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Chacterization of Paramecium Tetraurelia Ciliary Membrane Plasma Membrane Calcium Pumps and Lipid Rafts

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CHARACTERIZATION OF *PARAMECIUM TETRAURELIA* CILIARY MEMBRANE PLASMA MEMBRANE CALCIUM PUMPS AND LIPID RAFTS

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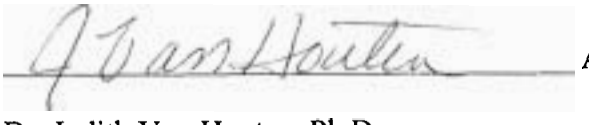

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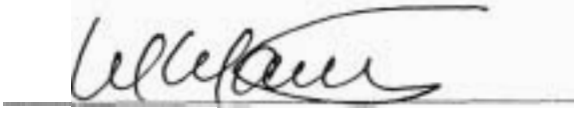

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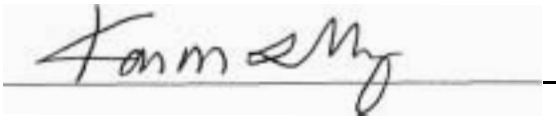

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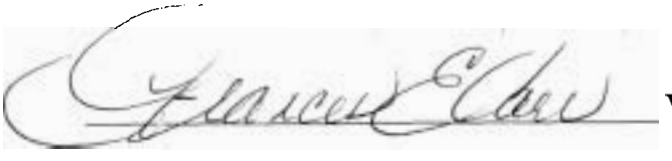

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ABSTRACT

Paramecium, a ciliate, is an important model for studying Ca^{2+} signaling and understanding chemoreception and signal transduction. There are several proteins, such as plasma membrane calcium ATPases (PMCAs)/ calcium pumps, SERCA pumps, calmodulin and Ca^{2+} channels that play an important role in maintaining intracellular Ca^{2+} level and signaling in *Paramecium*. Isoform 2 of PMCA has been identified in both the cilia and pellicle membranes of *Paramecium*, the activity of which leads to hyperpolarization. Plasma and ciliary membrane of *Paramecium* is made up of a variety of sterols and sphingolipids which constitute lipid rafts, demonstrated by the presence of detergent resistant membranes and their distribution in sucrose and Optiprep density gradients. PMCAs are important markers of lipid rafts and PMCA 2 is found to be localized in lipid rafts of both the cilia and somatic membrane of *Paramecium.* Methyl-β-cyclodextrin treatment can remove up to 42% of sterols from pellicle membranes but only about 12% from cilia. Sterol depletion of pellicle perturbs the distribution of PMCA 2 and other raft proteins in pellicle which is not observed in cilia as evident from western blot analysis and immunomicroscopic studies. There is evidence that selection of gradient medium for study of lipid rafts and its associated proteins is very important in *Paramecium*. Glutamate receptors and adenylyl cyclase, the upstream molecules of the signal transduction pathway through PMCA have also been identified in cellular cilia, indicating that these raft molecules forms a platform for signaling in *Paramecium* cilia.

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I am indebted to a number of people in my journey. I would like to thank my parents, my family and my friends who stood by me and supported all my decisions. I would like to thank my husband, who has been understanding and patient the last several years and has been a great asset in my life here at Burlington.

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CHAPTER ONE:

COMPREHENSIVE LITERATURE REVIEW

I. Introduction

Paramecium tetraurelia is a ciliated protozoon that can reach 150-200μm in length and is commonly found in ponds and other fresh bodies of water and feeds on bacteria. *Paramecium* supports 5000-6000 cilia, which are individually covered by a membrane that collectively accounts for about half of the total cell surface area. It contains two nuclei, a large somatic macronucleus, which contains many copies of the genetic material, is active in transcription and divides amitotically and a smaller micronucleus, which contains the genetic information, is inactive during transcription, divides mitotically during binary fission and undergoes meiosis. The life cycle of *Paramecium* consists of autogamy, conjugation and cytogamy. The genetic codon usage in *Paramecium* is unique; UAA and UAG encode glutamic instead of termination as in most other organisms (Allen, 1988).

Interestingly, like neurons, *Paramecium* has an excitable membrane that controls their swimming behavior. When the cell becomes hyperpolarized, mostly induced by posterior mechanical stimulation, chemical stimulation or manipulation of extracellular ions (Bonini et al, 1986), the cell swims faster forward with fewer turns. If the depolarization is strong enough, a calcium-based action potential is triggered, with an observable behavioral change. The intracellular Ca^{2+} concentration increases, which produce a change in the direction of the ciliary beating and an increased frequency, cause the cell to swim backwards (Eckert, 1972).

The primary role of the ciliary membrane which comprises about two-third of the total cell surface, is to provide a barrier between the locomotory apparatus and the external environment but it also plays a role as an ion-selective filter that is most important to the day to day survival of the unicell (Schultz, 1988).

Paramecium tetraurelia has more recently been developed as a system for understanding chemoreception and the nature of receptor binding and signal transduction in chemotaxis. Among the different receptors present on the cell surface that sense the presence of food are the glutamate, acetate, folate, biotin and cAMP. Interestingly, in the presence of glutamate, the amount of intracellular cAMP increases seven fold in 200ms in *Paramecium* (Yang et al.1997). Most likely this increase in cAMP activates PKA, which in mammalian system is known to activate the calcium pumps (James et al. 1989). Addition of cAMP and PKA increases the Ca^{2+}ATPase activity of the surface membrane preparations (Yang, 1997).

Ion channels in *Paramecium*

The distributions of ion channels in *Paramecium* are restricted to certain regions of the cell membrane in patterns (Plattner, 2002). When *Paramecium* encounters an anterior stimulus, a mechanoreceptor is activated and the cell becomes depolarized, which if large enough, an action potential arises resulting in backward swimming. The action potential has many similarities to that found in excitable cells, like neurons. The resting membrane potential of *Paramecium* is -40mV and can be depolarized. If the depolarization increases, an action potential occurs. However, unlike neurons, this action potential is graded and depends on the intensity of the stimulus. Additionally, the charge carrier during the initial depolarization phase in the action potential in *Paramecium* is Ca^{2+} instead of Na^{+}. This is very important as Ca^{2+} takes three times longer than Na^{+} to reach the peak of the action potential. This activates voltage gated K^{+} channels before the peak of the action potential resulting in a graded action potential. There are several important ions and ion channels that play a role in maintaining the resting membrane potential.

Ca^{2+} conductance and swimming behavior in *Paramecium*

Ciliary beating direction and frequency is mostly determined by the intracellular concentration of the free Ca^{2+} ion, causing the cell to swim in both forward and backward directions (Eckert, 1972). There are several calcium channels found in *Paramecium* including a leakage current, a fast voltage gated calcium channel that is inactivated by Ca^{2+}, a slow voltage gated current, a Ca^{2+} channel that is activated upon mechanical stimulation of the anterior, and a hyperpolarization induced current. The voltage dependent Ca^{2+} channels present in both the cilia and the somatic membrane is regulated by the Ca^{2+} binding protein calmodulin (CaM) (Plattner, 2002).

In depolarized cells, the ciliary reversal is triggered by Ca^{2+} based action potential, when intraciliary Ca^{2+} concentration increases (Eckert, 1972). The duration of backward swimming in *Paramecium* is longer when placed in K^+ rich solution when compared to an action potential. However, both type of backward swimming have been assumed to be induced by the Ca^{2+} influx through the same voltage gated Ca^{2+} channels (Oami and Takahasi, 2003). On the other hand chemical stimuli, manipulation of extracellular ions and posterior mechanical stimulation leads to hyperpolarization of cells leading to a faster forward swimming motion (Bonini et al., 1986). Studies have also shown that Paramecium cells have Ca^{2+} channels that are sensitive to temperature changes and are permeable to Ca^{2+}, Ba^{2+} and Sr^{2+} in the anterior soma membrane (Kutiu et. al., 1996).

Chemoattraction

Chemoattraction is a critical ability for the survival of *Paramecium*. Detection of chemicals in the environment, allows the organism to find mates, food or to detect dangerous environment. This ability to detect chemicals in the environment has been

found in bacteria and other lower eukaryotes like ciliates. Chemicals such as folate, biotin, glutamate, cAMP and NH_4Cl are known chemoattractant for *Paramecium.* There are apparently three signal transduction pathways in *Paramecium* which leads to hyperpolarization and ultimately swimming pattern of the cell (Van Houten, 1998). The first pathway involves acetate, biotin, cyclic AMP and folate. Specific binding sites, or receptors were found for all these chemical stimulants from binding kinetics, behavioral and electrophysiological studies (Sasner et al., 1989, Van Houten et al., 1991). Acetate, folate and biotin activate *Paramecium* PMCAs in a PKA independent manner, most likely through the binding of calmodulin as antisense to calmodulin disrupts chemoattraction to acetate among others (Yano et al., 1996). In the second pathway, the chemoattractant is glutamate, which was characterized by binding and behavioral studies. Glutamate binds to a receptor activating a K^+ channel, which in turn activates adenylyl cyclase (Yang et al., 1997). Adenylyl cyclase produces cAMP from ATP which then binds to protein kinase A (PKA). PKA activates the calcium pumps and phosphorylates the calmodulin binding domain (CBD) (Gannon-Murakami, 2004). NH_4Cl is involved in the third pathway, and is independent of any receptor.

Role of Ca^{2+} in *Paramecium*

Calcium plays a key role in the function and behavior of *Paramecium* including motility, trichocyst release for defense of predators, intracellular signaling, channel and pump regulation and chemoattraction. Therefore, maintaining the levels of calcium within the cell is challenging and critical to this single cell organism that encounters drastic changes of various ion concentrations present in the extracellular environment. There are several proteins that play a role in maintaining Ca^{2+} levels or intracellular

signaling through Ca^{2+} including, but not limited to, PMCAs, SERCA pumps, calmodulin and Ca^{2+} channels.

II. Introduction to cilia.

Cilia and flagella are interchangeable terms and are microtubule-based organelles found in single cell organisms and almost all cell types of higher organisms and are highly conserved structures. These structures are the major locomotory organelles for single cells like eukaryotes and sperms in higher organisms. In most organisms, they play a major role in cleaning the respiratory and reproductive tracts, excretion and water balance in some excretory organs, fluid circulation in coelomic cavity, brain and spinal cord, and selection and transport of food particles through gill surfaces. Apart from this, these organelles help in sensory functions, mating, mechanoreception, geotaxis, anchorage and chemoreception (Dentler and Witman, 1995).

Cilia constructed with a 9 + 2 microtubule pattern, generally are motile. The nine doublet microtubules surround a central pair of singlet microtubules. This central pair is important to generate force required for the ciliary motility (Davenport et al, 2005). Eukaryotic cells possess several hundreds of motile cilia in contrast to the non-motile cilia, which are usually solitary (Satir et al, 2007). Non-motile cilia or primary cilia, lack the central pair of microtubule and are referred to as 9 + 0 pattern. A third group of cilia, the nodal cilia, localized to the node of gastrulation – stage embryo, have a 9 + 0 architecture but possess the ability to move in a propeller – like fashion (Davenport et al, 2005) and help in the determination of left right asymmetry of the body (Satir et al, 2007). A fourth type of cilia containing the 9 + 2 arrangement but are non-motile have been identified as the kinocilium of hair cells in humans (Fliegauf et al, 2007). Cilia and

flagella are complex structures and are formed by specific protein targeting at the basal body area where pre-assembly of axonemal structures takes place. Intraflagellar transport (IFT) is responsible for the transport of proteins and protein precursors along the length of the axonemes to their functional assembly site. Loading of proteins onto IFT particles and their transport across the ciliary compartment border is known as compartmentalized ciliogenesis (Fligauf et al, 2007).

Cilia exert very different tissue specific functions during development, tissue morphogenesis and homeostasis, even though they have similar basic structure (Fligauf et al, 2007). Cilia related disorders or ciliopathies include a wide spectrum of pathologies, including polycystic kidney disease, hydrocephaly, ductal abnormalities in the liver and pancreas, embryonic and skeletal abnormality patterns and some modern day epidemic diseases like obesity, hypertension and diabetes (Davenport et al, 2005). However, most of the present understanding of the mammalian cilia depends upon the work on model microorganisms, like the ciliated protistans *Tetrahymena* and *Chlamydomonas* and recently on the model nematode *Caenorhabditis elegans* (Satir et al, 2007). Advancement in genomics along with mutant analysis has lead to the ciliary proteome of these organisms.

The most important feature of a living *Paramecium* cell is its ciliary beating. The cell possesses several thousand and they are motile with a 9 + 2 arrangement of axonemes. They are arranged in rows which follow the long axis over the dorsal surface and more or less curved around the oral region of the ventral surface (Allen, 1988). Ciliary beat in *Paramecium* takes place as a power stroke, when the cilium tends to be planar with the ciliary shaft largely straight in contrary to the return stroke when the cilia exhibits more circular motion with a curved ciliary shaft (Machemer, 1988).

It is assumed that Ca^{2+} can alter the regulation of frequency and ciliary beat orientation and is probably the primary candidate for the motor function because of its low concentration in the ciliary cytosol. In contrast, intracellular Mg concentration is maintained at a millimolar level and is impermeable to ciliary membrane, and its function is most likely restricted to competition or cooperativeness with intraciliary Ca (Machemer, 1988).

III. Proteins and Lipids of *Paramecium* ciliary membrane.

Lipids

Lipid composition is an essential factor, which affects membrane properties, in particular membrane fluidity. *Paramecium* requires sterol for growth. The ciliary membrane lipids are of special interest in view of the excitable properties associated with this organelle. Growth medium containing stigmasterol or sitosterol of plant origin supplements sterol requirement. Cholesterol as the only source of sterol does not support growth, however, when introduced, cholesterol is readily taken up and incorporated into cell and cilia membrane. This indicates that stigmasterol very specifically satisfies a sort of vitamin requirement of *Paramecium* (Allen, 1988).

When *Paramecium* cells are transferred to fresh medium, the rate of lipid uptake in the cell increases rapidly to almost about three times and as a result the lipid concentration increases to 7-13 ng/cell in the lag phase. The lipid content of the cell declines throughout the log phase and as a result during stationary phase, the lipid concentration in the cell is left to be only around 2-3 ng/cell. Both the cell and the ciliary membrane of *Paramecium* contain a variety of neutral and polar lipids

(Kaneshiro, 1987). Neutral lipids include triglycerides, free sterols, steryl esters and free fatty acids, whereas, the polar lipids are made up of ether lipids, sphingolipids and phosphonolipids. In the cell membrane changes in the neutral lipids is larger than the polar lipids in contrary to the ciliary membrane where the polar lipid fraction exhibit greater increase than the neutral lipids. Lipids of cilia when compared with the rest of the cell, the composition of the organelle has been found to be very distinct (Table 1) (Kaneshiro, 1987).

Cholesterol and glycosphingolipid enriched sub domains of cell membranes are recognized as lipid rafts in almost all types of cells. The highly saturated fatty acid side chain of the phospholipid allows the close packing of the saturated acyl chains of sphingolipids giving the rafts its characteristic lipid-ordered phase. (Pike, 2003). Lipid compositions of the two leaflets of the plasma membrane are very different. Most of the sphingolipids are present in the outer leaflet, while glycerophospholipids like phosphatidylinositol, phosphatidyl-ethanolamine and phosphatidylserine are enriched to inner leaflet (Munro, 2003). Though cholesterol can flip between the two leaflets spontaneously, preferably interacts with sphingolipids rather than unsaturated phospholipids (Remstedt and Slotte, 2002). The evidence of the high content of sterols and other lipids which are the characteristic lipids for raft formation in other organisms leads us to the hypothesis that the lipids of *Paramecium* plasma membrane is compartmentalized into raft like domains.

Paramecium has its distinctive lipid composition and metabolic pathways. The ciliary membrane of *Paramecium tetraurelia* contains a high concentration of ethanolamide sphingolipids. *Paramecium* takes up serine and other water-soluble substrates very slowly and catabolizes fatty acid rapidly to produce CO_2. *Paramecium* also contains sphingophospholipids, serine sphingophospho- and

sphingophosphonolipids along with six ethanolamine sphingophospho- and sphingophosphonolipids and some glycosphingolipids (Matesic et al, 1998). Ceramides have been found to be taken up rapidly by the cell by a phagosome independent process, which suggests that bulk transport is not the major mechanism for uptake of these components. Uptake of fatty acids is also competitively inhibited by other fatty acids, indicating that the transport involves a carrier-mediated mechanism (Kaneshiro, 1987). However, cold treatment decreased the rate of lipid uptake (Iwamoto, 2004).

Use of non-ionic detergents and hydrophobic drugs like local anesthetics could readily perturb the cell surface membrane most likely by their interaction with the membrane lipids. Alteration of membrane lipids also changed the activity of Ca^{2+} pumps and ion channels responsible for the generation of action potential (Forte et al, 1981).

Proteins

Recently about 539 ciliary membrane proteins have been detected using mass spectrometric analysis (Dr. Yano, personal communication). Proteomic studies have also shown the evidence of proteins that are abundant in the ciliary membrane like the class III Adenylyl cyclase, an isoform of PKA, plasma membrane calcium pump 18 and 19 and there are again less abundant proteins like the PMCA 3 and 4. Unfortunately we are still in search for proteins unique to the cilia. However, one and two-dimensional electrophoretic studies along with recent MS analysis have identified certain ciliary proteins which are thought of by analogy with other systems, to be involved in the regulation of vital functions in the cilia, such as excitability of the membrane or mechanical properties.

Guanylate cyclase

A guanylate cyclase is primarily localized in the ciliary membrane and is regulated most specifically by Ca ions (Linder et al, 2004). It has no activity at 100nM Ca^{2+}, which is the usual intraciliary calcium concentration. The cyclase is half-maximally activated at 10 μM Ca^{2+}. This guanylate cyclase is also detergent soluble. *Paramecium* guanylate cyclase is mediated only by calmodulin or a related Ca binding protein. Calmodulin is very much present in the cilia and the amino acid sequence shows the presence of trimethyllysine and dimethyllysine at positions 115 and 13 respectively. Guanylate cyclase being activated by Ca^{2+}, most likely is a intraciliary Ca sensor which can translate the incoming Ca signal during an action potential into a biochemical reaction sequence via cGMP-stimulated protein phosphorylation (Schultz, 1988).

Adenylate cyclase

The ciliary membrane from *Paramecium* contains a considerable fraction of the total cellular adenylate cyclase activity (Schultz and Klumpp 1983). The adenylate cyclase is a separate enzyme entity, not just a guanylate cyclase which happens to use ATP as substrate. Adenylate cyclase and guanylate cyclase are differentially localized in the ciliary membrane (Schultz and Klumpp 1983). *Paramecium* adenylate cyclase differs from most of the higher organisms in its activity being unaffected by GTP, forskolin, cholera toxin, and pertussis toxin. The protozoan adenylate cyclase is allosterically activated by Mg^{2+} via a single metal binding site separate from the substrate site with a Ks of 0.27mM (Schultz et al. 1987). Ca^{2+} inhibits in and uncompetitive manner, the extent of inhibition is dependent on the free Mg^{2+} concentration. Around the physiologically relevant and likely free Mg^{2+} levels of 1mM,

half-maximal inhibition is accomplished by 10μM Ca^{2+}, a meaningful Ca^{2+} concentration in the normal cellular setting (Schultz et al. 1987). With MgATP as substrate, K^+ very specifically activates adenylate cyclase at low millimolar concentration (Klumpp et al. 1984). In *Paramecium*, cAMP formation is stimulated by a potassium conductance, which is an intrinsic property of the adenylyl cyclase. The purified adenylyl cyclase homolog of *Paramecium* is a 96-kDa protein. The N-terminal 526 aa constituted a putative voltage-gated ion channel with six transmembrane helices. The C-terminal, the catalyst, is 339 aa long and consist of a single class III AC catalytic domain (CHD) linked to an individual C-terminal TPR (aa 802-835). In contrast to the S5-P-S6 pore-loop configuration, in *Paramecium* hydrophobicity and sequence analyses demonstrate a S5-S6-P arrangement, yielding a radically novel channel topology (Schultz et al. 2003).

