used fluorescence correlation spectroscopy (FCS) instead, to detect the fluorescence blinking caused by the reversible protonation reaction, and to determine both the fraction of fluorophore in each state and the associated relaxation time. We studied how various environmental conditions, pH, temperature, ionic strength and buffer concentration, influenced the blinking of pyranine. We found that knowledge of both the fraction of protonated pyranine and the blinking relaxation time can be used to measure two of these external variables at the same time, something that cannot be achieved with the ratiometric method. As a first application, we showed that pyranine can be used to measure both pH and temperature during the mixing of two solutions in a microfluidic channel. More relevantly for biological applications, this method can now be applied to measure both pH and ionic strength inside different cellular compartments.

2067-Pos Board B37

Components of intrinsic fluorescence revealed by Metabolic Modulation Matrix in isolated rat cardiac myocytes

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Aim: Recent developments in multi-wavelength fluorescence lifetime spectroscopy brought simultaneous measurement of fluorescence spectra and lifetimes in complex samples, namely cell and tissue intrinsic fluorescence. However, emission spectra of endogenous fluorophores are often superimposed in broad bands over the 400-600 nm wavelength region, making their separation particularly difficult. We therefore present a newly developed method of separation. Based on Metabolic Modulation Matrix approach, this method is built on evaluation of spectral modulation of endogenous fluorescence following changes in the cell metabolic state. **Methods:** Spectral fingerprints of time-resolved fluorescence are determined in isolated cardiomyocytes after excitation by 375-nm pulsed picosecond laser diode using SPC-830 TCSPC measurement system (Becker-Hickl on Zeiss Axiovert 200). Metabolic modulation was induced by respiratory chain and/or oxidative stress regulators. The number and profiles of the most significant spectral components were identified by time-resolved area-normalized emission spectroscopy and principal component analysis. **Results:** The Metabolic Modulation Matrix approach applied to time-resolved spectroscopy data in living cardiac cells demonstrated the presence of at least 3 significant spectrally-distinct components of NAD(P)H fluorescence corresponding to: i) NAD(P)H in water-like environment, ii) NAD(P)H in restricted-motion environment and iii) a flavin-type component. Lifetimes, revealed by fluorescence decay analysis showed values of τ_1~0.4 $\pm$ 0.1 ns and τ_2~1.0 $\pm$ 0.2 ns for component i), τ ~3.2 $\pm$ 0.8 ns, for component ii), and τ~2.5-5 ns for component iii). **Conclusions:** Presented Metabolic Modulation Matrix concept, in conjunction with spectrally-resolved fluorescence lifetime detection of the cell intrinsic fluorescence, is a promising, highly versatile tool for quantitative assessment of oxidative metabolism in living cells.

2068-Pos Board B38

Random Fluorescently Labeled Proteins: Label Distribution and Effect on Binding

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Proteins for fluorescence measurements are often labeled randomly by covalent linkage of fluorescent dyes to amine groups on the target protein and subsequently purified. Such labeling results in a heterogeneous population of protein molecules containing a varied number of labels, which may depend on the number and location of available lysine residues. We explore the extent to which protein labeling techniques result in a Poissonian distribution of protein-fluorophore complexes using fluorescence fluctuation spectroscopy (FFS). The fluctuation amplitude in an FFS measurement is related to the number of labeled proteins and is not sensitive to unlabeled protein. We model the expected fluctuation amplitude as a function of average incorporated fluorophores assuming the distribution is governed by Poissonian statistics. We experimentally fit the model by randomly labeling monoclonal antibody with fluorescent dye and show agreement for incorporation ratios up to ~ 1.5. For greater amounts of incorporated dye molecules, we use mass spectrometry to examine labeled $F(ab')_2$ fragments and show that the distribution is better described by a Gaussian profile. Finally, by performing quenching experiments on a steady-state fluorimeter, we show that randomly labeling antibodies and antigens does not affect measured affinity values within experimental uncertainty.

2069-Pos Board B39

Probing Variations In The Structural Environment Of A DNA Sequence Using Fluorescence Properties Of The Pteridine Analog Probes, 3MI and 6MI

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We explored two different microenvironments in the sequence; 5′-actaGagatccctcagacccttttagtcagtGtgga -3′ in single and duplex form using two similar nucleoside analogs. 3MI and 6MI were each investigated in two different environments, one flanked by thymines (PTRT) and the other, by adenines (PTRA)(shown by G's noted above). Each site is equidistant from a terminus. The probes differ only by the position of a methyl group in either the 3- (3MI) position or the 6- (6MI) position. Both time-resolved anisotropies and lifetimes of the probes depend upon local electrostatics which are impacted by duplex formation. 3MI shows less response to structural change as compared to 6MI. Integrals of lifetime curves compared with quantum yields of each sample reveal that each displays a ''dark'' component which we are unable to detect with TCSPC (e.g.,tau<70ps). For 6MI in the A environment this QSSQ ''quasi static quenching'' eliminates approximately half the molecules, whether in SS or DS form. 6MI in the T environment displays an unexpected increase in the quantum yield upon duplex formation (0.107 to 0.189) apparently the result of escape from QSSQ which simultaneously declines from 66% to 33%. Escape from the dark state is accompanied by doubling of steady state anisotropy of 6MI in PTRT in the duplex. Only 6MI in the T duplex displays a rotational correlation time over 7 ns. The DS A environment fails to constrain local motion and QSSQ remains the same as in SS; in contrast, the flanking T duplex environment restricts local motion and halves the QSSQ.

