

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

xii Contents

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. van der Ven</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 Nölte, James B. Pawley, and Lutz Hering</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 Baye 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	Overview	207
Working Distance	157	Telecentricity	207
Optical Materials	158	The Scanning System	208
Anti-Reflection Coatings	158	The Back-Focal Planes	210
Transmission of Microscope Objectives	158	Practical Requirements	210
Conclusion	160	Diffraction Limit	210
		Geometric Distortion	211
CHAPTER 8: THE CONTRAST FORMATION IN OPTICAL MICROSCOPY		Evaluation of the Illumination and Detection Systems	211
<i>Ping-Chin Cheng</i>		Influence of Optical Elements	211
Introduction	162	Errors	211
Sources of Contrast	163	Evaluation of Optical Arrangements	212
Absorption Contrast	163	Evaluation of Scanner Arrangements	213
Scattering and Reflection Contrast	167	Scanners	215
Phase Contrast	171	Attachment to Microscopes	217
Fluorescence Contrast	172	Merit Functions	217
Contrast Related to Excitation Wavelength Change	173	Multi-Fluorescence	217
Negative Contrast	173	Special Setups	218
Special Concerns in Ultraviolet and Near-Infrared Range Confocal Microscopy	174	Setups for Fluorescence Recovery After Photobleaching Experiments	218
Total Internal Reflection Contrast	177	Setups for Fluorescence Resonance Energy Transfer Experiments	218
Harmonic Generation Contrast	179	Setups for the Integration of Optical Tweezers	218
Geometric Contrast	180	Setups for the Integration of Laser Cutters	218
z -Contrast in Confocal Microscopy	180	Setups for the Observation of Living Specimens	219
Total Internal Reflection Fluorescence Contrast	180	Miniaturization and Computer Control	219
Fluorescence Resonant Energy Transfer	184	Thermal Stability	219
Fluorescence Recovery After Photobleaching (FRAP and FLIP)	187	Vibration Isolation	219
Structural Contrast	188	Conclusions and Future Prospects	219
Harmonic Generation Contrast	188		
Birefringence Contrast	188	CHAPTER 10: DISK-SCANNING CONFOCAL MICROSCOPY	
Derived Contrast (Synthetic Contrast)	188	<i>Derek Toomre and James B. Pawley</i>	
Ratiometric	189	Introduction	221
Deconvolution	189	Background	221
Movement Contrast (Subtraction of Previous Image)	190	Living Cell Imaging: Probing the Future	221
Spectral Unmixing and Color Reassignment	190	A Need for Speed and Less Photobleaching	222
Effects of the Specimen: Spherical Aberration and Optical Heterogeneity	192	Advantages and Limitations of Confocal Laser-Scanning Microscopes	222
Mounting Medium Selection	198	Other Imaging and Deconvolution	223
Artificial Contrast	201	Confocal Disk-Scanning Microscopy	223
Contrast Resulting from Instrument Vibration and Ambient Lighting	201	Nipkow Disk — An Innovation	223
Contrast Resulting from Interference of Cover Glass Surfaces	201	A Renaissance — Advantages of Disk-Scanning Confocal Imaging	223
Background Level and Ghost Images from the Transmission Illuminator	201	Disadvantages	224
Contrast Resulting from Differences in Photobleaching Dynamics	202	Critical Parameters in Pinhole and Slit Disks	224
Effect of Spectral Leakage and Signal Imbalance Between Different Channels	203	Fill Factor and Spacing Interval F	224
New Contrasts: Fluorescence Lifetime and Coherent Anti-Stokes Raman Spectroscopy	204	Lateral Resolution	225
