Confocal microscopy produces sharp images of structures within relatively thick specimens (up to several hundred microns). It is particularly useful for examining fluorescent specimens. Thick fluorescent specimens viewed with a conventional widefield fluorescent microscope appear blurry and lack contrast because fluorophores throughout the entire depth of the specimen are illuminated and fluorescence signals are collected not only from the plane of focus but also from areas above and below. Confocal microscopes selectively collect light from thin (∼1-µm) optical sections representing single focal planes within the specimen. Structures within the focal plane appear more sharply defined than they would with a conventional microscope because there is essentially no flare of light from out-of-focus areas. A three-dimensional view of the specimen can be reconstructed from a series of optical sections at different depths.

The confocal microscope is the instrument of choice for examining fluorescence-stained neurons in brain slices (Fig. 2.2.1) or small, intact organisms such as Drosophila and zebrafish embryos. It is also useful for localizing intracellular antigens in dissociated cells. Its sensitivity even allows fluorescence in living specimens to be monitored, making it feasible to follow the movements in living cells of fluorescent probes such as green fluorescent protein (GFP; Fig. 2.2.2), dyes for specific organelles, FM-143 (a marker for synaptic vesicle recycling), DiI (a dicarbocyanine dye that stains the plasma membrane), and Ca²⁺ indicators (e.g., Fluo-3, calcium green, and Indo-1; see UNIT 2.3). In addition, some types of confocal microscopes can be configured to perform photobleach experiments (Fig. 2.2.2C, D) and to photoactivate “caged” molecules (molecules that are inactive until released with UV illumination).

Neurobiologists use confocal microscopy in a number of creative ways that are beyond the scope of this article. The information presented herein is intended to provide background and practical tips needed to get started with confocal microscopy. An excellent source of practical information, and Confocal Microscopy (1990; edited by T. Wilson) for theoretical background.

THE BASIS OF OPTICAL SECTIONING

Confocal microscopes accomplish optical sectioning by scanning the specimen with a focused beam of light and collecting the fluorescence signal from each spot via a spatial filter (generally a pinhole aperture) that blocks signals from out-of-focus areas of the specimen. The physical basis of optical sectioning in fluorescence confocal microscopy is illustrated in Figure 2.2.3. A point light source (typically a laser) evenly illuminates the back focal plane of the objective, which focuses the light to a diffraction-limited spot in the specimen. The irradiation is most intense at the focal spot, although areas of the specimen above and below the focal spot also are illuminated. Fluorescent molecules excited by the incident light emit fluorescence in all directions. The fluorescence collected by the objective comes to focus in the image plane, which is conjugate (confocal) with the focal plane in the specimen. A pinhole aperture in the image plane allows fluorescence from the illuminated spot in the specimen to pass to the detector but blocks light from out-of-focus areas.

The diameter of the pinhole determines how much of the fluorescence emitted by the illuminated spot in the specimen is detected, and the thickness of the optical section. From wave optics we know that a point light source in the plane of focus of an objective produces a three-dimensional diffraction pattern in the image plane. The cross section at the image plane is an Airy disk (see Fig. 2.1.9), a circular diffraction pattern with a bright central region. The radius of the bright central region of the Airy disk in the reference frame of the specimen is given by

\[ R_{Airy} = 0.61\frac{\lambda}{\text{NA}} \]

where \( \lambda \) is the emission wavelength and NA is the numerical aperture of the objective (see UNIT 2.1 for a discussion of NA). At the image plane (the location of the pinhole aperture) the radius of the central region is \( R_{Airy} \) multiplied by the magnification at that plane (for a more complete explanation see Wilson, 1995).

Adjustment of the pinhole to a diameter slightly less than the diameter of the central
region of the Airy disk allows most of the light from the focal point to reach the detector and reduces the background from out-of-focus areas by ∼1000-fold relative to widefield microscopy (Sandison et al., 1995). The separation of the in-focus signal from the out-of-focus background achieved by a properly adjusted pinhole is the principle advantage of confocal microscopy for examination of thick specimens.

