

AN EVANESCENT PERSPECTIVE ON
CELLS

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ABSTRACT

We have optically sectioned living cells to a maximum depth of ~250 nm using a Variable Angle-Total Internal Reflection Fluorescence Microscope (VA-TIRFM). This yields 3D images of cell membranes and nearby organelles similar to that gained by confocal microscopes but with at least an order-of-magnitude greater depth resolution. It also enables cellular membranes to be imaged in near isolation from cell organelles. Key to achieving this resolution was integration of a controllable excitation laser micropositioner into a standard through-the-lens TIRF illuminator and development of a custom culture dish for re-use of expensive high index of refraction cover slips. Images are acquired at several penetration depths by varying the excitation laser illumination angles. At the shallowest penetration depth (~46 nm) just the membrane and a few internal puncta are imaged. As the penetration depth is increased up to 250 nm organelles near the membrane, such as the ER, are imaged as well. The sequence of images from shallow deep is processed to yield a z-stack of images of approximately constant thickness at increasing distance from the coverslip. We employ this method to distinguish membrane-localized fluorophores (α 4 GFP β 2 nicotinic acetylcholine receptors and pCS2:lyn-mCherry) at the plasma membrane (PM) from those in near-PM endoplasmic reticulum (ERTracker green, α 4 GFP β 2 nicotinic acetylcholine receptors), on a z-axis distance scale of ~45 to ~250 nm in N2a cells. In doing so we observe occasional smooth ER structures that cannot be resolved as being distinct from the membrane.

In a second project substantial progress has been made towards developing a Tip Enhanced Fluorescence Microscope (TEFM) capable of imaging wet biological samples with ~10 nm resolution. A TEFM combines a TIRFM with an Atomic Force Microscope (AFM) to modulate sample fluorescence through near-field dipole-dipole coupling.

In the third project the capability to consistently produce high quality nanotube AFM probes was developed and a technique for chemically functionalizing the tip of a nanotube AFM probe was invented.

TABLE OF CONTENTS

Acknowledgements	iii
Abstract	iv
Table of Contents	v
List of Illustrations and/or Tables	vi
Chapter 1: Introduction	1
Chapter 2: In-vivo distinction between plasma membrane and near-membrane organelles using Variable Angle Total Internal Reflection Fluorescence Microscopy (VA-TIRFM)	4
2.1 Variable Angle Total Internal Reflection Fluorescence Microscopy overview	4
2.2 VA-TIRFM image processing	10
2.3 Methods	20
2.4 Results	24
2.5 Discussion	39
2.6 Summary	42
Chapter 3: Towards imaging fluorescently labeled proteins with Tip-Enhanced Fluorescent Microscopy (TEFM)	46
3.1 Dry TEFM imaging	47
3.2 Wet TEFM imaging of live cells and membrane-bound proteins	57
3.3 Summary and conclusions	73
Chapter 4: Nanotubes, imaging and proteins	75
Appendix A: VA-TIRFM supporting information	110
Appendix B: Cell, cleaning and surface chemistry protocols	128
Appendix C: US patent 6,953,927	143
Appendix D: US patent 7,247,842	199
Appendix E: Multicolor TEFM (pictures, list of filters, etc.) and DAC	255
Appendix F: Hue-Saturation-Value representation and correlation of multispectral/multi-modal datasets	337
Appendix G: US patent 7,211,795	346
Appendix H: US patent 7,514,214	375

LIST OF FIGURES

<i>Number</i>	<i>Page</i>
2.1. An epifluorescence microscope was used in this study	5
2.2. Typical TIRF microscope cannot distinguish	9
2.3. A cell can be imaged in pure TIRF.....	10
2.4. A five image VA-TIRFM sequence is shown.....	13
2.5. Differenced images taken with small changes	18
2.6. A cell culture chamber was fabricated	21
2.7. A control experiment	26
2.8. A second control experiment.....	29
2.9. A 3D image of a N2a cell	30
2.10. A 3D image of cell structures.....	31
2.11. A 250 nm thick 3D VA-TIRFM image.....	32
2.12. Intricate detail is visible	33
2.13. Many regions of apparent contact or unresolved proximity	34
2.14. A different N2a cell is pictured	36
2.15. Two views are given from the perspective.....	37
2.16. Bright nAChR puncta on membrane	38
3.1 The major components in a TEFM are schematically shown.....	46
3.2 Emission is stimulated when the AFM probe tip is proximate.....	47
3.3 A hybrid AFM/Inverted Optical microscope	63
3.4 An AFM image of a wet membrane ghost	67
3.5 An AFM image	68
3.6 Measurement 20091106Wbd1.....	69
3.7 Measurement 20091106Wbd3.....	70
3.8 Measurement 20091106Wbd5.....	71
3.9 Measurement 20091106Wbd5 zoomed in.....	72
A.1 Angle of incidence as a function of beam offset	112
A.2 Prism based TIRF measurement fit and data.....	113
A.3 Prism TIRF data compared to a linear fit.....	114

