Chapter 5: Role of GPI anchor acyl chains in the plasma membrane organization of GPI-APs

5.1 Introduction

GPI anchored proteins (GPI-APs) are organized into immobile nanoscale clusters and monomers at the plasma membrane, the fraction being independent of the protein concentration (Sharma et al., 2004; Goswami et al., 2008; van Zanten et al., 2009). This suggests that the nanoclustering of GPI-APs is driven by an active and not a passive chemical equilibrium mechanism. Further studies show that the spatial distribution of GPI-AP nanoclusters are dependent on the membrane lipid composition specifically cholesterol at the outer leaflet and the acto-myosin activity beneath the inner leaflet (Sharma et al., 2004; Goswami et al., 2008). Nonetheless, the mechanism by which the GPI-AP, residing in the outer leaflet, cross talks with the actin cytoskeleton proximal to the bilayer is poorly understood.

Gowrishankar K, Subhasri G et al. (Gowrishankar et al., 2012) using theoretical calculations predicted the existence of short dynamic actin filaments, that along with the motor protein myosin can form transient and dynamic actin asters of the scale of nanometers. Additionally, any molecule capable of interacting either directly or indirectly with these actin asters can form dynamic nanoclusters. These predictions were verified by experiments from single molecule TIRF and FCS measurements, which provided evidence for the existence of short dynamic actin filaments and also proved that the direct interaction with actin (using a chimeric transmembrane protein with actin binding domain) can lead to the nanoclustering of molecules at the plasma membrane. Thus the nanoscale actin asters at
the adjacent to the cytoplasmic leaflet are capable of dictating the spatio-temporal organization of molecules at the plasma membrane. In addition to this, the theoretical calculations (Gowrishankar et al., 2012) verified that the spatial distribution and the aggregation-fragmentation dynamics of actin asters are consistent with that of GPI-APs at the plasma membrane (Goswami et al., 2008). Taken together these evidences, we propose that the organization of outer leaflet GPI-APs are driven by actin asters underneath the bilayer via either transbilayer acyl chain interactions with a distinct inner leaflet lipid or a transmembrane protein, each of which is a direct or indirect interactor of actin. However, independent FRET studies on chimeric folate receptor with a transmembrane domain instead of GPI anchor (Varma and Mayor, 1998) or aberrant GPI-APs containing unsaturated acyl chains in GPI biosynthetic lipid remodeling mutant cells (Raghupathy et al., 2015) indicate that the GPI anchor is indispensible for GPI-AP nanoclustering. These evidences rule out the possible involvement of an ectopic interaction of GPI-AP with a transmembrane protein to connect to the actin. Therefore the only possible mechanism that explains the relevant role of actin in GPI-AP nanoclustering is via transbilayer acyl chain interactions with inner leaflet lipids. According to this ‘Transbilayer coupling mechanism’, a putative inner leaflet lipid, capable of direct or indirect interactions with actin can interlink it to the GPI-APs in the outer leaflet via purely acyl chain based interactions. This transbilayer coupling is in turn dependent on the outer and inner lipid acyl chain length and saturation, membrane cholesterol levels and the interaction with actin. Thus, according to this hypothesis, any factor that perturbs the transbilayer coupling for example, replacing long and saturated acyl chains with short or unsaturated chains, depletion of cholesterol or actin activity can abrogate the nanoclustering of GPI-APs. The existence of transbilayer
coupling can be experimentally verified by measuring the nanoclustering capability of GPI anchors with varying acyl chain length or saturation. This challenge was overcome by utilizing synthetic fluorescently tagged GPI analogues containing either long or short and saturated or unsaturated acyl chains. The GPI analogues were incorporated into live cell membrane and its ability to form cholesterol and actin dependent nanoclusters in comparison to endogenous GPI-APs were determined by anisotropy measurements. As described in Chapter 3 (section 3.1.4) chemically synthesized head group tagged GPI analogues were utilized for this study. The two classes of head group tagged GPI analogues include the Bodipy™ (GPI\textsubscript{C16:0/C16:0} and GPI\textsubscript{C8:0/C8:0}) (Figure 5.1 A) and the fluorescein tagged GPI analogues (GPI\textsubscript{C18:0/C18:0} and GPI\textsubscript{C18:1/C18:1}) (Figure 5.1 B). The design of the length of GPI acyl chains is based on the previous reports that the GPI-APs in mammalian cells carry relatively long saturated acyl chains like C18:0 or C16:0 (Orlean and Menon, 2007). However one has to keep in mind that the term ‘long’ is used here in context of the GPI anchor alone, whereas it would mean C20:0 or longer acyl chains in case of other lipids present in the cell. The GPI analogues possess similar glycan structure except for the variation in the acyl chains and the fluorescent label. The fluorophore was specifically tagged at the head group to minimize its interference (if any) with lipid acyl chain mediated transbilayer coupling.

