in cell biology, namely the nature of molecular organization and its spatiotemporal regulation on the plasma membrane.

1588-Pos

Membrane Anchor Dependent Colocalization in Cellular Membranes Observed by Fluorescence Cross-Correlation Spectroscopy

Sara B. Triffo, Hector H. Huang, Adam W. Smith, Jay T. Groves.
University of California, Berkeley, Berkeley, CA, USA.

Membrane anchors exist on many proteins in a variety of combinations of enzymatically attached fatty acids and glypiations. These anchors play a part in protein trafficking within cells and in associating proteins with cell membranes. They are also frequently found on well-known signaling proteins. Given the variety of anchor composition, we question whether these anchors play a more significant role in the lateral sorting or dynamic colocalization of proteins within cell membranes. To observe this *in vivo*, we create fusion proteins of red and green fluorescent proteins with the consensus protein lipidation motif of various signaling proteins and express both red and green constructs in HEK293T cells. The dynamic colocalization of red and green fluorescent proteins, and therefore the dynamic colocalization of membrane anchors, can be directly observed using Fluorescence Cross-Correlation Spectroscopy (FCCS). FCCS allows us to observe dynamic colocalization on the nanometer length scale. Unlike FRET, FCCS can detect positive colocalization regardless of orientation and at lengths larger than 10nm. Recent results will be discussed.

1589-Pos

Growth of the E. Coli Outer Membrane

Eliane H. Trepagnier, Julie A. Theriot.
Stanford University, Stanford, CA, USA.

The outer membrane (OM) of E. coli is composed of four elements: lipopolysaccharide (LPS), phospholipids, OM proteins, and lipoproteins. Together these elements form a continuous protective layer, defending the bacterium against harsh environments and toxic chemicals. Maintenance of an intact OM requires that synthesis and insertion of new OM components keep pace with bacterial growth. At present, little is known about where new OM is incorporated, or how its growth is regulated. We use video microscopy to examine the behavior of fluorescently labeled LPS and specific OM proteins on the surfaces of growing bacteria. Initially, labeled LPS and OM proteins in an individual cell exhibit a uniform peripheral distribution. As the bacterium elongates, fluorescent spots emerge, subsequently drift apart from one another, and occasionally bifurcate. Arresting bacterial growth with Rifampin halts the motion of the fluorescent spots, resulting in a fluorescence pattern which remains stable over a period of hours. We hypothesize that the appearance and divergence of these fluorescent spots of labeled OM is due to insertion of newly synthesized, unlabeled OM components. We track the motion of these spots on the surfaces of E. coli, and measure the convergence and divergence of adjacent tracks on the periphery of the cell. Our data suggest that new OM is incorporated in patches and distributed non-uniformly, with the bulk of the new material inserted along the lateral walls of the cell and lower rates of insertion in the polar regions of the cell.

1590-Pos

Single-Molecule Study of the Dynamics of Lipid-Like Molecules in the E. Coli Outer Membrane

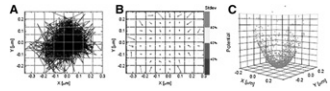
Alyssa J.C. Garrelts, Kenneth P. Ritchie, Onkar Sharma,
William A. Cramer, Yi-Ju Hsieh, Barry L. Wanner.
Purdue University, West Lafayette, IN, USA.

While there have been many studies on the diffusion of membrane lipids in eukaryotic cells, which have given insight into the structure and organization of these membranes, little is known to date of their mobility in bacterial membranes, specifically the Gram negative bacteria, *Escherichia coli*. The *E. coli* outer envelope consists of inner and outer lipid membranes that are separated by a periplasmic space containing the cell wall. The outer membrane is unique in that it is thinner than mammalian plasma membranes and consists of a phospholipid inner leaflet with a predominantly lipopolysaccharide (LPS) outer leaflet. Here we look at the diffusion of the fluorescent lipid analog 3,3′-dioctadecylindocyanine iodide (DiI(C_{18})) and Alexa488-LPS in the outer membrane of live E. coli cells using single molecule imaging/tracking techniques. The diffusion coefficient of DiI(C18) was found to be $(5.2 \pm 0.2) \times 10^{-11}$ cm^2/sat time scales of 0.33 *s*. By contrast, the diffusion coefficient of DiI(C18) in human epithelial cancer cells of the nasopharanyx (KB) is found to be $(1.94 \pm 0.2) \times 10^{-8}$ cm^2/s, in good agreement with previously measured diffusion coefficients of DiI(C18) in other mammalian cells. The mobility of LPS in the outer membrane and the implications of the slow diffusion of DiI(C_{18}) on the structure of the outer membrane of *E. coli* will be discussed.

