Probing localized neural mechanotransduction through surface-modified elastomeric matrices and electrophysiology

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Mechanotransduction of sensory neurons is of great interest to the scientific community, especially in areas such as pain, neurobiology, cardiovascular homeostasis and mechanobiology. We describe a method to investigate stretch-activated mechanotransduction in sensory nerves through subcellular stimulation. The method imposes localized mechanical stimulation through indentation of an elastomeric substrate and combines this mechanical stimulation with whole-cell patch clamp recording of the electrical response to single-nerve stretching. One significant advantage here is that the neurites are stretched with limited physical contact beyond their attachment to the polymer. When we imposed specific mechanical stimulation through the substrate, the stretched neurite fired and an action potential response was recorded. In addition, complementary protocols to control the molecules at the cell–substrate interface are presented. These techniques provide an opportunity to probe neurosensory mechanotransduction with a defined substrate, whose physical and molecular context can be modified to mimic physiologically relevant conditions. The entire process from fabrication to cellular recording takes 5 to 6 d.

INTRODUCTION

The ability to impose mechanical stimulation on living cells is critical in a diversity of physiological areas. One area in mechanobiology that has generated much attention recently is in neurobiology, in which the response of neural systems is known to be linked to mechanical stimulation, including the areas of axon outgrowth, neurodevelopment, neuroregeneration, hearing, touch and pain[1–6]. The ability to mechanically stimulate these living neural cells and then determine their response is critical to understand physiological responses. However, such studies are limited by the techniques that are currently available to impose mechanical stimulation. The techniques that have been developed to impose mechanical stimulation include atomic force microscopy, magnetic beads, optical tweezers and pipettes actuated by a hydraulic manipulator or a piezoelectric bimorph[7–10]. In these mechanobiology studies, the ability to stimulate whole cells and measure their responses has provided much insight, but to be able to locally probe cell response in neurobiology has significant implications for addressing challenging questions[11,12]. To impose controlled subcellular mechanical stimulation on neurons, we review previous work in neurosensory mechanotransduction and then approaches to probe and record neurosensory mechanotransduction.

Somatosensory mechanotransduction

Neural mechanotransduction research has largely focused on the sensory nervous system, though many other neural types are also mechanosensitive. One very important neural system commonly used in neurosensory studies is the dorsal root ganglia (DRG). These are extremely relevant in neurobiology, as the damage-sensing neurons (nociceptors), proprioceptive neurons and other tactile-sensing neurons have their cell bodies in the DRG, yet they also extend their peripheral processes into the skin, muscle, tendon, vessel and other deep tissues[4]. Although mechanotransduction occurs in nerve terminals, most studies in this regime have used neurtie-free DRG neurons to probe the neurosensory mechanotransduction. This approach fails to address important structurally related challenges, as the DRG neurons are cultured on hard substrates such as Petri dishes or coverslips, which have Young's (or elastic) moduli that are around 20 to several hundred times higher than their physiological surroundings. Differences in substrate elasticities have been linked to significant changes in neurite outgrowth[13–15], cell motility[16,17] and other complex biological functions including the differentiation of stem cells into bone or neural cells[18]. Beyond the elasticity-based mechanics questions, one of the current best ways to assess neurosensory mechanotransduction is in an *in vivo* or an *ex vivo* system, in which the nerve endings are still connected to targeted tissues[19–27]. An *in vitro* system culturing neurite-bearing DRG neurons on a physiologically relevant substrate is an approach that may readily mimic more physiological systems[11,18,28]. Furthermore, an *in vitro* culturing system will allow the molecular or genetic manipulations to probe the molecular contexts that constitute mechanosensitive ion channels and receptors, as well as related signaling pathways.

Mechanical input and cellular output

For examining neurosensory mechanotransduction *in vitro*, the choice of technique is critical for testing hypotheses grounded in mechanobiology. After imposing mechanical stimulation, the ability to assess cell response is important, as the link from the mechanical 'input' to the cellular 'output' directly influences the hypotheses to be tested. One of the methods that has been gaining much attention for controlling this input in many mammalian cell types is using

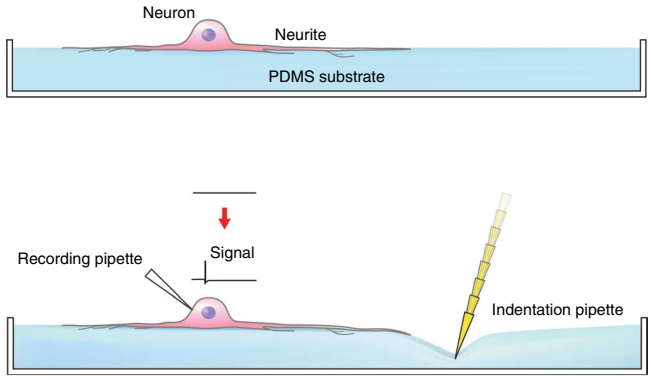

Figure 1 | Schematic of the mechanical stretching imposed on a neurite in the recording chamber. To create an *in vitro* model for probing the molecular mechanism of neurosensory mechanotransduction, DRG neurons are cultured and neurites are grown on a PDMS substrate that mimics a physiologically relevant elastic modulus and with specific extracellular matrix coating. In this condition, a single neurite can be stretched by substrate indentation without contacting the neurite. Whole-cell patch clamp recording is used to examine the electrical response to neurite stretching, which is conducted with our direct approach of indentation of a PDMS substrate at a location adjacent to the neurite with a pipette.

elastomeric polymers such as polydimethylsiloxane (PDMS). PDMS is widely used in various applications such as medical devices, microfluidics and microcontact printing[29–32], and also has the advantages of having low cost, scalability, cell compatibility and the ability to mimic topological features down to sub-micrometer scales[33–35]. The output response of neural systems has previously been probed using a variety of techniques including calcium imaging and whole-cell patch clamp recording. Fluorescent techniques such as calcium imaging have been developed to track dynamic responses in intracellular domains[36–38]. One of the most conventional techniques in neurobiology is to detect electric signals through patch clamping. Whole-cell patch clamp recording has been largely used to record the mechanically induced inward currents produced by using a blunt pipette to directly indent the neural soma[5,6,12,39–41]. Only recently, a few studies have begun to probe mechanotransduction on nerve terminals through the direct displacement of the terminals with a pipette or by imposing pressure jets with a pipette through the control of scanning ion conductance microscopy[3,19,37,42]. Although these studies imposed mechanical stimulation, the substrate interface with respect to the mechanical environment of elasticity, which is very physiologically relevant, was not addressed, and minimal control over molecular specificity was provided in these mechanical stimulation experiments.

