925-Pos
Novel Visualisation Techniques for Localisation Microscopy
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Localisation microscopy techniques based on localising single fluorophore molecules now routinely achieve accuracies better than 30 nm. Unlike conventional optical microscopy approaches, localisation microscopy experiments do not generate an image but a list of discrete coordinates of estimated fluorophore positions, typically involving 10,000 to 100,000 molecule coordinates. Data display and analysis therefore generally requires visualisation methods that translate the position data into conventional images. Here we investigate the properties of several widely used visualisation techniques and show that a commonly used algorithm based on rendering Gaussians may lead to a 1.44-fold loss of resolution. Additionally, existing methods typically do not explicitly take sampling considerations into account and thus may produce spurious structures. To overcome some of these issues we present two additional visualisation algorithms, an adaptive histogram method based on quad trees and a Delaunay triangulation based visualisation of point data. The new visualisation methods are designed to supress erroneous detail in poorly sampled image areas but avoid loss of resolution in well sampled regions. A number of criteria for scoring visualisation methods are developed as a guide for choosing among visualisation methods and are used to qualitatively compare various algorithms. We show how these algorithms can be extended to visualise 3D localisation data and demonstrate in practical cell labelling experiments that the effective resolution is typically sampling-limited. The visualisation techniques are illustrated with 2D and 3D localisation data obtained in cardiac ventricular myocytes stained for caveolin-3, ryanodine receptors and β-tubulin.

926-Pos
Nanometer-Scale Imaging of Collagen Fibers Using Gold Beads
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The 3D spatial position of a particle can be determined by scanning the excitation volume of a 2-photon microscope in a three-dimensional orbit around the particle and by subsequently analyzing the fluorescence intensity profile along the orbit. We track the movement of gold beads moving along collagen fibers by 3D particle tracking method. As the particle moves on the fiber, the particle trajectory maps the substrate with high-resolution (2-20 nm). When the particle moves in close proximity to the collagen, it locally couples and excites to the weak fluorophores on the collagen. This method provides the possibility to characterize the interactions between particle and substrate even further. We can obtain the dynamic structure information of collagen fibers with nanometer resolution in real time. More interestingly, the gold beads move not at random but in specific directions under two photon laser excitation. We were able to move the gold particle very fast along the collagen fibers parallel to the scanning line direction. Based on these results, we can control the velocity and direction of gold beads at our own will.

927-Pos
Optimizing Image Analysis for Subwavelength Fluorescence Microscopy with Palm and Storm
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Emerging super-resolution fluorescence microscopy techniques (e.g. PALM and STORM) are of growing significance in biophysical research, enabling high resolution imaging of live cells. Key structures imaged by these techniques include the cytoskeleton, membranes, and mitochondria. Recent theoretical work confirms that the experimentally achievable image acquisition rate and resolution in these techniques is limited by the performance of the rejection algorithm (used to distinguish single-fluorophore images from multi-fluorophore images) as much as by the physical performance of the imaging system. Better rejection algorithms may therefore yield faster and more accurate experiments as well as faster post-processing.
We benchmarked the performance of several shape-based rejection algorithms that require no a priori knowledge about the fluorescence efficiency or orientation of the probes, as these parameters are subject to considerable variation. We initially characterized an approach to rejection based on a process of (1) nonlinear curve fitting of the intensity map to an asymmetric Gaussian and (2) subsequent rejection or acceptance of images based on the ellipticity of the fitted function. Ellipticity is used to indicate the presence of multiple activated fluorophores that are separated by less than the wavelength of light and forming overlapping blurs with different centers. We found that the minimum separation for reliable rejection was approximately lambda/3. We then characterized an iterative noise-compensated linear curve-fitting algorithm and found its rejection performance to be nearly identical to the nonlinear approach, but significantly faster. Additionally, we have preliminary performance data for a novel rejection algorithm that employs center of mass estimation on different portions of the bright spot to infer ellipticity. These results are promising steps towards STORM/PALM image processing tools fast enough to enable real-time (rather than post facto) visualization of live cells during experimental manipulations.

928-Pos
Measuring the Evanescent Field in Tirf Microscopy Using Tilted Fluorescent Microtubules
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Total internal reflection fluorescence microscopy has become a powerful tool to study the dynamics of sub-cellular structures and single molecules near substrate surfaces. However, the penetration depth of the evanescent field, that is, the distance at which the excitation intensity has exponentially decayed to 1/e, is often left undetermined. This presents a limit on the spatial information about the imaged structures. Here, we present a novel method to quantitatively characterize the illumination in total internal reflection fluorescence microscopy using tilted, fluorescently labelled, microtubules. We find that the evanescent field is well described by a single exponential function, with a penetration depth close to theoretically predicted values. The use of in vitro reconstituted microtubules as nanoscale probes results in a minimal perturbation of the evanescent field; excitation light scattering is eliminated and the refractive index of the sample environment is unchanged. The presented method has the potential to provide a generic tool for in situ calibration of the evanescent field.

929-Pos
Modulation Particle Tracking
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In this study, we present a novel optical imaging method that makes use of high precision particle tracking of fluorescent particles to obtain images of nanometer size structures in live cells. Particle tracking not only provides the trajectory of the center of mass but also the particle orientation and size can now be observed, in vivo and real time with the nanometer resolution. This method helps in further understanding of the dynamics of the small particles in biological systems, which was hard to achieve by the current optical techniques. The method is based in rapidly modulating the position of the laser beam around small structures on the order of 100nm in size. When the laser spot oscillates in the direction toward the particle surface, the fluorescence of the particle is modulated. The modulation, which is the ratio of the alternating part to the average fluorescence intensity, is a function of the distance of the particle from the center of mass to the oscillation. In order to track the particle, we circularly moved the oscillating laser spot around the moving particle, and at the same time, analyzed the modulation in the frequency spectrum of the intensity along the orbit to perform a feedback loop updating the average laser position to the center of mass of the particle position. The size, shape and orientation information of the fluorescent structure can then be obtained by looking at the higher order modulations components. We explain the theory behind this method and we show the 3D reconstruction of nanometer microvilli structures on the apical membrane of OK cells.
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930-Pos
Fast Line Scan Confocal Microscope with Minimal Photobleaching
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We report on a custom-built high speed laser scanning confocal microscope that produces high quality images in the diffraction limit with minimal photobleaching. The image resolution and contrast are increased when the sample is scanned by a finely focused illumination source by a 50 μm confocal pinhole at the laser illumination source. With the use of high speed resonant scanning mirrors (8kHz), and given a scanning field of 200μm x 200μm and a laser spot size of 250nm at the diffraction limit, the mean exposure time for a single fluorophores is ~ 80ns (250nm/(2*8kHz*200μm)). The short exposure time due to the fast scanning decreases the probability of fluorophores to populate the dark triplet state (whose life time is on the order of microseconds) and minimizes photobleaching. During image acquisition, each line is scanned N (e.g., N = 64) iterations and the acquired data for each line are summed together before the next line is scanned. Compared to a single frame of image, such a