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Imaging Membrane Proteins Using Atomic Force Microscopy Techniques

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ABSTRACT

Thousands of different membrane proteins exist in the human body and reside in the membranes of cells. Structural information regarding these proteins can provide insight into the function of these proteins. Acquiring membrane protein structural information is difficult, especially when preparation techniques that are traditionally used for soluble proteins such as crystallizing and isolating proteins are not easily adapted for membrane proteins. The atomic force microscope (AFM) provides an alternative for imaging membrane proteins. The preparation required does not require crystallizing or isolating proteins, and can imagine membrane proteins in their native environment. Techniques are explored that can utilize the AFM for imaging proteins, including examining isolated cell membrane patches and membrane surfaces. One technique utilizes plasma membrane from *Xenopus laevis* oocytes as a model system to study membrane.

A novel technique is developed to isolate oocyte membranes by bursting the oocyte and depositing its membrane on a flat mica substrate. The flat surface membrane preparation allows high-resolution AFM images to be obtained, revealing a novel structure of densely packed particles. These particles exhibit a regular, repeating pattern of a lattice-like array with orderly packing, and are thus termed “lattice-like array particles” (LAPs). The LAPs are orderly yet imperfectly packed, are located in depressed pools, occur with a low frequency on the oocyte membrane surface, and have not previously been seen using other isolation and imaging methods. Histogram analysis of the center-to-center distance between LAPs suggest their size to be about 44 nm in diameter, considerably larger than other reported size estimates of IMPs. These results indicate that LAPs represent a novel membrane particle organization, which merits further study.
Future developments using this method or further studies to develop alternative membrane preparations may help elucidate membrane protein structure.
CHAPTER 1

Introduction
Structure-function relationships of biological specimens

Cell membranes contain a multitude of proteins. Highly diversified, these proteins provide cells with the ability to interact with one another and engage in other vital functions. It is estimated that the human genome codes for 10,000 distinct membrane proteins (Stahlberg H et al, 2001). These proteins can be membrane-associated or membrane-delimited. The importance of these proteins and their structure in proper physiological functioning cannot be underestimated. Structural features of proteins are the basis of their functional characteristics (e.g. the proteins’ surface grooves, ridges, and pockets). Three-dimensional structures of proteins can provide information on how they function normally or abnormally (i.e. faulty protein structures can cause disease). The proteins that span the cell membrane have been linked to diseases such as cystic fibrosis, hypertension, and diabetes. In fact, 70% of therapeutic drugs target membrane proteins.

Structural information of molecules can provide insight into protein function. Further, integrating structural and functional approaches in research can enhance the understanding of protein function at a molecular level. For instance, when the electroplaque membranes of Torpedo rays were examined using the electron microscope, the structure of the acetylcholine receptor (AchR) was resolved to 9 angstroms (Unwin N, 1993). This information enabled researchers to elucidate functional properties of the AchR, including determining the domains involved in ligand binding. More recently, the mechanism to preferential selectivity of potassium ions in potassium channels was clarified, after the crystal structure of the potassium channel KcsA was solved (Roux B et al, 1999; Jiang Y et al, 2000).
Understanding biological membrane function necessitates progress in membrane protein structure determination. Ultimately, this will provide the basis for better design of therapeutic agents.

**Determining membrane protein structure**

Membrane proteins are challenging to study structurally because they are difficult to purify, isolate, and crystallize, and image with traditional methods such as x-ray crystallography. Few of the estimated 10,000 membrane proteins have been resolved structurally. Though 30% of all sequenced genes appear to encode membrane proteins, only 37 of these have been resolved at atomic resolution (Stahlberg H et al, 2001), compared to more than 10,000 unique structures of soluble proteins.

Purifying membrane proteins presents many challenges. Most purification procedures are not suitable for lipophilic membrane proteins. Regardless, some proteins have been purified through these methods, though it is doubtful that the resulting structure resembles the structure in the native lipophilic environment.

Some membrane protein structures have been solved by crystallizing proteins and determining structure via electron microscopy or x-ray crystallography. Recently, in MacKinnon’s laboratory, the structure of the Streptomyces lividans potassium channel (KcsA) was solved. Crystals were obtained that allowed them to solve the structure at a resolution of 3.2 Angstroms (Doyle DA et al, 1998). With the exception of porins, whose structure has been solved by x-ray crystallography, all other channel proteins have only been studied using electron microscopy, since three dimensional crystals have not been developed. Other channels have been studied by purifying high density channels from
native membranes. For example, purification through immunoaffinity chromatography furthered the structure of the eel electroplax sodium channel by using electron microscopy to obtain images of the channel. The images were then averaged to determine channel structure (Sato C et al, 1998).

Besides resolving structure from crystallography, or imaging membranes using surface microscopy techniques it also can characterize membrane proteins. In order to resolve membrane protein structure, a microscope with the following properties would be useful: the ability to achieve atomic resolution; the ability to work in any environment, and the availability of techniques where biological specimens require little sample preparation or staining. Atomic force microscopes (AFM) possess these properties, and thus, provide unique advantages in the study of membrane proteins and cell surfaces. Moreover, the features of the atomic force microscope position it as an ideal instrument with which to image membrane proteins in their native environment at atomic resolution.

**Scanning probe microscopy**

A wide array of microscopes comprises this family and the AFM is but one member of the scanning probe microscope family. In biology, these microscopes have answered questions related to the structure of molecules adsorbed to inert or biological surfaces. The primary members of this family include the scanning tunneling microscope (STM) and the AFM (sometimes called the scanning force microscope or SFM in literature).

All scanning probe microscopes function with the same underlying principle: a sharp probe is scanned across the surface of a sample and probe-sample interaction is measured. They have no lenses. Instead, a probe is brought close to the sample surface,
and the interaction between the tip and the sample below is measured. These microscopes are characterized by the types of probes used to scan the surface of the sample and measure interaction between the probe and sample. When the probe consists of photons, this is called laser scanning confocal microscopy (Wilson T, 1989). The probe can be a sharp tip that conducts current, as in the STM. With the AFM, the probe is a stylus sensing changes in interaction. A schematic of the STM and AFM are presented in Figure 1.

The AFM is the most widely used of the SPM family. Its features include the ability to image at atomic resolution on a variety of specimens and environmental conditions. Additionally, unlike specimens for electron microscopy, specimens do not need heavy metal coatings. Samples are easier to prepare for imaging, and can be examined without the need for a vacuum.

Scanning probe microscopes have many advantages as compared to other high resolution microscopes. Whereas the scanning and transmission electron microscopes function only in a vacuum, the scanning probe microscopes work in vacuum, liquid, or atmospheric environments. Whereas the scanning electron microscope has a depth of field in the millimeter range, the scanning probe microscope is only limited by length and shape of tip and range of the piezoelectric actuator, which combine to result in a 100 mm limit. A disadvantage of scanning probe microscopes is the lack of abundant scientific knowledge that accompanies its short history. Whereas images and interpretation of images from the scanning and tunneling electron microscopes are well understood, scanning probe microscope artifacts are not. Improvements and advancements in the probe microscopy field will alleviate this limitation.
History of the AFM

Considered the precursor to the AFM, the topografiner was developed by two scientists at the National Bureau of Standards in 1971. Young and Teague originally described the topografiner in a paper published in 1972 (Young RD et al, 1972). Young and Teague recognized that the forces exerted by the tip on the sample could be used to map the topography of a sample. The topografiner used a non-contacting field emission probe to measure surface topography, and could image samples and perform tunneling. It had a horizontal resolution of 100 nm and a vertical resolution limit of 30 angstroms, much coarser than the resolution of the SEM. The topografiner had no better resolution than the SEM and less depth of field; though the instrument utilized a unique imaging method, it was not used due to these reasons. A schematic is presented in Figure 2.

The next development occurred in 1982, with the profilometer, the next generation of the surface scanning microscope, which had similar features to the AFM (Teague EC et al, 1982). The profilometer measured surface topology of hard surfaces by scratching the surface with a stylus.

In 1983, Gerd Binnig developed the scanning tunneling microscope (STM), the immediate precursor to the AFM (Binnig G et al, 1985; Gerber C et al, 1986; Binnig G et al, 2000). Improvements were attributed to accessible personal computers and improved piezo-electric crystals. This proved to be an important development—Binnig and Rohrer received the Nobel Prize in Physics in 1986 for their work on the STM. The STM, a near-field tunneling microscope, utilized the principles of constant current – the control unit maintains a constant tip-to-surface distance by monitoring tunneling current.
Atomic resolution was attained with the STM—vertical resolution reached 0.01 angstroms, and horizontal resolution reached 1 angstrom. The development of the STM advanced the progression of the atomic resolution study of surfaces from an ultrahigh vacuum surface physics specialty into a low-cost commercially available non-vacuum microscopy. The first images were the surface of a silica crystal. Early capabilities of the STM included tunneling, imaging, and observation of atomic scale features (Baro AM et al, 1986; Demuth JE et al, 1986; Leavens CR et al, 1986; Lang ND, 1986a; McCord MA et al, 1986a; Lang ND, 1986b; McCord MA et al, 1986b; Guckenberger R et al, 1987; Morita S et al, 1987; Marti O et al, 1987; Lindsay SM et al, 1987). Later, the STM was used for lithography, electron spectroscopy, and sample surface modification (Dobisz EA et al, 1991; Bourgoin JP et al, 1996; Perkins FK et al, 1996; Maruyama H et al, 1997).

In 1986, Binnig, Quate, and Gerber developed the AFM (Binnig G et al, 1986). The AFM evolved directly from the STM; it used an STM tip placed such that the sharp end of the tip was parallel to the surface, rather than normal to the surface. Hence, the tip was transformed from a tip to a cantilever. The AFM, or modified STM, measured the tiny deflections of the cantilever. This provided a revolutionary step in the advancement of scanning probe microscopy: since the AFM could measure slight cantilever deflections, it was useful for imaging of non-conductive materials at high resolution. SEM provided classical topographic information regarding surfaces, but this new technology enabled sample imaging without a conductive layer. This proved to be useful for future studies of biological materials, which were never naturally highly conductive, and studies began to explore this aspect using the AFM. Baro proved the usefulness of the AFM for resolving
structure of biological materials, by obtaining images of bacteriophage surfaces (Baro AM et al, 1985) using the AFM. However, this study continued to coat the surface with conductive materials. The first images from the AFM of non-conductive material were of boron nitride and a polymerized organic monolayer, showing images could be obtained from an organic system (Marti O et al, 1988).

**Previous biological studies using the AFM**

Since initial papers by Marti and Baro, many studies utilizing the AFM have been published. These include applications in cell biology: visualization of exocytotic pits (Spudich A et al, 1995); visualization of nuclear pores (Schneider S et al, 1995; Rakowska A et al, 1998; Danker T et al, 1999; Danker T et al, 2000); visualization of cytoskeleton rearrangement (Radmacher M et al, 1992); and the measurement of local viscoelasticity (Radmacher M et al, 1992). Images have been generated that reveal cell membranes with their underlying cytoskeletal structure from a human medulloblastoma cell line using STM (Ruppersberg JP et al, 1989).

Other cell biology applications utilized the AFM at lower resolution limits. These include rearrangements of plasma membrane or movements of sub-membrane filament bundles (Henderson E et al, 1992; Fritz M et al, 1994; Schoenenberger CA et al, 1994; Hoh JH et al, 1994; Barbee KA et al, 1994). Local visco-elastic properties have been studied, including an estimation of surface elastic modulus from measuring the local deformation of the surface under stress (Radmacher M et al, 1992). Gold particle labeling of cell surfaces has been used to identify or map surface proteins (Neagu C et al, 1994).

