Supplemental Materials

Molecular Biology of the Cell

Saha et al.
Supplemental Material

Diffusion of GPI-anchored proteins is influenced by the activity of dynamic cortical actin

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Here we provide a detailed discussion of the interpretation of our data regarding the temperature dependence of the diffusion of inert (e.g., short chain lipid probes) and passive molecules (e.g., GPI-anchored proteins and the transmembrane protein TM-ABD). We first discuss a general framework for an active composite cell surface, and then provide an explanation for the temperature-independent diffusion of passive molecules in small confocal volumes and the cross over to a temperature-dependent diffusion in large volumes.

General Framework

The active composite model (Gowrishankar et al., 2012; Rao and Mayor, 2014) regards the cell surface as a composite of the multi-component plasma membrane and the cortical actin configuration that it rests on. This cortical actin is composed of two species - one a static crosslinked meshwork and other a dynamic active fluid. The active cortical fluid consists of short, polar active filaments (of length \( \ell \)) driven by filament treadmiling and myosin motors whose activity is correlated over a time \( \tau \). The local composition (Gowrishankar 2012) dynamics and shape (Maitra et al., 2014) of the plasma membrane is affected by active currents and stresses generated by the contractility of actomyosin cortex. These currents and tangential stresses come from the coupling of dynamic actin filaments to the membrane; from active processes such as treadmiling of filaments and myosin motor driven motility. In what follows, we will see how active stresses and currents generated by the cortical actin fluid affects the tagged particle diffusion of passive molecules. A similar argument could be made when the active stresses arise from the myosin dependent deformations of the cortical actin mesh (see Figure 4 in Chaudhuri et al., 2011).
Diffusion of passive molecules in an active composite membrane

*Passive* cell-surface molecules like GPI-anchored proteins and TMABD, which can couple to cortical actin, can bind to these filaments with a binding/unbinding rate of $k^+/k^-$, with a mean duty ratio,

$$K_R = \frac{k^+C_R}{(k^+C_R + k^-)}$$

where $C_R$ is the mean concentration of actin filaments with the observation region $R$ (see Figure 8). The mean duty ratio $K_R$ characterizes the fraction of time spent by the tagged particle in the bound state over the FCS observation time within the observation region. Thus if $k^+C_R/k^- \gg 1$, which can be achieved by either having a high filament concentration or low unbinding rate, the duty ratio $K_R \rightarrow 1$, i.e., it is permanently bound. When the length of the filament $\ell$ is small as proposed by the active composite model, the tagged particle, though bound, will not be bound to the same filament. Instead, the molecule will switch between the bound and unbound state with a mean switching time of $t_{sw}$. The mean switching time describes the time taken to switch from a bound to an unbound state, and is large when $k^-$ is small. As noted above, when the filament concentration is high, the *passive* molecule hops from one filament to the next and so the effective unbinding rate $k^-$ is small. When bound to the filaments, they would be actively driven with an advection velocity ($V_a$) but they can switch to an unbound state and can diffuse randomly due to thermal kicks.

For a *passive* molecule switching between a bound and unbound state; the diffusion coefficient of the bound particle is given by the correlations of $V_a$, yielding an ‘active diffusion’ coefficient($D_a$). While in principle one expects a clear crossover behaviour from a purely ‘active diffusion’ when $K_R \approx 1$, to a thermal diffusion when $K_R = 0$, we must remember the diffusion behavior of *passive* molecule will probably be a combination of both, the amplitude of each component will depend strongly on the local concentration of the locally oriented polar filaments($\text{or } C_R$). The limit $K_R = 0$ has been discussed earlier - this occurs either when the particle does not interact with actin (inert particle) or when the concentration of actin $C_R$ is negligibly small - one simply obtains the thermal diffusion coefficient $D_t$. The limit $K_R \approx 1$, on the other hand, occurs when the filament concentration $C_R$ is high, which in the FCS experiments appeared to be the case for small confocal spot sizes $R$. As $R$ was increased beyond a scale $R_0$ over which the density of dynamic cortical actin falls appreciably, the value of $K_R$ would reduce considerably, leading to a crossover from purely ‘active diffusion’ to a diffusion which has significant contributions from thermal fluctuations.
When bound to filament or under conditions of $K_R \approx 1$, the active composite model predicts that passive molecules could be advected by both flows generated by active stress (due to acto-myosin contractility) and actively moving polar filaments. Actomyosin activity transduces about 20 $k_B T$ per ATP molecule, which is significantly larger than $k_B T$. To be consistent with our diffusion results on the passive molecules, GPI-anchored proteins and TM-ABD, it appears that the combination of parameters that enter into the ‘active diffusion’ $D_a$ should be weakly dependent on temperature to give rise to the observed temperature independent diffusion. This is not unreasonable, since $D_a$ can be written as a product of the active temperature and activity renormalized viscosity, both of which are related to active stress fluctuations, which are orders of magnitude larger than thermal fluctuations. Thus we expect that active fluctuations that drive passive molecule diffusion, will be relatively insensitive to changes in temperature within the physiological range.

