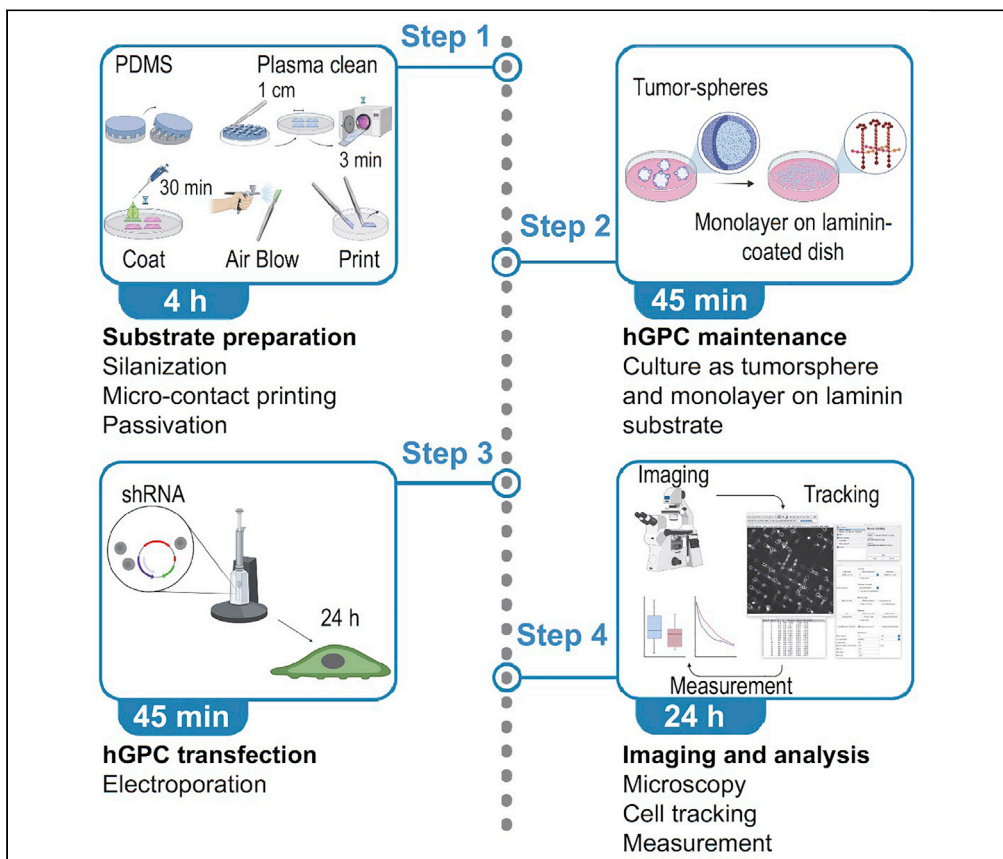


## Protocol

# Protocol to assess human glioma propagating cell migration on linear micropatterns mimicking brain invasion tracks



Glioblastoma (GBM) cells invade the brain by following linear structures like blood vessel walls and white matter tracts by using specific motility modes. In this protocol, we describe two micropatterning techniques allowing recapitulation of these linear tracks *in vitro*: micro-contact printing and deep UV photolithography. We also detail how to maintain, transfect, and prepare human glioma propagating cells (hGPCs) for migration assays on linear tracks, followed by image acquisition and analysis, to measure key parameters of their motility.

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**Highlights**  
 Micropatterning of  
 linear tracks on  
 imaging dishes

Maintenance and  
 preparation of human  
 glioma propagating  
 cells (hGPC) for  
 transfection

Transfection of hGPC  
 by electroporation  
 and preparation for  
 imaging

Imaging of hGPC  
 migration on linear  
 tracks, cell tracking,  
 and analysis

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## Protocol

## Protocol to assess human glioma propagating cell migration on linear micropatterns mimicking brain invasion tracks

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<https://doi.org/10.1016/j.xpro.2022.101331>

## SUMMARY

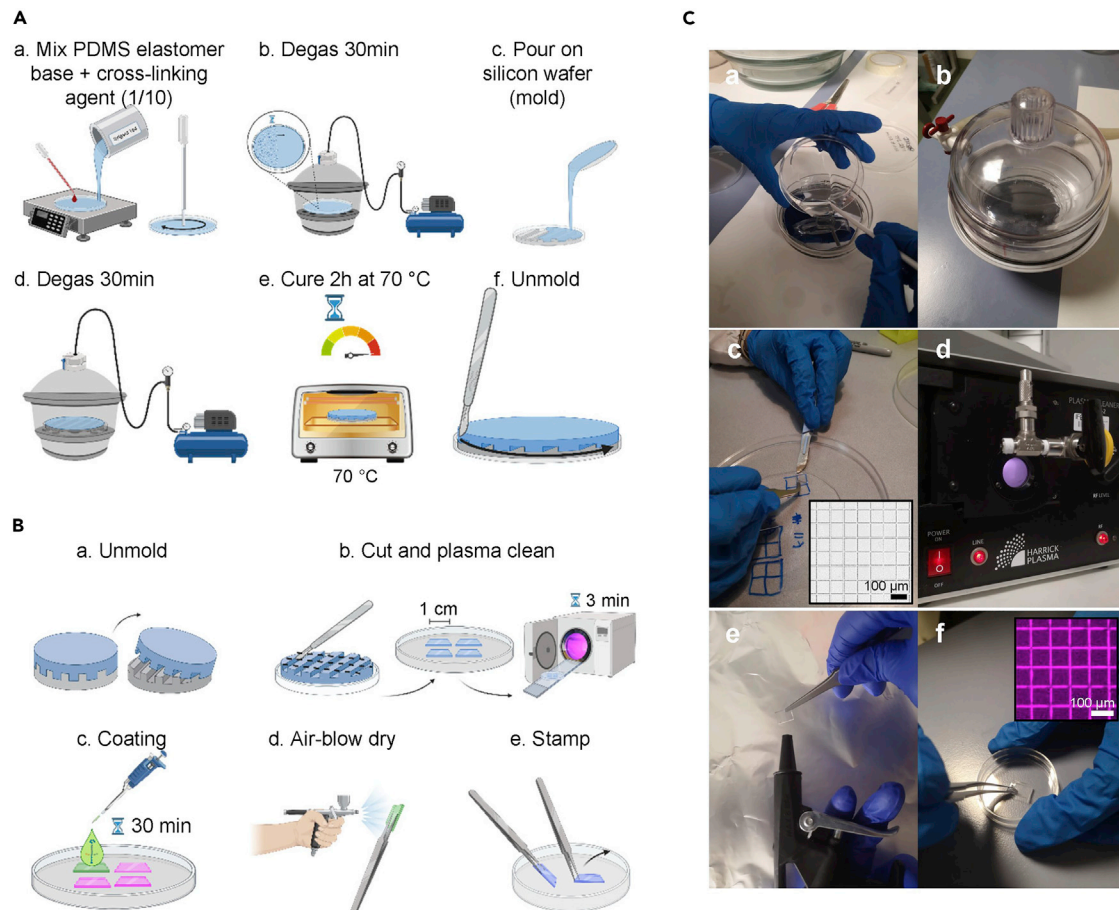
**Glioblastoma (GBM) cells invade the brain by following linear structures like blood vessel walls and white matter tracts by using specific motility modes. In this protocol, we describe two micropatterning techniques allowing recapitulation of these linear tracks *in vitro*: micro-contact printing and deep UV photolithography. We also detail how to maintain, transfect, and prepare human glioma propagating cells (hGPCs) for migration assays on linear tracks, followed by image acquisition and analysis, to measure key parameters of their motility. For complete details on the use and execution of this protocol, please refer to Monzo et al. (2016) and Monzo et al. (2021a).**

## BEFORE YOU BEGIN

Micropatterning techniques allow ‘printing’ matrix proteins on the surface of imaging dishes or coverslips to restrain cell adhesion onto specific patterns (Chen et al., 1997, 1998; Craighead et al., 2001; Singhvi et al., 1994). Several techniques have been used for micropatterning surfaces, including micro-contact printing or deep UV photolithography (Azioune et al., 2010; Csucs et al., 2003; Fink et al., 2007). When the technique of deep UV photolithography is used, the surface (i.e., the coverslip) is first ‘passivated’ with PLL-g-PEG and the pattern is then formed by burning the PLL-g-PEG through a photomask. The patterned area can then be coated with the molecule of interest. Photomasks can be obtained through various sources (Monzo et al., 2021b) (Monzo et al., 2021a).

