BOOK REVIEW

Handbook of Biological Confocal Microscopy, Third Edition

James B. Pawley, 985 pages +xxviii, ISBN 0-387-25921-x, illus., index, Springer, New York (2005), $129.00, hardcover.

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In the decade between the publication of the second and the third edition of the *Handbook of Biological Confocal Microscopy* there emerged a major advance in Nipkow disk-based confocal microscopes, as well as several seminal advances in both instrument development and imaging techniques that have had a major impact on biological optical microscopy. In the field of confocal microscopy, which is the main subject of the *Handbook*, a major technical innovation was the Ichihara microlens-disk scanning confocal microscope. Why is that significant? The key limitation of the Nipkow disk (arrays of conjugate pinholes on a rotating disk) is the low illumination efficiency, since the pinholes only cover one to two percent of the area of the illumination disk. For reflected light imaging this does not present a major problem; however, for weakly fluorescent objects the low signal-to-noise ratio limits its use in cell imaging. Ichihara and his colleagues cleverly solved this problem. Their key invention (commercialized by Yokogawa Electric Corporation) was to place a second disk that contained microlenses above and on axis with the Nipkow disk containing the pinholes. The lower pinhole disk is located in the focal plane of the microlens and both disks rotate together on the same axis. The effect of the array of microlenses is to significantly increase the illumination efficiency (to about 40%) of the pinholes in the Nipkow disk microscope. The result is a large increase in the signal-to-noise ratio of the detector, which permits the use of the Nipkow disk confocal microscope with objects that show extremely weak fluorescence.

The other important advances in instrument development as well as imaging techniques that occurred during the last decade are as follows. First, compact diode-pumped femtosecond lasers for both multiphoton excitation fluorescence microscopy and second- and third-harmonic generation (SHG, THG) microscopy became available to researchers. Second, new molecular probes became commercially available; they include new variants of green fluorescent proteins (GFP) and a wide variety of quantum dots (based on three-dimensional quantum confinement in nanocrystals). Third, the invention and commercial realization of optical microscopes that exceed the Abbe diffraction limit occurred, thereby providing improved transverse spatial resolution. Examples of these super-resolution optical imaging techniques include: nonlinear structured-illumination microscopy (a wide-field fluorescence microscopy); stimulated emission depletion microscopy (STED) that is based on the stimulated depletion of the excited state to form a subdiffraction volume of the fluorescent molecules; multicolor stochastic optical reconstruction microscopy (STORM) that depends on photoswitchable fluorescent probes; and a technique founded on photoactivatable fluorescent proteins that are bleached termed photoactivated localization microscopy (PALM). Fourth, researchers exploited live cell and *in vivo* applications of nonlinear optical microscopy; for example, SHG, THG, and coherent anti-Stokes Raman (CARS) imaging. SHG and THG microscopy offer several significant advantages over two-photon fluorescence microscopy. There is minimal photodamage since there is no absorption of energy; molecules are excited by scattering processes via virtual states. CARS provides chemical information from the specimen based on excitation of specific vibrational bands. Fifth, the use of adaptive optics (first implemented with astronomical telescopes) to correct wave-front aberrations has improved the resolution and the contrast of confocal microscopes for imaging human retinal photoreceptors *in vivo,* and may be significant for other imaging modalities. Sixth, new algorithms to automatically acquire, analyze, and data mine three-dimensional, multiterabit data sets from centimeter-sized specimens with nanometer resolution have been created. Seventh, new types of microscope objectives for nonlinear microscopy that combine high numerical aperture with long working distances are now available for *in vivo* imaging. Eighth, photodetectors have continued to improve their sensitivity and efficiency in the near-infrared regions; also, the availability of electron-multiplier charge-coupled devices (EM-CCD) has increased, and multianode photomultipliers offer enhanced sensitivity and multispectral imaging. Finally, a variety of slit scanning confocal microscopes are commercially available.

Does the *Handbook* accurately reflect and adequately describe these modern advances? The answer is yes. James Pawley has wisely removed several chapters that he deemed to be either of low interest or not essential for the reader; in their place, there are chapters that describe imaging techniques that are outside the realm of confocal microscopy.

I congratulate the publisher, Springer Verlag, in their decision to produce the *Handbook* in full color and maintain the price of the book at $129. The use of full color significantly enhances the understanding of the schematic illustrations as well as the false color images obtained with a panoply of imaging techniques.