Our earlier work on the dynamics of fragmentation-aggregation and movement of GPI-AP nanoclusters in CHO cells (Goswami et al., 2008), where we showed that the rates of fragmentation-aggregation, related to the parameters of actomyosin activity and active noise, and the diffusion of GPI-AP monomers was independent of temperature in the range 24-37°C. These arguments suggest that the actively driven diffusion coefficient may be independent of temperature in the physiological range. As the confocal spot size $R$ increases beyond a scale $R_0$, the density of dynamic cortical actin reduces and so does $K_R$ - thus the effective diffusion coefficient starts getting contributions from the temperature dependent diffusion coefficient $D_t$. This crossover would be interesting to study in some detail, and a detailed theoretical study is currently underway.

To summarize, the active composite model is consistent with our observed results for the diffusion of inert and passive molecules such as GPI-APs:

1. For small FCS spot sizes, the diffusion of passive molecules, is controlled by active actomyosin at the cell cortex and hence insensitive to temperature in the range 24-37°C. In contrast, the diffusion coefficient of inert molecules is Brownian and varies smoothly with temperature (Figure 1, 2).

2. Upon increasing the spot size, we see a crossover of the passive molecule diffusion to a temperature dependent behaviour (Figure 2).

3. Passive molecule diffusion is strongly affected when its interaction with cortical actin is reduced, since this reduces the binding rate $k^+$ of the passive molecules to cortical actin. In the case of GPI-APs, its interaction with cortical actin is mediated by
cholesterol, thus perturbations of cholesterol reduce the active contribution to diffusion (Figure 3). In the case of TM-ABD (see main text), the interaction with cortical actin is directly via the actin-binding domain; thus mutations of this into a non-binding counterpart also reduce the active contribution to diffusion (Figure 5). In both these cases, the diffusion coefficient reverts to being temperature dependent.

4. Passive molecule diffusion are strongly affected by perturbations of dynamic cortical actin, which reduces $C_R$ and hence $K_R$ (either by pharmacological treatments or by inducing blebs). This leads to a reduction in the relative contribution due to activity and the diffusion coefficient reverts to being temperature dependent (Figure 4, 6).

5. Passive molecule diffusion is strongly affected by reduction in actomyosin activity, which reduces $D_a$. This leads to a reduction in the relative contribution due to activity and the diffusion coefficient reverts to being temperature dependent (Figure 5).

**Experimental evidence supporting existence of short and dynamic actin filaments**

The existence of short actin filaments at the cell cortex is available in the literature on actin filaments over last few decades. Biochemical studies in polymorphonuclear leukocytes show that the dominant filament length in cells is $\approx 180$ nm (Cano *et al.*, 1991). Recent results have provided evidence for stabilized short actin filaments (50-150 nm) in-vitro (Volkmann *et al.*, 2014), Cryo-EM studies looking at the division furrow of Dictyostelium (Reichl *et al.*, 2008). Other EM based studies (Svitkina and Borisy, 1999; Puthenveedu *et al.*, 2010; Collins *et al.*, 2011) have provided evidence for the presence of short actin filaments at the lamellipodium and endosomes, respectively. Our own studies by FCS (Gowrishankar *et al.*, 2012) show the presence of short filaments ($\approx 150$-250 nm in length) in cortex of the cell. This pool of filaments is sensitive to the perturbations of filament remodeling and turnover, as blocking polymerisation by Latrunculin A treatment led to a loss of these filaments, while jasplakinolide was found to stabilize them (Gowrishankar *et al.*, 2012). Moreover, we showed that these filaments are recruited near the membrane by TIRF-single particle tracking. New cryo-EM studies (unpublished observations) provide data consistent with the length scales observed for these filaments.

