# Cold Spring Harbor Protocols

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# Fluorescence Microscopy

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Fluorescence microscopy is a major tool with which to monitor cell physiology. Although the concepts of fluorescence and its optical separation using filters remain similar, microscope design varies with the aim of increasing image contrast and spatial resolution. The basics of wide-field microscopy are outlined to emphasize the selection, advantages, and correct use of laser scanning confocal microscopy, two-photon microscopy, scanning disk confocal microscopy, total internal reflection, and super-resolution microscopy. In addition, the principles of how these microscopes form images are reviewed to appreciate their capabilities, limitations, and constraints for operation.

## INTRODUCTION

The common adage of "seeing is believing" is often cited (McGeown 2010) when encountering new phenomena, and where better its application than when trying to comprehend cell physiology using florescence microscopy? Microscopy has played an important role in determining the activity of cells, from the very early appreciation of living animalcules by Van Leeuwenhoek (Ford 1989) with a simple microscope, to details of cellular events with a variety of present-day sophisticated imaging systems (Hell 2009). The current drive is to watch living events with ever more spatial and temporal resolution.

The development of numerous transmitted light microscopy approaches, including techniques such as phase-contrast, differential interference contrast (DIC), and polarized microscopy, enhanced the inherent contrast of living specimens to make them more visible. However, the introduction of fluorescence microscopy, using a variety of fluorescent indicators (referred to as indicators hence-forth) that can be tailored in terms of their specificity for targets such as proteins, lipids, or ions (Giepmans et al. 2006; Palmer and Tsien 2006) has perhaps been the biggest step in allowing us to watch cell physiology. Indeed, there appears to be no limit when it comes to innovative indicator design using molecular approaches. But, like all techniques, fluorescence microscopy is subject to practical physical limitations, the most important of which is resolution (Hell 2003). Consequently, most of the recent advances with fluorescence microscopy have sought to improve image quality by addressing the fundamental problem of image resolution, which is determined by image contrast and the diffraction of light within optical systems.

Although users are always excited about using the most advanced or latest innovation associated with fluorescence microscopy, it must be clearly acknowledged that the best imaging can only be achieved by understanding the principles of fluorescence and microscopy, the methods of microscope alignment, the properties of light, the practicalities of wavelength selection, image recording tech-

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niques, and, finally, image analysis. Although commercial instruments can provide full access to fluorescence microscopy, it requires the user to understand what is "in the box" to correctly interpret the images collected. A good foundation is achieved by first considering wide-field fluorescence microscopy; this technique requires that we address all aspects noted above and allows us to subsequently highlight the advances of laser-scanning microscopy.

## PRINCIPLES OF FLUORESCENCE

This topic has been addressed in depth in many resources, because it is the fundamental phenomenon that makes fluorescence microscopy possible. New or inexperienced students of microscopy can find a wealth of information on many aspects of light and microscopy at several excellent interactive websites (http://www.olympusmicro.com, http://www.microscopyu.com, and http://zeiss-campus.magnet.fsu.edu/index.html).

The underlying process of fluorescence involves the absorption of light energy (a photon) by an indicator followed by the emission of some of this light energy (as another photon) a few nanoseconds later. Because some energy is lost in this process, the emitted photon has less energy than the absorbed photon. Light with a short wavelength (toward the blue) has higher energy than light with a long wavelength (toward the red). Therefore, light emitted from an indicator usually has a longer wavelength than that of the absorbed (excitation) light. This change is called the Stokes shift. The molecular transitions explaining these processes can be depicted in terms of Jablonski energy diagrams (Fig. 1).

### One- or Two-Photon Fluorescence

The above scenario applies to the absorption of single photons; each photon absorbed results in the release of a lower energy photon. However, it is also possible for an indicator to simultaneously absorb the energy from multiple photons but only emit one photon. Importantly, the energy of each of the two absorbed photons is less than energy of the emitted photon. Thus, red light can be used to generate green light. This principle underlies "two-photon" or "multiphoton" microscopy (Zinselmeyer et al. 2009). This process can only be achieved by having a very high spatial and temporal density of photons, because the two absorbed photons must arrive simultaneously, which requires a relatively specialized, high-powered, pulsed laser (Ti:Sapphire). The pulses serve to package the photons. There are considerable advantages of using red light instead of blue light as an excitation source. In particular, longer-wavelength light can penetrate deeper into tissues better than shorter wavelength light because it is scattered less (Helmchen and Denk 2005). The absorption of high-energy
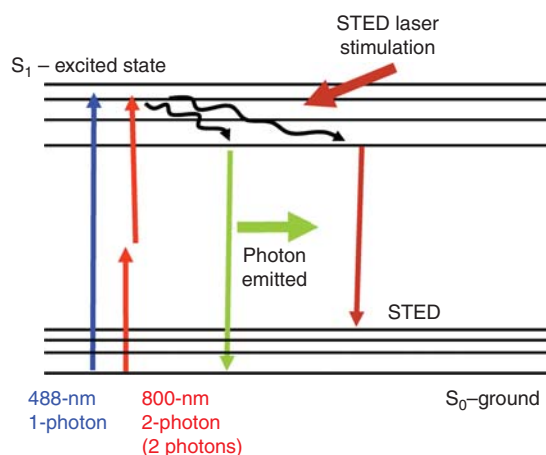


**FIGURE 1.** Changes in electron state of fluorescent indicators during photon excitation and emission (Jablonski profiles). Excitation (from $S_0$ to $S_1$) induced by 488 nm laser light (blue) requires one photon or by two-photon 800 nm light (red) requires two photons. After relaxation to the lowest energy levels, the reverse transition (from $S_1$ to $S_0$) releases a photon of longer wavelength (green). The incidence of photons at 592 nm (STED wavelength) induces the transition from $S_1$ to high levels of $S_0$ and suppression of fluorescence.

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blue light also has the disadvantage that is more likely to damage cells. Following the concept that red light is scattered less, two-photon excitation of thick tissues is best coupled with an indicator that fluoresces and emits photons in the red region.

In conventional imaging, spatial resolution is dependent on the wavelength of the excitation light and the microscope optics. In practice, with one-photon confocal imaging, this yields lateral and axial resolution of ∼200 and ∼500 nm, respectively. Super-resolution techniques, such as stimulated emission depletion (STED), can circumvent this "diffraction limit" and improve spatial resolution by an order of magnitude. As will be discussed in more detail, STED microscopy achieves this increase in spatial resolution by reducing the size of the excitation spot by overlapping the excitation laser with a second longer wavelength laser in the shape of a "donut" (see Fig. 10) (Hell 2007). The reduction in size of the excitation zone is achieved by a process that also exploits the Jablonski energy transitions. In the zone of laser overlap, the longer wavelength excitation light is used to quench undesired specimen fluorescence. When the excited indicator (resulting from the absorption of a photon at the excitation wavelength) is stimulated with additional photons at the STED wavelength, it is forced to back to the ground state and thereby loses the ability to fluoresce (Fig. 1).

## FLUORESCENCE LIGHT SEPARATION

The goal of the fluorescence microscope is to separate emitted light (dim) from excitation light (bright) (Fig. 2); fluorescence indicators with large Stokes shifts are advantageous for this. The separation of the light is generally achieved with optical filters and the key to successful imaging is their selection with respect to the indicators being used. Details of filter specifications and their selection relative to various indicators can be found at the manufacturer's webpage. Two excellent sources are Semrock (http://www.semrock.com) and Omega Optical (http://www.omegafilters.com/Products/Curvomatic).

Although it is often reported that a particular indicator has a single absorption and emission wavelength (a maximum peak value is often cited in the indicator specifications), in reality indicators, especially genetically encoded fluorescent proteins, have a broad spectrum of excitation and emission wavelengths. Moreover, the excitation and emission spectra of different indicators frequently overlap, thereby increasing the importance of considering the appropriate excitation and emission wavelengths when multiplexing indicators. Filter choice is generally a best compromise between passing emitted light and blocking excitation light.
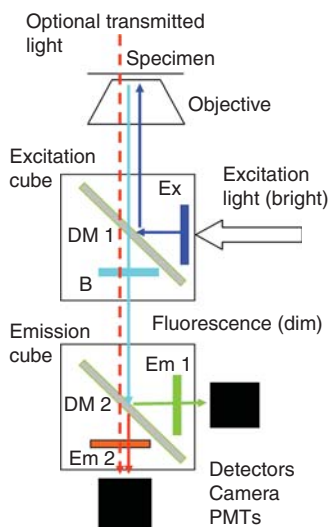


**FIGURE 2.** The basic light paths of a fluorescence microscope. An excitation filter cube containing a dichroic mirror (DM 1) directs excitation light (from a bulb or laser; filtered with an excitation filter) to the specimen and passes emitted fluorescence to the emission cube for further separation (multiple indicators). The barrier filter B prevents excitation light from reaching the detectors. Emitted fluorescence is separated by DM 2 into two beams. The emission filters (Em 1 and Em 2) block unwanted light. Fluorescence is detected by cameras (wide-field) or PMTs (laser-scanning). For transmitted illumination of a bright-field image, long wavelength light is selected.

## The Filter Cube

In many fluorescence microscopes, the filter cube is the conventional element used for aligning filters in the excitation and emission light path (Fig. 2). The traditional filter cube typically carries an excitation filter, a dichroic mirror at 45°, and a barrier/emission filter; the cube directs light from the excitation source to the specimen and from the specimen to the detector. An important issue when loading filters into the cube is their orientation. Because many filters work by reflected interference, it is important to ensure the filter is correctly orientated in the direction of the light path. The front surface of the dichroic mirror is the reflective side. In most cases, the dichroic mirror is marked in the corner on the reflective side (white dot or label number) and mounted filters have directional arrows. In many modern fluorescence imaging systems, excitation and emission filters are not located in a filter cube, but rather in an automated filter changer where they can be rapidly switched in and out of the light path. In addition, excitation light can be dynamically selected by devices such as a monochromator or an acoustooptic modulator, potentially avoiding the need for an excitation filter.

