INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

ProQuest Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

UMI
RICE UNIVERSITY

Membrane Tether Formation from Outer Hair Cells with
Optical Tweezers

By

Zhiwei Li

A THESIS SUBMITTED
IN PARTIAL FULFILMENT OF THE
REQUIREMENTS FOR THE DEGREE

Master of Science

APPROVED, THESIS COMMITTEE:

Bahman Anvari, Assistant Professor
Department of Bioengineering

William Brownell, Chair and Professor
Department of Otorhinolaryngology and Communicative
Sciences, Baylor College of Medicine

Kyriacos A. Athanasiou, Professor
Department of Bioengineering

Jorge H. Torres, Faculty Fellow
Department of Bioengineering

HOUSTON, TEXAS
MAY, 2001
ABSTRACT

Membrane Tether Formation from Outer Hair Cells with

Optical Tweezers

By

Zhiwei Li

The mechanical properties of the outer hair cells (OHCs) lateral wall are essential to elucidate the mechanism of OHC electromotility. Optical tweezers were used to characterize the mechanical properties of OHC plasma membrane (PM) by pulling tethers. A greater force was required for tether formations from OHC lateral wall (499 pN ± 152) than from OHC basal end (142 pN ± 49), consistent with the presence of a more extensive cytoskeleton support beneath the PM at the lateral wall. The apparent PM stiffness, which was estimated by measuring tether force at different tether length, was 3.71 pN/μm for OHC lateral wall and 4.57 pN/μm for OHC basal end. The apparent PM viscosity was measured by pulling tethers at different rates while continuously recording the tether force, and was estimated in the range of 13-33 pN·s/μm.
ACKNOWLEDGMENTS

I would like to give special thanks to my advisor, Dr. Bahman Anvari. He is a true mentor to me. His brilliance and dedication to research and teaching has been, and always will be a great inspiration to me. I would like to thank the following individuals for their assistance.

Dr. Brownell, for providing me the opportunity to work with outer hair cells and for his novel ideas and wonderful advice on this project.

Dr. Athanasiou for serving on my thesis committee and helping on the modeling of tether formation.

Dr. Torres for his numerous help on the experiments and revising this manuscript.

I greatly appreciate the contribution of Dr. Takashima and Peter Brecht to the static measurements of tether force.

I want to thank Maneesh and Kathryn for their help on the calibration of optical tweezers. Thank Dr. Hong-bo Zhao and Cindy Shope for their help on OHC isolation and fluorescence imaging.

This work was supported in part by grants from the National Institute on Deafness and other Communication Disorders (DC02775), and The Whitaker Foundation.
# TABLE OF CONTENTS

1 INTRODUCTION 1

1.1 Outer Hair Cells and OHC Electromotility 1
   1.1.1 Structure of the Ear and Organ of Corti 1
   1.1.2 OHC Electromotily 2
   1.1.3 OHC Lateral Wall Structure 3

1.2 Optical Tweezers 4
   1.2.1 Optical Tweezers and Their Applications 4

1.3 Using Optical Tweezers to Study Mechanical Properties of OHC Lateral Wall 6

2 MATERIALS AND METHODS 7

2.1 OHC Isolation 7

2.2 Experimental Setup 7

2.3 Calibration Procedure 9
   2.3.1 Trapping Force Calibration Using Stokes Law 9
   2.3.2 Calibrations of Escaping Force 9
   2.3.3 Calibrations of Trapping Force 10

2.4 Tether Force Measurements 11
   2.4.1 Static Force Measurements 11
   2.4.2 Dynamic Force Measurements 12

2.5 Tether Fluorescence Imaging 13
3 RESULTS

3.1 Tether Fluorescence Imaging

3.2 Static Force Measurements
   3.2.1 Tether Formation Force
   3.2.2 Static Tether Force

3.3 Dynamic Force Measurements
   3.3.1 Single Speed Pulling Force Measurements
   3.3.2 4-speed Pulling Force Measurements

3.4 Tether Relaxation Measurements

4 DISCUSSION

4.1 Optical Tweezers as a tool to Study Mechanical Properties of OHC Membrane

4.2 Tether Formation Force

4.3 Static Tether Force

4.4 Dynamic Tether Force
   4.4.1 PM Tether Model
   4.4.2 Viscous Tether Force and Membrane Apparent Viscosity

4.5 Local Heating by Optical Tweezers and Force Measurement Errors

4.6 Future Experiments

5 REFERENCES
6 APPENDIX

6.1 Appendix I  28
6.1 Appendix II  30
6.2 Appendix III  31

7 FIGURES  33
1 INTRODUCTION

1.1 Outer Hair Cells and OHC Electromotility

1.1.1 Structure of the Ear

The ear has three divisions: the external ear, the middle ear, and the inner ear (Figure 1a). The external ear collects sound waves and funnels them down the ear canal, where they vibrate the eardrum. Within the middle ear, the eardrum is connected to the middle ear bones. These are the smallest bones in the body, and they mechanically carry the sound waves to the inner ear. The eustachian tube connects the middle ear to the upper part of the throat, equalizing the air pressure within the middle ear to that of the surrounding environment. The inner ear contains the cochlea. This is the organ that converts sound waves into neural signals, which are passed to the brain via the auditory nerve.

