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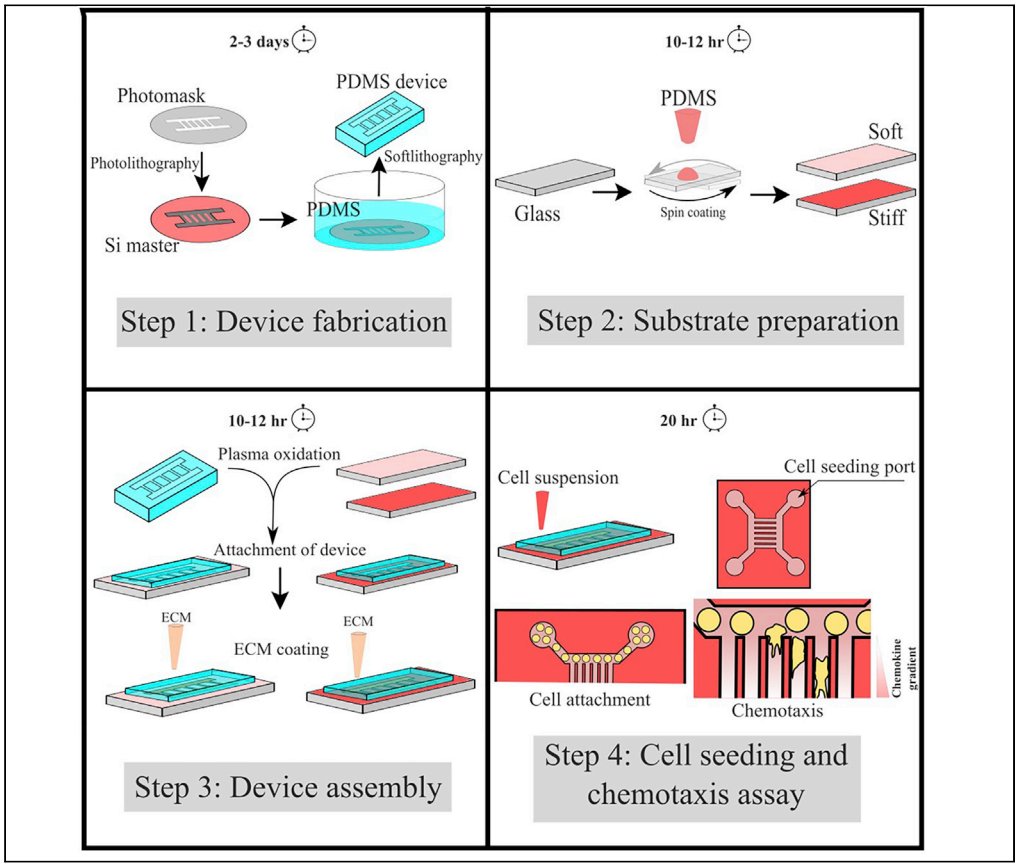
Fabrication of a microfluidic device for studying the combinatorial effect of physical and chemical cues on cell migration



In vivo cell migration is influenced by soluble factors as well as stiffness. Current *in vitro* strategies mostly account for one of these two factors to study cell migration. To understand the combinatorial effect of stiffness and chemokines on cell behavior, we have developed a microfluidic model to study stiffness-dependent chemotaxis of mesenchymal stem cells (hMSCs). A detailed description of our methodology will help researchers develop microfluidic models that combine these two factors influencing cell behavior.

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HIGHLIGHTS

Device allows independent control of substrate stiffness and chemokine gradient

Device allows maintenance of stable chemokine gradient for 12 h

PDMS substrates of varying stiffness can be incorporated into the integrated device

Device allows us to study stiffness-dependent chemotaxis of adherent cell types

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Fabrication of a microfluidic device for studying the combinatorial effect of physical and chemical cues on cell migration

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SUMMARY

***In vivo* cell migration is influenced by soluble factors as well as stiffness. Current *in vitro* strategies mostly account for one of these two factors to study cell migration. To understand the combinatorial effect of stiffness and chemokines on cell behavior, we have developed a microfluidic model to study stiffness-dependent chemotaxis of mesenchymal stem cells (hMSCs). A detailed description of our methodology will help researchers develop microfluidic models that combine these two factors influencing cell behavior.**

For complete details on the use and execution of this protocol, please refer to Saxena et al. (2018).