Organization of the protocol

This protocol focuses on a new technique that is able to locally probe neurosensory mechanotransduction at subcellular domains *in vitro*, while providing control over the elasticity and molecular interactions at the cell–substrate interface (**Fig. 1**). We have integrated the ability to use our mechanical stimulation approach with well-established techniques such as electrophysiology that are used in neurobiology. The goal is to mimic and monitor the response of nerve endings in a physiologically relevant environment. We first culture neurite-bearing DRG neurons on flexible, coated PDMS substrates. We then indent the substrate in local areas, thus transmitting mechanical stimulation to subcellular areas. To precisely stretch a single neurite and prevent cell damage caused by direct mechanical contact, the cell density on the PDMS should be kept low so that neurites of a neuron can be easily tracked and then stretched through substrate deformation. Determining the output is simultaneously accomplished through a whole-cell patch clamp recording setup, which measures stretch-activated electrical responses. As the mechanical stimulation on cells could easily disrupt the whole-cell conditions, ensuring the maintenance of a good seal during the recording/stretching is essential; this is discussed further in the PROCEDURE and ANTICIPATED RESULTS sections. A major advance of this *in vitro* setup is the ability to grow neurite-bearing neurons on a surface-modified elastomeric matrix, while providing defined control over mechanical stimulation and molecular interactions. Issues related to molecular specificity and control of the cell–substrate interface, indentation forces on the substrates and measuring the mechanical thresholds are discussed in the ANTICIPATED RESULTS section. In addition, a technique of modifying the PDMS for added control over cellular adhesion is described and illustrated in **Box 1**.

BOX 1 | SURFACE MODIFICATION OF PDMS WITH ALTERNATE MOLECULES

The utilization of PDMS coatings other than fibronectin with this overall protocol allows for more specific control of the cellular adhesion molecules involved in the attachment of the neurons to the PDMS surface. When coated on substrate surfaces, fibronectin allows cells to attach through several different transmembrane adhesion proteins, including members of both the integrin and syndecan families. As a result, it can be useful to conjugate an antibody on substrates to isolate the specific cell surface proteins (e.g., β1 integrin) through which the cells are attaching to the PDMS surface for studying molecule-specific cell adhesion. By utilizing this supplemental approach, researchers would have specific control over the molecules that are physically linking the cells to the PDMS during the indentation procedure. This box describes a recently published approach for conjugating antibodies to PDMS surfaces[50], derived from a general method for protein conjugation published by Malpass *et al.*[51]. The changes in the functional surface chemistry of the PDMS surface through each step of the procedure are depicted in **Figure 4a**.

Air or oxygen plasma treatment of the PDMS surface

- Treat the PDMS-coated coverslip with air or oxygen plasma in a commercial plasma generator (e.g., Harrick Plasma PDC-32G) for 15–20 s. If a commercial plasma generator is not available, a microwave oven-based method of oxygen plasma treatment works well[52].

(3-Aminopropyl) triethoxysilane (AMEO) treatment

- Place the PDMS-coated coverslip in a glass Petri dish containing 2% (vol/vol) AMEO (Sigma) solution in 100% ethanol for 24 h at room temperature; cover the dish to prevent evaporation.

BOX 1 | CONTINUED

- Uncover the dish and place it into a preheated oven (80 °C) for 8 h to dry the AMEO on the surface of the PDMS membrane and crosslink the AMEO molecules.
- Rinse the PDMS-coated coverslip twice in 95% ethanol to remove excess AMEO.
- Rinse the PDMS-coated coverslip three times in PBS to remove excess ethanol.

Antibody conjugation

- Place the PDMS-coated coverslip in a 0.08 mg ml^{-1} antibody solution (in PBS) for 24 h at 4 °C with gentle rocking.
- Rinse three times for 5 min with PBS to remove unbound antibody.

Blocking of nonspecific binding sites

- Place the PDMS-coated coverslip in a 1% bovine serum albumin (BSA) solution (in PBS) for 1 h at room temperature with gentle rocking. For best results, the BSA solution should be made fresh and filtered through a 0.2 µm syringe filter before use to ensure that no BSA aggregates exist in the solution. BSA aggregates can result in nonspecific cell adhesion to the PDMS surface.
- Rinse three times for 5 min with PBS to remove unbound BSA.

Sterilization

- In the cell culture hood, dip the entire PDMS-coated coverslip in 70% ethanol for 15–20 s to sterilize.
- Place the sterilized coverslip in a sterile Petri dish to dry.

Evidence of the effectiveness of this approach for conjugating antibodies to PDMS surfaces and supporting DRG neuron attachment is shown in **Figure 4**.

MATERIALS

REAGENTS

- Dulbecco's modified Eagle's medium cell culture medium (DMEM; Gibco, cat. no. 12800-017)
- Fetal bovine serum (Gibco, cat. no. 26140)
- Collagenase type Ia (Sigma, cat. no. C9891)
- 0.25% (wt/vol) trypsin solution (Gibco, cat. no. 25200)
- Penicillin/streptomycin (Gibco, cat. no. 15140)
- Fibronectin (BD Biosciences, cat. no. SI-F1141)
- Polydimethylsiloxane (PDMS; Dow Corning, cat. no. Sylgard 184)
- Sterile distilled water
- Ethanol (MERCK, cat. no. 1.00983.2500) **! CAUTION** This chemical is flammable. Keep this away from Bunsen burners.
- $CaCl_2$ (MERCK, cat. no. 1.02382.0500)
- EGTA (ethylene glycol tetraacetic acid; Sigma, cat. no. E0396)
- Glucose (MERCK, cat. no. 1.08342.1000)
- HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Sigma, cat. no. 83264)
- KCl (MERCK, cat. no. 1.04936.0500)
- KOH (Sigma, cat. no. P5958) **! CAUTION** This chemical is corrosive. Avoid skin contact.
- $MgCl_2$ (MERCK, cat. no. 1.05833.0250)
- NaCl (MERCK, cat. no. 1.06404.1000)
- Na_2-ATP (Sigma, cat. no. A6559)
- Na_3-GTP (Sigma, cat. no. G8877)
- NaOH (MERCK, cat. no. 1.06498.1000) **! CAUTION** This chemical is corrosive. Avoid skin contact.
- Adult mice 8–12 weeks old **! CAUTION** Researchers must adhere to national regulations and institutional guidelines regarding animal use.

