Fluorescence Microscopy: A Concise Guide to Current Imaging Methods

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ABSTRACT

The field of fluorescence microscopy is rapidly growing, providing ever increasing imaging capabilities for cell and neurobiologists. Over the last decade, many new technologies and techniques have been developed which allow for deeper, faster, or higher resolution imaging. For the non-expert microscopist, it can be difficult to match the best imaging technique to the biological question to be examined. Picking the right technique requires a basic understanding of the underlying imaging physics for each technique, as well as an informed comparison and balancing of competing imaging properties in the context of the sample to be imaged. This unit provides concise descriptions of a range of commercially available imaging techniques and provides a tabular guide to choosing among them. Techniques covered include structured light, confocal, total internal reflection fluorescence (TIRF), two-photon, and stimulated emission depletion (STED) microscopy. Curr. Protoc. Neurosci. 50:2.1.1-2.1.14. © 2010 by John Wiley & Sons, Inc.

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INTRODUCTION

Fluorescence microscopy is a powerful tool for modern cell and molecular biologists and, in particular, neurobiologists. It provides a window into the physiology of living cells at sub-cellular levels of resolution. This allows direct visualization of the inner workings of physiological processes at a systems level context in a living cell or tissue. Fluorescence microscopy enables the study of diverse processes including protein location and associations, motility, and other phenomena such as ion transport and metabolism. This versatility explains why thousands of papers describing variants of the many fluorescent microscopy techniques are published each year.

Many new techniques have been developed over the last decade that enable more comprehensive exploitation of light for biologic imaging. These advances include the widespread use of fluorescent proteins (for review, see Shaner et al., 2005), large number of new fluorophores available (for reviews see Eisenstein, 2006; Suzuki et al., 2007), growth of the utility of the basic confocal microscope, use of multiphoton microscopy to optically image deeper into tissues, and breaking of the diffraction limit for super-resolution. Many of the new advanced techniques are now being commercialized, opening their use to a large population of modern biologists.

However, for the biologist inexperienced in light microscopy, matching the best technique to the biological experiment can be difficult. Optimal use of fluorescence microscopy requires a basic understanding of the strengths and weaknesses of the various techniques, as well as an understanding of the fundamental trade-offs of the variables associated with fluorescent light collection. In a very simple form, the ideal light microscopy experiment can be viewed as optimizing the competing properties of image resolution (in the xy or lateral direction, as well as the z or axial dimension), imaging speed (and/or acquisition time), and the amount of signal collected from the fluorescing sample (Fig. 2.1.1). This is bounded by the limits imposed by photobleaching and/or phototoxicity. In many experiments, light levels at the diffraction limited spot (focused by the objective) can be very high. This can lead to destruction of the fluorophore and unwanted biological consequences leading to cell death or changes in the physiology of the cells or tissue being illuminated. Given such constraints, these variables are difficult to balance and require careful attention to detail and systematic empirical testing. On top of these basic variables, other secondary variables also can become important, such as the cost of the necessary equipment and the difficulty of the technique.
Avoidance of excessive photobleaching and/or phototoxicity

Figure 2.1.1 Diagram of some of the critical opposing factors in an imaging experiment. The best image is one that can balance these factors to obtain the necessary information while avoiding photobleaching or phototoxic effects. Table 2.1.1 outlines how these factors differ among the various commercialized microscopy techniques discussed in this unit.

In this unit, knowledge of the basics of fluorescence microscopy (including wide-field microscopy) is assumed. The objective is to provide non-expert microscopists a concise description and guide to select techniques that may have the widest appeal and that are, or will soon be, commonly used in most light microscopy core facilities or advanced biological research laboratories. The techniques discussed encompass the most basic (such as wide-field fluorescence microscopy), as well as cutting edge techniques like stimulated emission depletion (STED) microscopy. Emphasis is placed on explaining the strengths and weaknesses of these techniques in terms of balancing the variables discussed in Figure 2.1.1. Table 2.1.1 summarizes this discussion and should serve as a quick guide for choosing the appropriate imaging modality from among the techniques discussed.