However, we there is no visualization (super-resolution or EM images) of the configuration of dynamic actin filaments in this study or our older study. Finally, in this study, we show that these actin filaments are nucleated in cells by formins and not by Arp2/3 (Figure 6 B, C). Using the pharmacological drug SMIFH2 (Rizvi *et al.*, 2009), we inhibit the presence of the slowly diffusing filament-bound actin probe which corresponds to the 100-250 nm filament
in our earlier studies (Gowrishankar et al., 2012). Consistent with the role of filaments in regulating GPI-AP diffusion, this perturbation makes GPI-AP diffusion dependent on temperature (Figure 6 E, F).

**Relationship of our model for diffusion to the hop diffusion/picket fence model**

The picket fence model of membrane diffusion is used as an explanation of the corralled diffusion of membrane components (Kusumi et al., 2012). Hence, we comment on the relationship of our data on temperature-independent diffusion with the hop diffusion/picket fence.

In order to be succinct, we discuss this in a point-wise manner:

1) The cell surface is a multicomponent fluid membrane juxtaposed with a cortical actin. In the picket fence model the configuration of actin at the cortex appears to be a relatively stable, cross-linked meshwork consisting of branched filaments, nucleated by Arp2/3 (Higgs and Pollard, 2001). The actin meshwork displays a distribution of mesh-sizes that is typically cell type dependent (Murase et al., 2004; Morone et al., 2006).

2) Transmembrane proteins with a stronger affinity to the actin mesh get stuck to the filamentous mesh for a longer time, and thus impede the movement of other membrane molecules including lipids. These membrane molecules are caged for a while and get un-caged over a time scale corresponding to local structural reorganization of the meshwork and the other caged molecules. This gives rise to cage-hopping dynamics, well known in many physical systems just above glass transition. This has been called ‘hop-diffusion’ and the structural caging described above has been rechristened ‘picket-fences’ by Kusumi and others (Saxton and Jacobson, 1997; Kusumi et al., 2012). Note that in the absence of active cage-breakage from the contractile effects of myosin, the uncaging times (related to the so-called α-relaxation time) can be very large, but the dynamics will remain diffusive at late times, as a result of thermal fluctuations. Even so, the diffusion coefficient can be quite complex since it depends on a scale-dependent viscosity (which could be temperature dependent) as well as the nature of structural reorganization in the vicinity of the tagged particle. However, one might expect significant temperature variation above the glass transition temperature. Hence, the picket-fence model alone cannot provide an explanation for the temperature independence we observe for passive molecules.

3) To reconcile the active actin composite model with the picket fence observations we posit that there two kinds of cortical actin filaments. Those that make up the relatively static mesh and those that constitutes the dynamic filaments. Furthermore in addition to the ever-present thermal fluctuation there are active fluctuations arising from the coupling of molecules to the
dynamic pool of actin. The relative contribution from these two sources of fluctuations should depend on (i) the local concentration of dynamic actin and (ii) the binding affinity of the tagged molecule to actin and the concentration of myosin activity. If we park the FCS spot in a region with high concentration of dynamic actin, then the relative contribution to diffusion from active sources would be high. This is what we observe at small spot sizes. It is reasonable to assume that the concentration of dynamic actin falls off over a scale corresponding to the density correlation length (we do not know what its value is at this stage). Thus if the spot size is made larger than this density correlation length, then the observed tagged particle diffusion will start getting contributions from thermal fluctuations. This is consistent with the temperature dependence of the diffusion coefficient of GPI-APs, which resembles an inert molecule at the larger spot sizes. Our experiments suggest that this crossover has little to do with the mesh size of the stable cortical actin, where we - (i) change the mesh size by doing experiments on a different cell type (Figure 7) and (ii) by perturbing the nucleators of the stable actin mesh, namely Arp2/3 (Figure 6), and find no major effects on the nature of diffusion.
References

Cano, M. L., Lauffenburger, D. a., and Zigmond, S. H. (1991). Kinetic analysis of F-actin depolymerization in polymorphonuclear leukocyte lysates indicates that chemoattractant stimulation increases actin filament number without altering the filament length distribution. J. Cell Biol. 115, 677–687.

Chaudhuri, A., Bhattacharya, B., Gowrishankar, K., Mayor, S., and Rao, M. (2011). Spatiotemporal regulation of chemical reactions by active cytoskeletal remodeling. Proc. Natl. Acad. Sci. U. S. A. 108, 14825–14830.