When the microcontact printing technique is used, an elastomeric stamp (made of Polydimethylsiloxane, PDMS), with patterned relief structures, is first ‘inked’ with the molecule of interest such as laminin or fibronectin. The pattern is then printed onto the surface of an imaging dish by pressing the stamp onto the dish. The surface of the dish is then passivated to prevent cell attachment outside of the patterned area. To prepare the PDMS stamp, a silicon wafer with the negative features must be made using photolithography or laser engraving. This wafer will serve as a master mold from which positive PDMS replica will be obtained. Such wafers can be obtained from various commercial sources (Fink et al., 2007; Théry and Piel, 2009). Stamps are prepared by curing the PDMS onto the silicon wafer (Figure 1A) (Xia and Whitesides, 1998). To allow the unmolding of the PDMS, it is important to prevent its adsorption onto the wafer by silanizing the silicon wafer before its first use (Seed, 2000). After silanization, silicon wafers can be used multiple times without the need to be silanized again. Once cured, the molded PDMS can be kept at room temperature (20°C–25°C) for months.





**Figure 1. Microcontact printing**

(A) Preparation of PDMS (soft lithography). (a) elastomer base and cross-linking agent are mixed on a weighing board at 10:1 ratio. (b) PDMS is degassed under vacuum for 30 min. (c) PDMS is poured on the silicon wafer. (d) PDMS is degassed under vacuum for 30 min. (e) PDMS is cured for 2 h at 70°C. (f) PDMS is unmolded from the silicon wafer with a scalpel.

(B) Details of the various steps involved in microcontact printing. (a) PDMS is unmolded from the silicon wafer. (b) Stamps are cut in 1 cm<sup>2</sup> squares and plasma-cleaned for 3 min. (c) Stamps are coated with laminin for 30 min. (d) stamp is blown with an air-gun to remove the excess of laminin. (e) Stamp is leant and removed from the substrate to perform microcontact printing.

(C) Main steps of microcontact printing: (a) The mixture of elastomer base and cross-linking agent (PDMS 10:1, previously degassed) is poured on the silicon wafer (which has been silanized). (b) The PDMS on the silicon wafer is kept in a vacuum desiccator to remove all bubbles. (c) After curing, the PDMS is unmolded and cut into small stamps. The inset shows the gridded micropattern used in (Monzo et al., 2021a). (d) Stamps are plasma cleaned. (e) After incubation with laminin, the stamps are gently dried with an air-gun. (f) PDMS stamps are gently pressed onto the surface of the imaging dish to print laminin micropatterns (see inset). Scale bars are 100 μm.

### Silanization of the silicon wafer

⌚ Timing: 10 min + overnight incubation (12–20 h)

**Note:** Silane vapors are toxic and highly flammable. The steps involving silane should be conducted in a chemical fume hood.

1. Place the silicon wafer inside a plastic petri dish with the cap of an Eppendorf tube next to it.
2. Place the petri dish containing the silicon wafer and the cap inside a desiccator.
3. Place the desiccator under a chemical fume hood.
4. Add the compressed inert gas (such as nitrogen) in a beaker sealed with parafilm (keep adding the gas while aspirating with the syringe) and aspirate 100–200 μL of gas.

5. Punch the rubber cap of the hexamethyldisilazane bottle with the needle, turn the bottle upside down, inject the gas and withdraw 100–200  $\mu\text{L}$  of hexamethyldisilazane.
6. Transfer hexamethyldisilazane in the cap of the Eppendorf tube, close the desiccator, apply vacuum for 1 h, leave the desiccator sealed, and let the silane be deposited on the surface of the silicon wafer overnight (12 h–20 h).
7. The wafer is then transferred to a glass petri dish and is ready to use.

**△ CRITICAL:** Never open the silane solution vial. The solution is withdrawn from the vial by inserting a syringe needle through the rubber cap and exchanging an equivalent quantity of inert gas. This is to avoid oxidation that would prevent silane from binding to the silicone (Seed, 2000).

### Preparation of PDMS (Sylgard 184 silicone elastomer kit)

⌚ Timing: 3 h

The PDMS is prepared by mixing the elastomer base and the cross-linking agent in a ratio 10:1 (Sylgard 184 silicone elastomer kit).

8. In a weighing board or a 10-cm dish, pour 10 g of the elastomer base (Sylgard 184 silicone elastomer kit) and add 1 g of the cross-linking agent using a plastic Pasteur pipette (Figure 1A).
9. Stir vigorously the mixture using the same pipette.
10. Degas the mixture into a vacuum desiccator for 30 min or until all the bubbles have disappeared (Figure 1A).
11. Pour gently all the solution onto the wafer and degas again for 30 min or until all the bubbles have disappeared (Figures 1A and 1C-a-b).
12. Verify that no more bubbles are present and place the wafer in an oven at 70°C to cure for 2 h.
13. Unmold the PDMS by cutting around the edge with a scalpel and place it in a 15-cm plate with the features facing up (Figures 1A and 1B).

**Note:** PDMS can be stored for months at room temperature (20°C–25°C) protected from dust.

**△ CRITICAL:** Make sure no air bubbles are present when placing the wafer and PDMS to cure.

### Coating of untreated petri dishes

⌚ Timing: 45 min

For electroporation and migration assays, human Glioma Propagating Cells (hGPC) are cultured as monolayers on laminin-coated plates for 3–5 days. Laminin adsorbs better on untreated plastics like bacterial plates. We usually coat 6-cm bacterial plates to culture our hGPC and 35-mm bacterial plates to recover the cells after electroporation.

14. Under a biosafety cabinet, add 2 mL of DPBS in a 6-cm bacterial plate or 1 mL to a 35-mm bacterial plate.
15. Add laminin in the DPBS in order to obtain a 10  $\mu\text{g}/\text{mL}$  final laminin concentration. The amount of laminin depends on the concentration of the stock (e.g., dilute 17.2  $\mu\text{L}$  of laminin for a 1.16 mg/mL stock solution in 2 mL).
16. Rock the plate on a rocker at room temperature (20°C–25°C) for 30 min to ensure a homogeneous coating.
17. Aspirate the laminin solution and wash 2 times with 2 mL DPBS.

18. Replace the DPBS with 2–4 mL of GSC medium complemented with growth factor mix. The dish is now ready for cell seeding.

**△ CRITICAL:** To ensure an optimal coating of laminin, the dishes must not be tissue culture treated. Rocking allows the solution to cover the dish entirely. Due to the hydrophobicity of the bacterial plate, 2 mL of DPBS only will not spread as a flat layer over the surface. However, upon laminin adsorption, the 2 mL is enough to cover the surface of a 6-cm dish.

### Acid wash coverslips

⌚ Timing: 2 h

For deep UV photolithography, we wash our coverslips with nitric acid to remove any traces of oil that could impair the coating or the imaging.

**Note:** The steps involving nitric acid should be conducted in a chemical fume hood, with the appropriate PPE.

19. Fill a coverglass rack with the desired number of round coverslips and transfer it into a beaker.
20. Under a chemical fume hood add 20% Nitric acid to cover all the coverslips.
21. Cover the beaker with aluminum foil and incubate for 2 h.
22. Remove the rack with a tweezer, recycle back the acid in the acid bottle, put back the rack in the empty beaker, and rinse it 3 times with ddH<sub>2</sub>O.
23. Replace the ddH<sub>2</sub>O with a solution of ethanol at 70%, and keep covered to avoid evaporation.

