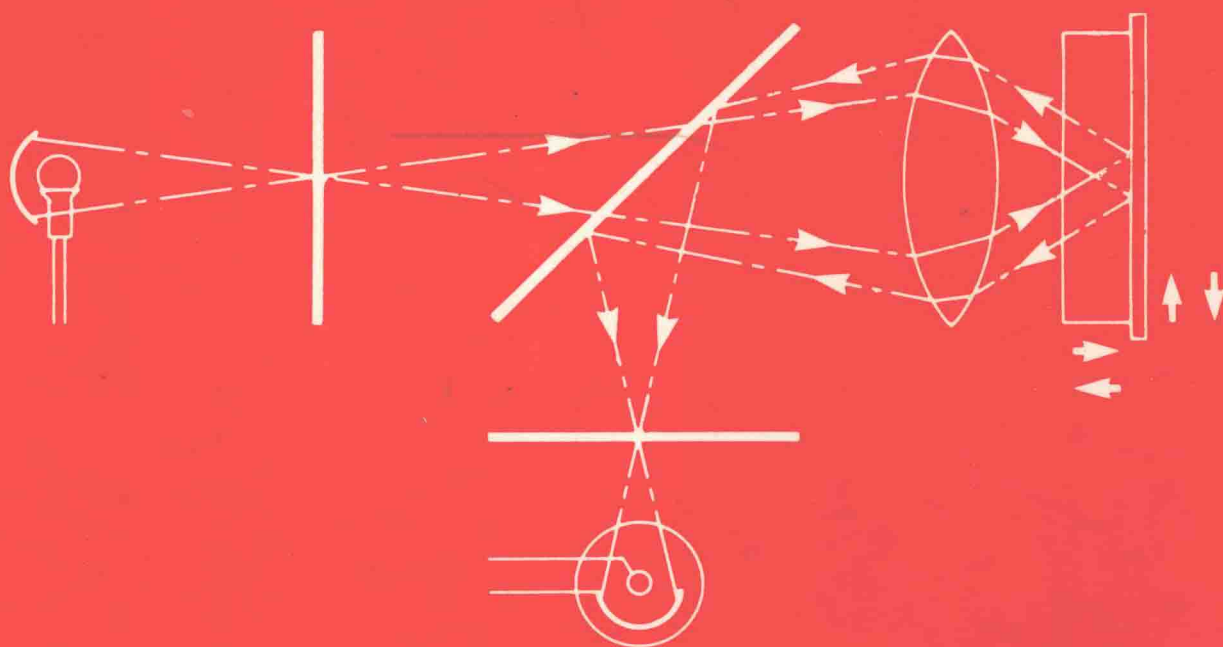


# HANDBOOK OF BIOLOGICAL CONFOCAL MICROSCOPY



Edited by  
**James B. Pawley**

# **HANDBOOK OF BIOLOGICAL CONFOCAL MICROSCOPY**

**Edited by  
James B. Pawley**

Integrated Microscopy Resource for Biomedical Research  
University of Wisconsin-Madison  
Madison, Wisconsin

**REVISED EDITION**

**PLENUM PRESS • NEW YORK AND LONDON**

ISBN 0-306-43538-1

Based on papers given at a confocal microscopy workshop, supported  
by the National Science Foundation and held at the Electron  
Microscopy Society of America meeting, August 8-9, 1989,  
in San Antonio, Texas

© 1990, 1989 Plenum Press, New York  
A Division of Plenum Publishing Corporation  
233 Spring Street, New York, N.Y. 10013

All rights reserved

No part of this book may be reproduced, stored in a retrieval system, or transmitted  
in any form or by any means, electronic, mechanical, photocopying, microfilming,  
recording, or otherwise, without written permission from the Publisher

Printed in the United States of America

# **HANDBOOK OF BIOLOGICAL CONFOCAL MICROSCOPY**



# Authors

**David A. Agard**

Department of Biochemical Science  
The Howard Hughes Medical Institute  
University of California, San Francisco  
San Francisco, CA 94143-0448

**Jonathan Art**

Department of Physiology  
University of Chicago  
947 East 58th Street  
Chicago, IL 60637

**Robert Bacallao**

Division of Nephrology  
University of California, Los Angeles  
Department of Medicine  
Center for the Health Sciences  
10833 LeConte Avenue  
Los Angeles, CA 90064-1736

**Morgane Bomsel**

Cell Biology Program  
European Molecular Biology Laboratory  
Postfach, 10.2209  
D-6900, Heidelberg  
Federal Republic of Germany

**Alan Boyde**

Department of Anatomy & Embryology  
University College London  
Gower Street  
London WC1E 6BT, UK

**G. J. (Fred) Brakenhoff**

Department of Molecular Cell Biology  
Section of Molecular Cytology  
University of Amsterdam  
Plantage Muidersgracht 14  
1018 TV Amsterdam  
Amsterdam, Netherlands

**Walter A. Carrington**

Physiology Department  
University of Massachusetts  
Medical School  
55 Lake Avenue North  
Worcester, MA 01655

**Hans Chen**

Department of Biochemical Science  
The Howard Hughes Medical Institute  
University of California, San Francisco  
San Francisco, CA 94143-0448

**Victor Chen**

K.H.C. Associates  
P.O. Box 21  
Amherst, NY 14226-0021

**Ping-Chin Cheng**

Advanced Microscopy Laboratory for  
Biomedical Sciences  
Department of Anatomical Sciences  
317 Farber Hall  
State University of New York  
Buffalo, NY 14214

**Jan De Mey**

Cell Biology Program  
European Molecular Biology Laboratory  
Postfach, 10.2209  
D-6900, Heidelberg  
Federal Republic of Germany

**C. Kathleen Dorey**

Eye Research Institute of Retina  
Foundation  
20 Staniford Street  
Boston, MA 02114

**Fredric S. Fay**

Physiology Department  
University of Massachusetts  
Medical School  
55 Lake Avenue North  
Worcester, MA 01655

**Kevin E. Fogarty**

Physiology Department  
University of Massachusetts  
Medical School  
55 Lake Avenue North  
Worcester, MA 01655

