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Dynamics of the Actin Cytoskeleton and Plasma Membrane at the Immunological Synapse Revealed using Live-Cell Super-Resolution Microscopy

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Filamentous (F)-actin and the plasma membrane (PM) both have a role in regulating molecular organisation and trafficking during cellular processes. In T-cells, this includes cell migration and the formation of the immunological synapse (IS). The IS is a specialised cell-cell junction crucial for antigen recognition. The molecular organisation during IS formation can influence downstream signalling and therefore the effectiveness of the antigen-mediated immune response.

Combining Total Internal Reflection Fluorescence (TIRF) with Structured Illumination Microscopy (SIM) provides live-cell super-resolution images and molecular dynamics of sub-diffraction structures using standard fluorophores. Using Spatio-Temporal Image Correlation Spectroscopy (STICS) we can extract directionality and velocity information from this data in the form of vector maps and histograms. We applied TIRF-SIM and STICS to T-cells during the formation of the Immunological Synapse (IS) to study the dynamics of the cortical F-actin meshwork and the PM itself.

At resolutions of approximately 100 nm both the cortical F-actin meshwork and the PM itself demonstrate retrograde flow during IS formation and in mature synapses. To establish what could be driving this retrograde flow we applied various drug treatments designed to alter the cytoskeletal meshwork in different ways such as Latrunculin-B and Cytochalasin-D which disrupt F-actin and Jasplakinolide which stabilises fibres. Using these reagents we were able to influence not only actin retrograde flow, but also the flow of the plasma membrane lipids themselves. This suggests F-actin polymerisation and retrograde flow may drive PM flow during IS formation through actin-membrane coupling.

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Full Field Nonlinear Structured Illumination Microscopy with STED

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Structured illumination microscopy (SIM) allows fast full-field imaging of biological samples. Linear SIM improves the lateral resolution limit by a factor of two. Once combined with a nonlinear effect of the fluorescence emission, SIM can further suppress the resolution limit to <100 nm, as reported by Photoswitchable and excitation saturated SIM (SSIM) [1, 2]. Here, we present a novel nonlinear SIM method named STED-SIM, which utilizes the stimulated emission depletion (STED) effect to achieve 4-fold resolution enhancement [3].

STED-SIM utilizes a 2D grating to generate a 2D structured illumination pattern. The grating is shifted at high speed by piezo stages. Therefore, the imaging speed of 2D STED-SIM in only limited by the speed of the camera. Compared with previously reported nonlinear SIM approaches, STED-SIM has the advantage of fast switching response, negligible stochastic noise in switching. In addition, the achievable resolution of STED-SIM in theory is unlimited.

STED-SIM microscope was first tested on fluorescent beads samples and achieved full field imaging over 10×10 [μm]2 at the speed of 2s/frame with 4-fold improved resolution. Imaging experiments of biological samples are under way.

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Reducing Photobleaching in STED Microscopy with Higher Scanning Speed

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Photobleaching is a major limitation of super-resolution STED microscopy. Using our custom-built resonant-scanning STED microscope with a large (50 x 50 μm) field of view, we now show that the photobleaching rate in STED microscopy can be slowed down by scanning with high linear speed. The effect of scanning speed on photobleaching is more remarkable at higher levels of depletion laser irradiance. With a depletion irradiance of 0.4 GW/cm2 (time average) and a 7-fold faster scanning (speed increased from 0.17 m/s to 1.38 m/s), we were able to slow down the photobleaching of the Atto 647N dye by 80%. Photobleaching is primarily caused by the depletion light acting upon the fluorophores in the excited states. Experimental data qualitatively agree with a theoretical model. Our results encourage to increase linear scanning speed further or to reduce continuous light exposure time to significantly reduce photobleaching in STED microscopy.

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DNA-Paint and Exchange-Paint for Multiplexed 3D Super-Resolution Microscopy

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While it's clear that technological and scientific advances often go hand-in-hand, it's generally difficult to identify which combinations will produce the most lasting impact. Here, we present an unlikely union of DNA engineering and optical microscopy that enables 3D super-resolution imaging with extreme color multiplexing [1]. In essence, DNA-PAINT utilizes thermally-driven binding and unbinding of engineered DNA sequences to induce fluorophore blinking, which ultimately yields images with sub-10-nm spatial resolution. Moreover, because binding sites and flurophores are connected on opposite strands of hybridized DNA, careful engineering of the base pair sequence allows for orthogonal selectivity between signal and target. Thus, EXCHANGE-PAINT goes beyond the single color detection of DNA-PAINT and enables an extreme form of color multiplexing. By combining this unique approach to target labeling with high-speed confocal imaging, we are now in position to move toward full 3D super-resolution imaging. In this work, we present recent progress toward this ultimate goal and demonstrate how such techniques can provide unique insights on molecular scale biophysical systems.

R.J., M.D., M.S.A., J.B.W. and P.Y. have filed a provisional US patent application regarding the current work.

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2408-Pos Board B545

Quantitative Multiplexed Super-Resolution Neuronal Synapse Imaging using DNA-Paint

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Neuronal synapses form critical junctions of communication in neuronal networks, mediating neuronal signal transmission and circuit function. Synapses consist of thousands of proteins organized on the sub-micron scale, and their dysregulation via genetic aberrations including copy number variations and site-specific mutations is associated with a large number of neurological and psychiatric diseases. Understanding how these genetic aberrations affect the localization and structural organization of synapse proteins at the single synapse level is crucial for understanding neuronal function and related pathogenesis. Super-resolution fluorescence imaging is a powerful approach to resolving nanometer-scale organization of synapse molecules. However, conventional super-resolution imaging is limited to simultaneous interrogation of only 2-4 proteins in a single synapse. As an alternative, here we apply DNA-PAINT (Points Accumulation for Imaging in Nanoscale Topography) that enables highly multiplexed super-resolution imaging of synaptic proteins. PAINT generally employs transiently binding imaging probes to molecular targets in order to generate target blinking while simultaneously allowing probe washout or exchange, thereby in principle enabling sequential imaging of arbitrary numbers of molecular targets using a single dye and laser source. Use of single-stranded DNA as the soluble fluorescent probe that targets complementary single-stranded DNAs on cognate antibodies facilitates arbitrary blinking events per spatial localization. We employ this approach to resolve the localization and organization of synaptic proteins simultaneously with cytoskeletal markers for microtubules and actin, and demonstrate how fluorescence correlation analysis can be used to quantify their copy number in a highly multiplexed manner at the single cell and synapse level.

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Single-Molecule Digital Imaging with Molecular Resolution using DNA-Paint

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Recent advances in super-resolution microscopy have allowed the circumvention of the classical diffraction limit of light, and demonstrated the ability of