Chapter 1

Introduction

1.1 The Surface of the Cell

The cell is a machine, using food to convert chemical energy for the various processes it requires for its running. New pieces of information about the sub-processes that keep the cell functioning are discovered everyday, along with a growing number of molecular players. The cell has evolved both a spatial compartmentalization, and a functional modularity, and it is evident that these two properties are highly correlated. The membrane bound Golgi apparatus, for instance, is mainly concerned with the sorting of proteins; the endoplasmic reticulum, with the production of proteins. The general consensus is that such spatial localization of function aids in the orchestration of a large number of events.

The plasma membrane is highly dynamic and has a range of functions as well. On account of being the physical barrier between the cytosol and the extracellular medium, it forms the via media for cross talk between the two. One can easily see that the plasma membrane must have the following properties: (1) It must be a highly selective barrier, capable of allowing molecules with a range of sizes, into the cell (2) It must be capable of transmitting information across. It is therefore quite conceivable that the plasma membrane has evolved a spatial organization similar in principle to the three dimensional cell, to perform these functions efficiently.

We explore the possible general routes that can enable “compartmentalization” on the membrane. The presence of physical barriers in the most obvious route. Analogous to the role the membrane plays in the 3D structure of the cell, the barriers can prevent
or restrict diffusion between different membrane regions. As illustrations of different mechanisms, we use three examples: (1) The clathrin coated pit (2) The cytoskeletal meshwork (3) Heterogeneities in the lipids.

1.1.1 Example 1: The Clathrin Coated Pit

Heterogeneity is highlighted at the plasma membrane during the process of clathrin-mediated endocytosis [1](Figure 1.1). Many large particles, like the LDL (Low Density Lipoprotein), enter the cell by first binding to a transmembrane receptor (the LDL receptor). The transmembrane receptor straddles both the leaves of the bilayer plasma membrane, and hence is physically connected to both the outside and inside of the cell. The cargo-receptor binding initiates further binding of a clathrin monomer, on the cytosolic side of the transmembrane receptor. On polymerization, clathrin has the ability to further cluster together cargo bound receptors. Polymerized clathrin forms a fully enclosed cage, and is eventually pinched off from the membrane by the action of dynamin, an ATP hydrolyzing machine. The agent leading to the transient heterogeneity in this case is the clathrin coated pit. Other pathways that make use of clathrin-like proteins, like COP-I, COP-II, are also found on the membranes of internal organelles like the Golgi, and are similar in principle to the clathrin mediated pathway. It is a pathway which is triggered by the cargo-receptor binding, and hence these heterogeneities are induced.

1.1.2 Example 2: The Cytoskeletal Meshwork

The cell cortex, can also act as a physical barrier. Interlinked actin filaments form a dense meshwork just below the plasma membrane. Morone et al [32] constructed a 3 dimensional tomography (Figure 1.2) of a ripped off piece of plasma membrane, along with the cortical actin adjacent to it. The images reveal that actin is aligned mostly horizontal to the membrane, forming a meshwork of typical meshsize ranging from 30 nm to 300 nm (in rat kidney cells; size may vary with cell type). Single molecule tracking of immuno-gold labeled surface molecules at a very high time resolution (25 microsecond) by the same lab [43], concluded that molecules perform fast thermal diffusion within, and slower hop diffusion in between compartments, whose size distribution matches that of the cortical actin mesh. Recent Fluorescence Correlation Spectroscopy (FCS) measurements from our lab (unpublished material) also show two kinds of diffusive behaviour, a fast
Figure 1.1: Clathrin mediated endocytosis: Clathrin and Dynamin dependent pathway: Trans-membrane cargo receptors (in maroon) bind to cargo (in gold) resulting in the binding of a clathrin “cap” (in black). Subsequently, bound receptors are brought together to form a fully enclosed vesicle. This vesicle is pinched off from the plasma membrane due to the ATP driven pinching action of Dynamin (in green). These vesicles are then taken to the Sorting endosome.

diffusion, and a slow diffusion, whose amplitudes and values dependent on the molecule, and the cell type (and hence the mesh size).