## Dichroic Mirror

Initially, fluorescence microscopy was performed with transmitted light, but because the emitted fluorescence is weak in comparison to the excitation light, it was necessary to view the fluorescence with filters capable of blocking very bright transmitted excitation light. Overall, this scheme was not very practical and a reflected optical design was adopted. With this epi-illumination, the dichroic mirror serves as the primary optical element separating the excitation and emission light. The common mode of operation is that the shorter excitation wavelength is reflected toward the specimen and the longer emitted wavelength is transmitted by the dichroic mirror. However, dichroic mirrors can also be used to reflect longer wavelengths and to transmit shorter wavelengths. Dichroic mirror specifications are also not perfect and are usually defined in terms of the wavelength that is reflected/transmitted at ~50%. The transition between reflection and transmission is rarely precise and tends to encompass a range of wavelengths. This problem is minimized if the indicator has a large Stokes shift and the peak absorption and emission wavelengths are well to either side of the dichroic mirror's transition zone. However, with a small Stokes shift, care must be used to ensure the dichroic mirror's transition zone lies between the indicator excitation and emission wavelengths. Newer dichroic mirror design (hard-coated filters) results in narrower transition zones; so, this problem is becoming less of a concern. In addition to transition zone imprecision, dichroic mirrors can also be reflective or transmissive at additional wavelengths above and below the center wavelength. When using monowavelength sources, such as a laser, this problem may not occur, but the use of a broad spectrum source, such as a mercury bulb, may result in the illumination of the specimen with spurious light. The full spectrum of the dichroic mirror (potentially ranging from 300 to 900 nm) should be evaluated before selection; spectra are available at the manufacturer's websites.

## Blocking Filters

To enhance the selection of appropriate excitation/emission wavelengths in addition to the wavelength discrimination by the dichroic mirror, it is common to add excitation and emission filters (Fig. 2). The excitation filter ensures only the required wavelengths are transmitted. Usually a band-pass filter with good blocking ($10^6$) of light outside the specified band-pass wavelengths is used. However, a key consideration of fluorescence microscopy is obtaining sufficient light to create a useful image; passing light through filters always results in a loss of intensity. Modern light sources have a high light intensity, minimizing this concern for the excitation filters, but it is an important criterion when assessing the suitability of emission filters. The bandwidth (spectral range that passes through a filter) and percentage of the light transmitted by the filter will determine image brightness. When using a single indicator, there is only one source of fluorescence. Thus, the emission filter needs only to block any spurious excitation light. Here, the choice of a long-pass filter has the advantage that all the light from emission spectrum can be collected. Blocking filters must be especially good for total

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internal reflection (TIRF) microscopy, because a large fraction of the excitation light is reflected directly back into the objective.

## Orientation of Dichroic Mirrors and Filters

Often manufacturers indicate the orientation of a filter by either an arrow that should point in the direction of the light propagation direction or some form of marking to indicate the front surface that should face the light source. This minimizes spatially translated reflection from the uncoated side. Orientation of interference filters (filters that operate by reflecting or transmitting light rather than absorbing light) is not important but in older filters that also incorporated absorptive elements, the reflective elements are best oriented facing high-intensity light sources.

## Multiple Fluorophores

Frequently, two or more indicators are utilized to perform comparative studies. In view of the relatively broad excitation and emission spectra of the indicators and less than perfect filter characteristics, the simultaneous monitoring of multiple indicators can be challenging because of "bleed through" or cross talk of wavelengths from one indicator to the other. The cleanest approach is to take separate, sequential images with filters specifically designed for each indicator, but if multiple indicators are in the preparation some cross talk is still likely. However, the sequential collection of images with different filter cubes requires the careful alignment of both the dichroic mirror and blocking filters if a shift in image position is to be avoided.

Nonetheless, many studies require simultaneous observation of multiple indicators within a sample, which complicates the selection of filters and dichroic mirrors. In the case of two indicators, the dichroic mirror has to reflect two peak excitation wavelengths while transmitting the emitted light between the peaks. The emitted light can be separated into different wavelengths using a second dichroic mirror together with an appropriate filter (Fig. 2). Using more than two indicators adds further complexity and requires a primary dichroic mirror with multiple reflection/transmission peaks. In theory, multiple indicators can be used, providing their excitation/emission characteristics allow them to be discretely resolved. It should be noted that as more indicators are used the bandwidth available for detecting emitted light becomes progressively narrower and the resultant image will be dimmer. Modern microscope systems with spectral imaging capacity use algorithms that allow even closely overlapping indicators to be mathematically separated (see Spectral Imaging). However, this technique is too laborious for kinetic experiments.

## WIDE-FIELD FLUORESCENCE MICROSCOPY

The basic fluorescence microscope is a common tool of modern cell biologists. In this instrument, a parallel beam of light simultaneously illuminates the whole specimen (or wide-field of view) to excite (via the filter block) the fluorophore(s) it contains. Traditionally, excitation light is provided by a mercury or xenon high-pressure bulb and the required wavelengths are selected with custom optical filters (as described above). In contrast, laser scanning microscopes illuminate only a small point of the specimen at any instant and the laser beam therefore needs to scan across the specimen to create an image. A new approach for excitation light that is rapidly being adopted is the use of bright, single-wavelength light-emitting diodes (LEDs). LEDs have the advantages of long life, fast switching (no shutters required), and tight wavelength control. Multiple LEDs may be required for different indicators although white LEDs could be used. LEDs may obviate the need for filter wheels. Mercury/xenon bulbs can also be replaced by longer-lasting fiber-coupled metal halide lamp systems.

The resulting fluorescence in the specimen is viewed by eye or captured electronically. The simplicity of wide-field microscopy is that all parts of the specimen are viewed simultaneously and an image can be easily captured with a camera (Fig. 2). However, this simplicity, in conjunction with diffraction-limited optics and the unavoidable projection of out-of-focus light onto the single image

plane of the camera, can result in images of low contrast and spatial resolution. Nonetheless, the wide-field fluorescence microscope is commonly used. The key to its successful use lies with the specimen selection.

Because image quality is degraded by examining specimens that have material outside the plane of focus (above and below), the use of thin specimens can readily generate informative images. Thus, layers of single cells are well suited to wide-field microscopy. Better still are macroscopic structures, such as organelles, that are very thin and often closely adhered to a glass surface. Another cause of reduced image resolution is the proximity of fluorescent objects to each other. Because of optical diffraction, each fluorescent object generates a relatively broad zone of fluorescence (cone shaped intensity profile defined as Airy rings) that is substantially bigger than the actual object. When these zones of fluorescence overlap, the neighboring objects cannot be resolved (the Rayleigh criterion). Thus, if there are many closely associated fluorescent objects, the resulting image will depict a bright blur with little structure rather than discrete objects. However, the examination of a series of more sparsely spaced samples of the fluorescence material can render valuable images, which is the basis for super-resolution microscopy using techniques called photoactivated localization microscopy (PALM) or stochastic optical reconstruction microscopy (STORM). In these techniques, fluorescence from individual indicators is separated by imaging their fluorescence at different times. More simply, separating the fluorescence indicators or objects can be achieved by effectively spreading out the observed material as might be the case with isolated structures. Alternatively, the indicator can be partitioned to selected sites, perhaps by specific antibody labeling or by site-specific trafficking of expressed fluorescent proteins. For example, the expression of a fluorescent protein with a mitochondrial targeting sequence will give relatively clear images of the mitochondrial distribution.

In contrast to imaging discrete objects, imaging ions (e.g., $Ca^{2+}$ or $H^+$) usually involves loading the cell cytoplasm with a soluble, diffusible indicator (e.g., Fluo-4 or SNARF). The rapid mobility of these indicators can increase image blurring as imposed by the Rayleigh criterion on fixed objects. Furthermore, $Ca^{2+}$ and $H^+$ signals are often transient and therefore not amenable to techniques involving signal averaging. For wide-field microscopy, the images from fluorescent ion indicators are greatly improved when monolayers of thin cells are used. For example, it is possible to resolve $Ca^{2+}$ waves that spread diffusively across, or between, cells. Although some of the details of these patterns (e.g., leading edge) or underlying elemental $Ca^{2+}$ release points (i.e., clusters of $InsP_3R$ or $RyR$ $Ca^{2+}$ channels) will not be sharp, the overall changes are readily observable. It is also important to appreciate that image sharpness can also depend on temporal resolution; for a propagating $Ca^{2+}$ wave, a relatively long exposure time will result in a blurred image. A balance between image brightness and exposure time must be reached to track dynamic $Ca^{2+}$ signals.

The loss of image contrast due to out-of-focus light can be compensated to some degree by reducing the objective numerical aperture (NA, closing the objective iris). Normally, the NA would be maximal to collect all the diffracted light to enable the best image resolution. However, a reduced NA can eliminate some of the out-of-focus light (thereby collecting only useful light) to increase image contrast. A balance between improved image quality and decreased signal-to-noise must be established. We have had success with this approach in recording intercellular $Ca^{2+}$ waves in cultured epithelial cells in real-time (Sanderson et al. 1990).

An advantage of wide-field microscopy is that all parts of the image are illuminated simultaneously, allowing for faster imaging. However, the rate of image acquisition is generally determined by the acquisition capabilities of the camera or photomultiplier system being used. In general, lower excitation intensities are used in wide-field imaging which should result in less photobleaching and phototoxicity. It is also common to use wide-field imaging to observe specimens in real-time by eye; this is a great benefit for user interaction and selection of fluorescent cells to image but care must be taken to minimize observation times and photobleaching, especially if the sample is to be imaged after manual observation. A major advantage of the wide-field microscope is the low cost, simplicity, and flexibility of the system. In contrast, some disadvantages of wide-field microscopy include low image resolution, potential for shading artifacts due to uneven illumination, and the alignment of different cameras to ensure pixel registration when using multiple indicators. The camera problem can be

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partially addressed with specialized beam-splitting devices or cameras that use the same detector, but the problem of pixel registration is not always eliminated.

### Combined Transmitted and Florescence Microscopy

An important requirement when presenting fluorescence images is to provide sufficient information to inform the viewer with regard to the orientation of the sample. A reference image could be of another fluorophore with a well-characterized distribution. Alternatively, the reference could be an overlay of the fluorescence image on a traditional transmitted microscopy image taken with DIC or phase-contrast optics. Such an image can be achieved by simply turning off the fluorescence system and changing optics. However, specimen movement must be avoided. Alternatively, especially if examining dynamic processes, the transmitted light image can be obtained simultaneously by exploiting different wavelengths of light that are reflected or transmitted by the dichroic filters in the microscope (Lansley and Sanderson 1999).

For this approach, long wavelength light (red) is used for the transmitted image in combination with shorter wavelength light (green) for the fluorescence image (Fig. 2). Both the reflected green and transmitted red light will pass the excitatory dichroic mirror. A subsequent dichroic mirror, much like that used to separate multiple indicators, reflects the green light to a high-sensitivity camera while the red light is transmitted to a second specialized camera (Fig. 2). Again, image registration is required. This procedure can be performed digitally. Imaging a group of small fluorescence beads with both cameras can be used to determine the offset in bead locations. All subsequent images can be corrected with this offset.

