proteins consistent with single-particle tracking data. For system (2), we perform drug-trial experiments on living COS-7 cells to show that kICS can quantify the degree to which the drugs will affect kinetic binding/unbinding rates. By labeling the glycolipid membrane receptor GM1 with a fluorescent cholera toxin B-subunit, we show that cytoskeleton perturbations with the drug cytochalasin D result in significant changes ($k_u = 0.013 \pm 0.004\ s^{-1}$ to $0.08 \pm 0.02\ s^{-1}$) to the undocking rate of GM1 to the actin cytoskeleton. With these two proof-of-concept experiments, we pave the way to using kICS as an efficient and reliable method to elucidate kinetic binding rates and transport dynamics parameters for studies in biomembranes.

3054-Pos Board B746

A New Efficient Implementation of 2PE-STED Microscopy

Ivàn Coto Hernàndez[1,2], Paolo Bianchini[1], Chiara Peres[1,2], Gustavo De Miguel[1], Alberto Diaspro[1], Giuseppe Vicidomini[1].
[1]Nanophysics, Istituto Italiano di Tecnologia, Genoa, Italy, [2]Department of Physics, University of Genoa, Genoa, Italy.

Time-gated detection substantially reduces the requested intensity for reaching effective sub-diffract resolution in stimulated emission depletion (STED) microscopy. When the time-gated detection is combined with STED beam operating in continuous-wave (CW), a simple, cheap, but also efficient, STED microscopy implementation is obtained, the so called gated CW-STED (gCW-STED) microscopy. The virtues of two-photons excitation (2PE) microscopy have been recently combines with those of STED microscopy. Two mainly implementations are explored. Chronologically, the first implementation relied on STED beams running in CW. Nevertheless, this implementation is relative simple, its resolution performance is strongly limited. The second implementation relied on pulsed STED beams. In this case the resolution performance improves but also the cost and the technical skills necessary for its routine use.

In this scenario, a natural solution is the combination of 2PE microscopy with gCW-STED microscopy. Although this implementation is rather straightforward, the small 2PE cross-section, which results in weak signals, conflicts with the major disadvantages of gCW-STED microscopy. Namely, time-gated detection surely reduces the signal, hence, in a situation of weak signal the images degrade in terms of signal-to-noise/background ratio (SNR and SBR).

Here, we propose a combination of different hardware- and software-based approaches for recovering both SNR and SBR. In particular, we explored the use of synchronous detection methods to remove all the background potentially induced by the STED beam. As well as, we improved the signal by accelerating the pulse repetition rate of the 2PE laser beam. As a side effect, this approach speeds up the acquisition time, reducing potential photodamage effects. The collection efficiency of the microscope is also optimized by using dedicated time gate electronics. Finally, raw images are processed by ad-hoc deconvolution algorithms to further enhance the SNR and SBR.

3055-Pos Board B747

Dual Color STED Microscopy with Ultrafast Photon Counting

Yong Wu, Xundong Wu, Rong Lu, Ligia Toro, Enrico Stefani.
Anesthesiology, UCLA School of Medicine, Los Angeles, CA, USA.

To more precisely quantify association between cardiac proteins, we built a dual-channel super-resolution stimulation emission depletion (STED) microscope with an ultrafast photon-counting acquisition system. STED microscopy is unique among super-resolution technologies due to its capability of optical sectioning and fast imaging speed. A major limitation of STED is the aggravated photobleahching, to which fast scanning speed is an excellent solution. In order to keep up with fast scanning speed and to reduce counting nonlinearity, we invented a photon counting acquisition system based on ultra-fast analog-to-digital conversion (ADC), pushing the readout rate to the gigahertz range. Incorporated into a resonant scanning confocal microscope, this system can acquire images at 16,000 lines per second and 58,000 pixels per line. By adding a continuous wave (CW) depletion laser and photon arrival time control elements, the confocal microscope was converted to a time-gated STED microscope. A second STED channel was built with picosecond depletion pulses. The dual-channel STED microscope is able to achieve a resolution of ~40 nm in biological samples which allows a more stringent analysis of colocalization in native systems.

3056-Pos Board B748

Efficient ROI Selection for Multi-Emitter Fitting Approaches in Single-Molecule Super-Resolution Microscopy

David Baddeley.
Cell Biology, Yale University, West Haven, CT, USA.