EQUIPMENT

- Stereoscopic dissecting microscope (e.g., 170AT-3D, Youlum Biotech)
- *Surgical instrument tools*: one pair of scissors (Fine Science Tools, cat. no. 14064-11), one pair of no. 5 forceps (Fine Science Tools, cat. no. 11252-23)
- *Tissue culture plastics*: conical centrifuge tubes (15 ml; BD Bioscience), Petri dishes (35 mm in diameter; BD Bioscience)
- *Glass wares for tissue culture*: 10 ml glass pipette, Pasteur pipettes
- 12 mm Coverslips (Marienfeld GmbH & Co.KG)
- Centrifuge for 15 ml conical tubes (e.g., Model 5800, Kubota)
- Bunsen burner (e.g., Touch-O-Matic, Water PIK)
- Cell culture incubator, humidified, 5% CO_2 (e.g., MCO-17AIC, SANYO)
- Biological safety cabinet (Esco Technology)
- Patch pipettes (64-0792, Warner Instruments)
- Microelectrode holder (W2 64-0839, Warner Instruments)
- Pipette puller (PP-830, Narishige)
- Blu-Tack (Bostik Australia)
- Vibration isolation table (e.g., VIBRAPLANE 1201, Kinetic Systems)
- Faraday cage (commercial or homemade, made of copper wire mesh)
- Micromanipulator for indentation pipette (EMM-3SV, Narishige)
  ▲ **CRITICAL**. Using a control box to drive the micromanipulator can significantly reduce vibrations introduced by moving probes manually. The control box should be located outside the Faraday cage and the vibration isolation table.
- Micromanipulator for recording pipette (MHW-3, Narishige)
- Inverted microscope (IX71, Olympus)
- Charge-coupled device (CCD) camera: black-and white with a pixel density of 768 × 494, chip size 7.95 × 6.45 mm (e.g., XC-ST50, Sony)
- Monitor (e.g., PVM-9L1, Sony)
- Preamplifier (CV-7B, Axon Instruments)
- Amplifier (Axopatch MultiClamp 700B, Axon Instruments)
- CED1401 converter (Cambridge Electronic Design)
- Rectangular open bath chamber (Warner RC-27, Warner Instruments)
- Ag-AgCl electrodes (WA30-5, Warner Instrument)
- Copper wires
- Polyethylene (PE)100 tubing (Becton Dickinson)

REAGENT SETUP

Penicillin/streptomycin-DMEM Sterilize the medium by passing DMEM through a 0.22 µm filter. Add 5 ml of penicillin/streptomycin to 495 ml of sterilized DMEM (PS-DMEM). Prepare fresh and store at 4 °C.

Serum-containing PS-DMEM (10% (vol/vol)) Add 50 ml of fetal bovine serum to 450 ml of PS-DMEM. Prepare fresh and store at 4 °C.

Fibronectin-containing solution Add 10 mg fibronectin to 1 ml PBS to obtain a final concentration of 10 mg ml^{-1}. Prepare fresh and store at 4 °C.

Collagenase type I Prepare the collagenase as a 20× stock solution by adding 100 mg collagenase to 4 ml distilled water to obtain a final concentration of 2.5% (wt/vol) and store at −20 °C. Make the 1× working solution fresh. Add 50 µl of the stock solution to 950 µl distilled water for digestion.

Artificial cerebrospinal fluid Add NaCl, KCl, glucose, $MgCl_2$, $CaCl_2$ to obtain a final concentration of (in mM) 130 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 glucose and 20 HEPES. Adjust pH to 7.4 with NaOH. Prepare the artificial cerebrospinal fluid (ACSF) as 10× stock solution and store at 4 °C for up to 2 weeks. ▲ **CRITICAL** The 1× ACSF solution should be freshly prepared.

Internal pipette solution Add KCl, EGTA, HEPES, $MgCl_2$ to a final concentration of (in mM) 100 KCl, 2 Na_2-ATP, 0.3 Na_3-GTP, 10 EGTA, 5 $MgCl_2$ and 40 HEPES. Adjust the pH to 7.4 with KOH. Osmolarity was

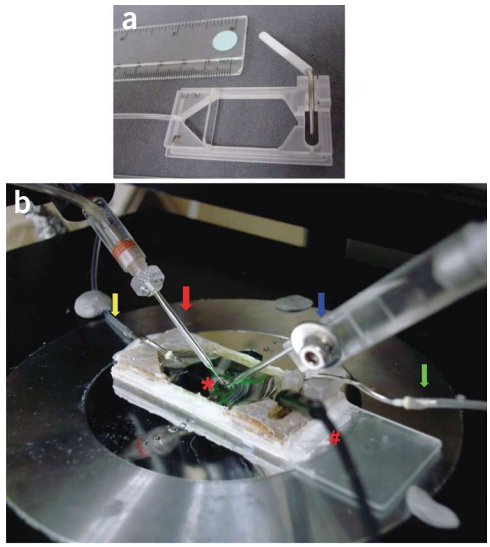

Figure 2 | Digital images of the recording chamber. (**a**) RC-27 rectangular open recording chamber. (**b**) Assembly of the recording chamber. The ACSF is perfused through a PE tube in the left region of the RC27 chamber. The superfluous ACSF is then aspirated off with negative pressure. The recording and indentation pipette are affixed to the pipette holder and actuated with a micromanipulator. A green light source is used to visualize the neuron culture while using phase contrast microscopy. The red arrow indicates the recording electrode and the blue arrow indicates the indentation pipette. The yellow arrow indicates the perfusion PE tube and the green arrow indicates the suction PE tube. The recording chamber is marked by * and the grounding electrode is marked by #.

~300 to 310 mOsm. ▲ CRITICAL This solution should be freshly prepared and used or have the aliquots stored at −20 °C for up to 2 weeks.

EQUIPMENT SETUP

Recording chamber Assemble the recording chamber with a rectangular open bath chamber mounted on a glass slide (76 × 26 mm) to create two compartments, a 4 mm deep hexagon main chamber (long axis, 38 mm; short axis, 12.5 mm) with a suction reservoir to connect to one of the PE tubes (**Fig. 2a**,**b**). Position the perfusion PE tubing at one end of the hexagon chamber opposite from the suction reservoir. If necessary, affix both PE tubes to the chamber with Blu-Tack.

