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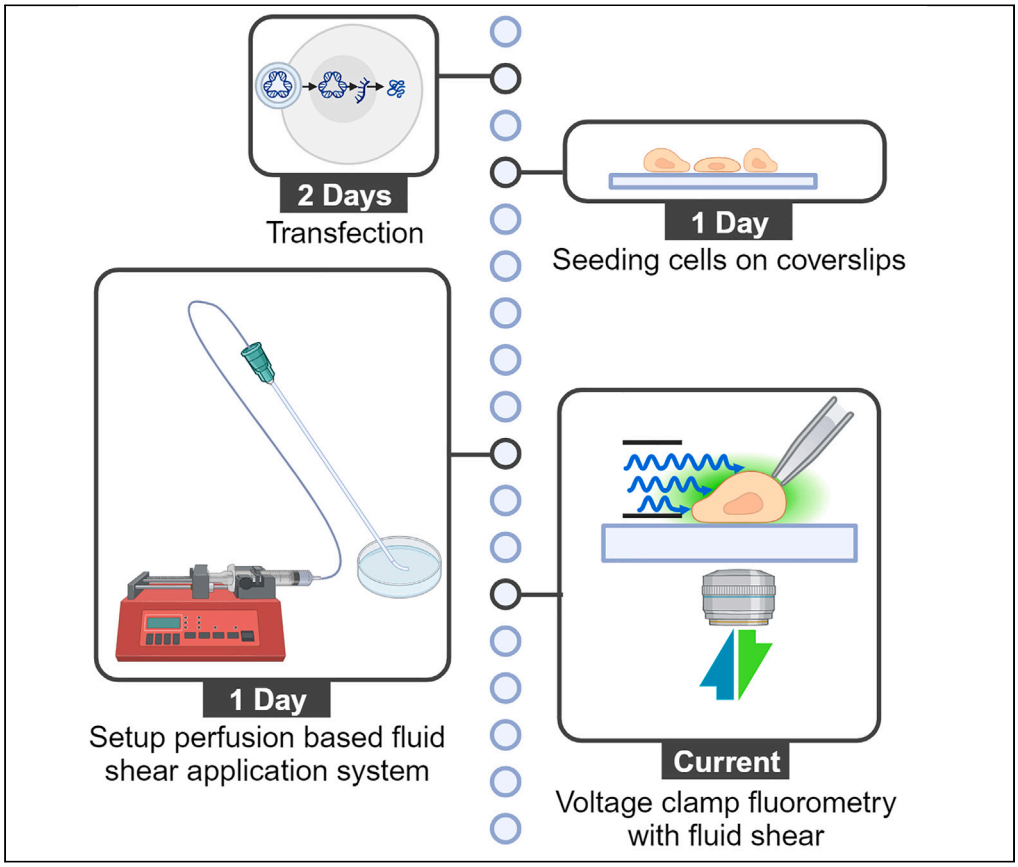
Protocol

Voltage-clamp fluorometry to record flow-activated PIEZO1 currents and fluorometric signals



PIEZO channels sense mechanical forces through conformational rearrangements of a mechanosensory domain called blade. To probe these rearrangements in real time, we have inserted conformational-sensitive cyclic-permuted GFP into several positions of PIEZO1's blade. Here, we describe the step-by-step experimental procedure we developed to simultaneously measure flow-activated ionic currents and fluorometric signals in cells expressing these engineered constructs. We describe steps for performing transfection, seeding cells on coverslips, setting up a perfusion-based fluid shear application system, and performing voltage-clamp fluorometry.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Multiplexing electrophysiology, fluorescence imaging, and mechanical stimulations

Detailed procedures from sample preparation to data acquisition

Flow-rate optimization assay to maximize fluorometric signals

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Voltage-clamp fluorometry to record flow-activated PIEZO1 currents and fluorometric signals

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SUMMARY

PIEZO channels sense mechanical forces through conformational rearrangements of a mechanosensory domain called blade. To probe these rearrangements in real time, we have inserted conformational-sensitive cyclic-permuted GFP into several positions of PIEZO1's blade. Here, we describe the step-by-step experimental procedure we developed to simultaneously measure flow-activated ionic currents and fluorometric signals in cells expressing these engineered constructs. We describe steps for performing transfection, seeding cells on coverslips, setting up a perfusion-based fluid shear application system, and performing voltage-clamp fluorometry.

For complete details on the use and execution of this protocol, please refer to Ozkan et al. (2023).[1]

BEFORE YOU BEGIN

Preparing Matrigel stock solutions

Timing: perform 3 days prior to the experiment

1. Place Matrigel on ice and place the ice box in a 4°C refrigerator overnight for thawing.
2. Make 22 μL aliquots of thawed Matrigel and put into prechilled 0.2 mL PCR tubes.
3. Store the tubes at −20°C until further use.

⚠ CRITICAL: Matrigel solutions can polymerize at room temperature. Handling the Matrigel stock solutions on ice, precooling pipette tips in refrigerator and using chilled media to make the Matrigel working solution is critical to prevent premature gelation.

Note: Keeping Matrigel stock solutions in small aliquots of 10–25 μL in 200 μL PCR tubes will allow fast thawing and re-freezing. This will help preserve their functionality and use up the entire stock solution in the tube before undergoing too many freeze-thaw cycles.

Transfecting cells

Timing: perform 2 days prior to the experiment

4. Pick three wells in a 12-well plate with HEK293T$^{\Delta PIEZO1}$ cells grown to ~70% confluency (5-7 x 10^5 cells per well).

