HANDBOOK OF BIOLOGICAL CONFOCAL MICROSCOPY

THIRD EDITION

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Editor

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To my wonderful wife, Christine, who is hoping that
we still get along once she begins to see me more often,
and to the friends and partners of all the 123 authors, similarly oppressed.

Preface to the Third Edition

Once the second edition was safely off to the printer, the 110 authors breathed a sigh of relief and relaxed, secure in the belief that they would "never have to do that again." That lasted for 10 years. When we finally awoke, it seemed that a lot had happened. In particular, people were trying to use the *Handbook* as a textbook even though it lacked the practical chapters needed. There had been tremendous progress in lasers and fiber-optics and in our understanding of the mechanisms underlying photobleaching and phototoxicity. It was time for a new book. I contacted "the usual suspects" and almost all agreed as long as the deadline was still a year away.

That was in 2002. Three years later, most of the old chapters have been substantially or totally rewritten. Although 12 of the chapters are on topics that have either been rendered obsolete by improvements in instrumentation or changes in research interest have been dropped, some have been replaced by chapters on similar topics. To make the *Handbook* of more use as a textbook, we have added an extended appendix about practical multiphoton imaging and another describing the operation of CCD cameras in some detail. There is a new series of practical chapters on confocal microscopy and the selection of dyes, as well as on ion imaging, and on methods for studying brain slices, embryos, biofilms and plants (two). There is also a new chapter describing in some detail how such components as interference filters, acousto-optical devices, and galvanometers are made and what parameters limit their performance. The single chapter on 3D image analysis now has the company of two more on automated 3D image analysis and a third on high-content screening and a fourth on database management. Chapters have been added describing techniques that have only recently come to the fore, such as patterned-illumination fluorescence microscopy, fluorescence resonance energy transfer (FRET) and the generation and detection of second- and third-harmonic signals. In addition, new imaging techniques such as stimulated emission depletion (STED), coherent anti-Stokes Raman (CARS) imaging and selected plane illumination (SPIM) now have their own chapters and there are also chapters that connect the world of 3D light microscopy to the larger world of micro-CT and micro-MRI and the smaller world revealed by the scanning and transmission electron microscopes. To round out the story we even have a chapter on what PowerPoint does to the results, and the annotated bibliography has been updated and extended.

As with the previous editions, the editor enjoyed a tremendous amount of good will and cooperation from the 124 authors involved. Both I, and the light microscopy community in general, owe them all a great debt of gratitude. On a more personal note, I would like to thank Kathy Lyons and her associates at Springer for their unstinting support on one of the biggest books they have done in microscopy and the assistance of her co-workers at Chernow Editorial Services, Barbara Chernow and Kathy Cleghorn. Helen Noeldner was again willing to work long hours to keep all the manuscripts straight in spite of my best effort to confuse them. Thanks are also due to Bill Feeny, the Zoology Department artist, for the innumerable figures that he rescued, reconstructed and otherwise returned to life.

If the hidden agenda of the first edition was photon efficiency, and of the second, spherical aberration, the message of the third edition is definitely that all raw, 3D data sets should be deconvolved (or at least 3D-Gaussian filtered) before being viewed or measured. Not only is this required to meet the Nyquist reconstruction criterion, it also greatly reduces the apparent effects of Poisson Noise by effectively averaging the signal over the 50–100 voxels needed to make a Nyquist-sampled, 3D image of a single point object. This last factor allows one to obtain acceptable images using much less excitation, thereby reducing the chance that studies of living cells will be compromised by artifacts caused by phototoxicity. As evermore studies in 3D light microscopy are carried out on living cells, nothing is more important. Now we need dyes that produce less toxicity because they do not cross to the triplet state and photodetectors that operate with lower noise and higher quantum efficiency! That will take another book.

James B. Pawley
January 2006

Preface to the Second Edition

Confocal microscopy is a good idea that was invented, forgotten and then reinvented about once every decade in the years between 1957 and 1985. However, when White and Amos demonstrated an instrument that was sufficiently user-friendly to become the ideal tool for the 3D localization of specific, fluorescent labels in biological specimens, the field finally took off. Soon after the publication of their 1985 article in the *Journal of Cell Biology*, requests to fund the purchase of similar equipment increased at such a rate that, in the fall of 1988, the U.S. National Science Foundation (NSF) realized that it needed some hard information about the capabilities of this new technique. They funded a two-day symposium on the subject as part of the August 1989 annual meeting of the Electron Microscope Society of America and also financed the publication of 18 papers by the participants as *The Handbook of Biological Confocal Microscopy* for free distribution at the meeting.

