planes at different depths in the sample. The other method introduces a cylindrical lens to the detection path[8], which causes astigmatism in the detected fluorescence[5]. This results in a stretch along one of the two lateral axes depending on the axial position of the fluorescent particle. This work determines the best optical parameters for each method in order to localize over the largest axial range with best possible uniformity in localization accuracy.

1. Hess, S. T. et al., *Biophys. J.* **91**, 4258 (2006).
2. Betzig, E. et al., *Science* **313** (5793), 1642 (2006).
3. Rust, M. J. et al., *Nat. Methods* **3** (10), 793 (2006).
4. Egner, A. et al., *Biophys. J.* **93** (9), 3285 (2007).
5. Huang, B. et al., *Science* **319** (5864), 810 (2008).
6. Juette, M. F. et al., *Nat Methods* **5** (6), 527 (2008).
7. Berg, H. C., *Rev Sci Instrum* **42** (6), 868 (1971).
8. Kao, H. P. et al., *Biophys J* **67** (3), 1291 (1994).

3283-Pos Board B330

Imaging Actin Filaments in Synaptic Spines Beyond the Diffraction Limit of Light

Ignacio Izeddin[1], Christian G. Specht[1], Xavier Darzacq[1], Antoine Triller[1], Christophe Zimmer[2], Maxime Dahan[1].
[1]École Normale Supï¿½rieure, Paris, France, [2]Institut Pasteur, Paris, France.

The development of novel physical tools to image biological samples at a resolution in the nanometer range is likely to revolutionize our current understanding of the spatial organization and compartmentalization of cells. The high-resolution analysis of biological macromolecular assemblies has long remained widely inaccessible by conventional optical microscopy due to the diffraction limit of light, which prevents structures finer than half of the wavelength of the light (typically ~300 nm) to be resolved. Recently, three independent studies have demonstrated that imaging of biological samples under the diffraction limit is however possible, by making use of photoactivatable proteins or dyes as fluorescent probes and in combination with computational image analysis and reconstruction [1, 2, 3].

Here, we show the high resolution imaging of actin filaments in synaptic spines using photoactivated localization microscopy (PALM). We expressed a tdEos-tagged actin-binding peptide (ABP-tdEos) in primary hippocampal neurons, to indirectly determine the structure of the cytoskeleton in spines, without interfering with the F-actin structure itself. A low density of tdEos molecules were photoactivated, imaged and bleached continuously, followed by image reconstruction, resulting in actin images with subdiffraction resolution . We also discuss how high resolution imaging of cytoskeletal elements can be extended to live cells, a key challenge to investigate how the synaptic structure is dynamically assembled, maintained over time, and altered in response to synaptic activity, to better understand the role of the spine cytoskeleton in synaptic plasticity.

[1] E. Betzig *et al.*, Science **313**, 1642-1645 (2006).
[2] S. T. Hess *et al.*, Biophys. J. **91**, 4258-4272 (2006).
[3] M. J. Rust *et al.*, Nat. Meth. **3**, 793-795 (2006).

3284-Pos Board B331

Paxillin focal adhesions, localization and implication: insight from Photo-Activated Localization Microscopy

Francesco Cutrale.
University of California Irvine, Irvine, CA, USA.

Photo-Activated Localization Microscopy (P.A.L.M.) as described by E. Betzig (2006) optically resolves selected subsets of photo-activatable fluorescent probes within cells at mean separations of less than 25 nanometers through serial photo-activation and subsequent photobleaching of numerous sparse subsets of photo-activated fluorescent protein molecules.

The position information from all subsets is then assembled into a super-resolution image, in which individual fluorescent molecules are isolated at high molecular densities. In this work COS-7 and ST14A tdEos-Paxillin transfected cells were used. We observed some features that limit the versatility of PALM, both in this setup and in its present version. It takes actually hours to go through the cycles of photo-activation and image acquisition, to collect all of data needed and to generate a single high-resolution image limiting the use to fixed specimens which precludes PALM.'s use for imaging of live cells. More important is the loss of data. Depending on the spatial concentration of the PA-FPs, most of the information about the position of molecules is lost during the photo-activation photobleaching phase, especially during the first cycles of data collection. From the biological point of view, we observe small paxillin clusters along the focal adhesions. Supported by U54 GM064346 CMC (MD, EG), NIH-P41-RRO3155 (EG, FC), P50-GM076516 (EG).
Sponsored by Enrico Gratton

3285-Pos Board B332

Super-resolution Imaging Of Ca2+ Flux Through IP3Rs With Millisecond Temporal Resolution And Nanometer Spatial Resolution

Steven M. Wiltgen[1], Ian F. Smith[1], Neil Beri[2], Ian Parker[1].
[1]University of California, Irvine, Irvine, CA, USA, [2]University of California Los Angeles, Los Angeles, CA, USA.

