Measuring Electrical Properties of the Tectorial Membrane

by

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Submitted to the Department of Electrical Engineering and Computer Science
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Abstract

The tectorial membrane is a gelatinous structure that plays a key role in stimulating the sensory hair cells in the inner ear. Recent research suggests that the material properties of the tectorial membrane depend on the charge groups attached to the macromolecules that make up the membrane. To investigate the importance of this fixed charge, this thesis describes methods to measure the Donnan equilibrium potential between the tectorial membrane and its bathing solution. Using a standard micro-electrode technique we achieved results that are consistent with previous work. However, the measured potentials were not stable, which made the results difficult to interpret. In an effort to improve the stability of the measurements, two new approaches for measuring electrical properties of the TM were developed: one based on the partitioning of charged dyes between the TM and bath, and one based on the potential difference between dissimilar solutions electrically connected via the tectorial membrane. Using the latter approach we achieved results which were stable over tens of minutes and repeatable between membranes from different mice. Based on the measurements, we estimate the fixed charge concentration of the TM to be $-232\pm20 \text{ mmol L}^{-1}$.

Thesis Supervisor: Dennis M. Freeman
Title: Associate Professor
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# Contents

1 Introduction ........................................... 10

2 Theory ................................................. 12
   2.1 Composition of the TM ............................... 12
   2.2 Polyelectrolytic gel model of the TM .............. 13
      2.2.1 Variable definitions ........................... 13
      2.2.2 Charge neutrality .............................. 13
      2.2.3 Electrodiffusive equilibrium ................. 14
      2.2.4 Equilibrium Condition ......................... 14
   2.3 Liquid Junction Potentials ........................ 15
   2.4 Predictions ...................................... 15

3 Micro-electrode method ................................ 18
   3.1 Motivation ........................................ 18
   3.2 Measurement Issues ................................ 18
      3.2.1 Silver Chloride to bath junction potential .. 19
      3.2.2 Tip resistance and the effects of leakage current 19
      3.2.3 Tip liquid junction potential ................ 20
   3.3 Method ........................................... 20
      3.3.1 Concept ........................................ 21
      3.3.2 Micro-electrode holder ......................... 22
      3.3.3 Data acquisition ............................... 22
   3.4 Implementation ..................................... 23
3.4.1 Test solutions ........................................ 27
3.4.2 Test gel protocol ..................................... 27
3.4.3 TM ...................................................... 28
3.4.4 Isolating the tectorial membrane .................... 28
3.5 Results .................................................. 28
3.5.1 Sample gels ............................................. 28
3.5.2 TM ...................................................... 30
3.6 Discussion .............................................. 34
3.6.1 Reference electrode .................................. 34
3.6.2 Visualization .......................................... 34
3.6.3 Measurement stability ................................ 35
3.6.4 Junction potentials .................................. 35
4  Molecular Probe method .................................. 36
4.1 Method .................................................. 36
4.1.1 Estimating point-spread function .................... 36
4.1.2 Preparing Dyes ....................................... 37
4.1.3 Fluorescent stimulus and filters .................... 39
4.1.4 Protocol ............................................... 39
4.2 Results ................................................ 40
4.3 Discussion .............................................. 45
5  Difference of interface potentials .......................... 46
5.1 Theory ................................................. 46
5.2 Chamber design ......................................... 51
5.3 Method .................................................. 57
5.3.1 Setup .................................................. 57
5.3.2 Dissection ............................................. 58
5.3.3 Testing ............................................... 58
5.4 Results ................................................ 60
5.5 Discussion .............................................. 66
6 Discussion
List of Figures

2-1 Plot of $d$ as a function of $C_{Cl}^o$ and $C_K^o$ .................................................. 16
2-2 Plot of interface potential as a function of $C_{Cl}^o$ and $C_K^o$ .......................... 17

3-1 Diagram of micro-electrode ................................................................. 19
3-2 Diagram of setup concept ................................................................. 21
3-3 Diagram of micro-electrode and pre-amplifier configuration ...................... 22
3-4 Diagram of grounding pellet position. .................................................. 23
3-5 Diagram of implementation of setup .................................................. 25
3-6 Picture of micro-electrode setup .......................................................... 26
3-7 Results from sample gel .................................................................. 29
3-8 Results from TM ............................................................................ 30
3-9 Results from TM continued ............................................................... 32

4-1 point spread in Z ........................................................................... 37
4-2 Typical image from fluorescent method ............................................. 40
4-3 R-123 results .................................................................................. 42
4-4 Data from LY experiments ............................................................... 43

5-1 One dimensional two compartment diagram ....................................... 47
5-2 Calculated concentration and potential profile for identical baths. ........ 48
5-3 Calculated concentration and potential profile. .................................... 50
5-4 Hole setup ....................................................................................... 52
5-5 Setup for membrane measurement ................................................... 53
5-6 Picture of hole chamber ................................................................. 54
| 5-7  | Wall setup diagram                      | 55 |
| 5-8  | Wall setup picture                      | 55 |
| 5-9  | Two trench setup                        | 56 |
| 5-10 | Puddle picture                          | 59 |
| 5-11 | TM on wall                               | 60 |
| 5-12 | Results from two trench chamber experiment 1 | 61 |
| 5-13 | Results from two trench chamber experiment 2 | 62 |
| 5-14 | Results from two trench chamber experiment 3 | 63 |
| 5-15 | Results from two trench chamber experiment 4 | 65 |
| 5-16 | Plot of expected difference in potential versus fixed charge for test solutions | 67 |
List of Tables

3.1 Tip junction potentials for test solutions ........................................ 20
3.2 Solution components ........................................................................ 27
4.1 Fluorescent Dye characteristics ...................................................... 38
5.1 Calculated and measured voltage shifts ........................................... 68
Chapter 1

Introduction

The tectorial membrane (TM) is a fascinating part of the inner ear. It is a gelatinous structure which lies above the sensory hair cells responsible for transducing sound induced motions of the ear to electrical signals. Given the TM’s interesting physical location, surprisingly little work has been done to understand its function.

In an effort to understand how the inner ear works, the Micromechanics group at the MIT Research Laboratory for Electronics has been doing work to determine the properties of the tectorial membrane. Previous work from this group includes the application of a polyelectrolytic gel model to describe the behavior of the TM [11] and a number of experimental protocols designed to probe physical characteristics of the membrane [2] [7] [1] [5].

The TM is a polyelectrolytic gel. It is mostly water: 97\% by weight. The rest of the gel is a network of protein and sugar macromolecules that have ionizable charge groups. This fixed charge is thought to contribute to the osmotic and mechanical properties of the TM.

The goal of this project is to directly determine whether fixed charge plays a role in determining the physiochemical properties of the TM. The fixed charge in the TM should give rise to a space charge layer surrounding the protein and sugar macromolecular matrix. The electric potential across the space charge layer should be a measurable quantity.

Chapter 2 summarizes a simple gel model for the electrical properties of the
tectorial membrane. Chapters 3, 4, and 5 report three different methods to measure the Donnan equilibrium potential between the TM and bath. The first method uses micro-pipettes to measure the TM potential, much as micro-pipettes are used to measure the electrical properties of cells. Results obtained using this method are similar to those reported by others [6]. However, the measured potentials are neither stable nor repeatable. Chapters 4 and 5 report new methods intended to provide more stable and repeatable results.
Chapter 2

Theory

This chapter introduces a model of the tectorial membrane that provides a context for interpreting experimental measurements.

2.1 Composition of the TM

Knowing the composition of the TM provides a starting point for speculating about its properties. Thalman et al. [9] have analyzed the composition of the TM and found it to be 97% water and 3% solids by weight. The solids fall into two main categories: glycosaminoglycans (GAGs) and proteins.

The GAGs and proteins found in the TM are common constituents of connective tissue. Both types have ionizable groups whose charge state depends on the pH of the surrounding solution.

The effect of fixed charge has been studied in other connective tissues, such as cartilage. Cartilage and the TM have very similar compositions of solids, both being composed mainly of GAGs and proteins in about the same proportion. Cartilage however is nearly half solids by weight [4].
2.2 Polyelectrolytic gel model of the TM

Based on the compositional similarity between the TM and other connective tissues, a polyelectrolytic gel model has been applied to the TM by Weiss and Freeman [11]. Polyelectrolytic gel models can be quite complex. The model as applied to the TM makes some simplifying assumptions. The model assumes an isotropic structure, charge neutrality in the bulk, constant fixed charge concentration, and no binding of the bath ions. This section will detail parts of the model that are relevant to measuring electrical properties.