The synthetic GPI analogues (Bodipy™ or Fluorescein tagged) were incorporated as γ-CD complexes (Chapter 4, Section 4.3.2) into the plasma membrane of CHO cells (IA2.2F cells) stably expressing folate receptor (a GPI-AP) and its anisotropy measured. For each class of analogues (Bodipy™ or fluorescein), GPI-APs in separate cells were marked with the corresponding fluorescent label (PLB™ or PLF) and used as a control.
Once incorporated into the cell, GPI analogues are retained in the outer leaflet of the plasma membrane as indicated by its cleavage due to exogenously added phosphatidylinositol specific phospholipase-C (PI-PLC) (Figure 5.2). As mentioned in Chapter 3 and 4, PI-PLC is known to cleave GPI anchor at the phosphodiester bond to break down it into inositol phosphate residue and diacylglycerol. Since the fluorescent tag is located at the head group of GPI analogues, the PI-PLC cleavage resulted in the loss of fluorescence intensity that was readily detected by fluorescence intensity measurements. Further, using both FCS (with help from Suvrajit Saha in the laboratory) and FRAP measurements, the diffusion properties of these GPI analogues were found to be similar to that of GPI-APs (Folate receptor). This result confirms that the synthetic GPI analogues are well incorporated (different methods) into the plasma membrane of cells (Figure 5.3 A and B).

5.2 Results:

Bodipy\textsuperscript{TMR} tagged GPI analogues (10-20 µM) with relatively long or short acyl chains (GPI\textsubscript{C16:0/C16:0} or GPI\textsubscript{C8:0/C8:0}, respectively) were independently incorporated into the plasma membrane of IA2.2F cells and its steady state anisotropy was measured on Andor spinning disc based confocal microscope (described in chapter 2, in section 2.5.1.4). Since the efficiency of lipid incorporation varies with the acyl chain length, GPI analogues (between the two analogues) were incorporated at different concentrations into the cell membrane such that they are in comparable range of fluorescence intensities to folate receptor (PLB\textsuperscript{TMR} labeled) expressed in IA2.2F cells.
In order to understand the experimental results better, it is important to envisage how lipid molecules organize at the plasma membrane with increase in its concentration (depicted in Figure 5.3. At relatively low concentrations, the lipid molecules are sparsely distributed at the membrane and hence do not undergo FRET. As the concentration of the lipid on the membrane increases, the intermolecular distance decreases and therefore the probability of FRET will increase (trivial FRET) which is detected by the decrease in its anisotropy. The aim of my study is to detect actively clustered GPIs and so I measured the anisotropy of GPI analogues at a low concentration regime (the red box in Figure 5.3 A) where there is ideally no trivial FRET (red box in Figure 5.3 B).

5.2.1 \( \text{GPI}_{16:0/16:0} \) exhibits nanoclustering very similar to that of GPI-AP

