Video Article

A Microbiomechanical System for Studying Varicosity Formation and Recovery in Central Neuron Axons

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Abstract

Axonal varicosities are enlarged structures along the shafts of axons with a high degree of heterogeneity. They are present not only in brains with neurodegenerative diseases or injuries, but also in the normal brain. Here, we describe a newly-established micromechanical system to rapidly, reliably, and reversibly induce axonal varicosities, allowing us to understand the mechanisms governing varicosity formation and heterogeneous protein composition. This system represents a novel means to evaluate the effects of compression and shear stress on different subcellular compartments of neurons, different from other *in vitro* systems that mainly focus on the effect of stretching. Importantly, owing to the unique features of our system, we recently made a novel discovery showing that the application of pressurized fluid can rapidly and reversibly induce axonal varicosities through the activation of a transient receptor potential channel. Our biomechanical system can be utilized conveniently in combination with drug perfusion, live cell imaging, calcium imaging, and patch clamp recording. Therefore, this method can be adopted for studying mechanosensitive ion channels, axonal transport regulation, axonal cytoskeleton dynamics, calcium signaling, and morphological changes related to traumatic brain injury.

Video Link

The video component of this article can be found at https://www.jove.com/video/57202/

Introduction

Varicosity formation, or swelling/beading, along axons, is a prominent feature of neurodegeneration observed in many disorders or injuries of the central nervous system, including multiple sclerosis, Alzheimer's disease, Parkinson's disease, and traumatic brain injury[1,2]. Despite the significant physiological impacts of axonal varicosities on action potential propagation and synaptic transmission[3], how the varicosities are generated remains unknown. Recently, using a newly-established microbiomechanical assay on cultured hippocampal neurons from rodents, we found that mechanical stimuli can induce varicosities in these neurons with highly intriguing features. First, varicosity induction is rapid (<10 s) and this process is unexpectedly reversible. Second, varicosity initiation depends on the strength of puffing pressure: the higher the pressure, the faster the initiation. Third, varicosity initiation depends on neuronal age. The axons of younger neurons appear more responsive to mechanical stress, compared to those of older neurons. Fourth, the varicosities form along the axons of hippocampal neurons, while the dendrites and initial axon segments of these neurons display no change under the same puffing condition. Thus, our study revealed a novel feature of neuronal polarity. These findings with the *in vitro* system are physiologically relevant. Using an *in vivo* model for mild traumatic brain injury (mTBI), we showed that axonal varicosities developed in a multi-focal fashion in the somatosensory cortex of the mice immediately after close-skull impact, consistent with our *in vitro* results[4]. It is important to note that our staining and imaging of the mTBI mice only provide a snap shot of neuronal morphological changes, since performing *in vivo* time-lapse imaging of neuronal morphology during a mechanical impact is still not feasible.

This fluid-puffing system allowed us not only to capture unique features associated with mechanical stress-induced varicosity formation, but also to determine the underlying mechanism. By testing different extracellular solutions, the blockers and openers of different candidates of mechanosensitive ion channels, and cellular electrophysiology, we identified that the transient receptor potential cation channel subfamily V member 4 (TRPV4) channel that is permeable to Ca^{2+} and Na^{+} and activated by puffing is mainly responsible for detecting initial mechanical stress during axonal varicosity formation[4]. This was further confirmed with an siRNA knockout approach. Taken together, this new assay system we have developed with hippocampal neuron culture, is highly valuable for studying the micromechanical properties of central neurons, especially in combination with other techniques.

This micromechanical system we have established is unique and differs from the previously-existing systems in several major aspects. First, in this system, the neurons experience out-of-plane mechanical stress in the forms of compression and shearing. During the mechanical impact, neuronal processes remain attached to the coverslip surface and do not move. This differs from other experimental systems that mainly involved bending and in-plane stretching (or tension), for instance, the deflection of bundled axons like moving strings[5,6] or stretching axons grown on

micropatterned channels and stretchable membranes[7,8]. Moreover, although axonal varicosities can also be induced in these assays like in our fluid-puffing system, the process in these settings takes much more time (from 10 min to several hours[6,7,8]) and appears irreversible. Finally, our system using local fluid puffing allows the examination of the spatial features of varicosity formation (*e.g.*, dendrites, dendritic spines, soma, axonal initial segments, axonal terminals), besides its temporal features. Using this system, we discovered several unexpected and unique features of axonal varicosity formation, especially rapid onset, slow reversibility, and axon-dendrite polarity.

