This book is concerned with optical microscopy; the principles, instruments, and limitations of far-field microscopes. Alternative types of microscopes operate in the near-field (in which the object-to-microscope distance is less than the wavelength of light), which provide another type of optical microscopy that exceeds the classical limit of resolution (Jutamulia, 2002).

Although the emphasis of this book has been on confocal microscopes and multiphoton excitation microscopes, the reader should not fall into the trap of using those instruments that are available, familiar, or that were successful in the past. Both types of microscopes are limited by the depth of penetration and a significant difference in the lateral and axial resolution.

As research problems evolve, there is always the possibility that other types of imaging may be more appropriate and provide unexpected solutions. The great advances made in optical microscopy, both in far-field and near-field optical microscopes, have a significant impact on our visualization and understanding of the microscopic world.

Similarly, nonoptical imaging modalities such as ultrasound, computerized x-ray tomography or x-ray computed tomography (CT), and magnetic resonance imaging are in continuous development and evolution. These techniques have been adapted to a wide range of specimens, from whole body imagers to microscopes for very small specimens. The reader should be open to new and to evolving techniques. The message is simple: the instrument used in the investigation must be appropriate to the questions asked and to the specimen under observation. Resolution and contrast are partial considerations; there are also factors of safety, specimen area and thickness, and image acquisition time. Optical microscopes offer high-resolution, high-contrast images, but only for a small area.

Optical microscopy is inherently two-dimensional: the focal plane is flat. Another consideration is that the axial and lateral resolutions are very different. The depth of penetration is limited by both the microscope and the specimen. The free-working distance of the microscope objective is a limit of the instrument. The absorption and scattering coefficients of the specimen limit the light penetration into the thickness of the specimen. There are many specimens whose diameter or thickness exceeds the depth of penetration of the light microscope. Further development and progress in the fields of optical biopsy and in vivo microscopy for biology and medicine may reach limits based on these considerations.

Fortunately, there are new and exciting advances to solve some of these problems. Optical projection tomography has been developed to provide high-resolution three-dimensional images of both fluorescent and nonfluorescent biological specimens with a thickness up to 15 mm (Sharpe et al., 2002). Another approach to imaging large, thick, live biological specimens is selective plane illumination mi-
Another approach that is emerging as an important diagnostic imaging tool is optical low-coherence reflectometry. This technique was originally developed in the telecommunications industry for testing fiber optic cables and integrated optical devices. In the last decade the field has been further developed and applied to biology and diagnostic medicine (Masters, 2001).

There are still obstacles on the path to imaging live cells, tissues, and organs. The depth of penetration is not sufficient for many specimens and for “optical biopsy.” The problem of phototoxicity is a major concern. Optical microscopes are inherently two-dimensional, but they are used to view a three-dimensional world.

Microscopes are tools. Humans are tool users, but they are also tool makers. In this book I have described the ingenious tools, from optical microscopes to fluorescent probes, that have been developed so we can image the invisible world of the microcosm. There is no reason to believe that this development of new tools will not continue. And with the new developments in microscopy will come increased knowledge and understanding of our world.
Appendix

Reference Materials and Resources


W. Becker, *Advanced Time-Correlated Single Photon Counting Techniques*, Springer Verlag, Berlin (2005). This is an important and practical book on the topic of single-photon counting techniques. The chapter on detectors for photon counting is both clear and comprehensive and thus highly recommended.


R. W. Boyd, *Nonlinear Optics*, 2nd ed., Academic Press, San Diego (2003). This is a very well written textbook that provides the theoretical foundation for modern nonlinear optics. This book provides a solid foundation to understand the fundamentals of nonlinear spectroscopy and microscopy.


experimental details in order that the reader can use these methods. There are several chapters on in vivo microscopy.

T. R. Corle and G. S. Kino, *Confocal Scanning Optical Microscopy and Related Imaging Systems*, Academic Press, San Diego, CA (1996). This book is a comprehensive introduction to the field of scanning optical microscopy, including the confocal scanning optical microscope and the optical interference microscope. It contains a very clear introduction to the theory of depth and transverse resolution. This is a good source of applications in the semiconductor industry and metrology. The theory of the confocal microscope is well written.


D. J. Goldstein, *Understanding the Light Microscope: A Computer-aided Introduction*, Academic Press, London (1999). This very practical book contains computer programs that allow students to simulate the effects of aperture, spherical aberration, and focus of the objective lens; the operation of bright-field and phase contrast microscopes; quantitative polarized-light microscopy; and a ray-tracing program that shows the effects of aberrations in simple and compound lenses. The book contains a good review of Abbe’s elementary diffraction theory and various techniques to form contrast in optical microscopy.


E. Hecht, *Optics*, 4th ed., Addison-Wesley, Reading, MA (2001). This is the standard work on optics for the undergraduate level. It offers a clear discussion of geometrical and physical optics. The numerous figures clearly illustrate the fundamental principles of optics.


D. B. Murphy, *Fundamentals of Light Microscopy and Electronic Imaging*, Wiley-Liss, New York (2001). This is a very good book to learn the fundamentals of microscopy. Each chapter includes practical demonstrations and exercises. It has a good balance between the theory and the practical aspects of optical microscopy. Several laboratory demonstrations of important principles are described.