2.2.1 Variable definitions

The following variables will be useful in explaining the model:

- $C_f$ is the net concentration of fixed charge in the membrane (which can be positive or negative).
- $C^0_k$ is the concentration of ion $k$ in the bath solution.
- $C^i_k$ is the concentration of ion $k$ in the membrane.
- $D_k$ is the diffusivity of ion $k$ in the membrane.
- $z_k$ is the charge of ion $k$ normalized to one proton.
- $V$ is the Donnan potential across the membrane interface.
- $q$ is the charge of one proton.
- $k$ is Boltzman’s constant.
- $T$ is the absolute temperature.

2.2.2 Charge neutrality

Charge neutrality tells us that in the bulk of the membrane at equilibrium the amount of positive charge equals the amount of negative charge. This condition is quantified as

$$C_f + \sum_k C^i_k z_k = 0.$$  \hspace{1cm} (2.1)
2.2.3 Electrodiffusive equilibrium

Electrodiffusive equilibrium describes the condition where the net flux of ions across a surface is zero due to the counter balancing of an electric drift flux and a diffusive flux. The fixed charge in the TM exerts an electro-motive force on the mobile ions in the bath solution, attracting ions of opposite charge and repelling ions of similar charge. The separation of charge is limited by a diffusive force which opposes the pulling or pushing due to the fixed charge.

The competing electro-motive and diffusive fluxes create a thin space charge layer around the surface of the membrane. The relation [10] between the concentration differences and the electric potential difference across the space charge layer is

\[ V = \frac{kT}{qz_k} \ln \left( \frac{C_k^o}{C_k^i} \right). \]  

(2.2)

If the bath solution contains multiple ions, every ion species will experience the same potential across the interface. The relation between the concentration inside and outside the membrane for each ionic species \( k \) is

\[ C_k^i = C_k^o \left( e^{\frac{V}{kT}} \right)^{z_k} = C_k^o d^{z_k} \]  

(2.3)

where \( d = e^{\frac{V}{kT}} \), and \( d^{z_k} \) is the ratio of \( C_k^i \) to \( C_k^o \).

2.2.4 Equilibrium Condition

Combining the equations for charge neutrality and electrodiffusive equilibrium yields an expression for \( d \) as a function of the bath concentration of the ions and the fixed charge inside the membrane,

\[ C_f + \sum_k z_k C_k^o d^{z_k} = 0. \]  

(2.4)
2.3 Liquid Junction Potentials

When two solutions interface in a small volume, diffusion is the dominant method of ion exchange. Ions have different diffusion coefficients so they travel through the interface volume at different rates. Since the ions diffuse at different rates, positive and negative charges can become separated. This charge separation is opposed by the resulting electric field which will try to pull the charged particles back together. The consequent potential difference between the two solutions of KCL at different concentrations [3] is

\[ V_l = \frac{kT}{q} \left( \frac{1 - \frac{D_K}{D_{Cl}}}{1 + \frac{D_K}{D_{Cl}}} \right) \ln \left( \frac{C^K_0}{C_{Cl}^0} \right) \]

where \( D_K = 1.96 \times 10^{-9} \text{m}^2/\text{s} \) and \( D_{Cl} = 2.03 \times 10^{-9} \text{m}^2/\text{s} \).

2.4 Predictions

Based on the molecular makeup of the TM, the expected fixed charge concentration is approximately \(-20 \text{mmol/L}\) at physiological pH [8]. This value will be used for the following calculations. We will start solving Equation 2.4 for \( d \) in terms of the concentration of potassium and chlorine in the bath solution. Substituting in the values of \( z_K \) yields

\[ C_f + C^K_0 d - \frac{C_{Cl}^0}{C^K_0} = 0, \]

which after multiplying through by \( d \) and dividing by \( C^K_0 \) can be re-written as

\[ d^2 + d \frac{C_f}{C^K_0} - \frac{C_{Cl}^0}{C^K_0} = 0. \]

Using the quadratic equation, we find

\[ d = \frac{-C_f}{2C^K_0} \pm \sqrt{\left( \frac{C_f}{2C^K_0} \right)^2 + \frac{C_{Cl}^0}{C^K_0}}. \]
and as the ratio of two positive numbers, $d$ must also be positive, so

$$d = \sqrt{\left(\frac{C_f}{2C_K^o}\right)^2 + \frac{C_{Cl}^o}{C_K^o} - \frac{C_f}{2C_K^o}}.$$

This fits our intuition because, if $C_f = 0$, then $d = 1$, which indicates that the concentration inside the membrane equals the concentration outside the membrane.

For our specific case where $C_f = -20\text{ mmol/L}$, and the concentration of potassium and chlorine in the bath solution are equal, and

$$d = \sqrt{\left(\frac{-20\text{ mmol}}{2C_K^o}\right)^2 + 1 - \frac{-20\text{ mmol}}{2C_K^o}}$$

which is plotted in Figure 2-1 for $C_K^o = C_{Cl}^o$ from 0.001 to 1 $\text{ mol/L}$.

![Figure 2-1: Plot of $d$ as a function of $C_{Cl}^o$ and $C_K^o$.](image)

The relation between $d$ and the voltage at the interface can be found from Equation 2.3, and is $V = \frac{kT}{q} \ln(\frac{1}{d})$. A plot of the voltage that would result for
the values of $d$ plotted in Figure 2-1 is shown in Figure 2-2.

Figure 2-2: Plot of interface potential as a function of $C_{C_I}$ and $C_K^0$
Chapter 3

Micro-electrode method

This chapter describes a method of using a micro-electrode inserted into the tectorial membrane to measure the electric potential inside the TM.

3.1 Motivation

This method is attractive because it is commonly used for cell electro-physiology. The characteristics of micro-electrodes are well understood and well modeled. Additionally, there is a previous experiment measuring the potential of the TM using this method. [6]

3.2 Measurement Issues

It is always important to understand how the parasitic effects of a measurement tool affect the measurement. In this case, a measurement tool refers to a voltage meter and all the peripheral components necessary to make a measurement of the TM, including the wires, pre-amplifiers, micro-electrodes and conducting solutions. As shown in chapter two, the predicted interface potential of the TM is on the order of a few millivolts and can be pushed up to tens of millivolts by using osmotically weaker bath solutions.

A number of issues are important when trying to measure a small potential: offsets
due to the measurement tool; offsets due to leakage currents; and electromagnetic interference. Leakage current refers to the small biasing current drawn by amplifiers in the measurement tool.

3.2.1 Silver Chloride to bath junction potential

A main source of offset in the measurement tool comes from the interface between the solid conductor and the liquid conductor in the micro-electrode. Figure 3-1 shows a diagram of a micro-electrode.

![Diagram of micro-electrode]

There is an electric potential between the silver chloride pellet and the solution in the electrode that can be modeled as

\[
V_{AgCl/KCl} = V_{AgCl/KCl}^0 - \frac{RT}{F} \ln (C_{Cl})
\]  

(3.1)

where \(V_{AgCl/KCl}^0\) is a reference voltage and \(C_{Cl}\) is the concentration of chloride ions in the electrode solution [10]. The fact that this junction potential is a function of the chlorine concentration of the electrode solution will be important later.

3.2.2 Tip resistance and the effects of leakage current

The leakage current from the pre-amplifier has an effect when there are large resistances in the measurement path. You will notice from Figure 3-1 that the tip of
the micro-electrode is drawn to a fine point. Typically the radius of the tip opening is on the order of one to fifty micrometers. Because the resistance of the tip is inversely proportional to the area of the opening, the small openings can lead to very high resistance. The tip conductivity is given [10] by

$$\sigma = \frac{a}{l} \left( \sum_k \mu_k c_k \right)$$

(3.2)

where $a$ is the area of the tip opening, $l$ is the length of the tip, $\mu_k$ is the mobility of ion $k$, and $c_k$ is the concentration of ion $k$ in the electrode.

To minimize the effect of leakage current, we use a pre-amplifier specified to have a leakage current in the pico-amp range so that, even for a tip resistance of $10 \text{M}\Omega$, the offset in the measurement voltage is only $10 \mu \text{V}$.