**Note:** The coverslips can be stored in ethanol at room temperature (20°C–25°C) for several months.

### KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
3-(trimethoxysilyl)-propyl methacrylate	Merck	Cat#M6514-25ML
Albumin from Bovine Serum (BSA), Alexa Fluor™ 647 conjugate	Thermo Fisher Scientific	Cat#A34785
B-27™ Supplement	Thermo Fisher Scientific	Cat#17504044
Bovine serum albumin (BSA)	VWR	Cat#1000-70-500G
DMEM/F-12, GlutaMAX™ Supplement	Thermo Fisher Scientific	Cat#31331093
Heparin sodium salt from porcine intestinal mucosa	Sigma-Aldrich	Cat#H3149-10KU
HEPES	Euroclone	Cat#ECM0180L
Hexamethyldisilazane	Sigma-Aldrich	Cat#440191
Laminin Mouse Protein, Natural	Thermo Fisher Scientific	Cat#23017015
Nitric Acid	Merck	Cat#84378-1L
Non-essential amino acid	Biowest	Cat#X-0557
Penicillin/streptomycin	Euroclone	Cat#ECB3001
PLL-PEG (PLL(20)-g[3.5]- PEG(2))	SuSoS	Cat#PLL(20)-g[3.5]- PEG(2)
Pluronic® F-127	Sigma-Aldrich	Cat#P2443-250G
QIAfilter Plasmid Maxi Kit	QIAGEN	Cat#19743
Recombinant Human EGF	PeproTech	Cat#AF-100-15
Recombinant Human FGF-basic (154 a.a.)	PeproTech	Cat#100-18B

(Continued on next page)

<b>Continued</b>		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Sylgard 184 silicone elastomer kit	Dow Corning	Cat#1064291
Sodium pyruvate	Biowest	Cat#L0642
Tris ultrapure	Euroclone	Cat#EMR045001
Trypsin-EDTA 1× in PBS	Euroclone	Cat#ECB3052D
<b>Deposited data</b>		
Programs for speed and persistence measurements	(Gorelik and Gautreau, 2014)	<a href="https://www.nature.com/articles/nprot.2014.131#Sec28">https://www.nature.com/articles/nprot.2014.131#Sec28</a>
<b>Experimental models: Cell lines</b>		
NNI-21	Carol Tang, National Neuroscience Institute (NNI)	N/A
NNI-24	Carol Tang, National Neuroscience Institute (NNI)	N/A
NNI-11	Carol Tang, National Neuroscience Institute (NNI)	N/A
<b>Software and algorithms</b>		
ImageJ	(Schindelin et al., 2012)	<a href="https://imagej.nih.gov/ij/download.html">https://imagej.nih.gov/ij/download.html</a>
Graph Pad Prism 9.0	GraphPad	<a href="https://www.graphpad.com/scientific-software/prism/">https://www.graphpad.com/scientific-software/prism/</a>
Leica Application Suite (LAS)	Leica Microsystems	LAS
Microsoft Excel	Microsoft	N/A
<b>Other</b>		
Chrome mask	PHOTOMASK PORTAL	<a href="https://www.photomaskportal.com">https://www.photomaskportal.com</a>
Engraved silicon wafer	Mechanobiology Institute	MBI#395
Fluorescence lamp	Leica Microsystems	Kubler codix
1× Neon™ Transfection System 100 µL kit	Thermo Fisher Scientific	Cat#MPK10096
Neon Transfection System	Thermo Fisher Scientific	Cat#MPK5000
Glass bottom dishes, 35 mm	Mattek	Cat#P35G-1.5-20-C
Greiner dishes, petri dish, 35 × 10 mm, triple vented	Merck	Cat#P5112-740EA
Greiner dishes petri dish, 60 × 15 mm, vented (6 vents)	Merck	Cat#P5237-600EA
Microscope air pump	Okolab	OKO-AP
Microscope CO2 unit	Okolab	DGTCO2BX
Microscope heating unit	Okolab	H201-T
Microscope stage controller	Okolab	MW tango
TIRF microscope	Leica Microsystems	Leica AM TIRF MC
Plasma Cleaner	Harrick Plasma	PDC-32G-2 (230V)
Porcelain coverglass rack	Thomas scientific	N/A
Precision cover glasses thickness No. 1.5H (tol. ± 5 µm) for high performance microscopes, 24 mm Ø	Marienfeld	Cat#117640
UVO cleaner	Jelight Company Inc.	342-220

## MATERIALS AND EQUIPMENT

**EGF and FGF solutions:** FGF and EGF powders (stored at  $-80^{\circ}\text{C}$ ) are centrifuged at  $16,000 \times g$  for 30 s and reconstituted in sterile water to a solution at 1 mg/mL as indicated by the manufacturer. After reconstitution, the solution is further diluted in a sterile solution of BSA (1 mg/mL in DPBS) to a final concentration of 100 µg/mL.

**Note:** FGF and EGF are stored in 20 µL aliquots at  $-80^{\circ}\text{C}$  for < 1 year.

**GSC medium:** DMEM/F-12 containing glutamax is supplemented with sodium pyruvate, non-essential amino acid, penicillin/streptomycin.

Reagent	Final concentration	Total quantity
DMEM/F-12-glutamax (1×)	1×	500 mL
Sodium pyruvate (100 mM)	1 mM	5 mL
Non-essential amino acid (100×)	1×	5 mL
Penicillin/streptomycin (100×)	1×	5 mL

**Note:** This medium is stored at 4°C for 1–2 months.

**GSC-BSA medium:** DMEM/F-12 containing glutamax is supplemented with sodium pyruvate, non-essential amino acid, penicillin/streptomycin, and 25 g of BSA. The bottle of medium is incubated for 30 min at 37°C until BSA dissolves. The medium is then filtered (0.2 µm filter), aliquoted in 40 mL aliquots, which can be stored at –20°C for < 1 year.

Reagent	Final concentration	Total quantity
DMEM/F-12-glutamax (1×)	n/a	500 mL
Sodium pyruvate (100 mM)	1 mM	5 mL
Non-essential amino acid (100×)	1×	5 mL
Penicillin/streptomycin (100×)	1×	5 mL
BSA (Powder)	5%	25 g

**50× Grow factor mix solution:** Add 20 µL EGF (100 µg/mL), 20 µL FGF (100 µg/mL) and 100 µL heparin (5 mg/mL) to 2 mL B27 supplement, mix gently by pipetting up and down with a 5-mL serological pipette.

50× grow factor mix	Final concentration	Total quantity
B27 supplement (50×)	50×	2 mL
EGF (100 µg/mL)	1 µg/mL	20 µL
FGF (100 µg/mL)	1 µg/mL	20 µL
Heparin (5 mg/mL)	250 µg/mL	100 µL

**Note:** This mix is stored at 4°C for 2 weeks maximum. The mix is added directly to the cells (80 µL of mix per 4 mL GSC medium for a 6-cm dish) to obtain GSC medium containing a final concentration of B27 supplement (1×), bFGF (20 ng/mL), EGF (20 ng/mL), and heparin (5 µg/mL).

**Note:** 2 mL aliquots of B27 supplement are stored at –20°C until the expiration date.

**Laminin stock solution:** Laminin is provided at a concentration close to 1 mg/mL (exact concentration of the lot is indicated on the vial). Laminin is thawed overnight (12 h–20 h) at 4°C, aliquoted, and stored at 4°C.

**Note:** the laminin aliquots can be stored at 4°C for < 1 month. Once thawed, we never freeze back the laminin.

**Pluronic solution:** Add 2 g of pluronic F-127 to 1 L of ddH<sub>2</sub>O. To dissolve pluronic F-127, incubate the solution overnight (12–20 h) at 4°C, then filter (0.45 µm filter).

**Note:** The solution can be stored at room temperature (20°C–25°C) for several years.

**PLL-g-PEG stock solution:** a 1 mg/mL stock solution is prepared by adding 20 mL of 10 mM HEPES to 20 mg of PLL-g-PEG.

**Note:** The solution is filtered (0.2  $\mu\text{m}$  filter pore), aliquoted, and stored at 4°C for < 1 year.

**PLL-g-PEG working solution:** a 0.1 mg/mL working solution is prepared by adding 36 mL of 10 mM HEPES to 4 mL of PLL-g-PEG stock solution.

**Note:** The solution can be stored at 4°C for < 1 year.

**Time-lapse microscopy:** Total Internal Reflection Fluorescence (TIRF) microscope (Leica) equipped with temperature, CO<sub>2</sub> control, a HCX PL APO 10  $\times$  /1.47NA oil immersion objective. The microscope is driven with LAS software (Leica).

**Note:** any microscope equipped with temperature, CO<sub>2</sub> control, 10 $\times$  phase objective, and an automated stage can be used.

