MINIREVIEW

Further Developments of the Laser Scanning Confocal Microscope in Biomedical Research[1] (44032)

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Abstract. **The laser scanning confocal microscope (LSCM) is a valuable research tool for imaging fluorescently labeled biological specimens. Rather than cutting sections of the tissue with a knife, it is now possible to produce relatively noninvasive "optical sections" using the LSCM as an imaging tool. This has made the imaging of living cells *in situ* more of a practical option. This minireview briefly describes some of the improvements made to the LSCM over the past 5 years and, in more detail, outlines many of the current biomedical applications of the LSCM, including single and multiple labeling of fixed and living specimens, physiological imaging, 3-dimensional imaging, and the use of the LSCM for lineage tracing and in correlative microscopy.** [P.S.E.B.M. 1996, Vol 213]

Over the past decade there has been an unprecedented growth in the popularity of the laser scanning confocal microscope (LSCM) among biomedical researchers (1). In fact, the LSCM is now fully established as an alternative to the conventional wide-field light microscope for producing improved images of relatively thick fluorescently labeled specimens (2).

The stunning images produced by the LSCM are a testament to its success but are now so abundant in the literature that a full coverage of the field is impossible in a short review article. Instead, this minireview will discuss some of the major advances that have taken place since my first review appeared in this organ in 1991 (3). The interested reader is referred to several recent compendia for more details of the technical aspects (4, 5) and the biological applications (6, 7) of the LSCM.

Historical Perspective

The current generation of LSCMs has evolved from the basic confocal principle set out in Marvin Minsky's patent some 35 years ago (8). Several landmark instruments have been introduced over the intervening years, and the evolution of the LSCM is a story of the incorporation of advances in laser technology, optics, and computer imaging into Minsky's basic design. All of the LSCMs currently used in a biological setting adopt the reflected light or, more specifically, the epifluorescent configuration (1, 4, 8).

The LSCM continues to develop largely through discussions between its users, inventors, and manufacturers. This has resulted in ever-more-sophisticated LSCMs becoming available to the biological community. Some recent improvements have concentrated on balancing the light budget and on improving the sensitivity of the instrument. These are important features

[1] This manuscript is an update of a previously published minireview entitled, "The Laser Scanning Confocal Microscope in Biomedical Research," Proc Soc Exp Biol Med **198**:772–780, 1991.

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0037-9727/96/2131-0024$10.50/0

for imaging weakly labeled samples and essential for imaging living cells, which are sensitive to light. Other improvements have been made in rendering the microscope more user-friendly, especially to the novice confocal microscopist. For example software control, rather than physical control, of many of the routine operating parameters such as selection of laser power, detector gain, and black level, and the positioning of the excitation and emission filters in the light path has been introduced into some of the newer confocal imaging systems.

One of the most dramatically improved features of the LSCM, which has arisen over the past few years, is in the display and analysis of the final images. This is extremely important since the increase in resolution achieved using the instrument is of little value if it cannot be reproduced and displayed in the final hard copy of the image. Since the images collected with the LSCM are most often in a digital form, they are compatible with computer programs and devices that have been developed by other sources—mainly the graphic arts industry—for the display and reproduction of high-resolution color images. Such high resolution color imaging devices are now widely available, and their cost has dropped drastically over the past 5 years. Thus, many laboratories are now able to produce respectable color hard copies in the "digital darkroom" using computer programs to manipulate the images and electronic printers to reproduce the images (9).

The quality of the final published image continues to improve as many journals will now accept digital images for publication, which eliminates the loss in resolution caused by the use of photographic copies or inter-negatives as part of the publication process. This is a dramatic improvement for publishing color images: the intended resolution and color balance is now faithfully reproduced directly from the computer file by the journals, relatively quickly and at a reduced cost to the author.