I applaud Pawley and his contributors for their ability to present modern microscopy (predominately confocal microscopy) in a didactic format to a wide audience of students, scientists, and engineers who work in the life sciences. The *Handbook* is highly suited for its intended audience. It succeeds as a teaching tool because it is clearly written and the level of exposition is readily understandable to readers who

are not trained in physics or mathematics. The pedagogical approach is predominately phenomenological and qualitative, based on the written word, and superbly augmented with well-designed figures and tables. In fact, many of the figures that illustrate the techniques are almost self-explanatory; the graphic contains the principle to be learned and the reader is able to grasp that principle without reading the figure legend.

I now enumerate several of the emerging topics that are now included in the *Handbook*. Fluorescence lifetime imaging microscopy (FLIM) is a vibrant research area that significantly evolved in the last decade. The emergence of numerous applications is in part based on the technique of time-correlated single-photon counting (TCSPC).

Live cell imaging is a major application of both confocal and nonlinear microscopy; it offers the researcher a window into the dynamic processes that occur in cells and tissues. Additionally, endoscopes that are based on confocal or nonlinear optical imaging methods, together with scanning units that are based on microelectromechanical systems (MEMS) devices, are not sufficiently described in the *Handbook*. Nevertheless, the *Handbook* is significantly enriched by several new chapters that describe the imaging of brain slices, living embryos, and plant cells, as well as automated confocal imaging and high-content screening for cytomics.

Three-dimensional imaging with optical microscopes is an evolving research area. The problems that exist include the following: how to speed up the acquisition of three-dimensional data sets that exhibit subcellular resolution and are of the volumes from 1 nm^3 to 1 cm^3, how to store these huge data sets, and how to use both manual and automated three-dimensional analysis on the complete data sets. The current commercial three-dimensional image analysis packages are not adequate nor are they of appropriate speed to perform these tasks. The important inclusion of a new chapter by Roysam and his collaborators points the way toward solving these important problems. The subject of digital deconvolution is adequately treated in several comprehensive chapters.

The *Handbook* presents a modern exposition of several new and significant advances in microscopy, specifically those that improve the speed of image acquisition or provide improved optical resolution. For example, Bewersdorf, Egner, and Hell (of the Göttingen group) contributed a very clear and comprehensive chapter on multifocal multiphoton microscopy (MMM). The goal is to increase the speed of image acquisition through parallelization. They carefully describe the advantages and the limitations of the different technical realizations of MMM.

The group of Stefan Hell has also developed new techniques to break the classical Abbe diffraction barrier [see the article on Abbe, by B. Masters, in *Optics and Photonics News*, "Ernst Abbe and the Foundation of Scientific Microscopes," Vol. 18(2), 18–23 (2007)] and thus provide super-resolution optical microscopy. Again, Bewersdorf, Egner, and Hell wrote a lucid chapter on 4Pi microscopy. In this technique, which is now commercially available from Leica, the specimen is coherently illuminated through two opposing microscope objectives; interference forms the basis for the three- to seven-fold improvement in the axial resolution.

The Hell group has developed both the theoretical foundations and the technical implementation of several different approaches to improve the transverse resolution of optical microscopes using reversible saturable optical fluorescence transition microscopy (RESOLFT). Stimulated emission depletion microscopy (STED) is being implemented in a commercial microscope by Leica. Super-resolution optical microscopy is an active field of research and we can expect to see continuous technical developments and many applications to cell biology.

The development of various types of nonlinear microscopies is rapidly emerging. Sunney Xie and his colleagues and former students have developed the theory, the instrumentation, and many of the advances in an important nonlinear optical microscopy: coherent anti-Stokes Raman scattering microscopy (CARS). A major advantage of CARS microscopy over other types of confocal and nonlinear microscopy is that CARS microscopy can produce images of live cells at high spatial resolution that is specific to the chemical constituents of cells. Specifically, the Raman shift can be tuned to lipid vibrations. Again, CARS microscopy provides images of unstained cells and tissues with both vibrational selectivity and three-dimensional spatial resolution.