Studies that focus on protein structure using the AFM have been performed. Using the AFM, pertussis toxin (Yang J et al, 1994), cholera toxin (Mou J et al, 1995b) were visualized, and the enzymes phosphorylase b and phosphorylase kinase were studied using the STM. Both images were similar to images taken with electron microscopy and x-ray crystallography (Edstrom RD et al, 1989; Elings VB et al, 1990). Serum albumin and lysozyme were imaged at low resolution in 1988 (Feng L et al, 1988; Feng L et al, 1989). Additionally, vicilin, a globular pea seed protein, was imaged by STM, with multiunit structure consistent with x-ray scattering data (Welland ME et al, 1989). The carbohydrate Beta-cyclodextrin derivatized with toluenesulfonylchloride (tosyl) was imaged in 2-dimensional arrays (Miles MJ et al, 1990). A Langmuir-Blodgett film of a mixed laurymethylether of cellulose was imaged (Rabe JP et al, 1990). Glycogen molecules from rabbit liver (Yang XR et al, 1990) were also studied using the AFM.

AFM has been used to study multimolecular complexes. The interaction of DNA with RNA polymerase (Rees WA et al, 1993), and the recA-DNA complex (Amrein M et al, 1989) was examined. The AFM is a convenient vehicle to study how proteins interact with other molecules. AFM provides a vehicle to study enzyme substrates, antigens,
antibodies, and receptor agonists. Binding domains can be identified, or how the interaction changes the protein’s structure and ability to function.

Purified native membrane fragments, including gap junction plaques (Hoh JH et al, 1991), nuclear pore complexes (Braunstein D et al, 1994), nuclear membrane from Xenopus oocytes (Holstein TW et al, 1992), the HPI layer of *Deinococcus radiodurans*, which forms natural two-dimensional crystals (Karrasch S et al, 1995; Karrasch S et al, 1996), have all been imaged using the AFM.

The AFM also has been used in non-imaging applications. These include using a large probe force to remove the outer layer of a nematocyte capsule to visualize underlying structures (Holstein TW et al, 1990; Hansma HG, 2001), measuring intermolecular forces (Butt HJ, 1991; Florin EL et al, 1994), and detecting of 1 nm fluctuations of single enzyme molecules during activity (Radmacher M et al, 1994a; Radmacher M et al, 1994b).

The wealth of information that can be derived from the use of the AFM shows the versatility of this instrument in elucidating novel structural and functional information in biology. Methods thus far have been pain-stakingly developed for each scientific question, and as yet, techniques in AFM have not been standardized to improve data-gathering for a wide variety of biological questions.

*Principles of the AFM*

The theory and principles of the AFM can be summarized by describing the interaction forces between a probe and a sample (see Figure 3). The AFM measures force
interactions between the atoms on the sample and on the tip; this measurement is
transduced through a sensitive cantilever. The interaction causes the cantilever to bend;
this deflection can be measured optically, and then converted to an electrical signal to be
processed by the microscope’s electronic controls. The three dimensional topography of
the sample is then scanned by the probe, and the interaction forces along the sample are
recorded. An image is generated by rastering the deflection data, which displays the
topography of the scanned sample.

The AFM complements other methods that provide information about three dimensional
structure and composition of matter. Other methods that provide information about
topographical structure include SEM, NMR, and X-ray crystallography. The AFM is
utilized extensively in structural biology. In addition to topographical structure, the
AFM can provide information regarding binding forces, attractive forces, and dynamic
and stochiometric studies. The AFM is capable of high-resolution imaging, reaching
atomic resolution limits, though vertical resolution is much higher than horizontal
resolution. Due to the softness of the sample and lack of rigidity, resolution in biological
samples has not been as sensitive as in other applications with rigid surfaces. Improving
rigidity may improve the resolution of biological samples closer to the theoretical limits
of resolution for the AFM.

Several attributes render the AFM advantageous over other high-resolution microscopes.
The AFM can be used to image in ambient conditions, including room temperature and at
atmospheric pressure. It can also be used to image in physiological solutions, and in
certain instances, can achieve higher resolution images in solution, as compared to
images acquired in air. It covers an enormous range of resolution from microns to
nanometers, which enable it to encompass the combined range of resolutions covered by light and electron microscopy. Moreover, the combination of these features allows it to image whole cells or single molecules.

**Components of the AFM**

A variety of parts comprise the AFM. These include the tip, the cantilever, a laser beam, a photodiode detector, and a piezo driver (Figure 4). The AFM also requires an array of supporting instrumentation. These include a vibration isolation system, control and feedback electronics, a data acquisition system, and image display. In addition, the microscope includes an optical microscope for alignment and gross visualization of the samples. The main part of the AFM is actually quite small, weighing only three kilograms. The supporting instrumentation, which includes the computer, interacts with Multimode IIIA (Digital Instruments, Santa Barbara CA) AFM. The Multimode provides feedback to the computer, which in turn controls the travel of the piezo and records the changes in height.

**The probe (tip).** On the AFM at the end of the cantilever, the probe is a super-sharp, mechanical tip (commercial tips are typically 10 nM), composed of an alloy. The probe can be made of diamond or a metal alloy like silicon nitride. In the case of a diamond tip, a diamond fragment is fractured, and then attached to a spring. The spring can be a cantilever, and typically has a spring constant near 1 N/m, which is similar to the spring constant of the Slinky®. Initially, the AFM probes were made of diamond chips glued onto wire springs. Currently, sharp tips can be mounted on cantilevers commercially or in the lab with proven techniques. Tips can also be made by electron beam deposition.
These have a sharp aspect ratio and a smaller curvature radius. Sharper tips have lower minimum forces. The probe systematically goes across scanned sample in a raster pattern; the vertical position of the probe is recorded.

**The cantilever.** The probe tip is mounted on the end of a cantilever. It is the deflection of the cantilever that is measured to indicate the interaction between the probe and the sample. Often, studies scan the surface of a sample with a sharp tip mounted on a very soft cantilever having a spring constant usually less than 0.5 N/m (Rugar D et al, 1990). The cantilever is monitored for changes in vertical position by measuring deflection of a laser light on its top surface. This reflection is recorded into a photodiode detector.

**Laser beam and photodiode detector.** The laser is reflected onto the cantilever and into the photodiode detector. Usually, the photodiode detector is composed of an upper (A) and a lower (B) half. Initially, the beam is centered so that half the light of the beam is on each half of the detector. Any changes in deflection of the tip, and therefore the cantilever, can be magnified and calculated by a computer comparing the signals from the (A) and (B) photodiodes. In order to determine travel, the computer will normalize the signal \((A-B)/(A+B)\), equivalent to amount of light on A/light on B.

**The piezoelectric driver and piezo unit.** Piezoelectric materials change their physical length in proportion to applied voltage. The sample can be mounted on the top of the piezo unit. When the piezo expands, it changes height, and the sample can be moved closer to or farther from the tip. Alternatively, the piezo can expand on one side, and contract on the other, effectively causing the sample to move in the horizontal direction.
The piezo is controlled by the computerized feedback control system, or can be over-
ridden by manual computer controls.

**Practical aspects of the AFM**

Small forces near the sample and tip interact, causing a deflection of the cantilever beam. Because the cantilever has a known spring constant, the force can be calculated. Several common forces are encountered. Attractive long-range forces include Van der Waals, capillary condensation, and electrostatic. Attractive short-range forces include hydration and adhesion. Repulsive, adhesive, and magnetic forces are also encountered.

The computer records interatomic forces between the apex of a tip and atoms in a sample as the tip scans over the sample surface (See Figure 5). Interatomic interactions on STM are closer to true atomic resolution – dependence of the tunneling current on the tip-to-sample separation is exponential; for AFM the dependence of cantilever deflection on tip-to-sample interaction is weaker than that in STM; therefore several atoms on the tip interact with several atoms on the sample. On the AFM there is a spread of interaction forces. The long-range Van der Waals attraction is balanced by the hard repulsion between tip and sample. Additional interactions include coulomb forces (Butt HJ, 1991), protein binding forces (Willemsen OH et al, 2000), adhesion forces (Benoit M et al, 2000), and hydration forces (Israelachivili JN et al, 1983). When the AFM is imaging in air, the meniscus force of a wetting water film may dominate attractive forces. The typical forces between probe and surface are less than 1nN.

Substrates are typically mounted to a metal disk. This disk can be attached to the piezo. Samples are supported by a variety of substrates, depending on the properties of the
sample in conjunction with the properties of the substrate. Various bonding materials to bind the substrate to the metal disk that is held in place in the AFM are used. These include double-sided tape, two-ton epoxy, 5-minute epoxy, CrazyGlue®, and Permaslip®. Mica is the predominant substrate. It is atomically flat and cleavable, so that there is a fresh, sterile layer for each sample. It is crystalline aluminum silicate, and occurs in natural mineral deposits. It is useful for imaging molecules less than a few nanometers high, has negative surface charge, is 1 nm/layer, and its unit cell is 2 nm. It is considered atomically flat but does not precisely achieve atomic resolution, though it is considered to be the 5 angstrom standard. It is composed of oxygen and silicon making a plane, with, a hydroxyl group below, and potassium ion above this plane. When potassium is removed, a negatively charged aluminum silicate surface is exposed. Charge can be reversed by incubating mica with magnesium. This technique is useful for binding DNA to mica. Modifications can be made to mica: silanes or APTES (amino propyl triethoxy silane) can modify mica, which is then crosslinked with ultraviolet light. Mica can be modified with polylysine; though it is a large protein with surface roughness. Sometimes muscle adhesive protein is used instead of poly-lysine, which is also rough but is smaller in size.

Glass substrates are also used. These are composed of 50-80% SiO₂ (otherwise known as beach sand), along with magnesium, and sodium. When well-cleaned, glass is negatively charged. The surface roughness is approximately 1-2 nm. It is good for imaging thicker biological samples like cells. Like mica, it has negative surface charge, and is made of silicon oxide. It has been used successfully in studies requiring Langmuir-Blodgett films. Sapphire (Al₂O₃) is also used as a substrate. At neutral pH, it is positively charged. There
are few substrates that are natively positively charged surfaces. Other substrates include silicon wafers. These can be made atomically flat by etching with hydrofluoric acid, and this layer will be hydrophobic. Silicon can be cleaned like glass. The wafer will oxidize top 2 nm into silicon. Highly ordered pyrolytic graphite (HOPG) is smoother than carbon films and have a hydrophobic surface. Though expensive, it is cleavable. Some specific-use studies have utilized filter membranes as substrates. The filter membranes are composed of membranes with small pores, in which cells can be mechanically trapped. The pore size of the membranes must be slightly smaller than the diameter of the cells.

The scanning AFM typically functions in one of three different modes. The three modes are called contact mode, non-contact mode, and tapping mode. Though each measures deflection and topography, they each have a different control or feedback mechanism, and are appropriate for different types of samples.

In contact mode AFM, the tip is lowered until it detects attractive Van der Waals forces, typically 10-20 angstroms from the surface of the sample. Hence, the AFM has limited functionality with soft, sticky samples. Here, the primary issue is force control, and damping the shear forces. In non-contact mode, the tip is oscillated at its resonance frequency. The oscillation amplitude must be small (10-20 angstroms) to be within the Van der Waals envelope. The major concern when in non-contact mode is feedback stability; the feedback control must move the sample away from the oscillating tip; if the tip touches fluid layer, it tends to become sticky, preventing it from accurately maintaining its forces, and resulting in an improper feedback and an image that does not accurately represent the surface of the sample. Previous studies have resolved this issue by performing non-contact AFM in a vacuum, then baking the fluid layer. The studies
that have successful results with non-contact thus far have been in vacuum; the ones in ambient conditions have described improvements in techniques. The third functioning mode is called tapping mode. Similar to non-contact mode, this mode oscillates the tip, but the amplitude of oscillation is much larger, with amplitudes 5-50 nm. This allows the tip to tap on the sample surface. As a result, a repulsive force is generated as the tip hits the sample, resulting in stiffening the sample surface. This tip has stored energy, and requires the use of a stiffer cantilever. The advantage of tapping mode is that there are no shear forces and no friction, which exist in non-contact mode.

