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Combining optical tweezers and patch clamp for studies of cell membrane electromechanics

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Abstract

We have designed and implemented a novel experimental setup which combines optical tweezers with patch-clamp apparatus to investigate the electromechanical properties of cellular plasma membranes. In this system, optical tweezers provide measurement of forces at piconewton scale, and the patch-clamp technique allows control of the cell transmembrane potential. A micron-size bead trapped by the optical tweezers is brought in contact with the membrane of a voltage-clamped cell, and subsequently moved away to form a plasma membrane tether. Bead displacement from the trapping center is monitored by a quadrant photodetector for dynamic measurements of tether force. Fluorescent beads and the corresponding fluorescence imaging optics are used to eliminate the shadow of the cell projected on the quadrant photodetector. Salient information associated with the mechanical properties of the membrane tether can thus be obtained. A unique feature of this setup is that the patch-clamp headstage and the manipulator for the recording pipette are mounted on a piezoelectric stage, preventing relative movements between the cell and the patch pipette during the process of tether pulling. Tethers can be pulled from the cell membrane at different holding potentials, and the tether force response can be measured while changing transmembrane potential. Experimental results from mammalian cochlear outer hair cells and human embryonic kidney cells are presented.

I. INTRODUCTION

Development of gradient force optical trapping, called optical tweezers, has made it possible to precisely manipulate microscopic objects and to measure forces in the piconewton range acting on these objects. Since the first demonstration by Ashkin in 1986, optical tweezers have become a versatile tool for research in various areas of science.^{1,2} Optical tweezers have found a multitude of applications in biology due to the noninvasive nature of light and the biologically relevant force range. Researchers have reported on the use of this technique

to study a variety of cellular activities such as vesicle transport, motion of single kinesin molecules along a microtubule track, stretching of single collapsed deoxyribose nucleic acid molecules, and measure tethering forces associated with cell membranes.^{3–7}

Patch clamp recording is a widely applied electrophysiological technique to study behavior of single ion channels, or macroscopic currents in small cells or macropatches.⁸ The whole-cell variation in concert with voltage clamp allows direct electrical control of the cell transmembrane potential.

Several experimental methods including micropipette aspiration, flow chamber, and optical tweezers have been used to study mechanical properties of cell membranes through the formation of membrane tethers.^{6,7,9,10} In these studies, the membrane tether was shown to behave as a viscoelastic material and mechanical properties such as membrane-cytoskeleton separation force, bilayer bending stiffness, and effective membrane viscosity were measured. Membrane tether investigations with optical tweezers have utilized microspheres or “beads” which are manipulated by a laser trap. A bead is first attached to the cell membrane, and then the cell and the bead are separated. In the procedure, a thin tether of membrane material linking the bead and the cell is stretched exerting a force on the bead. Bead displacement is usually monitored by a quadrant photodetector (QPD) for dynamic measurements of membrane tethering force with millisecond time resolution.⁷

We describe here a hybrid system, which combines optical tweezers and patch-clamp techniques to study membrane electromechanical properties of living cells, such as the outer hair cells (OHCs) of the mammalian cochlea. OHCs are specialized sensory cells with force generation capabilities that give rise to the exquisite sensitivity and frequency resolving ability of normal hearing. They actively change their length in response to changes in transmembrane potential.^{11,12} The “electromotile” mechanism resides in OHCs lateral wall. A membrane protein, prestin, is an essential component of this membrane-based motor mechanism that converts voltage to mechanical force. When transfected into model human embryonic kidney (HEK) cells, prestin endows these cells with electromotility.^{13,14} Recently, an alternate hypothesis for OHC electromotility has been proposed that transmembrane potential modulates membrane tension which results in the membrane movement.^{15,16}

We have previously investigated OHC membrane mechanics using optical tweezers in the absence of an applied transmembrane electrical potential.⁷ However, the voltage dependence of the mechanics has motivated us to integrate patch-clamp technique into our optical tweezers setup. With this setup, tethers can be pulled from plasma membranes held at different transmembrane potentials, and the tethering force can be measured while changing this potential. Experimental results showing dynamic tether force profiles and tether force changes in response to applied transmembrane potentials are presented.