This first edition of the *Handbook* differed from most of the many other compiled volumes on the subject in that, rather than each author concentrating on his or her own work, an outline for the entire book was written first, and then authors were solicited to cover particular aspects of the instrumentation or its use. Although the necessity of having a volume ready for distribution by August 1989 imposed stringent deadlines on the authors and required the typography and printing to be done locally, every effort was made to try to edit the chapters so that they fit together to form a cohesive whole. The success of the project was due almost entirely to the enthusiasm the authors had for sharing their knowledge of this fascinating subject with a wider audience. Manuscripts originally expected to be 10 pages in length ended up being more than twice this length, and several were more that 50 pages long.

The resulting volume included chapters that described and compared each of the component parts of the microscope itself (laser and conventional light sources, intermediate optics, alternative scanning systems, objectives, pinholes, detectors, and antecedent and related optical techniques), chapters that discussed the digital aspects of data acquisition (pixelation, digitization, and display and measurement of 3D data sets) and chapters that reviewed the properties of fluorescent dyes, the techniques of 3D specimen preparation, and the fundamental limitations and practical complexities of quantitative confocal fluorescence imaging. An annotated bibliography of the field was also included.

If this first book had any underlying theme, it was probably the importance of photon efficiency. This came about because, as the chapters came together, it became clear that technical limitations of the early instruments, in combination with suboptimal operating techniques, often had an effect such that the signal actually recorded was only about 1% of the expected signal. The *Handbook* included several concrete suggestions for increasing this fraction, and it is a pleasure to report that instruments incorporating many of these improvements now demonstrate an efficiency figure that is closer to 10–20%.

Because of the widespread acceptance of the NSF-sponsored volume by users of the confocal microscope, a revised edition (the "red book") was published by Plenum in 1990. Although this hardcover version included over 40 new figures, updated tabular information and over 1,400 typographical improvements, it was otherwise generally very similar to the initial offering.

However, the past five years has seen a virtual explosion in the field of biological confocal microscopy. As it became more and more evident that the original *Handbook* could no longer claim to cover the entire field, I contacted the original set of authors about producing an updated edition. Remembering the frantic urgency that had typified the production of the first edition, I did this with some trepidation; but I need not have worried. The response was uniformly enthusiastic, and several authors were not only willing to completely revise their original chapters but also volunteered to write additional chapters describing several new areas. The response from the 17 new authors was similarly enthusiastic.

The final product includes 37 chapters (15 updated from the first edition, 21 new ones, and an annotated bibliography) and is almost three times as long as the original. Chapters covering confocal operation in the UV, in the transmission mode, and when scanning at video rates using a variety of either point-scanning or line-scanning techniques have been added. The use of pulsed laser sources for both two-photon excitation and fluorescence-lifetime imaging is covered in depth, and there is an entire chapter on the functional principles of modern fiberoptic components and the manifold ways that these can be applied to confocal microscopy. In addition, chapters on the joys and perils of observing living specimens in the confocal microscope and on the detection of gold-conjugated labels now complement a revised version of the earlier chapter describing the preparation of dead specimens.

No less than 3 of the new chapters address the comparative advantages of the confocal and widefield/deconvolution methods of obtaining 3D data sets from biological specimens with the minimum possible damage. Although each of these chapters proceeds from a very different perspective (algebraic optics, actual measurements, and minimum-entrope image processing), I believe that together they give a balanced view of this complex and important subject and make it clear that the confocal microscope could still be improved if the present photodetector were replaced with one having a higher quantum efficiency. The longest chapter in the book describes the inner workings of the 17 currently available systems applicable to the analysis and display of 3D digital image data, and there is now also a chapter describing the features of all of the current hardware systems for the storage, display, and hard-copy output of 3D and 4D image data sets.

The subtext of this second edition is probably an increased recognition of the extent to which the resolution and signal strength of confocal images can be degraded by spherical aberration introduced whenever there is a refractive-index mismatch, such as that occurring when an oil-immersion objective is used with an aqueous specimen. Not only is an entirely new chapter devoted to the subject, but many other authors emphasize the same point in their chapters. Again, the manufacturers have responded with the introduction of a number of superb new water-immersion objectives to simplify confocal observations of living specimens; these are also described.

On the subject of optics there are also two chapters on real-time 3D imaging. In one, the approach is to combine a high speed slit scanner with rapid motion of the focus plane, while the other demonstrates the truth of the almost paradoxical premise that it can be useful to actually increase the chromatic aberration of an objective if it is to be used to examine surface height in the back-scattered light mode with "white" light. Of more interest to those wishing to improve axial resolution in the fluorescent mode is the chapter describing new, high-resolution techniques that combine either two or even three confocal objectives with two-photon excitation to improve resolution to a level heretofore believed to be impossible.