Single molecule super-resolution techniques such as PALM and STORM circumvent the diffraction limit by using fluorophore switching to isolate single emitters in space and time. The requirement that individual fluorophore signals are well separated, however, limits the speed of these methods. Recent advances have relaxed this requirement through the use of model functions which allow multiple overlapping emitters within one image region. Building on the methods used for sparse localisation, multi-emitter fitting methods typically extract fixed size rectangular regions of interest (ROIs) around candidate molecules and perform fitting within these regions. These ROIs will often intersect and it is common for molecules to be present in multiple ROIs and/or to only be partially enclosed within a ROI. This requires a number of empirical solutions to collate molecules and ensure that they are not over-counted, and to ensure convergence when attempting to fit partial molecules. Fitting molecules repeatedly within multiple ROIs also incurs significant computational overhead.

Rather than using fixed size rectangular ROIs at candidate molecule positions, an alternative approach can be constructed based on the selection of contiguous regions of pixels which have a non-zero information content. In short, the algorithm determines which pixels in the image contain useful information by performing a statistical test to determine if pixels (or groups of neighbouring pixels) are significantly higher than the background, dilates this mask to introduce a 'safety factor', and uses a labelling algorithm to identify contiguous groups of pixels within this mask. These contiguous, non-rectangular, regions become the new ROIs. The approach has the dual advantages of fitting each emitter only once, and incorporating the full information about every emitter within each ROI so as to avoid any convergence problems.

3057-Pos Board B749

Highly Efficient HIV-1 Entry Mediated by Nonspecific Virion-Cell Interactions Quantified by Real-Time Single Particle Imaging

Michael C. DeSantis, Jamie L. Austin, Wei Cheng.
Pharmaceutical Sciences, University of Michigan, Ann Arbor, MI, USA.

The apparent low infectivity of HIV-1 is purportedly explained by virion-cell interactions that take place prior to viral entry in which the virus must first diffuse to the cell surface and bind to specialized receptors; these interactions are speculated, from in vitro studies, to be the rate-limiting step involved in the infection process yet the underlying mechanisms are not completely understood. Furthermore, receptor binding, in addition to nonspecific interactions limiting infectivity, may dictate the subsequent steps, i.e. entry pathways, of infection. Using single-virus tracking methods with spatial and temporal resolutions of ~20 nm and 40 ms, respectively, we have quantified the dynamics of mCherry labeled HIV-1 virions with varied envelope (Env) glycoprotein incorporation interacting with TZM-bl cells, a HeLa-derived cell line expressing CD4 and co-receptors at high surface densities. The number of viral-cell touching events and corresponding contact lifetimes of most trajectories suggest few and transient interactions with the cell surface before permanent dissociation. The fraction of virions that become immobilized is heavily influenced by nonspecific interactions in both the presence and, to a lesser extent, absence of DEAE-dextran, contributing to receptor-independent endocytic entry as revealed by time-lapse and confocal imaging studies. Measured internalization efficiencies as high as 80% appear to be independent of Env content despite proportionally higher infectivities; thus, nonspecific binding and subsequent endocytosis may actually lower the apparent HIV-1 infectivity. Moreover, we directly observed a prevalence of these internalized virions to undergo recycling and/or transcytosis implicating this pathway as being involved, albeit indirectly, with infection. (Supported by NIH 1DP2OD008693).

3058-Pos Board B750

Development of Stable Small Quantum Dots for AMPA Receptor Tracking at Neuronal Synapses

En Cai[1], Pinghua Ge[2], Sang Hak Lee[1], Yong Wang[1], Okunola Jeyifous[3], Sung Jun Lim[4], Andrew M. Smith[4], William N. Green[3], Paul R. Selvin[1].
[1]Department of Physics and Center for the Physics of Living Cells, University of Illinois at Urbana-Champaign, Urbana, IL, USA, [2]Department of Physics, University of Illinois at Urbana-Champaign, Urbana, IL, USA, [3]Department of Neurobiology, University of Chicago, Chicago, IL, USA, [4]Department of Bioengineering, University of Illinois at Urbana-Champaign, Urbana, IL, USA.

AMPA receptors are important excitatory neurotransmitter receptors in the brain. Trafficking of AMPA receptors in and out of synapses affects synaptic plasticity. Quantum dots have been used as fluorescent probes for tracking diffusion of AMPA receptors. However, the size of commercially available quantum dots is ~20 nm, likely limiting their ability of accessing the synaptic cleft which is ~30 nm. Therefore, photo-stable fluorescent probes with smaller sizes are needed. In this study, we developed a new method for coating quantum dot cores to obtain stable quantum dots with diameters about 9 nm.

