Supplementary Information for

Active emulsions in living cell membranes driven by contractile stresses and transbilayer coupling

Suvrajit Saha, Amit Das, Chandrima Patra, Anupama Ambika Anilkumar, Parijat Sil, Satyajit Mayor and Madan Rao

Corresponding Author(s): mayor@ncbs.res.in & madan@ncbs.res.in

This PDF file includes:

- Supplementary text
- Figs. S1 to S6 (not allowed for Brief Reports)
- SI References
1. Simulation Methods

A. Details of the System. A minimal description of the nano-to-meso scale organization of lipids and GPI-AP in the multi-component asymmetric bilayer, driven by active contractile stresses and transbilayer coupling requires at least 5-components. Below we provide all the necessary details of the system.

A.1. The Components. We represent the components of a minimal asymmetric bilayer by an index $\alpha = 1, \ldots, 5$ (Fig. S2A)

1. GPI-AP in the upper-leaflet,
2. $lo$-component in the upper-leaflet, collectively comprising SM, cholesterol, ...,
3. PS in the lower-leaflet, together with cholesterol and other long-chain saturated lipids such as PIP2 (other $lo$ component in Fig. S2A),
4. $ld$-component in the upper leaflet, collectively comprising short chain, unsaturated lipids.
5. $ld$-component in the lower leaflet, collectively comprising short chain, unsaturated lipids.
6. transmembrane protein TMABD straddling both leaflets,

A.2. Lateral and Trans-bilayer Interactions of the Components. In our coarse-grained description, we represent the molecules of all components as spheres, that reside either on the upper or lower leaflet of the bilayer membrane, or straddling the two leaflets (partitioned according to Rules 1-6 above). The two leaflets of the bilayer are treated as two parallel 2-dimensional planes, separated by a distance $\sigma$, and lateral dimension $L \times L$. The total number of molecules in the two planes $N$, is high enough so that there are no large empty spaces in either plane. Periodic boundary conditions are applied on both X and Y directions.

Components that reside on the same leaflet of the bilayer membrane interact via a Lenard-Jones potential of the form,

$$V(X_1, X_2) = \infty, \text{ if } r_{ij}^{\alpha\beta} \leq \sigma_{\alpha\beta}$$

$$= -J_{\alpha\beta} \left( \frac{\sigma_{\alpha\beta}}{r_{ij}} \right)^{12}, \text{ if } r_{ij}^{\alpha\beta} > \sigma_{\alpha\beta}$$

[1] where $i$-th molecule of components $\alpha$ interacts with the $j$-th molecule of component $\beta$, with an attractive interaction strength $J_{\alpha\beta}$ expressed in units of thermal energy $k_B T$ ($k_B$ is the Boltzmann constant and $T$ is the absolute temperature). Here, $\sigma_{\alpha\beta}$ represents the hard-core length scale and $r_{ij}^{\alpha\beta} = |X_{i}^{\alpha} - X_{j}^{\beta}|$ is the distance between the two molecules. For simplicity, we take $\sigma_{\alpha\beta} = \sigma$, for all $\alpha, \beta$. Note that with the above ranking of the components, the only cross-term that is nonzero is $J_{12} > 0$, the rest are 0.

Components that reside on separate leaflets of the bilayer membrane do not have any steric interaction, and only interact via an attractive Lenard-Jones potential,

$$V_2(X_1, X_2) = -J_{\alpha\beta}^{12} \left( \frac{\sigma_{\alpha\beta}}{r_{ij}} \right)^{12}$$

[2] For simplicity, we take $\sigma_{\alpha\beta} = \sigma$, for all $\alpha, \beta$. With the above ranking of the components, only $J_{13}^{12} = J_{23}^{12} > 0$, the rest are 0.

The TMABD being transmembrane has just steric interactions with components on either leaflet.

A.3. Active Contractile Stresses. The second main ingredient is the contractile stresses applied by the cortical actomyosin adjoining the lower leaflet of the bilayer on the membrane components that bind to it - these are the lower leaflet PS ($\alpha = 6$) and the transbilayer protein TMABD ($\alpha = 6$), with binding affinities given by $K_{PB}$ and $K_{TB}$, respectively. These stresses are applied over a spatial region characterized by the correlation length scale $\xi$ of the active stress event. The events are considered stochastic with statistics given by a Poisson birth-death process with lifetime $t$ distributed exponentially: $\exp[-t/\tau]$ (1–3).

The regions are denoted as $\{\Omega_{x_n}\}$, with radius $\xi$. The contractile stresses give rise to centripetal forces applied radially towards the centre $x_n$, $\alpha = 1, \ldots, n$, i.e.

$$\Sigma(x, t) = -\sum_{n} \hat{r}_{x_n}, \text{ if } x \in \Omega_{x_n}$$

$$= 0, \text{ if } x \notin \Omega_{x_n}$$

[3] where $\hat{r}_{x_n}$ is the unit radial vector from the centre of the domain $\Omega_{x_n}$. We work in an ensemble where the number of such active stress events $n$ are held fixed (alternatively, we can specify the birth-death rates which fixes the mean $\langle n \rangle$). The coverage of nonzero active stress is $A = n\pi\xi^2/L^2$. One such region is illustrated in Fig. S2B.
B. Kinetic Monte-Carlo simulation. We have already described that we solve the Master equation for the time dependence of the probability distribution of the system through a Kinetic Monte-Carlo (MC) approach. The simulation method depends on updates of both positions of the molecules and the positions of the centres of the non-zero stress events. The MC moves on the molecules comprise both diffusive moves and active moves inside the regions of non-zero active stress. The configuration in each plane is updated by attempting an exchange of the positions of any chosen pair of neighbouring particles, \( \{X_i^\alpha\} \), \( \{X_i^\beta\} \). These position exchanges are determined both by equilibrium pair-potentials (Eqs. 1, 2) and by forces arising from the local contractile stress if the particles (PS and TMABD) are in the region \( \{\Omega_{\alpha}\} \) (Eq. 3). For numerical convenience, the membrane components on the two planes are restricted to lie on a square lattice with lattice spacing \( \sigma \) (the square lattice in the two planes are in complete registry). Every lattice site on the upper plane is occupied by one of the components \( \alpha = 1, 2, 4 \), similarly every lattice site on the lower plane is occupied by one of \( \alpha = 3, 5 \). The transmembrane protein \( \alpha = 6 \), when included, straddles both the planes.