Within these modes, there are two kinds of feedback that can be employed. In the first, constant height mode, the repulsive force is detected at each point, and the distance between the sample and the tip is held constant. In the second, constant force mode, the feedback mechanism maintains the repulsive force constant by varying the distance between the tip and the specimen at each point. Data is returned in contact mode in either height or deflection information. Height mode is most commonly utilized. This displays changes in sample height during scanning. It records calibrated height information about the sample surface. Deflection mode is also called error signal mode, which records small changes in cantilever deflection. Here the images resemble a derivative of the height image.

Ongoing improvements in techniques, instrumentation, and materials will improve resolution. Carbon-deposited super tips improve resolution to (Karrasch S et al, 1994) near 1 nm. Resolution is limited largely by the dimension of the tip. Though true at modest resolutions, it is not true at sub-nanometer resolution. Resolution may be improved by new developments in technique. Resolution on cell surfaces hover around
on DNA and soluble proteins, the AFM has been able to achieve nanometer resolution. What is the cause of this discrepancy? The softness of biological specimens is a major obstacle to higher spatial resolution. Low resolution on cell surfaces either in solution or dried occurs frequently. The cell membrane is too soft to withstand tip forces: the larger the contact area on the tip apex, the lower the lateral resolution. In previous studies, there was no marked improvement by using glutaraldehyde to fix cells. Some of the resolution problems can be partially circumvented by the use of solid supports beneath the membrane to mimic supported bilayers (Kunkel DD et al, 1997b). Sometimes images are not better than EM or X-ray crystallography. However, a few hours’ effort can determine the dimensions and shape of a protein.

Aims/Goals of the thesis

Currently available technologies have difficulty providing the information necessary to elucidate membrane protein structure at the molecular level. The atomic force microscope offers the structural biologist a new tool to resolve membrane protein structure at molecular resolution. However, the molecular resolution images that have been obtained thus far have not been as successful as has been suggested by the technological potential of this instrumentation. Marked improvement in this scientific arena requires the development of standardized protocols for sample preparation and imaging techniques. Thus, it is important to develop techniques which standardize sample preparation, improve imaging, and access the high-resolution capabilities of the AFM. The purpose of this thesis is to develop standard protocols for sample preparation and imaging using the AFM. If these protocols for sample preparation and AFM imaging
can be developed, then they can be adapted to elucidate the structure of any membrane protein. The protocols and cell systems can be used as tools in the structural biologists’ laboratories, providing novel information regarding protein structure, information that has been elusive, given currently available techniques. More importantly, the proteins that have historically been most difficult to render structural information – the membrane spanning proteins – can be studied more efficiently. This advancement would offer tremendous promise for new information.

Chapter 2 presents several approaches to improve sample preparation such that the preparative methods are repeatable, reliable, readily reconfigurable for a variety of proteins, and, upon imaging, can provide high-resolution information. Sample preparation protocols were developed; each protocol was evaluated for its ability to produce easily imaged samples using the AFM. The parameters that were adjusted in each protocol included cell membrane type, cell membrane preparation, and substrate preparation. Each experiment evaluated the quality of a single protocol. This chapter is composed of these independent experiments.

One of the protocols developed in Chapter 2 resulted in the ability to acquire high resolution AFM images of the intracellular *Xenopus* oocyte plasma membrane. Using this protocol, we have identified a structure that has not been previously reported in the literature. Chapter 3 describes the work related to developing the protocol for this experiment, imaging the oocyte membrane. Further, it characterizes the features of the observed structure and defines it as a “lattice-like array.” The discussion speculates on the identity of the array, as well as the questions that lead new research in this area.
Potential artifacts are identified, and methods to improve the reliability of membrane protein imaging are provided.

Chapter 4 discusses the results of these investigations and the implications for future studies. These protocols may contribute to the study of membrane protein structure. The limitations, potential avenues, and future directions suggested by these studies are discussed.
Figure 1. Schematic of A) STM and B) AFM.

In the STM, a feedback mechanism maintains a constant tunneling current and traces contours of constant electron density. Different atoms (shaded atom) in a surface will alter the path that the tip follows. In the AFM, feedback maintains a constant force, and the tip follows a path that is an accurate topograph of the surface; no current is needed, therefore the AFM can image nonconducting samples. Adapted from (Hansma PK et al, 1988).
Figure 2. An illustration of the topographiner, shown schematically. The topographiner was the precursor to the AFM. Instead of one piezo unit, it utilized three piezo drivers. The X and Y piezo drivers scanned the emitter parallel and directly above the sample surface. The emitter to sample voltage was determined by the constant current passing between them and by the space between them. The Z piezo was controlled by a system that maintains a constant voltage between the emitter and the sample. As a result, the amount that the Z piezo moves is a direct reflection of the surface profile. The topographiner lacked the resolution of other systems, and hence, was shelved. However, it was the development topographiner that directly preceded the development of the technology for the AFM. Adapted from: (Young RD, 1971).
Figure 3. TOP: Two dimensional schematic of the optical deflection technique. Small
deflections of the cantilever, caused by small changes in interaction between the tip and
sample can be magnified by deflecting a laser light off the back of the cantilever. The
laser diode is maneuvered so that the photodiode detector can record the deflections of
light. The amount of light in one half of the diode is registered compared to the amount
of light detected in the other half. Deflection is measured by the fractional difference of
the light on one half versus the other half of the diode. Source: Digital Instruments, Santa
Barbara, CA. BOTTOM: A: Force vs Lateral position curve (adapted from (Gardner DJ,
2002)). Shown are A: repulsive force, B: zero net force, C: maximum attractive force, D:
low contact attractive force. From large distances to B, we see a direct relation between
sample height and deflection in the photodiode detector; at smaller (submicron) distances,
attraction and repulsion of the tip to the sample will alter the information received by the
photodiode detector. Appropriate microscope manipulations must be made to account for
attractive and repulsive forces.
Figure 4. Schematic of the AFM instrumentation. Source: Digital Instruments. A schematic of the AFM head, specifically the Digital Instruments Multimode head. All major components are labeled. For an explanation of the role of each, see text.
Figure 5. Interaction forces between the tip and sample surface. Small forces near the sample interact with the tip to cause a deflection of the cantilever beam. A variety of forces can be measured. See text for more details on intermolecular forces encountered. Information adapted from (Heinz WF et al, 1999).
Van Der Waals Forces  
Ionic Repulsion (imaging AFM)  
Magnetic or electrostatic Forces  
Adhesion, Bonding, or Frictional Forces  
Elastic and plastic properties of surface
CHAPTER 2

Development of sample preparation protocols for improved image resolution during acquisition of topographic information using AFM
Abstract

The resolution capabilities of the AFM reach sub-nanometer levels. This level of resolution has the potential to resolve the structural topography of membrane proteins. Though the AFM possesses features that render it an attractive tool for precisely these types of studies, few studies have successfully utilized the full capabilities of the AFM to image membrane proteins in their native state. Each successful study has optimized the biological sample preparation specifically to the conditions of that particular membrane protein. Therefore, in order to fully realize the potential of using the AFM to image membrane proteins in their native state, advances must be made in (1) standardizing protocols to prepare membrane proteins for imaging, and (2) optimizing conditions to improve resolution of biological preparations.

This chapter presents a series of experiments designed to develop sample preparation in a manner such that the preparative methods are standardized. Experiments are designed to improve repeatability, reliability, and ease of reconfiguration for a variety of proteins. In addition, imaging conditions are optimized to provide high-resolution topographic information of membrane proteins.

This chapter reviews five independent experiments. These are technique-driven experiments; the goal of each experiment is to improve or develop a protocol that results in high-resolution AFM imaging. Each experiment attempts to improve a specific aspect of the techniques involved in imaging biological samples using the AFM. The parameters that were adjusted in each protocol included cell type, cell membrane
preparation, and substrate preparation. The cells and membranes chosen in these experiments have all been used to express recombinant proteins in plasma membranes.

Experiments 1 and 2 develop protocols to image plasma membranes of cells from high expressing cell lines. The cell lines used in these experiments are known to express high levels of recombinant protein. The AFM is then able to image the membranes that express recombinant protein, and identification of the protein of interest is facilitated. However, problems arose with the high expressing cell lines in Experiments 1 and 2. The cells in Experiment 1 were prone to lysis; the cells in Experiment 2 did not adhere well to its substrate. Therefore, the cell membranes proved to be difficult to image at high resolution.

Experiment 3 presents protocols to prepare cells from cell lines with stronger adhesion properties than those in Experiments 1 and 2. Stronger adhesion properties enable the cells to stay in place during scanning of the AFM probe. However, these live cells proved to have inherent softness, which prevented acquisition of topographic information at high-resolution. In order to increase rigidity of the cell membrane, two changes were included in Experiment 4 – cell membranes were examined after they were isolated from the cytosol, and membranes were examined after they were allowed to air dry.

Experiment 4 develops a protocol in which membrane patches were pulled from *Xenopus* oocytes, and then air-dried on a substrate. This incorporated the inherent rigidity of the substrate into the membrane patch. The rigidity is thought to enhance the imaging resolution of the AFM. However, this raised concerns regarding the integrity of the oocyte plasma membrane. Because the vitelline membrane is removed prior to obtaining
a plasma membrane patch, there were concerns that the plasma membrane patches were contaminated with vitelline membrane. This would result in acquiring images from the surface of the vitelline membrane, rather than the plasma membrane.

Experiment 5 develops a protocol for isolating vitelline membrane from oocytes and determining molecular structure of vitelline membrane.
Experiment 1: Development of Sf9 cell membranes for high-resolution imaging

Create a membrane preparation suitable for determining structure of membrane proteins using high-resolution AFM imaging. Develop a protocol that can utilize the AFM to identify membrane proteins in native membrane, using the cell line Sf9, which expresses high quantities of protein.

Abstract

Cells that express high levels of recombinant protein have the potential to present large numbers of membrane proteins on the cell surface. AFM, which is a surface microscopy, can reveal surface topography of cell membranes and embedded proteins. At its highest resolution limits, the AFM can obtain molecular resolution, providing a simple, yet effective means of determining membrane protein structure. The task of identifying the protein of interest can be facilitated by comparing the structural differences in cell membranes that express a large number of recombinant proteins to a similar cell that does not express recombinant protein. The baculovirus expression system provides an environment that is well suited to this type of study; it utilizes the Sf9 cell, which can express large quantities of recombinant protein. In this experiment, a protocol is developed; it compares Sf9 cells that do and do not express high levels of GIRK channel. These cells are then prepared for high resolution AFM imaging.

Background

Various cell lines have been used in imaging of cells using AFM (Choi YH et al, 1999; Schneider SW et al, 1999; Vie V et al, 2000; Tenidis K et al, 2000; Avery J et al, 2000;
Wangerek LA et al, 2001). Though Sf9 cells have not been used in AFM studies, these cells provide a desirable feature for the determination of membrane protein structure. Sf9 cells in the baculovirus system are typically used to express high levels of recombinant proteins. The Sf9 cells originated from the IPLBSF-12 cell line, derived from the pupal ovarian tissue of the fall army worm, *Spodoptera frugiperda* (Czuba M et al, 1994). Sf9 cells are spherical with some granular appearance and are regular in size. The small, regular size makes them exceptional for the formation of monolayers and plaques. These are suitable for transfection, plaque purification, plaque formation, and expression of recombinant proteins. Our experiment utilized the Sf9 cells to express membrane proteins. These cells were fixed to coverslips and prepared for imaging with the AFM.

