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Analysis of Mechanical Characteristics of Plasma Membranes Using Optical Tweezers

by

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ABSTRACT

Analysis of Mechanical Characteristics of Plasma Membranes Using Optical Tweezers

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In this thesis work an optical tweezers system was designed and used to characterize viscoelastic response of plasma membranes (PMs) to an applied stress under different environmental conditions. In order to perform accurate force measurements we analyzed dynamics of an optically trapped microsphere under altering external viscous drag force using Fourier methods. Next, using optically trapped fluorescent microspheres, we recorded tethering force vs. PM displacement profiles, which revealed the tether formation process, initiated with linear deformation of the PM, followed by a nonlinear regime and terminated with the local separation of PM. Tethering force vs. displacement profiles were used to estimate tether formation force and stiffness parameter of the PM. Integration of the force-displacement profiles yielded the work of tether formation, including linear and nonlinear components. We characterized mechanical properties of the outer hair (OHC) and human embryonic kidney (HEK) cell PMs perfused with 10 mM sodium salicylate (Sal), which is known to affect electromotility of OHCs as well as PM surface charge, morphology of erythrocytes, and PM lipid diffusion. Sal reduced tether formation force, PM stiffness parameter, and equilibrium tethering force in HEK cells, explained by enhanced PM/cytoskeleton compliance. The parameters estimated for OHCs remained the same after Sal perfusion,
which is consistent with the hypothesis that Sal induced reversible hearing loss appears to be more the result of its competition with essential anions and less the result of a change in PM mechanics. Finally, we found that cell perfusion with hypo- and hyperosmotic solutions did not influence equilibrium tethering force, attributed to cellular regulation of the effective PM tension. The observed reduction in magnitude of the fast tethering force relaxation process was related to the stress-minimizing redistribution of the PM lipids. Application of a non-specific transmembrane water transport blocker, mercury (II) chloride, increased steady-state and equilibrium tethering forces, and suppressed slow component of the tethering force relaxation. Temporal tethering force profiles obtained from the same PM tethers pulled in several repetitive cycles exhibited different behavior and resulted in reduced forces and time constants explained by essential irreversibility of PM tether elongation involving PM-cytoskeleton dissociation and/or plastic deformations.
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CHAPTER I. INTRODUCTION

A. Role of Plasma Membrane Mechanical Properties in Cell Physiology

Plasma membrane (PM) of a eukaryotic cell encloses the cell body and maintains necessary physiological differences between the cytosol and the extracellular environment. PM is a thin (about 5 nm thickness) film composed of a lipid bilayer matrix and protein molecules embedded into or associated with the lipids (Figure 1). The extracellular surface is covered with glycocalyx (carbohydrate-rich zone of the cell surface) and is anchored to the cytoskeletal network intracellularly. The PM lipid fraction constitutes about 50% of the total PM mass, and serves as relatively impermeable barrier for water and water-soluble molecules, whereas PM proteins participate in transmembrane molecular transport, signaling, and catalytic reactions, perform motor
functions, and support structural association with intracellular cytoskeletal cortex (Alberts et al., 1994).

Local mechanical properties of PM and underlying supporting cytoskeleton, largely determine shape-change resistance of the cell, which is important for many biological processes, including cell adhesion (Zhu et al., 2000; Stamenovic and Ingber, 2002), locomotion of amoeboïd cells (Christopher and Guan, 2000), deformation of blood cells in capillaries (Kamm, 2002), electromotility of outer hair cells (Brownell et al., 2001), and cell division (Robinson, 2001). Mechanically PM is considered to be an incompressible two-dimensional fluid, able to sustain surface bending deformations at constant PM area (Waugh and Hochmuth, 1987; Alberts et al., 1994; Hochmuth et al., 1996). Fluid-like in-plane behavior of PM is justified by the observed free lipid diffusion inside a monolayer, with lateral diffusion coefficient of PM lipids $D_{lip} \sim 10^{-8}$ cm$^2$/s (Alberts et al., 1994). For comparison, more massive transmembrane proteins have lateral diffusion coefficient 10-100 times smaller than that of lipids, and often they are confined together with specific lipids into particular PM domains (Alberts et al., 1994). On the other hand, PM surface deformations can be induced by external or internal forces, and are determined by PM surface tension, bending rigidity of constitutive lipid and protein molecules, and PM-cytoskeleton interactions (Waugh and Hochmuth, 1987; Hochmuth et al., 1996; Hwang and Waugh, 1997; Merkel et al., 2000). One particular example of PM surface bending, experienced by every cell under normal physiological conditions, is thermal fluctuations of PM, which influence effective PM tension and are the source of excessive PM surface area (Ramaswamy et al., 2000; Gov and Safran, 2004; Lin and Brown, 2004).
In a cell, the PM is associated with the cytoskeletal protein network through discrete attachment sites, which can be dynamic in nature and help to redistribute mechanical loads between PM and cytoskeleton (Gov et al., 2003; Gov and Safran, 2004). The cytoskeleton is usually much stiffer than the PM, and is considered a three-dimensional, solid-like structure (Stamenovic and Wang, 2000; Heidemann and Wirtz, 2004).

The fluid-like in-plane behavior of PM together with its bending resistance and mechanical reaction from the cytoskeleton lead to a complex viscoelastic response of the PM to the applied mechanical loads. The measurements and modeling of the PM viscoelastic response to a standardized mechanical perturbation can be used to elucidate and quantify physiologically-important material PM parameters, which will promote the fundamental understanding of mechanical phenomena in living cells, provide diagnostic criteria to assess normal and pathological cellular processes, and help to predict mechanical cell behavior under particular environmental conditions.

B. Methods of studying plasma membrane mechanics

There are several techniques, which have been used to study local plasma membrane mechanics, including optical tweezers (Raucher and Sheetz, 1999; Sleep et al., 1999; Li et al., 2002), micropipette aspiration (Sato et al., 1987; Sit et al., 1997; Hochmuth, 2000), atomic force microscopy (AFM) (Hoh and Schoenenberger, 1994; van der Mei et al., 2000; Sato et al., 2000), and biomembrane force probe (Evans et al., 2005; Heinrich et al., 2005).
1. Micropipette aspiration technique

Micropipette aspiration is a relatively inexpensive and simple method, often used by researchers to study cell mechanics (Sit et al., 1997; Oghalai et al., 1998; Hochmuth, 2000; Merkel et al., 2000). During the experiment, glass micropipette with the inner diameter about 3-4 µm is brought into tight contact with PM at the desired location. The controlled pressure inside the micropipette determines the force applied to the studied portion of PM. Deformation of the PM is determined by subsequent video image processing. Multiple vesiculations of PM (up to 20 vesicles per cell) were observed.

Figure 2. Micropipette aspiration of plasma membrane. A glass micropipette with inner diameter $R_p \approx 3-4$ µm is brought into tight contact with the plasma membrane. An internal negative pipette pressure is applied to aspirate plasma membrane in the form of a tongue. The tongue length $\Delta L_t$ is recorded as a function of the internal pipette pressure to estimate the stiffness parameter of plasma membrane.
when the aspiration pressure reached its cell-specific critical value of about \(-1\) nN/\(\mu\)m\(^2\) (Sit et al, 1997; Oghalai et al, 1998).

The stiffness parameter is the most common mechanical characteristic, obtained using micropipette aspiration technique. It is defined as \(K_s = \frac{\Delta P R_p^2}{\Delta L_t}\) where \(\Delta P\) is the pressure generated across the PM, \(R_p\) is the micropipette inner radius and \(\Delta L_t\) is the length of the aspirated element of the PM (Figure 2). According to the elastic shell theory \(K_s\) is related to the shear modulus of a composite PM-cytoskeleton structure, Poisson ratio, and cell geometry (Sit et al, 1997).

As an alternative approach to the aspiration of PM into a micropipette, a plastic microsphere can be used for force clamp experiments on PM isolated from the cell body, with external force controlled by the applied aspiration pressure (Waugh and Hochmuth, 1987; Hochmuth and Marcus, 2002).

2. Atomic force microscopy (AFM)

Atomic force microscopy (AFM) (Binnig and Quate, 1986) is a method that utilizes a sharp probe (radius of curvature at the tip of the probe 10-30 nm) made of silicon, carbon, or other materials (Siedlecki and Marchant, 1998) to assess the force-displacement profile as the probe tip is in contact with a PM. The force generated by AFM tip is proportional to its stiffness, which is much bigger than the apparent surface stiffness of a PM, allowing minimum measurable PM generated forces on the order of 10 pN. The AFM was used in the indentation mode (when the tip is continuously indented at the constant rate into the PM) to measure local cell elastic moduli (Weisenhorn et al.,
1993; Sato et al., 2000; Charras and Horton, 2002) or to create the whole cell elasticity maps (Rotsch and Radmacher, 2000), and, also, applying both indentation and retraction of a tip, which additionally allows characterization of interactions between AFM tip and PM (Hoh and Schoenenberger, 1994; van der Mei et al., 2000).

3. Biomembrane force probe

Biomembrane force probe (BFP) technique was recently introduced for studies of the PM mechanics (Evans et al., 2001; Evans et al., 2005; Heinrich et al., 2005). To produce controlled mechanical force authors used a micropipette-aspirated red blood cell, specifically attached to a biotinylated glass microsphere. During experiments, the microsphere of assembled BFP is attached to the studied PM and the forces generated by PM result in the displacement of the BFP, which is measured using high-resolution video analysis. The micropipette-controlled tension of the red blood cell allows calculation of the BFP stiffness, and subsequently the PM-generated forces with resolution ~1-10 pN.

C. Optical tweezers

Single-beam gradient force optical traps (optical tweezers) provide a technology to generate and measure mechanical forces as small as fractions of a piconewton up to several hundred piconewtons (Ashkin et al., 1986; Wright et al., 1994; Gauthier and Wallace, 1995; Simmons et al., 1996; Lang et al., 2002; Brouhard et al., 2003). When a highly converged laser beam interacts with an optically transparent small dielectric object it moves the object into the stable equilibrium position (optical trap) near the focal point,
which was first successfully demonstrated by Arthur Ashkin of AT&T Bell Laboratories in 1986 (Ashkin et al, 1986).

Depending on the size of a trapped particle, two approaches are generally used to explain this phenomenon. In the Rayleigh regime, where particle diameter \( d \) is much less than the laser wavelength \( \lambda \) (usually valid for \( d < 0.1\lambda \)), diffraction effects cannot be neglected and electromagnetic field theory is applied to describe the trap. In this approach (Ashkin et al, 1986), dielectric particles are treated as induced point dipoles and trapping force decomposes into two components: (1) scattering force, which is proportional to the radiant energy flux and coincides with the light propagation direction; and (2) gradient force, which is proportional to and oriented in the direction of intensity gradient (Figure 3). Thus, the gradient force pulls the particle both axially toward the

![Figure 3. Force and ray diagrams of a particle optically trapped with a Gaussian (TEM\(_{00}\) mode) laser beam (Ashkin, 1997). Scattering (\( F_{\text{scat}} \)) and gradient (\( F_{\text{grad}} \)) forces appear in the Rayleigh regime. Single rays \( \mathbf{a} \) and \( \mathbf{b} \) produce corresponding forces \( F_a \) and \( F_b \) in the Mie regime.](image-url)
laser focus and radially toward the center of the beam. For stable trapping to occur, the gradient force must overcome the scattering force (Kuyper and Chiu, 2002).

In the Mie regime, where the particle size is much greater than $\lambda$ ($d > 10\lambda$), diffraction is neglected and light is considered to be composed of separate beams. The discontinuity of the index of refraction at the boundary of the object leads to the partial transfer of the light momentum to the object, generating a net force directed (Figure 3) to the focal point of the laser beam (Ashkin, 1992).

In both Rayleigh and Mie regimes, the net force can be also divided into axial and transverse components, directed to the focal point of the laser beam, indicating the presence of a "negative" radiant pressure. The net force increases with displacement of the particle center from the focal point up to a distance on the order of the particle radius. Axial ($\partial F/\partial z$) and transverse ($\partial F/\partial x$) stiffness of an optical trap also increase towards the edge of the trapping zone (Ashkin, 1992). For intermediate sizes of trapped particles ($0.1\lambda < d < 10\lambda$) the generalized Lorenz-Mie scattering theory allows computational estimation of the trapping forces (Lock, 2004).

D. Optical tweezers in studies of cellular mechanics

Laser tweezers with the trapped polystyrene or silica beads are used for quantitative measurements of forces and displacements (Kuo and Sheetz, 1992). In this technique, a bead is either specifically (when particular interactions are studied) or non-specifically (to study mechanical properties of biological membranes) attached to the biological object. Generated forces are transferred to the bead and eventually calculated using the results of force calibration. Optical tweezers were used to measure forces and
displacements produced by a single kinesin and myosin molecules (Kuo and Sheetz, 1993; Finer et al, 1994; Svoboda and Block, 1994). A particularly high resolution was achieved, when the position feedback control was implemented (Finer et al, 1994; Lang et al, 2002). The similar technique was used to measure forces required to unfold single RNA molecules (Liphardt et al, 2001), to evaluate stiffness curves for a collagen molecule (Luo et al, 1997) and DNA (Wang et al, 1997), to quantify receptor-ligand interactions (Area et al, 2002a,b; Stout, 2001), and bacterial adhesion (Hicks-Simpson et al, 2003).

In the studies, which involve cell membrane mechanics, continuous temporal profile of the trapping force is generally recorded. This method was used to determine elastic properties of the erythrocyte cell wall (Henon et al, 1999; Sleep et al, 1999), viscoelastic characteristics of cultured fibroblasts (Raucher and Sheetz, 1999), mammalian outer hair cells (Li et al., 2002), and blebbing human melanoma cells (Dai and Sheetz, 1999). One advantage of using optical tweezers for studies of PM mechanics over micropipette aspiration or AFM is that the optical tweezers can be used in mechanical testing regimes with complex microsphere manipulation (Raucher and Sheetz, 1999; Li et al., 2002; Murdock et al., 2005). Although similar, in terms of the flexibility of mechanical manipulations to the BFP, optical tweezers provide higher force resolution, and do not require complex BFP assembling procedure. Additionally, optical tweezers are superior to the other methods in their ability to sense high frequency PM-generated forces, limited only by a viscous damping and inertia of the trapped microsphere.
A particular methodology of studying PM mechanics with optical tweezers involves formation and stretching of a thin strand (tether) of PM separated from the cell body using a trapped microsphere as a "handle". An example of a PM tether visualized by a fluorescent dye is shown in Figure 4. It is a long (up to 100 μm) cylinder of membrane with a diameter 10-200 nm, which is beyond optical resolution of a microscope. Displacement of the trapped microsphere from the trapping center is used to measure membrane-generated forces transduced over the microsphere-PM contact (patch) area. Tether pulling technique creates an artificial amplification of the stress-generated displacements of PM, and therefore significantly facilitates mechanical measurements. Normally undetectable very small PM displacements or surface area changes produce measurable changes of the tether length, allowing reliable estimates of some PM mechanical parameters (Raucher and Sheetz, 1999; Li et al., 2002; Murdock et al., 2003).
E. Significance

Mechanical properties of plasma membrane determine many essential processes in cell biology (Chapter I, Part A), sensitive to plasma membrane molecular composition, interaction of plasma membrane with cytoskeletal network, and intra- and extracellular physical and chemical conditions. Among the variety of techniques, allowing measurements of plasma membrane mechanical properties, optical tweezers present an accurate, versatile, and convenient technology.

One of the most interesting experiments performed on plasma membrane with optically trapped microspheres involves pulling long thin plasma membrane cylinders (tethers) under controlled pulling rate or force. This manipulation extends the mechanical testing capabilities to a pure plasma membrane, free of the cell body attachments and cytoskeletal interactions, allowing investigation of plasma membrane viscoelasticity. However, there are some limitations with traditional imaging that confine measurements only to long tethers, when optically trapped microsphere is at a large distance from the cell body. In such measurements, important processes happening just prior and during tether formation are hidden from the investigator.

This work presents studies aimed at obtaining novel information on mechanical properties of plasma membranes using optically trapped microspheres. It starts with the development of theoretical and experimental analyses of mechanical behavior of the trapped microsphere under external forces, which are essential for accurate force measurements. The following chapter describes a novel implementation of the optically trapped fluorescent microspheres for studies of plasma membrane mechanics in the proximity to the cell. Later on, the developed optical tweezers technology is used for
measurements of the outer hair and human embryonic kidney cell plasma membrane viscoelasticity using a multiple-rate tether pulling technique under normal physiological conditions and in presence of the amphipathic drug salicylate, known to affect hearing and some membrane properties. Finally, the influence of different osmotic conditions, suppressed transmembrane water transport, and repetitive tether pulling are investigated in the scope of measured plasma membrane mechanical parameters. Significant contribution of these studies to both theoretical and experimental science involving plasma membrane mechanics and biological applications of optical tweezers, make this work interesting and useful for a broad scientific audience.
CHAPTER II. DYNAMIC MEASUREMENTS OF TRANSVERSE OPTICAL TRAPPING FORCE IN STUDIES OF PLASMA MEMBRANE MECHANICS

Chapter overview

In this chapter we describe dynamics of the optically trapped object under the influence of external viscous drag force using Fourier analysis of the equation of motion. Triangular waveforms of different frequencies are used both in theoretical modeling and experiments to induce a force on a trapped object. We investigate the contribution of various factors including fluid viscosity, density and dimensions of the trapped object (by theoretical modeling), frequency of the external force, stiffness of the optical trap, and frequency response of the instruments used to control the motion of the viscous medium (by both theoretical modeling and experimental studies) towards the accuracy of the force measurements. The developed model can be adopted for calibration of the transverse trapping force, analysis of the trapped object motion, and reconstruction of a force profile during measurements of dynamic biological forces.
A. Introduction

All biological applications of laser tweezers rely upon a calibration of optical forces. Typically, the transverse trapping force is calibrated as a function of the trapped object displacement from the trapping center. In this case, the displacement is related to the differential output signal of a position-sensing detector (e.g., a quadrant photodetector), while an external viscous drag force is generated by either translating the trapped object within a stationary fluid, or passing fluid over the trapped object. The trap displacement or the fluid flow is induced by specific devices (e.g., acousto-optic modulators (Simmons et al., 1996; Lang et al., 2002), galvano-driven mirrors (Henon et al., 1999) or piezo-electric translators (PZTs) (Felgner et al., 1995; Raucher and Sheetz, 1999; Hicks-Simpson et al., 2003), operated with control electrical signals of appropriate waveforms.

There are some reports on factors influencing accuracy of the trapping force calibration. Felgner et al., 1995 analyzed the change of trapping efficiency with the distance of the trapping center from the coverslip. Wood et al., 2002 and Wright et al., 2002 studied the effects of the concentration of dielectric particles and fluid velocity profile. There are also some comments related to technical problems, leading to the errors in trapping force measurements (Svoboda and Block, 1994; Simmons et al., 1996; Mehta et al., 1998; Sterba and Sheetz, 1998).

Additionally, first, the control electrical signals are generally distorted by the utilized instruments, affecting the dynamic behavior of the trapped object during calibration. Second, the accelerated motion of the trapped object indicates inequality between trapping and external viscous drag forces.
B. Methodology

1. Theoretical concepts

The external, transverse force applied to a trapped object (Figure 5) displaces the object according to the equation of motion:

\[ m \frac{d^2 \vec{r}}{dt^2} = \vec{F} + \vec{F}_{\text{trap}} \]  

(2.1)

where \( \vec{F} \) is the external force, \( \vec{F}_{\text{trap}} \) is the transverse trapping force, \( \vec{r} \) is the transverse (radial) displacement of the trapped particle center from the center of the trap, \( m \) is the particle mass, and \( t \) is time. In this model, rotational motion and stochastic (e.g. thermal) fluctuations of the trapped object are neglected.

Although the external force can be an arbitrary function, during calibration of the trapping force it is usually a viscous drag force (Svoboda and Block, 1994; Felgner et al., 1995; Mehta et al., 1998) governed by the Stokes’ Law:

\[ F = F_{\text{drag}} = C_{\text{drag}} \cdot \left[ V_f(t) - \frac{d \vec{r}}{dt} \right] \]  

(2.2)

Figure 5. Force balance diagram for a trapped spherical object during calibration of optical tweezers.
where \( C_{\text{drag}} \) is the drag coefficient, equal to \( 3k\pi \eta d \) for a spherical object, \( k \) is a correction factor (Happel, 1983) that accounts for the proximity of the object \( (h) \) to a stationary solid plane surface (bottom of the Petri dish), \( \eta \) is fluid viscosity, \( d \) is a diameter of the object, and \( V_{\ell}(t) \) is the transverse component of the fluid velocity function. Note that equation 2.2 and all the following equations describing the dynamics of the trapped object are presented in the scalar form.

Using first-order approximation, the trapping force can be expressed as a linear function of the trapped particle displacement:

\[
F_{\text{trap}} = -b \cdot r
\]

(2.3)

where \( b \) is the optical trap transverse stiffness. Equations 2.1-2.3 will subsequently result in the final equation of motion for the trapped object during calibration:

\[
\frac{d^2 r}{dt^2} + p_1 \frac{dr}{dt} + p_2 \cdot r = p_1 \cdot V_{\ell}(t)
\]

(2.4)

with the initial conditions:

\[
r(0) = 0
\]

(2.5)

\[
\frac{dr}{dt} \bigg|_{t=0} = 0
\]

(2.6)

where \( p_1 = C_{\text{drag}}/m \) and \( p_2 = b/m \).

The trajectory of the trapped object, obtained using Fourier Transform analysis, is:

\[
r(t) = \Phi^{-1} \left\{ S_r(f) \cdot e^{i\phi_r(f)} \right\}
\]

(2.7)

where \( \Phi^{-1} \) is the inverse Fourier Transform operator, and \( S_r \) and \( \phi_r \) are the respective amplitude and phase spectrum of the trapped object trajectory, represented as:
\[ S_r(f) = \frac{p_1}{\sqrt{4p_1^2 \pi^2 f^2 + (p_2 - 4\pi^2 f^2)^2}} \cdot S_{v_r}(f) \]  

(2.8)

\[ \phi_r = -\tan^{-1}\left( \frac{2\pi p_1}{p_2 - 4\pi^2 f^2} \right) + \phi_{v_r}(f) \]  

(2.9)

and \( S_{v_r}(f) \) and \( \phi_{v_r}(f) \) defined as the amplitude and phase spectra of the fluid velocity, respectively.

Generally, the instruments controlling fluid velocity as well as the position-sensing module, which includes the position-sensing detector and analog-to-digital interface, alter the measured position of the trapped object according to their impulse responses, which in this case are convoluted with the input signal. The resultant changes of the position-sensing module output could be characterized in the frequency domain by the amplitude and phase response of the signal distorting instruments, \( S_{\text{inst}}(f) \) and \( \phi_{\text{inst}}(f) \), respectively. Therefore, equations 2.8 and 2.9 must be corrected for the distortion of the output signal by multiplying the right part of the equation 2.8 by \( S_{\text{inst}}(f) \), and adding \( \phi_{\text{inst}}(f) \) to the right part of the equation 2.9.