WIDE-FIELD FLUORESCENCE MICROSCOPY (WFFM) TECHNIQUES

In the most basic form, wide-field fluorescence microscopy (WFFM) involves exciting the fluorophore(s) in the sample of interest using a fluorescent light source, a microscope, excitation and emission filters, and an objective lens. The resulting emitted light, of longer wavelength, is observed through the microscope eyepieces or by a camera, followed by computer digitization (for reviews, see Inoue and Spring, 1997; Lichtman and Conchello, 2005). Over the last decade, developments in microscope and camera design, light filters, and in new techniques have greatly improved resolution and light collection for WFFM.

One of the most significant advancements has been the development of electron multiplied (EMCCD) and very low-noise, cooled CCD cameras. These cameras allow for fast detection of low-light fluorescence (EMCCD) or the gradual accumulation of fluorescence signal to be integrated with little noise (cooled CCD), while still maintaining high resolution. Both allow for faster imaging and better contrast at low signal levels, e.g., when the excitation light is purposefully minimized to prevent photobleaching or phototoxicity.

In addition to cameras, wide-field microscopy has also been improved by better light filters, mirrors, and objectives. Commercially available filters, e.g., from Chroma Inc. (Rockingham, VT), Omega (Brattleboro, VT), or Semrock (Rochester, NY), have very high transmittance or reflection values enabled through new sputter-coating technologies. In
<table>
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<tr>
<th>Technique</th>
<th>Resolution $xy$</th>
<th>Resolution $z$</th>
<th>Resolution temporal</th>
<th>Imaging depth</th>
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<tr>
<td>Wide-field diffraction limited</td>
<td>Weak</td>
<td>Best (ms/ frame, signal limited)</td>
<td>Best (ms/ frame, signal limited)</td>
<td>Worst</td>
<td>Simple</td>
<td>$</td>
<td>Best (usually μW)</td>
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<tr>
<td>Structured light</td>
<td>Varies (can be super-resolution)</td>
<td>Varies based on number of images needed</td>
<td>Varies based on number of images needed</td>
<td>Better</td>
<td>Varies with resolution needed</td>
<td>$  $</td>
<td>Varies with number of images needed</td>
</tr>
<tr>
<td>Laser-scanning confocal (LSC)</td>
<td>Diffraction limited</td>
<td>Typically slow (1 sec/frame)</td>
<td>Better (less than 100 μM)</td>
<td>Complex but most versatile</td>
<td>$$$</td>
<td>Can be bad (μW of power focused to spot)</td>
<td></td>
</tr>
<tr>
<td>Multi-point/slit confocal</td>
<td>Range to diffraction limit</td>
<td>Good (signal limited)</td>
<td>Better (same as LSC)</td>
<td>Better</td>
<td>$$$</td>
<td>Better (usually lower flux density than LSC)</td>
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<td>TIRF$^a$</td>
<td>Diffraction limited but low background</td>
<td>Best but only first 200-300 nm</td>
<td>Good (signal limited)</td>
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<td>$  $</td>
<td>Better</td>
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<td>Two-Photon (TPFM)</td>
<td>Diffraction limited</td>
<td>Typically slow (1 sec/frame)</td>
<td>Best (hundreds of μM)</td>
<td>Complex</td>
<td>$$$</td>
<td>Can be bad (mWs power focused to spot but limited to 1 plane)</td>
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<tr>
<td>STED$^c$</td>
<td>Super-resolution (&lt;70 nm)</td>
<td>Same as TPFM</td>
<td>Slowest</td>
<td>Sample specific</td>
<td>Most complex</td>
<td>Very high cost</td>
<td>Worst (second beam with many mW of power)</td>
</tr>
</tbody>
</table>

$^a$ Bold text denotes best in the category, and italic text denotes worst in the category.

$^b$ TIRF=Total Internal Reflection Fluorescence.

$^c$ STED=Stimulated Emission Depletion.

addition, these filters can have very sharp wavelength dependencies which enable multicolor discrimination. In the last decade, all of the major microscope companies (e.g., Leica, Nikon, Olympus, and Zeiss) have also improved microscope objectives. These new objectives have very flat fields (which decrease objective-induced gradients in intensity across an image), long working distances with good resolving power, and improved light transmission from the near UV to the infrared, and they are available in varieties that match the refractive index of the sample being imaged.

