Published in final edited form as:

Methods Mol Biol. 2016 ; 1427: 349–362. doi:10.1007/978-1-4939-3615-1_20.

Nanotechnology in Auditory Research: Membrane Electromechanics in Hearing

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Abstract

The soft, thin membranes that envelop all living cells are 2D, nanoscale, fluid assemblies of phospholipids, sterols, proteins and other molecules. Mechanical interactions between these components facilitate membrane function, a key example of which is ion flow mediated by the mechanical opening and closing of channels. Hearing and balance are initiated by the modulation of ion flow through mechanoreceptor channels in stereocilia membranes. Cochlear amplification by the outer hair cell involves modulation of ion movement by the membrane protein prestin. Voltage gated ion channels shape the receptor potential in hair cells and are responsible for the initiation of action potentials that are at the heart of sensory processing in the brain. All three processes require a membrane and their kinetics are modulated by the mechanical (ie. material) properties of the membrane. This chapter reviews the methodology for measuring the mechanics of cellular membranes and introduces a method for examining membrane electromechanics. The approach allows examination of electromechanically mediated interactions between the different molecular species in the membrane that contribute to the biology of hearing and balance.

Keywords

membrane electromechanics; optical tweezers; patch clamp; neurons; ion channels; outer hair cells; prestin; membrane/protein interactions; phospholipids; sterols

1. Introduction

Membrane molecules are amphipathic, having both hydrophilic and hydrophobic domains. The hydrophilic domains are located on the membrane surface, while the hydrophobic regions are located inside. The membrane is stabilized by the surface tension arising at both the inner and outer membrane/water interfaces. Living cells expend energy to maintain an asymmetry in the number of phospholipid-associated fixed charges on the inner and outer

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membrane surfaces. The electrical polarization resulting from the charge asymmetry sets the stage for force generation. Electromechanical force by the membranes of living cells is driven by a diffusion potential of ~100 mV. This potential is generated by the active pumping of ions to maintain electrochemical gradients. The resulting transmembrane electric field is huge (>10 MV/m – compare to the ~3 MV/m fields associated with atmospheric lighting) because the membrane is very thin (> 5 nm). Electromechanical coupling occurs naturally in simple lipid bilayers, where it is called the flexoelectric effect. Flexoelectricity is analogous to the electromechanical behavior of piezoelectric crystals and appears to be a universal property of all cell membranes.

Membrane mechanical properties, such as tension, bending stiffness, viscosity, and the active modulation of these properties by membrane electric potential, are important for many physiological functions. Mechanical connections with the cytoskeleton also alter membrane properties and important membrane proteins, such as ion channels and transporters that are anchored to the cytoskeleton. Changes in membrane tension in response to changes in membrane potential and subsequent dissipation of the tension by viscoelastic relaxation are relevant to synchronized gating and inactivation/closing of stretch-activated and voltage-gated ion channels and transporters. Synchronized gating or cooperative gating of ion channels at the axon initial segment permits rapid charge transfer and fast action potential initiation (1). The rapid onset of the action potential allows for phase locking at high frequencies and rapid temporal processing in the auditory system. Similarly, the cooperative gating of prestin may permit high frequency charge transfer in outer hair cells.

Several methods have been developed to probe the mechanical properties of cellular membranes, including atomic force microscopy, aspiration pipettes, optical and magnetic tweezers, cell stretching, and microelectromechanical systems (MEMs). Each method will be reviewed and its advantages and limitations with respect to the others identified. A new method will then be presented that combines optical tweezers and whole cell voltage clamp to characterize the physical mechanism underlying electromechanical force production by membranes. This new method allows investigation of outer hair cell electromotility, ion channel gating, and rapid temporal processing of auditory information.

The simplest way to investigate cell membrane mechanics is by attaching them to a flexible substrate and pulling or compressing the substrate. Cells are cultured directly on a thin polymer sheet, whose surface is coated with extracellular matrix molecules to encourage adhesion of the cells to substrate. Systematic altering of the mechanical properties of the substrate material can be made by changing the degree of cross link in the polymeric substrate and/or mechanical deformation of the substrate. The effects of mechanical forces, loading on the individual and collective interactions of the membrane with the substrate can be investigated. Several varieties of actuators, such as microelectromechanical systems (MEMs), have been used to improve the sensitivity and resolution of force measurement (2). When using MEMs, the substrate topography can be micro-manipulated and moved to apply and measure force. Further improvement can be achieved by fractionalization and micro-designing of the substrate with specific cellular matrix targets. Because the membrane of living cells is directly moved, the cells cannot be voltage clamped and the modulation of membrane mechanics by electric potential cannot be measured directly. Measurement of

