Real-time intermembrane force measurements and imaging of lipid domain morphology during hemifusion

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Membrane fusion is the core process in membrane trafficking and is essential for cellular transport of proteins and other biomacromolecules. During protein-mediated membrane fusion, membrane proteins are often excluded from the membrane–membrane contact, indicating that local structural transformations in lipid domains play a major role. However, the rearrangements of lipid domains during fusion have not been thoroughly examined. Here using a newly developed Fluorescence Surface Forces Apparatus (FL-SFA), migration of liquid-disordered clusters and depletion of liquid-ordered domains at the membrane–membrane contact are imaged in real time during hemifusion of model lipid membranes, together with simultaneous force–distance and lipid membrane thickness measurements. The load and contact time-dependent hemifusion results show that the domain rearrangements decrease the energy barrier to fusion, illustrating the significance of dynamic domain transformations in membrane fusion processes. Importantly, the FL-SFA can unambiguously correlate interaction forces and in situ imaging in many dynamic interfacial systems.
Lipid domains are clusters or two-dimensional aggregates of lipids whose molecular composition differs from the surrounding membrane. One commonly observed lipid domain, the sphingolipid and cholesterol (CHOL)-enriched domain, plays important roles in many biological membrane fusion processes. Lipid domains are associated with protein-binding sites during exo- and endocytosis, which are essential for transport of protein and vesicle cargo. In addition, ion channels for electrical signal transduction are localized in lipid domains. In extracellular processes, lipid domains are known to act as viral gateways or pathogen-binding sites in diseases such as Alzheimer’s, bovine spongiform encephalopathy (also known as ‘mad cow disease’) and HIV-1.

Previous studies on combined lipid and protein systems show that lipid domains localize SNARE proteins, and the formation of lipid domain/SNARE complexes is essential for lowering the energy barrier to fusion. Other studies on myogenic cells show that lipid domains dynamically cluster and disperse during different stages of fusion, contributing to cell adhesion and plasma membrane union. Furthermore, during and disperse during different stages of fusion, contributing to cell adhesion and plasma membrane union.

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**Results**

**Lipid domain visualization in the FL-SFA.** Figure 2a shows the schematic of the bilayer substrates used for the experiments. Briefly, asymmetric lipid bilayers were deposited on freshly cleaved mica surfaces using Langmuir–Blodgett (LB) deposition (see Methods). 1,2-dipalmitoyl-sn-glycero-3-phosphoethanolamine (DPPE) was deposited on mica as a supporting first monolayer (Supplementary Fig. 1a). As a second layer, a 1:1:1 mixture of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), Brain sphingomyelin (BSM) and CHOL, with a trace amount (1 wt%) of Texas Red 1,2-Dihexadecanoyl-sn-Glycero-3-Phosphoethanolamine, Triethylammonium Salt (TR-DHPE), was deposited on the DPPE monolayer (Supplementary Fig. 1b), and readily forms lipid domain structures (Supplementary Fig. 1c).

Using the FL-SFA, bilayers containing lipid domains were imaged inside the SFA (Fig. 2 and Supplementary Fig. 2). When the bilayers are positioned far apart (mica–mica separation distance D > 500 nm), only the upper bilayer is visible (Fig. 2b) because of the lower bilayer is out of focus. The bright regions indicate the lipid-ordered phase (Ld) of lipid bilayers, conventionally referred to as lipid domains, which are rich in BSM and CHOL. The bright regions, where TR-DHPE is selectively localized, are in the lipid-disordered phase (Lo) of lipid bilayers and rich in DOPC.

Noncircular and large domains are observed, while others observed circular and smaller domains in similar systems. The irregular domain shapes observed here are primarily because of the presence of calcium ions in the subphase during the LB deposition, which are known to bind strongly to the bilayer, induce phase separation, presumably make larger and irregularly shaped domains, and also lower the energy barrier to membrane fusion.

**Force and thickness measurements between hemifusing bilayers.** Interaction forces (F/R) between the bilayers were measured as a function of separation distance (D) with simultaneous fluorescence imaging (Fig. 3). Three distinct force runs (FRs) were performed where the bilayers were brought into contact under low compression (F/R = 8 mN m⁻¹) and then separated after a contact time (t): (i) FR1: t = 0 min, (ii) FR2: t = 19 h and (iii) FR3: t = 0 min, but at a previously hemifused contact region.