**Enrico Gratton**

Physics Department  
University of Illinois, Urbana-Champaign  
Laboratory for Fluorescence Dynamics  
1110 West Green Street  
Urbana, IL 60637

**Shinya Inoué**

Marine Biology Laboratory  
Woods Hole, MA 02543

**H. Ernst Keller**

Carl Zeiss Incorporated  
One Zeiss Drive  
Thornwood, NY 10594

**Gordon S. Kino**

Edward L. Ginzton Laboratory  
Stanford University  
Stanford, CA 94305-4085

**Charles J. Koester**

Department of Ophthalmology

Columbia University  
635 West 165th Street  
New York, NY 10032

**Larry Lifschitz**

Physiology Department  
University of Massachusetts  
Medical School  
55 Lake Avenue North  
Worcester, MA 01655

**James B. Pawley**

Integrated Microscopy Resource  
1675 Observatory Drive  
University of Wisconsin  
Madison, WI 53706

**David R. Sandison**

Developmental Resource for Biophysical  
Imaging and Optoelectronics  
Applied Engineering and Physics  
Clark Hall  
Cornell University  
Ithaca, NY 14853

**John W. Sedat**

Department of Biochemical Science  
The Howard Hughes Medical Institute  
University of California, San Francisco  
San Francisco, CA 94143-0448

**Ernst H. K. Stelzer**

Light Microscopy Group  
European Molecular Biology Laboratory  
Postfach, 10.2209  
D-6900, Heidelberg  
Federal Republic of Germany

**James Strickler**

Developmental Resource for Biophysical  
Imaging and Optoelectronics  
Applied Engineering and Physics  
Clark Hall  
Cornell University  
Ithaca, NY 14853

**Robert G. Summers**

Department of Anatomical Sciences  
317 Farber Hall  
State University of New York  
Buffalo, NY 14214

**Roger Y. Tsien**

Department of Pharmacology M-036  
School of Medicine  
University of California, San Diego  
LaJolla, CA 94720

**H.T.M. van der Voort**

Department of Molecular Cell Biology

Section of Molecular Cytology  
University of Amsterdam  
Plantage Muidergracht 14  
1018 TV Amsterdam  
Amsterdam, Netherlands

**Martin J. vandeVen**  
Physics Department  
University of Illinois, Urbana-Champaign  
Laboratory for Fluorescence Dynamics  
1110 West Green Street  
Urbana, IL 60637

**K. Visscher**  
Department of Molecular Cell Biology  
Section of Molecular Cytology  
University of Amsterdam  
Plantage Muidergracht 14  
1018 TV Amsterdam  
Amsterdam, Netherlands

**Alan Waggoner**  
Resident Biologist, Department  
of Biological Sciences and  
Center for Fluorescence Research in the  
Biomedical Sciences  
Carnegie-Mellon University  
4400 5th Avenue  
Pittsburgh, PA 15213

**Robert Webb**  
Eye Research Institute of Retina  
Foundation  
20 Staniford Street  
Boston, MA 02114

**Watt Webb**  
Developmental Resource for Biophysical  
Imaging and Optoelectronics  
Applied Engineering and Physics

Clark Hall  
Cornell University  
Ithaca, NY 14853

**K. Sam Wells**  
Developmental Resource for Biophysical  
Imaging and Opto-electronics  
Applied Engineering and Physics  
Clark Hall  
Cornell University  
Ithaca, NY 14853

**Tony Wilson**  
Department of Engineering Science  
University of Oxford  
Oxford, OX1 3PJ  
United Kingdom

# Preface

In 1987 the Electron Microscopy Society of America (EMSA) under the leadership of J. P. Revel (Cal Tech) initiated a major program to present a discussion of recent advances in light microscopy as part of the annual meeting. The result was three special LM sessions at the Milwaukee meeting in August 1988: The LM Forum, organized by me, and Symposia on Confocal LM, organized by G. Schatten (Madison), and on Integrated Acoustic/LM/EM organized by C. Rieder (Albany). In addition, there was an optical micro-analysis session emphasizing Raman techniques, organized by the Microbeam Analysis Society, for a total of 40 invited and 30 contributed papers on optical techniques.

Following this successful meeting, discussions among the participants revealed support for a slightly more focussed approach at the next meeting. The benefits of confocal techniques were now felt to be widely appreciated and it seemed time to really evaluate the actual performance of the various instruments and to compare this with theoretical benchmarks and so produce a consensus on where major improvements were likely to be possible in the future. It was felt important to shift from the assertion that "Confocal Works" to the matter of how to make it work better.

Because of the rapid pace of development in the field, we recognized that we were unlikely to be able to be totally definitive on all matters affecting the confocal microscope, but we also felt that the field would benefit from access to a good list of questions and as many answers as time permitted. To do this, it was decided to try to elicit a series of talks in which each one covered a single instrumental feature unique to confocal microscopy (particularly biological confocal microscopy). The initial list included 12 topics ranging from laser and conventional sources through scanning systems, objective lenses, chromophors, "the pinhole", photon detectors and 3D data display, as well as three overviews on the genesis of the confocal approach, its fundamental limitations and its quantitative capabilities.

In parallel with these developments at EMSA, Drs. J. Wooley and S. Pierce of the Instrumentation and Instrument Development Program at the National Science Foundation had followed a similar path. The Instrumentation and Instrument Development Program is responsible for supporting the purchase of major items of multi-user instrumentation for the conduct of basic research in the life sciences, particularly that which is supported by the NSF Divisions of Behavioral and Neural sciences, Cellular Biosciences, Molecular Biosciences, and BIOTIC Systems and Resources. For the past five years, the Program had emphasized three areas of activity: 1) New instruments that either extend current sensitivity or resolution, or provide new techniques for detection, quantification or observation of biological phenomena. 2) New computer software to enhance current or new instrumentation, and 3) Sponsored workshops in emerging areas of instrumentation or instrument development. They believed that it was clear that confocal microscopy and other new microscopical instrumentation was

going to drive important scientific discoveries across wide areas of physiology, cellular biology and neurobiology. They had been looking for a forum in which they could advance the state of the art of confocal microscopy, alert manufacturers to the limitations of current instruments, and catalyze progress toward new directions in confocal instrument development.