5. In each well, transfect 1 μg of plasmid (~0.15 μg per 10^5 cells) encoding PIEZO1 wild-type (WT), Lck-cpGFP (control construct carrying cpGFP) and PIEZO1-86-cpGFP using Lipofectamine 3000. Amounts of reagents mentioned below are per 1 μg DNA (per one well)
   a. Dilute plasmid stock solution in ultra-pure water to prepare 100 μL of 0.1 μg/μL plasmid working solution.
   b. In a 1.5 mL microcentrifuge tube, mix 3 μL P3000 with 37 μL Reduced Serum Medium (Opti-MEM).
      i. Add 10 μL of plasmid working solution to this mixture while flicking the tube with fingers to facilitate mixing.
      ii. Use a 100–200 μL micropipette to mix the Opti-MEM + P3000 + plasmid cocktail by pipetting the mixture up and down ~10 times.
   c. In a 1.5 mL microcentrifuge tube mix 3 μL Lipofectamine 3000 with 47 μL Opti-MEM.
   d. Incubate both tubes at 25°C for 5 min.
   e. Mix the contents of the two tubes together by pipetting up and down ~50 times using a 100–200 μL micropipette.
   f. Incubate the mixture for 20 min at 25°C.
   g. Carefully transfer the mixture into one well of the 12-well plate.

⚠ CRITICAL: Perform the transfection including the preparation of the transfection cocktail in a biosafety cabinet with laminar flow to avoid contamination.

6. Label the transfected wells and incubate the 12-well plate at 37°C, 5% CO2 and 100% relative humidity in a cell culture incubator.

Coating coverslips with Matrigel

Timing: perform 2 days prior to the experiment

7. Place five round coverslips (12 mm diameter) at the bottom of a 35 mm dish. The coverslips should not overlap to ensure maximum coating of the top side of the coverslips.
8. Thaw a Matrigel stock tube (From step 3) on ice and add 10 μL Matrigel into the center of the 35 mm dish.
9. Immediately add 1 mL of chilled cell culture media onto the Matrigel drop and gently pipette up and down to mix Matrigel in cell culture media.
10. Make sure all coverslips are at the bottom of the dish and are completely covered with the Matrigel + cell culture media mixture.
11. Label and store the 35 mm dish in a cell culture incubator.

Seeding transfected cells on to the coverslips

Timing: perform 1 day prior to the experiment

12. Aspirate out media from the transfected well and add 0.2 mL calcium and magnesium free Phosphate-buffered saline (PBS).
13. Aspirate out the PBS and add 0.2 mL of 1× TrypLE. Place the plate back in the incubator for 5 min.
14. Aspirate out the media from the 35 mm dish with Matrigel-coated coverslips (from step 11) and gently wash the dish with 3 mL calcium-free PBS.
15. Add 1.5 mL of cell culture medium (e.g., DMEM + 10% fetal bovine serum) to the dish.
16. Once the 5 min incubation (from step 13) is complete, take the 12-well plate out from the incubator.
17. Gently detach transfected cells from the well by slowly pipetting up and down using a 200 μL pipette tip.

18. Seed the detached cells (about 0.7 × 10^6 per dish) into the 35 mm dish containing the 5 Matrigel-coated coverslips. Gently swirl the dish to evenly spread the cells throughout the dish.
19. Make sure none of the coverslips are floating on media or overlapping on each other. If any floating/overlapping coverslips are observed gently push the coverslips back to the bottom of the plate with a disposable 1 mL pipette tip.
20. Label and store the 35 mm dish in a cell culture incubator.

Setting up voltage clamp fluorometry with perfusion control

Timing: 1 day before the experiment

Note: The TTL trigger inputs of syringe pump, camera, fluorescence illuminator, and poking probe controller are connected via BNC cables to the analog / digital TTL outputs of a digitizer Digidata 1550B (Molecular Devices) to synchronize triggering of each apparatus using the Clampex software.

21. Connecting the syringe pump (NE-1000) to the digitizer.
    a. Connect the DB-9 connector in the back of the syringe pump to the analog output of the digitizer *via* a DB-9-to-BNC cable according to the NE-1000 Syringe pump user manual. (Refer to the "Logic Interface: TTL Input and Output" section of the user manual).
    b. Configure the NE-1000 syringe pump to be triggered by a 5 V input signal provided by the digitizer. Detailed instructions on how to program the pump to be triggered *via* a DB-9 connector are available in the NE-1000 syringe pump user manual provided by the manufacturer (refer the "TTL I/O Operational Controls" section of the user manual).
22. Connecting and setting up the Zyla sCMOS camera (Andor).
    a. Connect the Rear 15-pin D type connector to the analog output of the digitizer *via* a manufacturer-provided 15-pin D type-to-BNC cable (ACC-ASE-13532).
    b. Open the Solis software (Andor).
    c. Set the "external exposure" setting to allow image exposure and frame rate to be controlled by the trigger signal created by pClamp and transmitted *via* the digitizer.
    d. Turn on "image spooling" and "automatic saving".
23. Connecting the light engine Spectra X (Lumencor)
    a. Connect the TTL inputs on the front panel of the light engine to digital outputs of the digitizer *via* BNC cables.

Note: Both the NE-1000 pump and sCMOS camera operate on 5 V (on) and 0 V (off) commands. Therefore, the 0–5 V digital outputs of the digitizer can also be used to control the syringe pump.

24. Optical Filtering Arrangement.
    a. Use cyan (475 nm) LED output of the light engine.
    b. Use a triple-band dichroic mirror (FF403/497/574, Semrock) to reflect the excitation light.
    c. Filter the emission light using a triple-band emission filter (FF01-433/517/613, Semrock)
25. Poking probe setup.
    a. Connect P-841 Preloaded piezoelectric actuator to an E-625 piezoelectric servo controller (Physik Instrumente).
    b. Connect the analog input of the servo controller to one of the analog outputs of the digitizer.