3.2.3 Tip liquid junction potential

The micro-electrodes were filled with AE. Placing the tip in baths with reduced ionic concentrations leads to a liquid junction potential through the tip. The tip liquid junction potential is described in Chapter 2. Table 3.1 shows the predicted potentials for test solutions which are described in Section 3.4.1.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Potential</th>
</tr>
</thead>
<tbody>
<tr>
<td>AE</td>
<td>0</td>
</tr>
<tr>
<td>K87</td>
<td>0.3mV</td>
</tr>
<tr>
<td>K43</td>
<td>0.6mV</td>
</tr>
<tr>
<td>K21</td>
<td>0.9mV</td>
</tr>
</tbody>
</table>

3.3 Method

This section goes over the measurement setup concept, setup implementation, test solutions, sample gels, and the TM experiment.
3.3.1 Concept

A schematic of the final measurement concept is shown in Figure 3-2. $V_1$ is the potential of the pellet inside the micro-electrode. $V_2$ is the potential of the solution in the micro-electrode. $V_3$ is the potential of the bath. $V_4$ is the potential inside the TM. $V_5$ is the potential of the grounding pellet. As you can see from the diagram, depending on whether the measuring electrode is in the TM or not, there are either three or four material interfaces in the conduction path.

The electric potential between the TM and the bath is $V_{43}$. $V_{43}$ is not the measured electric potential. $V_m$ is the measured electric potential, which is the difference between $V_1$ and $V_5$. If the micro-electrode is not in the TM, as shown in figure, the measured electric potential is given by

$$V_{m\text{out}} = V_{12} + V_{23} + V_{35}.$$  

If the micro-electrode is in the TM, the measured electric potential is given by

$$V_{m\text{in}} = V_{12} + V_{24} + V_{43} + V_{35}.$$  

Using the previously described setup you can eliminate some of the unknown electric potentials by taking the difference of $V_{m\text{in}}$ and $V_{m\text{out}}$:

$$V_{m\text{in}} - V_{m\text{out}} = V_{24} + V_{43} - V_{23}.$$
Since $V_{24}$ and $V_{23}$ are both liquid junction potentials and since the solution inside the TM is similar to the same as the solution outside the TM, the two electric potentials should be approximately equal, which reduces the previous equation to

$$V_{m_{in}} - V_{m_{out}} \approx V_{43}.$$

This difference is the interface potential between the TM and the surrounding bath. This demonstrates that by moving the electrode in and out of the TM over time, we can gain insight into effects due to the TM since “common mode” effects can be subtracted away.

### 3.3.2 Micro-electrode holder

A diagram of the micro-electrode, micro-electrode holder, and pre-amplifier configuration is shown in Figure 3-3. The micro-electrode is as described in Section 3.2.1. It is secured to the micro-electrode holder, which is attached to the pre-amplifier. The pre-amplifier (Axon Instruments, x1 HS-2 headstage) provides some initial gain and compensates for leakage current before passing the signal to the main amplifier and voltage meter (Axon Instruments, AxoClamp-2B-2).

### 3.3.3 Data acquisition

The output of the main amplifier was sent to an oscilloscope (Tektronix, 2445 150MHz oscilloscope) and to a 12 bit analog to digital converter (Data Translation, DT1492).
The data acquisition software was setup to take 32,768 points over a 2 second interval and average the data (A/D clock at 10MHz, pre-selector at 2, clock counter at 103).

3.4 Implementation

Designing the experimental setup involved making a chamber for the TM, setting up a perfusion system to regulate the bath, and making a manipulator for the microelectrode. The bath chamber consisted of an o-ring (0.75in diameter) epoxied to a glass microscope slide. Originally the grounding pellet was just placed into the bath, as shown in in the top part of Figure 3-4. As you can see the top of the pellet extends over the top of the o-ring, which means that the amount of the pellet that is submerged is dependent on the level of the solution in the chamber.

![A](image1)

B

![B](image2)

Figure 3-4: In case A, the wire is routed over the o-ring. In case B, the wire is routed under the o-ring

It was later found that the offset from the grounding pellet depended on how much of the pellet was submerged. To ensure that the grounding pellet would always be completely submerged, it was attached to the bottom of the chamber, and the wire was routed underneath the o-ring as shown in in the bottom part of Figure 3-4.

A perfusion system was set up to keep the composition of the bath from changing due to evaporation and to make sure that the solution in the bath stayed well mixed. The perfusion system consisted of a syringe pump, plastic tubing, and a peristaltic pump. The syringe pump delivered the desired solution to the experimental chamber, and the excess was drawn off by the peristaltic pump.
It became apparent, when working with the TM, that the micro-electrode would need to be very well controlled to avoid damaging the tissue. The micro-electrode and pre-amplifier were attached to a hydraulic piston that could be positioned with sub-micrometer accuracy. The hydraulic piston was attached to an xyz stage, which could then be mounted on the stage of a Zeiss Axioplan research grade microscope in order to ensure that the micro-electrode would move with the sample when focusing.

Figure 3-5 shows a diagram of the final micro-electrode measurement setup. The signal from the pre-amplifier is fed to the main amplifier, which then outputs the final signal to a capture board on the computer and an analog oscilloscope. By viewing the signal on the analog oscilloscope, you can observe noise that may be aliased by the digital sampling process. Figure 3-6 shows a picture of the measurement system without the perfusion system.
Figure 3-5: Diagram of implementation of setup
Figure 3-6: Picture of micro-electrode setup
3.4.1 Test solutions

The experiment is to observe how the junction potential varies with the osmolarity of the bath solution. The TM is normally surrounded by endolymph. Endolymph is a solution with a high concentration of potassium. Our test solutions are based on artificial endolymph (AE) which mimics the composition of endolymph. The easiest way to vary the osmolarity of the bath was to vary the amount of KCl in the AE. Table 3.2 shows the composition of the test solutions.

Table 3.2: Solution components

<table>
<thead>
<tr>
<th>Solution</th>
<th>KCl mmol L⁻¹</th>
<th>NaCl mmol L⁻¹</th>
<th>CaCl₂ mmol L⁻¹</th>
<th>Dextrose mmol L⁻¹</th>
<th>HEPES mmol L⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>AE</td>
<td>174</td>
<td>2</td>
<td>0.02</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>K87</td>
<td>87</td>
<td>2</td>
<td>0.02</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>K43</td>
<td>43</td>
<td>2</td>
<td>0.02</td>
<td>3</td>
<td>5</td>
</tr>
<tr>
<td>K21</td>
<td>21</td>
<td>2</td>
<td>0.02</td>
<td>3</td>
<td>5</td>
</tr>
</tbody>
</table>

3.4.2 Test gel protocol

The setup and protocol were tested on a synthetic gel that was physically larger than the TM and, consequently, easier to work with. The gels were made using 3 grams of research-grade gelatin(Cat. #16560, Electron Microscopy Sciences) in 100 mL of ultra-pure water(Milli-Q, Millipore Inc.).

For an experiment, one test gel (3 mm cube cut to size with a scalpel) was put in to the chamber and the perfusion system was activated to deliver AE to the chamber. Once the chamber had come to steady state, the micro-electrode was inserted about 2 mm into the gel and left there for a number of minutes. The micro-electrode was then removed from the gel so that the tip was exposed to the bathing solution. This process of inserting and removing the micro-electrode was repeated a number of times for each solution. The same process was repeated for the solutions K87, K43, and K21.
3.4.3 TM

The same protocol as described for the sample gel was used on the TM once the TM was in the chamber. The only difference is that the micro-electrode is inserted 10 μm into the TM as opposed to 2 mm into the gel. The TM is held stationary using Cell-Tak (Cat. #40240, Collaborative Biomedical Products).

3.4.4 Isolating the tectorial membrane

For this protocol the tectorial membrane was removed from euthanized adult male mice. The TM was removed within 30 minutes of death. The isolated TM was then placed in the experimental chamber.

3.5 Results

This section describes the data obtained from the sample gels and from the TM. The date that the data were obtained appears in the title of each plot.

3.5.1 Sample gels

Figure 3-7 shows the results from two experiments done with a sample gel. The plots are marked with solid vertical lines to indicate the approximate time when the bath solution was changed. The space between the lines is labeled with the bath type. An up arrow is shown where the micro-electrode was drawn out of the sample gel, and a down arrow is shown where the micro-electrode was inserted into the sample gel.

There is a shift in the measured potential when the bath is changed. This shift occurs because the junction potential between the grounding pellet and the bath depends on the concentration of chloride in the bath. The shift in voltage is approximately the same amount (18 mV) every time the bath solution is changed. Each solution decreases the chloride concentration by a factor of two. That a constant shift is seen is consistent with Equation 3.1, which shows that the pellet to bath junction potential is logarithmically dependent on the chloride concentration. This
shift occurs over a five to ten minute period as the new solution replaces the previous solution. The rate that the replacement occurs depends on the influx of new solution and the volume of the chamber.

