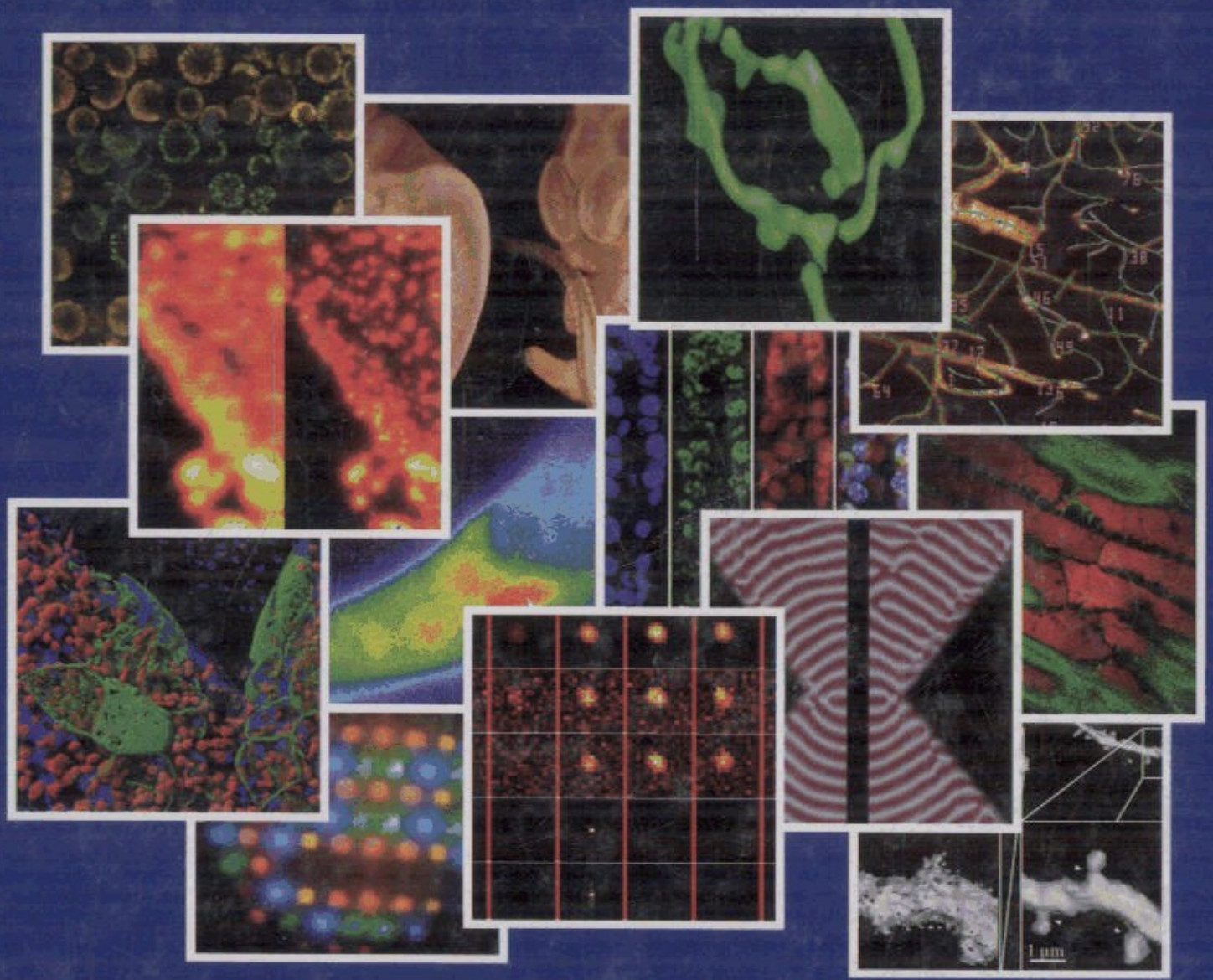


HANDBOOK OF
**BIOLOGICAL CONFOCAL
MICROSCOPY**

THIRD EDITION



James B. Pawley

Editor

Contents

Preface to the Third Edition	vii	Resolution: How Much Is Enough?	36
Preface to the Second Edition	ix	Can Resolution Be Too High?	36
Contributors	xxv	Limitations Imposed by Spatial and Temporal Quantization	37
		Practical Considerations Relating Resolution to Distortion	39
		Conclusion	41
CHAPTER 1: FOUNDATIONS OF CONFOCAL SCANNED IMAGING IN LIGHT MICROSCOPY		CHAPTER 3: SPECIAL OPTICAL ELEMENTS	
<i>Shinya Inoué</i>		<i>Jens Rietdorf and Ernst H.K. Stelzer</i>	
Light Microscopy	1	Introduction	43
Lateral Resolution	1	Regulating the Intensity	43
Axial Resolution	3	Wavelength Selective Filtering Devices	43
Depth of Field	4	Selecting the Wavelength of the Illumination and the Detected Light	44
Confocal Imaging	4	Separating the Light Paths	44
Impact of Video	5	Conventional Filters	45
Nipkow Disk	5	Interference Filters	45
Electron-Beam-Scanning Television	6	Dichroic and Polarizing Beam-Splitters	50
Impact of Modern Video	7	Filters and Dispersive Elements for Multi-Channel Detection	51
Lasers and Microscopy	7	Mechanical Scanners	51
Holography	7	Galvanometer Scanners	52
Laser Illumination	8	General Specifications	54
Laser-Illuminated Confocal Microscopes	9	Acousto-Optical Components	54
Confocal Laser-Scanning Microscope	9	Acousto-Optical Deflectors	56
Two- and Multi-Photon Microscopy	10	Acousto-Optical Modulators	56
Is Laser-Scanning Confocal Microscopy a Cure-All?	11	Acousto-Optical Tunable Filters	56
Speed of Image or Data Acquisition	11	Acousto-Optical Beam-Splitters	56
Yokogawa Disk-Scanning Confocal System	12	Electro-Optical Modulators	57
Depth of Field in Phase-Dependent Imaging	13	Piezoelectric Scanners	57
Other Optical and Mechanical Factors Affecting Confocal Microscopy	13	Polarizing Elements	57
Lens Aberration	13	Removing Excess Light	58
Unintentional Beam Deviation	15		
Contrast Transfer and Resolution in Confocal Versus Non-Confocal Microscopy	16	CHAPTER 4: POINTS, PIXELS, AND GRAY LEVELS: DIGITIZING IMAGE DATA	
Summary	16	<i>James B. Pawley</i>	
		Contrast Transfer Function, Points, and Pixels	59
		Pixels, Images, and the Contrast Transfer Function ...	59
		Digitization and Pixels	62
		Digitization of Images	62
		How Big Should a Pixel Be? Sampling and Quantum Noise	63
		The Nyquist Criterion	64
		Estimating the Expected Resolution of an Image ...	65
		The Story So Far	68
		Reality Check?	68
		Is Over-Sampling Ever Wise?	68
		Under-Sampling?	68
		Digitizing Trade-Offs	68
CHAPTER 2: FUNDAMENTAL LIMITS IN CONFOCAL MICROSCOPY			
<i>James B. Pawley</i>			
Introduction	20		
What Limits?	20		
Counting Statistics: The Importance of n	20		
Source Brightness	21		
Specimen Response: Dye Saturation	21		
A Typical Problem	24		
Practical Photon Efficiency	24		
Losses in the Optical System	25		
Detection and Measurement Losses	28		
Where Have All the Photons Gone?	33		

