2993-Pos

Microprisms for In Vivo Multiphoton Microscopy of Mouse Cortex

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Fluorescence microscopy of cortical slices, yielding ready access to all six layers of cortex, has proven to be a powerful technique in neurophysiology, however it lacks the context of in vivo experiments. In vivo microscopy, primarily multiphoton microscopy, provides this context but without ready access to deeper layers and typically involves imaging of a field-of-view that is roughly parallel to the cortical layers. Needle-like gradient index (GRIN) lenses have been used as invasive relay lenses to access deeper brain structures, however these lenses damage the apical dendrites of the neurons of interest during insertion into the cortex, and are therefore of limited use for functional cortical imaging.

We present here the use of micro-prisms for performing in vivo multiphoton microscopy of mouse cortex. Small (~1 mm) prisms with a reflective coating on the hypotenuse act as a miniature periscope, rotating the image plane from one parallel to the cortical layers to one that is perpendicular to the layers. This enables simultaneous imaging of the entire thickness of cortex, much as is done it cortical slice preparations, while maintaining a large degree of the in vivo context.

2994-Pos

Structured Illumination Microscopy in 2-D with Image Restoration

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The resolution of fluorescence microscopes is fundamentally limited by physics to about 200 to 250 nanometers. An approach to improving this resolution is to impose a pattern on the illumination exciting the fluorescent sample [Gustafsson, 2000]. This structured illumination approach doubles the resolution of the fluorscence microscope. A further doubling of the resolution of structured illumination images. is obtained using a computational approach, image restoration or 'deconvolution'. In this computational approach, the imaging process is quantitatively characterized and this quantitative knowledge is used to improve the resolution of the microscope data [Carrington, 1995].

This algorithm has been implemented in 2-d for structured illumination microscopy with good results. Simulations show that for a noise free image we obtain resolve point sources separated by 40 nanometers. In simulations of samples with noise typical of bright samples, two point sources separated by 56 nanometer are resolved. In simulations of point sources each of which is a single fluorophore, two fluorophores separated by 72 nanometer are resolved.

Carrington, W.A., Lynch, R.M., Moore, E.D.W., Isenberg, G., Fogarty, K.E. & Fay, F.S. (1995) Super-resolution three-dimensional images of fluorescence in cells with minimal light exposure. Science, 268, 1483-1487.

Gustafsson, MGL(2000) Surpassing the lateral resolution limit by a factor of two using structured illumination microscopy. Journal of Microscopy 198 (2), 82-87.

2995-Pos

Dual-Channel Single-Molecule Imaging of pRNA on phi29 DNA-Packaging Motor

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Bacteriophage phi29 DNA-packaging motor is geared by six packaging RNAs (pRNA). The pRNA molecules have been reported to serve as building blocks in RNA nanotechnology, and as vehicles for specific delivery of therapeutics to treat cancers and viral infections. The understanding of the 3D structure of pRNA and its location and positioning on the motor are both fundamentally and practically important. A customized single-molecule dual-color imaging system has been constructed to study the structures of pRNA molecules. The system is the combination of a low-temperature (−80 °C) sensitive electron multiplied CCD camera and the prism-type total internal reflection mechanism. A laser combiner was introduced to facilitate simultaneous dual-channel imaging. It has been applied to study the structure, stoichiometry, distance and function of the phi29 DNA packaging motor. Single molecule photobleaching analysis clarified the stoichiometry of pRNA on the motor and elucidated the mechanism of pRNA hexamer assembly. The feasibility of the single-molecule imaging system was demonstrated in single-molecule FRET studies. Distance rulers made of dual-labeled dsDNA and RNA/DNA hybrids were used to evaluate the system. The single-molecule FRET was also applied to the reconstructed the 3D structure of phi29 motor pRNA monomers and pRNA dimers. Ten pRNA monomers labeled with single donor or acceptor fluorophore at various locations were constructed, and eight partner pairs were assembled into dimers. FRET signals were detected and utilized to assess the distance between each donor/acceptor pair. The results provide the distance constraints for computer modeling of the motor. We have also re-engineered the energy conversion protein, gp16, of phi29 motor for single fluorophore labeling to facilitate the single molecule studies of motor mechanism. The potential applications of nanometer localization approaches (SHRImP and SHREC) to the study of the phi29 nanomotor were also investigated.