Point illumination and the presence of a pinhole in the detection light path also produces improved lateral and axial resolution relative to conventional microscopy (Table 2.2.1). The actual extent of improvement depends on the size of the pinhole. Near-maximal axial resolution is obtained with a pinhole radius $\sim 0.7 \times R_{\text{Airy}}$ whereas optimal lateral resolution is obtained with a pinhole less than $0.3 \times R_{\text{Airy}}$ (Wilson, 1995). However, a pinhole smaller than $\sim 0.7 \times R_{\text{Airy}}$ significantly reduces the total signal, a sacrifice that may not be worth the gain in resolution especially when imaging dim sam-

![Figure 2.2.1](https://www.currentprotocols.com/colorfigures)

**Figure 2.2.1** Laser-scanning confocal microscope images of mouse neuroepithelial precursor cells transplanted into the embryonic rat brain. (A) Projection of a stack of 25 confocal images collected at 0.5-μm intervals in the z axis. (B) Stereo view that, when observed with red/green or red/blue spectacles, is perceived as three-dimensional. Images were collected with a 40×, 1.2-NA objective on a Zeiss LSM 410 laser-scanning confocal microscope with a krypton-argon laser. Donor-derived cells are visualized with a mouse-specific antibody to M6 (Lund et al., 1985; Brüstle et al., 1995). Scale bar, 25 μm. *This black and white facsimile of the figure is intended only as a placeholder; for full-color version of figure go to [thp://www.currentprotocols.com/colorfigures](http://www.currentprotocols.com/colorfigures)*
ples. In fluorescence imaging, resolution also is influenced by the emission and excitation wavelengths (Table 2.2.1).

**TYPES OF CONFOCAL MICROSCOPES**

Several types of confocal microscopes are available, each having unique features and advantages. The types most commonly used for examining fluorescence specimens are laser-scanning confocal microscopes. These microscopes, as their name implies, use lasers as light sources and collect images by scanning the laser beam across the specimen.

Lasers provide intense illumination within a narrow range of wavelengths. The emission wavelengths of several types of lasers, together with the excitation spectra of familiar fluorophores, are illustrated in Figure 2.2.4. Mixed krypton-argon gas lasers are popular for multi-
wavelength confocal microscopy because they emit at three well-separated wavelengths (488, 568, and 647 nm) that can be used to simultaneously image two or three fluorophores (e.g., FITC, lissamine rhodamine, and Cy5). The disadvantage of krypton-argon lasers is that their life spans are short (∼2000 hr). Another way to obtain multiwavelength excitation is to combine the outputs of two or more lasers.

Several methods have been devised for scanning the sample with the laser beam to illuminate different positions in the specimen. The most common method employs a pair of galvanometer mirrors to both scan the laser beam across the specimen and collect the fluorescence emitted from the specimen (Fig. 2.2.5). One galvanometer mirror sequentially illuminates spots along the x axis, and the second mirror moves from line to line in the y axis. The fluorescence emission is separated from the illuminating beam by a dichroic beam splitter and is directed to a photomultiplier tube which collects the fluorescence produced as each spot in the specimen is illuminated. The photodetector output is converted to a digital image that can be displayed on a monitor or stored for later analysis. Most laser-scanning confocal microscopes have 8-bit digitizers which encode 256 gray levels although some recent models have 16-bit digitizers. Collection of a full-size image (typically 1024 × 1024 pixels) takes ∼2 sec. Laser-scanning microscopes that employ galvanometer mirror scanners sometimes are called “slow-scan” microscopes because of their relatively slow image acquisition rates.

The movements of the galvanometer mirrors in laser scanning microscopes are under the control of a computer, providing flexibility in the scanning pattern. For example, it is possible to “zoom” a region of interest (visualize it at higher magnification) by reducing the scan area and the distances between sample points. In

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**Figure 2.2.3** The basis of optical sectioning in confocal epifluorescence microscopy. Illumination from the point light source is reflected by the dichroic mirror and focused by the objective lens to a diffraction-limited spot within the specimen. Fluorophores within the focal spot as well as in the cone of light above and below it are excited, emitting fluorescence at a longer wavelength than the incident light. The fluorescence captured by the objective passes through the dichroic mirror because of its longer wavelength. The confocal pinhole allows fluorescence from the plane of focus in the specimen to reach the photodetector but blocks fluorescence from areas above and below the plane of focus. Redrawn from Shotton (1993).
addition, many laser-scanning microscopes have the ability to repetitively scan a single line or to “park” the scanner to monitor fluorescence at a single spot. The latter technique is particularly useful for studying rapidly changing fluorescence signals, such as those produced by a Ca²⁺ indicator in an active neuron. Laser-scanning microscopes are available that can collect images at video rates (30 frames/sec) or faster. Several methods for achieving rapid scanning rates have been employed (e.g., acousto-optical deflection devices, rotating mirrors or resonating mirrors; reviewed by Art and Goodman, 1993; Tsien and Bacskai, 1995). The gain in speed of imaging always comes at a cost, however. For example, rapid-scan confocal microscopes do not provide the degree of control over the scan pattern offered by top-of-the-line slow-scan microscopes, and some video-rate confocal microscopes are incapable of multiwavelength illumination. Video-rate microscopes that rely on slit apertures rather than pinhole apertures have slightly poorer lateral and axial resolution.