The difference in potential between times when the micro-electrode is out of the gel and when it is inserted into the gel gets larger as the bath osmolarity gets smaller. When the bath solution is AE, the shift is barely resolvable on the plot. As the osmolarity of the bath solution decreases, the change becomes more noticeable: 2 mV in K87, 5 mV in K43, and 10 mV in K21.
3.5.2 TM

Figure 3-8 shows data taken on the TM in early experiments. These early experiments were done using a dissecting scope to observe the experiment instead of a microscope, and the micro-electrode holder was attached to micrometer, as opposed to a hydraulic piston.

![Graph showing measured voltage versus time](image)

**Figure 3-8: Results from TM**

The top panel of Figure 3-8 shows the data gathered from an experiment done with a single bath solution. The micro-electrode was initially out of the TM. A down arrow indicates when the micro-electrode was inserted into the TM, and an up arrow indicates when the micro-electrode was removed from the TM. The shift at 28 minutes and at 44 minutes is due to the adjustment of a manual calibration knob on the amplifier. Had the manual calibration not shifted the data, it would...
be apparent that the measured voltage steadily decreased. This is not consistent with expectation: the voltage should remain constant while the micro-electrode is completely inside the TM or completely outside the TM. There is a shift in voltage the first time the micro-electrode is inserted at 14 minutes into the experiment. This is consistent with the expectation that the voltage inside the TM would be negative due to the fixed charge. When the electrode is removed, the measured voltage does not shift significantly but continues to move more negative.

The bottom panel of Figure 3-8 shows data gathered from an experiment done on the same setup, using four solutions. A vertical line is shown on the plot at the time when the solution was switched. The space between the lines is labeled with the bath type. The micro-electrode was initially out of the TM. A down arrow indicates when the micro-electrode was inserted into the TM, and an up arrow indicates when the micro-electrode was removed from the TM. A ‘z’ indicates a time when an abrupt shift is due to the adjustment of a manual calibration knob.

There is no shift when the solution is changed in this experiment because the grounding pellet was inside a micro-electrode. When the grounding pellet is inside a micro-electrode the change in the bath solution does not cause a shift in the measured potential.

There do not seem to be any substantial changes in the measured potential between times when the micro-electrode is in the TM and out of the TM with the first three bath solutions. The data becomes quite erratic when the bath is K21. It was at this point when I started looking at the amplifier output with an oscilloscope and noticed a large amount of noise on the measured voltage.

Figure 3-9 shows data taken on the TM with the setup shown in Figure 3-6 in two different experiments. The results shown are typical of the six experiments done using this setup. The changes made in the setup helped with visualization during the experiment, and the new character of the results facilitated data analysis. The setup was approximately the same as the setup used for the sample gels, with the exception that the sample gel experiments were done using a dissecting scope, and the TM measurements were done using a microscope.
In both experiments, as with the sample gel data, there is a shift in the measured potential when the bath is changed. This shift occurs because the junction potential between the grounding pellet and the bath depends on the concentration of chloride in the bath. The shift in voltage is approximately the same amount (15 mV) every time the bath solution is changed. Each solution decreases the chloride concentration by a factor of two. That a constant shift is seen is consistent with Equation 3.1, which shows that the pellet to bath junction potential is logarithmically dependent on the chloride concentration. This shift occurs over a five to ten minute period as the new solution replaces the previous solution. The rate that the replacement occurs depends on the influx of new solution and the volume of the chamber.

The top panel of Figure 3-9 shows data from an experiment done on a TM using three solutions. The bottom panel shows data taken from an experiment with four
solutions. The plots are marked with solid vertical lines to indicate the approximate time when the bath solution was changed. The space between the lines is labeled with the bath type. An up arrow is shown where the micro-electrode was out of the TM, and a down arrow is shown where the micro-electrode was inserted into the TM.

For the experiment whose results are shown in the top panel, the micro-electrode was not inserted into the TM in the last bath solution because the micro-electrode tip broke. When the bath solution is AE, there is no noticeable difference in the measured voltage between the time when the micro-electrode was inserted into the TM, and when the TM is out of the TM. When the bath solution was changed to K87 there was a noticeable difference the last two times the micro-electrode was inserted. The first time, there must have been a short. For the second two insertions there is a drop of approximately 4 mV in the measured voltage. This would correspond to a fixed charge concentration of approximately $-28 \text{ mmol/L}$. The measurements appear to be unstable, however, drifting up in the first case, and down in the second.

The bottom panel shows data taken from an experiment with four solutions. The oscillations when the bath solutions are AE and K87 are due to the water level rising and falling which exposed the grounding pellet varying amounts. I later did an experiment to confirm that the amount of the grounding pellet submerged affected the measured potential. With the K43, K21, and final AE bath I managed to maintain a near constant solution level in the chamber by adjusting the perfusion system. In this experiment there are no resolvable changes between when the micro-electrode was inserted into the TM and when the micro-electrode was out of the TM. When the bath solution was changed back to AE at the end of the experiment, the measured potential approached its original value. This is consistent with expectation, since the voltage is due only to the solutions and silver chloride pellets which should be stable over long periods of time.
3.6 Discussion

When measuring the electric potential a number of issues were explored, which dictated how later experiments would evolve.

3.6.1 Reference electrode

The configuration of the grounding pellet strongly influences that measured potential. If the grounding pellet is placed inside a micro-electrode then there is an electrical symmetry to the conduction path. This would be useful if the measurement electrode was inserted into the TM and remained there for an entire experiment. In that case, the bath solution could be changed and the measured voltage would just be the electric potential between the TM and the bath. The major disadvantage of configuring the grounding pellet in a micro-electrode is that it introduces a high resistance path between the bath solution and ground. Because of this, the bath and bath perfusion system is highly susceptible to noise from capacitive coupling.

By placing the grounding pellet directly in the bath, there is a low resistance path between the bath and ground which significantly reduces electrical noise. Another effect of having the pellet in the bath is that changes in the bath composition appear in the measured voltage. This has the advantage of allowing direct monitoring of the rate that the new solution replaces the old solution. It has the disadvantage of shifting the measured potential whenever the bath is changed. This disadvantage can be overcome by taking the difference of the measured potential between when the micro-electrode is inserted into the TM and when it is out of the TM.

3.6.2 Visualization

Since the micro-electrode needed to be inserted and removed a number of times during an experiment, it was necessary to be able to see what was happening. Early experiments were done using a dissecting microscope which was convenient because its long working distance permitted easy access to the chamber. Since the TM is nearly transparent, the dissecting microscope was unable to provide the contrast
necessary to allow observation of the micro-electrode being inserted into the TM. This necessitated moving the setup to a Zeiss Axioplan research grade microscope. The microscope facilitated observation of the micro-electrode insertion, but required the electrode placement and insertion angle to be more carefully thought out.

### 3.6.3 Measurement stability

As the data in Figure 3-9 shows, the data taken on the TM tended to drift when the micro-electrode was inserted into the TM.

Previous work on the TM using this method was done by Karen Steel [6]. She concluded that the fixed charge concentration in the TM was $-27\pm2\text{mmol/L}$. She notes in her paper that it was difficult to get a stable reading except in the thicker parts of the TM. She felt this was because the “TM gradually split[s] along the length of the fibres following the damage caused by impaling, which could allow the bathing fluid access to the electrode tip”[6]. I tend to agree with her hypothesis.

### 3.6.4 Junction potentials

On a deeper level, using a micro-electrode to measure the electric potential of the membrane may be inappropriate. The micro-electrode is a good tool for cell electrophysiology because a cell has a very distinct inside and outside. That is to say that the inside of the cell is separated from the outside by a lipid bi-layer membrane. A micro-electrode is an appropriate tool for cells because the membrane needs to be pierced to allow access to the inside.

The tectorial membrane is a very different sort of structure. From an electrical point of view, the inside and outside of the TM is defined by a surface space-charge layer that approximately encloses the physical limits of the membrane.

What it means to be inside the space charge layer poses an interesting question. Basically, the electrode does not need to be 'inside' the TM at all. The tip of the micro electrode just has to contact the space charge layer and ideally short it out in that area. This realization motivated the work in Chapter 5.
Chapter 4

Molecular Probe method

An optical method for determining the potential is particularly attractive, as it requires no physical interaction with the TM. Since there is no physical contact, there is no need to worry whether the properties of the TM are influenced by touching it.