Protein kinases

Protein kinases mediate actions of their respective Ca^{2+} and cyclic nucleotides. Cyclic nucleotide-dependent protein kinase like PKA, PKG and Ca^{2+} - (CaPK1 and CaPK2) has been purified from *Paramecium* (Walczak et al, 1993). A high ratio of cGMP-dependent to cAMP-dependent protein kinase activity was found in cilia and whole cell extracts (Miglietta et al, 1988). Microinjection of cyclic AMP and cyclic-GMP into cells modifies the ciliary beat of it (Lewis et al, 1981). Three different cyclic-nucleotide dependent protein kinases have been identified in unfractionated *Paramecium* cilia. Two of them are stimulated by cAMP and correlates to protein kinases type II and I. One protein kinase is activated preferably by cGMP over cAMP. The third type identified and partially purified in *Paramecium* is a Ca^{2+} stimulated protein kinase, which is not similar to the protein kinase C (Schultz et al, 1988). Like

mammalian protein kinase A, *Paramecium* PKA also contains regulatory subunits and catalytic subunits forming a holoenzyme. Two regulatory subunits of PKA have been found in cilia; a 70kDa and 220kDa (Hochstrasser, 1996). The regulatory subunit (R44) was cloned (Carlson and Nelson, 1996). Our lab also cloned a regulatory subunit of PKA and found several additional introns and changes in amino acid sequence, including ^{57}P-A, and ^{67}I-V (Sun, 2000).

The Plasma membrane Calcium ATPase

Plasma membrane calcium pump is ATP- driven and characterized by a high affinity and low capacity of Ca^{2+} ion transport compared to sodium calcium exchanger, which are involved in Ca^{2+} clearance at a very fast rate. Thus PMCA can bind to Ca^{2+} effectively even when the intracellular Ca^{2+} level is very low but higher than normal resting potential. PMCAs belong to the P-type primary ion transport ATPases. ATP is hydrolysed and transfers a phosphate to the PMCA which then forms an aspartyl phosphate intermediate. Ca^{2+}/calmodulin bind and activate the pumps further, increasing the affinity of the proteins Ca^{2+} binding site 20 to 30 times. Calmodulin also increases the rate of Ca^{2+} extrusion from the cell up to ten fold. The Ca^{2+} pumps are important in maintaining a very low concentration of Ca^{2+} in the cytosol (~10^{-7} M) against a much higher extracellular Ca^{2+} concentration (10^{-3} M). This steep Ca^{2+} gradient is maintained by the P-type Ca^{2+}-ATPase along with the Na^{+}-Ca^{2+} exchanger (an antiporter). This maintenance of low level of Ca^{2+} is important for many signaling pathway, since Ca^{2+} is an important second messenger (Chan et al, 1999).

One important key to controlling calcium levels in cells is the plasma membrane calcium ATPase (PMCA). Because the cell requires much lower concentrations of intracellular calcium than those in the extracellular environment, activation of the

PMCA plays a critical role in maintaining low levels of Ca^{2+} inside the cell, particularly in excitable cells. Activation of PMCA is energy dependent; ATP hydrolysis results in the movement of 1 Ca^{2+} ion up its concentration gradient across the plasma membrane. This removal of Ca^{2+} from the cytoplasm to the outside of the inevitably accumulates more positive charges outside the plasma membrane, resulting in cell hyperpolarization (Bonini et al, 1986).

The Ca ATP-ase activity is mostly described in red blood cells, since the calcium ATP-ase forms are predominant there. In this assay the enzymatic hydrolysis of ATP is determined by quantitation of the inorganic phosphate resulting from ATP hydrolysis from the pump. The Mg dependent, Ca^{2+} stimulated ATPase activity is calculated by subtracting the basal rate of ATP hydrolysis in the presence of Mg from the total rate in the presence of Ca^{2+} and Mg^{2+}.

Ca^{2+} enters paramecia during normal electrical activity, as a depolarizing inward current through the ciliary membrane. It is responsible for the momentary ciliary reversal. Ca^{2+} must then be sequestered and finally transported out of the cell (Lewis et al, 1980). *Paramecium* when deciliated using a Ca^{2+} shock, about 1% of the total cellular protein is released, which has a high Ca-dependent ATPase activity. Properties of ciliary membrane bound Ca^{2+} dependent ATPase is distinct from the soluble ATPase released by deciliation (Nelson et al. 1986). The ciliary membrane of *Paramecium* contains more than one ATPase, and has Ca^{2+} and Mg^{2+} stimulated ATPase activity that is firmly bound to the membrane. This membrane activity is distinct from the soluble Ca^{2+}-dependent ATPase of the deciliation supernatant (Nelson et al. 1986).

PMCAs are identified in *Paramecium*, and are thought to have 10 transmembrane domains, with a large intracellular component, with an ATP,

phospholipids and a C-terminal calmodulin-binding domain (CBD) (Figure A). Wright and Van Houten (1990) have found an ATPase localized in the cell body of *Paramecium*, which also binds to calmodulin. Vanadate, a general ATPase inhibitor, and calmodulin inhibitors in a Ca^{2+} ATPase assay inhibit the pPMCAs. Over 20 pPMCAs are found and five isoforms, pPMCA 2-4, 12 and 18 are cloned and expressed in *Paramecium* (Dr. Yano, personal communication); pPMCA1 is not been expressed. The pPMCAs are highly homologous to mammalian PMCAs (mPMCA) with conserved regions in the acylphosphate domain, the hinge region, the ATP binding site and the Ca^{2+} transport region. Like the mammalian PMCA, the C-terminus of *Paramecium* PMCAs contains potential sites for regulation including phosphorylation sites, calmodulin binding and protein- protein interacting.

Calmodulin, which is a Ca^{2+} binding protein, is a Ca^{2+} sensor of many molecules, which are simulated by Ca^{2+}. Western blot analysis of ciliary proteins of *Paramecium* using CaM labels identified up to 9 polypeptides in presence of Ca^{2+}. These include the axonemal and a soluble protein. In *Paramecium*, Doughty and Kaneshiro (1983) resolved 15 bands that had Ca ATP-ase activity in whole cell homogenates, 5 of which are present in cilia. Of the 5 ciliary Ca-ATPases, 2 represent axonemal dyenins that are of high molecular mass and are activated by Mg^{2+} over Ca^{2+}. One band appears to be a soluble activity often activated isolated in the deciliation supernatant. Therefore, of the 5 activity bands in cilia 2 are potential candicates for a Ca^{2+} pump. Along with the dynein ATP-ases, there could be other calcium transporting as well as other ion transporting ATPases.

The *Paramecium* plasma membrane calcium pump isoforms 1 and 2 were identified (Elwess et al. 1997) and found to be highly homologous to the mammalian pump with 42% identity and 65% similarity to the human PMCA 1 (Elwess and Van

Houten, 1997). We have cloned 2 more isoforms, making a total of 4. All of the isoforms have comparable similarity to the human sequence and all have CBDs, with highly conserved serines that were shown to be phosphorylated by PKA in the mammalian system (James, 1989). Isoform 1 probably is expressed at a very low level if ay all. I will work with the isoforms 2 and 3, which have a common antibody against them that binds to the calmodulin domain.

PMCA enzyme cycle

Plasma membrane calcium pump is ATP- driven and characterized by a high affinity and low capacity of Ca^{2+} ion transport compared to sodium calcium exchanger, which are involved in Ca^{2+} clearance at a very fast rate. Thus PMCA can bind to Ca^{2+} effectively even when the intracellular Ca^{2+} level is very low but higher than normal resting potential. PMCAs belong to the P-type primary ion transport ATPases. ATP is hydrolysed and transfers a phosphate to the PMCA which then forms an aspartyl phosphate intermediate (Carafoli, 1991). Ca^{2+}/calmodulin binds and activates the pumps further, increasing the affinity of the proteins Ca^{2+} binding site 20 to 30 times. Calmodulin also increases the rate of Ca^{2+} extrusion from the cell up to ten fold. The Ca^{2+} ATPases help to transport Ca^{2+} out of the cytoplasm as required by the cell. This maintenance of low level of Ca^{2+} is important for many signaling pathway, since Ca^{2+} is an important second messenger.

Coupling of Ca^{2+} with the ATP-ase is responsible for an ordered sequence, which occurs alternately at the cytoplasmic domain ATP site and at the calcium transport sites of the transmembrane domain of the pump. These two sites are distinctly apart and their conformational change plays an important role in their coordination. Thus in

P-type Ca^{2+} ATP-ases these two functional cycles are mainly described as E1 and E2 states. In the first step a high energy E1~P intermediate state with occluded Ca^{2+} is formed by the reversible ADP sensitive γ-phosphoryl transfer from ATP to the side chain of a conserved aspartic acid residue. A conformational change due to active cation transport across the membrane leads to the transition of the ATP-ase from the E1~P to the E2-P state. Translocation of a counterion through the cation conducting pathway in the opposite direction leads to the hydrolysis of the E2-P state. The cycle completes when the counterion is dissociated and the binding of the cytoplasmic cations and ATP is renewed (Oleson, 2004).

Paramecium ATPases:

Ciliary ATPases in *Paramecium* was expected to be present in the membrane due to its importance in maintaining intracellular Ca level in the somatic membrane and the cilia of most other organisms. Work has been done to find if the ciliary membrane contained enzymes similar or distinct from the rest of the cell and was found that ciliary membrane contains at least 2 major ATPases unique from the rest of the cell membrane (Doughty et al, 1983). Studies have been shown that ecto-ATPases and the activities found in cilia, pellicles and tricocysts are due to different enzymes. These enzymes were identified based on their electrophoretic mobilities on Triton-polyacrylamide gels. Ciliary Ca^{2+} ATPases exhibited affinity for $CaATP^{2-}$ and was inhibited by other divalent cations, La^{3+}, and phosphate but not by ADP or AMP. These suggest that the *Paramecium* ciliary membrane ATPase is distinct from other divalent cation dependent ATPase activity either in cilia or somatic membrane (Doughty et al, 1984).

This ciliary ATPase is maximally stimulated by millimolar concentrations of Ca^{2+} and is able to hydrolyze ATP and GTP and UTP and CTP and measurable rates. ATP is hydrolyzed in ciliary membrane at a 0.3μM Ca^{2+} concentration, which is unaffected by vanadate. It is thus possible that the activity of the Ca^{2+} transporting of ciliary membrane is too low to be detected over the high specific activity of the Ca^{2+} or Mg^{2+} ATPase (Travis et al, 1986). In the pellicle membrane of *Paramecium*, a putative Ca^{2+} transporting ATPase was first biochemically characterized (Wright, 1990). According to this study, calcium pumps are predominant in the somatic membrane and not in the cilia. The work done recently disproves this theory and has evidence that an active pump is also present at the ciliary membrane. However, its rate of activity and the pH sensitivity of the pump are yet to be understood.

IV. Lipid Rafts

Lipid rafts are small, specialized microdomains found in the plasma membrane of most of the cells. They are characterized by their high content of cholesterol and sphingolipids, cold non-ionic detergent insolubility (hence, known as detergent resistant membrane, DRM) and low buoyant density (Pike, 2003). Recent researchers believe that hydrogen bonding between the 3-OH group of cholesterol and the amide group of sphingolipids segregates these lipids away from the rest of the plasma membrane, helping them form tightly formed ordered domains, coalesced by GPI-anchored or transmembrane proteins cross-linked by ligands or antibodies (Ilangumaran, et. al. 1998) (Figure B). Such small rafts can sometimes be stabilized to form larger platforms through protein-protein interaction and protein-lipid interactions (Pike, 2006). Microtubules like actin and tubulin are resident in lipid rafts, which interact with raft proteins (Nebl et al, 2002) (Figure C). Although some recent studies

show that lipid rafts and actin are not directly related to protein-protein interaction, they could not deny the fact that rafts are important for their signaling capabilities.

The biochemical definition of rafts is their detergent insolubility. Rafts are recovered in low-density fractions after cold Triton extraction and density gradient centrifugation. Even though the use of Triton X-100 to isolate these detergent insoluble membranes is most common, a non-detergent based isolation is also being used. Use of sodium carbonate as a source of high pH, also segregates raft fractions in a similar way like the detergents does. Among these density fractions, the ones that are associated with membrane skeleton have higher characteristic density and are referred to as DRM-H compared to the DRM-L fractions. In other words, DRM-H are DRM-L fractions attached to membrane skeleton (Pike, 2006).

While rafts have a distinctive protein and lipid composition, all rafts do not appear to be identical in terms of either the proteins or the lipids that they contain. A variety of proteins, especially those involved in cell signaling, have been shown to partition in lipid rafts. As a result, lipid rafts are thought to be involved in the regulation of signal transduction. Growth factor signaling receptors like EGF, PDGF are localized in lipid rafts and are modulated by changes in cellular cholesterol content (Zurzolo et al, 2003). Multi-chain immune receptors like IgE, T-cell receptor and B-cell receptor are translocated to lipid rafts upon cross-linking, activating the Src-family kinases. Rafts also play an important role in cytoskeletal organization, protein transport and protein sorting by exocytosis and endocytosis. Many viruses use rafts as their entry and exit ports most likely because rafts make up a large portion of the cell surface and help in endocytosis, which is the first requirement for many of them (Ilangumaran, 1998).

When lipid rafts are perturbed through chemical or genetic manipulations, cells tend to loose their raft mediated signaling functions. This shows the requirement of raft

compartmentalization of signaling molecules. Since rafts are highly enriched in cholesterol, removal or reduction of its content sometimes redistributes proteins away from lipid rafts membrane domains. Some most common techniques for this approach are sequestration of cholesterol, its depletion and removal using methyl-β-cyclodextrin, inhibition of cholesterol synthesis or even genetic approaches like knockout animal models. In some studies, interestingly, signaling function is restored with the restoration of cholesterol in them (Simons, 2002).

In conclusion it can be said that despite of so much research in this field it is still hard to understand its structure and function adequately, partly because of their very small size and the inability to study them intact in live cells.

Based on our literature review, we know that intracellular Ca^{2+} concentration regulates exocytosis and ciliary beat reversal along with other major functions, and hence, maintenance of low level of Ca^{2+} is important for its proper functioning. We also know that PMCAs play an important role in the calcium extrusion from the cell body membrane in *Paramecium*, like many other organisms. In this study we took a complete biochemical approach to see if the plasma membrane calcium pumps are present in the ciliary membrane of *Paramecium*, and if so which isoforms are present. Again PMCAs have been found in the lipid rafts of the cell body membrane and their distribution in the rafts are perturbed when sterols are depleted from the membrane. We, therefore, hypothesize that the *Paramecium* ciliary membranes have active PMCAs and that these pumps are associated with the lipid rafts in this organelle.

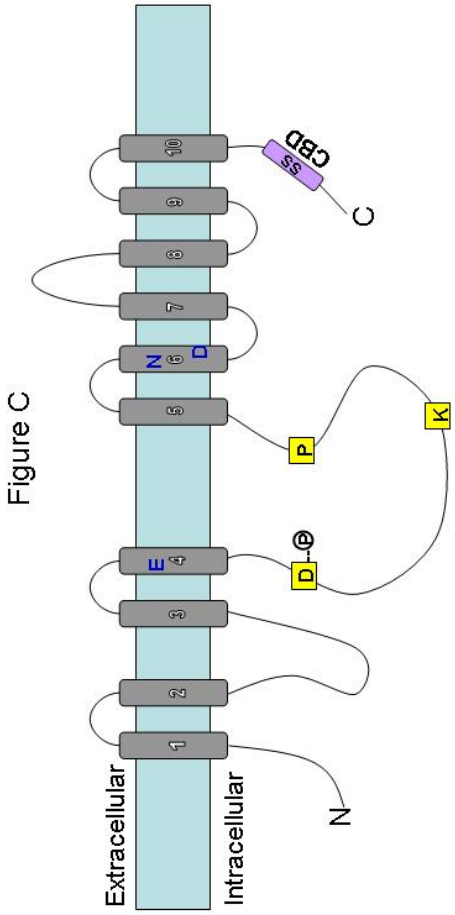
Table 1: Composition of *Paramecium tetraurelia* whole cell and ciliary lipids (Kaneshiro, 1987)

Lipids	***Weight % of Cell***	***Weight % of Cilia***
Cholesterol	3.6	5.2
Stigmasterol	9.7	10.3
Choline sphingolipids	2.1	2.5
Ethanolamine sphingolipids	3.8	15.5
Total neutral lipids	48.1	32.9
Total phospholipids	51.9	67.2

Figure A: Protein structure of *Paramecium* PMCA.

The protein model of *Paramecium* PMCA shows 10 transmembrane domains, the ATP and Ca^{2+} binding site and the calmodulin binding domain. Both the C and the N terminals of the protein are present in the intracellular region.

Figure A:

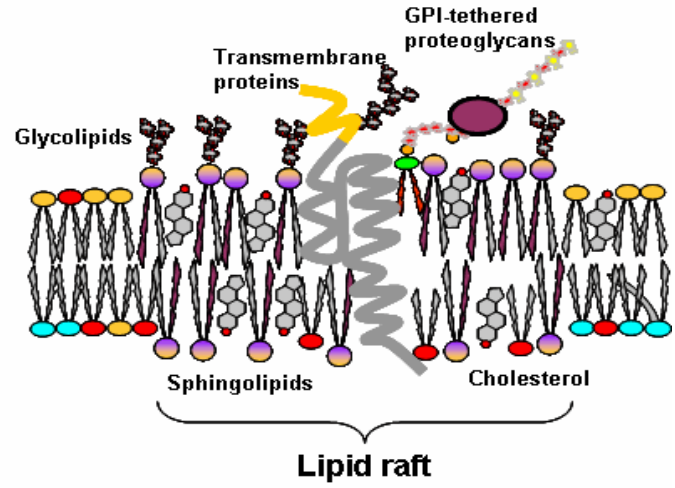


Courtesy Yunfeng Pan

Figure B: Model of lipid raft.

A typical model of membrane lipid raft showing the cholesterol and sphingolipid rich sub domain in the cell membrane. GPI anchored proteins and transmembrane proteins are associated with this raft along with other peripheral proteins.

Figure B:

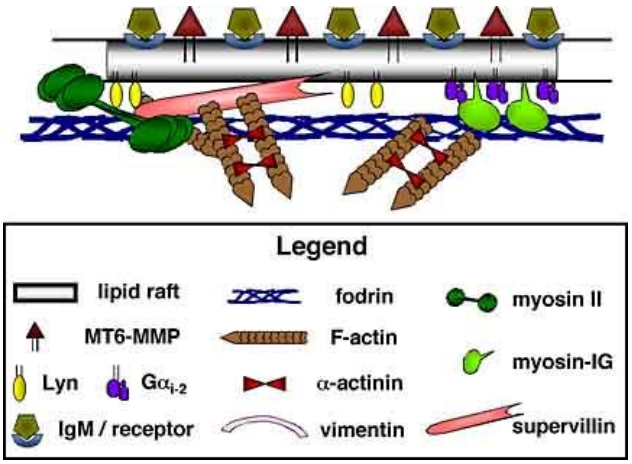


www.steve.gb.com/images/science

Figure C: Model of raft with cytoskeletal association.

Raft model showing the association of cytoskeletal proteins along with other integral membrane proteins. Some these cytoskeletal proteins are actin, myosin and supervillin.

Figure C:



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CHAPTER TWO:

IDENTIFICATION OF THE LIPID RAFTS IN CILIARY MEMBRANES AND THE DISTRIBUTION OF PUMPS IN THEM

INTRODUCTION

Paramecium tetraurelia is a single-celled ciliate that can reach 150-200μm in length. The cell body supports 5000-6000 cilia, which are individually covered by a membrane that collectively accounts for about half of the total cell surface area. The somatic and ciliary membranes are physically continuous differing in biochemical compositions. Its general habitat is fresh water and it feeds on bacteria (Allen, 1988). Interestingly *Paramecium* has an excitable membrane that controls their swimming behavior. When the cell becomes hyperpolarized, the cell swims faster forward with fewer turns; when the cells become depolarized enough to induce an action potential. *Paramecium* swim backward briefly during the depolarization and when the cell repolarizes, *Paramecium* swims forward in a new random direction (Schultz, 1988).

The primary role of the ciliary membrane is to provide a barrier between the locomotory apparatus and the external environment but it also plays a role as an ion-selective filter that is most important to the day to day survival of the unicell (Allen, 1988). *Paramecium tetraurelia* has more recently been developed as a system for understanding chemoreception and the nature of receptor binding and signal transduction in chemotaxis. Among the different receptors present on the cell surface that sense the presence of food are the glutamate, acetate, folate, biotin and cAMP. Interestingly, in the presence of glutamate, the amount of intracellular cAMP increases seven fold in 200ms in *Paramecium* (Yang et al.1997). Most likely this increase in cAMP activates PKA, which in mammalian system is known to activate the calcium pumps (James et al. 1989). Addition of cAMP and PKA increases the Ca^{2+}ATPase activity of the surface membrane preparations (Yang, 1995). The kinase inhibitors H7 and H8 were shown to disrupt glutamate chemoattraction and H89 inhibit Ca-ATPase activity of the *Paramecium* membrane (Elwess and Van Houten, 1995). These results

suggest that glutamate indirectly increases the cAMP levels and activates PKA, which then phosphorylates the calmodulin binding domain of the PMCA and helps maintain hyperpolarization of the cells.