### STEP-BY-STEP METHOD DETAILS

#### Substrate preparation: silanization, micro-contact printing, passivation, and deep UV photolithography

⌚ Timing: 2 h for step 1

⌚ Timing: 2 h for step 2

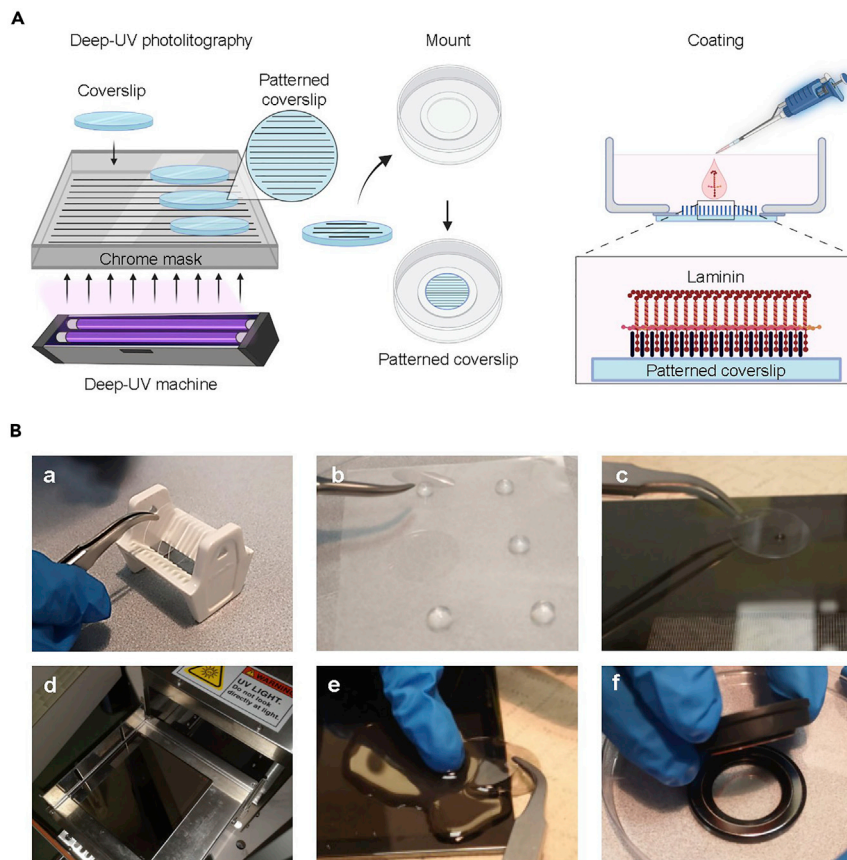
⌚ Timing: 4 h for step 3

The micropatterns can be obtained by microcontact printing: PDMS stamps are covered with a solution of laminin. Then, the excess of laminin solution is removed, and the stamps are carefully pressed on the surface of the imaging dish for imprinting the desired features on it (Figures 1B and 1C) (Fink et al., 2007; Xia and Whitesides, 1998). The rest of the surface is then passivated to prevent the cells from attaching outside of the stamped features. For routine migration assays, micropatterns are made on 35-mm plastic bacterial plates. For Total Internal Reflection Fluorescence (TIRF) or confocal imaging, micropatterns are made on glass-bottom dishes that are first silanized to help the laminin to adsorb better to the glass. The passivation on plastic is performed with pluronic solution while the passivation on glass is performed with PLL-g-PEG working solution. A good alternative to the microcontact printing on glass is the technique of deep UV photolithography that is also described here (Figure 2) (Azioune et al., 2009, 2010). Deep UV photolithography consists of first coating coverslips with PLL-g-PEG and then burning it with the desired features using a photo-mask and deep UV. The regions that have been burned can be subsequently coated with the desired substrate (in our case, laminin, Figure 2A). Deep UV photolithography is easier than the microcontact printing on glass and less prone to variations due to the experimenter. It is preferable when protein concentration has to be finely tuned, for substrate concentration comparison for example. However, since laminin adsorbs much better on plastic, micro-contact printing on plastic is preferred for long-term cultures or for large micropatterns where many cells need to adhere (Monzo et al., 2016). In this case, cells can stay attached to the patterns for many days whereas on glass, high-density cultures can rip off the laminin substrates rapidly.

**Note:** Sterility is not needed to perform the steps 1–3. However, at the very end of steps 2 and 3, dishes are transferred under a biosafety cabinet to be rinsed with sterile PBS and filled with culture medium under sterile conditions.

1. Silanization of glass bottom dishes:
  - a. Incubate the glass-bottom dishes in silane solution (1.2  $\mu\text{L}$  of 3-(trimethoxysilyl)-propyl methacrylate diluted in 2 mL methanol per dish) for 1 h at room temperature (20°C–25°C).
  - b. Remove silane solution and rinse three times with methanol for 5 min each time on a rocker.





**Figure 2. Deep-UV photolithography**

(A) Principles of deep-UV lithography. PEGylated coverslips are placed on the chrome mask, bubbles are removed and the PLL-g-PEG is burned through the micropatterns drawn on the photomask, by deep UV, for 10 min. The coverslips are then mounted either on an imaging chamber or on a 35-mm dish with a hole, and coated with laminin at 37°C for 1 h.

(B) Main steps of deep-UV lithography: (a) acid washed and air-dried coverslips are placed in a coverslip holder for plasma cleaning. (b) plasma cleaned coverslips are coated with PEG-g-PLL. (c) PEGylated coverslips are placed onto a drop of milliQ water covering the micropatterns on the chrome mask. (d) The chrome mask is placed in the deep-UV machine coverslips facing down. (e) The coverslips are lifted from the chrome mask using milliQ water. (f) Micropatterned coverslips are mounted in magnetic imaging chambers.

- c. Remove the methanol and air-dry the dishes under the chemical hood to evaporate the left-over methanol.
- d. Proceed with the stamping procedure as indicated in step 2.

△ **CRITICAL:** Silane vapors are toxic and highly flammable. The steps involving silane should be conducted in a chemical fume hood. Once silanized, the glass bottom dishes can be kept overnight (12–20 h), at room temperature (20°C–25°C), protected from dust, before micro-contact printing.

## 2. Micro-contact printing and passivation:

- a. Using a scalpel, cut out the desired features (stamps are around 1 cm<sup>2</sup>) and place them in a small culture dish (the features must be facing up).
- b. Remove the lid of the dish to expose the stamps and place it in the plasma cleaner. Start the vacuum pump, switch ON the plasma cleaner for 3 min (Figures 1B and 1C-d). This step makes the surface of the PDMS stamps hydrophilic and facilitates the coating of the stamps.

- c. Add 20  $\mu\text{L}$  of laminin solution (50  $\mu\text{g}/\text{mL}$  in DPBS) on top of the stamps, and incubate at room temperature (20°C–25°C) for 30 min (Figure 1B).

**Note:** Fluorescent BSA can be added to the laminin solution in order to visualize the stamped area at the microscope. We usually mix BSA-Cy5 at a final concentration of 7  $\mu\text{g}/\text{mL}$  with our laminin solution (Figure 1C-f) (Monzo et al., 2021a).

- d. Using a tweezer, hold the stamp, and air-blow dry all the laminin (Figures 1B and 1C-e), turn the stamp upside down and press it gently onto the imaging dish.

**△ CRITICAL:** No micro-droplets must be left on the edges of the stamp. Due to capillarity, they might flow in between the features of the stamp. However, an excessive air-blow dry of the stamp detaches the coating. Therefore, we recommend leaning the side of the stamp onto a kimwipe as a first step: this removes the majority of the coating solution. Then air-blow dry the stamp.

- e. After 1–2 min remove the stamp from the imaging dish in a single motion.

**△ CRITICAL:** The stamp must not be shaken while leaning. To remove it, we use two tweezers. This helps to leverage the stamp and avoids smearing the pattern. We never re-use the stamps and each stamp is meant for 1 dish.

- f. In case of plastic dishes, add 2 mL of pluronic solution per dish. In case of glass-bottom dishes, add 300  $\mu\text{L}$  of PLL-g-PEG working solution per dish. Incubate 1 h at room temperature (20°C–25°C).
- g. Transfer the dishes under a biosafety cabinet, aspirate the solutions of pluronic and PLL-g-PEG and replace with 2 mL of sterile DPBS, rinse 5 times with dPBS and replace with 2 mL GSC medium and 40  $\mu\text{L}$  of growth factor mix. The dishes are now ready for cell seeding.

**Note:** We never store the dishes with pluronic, PLL-g-PEG or DPBS, we recommend to store them in GSC medium at 37°C and to seed the cells within 24 hours (preferably the same day of micro-contact printing).