Summary	204	Pinhole/Slit Size	225
		Axial Resolution	225
CHAPTER 9: THE INTERMEDIATE OPTICAL SYSTEM OF LASER-SCANNING CONFOCAL MICROSCOPES		Types of Disk-Scanning Confocals	228
<i>Ernst H.K. Sielzer</i>		General Considerations	228
Introduction	207	Disk Scanners for Backscattered Light Imaging	228
Design Principles	207	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	Experimental Considerations	265
Applications and Examples of Confocal Disk-Scanning Microscopes	235	Pattern Generation	266
Comparison with Epi-Fluorescence Imaging	235	Computing Optical Sections from Structured-Illumination Data	268
Fast 3D/4D Imaging	235	Resolution Improvement by Structured Illumination	270
Blazingly Fast Confocal Imaging	235	Nonlinear Structured Illumination	276
Future Developments?	236	Summary	276
Summary	237		
CHAPTER 11: MEASURING THE REAL POINT SPREAD FUNCTION OF HIGH NUMERICAL APERTURE MICROSCOPE OBJECTIVE LENSES		CHAPTER 14: VISUALIZATION SYSTEMS FOR MULTI-DIMENSIONAL MICROSCOPY IMAGES	
<i>Rimas Jukaitis</i>		<i>N.S. White</i>	
Introduction	239	Introduction	280
Measuring Point Spread Function	240	Definitions	280
Fiber-Optic Interferometer	240	What Is the Microscopist Trying to Achieve?	280
Point Spread Function Measurements	241	Criteria for Choosing a Visualization System	281
Chromatic Aberrations	242	Why Do We Want to Visualize Multi-Dimensional Laser-Scanning Microscopy Data?	281
Apparatus	243	Data and Dimensional Reduction	281
Axial Shift	243	Objective or Subjective Visualization?	281
Pupil Function	245	Prefiltering	281
Phase-Shifting Interferometry	245	Identifying Unknown Structures	281
Zernike Polynomial Fit	245	Highlighting Previously Elucidated Structures	284
Restoration of a 3D Point Spread Function	247	Visualization for Multi-Dimensional Measurements	284
Empty Aperture	248	What Confocal Laser Scanning Microscopy Images Can the Visualization System Handle?	286
Miscellanea	248	Image Data: How Are Image Values Represented in the Program?	286
Temperature Variations	248	What Dimensions Can the Images and Views Have?	286
Polarization Effects	249	Standard File Formats for Calibration and Interpretation	288
Apodization	250	How Will the System Generate the Reconstructed Views?	290
Conclusion	250	Assessing the Four Basic Steps in the Generation of Reconstructed Views	290
		Loading the Image Subregion	290
 		Choosing a View: The 3D Image Display Space	291
CHAPTER 12: PHOTON DETECTORS FOR CONFOCAL MICROSCOPY		Mapping the Image Space into the Display Space	294
<i>Jonathan Art</i>		How Do 3D Visualizations Retain the z-Information?	296
Introduction	251	Mapping the Data Values into the Display	300
The Quantal Nature of Light	251	How Can Intensities Be Used to Retain z-Information?	304
Interaction of Photons with Materials	252	Hidden-Object Removal	304
Thermal Effects	252	Adding Realism to the View	306
Direct Effects	252	How Can I Make Measurements Using the Reconstructed Views?	312
Photoconductivity	252	Conclusion	312
Photovoltaic	252		
Photoemissive	254		
Comparison of Detectors	255	 	
Noise Internal to Detectors	256	CHAPTER 15: AUTOMATED THREE-DIMENSIONAL IMAGE ANALYSIS METHODS FOR CONFOCAL MICROSCOPY	
Noise in Internal Detectors	256	<i>Badrinath Roysam, Gang Lin, Muhammad-Arri Abdul-Karim, Omar Al-Kofahi, Khalid Al-Kofahi, William Shain, Donald H. Scarvesk, and James N. Turner</i>	
Noise in Photoemissive Devices	256	Introduction	316