A type of rapid-scan confocal microscope that deserves mention because of its lower cost, (among other reasons) uses a spinning disk with multiple pinholes (∼200,000) to simultaneously illuminate and detect emission from many spots in the specimen. The light source is the same type of broad-spectrum lamp used for conventional epifluorescence microscopy. The principle advantage of this type of confocal microscope is that it is capable of collecting images very rapidly (∼700 frames/sec at 5000 lines resolution; Kino, 1995). The images can be examined directly by eye or captured with a sensitive camera. The main disadvantage is that the disk transmits only ~1% of the available light because the holes in the spinning disk need to be widely spaced. The low transmission makes it difficult to obtain enough light for weakly fluorescent specimens. A new method for real-time confocal microscopy with a broad-spectrum light source has recently been described in which the spinning disk is replaced by a random-pattern mask that transmits ~50% of the light (Juskaitis et al., 1996).

Another form of laser-scanning microscopy that promises to be of great value to neurobiologists uses two-photon (and three-photon) excitation to induce fluorescence emission (Denk et al., 1995). Two-photon excitation occurs when a fluorophore simultaneously absorbs two photons, each having half the energy needed to raise the fluorophore to the excited state. The light intensities required for simultaneous absorption occur only at the focal point so only fluorophores at the focal point are excited. Therefore, two-photon excitation allows optical sectioning without a spatial filter in front of the detector. Moreover, since fluorophores outside the focal point are not excited, the specimen is less subject to photobleaching than in a conventional laser-scanning microscope. The wavelengths needed to excite standard visible light fluorophores by two-photon absorption are longer and penetrate tissue better than the wavelengths used for one-photon excitation, making it possible to look deeper into a specimen. In addition, UV fluorophores can be imaged without many of the problems that arise when UV wavelengths are used in conventional laser-scanning microscopes. A cur-
rent drawback of two-photon confocal microscopy is the high cost of an appropriate laser (∼$100,000).

PRACTICAL GUIDELINES

Sample Preparation: Immunofluorescence in Fixed Specimens

Fixation

The best fixative is one that accurately preserves the three-dimensional geometry of the specimen. The standard fixative for fluorescence microscopy (2% to 4% formaldehyde in PBS) is not ideal because it can cause blebbing of the plasma membrane, vesiculation of intracellular membrane compartments, and other alterations in cellular morphology. Moreover, some commercial preparations of formaldehyde contain methanol which shrinks cells. Techniques for optimizing formaldehyde fixation are described by Bacallao et al. (1995; also see UNIT 2.1). The buffer should be chosen to match the osmolality and pH of the specimen. Fixatives containing 0.125% to 0.25% glutaraldehyde in addition to formaldehyde preserve cellular morphology better than formaldehyde alone. Some investigators avoid using glutaraldehyde for fluorescence microscopy because it induces autofluorescence. However, autofluor-
Rescence can be reduced by treating the sample after fixation with NaBH₄ (1 mg/ml in PBS, pH 8.0, using two treatments of 5 min each for dissociated cells, longer for thicker samples). A more serious drawback of glutaraldehyde for immunofluorescence studies is that it destroys the antibody recognition sites of some antigens. An alternative fixation technique that preserves tissue better than chemical fixation is rapid freezing followed by freeze substitution (Bridgman and Reese, 1984).

**Choices of fluorophores**

Criteria to consider in selecting fluorophores for fluorescence microscopy are described in UNIT 2.1. The only additional consideration for confocal microscopy is to choose fluorophores that can be excited by the wavelengths provided by the available lasers. However, it is not essential for the excitation spectrum peak to precisely match the laser wavelength because the lasers on most microscopes are sufficiently powerful to maximally excite fluorophores at off-peak wavelengths. For experiments that depend on imaging two fluorophores, it is best to select fluorophores whose excitation and emission spectra have minimal overlap. Good choices for multi-wavelength imaging with a krypton-argon laser are: FITC/Bodipy/Oregon green for excitation at 488 nm; lissamine rhodamine/Cy3/Texas red for excitation at 568 nm; and Cy5 for excitation at 647 nm. UV fluorophores also are good for multicolor imaging (some of the best dyes for DNA are UV fluorophores) if a UV laser is available.