The main advantages of basic WFFM are that it is the least expensive technique, provides good $xy$ dimension resolution (the ability to distinguish fine detail in a specimen in the $xy$ dimension), can provide very fast temporal resolution (particularly with the new EM-CCD cameras), and in many cases requires the least amount of excitation light (Table 2.1.1). $xy$ resolution ($R_{xy}$) in wide-field microscopy is a function of the numerical aperture (NA) of the objective lens and the wavelength of the excitation light according to Ernst Abbe’s diffraction limit expression:

$$ R_{xy} = \frac{0.61 \lambda}{\text{NA}} $$

**Equation 2.1.1**

where $\lambda$ is the wavelength of the emitted light and NA is the numerical aperture of the objective.

For a high NA objective (e.g., NA 1.4) lens this limit is around 200 nm. All of the techniques listed in Table 2.1.1 are approximately limited to this type of $xy$ resolution except where super-resolution is indicated.

The main disadvantage of basic WFFM is that all of the emitted light is integrated through the sample in the $z$ dimension. Therefore, it is difficult to tell where the fluorescence from a point in the sample originated in the $z$-dimension. For samples that are thin or where $z$-discrimination is not critical this may not be a limiting factor. For thick samples,
such as live cells or tissues, where optical sectioning is critical or where out-of-focus light obscures details even in the \(xy\) plane, other techniques such as confocal or multi-photon microscopy may be more appropriate (see the following sections), although fluorescence deconvolution microscopy and structured light microscopy (SLM) are WFFM techniques that are commercially available.

Structured light microscopy (SLM) is a form of WFFM that enables optical sectioning. SLM works by inserting a moveable grid pattern into the optical path of the excitation light in the wide-field microscope. This produces a pattern in the images produced. The pattern is moved in the \(xy\) and even \(z\)-dimension and the way that the detected fluorescence from the sample interacts with the pattern is then analyzed using a simple mathematical formula to create the optical sections (Fig. 2.1.2). Many commercial systems are available for SLM, and moveable grating patterns are available for those wishing to modify existing WFFM microscopes. In the last few years, SLM has been shown capable of producing super-resolution images (≈ half the diffraction limit) (Gustafsson, 2000; Schermelleh et al., 2008).

The main advantage of SLM is that one can optically section using WFFM without the cost of expensive confocal systems and, in some cases, produce super-resolution images without the cost or technical complexity associated with other super resolution techniques such as stimulated emission depletion microscope (STED), discussed below. The main disadvantage of this technique is that multiple images must be acquired to produce optical sections. This can lead to photobleaching. In addition, the optical sectioning ability of SLM is negated if the sample moves while the different images are being captured (as would be the case in live cells).

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**Figure 2.1.2** The basic principles of structured light microscopy are shown in panels A, B, and C. If an unknown pattern (such as a biological sample) represented in (A) is multiplied by a known regular illumination pattern (B) then a beat pattern (moiré fringes) will appear (C). The pattern is the difference between the sample and the regular illumination pattern and is coarse enough to be seen through the microscope even if the original pattern in the sample was not resolvable. By moving the grid and the sample in space and computationally processing the resulting data an image can be generated that has resolution at least 2× better than a conventional wide-field image. Confocal (D) and structured light (F) images of the edge of a Hela cell showing the actin cytoskeleton. E and G show enlargements of the images in D and F. The apparent fiber diameters are 110 to 120 nm in the structured light images, compared to 280 to 300 nm in the confocal image. Panels A, B, and C are reproduced from Gustafsson (2005), and panels D and E are reproduced from Gustafsson (2000), with permission of the National Academy of Sciences U.S.A. Panels A to E were originally published in color and have been altered here to black and white.
Deconvolution fluorescence microscopy (DFM) is a form of WFFM. DFM requires prior knowledge of the point spread function (PSF), the 2D or 3D image resulting from a sub-resolution point-like object. In fluorescence microscopy this is typically measured using a fluorescent bead that is <0.2 μm in size. The PSF produced by the microscope system can be used to measure the achievable resolution and give information regarding aberrations inherent in the optics of the system.