Perfusion system Perfuse the recording chamber with ACSF at a rate of ~2 ml min^{-1} by using gravity-driven flow-through PE100 tubing. To achieve this, first put a bottle filled with ACSF on top of the Faraday cage and introduce the ACSF into the recording chamber with the tubing. Control the height of the ACSF bottle until the gravity produces a flow rate of 2 ml min^{-1}. Place a negative pressure on the PE tubing using a 10 ml syringe until the ACSF flows inside the PE tubing and then into the recording chamber. ▲ CRITICAL Bubbles inside the PE tubes can slow the perfusion rate and cause variations in the flow rate during the experiment. Carefully tap the PE tubing with fingers to reduce the bubbles inside the tubes, if any are present.

Recording electrode Pull a patch pipette by using a pipette puller. The diameter of the pipette tip is determined by the pulling conditions that can also be adjusted during the heating steps of the pulling. Fill the pipette with an internal solution before connecting it to a microelectrode holder. Place the pipette in the ACSF of the recording chamber (**Fig. 2b**). Test the resistance of the recording electrode by using a MultiClamp 700B. Discard the electrode if the resistance is not in a range between 1 and 5 MΩ. ▲ CRITICAL One adjustment that can be made is to increase or decrease the heating temperature that will change the diameters of the pulled pipette tips.

Indentation pipette Pull a patch pipette to a tip radius of ~0.5 to 1 µm by using the pipette puller. Flame the pipette tip with a Bunsen burner for 5–10 s. Examine the pipette tip under the microscope and ensure that the tip is blunt with a tip radius of 4–5 µm. Use a screw to affix the indentation pipette to the electrode holder of a three-axis motorized micromanipulator (Narishige, EMM-3SV) (**Fig. 2b**).

Issues of grounding Noise can be limited by grounding the system with an Ag-AgCl electrode and copper wire. To reduce the electrical noise in the recording chamber, we put a grounding (Ag-AgCl) electrode into the bath of the suction reservoir (**Fig. 2b**). Copper wire was used to ground the electrical noise generated outside the recording chamber, such as noise from the preamplifier, the amplifier, the microscope, the CCD camera or the motorized micromanipulator[43].

PROCEDURE

PDMS preparation ● TIMING 3 d before DRG culture

1| To prepare the PDMS, add 0.1 ml of the PDMS curing agent to 3.5 ml of the base agent in a weighing boat. Gently mix this solution using the cut head of a pipette tip (**Fig. 3a**) to minimize the introduction of bubbles into the 1:35 PDMS mixture.

▲ CRITICAL STEP Avoid creating bubbles in the PDMS during the mixing. If bubbles remain after mixing, degas the resulting solution to eliminate the bubbles. Degas the solution by putting the PDMS mixture in a Speed Vac chamber (SC110, Savant, NJ).

2| Place four 12-mm coverslips in a 35-mm Petri dish and pipette ~90 µl of the mixture onto the top of a 12-mm coverslip using a pipette (**Fig. 3b**).

3| Dry the PDMS coverslips in the laminar flow hood for at least 2 d and store them at room temperature (21–25 °C) until being used in experiments. The thickness of the PDMS should be 0.75 mm if possible.

▲ CRITICAL STEP PDMS that is formulated with a lower percentage of curing agent requires an equally longer time to cure. For dishes with PDMS with a lower percentage of curing agent, place the dishes in a biological safety cabinet for 3 d before using.

■ PAUSE POINT These PDMS coverslips can be stored for up to 1 week in a covered Petri dish.

4| Place the dishes in a laminar flow sterile hood and sterilize them by exposing them to ultraviolet light for 1 h. The PDMS-covered coverslips are higher in the center of the coverslips than at the edges (**Fig. 3c**).

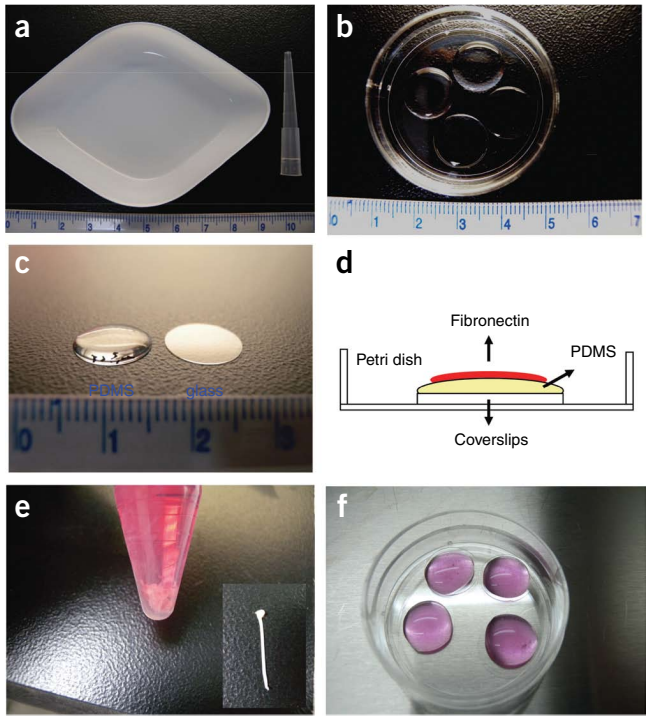

Figure 3 | Preparation of the DRG culture on fibronectin-coated PDMS-covered coverslips. (**a**) A weighing boat and a blunt tip are used for mixing the PDMS. (**b**) Four 12-mm coverslips have PDMS drops with a 1:35 PDMS mixture. These coverslips are placed in a 35-mm Petri dish. (**c**) A PDMS-covered coverslip and a glass coverslip with no PDMS. (**d**) A schematic of fibronectin coating on a PDMS-covered coverslip. (**e**) DRG suspension after treatment with collagenase and trypsin. The inset in the lower left corner is a freshly isolated DRG. (**f**) Seeding a DRG cell suspension on the top of the fibronectin-coated PDMS coverslips. The experimental protocol was approved by the Institute of Animal Care and Use Committee of Academia Sinica.

5| Sterilize the dish by using 70% (vol/vol) ethanol, as well by pipetting 70% ethanol on top of the PDMS and allowing it to cover the PDMS for 5 min. After this sterilization, wash the ethanol off by rinsing with PBS three times before proceeding further with the protocol.

