# Optical Microscopy and Super-Resolution Imaging I

**1607-Pos Board B558**
**An Automated Protocol for Performance Benchmarking a Widefield Fluorescence Microscope**
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Widefield fluorescence microscopy is a highly used tool for visually assessing biological samples and for quantifying cell responses. Despite its widespread use, few published methods exist for evaluating and benchmarking the analytical performance of a microscope. Easy-to-use benchmarking methods would facilitate the use of fluorescence imaging as a quantitative analytical tool in research applications. We describe and evaluate an automated method to characterize a fluorescence imaging system's performance by benchmarking the detection threshold, saturation and linear dynamic range to a reference material. The benchmarking procedure is demonstrated using two different materials as the reference material, uranyl-ion-doped glass and Schott 475 GG filter glass. Both are suitable candidate reference materials that are homogeneously fluorescent and highly photostable, and the Schott 475 GG filter glass is currently commercially available. In addition to benchmarking the analytical performance, we also demonstrate that the reference materials provide for accurate day to day intensity calibration. A script written in MicroManager, an open-source microscopy control software, has been developed to automate the procedure and return the benchmarked parameters. The MicroManager script is now publicly available. Ideally, this work will lead to common operating procedures that will help to assure that fluorescence microscopy results are meaningful, traceable, and comparable from day to day and between laboratories.

**1608-Pos Board B559**
**Respiration Rate Measurements of Single Bacterial Cells**
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One of the limitations of a population-based analysis method is that it averages over a large number of cells. While this helps average out fluctuations in the measured signal, it potentially can cover up subpopulations that could be functionally important. Using a single-cell approach, one can individually measure cells and look at the variation within the isogenic cell population.
Respiration can be an indicator of physiological state and therefore is an excellent target for analysis of heterogeneity within the sample. Single-cells are isolated in microwells containing Pt-porphyrin embedded microspheres on a glass chip. These microwells are diffusionally sealed with a lid actuator which can be raised at the end of the measurement to reoxygenate the sample. Since the phosphorescence lifetime of the Pt-porphyrin depends on the oxygen concentration, the Pt-porphyrin embedded microspheres can be used as an oxygen sensor. Monitoring the consumption of oxygen in the sealed microwells over time allows a respiration rate to be calculated for the individual cells. This method is compatible with other optical imaging techniques.

**1609-Pos Board B560**
**Development of Physiologically Based Pharmacokinetic Model (PBPK) of Cancer Treatment in Mice**
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In hormone-dependent cancers, tumor growth is driven by the binding of the hormone to its receptor. Vitamin D3 can have both preventive and therapeutic effects by regulating cell growth, cell cycle, apoptosis, and differentiation - a role much greater than earlier views that focused primarily on bone health and maintenance of calcium homeostasis. Epidemiological studies have found a significant association between low serum levels and low dietary intake of Vitamin D3 and the incidence, degree of malignancy, metastases, and mortality of cancers of the breast, prostate, colon, and ovaries. We used Semiconductor Nanoparticles (Quantum Dots, (QDs)) in conjunction with a binding procedure to develop a Calcitriol-QD conjugate. Based on this research a Physiologically-Based Pharmacokinetic (PBPK) model has been developed to determine steady-state distribution of Calcitriol-QD in mice. The multi-compartmental PBPK model represents relevant organ/tissues with physiological accuracy. Four processes characterized the change in the concentration of the protein in every compartment: blood flow in, blood flow out, protein turnover and receptor binding in the organ. The unique aspects of the model are the determination of elimination using receptor kinetics and generation using protein turnover. The model also predicts steady state concentrations of Calcitriol-QD in tissues in mice and may be used for possible scale-up of dosage regimens in humans. Due to the QDs fluorescent characteristics, our Calcitriol conjugates can be applied successfully to image the distribution and uptake of Calcitriol into cells. Our data show that we successfully generated a Calcitriol-QD conjugate that is biologically active and stable for at least 48h at RT. Additionally we were able to image the uptake and the distribution of Calcitriol in real time and determine its interaction with the cell membrane and accumulation in the cell nucleus.

**1610-Pos Board B561**
**Raising the Speed Limit on 3D-3Way FRET Microscopy**
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The spatial organization of protein-protein interactions inside of cells is an important component of cellular signaling. These interactions can be imaged using fluorescent resonance energy transfer (FRET) microscopy methods. We have described a generalized mathematical solution and method for any number of interacting FRET pairs (N-Way FRET). Here, we combine N-Way FRET with our three-dimensional FRET stoichiometry reconstruction (3D-FSR) to allow 3D-3Way-FRET image deconvolution. By developing a multi-camera instrument, we are able to acquire the 6 images needed per z-plane with sub-second time resolution, allowing observation of live cell dynamics. Furthermore, we implemented improved fast Fourier transforms and parallelized calculations to achieve a 10-fold decrease in the computational time required to reconstruct 3D-3Way-FRET data. This reduction has made it feasible to analyze dynamic 2Way and 3Way FRET data with 3D reconstructions, increase the number of reconstruction iterations, as well as explore stopping criteria for the algorithm.