Collins, A., Warrington, A., Taylor, K. A., and Svitkina, T. (2011). Structural organization of the actin cytoskeleton at sites of clathrin-mediated endocytosis. Curr. Biol. 21, 1167–1175.

Goswami, D., Gowrishankar, K., Bilgrami, S., Ghosh, S., Raghupathy, R., Chadda, R., Vishwakarma, R., Rao, M., and Mayor, S. (2008). Nanoclusters of GPI-Anchored Proteins Are Formed by Cortical Actin-Driven Activity. Cell 135, 1085–1097.

Gowrishankar, K., Ghosh, S., Saha, S., C, R., Mayor, S., Rao, M., Rumamol, C., Mayor, S., and Rao, M. (2012). Active remodeling of cortical actin regulates spatiotemporal organization of cell surface molecules. Cell 149, 1353–1367.

Higgs, H. N., and Pollard, T. D. (2001). Regulation of actin filament network formation through ARP2/3 complex: activation by a diverse array of proteins. Annu. Rev. Biochem. 70, 649–676.

Kusumi, A., Fujiwara, T. K., Chadda, R., Xie, M., Tsunoyama, T. A., Kalay, Z., Kasai, R. S., and Suzuki, K. G. N. (2012). Dynamic Organizing Principles of the Plasma Membrane that Regulate Signal Transduction: Commemorating the Fortieth Anniversary of Singer and Nicolson’s Fluid-Mosaic Model. Annu. Rev. Cell Dev. Biol. 28, 215–250.

Maitra, A., Srivastava, P., Rao, M., and Ramaswamy, S. (2014). Activating Membranes. Phys. Rev. Lett. 112, 258101.

Morone, N., Fujiwara, T., Murase, K., Kasai, R. S., Ike, H., Yuasa, S., Usukura, J., and Kusumi, A. (2006). Three-dimensional reconstruction of the membrane skeleton at the plasma membrane interface by electron tomography. J Cell Biol 174, 851–862.

Murase, K., Fujiwara, T., Umemura, Y., Suzuki, K., Iino, R., Yamashita, H., Saito, M., Murakoshi, H., Ritchie, K., and Kusumi, A. (2004). Ultrafine membrane compartments for molecular diffusion as revealed by single molecule techniques. Biophys. J. 86, 4075–4093.

Puthenveedu, M. A., Lauffer, B., Temkin, P., Vistein, R., Carlton, P., Thorn, K., Taunton, J., Weiner, O. D., Parton, R. G., and Mark Von Zastrow (2010). Sequence-dependent sorting of recycling proteins by actin-stabilized endosomal microdomains. Cell 143, 761–773.

Rao, M., and Mayor, S. (2014). Active organization of membrane constituents in living cells. Curr. Opin. Cell Biol. 29, 126–132.
Reichl, E. M., Ren, Y., Morphew, M. K., Delannoy, M., Effler, J. C., Girard, K. D., Divi, S., Iglesias, P. A., Kuo, S. C., and Robinson, D. N. (2008). Interactions between Myosin and Actin Crosslinkers Control Cytokinesis Contractility Dynamics and Mechanics. Curr. Biol. 18, 471–480.

Rizvi, S. A, Neidt, E. M., Cui, J., Feiger, Z., Skau, C. T., Gardel, M. L., Kozmin, S. A, and Kovar, D. R. (2009). Identification and characterization of a small molecule inhibitor of formin-mediated actin assembly. Chem. Biol. 16, 1158–1168.

Saxton, M. J., and Jacobson, K. (1997). Single-particle tracking: applications to membrane dynamics. Annu. Rev. Biophys. Biomol. Struct. 26, 373–399.

Sengupta, P., Hammond, A., Holowka, D., and Baird, B. (2008). Structural determinants for partitioning of lipids and proteins between coexisting fluid phases in giant plasma membrane vesicles. Biochim. Biophys. Acta - Biomembr. 1778, 20–32.

Sezgin, E. et al. (2012). Partitioning, diffusion, and ligand binding of raft lipid analogs in model and cellular plasma membranes. Biochim. Biophys. Acta - Biomembr. 1818, 1777–1784.

Svitkina, T. M., and Borisy, G. G. (1999). Arp2/3 complex and actin depolymerizing factor/cofilin in dendritic organization and treadmilling of actin filament array in lamellipodia. J. Cell Biol. 145, 1009–1026.

