with virus-membrane contact involving a multivalent interaction that is highly sensitive to membrane organization and lipid mobility. Therefore, membrane sterol composition modulates influenza virus-receptor binding in a manner closely correlated to nanoscale organization and dynamics. The resulting changes to receptor sensitivity may be important in controlling permissiveness to infection.

1237-Pos Board B214
New In Vitro Hemagglutinin Inhibitor Screening System Based on Single Vesicle Fusion Assay
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Hemagglutinins of influenza virus plays a pivotal role in the infection to host mammalian cell so is coming up as druggable target like neuraminidases of this virus. However, the researches regarding to influenza virus should be strictly restricted within the facilities guaranteed over BioSafety Level 3 because of the potential threats of the contagion of influenza virus. In order to develop new hemagglutinin inhibitor screening system without the contagion of influenza virus, we introduced single vesicle fusion assay with recombinant hemagglutinin. In this study, we show that recombinant hemagglutinin can exert the membrane fusion in single vesicle fusion assay and this single vesicle fusion assay using recombinant hemagglutinin can be applied as an in vitro inhibitor screening system against hemagglutinin of influenza virus.

1238-Pos Board B215
Formation and Mechanical Properties of Calcium-Stabilized Membrane Rolls
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Cochleates are spirally curving, tightly packed membrane rolls of negatively charged phospholipid bilayers stabilized by calcium ions. Previous studies led to a detailed model of their geometry, however, little is known about their formation, internal dynamics and mechanical properties. The aim of our study was to follow the evolution of cochleates from unilamellar liposomes, to assess and model their mechanical characteristics, using phase contrast and atomic force microscopy (AFM) imaging and force spectroscopy.
When calcium was added to dioleoyl phosphatydilserine liposomes, they first precipitated and formed aggregated multilamellar vesicles. Cochleates appeared only minutes to hours later, and it took up to several weeks until they became the prevailing phase of the sample. Partially rolled particles were found occasionally but no intermediate species were identified. Mechanical loading of cochleates resulted in a multiphase force curve with isolated force saw teeth superimposed on the progressively growing force response. The force dropped to zero upon retracting the AFM tip, which led to a large hysteresis. This indicates both the deformation of the roll (spring constants: 1.4-15.7 N/m) and the successive penetration of AFM probe through mechanically isolated membrane layers (cca. 10 nN average breakthrough force/layer). The line tension of free membrane edges (3-9 nN) were about 2-3 orders of magnitude greater than that of fluid bilayers. Ultimate tensile strength of cochleates was estimated to be 2-9 MPa. Thus, in cochleates lipid layers are stabilized in solid phase by forming a mechanically resilient overall structure. The lack of mechanical coupling between individual bilayers may play a key role in the evolution of the rolled geometry.

1239-Pos Board B216
Single-Particle Tracking of HIV-1 Virions Bearing an Extra-Viral Fluorescent pH Sensor Reveals Viral Entry occurs after Trafficking to an Acidic Cellular Compartment
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The HIV-1 infectious cycle begins with viral glycoprotein mediated fusion with the host cell membrane and subsequent entry of the viral capsid to the host cytosol. It has been proposed that HIV-1 fusion and productive entry occur at the plasma membrane (PM) because functionality of the HIV-1 fusion glycoprotein (Env) is pH-independent. Further supporting this hypothesis are the observations that HIV-1 mediates fusion between adjacent cells and that cell-cell fusion occurs between Env- and receptor/co-receptor-bearing cells. However, it was recently demonstrated that, after Env engagement of receptor/co-receptor at the cell surface, the virus traffics via an endocytic pathway before fusing with the endosomal membrane. Though convincing in its conclusion, this study was unable to reliably detect viral fusion at the PM. Here, we implement a virus labeling strategy that biases towards detection of virus fusion that occurs in a pH-neutral environment, at the PM or, possibly, in early pH-neutral vesicles. Virus particles are co-labeled with an intra-viral content-marker released upon viral fusion and an extra-viral pH sensor that fully quenches upon trafficking to a mildly acidic compartment, precluding subsequent detection of viral content release. In imaging viruses bound to living cells, we found that HIV-1 content release in neutral-pH environment was a rare event with efficiency not significantly greater than the background content-loss that occurs due to non-specific increases in viral membrane permeability. Our finding implies that the majority of HIV-1 entry events occur after the virus particle traffics to an acidic cellular compartment. Interestingly, this labeling strategy also reveals that HIV-1 particles occasionally shuttle between neutral and acidic compartments, suggesting that the viral particles interact with a cellular recycling pathway. This work supported by NIH R01 GM054787.