In addition, several pioneering journals are now available in electronic form, either on CD ROM disc or *via* the Internet. Consequently, the confocal micrographs can now be viewed using a laboratory microcomputer, theoretically at the same resolution as that attained using the confocal microscope itself. This is a far cry from the hazy images first produced on Minsky's display screen, an army surplus oscilloscope, which unfortunately did not reflect the resolution achieved in his microscope and perhaps was a reason why confocal microscopy failed to capture the imagination of more biologists at that time (8).

Imaging Fixed Tissues

Most applications of the LSCM in biomedical research continue to be for improved imaging of the distribution of various macromolecules in fixed and fluorescently labelled tissues (2). Indeed, the LSCM has become essential in many studies of relatively thick specimens because the exquisite specificity of immunofluorescence or fluorescent *in situ* hybridization (10) is often compromised by glare from structures outside the focal plane of interest, when imaged using the conventional wide-field light microscope.

Using the LSCM as an imaging device, the 3-dimensional (3-D) structure of tissues is not as much of a limiting factor for imaging bright fluorescently labelled specimens. Previously, biologists were reduced to imaging artificially flattened cells or tissue sections in order to observe immunofluorescently labeled specimens at an acceptable resolution. Using the LSCM to scan the fluorescence in the sample, a series of optical sections from a fluorescently labeled specimen can be produced at depths of 100–200 μm into the specimen. The number of optical sections collected is somewhat dependent upon the characteristics of the specimen (e.g., opacity, refractive index) and is also a function of the objective lens used (e.g., numerical aperture, working distance, flatness of field). When the LSCM is used, structures deep within the specimen are revealed that were not previously visible using a conventional wide-field epifluorescence microscope (Fig. 1).

The distribution of more than one macromolecule can be mapped in the same tissue sample using the LSCM. For a full review of the technical details of multiple labeling strategies, the reader is referred to the work of Brelje (11). The reduction of out-of-focus information by the LSCM is extremely important for multiply labeled specimens since structural details can be lost when two or more images are color coded and merged using the computer. Image merging is essential for the elucidation of the relative distribution of more than one molecule since overlapping distributions are clearly viewed as a change in color. For example, regions of rhodamine staining (red) that overlap regions of fluorescein staining (green) appear as yellow in both the fluorescence microscope and in a color-coded digital image (3).

To date, as many as three different fluorochromes have been imaged in the same specimen by using the LSCM. The images are usually collected at three different excitation wavelengths as single images, and subsequently merged into a red/green double label or a red/green/blue triple label image using relatively simple computer graphics techniques on the confocal workstation or using a second microcomputer. In the more recent LSCMs, double or triple label images are collected simultaneously, rather than sequentially. This method reduces the amount of photobleaching of the fluorophores, since each one is scanned by the laser beam once, rather than three times.

