correlation spectroscopy (Petersen et al., BJ 65:1135. 1993), with the improvement that the autocorrelation value g(0,0) was explicitly corrected for shot noise and CCD camera read noise.
Monovalent ligands in fluid membranes cause receptor clustering in T-cells and B-cells, in cytoskeleton-dependent processes. The high DNP concentration required for large-scale aggregation in RBLs suggests that degranulation signaling and large-scale aggregation may be two distinct cellular responses that depend differently on hapten concentration and presentation.
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Single Molecule Studies of FcεRI Dynamics Link Mobile Receptors with Signaling
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The high affinity IgE receptor, FcεRI, is the principal multi-subunit immunoreceptor on the surface of mast cells and basophils. These receptors bind circulating IgE with high affinity and are activated when multivalent allergen crosslinks IgE-bound receptors. Crosslinking initiates a complex signaling pathway that ultimately leads to degranulation and release of key mediators of allergic inflammation. To study the dynamic events that induce FcεRI signaling, we generated two novel quantum dot (QD) probes for single particle tracking: monovalent QD-IgE that binds FcεRI without crosslinking and multivalent DNP-QD that mimics allergen by crosslinking DNP-specific IgE. Previously, we provided direct evidence that actin filaments ''corral'' resting receptor motion and are involved in receptor immobilization (Andrews et al, *Nature Cell Biology* 10:955). Simultaneous imaging of single QD-IgE-FcεRI complexes and GFP-tagged actin revealed that membrane-proximal actin bundles form a dynamic labyrinth that restricts receptor diffusion. Real-time imaging revealed that receptors become immobilized within seconds of crosslinking by high doses (1 μg/ml) of multivalent antigen and that immobilization is delayed when actin is disrupted. We have now investigated further the relationship between receptor immobilization and activation. We find that the kinetics of immobilization are dose dependent and receptors remain mobile at antigen doses corresponding to maximal degranulation (0.001-0.01 μg/ml). Using a novel hyperspectral microscope with ~1 nm spectral resolution, we tracked up to five spectrally distinct QDs simultaneously. Multi-color tracking of QD-IgE-FcεRI complexes revealed that small crosslinked clusters (2-4 receptors) remain mobile under activation conditions. In addition, DNP-QDs were shown to induce degranulation, yet DNP-QD-crosslinked receptors continue to diffuse. These results indicate that receptor immobilization is a feature of highly aggregated receptors, depends upon an intact actin cytoskeleton, and is more likely involved in signal termination than activation.

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Protein movement between membrane domains: the Epidermal Growth Factor Receptor (EGFR) signaling cascade
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We have developed a novel procedure to isolate membrane domains as they exist at 37°C and are using it to follow the redistribution of membrane proteins upon stimulation of EGFR. TACE (Tumor necrosis factor-α Converting Enzyme, also known as ADAM17) is responsible in ~80% of the physiological cases for initiating the signaling cascade, via trans-activation, by phosphorylating EGFR (to p-EGFR). Trans-activation did not affect the location of TACE. Instead, a significant amount of p-EGFR moved from a fraction rich in caveolin-1 (Cav-1) to TACE-containing fractions. PKC which participates in TACE signaling also became more concentrated in the TACE fractions. The ectodomain of TGF-α, cleaved by TACE, is a physiologically important agonist for EGFR. Upon stimulation, TGF-α moved to the TACE fraction. We conclude that stimulation of the EGFR signaling cascade leads to colocalization of EGFR, TACE, and TGF-α along with PKC. By also measuring lipid compositions, we have arrived at our current mechanistic picture of protein redistribution upon EGFR stimulation: At rest, most of the EGFR is in caveolae. But 5-20% of the non-phosphorylated EGFR is in domains rich in sphingomyelin-rich domains concentrated in TACE and flottilin-2. EGFR continuously cycles between caveolae and the TACE domains. Upon stimulation, TGF-α moves into TACE domains and is thereby cleaved. The released ectodomain activates the EGFR that is in these domains. Independent of whether this initial hypothesis proves correct, our data shows that multiple signaling proteins are recruited to the same domains. These domains, containing proteins shared by separate cascades, may provide a means to functionally connect cascades to create integrated cellular processing.
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Investigating the Role of Receptor Clustering and Dynamics in Transmembrane Signaling by Functional Arrays of Bacterial Chemoreceptors
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Both clusters and conformational changes are thought to be important in the transmembrane signaling mechanism of bacterial chemotaxis receptors. Full signaling activity of these receptors requires assembly of a ternary complex with two other proteins, CheA and CheW. Weis & coworkers have recently developed an approach for assembling active arrays of the receptor cytoplasmic domain complexes at vesicle surfaces, and have demonstrated that the surface density modulates the activity of the receptor: high density increases the kinase activity and low density increases the methylation activity (Besschetnova et al, Proc. Natl. Acad. Sci. USA 105, 12289). In order to determine how ligand binding modulates this density-dependent equilibrium, we have reconstituted the intact receptor into membrane vesicles at a range of lipid:protein ratios. Preliminary results indicate that the kinase activity of the intact receptor exhibits a density dependence similar to that of the cytoplasmic fragment arrays: the fully methylated receptor shows little activity change with density but the demethylated receptor requires high density for full activity. Thus methylation state modulates the density-dependent equilibrium of the intact receptor and experiments are in progress to determine whether attractant ligand modulates it in an opposing manner. We are also assembling vesicle surface-associated functional arrays of the receptor cytoplasmic fragment for further biophysical studies: (1) solid-state NMR distance measurements to determine the array geometry, and (2) mass spectrometry measurements to determine whether receptor dynamics change with signaling state.
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Multi-color Single Quantum Dot Tracking To Characterize Membrane Receptor Interactions On Living Cells
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Single particle tracking (SPT) provides the necessary spatial and temporal resolution to acquire information on the diffusional and interaction dynamics of individual proteins. Quantum Dots (QDs) are bright and photostable probes that make long-term SPT possible and their large Stokes shifts facilitate multi-color SPT through single wavelength excitation. We developed two-color single QD tracking methods and analyses to characterize receptor dynamics as well as transient protein-protein interactions down to the ~10 nm scale. The analyses of receptor trajectories provide quantification of dynamic parameters that cannot be obtain from traditional biochemical techniques. Specifically, we developed a Hidden Markov Model (HMM) approach to identify and extract the lifetime and kinetic rates for dimerization and map regions of the plasma membrane explored by monomeric and dimeric receptors. We apply this technique to study the interplay between transmembrane receptor tyrosine kinases, erbB1 and erbB2, using QD-conjugated ligands, variable fragments of heavy-chain only antibodies (Nanobodies), and reduced monoclonal antibody fragments. Single-color QD-SPT is used to characterize the receptor diffusional dynamics in the presence or absence of ligands or inhibitors. The diffusion coefficients we measured by single QD tracking are consistent with published values obtained from tracking probes labeled with organic fluorophores. Using spectrally distinct QDs and simultaneous two-color imaging, we directly observe transient homo- and hetero-dimers even in the absence of ligand. Addition of EGF, the ligand for erbB1, prolonged dimer interactions. Current efforts seek to extract dimerization parameters from these two-color data sets using the HMM analysis.

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An Investigation Into the Membrane Diffusion and Organisation of Adenosine Receptor Homo-oligomers
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Oligomerisation of G-protein coupled receptors (GPCRs) is now a widely accepted phenomenon, although its effects on receptor signalling, pharmacology and organisation are still unclear. Using a combination of bimolecular