York (2005). This is a practical guide for the imaging of tissues and organisms of key importance for neuroscience and development. The tutorial on microscopy and microscope optical systems by Lanni and Keller is both clear and comprehensive.


**Journals**

*Applied Optics*

*Biophysical Journal*

*Journal of Biomedical Optics*

*Journal of Microscopy*

*Journal of Optical Society of America*

*Microscopy Research and Technique*

*Optics Communications*

*Optics Express*

*Optics Letters*

**Special Journal Issues on Multiphoton Microscopy**


**Internet Resources**

**Fluorescent Probes**

Molecular Probes, Inc.: http://probes.invitrogen.com/

This website contains links to many other Web resources: noncommercial, journal, commercial sites, conferences, and other meetings.

Their online catalog contains a useful tutorial on many aspects of fluorescence and an extensive catalog of fluorescence probes for labeling: ions, molecules, cells, tissues and organs. Their catalog is actually a wonderful, comprehensive reference containing application images, references, absorption and emission spectral data, and the chemical and photochemical properties of all their products under a variety of conditions. Detailed protocols are provided for loading cells, calibrating fluores-
cence intensity, the use of caging groups and their photolysis, the study of signal transduction, using potentiometric probes, and using dyes to determine ion concentration and pH. They also have books on microscopy and fluorescence techniques and a good variety of calibration systems. The protocols contained in the handbook cover the scale from membranes, cell organelles, cells, tissues, and organs to whole organisms used for studies of their developmental biology. The online version of the handbook is updated often and of great utility.

Information on confocal microscopy, multiphoton excitation microscopy, microscopes, lasers, image processing software, techniques, technical information on microscope objectives, light sources, microscope images from many types of microscopes, and a wide range of detailed technical application notes can be found here.

**Quantum Dot Fluorescent Probes**, Quantum Dot Corporation, Hayward, CA: http://qdots.com

**Microscopes and Tutorials on Microscopy**

**Florida State University’s Molecular Expressions**: http://micro.magnet.fsu.edu
Includes confocal and multiphoton microscopy Java tutorials.

**Leica**: http://www.leica-microsystems.com

**Nikon Instruments, Inc.**: http://www.nikonusa.com/, http://www.microscopyu.com
Interactive Java tutorials.

Interactive Java tutorials and Microscopy Resource Center, which contains a section on microscopy history, several websites showing collections of antique microscopes from around the world.

**Carl Zeiss**: www.zeiss.com, www.zeiss.de/lsm
Tutorials and application notes on all aspects of microscopy; searchable database.

**Lasers**

**Coherent, Inc.**: http://www.cohr.com

**Other Components**

**Physikinstrumente**: www.physikinstrumente.de
Tutorial on piezoelectrics in micropositioning devices; piezoelectricity and piezo actuators.
**Newport Corporation and Spectra Physics**: http://www.newport.com
A good source of CW and pulsed lasers and an optics tutorial.

**New Focus**: http://www.newfocus.com

**Detectors**

**Hamamatsu**: http://www.hamamatsu.com

**Becker & Hickl GmbH, Berlin, Germany**: http://www.becker-hickl.de/

**Photometrics**, a division of Roper Scientific, Inc.: www.roperscientific.com
This website contains technical information and application notes for cooled, back-illuminated, high quantum efficiency (90%) charge-coupled-device cameras with on-chip multiplication gain.

**Scanners**

Cambridge Technology, Inc.: www.camtech.com

**Optical Filters**

**Omega Optical Inc.**: http://www.omegafilters.com

**Chroma Technology Corporation**: http://www.chroma.com

**Microscopy Societies**

**Microscopy Society of America**: http://www.microscopy.org

**Royal Microscopical Society**: http://www.rms.org.uk/

**Image Processing Software**

**Patents**

Patents are an excellent source of information for the understanding, design, and construction of instruments. Here you can search by key words, patent inventor name, patent number.
**Other Websites**

**Professor Peter So laboratory**: http://web.mit.edu/solab/
This website provides a wealth of information on the engineering of novel microscopy instrumentation and the application of these new tools to biomedical problems. These new types of microscopic and spectroscopic instruments are designed to span the range from single molecule dynamics, to the cellular level, to the tissue level. There are useful links for optical instrumentation.

**Professor Stefan Hell laboratory**: www.4pi.de
Tutorial information on how to overcome the Abbe diffraction limit in light microscopy and achieve three-dimensional resolution in the 100 nm range. The group’s publications are available as PDFs. There are links to sites on the history of the microscope.

**References for Applications in Ophthalmology and Dermatology**

**Ophthalmology**

These references cover the development of instruments and the ex vivo and in vivo microscopic investigation of cells, tissues, and organs. There are instruments designed to use light microscopy to monitor cellular metabolism; optical techniques to provide three-dimensional microscopy of the cornea, the ocular lens, and the optic nerve in vivo; the development of clinical confocal microscopes for diagnostic “optical biopsy” of the living eye; the use of confocal microscopy to investigate redox metabolism is developed, as is the use of multiphoton excitation microscopy to monitor redox metabolism in the ex vivo cornea; correlative microscopy is demonstrated by the use of both confocal and electron microscopy on the same human lenses in the same regions.


**Dermatology**

These papers demonstrate the use of *in vivo* confocal microscopy and *in vivo* multiphoton excitation microscopy and spectroscopy to investigate the structure and function of *in vivo* human skin.


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