We considered cases with trapped object (microsphere) diameters \( d = 0.5 - 10 \mu m \), trap stiffness \( b = 0.15 \cdot 10^{-4} - 1.5 \cdot 10^{-4} \) N/m (utilized in the most optical tweezers systems), and viscosity of the surrounding fluid corresponding to the pure water at different ambient temperatures. Mass of a microsphere was calculated from its density \( \rho \).
2. Experimental technique

Our optical tweezers setup is shown in Figure 6. A continuous wave Titanium-

Sapphire laser (Model 3900S, Spectra Physics, Mountain View, CA), pumped by a 5W frequency-doubled Nd:YVO₄ laser (MilleniaTM V, Spectra Physics, Mountain View, CA), was tuned to 830 nm, a wavelength where minimal damage to living cells (Liang et al., 1996), bacteria (Neuman et al., 1999), and DNA (Mohanty et al., 2002) has been reported. Gaussian laser beam (TEM₀₀ profile) passed through a light-collimating system, which allowed us to control the divergence of the laser beam, and filled the rear pupil of a high numerical aperture (NA ≈ 1.3) microscope objective lens (Plan-Neofluar 100× Oil, Zeiss, Jena, Germany) to form an optical trap. Laser power was controlled by a manually operated attenuator (925B, Newport, Irvine, CA) and continuously measured using a power meter (PD300-3W, Ophir Optronics, Danvers, MA).
We trapped 4.5 \( \mu \text{m} \) diameter polystyrene beads (Polysciences, Warrington, PA) in an aqueous solution (viscosity \( \eta = 1.002 \cdot 10^3 \ \text{Pa}\cdot\text{s} \) at \( T = 20^\circ\text{C} \)) at a height \( (h) \) of 5 \( \mu \text{m} \) above the coverslip. The solution chamber was mounted on a 3-D controlled PZT (P-527.3CL, Physik Instrumente, Karlsruhe, Germany), and illuminated by a 100W quartz halogen microscope light source. The light from the halogen lamp passed through the microscope objective and was reflected by a dichroic mirror towards a beam splitter. A short-pass IR filter was used to cut off any remaining laser light with \( \lambda > 705 \ \text{nm} \). The beam splitter divided the imaging light in the ratio of 1/9. The larger fraction (90\%) was directed towards a quadrant photodetector (PD) (S4349, Hamamatsu, Bridgewater, NJ) where image of a trapped bead was formed by an imaging lens. The remaining 10\% was used to form an image on a CCD camera (CCD 100, Dage-MTI, Michigan City, IN). The alignment of the optical trap and the imaging channel are described in Appendices I and II. The physical parameters related to optical trap are described in Appendix III.

The PZT was used to induce a viscous drag force against the trapped particle by generating fluid motion. It was operated with triangular control signals of different frequencies and amplitudes supplied by a synthesized function generator (SFG) (DS 345, Stanford Research Systems, Sunnyvale, CA). The amplitudes of the control signals were adjusted according to the frequency to obtain the same maximum value of the first derivative of the waveform, which was proportional to the expected value of the maximum trapping force. For example, when we used a 1 Hz control signal with amplitude of 5 V, we adjusted the amplitude of a 2 Hz signal to 2.5 V to guarantee the same maximum value of the first derivative of the waveform as 20 V/s.
3. Signal processing

PZT and a digital second-order Butterworth filter with cutoff frequency $f_{cut} = 55$ Hz (at −3 dB level), used in the processing LabView™ software to reduce noise, were the two major sources of instrumental distortion in our set up. Frequency response of the PZT was obtained by measuring amplitude and phase of the voltage signal from the PZT positioning sensors in response to the sinusoidal input signals of different frequencies (Figure 7). Later, amplitude response of the PZT was multiplied by the amplitude response of the Butterworth filter, and phase response of the PZT was added to the phase

![Graph](a)

![Graph](b)

Figure 7. Frequency responses of the PZT: (a) amplitude, and (b) phase spectra.
response of the Butterworth filter in the processing MatLab\textsuperscript{TM} program to obtain $S_{\text{inst}}(f)$ and $\varphi_{\text{inst}}(f)$.

Displacement of the trapped bead from the trapping center caused the displacement of its image on the quadrant PD to produce the differential output signals as (see Figure 8):

$$V_x = (V_1 + V_4) - (V_2 + V_3) = K_{\text{im}} \cdot v_0 \cdot f(d_{\text{im}}, x, M)$$

$$V_y = (V_3 + V_4) - (V_1 + V_2) = K_{\text{im}} \cdot v_0 \cdot f(d_{\text{im}}, y, M)$$

(2.10)

(2.11)

where

$$f(d_{\text{im}}, x, M) = 2Mx \cdot \sqrt{\frac{d_{\text{im}}^2}{4} - (Mx)^2 + \frac{d_{\text{im}}^2}{2} \cdot sin^{-1}(\frac{2Mx}{d_{\text{im}}})}$$

(2.12)

and $V_i$ is the voltage signal from i-quadrant; $K_{\text{im}}$ is the spatially averaged image contrast, defined as:

$$K_{\text{im}} = 1 - \frac{v_{\text{im}}}{v_0}$$

(2.13)

in which parameters $v_{\text{im}}$ and $v_0$ are the spatially-averaged voltages per unit surface area generated by PD from the parts covered and free from the bead image respectively; $M$ is the magnification of imaging system; $d_{\text{im}}$ is the image diameter; and $x$ is the x-component.

Figure 8. Position sensing with quadrant photodetector.
of the bead displacement.

Functional relationship between differential PD voltage signal and displacement of the trapped object (Equations 2.10-2.12) is valid only when \( v_0 \) value calculated for the entire PD surface area is equal to those calculated for each quadrant. In reality, due to various reasons, such as inhomogeneous illumination pattern on the quadrant PD, \( v_0 \) values calculated for different parts of PD may not be the same. Particularly, this fact makes the use of equations 2.10 and 2.11 ineffective for initial centering of the bead image on the quadrant PD. In other words, zero values of \( V_x \) and \( V_y \) will not be achieved for the image of the trapped object centered on the quadrant PD. Moreover, different responses of individual quadrants will cause non-linearity in relationship between the differential PD signal and transverse displacement of the trapped object. To overcome this problem, and simultaneously account for the absolute value of the light illumination level, we normalized each individual quadrant signal without an image of the trapped object:

\[
\tilde{V}_i = \frac{1}{\pi D_{PD}^2} \cdot \frac{V_i}{v_{0i}} \quad (2.14)
\]

where \( \tilde{V}_i \) is the normalized voltage signal from i-quadrant, \( D_{PD} \) is the diameter of the sensitive area of quadrant PD (in our case, 1 mm), and \( v_{0i} \) is the voltage per unit surface area generated by i-quadrant when it is free from the bead image. The differential PD signals (Equations 2.10 and 2.11), even being normalized for each quadrant using Equation 2.14, are still vulnerable to the short-term fluctuations of imaging light. We used a highly stabilized power supply for the imaging light source to minimize the short-term light fluctuations, which result in fluctuations of \( v_{0i} \) to about 0.1-0.2 %.
During experiments, we observed a systematic decrease in the average of the normalized voltage signals from four quadrants with increased transverse bead displacement from the trapping center, which was associated with change of the contrast and size of the trapped bead image. This effect influenced the differential PD signal, which is directly proportional to the image contrast and has a complex relationship to the size of the trapped bead image (Equations 2.10-2.14). The systematic change of the average of the normalized voltage signals observed during experiments could reach 50% and more, thus introducing the major component of the signal error.

We attributed this phenomenon to the axial displacement of the trapped bead into a new equilibrium position, further from the focal plane of the trapping beam, when it is transversely displaced from the trapping center, a phenomenon previously described (Ashkin, 1992). When the trapped object is displaced in the direction parallel to the imaging light propagation, the scattering pattern of the imaging light changes, thus changing the size and contrast of the image formed on the PD (Figure 9).

![Figure 9](image_url)

Figure 9. Change of the image contrast (visualized by the CCD camera) due to the axial displacement of the trapped microsphere
To eliminate the influence of the image contrast, another signal processing algorithm was considered:

\[
\hat{V}_x = \frac{\bar{V}}{1 - \bar{V}} = \frac{4}{\pi \cdot d_{im}^2} \cdot f(d_{im}, x, M)
\]  

(2.15)

where \(\hat{V}_x\) is the contrast-corrected normalized differential PD signal, \(\bar{V}\) is the average of the normalized PD signals (Equation 2.14) from all four quadrants, and \(\hat{V}_x\) is the normalized differential voltage signal obtained from normalized voltage signals of each quadrant (\(\bar{V}_i\)). It is essential to notice that the parameter \(\hat{V}_x\) is related to the bead displacement from the trapping center in the same functional manner as \(V_x\). Due to the change of the image size, diffraction effects, and short-term light level changes (especially important in low-contrast images), \(\hat{V}_x\) still exhibits some variation, but much smaller than that in the case of \(V_x\). We have found the transformation given in equation 2.15 particularly useful in improving the linearity of differential PD signal, which in case of non-corrected signal is caused by the diffraction effects (Figure 10). In other words, the error, brought about by linearization of the calibration data will be much smaller and

![Figure 10. Improved linearity of the differential PD signal after implementation of the contrast-corrected signal-processing algorithm.](image-url)
less dependent upon the calibration range for the contrast-corrected algorithm.

The amplitude values of $\dot{V}_x$ were used to compare the maximum bead displacement from the trapping center, and consequently, the maximum trapping force, for control signals of different frequencies. To obtain the frequency response of the calibrated trapping force, we calculated the ratio of the differential voltage signal amplitude at a specified frequency $\dot{V}_x(f)$ to the differential voltage signal amplitude at 1Hz ($\dot{V}_{x_0}$). The PD response to a single-triggered triangular control signal has three characteristic regions, corresponding to the three peaks of PZT velocity (Figure 11). The

Figure 11. Waveforms corresponding to: (a) signal applied to the PZT, and (b) response of the quadrant photodetector.

first region is defined over the interval between $t = 0$ (onset of the PD response) to $t = 1/(4f)$; the second – from $t = 1/(4f)$ to $t = 3/(4f)$; and the third – from $t = 3/(4f)$ to $t = 1/f$. 

We used the maximum values of $\hat{V}_x(f)$ at each of those characteristic regions to calculate the frequency response.

4. Estimation of the trap stiffness

To estimate the stiffness of the optical trap we performed two separate series of measurements of the contrast-corrected normalized differential voltage signal ($\hat{V}_x$). First, we measured $\hat{V}_x$ for the trapped microsphere and PZT driven with 1Hz triangular waveform. The calculated maximum transverse trapping force ($F_{\text{trap}}^{\text{max}}$) was divided by the amplitude of the differential voltage signal ($\hat{V}_{a0}$), which was used as a normalization factor in calculation of the frequency response to obtain the force-voltage slope ($k_F$). Then, we measured $\hat{V}_x$ as a function of the controlled bead displacement using a microsphere adherent to the coverslip of a sample chamber. In order to do that, beads were incubated in distilled water solution inside the poly-D-lysine coated Petri dish overnight, which allowed them firmly adhere to the coverslip. The following day, the image of a trapped bead, and then the image of a bead adherent to the coverslip were centered on the PD. Vertical position of the Petri dish was finely tuned using PZT to image the adherent bead as it would be trapped, and the Petri dish with the attached bead was run with a single-triggered triangular waveform of the amplitude about a bead diameter. The contrast-corrected normalized differential PD voltage was recorded and analyzed as a linear function of the bead displacement. The resultant slope between the contrast-corrected normalized differential voltage signal and the bead displacement ($k_V$) was used together with $k_F$ to estimate the trap stiffness as $b = k_F k_V$. 
C. Results

Theoretical amplitude spectral functions of a microsphere displacement from the trapping center in response to an external drag force for different values of trap stiffness and microsphere size are shown in Figure 12. Amplitude of the bead displacement from the trapping center decreases with frequency. For a specified frequency, the amplitude decreases with increased bead size and reduced trap stiffness. For small bead sizes (\(d \approx 0.5 \, \mu m\)), the amplitude spectral function shows negligible decrease up to \(f = 100\) Hz, while for \(d = 4.5 \, \mu m\) and \(b = 15\) pN/\(\mu m\) it reduces to 0.8 at \(f = 30\) Hz. For \(d = 9.1 \, \mu m\) and \(b = 15\) pN/\(\mu m\), the displacement amplitude reduces substantially at frequencies as low as \(f = 5\) Hz.

Theoretical analysis of microspheres with various densities showed that the amplitude spectral function of a microsphere displacement was not significantly affected by density of the constitutive material. For example, theoretical amplitude spectral function of a microsphere displacement from the trapping center calculated for 4.5 \(\mu m\) diameter microsphere and trap stiffness 75 pN/\(\mu m\) at \(f = 500\) Hz, slowly increases by 0.2\% for microsphere density changing from 1,000 to 3,000 kg/m\(^3\). This effect is caused by inertia of a microsphere, which is dependent on density of the constitutive material, and is negligible in comparison to the total 60\% reduction of the amplitude spectral function at 500 Hz.
Figure 12. Theoretical amplitude spectral functions of a microsphere displacement within an optical trap calculated for microsphere diameters of: (a) 0.535 μm, (b) 4.537 μm, and (c) 9.14 μm. Values of the trap stiffness are 150 (solid line), 75 (dashed line), and 15 (dash-dotted line) pN/μm.
In Figure 13 the theoretical amplitude spectral functions of a microsphere displacement from the trapping center are shown for the case when it is trapped in fluids of different viscosities. Increased fluid viscosity leads to a proportional increase in the viscous drag on the trapped object, and lowers the theoretical amplitude spectral function.

![Graph](image)

Figure 13. Theoretical amplitude spectral functions of a microsphere displacement within an optical trap calculated for different fluid viscosities. Values of the fluid viscosity are 1002 (solid line), 788 (dashed line), and 547 (dash-dotted line) Pa·s, representing water at 20, 30 and 50 °C respectively. $b = 75$ pN/μm, $d = 4.537$ μm.

In Figure 14 both theoretically and experimentally obtained frequency responses of the calibrated trapping force are shown for transverse trap stiffness values of 38, 70, and 125 pN/μm, using triangular input signals to control the PZT movement when trapping a 4.5 μm diameter bead. The initial “overshoot” in the theoretical curves is caused by low-pass filtering of the differential PD signal. Similar negative trend is observed at high frequencies in both theoretical and experimental frequency responses. However, the deviations from the theory in the low-frequency range are quite large to be explained by statistical fluctuations during experiments. To understand this phenomenon, we examined the profile of experimentally measured differential PD signal in the time domain (Figure 11), which demonstrates periodic oscillations in the signal.
Inasmuch as the solution to Equation 2.4 indicates asymptotic behavior for all values of the parameters, no oscillations of a trapped microsphere are predicted.

We suggest that the oscillations may be induced by wave formation within the fluid during PZT motion. In this case, the observed oscillations of the differential PD signal can be explained by the direct mechanical impact of those waves on the trapped
object. The phase of the induced wave relative to the PZT velocity profile as well as the amplitude of the waves will be determinant factors of the output PD signal distortion. Note that local in-phase change in propagation of the imaging light through the oscillating fluid meniscus was compensated in our case by the contrast-correction algorithm.

The evidence in support of this hypothesis is as follows: The frequency of the PD signal oscillations was found to be about 11.1 Hz for a 13.5 mm diameter fluid meniscus and 14.9 Hz for a 10.6 mm diameter fluid meniscus, achieved using different sample chamber sizes, which implies its inverse relationship to the size of the liquid sample. The frequency of the PD signal oscillations was independent of microsphere diameter (2 and 4.5 μm), magnitude and frequency of the control signal applied to the PZT, direction of the piezostage motion, and trap stiffness in the range of 35-125 pN/μm. When x- and y-components of the differential PD signal were plotted against each other, the graph formed a typical pattern similar to that of an ellipsoidal Lissajou figure, which implies two-dimensional orthogonal oscillations with equal frequencies, expected of microsphere within a 2-D fluid wave field. Finally, during motion of the PZT we visually observed surface waves.

The difference between experimental and theoretically predicted frequency responses is minimal if the first characteristic region of the PD signal (Figure 11b) is used in calculations of both frequency responses. When PZT motion is just initiated, the wave process is not fully developed, and has a minimal impact on the dynamic behavior of the trapped object. The reduction in the amplitude spectrum profiles when using the second and third characteristic regions of the PD signal in comparison to the theoretical profiles
(Figures 14b and 14c) can be caused by the additional damping of the large amount of liquid during wave formation process inside the sample chamber.

Further, it is hypothesized that inertial properties of the liquid meniscus could make some contribution to the observed oscillative behavior of the differential PD signal (see Figure 11b). To check that, the frequency response of the calibrated trapping force was measured for trap stiffness of 57 pN/μm with a Petri dish full of water (diameter 33.9 mm), which made it impossible for a highly curved liquid meniscus to form. The differential PD signal showed the oscillative behavior. However, its fundamental frequency was reduced to 6.2 Hz, which further supports our wave formation hypothesis.

Experimental frequency response calculated using the first characteristic region in the PD response is shown in Figure 15. It follows the shape of the theoretically predicted

![Figure 15](image.png)

Figure 15. Modeled (dashed line) and experimentally measured (solid line) amplitude frequency responses during calibration of optical tweezers with triangular PZT control signals and external fluid without meniscus. Calculations are based on the first characteristic region in the PD response. $b = 57 \text{ pN/μm}$.

frequency response rather well except at low frequencies near 2 Hz. The behavior is similar when second or third characteristic regions in the PD response are used for the calculation of the frequency response curves. There is no significant improvement of the frequency response curves after removal of the liquid meniscus implying that its
mechanical properties, probably, are not important for the behavior of the trapped object during wave formation. Table 1 summarizes the findings and describes some factors, which influence calibration of the transverse trapping force and forces measured with optical tweezers system.

**Table 1** Factors influencing the force frequency response measured using optical tweezers

<table>
<thead>
<tr>
<th>Factor</th>
<th>Effect on the force frequency response</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diameter of the trapped object</td>
<td>Frequency response reduces faster for larger objects</td>
</tr>
<tr>
<td>Stiffness of the optical trap</td>
<td>Frequency response reduces faster for less stiff traps</td>
</tr>
<tr>
<td>Viscosity of the fluid</td>
<td>Frequency response reduces faster for more viscous media</td>
</tr>
<tr>
<td>Density of the trapped particles</td>
<td>Frequency response is negligibly affected</td>
</tr>
<tr>
<td>Standing waves in the fluid</td>
<td>Measured and theoretical frequency responses are different depending on the frequency of the control PZT signal</td>
</tr>
</tbody>
</table>

D. **Discussion and conclusions**

Both theoretical and experimental results demonstrate that when optical tweezers are calibrated to measure biological forces, the low frequency components of the transverse displacement signal are less attenuated than the high frequency components. In other words, the measured signal exhibits the behavior of a signal that is low-pass filtered. Increased microsphere diameter or surrounding fluid viscosity, as well as decreased trap stiffness reduce the cutoff frequency of the “apparent low-pass filter”. The amplitude and the phase spectra of the microsphere position signal will also depend on the distortion introduced by the motion-controlling instruments and position-sensing module.
Performing a dynamic analysis of the trapped object motion is important for the accuracy of the calibration of the transverse trapping force and subsequently the measurements of the biological forces during optical tweezers experiments. The information on motion-controlling signals, and expected output signals resulting from the microsphere displacement can be used in the model to estimate the sample fluid viscosity, size of the microspheres and trap stiffness, and choose the correct motion-controlling and position-sensing devices.

It was shown that the wave formation inside the fluid-filled sample chamber could have a noticeable impact on the measured microsphere displacement signal. Being independent from the frequency of the control signal, the waves can appear during any dynamic manipulation of an optical trap that involves use of a PZT. However, in applications when small accelerations of the PZT are used, or measurements are performed at least several seconds following the PZT acceleration, the wave energy is insufficient (in the second case the wave is largely damped) to have a noticeable effect on the measured microsphere displacement signal. The particular experimental conditions (mass, viscosity and size of the sample fluid, mechanical properties of the trapped object and the optical trap) will determine whether PZT kinematics satisfy that requirement.

In the case, when mechanical oscillations of the sample fluid cannot be neglected, three approaches can be used to increase the accuracy of the measured data. First, the wave formation process can be incorporated into the model of the dynamic behavior of the trapped object. Second, experiments, which will imitate the kinematics of the PZT motion during the biological force measurements, must be performed to calculate displacement of a trapped microsphere. The results of these experiments can be used to
estimate the contribution of the wave formation process to the motion of the trapped object during biological force measurements. Lastly, the problem of the wave formation in the sample fluid exists only when a PZT or any similar instrument is used to translate the sample chamber with a microsphere trapped by a stationary laser beam. The oscillations can be completely eliminated if during an experiment the trapped object is moved inside the surrounding media by translating the trapping laser beam using galvano-driven mirror or acousto-optic modulator. However, this approach is limited by the maximum travel distance of the trapped object on the order of several micrometers. If an experiment requires a trapped object to be moved large distances, PZT can be used to generate large-distance motion with a small acceleration, and the beam steering device will be responsible for the short accelerated displacements.

The analyzed model describes the behavior of a microsphere optically trapped in a moving viscous medium. An important application of the model is that experimentally measured signal related to the displacement of the trapped object can be used to reconstruct the true temporal profile of the external force acting on the trapped object, separated from the viscous drag and inertia. In this case, the transverse component of the external force \( F(t) \), will appear additionally to the fluid velocity component \( V_f(t) \), in the right side of the equation of motion for the trapped object:

\[
\frac{d^2r}{dt^2} + p_1 \frac{dr}{dt} + p_2 \cdot r = p_1 \cdot V_f(t) + \frac{1}{m} \cdot F(t) \tag{2.16}
\]

Using the known system parameters \( p_1, p_2 \) and \( V_f(t) \), together with measured displacement \( r(t) \), equation 2.16 can be solved to obtain \( F(t) \). To illustrate the effectiveness of this approach, the contribution of the inertia for 2 and 4 \( \mu m \) diameter polystyrene microspheres to the measured external force was estimated. In Figure 16, the
 inertia of a microsphere is calculated for its maximum acceleration when the microsphere velocity is exponentially changed to a final asymptotic value of 1000 $\mu$m/s with a time constant ranging from 1 to 100 $\mu$s. The inertia inversely increases with time constant, and reaches several piconewtons when time constant is about 10 $\mu$s. Moreover, according to the Stokes' Law, viscous drag on the microsphere increases linearly with microsphere velocity and can contribute tens of piconewtons to the measured value of the external force when microsphere velocity is about 1000 $\mu$m/s.

The suggested procedure for the external force reconstruction will be especially useful for those applications involving fast manipulations of an optical trap when the viscous drag force and microsphere inertia significantly contribute to the dynamics of a trapped microsphere and cannot be neglected. An example of such biological application can be the studies of intermolecular bond rupture at high loading rates, or manipulation of the trapped microsphere by acousto-optic modulator with rapid changes of a microsphere velocity.
Our results show that calibrating optical trapping forces with high accuracy is a challenging problem, which is particularly important for biological applications where fast low magnitude forces are measured. Interesting work has been recently published by Tolic-Norrelykke et al. (2004). The authors developed a computer program, which allowed highly accurate calibration of the small trapping forces using power spectrum of the trapped microsphere brownian motion (Berg-Sorensen and Flyvbjerg, 2004). Using QPD differential signals measured for a trapped microsphere subject to thermal fluctuations the program calculates transverse trapping stiffness by incorporating frequency dependent hydrodynamic drag, signal filtering by the position sensing devices, aliasing, and cross-talk between QPD channels. Unfortunately, the program takes about 15 minutes to complete and high accuracy of this kind of calibration is limited to external forces on the order of thermal fluctuations, which is often insufficient for measurements of PM mechanics. Some additional aspects related to accuracy and precision of force measurements with optical tweezers are discussed in Appendix IV.

