kinase to bind to the N lobe of the other kinase and activate it allosterically (*Cell*, 2006 *125*, 1030). Recent biophysical experiments, however, suggest EGFR dimers exist in the *absence* of ligand: FCS cross correlation (*Biophys. J*. 2007 *93*, 684) and brightness analysis (*Biophys. J*. 2007 *93*, 1021) measurements support prior claims of preformed dimers (e.g., *J. Mol. Biol*. 2001 *311*, 1011). Why aren't these preformed dimers active? We proposed the JM region, net charge = +8, binds to acidic lipids on the inner leaflet of the plasma membrane, which could prevent contact between kinase domains in a preformed dimer (*J Gen Physiol* 2005, *126*, 41). This suggests the "Twist model for ligand-dependent activation of preformed EGFR dimers" of Gadella and Jovin (*J. Cell Biol*. 1995 *129*, 1543) could be compatible with the elegant allosteric activation model from the Kuriyan laboratory (*Cell*, 2006 *125*, 1137). A combination of NMR, CD, infrared and fluorescence measurements on reconstituted TM + JM EGFR(622–660) peptides led us to 3 conclusions: the TM helix breaks at the membrane-solution interface; the unfolded JM region binds electrostatically to the membrane; binding of Ca^{2+}/calmodulin to the positively charged JM region can reverse its charge from +8 to −8 and release it from the membrane (*Biochemistry* 2006, *45*, 12704). Any factor that reduces the electrostatic interaction of the JM region with the plasma membrane should activate EGFR in the absence of ligand (*J. Biol. Chem*. 2007 *282*, 8474).

Platform J: Self-Assembled-Session: The Hidden Photophysics of Autofluorescent Proteins

108-Plat From the knowledge of protonation ground states to the development of photochromic Green Fluorescent Proteins

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Autofluorescent proteins (FPs), and particularly Green Fluorescent Proteins mutants (GFPs), have revolutionized biophysical and molecular biology studies owing to the genetic encoding of strong visible fluorescence in vivo (1). The optical properties of FPs originate from an organic emitter molecule buried inside a higly-ordered b-can tertiary structure. The engineering of FPs' optical properties by means of sequence mutation allows to obtain probes tailored for high-resolution intracellular studies.

Recently, scientific attention has been attracted by "hidden" photophysical properties of FPs (2). In this field, we have been involved in the development and application of photochromic GFPs mutants since 2001 (3–5) and we have shown that pH-dependent optical properties of most GFP mutants can be described by a general two-site ionization model (6). This model helped us to develop new ratiometric pH biosensors for intracellular use (7). Here, we shall show how the knowledge of the protonation ground states led us to generate new photochromic GFP mutants. Quantitative details on the photophysical mechanism underlying the observed photochromic behavior will be reported.

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109-Plat Pa-GFP Photoactivation Kinetics In Model And Cell Systems

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We present data related to the possibility to induce the photoactivation process on the photo-activatable green fluorescent protein paGFP, T203 variant (1), at different wavelengths, specifically in the (270–488) nm spectral range under linear conditions and in the infrared range under multi-photon absorption (2). In particular, exploiting UV irradiation to bring the molecules in the activated state it is possible to evaluate the absorption properties of activated and non activated form of the fluorescent protein in solution, without additional artefacts due to photobleaching or environmental conditions. These results were employed to separate the contribution of the two forms of the molecules via a home made linear unmixing algorithm. This analysis allowed to identify the kinetic behaviour of activated and non activated paGFP populations in more complex systems, like proteins layers and fixed cells while utilizing confocal and two-photon microscopy. In particular, a different fraction of molecules undergoing photoactivation was found depending on the energy delivered during the activation step. We demonstrate that this phenomenon is driven by the competition of photoactivation and photobleaching of the pre- and post-activated form of paGFPs.

In conclusion, we showed unreported photoactivation pathways of the proteins in the UV range that were useful for the comprehension of the nature and kinetics of photobleaching and photoactivation processes. These results are in tune with the broadening of two photon activation spectra (2) and with blue-photoactivation pathways (3) early reported.