### 3. Deep UV photolithography.

- a. Pick up the coverslips from the ethanol solution and dry them one by one with an air-gun. Transfer them to a clean rack (Figure 2B-a).
- b. Place the rack containing the dried coverslips in a plasma cleaner, start the vacuum pump, switch ON the plasma cleaner for 3 min.
- c. Drop 100  $\mu\text{L}$  PLL-g-PEG working solution (100  $\mu\text{g}/\text{mL}$ ) on the bottom of a petri dish previously covered with a layer of parafilm (Figure 2B-b).
- d. Flip the coverslips onto the drops and incubate 1 h at room temperature (20°C–25°C).
- e. Using a tweezer, rinse the coverslips in a beaker containing milliQ water, and let them dry on a paper towel, the PEG-PLL facing up.
- f. Clean the photomask with isopropanol and carefully dry it with a kimwipe or airflow.
- g. Place the photomask under deep UV for 5 min (golden side facing up) to make it more hydrophilic.
- h. Place the photomask (golden side facing up) onto the bench, drop 4  $\mu\text{L}$  of milliQ water onto the desired pattern and flip the coverslip on the drop. The PLL-g-PEG must be in contact with the mask (Figure 2B-c).
- i. Remove all the bubbles between the mask and the coverslip by gently pressing on the coverslip with the tip of the tweezer.

**△ CRITICAL:** Always wear protective nitrile gloves when manipulating the mask to avoid leaving fingerprints. Make sure to remove the bubbles since they interfere with the

mask design. Some user might find it easier to remove the bubbles by pressing gently the cover slip with the tip of the finger. If using a metallic tweezer, be careful to not touch the patterns of the mask with the tweezer as it may scratch easily. If possible use a plastic tweezer.

- j. Place the mask (golden side facing down) under deep UV for 10 min (Figures 2A and 2B-d).
- k. Place the mask, face-up, on a paper towel, spray milliQ water onto the coverslips and let them detach (do not force them to detach to avoid smearing the PLL-g-PEG, Figure 2B-e).
- l. Rinse the coverslips in milliQ water and transfer them to an imaging chamber.

**Note:** The imaging chamber can be a magnetic imaging chamber (Figure 2B-f) or can be fabricated by mounting the coverslip onto a 35 mm petri dish with a hole in the middle of the bottom surface. The mounting can be done with biocompatible glue such as dental glue.

- m. Transfer the dishes under a biosafety cabinet, coat the coverslip with 10  $\mu\text{g}/\text{mL}$  laminin diluted in sterile DPBS, incubate at 37°C for at least one hour.
- n. Rinse 2 times with DPBS and replace with 2 mL GSC medium and 40  $\mu\text{L}$  of growth factor mix. The dishes are now ready for cell seeding.

**Note:** Before coating (step m) micropatterned-coverslips can be stored at 4°C, face-up, in a dish sealed with parafilm for <1 week. After coating, we never store the dishes with DPBS, we recommend storing them in GSC medium at 37°C and seeding the cells within the next 24 hours.

### Maintenance of human glioma propagating cells (hGPCs)

⌚ Timing: 30 min, every 5 days

Patient-derived glioblastoma propagating cells are obtained from patient tumor samples and are not commercially available. Methods to initiate and propagate such lines from tumor tissues are well established (Gritti et al., 1996; Stringer et al., 2019). NNI-11, NNI-21 and NNI-24 cell lines analyzed in (Monzo et al., 2021a) were provided by the laboratory of Carol Tang (Singapore) (Chong et al., 2016; Tan et al., 2019). The maintenance and expansion of these cells are described here. hGPCs are cultured in 6-cm dishes as tumor-spheres (Figure 3A). Every 5 days, spheres are triturated and passaged according to their growth rate (Figure 3B). Before imaging or transfecting, cells are triturated and passaged on plates coated with laminin (Figure 3C).

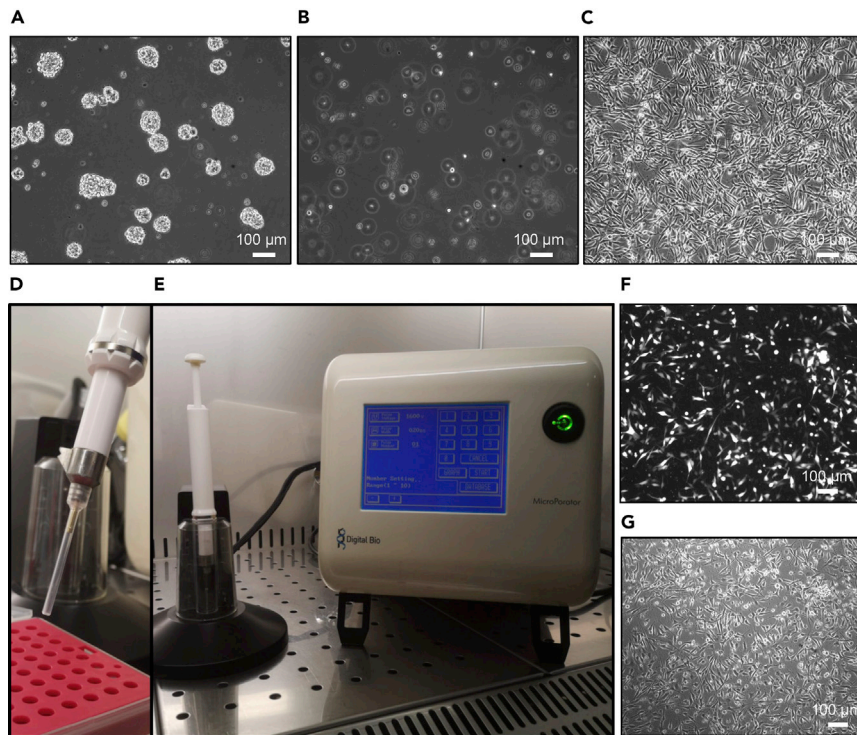
**Note:** The following steps should be conducted under a level 2 biosafety cabinet.

⚠ **CRITICAL:** hGPCs should be dissociated into single cells to ensure proper growth (Figure 3B).

4. Evaluate the density of the tumor-spheres at the microscope (see Figure 3A) and transfer the content of the dish into a 15-mL tube.
5. Centrifuge the sphere suspension at 200  $\times g$  for 5 min at room temperature (20°C–25°C).
6. Aspirate the supernatant, add 1 mL of GSC medium.
7. Triturate by pipetting the suspension up and down 100 times with a P1000 pipetman set at 800  $\mu\text{L}$ . After trituration, no sphere should remain (Figure 3B).

**Note:** If a large number of spheres remain after trituration, we recommend further triturating by pipetting 20–30 times. Passage number recommended: 10–15 times.

8. In function of the cell growth rate, transfer between 1/20 to 1/3 of the cell suspension volume to a new 6-cm culture dish containing 4 mL of GSC medium and 80  $\mu\text{L}$  of grow factor mix. Cells are also



**Figure 3. hGPC maintenance and transfection**

(A) hGPC tumor-spheres as seen under the microscope (10× objective) after 5 days in culture.  
 (B) hGPCs after trituration (no spheres are present).  
 (C) hGPCs as a confluent monolayer grown on a laminin-coated dish.  
 (D) Neon pipette and Neon tip.  
 (E) Neon Electroporator setup.  
 (F and G) hGPCs transfected with GFP control vector 24 h after electroporation (GFP channel and phase contrast).  
 Scale bars are 100 µm.

seeded on laminin-coated plates (Figure 3C) for transfection and/or migration assay (see ‘before you begin’ section for laminin coating).

9. After 5 days, passage the cells again.

### hGPC transfection by electroporation

⌚ Timing: 45 min

hGPCs are detached from their plate and counted. One to two million cells are electroporated with 10 µg of DNA using the Neon™ Transfection System 100 µL Kit and subsequently re-plated on a laminin-coated dish (Figures 3D–3G). For transfection, we use purified DNA (QIAfilter Plasmid Maxi Kit - Qiagen) adjusted at a concentration of 1 µg/µL in milliQ water.

**Note:** The following steps should be conducted under a level 2 biosafety cabinet.

10. Aspirate the medium and rinse the cells with DPBS.

11. Add 0.5 mL of trypsin/EDTA for a 6-cm dish and incubate for 2 min at 37°C.

**Note:** incubation time may vary according to the cell line. To avoid any toxicity effect due to over-exposure to trypsin, we advise monitoring cell detachment under the microscope.

12. Inactivate the trypsin with 3.5 mL of GSC-BSA medium and transfer the cell suspension in a 15-mL tube.

△ **CRITICAL:** To neutralize trypsin, a serum-containing medium must be avoided to prevent hGPC differentiation. We use BSA to neutralize the trypsin but other means such as trypsin inhibitor could be used.