This experiment utilizes the Sf9 cells to express the GIRK channel. These cells can accept a recombinant plasmid containing GIRK upon infection. The GIRK gene encodes for a subunit of a G-protein coupled inwardly rectifying potassium channel. Five GIRK genes have been identified, GIRK1 – GIRK5. Expression of GIRK2 using the baculovirus system has been shown to be efficient (Kamb A et al, 1992; Schwalbe RA et al, 1995; Gaymard F et al, 1996; Marten I et al, 1996). GIRK channels, similar to other inwardly rectifying K channels, are thought to form a heterotetramer (Li M et al, 1994; Corey S et al, 1998a; Corey S et al, 1998b; Mark MD et al, 2000), but no study has specifically determined the structure of GIRK channels. Two copies of each gene, GIRK1 and GIRK2, are hypothesized to form the four subunits of the GIRK channel.
Methods

GIRK2/Kir3.2 (GenBank L35771) cDNA was ligated into the Bsu36 I site of pBlueBac4 (Invitrogen, San Diego, California) and confirmed by nucleotide sequencing. Plasmid DNA was purified and desalted prior to transfection procedures. The ATG start site within the GIRK sequence was used for protein translation.

Sf9 cells were maintained at 17°C in Grace’s insect media. During log phase growth, cells were co-transfected with Ban-N-Blue DNA and the pBlueBac4 construct containing GIRK by cationic liposome mediated transfection. Upon viral infection of cells, approximately 2 days after infection, lysis occurred, and a supernatant containing active virus formed. Sf9 cells were plated prior to lysis, on day one, onto sterile cover slips, and covered with media. Cells were incubated at 17°C overnight. Cells were lightly rinsed in media, then were allowed to air-dry overnight. Cover slips were mounted onto metal pucks for subsequent AFM imaging.

AFM imaging of cells was performed in dry ambient conditions at room temperature using a Nanoscope IIIa (Digital Instruments, Santa Barbara, CA) equipped with a J-type 120 µm scanner. Scanning was performed in contact mode. 200 µm V-shaped silicon nitride cantilevers with a nominal force constant of 0.06 N m⁻¹ were used.

Results and Discussion

The techniques described in this experiment attempted to optimize protein expression of GIRK. GIRK expression was measured using the AFM by comparing membrane surfaces of cells that expressed protein to control cells that did not express recombinant
protein. However, because the baculovirus system produced high levels of protein expression through the lysis of Sf9 cells, the lysis made it impossible to deposit intact cells onto cover slips for subsequent AFM imaging. No images were confirmed to be Sf9 cell membranes. Additionally, depositing membranes of lysed cells onto cover slips was difficult and impractical. Following this experiment, it was determined that high expression of protein continued to be a priority. Development of this experiment included performing similar protein expression in cells that did not lyse. This is discussed in Experiment 2.
Experiment 2: Development of non-lytic S2 cell membranes for high-resolution imaging

Create a membrane preparation suitable for determining structure of membrane proteins using high-resolution AFM imaging. Develop a protocol that can utilize the AFM to identify membrane proteins in native membrane, using the Drosophila Schneider S2 cell line, which expresses high quantities of protein and does not require lysis for expression.

Abstract

Cells that express high levels of recombinant protein have the potential to present large numbers of membrane proteins on the cell surface. AFM, a surface microscopy, can reveal surface topography of cell membranes. The task of identifying the protein of interest can be facilitated—large numbers of similar structures are identified in cells that express recombinant protein, and are not present in cells that do not express recombinant protein. The Drosophila Schneider cell expression system utilizes S2 cells; these cells can express large quantities of recombinant protein but do not require lysis for protein expression. In this experiment, a protocol is developed. It attempts to achieve high levels of GIRK channel expression in S2 cells and then prepares the S2 cell membrane for high resolution AFM imaging. A construct containing the GIRK gene is expressed in S2 cells. Upon expression of GIRK, S2 cells are prepared for AFM imaging.
**Background**

Various cell lines have been used in imaging of cells with the AFM (Choi YH et al, 1999; Schneider SW et al, 1999; Vie V et al, 2000; Tenidis K et al, 2000; Avery J et al, 2000; Wangerek LA et al, 2001). Though S2 cells have not been used in AFM studies, these cells possess a desirable feature for the determination of membrane protein structure. The Drosophila S2 insect expression system is non-lytic and produces high levels of recombinant protein expression. Expression is more efficient than mammalian cells. This expression system uses small plasmid DNA vectors for expression of proteins. These cells spontaneously integrate hundreds of copies of the recombinant plasmid into their genome. In virus-based systems like the Sf9 system, cells that are expressing the recombinant protein are lysed by the virus. A wide range of proteins have been expressed in *Drosophila* S2 cells. It is especially well suited to express receptors. (From Invitrogen, San Diego, CA.)

This experiment utilizes the Schneider S2 cells to express the GIRK channel. These cells can accept a recombinant plasmid containing GIRK upon transfection. The GIRK gene encodes for a subunit of a G-protein coupled inwardly rectifying potassium channel. Five GIRK genes have been identified, GIRK1 – GIRK5. Expression of GIRK using the Schneider insect cell system has not been verified. GIRK channels, similar to other inwardly rectifying K channels, are thought to form a heterotetramer (Li M et al, 1994; Corey S et al, 1998a; Corey S et al, 1998b; Mark MD et al, 2000), but no study has specifically determined the structure of GIRK channels. Two copies of each gene, GIRK1 and GIRK2, are hypothesized to form the four subunits of the GIRK channel.
Methods

Plasmids pMT and pAT were kindly supplied by L. Cherbas (Indiana University, Bloomington, IN). Plasmid pAT contained an actin promoter; it was incubated with the restriction enzyme BamHI. A 1.6 kb cDNA fragment encoding GIRK2 was subcloned into the expression plasmid via this site. Properly oriented constructs were selected after restriction enzyme mapping. S2 cells were co-transfected via calcium phosphate transfection with 1 ug of pUCneo and 20 ug of pMT/GIRK2. S2 cells were maintained and induced to express recombinant protein. S2 cells were maintained at 27 C in Schneider media supplemented with 10% heat inactivated fetal calf serum, and 2% mM glutamine.

S2 cells expressing GIRK channel or non-transfected cells were plated onto cover slips for AFM imaging; cells were allowed to adhere for 24 hours prior to sample preparation. Cells were lightly rinsed in media, then were allowed to air-dry overnight. Cover slips were mounted onto metal pucks for subsequent AFM imaging.

Cover slip preparation: For treated cover slips, 5 ul Cell-Tak (Collaborative BioMedical Products, Bedford, MA) or 0.01% poly-l-lysine (Sigma, St Louis, MO) was placed on sterilized cover slips and allowed to dry. Treated or untreated glass cover slips of 15 mm in diameter were placed in cell culture dishes containing freshly passaged S2 cells. After 24 hours, the glass cover slips were mounted for AFM imaging.

AFM imaging of cells was performed (see Experiment 1).
Results and Discussion

Schneider S2 cells were placed upon glass cover slips to prepare samples for AFM imaging. Subsequent to the 24-hour incubation period, cells were taken to the AFM stage and imaging procedures were initiated. However, no images were collected, because it was found that the S2 cells were not adhering to the cover slips. This caused the cells to slip during imaging, resulting in an inability to acquire information regarding surface topography. Additives Cell-Tak and poly-l-lysine were placed on the cover slips to improve cell adhesion. These materials did not improve cell adhesion adequately to allow the acquisition of topographic information. It was determined that these cells would be inappropriate to use as vehicles for high levels of protein expression for AFM imaging.
Experiment 3: Development of cell membranes with adherent properties for high-resolution imaging

Create a membrane preparation suitable for determining structure of membrane proteins using high-resolution AFM imaging. Develop a protocol that can utilize the AFM to identify membrane proteins in native membrane, using cell lines that have adherent properties to substrates.

Abstract

The substrate causes those cells that adhere to it to be rigid. AFM, which is a surface scanning microscopy, can reveal surface topography of cell membranes. The resolution of the AFM is increased with greater sample rigidity. AV12, SK-N-SH, and SH-SY-5Y cells are mammalian cell lines that have adherent properties. Because of this tight adhesion, these cells are well suited to withstand the forces imposed on the cell during AFM imaging. In addition, AV12 cells have reasonably high levels of recombinant protein expression. In this experiment, a protocol is developed. It attempts to prepare these cell membranes for high resolution AFM imaging.

Background

Various cell lines have been used in imaging of cells using AFM (Choi YH et al, 1999; Schneider SW et al, 1999; Vie V et al, 2000; Tenidis K et al, 2000; Avery J et al, 2000; Wangerek LA et al, 2001). Though AV12 cells have not been used in AFM studies, these cells provide a desirable feature for the determination of membrane protein structure.
AV12 cells are known to tightly adhere to cover slips. They are hearty cells and express reasonably high levels of recombinant protein. This expression system uses small plasmid DNA vectors for expression of proteins. A wide range of proteins has been expressed in AV12 cells. It is especially well suited to express receptors.

SK-N-SH and SH-SY-5Y cells are neuroblastoma cell lines that have adherent properties similar to AV12. In addition, they express endogenous mu opioid receptors. This feature may be utilized if these cells are appropriate for high resolution imaging with the AFM. Recombinant GIRK may be expressed in these cells, and interactions between the receptor and the channel can be observed.

**Methods**

AV12 cells were kindly provided by Eli Lilly Corporation (Indianapolis, USA).

Sterilized glass cover slips of 15 mm in diameter were placed in cell culture dishes containing freshly passaged AV12 cells. The cells were cultured in DMEM media and incubated at 37°C with 5% CO₂. After 2 days, the glass cover slips were mounted for AFM imaging.

SK-N-SH and SH-SY-5Y cells were acquired through ATCC. Sterilized glass cover slips of 15 mm in diameter were placed in cell culture dishes containing freshly passaged neuroblastoma cells. The cells were cultured in MEM-alpha media and incubated at 37°C with 5% CO₂. After 2 days, the glass cover slips were mounted for AFM imaging.

AFM imaging of living cells was performed (see Experiment 2).
Results and Discussion

Images of cells are shown in Figure 6. AV12 cells have been imaged using the AFM. High quality AFM images that accurately reflect the topography of the membrane surface have been generated. Cellular structure, such as the cytoskeletal network is seen.

Images of SK-N-SH cells are shown in Figure 7. Similar to the AV12 cells, SK-N-SH cells have been imaged using the AFM. High quality AFM images that accurately reflect the topography of the membrane surface have been generated. Cellular structure, such as the cytoskeletal network is seen. However, due to the inherent softness of cells, resolution was not high enough to appropriately resolve membrane protein structure. It was determined that large, intact cells, would be too soft to attain the resolution needed to image proteins on the cell surface. It was determined that isolating membranes away from the intracellular contents would improve cell membrane rigidity on the substrate.

SH-SY-5Y cells were not imaged. Due to the low density of these cells, it was difficult to identify areas on the coverslip that contained cellular matter.
Figure 6. A typical sequence of deflection-mode AFM images (60 x 60 µm) obtained from a patch containing several AV12 cells. Scans were performed over a period of \(~42\) minutes.
Figure 7. Deflection-mode AFM images (80 x 80 µm) of SK-N-SH cells, showing the underlying cytoskeleton structure. Scans were performed over a period of ~100 minutes.
Experiment 4: Development of cell membrane patches for high-resolution imaging

Create a membrane preparation suitable for determining structure of membrane proteins using high-resolution AFM imaging. Develop a protocol that can utilize the AFM to identify membrane proteins in native membrane, using cell membrane patches that can express high quantities of protein and can be supported by a rigid substrate.

Abstract

Cells that express high levels of recombinant protein have the potential to present large numbers of membrane protein on its cell surface. AFM, which is a surface microscopy, can reveal surface topography of cell membranes. The task of identifying the protein of interest can be facilitated—large numbers of a similar structure are identified in cells that express recombinant protein, and are not present in cells that do not express recombinant protein. However, in previous experiments, AFM imaging achieving molecular resolution failed because cells were not rigid enough to allow for high-resolution imaging. We determined that isolating the cell membrane apart from the cellular contents would enable the cell membrane to become more rigid, thus enhancing the resolution. In addition, because oocytes are often used in functional assays for channel studies, we proposed to use a patch-clamp technique to initially measure channel activity, derive channel number from this activity, and deposit the patch clamped membrane onto a rigid surface. Further, we explored using a giant patch technique to facilitate the detection of the patch using the optical microscope prior to AFM imaging. Though channel activity
was successfully recorded, the methods used could not utilize this information; hence, this material has not been presented.