**Control samples**

Confocal microscopy uses electronic image enhancement techniques that can make even a dim autofluorescence signal or nonspecific background staining look bright. In order to be able to distinguish a real signal from back-
ground it is essential to prepare appropriate control samples. For immunofluorescence experiments with one primary antibody, the appropriate control samples are unstained specimens and specimens treated with the secondary antibody but no primary antibody. Experiments with two primary and secondary antibodies require additional controls to test whether the secondary antibodies cross-react with the "wrong" primary antibody. Other control experiments may be required to verify the specificity of labeling (see UNIT 2.1).

Mounting the specimen

The mounting medium should preserve the three-dimensional structure of the specimen. PBS (APPENDIX 2A) or a mounting medium consisting of 50% glycerol/50% PBS preserves the shapes of cells quite well, but Mowiol and gelvatol cause a 10% decrease in height (Bacallao et al., 1995). Adding an antioxidant to the mounting medium helps to alleviate photo-bleaching. One of the best antioxidants is 100 mg/ml 1,4-diazabicyclo[2,2,2]octane (DABCO; Sigma; Bacallao et al., 1995). n-propyl gallate (Giloh and Sedat, 1982) and p-phenylenediamine (PPD; Johnson et al., 1982) also are effective antibleaching agents, but the former may cause dimming of the fluorescence while the latter may damage the specimen (Bacallao et al., 1995).

The choice of mounting medium should take into account the type of microscope objective that will be used to observe the specimen. In order for an objective to perform optimally, the mounting medium should have the same refractive index as the objective immersion medium. Table 2.2.2 gives the refractive indexes of standard objective immersion media and mounting media. Mismatches in the refractive indexes produce spherical aberration leading to loss of light at the detector, as well as decreased z axis resolution and incorrect depth discrimination. Image deterioration caused by spherical aberration increases with depth into the specimen. Significant losses of signal intensity and axial resolution are apparent at distances of just 5 to 10 µm when an oil immersion objective is used to examine a specimen in an aqueous medium (Keller, 1995).

Most microscope objectives are designed for viewing specimens through a glass coverslip of a specific thickness (typically 0.17 µm, a no. 1 ½ coverslip). Correct coverslip thickness is especially critical for high-NA (>0.5) dry objectives and water immersion objectives (Keller, 1995). Use of a coverslip that differs from the intended thickness by only 5% causes significant spherical aberration. High-NA dry and water immersion objectives typically have an adjustable collar to correct for small variations in coverslip thickness.

The specimen should be mounted as close to the coverslip as possible especially for observation with immersion objectives, which have short working distances (~100 to 250 µm, depending on the type of objective). This also helps to avoid image deterioration due to spherical aberration. Fragile specimens should be protected by supporting the coverslip; for

<table>
<thead>
<tr>
<th>Table 2.2.2 Refractive Indexes of Common Immersion and Mounting Media</th>
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<tr>
<td><strong>Medium</strong></td>
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<tr>
<td>----------------</td>
</tr>
<tr>
<td><strong>Immersion media</strong></td>
</tr>
<tr>
<td>Air</td>
</tr>
<tr>
<td>Water</td>
</tr>
<tr>
<td>Glycerol</td>
</tr>
<tr>
<td>Immersion oil</td>
</tr>
<tr>
<td><strong>Mounting media</strong></td>
</tr>
<tr>
<td>50% glycerol/PBS/DABCO</td>
</tr>
<tr>
<td>5% n-propyl gallate/0.0025% p-phenylene diamine (PPD) in glycerol</td>
</tr>
<tr>
<td>0.25% PPD/0.0025% DABCO/5% n-propyl gallate in glycerol</td>
</tr>
<tr>
<td>VectaShield (Vector Labs)</td>
</tr>
<tr>
<td>Slow Fade (Molecular Probes)</td>
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</tbody>
</table>

<sup>a</sup>Data from Bacallao et al. (1995).
example, using a thin layer of nail polish, strips of coverslips, or a gasket made from a sheet of silicon rubber (Reiss; see SUPPLIERS APPENDIX). Sealing the edges of the coverslip—perhaps with nail polish or silicon vacuum grease (Dow Corning; see SUPPLIERS APPENDIX)—helps to prevent specimen desiccation and movement.

