Immunofluorescence Microscopy

Domenico F. Galati[1,3] and David J. Asai[2]

[1]Biology Department, College of Science and Engineering, Western Washington University, Bellingham, Washington
[2]Inclusive Learning Group, Science Education Department, Howard Hughes Medical Institute, Chevy Chase, Maryland
[3]Corresponding author: *nick.galati@wwu.edu*

Published in the Essential Lab Techniques section

Visualizing fluorescence-tagged molecules is a powerful strategy that can reveal the complex dynamics of the cell. One robust and broadly applicable method is immunofluorescence microscopy, in which a fluorescence-labeled antibody binds the molecule of interest and then the location of the antibody is determined by fluorescence microscopy. The effective application of this technique includes several considerations, such as the nature of the antigen, specificity of the antibody, permeabilization and fixation of the specimen, and fluorescence imaging of the cell. Although each protocol will require fine-tuning depending on the cell type, antibody, and antigen, there are steps common to nearly all applications. This article provides protocols for staining the cytoskeleton and organelles in two very different kinds of cells: flat, adherent fibroblasts and thick, free-swimming *Tetrahymena* cells. Additional protocols enable visualization with widefield, laser scanning confocal, and eSRRF super-resolution fluorescence microscopy. © 2023 Wiley Periodicals LLC.

Basic Protocol 1: Immunofluorescence staining of adherent cells such as fibroblasts
Basic Protocol 2: Immunofluorescence of suspension cells such as *Tetrahymena*
Basic Protocol 3: Visualizing samples with a widefield fluorescence microscope
Alternate Protocol 1: Staining suspension cells adhered to poly-L-lysine-coated coverslips
Alternate Protocol 2: Visualizing samples with a laser scanning confocal microscope
Alternate Protocol 3: Generating super-resolution images with SRRF microscopy

Keywords: cytoskeleton • fluorescence • immunofluorescence • microtubules • *Tetrahymena* • tubulin antibody

How to cite this article:
Galati, D. F., & Asai, D. J. (2023). Immunofluorescence microscopy. *Current Protocols*, *3,* e842. doi: 10.1002/cpz1.842

OVERVIEW

Living cells are dynamic and complex systems. Eukaryotic cells contain thousands of macromolecules that move between the cytoplasm, membranous organelles, and phase-separated droplets on the millisecond time scale (Lippincott-Schwartz et al., 2018; Shin & Brangwynne, 2017; Wu et al., 2018). To understand the biology of cells, we must visualize their macromolecules with specificity in four dimensions (Fig. 1): the three spatial dimensions (XYZ) and the fourth dimension of time (T). Where is the molecule?

Current Protocols e842, Volume 3
Published in Wiley Online Library (wileyonlinelibrary.com).
doi: 10.1002/cpz1.842

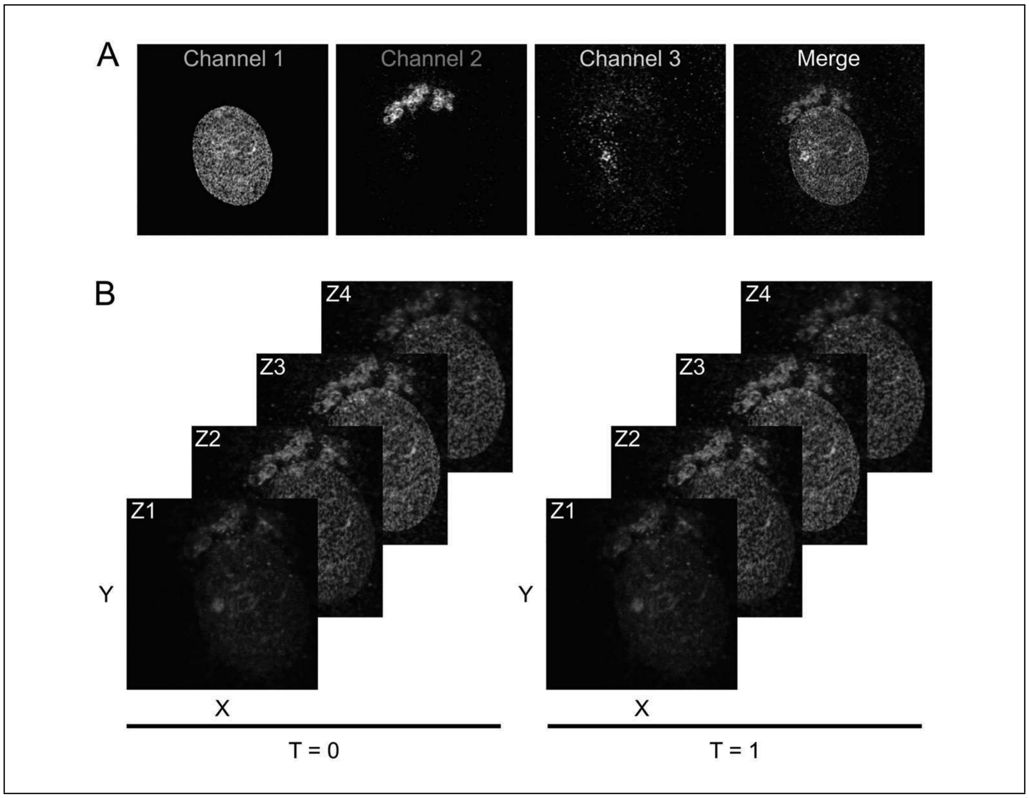

Figure 1 The multiple dimensions of fluorescent microscopy. (**A**) Different molecules of interest can be imaged as different channels in an immunofluorescent microscopy experiment. In this image, the nucleus (channel 1), the Golgi apparatus (channel 2), and the centrosome (channel 3) are shown individually in grayscale and as a false-colored merged image. B) Each image is composed of a 2-dimensional array of pixel intensities (the X-Y dimensions). Multiple X-Y dimensions acquired at different focal distances can be stacked together in the Z dimension. When imaging live cells, the X, Y, and Z dimensions can be acquired at different time intervals. The images in this figure are from hTERT-RPE1 cells immunostained with a primary antibody that binds to the Golgi protein Golph2 (Rhodamine Red X was conjugated to the secondary) and a primary antibody that binds to the centrosome protein PCM1 (Alexa 647 was conjugated to the secondary). The images were acquired with a Leica Stellaris 8 FALCON/FLIM laser scanning confocal microscope.

How much of it is present in a particular location? With what other molecules does it interact? How does this spatial pattern change with time? Many of these questions can be addressed through fluorescence microscopy of chemically preserved dead (i.e., fixed) cells.

Fluorescence microscopy of fixed cells uses chemically modified antibodies to indirectly visualize molecules of interest. This process is generally called immunofluorescence, and although there are limitations to analyzing fixed cells, immunofluorescence is still a powerful method to investigate cellular organization. This is because immunofluorescence does not require genome engineering, over-expression, or molecular cloning, which is required to visualize molecules of interest in live cells (e.g., GFP-tagged genes). Instead, the naturally expressed molecule of interest can be labeled via a rich repertoire of commercially available antibodies that bind to their target with high affinity.

From a training standpoint, immunofluorescence microscopy is a single technique that can be used to teach all facets of STEAM (**S**cience, **T**echnology, **E**ngineering, **A**rt, and **M**ath): the biology of cell structure and organization; the chemistry of detergents, fixation, and binding reactions; the physics of light and optics; the technology and engineering of modern automated research grade microscopes; and both the artistic quality and mathematical basis of digital images (Prameela et al., 2020; Lindsay, 2021).

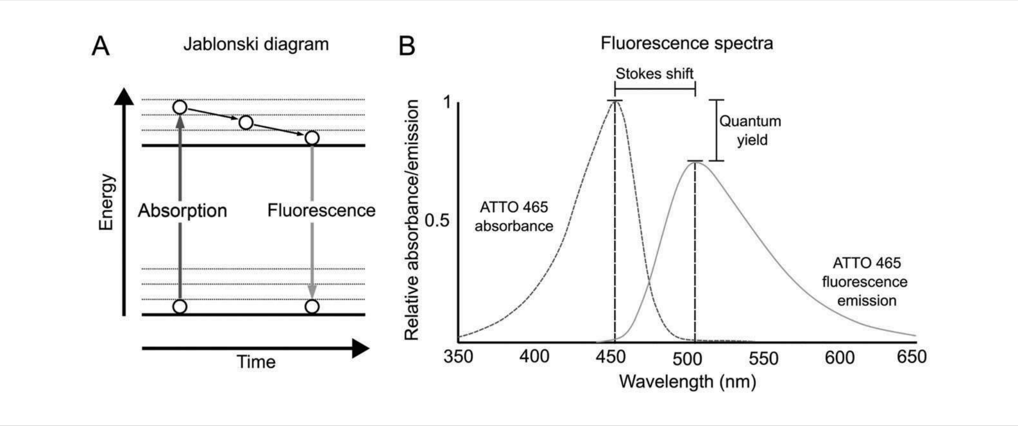

Figure 2 Basic properties of fluorescent molecules. (**A**) A simplified Jablonski-style diagram that shows the energy (y-axis) and time (x-axis)-dependent nature of fluorescence. A high energy photon (blue) is absorbed by the fluorophore, which raises an electron to a higher energy (excited) state. The excited electron dissipates energy through vibration and emits a lower energy photon (green) as it returns to the ground state. B) Absorbance and emission spectra for the commercial fluorophore Atto 465, which absorbs ~450 nm photons (blue dashed line) and emits ~500 nm photons (green dashed line) with the highest efficiency. The separation between peak absorbance (e.g., excitation) and peak emission is called the Stokes shift, which is ~50 nm for Atto 465. The ratio of absorbance to emission is the quantum yield of the fluorophore, which relates to the probability that the absorption of an excitation photon will lead to generation of an emission photon.

PRINCIPLES

Immunofluorescence microscopy requires a basic understanding of antibodies (i.e., "immuno"), fluorescence, microscopy, and the chemistry that enables antibody binding to the sample of interest. With a firm grasp of these basic principles, the investigator can apply the technique of immunofluorescence microscopy to understand cell structure, function, and dynamics. This section covers the basic principles and terminology associated with immunofluorescence microscopy. References that offer more detailed discussion of these basic principles are provided.

Fluorescence Microscopy Basics

Biological microscopy is generally divided into two complementary approaches: transmitted light microscopy and fluorescence microscopy. Transmitted light methods include brightfield, phase contrast, and differential interference contrast (DIC). These techniques capitalize on the fact that cellular structures alter the phase, polarization, and/or angle of light that is transmitted through a specimen (Ernst Keller & Watkins, 2013). Fluorescence microscopy is an umbrella term that applies to an expanding suite of methods that rely on labeling cells with molecules called fluorophores (Combs & Shroff, 2017). Fluorophores absorb light of one wavelength (the excitation light) and emit light of a different wavelength (the emission light). Fluorescence microscopy separates the emission light away from the excitation light to generate an image of the fluorophores on a dark background. This section covers the basic principles of fluorescence, how excitation and emission light are generated and separated, the basic principles of magnification and resolution, and the major fluorescence microscopy modalities. Subsequent sections describe how antibodies can be used to localize fluorophores to discrete cellular locations. For a more thorough discussion of all facets of biological microscopy, the reader is referred to (Inoue & Spring, 1997).

Fluorescence

Fluorescence is a photophysical process that involves the absorption and emission of photons by a subset of molecules called fluorophores. The events that underly fluorescence are often explained through a stepwise Jablonski diagram (Fig. 2A):

1) the fluorophore **absorbs** short-wavelength, high-energy **excitation** photons.
2) the high-energy excitation photons raise electrons from their ground state to an **excited** state.
3) the excited electrons dissipate a fraction of their energy through **vibration** (a non-radiative transition).
4) the excited electrons **emit** a lower energy **emission** photon as they return to their ground state (a radiative transition).

The energy lost through non-radiative vibration and radiative photon emission is equal to the energy of photon absorption. Therefore, the emission photon is generally a longer wavelength and lower energy than the excitation photon, which is called the **Stokes shift**. Fluorophores are typically characterized by their Stokes shift, quantum yield, excitation spectrum, emission spectrum, and fluorescence lifetime (Fig. 2B).

Illumination

Samples must be evenly illuminated with excitation photons of the appropriate wavelength. Even illumination is achieved using the Köhler principle, which produces a defocused (i.e., even) image of the light source on the specimen (Cole, 2016). Most conventional fluorescence microscopes achieve Köhler illumination through an epi-illumination configuration. In an epi-configuration, excitation light passes through the objective to the specimen, and emission light returns through the same objective to form an image of the sample. Early fluorescence microscopes utilized mercury lamps for illumination. Contemporary fluorescence microscopes utilize metal halide lamps, fast-switching light-emitting diodes (LEDs), or lasers tuned to specific wavelengths, which have numerous advantages over mercury lamps.

Separating excitation photons and emission photons

During fluorescence microscopy, excitation photons must be separated from the emission photons before reaching the image plane. This separation is typically achieved by a filter cube that contains an excitation filter, a dichroic beam-splitting mirror, and an emission filter. To understand how the filter cube works, it is easiest to describe the general light path of an epifluorescence microscope (Fig. 3):

1) The illumination source produces excitation photons across a range of the visible spectrum. The range of photons that are produced depends on the illumination source.
2) Excitation photons pass through an **excitation filter** to ensure that excitation photons of the correct wavelength reach the specimen plane. Photons whose wavelengths are too short or too long will be blocked by the excitation filter.
3) Filtered excitation photons reflect off a **dichroic beam-splitting mirror** and travel through the microscope objective toward the sample plane to stimulate the production of emission photons.
4) Emission photons travel through the microscope objective and pass through the dichroic beam-splitting mirror.

   This step relies on a special property of dichroic beam-splitting mirrors. Specifically, these mirrors reflect light below a specified wavelength and transmit (or pass) light of higher wavelengths. For example, a typical dichroic beam-splitting mirror may reflect blue excitation photons and transmit green emission photons.

5) Emission photons pass through an **emission filter**, which blocks any contaminating excitation photons from reaching the image plane.
6) If a sample is labeled with multiple different fluorophores, different filter cubes can be sequentially rotated into the optical path to capture images of each fluorophore. In this case, fluorophores need to be chosen carefully to ensure there is minimal overlap between their excitation and emission spectra.