Currently, we have successfully made small quantum dots with different emission wavelengths (527 nm, 615 nm, 620 nm and 655 nm), and functionalized them with streptavidin. Furthermore, we tested the small quantum dots on cultured neurons and found that all of them bind to AMPA receptors with high specificity. We found that the diffusion behavior of AMPA receptors labeled with our small quantum dots is different from that with commercial quantum dots, indicating that bigger quantum dots may have hindered the AMPA receptors' accessibility to the synaptic cleft. In addition, we identified micro-domains of approximately 100 nm x 100 nm where the AMPA receptors tend to bind and diffuse within these domains.

3059-Pos Board B751

Thermodynamically Driven Blinking for Super-Resolution Microscopy

Susan Gayda, Richard Haack, Joseph P. Skinner, Qiaoqiao Ruan, Richard J. Himmelsbach, Sergey Y. Tetin.
Abbott Diagnositics, Abbott Park, IL, USA.

Super-resolution microscopy enables imaging of structures smaller than the diffraction limit, defined by Abbe's law. Over the last years, a continuously increasing toolset of physical methods, fluorescent probes and labeling strategies has emerged, making this imaging approach accessible to a broad range of applications. Single-molecule based stochastic methods, as STORM, PALM and their derivates require the least complex instrumentation, which gained these methods fast growing popularity among scientist of very diverse backgrounds. However, the relative simplicity of the instrumentation comes with a trade-off in probe requirements. These methods rely on the partial suppression of the detectable on-state, which can be achieved by photo-activation of an initially dark state or reversible switching between a bright (on) and a dark (off) state. Most synthetic dyes allow for the latter activation scheme, but require special treatment in order to do so. Chemical additives, as reducing reagents and oxygen scavenger, complex illumination schemes, molecular scaffolds, high irradiation intensities or a combination of the above is usually required to optimize the ratio between on- and off-state that enables a maximal densely labeling of the structure of interest. Here, we report a rhodamine derivate, which converts between a bright and a dark state in response to pH changes. At pH7, an average of $>1\%$ is fluorescent and tumbles between the two states on the molecular level. The resulting blinking occurs on timescales of up to several seconds and can therefore be exploited for e.g. STORM without further sample treatment or complex illumination schemes. Its ease-of-use and its outstanding photo-stability and brightness render this dye an excellent tool for super-resolution imaging techniques based on stochastic read-out.

3060-Pos Board B752

Multiplexed Imaging of Osteocytes in Bone

LeAnn M. Tiede-Lewis, Yixia Xie, Sarah E. Dallas.
Oral and Craniofacial Sciences, University of Missouri Kansas City, Kansas City, MO, USA.

Osteocytes are embedded in bone and account for 90% of cells within mature bone. Although previously viewed as quiescent cells, recent research has revealed the importance of osteocytes in regulation of bone remodeling, both as a mechanosensor and a source of signaling molecules for regulating osteoclasts and osteoblasts. Many osteocyte imaging techniques rely on imaging the lacunocanalicular space around the cell rather than directly imaging osteocytes themselves. To better characterize differences in osteocyte morphology and structure in aging and disease, we have developed a multiplexed imaging technique with the goal of examining osteocytes in 3D within their bone environment. 50-100 micron-thick decalcified bone sections were prepared and a variety of imaging dyes were used in combination with confocal microscopy to image bone matrix, lacunocanalicular space, osteocyte cell membrane, nuclei, and cytoskeleton in various combinations. We have simultaneously imaged collagen matrix (using a transgenic mouse line expressing GFP-tagged-collagen), lacunocanalicular space (using a fixable Texas Red-labeled dextran), osteocyte cytoskeleton (using alexa-647-phalloidin) and nuclei (using DAPI). We have also employed DiO labeling of the cell membrane in addition to dextran labeling of the lacunocanalicular system and DAPI imaging of cell nuclei. DiI labeling of the membrane in combination phalloidin labeling of the cytoskeleton was problematic in bone slices with either incomplete penetration of DiI in the sample or loss of phalloidin specificity. Mounting of bone slices in 2-2'-thiodiethanol increased imaging depth and resolution but resulted in loss of GFP and phalloidin signal over time, therefore requiring imaging immediately after mounting. From multiplexed confocal image stacks it is possible to simultaneously study several aspects of osteocyte structure in demineralized bone matrix with sufficient resolution to render detailed 3-D reconstructions of imaged volumes. These methods can be applied to studying osteocyte structure/morphology in normal and diseased bone tissues.