These goals were so close to those of the EMSA project that the two groups decided to join forces with EMSA to provide the organization and the venue for a Confocal Workshop and NSF to provide the financial support for the speakers expenses and for the publication of extended abstracts.

The abstracts were initially envisioned as each being about 10–15 pages of camera-ready manuscript but, because of the generous and enthusiastic response of the many leaders of the confocal LM community who agreed to participate, the manuscripts actually submitted were up to fifty pages in length. In addition, scissions and additions increased the list of the topics covered to a total of 19, plus an annotated bibliography.

As the aim of the volume was to discuss the instrument rather than to describe specific applications, the biological emphasis emerges in most chapters as the need to use photons efficiently at every stage of the imaging process and thereby reduce the effects of bleaching and photo-damage to the specimen. In this context, several chapters in this volume emphasize for the first time limitations imposed by everything from fluorescence saturation and sub-optimal signal digitization to specimen preparation.

On a more general level, chapters were added on related instrumentation and on the often unrecognized limitations imposed by the process of pixelating the data contained in digitally recorded images. Several months of frenzied activity got the final mock-ups to the printer in early July.

The nineteen papers were presented at a two day workshop on August 8–9, 1989 at the EMSA Meeting in Houston, TX where the first, soft-cover edition of the Handbook was distributed at that time under the convenient but largely fanciful imprimatur of the IMR Press.

The response was so enthusiastic that it was decided to produce a second, hard-cover edition with an established publisher. This would permit wider distribution and would allow us to correct the errors associated with the short preparation time of the first edition. In addition, extra paragraphs and figures were added to fill gaps in the original or to take note of recent developments.

Taken as a whole, I believe these papers constitute the most complete consideration on the topic available at this time. I am sure that all of the other authors join me in the hope that it will prove to be a catalyst in the development of yet better instrumentation and techniques in the field of biological confocal microscopy. Indeed, improvements evident in the design of the Biorad MRC-600 and of the Leitz CLSM show some evidence of this trend.

Many people have contributed to the production of this volume starting with Drs. Pierce and Wooley and all of the authors.



In addition, I should like to single out R. and C. Moen and K. Hamele for their editorial assistance and C. Thomas, C. Ewing, K. Morgan and P. Henderson for help in retyping some of the manuscripts, A. Freidman and L. Moberly of University of Wisconsin-Madison Publications and W. Kasdorf and N. MacMiller of Impressions for their patience with the typesetting. Special thanks are also due to G. Benham of the Biorad Corporation, and V. Argiro of Vital Images who, when rising costs threatened to delay publication of the first edition, stepped

in to fund the printing of the colored cover. This gesture is noted here because, due to a printing mix-up, no mention of the source of the cover images or of the support was included in that edition.

My heartfelt thanks to you all.

James Pawley  
Editor  
12/89

# Contents

<b>CHAPTER 1: FOUNDATIONS OF CONFOCAL SCANNED IMAGING IN LIGHT MICROSCOPY</b>	<b>1–14</b>		
<i>Shinya Inoue</i>			
<b>Light microscopy</b>	1		
Lateral resolution	1		
Axial resolution	3		
Depth of field	3		
<b>Confocal imaging</b>	4		
<b>Impact of video</b>	5		
The Nipkow disk	5		
Electron-beam scanning TV	5		
Impact of modern video	6		
<b>Lasers and microscopy</b>	6		
Holography	6		
Laser illumination	7		
Laser-illuminated confocal microscopes	9		
<b>Laser scanning confocal microscope</b>	9		
<b>Is laser scanning confocal microscopy a cure-all?</b>	10		
Speed of image or data acquisition	10		
Depth of field in phase dependent imaging	11		
<b>Some other optical and mechanical factors     affecting confocal microscopy</b>	11		
Lens aberration	11		
Unintentional beam deviation	12		
<b>Note added in proof</b>	12		
<b>Acknowledgment</b>	13		
<b>References</b>	13		
 <b>CHAPTER 2: FUNDAMENTAL LIMITS IN CONFOCAL MICROSCOPY</b>	 <b>15–26</b>		
<i>James Pawley</i>			
<b>Introduction</b>	15		
What limits?	15		
Counting statistics	16		
Source brightness	16		
Specimen response	16		
A typical problem	16		
<b>Practical photon efficiency</b>	17		
Losses in the optical system	18		
Objectives	18		
Mirrors	18		
Pinhole	19		
Is the confocal pinhole a “good thing”?	19		
Features of the confocal pinhole	19		
Detection and measurement losses	20		
The detector	20		
The PMT	20		
Solid-state photon detectors	21		
Digitization	21		
Evaluating photon efficiency	21		
<b>Resolution: how much is enough?</b>	22		
Can resolution be too high?	22		
Limitations imposed by spatial and temporal quantization	23		
Aliasing	24		
Pixel shape	24		
Blind spots	24		
		Practical considerations relating resolution to distortion	24
		<b>Summary</b>	26
		<b>Acknowledgements</b>	26
		<b>References</b>	26
 <b>CHAPTER 3: QUANTITATIVE FLUORESCENCE IMAGING WITH LASER SCANNING CONFOCAL MICROSCOPY</b>			<b>27–39</b>
		<i>K. Sam Wells, David R. Sandison, James Strickler and Watt W. Webb</i>	
		<b>The promise of scanning confocal fluorescence     microscopy</b>	27
		<b>Optical transfer efficiency</b>	28
		Methods of measurement of optical transfer efficiencies	29
		Confocal spatial filtering for depth of field compromises optical transfer efficiency	30
		Optical aberrations in fluorescence LSCM	31
		Chromatic aberrations in fluorescence LSCM measurements	32
		<b>Photodynamic effects</b>	33
		Theory	34
		Population rate equations	34
		Experiment	35
		<b>Fluorescence photobleaching recovery with LSCM</b>	37
		<b>Conclusion</b>	38
		<b>Acknowledgments</b>	39
		<b>References</b>	39
 <b>CHAPTER 4: THE PIXELATED IMAGE</b>			<b>41–51</b>
		<i>Robert H. Webb and C. Kathleen Dorey</i>	
		<b>Introduction</b>	41
		Pixelation	41
		Optical resolution: the resel	41
		Pixel	42
		Gray level	42
		In between	42
		<b>Matching image spatial characteristics</b>	42
		The Nyquist theorem	42
		Hyper-resolution: oversampling	43
		The resel/pixel ratio	44
		Diagonal dropout	44
		Pixel shape distortion	45
		Aliasing	45
		<b>The mechanics of pixelation</b>	46
		<b>Matching image intensity characteristics</b>	49
		Gray scale	49
		Detection	49
		Display	49
		Color displays	50
		<b>Caveats</b>	50
		Spectral variation of detectors	50
		Automatic gain control	50
		<b>Strategy for magnification and resolution</b>	50
		<b>Acknowledgements</b>	50
		<b>References</b>	50