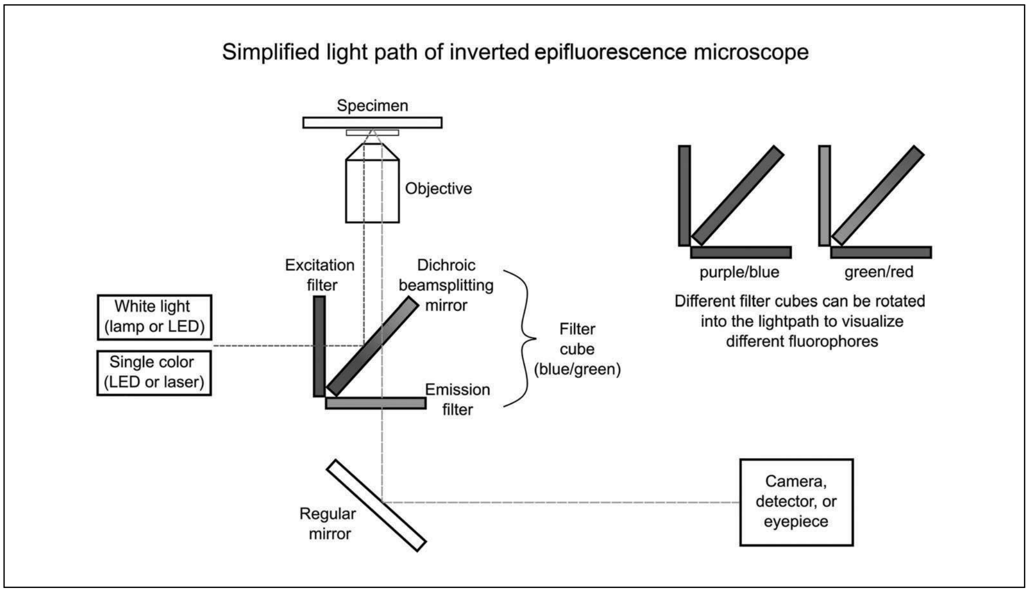

Figure 3 Simplified light path through an inverted epifluorescence microscope. Light produced by the illumination source (blue dashed line) passes through an excitation filter (blue rectangle) and is reflected at a 90-degree angle by the dichroic beam splitting mirror (blue-green tilted rectangle). The objective "defocuses" the excitation light at the specimen plane. Emission photons produced at the specimen plane are collected by the objective, which directs the emission photons through the dichroic beam splitting mirror and emission filter (green rectangle). Typically, a regular mirror reflects the emission light towards the eyepiece or digital detector.

Microscope objectives: numerical aperture, correction, and magnification

The quality of a microscopy image depends upon the objective that is used to form the image. Although most microscope objectives have a long list of specifications, the three most critical to image quality are the **numerical aperture** (**NA**), **magnification**, and the **correction** factors that are built into the objective (Fig. 4A). These critically important specifications are typically stamped onto the metal barrel of each microscope objective.

Numerical aperture

The NA is a singular property that determines the overall resolving power of the objective. The NA of an objective is defined by the equation:

(1) $\mathrm{NA} = \eta \sin \alpha$

where:

(2) η = refractive index = velocity of light in a vacuum/velocity of light in a medium
(3) α = the half-angle of light captured by the objective.

The resolving power (R) of a microscope objective is defined by the equation:

(4) $R = (0.61\lambda)/\mathrm{NA}$

where:

(5) λ = the wavelength of light being collected by the objective.

In essence, the above formulas dictate that the highest-resolution images will be formed when imaging short-wavelength light using objectives that have a short working distance (which increases α) and high refractive index materials (such as immersion oil) between the objective and the sample (which increases η). Indeed, the highest resolution objectives available have working distances of ~130 µm and are designed to be used with

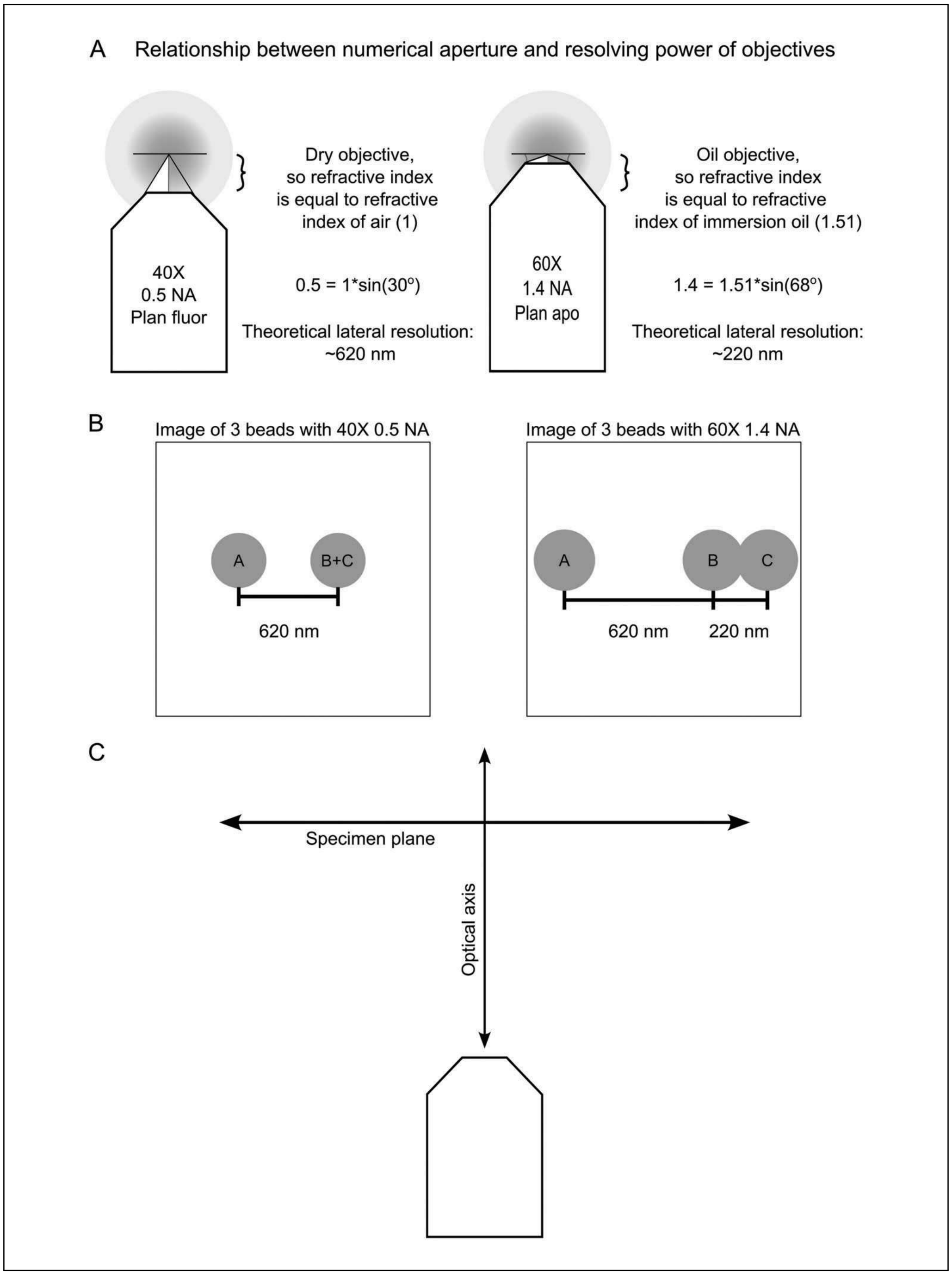

Figure 4 The numerical aperture of an objective determines resolution. (**A**) On the left is a 40× magnification objective with a numerical aperture of 0.5 and plan fluor correction. This is a “dry” objective with a longer working distance, which means that the space between the objective front lens and the specimen is filled with air (refractive index =1) and the objective can capture a maximum of 60° of light (30° half angle shaded white). On the right is a 60× magnification objective with a numerical aperture of 1.4 and plan apo correction. This is an “oil” objective with a shorter working distance, which means that the space between the objective front lens and the specimen is filled with oil (refractive index =1.51) and the objective can capture a maximum of 134° of light (68° half angle shaded white). Notice that the theoretical resolution of the 60× objective is 3 times greater than the 40× objective, but the magnification is only 1.5 times greater. (**B**) A simplified diagram showing what one would expect when using either objective to capture an image of three fluorescent beads. Bead A is separated from Bead B by 620 nm and Bead B is separated from Bead C by 220 nm. The higher numerical aperture, not the higher magnification, allows Bead B to resolved from Bead C. (**C**) A schematic that shows the specimen plane perpendicular to the optical axis. Field curvature aberrations lie in the specimen plane. Spherical and chromatic aberrations lie along the optical axis.

immersion oils that have refractive indices of ~1.51. When imaging visible light, these objectives can achieve a resolution of ~220 nm, which means that two fluorescent objects will appear separate if they are greater than 220 nm away from one another (Fig. 4B).

Correction

Microscope objectives have an optical axis that is perpendicular to the specimen plane and passes directly through the center of the objective (Fig. 4C). Since lenses are curved, light that passes along the optical axis will have a different light path than light that passes through the objective away from the optical axis. Similarly, since the focal point of light that passes through a lens depends on wavelength, light of different wavelengths will come to a focus at slightly different places. Three major consequences arise due to this unavoidable property of lenses.

1) Image aberrations increase with increasing distance from the center of the field of view. This leads to **field curvature aberrations** whereby image distortion increases at the periphery of the field of view.
2) Image aberrations are often more severe on one side of the focal plane than the other. This leads to asymmetric image distortion along the optical axis, which is called **spherical aberration**.
3) Different wavelengths of light will come to focus at different distances along the optical axis, leading to color-dependent differences in the focal plane for multicolor images, known as **chromatic aberration**.

Many microscope objectives correct for these consequences by incorporating additional lens elements into the barrel of the objective. The most common fluorescence microscopy corrections include:

1) **Plan** correction to minimize field curvature.
2) **Achromatic** correction to minimize spherical aberration and chromatic aberration for one or two wavelengths of light.
3) **Fluorite** correction to minimize spherical aberration and chromatic aberration for two to four wavelengths of light.
4) **Apochromatic** correction to minimize spherical aberration and chromatic aberration for three to five wavelengths of light.

In other words, a **Plan Apochromatic** objective will achieve the highest level of field curvature, spherical, and chromatic aberration correction for up to five wavelengths of light. In contrast, a **Plan** objective will correct for field curvature aberration, but will suffer from spherical and chromatic aberration across the visible spectrum.

Magnification

The magnification (M) of an objective is defined as the extent to which images produced by the objective appear larger than they are. The useful range of magnification is 500 to 1000 times the NA. The brightness of an image decreases as the magnification increases. In fluorescence microscopy, the overall brightness is proportional to NA^4/M^2. Thus, the brightest fluorescence imaging will be accomplished by using an objective with the highest NA and the lowest magnification. However, as described in the Strategic Planning section, the ideal objective for a particular immunofluorescence experiment depends upon the question that is being asked.

Widefield, confocal, and super-resolution fluorescence microscopy

Regardless of the objective used, there are three general methods of fluorescence microscopy for immunofluorescence experiments: widefield, confocal, and super-resolution microscopy.

1) Widefield microscopy excites and captures emission from the entire field of view. Widefield microscopy generally uses lamps or LEDs as an excitation source and digital cameras to capture an image of the emission. Although relatively simple and cheap, widefield microscopy does suffer from blur. Specifically, the image plane contains information from the specimen plane that is in focus (which is good), but it also contains information that originates from out-of-focus specimen planes at different distances along the optical axis (which is bad).
2) Confocal microscopy excites and captures emission sequentially from individual spots throughout the field of view. Confocal microscopy generally uses high-intensity lasers as an excitation source and either a digital camera (spinning disk confocal microscopy (SDCM)) or a photomultiplier tube (laser scanning confocal microscopy (LSCM)) to create an image of the emission. The major advantage of confocal microscopy is that "out-of-focus" information is blocked prior to reaching the detector, so the image does not suffer from blur (Elliott, 2020).
    a. SDCM creates spots by passing a column of excitation light through a rotating disk that contains pinholes. In-focus emission light than passes back through the pinholes to the camera, while out of focus emission light is blocked by the non-pinhole portion of the disk. Multiple "spotty" images are added together to create a final image constructed from all the spots.
    b. LSCM creates spots by using mirrors to scan a focused laser beam point by point across the image. In-focus emission light then passes back through a pinhole in the center of a non-rotating disk, while out-of-focus emission light is blocked by the disk.
3) Although confocal microscopy can reduce blur, the fundamental limit to resolution is still dictated by the NA of the objective, the refractive index of medium between the objective and the sample, and the wavelength of the light being imaged. Despite these constraints, the new "super-resolution" techniques overcome this fundamental problem (Kasuboski et al., 2012). The major super-resolution techniques are:
    a. PALM/STORM (**P**hoto **a**ctivation **L**ight **M**icroscopy/ **S**tochastic **O**ptical **R**econstruction **M**icroscopy) creates a super-resolution image by sequentially imaging individual fluorophores that are spatially separated from one another. This method can achieve a resolution of 20-50 nm but requires special fluorophores, and it takes a relatively long time to capture a complete image (Xu et al., 2017).
    b. SIM (**S**tructured **I**llumination **M**icroscopy) uses spatially patterned illumination widefield fluorescence microscopy combined with image reconstruction to reveal higher-resolution features in an image. This method can double the resolution of a widefield microscope and is relatively quick (Gustafsson, 2000).
    c. STED (**St**imulated **E**mission **D**epletion) microscopy uses LSCM with the addition of a depletion laser that reduces the size of the confocal spot. This method can also achieve a resolution of 20-50 nm but requires special fluorophores and generally high laser power (Sahl & Hell, 2019).
    d. SRRF (**S**uper- **r**esolution **R**adial **F**luctuations) microscopy is like PALM/STORM in that it requires taking sequential images of the same field of view. However, SRRF is a purely computational approach that does not require any special optical components or fluorophores, which makes it accessible to a wider range of laboratories (Gustafsson et al., 2016).
    e. Expansion microscopy is a super-resolution technique that does not require optical or computational tools. Rather, expansion microscopy relies on infiltrating specimens with polymers that expand in a controlled isotropic fashion. This allows the specimen to be enlarged and imaged with a regular

microscope. For example, a 2-fold specimen expansion can theoretically lead to a 2-fold increase in resolution (Zhang et al., 2020).