Preparing voltage clamp fluorometry protocols

Timing: 1 day before the experiment

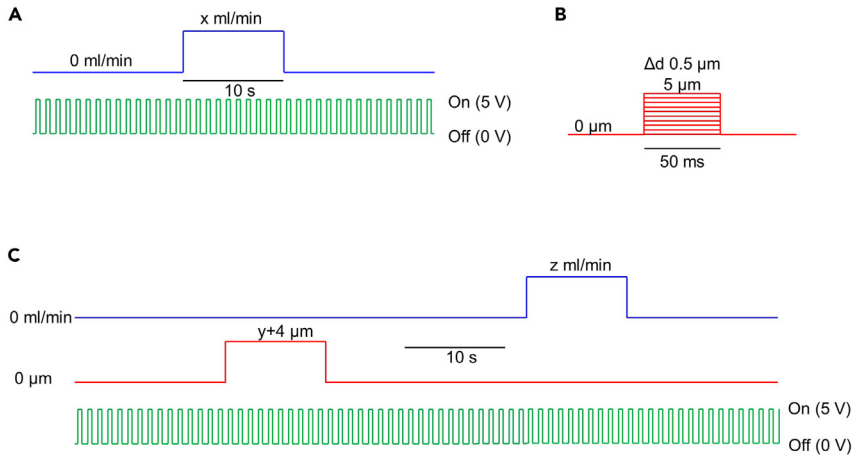

Figure 1. Patch Clamp and pump protocols

(A) Flow rate optimization protocol composed of a pump protocol (top, blue) and camera + fluorescence illumination protocol (bottom, green). Flow rates to be used here (shown as X ml/min) are 0.036, 0.090, 0.360, 0.900, 3.600 and 9.000 mL/min. Each cell is tested only once using a single flow rate value (see steps 6–14 for more details).

(B) Poking probe calibration protocol. The interval between two consecutive pulses is 15 s (to allow PIEZO1 to fully recover from inactivation). The poking distance increment between two pulses (Δd) is 0.5 μm.

(C) The voltage clamp fluorometry protocol consists of a pump protocol (top, blue), poking protocol (middle, red) and camera + fluorescence illumination protocol (bottom, green). The optimum flow rate calculated using protocol A is used as the flow rate (z) in protocol C. The poking distance that generates the first instance of poking current in protocol B (y μm) will be used to adjust the poking distance of protocol C. Detailed instructions on adjusting the poking distance is shown in step 24. Time scales are shown in black. Both camera and fluorescence illuminator are triggered simultaneously.

Note: Here we show how to design voltage clamp fluorometry protocols using commercial software suites designed for the equipment used in this protocol. However, alternative equipment and software suites may be used instead. Therefore, we show a detailed overview of the protocols we used in Figure 1 so that experimenters can design identical protocols using other software.

26. Set up flow rate optimization protocol.
    a. Set the syringe pump input units of volume and rate to mL and mL/min, respectively. Flow rates and volumes to be used during the experiments are mentioned in later steps.
    b. Open the Solis software and select kinetic series + external exposure configurations. Set the number of acquisitions to 40 (40 frames total)
    c. Setup Clampex protocol (Figure 1A).
        i. Initiate a new patch clamp episodic protocol with 0.6 s long sweeps.
        ii. Set the number of sweeps to 40 and the start-to-start interval to 1 s (i.e., 1 frame per sec).
        iii. In the analog waveform tab, select the output signal to the camera.
        iv. Add 0 V for 50 ms in the first epoch, 5 V for 400 ms in the second epoch, and 0 V for 50 ms in the third epoch.
        v. In the same tab, set the digital output for cyan light to 1 in second epoch in order to trigger the cyan light from the light engine together with image acquisition by the camera.
        vi. In the analog waveform tab, select the output signal to the Pump and set the first epoch (epoch A) to 50 ms.
        vii. Go to "stimulus tab" and select the channel for Pump.
        viii. Enable "user list" and select the epoch A level (this defines the output voltage for first epoch during each sweep).
        ix. Set the epoch value to 0 in all sweeps except the 16th sweep where the pump must be triggered. Set the value of the 16th sweep to 5 V.

x. Save the protocol.

Note: See the "Axon Guide" (Molecular Devices) to get detailed instructions on how to edit the "stimulus tab" to customize a patch clamp protocol.

Note: Clampex is used to trigger all the equipment in synchrony. Therefore, Clampex will be used to run the protocols even when no electrophysiology data is being recorded.

27. Setup Voltage clamp fluorometry protocol.
    a. Open the Solis software and select kinetic series + external exposure configurations. Set the number of acquisitions to 70 (70 frames total).
    b. Setup Clampex protocol (Figure 1C).
        i. Initiate a new patch clamp episodic protocol with 0.6 s long sweeps. Set the number of sweeps to 70 and start-to-start intervals to 1 (1 framer per sec).
        ii. In the analog waveform tab, select the output to the camera. Add 0 V for 50 ms (first epoch), 5 V for 400 ms (sec epoch), and 0 V for 50 ms (third epoch).
        iii. In the same tab, change the digital bit pattern in the second epoch to trigger cyan light, in order to trigger the cyan light from the light engine together with camera.
        iv. In the analog waveform tab, select the output to the poking probe. Set duration for epoch A (first epoch) to 50 ms. Set the inter sweep holding level to "use last epoch".
        v. Go to "stimulus tab" and select the channel for poking probe. Enable user list and select the epoch A level (this defines the poking distance for first epoch).
        vi. Set the epoch value to 0 in all sweeps except the 16^{th}–26^{th} sweeps where the poking probe must keep poking the cell. The poking distance to be used in 16^{th}–26^{th} sweeps will be decided from a calibration protocol described at steps 24 in the "step by step" protocol section.
        vii. In the analog waveform tab, select the output to the pump and set first epoch to 50 ms.
        viii. Go to "stimulus tab" and select the channel for Pump. Enable user list and select the epoch A level (This defines the output voltage for first epoch).
        ix. Set the epoch value to 0 in all sweeps except the 46^{th} sweep where the pump must be triggered. Set the value at 46^{th} sweep to 5 V.

Preparing patch clamp saline solutions

Timing: 1 day before the experiment

28. Make a 50 mL pipette solution containing 140 mM KCl, 10 HEPES, 10 mM TEA, and 2 mM EGTA (pH 7.4 with KOH). Create 0.5 mL aliquots in 1.5 mL Eppendorf tubes and store the aliquots in a −20°C freezer until use.