2996-Pos

High-Resolution Intracellular Viscosity Measurements using Time-Dependent Fluorescence Anisotropy

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A characteristic of living cells is that they continuously respond to changes in their environment. Our ability to observe and measure these responses on micro- and nanoscale levels gives us insight into the internal organization of the cell, and allows us to formulate a more complete model of cell physiology. We have developed a technique for makinghigh-resolution, sub-micron measurements of intracellular viscosity *in vivo*. A low-cost pulsed laser is used in conjunction with a homebuilt confocal laser-scanning epifluorescence microscope with submicron lateral and axial spatial resolution to measure fluorescence anisotropy at specific locations within a mouse J774 microphage cell. Global deconvolution techniques are used to determine rotational correlation times for fluorophores in those locations. In order to effectively determine the quantitative viscosity of the selected intracellular region, we first measure molecular rotational correlation times of our chosen fluorophore (HPTS, or pyranine) in known viscosity solutions of trehalose in water. We then construct a calibration curve relating the rotational behavior of the fluorophore to viscosity. This calibration curve is used to generate quantitative viscosity measurements for the measured intracellular rotational correlation times. The data show that local viscosities within the cell are not uniform. In the cytoplasmic areas measured, rotational correlation times of HPTS ranged from 0.144 ns to 0.320 ns, and viscosities ranged from 1.00 to 2.21 cP. We will compare the use of time-dependent fluorescence anisotropy with fluorescence correlation spectroscopy techniques used to determine intercellular viscosity, and identify the conditions under which each technique is most beneficial.

2997-Pos

Goodbye to Foerster Constant RO: FRET Imaging By K_f/k_t

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Most FRET methods based on relative donor characteristics involve an expression for the FRET "efficiency" E of a given DA pair:

$$k_t = \frac{(R_0/r)^6}{\tau_D};\ E \equiv k_t\tau_{DA} = \left[1+(r_{DA}/R_0)^6\right]^{-1};\ R_0^6 = c_o J\kappa^2 n^{-4} Q_o \qquad [1]$$

$$Q_o = k_f\tau_D;\ \tau_D^{-1} = k_f + k_{ic} + k_{ec} + k_{pc};\ \tau_{DA}^{-1} = \tau_D^{-1} + k_t$$

Thus, E is defined as the product of the energy transfer rate constant, k_t, and the fluorescence lifetime, τ_{DA}, of the quenched donor. The other quantities in Eq. 1 are the DA overlap integral, J; refractive index of the transfer medium, n; orientation factor, κ^2; and unperturbed donor quantum yield, Q_o. Because of the latter, τ_D appears both in the numerator and denominator of k_t and cancels out. Thus, τ_D is absent in the more fundamental expression representing the essence of the Förster relationship, namely the ratio of the rate of energy transfer, k_t, to the radiative rate constant, k_f: $k_t/k_f = (\Gamma_o/r_{DA})^6$; $\Gamma_o^6 = c_o J\kappa^2 n^{-4}$.

The simplified reduced Förster constant we denote as Γ_o is better suited than R_0 in measurements based on *acceptor* (≡ donor) properties in that it (i) avoids the arbitrary introduction into the definition of R_0 of τ_D, which can vary from one position to another in an unknown and indeterminate manner; and (ii) finesses the absolute requirement for an estimation of E (Eq. 1). k_t can be expressed directly in terms of measured experimental parameters without the need for estimating E. That is, excluding bleed-through corrections, k_t/k_f at every image position is given by: $k_t/k_f = Q_{o,A}^{-1}(d_{A,D}/d_{A,A})(f_{DA}/f_{DD})$, in which d is a relative detection efficiency ($d_{A,D}$ Denotes Acc Emission Ind) and f is a corresponding measured signal. The "k_t/k_f" image provides a direct measure of $(\Gamma_o/r_{DA})^6$. The acceptor quantum yield $Q_{o,A}$, unlike the donor, can be checked pixel-by-pixel by lifetime determinations using direct excitation.