## CHAPTER 5: LASER SOURCES FOR CONFOCAL MICROSCOPY

*Enrico Gratton and Martin J. vandeVen*

<b>Introduction</b>	53
<b>Laser power requirements</b>	53
<b>The basic laser</b>	54
Principle of operation	54
Laser modes: longitudinal and transversal	54
Polarization	55
Coherent properties of laser light	55
Temporal coherence	55
Coherence length	55
Spatial coherence	55
Coherence surface	55
Coherence volume	55
Pumping power requirements	55
Heat removal	56
Other installation requirements	56
<b>Types of lasers</b>	56
<b>Continuous wave (cw) lasers</b>	56
Gas lasers	58
Argon-ion	58
Krypton	59
Helium-neon	59
Helium-cadmium	59
Dye lasers	60
Solid state lasers	60
Semiconductor or diode injection lasers	60
Diode-pumped lasers	60
Tunable solid state laser	61
<b>Pulsed lasers</b>	61
Nitrogen lasers	61
Excimer lasers	61
Metal vapor lasers	61
Q-switched lasers	61
Trends in time-resolved spectroscopy applied to microscopy	63
<b>Wavelength expansion through non-linear techniques</b>	63
<b>Spatial beam characteristics</b>	63
<b>Intensity fluctuations of cw lasers</b>	64
<b>Maintenance</b>	64
Maintenance of active laser media	64
Laser tubes	64
Gases	64
Dyes	64
Laser rods	65
Maintenance of pumping media	65
Maintenance of the optical resonator	65
Maintenance of other system components	65
Cooling water	65
External optics	65
<b>Safety precautions</b>	65
Curtains	65
Screens	65
Beam stops	66
<b>Acknowledgement</b>	66
<b>References</b>	66

## CHAPTER 6: NON-LASER ILLUMINATION FOR CONFOCAL MICROSCOPY

*Victor Chen*

<b>Introduction</b>	69
Why use non-laser sources?	69
<b>Wavelength</b>	69
<b>Coherence</b>	70

<b>Which types of confocal microscope can use non-laser sources?</b>	70
<b>Characteristics of non-laser light sources</b>	72
Wavelengths available	72
Source radiance	72
Source stability	72
Source coherence	72
Source distribution	72
<b>Collecting the light and relaying it to specimen</b>	72
Illumination of the specimen: a basic part of microscopy	72
Tandem scanning: basic description	72
Single-sided disk scanning: basic description	72
How do you uniformly illuminate both the objective back focal plane and the intermediate image plane?	73
Scrambling and filtering the light	74
<b>Measuring what comes through the illumination system</b>	75
Exposure time and source brightness	75
Stationary specimens	75
What if the specimen is moving or changing?	75
<b>Incoherent laser light sources for confocal microscopy</b>	76
<b>References</b>	76

## CHAPTER 7: OBJECTIVE LENSES FOR CONFOCAL MICROSCOPY

*H. Ernst Keller*

<b>Abstract</b>	77
<b>Aberrations of refractive systems</b>	77
Defocusing	78
Monochromatic aberrations	79
Spherical aberrations	79
Coma	80
Astigmatism	80
Flatness of field	80
Distortion	81
Chromatic aberrations	82
Longitudinal chromatic aberration	82
Lateral chromatic aberration (LCA) or chromatic magnification difference	83
<b>Finite versus infinity optics</b>	84
<b>Optical materials</b>	85
<b>Anti-reflection coatings</b>	85
<b>Conclusion</b>	86

## CHAPTER 8: SIZE AND SHAPE OF THE CONFOCAL SPOT: CONTROL AND RELATION TO 3D IMAGING AND IMAGE PROCESSING

*G.J. Brakenhoff, K. Visscher and H.T.M. van der Voort*

<b>Abstract</b>	87
<b>Introduction</b>	87
<b>Pinholes and optical probe formation</b>	88
<b>Practical use of variable pinholes</b>	88
<b>Experimental axial confocal response</b>	89
<b>Comments and conclusions</b>	90
<b>References</b>	90