Lens chromatic aberration may be detrimental to image resolution in this technique. Because most objectives have a focal length that is calibrated for mid-range wavelengths ($\sim$550 nm), images obtained with the same objective, but with different light wavelengths, could be in focus at slightly different planes. If the same camera is used to record these images, some blurring of one image may occur. Refocusing after each wavelength change will alleviate this issue, but it will greatly slow image capture. Fortunately, modern apochromatic objectives are well corrected and largely circumvent this problem.

In a related method, fluorescence images from different planes of a specimen can be simultaneously obtained using one objective and two separate cameras that are independently focused. We have exploited this approach to focus on different parts of the specimen to examine ciliary activity with respect to changes in cellular $Ca^{2+}$ (Lansley and Sanderson 1999). These two processes occur at different locations; the cilia project above the cell for up to 5–8 μm, whereas the $Ca^{2+}$ changes occur within the cell body at the base of the cilia. To record the ciliary activity, we used a high-speed video camera (recording 200 fps) focused near the tips of the cilia (Lechtreck et al. 2009). To record the $Ca^{2+}$, we used a low-light-sensitive camera (recording at 30 fps) focused at the cell level. Focusing for one camera is achieved conventionally by moving the objective position; focusing of the second camera at a different plane is achieved by moving the camera. Video synchronization is also required in the time domain and is achieved by the use of a visible event marker (white light flash) in both image sequences.

## CONFOCAL MICROSCOPY

The use of thin or sparse specimens is a severe limitation to many research endeavors, but with the addition of specimen thickness, increased out-of-focus light severely hinders imaging. Fortunately, a solution can be found with laser-scanning confocal microscopy (LSCM) (Pawley 2006). All of the major concepts of fluorescence excitation and emission also apply to LSCM. The major differences are the excitation source (a bright point source, i.e., laser), a sequential scanning method of the point source of illumination and the detection of emitted light intensity with a photomultiplier tube (PMT). The principles of operation of a spinning disk confocal microscope are similar, but this microscope records light intensity with a camera as will be discussed later.
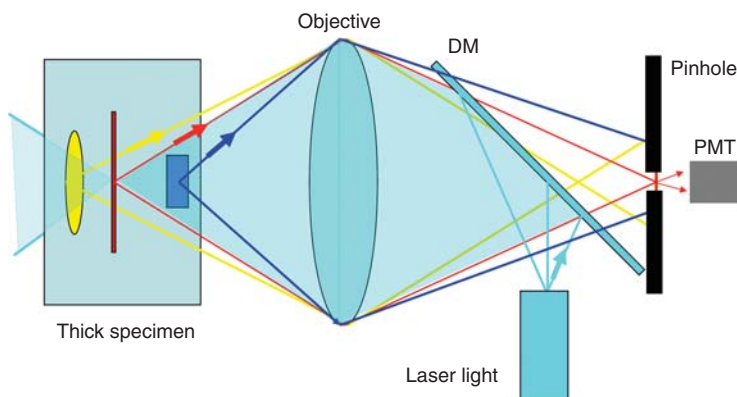
**FIGURE 3.** Principle of operation of a laser scanning confocal microscope. Laser light is focused on the thick specimen by reflection from the dichroic mirror (DM) and the objective lens. The laser excites fluorescence throughout the specimen that passes through the DM and is focused onto the image plane. A pinhole only allows light from the confocal plane of the specimen to reach the photomultiplier tube (PMT).

The goal of confocal microscopy is to reject out-of-focus light from the image. This is achieved with a pinhole aperture (Fig. 3) that ensures that light reaching the detector comes only from the equivalent (confocal) point in the specimen where the excitation light was focused. By scanning the confocal excitation and detection point across the specimen, an image can be sequentially compiled, pixel by pixel, by recording the fluorescence intensity at each position (Fig. 4). The laser and pinhole remain stationary, but the location on which they are focused is optically moved across the specimen by an orthogonal pair of oscillating mirrors.
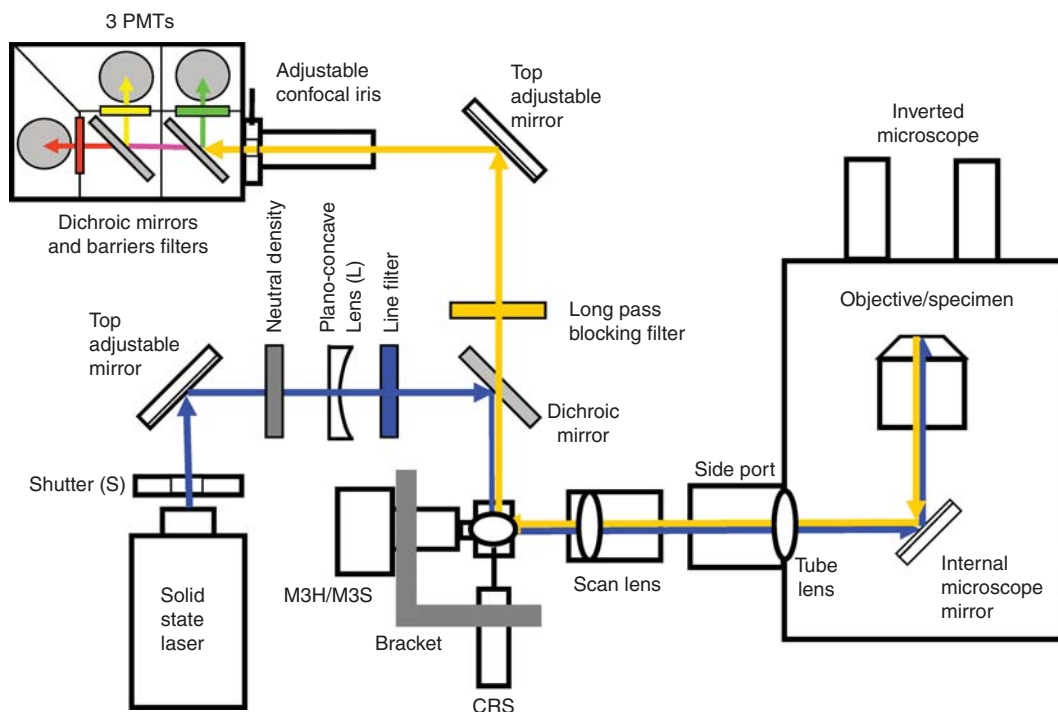


**FIGURE 4.** Basic elements of a laser scanning confocal microscope. Laser light is directed to the scan mirrors via a dichroic mirror. The laser is scanned across the specimen by the scan mirrors and the returning emitted fluorescence is descanned by the same mirrors and transmitted by the dichroic mirror. The fluorescence passes through the barrier filter and is focused on to the pinhole (adjustable iris) before reaching the PMTs. Additional dichroic mirrors can be used to separate fluorescence wavelengths.

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Most of the complications of a confocal microscope are centered on the scanning optics, image creation from intensity detection and the packaging of the optical train into a small box. Our approach has been the construction of a "home-built" microscope (Sanderson and Parker 2003); perhaps surprisingly the complications are not as daunting as might be expected (Fig. 4). However, our focus here is not construction (detailed elsewhere [Sanderson and Parker 2003]) but rather on the use of LSCM and the relevant points to understand image formation.

### Dwell Time

This simply refers to the time that the laser spot remains in one location within the image/specimen. As described above, the laser beam is continually scanned across and down the sample and the image is constructed from discrete pixels that are compiled as the laser scan proceeds. The pixel intensity results from the time-averaged response of the PMT to the emitted fluorescence as the laser spot moves across this small unit of distance.

The dwell time is a function of image speed (images recorded per second) and pixel resolution. For example, if a system records at 30 images per second, and each image has 512 horizontal pixels and 512 vertical lines, the dwell time would be in the order of 127 ns (i.e., 33 ms/(512 × 512); this assumes no losses in time between each image). Clearly, each part of the image is only illuminated for a very short time in comparison to wide-field microscopy where each part of the specimen would be exposed for ∼33 ms (at 30 images per second). Hence, with confocal microscopy, there is a need to have a bright excitation source and very sensitive detection modules. An increase in dwell time will increase the numbers of photons that can be captured and this will increase image brightness and may improve signal to noise. At the same time, increased dwell time can contribute to indicator bleaching or cellular photodamage.

### Pinhole Size

This is a key determinant in image resolution; the smaller the diameter of the pinhole, the greater the exclusion of out-of-focus light (Figs. 3 and 5). Reducing the pinhole size also reduces the image intensity, as fewer photons can be captured in the given dwell time. The usual compensation is to increase laser illumination, which can result in bleaching as noted above. Alternatively, PMT gain can be increased but such an increase will also enhance shot noise. Thus, a compromise between image
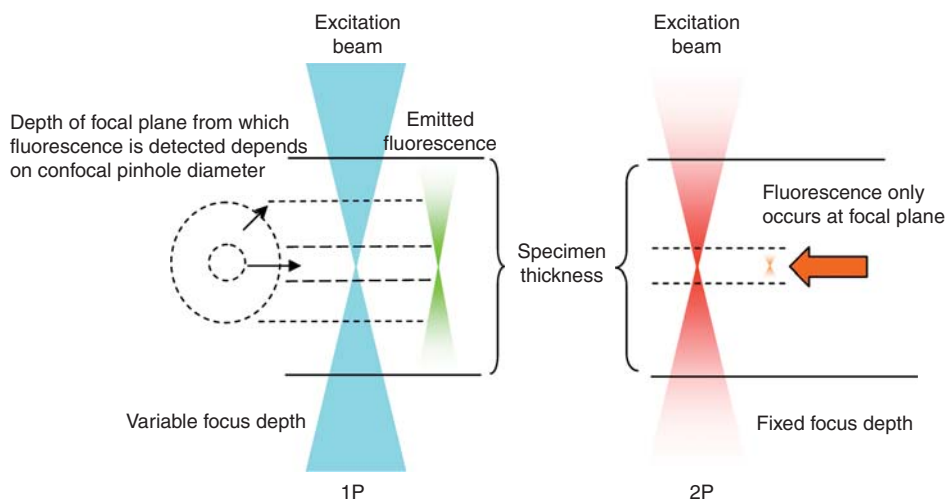


**FIGURE 5.** Determination of depth-of-field and bleaching in one- or two-photon microscopy. In one-photon microscopy (1P, blue), a cone of excitation light on either side of the focal point illuminates and excites the fluorescence indicator throughout the specimen. The size of the pinhole determines the depth of fluorescence detection. In two-photon microscopy (2P, red), the photon density is only sufficiently high at the focal plane to induce indicator fluorescence. This beam has a fixed depth of excitation.

resolution and signal-to-noise will dictate the useable pinhole size. Most microscopes have a selection of pinhole sizes that can be changed as required.

