82-Plat Cysteine Substitution Reveals Novel Inter-subunit Interactions In The Iks Potassium Channel

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The I_{KS} potassium channel, which consists of the alpha subunit (KCNQ1) and beta subunit (KCNE1), is essential for control of action potential duration in the human heart. Loss of function mutations in either subunit can cause long QT syndrome. Gain of function mutations in the S1 helix of KCNQ1 cause atrial fibrillation (AF) and functionally are dependent on association with KCNE1. Previous investigations of inter-subunit KCNE1/KCNQ1 interactions have concentrated primarily on the S6, P-loop, and S4 helices as well as the intracellular termini of KCNQ1 as possible sites of KCNE1 interaction. Here we report novel KCNE1/KCNQ1 interactions revealed by experiments in which we used the spontaneous formation of disulfide bonds by subunit-specific cysteines to report inter-subunit proximity. Cysteines (Cys) were introduced into the extracellular juxtamembrane regions of both KCNQ1 and KCNE1 and crosslinking was determined by DTT-sensitive gel shifts detected under non-reducing conditions on Western blots. Spontaneous crosslinking was tested for 96 permutations of Cys-substituted subunits. We find that Cys substitutions into the extracellular juxtamembrane regions of KCNE1 and S1 and S6 of KCNQ1 cause a gel shift consistent with a KCNE1-KCNQ1 heterodimer whereas substitutions into S2, S3–4, and S5 do not. Whole cell patch clamp analysis of transiently transfected CHO cells expressing selected crosslinking pairs at S1 or S6 revealed a DTT-reversible phenotype of channels that are either constitutively open or once opened, reluctant to close. These data, in concert with the crystal structure of the Kv1.2 K^+ channel, imply that the transmembrane portion of KCNE1 is located between the S1 and S6 helices of two intercalated KCNQ1 subunits and that relative movements between S1/E1 or S6/E1 are important for channel gating. This provides a structural basis to understand the inherited AF mutations of KCNQ1.

83-Plat Doxazosin Induces Apoptosis Of Cells Expressing Herg K^+ Channels

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The antihypertensive drug doxazosin is associated with an increased risk for congestive heart failure and cardiomyocyte apoptosis. Human ether-a-go-go-related gene (hERG) K^+ channels, previously shown to be blocked by doxazosin at therapeutically relevant concentrations, represent plasma membrane receptors for the antihypertensive drug. To elucidate the molecular basis for doxazosin-associated pro-apoptotic effects, cell death was studied in human embryonic kidney cells using three independent apoptosis assays. Doxazosin specifically induced apoptosis in hERG-expressing HEK cells, while untransfected control groups were insensitive to treatment with the antihypertensive agent. An unexpected biophysical mechanism has emerged: binding of doxazosin to its novel membrane receptor, hERG, triggers apoptosis, possibly representing a broader biological strategy in drug-induced heart failure.

84-Plat Characterization Of Action Potential And Inward Currents In Freshly Isolated Ventricular Myocytes From Zebrafish (*Danio rerio*)

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The Zebrafish is a tropical teleost fish that has been the focus of an increasing number of developmental studies. Physiological interest in this species has been spurred on by the ease of mutants which can be induced by chemomutagenesis. However, our understanding of the basic physiology of Zebrafish, in particular cardiac excitation-contraction coupling is limited. Indeed, there is currently no information about the electrical activity of single myocytes freshly isolated from Zebrafish ventricle; this study addressed this point. Viable ventricular myocytes were obtained by enzymatic digestion. The whole cell configuration of the patch clamp was used to record Na current (INa), Ca current (ICa) and action potential (AP). Results are presented as mean±SE and analyzed with paired t-test. Single ventricular myocytes from Zebrafish are long and thin, as described for other fish species. Cell capacitance was 26.0±1.1 pF (n=69). ICa density (test pulse to 0 mV) was −11.4±3.4 pA/pF and INa density (test pulse to −40 mV) was −104±22 pA/pF (n=6). Resting membrane potential was −66±2 mV (n=19). At 0.1 Hz stimulation frequency, AP duration at 25, 50, and 90% repolarization was 50±17, 105±27, 137±35 ms, respectively (n=12), indicating the presence of a plateau phase. Increasing stimulation frequency to 2 Hz significantly decreased AP duration (n=6, $p<0.05$). To conclude, we have developed a method to obtain viable isolated ventricle myocytes from Zebrafish heart. Ionic currents studied present characteristics similar to other fish species. The presence of a plateau during the AP suggests that this species might be appropriate for ion channels related mutation screening of cardiac alteration.