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110-Plat Fluorescence Lifetimes Of Cyan Fluorescent And Yellow Fluorescent Proteins In Vitro And In Vivo: Implications For The Use Of Fret-based, Genetically-encoded Sensors

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The use of paired autofluorescent proteins capable of FRET has been a great promise for thedevelopment of intracellular probes - from simple cells to whole animals. Although the variety (and spectral range) of autofluorescent proteins is enlarging every year, the mainlyused couple consists of autofluorescent proteins belonging to the cyan (donor) and yellow (acceptor) fluorescent proteins, respectively. Among the many established sensors those for calcium and chloride are of particular interest. Data from in vitro as well as in vivo (transfected HEK293 cells and retina slices from genetically modified rodents) will be presented. The nvironmental influence on the properties of the autofluorescent proteins - especially on the donor - and the consequences for the FRET-based sensor function is studied and discussed in this contribution.

111-Plat Nanoscale Live Cell Imaging of Hemagglutinin Rafts by Fluorescence Photoactivation Localization Microscopy

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Photoactivation of non-fluorescent molecules into fluorescent ones allows control over the number of molecules within a microscopic region of interest in a biological sample. This control can be exploited to achieve a sparse distribution of fluorophores at a given time, which can be imaged and analyzed to determine molecular positions by localization. Since molecules can be localized with nanometer precision, if a large enough number of molecules can be individually localized within the sample, an image of the sample can be reconstructed with higher resolution than the diffraction limit would ordinarily allow. Recently developed methods such as Fluorescence Photoactivation Localization Microscopy (FPALM) use such a scheme to image biological and non-biological samples with resolution in the tens of nanometers. FPALM has been used to image living cells expressing photoactivatable green fluorescent protein attached to hemagglutinin (HA), the fusion protein from influenza virus, with resolution of better than 40 nm. The distribution of HA was found to be inconsistent with several models of lateral heterogeneity (i.e. “rafts”) in the plasma membrane, and consistent with at least one other model. Rafts are controversial membrane clusters proposed to exist in live cell membranes and shown by biochemical methods to be enriched in cholesterol, glycosphingolipids, and certain proteins, including HA. Rafts were previously too small to image by normal light microscopy. However, these results, exploiting the ~six-fold enhanced resolution of FPALM, provide for the first time a view of HA rafts in live cell membranes.

112-Plat Stroboscopic Approach To Fast Photoactivation Localization Microscopy With Dronpa And Mutants

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The photophysical properties and photoswitching scheme of the reversible photoswitchable GFP-like fluorescent proteins Dronpa-2 and Dronpa-3 were investigated by means of ensemble and single-molecule fluorescence spectroscopies, and compared to the precursor protein Dronpa. The faster response to light and the faster dark recovery of the new mutants observed in bulk holds at the single-molecule level. Analysis of the single-molecule traces allows us to extract the efficiencies and rate constants of the pathways involved in the forward and backward switching, and we find important differences when compared to Dronpa. We rationalize our results in terms of a higher conformational freedom of the chromophore in the protein environment provided by the β-can. The thorough understanding of the photophysical parameters has allowed us to optimize the acquisition parameters for camera-based subdiffraction-limited imaging. We show that Dronpa and its mutants are useful for fast photoactivation localization microscopy (PALM) using common wide-field microscopy equipment, where individual Dronpa molecules can be localized several times. We provide a new approach to achieve fast photoactivation-localization microscopy by introducing simultaneous two-color stroboscopic illumination (S-PALM).

113-Plat Lifetime Microscopy For Uncovering Photoconversion In Autofluorescent Proteins

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Autofluorescent proteins, in which the fluorescent moiety is formed out of several amino acids of the protein sequence, became versatile tools in the life sciences. While in the beginning labelling was the main application, purposeful photochemical transformations are gaining interest for tracking protein diffusion or high resolution microscopy.

Accidental occurring photochemical reactions, however, are detrimental to the brightness of autofluorescent proteins in microscopy: light-driven isomerization diminishes the fraction of fluorescent species, and photoconversion reduces the fluorescence lifetime.