The force curve (FR1) shows no hysteresis between approach and separation, and a steric (hard) wall thickness (D) similar to the thickness, T, of two bilayers (T = 2D). The approach run of FR2 is similar to FR1 with the same steric wall thickness; however, during 19 h of contact, slow hemifusion of the bilayers is observed. The thickness of two bilayers (T = 2D = 8.7 nm) decreases down to one bilayer thickness (T = D = 4.4 nm) over time (Fig. 3b). The thickness decrease was fitted with an exponential decay equation: T = C₀ + C₁ exp(-t/τ), where C₀ and C₁ are constants and exhibit two different regimes (Fig. 3b). In the first regime (t < 200 min), the thickness decreases with a characteristic time, τ, of 56 ± 12 min (± values are the s.d. of at least three different replicates), while the second regime (t > 200 min) has
\[ \tau = 510 \pm 100 \text{ min.} \] The first regime is governed by the compression and thinning of the outer monolayer, while the second regime is likely related to the hydrophobic interaction and hemifusion of the lipid bilayers.

The approach curve (FR2) was fitted to a previously developed interaction potential between two bilayers\(^{13}\), which includes electrostatic, Van der Waals and hydrophobic interaction potentials (Supplementary Fig. 3). Comparison of the theoretical model with previous work (see Supplementary Note 1) indicates that bilayer thinning and hydrophobic interactions lead to fusion\(^{13}\). Separation after slow hemifusion of the bilayers leads to an adhesion force of \( F_{ad}/R = -24 \pm 3 \text{ mNm}^{-1} \), which can be converted to adhesion energy using the Johnson–Kendall–Roberts (JKR) model\(^{24,25}\), \( W_{ad} = F_{ad}/1.5\pi R = -5.0 \pm 0.7 \text{ mJm}^{-2} \).

The interleaflet hydrophobic attraction energy is much smaller compared with the expected value for fully hydrophobic surfaces \(-100 \text{ mJm}^{-2} \) (ref. 26), which is because of segregation of curvature-favouring lipids (that is, DOPC) at the boundaries of the stalks, as discussed later. After FR2, a third FR on the same contact revealed that the steric hard wall was shifted down from 8.7 to 5.6 nm, slightly larger than the thickness of a single bilayer. However, during separation, no adhesion force was measured, indicating that lipid molecules partially mended the damaged bilayers.

After FR3, a high compression \((F/R = 1,150 \text{ mNm}^{-1})\) experiment was performed (Supplementary Fig. 4) with \( t_c = 23 \text{ h} \). High compression induces fast hemifusion, which was completed in 1 h (from Fig. 4g to j). Immediately after compression, the central,
Figure 2 | Schematic of the experimental set-up and obtained lipid domain images. (a) Two asymmetric bilayers deposited on mica surfaces using LB deposition technique and (b–d) images of lipid domains obtained with FL-SFA. (b) Lipid domains of only upper bilayer, (c) lipid domains in both bilayers and (d) outlined and shaded lipid domains.

Lipid domain rearrangements during hemifusion. From the initial membrane–membrane contact to the complete hemifusion of lipid bilayers, significant reorganization of lipid domains (Lₜₜ) was observed as displayed in Figs 3c and 4f,i,l. Both low and high compressions display similar domain reorganization behaviour, although the timescale of hemifusion is different. The reorganization of lipid domains (Lₜₜ) during high compression was investigated in detail (see Fig. 5, Supplementary Movies 2 and 3), and can be summarized as follows: (1) first, the Lₜₜ phase is depleted from the contact (in at least one bilayer), rapidly diffusing out and forming a dark (Lₜₜ–Lₒ) rim surrounding the bright (Lₜₜ–Lₜₜ) and grey (Lₜₜ–L₋ₜₜ) contact and also slowly disappearing by lipid molecules mixing with the Lₜₜ phase. The average Lₒ phase disappearance rate was ~100 µm² min⁻¹ (see Supplementary Fig. 6a) after applying a constant load (L = 23 mN) and at tₛ = 14 min, the Lₒ phase was fully depleted from the contact in at least one bilayer. (2) The hemifusion of two lipid bilayers initiates near the centre of the contact, where the stress is highest and the two Lₜₜ phases (Lₜₜ–L₋ₜₜ, which has the lowest-energy barrier for fusion) were in contact. The hemifused region reveals itself as a dark spot inside the contact, which is surrounded by a bright (Lₜₜ–L₋ₜₜ) rim. (3) The hemifused area propagates and grows logarithmically with tₛ (see Supplementary Fig. 6b) to the size of the initial contact (or even slightly larger because of higher adhesion), which results in completely hemifused bilayers. The final image shows the dark ellipsoidal (or circular) contact with a bright (L₋ₜₜ–L₋ₜₜ) rim surrounding it.