13. Transfer 10  $\mu$ L of cell suspension and count the cells in a cell counting chamber (or the volume necessary for the available cell counting machine).
14. Centrifuge for 5 min at  $200 \times g$ .
15. Aspirate the supernatant, add 4 mL of DPBS to the cell pellet, and centrifuge for another 5 min at  $200 \times g$ .
16. Aspirate the supernatant and re-suspend the cell pellet with buffer R (that is contained in Neon™ Transfection System 100  $\mu$ L Kit) in order to obtain  $1\text{--}2 \times 10^6$  cells / 100  $\mu$ L.
17. Place the cuvette in the Neon electroporator and add 3.5 mL of buffer E2 (that is contained in Neon™ Transfection System 100  $\mu$ L Kit).

△ **CRITICAL:** Make sure the cuvette “clicks” inside the slot in the Neon electroporator. Clicking means that all the electrical pads that provide the voltage pulse make contact correctly. Avoid wetting the electrical pads located between the cuvette and the Neon electroporator.

18. Add 12  $\mu$ L of DNA to a 1.5 mL sterile Eppendorf tube, add 120  $\mu$ L of the cell suspension in buffer R. The volume of buffer R has to be 10 times greater than the volume of DNA. Mix gently up and down the cells with the DNA. Avoid bubble formation as much as possible as it will prevent the conduction of the electroporating stimulus.
19. Set the following parameters on the Neon electroporator. Voltage: 1,600 V, pulse duration: 20 ms, number of cycles: 1.

**Note:** The optimization of the electroporator parameters might vary according to the cell line. We recommend a dedicated experiment to find the parameters that maximize transfection efficiency and cell viability. These settings correspond to the ones we used for the hGPC line NNI-21 (Monzo et al., 2021a).

20. With the Neon pipette pick up the tip for electroporation (Figure 3D).

**Note:** We mix 120  $\mu$ L buffer R + 12  $\mu$ L of DNA to have an extra volume. This is to avoid bubble formation when we pipet the solution with the 100  $\mu$ L-neon tip.

21. Take 100  $\mu$ L of cell suspension - DNA mixture and insert the Neon pipette along with the tip in the cuvette.
22. Hit “Run” on the Neon electroporator. The machine indicates if the electroporation occurred or if there is a connection problem. Also, at the time of the choc, bubbles can be observed at the level of the resistance.
23. Transfer the electroporated cells directly to the 35-mm bacterial plate that was previously coated with laminin.

**Note:** If cells are well adherent on the dish, it is advisable to change the medium after 6/8 hours from transfection. This helps to improve cell viability.

24. The day after, check the cells and change the medium. Cells can also be trypsinized and transferred to another dish. Cells can start expressing the construct within the next 6–8 h (Figure 3F)

and 3G). For shRNA experiments, we typically wait 3 days after transfection to do the migratory experiment and the corresponding cell lysate for protein assay.

△ **CRITICAL:** For imaging and transfection, hGPCs are first cultured on a laminin dish. In fact, cells survive better after trypsinization than trituration which can lead to massive cell death. Also, this step allows a better estimation of the cell number upon counting.

### Imaging, cell tracking, and measurement of mean speed and persistence

⌚ **Timing:** 2 h

For migration assays (measure of speed and persistence), cells seeded on micropattern lines or grids are imaged in phase contrast using a 10× objective on a microscope equipped with temperature, humidity and CO<sub>2</sub> control (Figures 4A and 4B). Acquisitions are typically obtained over a period of 12–24 h.

#### 25. Cell seeding on micropatterns:

- Aspirate the medium from the cells previously seeded on laminin, rinse once with DPBS and incubate cells with 500 μL of trypsin/EDTA for 1–5 min at 37°C.
- Resuspend the cells with 3.5 mL of GSC-BSA medium to neutralize the trypsin and centrifuge at 200 × g for 5 min at room temperature (20°C–25°C).
- Aspirate the supernatant and re-suspend the cells in 4 mL of GSC medium.
- Count 10 μL of cells using a cell counting chamber.
- Seed 2,000–100,000 cells on the patterned dishes depending on the pattern and the size of the cells (we seed 2,000 cells on lines and 30,000 cells on grids with our NNI-21 with 1 cm<sup>2</sup> patterned area).
- Incubate at 37°C for 3–4 h, then gently rinse with warmed medium to get rid of the floating cells before imaging.

**Note:** The incubation time may be increased to 24–48 h depending on the cell's ability to attach and the type of patterns.

#### 26. Migration assay:

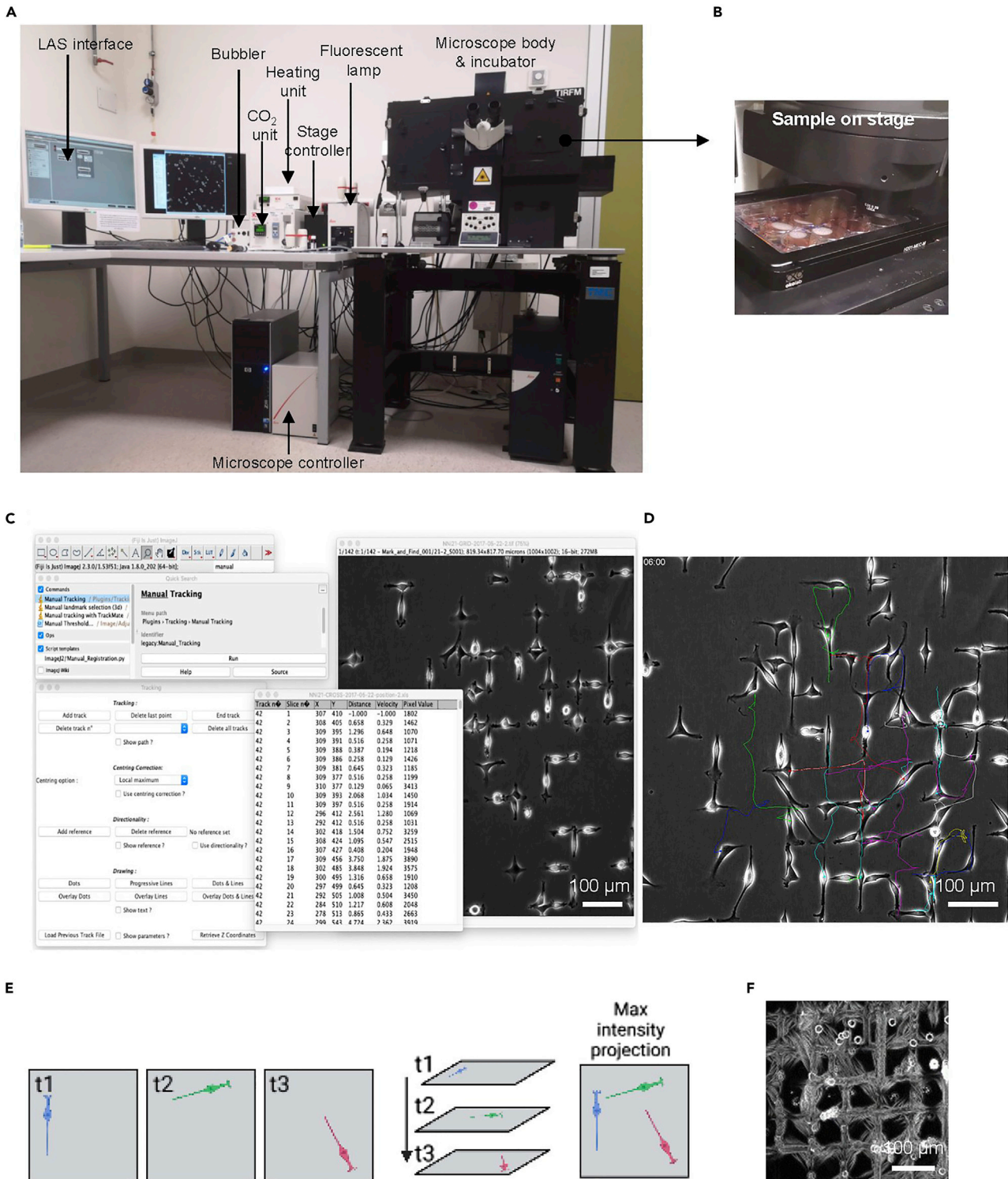
- Turn on the microscope, camera, CO<sub>2</sub> supply, the water pump and the heating unit at least 1 h before use. The heating unit temperature is set to 37°C. Set the 10× phase objective (Figure 4A).
- Place the imaging dishes in the plate holder and allow cells to stabilize for at least 30 min before recording (Figure 4B).
- Using the software interface, select the positions to record, adjust the focus using the camera, the exposure time, the light power, the time length of the acquisition, and the acquisition rate, and start imaging.