Here, we present data on phototransformations of a photoactivatable GFP in confocal microscopy. Photoconversion is achieved solely by excitation of the barely absorbing anionic chromophore state R_{eq}^{-} in the GFP mutant Thr203Val. Besides the well-known shift of the equilibrium between the neutral chromophore state RH

and R_{eq}^-, the photoconverted anionic chromophore R_{pc}^- exhibits a reduced fluorescence lifetime of 2.2 ns. The fluorescence lifetime which is measured in fluorescence lifetime imaging microscopy with spatial resolution depends however on the excitation conditions and history. The underlying photochemistry is described by the kinetic scheme of consecutive reactions, $R_{eq}^- -> R_{pc}^- -> P_{dark}$, in which the anionic chromophore species and the dark protein P_{dark} are coupled by photoconversion and photobleaching. Time correlated single photon counting in a confocal geometry of diffusing species is used to compute the quantum yields for photoconversion and photobleaching for the anionic chromophore species.

114-Plat Novel Two-Photon Absorption Bands in Fluorescent Proteins

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Despite a rather extensive use in high-resolution imaging of living cells and tissues [1], two-photon excitation properties of Fluorescent Proteins still need to be thoroughly investigated and characterized over a wide range of excitation wavelengths and external conditions. An optimal two-photon excitation wavelength can be often obtained by doubling the maximum one-photon excitation, and normally the two-photon excitation profile of FPs at about halved wavelength is found to mimic the one-photon excitation [2]. However, other spectral regions that are inactive at one-photon excitation can become active when accessed by two-photon excitation. DsRed, in particular, displays such "anomalous" behavior: a strong two-photon band appears at wavelengths shorter than 780nm. At the corresponding one-photon region (370nm) the one-photon absorption spectrum is almost completely flat [2,3]. By means of Density Functional Theory techniques, we explain here the presence of this band as stemming from transition to a higher excited state, through coupling with the HOMO-LUMO one-photon excitation [4]. We shall argue that two-photon excitation bands in the 500–700nm region are ubiquitous among FPs, and might provide interesting spectral windows for multiphoton fluorescence imaging.

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115-Plat Singlet Oxygen Production By Proteins From The GFP Family

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Proteins from the green fluorescent protein (GFP) family are increasingly being used as genetically-encoded reporters for intracellular dynamics, protein expression and protein-protein interaction studies based on fluorescence microscopy. Extended observation of GFPs is unfortunately limited by photobleaching/photoconversion of the chromophore or light-induced damage of the surrounding biological medium. Photoproduction of reactive oxygen species are thought to play a key role in this limitation. The protein photophysics can be fine-tuned by modification of the chromophore's environment and recently a GFP mutant has been engineered for genetically-targeted chromophore-assisted light inactivation. The prospect of using genetically-encoded photosensitizers for mechanistic and eventually therapeutic purposes has lead us to study their ability to photosensitize the production of singlet oxygen. The results of these studies reveal the role of the beta can in the photosensitization process.

Platform K: Voltage-gated K Channels, Voltage-Dependence & Gating

116-Plat Engineering Atomic Constraints Between the Voltage Sensor and the Pore Domain of a Voltage-Gated K^+ Channel of Known Structure

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Voltage-gated K^+ channels (Kv) are tetrameric subunit complexes surrounding an aqueous pore, each subunit comprising six transmembrane segments (S1–S6) with S1–S4 constituting the voltage-sensor domain (VSD) and S5–S6 the pore-forming region. Membrane depolarization promotes structural re-orientation of each of the four voltage-sensor domains allowing pore opening. Although the crystal structure of Kv1.2 provided the first atomic resolution view of a eukaryotic Kv channel, several components of the VSD remain poorly resolved. In particular, the position and orientation of the charged arginine side chains in S4 during voltage-dependent channel activation remains controversial. Here, utilizing electrophysiological analysis of intersubunit metallic bridge formation as employed previously in *Shaker*, in combination with all-atom molecular dynamic simulations of the Kv1.2 channel in an explicit membrane, we demonstrate that S4 of Kv1.2 interacts directly with the pore domain in the open-activated conformation, and we provide a structural model for this conformation. The results from electrophysiology indicate that residues R294 and A351 are closer than the 13.7 Å predicted in the Kv1.2 crystal structure, consistent with the molecular dynamics simulations.