Antibody Basics

Immunofluorescence is an antibody-mediated process that can be used to detect the presence, quantity, and subcellular location of an antigen. Immunofluorescence is generally categorized as direct or indirect. During direct immunofluorescence, a fluorophore-conjugated primary antibody binds to a protein of interest. During indirect immunofluorescence, a primary antibody binds to a protein of interest, and a secondary antibody that is conjugated to a fluorophore binds to the primary antibody. Therefore, generating antibodies that have high binding affinity (e.g., binds tightly) and specificity (e.g., binds tightly only to its target) is fundamental to all immunofluorescence experiments. This section covers the basic process by which antibodies are generated, the basic structure of common antibodies, and emerging antibody tools that are broadening immunofluorescence approaches. For a more thorough discussion of all facets of antibody production and uses, the reader is referred to (Greenfield, 2013).

Primer on the vertebrate immune response

Antibodies are proteins that are produced by the immune systems of all vertebrates (from sharks to humans). To understand how antibodies are used for indirect immunofluorescence, it is necessary to summarize how the vertebrate immune system produces primary antibodies that bind to a protein of interest (Fig. 5A) or secondary antibodies that bind to primary antibodies (Fig. 5B).

1) A protein of interest (either full length or a fragment) is injected into the bloodstream of a vertebrate animal called the **host**. The host bloodstream generally contains red blood cells, white blood cells, and protein-rich serum.
2) If the host recognizes the protein as a non-self, there is a humoral immune response to neutralize it. The non-self protein is now called an **antigen** because it caused an immune response.
3) The humoral immune response generates several clonal B cell populations. Each B cell clone secretes many copies of a single antibody (also called an immunoglobulin) that have high affinity for a single epitope (generally several amino acids) on the antigen.
4) The bloodstream of the host animal now contains a polyclonal population of B cells and serum with antibodies that bind to several epitopes on the antigen (Fig. 6).
    a. If the serum is separated from the blood cells it can be used as a **polyclonal antiserum**. It is called polyclonal because the antiserum contains a mixture of antibodies from multiple B cell clones that bind to various epitopes on the protein of interest (Fig. 6A).
    b. If an individual clone of B cells is isolated and immortalized as a hybridoma, then the clone can be used to produce a **monoclonal antiserum.** It is called monoclonal because the antiserum contains a single population of antibodies that bind to a single epitope on the protein of interest (Fig. 6B).

Antibody structure

Antibodies are Y-shaped heterotetramers composed of two identical heavy chains and two identical light chains (Fig. 7A). Each light chain (L) has two domains: a variable region (V_L) and a constant (C_L) region. Each heavy chain (H) has up to five domains: a variable region (V_H) and up to four constant regions ($C_{H1\text{-}4}$). The variable regions (V_L and V_H) form the antigen-binding sites. The antibody fragment that contains V_L, V_H, C_L, and C_{H1} is called the “ **f**ragment that is **a**ntigen **b**inding (Fab’)”. Constant regions C_{H2}-$C_{H3/4}$ dimerize to create the “ **f**ragment that is **c**rystallizable (Fc)”. There is mirror symmetry across the vertical plane of the Y-shaped antibody. Therefore, antibodies have a single Fc domain and a variable Fab domain that has two identical antigen binding sites.

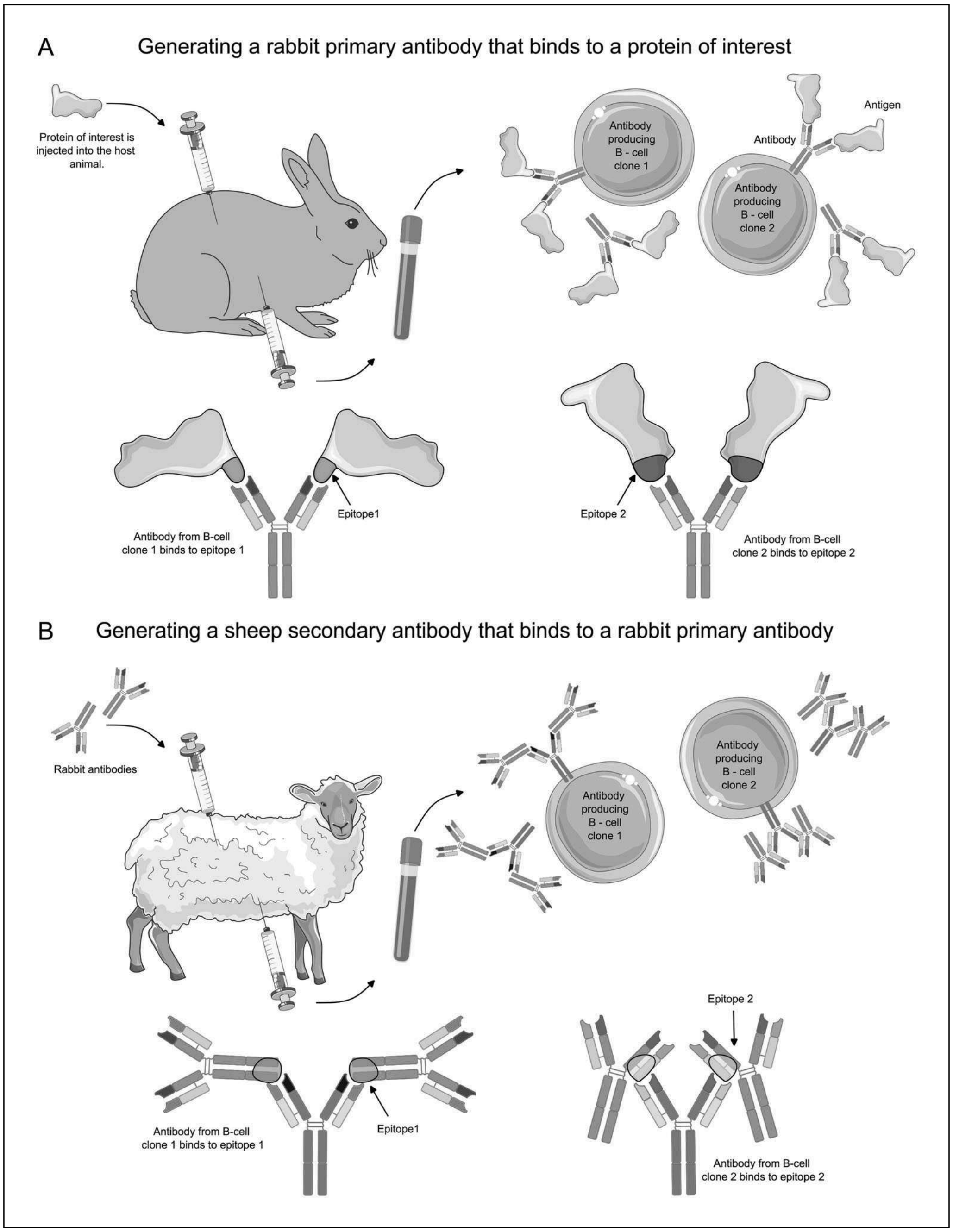

Figure 5 Generating primary and secondary antibodies. (**A**) To generate rabbit primary antibodies against protein of interest (yellow), the protein of interest is injected into a rabbit. The rabbit mounts an immune response against the protein of interest (now called an antigen). This leads to the production of B-cell clones that produce antibodies that bind to specific epitopes on the antigen. (**B**) To generate sheep secondary antibodies, rabbit antibodies are injected into a different species (in this case sheep). The sheep mounts an immune response against the rabbit antibodies. This leads to the production of B-cell clones that produce sheep antibodies that bind to rabbit antibodies. The vector graphics in this figure were generated with the open source repository BioIcons (*www.bioicons.com*).

Antibody isotypes and subtypes

There are five immunoglobulin isotypes (IgM, IgG, IgA, IgE, and IgD) that serve specific functions in the host immune response (Fig. 7B). The constant region of the heavy chain generally determines the isotype.

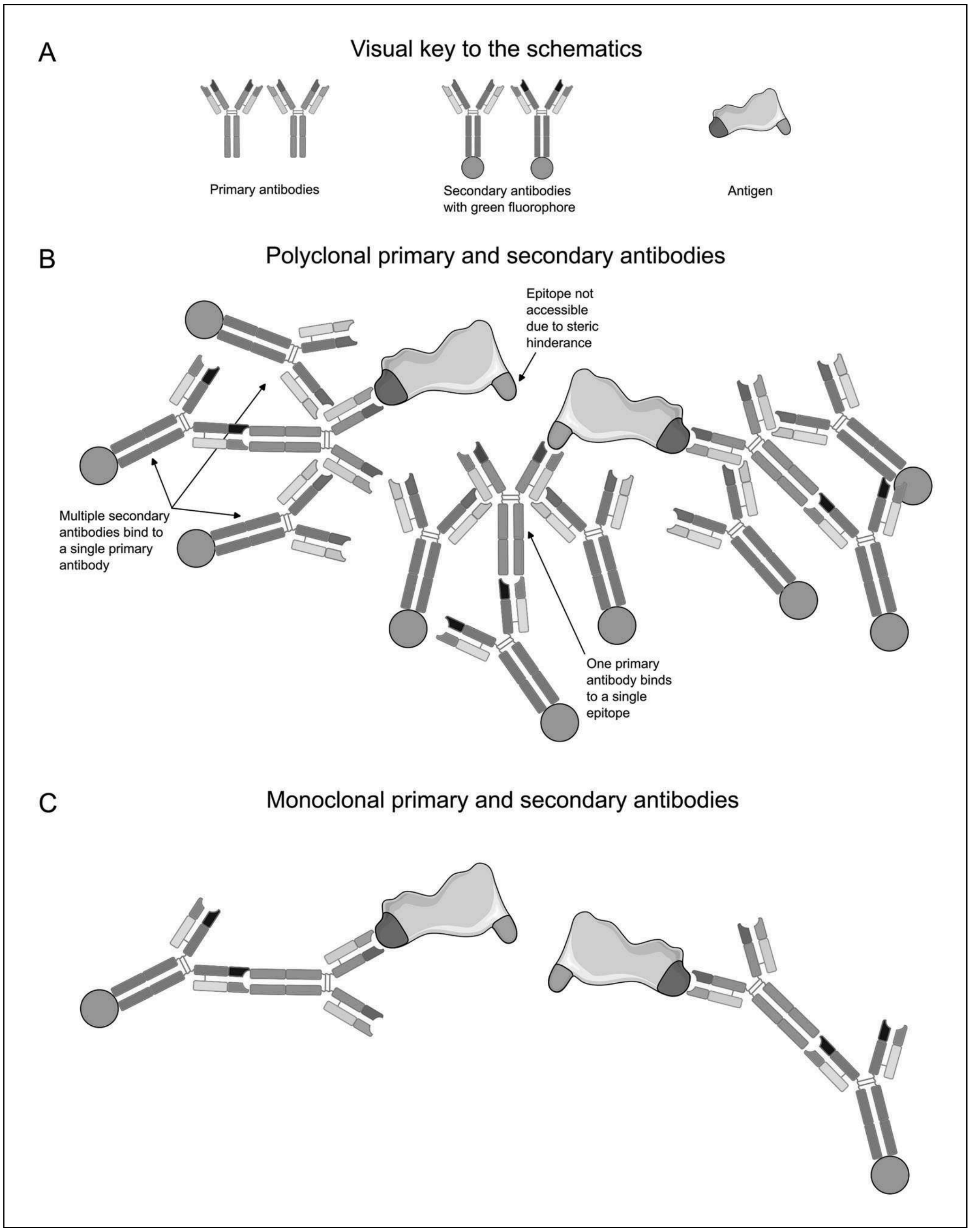

Figure 6 Polyclonal antibodies can produce a stronger fluorescence signal than monoclonal antibodies. (**A**) When polyclonal antibodies are used in an immunofluorescence experiment, each antigen can bind multiple primary antibodies and each primary antibody can bind multiple secondary antibodies. (**B**) When monoclonal antibodies are used, each antigen can bind to a single antibody. This generally reduces the overall signal relative to polyclonal antibodies. The vector graphics in this figure were generated with the open source repository BioIcons (*www.bioicons.com*).

1) IgG is usually the major class that is present in serum. IgG is a single tetramer with two antigen-binding sites.
2) IgM is the major cell surface antigen receptor and plays a key role in the early stages of the humoral immune response. IgM is pentamer of tetramers with ten antigen-binding sites.
3) IgA is present in secretions and mucus membranes. IgA exists as a dimer of tetramers with four antigen-binding sites.
4) IgE is the antibody that triggers mast cells to dump histamines in the allergic response. IgE is a single tetramer with two antigen-binding sites.