⚠ CRITICAL: Leave at least 50% of headspace in Eppendorf tubes to allow expansion of the saline when freezing. This will prevent the tubes from popping in the freezer.

29. Store HBSS bath solution at 25°C until use.

Setting up the perfusion nozzle

Timing: 1 day before the experiment

30. Bend the tip of an 18-gauge canula by 50 degrees (Figure 2A).
31. Mount the canula to a micromanipulator (e.g., MP-285, Sutter) at a 50-degree angle so that the canula output will be parallel to the flat dish bottom (Figures 2B–2D).

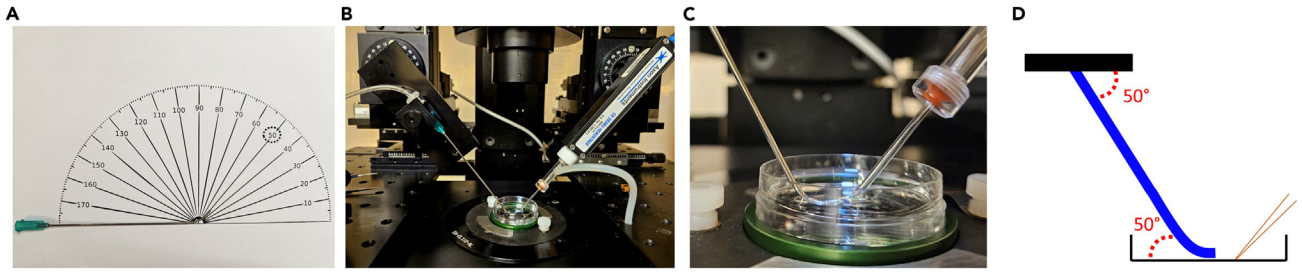

Figure 2. Shear nozzle setup

(A) Tip of the shear nozzle bent at a 50-degree angle.

(B) Shear nozzle (left) and patch pipette (right) mounted to MP-285 manipulators with rotating dovetail clamps.

(C) Zoomed view of the shear nozzle (left) and patch pipette (right).

(D) A 50-degree mounting angle of a 50-degree bent nozzle results in nozzle output parallel to the bottom of the coverslip.

⚠ CRITICAL: Angle of the saline nozzle changes the fluorometric response of the probes. Therefore, ensure that the nozzle outlet is perfectly parallel to the bottom of the preparation.

Note: The MP-285 micro manipulator has a rotating bezel with angle measurements. This can be used to precisely measure the angle of the mounted apparatus.

32. Mount a 30 mL syringe to the syringe pump and input the syringe internal diameter to the syringe pump system according to the manufacturer's guidelines.

⚠ CRITICAL: The syringe internal diameter is used by the syringe pump's electronics to control the output flow. Therefore, carefully check the accuracy of the diameter value entered. Re-enter the diameter of the syringe each time a different type of syringe is used. A pump test protocol (Step 2 of the "step-by-step" protocol) can be used to verify the accuracy of the programmed flow rate.

33. Connect the canula to the 30 mL syringe using a 30 cm long tube.

⚠ CRITICAL: tension in the tubing will transfer mechanical noise/vibrations from the pump to the canula. Any shaking/vibration of the canula may compromise the integrity of the patch.

Note: The length of the tube connecting the nozzle to the pump depends on the dimensions of the patch clamp rig. The main point is that the tubing should be long enough to connect the nozzle to the pump without generating tension in the tube.

Pulling patch pipettes and poking probes

Timing: 3 h before the experiment

34. Use borosilicate capillaries with Filament (BF150F-3, Harvard Apparatus) to pull both patch pipettes and poking probes.
35. Pull patch pipettes using Narishige PC-100 Vertical Puller with a two-step protocol.
    a. Set heat value for the first step to 59.6.
    b. Set heat value for the second step to 47
    c. Set the pull distance to 6 mm.
    d. Load the capillary to the puller and press "pull" button.

Note: This protocol yields patch pipettes with 3–4 MΩ when filled with saline mentioned in this protocol. Other pipette pullers may be used to produce pipettes with a similar resistance.

36. Fire polish the tip of the poking probes using a microforge (e.g., Narishige mf-900) to form a blunt tip (Methods video S1).

Note: We did not fire-polish the patch pipettes in these experiments as this step did not seem to significantly improve the formation of a gigaohm seal.

Preparing high viscous HBSS saline

Timing: 2 h before the experiment

37. Pour 20 mL of HBSS saline into a 50 mL capped centrifuge tube and place the tube in 37°C water bath for 10 min to warm up the saline.
38. Measure 0.4 g of high molecular weight polyvinylpyrrolidone (PVP).
39. Put PVP in to the prewarmed HBSS containing tube and vortex for 30 s.
40. Place the tube in a 37°C shaker incubator at 250 rpm for 30 min.

Note: PVP can be harder to dissolve and may form clumps. Undissolved clumps of PVP can block the perfusion system. Preheating saline and/or using stirring facilitate proper solvation thus help eliminate PVP clumping.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Potassium chloride	MilliporeSigma	P3911-25G
HEPES	MilliporeSigma	H3375-25G
Tetraethylammonium chloride	MilliporeSigma	T2265-25G
EGTA	MilliporeSigma	324626-25GM
KOH	MilliporeSigma	221473-25G
HBSS	MilliporeSigma	H8264-500ML
Polyvinylpyrrolidone (PVP)	MilliporeSigma	PVP360-100G
Matrigel	Corning	354277
Lipofectamine 3000	Thermo Fisher Scientific	L3000015
P3000	Thermo Fisher Scientific	L3000015
DMEM	Thermo Fisher Scientific	10564011
PBS	Thermo Fisher Scientific	10010023
HBSS	Thermo Fisher Scientific	14025092
Opti-MEM reduced serum medium	Thermo Fisher Scientific	31985062
TrypLE	Thermo Fisher Scientific	12605010
Deposited data		
Force-induced motions of the PIEZO1 blade probed with fluorimetry	Cell Reports	https://doi.org/10.1016/j.celrep.2023.112837
Experimental models: Cell lines		
PIEZO1 knockout HEK293T (HEK293T$^{\Delta PIEZO1}$)	ATCC	CRL-3519
Software and algorithms		
pCLAMP 11 software suite	Molecular Devices	V11.2.2.17
Solis software	Andor Technology	Solis 64
Other		
12 Well tissue culture plate	CELLTREAT	229111