The details of the transition rates associated with the exchange moves are described below and in Fig. S2B. Note that one time step of this kinetic Monte-Carlo scheme, \( \Delta t \) is given by \( N \) attempts at position exchanges (\( N \) is the total number of particles in the two planes). We choose \( \Delta t \), so that \( \sigma^2/(4\Delta t) \) is the typical diffusion coefficient of a protein on the membrane.

B.1. Equilibrium exchange moves. Choosing a neighbouring particle pair in the same plane - \( i \) (of component \( \alpha \)) and \( j \) (of component \( \beta \)) - with uniform probability, we attempt an exchange with a probability,

\[
\omega = 1, \quad \text{if } \Delta \xi \leq 0 \\
= e^{-\Delta \xi/k_B T}, \quad \text{if } \Delta \xi > 0
\]

where \( \Delta \xi \) is the difference in energy before and after the exchange, and is obtained from the inter-particle potentials Eqs. 1, 2. This is the usual Metropolis transition probability and obeys detailed balance (4).

B.2. Active advection moves. This applies only to PS (\( \alpha = 3 \)) and TMABD (\( \alpha = 6 \)) when included. When these particles are within the contractile regions \( \Omega_{\alpha} \) and bound to it with affinities, \( K_{PS} \) and \( K_{TM} \), respectively, they move preferentially towards the centre \( x_a \) with a radial velocity proportional to \( T_0 \) (Eq. 3), by attempting a series of biased exchange moves. This detailed balance violating move is shown in Fig. S2B - for details of the implementation see (3).

C. Choice of Parameters and simulation units.

C.1. Simulation parameters. In the simulations we vary the parameters: (i) total coverage of the contractile regions where active stress is nonzero, \( A \); (ii) life time of the active stress events \( \tau \); (iii) binding affinities, \( K_{PS} \) and \( K_{TM} \); and (iv) transbilayer coupling tuned via \( \beta_{2x} \). The rest of the parameters of the model, such as overall composition of the components and temperature \( T \), are held fixed. The qualitative results we obtain are independent of these parameters across a large range. In our simulations, the upper-leaflet is populated with 11.1% concentration of GPI-AP and rest by equal amounts of \( lo \) and \( ld \) components. In the lower leaflet, we have 22.2% occupied by PS and other \( lo \) components and the rest by the \( ld \) component. The TMABD concentration, when present, is kept fixed at 11.1%. This composition approximates the observed composition in a typical patch of membrane in wild-type CHO cells (5).

C.2. Units of energy, length and time. In our simulations, the interaction energy scale between \( lo \) components sets the scale of energy. In these units, the simulations are done at \( T = 1.05T_c \) (i.e. \( T > T_c \)) where \( T_c \) is the critical phase segregation temperature of the \( lo - ld \) mixture (3). This temperature was chosen because it corresponds to 310 K \( \approx 37^\circ \)C, the physiological temperature of the cell. We set \( \beta_{2x} = 0.5k_B T \) so that PS does not phase segregate in the lower leaflet. In addition, we keep the mutual attraction of GPI-AP and TMABD’s small with \( J_{23} = J_{66} = 0.15k_B T \) to avoid any spontaneous clustering. We emphasize that our choice of relative concentration and temperature is not unique and the results hold for a wide range of relative concentrations and temperatures above \( T_c \) (6).

To express the results of our simulations in physical units, we note every length scale in our simulation in units of \( \sigma = 10\text{nm} \), a typical molecular scale. This gives us the spatial scale of contractile stress \( \xi = 40 \text{nm} \) and the lateral size of each bilayer patch \( L = 600 \text{nm} \). We fix the unit of time in our simulations, using a molecular diffusion coefficient of \( D \approx 1 \mu\text{m}^2/\text{s} \) (7). This corresponds to a Monte-Carlo timestep \( \Delta t = 0.025 \text{ms} \) which is also the time taken by any molecule to travel a distance \( \sigma \) at the cell surface. In these units, a typical simulation run of \( 10^6 \) MC steps corresponds to \( 25 \text{s} \). The local order parameter \( \phi \) is calculated on a two-dimensional grid with spacing 0.03 \mu m overlaid on the bilayer patch. The simulations are performed with a fixed value of the active radial velocity, implemented as an attempt probability, \( \Sigma_0 = 0.8 \) (illustrated in Fig. S2b). Majority of our simulations with activity are performed with \( A = 30\% \) and \( \tau = 1600 \text{MC steps} \approx 0.04 \text{s} \), unless mentioned otherwise.