Triple labeling has been used extensively for im-

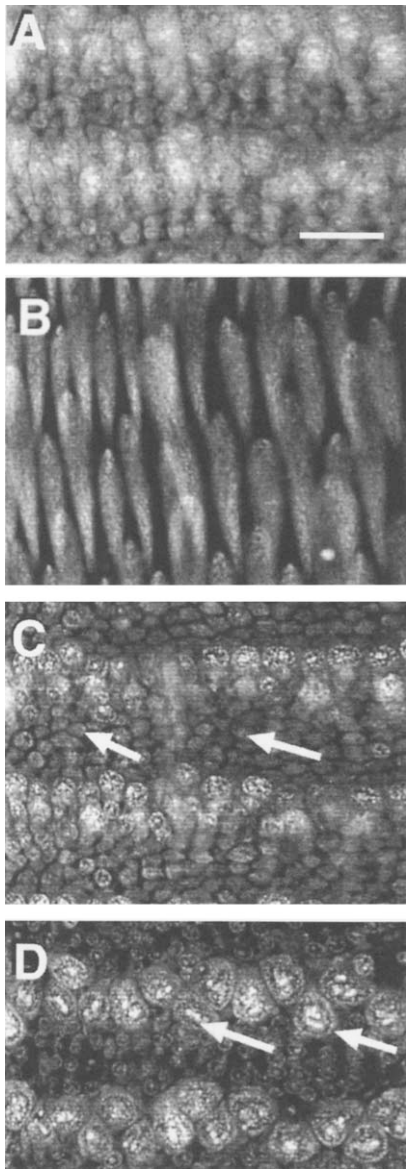

Figure 1. Serial optical sections (*z* series) collected from a whole mount of the pupal wing epithelium of the buckeye butterfly *Precis coenia*, fixed, and stained with propidium iodide. (A) Several optical sections have been digitally merged to represent the image obtained using a conventional wide field epifluorescent light microscope. (B–D) Individual optical sections displayed from the *z* series yield more cellular details within the epithelium. For example, in Panel B rows of emerging scales emerge at a different level in the epithelium to the small epithelial cell nuclei; white arrows in Panel C, and the larger scale forming cell nuclei white arrows in Panel D. Scale bar, 25 μm.

aging a variety of tissues, including developing *Drosophila* tissues (Fig. 2). For example three different gene products have been localized in the same *Drosophila* embryo at the cellular blastoderm stage (12). Triple labeling has been used extensively to image gene products responsible for patterning various *Drosophila* appendages including the eyes (13) and the wings (14).

The synthesis of bright fluorescent probes that have their excitation and emission spectra more closely matched to the specific wavelengths delivered by the lasers attached to the LSCM continue to be vital for successful imaging of multiple label specimens (11, 15). The cyanine dyes in particular have made multiple labeling strategies more practical (16). For example Cyanine 3 is a brighter alternative to rhodamine, and Cyanine 5 (CY5) is useful as the third fluorophore, together with fluorescein and rhodamine, in triple label strategies (17). Moreover, since CY5 is excited at a longer wavelength than either fluorescein or rhodamine, its use enables the images to be collected from deeper within the specimen, albeit at a slightly reduced resolution compared with those collected at shorter wavelengths. In addition, most biological tissues have much reduced autofluorescence when excited in the red CY5 wavelength, which reduces the signal from background tissue autofluorescence resulting in an improved signal-to-noise ratio (18).

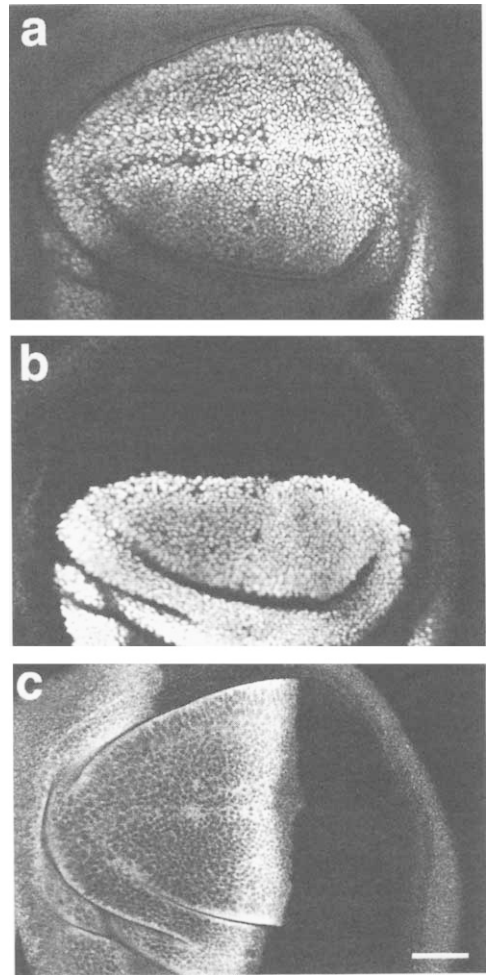

Figure 2. Triple-labeled *Drosophila* third instar wing imaginal disc. The images were collected using a BioRad MRC 600 LSCM with a 25 mW Krypton/Argon laser source, which has three lines at 488 nm (blue), 568 nm (yellow), and 647 nm (red). The three fluorochromes used were fluorescein ex. 496 nm, em. 518 nm, Lissamine rhodamine ex. 572 nm, em. 590 nm, and CY5 ex. 649 nm, em. 666 nm. The images were collected in sequence as single optical sections and show the expression of three wing patterning genes: *vestigial* (a), *apterous* (b), *CiD* (c). For more details see Ref. 14. Scale bar, 75 μm.