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
12 mm coverslips	Thomas Scientific	1203J81
Syringe pump	New Era Pump Systems	NE-1000
Patch-clamp digitizer	Molecular Devices	1550B
Patch-clamp amplifier	Molecular Devices	200B
Patch-clamp headstage	Molecular Devices	CV-203BU
Camera	Andor Technology	Zyla 4.2 PLUS sCMOS
Light engine	Lumencor	Spectra X
Preloaded piezoelectric actuator	Physik Instrumente, USA	P-841
Piezo servo controller	Physik Instrumente, USA	E-625SR
Canula	Dispense All Store (Amazon.com)	DA-4I-ALLG-10
Multi-micromanipulator system	Sutter Instrument	MPC-325
30 mL syringe	WERTYCITY	30 mL-caps-10
Glass capillaries	Sutter Instrument	BF150F-3
Pipette puller	Narishige	PC-100
Microforge	Narishige	MF-900
Tubing	Tygon	VE-ACF00002
Triple-band dichroic mirror	Semrock	FF01-403/497/574
Triple-band emission filter	Semrock	FF01-433/517/613

MATERIALS AND EQUIPMENT

50 mL Patch Pipette Solution		
Reagent	Final concentration (mM)	Amount
KCl	140	0.5219 g
HEPES	10	0.1192 g
TEA	10	0.0829 g
EGTA	2	0.0380 g
KOH	Titrate to get 7.4 pH	variable
ddH_2O	N/A	50 mL
Total	**N/A**	**N/A**

Note: Use saturated KOH saline to adjust pH to minimize the change in total saline volume

STEP-BY-STEP METHOD DETAILS

Setting up the perfusion system

Timing: 1 h before the experiment

This section describes how to prepare and properly load the high viscous HBSS saline to the perfusion system.

1. Load 2% PVP HBSS saline to the shear flow system.
   a. Remove the 30 mL syringe from the pump and manually fill the 30 mL syringe with 2% PVP HBSS saline.
   b. Wait 2 min for air bubbles to rise and remove them by tipping the syringe upwards and tapping the syringe barrel.

⚠ CRITICAL: Air bubbles may form or become trapped in the solution along the tubing. If not eliminated, air bubbles exiting the canula will flush out cells and prematurely terminate the experiment. The high viscosity of the PVP saline makes it harder to eliminate air bubbles, therefore do not rush this step.

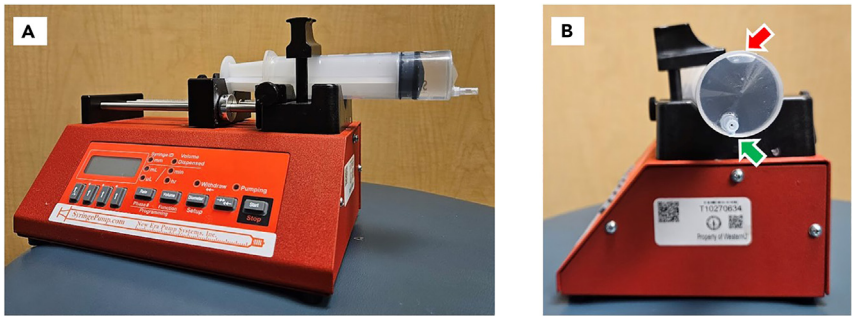

Figure 3. Mounting syringe to the pump

Front (A) and side (B) view of an eccentric syringe mounted with its tip in a proper "down" position. Note that air bubbles (red arrow) accumulate at top of the barrel, far away from the outlet (green arrow).

c. Connect the syringe to the canula via the tubing and press the plunger until saline comes out from the canula.
d. Mount the syringe to the pump.

Note: If the syringe outlet is off to the side and not in the center (eccentric syringe), it could enable air bubbles to enter the tubing. To prevent this risk, rotate the syringe to keep the eccentric outlet in a down position (Figure 3).

e. Place an empty beaker beneath the nozzle tip and run the pumping protocol once to make sure the perfusion system is ready to use.

Note: The syringe pump drives a piston to push the syringe plunger through the barrel of the syringe, the latter remaining stationary (Figure 3A). Running the pump protocol once will ensure no physical gap exists between the piston and the plunger, thus reducing the delay between the pump trigger and the delivery of the shear stress stimulus to the cell.

Testing the accuracy of the pump protocol

Timing: 30 min before the experiment

The syringe pump system uses a piston to push the syringe plunger through the barrel of the syringe. The piston is driven by a rotating screw. Therefore, the flow rate is decided by the rate of rotation of this screw. Syringe pump is not equipped to measure the fluid output or self-calibrate. Therefore, we use a pump accuracy test protocol to make sure the actual rate of output tallies the input flow rate values.

2. Input the following "Pump test" protocol to the syringe pump.
   a. Set flow rate to 10 mL/min and pump volume to 10 mL.

   Note: The NE-1000 syringe pump can be programmed using inputs of flow rate and volume, but not duration. The flow duration can be calculated by dividing flow volume by flow rate.

3. Place the shear nozzle inside a 15 mL conical tube (or a 10 mL volumetric flask).
4. Run the protocol. After the protocol has run, remove the nozzle from the conical tube (or the 10 mL volumetric flask).
5. Measure the output volume and compare this volume to the programed flow volume. If there is a significantly large difference between the two values, the pump may need to be re-calibrated by the manufacturer.