Nyquist Reconstruction: "Deconvolution Lite"	68	Modulated Diode Lasers	112
Some Special Cases	70	Diode Pumped Solid State Laser in Pulsed Mode	112
Gray Levels, "Noise," and Photodetector		Ultrafast Diode Pumped Solid State Lasers	112
Performance	71	Titanium-Sapphire and Related Ultrafast Lasers	112
Optical Density	71	White Light Continuum Lasers	113
The Zone System: Quantified Photography	71	Ultrafast Fiber Lasers	113
Linearity: Do We Need It?	72	Wavelength Expansion Through Non-Linear	
Gray Levels in Images Recorded Using		Techniques	114
Charge-Coupled Devices: The Intensity Spread		Second and Higher Harmonic Generation: SHG,	
Function	74	THG, FHG Label-Free Microscopy	114
What Counts as Noise?	74	Sum or Difference Mixing	114
Measuring the Intensity Spread Function	75	Optical Parametric Oscillators and Optical Parametric	
Calibrating a Charge-Coupled Device to Measure		Amplifiers	114
the ISF	75	Pulse Length Measurement	115
"Fixed-Pattern" Noise	76	Maintenance	115
Gain-Register Charge-Coupled Devices	76	Maintenance of Active Laser Media	115
Multiplicative Noise	77	Maintenance of Pumping Media	116
Trade-Offs	79	Maintenance of the Optical Resonator	116
		Maintenance of Other System Components	116
		Troubleshooting	117
CHAPTER 5: LASER SOURCES FOR		Safety Precautions	117
CONFOCAL MICROSCOPY		Beam Stops	118
<i>Enrico Gratton and Martin J. vandeVen</i>		Curtains	118
Introduction	80	Laser Goggles	118
Laser Power Requirements	80	Screens	118
The Basic Laser	81	Exposure Effects, Warning Signs, and Interlocks	118
Principle of Operation	82	Infrared Paper	118
Pumping Power Requirements	82	Conclusion	118
Laser Modes: Longitudinal (Axial) and			
Transverse	82	CHAPTER 6: NON-LASER LIGHT SOURCES	
Polarization	83	FOR THREE-DIMENSIONAL MICROSCOPY	
Coherent Properties of Laser Light	83	<i>Andreas Nolte, James B. Pawley, and Lutz Höring</i>	
Phase Randomization: Scrambling the Coherence		Introduction	126
Properties of Laser Light	84	General Remarks on Choice of Excitation Light	
Measures to Reduce the Coherence Length of		Sources	126
Laser Light	84	Scrambling and Filtering the Light	131
Heat Removal	84	Types of Sources and Their Features	132
Other Installation Requirements	85	Structure	132
Attenuation of Laser Beams	85	Wavelength	135
Stabilization of Intensity, Wavelength, and Beam		Stability in Time and Wavelength	136
Position in Lasers	85	Radiance	137
Sources of Noise in Lasers	85	Control	138
Spatial Beam Characteristics	89	Measuring What Comes Through the	
Laser Requirements for Biological Confocal Laser		Illumination System	139
Scanning Microscopy-Related Techniques	89	The Bare Minimum	139
Optical Tweezers	89	Types of Confocal Microscopes That Can Use	
Total Internal Reflection Microscopy	89	Non-Laser Light Sources	141
Confocal Raman Confocal Laser Scanning Microscopy		Tandem Scanning: Basic Description	141
for Chemical Imaging	90	Single-Sided Disk Scanning: Basic Description	141
Non-Linear Confocal Microscopy	90	Exposure Time and Source Brightness	141
Nanosurgery and Microdissection	90	Future Trends	143
Types of Lasers	90		
Continuous Wave Lasers	90	CHAPTER 7: OBJECTIVE LENSES FOR	
Gas Lasers	90	CONFOCAL MICROSCOPY	
Dye Lasers	103	<i>H. Ernst Keller</i>	
Solid-State Lasers	103	Introduction	145
Thin Disk Lasers	109	Aberrations of Refractive Systems	146
Pulsed Lasers	110	Defocusing	146
Classification of Pulsed Laser Systems	111	Monochromatic Aberrations	147
Nitrogen Lasers	112	Chromatic Aberrations	152
Excimer Lasers	112		
Metal Vapor Lasers	112		
Dye Lasers	112		

Finite Versus Infinity Optics 156
 Working Distance 157
 Optical Materials 158
 Anti-Reflection Coatings 158
 Transmission of Microscope Objectives 158
 Conclusion 160

**CHAPTER 8: THE CONTRAST FORMATION
 IN OPTICAL MICROSCOPY**

Ping-Chin Cheng

Introduction 162
Sources of Contrast 163
 Absorption Contrast 163
 Scattering and Reflection Contrast 167
 Phase Contrast 171
 Fluorescence Contrast 172
 Contrast Related to Excitation Wavelength
 Change 173
 Negative Contrast 173
 Special Concerns in Ultraviolet and Near-Infrared
 Range Confocal Microscopy 174
 Total Internal Reflection Contrast 177
 Harmonic Generation Contrast 179
Geometric Contrast 180
 z-Contrast in Confocal Microscopy 180
 Total Internal Refraction Fluorescence Contrast 180
 Fluorescence Resonant Energy Transfer 184
 Fluorescence Recovery After Photobleaching
 (FRAP and FLIP) 187
Structural Contrast 188
 Harmonic Generation Contrast 188
 Birefringence Contrast 188
Derived Contrast (Synthetic Contrast) 188
 Ratiometric 189
 Deconvolution 189
 Movement Contrast (Subtraction of Previous
 Image) 190
 Spectral Unmixing and Color Reassignment 190
 Effects of the Specimen: Spherical Aberration and
 Optical Heterogeneity 192
 Mounting Medium Selection 198
Artificial Contrast 201
 Contrast Resulting from Instrument Vibration and
 Ambient Lighting 201
 Contrast Resulting from Interference of Cover
 Glass Surfaces 201
 Background Level and Ghost Images from the
 Transmission Illuminator 201
 Contrast Resulting from Differences in
 Photobleaching Dynamics 202
 Effect of Spectral Leakage and Signal Imbalance
 Between Different Channels 203
**New Contrasts: Fluorescence Lifetime and Coherent
 Antistokes Raman Spectroscopy** 204
Summary 204

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

Ernst H.K. Stelzer

Introduction 207
Design Principles 207

Overview 207
 Telecentricity 207
 The Scanning System 208
 The Back-Focal Planes 210
Practical Requirements 210
 Diffraction Limit 210
 Geometric Distortion 211
**Evaluation of the Illumination and Detection
 Systems** 211
 Influence of Optical Elements 211
 Errors 211
 Evaluation of Optical Arrangements 212
 Evaluation of Scanner Arrangements 213
 Scanners 215
 Attachment to Microscopes 217
 Merit Functions 217
Multi-Fluorescence 217
Special Setups 218
 Setups for Fluorescence Recovery After
 Photobleaching Experiments 218
 Setups for Fluorescence Resonance Energy Transfer
 Experiments 218
 Setups for the Integration of Optical Tweezers 218
 Setups for the Integration of Laser Cutters 218
 Setups for the Observation of Living Specimens 219
Miniaturization and Computer Control 219
Thermal Stability 219
Vibration Isolation 219
Conclusions and Future Prospects 219

