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.

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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