At the same concentration range or fluorescence intensities, the steady state anisotropy values of \( \text{GPI}_{16:0/16:0} \) is significantly different [Kolmogorov-Smirnov (KS) test \( p<0.001 \)] and low as compared to the \( \text{GPI}_{8:0/8:0} \) (Figure 5.4 B). This result suggests that the \( \text{GPI}_{16:0/16:0} \) exhibits high FRET (is clustered) compared to \( \text{GPI}_{8:0/8:0} \). Anisotropy of PLB\text{TM}R bound folate receptor (GPI-AP) was used as a control in the measurement (Figure 5.5A). Photobleaching of \( \text{GPI}_{16:0/16:0} \) confirmed the presence of Homo-FRET (Figure 5.5 B & C). As described previously (Sharma et al, 2004), in a photobleaching experiment the fluorophores are bleached continuously and the anisotropy of this fluorophore is measured in equally spaced time intervals. Photobleaching effectively reduces the population of fluorophores that are undergoing FRET thereby causing an increase in the anisotropy measured. \( \text{GPI}_{16:0/16:0} \) on photobleaching exhibits an increase in anisotropy very similar in trend to that observed for PLB\text{TM}R bound folate receptor, verifying that the low values of anisotropy is indeed due to FRET. This result confirms that the \( \text{GPI}_{16:0/16:0} \) are
nanoclustered like the GPI-APs (folate receptor in this case). However, in case of GPI\(_{C8:0/C8:0}\) the anisotropy remains constant throughout out photobleaching confirming the absence of FRET (Figure 5.5 B & C). The above results taken together suggest that GPI\(_{C16:0/C16:0}\) form nanoclusters like GPI-AP whereas GPI\(_{C8:0/C8:0}\) does not. Next I tested the dependence of GPI\(_{C16:0/C16:0}\) nanoclusters on cholesterol and actin activity.

### 5.2.2 GPI\(_{C16:0/C16:0}\) and not GPI\(_{C8:0/C8:0}\) forms cholesterol sensitive nanoclusters similar to GPI-AP at the plasma membrane

To test the dependence on membrane cholesterol levels, the GPI\(_{C16:0/C16:0}\) or GPI\(_{C8:0/C8:0}\) was incorporated into live IA2.2F cell membrane and then subjected to saponin (a glycosides used to extract cholesterol from plasma membrane) treatment (Figure 5.6A & B). On depleting membrane cholesterol levels, as previously reported (Sharma et al., 2004; Goswami et al., 2008) for GPI-APs (folate receptor labeled with PLB\(^{TMR}\)) the GPI\(_{C16:0/C16:0}\) also exhibited a loss of nanoclusters, as indicated by increase in its anisotropy values [Kolmogorov-Smirnov (KS) test \(p<0.001\)] when compared to saponin untreated (control) cells. In a similar experiment, the GPI\(_{C8:0/C8:0}\) did not show significant change in its anisotropy when treated with saponin. These results suggest that GPI\(_{C16:0/C16:0}\) and not GPI\(_{C8:0/C8:0}\) can form cholesterol dependent nanoclusters.

### 5.2.3 GPI\(_{C16:0/C16:0}\) and not GPI\(_{C8:0/C8:0}\) forms actin sensitive nanoclusters similar to GPI-AP at the plasma membrane

In order to test the dependence of GPI\(_{C16:0/C16:0}\) nanoclusters on the activity of actin, the clustering efficiency of this analogue on membrane blebs (Goswami et al., 2008) were measured. Blebs are spherical membrane outgrowth of the plasma membrane that is devoid
of actin. It is formed when the cortical actin contact sites with the plasma membrane are decoupled. The \( \text{GPI}_{\text{C16:0/C16:0}} \) or \( \text{GPI}_{\text{C8:0/C8:0}} \) was incorporated into plasma membrane of IA2.2F cells and treated with jasplakinolide (a drug that stabilizes actin, described in Chapter 2 in Section 2.4.4) to produce membrane blebs. Very similar to the GPI-APs, anisotropy of \( \text{GPI}_{\text{C16:0/C16:0}} \) indicated an increase in anisotropy values when compared to that on the flat membrane (without jasplakinolide) [Kolmogorov-Smirnov (KS) test \( p<0.001 \)](Figure 5.6A and B). However the average anisotropy of \( \text{GPI}_{\text{C8:0/C8:0}} \) does not change neither on the blebs nor on the flat membrane. These results suggest that the \( \text{GPI}_{\text{C16:0/C16:0}} \) requires an active actin machinery for the formation of nanoclusters whereas \( \text{GPI}_{\text{C8:0/C8:0}} \) does not. In short, these measurements suggest that actin can interlink with GPI-AP only if it contains long acyl chains (greater than or equal to C16:0).