New probes are also available for imaging DNA and nuclei using the LSCM (19). These include dimeric nucleic acid dyes such as TOTO-1 and YOYO-1. It should be noted that dyes such as Hoechst 33342 and DAPI have excitation spectra (max. 346 and 359 nm, respectively) that are too short for most of the lasers supplied with the commercially available LSCMs, other than specialized ultraviolet systems (20). Other probes, such as fluorescently labeled phalloidin, can be used to label cell outlines (21), and specific cellular organelles can be labeled. For example, rhodamine 123 or JC-1 can be used to label mitochondria (22); NBD-Ceramide or Bodipy-Ceramide, to label the Golgi apparatus (23), and DiI, to label endoplasmic reticulum (24).

Imaging Living Tissues

Two of the pioneers of confocal microscopy, Minsky and Mohimar Petran were stimulated by the potential of imaging living cells *in situ* when they first designed their confocal microscopes. However, the early LSCMs were rather wasteful of photons, which made live cell imaging problematic. Their dreams of viewing dynamic developmental events are now attainable using today's highly sensitive LSCMs. Over the past 5 years, major advances have been made in the LSCMs for the imaging of living tissues. The major problem is that living cells and tissues that are loaded with fluorescent dyes are sensitive to photodamage and may change their behavior or even die as a result of exposure to elevated light levels (25).

Some of the more recent improvements to the LSCM that have alleviated a number of these problems include the use of improved laser sources (biological molecules [e.g., proteins and nucleic acids] do not absorb in the red), more sensitive detectors (photomultiplier tubes or CCDs), more efficiently transmitting mirrors within the scan-heads of the LSCMs, and improved digitizing techniques including photon counting. In addition, brighter, more photostable dyes have been developed so that exposure of the tissues to reduced laser light levels is required to produce a detectable signal in order to form acceptable images. These improvements have contributed to making confocal imaging of photosensitive fluorescently labeled living cells more feasible.

An example of the progress made in improving the LSCM for imaging living cells can be seen in the instrument's successful imaging of fluorescently labeled proteins injected into living cells—for instance, of rhodamine-tubulin injected into living *Drosophila* embryos (26). This method is especially sensitive to photobleaching since the fluorophore is bound directly to tubulin, which is incorporated into the cytoskeleton, and therefore the fluorescence is not replenished during the experiment.

This method was first introduced using computer deconvolution algorithms applied to epifluorescent images collected with a conventional wide-field microscope, using the sensitivity and dynamic range of a cooled CCD camera for recording the images (27). As the sensitivity of modern LSCMs improves, and the speed of computers and deconvolution algorithms is increased, it is becoming more practical to apply both techniques simultaneously for imaging living specimens.

The new generation of LSCMs has been extensively utilized for imaging living neurons *in vivo,* which, interestingly, was Minsky's goal when he first designed his confocal microscope. Confocal imaging has, for example, been used to image developing neuronal dendrites in tissue slice and cell culture preparations stained with DiI or DiO (28). Here the improved resolution and optical sectioning power of the LSCM is necessary in order to follow the behavior of the fine filopodial protrusions on dendrites and the subsequent development of synaptic contacts.

Green fluorescent protein (GFP) is a recently developed probe for imaging gene expression and protein localization in living tissues (29). GFP was isolated by the cloning of a gene from the jellyfish *Aequorea victoria,* which is responsible for its chemiluminescence. GFP fluoresces at 395 and 470 nm; the latter wavelength is close to fluorescein excitation, which means that GFP can be imaged using a standard LSCM (30). GFP can be added to a coding sequence or behind a promoter of interest and produces a fluorescent product correlated with the expression of the gene of interest—the cell literally stains itself. In one study, the GFP coding sequence was placed under the control of the *mec-seven* tubulin gene in *C. elegans,* and the patterns of tubulin expression were similar to those seen previously in fixed animals (29).

