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FCS and Sub-diffraction Resolution Fluorescence Imaging of Membrane Receptors in Living Organelles

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Fluorescence microscopy is a standard tool in molecular biophysics, but even the best resolution obtained by diffraction-limited conventional optical techniques misses the molecular level by two orders of magnitude. In order to overcome the classical diffraction limit, several sub-diffraction resolution imaging methods have been introduced so far.

Direct stochastic optical reconstruction microscopy (dSTORM) (1) and PAINT (points accumulation for imaging in nanoscale topography) (2) have the potential to shed light on the intracellular organization of cells with near-molecular resolution.

These techniques will be used to localize labelled peptides binding to receptors located in the membrane of protoplasts of the flowering plant *Arabidopsis* (a model organism for plant research). Binding dynamics will be studied by fluorescence correlation spectroscopy (FCS).

References:

(1) M. Heilemann et al., Angew. Chem. Int. Ed. 2008 **47** p. 6172.
(2) M. Hochstrasser et al., PNAS **103** p. 18911 (2006).

134-Pos   Board B13

Single molecule image deconvolution. I. Standard deviation analysis of immobile fluorescent molecules

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Single molecule fluorescence imaging has been a powerful technique in studying individual processes not accessible by bulk, ensemble-averaged measurements [1]. Improvements in image analysis are required for high temporal and spatial precision in the localization of single fluorescent molecules. We present the first thorough standard deviation analysis for point spread functions (PSFs) of single immobile fluorescent molecules. Using this new single molecule image deconvolution (SMID) method, we show that 3D localization of individual molecules with sub-nanometer precision can be achieved. We have derived an expression estimating the standard error of the PSF's standard deviation, incorporating experimental effects of the number of collected photons, finite pixel size, and background noise. The localization precision obtained via this expression is approximately 1.5 times better than the current available methods. The use of SMID to extract subexposure dynamics of mobile molecules will also be discussed.

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135-Pos   Board B14

EstimationTool And FandPLimittool - User-friendly Software Packages For Single Molecule Localization/Resolution And Accuracy Calculations.

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The 2D/3D localization and resolution of single molecules from their images has the potential to provide much biological insight. However, they are complex and challenging tasks that involve fitting various model profiles to single molecule images using various parameter estimation algorithms. We developed the EstimationTool (1) to facilitate this process. Through a user-friendly graphical user interface, the tool allows a user to select from a variety of localization/resolution tasks, select from a variety of image models (Gaussian, Airy, Born-Wolf), noise models (Poisson, Gaussian), and estimation algorithms (maximum likelihood, least squares), and customize every aspect of the associated calculations. The accuracy of results from the various localization/resolution tasks varies based on the estimation algorithm, noise model, and image profile used. Therefore it is important to know the best possible accuracy with which the location of a single molecule or the distance between closely-spaced single molecules can be estimated. Various localization/resolution measures have been developed for this purpose (2,3,4,5). The FandPLimitTool (Fundamental and Practical Limit Tool) is a software package that calculates these localization/resolution measures for various estimation scenarios (6). Together, the EstimationTool and FandPLimitTool provide significant assistance in the quantitative analysis of single molecule data.

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Maximum-Likelihood Position Sensing of Single Molecules in a Confocal Microscope

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In wide-field microscopy, the location of a single molecule can be measured to within tens of nanometers by imaging the point spread function over a number of camera pixels and finding the center of the image. However, for many single-molecule applications, confocal microscopy is preferable to wide-field imaging, as it provides improved signal-to-noise ratio due to the very small detection volume; it is necessary for two-photon excitation, which offers potential advantages for intracellular studies; and it facilitates monitoring of sub-millisecond dynamics and fluorescence lifetimes by use of a single-photon avalanche diode (SPAD) detector for time-resolved single-photon counting. Here, we report studies of the capabilities for sub-diffraction, single-nanoparticle position determination in a confocal two-photon microscope. To measure the position, the beam from a femtosecond laser is split and recombined at beam splitters to produce four beams that are focused to slightly offset spatial positions centered at the vertices of a tetrahedron, and with pulses that are temporally offset so as to yield pulse-interleaved excitation at the four overlapping focal volumes. Two-photon-excited fluorescence is collected from the entire four-beam excitation volume onto just one SPAD detector. Time-gated photon detection provides information on the volume from which each photon was most likely emitted and hence, the most likely position of the single particle. As this form of position sensing requires only a single SPAD, it is scalable to multiple detectors for multi-color observations, and can thus be used to find the separations of differently colored molecules over a distance range that is complementary to that achievable by FRET.

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Quantitative Study Of Single Molecule Localization Techniques

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Among the various techniques by which the locations of single molecules can be estimated from microscopy images, the question arises as to which produces the most accurate results. The accuracy of an estimation technique is measured by the standard deviation of its estimates. The estimated location of a single molecule can deviate from its true location because of the stochastic nature of the photon emission/detection process, extraneous additive noise, and pixelation. Here we examine the estimates from the maximum likelihood and the non-linear least squares estimators when fitting Airy and Gaussian profiles to single molecule images. We see that on average both estimators recover the true location of the single molecule. Comparing the standard deviations of the estimates from both estimators, the maximum likelihood estimator appears to be generally more accurate. Since the accuracy of estimation techniques varies, it is important to know the best possible accuracy that can be achieved for a given set of imaging conditions. We have previously developed a method by which this can be determined (1). We find that the accuracy of the maximum likelihood estimator is typically close to the best possible accuracy. We also observe that localization accuracy is dependent on the specific image profile used to fit the data. Gaussian profiles are often used to fit single molecule images even though the image of an in-focus point source may be modeled more accurately by an Airy profile in many cases. We explore the effect on localization accuracies of performing estimations with such a model mismatch.

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Creation and Mixing of Monodisperse Sub-femtoliter Bioreactors

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We have developed and describe a method to generate monodisperse optically-trappable aqueous emulsion droplets ("hydrosomes") on demand by piezoelectric injection. The droplets have been measured to have radii as small as 368 nm $\pm$ 16 nm, corresponding to a volume of 212 aL $\pm$ 27 aL. The hydrosomes are injected into a perfluorocarbon continuous phase. The refractive index of the