

Membrane Receptors and Signal Transduction III

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Proteomic Imaging of Plasma Membranes of Antigen-Activated B Lymphocytes

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Dense protein complexes such as those found in the immunological synapses of antigen-activated lymphocytes are sites of critical biological activity, but their study is complicated by the large number of protein species present. Array tomography is a proteomic imaging method capable of addressing this high-dimensional problem through iterative immunostaining, but would be difficult to apply to spatially-restricted regions of interest such as complexes within the immunological synapse that form in the area of contact between lymphocytes and antigen-presenting surfaces. We have developed a novel variant of this technique which embeds a thin (~500 nm) slice of material in LR White, making proteomic multiplexing possible. We are using this method with physically unroofed resting and activated B cells to study signaling in antigen-receptor microclusters in B cell plasma membranes. Here we present this method, and our current work in its application to the B cell membrane.

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T-Cell Receptor-CD3 Signaling Complex Extracellular Interactions Characterized by Genetic Incorporation of Unnatural Amino Acid Photo-Crosslinkers

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T-cells play a central role in the adaptive immune response to invading pathogens and cancer while largely avoiding self-reactivity. The interaction of T-cell receptors (TCRs) displayed on T-cells with peptide-major histocompatibility complexes (pMHCs) on the surface of antigen presenting cells leads to signaling through the TCR-CD3 signaling complexes, which result in the initiation of cellular immune responses. However, how molecular and structural events in TCR-recognition translate through TCR-CD3 complex and generate differences in intracellular signaling is still not clear. Previous studies have shown that the extracellular TCR-CD3 subunits interactions are important for T-cell responses to TCR engagement. The objective of this research is to understand structural organization and specific interactions among TCR and CD3 subunits, and to provide insights into how structural features and mechanisms initiating TCR-triggering determine the outcome of immune responses.

To monitor interactions among TCR-CD3 subunits, we developed a system to site-specifically incorporate an unnatural amino acid photo-crosslinker *p*-Azido-L-phenylalanine (pAzpa) or *p*-benzoyl-L-phenylalanine (pBpa) into TCR expressed on cell surface. Subsequently, after activation of TCR with pMHC, photo-crosslinked TCR-CD3 complexes were immunoprecipitated and analyzed by Western Blotting. Our analysis showed that specific residues in the TCR DE, CC, FG loops and G strand play critical roles in TCR-CD3 subunit interactions. Moreover, cross-linked high molecular weight complexes suggested TCR-TCR dimerization during the signaling process. Together our results 1) reveal how TCR-CD3 signaling subunits are positioned together to perform their function, 2) suggest how the antigen specific recognition information gets transferred from outside to the signaling molecules inside cell to determine immune responses, 3) provide potential targets for modulating outcome of immune response.

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The Molecular Mechanisms of Gradient Sensing by CXCR4

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The chemokine receptor CXCR4 is essential for many physiological processes, including stem cell homing, organogenesis, inflammation and tissue repair. Expression of CXCR4 in malignant cells may influence the biology of cancer, in modulating tumor survival, growth and angiogenesis; CXCR4 plays an important role in directing metastatic CXCR4-expressing tumor cells to organs that express CXCL12. Directional cell migration towards increasing concentration of the chemokine ligand CXCL12 is result of *Gzi* and *Gzq* pathways activation. These biochemical pathways are well studied, however the mechanism of gradient sensing is not yet understood.

In this study we use Ewing's sarcoma derived cells to unravel the processes that are involved in CXCR4 related metastasis formation. Cells are transformed with CXCR4-eYFP enabling use of single-molecule imaging to access diffu-

sive dynamics of CXCR4 in the plasma membrane under different conditions. We find that receptor diffusion is not homogeneous and is present as both mobile and immobile (~20%) receptors. Mobile receptors show free diffusion with diffusion coefficient $D=0.19 \pm 0.01 \mu\text{m}^2/\text{s}$. Stimulation with CXCL12, does not change receptor mobility, however it leads to changes in the ratio of mobile and immobile receptor fractions in a concentration dependent manner. Further experiments show that actin cytoskeleton depolymerization causes an unexpected drop in mobile receptor fraction. Notably, this effect is stimulation independent. Endocytosis inhibition results in an extra receptor fraction, which shows confined diffusion and significantly increases upon stimulation. Interestingly, G-proteins and dimerization do not influence diffusive behavior of CXCR4 in the plasma membrane. These findings together with whole cell migration results highlight new molecular insights into chemokine gradient recognition.