II. INSTRUMENT DESIGN

Figure 1 shows a schematic drawing of the entire measuring system, which consists of three major units: optical tweezers, patch-clamp device, and fluorescence imaging optics. In the following, we describe in detail the construction of each unit.

A. Optical tweezers

The optical tweezers setup comprises a continuous wave, tunable (650–1100 nm) titanium–sapphire laser (Spectra-Physics, 3900S) pumped by a 5 W solid state, frequency doubled Nd:YVO₄ laser (Spectra-Physics, Millennia V). The titanium–sapphire laser is tuned to 830 nm where no or minimal damage to cells has been reported.^{17,18} In our experiments, there

is no evidence of damage to the living cells, nor are thermally induced length changes in OHCs observed. The laser beam is collimated and passed through a 100 $\times$ oil (type A) immersion objective (Zeiss, Plan-Neofluar, numerical aperture (NA) = 1.3), achieving a high convergence angle required for optical trapping. Note that stiffness of the laser trap formed by oil immersion objective decreases rapidly with the increasing distance of the trapping position from the coverslip. Therefore, a water immersion objective could be used when trapping an object far from the coverslip surface.^{19,20} The laser beam is directed by two highly reflective mirrors towards the bottom port of an inverted microscope (Zeiss, Axiovert S100TV). A dichroic mirror placed before the bottom port transmits the laser light into the microscope, and reflects the emitted light (<650 nm) from the sample towards a beam splitter, which in turn, transmits 10% of that light to a charge coupled device (CCD) camera (DAGE-MTI, CCD100) for image collection, and the remaining 90% to a QPD (Hamamatsu, S1557) mounted on a micrometer stage.

The image of the trapped bead is projected onto the QPD active area. A QPD sum and difference amplifier (Pacific Silicon Sensor, QP50-6SD) with 257 kHz maximum bandwidth provides right-minus-left and top-minus-bottom difference voltage signals, and a signal that is the sum of all four quadrant output voltages. The signals are digitized with an analog to digital (A/D) converter (Iotech, Wavebook512), and subsequently analyzed by a custom-made LabVIEW (National Instruments, v6.0) program. The difference signals contain the information about the position of the bead in the XY plane (specimen plane) with respect to the center of the QPD, and the displacement of the bead from the trapping center is proportional to the tethering force.

Petri dish containing the living cells is placed onto a rotation stage (Newport, RSP-2T) mounted on a piezoelectric translation stage (Physik Instrumente, P-527.3CL). The piezoelectric stage is positioned on a manual translation stage. Movement of the piezoelectric stage is controlled by a function generator (Stanford Research Systems, DS345). The resolution of the piezoelectric stage is 2 nm in the XY plane and 0.1 nm in the Z direction (laser beam propagation direction).

B. Patch-clamp device

To pull a membrane tether from a cell, either the cell or the trapped bead should remain stationary while the other is moved away. The position of the trapping beam can be scanned by the use of computer-controlled acousto-optic deflectors or galvo-scanner mirrors. The deflection angle of the diffracted light and therefore the trapping position are determined by the acoustic frequency driving a crystal. The displacement range of the trapping beam in the specimen plane is usually limited to less than 20 μm , which is shorter than the 30 μm long tethers in our experiments. Therefore, we pull tethers by moving the cell away from the trapped bead using the piezoelectric stage. Under this condition, the patch pipette should move simultaneously with the cell, which requires the patch pipette holder, together with a three-dimensional manipulator and the patch-clamp headstage, to be fixed on the piezoelectric stage.