Statistics of Photon Flux and Detectors	257	Types of Automated Image Analysis Studies	318
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			
<i>Rainer Heintzmann</i>			
Introduction	265		

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

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 21: INTERACTION OF LIGHT WITH BOTANICAL SPECIMENS	
		<i>Ping-Chün Cheng</i>	
		Introduction	414
		Light Attenuation in Plant Tissue	414
		Linear Absorption	414
		Nonlinear Absorption	416
		Scattering	417
		Refractive Index Heterogeneity	418
		Birefringent Structures in Plant Cells	420
		Fluorescence Properties of Plants	421
		Changes in Emission Spectra Depending on One- Versus Two-Photon Excitation	421
		Microspectroscopy	421
		Light-Specimen Interaction (Fluorescence Emission)	425
		Harmonic Generation Properties	428
		The Effect of Fixation on the Optical Properties of Plants	428
		Living Plant Cells	429
		Callus, Suspension Culture Cells and Protoplasts	429
		Meristem	430
		Stem and Root	430
		Microspores and Pollen Grains	431
		Cuticles, Hairs, and Waxes	434
		Storage Structures	435
		Mineral Deposits	436
		Primary and Secondary Cell Walls	438
		Fungi	438
		Conclusion	439
		CHAPTER 22: SIGNAL-TO-NOISE RATIO IN CONFOCAL MICROSCOPES	
		<i>Colin J.R. Sheppard, Xiaosong Gan, Min Gu, and Maitreyee Roy</i>	
		Introduction	442
		Sources of Noise	442
		Shot Noise and Quantum Efficiency	442
		Background Noise	443
CHAPTER 19: CONFOCAL MICROSCOPY OF LIVING CELLS			
<i>Michael E. Dailey, Erik Manders, David R. Söll, and Mark Terasaki</i>			
Introduction	381		
Overview of Living-Cell Confocal Imaging			
Techniques	382		
Time-Lapse Fluorescence Imaging	382		
Multi-Channel Time-Lapse Fluorescence Imaging ..	382		
Spectral Imaging and Linear Unmixing	382		
Fluorescence Recovery After Photobleaching	382		
Fluorescence Loss in Photobleaching	382		
Fluorescence Resonance Energy Transfer	382		
Fluorescence Lifetime Imaging	382		
Fluorescence Correlation Spectroscopy	383		
Fluorescence Speckle Microscopy	383		
Photo-Uncaing/Photoactivation	383		
Optical Tweezers/Laser Trapping	383		
Physiological Fluorescence Imaging	383		
Combining Fluorescence and Other Imaging Modalities	383		
General Considerations for Confocal Microscopy of Living Cells	386		
Maintenance of Living Cells and Tissue Preparations	387		
Fluorescent Probes	387		
Minimizing Photodynamic Damage	389		
The Online Confocal Community	390		
A Convenient Test Specimen	390		
Specific Example I: Visualizing Chromatin Dynamics Using Very Low Light Levels	390		
Phototoxicity	390		
Reduction of Phototoxicity	391		
Improving Image Quality in Low-Dose Microscopy	391		
Low-Dose Imaging Conclusion	391		
Specific Example II: Multi-Dimensional Imaging of Microglial Cell Behaviors in Live Rodent Brain Slices	392		
Preparation of Central Nervous System Tissue Slices	393		
Fluorescent Staining	393		
Maintaining Tissue Health on the Microscope Stage	393		
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	More Examples	480
Signal-to-Noise Ratio for Confocal Microscopes	445	Blind Deconvolution and Spherical Aberration	480
Q, N1, and Stain Level	445	Widefield Fluorescence Simulation	481
N2 and Detectability	446	Spinning-Disk Confocal	481
Multi-Photon Fluorescence Microscopy	447	Two Photon	481
Designs of Confocal Microscopes	447	Speed	482
Sampling	448	Future Directions	483