The model describing the behavior of a trapped object under viscous drag force that accounts for the distortion of the measured position signal introduced by the motion controlling instruments and position-sensing module has been developed in these studies. Theoretical frequency response of the calibrated trapping force, showed a negative trend with size and viscosity of the surrounding fluid, and a positive trend with transverse stiffness of optical trap. It was demonstrated theoretically that the density of the trapped object has a negligible effect on the frequency response of the calibrated trapping force. The experimentally measured frequency responses followed the theoretical profiles with some deviations explained by a wave formation process in the fluid-filled sample.
chamber. The analysis of the equation of motion for the trapped microsphere is particularly important in the processing of the data obtained from the experimental studies of cellular and molecular mechanics using optical tweezers. Such an approach allows the reconstruction of the temporal profile for the external biological forces exerted on a trapped microsphere, and analysis of the dynamics of the trapped object under the known external force.
CHAPTER III. STUDIES OF PLASMA MEMBRANE MECHANICS AND PLASMA MEMBRANE – CYTOSKELETON INTERACTIONS USING OPTICAL TWEEZERS AND FLUORESCENCE IMAGING

Chapter Overview

This chapter demonstrates the advantages offered by the use of optically trapped fluorescent microspheres for studies of cellular mechanics. We use optical tweezers in conjunction with an optical position-sensing system, which spectrally filters signals generated by a trapped fluorescent microsphere to study PM mechanics and its interactions with cytoskeleton. We validate the accuracy of this technique through the comparison of the temporal tethering force profiles recorded in the fluorescence mode and the values of calculated parameters, related to the mechanical properties of PM, with those measured previously using transillumination mode. Recorded tethering force vs. PM displacement profiles, revealed the tether formation process, initiated with linear deformation of the PM, followed by a nonlinear regime and terminated with the local separation of PM. Tethering force vs. displacement profiles were used to estimate tether formation force and stiffness parameter of the PM. Integration of the force-displacement profiles yielded the work of tether formation, including linear and nonlinear components. Our results demonstrate that spectral filtering of the optically trapped fluorescent microsphere image formed on the position-sensing system overcomes the artifacts introduced by transillumination imaging and allows accurate measures of PM mechanics before and during the initial stages of tether formation.
A. Introduction

The transillumination mode of microsphere imaging on the QPD has been successfully used in studies of PM mechanics by pulling long (10–100 µm) PM tethers (Simmons et al., 1996; Mehta et al., 1998; Li et al., 2002). However, if the trapped microsphere is within proximity of the cell (about two microsphere radii or less), the cell image formed on the QPD contributes to a large optical artifact, introducing an error on the estimated mechanical parameters (Qian et al., 2004). A particular approach to overcome the problem of the cell image artifacts during transillumination mode is based on spectral separation of the images of the trapped microsphere and the cell using fluorescence techniques. Recent advances in manufacturing of fluorescent microspheres make it possible to utilize narrow-band fluorescence of an optically trapped object, which, after being spectrally filtered, allows high signal-to-noise ratio measurements.

B. Methodology

1. Experimental setup and conditions

The optical tweezers setup, described in details in Chapter II, Part B2, was combined with a fluorescence imaging system (Figure 17). The light from a halogen light source was used for visualization of the cells and the trapped object on the CCD camera. A band-pass filter (15) was used to allow the transmission of the visualizing light in the 450 – 510 nm spectral range through a dichroic mirror (8) with a transparency band between 360 and 510 nm towards a beam splitter. The beam splitter directed 10 % of the light to the CCD camera, while the remaining 90 % was blocked by a filter (21) with spectral band-pass of 605 ± 27.5 nm (see Appendix V).

We optically trapped a 4 μm diameter sulfate-modified fluorescent polystyrene microspheres (F-8858, Molecular Probes, Eugene, OR) with excitation spectrum between 480 and 590 nm and maximum fluorescence emission at 605 nm. A 75W xenon short arc lamp was used for the fluorescence excitation. The fluorescence excitation light passed through an excitation band-pass (535 ± 25 nm) filter, and was reflected by the dichroic mirror (8) towards the trapped fluorescent microsphere. The fluorescent light emitted from the trapped microsphere was split by the 1/9 beam splitter, with its largest portion spectrally separated from the visualizing light by the emission band-pass filter and directed towards a QPD (QP 1.1-6-TO18, Pacific Silicon Sensor, Westlake Village, CA).

A glass bottomed Petri dish (P35GC-0-10-C, MatTek Corporation, Ashland, MA) solution chamber was positioned on the PZT. The movement of the PZT was controlled
either by the SFG or using a custom-designed computer program (LabView, National Instruments) for complex functions unavailable on the SFG. The chamber was continuously perfused with normal extracellular solution (NES, see Appendix VI). Viscosity of the solution, measured using a viscometer (Rheolyst AR 1000, TA Instruments, New Castle, DE), was 1.28 mPa·s assuming a Newtonian fluid and shear rates between 10 and 100 s⁻¹.

Displacement of the trapped microsphere from the trapping center resulted in the displacement of its image on the QPD (Figure 8). The image displacement was related to the differential voltage signals from the QPD corrected for the changes in fluorescence intensity in the same functional manner as described by Eq. 2.15:

\[
V_{PDx} = \frac{V_x}{V_\Sigma} = \frac{4}{\pi d_{im}^2} \cdot f(d_{im}, x, M) \quad (3.1)
\]

\[
V_{PDy} = \frac{V_y}{V_\Sigma} = \frac{4}{\pi d_{im}^2} \cdot f(d_{im}, y, M) \quad (3.2)
\]

where \( V_{PDx} \) & \( V_{PDy} \) are the x & y-components of the QPD differential voltage signal, corrected for the changes in fluorescent intensity, respectively; \( V_x \) & \( V_y \) are the x & y-components of the non-corrected QPD differential voltage signal, respectively; \( V_\Sigma \) is the QPD sum voltage signal; function \( f \) is described by Eq. 2.12; \( d_{im} \) is the image diameter; \( M \) is the magnification of imaging system.

The high and stable contrast of the fluorescent microsphere image eliminates the problem of contrast fluctuations appeared in the transillumination mode due to the axial displacement of the microsphere (Chapter II). Therefore, only fluorescence intensity correction, which is efficiently performed in Equations 3.1 and 3.2, is required to reduce the error associated with imaging conditions.
Calibration of transverse microsphere displacement was performed using a technique based on the controlled positioning of a stationary microsphere using the PZT, as described in Chapter II, Section B4. To calibrate the QPD output voltage vs. transverse trapping force a PZT was used to induce a viscous drag force against the trapped microsphere by moving the fluid-filled sample chamber. The PZT was operated with control electrical signals of triangular waveforms, 5V peak-to-peak amplitude, and frequencies in the range from 1 to 6 Hz, where amplitude response of the trapping force is close to 1 (see Chapter II). Calibration was performed before every experiment in the range of 25-140 pN. The fluorescence intensity corrected differential voltage signal measured for the minimal calibrated trapping force ($V_{PD}^{\text{min}}$) (corresponding to the drag force of 24.8 pN) was taken as a lower limit for the force calibration. All the measured signals with magnitudes less than $V_{PD}^{\text{min}}$ were converted into trapping force using linear interpolation between 0 and $V_{PD}^{\text{min}}$.

2. Preparing test cells and sample chamber coating

We tested our technique by measuring mechanical properties of HEK-293 cells (Advanced Cell Technology, Worcester, MA) PMs. On the day of experiments, cultured HEK cells were passaged into the sample chamber and left in an incubator for 2 hours. After the cells became adherent to the bottom of the sample chamber, the culture medium was substituted with 1.5 ml of NES, and microspheres were added. A medium size (population distribution: 10-20 μm in diameter) HEK cell was selected for measurements if it had rounded shape without any PM blebs or protrusions, the cytoplasm was
agranular, and the cell did not develop a significant network of stress fibers. All experiments on HEK cells were performed within 2 hours of removal from the incubator.

Sulfate modified fluorescent microspheres are very hydrophobic, and as a result, they adhere firmly to the glass surface of a sample chamber. Our optical tweezers setup did not have enough power to detach the microspheres unless they were suspended in distilled water. From our own observations during experiments, we discovered that the antimycotic solution (Fungizone, Invitrogen, Carlsbad, CA), a component of the HEK cell culture medium, could be used to reduce interactions between a fluorescent microsphere and the sample chamber bottom (See Appendix VII). The success rate of a microsphere detachment from the coverslip of a sample chamber using optical tweezers increased from 0% to 70-80% after sample chamber was coated with antimycotic. We incubated 0.5 ml of antimycotic overnight inside a sample chamber at room temperature. On the following day, and immediately prior to an experiment, antimycotic was substituted with 1.5 ml of NES. The antimycotic was not toxic to the HEK cells, and did not change their adhesive properties.

3. Experimental procedure

Microscope manipulators were used to move an HEK cell, firmly attached to the bottom of the sample chamber, towards the optically-trapped microsphere until they were in physical contact. The cell movement was stopped as soon as the QPD differential signal deviated from the initial zero value. A non-zero QPD differential signal indicated the presence of reactive forces from the cell acting on the trapped microsphere, which implied initiation of the PM-microsphere physical contact. We allowed the microsphere
to adhere to the PM for about 20 seconds and subsequently initiated the PZT movement. PZT moved the cell away from the trapped microsphere at 1 μm/s speed for 10 seconds. During that time a PM tether was formed. PZT was subsequently stopped, and the relaxation of tethering force was recorded for 60 seconds.

4. Analysis of the temporal tethering force profiles

We analyzed temporal tethering force profiles recorded at 1 kHz sampling rate to obtain the parameters related to the viscoelastic behavior of PM tethers (Figure 18). Last

![Diagram](image)

**Figure 18.** Typical temporal PM tethering force profile, showing tether formation (TF), steady-state (SS), and relaxation (REL) segments for an HEK cell. $F_{\text{max}}$ — tether formation force; $F_{\text{ss}}$ — steady-state tethering force; $F_{\text{eq}}$ — equilibrium tethering force.

100 samples of the tethering force signal immediately prior to relaxation were averaged to estimate the steady-state tethering force ($F_{\text{ss}}$). The force relaxation part was analyzed using bi-exponential model (Murdock et al., 2005):

$$F(t) = F_{\text{eq}} + F_{\text{long}} e^{-\frac{t}{\tau_{\text{long}}}} + F_{\text{short}} e^{-\frac{t}{\tau_{\text{short}}}}$$  \hspace{1cm} (3.3)

where $F_{\text{eq}}$ is equilibrium tethering force, estimated as the average of tethering force values during last 2 s of relaxation (Figure 18); $t$ is the time from the beginning of
relaxation; $F_{\text{long}}$ and $\tau_{\text{long}}$ are the amplitude and time constant of the slower force relaxation component, reflecting lipid flow from the cell body to the tether, and $F_{\text{short}}$ and $\tau_{\text{short}}$ are the respective amplitude and time constant of the faster force relaxation component, indicating lipid rearrangement inside the tether (Murdock et al., 2005). The model, described by Eq. 3.3 is sensitive to initialization of four independent fitting parameters, which must be estimated from the relaxation data fitting (equilibrium tethering force, two time constants and either one of the relaxation amplitudes). The remaining relaxation amplitude can be related to the estimated amplitude and other parameters as:

$$F_{\text{long}} + F_{\text{short}} = F_{\text{ss}} - F_{\text{eq}}$$

(3.4)

The tethering force relaxation data were fit using three steps. Initially we fit the slower exponential component of the relaxation data with a long time constant (>5s) using data starting 5s after the beginning of relaxation. This is justified because of the negligible contribution from the fast relaxation process in that time interval. The slower process was then extrapolated to the initial 5s of relaxation segment (Figure 19a) and subtracted from the measured relaxation data (Figure 19b), and the resultant was fit with a fast exponential yielding a complete bi-exponential relaxation model (Figure 19c). This procedure allowed splitting of one four-parametric fitting procedure into two bi-parametric fitting procedures with better fitting accuracy. Splitting the fitting process using two single-exponents allows a “good” initial guess for the relaxation amplitudes just by observing the temporal relaxation, which further reduces the error of the estimated relaxation parameters.
Figure 19. Bi-exponential fit of the tethering force relaxation segment. (a) A single exponential fit with a slower component; (b) The difference signal obtained by subtraction of the slower exponent (a) from the original tethering force relaxation data and its fit with a faster exponential process; (c) Resultant bi-exponential model of the tethering force relaxation.
5. Analysis of the tether formation segment

Tether formation segment was defined as the part from the beginning of tether pulling to the point where tethering force reached its steady-state value (Figure 18). The maximum value of the tethering force during this time interval was defined as the tether formation force \( F_{\text{max}} \). The part of the tether formation segment from the beginning of tether pulling to the tether formation force contains the information about mechanical behavior of the PM-cytoskeleton composite just before the PM is separated from the cell body. We analyzed that part by examining the tethering force as a function of the PM displacement, calculated as (Figure 20):

\[
x_{\text{PM}} = x_{\text{PZT}} + x
\]

(3.5)

where \( x_{\text{PM}} \) and \( x_{\text{PZT}} \) are respectively the PM and PZT displacements from the initial PM position (in the absence of an external force), and \( x \) is the transverse displacement of the PZT stage.

Figure 20. A diagram explaining the calculation of plasma membrane displacement \( x_{\text{PM}} \) from the controlled displacement of piezoelectric microscope stage \( x_{\text{PZT}} \) and measured transverse displacement of the trapped microsphere from the trapping center \( x \). Initial (no external force applied) position of plasma membrane and trapped microsphere are shown using dashed lines.
trapped microsphere from the trapping center.

C. Results

Figure 21 displays a typical tethering force – PM displacement profile. We divided it into two distinct regions of linear and nonlinear deformations. The point where first nonlinear deformations appeared was determined as the point where the derivative $\frac{dF_{\text{teth}}}{dx_{\text{PM}}}$ started to reduce. We divided the work of tether formation into linear ($W_l$) and nonlinear ($W_{\text{nl}}$) regimes, calculated as the areas under the tethering force curve on the corresponding regions (Figure 21) using numerical integration. The total area under the force curve prior to tether formation was the work done to form a tether ($W_{\text{teth}}$). The linear part of the force curve was used to estimate the slope $k_{\text{PM}}$ as the stiffness parameter of the PM.

Material parameters calculated for HEK cells are presented in Table 2. The tethering force-PM displacement profiles (Figure 21) allowed the first determination of
tether formation force, stiffness parameter of the PM, and energetic parameters of the
tether formation ($W_{\text{teth}}$, $W_i$ and $W_{\text{nl}}$) using optical tweezers – shown in the shaded portion
of Table 2.

**Table 2** Material parameters of the HEK cell plasma membrane calculated from the
tether-pulling experiments
(shaded area indicates parameters estimated for the first time using optical tweezers)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>$F_{\text{max}}$</th>
<th>$k_{\text{PM}}$</th>
<th>$W_{\text{teth}}$</th>
<th>$W_i$</th>
<th>$W_{\text{nl}}$</th>
<th>$F_{\text{ss}}$</th>
<th>$F_{\text{eq}}$</th>
<th>$F_{\text{short}}$</th>
<th>$F_{\text{long}}$</th>
<th>$\tau_{\text{short}}$</th>
<th>$\tau_{\text{long}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dimension</td>
<td>pN</td>
<td>pN/μm</td>
<td>$10^{-18}$ J</td>
<td>$10^{-18}$ J</td>
<td>$10^{-18}$ J</td>
<td>pN</td>
<td>pN</td>
<td>pN</td>
<td>pN</td>
<td>s</td>
<td>s</td>
</tr>
<tr>
<td>Average value</td>
<td>246.3</td>
<td>311.4</td>
<td>234.1</td>
<td>69.8</td>
<td>184.5</td>
<td>73.22</td>
<td>42.06</td>
<td>12.75</td>
<td>20.21</td>
<td>1.42</td>
<td>14.74</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>87.6</td>
<td>181.0</td>
<td>171.1</td>
<td>78.6</td>
<td>162.7</td>
<td>20.46</td>
<td>14.15</td>
<td>3.44</td>
<td>11.71</td>
<td>0.52</td>
<td>6.49</td>
</tr>
<tr>
<td>Sample size</td>
<td>15</td>
<td>15</td>
<td>14</td>
<td>14</td>
<td>14</td>
<td>15</td>
<td>15</td>
<td>12</td>
<td>15</td>
<td>12</td>
<td>15</td>
</tr>
</tbody>
</table>

**D. Discussion and conclusions**

Fluorescence imaging of the optically trapped microspheres has two important
advantages over transillumination imaging. The first is the ability to perform accurate
measurements of the mechanical behavior of a PM-cytoskeleton composite and PM tether
formation process when the microsphere is close to the cell. Qian et al. (2004) showed
that a large artifact (> 70 pN) is introduced with transillumination when forces are
measured within several microns of the cell. This artifact is completely eliminated by
using spectral separation of the position sensing and visualization signals (Qian et al.,
2004). The only imaging concern in case of the fluorescent microspheres is associated
with parasitic signals from other fluorescent microspheres, floating nearby an optical
trap. In our setup the parasitic signals were reduced to a 1 pN force level when a
microsphere was in the focal plane of the microscope objective and 17 μm off the optical
axis, or on the optical axis and 14 μm off the focal plane of the objective (see Appendix
VIII. The second advantage of the fluorescent microspheres is related to the very high and stable contrast of fluorescent microsphere image, which allows a simple intensity correction algorithm to be implemented for sufficient reduction of the error associated with imaging conditions (Chapter II, Section B3). Figure 22 shows measured relationship between the fluorescence intensity corrected differential QPD voltage signal and transverse displacement of a fluorescent microsphere. High signal linearity in the working range of the transverse microsphere displacement can be observed (see also Figure 22. Fluorescence intensity corrected differential QPD voltage signal as a function of the transverse displacement of the fluorescent microsphere.

Appendix IX).

The temporal tethering force profiles obtained using optically-trapped fluorescent microspheres are similar to those measured in transillumination mode (Li et al., 2002; Murdock et al., 2004), with gradual force increase up to the maximum value, defined as the tether formation force, followed by the sudden drop to the steady-state tethering force, and tethering force relaxation.

We were able to record force-displacement profiles before the PM separation in the form of a tether. Similar profiles have been recently measured on giant vesicles using
optical tweezers and DIC microscopy (Koster et al., 2005) and from leukocytes using a biomembrane force probe (Evans et al., 2005; Heinrich et al., 2005). Prior to $F_{\text{max}}$, the profiles are similar to those of a solid material, which expresses linear behavior up to a certain strain, followed by a nonlinear plastic regime, with increasing deformation rate. The linear deformations of the PM have been observed using micropipette aspiration (Sit et al., 1997; Oghalai et al., 1998; Hochmuth, 2000) and PM indentation with AFM (Hoh and Schoenenberger, 1994; van der Mei et al., 2000; Sato et al., 2000). If the applied pressure in the micropipette aspiration experiments was increased beyond a certain critical value, PM became separated from the cell cortex (Sit et al., 1997; Merkel et al., 2000).

Tether formation force ($F_{\text{max}}$) is the force required to separate a part of lipid bilayer bounded by the microsphere-PM contact (patch) area from the deformed cell body. The PM-cell body separation process involves spontaneous local PM reshaping from the tent-like surface, sustained in the PM-cytoskeleton composite structure by the cytoskeletal elastic properties, into a cylindrical tether with diameter defined by the externally-applied stress and bending properties of a lipid bilayer (Waugh and Hochmuth, 1987). The mean and standard deviation for $F_{\text{max}}$ measured in this work for HEK cells were $246.3 \pm 87.6$ pN. The mean is much larger than the $F_{\text{max}}$ measured by D.R. Murdock in his preliminary tether pulling experiments on giant unilamellar lipid vesicles (10-40 pN) and data obtained by Koster et al. (2005). The difference is consistent with the energy of interactions between cytoskeletal network and PM dominating the PM bending energy (Hochmuth et al., 1996; Hwang and Waugh, 1997; Hochmuth and Marcus, 2002).
The stiffness parameter ($k_{PM}$) gives information about the compliance of PM-cytoskeleton composite structure (Sit et al., 1997). The value of $k_{PM}$ measured in our work (311.4 pN/μm) is close to the ones measured using micropipette aspiration technique on macrophages (303 pN/μm) and endothelial cells (377 pN/μm) (Mege et al., 1987; Sato et al., 1987), suggesting similar PM-cytoskeleton compliance among these cell types.

The work required to form a tether ($W_{teth}$) includes linear ($W_l$) and plastic ($W_{pl}$) components. Since $W_{pl} \approx 2.5 W_l$ (Table 2), nonlinear effects must play a major role during tether formation. They may result from PM/cytoskeleton plastic deformations. PM separation may also contribute to $W_{pl}$. Nonlinear events as manifested by multiple peaks in the profile were observed in ~10% of the tethers (see Appendix X). The multiple peak profiles were not included in our analysis.

Assuming thermodynamically reversible tether formation process, Hwang and Waugh (1997) and Hochmuth and Marcus (2002) estimated the adhesion energy per unit area between PM and cytoskeleton to be 0.50-1.05*10^{-4} J/m² for red blood cells and 1.3*10^{-4} J/m² for neutrophils. In another micropipette study, the adhesion energy per unit area (0.6-11*10^{-3} J/m²) was estimated for Dictyostelium discoideum cell wall (Merkel et al., 2000). In our experiments the adhesion energy per unit area was calculated ≥1.5*10^{-3} J/m² (see Appendix XI). This value is an upper estimate, and lies within the range calculated by Merkel et al. (2000), although in our case the contribution from the plastic deformations of cytoskeletal and PM proteins needs to be investigated separately. The important advantage of our approach for the studies of PM-cytoskeleton interactions is the ability to directly measure the work spent at any time prior to the tether formation.
using the force-PM displacement profiles, which eliminates a necessity for specific theoretical assumptions, and allows more thorough analysis of the tether formation process.

The measured steady-state tethering force and force-relaxation parameters have been also estimated for HEK cells using transillumination technique (Murdock et al., 2004). Both $F_{ss}$ and $F_{eq}$ measured in this study are higher than those reported by Murdock et al (2004). Using different microsphere sizes (4 μm vs. 4.5 μm) or microsphere surface hydrophobicity could cause different patch areas, contributing to the observed deviations.