### 5.2.4 \( \text{GPI}_{\text{C18:0/C18:0}} \) but not \( \text{GPI}_{\text{C18:1/C18:1}} \) form cholesterol and actin sensitive nanoclusters similar to GPI-AP at the plasma membrane

In mammalian cells, phosphatidylinositol (PI) forms the building block for GPI biosynthesis, which inherently possesses an unsaturated lipid tail at \( sn-2 \) position. However during an intermediate step at GPI biosynthesis, cells specifically remodel the GPI-APs containing unsaturated lipid tails and replace it with a long, saturated acyl chain (either C16:0 or C18:0). The physiological relevance of this lipid remodeling which is conserved across species is unknown (see Chapter 1, section1.2.2). In this context, the importance of saturated acyl chains of GPI anchor was tested using GPI analogues containing unsaturated acyl chains. \( \text{GPI}_{\text{C18:0/C18:0}} \) (30 \( \mu \)M) and \( \text{GPI}_{\text{C18:1/C18:1}} \) (1 \( \mu \)M) were incorporated into IA2.2F cells as \( \gamma \)-CD complexes to comparable fluorescence intensities of GPI-APs (PLF bound folate receptor) at the plasma membrane of independent IA2.2F cells. The average
anisotropy of GPI_{C18:0/C18:0} was measured to be low as compared to GPI_{C18:1/C18:1} suggesting that the GPI_{C18:0/C18:0} is more nanoclustered than GPI_{C18:1/C18:1} (Figure 5.7B and C). Once again this result was confirmed by photobleaching (results not shown). Since GPI_{C18:0/C18:0} formed nanoclusters, it was important to test if this clustering required cholesterol and actin for its formation. Consequently, up on cholesterol depletion (mβCD) or on actin perturbation (jasplakinolide), the steady state anisotropy of GPI_{C18:0/C18:0} increased significantly whereas that of GPI_{C18:1/C18:1} does not exhibit a significant change in anisotropy. This result suggests that the GPI_{C18:0/C18:0} forms cholesterol and actin dependent nanoclusters like a GPI-AP (folate receptor in this study). Together with the previous results, the results presented so far indicate that the GPI analogues containing 'long' and 'saturated' but not short or unsaturated acyl chains are a pre-requisite to recapitulate the nanoclustering of GPI-AP in vivo and therefore these results confirm that the GPI anchor acyl chains have an important role in organizing GPI-APs into nanoclusters at the plasma membrane. These results support the transbilayer coupling mechanism.

5.2.5 GPI in PGAP2/3 mutants: to test if the cell membrane can support nanoclustering

As discussed earlier (in chapter 1 section 1.4.1), the GPI-AP in lipid remodeling deficient mutants (PGAP2/3 mutant cells) (Maeda et al., 2007) carry an unsaturated lipid tail at sn-2 position (Figure 5.10 A) and was found to be less efficiently clustered (Raghupathy et al., 2015) at the plasma membrane. In this case, the GPI analogues were utilized to measure the clustering ability of the plasma membrane in PGAP2/3 mutant cells. GPI_{C16:0/C16:0} was incorporated into these cells and its ability to nanocluster in comparison to that of wild type (3B2A) cells were studied by anisotropy measurements. GPI_{C16:0/C16:0} exhibited similar
anisotropy values in both the wild type and mutant cells (Figure 5.10 B). Interestingly, the extent of nanoclustering was found to be similar as observed from the extent of increase in anisotropy on cholesterol depleted cells. This result suggests that the plasma membrane of the PGAP2/3 mutants can support nanoclustering and therefore the less efficient nanoclustering of endogenous GPI-APs in the mutant cells were attributed to the presence of an unsaturated acyl chain on its GPI anchor.