Membrane rafts are characterized as small (10-200 nm), heterogeneous, highly dynamic, sterol- and sphingolipids-enriched domains that compartmentalize cellular processes. Protein-protein and protein-lipid interactions helps to form stable large platforms originating from small raft domains (Pike, 2006). They are resistant to solubilization by the non-ionic detergent Triton X-100 and sensitive to cholesterol depletion (Munro, 2003). While rafts have a distinctive protein and lipid composition, all rafts do not appear to be identical in terms of either the proteins or the lipids that they contain. A variety of proteins, especially those involved in cell signaling, have been shown to partition in lipid rafts. As a result, lipid rafts are thought to be involved in the regulation of signal transduction. Rafts may contain incomplete signaling pathways that are activated when a receptor or other required molecule is recruited into the rafts (Pike, 2003). Rafts also play important roles in membrane trafficking, cytoskeletal organization, and pathogen entry. A variety of proteins have been found to be enriched in lipid rafts. The major resident raft proteins are those attached to the bilayer by covalent linkage to glycosylphosphatidylinositol (GPI) lipid anchors (Munro, 2003). Other proteins like caveolins, flotillins, PMCAs, low molecular weight and heterotrimeric G proteins, src family kinases, EGF receptors, platelet derived growth factor receptors, endothelin receptors, MAP kinase, protein kinase C are also raft marker proteins (Pike, 2003).

In this study we take a purely biochemical approach to identify lipid rafts in *Paramecium* ciliary membrane. We also suggest that like other organisms lipid rafts

here forms a platform for glutamate receptors, adenylyl cyclase and PMCA which all together help maintain the intraciliary Ca^{2+} level which results in smooth forward swimming.

Materials and methods:

Maintaining 51-S wild type and PMCA transformed Paramecium cells:

Stocks of 51-S wild type cells were maintained at room temperature in a culture media containing regular wheat grass (Pines, Lawrence, KS), 2.0gm/lit sodium phosphate ($Na_2HPO_4.7H_2O$) and 5mg/ml stigmasterol (Sasner and Van Houten, 1989). *Aerobacter aerogenes* was added to the media as the main source of food for the *Paramecium*. For transformed PMCA-HA tagged cells, ND 6 cells (ND6 are non trichocyst discharge mutants with similar electrophysiological properties as wild type cells (Dr. Yano, personal communication)) were microinjected with the PMCA 2, 3 or 4 plasmid tagged with Hemagglutinin epitope tag (HA), by Dr. Junji Yano. Stocks of these transformed cells were maintained in 10ml of bacterized regular wheat grass media containing 2 mg/ml Paromomycin sulfate (Sigma) and grown at 22°C. Every alternate day about 20 cells were transferred to a fresh 10ml culture to make sure that the cells are well fed. pGlu-GFP transformed cells are maintained in a similar way as the PMCA transformed cells are.

Paramecium Genomic DNA Isolation:

Paramecia were grown in 100 ml wheat grass medium at 28°C till stationary growing phase, obtained from cell counts. Cells were harvested by filtering through Kimwipes tissue and pelleted by centrifugation at 350xg for 2 minutes in IEC HN-DII

centrifuge. Cells were washed twice in 10 ml Dryl's solution (1 mM $NaH_2PO_4 \cdot H_2O$, 1 mM $Na_2HPO_4 \cdot 7H_2O$, 2 mM sodium acetate, 1.5 mM $CaCl_2$, pH6.8). Each time the cells were pelleted by centrifugation at 200xg for 2 minutes in the IEC Clinicla Centrifuge. The cell pellet was suspended in 100 μl Dryl's solution and transferred into 2 ml Microfuge tube. To the cell suspension, 200 μl Denaturing solution (Promega) was added, mixed well by inversion several times and followed by addition of 30 μl 3 mM sodium acetate (pH 5.2), and mixed well. An equal volume of phenol/chloroform/isoamyalcohol (25:24:1) was added to the sample, mixed well and kept on ice for 10 minutes. The sample was centrifuged at 16,000xg for 20 minutes in the Eppenddorf centrifuge 5414C at 4°C. The aqueous phase (upper layer) was transferred to a new 1.5 ml Microfuge tube, followed by the addition of same volume of 100% isopropanol and mixed well. The sample was kept at -20°C for 30 minutes to precipitated DNA. The sample was centrifuged at 16,000xg for 20 minutes at 4°C to pellet DNA. The DNA pellet was washed 3 times with 500 μl 70% ethyl alcohol. Each time, the sample was centrifuged at 16,000xg for 5 minutes at 4°C, to discard the supernatant. The sample was placed in fume hood for 20 minutes to vaporize the remaining ethanol. The genomic DNA was dissolved in 50 μl of sterilized MilliQ H_2O and stored at -20°C for future use. The concentration of the DNA was measured by using the Nanodrop quantitation (Nanodrop).

Isolation of whole cilia from Paramecium:

For 51-S cells, about 500μl of the stock was added to 100ml of bacterized regular wheat grass culture media and was incubated at 28°C for the cells to reach logarithmic phase of growth. Cells were filtered, centrifuged in pear shaped tubes using IEC HN-SII centrifuge and cleaned for an hour in 10ml Dryl's solution containing 100μl of

50mg/ml Gentamycin (Invitrogen) in a centrifuge tube. The tube was centrifuged using IEC Clinical centrifuge and the cleaned cell pellet was transferred to 250ml of bacterized regular wheat culture medium. After two days, the cells were cleaned again in Dryl's and gentamicin in a method described above, to move to an even higher volume of 500ml of regular wheat culture. Finally the cells were transferred to a 1.5 L bacterized flask of "Improved" wheat culture (wheat grass, proteose peptone (Oxoid), TrisHCl, Dibasic anhydrous sodium phosphate ($Na_2HPO_4.7H_2O$), Monobasic monohydrate sodium phosphate ($NaH_2PO_4.H_2O$), and 5mg/ml stigmasterol). Cells were counted every day and were allowed to grow for 2-3 days or until they reach a density of around 15,000/ml.

For transformed cells, 500µl of the stock was added to 100ml of bacterized regular wheat grass culture media and was incubated at 22°C for the cells to reach its logarithmic phase of growth. The cells were not cleaned and directly added to 1.5 L bacterized flask of "Improved" wheat culture and allowed to grow at 22°C for it to reach a density of 10-12,000/ml.

For all types of cells when they reached the logarithmic phase, determined by the cell count (15,000/ml for wild type cells and 10,000/ml for transformed cells), the cells were harvested from a total volume of 6 liters. Cells were filtered through cheesecloth and Kimwipes and concentrated using a continuous flow IEC clinical chemical centrifuge at a speed of 200g. Concentrated cells were washed three times with Dryl's solution at room temperature. IEC HN-SII centrifuge was used to concentrate the cells between each wash. The cells were then resuspended in 50ml Dryl's and 50ml ice cold STEN (0.5 M sucrose/ 20mM Tris-HCl/ 2mM Na_2EDTA/ 6mM NaCl (pH 7.5)) buffer and placed on ice for 10 minutes till the cells were immobilized. The cells were deciliated by the addition of $CaCl_2$ and KCl to final concentrations of 10 mM $CaCl_2$ and

30mM KCl. Deciliation was monitored by phase contrast microscope, to make sure that most of the cells are intact and only cilia was removed, and the process was normally completed within 5 minutes. Cell bodies were removed by centrifuging the suspension at a speed of 850 x g. The supernatant was carefully removed and spun again to make the cilia suspension completely free of cell bodies. The supernatant was transferred to clear 30ml Corex glass tubes and centrifuged at 28,000g in s JA-20 rotor of a Beckman centrifuge for 20 minutes at 4ºC. The pellet is resuspended in 500µl of Membrane buffer (0.1mM Tris HCl, 8mM Tris base, 50mM KCl, 5mM $MgCl_2.6H_2O$ (Sigma), and 1mM Na_2 EGTA). A cocktail of protease inhibitor containing 1mg/ml phenylmethylsulfonyl fluoride (PMSF) (Sigma-Aldrich), 0.1mg/ml of Pepstatin (Research Products International) and 0.1mg/ml Leupeptin (Research Products International) was added to prevent protein degradation. The protein was stored at -80°C for up to two weeks.

Isolation of crude pellicle from Paramecium:

Cells were cultured and harvested in a similar way as for the whole cilia preparation. Concentrated cells were washed twice using HM buffer (20mM Tris Maleate and 1mM Na_2 EDTA, pH 7.8) and spun in pear shaped tubes at 200 x g. Clean pellet was then washed in HMI buffer(HM buffer containing protease inhibitors 1mg/ml PMSF, 0.1mg/ml Leupeptin and 0.1mg/ml Pepstatin) and spun again. The pellet was then transferred to a cold Potter Elvajehm homogenizer and the volume was brought up to 15ml by adding more HMI buffer. The pestle was driven by an electric drill was used to create suction in the homogenizer and cells were deciliated and lysed open. The homogenate was observed periodically under a phase microscope to assure that almost 75% of the cells were lysed open. The homogenate was washed in

HMI buffer for at least 6 times and each time the suspension was centrifuged at a speed of 3,440 x g for 5 minutes using the JA-20 rotor at 4°C. The final pellet was resuspended in 1ml of Membrane Buffer containing the protease inhibitors 1mg/ml PMSF, 0.1mg/ml Leupeptin and 0.1mg/ml Pepstatin. The protein was stored at -80°C for up to one week.

Triton X-100 solubilization of crude pellicle and whole cilia:

Crude pellicle or whole cilia was added to cold 1% Triton X-100 (Sigma-Aldrich, Ca # T9284 – 100ml), vortexed thoroughly, and incubated on ice with agitation for 1 hour. The solubilized homogenate was then spun in a microfuge tube at 13,100g at 4°C for 10 minutes. The Triton soluble supernatant was removed from the Triton insoluble pellet by pipetting it out very carefully. The insoluble pellet was saved for immediate use for density gradient studies and the soluble supernatant was subjected to acetone precipitation.

Sodium Carbonate Extraction of Pellicle Proteins:

Pellicle preparations (500 μg) were suspended in 1 ml 0.1 M Na_2CO_3 (pH11.5) and rocked at 4°C for 1 hour. The sample was centrifuged at 13,100xg at 4°C for 8 minutes (Eppendorf centrifuge 5415c) to pellet the protein. The pellet was resuspended in one volume of 2x SDS/sample buffer (0.5 M Tris-HCl, 20% glycerol, 4% SDS, 0.2 M DTT, 0.001% bromophenol blue, pH 6.8) and analyzed by SDS-PAGE and Western blotting.

Isolation of pure pellicle from Paramecium:

Cells were cultured and harvested in a similar way as for the whole cilia preparation. Concentrated cells were washed twice using cold Dryl's solution and spun in pear shaped tubes at 200 x g. The cells were then resuspended in 50ml Dryl's and 50ml ice cold STEN (0.5 M sucrose/ 20mM Tris-HCl/ 2mM Na_2EDTA/ 6mM NaCl (pH 7.5)) buffer and placed on ice for 10 minutes till the cells were immobilized. The cells were deciliated by the addition of $CaCl_2$ and KCl to final concentrations of 10 mM $CaCl_2$ and 30mM KCl. Deciliation was monitored by phase contrast microscope, to make sure that most of the cells are intact and only cilia was removed, and the process was normally completed within 5 minutes. Supernatant containing the cilia was removed by centrifuging the suspension at a speed of 850 x g. Cell pellet was then washed in HMI buffer(HM buffer containing protease inhibitors 1mg/ml PMSF, 0.1mg/ml Leupeptin and 0.1mg/ml Pepstatin) and spun again. The pellet was then transferred to a cold Potter Elvajehm homogenizer and the volume was brought up to 15ml by adding more HMI buffer. The pestle was driven by an electric drill was used to create suction in the homogenizer and cells were deciliated and lysed open. The homogenate was observed periodically under a phase microscope to assure that almost 75% of the cells were lysed open. The homogenate was washed in HMI buffer for at least 6 times and each time the suspension was centrifuged at a speed of 3,440 x g for 5 minutes using the JA-20 rotor at 4ºC. The final washed pellet was resuspended in HMI and mixed 1:1 with 2.1 M sucrose dissolved in HMI. The pellicles were purified further through a discontinuous 1.72/2.1 M sucrose gradient dissolved in HMI by centrifugation at 1950 x g for 3 hours at 4ºC. The pellicles were collected from the 1.72/2.1 interface, diluted 1:1 in membrane buffer (0.1mM Tris HCl, 8mM Tris base, 50mM KCl, 5mM $MgCl_2.6H_2O$ (Sigma), and 1mM Na_2 EGTA) and centrifuged for 10

minutes at 6750 x g. The pellet was resuspended in 1 ml membrane buffer, aliquoted in 0.2ml portions in microfuge tubes and stored at -20ºC, which was stable for a week.

Sucrose gradients for Lipid rafts:

The insoluble crude pellicle or cilia pellet was mixed with equal volumes of cold 80% sucrose solution made in TNE buffer (5mM Tris base, 1M Na_2 EDTA, 5M NaCl) to get a final concentration of 40% sucrose in the insoluble pellet. 500µl of this pellet was put into the bottom of six ice-cold Ultra-clear (11 x 60 mm) ultracentrifuge tubes of 4.5ml volume. It was overlaid with 4ml of 5-40% continuous linear sucrose gradient, made using a continuous gradient maker and a Buchler Duostaltic pump. The tubes were balanced by brining them up to volume using the remaining 5% sucrose solution in TNE. The ultracentrifuge tubes were then placed in ice-cold Beckman SW 60 Ti rotor swinging buckets and the tubes were spun at a speed of 160,000xg at 4ºC for 4 hour in a Beckman L8-70M preparative ultracentrifuge. The contents of each of the six ultra clear tubes were divided into nine fractions of 500µl each. Similar fractions from all the tubes were pooled together. About 100µl of each fraction was set aside for density analysis and protein assay. Fractions were dialyzed against cold distilled water at 4ºC for almost 24 hours and were then subjected to protein precipitation using four times the volume 100% acetone at -20ºC overnight. The protein pellet was obtained by centrifugation using JA-17 rotor of the Beckman centrifuge and then washed with 90% acetone and further centrifugation. Final pellets were air dried and resuspended in 25µl membrane buffer, 25µl 2X SDS sample buffer (2X Tris HCl, glycerol, SDS, Bromophenol blue, pH 6.8) and 2µl β-Mercaptoethanol (BME) (Sigma-Aldrich).

Optiprep gradients for Lipid rafts:

The Triton X-100 insoluble pellet from whole cilia or pellicle was mixed with twice the volume of Optiprep solution (Axis Shield Ca # 1030061). Optiprep is a density gradient medium containing 60% (w/v) solution of iodixanol in water. 600µl of the suspension was taken into each of the six ultra clear centrifuge tubes. Optiprep is diluted in Solution D containing 150mM NaCl, 5mM Dithiothreitol, 5mM EDTA, 25mM Tris HCl, 1% Triton X-100, pH 7.4, to get 35%, 30%, 25% and 20% (w/v) Optiprep. The sample was overlaid with 600µl of all the four gradients starting the highest at the bottom, forming a step gradient of Optiprep. Tubes were filled and balanced with solution D and centrifuged in a similar way like the Sucrose gradient. To each of the nine pooled fractions, 10-12 mls of ice cold solution D was added and centrifuged in JA-20 rotor of Beckman at 19,000rpm (44,000g) for 30 minutes. Final pellets were air dried and resuspended in 25µl membrane buffer, 25µl 2X SDS sample buffer and 2µl β-Mercaptoethanol (BME).

Densitometry analysis of sucrose gradients:

The refractive index of the nine lipid raft fractions obtained from sucrose gradient was calculated using the Reichert-Jung ABBE MARKII digital refractometer. 5µl of the control and the sample were aliquoted onto the flat surface of the machine separately. The sample was covered and the reading was taken at normal settings and was compared to the standard densities. The standard densities to compare the samples were made from 40% and 5% sucrose. The corresponding temperature values for each sample were also taken. The entire process was repeated two times for each set of sucrose density gradient samples.

BCA Protein assay:

The concentrations of the proteins isolated from *Paramecium* were determined using a BCA Protein Assay Reagent (Pierce, Ca # 23223) kit. The standard curve was obtained from 6 dilutions, 50, 100, 150, 200, 250 and 300µg/µl of the Bovine Serum Albumin (BSA 2mg/ml, provided in the BCA protein assay kit). Protein samples were diluted in the ratio of 1:10 and 1:20. Reagent A and B from the kit was mixed in a ratio of 49:1. 2ml of the dye mixture was added to all the standards and the samples and mixed thoroughly. The tubes were then incubated at 37ºC for 30 minutes. The absorbance of the standards and samples was read at 562nm using a spectrophotometer (Agilent). Protein concentration of the samples was then calculated from the standard plot.

Cholesterol depletion:

Paramecium cells grown at 28ºC or 22ºC were concentrated using the continuous flow centrifuge. The concentrated cells were then incubated in 300ml of chemokinesis buffer (1mM Calcium citrate, 1mM Tris base, 2mM NaCl, pH 7.05) containing 1.8g methyl- β cyclodextrin (MBCD) for 2 hours at 28ºC after which the cells were washed with Hm and HMI buffer as described earlier. The cells were harvested and crude membranes were obtained as explained before. These membrane preparations were then used for protein assay, cholesterol assay, and lipid raft analysis.

Cholesterol assay:

50µl of various concentrations (0, 1, 2, 3, 4, 5, 6, 7 and 8µg/ml) of cholesterol reference standard (Amplex Red Kit, Molecular Probes, Ca # A-12216) were made

using sterile MQ water. From the protein assay result, 60µg of the cholesterol-depleted sample was pipetted in to a well of the plate and brought to 50µl volume using sterile MQ water. Hydrofen peroxide provided in the kit was used as the positive control. The 96 well plate with the cholesterol standard, samples and the hydrogen peroxide was treated with 50µl of 300 µM Amplex Red reagent containing ~20mM Amplex Red reagent stock, 2U/ml HRP, 2U/ml cholesterol oxidase, and 0.2U/ml cholesterol esterase and was incubated at 37ºC for 30 minutes away from light. The plate was read using a fluorescent microtiter plate reader (excitation 530nm, emission 590nm) and standard curves were plotted. The concentration of cholesterol was obtained from the standard plot and the percent of cholesterol depleted was then calculated.

Sodium Dodecyl Sulfate – Polyacrylamide Gel Electrophoresis (SDS-PAGE):

The proteins were mixed with 2X sample buffer (2X Tris HCl, glycerol, SDS, Bromophenol blue, pH 6.8) and β-Mercaptoethanol (BME) (Sigma-Aldrich) was added to it. Samples were boiled for 5 minutes in water and placed on ice immediately. They were then loaded on to a 8-15% SDS polyacrylamide gel containing a 3.9% stacking gel. Benchmark Pre-Stained Protein Ladder was used to run along with the samples. The gel was run at a constant voltage of 170 till the dye reaches the bottom of the gel.

Transfer of proteins to Nitrocellulose Paper:

Once the protein migrates to the bottom of the gel, they are ready to be transferred to the nitrocellulose paper. The gel is placed in contact with the paper and they are placed between stacks of blotting paper and foam. The whole set up is placed in Transfer buffer (0.04M Glycine, 0.05M Tris base, and 20% Methanol) and the transblot was set up at 250 milliamps for 60 minutes followed by 390 milliamps for 90

minutes. The nitrocellulose paper was immediately removed, dried and saved at -20ºC for later use.

Antibodies:

Anti-CBD is an affinity purified rabbit polyclonal antibody, which is against KLH peptide (KPSFILELRRGSSLRKK) of PMCA isoform 2 (Biosource Caramillo, CA). Anti-N-terminus 3 (N-3), a rabbit polyclonal antibody, is made against a GST- fusion protein with the sequence of MNQSALKTDVNVTVREEEMRLNMGG, which was then purified to remove anti-GST (Bethyl Lab Montgomery, TX). Anti-acetylated tubulin, a mouse monoclonal antibody, is purchased from Sigma (St. Louis, MO). Anti-pPCM, a rabbit polyclonal antibody, is made against a GST-fusion protein with the sequence of 90 amino acids from the homologous region of *Paramecium* PCM protein by Lampire Biological Lab Inc. (Pipersville, PA). Anti-surface antigens, a rabbit polyclonal antibody, donated by Dr. Jim Forney, is made against the salt-ethanol wash proteins of *Paramecium,* which expresses surface antigen A or B. Antibody against the N-terminal of *Paramecium* adenylyl cyclase (pAC-N) was used.

