rat show a group of cells much brighter than those observed in non-Nigral control regions. They contain hundreds of dopamine vesicles/vesicle clusters in the soma, akin to the serotonergic neurons from the Raphe imaged earlier. Drugs such as amphetamine are thought to cause non-exocytotic release of dopamine from the dopaminergic neurons but measuring such dynamics has been difficult. Our time-lapse imaging shows that 100 μM para-chloroamphetamine depletes the total dopamine content of SN neurons by about 25% over a time scale of fifteen minutes without substantial translocation of the vesicles. Imaging neurotransmitters in live cells also allows us to monitor the differentiation of stem-cells into serotonergic neurons over a period of many weeks. Interestingly, we observe that the ability to exocytose serotonergic vesicles arises several days after serotonin starts expressing in these cells. Our imaging techniques therefore provide a visual assay for a more functionally relevant maturation point for the differentiation of these cells, a fact with possible clinical significance for the treatment of various neurodegenerative and injury-related disorders.

2983-Pos

Late Endosomal Degradation of Low-Density Lipoprotein Probed with Multi-Color Single Particle Tracking Fluorescence Microscopy

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The vesicle-mediated degradation of low-density lipoprotein (LDL) is an essential cellular function due to its role in cellular membrane biosynthesis. Using multi-color single particle tracking fluorescence microscopy, we have probed the intracellular degradation of low-density lipoprotein in living cells. The unique aspect of our experiments is the direct observation of LDL degradation using an LDL-based probe that increases fluorescence intensity upon degradation. Specifically, individual LDL particles are labeled with multiple fluorophores resulting in a quenched fluorescent signal. Control experiments demonstrate that enzymatic degradation of the LDL particle results in an increase in fluorescence. The ability to directly observe LDL degradation allows us to determine which vesicle is responsible for degradation and quantify the vesicle dynamics involved in LDL degradation. Visualization of early endosomes, late endosomes and lysosomes is accomplished by fluorescently labeling vesicles with variants of GFP. Transient colocalization of LDL with specific vesicles and the intensity of the LDL particle are measured simultaneously. The measured colocalization durations are then correlated with changes in fluorescence intensity due to LDL degradation. We observe that degradation of LDL occurs in the late endosome. While there are a broad distribution of colocalization durations of LDL with Rab7, a late endosomal protein, only relatively long (>420 s) colocalization leads to the degradation of LDL. These studies, which are the first to directly observe the degradation of LDL within a cell, support a model in which late endosomes are the site of degradation with lysosomes serving as enzyme storage vesicles.

2984-Pos

Hotspots of GPI-Anchored Proteins and Integrin Nanoclusters Function as Nucleation Sites for Cell Adhesion

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Lymphocyte function-associated antigen-1 (LFA-1) is a leukocyte specific integrin that mediates migration across the endothelium and the formation of the immunological synapse. Association of LFA-1 into specific cholesterol enriched microdomains, called lipid rafts, is thought to regulate its activity. These 20 to 200 nm lipid rafts are abundantly present on the cell membrane. However, these length-scales are not available for conventional light microscopy, requiring higher resolution. Near-field scanning optical microscopy (NSOM) uses a sub-wavelength aperture probe to locally excite fluorophores thus providing optical resolution at the nm-scale [1]. Here, we have used single-molecule sensitive NSOM to capture the spatiofunctional relationship between LFA-1 and raft components (GPI-APs) on immune cells. Direct dual-color nanoscale imaging revealed the existence of a GPI-AP subpopulation organized in nanodomains that further concentrated in regions smaller than 250nm, suggesting a hierarchical pre-arrangement of GPI-APs. In addition, integrin nanoclusters reside proximal to these GPI-AP nanodomains, forming cholesterol sensitive hotspots on the cell surface [2,3]. These hotspots function as essential intermediates in nascent cell adhesion, driving the formation of large-scale macrodomains that facilitate firm adhesion [3,4]. Altogether, this well-defined pre-assemblage of proteins might constitute a prominent mechanism exploited by the cell to rapidly and efficiently aggregate distinct nanodomains into larger functional cell surface assemblies.