**CHAPTER 10: DISK-SCANNING CONFOCAL
 MICROSCOPY**

Derek Toomre and James B. Pawley

Introduction 221
Background 221
 Living Cell Imaging: Probing the Future 221
 A Need for Speed and Less Photobleaching 222
 Advantages and Limitations of Confocal
 Laser-Scanning Microscopes 222
 Other Imaging and Deconvolution 223
Confocal Disk-Scanning Microscopy 223
 Nipkow Disk — An Innovation 223
 A Renaissance — Advantages of Disk-Scanning
 Confocal Imaging 223
 Disadvantages 224
Critical Parameters in Pinhole and Slit Disks 224
 Fill Factor and Spacing Interval F 224
 Lateral Resolution 225
 Pinhole/Slit Size 225
 Axial Resolution 225
Types of Disk-Scanning Confocals 228
 General Considerations 228
 Disk Scanners for Backscattered Light Imaging 228
 CARV, DSU, and Other Disk-Scanning Confocal
 Microscopes 229
 The Yokogawa Microlens — An Illuminating
 Approach 231
 New Fast Slit Scanner — Zeiss LSM510 LIVE 231
New Detectors — A Critical Component 232
 Image Intensifiers 232
 On-Chip Electron Multiplying Charge-Coupled
 Device 233

Electron Multiplication Charge-Coupled Devices and Disk Scanners	234
Applications and Examples of Confocal Disk-Scanning Microscopes	235
Comparison with Epi-Fluorescence Imaging	235
Fast 3D/4D Imaging	235
Blazingly Fast Confocal Imaging	235
Future Developments?	236
Summary	237

CHAPTER 11: MEASURING THE REAL POINT SPREAD FUNCTION OF HIGH NUMERICAL APERTURE MICROSCOPE OBJECTIVE LENSES

Rimas Juškaitis

Introduction	239
Measuring Point Spread Function	240
Fiber-Optic Interferometer	240
Point Spread Function Measurements	241
Chromatic Aberrations	242
Apparatus	243
Axial Shift	243
Pupil Function	245
Phase-Shifting Interferometry	245
Zernike Polynomial Fit	245
Restoration of a 3D Point Spread Function	247
Empty Aperture	248
Miscellanea	248
Temperature Variations	248
Polarization Effects	249
Apodization	250
Conclusion	250

CHAPTER 12: PHOTON DETECTORS FOR CONFOCAL MICROSCOPY

Jonathan Art

Introduction	251
The Quantal Nature of Light	251
Interaction of Photons with Materials	252
Thermal Effects	252
Direct Effects	252
Photoconductivity	252
Photovoltaic	252
Photoemissive	254
Comparison of Detectors	255
Noise Internal to Detectors	256
Noise in Internal Detectors	256
Noise in Photoemissive Devices	256
Statistics of Photon Flux and Detectors	257
Representing the Pixel Value	258
Conversion Techniques	259
Assessment of Devices	260
Point Detection Assessment and Optimization	260
Field Detection Assessment and Optimization	261
Detectors Present and Future	262

CHAPTER 13: STRUCTURED ILLUMINATION METHODS

Rainer Heintzmann

Introduction	265
-------------------------------	------------

Experimental Considerations	265
Pattern Generation	266
Computing Optical Sections from Structured-Illumination Data	268
Resolution Improvement by Structured Illumination	270
Nonlinear Structured Illumination	276
Summary	276

CHAPTER 14: VISUALIZATION SYSTEMS FOR MULTI-DIMENSIONAL MICROSCOPY IMAGES

N.S. White

Introduction	280
Definitions	280
What Is the Microscopist Trying to Achieve?	280
Criteria for Choosing a Visualization System	281
Why Do We Want to Visualize Multi-Dimensional Laser-Scanning Microscopy Data?	281
Data and Dimensional Reduction	281
Objective or Subjective Visualization?	281
Prefiltering	281
Identifying Unknown Structures	281
Highlighting Previously Elucidated Structures	284
Visualization for Multi-Dimensional Measurements	284
What Confocal Laser Scanning Microscopy Images Can the Visualization System Handle?	286
Image Data: How Are Image Values Represented in the Program?	286
What Dimensions Can the Images and Views Have?	286
Standard File Formats for Calibration and Interpretation	288
How Will the System Generate the Reconstructed Views?	290
Assessing the Four Basic Steps in the Generation of Reconstructed Views	290
Loading the Image Subregion	290
Choosing a View: The 5D Image Display Space	291
Mapping the Image Space into the Display Space	294
How Do 3D Visualizations Retain the z-Information?	296
Mapping the Data Values into the Display	300
How Can Intensities Be Used to Retain z-Information?	304
Hidden-Object Removal	304
Adding Realism to the View	306
How Can I Make Measurements Using the Reconstructed Views?	312
Conclusion	312

CHAPTER 15: AUTOMATED THREE-DIMENSIONAL IMAGE ANALYSIS METHODS FOR CONFOCAL MICROSCOPY

Badrinath Roysam, Gang Lin, Muhammad-Amri Abdul-Karim, Omar Al-Kofahi, Khalid Al-Kofahi, William Shain, Donald H. Szarowsk, and James N. Turner

Introduction	316
Types of Automated Image Analysis Studies	318

Common Types of Biological Image Objects	319	Oxygen Sensor	347
Specimen Preparation and Image Preprocessing		cAMP Indicators	347
Methods	319	Fatty Acid Indicator	347
Data Collection Guidelines for Image Analysis		Genetically Expressed Intracellular Fluorescent	
Purposes	319	Indicators	348
Image Preprocessing Methods	320	Green Fluorescent Protein	348
General Segmentation Methods Applicable to		Ligand-Binding Modules	348
Confocal Data	321	Ion Indicators	348
Bottom-Up Segmentation Methods	321	Future Developments	348
Top-Down Segmentation Methods	322		
Hybrid Segmentation Methods Combining Bottom-Up			
and Top-Down Processing	322		
Example Illustrating Blob Segmentation	322	CHAPTER 17: PRACTICAL CONSIDERATIONS IN	
Model-Based Object Merging	323	THE SELECTION AND APPLICATION OF	
Example Illustrating Segmentation of Tube-Like		FLUORESCENT PROBES	
Objects	324	<i>Iain D. Johnson</i>	
Skeletonization Methods	324	Introduction	353
Vectorization Methods	324	Selection Criteria for Dyes and Probes	353
Example Combining Tube and Blob		Organic Dyes	353
Segmentation	328	Fluorescent Proteins: Green Fluorescent Protein and	
Registration and Montage Synthesis Methods	328	Phycobiliproteins	356
Methods for Quantitative Morphometry	331	Quantum Dots	357
Methods for Validating the Segmentation and		Multi-Photon Excitation	357
Making Corrections	333	Introducing the Probe to the Specimen	358
Analysis of Morphometric Data	334	Loading Methods	358
Discussion, Conclusion, and Future Directions	335	Target Abundance and Autofluorescence	
		Considerations	360
CHAPTER 16: FLUOROPHORES FOR CONFOCAL		Interactions of Probes and Specimens	361
MICROSCOPY: PHOTOPHYSICS AND		Localization and Metabolism	361
PHOTOCHEMISTRY		Perturbation and Cytotoxicity	362
<i>Roger Y. Tsien, Lauren Ernst, and Alan Waggoner</i>		Under the Microscope	362
Introduction	338	Photobleaching	362
Photophysical Problems Related to High Intensity		Phototoxicity	363
Excitation	338	Summary	364
Singlet State Saturation	338		
Triplet State Saturation	339		
Contaminating Background Signals	339	CHAPTER 18: GUIDING PRINCIPLES OF	
What Is the Optimal Intensity?	340	SPECIMEN PRESERVATION FOR CONFOCAL	
Photodestruction of Fluorophores and Biological		FLUORESCENCE MICROSCOPY	
Specimens	340	<i>Robert Bacallao, Sadaf Sohrab, and Carrie Phillips</i>	
Dependency on Intensity or Its Time Integral?	340	Introduction	368
Strategies for Signal Optimization in the Face of		Characteristics of Fixatives	368
Photobleaching	341	Glutaraldehyde	369
Light Collection Efficiency	341	Formaldehyde	369
Spatial Resolution	341	Fixation Staining and Mounting Methods	370
Protective Agents	341	Glutaraldehyde Fixation	370
Fluorophore Concentration	342	pH Shift/Formaldehyde Fixation	370
Choice of Fluorophore	342	Immunofluorescence Staining	371
Fluorescent Labels for Antibodies, Other Proteins,		Mounting the Specimen	371
and DNA Probes	342	Critical Evaluation of Light Microscopy Fixation and	
Fluorescent Organic Dyes	342	Mounting Methods	371
Phycobiliproteins	343	Use of the Cell Height to Evaluate the	
DNA Probes	343	Fixation Method	372
Luminescent Nanocrystals	343	Use of Cell Height to Evaluate Mounting	
Fluorescent Lanthanide Chelates	345	Media	373
Fluorescent Indicators for Dynamic Intracellular		Well-Defined Structures Can Be Used to Evaluate	
Parameters	346	Fixation Methods	373
Membrane Potentials	346	Comparison of <i>In Vivo</i> Labeled Cell Organelles with	
Ion Concentrations	346	Immunolabeled Cell Organelles	374
pH Indicators	346	General Notes	374
Ca ²⁺ Indicators	346	Labeling Samples with Two or More Probes	375