Cloning and expression of PCM and generation of a polyclonal antibody against it:

An antigenic epitope which is common for all the four isoforms of PCMs are selected and about 250bp was amplified by PCR. The product and pGEX-2TK (Amersham Pharmacia) was simultaneously digested using restriction enzyme BamH1 (Invitrogen). Linearized pGEX vector was dephosphorylated. The samples were resolved in an agarose gel and the band of interest was excised and extracted from the gel using the Zymoclean gel DNA recovery kit (Zymo research, California). Gel

eluted DNA samples were then ligated using the clonables ligation mix (Novagen). The ligation mixture was then transformed to one-shot competent cells (Invitrogen). Transformed cells were then plated on LB-AMP plates and later plasmids from positive colonies were sequenced at the Vermont Cancer Center.

pPCM expression:

pPCM in pGEX-2TK plasmid was transformed in BL-21 competent cells (Novagen). A single colony was picked and was grown in 1 liter of LB-AMP medium induced with 1mM IPTG. The transformed pPCM-GST fusion protein, from the cells was extracted and purified using a Glutathione-sepharose bead column. The protein concentration of the eluted samples was determined using Bradford assay (BioRad). Proteins were resolved in a 15% SDS-PAGE and visualized using Coomassie blue stain (0.1% Coomassie blue R-250, 10%acetic acid, 50% methanol, 40% distilled water). The pPCM-GST band was excised from the SDS gels and about 6mg of protein was sent to the Lampire Biologicals for the antibody production in rabbit.

Western blot:

Nitrocellulose papers were blocked using 5% non-fat dry milk in Tris buffer saline and tween (TBS-T, 0.16M Tris HCl, 0.04M Tris base, 1.4M NaCl and 1% Tween, pH 7.5) or in 5% non-fal dry milk, 2% Teleost gelatin (Sigma, Ca # G7765) and 3% goat serum (Vector laboratories, Inc., Ca # S1000) in TBS-T for 1 hour. The blot was cut according to need based on the pre stained protein ladder and probed for proteins of interest with primary antibodies of suitable dilution (Rabbit anti-AB 1:5000, rabbit anti-FBP 1:2000, rabbit anti-PCM 1:5000, rabbit anti-CBD 1:5000, mouse anti-aTubulin 1:10,000) at 4ºC overnight. The next day the blot was washed several times in TBS-T

and incubated in its suitable secondary antibody (anti rabbit or anti mouse IgG 1:10,000) conjugated to alkaline phosphatase and after an hour washed again at least 6 times with enough TBS-T. this was followed with development using nitroblue tetrazolium/ 5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP, Moss Inc., Pasadena, Maryland). The blots were also subjected to enhanced chemiluminescence (ECL, Amersham, Ca # RPN 2108) following the manufacturer's instructions.

Phosphoenzyme Intermediate analysis:

In order to identify the activation of Ca^{2+} stimulated ATPase, pure pellicle membrane or whole cilia was isolated from 51-S Paramecium cells. Membranes were incubated with γ-32 ATP under conditions favorable for the formation of a stable phosphorylated enzyme intermediate. About 200µg of protein was incubated in microcenrifuge tubes at 0ºC for 30 seconds in a reaction mixture containing 20mM Mops Tris (pH 7.0), 25mM KCl, 25µM EDTA, 5µM ATP with 15µCi/nmol γ-32 ATP and 1mM EGTA or 50µM Ca^{2+}, making up a total volume of 0.2ml. The reaction was started by the addition of γ-32 ATP to the suspension and terminated with the addition of 0.5ml ice cold 10% Trichloroacetic acid (TCA) containing 1mM ATP and 50mM H_2PO_4. After 10 minutes on ice the TCA precipitated proteins were pelleted by centrifugation for 4 minutes in a Beckman microfuge at maximum speed. The pellets were three times by resuspending them in 0.5ml of the TCA solution followed by centrifugation. The pellets were finally rinsed with 1ml ice cold sterile MQ water. If the proteins were tested for hydroxylamine sensitivity, the water rinsed pellets were suspended in 0.5ml of either a control solution containing 0.2 m NaOAc plus 150 mM Tris-HCl (pH 6.0) or 0.2 M NaOAc plus 150 mM Hydroxylamine (pH 6.0) for 10 min at room temperature. The proteins were then precipitated with TCA, pelleted and rinsed

with water. The pellets were then dissolved in 25 µl of sample buffer which contains 50 mM $NaPO_4$ (pH 6.3), 10% glycerol, 2% $NaDodSO_4$, 5% β-mercaptoethanol and 10 µg/ml pyronin Y and incubated at room temperature for 10 mins. Subsequently 20 µl samples were loaded to the 7.5% polyacrylamide slab gel (10 cm2 x 1.5 mm) which was buffered with 100 mM $NaPO_4$ (pH 6.3) and contained 0.1% $NaDodSO_4$. The gel was allowed to run at 20 mA for the first 15 min and then at 100mA until the dye front had just migrated from the gel. The gel was dried in the gel drier (courtesy Dr. Rincon's laboratory) for at least 2 hours at a temperature of 80ºC. Autoradiography was performed by exposing the dried gels to Kodak film at -70^{o}C. The apparent molecular weight of labeled proteins was estimated by comparison with the relative mobilities of Sigma prestained molecular weight standards run simultaneously on each gel.

To assure that enough protein was loaded onto each gel, a western blot was performed simultaneously using the same protein samples prepared for the phosphoenzyme intermediate assay. About 5µl of the remaining protein from each sample prepared was loaded onto a similar acid gel and allowed to run along with the other gel. Later it was transferred to a nitrocellulose paper using the Hoefer Semidry Transfer apparatus. The nitrocellulose paper was used to probe with anti CBD and developed in a similar process described earlier.

Results:

Presence of PMCAs in wild type ciliary membrane

Whole cilia proteins from wild type cells were analysed in an 8-15% gradient SDS-PAGE gel. About 80µg of protein was loaded onto each well and transferred in nitrocellulose paper in a method described above. Primary antibody against the calmodulin binding domain (CBD) of *Paramecium* was used to probe the blot. The development was completed using an anti-rabbit secondary antibody. Blots showed two distinct bands around the 115kDa region of molecular marker as evident from the prestained protein marker (Figure 1). As a positive control, one lane of the gel containing an equal amount of protein is probed with anti α-tubulin. This control blot was also developed simultaneously with blots using anti-CBD primary antibody using anti-mouse secondary antibody and finally developed using NBT-BCIP. A similar banding pattern was also obtained from the pure ciliary membrane isolated from *Paramecium*. A typical representation of the doublet protein bands obtained from the whole and pure ciliary membrane of the wild type cells is given, which shows the upper band around 120kDa and a lower band around 110kDa (Figure 1a). However, when anti PMCA 3 antibody is used in the same blot, both for whole and pure ciliary membrane, no bands showed up, indicating that either PMCA 3 is absent in the cilia or it is present in a very low abundance which cannot be detected by the anti-N3 antibody. It could also be possible that the protein is not recognized by the antibody in its current confirmation. These results show that either PMCA 2 or 4 is present in wild type ciliary membrane or the band is a representation of other isoforms of the PMCAs like the PMCA 18 or PMCA 19 which are very much abundant in the cilia.

Distribution of PMCAs in transformed cells

PMCA 2, 3 and 4 transformed cells were cultured and proteins were isolated from whole cilia in a method described earlier. About 80µg of protein from different cell types were loaded onto an 8-15% gradient SDS-PAGE for analysis. This time the isolated protein was loaded in triplicates to use the additional lane for probing using anti-HA antibody. This is because the transformed cells were tagged with HA and the HA protein will be recognized, using anti-HA, in the similar region as the PMCA isoform it is tagged to. When we used transformed cells with any one of the three isoforms, tagged with HA, we get an observation very different from wild type cells. When PMCA 2 is transformed in cells, we lose the lower band of the doublet in western blot analysis. Anti HA antibody recognizes the upper band suggesting that this band is PMCA 2 (Figure 2).

When PMCA 3 is transformed in cells, we get a band corresponding to the lower band of the doublet in western blot, when anti-CBD is used. Anti HA antibody does recognize this lower band well (Figure 3). When a specific antibody against N-terminus of PMCA3 was used, it did not recognize the lower band, indicating that the PMCA band recognized by anti-CBD may not be specific for the isoform 3. Other possibilities could be either the specific antibody could not recognize the antigenic epitope or the protein is present in a very low abundance and as a result it could not be recognized.

Since PMCA 3 is partially soluble in carbonate extraction as evident from previous studies, in order to solve this problem, 500µg of the transformed protein was solubilized using the sodium carbonate extraction method. To do a comparison study, whole cilia proteins were isolated and were subjected to the same carbonate extraction under the same conditions. Results from this experiment are as follows. In the wild type (51-S) cell proteins, anti-CBD recognizes the doublet band around the 115.5kDa

molecular marker region in all the three lanes (C= Whole ciliary proteins, P= Na_2CO_3 extracted pellet, S= soluble supernatant from Na_2CO_3 extraction) (Figure 4a). However, when these three lanes were reprobed with anti-N3, there were no bands recognized at the expected region (Figure 4b). Interestingly, when a Na_2CO_3 extraction is done using ciliary preps from PMCA 3 transformed cells, PMCA 3 gets partially solubilized and thus gives a band both in the supernatant and the pellet. Anti-CBD and anti-N3 (Figure 4c) probed blots from PMCA 3 transformed cells were developed both by the alkaline phosphatase and ECL method. This resulted in development of similar bands, which contributed to the confidence that the band is PMCA 3.

In case of PMCA 4 transformed cells, a similar banding pattern like PMCA 3 is observed, where the lower band of the doublet is very prominently recognized by anti-CBD (Figure 5). The upper band, however, is not seen in the developed blots and anti-HA does not recognize the lower band indicating this lower band may not be PMCA 4 isoform. Anti-N3 did not recognize any band in wild type or PMCA 2 or 4 transformed cells, which most likely indicates that PMCA 3 is only present in the PMCA 3 transformed cells.

Detection of the activity of the calcium pump in cilia

To determine if the calcium pumps of the cilia are active or not, a phosphoenzyme intermediate assay was performed. The assay was performed in a process described previously and here pure pellicle membrane was used as a positive control. Since it has been previously established (Wright, 1990) that the calcium pumps of the pure pellicle membrane is active, the data were used to compare the presence and activity of ciliary pumps. When pure pellicle membranes were resolved in a pH 6.3, 7.5% $NaPO_4$-PAGE, proteins with EGTA had no bands, since there was

no phosphorylation in the absence of divalent cations, around the expected molecular weight region as evident from the protein marker in the autoradiography. Samples incubated with Ca^{2+} had a robust band around 133kDa, indicating that the phosphoenzyme intermediate was formed. To distinguish this specific band formed between the intermediate and the protein kinase substrates, hydroxylamine was used to hydrolyze ^{32}P from the labeled protein. This treatment resulted in the loss of the 133kDa protein band concluding that the band was from a Ca^{2+} specific, hydroxylamine-sensitive phosphoprotein is the phosphoenzyme intermediate of the Ca^{2+}-ATPase. This data were very similar to the data obtained previously (Wright, 1990) in our laboratory.

When whole cilia are used however, the data were not very conclusive. There was no band with EGTA added to the sample, and a band with Ca^{2+} incubation. However, instead of reduction in the density of the 133kDa band with hydroxylamine it was actually increased (Figure 6). This result indicates that the 133kDa band in the ciliary proteins, is not a hydroxylamine-labile protein, but is a result from some other Ca^{2+} dependent protein kinase. A western blot analysis was simultaneously performed with the experiment, using the same protein samples. A smaller but equal volume of proteins were loaded on to a similar acid gel, resolved simultaneously and transferred (Figure 7). Results from the blot showed that the protein used for the intermediate assay does react with CBD, indicating that the total protein does contain pumps in them. Western blot result also indicates that the ciliary protein contains lot less of the pumps when compared to an equal volume of the pellicle proteins.

Analysis of detergent resistant membranes from whole cilia in Optiprep density gradient

In order to determine if lipid rafts are present in the cilia of *Paramecium tetraurelia*, whole cilia was isolated from 51-S wild type cells and approximately 8mg of the total protein was solubilized in cold 1% Triton X-100 to begin with the experiment. Lipid raft proteins are supposed to be associated with the Triton insoluble pellet. Lipid rafts were extracted from a gradient of 20%, 25%, 30%, 35% and 40% of Optiprep after ultracentrifugation. A total of 9 fractions were collected from each of the six, 4.5ml of ultra clear tubes. Similar fractions were pooled together and 100ul of it was saved for the determination of the protein concentration.

Proteins from each of the pooled fractions were purified by a process described earlier. Total protein collected from each fraction was then resolved by 8-15% gradient SDS-PAGE followed by transfer to nitrocellulose membrane. The nitrocellulose paper with the transferred protein was cut in different portions based on the molecular weight of the prestained protein marker and immunoprobed with different antibodies.

GPI anchored surface antigens were observed to be present from fractions 3-9, while *Paramecium* calcium pumps (PMCA) was observed in fractions 4-8. The 75kDa *Paramecium* PCMs were observed in fractions 5 and higher. *Paramecium* PCMs are predicted to be GPI anchored proteins and are found consistently in the lipid rafts of the cell body membrane. Their distribution in the rafts remains unaltered in the somatic membrane of the sterol depleted cells. Similarly, tubulin, another know raft associated cytoskeletal protein, was also associated with the higher fractions of the lipid rafts starting at 5 and above, which suggests that cytoskeletal proteins are associated with lipid rafts (Figure 8).

For all the trials of the above described protocol, a huge amount of protein was present in the fraction 9 suggesting that not all the proteins floated up in the lipid raft lighter density fractions. For all the trials the protein concentration of all the fractions were measured using the BCA kit, from the saved samples. The data was pooled together later for both the mock and the cholesterol depleted cell cilia and was plotted onto a graph.

Distribution of rafts in cilia of cholesterol depleted cells, using Optiprep density gradient

Lipid rafts are enriched in sterols and sphingolipids, which give them their characteristic detergent resistant property. Depletion of cholesterol results in disruption of lipid rafts in other organisms. In *Paramecium*, stigmasterol is present instead of cholesterol. Though we know that Methyl-beta cyclodextrin can also remove stigmasterol from the plasma membrane, however, we are not sure of how effectively it does so, as compared to cholesterol. An attempt was made to deplete the sterols that are present in the cilia of the cells, using methyl-β-cyclodextrin, used in most other lipid raft studies. However, only up to 12 $\pm$ 0.12% of sterols could be successfully removed from the cilia unlike the pellicle membrane where the sterol is depleted up to 40 $\pm$ 0.25% (Table 2). This may be due to the distinct sterol composition of cilia as compared to the pellicle membrane. To deplete sterol level in *Paramecium*, cells were treated with 5mM MβCD at 28^{o}C for 2 hours. After treatment, the sterol level from whole cilia was measured using the cholesterol assay kit and a process described earlier. Whole ciliary proteins were then isolated from these sterol-depleted cells and was Triton solubilized.

The insoluble pellet was subjected to an Optiprep density gradient study using the similar protocol for wild type cells. To compare the data of the sterol depleted cells, a set of mock treated cells, where the cells were incubated in the same buffer for 2 hours at 28^{o}C, were used simultaneously used for the entire density gradient study. When the western blot analysis of the mock and the treated cells were compared, there was hardly any noticeable change (Figure 9). This was expected, since the percent of sterol depletion from the cilia was not significant enough.

The protein concentration of all the nine fractions from both the mock treated cells and the sterol depleted cells were determined and plotted onto a graph. Protein concentrations do not show a significant deference between the two treatments (Figure 9a).

Analysis of detergent resistant membranes from crude pellicle in sucrose density gradient

Triton insoluble proteins are raft-associated proteins and are recovered by floatation in a continuous sucrose density gradient in other organisms. To find out if *Paramecium* cell body membrane proteins are associated with lipid rafts, the Triton-insoluble crude pellicle proteins were recovered in 5%-40% sucrose linear gradient through ultracentrifugation. Every 500 µl of supernatant was taken as one fraction. A total of 9 fractions were collected from each of the six, 4.5ml of ultra clear tubes.

Similar fractions were pooled together and 100µl from each fraction was saved for densitometric analysis. Each fraction was dialyzed in MQ water for at least 20 hours. Each dialyzed fraction was then subjected to 100% acetone precipitation and pelleted. Pelleted proteins were rewashed in 90% acetone and then air dried on ice. Proteins from each fraction were resolved by a 8-15% gradients SDS-PAGE followed

by Western Blot analysis. The blots were cut into several pieces according to the molecular weight and immunoprobed with several different antibodies.

GPI anchored surface antigens were observed to be present from fractions 3-9, while *Paramecium* calcium pumps (PMCA) was observed in fractions 4-8. The 75kDa *Paramecium* PCMs were observed in fractions 5 and higher. Similarly, tubulin was also associated with the higher fractions of the lipid rafts starting at 5 and above, which suggests that cytoskeletal proteins are associated with lipid rafts (Figure 10). For all the trials of the above described protocols, a huge amount of protein was present in the fraction 9 suggesting that not all the proteins did float up in the lipid raft lighter density fractions.

Distribution of rafts in crude pellicle of cholesterol depleted cells using sucrose density gradient

Since cholesterol is considered to be a major component of the lipid raft, the study of the effects of cholesterol depletion from membranes was found to be interesting. Although, it is noteworthy here, that the plasma membrane of *Paramecium* is made up of Stigmasterol, a phytosterol form of the sterol as opposed to cholesterol. Fortunately, MβCD was found to remove stigmasterol along with cholesterol from the cells. To deplete sterol level in *Paramecium*, cells were treated with 5mM MβCD at 28ºC for 2 hours. After treatment, the sterol level from whole cilia was measured using the cholesterol assay kit by a process described earlier. The sterol content of crude pellicle in the treated cells was compared to mock treated (control) cells, which showed that the sterol level in the pellicle membrane reduced to up to 42 %.

Triton insoluble pellet of crude pellicle from the sterol-depleted cells were subjected to sucrose density gradient, in a similar way like the wild type cells were. To

compare the data with mock treated cells and as a control, the insoluble pellet of the mock treated cells was used in a similar sucrose density gradient protocol. The immunoprobed results from the treated cells were very different to the mock type cells. This was very much expected since the sterol depletion of the treated cells was high and was comparable to previous work done. At about 40% of sterol depletion from cells, the rafts proteins from the cell membrane was found to be perturbed in the sucrose density gradient and a similar result was reproduced here.

The distribution of the GPI-anchored surface antigens was shifted to the high buoyant density fractions only (7-9). PMCA 2 was completely removed from the buoyant density fractions and was found in the pellet of the ultracentrifugation tube (Figure 11a), which shows that they are the most affected proteins of lipid rafts when sterols are depleted. Interestingly the distribution of PCMs remained unaffected due to cholesterol depletion, which suggests that these proteins are migrated in a cholesterol independent method (Figure 11). This result is also supported by immunomicroscopic studies of cholesterol-depleted cells, where proteins are found to be disrupted in MβCD treated cells.

About 5ul of the samples from all the nine pooled fractions, after sucrose density analysis was used to measure the density of each fraction by the method described earlier (Table 3). The densities when compared with the densities obtained from fractions using Optiprep which was done previously in the lab was compared, it did not show any significant change in each fraction.

Analysis of detergent resistant membranes from whole cilia in sucrose density gradient

Although many whole cilia preparations were pooled together to analyze lipid rafts using sucrose as the density gradient, the experiment was not very successful. This probably suggests that there is a significant loss of protein in the process of isolation of the protein from each of the nine fractions after the ultra centrifugation in a continuous gradient of 5-40% sucrose.

Distribution of rafts in crude pellicle of mock and cholesterol depleted cells using optiprep density gradient

When Optiprep was used instead of sucrose to analyze the raft proteins of pellicle to compare the data with the Optiprep experiments of ciliary proteins, the results were not very satisfactory. The western blots of mock treated cells were similar to the results when sucrose is used (Figure 12), but interestingly, when sterol depleted cells were used for the Optiprep density studies, there was hardly any difference as compared to the mock treated cells (Figure 13). This observation is very interesting because, even though almost 40% percent of sterol is depleted from the pellicle in this analysis, it is not evident from the western blot analysis. The most probable explanation to such an observation is that there is some interaction between sucrose and sterol, which helps in the flotation of rafts to low buoyant density fractions.