## CHAPTER 9: THE INTERMEDIATE OPTICAL SYSTEM OF LASER-SCANNING CONFOCAL MICROSCOPES

*Ernst H.K. Stelzer*

<b>Design principles of confocal systems</b>	93
Overview	93

Microscope objectives	93	<b>CHAPTER 12: PHOTON DETECTORS FOR CONFOCAL MICROSCOPY</b>	<b>127–139</b>
Position of the pivot point	93	<i>Jonathan Art</i>	
Position of the detector pinhole	94	<b>Introduction</b>	127
<b>Practical requirements</b>	95	<b>The quantal nature of light</b>	127
Illumination	95	<b>Interaction of photons with materials</b>	127
Detection	96	Photoconductivity	128
Distortion	96	Photovoltaic	128
<b>Evaluation of illumination/detection systems</b>	96	<i>Charge coupled devices</i>	129
Influence of optical elements on the properties of light	96	Photoemissive	130
Errors caused by optical elements	96	<i>Image dissector</i>	130
Evaluation of optical arrangements	97	<i>Micro channel plate</i>	130
Evaluation of scanner arrangements	98	<b>Noise internal to detectors</b>	131
Disk scanners	99	Statistics of photon flux and detectors	132
Object scanners	100	Representing the pixel value	133
Attachment to microscopes	100	<b>Conversion techniques</b>	134
Merit functions	100	<b>Assessment of devices</b>	135
<b>Requirements for multi-fluorescence experiments</b>	101	Point detection optimization	135
<b>Special optical elements</b>	101	Field detection optimization	136
Multi-mode optical glass fibers	101	<b>Detectors present and future</b>	137
Single-mode polarization-preserving glass fibers	101	<b>References</b>	138
Polarizing elements	101		
Mechanical scanners	102		
Acousto-optical scanners	102		
<b>Conclusions</b>	102	<b>CHAPTER 13: MANIPULATION, DISPLAY, AND ANALYSIS OF THREE-DIMENSIONAL BIOLOGICAL IMAGES</b>	<b>141–150</b>
<b>Acknowledgements</b>	103	<i>Hans Chen, John W. Sedat and David A. Agard</i>	
<b>References</b>	103	<b>Introduction</b>	141
		<b>Storage of three-dimensional image data</b>	141
<b>CHAPTER 10: INTERMEDIATE OPTICS IN NIPKOW DISK MICROSCOPES</b>	<b>105–111</b>	<b>Image enhancement</b>	142
<i>Gordon S. Kino</i>		Linear filters	142
<b>The tandem scanning reflected light microscope   (TSRM)</b>	105	Median filters	142
<b>The real-time scanning optical microscope (RSOM)</b>	106	Local contrast enhancement	143
<b>Images of the eye</b>	106	Gradient method	143
<b>Pinhole size</b>	106	<b>Processing methods for displaying 3D data</b>	144
<b>Pinhole spacing</b>	109	Stereo images	144
<b>Illumination efficiency and reflection from the disk</b>	110	3D rotations	144
<b>Internal reflections</b>	110	Rotated projections	145
<b>Acknowledgements</b>	111	Pixar displays	145
<b>References</b>	111	Contour surface representation	145
		<b>Graphic system for 3D image display and analysis</b>	145
<b>CHAPTER 11: THE ROLE OF THE PINHOLE IN CONFOCAL IMAGING SYSTEMS</b>	<b>113–126</b>	Details of PRISM's design and implementation	146
<i>Tony Wilson</i>		The window system	146
<b>Introduction</b>	113	Digital movies	147
<b>The optical sectioning property</b>	113	Choice of display hardware	147
<b>The optical sectioning property with a finite-sized   circular detector and coherent light</b>	113	Model building in PRISM	148
<b>Lateral resolution as a function of effective   detector size</b>	117	Model building	149
<b>The role of aberrations</b>	117	Superimposing the model on a background image	150
<b>Images with a finite-sized detector</b>	118	Future development and discussion	150
<b>Extended-focus and auto-focus imaging with a   finite-sized detector</b>	118	<b>Acknowledgements</b>	150
<b>Height imaging with a finite-sized pinhole</b>	118	<b>References</b>	150
<b>Alternative detector geometries</b>	119		
<b>Noise</b>	121	<b>CHAPTER 14: THREE-DIMENSIONAL IMAGING ON CONFOCAL AND WIDE-FIELD MICROSCOPES</b>	<b>151–161</b>
<b>Fluorescence imaging</b>	123	<i>Walter A. Carrington, Kevin E. Fogarty, Larry Lifschitz, Fredric S. Fay</i>	
<b>Conclusions</b>	125	<b>Introduction</b>	151
<b>References</b>	125	<b>Signal to noise ratio and resolution</b>	151
		Signal strength, photo-damage and photo-bleaching	151
		Optical transfer function, resolution and noise	152
		<b>Three-dimensional image restoration and confocal   microscopy: a comparison</b>	152

Image restoration methodology	153
Requirements	154
Results	154
Multiple detector confocal microscopes	156
Conclusions and recommendations	156
<b>Computer graphics 3D visualization</b>	156
Display of 2D slices of 3D data	156
Volume displays	157
Surface model displays	157
3D perception from 2D displays	157
User interaction and analysis	157
<b>Automated image analysis: feature extraction and computer vision</b>	157
Thresholding	157
Human interaction and partial automation	158
Fully automated analysis	158
<b>Computer hardware considerations</b>	158
Image acquisition	158
Image restoration	159
Image analysis and display	159
Archival storage	159
<i>Magnetic disks</i>	159
<i>Cartridge magnetic tape</i>	159
<i>Reel magnetic tape</i>	159
<i>Optical disk</i>	159
Networking	159
<b>Conclusion</b>	160
<b>References</b>	160

**CHAPTER 15: DIRECT RECORDING OF STEREOSCOPIC PAIRS OBTAINED DIRECTLY FROM DISK SCANNING CONFOCAL LIGHT MICROSCOPES** 163–168  
*Alan Boyde*

<b>Summary</b>	163
<b>Introduction</b>	163
<b>Use of a confocal microscope to reduce the depth of field</b>	163
<b>Optical sectioning in the TSRLM</b>	164
Direct photographic recording of the stereo-pair	164
Means for stereo imaging of a layer inside a bulk	164
<i>Fixed tilt angle difference: hand-operated device</i>	164
<i>DC micromotor-controlled stage</i>	164
<i>Piezo-electric control of the lens</i>	165
Top or bottom overlap?	165
<b>Stereopairs generated from one through-focus pass</b>	165
Topographic mapping	165
Color coding without a computer	165
Depth limitation	166
Particle counting	167
Geometric properties of the stereo images	167
<b>Discussion: TSRLM or LSCM?</b>	167
<b>Acknowledgements</b>	168
<b>References</b>	168

**CHAPTER 16: FLUOROPHORES FOR CONFOCAL MICROSCOPY: PHOTOPHYSICS AND PHOTOCHEMISTRY** 169–178  
*Roger Y. Tsien and Alan Waggoner*

<b>Introduction</b>	169
<b>Photophysical problems related to high intensity excitation</b>	169