In DFM, images of multiple xy sections are acquired through the sample in the z-dimension. The resulting stack of images, still lacking z-dimension discrimination, are then analyzed using an empirical or idealized mathematical model of the PSF created by the microscope optics. This analysis results in a volumetric recreation of the sample in 3D space. In the same way, deconvolution techniques can also be used to enhance other microscopy techniques (e.g., confocal microscopy and two-photon microscopy), as long as the PSF is known or can be estimated based on the optics used. Software for image deconvolution is available commercially or through plug-ins for the free image analysis program ImageJ (http://rsbweb.nih.gov/ij/, NIH). Although DFM is a powerful technique when used in capable hands (reviews include Wallace et al., 2001; Boccacci and Bertero, 2002), the novice user should be warned that optical sectioning is done indirectly, using a mathematical model. Knowledge of the limitations of the model used for DFM is critical to understanding the images produced and interpretation of the data. Given such constraints, consideration of DFM is beyond the scope of this unit, and it is not listed in Table 2.1.1.

MODERN CONFOCAL MICROSCOPY

The laser scanning confocal microscope (LSCM) remains a key piece of equipment in most imaging laboratories. Most modern LSCM systems offer a variety of advantages and are equipped with software to perform complex 3D (z-stack, or xy images taking sequentially from top to bottom of the sample), 4D (z-stack over time), or even 5D (z-stack over time including spectral imaging) experiments. These microscopes often include software to facilitate data acquisition for complex methodologies such as spectral deconvolution, fluorescence recovery after photobleaching (FRAP), and fluorescence resonance energy transfer (FRET). There have been many reviews written about confocal microscopy, but we recommend the comprehensive information regarding all forms of confocal microscopy (as well as other microscopy techniques) in Hibbs (2004) and Pawley (2006).

In the last few years, many changes have been made to improve these microscopes, but the fundamental design for optical sectioning remains largely unchanged. Figure 2.1.3 shows a simplified diagram of the light path of an LSCM. This figure shows that laser light is directed to the sample through collimating and beam-steering optics, scanning mirrors (which sweep the laser beam over the field of view), and an objective that focuses the light to a diffraction limited spot in the sample. Emission light from the sample is directed to light-sensing detectors (typically, photomultiplier tubes, also known as PMTs) through a pinhole that is in the conjugate image plane to the point of focus in the sample. After spatial filtering by the pinhole, the light is sensed by the detectors, and a proportionate voltage is produced, amplified, and converted into digital levels for image display and storage.

At the heart of the confocal microscope is the pinhole. When placed in the conjugate image plane to the point of focus on the sample, it enables optical sectioning (Fig. 2.1.3). The pinhole optically sections by acting as a barrier to light originating from other focal planes in the sample. Although the pinhole facilitates optical sectioning, it must be understood that the axial resolution is still worse than the xy resolution (which is similar to WFFM). Axial resolution (R_z) in the confocal microscope is set by the expression:

\[ R_z = 1.4λ\eta/(NA)^2 \]

Equation 2.1.2

where \( λ \) is the wavelength of the emission light, \( \eta \) is the refractive index of the mounting medium, and NA is the numerical aperture of the objective. An intermediate emission wavelength, coupled with a pinhole and a high NA lens, would enable an ideal axial resolution of \( \sim 0.6 \) μm. In practical terms, axial resolution is likely to be between 0.6 and 1.0 μm. The difference between the xy and z dimensions leads to a resolution limit that is ellipsoidal in shape in 3D space.

Most LSCM manufacturers also offer a spectral imaging option that will allow for either variable band-pass emission filtering or spectral detection on a per pixel basis. This works by placing either a diffraction grating...
or a prism in the light path before the PMT detectors. In many cases, polychromatic (spectral) light is passed to a PMT array to detect a range of wavelengths either sequentially or simultaneously, depending on the range of wavelengths desired. An example of this type of imaging is shown in Figure 2.1.4, where many fluorescent proteins are simultaneously imaged in a sample. Although this option allows for more versatility and direct selection of the emission range, it can come at the cost of less sensitive detection, due to the light loss in the additional optics required and in the spreading out of the light over a series of detectors to enable the spectral detection.

Table 2.1.1 shows the main advantages and disadvantages of LSCM. The main advantage is that one may optically section while still doing complex experiments. Another advantage is the versatility of imaging capabilities and types of experiments they enable. Most of these systems have multiple channels for multicolor, variable pinhole sizing for selecting the desired optical section thickness
Figure 2.1.4 Maximum projection reconstruction from confocal images obtained through a 65-μm stack of mouse cerebellum labeled with a combination of fluorescent proteins. In this image one can see the unique colors produced and spectrally detected by the genetic combinations of individual fluorescent proteins (XFPs). These colors were used to trace and map the various synaptic circuits. This panel of the figure was reproduced from Livet et al. (2007), with permission of the Nature Publishing group. For the color version of this figure go to http://www.currentprotocols.com/protocol/ns0201.