mechanical properties of voltage clamped cells can be made using atomic force microscopy (AFM) (3). AFM uses a sharp tip at the end of flexible cantilever to generate a local deformation to the cell surface. Changes in membrane tension will move the cantilever and the membrane tension can be quantified using a calibrated cantilever tip. The measured force includes contributions from the cytoskeleton and does not allow direct quantification of membrane mechanical properties. AFM sensitivity is however great and the technique allows determination of the force required to pull a protein from a membrane or the force that will break bonds and allow a protein to unfold.

The separation of the membrane from the cytoskeleton and direct measurement of mechanical properties of cell membranes can be accomplished using aspiration micropipettes (4-6) or optical tweezers (7; 8). Aspiration pipettes suck a portion of a cell in to the micropipette. By measuring the force required to pull a “tongue”, the force required to separate the membrane from the cytoskeleton and estimates of stiffness and tension can be obtained. This method permits the measurements of forces as small as 1 pN, but does not allow simultaneous voltage clamping. Measures of membrane force in response to electrical stimulation can be achieved using optical tweezers combined with whole-cell patch-clamp, which is the topic of this chapter.

Optical tweezers (**Fig. 1**) consist of an optically trapped bead that function as “tweezers,” attaching to and deforming components of the cell. A powerful, highly focused laser beam produces an attractive force on dielectric beads, having a high index of refraction (**Fig. 2A**). The force required for the deformation pulls or pushes the bead from the center of the trap. The force is proportional to the displacement of the bead from the center of the trap resulting in a sensitive force biosensor. An isolated cell that is adherent to the glass surface of a petri dish is brought into contact with a trapped bead by moving the microscope stage. The beads are fluorescent and coated with sulfate surface groups to facilitate their adhesion with cell membranes. When the cell is pulled away after attachment, the force pulling on the bead increases until a maximum is reached and the membrane separates from the cytoskeleton. A membrane nanotube (tether) is then extracted from the cell. The force required to hold the tether is less than that required to break the cytoskeletal adhesion. Measurement of the tether force can be used to compute mechanical properties of the membranes such as bending rigidity, surface tension and viscosity. An analysis of the force measurement allows the understanding of the membrane-cytoskeleton adhesion and modulation of membrane mechanics by electric potential or pharmacological agents. Fundamental energy considerations allow membrane mechanical properties to be calculated from measurements of the tether force and the tether diameter (8; 9).

Electrical stimulation of the cell membrane while membrane tether force is measured is accomplished by maintaining the cell in whole-cell, tight-seal, voltage-clamp (**Fig. 1**). The materials and methods for patch-clamp experiments are presented in chapters of the present volume (see Goutman & Pyott in this edition for details). The only variation on standard techniques arises from the fact that the cell is moved during tether formation and the patch pipette must remain attached to the cell. This is achieved by mounting a low mass micro-manipulator on the microscope stage. Tethers are pulled from plasma membranes, while the membrane is held at different holding potentials, and the tether force is measured, while

changing the membrane potential. Measuring biologically relevant mechanical properties typically involves applying mechanical, chemical and electrical stimuli and recording the evoked mechanical response. Lipid protein interactions convey the mechanical force resulting from changes in the transmembrane electric field and contribute to ion channel cooperative gating and transport. Electromechanical force production by the membrane is altered on application of a variety of pharmacological agents or changing cytoplasmic pressures. These treatments also alter ion channel kinetics. Testing for concomitant variation between electromechanical force and channel function can provide experimental support on the membrane's role in ion channel gating.

2. Materials

2.1. Hair Cell and Neuron Culture Preparations

1. Neurobasal medium containing B27 supplement.
2. Glass bottom 35mm Petri dish, 14mm Micro-well, No. 1.5 cover glass (0.16-0.19 mm) poly-d-lysine coated.
3. Incubators, biological safety hoods, freezers and other equipment.
4. Trypsin, dissection and maintenance media.
5. Dissection tools.