2. Details of Experimental Methods

A. Plasmids, cell culture and labeling. The following constructs were used (with references): 1) Human GPI-anchored Folate Receptor (FR-GPI) described in detail earlier (8). 2) EGFP-GPI, a chimeric model GPI-AP, where the EGFP was fused to the GPI signal from FR-GPI (9). 3) TMABD/ABD*, a chimeric model transmembrane actin (/ABD* non) binding probe, where the transmembrane form of the folate receptor is fused to a cytosolic actin-binding domain from Ezrin (c-terminal Ezrin ABD) or its mutant non-binding counterpart (2). 4) LactC2-Ez-YFP, a fusion construct of LactC2 domain with the actin-filament binding domain of Ezrin and YFP (10).
Chinese hamster Ovary (CHO) cell lines stably expressing either GPI-anchored GFP (EGFP-GPI, GPI signal from FR-GPI), Folate receptor (FR-GPI) and transmembrane (FR-TMABD/+) constructs were maintained in Ham’s F12 media (Hi Media, India) supplemented with 10% fetal bovine serum (FBS, Gibco, USA). PSA3 cell line for phosphatidylserine (PS) perturbation experiments were maintained in Ham’s F12 medium in the presence of 10mM ethanolamine and 10% FBS. Cells expressing GFP-tagged membrane proteins were treated with 75 µg/ml of cycloheximide for 3 hrs at 37°C to clear Golgi associated fluorescence before imaging (11). FR-GPI and FR-TMABD expressing cells were cultured in folic-acid free Ham’s F-12 and supplemented with 10% dialyzed FBS to allow efficient labeling with fluorescence analogs of folic acid. Na-pteroyl-Ne-BodipyTMR-L-lysine (PLB-TMR) was used to label the cell surface at saturating concentration (~400 nM) on ice for 1 hr or for 5 mins at 37°C. Transient transfections reported here were carried out ~12–16 hours before preparing them for the experiments. The cells were transferred to pre-warmed buffers (150 mM NaCl, 20 mM HEPES, 5 mM KCl, 1 mM CaCl2, 1 mM MgCl2, pH 7.2-7.4 also referred to as Buffer M1; supplement with 2mg/ml glucose, M1-Glc) before placing on a temperature-controlled microscope stage and imaged within 30 min (7, 12).

B. Treatments and Perturbation. Cholesterol depletions were carried out by treating the cells with 10 mM MβCD at 37°C for 30 mins, followed by exogenous labeling of fluorescent probes. Cells were depleted of sphingomyelin by growing cells in culture media containing 40 µg/ml of Fumonisin B1 (FB1) for 72 hrs at usual culture conditions leading to 40-50% reduction in sphingolipids levels (13). PS depletion (PS- condition) was carried out by growing PSA3 cells in media devoid of ethanolamine (using 10% dialysed FBS) for 36-48 hrs (10). PS levels were restored by growing them in cell culture media with normal serum (PS+ condition). Perturbation of formin activity was carried out by pre-treatment of cells with the formin inhibitor SMIFH2 at 25µM for 2hr at 37°C. Arp2/3 activity was perturbed by treating cells with CK-666 at 50µM for 2 hr at 37°C. Unless otherwise mentioned, inhibitors with reversible effects were maintained in the buffer during the imaging session.

C. Steady-state anisotropy measurements. Cells singly labeled with PLB-TMR or expressing EGFP-GPI or dually labeled with PLB-TMR and EGFP were imaged using a spinning disk confocal system (Andor Technologies, Belfast, Northern Ireland) custom adapted for fluorescence emission anisotropy (14). The labeled cells were excited with 488/561 nm laser illumination after which parallel and perpendicular polarized fluorescence emission was resolved by passing the emission tight band-pass emission filters, 500-550 nm (for imaging GFP) and 570-610 nm (for imaging PLB) followed by a nanowire polarizing beam splitter and collected using two EMCCD detectors. Dual color anisotropy imaging was carried out on the same setup by sequential imaging of cells to avoid spectral crosstalk and signal bleed-through. Image processing, analysis, and quantification were performed using Metamorph 7.0 (Molecular Devices Corporation, CA, USA), MATLAB (Mathworks, USA) and Image J (NIH, USA) as described earlier (14, 15).

D. Membrane ordering measurements.

D.1. Laurdan General Polarisation (GP). Laurdan (6-lauryl-2-dimethylamino-napthalene) is a polarity sensitive probe to estimate membrane ordering (or fluidity). Laurdan generalized polarization (GP), a ratiometric measurement based on the fluorescence intensity collected at the two spectral channels allows a quantitative estimation of the membrane order. Laurdan imaging and GP measurements was implemented on the confocal spinning disk anisotropy platform to monitor the protein clustering and membrane order at high spatial resolution. Imaging Laurdan on a confocal spinning disk also offer other unique advantages like comparatively lesser photobleaching and ability to measure GP close to the basal membrane plane and avoid contamination from internal pools of Laurdan. Co-imaging of Laurdan GP and anisotropy necessitated some modifications to existing protocols of Laurdan labeling and imaging (16). Laurdan dye stocks (10 nM) were prepared in DMSO and stored in an airtight and lightproof clean glass vial. CHO cells expressing FR-GPI and TMABD were plated on clean glass coverslip dishes, grown in presence of dialyzed serum (for folate analog binding), and grown for 2 days before imaging was done. The dishes were washed twice gently with M1-glucose buffer before the central well with cells was incubated with labeling mix containing both Laurdan (at 10 µM) and folate analog PLB (400 nM) at 37°C for 5 mins. This allows quick labeling of cell-surface and prevents the buildup of internal pools of Laurdan associated with labeling Laurdan at 37°C for 30 mins (the usual staining protocol for cells). Post-labeling, the dishes were washed thoroughly to reduce overall background fluorescence in images. Laurdan labeled samples were excited using 405 nm laser line and fluorescence was recorded at the two spectral channels 410-460 nm (Ch1) and 470-530 nm (Ch2). However, co-imaging of Laurdan along with anisotropy imaging requires placing the polarizing beam splitter at the secondary dichroic position. This splits up the emission into two orthogonal polarization components for both the channels. The total intensities for both Ch1 and Ch2 was obtained using similar image analysis procedure used for processing anisotropy images. The background intensities were estimated from M1-Glucose buffer imaged under similar imaging condition as the cells. Additionally, a dilute sample of Laurdan in DMSO (~1µM) was also imaged at similar conditions to determine the Laurdan g-factor as described earlier (16, 17). The Laurdan GP and g-factor was computed using the following formula