Volkmann, N., Page, C., Li, R., and Hanein, D. (2014). Three-dimensional reconstructions of actin filaments capped by Arp2/3 complex. Eur. J. Cell Biol. 93, 179–183.
TABLE S1. Temperature sensitivity of diffusion for different lipid probes and their corresponding anomalous exponent ($\alpha$). The partitioning of the various probes in GPMVs is based on published data (Sengupta et al., 2008; Sezgin et al., 2012). We fit the FCS data to the form appropriate to anomalous diffusion to $\langle r^2 \rangle \propto t^\alpha$ (described in Methods), and find that $\alpha$ is close to 1 for all the probes, suggesting simple diffusion. However the diffusion could be either thermal or active.

| Lipid Analogs/cDNA constructs (Cell type) | Partitioning in GPMV (based on) | Temperature sensitivity at $\omega^2 \cdot 3 \times 10^4 \text{nm}^2$ | Anomalous Diffusion Exponent ($\alpha$) (±SD) | Temperature sensitivity at $\omega^2 \cdot 6 \times 10^4 \text{nm}^2$ |
|-----------------------------------------|---------------------------------|-------------------------------------------------|-----------------------------------------------|-------------------------------------------------|
| EGFP-FR-GPI (CHO)                       | Liquid ordered (Lo)              | Independent                                     | $1.12 \pm 0.02$                               | Dependent                                       |
| FR-GPI (CHO)                            | Liquid ordered (Lo)              | Independent                                     | $1.08 \pm 0.09$                               | --                                              |
| EGFP-CD52-GPI (CHO)                     | --                              | Independent                                     | $1.06 \pm 0.14$                               | --                                              |
| BODIPY C5/C12-SM (CHO/RBL)              | Liquid ordered (Lo)              | Dependent                                       | $0.99 \pm 0.05$                               | Dependent                                       |
| BODIPY C5/C12-PC (CHO/RBL)              | Liquid disordered (Ld)           | Dependent                                       | $1.10 \pm 0.1$                                | Dependent                                       |
| EGFP-CD52-GPI (Jurkat T)                | --                              | Independent                                     | $1.06 \pm 0.1$                               | Dependent                                       |
| EGFP-CD52-GPI (NRK)                     | --                              | Independent                                     | $1.07 \pm 0.2$                                | Dependent                                       |
Table S2. Statistical Analysis of Pair-wise Q_{10} Comparisons (for GPIs)

| Comparisons (A v/s B)                                                                 | Q_{10} Difference B-A | Significant (at p<0.05) | Summary | P Value | Figure Ref. |
|---------------------------------------------------------------------------------------|-----------------------|-------------------------|---------|---------|-------------|
| **GPI Controls (with spot sizes in 10^4 nm^2)**                                        |                       |                         |         |         |             |
| EGFP-GPI(3) vs. EGFP-GPI(4.5) in CHO cells                                            | 0.13                  | No                      | ns      | 0.76    | 2 A,C       |
| EGFP-GPI(3) vs. EGFP-GPI(6) in CHO cells                                              | 0.39                  | Yes                     | **      | <0.009  | 2 A,C       |
| EGFP-GPI(3) vs. CD52(3) in CHO cells                                                 | -0.06                 | No                      | ns      | 0.99    | 2 C         |
| EGFP-GPI(3) vs. FR-GPI(3) in CHO cells                                               | 0.04                  | No                      | ns      | 0.99    | 2 C         |
| CD52(3) v/s CD52(6) in Jurkat cells                                                  | 0.9                   | Yes                     | ****    | <0.0001 | 2 B         |
| **Cholesterol perturbations in CHO cells**                                            |                       |                         |         |         |             |
| EGFP-GPI vs. EGFP-GPI(MBCD)                                                          | 0.5                   | Yes                     | ***     | 0.001   | 3B          |
| FR-GPI vs. FR-GPI(MBCD)                                                               | 0.25                  | Yes                     | **      | 0.004   | 3C          |
| **Acto-myosin Perturbations in CHO cells**                                            |                       |                         |         |         |             |
| FR-GPI vs. FR-GPI(Latrunculin A)                                                     | 0.1                   | Yes                     | **      | 0.01    | 4C          |
| FR-GPI vs. FR-GPI(ML7+Y27 cocktail)                                                   | 0.15                  | Yes                     | **      | 0.003   | 4C          |
| FR-GPI vs. FR-GPI(Bleb)                                                              | 0.29                  | Yes                     | ***     | 0.0002  | 4C          |
| **Transmembrane Actin Binding Probe**                                                |                       |                         |         |         |             |
| TM-ABD vs. TM-ABD*                                                                   | 0.23                  | Yes                     | **      | 0.003   | 5C          |
| **Actin Nucleator Perturbations**                                                    |                       |                         |         |         |             |
| EGFP-GPI vs. EGFP-GPI (Formins by SMIFH2)                                             | 0.38                  | Yes                     | **      | 0.007   | 6 E,F       |
| EGFP-GPI vs. EGFP-GPI (Arp2/3 by CK-666)                                             | -0.025                | No                      | ns      | 0.95    | 6 E,F       |
| **Actin Meshwork Changes (spot size in 10^4 nm^2)**                                   |                       |                         |         |         |             |
| CD52 (CHO) v/s CD52 (NRK) at 3x10^4 nm^2                                              | -0.1                  | No                      | ns      | 0.962   | 2C,7C       |
| CD52(3) v/s CD52(6) in NRK                                                            | -0.3                  | Yes                     | *       | 0.02    | 7C          |