Advanced imaging techniques such as PALM and STORM have broken the diffraction limit of conventional optical microscopy through their ability to turn fluorescent molecules on and off at low enough densities such that the positions of single molecules can be determined, one at a time, with a precision of ~10 nm (Gustafsson, 2008). However, these techniques involve the use of fluorescently tagged proteins or antibodies, which may alter protein properties and provide only positional, not functional information. Thus, we have developed a technique termed Single Channel Ca^{2+} Nanoscale Resolution (SCCaNR), based on similar principles except that it generates a super-resolution image by using Ca^{2+} sensitive fluorescent dyes to image the stochastic openings and closings of Ca^{2+} permeable ion channels. Subsequently, the point spread function resulting from the diffusion of calcium bound to the indicator dye can be fit to a 2-D Gaussian function, allowing the position of functional calcium channels to be localized with much higher precision (~40 nm) than previously possible.

The inositol triphosphate receptor (IP_3R) is an ER Ca^{2+} channel that is both facilitated and inhibited by Ca^{2+} itself. This property enables a functional coupling between IP3Rs, which underlies the generation of localized Ca^{2+} events known as puffs (Yao, et al, 1995). This same property makes IP_3Rs highly dependent on their spatial proximity to one another. Using our SCCaNR technique, we have found that the concerted opening of 4-10 IP_3R channels likely underlies the generation of Ca^{2+} puffs in SH-SY5Y neuroblastoma cells. These puffs arise from clusters of IP_3Rs approximately 300 nm in diameter, a dimension below the resolution limit of conventional optical microscopy.

3286-Pos Board B333

Overcoming the Nyquist limit with intensity modulation spectral analysis

Ken Halvorsen, Wesley P. Wong.
Harvard University, Cambridge, MA, USA.

Power spectral density measurements of any sampled signal are typically restricted by both acquisition rate and frequency response limitations of instruments. We present a new method called Intensity Modulation Spectral Analysis (IMSA) that circumvents these limitations, extending the effective bandwidth of potentially any measurement device. We demonstrate this for the specific case of video imaging, where oscillating an LED illumination source allows us to quantify fluctuations of an optically-trapped microsphere at frequencies over 10 times higher than the Nyquist limit.

3287-Pos Board B334

Optimizing Fluorophores For Super-resolution Fluorescence STED Microscopy

Kyu Young Han[1,2], Eva Rittweger[1], Scott E. Irvine[1], Christian Eggeling[1], Stefan W. Hell[1].
[1]Max-Planck-Institute for Biophysical Chemistry, Göttingen, Germany, [2]Department of Chemistry, Seoul National University, Seoul, Republic of Korea.

Far-field fluorescence nanoscopy is an emerging field, surpassing the diffraction barrier of conventional far-field microscopy and visualizing biological specimen in three dimensions, in principle, with molecular resolution. Stimulated emission depletion (STED) microscopy is a well-established nanoscopy platform which can be applied to conventional organic fluorophores and fluorescent proteins. A major bottleneck of fluorescence microscopy including STED microscopy is the photobleaching of fluorophores which limits both brightness and observation time. Therefore, we have assessed several photostable fluorophores and nanoparticles for their suitability and applied them to STED microscopy. Imaging with continuous wave laser as well as with high repetition rates of 80 MHz offers sub-diffraction resolution with strongly improved photostabilities.

3288-Pos Board B335

Ultra Resolution Direct Imaging Optical Microscope

Peter R.H. Stark.
Harvard University, Boston, MA, USA.

The size of the smallest detail visible in conventional microscopy is determined by the wavelength of the light used to image a specimen. For state-of-the-art optical imaging, this diffraction limit is 200-300 nm, leaving a considerable 'blind spot' between the angstrom-scale molecular details visible by X-ray crystallography and the those accessible by visible light microscopy. Recently, a number of developments have been reported that allow fluorescence imaging of samples with resolutions of an order of magnitude below the diffraction limit.