The system that we discuss in this paper is compatible with many techniques of molecular and cell biology. For instance, to study the effects of mechanical stress on neuronal morphology and function, it can be used together with myelin coculture, time-lapse imaging of fluorescent resonance energy transfer (FRET) and total internal reflection fluorescence (TIRF), calcium imaging, and patch clamp recording. In this paper, we focus on the core components of the system. Hippocampal neuron culture, the fluid-puffing setup, high-resolution time-lapse imaging for axonal transport, and calcium imaging are illustrated step-by-step below.

Protocol

All methods described below have been approved by the Institutional Animal Care and Use Committee (IACUC) of the Ohio State University.

1. Coverslip Preparation

1. Place one or more boxes of 12 mm or 25 mm coverslips into a glass beaker containing 70% nitric acid and incubate the coverslips at room temperature overnight.
   NOTE: Do not wash the 25 mm coverslips in the same beaker as the 12 mm coverslips. It is better to wash them separately.
2. Transfer all coverslips into a 4 L beaker filled with 2.5 L of ddH_2O and shake the beaker containing the coverslips for 1 h at 100 rpm. Use rubber bands to hold the beaker during shaking. Repeat the rinse with fresh 2.5 L ddH_2O four times.
3. Transfer cleaned coverslips to a dry flask with a metal cover. Bake the coverslips in an oven at 225 °C for 6 h and allow them to cool to room temperature.
   NOTE: The 25 mm coverslip is prone to breaking. Separate coverslips one by one and air dry, then bake them in an oven.
4. Store the dried and cleaned coverslips at room temperature in a sterilized plastic container until usage.
5. **Coat the coverslips as follows:**
   1. Prepare fresh coating solution in a centrifuge tube.
   2. Place each coverslip into one well of a 24-well (or 6-well) plate. Place 30 (or 100) µL of coverslip coating solution (**Table 1**) onto a 12 mm (or 25 mm) coverslip and swirl. Add 1 mL of sterile phosphate-buffered saline (PBS, tissue culture grade) to the well. These plates can either be used immediately or stored at 4 °C in sterile PBS for up to a week.
   3. On the day of dissection, remove the PBS. Air-dry and place the plates under UV light (30W) for 2-4 h.
      NOTE: The last step should be completed with the UV light on in a cell culture laminar flow hood. The door of the hood should be lifted slightly to allow air flow for drying the coverslips.

2. Dissection, Dissociation, and Culture of Hippocampal Neurons from Pregnant Mouse/Rat

1. **Dissection and dissociation of hippocampal neurons for culture**
   1. Thaw protease enzyme solution (3 mg/mL protease 23 in SLDS, **Table 1**) and pre-warm plating media (**Table 2**) at 37 °C.
   2. Dissect hippocampus and rinse with SLDS, according to the methods described in previous publication[14].
      NOTE: Four hippocampi provide enough cells for one 24-well or one 6-well plate.
   3. Remove SLDS, add 2 mL of protease enzyme solution, and incubate the sample at 37 °C, 5% CO_2 for 15 min.
   4. Wash the hippocampus explants with 5-10 mL of plating medium twice. Add 5 mL of plating medium. Dissociate hippocampal explants into single cells by generating a small vortex using a pipette with a 1-mL plastic tip. Pipette up and down about 40 times, avoiding the formation of oxygen bubbles, until the solution becomes cloudy and no large pieces of explant remain.
      NOTE: Remember to leave some media during washes, the hippocampal explants should remain submerged in the medium during the whole process.
   5. Centrifuge the cells at 1,125 x g for 3 min. Remove the supernatant with the cells submerged in media. Resuspend the cells in 145 mL of plating media. Add 1 mL/well of the cell suspension to a 24-well plate (3 mL/well to a 6-well plate).
      NOTE: 145 mL of plating media is used to produce a cell suspension that has a low cell density. This volume will allow the plating of cells into six 24-well plates, eight 6-well plates, or various combinations of 24- or 6-well plates depending on the experiments to be conducted. Each well of the 24-well plate will have about 3 x 10^4 cells[9].
   6. Incubate the cells in the plating media at 37 °C, 5% CO_2 for 2-4 h, then remove all plating media and replace it with fresh maintenance media.
   7. After 2 days (2 days *in vitro*, 2DIV), replace half of the media with 2 µM Ara-C (arabinosylcytosine) dissolved in maintenance media which inhibits growth of fibroblasts, endothelial, and glial cells. After exposing cells to Ara-C for two days, replace the media with fresh maintenance media.

