identical. Isoproterenol (100 nM) shifted the activation curves nearly identically for both groups, but the ability of isoproterenol to enhance the current was decreased from a 4.1 ± 0.48 fold increase to a 2.64 ± 0.37 ($p < 0.05$). Finally, the recovery rate for calcium current was reduced by hibernation, reflecting an approximate 20 mV shift. These changes in L-type calcium current and isoproterenol response may explain the reduced contractility of hibernating myocytes and the increased the likelihood of sudden cardiac arrhythmias.

Platform AD: Fluorescence Spectroscopy

2130-Plat

Proton Transfer and Hydrogen-Bond Interactions Determine the Fluorescence Quantum Yield of Bacteriophytochrome, a Novel Deep-Tissue Fluorescent Probe

K.C. Toh[1], Emina Stojkovic[2], Ivo H.M. van Stokkum[1], Keith Moffat[2], **John T. Kennis[1]**.
[1]Vrije Universteit, Amsterdam, Netherlands, [2]University of Chicago, Chicago, IL, USA.

Phytochromes are red-light photoreceptors that regulate a variety of responses and cellular processes. The phytochrome light activation mechanism involves isomerization around the C15=C16 double bond of an open-chain tetrapyrrole chromophore, resulting in a flip of its D-ring. In an important new development, bacteriophytochrome (Bph) has been engineered for use as a fluorescent marker in mammalian tissues (Shu et al. Science 2009). Bph fluoresces at ~720 nm, a wavelength less prone to scattering that can penetrate more deeply into tissue than light emitted by GFP-derived fluorescent proteins. The Bph chromophore biliverdin is a naturally occurring cofactor in mammalian tissue that covalently binds to a conserved cysteine in Bph, and hence BPhs can readily be genetically encoded. BPh photochemistry is thus of considerable significance for biomedical technology. Here we report that an unusual Bph, P3 from Rps. palustris, is highly fluorescent. We identify the factors that determine the fluorescence and isomerization quantum yields of P3 through the application of ultrafast spectroscopy to wild-type and mutants of P3 and a classical Bph, P2. The excited-state lifetime of biliverdin in P3 was significantly longer at 330 - 500 ps than in P2, and accompanied by a significantly reduced isomerization quantum yield. H/D exchange reduces the rate of decay from the biliverdin excite state by a factor of 1.4 and increases the isomerization quantum yield. Comparison of the properties of the P2 and P3 variants in relation to X-ray structures shows that the quantum yields of fluorescence and isomerization are determined by excited-state deprotonation of biliverdin at the pyrrole rings, in competition with hydrogen-bond rupture between the biliverdin D-ring and the apoprotein. This work provides a basis for structure-based conversion of BPh into an efficient near-IR fluorescent marker.

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Fine Tuning the Optical Properties of Green to Red Photoconvertible Fluorescent Proteins

Karin Nienhaus[1], Virgile Adam[2], Dominique Bourgeois[2], G. Ulrich Nienhaus[1].
[1]Karlsruhe Institute of Technology, Karlsruhe, Germany, [2]European Synchrotron Radiation Facility, Grenoble, France.

Dendra2 is an engineered, monomeric GFP-like protein that belongs to a subclass of fluorescent proteins undergoing irreversible photoconversion from a green- to a red-emitting state upon exposure to purple-blue light. We have measured the X-ray structure of the green species of Dendra2 and performed a comprehensive characterization of the optical absorption and fluorescence properties of the protein in both its green and red forms. The structure, which is very similar to those reported for the closely related proteins EosFP and Kaede, revealed a local structural change next to the chromophore, involving mainly Arg66 and a water molecule. We propose that this structural change explains the blue shift of the absorption and emission bands, as well as the markedly higher pKs of the hydroxyphenyl moiety of the chromophore, which were determined as 7.1 and 7.5 for the green and red species, respectively. The 20-fold enhancement of the neutral species in Dendra2 at physiological pH accounts for the observed higher photoconversion yield of this protein in comparison to EosFP.

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Color Hues in Fluorescent Proteins with the Same Chromophore are due to Internal Quadratic Stark Effect

Mikhail Drobizhev, Shane Tillo, Nikolay S. Makarov, Aleksander Rebane, Thomas Hughes.
Montana State Univ, Bozeman, MT, USA.