Comparative Performance of Fluorescence Microscopes	448	Summary of Main Points	483
Bleaching-Limited Performance	448		
Saturation-Limited Performance	450	CHAPTER 25: IMAGE ENHANCEMENT BY DECONVOLUTION	
Effects of Scanning Speed	450	<i>Mark B. Cantwell, Angus McMorland, and Christian Soeller</i>	
3D Imaging	451	Introduction	488
Summary	451	Background	488
		Image Formation	489
CHAPTER 23: COMPARISON OF WIDEFIELD/DECONVOLUTION AND CONFOCAL MICROSCOPY FOR THREE- DIMENSIONAL IMAGING		Forwards: Convolution and the Imaging System ..	490
<i>Peter J. Shaw</i>		Properties of the Point Spread Function	492
Introduction	453	Quantifying the Point Spread Function	492
The Point Spread Function: Imaging as a Convolution	453	The Missing Cone Problem	494
Limits to Linearity and Shift Invariance	457	Noise	495
Deconvolution	457	Deconvolution Algorithms	495
Practical Differences	458	Nearest-Neighbor Deconvolution	495
Temporal Resolution	458	Wiener Filtering	496
Combination of Charged-Coupled Device and Confocal Imaging	458	Nonlinear Constrained Iterative Deconvolution Algorithms	496
Integration of Fluorescence Intensity	459	Comparison of Methods	497
Resolution, Sensitivity, and Noise	459		
Fluorescence Excitation	459	CHAPTER 26: FIBER-OPTICS IN SCANNING OPTICAL MICROSCOPY	
Fluorescent Light Detection	459	<i>Peter Delaney and Martin Harris</i>	
Gain Register Charge-Coupled Devices	460	Introduction	501
Out-of-Focus Light	461	Key Fiber Technologies Relevant to Scanning Microscopy	501
Model Specimens	461	Glass Made from Gas and Its Transmission Properties	501
The Best Solution: Deconvolving Confocal Data ..	461	Step Index and Gradient Index Optical Fibers	501
Practical Comparisons	463	Modes in Optical Fibers	502
Conclusion	466	Evanescent Wave and Polarization Effects in Optical Fibers	503
Summary	467	Polarization-Maintaining Fibers	503
		Fused Biconical Taper Couplers: Fiber-Optic Beam-Splitters	503
CHAPTER 24: BLIND DECONVOLUTION		Microstructure Fibers	504
<i>Timothy J. Holmes, David Biggs, and Asad Abu-Tarif</i>		Fiber Image Transfer Bundles	504
Introduction	468	Key Functions of Fibers in Optical Microscopes ..	505
Purposes of Deconvolution	468	Optical Fiber for Delivering Light	505
Advantages and Limitations	468	Optical Fiber as a Detection Aperture	506
Principles	472	Same Fiber for Both Source and Confocal Detection	506
Data Collection Model	472	Fiber Delivery for Nonlinear Microscopy with Femtosecond Lasers	507
Maximum Likelihood Estimation	472	Large Core Fibers as Source or Detection Apertures	507
Algorithms	472	Benchtop Scanning Microscopes Exploiting Fiber Components	507
Different Approaches	475	Miniaturized Scanning Confocal Microscope Imaging Heads	508
3D	475	Miniature Confocal Imaging Heads Based on Coherent Imaging Bundles	508
2D Image Filtering	476		
Data Corrections	477		
Light Source and Optics Alignment	477		
Newest Developments	478		
Subpixel	478		
Polarized Light	479		
Live Imaging	480		

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. Agronkaia</i>			
Introduction	516	CHAPTER 29: MULTIFOCAL MULTI-PHOTON	
Fluorescence, Lifetime, and Quantum		MICROSCOPY	
Efficiency	516	<i>Jörg Bewersdorff, 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>Wolfgang Denk, David W. Piston, and Watt W. Webb</i>			
Introduction	535	CHAPTER 30: 4PI MICROSCOPY	
Physical Principles of Multi-Photon Excitation		<i>Jörg Bewersdorff, 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		Coherent Anti-Stokes Raman Scattering Correlation Spectroscopy	602