Triple Labeling	375	CHAPTER 20: ABBERATIONS IN CONFOCAL AND MULTI-PHOTON FLUORESCENCE MICROSCOPY INDUCED BY REFRACTIVE INDEX MISMATCH	
Preparation of Tissue Specimens	376	<i>Alexander Egner and Stefan W. Hell</i>	
Labeling Thick Sections	376	Introduction	404
Refractive Index Mismatch	377	The Situation	404
Screening Antibodies on Glutaraldehyde-Fixed Specimens	377	Theory	404
Microwave Fixation	377	Results of Theoretical Calculations	407
Conclusion	378	Experiments	409
		Other Considerations	410
		Dry Objectives	410
		Refractive Index, Wavelength, and Temperature	411
		Spherical Aberration Correction	411
		Conclusion	412
		Consequences	412
		Practical Strategies to Reduce Refractive Index Mismatch	412
CHAPTER 19: CONFOCAL MICROSCOPY OF LIVING CELLS		CHAPTER 21: INTERACTION OF LIGHT WITH BOTANICAL SPECIMENS	
<i>Michael E. Dailey, Erik Manders, David R. Soll, and Mark Terasaki</i>		<i>Ping-Chin Cheng</i>	
Introduction	381	Introduction	414
Overview of Living-Cell Confocal Imaging		Light Attenuation in Plant Tissue	414
Techniques	382	Linear Absorption	414
Time-Lapse Fluorescence Imaging	382	Nonlinear Absorption	416
Multi-Channel Time-Lapse Fluorescence Imaging	382	Scattering	417
Spectral Imaging and Linear Unmixing	382	Refractive Index Heterogeneity	418
Fluorescence Recovery After Photobleaching	382	Birefringent Structures in Plant Cells	420
Fluorescence Loss in Photobleaching	382	Fluorescence Properties of Plants	421
Fluorescence Resonance Energy Transfer	382	Changes in Emission Spectra Depending on One- Versus Two-Photon Excitation	421
Fluorescence Lifetime Imaging	382	Microspectroscopy	421
Fluorescence Correlation Spectroscopy	383	Light-Specimen Interaction (Fluorescence Emission)	425
Fluorescence Speckle Microscopy	383	Harmonic Generation Properties	428
Photo-Uncaging/Photoactivation	383	The Effect of Fixation on the Optical Properties of Plants	428
Optical Tweezers/Laser Trapping	383	Living Plant Cells	429
Physiological Fluorescence Imaging	383	Callus, Suspension Culture Cells and Protoplasts	429
Combining Fluorescence and Other Imaging Modalities	383	Meristem	430
General Considerations for Confocal Microscopy of Living Cells	386	Stem and Root	430
Maintenance of Living Cells and Tissue Preparations	387	Microspores and Pollen Grains	431
Fluorescent Probes	387	Cuticles, Hairs, and Waxes	434
Minimizing Photodynamic Damage	389	Storage Structures	435
The Online Confocal Community	390	Mineral Deposits	436
A Convenient Test Specimen	390	Primary and Secondary Cell Walls	438
Specific Example I: Visualizing Chromatin Dynamics Using Very Low Light Levels	390	Fungi	438
Phototoxicity	390	Conclusion	439
Reduction of Phototoxicity	391		
Improving Image Quality in Low-Dose Microscopy	391	CHAPTER 22: SIGNAL-TO-NOISE RATIO IN CONFOCAL MICROSCOPES	
Low-Dose Imaging Conclusion	391	<i>Colin J.R. Sheppard, Xiaosong Gan, Min Gu, and Maitreyee Roy</i>	
Specific Example II: Multi-Dimensional Imaging of Microglial Cell Behaviors in Live Rodent Brain Slices	392	Introduction	442
Preparation of Central Nervous System Tissue Slices	393	Sources of Noise	442
Fluorescent Staining	393	Shot Noise and Quantum Efficiency	442
Maintaining Tissue Health on the Microscope Stage	393	Background Noise	443
Imaging Methods	394		
Imaging Deep Within Tissue	395		
Keeping Cells in Focus	395		
Handling the Data	395		
Results	396		
Conclusion	396		
Future Directions	398		