To date, GFP has been used to monitor gene expression in several different cell types including living *Drosophila* oocytes (31) and in plants (32). Moreover, various mutant forms of GFP with different fluorescent excitation/emission maxima and minima are now being isolated, which opens up the exciting possibility of imaging the expression of more than one gene product in living cells (33).

Two-photon microscopy holds much promise for imaging living tissues (34). Here photobleaching is much reduced because excitation of the fluorophore occurs only in the region-of-interest in the sample, whereas some excitation of the fluorophore occurs along the entire path of the laser beam in the LSCM. The same method of scanning using a commercially available confocal scan-head is used for both techniques. In addition, the pulsed red light, which is typ-

ically used in a two-photon system, penetrates more deeply into many tissues, enabling useful information to be gained from lower levels in the specimen, than can be collected using a regular LSCM. Multiple labeling is also practical using two- and three-photon excitation of different fluorophores with a single laser as the source, and by subsequently separating the emitted wavelengths with optical filters (35).

It is not always practical to image living tissues using the LSCM. Perhaps the tissues of interest are not accessible to the objective lens of the microscope, or they will not grow in culture, or the probes, especially multiple labels for living cells, are not available. In these scenarios, the tissue of interest is usually dissected from several different animals at progressively later stages of the development. The tissue is subsequently fixed and stained with one or more immunofluorescent probes, and viewed using the LSCM. The resulting images are then placed in a time sequence that represents development. Since the confocal images are in a digital form it is relatively easy, and can be instructive, to produce digital animations of such developmental sequences. For example, the computer graphics technique of morphing lends itself well to such digital images of developmental specimens and has been applied to double- and triple-label specimens of *Drosophila* embryogenesis and imaginal disc development (36).

Physiological Imaging

All of the conditions that apply to the qualitative aspects of imaging living tissues also apply to the quantitative aspects of physiological events using the LSCM. The advantages of confocal imaging for performing physiological measurements include the ability to produce optical sections of thick tissues such as embryos and the preservation of image registration for dual emission images. Many novel probes continue to be synthesized specifically for measuring physiological parameters using the LSCM. Most of the probes change their fluorescence characteristics in the presence of different concentrations of specific ions (37). Such physiological parameters as calcium, pH, membrane potential, cAMP, and protein kinase C can be measured using specific fluorescent probes (6, 38, 39). In order to be confident in the measurements collected using the LSCM, it is usually necessary to employ ratio imaging to control for experimental fluctuations such as photobleaching of the probe by the laser.

Many physiological events occur faster than the image acquisition speed of most LSCMs, which is usually in the order of a single frame per second. Fast scanning LSCMs that use an acousto-optical device and a slit to scan the specimen, rather than the slower galvanometer-driven, point scanning systems, have been introduced to address some of the problems associated with the acquisition speed of the instrument compared with the speed of the physiological event of interest (40). This design has the advantage of good spatial resolution coupled with good temporal resolution (i.e., full-screen resolution images collected at 30 frames per second (near video rate).

The galvanometer-driven mirror, point scanning LSCMs can be used for scanning events that occur at rates much faster than one frame per second using a line-scan mode. Here, spatial resolution must be sacrificed in order to achieve the required temporal resolution (although images at a higher resolution can be collected at infrequent time points during the experiment). For example spontaneous local increases in calcium, termed calcium sparks, have been analyzed in cultured rat heart cells. Each line scan was acquired in 2 ms, and each spark was restricted to a radius of 1.5 μm (41). Estimates of the calcium flux associated with the sparks suggest that they result from spontaneous opening of single sarcoplasmic reticulum calcium release channels.