(usually sacrificing z-resolution for signal intensity), and software for variable ROI (region of interest) selection. Another advantage would be the ability to separate spectrally overlapping fluorescent proteins with spectral detection and spectral deconvolution methods. In addition, these LSCM systems, particularly in the inverted microscope configuration, can accommodate live or fixed cells or tissue. Many manufacturers also provide options for small-stage incubation systems, which allow relatively long-term experiments, particularly when coupled to automated acquisition software that enables auto-focusing algorithms in tandem with precise xyz stage movement.

Disadvantages of a modern LSCM system include the relatively low scan speed (as the beam must be swept through each pixel in the field of view), relatively high price, and amount of light impinging on the sample. The flexibility of the LSCM does mitigate many of the disadvantages, and in many instances, one can balance the imaging conditions among the variables listed in Figure 2.1.1 to get the most out of a given experiment. For instance, if full-frame imaging speed is too slow to capture a physiological event in a live cell experiment, one might use a small ROI to increase temporal resolution. Despite this flexibility, one concern always remains and should be considered when conducting LSCM, i.e., keeping the light levels low enough to avoid killing or bleaching the sample.

Another type of confocal microscopy is multipoint confocal microscopy, which includes Nipkow spinning disk, swept-field, and slit line scanning microscope systems. Each of these types of microscope systems shares the characteristic that multiple parts of the sample are imaged at once, thus increasing imaging speed. In the case of the Nipkow spinning disk and swept field systems, a sensitive camera (typically an EMCCD) is also employed. This allows for fast (usually tens to hundreds of milliseconds vs. the seconds timeframe of the LSCM), relatively low-light, confocal imaging. A drawback of Nipkow scanning systems is that confocal sectioning can only occur with relatively high NA objective lenses and the pinhole size is fixed. In the case of the slit-scanning confocal microscopes, there is also a modest decrease in resolution for the X or Y dimension. All of these systems are usually less expensive than a LSCM system but can become relatively expensive if a very sensitive camera is also included.
TOTAL INTERNAL REFLECTION FLUORESCENCE (TIRF) MICROSCOPY

TIRF microscopy provides very good axial resolution (z-direction, along the axis of illumination) to levels of ~200 nm (for review see Toomre and Manstein, 2001). Not only does this provide better axial resolution than most other techniques but this also can greatly reduce background light (thus increasing the signal to noise ratio) that can obscure fine details. The setup for TIRF microscopy is very simple and is similar to wide-field microscopy except that it employs an oblique angle for the excitation light impinging on the sample. When the incidence angle is set to a critical angle relative to the coverslip, the excitation light is totally internally reflected (Fig. 2.1.5A). This generates an electromagnetic field at the interface, called an evanescent wave, which excites fluorophores in nearly the same manner as conventional fluorescence excitation light. The key here is that the evanescent wave propagates only a short distance above the coverslip (Fig. 2.1.5B). Therefore, only fluorescent molecules in close proximity to the coverslip are excited. Figure 2.1.5C and D show a wide-field and a TIRF image.
respectively, of the fluorescence from EGFP-labeled myosin in *Drosophila* embryo hemocytes. As can be seen in Figure 2.1.5D, only myosin molecules in portions of the cell near the coverslip are excited, showing where the cell is closest to or touching the coverslip.

As mentioned above, the decay of the evanescent wave is exponential with the distance above the coverslip. This relationship can be expressed as:

\[
I(z) = I(o) e^{-z/d}
\]

**Equation 2.1.3**

where \(I(z)\) represents the intensity \(I\) at a given distance \(z\) from the coverslip, \(I(o)\) is the intensity at the coverslip, and \(d\) is the penetration depth in microns. The penetration depth \(d\) decreases as the reflection angle of the incident \(\theta_c\) in Fig. 2.1.5) beam grows larger. This value is also dependent on the illumination wavelength and on the refractive index of the medium present at the interface. In a typical commercially available objective-based TIRF system, the reflection angle of the excitation light can be changed mechanically on a special illumination module attached to the epifluorescence port of a wide-field microscope. Turning of the micrometer changes the position of the beam traveling in the periphery of the objective back aperture, resulting in a change in the angle of the beam exiting the front element.