Discussion
Under low compression, hemifusion took almost 19 h to complete, while under high compression the bilayers hemifused in 2.5 h (1 h for the previously fused bilayers, Fig. 4; and 2.5 h for the pristine bilayers Fig. 5 and Supplementary Movies 2 and 3). The dynamics of domain rearrangements contribute to slow hemifusion. Localization of the L₋ₜₜ phase at the contact lowers the energy barrier for hemifusion because of a larger area per molecule exposing more hydrophobic groups. The rate of the Lₒ phase depletion is proportional to the applied load. Nevertheless, the hemifusion processes here are much slower than in vivo during membrane trafficking that takes milliseconds to minutes. The difference in the timescale of fusion originates from the differences in the energy barrier to fusion, which is significantly affected by the membrane curvature diameter (centimetres versus tens of nanometres), the mobility of lipids (supported versus free-standing), and the temperature (room temperature versus body temperature).
imaged in real time during hemifusion. The migration of the Ld
stabilizes the energetically unfavourable stalk edge.

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Figure 3 | Low compression FL-SFA results. (a) Force–distance (F–D) curve measured between two bilayers performed under low compression (F/R = 8 mN m⁻¹). The error value is the s.d. from the repeats of at least four experiments performed with different bilayers and contacts. (b) The bilayer thickness change, during slow hemifusion of two bilayers during a contact time of 1,140 min and (c) lipid domain reorganization during hemifusion.

The Ld phase, which forms within 2.5 h at the edge of the stalk, as indicated by the bright rim in Fig. 5, is stable (or at least metastable) for more than 12 h, so long as the bilayers are kept under pressure in the hemifused state, that is, not detached from each other. If the bright rim observed after the hemifusion was just a pile-up of lipids (which includes dye-containing lipids), the thick pile-up would be easily observable as a deformation of fringes of equal chromatic order (FECO). However, the FECO show no noticeable deformation (Fig. 4j); thus, we conclude that the bright rim is indeed a selective localization of the Ld phase. Previous studies on lipid membranes have shown that BSM-rich membranes (L0) have a higher bending rigidity compared with the DOPC-rich membranes (Ld). In order to lower the bending energy, the Ld phase is enriched in high-curvature membrane regions, as observed by the formation of the bright rim around the edge of the contact region (Fig. 5). When the hemifused bilayers are separated and relaxed, the bright Ld phase rim becomes delocalized and disappears (Supplementary Fig. 7), providing further evidence that the bright Ld phase rim stabilizes the energetically unfavourable stalk edge.

Here using the FL-SFA, domain reorganization has been imaged in real time during hemifusion. The migration of the Ld region to the edge of the contact zone, combined with the small measured values for Wad, shows that the domains (L0) rearrange into their lowest-energy state during fusion. The fusion rate (and rate of rearrangement of the domains) is much faster at larger applied pressures, suggesting that the extra energy input into the system activates faster mixing of the leaflets. These results highlight the role and molecular mechanisms of lipid domains (L0) during hemifusion of model membranes, indicating that domains can rearrange to decrease the energy barrier and increase the rate of fusion in membrane processes.

The use of FL-SFA can be extended further to monitor dynamic transformations in systems where lipid domains are likely or known to occur (including pathological biological membranes) and gather previously unobtainable fundamental insights. In addition to model membrane systems, the FL-SFA has a wide range of potential applications for studying dynamic rearrangements/adsorption and forces of various interacting/noninteracting materials during and after confinement. These materials could include surfactant mono- and bilayers, biomolecules, colloidal particles, nanoparticles, polymers and smart materials. In these natural and engineered systems, close proximities and dynamic changes often occur, which can now be studied in greater detail using the FL-SFA.