Platform H: Fluorescence Spectroscopy

85-Plat Fluorescence Correlation Spectroscopy in Zebrafish Embryos

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Fluorescence Correlation and Cross-correlation Spectroscopy (FCS and FCCS) have developed into routine tools for the determination

of molecular processes in live cells. However, for the study of medically relevant parameters, the measurements on 2D cell cultures are important but not sufficient. In recent years strong doubts have surfaced whether cellular responses in 2D cultures can be indicative of cell functions in their natural 3D matrix. It is therefore important to develop the existing spectroscopic tools for measurements in organisms. A promising vertebrate organism for this research is zebrafish (danio rerio) which can be easily studied due to its transparency in its embryonic stage.

Here we investigate the feasibility to perform FCS in live zebrafish embryos. We first characterize the one- and two-photon autofluorescence of zebrafish embryos before measuring one FCS curves of EGFP in the cytoplasm of cells in dependence of depth within the organisms to determine the viscosity of the cytoplasm of different cell types. We express a transmembrane receptor, cxcr4-EGFP, to measure its membrane dynamics. This receptor is involved in cell differentiation and migration during embryo development and is a co-receptor for HIV-1. We then show that using transgenic lines, the systolic and diastolic blood flow in a specified organ, here the liver, can be determined in dependence of location and development time. And lastly, we perform FCCS measurements on different fluorescent protein constructs (EGFP, mRFP, mCherry, EGFP-mRFP and EGFP-mCherry) to show that even molecular interactions can be accurately determined. This work demonstrates that FCS can be performed in living zebrafish embryos to determine physiological important data (blood flow), cellular characteristics (viscosity of cytoplasm), and even molecular interactions (protein-protein interactions using FCCS). FCS/FCCS thus gives access to a range of different physiological parameters over different scales.

86-Plat Brightness Analysis in two-dimensional Model systems

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Giant Unilammellar Vesicles (GUVs) have been extensively studied and widely used as model membranes. Though diffusion behavior of different probes has been systematically studied on such model systems, a systematic analysis of the measurement of the brightness of fluorophores has not yet been done in GUVs. In previous work, we developed a method to determine the sizes and distribution of clusters of fluorescent proteins on membrane of living cells under physiological conditions (Saffarian, 2007). Here we report the application of brightness analysis on well defined GUV model systems. Fluorescent probes with different brightness properties have been incorporated both individually and in mixtures into GUV membranes. We studied the effect of bin time, integration time and single molecule brightness on the accuracy of Fluorescence Intensity Distribution Analysis (Kask, 1999) for determining the number and brightness of molecules in the membrane from measured photon count histograms. We compare the accuracy of the data analysis obtained with different implementations of this method.

References

Kask, P., K. Palo, D. Ullmann and K. Gall (1999). "Fluorescence-intensity distribution analysis and its application in biomolecular detection technology." *Proc Natl Acad Sci U S A* ***96***(24): 13756–61.

Saffarian, S., Y. Li, E. L. Elson and L. J. Pike (2007). "Oligomerization of the EGF receptor investigated by live cell fluorescence intensity distribution analysis." *Biophys J* ***93***(3): 1021–31.