1591-Pos

Investigation of the Confining Potential of Toxin Receptors in Membrane Microdomains by Single Molecule Tracking with Lanthanide-Doped Nanoparticles

Silvan C. Türkcan[1], Jean Baptiste Masson[2], Didier Casanova[1], Guillaume Voisinne[2], Genevieve Mialon[3], Thierry Gacoin[3], Jean Pierre Boilot[3], Michel Popoff[4], Massimo Vergassola[2], Antigoni Alexandrou[1].
[1]Ecole Polytechnique, Laboratoire d'optique et bioscience, Palaiseau, France, [2]Unité de Génétique in Silico, Institut Pasteur, Paris, France, [3]Ecole Polytechnique, Laboratoire de Physique de la Matière Condensée, Palaiseau, France, [4]Unité Bactéries Anaérobies et Toxines, Institut Pasteur, Paris, France.



We coupled photostable and non-blinking $Y_{0.6}Eu_{0.4}VO_4$ nanoparticles to epsilon toxins produced by *Clostridium perfringens* type B and D, which bind to a specific receptor on MDCK cells. Single-molecule tracking using these labels shows that the toxin receptor exhibits confined motion within microdomains.

To analyze the receptor trajectories, we introduced a novel approach based on an inference method [1]. Our only assumption is that the receptor moves according to the Langevin equation of motion. This method fully exploits the information of the ensemble of the trajectory (Fig. A), in contrast to the usual mean square displacement analysis, which focuses only on a single observable, the second-order moment. Applying both techniques to collected trajectories, we can highlight the difference in extracted parameters.

From the shape of the confining potential (Fig. C), which is obtained by mapping the forces (Fig. B) inside domains, we can deduce information about the mechanism of confinement. In combination with experiments on cholesterol depletion and cytoskeleton depolymerization, this technique will shed light into the nature of the membrane micropatterning.

[1] J.-B. Masson et al, *Phys. Rev. Lett.***102**, 048103 (2009).

1592-Pos

Mechanisms Regulating the Diffusion of the Lipid Raft Marker Cholera Toxin B Subunit

Charles A. Day, Kimberly R. Drake, Minchul Kang, **Anne K. Kenworthy**.
Vanderbilt University, Nashville, TN, USA.

The B subunit of cholera toxin (CTXB) is generally accepted as a marker of lipid rafts. Compared to other raft markers or lipid-anchored proteins, CTXB exhibits relatively slow diffusion. A variety of mechanisms could potentially account for this slow diffusion of CTXB, including crosslinking of small raft domains, confinement by the actin cytoskeleton, association with caveolae, incorporation into actively maintained domains, or molecular crowding effects in response to elevated membrane protein density. We evaluated the role of each of these mechanisms in controlling the lateral diffusion of CTXB in the current study by employing fluorescence recovery after photobleaching (FRAP) of fluorescently labeled CTXB following actin depolymerization, ATP depletion, cholesterol depletion, labeling across a range of CTXB concentrations, or in caveolin-1 knockout MEFs. Of these conditions, only cholesterol depletion significantly altered the diffusional mobility of CTXB. Furthermore, we tested whether the slow diffusion of CTXB is an intrinsic property of its receptor by examining the effects of CTXB on the diffusion of a fluorescent GM1 analog. The results of this experiment showed that CTXB slows the diffusion of its receptor. However, binding of CTXB to cells did not affect the diffusion of another raft marker (YFP-GL-GPI), a non-raft marker (YFP-GT46), or a fluorescent lipid analog (DiIC16). Taken together, these data suggest that CTXB diffusion is not limited by actin corrals, caveolae, molecular crowding effects, or the intrinsically slow diffusion of GM1. In addition, they suggest that crosslinking of small rafts induced by CTXB binding does not substantially alter the dynamics of membrane domains enriched in other types of raft or non-raft proteins or lipids.

1593-Pos

Direct Observation of Hop Diffusion of Lipid and Protein Molecules in the Plasma Membrane by High-Speed Single Fluorescent-Molecule Imaging

Takahiro K. Fujiwara[1], Shinji Takeuchi[2], Yosuke Nagai[2], Kazuhide Hanaka[2], Kokoro Iwasawa[1], Kenichi G.N. Suzuki[1,3], Akihiro Kusumi[1].
[1]ICORP-JST, Institute for Integrated Cell-Material Sciences (iCeMS) and Institute for Frontier Medical Sciences, Kyoto University, Kyoto, Japan, [2]Photron Limited, Tokyo, Japan, [3]PRESTO-JST, Kyoto, Japan.