Coating of the PDMS-covered coverslips with fibronectin ● TIMING 2 h (1 d before cell culture). All steps are conducted in a biological safety cabinet

6| To coat fibronectin on the PDMS-covered coverslips, Q4 place four PDMS-covered coverslips in a 3.5-cm Petri dish (PDMS side up). PDMS membranes can alternatively be coated with other molecules of interest to generate adhesion-specific surfaces. See **Box 1** and **Figure 4** for more information and the associated protocol.

7| With a pipette loaded with 100 µl of fibronectin, position the pipette at the center of the PDMS coverslips and then Q10 slowly release the fibronectin liquid until the resulting solution pool covers the entire PDMS region (**Fig. 3d**). Incubate the plates for at least 2 h in the biological safety cabinet before seeding with cells.

▲ **CRITICAL STEP** Controlling the amount of fibronectin that is pipetted onto the middle of the PDMS-covered coverslips is important. The cells have a better survival rate at the center of the coverslips than at the edge, when the coating is not uniform across the entire PDMS-covered coverslip.

Washing of PDMS coverslips ● TIMING 1 h (1 d before cell culture)

8| Aspirate the fibronectin with negative pressure and wash the PDMS coverslips twice with sterile water. Remove the water after the final wash and allow the dishes to remain in the biological safety cabinet until further use.

▲ **CRITICAL STEP** Long-term storage is not recommended. After 1 week of storage, the survival rates of neurons when culturing them on these substrates decrease significantly.

■ **PAUSE POINT** Fibronectin-coated PDMS coverslips can be stored at room temperature for up to 1 week.

Harvesting DRG neurons ● TIMING 20–30 min

9| Add 15 ml of DMEM to a 15 ml conical centrifuge tube.

10| Kill an adult mouse (8–12 weeks old) using CO_2 and thoroughly rinse the skin of the mouse back with 70% ethanol. Clean the surgical instruments (scissors and no. 5 forceps) with 70% ethanol, remove the skin of the mouse and dissect out the spine before carefully removing the muscle tissue with forceps and scissors from the isolated spine.

▲ **CRITICAL STEP** Researchers must adhere to the institutional guidelines regarding animal use.

11| Remove and clean the spinal cord. Remove the membrane over the DRGs using sterile no. 5 forceps. Cut the fiber connection to the ganglion and transfer the ganglion to the DMEM-filled 15 ml conical centrifuge tube. Additional details for the process of DRG isolation can be found in these protocols[44,45].

DRG primary culture ● TIMING ~5 h

12| Centrifuge the ganglion suspension at 190*g* for 3 min to pellet the ganglia and remove the supernatant. Resuspend the ganglion pellet with 1 ml DMEM containing 0.125% (wt/vol) type IA collagenase and incubate it at 37 °C for 1.5 h (**Fig. 3e**).

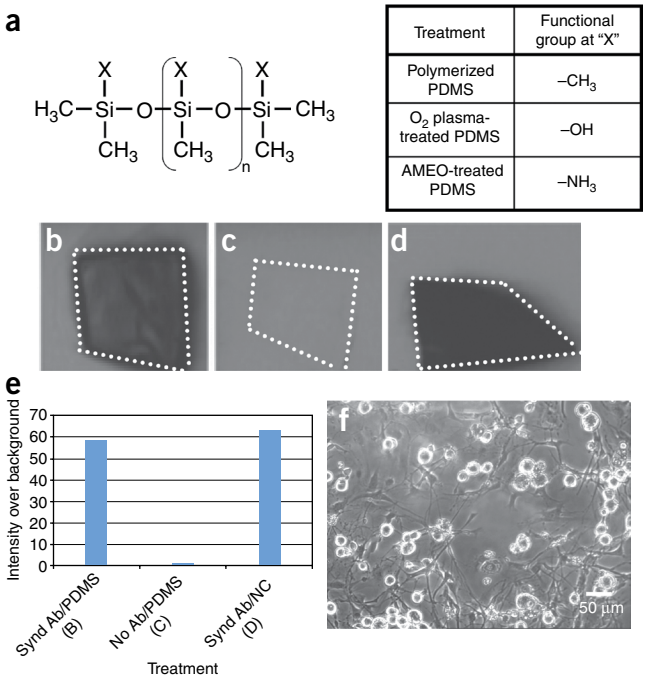

Figure 4 | Modification of the PDMS substrate with antibodies against cell surface proteins, an alternative procedure to fibronectin coating. (**a**) The structure and chemical surface modifications of PDMS used in this protocol to prepare the surface of PDMS for antibody absorption. (**b**–**e**) The effectiveness of conjugating antibodies to treated PDMS membranes was tested using a secondary antibody-based assay. (**b**) PDMS coated with rat IgG molecules (i.e., antibodies) with the AMEO modification approach. (**c**) PDMS with the AMEO modification approach, but with no antibody in the coating solution and (**d**) Rat IgG molecules absorbed on nitrocellulose. For **b**–**d**, antibody conjugation was probed by incubating the membrane pieces with goat anti-rat HRP secondary antibody, followed by detection with enhanced chemiluminescence. The shape and position of the PDMS (**b** and **c**) and nitrocellulose (**d**) pieces are marked with dashed lines. (**e**) Quantitative analysis of the results shown in **b**–**d**. The average signal intensity outside each sample area was subtracted from the average signal intensity within the sample area, as determined by ImageJ. (**f**) A 2-d DRG culture on the AMEO-modified PDMS coated with anti-β1 integrin antibodies. A total of 18,000 cells were seeded on the substrate. The appearance of shiny, round neurons in this image indicated that DRG cultures can be readily established through this approach on PDMS coated with antibodies for cell surface proteins.

Q7

13| Add 5 ml of DMEM and mix the suspension with finger tapping before centrifuging and pelleting again at 190*g* for 3 min. Remove the supernatant and resuspend this pellet in 1 ml DMEM containing 0.25% trypsin. Incubate this solution at 37 °C for 20 min.

14| Add 10 ml DMEM and mix the ganglion suspension before centrifuging and pelleting (190*g* for 3 min). Remove the supernatant while keeping the pellet. Add 10 ml DMEM and repeat this step (Step 14) once.

15| Resuspend the ganglia in 800 µl DMEM.

16| Freshly prepare a flamed polished Pasteur pipette for trituration. To create the desired Pasteur pipette tip diameter, control the time that the tip is exposed to the flame of the Bunsen burner until the pipette opening is around 0.5–0.7 mm in diameter.

17| Triturate the ganglion suspension through the flamed polished Pasteur pipette five to eight times until the suspension becomes visually clear.