Signal Level in Confocal Microscopes 444

Signal-to-Noise Ratio for Confocal
Microscopes 445

Q_E , N1, and Stain Level 445

 N2 and Detectability 446

 Multi-Photon Fluorescence Microscopy 447

Designs of Confocal Microscopes 447

Sampling 448

Comparative Performance of Fluorescence
Microscopes 448

 Bleaching-Limited Performance 448

 Saturation-Limited Performance 450

 Effects of Scanning Speed 450

 3D Imaging 451

Summary 451

**CHAPTER 23: COMPARISON OF
WIDEFIELD/DECONVOLUTION AND
CONFOCAL MICROSCOPY FOR THREE-
DIMENSIONAL IMAGING**

Peter J. Shaw

Introduction 453

The Point Spread Function: Imaging as a
Convolution 453

 Limits to Linearity and Shift Invariance 457

Deconvolution 457

Practical Differences 458

 Temporal Resolution 458

 Combination of Charged-Coupled Device and Confocal
 Imaging 458

 Integration of Fluorescence Intensity 459

Resolution, Sensitivity, and Noise 459

 Fluorescence Excitation 459

 Fluorescent Light Detection 459

 Gain Register Charge-Coupled Devices 460

 Out-of-Focus Light 461

 Model Specimens 461

 The Best Solution: Deconvolving Confocal Data 461

Practical Comparisons 463

Conclusion 466

Summary 467

CHAPTER 24: BLIND DECONVOLUTION

Timothy J. Holmes, David Biggs, and Asad Abu-Tarif

Introduction 468

 Purposes of Deconvolution 468

 Advantages and Limitations 468

Principles 472

 Data Collection Model 472

Maximum Likelihood Estimation 472

 Algorithms 472

Different Approaches 475

 3D 475

 2D Image Filtering 476

Data Corrections 477

Light Source and Optics Alignment 477

Newest Developments 478

 Subpixel 478

 Polarized Light 479

 Live Imaging 480

More Examples 480

 Blind Deconvolution and Spherical Aberration 480

 Widefield Fluorescence Simulation 481

 Spinning-Disk Confocal 481

 Two Photon 481

Speed 482

Future Directions 483

Summary of Main Points 483

**CHAPTER 25: IMAGE ENHANCEMENT BY
DECONVOLUTION**

Mark B. Cannell, Angus McMorland, and Christian Soeller

Introduction 488

Background 488

Image Formation 489

Forwards: Convolution and the Imaging System 490

Properties of the Point Spread Function 492

Quantifying the Point Spread Function 492

The Missing Cone Problem 494

Noise 495

Deconvolution Algorithms 495

 Nearest-Neighbor Deconvolution 495

 Wiener Filtering 496

 Nonlinear Constrained Iterative Deconvolution
 Algorithms 496

 Comparison of Methods 497

**CHAPTER 26: FIBER-OPTICS IN SCANNING
OPTICAL MICROSCOPY**

Peter Delaney and Martin Harris

Introduction 501

Key Fiber Technologies Relevant to Scanning
Microscopy 501

 Glass Made from Gas and Its Transmission
 Properties 501

 Step Index and Gradient Index Optical Fibers 501

 Modes in Optical Fibers 502

 Evanescent Wave and Polarization Effects in
 Optical Fibers 503

 Polarization-Maintaining Fibers 503

 Fused Biconical Taper Couplers: Fiber-Optic
 Beam-Splitters 503

 Microstructure Fibers 504

 Fiber Image Transfer Bundles 504

Key Functions of Fibers in Optical Microscopes 505

 Optical Fiber for Delivering Light 505

 Optical Fiber as a Detection Aperture 506

 Same Fiber for Both Source and Confocal
 Detection 506

 Fiber Delivery for Nonlinear Microscopy with
 Femtosecond Lasers 507

 Large Core Fibers as Source or Detection
 Apertures 507

Benchtop Scanning Microscopes Exploiting Fiber
Components 507

Miniaturized Scanning Confocal Microscope
Imaging Heads 508

 Miniature Confocal Imaging Heads Based on
 Coherent Imaging Bundles 508

Resolution and Optical Efficiency of Bundles	509	Chromophores (Fluorophores and Caged	
Bundle Imagers for <i>In Vivo</i> Studies in Animals	509	Compounds)	543
Scan Heads Based on Single Fibers with Miniature		Two-Photon Absorption Cross-Sections	543
Scanning Mechanisms	510	Caged Compounds	544
Vibrating the Fiber Tip	510	Cell Viability During Imaging	544
Vibrating the Lens and Fiber	510	Applications	545
Scanning with Micromirrors	511	Calcium Imaging	545
Scanning Fiber Confocal Microscopes for <i>In Vivo</i>		Uncaging and Photobleaching	545
Imaging in Animals	512	Autofluorescence	545
Implementations for Clinical Endomicroscopy	513	Developmental Biology	545
Summary	513	<i>In Vivo</i> (Intact Animal) Imaging	545
		Outlook	545
CHAPTER 27: FLUORESCENCE LIFETIME IMAGING IN			
SCANNING MICROSCOPY			
<i>H.C. Gerritsen, A. Draaijer, D.J. van den Heuvel,</i>			
<i>and A.V. Agronskaia</i>			
Introduction	516	CHAPTER 29: MULTIFOCAL MULTI-PHOTON	
Fluorescence, Lifetime, and Quantum		MICROSCOPY	
Efficiency	516	<i>Jörg Bewersdorf, Alexander Egner, and Stefan W. Hell</i>	
Fluorescence Lifetime Spectroscopy	516	Introduction	550
Fluorescence Lifetime Imaging Applications	516	Background	550
Fluorescence Resonance Energy Transfer	517	Determination of the Optimum Degree of	
Fluorescence Lifetime Imaging Methods	518	Parallelization	550
Introduction	518	Experimental Realization	551
Lifetime Sensing in the Frequency Domain	518	A Multi-Focal Multi-Photon Microscopy Setup Using	
Fluorescence Lifetime Sensing in the Time		a Nipkow-Type Microlens Array	551
Domain	520	Resolution	552
Comparison of Confocal Fluorescence Lifetime		Time Multiplexing as a Solution to Interfocal	
Imaging Methods	523	Crosstalk	553
Applications	527	Alternative Realizations	554
Multi-Labeling and Segmentation	527	Advanced Variants of Multi-Focal Multi-Photon	
Ion-Concentration Determination	528	Microscopy	555
Probes for Fluorescence Lifetime Microscopy	530	Space Multiplexing	555
Summary	532	Fluorescence Lifetime Imaging	555
		Second Harmonic Generation Multi-Focal Multi-Photon	
		Microscopy	556
		Multi-Focal Multi-Photon Microscopy-4Pi	
		Microscopy	556
		Imaging Applications	556
		Limitations	556
		Current Developments	558
		Summary	559
CHAPTER 28: MULTI-PHOTON MOLECULAR			
EXCITATION IN LASER-SCANNING			
MICROSCOPY			
<i>Winfried Denk, David W. Piston, and Watt W. Webb</i>			
Introduction	535	CHAPTER 30: 4Pi MICROSCOPY	
Physical Principles of Multi-Photon Excitation		<i>Jörg Bewersdorf, Alexander Egner, and Stefan W. Hell</i>	
and Their Implications for Image Formation	535	Introduction	561
Physics of Multi-Photon Excitation	535	Theoretical Background	562
Optical Pulse Length	537	The Point Spread Function	562
Excitation Localization	538	The <i>z</i> -Response and the Axial Resolution	563
Detection	538	The Optical Transfer Function	563
Wavelengths	538	Multi-Focal Multi-Photon Microscopy-4Pi	
Resolution	539	Microscopy	563
Photodamage: Heating and Bleaching	539	Space Invariance of the Point Spread Function	564
Instrumentation	540	Live Mammalian Cell 4Pi Imaging	564
Lasers and the Choice of Excitation Wavelengths	540	Type C 4Pi Microscopy with the Leica TCS 4Pi	565
Detection	541	Resolution	567
Optical Aberrations	542	Type C 4Pi Imaging in Living Cells	568
Pulse Spreading Due to Group Delay Dispersion	543	Summary and Outlook	568
Control of Laser Power	543		
Resonance and Non-Mechanical Scanning	543		

CHAPTER 31: NANOSCALE RESOLUTION WITH FOCUSED LIGHT: STIMULATED EMISSION DEPLETION AND OTHER REVERSIBLE SATURABLE OPTICAL FLUORESCENCE TRANSITIONS MICROSCOPY CONCEPTS

Stefan W. Hell, Katrin I. Willig, Marcus Dyba, Stefan Jakobs, Lars Kastrup, and Volker Westphal

The Resolution Issue	571
Breaking the Diffraction Barrier: The Concept of Reversible Saturable Optical Fluorescence Transitions	571
Different Approaches of Reversible Saturable Optical Fluorescence Transitions Microscopy	573
Stimulated Emission Depletion Microscopy	574
Challenges and Outlook	577

CHAPTER 32: MASS STORAGE, DISPLAY, AND HARD COPY

Guy Cox

Introduction	580
Mass Storage	580
Data Compression	580
Removable Storage Media	585
Random-Access Devices	586
Solid State Devices	588
Display	588
Monitors	588
Liquid Crystal Displays	589
Data Projectors	590
Hard Copy	590
Photographic Systems	590
Digital Printers	591
Conclusion	593
Summary	593
Bulk Storage	593
Display	594
Hard Copy	594