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2985-Pos

Imaging α-Cell Calcium Dynamics

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Glucagon is released into the bloodstream when glucose reaches threateningly low levels. Its secretion from α-cells, within pancreatic islets of Langerhans, stimulates hepatic glucose release and, therefore, restores proper glycemia. Once normoglycemia is reestablished, glucagon release is inhibited. Impairment of this inhibition has been observed in diabetes mellitus where greater amount of glucagon worsens the chronic hyperglycemic state. However, the mechanisms mediating this glucose suppression of glucagon secretion are poorly understood. Two models have been proposed: direct inhibition by glucose, or paracrine inhibition from non α-cells within pancreatic islets. We report here the use of transgenic mouse lines that specifically express Red Fluorescent Protein within α-cells. This strategy makes it possible to easily identify α-cells and study their intracellular calcium ($[Ca^{2+}]_i$) dynamics by Fluo4 imaging. Our glucagon measurements from flow-sorted α-cells indicate that glucose does not directly inhibit α-cells (+57% increase in glucagon secretion from 1 to 20mM, $p<0.05$, ANOVA). This observation supports the paracrine inhibition model. Influx of calcium ions is a trigger for exocytosis of neuroendocrine vesicles. Since glucagon release from islets is inhibited by increasing levels of glucose, one would naively expect that α-cell $[Ca^{2+}]_i$ would decrease. Imaging of calcium dynamics by fluorescence microscopy demonstrates that glucose mediates an increase in α-cell $[Ca^{2+}]_i$ in intact islets (24.7 ± 3.4% increase in fluorescence intensity from 1 to 20mM glucose, compared to +51.5 ± 5.0% for insulin-secreting β-cells). Meanwhile the percentage of α-cells exhibiting calcium oscillations is comparable at low and high glucose levels (44% of α-cells oscillate during a 5-minute observation period). Furthermore, the shape, frequency, and amplitude distribution of these $[Ca2+]_i$ signals were not modified either. Taken together, these results suggest that suppression of glucagon secretion occurs downstream from α-cell calcium influx, likely at the level of vesicle trafficking or exocytotic machinery.

2986-Pos

DNA Stabilizes Fluorescent Few-Atom Silver Clusters with Unique Photophysical Properties

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Recent studies of DNA-stabilized few-atom Ag clusters (Ag:DNA) indicate that these fluorophores may be well suited for high-resolution imaging techniques requiring optically modulated fluorescence, as well as fluorescence applications using two photon excitation. Ag:DNA exhibit sequence-dependent fluorescence from the blue to the near-infrared, thus DNA sequence presents an enormous parameter space for creating new emitters with optimized properties for fluorescence imaging. Even a very small search through sequence space reveals that single-base mutations in oligonucleotides can change the fluorescence intensity of Ag:DNA solutions by 3000%. We address some basic features of Ag:DNA that will help guide future large-scale searches through sequences space. By correlating fluorescence and mass spectroscopy, we identify emitters comprised of around 10 Ag atoms. We also observe absorbance by the DNA bases as an excitation pathway common to all Ag:DNA emitters. Finally, we discuss our efforts towards producing pure solutions of Ag:DNA at high enough concentrations for structural characterization by NMR.

2987-Pos

Multiphoton Microscopy of Entire Intact Mouse Organs

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Three-dimensional datasets from tissue biopsies may provide critical morphological information that is not readily obtained from traditional approaches to histology using thin physical sections of tissue. Multiphoton microscopy (MPM) provides optical sectioning with penetration into highly scattering materials, ready excitation of intrinsic tissue fluorescence, and access to nonlinear signals such as second harmonic generation (SHG). However, the penetration depth of MPM is typical limited to ~200 microns in many tissues. We present MPM of entire intact, fixed and optically cleared mouse organs. Clearing of tissue is typically incomplete for large tissue samples, however, MPM has sufficient tolerance to scattering to image entire mouse organs. Using macro lenses

with 5x magnification and 0.5 numerical aperture enables the generation of high resolution, large field-of-view datasets with imaging depths of several millimeters, sufficient to generate 3D image sets of mouse intestine, heart, lung, brain and other organs.