Another requirement for the typical objective-based TIRF system is that high numerical oil objectives (>1.4 NA) are required to generate the necessary reflection angles to establish the evanescent wave.

As is shown in Table 2.1.1, the main advantage of TIRF is enhanced z-resolution. xy resolution benefits from a reduction in background fluorescence. Compared to confocal and two-photon techniques, a commercial turn-key, objective-based TIRF microscope system is inexpensive, requiring only a microscope, special illuminators, lasers, camera, and high NA objective lens. The main disadvantage of TIRF is related to its main advantage. Only fluorophores in the first 200 to 300 nm can be excited, which obviously limits imaging to near the coverslip but enables a z-resolution to the same depth as the penetration of the evanescent wave. Also, because the intensity of the evanescent wave decreases according to this relationship, fluorescence intensity will be a function of distance from the coverslip, as well as the concentration of the fluorescent molecules. This makes quantification of depth from the coverslip or comparisons of molecular concentration difficult from TIRF images.

**TWO-PHOTON FLUORESCENCE MICROSCOPY (TPFM)**

TPFM is a type of laser scanning microscopy that is particularly useful for imaging thick samples both in vitro and in vivo. It has been used to image hundreds of microns into tissues (for reviews, see Diaspro et al., 2006; Svoboda and Yasuda, 2006). An example of this type of imaging is shown in Figure 2.1.6C. Deep imaging is achieved by using pulsed near-infrared excitation light. Infrared light penetrates much deeper into tissue than the visible wavelengths used in standard confocal and wide-field microscopy, due to decreased scattering and absorption. This technique is also good for limiting the excitation (and often photobleaching and possible phototoxicity) to just one focal plane.

TPFM has the added benefit of eliminating the need for a pinhole aperture for optical sectioning. In confocal microscopy, the pinhole is used to reject out-of-focus emission light from reaching the photo-sensor (photo-multiplier tube or camera), effectively selecting only a small portion of the emission light to achieve optical sectioning with much of the emission light “thrown away.” In TPFM it is the excitation pulse that provides the optical sectioning; therefore, all of the light can be collected from the excited focal spot and none of the scattered or ballistic emission light photons need be wasted during collection. TPFM is a form of multi-photon imaging, where more than one photon at a time is used to excite a fluorophore. Other examples of multiphoton imaging are second harmonic generation (SHG) imaging and coherent anti-stokes Raman (CARS) microscopy. However, CARS and SHG are not fluorescence techniques and are outside the scope of this unit.

TPFM excitation is generated when a fluorophore absorbs two photons essentially simultaneously. This roughly doubles the amount of energy absorbed by the fluorescent molecules, which drives their excited electrons to the same energy level as would the absorption of one photon at one-half the two-photon excitation wavelength (Fig. 2.1.6A). An example would be the excitation of GFP (typically excited at \(\sim488\) nm in a confocal experiment) at \(\sim960\) nm using a pulsed laser. This is an oversimplification, as the actual TPFM absorption spectra for many fluorophores are over 100 nm broad, and “selection rules” that govern the relative strengths of
Absorption bands vary between one-photon and two-photon excitation, but in most cases that is a good starting point for guessing where the maximal TPFM excitation occurs. The broad spectral absorption range of the typical two-photon fluorophore allows for multiple fluorophores in a sample to be simultaneously excited at one wavelength. Corresponding emission wavelengths for each fluorophore are then separated in different channels with the appropriate set of dichroic and emission filters or with spectral detection.

The inherent optical sectioning ability of two-photon excitation is due to increased probability of two-photon absorption occurring at the diffraction limited spot because of spatial energy crowding (Fig. 2.1.6B).