Theory predicts that the fixed charge in the tectorial membrane will cause the concentration of charged species to be different inside and outside the membrane. A charged fluorescent dye would be influenced by these forces as well. It may be possible to use a charged fluorescent dye and use the measured light intensity inside and outside of the TM to determine the ratio of the concentrations and the potential difference that would correspond to the difference in concentration.

4.1 Method

A key to making this idea work, is knowing where the light collected by the microscope is coming from. It is also important to pick charged dyes that will not bind to the TM.

4.1.1 Estimating point-spread function

Measurements were done on a Zeiss Axioplan 1 microscope equipped with a Zeiss 63x water immersion lens. Fluorescent beads with a radius of 0.2μm attached
to carboxylated beads with a radius of 2\(\mu\)m were used as point sources for the measurement. The beads were illuminated with reflected light from a tungsten bulb. A volume of images was taken over 20\(\mu\)m vertical space with increments of 0.5\(\mu\)m.

Figure 4-1 shows a plot of light intensity versus vertical position, with the bead centered at \(z=9\mu\)m. From the plot, we can determine that 90\% of the light collected by the camera comes from a 7.5 micrometer vertical section. This is important because 7.5 micrometers is small compared with the thickness of the TM (typically 50\(\mu\)m). If the focal plane is centered relative to the TM, then there is no need to worry that the solution between the top of the TM and the objective lens is contributing to the observed brightness.

4.1.2 Preparing Dyes

In this section we discuss some of the issues of selecting molecular probes. A molecular probe is a chemical compound designed to interact with other chemicals in a specific and detectable manner. Fluorescent dyes are one type of molecular probe.

Electrically sensitive dyes have been designed for cell electro-physiology. One class of electrically sensitive dyes dissolve into the membranes of cells where they are
sensitive to the differences in potential between the inside and outside of the cells. Since the TM is hydrophyllic, these dyes are not useful to measure TM potential.

The dyes selected were chosen based on charge polarity, molecular weight, solubility, adsorption spectrum, and emission spectrum. The fixed charge in the TM should attract ions of opposite charge and repel ions of similar charge. By using one positively charged dye and one negatively charged dye, there is a built in consistency check.

We chose dyes with low molecular weight because they are generally smaller, which means they would have faster diffusion times. A drawback of small dyes is that they may bind more easily to the TM.

Since the TM is a physiological membrane, we needed to make sure that the dyes could be prepared in a way which did not destroy the membrane, i.e., the dyes needed to be prepared in an aqueous solution.

To be compatible with our fluorescence setup, the dyes needed to absorb light in the violet or ultraviolet range and emit light with a wavelength longer than 500 nm.

The dyes we elected to test were Rhodamine 123 (R-123), Lucifer Yellow (LY), and Nuclear Yellow (NY). Unfortunately, I could not get the Nuclear Yellow dye to dissolve properly. The important characteristics of the other two dyes are shown in Table 4.1.

<table>
<thead>
<tr>
<th>Name</th>
<th>Molecular weight</th>
<th>Net Charge</th>
<th>Absorption center wavelength (nm)</th>
<th>Emission center wavelength (nm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rhodamine 123</td>
<td>381</td>
<td>+1</td>
<td>507</td>
<td>529</td>
</tr>
<tr>
<td>Lucifer Yellow</td>
<td>522</td>
<td>-2</td>
<td>427</td>
<td>535</td>
</tr>
</tbody>
</table>

Stock solutions of R-123 and LY were made using 1 mg of dye in 100 mL of ultra pure water. For an experiment, 3 mL of stock solution was added to 15 mL of either AE or K43.
4.1.3 Fluorescent stimulus and filters

A xenon arc lamp was used as the light source for epitaxial illumination of the sample. The filter used for the Rhodamine 123 dye let light with wavelengths from 450 nm to 490 nm reach the sample and allowed wavelengths longer than 520 nm go to the camera. The filter used for the Lucifer yellow dye let light with wavelengths from 380 nm to 440 nm reach the sample and allowed wavelengths longer than 470 nm go to the camera.

4.1.4 Protocol

The chamber and profusion system from the micro-electrode method were used for this method. The TM was not bound to the chamber using Cell-Tak since the dyes bound to Cell-Tak, and there was no need to immobilize the membrane.

Once the TM was in the chamber, it was important to figure out where the top and bottom of the TM were in order to make sure that I would know when all the light collected by the microscope was coming from the TM.

After figuring out where the TM was, I centered the field of view on the edge of the TM, so that half the field of view was filled by the TM and half the field of view was filled by the bath. With this setup, I could sweep through a 80 μm vertical section using the piezo focusing tool (PIFOC, Polytec PI) and record intensities from the TM and the bath simultaneously. Since the TM is between 30 and 40 μm thick, sweeping through 80 μm insures that there will be a set of focal planes above and below the TM that do not contain the TM. For each focal plane, 10 images were taken and the average image was stored.

In analyzing the data a vertical section that contained only the bath was compared to a vertical section that contained the TM. Each vertical section had a cross-sectional diameter of 10 pixels and the average of all the pixels in the area was taken.
4.2 Results

A typical picture is shown in Figure 4-2. The brighter area is the TM, and the dimmer area is the bath. When the TM was viewed using transmitted light illumination from a green LED, the entire field was illuminated and the fibrillar structure of the TM was clearly visible.
Figure 4-3 shows data obtained from an experiment using R-123. The bottom of the TM was at 30 $\mu$m, and the top of the TM was at 60 $\mu$m. The bath solution was initially AE. After 30 minutes, the bath solution was changed to K43. 30 minutes later, the bath solution was changed back to AE. The left hand panels in the figure show plots of brightness in the TM and in the bath versus vertical distance. The right hand panels show plots of the ratio of the TM brightness to the bath brightness as a function of vertical distance. Time is increasing as the plots go from top to bottom.

The plots of brightness versus vertical distance show that the measured brightness increases as the objective moves away from the TM. This makes sense for values of focal depth between 0 and 33$\mu$m because for those depths the focal plane is either entirely or partially in the microscope slide which does not contain any fluorescent material. It is not intuitive that the brightness continues to increase for larger values of the focal depth.

The plots of the ratio of the intensities are shown in the right hand panels of Figure 4-3. The plots show that when the focal plane is above and below the TM the ratio of brightness approaches one. This fits the expectation that when the focal plane includes only the bath (or only the microscope slide) the intensity should be nearly constant across the field of view.

The ratio plots peak at approximately 45$\mu$m which is near the vertical center of the TM. This fits the expectation that the greatest difference would be noticeable far within the TM. The ratio inside the TM increases with time much more substantially then the ratio above and below the TM, as the solution is changed from AE to K43 and back to AE. This indicates that the dye was binding to the TM.
Figure 4-3: R-123 results

- Average Brightness vs. focal depth with AE Bath
- Ratio of TM intensity to bath intensity vs. focal depth with AE Bath
- Average Brightness vs. focal depth with K43 Bath
- Ratio of TM to bath value vs. focal depth with AE Bath
- Average Brightness vs. focal depth with A1 Bath again
- Ratio of TM to Bath value vs. focal depth with AE Bath again
Figure 4-4: Data from LY experiments

Figure 4-4 shows data from the LY experiments. The plots are arranged in the same manner as the R-123 results. The top panels show the data from the initial AE bath, and the bottom panels show the data from the K43 bath. The bath was changed 30 minutes into the experiment. The bottom of the TM was located at 20\(\mu\)m and the top of the TM was located at 50\(\mu\)m.

The plots of the brightness as a function of focal depth have the counter intuitive property that they decrease with increasing focal depth. This is counter intuitive because the brightest values are recorded when the focal plane is in the micro-scope slide which should contain no fluorescent material. This is not too troubling since only the ratio of intensities between the vertical section that contains the TM and the vertical section that contains only the bath is important. The ratio plots show
that the ratio is nearly one through the whole vertical section. This indicates that the TM has very little effect on the concentration of dye.
4.3 Discussion

The results for R-123 suggest that the dye binds to the TM. For the R-123 experiments, as time progressed, the ratio of light intensity emitted from the TM relative to light intensity in the bath increased, seemingly independent of the bath solution. Since R-123 is a positively charged dye, the fact that it binds to the TM suggests that there is negative fixed charge in the membrane.