**Background**

Biological samples imaged using the AFM have shown to have inherent softness, which reduces image quality and resolution of the images. We propose to develop a protocol that improves rigidity of the samples to improve resolution. This is accomplished by isolating native membrane away from the cytosolic contents of the cell and mounting the membrane directly onto a rigid substrate, such as a cover slip or mica. Because oocytes are easy to manipulate and work with, we initially targeted these membranes for this experiment. Additionally, oocytes are often used to express recombinant membrane proteins. Several experiments utilize Xenopus oocyte membranes in AFM imaging studies (Bron P et al, 1999; Steinacker P et al, 2000; Schillers H et al, 2001).

Using the AFM to image oocyte membrane patches, the objective was to observe whether a significant difference existed in structural features on the surface of one membrane patch that is known to have large amounts of protein expression compared to a membrane patch that is known to have lower amounts of protein expression. However, it is difficult to correlate whole cell channel number with these images. The macroscopic current observed for an oocyte may not be a proper tool to correlate to structural features observed in a small area of membrane. Instead, identifying current in a small patch of membrane and comparing the expected channel number to the AFM image features appears more appropriate and conclusive. Several studies have utilized membrane

This procedure allows for (1) an initial calculation of channel number by electrophysiologically measuring channel activity in the patch, (2) an examination of membranes which have large amounts of protein expressed on the surface, and (3) a comparison to membranes which have lower amounts of protein expressed. The amount of protein expressed can be manipulated by altering the amount of RNA (or DNA, in the case of nuclear injected oocytes) that is injected into the oocytes. By altering the channel number (via expressing channel through various injection techniques), membrane patches that have high channel number can be compared with patches with low channel number. Structural differences in AFM images that correlate with the electrophysiologically measured functional difference may then be observed.

Methods

Oocyte Patch Preparation. Oocytes from adult female *Xenopus laevis* (Xenopus I, Dexter, Michigan) were harvested essentially as described (Mestek A et al, 1995). Individual oocytes were placed in hypertonic bath (100 mM K-aspartate, 20 mM MgCl₂, 5 mM EGTA, and 10 mM HEPES, pH 7.4) for five minutes. Using fine forceps, the oocytes were manually defolliculated (if follicles still present) and the vitelline membrane peeled away. The oocyte was then transferred to a dish containing a high potassium bath solution (110 mM KCl, 2 mM CaCl₂, 5 mM HEPES (pH7.5)). The pipette solution was identical expect for the addition of mM MgCl₂. Patch pipettes were
pulled from hematocrit glass (Drummond, Broomall, PA), on a two-stage puller (Kopf). Pipettes were fire polished prior to use. Resistance averaged 5 MOhms.

Patch transfer. Patches were transferred as described in (Larmer J et al, 1997). This procedure is shown in graphic form in Figure 8. After obtaining a gigaseal and excising the patch, channel activity was recorded. Patch pipettes were then manipulated to a submerged, freshly cleaved mica disk. The disk was submerged and was positioned at an angle perpendicular to the patch pipette. The pipette was gently brought to the surface of the mica. Contact was confirmed by optical microscopy. Light pressure was placed on the end of the pipette to eject the patch from the end. Thus, the patch was attached to the mica, ready for AFM imaging.

Giant patch transfer. Giant patches were generated in a fashion similar to standard patches. Giant patch pipettes were pulled from hematocrit glass and fire polished prior to use. Resistance averaged 1-2 MOhms.

Results and Discussion

Electrophysiological techniques were used to measure channel expression in pulled patches. Patches were then deposited onto mica. Standard patches (approximately 1um) were difficult to identify and locate on the AFM mounting surface. The giant patch method was developed because of the larger size of the patches; they were easier to manipulate, and were easier to find prior to AFM imaging. These techniques resulted in a low success rate of deposited patches. Many patches were destroyed during the transfer process. Because the membrane resistance is high in a gigaseal patch, it was difficult to dislodge the patch without destroying the membrane patch. Additionally, removing the
attached patch and mica substrate from the bath solution often caused the patch to bunch, rendering the flat membrane inadequate for imaging.
Figure 8. Schematic showing the patch transfer method. Oocytes are cleared of vitelline membrane in isotonic solution. A seal is obtained and the currents are recorded. Finally, the pipette remains in solution where the target substrate is waiting, mounted on flexible tape. The tip is engaged to the substrate under microscopic control, and the patch is blown off the pipette by applying positive pressure. Figure adapted from Danker et al., 1997.
**Experiment 5: Development of Xenopus plasma membrane patches for high-resolution imaging**

Using the AFM, image the vitelline membrane of *Xenopus* oocytes. Confirm that the preparation protocol can produce images of the vitelline membrane, and that these images resemble those of vitelline membrane prepared using other techniques.

**Abstract**

The vitelline membrane is a clear, thin protective membrane that surrounds each oocyte. This vitelline membrane is removed with forceps during oocyte sample preparation. Since the vitelline membrane may not be entirely removed from the samples, it was a concern that vitelline membrane (and not oocyte plasma membrane) might be imaged. Thus vitelline membrane was isolated and imaged independently of the oocyte membrane.

**Background**

Two previous studies have imaged the vitelline membrane using electron microscopy (Grey RD et al, 1974; Larabell C et al, 1991).

**Methods**

Vitelline Membrane Preparation. Oocytes from adult female *Xenopus laevis* (Xenopus I, Dexter, Michigan) were harvested essentially as described (Mestek A et al, 1995).
Individual oocytes were placed in hypertonic bath (100 mM K-aspartate, 20 mM MgCl₂, 5 mM EGTA, and 10 mM HEPES, pH 7.4) for five minutes. Using fine forceps, the oocytes were manually defolliculated (if follicles still present) and the vitelline membrane peeled away. While in hypertonic solution, the vitelline membrane was transferred to a freshly cleaved and submerged 12 mm diameter ruby mica disk (Asheville Schoonmaker, Newport News, VA). Care was taken to ensure that the vitelline membrane was mounted as a single membrane layer on the mica. The solution was removed and the prepared sample was left to air-dry overnight in a dust-free environment.

Imaging Techniques. AFM imaging of vitelline membrane was performed in dry ambient conditions (see Experiment 1).

**Results and Discussion**

The vitelline membrane can, in fact, be imaged; these images do not contain specific structural landmarks or features. The vitelline membrane does not resemble the structure of oocyte membrane. However, it resembles other images of the vitelline, obtained using electron microscopy techniques. Images of vitelline membrane are shown in Figure 9.
Figure 9. Vitelline membrane of Xenopus oocyte as imaged by AFM. Left panels: AFM images depicting topographic information in height mode; right panels: AFM images in deflection mode. Scan rates 1.6-2.0 Hz. Vitelline membrane images do not contain specific structural landmarks or features. Images 700 nm across.
CHAPTER 3

Lattice-like array particles on *Xenopus* oocyte plasma membrane
Abstract

Plasma membrane from *Xenopus laevis* oocytes has been used as a model system to study membrane structure and particle components, including native and exogenously expressed proteins. Previous studies by electron microscopy (EM) and atomic force microscopy (AFM) compared intramembrane particles (IMPs) on uninjected oocyte membranes to oocytes expressing proteins of interest. These studies observed randomly distributed IMPs on the surface of the oocyte plasma membrane. In this paper, we introduce a novel technique to isolate oocyte membranes by bursting the oocyte and depositing its membrane on a flat mica substrate. The flat surface membrane preparation allows high-resolution AFM images to be obtained, revealing a novel structure of densely packed particles. These particles exhibit a regular, repeating pattern of a lattice-like array with orderly packing, and are thus termed “lattice-like array particles” (LAPs). The LAPs are orderly yet imperfectly packed, are located in depressed pools, occur with a low frequency on the oocyte membrane surface, and have not previously been seen using other isolation and imaging methods. Histogram analysis of the center-to-center distance between LAPs suggest their size to be about 44 nm in diameter, considerably larger than other reported size estimates of IMPs. These results indicate that LAPs represent a novel membrane particle organization, which merits further study.
Introduction

The study of cell surface topology and membrane components contributes to our understanding of the structural basis of transmembrane signaling and how cells interact with one another. These structural properties, including membrane particle localization, spatial distribution, clustering, and size can be studied using electron microscopy (EM) or atomic force microscopy (AFM), and indeed, several studies have been published using these microscopic techniques to elucidate membrane particle structure (Ehrenhofer U et al, 1997; Muller DJ et al, 1997; Bayburt TH et al, 1998; Czajkowsky DM et al, 1998; Eskandari S et al, 1998; Zampighi GA et al, 1999; Bron P et al, 1999; Oesterhelt F et al, 2000).

Xenopus laevis oocytes provide a convenient cellular model for studying membrane structure and protein function. Using scanning electron microscopy (EM) to examine freeze-fractured oocyte membranes, the membrane is observed to be sparsely populated with intramembrane particles (IMPs) but is otherwise devoid of prominent structural features (Bluemink JG et al, 1983; Zampighi GA et al, 1995; Eskandari S et al, 1998; Zampighi GA et al, 1999; Bron P et al, 1999). IMPs are randomly distributed on the exoplasmic (E) and protoplasmic (P) faces of the membrane lipid bilayer, are not clustered, and are consistent in size. Several pieces of evidence suggest that IMPs represent membrane-spanning proteins. IMPs appear as protrusions on one face and as pits in the opposite face (Zampighi GA et al, 1995). Additionally, in mRNA-injected oocytes, the number of IMPs is often ten-fold greater than those in uninjected oocytes (Zampighi GA et al, 1999). The increase in IMPs is accounted for primarily by P face protrusions, whereas E face protrusion number remains constant (Zampighi GA et al,
This observation suggests that the increase in P face protrusions represents exogenously expressed integral membrane proteins. Particle analysis of the EM images indicates that the IMPs are approximately 7 nm in diameter in the P face (Zampighi GA et al, 1995; Bron P et al, 1999), and are 10-14 nm in diameter in the E face (Bluemink JG et al, 1983; Zampighi GA et al, 1995).

AFM studies of particles on the oocyte membrane provide different results than those by the EM freeze-fracture technique. IMPs are observed to be present in much greater number on the membrane surface, and the surface does not resemble the sparsely featured surface as seen in EM images (Schillers H et al, 2000; Schillers H et al, 2001). AFM images show an oocyte membrane surface rich in IMPs, consisting of particles of varying heights and diameters. However, in agreement with EM studies, the AFM data suggest that IMPs are randomly distributed and are not clustered (Schillers H et al, 2000). Moreover, the IMPs are susceptible to trypsin digestion, suggesting that the IMPs are indeed proteins (Schillers H et al, 2000).

Regarding the topographical distribution and number of integral membrane proteins, the conflicting results from EM versus AFM studies may be due to differences in membrane preparation or method of imaging. EM studies suggest there is a low density of IMPs and that few particles span the lipid membrane, resulting in a sparsely decorated bilayer (Zampighi GA et al, 1995); AFM studies suggest that the membrane surface is rich in proteins and topographic diversity (Schillers H et al, 2000; Schillers H et al, 2001). Though there is disagreement regarding the density of proteins, all studies to date suggest that the membrane surface is composed of a flat lipid bilayer and has protein particles that are randomly distributed.
In order to gain a better understanding of the constituents and topography of the membrane, we have developed a novel method for oocyte membrane preparation, resulting in flat membrane suitable for high resolution AFM imaging. AFM images of the intracellular surface of the native oocyte plasma membrane confirm the presence of IMPs on the intracellular surface of the oocyte membrane. The current study further characterizes the oocyte membrane surface. Furthermore, we report a lattice-like structure composed of lattice-like array particles (LAP), not previously reported in other published findings of native oocyte membrane surfaces.