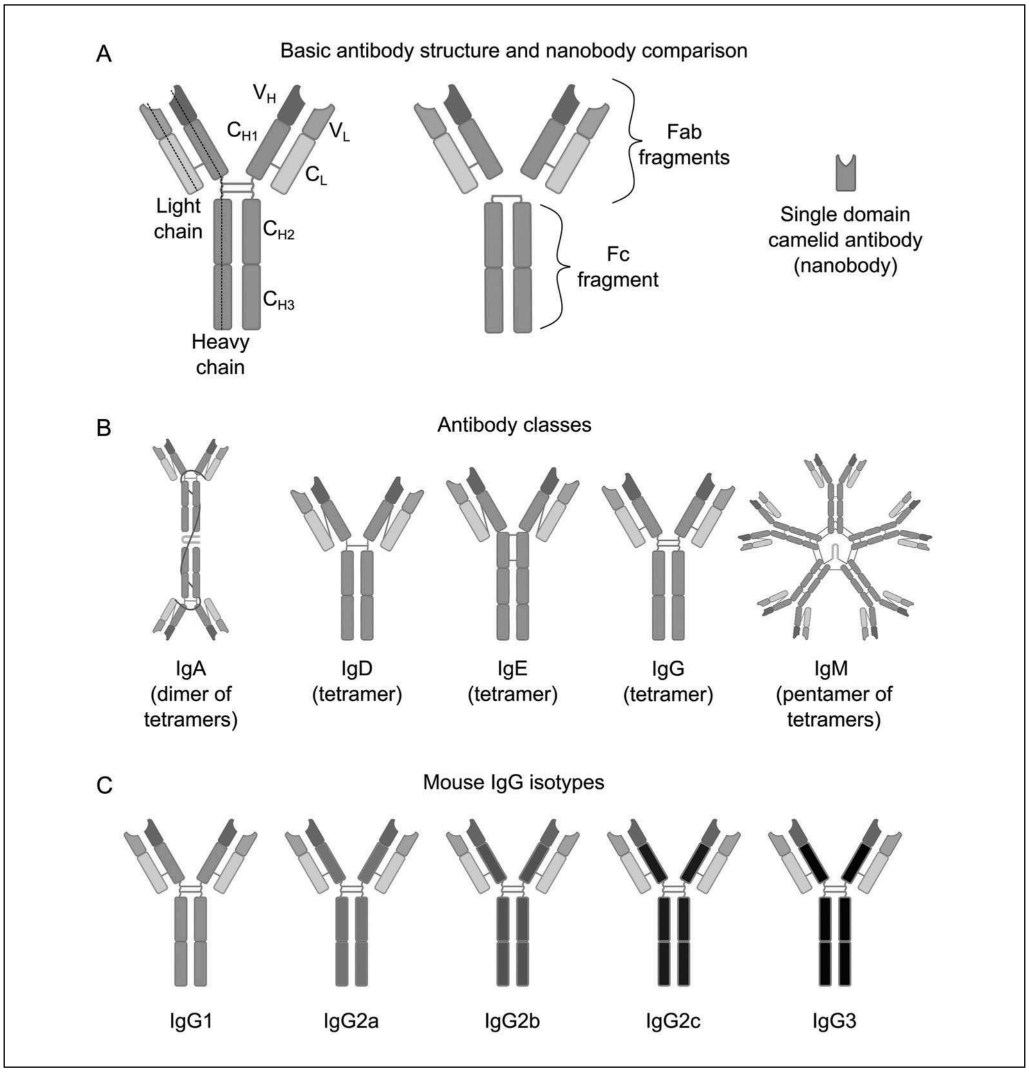

Figure 7 Polyclonal antibodies can produce a stronger fluorescence signal than monoclonal antibodies. (**A**) When polyclonal antibodies are used in an immunofluorescence experiment each antigen can bind multiple primary antibodies and each primary antibody can bind multiple secondary antibodies. (**B**) When monoclonal antibodies are used, each antigen can bind to a single antibody. This generally reduces the overall signal relative to polyclonal antibodies. The vector graphics in this figure were generated with the open source repository BioIcons (*www.bioicons.com*).

5) IgD mediates the hyperimmune response that amplifies production of IgG molecules when an animal is repeatedly exposed to antigen. IgD exists as a single tetramer with two antigen-binding sites.

For some host species, the isotypes can be further classified into subtypes. In mice, the IgG isotype has IgG1, IgG2a, IgG2b, IgG2c, and IgG3 subtypes (Fig. 7C). In humans, The IgG isotype has IgG1, IgG2, IgG3, and IgG4. In general, the various IgG isotypes for a particular species all have the basic IgG structure, but they have subtle differences in their constant regions. These subtle differences can be advantageous when designing multi-color immunofluorescence experiments.

Antibody diversity is necessary to neutralize the nearly infinite number of pathogens that can infect animals. In immunofluorescence, antibody diversity within and across host species enables elegant multi-color immunofluorescence experiments that can reveal the presence, quantity, and subcellular location of many proteins of interest at the same time. Practical guidelines for choosing antibodies to maximize signal and minimize interference between antibodies is discussed in the Strategic Planning section.

Emerging antibody tools in cell biology

Traditional antibody reagents for cell biology have been generated in a few common species and isolated using a few common techniques. However, new antibody species and more sophisticated production methods are gaining traction in cell biology. Some emerging antibody tools and the motivations for using them are briefly summarized below.

Nanobodies

Antibodies from common host species are relatively large, with an average molecular weight of ~150 kDa and an average hydrodynamic radius of ~40 nm. Large antibodies present two challenges to immunofluorescence. First, they do not easily penetrate thick cells and tissues. Second, the combined length of primary and secondary antibodies will alter the apparent location of a protein when visualized with super-resolution fluorescence microscopy methods, such as STED and PALM/STORM. Some of these issues can be overcome using individual Fab fragments or single scFv fragments, which are cleavage products of traditional antibodies. However, the preparation of these fragments is time consuming and costly.

One alternative to Fab and scFv fragments are antibodies from camelid species that produce highly specific antibodies from a single variable domain that is only ~13 kDa and 2 nm in length. These camelid antibodies are called "nanobodies", and they are substantially less massive and shorter than scFv fragments (~30 kDa/3 nm), Fab' fragments (~48 kDa/3 nm), and full-length antibodies (~150 kDa/40 nm). In addition, nanobodies are less prone to aggregation than traditional antibodies and they can even be expressed within nonimmune cells, which allows immunofluorescence to be performed in live cells. Thus, nanobodies represent an emerging tool that has several key advantages over antibodies from traditional host species (Gavira-O'Neill et al., 2021).

Recombinant antibodies

Traditional antibody production methods rely on host animals and/or immortalized hybridoma cell cultures. These production methods have several limitations. First, animals and cells change over time and so there can be time-dependent variability in antibody preparations. Second, it is difficult to control the isotype and subtype of antibodies naturally produced in animals. Third, the generation of polyclonal and monoclonal antibodies requires the repeated injection of animals, which can harm them.

Recombinant antibodies and nanobodies overcome these limitations. Specifically, once a useful antibody is generated, its gene sequence can be recovered from the immune cells that made it. This allows the cloned antibody gene to be expressed indefinitely in bacteria, yeast, or human cell cultures without the need for further manipulation of the animal. Moreover, the regions of the antibody gene that encode the antigen-binding domains can be spliced to Fc regions from different isotypes, subtypes, or even different species. Collectively, these advantages allow recombinant antibodies to be a stable source of high-quality antibodies that can be easily manipulated for different experimental purposes (Trimmer, 2020).

STRATEGIC PLANNING

Key Decisions when Designing Immunofluorescence Experiments

The goal of an immunofluorescence experiment is to label proteins of interest in their native state with a high-specificity fluorescent signal while achieving low nonspecific background. Since all samples are unique, and so are the antibodies used to stain them, many factors must be considered when establishing an immunofluorescence protocol. This section describes some of the key decisions that must be made when embarking on an immunofluorescence experiment.

Preparing your source material

There are a variety of samples that can be stained via immunofluorescence. The most common samples are whole animals (usually embryos), embryonic or adult tissues, and cultured cells. It is important to understand the nature of your sample and how you will prepare it for the immunofluorescence workflow and microscopy. In general, whole animals can be stained as free-floating specimens, tissues can be cut into thin sections and stained as free-floating specimens, adherent cells can be grown directly on glass coverslips, and suspension cells can be stained in suspension and deposited onto glass coverslips at some point during the staining process.

Coverslips are usually either round or square and come in a variety of sizes. In general, coverslip size can be matched to the culturing vessel. For example, 12-mm diameter round coverslips fit nicely within the wells of a 24-well culture dish, while 35-mm diameter round coverslips can be used with 6-well culture dishes. Coverslips generally come in four thicknesses (0 (100 μm), 1 (150 μm), 1.5 (170 μm), and 2 (220 μm)). When choosing a coverslip thickness, it is important to consider the microscope and objectives that will be used to image the samples. Most objectives are corrected to be used with a specific thickness, which is usually stamped on the barrel of the objective.

Fixation

Fixation “freezes cells in time” by simultaneously stopping cell processes and preserving the position of macromolecules. There are many chemicals that can serve as a “fixative”, and each chemical has advantages and disadvantages. The most common fixation methods include aldehyde crosslinking and precipitation with alcohols or ketones.

1) Aldehydes covalently crosslink primary amines on neighboring proteins. Two common aldehyde fixatives are glutaraldehyde and paraformaldehyde. Glutaraldehyde provides stronger crosslinking than paraformaldehyde, but penetrates cells more slowly. To achieve a balance of crosslinking strength and speed, one may combine glutaraldehyde and paraformaldehyde.
2) Some alcohols and ketones, such as methanol and acetone, can simultaneously precipitate and denature proteins while extracting lipids, which can help to expose hidden epitopes. Compared to aldehyde fixation, methanol and acetone fixations are rapid but tend to dehydrate and shrink cells.

Permeabilization

Permeabilization removes the plasma membrane and organelle membranes depending on the permeabilization strategy that is used. Permeabilization is typically achieved with nonionic detergents (e.g., Triton X-100 or digitonin), alcohols (e.g., methanol), or ketones (e.g., acetone). In the case of methanol and acetone, fixation and permeabilization proceeds simultaneously. Regardless of the permeabilization method, it is important to optimize the duration of permeabilization, the concentration of the permeabilization detergent, and when permeabilization is achieved.

1) How long and what concentration of detergent? Longer permeabilization time with a higher concentration of detergent will increasingly solubilize and denature weakly fixed proteins. This can diminish the presence of certain antigens and expose hidden epitopes. Alternatively, insufficient permeabilization will limit antibody access to the inside of the cell, which can reduce the detection of intracellular antigens.
2) Methanol extraction after aldehyde fixation? In some cases, methanol extraction can be performed after aldehyde fixation. This can denature fixed proteins and extract lipids without the need for additional detergents.

3) When to permeabilize? Specimens can be permeabilized before, during, or after fixation. Some staining protocols even skip permeabilization altogether. Some considerations for choosing when to permeabilize are:

   a. Permeabilizing before or during fixation is beneficial if soluble antigen masks the signal of antigen that is incorporated into more stable structures. For example, this is the case when a cell is filled with soluble tubulin dimers and polymerized microtubules. If the sample is permeabilized before it is completely "fixed", then tubulin dimers have time to diffuse out of the cell while microtubule polymers are fixed in place.
   b. Permeabilizing after fixation is beneficial if detergent disrupts the location of antigen in live cells. For example, this is the case when microtubule motor proteins are bound to microtubules. If detergent is added before or during fixation, the detergent can disrupt interactions between motor proteins and microtubules. In this case it may be beneficial to fix the motor proteins to the microtubules first and then to permeabilize.
   c. Skipping permeabilization altogether is beneficial if the antigen of interest is on the extracellular surface. For example, this is the case when trying to specifically label the population of receptors at the cell surface instead of the population that is trafficking through the secretory pathway. If permeabilization is skipped, antibody will only have access to the surface population.

Considering the combined effect of fixation and permeabilization

The binding of antibodies to antigens is an equilibrium reaction. Since fixation and permeabilization alter the abundance and chemical structure of antigens, fixation and permeabilization will impact the antibody/antigen equilibrium. Therefore, one must determine the optimal fixation and permeabilization conditions for each new antibody. For example, some antibodies react only with antigen that has been fixed with glutaraldehyde. Other antibodies react best when the antigen is denatured. Some antibodies—especially antibodies raised against a short peptide located in the middle of the protein sequence—react well on immunoblots of SDS-denatured proteins but do not bind their antigens in fixed cells because the epitope is hidden in the tertiary structure of the aldehyde-treated protein. While optimizing a protocol, it is important to remember that the apparent location of an antigen in an immunofluorescence experiment can be drastically influenced by the fixation and permeabilization conditions (Melan & Sluder, 1992).

Blocking

Regardless of how samples are fixed and permeabilized, samples contain many sites that can bind nonspecifically to primary and secondary antibodies. If these nonspecific binding sites are exposed when primary or secondary antibodies are added, they will nonspecifically bind to the antibodies and increase background. Therefore, it is important to "block" nonspecific binding sites by incubating samples with a blocking agent, such as bovine serum albumin (BSA) or normal nonimmune serum from the host species that made the secondary antibodies. These blocking agents contain an abundant source of serum proteins that will saturate the nonspecific binding sites without interfering with subsequent antibody/antigen interactions.

Choosing primary and secondary antibodies

The primary antibodies chosen for an immunofluorescence experiment depend on the experimental question being asked. The choice of secondary antibodies depends on the primary antibodies that are used. When there is flexibility in antibody choice, it is important to consider the host species, whether the antibody is monoclonal or polyclonal, and the antibody isotype and subtype.

1) Host species? The host species of the antibody is critical when staining multiple targets with multiple primary antibodies. For example, if one wishes to stain microtubules with a mouse primary antibody, they may want to choose a rabbit primary antibody to stain an organelle. This would allow one to use an anti-mouse secondary antibody with a red dye and an anti-rabbit secondary antibody with a green dye.
2) Monoclonal or polyclonal? Monoclonal antibodies all bind the same epitope with precisely the same affinity. This can be advantageous when localizing a single epitope, such as a posttranslational modification of a protein (e.g., acetylated tubulin). However, monoclonal antibodies may cross-react with other proteins that have similar epitopes, and it may be difficult or impossible to eliminate this unwanted cross-reactivity. Polyclonal antibodies contain a mixture of antibodies that bind to several different epitopes all on the same antigen. Although one epitope may be found on a cross-reactive protein, it is unlikely that all the epitopes are found on the same cross-reactive protein. In some cases, this allows polyclonal antibodies to reduce the problem of cross-reactivity by averaging the cross-reactivity across many nonspecific proteins. A similar effect can be achieved by mixing several different monoclonal antibodies that bind to different epitopes on the same antigen.
3) Monoclonal antibody isotype and subtype? If monoclonal antibodies have different isotypes and/or subtypes, multiple targets can be stained with primary antibodies from the same species. For example, if a mouse monoclonal antibody with isotype IgG1 is used to stain microtubules, a mouse monoclonal antibody with isotype IgG2a could be used to stain an organelle. This would allow one to use an anti-mouse IgG1 secondary antibody with a red dye and an anti-mouse IgG2a secondary antibody with a green dye.

Choosing fluorescent dyes

Secondary antibodies can be covalently conjugated to a large range of bright and stable fluorescent dyes that span the UV-VIS-IR spectra. When choosing fluorescent dyes, the main consideration is the excitation and emission filters of the fluorescence microscope that will be used to image the samples. At a minimum, most research-grade fluorescence microscopes have “blue”, “green”, and “red” filter sets, which could be used to image DAPI (blue), Alexa 488 (green), and Alexa 594 (red). When imaging multiple fluorescent dyes, it is important to ensure that the dyes have spectrally distinct excitation and emission profiles. If the excitation and emission profiles overlap substantially it will lead to “crosstalk” and/or “bleed trough”. Crosstalk results when dyes have overlapping excitation spectra. For example, Alexa 488 and Alexa 514 will both be excited by 505 nm light, and so there would be crosstalk if these dyes were imaged on a microscope with a 505 nm excitation source. Bleed-through results when dyes have overlapping emission spectra. For example, Alexa 488 and Alexa 514 both emit light at 530 nm, and so there would be bleed-through if these dyes were imaged on a microscope with an emission filter that passes 530 nm light. By carefully matching the fluorescent dyes to the microscopy system, crosstalk and bleed-through can be minimized. It is also important to note that the human eye does not generally detect infrared light and so far-red dyes (such as Alexa 647) will not be observable through the eye piece although they can be captured with digital cameras. The internet resource *www.fpbase.org* provides an excellent overview of common fluorescent dyes and simple tools to evaluate their effectiveness for a particular microscopy setup.