The protein concentration of all the nine fractions from pellicle raft study using the Optiprep gradient in both the mock treated cells and the sterol depleted cells were determined and plotted onto a graph. Protein concentrations do not show a significant deference between the two treatments (Figure 13a).

Distribution of pGlu-GFP in lipid rafts of whole cilia using Optiprep

Transformed cells containing *Paramecium* glutamate receptor tagged with Green Fluorescence protein (GFP) (pGlu-GFP) plasmid were cultured in a method described

earlier. This glutamate receptor is a NMDA like receptor. Cells were harvested and the total protein of whole cilia was obtained. Before subjecting the proteins to density gradient and analyze lipid rafts, the total ciliary proteins from pGlu-GFP expressing cells were compared to equal volumes of total protein of whole cilia from 51-S wild type cells. Proteins from both types of cells were resolved in the same 8-15% gradient SDS-PAGE followed by western blot analysis. Anti rabbit GFP was used as the primary antibody to probe the blots. GFP recognized a band around 78kDa in the lane containing the pGlu-GFP protein but not in the wild type cells. These data suggest that this band is not from an endogenous protein product but, the product of the microinjected construct in the transformed cells.

Six liters of cells were then harvested and solubilized using cold 1% Triton X-100. The insoluble pellet was subjected to a gradient of 20%, 25%, 30%, 35% and 40% of Optiprep and lipid rafts were extracted after ultracentrifugation. 9 fractions from each ultra clear tube were processed in a way similar to the wild type cells. Proteins were resolved in a gradient gel followed by western blot analysis. The nitrocellulose membrane was cut into three parts. The antibodies used were against the GPI-anchored surface antigens A and B, anti-GFP to recognize the glutamate receptors and anti-α-tubulin. The surface antigens were observed in the light through heavy buoyant density fractions like the wild type cells. Glutamate receptors were observed in the somewhat heavy fractions starting from fraction 5 through 8. The cytoskeletal element tubulin was distributed like it does in the wild type cells in the heavy buoyant fractions (Figure 14).

pGlu-GFP cells were also subjected to sterol depletion and then whole cilia were isolated from these sterol-depleted cells. Like wild type cells, the percent of sterol depletion was only about 10% and this was evident from the cholesterol assay. As a

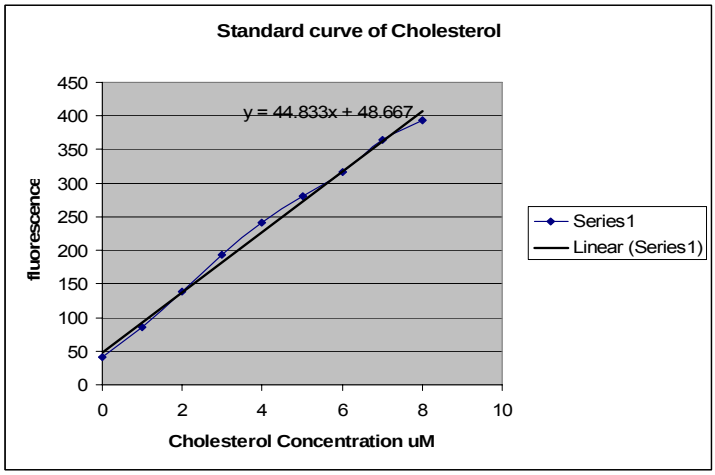
result, the distribution of proteins in the raft fractions remained unaltered (Figure 15). This result shows that the glutamate receptors are associated with the lipid rafts but their involvement with the lipids, specifically sterols is still unknown in *Paramecium* cilia.

The protein concentration of all the nine fractions from the cilia rafts of pGlu-GFP of both the mock treated cells and the sterol depleted cells, using Optiprep were determined and plotted onto a graph. Protein concentrations do not show a significant deference between the two treatments (Figure 15a).

This result was expected since NMDA like glutamate receptors are found to be associated with lipid rafts in other organisms. In case of *Paramecium*, lipid rafts are now found to be present in the ciliary membrane and its association with adenylyl cyclase and the plasma membrane calcium pumps. Along with the above proteins, glutamate receptor is a component of the same signal transduction pathway and is expected to be present in the lipid rafts, which serves as a platform for the signal transduction pathway.

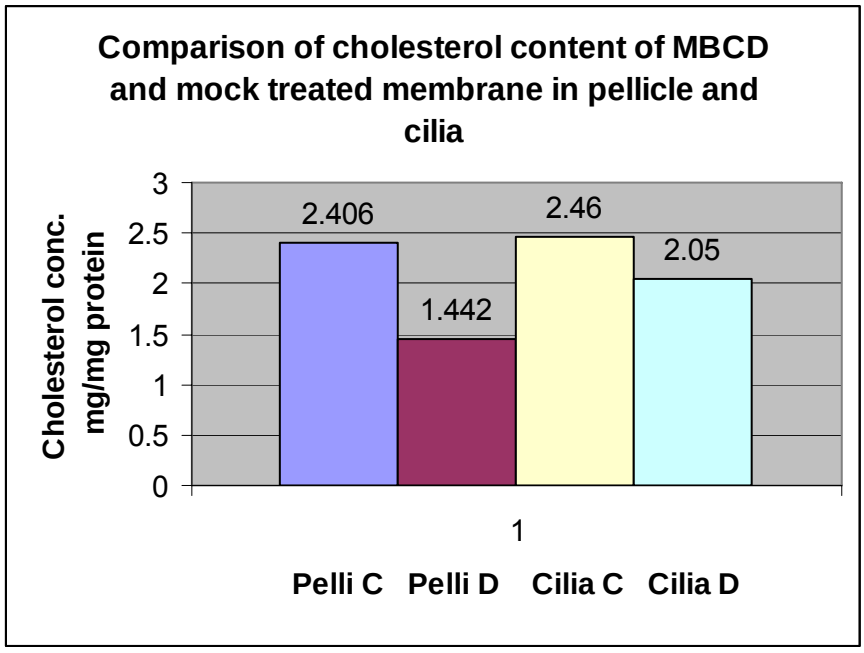
Table 2:

Fluorescence from Cholesterol standards								
0	1	2	3	4	5	6	7	8
0	5772	11875	19514	23925	30312	34131	38970	43410
0	6399	13447	20055	26657	31071	36151	41320	46080
0	4095	9549	14974	19031	22191	26886	32874	36740
0	3774	9022	14281	18399	23337	26795	30685	35708
0	3845	8794	14382	17780	21664	26210	29597	33767
0	2755	6774	10990	14937	18498	22797	24760	28486
0	2366	7205	11548	15842	19620	22884	26509	31239
0	1763	6312	10417	14575	19421	22524	27170	32236
0	3846.1	9122.3	14520	18893	23264	27297	31486	35958



a. Table and standard plot representing the linear axis obtained from the standards of the cholesterol assay kit, based on which the sterol concentration of pellicle and cilia are measured. This plot is obtained from the average reading of 9 standard readings.

	Pellicle		Cilia	
	Control	Depleted	Control	Depleted
	2.19	1.45	2.16	1.93
	1.79	0.92	2.48	2.26
	2.98	1.92	2.28	2.05
	2.95	1.83	2.35	1.98
	2.12	1.09	3.03	2.03
Avg	2.406	1.442	2.46	2.05
S.D.	0.53	0.44	0.34	0.12
	Depleted sterol			
	Pellicle	40.07%	Cilia	9.25%



b. Table and chart showing the sterol depletion of the pellicle and the cilia membrane. Data is obtained based on the linear equation given above and is the average from five experiments.

Table 3:

Standards of Refractometer readings

	Water	5% Sucrose	10% Sucrose	20% Sucrose	40% Sucrose
	1.3651	1.3401	1.3452	1.3553	1.3683
	1.3615	1.3395	1.3445	1.3572	1.3701
Avg.	1.3633	1.3398	1.34485	1.35625	1.3692
Temp.	24.6	24.6	24.6	24.6	24.6

Refractometer readings of the nine samples of sucrose density gradient

Fraction #	1	2	3	4	5	6	7	8	9
Reading	1.3607	1.3617	1.3642	1.3654	1.3688	1.3718	1.3751	1.3822	1.3959
	1.3607	1.3617	1.3641	1.3661	1.3688	1.3711	1.3753	1.3822	1.3959
Avg.	1.3607	1.3617	1.3642	1.3658	1.3688	1.3715	1.3752	1.3822	1.3959
Temp.	24.6	24.7	24.8	24.9	25.2	25.3	25.4	25.5	25.6

Discussion:

The presence of plasma membrane calcium pumps in the cilia of Paramecium:

Like all other cell types, in *Paramecium*, a low resting level of internal calcium is maintained. Effects of calcium concentration fluctuations are very much evident on its ciliary activity. Wright and Van Houten (1990) reported that, in the pellicle membrane of *Paramecium*, there is a putative Ca^{2+} transporting ATPase is present along with other voltage sensitive Ca^{2+} channels, which is not true for the cilia. However, in our current work, we find that native and tagged proteins react with the antibody against PMCA 2 in both the pellicle and the ciliary membrane, which is supported by both western blot analysis and immunomicroscopy studies. Anti-CBD antibody in whole and pure ciliary membrane recognizes a doublet of protein band around the 115kDa molecular weight. Although the affinity purified anti-CBD antibody is against the calmodulin binding domain (CBD) of PMCA 2, it can also recognize the CBD of PMCA 3 and PMCA 4 (Gannon-Murakami, 2005). Recently it is reported that along with PMCA 2, 3 and 4, anti-CBD can also recognize the CBDs of PMCA 18 and 19, and that all these five isoforms are present in cilia as evident from MS analysis (Dr. Yano, personal communication). Again, the molecular weights of these isoforms are so close that it is very difficult to distinguish them individually from a resolved 8-15% gradient gel. I attempt here, to determine, was which isoforms of PMCAs are represented by the doublet protein band in western blot analysis of whole ciliary proteins and whether PMCAs are active in the cilia.

Transformed cells with HA-tagged PMCA 2, 3 or 4 in separate transformed cells were used to identify the protein localization. Interestingly, when any one of the isoform was transformed in the cell, expressions of other isoforms were most likely

reduced and were not normally determined by western blot analysis. In case of PMCA2 transformed cells, the band of higher molecular weight is recognized by anti-CBD. Anti-HA when used recognizes the same higher band of molecular mass indicating that the higher molecular weight band if 120kDa probably represents PMCA isoform 2.

When PMCA 3 transformed cells are used for the study, the lower molecular mass band of 110kDa weight became evident using both anti-CBD and anti-HA antibodies, while there is no comparable band in control or wild type cells ciliary membrane. When the specific antibody generated against the N-terminal peptide of PMCA 3 is used in western blot analysis of Na_2CO_3 solubilized PMCA 3 transformed cells, it recognizes the band of 110kDa, both in the supernatant and the pellet of the solubilized protein. These data indicates that the lower molecular mass protein band is represented by PMCA 3 in PMCA 3 transformed cells. However, in wild type 51-S cells, Na_2CO_3 solubility studies using anti-N3 does not recognize a band around 110kDa in either of the supernatant or the pellet fraction of the whole cilia preparation. This result is contradictory to the recent findings of PMCA 3 in cilia from MS analysis. One possible explanation for this is, in wild type cells, the quantity of PMCA 3 expressed protein is not high enough to be recognized in a western blot analysis or Na_2CO_3 solubility study.

In case of PMCA 4 transformed cells, a protein band of lower molecular mass around 110kDa was observed in western blot analysis, when the lower molecular weight protein band is identified both by anti-CBD and anti-HA antibodies. From the above overexpression studies in the cilia of *Paramecium*, it can be concluded that the higher 120kDa protein band represents PMCA 2 and the lower band represents PMCA 4, most likely in wild type cells. These findings are similar to the findings of pellicle

membrane, where the higher molecular weight protein band is also recognized as PMCA 2 isoform (Pan, 2007), but unfortunately, nothing could be concluded about the lower molecular weight protein band.

Activity of PMCA in cilia:

Our next question in this study was to find if these calcium ATPases in ciliary membrane are active. To address this question we tried to examine the Ca^{2+} dependent phosphoenzyme intermediate formation of the ciliary pumps. Previous work have established the presence of a Ca^{2+} dependent, hydroxylamine-labile phosphoprotein Ca^{2+} -pumping ATPase in *Paramecium*, of approximately 133kDa molecular weight (Wright and Van Houten, 1990). We used pellicle membrane as a positive control for the phosphoenzyme intermediate assay used in this study. However, when whole cilia was used to determine the phosphoenzyme intermediate, it indicate that the protein band corresponding to the pumps in pellicle could be any Ca^{2+} dependent protein kinase, but its activity in the presence of hydroxylamine increased. Since whole ciliary proteins were used for the intermediate investigation, one possible explanation could be the presence of other Ca^{2+} dependent, ATP sensitive that does not exhibit hydroxylamine-labile property. Thus these data indicate that the 133kDa band in the ciliary proteins, is not a hydroxylamine-labile protein, but is a result from some other Ca^{2+} dependent protein kinase.

Lipid rafts in Paramecium cilia:

A biochemical approach had been made to identify the lipid rafts in *Paramecium*. Pellicle membranes when solubilized in cold non-ionic detergents resulted in the isolation of detergent resistant membranes. These membranes are expected to house

the lipid rafts and its associated proteins. A continuous sucrose density gradient followed by ultracentrifugation had been used to characterize lipid rafts of *Paramecium* pellicle membranes (Chandran, 2004 and Pan, 2007). A similar approach had been taken to identify the rafts in the cilia of *Paramecium*. It has also been shown that when wild type cells were treated with methyl-β-cyclodextrin (MβCD) followed by gradient centrifugation, the distribution of raft associated proteins was no longer similar to the non-treated cells (Pan, 2007). This result suggests that pellicle membrane rafts are sensitive to MβCD which efficiently removes up to 42% of sterols from *Paramecium* pellicle membranes. To confirm these previous observations, the work was repeated with crude pellicle membrane from both mock and MβCD treated cells. In cholesterol depleted cells, the GPI anchored surface antigens and tubulin cytoskeleton appeared only in the high buoyant density fractions in sucrose gradient. Distribution of PCM, however, remained unaffected due to the MβCD treatment in pellicle membranes. Since we expect that different types of rafts containing differential sterol amount is present in the cell membrane, the presence and floatation of the PCM in raft fractions in sterol depleted cells is due to its association with certain sterols that are not affected by MβCD treatment.

Plasma membrane calcium pumps did not float up at all in the 9 fractions but stayed in the pellet of the ultracentrifugation tube. When the pellet was resuspended and resolved in a 8-15% gradient SDS-PAGE, the PMCAs were found to be present in it. These data were further supported by complete disappearance of PMCAs from the MβCD treated cells in immunocytochemical studies (Pan, 2007).

Sucrose density was initially used for the separation of cilia raft proteins. Unfortunately, sucrose was not a very effective method for raft protein separation in cilia. Even after the method was repeated with a huge amount of total cilia protein to

start with, there was probably a significant loss in the process of dialysis and protein precipitation, which led to no results after western blot analysis. Later a step 40-20% Optiprep gradient was used instead of the sucrose. Raft proteins were isolated from all the nine fractions and analyzed by western blot detection. Results showed that GPI anchored raft marker proteins were associated with both the low and the high density fractions. PMCAs were found to be associated with fractions 4-7. Again, cytoskeletal proteins like tubulin were associated with high buoyant density fractions only, in accordance to findings in other raft research. MβCD treatment of cells could deplete only up to 12% of the ciliary sterols. Since this percent of sterol depletion was not significant enough, the result of raft experiments from cilia of treated cells using Optiprep was as expected. There was no noticeable shift of proteins in the ciliary raft fractions.

As opposed to sucrose when Optiprep was used as the density gradient medium for the isolation of rafts from the pellicle, there was no change in the raft protein distribution of mock treated and MβCD treated cells. Although the sterol depletion in the pellicle membrane was up to 40%, compared to cilia, which was only 12%, there was no noticeable shift in the protein distribution in the Optiprep density fractions. As a result these data could not be considered as a control data obtained for the lipid raft studies on cilia.

This observation could be explained by the fact that, Optiprep helps in the floatation of lipid rafts by a mechanism independent of the involvement of cholesterol. Hence, when cholesterol is depleted significantly from pellicle, Optiprep helps rafts to float up by the aid of other lipids. Whereas, in sucrose, the rafts float up to the low buoyant density fractions only when there is enough cholesterol associated with them. An alternate explanation would be, the interaction of sucrose and cholesterol is

important fro raft proteins for it to move to a low buoyant density fraction, which is not the case for Optiprep. Since our approach is completely biochemical and we are unaware of exactly what lipids are involved in Paramecium rafts, it is difficult to come to a definitive conclusion. However, it can be said that for this model, sucrose is most likely a better density gradient medium for detection of lipid rafts.

Association of Glutamate receptors, adenylyl cyclase and PMCAs in ciliary lipid rafts:

Rafts isolated from wild type 51-S cells have the evidence of the presence of adenylyl cyclase in fractions 3-7. presence of adenylyl cyclase in the raft is important because it is one of the important signaling molecules of the pathway through the glutamate receptor. When an Optiprep density analysis of rafts was done using transformed cells with pGlu-GFP expressing cells, the expressed protein was shown to float in lighter buoyant fractions of lipid rafts in the gradient. Other endogenous expressed proteins like GPI-anchored surface antigens and cytoskeletal proteins separated out in fractions where they are normally found in both 51-S cells and mock treated cells. When cholesterol was depleted from pGlu-GFP cells using MβCD, there was no significant shift in the protein distribution since the percent of cholesterol removal from cilia remained low (10%). Previously it has been shown through multiple trials that PMCAs are associated with lipid rafts of the cilia. Put together all these data, it can be hypothesized that, the lipid rafts serves as a platform for the glutamate receptors, the adenylyl cyclase, PKA and eventually the calcium pumps, which altogether takes part in the chemosensory signal transduction pathway of *Paramecium*.

Figure 1: The presence of PMCA in whole ciliary proteins.

The ciliary proteins from wild type cells were isolated by deciliation and analyzed through Western Blot. The blot was immunoprobed by anti-CBD (Primary, 1:5,000) and alkaline phosphatase conjugated rabbit anti-IgG (Secondary, 1:10,000)

Arrows point the doublet around 115.5kDa molecular weight, representing the doublet of PMCA protein band. Middle panel shows the presence of PMCA recognized by anti CBD antibody in pure ciliary membrane. Anti N-3 does not recognize any band.

Figure 1:

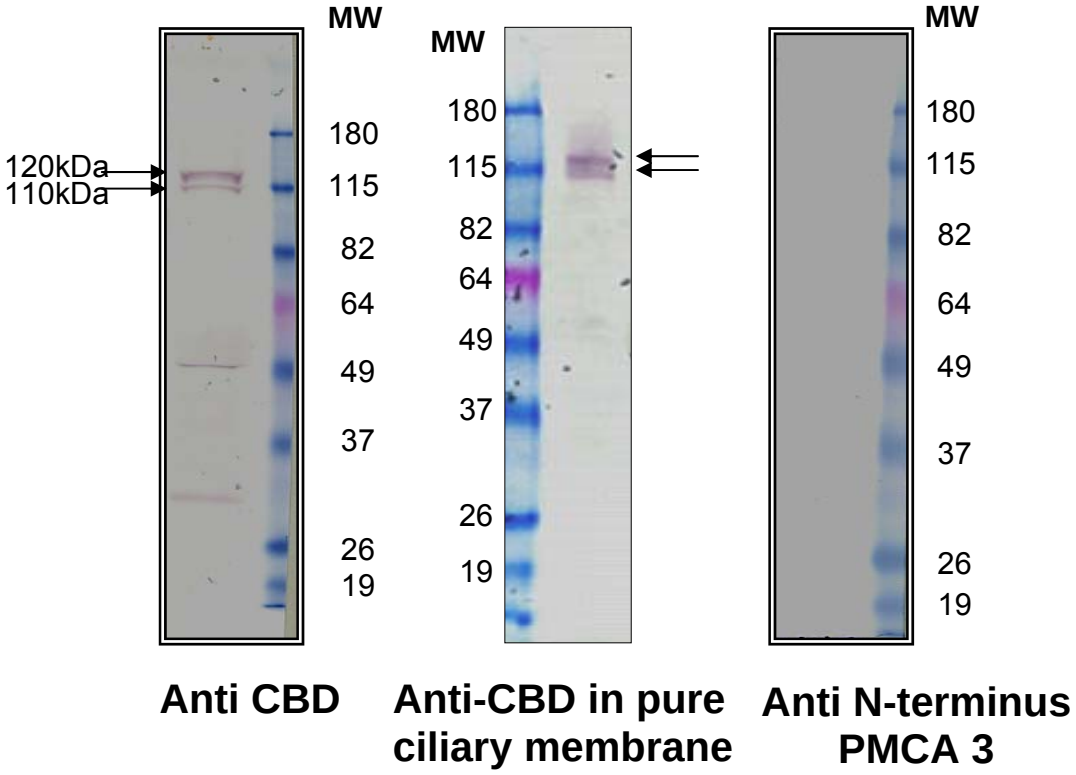

MW
180
115
82
64
49
37
26
19
120kDa
110kDa
MW
180
115
82
64
49
37
26
19
MW
180
115
82
64
49
37
26
19
Anti CBD
Anti-CBD in pure ciliary membrane
Anti N-terminus PMCA 3

Figure 1a: Schematic diagram of the protein bands of PMCAs from western blot analysis.

