Optical Sectioning—Slices of Life

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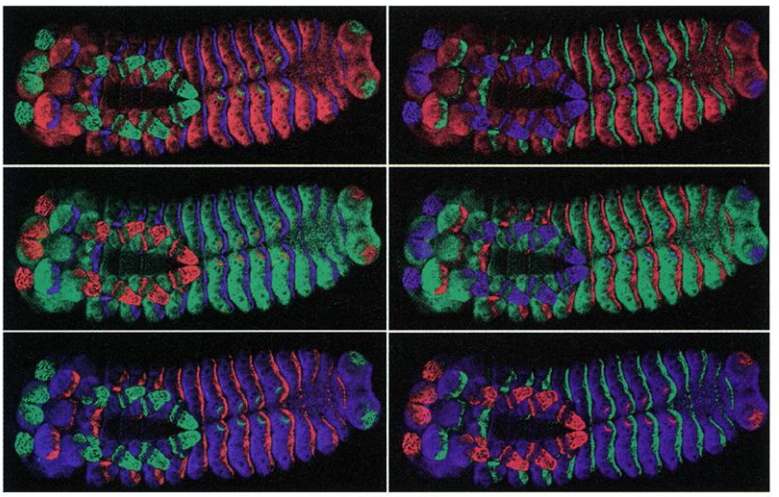
Since its introduction, the confocal microscope has contributed to many fields of contemporary biomedical research and enhanced the display of biological information in a most aesthetically pleasing manner. Confocal microscopy came of age in the early 1980s with the introduction of laser scanning confocal microscopes for biology (*1*). Before this time, images of relatively thick, fluorescently labeled biological specimens such as embryos were technically challenging to capture with the use of a conventional wide-field epifluorescence light microscope. With this latter type of microscope, background from labeled structures within the specimen can mask the signal from the focal plane of interest because the entire specimen is flooded with light. The power of the confocal approach lies in its ability to collect images in optical sections at different excitation wavelengths from intact fluorescently labeled biological specimens.

The images produced by a confocal microscope are called optical sections because the instrument effectively reduces the background signal by scanning the specimen with discrete points of light. For example, the laser scanning confocal microscope (LSCM) produces optical sections by using a focused laser beam to scan the specimen. A spatial filter, usually an adjustable iris, blocks the passage of any background fluorescence from above and below the focal plane. The LSCM does not create a real image; rather, a light-sensitive photomultiplier tube (PMT) detects light that passes through the iris, and the resulting signal is sent to a digital imaging system that constructs it into an image (*1*, *2*). Thus, a confocal microscope can yield images of sections of a specimen without the need to physically slice it, as would be necessary for conventional microscopy.

Advancements have been made in recent years to the instrumentation for imaging multiple probes in specimens prepared by immunofluorescence, fluorescence in situ hybridization (FISH), or direct labeling (*2*). The lasers of the commercially available LSCMs excite fluorophores attached to probes that label distinct structures (see figure, right) and can be used in a plethora of applications for the analysis of the spatial distribution of macromolecules in cells and tissues. Many instruments produce images of two, three, and sometimes four different fluorescent probes in a specimen, and a spectral detector has now been developed that could theoretically distinguish among as many as 32 different probes labeling a specimen (*3*).

If an emission wavelength of one fluorophore "bleeds through" into that of another, it can interfere with the interpretation of images collected from multiply labeled specimens. Because the emission wavelengths of many fluorophores overlap, bleed-through most often occurs when a single laser is used to simultaneously excite more than one fluorescent probe (*4*). Therefore, fluorophores for an individual specimen must be chosen judiciously to counteract bleed-through and to avoid fluorescence from naturally occurring molecules in the specimen (known as autofluorescence). Some of the more traditional probes in immunochemistry, such as fluorescein and rhodamine, are now passed over in favor of brighter, more photostable alternatives that have narrower excitation and emission curves, such as the cyanine and the Alexa dyes (*5*). Nanocrystals of semiconducting materials, also known as quantum dots, are promising alternatives to conventional probes because they can be engineered to fluoresce at very specific wavelengths of light (*6*).

Technological improvements have been made to the LSCM to control bleed-through. Some scan several fluorophores sequentially so that the filter combinations which more precisely match the fluorescent emissions of the probes are used. In the early instruments, sequential imaging was achieved by physically changing filters, which took time, exposed the specimen to more light, and potentially resulted in registration problems when the images were merged digitally. Furthermore, glass filters are now being replaced with an acousto-optical tunable filter (AOTF) that not only better matches the excitation wavelength from the laser to each of the fluorophores but also controls the laser power to each channel (*7*).



Confocal imaging of a triple-labeled butterfly embryo. The specimen was fixed, immunofluorescently labeled, and imaged with an LSCM equipped with a krypton/argon laser, and the three probes were imaged simultaneously (*2*, *17*). The subsequent images are different color combinations produced by rearranging the red, green, and blue channels in the image, also known as "channel surfing" (*18*). In the image at top left, the segment polarity genes *cubitus interruptus* (red) *and engrailed* (blue) are expressed in the anterior and posterior compartments of each segment of the embryo, respectively, and the *Distal-less* gene (green) is expressed in the distal region of each of the developing appendages. Image courtesy of J. Selegue, C. Brunetti, and S. Carroll.

In addition to the LSCM (*1*, *2*), other options for optical sectioning include the spinning disk microscope (*8*), the multiple-photon microscope (*9*), and deconvolution of images collected with a wide-field epifluorescence microscope (*10*). Many applications can be performed with any one of these systems, although some run more optimally with the use of a particular microscope.

