Supporting Information

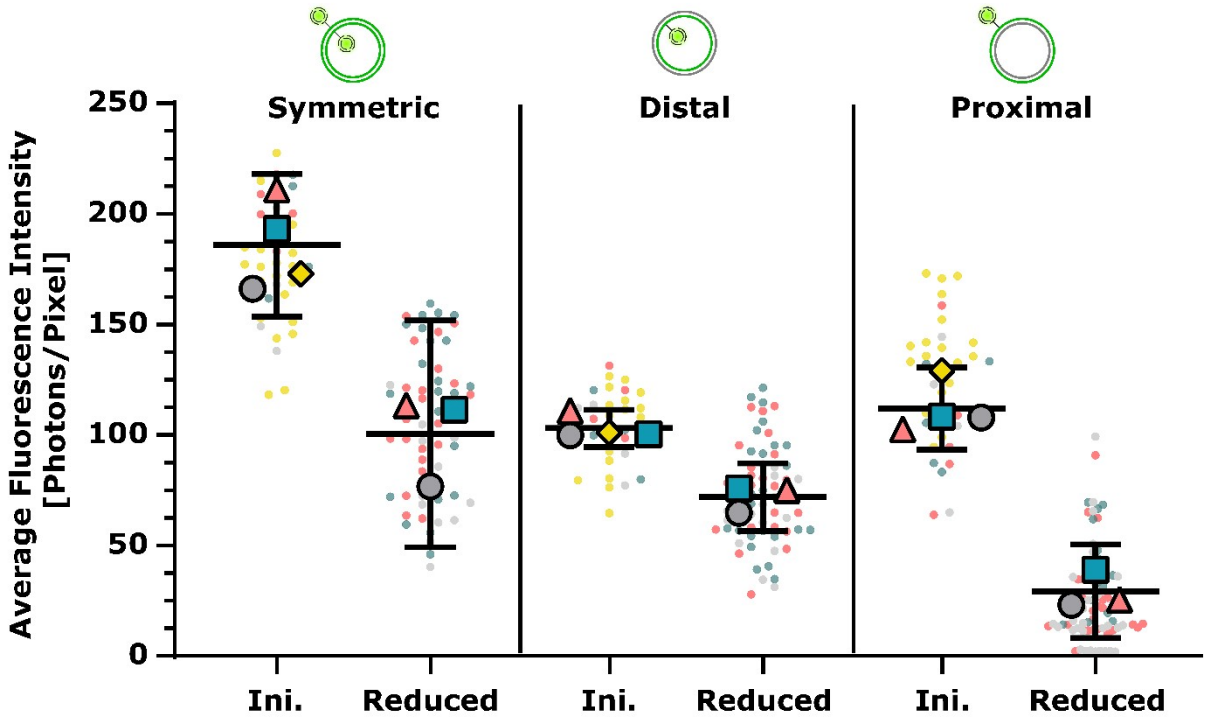
Estimation of red blood cell's monolayers spontaneous curvatures

Lipid Type	ζ_i [nm^{-1}]	Inner	Outer
PC	-0.031	21.43	43.14
SM	-0.134	2.21	48.87
PS	0.007	26.81	1.73
PE	-0.375	39.51	0.93
Others	0	10.04	5.33

Table S1 - Estimation of $\bar{\zeta}$ (Eq. 4) and $\Delta\zeta$. (Eq. 3, here defined as $\Delta\zeta = \frac{1}{2}(\bar{\zeta}_{out} - \bar{\zeta}_{in})$) based on the lipidomic data of the plasma membrane of red blood cells published in J. Lorent et al. (Nature Chemical Biology, 2020, Figure 1). The table represents the phospholipid mole fraction. Since the lipidomic data contain hundreds of different lipid species, most of which have unknown intrinsic curvature, ζ_i, the lipids are grouped based on their head group type: The intrinsic curvature of PC is the averaged value of DOPC (-0.1 nm^{-1}), DPPC (0.068 nm^{-1}), DSPS (-0.1 nm^{-1}) and POPC (-0.022 nm^{-1}) and SOPC (-0.01 nm^{-1}). Similarly, the intrinsic curvature of PE is the average of DOPE (-0.399 nm^{-1}) and POPE (-0.316 nm^{-1}). The intrinsic curvature of SM and PS is even less well-studied. Therefore, the curvatures of eggSM (-0.134 nm^{-1}) and DOPS (0.007 nm^{-1}) are used. The cholesterol mole fraction is 40% in each monolayer. Its intrinsic curvature is taken as -0.42 nm^{-1}. All the lipid intrinsic curvature values are taken from Kollmitzer et al. (Soft Matter, 2013), except that of DOPS taken from Kooijman et al. (Biochemistry, 2005). The 'Others' lipid type subgroup represents all the other phospholipids components whose head groups are not PC, SM, PS, or PE, and species with abundance lower than 0.1%. The monolayer spontaneous curvature is assumed to be the mean of its constituting lipids' intrinsic curvatures

$$\zeta_{sm} = \sum_{i=1}^{N} \zeta_i \phi_i .$$

N is the number of lipid species in the monolayer, ζ_i and ϕ_i are the intrinsic curvature (Fig. 1A) and the area fraction of the *i*'th lipid component, respectively.