Finally, there is a tutorial chapter intended for the novice user, as well as two appendixes. The first appendix describes the relationship between real-space and optical coordinates, while the second provides a compilation of the optical path layouts of the major commercial confocal instruments.

The topics in this book cover a very wide range of disciplines. While this is good in that it shows the integrating nature of the field, it can lead to problems with notation when optical physicists, experts on information theory, microscope designers, and just plain biologists have to try to agree on a common system of notation. In the first edition, we did not even try to overcome this problem. Although this led to some confusion, I must confess that my efforts to remedy the problem in the present volume have not been totally successful. Index of refraction has been rendered as η, so that **n** can be reserved for the number of quantum events; where *t* has been used for thickness, we have tried to use italics, so that **t** could be used for time as a variable and T for temperature, while specific times (lifetimes, pulse times) are shown as T or τ; wherever *x*, *y* and *z* are used as directions, we have italicized them, while we have tried to keep r as actual dimensions in the *x*–*y* plane (r_p = pinhole radius, r_s = slit width, r_d = detector diameter, etc.); and numerical aperture appears almost everywhere as NA but becomes A_{NA} in some equations. Perhaps most debatable was my decision to try to save space by replacing the word "wavelength" with λ in the body of the text. On reflection, this change probably did not repay, in space, the interruption of the reader that it produces, but, unfortunately, by the time this became evident, it was too late to change it. In spite of our best efforts, problems arose because, while authors wanted to fit in with the book as whole, they also, understandably, wished to remain consistent with their previous publications. I would like to thank them all for their cooperation on this complex issue, and I hope that our efforts at consistency have not introduced any errors into the text.

This brings us to the Index. There was not enough time to prepare an index for the NSF version. One was put together for the "red book," but it was somewhat less extensive than one might have wished for a handbook. This time, when faced with the need to do it all again, and also having all of the text in electronic form, I was mindful of the two opposing indexing concepts currently pervasive in the popular culture. What one might call the minimalist view of indexing comes from the Douglas Adams book *The Hitchhiker's Guide to the Galaxy*, where the original entry for Earth is "Harmless;" this is only slightly improved later by being updated to "Mostly Harmless." The opposing view was crystallized by Barry Commoner as: "Everything is connected to everything else" — a concept amply demonstrated within the field of confocal microscopy. Trying to steer a middle course between these two extremes, I have concocted a new Index that is over twelve times the size of the previous one (now with nearly 7,000 topics and about twice that many page listings), while the book itself has almost tripled. This Index contains entries for almost every diagram, plot, image, and table in the book. It also lists under "Summaries" the pages of the summary sections that conclude most chapters and contain their "take-home lessons." The listing "chapter" refers to an entire chapter starting on the page noted and dealing predominantly with the listed topic. Although subjects in the text are extensively cross-indexed, literally "connecting everything to everything else" would have required another book. I settled for making sure that each text topic appeared at least once under all of the Index topics that seemed appropriate, but I did not attempt to list all the pages in each chapter on which a term was mentioned. As a result, the reader would probably be well-advised to look for additional information on the pages adjacent to (usually following) those pages listed in the Index. I beg indulgence for all of the "inevitable omissions."

Confocal microscopy is not the only technology to have developed over the last five years. Constant improvements in the international digital communication network have brought e-mail and electronic file transfer into the normal working lives of most of the authors, and this made the editing of the present edition much more of a two-way process. Chapters could be modified to fit better with their neighbors, returned, checked, and resubmitted all in a matter of days, even when the authors concerned were in Australia, Taiwan, and Europe. Although this process added a welcome level of flexibility not present for the earlier book, it also imposed an additional strain on the authors, who often were just congratulating themselves on finally getting their chapter "out the door" only to have them reappear with a lot of suggested changes and requests for expansion to cover additional areas. Again, the authors responded to this challenge in the most positive manner possible, and this seems the most appropriate place to record my sincere thanks to them for the cooperative spirit that they invariably displayed. Thanks are also due to Helen Noeldner, who provided the order and secretarial assistance without which we could not have succeeded; to Mary Born, my editor at Plenum, whose kind voice prevented me from jumping out of my twelfth-floor window on several occasions; to those manufacturers who provided support for publishing some of the color figures and to their representatives for providing the diagrams and other information included in Appendix 2; to NSF, which provided me with grant DIR-90-17534, to my wife; Christine, who toiled many late nights on the Index; and to my family (and doubtless the families of the authors), who gave me their precious time to help get this project finished.

All of these contributed everything that they could in an effort to make this the most comprehensive, accurate, and useful volume on the subject possible. We all hope that you will think we have succeeded.

James B. Pawley
January 1995

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