BEFORE YOU BEGIN

Preparation for fabricating the PDMS device

Timing: 2–3 days

1. Prepare master of the desired microfluidic device design on a silicon wafer before starting the experiment. Designing and fabrication of a microfluidic device include the following steps:
   a. Prepare photomask of the desired device design with appropriate dimensions by either printing on a transparency sheet or laser writing on a chrome-coated glass slide. In our experiments, we have used the following design with the given dimensions and printed on a transparency sheet using a printer with at least 600 dpi resolution (Figure 1).
   b. Device is made by the process called photolithography (Figure 2) using SU-8 (2050) photoresist (MicroChem).
      i. Spin coat 2 mL of negative photoresist on a 2-inch silicon wafer at 500 rpm for 10 s, followed by 2,000 rpm for 30 s.
      ii. After coating, soft bake the wafer on a hot plate at 65°C for 5 min and then at 95°C for 12 min.
      iii. Spin-coated SU-8 on the wafer is then exposed to Ultraviolet (UV, 350 nm) light through the photomask with an energy of 180 mJ/cm^2 using MJB4 mask aligner (Karl Suss, Garching, Germany), yielding the desired design on the wafer.
      iv. After the alignment and exposure process, post-bake the wafer on a hot plate at 65°C for 5 min and then at 95°C for 7 min.

v. After post-bake, immerse the wafer in SU-8 developer solution for 5–10 min, followed by washing with isopropanol.
vi. Air-dry the developed wafer and hard-bake for 15 min at 120°C. Pattern height should be measured using a profilometer (Dektak 150, Veeco Instruments, Inc.).

2. Clean all the glass slides/coverslip by sonication in 1 N NaOH and further washing with deionized water followed by drying.

⚠ CRITICAL: Always carry out Photolithography process in a clean room with protective clothing, gear, mask, and safety glasses on.

Cell culture maintenance for later use in the device

Timing: 3–4 days

3. Prepare complete media for hMSC culture – DMEM low glucose, 16% FBS, 1% Glutamax and 1% Antibiotic.
4. Seed hMSCs at a concentration of 10^5 cells in complete media in a T75 flask and incubate at 37°C in 5% CO_2 incubator.
5. For experiment, wash the cells with sterile phosphate buffer saline (PBS) after removing the media. Trypsinize the cells by adding 2 mL of Trypsin-EDTA for 5 min. Once detached, neutralize trypsin by adding 2 mL of complete media. Collect the trypsinized cells in a centrifuge tube and spin down at 500 × *g* for 5 min. Resuspend the cell pellet in 1 mL of complete media and count the cells with a hemocytometer. For seeding the cells in device, the recommended concentration is 10^6 cells/mL.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals		
SU8-2050	Microchem	Y111072
SU8-Developer	Microchem	Y020100
PDMS (Sylgard 184 and 527)	Dow Corning	NA
Collagen I (rat tail protein)	Thermo Scientific	A104830
DMEM low glucose (DMEM-LG)	Hi-Media	AL006
Fetal bovine serum (FBS)	Thermo Scientific	12662029
Phosphate buffer saline (PBS, without divalent cations)	Hi-Media	TS1120
Glutamax	Thermo Scientific	35050061
Trypsin-EDTA (0.25% Trypsin and 0.02% EDTA)	Hi-Media	TC1070
Antibiotic	Thermo Scientific	15140122
Hoechst 33342	Life Technologies	62249
Epidermal growth factor (EGF)	Sigma	E9644
Experimental models: cell lines		
hMSC	Bone marrow	Lonza (PT-2501)
Other		
MJB4 mask aligner	Karl Suss, Garching, Germany	NA
Spin coater	spinNXG-P2, Apex India	NA
Hot air oven	Custom made	NA
Inverted fluorescence microscope	EVOS FL Auto cell imaging system	NA
Plasma cleaner	Harrick-Plasma	PDC-32G
Biosafety cabinet	Custom made	NA
Glass slides/coverslips	Blue star	NA
Biopsy puncher (3 mm)	NA	NA

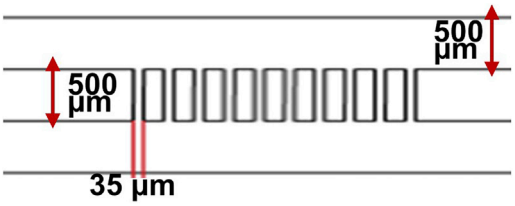

Figure 1. Top view of the microfluidic device used for studying human mesenchymal stem cell (hMSC) chemotaxis and homing

The device comprises two side-channels (500 µm width) connected by multiple transverse channels (35 µm width).