Singlet state saturation	169
Triplet state saturation	172
Contaminating background signals	172
<i>Rayleigh and Raman scattering</i>	172
<i>Autofluorescence from endogenous fluorophores</i>	172
<i>What is the optimal intensity?</i>	172
<b>Photodestruction of fluorophores and biological specimens</b>	172
Dependency on intensity or its time integral?	173
<i>Theory</i>	173
<i>Experiment</i>	173
Protective agents	174
<b>Strategies for signal optimization in the face of photobleaching</b>	174
Light collection efficiency	174
Spatial resolution	175
Fluorophore concentration	175
Choice of fluorophore	175
<b>Fluorescent indicators for dynamic intracellular parameters</b>	175
Membrane potentials	175
Ion concentrations	176
<i>Wavelength ratioing</i>	176
<i>pH indicators</i>	176
<i>Ca<sup>2+</sup> indicators</i>	176
<i>Other forms of ratioing</i>	177
<b>Future developments?</b>	177
<b>Acknowledgments</b>	177
<b>References</b>	177

**CHAPTER 17: IMAGE CONTRAST IN CONFOCAL LIGHT MICROSCOPY** 179–195  
*P.C. Cheng and R.G. Summers*

<b>Introduction</b>	179
<b>Sources of contrast</b>	179
<b>Confocal microscopy in back scattered mode</b>	180
Signal formation	180
Backscattered light contrast on stained specimens	181
Reflection contrast on non-biological specimens	182
Backscatter contrast on living specimens	182
The effect of overlying structures	182
Absorption contrast	184
Artificial contrast	184
<b>Transmitted confocal image</b>	186
<b>Confocal microscopy in epi-fluorescent mode</b>	189
Countermeasures	193
<b>Acknowledgement</b>	195
<b>References</b>	196

**CHAPTER 18: GUIDING PRINCIPLES OF SPECIMEN PRESERVATION FOR CONFOCAL FLUORESCENCE MICROSCOPY** 197–205  
*Robert Bacallao, Morgane Bomsel, Ernst H.K. Stelzer and Jan De Mey*

<b>Introduction</b>	197
<b>Critical evaluation of fixation and mounting methods</b>	197
Theoretical considerations	197
The use of the cell height to evaluate the fixation method	198
The use of cell height to evaluate mounting media	199
Well defined structures can be used to evaluate fixation methods	199
Comparison of <i>in vivo</i> labeled cell organelles with immunolabeled cell organelles	200

<b>Fixation methods</b>	201	<b>Optical Sectioning: Experimental and Theoretical</b>	210
Glutaraldehyde fixation	201	Scanning Mirror/Slit System	210
<i>Stock solutions</i>	201	Confocal Pinhole System	211
<i>Preparation of stock solutions</i>	201	Theoretical Comparison of Slit and Pinhole	
<i>Fixation protocol</i>	201	Systems	211
The pH shift/paraformaldehyde fixation	201	<b>A Possible Improvement in Pinhole Confocal</b>	
<i>Stock solutions</i>	201	<b>Systems</b>	212
<i>Preparation of the stock solutions</i>	201	<b>A Possible Improvement in the Slit Scanning</b>	
<i>Fixation protocol</i>	201	<b>System</b>	213
Immunofluorescence staining	202	<b>Examples of Images Obtained with the Divided</b>	
Mounting the specimen	202	<b>Aperture Scanning Slit System</b>	213
<b>General notes</b>	202	<b>Summary Comparison of Slit and Pinhole Confocal</b>	
Labeling samples with two or more probes	203	<b>Systems</b>	213
Ramifications of techniques to preserve the		Slit system	213
specimens		<i>Inherent advantages</i>	213
Conclusion	204	<i>Practical advantages of divided aperture system</i>	
<b>Acknowledgements</b>	204	<i>as described</i>	214
<b>References</b>	204	Single pinhole confocal systems	214
		<i>Inherent advantages</i>	214
		<b>References</b>	214
 <b>CHAPTER 19: A COMPARISON OF VARIOUS</b>			
<b>OPTICAL SECTIONING METHODS: THE</b>			
<b>SCANNING SLIT CONFOCAL MICROSCOPE</b>	207–214	<b>BIBLIOGRAPHY ON CONFOCAL MICROSCOPY</b>	215–227
<i>Charles J. Koester</i>		<i>Robert H. Webb</i>	
<b>Introduction</b>	207		
<b>Non-Confocal Optical Sectioning</b>	207		
<b>Confocal Optical Sectioning</b>	208		
Scanning Mirror/Slit Microscope	209	<b>INDEX</b>	229–231

# Chapter 1

## Foundations of Confocal Scanned Imaging in Light Microscopy

SHINYA INOUÉ

Marine Biological Laboratory, Woods Hole, MA 02543

The preparation of this article was supported in part by NIH grant R37 GM 31617-07 and NSF grant DCB 8518672

Seldom has the introduction of a new instrument generated as instant an excitement among biologists as the laser-scanning confocal microscope. With the new microscope one can slice incredibly clean, thin optical sections out of thick fluorescent specimens; view specimens in planes running parallel to the line of sight; penetrate deep into light-scattering tissues; gain impressive 3-dimensional views at very high resolution; and improve the precision of microphotometry.

While the instruments that engendered such excitement mostly became commercially available in 1987, the optical and electronic theory and the technology that led to this sudden emergence had been brewing for several decades. The development of this microscope stems from several roots, including light microscopy, confocal imaging, video and scanning microscopy, and coherent or laser-illuminated optics (see historic overview in Table 1). In this chapter, I will review these developments as they relate to the principles and use of the confocal microscope, and then end with some general remarks regarding the new microscope.