5.2.6 GPI in PS mutant cells: to test if nanoclustering requires PS at the inner leaflet.

Transbilayer coupling is a co-operative effect produced by acyl chain mediated interactions of lipids residing in the outer and inner leaflet. This suggests that specific long acyl chain containing lipids at the inner leaflet are involved in effecting this coupling. Therefore it was important to identify this inner leaflet lipid. In our laboratory Anupama Ambika Anilkumar, a PhD student (Raghupathy et al., 2015) has screened for various inner leaflet lipids (phosphatidylinositol and phosphatidylserine) known to interact with actin, which could potentially link outer leaflet GPI-APs to actin. Her results indicate that the long acyl chain containing phosphatidylserine (PS) is a possible candidate for transbilayer coupling with GPI-APs. To determine if the transbilayer coupling of GPI-analogues require PS at the inner leaflet, it was important to test if the GPI analogues can undergo nanoclustering in mutant cells where a key enzyme involved in PS synthesis (PSA3 cells; refer Chapter-2, section 2.2) is depleted. PSA3 cells have a mutation in the PSS1 enzyme and depend on phosphatidylethanolamine (PE) to synthesize PS, thus making the cellular levels of PS completely dependent and sensitive to the levels of PE in the cell. PE synthesis is in turn dependent on exogenous supply of ethanolamine and therefore allows PS levels to be controlled by exogenous addition of ethanolamine (EA). $\text{GPI}_{C16:0/C16:0}$ or $\text{GPI}_{C8:0/C8:0}$ was
incorporated in to PS mutant cells grown in presence of EA (cells synthesize PS denoted as PS⁺) or absence of EA (cells cannot synthesize PS denoted as PS⁻) and anisotropy measured. In PS sufficient (control) conditions (PS⁺), GPI₁₆:₀/₁₆:₀ exhibits low anisotropy values as compared to GPI₈:₀/₈:₀ which is suggestive of the formation of nanoclusters. But in PS deplete conditions (PS⁻), the average anisotropy values of GPI₁₆:₀/₁₆:₀ is higher and close to that of cholesterol depleted control cells (GPI₁₆:₀/₁₆:₀ in PS⁺), suggesting a loss of nanoclusters when PS is absent (Figure 5.10 A). However, the anisotropy values of GPI₈:₀/₈:₀ in PS control and PS depleted conditions remain unchanged clearly indicating that it is not clustered (Figure 5.10 B). These results suggest that PS is indeed the inner leaflet candidate that couples GPI to actin.

Thus the results detailed in this chapter, confirms the existence of a transbilayer coupling mechanism for nanoclustering GPI-APs. Results from PS mutant cells, confirms that PS at the inner leaflet is the key lipid involved in coupling GPI-APs to actin.

5.3 Conclusions:

1) In stark contrast to GPI₈:₀/₈:₀, GPI₁₆:₀/₁₆:₀ forms nanoscale clusters at the plasma membrane of live cells. These GPI₁₆:₀/₁₆:₀ nanoclusters requires membrane cholesterol and actin activity for its formation

2) Similarly, GPI₁₈:₀/₁₈:₀ forms cholesterol and actin dependent nanoclusters at the plasma membrane of live cells whereas the GPI₁₈:₁/₁₈:₁ does not.

3) In PS mutant cells, GPI₁₆:₀/₁₆:₀ formed nanoclusters only under conditions were PS was synthesized (PS⁻) indicating that PS in the inner leaflet is the candid molecule for coupling actin to outer leaflet GPI.
From the above observations, I conclude that only GPI-analogues with long (equal to or above C16:0) and saturated acyl chains can undergo nanoclustering at the plasma membrane of cells. This evidence favours a Transbilayer coupling mechanism.

3) All the GPI analogues \( \text{GPI}_{\text{C16:0/C16:0}} \) and \( \text{GPI}_{\text{C18:0/C18:0}} \) that formed nanoclusters lacked the protein and the mannoses in the glycan head group. This suggests that neither the protein nor the mannose residues have a role in GPI-AP nanoclustering.

4) Finally, the GPI analogues \( \text{GPI}_{\text{C16:0/C16:0}} \) and \( \text{GPI}_{\text{C8:0/C8:0}} \) were utilized as tool to determine the clustering ability of the membrane in PGAP2/3 mutant and the requirement of PS for transbilayer coupling to actin in PSA3 mutant cells.
Chemical structure of head group labeled GPI analogues. A. Represents the structure of Bodipy<sup>TM</sup> labeled GPI analogues. These analogues contain variation in the lipid chain length. 1. Represents the GPI analogue containing di-hexadecanoyl (GPI<sub>C16:0/C16:0</sub>) and 2. dioctanoyl (GPI<sub>C8:0/C8:0</sub>). B. Represents chemical structure of Fluorescein labeled GPI analogues. These analogues contain variation in the lipid saturation. 1. Represents the GPI analogue with saturated GPI<sub>C18:0/C18:0</sub> and 2. unsaturated lipid tails GPI<sub>C18:1/C18:1</sub>. 