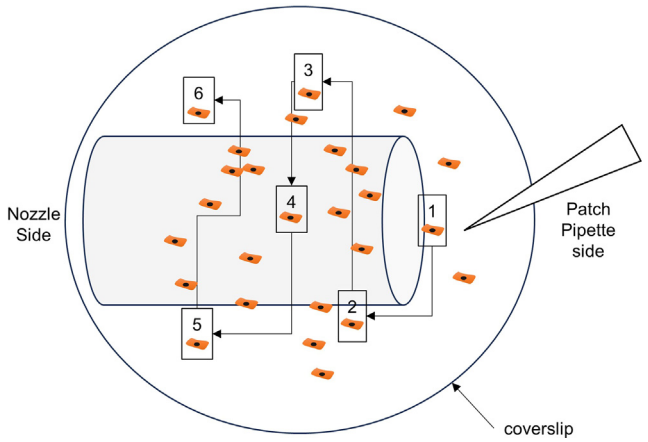

Figure 4. Guide to chronologically select cells for experiments

Start with a transfected cell located near the right edge of the coverslip and move backward and left/right with respect to flow direction for subsequent recordings. This sequence ensures that each new cell being recorded was not inadvertently exposed to fluid flow during a prior recording.

Note: The maximum flow rate of perfusion system decreases with increasing saline viscosity. The pump will not be able to push the syringe beyond a certain flow rate depending on the viscosity and the specific dimensions of the tubing being used due to backpressure from flow resistance. Therefore, the pump should be tested at the highest intended flow rate and viscosity.

Optimizing flow rate to maximize fluorometric signals

Timing: immediately before the experiment

We found that our PIEZO1-cpGFP probes sense low intensity shear stimuli.[1] In addition, the maximal amplitude of the cpGFP signal decreases at higher flow rates, creating a bell-like curve for the flow rate vs. fluorescence signal curve (see expected results). This step identifies the optimum flow rate that generates the largest fluorometric signals.

6. Take one coverslip with seeded cells from the incubator and place it in a fresh 35 mm cell culture dish filled with 1 mL HBSS saline.
7. Gently wash the cells with HBSS saline by rocking the dish to remove debris and cell culture media that may be on the cells.
8. Place the dish on the microscope stage.
9. Switch to a 40× objective and turn on the light engine.
10. Identify a positively transfected (green) cell toward the rightmost edge of the coverslip (Figure 4).
11. Position the shear nozzle (canula) ~100 μm away from the cell and bring the nozzle toward the bottom of the coverslip until contact is made (a slight displacement of the coverslip can be seen when contact is made).

⚠ CRITICAL: Use the same distance between the nozzle and the cell throughout all experiments to ensure consistent flow stimulation.

12. Run the "Flow rate optimization" protocol using Clampex software, starting at 0.036 mL/min.
13. Lift the shear nozzle up from the coverslip and move the coverslip toward an area that was not previously exposed to the solution flowing from the canula (Figure 4).

⚠ CRITICAL: After measuring a cell, move the nozzle backward and left/right relative to the direction of flow. This will allow the experimentalist to uncover cells that were not previously exposed to fluid shear stress stimuli. This enables measuring multiple cells from the same coverslip, saving time and effort. Follow the pattern shown in Figure 4 during nozzle shear experiments.

14. Repeat steps 11–13 on new cells using flow rates of 0.090, 0.360, 0.900, 3.600 and 9.000 mL/min.

Note: The flow rate values were chosen to be equally spaced onto a logarithmic scale. The flow volumes were chosen to precisely yield a total pump duration of 10 sec. E.g., (0.006 mL / 0.036 mL min^{-1}) * 60 = 10 sec.

15. Using an image analysis software, plot the time-course of the mean fluorescence intensity of the pixel region covering the measured cell before, during, and after flow stimulation.
16. Determine the maximal relative change of fluorescence intensity, $(F_{max}- F_0)/F_0$, and plot this value (fluorescence signal) as a function of flow rate.
17. Identify the flow rate that generates the highest fluorescence signal and use this flow rate during the next experiments.

Voltage clamp fluorometry

Timing: current

Here we measure the flow induced current and fluorescence signal together with poking induced current by PIEZO1.

18. Repeat steps 6–11 to position the shear nozzle.
19. Place the grounding electrode in the dish and connect the electrode to the grounding port located in the back of the headstage *via* a conducting cable.
20. Mount the poking probe onto the piezoelectric actuator.
21. Approach the poking probe toward a cell until a physical contact is established. A slight deformation of the membrane can be seen at magnification 40× and above when the poking probe touches the cell (Methods video S2). Take precautions not to send the poking probe too deep in to the cell which can damage the cell (Methods video S2).
22. Slightly retract the poking probe until it no longer contacts the cell.
23. Using the patch pipette, make a whole cell patch (Figure 5).
24. Estimate the distance between the poking probe and the cell.
    a. Run the poking distance calibration protocol (Figure 1B) until the first occurrence of poke-induced current is detected.
    b. Mark the distance at which the first poke-induced current is seen as a +1 μm poking displacement in the voltage clamp fluorometry protocol (Figure 1C). E.g., if the first instance of poking-induced current corresponds to a 3 μm poke displacement, the poking distance should be adjusted to 7 μm in the protocol to yield a net 5 μm poking distance.

Note: The initial position of the poking probe relative to the cell may vary across experiments due to human errors in estimating the probe position relative to cell. Therefore, the step 24 is used to arbitrarily normalize the threshold for poke-induced PIEZO1 current at +1 μm poking displacement, enabling comparison across multiple experiments.

⚠ CRITICAL: Repeat this step to readjust the poking distance in the voltage clamp fluorometry protocol each time a new cell is patched.