**** p<0.0001
*** p<0.001
** p<0.01
* p<0.05

Table S2. Statistical Analysis of Q_{10} between controls and test scenario (changing spot size or pharmacological treatments). Mean comparison (of Q10) was done by either Student’s T-test (with Welch’s Correction for unequal variances for comparing two samples) or One-way Anova (with Tukey’s multiple mean comparison test for comparing more than 2 samples) using GraphPad Prism 6 software. We have used confidence level of p<0.05 to determine significance in mean comparisons (col. 3) while the raw p-values and individual figure references have been shown.
Table S3. Statistical Analysis of linear slopes (for GPIs)

| Protein/Lipids (Treatment/Spot size) | Cell type | Linear Slope | Deviation from linearity (p-value) | Is slope significantly non-zero? (p-value) | Figure Ref. |
|-------------------------------------|-----------|--------------|-----------------------------------|-------------------------------------------|-------------|
| GPI Controls (at different spot sizes in 10^4 nm²) | | | | | |
| EGFP-GPI (3 x 10^4 nm²) | CHO | 0.0016 ± 0.010 | ns (0.5) | ns (0.8914) | 2A |
| EGFP-GPI (4.5 x 10^4 nm²) | CHO | 0.0137 ± 0.009 | ns (1) | ns (0.2104) | 2A |
| EGFP-GPI (6 x 10^4 nm²) | CHO | 0.039 ± 0.008 | ns (0.5) | * (0.0179) | 2A |
| FR-GPI (3 x 10^4 nm²) | CHO | 0.0141 ± 0.006 | ns (0.5) | ns(0.1050) | 1C |
| CD52-GPI (3 x 10^4 nm²) | CHO | 0.0025 ± 0.004 | ns (1) | ns (0.6204) | 1C |
| CD52-GPI (6 x 10^4 nm²) | Jurkat | 0.0269 ± 0.012 | ns(0.5) | ns (0.1048) | 2B |
| Cholesterol perturbation | | | | | |
| EGFP-GPI (MBCD) | CHO | 0.0332 ± 0.002 | ns (0.9) | ***(0.0005) | 3B |
| FR-GPI (MBCD) | CHO | 0.0358 ± 0.007 | ns (1) | *(0.0135) | 3C |
| Actomyosin Perturbations | | | | | |
| FR-GPI (Lat A) | CHO | 0.0227 ± 0.005 | ns (0.9) | *(0.022) | 4B |
| FR-GPI (ML7 + Y27) | CHO | 0.028 ± 0.006 | ns (0.5) | *(0.0172) | 4B |
| FR-GPI (Bleb) | CHO | 0.039 ± 0.008 | ns (0.5) | *(0.0179) | 4B |
| Transmembrane Actin Binding Probe | | | | | |
| TM-ABD | CHO | 0.0015 ± 0.006 | ns (0.667) | ns (0.8408) | 5B |
| TM-ABD* | CHO | 0.0226 ± 0.003 | ns (0.667) | *(0.0178) | 5B |
| Actin Nucleator Perturbations | | | | | |
| EGFP-GPI (SMIFH2) | CHO | 0.0361 ± 0.003 | ns (1) | ***(0.0016) | 6E |
| EGFP-GPI (CK 666) | CHO | 0.0104 ± 0.005 | ns (0.9) | ns (0.1513) | 6E |
| Actin Meshwork Changes | | | | | |
| CD52-GPI (3 x 10^4 nm²) | NRK | 0.0139 ± 0.007 | ns (0.5) | ns (0.1774) | 7B |
| CD52-GPI (6 x 10^4 nm²) | NRK | 0.0188 ± 0.003 | ns (0.5) | *(0.0127) | 7B |