87-Plat Nuclear Receptor and Coactivator Interaction Probed by Fluorescence Fluctuation Spectroscopy inside Living Cells

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The actions of nuclear receptors (NR) on gene transcription require a highly coordinated interaction with coactivators. Since NRs bind DNA as homo- or hetero-dimer, it is generally thought that each coactivator molecule recruits two NRs. However, coactivators, such as the transcription intermediate factor 2 (TIF-2), have three NR interaction domains. An experimental investigation of the interaction model between NRs and coactivators is still lacking. In this work, we apply dual-color time-integrated fluorescence cumulant analysis (TIFCA), a fluorescence fluctuation spectroscopy technique, to investigate the interaction between the coactivator TIF-2 and two nuclear receptors, retinoid acid receptor (RAR) and retinoid X receptor (RXR), directly in living cells. We first focus on the ligand binding domain (LBD) of RAR (RARLBD) and RXR (RXRLBD), because the LBD is the only NR domain known to interact with the coactivator. Our experiments show that the oligomeric state of NRs changes in the presence of coactivators. Surprisingly, we find that all three binding sites of TIF-2 can be occupied by different combinations of nuclear receptors. We then proceed to measure full length RAR and RXR and find that TIF-2 only recruits dimers of full length NRs. This result indicates that the other domains of the NR, although not directly interacting with the coactivator, play a crucial role in regulating the binding stoichiometry. This study also demonstrates that TIFCA is able to quantitatively reveal the composition of hetero complexes involving three proteins directly inside living cells.

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88-Plat Two-protein PCH Analysis For Simultaneous Measurement Of Stiochiometry And Interactions Of SAM Domain Proteins In Live Yeast

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We apply the photon counting histogram (PCH) to live yeast to extract simultaneously stoichiometry and interactions of MAPK signaling partners Ste11 and Ste50 using a single autofluorescent protein, GFP. Ste11 and Ste50 both have well characterized sterile-alpha-motif (SAM) domains that facilitate their interaction, which is key to signaling in yeast pathways such as MAPK, HOG, and

invasive growth. Numerous biochemical structural studies have examined binding affinity and stiochiometry of this interaction, with varied results. In this study, we apply PCH to live yeast to first detail stiochiometry of Ste11 and Ste50 separately, and the effect of pheromone response on the observed distribution of oligomers. Results are compared to endogenously expressed monomers, dimers and trimers of GFP in live yeast. Then, we examine PCH of a yeast strain with both Ste11 and Ste50 tagged with GFP, which, in comparison with the one-protein PCH measurements, provides a simultaneous measure of both the interaction of these proteins, and stiochiometry of the large protein complex using only GFP, without the need for a second color autofluorescent protein. Mutation of two amino acids in the Ste50 SAM domain abolishes the interaction, as measured by two-protein PCH. The accuracy of the two-protein PCH measurements for examining protein interactions, for both wt proteins, and with the Ste50 SAM domain mutant, are verified by live-cell fluorescence cross-correlation spectroscopy using GFP and mCherry tagged proteins. In general, we provide evidence for the ability to simultaneously examine stiochiometry and interactions in live yeast using a single autofluorescent protein. This method is compared and contrasted to other live cell fluorescence fluctuation methods for examining stiochiometry and binding.

89-Plat Dark States in Monomeric Red Fluorescent Proteins studied by Fluorescence Correlation and Single Molecule Spectroscopy

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Monomeric red fluorescent proteins (mRFPs) have become indispensable tools for studying protein dynamics, interactions and functions in the cellular environment. Their emission spectrum can be well separated from other fluorescent proteins and their monomeric structure preserves the natural function of fusion proteins. However, previous photophysical studies of some RFPs have shown the presence of light-induced dark states that can complicate the interpretation of cellular experiments.