**Methods**

**Oocyte Membrane Preparation.** Oocytes from adult female Xenopus laevis (Xenopus I, Dexter, Michigan) were harvested essentially as described (Mestek A et al, 1995). Individual oocytes were placed in hypertonic bath (100 mM K-aspartate, 20 mM MgCl₂, 5 mM EGTA, and 10 mM HEPES, pH 7.4) for five minutes. Using fine forceps, the oocytes were manually defolliculated (if follicles still present) and the vitelline membrane peeled away. The oocyte was then transferred to a dish containing isotonic ND96 and a freshly cleaved 12 mm diameter ruby mica disc (Asheville Schoonmaker, Newport News, VA). After the oocyte recovered from the hypertonic solution (approximately five minutes), the oocyte was then lifted from the solution using a 100 μl glass capillary pipette (Drummond, Broomall, PA), and allowed to fall into the solution droplet at the end of the pipette, where it rested and became exposed to air. The glass capillary containing the oocyte was then brought to the surface of the isotonic solution, and the oocyte was transferred from the capillary to the bath solution. This transfer
exposed the oocyte to the air/water interface, often causing the oocyte to burst. The oocyte’s cytosolic contents drained away from the oocyte membrane with time. Using a dissecting microscope, the cytosolic contents were observed, and after five minutes or when little cytosolic movement was observed to remain, the bath solution was drained from the dish. This allowed the oocyte membrane to be deposited on the mica disk. The prepared sample was left to air-dry overnight in a dust-free environment.

**Imaging Techniques.** AFM scanning was performed in contact mode. Mica disks with oocyte membrane deposits were glued to 15 mm stainless steel stubs (Ted Pella, Redding, CA) using two-ton epoxy. AFM imaging of oocytes was performed in dry ambient conditions at room temperature using a Nanoscope IIIa (Digital Instruments, Santa Barbara, CA) equipped with a J-type 120 μm scanner. 200 μm V-shaped silicon nitride cantilevers with a nominal force constant of 0.06 N m⁻¹ were used. AFM scan regions are typically 2-4 μm across. The image data were collected in the height and deflection mode with a loading force of typically 1 nN and at a scan rate of 1-2 Hz. Images with a height range of no more than 60 nm were chosen for further study. For optical microscopy images, a one-third inch CCD camera (Nikon, Japan) was attached to the AFM camera port, and optical images were captured and downloaded into NIH Image software (Scion, Frederick, MD).

**Image Processing.** For center-to-center distance measurements, data was acquired using Nanoscope IIIa software and processed using SPIP (Imagemet, Denmark). Images were processed through a standard deviation of the mean with 5x5 kernel filter. Filtered images were transferred to Metamorph software (Universal Imaging, Downington, PA) to
measure center-to-center distances. Centers were digitized and distance measurements were compiled into histogram format.

**Results**

We have developed a novel method to prepare oocyte membranes for high-resolution AFM imaging. By exposing the oocyte to the air-water interface, it bursts and floats on the solution surface, often with the intracellular side facing up and exposed to air. Cytosolic contents diffuse into the solution and away from the plasma membrane over time. As a result, the plasma membrane rests on the surface of the solution as a bilayer, relatively devoid of cytosolic material. When the bath solution is removed, the oocyte membrane settles onto the surface of freshly cleaved mica. Thus, our preparation method removes the cytosolic contents of the oocyte and provides a flat membrane sample as a single bilayer on a rigid mica substrate. This results in a membrane sample with the flatness and rigidity similar to synthetic bilayers, allowing high-resolution AFM images of native membranes to be obtained.

**Optical Microscopy of Oocyte Membrane Preparation.** Examination of the oocyte membrane preparation with a light microscope shows that the mica substrate is covered with membrane patches and particulate matter from the oocyte (Figure 10), with three regions of distinct appearances. The bulk of the oocyte membrane resides in the “membrane” region in the center of the sample. This region is covered with contiguous oocyte membrane and displays considerable height variability due to the remnant cytosol adherent to the oocyte membrane. “Mix” is a region radially surrounding the “membrane” region, with discontinuous membrane fragments of lesser height variation.
The “mica” region is the most peripheral region of the membrane preparation, consisting of mostly bare mica surface and decorated with small, flat particles of oocyte membrane. The pieces of membrane are much smaller in the “mica” region than in the “mix” region. These small membrane pieces in the “mica” region are relatively flat, with the least variation in height; therefore, they are more suitable to producing high-resolution AFM images because of the narrow height range in which the AFM instrument can maneuver. The burst oocyte method of our preparation results in a radial distribution of these three regions; the sum of the membrane regions spans approximately $4 \text{ mm}^2$, in agreement with the calculated surface area for an oocyte of 1.1 mm-1.2 mm in diameter.

**Lattice-Like Array Particles (LAPs) Identified in AFM Imaging.** AFM scanning of oocyte membrane reveal novel structure consisting of densely packed particles (Fig. 11). The structure exhibits a regular, repeating pattern of particles that resembles a lattice-like array with orderly packing; hence, we term the particles in the novel structure as lattice-like array particles (LAPs). Many, but not all, LAPs were found in crater-like depressions on the membrane surface. These depressions had a depth of approximately 5 nm. In areas outside the depression, there is no orderly packing pattern (Fig. 11A, right panel, top right corner). Sometimes, particles larger than LAPs are present, randomly interspersed among the LAPs (Fig. 11).

**Center-To-Center Distances of LAPs.** To quantify the orderly packing of LAP structure, we measured the center-to-center distances between a LAP and each of its close neighbors. Figure 12 shows the results in histogram format from measurements of three separate oocyte samples. The results indicate a normal distribution, with the mean center-to-center distance of $44 \pm 0.2 \text{ nm}$. Given the orderly dense packing of LAPs, the
44 nm center-to-center distances may represent the upper limit of LAP size, and the actual size of LAPs may be closer to this size or slightly smaller. The 44 nm size is considerably larger than both the 7 – 14 nm IMPs determined by EM (Zampighi GA et al, 1995; Bron P et al, 1999) or the estimates determined by AFM (Schillers H et al, 2000), further suggesting the LAPs to be a novel structure.

**LAP Structure Prevalence on Oocyte Membrane.** We usually encountered a LAP structure after scanning multiple membrane sample areas, suggesting that LAP structure is not commonly encountered. To estimate the prevalence of LAPs, we performed a survey with three oocyte samples, and scanned eight or nine blocks of 16 µm² area for a total of 128 µm² or 144 µm² (Fig. 10C) for each of the three regions of “membrane,” “mix,” and “mica.” The total surface area scanned and percentage of scanned area with LAP presence are summarized in Table 1. The survey results indicate that the areas of LAP structure are rare on the oocyte membrane, with LAPs accounting for a low percentage for the total area surveyed. This may be one reason why LAPs have not been reported previously.

**Imperfect Packing of LAP Structure.** A distinct feature of the LAP structure is its highly ordered, yet imperfect, packing (Fig. 13). As shown in Fig. 13A, a clear demarcation existed for the crater-like pool of LAPs. Although the LAPs are tightly packed in an orderly fashion, the packing appeared to be imperfect (Fig. 13B). In some areas the packing pattern shifts, resulting in a change of direction in the LAP packing marked by the white lines in Fig. 13C. The imperfect nature of LAP packing suggests
that the LAP structure is likely to represent a biological phenomenon, rather than imaging artifacts.

**Discussion**

We have developed a method to flatten and expose the intracellular side of the oocyte plasma membrane, enabling the use of AFM to examine native membrane structure at high resolution.

Of particular note is that we observed a lattice-like structure (Figs. 11 and 13). The arrangement of lattice-like array particles (LAPs) in the structure suggests that the LAPs are tightly packed. Close packing of particles on a surface results in tetragonal or hexagonal arrays (Stewart I, 1991), while loosely packed particles or sparsely distributed particles do not form any array patterns. Dense packing has been observed in other native membranes: acetylcholine receptors form a packed array in the electric organ of *Torpedo californica* (Bridgman PC et al, 1987); purple membranes from helobacteria are composed of nearly crystalline arrays of bacteriorhodopsin proteins (Michel H et al, 1980; Sabra MC et al, 1998); tetragonally packed arrays can be observed with the calcium pump protein in the isolated sarcoplasmic reticular membrane (Napolitano CA et al, 1983); and native hexagonal arrays have been observed in freeze-fracture images of hydra gap junctions (Wood RL, 1974). Often, the densely packed proteins in a membrane subserve a specialized function. Protein clustering in the membrane allows the protein to function in a way that sparsely distributed proteins cannot. The cystic fibrosis transmembrane conductance regulator (CFTR) by itself transports ATP across the apical membrane; however, clustering of CFTR and associated proteins create locally
high ATP concentrations, which act upon G-protein coupled receptors to regulate phospholipase activity and other ion channels (al Awqati Q, 1995). Similarly, individual sodium channels can transport sodium across the synaptic membrane, but clustering of sodium channels in the neuromuscular junction is required to supply sufficient current to initiate an action potential (Caldwell JH, 2000). Thus, densely packed LAPs may also subserve a specialized function.

In our survey to determine the LAP prevalence, LAP structure was observed in only a low percentage of the surveyed areas, consistent with our experience of performing multiple AFM scans before encountering a LAP structure. The relatively low prevalence of LAPs may explain why they were not encountered in previous studies (Schillers H et al, 2000; Schillers H et al, 2001). In our survey, we scanned an adequate number of membrane areas to achieve a 95% confidence interval for our results. In our experience, LAP structure was observed more often on flat membrane fragments in the “mica” region, though LAPs have been observed in all three regions of the oocyte (unpublished observations.) This could be attributed to the fact that the membrane fragments in the “mica” region are much flatter and more uniform in height; therefore subtle changes in surface topology can be more readily detected by AFM. Additionally, the membrane fragments in the “mica” region are devoid of adherent cytosolic contents, allowing high resolution imaging of the intracellular side of the oocyte plasma membrane.

The center-to-center distance measurement (Fig. 12) provides estimates of the size of LAPs. The LAPs in our measurement have an average nearest neighbor, or center-to-center, distance of 44 nm. Particle size cannot be larger than the center-to-center distance in the LAP structure, since they are unable to pack more tightly than their own
dimensions; therefore, particles greater than 44 nm in diameter would not form the arrays we observed. Similarly, particles with a diameter much smaller than 44 nm would likely to either form orderly packed clusters with smaller center-to-center distances or maintain the 44 nm distances without the orderly packing. Based on these considerations, we propose the LAP size to be close to 44 nm in diameter.

We questioned whether the LAPs are actually a membrane structure, or whether they are the artifact of sample preparation or AFM imaging system. We considered the possibility of artifacts in several ways. First, we imaged mica substrate without the oocyte membrane. We did not observe LAPs or any discernable features (data not shown), only the atomically flat surface as reported in previous studies (Yokota H et al, 1998). Thus, the LAP structure is unlikely to represent an artifact of the mica surface. Second, we considered the vitelline membrane. This membrane was removed during our oocyte membrane preparation; however, it may be possible that small pieces of vitelline might remain on the sample, and the LAPs may be a component of the vitelline membrane. Thus, we isolated the vitelline membrane from oocytes and mounted it on mica substrate for AFM imaging. We observed a feature-less surface without any LAPs (data not shown), suggesting that the LAP structure is unlikely to be a vitelline artifact. Next, we considered the possibility that LAPs may exist within the oocyte cytosol or on the nuclear membrane. Because the LAPs were observed on very flat surface areas, it is unlikely that the LAP structure is part of the cytosolic contents of the oocyte, which tend to show considerable height variations. Our attempts to image the nuclear membrane from oocytes were unsuccessful due to the large height variations. Previous AFM studies of nuclear membrane (Horber JK et al, 1995; Danker T et al, 1997; Rakowska A et al, 1998;
Danker T et al, 2000) did not report structures similar to the LAP; the predominant particle is the nuclear pore with a diameter of 80 nm (Danker T et al, 2000), significantly larger than the 44 nm center-to-center distance of LAPs (Fig. 12) we have observed. Thus, the LAPs are unlikely to be nuclear pores.