\[
GP = \frac{I_{Ch1} - G.I_{Ch2}}{I_{Ch1} + G.I_{Ch2}}
\]

where G is the g-factor calculated using,

\[
G = \frac{GP_{ref} + GP_{max} GP_{max} - GP_{ref}}{GP_{max} + GP_{ref} GP_{max} - GP_{ref}}
\]

109

110

111

112

113

114

115

116

117

118

119

120

121

122

123

124

125

126

127

128

129

130

131

132

133

134

135

136

137

138

139

140

141

142

143

144

145

146

147

148

149

150

151

152

153

154

155

156

157

158

159

160

161

4 of 13 Suvrajit Saha, Amit Das, Chandrima Patra, Anupama Ambika Anilkumar, Parijat Sil, Satyajit Mayor and Madan Rao
Here \( GP_{\text{max}} \) is the estimated GP value of the Laurdan in DMSO (the g-factor sample). \( GP_{\text{ref}} \) is a reference value for Laurdan GP which is conventionally fixed at 0.207 (16). The empirically estimated g-factor of 0.5 was uniformly applied across all experiments to calculate Laurdan GP and obtain spatial Laurdan GP maps of membrane order. Laurdan imaging was carried out at room temperature as at 37°C we saw a rapid equilibration of Laurdan to internal membrane pools and its loss from cell surface (Fig.S5 A,B). The internalization of Laurdan (Fig. S5 A,C and higher temperature (37°C compared to 22°C; Fig. S5 B,D) both led to lowering of the Laurdan GP values (and decrease in dynamic range) as reported earlier (18, 19) and verified independently by us (Fig. S5 A-D).

**D.2. NR12S Blue/Red Ratiometric Imaging.** The cells (untreated or post-perturbation) were labelled with 1 \( \mu \text{M} \) NR12S (20, 21) in M1-Glc for 5 mins at 37°C, washed gently and imaged in M1-Glc. Cells were imaged on a spinning disk confocal microscope with 100X objective (representative images in Fig. S6 A), illuminated with 561 nm excitation laser and emission output was collected using bandpass filters collecting light in the two spectral windows - 565-585 nm emission filter (blue emission) and 593-643 nm (red emission). The background intensities were estimated from M1-Glc buffer imaged under similar imaging condition as the cells and images were background corrected before computing and quantification of Blue/Red ratiometric images.

**E. Imaging and data analysis.**

**E.1. Quantification of domain abundance and size.** We have established a systematic and consistent approach in quantifying the features of mesoscale domains and extended the same to study mesoscale organisation of the different cell surface probes (in control and upon perturbations) reported here. Multiple (≥ 20) 6-10 \( \mu \text{m}^2 \) patches were selected from the anisotropy maps of probes (representative image in Fig. S3A) from multiple cells (~ 10 – 15) pooled across independent replicates were pooled to generate a typical pixel anisotropy distribution for each probe (or any given condition). Anisotropy thresholds (typically 1 standard deviation less than peak of the distribution; black line in Fig. S3A for FR-GPI) was set to binarize the maps to show only those pixels with anisotropies below this threshold (Fig. S3A). These pixels are highly enriched in nanoclusters and a criterion of contiguous set of pixels (≥ 5 pixels, 1 pixel: 100 nm or 0.01 \( \mu \text{m}^2 \)) was used to define individual mesoscopic domains. The same cut-off that was set for control condition was used for perturbation to compare both. The domains maps were quantified using ‘Analyze particles’ routines of ImageJ to extract the relative abundance (or domain area fraction) and average domain area (in pixel, area: 0.01 \( \mu \text{m}^2 \)) for each analysed patch of the membrane. The data was plotted as notched box-plot and statistically compared using appropriate parametric or non-parametric statistical tests.

**E.2. Analysis of domain size distribution.** We binarize the thresholded segregation order parameter matrix (simulation) and density profile matrix (experiment). The thresholds selected are shown in Supplementary Table 1. We use MATLAB function bwlabel to detect all connected objects (domain) and count the number of pixels in a given object which is denoted as the domain area \( s \). For the experimental data we have considered all objects with \( s \geq 3 \). This procedure is repeated for many simulated realizations of the segregation order parameter matrix or density profile matrix to generate the probability density function of domain area \( P(s) \).

Following analysis described in (3), we fit the domain area distribution to

\[
P(s) \sim s^{-\theta} \exp[-s/s_0],
\]

with power law exponent \( \theta \) that is exponentially cutoff at a scale \( s_0 \). Similar domain distributions have been used in other contexts of activity (22, 23). We fit the simulation/experimental \( P(s) \) to the above form using a custom MATLAB program to implement a Monte-Carlo method to minimize the residual sum of squares (24).

