Confocal Microscopy and Multiphoton Excitation Microscopy

The Genesis of Live Cell Imaging

Barry R. Masters

SPIE
PRESS
Bellingham, Washington USA

Library of Congress Cataloging-in-Publication Data

Masters, Barry R.
Confocal microscopy and multiphoton excitation microscopy : the genesis of live cell imaging / Barry R. Masters.
p. cm.
"Press monographs v. PM161"—Provided by publisher.
Includes bibliographical references and index.
ISBN 0-8194-6118-0 (alk. paper)
1. Confocal microscopy. 2. Multiphoton excitation microscopy. I. Title.

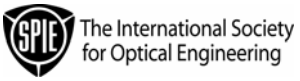
QH244.M37 2005
502'.82—dc22

2005026105

Published by

SPIE—The International Society for Optical Engineering
P.O. Box 10
Bellingham, Washington 98227-0010 USA
Phone: +1 360 676 3290
Fax: +1 360 647 1445
Email: spie@spie.org
Web: http://spie.org

Printed in the United States of America.



Cover image: Confocal microscopy of a fixed, stained, vertical section of human skin. This is a biopsy specimen from the upper arm. The horizontal field width is 1400 μm.

To our teachers who taught us,
so that we can teach others

On looking back to this event, I am impressed by the great limitations of the human mind. How quick are we to learn, that is, to imitate what others have done or thought before. And how slow to understand, that is, to see the deeper connections. Slowest of all, however, are we in inventing new connections or even in applying old ideas in a new field.

Frits Zernike, Nobel Lecture, December 11, 1953

Contents

List of Abbreviations

AOTF	acousto-optical tunable filter
APD	avalanche photodiode
CCD	charge-coupled device
CRT	cathode-ray tube
CT	computed tomography
CSLM	confocal scanning laser microscope
DIC	differential interference contrast
DOF	depth of focus
DPH	diphenylhexatriene
FISH	fluorescence *in situ* hybridization
FLIM	fluorescence lifetime imaging
FRAP	fluorescence recovery after photobleaching
FRET	fluorescence resonance energy transfer
fs	femtosecond
GFP	green fluorescent protein
LED	light-emitting diode
LSCM	laser scanning confocal microscope
MIAM	multiple imaging axis microscopy
NA	numerical aperture
OCT	optical low-coherence tomography
PDT	photodynamic therapy
PMT	photomultiplier tube
ps	picosecond
PSF	point spread function
RMS	Royal Microscopical Society
SHG	second-harmonic generation
SNR	signal-to-noise ratio
SPAD	single-photon avalanche photodiode
STED	stimulated emission depletion

Preface

This text explains the fundamentals of confocal microscopy and multiphoton excitation microscopy. It presents the big picture of technological development in optical microscopy and provides insight into the origins, development, modification, and application of confocal and multiphoton excitation microscopes and their use in biology and medicine. This insight is presented in light of the key problems that each new invention attempted to solve, the various paths to the solution, the myriad interactions of various inventors and their associated technologies, and the practical limitations of each step of discovery and technological development. The human side of these technological developments is also revealed by describing the individual motivations that drove different scientists to their inventions, as well as the parallel developments that preceded each stage of technological development.

The repeated convergence of disparate techniques, instruments, theoretical studies, inventions, and reinventions from a wide variety of disciplines partially solved a series of problems in the field of microscopy and produced the current renaissance in modern optical microscopy. Innovative ideas and technical developments came from many individuals living and working in several countries around the world. Innovation evolves from a broad knowledge base, an awareness of advances in disparate fields of science, the courage to radically depart from mainstream thinking, and a clear understanding and statement of the problem to be solved. In many cases, innovations arise from technology transfer and not true invention.

Only recently have technical developments in many separate fields—for example, medical imaging and cell biology—spread across disciplines. There are many more examples of advances in different fields finding applications in optical microscopy. The field of digital image processing was first developed for air and space imaging applications. The field of adaptive optics, now being developed into optical microscopes and medical laser imaging devices, was first developed in the fields of astronomy and military laser weapons. Finally, the emerging medical imaging field of optical low-coherence reflectometry and tomography was first developed for the telecommunications industry as devices for checking fiber optics and integrated optical devices.

The biomedical applications of optical microscopy constitute an emerging field driven by spectacular advances in the field of *in vivo* microscopy. Advances in confocal microscopy are providing new and important technical solutions in the fields of endoscopy, minimally invasive surgery, dermatology, and ophthalmology. New technical advances in the fields of neurobiology and developmental biology build on the instruments described in this book. In many cases, the solutions to these problems required the optimization of one or more other solutions; typically, designs compromised one or more parameters (resolution, contrast, time for image acquisition) to serve a specific purpose. Optical microscopy began with the obser-

vation of living specimens, and recently there has been a revolution to return to the observation of *in vivo* specimens. The combination of spectroscopic techniques and optical microscopy has resulted in important advances in the field of "optical biopsy." Furthermore, these promising new diagnostic techniques are transitioning from the laboratory to the clinic.