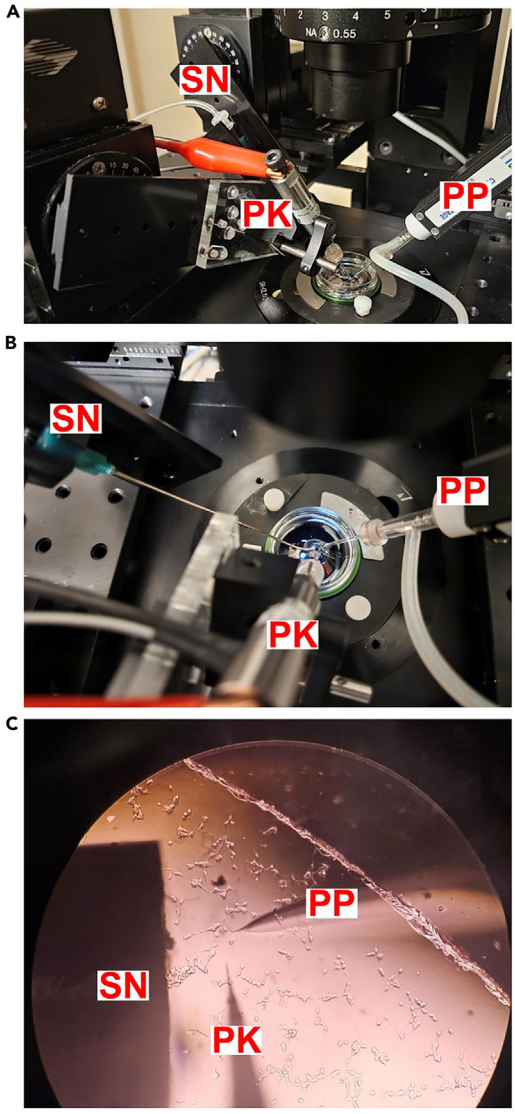

Figure 5. Positioning of the shear nozzle, poking probe, and patch pipette

The poking probe (PK), shear nozzle (SN) and patch pipette (PP) are shown from front two different vantage points near the microscope (A, B).

(C) The poking probe (bottom), shear nozzle (left) and patch pipette (right) shown through the eyepiece using a 10× objective.

25. Start the AxoScope data acquisition and run the voltage clamp fluorometry protocol.

    Note: The AxoScope records all gap free current events, therefore is used to visualize all the current events throughout the experiment. The Clampex protocol is an episodic protocol which records current events during sweeps, but not between sweeps.

26. Rename a folder with the Clampex file name and move the acquired images, Clampex recording and AxoScope recording into the folder.

EXPECTED OUTCOMES

Plotting the maximal fluorescence signal as a function of the flow rate should generate a bell-shaped curve. For unclear reasons, the peak of the bell curve appears to shift toward higher flow rate with

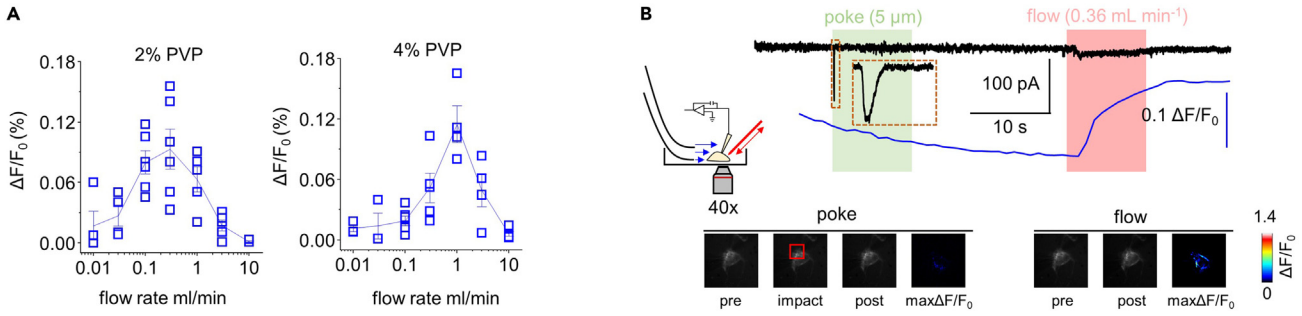

Figure 6. Expected results

(A) Results of optimum flow rate calibration at 2% PVP (left) and 4% PVP (right) using PIEZO1-86-cpGFP. Error bars represent standard error of mean.
(B) Voltage clamp fluorometry results from cells expressing PIEZO1-86-cpGFP. All tested cells were mechanically stimulated twice, first with a 5 μm poke, then with a 0.36 mL min^{-1} flow stimulus. Representative images from one cell at different times during the double stimuli protocol are shown. The poking probe impact can be seen in the area marked with the red box. The scale bars represent 100 pA (current) and 0.1 $\Delta F/F_0$ (fluorescence).

increasing viscosity of the flow solution (Figure 6A). In our two tested constructs (cpGFP86 and cpGFP1591), the poking stimulus generate rapidly-activating and -inactivating currents characteristics of WT PIEZO1 (Figure 6B).[2] The poking stimulus, however, does not generate a fluorometric signal in these cpGFP constructs. In contrast, the flow stimulus generates a robust fluorometric signal in cells transfected with our two tested constructs (Figure 6B).[1] Noticeably, the flow-induced shear stimulus generates a slowly-activating and -inactivating current compared to poke-evoked currents, presumably because the flow stimulus is delivered more slowly (or more gently) to the cell as compared to the poke stimulus.

LIMITATIONS

This protocol requires cells to be attached to a coverslip. If cells in suspension are patched, the fluid flow from the canula can detach the cell from the patch pipette. Therefore, this protocol is best suited to test fluid shear dependent activation of PIEZO1 in adhesive cells using low to moderate flow rates.