Figure: 5.1
Figure: 5.2

A

<table>
<thead>
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<th>Control</th>
<th>PI-PLC treated</th>
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</tr>
<tr>
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<td><img src="image3" alt="Image" /></td>
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<tr>
<td>GPI&lt;sub&gt;C8:0/C8:0&lt;/sub&gt;</td>
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B

<table>
<thead>
<tr>
<th>Control</th>
<th>PI-PLC treated</th>
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</thead>
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</tr>
<tr>
<td>GPI&lt;sub&gt;C18:0/C18:0&lt;/sub&gt;</td>
<td><img src="image9" alt="Image" /></td>
</tr>
<tr>
<td>GPI&lt;sub&gt;C18:1/C18:1&lt;/sub&gt;</td>
<td><img src="image11" alt="Image" /></td>
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PI-PLC treatment GPI lipids incorporated 1A2.2 F cells. A. Images of IA2.2F cells labeled with either PLB\textsuperscript{TMR} or Bodipy\textsuperscript{TMR} tagged GPI analogues pre and post PI-PLC treatment. On PI-PLC treatment, the folate receptor loses 95% of fluorescent signals whereas GPI\textsubscript{C16:0/C16:0} and GPI\textsubscript{C8:0/C8:0} to background signal level very similar to GPI-AP. This result suggests that the GPI analogues are retained in the outer leaflet of plasma membrane. B. Images of IA2.2F cells labeled with either PLF or Fluorescein tagged GPI analogues pre and post-PI-PLC treatment. Similar to the Bodipy\textsuperscript{TMR} tagged GPI analogues, the fluorescein tagged GPI analogues also loss their fluorescence on PI-PLC treatment. While the folate receptor loses 97% of its fluorescent signals, the GPI\textsubscript{C18:0/C18:0} loses 91% and GPI\textsubscript{C18:1/C18:1} 97% suggesting that all the head group labeled GPI analogues similar to the GPI-AP is retained in the outer leaflet of the plasma membrane.
Figure: 5.3

### Table: Diffusion Coefficients and Mobile Fractions

<table>
<thead>
<tr>
<th>System</th>
<th>n</th>
<th>τ(D) (ms)</th>
<th>D(μm²/s)</th>
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</thead>
<tbody>
<tr>
<td><strong>PLB\textsuperscript{TMR}-FR-GPI</strong></td>
<td>6</td>
<td>12.4 (±1.3)</td>
<td>0.92 (± 0.09)</td>
</tr>
<tr>
<td><strong>GPI\textsubscript{C16:0/C16:0}</strong></td>
<td>7</td>
<td>11.8 (± 2)</td>
<td>0.97 (± 0.16)</td>
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Diffusion properties of GPI analogues: A. The diffusion coefficients were measured and mobile fraction $n$ of PLB$^{TMR}$ bound FR-GPI or GPI analogues in IA2.2F cells were measured from FRAP measurements. The data shown here suggests that the exogenously incorporated lipids behave very similar to the endogenous GPI-anchored proteins. d) Diffusion coefficient was also measured using FCS. The plot represents the autocorrelation curves and fits obtained from MEM analysis for both PLB$^{TMR}$-FR-GPI and GPI$_{C16:0/C16:0}$. The diffusion time scales $\tau(D)$ and diffusion coefficient $(D)$ obtained from the MEM analysis above are given in the table below. $n$=number of independent measurements and values in parenthesis denote standard deviation.
Figure: 5.4

A

No FRET

Concentration regime of interest

concentration of lipids (C)

B

No FRET

Concentration regime of interest

concentration of lipids (C)

Actively clustered
Cartoon representation of FRET expected from incorporation of increasing amounts of labeled lipids in the cell membrane. A. Cartoon represents the anisotropy behavior of lipids with increase in its concentration. Here the profile of lipid anisotropy at 3 concentrations is depicted in boxes above. At very low concentration, the lipid molecules do not FRET. At some intermediate concentration the lipid begins to FRET and at very high concentrations the FRET is due to random collisions. The concentration region of interest marked as red box is the intermediate concentration of lipids at which measureable FRET is observed. B. Represents the aim of the experiment to differentiate active V/s concentration dependent clustering. As depicted in the boxes, the idea is to differentiate the anisotropy exhibited by lipid A and B.
Figure: 5.5

A

Anisotropy vs. Total intensity for PLB<sup>TM</sup>.

B

Anisotropy vs. Total intensity for GPI<sub>C8:0/C8:0</sub> and GPI<sub>C16:0/C16:0</sub>.