### LIGHT MICROSCOPY

#### Lateral Resolution

The foundation of light microscopy was established, a century ago, by Ernst Abbe (1873, 1884). He demonstrated how the diffraction of light by the specimen and by the objective lens determined image resolution, defined the conditions needed to design a lens whose resolution was diffraction limited (rather than limited by chromatic and spherical aberrations), and established the role of objective lens and condenser numerical apertures on image resolution (Equation 1). Thus,

$$d = 1.22 \times \lambda_o / (NA_{obj} + NA_{cond}) \quad (1)$$

where  $d$  is the minimum distance that the diffraction images of two points in the specimen can approach each other laterally before they merge and can no longer be resolved as two separate points (in accordance with Rayleigh's criterion for visually resolving two nearly equally bright points).  $d$  is expressed as distance (within the focused plane) in the specimen space;  $\lambda_o$  is the wavelength of light in vacuum; and  $NA_{obj}$  and  $NA_{cond}$  the numerical apertures (NA)s of the objective and condenser lenses respectively. The NA is the product of 'the sine of the half angle of the cone of light either acceptable by the objective lens or emerging from the condenser lens' and 'the refractive indexes of the imbibition medium between the specimen and the objective or condenser lens, respectively.' [The impact of the qual-

ity and NA of the condenser lens on image resolution are considered from a more precise, theoretical standpoint by Zernicke and Hopkins (see Born and Wolf 1980). Their derivations lead to a more complex relationship which is somewhat at variance with Equation (1) or with the alternate Sparrow criterion.]

Using the Rayleigh criterion for resolution, the value for  $d$  equals the radius of the Airy disk, namely the radius of the first minimum of a unit diffraction image. The unit diffraction image is the diffraction pattern (produced in the image plane under the particular conditions of observations) of an infinitely small point in the specimen space.

In addition to the wavelength and NA of the objective and condenser lenses, three additional factors or conditions affect the unit diffraction image and image resolution in the microscope. The first factor is the degree of coherence of the light waves. Equation (1) assumes that one is dealing with points (or periodic objects) in the specimen that emit or scatter light waves whose coherence varies with the condenser NA. For objects that are illuminated fully coherently (a condition that pertains when  $NA_{cond}$  approaches 0, namely when the condenser iris is closed down to a pinhole), the minimum resolvable distance becomes  $2d$ ; in other words, the resolution decreases by a factor of two compared to the case when adjoining specimen points are illuminated incoherently. As the condenser iris is opened up and  $NA_{cond}$  becomes larger, the illumination becomes progressively less coherent. [Note, however, that laser beams tend to illuminate objects coherently even when the condenser iris is not closed down (see "LASERS AND MICROSCOPY").]

The second factor is the field size. Equation (1) holds true only when the field of view is not extremely small. When the field of view is extremely small, as in confocal microscopy, the resolution can in fact be greater than when the field of view is not limited. We shall return to this point later.

The third, and equally important, condition is that the resolution criterion applies only to objective lenses used under conditions in which the image is free from significant aberrations. This implies several things: a well-corrected, clean objective lens is used within the wavelengths of light and diameter of field for which the lens was designed (in many cases in conjunction with specific oculars); the refractive index, dispersion, and thickness of the immersion media and cover slip are those specified for the particular objective lens; the correct tube length and ancillary optics are used and the optics are axially aligned; the full complement of image-forming rays and light waves leaving the objective aperture converge to the image plane without obstruction; the condenser aperture is homogeneously and fully illuminated; and the condenser is properly focused (see Chapters 6, 7, 8, 9, and 11; also Inoué, 1986, Chapter 5).



TABLE 1 HISTORIC OVERVIEW

Confocal Microscopy	Microscopy	Video (Microscopy)
	Abbe (1873, 1884) <sup>a</sup> Berek (1927) <sup>d</sup> Zernicke (1935) <sup>a,c</sup> Gabor (1968) <sup>a</sup> H.H. Hopkins (1951) <sup>a</sup> Linfoot & Wolf (1953) 3-D diffraction by annul. apert. <sup>a,d</sup> Tolardo di Francia (1955) Limited field <sup>b</sup> Nomarski (1955) <sup>c</sup> Linfoot & Wolf (1956) 3-D diffraction pattern <sup>a,d</sup> Ingelstam (1956) Resolution and info. theory <sup>a</sup>	Nipkow (1884)  Zworykin (1934)  Flory (1951) Young/Roberts (1951) Flying spot <sup>c</sup> Montgomery et al (1956) Flying spot UV <sup>c</sup>
Minsky Patent (1957) Insight <sup>a,b,c,d</sup> Stage scanning <sup>f</sup>	Kubota & Inoué (1959) <sup>a</sup> Smith & Osterberg (1961) <sup>a</sup> Harris (1964) <sup>a,b</sup>	Freed & Engle (1962) Flying spot <sup>c</sup>
Petráň et al (1968) Tandem scanning <sup>d</sup> Davidovits & Egger (1971) Laser illumination, Lens scan <sup>d</sup>	Ellis (1966) Holomicrography <sup>c,d</sup>  Hoffman & Gross (1975) Modulation contrast <sup>c</sup>	
Sheppard & Choudhury (1977) Theory <sup>a,b,d</sup> Sheppard et al (1978) Stage scanning	Ellis (1978) Single sideband edge enhancement microscopy <sup>c</sup>	
Brakenhoff et al (1979) Specimen scan <sup>e</sup> (1985) Koester (1980) Scanning mirror <sup>d</sup>	Quate (1980) Acoustic microscopy <sup>a,c</sup>	Castleman (1979) Digital image processing <sup>a,c,e</sup>
Boyde (1985a) Nipkow type <sup>d,e</sup> Cox & Sheppard (1983) Digital recording <sup>d,e</sup> Åslund et al (1983) 2-mirror laser scanning <sup>d</sup> Hamilton et al (1984) Differential phase <sup>c,d</sup> Wilson & Sheppard (1984) Extended depth of field <sup>c,d,e,f</sup> Carlsson et al (1985) Laser scan Stacks of confocal images <sup>d,e</sup> Wijnaendts van Resandt et al (1985) x-z view <sup>d,e</sup> Suzuki-Horikawa (1986) Video rate laser scan, Acousto optical modulator, No exit pinhole Xiao & Kino (1987) Nipkow type <sup>d</sup> McCarthy & Walker (1988) Nipkow type <sup>d</sup> Amos et al (1987) <sup>c,d,e</sup>		Inoué (1981) <sup>a,c</sup> Allen et al (1981a,b) <sup>a,c</sup> Fuchs et al (1982) <sup>f</sup> Agard & Sedat (1983) <sup>a,c,d</sup>
	Ellis (1985) Light scrambler <sup>d</sup>  Cox & Sheppard (1986) <sup>b</sup>	Sher & Barry (1985) <sup>f</sup>  Inoué (1986) Overview, how to <sup>a,c,c,f</sup>  Fay et al (1985) <sup>a,c,d</sup>  Castleman (1987) <sup>a</sup>
	Ellis (1988) Scanned aperture phase contrast <sup>a,c,d</sup>	

a—Diffraction theory; b—Superresolution; c—Contrast modes; d—Optical sectioning/depth of field; e—Stereo; f—3-D in objective space



## Axial Resolution

We now turn to the axial (z-axis) resolution, measured along the optical axis of the microscope, i.e., perpendicular to the plane of focus.