The schematic diagram here represents a typical western blot analysis of the doublet protein band obtained from whole cilia and ciliary membrane proteins of wild type 51-S cells. When the blot is probed with anti CBD as the primary antibody followed by anti rabbit as the secondary antibody, after development two protein band s are observed. The upper, more intense band is around 120kDA whereas the less intense lower band is around the 110kDa. They represent different isoforms of PMCA, but we are not sure of which band represents what.

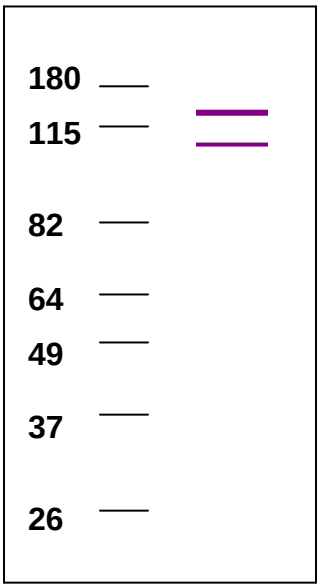
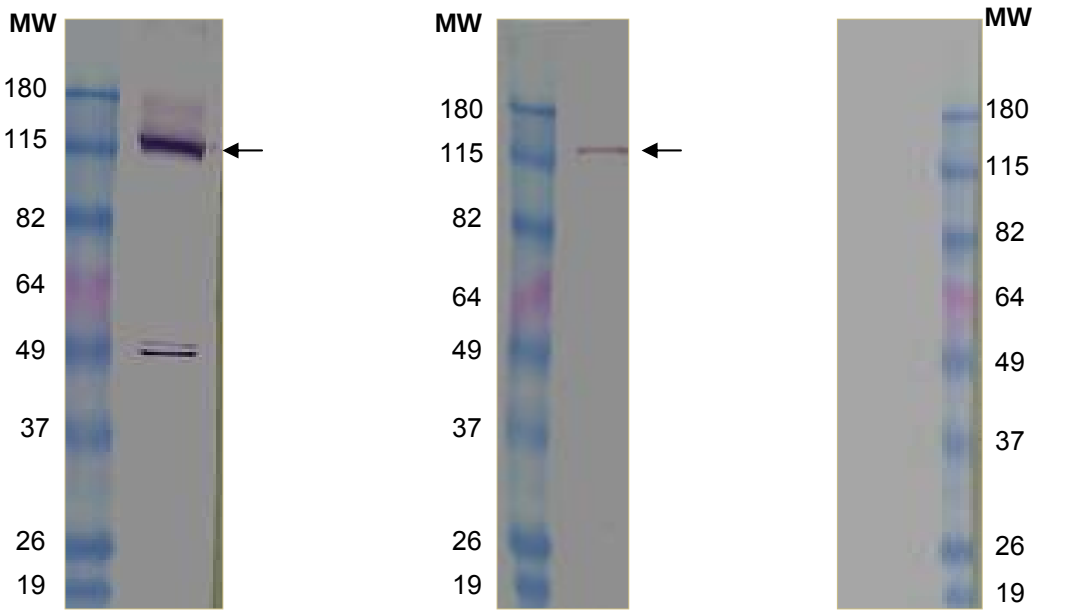


Figure 1a :

Figure 2: The presence of PMCA 2 in whole ciliary proteins of PMCA 2 transformed cells.

The ciliary proteins from transformed cells were isolated by deciliation and analyzed through Western Blot. The blot was immunoprobed by anti-CBD (Primary, 1:5,000) and anti-HA (1:2,000) and alkaline phosphatase conjugated rabbit anti-IgG (Secondary, 1:10,000). Arrow points to the upper band of the doublet around 115.5kDa molecular weight, representing the presence of PMCA 2 protein band. HA also recognizes the corresponding upper band. Anti N-3 does not recognize any band.

Figure 2:

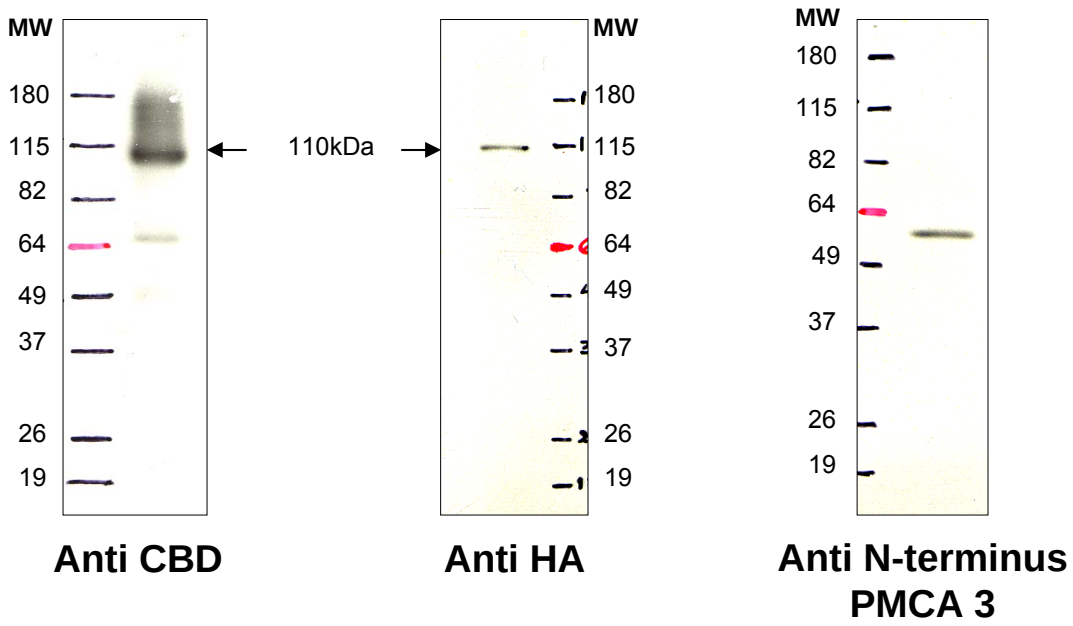


MW = Molecular weight

Figure 3: The presence of PMCA 3 in whole ciliary proteins of PMCA 3 transformed cells.

The ciliary proteins from transformed cells were isolated by deciliation and analyzed through Western Blot. The blot was immunoprobed by anti-CBD (Primary, 1:5,000) and anti-HA (1:2,000) and alkaline phosphatase conjugated rabbit anti-IgG (Secondary, 1:10,000). Arrow points to the lower band of the doublet around 115.5kDa molecular weight, representing the presence of PMCA 3 protein band. HA also recognizes the corresponding lower band of the doublet. Anti N-3 does not recognize any band around 115.5kDa. This most likely indicated that the expression of PMCA 3 protein is very less in the wild type cells as well as the transformed cells to be detected by the specific anti-N3 antibody.

Figure 3:



MW = Molecular weight

Figure 4: The presence of PMCA 3 in whole ciliary proteins extracted by Na_2CO_3, of PMCA 3 transformed cells.

To solve the problem detected in the previous experiment, the ciliary proteins from transformed cells as well as wild type cells were isolated by deciliation and treated with Na_2CO_3 followed by analysis through Western Blot. The blot was immunoprobed by anti-CBD (Primary, 1:5,000), anti N3 and alkaline phosphatase conjugated rabbit anti-IgG (Secondary, 1:10,000).

a. Anti-CBD recognizes the doublet band around 115.5kDa both in the supernatant (S) and the pellet (P) as well as the whole ciliary protein (C), indicating that all the PMCAs are partially solubilized in Na_2CO_3. M is the molecular marker lane.

b. Anti N-3 does not recognize bands in Na_2CO_3 solubilized ciliary proteins, indicating that PMCA 3 is below detection in wild type cell cilia [supernatant (S), pellet (P), whole ciliary protein (C)].

c. Anti N-3 recognizes the PMCA 3 band in the pellet and partially solubilized supernatant [supernatant (S), pellet (P)].

d. Anti CBD recognizes all the pumps in both the pellet and the partially solubilized supernatant, indicating that the bands are specific for pumps and not artifacts.

Figure 4:

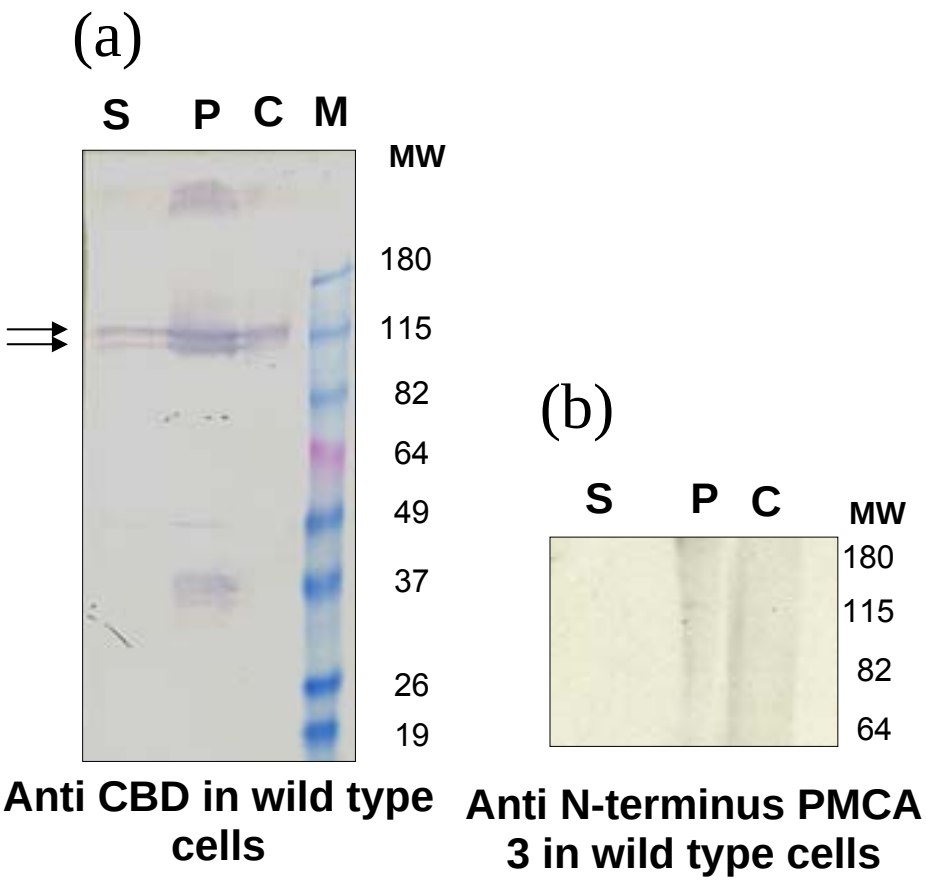

(a)
S
P
C
M
MW
180
115
82
64
49
37
26
19
Anti CBD in wild type cells
(b)
S
P
C
MW
180
115
82
64
Anti N-terminus PMCA 3 in wild type cells

Figure 4:

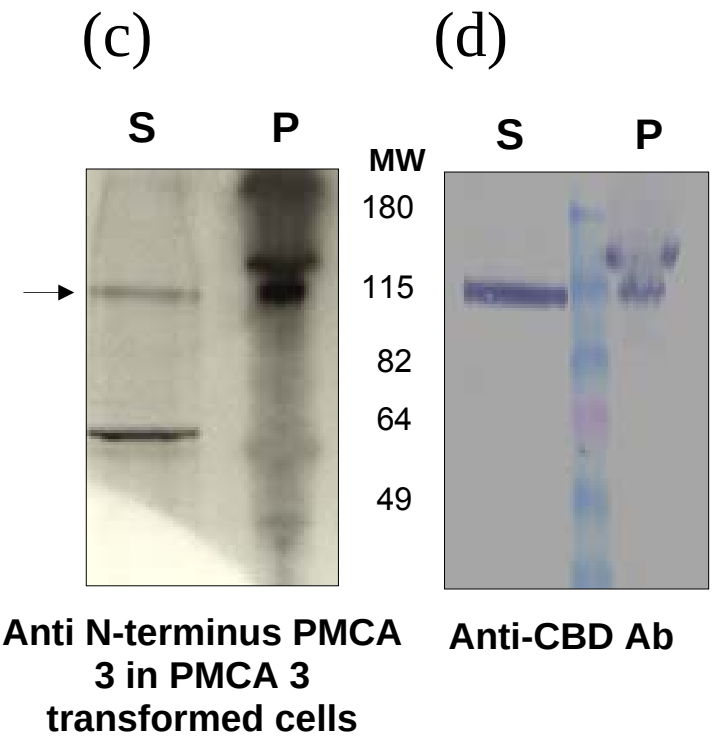

(c)
(d)
S
P
MW
S
P
180
115
82
64
49
Anti N-terminus PMCA 3 in PMCA 3 transformed cells
Anti-CBD Ab

Figure 5: The presence of PMCA 4 in whole ciliary proteins of PMCA 4 transformed cells.

The ciliary proteins from transformed cells were isolated by deciliation and analyzed through Western Blot. The blot was immunoprobed by anti-CBD (Primary, 1:5,000) and anti-HA (1:2,000) and alkaline phosphatase conjugated rabbit anti-IgG (Secondary, 1:10,000). Arrow points to the lower band of the doublet around 115.5kDa molecular weight, representing the presence of PMCA 4 protein band. HA, however, does not recognize the corresponding lower band, which probably indicates that the PMCA 4 band is not very specific. Anti N-3 does not recognize any band.

Figure 5:

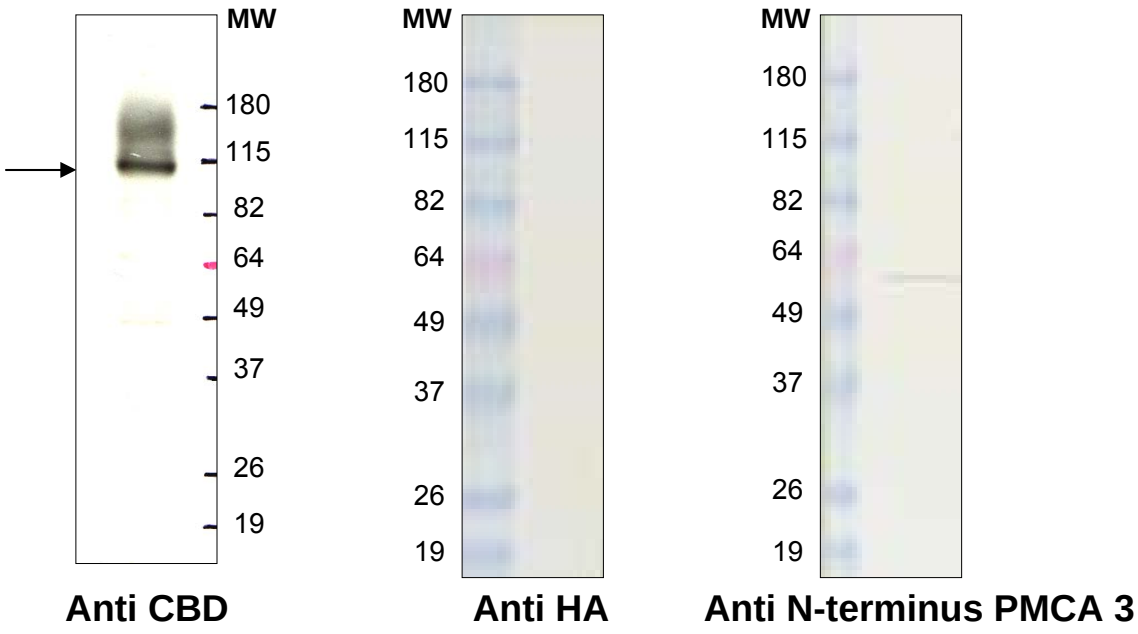

MW
180
115
82
64
49
37
26
19
Anti CBD
MW
180
115
82
64
49
37
26
19
Anti HA
MW
180
115
82
64
49
37
26
19
Anti N-terminus PMCA 3

Figure 6: Phosphoenzyme Intermediate assay of pure pellicle and whole cilia.

Lanes from left to right represents as follows:

1. Pure pellicle with EDTA, shows no activity of the pump.
2. Pure pellicle in presence of calcium, has the phosphoenzyme intermediate around the 133kDa protein band.
3. PMCA being hydroxylamine-labile, disappears, when hydroxylamine is added to pure pellicle protein.
4. Whole ciliary protein in presence of EDTA does not have the active pump but there is presence of other enzymes of lower molecular masses.
5. Addition of calcium to the ciliary protein shows the presence of the 133kDa protein band, representing the pump intermediate.
6. Addition of hydroxylamine does not decrease the intensity of the protein bands of whole ciliary protein.

The difference between the left and the right panel is only the exposure time.

The data shown is a representative of three experiments.

Figure 6:

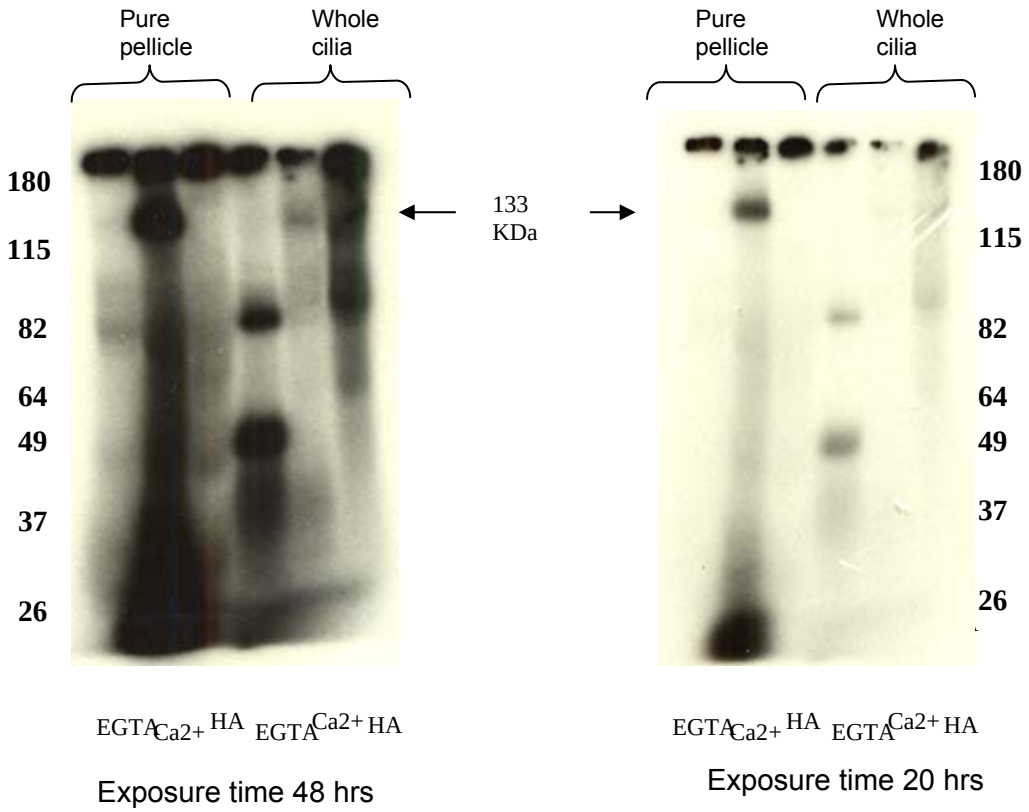

Pure pellicle
Whole cilia
180
115
82
64
49
37
26
133 KDa
Pure pellicle
Whole cilia
180
115
82
64
49
37
26
EGTA Ca2+ HA EGTA Ca2+ HA
Exposure time 48 hrs
EGTA Ca2+ HA EGTA Ca2+ HA
Exposure time 20 hrs

Figure 7: Proteins from Phosphoenzyme Intermediate assay of pure pellicle and whole cilia in western blot analysis.