2.2. Optical Trapping Using Optical Tweezers and Tether Pulling

1. Sound insulated room to house the device as well AVAC for temperature stability.
2. An optical table with a STACIS active damping system.
3. Continuous wave, tunable (650–1100 nm) titanium–sapphire laser (Spectra-Physics, 3900S), with a frequency doubled Nd: YVO4 laser (spectra-physics, Millennia V).
4. Inverted microscope (Nikon Eclipse Ti-U).
5. 60× oil immersion objective, high numerical aperture: NA 1.4
6. A piezoelectric translation stage (Physik Instrument, P-527.3CL).
7. Other optical instruments: Beam expanders and attenuators (lambda quarter plate with polarized beam splitter should be used as an attenuator to avoid damaging cells), focusing lenses (2-f 250, 2-f150, 1 f-75mm lenses in this specific case), 10 mirrors (in kinematic mount with vertical drive, mounting base and, 1 dichroic mirror).
8. Polyethylene fluorescent dielectric beads with a sulfate surface group and a diameter of 4.5 μm (Poly-Sciences, Niles, IL).
9. 300W light emitting diode (TLED) lamp (Sutter Instrument, Novato, CA).

10. Position-sensing device, a quadrant photodetector (QPD) placed at the Right port, 1 focusing lens
11. Personal computers, Field programmable gated Array (FPGA, National Instruments PCIe-7852R, Austin, TX), Data Acquisition card (DAQ, National Instruments PCIe-6363), DAQ device drivers (NI-DAQmx).
12. LabVIEW (Ver. 6.0; National Instruments, Austin, TX), Matlab.
13. Calibration equipment: Optical power meter and Acousto-optic deflectors.
14. Imaging optics for bright field microscopy: 100W Halogen Lamp for bright field microscopy and visualization of cells and Charge-coupled device CCD camera.

3. Methods

3.1. Alignment and Calibration of Optical Tweezers

This procedure is to be performed for each bead used. Begin by aligning the laser.

1. Turn on the solid-state laser and wait for ~30 min until the pump laser reaches 5W.
2. Prepare ~ 0.2% stock bead solution in PBS.
3. Turn on the AODs to allow the first order beam to pass (*see Note 1*) and the run application program (*see Note 2*).

¹We developed a calibration scheme utilizing Acousto-optical deflectors (AODs) to control the position of the trapping beam. A radio frequency signal is applied to solid crystal through a piezoelectric transducer of AODs to control the diffraction of light. Several properties of diffracted light are independently controlled with AODs, including the angle, amplitude and frequency. The diffraction angle is proportional to the sound frequency and the intensity of the diffracted beam is proportional to the sound amplitude. The ability to control the refraction angle allows the beam to be moved rapidly ($< 10 \mu\text{s}$). Two orthogonally oriented AODs, AODX and AODY are used to scan the laser beam in the XY plane. The AODs are driven by RF signals 40-60 mHz generated by the FPGA (*see Notes 4*) and the frequency and amplitude of the RF pulse is determined by two voltage inputs generated by the FPGA. The position of the bead at the objective is determined by the center frequency of the AOD (50 mHz). The AOD is designed such that the zeroth order beam has the highest efficiency at about 80% of the incident light and the first order beam has about 12.5% of the incident center frequency. Drive the AODs using voltage signals (0.5 V, 6.2 V to control the amplitude and frequency of the RF wave at center frequency of the AOD) to provide at least 530-570 mW in the first order beam of the AOD crystal (yielding 130 mW at the objective). But as the acoustic wave frequency deviates from its center value, diffraction efficiency decreases, causing a decrease in laser power. Spatial filters are used to block zeroth order beam and the first order beam is deflected by slightly changing the frequency of the RF pulse from the center frequency of the AOD.

²Integration of computer with instruments - automation of measurements. The extensive data collection protocol and limited time for maintaining cellular integrity requires automation of the movement of the piezoelectric stage (to elongate tether) while recording the displacement of the bead for further analysis. Patch clamp amplifiers, piezoelectric stages and QPDs have features that allow extensive interaction with the laboratory computer. Some are inputs and some are outputs. The interfacing of these devices with a computer can be achieved with data acquisition (DAQ) or field programmable-gated array (FPGA) systems. Using a terminal block, cables and a DAQ device, the measured signal can be transferred, digitized and stored in the computer for later analysis. From the DAQ device, the signal can be transferred to the computer through a variety of different bus structures that plug into the PCI bus of a computer. Most DAQ devices have four standard elements— analog input, analog output, digital I/O, and counters. Analog input is used to measure the voltage (mV) signal from the QPD device and the current delivered to the cell by the patch-clamp amplifier. The analog signal is digitized by the analog to digital converter circuit of the DAQ so that a computer can store and processes it. Although the DAQ device provides analog filtering options, the signals may be acquired at higher bandwidths and digitally filtered at a later time. Analog output is used to deliver voltage signals from the DAQ device to the patch clamp amplifier and piezoelectric stage as external command. Complex voltage protocols are required to automate the electrical stimulation of the membrane and movement of the PI stage. Application software is required to interface the user with the DAQ device. LabVIEW is commonly used for this purpose because it contains extensive support for interfacing to devices and instruments. DAQ device requires a driver engine that communicates between the device and application software by performing low-level register writes and reads on the DAQ device, while exposing the application program interface (API) to user applications software. We chose a NI-DAQmx device driver engine because it includes a DAQ assistant for configuration of channels, automation and measurement tasks with simpler API for creating DAQ applications.