<i>Stefan W. Hell, Katrin I. Willig, Marcus Dyba, Stefan Jacobs, Lars Kantrup, and Volker Westphal</i>		Coherent Anti-Stokes Raman Scattering Microscopy Imaging of Biological Samples	603
The Resolution Issue	571	Conclusions and Perspectives	604
Breaking the Diffraction Barrier: The Concept of Reversible Saturable Optical Fluorescence Transitions	571		
Different Approaches of Reversible Saturable Optical Fluorescence Transitions Microscopy	573	CHAPTER 34: RELATED METHODS FOR THREE-DIMENSIONAL IMAGING	
Stimulated Emission Depletion Microscopy	574	<i>J. Michael Tyszka, Seth W. Ruffins, Jamey P. Weichert, Michael J. Paulus, and Scott E. Fraser</i>	
Challenges and Outlook	577	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 32: MASS STORAGE, DISPLAY, AND HARD COPY			
<i>Guy Cox</i>		CHAPTER 35: TUTORIAL ON PRACTICAL CONFOCAL MICROSCOPY AND USE OF THE CONFOCAL TEST SPECIMEN	
Introduction	580	<i>Victoria Centonze and James B. Pawley</i>	
Mass Storage	580	Introduction	627
Data Compression	580	Getting Started	627
Removable Storage Media	585	Bleaching — The Only Thing That Really Matters	627
Random-Access Devices	586	Getting a Good Confocal Image	629
Solid State Devices	588	Simultaneous Detection of Backscattered Light and Fluorescence	631
Display	588	New Controls	631
Monitors	588	Photon Efficiency	631
Liquid Crystal Displays	589	Pinhole Size	631
Data Projectors	590	Siry Light	632
Hard Copy	590	Is the Back-Focal Plane Filled?	633
Photographic Systems	590	Pinhole Summary	633
Digital Printers	591	Statistical Considerations in Confocal Microscopy	633
Conclusion	593	The Importance of Pixel Size	634
Summary	593	Measuring Pixel Size	635
Bulk Storage	593	Over-Sampling and Under-Sampling	635
Display	594	Nyquist Reconstruction and Deconvolution	635
Hard Copy	594	Pixel Size Summary	636
CHAPTER 33: COHERENT ANTI-STOKES RAMAN SCATTERING MICROSCOPY			
<i>X. Sunney Xie, Ji-Xin Cheng, and Eric Potma</i>			
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		

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: PHOTBLEACHING	
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	
Image Collection for Colocalization	667	Imaging	706
Quantifying Colocalization	668	Nonlinear Optical Microscopy Setup	708
Spatial Deconvolution in Colocalization Studies	668	Optically Active Biological Structures	710
Discussion	670	Optically Active Structures in Plants	710
		Optically Active Structures in Animal Tissues	714
CHAPTER 37: SELECTIVE PLANE ILLUMINATION MICROSCOPY		Polarization Dependence of Second Harmonic Generation	717
<i>Jan Huiskens, Jim Swoger, Steffen Lindke, and Ernst H.K. Stelzer</i>		Summary	719
Introduction	672		
Combining Light Sheet Illumination and Orthogonal Detection	672	CHAPTER 41: IMAGING BRAIN SLICES	
Selective Plane Illumination Microscopy Setup	673	<i>Ayumu Tashiro, Gloster Aamn, Dmitriy Aronov, Rosa Cossart, Daniela Dumitriu, Vivian Fenstermaker, Jesse Goldberg, Farid Hamzei-Sichani, Yuji Ikegaya, Sıla Konur, Jason MacLean, Boaz Nemet, Volodymyr Nikolenko, Carlos Portera-Cailliau, and Rafael Yuste</i>	
Lateral Resolution	674	Introduction	722
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
Bioctylin Protocol	730	Depicting Embryos in Time and Space: 2D + Time Versus 3D + Time	762
Anatomy with a Two-Photon/NeuroTrace 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		