2. **Transfection for the visualization of cell morphology**
   NOTE: Transfect the cells with fluorescent constructs of green fluorescent protein (GFP), yellow fluorescent protein (YFP), mCherry, *etc.* to illuminate the morphology of individual neurons.
   1. Dilute the constructs from stock (1 µg/µL) into Opti-MEM media (0.8 µg of the construct in 50 µL of Opti-MEM media) and vortex. Prepare 1 construct dilution for each well.
      NOTE: A 24-well plate requires 0.8 µg of construct in 50 µL of Opti-MEM media. A 6-well plate requires 2.4 µg of construct in 150 µL Opti-MEM media.

2. Dilute liposome-mediated transfection reagent into Opti-MEM media (1.5 µL of the transfection reagent in 50 µL of Opti-MEM media) and vortex. Prepare 1 transfection dilution for each well.
   NOTE: A 24-well plate requires 1.5 µL of transfection reagent in 50 µL of Opti-MEM media. A 6-well plate requires 4.5 µL of transfection reagent in 150 µL of Opti-MEM media.
3. Prepare a 1:1 mixture (100 µL) of the construct in Opti-Mem media and transfection reagent in Opti-MEM media and vortex. Incubate the mixture at room temperature for 20 min.
   NOTE: Per well, there should be about 100 µL of solution containing construct, transfection reagent, and Opti-MEM media if using a 24-well plate (300 µL for 6-well plate).
4. Remove half of the media (500 µL for 24-well plate, 1.5 mL for 6-well plate) from each well and transfer this media into a clean conical tube. Keep this tube in the incubator. Then add the 100 µL mixture (step 2.2.3) to the remaining media in the well. Allow the cells to incubate at 37 °C for 20-30 min.
5. During the incubation (step 2.2.4), add one volume of fresh maintenance media to the media in the conical tube (step 2.2.4). Place the mixture in the same incubator (37 °C and 5% CO_2).
6. Following the incubation, remove all media containing the transfection solution from the wells and replace it with the 1:1 mixture of old and fresh maintenance media in the conical tube (step 2.2.5).
   NOTE: Depending on the size of the constructs, cells may begin expressing within 12 h; for larger constructs, 3-4 days may be needed before the peak expression of the constructs is reached.

3. Setting Up the Puffing Pipette Apparatus

NOTE: There are five basic components to this set-up: the glass pipette, the tubing, the syringe, the micromanipulator, and the buffer (Hank's). Any buffer that has physiologically relevant salt concentrations could be used in this set-up.