**E.3. Quantitative relation between clustering of (two probes) and with lipid order.** We used the following approach to analyze the mesoscale sorting behavior of probes. The two color spatial anisotropy maps of several (≥ 20) 6-10 \( \mu \text{m}^2 \) square membrane patches allowed us to manually identify and compute the anisotropy values of a very small patch of membrane (typically of similar size as the domains of the GPI-AP or TMABD probes). For most cases, we have integrated pixel anisotropies of 4 x 4 pixel ROI boxes (pixel size: 100 nm) to calculate the overall average anisotropy of the cluster-enriched and cluster-deficient hotspots on the cells. Anisotropy was computed from ROIs marking the cluster-enriched (hence low anisotropy) and cluster-sparse (high anisotropy) regions from the anisotropy maps obtained for one protein (say FR-GPI in the case of Fig.S4 A-C). The same sets of regions were then transferred to the complementary EGFP-GPI spatial anisotropy map from the same cell, and the anisotropy values of the domains were calculated. Several such ROIs (at least 500) were quantified for both proteins from multiple cells (n=15-20 cells across independent replicates) and the data was pooled to build a scatter plot to show the trend in the data and compute the Pearson’s correlation coefficient (\( R=0.55, p<0.01 \) between FR- and EGFP-GPI; Fig S3C) for entire dataset. Next we check the correlation coefficient obtained for each individual probe across two sequential frames of acquisition (indicated as T1 and T2). A high correlation coefficient (see GFP-GPI T1-T2 and FR-GPI T1-T2 in Fig. S4 D,E) indicates there is a significant preservation of domains between two sequential frames and a low correlation values observed between two probes (say GPI-AP and LactC2-ABD) in our experiments is not due to the dissolution of the mesoscopic domains over the timescale of imaging (~ 2sec). We translated the same approach to analyse the correlation between the values of anisotropy and laurdan GP of domains. First, cluster rich (and poor) domains were identified from the anisotropy maps of the membrane probe (say FR-GPI or TMABD) and the same pool of ROI were transferred to the corresponding Laurdan GP map of the same membrane patch and GP values were extracted (Fig. 4C, S5F). The data is displayed as a scatter plot of GP v/s anisotropy to qualitatively observe the trend and quantify the Pearson’s Correlation coefficient (with statistical significance).
Fig. S1. Lipid perturbations abrogate clustering of GPI-AP but not TMABD (Related to Figure 1). (A, B) Representative confocal spinning disk images shows the intensity (top grayscale image) and anisotropy maps (bottom color map image) of CHO cells expressing GFP-GPI (A) or FR-TMABD labelled with PLB (B) in untreated (Control), depleted of cholesterol (MBCD) or sphingolipids (Fumonisin B1; FB1). Quantification of these experiments are presented in Figure 1 F, G. LUT bar indicates the anisotropy scale. Scale bar: 10 µm.
Fig. S2. Kinetic Monte Carlo simulation model showing active segregation in a multicomponent, asymmetric bilayer. (a) Schematic of the multicomponent asymmetric bilayer showing the components. (b) Kinetic Monte-Carlo simulation moves: Left – equilibrium moves governed by Kawasaki exchange dynamics (Eq. 4). Right – Active moves on PS and TMABD components when they are within the contractile regions $\Omega_{xa}$ and bound to actomyosin with an affinity $K_{PS}$ and $K_{TM}$, respectively. The active moves result in preferentially advecting the particles towards the centre $x_a$ with a radial velocity proportional to $\Sigma_0$ (Eq. 3). These moves violate detailed balance. Details of implementation in (3). (c) Probability density functions of lo-ld segregation parameter $\phi$ with increasing $K_{PS}$ when TMABD is included in the simulation. We take $K_{TM} = 0.6$. (d) Spatial profile of number density of TMABD calculated following the same protocol as in Fig. 2b or 2e, when PS-actin interaction is switched off ($K_{PS} = 0$). Scale bar 0.1 $\mu$m. (e) Time averaged profiles in the presence of TMABD, of $\phi$ (top row), number densities of GPI-AP (second row), PS (third row) and TMABD (bottom row), at three different values of actin binding affinity of PS.
Fig. S3. Measuring spatial features of mesoscale domains from high-resolution anisotropy maps and the effect of perturbations (related to Figure 3). A) Analysis approach to measure area fraction and size distribution from anisotropy maps. To generate spatial maps of cluster rich regions ROIs sized 6 µm × 6 µm (left panel) taken from high resolution PLB-labeled FR-GPI anisotropy image, the pixel anisotropy distribution for the entire dataset (middle panel; from at least 30-40 patches derived from 10-20 cells) was used to set an anisotropy cut-off (mean - standard deviation; dashed line in plot). Anisotropy maps were thresholded to obtain binary domain maps (right panel) consisted only of pixels enriched in clusters. We next quantify the abundance (area fraction) and size distribution of individual domains at least 5 pixels in size (red circles on binary maps; > 5 pixel area; pixel = 0.01 um2). (B-D) Representative anisotropy and domain maps of GPI-AP proteins (GFP-GPI or PLB-labelled FR-GPI) from 6 µm × 6 µm boxes (B) show very similar characteristics of mesoscopic domain abundance (notched box plots in C; note GFP-GPI data used for comparison is the same as depicted in Figure 3A) and distribution of domain area (D); solid lines represent the corresponding fits to a model distribution, $A_0 s^{-\theta} \exp\left[-s/s_0\right]$. (E-G) Representative anisotropy and domains maps (E; 6 µm × 6 µm boxes) of PLB-labelled FR-TMABD in untreated (Control) or depleted of cholesterol (MBCD) or sphingomyelin (FB1) show that both domain abundance (notched box plots in F) and distribution of domain area (G) are insensitive to these perturbations. Fits to domain distribution yield a characteristic domain area of $0.1 \mu m^2$ which translates to a domain diameter of 360 nm for TMABD (Control, dashed line on plot G). (H-J) Representative anisotropy and domain maps (H; 10 µm × 10 µm boxes) of PLB-labelled FR-TMABD and actin binding mutant version, PLB-TMABD* show that loss of actin interaction capacity results in a significant decrease in domain area fraction (notched box plots in I, two-sample t-test $p < 0.001$) and a marked shift towards smaller domain sizes (J; dashed line on J shows characteristic domain diameter of $\sim 365$ nm). (K-M) Representative anisotropy and domain maps (K; 6 µm × 6 µm boxes) for PLB-labelled FR-TMABD in untreated (Control) or cells treated with inhibitors of formins (SMI; red) and Arp2/3 (CK666; blue) show that formin inhibition but not Arp2/3 led to significant drop in TMABD domain abundance (notched box plots in L; $p < 0.001$ by one-way ANOVA with Tukey means comparison) and a shift towards smaller domain sizes (M; dashed line on M indicates domain diameter of $\sim 375$ nm). The dataset presented here is pooled from multiple independent replicates (2-3) comprising of at-least n = 20-50 anisotropy patches collected from 10 – 20 cells for each condition.
**Fig. S4. Correlation of properties of mesoscopic regions (Related to Figure 4).** A-E) CHO cells stably expressing FR-GPI (and labeled with PLB; A, i) and transiently expressing GFP-GPI (A, ii) were imaged using a spinning disk confocal microscope to generate representative intensity and anisotropy maps of PLB-labeled FR-GPI (A i') and GFP-GPI (A ii'). The expression of two GPI-APs in the same membrane at comparable levels dilutes the nanoclusters of the individual proteins. To select the region of relative expression levels where the contribution of homo-FRET dilution is minimal, the anisotropy of PLB-labeled FR-GPI is plotted against GFP/PLB ratio (B; anisotropy +/- standard error of mean calculated from 650 ROIs from n=20 cells). The blue-band (B) outlines the regime where the two probes don't show homo-FRET dilution with expression levels of FR-GPI and GFP-GPI that allowed us to probe the colocalisation of cluster rich domains of GFP-GPI and FR-GPI. A $6\mu m \times 6\mu m$ patch from the anisotropy maps of FR-GPI (top) and EGFP-GPI (bottom) was outlined from the same cell (dashed box in A i' and ii'; right panel). Mean anisotropy values quantified from corresponding ROIs (4x4 pixels, dashed boxes) on both images were used to generate scatter plots of anisotropy values of PLB-labeled FR-GPI and GFP-GPI (C; 206 ROIs from n=8 cells). Scatter plot of anisotropy values from a similar analysis of two sequential frames (T1 and T2) of the same GPI-AP probe; PLB-labeled FR-GPI (D; 337 ROIs from n=10 cells) and GFP-GPI (E; 332 ROIs from n=10 cells) shows high Pearson’s correlation coefficient (on individual plots), indicating the preservation of domain maps over the timescale of the sequential multi-colour imaging routine (2-3 sec). F) YFP-LactC2-ABD, a synthetic linker construct that links PS (via the LacC2 domain) to cortical actin (via the ABD from Ezrin) was expressed in CHO cells and subject to photobleaching while collecting images for anisotropy imaging. Photobleaching led to a gradual increase in anisotropy (+/- SD) (data from n = 11 cells), consistent with YFP-LactC2-ABD exhibiting significant homo-FRET as a consequence of its nanoscale clustering. G) Table shows Pearson’s correlation coefficient values taken from scatter plot of anisotropy data from a anisotropy correlation analysis (analyzed as in D, E) of two sequential frames of YFP-LactC2-ABD or PLB-labeled FR-TMABD . Note these are also well correlated across two consecutive frames of imaging the same fluorescent molecule. (H, I) Representative TIRF images of fluorescence intensity and anisotropy (H) of cells expressing FR-TMABD (labeled with PLB; upper panel in H) and transiently transfected with YFP LactC2-ABD (lower panel in H). Identical $7\mu m \times 7\mu m$ sized patches (black boxes in H, middle panel) chosen from the anisotropy images of FR-TMABD and LactC2-Ez and shown as Analysis ROI (iii, iii'). Scatter plots (I) exhibit significant positive correlation (R = 0.28, p < 0.01) between the anisotropy of small mesoscale domain sized ROIs ($400 \times 400 \text{nm}$) drawn around cluster-rich and cluster-poor hotspots of FR-TMABD and LactC2-ABD (H; iii, ii', dashed boxes). Scatter plot (I) report on 600 ROIs collected from cells (n=21) pooled from two independent replicates. Scale bar for images (A, H) : 10$\mu m$. 

