

Contents

Preface XIII

List of Contributors XVII

1	Introduction to Optics and Photophysics	1
	<i>Rainer Heintzmann</i>	
1.1	Interference: Light as a Wave	2
1.2	Two Effects of Interference: Diffraction and Refraction	7
1.3	Optical Elements	14
1.3.1	Lenses	14
1.3.2	Metallic Mirror	17
1.3.3	Dielectric Mirror	18
1.3.4	Pinholes	18
1.3.5	Filters	19
1.3.6	Chromatic Reflectors	20
1.4	The Far-Field, Near-Field, and Evanescent Waves	20
1.5	Optical Aberrations	23
1.6	Physical Background of Fluorescence	24
1.7	Photons, Poisson Statistics, and AntiBunching	30
	References	31
2	Principles of Light Microscopy	33
	<i>Ulrich Kubitscheck</i>	
2.1	Introduction	33
2.2	Construction of Light Microscopes	33
2.2.1	Components of Light Microscopes	33
2.2.2	Imaging Path	34
2.2.3	Magnification	36
2.2.4	Angular and Numerical Aperture	38
2.2.5	Field of View	38
2.2.6	Illumination Beam Path	39
2.3	Wave Optics and Resolution	42
2.3.1	Wave Optical Description of the Imaging Process	43

2.3.2	The Airy Function	47
2.3.3	Point Spread Function and Optical Transfer Function	50
2.3.4	Lateral and Axial Resolution	52
2.3.5	Magnification and Resolution	59
2.3.6	Depth of Field and Depth of Focus	60
2.3.7	Over- and Under Sampling	61
2.4	Apertures, Pupils, and Telecentricity	61
2.5	Microscope Objectives	64
2.5.1	Objective Lens Design	64
2.5.2	Light Collection Efficiency and Image Brightness	68
2.5.3	Objective Lens Classes	73
2.5.4	Immersion Media	73
2.5.5	Special Applications	77
2.6	Contrast	78
2.6.1	Dark Field	80
2.6.2	Phase Contrast	81
2.6.3	Interference Contrast	86
2.6.4	Advanced Topic: Differential Interference Contrast	89
2.7	Summary	94
	Acknowledgments	94
	References	95
3	Fluorescence Microscopy	97
	<i>Jurek W. Dobrucki</i>	
3.1	Features of Fluorescence Microscopy	98
3.1.1	Image Contrast	98
3.1.2	Specificity of Fluorescence Labeling	101
3.1.3	Sensitivity of Detection	102
3.2	A Fluorescence Microscope	103
3.2.1	Principle of Operation	103
3.2.2	Sources of Exciting Light	107
3.2.3	Optical Filters in a Fluorescence Microscope	110
3.2.4	Electronic Filters	111
3.2.5	Photodetectors for Fluorescence Microscopy	112
3.2.6	CCD–Charge-Coupled Device	113
3.2.7	Intensified CCD (ICCD)	116
3.2.8	Electron-Multiplying Charge-Coupled Device (EMCCD)	117
3.2.9	CMOS	119
3.2.10	Scientific CMOS (sCMOS)	120
3.2.11	Features of CCD and CMOS Cameras	121
3.2.12	Choosing a Digital Camera for Fluorescence Microscopy	121
3.2.13	Photomultiplier Tube (PMT)	121
3.2.14	Avalanche Photodiode (APD)	122