The unique possibility to investigate mechanical properties of an intact PM-cytoskeleton composite and PM tether formation process, in combination with accurate force measurements on long PM tethers, makes the described technique of optically-trapped fluorescent microspheres an attractive tool in studies of cellular mechanics. The spectral separation of cell imaging and position sensing of the trapped fluorescent microsphere can be also utilized in studies of receptor-ligand interactions or any other experiments which involve large imaging artifacts projected on the position-sensing detector.
CHAPTER IV. EFFECTS OF SALICYLATE ON MECHANICAL PROPERTIES OF OUTER HAIR AND HUMAN EMBRYONIC KIDNEY CELLS

Chapter Overview

This chapter describes the use of the optical tweezers technology developed in Chapters II and III to characterize mechanical properties of the outer hair (OHC) and human embryonic kidney (HEK) cell PMs with and without extracellular perfusion of 10 mM sodium salicylate (Sal), which is known to affect OHC electromotility, morphology of red blood cells, PM surface charge, and lateral lipid diffusivity. Mechanical characteristics of tether formation and tethering force relaxation were estimated for Sal-treated HEK cells using the tether pulling methodology described in Chapter III. Sal-induced reduction in tether formation force and PM stiffness parameter was explained from the increased PM/cytoskeleton compliance. Sal-induced reduction in equilibrium tethering force was explained by reduced effective PM tension. Effective tether viscosity (ηeff), steady-state tethering force extrapolated to zero pulling rate (Fss(0)), and time constant for tether growth (τg) were estimated from the measurements of the instantaneous tethering force at different tether pulling rates. The measured values of Fss(0) for OHC lateral wall were statistically different from Fss(0) for OHC basal end and HEK cells, which was explained by the large number of lateral wall associated PM proteins, increasing effective PM tension, and additional voltage-dependent component of PM stiffness present in OHC lateral wall. Fss(0) was also statistically different for OHC basal end and HEK cells, presumably attributed to large amounts of cholesterol previously found in OHC basal end PM. The force- and viscosity-related mechanical
parameters ($F_{max}$, $F_{ss}$, and $\eta_{eff}$) measured in our studies were an order of magnitude bigger than those measured on lipid vesicles, which provides evidence for important mechanical contribution of PM-cytoskeleton interactions and dominating effects of PM-associated proteins on effective PM tension. Average values of $\eta_{eff}$, $F_{ss0}$, and $\tau_8$ for the OHC lateral wall plasma membrane and control cell plasma membrane remained the same after Sal perfusion, which is consistent with the hypothesis that Sal induced reversible hearing loss appears to be more the result of its competition with essential anions and less the result of a change in plasma membrane mechanics.
A. Introduction

1. Outer hair cells

Cochlear outer hair cells (OHCs) (Figure 23) are specialized sensory/motor cells capable of producing electrically evoked length changes (Brownell 1984; Brownell et al. 1985). The force responsible for this electromotility provides additional mechanical energy to the vibrating structures of the inner ear, and functions to narrow the bandpass filtering at a given location along the length of the cochlear partition (Brownell et al. 1985-2001; Ashmore 1987; Dallos and Corey 1991). The necessity for active filtration of the acoustic waves in the inner ear comes from the fact that mammalian hearing organ is filled with fluid, which leads to viscous damping and distortion of auditory signals, especially at high frequencies. Unique active tuning, performed by the OHCs, corrects for those undesirable effects.

Figure 23. An outer hair cell, isolated from a guinea pig organ of Corti (modified from Brownell et al, 1985). (A) stereocilia (B) cuticular plate (C) lateral wall (D) nucleus (E) basal end.
The maximum OHC length change is approximately 4%, and is related to change in transmembrane potential rather than current. PM hyperpolarization causes OHC to increase in length while depolarization causes it to shorten with the frequencies up to 50 kHz and above, which suggests that the cell movement is indeed rapid enough to participate in audition at high frequencies (Frank et al, 1999). Short time constants (about 10 μs) of the electromechanical response as well as the voltage (but not current) dependence of the phenomenon argue in favor of piezoelectric-like mechanism of electromotility (Brownell et al, 2001). Electromotility was shown to be independent of calcium (Brownell et al, 1985) and intracellular ATP (Kachar et al, 1986). Electromotile response weakens when the cell’s turgor pressure is decreased (Santos-Sacchi, 1991).

OHC (Figure 23) has a cylindrical shape with uniform diameter of about 9 μm, eccentrically located nucleus and stereociliar bundle at the opposite end of the cell. OHCs significantly vary in length, from 15 μm to more than 90 μm, depending on their location in cochlea. Low water permeability of an OHC makes it a cellular hydrostat with the cytoplasm turgor of about 1-2 kPa (Ratmanather et al, 1993). The hydraulic support facilitates rapid shape change, with high compressive resistance at the same time (Brownell et al, 1985; Brownell, 1990; Chertoff and Brownell, 1994).

Three regions of OHC – flat apex, hemispheric base, and lateral wall (Figure 17) – perform various functions (Brownell et al., 2001). Bending of stereocilia (thin cellular protrusions) at the apex of OHC, convert acoustic energy into electrical currents. Synaptic structures at the base of the cell use electrical energy to modulate the release of neurotransmitters activating the 8th nerve fibers contacting the cell. Studies with
microbead displacements (Kalinec and Kachar, 1995) revealed that the electromotile force generation mechanism resides in the lateral wall of the cell.

The OHC lateral wall (Figure 24) is a 100-nm thick tri-laminate structure consisting of the PM, cytoskeletal cortical lattice (CL) and subsurface cisterna (SSC) (Brownell and Popel, 1998). Electron microscopy revealed that OHC PM has nanoscale ripples (Dieler et al. 1991; Holley 1996), which may provide the necessary reservoir for cell length changes. The CL is organized into microdomains composed of parallel 6-7 nm thick F-actin filaments that on average run circumferentially. The actin filaments are cross-linked with 3-4 nm thick and 40-50 nm long spectrin molecules (Forge, 1991; Holley et al. 1992; Brownell et al. 2001). The SSC is composed of concentric layers of flattened membranes that form the innermost layer of the lateral wall. It resembles both Golgi apparatus and endoplasmic reticulum (Pollice and Brownell, 1993).
The transmembrane OHC protein, prestin, has been shown to endow rudimentary electromotility in transfected cells (Zheng et al. 2000). The manner by which prestin enhances the piezoelectric-like membrane behavior (Ludwig et al. 2001; Dong et al. 2002; Qian et al. 2004; Brownell 2005) is not clear; however, it must interact with the membrane in which it resides in order to function. The PM interacts with an underlying CL through radially oriented pillars (Arima et al., 1991), which are thought to transfer the forces generated in the PM to the CL. The pillars have an unknown molecular composition.

Prestin-transfected human embryonic kidney (HEK) cells have been used in functional expression studies of electromotility (Zheng et al. 2000; Ludwig et al. 2001). HEK cells have a different morphology than the cylindrically shaped OHCs, lack the orthotropically organized CL of OHCs, and do not have pillars linking the PM to the cytoskeleton.

2. Effects of salicylate on outer hair cell physiology and plasma membrane mechanics

Models of membrane-based electromotility (Raphael et al. 2000; Brownell et al. 2001; Petrov and Sachs 2002) depend on the mechanical properties of the PM. Therefore, chemical agents, which can alter mechanical characteristics of PM, may also affect electromotility.

Amphipaths are agents that are both water and lipid soluble. Salicylate (Sal), the anionic amphipathic metabolite of aspirin, has long been known to increase the local outward bending of the red blood cell PM (Sheetz and Singer 1976). Other early experiments suggested that it introduces an extra negative surface potential (McLaughlin
There is also a Sal induced reduction in membrane capacitance (Tunstall et al., 1995) and lipid lateral diffusion (Oghalai et al. 2000) in the OHC lateral wall PM. Recent studies demonstrated that Sal reduces electrically evoked force generation in PM tethers from wild type HEK cells (Anvari et al. 2005), and reduces bending stiffness of pure lipid vesicles (Zhou and Raphael, 2005).

In pharmacological doses aspirin results in reversible full frequency range hearing loss of up to 40 dB (Stypulkowski, 1990) and diminishes otoacoustic emissions (Long and Tubis, 1988). Sal suppresses electromotility of OHCs (Dieler et al. 1991; Shehata et al. 1991; Kakehata and Santos-Sacchi 1996) and prestin-transfected HEK cells (Zheng et al. 2000). Sal may also compete with intracellular Cl\textsuperscript{−} ions, which have been found essential for OHC electromotility (Oliver et al. 2001).

There are two studies that report an effect of Sal on electromotility but not on cell mechanics: 1) Hallworth (1997) demonstrated a reduction in electrically-evoked force production without an effect on OHC axial stiffness; 2) Zhang et al. (2001) used atomic force microscopy on wild type HEK cells and found that Sal produced no change in membrane indentation profiles (force-displacement functions in the absence of electrical stimulation) but did reduce electromotility.

B. Methodology

1. Experimental setup and studied cells

The optical tweezers setup (Figure 17), trapping force calibration, preparation of HEK cells, and coating of the sample chamber have been described in details in Chapter III, parts B1 and B2. Pigmented guinea pigs of either sex weighing 200-250 g were
decapitated following an institutionally approved protocol at the Baylor College of Medicine (protocol # AN1105). Both cochlea were dissected from temporal bones (See Appendix XII), and placed into the NES or Sal (See Appendix VI) solution. The particular concentration of Sal (10 mM) was used since in OHCs it induces maximum reduction of the peak nonlinear capacitance, associated with electromotility (Kakehata and Santos-Sacchi 1996). A medium size OHC (population distribution: 55-85 μm long and 8-10 μm in diameter) firmly adherent to the bottom of the sample chamber was selected for measurements if it exhibited a cylindrical shape with a basally located nucleus, with limited osmotic swelling and no cytoplasmic particles exhibiting Brownian motion. All OHCs were used within 4 hours of the animal sacrifice.

We found that OHCs deteriorated quickly after being dispersed in the sample chamber coated with antimycotic. Therefore, in experiments using multiple tether pulling rates, which involved OHCs, we coated fluorescent microspheres with bovine serum albumin (BSA) by incubating 50 μl of fluorescent microspheres with 1 ml of 10 mg/ml aqueous BSA (Fisher Scientific, Hampton, NH) solution overnight under gentle mixing. The success rate of a microsphere detachment from the coverslip of a sample chamber using optical tweezers increased from 0% to more than 10% after microspheres were coated with BSA.

2. Experimental procedure

During experiments, an OHC or HEK cell, firmly attached to the coverslip, was identified by microscopic scanning through the sample chamber. A physical contact between trapped fluorescent microsphere and the studied cell was initiated as described in
Chapter III, Part B3, followed by the controlled PZT movement. Only Sal-treated HEK cells were used in tethering force relaxation experiments performed according to the protocol described in Chapter III, Part B3, while both HEK cells and OHCs perfused with NES or Sal were used in experiments with multiple tether pulling rates. In those experiments the cell was moved away from the trapped microsphere at 1 μm/s speed for 10 seconds. During this time a PM tether was usually formed. Speed of the PZT was subsequently increased every two seconds by 1 μm/s until it reached 6 μm/s. In case of OHCs, PM tethers were pulled from either lateral wall or basal end in one of the orthogonal directions, allowed by PZT, which had a minimal angle (≤45°) with the normal to the PM patch site.

3. Data analysis

Tether formation segment and tethering force relaxation were analyzed as described in Chapter III, Parts 4 and 5. We analyzed temporal tethering force profiles recorded with multiple tether pulling rates (Figure 25a) to obtain additional parameters related to the viscoelastic behavior of the PM tether. Last 100 values of the tethering force signal recorded for 1 μm/s pulling rate were averaged to estimate the steady-state tethering force at 1 μm/s pulling rate. Each individual part of a tethering force signal recorded for the \(i^{th}\) pulling segment at a specific pulling rate, starting with 2 μm/s, was fit with an exponential function (Figure 25b) with unknown change in steady-state tethering force (\(ΔF_{ss(i)}\)), and the time constant for tether growth (\(τ_{tg(i)}\)):

\[
F_{teth(i)}(t) = F_{teth(i-1)end} + ΔF_{ss(i)} \cdot (1 - \exp(-\frac{t - t(i-1)end}{τ_{tg(i)}})) \quad i = 2, 3, \ldots, 6
\]  

(4.1)
where \( t \) is the time elapsed from the beginning of pulling; \( F_{\text{teh}(i)}(t) \) is the tethering force measured at time \( t \) for the \( i^{th} \) pulling segment; \( F_{\text{teh}(i-1)\text{end}} \) is the last value of the tethering force measured for the \((i-1)^{th}\) pulling segment; and \( t_{(i-1)\text{end}} \) is the time corresponding to the last value of the tethering force measured for the \((i-1)^{th}\) pulling segment. The exponential increase of the tethering force after sudden increase in the tether pulling rate was first reported by Li et al., 2002.

Using the obtained values of \( \Delta F_{\text{ss}(i)} \), we calculated the steady-state tethering forces as:

\[
F_{\text{ss}(i)} = F_{\text{teh}(i-1)\text{end}} + \Delta F_{\text{ss}(i)}
\]

where \( F_{\text{ss}(i)} \) is the asymptotic value of the steady-state tethering force calculated for the \( i^{th} \) pulling segment \((i = 2, 3, \ldots, 6)\).
The calculated data for the steady-state tethering forces at each tether pulling rate ($V_{\text{pull}}$) were fit (Figure 26) with a linear function (Hochmuth et al. 1996) to estimate the unknown steady-state tethering force extrapolated to the zero pulling rate $F_{ss(0)}$ and effective tether viscosity ($\eta_{\text{eff}}$):

$$F_{ss}(V_{\text{pull}}) = F_{ss(0)} + 2\pi \eta_{\text{eff}} V_{\text{pull}}$$  \hspace{1cm} (4.3)

Figure 26. Plot of steady-state tethering forces vs tether pulling rates. $F_{ss(0)}$ and $\eta_{\text{eff}}$ were calculated from a linear fit (Eq. 4.3) to the data points: $r^2 = 0.9835$, $F_{ss(0)} = 87.4$ pN, $\eta_{\text{eff}} = 2.55$ pN/(µm/s). Steady-state forces were calculated from data shown in Fig. 25a, using Eq. 4.1 and 4.2.

Values of $F_{ss(0)}$, $\eta_{\text{eff}}$, and $\tau_{\text{tg}}$ averaged over the all pulling segments as well as the other PM mechanical characteristics described in Chapter III were used in our studies to compare the viscoelastic properties of the PM for different cells and experimental conditions. Statistical analysis of the differences in the mean values of calculated parameters was performed on OHCs and HEK cells, and on cells of the same type perfused with NES and Sal solution. We used unpaired t-test, assuming normal populations with unknown variances, and statistical significance was accepted if the $p$-value did not exceed 0.05.
C. Results

1. Mechanical characteristics measured using multiple rate tether pulling

Our preliminary experiments done on cells treated with NES or 10 mM NaSal (salicylate was simply added to NES causing 320 mOsm of the solution osmolarity) using multiple rate tether pulling and transillumination position sensing (See Appendix XIII) showed the following results: 1) The differences in the mean values of $F_{ss(0)}$ for the cells perfused with NES were statistically significant for OHC lateral wall and basal end ($p < 0.01$), OHC lateral wall and HEK cells ($p < 0.01$), and OHC basal end and HEK cells ($p = 0.02$). No statistically significant difference was found in $F_{ss(0)}$ for cells perfused with NES and 10 mM NaSal. 2) No statistically significant differences in the mean values of $\eta_{eff}$ were found when analyzed for OHCs and HEK cells, and for cells perfused with NES and 10 mM NaSal. 3) The mean values of $\tau_{ig}$ for the cells perfused with NES were statistically different for OHC lateral wall and basal end ($p < 0.01$), and OHC basal end and HEK cells ($p < 0.01$). A statistically significant difference ($p < 0.03$) was also found for the mean values of $\tau_{ig}$ in OHC basal end for cells perfused with NES and 10 mM NaSal.

Using the same multiple rate tether pulling methodology with optically-trapped fluorescent microspheres, and osmolarity of Sal solution adjusted to 300 mOsm resulted in the mean values of the estimated parameters within the error of those measured using transillumination technique, and yielded the same statistical significances.

Average steady-state tethering forces extrapolated to zero pulling rate (±SD) for OHC lateral wall, OHC basal end, and HEK cells, perfused with NES, were 103.4 ± 28.4
\( (N = 17), 81.5 \pm 22.9 \ (N = 11), \) and \( 43.1 \pm 13.6 \ (N = 15) \) pN, respectively (Figure 27). The presence of NaSal resulted in \( F_{ss(0)} \) mean values of \( 99.2 \pm 25.6 \ (N = 13), \) \( 79.7 \pm 29 \ (N = 14), \) and \( 42.3 \pm 9.9 \ (N = 7) \) pN for OHC lateral wall, OHC basal end, and HEK cells, respectively (Figure 27). The differences between the mean values of \( F_{ss(0)} \) for the cells perfused with NES were statistically different for OHC lateral wall and basal end \( (p = 0.034), \) OHC lateral wall and HEK cells \( (p < 0.001), \) and OHC basal end and HEK cells \( (p < 0.001). \) No statistically significant differences in the mean values of \( F_{ss(0)} \) were found between the cells perfused with NES and 10 mM NaSal (Figure 27).

![Figure 27. Steady-state tethering force extrapolated to zero tether pulling rate, \( F_{ss(0)} \) ± SD (pN). □ - cells perfused with normal extracellular solution: OHC lateral wall 103.4 ± 28.4 \( (N = 17), \) OHC basal end 81.5 ± 22.9 \( (N = 11), \) HEK cells 43.1 ± 13.6 \( (N = 15); \) ■ - cells perfused with 10 mM NaSal: OHC lateral wall 99.2 ± 25.6 \( (N = 13), \) OHC basal end 79.7 ± 29 \( (N = 14), \) HEK cells 42.3 ± 9.9 \( (N = 7). \) The mean values of \( F_{ss(0)} \) for the basal end and HEK cell were both statistically different than that for the lateral wall; the HEK cell value was also statistically different than the basal end \( (p < 0.05 \) for all comparisons) The differences between the normal and Sal treated membranes were not significant.

Average effective tether viscosities \((±SD)\) for OHC lateral wall, OHC basal end, and HEK cells, perfused with NES, were \( 1.70 ± 0.58 \ (N = 17), \) \( 1.71 ± 0.64 \ (N = 11), \) and \( 1.39 ± 0.56 \ (N = 15) \) pN/(μm/s), respectively (Figure 28). The presence of NaSal resulted in \( \eta_{eff} \) mean values of \( 1.69 ± 0.62 \ (N = 13), \) \( 1.65 ± 0.78 \ (N = 14), \) and \( 1.31 ± 0.34 \ (N = 7) \)
pN/(μm/s) for OHC lateral wall, OHC basal end, and HEK cells, respectively (Figure 28). No statistically significant differences in the mean values of $\eta_{\text{eff}}$ were found between OHCs and HEK cells, and between cells perfused with NES and 10 mM NaSal (Figure 28).

![Bar graph](image)

Figure 28. Effective tether viscosity, $\eta_{\text{eff}} \pm $ SD (pN/(μm/s)). □ – cells perfused with normal extracellular solution: OHC lateral wall $1.70 \pm 0.58$ ($N = 17$), OHC basal end $1.71 \pm 0.64$ ($N = 11$), HEK cells $1.39 \pm 0.56$ ($N = 15$); ■ – cells perfused with 10 mM NaSal: OHC lateral wall $1.69 \pm 0.62$ ($N = 13$), OHC basal end $1.65 \pm 0.78$ ($N = 14$), HEK cells $1.31 \pm 0.34$ ($N = 7$). The differences between the normal and Sal treated membranes and between the different groups were not significant.

Average time constants for tether growth (±SD) for OHC lateral wall, OHC basal end, and HEK cells, perfused with NES, were $0.92 \pm 0.44$ ($N = 18$), $1.51 \pm 0.60$ ($N = 14$), and $1.07 \pm 0.52$ ($N = 15$) s, respectively (Figure 29). The presence of NaSal resulted in $\tau_g$ mean values of $1.08 \pm 0.82$ ($N = 15$), $0.94 \pm 0.46$ ($N = 16$) and $0.99 \pm 0.42$ ($N = 7$) s for OHC lateral wall, OHC basal end, and HEK cells, respectively (Figure 29). The mean values of $\tau_g$ for the cells perfused with NES were statistically different between OHC lateral wall and basal end ($p = 0.005$), and OHC basal end and HEK cells ($p = 0.046$). There was no significant difference for the OHC lateral wall PM and HEK cell PM as well as for the OHC lateral wall and HEK PM in the absence and presence of Sal.
A statistically significant difference \((p = 0.008)\) was found for the mean values of \(\tau_{tg}\) in OHC basal end between cells perfused with NES and 10 mM NaSal.

![Graph showing \(\tau_{tg}\) values for OHC lateral wall, OHC basal end, and HEK with error bars and sample sizes indicated.]

Figure 29. Average time constant for the tether growth, \(\tau_{tg} \pm SD\) (s). \(\square\) – cells perfused with normal extracellular solution: OHC lateral wall 0.92 ± 0.44 \((N = 18)\), OHC basal end 1.51 ± 0.60 \((N = 14)\), HEK cells 1.07 ± 0.52 \((N = 15)\); ■ – cells perfused with 10 mM NaSal: OHC lateral wall 1.08 ± 0.82 \((N = 15)\), OHC basal end 0.94 ± 0.46 \((N = 16)\), HEK cells 0.99 ± 0.42 \((N = 7)\). The mean value of \(\tau_{tg}\) for the basal end was significantly larger than for the other non-Sal treated cell groups. There was no significant difference between the Sal treated groups. The difference between the normal and Sal treated membranes within groups was not significant except for the basal end, which is indicated with a star (*) with \(p < 0.05\).

2. Tether formation and tethering force relaxation of Sal-treated HEK cells

Temporal tethering force profiles and force-PM displacement profiles measured on Sal perfused HEK cells were similar to those measured on NES perfused HEK cells (See Figures 18 and 21). Material parameters calculated for Sal perfused HEK cells are presented in Table 3. The differences in the mean values for the cells perfused with NES and 10 mM Sal were statistically significant for \(F_{\text{max}}\) \((p = 0.001)\), \(k_{PM}\) \((p = 0.017)\), and \(F_{eq}\) \((p = 0.0025)\). Two out of 22 investigated tethers were formed without a distinctive peak \(F_{\text{max}}\) in the tethering force, which might be due to the dominance of plastic PM/cytoskeleton deformations in those measurements. The results from those cells were not used in calculations of the tether formation parameters. Eight tethers did not exhibit
fast exponential tethering force relaxation, and two cells did not exhibit slow exponential tethering force relaxation.

Table 3 Material parameters of the HEK cell plasma membrane perfused with 10 mM sodium salicylate (parameters, significantly different from those measured in NES treated cells (p < 0.05) are shaded)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>$F_{\text{max}}$ (pN)</th>
<th>$k_{PM}$ (pN/µm)</th>
<th>$W_{\text{eth}}$ ($\times 10^{-18}$ J)</th>
<th>$W_{i}$ ($\times 10^{-18}$ J)</th>
<th>$W_{\text{nl}}$ ($\times 10^{-18}$ J)</th>
<th>$F_{ss}$ (pN)</th>
<th>$F_{eq}$ (pN)</th>
<th>$F_{\text{short}}$ (pN)</th>
<th>$F_{\text{long}}$ (pN)</th>
<th>$\tau_{\text{short}}$ (s)</th>
<th>$\tau_{\text{long}}$ (s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dimension</td>
<td>150.7</td>
<td>184.8</td>
<td>137.8</td>
<td>55.4</td>
<td>82.4</td>
<td>60.06</td>
<td>29.94</td>
<td>12.01</td>
<td>22.16</td>
<td>1.49</td>
<td>18.1</td>
</tr>
<tr>
<td>Average value</td>
<td>150.7</td>
<td>184.8</td>
<td>137.8</td>
<td>55.4</td>
<td>82.4</td>
<td>60.06</td>
<td>29.94</td>
<td>12.01</td>
<td>22.16</td>
<td>1.49</td>
<td>18.1</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>69.4</td>
<td>93.8</td>
<td>120.5</td>
<td>66.1</td>
<td>118.8</td>
<td>29.34</td>
<td>17.68</td>
<td>6.7</td>
<td>9.7</td>
<td>0.56</td>
<td>12.67</td>
</tr>
<tr>
<td>Sample size</td>
<td>20</td>
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<td>17</td>
<td>17</td>
<td>17</td>
<td>22</td>
<td>22</td>
<td>14</td>
<td>20</td>
<td>14</td>
<td>20</td>
</tr>
</tbody>
</table>

D. Discussion and conclusions

The PM tether is a structure held at equilibrium between photonic forces acting on the trapped bead and those forces resulting from the effective cell membrane tension. The effective cell membrane tension refers to the mechanical tension generated in the PM by the combination of osmotic conditions, PM-cytoskeleton interaction at discrete PM confinement sites (Fournier et al. 2004; Gov et al. 2003, 2004), local and nonlocal PM curvature elasticity (Hochmuth et al. 1996; Waugh et al. 1992).