▲ **CRITICAL STEP** Excessive trituration for more than eight times is not recommended, because DRG neurons will be damaged by the shear force causing a low survival rate.

? TROUBLESHOOTING

18| Pipette 100–150 µl of cell suspension onto the fibronectin-coated PDMS coverslips using a new, sterile Pasteur pipette (**Fig. 3f**).

▲ **CRITICAL STEP** A low cell density is desired. Because this is a neuron–glial cell coculture, a high cell density can lead to a large population of glial cells and neurons on the PDMS systems. If this occurs, the neurons will grow extensive neurites, which creates significant challenges for accessing and then indenting the substrate. A typical cell density for seeding is in the range of 10,000–18,000 dissociated cells for a 100 µl cell suspension. To control the cell density, use a hemocytometer to determine the cell density and then apply the appropriate trituration for seeding. A total of 40–50 ganglia can usually be used to seed all of the eight PDMS-covered coverslips.

? TROUBLESHOOTING

19| Carefully transfer the dish containing cell-seeded coverslips to a 37 °C and 5% CO_2 incubator.

20| After 1 h of incubation, add 2 ml DMEM to the dish. To prevent the PDMS coverslips from floating on top of the solution, slowly add media to the top of each coverslip. Return the cell-seeded coverslips to the incubator and incubate for 1 to 2 d. The time for DRG neurons to grow into neurites is 1 to 2 d.

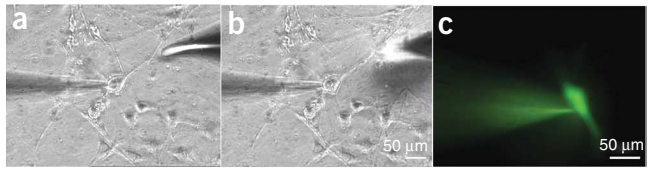

Figure 5 | Imposing mechanical stretching on a single neurite of a patched neuron. (**a**) A neuron with a trackable neurite is patched with a recording pipette. An indentation pipette is positioned 100 μm away from the patched neuron and 10–15 μm away from the neurite. (**b**) Indenting the pipette into the PDMS substrate stretches the neurite and the electrical response is examined under current clamp mode. (**c**) The usage of Lucifer yellow in the recording pipette allows the patched neuron to be more easily visualized with UV light and also the whole-cell conditions to be monitored during the stretching. Panel **c** was published previously in Lin *et al.*[11].

Establishing recording condition ● TIMING ~1–10 min

21| Prepare patch pipettes with resistances of 1–5 MΩ.

22| Prepare a recording electrode by filling the inside of a patch pipette with the internal pipette solution and then connecting the pipette to a microelectrode holder (**Fig. 2b**, indicated with a red arrow).

23| Perfuse the bath with an external solution (the solution outside of the pipette) of (in mM) 130 NaCl, 5 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 glucose and 20 HEPES, adjusted to a pH of 7.4 with NaOH.

24| Place the coverslip (with the DRG culture) into the recording chamber and fasten the coverslip in the chamber by topping with a screw nut.

25| Visualize the coverslips under the microscope. Manually move the stage with the coverslip until the DRG neurons are in the field of view. Next, use the micromanipulator to move the recording electrode onto a neuron containing trackable neurites that are not attached to other cells (**Fig. 5a**).

▲ **CRITICAL STEP** It is important to select neurons that have isolated neurites. These neurons must not be connected to other neurons or surrounded by glial cells. Maintaining a DRG culture for more than 5 d is not recommended because the glial cells will grow over the entire PDMS substrate surface leaving no area for the pipette indentation.

Obtaining whole-cell patch clamp recording for neurite stretching ● TIMING From 10 min to several hours

26| Actuate the recording pipette near the selected neuron. When the recording pipette contacts the neuron, the resistance is significantly increased. Use a syringe to apply a negative pressure on the neuron to get whole-cell recording. During this time, the recording electrode should become fused with the neuron with a giga Ω seal.

27| Use a current clamp mode to record action potentials (APs) evoked by current injection. Repeat this step after a series of mechanical indentations, so that the response of the patched neuron can be examined and it can be determined whether or not it remained reliable. Throughout the recording period, monitor the series and input resistance carefully by applying a 100 msec negative pulse of 50 pA at 0.1 Hz. The input resistances are acceptable in the range of 400–600 MΩ and the series resistance can be compensated by 70–80% through using Axopatch 700B compensation circuitry. Additional details for the current clamp recording approach can be found in this protocol from Cummins *et al.*[46].

? TROUBLESHOOTING

28| Use the flamed polished pipette to indent the PDMS substrate with the micromanipulator at a distance of ~100 μm away from the recording neuron and 10 μm away from the neurites (**Fig. 5b**). To obtain the whole-cell recording for neurite stretching, gently indent the PDMS substrate with the micromanipulator. Typically, the indentation velocity should be ~150 μm s^{-1}.

▲ **CRITICAL STEP** To precisely position the indentation pipette, use a CCD camera connected to a monitor to visual the patched neuron and guide the micromanipulator. First, move the indentation pipette to the general field of view for the patched neuron and then fine-tuned the location of pipette to the desired position on the monitor. In our case, the 100 μm distance equaled 12.5 cm on the monitor screen.

? TROUBLESHOOTING

29| After the indentation, carefully monitor the whole-cell conditions with an amplifier and monitoring system (Axonclamp 700B drive with Signal 3.0 software). Stop and discard the recording if the series resistance varies >30% from the original values during the experiment.

▲ **CRITICAL STEP** The whole-cell condition can be monitored during the indentation by adding Lucifer yellow (2 mg ml^{-1}) in the recording pipette (**Fig. 5c**). Lucifer yellow will leak out of the neuron and will be observed in the media with UV light when whole-cell viable conditions are disrupted.

? TROUBLESHOOTING

30| For multiple indentations, use at least a 30-s interval between each indentation.