Into the Depths: Embryos Are Thick, Refractile, and Susceptible to Photodamage	746		
Imaging Embryos Often Requires "4D" Imaging	746		
		CHAPTER 44: IMAGING PLANT CELLS	
		<i>Nuno Moreno, Susan Bougourd, Jim Haseleff, and José A. Feijó</i>	
		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 In Vivo 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	Metadata Structure	867
Motion Picture Artifacts	839	Digital Rights Management	867
The MPEG Formats	840	Future Prospects	867
MPEG Display Formats	840		
Very High Resolutions	841		
Movie Compression and Entropy	841	CHAPTER 51: CONFOCAL MICROSCOPY OF BIOFILMS — SPATIOTEMPORAL APPROACHES	
Performance Benchmark	841	<i>R.J. Palmer, Jr., Janus A.J. Haugensen, Thomas R. Neu, and Claus Sternberg</i>	
Storing Your Presentation for Remote Use	842		
Taking Your Presentation on the Road: Digital Rights Management and Overlaying	844	Introduction	870
		Sample Presentation	870
CHAPTER 49: WHEN LIGHT MICROSCOPE RESOLUTION IS NOT ENOUGH: CORRELATIONAL LIGHT MICROSCOPY AND ELECTRON MICROSCOPY		Flowcells and Other Perfusion Chambers	870
<i>Paul Sims, Ralph Albrecht, James B. Pawley, Victoria Centonze, Thomas Doerneck, and Jeff Hardin</i>		Water-Immersible Lenses	872
Introduction	846	Upright Versus Inverted Microscopes	872
Early Correlative Microscopy	846	Setup of a Flow Chamber System Setup — A Practical Example	872
Early 4D Microscopy	846	Making Bacteria Fluorescent	873
Correlative Light Microscope/Electron Microscope Today	846	Fluorescent Proteins	873
Light Microscope and Electron Microscope Have Different Requirements	846	Stains	874
Finding the Same Cell Structure in Two Different Types of Microscope: Light Microscope/Scanning Electron Microscope	850	Nucleic Acid Stains	874
Finding the Same Cell Structure in Two Different Types of Microscope: Light Microscope/Transmission Electron Microscope	852	Live/Dead Stain	875
Cryo-Immobilization Followed by Post-Embedding Confocal Laser Scanning Microscopy on Thin Sections	856	Fluorescence <i>In Situ</i> Hybridization	875
Tiled Montage Transmission Electron Microscope Images Aid Correlation	858	General Procedure for Embedding of Flowcell-Grown Biofilms for Fluorescence <i>In Situ</i> Hybridization ...	876
Conclusion	860	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 50: DATABASES FOR TWO- AND THREE-DIMENSIONAL MICROSCOPICAL IMAGES IN BIOLOGY		CHAPTER 52: BIBLIOGRAPHY OF CONFOCAL MICROSCOPY	
<i>Steffen Lindk, Nicholas J. Salmon, and Ernst H.K. Stelzer</i>		<i>Robert H. Webb</i>	
Introduction	861	A. Book and Review Articles	889
Data and Metadata Management in Microscopes	861	B. Historical Interest	889
Recent Developments	861	C. Theory (Mostly)	890
Image Information Management	862	D. Technical	891
The Aims of Modern Microscope System Design ...	862	E. General	891
Instrument Database Model	864	F. Adaptive Optics	892
System Requirements	864	G. Differential	892
Image Database Model	864	H. Display	892
Selected Projects	865	I. Fiber-Optic Confocal Microscopes	893
BiImage	865	J. Index Mismatch	893
Biomedical Image Library	866	K. Multiplex	894
Scientific Image DataBase	866	L. Nonlinear	894
Other Projects	866	M. Polarization	894
Criteria and Requirements for Microscopy Databases	866	N. Profilometry	895
User Interface	866	O. Point Spread Function	895
Query by Content	866	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. Cressell, 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