Qualitatively, the two kinds of diffusion seem to be independent of the nature of the tagged molecule: they could be upper or lower leaflet lipids or proteins, all the molecules feel the effect of the actin meshwork. The interactions of membrane molecules and the cytoskeleton are yet uncharacterized. They could arise from steric hindrance or scale dependent hydrodynamic dissipation of membrane molecules, however these properties are derivable from the the static properties of the meshwork: they need not depend on its dynamics.

1.1.3 Example 3: Lipid Heterogeneities

The two instances cited so far both assume that the environment in which diffusion, or sequestering, of molecules takes place is a passive player, merely acting as a source of fluctuations and a sink of dissipation. This is the basis of the Fluid Mosaic model of Singer and Nicholson [1], which likens the plasma membrane to an artificial multicomponent membrane. In the fluid mosaic, it is possible that a few the molecules associate with each other to form energetically favourable configurations. In particular, Cholesterol and Sphingolipids are shaped such that their packing maximizes Van Der Waals Interactions,
Figure 1.2: **Cortical Actin Meshwork.** Two dimensional projections of a cryo electron micrograph of the underside of the membrane. Left Panel: Normal rat kidney fibroblasts. Attached to the membrane are found (1) Clathrin Coated Pits (red circle), (2) A meshwork mostly horizontal to the membrane (brown circle) (2) Filaments of actin pointing towards the membrane (green circle). The mean mesh size is ~230 nm. Right panel: Fetal rat skin keratocytes, with a much small mesh size ~50 nm (Courtesy Morone et al, http://www.nanobio.frontier.kyoto-u.ac.jp/lab/slides/4/e.html) (Scale bar = 200 nm)

at a cost to entropy. Cholesterol, a small almost planar molecule with a very small headgroup, occupies the interstices in between sphingolipids, that have long saturated fatty acid chains and a large head group. This arrangement is energetically favourable as it maximizes interactions between the polar groups of the lipids. Fluorescence imaging of Giant Unilamellar vesicles (GUVs) (Figure 1.3 (b)) containing appropriate proportions of cholesterol, saturated lipids (DPPC) and other unsaturated lipids (DOPC), do show large phase segregated domains rich in cholesterol and sphingolipids, separated from the unsaturated lipids (green) [21]. When phase segregation occurs, the long saturated tails of the sphingolipids increases the local thickness of the membrane, such a region of low entropy and low energy is referred to as the Liquid Ordered phase, and coexists on artificial membranes, with the liquid disordered phase made up of short, unsaturated lipids. Single molecule tracking measurements show that the diffusion constant is much larger in the LD phase than in the LO phase. The phase diagram at 23°C (Figure 1.3 (a)) for such a ternary mixture (cholesterol + PSM (unsaturated lipid) + POPC (saturated lipid) ), reflects the coexistence of the LO and the LD phases at certain proportions of the three. The mole fractions of the three species are plotted along the three sides of the triangle, reaching 100% at the vertices. In the coexistence region, as the cholesterol percentage increases from 10% to 35%, the size of the LO domains in the co-existence...
region progressively increases from nanometers to the order of microns.

The plasma membrane contains a large amount of cholesterol (~40%) and sphingolipids (~20%). In order for the above mechanism to be a candidate for lipid-based heterogeneities, the cell is expected to be in the coexistence regime of the phase diagram, corresponding to micron sized domains. However, this has not been detected in the plasma membrane.

![Diagram](image)

**Figure 1.3: Cholesterol Induced Partitioning on GUVS:** (a) The LO-LD phase diagram shows regions of Coexistence of the LO and LD phases (in blue). (b) Fluorescence images of GUVs labeled with dyes that preferentially partition into LO (orange) and LD (green) phases.

The mechanisms we have suggested so far are all passive: in the clathrin coated pit, we come across a situation wherein proteins recruit membrane molecules to create local heterogeneities; in example II, the static actin cortex, thermally driven diffusion in the presence of actin corral results in compartmentalization; finally in example III, lipid heterogeneities drive compartmentalization.

