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

''line scan'' scanning scheme increases the contrast (signal to noise ratio) of the image by sqrt(N). It also improves the image resolution because it eliminates the mechanical backlash of the vertical scanning mirror when each frame is scanned multiple times in regular raster scanning schemes. Supported by NIH grant HL088640.

931-Pos

High Throughput High Sensitivity Depth Resolved Wide Field Microscopy

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3D optical microscopies including confocal microscopy, two-photon excitation microscopy, and coherent anti-Stokes Raman scattering microscopy have optical sectioning capability, but their image acquisition is relatively slow due to the sequential nature of raster scanning. Recently, scanningless nonlinear microscopy based on temporal focusing was introduced as an alternative to using the diffraction-limited spot. However, comparable optical sectioning has not been proved without optimizing the optical design and high-throughput capability has not been achieved due to the optical power limitation. In this presentation, high-throughput high-sensitivity depth-resolved wide-field two-photon microscopy is proposed. To quantify depth discrimination capability, a comprehensive mathematical model for depth-resolved wide-field illumination is derived and experimentally validated. By optimizing optical design parameters through numerical simulation, the best 3D resolution is shown to be close to diffraction limit. In addition, single particle detection sensitivity and high-throughput imaging capability are demonstrated by incorporating quantum dots, which are known to have high two-photon cross section, as a contrast agent into the proposed system. Finally, depth-resolved single particle tracking is evaluated to study the transport process in the cells with the developed microscopy, which confirms that this microscopy holds the potential in the fields of biology and medicine where both sensitivity and throughput are required.

932-Pos

Optimizing Multi-Photon Fluorescence Microscopy Light Collection from Living Tissue by Non-Contact Total Emission Detection (TEDII)

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A benefit of multiphoton fluorescence microscopy is the inherent optical sectioning that occurs during excitation at the diffraction-limited spot. The scanned collection of fluorescence emission is incoherent; i.e., no real image needs to be formed on the detector plane. The isotropic emission of fluorescence excited at the focal spot allows for new detection schemes that efficiently funnel all attainable photons to detector(s). We previously showed (JOM v.228, p.330-7, 2007) that parabolic mirrors and condensers could be combined to collect the totality of solid angle around the spot for tissue blocks, leading to ~8-fold signal gain. We now apply a version of this Total Emission Detection instrument modified to make non-contact images inside tissue *in vivo*. The device is mounted on a periscope (LSM Tech) to avoid touching tissue and is simpler, and in some cases more effective, than hybrid objective and fiber optic ring based systems for emission collection enhancement. Images of live brain and kidney show that the gain using this optical scheme varies as a function of imaging depth and the characteristics of the sample being imaged. Brain imaging (through a tiny region of thinned skull) of GFP labeled microglia showed up to a 1.8 fold increase in emission collection, while the gain in whole *ex vivo* brain samples showed up to a 2.5 fold increase (vs. light collected by a 20X water 0.95NA lens alone). Rat kidney imaging of blood vessels labeled with anneps (Invitrogen) *in vivo* showed up to a 2 fold enhancement in emission collection. These results show that multi-photon imaging using the TEDII device will permit scanning at twice the rate with the same SNR in these tissues or allow reduction of laser power by 60% to reduce photo-damage.

933-Pos

Sub-Diffraction Limited Wide Field Imaging and Microfabrication Based on Surface Plasmons

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Standing-wave surface plasmon resonance fluorescence (SW-SPRF) is a microscopy technique combining standing-wave total internal reflection fluorescence (SW-TIRF) microscopy and surface plasmon resonance (SPR) or surface plasmon-coupled emission (SPCE). Previous studies have shown that SW-TIRF technique can enhance lateral image resolution by more than twice utilizing standing evanescence waves. Further improvement may be generated using surface plasmons by reflecting light on the gold surface through the cover glass at a specific angle inducing collective excitation of electrons in the metal. In this study, we developed imaging and lithography method with less than 100 nm resolution by applying SW-SPRF microscopy with corrugated gold surface. We used corrugated gold surface to induce surface plasmon waves with larger wave number compared to uncorrugated one. This matching process requires proper optimization of parameters including grating constant, perturbation depth, incidence angle of the beam, and excitation wavelength. The fabrication of the corrugated gold surface was done by e-beam etching. For imaging, sub-diffraction size fluorescent particles were used to measure point spread function. For lithography, nano-patterns were produced by the exposure of interfering evanescent waves on azo dye (Congo-Red) thin films produced by spin-coating. The resultant patterns were measured with AFM. We gratefully acknowledge funding from the Singapore-MIT Alliance (SMA-2), the Singapore-MIT Alliance for Research and Technology (SMART), and the Samsung Scholarship.

934-Pos

High Resolution Wide Field Stimulated Raman Scattering Microscopy

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Fluorescent imaging modalities, such as STED, PALM and STORM, has demonstrated the feasibility of super-resolution imaging. However, no comparable super-resolution imaging has been achieved based on non-fluorescent contrast mechanisms. We present a novel super-resolution approach based on incorporating stimulated Raman scattering (SRS) contrast into a standing-wave (SW) total internal reflection microscope. SW-SRS microscopy has the potential to improve the lateral resolution of current SRS microscopy in total internal reflection geometry. There is a critical difficulty to implement SW-SRS microscopy. Stimulated Raman gain, SRG, is a weak modulation of the intensity of the Stokes beam. The ratio of the SRG to Stokes beam intensity is a function of pump beam instantaneous intensity. The need for wide field imaging further reduces pump beam flux resulting in very unfavorable SRG to Stokes beam intensity ratio. As an example, using a standard Ti-Sapphire laser exciting a 100×100 micron square region, SRG to Strokes beam intensity can be as low as 10^-9. This low signal to noise ratio is particularly challenging for wide field imaging that requires area detectors, such as CCD cameras, with limited dynamic range. To overcome these difficulties, we show that SRG to Stokes beam ratio can be improved to 10^-4 by utilizing mJ pulses using a regenerative amplifier, optimization of pulse durations and bandwidths, and destructive interference of the Strokes beam background. We gratefully acknowledge funding from the Singapore-MIT Alliance (SMA-2), the Singapore-MIT Alliance for Research and Technology (SMART), and the Samsung Scholarship.

935-Pos

Single Point FCS on a Commercial Confocal Laser Scanning Microscope with Analog Detectors

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Fluorescence Correlation Spectroscopy is a technique invented in the early 1970s to measure diffusion coefficient, chemical reaction rates and photo physical processes. It is a common belief that in order to obtain single point FCS data, one needs either a sophisticated FCS instrument with photon counting detectors or avalanche photon detectors or an instrument custom made for this type of experiments. Here we show that we can obtain single point FCS data on a commercial confocal laser scanning microscope without any modifications (Nikon C1). We successfully measured the diffusion coefficient and the concentration of Rhodamine B in solution for concentrations ranging from 5 nM to 280 nM. We also determined the diffusion coefficient of two different labeled lipid analogs (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate and BODIPY TMR phosphatidylinositol (4,5) bisphosphate) incorporated in the membrane of giant unilamellar vesicles. The results obtained for these lipid analogs are in good agreement with previously published data. Finally, we highlighted the fact that the actual proportion of labeled lipid analogs incorporated in the membrane of the giant unilamellar vesicle (formed by the electroformation method) is significantly different than the proportion of these lipids in the organic solvent stock solution.

936-Pos

Computational and Statistical Limits to Palm, Storm, and Related Sub-Diffraction Fluorescence Microscopy Techniques

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Techniques such as PALM and STORM enable fluorescence microscopy with subwavelength resolution, using molecules that can be activated from a dark