**Suhrajit Saha, Amit Das, Chandrima Patra, Anupama Ambika Anilkumar, Parijat Sil, Satyajit Mayor and Madan Rao**
Fig. S5. Membrane order measurements in living cells using Laurdan (Related to Figure 4). (A, B) Laurdan intensity and GP image (A) of same cell (also represented in Fig. 5B) at the basal surface (cell surface) and an internal plane (intracellular). Internal membrane pools show distinct Laurdan staining (A; intensity) but register significantly lower GP values (A; Laurdan GP map) as shown in the linescan of GP values (B) of the same ROI (red dashed-line) in both images. The dynamic ranges of Laurdan GP reported in Figures 5 clearly indicate that the measurements are specific to the cell-surface pool and hence faithfully report on cell membrane lipid ordering. (C, D) Laurdan GP distribution is extremely sensitive to temperature of labelling and measurement. At 22°C, Laurdan intensity images show more prominent cell membrane specific labeling (C i) but at 37°C, it is internalized rapidly and builds up intracellular pools (C ii). Moreover, the Laurdan GP values reduce drastically at higher temperatures (C ii') as quantified in box-plot (D); at least 250 ROIs were considered for each condition from n=10-12 cells. Hence, the Laurdan GP measurements were conducted at room temperature (22°C). (E-G) High-resolution confocal fluorescence and anisotropy images of CHO cells expressing FR-TMABD (labeled with PLB, E, top panel) and co-labelled with Laurdan (E, bottom panel, Intensity) to obtain Laurdan GP maps. Identical 6µmx6µm sized boxes (black squares) were chosen across the anisotropy and Laurdan GP map and shown on the right (Analysis ROI; E iii, iii', respectively). Scatter plot (F) of local Laurdan GP and anisotropy computed for small mesoscale domain (400 x 400 nm) from identical ROIs depicted in the Analysis ROI for PLB-labeled FR-TMABD (E ii, dashed boxes) and Laurdan GP (E iii', dashed boxes). Laurdan GP and anisotropy of PLB FR-TMABD show a small but significant positive correlation (Pearson’s R = 0.2, *p* < 0.01). Table (in G) shows Pearson’s correlation coefficients (R) obtained from scatter plots of anisotropy values of PLB-labeled FR-GPI-AP or PLB-labeled TMABD against their local Laurdan GP (rows, 1, 2). Note high degree of correlation of clustering of GPI (row 3), TMABD (row 4) and local lipid order (Laurdan GP; row 5) across two sequential frames. Individual scatter plot (F) report on at least 500 ROIs collected from cells (n=20 cells) pooled from two independent replicates. Scale bar for images (A,C & E) : 10µm.
Fig. S6. Steady-state lipid order of the outer-leaflet of cell surface probed by solvatochromic probe, NR12S (Related to Figure 5)  