1240-Pos Board B217
Dynamics and Energetics of SNARE Zippering in Membrane Fusion
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Membrane fusion is crucial for many physiological processes, including protein and membrane trafficking, hormone secretion and neurotransmitter release. An evolutionarily conserved 4-helix protein complex, the SNARE complex, plays a key role in mediating membrane fusion. Having one of its two transmembrane domains (TMDs) anchor to a vesicular membrane and the other to a target membrane, the SNARE complex exerts a force to join the two membranes by associating/zippering its TMDs together. In order to understand the molecular mechanism of how SNARE exerts force on the membranes, one needs to know the dynamic structure of SNARE in membrane fusion. We characterized the SNARE structure during the zippering process between two membranes, and calculated the free-energy landscape of the structural transition, by employing molecular dynamics in the form of a hybrid-resolution model with atomistic protein and coarse-grained lipid descriptions. In altogether ~ 1.2 ms simulations with umbrella sampling and replica exchange, all SNARE helices remained mostly helical. The SNARE linker domain that connects the two TMDs has a helix propensity of 61% although the domain helix is highly bent. Trp89 and Trp90 in SNARE VAMP2 were identified to be important in inducing membrane fusion. The two residues are located in the most bent region of the linker domain helix and interact strongly with membrane lipid tails. Along with stretching motions in the bent linker domain helix, these two residues bring lipid tails to the membrane surface and, thus, enhance lipid-lipid interactions between the two membranes and facilitate membrane stalk formation. The whole SNARE zippering process provides 32 kcal/mol free energy, which is sufficient to overcome the membrane fusion barrier.

1241-Pos Board B218
Effects of Sterol Substitution on Influenza Viral Membrane Fusion
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Influenza virus membranes are enriched in cholesterol in comparison to the plasma membrane, as cholesterol can comprise as much as 50% of viral envelope lipids. This enrichment in sterol content likely plays an adaptive role for the virus. Prior work has shown that influenza viral fusion rates vary with cholesterol concentration of both viral and target membranes. To understand which cholesterol properties affect influenza fusion, we measured fusion kinetics between viral and liposomal membranes where cholesterol was replaced with different sterols. Influenza fusion rates are surprisingly invariant across different sterols in the target membrane (all tested at the same mol %) and across a fairly broad range of sterols in the viral membrane. Two sterols–lanosterol and 25-hydroxycholesterol—did slow fusion moderately when introduced into the viral membrane, while substitution of cholesterol with polar sterols greatly slowed fusion. No other sterols tested showed a significant perturbation in fusion rates, including ones altering membrane phase behavior. Our findings suggest that the rate-limiting step in influenza viral fusion may be sensitive to membrane bending moduli but does not depend strongly on sterol-mediated changes to liquid-liquid phase coexistence in membranes.

1242-Pos Board B219
Membrane Fusion via Snare Mimetics Spatially Confined to Intramembrane Domains
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Membrane fusion is an essential step for many cellular functions. Moreover, cells must be able to confine specific fusion events to certain organelles. Therefore, it is of great interest to demonstrate and study spatially targeted membrane fusion events in vitro. In eukaryotic cells, processes such as neurotransmission, exocytosis and viral infection require membrane fusion and are mediated by rather complex SNARE proteins. Fusion has also been observed using SNARE mimetics such as DNA-lipid conjugates [Chan et al., Biointerphases, 3:FA17, 2008] and lipidated peptides [Pähler et al., Biophysical Journal, 103:2295, 2012]. Here, we demonstrate the fusion of large unilamellar vesicles (LUVs) to a specific location on giant unilamellar vesicles (GUVs). As fusion mediators we use coiled-coil peptide heterodimers or hybridized DNA, both of which are conjugated to lipid anchors. These molecules are spatially confined to phase domains within the GUV membranes. We observe liquid-ordered-specific partitioning for the DNA-lipid conjugate as well as liquid-disordered-specific docking and hemi-fusion events using lipid anchored peptides. This work is pursued within the MaxSynBio consortium which is jointly funded by the Federal Ministry of Education and Research of Germany and the Max Planck Society.

1243-Pos Board B220

SNARE-Mediated Transient Fusion of Liposomes to Supported Bilayers Probed by Two-Color pTIRFM

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In membrane fusion opening of a fusion pore establishes the first connection between two compartments. During neurotransmitter or hormone release via exocytosis, fusion pores can transiently open and close repeatedly (flicker) before resealing ('kiss-and-run') or dilating irreversibly (full fusion). Pore dynamics regulate the amount and size of released cargo and determine the mode of vesicle recycling, yet what governs pore dynamics is poorly understood. We established an assay to monitor membrane fusion by polarized total internal reflection (pTIRF) microscopy with single molecule sensitivity and ~15 ms time resolution in a biochemically well-defined system. In our assay, fusion of small unilamellar vesicles containing v-SNARE proteins (v-SUVs) fuse to a flat bilayer reconstituted with cognate t-SNAREs, supported on a soft polymer cushion (t-SBL). Exploiting the signal enhancement when a label transfers from the SUV to the SBL membrane in pTIRF, we could monitor the release kinetics of vesicular lipid labels into the SBL upon fusion. We found release times were much longer than would be expected for permanently open pores. Our sensitivity to single fluorescent lipids allowed us to calculate the lipid diffusivity and SUV size for each fusion event. This was used in a model we developed assuming the retardation was due to flickering pores, to estimate pore openness P0, the fraction of time a pore remains open during fusion. Increasing cholesterol levels increased P0. However, retardation of lipid release could also be due to steric hindrance of lipid diffusion through the walls of the fusion pore by SNARE proteins. To exclude this, we encapsulated soluble markers into the SUVs and simultaneously monitored lipid and contents release. Release of both lipid and soluble cargo started simultaneously. Interestingly, many pores resealed after partially releasing their contents.