1. Pull a borosilicate pipette to have around 45-µm-diameter opening tip using a micropipette puller and the specifications in **Table 3**.
   NOTE: It was found that 45 µm is a suitable size for the diameter of the pipette opening under experimental conditions. Slight variations in the opening size should not significantly affect results. It is important to note that larger departures from this number should still work for varicosity induction by adjusting the height of the syringe that is linked to the pipette via tubing, but this will require recalibration of the pressure value.
2. Insert the un-pulled end of the pipette into thin rubber tubing.
   NOTE: The tubing wall should be thin and rigid to minimize the fluid resistance, ensuring that the height of the syringe is directly proportional to the pressure of fluid flowing through the pipette with negligible resistance. The tubing should be no longer than about 0.6 m in length to further minimize the resistance.
3. Connect the other end of the tubing to a 10-mL plastic syringe through a connector with a turn-able valve.
   NOTE: The plastic syringe needs to be held at a constant, measurable height to ensure an accurate measure for the assumed pressure flowing from the pipette. A height of 190 mm was utilized, since this provides minimal pressure but reliably induces varicosities in axons. Other height values may also be used if the pressure cause the cells to detach from the coverslip surface. It is recommended to use the same setting throughout the experiment.
4. Insert the pipette into the screw top of the micromanipulator to hold the pipette in place. Screw the metal, flat topped screw attached to the micromanipulator arm so that it holds the un-pulled end of the pipette just above where the rubber tubing is attached. Angle the pipette at a 45° angle with the surface of the neuron-containing coverslips.
   NOTE: The micromanipulator should be constructed so that the pipette can be held without constricting the rubber tubing. The micromanipulator must allow motion of the pipette in the x, y, and z directions to accurately position it within range of the cells. A graphic as well as a photograph of the apparatus is provided below (**Figure 1**).
5. Turn on a fluorescence filter, open the aperture, and ensure a fluorescent spot can be seen coming through the objective. Using the micromanipulator, move the pipette into a position that lines up the tip with the fluorescent spot and lower the pipette into a position just above the height of the cell culture dish (35 mm x 10 mm). Once it is in position, turn on the transmitted light and turn off fluorescence.
6. Using tweezers, add one coverslip (with the cells facing up toward the pipette) from the cell culture plate to the cell culture dish containing about 2 mL of Hank's buffer at room temperature.
7. Place the culture dish containing the coverslip onto the scope.
8. Using the fine focus knob and 20X objective, focus on a plane about four full turns above the cells (about 0.4 mm). Use the micromanipulator to position the pipette tip so that it is focused and in the center, left-hand side of the plane as seen through the eye-pieces.
   NOTE: Dendrites are projections that should be in the same frame as the cell body they originate from. To induce axonal varicosities, one should follow longer projections from the cell body to medial and distal axons.

4. Calibrating the Pressure of the Pipette Utilizing a Stretchable-membrane System

1. Setup the puffing pipette apparatus with the exact same parameters as described above over a system containing a microfabricated silicone membrane (500 µm in diameter and 50 µm thick) and focus the microscope onto the surface of the membrane.
   NOTE: This system was designed to measure small pressure values and an explanation of the system has been published before[10]. This measurement only needs to be done once for puffing experiments if the exact same setting of puffing is used.
2. Focus the microscope on the arrays of microdots (4 µm in diameter and 10 µm apart, as measured between centers). Turn the stop valve and allow fluid to flow out of the pipette. Examine the deflection of the membrane in response to the fluid pressure with a confocal microscope.
3. Utilize a linear model to determine the corresponding pressure associated with deflection of the membrane.
   NOTE: While a recent study found that a deflection of w= -3.143 ± 0.69 µm was obtained for 190 mm syringe height, this produced a pressure of 0.25 ± 0.06 nN/µm^2 from the current pipette setup, which is within the physiological and pathological range, though the syringe height is not the sole determinant. Other factors influence the exact pressure value on the neurons as well, including pipette opening, positioning (distance and angle) of the pipette tip relative to the cells, and even the flexibility of tubing[4,10].