Refs: Jares-Erijman EA, Jovin TM: (2006) *Curr. Opin. Chem. Biol.* **10**:1-8; (2009) Reflections on FRET imaging: formalism, probes, and implementation *In* FRET and FLIM Imaging Techniques (Ed. D. Gadella Jr.). Academic P., pp. 475-517.

2998-Pos

Monitoring Changes in Bacteriorhodopsin Conformation using Fluorescence Lifetime FRET with a Maximum Likelihood Estimator

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Protein folding is a fundamental process within biological systems and changes of the protein conformation upon folding and unfolding can be studied by

performing Förster resonance energy transfer (FRET) measurements. Unlike fluorescence intensity which has been traditionally used in FRET experiments, fluorescence lifetimes do not depend on fluorophore concentration, detection efficiency, illumination intensity and uniformity. Therefore, lifetime FRET overcomes the artefacts arising from intensity measurements and provides improved precision in investigating the protein unfolding procedure. Herein, we developed a novel technique for performing lifetime FRET using a maximum likelihood estimator (MLE) adapted from single molecule studies. We demonstrate the feasibility of our detection technique by monitoring the unfolding procedure of the membrane protein Bacteriorhodopsin (bR) labelled with the FRET pair Alexa Fluor 488 (donor) and Alexa Fluor 647 (acceptor). A home-built laser scanning confocal microscope and two avalanche photodiode detectors (APDs) are used for detection with high sensitivity and the fluorescence decays are collected using time correlated single photon counting (TCSPC). When determining a fluorescence lifetime with less than 2000 photons, the conventionally used least squares approach is not appropriate. Therefore, a MLE previously developed in our lab defined by multinomial statistics is used to accurately extract molecular fluorescence lifetimes from as little as 10 photons. These are then used to calculate FRET efficiencies and hence the conformational state of bR.

2999-Pos

Exploiting the Rise Time of Acceptor Fluorescence by FRET-FLIM in Living Cells

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Previously (Borst et al. (2008) Biophys. J. 95) a time-resolved fluorescence methodology has been described for quantitative determination of Förster resonance energy transfer (FRET) between donor-acceptor pairs in biological macromolecules by analyzing the time resolved rise of acceptor fluorescence upon donor excitation. The main advantage is that only those molecules are monitored involved in the energy-transfer process. This contrasts with the more conventional method that measures time-resolved fluorescence of donor molecules and thereby probing a mixture of FRET-active and FRET-inactive populations. We have extended the determination of rise times of acceptor fluorescence to measure FRET in living cells with fluorescence lifetime imaging microscopy (FLIM). Parameters describing the rise of acceptor fluorescence and the decay of donor fluorescence can be determined via simultaneous global analysis of multiple FLIM images thereby increasing the accuracy of the recovered parameters. In the present study, plant protoplasts were transfected with a visible-fluorescent-protein fusion composed of a 6-amino-acid peptide flanked by enhanced GFP (eGFP) and mCherry for illustration of the new data analysis method. It is demonstrated that the distances estimated with the present method are substantially smaller (and more realistic) than those estimated from average donor fluorescence lifetimes. The latter over-estimation is due to a fraction of non-transferring donor molecules, which makes the average fluorescence lifetime of the donor longer. In addition, combining fit results of fluorescence kinetics at different detection wavelengths allows correcting for the contribution of molecules that are not able to transfer their excitation energy. The high fraction of non-transferring eGFP in the construct is explained by taking the presence of non-matured mCherry into account. Software to reproduce the presented results is provided in an open-source and freely available package called "TIMP" for "The R project for Statistical Computing".

3000-Pos

Macrophages Create a Lysosomal Synapse to Digest Aggregated Lipoproteins

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Atherosclerosis is the underlying cause of the majority of heart attacks and strokes. Lipoprotein accumulation and degradation by monocyte-derived macrophages is a central event in the pathogenesis of atherosclerosis. Data show that the vast majority of low density lipoprotein (LDL) in atherosclerotic plaques is aggregated and avidly bound to the subendothelial matrix.