C

Cumulative Frequency vs. Anisotropy for GPI<sub>C16:0/C16:0</sub> and GPI<sub>C8:0/C8:0</sub>.
Anisotropy of \( \text{GPI}_{16:0/16:0} \) in comparison to \( \text{GPI}_{8:0/8:0} \): The graph represents the steady state anisotropy of A. PLB\(^{\text{TMR}} \) bound folate receptor B. \( \text{GPI}_{16:0/16:0} \) in comparison to \( \text{GPI}_{8:0/8:0} \) for the same concentration range on the plasma membrane of IA2.2F cells. For this the \( \text{GPI}_{16:0/16:0} \) was complexed with \( \gamma \)-CD at 2\( \mu \)M and \( \text{GPI}_{8:0/8:0} \) 20\( \mu \)M concentration and incubated on cells for 30 minutes at 37\(^\circ\)C followed by replating on to fibronectin coated dishes. Each point in the graph represents the average and standard deviation of anisotropy values in that particular intensity bin. As evident from the graph the average anisotropy for \( \text{GPI}_{16:0/16:0} \) is lower than \( \text{GPI}_{8:0/8:0} \) indicating that the former one is undergoing more homo-FRET as compared to the latter. C. The same result in B is plotted as a cumulative frequency distribution (CFD).
Figure: 5.6

A

PLB<sup>TMR</sup>

0.25

Anisotropy

0.2

0.15

0.1

B

GPI<sub>C16:0/C16:0</sub>

0.25

Anisotropy

0.2

0.15

0.1

C

GPI<sub>C8:0/C8:0</sub>

0.25

Anisotropy

0.2

0.15

0.1

Normalized intensity I/I<sub>0</sub>
Change in anisotropy on photobleaching the GPI<sub>C16:0/C16:0</sub> and GPI<sub>C8:0/C8:0</sub>. The different fluorophores at the same intensity range was photobleached till the signals reduced to the background signal intensity. Graphs represent the anisotropy change with photobleaching of A. PLB<sup>TMR</sup> labeled folate receptor B. GPI<sub>C16:0/C16:0</sub> and C. GPI<sub>C8:0/C8:0</sub>. With photobleaching, the anisotropy of PLB<sup>TMR</sup> and GPI<sub>C16:0/C16:0</sub> increases to the same extent where as GPI<sub>C8:0/C8:0</sub> remains constant.
Figure: 5.7

A

Cumulative frequency, f(r)

Anisotropy, r

B

Control | Saponin | Jasplakinolide

- Control
- Saponin
- Jasplakinolide
Membrane organization of GPI$_{C16:0/C16:0}$ and GPI$_{C8:0/C8:0}$ in response to cholesterol depletion and actin perturbation. A. Represents the CFD plots for IA2.2F cells labeled with PLB$^{TMR}$ or GPI$_{C16:0/C16:0}$ or GPI$_{C8:0/C8:0}$ pre and post saponin (cholesterol sequestering agent) or jasplakinolide treatment. As reported earlier, the anisotropy of PLB$^{TMR}$ labeled folate receptor increases with the cholesterol depletion and on blebs. The same result is observed for GPI$_{C16:0/C16:0}$. However the GPI$_{C8:0/C8:0}$ does not observe any change in the anisotropy for the same treatments. B. Images of IA2.2F cells labeled with either PLB$^{TMR}$ or GPI$_{C16:0/C16:0}$ or GPI$_{C8:0/C8:0}$ pre and post the treatments and the corresponding anisotropy maps.
Figure: 5.8

A

![Graph A](image)

B

![Graph B](image)

C

![Graph C](image)
Membrane organization of GPI\textsubscript{C18:0/C18:0} and GPI\textsubscript{C18:1/C18:1} in comparison to PLF bound folate receptor. The graph represents the steady state anisotropy of A. PLF bound folate receptor B. GPI\textsubscript{C18:0/C18:0} in comparison to GPI\textsubscript{C18:1/C18:1} for the same concentration range on the plasma membrane of IA2.2F cells. Each point in the graph represents the average and standard deviation of anisotropy values in that particular intensity bin. As evident from the graph the average anisotropy for GPI\textsubscript{C18:0/C18:0} is lower than GPI\textsubscript{C18:1/C18:1} indicating that the former one is undergoing more homo-FRET as compared to the latter. C. The same result in B is plotted as a cumulative frequency distribution.
Figure: 5.9

A.  