1244-Pos Board B221

Using Single-Virion Fusion Assay to Study Hemifusion Kinectics of Influenza a Viruses and Influenza Pseudotypes

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Influenza A viruses are membrane-enveloped viruses and infect host cells via clathrin-mediated endocytosis. In late endosome, pH drop triggers conformational change of influenza hemagglutinin (HA) result in the fusion of viral and endosomal membrane and further form a pore that allows the viral RNAs to be released into the cytosol. Neuraminidases (NA) are enzymes that cleave sialic acid groups from glycoproteins during viruses egress. Typically, to study entry of highly pathogenic strains of influenza like avian flu and H5N1, viral pseudotyping systems are employed, whereby the fusion proteins are expressed on the backbone of a low pathogenic virus such as vesicular stomatitis virus (VSV). However, few studies have been carried out which compare the entry kinetics of native viruses and their pseudotyped analogs. In this study, we exploit influenza viruses since they require both HA and NA to become infectious. We produced various VSV pseudotypes containing H3 of influenza virus (A/Aichi/68-X31(H3N2)) and N2 from influenza virus (A/X31(H3N2)), influenza virus (A/Japan/305/57) or influenza virus (A/chicken/Korea/MS96/96). We performed single-virion fusion assay to determine the fusion kinetics using the total internal reflective fluorescence microscopy. Results illustrate that matching pseudoparticles H3N2 (X31/X31) behaved similar to native influenza X31. However, pseudoparticles H3 (X31), pseudoparicles H3N2 (X31/Japan) and pseudoparticles H3N2 (X31/MS96) fuse significantly slower than native influenza X:31 at all pHs. The quantitative western blot was then used to determine HA density on viral membranes of pseudoparicles. As expected, HA density of matching pseudoparticle H3N2 (X31/X31) is the highest among four samples. Immunofluorescence assay (IFA) was used to clarify the HA trafficking and expression in HA/NA-transfected cells. The results indicate native HA/NA pair is required for successfully producing effective pseudovirus. The study provides better insight on the evolution of influenza virus.

1245-Pos Board B222

Using Giant Plasma Membrane Vesicles from Cells to Form Supported Lipid Bilayers

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Previous studies have established chemical vesiculation and osmotic stress methods to obtain giant plasma membrane vesicles (GPMVs) from cells. The GPMVs are the blebs directly from the cell plasma membrane and contain most of the biological components in the plasma membrane including the lipids and the embedded membrane proteins. Being able to use GPMVs to form supported lipid bilayers can allow numerous biosensing applications. However, GPMVs do not easily rupture on conventional supports to form supported lipid bilayers because of their high protein and cholesterol contents. Here, we demonstrate the possibility to use air-water interface to break GPMVs to form supported lipid bilayers. We constructed micro-grating structured features on the support to control the air-water interface in a way so that the interface can rupture the GPMVs but does not peel off the deposited GPMV patches. The method provides us a way to form supported lipid bilayers with native plasma membrane proteins. Incorporating the built platforms with some surface analytical tools could allow us to study membrane proteins in their native environment.

1246-Pos Board B223

Structural Analysis of Hemagglutinin-Induced Hemifusion by Volta Phase-Plate Cryo-Electron Tomography

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Enveloped viruses feature metastable fusion proteins, which upon their conformational change transfer configurative energy into membrane fusion. To overcome the initial and substantial energetic barrier due to hydration repulsion of phospholipids, membrane fusion is thought to proceed through a low energy intermediate called hemifusion; yet for viral fusion the hemifusion structure has never been characterized. Variations of membrane curvature theory predict hemifusion diaphragms of varying dimensions. We study the hemifusion intermediate catalyzed by wild-type and G1S hemifusion mutant influenza hemagglutinin (HA) incorporated into virus-like particles (VLP) using Volta phase-plate cryo-electron tomography. We found ''Y'' shaped lipidic junctions with liposomal membrane inserted into the VLP membrane as suggested previously (Lee K. K., EMBO, 2010). Lipidic junctions and hemifusion diaphragms (HD) were typical membrane structures in both mutated and wild-type HA mediated fusion. Dimensions of HD and lipidic junctions ranged widely with their variance controlled by the rigidity imposed by the matrix layer, in agreement with our continuum mechanics model. We observed HA in close proximity to free membrane edges of ruptured liposomal membrane and lipidic junctions, suggesting that HA is capable of rupture, stabilization and resealing of membrane compartments in a step-wise manner permitting HD formation by