4. Open the shutter and measure the laser power (~ 1.5 W) and spectrum of the bead.
5. Align the laser by adjusting the mirrors (*see Note 3*).
6. Measure the power of the laser at the objective by placing the power meter directly at the objective lens of the microscope. Adjust AODs to produce at least 130-140 mW power at the objective. This is required to trap $4\text{ }\mu\text{m}$ beads with sufficient restoring force to maintain beads in stable equilibrium without thermal damage to the cell.
7. Turn on the piezoelectric controller and set the (x, y, and z) coordinates for the piezoelectric stage at ($100\text{ }\mu\text{m}$, $100\text{ }\mu\text{m}$, and $70\text{ }\mu\text{m}$). The coordinates are specific to our microscope and may vary from configuration to configuration.
8. Focus the laser center at about $5\text{ }\mu\text{m}$ above the bottom of the dish. This will place the high convergence angle required for trapping at $5\text{ }\mu\text{m}$ above the coverslip when oil immersion objective is used.
9. Place a bead at the bottom of the dish. Turn on the LED light to excite the bead and turn off other source of light including the room light.
10. Align the fluorescence of the bead at the center QPD after the trapping laser is aligned at the center of the objective.

3.2. Calibrate QPD Output and Stiffness of Optical Tweezers

1. Calibrate the position sensor by moving the microscope stage a known distance ($\pm 2500\text{ nm}$, $\pm 2000\text{ nm}$, $\pm 1500\text{ nm}$, $\pm 1000\text{ nm}$, and $\pm 500\text{ nm}$) and recording the output (in mV). The QPD signal varies linearly with bead displacement up to 2000 nm from the center of the QPD. The slope of this relation is used to convert the QPD signal to displacement of the bead in nanometers.
2. Apply radio frequency signal 50 MHz to the Acousto-optical deflectors (AODs). The AODs have two voltage inputs for the amplitude and frequency of the RF wave (0.5 V and 6.2 V , respectively) (*see Note 2*).
3. The tweezers are stepped for $\pm 500\text{ nm}$ for 40 ms every 80 ms for 1 min . To move the beam 1 nm , change voltage of the frequency input by 0.178 mV in X or 0.189 mV in Y.
4. Take periodic average of the time course and the magnitude of the bead position to enhance the signal to noise ratio of the QPD output. (QPD

³Laser Alignment. The alignment of the laser is verified by observing the diffraction pattern of the beam from the adjustable circular aperture. Adjust the angle of mirrors until the laser is aligned. Place the adjustable circular aperture at a right angle to the incident laser beam and observe the diffraction pattern of the beam by placing a piece of paper near the aperture. When the circular aperture is uniformly illuminated, the diffraction pattern has a bright region in the center, known as the Airy disk with a series of concentric bright rings around it called the Airy pattern. Carefully narrow the width of the aperture until the airy patterns disappear. When the bead is aligned, the intensity of the airy disk will be uniform.

output contains high frequency noise and displacement of the bead cannot be readily quantified from a single trace).

5. Fit the measured movement of the bead to a stepwise deflection of the trap with an analytic expression for an over-damped harmonic oscillator.
6. Estimate the decay time constant (τ , which is proportional to the ratio of viscosity over the stiffness ($\tau = b/k$)) using fitting algorithm. Use a fitting routine such as polyfit in matlab.
7. Compute the trapping stiffness. The drag coefficient is known ($b = 6\pi R\eta$, where R is stokes radius = bead radius and η is the viscosity of the solution). Trap stiffness can be solved for from the time constant and drag coefficient. We find that it varies with the power of the laser as a linear function of laser intensity (**Fig. 2**).

3.3. Hair Cell and Neuron Cultures

All procedures using animals should be approved by the Institutional Animal Care and Use Committee according to the guidelines set forth by the NIH.

