to use and that they have incubation systems for keeping cells alive during a long time lapse if that is needed for your experiment.

The relative performances of different imaging modalities are compared in Table 1. In general, for routine imaging, widefield epifluorescence is a good starting point: it is easy to use and has relatively good sensitivity and speed. For samples thicker than ~20 µm, it is worth considering confocal microscopy, particularly if

Microscope	Maximum sample thickness	Speed	Sensitivity	Phototoxicity
Widefield	20 µm	++	++	++
Laser-scanning confocal	100–200 µm	+	+	+
Spinning-disk confocal	30–50 µm	+++	+++	++
TIRF	At coverslip	+++	+++	+++
Light sheet	>1 mm	+++	++	+++

Speed, sensitivity, and phototoxicity are rated +, ++, and +++ from worst to best, respectively. Maximum sample thickness is a guideline, and with careful sample preparation, thicker samples can often be imaged.

TABLE 1: Performance of imaging modalities.

high resolution in *Z* is required or if the samples are densely stained. Laser-scanning confocal microscopy is excellent for rejecting out-of-focus light and acquiring 3D image data, but in general it is less sensitive and more phototoxic than spinning-disk confocal microscopy. For live specimens for which 3D information is required, spinning-disk confocal microscopy should be considered. Spinning-disk confocal microscopy is also excellent for obtaining high-resolution 3D information on small objects, as in imaging yeast with a 100×/1.4 NA oil objective. For membrane imaging, or for any other sample that is within a few hundred nanometers of the coverslip, TIRF microscopy should be considered. Finally, should you have access to a light sheet microscope or other specialized microscope, learn its capabilities and consider whether it is a good fit for your experiments.

The performance of a microscope can depend heavily on the details of its components and how it is configured. For this reason, it often makes sense to try multiple systems when trying a new imaging experiment to determine which one will best meet your needs. Local microscopy experts, such as a core director, can often help you choose an appropriate microscope. You may also need to consider changing the fluorescent labels you use, or otherwise adjusting your experimental design, to make best use of the microscopes to which you have access.

ADDITIONAL RESOURCES

Microscopy is a large and diverse field with an active research community, so for a newcomer to the field, the amount of information available can easily seem overwhelming. Fortunately, there are some excellent resources for the novice microscopist. I recommend two books in particular: *Fundamentals of Light Microscopy and Electronic Imaging* (Murphy and Davidson, 2012) provides an excellent introduction to the subject, and the *Handbook of Biological Confocal Microscopy* (Pawley, 2006) provides a much more detailed reference to many aspects of microscopy, particularly confocal microscopy. There are also two excellent websites that have information on a wide range of microscopy topics: microscopy.com and micro.magnet.fsu.edu. Finally, a comprehensive set of lectures on microscopy is available online at ibiomicroscopy.com.

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