Coiling around the inside of the cochlea, the organ of Corti (Figure 1b) contains the cells responsible for hearing, the hair cells. There are two types of hair cells: inner hair cells and outer hair cells. Their name was derived from the fact they have thin cellular projections (stereocilia) at the cell apex. The bottom of these cells are attached to the basilar membrane, and the stereocilia are in contact with the tectorial membrane. Inside the cochlea, sound waves cause the basilar membrane to vibrate up and down. This creates a shearing force between the basilar membrane and the tectorial membrane, causing the hair cell stereocilia to bend back and forth. This leads to internal changes within the hair cells that creates electrical signals. Auditory nerve fibers rest below the hair cells and pass these signals on to the brain. So, the bending of the stereocilia is how hair cells sense sounds.
1.1.2 OHC Electromotility

The cochlea outer hair cells are the sensory receptor cells of hearing and balance. The OHCs are shaped cylindrically and have a diameter of about 9 micrometers (Figure 2). Three regions (flat apex, hemispheric base, and lateral wall) of OHC perform various functions. The stereocilia at the apex of the cell convert mechanic energy of sound into electrical energy. Synaptic structures at the base of the cell convert electrical energy into chemical energy by modulating the release of neurotransmitters that activate the 8th nerve fibers contacting the cell. The lateral wall is believed to be responsible for electrically induced length changes known as electromotility (Brownell et al., 1985). Electromotility is unique to OHCs and no other cell is able to change its length in response to electrical stimulation.

The OHC expresses two types of motile response. The first is a high-frequency length change of up to 5% that can be driven at acoustic frequencies and that is not directly dependent on ATP or any other chemical intermediate. Direct experimental observations suggest that this mechanism is both fast enough and strong enough to sharpen the sound-induced mechanical displacements of the basilar membrane (Holley, 1996). The second response is a much larger but slower cell length change that is directly dependent upon ATP and that probably involves several other chemical intermediates. Its function may be to alter the sensitivity of the ear by modulating the mechanical coupling of the high-frequency mechanism.

When the organ of Corti begins to vibrate in response to the incoming sound, OHC will sense the vibration through the bending of its stereocilia. The bending results in a change in the OHCs internal electrical potential which drives electromotility. The OHC
length change exerts a force against the tectoral membrane, amplifying the basilar membrane vibration and refining the sensitivity and frequency selectivity of Cochlea mechanical vibrations (Ashmore, 1987; Brownell, 1990; Dallas and Corey, 1991; Holley, 1996).

1.1.3 OHC Lateral Wall Structure

The exact mechanism of OHC’s electromotility is not clearly elucidated. The current popular hypothesis is that electromotility is due to a conformational change of a motor molecule in the lateral wall leading to changes in lateral wall surface area (Isawa and Chadwick, 1992; Kalinec et al., 1992). The OHC lateral wall is a 100-nm thick trilaminated structure consisting of the plasma membrane, cortical lattice (CL) and subsurface cisterna (SSC) (Figure 3) (Brownell and Popel, 1998). Electron microscopy revealed that the plasma membrane has nanometer scale ripples (Dieler et al., 1991; Holley, 1996) which may provide the necessary reservoir for cell length changes. An alternative hypothesis of electromotity is that changes in transmembrane potential alter the curvature of nanoscale ripples (Raphael et al., 2000).

A distinguishing feature of OHC plasma membrane is the high density of integral membrane proteins (Forge, 1991; Kalinec et al., 1992). The putative motor proteins, which are proposed to be responsible for the electromotility, are thought to reside in the plasma membrane. Prestin was recently identified as the motor protein of outer hair cells which endows rudimentary electromotility in transfected kidney cells (Zheng et al., 2000).

The cortical lattice, sandwiched between SSC and PM, is composed of cytoskeletal microdomains of parallel actin filaments cross-linked with spectrin. Ultrastructural
studies have revealed the presence of pillars about 25 nm in length linking the cortical lattice to the plasma membrane (Arima et al., 1991; Holley, 1992). The function of the pillar is probably the mechanical coupling of PM to CL through which the force generated by the motor protein can be transferred to CL and SSC. The SSC is composed of concentric layers of flattened membranes that form the innermost layer of the lateral wall. It shares common features with both Golgi apparatus and endoplasmic reticulum (Pollice and Brownell, 1993).