The naturally occurring arrays known to date, all composed of membrane proteins, are all significantly smaller than the predicted size of the LAP. Acetylcholine receptor-injected oocytes form hexagonal arrays with 10 nm spacing (Kunkel DD et al, 1997b); the measured center-to-center distances of bacteriorhodopsin proteins are 9 nm (Sabra MC et al, 1998); IMPs in hydra form a hexagonal lattice with a particle spacing of 10 nm (Wood RL, 1974); and gap junctions form hexagonal arrays with a center-to-center spacing of 9 nm. In these published findings of membrane particle arrays, none display a center-to-center distance that approaches the 44 nm of LAPs. Given the size of the individual LAPs, their molecular identity remains unknown at the present time. Further studies can help illuminate the properties and characteristics of LAPs that comprise this lattice-like array. Clearly, this closely packed array represents a novel particle organization, which merits further study.
Figure 10. A sample of a prepared oocyte membrane on mica substrate. A) The sample deposited on mica as viewed through an optical microscope. Areas for AFM scanning are chosen from three regions: “membrane” consists of mostly membrane with considerable height variations and contains the bulk of the oocyte sample; “mix” consists of both membrane and mica surfaces; “mica” is mostly mica surface, with small, flat membrane fragments. B) A higher magnification of an area chosen for scanning, illustrating in more detail the fragments of membrane that are found in the “mix” region. Dark patches are membrane. Light areas are mica. The arrow points to a typical AFM scan area of 16 µm². C) During survey scans, eight or nine blocks of 16 µm² areas are examined to estimate the prevalence of LAP. These survey scans are arranged in three rows of three scans per row for each of the three oocyte samples surveyed.
Figure 11. High magnification of lattice-like array particles (LAPs) on oocyte membrane as imaged by AFM. Left panels: AFM images depicting topographic information in height mode; right panels: AFM images in deflection mode. Bars are 200 nm. Scan rates 1.6-2.0 Hz. A) LAPs are observed in a crater-like depression on the membrane surface. There are also larger particles interspersed among the LAPs. B) Higher magnification of LAP structure for the area in the white square box in A). The orderly nature of LAP packing is clearly seen.
Figure 12. Center-to-center distances of LAPs. Distances from the center of each LAP to the next closest LAP was measured. The results from three separate oocyte samples (open, solid, and hatched bars) were tabulated in this histogram graph, indicating a normal distribution for the center-to-center distances. Mean distance = 44 ± 0.2 nm (n=1584).
Figure 13. Imperfect packing of LAPs on oocyte membrane. Left panels: AFM images depicting topographic information in height mode; right panels: AFM images in deflection mode. Bars are 200 nm. Scan rates 1.6-2.0 Hz. A) Similar to Fig. 11, the LAP structure is observed in a crater-like depression on the membrane surface; areas outside the depression do not have an orderly packing. B) Higher magnification of the area in the white square box in A), showing orderly packing of LAPs. C) The imperfect nature of LAP packing is highlighted, with the white lines marking the shifts in LAP packing.
Table 1: LAP frequency in oocyte membrane. A survey of areas within the three regions of the oocyte preparation, “membrane,” “mix,” and “mica,” was performed to estimate the prevalence of LAP pools. Three oocyte samples were surveyed. The numerator of each value represents the square area with observed LAP, and the denominator represents the surveyed area, both in microns.

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CHAPTER 4

Discussion
**Summary of thesis**

There were several objectives to this thesis:

1) Develop improved sample preparation protocols to enhance high-resolution imaging through AFM.

2) Develop sample preparation protocols that can be utilized in a standardized manner when imaging membrane proteins.

3) Develop the techniques in imaging with the AFM to fully utilize the features of the AFM to determine membrane protein structure at the molecular level in its native state.

4) Utilize one of the protocols to determine native oocyte membrane structure.

Using two insect cell lines, I presented protocols to utilize these high protein expressing cell types to express high levels of recombinant membrane protein. I determined that neither one of these cell lines were suitable for AFM imaging. In Experiment 1, cells lysed, rendering it difficult to isolate intact membrane patches. In Experiment 2, cells did not adhere to the substrate, which is necessary to image using the AFM. Next, I presented three cell lines that adhered to substrates, and evaluated these cells for their potential as vehicles to express protein and be imaged at high resolution. Three cell lines were evaluated – AV12 cells, SK-N-SH, and SH-SY-5Y cells. All cells adhered well to substrates, and were able to be imaged using the AFM, but due to inherent cellular softness, were not able to be imaged at resolution limits that were adequate for resolving structure of membrane proteins. Experiment 4 presented a protocol for isolating oocyte membranes and determining functional capabilities of the membrane prior to imaging,
using traditional patch clamp and giant patch techniques. These techniques are most often used in functional electrophysiological assays. Due to the fact that patches of membrane, in this case oocyte membrane, were literally pulled from the cell, whether these patches could be deposited onto a substrate was explored, then imaged using the AFM. Though this experiment remains a work in progress, it provides a potentially excellent vehicle in which to have expressed proteins in membrane that is rigidly supported and can provide molecular resolution of those proteins. The final experiment in Chapter 2 presents a protocol to isolate vitelline membrane. Indeed, with the rigid mica substrate and appropriate sample preparation, this protocol was able to provide images of the membrane surface that neared molecular resolution.

The final experiment presented in this thesis is presented in Chapter 3. This protocol develops a method for utilizing the plasma membrane of *Xenopus* oocytes as a vehicle to resolve membrane protein structure. In fact, molecular structure of the plasma membrane surface is rendered in Chapter 3. This results in the identification of a structure not previously seen in oocyte membrane studies.

Based on these experiments and the study in Chapter 3, this chapter discusses the contribution to the structural biology community that is offered with these studies.

**Cells as vehicles for membrane protein structural studies**

Using cells as vehicles for membrane protein structure studies is attractive for several reasons. Cells can produce large quantities of recombinant protein on their plasma membrane, providing the native environment required to form functional channels. Cells
have the machinery needed to generate proteins, including associative proteins needed for membrane protein trafficking and expression. Recombinant proteins can be easily expressed upon transfection. Alterations to protein molecular structure can be analyzed with minor changes to genetic code of protein. Indeed, several studies have utilized cells to produce membrane proteins and studied the membrane surface (Schabert FA et al, 1994; Horber JK et al, 1995; Boujrad N et al, 1996; Ohnesorge FM et al, 1997; Danker T et al, 1997; Ehrenhofer U et al, 1997; Larmer J et al, 1997; Ziegler U et al, 1998; Bushell GR et al, 1999; Henderson RM et al, 2000).

Because *Drosophila* Sf9 and Schneider S2 cells are known to be high expressing cell lines, these were examined as potential vehicles for protein expression. Higher levels of protein expression enable more of the protein of interest to be expressed on the cell surface. During imaging, the higher numbers of protein on the cell surface results in facilitated identification of the proteins of interest. For instance, control cells that do not express the protein of interest can be imaged; the topography of a cell that expresses recombinant protein can be imaged and compared to the control cell. Physical features that appear in the transfected cell but do not appear in the control cell can be conceivably due to the expression of recombinant protein. Structures on the cell membrane can be attributed to the membrane protein, and high resolution of these structures may provide a basis for the structure of that membrane protein.

Cells also provide natural environment of a lipid bilayer. Reconstituted membrane proteins may not form in artificial bilayers as the native, functional protein.
Additionally, studying membrane protein function can be performed with AFM imaging. If the resolution is great enough, changes in membrane protein primary structure – a mutation, a deletion – can be incorporated into the recombinant plasmid. Any structural changes can be evaluated and correlated back to the functional differences.

A third advantage of studies in cells includes the potential for dynamic, real time studies. Several studies, in fact, have been performed in real time on cells (Ladoux B et al, 2000; Martin AL et al, 2000; Viani MB et al, 2000; Pereira RS, 2001; You HX et al, 2001). However, these studies have not had the resolution necessary to study dynamics of membrane proteins. If samples could be prepared that provide higher resolution than these current studies, pore changes, receptor binding alterations, kinetics, can all be evaluated and explored. These types of experiments require the endogenous contents of cells and real cells to perform. Ligands can be added to the fluid surrounding the cells under study.

Stoichiometry studies. Stoichiometric studies can also be performed. Though no studies have been done to this effect, a true contribution to this area would include the ability to identify interacting proteins, and determine the stoichiometric relationship between them. For instance, the mu opioid receptor is known to activate the GIRQ channel. Due to the kinetics of the reaction as measured electrophysiologically, it is known that the distance between the receptor and channel is small. However, it is unknown if one receptor can activate several channels, or perhaps several receptors activate a single channel. The ability to identify and differentiate receptor from channel and determine interactions, either in real time or in static cells, would provide new information to the structural biology community.
GPCR structure. To date, no study has determined the structure of G protein-coupled receptors (GPCR), other than hydrophobicity or biochemical studies. Similar to other membrane proteins, the structure of GPCRs can be resolved, given that the membrane preparation and upper limit of AFM resolution is available. Determining which cells would be more amenable to AFM imaging and the expression of membrane proteins would be a great contribution to determining receptor structure. Providing a standard protocol that adapts to a variety of proteins would enhance the utility of AFM imaging in determining membrane protein structure.

Isolating membrane patches for improved high resolution imaging using the AFM

Apart from imaging cells directly, imaging membrane patches may be a good stepping-stone to imaging cells. Imaging whole cells has a lot of advantages. However, imaging patches of natural membrane may provide information that currently cells cannot. First, membrane patches have a rigidity that enhances resolution capabilities of the AFM. Since the stylus tip grazes the surface of the sample soft samples such as cells, especially living cells, cells deform, which decreases the potential resolution of the resulting images. This loss in resolution is greater than can be accommodated for molecular resolution for membrane proteins. Hence, forming a rigid bilayer support is essential to harness the potential resolution of the AFM.

Isolating a membrane patch and fixing the patch to a rigid substrate can improve imaging resolution. Hence, in experiments in Chapter 2 we attempt to isolate patches using proven patch clamp methods. We evolve this technique into isolating oocyte membranes
through the burst oocyte methods, and ultimately, this method provides a great deal of information about the structure of the oocyte plasma membrane (Chapter 3).

There remains a lot of potential with the patch clamp and giant patch techniques. These methods have proven effective in other studies (Horber JK et al, 1995; Danker T et al, 1997; Larmer J et al, 1997; Franco-Obregon A et al, 2000). Unfortunately, they were not successful in oocyte membrane patches. Additional effort can be placed in this area to further improve these techniques. If this can be done, this will provide an excellent sample preparation. Expressing proteins in oocytes has become a trivial and stock technique. In addition, this method provides a way to initially measure channel activity prior to depositing the patch; this would give an indication of the number of channels in a patch, and can be correlated to the images that are generated from the same patch.

A variation of this method has been applied previously (Bustamante JO et al, 1995); a group has designed a combined AFM/electrophysiological setup, which images the patch without removing it from the pipette. Though in their study this provided information to describe TATA binding protein interaction with the nuclear pore complex, I believe the resolution is greatly compromised because of the lack of support for the center of the membrane sample. This inherently seems to be an even softer sample than the live cell, and can be substantiated by the low resolution exhibited in this study.

**Structural topography of oocyte plasma membrane**

In an attempt to create a suitable membrane preparation that can be used for a variety of membrane protein studies, this thesis worked on developing a rigidly supported oocyte membrane preparation. This was partially successful, and the developing protocols are
discussed in Chapter 2. In Chapter 3, one of these protocols is utilized to identify a previously unreported structure.