In the case of lateral resolution, i.e., the resolution in the plane of focus, we defined resolution in terms of the minimum distance that the diffraction images of two point sources in the specimen could approach each other and still visually be distinguished as two. Using the Rayleigh criterion, this minimum distance equaled the radius of the first minimum of the unit diffraction image.

Similarly, axial resolution can be defined using two criteria, either the minimum distance that the diffraction images of two points can approach each other along the axis of the microscope and still be seen as two, or by the radius of the first minimum of the diffraction image of an infinitely small point object. I shall now expand on the latter point.

The precise distribution of energy in the image-forming light above and below focus, especially for high NA objective lenses, cannot be deduced by geometric ray tracing, but must be derived from wave optics. The wave optical studies of Linfoot and Wolf (1956) show that the image of a point source produced by a diffraction-limited optical system (such as a properly constructed and used microscope) is not only periodic around the point of focus in the focal plane, but is also periodic above and below the focal plane along the axis of the microscope. [Such 3-dimensional diffraction images (including those produced in the presence of lens aberrations) are presented photographically by Cagnet et al. (1962). The intensity distribution calculated by Linfoot and Wolf for an aberration-free system is reproduced in Born and Wolf (1980); also in Inoué (1986, Fig. 5–21). The 3-dimensional pattern of a point source formed by a lens possessing an annular aperture was calculated by Linfoot and Wolf (1953).]

Near the plane of focus, the axial magnification of the microscope rises as the square of the lateral magnification, and the distance from the center of the diffraction image to the first minimum along the microscope axis turns out to be approximately twice as far as it is to the first minimum in the plane of focus (again both translated into dimensions measured in the specimen space). The central zone of the unit diffraction image is thus stretched along the z-axis of the microscope like an American football, whose major radius is twice as long as its minor radius.

For an incoherent source or scatterer, the cross section through the middle of the football (transverse to its axis of elongation) yields the familiar Airy disk. Therefore, the minimum distance that one can resolve axially is twice as large as the minimum distance that one can resolve transversely. In other words, the axial resolving power is approximately one half of the lateral resolving power.

The axial resolution of the microscope also gives rise to its “axial setting accuracy.” The axial setting accuracy is defined as the distance that one has to shift the fine focus of the microscope before the image of an infinitely thin object changes perceptibly. According to Françon (1961), the axial setting accuracy ( $2\zeta$ ) is given by:

$$2\zeta = \lambda / \{4n \times \sin^2(u/2)\} \quad (2)$$

where,  $\lambda$  is the wavelength of light,  $n$  the refractive index of the immersion medium, and  $u$  the half angle of the cone of light that is captured by the objective lens. Françon points out that

measurements of the axial setting accuracy is influenced by several factors, and that a value as small as  $2\zeta$  is seldom achieved in practice.

Regardless of whose equation is chosen for calculating the axial resolution, it is important to note that the axial resolution (and the related axial-setting accuracy and the shallowness of depth of field) rises with the square of the NA, in contrast to the lateral resolution which rises with the first power of the NA [cf. Eqs. 2, 1].

## Depth of Field

The depth of field of a microscope is the depth of the image (measured along the microscope axis translated into distances in the specimen space) that appears to be sharply in focus at one setting of the fine focus adjustment. In bright field microscopy, this depth should be approximately equal to the axial resolution, at least in theory. The actual depth of field has been measured, and the contribution of various factors that affect the measurement have been explored by Berek (1927).

According to Berek, the depth of field is affected by several factors, including (a) the geometric spreading, above and below the plane of focus, of the light beam that arose from a single point in the specimen; (b) accommodation of the observer's eye; and (c) the final magnification of the image. The second factor becomes irrelevant when the image is not viewed directly through the ocular but is instead focused on a thin detector (in the absence of an auto-focusing device). The third factor should also disappear once the total magnification is raised sufficiently so that the unit diffraction image becomes significantly larger than the resolution unit of the detector (see e.g., Castleman, 1987; Hansen in Inoué, 1986).

Many other authors have calculated the depth of field, but unlike Equation (1) that specifies the lateral resolution, no equation is generally accepted as specifying the depth of field. One reason that several equations have been proposed for the depth of field is that different criteria have been used for what is “in focus.”

In conventional fluorescence and dark field microscopy, the light that makes up each point of the image spreads in a solid cone that can reach a significant distance above and below focus (as seen in the point spread functions for these modes of microscopy; see e.g., Streibl, 1985, and also Chapters 8, 9, 10, 11, 13 and 14). The spreading cone of light blurs the focused image of the specimen. Also, fluorescent (or light-scattering) objects that are out of focus can inject unwanted light and further reduce the contrast of the specimen region in focus.

For these reasons, the depth of field may be difficult to measure or even to define precisely in fluorescence and dark field microscopy. Or, one could say that the apparent depth of field is very much greater than the axial resolution when objects that are not infinitely thin are observed in conventional fluorescence and dark-field microscopy.

The unwanted light that expands the apparent depth of field is exactly what confocal imaging eliminates. Thus we can view only those fluorescent and light-scattering objects that lie within the depth that is given by the axial resolution of the microscope and attain the desired shallow depth of field.

As mentioned earlier, the lateral resolution of a microscope is a function of the size of the field (observed at any one instant). Tolardo di Francia (1955) suggested, and Ingelstam (1956) argued on the basis of information theory that one gains lateral