The parameter $F_{ss(0)}$ is an approximation of the equilibrium tethering force extrapolated from the steady state tethering forces measured at known tether pulling rates. Our values of $F_{ss(0)}$ for outer hair cells are in the range reported by Li et al. (2002) for static tether forces, which were measured with optically trapped microspheres using a different protocol.

OHCS are cellular hydrostats (Brownell 2005) making them sensitive to changes in the osmolarity of the bathing media. We were careful to maintain the NES osmolarity
at the same value and any change in the bathing osmolarity during the experiment would be immediately obvious by changes in cell length. Therefore the major contribution to the differences in $F_{ss(0)}$ among OHCs and HEK cells comes from cell-specific internal osmotic conditions, PM-cytoskeleton interactions, and/or bending properties of the PM.

Cholesterol may increase PM bending resistance, and consequently the $F_{ss(0)}$ value, by increasing the lipid bilayer rigidity. There is evidence suggesting membrane cholesterol is at a higher concentration in the OHC basal end than in the OHC lateral wall (Nguyen and Brownell 1998). The 68% greater $F_{ss(0)}$ for the OHC basal end when compared to HEK cells might be explained by a greater cholesterol concentration in the OHC basal membrane than in the HEK PM. However the $F_{ss(0)}$ for the lateral wall PM is 56% greater than the $F_{ss(0)}$ for basal PM. Therefore, factors other than cholesterol concentration must contribute to the $F_{ss(0)}$ in the lateral wall PM. These may include large amounts of PM-associated proteins not found in the OHC basal end (Forge, 1991), including prestin, glucose transporters, as well as membrane interaction with the pillars. Significant influence of PM-associated proteins on $F_{ss(0)}$ is also supported by the fact that steady-state tethering forces at low (0.5 μm/s) pulling rates measured on pure lipid vesicles (Koster et al., 2005) are an order of magnitude smaller than $F_{ss(0)}$ measured on OHCs or HEK cells. Additional voltage-dependent component of OHC lateral wall PM stiffness (He et al., 2003) corresponding to the OHC transmembrane potential established during our experiments (we did not control or measure it) can also contribute to high values of $F_{ss(0)}$ measured from OHC lateral wall PMs.

The non-zero value of $\eta_{eff}$ implies the existence of viscous-like dissipation during tether pulling also revealed during tethering force relaxation (Figure 19). In our studies
we found $\eta_{\text{eff}}$ to be on the order of 2.2-3.2 pN/(\mu m/s) with no statistically significant differences among the OHC lateral wall, OHC basal end, and HEK cells. The values are consistent with those previously measured on the OHC lateral wall (Li et al. 2002) and HEK cells (Murdock et al. 2003), using similar techniques, and two orders of magnitude greater than the reported $\eta_{\text{eff}}$ for pure phospholipid vesicles (Waugh 1982; Evans and Yeung 1994), which supports the hypothesis (Hochmuth et al. 1996; Li et al. 2002) that in cells $\eta_{\text{eff}}$ is dominated by interactions between the PM and underlying cytoskeleton.

The parameter $\tau_{\text{tg}}$ provides information on the rate of change in tethering force with a sudden increase in the pulling rate. The bigger $\tau_{\text{tg}}$, the slower tethering force will be rising. It is related to the viscoelastic properties of the PM, mass of the trapped microsphere, viscosity of the extracellular solution, and transverse stiffness of the optical trap. Smaller effective cell membrane tension or larger $\eta_{\text{eff}}$ increase $\tau_{\text{tg}}$, similar to the way it happens in a model composed of a dashpot and a spring connected in parallel. Therefore, both smaller value of $F_{\text{ss}(0)}$ (directly related to the effective PM tension) and larger value of $\eta_{\text{eff}}$ (representing viscous dissipation processes) for OHC basal end compared to the lateral wall would contribute to the observed increased value of $\tau_{\text{tg}}$ for OHC basal end. On the other hand, the smaller value of $\eta_{\text{eff}}$ for HEK cells in comparison to the OHCs could counteract the increase of $\tau_{\text{tg}}$ anticipated because of the smaller values of $F_{\text{ss}(0)}$.

The 29% reduction in $F_{\text{eq}}$ and 49% reduction in $k_{\text{PM}}$ for Sal treated cells imply the reduced effective PM tension and enhanced compliance of the PM/cytoskeleton composite, consistent with Sal-induced morphological changes of OHCs observed by Morimoto et al. (2002), and decreased bending stiffness and apparent area
compressibility modulus of pure lipid membranes (Zhou and Raphael, 2005). Apparent area compressibility modulus is derived from the linear slope of the high-tension domain on the tension-fractional area change diagram measured using micropipette aspiration technique (Zhou and Raphael, 2005), similar to our force-PM displacement diagram, and, therefore, is directly related to $k_{\text{PM}}$. The bending stiffness of a lipid bilayer contributes to the effective PM tension (Gov et al., 2003; Gov and Safran, 2004), and, therefore, positively correlates with $F_{\text{eq}}$.

Different statistical inferences obtained for $F_{\text{ss}(0)}$ and $F_{\text{eq}}$ on salicylate treated HEK cells can be understood by recalling the procedure of the measurements of $F_{\text{ss}(0)}$ and $F_{\text{eq}}$, with the former parameter only approximately estimating the equilibrium tethering force from the linear regression of the steady-state data. In addition to the relaxed equilibrium tethering force, $F_{\text{ss}(0)}$ incorporates processes happening during tether pulling, which may dominate over the difference in effective PM tension. Another study (Zhang et al., 2001) showed no change in the force-indentation depth curves measured with AFM in cells perfused with Sal. However, two distinctive mechanical regimes are involved in AFM and tether pulling studies. The former uses compression of the PM/cytoskeleton composite, and is strongly influenced by cell turgor and viscoelasticity of the cytoplasm, which may dominate actual PM/cytoskeleton compliance. From the other hand, tether pulling utilizes extension of PM/cytoskeleton composite, and may be less affected by the mechanics of intracellular fluid.

A Sal-induced 39% reduction in tether formation force for HEK cells may be a consequence of increased PM/cytoskeleton compliance. While the PM/cytoskeleton adhesion energy measured with mechanical loads applied normally to PM (during
micropipette aspiration, the aspiration pressure is equal to the normal stress of the PM at the extracellular interface) may remain the same for NES and Sal treated cells (Morimoto et al., 2002), the large PM/cytoskeleton compliance leads to significant tent-like PM deformations of the patch site during initial stages of tether pulling. Due to axial orientation of the mechanical load applied to PM during tether pulling, the tent-like portion of the PM experiences significant shear forces over the PM-cytoskeleton interface, which may greatly facilitate tether formation process.

The data show that addition of Sal does not cause significant changes in the calculated mechanical parameters of OHC lateral wall PM. Using micropipette aspiration technique Morimoto et al. (2002) showed no effect of Sal on PM vesiculation pressure, a parameter related to the strength of PM-cytoskeleton interaction. Our experiments showed that Sal does not affect $n_{\text{eff}}$ of OHCs and HEK cells, which is consistent with findings by Morimoto et al. (2002). Our results are compatible with those showing no effect of Sal on OHC lateral wall active and passive stiffness components (Batta et al., 2003), and axial compliance (Hallworth 1997).

The absence of a change in OHC membrane mechanics supports the concept that Sal influences OHC electromotility through interaction with surface charge of the membrane. There is evidence that Sal anions compete with intracellular Cl$, decreasing the availability of Cl$ required for electromotility (Oliver et al. 2001). Salicylate’s phenol group is thought to partition into the hydrophobic phase of a plasma membrane while its hydroxyl group produces an additional negative surface charge by partitioning into the phospholipids headgroup region (McLaughlin 1973, Song and Baker 2005). McLaughlin (1973) has argued that the salicylate-induced change in surface charge
contributes to observed decreased anion permeability and increased cation permeability. A decreased membrane permeability for the Cl anions is of particular importance for OHCs, since at a constant holding electrical potential it would lead to depletion of the intracellular chloride pool, with subsequent reduction of electromotility. These observations all suggest that Sal reduces OHC electromotility via an electrochemical interaction involving chloride.
CHAPTER V. EXPERIMENTAL CHARACTERIZATION OF PLASMA MEMBRANE TETHERS BY OSMOTIC CHALLENGE, SUPPRESSED TRANSMEMBRANE WATER TRANSPORT, AND REPETITIVE TETHER ELONGATION

Chapter Overview

In this chapter we used the technique of optically-trapped fluorescent microspheres, described in the Chapter III to further investigate mechanical properties of HEK PM tethers. Cell perfusion with hypo- and hyperosmotic bathing solution did not influence equilibrium tethering force, which was attributed to cellular regulation of the effective PM tension by the cytoskeletal confinement. Additionally, a statistically significant reduction in the magnitude of the fast tethering force relaxation process was observed and referred to the stress-minimizing redistribution of PM tether lipid molecules. Extracellular application of 1 mM mercury (II) chloride increased steady-state and equilibrium tethering forces, and suppressed bi-exponential tethering force relaxation process, which was attributed to the increased effective PM tension and reduced transmembrane water transport. The temporal tethering force profiles obtained from the same PM tethers elongated in several repetitive cycles exhibited different behavior explained by the essential irreversibility of PM tether elongation process. Particularly, generally observed trend of statistically significant reduction in the measured forces and time constants after successive tether elongations was an indicator of plastic PM deformations associated with irreversible PM dissociation from the cell body.
A. Introduction

The mechanical PM characteristics described in Chapter III may be affected by different extracellular chemical or physical environment. Osmolarity influences boundary conditions for the normal stress components at the PM/cytosol or PM/extracellular fluid interface, leading to the change of the effective membrane tension. Lower values of extracellular osmolarity decreased the PM diffusion coefficient of the OHC lateral wall PM (Oghalai et al., 2000) and induced OHC swelling (Ratnanather et al., 1996).

Mercury (II) chloride is a cytotoxic chemical agent, which nonspecifically blocks transmembrane water transport (Belyantseva et al., 2000; Verkman, 2000). Application of 1 mM HgCl$_2$ solution was shown to inhibit osmotically induced swelling of OHCs by about 78% (Belyantseva et al., 2000).

Currently many theoretical analyses of PM tether mechanical behavior are based on equilibrium thermodynamics (Bozic et al., 1992; Hochmuth et al., 1996; Heinrich et al., 1999; Hochmuth and Marcus, 2002), considering tether pulling as a reversible process. However, it is intuitive that at least the processes of PM dissociation from the cell body and possible plastic deformations of the cytoskeleton during tether formation should be irreversible. Additionally, considering large deformations of PM involved in the tether elongation process there may be an irreversible component during tether elongation as well. Therefore, experiments were carried to reveal the contribution of irreversible processes to the mechanical behavior of PM tethers.

In this chapter we use optically-trapped fluorescent microspheres to obtain temporal tethering force profiles, force-PM displacement profiles, and estimate
mechanical parameters of HEK cells as they influenced by extracellularly perfused 250/350 mOsm solution or 1 mM HgCl₂, and by repetitive tether elongations.
B. Methodology

We used optical tweezers setup and methodology described in Chapter III, Part B to perform on each HEK cell a single PM tether pulling to a distance of 10 μm at 1 μm/s pulling rate with subsequent tethering force relaxation during a 60s pause. The cells were perfused with hypo- (250 mOsm), hyper- (350 mOsm) osmotic or 1 mM HgCl₂ extracellular solutions (see Appendix VI).

HEK cells perfused with NES were used in studies of tether elongation reversibility. In those experiments following the tethering force relaxation PZT moved backwards with the speed of 1 μm/s for 4 seconds. The elongation-relaxation-retraction cycle was repeated 4 times to assess the reversibility of tether elongation process. We analyzed the changes of steady-state tethering forces and force relaxation parameters after repetitive tether elongations. Each parameter at i-cycle (i = 2...4) was normalized to the one calculated for the first tether elongation cycle. We used t-test to compare 1 and the mean normalized value of each parameter for every tether elongation cycle. Statistical significance of the mean being different from 1 was accepted if the calculated p-value did not exceed 0.05.

C. Results and Discussion

1. Hypo- and hyperosmotically treated HEK cells

Temporal tethering force profiles and force-PM displacement profiles measured on HEK cells perfused with hypo- or hyperosmotic extracellular solution were similar to those measured on NES perfused HEK cells (See Figures 18 and 21). Material
parameters calculated for HEK cells perfused with 250 and 350 mOsm extracellular solutions are presented in Tables 4 and 5, respectively.

**Table 4** Material parameters of the HEK cell plasma membrane perfused with hypoosmotic (250 mOsm) extracellular solution
(parameters, significantly different from those measured in NES treated cells ($p < 0.05$) are shaded)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>$F_{\text{max}}$</th>
<th>$k_{PM}$</th>
<th>$W_{\text{leth}}$</th>
<th>$W_{i}$</th>
<th>$W_{\text{el}}$</th>
<th>$F_{\text{ss}}$</th>
<th>$F_{\text{eq}}$</th>
<th>$F_{\text{short}}$</th>
<th>$F_{\text{long}}$</th>
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<tbody>
<tr>
<td>Dimension</td>
<td>pN</td>
<td>pN/µm</td>
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<td>$\times 10^{-18}$ J</td>
<td>$\times 10^{-18}$ J</td>
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<td>pN</td>
<td>pN</td>
<td>pN</td>
<td>s</td>
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<tr>
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<td>11</td>
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**Table 5** Material parameters of the HEK cell plasma membrane perfused with hyperosmotic (350 mOsm) extracellular solution

<table>
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<tr>
<th>Parameter</th>
<th>$F_{\text{max}}$</th>
<th>$k_{PM}$</th>
<th>$W_{\text{leth}}$</th>
<th>$W_{i}$</th>
<th>$W_{\text{el}}$</th>
<th>$F_{\text{ss}}$</th>
<th>$F_{\text{eq}}$</th>
<th>$F_{\text{short}}$</th>
<th>$F_{\text{long}}$</th>
<th>$\tau_{\text{short}}$</th>
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<tbody>
<tr>
<td>Dimension</td>
<td>pN</td>
<td>pN/µm</td>
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<td>$\times 10^{-18}$ J</td>
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<td>24.31</td>
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<tr>
<td>Standard deviation</td>
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</tbody>
</table>

The difference in the mean value of $F_{\text{short}}$ for the cells perfused with NES and 250 mOsm was statistically significant ($p = 0.0063$). A 34% reduction in $F_{\text{short}}$ for 250 mOsm treated cells imply the diminished role of a fast tethering force relaxation processes in hypoosmotic solution. A lipid bilayer separated by aqueous solutions of different osmolarities develops nonzero tension proportional to the osmotic pressure difference. Higher extracellular osmolarities tend to increase intracellular hydrostatic pressure and membrane tension. Because $F_{\text{eq}}$ was not significantly affected by the different osmotic
conditions, we think that in the investigated range of extracellular osmolarity (250-350 mOsm) the effective PM tension in HEK cells is carefully regulated by the dynamic cytoskeletal confinement, the phenomenon described theoretically in several papers (Gov et al., 2003; Gov and Safran, 2004). However, the PM tether itself is free from the cytoskeleton and the equilibrium stresses inside the tether should be affected by osmotic difference. Following this reasoning, we attribute the reduced magnitude of the fast tethering force relaxation process to the stress-minimizing rearrangement of PM tether lipids. Lower extracellular osmolarity then leads to a more stressed mechanical equilibrium of PM tether, and smaller difference between the steady-state and equilibrium mechanical tether states. Our hypothesis is consistent with that of Murdock et al. (2005) derived from the tether pulling experiments on chlorpromazine-treated OHCs.

2. HEK cells treated with mercury (II) chloride

Force-PM displacement profiles measured on HEK cells perfused with 1 mM HgCl₂ solution were similar to those measured on NES perfused HEK cells (See Figure 21). However, temporal tethering force profiles exhibited different behavior during the relaxation segment (Figure 30). Namely, the steady-state and equilibrium tethering forces were abnormally large and the bi-exponential relaxation process was significantly suppressed. Material parameters calculated for 1 mM HgCl₂ perfused HEK cells are presented in Table 6.
Figure 30. Temporal tethering force profiles recorded with optically-trapped fluorescent microspheres for HEK cells perfused with NES and 1 mM HgCl₂. Considerable increase in steady-state and equilibrium tethering forces as well as the lack of bi-exponential component of the tethering force relaxation are observed for HgCl₂.

HEK cells treated with HgCl₂ quickly developed blebs (spherical outward protrusions of PM) that is associated with increased effective PM tension. Although we only used cells which did not yet develop blebs, the process of effective PM tension increase must have been on its way, reflected in statistically significant increase of $F_{eq}$ (more than two times!) and $F_{ss}$ (by 60%). Eleven out of sixteen (69%) tethers did not exhibit bi-exponential tethering force relaxation process, which was caused either directly by the reduced transmembrane water transport (in case of non-isometric tether elongation) or through the increased effective PM tension, leading to the smoothening of
PM undulations and reduced lipid flow from the cell body to the tether (Murdock et al., 2005).

**Table 6** Material parameters of the HEK cell plasma membrane perfused with 1 mM mercury (II) chloride (parameters, significantly different from those measured in NES treated cells ($p < 0.05$) are shaded)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>$F_{max}$</th>
<th>$k_{PM}$</th>
<th>$W_{teth}$</th>
<th>$W_{t}$</th>
<th>$W_{el}$</th>
<th>$F_{as}$</th>
<th>$F_{eq}$</th>
<th>$F_{short}$</th>
<th>$F_{long}$</th>
<th>$\tau_{short}$</th>
<th>$\tau_{long}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dimension</td>
<td>pN</td>
<td>pN/µm</td>
<td>$\times 10^{-18}$ J</td>
<td>$\times 10^{-18}$ J</td>
<td>$\times 10^{-18}$ J</td>
<td>pN</td>
<td>pN</td>
<td>pN</td>
<td>pN</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Average value</td>
<td>234.2</td>
<td>445.3</td>
<td>148.5</td>
<td>46.98</td>
<td>145.7</td>
<td>117.2</td>
<td>94.53</td>
<td>16.38</td>
<td>24.63</td>
<td>2.44</td>
<td>15.11</td>
</tr>
<tr>
<td>Standard deviation</td>
<td>118.5</td>
<td>260.6</td>
<td>145.8</td>
<td>39.15</td>
<td>141.2</td>
<td>46.8</td>
<td>34.62</td>
<td>11.88</td>
<td>17.69</td>
<td>1.16</td>
<td>5.29</td>
</tr>
<tr>
<td>Sample size</td>
<td>16</td>
<td>16</td>
<td>16</td>
<td>16</td>
<td>11</td>
<td>16</td>
<td>16</td>
<td>15</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>

Unfortunately, in our experiments on HgCl$_2$ treated HEK cells, it was impossible to answer which of two phenomena (water transport or reduction of extra PM area due to increased effective PM tension) was dominant for the slow tethering force relaxation. One approach to separate effects from transmembrane water transport and effective tension may involve controlling the effective PM tension with micropipette aspiration while pulling tether using optical tweezers. However, cell regulation of the effective PM tension, similar to that observed on the HEK cells in osmotically challenged medium (Chapter V, Part C1) may appear in micropipette aspirated cells. Another possibility would be a transfection of a cultured cell line with large amounts of water transporting protein, e.g. a member of the aquaporin family (Verkman et al., 1996; Verkman and Mitra, 2000) and measuring the tethering force profiles from such cells. If water transport is a dominant component, the slow tethering force relaxation should become significantly faster than in control cells.
3. Repetitive PM tether elongation

We found that, the synchronized temporal tethering force profiles recorded at tether pulling cycles two through four were different from those recorded at the first tether pulling cycle (Figure 31). Specifically, steady-state tethering force value, observed

![Figure 31. Typical temporal tethering force profiles at the first through fourth tether pulling cycles. For temporal synchronization, force profile at the first tether pulling cycle is shown starting at the steady-state segment. Tether formation segment is not shown.](image)

for the first tether pulling cycle was not reached at most of the subsequent tether pullings. Instead, a continuous growth of the tethering force was observed up to the beginning of the tethering force relaxation (Figure 31). Therefore, we used the tethering force value averaged over the last 0.5 s before tethering force relaxation as an estimate of the steady-
state tethering force at the cycles 2-4. Tethering force relaxation parts at all four cycles looked similar.

The changes of mechanical PM parameters calculated for the steady-state and relaxation segments at the cycles 2-4 in comparison to the cycle 1 are shown in the Figure 32. Average steady-state tethering forces calculated for 2,3 and 4 tether pulling cycles dropped relative to the cycle 1 to $0.92 \pm 0.2$ ($n = 12$), $0.87 \pm 0.25$ ($n = 9$), and $0.76 \pm 0.19$ ($n = 5$), respectively (Fig. 32a). The mean value of the steady-state tethering force calculated for the fourth cycle relative to the first cycle was statistically different from 1 ($p = 0.023$). Average equilibrium tethering forces calculated for 2,3 and 4 tether pulling cycles dropped relative to the cycle 1 to $0.95 \pm 0.09$ ($n = 13$), $0.88 \pm 0.17$ ($n = 10$), and $0.75 \pm 0.1$ ($n = 5$), respectively (Fig. 32b). The mean values of the equilibrium tethering force calculated for the 2,3 and 4 cycles relative to the first cycle were statistically different from 1 ($p = 0.037$, 0.032, and 0.0023 respectively). Average amplitudes of the fast tethering force relaxation calculated for 2,3 and 4 tether pulling cycles increased relative to the cycle 1 to $1.49 \pm 0.36$ ($n = 6$), $1.21 \pm 0.45$ ($n = 7$), and $1.3 \pm 0.13$ ($n = 2$), respectively (Fig. 32c). The mean value of the fast tethering force relaxation amplitude calculated for the second cycle relative to the first cycle was statistically different from 1 ($p = 0.0098$). Average amplitude of the slow tethering force relaxation (Fig. 32d) calculated for the second tether pulling cycle decreased relative to the cycle 1 to $0.81 \pm 0.21$ ($n = 11$, $p = 0.014$), and returned close to 1 again for the cycles 3 and 4 ($1.05 \pm 0.77$ ($n = 7$) and $0.92 \pm 0.45$ ($n = 4$) respectively). Average time constants of the fast tethering force relaxation calculated for 2,3 and 4 tether pulling cycles decreased relative to the cycle 1 to $0.65 \pm 0.69$ ($n = 8$), $0.69 \pm 0.46$ ($n = 6$), and $0.69 \pm 0.19$ ($n = 2$), respectively.
Figure 32. Change of plasma membrane mechanical parameters after repetitive tether pullings. The value of each parameter was normalized to the one calculated for the initial tether. (a) Steady-state tethering force at 1 μm/s tether pulling rate and 10 μm tether length; (b) Equilibrium tethering force at 10 μm tether length; (c) Fast tethering force relaxation amplitude; (d) Slow tethering force relaxation amplitude; (e) Fast tethering force relaxation time constant; (f) Slow tethering force relaxation time constant. Statistically different from 1 parameters are indicated with (*) with p < 0.05.