1. Guinea pig outer hair cells (OHCs) are easy to isolate, their hearing is similar to that of humans, and the methods are well characterized (5; 10; 11).
2. Dissect the ventral cochlear nucleus from the fetal animal brain (usually at gestation day 16-18) following decapitation. Neurons are then trypsinized, dissociated, and plated onto the glass bottom of poly-d-lysine-coated microwell petri dishes (MatTek, Ashland, MA) and maintained in Neurobasal medium containing the B27 supplement. Embryonic neurons can be maintained well in culture for up to about 4 weeks and will undergo distinct stages of differentiation and eventually form synaptic connections (see Note 4).
3. Bushy cells are the principal cells of the VCN. If other cell types are found in the culture system, differentiate them based on their morphology and ion channel profile.

3.4. Pull Membrane Tether and Measure Force

1. A tether is formed and elongated by placing the cell in contact with an optically trapped bead and moving the cell away from the trapped bead at

⁴Primary Cultures of neurons and hair cells. Membrane mechanics experiments on living cells require unrestricted access to their surface. This precludes *in vivo* or slice preparations. Isolated cells, ideally in a long-term culture system that is less complex than found *in vivo* are maintained *in vitro* under cell culture conditions. Hair cells should be used in < 4 h after removal from the cochlea - make sure all dissection tools, solution and instrumentation is ready before sacrificing the Guinea pig. The acute isolation of neurons from the ventral cochlear nucleus in adult guinea pigs was described first by Manis & Marx (12). Early developmental stage neurons are less susceptible to damage during the processing required for their culture. Glial cells are the major source of contamination in neuronal cultures and early developmental cultures contain fewer glial cells. Protocols for embryonic stage (E14-18) hippocampal neurons were described elsewhere (13). These protocols in conjunction with the knowledge of rodent brainstem anatomy gained from the adult guinea pig experiments can be applied to embryonic rats and mice to harvest, culture, and isolate embryonic bushy cells from the VCN.

a preset velocity. Open the application software to generate the voltage protocol to automate the movement of the piezoelectric stage and tether formation (*see Note 2*).

2. Enter inputs of the application software that include: 1) The distance of the cell from the trapped bead (usually 25-30 μm); 2) The move in speed of the cell towards the trapped bead (0.1-1 $\mu\text{m/s}$), 3) The hold time between the cells (1-5 s), 4) The move away speed of the cell from the bead and (0.1-1 $\mu\text{m/s}$) and 5) the final distance of the cell from the bead (25-30 μm).
3. Place dish containing cultured cell in the piezoelectric stage and focus the trapping beam at $\sim 5\mu\text{m}$ over the petri dish cover slide.
4. Add florescent beads to cell culture solution. Add $\sim 2\mu\text{l}$ of 0.2% bead solution in to one corner of the square plate of the petri dish. This addition will help prevent the entrance of multiple beads into the field of view of the QPD when a bead is trapped.
5. Search for floating beads using manual or coarse control of the piezoelectric stage, and move the floating bead towards the center of the beam very fast by moving the piezoelectric stage very fast. If the bead is trapped, it will not move.
6. Search for healthy looking cells and bring the cell or (voltage-clamped cell is electromechanical force measurement is being made) near the trapped bead by moving the piezoelectric stage manually. Bring the cell close, $\sim 20\text{-}30\mu\text{m}$, to the bead and note the precise distance.
7. Run the application program (*see Note 2*).

3.5. Measurement of Passive Membrane Mechanics

1. The measurement of bending stiffness requires a measurement of tether radius. Achieve this measurement by taking a microscopic image of the tether and analyzing the tether morphology from the saved image.
2. To measure membrane viscosity, pull membrane tethers at several speeds (Typically, 1, 2, 3 & 4 $\mu\text{m/s}$). Move the cell away from the trapped bead at 1 $\mu\text{m/s}$ for 30s as described above, then increase the pulling rate every 3 s by 1 $\mu\text{m/s}$ until it reaches 5 $\mu\text{m/s}$.

3.6. Measurement of Membrane Electromechanics

1. Pull, coat, and fill pipette electrode prior to starting the experiment (*see Goutman & Pyott in this edition for details*).
2. Voltage-clamp the cells prior to forming and pulling tethers at different holding potentials (-120 mV , -60 mV , 0 mV , 60 mV , 120 mV) during electromechanical force measurements.
3. Stage movement is stopped when tether length reaches a preset value and allows the tether to relax for 60 s.

