nanoparticles including asymmetric quantum dots as non-bleaching probes of the rotation of individual cell surface proteins. Invitrogen QD655s conjugated to A2 DNP-specific IgE allow examination of slow rotation of the Type I Fcε receptor (FcεRI) on RBL-2H3 cells. Fluorescence from cell-bound quantum dots is excited by illumination polarized at 45 deg and a Princeton Instruments Dual View equipped with a polarizing beam splitter allows recording image sequences containing simultaneous x- and y-polarized sub-images in each frame. For individual quantum dots, we calculate the time-autocorrelation functions for fluorescence polarization fluctuations. Decays of these fluctuations extend down to the ms timescale, as implied by time-resolved phosphorescence anisotropy results. Treatment effects suggest that such slow decay may be a property of the membrane itself, perhaps reflecting large-scale fluctuations of mesoscale membrane regions. To examine rotational correlation function decays *faster* than imaging measurements permit, we have applied time-tagged single photon counting to individual quantum dot fluorescence and analyzed data down to correlation times below 1μs. These measurements *should* thus include the 80μs hydrodynamic rotation of single FcεRI molecules, but significance of current data is limited by quantum dot emission rates. Other probes are therefore being explored as are the consequences of the two-dimensional transition dipoles exhibited by quantum dots. Supported by NSF grant MCB-1024668.

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Fluorescence Fluctuation Spectroscopy of Ternary Protein Interactions in Living Cells

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Because many proteins contain multiple interaction sites, the formation of ternary or higher-order protein complexes are frequently encountered and play a critical role in many cellular processes. Fluorescence fluctuation spectroscopy (FFS) characterizes the brightness of fluorescently-labeled proteins, which provides a quantitative and noninvasive method to characterize protein interactions in living cells. Here we focus on extending two-photon brightness microscopy to the characterization of three interacting protein species, with each species carrying a differently colored tag. The fluorescence signal is split into three detection channels by optical filters. We discuss the choice of tags and filters that optimize the signal-to-noise ratio of resolving species, and the influence of spectral cross-talk on data analysis by time-integrated cumulants of the photon counts. While proof of principal experiments with dyes are useful, our main challenge remains the limited choice of fluorescence proteins that have simple photophysics and good color separation for cellular applications. We characterize different combinations of fluorescent proteins and evaluate their potential for three-color FFS studies of ternary protein interactions. This work has been supported by grants from the National Institutes of Health (GM64589) and the National Science Foundation (PHY 0346782).

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Brightness Characterization of Internal Endomembrane Proteins by Z-Scan Fluorescence Fluctuation Spectroscopy

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Traditionally, fluorescence fluctuation spectroscopy (FFS) has been used to quantify the stoichiometry of soluble proteins in the nucleus and the cytoplasm of mammalian cells by brightness analysis. The development of z-scan FFS broadened the capability of brightness analysis to include proteins distributed across stratified layers, such as the cytoplasm and the plasma membrane. In this work, we further extend z-scan FFS to study proteins that reside on or within internal endomembranes, including the endoplasmic reticulum (ER) membrane/lumen and the nuclear envelope/perinuclear space. Experimentally, we place a 20 amino acid ER signal sequence in front of EGFP (SS-EGFP); the complex is translated into the lumen of the ER where of the signal sequence is cleaved, leaving EGFP to diffuse within the ER and nuclear envelope. The brightness of SS-EGFP is determined by performing z-scan FFS measurements where corrections are applied for both the thin layer geometry and coexcitation of adjacent layers. Additionally, we create and test a tandem dimeric EGFP protein (SS-EGPF2) to establish a model for calibrating brightness and stoichiometry. Finally, we test the limits of our technique and apply z-scan FFS to characterize the brightness of other proteins found within the endomembrane system. This research was supported by grants from the National Institutes of Health (R01GM064589) and the National Science Foundation (PHY-0346782).

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The Functional Incorporation of Tyr-Coumarin Into Proteins Synthesized by HEK293T Cells

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The generation of novel tools for the observation of natural phenomenons it's a constant (pre)occupation for researchers of all areas. Therefore engineering of new methodological approaches capable of alter the biochemical properties of proteins in vivo will be always beneficial for the broad fields of natural sciences. The use of un-natural amino acids (UAAs) into proteins in response of TAG codon recognition had emerged as a promising strategy to manipulate protein structure in vitro and in vivo. One of the strategies for UAA incorporation is one that implicate the evolution of wild type aatRNA/aaRS pairs to a unique pair able to recognize specifically the UAA for its introduction into the TAG mutant on living systems. Our laboratory proposed to engineer a tRNA/RS pair allowing for the incorporation of a version of tyrosin, modified with the coumarin fluorophore (Tyr-coum) into proteins synthesized by mammalian cells.

To engineer the eukariotic RS we used a bacterial version as template. Rational design was guided by molecular modelling and mutations were incorporated by site-directed mutagenesis. The efficiency of incorporation was evaluated by microscopy at the level of single cell.

Our work show that the seven point mutations made over the tyrosil-tRNA synthetase are enough to modify the enzyme performance, now allowing the functional incorporation of Tyr-coum into TAG mutant proteins synthesized by HEK293T cells. Single molecule imaging was obtained and evaluated.

One of the mayor projections of this work is related with the acquisition of structural information from coumarin's fluorecence fluctuations on living systems with a high signal-to-noise ratio.