3.3	Types of Noise in a Digital Microscopy Image	123
3.4	Quantitative Fluorescence Microscopy	127
3.4.1	Measurements of Fluorescence Intensity and Concentration of the Labeled Target	127
3.4.2	Ratiometric Measurements (Ca ⁺⁺ , pH)	130
3.4.3	Measurements of Dimensions in 3D Fluorescence Microscopy	131
3.4.4	Measurements of Exciting Light Intensity	132
3.4.5	Technical Tips for Quantitative Fluorescence Microscopy	132
3.5	Limitations of Fluorescence Microscopy	133
3.5.1	Photobleaching	133
3.5.2	Reversible Photobleaching under Oxidizing or Reducing Conditions	134
3.5.3	Phototoxicity	135
3.5.4	Optical Resolution	135
3.5.5	Misrepresentation of Small Objects	137
3.6	Current Avenues of Development	139
	References	139
	Further Reading	141
	Recommended Internet Resources	142
	Fluorescent Spectra Database	142
4	Fluorescence Labeling	143
	<i>Gerd Ulrich Nienhaus and Karin Nienhaus</i>	
4.1	Introduction	143
4.2	Principles of Fluorescence	143
4.3	Key Properties of Fluorescent Labels	144
4.4	Synthetic Fluorophores	149
4.4.1	Organic Dyes	149
4.4.2	Fluorescent Nanoparticles	150
4.4.3	Conjugation Strategies for Synthetic Fluorophores	152
4.4.4	Nonnatural Amino Acids	155
4.4.5	Bringing the Fluorophore to Its Target	156
4.5	Genetically Encoded Labels	158
4.5.1	Phycobiliproteins	158
4.5.2	GFP-Like Proteins	159
4.6	Label Selection for Particular Applications	163
4.6.1	FRET to Monitor Intramolecular Conformational Dynamics	163
4.6.2	Protein Expression in Cells	167
4.6.3	Fluorophores as Sensors inside the Cell	167
4.6.4	Live-Cell Dynamics	168
4.7	Conclusions	168
	References	169

5	Confocal Microscopy	175
	<i>Nikolaus Naredi-Rainer, Jens Prescher, Achim Hartschuh, and Don C. Lamb</i>	
5.1	Introduction	175
5.1.1	Evolution and Limits of Conventional Widefield Microscopy	175
5.1.2	History and Development of Confocal Microscopy	177
5.2	The Theory of Confocal Microscopy	180
5.2.1	The Principle of Confocal Microscopy	180
5.2.2	Radial and Axial Resolution and the Impact of the Pinhole Size	182
5.2.3	Scanning Confocal Imaging	189
5.2.4	Confocal Deconvolution	194
5.3	Applications of Confocal Microscopy	196
5.3.1	Nonscanning Applications	196
5.3.2	Advanced Correlation Techniques	200
5.3.3	Scanning Applications Beyond Imaging	205
	Acknowledgments	210
	References	210
6	Fluorescence Photobleaching and Photoactivation Techniques	215
	<i>Reiner Peters</i>	
6.1	Introduction	215
6.2	Basic Concepts and Procedures	216
6.2.1	Putting Photobleaching to Work	216
6.2.2	Setting Up an Instrument	219
6.2.3	Approaching Complexity from Bottom Up	220
6.3	Fluorescence Recovery after Photobleaching (FRAP)	221
6.3.1	Evaluation of Diffusion Measurements	222
6.3.2	Binding	225
6.3.3	Membrane Transport	226
6.4	Continuous Fluorescence Microphotolysis (CFM)	228
6.4.1	Theoretical Background and Data Evaluation	229
6.4.2	Combination of CFM with Other Techniques	231
6.4.3	CFM Variants	232
6.5	Confocal Photobleaching	233
6.5.1	Use of Laser Scanning Microscopes (LSMs) in Photobleaching Experiments	233
6.5.2	New Possibilities Provided by Confocal Photobleaching	234
6.5.3	Artifacts and Remedies	237
6.6	Fluorescence Photoactivation and Dissipation	238
6.6.1	Basic Aspects	238
6.6.2	Theory and Instrumentation	239
6.6.3	Reversible Flux Measurements	239

