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 (α4 GFP β2 nicotinic acetylcholine receptors and pCS2:lyn-mCherry) at the plasma membrane (PM) from those in near-PM endoplasmic reticulum (ERTracker green, α4 GFP β2 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.

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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 stochiometries are possible as the α subunits exist in at least ten different subtypes (α1 through α10) and the β subunits exist in at least four subtypes (β1 through β4).[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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