Three-Dimensional Imaging

The series of optical sections produced using the LSCM can be constructed into a 3-D representation of the specimen using computer volume visualization techniques. Over the past 5 years, many 3-D reconstruction programs have become available to the confocal microscopist (42). These programs were initially written for the medical imaging market, specifically for the reconstruction of CT scan images (anatomy) and subsequent merging with PET scan images (physiology). Programs are now available for processing confocal images that run either on extremely fast workstations (up to 50 million voxels per second) or on slower, but more affordable, personal computers. Indeed, with the introduction of faster computer chips, simple 3-D reconstruction of moderately sized z series can now be achieved using the workstation of the confocal microscope in a respectable time.

This approach is now used to elucidate both the qualitative and the quantitative relationships between the 3-D structure and function of tissues. It is often conceptually difficult for many investigators to visualize complex interconnected structures from a series of 200 or more optical sections collected from a developing structure with the LSCM. This is especially true for structures with complex branching 3-D architectures such as nerve cells. Since any z series of images produced with the LSCM maintains the registration of structures within the specimen (assuming the specimen itself does not move during the period of image acquisition), and is in a digital form, they can be processed with relative ease into a 3-D representation of the specimen. The result is a single 3-D image, a stereo image (digital offset or color anaglyph), a stereo movie

or a movie sequence of single-, double-, or triple-labeled specimens rotating on the screen. Moreover, specific parameters of the 3-D image such as opacity can be interactively changed using volume rendering algorithms to highlight spatial relationships between structures of interest at various depths within the specimen.

Volume visualization techniques are often used to display single optical sections from a time-lapse series as a 3-D representation of the data set. Here, time rather than structure contributes the *z* axis. This approach is especially useful as a method for visualizing physiological changes over developmental time, such as calcium dynamics within developing echinoderm embryos (43).

Now that the sensitivity of the LSCM has improved, time-lapse sequences of *z* series can be collected from a living specimen at specific time points. A 3-D reconstruction of each of the *z* series is subsequently constructed, and a so-called 4-D movie specimen can be produced (i.e., three spatial dimensions, *x*, *y*, and *z*, with time as the fourth dimension). Here structures of interest can be followed through the tissue not only in *x* and *y* but also in the *z* axis. Such 4-D imaging has recently been used to follow individual axons over time in the retinae of *Xenopus* tadpoles (44). Specific neurons were injected with DiI and a series of optical sections was collected from the same animal at various times during development. Here, the action of specific neurotrophins causing axon arborization was traced using 3-D reconstruction of the confocal images. In addition, morphed animations of such timed 3-D specimens can form effective instructional videos (36).

Lineage Tracing

The LSCM has been used extensively for the elucidation of cell lineages. For this, a few cells within a developing embryo are labeled with a fluorescent probe, and the position or fate of the labeled cell (or cells) is determined later in development. It is essential that the fluorescent probe does not interfere with normal cell behavior and is confined to the labeled cells during the time course of the experiment, which can be in the order of hours, days, weeks, or even years. Development is allowed to proceed, and the positions of the labeled cells are subsequently imaged deep within the developing tissue using the LSCM. This approach has been used to trace the migration of DiI-labeled cells in many tissues including neural crest cells in the mouse embryo (45) and rhodamine-dextran–labeled cells in sea urchin embryos (46).

The success of lineage tracing depends upon mapping the spatial and temporal components of the migration of specifically labeled cells within an unlabeled population of cells. It is often necessary to merge a transmitted light image of the embryo (total cell population) with a confocal fluorescence image of the subset of specifically labelled cells. Virtually any form of light microscope image, including phase contrast, DIC, polarized light, or dark field, can be collected using a transmitted light detector attached to the LSCM. This is a device that collects the light passing through the specimen, and transfers it to the scanning unit of the LSCM; the transmitted light image is constructed in the same way as the confocal fluorescence images. Since confocal epifluorescence images and transmitted light images are collected simultaneously using the same laser beam, image registration is preserved, so that localization of fluorescently labeled cells within the unlabeled tissues can be accurately mapped.