The results for LY indicate that the change in potassium concentration had little effect on the concentration of dye inside and outside the TM. Assuming that the TM contains negative fixed charge, you would expect that the concentration of LY would be less inside the TM than in the bath. This does not seem to be the situation when the bath is AE or K43. This could indicate a number of things: error in fluorescent technique; positive fixed charge; not using a low enough osmolarity solution; dye was not charged, etc.

To continue exploring the method described in this chapter, it would be necessary to test the dyes available (on the order of one to two hundred). Given the cost of the dyes and the uncertainty of finding the right dye and since none of the three dyes purchased worked as hoped, the method was not pursued further. Although none of the 3 dyes were useful for measuring TM potential, the dye method remains attractive because the optical detection method requires no physical contact with the TM. Given that there are hundreds of commercially available dyes, it remains possible that one or more could have the required properties.
Chapter 5

Difference of interface potentials

The most important limitation of the micro-electrode method described in Chapter 3 is that the electric potentials measured with the micro-electrode in the TM were not stable. While working on ways to make the method more stable, we were motivated to think through the basic mechanism that generate the electric potential between the TM and the bath, which lead to a new approach to measuring the electric potential. The electric potential between the TM and the bath is a junction potential. By exposing the TM to two different solutions, we can create two junction potentials. Measuring the difference between the two junction potentials is easy and informative.

The new approach is similar to an approach described to me by scientists studying ion transport through cartilage. This chapter describes the theory, some implementations, and results using the new approach.

5.1 Theory

This section details the concepts and assumptions for this method. The general idea is that it is easy to measure the difference between two junction potentials, so if one junction potential can be forced to zero, the difference corresponds to the remaining junction potential.

The model reviewed in Chapter 2 predicts that some electric potential will develop between the TM and the solution around it, depending on the fixed charge...
concentration in the TM and the composition of the solution. We further understand that Equation 2.3 tells us something about the concentration of ions on either side of the TM surface. Let us examine the one dimensional case illustrated in Figure 5-1, where the membrane is placed between two solutions. If we assume that the membrane

<table>
<thead>
<tr>
<th>Solution 1</th>
<th>Membrane with fixed charge Cf</th>
<th>Solution 2</th>
</tr>
</thead>
</table>

+V1- +V2-

Figure 5-1: One dimensional two compartment diagram

is much thicker than a Debye length, the potential V1 will be determined by the interaction between the ions in solution 1 and the fixed charge, and the potential V2 will be determined by the interaction between the ions in solution 2 and the fixed charge.

We will start by examining the case in which the solutions on either side of the membrane are the same. In this case, given the isotropic gel model described in chapter 2, the system will come to electro-diffusive equilibrium. The concentration profiles in the baths and in the membrane will be constant. The concentration profile in the membrane relative to the bath will be shifted based on the amount and polarity of the fixed charge in the membrane. Figure 5-2 shows a concentration and electric potential profile that would be expected in this case. Bath 1 is on the left and extends into negative values of x, and bath 2 is on the right and extends for x greater than 30μm. The concentrations are calculated using the charge neutrality effects described in Chapter 2. The electric potential shifts are calculated using the Donnan ratio, also described in Chapter 2. The concentration profiles are constant everywhere and there is a shift inside the membrane. The bottom panel illustrates the difference in potential between the two solutions is zero. This is a result of having symmetric
Figure 5-2: Calculated concentration and potential profile for identical baths. $C_f$ is $10 \text{mmol} \text{L}^{-1}$, $C_{K1}$ is $200 \text{mmol} \text{L}^{-1}$, $C_{Cl1}$ is $200 \text{mmol} \text{L}^{-1}$, $C_{K2}$ is $200 \text{mmol} \text{L}^{-1}$, $C_{Cl2}$ is $200 \text{mmol} \text{L}^{-1}$.

Junction potentials, the potential increases when crossing one and decreases when crossing the other.

Now let us explore what happens as we change the concentration of one of the baths. As the concentration of ions in bath 2 decreases, there will be a flow of ions out of the membrane, and the concentration of ions on the right side of the membrane will decrease. After some amount of time described by the steady state time constant, the system will come to a steady state where there is a flow of ions from bath 1 to bath 2 through the membrane. The steady state time constant is given by

$$\tau_{ss} = \frac{d^2}{\pi^2 D_k}$$

where $d$ is the width of the membrane and $D_K = 1.96 \times 10^{-9} \text{m}^2 \text{s}^{-1}$. For $d = 30\mu\text{m}$, $t_{ss} = 0.045\text{s}$. For $d = 500\mu\text{m}$, $t_{ss} = 12.66\text{s}$. Since $\tau_{ss}$ is proportional to $d^2$, it increases very rapidly with size. For distances on the order of the thickness of the TM the
system comes to steady state in well under a second. For distances on the order of the length of the TM, the system comes to steady state on the order of a minute.

After a few time constants we expect the system to be fairly constant. The next question is, what are the boundary conditions at the edge of the membrane. The only solution that satisfies electro-neutrality and electro-diffusive equilibrium is that the relation between the ion concentrations just inside the membrane and the ion concentration outside the membrane have to satisfy Equation 2.3 for every ion species. As long as the baths are separated by more than a few Debye lengths (10 nm to 100 nm depending on the osmolarity of the solution), this result holds.

Given the boundary conditions, and that the difference between positive and negative ions is equal to the fixed charge concentration, a linear concentration gradient connecting the boundary concentrations is a self consistent result.

As discussed in chapter 2, if the concentration of ions in the solution is significantly larger then the concentration of fixed charge, the predicted interface potential is fairly small. Keeping one potential small while varying the other potential would allow us to measure the varied potential. Figure 5-3 shows a plot of the predicted ion concentrations and potential as a function of position for a membrane cross-section that starts at $x = 0$ and ends at $x = 30\mu$m.
Figure 5.3: Calculated concentration and potential profile. 
$C_f$ is $10 \text{ mmol} \text{ L}^{-1}$, $C_K^1$ is $200 \text{ mmol} \text{ L}^{-1}$, $C_{Cl}^1$ is $200 \text{ mmol} \text{ L}^{-1}$, $C_K^2$ is $10 \text{ mmol} \text{ L}^{-1}$, $C_{Cl}^2$ is $10 \text{ mmol} \text{ L}^{-1}$.
Figure 5-3 illustrates three key points: charge separation in the membrane, equal concentration gradients in the membrane, and the difference in potential between bath 1 and bath 2. In this case the fixed charge is positive, so the potassium ions are forced out of the membrane. The concentration gradients are the same because the difference between the two concentrations is equal to the fixed charge concentration as dictated by electro-neutrality.

Since there is a diffusive flux of ions, we have to consider the possible effects of liquid junction potentials on the measured potential. The same argument used to explain why liquid junction potentials were negligible for the micro-electrode case holds here. For the solutions used, potassium and chloride are the only ions altered and since they have approximately equal diffusion coefficients, they do not generate a substantial liquid junction potential (on the order of one millivolt for a factor of ten difference in the concentrations on either side of the membrane).

5.2 Chamber design

I explored a number of possible ways to use the TM to separate two baths. Each has distinct advantages and disadvantages.

Using the TM as a membrane

Figure 5-4 shows the TM placed over a hole that separates two baths. In this case diffusion between the baths occurs in the direction of the TM’s smallest dimension. There were a number of incarnations of this basic design. We began by using an Argon-Ion laser to drill 20 μm holes in pieces of acetate. The sheets where then placed between two pieces of machined plexi-glass so that different solutions could be applied to either side as shown in Figure 5-5. An issue with this setup is how to determine whether an effect being measured was due to the TM or was simply a function of changing one of the bath solutions. One solution to this problem is also shown in Figure 5-5. In one chamber, the TM can be placed over the hole while in the other chamber the hole is left uncovered. Using this setup, we can duplicate
Figure 5-4: Hole setup

the proposed advantage of systematically probing the TM and bath solution and
determine that the difference between the two measurements is due to the TM.

Figure 5-6 shows a picture of one of the hole type setups. The lower bath, shown
in blue, is the grounding solution. The tubes used for the profusion system are shown
in red. The test chamber and reference chamber are labeled in the picture.

The original construction had three main problems: the 63x water immersion lens
didn’t fit in the opening to the chamber, so you couldn’t see the TM; the TM didn’t
stick to the hole; and air bubbles would get trapped in the hole. The lens problem
was easy to fix; I simply made the TM chamber bigger. The air bubble problem
was initially difficult to isolate because a 20 μm hole with an air bubble trapped in
it looks pretty much like a clear 20 μm hole. The problem manifested itself in that
the voltage measured would be unstable or the path resistance would be very high.
Once I figured out that there was an air bubble in the hole it was just a matter of
determining how to get rid of it. I used an etched tungsten wire to push the air
bubble away from the hole. This did not actually eliminate the air bubble (it was on
the bottom side of the acetate), but it did get it away from the hole. Once the air
bubble was away from the hole, it did not drift back in as long as the setup remained
level.