In this article, we extend these studies to mRFP1, mCherry and mStrawberry and prove that this light-driven intensity flickering also occurs in these proteins, using fluorescence correlation spectroscopy (FCS). Furthermore, we show that the flickering in these proteins is pH-dependent. Single molecule spectroscopy revealed reversible transitions from a bright to a dark state in several timescales, even up to seconds. Time-resolved fluorescence spectroscopy showed a bi-exponential decay, proving that conformational changes can occur.

Based on these experimental results, we offer a structural basis for the fluorescence flickering using known crystal structures. We apply dual-colour FCS inside live cells to prove that this flickering can seriously hamper cellular measurements if the timescales of the flickering and diffusion are not well-separated.

90-Plat Time-Dependent Distributions of Fluorescently Labeled Proteins in Solution by Molecular Fourier Imaging Correlation Spectroscopy

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The ability to non-invasively observe protein structural changes in the presence of spatial diffusion is important to many biophysical problems, ranging from intracellular trafficking to signal transduction. We describe a new experimental method, called molecular Fourier imaging correlation spectroscopy (MFICS), to simultaneously study the distributions of protein center-of-mass and anisotropy fluctuations. We demonstrate our approach using the fluorescent protein DsRed, which is a tetrameric complex of fluorescent protein subunits. In our experiments, we monitor the fluctuating fluorescence signal resulting from the spatial overlap of a polarized and phase-modulated excitation fringe pattern with a small number of diffusing molecules. The emission is separated into orthogonal polarization channels, and each channel is continuously recorded in terms of its amplitude and phase. The phase selectivity of our measurement enables us to detect signal fluctuations arising from nanometer scale molecular center-of-mass displacements. From our data, we construct fluctuating signals proportional to the Fourier component of the collective number density, and to the fluorescence anisotropy. From these quantities, we compute space-time distributions and correlation functions that characterize molecular center-of-mass displacements and anisotropy fluctuations. We find that the translational dynamics of the DsRed molecules are well characterized in terms of Fickian diffusion. We interpret the anisotropy fluctuations as due to incoherent electronic energy transfer between adjacent protein subunits of the DsRed complex, which are slowly modulated by relaxations of its quaternary structure.

91-Plat A Novel Microfiberoptic Method To Measure Diffusion In The Extracellular Space Deep In Tissues In Vivo

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Diffusion in the extracellular space (ECS) of solid tissues such as brain plays an important role in the transmission of ions, messenger molecules, metabolites and drugs. To overcome the limited penetration of light in solid tissues, we recently developed a microfiberoptic epifluorescence photobleaching method in which an optical microfiber with micron-sized tip is introduced deep into living tissues. We found marked hindrance to diffusion deep in tumors, which could be enhanced substantially by digestion of tumor

collagen and its associated proteoglycan decorin (Magzoub et al., Faseb J. 2008). Our studies also revealed an unexpected dependence of diffusion in deep brain on the size of the diffusing macromolecule (Zador et al., Faseb J. 2008). Here, we report a new method to measure diffusion in the ECS using a dual-lumen device consisting of a micropipette for fluorescent dye delivery in close proximity to a guide barrel for introduction of the optical microfiber. Small quantities of extracellular fluorescent dyes are delivered by iontophoresis or pulsed pressure injection, and the kinetics of dye diffusion measured with the optical microfiber. With this method we found slowed diffusion of a small dye, calcein, in tissue vs. saline, D_o/D, of 3.9 ± 0.5 in brain cortex, in agreement with prior data, with substantial slowing of ECS diffusion in skeletal muscle (D_o/D = 229 ± 21) and kidney (D_o/D =134 ± 10). Relative diffusion of a larger dye, 70 kDa FITC-dextran, was similar in brain cortex to that of calcein ($D_o/D = 4.4 \pm 0.5$), though different in kidney ECS (D_o/D = 357 ± 16). This new microfiberoptic method has utility for measurement of diffusion of any fluorescent molecule deep in living tissue as well as for measurements of anisotropic diffusion.