5. Puffing to Induce Varicosities

1. Once the pipette is in position, focus the microscope on a plane that contains a region of interest. Position the cells and processes in the left half of the imaging field (seen by live capture of the camera) within the puffing area, while the right half is outside of puffing area.
   NOTE: Due to extensive outgrowth of axons and dendrites of neurons, it is unlikely that all the processes of a neuron are within the same puffing area. This is actually one advantage of this system, which allows the examination of the local effect of puffing. There are two types of puffing that can be utilized to induce varicosities: long duration puffing and pulse puffing.
2. **Puffing**
   1. Long duration puffing
      1. Position the syringe at the height, 190 mm above the coverslip containing cultured neurons[4]. Begin time-lapse imaging of area of interest with an optimized exposure time revealing clear neuronal morphology. Capture at least 15 frames at a 2 s interval (30 s total) as the baseline. The interval can be adjusted based on the experiment. At frame 16, open the turn-able stop valve of the syringe and allow fluid to flow for 75 frames (150 s). At frame 75, turn the valve off so that fluid flow stops. Stop video capture.
         Note: This should induce axonal varicosities in neurons 7-9 DIV within 5-10 s, whereas older neurons take longer to form varicosities.

   2. Pulse (2 s duration) puffing
      1. Position the syringe at the proper height (190 mm). Begin time-lapse imaging and capture at least 15 frames (30 s) at a 2 s interval. At frame 16, open the valve of the syringe and allow fluid to flow, then close the valve after 2 s. Wait 5-20 s (depending on the frequency to be tested) before opening the valve again. Repeat opening and closing of the valve to create fluid pulses, and continue for a total period of 150-300 s. Continue time-lapse imaging for 20 min to capture the recovery stage.
         NOTE: When the interval is longer than 10 s, the pulses induce drastically less varicosities. At a 20 s interval, no varicosities can be induced by the pulses[4]. To obtain reliable, consistent varicosity formation, it is advised the syringe height should be around 190 mm. Syringe height and fluid pressure should not be raised to a point where cells begin detaching from the coverslip surface.

   3. Before a day of use, clean the set-up (pipette, tubing, and syringe) by flowing about 10 mL 70% Ethanol through the set-up. Following the ethanol wash, flow 10 mL of Hank's buffer (or desired buffer) through the set-up to prime the system for that day's use. After finishing experiments for the day, clean the set-up as previously described with ethanol to prevent growth of contaminants overnight.

6. Complimenting Methods

NOTE: The puffing assay can be combined with a variety of different techniques that are discussed in the Introduction section. Here, the focus is on the core techniques that are most frequently used together with the puffing assay, including high-resolution fluorescence timelapse imaging, calcium imaging, and recovery assays. These are discussed below.

1. **Conduct high-resolution fluorescent time-lapse imaging by following the protocol below**
   1. Track the movement of fluorescently-tagged proteins within neurons with a 100X oil lens. Neurons are transfected with the proper cDNA construct. Capture for 150 frames (total 300 s) with a 2 s interval.
      NOTE: Sometimes, multiple-color time-lapse imaging is performed to image the movement of more than one fluorescently-tagged protein simultaneously.
   2. Generate a kymograph either through the video capturing software or through ImageJ (NIH).
      NOTE: The speed and directionality of travel can be determined using the kymograph. *Be sure to note the direction the soma lies from the region captured. Travel towards the soma is retrograde, while travel away from the soma to the axonal tip is anterograde.
   3. Repeat this process following puffing, or a kymograph can be created using the image capture during the puffing assay.
      NOTE: The effects of puffing on axonal transport of mitochondria and synaptic proteins were shown in a recent paper[4].

2. **Calcium imaging**
   1. Add 1 µL (for a 24-well plate) or 3 µL (for a 6-well plate) of 5 mM Fluo-4 AM to the culture media in one well and incubate at 37 °C for 30 min.
   2. Wash the cells twice with 1 mL Hank's buffer.
      NOTE: Loaded neurons emit green fluorescence through a FITC filter set. Loci denote regions of appreciable calcium within the cell. When combined with puffing, an increase in intensity (increase in internal calcium level) can be seen.

3. **Varicosity recovery**
   NOTE: Immediately following a puffing experiment, a 20 min recovery experiment can be carried out.
   1. Capture images for 300 frames (1,200 s) with a 4 s interval.
      NOTE: A cell transfected with soluble YFP/mCherry/GFP should show a moderate level (less than 50%) of recovery by the end of the imaging session. Axonal recovery depends on a number of factors, such as the developmental stage of cultured neurons[4]. This can also be combined with high-resolution fluorescent imaging and calcium imaging described above.