The spinning disk microscope uses a different scanning system from the LSCM (*8*). Multiple spots of light that are produced by a spinning disk with many small holes (called a Nipkow disc) scan the specimen, and each spot of fluorescent light emitted from the speci-

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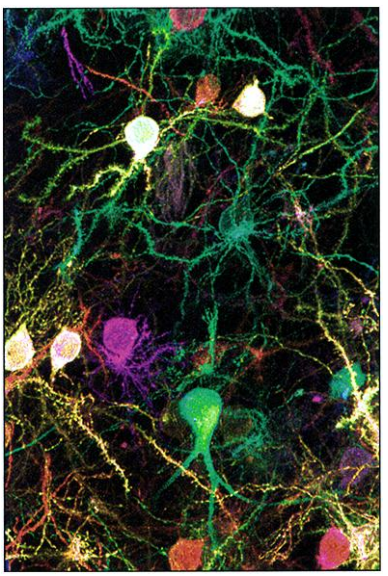
men is detected simultaneously. Optical sections are viewed in real time either by eye or, more commonly, with a charge-coupled device (CCD) camera. Early models of this instrument were only useful for imaging the brightest specimens because so much light was lost through the instrument. Modern spinning disk microscopes have been improved by the addition of laser light sources, better light transmission through the optical path, and high-quality CCD detectors.

Spinning disk systems are generally chosen for experiments where both spatial and temporal resolution are required. For example, a spinning disk microscope has recently been used for fluorescent speckle imaging of the interactions between purified microtubules and F-actin bundles (*11*). Good temporal resolution can be achieved with the LSCM but at the sacrifice of spatial resolution. Using the LSCM with the line scan mode, a time resolution of 2 milliseconds can be achieved, whereas full spatial resolution images are collected at less frequent time intervals (*12*).



Multicolor confocal image of a brain slice from a mouse. The specimen was loaded with seven combinations of fluorescent lipophilic dyes using the "DiOlistic" method and imaged with an LSCM (*19*). The image is a projection of 132 optical sections that represents approximately 50 μm in depth. Image reprinted with permission from Elsevier Science from (*19*), courtesy of J. Lichtman.

Multiple-photon microscopes have evolved from confocal instrumentation; many of them use the same scanning systems and may even use an adjustable iris in front of the detector for further improvement of the quality of the optical sections (*9*, *13*). The light source in a multiple-photon microscope is a high-energy pulsed laser with tunable wavelengths, and the fluorophores are excited by multiple, rather than single, photons. Optical sections are produced simply by focusing the laser beam in the specimen. Multiple-photon excitation of the fluorophore is confined to the region where energy levels are statistically high enough at the point of focus.

The multiple-photon microscope improves the imaging of live cells because only those fluorophores in the focal plane are excited. Moreover, cells are usually less susceptible to photodamage by the longer wavelength red light delivered by the laser (unless they happen to contain pigments that absorb in the red). In a recent study of hamster embryonic development, most of the embryos died after 8 hours of illumination in the LSCM; they developed normally after 24 hours of illumination with 1047-nm light from a multiple-photon system (*14*).

Optical sections can also be computed from conventional wide-field microscope images by using deconvolution to subtract the out-of-focus information from the digital image (*10*). Early versions of the method were relatively slow; for example, it could take hours for some algorithms to compute a single optical section that, at the time, took seconds to produce with the LSCM. Deconvolution has become much faster because of improved computers and software, and it is now practical for multiple-label imaging of both fixed and living cells. Additional background from images that were collected

with the LSCM, the spinning disk microscope, or a multiple-photon microscope can also be removed with deconvolution methods.

Though confocal imaging of fixed and multiply labeled specimens has become relatively routine, many advancements have been made in the confocal imaging of multiple probes in living specimens (see figure, opposite page). Experiments that previously were practical only for fixed and immunofluorescently labeled tissues can now be performed with live tissue. These advancements have been dependant on the introduction of new fluorescent probes as well as technological improvements to the instruments themselves. The engineering of the green fluorescent protein (GFP) and its spectral variants together with DsRed, a novel red fluorescent protein recently isolated from sea anemones, have enabled multiple-label imaging of living cells (*15*).

The development of the ability to collect a series of optical sections from multiply labeled living specimens by confocal microscopy, deconvolution, or multiple-photon imaging and to maintain registration between successive images has made multidimensional imaging a practical option. Four-dimensional (4D) imaging is the collection of optical sections (in the x and y dimensions) from different depths (the z dimension) in the specimen at different points in time (the fourth dimension). Naturally, the time frame within which the phenomenon under study occurs should not be faster than the time taken to acquire a single z series. Also, the structures of interest should be within the resolution of the microscope. Using a 60× objective lens with a numerical aperture of 1.4, the maximum lateral resolution is ~0.1 μm and the maximum vertical resolution is ~0.2 μm (*2*).

Multidimensional experiments can present problems in data handling because a single 4D imaging experiment can amass gigabytes of information. File sizes are dramatically increased when multiply labeled specimens are imaged in 4D, especially with the recent development of a spectral detector coupled with a 32 PMT array that can separate as many as 32 channels of emission data simultaneously (*3*). Movement of a structure through the consecutive stacks of images can be traced, changes in volume of a structure can be measured, and the 4D data sets can be displayed as movies in stereo (*16*).

For successful optical sectioning, whether by confocal, spinning disk, multiple-photon, or wide-field microscopy, a minimum number of photons should be used to efficiently excite each fluorophore labeling the specimen. As many of the emitted photons from the fluorophores as possible should make it through the light path of the instrument to the detector. Or, to paraphrase from a song in Monty Python's "The Meaning of Life," "Every photon is sacred!" Following this maxim, scientists may soon be able to avoid photobleaching probes and photodamage to living specimens and, thus, to collect the maximum amount of biological information.

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