An important question is how the motor protein directs its force to induce electromotility. The mechanical properties of each of the three lateral wall layers and the force coupling among them are central to this question. Additionally, the mechanical properties are important parameters in development of nanoscale model to describe and eventually elucidate the mechanism of OHC electromotility.

1.2 Optical Tweezers

1.2.1 Optical Tweezers and Their Applications

Single beam gradient laser traps or optical tweezers provide an advanced technique for cellular manipulation and biological force measurements. An optical tweezers system is constructed by focusing collimated laser light through a microscope objective with a high numerical aperture (e.g. 100x N.A. = 1.3). The objective serves two purposes, both imaging the object under investigation/manipulation as well as focusing the laser beam to a small spot in the objective plane. A small particle (100 nm – 100 µm) can be ‘optically trapped’ in the waist of the beam. If the force exerted by the light beam is sufficiently high, it is possible to manipulate objects which great precision by moving the beam.
NOTE TO USERS

Page(s) not included in the original manuscript are unavailable from the author or university. The manuscript was microfilmed as received.

Pages 5 - 10

This reproduction is the best copy available.
velocities which corresponded to different trapping force. One typical recording is shown in Figure 10. In this study, we did the calibration when the pumping laser was set at maximum output of 5 W which resulted a laser power of 500 mW after the objective. The calibration of trapping force as a function of $V_z$ for 4.5 μm polystyrene bead placed at a height of 5 μm from the coverslip is shown in Figure 11. This calibration was obtained when the output of each quadrant ($V_I - V_4$) was set to an initial 0.6 V (in response to the level of microscope illumination) with the bead trapped and in the absence of flow. The light level during experiments was kept at the same level as in the calibration procedure. A step-by-step procedure for the calibration of trapping force is provided in Appendix III.

2.4 Tether Force Measurements

2.4.1 Static Force Measurements

A firmly attached OHC (or a supporting Deiters or Hensen cell) to the coverslip was identified and brought in contact with an optically trapped beam by moving the stage. Following a five seconds contact time, the cell was moved away to form a plasma membrane tether. If the laser power was not large enough, the trapping force exerted on the bead could not overcome the attachment force of PM to CL, and as a result the bead would be moved out of the trap by the cell. The laser power was increased in a stepwise fashion by increasing the light transmission through the attenuator, and pulling was repeated until detachment of bead from the cell was achieved. The presence of PM tether was indicated as the bead rapidly returned towards the lateral wall once laser light was
cut off. The tether formation force was measured for OHC lateral wall, OHC basal end and supporting cells (Deiters cells and Hensen cells).

Once a tether was formed, the cell was moved away at a speed of 2 μm/s until the tether was extended to a length ranging between 5-20 μm. At a given tether length, laser power was then decreased gradually until the tether force exceeded the strength of the optical trap, causing a rapid return of the bead to the cell wall. We refer to the tether force measured by this method as the static tether force.

2.4.2 Dynamic Force Measurements

The instantaneous tether force was measured using the quadrant photodetector which recorded the shadow of the trapped bead projected on its quadrants. After the cell was in physical contact with the trapped bead for 5 seconds, it was moved away at a constant speed ranging between 0.5-4 μm/s. As the piezoelectric stage moved the cell, the photodetector measured the amount of the change in the shadow of the trapped bead which was induced by displacement of the bead from the trap center. A continuous curve of $V_s$ (where $V_s$ is a measure of the tether force exerted on the bead) against time was obtained while the tether was elongating and displacing the trapped bead. Using this curve together with the trapping force calibration curve in Figure 11, the instantaneous tether force was measured. Some tethers were pulled at increasing rates during one recording. In such experiments, the cell was initially moved at 0.5 μm/s for 20 seconds, followed by movements at 1 μm/s for 10 seconds, 2 μm/s for 7.5 seconds, and 4 μm/s for 7.5 seconds. Total displacement for tether elongated length was 65 μm and lasted for 45 seconds. In several experiments, the tether force relaxation was measured in which the
tether was pulled and maintained at a constant length for an extended time while tether force was continuously monitored by the photodetector.