(Fig. 32e). Lastly, average time constants of the slow tethering force relaxation calculated for 2,3 and 4 tether pulling cycles decreased relative to the cycle 1 to 0.79 ± 0.38 (n = 11), 0.78 ± 0.26 (n = 8), and 0.7 ± 0.25 (n = 4), respectively (Fig. 32f). The
mean values of the time constants of the slow tethering force relaxation calculated for 2, 3 and 4 cycles relative to the first cycle were statistically different from 1 ($p = 0.046, 0.021,$ and $0.047$ respectively).

Tethering force profiles obtained from the consecutive tether pullings look similar to those obtained from lipid vesicles, without obvious steady-state tethering force and showing sharp tethering force drop after pulling is stopped. This fact supports the hypothesis that during successive tether pullings plasma membrane was already separated from the cytoskeleton. Additionally, significant changes of the measured mechanical parameters after successive tether pullings, with generally observed reduction of the measured forces and relaxation time constants and increase of the fast relaxation amplitudes, prove the irreversibility of the processes happening during tether pulling through irreversible PM dissociation from the cell body and/or large PM deformations beyond its elastic limits.
CHAPTER VI. SUMMARY AND PERSPECTIVES

We have investigated the broad capabilities of using optical tweezers for measurements of plasma membrane mechanics. It was shown that by proper solution of the dynamic equation describing mechanical behavior of the optically trapped microsphere it is possible to measure instantaneous forces exerted by the stressed plasma membrane on the trapped microsphere. Several approaches have been suggested to improve the accuracy of the force measurements using optical tweezers and remove intrinsic limitations provided by the control equipment. For example, our work provides a solid foundation for a rapidly growing research field, which involves studies of fast dynamic biological processes such as electromotility, intermolecular bond rupture and initiation, etc. This study shows that dynamic measurements in the range of milliseconds will encounter difficulties, which can be resolved only by rigorous dynamic analysis of a microsphere optically trapped inside a viscous fluid under unknown external forces.

Another advancement was made in the processing of position sensing signals. It was shown that correction for the contrast of the microsphere image on the quadrant photodetector could significantly improve linearity of the position-sensing curve, and remove some artifacts intrinsic to transillumination position sensing. Better position sensing may involve use of fluorescent microspheres. The investigated technique of optically-trapped fluorescent microspheres indeed showed the superior position sensing capabilities, including high linearity of position-sensing curve, stability of the microsphere image contrast, and removal of the cell image artifacts.
In this work we also used the developed technologies based on optical tweezers to study mechanical properties of cell membranes. We investigated two cell types: mammalian outer hair cells, which are highly specialized cells of the hearing organ, and cultured human embryonic kidney cells. Several mechanical parameters were obtained under different cell treatments by pulling plasma membrane tethers, and each was attributed to a specific mechanical property or phenomenon associated with plasma membrane or cytoskeleton. We showed that extracellular perfusion of salicylate increases compliance of PM/cytoskeleton composite and reduces effective PM tension without influence on PM-cytoskeleton adhesion energy. Mechanical parameters measured on outer hair cells were insensitive to salicylate supporting the hypothesis of electrochemical mechanisms behind salicylate-induced reduction of electromotility. Using extracellular solutions of different osmolarities we identified the phenomena of cell regulation of effective PM tension and lipid rearrangement inside the PM tether. Use of a mercury chloride helped to realize the putative role of transmembrane water transport in the slow tethering force relaxation process, which is important for interpretation of data obtained from PM tether pulling experiments. Finally, we showed that tether elongation is essentially an irreversible process related to PM dissociation and/or large plastic PM deformations. Despite the vast information on mechanical properties of the cells obtained from PM tethers, the empirical assignment of the studied parameters to material properties of the cell plasma membrane or cytoskeleton still needs to be investigated in details using theoretical approaches of the cell mechanics.

In summary, our thesis work provides novel methodologies based on optical tweezers system, which can be used in studies of cellular or molecular mechanics. We
implemented them in studies of mechanical properties of plasma membranes resulted in additional scientific information, which increased the knowledge accumulated in the intriguing field of cell biology.
REFERENCES


SPIE: Nanobiophotonics and Biomedical Applications, edited by Cartwright AN 5331: 118-125.


LIST OF PUBLICATIONS


LIST OF CONFERENCE PRESENTATIONS


10. S. Ermilov, W. Brownell, and B. Anvari, “Effects of salicylate on mechanical characteristics of outer hair and human embryonic kidney cell plasma membrane”


APPENDICES

Appendix I: Alignment of the optical trap

The laser beam is located and the first lens (closest to the laser) of the collimating system (5) (Figure 17) is placed coaxially with the beam. Transverse position of the lens is finely adjusted until the output laser beam is propagated along the same optical axis (identified by two concentric marks on the photosensitive paper placed into two different positions along the optical axis). A beam shutter is placed between two mirrors (6), but close to the second mirror in order to prevent damage from the focused laser beam upon introduction of the second collimating lens. The second lens of the collimating system is placed just beyond the double focal distance from the first lens. Transverse position of the second lens is finely adjusted until the output laser beam is incident on the middle part of the first mirror (6) (use fluorescent IR indicator). The desired divergence of the laser beam is checked by the beam diameter (use photosensitive paper) near the second mirror (6), and tuned by axial positioning of the second lens (5). The second mirror (6) is positioned under the microscope objective, and the first mirror (6) is adjusted that the laser beam is incident at the middle of the second mirror (6) (use fluorescent IR indicator). The microscope objective is removed and the second mirror (6) is adjusted that the laser beam is passing through middle of the objective port (use fluorescent IR indicator). An alignment mirror is placed with its flat reflective surface on the microscope objective port. A fluorescent indicator with an aperture of about 2 mm is fixed on a 2-D positioning system perpendicular to the optical axis somewhere between two mirrors (6) with sensitive surface facing the alignment mirror. The aperture is
positioned in the middle of the laser beam (use another fluorescent IR indicator to check the beam position relative to the aperture). The laser beam passing through the aperture of fluorescent IR indicator and reflected back by the alignment mirror should be now visible in the darkness on the sensitive surface of the fluorescent IR indicator. Adjust the second mirror (6) to bring the reflected beam to the middle of the aperture. Install the objective into the port. Position the powermeter right on the objective. Use translation of the second mirror (6) along horizontal part of the optical axis to maximize the laser power after objective. Use translation of the first mirror (6) along the optical axis of the collimating system (5) to maximize the laser power after objective. The last two manipulations must be repeated until the power after objective will be at absolute maximum. Good alignment of the optical trap results in power after objective >200 mW. Eventually the laser trap quality has to be checked by measuring the escaping force and stability of the trap (especially in the axial direction).

**Appendix II: Focusing of the CCD imaging channel**

The image of the trapped microsphere should be centered on the screen of the monitor (20) (Figure 17). The microscope objective is focused on a piece of mud at the bottom of the Petri dish, which is moved into the trapping center. Then the microscope objective is defocused until the air bubble forms from the heat produced by the laser beam focused on the piece of mud. The laser is blocked and the imaging lens (18) is moved until the same piece of mud is sharply imaged on the monitor. Then the imaging lens is fixed on the optical rail.
The simpler, but less accurate way to do the alignment is to focus sharply an object, using microscope eyepieces, and then move the imaging lens for a sharp image on a video monitor.

**Appendix III: Parameters of the optical trap**

Microscope objective working distance is 200 μm and back focal length is 1.645 mm.

The convergence inside the bathing solution is:

\[ \alpha = \sin^{-1}\left(\frac{NA}{n_{sol}}\right) \]  

(III.1)

where \( NA \) is the numerical aperture of the microscope objective, and \( n_{sol} \) is the refractive index of the bathing solution. Our microscope objective had \( NA = 1.3 \) and \( n_{sol} \approx n_{H2O} \approx 1.33 \). Therefore, according to (III.1), \( \alpha \approx 78^\circ \).

Diffraction limits the focused laser spot to a diameter approximately equal to wavelength:

\[ d' \approx \lambda \]  

(III.2)

Our optical tweezers setup operated at \( \lambda = 830 \) nm.

The average optical flux at the focal spot of optical tweezers is:

\[ \Phi_{av} = \frac{4P}{\pi d'^2} \]  

(III.3)

where \( P \) is the laser power inside the bathing solution. According to (III.3), in our optical tweezers setup, the average optical flux was about \( 0.75 \times 10^{12} \) W/m² for \( P \approx 400 \) mW (maximal laser power measured after microscope objective in our setup).
Maximum theoretical transverse trapping force can be estimated as (Ashkin, 1992):

\[ F_m = Q_m \frac{n_{\text{sol}} P}{c} \] (III.4)

where \( Q_m \) is maximum of the dimensionless parameter, determining the quality of optical trap, and \( c \) is the speed of light in vacuum. Ashkin (1992) calculated \( Q_m \approx 0.3 \) for a polystyrene microsphere, resulting in \( F_m \approx 532 \) pN. Some of the tether formation forces measured in this thesis work exceeded 300 pN.

**Appendix IV: Accuracy and precision of the force measurements using optical tweezers**

There are several factors, which introduce significant variation of the PM mechanical parameters measured using optical tweezers:

1) The electronic noise of the system is mostly the PD dark noise, and the digital noise of the analog-to-digital converter (ADC). During data processing the electronic noise is transformed to the force error, and the higher the trap stiffness of the optical tweezers, the lower force precision will be achieved. The utilized values of trap stiffness (100-200 pN/μm) and system alignment allowed us to work with the trapping force mean square error (MSE) of 1-2 pN, due the electronic noise alone.

2) Accuracy of the SFG, which is very important during calibration of the trapping force, was ± 5% for the amplitude and ± 0.5 μHz for the frequency of the PZT control signal.

3) The size of the trapped microspheres is regulated by the manufacturer with about ±10% precision.
4) The distance from the trapped object to the bottom of Petri dish is controlled by the microscope manipulator with ±1 μm precision.

5) During calibration, the Stokes’s law is used to calculate the trapping force (See equation (2.2)). The error due to the calculation of trapping force can be determined by:

$$\frac{dF_{\text{trap}}}{F_{\text{trap}}} = \frac{d\eta}{\eta} + \frac{dd}{d} + \frac{dV_{\text{max}}}{V_{\text{max}}} + \frac{dk}{k}$$ \hspace{1cm} (IV.1)

$$\frac{dV_{\text{max}}}{V_{\text{max}}} = \frac{dV_{\text{SFG}}}{V_{\text{SFG}}} + \frac{df_{\text{SFG}}}{f_{\text{SFG}}} \hspace{1cm} (IV.2)$$

where $V_{\text{SFG}}$ is the amplitude of the control PZT signal generated by the SFG and $f_{\text{SFG}}$ is the frequency of that signal. Using the relationship for $k$ (Happel, 1983), the associated error can be estimated as 6.6 %. Finally, substitution of all the error values into (IV.1) gives the error of the trapping force calculation of about 21 %, comparable to 19-24 % reported by Capitanio et al. (2002).

During optical tweezers experiments, stochastic errors from the force calibration are overlapped with stochastic errors from the PD voltage measurements and must be considered in the appropriate statistical design.
Appendix V: Spectral filtering of the optically trapped fluorescent microsphere image formed on the position-sensing system.

(a) Fluorescence excitation spectra. (b) Spectral regions for visualization and position sensing.

Appendix VI: Extracellular solutions

Normal extracellular solution (NES) contains (mM of): 142 NaCl, 5.37 KCl, 1.47 MgCl₂, 2 CaCl₂·2H₂O, and 10 HEPES. The osmolarity is adjusted with raffinose to 300 ± 5 mOsm.

10 mM sodium salicylate solution (Sal) contains (mM of): 132 NaCl, 5.37 KCl, 1.47 MgCl₂, 2 CaCl₂·2H₂O, 10 NaSal, and 10 HEPES. The osmolarity is adjusted with raffinose to 300 ± 5 mOsm.

250 mOsm extracellular solution (250 mOsm) contains (mM of): 112 NaCl, 5.37 KCl, 1.47 MgCl₂, 2 CaCl₂·2H₂O, and 10 HEPES. The osmolarity is adjusted with raffinose to 250 ± 5 mOsm.
350 mOsm extracellular solution (350 mOsm) contains (mM of): 167 NaCl, 5.37 KCl, 1.47 MgCl\(_2\), 2 CaCl\(_2\cdot2\)H\(_2\)O, and 10 HEPES. The osmolarity is adjusted with raffinose to 350 ± 5 mOsm.

1mM HgCl\(_2\) extracellular solution (HgCl\(_2\)) contains (mM of): 142 NaCl, 5.37 KCl, 1.47 MgCl\(_2\), 2 CaCl\(_2\cdot2\)H\(_2\)O, 10 HEPES, and 1 HgCl\(_2\). The osmolarity is adjusted with raffinose to 300 ± 5 mOsm.

The pH of the each solution is adjusted to 7.2 - 7.4 using 1M NaOH.

**Appendix VII: Adhesion of fluorescent microspheres to the bottom of the sample chamber**

Table VII.1 Studies of the NES components

<table>
<thead>
<tr>
<th>Solution</th>
<th>pH</th>
<th>Osmolality (mOsm)</th>
<th># of trapped beads (5 min)</th>
<th># of trapped beads (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DI H(_2)O</td>
<td>7.00</td>
<td>n/a</td>
<td>0/10</td>
<td>10/10 (85)</td>
</tr>
<tr>
<td>DI H(_2)O + 142 mM NaCl</td>
<td>6.45</td>
<td>273</td>
<td>2/10(^1)</td>
<td>0/10 (124)</td>
</tr>
<tr>
<td>DI H(_2)O + 71 mM NaCl</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>0/10 (10)</td>
</tr>
<tr>
<td>NES – raffinose – HEPES – NaOH</td>
<td>6.02</td>
<td>288</td>
<td>3/10(^1)</td>
<td>0/10 (70)</td>
</tr>
<tr>
<td>NES – raffinose – NaOH</td>
<td>5.35</td>
<td>295</td>
<td>3/10(^1)</td>
<td>0/10 (93)</td>
</tr>
<tr>
<td>NES</td>
<td>7.32</td>
<td>300</td>
<td>0/10</td>
<td>0/10 (85)</td>
</tr>
<tr>
<td>NES – raffinose + NaCl</td>
<td>7.36</td>
<td>357</td>
<td>0/10</td>
<td>0/10 (91)</td>
</tr>
<tr>
<td>NES – raffinose + NaCl (saturated)</td>
<td>7.42</td>
<td>1513</td>
<td>2/10(^1)</td>
<td>0/10 (133)</td>
</tr>
<tr>
<td>NES – raffinose – NaOH + NaHCO(_3)</td>
<td>7.24</td>
<td>358</td>
<td>3/10(^1)</td>
<td>0/10 (65)</td>
</tr>
</tbody>
</table>
– microspheres were optically trapped but stuck to the bottom of the sample chamber upon the contact.

Table VII.1 shows that even the small concentration of NaCl makes microspheres sticky.

<table>
<thead>
<tr>
<th>Solution</th>
<th>Sample chamber coating</th>
<th>Beads coating</th>
<th># of trapped beads (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DI H₂O</td>
<td>uncoated</td>
<td>uncoated</td>
<td>30/30 (180)</td>
</tr>
<tr>
<td>NES</td>
<td>uncoated</td>
<td>uncoated</td>
<td>0/10 (180)</td>
</tr>
<tr>
<td>NES</td>
<td>Poly-D-lysine</td>
<td>uncoated</td>
<td>1/20</td>
</tr>
<tr>
<td>HEK medium</td>
<td>Poly-D-lysine</td>
<td>uncoated</td>
<td>4/10 (180)</td>
</tr>
<tr>
<td>DMEM</td>
<td>Poly-D-lysine</td>
<td>uncoated</td>
<td>2/10 (180)</td>
</tr>
<tr>
<td>DMEM + HEPES</td>
<td>Poly-D-lysine</td>
<td>uncoated</td>
<td>0/10 (180)</td>
</tr>
<tr>
<td>DMEM + HEPES + NaHCO₃</td>
<td>Poly-D-lysine</td>
<td>uncoated</td>
<td>1/10 (180)</td>
</tr>
<tr>
<td>DMEM + HEPES + NaHCO₃ + Antibiotic</td>
<td>Poly-D-lysine</td>
<td>uncoated</td>
<td>0/10 (180)</td>
</tr>
<tr>
<td>DMEM + HEPES + NaHCO₃ + Antibiotic + Antimycotic</td>
<td>Poly-D-lysine</td>
<td>uncoated</td>
<td>2/10 (180)</td>
</tr>
<tr>
<td>NES</td>
<td>HEK medium</td>
<td>uncoated</td>
<td>3/10 (30-40)</td>
</tr>
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<td>HEK medium + Poly-D-lysine</td>
<td>uncoated</td>
<td>5/10</td>
</tr>
<tr>
<td>NES</td>
<td>DMEM</td>
<td>uncoated</td>
<td>0/10 (30)</td>
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<td>NES</td>
<td>DMEM + Poly-D-lysine</td>
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<td>0/10</td>
</tr>
<tr>
<td></td>
<td>lysine</td>
<td>uncoated</td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>-------------------------</td>
<td>----------</td>
<td>---</td>
</tr>
<tr>
<td>NES</td>
<td>Antibiotic</td>
<td>uncoated</td>
<td>0/2</td>
</tr>
<tr>
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<td>Antibiotic + Poly-D-lysine</td>
<td>uncoated</td>
<td>0/10</td>
</tr>
<tr>
<td>NES</td>
<td>Antimycotic</td>
<td>uncoated</td>
<td>2/10</td>
</tr>
<tr>
<td>NES</td>
<td>Antimycotic + Poly-D-lysine</td>
<td>uncoated</td>
<td>8/10</td>
</tr>
<tr>
<td>NES</td>
<td>FBS</td>
<td>uncoated</td>
<td>0/10</td>
</tr>
<tr>
<td>NES</td>
<td>FBS + Poly-D-lysine</td>
<td>uncoated</td>
<td>2/10</td>
</tr>
<tr>
<td>NES</td>
<td>Antibiotic</td>
<td>Antibiotic</td>
<td>0/10 (20)</td>
</tr>
<tr>
<td>NES</td>
<td>Antibiotic + Poly-D-lysine</td>
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<td>0/10 (20)</td>
</tr>
<tr>
<td>NES</td>
<td>Antimycotic</td>
<td>Antimycotic</td>
<td>7/10(20)</td>
</tr>
<tr>
<td>NES</td>
<td>Antimycotic + Poly-D-lysine</td>
<td>Antimycotic</td>
<td>8/10 (30)</td>
</tr>
</tbody>
</table>
Appendix VIII: Fluorescence cross-talk signal

as a function of the transverse (a) and axial (b) displacement of the fluorescent microsphere from the trapping center

The only imaging concern in case of the fluorescent microspheres was associated with parasitic signals from other fluorescent microspheres, floating nearby the optical trap. In our setup the parasitic signals were reduced to a 1 pN force level when a microsphere was in the focal plane of the microscope objective and 17 μm off the optical axis (Figure (a)), or on the optical axis and 14 μm off the focal plane of the objective (Figure (b)).

To measure the fluorescence cross-talk signals we attached a fluorescent microsphere to the bottom of the sample chamber and moved it using the PZT to the center of the field of view where the sum signal from four quadrants of the QPD was maximal. Then a control triangular waveform was applied to move PZT with maximum displacement of 20 μm perpendicular to the optical axis of microscope objective while recording the sum QPD signal. The ratio of sum QPD signal at a specified transverse displacement to the initial sum QPD signal (zero displacement) is plotted in figure (a). Figure (b) was obtained similarly, by moving the microscope objective with the focusing knob, which simulated
axial displacement of the microsphere. In order to get the 1pN tethering force level, we applied the force calibration to the non-normalized sum QPD signal.

Appendix IX: Linearity and sensitivity of the fluorescent QPD position sensing

Sensitivity of the position sensing can be found by differentiation of the Eq. 3.1 with respect to displacement of the trapped object ($x$):

$$
\frac{dV_{PDx}}{dx} = \frac{8}{\pi d} \sqrt{1 - \left(\frac{2x}{d}\right)^2}
$$

(IX.1)

where $d$ is a diameter of the microsphere. The relationship (VIII.1) shows that for small displacements of the trapped microsphere ($x \ll d/2$), the sensitivity is almost constant (linear regime) and equal to $8/(\pi d)$, which is about 0.64 µm$^{-1}$ for a 4 µm diameter microsphere. Therefore, a linear part of the sensitivity may be reliably estimated without calibration. For bigger displacements the sensitivity is continuously decreasing (nonlinear regime), until it goes to zero at $x = d/2$. 
Appendix X: Tether formation segment showing three local maxima indicating three PM detachment events.

Regression of linear parts of the force-displacement profile resulted in three slopes: $k_{PM} > k_1 > k_2$. All three linear regression lines have the same intercept with abscissa.

Appendix XI: Estimation of PM/cytoskeleton adhesion energy

In order to estimate the adhesion energy per unit area in our experiments, we need to know the patch area. Assuming the indentation depth of a $d = 4$ μm diameter microsphere in the PM before tether formation to be $\Delta h \leq 10$ nm (during experiments we moved a cell towards an optically-trapped microsphere at 10 nm steps until a non-zero differential QPD signal appeared indicating the presence of reactive forces from the PM)
we estimate a patch area $A_p \approx \pi d \Delta h$ or $A_p \leq 0.126 \, \mu m^2$. Using the nonlinear part of the tether formation work as an estimate of the PM-cytoskeleton adhesion energy (plastic deformations of cytoskeletal and PM proteins are neglected), we obtain the adhesion energy per unit area to be $\geq 1.5 \cdot 10^{-3} \, J/m^2$.

**Appendix XII: Preparation of outer hair cells**

1. Check in advance: Small tissue forceps. Fine micro dissecting forceps. #10 rounded blade scalpel. #11 pointed blade scalpel. 100 µl microsyringe with a plastic tip. All instruments should be sharp and clean. NES or other extracellular solution – no older than 1 month. Microspheres diluted to 1 drop/ml in extracellular solution. 500 µl of 0.5 mg/ml trypsin. 60 mm in diameter Petri dish for dissection of cochlea. 35 mm in diameter uncoated glass-bottomed Petri dish for trypsonization of OHCs. 35 mm in diameter poly-D-lysine coated glass-bottomed Petri dish for experiments.

2. Before the dissection put the trypsin into warm water bath. Clean the tools with DI water and extracellular solution.

3. Clean the cochlea using #10 scalpel and holding the bone with tissue forceps under 2X microscope objective inside the 60 mm Petri dish filled with extracellular solution. Carefully, without damaging organ of Corti, excise the cochlea at the base using #10 scalpel, and using fine micro dissection forceps transfer it into the cup of 60 mm Petri dish filled with extracellular solution.