2988-Pos

The Role of the Protein Matrix in GFP Chromophore Biosynthesis: A Molecular Dynamics Study

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Spontaneous chromophore formation is probably the key feature for the remarkable success of GFPs (Green Fluorescent Proteins) and related fluorescent proteins in bioimaging and fluorescence microscopy. Though a quantitative analysis of the energetics of chromophore biosynthesis still remains elusive, substantial progress has been made [1] in identifying the various steps involved in the reaction and in unveiling the role played by the protein scaffold and by individual residues. A mechanical compression was initially proposed as a determinant factor in triggering backbone cyclization (the first step in chromophore formation) of the chromophore-forming tripeptide [2]. This compression was later ruled out on the basis of X-ray and mutagenesis studies, leading instead to the formulation of a conjugation-trapping mechanism, where the endothermic cyclization product is trapped by subsequent oxidation [3]. Here, by molecular dynamics simulations and potential of mean force calculations, we shall present an estimate of the contribution of the protein scaffold in promoting the proximity of the reacting atoms (a backbone amide and a carbonyl group) - and hence backbone cyclization - by a sort of compression mechanism. Comparing several mutants we shall highlight the role of some residues within or surrounding the chromophore-forming tripeptide. Finally, we shall analyze the case of the HAL (Histidine Ammonia-Lyase) enzyme active site, which undergoes a cyclization reaction analogous to the one in GFP.

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2989-Pos

Metabolism-Excitation Coupling in a Model of K_{ATP} Channel Neonatal Diabetes Mellitus

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The K_{ATP} channel is critical to pancreatic beta cells, linking glucose metabolism with insulin secretion. K_{ATP} channel mutations that reduce ATP-sensitivity lead to neonatal diabetes mellitus (NDM). Expression of these K_{ATP} channel mutations in mice also leads to severe diabetes. Beta cell mass and insulin content are initially preserved, but both decrease with time following the prolonged hyperglycemia. When glycemic control is imposed by transplantation of exogenous islets, this secondary loss of beta-cell mass and insulin content is avoided. We have examined various steps of the glucose stimulated insulin secretion pathway in islets that express ATP-insensitive K_{ATP} channels, which are either unprotected from hyperglycemia or protected by islet transplantation. In protected islets, expression of mutant K_{ATP} channels leads to a severe blunting of glucose stimulated $[Ca^{2+}]$ activity. There remains some sulfonylurea stimulated $[Ca^{2+}]$ and normal KCl stimulated $[Ca^{2+}]$, with insulin secretion following a similar pattern. In unprotected islets, isolated from severely diabetic mice, very similar patterns of $[Ca^{2+}]$ activity are measured but insulin secretion is markedly decreased.

Using two-photon microscopy of NAD(P)H we find glucose metabolism is unaltered in protected mutant islets. This suggests that the reduced $[Ca^{2+}]$ elevation and thus mitochondrial Ca^{2+} uptake has little effect on mitochondrial metabolism. However in unprotected islets, there is elevated basal NAD(P)H and an absence of glucose stimulated NAD(P)H increase, suggesting a disruption of glycolysis and/or mitochondrial metabolism.

Finally the low level of glucose stimulated $[Ca^{2+}]$ in mutant islets is partially synchronized, indicating coupling is present. Reduction of gap junction conductance via chemical inhibitors or islet dispersal leads to elevated glucose stimulated Ca^{2+}. This indicates an important role for gap junctions in regulating Ca^{2+} triggering in the presence of K_{ATP} channel mutations.