2.5 Tether Fluorescence Imaging

Inasmuch as tethers from OHC were not visible under normal light microscopy, we labeled OHC plasma membrane with Di-8-ANEPPS (Molecular probes, D-3167) which is a styryl dye that specifically binds to plasma membrane. This dye is a molecule with a nonpolar region that inserts into membranes, and a fluorescent polar region. When bound to phospholipid vesicles, Di-8-ANEPPS has absorption/emission maxima of ~467/631 nm (Figure 12). It stains OHC plasma membrane very well, and does not internalize significantly (Fluhler et al, 1985). The fluorescent dye was excited by the light from a Halogen lamp passing through a band pass filter (480±20nm). The fluorescence was collected by the CCD camera through an emission filter (635±20nm).
3 RESULTS

3.1 Tether Fluorescence Imaging

A fluorescent image showing the OHC plasma membrane labeled with Di-8-ANEPPS is shown in Figure 13. The figure shows the lateral wall and apex of the OHC (basal end is not shown). The thin strand linking the lateral wall and the bead is PM tether. The diameter of the tether was estimated about 40 nm by comparing it to the diameter of OHC and the bead.

3.2 Static Force Measurements

3.2.1 Tether Formation Force

Average tether formation forces (± standard deviation) for OHC lateral wall, OHC basal end, Deiters cells, and Hensen cells were 499±152 (n = 44), 142±49 (n =16), 146±44 (n = 13), and 116±15 (n = 13) pN, respectively (Figure 14). The tether formation force for the basal end was about 28% of that for the lateral wall.

3.2.2 Static Tether Force

The static measurements of the tether force as a function of tether length for lateral wall and basal end are shown in Figure 15. The tether force for the lateral wall was larger than that of the basal end, but increased with tether length for both the lateral wall and the basal end. The apparent stiffness of the tether estimated from the slope of tether force vs. tether length was 3.71 pN/μm for the lateral wall, and 4.57 pN/μm for the basal end.
3.3 Dynamic Force Measurements

3.3.1 Single Speed Pulling Force Measurements

A typical dynamic tether force measurement is shown in Figure 16. In this measurement, the cell was moved 40 μm away from the bead at a pulling rate \(v\) of 1 μm/s. Note that the force was negative prior to the beginning of pulling since the cell pushed the bead in the direction opposite to that of the stage movement. Membrane tether was formed when the cell was moved about 3.4 μm away and the tether formation force was approximately 300 pN. Once the tether was formed, the tether force was reduced to about 100 pN, and gradually increased to an equilibrium force \(F_e\) of 115 pN where it remained approximately constant with increased tether length.

Dynamic tether force measurements were made at pulling rates of 0.5, 1, 2 and 4 μm/s. We observed that the equilibrium force tended to become larger with increased pulling rates, but was quite scattering at each pulling rate.

3.3.2 4-speed Pulling Force Measurements

To eliminate the large variability in the response of different cells subject to different pulling rates, we changed the pulling rate on the same cell. The cell was moved at 0.5 μm/s for 20 seconds, 1 μm/s for 10 seconds, 2 μm/s for 7.5 seconds, and 4 μm/s for 7.5 seconds. As a result, the equilibrium forces were obtained at different pulling rates on the same tether.

A recording using this type of pulling is shown in Figure 17. The broken line represents the pulling rate and the continuous line represents the tether force during pulling. In this recording, the tether force dropped from 290 pN to 100 pN after the tether was formed, increased to 150 pN, and dropped again to 10 pN. This type of response
maybe due to additional membrane pulled off from the cell after the initial tether formation. Figure 17 shows a clear trend in that the tether force began to increase when the pulling rate was increased, and reached a final equilibrium value.

Figure 18 shows the equilibrium tether force at different pulling rates for the tether shown in Figure 17. The slope of the fitted line which corresponds to membrane apparent viscosity is 13.53 pN·s/μm. In some of the 4-speed pulling experiments, the tether broke before it reached a full length of 65 μm. These recordings were not used in estimating the slope of the $Fe-v$ curve. Ten full-length tethers were obtained using the 4-speed pulling signal and the slopes of $Fe-v$ curve were in the range of 13-33 pN·s/μm. The average tether formation force was 492 pN for these 10 tethers and the value was consistent with that (499 pN) obtained from static measurements.