Antibody specificity and important controls

The reliability of the information obtained by immunofluorescence microscopy is only as good as the specificity of the antibody. Regardless of whether an experiment utilizes custom antibodies or commercially available antibodies, there are several ways to evaluate antibody specificity. Some common strategies are described below.

1) Antibody concentration—The concentration of primary and secondary antibodies must be empirically determined in preliminary experiments. When antibodies are used at too high of a concentration, there can be high non-specific staining. Conversely, when antibodies are used at too low of a concentration, there can be low specific staining. Ideally, one begins a new immunofluorescent staining experiment by testing a range of dilutions of both the primary and secondary antibodies using the same fixation, permeabilization, and blocking conditions.
2) Immunoblotting and knockout verification—Even if a new antibody gives beautiful immunofluorescent signal, it does not mean that the antibody is specifically binding to the protein target of interest. Two common ways to determine if an antibody is specific are immunoblotting and knockout verification. If the antibody is specific, it should produce a single distinct band at the correct molecular weight when analyzed via SDS-PAGE immunoblotting. However, SDS-PAGE completely denatures proteins, so antibodies that work in immunofluorescence may not work in SDS-PAGE and vice versa. To circumvent this issue, one can stain "knockout" cells or tissue. If the antibody is specific, the immunofluorescence signal should disappear when used to stain samples that lack the protein target.
3) Preimmune and nonimmune serum—Unless antibodies are made recombinantly or extensively purified, they may be contaminated with other non-specific antibodies. To ensure that the immunofluorescent signal results from the specific antibody, samples should be stained with the appropriate nonspecific source material. For polyclonal antibodies isolated from host animals injected with antigen and monoclonal antibodies isolated from mouse ascites fluid, it is best to obtain serum from that animal prior to injecting the antigen (called preimmune serum). For monoclonal antibodies isolated from hybridoma cultures, it is best to obtain cell culture media used to grow the hybridomas. Although less ideal, one could also use serum from a different animal of the same species (called nonimmune serum) or similar cell culture media.
4) No primary antibody—Secondary antibodies can nonspecifically stain cellular structures. To determine if a secondary antibody is binding nonspecifically, one can omit the primary antibody, which should cause the fluorescent signal to disappear.
5) No secondary antibody—Some cells and tissues have strong autofluorescence even in the absence of secondary antibodies. To determine if a sample has autofluorescence, omit the secondary antibody. If the sample has strong fluorescence without secondary antibody, the signal can be attributed to autofluorescence.
6) Affinity purification and/or absorption of the antibody—If the antibody of interest is in a complex mixture, such as serum, the antibody can be purified using affinity chromatography with the antigen that was used to generate the antibody. If the antibody is specific, affinity purification of the antibody should not alter its staining pattern. Conversely, one should be able to eliminate the specific signal by pre-absorbing the antibody with excess antigen.

Supplementing immunofluorescence with fluorescent stains

In addition to antibody-mediated detection, many immunofluorescence experiments utilize non-antibody fluorescent dyes to stain intracellular structures. For example, DAPI is a blue, fluorescent molecule that intercalates into DNA thereby allowing nuclear staining without antibodies. Other fluorescent dyes can stain mitochondria, secretory organelles, plasma membrane, filamentous actin (phalloidin), and microtubules. The main consideration when choosing a non-antibody fluorescent dye is whether the dye is impacted by the fixation and permeabilization conditions of your experiment and whether the excitation and emission spectra of the dye are compatible with your microscope and the secondary antibodies used.

Choosing a microscopy method

Widefield and confocal fluorescence microscopy are the most common ways to image immunostained cells. Widefield fluorescence microscopy with a high numerical aperture objective is sufficient to generate high-contrast images of most cell cultures (which are typically less than 15 μm thick). If the samples are thick and/or if the biological question requires higher-contrast images, confocal microscopy is preferred. If the biological question requires super-resolution, it is important to consider that some techniques only work with certain dyes (e.g., STED and PALM/STORM) and some super-resolution methods can resolve the length of individual antibodies. For example, if you aim to perform STED microscopy, you should use secondary antibodies that are optimized for STED and you may want to consider using Fab fragments or nanobodies to avoid localization error due to the length of multiple IgG molecules.

SAFETY CONSIDERATIONS

The chemical fixatives used in these protocols (glutaraldehyde and formaldehyde) are extremely toxic and will damage exposed tissues, including eyes and airways. Always work with these chemicals in a properly exhausting fume hood and properly dispose of solutions. The high intensity of illuminating light (e.g., from a 100-W mercury lamp or a laser) will injure the retina if viewed directly.

CAUTION: Never look directly at the illuminating light source.

NOTE: All protocols involving animals must be reviewed and approved by the appropriate Animal Care and Use Committee and must follow regulations for the care and use of laboratory animals.

PROTOCOLS

The following protocols illustrate the methods of indirect immunofluorescence for staining two kinds of cells that have very different morphologies: mammalian fibroblasts (which are flat and grow on glass coverslips) and *Tetrahymena thermophila* cells (which are much thicker and grow in suspension). Our methods for staining microtubules with tubulin antibodies are described. Microtubules are very labile and are readily disassembled by cold temperature or calcium ions; therefore, the protocols illustrate the importance of controlling the conditions for fixation and permeabilization. Similar methods work for many other antigens, but the investigator will need to fine-tune the protocols for their particular antigen, cell type, and antibody.

BASIC PROTOCOL 1

IMMUNOFLUORESCENCE STAINING OF ADHERENT CELLS SUCH AS FIBROBLASTS

This protocol describes how to perform immunofluorescence labeling of microtubules in mammalian cells that have been grown on glass coverslips (e.g., adherent cells). The general process involves sequential incubation in fixative solution, permeabilizing solution, blocking solution, primary antibody that binds to the protein target of interest, and secondary antibody that binds to the primary antibody. Although this protocol is optimized for staining microtubules (Fig. 8, Table 1), it can be used to stain a variety of structures.

NOTE: The cold methanol step will prevent phalloidin from binding to actin filaments; methanol should be avoided if the investigator is using phalloidin as one of the cytoskeleton probes.

Materials

Mammalian fibroblasts: grown to ~50% confluence (typically one day, depending on initial cell density) on autoclaved no. 1, 22 mm × 22 mm glass coverslips

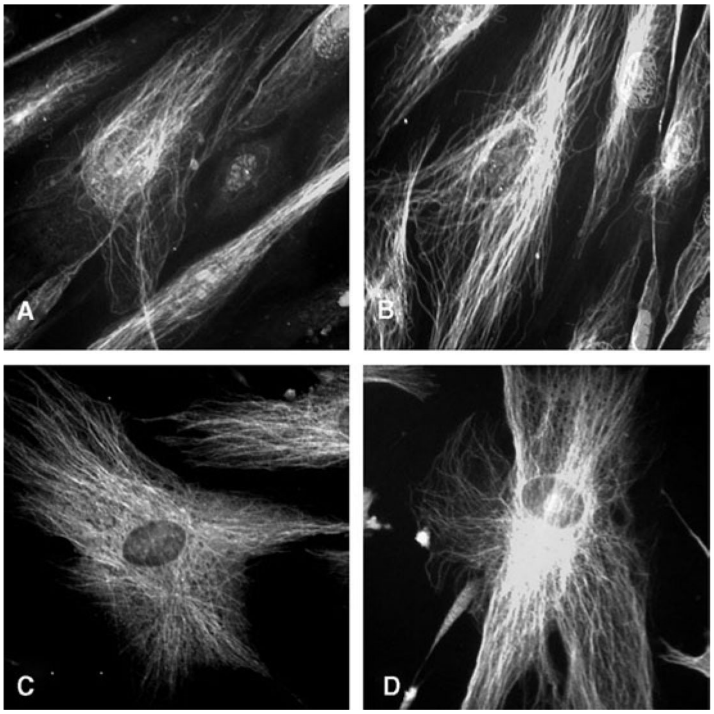

Figure 8 Microtubule arrays in cultured fibroblasts. Human corneal fibroblasts were grown on sterilized glass coverslips and processed for immunofluorescence microscopy. All slides were stained with a mouse monoclonal (IgM) antibody to β-tubulin (Asai et al., 1982) followed by a rhodamine-conjugated anti-mouse IgM antibody (Molecular Probes). Four different fixation and permeabilization protocols were used as described in Table 1. Fewer microtubules were preserved when the first step was a prolonged treatment in detergent (panel A). The cultured fibroblasts were a gift from Dr. Elizabeth Orwin's laboratory.

Table 1 Treatments Used to Generate Panels Shown in Figure 8

Figure panel	Treatment
8 A	(i) Triton X-100, 10 min; (ii) formaldehyde, 10 min; (iii) cold methanol, 10 min
8 B	(i) Triton X-100, 2 min; (ii) formaldehyde, 10 min; (iii) cold methanol, 10 min
8 C	(i) formaldehyde, 10 min; (ii) cold methanol, 10 min (no detergent step)
8 D	(i) formaldehyde, 10 min; (ii) Triton X-100, 5 min; (iii) cold methanol, 10 min

(five to six) on the bottom of a sterile, plastic 150-mm petri dish using standard cell culture techniques (e.g., see Phelan & May, 2015)

The coverslip size and format can be modified.

Phosphate-buffered saline (PBS; see recipe), 37°C

0.1% (v/v) Triton X-100 in 1× microtubule stabilizing buffer (MTSB; see recipe)

3.7% (v/v) formaldehyde/1× MTSB (see recipe), 37°C

100% methanol, −20°C (optional)

Primary antibody, diluted (see Strategic Planning) to working concentration in 0.1% PBSA (see recipe)

Secondary antibody, diluted (see Strategic Planning) to working concentration in 0.1% PBSA (see recipe)

Other stains: e.g., 0.5 μg/ml 4′,6-diamidino-2-phenylindole (DAPI), 0.01 mM SYTOX (Molecular Probes), fluorescein isothiocyanate (FITC), or rhodamine

Mounting medium (see recipe)

Nail polish
Ceramic coverslip rack (Coors; Thomas Scientific)
Fine-tipped jeweler's forceps
250-ml beakers
Humidified chamber (e.g., Tupperware box with moistened paper towel) with grid (e.g., plastic gel spacers)
Microscope slides (Gold Seal; VWR)

1. Remove the coverslips from the medium and place in the ceramic coverslip rack. Keep track of which side of the coverslip has the cells!

   A curved fine-tipped pair of jeweler's forceps is useful for manipulating the coverslips.

2. Rinse the cells briefly with 37°C PBS by placing the rack containing the coverslips into a 250-ml beaker containing 100 ml solution.

3. Demembranate the cells by incubating 5 min in prewarmed 100 ml 0.1% Triton X-100 in 1× MTSB at 37°C.

4. Fix the cells 10 min in 100 ml prewarmed 3.7% formaldehyde/1× MTSB at 37°C.

   CAUTION: Formaldehyde is extremely toxic and will damage exposed membranes (eyes, nose, throat). Always work in a properly exhausting fume hood and properly dispose of solutions.

   Better preservation of cellular structures can be achieved by reversing steps 3 and 4, i.e., fixing the cells first in formaldehyde (step 4) followed by detergent extraction (step 3); however, the background can be higher in cells fixed before permeabilization. A good compromise may be achieved by reducing the time in the detergent.

   Figure 8 shows microtubule staining of fibroblasts fixed and permeabilized in different ways as described in Table 9.2.2.

5. *Optional*: Extract 10 min with 100 ml prechilled 100% methanol at −20°C.

   IMPORTANT NOTE: If phalloidin will be used to stain the actin microfilaments, do ***not*** *use methanol for the extraction step.*

   The cold methanol step serves to further extract the cells, usually resulting in a lower staining background. It is also possible to use only cold methanol or cold 100% acetone to permeabilize and fix the cells, i.e., skipping the detergent and aldehyde steps.

   In our hands, cold methanol alone tends to fragment the microtubules, presumably because the cells are being rapidly dehydrated.

6. Rinse coverslips briefly in 100 ml PBS.

7. Remove each coverslip from the rack, draw off excess fluid by touching the edge to a Kimwipe, and lay the coverslip cell side up on a grid in a humidified chamber.

   We use a plastic box lined with a moistened paper towel.

   The coverslips are placed cell side up on plastic strips (we use SDS-PAGE gel spacers).

   It is important to keep track of the arrangement of the coverslips.

8. Pipet 30 to 50 µl of the diluted primary antibody onto the coverslip, resulting in a drop of antibody solution on the coverslip.

   See Strategic Planning for a discussion of antibody dilutions.

   If the cells are to be double-stained (i.e., two different antibodies), it is convenient to mix the primary antibodies and perform this step as a simultaneous co-incubation with both antibodies at 37°C for >1 hr.

This is a good stopping point. The primary antibody incubation can extend for more than a day, as long as the coverslips are in a closed and moistened chamber. For primary antibody incubations overnight or longer, we recommend incubating at 4°C.

9. Replace the coverslips carefully into the ceramic rack. Rinse three times (5 min each) in 100 ml PBS at 37°C.
10. Remove each coverslip from the rack, draw off excess fluid with a Kimwipe, and lay the coverslip cell side up in the humidified chamber.
11. Pipet 30 to 50 μl of the diluted secondary antibody onto each coverslip.

    If double-staining, both secondary antibodies can be simultaneously applied.

    Instead of or in addition to two different antibodies, the investigator may wish to include a stain for organelles (e.g., DAPI or SYTOX for nuclear DNA, wheat germ agglutinin for Golgi apparatus, or phalloidin for actin). For example, if the microscope is fitted with the appropriate filters, the investigator may wish to stain the cells with (1) DAPI to visualize the nuclei, (2) anti-tubulin (e.g., FITC-tagged) to visualize the microtubules, and (3) fluorescence tagged (e.g., rhodamine) phalloidin to visualize the actin stress fibers.
12. Incubate 1 hr at 37°C, in the dark.