**Note:** We use a Leica microscope with the dedicated software Leica Application Suite (LAS). Any microscope equipped with temperature, CO<sub>2</sub> control, 10× phase objective, and an automated stage can be used. We typically image the cells at 1 image every 6 min for 12–20 h (overnight). Moreover, if cells were transfected with a fluorescent construct, the user should acquire the corresponding fluorescent channel to track the transfected cells.

#### 27. Cell tracking (Figure 4C):

- Open one imaging file with Fiji (Schindelin et al., 2012).
- In Plugins, go to tracking and manual tracking.

**Note:** Because glioma cells can move in antiparallel streams, change shapes, and bump frequently in each other, automated tracking programs cannot be used to determine speed



**Figure 4. hGPC imaging and manual tracking**

(A and B) Setup used for time-lapse acquisition.

(C and D) Manual tracking plugin in ImageJ/Fiji and tracks. Bars are 100  $\mu$ m.

(E) Maximum intensity projection: the images of each slice of the timelapse are overlaid on top of each other with ImageJ: Image>Stack>Z-project>MAX intensity.

(F) Resulting maximum intensity projection of the temporal stack of hGPCs migrating on the gridded micropattern over 7 h movie. Bar is 100  $\mu$ m.

and persistence accurately. Cell bodies are manually tracked using the manual tracking plugin of ImageJ/Fiji (Figure 4C). The tracks are then pasted in Excel and calculations are made to extract the speed and persistence.

- c. In the tracking window, hit the button “Add track”.
- d. Click on the cell to track: after each mouse click, the following image of the temporal stack is visualized until the last image is reached or the “End track” button is pressed.
- e. The track files are then saved and opened in excel for analysis.

**Note:** We usually track the first 6 hours of the movie, for statistical reasons and to avoid bias, we track all the cells that are present in the first frame of each movie. The following link gives detailed indications on how the manual tracking plugin works: <https://imagej.nih.gov/ij/plugins/track/track.html>. Moreover, in the case of transfected cells, double check with the fluorescent channel in order to be sure to track only cells that were successfully transfected.

- f. Under “Image > Stack > Z project > MAX intensity” the maximum intensity projection of the temporal stack can be obtained (Figures 4E and 4F). It is a way to extrapolate qualitative phenotypes from motile cells, such as hurdling or gliding (Monzo et al., 2021a).

#### 28. Measurement of speed and persistence:

- a. To extrapolate values of mean speed and persistence, we used 2 programs developed in (Gorelik and Gautreau, 2014) that can be integrated with Microsoft Excel.
- b. Open a Microsoft Excel workbook and activate the Developer tab. For Windows, click “File”, “Options”, “Customize Ribbon”, and flag “Developer”, then click “Ok”. For macOS, click on “Excel” in the menu bar, “Preferences”, “Ribbon & Toolbar”, then flag “Developer” and save (Figure 5A).
- c. For Windows, select “Macro Security” in the Developer tab. In the window that opens, flag “Enable all Macros” and “Trust access to the VBA project object model”. Click “Ok”. For macOS, click on “Excel” in the menu bar, “Preferences”, “Security”, then flag “Enable all macros” and “Trust access to the VBA project object model” and save (Figure 5B).
- d. Select “Visual Basic” in the Developer tab. The Visual Basic Editor appears.
- e. On the top left, click on “Insert Module”. A blank window appears (Figure 5C).
- f. Go on [https://static-content.springer.com/esm/art%3A10.1038%2Fnprot.2014.131/MediaObjects/41596\\_2014\\_BFnprot2014131\\_MOESM399\\_ESM.txt](https://static-content.springer.com/esm/art%3A10.1038%2Fnprot.2014.131/MediaObjects/41596_2014_BFnprot2014131_MOESM399_ESM.txt). Select all the text, copy and paste it in the blank window. This is the program for mean speed (Figure 5C).

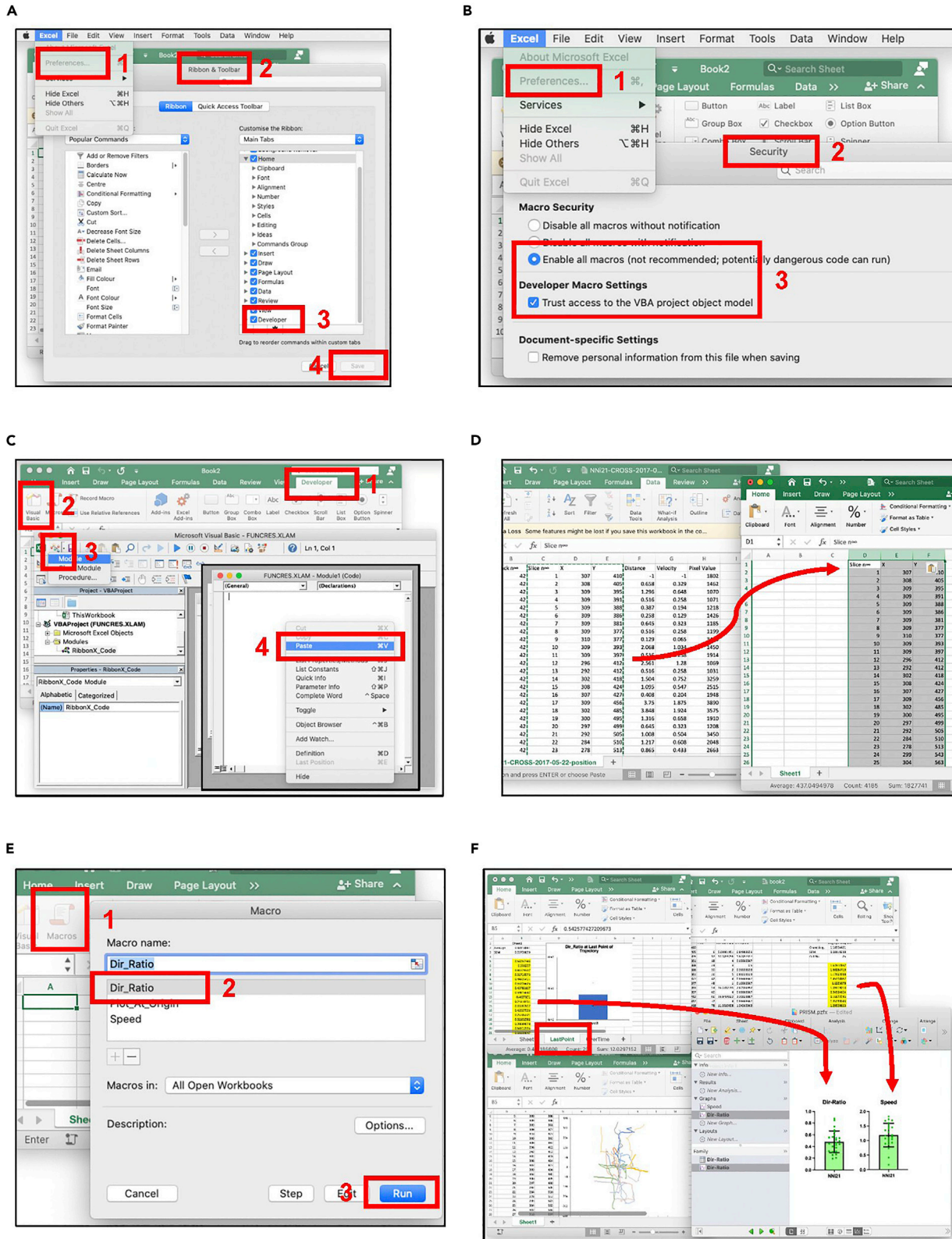
**△ CRITICAL:** Make sure to select all the text: use the shortcut “CTRL-A” (Windows) “command-A” (macOS).

- g. Repeat steps e and f with the following links:
  - i. [https://static-content.springer.com/esm/art%3A10.1038%2Fnprot.2014.131/MediaObjects/41596\\_2014\\_BFnprot2014131\\_MOESM400\\_ESM.txt](https://static-content.springer.com/esm/art%3A10.1038%2Fnprot.2014.131/MediaObjects/41596_2014_BFnprot2014131_MOESM400_ESM.txt). This is the program for cell persistence.
  - ii. [https://static-content.springer.com/esm/art%3A10.1038%2Fnprot.2014.131/MediaObjects/41596\\_2014\\_BFnprot2014131\\_MOESM394\\_ESM.txt](https://static-content.springer.com/esm/art%3A10.1038%2Fnprot.2014.131/MediaObjects/41596_2014_BFnprot2014131_MOESM394_ESM.txt). This is the program for Plot at origin.