Approximately 5µl of sample was loaded from the left over proteins of the enzyme assay. The proteins were resolved in a similar acid gel and transferred. The blot was immunoprobed by anti-CBD (Primary, 1:5,000) and alkaline phosphatase conjugated rabbit anti-IgG (Secondary, 1:10,000). Protein bands representing PMCAs are observed in each and every lane (1-6) indicating that there is enough protein loaded on to the acid gel to be detected by the anti-CBD antibody.

The data shown is a representative of three experiments.

Figure 7:

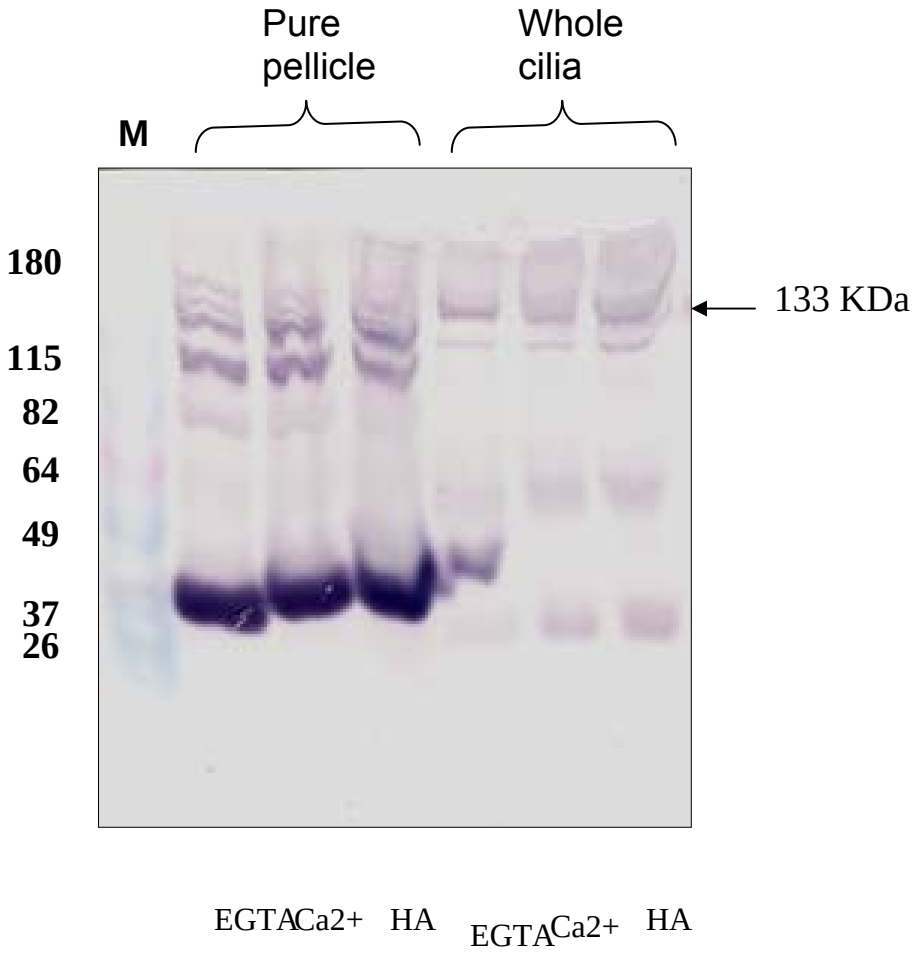

Pure pellicle
Whole cilia
M
180
115
82
64
49
37
26
133 KDa
EGTA
Ca2+
HA
EGTA
Ca2+
HA

Figure 8: Distributions of whole cilia proteins from 51-S wild type cells in optiprep density gradient.

The whole ciliary proteins (~8 mg) were extracted with 1% Triton X-100 and pelleted proteins were recovered in 40%-20% linear optiprep density gradient. Lane 1-9 represents 9 factions of optiprep density gradient. Lane M is protein marker.

A. The blot was immunoprobed by anti-surface antiges (1:5,000 diluted). GPI-anchored surface antigens (220-250 kDa) distributed in fractions 3-9.

B. The blot was immunoprobed by anti-*Paramecium* adenylyl-cyclase (1:5,000). PAC-N terminal (80 kDa) distributes in fraction 3 to 8.

C. The blot was immnoprobed by anti-*Paramecium* (1:2,000) PCM (65 kDa) distribute in fractions 3-9.

D. The blot was immnoprobed by anti-tubulin (1:10,000 diluted). Tubulin (54 kDa) distributes mostly in the heavy buoyant fractions.

The blots represent >4 experiments.

Figure 8:

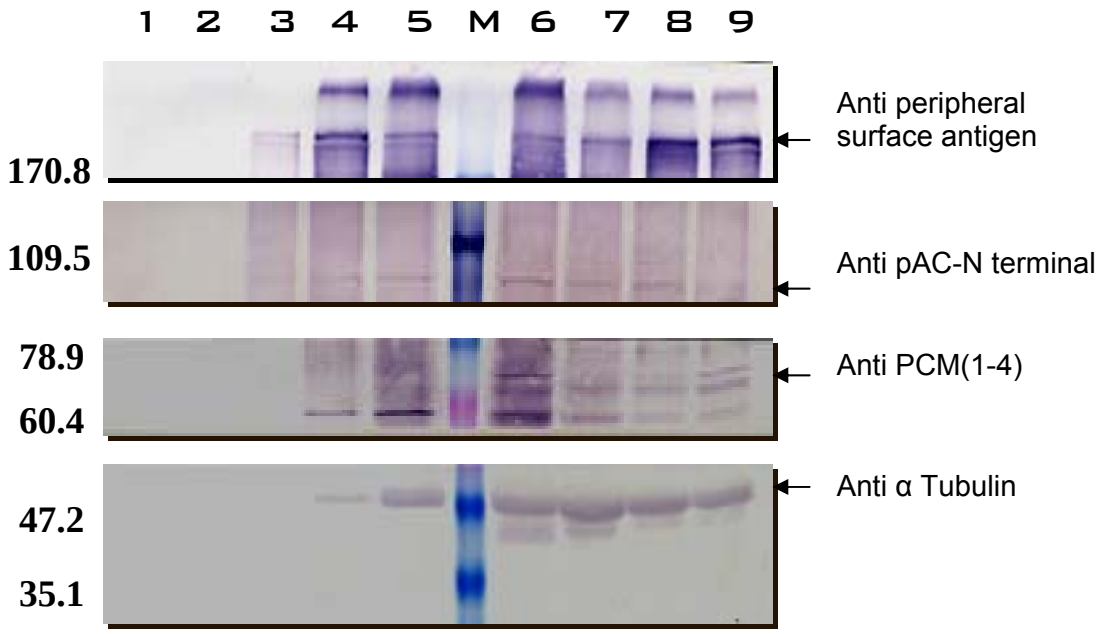

1 2 3 4 5 M 6 7 8 9
170.8
109.5
78.9
60.4
47.2
35.1
Anti peripheral surface antigen
Anti pAC-N terminal
Anti PCM(1-4)
Anti α Tubulin

Figure 9: Distributions of whole cilia proteins from cholesterol depleted 51-S wild type cells in optiprep density gradient.

The whole ciliary proteins (~8 mg) were extracted with 1% Triton X-100 and the pelleted protein was recovered in 40%-20% linear optiprep density gradient. Lane 1-9 represents 9 factions of optiprep density gradient. Lane M is protein marker.

A. The blot was immunoprobed by anti-surface antiges (1:5,000 diluted). GPI-anchored surface antigens (220-250 kDa) distributed in fractions 4-9.

B. The blot was immunoprobed by anti-*Paramecium* CBD (1:5,000). PMCA2 (125 kDa) distributes in fraction 4 to 7.

C. The blot was immnoprobed by anti-*Paramecium* (1:2,000) PCM (65 kDa) distribute in fractions 5-9.

D. The blot was immnoprobed by anti-tubulin (1:10,000 diluted). Tubulin (54 kDa) distributes mostly in the heavy buoyant fractions (6-9).

The blots are representative of 3 experiments.

Figure 9:

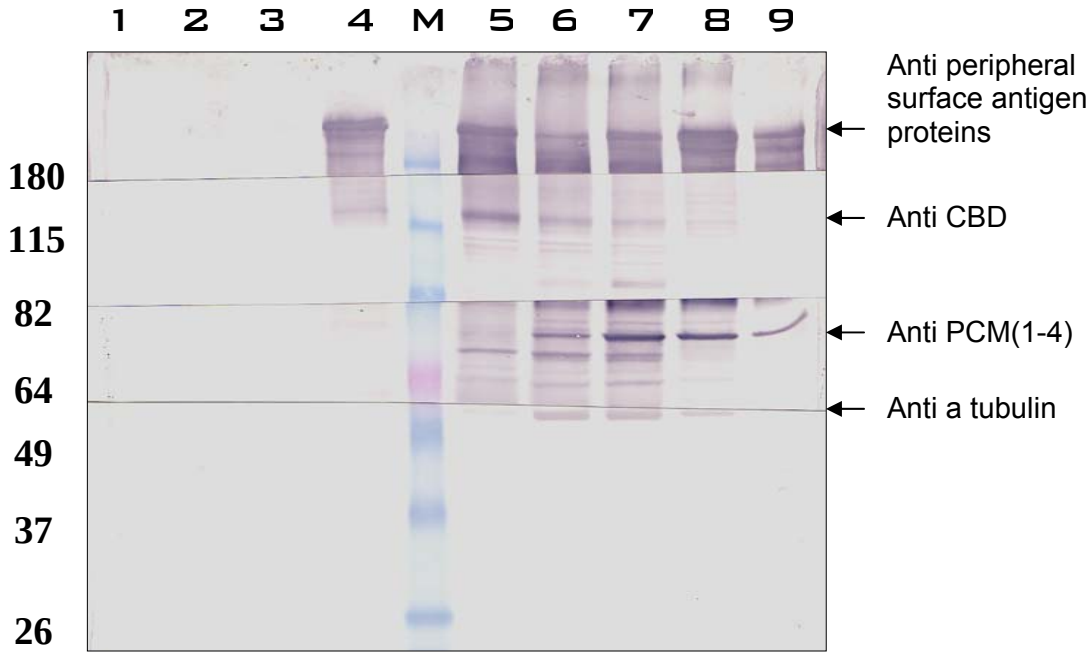

1 2 3 4 M 5 6 7 8 9
180
115
82
64
49
37
26
Anti peripheral surface antigen proteins
Anti CBD
Anti PCM(1-4)
Anti a tubulin

Figure 9a: Comparison of protein concentrations from mock treated and sterol depleted cilia from optiprep density gradient.

The protein samples saved from the nine pooled fractions were assays using the BCA protein assay kit and the results of both the mock and the sterol depleted cilia samples from the nine fractions of optiprep density gradient is shown by a bar graph here.

The protein concentration is in ug/ul.

Figure 9a:

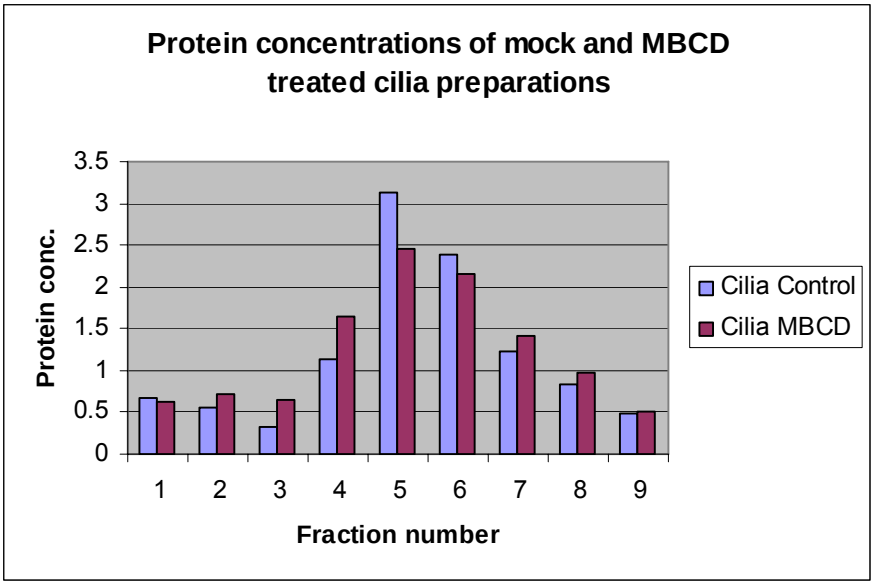

Protein concentrations of mock and MBCD treated cilia preparations
Protein conc.
3.5
3
2.5
2
1.5
1
0.5
0
1
2
3
4
5
6
7
8
9
Fraction number
Cilia Control
Cilia MBCD

Figure 10: Distributions of pellicle proteins from mock treated 51-S wild type cells in sucrose density gradient.

The pellicle proteins (~30 mg) were extracted with 1% Triton X-100 and recovered in 40%-5% continuous sucrose density gradient. Lane 1-9 represents 9 factions of sucrose density gradient. Lane M is protein marker.

A. The blot was immunoprobed by anti-surface antiges (1:5,000 diluted). GPI-anchored surface antigens (220-250 kDa) distributed in all 9 fractions.

B. The blot was immunoprobed by anti-*Paramecium* CBD (1:5,000). PMCA2 (125 kDa) distributes in fraction 2 to 7.

C. The blot was immnoprobed by anti-*Paramecium* (1:2,000) PCM (65 kDa) distribute in all fractions starting from 1-9.

The blots are representative of 3 experiments.

Figure 10:

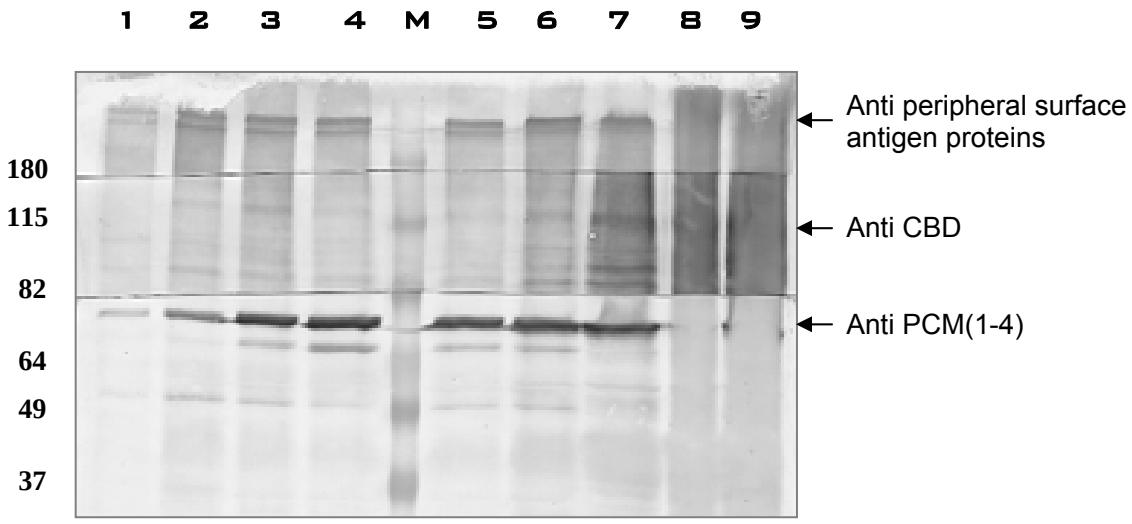

1 2 3 4 M 5 6 7 8 9
180
115
82
64
49
37
Anti peripheral surface antigen proteins
Anti CBD
Anti PCM(1-4)

Figure 11: Distributions of pellicle proteins from cholesterol depleted 51-S wild type cells in sucrose density gradient.

The pellicle proteins from cholesterol depleted cells (~30 mg) were extracted with 1% Triton X-100 and recovered in 40%-5% continuous sucrose density gradient. Lane 1-9 represents 9 factions of sucrose density gradient. Lane M is protein marker.

A. The blot was immunoprobed by anti-surface antiges (1:5,000 diluted). GPI-anchored surface antigens (220-250 kDa) distributed in only heavy bouyant fractions (7-9).

B. The blot was immunoprobed by anti-*Paramecium* CBD (1:5,000). PMCA2 (125 kDa) distributes in none of the fractions.

C. The blot was immnoprobed by anti-*Paramecium* (1:2,000) PCM (65 kDa) distribute in heavy fractions only starting from 6-9.

D. The blot was immnoprobed by anti-tubulin (1:10,000 diluted). Tubulin (54 kDa) distributes mostly in the heavy buoyant fractions (7-9).

The blots are representative of 3 experiments.

Figure 11:

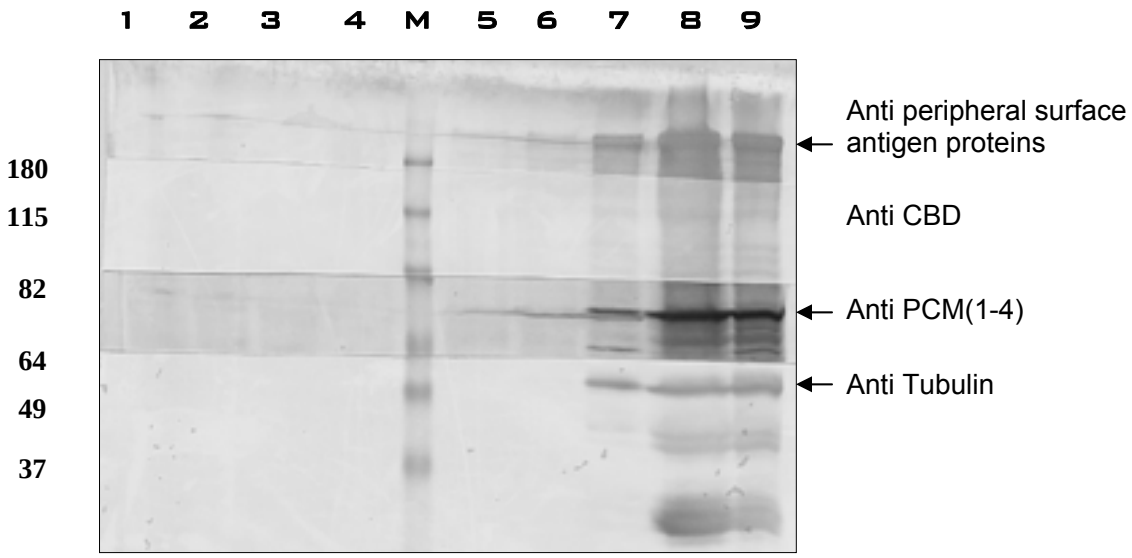
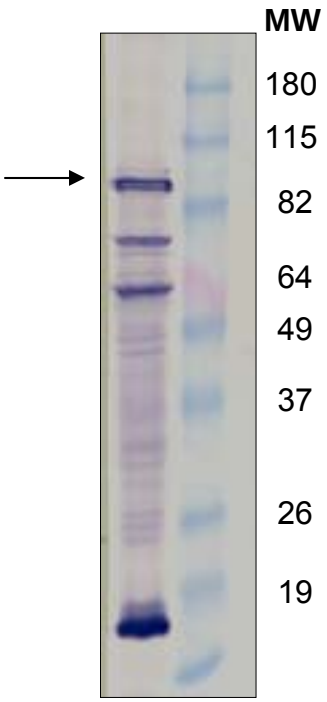

1 2 3 4 M 5 6 7 8 9
180
115
82
64
49
37
Anti peripheral surface antigen proteins
Anti CBD
Anti PCM(1-4)
Anti Tubulin

Figure 11a: Presence of the pumps in the pellet of the ultracentrifugation tube of the sterol depleted cells in sucrose gradient.

This western blot here shows the evidence of the presence of pumps in the pellet of the ultracentrifuged tube. The protein sample was obtained from the pellicle pellet of sterol depleted cells using sucrose as the density gradient. The blot was probed with anti-CBD primary antibody followed by anti rabbit secondary. This blot is a representative of three experiments.

Figure 11a:



Anti CBD

Figure 12: Distributions of pellicle proteins from mock treated 51-S wild type cells in optiprep density gradient.

The pellicle proteins (~30 mg) were extracted with 1% Triton X-100 and recovered in 40%-20% linear optiprep density gradient. Lane 1-9 represents 9 factions of optiprep density gradient. Lane M is protein marker.

A. The blot was immunoprobed by anti-surface antiges (1:5,000 diluted). GPI-anchored surface antigens (220-250 kDa) distributed in all 9 fractions.