Previously, using single-particle tracking at a temporal resolution of 0.025 ms, employing a 40-nmΦ colloidal gold probe, we have shown that virtually all of the lipid and protein molecules incorporated in the plasma membrane undergo hop diffusion. Based on this and many other observations, we proposed a model in which the entire plasma membrane is parcelled up into apposed domains due to the presence of the actin-based membrane skeleton (fence) and its associated transmembrane proteins (pickets), and membrane molecules undergo short-term confined diffusion within a domain (compartment), and long-term hop diffusion between the compartments. However, due to technological limitations, the observation of hop diffusion was only possible with a 40-nmΦ-colloidal gold probe, which might artifactually induce hop diffusion. To circumvent this problem, here, we developed a new high-speed, high-sensitivity CMOS camera system, which allowed us to track single fluorescently (0.5-nmΦ)-labeled molecules at a temporal resolution of 0.1 ms, the fastest single fluorescent-molecule imaging ever made. This camera system gave the position determination accuracy for single fluorescent molecules of ≈35 nm at a 0.1-ms time resolution. Virtually, all molecules of a phospholipid (DOPE) and a transmembrane protein, transferrin receptor, were found to undergo hop diffusion over the 110-nm compartments with median residency times of 9 ms and 33 ms, respectively, in the plasma membrane of a human epithelial T24 cell line. Meanwhile, in the actin-depleted, blebbed membrane, all of the DOPE and transferrin receptor molecules exhibited simple-Brownian diffusion. These results are in an excellent agreement with the previous high-speed gold-particle tracking data, and clearly indicate the necessity for the paradigm shift for the plasma membrane structure and dynamics, from the single continuous fluid model to the partitioned fluid model.

1594-Pos

Cluster Size Formation and its Effect on Protein Sorting in the Immunological Synapse

Niña C. Hartman[1], Jeffrey A. Nye[1], Cheng-Han Yu[1], Wan-Chen Lin[1], Jay T. Groves[1,2].
[1]UC-Berkeley, Berkeley, CA, USA, [2]Howard Hughes Medical Institute, Berkeley, CA, USA.

Micron-scale assemblies of molecules is thematic in biology, although oftentimes their exact function and mechanism of formation are unknown. A hallmark example is the immunological synapse (IS). T cell detection of pathogenic invasion on an antigen-presenting cell leads to the arrangement of receptor-ligand pairs into well-defined concentric zones. Specifically, T cell receptors (TCR) bound to peptide-presenting major histocompatibility complex (MHC), occupy the central zone surrounded by a ring of leukocyte function associated antigen-1 (LFA-1) bound to intercellular adhesion molecule-1 (ICAM-1). We postulate that the differences in cluster sizes between large TCR:pMHC micro-clusters and small LFA-1:ICAM-1 complexes prior to centripetal actin transport determine their differential sorting. To study this, we increase the LFA-1 cluster size two additional degrees beyond its native state by crosslinking LFA-1 or ICAM-1 on the supported membrane with a bivalent or tetravalent crosslinker. Progressively more central localization of LFA-1 proportional to the degree of crosslinking results until LFA-1 occupies the central zone with TCR. The different clustering states are identified using fluorescence correlation spectroscopy (FCS). Furthermore, the addition of a costimulatory interaction also increases radial transport. Thus, we demonstrate that the well-regulated event of clustering is a critical parameter in determining spatial patterning in the IS. We propose a sorting mechanism based on frictional protein coupling to actin, which is consistent with our observations and may be generalized to all membrane proteins in the IS.

1595-Pos

Flow Induced Protein Reorganization on Cell Surfaces

Eric Stellamanns[1], Sravanti Uppaluri[1], Niko Heddergott[2], Markus Engstler[2], Thomas Pfohl[3,1].
[1]Max Planck Institute for Dynamics and Self-Organization, Göttingen, Germany, [2]Department of Zoology, University of Würzburg, Würzburg, Germany, [3]Department of Chemistry, University of Basel, Basel, Switzerland.

Living in an environment of continuous flow the unicellular human bloodstream parasite Trypanosoma brucei utilizes shear forces to remove hostile antibodies from its surface. African trypanosomes are spread via the bite of the Tsetse fly and cause the so called sleeping sickness in human and various diseases in animals as well. Their motility is driven by a single flagellum attached along the whole length of the cell and is essential for survival within the hostile immune system.