Representative Results

Prior to puffing, axons normally show little varicosity formation. Following puffing with our standard pressure (190 mmH_2O height), the axons start to develop many bead-like varicosities. The formation of varicosities is partially reversible, as shown by regions of the axon returning to their pre-puffed state following a 10 min recovery period (**Figure 2A-B**). After a longer period of recovery (>20 min), some axons completely recover. Suboptimal positioning of the puffing pipette as well as accurate positioning of the syringe at 190 mm above the stage may not generate varicosities rapidly. Optimal conditions should result in varicosity formation in the axons of young neurons (around 7 DIV) in about 5 s[4].

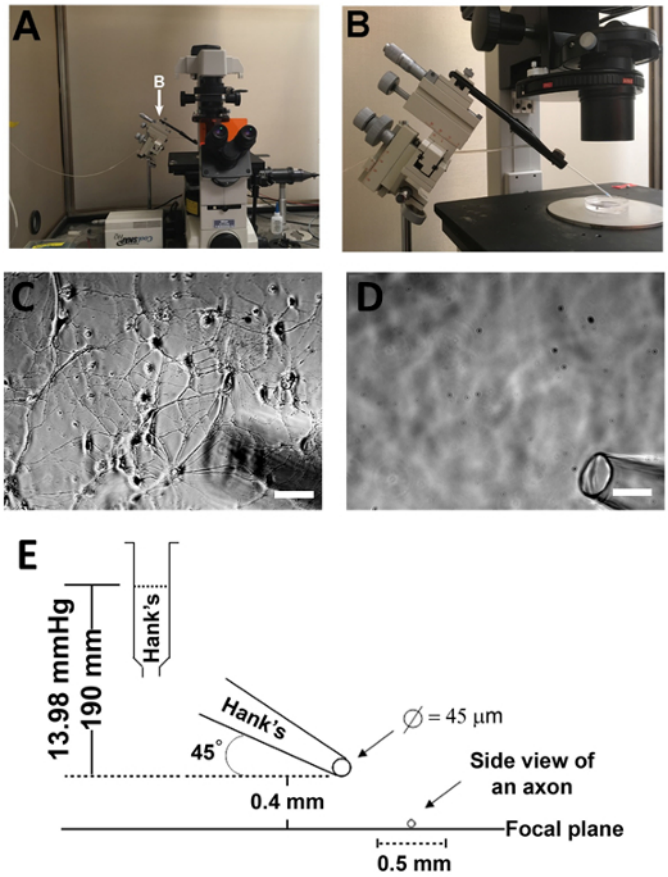

Figure 1: Schematic and photograph of puffing apparatus. (**A**) Photographs showing overall microscope set-up. (**B**) Puffing apparatus showing the glass pipette held by the micromanipulator and connected to the rubber tubing. (**C**) Phase contrast images with focus in plane of primary hippocampal neurons showing shadow of pipette in bottom right. (**D**) Out of cell plane focused on puffing pipette tip. (**E**) Schematic of distances and calculated pressures for inducing varicosities. Scale bar = 50 µm. Please click here to view a larger version of this figure.