2990-Pos

ATP Gradient Across the Innermitochondrial Membrane

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Adenosine 5'-triphosphate (ATP) is one of the most important biological molecules, playing roles as an energy-currency and also as an intracellular and extracellular signal transducer of the cells. In spite of its importance, however, how ATP distribute inside a cell is not clear, because cellular ATP is usually measured after disruption of numbers of cells. Recently, we have developed genetically-encoded Förster resonance energy transfer (FRET)-based indicators specific to ATP, which are called ATeams. These indicators enabled us to monitor ATP concentrations at any desired place inside living cells. To investigate whether there is any biased ATP distribution inside living cells, we compare ATP level of cytoplasm, nucleus and mitochondria using ATeams. ATP levels of cytoplasm and nucleus were almost the same, suggesting that ATP can freely pass through nucleic pores. On the other hand, we observed that ATP level of mitochondria matrix was significantly lower than those of cytoplasm and nucleus. Therefore, there is a gradient of ATP across inner mitochondrial membrane. When the loss of membrane potential of mitochondria was induced by an uncoupling reagent, CCCP, mitochondrial ATP level elevated and the gradient of ATP across the inner mitochondrial membrane disappeared. In addition, loss of membrane potential of mitochondria during apoptosis also resulted in the elevation of mitochondrial ATP. Our results suggest that membrane potential-dependent unidirectional exchange of ATP and ADP by ATP:ADP carrier proteins occurs very rapidly, enough to keep intramitochondrial ATP lower than outside.

2991-Pos

Alpha Hemolysin Induces an Increase of Erythrocytes Calcium: a Fluorescence Lifetime Imaging Microscopt Study

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α-hemolysin (HlyA) from *Escherichia coli* is considered as the prototype of a family of toxins called RTX (repeat in toxin), a series of proteins that share genetic and structural features. HlyA is an important virulence factor in *E. coli* extraintestinal infections, such as meningitis, septicemia and urinary infections. High concentrations of the toxin causes the lysis of several cells as erythrocytes, granulocytes, monocytes, endothelial cells and renal epithelial of different species and low concentrations induces the production of cytokines and apoptosis. Eriptosis, the apoptosis process in erythrocytes, can be induced by several toxins and the increase in calcium concentration inside the cell is being postulated as the trigger of this process. In this context, we followed the calcium concentration inside the erythrocytes while incubating with sublytic concentrations of HlyA; calcium concentration was monitored following the changes in lifetime of the calcium indicator Green 1 using fluorescence lifetime imaging microscopy (FLIM). Data were analyzed using the phasor representation.

In this report we present evidences that, at sublytic concentrations, HlyA induces an increase in Calcium concentration in rabbit erythrocytes in the first 5 minutes. Results are discussed in relation to the difficulties of measuring Calcium concentrations in erythrocytes where hemoglobin is present, the contribution of the background, the heterogeneity of the response observed in different cells and how the phasor approach for lifetime measurements analysis can solve these challenges successfully.

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2992-Pos

Measuring Diffusion Coefficients in Confined Systems Via Multi-Photon Fluorescence Recovery after Photobleaching

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Multi-photon fluorescence recovery after photobleaching (MP-FRAP) is a microscopy technique used to measure the diffusion coefficient of macromolecules in both in vitro and in vivo biological systems. As MP-FRAP is introduced into more systems in vivo, the need arises to expand the technique for application to a wider range of physiological situations. In this poster, we present our investigations into measuring diffusion coefficients via MP-FRAP in bounded systems. We begin by modeling both diffusion and the fluorescence recovery process within a bounded system via Monte Carlo simulations. We then move in vitro, taking and analyzing fluorescence recovery curves in the presence of one and two boundaries. From our results, we determine three limiting cases: 1) boundaries are sufficiently far away to allow the use of MP-FRAP as currently formulated, 2) boundaries are so close as to prevent the use of MP-FRAP entirely, and 3) boundaries are located in a range between these limits, and MP-FRAP is applicable, with modifications to the analysis.