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.

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

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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.

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

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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

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

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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.

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Overcoming the Nyquist limit with intensity modulation spectral analysis

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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.

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Optimizing Fluorophores For Super-resolution Fluorescence STED Microscopy

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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.

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Ultra Resolution Direct Imaging Optical Microscope

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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.