STEP-BY-STEP METHOD DETAILS

Timing: preparation time of substrates to a complete migration experiment requires 2 days

Preparation of PDMS substrates of varying stiffness

Timing: 10–12 h

Preparation of substrates of varying stiffness, which will serve as a base stiffness during cell migration (Figure 3).

Note: Use two different PDMS (Sylgard 184 and 527) for preparing substrates of varying stiffness (Palchesko et al., 2012).

1. Prepare Sylgard 527 by vigorously mixing equal mass of Part A and Part B provided by the manufacturer. Prepare Sylgard 184 by mixing 10 parts of base to one part of curing agent, followed by vigorous mixing and subsequent degassing for 20–30 min.
2. To make substrates of two different stiffnesses, mix above-prepared solutions of Sylgard 527 and Sylgard 184 in ratios of 5:1 and 50:1, respectively. The obtained stiffness from these two ratios are 30 kPa and 3 kPa, respectively.

Note: Other ratios can also be prepared for getting a wide range of stiffnesses.

3. For fabricating 2D PDMS substrates of these two different stiffnesses, spin coat the PDMS mixture (mix of Sylgard 527 and 184) on glass coverslips/slides using a spin coater (spinNXG-P2, Apex India) at 600 rpm for 30 s to obtain a uniform surface coating of 150 µm. To cure the coated PDMS, heat PDMS coated glass coverslips at 70°C in a hot air oven for 10–12 h.