3.4 Tether Relaxation Measurements

We also did some tether relaxation measurements in which the tether was pulled and maintained at a constant length for an extended time. One such measurement is shown in Figure 19. In this measurement the tether was pulled to 10 μm at 0.5 μm/s and subsequently allowed to relax. The upper panel shows the whole procedure including tether formation, pulling and relaxation. The lower panel shows the recording from the moment that tether length reached 10 μm to the moment that tether force reached equilibrium. The two spikes on the relaxation curve in the upper panel are due to some debris entered the optical trap. This cell exhibited relaxation time constant (the time for the tether force relaxed to an equilibrium value) of 50 seconds and equilibrium force of 60 pN.
4 DISCUSSION

4.1 Optical Tweezers as a tool to Study the Mechanical Properties of OHC Membrane

Optical tweezers have been used to characterize the mechanical properties of the cell membrane by pulling plasma membrane tethers with microspheres (Dai and Sheetz, 1995; Dai and Sheetz, 1999; Raucher and Sheetz, 1999). Most of these studies have studied membranes without cytoskeleton support (cell bleb membrane) or with very simple cytoskeleton (neuronal growth cone and fibroblast). Little is known about the mechanical properties of cell membrane of more complex cells (such as outer hair cells) with extensive cytoskeleton support. With the high laser power (500 mW at the trapping plane) used in our optical tweezers system, we have achieved trapping force larger than 600 pN which has enabled us to study the mechanical properties of the OHC plasma membrane.

4.2 Tether Formation Force

Previous studies have demonstrated that the membrane tether contains only plasma membrane so the tether formation involves separating the plasma membrane from the underlying cytoskeleton. In this study we measured the tether formation force from the lateral wall and the basal end of OHC as well as its supporting cells (Deiters cells and Hensen cells). The force required to form a tether was significantly higher for the lateral wall (499 pN in static measurements and 492 pN in dynamic measurements) than those for the basal end (142 pN), Deiters cells (146 pN) and Hensen cells (116 pN). The larger value of the force required to pull tethers from the lateral wall reflects the influence of the
OHC cortical lattice. The lateral wall plasma membrane is attached to the cortical lattice which is absent in the basal end of OHC and supporting cells.

Other investigators have measured the tether formation force for a variety of cell types using optical tweezers as well as micropipette aspiration and microcantilever techniques. The respective values for a leukemic rat cell (Sheetz, 1998), red blood cells (Waugh and Bauserman, 1995), neutrophils (Shao and Hochmuth, 1996), and chick fibroblasts (Raucher and Sheetz, 1999) have been reported as 70, 50, 45, and 35 pN. These values are lower than what we have measured for the OHC, and its supporting cells suggesting the tight attachment of the PM to the underlying cytoskeleton in these cochlea cells specially at the site of the OHC's lateral wall. The results from this study suggest that the plasma membrane-cytoskeleton adhesion force is the major contributor to the tether formation force.

4.3 Static Tether Force

We measured the static tether force for tether lengths ranging between 5-20 μm from the lateral wall and basal end of OHC. The apparent stiffness of the tether estimated from the slope of tether force vs. tether length was 3.71 pN/μm for the lateral wall, and 4.57 pN/μm for the basal end. The difference of PM stiffness may be due to different cholesterol distribution in the PM of the lateral wall and the basal end. A previous study has demonstrated that more cholesterol was present in the basal end than in the lateral wall (Nguyen and Brownell, 1998). The lateral wall stiffness parameter of OHCs treated with water-soluble cholesterol (0.76 dyn/cm) was significantly higher than that of controls (0.46 dyn/cm).
During the measurements of static tether force, the laser power was gradually decreased until the static tether force exceeded the strength of the optical trap. From the relaxation experiments, we observed that the tether relaxed immediately after the pulling was stopped. In the process of decreasing laser power, the bead moved toward the cell so that the tether relaxed even more. As a result, the static tether force should be smaller than dynamic tether force, and this is what we observed.

4.4 Dynamic Tether Force

4.4.1 PM Tether Model

Previous studies have indicated that membrane tether behaves like a viscoelastic material which possesses both elastic and viscous properties, and plasma membrane continuously flows into the tether when a membrane tether is forming (Evans and Hochmuth, 1976a,b). The viscoelastic behavior of the membrane tether can be modeled using the Maxwell model which is composed of a linear spring and a dashpot (Figure 20). The tether force is the sum of elastic and viscous forces: \( F = k\Delta x + \eta \nu \), where \( k \) is PM apparent stiffness, \( \Delta x \) is tether length change, \( \eta \) is membrane apparent viscosity and \( \nu \) is tether formation rate (pulling rate). The viscous force could result from the interactions between: 1) the PM leaflets; 2) PM and cytoskeleton; and 3) PM and membrane integral proteins.

The elastic component of the tether force \( (k\Delta x) \) is the \( y \)-intercept of the \( F - \nu \) curve. The apparent PM stiffness can be derived if tether length change \( (\Delta x) \) is known. During the pulling process, plasma membrane continuously flows into the tether which complicates the process of tether formation, and hence, estimating the true apparent PM stiffness.
4.4.2 Viscous Tether Force and Membrane Apparent Viscosity

Membrane viscosity is an important factor in determining the rate at which the membrane can undergo deformation on a macroscopic scale, and it also influences the rate at which particles can diffuse in the plane of the surface (Waugh, 1982b). In this study, we estimated the membrane viscosity of OHC from the slope of \( F_e - v \) curve by measuring the equilibrium tether force at different pulling rates. The calculated membrane viscosity was in the range of 13-33 pN·s/μm.