**** p<0.0001  
*** p<0.001  
** p<0.01  
* : p<0.05

Table S3. Statistical analysis of linear slopes. We have performed a linear regression and fitting of the D v/s T plot. A linear fit of the data gives us the overall (global) slope for individual controls and different perturbations across cell types. Both significant deviations from linearity (col.4) and significant difference between slopes as compared to a zero slope is determined using GraphPad Prism 6 software. The p-values for both these comparisons is shown along with figure references.
Supplemental Figure Legends.

Figure S1. Systematic fitting procedure to obtain the typical diffusion coefficient D. (A) Representative auto-correlation plots from measurements taken at different temperatures from cells labeled with C5-BODIPY FL-SM. These were found to fit well to a single component 2D diffusional model (see discussion in Methods) from which we extracted diffusional timescales ($\tau_d$) and the corresponding diffusion coefficient, D (B) We generated a distribution of D for every temperature sampled between 20–37 °C, each of which were fitted to an error function to obtain the most-probable (or typical) value of D.

Figure S2. Inert lipids exhibit temperature dependent diffusion on RBL cells. (A) Typical value of the diffusion coefficient, D, extracted from FCS measurements made on inert lipids in RBL2H3 cells, increased smoothly across the temperature range 20-37°C. Here two different inert probes, C12-BODIPY-SM (B-SM) and C12-BODIPY-PC (B-PC) exhibit a distinct temperature dependence when measured at a larger confocal spot size ($\omega^2=6\times10^4$ nm$^2$). (B) Analysis of Q$_{10}$ temperature coefficient also indicates the extent of temperature dependence. Error bars are standard errors.

Figure S3. Inert molecule (C5-BODIPY FL-SM) diffusion on cholesterol depleted cells, membrane blebs and on actomyosin perturbed cells. (A) The diffusion of the inert molecule (C5-BODIPY FL-SM) on cholesterol depleted cells shows a reduced $D$, but retains a temperature-dependent increase. (B) This is verified by measuring Q$_{10}$. Error bars are standard error. (C) Bleb diffusion is highly enhanced for C5-BODIPY FL-SM and remains strongly temperature dependent. (D) Upon perturbations of actomyosin, the diffusion of the inert probe is still temperature dependent, although the extent of the temperature dependence appears to have been affected. (E) These results are verified by measuring Q$_{10}$. Error bars are standard error. 10-12 cells for each temperature from two experiments. *p<0.05, **p<0.01 (one-way ANOVA, Tukey’s mean-comparison test).

Figure S4. FCS measurements on GFP-GPI show an intracellular component. Autocorrelation plot of FCS measurements of GFP-GPI (A) and its corresponding diffusion timescale distribution from MEM analysis (B), shows two-component diffusion with a prominent contribution from internal pool, presumably the soluble EGFP pool in endoplasmic...
reticulum or other cytoplasmic membranes (red bar). The higher timescale component reflects membrane diffusion and was used for extracting the diffusion timescales for the calculation of diffusion coefficients (blue bar). (C) The membrane diffusion timescale was also confirmed by labeling only the surface accessible membrane pool of EGFP-GPI using anti-GFP fab (conjugated to BODIPY-TMR) and monitoring its diffusion by FCS. The MEM analysis of such autocorrelation data clearly shows single component diffusion, with similar timescales as obtained from EGFP-GPI (blue bar).