I now highlight some other topics that represent ancillary imaging techniques for three-dimensional imaging: new nonlinear techniques such as harmonic generation imaging, a review of photo and thermal damage during image acquisition, and new chapters that stress other aspects of the confocal imaging, i.e., automated confocal imaging and high-content screening for cytomics. Förster's resonance energy transfer (FRET) provides imaging techniques to investigate molecular interactions within cells, i.e., drug-receptor binding and signal transduction pathways.

A reasonable goal is to have a variety of imagers that span the spatial resolution from the electron microscope to optical microscopes; they include disparate types of imagers: photoacoustic microscopes, ultrasound imagers, magnetic resonance imagers, and computerized three-dimensional x-ray imagers. These imaging devices provide new investigative tools for life scientists, as well as new diagnostic instruments; for example, endoscopes, ophthalmic imagining devices, and imagers for use in cancer detection, gastroenterology, neurobiology, and dermatology. The inclusion in the *Handbook* of a chapter on nonconfocal methods by Fraser supports the dictum that the imaging technique should be optimized and selected for both the specimen and the question posed. Is the specimen live or fixed, stained or unstained, human patient or animal? What temporal resolution and spatial resolution are required to answer the question at hand? Is the image acquisition a single event or a reoccurring event over a period of months, i.e., brain imaging to study neuronal plasticity? Fraser and his colleagues introduce the reader to the following techniques: surface imaging microscopy (SIM), selective plane illumination microscopy (SPIM), optical low-coherence tomography (OCT), as well as two nonoptical techniques, microscopic

magnetic resonance imaging (μMRI) and microscopic computed tomography (CT). An optical imaging technique similar to SPIM is selective plane illumination microscopy. SPIM can image inside of large embryos that are either fixed or living and provide three-dimensional images of the entire organism. In some cases the combined use of two different imaging modalities can provide three-dimensional information that a single technique is unable to yield. I would add that high-resolution ultrasound imaging and positron emission tomography are two other imaging modalities that will see further utility and development for imaging embryos and small animals.

All types of microscopes produce various types of artifacts. The goal of the careful microscopist is to understand their origins, to mitigate their effects, and to validate and calibrate the mapping of the original specimen and the final image obtained from the instrument. For both live cell studies and long-term studies of tissues and organisms the issue of cell damage induced by the imaging process is paramount. König provides a brief overview of cell damage during multiphoton excitation microscopy.

Photobleaching is another serious problem in both confocal and multiphoton microscopes, and Diaspro and his colleagues describe the molecular mechanisms that are the cause and techniques to reduce photobleaching. Photodamage and photobleaching can be eliminated by using a nonlinear imaging technique such as SHG or THG microscopy; there is no absorption of a photon in the transition to the excited state. Consequently no photodamage or photobleaching is produced; however, in the presence of absorbers photodamage can still occur. SHG and THG microscopy provides contrast based on structures within the specimen, i.e., collagen, membranes, microtubules. As with multiphoton excitation fluorescence microscopy, the optical sectioning capability of SHG and THG is due to the physics of the nonlinear process.

Unfortunately, some of the chapters from the second edition are almost identical with those in the third edition of the *Handbook*, the changes being the placement of the previous figures and the addition of a few new references. For example, Chapter 12 on "Photon Detectors for Confocal Microscopy" by Jonathan Art is almost unchanged and is not current. More specifically, there is no discussion of Hamamatsu's new multianode photomultipliers that are used in multispectral imaging, and the Hamamatsu multipixel hybrid photodetectors with high quantum efficiency and gain. These hybrid photodetectors (HPD) consist of a photocathode and a multipixel avalanche diode (MP-AD). These HPD detectors have a gain of 5×10^4 that is sufficient to detect single photons with a timing resolution that is better than 100 ps.

In summary, Pawley and his contributors have produced a very clear, eclectic, and practical *Handbook* that extends beyond the field of biological confocal microscopy to include other imaging techniques. The audience for this book is students and scientists who work or plan to work in the life sciences. The publisher produced a well designed, carefully and beautifully illustrated book. The reader will not find detailed physical theories of physical optics, digital imaging, nor molecular spectroscopy, but that is the intent of the editor. Other sources such as monographs, textbooks, reviews, and the published literature will augment the treatment of the topics in the *Handbook*. Again, I encourage the reader to peruse the published literature and the original patents. With these caveats, I highly recommend the *Handbook* as a superb teaching and reference book.