92-Plat Limiting Calmodulin Revealed by Image Correlation Spectroscopy

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Protein-protein interactions depend on the ability of proteins to move from one place to another in search of their respective targets. The passive transport of signaling molecules is potentially hindered by obstacles or by its amount of available (mobile) pool. Calmodulin (CaM) a Ca^{2+} sensor protein searches among a wide number of targets, posing the question on whether there is enough non-bound CaM that can move freely through the cytoplasm to activate all of its targets. To address this question, we have used Raster Image Correlation Spectroscopy (RICS) to study the spatial distribution and mobility of CaM inside cells at rest and following elevation of intracellular Ca^{2+}. RICS can be performed on data collected with a standard confocal microscope and allows one to quantify the availability and diffusivity of a tagged protein from fluorescent fluctuations over space and time. Diffusion coefficients from a few hundred $\mu m^2/s$ to less that 1 $\mu m^2/s$ can be resolved using RICS. At the whole cell level on cells expressing GFP tagged CaM, only ~ 15% of the fluctuations generate a diffusion rate of > 2 $\mu m^2/s$, while the rest come from the immobile pool or the background motion of the cell. This rate is comparable to the diffusion rate of a target protein CaM Kinase II tagged with GFP (GFP-CaMKII). A non-interactive molecule like GFP, which diffuses at a similar rate as GFP-CaM in solution, has a diffusion coefficient of ~ 20 $\mu m^2/s$ when measured inside the cells. We conclude that the intracellular diffusion of GFP-CaM is consistent with the hypothesis that CaM is sequestered by the wide number of targets and its availability for signal transduction is limited.

Platform I: Cell Mechanics & Motility

93-Plat Uniaxial Loading Of Neurons Results In Cyoskeletal Reorganization

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Understanding how mechanical forces affect growth and signaling of neurons is vital to regenerative therapies used for the treatment of neurodegenerative disease or trauma. Research on the mechanical properties of neurons has largely been restricted to studies on the effect of surgical procedures, such as limb lengthening, at the tissue level. Consequently, there is a dearth of information on the response of individual neurons to mechanical loading. Additionally, while there are several commercially available cell-stretchers, many are not intended for high magnification fluorescence microscopy, and are extremely costly. In order to address these issues, we have designed and fabricated a cell stretching device capable of uniaxially loading cells. Design criteria include the ability to image both live cell morphology and the movement of fluorescently labeled subcellular organelles during loading Cells are cultured on a flexible lysine-coated silicone membrane and inverted onto a glass-bottomed aluminum culture chamber. The membrane is attached to multidirectional actuators, which allow its positioning within the culture chamber and also control membrane strain. In this study, we probed the response of mouse neuroblastoma cells to static mechanical loading (n=42). Immediately upon loading, cells stretch with the substrate, thereby exhibiting viscoelastic behavior. This is followed within 10–15 minutes by an active response phase during which the cell body shrinks and neurites retract and/or elongate both parallel and perpendicular to the axis of strain. These results strongly indicate dramatic cytoskeletal reorganization in response to mechanical loading. The effect of chronic loading on the directionality and dynamics of neurite growth is currently being investigated with a focus on this reorganization.

94-Plat The Limits of Filopodium Stability

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Filopodia are long, finger-like membrane tubes supported by cytoskeletal filaments, that are thought to play a mechanosensing role in cell motility and axon growth. Their physical shape is determined by the properties of the stiff actin filament bundles found inside them and by the interplay between membrane surface tension and rigidity.

Although one might expect the Euler buckling instability to limit the length of filopodia, we show, through simple energetic considerations, that this may not be the case. By further analyzing the statics of filaments inside membrane tubes, and through computer simulations that capture membrane and filament fluctuations, we show under which conditions filopodia of arbitrary lengths are stable, and that in these filopodia the filaments adapt a helical shape (the attached figure an unstable filopodium in the process of