To this end, we have designed and constructed a compact device to eliminate the relative movements between the patch pipette and the voltage-clamped cell (Fig. 2). The device combines two separate micromanipulators into one unit, which enables manual control in the XY plane, and motorized control in the Z direction. It also offers the merits of low weight and small size. One micromanipulator (National Aperture, MM-3M-EX) driven by its positioning controller (National Aperture, MC-4B) is motorized in one direction, and has a resolution of 0.5 μm . It is fixed on the piezoelectric stage perpendicular to the XY plane through three custommade adaptors. Three stacked manual mini-stages (National Aperture, MM-1) compose the other three-axis micromanipulator, the size of which is 26.2 mm $\times$ 26.2

mm $\times$ 36.3 mm. The two micromanipulators are integrated by mounting the second one onto the slider of the first one through a fourth custom-made adaptor. Collectively, the manipulator unit weights only 80 g.

Clamped onto the manual micromanipulator, a manifold holder (Warner Instruments, MHH-25) is used to mount the patch-clamp electrode holder on its head. The holder head can be pivoted $\pm 90^\circ$ from its axial position and rotated 360° about the axis, enabling the easy positioning of the patch pipette. The patch-clamp headstage connects to the electrode holder with a 7 cm long flexible silicon tube, and is mounted on the piezoelectric stage through a fifth custom-made adaptor. With the patch-clamp electrode inside, the tube also prevents air escape to make suction applicable. Whole-cell voltage-clamp recording is done with a patch-clamp amplifier (Axon Instruments, Axopatch 200 B). Output is passed through the A/D converter that allows for the simultaneous digitization of transmembrane potential and resulting tether force response.

C. Fluorescence imaging optics

In our optical tweezers setup, the image of the trapped bead is projected onto the QPD to measure the tethering force. During the bead-membrane detachment phase, the trapped bead is directly adjacent to the voltage-clamped cell membrane, resulting in the image of both the bead and cell on the QPD. Presence of the cell image, therefore, causes inaccurate force measurements. To illustrate the effect of the artifact due to the cell image, a bead is trapped and imaged on the QPD using a microscope transmission illuminator (100 W halogen lamp) and a condenser (NA = 0.3) with bright field. The distance between the bead and an isolated OHC is controlled with the piezoelectric stage, and shown in Fig. 3(a). The closest separation distance between the trapped bead and the cell is $\approx 0.5 \mu\text{m}$. Figure 3(b) shows the output signal from the QPD during this procedure, which has been converted into force through the calibration method as described later. Note that the shadow of the cell generates about 75 pN of force artifact.

We utilize fluorescent beads combined with fluorescence imaging optics to overcome the shadow artifact. For fluorescence excitation (XBO xenon short-arc lamp, 75 W), only the image of the trapped fluorescent bead is projected on the QPD. The fluorescent beads are $4.0 \mu\text{m}$ diameter sulfate-derivatized polystyrene microspheres (F-8858, Molecular Probes) with peak excitation and emission wavelengths of 580 and 605 nm, respectively. Fluorescence illumination passes through an excitation filter (Chroma Technology, D535/50), and then is reflected by a dichroic mirror (Chroma Technology, 565DCLP) into the objective to illuminate the trapped fluorescent bead. The fluorescent light emitted from the bead is recollected by the objective, and separated from the excitation light by the dichroic mirror. A filter (Chroma Technology, D606/55) is placed in front of the QPD to specifically select the fluorescent light from the bead. Figure 3(c) shows the force measured when the trapped fluorescent bead is moved according to the position function shown in Fig. 3(a). The constant signal indicates a negligible effect of the cell image on force measurement when using the fluorescence technique.

III. EXPERIMENTAL PROCEDURE

A. Calibration

Calibration of trapping force (F) is performed by flowing a solution past a trapped fluorescent bead at a known velocity and using Stokes' law ($F = 6\pi r\eta vk$) to calculate the induced drag force, where r is the radius of the trapped bead, η is the solution viscosity, v is the velocity of the piezoelectric stage, and k is a factor that accounts for the viscous coupling when the trapped bead is at a given height in close proximity to a flat surface (petri dish

coverslip) during this process.^{20,21} The piezoelectric stage is displaced to a specific distance at a known velocity (v), and the bead's induced deviation from the trapping center by the fluid motion is monitored by the QPD. The process is repeated for increasing fluid velocities (kv), and the drag force is subsequently plotted against the QPD voltage change for each velocity. The slope of the linear fit (in pN/mV) is then used to calculate tether force from the QPD voltage signals recorded during each experiment. The calibration process is automated by use of a LabVIEW program and carried out prior to each experiment.