**Living Specimens**

Confocal microscopy of living preparations is challenging for several reasons. The specimen must be mounted in a chamber that keeps it healthy and immobile while at the same time providing access for the objective. For high-resolution transmitted-light imaging (e.g., by laser-scanning differential interference contrast microscopy), the chamber must be thin enough to accommodate a high-NA (oil immersion) condenser. Fluorescence signals in living specimens generally are weak and the light levels needed to detect them can be damaging to the specimen. Photobleaching inevitably is a problem for experiments that require collecting many images. Temperature fluctuations in specimens kept at nonambient temperatures make it difficult to maintain adequate focus.

A simple chamber for culture preparations grown on glass coverslips can be made by forming a well on a glass slide with a gasket cut from a sheet of silicon rubber or a plastic ruler. To prevent the well from leaking, it should be sealed with silicon vacuum grease, a mixture of melted paraffin and petroleum jelly, or Sylgard (Dow Corning; see SUPPLIERS APPENDIX). The well is filled with medium and then the coverslip with attached cells is placed, cell side down, on top of the well. The preparation can be kept warm during observation on the microscope with a heated air blower (e.g., a hair dryer with variable power source) or with infrared lamps. More elaborate chambers, some of which have built-in heaters and ports for changing solutions, are available from commercial sources (see Terasaki and Dailey, 1995, for a partial listing of manufacturers). An important factor to consider in choosing a chamber is whether it maintains the desired temperature while in contact with an immersion objective that acts as a heat sink. One solution to this problem is to heat the objective as well as the chamber. A heated chamber and objective warmer designed for microscopy with a high-NA objective and condenser are available from Bioptechs (see SUPPLIERS APPENDIX).

Addition of an oxygen quencher to the medium can help to alleviate photobleaching of the fluorophores. Photobleaching not only leads to dimming of the signal but also to generation of oxygen radicals that can damage cells. Several oxygen quenchers have been reported to be effective, including oxyrase (0.3 U/ml; Oxyrase [see SUPPLIERS APPENDIX]; Waterman-Storer et al., 1993); ascorbic acid (0.1 to 3.0 mg/ml; Sigma; Terasaki and Dailey, 1995); a mixture of Trolox (10 μM; Aldrich) and N-acetyl cysteine (50 μM; Sigma; M. Burack and G. Banker, pers. comm.;) and crocetin (Tsien and Waggner, 1995).

**Optimizing Imaging Parameters**

**Choice of objectives**

High-NA objectives generally are preferable for fluorescence microscopy because they collect more light than low-NA objectives (brightness is proportional to NA4). Most high-quality high-NA objectives have >80% transmission at visible wavelengths, but some have low transmission at UV wavelengths (Keller, 1995).

Water immersion objectives are the best choice for visualizing specimens in aqueous solutions (e.g., living specimens). Several microscope manufacturers recently have introduced high-NA water immersion objectives specifically designed for confocal microscopy of biological specimens. These objectives differ from previously available types of water immersion objectives in that they are intended for viewing specimens mounted under a coverslip. They have working distances of ∼250 μm.

Oil immersion objectives can have higher NAs than water immersion objectives. Most have fairly short working distances (∼100 μm) although some recently introduced oil objectives have working distances of ∼200 μm. A long-working-distance oil objective will be useful only if the specimen is mounted in a medium that matches the refractive index of immersion oil (η = 1.518). If an aqueous mounting medium is used, images from depths at more than ∼20 μm into the specimen will be noticeably degraded by spherical aberration. Also, distance measurements in the z axis will need to be corrected. The actual movement of the focal plane in the specimen (dz) produced by a movement of the objective (dobj) depends on the ratio of the refractive indexes of the specimen and immersion medium. A reasonable approximation (Majlof and Forsgren, 1993) of the relationship is given by:

\[ \frac{d_z}{d_{obj}} = \frac{\eta_s}{\eta_{obj}} \]
**Pinhole size**

As was explained in the Basis of Optical Sectioning, the size of the pinhole has a critical influence on image quality. A pinhole with a radius equal to the radius of the first minimum of the Airy disk (which is approximately equivalent to the diameter at half maximal intensity; Amos, 1995) will let most of the light from the plane of focus reach the detector, while blocking most of the out-of-focus flare. The lateral resolution will be –20% better than that obtainable by conventional microscopy with the same optics (Centonze and Pawley, 1995), but not as good as can be achieved with a smaller pinhole. Lateral resolution continues to improve as pinhole radius is decreased down to a pinhole size of \( -0.2 \times \) Airy disk radius, but a pinhole this small excludes \( -95\% \) of the signal (Wilson, 1995). Axial resolution improves as pinhole size decreases, down to \( -0.7 \times \) Airy disk radius, then levels off. The best trade-off between signal intensity and resolution will depend on the characteristics of the sample and aims of the experiment.