**Figure S5.** Representative FCS data with fits and residuals under different conditions for GPI-AP probes. (A) EGFP-FR-GPI on CHO cells at confocal spot size $\omega^2=3\times10^4$ nm$^2$ across 20–37°C. The FCS curves are mostly overlapping suggesting a very small difference in the diffusion timescales at different temperatures, resulting in effective temperature independence observed in Figure 2B. (B) FR-GPI on CHO cells at confocal spot size $\omega^2=3\times10^4$ nm$^2$ also shows a temperature independent trend, as shown in Figure 2A. (C) FCS curves for EGFP-FR-GPI shows a distinct temperature dependence upon cholesterol depletion using MBCD. The curves (and fits) obtained from cholesterol depleted cells shift to lower diffusion timescales on increasing the temperature, resulting in a temperature dependence of $D$, as in Figure 3B. (D) FCS curves for FR-GPI on blebs also show a clear temperature dependent shift from slower to faster diffusion timescales upon increase in temperature, data corresponds to Figure 4B. (E) FCS curves for EGFP-FR-GPI upon formin perturbation by SMIFH2, data corresponds to Figure 6E. (F) FCS curves for TM-ABD/* at 37°C suggest a faster correlation timescales for TM-ABD* than TM-ABD, highlighting the differences shown in Figure 5B.

**Figure S6.** Spot-variation FCS (sv-FCS) analysis of EGFP-GPI data (at 37°C) . The diffusion timescales was plotted against the spot-size and a linear fit was performed as described earlier (Lenne et. al, 2006). The y-intercept suggests whether the type of diffusion is free, nanoscale domain partitioned or actin-mesh confined. Here we show two fit-lines, one including all the three data points(blue) and the other one(red) only considering only the latter two points at spot size 4.5 and 6 (x 10$^4$ nm$^2$), since earlier data from Lenne et. al. were from similar lengthscales. Both the fit-lines show a similar positive y-intercept as reported earlier, suggesting a dynamic partitioning model.
Figure S7. Analysis of local slopes in typical D v/s T traces. (A) As an example, we used the typical D v/s T plot for EGFP-GPI (in CHO cells) and fit it to a piecewise quadratic spline (poly fits for a moving window of 3 temperature data points). (B) Local slope was calculated from the local derivative dD/dT and plotted across the indicated temperature range. When the slopes are consistently above zero over this temperature range, we declare it as a temperature dependent diffusion. When the slopes take on both positive and negative values over this temperature range, i.e., when they oscillate about zero, we declare it as temperature independent diffusion. In this case, EGFP-GPI shows a temperature independent diffusion coefficient within the temperature range. (C) On the other hand, the local slopes for inert lipids are consistently positive, indicating temperature dependence of D. (D) The EGFP-GPI shows slopes that alternate in sign about zero, for smaller confocal spot area ($3 \times 10^4$ nm$^2$) indicating that the measured diffusion coefficient is temperature insensitive. At larger spot areas ($6 \times 10^4$ nm$^2$) the local slopes dD/dT start to consistently show positive values indicating a crossover to a temperature dependent behaviour. (E) Consistently positive values of dD/dT for EGFP-GPI in cholesterol depleted cells compared to the control, indicates a crossover to temperature dependent behaviour. (F) Local slope dD/dT analysis shows that transmembrane actin binding molecule (TM-ABD) has a temperature independent diffusion coefficient, in stark contrast to the non-actin binding mutant (TM-ABD*) (G) Likewise, FR-GPI (control, blue circles) shows temperature independent diffusion and a crossover to temperature dependent behaviour upon perturbations of cortical actomyosin and in blebs. (H) Slopes obtained for CD52 in NRK cells at two different spot-sizes. (I) Diffusion of EGFP-FR-GPI in SMIFH2 treated cells show consistently positive values suggesting temperature dependence, which is in contrast to CK-666 treated scenario.
Figure S5

(A) Normalised $G(t)$ for EGFP-FR-GPI with $\omega^2=3\times10^4$ nm$^2$, showing data at 20°C, 24°C, 28°C, 32°C, and 37°C with a fit.

(B) Normalised $G(t)$ for FR-GPI with $\omega^2=3\times10^4$ nm$^2$, showing data at 20°C, 24°C, 28°C, 32°C, 37°C, and 37°C with a fit.

(C) Normalised $G(t)$ for EGFP-FR-GPI (with MBCD) showing data at 20°C, 28°C, 32°C, 37°C, and 37°C with a fit.

(D) Normalised $G(t)$ for FR-GPI (BLAB) showing data at 20°C, 24°C, 28°C, 32°C, and 37°C with a fit.

(E) Normalised $G(t)$ for EGFP-FR-GPI (with SMIFH2) showing data at 20°C, 28°C, 37°C, and 37°C with a fit.

(F) Normalised $G(t)$ for 37°C showing data for TM-ABD, TM-ABD* with a fit.