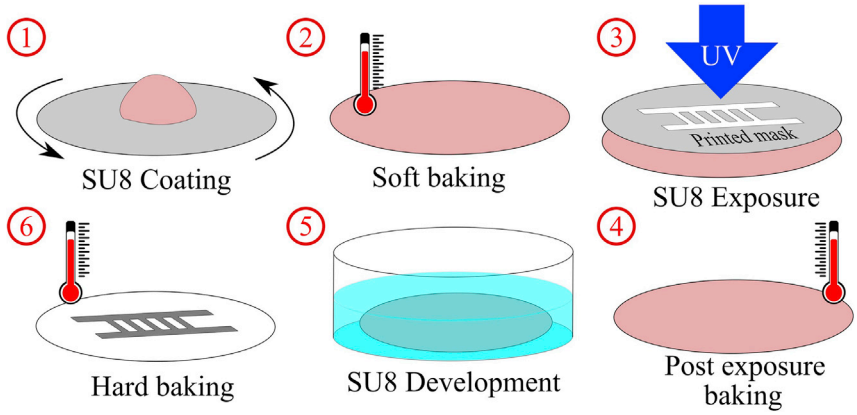

Figure 2. Steps for fabricating a microfluidic device master

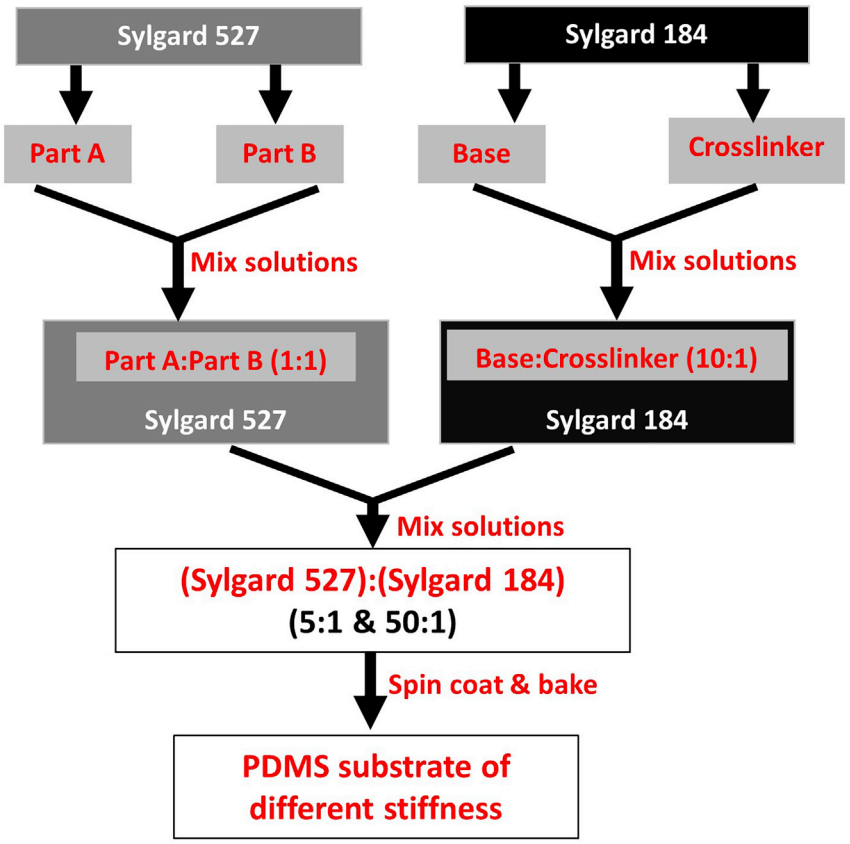

Figure 3. PDMS substrate preparation

Prepare Sylgard 527 by mixing part A and part B in a 1:1 ratio. Also, prepare Sylgard 184 by mixing base:crosslinker 10:1. Mix the two Sylgards in 5:1 and 50:1 ratios. After mixing, coat the prepared Sylgard mixture (PDMS mixture) on a glass coverslip and bake at 70°C for 10–12 h.

Note: Always cover the spin-coated surface to avoid dust and particle deposition. One would need to adjust speed to obtain coatings of different thicknesses.

4. Next day, the cured PDMS substrates of different stiffnesses are ready for use.

Note: Substrate stiffness and surface property could be characterized using a rheometer and a goniometer, respectively.

Fabrication of the microfluidic device

Timing: 6–7 h

Preparation of microfluidic device for chemotaxis assay (Figure 4).

Note: Use the device master prepared by photolithography (as described in Before you begin section) is used to fabricate the PDMS-based microfluidic devices.

5. Prepare Sylgard 184 for fabricating microfluidic devices:
   a. Prepare PDMS (Sylgard 184) by mixing 10 parts of the base to one part of the curing agent, followed by subsequent degassing.

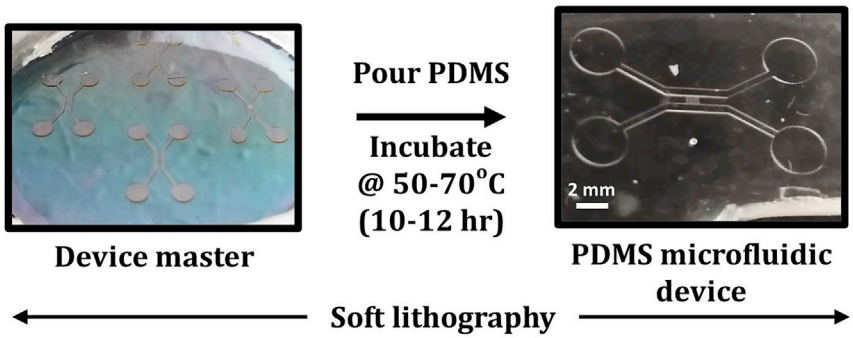

Figure 4. Soft-lithography process

Pour PDMS (Sylgard 184) on a silicon master containing the desired design. After PDMS addition, incubate the wafer at 50°C–70°C in a hot air oven for 6–7 h. After 7 h, peel off the PDMS device (top view) bearing the design from the master.

b. Pour the above degassed PDMS on the silicon wafer master (degas again if air bubbles appear post pouring). Heat at 50°C–70°C for 6–7 h in a hot air oven.

Note: This process of making a PDMS-based microfluidic device is called soft-lithography.