CHAPTER 33: COHERENT ANTI-STOKES RAMAN SCATTERING MICROSCOPY

X. Sunney Xie, Ji-Xin Cheng, and Eric Potma

Introduction	595
Unique Features of Coherent Anti-Stokes Raman Scattering Under the Tight-Focusing Condition ..	596
Forward and Backward Detected Coherent Anti-Stokes Raman Scattering	597
Optimal Laser Sources for Coherent Anti-Stokes Raman Scattering Microscopy	599
Suppression of the Non-Resonant Background	600
Use of Picosecond Instead of Femtosecond Pulses	600
Epi-Detection	600
Polarization-Sensitive Detection	600
Time-Resolved Coherent Anti-Stokes Raman Scattering Detection	600
Phase Control of Excitation Pulses	600
Multiplex Coherent Anti-Stokes Raman Scattering Microspectroscopy	602

Coherent Anti-Stokes Raman Scattering Correlation Spectroscopy	602
Coherent Anti-Stokes Raman Scattering Microscopy Imaging of Biological Samples	603
Conclusions and Perspectives	604

CHAPTER 34: RELATED METHODS FOR THREE-DIMENSIONAL IMAGING

J. Michael Tyszka, Seth W. Ruffins, Jamey P. Weichert, Michael J. Paulus, and Scott E. Fraser

Introduction	607
Surface Imaging Microscopy and Episcopic Fluorescence Image Capture	607
Optical Coherence Tomography	609
Optical Projection Tomography	610
Light Sheet Microscopy	613
Optical Setup	613
Micro-Computerized Tomography Imaging	614
Operating Principle	614
Contrast and Dose	614
Computed Tomography Scanning Systems	615
Magnetic Resonance Microscopy	618
Basic Principles of Nuclear Magnetic Resonance	618
Magnetic Resonance Image Formation	619
Magnetic Resonance Microscopy Hardware	622
Strengths and Limitations of Magnetic Resonance Microscopy	622
Image Contrast in Magnetic Resonance Microscopy ..	622
Magnetic Resonance Microscopy Applications	623
Future Development of Magnetic Resonance Microscopy	624
Conclusion	624

CHAPTER 35: TUTORIAL ON PRACTICAL CONFOCAL MICROSCOPY AND USE OF THE CONFOCAL TEST SPECIMEN

Victoria Centonze and James B. Pawley

Introduction	627
Getting Started	627
Bleaching — The Only Thing That Really Matters	627
Getting a Good Confocal Image	629
Simultaneous Detection of Backscattered Light and Fluorescence	631
New Controls	631
Photon Efficiency	631
Pinhole Size	631
Stray Light	632
Is the Back-Focal Plane Filled?	633
Pinhole Summary	633
Statistical Considerations in Confocal Microscopy	633
The Importance of Pixel Size	634
Measuring Pixel Size	635
Over-Sampling and Under-Sampling	635
Nyquist Reconstruction and Deconvolution	635
Pixel Size Summary	636

Using a Test Specimen	636	Absorbers and Targets in Biological Specimens	682
Why Use a Test Specimen?	636	Laser Exposure Parameters	682
Description of the Test Specimen	636	Evidence for Near Infrared-Induced Reactive Oxygen Species Formation	683
Using the Test Specimen	637	Evidence for Near Infrared-Induced DNA Strand Breaks	684
The Diatom: A Natural 3D Test Specimen	638	Photodynamic-Induced Effects	684
Reasons for Poor Performance	640	Photothermal Damage	685
Sampling Problems	640	Damage by Optical Breakdown	685
Optical Problems	640	Modifications of Ultrastructure	685
Imaging Depth	643	Influence of Ultrashort Near Infrared Pulses on Reproductive Behavior	686
Singlet-State Saturation	643	Nanosurgery	686
Which 3D Method Is Best?	644	Conclusion	687
Optimal 3D Light Microscopy Summary	646		
Things to Remember About Deconvolution	646	CHAPTER 39: PHOTOBLEACHING	
Decision Time	646	<i>Alberto Diaspro, Giuseppe Chirico, Cesare Usai, Paola Ramoino, and Jurek Dobrucki</i>	
Multi-Photon Versus Single-Photon Excitation	646	Introduction	690
Widefield Versus Beam Scanning	647	Photobleaching	691
Summary	647	Photobleaching Mechanisms	691
		Reducing Photobleaching	693
CHAPTER 36: PRACTICAL CONFOCAL MICROSCOPY		Photobleaching at the Single-Molecule Level	696
<i>Alan R. Hibbs, Glen MacDonald, and Karl Garsha</i>		Photobleaching of Single Molecules	697
The Art of Imaging by Confocal Microscopy	650	Photobleaching and Photocycling of Single Fluorescent Proteins	698
Balancing Multiple Parameters	650	Bleaching and Autofluorescence	698
Monitoring Instrument Performance	650	Other Fluorescent Proteins	698
Illumination Source	650	Conclusion	699
Scan Raster and Focus Positioning	651		
Optical Performance and Objective Lenses	652	CHAPTER 40: NONLINEAR (HARMONIC GENERATION) OPTICAL MICROSCOPY	
Signal Detection	660	<i>Ping-Chin Cheng and C.K. Sun</i>	
Optimizing Multi-Labeling Applications	663	Introduction	703
Control Samples Establish the Limits	663	Harmonic Generation	704
Separation of Fluorescence into Spectral Regions	664	Second Harmonic Generation	704
Sequential Channel Collection to Minimize Bleed-Through	664	Third Harmonic Generation	705
Spectral Unmixing	664	Multi-Photon Absorption and Fluorescence	705
Colocalization	667	Light Sources and Detectors for Second Harmonic Generation and Third Harmonic Generation Imaging	706
Image Collection for Colocalization	667	Nonlinear Optical Microscopy Setup	708
Quantifying Colocalization	668	Optically Active Biological Structures	710
Spatial Deconvolution in Colocalization Studies	668	Optically Active Structures in Plants	710
Discussion	670	Optically Active Structures in Animal Tissues	714
		Polarization Dependence of Second Harmonic Generation	717
CHAPTER 37: SELECTIVE PLANE ILLUMINATION MICROSCOPY		Summary	719
<i>Jan Huisken, Jim Swoger, Steffen Lindek, and Ernst H.K. Stelzer</i>			
Introduction	672	CHAPTER 41: IMAGING BRAIN SLICES	
Combining Light Sheet Illumination and Orthogonal Detection	672	<i>Ayumu Tashiro, Gloster Aaron, Dmitriy Aronov, Rosa Cossart, Daniella Dumitriu, Vivian Fenstermaker, Jesse Goldberg, Farid Hamzei-Sichani, Yuji Ikegaya, Sila Konur, Jason MacLean, Boaz Nemet, Volodymyr Nikolenko, Carlos Portera-Cailliau, and Rafael Yuste</i>	
Selective Plane Illumination Microscopy Setup	673	Introduction	722
Lateral Resolution	674		
Light Sheet Thickness and Axial Resolution	674		
Applications	675		
Processing Selective Plane Illumination Microscopy Images/Multi-View Reconstruction	675		
Summary	678		
CHAPTER 38: CELL DAMAGE DURING MULTI-PHOTON MICROSCOPY			
<i>Karsten König</i>			
Introduction	680		
Photochemical Damage in Multi-Photon Microscopes	682		