A) Confocal images showing the intensity and Blue/Red Ratio images of CHO cells labelled with 1 µM NR12S in untreated (CON/control) condition or upon perturbations of formins (SMI), Arp2/3 (CK), actin polymerization (LAT) and cholesterol (MBCD). Steady-state outer-leaflet membrane order are reflected in the ratio changes observed in these images (quantified in B; scale bar : 10 µm).  

B) Box-plots of NR12S Blue/Red ratio for the different perturbation conditions. Perturbation of Formin (SMI) and Latrunculin (LatA) but not Arp2/3 (CK666) lead to loss of outer-leaflet membrane order. The difference between untreated (Control) and cholesterol depleted (MBCD) cells establish the dynamic range captured by NR12S in this assay. At-least 200 ROIs were considered for the analysis from 40-50 cells each condition across 2 independent experiments. Statistical significance of difference between control and each perturbation was determined by one-way ANOVA with Tukey mean comparison.
### Supplemental Table 1: Fit Parameters for Domain Size Distribution

| Experiment | Membrane Probe | Fitted Values ± errors | s0 (x 0.01 μm²) |
|------------|----------------|------------------------|-----------------|
| **Tuning f-actin affinity** | | | |
| F-actin binding | TM-ABD | 3.634 ± 0.020 | 2.105 ± 0.002 | 38.394 ± 0.931 |
| No F-actin binding | TM-ABD* | 5.181 ± 0.067 | 1.917 ± 0.008 | 7.305 ± 0.057 |
| **Lipid Perturbations** | | | |
| GPI-AP control | GPI | 4.264 ± 0.097 | 2.205 ± 0.003 | 40.412 ± 1.758 |
| GPI-AP Sphingomyelin Depletion | GPI (FB1) | 10.881 ± 0.426 | 2.380 ± 0.041 | 7.260 ± 0.654 |
| GPI-AP Cholesterol Depletion | GPI (MBCD) | 18.615 ± 1.357 | 2.430 ± 0.115 | 4.059 ± 0.552 |
| TM-ABD control | TM-ABD | 4.855 ± 0.024 | 2.287 ± 0.003 | 73.689 ± 3.130 |
| TM-ABD Sphingomyelin Depletion | TM-ABD (FB1) | 3.922 ± 0.020 | 2.106 ± 0.002 | 46.650 ± 2.298 |
| TM-ABD Cholesterol Depletion | TM-ABD (MBCD) | 4.142 ± 0.015 | 2.203 ± 0.001 | 93.551 ± 2.701 |
| **Formin Perturbations** | | | |
| GPI Control | GPI | 3.263 ± 0.021 | 2.004 ± 0.003 | 46.127 ± 2.950 |
| GPI Formin Inhibition | GPI (SMIFH2) | 8.657 ± 0.574 | 2.236 ± 0.073 | 7.175 ± 0.384 |
| TM-ABD control | TM-ABD | 3.395 ± 0.021 | 2.005 ± 0.002 | 37.611 ± 1.801 |
| TM-ABD Formin Inhibition | TM-ABD (SMIFH2) | 4.557 ± 0.282 | 2.017 ± 0.012 | 10.752 ± 0.106 |
| **Two GPI Comparison** | | | |
| GFP-GPI | 3.263 ± 0.021 | 2.205 ± 0.003 | 46.127 ± 2.950 |
| FR-GPI | 4.264 ± 0.097 | 2.004 ± 0.003 | 40.412 ± 1.758 |
| **Simulation** | Tunable Parameter | A0 | θ | s0 |
| | | | | |
| Tuning Binding Affinity of PS | K,PS 0.9 | 0.561 ± 0.001 | 1.602 ± 0.003 | 17.919 ± 0.049 |
| | | 0.3 | 0.700 ± 0.001 | 1.795 ± 0.003 | 11.876 ± 0.075 |
| | | 0.1 | 0.849 ± 0.001 | 1.995 ± 0.003 | 6.939 ± 0.033 |
| | | 0.001 | 1.126 ± 0.007 | 1.939 ± 0.010 | 2.905 ± 0.050 |
| Tuning Area Fraction of Aster | A* 0.07 | 0.799 ± 0.001 | 1.994 ± 0.003 | 9.896 ± 0.057 |
| | | 0.154 | 0.650 ± 0.001 | 1.599 ± 0.001 | 13.521 ± 0.437 |
| | | 0.238 | 0.568 ± 0.001 | 1.600 ± 0.001 | 18.289 ± 0.558 |
| | | 0.349 | 0.550 ± 0.001 | 1.593 ± 0.007 | 19.864 ± 0.042 |