Validation of membrane asymmetry

Figure S1 – Membrane asymmetry validation with NBD reduction protocol. The average fluorescence intensity of GUVs is shown prior ('Ini.') and after ('Reduced') incubation with 5 mM of dithionite. NBD (7-Nitro-2,1,3-benzoxadiazol-4-yl) labelled monolayers have a composition of 98% DOPC and 2% NBD-DSPE (1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-(7-nitro-2-1,3-benzoxadiazol-4-yl)), and non-fluorescent monolayers in asymmetric conditions are pure DOPC. Under the symmetric condition, the average fluorescence decreased by 45.8% from 185.8 ± 32.4 [photons/pixel] to 100.5 ± 51.2 [photons/pixel]. In the case of asymmetric membranes with NBD in the distal monolayer, the fluorescence decreased from 103.0 ± 8.36 [photons/pixel] to 72.0 ± 15.2 [photons/pixel], resulting in a 30.1% reduction of the total fluorescence, while in the opposite asymmetry condition, the decrease was from 112.0 ± 18.6 [photons/pixel] to 29.3 ± 21.2 [photons/pixel] -a 73.9% reduction. Based on the fluorescence reduction ratio, the interleaflet mixing is ~28% for the NBD-DSPE lipid. In the graph, small color-coded dots represent a single measurement, and large symbols

of the same color show the average of the experiment. The error bars correspond to 5%-95% confidence intervals (n=4 for the initial condition and n = 3 for the reduced condition).

Experimental Materials and Methods

Asymmetric GUV formation

1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-dioleoyl-sn-glycero-3-phospho-L-serine (DOPS), 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP), 1-myristoyl-2-hydroxy-sn-glycero-3-phosphocholine (LPC), Rhodamine-DOPE (Rh-PE), and NBD-DSPE (NBD-PE) were purchased from Avanti Polar Lipids.

The lipid chloroform stock solution was mixed in HPLC grade chloroform (Sigma) and diluted with sigma light mineral oil for molecular biology (Sigma) for the proximal monolayer or with Roth light mineral oil (Carl Roth) for the distal monolayer to a final concentration of 0.4 mM. The chloroform-oil mix was inserted into an oven at 80 ℃ for 1 hour to evaporate excess chloroform and kept under mild vacuum for up to 16 hours. Glucose and sucrose buffers of the same osmolarity were prepared (20 mM HEPES, 150 mM NaCl, ~380 mM Glucose or ~370mM Sucrose, 7.4 pH, 700 mOsmol) and filtered through a 0.45 µm filter (Ahlstrom). The water-oil interface was formed in a 0.5 ml centrifuge tube by sequential insertion of 200 µL of the glucose buffer and 100 µL of the Sigma lipid stock. The tube was left at 4 ℃ overnight, followed by centrifugation at 400 g for 15 minutes to ensure the formation of a lipid layer in the interface. The upper emulsion layer was formed by inserting a sucrose buffer droplet into the Roth oil stock in a 1:50 volume ratio. The emulsion was formed by tapping and vortexing the test tube with consequent incubation at 50 ℃ for 30 minutes. Next, 75 µL of the emulsion solution was gently added to the water-oil interface tube and centrifuged at 1,200 g for 8 minutes.

The oil layer was carefully pipetted out, and the GUVs were collected from the bottom of the tube for consequent experiments. The GUVs were used on the same day within 6 hours from formation.

Bead coating

DOPC and DOTAP were mixed at a final lipid concentration of 0.25 mM in HPLC-grade chloroform. The solvent was evaporated under an argon stream, followed by desiccation under a mild vacuum overnight. Next, the lipids were rehydrated with 1 mL of glucose buffer (same composition and osmolarity as in the GUV formation method). Liposomes were produced by five freeze-thaw cycles; the lipid test tube was held in liquid nitrogen for 30 seconds, followed by thawing at 65 ℃ [1]. Polystyrene nonporous particles (beads; Spherotech Inc.) of 3.15 μm diameter were suspended in glucose buffer and washed through 3 cycles of vortexing followed by centrifugation for 5 min at 1200 g. Washed beads were introduced into a 600 μL liposome dispersion and continuously mixed on a rotator overnight to form a continuous lipid bilayer of the beads [2]. Next, beads were washed three times with a glucose buffer to remove any free liposomes.