2070-Pos Board B40

A Fluorescence Polarization Displacement Assay for Aggrecanase-1 and -2

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Fluorescence polarization is a valuable technique for characterizing small molecule inhibitors. Competition fluorescence polarization assays involve the displacement of a fluorescent ligand from the enzyme with increasing amounts of a competing compound. The technique allows the determination of inhibitor dissociation constants (Ki values) and evaluation of whether the data fit is consistent with competitive and stoichiometric binding, which enables the potency of small molecule inhibitors to be ranked. Fluorescence polarization assays have the advantage of being robust, non-radioactive, and formatted for 384-well plates. Inhibitor dissociation constants can be obtained without the necessity of separating bound and unbound species. Here we describe the development and characterization of a fluorescent ligand for the Aggrecanase-1 and Aggrecanase-2 enzymes, and present measured competition IC_{50} values and Ki values for hit compounds in the Aggrecanase program that aided the team in lead optimization.

2071-Pos Board B41

Optical force measurements utilizing Lanthanide Binding Tags

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The Lanthanide Binding Tag (LBT) is a motif comprised of 17 amino-acids with the sequence YIDTNNDGWYEGDELLA. The LBT has been crystallized in the presence of Tb^{3+} and thus it is known that this motif forms a loop in which the center is occupied by the Tb^{3+} ion, with the ion held in place by negatively charged amino-acids. Comparison with the highly related structure of an EF-hand revealed that only in the case of the LBT, water molecules are completely excluded from the interior of the loop (Nitz et al, 2004). This explains the high quantum yield of Tb^{3+} bound to the LBT.
However, when encoded into a large ''host molecule'' the environment of the LBT is more constrained and it is possible that if inserted in the correct position, it will sense forces originating from conformational changes within the host. The forces exerted by these conformational changes may lead to a deformation or an unfolding of the motif, which would consequently change the emission properties of the bound Tb^{3+} ion. Here we show that Tb^{3+} emission from LBTs inserted on a particular position of the voltage sensor of Shaker K channels can be quenched by voltage dependent conformational changes. In order to understand this phenomenon, and with the attempt to calibrate this system for optical force measurements, we have employed molecular dynamic simulations. In these simulations an artificial force was applied to the ends of

the LBT motif and the probability of water molecules interacting with the Tb^{3+} ion as a function of the applied force is used as a parameter related to the observed quenching of the emission.
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2072-Pos Board B42
Random Insertion of Split-can Venus into Kv1.4 Yields Voltage Sensitive Fluorescent Probes
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In order to increase the optical signal of genetically encoded voltage sensors, we have utilized a transposon reaction to randomly insert the front and back halves of venus into separate subunits of a voltage gated potassium channel, Kv1.4. This split fluorescent protein approach was used with the rationale that only properly folded subunits will produce a functional, fluorescent channel at the cell surface.
We tested 27 combinations for optical signals using voltage clamp fluorometry in HEK293 and NIE115 (mouse neuroblastoma) cell lines. 14 combinations show fluorescence only on the plasma membrane and achieve the goal of the split can design. The best sensitivity is -0.9% in $\Delta F/F$ for a 100 mV depolarization. The on rate during depolarization is on the scale of ms, but the off rate during repolarization is very slow, on the scale of 100 ms.
One combination yielded a surprising optical signal upon depolarization in NIE115 cells. The fluorescence decreased at the edge of the cell, but increased at the cell top and bottom. This phenomenon provides a clue for us to further study the mechanism of the probe's voltage sensitivity. (Funded by NIH grant 1U24NS057631-01A1)

2073-Pos Board B43
Use of Fluorescence Anisotropy to Explore the Subunit Composition of Ca2+/Calmodulin Protein Kinase II Holoenzymes
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Ca/calmodulin dependent protein kinase II (CaMKII) is a serine/threonine kinase which is highly enriched in the CNS and thought to be important in the development of long term potentiation. Several variants of CaMKII exist but alpha and beta subunits predominate in brain tissue. Though it is commonly believed that alpha and beta subunits combine to form heteromeric holoenzymes comprised of 12 subunits, this has not been directly demonstrated in vivo. Therefore, we used FRET imaging to directly examined the proximity of alpha and beta isoforms when they assemble to form heterologous holoenzymes. Specifically we used both time-resolved and steady-state fluorescence anisotropy measurements. Energy migration FRET (emFRET; FRET between like fluorophores) is observed in holoenzymes in which the C termini have been tagged with Venus. Fluorescence anisotropy is one of the few techniques which can detect emFRET and provide information of the number of fluorophores undergoing energy transfer. Venus tagged alpha isoforms were coexpressed with Amber (a point mutation in Venus that destroys the fluorophore) tagged beta isoforms and energy migration was seen to decrease indicating that holoenzymes are composed of both alpha and beta subunits in close proximity. Fluorescence anisotropy signatures of Venus tagged holoenzymes were invariant across a broad range of expression levels suggesting that inter-holoenzyme emFRET was not occurring. We used anisotropy ''standards'' consisting of concatamers of Venus and Amber to facilitate the interpretation of anisotropy curves in terms of the number of fluorescent proteins participating in emFRET. Together, these experiments indicate that alpha and beta subunits can coexist in the same holoenzyme. The availability of emFRET ''standards'' to the general scientific community should aid in the interpretation of anisotropy decay data.

