



Simultaneous dual-color imaging on single-molecule level on a Widefield microscope and applications

Ralph Ledesch

Schlüsseltechnologien / Key Technologies
Band / Volume 178
ISBN 978-3-95806-348-8

Forschungszentrum Jülich GmbH
Institute of Complex Systems
Molekulare Biophysik (ICS-5)

Simultaneous dual-color imaging on single-molecule level on a Widefield microscope and applications

Ralph Ledesch

Schriften des Forschungszentrums Jülich
Reihe Schlüsseltechnologien / Key Technologies

Band / Volume 178

ISSN 1866-1807

ISBN 978-3-95806-348-8

Contents

1	Introduction	1
1.1	The development of Fluorescence microscopy	1
1.2	Intents and structure of this work	3
2	Theoretical background	5
2.1	Fluorescence	5
2.1.1	Electronic states and the Jablonski diagram	5
	Excitation	6
	Excited state lifetime	6
	Fluorescence	6
	Inter-system crossing and Phosphorescence	6
	Photo-destruction	7
	Rate equations and the fluorescence quantum yield	7
	Fluorescence rate and the molecular brightness	8
	Quenching	8
2.2	Resolution in light microscopy	9
2.2.1	Gaussian approximation of the diffraction pattern	10
3	Sample preparation	13
3.1	Preparation of the Tetraspeck bead slides	13
3.2	Buffers	13
3.3	Single Alexa and Cyanine dyes in a polymer film	14
3.4	Single GFPem on a plasma cleaned surface	14
3.5	Photoprotection systems	14
3.5.1	GOC O ₂ -scavenging system	15
3.5.2	PCD/PCA O ₂ -scavenging system	15
3.5.3	Trolox solution	15
3.6	Double-stranded DNA sample	15
3.7	Ribosome samples	16
3.7.1	Multi-labeled ribosomes	16
3.7.2	Cell-free protein synthesis kit	16
3.7.3	Single-labeled re-associated ribosomes	17
4	The Widefield microscope	19
4.1	Introduction	19
4.1.1	Excitation path	19
4.1.2	Objective	21
4.1.3	Emission path	22
	Image splitter	22
	EMCCD camera	22
4.1.4	Nyquist criterion	23
4.1.5	Signal-to-noise-ratio and noise sources	24
4.1.6	Filter set spectra and the setup's transmission function	26

4.2	Getting started with imaging	29
4.2.1	Excitation intensity at the cover slide surface	29
4.2.2	Alignment check with fluorescent beads	32
4.2.3	Resolving performance of the setup	32
4.2.4	Chromatic aberration	36
4.2.5	Measurements in solution with a confocal microscope	37
5	Data analysis	41
5.1	Introduction	41
5.2	The recorded image	41
5.3	The analysis routine	42
6	Imaging on single-molecule level	47
6.1	Introduction	47
6.2	Structure and photophysical properties of the employed fluorophores	47
6.2.1	Cy3 and Cy5	47
6.2.2	Alexa488 and Alexa647	49
6.2.3	GFP emerald	49
6.3	Parameter optimization for the imaging of single fluorophores	50
6.3.1	Observing single fluorophores on the surface	50
6.3.2	Preliminary considerations	52
6.3.3	Varying the illumination time	53
Measurements	53	
Results and comments	54	
6.3.4	Power-series	55
Observations and comments	58	
6.3.5	EM-gain series	59
Preliminary considerations	59	
Measurements	60	
Results and comments	61	
6.4	Photoprotection	63
6.4.1	Oxygen-scavengers	63
6.4.2	The O ₂ -scavengers' impact on the observation time	64
The labeled streptavidin-conjugates	65	
GFP emerald	67	
6.4.3	Comments	68
7	Surface preparation: Tethering single molecules	69
7.1	Introduction	69
7.2	Slide preparation protocol	69
7.3	The slide background	73
7.4	Testing the slide performance with Alexa647-streptavidin	74
7.5	Testing the linker accessibility with Atto655-conjugated biotin	78
7.6	Testing the slide performance with Cy5-multilabeled bioCAN-ribosomes and GFPM	79
7.7	Observations and comments	79
8	Results: Observing functional ribosomes on single-molecule level	81
8.1	Introduction: Colocalization measurements on the surface	81
8.2	Preliminary measurements with double-labeled DNA	81
8.2.1	Measurement	81

8.2.2	Data analysis	82
8.2.3	Results and comments	82
8.3	Surface measurements of GFPem synthesized by exogenous modified ribosomes	84
8.3.1	Introduction to the project	84
8.3.2	Negative controls	85
8.3.3	Measurements with the confocal microscope and co-precipitation FCS and TCCD measurements	85
8.3.4	Co-precipitation measurements	87
8.3.4	<i>In vitro</i> measurement on plasma cleaned glass slides	87
8.3.5	Data analysis and results	89
8.4	Measurements on bioPEG-slides	90
8.4.1	<i>In vitro</i> measurement	90
8.4.2	<i>In situ</i> measurement	90
8.4.3	Results and comments	91
8.5	Re-associated ribosomes	94
8.5.1	The measurements	94
8.5.2	Results	94
8.5.3	Stability of the re-associated ribosomes	95
8.5.4	Results and comments	96
9	Conclusions	99
9.1	Imaging on single-molecule level	99
9.2	The WFM as a tool for quantitative surface measurements on single-molecule level	101
A	Appendix	103
A.1	Filter spectra	104
A.2	Convolution of the setup's transmission function with the emission spectra of the employed fluorophores	105
A.3	Illumination intensity	107
A.4	Determination of the molecular brightness	107
Acknowledgements		109
Bibliography		111

Schlüsseltechnologien / Key Technologies
Band / Volume 178
ISBN 978-3-95806-348-8

Mitglied der Helmholtz-Gemeinschaft