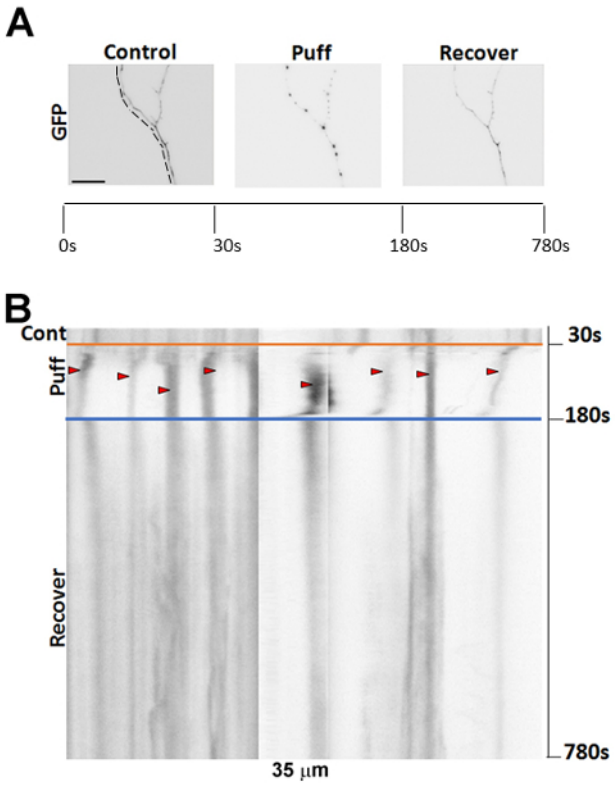

Figure 2: Representative images of varicosity formation following puffing. (**A**) Imaging of 7DIV, GFP transfected mouse hippocampal neurons showing an axon pre- and post-puffing as well as following a 10 min recovery period. (**B**) Kymograph of time-lapse imaging of neuron in (**A**). Red arrows indicate varicosities that have formed following 150 s of 190 mmH_2O puffing (beginning at 30 s). Scale bar = 15 µm. Please click here to view a larger version of this figure.

Coverslip coating solution	
780 µL	Acetic acid17 mM stock: 50 µL acetic acid in 50 mL of H_2O
200 µL	Poly-D-lysine (0.5 mg/mL stock)
20 µL	Rat tail collagen (3 mg/mL stock)
The total volume is determined based on the number of coverslips to be coated.	

Table 1: Coverslip coating solution. Provides the recipe for preparing the coverslip coating solution used to adhere cultured cells to the coverslips.

1x Slice dissection solution (SLDS), filtered, pH= 7.4, store at 4 °C	
1 L	ddH_2O
82 mM	Na_2SO_4
30 mM	K_2SO_4
10 mM	HEPES (free acid)
10 mM	D-glucose
5 mM	$MgCl_2$
0.00%	Phenol red (optional)
Plating Media (PM, filter, store at 4 °C)	
439 mL	MEM Earle's Salts
50 mL	FBS
11.25 mL	20% D-glucose
5 mL	Sodium pyruvate (100 mM,)
62.5 µL	L-glutamine (200 mM)
5 mL	Penicillin/Streptomycin (P/S, 100x)
Maintenance Media (MM, filter, store at 4 °C)	
484 mL	Neurobasal
10 mL	B27 50x supplement
1.25 mL	L-glutamine (200 mM)
5 mL	100x P/S
All solutions are sterilized using a filter with 0.2 mm pore size.	

Table 2: Cell culture media recipes. Provides the recipes for preparing media utilized in the culturing of primary hippocampal neuron cells.

Hank's buffer (filter, pH = 7.4, store at 4 °C	
150 mM	NaCl
4 mM	KCl
1.2 mM	$MgCl_2$
1 mM	$CaCl_2$
10 mg/mL	D-glucose
20 mM	HEPES

Table 3: Hank's buffer recipe. Provides the recipe for preparing the buffer utilized in the puffing protocol and during live-cell imaging.

Pipette pulling parameters					
Heat	Pull	Velocity	Delay	Pressure	Ramp
501	0	15	1	500	530

Table 4: Pipette pulling parameters. Provides the parameters to be set on the pipette puller to obtain an opening that is around 45 µm in diameter.

Discussion

The procedure of this microbiomechanical assay is straight forward. It will produce reliable results, if all its steps are carefully carried out. There are several key steps that, if improperly performed, will hinder successful data collection. The critical steps begin upstream of the actual application of the puffing stimulus. Careful dissection, culturing, and care of the primary neuron culture are paramount. If the cultured neurons are not healthy, they will not react consistently, since they might have already been primed for stress. Downstream of the culture, the initial set-up and calibration of the puffing apparatus to provide consistent pressure, that will induce varicosities but will not cause detachment of the cells from the coverslip surface, must be performed with patience. Accurate calibration of the set-up may take 1 h due to pipette clogging, tubing, or valve issues. It is critical to ensure that fluid flows out of the pipette with minimal resistance before moving forward with placing the cells in the dish and positioning the pipette tip. As far as modifications to the set-up are concerned, one can pair the puffing apparatus with perfusion, patch clamping, and any other microscope-based instrument, mostly dependent on the physical space free on the sides of the microscope.