**Zoom factor**

The zoom setting on a confocal microscope determines the size of the scan region and the apparent magnification of the image. A zoom factor of 2 will scan an area half as long and wide as a zoom factor of 1. Images are made up of the same number of samples (points along the horizontal axis, lines along the vertical axis) and are displayed on the image monitor by a fixed number of pixels regardless of the zoom factor. Therefore, the pixels in a zoom-2 image will represent areas within the specimen half as large in each dimension as the areas represented by the pixels at zoom 1. If the pixel size for an objective at zoom 1 represents \( 0.25 \mu m \times 0.25 \mu m \), then the pixel size at zoom 2 will be \( 0.125 \times 0.125 \mu m \). The pixel dimensions (referring to the specimen) are inversely related to the zoom setting.

For each objective, there is an optimal zoom setting which yields pixel dimensions small enough to take advantage of the full resolution of the objective but large enough to avoid oversampling. In order for the minimum resolvable entity to be visible on the display monitor, the pixel dimensions need to be smaller than (less than one-half) the optical resolution. However, if the pixel size is made too small by using a higher-than-optimal zoom factor, the specimen is subjected to more irradiation than necessary with an increased risk of photobleaching. The rate of photobleaching increases proportionally to the square of the zoom factor (Centonze and Pawley, 1995). A guideline for selecting an appropriate zoom factor derived from information theory (the Nyquist Sampling Theorem) states that the pixel dimensions should be equal to the optical resolution divided by 2.3 (see Webb and Dorey, 1995). However, pixel dimensions smaller than this may produce more informative images.

**Z axis sectioning interval**

In order to study the three-dimensional structure of a specimen, images are collected at a series of focal levels at intervals determined by the settings sent to the focus motor. The most straightforward way to ensure that the reconstructed images have correct proportions in the \( x, y, \) and \( z \) axes is to collect optical sections at \( z \) axis intervals equal to the \( x, y \) pixel dimension. However, the interfocal plane interval needed to adequately sample the specimen in the \( z \) axis is not as small as the \( x, y \) pixel dimension because the axial resolution is poorer than the lateral resolution (see Table 2.2.1). The optimal interfocal plane interval (according to the Nyquist Sampling Theorem) is equal to the axial resolution divided by 2.3. Collecting images at shorter intervals results in oversampling with an increased risk of photobleaching.

**Illumination intensity**

Fluorescence emission increases linearly with illumination intensity up to a level at which emission saturates. Optimal signal-to-background and signal-to-noise ratios are obtained with illumination levels well below saturation (Tsien and Waggoner, 1995). The illumination intensity on a laser-scanning microscope can be adjusted by inserting neutral-density filters into the light path and/or by operating the laser at submaximal power. In general, the best images are obtained with illumination levels as high as possible without producing unacceptable rates of photobleaching.

**PMT black level and gain**

The contrast and information content of confocal images are influenced by the black level and gain of the photomultiplier tube (PMT) amplifiers. To obtain maximal information, the black level and gain should be adjusted to take advantage of the full dynamic range of the PMTs. The appropriate black level setting can be found by scanning while the light path to the PMT is blocked. The image that appears on the display monitor should be just barely brighter than the background, which is black (gray level = 0). To set the gain, scan the specimen
and adjust the gain so that the brightest pixel in the image is slightly below white (gray level = 255). Selecting black level and gain settings which ensure that all signals fall within the dynamic range of the PMT is important for quantitative imaging experiments. The software provided with many confocal microscopes includes a pseudocolor image display mode that facilitates selection of appropriate black level and gain settings by highlighting pixels with intensity values near absolute black and absolute white.