4. Holding the cochlea at the base with fine micro dissection forceps insert the tip of #11 scalpel between organ of Corti and the bone. Applying small pressure, make a crack in the cochlea with #11 scalpel.
5. Using #11 scalpel and fine micro dissection forceps or two fine micro dissection forceps separate the spiral part of cochlea from the cracked bones and spiral ligament.

6. Using fine micro dissection forceps transfer spiral part of the cochlea into the 35 mm uncoated Petri dish with 1 ml of 0.5 mg/ml trypsin.

7. Switch to 4X microscope objective and wait for 5 minutes.

8. Using microsyringe transfer OHCs from the spiral part of cochlea and those floating in the solution into the poly-D-lysine coated Petri dish filled with 2 ml of extracellular solution.

9. Wait for 10 minutes.

10. Add 5 μl of microspheres. Observe OHCs to estimate the quality of dissection and note the approximate location of OHCs.

11. Clean the tools with 70% alcohol and distilled water.
Appendix XIII: Studies of salicylate treated outer hair and human embryonic kidney cells using multiple rate tether pulling and transillumination position sensing

Figure XII1. Steady-state tethering force extrapolated to zero tether pulling rate, $F_{\text{ss}(0)}$ ± SD (pN). □ – cells perfused with normal extracellular solution: OHC lateral wall 115.4 ± 49.9 ($N = 30$), OHC basal end 76.5 ± 44.6 ($N = 19$), HEK cells 45.5 ± 31.5 ($N = 38$); ■ – cells perfused with 10 mM NaSal: OHC lateral wall 126.4 ± 64.4 ($N = 24$), OHC basal end 93.3 ± 61.4 ($N = 10$), HEK cells 43.6 ± 27.0 ($N = 40$).

Figure XII2. Effective tether viscosity, $\eta_{\text{eff}}$ ± SD (pN/(µm/s)). □ – cells perfused with normal extracellular solution: OHC lateral wall 2.79 ± 1.56 ($N = 30$), OHC basal end 3.23 ± 2.24 ($N = 19$), HEK cells 2.26 ± 1.34 ($N = 38$); ■ – cells perfused with 10 mM NaSal: OHC lateral wall 2.10 ± 1.31 ($N = 24$), OHC basal end 2.51 ± 1.60 ($N = 10$), HEK cells 2.89 ± 1.97 ($N = 40$).
Figure XII.3. Average time constant for the tether growth ($\tau_{tg}$). □ - cells perfused with normal extracellular solution: OHC lateral wall 0.85 ± 0.49 ($N = 37$), OHC basal end 1.65 ± 1.02 ($N = 25$), HEK cells 0.78 ± 0.45 ($N = 45$); ■ - cells perfused with 10 mM NaSal: OHC lateral wall 0.87 ± 0.53 ($N = 32$), OHC basal end 0.81 ± 0.61 ($N = 14$), HEK cells 1.00 ± 0.74 ($N = 48$).

Appendix XIV: Some MatLab™ programs used in the thesis

1) The following Matlab program was used to process the QPD signals recorded during calibration of optical tweezers. The file containing 4 columns of data: $V_x$, $V_y$, $V_\Sigma$, and TRIG is read. Depending on the direction of calibration (x or y) the normalized differential QPD signal is formed and its magnitude together with calculated maximum viscous drag force are recorded to calibration file "caldata.bin".

```
%Ftrap calibration (fluorescent microspheres, normalized for the sum PD signal)
%version from 11/22/04
clear all;
fsampADC = 1000; %ADC sampling frequency, Hz;
Vpp = 5; %control voltage of the SFG, Vpp
etta = 1.28*10^-3; %solution viscosity, Pa*s
d = 4; %bead diameter, mkm
z = 5; %distance from the coverslip, mkm
deltat = 1/fsampADC; %time step, s
Vpd = load('D:\Sergey\Last experiments\control fl OHCs\10 mM Sal\4_12_5\cal\6Hz5');
m = size(Vpd,1); %total number of data recorded to Vpd
i = 1;
while Vpd(i,4)<4
    i = i+1;
```

2) The following Matlab program reads 'caldata.bin' file with values of trapping forces and corresponding QPD voltages (see XIV.1) and performs linear regression analysis yielding p1(2) – a transverse trapping force calibration factor.
% calibration statistics
clear all;
fid = fopen('D:\Sergey\water calibration\caldata.bin','r'); %open cal data for reading
[FvsV,count] = fread(fid,inf,'double'); %read the calibration data to an array
status = fclose(fid); %close the file;
for i = 1:(count/2)
    FvsV1(i) = FvsV(2*(i-1)+1); %voltage, arb units
    FvsV2(i) = FvsV(2*i); %Ftrap, pN
end
Vpdmin = FvsV1(1); %minimal calibrated differential signal
[p1,S1] = polyfit(FvsV1,FvsV2,1); %line of the linear regression for FvsV
polytool(FvsV1,FvsV2,1) %linear regression analysis with 95% confidence interval

3) The following Matlab program reads a .bin file and displays its content. In the
appearing dialog user can choose which elements need to be permanently deleted from
the file. The program performs the deletion and saves the file. The program was used to
remove outliers from the calibration data.

% correction of the data from bin-file
clear all;
fid = fopen('D:\Sergey\water calibration\caldata.bin'); %open f-r data for reading
[parameters,count] = fread(fid,inf,'double'); %read the data to an array
parameters %shows data
elemdelete = input('which elements you want to delete permanently?');
if elemdelete==0
    parameters(elemdelete) = [];
fclose(fid);
    fid = fopen('D:\Sergey\water calibration\caldata.bin','w'); %open data file
count = fwrite(fid,parameters,'double'); %record changed data
end
fclose(fid);

4) The following Matlab program was used to calculate theoretical frequency
response of a trapping force for a triangular PZT position-controlling signal. The
resultant outputs are: 1) S and Phi – amplitude and phase responses of the trapped
microsphere displacement from the trapping center; 2) Xbead – temporal signal of the
microsphere displacement from the trapping center in response to PZT movement with
triangular controlling signal; 3) Maximum value of the microsphere displacement from
the trapping center (Xbeadmax1-3) for each of three regions of the peak PZT velocity for
a particular frequency (f-sign) of a triangular PZT controlling signal

%Theoretical frequency response of the triangular control PZT signal
clear all;
fsampl = 1000; %sampling frequency, Hz
fsign = 50; %triangle waveform frequency, Hz
Vp_p = 0.1; %control signal amplitude in V/pulse
fbutter = 55; %cut off frequency of the Butterworth filter, Hz
deltat = 1/fsampl; %time step, s
etta = 1.002*10^-3; %water viscosity at 20C, Pa*s
d = 4.537; %bead diameter, mkm
%ro = 2330; %silicon density, kg/m^3
%ro = 2600; %glass density, kg/m^3
ro = 1050; %polystyrene density, kg/m^3
b = [0.38*10^-4 0.57*10^-4 1.25*10^-4]; %trap stiffness, N/m
k = 1/(1-9*0.1*d/16+(0.1*d)^3/8-45*(0.1*d)^4/256-(0.1*d)^5/16); %perturbation
coefficient
Cdrag = 3*pi*etta*k*d*10^-6; %dragging coefficient, N*s/m
m = 4*pi*d^3*ro*10^-18/24; %bead mass, kg
p1 = Cdrag/m; %coefficient at dx/dt, s^-1
p2 = b/m; %coefficient at x, s^-2
T = 1/ftsign; %period of the control signal
m = 32768; %number of points in the FFT
t = 0:deltat:3*T; %time axis, s
D(1:(T/deltat)) = 0;
D((T/deltat+1):(1.25*T/deltat)) = 2*Vp_p*t(1:0.25*T/deltat)*ftsign;
D((1.25*T/deltat+1):(1.75*T/deltat)) = Vp_p/2-2*Vp_p*t(1:0.5*T/deltat)*ftsign;
D((1.75*T/deltat+1):(2*T/deltat)) = -Vp_p/2+2*Vp_p*t(1:0.25*T/deltat)*ftsign;
f = (0:m/2)*fsampl/m; %frequency axis, Hz
Fsign = fft(D,m); %FFT of the control signal
clear D;
Ssign = abs(Fsign); %amplitude response of the input signal
Phisign = angle(Fsign); %phase response of the input signal
clear Fsign;
FPZT = load('C:\Documents and Settings\Administrator\Desktop\PZT_res.txt'); %PZT
transfer function
SPZT1 = interp1(FPZT(:,1),FPZT(:,2),f,'spline',0); %PZT amplitude response for real frequencies
PhiPZT1 = interp1(FPZT(:,1),FPZT(:,3),f,'linear','extrap'); %PZT phase response for real frequencies
clear FPZT;
SPZT2 = flipr(SPZT1); %PZT amplitude response for imaginary frequencies
PhiPZT2 = -flipr(PhiPZT1); %PZT phase response for imaginary frequencies
SPZT = [SPZT1 SPZT2(2:m/2)]; %complex amplitude response of the PZT
PhiPZT = [PhiPZT1 PhiPZT2(2:m/2)]; %complex phase response of the PZT
Sx = Ssign.*SPZT; %amplitude response of the output control signal
Sv(1:(m/2+1)) = 40*2*pi*f.*Sx(1:(m/2+1)); %real part of the amplitude response of the output PZT velocity
fcomplex = flipr(f); %complex frequency
Sv(m/2+2:m) = 40*2*pi*f.*fcomplex(2:m/2).*Sx((m/2+2:m)); %imaginary part of the amplitude response of the output PZT velocity
clear SPZT Sx;
Phix = Phisign + PhiPZT; %phase response of the output control signal
Phiv(1:(m/2+1)) = Phix(1:(m/2+1)) + pi/2; %real part of the phase response of the output PZT velocity
Phiv(m/2+2:m) = Phix((m/2+2:m)) - pi/2; %imaginary part of the phase response of the output PZT velocity
Phiv(1:(m/2+1)) = Phix(1:(m/2+1)) + pi/2; %real part of the phase response of the output PZT velocity
Phiv(m/2+2:m) = Phix((m/2+2:m)) - pi/2; %imaginary part of the phase response of the output PZT velocity
Phiv = Phiv + Phiv; %phase response of the trapped bead
S = Sbend.*Sv; %amplitude response of the trapped bead
Phibead(1:(m/2+1)) = -atan(2*pi*f.*f./(p2*(stiffness)-4*pi^2*f.^2)); %real part of the phase response of the optical trap
Phibead(m/2+2:m) = atan(2*pi*f.*fcomplex(2:m/2)./(p2*(stiffness)-4*pi^2*fcomplex(2:m/2).^2)); %imaginary part of the phase response of the optical trap
Phi = Phibead + Phiv; %phase response of the trapped bead
clear Sv Phiv Sbend Phibead;
i = sqrt(-1);
F = S.*exp(i*Phi); %Fourier image of the trapped bead behavior
Xout = ifft(F); %bead position relevant to the center of the trap without Butterworth filter
Wn = (2*butter)/fsamp; %normalized cut off frequency of the Butterworth filter
[a1,a2] = butter(2,Wn); %second order digital Butterworth filter
Xbend(1) = 0; Xbend(2) = 0; %initialization of the bead position
for i=3:m
Xbend(i) = real(a1(1)*Xout(i)+a1(2)*Xout(i-1)+a1(3)*Xout(i-2)-a2(1)*Xbend(i-1)-a2(3)*Xbend(i-2)); %bead position relative to the trap center
end
hold on;
plot(t,Xbead(1:(3*T/deltat+1)),'-b');
plot(t,Vp_p*40*2*fsign*Cdrag/b(stiffness),'-r');
plot(t,-Vp_p*40*2*fsign*Cdrag/b(stiffness),'-r');
if fsign == 1
Xbeadmax1 = mean(Xbead((T/deltat+50):(T/deltat+200))); %first peak of x-signal
Xbeadmin2 = mean(Xbead((T/deltat+300):(T/deltat+700))); %second peak of x-signal
Xbeadmax3 = mean(Xbead((T/deltat+800):(T/deltat+950))); %third peak of x-signal
else
Xbeadmax1 = max(Xbead((T/deltat):round(1.75*T/deltat))); %first peak of x-signal
Xbeadmin2 = min(Xbead); %second peak of x-signal
Xbeadmax3 = max(Xbead(round(1.75*T/deltat+1):round(3*T/deltat))); %third peak of x-signal
end

5) The following Matlab program was used to process data from multiple rate tether pulling experiments. The program plots temporal tethering force profile. It fits individual regions of constant pulling rate with rising exponential model (tetherfit1.m) and calculates steady-state tethering forces - a(i), and time constants for tether elongation - tau(i).

%Multi-speed pulling tether force (steady-state approximation v. from 5/27/03)
%The calculated parameters are
%a(i) - steady-state components of the tether force, pN
clear all;
global FIN
v = [1 2 3 4 5 6]; %speed of pulling, mkm/s
tpull = [10 2 2 2 2 2]; %time of pulling with each specific velocity
fsamplADC = 1000; %ADC sampling frequency, Hz;
kF = 800; %force calibration factor, pN/V
deltat = 1/fsamplADC; %time step, s
Vpd = load('D:\Sergey\Last experiments\control NES fl HEKs\2_19_5\1');
m = size(Vpd,1); %total number of data recorded to x
i = 1;
while Vpd(i,4)<4
    i = i+1;
end
Vpdx = Vpd(i:m,:); %record of the triggered x and y-signals
m = size(Vpdxy,1); %total number of data triggered
clear Vpd;
Kpower = 1;%power correction factor
Vpd0(:,1) = mean(Vpdxy(1:100,1)); %x-signal offset
Vpd0(:,2) = mean(Vpdxy(1:100,2)); %y-signal offset
Vpdxycorr(:,1) = Kpower*(Vpdxy(:,1)-Vpd0(:,1))./Vpdxy(:,3); %record of the corrected x signal
Vpdxycorr(:,2) = Kpower*(Vpdxy(:,2)-Vpd0(:,2))./Vpdxy(:,3); %record of the corrected y-signal
clear Vpdxy;
Ftrapyx = kF*Vpdxycorr; %trapping force
clear Vpdxycorr;
netforce = sqrt(Ftrapyx(:,1).^2+Ftrapyx(:,2).^2); %net-component of the trapping force
tetherangle = 180.atan(Ftrapyx(:,1)/Ftrapyx(:,2))/pi; %vertical angle of the tether force, degrees
t = 0:deltat:(m-1)*deltat; %time during pulling, s
if tspull(1)/deltat < m
l(tspull(1)/deltat) = v(1)*t(1:tspull(1)/deltat); %distance travelled by the trap with v = 1 mkm/s, mkm
if (tspull(1)+tspull(2))/deltat < m
l(((tspull(1)+tspull(2))/deltat+1):((tspull(1)+tspull(2))/deltat+1)) = v(2)*t((tspull(2))/deltat+1)); %distance travelled by the trap with v = 2 mkm/s, mkm
if (tspull(1)+tspull(3))/deltat < m
l(((tspull(1)+tspull(3))/deltat+1):((tspull(1)+tspull(3))/deltat+1)) = v(3)*t((tspull(3))/deltat+1)); %distance travelled by the trap with v = 4 mkm/s, mkm
if (tspull(1)+tspull(2)+tspull(3))/deltat < m
l(((tspull(1)+tspull(2)+tspull(3))/deltat+1):((tspull(1)+tspull(2)+tspull(3))/deltat+1)) = l(((tspull(1)+tspull(2)+tspull(3))/deltat+1):((tspull(1)+tspull(2)+tspull(3))/deltat+1)); %distance travelled by the trap with v = 6 mkm/s, mkm
if (tspull(1)+tspull(2)+tspull(3)+tspull(4))/deltat < m
l(((tspull(1)+tspull(2)+tspull(3)+tspull(4))/deltat+1):((tspull(1)+tspull(2)+tspull(3)+tspull(4))/deltat+1):((tspull(1)+tspull(2)+tspull(3)+tspull(4))/deltat+1)); %distance travelled by the trap with v = 8 mkm/s, mkm
l(((tspull(1)+tspull(2)+tspull(3)+tspull(4)+tspull(5))/deltat+1):((tspull(1)+tspull(2)+tspull(3)+tspull(4)+tspull(5))/deltat+1)); %distance travelled by the trap with v = 10 mkm/s, mkm
else
l(((tspull(1)+tspull(2)+tspull(3)+tspull(4))/deltat+1):m) = l(((tspull(1)+tspull(2)+tspull(3)+tspull(4))/deltat) + v(6)*t((tspull(5))/deltat+1)); %distance travelled by the trap with v = 10 mkm/s, mkm
end
else
l(((tpull(1)+tpull(2)+tpull(3))/deltat+1):m) = l((tpull(1)+tpull(2)+tpull(3))/deltat) +
  v(4)*t(2:(m+1-(tpull(1)+tpull(2)+tpull(3))/deltat)); %distance travelled by the trap with v = 6 mkm/s, mkm
end
else
  l(((tpull(1)+tpull(2))/deltat+1):m) = l((tpull(1)+tpull(2))/deltat) + v(3)*t(2:(m+1-
  (tpull(1)+tpull(2))/deltat)); %distance travelled by the trap with v = 4 mkm/s, mkm
end
else
  l((tpull(1)/deltat+1):m) = l(tpull(1)/deltat) + v(2)*t(2:(m+1-tpull(1)/deltat)); %distance
  travelled by the trap with v = 2 mkm/s, mkm
end
else
  l(1:m) = v(1)*t(1:m); %distance travelled by the trap with v = 1 mkm/s, mkm
end

titl = input('input title of the plot', 's');
subplot(2,1,1), plot(t,netforce), axis([0 t(m) 0 300])
xlabel('t, s'); ylabel('Ftrap, pN'); title(titl);
subplot(2,1,2), plot(t,tetherangle), axis([0 t(m) -90 90])
xlabel('t, s'); ylabel('Phi, degrees');
qualstr = input('Do you wanna include the results into the overall statistics? yes/no:', 's');
qual = strcmp quali str, 'yes'); %check the input
if qual == 1
  start(1) = input('Input the starting time for processing the steady-state tether force at v = 1
  mkm/s, or 0 if the region is to be unprocessed, st1=');
avtime(1) = input('Input the time (s) allowed for processing, t1=');
  start(2) = input('Input the starting time for processing the steady-state tether force at v = 2
  mkm/s, or 0 if the region is to be unprocessed, st2=');
avtime(2) = input('Input the time (s) allowed for processing, t2=');
  start(3) = input('Input the starting time for processing the steady-state tether force at v = 4
  mkm/s, or 0 if the region is to be unprocessed, st4=');
avtime(3) = input('Input the time (s) allowed for processing, t4=');
  start(4) = input('Input the starting time for processing the steady-state tether force at v = 6
  mkm/s, or 0 if the region is to be unprocessed, st6=');
avtime(4) = input('Input the time (s) allowed for processing, t6=');
  start(5) = input('Input the starting time for processing the steady-state tether force at v = 8
  mkm/s, or 0 if the region is to be unprocessed, st8=');
avtime(5) = input('Input the time (s) allowed for processing, t8=');
  start(6) = input('Input the starting time for processing the steady-state tether force at v = 10
  mkm/s, or 0 if the region is to be unprocessed, st10=');
avtime(6) = input('Input the time (s) allowed for processing, t10=');
  start0 = input('Input the starting time for averaging the initial steady-state tether force,
  st0=');
avtime0 = input('Input the time (s) allowed for averaging, t0=');
j = 0;
for i = 1:6
if start(i)==0 %process only required regions
    j = j+1;
    st(j) = start(i);
    avt(j) = avtime(i);
    speed(j) = v(i);
end
end
\[\text{startpoint0} = \text{fsamplADC} \times \text{start0} + 1; \text{endpoint of first-range data}\]
\[\text{endpoint0} = \text{startpoint0} + \text{fsamplADC} \times \text{avtime0}; \text{endpoint of first-range data}\]
\[a(1) = \text{mean(netforce(startpoint0:endpoint0))}; \text{initial steady-state tether force, pN}\]
\[\text{startpoint} = \text{fsamplADC} \times \text{st} + 1; \text{start point of data processing}\]
\[\text{endpoint} = \text{startpoint} + \text{fsamplADC} \times \text{avt}; \text{endpoint of data processing}\]
for i = 2:j
    ttether = t(startpoint(i):endpoint(i)) - t(startpoint(i)); %time at each pulling range, s
    deltaF = netforce(startpoint(i):endpoint(i)).' - netforce(startpoint(i)); %tether force difference, pN
    b0 = input('Input initial guess for nonlinear tether fit [steady tau] =');
    p = nlinit(ttether,deltaF,@tetherfit1,b0); %nonlinear regression for Fvst
    tau(i) = p(2); %time constant for tether stretching, s
    a(i) = netforce(startpoint(i)) + p(1); %steady-state tether force, pN
end
end

6) The following Matlab function describes the rising exponent used in multiple rate tether pulling data processing program (XIV.5).

```matlab
function f = tetherfit1(b,t) %fit of the tether force experiments without cell elasticity, v.1 from 12/12/02
f = b(1)*(1-exp(-t/b(2)));
```

7) The following Matlab program processes repetitive tether pulling data. For each tether pulling cycle: First, program plots temporal tethering force profile. Then it plots force-PM displacement profile and calculates tether formation force, PM stiffness parameter, work of the tether formation and its components. Next, the program process
steady-state tether pulling region. Finally it fits tethering force relaxation with bi-

%Study of the histeresis of tether parameters
%with fluorescent microspheres (v.6 from 11/18/04)
%NEW:
%tether force was calibrated vs Vpd with zero intercept
%Vpdvsx is const from the theory for 4 mkm microspheres and equal to 0.503
%mkm^-1, calculated as average of the measured data