Note: All distributions are generated by thresholding binary images for a domain size >= 3 pixels.
1. Chaudhuri A, Bhattacharya B, Gowrishankar K, Mayor S, Rao M (2011) Spatiotemporal regulation of chemical reactions by active cytoskeletal remodeling. *Proceedings of the National Academy of Sciences of the United States of America* 108(20):14825–14830.

2. Gowrishankar K, et al. (2012) Active remodeling of cortical actin regulates spatiotemporal organization of cell surface molecules. *Cell* 149(6):1353–1367.

3. Das A, Polley A, Rao M (2016) Phase segregation of passive advective particles in an active medium. *Physical Review Letters* 116(6):068306.

4. Landau DP, Binder K (2005) *A Guide to Monte Carlo Simulations in Statistical Physics*.

5. Lorent J, et al. (2020) Plasma membranes are asymmetric in lipid unsaturation, packing and protein shape. *Nature chemical biology* 16(6):644–652.

6. Bansal A, Das A, Rao M (2022) Active segregation dynamics in the living cell. *Indian Journal of Physics* pp. 1–10, doi:10.1007/s12648–022–02298–z.

7. Saha S, et al. (2015) Diffusion of gpi-anchored proteins is influenced by the activity of dynamic cortical actin. *Molecular biology of the cell* 26(22):4033–45.

8. Varma R, Mayor S (1998) Gpi-anchored proteins are organized in submicron domains at the cell surface. *Nature* 394(6695):798–801.

9. Sharma P, et al. (2004) Nanoscale organization of multiple gpi-anchored proteins in living cell membranes. *Cell* 116(4):577–89.

10. Raghupathy R, et al. (2015) Transbilayer lipid interactions mediate nanoclustering of lipid-anchored proteins. *Cell* 161(3):581–94.

11. Sabharanjak S, Sharma P, Parton RG, Mayor S (2002) Gpi-anchored proteins are delivered to recycling endosomes via a distinct cdc42-regulated clathrin-independent pinocytic pathway. *Developmental Cell* 2(4):411–423.

12. Lee IH, et al. (2015) Live cell plasma membranes do not exhibit a miscibility phase transition over a wide range of temperatures. *The Journal of Physical Chemistry B* 119(12):4450–4459.

13. Chatterjee S, Smith ER, Hanada K, Stevens VL, Mayor S (2001) Gpi anchoring leads to sphingolipid-dependent retention of endocytosed proteins in the recycling endosomal compartment. *EMBO J* 20(7):1583–1592.

14. Ghosh S, Saha S, Goswami D, Bilgrami S, Mayor S (2012) Dynamic imaging of homo-fret in live cells by fluorescence anisotropy microscopy. *Methods in enzymology* 505:291–327.

15. Saha S, Raghupathy R, Mayor S (2015) Homo-fret imaging highlights the nanoscale organization of cell surface molecules. *Methods in molecular biology (Clifton, N.J.)* 1251:151–73.

16. Owen DM, Rentero C, Magenau A, Abu-Siniyeh A, Gaus K (2012) Quantitative imaging of membrane lipid order in cells and organisms. *Nature Protocols* 7(1):24–35.

17. Gaus K, Zech T, Harder T (2006) Visualizing membrane microdomains by laurdan 2-photon microscopy. *Molecular membrane biology* 23(February):41–48.

18. Gaus K, et al. (2003) Visualizing lipid structure and raft domains in living cells with two-photon microscopy. *Proceedings of the National Academy of Sciences of the United States of America* 100(26):15554–9.

19. Jaureguiberry MS, et al. (2010) Membrane organization and regulation of cellular cholesterol homeostasis. *Journal of Membrane Biology* 234:183–194.

20. Iaea DB, Maxfield FR (2017) Membrane order in the plasma membrane and endocytic recycling compartment. *PLOS ONE* 12(11):e0188041.

21. Kucherak OA, et al. (2010) Switchable nile red-based probe for cholesterol and lipid order at the outer leaflet of biomembranes. *Journal of the American Chemical Society* 132(13):4907–16.

22. Truong Quang BA, Mani M, Markova O, Lecuit T, Lenne PF (2013) Principles of e-cadherin supramolecular organization in vivo. *Current Biology* 23(22):2197–2207.

23. Zhang HP, Be’er A, Florin EL, Swinney HL (2010) Collective motion and density fluctuations in bacterial colonies. *Proceedings of the National Academy of Sciences of the United States of America* 107(31):13626–30.

24. Draper NR, Smith H (1998) *Applied regression analysis*. (John Wiley & Sons) Vol. 326.

Suvarjit Saha, Amit Das, Chandrima Patra, Anupama Ambika Anilkumar, Parijat Sil, Satyajit Mayor and Madan Rao 13 of 13