accuracy in distance measurements between two different color dye-molecules attached at known positions along a surface tethered bio-molecule. The statistical uncertainty in the mean for an ensemble of N~10 identical single molecule samples is limited only by the total number of collected photons to ~0.3nm, or ~0.002 of the width of the optical PSF. We further show how our method can be used to improve the resolution of many sub-wavelength, far-field imaging methods such as those based on co-localization of stochastically excited fluorescent molecules.
Conclusion: We demonstrate sub-nanometer resolution in measurements of molecular-scale distances using far-field fluorescence imaging optics, at room temperature and in physiological buffer conditions. The improved resolution will allow deciphering in real-time, at the single molecule level the structure and dynamics of large, multi-subunit biological complexes.

**1984-Plat**
**Visualizing Single-proteins On A Single DNA Molecule With Super-resolution**
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DNA-proteins interactions can be studied in great detail when precise and dynamic control of the mechanical state of a single DNA molecule can be achieved together with direct visualization of proteins interacting with the DNA. Our custom-built experimental setup combines optical trapping and wide-field epifluorescence microscopy. In this setup we can visualize single DNA-bound proteins (see figure) with an accuracy of tens of nanometers while at the same time we can control the tension applied on the DNA at a sub-PicoNewton level.
Here we report the limit of localization accuracy in combined DNA-trapping/fluorescence experiments by fitting a point-spread function to the fluorescence image of single DNA-bound proteins.
We investigate the impact of DNA dynamics on the maximum attainable accuracy for the localization of these DNA-bound fluorescent proteins (in this case a Ca2+ inactivated restriction enzyme EcoRV). In particular, we study the effect of tension on the DNA and identify the force regime in which single-proteins can be localized with super-resolution.

**1985-Plat**
**Visualizing the Receptor Assembly Into Clathrin-coated Pits with Super-resolution Two-color Palm and sptPALM**
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The dynamic assembly of receptors into endocytic structures such as clathrin-coated pits underlies cellular response to many external signals. However, the biophysical mechanisms for the regulated uptake of select receptors are unresolved. While commonly used bulk measurements access only ensemble-averaged behaviors, single molecule measurements have largely been limited to looking at only a few molecules in a single cell, lacking true ensemble information. Thus, until recently, the study of receptor capture by clathrin-coated pits has been limited by a scarcity of experimental methods capable of accessing information on ensembles of individual molecules within an individual cell. We addressed this by combining two recent technological advances to image receptors and clathrin-coated pits in living cells: two-color imaging with the photoswitchable PA-mCherry fluorescent label, and single particle tracking photoactivated localization microscopy (sptPALM).
As compared to other monomeric red photoactivatable proteins, PAmCherry1 has higher pH stability, faster maturation and photoactivation kinetics, better photostability, and similar number of photons in single-molecule imaging. Furthermore, lack of background green fluorescence makes PAmCherry1 an advanced probe for two-color diffraction-limited microscopy and super-resolution techniques such as PALM. Two-color PALM imaging of PAmCherry1 tagged to the transferrin receptor (TfR) and PAGFP fused with the clathrin light-chain (CLC) were performed. Pair correlation analysis suggests clusters of less than 200 nm in size with both distinct and overlapping distributions of the TfR and CLC chimeras at sub-diffraction 25 nm resolutions. In addition, sptPALM was used to create spatially resolved maps of the trajectories of single receptor motions in conjunction with SPT of diffraction-limited clathrin-coated pits.

**1986-Plat**
**A Single-Molecule Study of Gene Regulation in Real Time**
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Gene expression is inherently stochastic, yet must be tightly regulated to properly carry out essential cellular processes. However, many of the transcription factors responsible for this precise regulation are expressed at extremely low copy numbers in cells. To understand how noise in gene expression is controlled to overcome this challenge, it is important to directly monitor gene regulation in real time at the single-molecule level.
In this work, we use the genetic switch of lambda phage as a model system to demonstrate continuous monitoring of autoregulation of lambda repressor CI in living *E. coli* cells. CI exhibits both positive and negative feedback on its own expression at low and high intracellular concentrations, respectively. We generated a single-molecule, gene-expression reporter that enables counting of the exact number of CI molecules expressed in real time without compromising CI's regulatory activity. Over several cell generations, we observed highly clustered expression of CI separated by relatively long periods of low expression, which we attribute to the alternating positive and negative autoregulation of CI. Our results suggest that the noise in CI expression is controlled by coupled positive and negative feedback, a mechanism also implicated in regulation of the eukaryotic cell cycle and circadian rhythm. This method should be applicable to similar studies probing gene regulation in other systems.