▲ **CRITICAL STEP** The timing and depth of the indentation can be monitored and controlled by the EMM-3SV micromanipulator. The use of the micromanipulator provides the advantage of having control over the timing of the indentation and also can

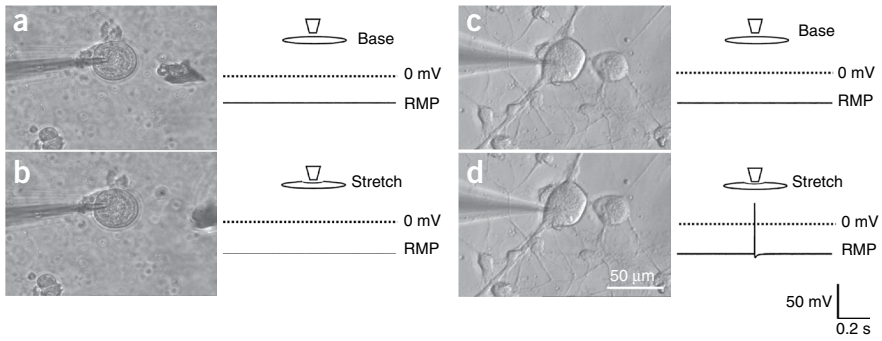

Figure 6 | Effects of distal indentation on neurite-free and neurite-bearing neurons. (**a**) A patched neurite-free neuron before PDMS indentation. (**b**) The neurite-free neuron showed no Vm change during the indentation. (**c**) A patched neurite-bearing neuron before the PDMS indentation. (**d**) The neurite-bearing neuron showed an AP response to indentation. RMP, resting membrane potential. (All neurons here are patched with the recording electrode at the left region.)

minimize the effect of unwanted human error such as when manually actuating the recording chamber. This further stabilizes the whole-cell conditions for the experimental procedure.

? TROUBLESHOOTING

● TIMING

Steps 1–5, PDMS preparation: 3 d
Steps 6–8, Coating of the PDMS-covered coverslips: 2 h + 1 h washing
Steps 9–11, Harvesting DRG neurons: 20–30 min
Steps 12–20, DRG primary culture: ~5 h + 1–2 d for neurite outgrowth
Steps 21–25, Establishing recording conditions: ~1–10 min
Steps 26–30, Obtaining whole-cell recording for neurite stretching: 10 min to hours
Total steps required ~5 d

? TROUBLESHOOTING

When there is a low survival rate for neuron growth on PDMS

The neuron survival rate is usually low when culturing on PDMS substrates as compared with glass. In DRG primary culturing, the trituration step (see Step 17) is the most damaging step and thus very critical for neuron surviving. One can practice the DRG culture on fibronectin-coated glass surface first to examine whether an approximate trituration is developed before seeding on PDMS-covered coverslips. It is also important to accurately control the cell density for seeding on the PDMS surface (see Step 18). Neurons have an extremely low survival rate at low cell densities. Furthermore, the low culture rates on the PDMS-covered coverslips may have challenges, as during the incubation and attachment periods, the cells are suspended in a pool of media that is not completely constrained to only the PDMS surface. This can be exacerbated by movement of the coverslips before the cells have attached (see Step 19). To confine the cell suspension for improving cell attachment, a plastic ring can be made by cutting the top of the cap of a 15 ml conical centrifuge tube; this helps constrain the cell suspension to the center of PDMS coverslips.

Electrical noise

The environmental noise can be largely reduced by using a Faraday cage. The source of electrical noise in these systems could be generated from electrical devices inside the Faraday cage including the light source, the CCD camera, the microscope or the manipulator (see Steps 28 and 30). Using copper wires to ground electrical devices helps to reduce the noise.

When whole-cell conditions are disrupted during the indentation

Selection of healthy neurons is essential for a stable whole-cell recording (see Steps 27 and 29). A healthy DRG neuron is shiny, has a smooth membrane and has a round or oval shape when visualized using phase contrast microscopy (as seen in **Figs. 5a** and **6c**). A neuron with a rough or dark (or even broken) membrane should not be used. To avoid jeopardizing the whole-cell conditions during indentation, it is important to use a vibration isolation table and a computer-controlled manipulator (see Steps 28 and 30). In addition, the perfusion rate should be kept at a low and steady rate (we used 2 ml min^{-1}) to minimize the influence on whole-cell responses. Make sure there are no bubbles in the perfusion tube, as this will cause significant issues.

ANTICIPATED RESULTS

Localized indentation-induced mechanical perturbations on the PDMS substrates are repeatable

When we used a pipette to indent the PDMS substrate, we generated a deformation that is correlated with the indentation depth. Our system will work if the indentation-induced substrate deformation is repeatable. To track the displacements in the substrate during indentation, we mixed 10 μg of fluorescent beads with 1 mg of PDMS. When we imposed a 100 μm deep

indentation at the same position on the substrate, the displacements of the fluorescent beads were consistent for many iterations (ten indentations were conducted) (**Supplementary Fig. 1**). This indicates that the localized indentations can repeatedly generate the same substrate deformation and associated bead displacement.

The indentation force is correlated with the indentation depth

When we imposed an indentation on the PDMS substrate, we generated a force to stretch the adjacent neurites (**Fig. 1**). Along with experimentally determining the response of the PDMS, we also modeled this approach using a Finite Element method (ANSYS)[47–49] as shown in **Supplementary Figures 2–4**. The dimensions that were used in this modeling were the radius of the glass pipette (4.2 μm), a Young's modulus of the PDMS of 90 kPa and a Poisson's ratio for the PDMS of 0.25. The Young's modulus of the PDMS was determined by the ratios of the curing agent to the base agent[34,35]. Indenting the pipette into a 1-mm thick PDMS slab to a depth of 125 μm is accomplished through a 57 μN force, as shown in **Supplementary Figure 2**. Furthermore, PDMS has a very low Young's modulus compared with glass (~70 GPa). The needle vertically displaces the PDMS surface down, and due to the much higher Young's modulus of the needle (over six orders of magnitude), the compression of the tip is extremely small, which is also observed in the ANSYS analysis. Because of this, the displacement of the needle in the actuation system is assumed to be identical to the actual needle tip displacement. In addition, the thickness of the PDMS will affect the displacement of the needle. We have done additional Finite Element modeling to examine the displacement with respect to the thickness of the PDMS (**Supplementary Fig. 3**). When increasing the thickness of the PDMS from 500 μm to 2 mm, the amount of force that is related to the indentation of the pipette to achieve a depth of 125 μm increases from 47 to 59 μN. We also provide a relationship that is curve fit to a line (R = 0.9999) based on additional ANSYS modeling. This relationship allows one to use this approach and determine the indentation force from the displacement depth for a PDMS slab with a thickness of 0.75 mm (**Supplementary Fig. 4**). In practice, we do not recommend an indentation greater than 125 μm, because we have found that this causes issues with maintaining the whole-cell patch conditions.