One such method makes use of the stochastic fluorescence emission of individual molecules. Massive oversampling of the fluorescence emission of these particles allows the determination of their positions with high accuracy and, thus, the construction the image of a fluorescently labeled, biological sample with a resolution below the diffraction limit. This and related techniques, however, are limited to the imaging of fixed samples and require many minutes or hours to construct a single image. Another approach is making use of certain photophysical properties of fluorophores and a combination of illumination lasers to decrease the size of the excitation focus in confocal microscopy. Disadvantages of this method include the need for sophisticated laser equipment, very specific requirements for the fluorescent labels, and long times to obtain images. In general, live cell imaging at the timescales required to study the dynamics of intracellular processes is impractical with these newly developed super-resolution techniques.
Here, we present a drastically different approach to sub-diffraction-limited imaging that utilizes a propagating, nanoscopic beam of visible light with a diameter of a few 10s of nm. This phenomenon relies on the resonance of surface plasmons with the photons at the dielectric/metal interface. The width of the transmitted photon beam is independent of wavelength remains constant over length scales of 100s of nm.

3289-Pos Board B336
UV Ratiometric Imaging Of Isolated Ventricular Cardiomyocytes Using An LED Based Illuminator
Martyn Reynolds[1], Niall MacQuaide[2], Martin Thomas[1], Godfrey Smith[2].
[1]Cairn Research Ltd, Faversham, United Kingdom, [2]University of Glasgow, Glasgow, United Kingdom.
Ratiometric fluorescence microscopy methods allow researchers to obtain calibrated images of dynamic changes in the physical properties of cells and tissues independent of dye concentration.The wavelength changes required for ratiometric imaging are routinely achieved using a short-arc source in combination either with a diffraction grating or interference filters mounted in a filter wheel. Filter wheels typically switch positions in around 50ms, scanning monochromators can achieve wavelength changes within a ms. These approaches generally limit ratio imaging rates to a little over 10Hz, and have the inherent drawbacks of short lamp life and high thermal emissions. An LED based system has much higher stability than a short-arc source, and with sub-microsecond wavelength switching times allows the very highest speeds to be obtained. Here we made use of the popular Ca2+ probe Fura-2 to record images of intracellular [Ca2+] in isolated ventricular cardiomyocytes at frame rates in excess of 100Hz using a simple and inexpensive LED based system.
Transient spatial gradients of Ca2+ can exist in single ventricular cardiomyocytes during spontaneous release of Ca2+ from the sarcoplasmic reticulum. This can result intracellular Ca2+ waves traveling at 100-200microns/s along the length of the cell. Rapid imaging is required to resolve the time course and pattern of intracellular Ca2+ release.
Conventionally Fura-2 ratios are calculated by monitoring the fluorescence signal elicited from excitation at 340 and 380nm, however short wavelength LEDs (340nm) are not available currently. Alternatively, reliable ratiometric measurements can be made by exciting Fura-2 at its isobestic point (360nm) and 380nm. We have followed fast spatial changes in Ca2+ by switching wavelength in the microsecond time domain using commercial LEDs emitting at peak wavelengths of 365nm and 385nm.

3290-Pos Board B337
Theoretical Analysis of Nano-scale Imaging by Ion Conductance Microscopy
Samantha J. L. Lee[1], Eero J. Willman[1], David Klenerman[2], F. Anibal Fernández[1], Guy W. J. Moss[1].
[1]University College London, London, United Kingdom, [2]Cambridge University, Cambridge, United Kingdom.
Ion conductance microscopy (ICM) is a powerful new technique that allows non-contact, nano-scale imaging of the topography of living cells in physiological solutions (Hansma et al., *Science* 243:641-643, 1989; Korchev et al., *Biophys. J.* 73:653-658, 1997). ICM works by measuring the reduction of current that occurs when a nano-pipette probe, in a conducting solution, approaches a non-conducting surface, such as the cell membrane. In ICM a point is chosen in the x-y plane and the probe is lowered towards the surface. The z-position at which the probe current is reduced by a specified amount (determined by the current set-point) indicates the relative height of the sample at that location. Measuring such heights at each position as the sample is scanned in the x-y plane allows the cell topography to be determined. We have developed a finite element-based computational model that can simulate nano-scale ICM imaging for small (~100 nm) probes. This model allows us to make a systematic study of how objects of arbitrary size and geometry appear in images obtained using a variety of current set-points and/or probes of different shapes. The implications of these simulations for producing an optimized scanning routine and the feasibility of using data from simulations to correct the recorded image so that it represents a true topography are also examined.