This system has two intrinsic limitations that need attention. The set-up can consistently initiate the formation of reversible varicosities in axons of cultured hippocampal neurons through puffing fluid to the cells. However, it is important to note that the exact pressure values onto the neurons within the same puffing field are not identical. The fluid pressure that impacts the cells, calculated based on pressure load measurement from a microfabricated silicone membrane[4,10], represents the average pressure in the center of the puffing field. Thus, the exact puffing pressure is the highest in the center of the puffing field and the lowest around the edge of the field. We showed that the pressure strength correlates with the onset, the size, and the number of varicosities induced[4]. Higher pressure resulted in faster onset, bigger, and more abundant varicosities[4]. In our recent studies, we used a minimal pressure (190 mmHg at the tip of puffing pipette) that can still reliably induce varicosities in most axons[4]. Under this condition, varicosity onset for young neurons is normally about 5 s. It is important to note that with the exact same puffing system, the value of the onset time can vary, which not only depends on neuronal type, age, and subcellular compartment, but can also be influenced by the position of the neuron in the puffing field. Despite the variation of puffing pressure received by neurons, this assay system still provides a reliable means for studying mechanical effects on central neurons, and yields highly consistent and reproducible experimental results.

The second limitation of the system is its clogging issue. To achieve physiologically relevant pressure, the opening of the pipettes is small, around 45 µm. However, the small opening tends to clog with debris from the plastic tubing and the debris in the pipette tip can be clearly seen under the microscope. This increases the amount of time prepping the set-up, in the event of a pipette clog. Therefore, all solutions are carefully filtered before being put into the syringe, tubing, and pipette. It is also important to wash the puffing system with 70% ethanol at the start and end of the day, followed by washing with filtered Hank's buffer. To solve the potential clogging problem, we suggest trying to pull multiple pipettes. It is essential to confirm that the solution is actually puffed out of the pipette through visualization before starting the experiments. Once a good puffing system is established, it usually can last for the rest of the day.

This biomechanical assay, combined with neuron culture, presents a unique opportunity for studying central neuron mechanosensation mimicking physiological and/or pathophysiological conditions. This system allows us to examine the effects of compression and shearing on neurons. In contrast, other systems were used to examine in-plane stretching[5,6,7]. The average pressure received by the neurons in the center of the puffing areas is 0.25 ± 0.06 nN/µm^2 in our system[4]. This pressure exerted on cells is comparable to the elastic modulus of hippocampal neurons as measured previously utilizing scanning force microscopy and bulk rheology, to identify optically induced deformation[13,14], as well as pressures used to alter the growth of neurite leading edges *in vitro,* measured by scanning force microscopy (0.27 ± 0.04 nN/µm^2)[16]. This pressure does not exceed the pressure that can be generated naturally by mesenchymal cells in an E11 embryo, as measured using mechanically specific fluorescent cell-sized oil microdroplets (1.6 ± 0.8 nN/µm)[2,14].

This system has already been successfully used in combination with calcium imaging, patch clamping, electronic microscope, and cellular analysis of a closed skull mTBI mouse model[4]. The assay can also be used in combination with microscope-based experiments, such as fluorescence recovery after photobleaching (FRAP), to examine protein turn-over within varicosities, as well as FRET to study if protein-protein interactions are altered during varicosity formation. Essentially, any method that can be done using a microscope and a live cell imaging set-up can be paired with this puffing assay. The versatility of this assay is highly valuable in studying the molecular mechanisms underlying various effects on neuronal morphology and functions by micromechanical stress.

Disclosures

The authors have nothing to disclose.

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