Membrane viscosity has been estimated for phospholipid vesicles (Waugh, 1982a,b) and red blood cells (Evans and Hochmuth, 1976a,b) using the tether formation method. The values of surface viscosity for membrane bilayer are reported in the range of 5-13 × 10⁻¹ pN·s/μm. Membrane viscosity for red blood cell membrane is 1 pN·s/μm when the membrane behaves in an elastic solid manner (before reaches the plastic failure) and is 10 pN·s/μm when the membrane behaves in a plastic flow manner. Since membrane viscosity of the phospholipid vesicles is 3-4 orders of magnitude smaller than that of red blood cells and OHC, we can conclude the PM leaflets shearing is much smaller than the viscous interactions between the lipid membrane and cytoskeleton or membrane proteins. The viscous force produced when pulling a membrane tether from OHC is due mainly to the viscous interactions between the PM and the tips of cortical lattice pillars or between the PM and membrane integral proteins.
4.5 Local Heating by Optical Tweezers and Force Measurement Errors

In our setup the laser was tuned to 830 nm, a wavelength which is shown to cause minimal damage to the Escherichia Coli within the optical trap (Neuman et al., 1999). We have observed that the optical trap can damage the red blood cells if the power is sufficiently high (>300 mW); however there was no observable damage to the OHCs even with the maximum power (500 mW). There may still be a possibility that the laser can cause local heating on the membrane attached to the trapped beads.

When pulling the tethers, the drag force induced by the surrounding medium would affect the force measurements. With the low pulling rates of several microns per second applied in our study, the drag force on the bead was less than one piconewton which is negligible compared to the much larger tether force. The trap could also exert some force directly on the tethers. Since the tether has a smaller diameter, and is further separated from the trap compared to the bead, it is unlikely that the direct force on the tether is more than 20% of that on the bead (Dai and Sheetz, 1995).

4.6 Future Experiments

Amphipathic drugs such as salicylate (SAL) and chlorpromazine (CPZ) can alter membrane curvature by preferentially partitioning into either the outer or inner leaflet of the phospholipid bilayer, selectively increasing the leaflet’s surface area. SAL interacts with the PM of the red blood cell resulting in an outward membrane buckling (crenation), while CPZ bends the red blood cell membrane inward (cupping) (Sheetz and Singer, 1974; Sheetz et al., 1976). Agents that alter membrane curvature would be expected to affect the mechanical properties of the membrane. In our future experiments, we will
study the effects of SAL and CPZ on tether formation from the lateral wall of the outer hair cell using optical tweezers.
REFERENCES


APPENDIX I

A Report on the Frequency Response of the Piezoelectric Stage

During the trapping force calibration and OHC tether experiments, a piezoelectric translation stage (Physik Instrumente, Model P-527.C3L) was used to achieve movement with known velocity. For general description of the piezoelectric stage and its controller please refer to the operating manuals provided by Physical Instrumente (PI). This report provides the information about the frequency response of the stage that is not mentioned in the manuals.

The movement of the stage can be controlled either by Pzmove software or an external function generator. To use which control method depends on what kind of movement you want to achieve. Pzmove software is better for the displacement of the stage (if you don’t care the speed of the movement). By using the software you can move the stage in three dimensions at any distance from 100 nm to 220 μm (for X and Y axis) or 100 nm to 22 μm (for Z axis) by just clicking the mouse. The software does provide a way to achieve movement follow some waveforms such as triangle wave and sinewave. One major limitation of the software is that it cannot provide enough speed to the stage that is necessary for the calibration of the trap. For the calibration of the trapping force, an external function generator which can generate complicated waveform to move the stage at different speed is needed.

One way to calibrate the escaping force of the trap is to apply a triangle wave to the stage. One can begin with a relative low speed which is not high enough to cause the bead to escape from the trap, then increase the speed gradually until the escaping velocity is reached. One way to change the speed is to set the amplitude of the triangle wave to a
certain voltage such as 4 volts (which corresponds to a one way displacement of 160 μm for the stage) and vary the frequency of the triangle wave. The reality is that the stage cannot move a full 320 μm (the total movement is 320 μm) especially at high frequencies. The actual movement of the stage can be monitored by collecting signal from the 'sensor monitor' port on the piezoelectric stage controller using an oscilloscope. Figure 21 shows the actual displacement of the stage at different frequencies for both X and Y directions. The actual displacement decreases linearly with frequency increase. The actual speed of the stage at different frequency is shown in Figure 22. The velocity increase linearly with frequency up to 50 Hz and peaked at 55 Hz, then decrease with the frequency increases. The peak velocity is about 10000 μm/s.
APPENDIX II

A Step-by-step Procedure for the Calibration of Escaping Force

1. Generate the signal (Figure 8) to move the stage in Excel and load it to the function generator. Fix the sample frequency (≤ 6000 points/second since the piezoelectric stage may not moving that fast beyond 6000 points/second).