3291-Pos Board B338
Enhancing Signal to Noise Ratio in linear and non-linear excitation microscopy
Emiliano Ronzitti[1,2], Valentina Caorsi[1], Francesca Cella[1], Alberto Diaspro[1,2].
[1]LAMBS-MicroSCoBiO, Department of Physics, University of Genoa,, Genoa, Italy, [2]IFOM-SEMM, Foundation FIRC Institute for Molecular Oncology, Milan, Italy.
The resolution capability of an optical system can be completely characterized by the vectorial diffraction theory[1], which defines the intensity distribution of a point like source imaged by a lens assuming ideal imaging conditions. Unfortunately, these conditions can not be completely reached as noise affects a recorded microscope image. A detailed characterization of the imaging process in linear and non-linear fluorescence microscopy allows to evaluate the noise deterioration effect on the resolution capability.
In this work we propose optical set-up schemes towards an image quality improvement in terms of Signal to Noise Ratio. In order to reach this aim, we insert a proper amplitude ring filter on the illumination arm of the microscope[2]. The effect induced by the filter results in a redistribution of the spatial frequencies of the OTF, in particular with high frequencies information collected at improved SNR[3].
The optical system response of the proposed scheme has been characterized in the spatial and in the frequency domain by using a computational simulation mainly based on a vectorial approach.
Analysis reveals that, the practical imaging quality in presence of noise can be significantly improved in the ring filtering scheme. Further improvements can be reached by the usage of the proposed annular filters in combination with image restoration[4].
[1] B. Richards and E. Wolf, *Proc. of the Royal Society of London. Series A* **253**, 358-379 (1959).
[2] C.Caballero et al., *Optical Engineering* **45**, 980031-980036 (2006).
[3] B. J. Davis et al., *Optics Express* **12**, 4150-4156 (2004).
[4] P.P.Mondal et al., *Journal of Applied Physics* **102**, 44701, (2007).

3292-Pos Board B339
FRET Imaging Through A White Light Laser (wll) Source
Valentina Caorsi[1], Paolo Bianchini[1,2], Emiliano Ronzitti[1,2], Giuliano Colombetti[3], Alberto Diaspro[1,2].
[1]LAMBS-MicroSCoBiO, Department of Physics, University of Genoa, Genoa, Italy, [2]IFOM-SEMM, Foundation FIRC Institute for Molecular Oncology, Milan, Italy, [3]Institute of Biophysics, CNR, Pisa, Italy.
FRET (Forster Resonance Energy Transfer) imaging has been largely used as a useful tool to investigate intermolecular interactions, allowing to measure distances in 1-10nm range. A large variety of methods, based on both steady state and lifetime measurements, has been developed to carry out quantitative results in the evaluation of the FRET efficiency1. However a quantitative data interpretation can be difficult due to donor-acceptor spectral overlap which leads to contaminations of the FRET signal2. These contaminations are worsen by the limited choice of excitation wavelengths available on conventional microscopes. Recently, a new generation of laser sources has been proposed, mainly based on supercontinuum laser technology3 . We recently tested a Koheras SuperK compact WLL combined with a Leica TCS SP5 AOBS system, to analyse FRET imaging. Moreover, lifetime measurements have been performed coupling the system with a time-correlated single-photon counting (TCSPC) electronics (Becker and Hickl), exploiting the 90 MHz repetition frequency in the visible excitation range. In all cases, we benefited of the flexibility of the WLL excitation wavelength choice as well as the capability of performing excitation spectra directly on the sample under investigation: on one hand it has been possible to fully characterize the FRET couple; on the other it has been possible to optimize the excitation wavelengths thus limiting the spectral contaminations.
(1) Chen Y. et al.2008. Journal of Microscopy 228: 139-152.
(2) Caorsi, V. al. 2007 Microsc Res Tech 70(5): 452 - 458.
(3) McConnell, G. 2005 Applied Physics B: Lasers and Optics 81(6): 783-786.

3293-Pos Board B340
Cell Image Quality and Point Spread Function Depends on Photomultiplier Sensitivity in a Fast Frame-Rate Multi-color Laser-Scanning Confocal Fluorescence Microscope
Yi-Kuang Liu, Mansoureh Eghbali, Enrico Stefani.
UCLA School of Medicine, Los Angeles, CA, USA.