We emulate the bloodstream environment with PDMS microfluidic devices by means of flow velocity, shear gradients and confinement to examine the effect on trypanosome surface protein sorting and motility. Using highly fluorescent quantum dots (Qdots) to mimic antibodies and optical tweezers to control the cells, we are able to observe protein sorting in real time in order to quantify hydrodynamic drag driven protein reorganization in living cell membranes.

1596-Pos

Different Types of Lateral Diffusion Measurements Reveal that Unlike HA, Dc-SIGN is Immobilized in Microdomains

Michelle S. Itano[1], Aaron K. Neumann[1], Feng Zhang[2], Wolfgang J. Parak[2], Nancy L. Thompson[1], Ken Jacobson[1].
[1]University of North Carolina, Chapel Hill, NC, USA, [2]Philipps Universitat Marburg, Marburg, Germany.

Hemagglutinin (HA), from influenza virus, and DC-SIGN, a dendritic cell C-type lectin that binds many pathogens including viruses, bacteria, and fungi, form microdomains on the plasma membrane. We investigated the dynamics of these proteins using Scanning FCS (S-FCS), defined valency quantum dot-based Single Particle Tracking (SPT) and Fluorescence Recovery After Photobleaching (FRAP). Using FRAP we verified that HA has a large mobile fraction (~80%) that is characterized by a diffusion coefficient of ~0.09 um^2/sec and that it exchanges with the surround (Ellens et al. (1990) *Biochemistry,* **29**(41): 9697-9707). By contrast, neither DC-SIGN or Δ35-DC-SIGN, a DC-SIGN mutant missing the cytoplasmic tail, recovered after photobleaching, even after many minutes, indicating that these molecules do not exchange significantly with the surround. In order to determine the dynamics of molecules within the domains, we utilized a confocal line S-FCS method that permits the autocorrelation function between the same pixel in successive linescans (acquired in the form of a carpet or kymograph) to be calculated on the ms timescale. HA lateral motion within its microdomain is characterized by a diffusion coefficient of ~0.10 um^2/sec, similar to that measured outside the domain by FRAP. On the other hand, DC-SIGN and Δ35-DC-SIGN do not diffuse within the domain. We are currently using quantum dots that have been conjugated to a *single* streptavidin in order to examine lateral dynamics within these domains. Such nanoparticles will obviate a consistent interpretative limitation for gold and quantum dot SPT; namely, that particle valency cannot be specified with absolute certainty for the particle whose motion is tracked. Further characterization of these surprisingly stable DC-SIGN domains that are important for pathogen entry into dendritic cells is in progress.

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1597-Pos

Psychostimulants Affect Dopamine Transporter Lateral Mobility and Membrane Microdomain Distribution

Jeffrey S. Goodwin[1], Tina Patel[1], Anne K. Kenworthy[2], Habibeh Khoshbouei[1].
[1]Meharry Medical College, Nashville, TN, USA, [2]Vanderbilt University, Nashville, TN, USA.

Neurotransmitter reuptake by transporters is a major mechanism for terminating synaptic transmission. The human dopamine transporter (hDAT) is one of the main targets for psychostimulants, and is critical to DA homeostasis. Lipid rafts are specialized membrane microdomains that serve as organizing centers to regulate different cellular processes such as neurotransmission and trafficking. To begin to understand how psychostimulants, methamphetamine (METH) and amphetamine (AMPH), affect hDAT microdomain association, we utilized fluorescence recovery after photobleaching (FRAP) and density-gradient centrifugation. Our FRAP studies revealed significant changes in the rate (D) and extent (Mf) of the fluorescence recovery into the bleached region of the plasma membrane in cells expressing YFP-hDAT in the presence of METH but not AMPH. Substitution of five N-terminal Ser with Ala, (cannot be phosphorylated) or Asp (pseudo-phosphorylated), and removal of the 22 N-terminal amino acids restored the diffusion rate of the transporter to control levels. Using density-gradient centrifugation, we found that YFP-hDAT is distributed into both, classically defined, membrane raft and non-raft fractions. Incubating with METH and AMPH shifted YFP-hDAT from non-raft to raft fractions. We have previously shown that METH uniquely modulates the biophysical properties of DAT. Our present findings suggest that METH and AMPH cause hDAT to partition into lipid raft membrane microdomains, and the decrease in the hDAT diffusion rate evoked by METH- vs. AMPH-occupied DAT could suggest that the N-terminal domain of the transporter is associated with a distinct group of proteins when exposed to these psychostimulants, and may thus describe an underlying mechanism behind the addictive biological differences between these psychostimulants.