This structure remains unidentified. As the discussion in Chapter 3 mentions, the lattice-like array particles are larger than lipids and most proteins. Gap junctions are similarly sized, as are nuclear pores, but these are large structures. The LAP is easily identified because of the clustering property we observed in the images.

Given the size of the individual LAPs, their molecular identity remains elusive. The naturally occurring arrays mentioned previously are all composed of membrane proteins; the size of individual particles are all significantly smaller than the predicted size of the LAP in our membrane preparation. In other published findings of membrane arrays, none display a center-to-center distance that approaches 44 nm. Acetylcholine receptor-injected oocytes form hexagonal arrays with 12 nm spacing (Kunkel DD et al, 1997a); the measured center-to-center distances of bacteriorhodopsin proteins are 7 nm (Muller DJ et al, 1995); IMPs in hydra form a hexagonal lattice with a particle spacing of 10 nm (Wood RL, 1974); and gap junctions form hexagonal arrays with a center-to-center spacing of 9 nm (Hatton JD et al, 1982).

Several samples were to determine the prevalence of the lattice-like structure on the surface of the oocyte membrane, and to determine if the frequency was consistent among the three regions of the oocyte. The lattice-like structure was observed to span 1.2% of the scanned area in one oocyte. The array has been seen on a number of oocyte samples, suggesting that this phenomenon is not due to an unusual oocyte phenotype or due to an imaging artifact. An adequate number of areas were scanned to achieve a 95%
confidence interval for our results. The array spans an area of 2-5 \( \text{um}^2 \), and was observed with the highest frequency in the mica region, though LAPs have been observed in all three regions of the oocyte (unpublished observations). Differences in prevalence could be attributed to the fact that the membrane fragments in the mica region are much flatter and uniform in height; therefore subtle changes in surface topology can be more easily discerned. Additionally, the membrane fragments in the mica region lack adherent cytosolic contents, which hinder the ability to image the membrane.

It was questioned whether the LAPs are actually on the membrane itself, or whether it is an artifact of the sample preparation or an artifact of the imaging system. Because the AFM images topographical structure, the structure of the underlying the sample could be producing the images. The possibility was addressed that the underlying mica substrate is the origin of the lattice-like structure by imaging bare mica. When mica is imaged without the membrane, no LAPs are observed. In fact, as reported in previous studies, using the imaging mica results in atomically flat images (Lyubchenko YL et al, 1992; Jacoboni I et al, 1999). It was speculated that the LAPs might exist on the vitelline membrane. This membrane is removed during oocyte membrane preparation; however, small pieces of vitelline may remain on the sample, and the LAPs may be a component of the vitelline membrane. The vitelline membrane was isolated from the oocyte samples and mounted the vitelline membrane for AFM imaging. The vitelline membrane itself did not show any LAPs (See Chapter 2).

It was not possible to rule out the possibility that the LAPs exist within the oocyte cytosol or on the nuclear membrane. However, because the area that is imaged is very flat, it is unlikely that the structure is part of the cytosolic contents of the oocyte. It is also
possible that the LAPs arise from the nuclear membrane; however, attempts to image the nuclear membrane were unsuccessful, due to the large height variations. However, previous studies have used the AFM to image nuclear membrane (Danker T et al, 1997; Danker T et al, 1999; Danker T et al, 2000; Jimenez-Garcia LF et al, 2000). These studies did not report structures similar to the one described here. The predominant particle is the nuclear pore, which has a diameter of 80 nm, significantly larger than the 40 nm center-to-center distance that was observed.

Previous AFM papers describing oocyte membrane structure did not report LAPs in their samples. This can be attributed to differences in membrane preparation. Prior membrane preparation methods likely resulted in the removal of proteins attached to the cytoskeleton and/or removal of proteins loosely associated with the membrane. If the LAP is associated with cytoskeletal elements, this may explain why no other reports of this structure exist in other AFM studies.

It is curious that the previous studies using freeze fracture on oocyte membranes have observed very little surface structure, yet both a previous AFM paper and this study report an intracellular membrane rich in surface landmarks. Perhaps the surfaces between the E and P faces of the bilayer have fewer landmarks, or the difference in results is due to preparation and imaging techniques. It is possible that there are more membrane particles that do not cross through the bilayer, which would account for the difference in membrane structure particles seen with EM compared to AFM.
Further studies can help illuminate the properties and characteristics of the LAPs that compose this lattice-like array. Clearly, this closely packed hexagonal array represents a novel subparticle organization, which merits further study.

**Limitations of developed protocols**

There are limitations to these protocols. Currently, only the burst oocyte protocol produced any concrete, high-resolution images. This protocol can be expanded; however, it is limited inherently by the ability of the AFM to scan the surface of the membrane. Because it can only look at a piece of a membrane at a time, features that appear in only small percentages of the membrane will be difficult to locate. The AFM has the ability to scan larger areas at lower resolution. However, the feature of interest must be identifiable at the lower resolution. This limitation works well when the feature of interest creates a pool or cluster of similarly shaped structures. In fact, this is exactly why the LAP was initially identified.

**Confirming the presence of molecular structure using other imaging methods**

It would be good to repeat these experiments and try to verify the existence of LAP’s using the tunneling or scanning electron microscope (TEM, SEM). However, the true advantages of the AFM in simple sample preparation will be lost once samples are prepared for TEM and SEM. Most samples that may be suitable for one imaging technique may not be suitable for another. We did in fact, attempt to perform TEM studies to discover the LAP; however, due to the current methods of preparing oocyte samples, the freeze replica was not suitable for oocyte samples that contained a great deal
of cytosolic contents. The complete changes are necessary for TEM with the sample preparation.

Since Binnig’s (Binnig G et al, 1986) introduction of the scanning probe microscope, many studies have taken advantage of AFM to image sample surfaces at high resolution. Scientists have been able to resolve previously unseen biological structures. Additionally, AFM provides a wide array of advantages, including simple sample preparation, the ability to image in real time, in liquid environments, and in ambient conditions without much sample manipulation or any need for fixative. However, the AFM is not suited to doing large area surveys; its strength lies in its ability to focus closely on small, micron-sized areas, and resolving membrane topological structure with nanometer resolution.
REFERENCES CITED


Baro, AM; Miranda, R; Alaman, J; Garcia, N; Binnig, G; Rohrer, H; Gerber, C; and Carrascosa, JL. (1985) Determination of surface topography of biological specimens at high resolution by scanning tunnelling microscopy. *Nature*, 315:253-254.


Bushell, GR; Cahill, C; Clarke, FM; Gibson, CT; Myhra, S; and Watson, GS. (1999) Imaging and force-distance analysis of human fibroblasts in vitro by atomic force microscopy. Cytometry, 36:254-264.


Lyubchenko, YL; Gall, AA; Shlyakhtenko, LS; Harrington, RE; Jacobs, BL; Oden, PI; and Lindsay, SM. (1992) Atomic force microscopy imaging of double stranded DNA and RNA. *J.Biomol.Struct.Dyn.*, 10:589-606.


Martin, AL; Davies, MC; Rackstraw, BJ; Roberts, CJ; Stolnik, S; Tendler, SJ; and Williams, PM. (2000) Observation of DNA-polymer condensate formation in real time at a molecular level. *FEBS Lett.*, 480:106-112.


Miles, MJ; McMaster, T; Carr, HJ; Tatham, AS; Shewry, PR; Field, JM; Belton, PS; Jeenes, D; Hanley, B; Whittam, M; Cairns, P; Morris, VJ; and Lambert, N. (1990) Scanning tunneling microscopy of biomolecules. *Journal of Vacuum Science & Technology A-Vacuum Surfaces and Films*, 8:698-702.


Oesterhelt, F; Oesterhelt, D; Pfeiffer, M; Engel, A; Gaub, HE; and Muller, DJ. (2000) Unfolding pathways of individual bacteriorhodopsins. *Science*, 288:143-146.


Schwalbe, RA; Wang, Z; Wible, BA; and Brown, AM. (1995) Potassium channel structure and function as reported by a single glycosylation sequon. *J.Biol.Chem.*, 270:15336-15340.


Viani, MB; Pietrasanta, LI; Thompson, JB; Chand, A; Gebeshuber, IC; Kindt, JH; Richter, M; Hansma, HG; and Hansma, PK. (2000) Probing protein-protein interactions in real time. *Nat.Struct.Biol.*, 7:644-647.


Willemsen, OH; Snel, MM; Cambi, A; Greve, J; de Grooth, BG; and Figdor, CG. (2000) Biomolecular interactions measured by atomic force microscopy. *Biophys.J.*, 79:3267-3281.


Yang, XR; Miller, MA; Yang, R; Evans, DF; and Edstrom, RD. (1990) Scanning tunneling microscopic images show a laminated structure for glycogen molecules. *FASEB J.*, 4:3140-3143.


Young, RD. (1971) 24:42-

Young, RD; Ward, J; and Scire, FE. (1972) The Topografiner: an instrument for measuring surface microtopography. *Review of Scientific Instruments*, 43:999-


Ziegler, U; Vinckier, A; Kernen, P; Zeisel, D; Biber, J; Semenza, G; Murer, H; and

## APPENDIX

### List of materials and sources

#### Instrumentation

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<td>NIH image</td>
<td>Scion, Frederick, MD</td>
</tr>
</tbody>
</table>

#### Materials

<table>
<thead>
<tr>
<th>Item</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stainless stubs</td>
<td>Ted Pella, Redding, Ca</td>
</tr>
<tr>
<td>Two ton epoxy</td>
<td>Devcon, Danvers, MA</td>
</tr>
<tr>
<td>Ruby Mica</td>
<td>Asheville Schoonmaker, Newport News, VA</td>
</tr>
<tr>
<td>Cover slips</td>
<td>Sigma, St. Louis MO</td>
</tr>
<tr>
<td>200 um V-shaped SiN</td>
<td>Digital instruments, Santa Barbara, CA</td>
</tr>
<tr>
<td>cantilevers</td>
<td>Drummond, Broomall PA</td>
</tr>
<tr>
<td>Hematocrit glass</td>
<td>Kopf, Tujunga, CA</td>
</tr>
<tr>
<td>Two stage puller</td>
<td></td>
</tr>
</tbody>
</table>
### Supplies

<table>
<thead>
<tr>
<th>Item</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBLUEBAC4</td>
<td>Invitrogen, San Diego CA</td>
</tr>
<tr>
<td>pMT</td>
<td>L.Cherbas, Indiana University</td>
</tr>
<tr>
<td>pAT</td>
<td>L.Cherbas, Indiana University</td>
</tr>
<tr>
<td>SF9 cells</td>
<td>Invitrogen, San Diego CA</td>
</tr>
<tr>
<td>S2 cells</td>
<td>L.Cherbas, Indiana University</td>
</tr>
<tr>
<td>AV12 cells</td>
<td>ATCC</td>
</tr>
<tr>
<td>SK-N-SH cells</td>
<td>ATCC</td>
</tr>
<tr>
<td>SH-SY-5Y cells</td>
<td>ATCC</td>
</tr>
<tr>
<td>Xenopus laevis</td>
<td>Xenopus I, Dexter, MI</td>
</tr>
<tr>
<td>MEM-alpha media</td>
<td>Sigma, St Louis, MO</td>
</tr>
<tr>
<td>HEPES buffered cell</td>
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<tr>
<td>culture media (no serum)</td>
<td>Sigma, St Louis, MO</td>
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<tr>
<td>Grace’s insect media</td>
<td>Sigma, St Louis, MO</td>
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<tr>
<td>Schneider media</td>
<td>Sigma, St Louis, MO</td>
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<tr>
<td>Fetal calf serum</td>
<td>Sigma, St Louis, MO</td>
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<tr>
<td>Glutamine</td>
<td>Sigma, St Louis, MO</td>
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<tr>
<td>Cell tak</td>
<td>Collaborative BioMedical Products</td>
</tr>
<tr>
<td>Poly-l-lysine</td>
<td>Sigma, St Louis, MO</td>
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</tbody>
</table>