We employ various microscopy and biochemical techniques in both cell culture and animal models to investigate the mechanism of uptake of aggregated LDL (agLDL) by macrophages. We show that macrophages create an extracellular, acidic compartment where the cells contact the agLDL, and lysosomal contents are delivered to these compartments, thereby forming an extracellular hydrolytic compartment - a lysosomal synapse. Active acidification of these specialized compartments and aggregate catabolism were observed by fluorescence ratiometric time-lapse imaging of an *in vitro* cell culture model. Biochemical assays, employing radiolabeled agLDL demonstrated an increase in free cholesterol in aggregates contained in the lysosomal synapse. This cholesterol can be delivered to the cell, initiating the process of macrophage cholesterol loading and ultimately causing progression of the atherosclerotic plaque.

Although *in vitro* systems provide insight into macrophage uptake of lipoproteins, ultimately we must strive to understand the function and behavior of macrophages in intact animals. Relatively little is known of the fate of monocytes once they migrate into atherosclerotic lesions. To this end, we employ intravital multiphoton microscopy in mouse models of atherosclerosis to allow observation of macrophages within the artery wall.

Our studies elucidate the mechanism of a novel pathway for catabolism of agLDL by macrophages. Better understanding of the mechanisms by which macrophages interact with lipoproteins in the subendothelium may lead to new approaches to inhibit lipid accumulation in macrophages and thus, may be of therapeutic value in preventing atherosclerosis.

3001-Pos

Broad-Beam Fluctuation Spectroscopy for Non-Flow Cytometry and Clinical Diagnostics

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Fluorescence fluctuation spectroscopy (FFS) and related techniques such as FCS, PCH and FIDA have been valuable tools for biophysical research, but have not found widespread clinical application. Many clinical blood diagnostics measure the concentration of particular cell types in the blood. These tests are typically performed using fluorescent antibodies and flow cytometry. We present a novel scanning FFS system, which we term Broad-beam Scanning Fluctuation Spectroscopy (BSFS), with application to cytometry. BSFS uses a much larger (~1 nL) observation volume than conventional FCS, so that the fluctuations measured result from cells, rather than individual molecules. This technique is well suited to the measurement of cell concentration, as the correlation analysis also yields a measurement of the sample volume, allowing an absolute concentration to be determined. BSFS is a viable alternative for a variety of cell-based clinical diagnostics, while lacking the optical and fluidic complexity of a flow system.

3002-Pos

Mobility Analysis in Living Yeast using 4Pi CFM

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Continuous fluorescence microphotolysis (CFM) is a powerful tool to analyze molecular mobilities and association reactions in single living cells but has mostly been restricted to diffraction limited focal volumes. Recently, we introduced the theoretical and experimental framework to combine CFM with super-resolution microscopy by utilizing a 4Pi point spread function in mobility analysis.

Here we show that this methodology can be readily applied to living yeast cells under physiological buffer conditions using water-immersion lenses. Yeast cells are relatively small with a typical diameter of 5 μm and the intracellular individual compartmentalization with a large vacuole and the nucleus results in small volume elements with unhindered diffusion. Therefore diffusion measurements with an engineered point spread function should be beneficial to recover the diffusion coefficient as this approach is less susceptible to improper positioning of the laser.

Using 4Pi CFM we were able to clearly recover the diffusion coefficient of GFP in the cytoplasm and the nucleus of living yeast cells. Additionally, the mobility of GFP-tagged proteins involved in nucleo-cytoplasmatic transport was analyzed. While the diffusion coefficient of a GFP-tagged cargo was determined to be in a range expected for a molecule of this respective size, we found evidence that the diffusion coefficient of a GFP-tagged transport receptor was reduced compared to the expected value for purely free diffusion. This might indicate that the molecule is subject to a certain degree of unspecific binding in the cytoplasm of yeast.

3003-Pos

Scanning Laser Image Correlation (SLIC) Measurements in Zebra Fish Larvae

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