**1611-Pos Board B562**
**Stretched, Oriented DNA Arrays (SODA) for Fluorescence Based Single-Molecule Experiments in Complex Environment**
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All biochemical and biophysical processes that support cellular activity take place in complex, crowded environments. Up to 30% of the weight of a cell consists of proteins, DNA and other large biological macromolecules. Consequently, 1-dimensional protein motions along DNA while replication or transcription have to be studied and understood in the context of a DNA molecule that is not naked, but instead bound by a wide variety of obstacles - roadblocks. Inspired by previous, pioneering work on DNA curtains, we used the intrinsic propriety of some macromolecules and polymers to create self-assembled, organized structures, adapted for visualization using TIRF microscopy of interactions between genome processing enzymes and roadblocks in crowded environment.

**1612-Pos Board B563**
**Dual-Switching FRET (dsFRET) Imaging Based on Photoswitchable Donor-Acceptor Pair**
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Fluorescence resonance energy transfer (FRET), especially with fluorescent proteins of donor and acceptor, has been widely used to measure biomolecular interactions. To overcome limitations of existing approaches for quantitative FRET, we here put forward a novel platform of dual-switching FRET (dsFRET), with a photoswitchable donor as well as a photoswitchable acceptor. With the photoswitchable capability from the FRET pair we constructed, neither donor-only nor acceptor-only samples would be required as control reference for calculation. Experiments of dsFRET and traditional $3^3$-FRET were conducted and compared in both dimer and two-hybrid forms. Our data demonstrate that dsFRET has higher accuracy and stability than $3^3$ -FRET, mainly benefited from *in-situ* references. Further development of dsFRET has been pursued, to enhance the performance of this new methodology, and also to extend its applications, such as in subcellular FRET and *in-vivo* FRET.

**1613-Pos Board B564**
**Dynamic Turnover of FtsZ-Ring in Live Cell**
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In bacterial, cell division is initiated at the midcell by the formation of a ring-like structure called Z-ring. The Z-ring is assembled by the polymerization of a tubulin homolog, FtsZ, and serves as a scaffold to recruit more than twenty other division proteins. Like tubulin, FtsZ is a GTPase. Thus, a model has been proposed wherein nucleotide-dependent changes in the conformation of

membrane-associated FtsZ polymers generate a mechanical force for membrane constriction. However, some FtsZ GTPase mutants are viable at permissive temperatures, raising the question of what the role of FtsZ's GTPase activity is in cell division. Previously, fluorescence recovery after photobleaching (FRAP) experiments showed the Z-ring is highly dynamic, and it constantly turns over with cytoplasm FtsZ pool[1]. In this work we used total internal reflection fluorescence microscope (TIRFM) to monitor the dynamics of FtsZ-GFP labelled Z-ring in live E coli cells. We observed that matured Z-rings exhibit highly dynamic oscillatory behaviours in live wild type background cells. These dynamic oscillations are larger in magnitude than the constant individual subunit turnover reported by FRAP. It is also distinct from the previously reported FtsZ oscillation along the cell length of newborn cells before Z-ring's assembly. We further investigated cellular factors and FtsZ properties that could influence this dynamic behaviour.

Reference
1. Stricker, Jesse, et al. *PNAS*, 2002, **99**, 3171-3175.

**1614-Pos Board B565**

**Counting Molecules in Non-Muscle Myosin II Filaments**

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Non-muscle (NM) myosin II is involved in many important cellular processes, including migration, adhesion and cytokinesis. NM myosin II assembles into bipolar filaments; this multimerization is thought to be essential for actomyosin contractility. Since each head in a myosin filament can bind F-actin, the number of heads per filament will affect the balance among its bundling, crosslinking and sliding activities, as well as the load-dependent feedback on these activities. Electron microscopy studies of interphase cells from different species have shown NM myosin II filaments contain 16-56 heads, but the size of filaments has never been determined in cytokinesis.

We first studied the number of NM-myosin II in meiotic and mitotic C. elegans zygotes. GFP tagged myosin was imaged by TIRF microscopy until the fluorescence signal was completely bleached. We developed a novel, yet very simple molecule counting method CoMPaS (counting molecules with photobleaching and subtraction) to measure the number of bleaching events in the fluorescence decay curves from cells with different level of GFP expression. We found that the total number of myosin molecules in a single filament is 100 - 150. As an independent assessment of myosin filament size, we also measured filament length in cultured Drosophila cells using super-resolution microscopy. We measured the distance between diffraction-limited foci containing GFP-tagged regulatory light chain (RLC) at the two ends of myosin filaments, with nm accuracy. On average, myosin filaments are approximately 380 nm long. This method also allowed us to determine the orientation and size of the RLC myosin head foci. Interestingly, increased myosin filament length correlated with more parallel filament head foci. We will combine these measurement techniques with genetic perturbations to understand how NM myosin II filament assembly is controlled and how interaction with actin influences myosin filament structure.