4. Apply a sinusoidal electrical stimulus after the tether force reaches steady state. The sinusoidal voltage (± 20 mV, [10 Hz-5 kHz]) is superimposed on the holding potentials for 10 s and the mechanical response of the tether measured.
5. When measuring tether electromechanical force, whole-cell currents may be simultaneously recorded to monitor the tight seal during the entire force measurements.

3.7. Data Processing and Analysis

1. Take a running average of the QPD signal to remove high frequency noise.
2. Convert QPD signal in (mV) to displacement of the bead from the center of the QPD (in nm) (*see Section 3.2, step 1*).
3. Multiply bead displacement by trap stiffness to compute tether force. The tether formation force is at the peak of the tether force profile, when the membrane has detached from the cytoskeleton and a tether is formed (**Fig. 3**). The electromechanical tether force at each holding potential should be normalized to tether force at 0mV. High frequency electromechanical force measurement requires normalization (*see Note 5*).
4. Calculate the membrane-cytoskeleton adhesion energy using measurements of tether force on a region of a cell where the membrane is supported by the cytoskeleton and on blebs lacking cytoskeleton support (i.e., where the adhesion term equals 0).
5. Calculate bending stiffness (B) with the equilibrium force required to hold the elongated tether (F_{eq}) and the tether diameter (d) using the relation $B = 2\pi * F_{eq} * d$.
6. Calculate membrane viscosity. Fit the data for different pulling rates, V_{pull} , to an exponential function to obtain the asymptotic value of the steady state force for the different pulling segments (*see Note 6*). Fit a set of steady state force and the pulling rate to the linear function $F_{ss}(V_{pull}) = F_{ss}(0) + 2\pi\eta V_{pull}$, to get an estimation of the steady-state force at zero pulling rate $F_{ss}(0)$ and the viscosity (η).
7. Assess the role of the membrane force generation in channel gating and transport. Add [1 mM, 5 mM, 10 mM, 50 mM, 100 mM] salicylate to the bath solution and measure the changes in electromechanical gain and

⁵Viscous damping of the bead displacement is negligible at low frequencies, becoming greater as the frequency increases. Limitations on high-frequency electromechanical force measurements, resulting from viscous damping, are similarly determined by applying the above stimulus and observing bead displacement above the noise floor. Calibration of the trap allows for the correction of the frequency dependent decreases in magnitude and increase in phase lag.

⁶Membrane viscosity measurements. Tethers also retracted rapidly when the trap was turned off, suggesting that these tethers have the same viscous properties as tethers pulled from lipid vesicles without cytoskeleton (14). (Waugh and Bauserman 1995). For each pulling speed, the tether force increases exponentially and approaches a specific steady-state value (F_{ss}) where a faster pulling rate increases the steady state value.

channel function. Whole cell membrane currents are used to assess channel function.

Acknowledgements

This work was supported by NIH grants NIH/NIDCD grants R01 DC000354 & DC002775. Dr. Brenda Farrell and Vivek Rajasekharan have generously provided help in building, learning and mastering the optical tweezers system.

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Fig. 1. Cartoon showing membrane tether formation with optical tweezer from voltage-clamped cell

Both constant and sinusoidal electrical stimuli are delivered to the cell through the whole-cell tight-seal patch electrode. Electromechanical force is quantified by measuring the bead displacements along the tether axis using the quadrant photodetector (11).

Fig. 2. Cartoon of optical trap and plot of trap stiffness as a function of laser power

(a) Dielectric objects are attracted to the center of focused beam. The trapping force applied on the bead depends linearly on its displacement from the trap center just as with a simple spring system. (b) Trapping stiffness as a function of laser power. Regression line shown in green was obtained by fitting the stiffness of optical trap as function of laser power, indicating that the stiffness of the optical trap varies linearly with laser power ($R^2 = 0.99$). The force that results from a bead displacement X is equal to the stiffness (k) times X .

Fig. 3. Representative force profile showing tether formation

The soma of an isolated neuron is brought into contact with the bead and pushes it in the negative direction (downward deflection). The neuron is then moved away from the bead at a constant velocity ($0.1 \mu\text{m/s}$) for the remainder of the trace. The membrane is attached to the cytoskeleton until a peak force is reached at which point the membrane separates and the force on the bead drops as membrane flows into the resulting tether. The peak value is the tether formation force and is proportional to the cytoskeleton–plasma membrane adhesion energy (8; 15).