**1987-Plat**
**Pitfalls In Single Particle Tracking In Living Cells**
**Ralf Metzler**[1], Yong He[2], Stas Burov[2], Eli Barkai[2].
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An increasing body of evidence for subdiffusion of biopolymers under typical in vivo conditions has been reported recently. The physical foundation of this subdiffusion remains unidentified although it is commonly ascribed to molecular crowding. Single particle tracking provides crucial information on the mechanisms behind the subdiffusion. In several such experiments the measured mean squared displacement shows a characteristic scatter (e.g., [1,2]).
Using the widely accepted continuous time random walk framework we demonstrate that pronounced scatter in time averaged quantities such as the mean squared displacement is no artefact but arises naturally from the nonexistence of a characteristic time scale separating microscopic and macroscopic events [3,4]. An expression for the broad distribution of diffusion coefficients in such measurements is derived and confirmed by simulations. The most crucial finding from our theory is that the subdiffusive nature of the particles will be masked in the time averages: What looks like normal diffusion in an experiment may in reality be subdiffusion in an ageing system. Interpretations of the reported data in [1,2] will be discussed. We provide general guidelines to properly intepret single molecule tracking data.
We also argue that ageing properties in biopolymer diffusion in living cells may be advantageous for the accuracy of genetic regulation at minimal concentrations of transcription factors. The physical picture emerging from our theory provides additional support for a more local picture of gene regulation and confirms the importance of colocalisation in the genome.
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**1988-Plat**
**New Single Molecular Detection System from Three-Dimensional Tracking of Single Nanocrystals using Scanning Electron Microscope**
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Recent technological progress in dynamical observations of individual functional single protein molecules in living cell has been achieved with several single molecular techniques and systems. In order to improve monitoring precisions and stability of the signal intensity from single molecular units under physiological conditions, we have proposed that single molecular techniques using shorten wavelength, for example, X-rays, electrons, neutron, and other accelerated ion probes. In this work, we demonstrate three-dimensional tracking of single nanocrystals using Scanning Electron Microscope. We called Diffracted Electron Tracking (DET).
Diffracted X-Ray tracking (DXT) [1] has been developed for obtaining the information about the dynamics of single molecules. This method can observe the

rotating motion of an individual nanocrystal, which is linked to specific sites in single protein molecules, using a time-resolved Laue diffraction technique. This method needs a very strong X-ray source, such as the SPring-8, so we began to develop a compact instrument for monitoring the rotation of the single protein molecules, using the electron beam instead of the X-ray.
Instead of the Laue diffraction using white X-ray, the Electron Back-Scattered Diffraction Pattern (EBSP) is adopted to monitor the crystal orientation of the nano-crystals linked to the single protein molecules. For this purpose, it is necessary to realize (1) wet cell with very thin sealing film, (2) EBSP system with high sensitivity, (3) damage-less electron irradiation technique and (4) perfect gold nano-crystals.
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**1989-Plat**
**Calibration of Holographic Optical Tweezers for Force Measurements on Biomaterials**
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Simon Fraser University, Burnaby, BC, Canada.
Optical tweezers have been widely applied in the field of single-molecule biophysics, as the picoNewton forces that can be exerted and measured with this noninvasive technique lie in the force range of many biomolecular properties and events. By using trapped micrometer-sized particles as handles, the force-extension relations of macromolecules such as DNA and proteins have been probed. When probing more complex systems, however, such as cells or protein networks, the 3D character of these materials requires more flexibility in manipulating particles. With holographic optical tweezers, multiple optical traps can be manipulated independently in three dimensions in real time, adding this necessary flexibility to the interactive control over multiple particles. Thus far, however, holographic tweezers have not been an accepted tool in the biophysics community, in large part due to lack of evidence as to how exerted forces vary as the positions of holographic traps are changed.
To perform quantitative force measurements, parameters such as trap stiffness and its position dependence, range of trap steering, and minimum step size are of key importance. Here, we systematically characterize the stiffness of traps within our holographic tweezers setup, in which high-speed (>kHz) camera imaging is used for particle position detection. We create multiple traps and steer one or more over small and large distances, and find that over a range of ~25 μm the trap stiffness does not change significantly. Also, we determine the efficiency with which the laser power is directed towards intended traps. In addition, we control and detect trap displacements to ~1 nm, comparable to the position detection limit of our system. Our results suggest that after full characterization, holographic optical tweezers can be successfully employed in quantitative experiments on biomaterials, e.g., probing elastomeric properties of structural protein networks.