    The secondary antibody staining step should not extend for more than a few hours.
13. Replace the coverslips carefully in the ceramic rack. Rinse the coverslips 3 times (5 min each) in PBS at 37°C.
14. After the last PBS wash, place the racked coverslips in a beaker of deionized water for a few minutes to remove salts.
15. Draw off the fluid from the coverslip with a Kimwipe and mount each coverslip on a cleaned glass microscope slide, inverting the coverslip (cell side down) onto a 10-μl drop of mounting medium.

    The mounting medium is a cryopreservative (glycerol) and inhibits fading of the fluorescence (because of its high pH and N-propylgallate).
16. Press gently (the eraser end of a pencil works well here) to squeeze out bubbles. Seal the coverslip to the slide with nail polish to prevent evaporation and the introduction of oxygen.
17. Store the well-sealed slides up to 6 months in the refrigerator, in the dark.

    Although slices can be stored for several months, they will degrade over time and should be imaged as soon as possible.

BASIC PROTOCOL 2

IMMUNOFLUORESCENCE OF SUSPENSION CELLS SUCH AS TETRAHYMENA

Tetrahymena cells are much larger than cultured fibroblasts and require a different protocol for solubilization, fixation, and staining. The principle is the same: (1) remove the plasma membrane with nonionic detergent, (2) chemically fix the cells, (3) add primary antibodies, (4) add secondary antibodies, and (5) mount and visualize. An example of stained *Tetrahymena* cells imaged with an LSCM is provided (Fig. 9). For additional information on culturing *Tetrahymena*, the reader is referred elsewhere (Cassidy-Hanley, 2012).

Materials

Tetrahymena cells or other thick cells (e.g., sea urchin embryo cells)
PHEM buffer (see recipe)
10% (v/v) Triton X-100 in PHEM buffer

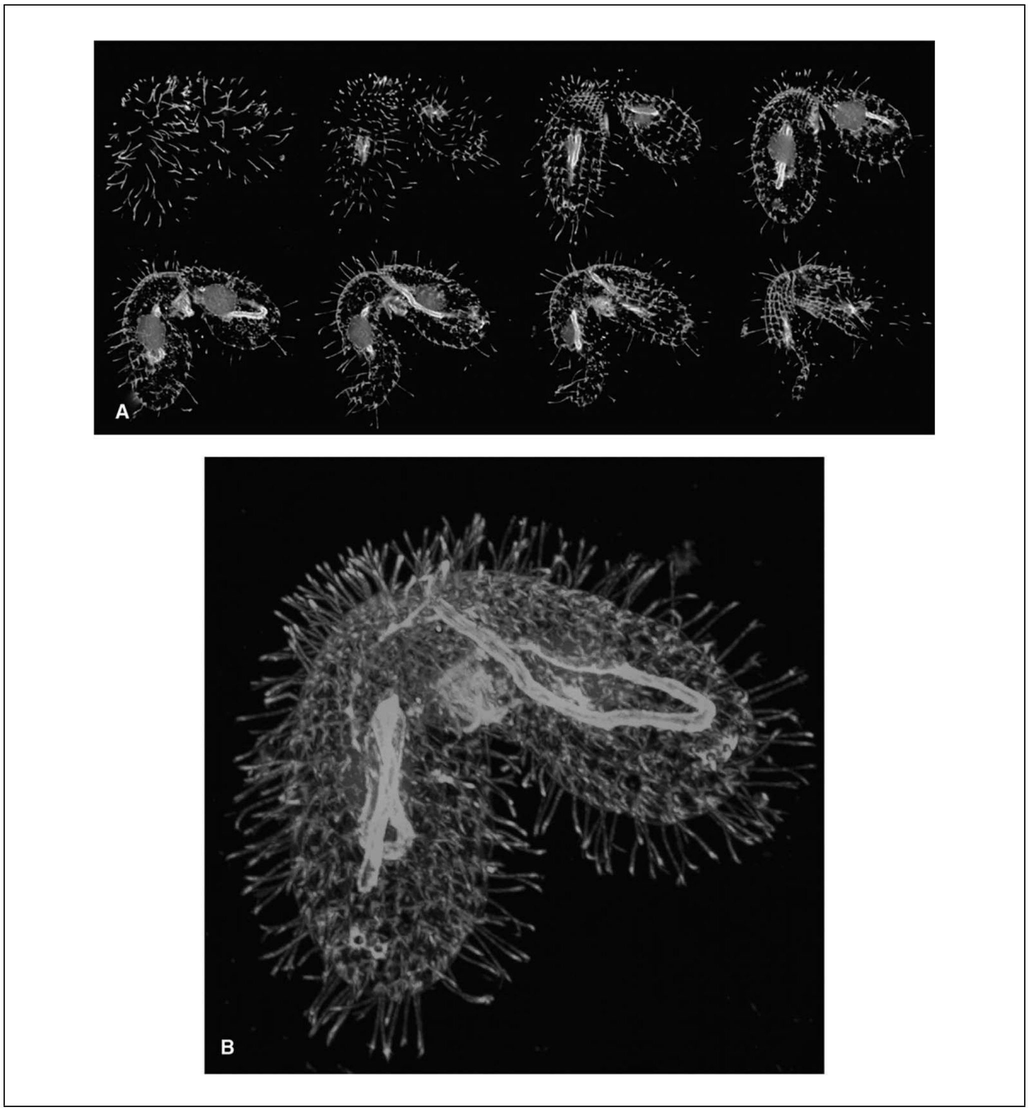

Figure 9 Example of optical sectioning by confocal microscopy. A conjugating pair of *Tetrahymena* cells, captured in prophase I (crescent stage), were fixed and stained with a cocktail of monoclonal antibodies to α-tubulin (Asai et al., 1982); the secondary antibody was rhodamine-conjugated anti-mouse IgG antibody (Molecular Probes). Mixed into the secondary antibody was SYTOX Green (Molecular Probes). The cells were then examined using confocal laser scanning fluorescence microscopy (Zeiss LSM510). (**A**) Selected optical sections through the pair of cells. (**B**) Projection of all thirty of the optical sections.

10% (w/v) paraformaldehyde in PHEM buffer: store up to 1 year at room temperature in a dark container in a fume hood
0.1% and 0.5% PBSA (see recipes)
Primary antibody, diluted (see Strategic Planning) to working concentration in 0.1% PBSA (see recipe)
Secondary antibody, diluted (see Strategic Planning) to working concentration in 0.1% PBSA (see recipe)
Nuclear stain: 0.5 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) or 0.01 mM
SYTOX (Molecular Probes), optional
Mounting medium (see recipe in Reagents and Solutions)

Nail polish
Tabletop centrifuge (e.g., VWR Galaxy Ministar personal centrifuge)
1.5-ml microcentrifuge tubes
Microscope slides

NOTE: All the centrifugations in this protocol are performed for 2 min at 1000 to 2000 × *g* and room temperature.

1. Centrifuge cells in 1.5-ml microcentrifuge tubes.

   Throughout this protocol, cells are collected by centrifugation. We use a simple tabletop centrifuge (e.g., VWR Galaxy Ministar personal centrifuge).

2. Quickly but gently decant the supernatant before the cells swim out of the pellet.

3. Gently resuspend the cells in 950 µl PHEM buffer at room temperature.

4. Add 50 µl of 10% Triton X-100 in PHEM buffer (final concentration 0.5% Triton X-100). Gently mix by inverting the closed microcentrifuge tube several times. Incubate 5 min at room temperature.

5. Add 250 µl of 10% paraformaldehyde in PHEM buffer (2% final concentration) and mix gently. Incubate 10 min at room temperature.

   CAUTION: Paraformaldehyde is toxic and will damage exposed tissues (including eyes, nose, throat). Handle in a fume hood.

6. Centrifuge the cells and decant the supernatant.

7. Resuspend the cells in ∼100 to 200 µl of 0.5% PBSA. Mix gently and incubate 2 min at room temperature.

   This step blocks any unoccupied reactive sites of the paraformaldehyde with bovine serum albumin, which should not react with the subsequent antibodies.

8. Repeat steps 6 and 7 and centrifuge the cells again.

   See Alternate Protocol for an alternative method for the following steps.

9. Decant as much fluid as possible and gently resuspend the cells in ∼100 µl diluted primary antibody.

   See Strategic Planning for a discussion of how to determine the appropriate working dilution of the primary antibody.

   If double staining is being performed, both primary antibodies can be mixed together and simultaneously incubated with the cells.

10. Incubate cells in the closed microcentrifuge tubes 1 hr at 37°C.

    This is a good stopping point. The primary antibody incubation can extend for more than a day, as long as the cells are in a closed tube. For overnight (or longer) incubations with primary antibody, incubation at 4°C is recommended.

11. Centrifuge the cells and decant the supernatant.

12. Wash the cells 3 times using 0.1% PBSA by incubating each wash 1 min at room temperature, with gentle mixing by inverting the tube. Centrifuge after each wash and discard the supernatant.

13. Resuspend the cells in ∼100 µl diluted secondary antibody.

    If double-staining, then both secondary antibodies can be simultaneously applied. Instead of or in addition to two different antibodies, the investigator may wish to include a stain for organelles (e.g., DAPI or SYTOX for nuclear DNA).

14. Incubate the cells in closed tubes 1 hr at 37°C, in the dark.

    The secondary antibody staining step should not extend for more than a few hours.

15. Collect the cells by centrifugation and decant the supernatant.

16. Wash the cells three times with 0.1% PBSA for 1 min at room temperature, with gentle mixing by inverting the tube. Centrifuge after each wash and discard the supernatant. After the last wash, decant as much fluid as possible.
17. Resuspend the cells in ~20 µl mounting solution. Place 10 µl of the resuspended cells onto a clean glass microscope slide, drop a coverslip onto the drop, and press down gently (the eraser end of a pencil works well).
18. Seal the coverslip to the slide with nail polish. Store the well-sealed slides up to several months at 4°C in the dark.

ALTERNATE PROTOCOL 1

STAINING SUSPENSION CELLS ADHERED TO POLY-L-LYSINE-COATED COVERSLIPS

An alternative to the previous protocol in which large cells are repeatedly washed and collected by centrifugation is to adhere the cells to poly-l-lysine-coated coverslips. After fixing the cells in paraformaldehyde and washing them (Basic Protocol 2, steps 1 to 8), the cells can be adhered to previously prepared coverslips coated with poly-l-lysine and stained. This is convenient if there is a very small quantity of cells. After adhering the cells to the coverslips, the coverslips are processed as described above for fibroblast staining (Basic Protocol 1). Washes are performed by placing the coverslips in the ceramic rack as described in that protocol.

Additional Materials (also see Basic Protocols 1 and 2)

Coverslips coated with poly-l-lysine (see recipe): prepared before beginning to work with the cells

1. Perform Basic Protocol 2, steps 1 to 8.
2. Decant as much fluid as possible.
3. Pipet the cells onto a coverslip coated with poly-l-lysine.
4. Allow the cells to settle onto the poly-l-lysine-coated coverslips for 30 to 60 min; keep covered to protect from dust and prevent evaporation.
5. Proceed as in Basic Protocol 1, steps 5 to 17.

BASIC PROTOCOL 3

VISUALIZING SAMPLES WITH A WIDEFIELD FLUORESCENCE MICROSCOPE

Regardless of the final imaging goal, it is important to evaluate the outcome of your staining with a basic widefield fluorescence microscope using a dry objective. Starting with a simple microscopy approach can help determine whether your immunofluorescence worked and whether there are any substantial issues with your specimen. The protocol below is a general description of how to acquire simple digital images using a generic fluorescence microscope equipped with a digital camera.

Equipment and software

Fluorescence microscope equipped with appropriate filters for imaging the dyes used in your immunofluorescence staining.

Optional: A microscope that has DIC or phase contrast available as a transmitted light technique is beneficial.

Commercial microscope acquisition software and/or the open-source microscope control software Micro-Manager (Edelstein et al., 2014).

Commercial microscope image viewer software and/or the open-source image viewing software ImageJ/FIJI (Schindelin et al., 2012).

1. Set up microscope filters and light source to view the largest and brightest structure in your sample.

   If samples are labeled with DAPI, the nucleus should be large and bright, so the DAPI filter is a good starting point for your initial focusing. If you have phase contrast or DIC available on your microscope, these are great alternatives to using fluorescence for your initial focusing.

2. Place slide on the microscope stage with the coverslip facing the objective.

   Whether this is coverslip up or coverslip down depends on whether you are using an upright or inverted microscope.

3. Focus on the specimen with the lowest magnification objective that will visualize labeled structures.

   DAPI-stained nuclei in eukaryotic cells are easily viewed with 10× magnification. To get close to the correct focal plane, it can be advantageous to bring the coverslip edge into focus using transmitted light. Focusing on the edge of the coverslip is a reliable way to get within 100-200 µm of the biological sample. It is also important to determine whether the focusing mechanism for your microscope moves the stage or the objective. Most commercial microscopes focus by moving the objectives, but some automated stages have Z-motion built into the stage top. Knowing the mechanical elements of the focusing process can help ensure you do not crash an objective into the specimen, which can damage the objective.

4. Switch to the desired magnification for your experiment.

   Most microscopes are parfocal so that all the objectives will be focused on approximately the same focal plane. As the magnification and numerical aperture (NA) increase, the depth of field decreases. Thus, the entire cell may appear to be in focus when viewed with a low-power lens, and only part of it in focus at a higher magnification.

   If switching from a dry objective (low resolution) to an immersion objective (high resolution), immersion fluid (e.g., oil) will need to be applied. Different labs use different methods to apply and remove immersion fluid. Consult a trained microscope user to learn the preferred method for your lab. Regardless of the method, there are some general guidelines to follow:

   - *Do not go back to a dry lens without fully removing the immersion fluid from both the lens and the slide. It is best to use immersion lenses after evaluating your sample with a dry objective.*
   - *Different brands of oil are not necessarily compatible with one another, and mixing oils can produce distressing cloudiness.*
   - *The first step in removing immersion fluid from slides and lenses is to gently blot the glass surface with lens paper. Do not wipe lens paper across the surface because wiping can scratch the glass.*
   - *Use glass cleaner or 95% ethanol to remove immersion oil residue from a slide. Do not apply glass cleaner or solvent to the objective lens without first discussing it with the person in charge of managing the microscope.*

5. Optimize illumination (e.g., LED intensity) and detector settings (e.g., camera exposure and gain) to acquire a non-saturated image for each channel.