2. Set the pumping laser power at 5 W and the attenuation unit to 100.

3. Trap a bead and place it 5 μm away from the coverslip. You can achieve this by adjusting the voltage input to the Z channel of the piezoelectric stage from a power supply.

4. Set the amplitude of the signal at a low voltage (which will not cause the bead escape from the trap) and apply the signal to the stage.

5. Repeat 4 with increasing amplitude until the threshold amplitude is reached which caused the bead to escape from the trap.

6. Repeat step 3, 4 and 5 for 4 times.

7. Repeat step 3, 4, 5 and 6 for other attenuation unit. You can stop at attenuation unit of 50 where the trapping force is about several pN.

8. Calculate the force according to Stokes law for each attenuation unit (for a bead 5 μm away from the coverslip the correction factor k is 1.34 from Figure 6).

9. Measure the laser power after the microscope objective with a power meter for the attenuation unit you used in step 6 and 7 (one such measurement is shown in Figure 7).

10. Plot escaping force against power after the objective (one such calibration curve is shown in Figure 9).
APPENDIX III

A Step-by-step Procedure for the Calibration of Trapping Force

1. Generate the signal (Figure 8) to move the stage in Excel and load it to the function generator. Set the amplitude of the signal to 5 volts (which corresponds to the stage movement of 200 μm).

2. Set the pumping laser power at 5 W and the attenuation unit to 100.

3. Trap a bead and place it 5 μm away from the coverslip.

4. Set the sample frequency of the signal at a low number (such as 100 points/sec).

5. Open program pd.vi (C:\photodetector\pd.vi). This program can track the position of the bead inside the trap dynamically. Start the program and place the bead at (0,0) on the XY plot by moving the micrometer that carried the quadrant photodetector. Adjust the illumination level so that the response of each quadrant is set to 0.6 V (you can choose a different voltage but please keep this the same for each trial).

6. Stop pd.vi and open pd_trigger.vi (C:\photodetector\pd_trigger.vi). Input the file name that you want to store the recording. Start the program and trigger the movement of the stage by pushing the ‘trigger’ button on the function generator.

7. After the movement is completed stop pd_trigger.vi.

8. Open the file you generated in 6 with Excel and plot the first column and you should get a figure like Figure 10. Figure 10 is obtained for 4.56 μm bead 5 μm away from the coverslip with a sample frequency of 2000 points/second (corresponds to a drag force of 230 pN). The displacement of the stage caused a 0.081 V (Vx) signal on the photodetector.

9. Repeat step 5, 6, 7 and 8 for bigger sample frequencies.
10. Generate the calibration curve by plotting the trapping force against $V_x$. One such calibration curve is shown in Figure 11.
Anatomy of the Ear

The Organ of Corti

Figure1 Structure of the ear and the Organ of Corti.
Figure 2 An outer hair cell isolated from a guinea pig organ of Corti, displaying the elongated cylindrical shape and the eccentrically placed nucleus.
Figure 3 Diagram of OHC (lower left) with detail of lateral wall components. The OHC lateral wall is a 100-nm thick trilaminated structure consisting of the plasma membrane (PM), cortical lattice (CL) and subsurface cisterna (SSC). The CL is composed of cytoskeletal microdomains of parallel actin filaments cross-linked with spectrin. Axial core (Ax) is the center of the cell, and the extracisternal space (ECiS) is the fluid space between the SSC and the PM, in which lies the CL. The stereocilia are rooted in the cuticular plate (CP).
How Do Tweezers Trap Particles?
Gradient Force vs Scattering Force