**1990-Plat**
**Measuring the Molecular Scale Dynamics of Protein Receptor Endocytic Trafficking in Neural Cells using Quantum Dot Bioconjguate Probes**
**Tania Q. Vu**.
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Protein trafficking is critical in neurons since neurons must orchestrate the movement of a plethora of discrete intracellular signaling proteins from the cell body to the ends of their axons, over distances that may span up to several meters. A problem in studying protein trafficking has been a lack of tools to visualize the movement of discrete proteins inside live neurons, in real time. An integrated understanding of endocytic trafficking at the level of single or small numbers of receptor complexes inside live cells is currently hampered by technical limitations. Here, we develop and apply quantum dot QD bioconjugates for imaging discrete receptor endocytic events inside live neural cells. QD probes can bind with specific cognate receptors consequently cell signaling cascades to regulate neural sprouting. Furthermore, QD-receptor complexes are internalized by cells dynamically traffic discrete receptor bound QDs on their membrane surface as well as along vast distances along intracellular microtubule tracks of neural processes. Using single particle tracking and immuno-colocalization, we illustrate and validate the use of QD-receptor complexes for imaging receptor trafficking at synchronized time points after QD-receptor binding and internalization (t= 15-150 minutes). The unique value of these probes is illustrated by new dynamic observations: 1) that endocytosis proceeds at strikingly regulated fashion, and 2) that diffusive and active forms of transport inside cells are rapid and efficient. QDs are powerful intracellular probes that can provide investigators with new capabilities and fresh insight for studying endocytic receptor signaling events, in real time, and at the resolution of single or small numbers of receptors in live cells.

# Platform AN: Actin & Actin-binding Proteins

**1991-Plat**
**Depletion of F-actin Near Surfaces**
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Proximity to membranes is required of actin networks for many key cell functions, including mechanics and motility. However, F-actin rigidity should hinder a filament's approach to surfaces. Using confocal microscopy, we monitor the distribution of fluorescent actin near non-adherent glass surfaces. Initially uniform, monomers polymerize to create a depletion zone where F-actin is absent at the surface but increases monotonically with distance from the surface. At its largest, depletion effects can extend >35 μm, comparable to mass-weighted filament lengths. Increasing the rigidity of actin filaments with phalloidin increases the extent of depletion, whereas shortening filaments using capping protein reduces it proportionally. In addition, depletion kinetics are faster with higher actin concentrations, consistent with faster polymerization and faster Brownian-ratchet-driven motion. Conversely, the extent of depletion decreases with actin concentration, suggesting that entropy is the thermodynamic driving force. Quantitatively, depletion kinetics and extent match existing actin kinetics, rigidity and lengths. However, explaining depletion profiles and concentration-dependence (power-law of -1) requires modifying the rigid rod model. Dynamically crosslinked and dendritic (ARP2/3) networks either slow or enhance the extent of depletion, respectively. In cells, surface depletion should slow membrane-associated F-actin reactions another ~10-fold beyond hydrodynamic considerations, and to favor membrane invaginations by decreased surface tension. Similar depletion principles underlie the thermodynamics of all surface-associated reactions with mechanical structures, ranging from DNA to filaments to networks. For various functions, cells must actively resist the thermodynamics of depletion.

**1992-Plat**
**Interactions of WASp Nucleation Promoting Factors with Fission Yeast Arp2/3 Complex**
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Arp2/3 complex mediates the formation of actin filament branches during endocytosis and at the leading edge of motile cells. The minimum requirements to reconstruct Arp2/3 complex mediated nucleation in vitro are actin monomers, a nulceation promoting factor (NPF), mother actin filaments and Arp2/3 complex. Although several reaction parameters have been measured, the pathway of branch formation from these reactants is still ambiguous owing to missing parameters. We use the CA motifs from the C-terminus of the fission yeast NPF, Wsp1p, to investigate the effect of actin filaments on the interaction between Arp2/3 complex and VCA. Actin filaments increase the affinity of CA for Arp2/3 complex 6-fold (Kd=300nM without filaments and 50nM with filaments). Equilibrium binding experiments and isothermal titration calorimetry both indicate that Arp2/3 complex binds two CAs with different affinities. These results show that the mechanism of Arp2/3 complex mediated actin nucleation involves CA binding to two different sites.

**1993-Plat**
**Nanotether Extrusion to probe Membrane-Cytoskeleton Interaction in Model Systems**
**Clément Campillo**, Léa-Laetitia Pontani, Pierre Nassoy, Patricia Bassereau, Cécile Sykes.
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We have recently introduced a method allowing the reconstitution of a dynamic actin cortex inside a liposome (Pontani et al., Biophysical Journal, in press). Liposomes encapsulating the cellular machinery required for actin polymerization are prepared with the inverse emulsion technique and actin polymerization is triggered at the liposome internal membrane. Those liposomes can thus model the cellular cortex. To extrude membrane tethers from liposomes, we use a novel experimental setup combining micropipette aspiration and optical tweezers in a confocal microscope. The variation of the tether extrusion force at various membrane tension allow the measurement of the adhesion energy between the membrane and the cytoskeleton. We can also measure the elastic properties of the actin cortex by monitoring its deformation as a function of the aspiration pressure in the micropipette. This mechanical characterization of liposomes encapsulating an actin cortex opens the way to their further use as model systems to study cellular plasma membrane.