A.4 Full scale fit.....	115
A.5 Beam angle as a function of excitation beam position.....	116
A.6 The measured field intensity was about 10% (on average) lower	117
A.7 The measured field normalization factor.....	119
A.8 Cell culture chamber parts	122
A.9 An assembled cell culture dish	123
A.10 A fully assembled culture dish	124
A.11 The complete Olympus IX71 microscope.....	125
A.12 Laser alignment and positioning assembly.....	126
A.13 View of laser positioning assembly.....	127
E.1 The complete microscope from three sides	256
E.2 Three excitation lasers are combined and expanded	257
E.3 Two methods for achieving p-polarization of the evanescent field ..	258
E.4 In the foreground is the dichroic wheel #2	259
E.5 Shows the sample stage, AMF and TIRF microscope.....	260
E.6 Shows the AFM and TIRFM from the side	261
E.7 Shows the microscope objective, sample scanning stages	262
E.8 Dichroic wheels	263
E.9 APD #1	264
E.10 This shows the 543 nm HeNe laser, AOM	268
E.11 The 502/514 nm adjustable Argon Ion laser, AOM	269
E.12 The 442 nm HeCd laser, AOM	269
E.13 Several of the DAC electronics boxes	270
E.14 Top right can be seen the HeCd power supply	271
E.15 The tip-excitation laser alignment monitor.....	271
F.1 Block diagram from combining two datasets	344
F.2 Comparison of RGB-based and HSV-based image combinations	344
F.3 Top right can be seen the HeCd power supply	345

LIST OF TABLES

<i>Number</i>	<i>Page</i>
2.1 The index of refraction of several cellular components.....	8
2.2 Five image sequence with each image being the same thickness.....	16
2.3 Five image sequence-logarithmic	17
A.1 Angle as a function of beam position	111
A.2 Evanescent field intensity at the coverslip surface.....	118
A.3 Images selected and averaged for z-stack.....	120

Chapter I

INTRODUCTION

Over the course of my studies at the California Institute of Technology I've had the opportunity to work on three lines of research. All of these efforts employed novel imaging techniques to query the organization and composition of surface proteins involved in intercellular signaling. Most of this work was focused on nicotinic acetylcholine receptors (nAChRs).

Neuronal signals are transmitted across a synapse via transmitter-gated ion channels to either skeletal muscle cells or to neuronal cells. Nicotinic acetylcholine receptors are found in many central nervous system and nerve-skeletal muscle postsynaptic membranes. The nAChR has a (pseudo)symmetric pentameric structure comprised of homologous subunits. A wide variety of nAChR stoichiometries are possible as the α subunits exist in at least ten different subtypes ($\alpha 1$ through $\alpha 10$) and the β subunits exist in at least four subtypes ($\beta 1$ through $\beta 4$).¹ nAChRs can be activated by both acetylcholine and nicotine. Chronic exposure to nicotine has been found to cause upregulation of functional nAChRs with a preferred $(\alpha_4)_2(\beta_2)_3$ stoichiometry.^{2, 3, 4, 5, 6, 7}

The capability to image the distribution and composition of nAChRs pre-and post-synaptically in pulse-chase experiments would be very helpful to attempts to untangle the regulatory mechanisms behind nicotine induced upregulation.⁸ It would be particularly useful to do so if the functional nAChR composition and distribution (in the membrane) could be resolved or isolated from the composition and distribution of nAChRs sequestered in nearby organelles of living cells.⁹ From a larger perspective this is capability would be useful for conducting pulse-chase experiments to illuminate trafficking and regulatory mechanisms of many types of functional surface receptors.

So motivated, I collaborated with the Henry Lester group, to extend Total Internal Reflection Microscopy (TIRFM) to achieve z-axis resolution sufficient to discriminate cellular membranes from nearby organelles via Variable Angle Internal Reflection

Microscopy (VA-TIRFM). We also took initial steps towards using the information acquired while VA-TRIFM imaging to construct three-dimensional images of cell membranes and nearby cellular organelles. We then successfully utilized this technique to image murine neuroblastoma cells (N2a) that had been transfected with fluorescently labeled nAChRs. This technique and our results are described in Chapter 2.

In the second project, we developed a Tip-Enhanced Fluorescence Microscope (TEFM) with the capability to resolve single molecules with <10 nm separation in collaboration with the Quake group.^{10,11} Later I attempted to use this technique to image surface proteins in a biological environment (warm and wet) as part of the Scott Fraser group. This effort and its results are described in some detail in Chapter 3.

In the third project, conducted primarily in collaboration with the Pat Collier group, we developed the capability to fabricate nanotube Atomic Force Microscope (AFM) probes.¹² The mechanisms behind nanotube adhesion, and the surprisingly high AFM imaging resolution achieved with nanotube AFM probes, was illuminated through atomistic modeling.¹³ Finally we explored utilization of such probes for molecular patterning.^{14,15} To enable substrate patterning we invented a novel technique for uniquely functionalizing the end of a nanotube probe. This work is described in Chapter 4.

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