Making Brain Slices	722	The Quest for Better Resolution: Aberration and the Challenge of Imaging Thick Embryos	747
Acute Slices	722	Embryos Are Highly Scattering and Refractile Specimens	747
Cultured Slices	724	Imaging Embryos Involves Inherent Trade-Offs	747
Labeling Cells	724	Common Themes in Living Embryo Imaging Have System-Specific Solutions	748
Biolistic Transfection	724	Dealing with Depth: Strategies for Imaging Thick Specimens	748
Genetic Manipulation with Dominant-Negative and Constitutively Active Mutants	725	Avoiding the Thickness Dilemma: Going Small	748
Diolistics and Calistics	726	Grazing the Surface: Superficial Optical Sections Are Often Sufficient	748
Dye Injection with Whole-Cell Patch Clamp	726	Up from the Deep: Explants Can Reduce the Thickness of Specimens Dramatically	748
Slice Loading and “Painting” with Acetoxymethyl Ester Indicators	726	Multi-Photon Microscopy Can Penetrate More Deeply into Specimens	749
Green Fluorescent Protein Transgenic Mice	727	Selective Plane Illumination Can Provide Optical Sectioning in Very Thick Specimens	751
Imaging Slices	727	Deconvolution and Other Post-Acquisition Processing	751
Two-Photon Imaging of Slices	727	Striving for Speed: Strategies for Reducing Specimen Exposure	753
Slice Chamber Protocol	727	Simple Solutions: Reducing Image Dimensions, Increasing Slice Spacing, and Scan Speed	753
Choice of Objectives	727	Disk-Scanning Confocal Microscopy Allows High-Speed Acquisition	754
Beam Collimation and Pulse Broadening	728	Additional Hardware Improvements Can Increase Acquisition Speed	754
Image Production, Resolution, and z-Sectioning	729	Localizing Label: Strategies for Increasing Effective Contrast in Thick Specimens	755
Choice of Indicators for Two-Photon Imaging of Calcium	729	Addition of Labeled Proteins to Embryos	756
Photodamage	729	Expressing Green Fluorescent Protein and mRFP Constructs in Embryos Allows Dynamic Analysis of Embryos at Multiple Wavelengths	756
Second Harmonic Imaging	729	Using Selective Labeling to Reduce the Number of Labeled Structures	757
Silicon-Intensified Target Camera Imaging	730	Bulk Vital Labeling Can Enhance Contrast	760
Morphological Processing and Analysis	730	Seeing in Space: Strategies for 4D Visualization	761
Biocytin Protocol	730	Depicting Embryos in Time and Space: 2D + Time Versus 3D + Time	762
Anatomy with a Two-Photon/NeuroLucida System	731	Other Uses for Confocal and Multi-Photon Microscopy in Imaging and Manipulating Embryos	764
Correlated Electron Microscopy	731	Multi-Photon–Based Ablation	764
Morphological Classification of Neurons Using Cluster Analysis	731	Fluorescence Resonance Energy Transfer	764
Image Processing	732	Conclusions: A Bright Future for 3D Imaging of Living Embryos	766
Compensation for the Drift and the Vibration of the Slices	732		
Alignment Based on the Overlap Between Images	732		
Alignment Based on the Center of Mass	732		
Online Cell Detection of Neurons	733		
Image De-Noising Using Wavelets	734		
Summary	734		
CHAPTER 42: FLUORESCENT ION MEASUREMENT			
<i>Mark B. Cannell and Stephen H. Cody</i>			
Introduction	736		
The Limiting Case	736		
Choice of Indicator	737		
Introducing the Indicators into Cells	738		
Care of Fluorescent Probes	739		
Interpretation of Measurements	740		
Kinetics	741		
Calibration	742		
Conclusion	745		
CHAPTER 43: CONFOCAL AND MULTI-PHOTON IMAGING OF LIVING EMBRYOS			
<i>Jeff Hardin</i>			
Introduction	746	CHAPTER 44: IMAGING PLANT CELLS	
Into the Depths: Embryos Are Thick, Refractile, and Susceptible to Photodamage	746	<i>Nuno Moreno, Susan Bougourd, Jim Haseloff, and José A. Feijó</i>	
Imaging Embryos Often Requires “4D” Imaging	746	Introduction	769
		The Ever Present Problem of Autofluorescence	770
		Single-Photon Confocal Microscopy	772
		Staining Plant Tissues	774
		Clearing Intact Plant Material	774
		3D Reconstruction	775
		3D Segmentation	776
		Two-Photon Excitation: Are Two Better Than One?	778
		Improved Signal-to-Noise Ratio and Dynamic Range	778

Imaging Thick/Opaque Specimens	779	Transfection Reagents	803
Fading, Vital Imaging, and Cell Viability	779	Microinjection	803
Two-Photon Imaging of Plant Cells and Organelles	782	Future Perspectives: 3D Microscopy, Biological Complexity, and <i>In Vivo</i> Molecular Imaging	804
Two-Photon Excitation Imaging of Green Fluorescent Protein	782	<i>In Vivo</i> Molecular Imaging	806
Dynamic Imaging	783	CHAPTER 46: AUTOMATED CONFOCAL IMAGING AND HIGH-CONTENT SCREENING FOR CYTOMICS	
Deconvolution	784	<i>Maria A. DeBernardi, Stephen M. Hewitt, and Andres Kriete</i>	
Conclusion	785	Introduction	809
CHAPTER 45: PRACTICAL FLUORESCENCE RESONANCE ENERGY TRANSFER OR MOLECULAR NANOBIOSCOPY OF LIVING CELLS		Platforms Used for Automated Confocal Imaging	810
<i>Irina Majoul, Yiwei Jia, and Rainer Duden</i>		Types of Assays	811
Introduction	788	3D Cell Microarray Assays	815
How to Make a Good Science	788	Data Management and Image Informatics	816
Beauty, Functionality, Cell Cycle, and Living-Cell Imaging	790	Conclusion	817
Fluorescence Resonance Energy Transfer Theory	790	CHAPTER 47: AUTOMATED INTERPRETATION OF SUBCELLULAR LOCATION PATTERNS FROM THREE DIMENSIONAL CONFOCAL MICROSCOPY	
Fluorescent Proteins and Fluorescence Resonance Energy Transfer	794	<i>Ting Zhao and Robert F. Murphy</i>	
Qualitative Analysis	795	Introduction	818
Preparation	795	Protein Subcellular Location	818
Nanobioscopy of Protein-Protein Interactions with Fluorescence Resonance Energy Transfer	795	Overview of 2D Dataset Analysis	818
Methods of Fluorescence Resonance Energy Transfer Measurement	795	High-Resolution 3D Datasets	820
Sensitized Emission of Acceptor	795	3DHeLa	820
Donor Fluorescence	796	3D3T3	820
Acceptor Bleach	797	Image Acquisition Considerations When Using Automated Analysis	821
Fluorescent Proteins as Fluorescence Resonance Energy Transfer Pairs	798	Image Processing and Analysis	822
Cyan Fluorescent Protein and Yellow Fluorescent Protein — The Commonly Used Fluorescence Resonance Energy Transfer Pair	798	Segmentation of Multi-Cell Images and Preprocessing	822
Cyan Fluorescent Protein or Green Fluorescent Protein Forms a Fluorescence Resonance Energy Transfer Pair with mRFP1	798	3D Subcellular Location Features	822
Fluorescence Resonance Energy Transfer-Based Sensors	798	Automated Classification of Location Patterns	824
Fluorescence Resonance Energy Transfer and Other Complementary Methods	799	Classification of 3DHeLa Dataset	824
Fluorescence Resonance Energy Transfer and Fluorescence Lifetime Imaging Microscope	799	Downsampled Images with Different Gray Scales	824
Fluorescence Recovery After Photobleaching and Fluorescence Loss in Photobleaching	801	Clustering of Location Patterns: Location Proteomics	825
Fluorescence Resonance Energy Transfer and Fluorescence Correlation Spectroscopy	801	Exclusion of Outliers	825
Fluorescence Resonance Energy Transfer and Total Internal Reflection Fluorescence	801	Determination of Optimal Clustering	825
Quantum Dots and Fluorescence Resonance Energy Transfer	801	Statistical Comparison of Location Patterns	826
Cloning and Expression of Fluorescent Constructs for Fluorescence Resonance Energy Transfer	801	Image Database Systems	827
Cloning of Fluorescent Chimeras	801	Future Directions	827
Functional Activity of Expressed Constructs	802	CHAPTER 48: DISPLAY AND PRESENTATION SOFTWARE	
Expression and Over-Expression	802	<i>Felix Margadant</i>	
Methods for Introducing Chromophores into Living Cells	803	Introduction	829
Electroporation	803	Testing	830
		“Static” Image Performance	831
		Brightness	832
		Resolution: Changing the Display Size of Your Images	832
		Compression	835
		Motion Pictures	836
		Coding Limitations	838