There is an advantage to staying aware of the theoretical and technical advances of disparate fields of science. Such awareness may prove to be useful in the development of techniques that seem far from the problem at hand. Being aware of current and interesting problems in the biomedical area as well as advances in modern techniques of imaging, signal processing, nanotechnology, and integrated optics creates the conditions for success in interdisciplinary research. I hope the reader will find these themes useful for stimulating developments in new instrumentation as well as innovative, clinically useful applications in the exciting field of optical microscopy.

Since many excellent books, courses, and Internet resources are available that describe many aspects of modern microscopy, is there anything new to write on this subject? I delayed the completion of this book over a period of years while I attempted to answer this question myself. I believe the answer is a definite yes. In this book, I present several new ways of approaching these two topics. First, I present the recent developments as partial solutions to existing long-term problems. Second, I show that many developments are advances on previous instruments and techniques; there was an intellectual lineage in the development of modern microscopes. Third, I connect the developments of unique types of microscopes in disparate fields of science and medicine, and demonstrate their similarities. Fourth, I indicate the problems, limitations, artifacts, and experimental difficulties with modern microscopes. And fifth, I describe the techniques that use optical microscopes for studies on living tissue and organs and explain why the new types of microscopes are emerging as important clinical tools for medical diagnostics. *In vivo* microscopy and optical biopsy are active fields of research. This is evident from the exciting research in developmental biology, ophthalmology, dermatology, oncology, and brain imaging.

Another unique feature of this text is the discussion of the historical developments of optical microscopy and the technology's critical impact on the fields of biology and medicine. The reader may ask why this is necessary for an understanding of the modern instruments and their applications. There are several reasons. First, an appreciation of the chain of invention may serve to correct some incorrect attributions of priority and rediscovery of previous inventions. Second, an understanding of the historical development of both instruments and techniques has an important educational value in demonstrating serial and parallel approaches to problem solving in optical microscopy. Third, the study of the antecedents to various technical developments can put each invention and advance in its proper perspective and perhaps stimulate innovation. So many excellent books focus on applications that I decided it would be redundant to present extensive reviews of applications. Application notes are available online from the companies that manufacture confocal and multiphoton microscopes.

The projected audience for this text includes those who wish to gain insight into confocal microscopy and multiphoton excitation microscopy, and who intend to apply these techniques to biology and medicine. Therefore, it cannot be stated that this book was written for a single group of individuals. The projected audience includes undergraduate students who seek a global insight into the field of modern optical microscopy, graduate and postdoctoral students who will work with these instruments, and physicians who work with engineers and scientists to design and develop new, noninvasive, diagnostic instruments based on confocal or multiphoton excitation microscopy.

Optical microscopy is a nexus of theory, techniques, and devices from a wide variety of sources and disciplines, and the organization of this book reflects this fact. The text is divided into four parts. The largest part is devoted to confocal microscopy, with an introduction and a part devoted to multiphoton excitation microscopy. The emphasis is not on the main types of optical microscopes, but on how various technical developments served to solve the common problems of optical microscopy. However, throughout the book there are common themes, connections, and technical solutions to the problems of light microscopy that necessitated the deliberate repetition of some central concepts and ideas.

Each chapter of this text begins by introducing the materials to be covered and explaining their role in the book. A summary of key points at the end of each chapter reinforces those critical points. Hopefully the text contains everything that is essential and excludes those topics and details that are not critical for an understanding of the principles and their applications in microscopy. Further insight into the theory and practice of optical microscopy may be garnered by perusal of the printed and electronic resources that I have recommended in the appendix.

Part I covers the background, significance, and principles of the optical microscope. Chapter 1 presents a history of the microscope and the development of fluorescent microscopy, and describes the role of microscopy in the advancement of biology and medicine. Chapter 2 introduces the reader to the optical microscope by describing its chief components and limitations. Chapter 3 describes the contributions of Abbe, including the Abbe theory of image formation in an optical microscope. Chapter 4 discusses optical resolution in a microscope. When the major problems of optical resolution and optical aberrations were solved, the new primary problems concerned the development of techniques to provide contrast. These techniques resulted in the emergence of live cell imaging in optical microscopy. Chapter 5 explains both the nonoptical and optical techniques (phase contrast and differential interference contrast microscopy) that provide contrast.

