''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

Daekeun Kim, Hyungsuk Lee, Yongdae Shin, Peter T. C. So.
Massachusetts Institute of Technology, Cambridge, MA, USA.

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)

Christian A. Combs, Aleksandr Smirnov, David Chess, Dorian McGavern, Merav Luger-Hammer, Jay R. Knutson, Robert S. Balaban.
NIH, Bethesda, MD, USA.

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

Yang-Hyo Kim[1], Euiheon Chung[2], Xihua Wang[3], Shyamsunder Erramilli[4], Peter T. C. So[1].
[1]Massachusetts Institute of Technology, Cambridge, MA, USA, [2]Massachusetts General Hospital, Boston, MA, USA, [3]University of Toronto, Toronto, ON, Canada, [4]Boston University, Boston, MA, USA.

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

Yang-Hyo Kim[1], Daekeun Kim[1], Shyamsunder Erramilli[2], Peter T.C. So[1].
[1]Massachusetts Institute of Technology, Cambridge, MA, USA, [2]Boston University, Boston, MA, USA.

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

Iyrri L. Salvemini[1], Enrico Gratton[2], **Pierre D.J. Moens[1]**.
[1]The University of New England, Armidale, NSW, Australia, [2]University of California, Irvine, CA, USA.

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

Alexander R. Small, Forrest Hippensteel, Edward Shore.
California State Polytechnic University, Pomona, Pomona, CA, USA.

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

state to a fluorescent state. Only a small fraction of the molecules are activated at any given time; giving a very low probability of nearby molecules being simultaneously activated and forming overlapping blurs in the image. The fluorescent molecules are imaged individually, their positions are determined from the images, and then a new set of molecules is activated to the fluorescent state. However, there is a tradeoff between image quality (improved by minimizing the activation probability per molecule, for fewer overlapping blurs) and speed (improved by increasing the activation probability per molecule, to reduce the risk of image cycles with zero activated molecules). One method of dealing with this tradeoff is to increase the activation probability and use algorithms (called rejection algorithms) that identify and remove spots formed by overlapping blurs from more than 1 molecule.

We performed a theoretical analysis to relate rejection algorithm performance with achievable resolution and image acquisition speed. We predict the existence of a minimum acquisition time independent of algorithm performance, and an algorithm-dependent maximum error rate. We have characterized the performance of commonly-used procedures for identifying multi-molecule spots via their shape (including linear and non-linear curve fitting), and show that procedures of widely varying complexity and speed have comparable performance, pointing to ways of reducing acquisition and post-processing time with optimized rejection algorithms. Additionally, we analyzed errors when molecules produce overlapping blurs and are then bleached. With proper control of activation probability and the photobleaching rate, bleaching can actually be used to enable faster acquisition of an image with subwavelength resolution, with implications for the design of photoswitchable fluorescent proteins.

937-Pos

Imaging Total Internal Reflection Fluorescence Cross-Correlation Spectroscopy (ITIR-FCCS)

Jagadish Sankaran[1], Manoj Manna[2], Lin Guo[2], Rachel Kraut[3], Thorsten Wohland[1].
[1]Singapore-MIT alliance and Department of Chemistry, National University of Singapore, Singapore, Singapore, [2]Department of Chemistry, National University of Singapore, Singapore, Singapore, [3]School of Biological Sciences, Nanyang Technological University, Singapore, Singapore.

General imaging approaches, in which contrast is not given by time averaged intensities but by other fluorescence parameters, for instance lifetime, anisotropy or parameters of correlation functions, promise to give new insights to biologists. We have recently shown that Fluorescence Correlation Spectroscopy (FCS) can be performed using EMCCD cameras if used with an objective type total internal reflection illumination scheme. This so called Imaging Total Internal Reflection-Fluorescence Correlation Spectroscopy (ITIR-FCS), allows the measurement of autocorrelation functions (ACF) on thousands of pixels independently on 2D surfaces. In this work we extend this technique to ITIR-FCCS (Fluorescence Cross-correlation Spectroscopy) to perform spatial cross-correlation for the measurement of general translational processes. A generalized expression was derived for auto- and cross-correlations of arbitrarily shaped areas on a CCD for diffusion and flow processes. ITIR-FCCS was able to precisely and accurately determine flow velocities and diffusion coefficients of model systems.

To address the translational processes and the organization of cell membranes we calculated the differences between spatial forward and backward cross-correlations to yield so called ΔCCF images. The ΔCCF imaging approach was successfully demonstrated on GUVs and mixed lipid bilayers by demarcating phase boundaries. It was then used to track the changes in heterogeneity of two cell membrane markers, a liquid-ordered phase marker (sphingolipid binding domain (SBD) derived from the amyloid peptide Aβ) and a liquid-disordered phase marker (DiI) on live neuroblastoma cells under conditions of cholesterol depletion and cytoskeletal disruption. Our findings from auto- and cross-correlation and ΔCCF analysis indicate that SBD is influenced by the cholesterol content and to a larger extent by the integrity of the cytoskeleton. DiI, by contrast, shows little dependence on both.