B. The blot was immunoprobed by anti-*Paramecium* CBD (1:5,000). PMCA2 (125 kDa) distributes in fraction 5 to 9.

C. The blot was immnoprobed by anti-*Paramecium* (1:2,000) PCM (65 kDa) distribute in heavy fractions starting from 4-9.

D. The blot was immnoprobed by anti-tubulin (1:10,000 diluted). Tubulin (54 kDa) distributes mostly in the heavy buoyant fractions (5-9).

The blots are representative of 3 experiments.

Figure 12:

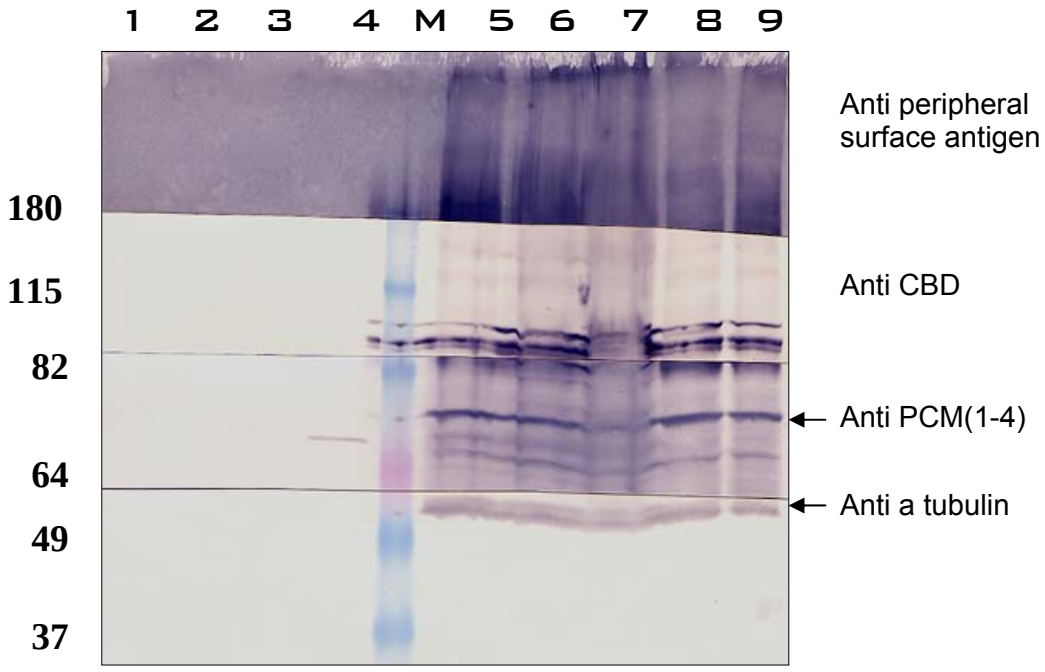

1 2 3 4 M 5 6 7 8 9
180
115
82
64
49
37
Anti peripheral surface antigen
Anti CBD
Anti PCM(1-4)
Anti a tubulin

Figure 13: Distributions of pellicle proteins from cholesterol depleted 51-S wild type cells in optiprep density gradient.

The pellicle proteins from cholesterol depleted cells (~30 mg) were extracted with 1% Triton X-100 and recovered in 40%-20% linear optiprep density gradient. Lane 1-9 represents 9 factions of optiprep density gradient. Lane M is protein marker.

A. The blot was immunoprobed by anti-surface antiges (1:5,000 diluted). GPI-anchored surface antigens (220-250 kDa) distributed in all 9 fractions.

B. The blot was immunoprobed by anti-*Paramecium* CBD (1:5,000). PMCA2 (125 kDa) distributes in fraction 5 to 9.

C. The blot was immnoprobed by anti-*Paramecium* (1:2,000) PCM (65 kDa) distribute in heavy fractions starting from 5-9.

D. The blot was immnoprobed by anti-tubulin (1:10,000 diluted). Tubulin (54 kDa) distributes mostly in the heavy buoyant fractions (5-9).

The blots are representative of 3 experiments.

Figure 13:

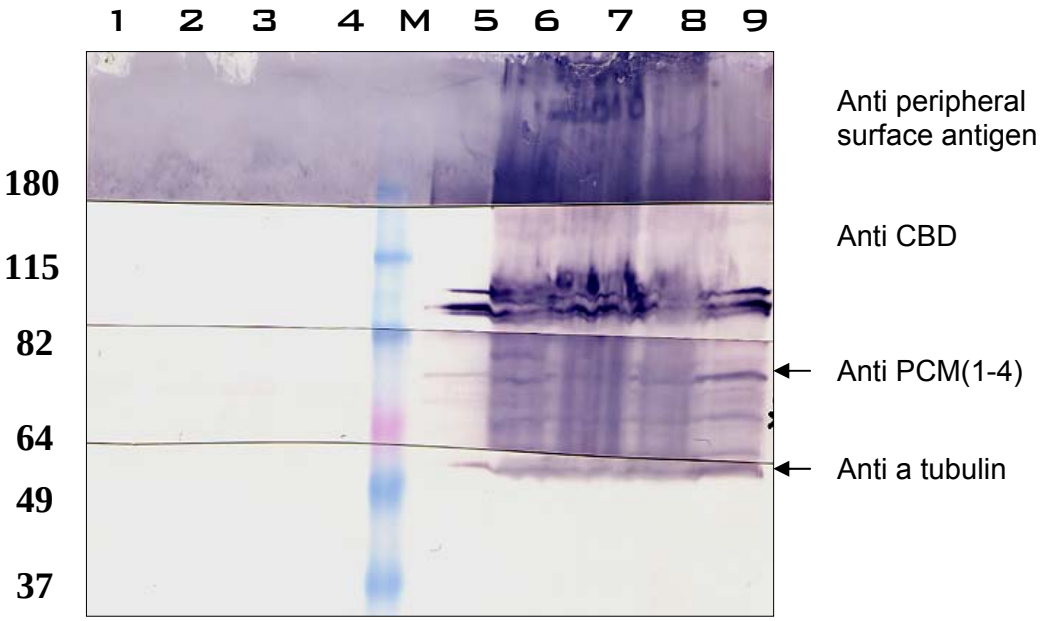

1 2 3 4 M 5 6 7 8 9
180
115
82
64
49
37
Anti peripheral surface antigen
Anti CBD
Anti PCM(1-4)
Anti a tubulin

Figure 13a: Comparison of protein concentrations from mock treated and sterol depleted pellicle from optiprep density gradient.

The protein samples saved from the nine pooled fractions were assays using the BCA protein assay kit and the results of both the mock and the sterol depleted pellicle samples from the nine fractions of optiprep density gradient is shown by a bar graph here.

The protein concentration unit is ug/ul.

Figure 13a:

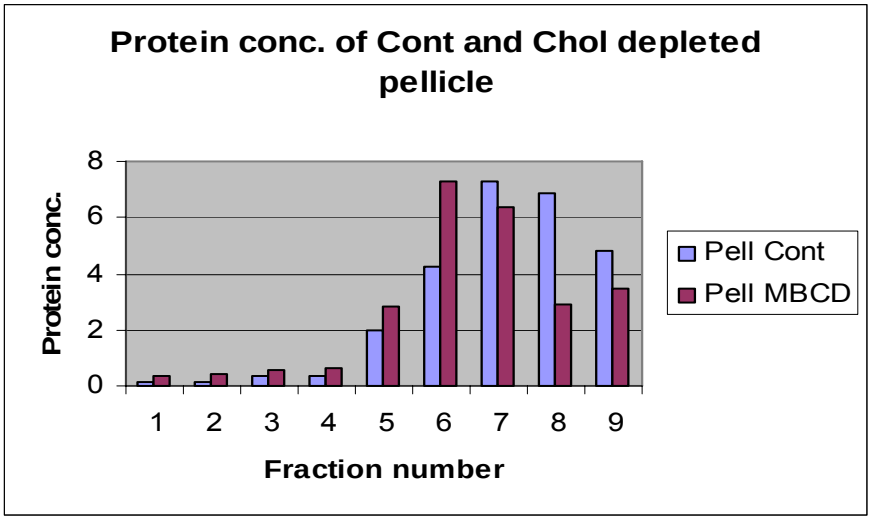

Protein conc. of Cont and Chol depleted pellicle
Protein conc.
0
2
4
6
8
1
2
3
4
5
6
7
8
9
Fraction number
Pell Cont
Pell MBCD

Figure 14: Distributions of whole cilia proteins from mock treated pGlu-GFP transformed cells in optiprep density gradient.

The whole ciliary proteins from the transformed cells(~8 mg) were extracted with 1% Triton X-100 and recovered in 40%-20% linear optiprep density gradient. Lane 1-9 represents 9 factions of optiprep density gradient. Lane M is protein marker.

A. The blot was immunoprobed by anti-surface antiges (1:5,000 diluted). GPI-anchored surface antigens (220-250 kDa) distributed in fractions 3-9.

B. The blot was immunoprobed by anti-GFP (1:5,000). pGlu-GFP (80 kDa) distributes in fraction 4 to 7.

C. The blot was immnoprobed by anti-tubulin (1:10,000 diluted). Tubulin (54 kDa) distributes mostly in the heavy buoyant fractions from 4-9.

The blots are representative of 2 experiments.

Figure 14:

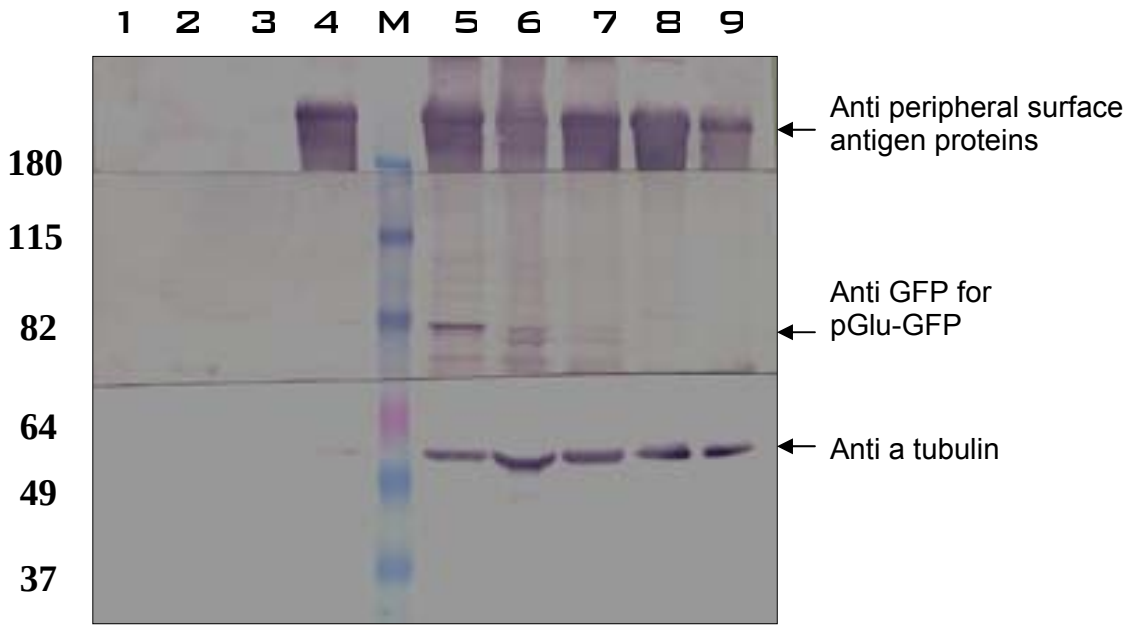

1 2 3 4 M 5 6 7 8 9
180
115
82
64
49
37
Anti peripheral surface antigen proteins
Anti GFP for pGlu-GFP
Anti a tubulin

Figure 15: Distributions of whole cilia proteins from cholesterol depleted pGlu-GFP transformed cells in optiprep density gradient.

The whole ciliary proteins from the cholesterol depleted transformed cells(~8 mg) were extracted with 1% Triton X-100 and recovered in 40%-20% linear optiprep density gradient. Lane 1-9 represents 9 factions of optiprep density gradient. Lane M is protein marker.

A. The blot was immunoprobed by anti-surface antiges (1:5,000 diluted). GPI-anchored surface antigens (220-250 kDa) distributed in fractions 3-9.

B. The blot was immunoprobed by anti-GFP (1:5,000). pGlu-GFP (80 kDa) distributes in fraction 4 to 7.

C. The blot was immnoprobed by anti-tubulin (1:10,000 diluted). Tubulin (54 kDa) distributes mostly in the heavy buoyant fractions from 4-9.

The blots are representative of 2 experiments.

Figure 15:

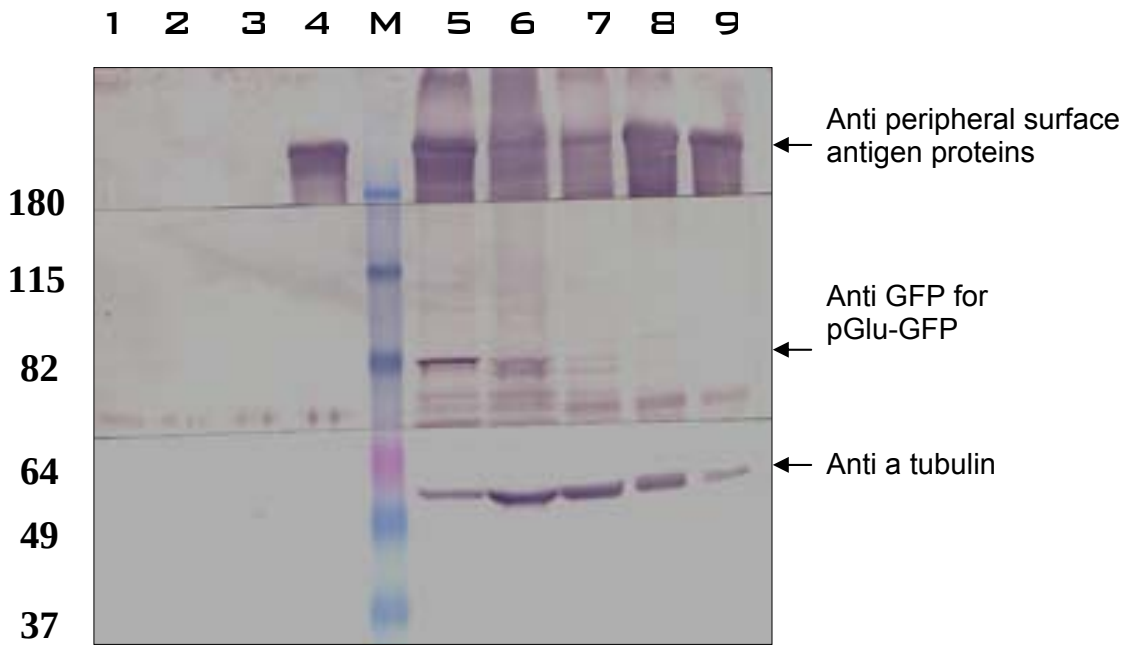
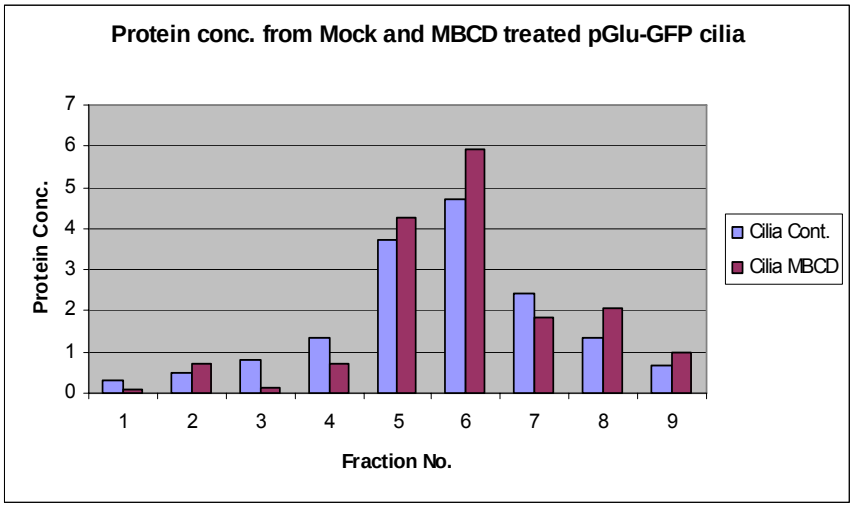

1 2 3 4 M 5 6 7 8 9
180
115
82
64
49
37
Anti peripheral surface antigen proteins
Anti GFP for pGlu-GFP
Anti a tubulin

Figure 15a: Comparison of protein concentrations from mock treated and sterol depleted cilia from pGlu-GFP cells obtained from optiprep density gradient.

The protein samples saved from the nine pooled fractions were assays using the BCA protein assay kit, and the results of both the mock and the sterol depleted cilia of the transformed cell samples, from the nine fractions of optiprep density gradient is shown by a bar graph here. The protein concentration unit is ug/ul.

Figure 15a:


Protein conc. from Mock and MBCD treated pGlu-GFP cilia
Protein Conc.
0
1
2
3
4
5
6
7
1
2
3
4
5
6
7
8
9
Fraction No.
Cilia Cont.
Cilia MBCD

CHAPTER 3:

FUTURE DIRECTIONS

Paramecium, a ciliate, is an important model for studying Ca^{2+} signaling and understanding chemoreception and signal transduction pathways. It has a wide variety of receptor proteins and they are attracted toward glutamate, folate, cAMP and acetate. In this work it has been established that both the pellicle and the cilia membrane of *Paramecium* contains the calcium pumps that are involved in Ca^{2+} extrusion. PMCA 2 in cell body and ciliary membrane was found to be insoluble in Triton X-100, however, other isoforms like the PMCA 3 and 4 are solubilized in cold non-ionic detergents. This indicates that PMCA 2 in the cell and cilia is most likely associated with the lipid rafts, which indicate they probably play a major role in the signal transduction pathway and maintenance of internal Ca^{2+} balance of the cilia. Cholesterol being and important component of lipid rafts, efforts of depletion of sterol from rafts in both cell body and cilia gave interesting results. Though MβCD is supposed to remove cholesterol, it has been found by other groups that it also remove other sterols like stigmasterol. Proteins studied after sterol depletion showed that the distribution of lipid raft marker proteins is not uniform, which means not all proteins are affected by sterol depletion. Proteins like PMCAs are distributed in MβCD sensitive rafts and other proteins like PCM and are present in MβCD insensitive rafts. Some proteins like the GPI anchored surface antigens A and B are present in both the MβCD sensitive and insensitive rafts. The phosphoenzyme intermediate analysis using whole cilia, however, did not support these ciliary pumps are hydroxylamine-labile. Since whole cilia were used in the study, there might have some interference in the method that changed the pH balance of the hydroxylamine and as a result there are bands present in the lane when hydroxylamine was added. In future studies, it would be a very interesting study to observe the enzyme intermediate from pure ciliary membrane, since we already know that the pumps are present in the ciliary membrane. Also addition of a protein kinase

inhibitor to the protein may help in the determination of the band observed as a pump or just a product from other protein kinase.

Both *Paramecium* pellicle and cilia contains lipid rafts as evident from the present and previous Triton insoluble density gradient studies. The observation that Optiprep is a better gradient for protein separation for cilia as compared to sucrose was evident. But the results of pellicle membrane in sucrose compared to Optiprep clearly shows that for lipid rafts analysis, sucrose can detect the absence and presence of cholesterol in rafts, which is a main component of lipid rafts. In future studies a better way of protein purification like ultracentrifugation of fractions for protein pellet, could be applied instead of dialysis in MQ water. This could most likely eliminate the fact that there is a substantial amount of loss of protein from ciliary rafts during dialysis. Sucrose could then be used to study the ciliary rafts of cholesterol-depleted cells and as a result it would be a better control to compare the raft protein localization as opposed to the pellicle membrane. It would also be interesting to analyse the lipids and proteins that are present in each fraction of the rafts from both the cilia and pellicle in details using lipid analysis and mass-spectometry analysis.

NMDA like glutamate receptors which are important marker proteins in other organisms are found to be associated in raft proteins in *Paramecium* ciliary membrane. Since the receptor is expressed from the microinjected transformed cells containing the pGlu-GFP construct, there might be some hindrance in its trafficking as it is not an endogenous protein. Also use of the anti-GFP antibody is not a very satisfactory analysis to identify the protein. Glutamate receptors are part of the signaling pathway involving adenylyl cyclase and calcium pumps. Since we already know from western blot analysis and recent mass spec results that there is an abundance of the above mentioned proteins in cilia, the finding of an endogenous glutamate receptor in cilia is

very likely, since they all together can complete the glutamate signaling pathway. Development of an antibody against the glutamate receptor of *Paramecium* would be much better option and could lead to some definite results.

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