Calibration is made using beads with their centers located approximately $5\text{ }\mu\text{m}$ from the coverslip. According to the Stokes' law, $1\text{ }\mu\text{m}$ bead position deviation at this depth introduces less than 10% change in the calibrated force. The powers of light sources for transmission illumination and fluorescence excitation are set to 75 W during calibration and experiments, and the corresponding sum voltage signals from the QPD are about 0.5 and 1.2 V, respectively. The X and Y position signals from the QPD amplifier are normalized to the total intensity to cancel out the effects of light level variation.

B. Whole-cell voltage clamp

Normal bath solution contains (in mM): 142 NaCl, 5.37 KCl, 1.47 MgCl_2 , 2 CaCl_2 , and 10 HEPES. Patch pipettes are filled with 140 KCl, 5 EGTA, 2 MgCl_2 , and 10 HEPES. All solutions are adjusted to a pH of 7.2 and an osmolarity of 295–305 mOsm/kg.

Patch pipettes with typical resistances of 2–4 M Ω are pulled from borosilicate capillary tubings (World Precision Instruments, TW100-4) with a carbon dioxide laser puller (Sutter Instruments, P-2000). After cell isolation, the dish containing the living cells and fluorescent beads is mounted on the rotation stage. The cell to be patched is then rotated to a desired orientation, followed by the placement of a bead in the vicinity of the cell using the optical tweezers. Due to the use of the 100 $\times$ objective and the external imaging optics, the field of view through the CCD camera is about $40\text{ }\mu\text{m} \times 40\text{ }\mu\text{m}$, which makes the approach of the pipette tip to the cell difficult to visualize. When the pipette tip is placed near the cell, a shadow created by the pipette can be seen easily through eyepiece when using dark-field illumination. The manual micromanipulator can then be used to locate the pipette and position it several microns above the cell. The motorized micromanipulator is used subsequently to move the pipette into contact with the cell. After a seal with resistance of $>1\text{ G}\Omega$ is formed, a voltage pulse or gentle suction is applied to break the membrane patch in order to enter whole cell.

C. Tether formation

Once the whole-cell recording mode is established, the cell membrane is set to a holding potential and the bead in proximity of the cell is captured with the optical tweezers. The bead is then placed against the cell plasma membrane for approximately 10 s, followed by moving the cell away from the bead at a constant velocity of $1\text{ }\mu\text{m/s}$, causing tether formation and elongation. Stage movement is stopped when the tether length reaches a preset value (20 or $30\text{ }\mu\text{m}$), allowing the tether force to relax. When the tethering force reaches a steady state value, transmembrane voltage is changed following predetermined patch-clamp protocols, and the resulting tether force response is continuously recorded by the QPD.

IV. EXPERIMENTAL RESULTS

Tether formation between an optically trapped bead and a voltage-clamped OHC is shown in Fig. 4. The tether is not visible because of its nanometer range diameter, but it has been visualized using a fluorescent membrane stain in another study from our group.⁷ After

entering the whole-cell recording mode, the holding potential is set to -40 mV. Tether is elongated at $1 \mu\text{m/s}$ and then kept at $30 \mu\text{m}$ length while the tether force relaxes.

Figure 5(a) shows the entire force profile of an OHC plasma membrane during tether formation, elongation and relaxation phases. The signals are digitally filtered by a low-pass Butterworth filter with 20 Hz cutoff frequency in the data acquisition program. A tether is formed at 3.9 s after the onset of pulling at a force of ≈ 126 pN. The sudden reduction in the force indicates the detachment of the bead from the cell. During tether elongation phase, the tether force increases from 63 to 77 pN. Figure 5(b) shows the recording from the moment that tether length reaches $30 \mu\text{m}$ to the moment that tether force reaches a nearly steady state value. The exponential decay fit in this figure provides the respective values of 50.4 pN and 17.3 s for the asymptotic steady state force (F_{ss}) and the relaxation time (τ).

