Imaging & Optical Microscopy III

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Imaging the Glucagon-Secreting Alpha Cells

Sylvain J. Le Marchand, David W. Piston.
Vanderbilt University, Nashville, TN, USA.

Pancreatic islets secrete insulin and glucagon, two critical hormones for the blood glucose homeostasis. Islets are composed mainly of insulin-secreting beta-cells (~80%) and glucagon-secreting alpha-cells (10-15%). Typically, insulin is secreted after a meal to reduce blood glucose levels, whereas glucagon is released in times of starvation. In order for insulin and glucagon to exert their opposite effects, glucose must also have an inverse effect on the secretory function of the two cell types. While the mechanisms of glucose-stimulated insulin secretion by beta-cells are fairly well understood, the study of the mechanisms underlying glucagon secretion has been hindered by the lack of reliable methods to distinguish between alpha- and beta-cells. Thus, it is not clear how glucose suppresses glucagon secretion. There is still little agreement as to whether this inhibition is a direct effect on alpha-cells or mediated by a paracrine inhibition from beta-cells via secretion of molecules such as insulin, Zn^{2+}, GABA.

Recently, a fragment of a rat glucagon promoter has been successfully used to specifically drive the expression of fluorescent proteins in mouse alpha-cells. Thus, it is now possible to easily identify glucagon-secreting cells within isolated islets or to flow sort a purified population of alpha-cells. Here, we report the use of dynamic fluorescence imaging techniques on these transgenic islets and cells. In particular, we investigated how glucose affects 1) alpha-cell metabolism via measurement of NAD(P)H autofluorescence by two-photon excitation microscopy; 2) alpha-cell intracellular calcium concentrations, via the loading of alpha-cells expressing red fluorescent proteins (tdRFP) with the calcium indicator dye Fluo4-AM; and 3) alpha-cell membrane potential, via application of the fluorescent probe $DiSBAC_2(3)$. The results of these biophysical measurements will also be compared to parallel glucagon secretion in response to glucose from both intact islets and sorted alpha-cells.

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Probing the Interaction of RecA and a dsDNA Segment via Optical Tweezers

Arthur E. Chiou[1], Chia-Hui Lien[1], Ming-Tzo Wei[1], Sung-Hua Tsai[1], Chung Wang[2], Ting-Fang Wang[2].
[1]National Yang-Ming University, Taipei, Taiwan, [2]Academia Sinica, Taipei, Taiwan.

RecA plays an important role in homologous recombination of DNA. When RecA combines with dsDNA to form RecA-dsDNA nucleofiliment, it unwinds dsDNA and changes the dsDNA structure. We measured with two complementary techniques, namely stationary optical tweezers and oscillatory optical tweezers, the force-extension relation and the elastic constant of a segment (~4μm) of dsDNA as a function of the stretching force before and after its interaction with RecA. The dsDNA sample was attached to two polystyrenes particles, one at each end; the smaller particle (diameter = 2μm) at one end was trapped by optical tweezers while the larger particle (diameter = 20μm) at the other end was fixed to the cover glass of the sample chamber which was filled with an appropriate buffer solution and was mounted on a PZT-driven translational stage. In consistent with the description of the worm-like chain (WLC) model, the elastic constant increased monotonically from approximately 8.6pN/μm to 35.9pN/μm when stretching force varied from 1.8pN to 17.0pN, and reached a constant value of approximately 41pN/μm for stretching force in the range of 20.0pN and 33.6pN (the enthalpic regime in the WLC model). After fully interacted with RecA, the elastic constant of the resulting RecA-dsDNA filament was determined to be approximately 47.3pN/μm in the enthalpic regime.

We also studied the dynamics of the interaction of dsDNA with RecA protein in terms of the elastic constant as function of time while the DNA was stretched at a constant stretching force of 33.6pN and allowed to interact with RecA (by injecting a solution containing RecA protein and ATPγS) and subsequently to dissociate with RecA (by injecting de-ionized distilled water into the chamber to wash off the ATPγS). The association rate increased with increasing concentration of RecA.

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Extending The Resolution In Total Internal Reflection Fluorescence (TIRF) Microscopy

Reto P. Fiolka, Andreas Stemmer.
ETH Zürich, Zürich, Switzerland.