The relationship of the variables to the intensity of the collected two-photon light can be seen in the equation for time averaged two-photon fluorescence intensity ($I_f$):

$$I_f = \delta \eta (P_{2\text{ave}}/\tau_{\text{pulse}})(NA^2/hc \lambda_{\text{exc}})^2$$

\textbf{Equation 2.1.4}
where $\delta_2$ is the two-photon cross-section for the fluorophore, $\eta$ is the quantum yield of the fluorophore, $P$ is the intensity (power) of the excitation light, $\tau_p$ is the pulse width of the excitation pulses, $f_p$ is the repetition rate of the laser, $NA$ is the numerical aperture of the objective, $h$ and $c$ are Planck's constant and the speed of light respectively, and $\lambda_{exc}$ is the wavelength of the excitation light (Diaspro et al., 2006). In fact, the probability of two-photon absorption decreases as the fourth power of distance away from this focal region along the z-axis (as can be seen by the NA dependence in Eq. 2.1.4) and increases as the square of the intensity (mW of power are typically required). Another variable is the temporal pulse width, $\tau_p$, of the excitation light pulse as it reaches the sample. In general, short pulse widths (on the order of 100 fsec) are optimal for two-photon excitation.

Commercially available turn-key TPFM systems usually consist of a modified point-scanning confocal microscope, which includes a Ti:Sapphire pulsed laser (often automatically tunable over a broad range of wavelengths) and non-descanned detector channel(s). The non-descanned detector is mounted on the microscope in a position that is close to the sample where the emission light does not travel back through the scan-head. Since no pinhole is necessary, this configuration can be employed to reduce light losses that would occur if the emission light passed back through the scan-head. Typically, commercially available pulsed lasers produce approximately 100 fsec pulses at a rate of 80 mHz. Dispersion in the optics of the microscope and objective will lengthen these pulse widths by at least a factor of two. Some commercial lasers now optionally include an additional unit for precompensation of this dispersion, which can reduce the pulse length at the sample and thus restore two-photon fluorescence efficiency.

In summary, as is shown in Table 2.1.1, the main advantage of TPFM is the depth of imaging (hundreds of microns) into the sample. Another important advantage is that the bleaching and phototoxicity are limited to the focal plane; however, in the focal plane the damage can be greater due to the higher light intensities (mW compared to $\mu$W in confocal) needed for TPFM. TPFM typically requires the same time frame for acquisition as traditional CLSM (on the order of 1 sec/frame). One big disadvantage is the cost due to the need for a point-scanning microscope and a tunable pulsed Ti:Sapphire laser. This cost increases substantially if one also adds a precompensation unit to correct dispersion in excitation pulse lengths or selects higher power lasers whose gain enables lasing at the regions approaching 700 nm or somewhat above 1000 nm, regions in which it is notoriously difficult to tune.

**STIMULATED EMISSION DEPLETION (STED)**

**FLUORESCENCE MICROSCOPY**

STED microscopy, developed by Stefan Hell and colleagues, is a relatively new super-resolution technique that has been shown to improve fluorescence microscopy resolution by approximately an order of magnitude over traditional diffraction limited techniques such as LSCM. STED can produce optical resolution to levels that were previously thought possible only with electron microscopy, and it has been used to examine key biological processes that no other technique could have examined (Willig et al., 2006b; Kellner et al., 2007). STED improves resolution by a direct reduction in the emission spot size by using a second laser beam (the STED beam; see Fig. 2.1.7 and below). It is important to note that the improvement in resolution is achieved directly without the need for post processing and mathematical redistribution of the light, as is done in deconvolution microscopy or by combining multiple images taken with respect to a moveable grid pattern, as is done in structured light microscopy. STED is so straightforward that to the user this seems like a normal point-scanning technique such as LSCM or TPFM. In fact, Leica Microsystems (Wetzlar, Germany) has now commercialized this system on their current point-scanning microscope stand.

Figure 2.1.7A shows the setup for the STED technique. In the commercially available system (Leica Microsystems), two pulsed (picosecond time-domain) lasers are included. One excites the fluorophore as in a LSCM experiment. The second, longer wavelength laser is used for the patterned quenching of the focal spot (STED beam). Specialized optics in the scanhead spatially shape the phase of the STED beam wave-front to form a doughnut pattern (with a sharply decreased laser intensity in the central portion of the doughnut) at the focal spot. The STED wavelength must be red shifted (longer wavelength) such that it does not overlap the absorption spectrum of the fluorophore, but does overlap with its emission...
Figure 2.1.7  Technical principals of stimulated emission depletion (STED) microscopy. (A) The combination of the normal excitation beam with the phase modulated STED beam produces a sub-diffraction emission spot. The images on the right in (A) show the doughnut pattern produced by the phase modulation of the STED beam. This beam, when overlapped with the diffraction-limited excitation spot, quenches emission where the beams overlap leaving the middle, sub-diffraction sized, spot for spontaneous fluorescence. (B) Comparison of confocal (left) and STED (right) images of a labeled preparation reveals a marked increase in resolution by STED because more labeled particles are visualized. Scale bar, 500 nm. Figure reproduced from Willig et al. (2006b) with permission of the Nature Publishing group.