Recently, a real color transmitted light detector has been introduced. The device utilizes the ability to mix digitally the red, green, and blue components from the specimen in real time. This approach will be of use for viewing specimens where the real color is important, for example, in specimens prepared for *in situ* hybridization combined with immunofluorescence. Many pathologists who are more familiar with viewing real colors in their stained specimens may also find this device extremely useful for interpreting confocal images.

Lineage analysis can also be performed using double- and triple-label fluorescence microscopy where at least one of the probes acts as a landmark. For example, the relative distribution of *wingless* and *engrailed* expression in fixed and stained *Drosophila* embryos is highly predictable and has been used as a landmark for mapping the distribution of an enhancer trap protein at single-cell resolution in the developing *Drosophila* nervous system (47). More sophisticated methods of lineage tracing that employ photoactivatable probes (48) or the FLP recombinase system (49) are also well suited to imaging using the LSCM or two-photon microscopy, especially if cell viability or photobleaching is of concern.

Correlative Microscopy

Since the resolution achieved with the LSCM is a little better than that achieved with a conventional wide-field light microscope (0.2 μm) but not as great as that of the electron microscope (2 nm), it has bridged an apparent gap between these two commonly-used techniques. This is especially true for thick fluorescently labeled specimens where 0.2 μm resolution can only be achieved using the LSCM to eliminate out-of-focus fluorescence. Confocal microscopy has been used increasingly in conjunction with other microscopical techniques including video microscopy (50), electron microscopy (51), and even flow cytometry (52).

Such correlative or "integrated microscopy" is based upon using more than one microscopic technique to view the same region of a specimen in order to collect structural information at different magnifications and resolutions, but it can add extra demands on specimen preparation. An early example of correlative microscopy was the observation of the relationship between cell substratum adhesions and the actin-containing microfilament system of the cell, using interference reflection light microscopy to view the adhesions, and high-voltage transmission electron microscopy (HVEM) to view the microfilament bundles associated with the adhesions (53). This observation was recently repeated using the LSCM in a reflected light mode as a simple interference reflection microscope and the HVEM (54).

The introduction of novel probes has again provided insights into the localization of specific proteins within fixed tissues using correlative microscopy. In this case, an old probe, eosin, has been used in a novel way for integrated microscopy using the LSCM and the transmission EM. Eosin is used as a fluorochrome for immunofluorescence and, secondly, for the photooxidation of DAB for subsequent electron microscopy (51). Here microtubules were imaged using eosin conjugated to a secondary antibody in the LSCM in the fluorescence mode; and the same microtubules were subsequently imaged in the transmission EM after photooxidation of DAB by eosin to produce an electron dense stain.

Conclusions

The LSCM has been significantly improved over the past 5 years. Its development is the result of a combination of several factors. The incorporation of technological innovations into the design of the instrument itself, the synthesis and, occasionally, the discovery of novel bright and more efficient fluorescent probes, and the imagination of a plethora of biomedical researchers in applying the technology to solve specific biological problems. The instrument manufacturers have also played an important role in the LSCM's development by responding to the demands of the increasingly sophisticated user base.

The major advantages of the LSCM over the conventional light microscope for imaging biological specimens are a reduction in glare from out-of-focus fluorescence and a slight increase in both lateral and axial resolution. The LSCM is still mainly used by biologists to visualize fixed and fluorescently labeled specimens, although the exciting potential of imaging living tissues is being realized.

The use of the LSCM as an imaging tool continues to reveal a new level of biological structure at a resolution between the light microscope and the electron microscope. The "optical section" has now achieved its place along with the "frozen" or "paraffin section" and the "thin section" in the vocabulary of biology.

I wish to thank Leanne Olds for expert graphics assistance, Jim Williams for preparing the specimen for Figure 2, Diana Wheeler for proofreading the manuscript, Sean Carroll for our continued collaboration, and the Howard Hughes Medical Institute for their support.

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