The fluorescence signals generated with this nozzle shear were much smaller compared to signals generated using commercially available enclosed flow chambers (ibidi). This is likely due to the fact that the presence of two solid boundaries (walls) in the enclosed chambers enables the flowing saline to produce a larger wall shear stress compared to our system which contains a single solid boundary (bottom of the dish). Our open system, however, enables a patch pipette to access the cell.

Our perfusion system is powered by a single pump, driving a single syringe. Therefore, the system is limited to using a single type of flow solution, preventing testing effects of different solutions using pairwise measurements.

TROUBLESHOOTING

Problem 1

The shear nozzle, the poking probe or the patch pipette touch the sidewall of the 35 mm dish (steps 18–26). This is due to the crowding of the working area by patch pipette, poking probe and shear nozzle (Figures 7A–7D).

Potential solution

- Increase the angle of the patch pipette and poking probe. This can be done by rotating the dovetail of the micromanipulator (Figures 7A–7D).

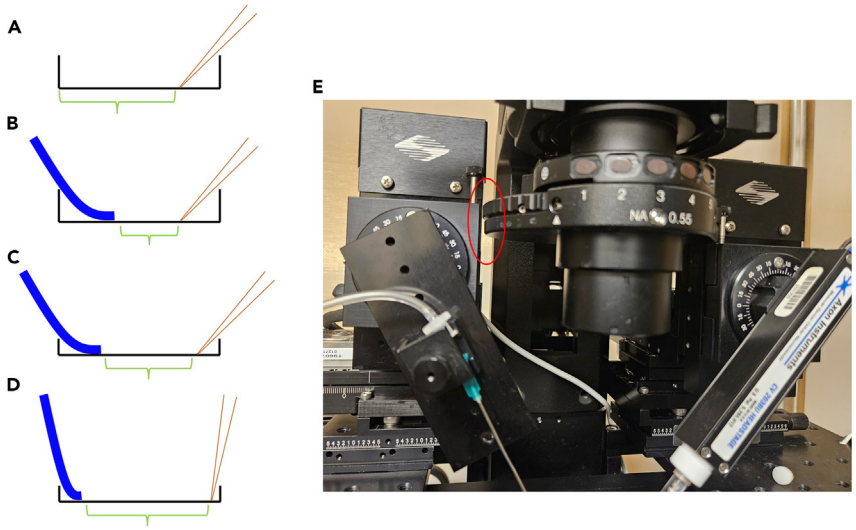

Figure 7. Means to increase workable area

(A) A patch pipette alone has a large working area (shown as green braces).

(B) The shear nozzle requires space to access cells, decreasing the working area.

(C) A low-wall cell culture dish increases the available work area since the probes can travel further without touching the walls of the dish.

(D) Using a steeper-angled patch pipette and shear nozzle can further increase the working area.

(E) The manipulator assembly nearly touches the microscope's light condenser (red circle).

- Use a wider dish (e.g., 60 mm dish) is a viable option which would greatly widen the area within which the probes can move freely. However, this would consume more saline per experiment.

Problem 2

The micromanipulator touches the condenser of the microscope (steps 18–23). This issue can be caused by one of the solutions to problem 1. A steeper (more vertical) angle for the patch pipette and poking probe would require moving the micromanipulator toward the center of the 35 mm dish, causing the micromanipulator to bump onto the light condenser located directly above the working area (Figure 7E).

Potential solution

- A bent pipette holder with angled body (e.g., Warner Instrument model Q45W-A17N) will provide a steeper approach angle without the need to bring the micromanipulator closer to the center of the dish.

Problem 3

Fluid flow causes the cells to detach from the coverslip (steps 6–17).

Potential solution

- Ensure that the Matrigel is not polymerized and that the coating is done properly.
- Do not seed cells at a high density onto the coverslips. Groups of connected cells tend to detach easily. Therefore, seed cells at a lower density so that they do not physically touch each other. Another possible option is to seed the cells on the coverslips on the day of the experiments and wait ~4 h for the cells to adhere on to the coverslips but not grow too much to connect each other.

Problem 4
Loss of patch integrity during poking (step 24).

Potential solution

- Increase the distance between the patch pipette and poking probe. Poking events can loosen the patch integrity if the poking probe is too close to the patch.

Problem 5
No flow dependent fluorometric signal (steps 6–17).

Potential solution

- The fluorometric signal is exquisitely sensitive to the flow direction (the flow must be parallel to the bottom of the dish) and the rate of flow. Therefore, make sure that the nozzle tip is parallel to the coverslip.
- The next step is to run the optimum flow rate calibration. The optimum flow rate changes with fluid viscosity (percentage of PVP in the saline), nozzle gauge, and variations in the nozzle angles. Therefore, we highly recommend conducting the optimum flow rate calibration every time a major change is made to the experimental setup.

Problem 6
Fluctuations in background fluorescence with fluid flow (steps 6–25).

Potential solution
To solve this, do a background correction for each image. Measure the fluorescence of an empty background area near the cell and subtract it from the Fluorescence value obtained for the cell.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and fulfilled by the lead contact, Jerome Lacroix (jlacroix@westernu.edu).

Technical contact
Technical questions on executing this protocol should be directed to and will be answered by the technical contact, TharakaWijerathne (twijerathne@westernu.edu).

Materials availability
PIEZO1-cpGFP constructs will be available upon request from the lead contact.

Data and code availability
A set of raw data obtained using this protocol is available upon request from technical contact.

SUPPLEMENTAL INFORMATION
Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2023.102789.

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AUTHOR CONTRIBUTIONS

J.L. conceived the project associated with the protocol. T.W. and J.L. designed and troubleshot the protocols. T.W. tested the protocols.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Ozkan, A.D., Wijerathne, T.D., Gettas, T., and Lacroix, J.J. (2023). Force-induced motions of the PIEZO1 blade probed with fluorimetry. Cell Rep. *42*, 112837.

2. Lewis, A.H., and Grandl, J. (2020). Inactivation kinetics and mechanical gating of Piezo1 ion channels depend on subdomains within the cap. Cell Rep. *30*, 870–880.e2.