**1615-Pos Board B566**

**Z-Profiling of CFTR Oligomerization State Distributions via Single Molecule Step Photobleaching Analysis in Epithelial Cells**

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The cystic fibrosis transmembrane conductance regulator (CFTR) is an anion channel which conducts chloride and bicarbonate ions through the apical plasma membrane of epithelial cells. CFTR interacts with other proteins which form a macromolecular complex that regulates its activity at the cell surface. The number of CFTR molecules within these complexes remains uncertain due to cell variability and the difficulty of measuring this parameter in situ. We show a single molecule step photobleaching method which measures the oligomerization state of resolved clusters and then maps the z-position profile of individual CFTR molecules within the cluster based on imaging data collected by TIRF microscopy. DNA nanorods with defined fluorescent label spacing were used as controls to validate the analysis and to recover the oligomerization distribution that would be expected for a 24.5 nm building block length ($24.6 \pm 2.5$ nm, measured), and for the 13.5 nm height of the base layer of streptavidin on the coverslip ($12.9 \pm 1.7$ nm). Studies of primary airway epithelial cells expressing GFP-CFTR indicate that CFTR clustering state increases linearly with the z-position from the inside to the outside of the cell. Plots of z position versus oligomer subunit count yielded the same slope under control conditions ($6.0 \pm 1.4$ nm/count) and during protein kinase C stimulation (PKC; $6.0 \pm 3.7$ nm/count). However, the number of counts per cluster was larger in PKC stimulated cells. Our results demonstrate that CFTR aggregation state varies with depth from the cell surface, and may be related to the mechanism of cluster assembly inside the cell and/or its function at the plasma membrane. We anticipate that our method can be generalized to monitor the z-position profile or oligomerization states of other membrane receptors and channel to determine structure/function relationships for such membrane complexes.

**1616-Pos Board B567**

**Dimerization of EphA2 in Cell Membranes**

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EphA2 is a single pass transmembrane protein and a member of the largest Receptor Tyrosine Kinase (RTK) family. EphA2 regulates cell-cell interactions during embryonic development in humans and is known as an oncoprotein as well as a tumor suppressor. The Eph receptors differ from other RTKs since they form oligomers when they bind their ligands. However, the interactions between the EphA2 receptors in the absence of ligand have not been investigated. We used spectral FRET in conjunction with two photon microscopy to probe unliganded EphA2 dimerization in HEK 293 cells. We measured the FRET efficiency with high precision over a concentration range that spans three orders of magnitude and includes the physiological range of receptor expressions. We show that EphA2 forms dimers in the absence of ligand. In addition, mutagenesis studies reveal that contacts between the EphA2 receptors that are important for ligand-mediated clustering are also important for unliganded dimerization. We therefore propose that unliganded EphA2 dimers are an important intermediate in EphA2 signal transduction.

**1617-Pos Board B568**

**Single Molecule Analysis Reveals Coexistence of Stable Serotonin Transporter Monomers and Oligomers in the Live Cell Plasma Membrane**

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The human serotonin transporter (hSERT) is responsible for the termination of synaptic serotonergic signaling. The formation of SERT oligomers in the plasma membrane has already been indicated by various approaches, including optical methods such as Förster resonance energy transfer (FRET) and classical biochemical ensemble analysis. However, neither application is suitable to yield quantitative interpretation and decipher the exact configuration of the oligomerization states; additionally, biochemical methods do not take the influence of the membrane environment into account. Here we used single molecule fluorescence microscopy to obtain the oligomerization state of SERT via brightness analysis of single diffraction limited fluorescent spots. The techniques applied in this study allow for identification and quantitative evaluation of subpopulations of SERT complexes exhibiting different degrees of oligomerization in a living cell. We found a variety of oligomerization states of membrane-associated transporters, revealing molecular associations at least up to pentamers and demonstrating the coexistence of different degrees of oligomerization in a single cell. The oligomerization was found to be independent of SERT surface density, and the interactions were stable over several minutes. Together, these results indicate kinetic trapping of preformed SERT oligomers at the plasma membrane. Next, we developed a strategy for single molecule analysis at the membrane of the endoplasmic reticulum. By evaluating the oligomerization of SERT in the ER we found that the oligomerization process is chemically equilibrated at ER membranes; after trafficking to the plasma membrane, the SERT stoichiometry remains fixed.

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**1618-Pos Board B569**

**RFP Tags for Labeling Secretory Pathway Proteins**

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Many RFPs are prone to form artificial puncta when labeling proteins in secretory pathway, which may severely impede their further uses in living cell