6. After 7 h, peel off the cured PDMS from the silicon master and cover it with scotch tape until further use to avoid dust deposition.

Note: Fabricated devices need to be free of dust particles to avoid blockages in the channels. Use Scotch tape to clean the devices.

7. Before use, punch the inlets and outlets in the device using an appropriate biopsy punch (we have used 3 mm).

Note: Inlets and outlets can be punched immediately after peeling off the PDMS device.

Optional: For long-duration cell culture purposes, autoclave microfluidic devices to avoid contamination during culture time.

Pause point: The prepared microfluidic devices can be stored at room temperature (22°C–28°C) until further use.

Assembling PDMS substrates and microfluidic devices

Timing: 5–10 min

Assembly of microfluidic device and PDMS substrates of varying stiffness for studying the combined effect of stiffness and chemical gradient on cell behavior (Figure 5).

8. Check the devices and substrates under an inverted microscope prior to assembly to avoid dust/dirt/particle deposition (clean with scotch tape if required).
9. To irreversibly bind the microfluidic device and different stiffness substrates, expose both of them to reactive oxygen plasma (Harrick-Plasma, PDC-32G) (exposure duration: 1 min).

Extracellular matrix (ECM) coating of the assembled microfluidic device

Timing: 10–12 h

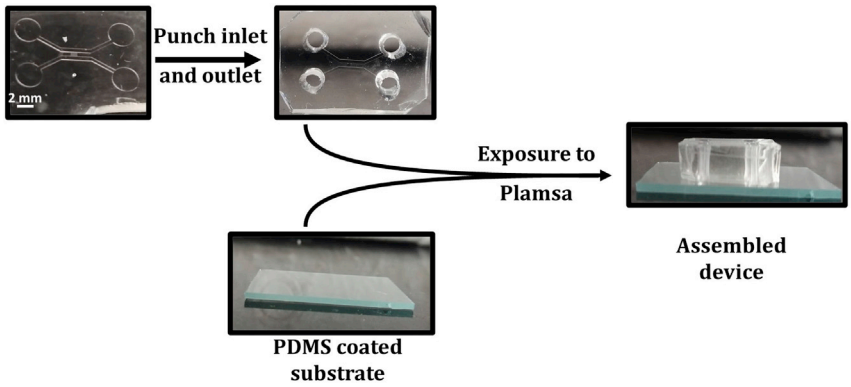

Figure 5. Device assembly

Punch the prepared PDMS device at the inlet and outlet using a biopsy puncher. Expose the punched device and prepared substrates to plasma for 1 min. After 1 min, the device and substrate are bonded together to form a complete device (side view).

ECM protein coating of assembled microfluidic device for cell attachment.

10. Add ECM protein (i.e., collagen I, 50 μg/mL) to the above assembled microfluidic device through one inlet port. Incubate the coated device for 10–12 h at 4°C.

⚠ CRITICAL: Devices need to be coated with ECM protein within 15 min of assembly; otherwise, devices will gain hydrophobicity. Once the PDMS regains hydrophobicity, it is difficult to perfuse the ECM protein solution through the device.

Note: Different concentrations of collagen I ranging from 5 μg/mL to 50 μg/mL works in this system (Saxena et al., 2018). According to available literature, various other ECM proteins (laminin, fibronectin etc.) can also be used for coating PDMS-based substrates (Jastrzebska et al., 2018; Wang et al., 2010).

11. Next day, before cell seeding, wash the device with PBS two times, followed by media perfusion through the inlet ports.

Note: Washing step is necessary to remove unbound ECM protein.

Cell seeding and experimental setup for chemotaxis assay

Timing: 8 h (cell seeding and adhesion) + 12 h for motility

Seeding of cells inside the ECM coated device to investigate the combinatorial effect of stiffness and chemokine gradient on cell migration (Figure 6).

12. Human mesenchymal stem cells (hMSCs) are cultured in a tissue culture flask with Dulbecco's modified Eagle's medium (DMEM) supplemented with 16% fetal bovine serum (FBS).

Note: Culture media and FBS may vary for different cell types.

13. Take trypsinized single-cell suspension and count the number of cells.

Note: Steps 14 and 15 need to be carried within small duration of time to keep the cells on one side of the channel.