%The calculated parameters are
%Ftform - tether formation force, pN
%kwall - stiffness parameter of the cell wall, pN/mkm
%Wtform - tether formation energy, *10^-18 J
%WEldef - work spent on elastic deformations, *10^-18 J
%Wplast - work spent on plastic deformations, *10^-18 J
%Fss - steady-state tethering force at 1 mkm/s pulling rate, pN
%Feq - equilibrium tethering force after 1 min of relaxation, pN
%relaxation amplitudes and time constants

clear all;
global FIN DIN t0 lteth Dss Feqglob; %global value for initial tether force for the fitting model, pN
fsamplADC = 1000; %ADC sampling frequency, Hz;
Vpzt = 1; %PZT speed, mkm/s
lteth = 10; %tether length (PZT travel distance), mkm
kF = 426.39; %force calibration factor pN
kV = 0.503; %proportionality coefficient between differential voltage and microsphere displacement, 1/mkm
ktrap = kF*kV; %estimated trap stiffness, pN/mkm
deltat = 1/fsamplADC; %time step, s
Vpd = load('D:\Sergey\Fluor histeresis\HEKs\NES\4_28_4\3');
pulldir = input('direction of pulling (x = 1, y = 2), pulldir =');
Vpd0 = mean(Vpd(1:100,pulldir)); %signal offset
Vpdcorr(:,1) = (Vpd(:,pulldir)-Vpd0)./Vpd(:,3); %record of the corrected signal
Vpdcorr(:,2) = Vpd(:,4); %record of the trigger signal
clear Vpd;
ncycles = input('number of recorded cycles, ncycles =');
for k = 1:ncycles
m = size(Vpdcorr,1); %total number of data recorded
i = 1;
while Vpdcorr(i,2)==0
i = i+1;
end
j = i;
while (j<=m) & (Vpdcorr(j,2)<0)
    j = j+1;
end
Vpdcut = Vpdcorr(i:(j-1),1); %recorded signal cut to the triggered data of k-cycle
Vpdcutmed(:, :) = Vpdcorr(j:m, :); %recorded data for the remaining cycles
clear Vpdcut;
Vpdcut = Vpdcutmed; %data reassignment
clear Vpdcutmed;
m = length(Vpdcut); %total number of data recorded at k-cycle
netforce = kF*Vpdcut; %the trapping force, pN
clear Vpdcut;
t = 0:deltat:(m-1)*deltat; %time during pulling, s
titl = input('input title of the plot','s');
plot(t,netforce), axis([0 t(m) -100 400])
xlabel('t, s'); ylabel('Ftrap, pN'); title(titl);
flipsignstr = input('Do you wanna flip the force profile? yes/no:','s');
flipsign = strcmp(flipsignstr,'yes');
if flipsign == 1
    netforce = -netforce; %flip the force profile
    plot(t,netforce), axis([0 t(m) -100 400])
xlabel('t, s'); ylabel('Ftrap, pN'); title(titl);
end
qualstr = input('Do you wanna include the cycle in to the overall statistics? yes/no:','s');
qual = strcmp(qualstr,'yes'); %check the input
if qual == 1
    qualstr = input('Do you wanna process the tether formation region? yes/no:','s');
    qual = strcmp(qualstr,'yes'); %check the input
if qual == 1
    %analysis of the tether formation region
    [Ftform(k), iftform] = max(netforce(1:lengthADC/Vpzt)); %measured tether formation force and corresponding array index
    PosFtrIndAr = find(netforce(1:iftform)>0); %array of positive Ftr values
    NegFtrIndAr = find(netforce(1:iftform)<0); %array of negative Ftr values
    NegFtrIndArDim = size(NegFtrIndAr,1); %size of the negative Ftr array
    if NegFtrIndArDim==0
        ZeroFtrInd = 2; %index of zero Ftr
    else
        ZeroFtrInd = round(0.5*(max(NegFtrIndAr)+min(PosFtrIndAr))); %index of zero Ftr
    end
    clear PosFtrIndAr NegFtrIndAr;
    Xpzt = Vpzt*t; %PZT displacement, mkm
    cellwallpos = Xpzt.-Xpzt(ZeroFtrInd)-netforce/ktrap; %position of the cell wall relative to the one of zero force, mkm
    clear Xpzt;
%plot(cellwallpos(1:iFform),netforce(1:iFform)); %plot tether formation region as a function of cell wall position
%xlabel('xwall, mkm'); ylable('Ftrap, pN'); title(titl);
plot(netforce(1:iFform)); %plot tether formation region as an array index
stEL = input('Input the starting index for elastic deformation fitting, stEL=');
leEL = input('Input the number of points allowed for elastic deformation fitting, leEL=');
stP = input('Input the starting index for plastic deformation fitting, stP=');
kwal = polyfit(cellwallpos(stEL:(stEL+leEL)),netforce(stEL:(stEL+leEL)),1); %cell wall stiffness parameter, pN/mkm
for i = 2:iFform
dW(i-1) = 0.5*(netforce(i)+netforce(i-1))*(cellwallpos(i)-cellwallpos(i-1)); %elementary work on the cell wall deformation, *10^-18 J
end
clear cellwallpos;
Wtform = sum(dW((ZeroFtrInd):(iFform-1))); %mechanical work done to form a tether, *10^-18 J
WPdef = sum(dW(stP:(iFform-1))); %mechanical work done on the plastic deformation of the cell wall, *10^-18 J
WELEdef = Wtform-WPdef; %mechanical work done on the elastic deformation of the cell wall, *10^-18 J
clear dW;
end %of the tether formation analysis
qualstr = input('Do you wanna process the steady-state tether region? yes/no:','s');
qual = strcmp(qualstr,'yes'); %check the input
if qual == 1
%analysis of the steady-state tethering force
stt = input('Input the starting time for averaging steady-state tethering force (s):');
durt = input('Input the time allowed for averaging steady-state tethering force (s):');
Fss(k) = mean(netforce((stt*fsmplADC):(stt+durt)*fsmplADC)); %steady-state tethering force at k segment, pN
end %of the steady-state tether force analysis
qualstr = input('Do you wanna process the tether relaxation region? yes/no:','s');
qual = strcmp(qualstr,'yes'); %check the input
if qual == 1
%analysis of the tether relaxation region
stt = input('Input the starting time of relaxation (s):');
durt = input('Input the time allowed for relaxation fit (s):');
trel = t((stt*fsmplADC):(stt+durt)*fsmplADC) - t((stt*fsmplADC)); %time array for tether relaxation, s
Feq(k) = mean(netforce(((stt+durt-2)*fsmplADC):((stt+durt)*fsmplADC))); %equilibrium tethering force in the end of relaxation, pN
Frel = netforce((stt*fsmplADC):(stt+durt)*fsmplADC)); %tethering force during relaxation, pN
clear t netforce;
%force double-exponential data fit
FIN = mean(Frel((5*fsamplADC-100):(5*fsamplADC))); %reassignment to the global variable
tlong = trel((5*fsamplADC):(durt*fsamplADC)); %time array for long term tether relaxation, s
Flong = Frel((5*fsamplADC):(durt*fsamplADC)); %tether force during long term relaxation, pN
b0 = input('Input initial guess for long-term relaxation fit [Feq tauL] =');
t0 = tlong(1); %initial time of the long-term relaxation, s
p1 = nlinit(tlong,Flong,@tetherfit2,b0); %non-linear long-term relaxation regression
%nlintool(tlong,Flong,@tetherfit2,b0); %confidence interval for the
%long-term relaxation fit
clear Flong;
Fequ = p1(1); %equilibrium tethering force from the long-term exponential fit, pN
TAUreL(k) = p1(2); %long-term relaxation time constant, s
Flongfit = tetherfit2(p1,trel); %fitted tether force due to long term relaxation, pN
%plot(trel,Frel,-'r',trel,Flongfit,-'b');
Along(k) = Flongfit(1)-Fequ; %magnitude of the long-term relaxation, pN
Frdiff = Frel - Flongfit'; %differential force data for short-term relaxation, pN
%plot(trel,Frdiff); %goodness of a single exponential fit
Ashort(k) = Frdiff(1); %magnitude of the short-term relaxation, pN
FIN = Ashort(k); %reassignment to the global variable
t0 = 0; %initial time of the short-term relaxation, s
b0 = input('Input initial guess for short-term relaxation fit [0 tauS] =');
p1 = nlinit(trel,Frdiff,@tetherfit2,b0); %non-linear short-term relaxation regression
TAUreS(k) = p1(2); %short-term relaxation time constant, s
Feq1(k) = Fequ+p1(1); %equilibrium tethering force from the double exponential fit, pN
Frdifffit = tetherfit2(p1,trel); %fitted tether force due to short-term relaxation, pN
%plot(trel,Frdiff,-'r',trel,Frdifffit,-'b'); %short-term exponential function and data
%nlintool(trel,Frdiff,@tetherfit2,b0); %confidence interval for the short-term exponential fit
end %of analysis of the tether relaxation region
end %of the cycle
end

8) The following Matlab function describes the falling exponent used in tethering force relaxation data processing program (XIV.7).

function f = tetherfit2(b,t) %fit of the tether relaxation force, v. from 3/12/04
global FIN t0
f = b(1)+(FIN-b(1))*exp(-(t-t0)/b(2));
## Appendix XV: Mechanical characteristics measured on cells and lipid vesicles

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
<th>CHAPTER VII. CELL/ VESICLE (TREATMENT)</th>
<th>Average ± St.D. [dimensions]</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tether formation force ($F_{\text{max}}$)</td>
<td>Maximum force required to form a membrane tether. Related to the energy of PM-cell body dissociation and/or membrane bending stiffness</td>
<td>HEK cells (NES) HEK cells (10 mM NaSal) HEK cells (250 mOsm) HEK cells (350 mOsm) HEK cells (1 mM HgCl₂) OHC lateral wall (NES) OHC basal end (NES) Deiters cells (NES) Hensen cells (NES) Giant vesicle</td>
<td>[pN] 246.3 ± 87.6¹ 150.7 ± 69.4¹ 204.9 ± 116.8¹ 207.1 ± 83.1¹ 234.2 ± 118.5¹ 499 ± 152⁹ 142 ± 49⁹ 146 ± 44⁹ 116 ± 15⁹ 5-45⁶⁴</td>
<td>OT</td>
</tr>
<tr>
<td>Stiffness parameter ($k_{\text{PM}}$)</td>
<td>Linear slope on the force – membrane displacement diagram. Related to the out-of-plane membrane rigidity</td>
<td>HEK cells (NES) HEK cells (10 mM NaSal) HEK cells (250 mOsm) HEK cells (350 mOsm) HEK cells (1 mM HgCl₂) Human neutrophil PMN OHC lateral wall (normal) OHC lateral wall (normal) OHC lateral wall (normal) OHC lateral wall (normal) OHC lateral wall (calcium-free medium) OHC lateral wall (2 mM Mg²⁺) OHC lateral wall (NES) OHC lateral wall (NES) OHC lateral wall (NES) OHC lateral wall (cholesterol-treated cells) OHC plasma membrane (NES) OHC lateral wall (10 mM NaSal)</td>
<td>[pN/µm] 311.4 ± 181.0¹ 184.8 ± 93.8¹ 201.1 ± 80.4¹ 223.0 ± 123.0¹ 445.3 ± 260.6¹ 200-300²² 1090 ± 250⁸ 1350 ± 230¹² 1070 ± 240¹⁵ 1910 ± 230¹² 1270 ± 200¹²</td>
<td>OT</td>
</tr>
<tr>
<td><strong>Initial (small deformations) indentation stiffness ($k_{ind}$)</strong></td>
<td>Linear coefficient from the quadratic regression of the force-membrane indentation profile.</td>
<td>Bovine aortic endothelial cells (normal)</td>
<td>$680 \pm 260^8$</td>
<td>AFM</td>
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<tr>
<td><strong>Nonlinearity of the force-indentation curve ($k_{nl}$)</strong></td>
<td>Second-order coefficient from the quadratic regression of the force-membrane indentation profile.</td>
<td>Bovine aortic endothelial cells (normal)</td>
<td>$[\text{pN/mm}^2]$ 0.5-2.5$^4$</td>
<td>AFM</td>
</tr>
<tr>
<td><strong>Apparent area compressibility modulus ($K_{app}$)</strong></td>
<td>Linear slope on the tension – areal strain diagram (high tension &gt;0.5 nN/µm domain).</td>
<td>35-100% POPC vesicles, Lα phase (15-45°C) SOPC giant unilamellar vesicles (normal) SOPC giant unilamellar vesicles (10 mM NaSal) diacyl-PC vesicles (different saturation)</td>
<td>$[\text{nN/µm}]$ 150-220$^9$ 212 ± 23$^7$ 138 ± 32$^7$ 150-244$^{17}$</td>
<td>MA</td>
</tr>
<tr>
<td><strong>Elastic area compressibility modulus ($K_A$)</strong></td>
<td>Apparent area compressibility modulus adjusted for thermal fluctuations</td>
<td>SOPC giant unilamellar vesicles (normal) SOPC giant unilamellar vesicles (10 mM NaSal) diacyl-PC vesicles (different saturation) Red blood cell cytoskeleton</td>
<td>$[\text{pN/µm}]$ 0.25 ± 0.03$^7$ 0.19 ± 0.02$^7$ 0.23-0.27$^{17}$ 9.7 ± 3.4$^{23}$</td>
<td>MA OS</td>
</tr>
<tr>
<td><strong>Bending rigidity ($k_c$)</strong></td>
<td>Quantifies resistance of membrane to outward bending. Calculated from low tension domain ($\leq 0.5$ nN/µm) of the tension – areal strain diagram</td>
<td>SOPC giant unilamellar vesicles (normal) SOPC giant unilamellar vesicles (10 mM NaSal) diacyl-PC vesicles (different saturation) Dictyostelium discoideum</td>
<td>$[\text{aJ}]$ 0.09 ± 0.02$^7$ 0.2$^{20}$ 0.05 ± 0.02$^7$ 0.04-0.12$^{17}$ 20$^{18}$</td>
<td>MA</td>
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<tr>
<td><strong>Shear</strong></td>
<td>Quantifies</td>
<td></td>
<td>$[\text{pN/µm}]$</td>
<td></td>
</tr>
<tr>
<td>modulus ((\mu))</td>
<td>resistance to shear deformations</td>
<td>Human erythrocyte (discoid)</td>
<td>Human erythrocyte (spheroid)</td>
<td>Red blood cell ghosts (intact)</td>
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<tr>
<td>Membrane Young's modulus ((\gamma))</td>
<td>Last recorded tension preceding membrane rapture</td>
<td>Red blood cell Cortex of Dictyostelium discoides</td>
<td>[MPa] (0.73-30) (^{16}) (0.03) (^{18})</td>
<td>MA</td>
</tr>
<tr>
<td>Critical (lysis) tension ((\pi_c))</td>
<td>Strain preceding membrane rupture</td>
<td>[nN/(\mu)m] (6-13) (^3)</td>
<td>MA</td>
<td></td>
</tr>
<tr>
<td>Critical (lysis) strain ((S_c))</td>
<td>Quantifies frequency of the first process during membrane lysis</td>
<td>SOPC giant unilamellar vesicles (normal)</td>
<td>SOPC giant unilamellar vesicles (10 mM NaSal)</td>
<td>[s(^{-1})] (0.32) (^7)</td>
</tr>
<tr>
<td>Frequency of defect formation ((v_b))</td>
<td>Quantifies frequency of the second process during membrane lysis</td>
<td>SOPC giant unilamellar vesicles (normal)</td>
<td>SOPC giant unilamellar vesicles (10 mM NaSal)</td>
<td>[(\mu)s(^{-1})] (1.0) (^7)</td>
</tr>
<tr>
<td>Tension of defect formation ((\sigma_b))</td>
<td>Membrane tension causing defect formation</td>
<td>SOPC giant unilamellar vesicles (normal)</td>
<td>SOPC giant unilamellar vesicles (10 mM NaSal)</td>
<td>[nN/(\mu)m] (3.2) (^7)</td>
</tr>
<tr>
<td>Tension of cavitation ((\sigma_c))</td>
<td>Membrane tension causing cavitation</td>
<td>SOPC giant unilamellar vesicles (normal)</td>
<td>SOPC giant unilamellar vesicles (10 mM NaSal)</td>
<td>[nN/(\mu)m] (129) (^7)</td>
</tr>
<tr>
<td>Tether formation</td>
<td>Mechanical work spent on tether</td>
<td>HEK cells (NES)</td>
<td>[aJ] (234.1 \pm 171.1) (^1)</td>
<td>OT</td>
</tr>
<tr>
<td>work ( (W_{\text{eth}}) ) formation. Related to the work of elastic/plastic membrane deformations and work of PM-cell body dissociation</td>
<td>HEK cells (10 mM NaSal)</td>
<td>137.8 ± 120.5&lt;sup&gt;1&lt;/sup&gt;</td>
<td>HEK cells (250 mOsm)</td>
<td>223.0 ± 175.9&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Work of linear membrane deformations ( (W_l) ) Mechanical work spent on linear membrane deformations during tether formation</td>
<td>HEK cells (NES)</td>
<td>[aJ]</td>
<td>HEK cells (10 mM NaSal)</td>
<td>69.8 ± 78.6&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Work of nonlinear membrane deformations ( (W_n) ) Mechanical work spent on nonlinear membrane deformations and PM-cell body dissociation during tether formation</td>
<td>HEK cells (NES)</td>
<td>[aJ]</td>
<td>HEK cells (10 mM NaSal)</td>
<td>184.5 ± 162.7&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>PM-cytoskeleton adhesion energy ( (F_{\text{PM}}) ) Estimates energy per unit area required to separate plasma membrane from the cell body</td>
<td>HEK cells (NES)</td>
<td>[mJ/m²]</td>
<td>Dictyostelium discoideum</td>
<td>1.5&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Equilibrium tethering force ( (F_{eq}) ) Asymptotic tethering relaxation force. Related to the effective membrane tension</td>
<td>HEK cells (NES, 10μm)</td>
<td>[pN]</td>
<td>HEK cells (10 mM NaSal, 10μm)</td>
<td>42.1 ± 14.2&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Mouse fibroblasts NIH 3T3 (normal)</td>
<td></td>
<td></td>
<td>94.5 ± 34.6&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Effective membrane tension ($T_{eff}$)</td>
<td>Characterizes in-plane stresses of the membrane. Calculated from the equilibrium tethering force</td>
<td>Rabbit proximal tubules (apical region)</td>
<td>[pN/μm]</td>
<td>OT</td>
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<td>Rabbit proximal tubules (basal region)</td>
<td>9(^{21})</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Rabbit proximal tubules (apical blebs)</td>
<td>3(^{21})</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Rabbit proximal tubules (basal blebs)</td>
<td>3(^{21})</td>
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<td></td>
<td></td>
<td>Human melanoma cells M2</td>
<td>32(^{21})</td>
<td></td>
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<td></td>
<td></td>
<td>Human melanoma cells A7</td>
<td>43(^{21})</td>
<td></td>
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<td></td>
<td></td>
<td>Human melanoma cells M2 (blebs)</td>
<td>11(^{21})</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Human melanoma cells A7 (blebs)</td>
<td>12(^{21})</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Fast tethering force relaxation magnitude ($F_{short}$)</th>
<th>Is derived from bi-exponential tethering force relaxation at constant tether length. Related to</th>
<th>HEK cells (NES)</th>
<th>[pN]</th>
<th>OT</th>
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</thead>
<tbody>
<tr>
<td></td>
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<td>12.8 ± 3.4(^{4})</td>
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<td></td>
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<td>12.0 ± 6.7(^{1})</td>
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<tr>
<td></td>
<td></td>
<td>8.5 ± 3.3(^{1})</td>
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<tr>
<td>Slow tethering force relaxation magnitude ($F_{long}$)</td>
<td>Is derived from bi-exponential tethering force relaxation at constant tether length. Related to the magnitude of the membrane stress relaxation due to transmembrane water transport or redistribution of surface membrane undulations</td>
<td>HEK cells (NES)</td>
<td>HEK cells (10 mM NaSal)</td>
<td>HEK cells (250 mOsm)</td>
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<tr>
<td>Fast tethering force relaxation time constant ($τ_{short}$)</td>
<td>Is derived from bi-exponential tethering force relaxation at constant tether length. Related to the rate of the membrane stress relaxation due to rearrangement of tether lipids.</td>
<td>HEK cells (NES)</td>
<td>HEK cells (10 mM NaSal)</td>
<td>HEK cells (250 mOsm)</td>
</tr>
<tr>
<td>Slow tethering force relaxation time constant ($τ_{long}$)</td>
<td>Is derived from bi-exponential tethering force relaxation at constant tether length. Related to the rate of the membrane stress relaxation due to transmembrane water transport or redistribution of surface membrane</td>
<td>HEK cells (NES)</td>
<td>HEK cells (10 mM NaSal)</td>
<td>HEK cells (250 mOsm)</td>
</tr>
<tr>
<td>Parameter</td>
<td>Value</td>
<td>Method</td>
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<td>--------------------------------------------------------------------------</td>
<td>----------------------------------------------------------------------</td>
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<tr>
<td>Steady-state tethering force extrapolated to zero pulling rate ($F_{sa(0)}$)</td>
<td>HEK cells (NES) 43.1 ± 13.6&lt;sup&gt;1.2&lt;/sup&gt; 42.3 ± 9.9&lt;sup&gt;1.2&lt;/sup&gt; HEK cells (10 mM NaSal) 103.4 ± 28.4&lt;sup&gt;1.2&lt;/sup&gt; 99.2 ± 25.6&lt;sup&gt;1.2&lt;/sup&gt; OHC lateral wall (NES) 81.5 ± 22.9&lt;sup&gt;1.2&lt;/sup&gt; 79.7 ± 29.0&lt;sup&gt;1.2&lt;/sup&gt; OHC lateral wall (10 mM Sal)</td>
<td>OT</td>
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</tr>
<tr>
<td>Membrane tether stiffness ($k_{teth}$)</td>
<td>OHC lateral wall (NES) 3.71&lt;sup&gt;9&lt;/sup&gt; OHC basal end (NES) 4.57&lt;sup&gt;9&lt;/sup&gt;</td>
<td>OT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Effective tether viscosity ($\eta_{eff}$)</td>
<td>HEK cells (NES) 1.4 ± 0.6&lt;sup&gt;1.2&lt;/sup&gt; HEK cells (10 mM NaSal) 1.3 ± 0.3&lt;sup&gt;1.2&lt;/sup&gt; OHC lateral wall (NES) 1.7 ± 0.6&lt;sup&gt;1.2&lt;/sup&gt; OHC lateral wall (10 mM Sal) 2.39-5.25&lt;sup&gt;9&lt;/sup&gt; OHC basal end (NES) 1.7 ± 0.6&lt;sup&gt;1.2&lt;/sup&gt; OHC basal end (10 mM Sal) 1.7 ± 0.8&lt;sup&gt;1.2&lt;/sup&gt;</td>
<td>OT</td>
<td></td>
<td></td>
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<tr>
<td>Surface membrane viscosity ($\eta_{surf}$)</td>
<td>Multilamellar EPC vesicles 0.005-0.17&lt;sup&gt;19&lt;/sup&gt;</td>
<td>FC</td>
<td></td>
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</tr>
<tr>
<td>Lateral lipid diffusion coefficient ($D$)</td>
<td>OHC lateral wall (NES) 54.6&lt;sup&gt;10&lt;/sup&gt; OHC lateral wall (salicylate) 25.8&lt;sup&gt;10&lt;/sup&gt; OHC lateral wall (chlorpromazine) 36.4&lt;sup&gt;10&lt;/sup&gt; OHC plasma membrane (NES) 42-61&lt;sup&gt;14&lt;/sup&gt; OHC subsurface cisternae (NES) 2.5-12.7&lt;sup&gt;14&lt;/sup&gt; Cultured rat hippocampal neurons (normal) 35.5-35.9&lt;sup&gt;10&lt;/sup&gt;</td>
<td>FRAP</td>
<td></td>
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</tr>
<tr>
<td>Time constant from the</td>
<td>HEK cells (NES) 1.1 ± 0.5&lt;sup&gt;1.2&lt;/sup&gt;</td>
<td>OT</td>
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</tbody>
</table>
for the tether growth ($\tau_{tg}$) exponential fit of the tethering force approaching its steady-state after sudden increase in pulling rate. Related to viscoelastic properties of the membrane, viscosity of the extracellular solution, mechanical parameters of the force transducer.

| HEK cells (10 mM NaSal) OHC lateral wall (NES) OHC lateral wall (10 mM Sal) OHC basal end (NES) OHC basal end (10 mM Sal) | 1.0 ± 0.4$^{1,2}$ 0.9 ± 0.4$^{1,2}$ 1.1 ± 0.8$^{1,2}$ 1.5 ± 0.6$^{1,2}$ 0.9 ± 0.5$^{1,2}$ |


OT – optical tweezers; MA – micropipette aspiration; AFM – atomic force microscopy; OS – optical stretcher; FRAP – fluorescence recovery after photobleaching; FC – flow chamber;