It is important to note that although the pinhole might be small, and thereby provide good axial resolution, the specimen remains fully illuminated by the cone of excitation light throughout its thickness (Figs. 3 and 5); the pinhole restricts light getting to the detector, but not to the specimen. Therefore, photobleaching occurs continuously in all planes of the specimen. Again, a compromise between image resolution, illumination intensity, and photobleaching rates is required.

## Image Speed

With wide-field microscopy, the rate of image capture is determined by the image acquisition technology and image brightness. A variety of camera types exist, ranging from high-resolution devices with large pixel array detectors, to high sensitivity electron-multiplying cameras (EMCCD). Image acquisition is usually mediated by a digital transfer to computer memory. The computer hardware can also be a limiting factor for image capture speed.

With confocal microscopy, another major limiting factor in imaging rate is the speed of the laser raster scan to create the image (assuming sufficient brightness can be achieved at each pixel). With point-source laser scanning, two oscillating mirrors generate the $x$, $y$ scan (Fig. 4). The two mirrors move at different rates. The vertical scan ($y$) mirror only moves a small distance (and therefore moves more slowly) at the end of each scan line. The mirror generating the horizontal ($x$) scan has to move much more quickly across the image and then rapidly reset to begin another scan. Unfortunately, the speed of the horizontal mirror is severally hindered by its inertia, making it difficult to quickly stop and start the mirror and achieve a linear scan essential for undistorted images.

Because of this scan limitation, many confocal systems that use standard galvanometer scanners can only acquire five to 10 images per second, increasing the dwell time substantially. Although this rate is sufficient for many applications, faster imaging may be needed for studying $Ca^{2+}$ dynamics. Faster imaging has been achieved in some confocal microscopes by scanning the horizontal beam using an acoustooptic modulator rather than a mirror or by scanning a laser line rather than a point source. Doing so greatly speeds up the imaging rate, but some resolution is lost because of adjacent pixel illumination.

Another approach to achieve fast imaging rates is using a resonance scanner in place of a galvanometer scanner for the horizontal laser sweep. The resonance scanner has lower inertia and can oscillate at high frequencies (various models range from ~4 to ~16 kHz) and scans a horizontal line (forward direction) in 62.5 μs at 8 kHz. By utilizing the forward and backward oscillation for scanning, the resonance scanner can produce 30 images per second with 512 scan lines. Unfortunately, the resonance scanner can only oscillate forward and backward with the same period and the angular velocity during this period follows that of a sine wave. This has the effect of stretching the image at its edges as the speed of the laser scan slows relative to a fixed sampling rate of the pixel clock (Fig. 6). On the other hand, the relationship between laser position and time is well defined, stable, and easily corrected using appropriate software in real-time by a pixel relocation look-up table (Sanderson 2004). As a result, high-speed linear images can be observed at any magnification (Fig. 6). Image speed can be increased at the expense of image height. A total of 512 lines per 1/30 sec are available; thus the microscope can run at 1024 lines at 1/15 sec, 256 lines at 1/60 sec, or 128 lines at 1/120 sec. In practice, a loss of numerous lines occurs to accommodate the resetting of the vertical mirror. The
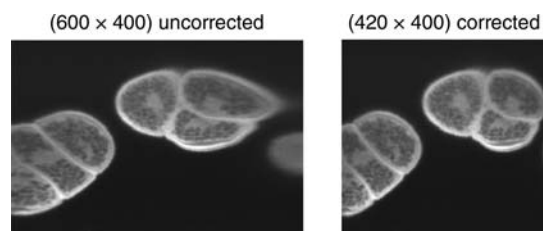
(600 × 400) uncorrected    (420 × 400) corrected



FIGURE 6. Images collected with a resonant scanner. *Left* image shows distortion resulting from the slowing of the scan toward the image edges. *Right* image shows the same image after correction that linearizes the scan rate to remove edge distortion.

speed of the laser scan remains the same—thus, horizontal resolution and dwell time remain equal at all frame rates.

The advantages of fast imaging are reflected by the short dwell time. It must be remembered that short dwell times limit the excitation of the indicator and hence the emitted signal. However, the averaging of images increases signal to noise with less risk of bleaching. The high-speed imaging rate also means that the microscope is interactive in real-time making focusing, gain control, pinhole size adjustment, and specimen searching easier. With the collection of multiple images by high-speed imaging, the nature of the averaged images can be manipulated by off-line processing. Simple averages can be computed by adding multiple frames. Running averages can be created to smooth noise with less effect on dynamic events. Often, much of the image is not of particular use (e.g., a long cell); by orientation of the cell with the horizontal scan, more images of the relevant area can be achieved.

## Line Scan

For extremely fast recording, a line scan option allows data to be collected at the speed of the resonance scanner (62.5 μsec/line). Line scanning can also be performed with galvanometer scanners but at a slower line rate. The position of the line scan is determined by the adjustable, but stationary position of the vertical scanning mirror. This recording format produces images with a spatial dimension on one axis and time on the other axis, which are very useful for tracking fast events. The trick of course is to get the site of interest located on the line. The dwell time for each pixel on the line is identical to that when using two-dimensional imaging. Line scanning can also be performed on commercial microscopes with user-defined lines (nonlinear and in any direction). Because this type of scanning requires coordinate positioning of the two scanning mirrors, it is likely to be slower than horizontal (single mirror) line scanning.

## Photomultiplier Gain

PMTs are the detection device of the confocal microscope mainly because of their speed of response (note that spinning disk confocal microscopes use cameras, not PMTs; see below). In terms of their quantum efficiency, PMTs are relatively poor. However, this technology is constantly changing for enhanced sensitivity; newer detection devices with higher sensitivities and quantum efficiency include avalanche PMTs or InGaAsP PMTs. For the most part, the user cannot control the type of PMT in an instrument. However, it is useful to understand some aspects of their function. Increased temperature has the effect of increasing background noise and PMTs should be operated at a cool temperature if possible. The sensitivity range of the PMT is not constant across the light spectrum, which may be important for comparative intensity measurements; PMT sensitivity can rapidly decline >650 nm, which may impact the use of long-wavelength indicators.

As mentioned above, sufficient pixel brightness is vital for good images and is generally expressed in terms of high signal to noise. Image brightness is directly related to the number of photons reaching the detector within the dwell time. The brightness of the recorded digital image can be adjusted by altering the gain of the PMT, but this should be set with care. There are several points to consider. First, the dynamic range of the image should be adjusted such that it does not saturate the detector or the image digitizer. In most 8-bit systems, the responsive combination of the detector and digitizer provides 256 gray levels. If the image is relatively static, the brightest value should not saturate the detector (i.e., the brightest pixel values should be close to, but <255). More care is required if any increase in image brightness is expected. In $Ca^{2+}$ imaging, an increase in image brightness would typically occur because of $Ca^{2+}$ increase. Some idea of the expected intensity change and therefore gray-level range is required. Thus, at the beginning of an experiment, pixels values may only be spread across a few low gray scale levels whereas the pixel values may subsequently increase up to 255.

A constant challenge is to obtain the required number of images over time with minimal phototoxicity and bleaching. More images will be possible with the system at its most sensitive so that it can detect the maximum number of photons. The simplest method to avoid phototoxicity and decrease bleaching is a reduction in laser intensity. This reduction can be achieved by decreasing the laser power

or by the insertion of neutral density filters into the excitation path. However, doing so will also decrease the numbers of photons emitted, so it is essential to initially optimize the system to capture all photons that are available. This requires, when possible, the use of high NA objectives, the minimization of light losses (a simplified optical pathway with as few elements or filters as possible), and high quantum efficiency detectors. Following this, the PMT gain should be set at a level to be able to see minimal shot noise when viewing a nonfluorescent field of view. It may be tempting to reduce the gain setting to have a noise-free, black background, but this is incorrect. Setting the PMT gain too low will result in some of the fluorescence in the sample not being detected. It is possible that when the PMT gain is set to see minimal shot noise as described above, fluorescent areas within the specimen may be very bright and could potentially saturate the available gray levels. If so, laser intensity must be attenuated. The most beneficial method is to decrease laser intensity, because this will reduce bleaching. Alternatively, the indicator concentration in the cell may be unnecessarily high. Although most indicator loading protocols call for a standard indicator concentration and incubation time, it is likely that many different cell types have unique indicator uptake or retention rates. It is therefore recommended that indicator loading be titrated against the sensitivity of the user's system. If the microscope has inherent low light sensitivity or an inadequate PMT gain setting, the risk of indicator overloading is high. In terms of $Ca^{2+}$ signaling, this should be avoided as the indicator can serve as a buffer to alter the $Ca^{2+}$ signals being measured. However, reducing indicator loading too much will reduce the indicator reservoir that normally can help compensate for indicator losses due to bleaching or extrusion.

## Lasers

A key part of the confocal microscope is the excitation lasers. The most common excitation wavelengths on commercial confocal systems include 488, 543, 592, and 635 nm. These wavelengths are suitable for many fluorescent proteins, and visible-wavelength $Ca^{2+}$ indicators such as Fluo-4, Rhod-2, and Fura Red. Although some lasers can provide multiple excitation lines, combinations of lasers are often required for simultaneous imaging of multiple indicators. Excitation lines from multiple lasers must be aligned to emit from a confocal point and follow the same optical path, which is most easily achieved by merging the laser sources into a single optic fiber. The choice of laser is primarily dictated by the required excitation wavelength. However, there are other considerations. The utility of ion lasers that have multiple excitation lines is overshadowed by their heat production and short life span. More reliable and long-lasting solid-state lasers are available in a wide range of wavelengths. The ability to individually steer different laser beams into a microscope allows for greater flexibility and the use of a wider range of indicators. With a fixed laser choice, indicator selection is predicted by the lasers available. Laser brightness control involves a direct adjustment of laser power or by the attenuation of the beam with neutral density filters. A polarizing filter may be used to attenuate laser power. Because lasers are very narrow in their wavelength output, an excitation filter is not usually required and if a single indicator is being used, a laser-line blocking filter can be used to allow the maximal amount of emitted fluorescence to be collected.

