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cryo-electron tomography. This technique ascertains that rhodopsin is preserved in a close-to-native state. Briefly: retina is fixed by high-pressure freezing, ultra-thin sectioned and visualized by cryo-electron tomography. In the reconstructed and processed tomograms the organization of rhodopsin molecules becomes visible. We identify three levels of hierarchical supramolecular organization. Rhodopsin forms dimers; the dimers form rows; and rows come in special pairs like rail tracks. Rows are aligned parallel to the disk incisure. A row is comprised of at least 10 dimers and is about 50 nm in length. The distance between rows within a track is 5 nm; and tracks are separated by 15 nm. We propose that rhodopsin tracks provide a template that organizes the spatio-temporal interaction of preassembled signalling components on the disk surface. Aligned rows of immobile rhodopsin renders photoreceptors highly dichroic and might provide the structural basis for detection of polarized light. We envisage that rhodopsin-like type A GPCRs, which are highly homologous, also entertain a supramolecular organization.

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Protein-Protein Interactions of the β-adrenergic Receptor and Kinetic Analysis of Intracellular Binding Partners via μ-Patterned Surfaces

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β-adrenergic (βAR) receptors belong to the class of G protein-coupled receptors (GPCRs) and generally administrate the physiological response of adrenaline and noradrenaline. They can be classified into three receptor subtypes (β1, β2, β3) which are major drug targets for some of the most commonly prescribed drugs in the history of medicine. The various βAR subtypes also interact differentially with a huge diversity of cytoplasmic (G-proteins, arrestin, adenylate cyclase,···) and transmembrane proteins (e.g. receptor homo-/hetero-multimerization, ion channels,···) leading to distinctive receptor signaling pathways. Studying such interaction processes of plasma-membrane localized receptor proteins is key for a better understanding of cellular processes. In the past few years, many studies indicated that βAR dimerization can occur between two identical receptors (homodimerization), between two different receptor subtypes of the same family or between receptors of different families (heterodimerization). The physiological role of such di-/multimerizations remains a matter of debate. Here we describe an assay combining TIR (total internal reflection) microscopy and micro-patterned surfaces, which can be used for the detection of protein-protein interactions in and near the cell membrane in-vivo. We studied the homo- and heteromultimerization of β1- and β2-adrenergic receptors. Furthermore we investigated the interaction of βARs with different cytoplasmic signaling proteins such as β-arrestin, G-protein and adenylate cyclase. We unequivocally show that β1ARs as well as β2ARs form homo- and hetero multimers in living CHO-K1 cells. We could also proof the interaction of βARs with β-arrestin, G-protein and adenylate cyclase. In addition we conducted FRAP (fluorescence after photobleaching) experiments for further characterization of these interaction processes. In general, our system is of great interest for a fast and straightforward analysis of membrane-receptor protein interactions.

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Site-Specific Bioorthogonal Labeling of a G Protein-Coupled Receptor at a Genetically Encoded Azido Amino Acid

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We have developed a strategy for site-specific bioorthogonal labeling of expressed G protein-coupled receptors (GPCRs) to facilitate single-molecule fluorescence imaging studies. We employed a two-step method that is highly efficient and specific and does not perturb receptor function. First, we used the amber codon suppression technique to introduce p-azido-L-phenylalanine (azF) at single sites into rhodopsin, a prototypical class A GPCR. We then ligated a fluorophore to the unnatural amino acid by strain-promoted azide-alkyne cycloaddition. We showed that two types of dibenzocyclooctyne reagents, DIBO and DBCO, gave robust stoichiometric labeling of rhodopsin with satisfactory kinetics. Moreover, the extent of non-specific covalent labeling was negligible. To evaluate whether this approach is general, we generated a series of azF-containing rhodopsin mutants and observed consistent labeling for most of them. Curiously, the dibenzocyclooctyne reagents readily labeled residues in the transmembrane region as well as the aqueous exposed extracellular and cytoplasmic receptor domains. To characterize the functionality of the labeled rhodopsins, we devised an energy transfer-based fluorescence assay to assess the 11-cis-retinal uptake kinetics. This assay both confirmed the functional integrity of the samples and provides a general scheme for studying receptor-ligand binding. We are currently characterizing the labeled receptors using single-molecule TIRF microscopy. In the long term, this strategy will serve as a key enabling technology to monitor the dynamics and allosteric regulation of GPCR signaling complex. Further, the approach described here can, in principle, be applied to other membrane or cellular proteins.

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Restricting EphA2 Receptor Movement Affects Internalization and Signaling in Living Cells

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The importance of spatial reorganization of receptors and ligands that occurs at the cell-cell interface is becoming increasingly evident in a variety of cell systems. Our lab has developed a unique experimental platform in which cells are interfaced with supported lipid membranes displaying ligands creating a controllable and laterally mobile ligand presentation system that recapitulates essential aspects of the cell-cell contact. To probe how protein organization in the membrane influences cell signaling events, we use a spatial mutation strategy in which we pattern supported lipid bilayers into microscale corrals to restrict protein movement within the corrals and then measure alterations in cell signaling. When MDAMB231 breast cancer cells expressing the receptor tyrosine kinase EphA2 are interfaced with a supported lipid bilayer containing the ephrinA1 ligand, EphA2 binds to ephrinA1 and the complex reorganizes into large clusters. When receptor-ligand movement is disrupted with the spatial mutation, the reorganization of EphA2-ephrinA1 clusters results in alterations of downstream signaling. In particular, we found that recruitment of the metalloprotease ADAM10 to the clusters is inhibited, indicating that EphA2 signaling is sensitive to the spatial organization of ephrinA1. ADAM10 has been implicated in the cleavage and endocytosis of other ephrins, suggesting that ADAM10 may be necessary for efficient endocytosis of EphA2-ephrinA1. Therefore, our research is probing how endocytosis is affected by the spatial organization of EphA2. Here, we report the development of a novel and highly quantitative endocytosis assay. Using this assay, we found that the spatial organization of EphA2-ephrinA1 does alter ephrinA1 endocytosis. We also found that Pitstop2, a small molecular inhibitor of clathrin-mediated endocytosis (CME), negatively effects ephrinA1 endocytosis, indicating that CME is important in ephrinA1 internalization. The implications of these observations for spatial regulation of EphA2 signaling will be further discussed.

Platform: DNA and RNA Structure

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High-Precision FRET to Analyze the Architecture and Heterogeneity of RNA Junctions

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Like for many other non-coding RNAs, helical four-way and three-way junctions (4WJs and 3WJs) are an essential structural motif of the for functional RNA structures. Using FRET restrained high-precision structural modeling as a hybrid tool we resolve the structures of three coexisting conformers of a fully Watson-Crick base paired RNA4WJ based on the hairpin ribozyme. 51 different FRET-pairs were measured using single-molecule multi-parameter fluorescence detection (smMFD). For each dataset, the single-molecule approach allowed for the simultaneous extraction of three distances (and their corresponding errors) belonging to one major FRET state and two minor states. Distinct Mg^{2+}-affinities were used for the assignment of the two minor states to the corresponding conformers. Rigid body models for the major and both minor conformers were obtained by docking rigid ds A-RNA helices explicitly taking into account dye position distributions. The three rigid body models were refined by all-atom MD simulations and coarse-grained RNA folding using FRET-restraints. A cluster analysis gives confidence levels for the proposed ensemble of models, and the precision was assessed via bootstrapping. The achieved precisions are significantly better than the uncertainty of the dye