   Increasing illumination intensity and camera exposure will increase pixel intensity in your digital image, but they will also increase photobleaching of your sample. Increasing gain will increase pixel intensity, but it will also increase noise in your image. Regardless, ensure that the brightest pixels do not exceed the dynamic range of your detector (e.g., saturation). Ideally, your setting will produce an image that utilizes 50% to 75% of the dynamic range of your detector. If the image data will be quantified, it is important to ensure that the same settings are used for all samples in your experiment and that all samples that will be compared are imaged on the same day.

Optimizing illumination and detector settings for a confocal spinning disk is like optimization for widefield fluorescence since both approaches use digital cameras. Optimizing acquisition for a laser scanning confocal is conceptually different and is covered in Alternate Protocol 2 below.

6. Optional: Establish the starting slice, ending slice, and slice interval for your acquisition if you are acquiring a Z-stack.

   Each microscope has a different software interface for automatically capturing multiple XY images acquired at sequential focal planes (called a Z-stack). The optimal spacing between focal planes (slice interval) is often calculated by the software.

7. Capture digital images of your sample.

   The number of digital images produced depends on the number of channels and the Z-stack size. For example, a 3-channel image acquired over 10 Z slices will produce 30 individual digital images. This is a substantial amount of data that can be viewed with commercial software or ImageJ/FIJI.

ALTERNATE PROTOCOL 2

VISUALIZING SAMPLES WITH A LASER SCANNING CONFOCAL MICROSCOPE

Confocal microscopy reduces image blur by preventing out-of-focus light from reaching the detector. LSCM uses photomultiplier tube detectors rather than cameras. The general process to optimize laser scanning confocal acquisition is described below. The order of optimization is instrument dependent - the steps described below are general guidelines rather than absolute sequential steps.

Additional Equipment (also see Basic Protocol 3)

Laser scanning confocal microscope equipped with laser lines and detectors that match the fluorescent dyes used to stain your sample.

1. Perform Basic Protocol 3, steps 1 to 4 to focus on your sample since most laser scanning confocal microscopes use widefield fluorescence to produce an image at the eyepiece.

2. Activate the laser lines and detectors needed to visualize the dyes that are present in your sample.

   Laser scanning confocal microscopes often have multiple lasers and multiple detectors. For each channel you wish to acquire, you must establish a laser wavelength to excite the dye and an emission window (in nm) to collect the emitted photons. If visualizing multiple colors, it is critical to establish the laser lines and emission windows to minimize bleed through and crosstalk.

3. Optimize the format of your digital image by choosing an image size, zoom factor, and pixel size. These parameters are related and changing one will impact the other.

   Laser scanning confocal microscopes can often capture images that are various sizes. These sizes range from single lines of data that are 1 pixel by 4096 pixels through large images that are 4096 pixels by 4096 pixels. In general, larger images take more time to capture. Laser scanning confocal data can be digitally enlarged via a zoom factor accessed through the software. In general, increasing the zoom factor will digitally enlarge your sample while reducing the field of view and decreasing the size of individual pixels.

4. Optimize the scan speed of the laser.

   The scan speed of the laser determines the pixel dwell time. Slow scan speeds will produce long pixel dwell times and brighter images and vice versa. However, slow scan speeds will increase image acquisition time and there will be more photobleaching since the laser spends more time exciting each area of the sample.

5. Optimize the laser intensity and digital gain of the detector.

   High laser power will increase image brightness, but there will also be increased photobleaching. High detector gain will increase image brightness, but there will also be increased noise in the image.

6. Perform Basic Protocol steps 6 and 7.

GENERATING SUPER-RESOLUTION IMAGES WITH SRRF MICROSCOPY

ALTERNATE PROTOCOL 3

SRRF is an open-source algorithm that generates super resolution images by capitalizing on fluorescence intensity fluctuations that occur over time. A major advantage of SRRF compared to other super-resolution techniques is that it can be accomplished with a basic widefield fluorescence microscope equipped with a digital camera. The general process of creating an optimized SRRF image using the latest version (called eSRRF) is described below. An example of a properly optimized eSRRF image of *Tetrahymena* basal bodies and mitochondria is provided (Fig. 10).

Additional Equipment (also see Basic Protocol 3)

A computer with FIJI and the latest version of eSRRF installed. eSRRF is computationally intensive and can be accelerated if used on a computer with a video card.

Note: The eSRRF GitHub page (currently at: *https://github.com/HenriquesLab/NanoJ-eSRRF*) provides extensive information on how to install eSRRF.

1. Perform Basic Protocol 3, steps 1 to 5 to focus on your sample and optimize the illumination and detector settings.

2. Capture 10 to 1000 frames using the time-lapse feature of the microscope control software.

   Capturing multiple channels can lead to a lengthy acquisition. Setting the acquisition to acquire all the frames for a single channel before capturing all the frames for the next channel can be advantageous. This will limit the number of filter changes, which can slow acquisition. The number of frames necessary for SRRF must be empirically determined for each sample.

3. Convert your image data into individual time series for each channel. This can be done using commercial microscope control software or with FIJI using the “Split Channels” and “Hyperstacks” commands.

   As an input, SRRF requires a single image stack where each image in the stack is a single channel (C) image acquired at a different time (T). For example, C1T1, C1T2, C1T3, C1T4… is correct. C1T1, C2T1, C1T2, C2T2… is incorrect.

4. Optional: Crop out a small region of your image that contains the biological structure of interest.

   Since SRRF performs calculations on every pixel in an image, it can be faster to optimize the SRRF algorithm with smaller images that contain fewer pixels.

5. Perform an eSRRF parameter sweep using the default parameter ranges.

   The eSRRF parameter sweep allows a user to determine the algorithm settings that produce the highest quality super-resolution reconstruction. The ideal parameters improve resolution without increasing radial intensity artifacts.

6. Use the optimal settings from the parameter sweep to generate a full eSRRF reconstruction of your entire image.

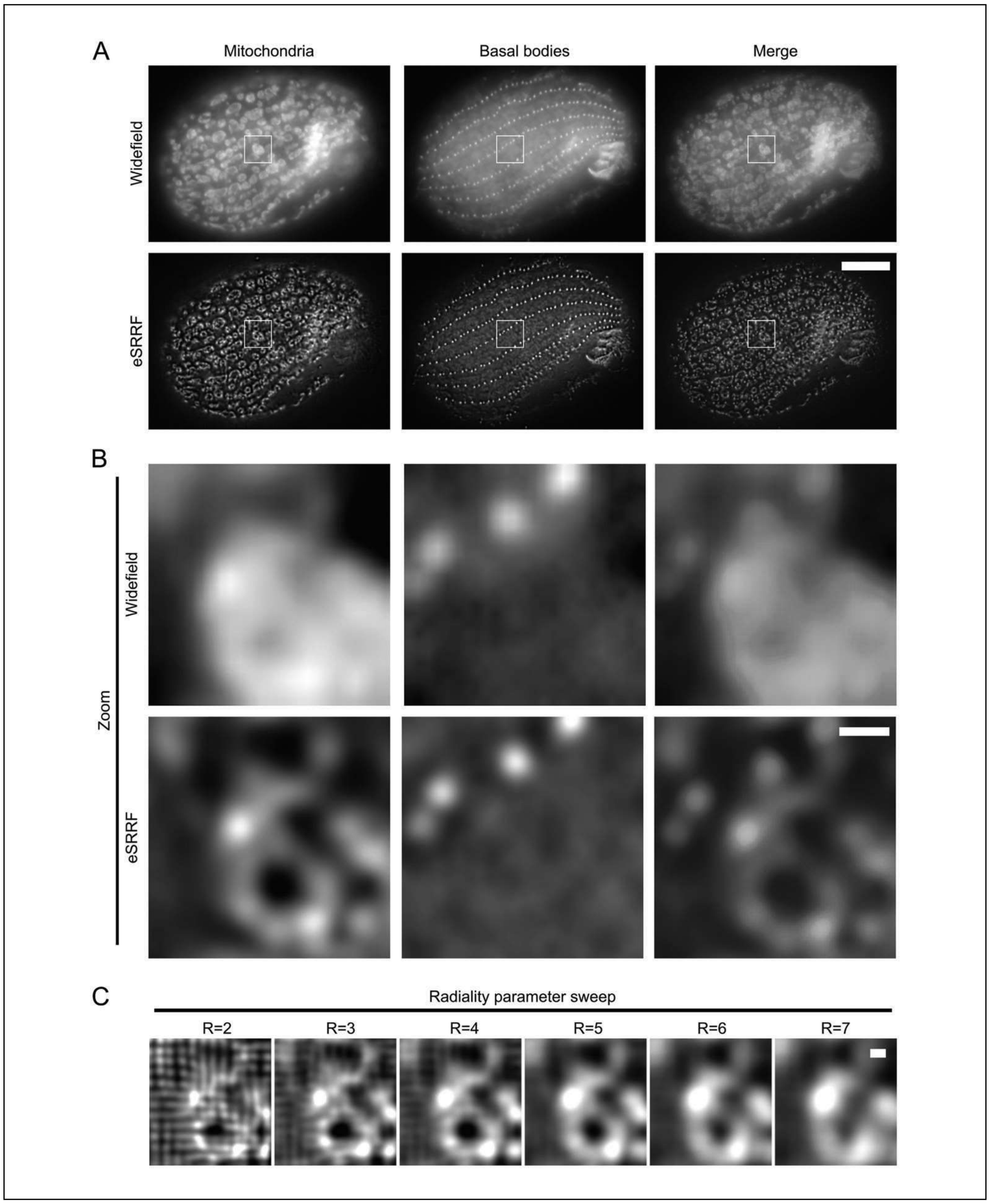

Figure 10 Using eSRRF to generate a super-resolution image from with a basic widefield microscope. (**A**) The average widefield image (top) and eSRRF image (bottom) that results from 50 consecutive frames of widefield fluorescence image data. The mitochondria are stained with an ATP5A antibody (cyan) and a centrin antibody (magenta) that recognizes a protein that localizes to basal bodies. Scale bar is 10 μm. (**B**) Digitally enlarged images of the widefield and eSRRF data from A. Scale bar is 0.5 μm. (**C**) A sample of eSRRF optimization data showing how altering the "Radiality" parameter alters eSRRF reconstructions. Obvious "ringing" occurs when the Radiality is set to 4 and below. For this data, a Radiality of 5 is optimal. Scale bar is 0.5 μm.

When generating any super resolution image, it is important to verify that the resulting image does not contain artifacts. Prior to interpreting the eSRRF image it is important to verify the super-resolution localization through an independent method, such as electron microscopy or a different super-resolution approach.

REAGENTS AND SOLUTIONS

Coverslips coated with poly-l-lysine

Holding the coverslip with a pair of sturdy forceps, pass the coverslip several times through the flame of a Bunsen burner. This deposits a thin carbon coating on the glass. Place the coverslips flat in a clean petri dish; pipet onto each coverslip a drop of 0.1% (w/w) poly-l-lysine. Incubate coverslips under poly-l-lysine for 30 to 60 min at room temperature. Remove coverslips and rinse in water. Store the poly-l-lysine solution up to 3 months at 4°C. The poly-l-lysine solution can be reused.

Formaldehyde, 3.7% (v/v)/MTSB, 1×

Dilute 37% formaldehyde stock (Sigma) 10-fold in microtubule stabilizing buffer (MTSB; see recipe) to give final concentrations of 3.7% formaldehyde and 1× MTSB. Prepare fresh before use.

Microtubule stabilizing buffer (MTSB), 2×

Combine the following in a final volume of 1 L:
70 g disodium PIPES (0.2 M PIPES)
3.04 g EGTA (2 mM)
80 g PEG, MW 6000 (8%)
0.5 g sodium azide (0.05%)
1.97 g $MgSO_4$, heptahydrate (8 mM)
Adjust to pH 6.8 using 1 M NaOH
Store up to 1 year at 4°C
Dilute as required with water to make 1× MTSB
CAUTION: Azide is toxic!

Final concentrations are given in parentheses.

Mounting medium

50% glycerol
50% 0.2 M borate, pH 9
0.7% *N*-propylgallate (antifade agent)
Store up to 6 months at 4°C in a dark container

If glycerol/borate mounting medium is used, the coverslips must be sealed with nail polish.

PBSA

10% (w/v) BSA in 1× PBS (see recipe). Store up to 1 year at 4°C. Dilute as required with 1× PBS to make 0.1% and 0.5% PBSA.

PBS, 10× stock solution

Combine the following in a final volume of 1 L:
350.65 g NaCl (1.5 M)
2.64 g NaH_2PO_4, monohydrate (0.02 M)
11.5 g Na_2HPO_4, anhydrous (0.08 M)
5 g sodium azide (0.5%)
Adjust to pH 7.4 with phosphoric acid or 1 M NaOH, if necessary
Store up to 1 year at room temperature
Dilute to 1× with water before use
CAUTION: Azide is toxic!

We add sodium azide as a preservative. The azide does not affect these methods, although the PBS with azide is not appropriate for washing living cells.

Final concentrations are given in parentheses.

PHEM buffer

Combine the following in a final volume of 500 ml:
9.1 g PIPES (60 mM)
2.98 g HEPES (25 mM)
1.9 g EGTA (10 mM)
0.2 g $MgCl_2$ (2 mM)

Adjust to pH 6.9 with 1 M NaOH
Store up to 6 months at 4°C

This buffer contains no azide.

Final concentrations are given in parentheses.

COMMENTARY

Understanding Results

"A picture is worth a thousand words." (Attributed to Fred Barnard in Printer's Ink, December 8, 1921).

The ability to gaze into the cell and determine the locations of specific molecules is the obvious allure of immunofluorescence microscopy. But it also has its potential pitfalls: a seductive invitation to replace scientific rigor with pretty pictures. Investigators—especially students who may be visualizing their favorite molecules for the first time—should be reminded what an immunofluorescence image is **not**. It is not dynamic: it is important not to over-interpret the history or the future of a molecule based on a snapshot of its present location. It is not readily quantifiable: brighter does not necessarily mean "more" because the intensity of the signal depends on many things including the avidity of the antibodies, chemistry of the fluorophore, and optics of the microscope. It is not imaginary: what you see is what you get.

As summarized in this chapter, there are many factors the investigator should consider when interpreting their results. Here are five questions to guide the analysis:

1) *Is the signal specific?* Is the investigator convinced about the specificities of the antibody reagents? What is the background fluorescence? Is there autofluorescence (i.e., the specimen fluoresces without any added fluorophore)? It is important to perform all the appropriate antibody controls before concluding that the fluorescent signal is specific.