3061-Pos Board B753

Structural Studies by Correlative Stochastic Optical Reconstruction Microscopy and Electron Microscopy

Doory Kim, Miriam Bujny, Xiaowei Zhuang.
Harvard Univ., Cambridge, MA, USA.

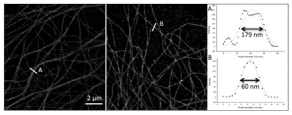
In cell biology, visual techniques such as light and electron microscopy are essential tools for analysis of cellular structures and function. The development of a super-resolution fluorescence microscopy method, stochastic optical reconstruction microscopy (STORM), has allowed multicolor and three-dimensional imaging with nanometer-scale resolution and enabled the study of cellular processes at the molecular scale. Electron microscopy (EM) can reveal not labeled structures, like organelles, membranes, macromolecules in higher resolution. By combining STORM with EM, we were able to understand cellular complexity and localization of molecules of interest in relation to other structures in high resolution. Here, we demonstrate that this method can be used to elucidate the ultrastructural details of cellular events by bridging the gap between light and electron microscopy in cell biology applications.

3062-Pos Board B754

4D Multiplexed Functional Imaging in Deep Tissue

Ming Zhao[1], Xiaoyang Wan[2], Weibin Zhou[2], Leilei Peng[1].
[1]College of Optical Sciences, The University of Arizona, Tucson, AZ, USA, [2]Department of Pediatrics and Communicable Diseases, University of Michigan, Ann Arbor, MI, USA.

We present a deep tissue multiplexed functional imaging method that probes multiple cellular conditions in live model organisms. The method uses FRET-based biosensors to sense cellular conditions such as calcium and cAMP concentration levels, and uses fluorescence lifetime quantification to interpret the cellular conditions reported by the FRET biosensors. The method is based on Fourier lifetime excitation-emission matrix (FLEEM) spectroscopy [1] that simultaneously measures fluorescence lifetimes at multiple excitation and emission wavelengths within 23 microseconds, allowing quantifications on multiple FRET biosensors simultaneously in live model organisms. Samples are imaged in 3D by combining FLEEM spectroscopy with scanning laser optical projection tomography (SLOT) [2]. We demonstrate the method in zebrafish embryos transiently expressing cAMP FRET biosensor, which showed an increase in cAMP concentration upon physiological stimulus with forskolin and IMBX. Calcium and cAMP concentration levels during zebrafish embryonic development were monitored with time lapse 3D functional imaging of the embryo from 12 hours to 22 hours post fertilization. The method opens the door to multiplexed functional imaging of cellular biochemistries in whole live organisms.



[1] M. Zhao and L. Peng, Optics Letters. 35,2910. (2010).
[2] R. A. Lorbeer, et al., Optics Express. 19,5419. (2011).

3063-Pos Board B755

Design and Implementation of 3D Focus Stabilization for Fluorescence Microscopy

Karl Bellve, Clive Standley, Lawrence Lifshitz, Kevin Fogarty.
Program in Molecular Medicine, University of Massachusetts Medical School, Worcester, MA, USA.

Focus stabilization is critical for many imaging modalities like TIRF, PALM and STORM. The focus stabilization device presented here, named pgFocus, is an open source and open hardware solution that can be integrated into microscopes with an existing objective positioner. pgFocus is a programmable and inexpensive circuit board consisting of a micro-controller, linear sensor array, DAC and an ADC. While pgFocus can stabilize on a single focal plane within ± 3nM at 30Hz, it can also follow and correct 3D focus changes when imaging multiple Z positions. pgFocus works by monitoring the reflection of an 808nm laser beam that is internally reflected at a glass/water interface. The translation of the reflected laser beam is converted into Δ distance change between the objective and the glass/water interface. The relationship between movement of the objective and the translation of the return laser beam is determined through an calibration procedure. This Δ distance change measurement is used to modify and adjust a pass-through voltage signal that is directed to a piezo objective positioner. The pass-through voltage is continually adjusted to move the reflected laser beam back to the original focus position.