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Single Molecule Observation of TCR Signaling Complexes in Living T Cells

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T cell's main function is to detect foreign peptides in a sea of self-peptides displayed on antigen presenting cells via T cell receptors (TCR). Individual TCRs bind to foreign peptides with affinities that differ only slightly from that of self-peptides. As a result, each TCR is likely to bind to a subset of self-peptides whose ever-present signal can overwhelm the signals from genuine foreign peptides. However, previous results demonstrate that T cells can be triggered by a few foreign peptides among thousands of self-peptides. In fact, erroneous triggering of T cells by self-peptides results in autoimmune diseases such as rheumatoid arthritis, Crohn's disease, and some forms of diabetes. This implies that the decision to activate at the cellular level is highly sensitive and selective, and the decision is likely to be based on a combination of factors, such as binding affinity, number of interactions, and spatial and temporal distribution of the signal. While the molecular interaction of TCR and its ligand has been well studied *in vivo*, any correlations between the binding kinetics and cellular responses are lost within population behaviors. Thus, the precise physical mechanism by which TCR-peptide binding leads to the triggering of T cells remains unclear. Elucidating the molecular mechanism of this process requires simultaneous measurement of the binding kinetics, stoichiometry, and movement of individual signaling molecules in living T cells. In this project, we directly observe molecular interactions within TCR-ligand complexes with single molecule resolution and correlate it with subsequent single cell responses in hybrid junctions between live primary T cells and supported lipid membranes.

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Exploring the Spatio-Mechanosensitivity of Eph Receptor in Stem Cells

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The Eph receptors constitute the largest family of receptor tyrosine kinases (RTK), and their ligands, ephrins, are glycoposphatidylinositol-linked (ephrinA1-10) or transmembrane (ephrinB1-3) proteins which bind to the Eph receptors on the opposing cell membrane to initiate bidirectional juxtacrine signalling [1]. Our collaborators in the Schaffer group have uncovered a previously unknown role of EphB4 signalling in regulating neural stem cell fate commitment. In particular, antibody-clustered ephrinB2 in solution induces neuronal differentiation *in vivo* and *in vitro* [2]. Experiments in solution [2], however, cannot reflect the microenvironment of cell-cell junction. Because ephrin ligands and Eph receptors are membrane bound, Eph:ephrin signalling is cell-cell contact dependent. The Groves group has developed a unique platform which is ideally suited for investigating the role of the physical microenvironment on cell-cell signalling. This hybrid system simulates the cell-cell contact geometry by combining live cells with a supported lipid bilayer_a synthetic cell membrane formed on a glass substrate which can be functionalised with cell surface ligands. By fabricating microstructures on the glass substrate, one can induce a spatial mutation, which is the orchestrated mechanical disruption of the spatial patterning of proteins on the lipid bilayer. Spatial mutations have been shown to have a demonstrably significant impact on EphA2-ephrinA1 signalling in cancer cells which alters the downstream molecule recruitment [3]. Altogether, these results motivate us to explore the effect of spatial-reorganisation of EphB receptor in stem cell functions by reconstituting the cell-cell contact geometry on a supported lipid bilayer.

[1] Pasquale, E.B. *Nat. Rev. Mol. Cell Biol.* 6, 462-475 (2005).

[2] Ashton, R.S. et al. *Nat. Neurosci.* 15, 1399-1406 (2012).

[3] Khalid S. et al. *Science.* 327, 1380 (2012).