Part II describes the partial solutions to the following problem: how to image thick, highly scattering specimens with an optical microscope. The invention of the confocal microscope, with its many technical variants, provided one partial solution. Confocal microscopy improves the resolution, contrast, and optical sectioning capability of the light microscope. The connecting theme in Part II is that a variety of techniques were invented and reinvented to solve the same problem: how to construct an optical microscope that has depth discrimination, and thus provide a mi-

croscope with the capability to "optically section" thick, scattering specimens. Chapter 6 formulates this problem and then describes several early antecedents to the development of confocal microscopy. Chapter 7 analyzes the myriad solutions to the problem of depth discrimination: the various types of confocal microscopes and their limitations.

Chapter 8 describes the development of scanning-slit confocal microscopes, which were developed in disparate fields: ophthalmology, neurobiology, and cell biology. Chapter 8 also plays a special pedagogical role in this book. While superficially it may seem that the theme is of interest only to ophthalmologists because the applications are predominantly imaging of the *in vivo* eye, there is a much deeper motivation to include these topics. This chapter demonstrates the linkages, connections, and technology transfers from numerous sources in the progression of technological development of the confocal microscope. For example, the inventions of Baer were motivated by the desire to develop a confocal microscope for cell biology, and the inventions of Svishchev were motivated by the desire to develop a confocal microscope to study neurobiology.

The primary message contained in Chapter 8 is that technical problems are solved by building on the previous and parallel work of others. The insights exposed in this chapter were derived not only from reading the published papers and patent literature, but also from personal conversations with Maurice, Svishchev, Petràn, Hadravsky, Baer, Koester, Kino, and Thaer. I also gained insight from working in the laboratory with Kino, Maurice, and Thaer. This chapter also provides an important lesson: teachers should teach not only those techniques that are popular; they must have a larger objective to teach how to solve problems by devising creative solutions. Many of the technical advances developed in Chapter 8 have found their applications in modern biomedical confocal instruments: scanning-slit confocal microscopes to investigate the cochlea, study *in vivo* human skin, and study the normal and pathological eye. Confocal microscopes based on slits are also being developed to image large embryos and study their development.

Chapter 9 describes the components of a confocal microscope. Even with the invention and development of the many types of confocal microscopes, problems remain. First, the ultraviolet excitation light used to excite many fluorescent dyes in molecular biology, ion indicator dyes, and endogenous molecules such as NAD(P)H and neurotransmitters, with absorption bands in the ultraviolet, is toxic to live cells, tissues, and organisms. Second, the depth of penetration of thick, scattering specimens is a few hundred microns and therefore precludes the imaging of thicker specimens. Third, the highly intense visible and short wavelength light causes photobleaching of the specimens during observation. The partial solution to these problems came with the invention and development of nonlinear microscopy.

Part III describes nonlinear optical microscopy with an emphasis on multiphoton excitation microscopy. Chapter 10 presents the development of nonlinear spectroscopy and microscopy—in particular, the seminal role played by the invention of the laser. Chapter 11 presents a detailed description of multiphoton excitation microscopy, from the Göppert-Mayer theory (Maria Göppert, 1929) to the Denk,

Strickler, and Webb 1990 *Science* publication. Chapter 12 summarizes the theory behind and describes the instrumentation of multiphoton excitation microscopy.

Part IV discusses the path to imaging live cells, tissues, and organs. Chapter 13 sets out the remaining problems and describes the limitations of nonlinear microscopy. Chapter 14 presents future directions for confocal and multiphoton excitation microscopy. Chapter 15 addresses the important topic of laser safety and includes a section on how to clean optics. An epilogue discusses humans as tool makers and tool users.

The book concludes with an appendix containing an annotated listing of carefully selected reference materials and resources. They present applications in great detail as well as experimental protocols. The appendix also contains a partial listing of the author's publications in ophthalmology and dermatology that illustrate the benefits of confocal and multiphoton microscopy in clinical medicine.

This book tells the story of the development of solutions to formidable problems in optical microscopy. It also tells the story of the limitations of optical microscopy: optical aberrations, optical artifacts, fundamental physical limitations of signal and noise, the quantum nature of light, stray light, background fluorescence, and light damage to the specimen. The information in this book will be an ongoing story—microscope development continues as an active field of progress toward the partial solution of the following problems: resolution, contrast, and optical microscopy of live cells, tissues, and organisms with minimal toxic and destructive effects. There is much work to be done, as we have only partial solutions to these problems. The state of the art is a moving target.

Finally, I gladly thank Margaret Thayer and Sharon Streams of SPIE for their help with the manuscript.

Confocally yours,

Barry R. Masters
November 2005

Part I

Optical Microscopy