## Spectral Imaging

An alternative way to address the issue of fluorescence cross talk when using multiple indicators is spectral imaging in combination with image analysis often called "linear uncoupling." With this approach, the contribution to image intensity by each indicator can be determined on a pixel-by-pixel basis and separated. This capability is available on commercial microscopes. The technique requires that a series of images at different emission wavelengths are collected—a lambda stack. There are a variety of ways this can be done but generally involves the spectral dispersal (with a prism or diffraction grating) of the light passing through the confocal pinhole before detection with the PMT. A movable slit can select the emission wavelength to be viewed. Alternatively, a multiport PMT can be used to capture the different wavelengths (∼10 nM bandwidths). A set of reference lambda stacks obtained under identical conditions is required for each indicator used in the exper-

imental image. From these two data sets, the analysis software computes the weighted contribution of each indicator at each pixel location and removes the cross-talk contribution. This approach is very useful for multi-indicator imaging but has limited application if speed is required.

## Spinning Disc Confocal Microscopy

Because acquisition speed has been a major concern for the study of cell physiology, alternatives to slow image acquisition confocal microscopes have been sought. We have described how the use of a resonant scanner can increase image acquisition rates but this approach has some disadvantages because the dwell time can be very short, which may require higher illumination intensities as well as limiting photon detection time.

An alternative approach is based on the use of multiple scanning points rather than a single-point scanner. In this technique, the specimen is both illuminated and viewed through a spinning disk that has rows of pinholes aligned with each other in a repeat pattern of an Archimedean spiral. As the disk spins, each pinhole serves as a point source of light that scans across the specimen. The returned fluorescence must also pass through a corresponding pinhole before being separated by a dichroic mirror in manner similar to all the microscopes described. For increased light transmission, a micro-lens associated with each pinhole focuses the light on the pinhole.

By scanning with multiple pinholes, the dwell time for each pixel is considerably longer even through the image acquisition rate is fast. Therefore, the illumination intensity can be reduced to decrease photobleaching. Another advantage is the generation of real-time, color confocal images that can be observed with the eye or a camera. The camera is usually an electron-multiplying charge coupled device (EMCCD) that has high sensitivity for fast imaging rates. These qualities have made the spinning disk confocal microscope popular for dynamic fluorescence measurements, such as $Ca^{2+}$ signals. However, the spinning disk confocal microscope has some disadvantages. These include some loss of resolution because of cross-talk between multiple florescence points in the specimen, limitations in its zoom capacity, and the inability to perform spectral imaging. In addition, the pinholes are optimized for a specific objective (typically 60×), and cannot be altered unless the disk can be changed. The design is also not compatible with two-photon microscopy.

## TWO-PHOTON MICROSCOPY

The confocal microscope improves image resolution by reducing the detection of out-of-focus light but is still limited in depth penetration of thicker tissues by the use of relatively short wavelength light caused by scattering and absorption. Hence, the quest for deeper tissue penetration and the development of intra-vital microscopy turned to the use of pulsed, longer wavelength lasers (Helmchen and Denk 2005). However, a second key feature of pulsed, long-wavelength laser excitation is the manner by which it excites fluorescence. As shown in Figure 1, indicator excitation can occur when two photons of long wavelength are absorbed simultaneously. For this to occur, the photon density has to be very high. Such high photon densities are achieved only when the laser is focused to a diffraction-limited spot centered on the focal plane of the objective (Fig. 5). The high photon density is also created by emitting short pulses of high power (~100 fsec pulse at 80 MHz). Importantly, the photon density increases as a quadratic function approaching the focal point and the necessary photon density only extends across a short distance (Fig. 5). It is only within this narrow zone of the focal point that indicator fluorescence can be stimulated. Thus, the two-photon mode of excitation mimics the effect of the confocal pinhole by eliminating any image-degrading effects of out-of-focus light, but in the two-photon microscope there is little out-of-focus light and no need for a pinhole.

The depth of the excitation plane is fixed in the two-photon microscope as it is a function of the focusing of the laser. In contrast, in the one-photon confocal microscope, the indicator excitation occurs continuously throughout the specimen, but the depth of the detected fluorescence emission

plane is determined by the size of the pinhole; by increasing or decreasing the pinhole size, the detected emission plane can be made deeper or shallower, respectively (Fig. 5).

## Laser Damage

Caution required with two-photon microscopy relates to the laser power at the specimen. It is not uncommon to have ~5–15 mW of average power. This compares to <1 mW for the one-photon confocal microscope. Fortunately, most of this power is restricted to the pulse, and thermal effects are minimized by the relaxation time between pulses. However, it is easy to turn up the laser power to compensate for poor indicator loading, incorrect wavelength selection, or pulse broadening caused by optical elements. With these higher powers, tissue damage quickly occurs and might even be mistaken for biological effects if it is not too severe. Again, the operating code is to work at the minimum levels that are practical.

## Implications for Two-Photon Microscope Design

In view of its reputation as an advanced imaging approach, it may be a surprise to learn that the two-photon microscope is essentially identical to a confocal microscope (Fig. 7). Image formation and detection is achieved in the same way with scanning mirrors and PMTs. A major advantage is that because fluorescence arises only from the focal plane, all photons that are captured can be attributed to an emission point source, irrespective of their scatter. Therefore, the PMTs used to capture the photons do not require image formation and can be moved closer to the specimen (Fig. 7), enhancing the sensitivity of the instrument as more photons can be collected. One aspect of two-photon excitation that may affect image intensity is a process called group velocity dispersion (GVD). GVD generally results from the inclusion of multiple optical elements in the excitation pathway and has the effect of broadening and reducing the peak power of the laser pulses. Fortunately, the inclusion of additional optical elements can compensate for this effect. All other microscope controls and optical considerations are essentially identical to the one-photon confocal microscope (Fig. 7).
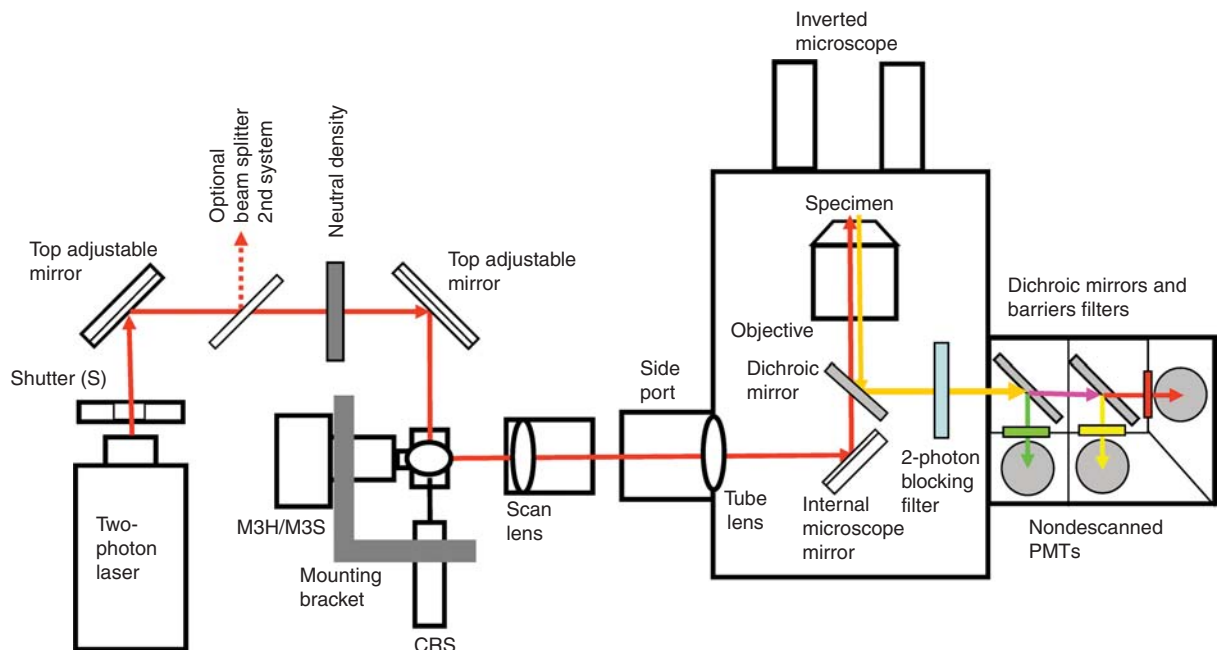
FIGURE 7. Basic elements of a two-photon microscope. PMTs detectors do not require pinholes or focused images and are located immediately below the objective. The design is for the most part similar to that of the one-photon laser scanning confocal microscope; see Figure 4.

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## Excitation Wavelength Selection

Another advantage of using two-photon excitation is that many indicators have a broad excitation spectrum (called two-photon cross section), which allows for multiple indicators to be simultaneously used without the need to change laser wavelength or switch filters. While the most recent versions of the Ti:Sapphire laser can be auto-tuned via a computer interface, the wavelength selection rate is not sufficiently fast to allow dynamic changes during experiments. Additional restrictions are also associated with wavelength selection. The most notable is a loss of excitation power; peak laser power occurs ~800 nm and significantly falls to its minimum ~1080 nm, the maximal wavelength of most Ti:Sapphire lasers. The actual power levels are dependent on the size of the pump laser driving the Ti:Sapphire laser; these range from 5 to 18 W. To image fluorescent protein indicators, it is common to work in the 900–1040 nm range with >300 mW of power.

The excitation wavelength (usually >780 nm) of the two-photon laser must still be separated from the emitted fluorescence (<700 nm) and a long-pass dichroic mirror is used. Because of the very bright intensity of the two-photon laser, the blocking specifications of the dichroic mirror and the accompanying barrier filter need to be very high, otherwise substantial background illumination can occur and reduce image contrast. The indicators excited in a two-photon system behave similarly as if they were excited by light in a one-photon microscope. The emission spectra of the indicators are identical to those observed with one-photon microscopy and the requirements and use of emission filters and dichroic mirrors to separate different emitted fluorescence wavelengths remains the same.

The selection of the correct wavelength for excitation is often a matter of tuning the laser through its spectrum and observing and selecting the best image brightness. If multiple indicators are used, it is likely that the wavelength selected will be a compromise giving the best excitation for each indicator. The idea that the two-photon wavelength selected should be equal to twice the one-photon wavelength (therefore same energy) for a particular indicator is not exact. For example, we frequently use Oregon Green which has a one-photon excitation maximum ~488 nm but we find that a two-photon excitation of 780 nm is ideal (Bai et al. 2009). However, wavelength selection is also associated with a change in laser power (peak power at ~800 nm). Therefore, optimal indicator brightness will be a function of wavelength and laser power and may vary between systems. Fluorescent proteins may have multiple excitation wavelengths; for example mCherry is excited ~760–780 nm but not again until the wavelength is >920 nm (Drobizhev et al. 2011). If the system is to be used at these higher wavelengths, it is essential to have sufficient laser pump power so that several hundred mWs of excitation light is available.