Optical Tweezers (OT) and Confocal Fluorescence Microscopy

The experiments were performed using a C-trap confocal fluorescence optical tweezers setup (Lumicks) made of an inverted microscope based on a water-immersion objective (NA 1.2) with a condenser top lens. The optical trap is generated by a 10 W 1064 nm laser. The displacement of the optically trapped beads from the center was measured and converted into a force signal by back-focal-plane interferometry of the condenser lens with a position-sensitive detector. The samples were illuminated by a bright field 850 nm LED and imaged in transmission onto a metal-oxide-semiconductor camera (CMOS).

The C-trap includes three fiber-coupled excitation lasers with 488 nm, 561 nm, and 638 nm wavelengths. Scanning is performed using a fast tip/tilt piezo mirror. For confocal detection, the emitted fluorescence was descanned, separated from the excitation by a dichroic mirror, and filtered using emission filters (blue: 500–550 nm; green: 575–625 nm; red: 650–750 nm). Photons were counted by using a fiber-coupled single photon counting module. The multimode fibers serve as pinholes, providing background rejection.

Experimental Chamber and Micropipette Aspiration

PDMS walls were placed on a glass slide (0.13–0.17 mm; BAR-NAOR Ltd.) and mounted onto an automated XY-stage. GUVs and coated beads were added into the

chamber containing glucose buffer. The 488 nm and 561 nm lasers were used for confocal imaging to excite NBD-PE and RH-PE (respectively), with emission detected in three channels (blue, green, and red).

A micropipette aspiration setup, including a micromanipulator (Sensapex) holding a capillary of 5 μm diameter (BioMedical Instruments) connected to a pump (EZ-25; Fluigent), was integrated into our optical tweezers instrument. By controlling the aspiration pressure, membrane tension on the GUV was modified according to [3]

$$\gamma_{asp} = \frac{\Delta P \cdot R_{pip}}{2(1 - \frac{R_{pip}}{R_{Ve}})}.$$

γ_{asp} is the aspiration tension, ΔP is the micropipette suction pressure, R_{Ve} is the vesicle radius, and R_{pip} is the micropipette radius. Before each experiment, the zero-suction pressure was found by aspirating a bead into the pipette and reducing the suction pressure until the bead stopped moving.

Lipid mixing assay

GUVs with membrane composition of 90% DOPC, 8% DOPS, 1% LPC, and 1% RhPE were formed under three symmetry conditions: 1% of the LPC in the distal monolayer, 1% in the proximal monolayer, and symmetric condition with 0.5% LPC in both monolayers. The GUVs were captured with a micropipette, and their tension was adjusted to 0.041 ± 0.014 mN/m, ensuring that the aspirated tongue is stable, and its length exceeds the radius of the micropipette. Next, an optically trapped bead was brought into contact with the GUV, and the fluorescence profile was monitored. The lipid mixing time delay was determined from the time stamp difference between the contact point and the first frame in which the fluorescence intensity increases on the bead (2.8 seconds per frame).

Asymmetry validation with NBD reduction assay

Asymmetric GUVs of 98% DOPC and 2% NBD-PE membrane composition were formed with the method described above. The NBD-PE was either localized in the proximal or distal monolayer. Following their formation, the vesicles were introduced

into the experimental chamber containing glucose buffer and fluorescence images of the vesicles prior to any treatment were taken. Next, freshly prepared dithionite (DTI, Sigma) in Tris-HCl buffer (1 M, pH 10) was added into the solution to a final concentration of 5 mM (20-fold dilution). After 4 minutes of incubation, additional fluorescence images of freely floating and non-deformed GUVs were taken. The imaging time did not exceed 15 minutes after the addition of DTI to minimize the effect of DTI permeation of the membrane.

At this condition, the assay primarily induces NBD-DSPE reduction on the proximal monolayer [4, 5]. As a result, this allows a quantitative verification of the NBD asymmetry on the membrane and validation of the GUV formation protocol.

Experimental Data Analysis

Data analysis was carried out using Bluelake, a commercial software by Lumicks. The software stores experimental data acquired during the experiments in HDF5 file format, which can be processed by using Lumicks' Pylake python package. Images of the confocal scans were reconstituted from the photon count per pixel data. All data analysis was performed with custom-written Python scripts.

References

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