2) *Is the image real?* The human mind tends to focus on structures and ignores the lack of organization. The interesting location of the antigen may, in fact, be diffused throughout the cell with no apparent organization. It is important to remember that the digital image is simply an array of pixel intensities that should be interpreted objectively. When dealing with super-resolution images, such as eSRRF, it is also important to validate any localization patterns with an independent method.

3) *Is the result reproducible?* How is the staining pattern affected by the fixation and permeabilization conditions? The weather? The time of day? The person who does the staining? Avoid making biological conclusions based on an immunofluorescence experiment until the specificity of the antibodies are proven, the image acquisition settings are optimized, and the localization has been reproduced on biological replicates.

4) *What is the magnification of the image?* It is always useful to determine the magnification as a check that the investigator understands the experimental system and the optics of the microscope. Although most microscope control software can export a scale bar, one should periodically image a stage micrometer to ensure that the microscope system is calibrated.

5) *How are the data recorded and reported?* Whether the image was captured with a digital camera (widefield or SDCM) or photomultipliers (LSCM), the image is affected by the conditions selected by the investigator. Further, after the image is captured, it often must be manipulated using software (Hartig, 2013). Subjectivity is unavoidable; thus, the key is not to discourage manipulations—rather, it is important to be consistent when capturing and reporting the images. If the end goal of an immunofluorescence experiment is to quantify image intensity or localization it is critical to consult a trained image analyst to ensure that the imaging process is amenable to quantification. One general rule of thumb is that if the investigator does not know the image file type and bit-depth of their raw data, the investigator is not ready to begin image quantification.

Troubleshooting

Immunofluorescence microscopy is technically challenging, and it is inevitable that something will go wrong, no matter how experienced the investigator. Therefore, before embarking on an immunofluorescence experiment with new antibodies, it is a good idea to practice the technique with protocols and reagents that have been successfully used by others. Once an investigator can reproduce a robust immunofluorescence procedure, they can be confident developing their own immunofluorescence experiments. As is the case for all experimental techniques, the key to troubleshooting immunofluorescence microscopy is to approach the problem logically and, as much as possible, dissect it one variable at a time.

Fluorescence is not observed through the eye piece of the microscope

Can the cells be found using transmitted light microscopy? If so, this indicates that the problem is with the fluorescence staining and/or the operation of the fluorescent microscope. To begin troubleshooting, it can be useful to reproduce a robust immunofluorescence staining protocol that has worked well for others in your lab, seek microscope training from an experienced user, and/or optimize the staining procedure as described in the next section. If the cells cannot be found using transmitted light, then the cells were either lost during processing (this includes insufficient fixation or upside-down coverslips) or they were not there to begin with. To troubleshoot this, thoroughly document the samples at each stage of the staining process using a simple low-powered transmitted light microscope to determine where the cells are being lost.

Fluorescence is observed through the eye piece of the microscope but it is not brighter than the background

Are the background and specific fluorescence signals both dim? If so, this may indicate that the staining procedure is working but that it needs to be optimized. Some strategies include increasing the antibody concentration to ensure the antibody is in vast excess of the antigen, altering the timing and method of fixation, trying a different antibody that binds to the same protein of interest, and checking the reactivity of the antibody by immunoblotting (Ni et al., 2016) the proteins extracted from the same kind of cells that are being stained. By altering one variable at a time, the investigator can usually arrive at a protocol that produces sufficiently bright samples.

Are the background and specific fluorescence signals both bright? If so, this may indicate that the primary and/or secondary antibody concentrations are too high leading to non-specific binding, that the samples were not adequately blocked, and/or that that the sample has high autofluorescence. These issues can usually be resolved by performing a small experiment in which the amount of primary antibody is held constant and the amount of fluorescent secondary antibody is varied (and vice versa), by increasing the length of the blocking step, increasing the concentration of the blocking agent, and/or by omitting the secondary antibody altogether to evaluate autofluorescence.

Is background signal present in areas where there is no biological material? If so, this may indicate that either the glass coverslip or glass slide is coated with a fluorescent material. To overcome this problem, try cleaning the coverslips and slides prior to use and/or try switching coverslip/slide suppliers.

The fluorescent signal is bright in some areas of the sample but dim in other areas

Do the relatively bright and dim areas appear in the same place within the field of view? For example, is the upper left quadrant always dim, but the lower right quadrant is always bright? If so, this may indicate that the illumination source is out of alignment or there is something incorrectly placed in the optical path. To fix this, ensure that all the optical elements are fully inserted or removed from the optical path and that the illumination source is properly aligned. Often, troubleshooting these problems requires the assistance of a fully trained microscope user—do not start randomly adjusting sliders and optical elements on the microscope.

Do the relatively bright and dim areas appear in different places within the field of view? If so, this usually indicates that the sample was stained unevenly. If adherent cells on coverslips were the starting material (Basic Protocol 1), uneven staining may result from air bubbles between the coverslip and staining solution. If suspension cells were the starting material (Basic Protocol 2), uneven staining may result from antibody incompletely penetrating cell pellets. These problems can be corrected by avoiding air bubbles and completely resuspending cell pellets during the staining process.

The fluorescent signal appears bright and specific but it is blurry

Is the blurriness present with all the objectives? If so, this may indicate that the wrong coverslip thickness is being used, the sample is oriented with the glass slide facing the objective instead of the coverslip, and/or optics common to all the objectives (e.g., filters, condenser lens, etc.) may be dirty. If the coverslip thickness and orientation are correct, try imaging the sample on a different microscope. If the sample is not blurry on a different microscope, your microscope has dirty optics, and you should consult the owner/operator of the microscope to discuss the preferred cleaning method.

Is the blurriness present with only a single objective? This may indicate that a single objective is dirty. One common problem is that a dry (i.e., non-immersion) objective is soiled with immersion oil. Typically, this problem can be solved by gently de-oiling the objective with lens paper and solvents. Prior to de-oiling a dry objective, you should consult the owner/operator of the microscope.

The fluorescent signal appears bright, specific, and crisp through the eye piece but not through the camera software

Is the camera image totally black? First, confirm that the camera image is truly black. Most camera software will display the XY coordinates and pixel intensity information as the cursor moves across the image (alternatively, check the pixel intensity histogram). If all pixels have a 0 intensity, either the image is forming at the eye piece, which can be verified, or there is a communication issue between the camera and the software.

Is the camera image dim compared to the perceived brightness through the eye piece? If the camera image is dim rather than black, the software settings may not be optimized for digital imaging. Some common troubleshooting targets include camera exposure, camera gain, fluorescence lamp intensity (if it is adjustable), dynamic range (e.g., 12-bit versus 16-bit), and the relationship between the image histogram and look-up table. These last two issues (dynamic range and the lookup table) are related and can cause confusion. For example, if the camera generates 16-bit images (2^{16} or 65,536 intensity units) and the brightest pixels in the image are at 5000 intensity units, the image may appear dark because the full dynamic range of the 16-bit image is not being used.

Is the camera image "pixelated" compared to the image through the eyepiece? This is unavoidable and the "pixelation" will be more prominent as one "zooms" into the image to look for smaller details. If the pixelation seems more noticeable, one can check the camera binning feature. As camera binning increases, pixel size also increases, which can accentuate the "pixelation". Regardless, it is important to remember that digital images are simply multi-dimensional arrays of pixel intensities.

Acknowledgments

Research in the Galati lab is supported by the National Science Foundation, through a CAREER award (NSF MCB 2146516) and a Major Research Infrastructure award for a laser scanning confocal microscope (NSF DBI 2019228), Research Corporation Scialog to Advance BioImaging, and WWU startup funds. DFG would like to thank the members of the Galati lab who read early versions of the manuscript. Work in the Asai laboratory has been generously supported by the National Science Foundation.

Author Contributions

Domenico F. Galati: Conceptualization; methodology; visualization; review and editing. **David J. Asai**: Conceptualization, methodology, original draft.

Conflict of Interest

The authors do not have any conflicts of interest to declare.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

Literature Cited

Cassidy-Hanley, D. M. (2012). Tetrahymena in the laboratory: Strain resources, methods for culture, maintenance, and storage. *Methods in Cell Biology*, *109*, 237–276. https://doi.org/10.1016/B978-0-12-385967-9.00008-6

Cole, E. S. (2016). Conventional light microscopy. *Current Protocols Essential Laboratory Techniques*, *12*(1), 9.1.1–9.1.29. https://doi.org/10.1002/9780470089941.et0901s12

Combs, C. A., & Shroff, H. (2017). Fluorescence microscopy: A concise guide to current imaging methods. *Current Protocols in Neuroscience*, *79*(1), 2.1.1–2.1.25. https://doi.org/10.1002/cpns.29

Edelstein, A. D., Tsuchida, M. A., Amodaj, N., Pinkard, H., Vale, R. D., & Stuurman, N. (2014). Advanced methods of microscope control using μManager software. *Journal of Biological Methods*, *1*(2), e10. https://doi.org/10.14440/jbm.2014.36

Elliott, A. D. (2020). Confocal microscopy: Principles and modern practices. *Current Protocols in Cytometry*, *92*(1), e68. https://doi.org/10.1002/cpcy.68

Ernst Keller, H., & Watkins, S. (2013). Contrast enhancement in light microscopy. *Current Protocols in Cytometry*, *63*(1), 2.1.1–2.1.9. https://doi.org/10.1002/0471142956.cy0201s63

Eswarappa Prameela, S., McGuiggan, P. M., Brusini, A., Glenn, T. W., & Weihs, T. P. (2020). Looking at education through the microscope. *Nature Reviews Materials*, *5*(12), 865–867. https://doi.org/10.1038/s41578-020-00246-z

Gavira-O'Neill, C. E., Dong, J.-X., & Trimmer, J. S. (2021). Correction: Development, screening, and validation of camelid-derived nanobodies for neuroscience research. *Current Protocols*, *1*(7), e193. https://doi.org/10.1002/cpz1.193

Greenfield, E. A. (2013). *Antibodies a laboratory manual* (2nd ed.). Cold Spring Harbor Laboratory Press.

Gustafsson, M. G. L. (2000). Surpassing the lateral resolution limit by a factor of two using structured illumination microscopy. *Journal of Microscopy*, *198*(2), 82–87. https://doi.org/10.1046/j.1365-2818.2000.00710.x

Gustafsson, N., Culley, S., Ashdown, G., Owen, D. M., Pereira, P. M., & Henriques, R. (2016). Fast live-cell conventional fluorophore nanoscopy with ImageJ through super-resolution radial fluctuations. *Nature Communications*, *7*(1), 1–9. https://doi.org/10.1038/ncomms12471

Hartig, S. M. (2013). Basic image analysis and manipulation in ImageJ. *Current Protocols in Molecular Biology*, *102*(1), 14.15.1–14.15.12. https://doi.org/10.1002/0471142727.mb1415s102

Inoue, S., & Spring, K. R. (1997). *Video microscopy: The fundamentals* (2nd ed.). Springer.

Kasuboski, J. M., Sigal, Y. J., Joens, M. S., Lillemeier, B. F., & Fitzpatrick, J. A. J. (2012). Super-resolution microscopy: A comparative treatment. *Current Protocols in Cytometry*, *62*(1), 2.17.1–2.17.24. https://doi.org/10.1002/0471142956.cy0217s62

Lindsay, S. M. (2021). Integrating microscopy, art, and humanities to power STEAM learning in biology. *Invertebrate Biology*, *140*(1), e12327. https://doi.org/10.1111/ivb.12327

Lippincott-Schwartz, J., Snapp, E. L., & Phair, R. D. (2018). The development and enhancement of FRAP as a key tool for investigating protein dynamics. *Biophysical Journal*, *115*(7), 1146–1155. https://doi.org/10.1016/j.bpj.2018.08.007

Melan, M. A., & Sluder, G. (1992). Redistribution and differential extraction of soluble proteins in permeabilized cultured cells. Implications for immunofluorescence microscopy. *Journal of Cell Science*, *101*(4), 731–743. https://doi.org/10.1242/jcs.101.4.731

Ni, D., Xu, P., Sabanayagam, D., & Gallagher, S. R. (2016). Protein blotting: Immunoblotting. *Current Protocols Essential Laboratory Techniques*, *12*(1), 8.3.1–8.3.40. https://doi.org/10.1002/9780470089941.et0803s12

Phelan, K., & May, K. M. (2015). Basic techniques in mammalian cell tissue culture. *Current Protocols in Cell Biology*, *66*(1), 1.1.1–1.1.22. https://doi.org/10.1002/0471143030.cb0101s66

Sahl, S. J., & Hell, S. W. (2019). High-resolution 3D light microscopy with STED and RESOLFT. In J. F. Bille (Ed.), *High resolution imaging in microscopy and ophthalmology: New frontiers in biomedical optics*. Springer. http://www.ncbi.nlm.nih.gov/books/NBK554055/

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J.-Y., White, D. J., Hartenstein, V., Eliceiri, K., Tomancak, P., & Cardona, A. (2012). Fiji: An open-source platform for biological-image analysis. *Nature Methods*, *9*(7), 676–682. https://doi.org/10.1038/nmeth.2019

Shin, Y., & Brangwynne, C. P. (2017). Liquid phase condensation in cell physiology and disease. *Science*, *357*(6357), eaaf4382. https://doi.org/10.1126/science.aaf4382

Trimmer, J. S. (2020). Recombinant antibodies in basic neuroscience research. *Current Protocols in Neuroscience*, *94*(1), e106. https://doi.org/10.1002/cpns.106

Wu, H., Carvalho, P., & Voeltz, G. K. (2018). Here, there, and everywhere: The importance of ER membrane contact sites. *Science*, *361*(6401), eaan5835. https://doi.org/10.1126/science.aan5835

Xu, J., Ma, H., & Liu, Y. (2017). Stochastic Optical Reconstruction Microscopy (STORM). *Current Protocols in Cytometry*, *81*(1), 12.46.1–12.46.27. https://doi.org/10.1002/cpcy.23

Zhang, C., Kang, J. S., Asano, S. M., Gao, R., & Boyden, E. S. (2020). Expansion microscopy for beginners: Visualizing microtubules in expanded cultured HeLa cells. *Current Protocols in Neuroscience*, *92*(1), e96. https://doi.org/10.1002/cpns.96