![Graph](image)

B.  

![Graph](image)

Change in anisotropy on photobleaching the GPI$_{\text{C18:0/C18:0}}$. The different fluorophores at the same intensity range was photobleached till the fluorescent signals reduced to the background signal intensity. Graphs represent the anisotropy change with photobleaching for A. PLF bound folate receptor B. GPI$_{\text{C18:0/C18:0}}$. The anisotropy changes in a similar fashion for both GPI-AP and GPI analogue.
Figure: 5.10

A

Cumulative frequency, $f(r)$

B

Control | mβCD | Jasplakinolide

![Images of cell structures under different conditions]
Membrane organization of $\text{GPI}_{\text{C}16:0/\text{C}16:0}$ and $\text{GPI}_{\text{C}8:0/\text{C}8:0}$ GPI lipids in response to cholesterol depletion and actin perturbation. A. Represents the CFD plots for IA2.2F cells labeled with PLF or $\text{GPI}_{\text{C}18:0/\text{C}18:0}$ or $\text{GPI}_{\text{C}18:1/\text{C}18:1}$ pre and post saponin (cholesterol sequestering agent) or jasplakinolide treatment. As expected the anisotropy of PLF labeled folate receptor increases with the cholesterol depletion and on blebs. The same result is observed for $\text{GPI}_{\text{C}18:0/\text{C}18:0}$. However the $\text{GPI}_{\text{C}18:1/\text{C}18:1}$ does not observe any change in the anisotropy for the same treatments. B. Images of IA2.2F cells labeled with either PLB$^{\text{TMR}}$ or $\text{GPI}_{\text{C}18:0/\text{C}18:0}$ or $\text{GPI}_{\text{C}18:1/\text{C}18:1}$ pre and post the treatments and the corresponding anisotropy maps.
Figure 5.11

**A**

A diagram illustrating the remodeling of GPI anchor in the Golgi. The unsaturated fatty acyl chain at the sn-2 position of the immature GPI is de-acylated by PGAP3 enzyme and is replaced with a saturated fatty acyl chain by PGAP2 enzyme. On mutation of PGAP2 and 3 enzymes, the GPI-AP synthesized retains the unsaturated lipid tail at sn-2 due to deficiency in lipid remodeling and are trafficked to the plasma membrane intact.

**B**

Cumulative Frequency Distribution (CFD) plots:

Average anisotropy of GPI\textsubscript{C16:0/C16:0} in wild type and mutant cells pre and post cholesterol depletion with saponin indicates similar values. This suggests that GPI\textsubscript{C16:0/C16:0} clusters to a similar extent in both the wild type and mutant cells.
Figure: 5.12

A

GPI_{C16:0/C16:0}

Cumulative frequency, f(r)

0.00 0.05 0.10 0.15 0.20 0.25

Anisotropy

B

GPI_{C8:0/C8:0}

PS^+  PS^-  PS^- saponin

C

GPI_{C16:0/C16:0}  PS^+  20 μm  PS^-

GPI_{C16:0/C16:0}  PS^- saponin  PS saponin

GPI_{C8:0/C8:0}  PS^+  PS^-

Legend:

- PS^+: Red
- PS^-: Black
- PS^- saponin: Green

Color Scale:

0.0  0.05  0.1  0.15  0.2  0.25  0.3
**PS is necessary for nanoclustering GPI-analogues:** The GPI$_{C16:0/C16:0}$ or GPI$_{C8:0/C8:0}$ is incorporated into the PSA3 cells in PS+ and PS- conditions and measured anisotropy. A. In the CFD plot, the anisotropy of GPI$_{C16:0/C16:0}$ in PS+ condition is lower compared to that in PS- condition. The anisotropy of the analogue in PS- conditions coincides with that of saponin treated PS+ cells. This suggests that GPI$_{C16:0/C16:0}$ clusters only when the PS is present in the inner leaflet. B. CFD plots for the anisotropy of GPI$_{C8:0/C8:0}$ in PS+ and PS- conditions. The mean anisotropy does not differ much in both the cases. C. Images and corresponding anisotropy maps of PSA3 cells incorporated with GPI$_{C16:0/C16:0}$ and GPI$_{C8:0/C8:0}$ in PS+ and PS- conditions.