The problem of keeping the TM covering the hole was never solved satisfactorily.
The cellular adhesive that works well on glass did not work well at all on acetate or
plexi-glass. I also tried an idea inspired by cartilage experiments in which researchers
mash a piece of cartilage between two plates with holes in them. A big difference between cartilage and the TM is that cartilage comes in 1 cm by 1 cm by 0.5 mm slices, and the pieces of TM I extract are generally 3 mm by 0.1 mm by 0.03 mm. To accommodate the small size of the TM, I made a thin slice of PEEK tubing and attached it to the end of a glass micro-manipulator. With the TM covering the hole, I could then maneuver the circular fitting over the TM and hold it down. The ring of PEEK tubing severely obscured the view of the TM, which was very frustrating since I really needed to know whether the TM was completely over the hole when making these measurements.
Using the TM as a bridge

The hole setup does not take advantage of the fact that the TM sections have one very long dimension. In the hole setup, the hole has to be small since the TM is only 100 μm wide. Since the TM is 10 to 40 times longer than it is wide (depending on the dissection), it makes sense to take advantage of it.

In Figure 5-7, the TM was placed in the experimental chamber, and an insulating sheet was brought down over the TM to separate the two baths. The bottom edge of the insulating sheet is coated with a gooey insulating material (Silicon Vacuum Grease) that will form around the TM and form an insulating layer between the sheet and the chamber bottom, the TM is then the only path through which ions may travel between the two baths. Figure 5-8 shows a picture of an implementation.
of the wall setup. The two baths are shown in different colors. It was more difficult than expected to get an adequate seal between the vertical sheet and the bottom of the chamber. The vertical sheet also obscured the view terribly, in particular because the water on either side of the sheet has a sharp curvature which distorts the image.

An alternative to putting the TM underneath a sheet is to lay the TM over the top of one. A diagram of a setup that uses this idea is shown in Figure 5-9. In this setup, the two bath solutions are on either side of a thin wall (200 μm). This setup also takes advantage of the way water behaves in small containers. Water has some properties that make it difficult to work with: it sticks to itself fairly strongly,
and surface tension was a big problem in controlling the volume of bath solution in many of the experimental chambers. The chamber in Figure 5-9 takes advantage of capillary action to keep the two baths from interacting. The TM is placed on top of a wall that separates two trenches. Since the trenches on either side of the wall are thin, surface tension holds water in them. They hold water with enough force that flipping the chamber over and shaking it does not take the water out. This is very advantageous, in that it ensures that the only electrical path between the two baths is through the TM, and that any excess solution is drawn into one of the trenches.

A micro-electrode filled with AE is placed in each trench to act as an electrical bridge between the bath in a trench, and a silver chloride pellet. The tip diameter of the micro-electrode in the test trench has to be small enough to prevent the test
solution from affecting the micro-electrode solution. In the final setup, the test micro-electrode tips are less than 5 μm in diameter. The grounding micro-electrode tip diameter is not as important since the same solution is in both the grounding trench and grounding micro-electrode. In the setup the grounding electrode tip was approximately 0.25 mm.

Using this setup, a solution of AE could perfuse through one trench which was at electrical ground, and test solutions could perfuse through the other trench. With only the TM between the two baths, the measured voltage would correspond to the difference in junction potentials of the TM with each bath. To test if an effect is due to the TM, an electrical shunt can be placed between the two baths using a simple cotton fiber to act as a salt bridge, which will effectively short the two baths together.

5.3 Method

Successful experiments were done using the two trench setup. This section details the experimental protocol used to acquire data. The whole procedure (surgery and experiment) was done using a dissecting scope for observation.

5.3.1 Setup

The same test solutions that were used in the micro-electrode method were used for this method. At the beginning of each experiment, the pH of each solution was measured and recorded. With the test solutions and grounding solution in syringes, the perfusion system was started to fill the perfusion tubes with fresh solution before they were attached to the experimental chamber. The first test solution was always AE.

The trenches of the experimental chamber and the micro-electrodes were filled with AE. Filling the trenches by hand (as opposed to letting the perfusion system push water through) prevented overflows that would otherwise happen at intersections between two trenches or near where the perfusion tubes fed into the trenches. It was also necessary to fill the trenches with solution so that the TM can be manipulated.
on the setup. When the TM is removed from solution, it balls up on itself and is nearly impossible to work with.

With the solutions in the trenches and the micro-electrodes filled, the grounding and measurement silver chloride pellets were inserted into their respective micro-electrodes. The trenches were shorted using a fiber to create a salt bridge and the measured potential was observed. This process was used to determine if there were any open circuits in the measurement path. Assuming there were none, the pellets were removed from the micro-electrodes and the chamber was set aside so the dissection could take place.

5.3.2 Dissection

The TM was removed from euthanized adult male mice as per lab protocol. It was particularly important to remove long pieces of TM. Pieces that were exceptionally long were cut in half in case one piece was lost in transfer between the dissection petri dish and the two trench setup (the transfer process has about a 25% success rate).

Once the TM was transfered into one of the trenches, it had to be positioned across the wall separating the trenches. Since the TM cannot be maneuvered out of solution, a puddle needs to be created on the wall. This can be done by placing two fibers across the wall and drawing solution up onto the wall using a fine and flexible manipulator (such as an eye lash). A picture of the puddle and TM is shown in Figure 5-10.

With the TM positioned on the wall so that each end is in one trench, the two fibers can be removed. With the fibers removed, the puddle collapses and the TM is left as shown in Figure 5-11.

5.3.3 Testing

With the TM on the two trench chamber, the perfusion system is connected, and the silver chloride pellets are inserted into the appropriate micro-electrodes. The measured voltage is checked to determine if the TM is connecting the two baths.
With the positioning of the TM confirmed, the perfusion system is turned on. The grounding solution was kept in a 60 mL syringe with the syringe pump set between 3 and 5. The test solutions were kept in a 20 mL syringe with the syringe pump set between 10 and 20.

After letting the system stabilize (nominally 5 minutes) the measured voltage is recorded using the same setup described in Chapter 3. While one solution is being tested, there are periods of time when the TM is the only connection between the two baths, and there are periods of time where a salt bridge is placed across the wall in addition to the TM.

After measurements are taken in both configurations, the test solution is switched. In a single experiment, the TM is exposed to the test solutions multiple times (for instance AE → K43 → K21 → AE → K21 → K43 → AE).

At the very end of the experiment, the TM is removed. This serves two purposes: with the TM removed there should be an open circuit in the measurement loop, and
when removing the TM it is possible to tell if it had dried up. If the TM sticks to the wall, or seems brittle, it has probably dried up.

5.4 Results

This section describes the results obtained with the two trench chamber, and the protocol described above. Figure 5-12, Figure 5-13, and Figure 5-14 show results obtained on TM samples removed from three different mice. All the figures have vertical lines indicating when the test solution was changed. There is a label between each set of vertical lines indicating the test solution at that time. Horizontal lines indicate an average voltage for a given solution. The difference in the potential is indicated on the far right between the horizontal lines. An up arrow indicates the time when a salt bridge was placed across the wall. A down arrow indicates the time when the salt bridge was removed. A “d” indicates a time when a drop of AE was applied directly to the TM.

Figure 5-12 shows data from the first experiment. Figures 5-10, and 5-11 are pictures taken of the TM during this experiment. One observation about the data is that the lower osmolarity solutions elicit a higher measured potential. A second
observation is that each time the two trenches are connected via a salt bridge, the measured potential returns to approximately the same potential. This potential is also the same as the potential when the solutions in both trenches are the same. Since the voltage returns to the same value whenever the TM is 'shorted' with a salt bridge, it is reasonable to conclude that the increased measured voltage with lower osmolarity solutions is due to the TM.

It also fits our expectation that the voltage change from AE to K43 is nearly twice the voltage change from K43 to K21. AE has four times the osmolarity as K43. K43 has twice the osmolarity of K21. In Chapter 2 it was explained that the change in voltage between the TM and bath would be proportional to the log of the ratio of the concentrations of the two solutions, for solutions whose ion concentration was less than the fixed charge concentration. In this experiment, there is a 12mV change for every factor of two change in concentration (25mV difference between AE and K43, 37mV difference between AE and K21).
The data shows that when a drop of AE is applied to the TM, the measured voltage drops suddenly and the recovers slowly. This fits with our intuition that at the moment when the drop is applied to the TM the effect of the TM is shorted, and then as the trenches pull the excess solution off of the TM, the measured voltage is dominated by the junction potentials between the TM and the bath solutions.