The desire to image deeper into tissues has driven the development of even longer wavelength lasers (up to 1600 nM). These lasers often use an optical parametric oscillator (OPO) to extend the wavelength range of the Ti:Sapphire laser. To reduce the amount of light scatter from the numerous interfaces encountered in tissues, fixed samples can be impregnated with a specialized media (Sca/e) to render the tissue more reflectively homogenous (Hama et al. 2011). This media is obviously of no benefit when imaging living tissues.

A recent advance in two-photon microscopy is the creation of images by scanning sheets of light rather than a spot of light. The detector (a camera) views the specimen at right angles to the scanned light. Again, because two-photon excitation limits the plane of excitation, only a thin plane of tissue is observed. However, the speed of scanning is greatly increased by the sheet approach (Truong et al. 2011).

## Photobleaching in the Two-Photon Microscope

Another benefit of the highly restricted zone of fluorescence in two-photon microscopy is that only a limited amount of the indicator is used to generate the image. Indicator outside of the focal plane is not excited and is therefore not subject to photobleaching. In terms of diffusible indicator, this means there is a large pool of indicator available to replenish the excitation zone for extended recording.

## Auto-Fluorescence

Clearly, two-photon microscopy is often the technique of choice for imaging intact tissue. As a result, auto-fluorescence is more of a problem than in other types of microscopy, because whole tissues contain the extracellular matrix (e.g., collagen and elastin), which is commonly auto-fluorescent. However, auto-fluorescence also occurs within cells. For example, when we examine lung slices, the epithelial cells strongly display auto-fluorescent vesicles. These vesicles are poorly visible with one-photon confocal microscope. Auto-fluorescence can interfere with imaging because it can mislead the observer to believe the fluorescent structure is related to the indicators used. A careful selection of filters can discriminate between fluorescence wavelengths, which might help determine the source. However, if the auto-fluorescence is of a similar wavelength to that of the indicator used, separation will be impossible. On the other hand, static auto-fluorescence can be useful for orientation, by providing an outline of the tissue structure. With dynamic responses such as $Ca^{2+}$ oscillations, static auto-fluorescence can be easily discerned. A key approach is to examine tissue in the absence of indicator to determine the extent and wavelength of auto-fluorescence. Doing so can help in terms of the sensitivity settings of the microscope and the choice of indicator. Spectral imaging can also be used to discriminate between indicator- and auto-fluorescence.

## Second-Harmonic Imaging

A special form of inherent tissue "fluorescence" can be viewed as a result of a second-harmonic transformation of the input laser wavelength. In this process, the wavelength of the input laser light is halved or the frequency doubled. Photons are not absorbed or released so true fluorescence is not occurring, rather this transformation is induced primarily by crystalline structures. As a result, regular repetitive structures such as collagen or striated muscle can be readily seen as bright entities when viewed with the appropriate filters. Although the second-harmonic light can be detected in the reflected mode (similar to fluorescence signals), the phenomenon is more prominent in the direction of light propagation and is therefore best detected by trans-illumination (Fig. 8).

## Trans-Illumination Imaging

In conventional microscopes, the optical element opposite the objective, on the far side of the specimen, is the condenser lens. This lens is generally of low NA in the fluorescence microscope and has a long working distance (especially with inverted microscopes). Although these parameters are intended for adjustment of bright-field illumination, they can be used to obtain acceptable trans-illuminated images of the specimen for reference purposes. Most of the excitation laser light passes through thin specimens and can be collected by the condenser. By directing this light to a PMT (which need not be of high sensitivity as there is excessive light) and collecting it in synchrony with the reflected fluorescence signal (simply by adding an extra channel to the frame grabber), a perfectly aligned, trans-illumination image is simultaneously obtained (Fig. 8). Because the laser is polarized, this image can be constructed using DIC optics if the appropriate polarization and analyzer optics are included. Although the image is collected by the same scanning technique, this image is a conventional wide-field image. Obviously, this technique does not apply to very thick opaque specimens such whole organs or animals.

While this arrangement works well with transmitted laser light, it is not well suited for the collection of low-intensity fluorescence light and for high-resolution images. However, the replacement of the condenser lens with a high-NA objective can provide greatly improved fluorescence images. The magnification is less important than working distance (for convenience) and NA (ability to collect light). The magnification of the collected image is determined by the excitation objective as this determines the size of the raster scan. There is a risk of detecting stray light when monitoring transmitted light, but this is also a common problem with the normal implementation of two-photon microscopy using non-de-scanned detectors (PMTs). The general solution to both problems is to surround the microscope in a light-tight enclosure. Doing so eliminates stray light
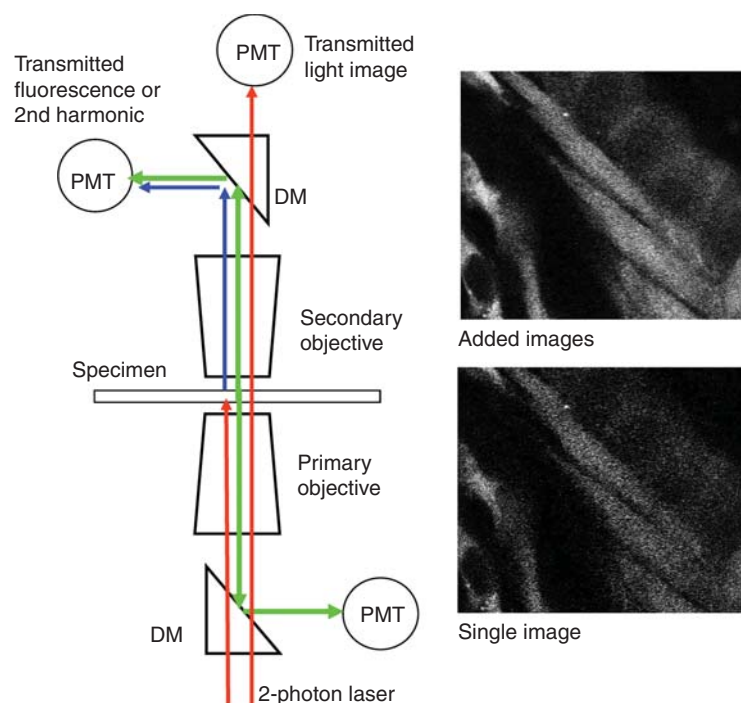
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**FIGURE 8.** Concept of trans-illumination: for fluorescence, second-harmonic, and conventional light imaging. Two-photon (red) excitation illuminates the specimen. Second harmonic (blue) or fluorescence (green) light are transmitted and collected by PMTs after separation by a dichroic mirror (DM). Two-photon light is transmitted by the DM to produce a transmitted light image. Reflected fluorescence is detected after DM (*bottom right* image). *Top* image is the combined transmitted and reflected fluorescence image with increased signal-to-noise ratio.

problems, but often presents other problems in terms of microscope access and monitoring of experiments.

For second-harmonic imaging, a dichroic mirror helps separate the untransformed light from the second-harmonic light and a good barrier eliminates any other fluorescence signals. When imaging with 800-nm light, the barrier filter would be narrowly centered on 400 nm.

Because fluorescent light is emitted from the specimen in all directions, the typical collection of only reflected light wastes ~50% of the available signal. By using a high-NA objective as a trans-illumination detector, a lot of additional fluorescence can be collected. Again, the resulting image is in precise alignment with the reflected images and the two images can be summed to increase the signal-to-noise ratio (Fig. 8). This approach can only be used with two-photon excitation, because the detector does not require a pinhole; the plane of focus is determined by the laser focus. Again, these approaches are not possible with thick opaque specimens.

## TOTAL INTERNAL REFLECTION FLUORESCENCE (TIRF) MICROSCOPY

A major advantage of confocal and multiphoton microscopy is that they provide optical sectioning in the *z*-axis. However, both approaches are diffraction-limited and provide an axial resolution of no better than ~500–800 nm. Total internal reflection fluorescence (TIRF) microscopy is a near-field imaging technique that offers substantially better optical sectioning, but with the disadvantage that the section is confined to a single-plane immediately adjacent to the cover glass.

TIRF microscopy works by directing excitation light through a glass substrate toward an aqueous specimen at a sufficiently shallow angle that total internal reflection occurs because of the refractive index decrease at the glass/water interface. A very thin electromagnetic field (evanescent wave) is created in the liquid with the same wavelength as the incident light and decays exponentially with

distance from the interface. The penetration distance (thickness of the "optical section") depends on the wavelength of the light, the change in refractive index between the media, and the incident illumination angle. Because this field is only able to excite fluorophores near the interface, it provides an "optical sectioning" effect similar to, but narrower (∼100 nm or less) than that achieved by a confocal microscope.

The use of total internal reflection to illuminate cells was first described by (Ambrose 1956) and extended and popularized by Axelrod (2003). However, TIRF microscopy has only become widely used in the last several years, following the development of specialized TIRF objectives and commercial microscope systems.

## Prism-Based TIRF

TIRF microscopy can be accomplished most simply in a "trans-illumination" mode, using a prism to introduce excitation light at an angle that results in total internal reflection at a surface to which cells adhere in an aqueous medium. The resulting fluorescence excited within the evanescent field is viewed through an objective lens facing the prism. This approach has advantages that the excitation light does not pass through the objective lens, thereby eliminating problems of fluorescence in the glass or immersion oil and blocking of excitation light by the emission filter. With prism-based TIRF, it is possible to use conventional excitation light sources (e.g., arc lamp) rather than lasers and it can be easily and inexpensively adapted to existing inverted or upright microscopes. On the other hand, the fluorescence must be viewed through the entire thickness of the specimen which degrades image quality and restricts its application to thin, transparent samples. Moreover, the specimen is trapped between glass in a thin aqueous layer, greatly hindering access. Consequently, prism-based TIRF has seen relatively little use for live cells.

## Through-the-Objective TIRF

In this method, the objective lens is used to direct light to the coverslip–specimen interface as well as to image the resulting fluorescence (Fig. 9A). To achieve total internal reflection, the incidence angle must be greater than the critical angle, which necessitates use of objectives of high numerical aperture (1.45 or higher). Thus, objective-based TIRF has hinged on the development of high-NA objectives, which are typically designated as TIRF objectives. Objectives with NAs as high as 1.49 are now available for use with regular coverslips and immersion oil. Even higher NA (1.65) is possible, but requires specialized, volatile immersion fluid and expensive sapphire coverslips. Because the cell cytosol has a refractive index of ∼1.38, the objective NA must exceed this value. Although in principle, a 1.4 NA objective could be used for TIRF, only a tiny annulus at the periphery of the objective can be utilized for total internal reflection, and the critical angle can only be marginally surpassed. Therefore, the coupling of the excitation light into the rear aperture of the objective is very challenging. However, objectives of higher NA provide a wider range of angles exceeding the critical angle and allow for angle adjustment and control of the evanescent field penetration depth.