Methods

Materials. Lipids used in this study that were purchased from Avanti Polar lipids (Alabaster, AL) were as follows: DPPE (16:0, Powder), DOPC (18:1, Chloroform), BSM (predominant 18:0, Porcine, Chloroform) and CHOL (ovine wool, ≥98%). For the fluorescence imaging, Texas Red 1,2-Dihexadecanoyl-sn-Glycero-3-Phosphoethanolamine, TR-DHPE was purchased from Invitrogen (Carlsbad, CA). DPPE was dissolved in a solvent, which is a 3:1 (vol/vol) mixture of chloroform...
High compression (fast hemifusion)

Figure 4 | Optical and FL imaging of compressed bilayers in the SFA. Simultaneous monitoring of the FECO (a,d,g,i), normal optical microscope showing Newton’s rings (c) and lipid domain localization (f,l) using FL-SFA before and during the contact time of FR4 (see Fig. 3b), and their schematics (b,e,h,k). (a-c) Bare mica-mica contact; (d-f) two bilayers before FR2; (g-i) hemifused bilayers right after high compression; and (j-l) hemifused bilayers at \( t_c \sim 70\) min.

Figure 5 | Fluorescence image of a contact (Sample no. 2, contact no. 1 from Supplementary Fig 2) as a function of time, immediately after high compression \( (F/R = 1,150\, \text{mN m}^{-1})\).
FL-SFA. A standard SFA2000 system (Surflorce LLC, Santa Barbara) was modified in order to enable simultaneous fluorescence imaging with the force–distance profiling. The two most critical modifications were (i) replacing the reflective layer of silver with a hard quartz wave plate coating to allow for wavelength-specific reflective and transmissive regions (see below and Supplementary Fig. 8) and (ii) modifying the optical paths to allow for the necessary filters and mirrors for fluorescence imaging. Figure 1 shows the FL-SFA set-up where the fluorescence light is illuminated from above and the white light for force–distance profiling is from below.

Substrate preparation. Atomically smooth mica surfaces of thickness 2–4μm were freshly cleaved in a laminar flow hood and immediately attached to a large and flat fluoropolymer film (Teflon) sheet of mica for storage, which prevents the mica surfaces from contamination. The back side of the mica surfaces was coated with a quartz wave plate using the Ion Beam Deposition technique. Alternating layers of 1 wt% of TR-DHPE was added to the mixture for imaging purposes. All lipids were purchased from Sigma-Aldrich (St Louis, MO), mixed and dissolved in Milli-Q water (Millipore, Billerica, MA) at final concentrations of 100 mM Sodium nitrate (Reagent Plus, purity ≥ 99.0%), 10 mM Tris(hydroxymethyl)aminomethane (ACs reagent, purity ≥ 99.8%) and 2 mM Calcium nitrate tetrahydrate (purity ≥ 99.0%) at a pH of 7.5.

FL-SFA experiments. The FRs were performed statically using a fine control motorized micrometre, with step sizes of 2–3 nm and equilibrium time of 5–10 s at each point. During the high compression experiment, after approaching surfaces to an F/R value of 8 nN m–1 with a fine control motorized micrometre, a medium control micrometre was used to compress the surfaces even further (30 nm, which corresponds to F/R = 1,150 nN m–2). During the separation after high compression, the medium control micrometre was used to separate the surfaces and measure adhesion force. FRs were performed in the order as mentioned in the main text, followed by four or more repeat experiments (see Supplementary Figs 2, 4 and 5) with different bilayers and/or contacts. Fluorescence imaging was performed simultaneously with FRs, especially focusing on the focusing on the images before compression, right after compression, during hemifusion and after separation. During the waiting time (under compression), FECO and fluorescence images are continuously monitored (when drastic fast changes in the bilayer images are observed). Figure 5, Supplementary Movies 2 and 3, intermittently imaged every 30–60 min (when slow changes are observed). Optimized fluorescence images required 5–10 s of exposure time. During the time Between imaging, the mercury light for fluorescence imaging and white light for FECO imaging were blocked to protect the fluorophore from photobleaching. All experiments were performed at room temperature.

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Author contributions
D.W.L. and K.K. designed the research. D.W.L., K.K., S.H.D. and N.C. performed the experiments. D.W.L., S.H.D. and N.C. analysed the data. D.W.L., K.K., X.B. and J.N.I. designed the FL-SFA. D.W.L., K.K., S.H.D. and N.C. wrote the paper. D.W.L. and J.N.I. supervised the project. All authors participated in the discussion of the data and in production of the final version of the manuscript.

Additional information
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