using biochemical methods. We used fluorescent moment image analysis and spatial intensity distribution analysis (SpIDA) to study the oligomeric state of NBCe1-A in cultured cells expressing the cotransporter and in rat kidney tissue. Both methods allow for quantitative measurement of fluorescent particle densities and oligomerization states within individual images acquired with laser-scanning microscopy. Initially we examined basal membranes of highly adherent CHO K1 cells expressing eGFP-tagged NBCe1-A because of their large surface area. As an independent control of monomeric brightness, we used cells expressing monomeric eGFP anchored to the membrane. Taking into account the recovered values of the monomeric eGFP quantal brightness, we show that NBCe1-A exists in monomeric and dimeric states on the cell membrane. We also used an Alexa488-alpha-bungarotoxin conjugate to label cells expressing an NBCe1A-bungarotoxin binding mutant. As a monomeric control, we immobilized Alexa488 dye on cover slips. The spatial fluorescence intensity fluctuation analysis revealed a similar distribution of aggregates as shown for eGFP data. Moreover, we immunolabeled NBCe1-A in rat kidney tissues as well as in cultured HEK293 cells expressing the cotransporter demonstrating the NBCe1-A is present in monomeric, dimeric and rarely in higher order oligomeric states. These experiments demonstrate for the first time the in situ oligomeric state(s) of NBCe1-A.

2978-Pos

Assessing the Mutagenicity Potential of Multiphoton Excitation during Imaging of Intrinsic Fluorescence from Cells and Tissues

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Multiphoton-excited intrinsic fluorescence emission signals from cells and tissues can be used for metabolic imaging and studies of disease morphology in chronic animal imaging experiments, and has diagnostic potential as a future in vivo clinical imaging tool. However, the signals are generally weak and require high laser intensities for imaging. Consequently, not only are viability studies important, but an investigation into the extent that multiphoton excitation is a mutagenic agent is critical. Information on the magnitude of permissible intensity levels of femtosecond pulsed near-IR light is vital to human clinical applications, yet there are currently no regulations that specifically indicate such parameters. This study is directed towards determining whether multiphoton imaging of cellular autofluorescence using 700 - 800 nm wavelength excitations causes mutations in mammalian cells. The induction of mutation by pulsed laser radiation employed for multiphoton imaging entails a risk of carcinogenicity in living tissue. The assessment of potential laser illumination toxicity was carried out by the hypoxanthine-guanine phosphoribosyl (HPRT) mammalian cell gene mutation assay, which measures mutation at the HPRT gene locus in cells, and is one of a handful of reporter loci that have been used as molecular biomarkers for both human and rodent exposure to mutagens and UV light. Experiments were performed to assess possible mutagenic effects of various intensities of 755 nm, 100 fs laser irradiation. Laser powers ranging from 20 to as high as 100 mW delivered as raster scanned excitation through a 0.7 NA objective for 20 seconds was found to be nonmutagenic to the HPRT gene locus test system, while higher laser powers initiated mutagenic responses.

2979-Pos

Optical Analysis of Calcium Channels at the First Auditory Synapse

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Transmitter release at the first auditory synapse, the ribbon synapse of cochlear inner hair cells (IHCs), is tightly regulated by Ca^{2+}. Using fast confocal Ca^{2+} imaging, we have recently described pronounced differences in presynaptic Ca^{2+} signals between single synapses within the same cell. These Ca^{2+} microdomains differed both in their amplitude and voltage-dependence of activation.

As for the mechanism behind the amplitude heterogeneity, we provided indirect evidence for differences in the Ca^{2+} channel complement, pointing towards a differential regulation of Ca^{2+} channel number (N_{Ca}) across synapses. Moreover, a very simplistic model reveals potential consequences of different Ca^{2+} channel complements for sound encoding at different synapses

In order to directly study synaptic Ca^{2+} channels, we are currently implementing an optical fluctuation analysis approach. Here, we present preliminary results along potential caveats. This work provides a framework of how to further dissect presynaptic Ca^{2+} microdomain heterogeneity - likely being involved in determining the diverse responses of the postsynaptic neurons, which, as a population, encode the huge range of perceived stimulus intensities (sound pressure varying over 6 orders of magnitude).