Measuring the alteration of membrane potential by stretching neurite-free or neurite-bearing neurons

With an indentation depth of 125 μm, the stretching force has minimal effect of Vm changes on neurite-free neurons that are ~100 μm away (**Fig. 6a**,**b**). The average Vm change (δ) was 2 mV, which is far below the threshold to evoke an AP[11]. At a closer indentation distance of 30 μm, an increase in Vm can occur to values of ~8–10 mV in neurite-free neurons. These values are not enough to evoke an AP in the DRG neurons that we recorded. In contrast, the same indentation depth will elicit AP response in ~44% of neurite-bearing neurons that are within a 100 μm radius (**Fig. 6c**,**d**). Therefore, the stretch-activated AP is dependent on neurite stretching. The stretch-activated AP response is also very reproducible. When we indented the PDMS multiple times with the same indentation depth, we recorded an AP response as long as the whole-cell conditions remained intact.

Measuring the mechanical threshold to fire an AP on a single neurite

As the stretch-activated AP response is reproducible in this system, we could determine the mechanical threshold to evoke an AP by gradually increasing the indentation depth on the PDMS substrate (**Fig. 7**). Before reaching the threshold, the indentation has limited effect on the resting membrane potential (**Fig. 7a**,**b**). When the indentation was in a sub-threshold range, we did observe a small change in membrane potential, although the depolarization was not enough to activate voltage-gated sodium channels (**Fig. 7c**). The average indentation depth to evoke the stretch-activated APs in neurite-bearing neurons was ~80 μm, which according to our ANSYS analysis was equal to a downward force of 33 μN for a 0.75-mm thick PDMS substrate. The mechanical thresholds vary in range from 40 to 120 μm.

Examining the molecular specificity of the stretch-activated mechanotransduction in neurites

Coating these PDMS substrates with fibronectin enabled increased neuron survival and neurite outgrowth (**Fig. 8**). These neurons were also mechanically sensitive to distal stretch. As fibronectin is an RGD-containing extracellular matrix protein, the neurite outgrowth is likely linked to integrins and also the

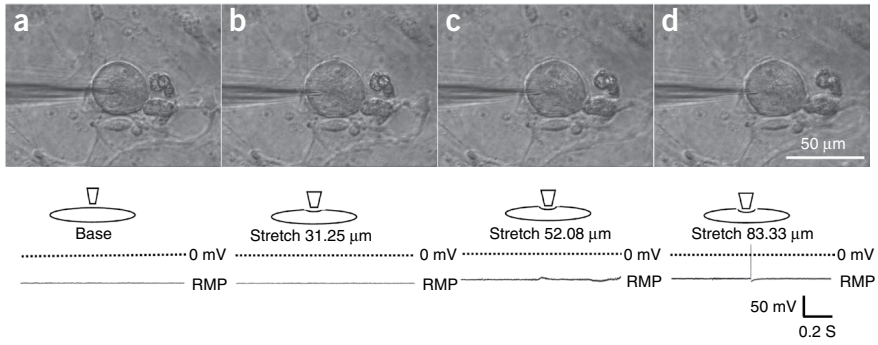

Figure 7 | Measuring the mechanical threshold in neurite stretching, which will evoke an action potential. (**a**) A patched neurite-bearing neuron before PDMS indentation. (**b**) The neuron showed no change in membrane potential when the indentation pipette indented the PDMS to a 31 μm depth. (**c**) A further indentation to 52 μm induced a small change in the membrane potential. (**d**) An AP was evoked when the indentation depth reached 83 μm.

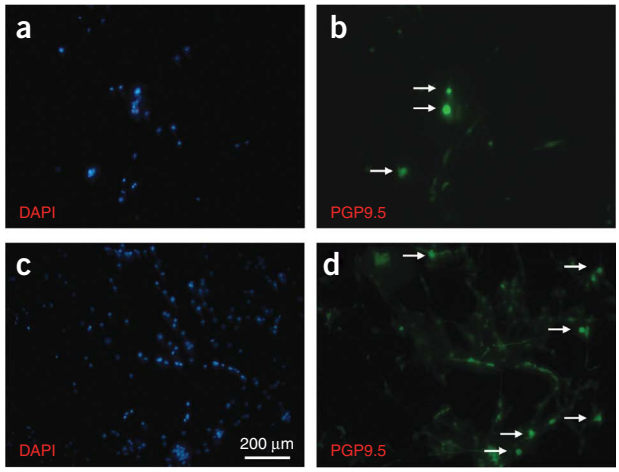

Figure 8 | Effects of fibronectin on neurite outgrowth for PDMS substrates. (**a**) DAPI staining indicating the number of cell nuclei in a 2-d DRG culture on a PDMS substrate without fibronectin coating. (**b**) Protein gene product 9.5 (PGP9.5) staining indicating the position of the neurons on the same fibronectin-free PDMS substrate. (**c**) DAPI staining for cell nuclei in a 2-d DRG culture on a PDMS substrate coated with fibronectin. (**d**) PGP9.5 staining for neurons on the same fibronectin-coated PDMS substrate. Arrows indicate the neurons with PGP9.5 staining.

cytoskeleton. Consistent with this idea, we observed that the PDMS stretching-evoked AP response was abolished when the recording chamber was incubated with agents that disrupted the cytoskeleton architecture such as with nocadazole and cytochalasin D and latrunculin A[11]. The PDMS stretching-evoked APs in neurite-bearing DRG neurons are influenced by both extracellular matrix and cell architecture.

Q6

Potential applications of the protocol

The idea of using an elastomeric substrate and an indentation probe to elicit a mechanical response could be used to study a diversity of additional areas including among others, probing mechanotransduction in a range of cell types, the specific role of extracellular matrix proteins under loading and subcellular signaling pathways due to localized mechanical stimulation. This would be of interest to a diversity of fields including biology, medicine, physics, material science and engineering.

Note: Supplementary information is available via the HTML version of this article.

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AUTHOR CONTRIBUTIONS C.-M.C., P.R.L. and C.-C.C. conceived and designed the experiments. C.-M.C., Y.-W.L., R.M.B., Y.-R.C. and R.L.S. performed the experiments. C.-M.C., Y.-W.L., R.M.B., Y.-R.C. and R.L.S. analyzed the data. C.-M.C., Y.-W.L., R.M.B., P.R.L. and C.-C.C. contributed reagents/material/analysis tools. R.M.B., P.R.L. and C.-C.C. wrote the paper.

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