Another requirement to perform TIRF imaging on a fluorescence microscope is an illuminator to introduce excitation light restricted to the outer periphery of the back aperture of the objective. One approach is to reimage an opaque disc onto the back aperture (back focal plane) such that light from a standard arc lamp illuminates only a peripheral annulus where the critical angle is exceeded at the specimen. This approach has the difficulties of alignment, the need to suppress stray light incident on the central part of the back aperture, and the fact that most of the light is blocked by the opaque disc.

A far more common configuration focuses a laser beam to a spot at the back aperture of the objective, which can be moved radially across the aperture to control the incident angle of light at the specimen. Thus, the microscope can be used in a quasi-wide-field mode with the laser spot positioned toward the center of the back aperture, or in TIRF by moving the spot toward the outer annulus (see Fig. 9B). This design of a TIRF illuminator is relatively simple, making it attractive for "home-brew" construction; however, commercial illuminators are relatively inexpensive. Key components are a
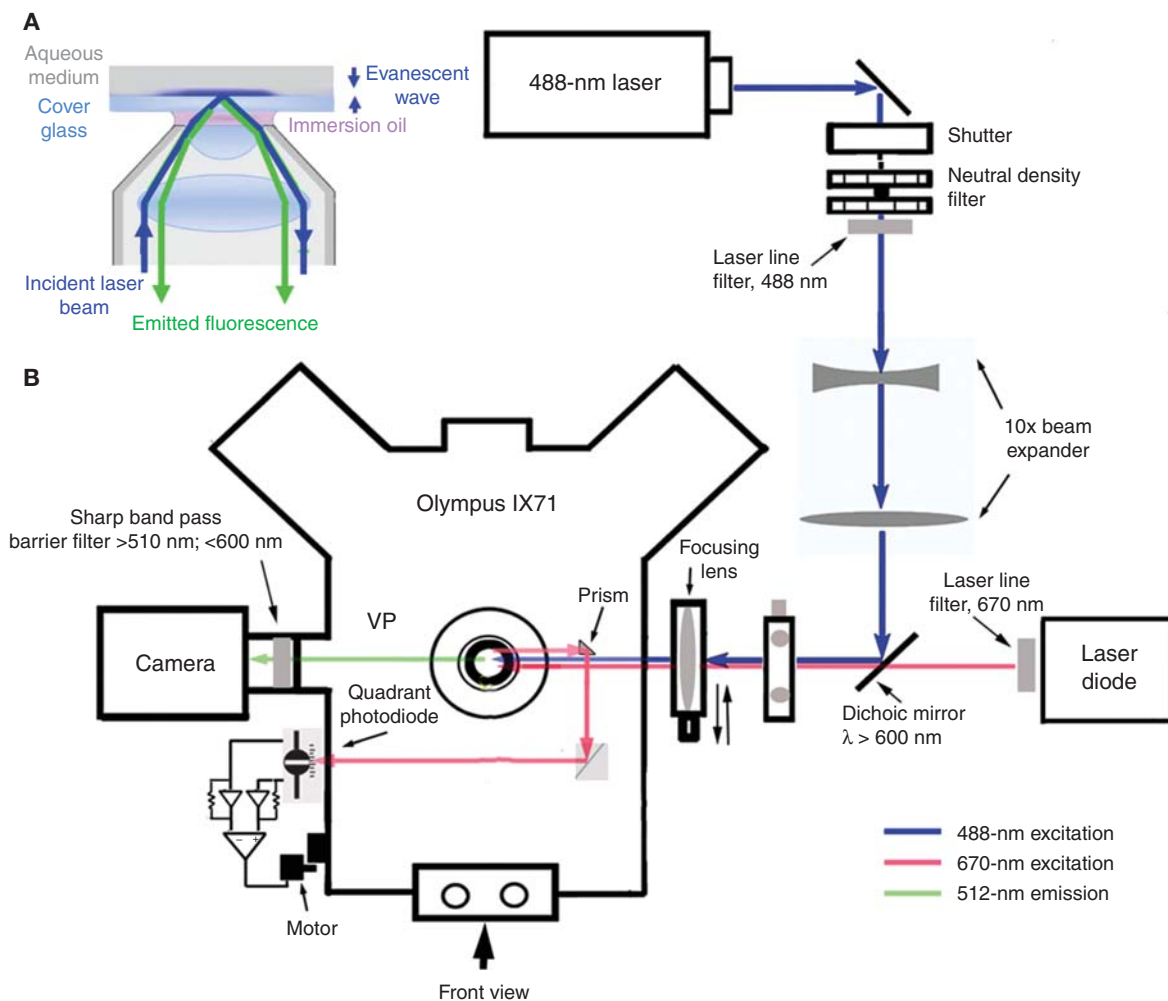
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**FIGURE 9.** Through-the-objective TIRF microscopy: (*A*) schematic illustrating the formation of an evanescent wave by a laser beam directed through a high-NA objective to undergo total internal reflection at the interface between a glass coverslip and aqueous medium. Fluorescence emitted by a fluorophore excited by the evanescent wave is collected back through the same objective. (*B*) Layout of a TIRF imaging system based on an inverted microscope. Translation of the focusing lens is used to control the position of the excitation laser spot at the objective back aperture and thereby alter the angle of incidence at the interface between the coverslip and aqueous medium. The system includes a separate deep-red laser for feedback control of microscope focus.

laser beam expander to expand the laser beam at the image plane of the microscope to a width greater than the field imaged by the camera, a lens to focus the beam to a spot at the back aperture of the objective, and a means to position the spot (e.g., by mounting the focus lens on a translation slide). The laser beam is usually directed into the objective using a dichroic mirror, but if multiple laser wavelengths are desired the use of different, well-aligned cubes with appropriate dichroics, or use of a specialized multiband dichroic designed to selectively reflect two or three specific wavelengths is required. Alternatively, we have constructed a system in which laser light is reflected at 90° by a small silvered prism located just under the objective. A major advantage is that because the light is reflected by a mirror, it is equally effective at all wavelengths. The only drawback is that a small fraction of the emitted fluorescent light is blocked by the prism.

Through-the-objective TIRF on an inverted microscope allows rapid and easy access to the sample for changes to the media or drug addition, and exchange of samples is similarly quick and simple. Such systems are also more user-friendly, requiring minimal maintenance and alignment. This configuration is most commonly used by cell biologists.

## Safety Issues with TIRF

Commercial instruments take care of safety issues with interlocks and covers that may complicate the efficient use of the microscope, but are important for safety. The laser light emerges from the objective as a collimated beam and presents an appreciable eye safety hazard, unlike the highly divergent beam from a confocal microscope. Thus, especially in "home-brew" configurations, there should be no possibility of personnel being exposed to a refracted beam. Correspondingly, when the beam undergoes total internal reflection most of laser power is reflected back into the microscope. This arrangement places stringent demands on the emission filter used to block laser light from reaching the camera, and it is important to ensure that laser light cannot be transmitted to the eyepieces. If it proves necessary to stack two reflective interference filters to achieve sufficient blocking, these should be angled slightly relative to each other to avoid multiple cross reflections, and preferably located at different positions along the optical path in the microscope.

A sparse suspension of small (50- or 100-nm) fluorescent beads in water provides a good specimen for testing a TIRF microscope; some beads will adhere to the cover glass, while others undergo Brownian motion suspended at various heights above the cover glass. When the incident laser beam is below the critical angle both adherent and suspended beads are visible, the latter coming in and out of focus as they randomly move. After adjusting the beam to achieve TIRF, only adherent beads should be visible, which will blur as the microscope is focused into the water droplet without suspended beads becoming visible.

## TIRF Imaging

Because the evanescent wave simultaneously excites the specimen across an $x$–$y$ plane, imaging is achieved using a camera in the same way as for wide-field fluorescence. The choice of camera depends largely on the biological specimen and topic being addressed. Unlike point-scanning confocal and multiphoton microscopy, the imaging speed is determined only by the frame rate of the camera, and by shot noise degradation of signal-to-noise ratio at high frame rates. A camera with high sensitivity is desirable, as fluorescence arises only from the optical "section" created by the evanescent field. EMCCD cameras are the best choice for imaging fast dynamic processes such as local calcium signals, where only a few photons are captured at each pixel in a single frame. For other applications the new generation of scientific complementary metal oxide semiconductor (CMOS) cameras offers higher pixel resolution and faster readout at lower cost.

For several reasons, it is difficult to make quantitative measurements of fluorescence intensity from TIRF images. First, the evanescent field decays exponentially, so that small differences in axial distance of fluorophores from the coverslip will cause large changes in apparent brightness. Second, the excitation intensity is typically uneven across the illuminated area. The Gaussian profile of the laser beam means that there will be a falloff in intensity from the center of the field. This falloff can be minimized by overexpanding the beam, at the cost of reduced maximum intensity. Finally, the coherent laser beam can produce interference fringes, apparent as alternating light/dark patterns across the image frame.

The fluorescence emitted in TIRF microscopy is confined exclusively to the extremely thin plane excited by the evanescent field immediately adjacent to the cover glass. Thus, the optical section in the specimen is defined by the position of the cover glass, not by the focal plane of the objective lens. This sets a stringent requirement for axial stability of the microscope, particularly during long recordings such as during acquisition of STORM images. With high NA objectives, a focus drift of only one or two hundred nanometers causes appreciable blurring of the image, whereas the same drift in a confocal or multiphoton microscope would merely result in a slight displacement of the optical section within the specimen. A solution to this problem is to use a "focus lock" device providing feedback to maintain a constant distance between the objective and the cover glass (Fig. 9B). Changes in this distance result in a shift of a laser beam reflected at interface of the cover glass, which is detected by a position-sensing photodiode and used to provide a feedback signal to the microscope focus

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(Demuro and Parker 2005). A related issue is that TIRF objectives need to be corrected for flat-field imaging, and optimal image quality may be obtained only within a small central part of the entire field.

A TIRF microscope can also be used in a mode colloquially known as "semi" or "dirty" TIRF, which restricts fluorescence to a section a few micrometers thick near the cover glass. In this technique the incident laser beam is adjusted slightly below the critical angle, so that a narrow sheet of light skims above and almost parallel to the cover glass. This mode provides a useful reduction in background fluorescence, while enabling imaging of deeper structures in the cell that lie beyond the evanescent field.