Total internal reflection fluorescence (TIRF) microscopy is the method of choice to image structures close to the cell-substrate interface. Illuminating the specimen with evanescent light decaying exponentially in axial direction results in essentially two-dimensional images without interference from regions deeper within the specimen. However, the lateral resolution of TIRF microscopy remains diffraction-limited to about 240 nm for green emission. Lateral resolution can be increased by up to a factor of 2.5 when illuminating the sample with evanescent standing waves [1].

Here we report a compact and versatile illumination set-up employing two beam splitters and two piezo-actuated mirrors featuring two angular degrees of freedom. The piezo-actuated mirrors provide full control over the orientation and penetration depth of the evanescent standing wave. In contrast to grating-projection set-ups [1], beam steering via mirrors facilitates simultaneous excitation at multiple wavelengths since the angle of incidence is set by reflection off the mirrors and does not vary for different wavelengths. To obtain a two-dimensional image with extended resolution, the specimen is illuminated with at least two differently oriented standing wave patterns. More directions lead to higher isotropy of the extended optical transfer function (OTF). In practice, however, we found that the cloverleaf-shaped OTF resulting from two illumination directions yields images of comparable morphology as the more isotropic OTF resulting from three illumination directions. Using only two directions increases imaging speed, whereas three illumination directions lead to more spectral overlap within the extended OTF, which facilitates image reconstruction. We further demonstrate the potential of this technique for biological imaging on examples including HeLa cells expressing GFP-actin.

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Multicolor Three-dimensional Whole-cell Imaging With Nanometer Scale Resolution

Sara A. Jones[1], Bo Huang[1,2], Boerries Brandenburg[1,2], Xiaowei Zhuang[1,2].
[1]Harvard University, Cambridge, MA, USA, [2]Howard Hughes Medical Institute, Chevy Chase, MD, USA.

The ability to directly visualize nanoscopic cellular structures and their spatial relationship in all three dimensions will greatly enhance our understanding of molecular processes in cells. In this work, we have developed multicolor three-dimensional (3D) stochastic optical reconstruction microscopy (STORM) as a tool to probe molecular structures and their interactions on sub-diffraction length scales. STORM achieves sub-diffraction limit image resolution by using photoswitchable fluorescent probes to separate the spatially overlapping images of individual probes in time. Only a small subset of probes was activated at any given time, allowing us to resolve individual activated probes and determine their positions with high precision. A super-resolution image was then constructed by plotting the measured probe positions accumulated over time. With this we have generated 3D whole cell images, several micrometers thick, with a spatial resolution of 20 - 30 nm and 60 - 70 nm in the lateral and axial dimensions, respectively. Using this approach, we imaged the entire mitochondrial network in mammalian cells and studied the spatial relationship between mitochondria and the microtubule cytoskeleton. The 3D STORM images clearly resolved the hollow mitochondria outer membrane structures obscured in conventional fluorescence images. Distinct mitochondrial morphologies were observed, ranging from thin elongated tubes to globular compartments. Interestingly, while globular mitochondria are relatively dispersed in size, from 200 nm to 1500 nm, the tubular structures are more uniform in diameter, taking a narrow distribution around 200 nm. The images also displayed several distinct interaction modes between mitochondria and microtubules. Notably, elongated mitochondria were observed to ''inchworm'' along microtubules with discrete attachment sites, while such an interaction mode was completely unresolvable with conventional fluorescence. Super-resolution optical microscopy techniques such as STORM promise to significantly expand the understanding of biological structures and their interactions on a molecular level.

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3D-Resolution In FPALM/PALM/STORM

Michael J. Mlodzianoski, Joerg Bewersdorf.
Institute for Molecular Biophysics, The Jackson Laboratory, Bar Harbor, ME, USA.

Light microscopy is one of the most widespread imaging techniques used by biologists, but even the best traditional microscopes are limited by diffraction to a resolution of about 250nm, leaving many sub-cellular structures in an unresolved blur. Over the last few years, several groups have pioneered localization techniques to surpass the resolution limit[1-4]; however those techniques were limited to two dimensions. Recently, two techniques have been employed that extend localization into the third, axial dimension[5,6]. Biplane fluorescent photoactivatable localization microscopy[6] (BP-FPALM) splits the detected fluorescence into two paths[7], one slightly longer than the other to image two

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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5. Huang, B. et al., *Science* **319** (5864), 810 (2008).
6. Juette, M. F. et al., *Nat Methods* **5** (6), 527 (2008).
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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).

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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).
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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.

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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.

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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.

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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.