Figure 4 The forces exerted by light are called "radiation pressure". Radiation pressure has two components: the scattering force and the gradient force. A TEM00 focused laser beam has a Gaussian intensity profile, with the region of more intense light toward the propagation axis. If the beam is strongly focused, for example, through a microscope objective, the brightest region is in the focal plane. Thus, the gradient force pulls the microsphere toward the laser focus. For micrometer-sized dielectric particles, like latex or silica microspheres, the gradient force is always greater than the scattering force, so they are trapped in the region near the focus.
Figure 5 Optical tweezers setup. The solid lines represent the laser pathway and the broken lines represent imaging pathway.
Figure 6 Stokes law correction factor $k$ as a function of distance from the coverslip for 4.5 $\mu$m polystyrene bead.
Figure 7 Laser power after microscope objective at different attenuation units. The pumping laser power was set at 5 W.
Figure 8 Signal to move the piezo-electric stage during the calibration.
Figure 9 Calibration of escaping force as a function of laser power after the microscope objective. Four and half micron polystyrene beads were held with the optical trap 5 μm above the coverslip. The trapping stiffness was approximately 1.3 pN/mW.
Figure 10 A typical measurement of Vx. This measurement was obtained when the movement of the stage was 200 μm and sample rate of the function generator was 2000 points/sec. The first spike (0.081 V) was caused by the fluid flow generated by the first ramp in Figure 9 and the second spike was caused by the second ramp. The velocity of the fluid of the first ramp in Figure 9 was 4000 μm/s which corresponded to a dragging force of 230 pN.
Figure 11 Calibration of trapping force as a function of $V_x$. $V_x = (V_i + V_4) - (V_2 + V_3)$ was a measure of bead displacement in the direction of fluid flow, where $V_i$-$V_4$ was the voltage signal of the four quadrants on the photodetector generated by the bead shadow. The calibration was done when the pumping laser was set at maximum output of 5 W and the bead was placed at a height of 5 μm from the coverslip.
Figure 12 Absorption and emission spectra of Di-8-ANEPPS. It has absorption/emission maxima of ~467/631 nm.
Figure 13 Fluorescent image of an outer hair cell and the plasma membrane tether. The PM was labeled with Di-8-ANEPPS.
Figure 14 Tether formation forces for Hensen cells, Deiters cells, OHC basal end and OHC lateral wall. Average tether formation forces (± standard deviation) for Hensen cells, Deiters cells, OHC basal end, and OHC lateral wall were 116 ± 15 (n = 13), 146 ± 44 (n = 13), 142 ± 49 (n = 14), and 499 ± 152 (n = 44) pN, respectively.
Figure 15 Static tether force as a function of tether length for OHC lateral wall and basal end. The apparent stiffness of the tether was 3.71 pN/µm for the lateral wall, and 4.57 pN/µm for the basal end.
Figure 16 Dynamic measurement of tether force as a function of time. The OHC was moved 40 μm away from the bead at a constant pulling rate of 1 μm/s. Tether formation force was approximately 300 pN for this measurement. Once the tether was formed, the force was reduced to about 100 pN, and gradually increased to an equilibrium value of 115 pN. After reaching this value, there was no further change in tether force with increasing tether length.
Figure 17 Dynamic tether force as a function of time from a single tether at 4 different pulling rates (0.5, 1, 2, and 4 μm/s). The broken line represents the pulling signal and the continuous line represents the tether force during pulling. The dotted lines mark the transitions from a lower pulling rate to a higher pulling rate. In this measurement, the cell was initially moved at 0.5 μm/s for 20 seconds, followed by movements at 1 μm/s for 10 seconds, 2 μm/s for 7.5 seconds, and 4 μm/s for 7.5 seconds. Total displacement for tether elongated length was 65 μm and lasted for 45 seconds.
Figure 18 Equilibrium tether force as a function of pulling rates for the tether shown in Fig. 9. The slope of the fitted line is 13.53 pN·s/μm.
Figure 19 Tether force as a function of time during tether relaxation.

The tether was pulled to 10 μm at 0.5 μm/s and subsequently allowed to relax for an extended time. The upper panel shows the whole procedure including tether formation, pulling and relaxation. The lower panel shows the recording from the moment that tether length reached 10 μm to the moment that tether force reached equilibrium. This cell exhibited relaxation time constant of 50 seconds and equilibrium force of 60 pN. The two spikes on the relaxation curve in the upper panel are due to debris entering the optical trap.
\[ F = k \cdot x + \eta \cdot v \]

Figure 20 The membrane tether model which is composed of a linear spring and a dashpot. Tether force is the sum of elastic force and viscous force: \( F = k \cdot x + \eta \cdot v \).
Figure 21 Actual stage displacement as a function of input frequency when the amplitude of the triangle wave was set to 160 µm. The actual movements of the stage decreased almost linearly with input frequency for both X-axis and Y-axis.
Figure 22 The velocity of the piezoelectric stage as a function of input frequency. The velocity increase linearly with frequency up to 50 Hz and peaked at 55 Hz, then decrease with the frequency increases for both X and Y axis. The movement in Y direction was a little faster than that in X direction above 30 Hz.