2980-Pos

Reactive Oxygen Species as Essential Mediators of Cell Adhesion and Migration

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In recent years reactive oxygen species (ROS) and by extension changes in the intracellular reductive/oxidative (redox) balance have come into focus as major regulators of key cellular functions in both physiological and pathological settings. Traditionally viewed as mediators of cell damage by exogenous noxae, oxygen intermediates have been also recognized of signaling roles downstream of cytokine and mitogen receptors, activated oncogenes, nutrient sensors and pro-apoptotic stimuli, when endogenously generated by a number of intracellular biochemical sources. The signaling properties of ROS are largely due to the reversible oxidation of redox-sensitive target proteins, and especially of protein tyrosine phosphatases, whose activity is dependent on the redox state of a low pKa active site cysteine. Cell spreading, adhesion and migration requires ROS generation and interaction with protein tyrosine phosphatases downstream of adhesion molecules. We have taken advantage of a redox-sensitive mutant of the Yellow Fluorescent protein (rxYFP), employed ratiometrically, to draw real-time redox maps of adhering and migrating cells. A quantitative analysis of redox maps allows to disclose a peculiar spatial organization of the redox environment, providing evidence that intracellular ROS are generated after integrin engagement and that these oxidant intermediates are necessary for integrin signaling during cell spreading, adhesion and migration. Taken together these observation support the application of rxYFP in the subcellular mapping of physiological dynamic redox phenomena involved in signal transduction.

2981-Pos

Real Time Imaging of Endogenous MRNAs during Stress

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During stress, cytoplasmic mRNAs aggregate to RNA granules such as stress granule (SG) and processing body (PB), where they are sorted and remodeled for reinitiation of translation, degradation or storage. Till now, stress-induced assembly of these granules is mainly studied indirectly by using protein markers, and the real time behavior of endogenous mRNAs in living cells has not been detected directly yet and remained uncertain.

Here we used a linear antisense probe to visualize endogenous cytoplasmic mRNAs in living mammalian cells in order to investigate the dynamics of mRNAs under stress. A Cy3 and biotin labeled poly$(U)_{22}$ 2'-*O*-methyl RNA probe was prepared for the detection of poly$(A)^+$ mRNAs. The probe combined with streptavidin was microinjected into the cytoplasm of COS-7 cells, followed by the inducement of 0.5 mM arsenite stress. We also transfected TIA-1-GFP plasmid into the cells by FuGENE 6 to determine SG. As a result, mRNAs visualized by the antisense probe aggregated to granules during stress and the granules colocalized with SG marked by TIA-1-GFP. Next, the number and size of the granules were studied by real time imaging. mRNAs rapidly aggregated to form clusters within 20 min in response to stress. A large amount of small granules first emerged, gradually gathered to bigger ones about 30 min after the inducement of stress.

In this study, the aggregation of endogenous mRNAs to SG was successfully visualized by using the linear antisense probe. The behavior of endogenous mRNAs in SG will be revealed.

2982-Pos

Imaging Dopamine and Serotonin in Live Neurons with Multi-Photon Excited Ultraviolet Auto-Fluorescence

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Monoamine neurotransmitters are implicated in mood, aggression, reward, and addiction pathways in the mammalian brain. However, visualizing them in live neurons with sub-micron resolution has remained a challenge. It is difficult to label them fluorescently, and their intrinsic ultraviolet fluorescence is difficult to access. Unlike serotonin which can be imaged with three-photon microscopy, dopamine presents a special challenge due to its shorter wavelength (~300nm) emission. We now show that dopamine can be imaged with sub-micron resolution in live brain slices with a combination of a non-epifluorescent collection design, special optical elements, and two-photon excitation with a visible femtosecond laser. Substantia Nigra (SN) tissue sections from the

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.

[1] van Zanten et al. BBA-Biomembranes (2009)
[2] Cambi et al. Mol. Biol. Cell (2006)
[3] van Zanten et al. PNAS (accepted)
[4] Diez-Ahedo et al. SMALL (2009)

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