## Sample Preparation

TIRF imaging is ideal for imaging structures and events at or near the plasma membrane of cells that are closely adherent to the cover glass. TIRF microscopy is therefore most readily applicable to study of cultured cell lines, although other cells can be used if the cell can be cleaned of any surrounding material; for example, by removal of the vitelline envelope around *Xenopus* oocytes (Demuro and Parker 2005). In many cases, cells will adhere well to a cleaned cover glass, but it may be necessary to coat coverslips with extracellular matrix molecules or substances such as poly-L-lysine or collagen to ensure cell adherence. Analogous to multiphoton microscopy, the restriction of excitation to a thin section within the cell minimizes problems of photobleaching and phototoxicity. But the reduction of bleaching of soluble fluorophores that can diffuse into and out of the evanescent field does not apply to fluorophores that are localized to the plasma membrane.

## SUPER-RESOLUTION MICROSCOPY

An exciting recent advance in optical microscopy has been the ability to circumvent the traditional diffraction limits of optical microscopy. For over a century it has been known that the wave nature of light imposes a limit to the resolution between closely apposed sources of light (e.g., cellular organelles or individual fluorophore molecules). This limit depends on the numerical aperture of the objective lens and the wavelength of light, and is somewhat arbitrarily defined, but in practice the best resolution that can be achieved by "classical" light microscopy is ∼200–300 nm in the lateral dimensions and 500–800 nm in the axial dimensions (Abbe 1873). However, recent innovations now side-step this limit, providing resolution that is in principle unlimited and in practice a few tens of nanometers. The new techniques do not break this law of physics, but circumvent it in two ingenious, but very different ways.

## PALM/STORM Super-Resolution Imaging

This method uses the approach of separating the fluorescence from individual fluorophore molecules by temporally separating their emission times. This technique was independently developed by several groups and named photoactivated localization microscopy (PALM) (Betzig et al. 2006), fluorescence PALM (fPALM) (Hess et al. 2006), and stochastic optical reconstruction microscopy (STORM) (Rust et al. 2006).The basic principle is essentially the same in each case. STORM and PALM techniques presently give the best spatial resolution of any optical microscopy technique (∼20-nm lateral and 50-nm axial resolution), but have relatively poor time resolution because images must be constructed from sequential frames captured over several seconds to minutes.

The basic principles that underlie these super-resolution techniques rely on the fact that a single fluorescent source (e.g., a fluorescent molecule) can be located with high precision by fitting a two-dimensional Gaussian function to determine the centroid of the blurred spot (point-spread function) formed by the microscope. The precision with which the centroid can be determined depends only on the number of photons collected; in practice, this can be a few tens of nanometers or better. However,

such precision requires that the density of the detected fluorescent molecules is sufficiently low so that the point-spread functions of two fluorophores are unlikely overlap.

STORM is essentially a super-resolution variant of immunofluorescence imaging, accomplished by utilizing photoswitchable fluorescent dyes such as Alexa Fluor 647 (A647; known as the reporter dye) conjugated to secondary antibodies. In this process, red laser light is used to evoke strong but transient fluorescent emission from an A647 molecule, which quickly switches back to a stable dark state. This reporter dye can be efficiently reactivated if an activator dye (e.g., Alexa Fluor 488) is located in close proximity and is illuminated with a laser that matches the excitation peak of the activator (in this case 488 nm). Reactivated A647 molecules can then be localized before they switch back to a stable dark state again. The intensity of this activating light (which may be pulsed or continuous) is adjusted so that in each imaging cycle, only a small fraction of the fluorophores in the field of view are switched on with the result that individual active fluorophores are optically resolvable from the rest. Each A647 molecule can be switched on and off many times before eventually photobleaching. Localizations from thousands of such on–off cycles are combined to reconstruct a super-resolution image. Multi-color STORM is achieved by conjugating other activator dyes (such as Alexa Fluor 405 or Cy3) with a different secondary antibody together with A647. The A647 reporter molecules of these combinatorial pairs (such as A405-A647 or Cy3-A647) can be selectively reactivated by illuminating with a wavelength that matches the excitation peak of the activator (405 and 562 nm, respectively for A405 and Cy3).

Whereas STORM utilizes photoactivatable organic dyes as a form of super-resolution immunostaining in fixed specimens, (f)PALM and PALM rely on the expression of photoswitchable genetically encoded proteins (PA-FPs) fused to a protein of interest. The PA-FP is selected on the basis of showing a substantial change in fluorescence properties in response to illumination with light of a specific wavelength. Some PA-FPs display photoswitching from a dark (nonfluorescent) form to a brightly fluorescent form (e.g., PAmCherry1, PAmRFP1, PA-GFP), whereas others (e.g., mEos2, Dendra, Kaede) undergo photoswitching of their fluorescence emission wavelength. Following expression of the PA-FP fusion protein, cells are fixed and immobilized on the microscope. Super-resolution imaging with these PA-FPs in fixed cells is essentially the same as for STORM: (i) sparse activation of a subset of fluorophores that are optically resolvable, (ii) determination of the positions of these fluorophores with subdiffraction-limit precision, and (iii) repetition of this process to allow multiple subsets of fluorophores to be localized and thus generate a super-resolved image.

In all super-resolution approaches, the quality of the final image requires a sufficient density of fluorophores to fully map fine details of a labeled structure. This relationship is described by the Nyquist sampling theory which requires a minimum of two data points per resolution unit (Huang et al. 2009). In creating a super-resolution image a fundamental requirement is that there must be no movement in the specimen during the time of image acquisition, thus typically necessitating the use of fixed preparations. However, PA-FPs can further be used to track the movements of single molecules within live cells. Single particle tracking PALM (sptPALM) experiments again follow the same basic principles as STORM/PALM with respect to sparse activation and localization, but in this case the locations of closely adjacent fluorescent spots in successive frames are linked together by the analysis algorithm to create particle tracks (Manley et al. 2008).

As described above, PALM and STORM provide only two-dimensional imaging. However, they can be readily adapted to provide super-resolution localization in the $z$-axis by the simple expedient of placing a weak cylindrical lens into the imaging path before the camera. Doing so introduces astigmatism, so that the diffraction-limited spot at the camera becomes elongated into an ellipse extended along the $x$-axis if it arises from a fluorophore below the plane of focus, and extended along the $y$-axis if above the plane of focus. By fitting images with a two-dimensional Gaussian function in which the widths (standard deviations) are allowed to vary independently in $x$- and $y$-axis it is thus possible to localize the position of a fluorophore molecule in the $z$-axis with high precision by reference to a look-up calibration function.

Constructing your own single molecule imaging microscope is relatively straightforward and cost-effective. Most TIRFM microscopes such as those in Figure 9 can be easily adapted to image single

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molecules by the addition of a highly sensitive EMCCD. One of the main complexities of STORM/PALM imaging involves fluorophore localization and fitting algorithms although software such as QuickPALM (Henriques et al. 2010) and the rapidSTORM project (http://www.super-resolution .biozentrum.uni-wuerzburg.de/home/rapidstorm/) Wolter et al. (2010) offer relatively simple open-source software for both hardware control that includes custom laser switching capabilities for multicolor experiments, methods to correct for sample drift during acquisition, and software to generate super-resolved images.

## STED Super-Resolution Imaging

A second, more dynamic approach is that of stimulated emission depletion (STED) microscopy (Hell 2009). The advantage of this technique is that it is compatible with the concept of scanning confocal one- or two-photon microscopy as well as second harmonic imaging and thereby has great potential for examining living tissue. The principle used by STED microscopy to separate indicators is a reduction in the size of the excitation spot, which results in individual indicators being activated separately. Although the microscope optics remain diffraction limited, a reduced spot size is achieved by overlapping two laser light sources and allowing the resulting fluorescence depletion to create the reduced excitation spot (Figs. 1 and 10).

The key fluorescence process of STED is indicated in Figure 1. However, the technique also requires that the STED laser is formed into a doughnut shape to concentrically overlay the excitation laser (Fig. 10). In the overlay region, the STED laser suppresses fluorescence of the indicator to reduce the excitation spot size. The resolution of the image is proportional to the size of the spot, which can be controlled with the intensity of the STED laser power. The doughnut shaping of the STED laser is achieved with a specialized "phase plate" or vortex that introduces a 0 to $2\pi$ phase delay in the laser. In conjunction with the objective, this phase delay invokes light interference that results in the required shape. Clearly, accurate concentric beam alignment is required. However, a newer phase plate has been constructed that can be inserted in the path of prealigned lasers (emitting from the same optic fiber) that modulates the STED wavelength into the doughnut shape while leaving the center excitation beam unchanged (Reuss et al. 2010).

STED microscopy has quickly advanced from a complex approach of synchronized pulsed lasers to one using simple (and cheaper) continuous wavelength lasers with the addition of a relatively simple optical element to a standard confocal microscope.
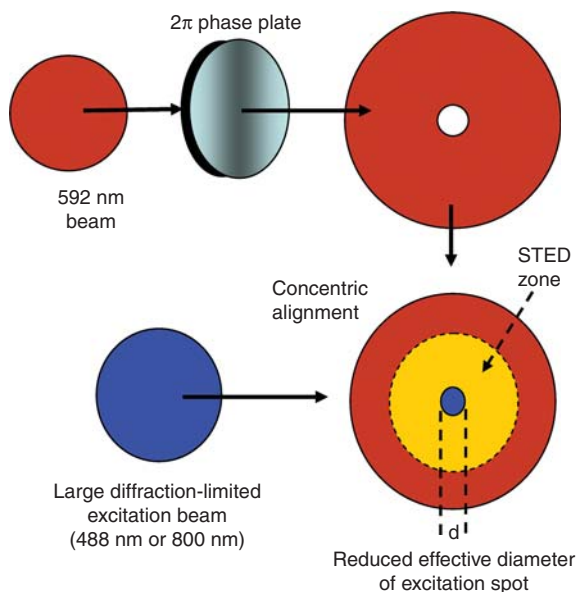
**FIGURE 10.** Creation of reduced excitation spot for STED microscopy. The STED (592-nm) laser beam (red) is shaped by a $2\pi$ phase plate into a doughnut and concentrically overlaid with the 488-nm excitation laser (blue). The process of STED occurs in the yellow zone to reduce the zone of fluorescence. Increased STED laser power decreases the diameter, d, of the excitation spot.

Labels in figure: 2π phase plate; 592 nm beam; Concentric alignment; STED zone; Large diffraction-limited excitation beam (488 nm or 800 nm); Reduced effective diameter of excitation spot; d

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