We also pull tethers from prestin-transfected HEK cells. Figure 6(b) shows the tether force response of a prestin-transfected HEK cell with a -40 mV holding potential while changing the transmembrane potential using the voltage protocol shown in Fig. 6(a). Tether force increases with hyperpolarization and decreases with depolarization. The electromechanical gain is about 0.03 pN/mV for this cell at the holding potential of -40 mV.

V. DISCUSSION

We have introduced an instrument integrating optical tweezers and patch-clamp techniques to study membrane electromechanical properties of living cells. The unique feature of this system is the ability to pull tethers from cell membranes at different transmembrane potentials, and measure tether force as a function of transmembrane potential. The relative movement between patch pipette and voltage-clamped cell during the process of tether pulling is eliminated by building a compact patch-clamp device and mounting it onto a piezoelectric stage. The use of fluorescent beads makes the bead-membrane interaction measurable during the tether-formation phase.

To investigate membrane electromechanics, it is necessary to rule out the role of additional factors on tether force response. When an external electric field is applied through a borosilicate pipette on a bead with negatively charged sulfate group, resulting electro-osmotic flow at the pipette tip and/or electrophoretic force may act on the bead, leading to its movements. To test the electrokinetic effects, a fluorescent bead was trapped and placed about $2 \mu\text{m}$ away from an open pipette tip with resistance of ~ 2 M Ω . The pipette tip was not imaged on the QPD due to the fluorescence imaging optics. When a ± 200 mV peak-peak sinusoidal voltage stimulus was applied, no force exerted on the bead was detectable above a noise level of 0.5 pN. When the test was carried out in distilled water instead of normal bath solution, up to 2 pN electrokinetic force was detected. Therefore, there are no electrokinetic forces or the forces are too weak to affect the patched cells. The same observations by atomic force microscopy have been reported.²²

As another control experiment, a trapped fluorescent bead was placed $1 \mu\text{m}$ away from a prestin-transfected HEK cell without a tether linking them. A ± 100 mV peak-peak sinusoidal voltage stimulus was applied to the cell when a seal with resistance > 5 G Ω was formed, and again when the cell had entered whole cell. No bead movements in response to the stimulus were observed.

Our experimental results from OHCs and prestintransfected HEK cells have shown the utility of an integrated optical tweezers and patch-clamp system in investigating membrane electromechanics. Further, the combination of various tether-pulling and patch-clamp protocols makes this measuring system versatile for such investigations. For example, membrane viscosity is an important factor in determining the rate of membrane deformation

and the movement of molecules within the phospholipid bilayer. The voltage-dependence of the membrane effective viscosity can be estimated by measuring the steady-state tether force at different pulling rates and transmembrane potentials. With this measuring system, the effect of amphipathic agents such as salicylate and chlorpromazine on the membrane electromechanics can be experimentally determined.

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FIG. 1. Schematic of optical tweezers setup in combination with patch-clamp device. Two dichroic mirrors are denoted by DM_1 and DM_2 .

(a)

(b)

FIG. 2. Schematic (a) and photograph (b) of the patch-clamp device mounted on the piezoelectric specimen stage. Custom-made adaptors are denoted by A_1 – A_5 .

FIG. 3.

(a) The distance of the trapped bead from an isolated OHC as a function of time; (b) the resulting force signal when microscopic transmission illumination is used for bead imaging. The shadow of the cell causes approximately 75 pN force artifact; (c) the resulting force when a fluorescent bead is trapped and imaged by fluorescence illumination. The shadow artifact seen in panel (b) has been eliminated.

FIG. 4.

Image of plasma membrane tether formation (tether is not visible) between the bead and voltage-clamped OHC.

FIG. 5.

(a) Membrane tethering force as a function of time; (b) measured tether force relaxation is fit with an exponential decay function.

(a)

(b)

FIG. 6. (a) Transmembrane potential protocol applied to a prestin-transfected HEK cell; (b) the resulting tether force response.