### 1.1.4 A route to nano-scale compartmentalization

In Chapter 2 we describe the standardization of a non-invasive probe of lipid heterogeneities ([38][19]), which makes use of the phenomenon of Fluorescence Resonant Energy Transfer. The molecular probe used is the GPI-Anchored protein. This protein is
attached to a saturated lipid anchor glycosylphosphatidylinositol (GPI) via an oligosaccharide backbone that binds to the C terminus of the protein, and is present on the upper (exoplasmic) leaflet of the plasma membrane. Many kinds of GPI-APs exist on the cell membrane, and they are functionally very varied. However, their lipid anchor dictates their behaviour on the membrane. In chapter 2, we summarize the results of Sharma et al, that suggest that the GPI-APs are organized in nanometer sized domains. The experiments discussed suggest that the formation and maintenance of heterogeneities of these molecules is an active process, highly dependent on the dynamics of the actin cytoskeleton.

GPI-APs are interesting functionally because they are endocytosed by a pathway which is independent of both clathrin and dynamin. Nascent endocytic vesicles enclosing GPI-APs form a compartment called GEEC (GPI-Enriched Endocytic compartment) [36]. Much of the fluid uptake of the cell is via this pathway. The GEEC pathway is unique in the following ways: (1) Unlike the clathrin mediated pathway, it does not need an external trigger like the binding of cargo and receptors and occurs even in the absence of GPI-APs.(2) The vesicle formed in this pathway have a range of sizes, unlike vesicles in the clathrin mediated pathway, whose radius is equal to the radius of the pit. The internalization of GPI-APs via this pathway is dependent on a specifically maintained organization of these proteins, characteristic of their lipid anchor. This pathway also crucially depends on actin machinery[13]. In chapter 2, we make attempts to characterize this organization.

Considerable evidence points to the existence of small, transient regions, enriched in cholesterol and sphingolipids, known in general as rafts. Proteins that attach on to the
plasma membrane with a saturated lipid tether, which have the capacity to associate with cholesterol in the way discussed above, are markers of these regions. Analysis of the single molecule tracks of CD59, one such molecule, by Kusumi et al [42], showed that a few of these (3-9) molecules undergo actin dependent temporary immobilization for about 0.57 seconds. Such entrapments was shown to cause further signaling into the cytosol. Thus these regions can act as signaling stations.

![Figure 1.5: The motion of CD59 on the membrane](image)

We will see that the examples discussed previously are insufficient to explain the nanoscale organization of the plasma membrane, and we need to invoke the presence of the active actin cytoskeleton. The lipid matrix in which all membrane action takes place has an actively held apriori organization.

### 1.2 The Cell Cortex

#### 1.2.1 Assembly of Actin

Actin is one of three members of the filaments making up the cytoskeleton, the others being microtubules and intermediate filaments. It is present in abundance just below the plasma membrane, and one its primary roles is to act as a scaffolding that imparts rigidity to the plasma membrane. It is a long stiff polymer, with individual monomeric
sub units arranging themselves in a helical manner. Actin subunits have a bound ATP molecule, whose hydrolysis is more probable when the subunit is part of the polymer. On hydrolysis, the subunit changes its configuration to one that is reactively asymmetric: it offers different binding sites on its two faces. This results in the actin filament having an overall polarity, and we can distinguish its ‘plus’ end from its ‘minus’ end. In its hydrolyzed form, a subunit can un-bind from a filament more easily, while ATP bound monomers can add on to the filament more easily.

The ATP hydrolysis step distinguishes actin from other polymers as the two ends of the filament have different polymerization and depolymerization rates because of this step. This results in the plus of the filament growing at a much faster rate than the minus end. An important consequence of this is treadmilling: local differences in monomer concentration at the two ends of a growing filament arise if the the two ends polymerize and polymerize at different rates. If the rates of polymerization (depolymerization) are $k^+_p (k^-_d)$ for the plus end of the filament, and $k^-_p (k^+_d)$ at the minus end, the local steady state monomer concentration at the two ends will be,

$$C^+_m = k^+_d / k^+_p$$
$$C^-_m = k^-_d / k^-_p$$

By maintaining this difference in concentration, the filament is able to maintain a steady state length.

In the cell, actin polymerization is a controlled process involving nucleation, capping and decapping of actin monomers. The cell contains different types of nucleators of actin polymerization such as the ARP complex, formin proteins and spire, which can each result in different assemblies of actin [16]. The ARP complex for instance, nucleates actin by binding to one face of a subunit of the subunit, presenting the minus end for further polymerization. One binding site of the molecule sits on the side of a pre-existing filament, and another binds an actin monomer, thereby cross linking actin filaments at angles of roughly $70^\circ$.