Up-Sampling or Frame Rate Matching 838
 Motion Picture Artifacts 839
 The MPEG Formats 840
 MPEG Display Formats 840
 Very High Resolutions 841
 Movie Compression and Entropy 841
 Performance Benchmark 841
 Storing Your Presentation for Remote Use 842
 Taking Your Presentation on the Road: Digital Rights Management and Overlaying 844

CHAPTER 49: WHEN LIGHT MICROSCOPE RESOLUTION IS NOT ENOUGH: CORRELATIONAL LIGHT MICROSCOPY AND ELECTRON MICROSCOPY

Paul Sims, Ralph Albrecht, James B. Pawley, Victoria Centonze, Thomas Deerinck, and Jeff Hardin

Introduction 846
 Early Correlative Microscopy 846
 Early 4D Microscopy 846
Correlative Light Microscope/Electron Microscope Today 846
 Light Microscope and Electron Microscope Have Different Requirements 846
 Finding the Same Cell Structure in Two Different Types of Microscope: Light Microscope/Scanning Electron Microscope 850
 Finding the Same Cell Structure in Two Different Types of Microscope: Light Microscope/Transmission Electron Microscope 852
 Cryo-Immobilization Followed by Post-Embedding Confocal Laser Scanning Microscopy on Thin Sections 856
 Tiled Montage Transmission Electron Microscope Images Aid Correlation 858
Conclusion 860

CHAPTER 50: DATABASES FOR TWO- AND THREE-DIMENSIONAL MICROSCOPICAL IMAGES IN BIOLOGY

Steffen Lindek, Nicholas J. Salmon, and Ernst H.K. Stelzer

Introduction 861
Data and Metadata Management in Microscopes 861
 Recent Developments 861
Image Information Management 862
 The Aims of Modern Microscope System Design 862
Instrument Database Model 864
System Requirements 864
Image Database Model 864
Selected Projects 865
 BioImage 865
 Biomedical Image Library 866
 Scientific Image DataBase 866
 Other Projects 866
Criteria and Requirements for Microscopy Databases 866
 User Interface 866
 Query by Content 866

Metadata Structure 867
 Digital Rights Management 867
Future Prospects 867

CHAPTER 51: CONFOCAL MICROSCOPY OF BIOFILMS — SPATIOTEMPORAL APPROACHES

R.J. Palmer, Jr., Janus A.J. Haagensen, Thomas R. Neu, and Claus Sternberg

Introduction 870
Sample Presentation 870
 Flowcells and Other Perfusion Chambers 870
 Water-Immersible Lenses 872
 Upright Versus Inverted Microscopes 872
 Setup of a Flow Chamber System Setup — A Practical Example 872
Making Bacteria Fluorescent 873
 Fluorescent Proteins 873
Stains 874
 Nucleic Acid Stains 874
 Live/Dead Stain 875
 Fluorescence *In Situ* Hybridization 875
 General Procedure for Embedding of Flowcell-Grown Biofilms for Fluorescence *In Situ* Hybridization ... 876
 Antibodies 877
 Preparation of Labeled Primary Antibodies 878
 Imaging Bacteria Without Fluorescence 879
Imaging Extracellular Polymeric Substances in Biofilms 879
Application of Two-Photon Laser-Scanning Microscopy for Biofilm Analysis 882
 Limitations of Confocal Laser Scanning Microscopy and Two-Photon Laser-Scanning Microscopy in Biofilm Analysis 884
Temporal Experiments 885
 Time-Lapse Confocal Imaging 885
Summary and Future Directions 887

CHAPTER 52: BIBLIOGRAPHY OF CONFOCAL MICROSCOPY

Robert H. Webb

A. Book and Review Articles 889
B. Historical Interest 889
C. Theory (Mostly) 890
D. Technical 891
E. General 891
F. Adaptive Optics 892
G. Differential 892
H. Display 892
I. Fiber-Optic Confocal Microscopes 893
J. Index Mismatch 893
K. Multiplex 894
L. Nonlinear 894
M. Polarization 894
N. Profilometry 895
O. Point Spread Function 895
P. Pupil Engineering 896
Q. Thickness 896
R. Turbidity 896
S. Variants on the Main Theme 897

APPENDIX 1: PRACTICAL TIPS FOR TWO-PHOTON MICROSCOPY

*Mark B. Cannell, Angus McMorland,
and Christian Soeller*

Introduction	900
Laser Safety	900
Laser Alignment	900
Testing Alignment and System Performance	900
Laser Settings and Operation	901
Monitoring Laser Performance	901
Power Levels and Trouble-Shooting	903
Choice of Pulse Length	903
Controlling Laser Power	903
Am I Seeing Two-Photon Excited Fluorescence or ...	904
Stray Light and Non-Descanned Detection	904
Laser Power Adjustment for Imaging at Depth	904
Simultaneous Imaging of Multiple Labels	904
Minimize Exposure During Orientation and Parameter Setting	905
Ultraviolet-Excited Fluorochromes	905

APPENDIX 2: LIGHT PATHS OF THE CURRENT COMMERCIAL CONFOCAL LIGHT MICROSCOPES USED IN BIOLOGY

James B. Pawley

Introduction	906
BD-CARV II	907
LaVision-BioTec TriM-Scope	907
Leica TCS SP2 AOBs-MPRS	910
Nikon C1si	911
Olympus Fluoview 1000-DSU	912
Visitech VT Infinity-VT-eye	914
Yokogawa CSU 22	915
Zeiss LSM-5-LIVE Fast Slit Scanner-LSM 510 META-FCS	916

APPENDIX 3: MORE THAN YOU EVER REALLY WANTED TO KNOW ABOUT CHARGE-COUPLED DEVICES

James B. Pawley

Introduction	919
Part I: How Charge-Coupled Devices Work	919
Charge Coupling	919
Readout Methods	920
What Could Go Wrong?	920
Quantum Efficiency	920
Edge Effects	921
Charge Loss	921
Leakage or "Dark Charge"	921
Blooming	921
Incomplete Charge Transfer	923
Charge Amplifiers	923
What Is a Charge Amplifier?	923
FET Amplifier Performance	924
Noise Sources in the Charge-Coupled Device	924
Fixed Pattern Noise	924
Noise from the Charge Amplifier	925
Where Is Zero?	925
A New Idea: The Gain Register Amplifier!!	925
Of Course, There Is One Snag!	926
Part II: Evaluating a Charge-Coupled Device	927
A. Important Charge-Coupled Device Specs for Live-Cell Stuff!	927
B. Things That Are (Almost!) Irrelevant When Choosing a Charge-Coupled Device for Live-Cell Microscopy	929
C. A Test You Can Do Yourself!!!	930
D. Intensified Charge-Coupled Devices	930
Index	933