In this way, it quenches the emission of the fluorophore (forces the excited electrons into a lower energy state without emitting fluorescent light) in the area of the spot where the STED beam overlaps the excitation beam. This reduces the ultimate emitting region to that of the middle of the doughnut. The size of this region is related to the power of the STED beam according to the following expression:

\[ R \approx \lambda [2NA(1 + \zeta)0.5] \]

**Equation 2.1.5**

where \( R \) is the lateral (\( xy \)) resolution, \( \lambda \) is the wavelength of the excitation light, \( NA \) is the numerical aperture of the objective, and \( \zeta \) describes the intensity of the STED beam. Specifically, \( \zeta \) stands for \( I/I_s \), which is a saturation factor for the dye used. \( I_s \) is the intensity value at which the dye falls to 1/e of its initial value. Therefore, the gain in resolution of this technique is both a function of laser intensity of the STED beam and the quenching characteristics of the dye used. Stefan Hell and colleagues have reported limiting the excitation size to around 30 nm, or almost the size of a few fluorescent molecules, in this manner (Willig et al., 2007).

The main disadvantages of the STED approach are the cost of the system and the amount of power that impinges on the sample (Table 2.1.1). The cost of the system is relatively high because two pulsed lasers are needed in addition to the already expensive laser scanning microscope system and very sensitive emission detectors (avalanche photodiodes required to produce fast electronics and having sensitivity in the far-red portion of the spectrum).
Another disadvantage is that the amount of power used in a STED system is high (tens of mW for the second beam). Since there is the potential for destruction of the probe or sample, only certain very photostable probes can be used. The list of probes that have been used to date are LDS721, certain ATTO dyes (AttoTech), and fluorescent proteins (Willig et al., 2006a). While the current commercial system can only improve lateral resolution, axial resolution can also be improved (Hell, 2007). It remains unclear how deep into tissue this technique remains effective. It is likely that that the loss of coherence of the shape of the STED beam with depth is a limiting factor and will be tissue-dependent. In general, STED is most effective for fixed tissue as movement or diffusion of the fluorescent marker during scanned imaging will negate any gains in resolution.

FINAL CONSIDERATIONS

Many of the microscope systems available from manufacturers have become very easy to use. While this provides easy image acquisition for many of the techniques listed in Table 2.1.1, the danger remains that an incomplete understanding of the fundamental physics and limitations of the techniques can result in wrong, incomplete, or biased data (as recently noted in Pearson, 2007). It should be noted that Table 2.1.1 is only a rough guide to the commercially available fluorescence microscopy techniques and the reader should consult other sources to achieve a more complete understanding. There are many excellent reviews and many good Web-based resources, such as Molecular Expressions: Exploring the World of Optics and Microscopy (http://www.microscopy.fsu.edu/), The Molecular Probes Handbook (http://probes.invitrogen.com/handbook/), and the Confocal Listserv (http://listserv.acsu.buffalo.edu/cgi-bin/wa?A0=CONFOCAL&D=0&F=P&T=0).

Finally, remember that one must also be able to analyze the data collected. There are many good commercially available image processing software packages available, as well as the comprehensive free program ImageJ (http://rsbweb.nih.gov/ij/). The mention of any company producing microscopy related products in this work is in no way intended as an endorsement of them by the National Institutes of Health or the author.

Recently, two techniques have been commercialized allowing super-resolution imaging. The first is PhotoActivated Localization Microscopy (PALM). This technique builds a super-resolution image from many image cycles, using low level activation to create sets of well-separated spots from photoactivatable probes localized with nm accuracy, followed by photobleaching. A composite image is made by fitting the individual spots from the many images. The second technique is a variant of STED microscopy discussed above. Instead of using pulsed lasers, it uses standard continuous wave (CW) lasers. This requires more laser power but has the advantage of a broader selection of dyes for the CW-STED imaging and is less expensive than the pulsed laser STED. CW-STED has been commercialized by Leica Microsystems. There is literature on both of these techniques and the interested reader is encouraged to explore it more fully.

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