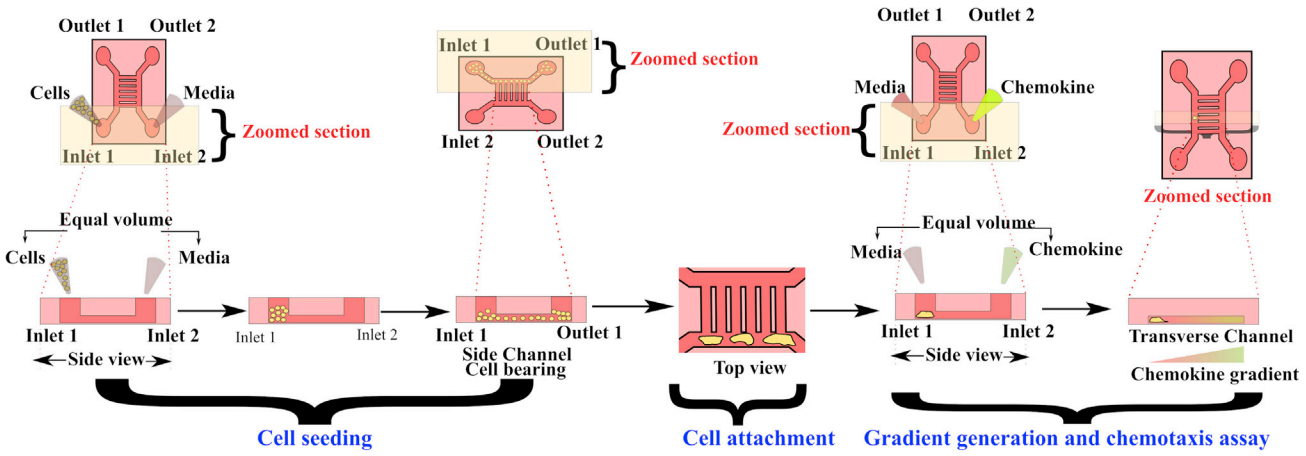

Figure 6. Cell seeding and chemotaxis assay

Seed hMSCs from the cell seeding port (inlet1) on one side of the channel. Allow cells to adhere for around 6 h with continuous replenishment of media after every 1–2 h. Once attached, generate the EGF (chemokine) gradient in the device. Capture images for 12 h at 15 min intervals using an inverted microscope equipped with an onstage incubator.

14. Take 10 μL of resuspended suspension media containing ~10,000 cells and introduce through one of the inlets.
15. From the other inlet, add 10 μL of complete media only and wait for 5 min to obtain pressure balance and incubate the device at 37°C.

⚠ CRITICAL: For performing chemotaxis assay, it is essential to have a sizable cell population on one side of the channel (if using a device design similar to ours).

16. Once the cells have adhered to the substrate (after ~6 h), introduce EGF as a chemokine through opposite channel inlet (in the cell-free side) for chemotaxis assay.

Note: Cells can be stained with Hoechst 33342 for 20 min before starting the experiment (as nucleus stain helps in cell tracking inside the channels).

17. To capture cell motility in the presence of EGF gradient and substrate stiffness, perform time-lapse microscopy for 12 h and capture images at a 15-minute time interval. For this time-lapse study, use an inverted microscope equipped with an onstage incubator to maintain 370 C and 5% CO_2 (Evos FL Auto, Life Technologies).
18. To quantify the cell motility, use manual cell tracker plugin of Image J (NIH).

EXPECTED OUTCOMES

At the end of the protocol, the time-lapse data can be analyzed to measure the combined effect of stiffness and chemokine on the morphology and motility of cells (Figure 7).

LIMITATIONS

Fabrication of substrates softer than 3 kPa stiffness is possible; however, lower stiffness substrates are sticky and tend to form surface deformations on the substrates during bonding the microfluidic devices. These deformations can lead to the blockage of microfluidic channels.

For maintaining chemotactic gradient longer than 12 h, a syringe pump has to be used.