6.7	Summary and Outlook	241
	References	241
7	Förster Resonance Energy Transfer and Fluorescence Lifetime Imaging	245
	<i>Fred S. Wouters</i>	
7.1	General Introduction	245
7.2	FRET	246
7.2.1	Historical Development of FRET	246
7.2.2	Requirements	254
7.2.3	FRET as a Molecular Ruler	258
7.2.4	Special FRET Conditions	262
7.3	Measuring FRET	265
7.3.1	Spectral Changes	266
7.3.2	Decay Kinetics	272
7.4	FLIM	280
7.4.1	Frequency-Domain FLIM	282
7.4.2	Time-Domain FLIM	283
7.5	Analysis and Pitfalls	285
7.5.1	Average Lifetime, Multiple Lifetime Fitting	285
7.5.2	From FRET/Lifetime to Species	286
	Summary	287
	References	288
8	Single-Molecule Microscopy in the Life Sciences	293
	<i>Markus Axmann, Josef Madl, and Gerhard J. Schütz</i>	
8.1	Encircling the Problem	293
8.2	What Is the Unique Information?	295
8.2.1	Kinetics Can Be Directly Resolved	295
8.2.2	Full Probability Distributions Can Be Measured	296
8.2.3	Structures Can Be Related to Functional States	297
8.2.4	Structures Can Be Imaged at Superresolution	298
8.2.5	Bioanalysis Can Be Extended Down to the Single-Molecule Level	300
8.3	Building a Single-Molecule Microscope	301
8.3.1	Microscopes/Objectives	301
8.3.2	Light Source	304
8.3.3	Detector	310
8.4	Analyzing Single-Molecule Signals: Position, Orientation, Color, and Brightness	316
8.4.1	Localizing in Two Dimensions	316
8.4.2	Localizing along the Optical Axis	318
8.4.3	Brightness	320
8.4.4	Orientation	321
8.4.5	Color	322

8.5	Learning from Single-Molecule Signals	323
8.5.1	Determination of Molecular Associations	323
8.5.2	Determination of Molecular Conformations via FRET	325
8.5.3	Superresolution Single-Molecule Microscopy	329
8.5.4	Single-Molecule Tracking	332
8.5.5	Detecting Transitions	332
	Acknowledgments	334
	References	334
9	Super-Resolution Microscopy: Interference and Pattern Techniques	<i>345</i>
	<i>Gerrit Best, Roman Amberger, and Christoph Cremer</i>	
9.1	Introduction	345
9.1.1	Review: The Resolution Limit	346
9.2	Structured Illumination Microscopy (SIM)	347
9.2.1	Image Generation in Structured Illumination Microscopy	349
9.2.2	Extracting the High-Resolution Information	352
9.2.3	Optical Sectioning by SIM	353
9.2.4	How the Illumination Pattern is Generated	355
9.2.5	Mathematical Derivation of the Interference Pattern	355
9.2.6	Examples for SIM Setups	358
9.3	Spatially Modulated Illumination (SMI) Microscopy	362
9.3.1	Overview	362
9.3.2	SMI Setup	363
9.3.3	The Optical Path	364
9.3.4	Size Estimation with SMI Microscopy	366
9.4	Application of Patterned Techniques	368
9.5	Conclusion	372
9.6	Summary	372
	Acknowledgments	373
	References	373
10	STED Microscopy	<i>375</i>
	<i>Travis J. Gould, Patrina A. Pellett, and Joerg Bewersdorf</i>	
10.1	Introduction	375
10.2	The Concepts behind STED Microscopy	376
10.2.1	Fundamental Concepts	376
10.2.2	Key Parameters in STED Microscopy	380
10.3	Experimental Setup	384
10.3.1	Light Sources and Synchronization	384
10.3.2	Scanning and Speed	385
10.3.3	Multicolor STED Imaging	386
10.3.4	Improving Axial Resolution in STED Microscopy	388
10.4	Applications	388
10.4.1	Choice of Fluorophore	388

10.4.2	Labeling Strategies	389
	Summary	390
	References	391
A	Appendix: Practical Guide to Optical Alignment	393
	<i>Rainer Heintzmann</i>	
A.1	How to Obtain a Widened Parallel Laser Beam	393
A.2	Mirror Alignment	395
A.3	Lens Alignment	396
A.4	Autocollimation Telescope	396
A.5	Aligning a Single Lens Using a Laser Beam	397
A.6	How to Find the Focal Plane of a Lens	399
A.7	How to Focus to the Back Focal Plane of an Objective Lens	400
	Index	403