**Averaging**

Confocal images of dimly fluorescent specimens captured at typical scan rates (1 to 2 sec/frame for a slow-scan confocal microscope) appear noisy because of the small numbers of photons collected from each spot. In some instances, it may be possible to improve the signal-to-noise ratio by scanning the specimen at slower rates. Another way to obtain a better image is by summing and averaging the signals obtained in multiple scans (frame averaging). Some confocal microscopes provide a second averaging method (line averaging), in which individual lines are repeatedly scanned and averaged. Line averaging generally produces sharper images than frame averaging (which averages full frames) because there is less risk of blurring due to movements or changes in the specimen.

**Image display**

Commercial confocal microscope packages provide software for some types of image enhancement and display. The display options for three-dimensional datasets typically include “z projections” (see Fig. 2.2.1A) which are two-dimensional displays formed by superimposition of stacks of optical sections, and stereoscopic views (see Fig. 2.2.1B and Fig. 2.2.2A) which are made by combining two image stacks, one aligned in the z axis and the other with a displacement between successive images. Many systems also have the capability to compute cross-sections (see Fig. 2.2.2C, D) and projections of the specimen from varying angles. Computed projections for a sequence of view angles can be played as a movie in which the specimen appears to rotate around an axis. Such movies give the viewer a striking impression of the three-dimensional geometry of the specimen. Additional display options are available in various integrated software/hardware packages specifically designed for visualization and analysis of three-dimensional images.

**Anticipated Results**

Fluorescence in fixed specimens protected with an antifade agent is often sufficiently bright and resistant to photobleaching to make it possible to reconstruct three-dimensional images using imaging parameters that provide optimal resolution. Superb three-dimensional views may be obtained of structures as small and complex as the neuronal cytoskeleton or the terminal arbor of an axon. The maximum depth in the specimen at which adequate images can be obtained depends on a number of factors (e.g., the match in refractive indexes of the immersion and mounting media, the wavelength of light, and the extent of scattering and absorption by the specimen). Under optimal conditions, it may be possible to image structures at depths near the limit allowed by the working distance of the objective; in practice, image quality usually deteriorates at depths in the range of a few hundred micrometers or less.

Although confocal microscopy on living cells is more difficult and damage to the tissue may preclude extensive three-dimensional reconstruction, the added time dimension and confidence in the reality of the images makes it well worth the effort. In addition, it is possible to study dynamic processes lasting for hours by collecting sequences of time lapse images (see, for example, O’Rourke, 1992; Dailey et al., 1994). Robust fluorophores such as DiI and variants of GFP can be imaged repeatedly with minimal loss of fluorescence. In addition, modern laser-scanning confocal microscopes provide a versatile optical bench and sophisticated specimen positioner which permit a wide range of experiments with the controlled application of laser light to living tissues. Current examples of these approaches are photobleaching (Cole et al., 1996) and release of caged compounds (Callaway and Katz, 1993; Svoboda et al., 1996). These are only the harbingers of many future applications of light probe physiology made possible by the versatility of the confocal microscope.

**Resources Available via Internet**

NIH Image, a powerful image analysis program for Macintosh computers developed by W. Rasband (Research Services Branch, National Institute of Mental Health, NIH), has many useful tools for analysis of confocal images. It can be downloaded from http://rsb.info.nih.gov/nih-image/ or obtained via FTP from zippy.nimh.nih.gov. A version of NIH Image modified for operation under Windows also is available. Confocal Assistant, a
program designed for operating on images from Biorad confocal microscopes, is available via FTP from genetics.bio-rad.com/public/confocal/cas/cas255.zip.

Many topics of interest to confocal microscopists are discussed on the confocal e-mail listserver network. To subscribe to the list, send the message “subscribe confocal-your name” to listserv@ubvm.cc.buffalo.edu.

LITERATURE CITED


KEY REFERENCES


Covers basics of light microscopy, video microscopy, and much more.


Good source of practical information.


Excellent source of theoretical and technical information.


Excellent source of information about digital image processing.


Covers many aspects of light microscopy, including confocal microscopy.


Good source of theoretical background information.

INTERNET RESOURCES

zippy.nimh.nih.gov

Use to obtain NIH Image via FTP.

genetics.bio-rad.com/public/confocal/cas/cas255.zip

Use to obtain Confocal Assistant.

http://optics.jct.ac.il/~aryeh/Spectra

Source of excitation emission spectra for common fluorophores.

http://rsb.info.nih.gov/nih-image/

Use to obtain NIH Image.

listserv@ubvm.cc.buffalo.edu

Confocal e-mail listserv network.

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