938-Pos

Optical Nanoscopy Far-Field Approaches to Cellular and Molecular Biophysics

Alberto Diaspro[1], Paolo Bianchini[1], Francesca Cella[2], Emiliano Ronzitti[2,3], Silvia Galiani[2], Mattia Pesce[1], Zeno Lavagnino[2], Gaser Abdelrasoul[1].
[1]IIT - Italian Institute of Technology, Genoa, Italy, [2]IFOM MICROSCOBIO and Dept.Physics-University of Genoa, Genoa, Italy, [3]SEMM-IFOM-IEO University of Milano, Milano, Italy.

Fluorescence optical far-field microscopy fostered the design and realization of crucial experiments in cellular and molecular biophysics, although the limited spatial resolution dictated by diffraction. Recently, an emerging family of fluorescence microscopy approaches exploiting the photo physical properties and the switching abilities of fluorescent markers allowed to achieve the surpassing of the diffraction barrier down to 10 nm resolution scale. Super-resolution microscopy and optical nanoscopy are the modern terms related to optical far-field methods opening a new window for the understanding of molecular interactions within the biological cell (A. Diaspro (ed.) (2009) ''Nanoscopy and Multidimensional Optical Fluorescence Microscopy '', Chapman and Hall). Within this framework, focusing on the saturated depletion of the markers' fluorescent state by stimulated emission we have pointed our attention to different modalities for realizing STED (stimulated emission depletion) approach. In particular we are interested in the excitation modalities (including phase modeling, intensity control and scanning speed) and in the possible photo-bleaching/toxical effects as function of the light intensity levels needed. To this end we are working on the ''classical'' solution using ps laser pulses both using white light laser generation and multi-photon based schemes as compared to the continuous wave (CW) excitation and depletion achieved by means of CW laser sources. In parallel we are also approaching optical super-resolution using the FPALM (fluorescence photoactivatable localization microscopy) scheme coupled to two different ways for switching on the fluorescent proteins involved. The former being classical, in order to have a comparison with the STED approach in terms of possible photo-bleaching and photo-toxical effects, and the latter based on the utilization of the single plane illumination microscopy (SPIM) concept to extend far-field optical nanoscopy methods to large samples.

939-Pos

Recognition of Protein Binding Events by Polarity-Sensitive Probes

Ranieri Bizzarri[1,2], Giovanni Signore[3], Riccardo Nifosi[2], Lorenzo Albertazzi[1,3], Barbara Storti[1,3].
[1]NEST, Scuola Normale Superiore, Pisa, Italy, [2]NEST, CNR-INFM, Pisa, Italy, [3]NEST @ Italian Institute of Technology, Pisa, Italy.

Polarity-dependent fluorescent probes are recently attracting interest for high-resolution cell imaging. The fluorescence enhancement of the solvatochromic dye, ideally located in a domain where polarity changes occur upon binding, allows for a fine detection of molecular recognition events even between non overexpressed proteins.1 We developed a toolbox of new solvatochromic coumarin derivatives, characterized by a donor-(coumarin core)-acceptor structure, tailored to in vivo imaging applications.

After a preliminary screening by computational methods, we adopted a synthetic procedure tuneable on the substitution patterns to achieve. Our probes possess excellent fluorescence quantum yields (up to 0.95), high molar extinction coefficients (up to 46,000 M-1cm-1), and large Stokes shifts. Furthermore, they display strong solvatochromism, being almost non emissive in water and very fluorescent in less polar media (up to 780-fold enhancement in brightness). When tested on cultured cells, the developed coumarins resulted not harmful and their photophysical properties were unchanged compared to free solution. Due to both their strong solvatochromic properties, and their lipophilic character, the coumarin did fluoresce only in the most lipophilic environments of the cell. In particular, colocalization experiments with standard markers evidenced staining in ER, membranes and lysosomes, depending on the chemical structure of the solvatochromic probe.

Finally, one compound (3-benzothiazenyl-4-ciano-6,7-dimethoxy coumarin) showed monoexponential decay of fluorescence with a lifetime which is linearly dependent on solvent polarity. This feature promotes its use as ratiometric indicator of cell polarity at nanoscale level. The prepared compounds are remarkable tools to investigate subtle biochemical processes in the cell environment after appropriate conjugation to biomolecules, and at the same time constitute the basis for further engineering of a new generation of biosensors.

1) Nalbant, P.; Hodgson, L.; Kraynov, V.; Toutchkine, A.; Hahn, K. M. Science 2004, 305, 1615-1619.

940-Pos

Superresolution Microscopy with Conventional Organic Fluorophores

Mike Heilemann[1], Sebastian van de Linde[1], Ulrike Endesfelder[1], Anindita Mukherjee[1], Steve Wolter[1], Markus Sauer[2].
[1]Bielefeld University, Bielefeld, Germany, [2]Julius-Maximilians-University Wuerzburg, Wuerzburg, Germany.

Fluorescence microscopy is a sensitive and non-invasive tool to study biomolecular structure and interactions. However, a diffraction limit resulting from the wave nature of light limits resolution to ~200 nm in lateral and ~700 nm in axial direction.

Out of a large set of methods that bypass the resolution limit and open the door for diffraction resolution microscopy, many of them rely on the use of photoactivatable or photoswitchable molecules, combined with precise single-molecule localization and image reconstruction. This concept has recently been extended to a large set of commercially available fluorophores (1-3). Key issues that need to be addressed are (i) controllable switching rates, (ii) live-cell