2074-Pos Board B44
Backbone Fluorescent DNA Modifications: Reducing Uncertainties In FRET
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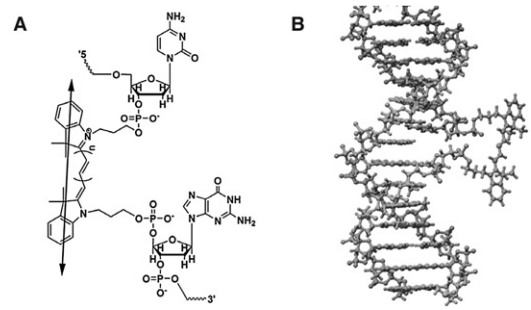
The kappa square factor is one of several uncertainties that preclude FRET from being a true quantitative technique. Fluorescent backbone modification of DNA structure can constrain the rotational flexibility of the dyes and the orientation can be predicted from the DNA structure. These constraints also reduce the dye-DNA interaction and the uncertainties associated with measuring donor acceptor distances for flexible linkers. In this work we show that the



FRET can be observed at very long distances because of favorable orientation which would have been impossible for freely rotating donor-acceptor pair and also that the assumption that kappa square = 2/3 may lead to large errors in distance measurements.

2075-Pos Board B45
In The Quest Of The Best Fluorescent Protein Couple For Quantitative Fret-flim
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Quantification of protein-protein interactions in living cells by using a fluorescent-protein based FRET approach is a powerful method, especially combined with Fluorescence Lifetime Imaging Microscopy (FLIM), since the fluorescent lifetime does not depend on fluorophore concentration or the excitation light path. In that respect, it is convenient to use a donor protein with a single exponential decay with no detectable photo-conversion. Single lifetime decays allow simplifying the calculation of the fraction of interacting donor-protein (fD). Moreover, in the case of single exponential donor fluorophores the lifetime is not influenced by light induced changes, i.e. photo-bleaching. At first, Green Fluorescent Protein (GFP) was taken as an appropriate donor because of its fluorescence decay can be convincingly fitted to a single exponential model and no photo-conversion was observed. GFP-Red tandems (GFP-mRFP, GFP-mStrawberry, GFP-HaloTag (TMR) and GFP-mCherry) were quantitatively studied by FRET-FLIM obtaining fD values far from the ideal 100%. The maximum value of FRET percentage (fD), was only around 50% for the couple GFP-mCherry. This relatively low percentage could be due to the dark states of the acceptor and/or misfolding of the red proteic domain as well as cleavage in the tandem. Aiming to improve the amount of donor protein engaged in FRET we have tested mTFP1 as a single exponential donor. mTFP1 lifetime remained constant when performing light induced fluorescent changes and no photo-conversion was detected. The percentage of FRET when combined to YFP as an acceptor turned out to be around 70%. Although higher percentages would be desirable mTFP1-YFP stands alone as the best FRET-FLIM standard because of its high transfer percentage (fD) and the single exponential behavior of mTFP1 as a donor.

2076-Pos Board B46
Improved FRET Sensing Of Membrane Voltage With Voltage Sensitive Phosphatase And New Coral Fluorescence Proteins
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Ciona voltage sensitive phosphatase (Ci-VSP) is an unique enzyme discovered first in asicdian genome, in which phosphatase activity is regulated by transmembrane potential. Ci-VSP consists of a phosphatase domain and a preceding voltage sensing domain (VSD) which is homologous to the S1-S4 transmembrane domain found in conventional voltage-gated ionic channels. Possible mechanism of Ci-VSP should be that changes in transmembrane potential elicit conformational changes in the VSD, which then induce conformational changes in the phosphatase domain, regulating enzymatic activity. Analogously, by replacing the phosphatase domain with two fluorescent proteins that act as fluorescence resonance energy transfer (FRET) donor and acceptor, it is expected that transmembrane potential can be optically probed as FRET readout. Using two new coral fluorescent proteins, we developed such a membrane potential reporter, named Mermaid, that displays 40% changes in emission ratio per 100 mV change, allowing for visualization of spatiotemporal dynamics in electrical activities of excitable cells. Notably, Mermaid has fast on-off kinetics at warm temperatures and can report voltage spikes comparable to action potentials.