Intrinsically fluorescent proteins (FPs) exhibit broad variations of absorption and emission colors and are available for different imaging applications. The physical cause of the absorption wavelength change in series of mutants with the same chromophore structure, but different surrounding, is however not understood. Here we study the FP series with acylimine-containing red chromophore, in which the absorption maximum varies from 540 nm to 590 nm, and a series of green FPs with phenolate chromophore, where the absorption peak shifts from 450 to 500 nm. We use two-photon absorption spectroscopy to show that the different colors in each series can be explained by quadratic Stark effect due to variations of the strong local electric field within the beta barrel. The model allows us to experimentally access the chromophore parameters, such as vacuum transition frequency (ν_0) and vacuum changes of permanent dipole moment ($\Delta\mu_0$) and polarizability ($\Delta\alpha_0$) upon excitation. Using this purely experimental and all-optical approach, we estimate, for the first time to our knowledge, the amplitudes of the internal electric field (namely its projection on $\Delta\mu_0$) in a protein. These values amount 10 to 100 MV/cm in the mFruits series. Although these fields appear to be very large, they fall well in the range previously estimated theoretically for different other proteins, and are still $1-2$ orders of magnitude smaller than the fields required to ionize the chromophore. Our model brings simplicity to a bewildering diversity of fluorescent protein properties, and it suggests a new way to sense electrical fields in biological systems. On the other hand, it opens up the way to create two-photon brighter FP probes by tuning internal electric field with smart mutagenesis around the chromophore.

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TIRF-Based FRET with One Base-Pair Resolution

Seamus J. Holden, Stephan Uphoff, David Yadin, Johannes Hohlbein, Ludovic Le Reste, Oliver J. Britton, Achillefs N. Kapanidis.
University of Oxford, Oxford, United Kingdom.

Single-molecule FRET (smFRET) is commonly used as a ''nanoscale ruler'' for the measurement of biomolecular distances and distance changes. However, the limits of FRET resolution for measurements on surface-immobilized molecules have not been rigorously explored. Using total-internal reflection fluorescence (TIRF) microscopy on a set of DNA standards and advanced image analysis software, we have quantified and extended the limits of FRET resolution associated with the use of electron-multiplying CCD (EMCCD) cameras. For such measurements, we derived a novel theoretical description of the major sources of noise (photon shot noise, background, CCD noise and pixelation effects); we find excellent agreement between our experimental results and predictions from theory and Monte Carlo simulations. For FRET measurements on a truly single-molecule basis (as opposed to measurements on an ensemble of single molecules), analysis of the experimental noise allows us to predict a resolution of 4% FRET within the linear FRET range (20-80%), sufficient to directly observe a distance difference equivalent to one DNA base-pair separation (3.4 Å). For FRET distributions obtained from an ensemble of single molecules (which exhibits broadening due to presence of static heterogeneity), we demonstrate the ability to distinguish between distances differing by as little as 2 base pairs (~7Å). Current work focuses on real-time observation of single-base-pair translocation steps of Escherichia coli RNA polymerase within single early transcription-elongation complexes; such observations are crucial for understanding the mechanisms of DNA and RNA polymerases.
Our work paves the way for ultra-high resolution studies of processes involving conformational changes and protein translocation on nucleic acids.

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Single-Molecule STED with Photostable Fluorophores

Robert Kasper.
Bielefeld University, Bielefeld, Germany.

With recent advantage in the development of far-field super-resolution microscopy, further development depends crucially on improved fluorescent probes. We combined STED microscopy with fluorophore stabilization through a reducing and oxidizing system (ROXS) and demonstrate significant improvement of photostability. We show that this improvement can be exploited either for repetitive measurements necessary for 3D or dynamic STED imaging or for resolution enhancement through the application of higher STED beam intensities. Accordingly, a lateral resolution below 30 nm is demonstrated for single organic fluorophores immobilized in aqueous buffer.

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Fluorescence Correlation Spectroscopy Elucidates the Pathway of RNA Interference

Jörg Mütze[1], Thomas Ohrt[2], Wolfgang Staroske[1], Lasse Weinmann[3], Julia Höck[3], Gunter Meister[3], Petra Schwille[1].
[1]TU Dresden, Dresden, Germany, [2]MPI for Biophysical Chemistry, Göttingen, Germany, [3]MPI for Biochemistry, Munich, Germany.