Monomers can be prevented from entering a filament by binding to a ‘cap’ (eg: thymosin) which prevent them from interacting with either the plus or minus end of a filament. Most of the monomeric actin in the cell is in this form, and decapping of actin occurs only when initiated by some other factor. The capping of monomers is affected by the presence of
Figure 1.6: ARP Activity (Courtesy [16]): the ARP complex (in orange) binds to an existing actin filament (in blue), enabling cross linking of filaments, and initiating polymerization.

a third player, a protein (eg: profilin) that competes with the cap to bind to one face of the monomer, leaving the other face free to add on to a pre-existing filament. This competition between capping and inhibition of capping, sets the rate at which nucleated filaments grow.

1.2.2 Forces generated by actin

Polymerizing actin generates normal forces at a fluctuating surface. The mechanism of force generation has been understood in terms of the rectifying action of monomer addition [31]. As the surface thermally fluctuates, it is likely to create gaps between itself and the filament for a short time. In this short time, monomeric units can add on to the existing filament, thereby rectifying the motion of the surface.

Actin filaments also interact with the myosin family of motors, that transiently cross-link many actin filaments and affect their movement. Myosin II, one such motor, is a protein composed of two globular heavy chains (heads of myosin), and two light chains forming a coiled coil, as indicated in the figure 1.7. The heads of myosin can bind to two actin filaments, cross linking them, and hydrolyze ATP to walk from the minus end to the plus end of the filaments. The action of myosin gives a momentum to the bound parallel filament pair and makes an antiparallel pair of filaments slide with respect to one another in opposite directions. We discuss the consequences of actin-myosin interaction in greater detail in Chapter 4.
1.2.3 Actin near the membrane

Many molecular players exist that initiate actin polymerization at the cell membrane. Cdc42, a GTPase, is one such molecule. It is mostly present in its GDP-bound form in the cytosol. On activation, it brings about the polymerization of actin close to the membrane by recruiting the ARP complex. We will encounter this molecule in Chapter 3 as well, where we reason that it is one of the major players in recruiting actin that is predominantly vertically aligned with respect to the membrane. The recruitment of the polymerization machinery of actin to the surface is instrumental for cell locomotion and adhesion. The formation of cellular 'appendages' like lamellipodia, filopodia etc are triggered by the recruitment of the actin polymerizing machinery at the plasma membrane.

In the cell, actin has different arrangements according to the function that it serves. Most of the filaments in the 3D bulk of the cell form contractile bundles, serving as stress fibres (Figures 1.8 and 1.10) that adhere the cell to a substrate at focal points. Near the the plasma membrane, actin forms a cross linked 3D intermeshed shell, of about a micron in thickness. This shell in broken in parts to accommodate protrusive bundles of actin.
There is considerable evidence to suggest that membranal appendages such as microvilli, filopodia, lamellipodia, ruffles etc contain protrusive bundles of polymerizing actin. [16]. The force exerted on the membrane by these bundles of actin is presumably a combination of both polymerization as well as myosin activity.
1.3 The Active Composite

With this general introduction to the actin meshwork and its properties, we can now go on to understand its effect on the plasma membrane. Both polymerization and myosin cross linking of actin make it a machine capable of generating forces. Hydrodynamic theories of acto-myosin suspensions view the actin-myosin complex as a force dipole due to this property, exerting active forces proportional to the orientation (the active stress is given by $\sigma_{ij} \sim p_i p_j$, $p$ being the orientation) [40]. We have seen that actin is capable of directed force generation, which gives rise to shape changes. We now propose that such forces can also aid in molecular organization on the cell, by giving rise to directed fluxes of particles.

In chapter 2 we will describe a set of experiments probing the organization and dynamics of GPI-APs on the plasma membrane, and we conclude that this organization is highly dependent on the active forces exerted by a special architecture of cortical actin, organized tangentially (as opposed to vertically) with respect to the plasma membrane.

In chapter 3, we discuss the possible mechanisms by which cortical actin can change its architecture, thereby modulating its shape and composition.
Finally in chapter 4, we propose a coarse grained model of actively cross linked filaments, giving rise to directed fluxes at a surface. We suggest that these forces are responsible for the nanoscale organization of GPI-APs.