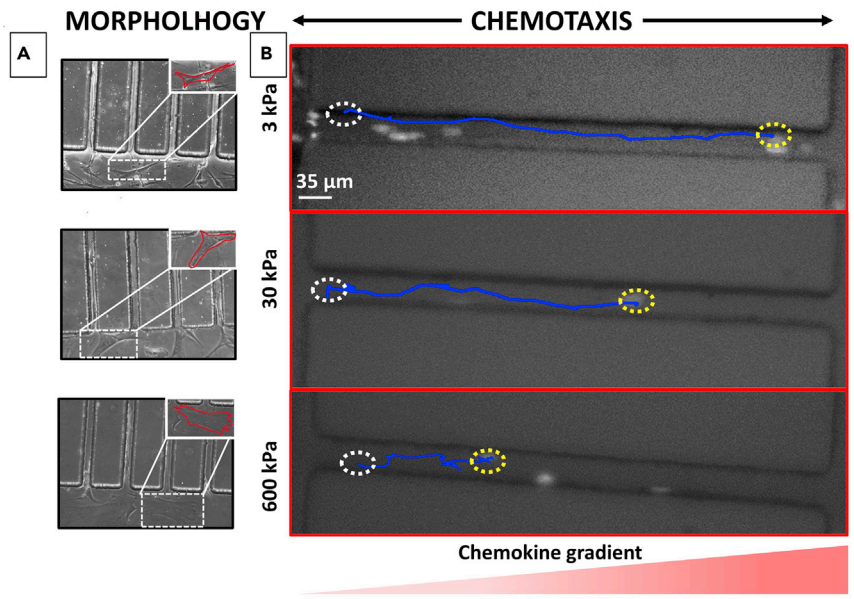

Figure 7. Cellular morphology and migration

(A) Representative images of hMSC morphology on substrates of varying stiffness.

(B) Representative images of hMSC chemotaxis on substrates of varying stiffness. White/yellow dotted ellipses depict the starting/ending position of the cells. Blue lines depict the trajectory of the cells based on tracking of the nucleus centroid.

TROUBLESHOOTING

Problem 1

Presence of dust particles on the surface of PDMS.

Potential solution

Always prepare substrates and PDMS devices in a dust free environment. After soft-lithography, clean the devices using scotch tape. Check under a microscope before and after assembling the device and avoid using the devices with blocked channels.

Problem 2

Improper bonding of device to substrate.

Potential solution

Plasma-based bonding is irreversible. Improper exposure time or energy of plasma may affect the bonding efficiency leading to leakage in the devices. If using a Harrick-plasma machine, always clean the chamber with isopropyl alcohol after use. This will help to maintain the required plasma energy essential for bonding. If using a different plasma machine, follow the manufacturer's protocol.

Always check devices under a microscope after adding the ECM protein if there is a leakage. Discard leaky devices.

Problem 3

PDMS regaining hydrophobicity.

Potential solution

It is critical to add ECM protein to the assembled devices within 15 min of bonding. Add sufficient amount of ECM protein (~60 μL) to the device to minimize drying inside the devices.

Problem 4
Cell survival.

Potential solution
The volume of media inside the device is in small quantity along with ~10,000 cells. It is difficult for the cells to attach and spread initially. To facilitate cell attachment and spreading, add fresh media to the device every 1 or 2 h for the initial 6 h duration.

Problem 5
Since the transverse channel size is 35 μm (the viewing chamber), it is difficult to figure out the cell boundary.

Potential solution
To address this, we have stained cells with Hoechst 33342 to track the cell movement by following its nucleus. Alternatively, the cell itself can be stained using any cell tracking dye (CellTracker CMTPX Dye) to visualize their morphology and movement.

Problem 6
Difficulty in having cells at the required viewing area and on one side of the channel.

Potential solution
Since there are two inlets and two outlets, the cells always tend to remain at inlets or outlets. Therefore, the key to it is to have a proper pressure drop across all the ports to have a significant number of cells in the side channel. Always use less volume of media to obtain pressure balance.

RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact Shamik Sen (shamiks@iitb.ac.in).

Materials availability
This study did not generate new unique reagents.

Data and code availability
The published article includes all details required for this study.

ACKNOWLEDGMENTS
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AUTHOR CONTRIBUTIONS
N.S., S.J., and S.S. conceived the idea. N.S. executed the idea, N.S., S.J., and S.S. wrote the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

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