![Measured voltage versus time](image)

**Figure 5-13: Results from two trench chamber experiment 2**

Figure 5-13 shows the data from the second experiment. Again, each time the two trenches are connected via a salt bridge, the measured potential returns to approximately the same potential. This potential is also the same as the potential when the solutions in both trenches is the same. Since the voltage returns to the same value whenever the TM is 'shorted', it is reasonable to conclude that the effect is due to the TM. The experiment starts out very similar to the first experiment in that the voltage rises by the same amount when the bath is initially switched from AE to K43 in both experiments. Something strange happens when the drop of AE is applied to the TM at 50 minutes into the experiment. From that point on, the
data gets very noisy and does not return to the measured voltage that was recorded before the shorting and drop. When the solution is switched from K43 to K21 there is a definite increase in the average measured voltage, which is consistent with our expectations and the first experiment.

At the end of the second experiment, 1 mm of the grounding micro-electrode tip was removed. This widened the tip opening to approximately 0.25 mm, which fixed the noise problems that were recorded in the second experiment.

![Measured voltage versus time](image)

**Figure 5-14:** Results from two trench chamber experiment 3

Figure 5-14 shows data collected from the third experiment using the two trench chamber. This data has the same trends as the data from the first two experiments, and is much less noisy. In this experiment the order that the solutions were presented to the TM was altered, which did not seem to affect the results. The data matches the data from the first experiment quiet well.

One interesting, and seemingly inexplicable, tendency of the data is the 7mV shift in the nominal value of the measured voltage (the voltage measured when the
trenches are shorted together). A drop of AE was applied to the TM at the end of the experiment (178 minutes into the experiment). After the drop was applied, there is a jump in the measured potential.

These three experiments demonstrate that the measured potential is stable to within a millivolt over periods as long as 10 minutes. The absolute differences as a function of solution are also repeatable to within 1 mV between experiments 1 and 3. The measured shifts indicate that the fixed charge is much larger than originally anticipated. To try to get a better idea for the actual amount of fixed charge another experiment was done using a modified protocol. In the last experiment, two solutions were added, K696 and K87. K696 is derived from AE in the same fashion as K87, K43 and K21 as described in Chapter 3. K696 was used as the grounding solution in this experiment since it had the greatest concentration of potassium.

Figure 5-15 shows data from the fourth experiment. This experiment is very similar to the previous experiments. Looking at only AE, K43, and K21, there is a 25.5 mV shift between AE and K43 and a 14 mV shift between K43 and K21. Both of those shifts are within two millivolts of the earlier experiments.

It is also important to note that there is a factor of 4 change in osmolarity between K696 and AE, where there is 12.5 mV shift in the measured potential. For the lower osmolarity solutions, there is approximately a 14 mV shift for every factor of 2 change in osmolarity.
Figure 5.15: Results from two trench chamber experiment 4
5.5 Discussion

The design of the two trench chamber is nearly as important as the results obtained using it. The chamber is very easy to work with. Placing the micro-electrodes in the trenches without breaking their tips is facilitated by the micro-electrode holders, which also make filling the micro-electrodes a simple task. Keeping the water level in the trenches constant is trivial given the layout of the trenches and the pool termination (the connection between the pool and the trenches provides a good deal of mechanical isolation). The TM is fairly easy to maneuver in the trenches, and there are no moving parts in the chamber which can mutilate the membrane.

Since the fourth experiment is consistent with the other experiments and provides additional information, it will be used to estimate the amount of fixed charge in the membrane. To estimate the fixed charge we can use the following two equations that were introduced in Chapter 2. The first gives the Donnan ratio as a function of $C_f$ and $C_p^o$,

$$d = \sqrt{\left(\frac{C_f}{2C_p^o}\right)^2 + 1 - \frac{C_f}{2C_p^o}}$$

where $C_p^o$ is the net concentration of monovalent positively charged ions in the solution. For the solutions used, this is equal to the potassium concentration plus $5 \text{ mmol L}^{-1}$. The second equation relates the Donnan ratio to the resulting electric potential,

$$V = \frac{kT}{q} \ln\left(\frac{1}{d}\right).$$

From the measured shifts in voltage and the values of $C_p^o$, the fixed charge can be estimated. Figure 5-16 shows a plot of the expected difference in electric potential for the test solutions. From this plot and the measured voltages, an estimate of the fixed charge concentration can be extrapolated. For example, using the plot shown in Figure 5-16 the 11.5 mV change between AE and K87 corresponds to a fixed charge concentration of $-232 \text{ mmol L}^{-1}$. The 1 mV uncertainty in the measurement corresponds to approximately a $\pm 20 \text{ mmol L}^{-1}$ uncertainty in the fixed charge concentration. This value of the fixed charge predicts the voltage shifts shown in Table 5.1, which shows
Figure 5-16: Plot of expected difference in potential versus fixed charge for test solutions

a nice agreement between theory and measurement.
Table 5.1: Calculated and measured voltage shifts

<table>
<thead>
<tr>
<th>Solutions</th>
<th>Calculated shift (Volts)</th>
<th>Measured shift (Volts)</th>
</tr>
</thead>
<tbody>
<tr>
<td>K696 → AE</td>
<td>0.0114961</td>
<td>0.0125</td>
</tr>
<tr>
<td>AE → K87</td>
<td>0.0114943</td>
<td>0.0115</td>
</tr>
<tr>
<td>K87 → K43</td>
<td>0.0145</td>
<td>0.014</td>
</tr>
<tr>
<td>K43 → K21</td>
<td>0.0151</td>
<td>0.014</td>
</tr>
</tbody>
</table>
Chapter 6

Discussion

The biochemical composition of the TM suggests the existence of fixed charge in the membrane. Recent experiments suggest that the fixed charge influences the mechanics of the TM. The goal of this work was to more directly measure the affect of fixed charge by measuring the electric potential between the bulk of the membrane and a surrounding bath solution.

Micro-electrode based methods seemed like a reasonable approach to take. Micro-electrodes are often used for probing the electric potential in small objects, like cells for instance. Most cells are much smaller than the TM which should make micro-electrode work even easier. Unfortunately there are some significant differences between cells and the TM that make a micro-electrode method inappropriate. The TM seems prone to split apart, probably since the macromolecules that constitute it are stacked in a regular, non-interlocking way. This structuring is visible when the TM is viewed under a microscope. When the micro-electrode tip is inserted into the membrane it must act as a wedge which separates the fibers of the TM and allows solution direct access from the bath to the tip, thereby shorting out the voltage that is being probed. The instability of the voltage measured with the micro-electrode inserted into the TM provides good evidence for this conclusion.

The fluorescence method was pursued because we had a good theory for how fixed charge affects the partitioning of charged mobile molecules between the inside and the outside the membrane. An optical method also has the advantage that there is
no physical strain on the membrane. The problem with this method was in finding an appropriate dye or dyes. The dyes tested in this thesis either bound to the TM, or seemed unaffected by the TM.

The method of measuring the difference of interface potentials came about after thinking about how the potential inside the TM was generated. From an electrical point of view, the TM is just a three dimensional array of charge fixed in space. When the array is placed in solution or solutions with mobile ions, the ions interact with the array and concentration profiles are set up that minimize the amount of isolated charge. The remaining isolated charge is localized at the interface between the array and the solution, and is the source of the potential difference between that solution and the bulk of the array. We can exploit this interaction and create two interfaces with solutions on spatially separated sections of the array. If one of the solutions is varied in a controlled manner and the electric potential difference between any two solutions is recorded, the amount of fixed charge can be calculated based on the relation between the ionic strength of two solutions and the measured shift in electric potential between them when compared with shifts of other pairs of solutions.

As reported in Chapter 5, using the method of measuring the difference of interface potentials provided stable and repeatable measurements. In a single experiment, the voltage recorded for a given solution varied less than 2 mV over periods greater than 10 minutes. Repeatability between experiments is good: the average shifts in voltage from two given solutions are nearly within a millivolt of each other. With simple analysis, a fixed charge concentration of $-232 \text{mmol} \text{L}^{-1}$ fits the theory to the data with less than 10% error.
Bibliography