**Note:** Other programs for further analysis can be downloaded from <https://www.nature.com/articles/nprot.2014.131>. In this protocol, we focused on speed, persistence and, plot at the origin.

- h. From the Visual Basic Editor, save the workbook as Excel Macro-Enabled Workbook (.xlsm). This format includes all the macros that have been pasted so far in the excel file.
- i. Make a copy of the .xlsm file just saved: we will paste cell trajectories on the copy and keep the original as a backup (select the file from the directory and copy-paste).





**Figure 5. From manual tracks to mean speed and persistence calculation**  
(A–F) visual instructions for mean speed, persistence and plot at origin representation. Error bars are S.D.

- j. From the trajectory file, copy the slice number of the trajectory, the X and Y coordinates and, paste them respectively in column D (or 4), E (or 5), and F (or 6) of a worksheet of the .xslm file just saved (Figure 5D). Numbers must start from 1, have no space between rows, and increase monotonically. When a new trajectory begins, the program will detect the change from, for instance, 43 to 1. At this step, it automatically generates a new trajectory.
- k. Rename the current worksheet (e.g., "Condition 1"), and repeat step j in new worksheets if additional conditions are present. The name of the worksheet is detected from the program and used in the graphs.
- l. From the Developer tab, click on "Macros." Select one of the installed programs and hit "Run". Results are displayed in a new worksheet (Figure 5E).
- m. To plot the data, copy and paste the data in a column tab in GraphPad Prism 9.0 (Figure 5F). For cell persistence, we focused on the last point: in the Excel workbook, go under the worksheet "LastPoint" and copy the data highlighted in Figure 5F.

### EXPECTED OUTCOMES

This protocol describes a way to design simple and controlled environments that mimic *in vivo* topography and that can be easily manipulated. We have used it to 1) analyze the motility modes of cells (glioma or other cells) and quantify their speed and persistence; 2) to compare transfected cells in order to define the role of a given protein in cell motility; 3) to generate high time-resolution kymographs to carefully describe a given motility mode; 4) to analyze the mechanoproperties of the cells on specific topographic environments; 5) to analyze the localization and function of molecules by high-resolution microscopy.

With this protocol of micropatterning, we have compared the motility modes of rat C6 glioma cells migrating on 2D and laminin strips of various widths (Monzo et al., 2016). We found that when C6 cells were migrating on thin lines (<10  $\mu\text{m}$ ), their motility was similar to the one observed *in vivo*, on brain blood vessels. They were moving in a 2 phase motion with one phase where cells were elongating in the 2 directions and one phase where the tail retracted (Monzo et al., 2016). Kymographs from high time-resolution movies (1 image/30 s) could be generated which allowed us to model this one-dimension type of motility (Ron et al., 2020). TIRF microscopy was used to analyze adhesion dynamics during this linear motility (Monzo et al., 2016). Moreover, we also observed antiparallel streaming when cells reached high density on larger strips which are similar to the currents observed later on, in neural progenitor cell cultures (Kawaguchi et al., 2017; Monzo et al., 2016). The motility of these cells was dependent on actin dynamics, myosin 2 and formins but was independent on Arp2/3 when using the Arp2/3 inhibitor CK666 which blocked motility on 2D but not on linear tracks. Finally, we could demonstrate the role of the formin FHOD3 in this linear motility (Monzo et al., 2016).

More recently, we have been able to compare the motility of several patient-derived cell lines on 3 different settings: 2D, grids and lines. We found that grids were the closest decoy to mimic the brain blood vessels, the most common tracks that these cells use when navigating the brain. We measured the speed and persistence of these cells and found that the most aggressive cells in the mouse brain were the fastest and the least persistent on 2D and grid. By using our micropatterned grids we have been able to describe two types of motility that we named gliding and hurdling (Monzo et al., 2021a). Gliders were cells that were persistently following the grids while the hurdlers were faster and 'jumped' from one line to another. These behaviors were easily observable by projecting the stacks of the time-lapses as explained in step 27-f. By manipulating the size of the grids, we could show that motility behaviors were intrinsic to the cells (Monzo et al., 2021a).

Using this protocol, we also analyzed the motility of other cell lines (cancer of various origin and normal fibroblast) and sorted them in function of their behavior. Moreover, through the function 'multiple stage positioning', we could test multiple drugs at different doses and tested also control and shRNA transfected cells simultaneously (Monzo et al., 2021a).

This protocol allowed us to compare the mechanoproperties of our patient-derived cell lines when migrating on linear tracks. Micropatterning on glass-bottom dishes allowed us to analyze cell adhesion (size, dynamic, number) by TIRF and Fluorescence Recovery After Photobleaching (FRAP). Deep UV photolithography allowed us to analyze traction forces (Hennig et al., 2020) and to measure cell stiffness by AFM. We found that the hurlers displayed adhesions that were larger in size and more dynamic. These cells were also the stiffest and displayed higher traction forces (Monzo et al., 2021a). Finally, micropatterning on glass allowed us to analyze the localization and the function of the formin FMN1 in the hurler migrating on linear tracks by high resolution microscopy (Monzo et al., 2021a).

## QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis can be done using GraphPad Prism 9.0 (Figure 5F). For statistical significance, we recommend doing t-test or one-way ANOVA test for mean speed. For the directionality ratio, we performed the Mann-Whitney test.

## LIMITATIONS

This protocol provides a quantitative way to evaluate the motility of hGPCs (or other cell lines) on an *in vitro* platform. The *in vitro* platform is a Petri dish (plastic or glass) that is functionalized with a gridded micropattern. This micropattern aims to reproduce linear tracks while forcing the cells to make a choice when encountering a junction: either turning 90 degrees or going straight. With this tool, we reproduce with a reasonable degree of complexity the blood vessel network that glioma cells follow as they invade the brain (Cuddapah et al., 2014). However, the gridded micropattern is made of a single protein (laminin in our case) deposited as a 7  $\mu\text{m}$  wide stripe onto a  $\sim\text{GPa}$  stiff substrate: we created a setup that is bi-dimensional with no consideration of the three-dimensional confinement of the brain.

*In vivo*, glioma cells migrate on the abluminal side of the blood vessels that contains laminin but also collagen IV and collagen V as well as fibronectin in some conditions (Bellail et al., 2004; Bellon et al., 1985; Charles et al., 2012; Giese and Westphal, 1996; Gladson, 1999; Knott et al., 1998; McComb and Bigner, 1985; Paulus et al., 1988). These substrates are not taken into account in our experimental setup since we have found that laminin only potentiated the motility of our glioma cells (Monzo et al., 2016, 2021a).

Moreover, by using stripes on plastic or glass, we do not reproduce the curved topography of the blood vessels. Other biophysical tools can take into account the tubular geometry of the blood vessels, such as electrospun nanofibers (Guetta-Terrier et al., 2015) (Monzo et al., 2021b) and brain slice assays (Polleux and Ghosh, 2002) that, however, lack controllability. In Monzo et al. (2021a), we utilized these 2 methodologies as an approach to complement what we observed on the gridded micropatterns. We exploited electrospun fibers to study the motility of the cell lines NNI-11, NNI-21, NNI-24, and the brain slice assay to validate the impact of the formin FMN1 on the invasion of NNI-21 (Monzo et al., 2021a).

Finally, our experimental setup does not take into account the mechanical confinement imposed by the brain, or its dimensionality. While migrating on the gridded micropattern, cells are laterally confined but the third dimension is not considered. Alternative approaches such as cellular embedding in soft hydrogels or brain slice assays are a closer mimicry of the mechanical confinement that the cells experience *in vivo*. In these cases, the environmental stiffness is in the same order of magnitude (from few to tens kilopascals, depending on the cross-linking) as the native one (the brain young's modulus ranges from several hundred pascals to several kilopascals) (Elkin et al., 2007; Franze, 2013; Hrapko et al., 2008).

## TROUBLESHOOTING

### Problem 1

Unmolding PDMS from the silicon wafer destroys the master (step 13 in “before you begin”).

### Potential solution

Most likely the silanization procedure of the wafer has failed. To overcome this, check that the silane is still functional by repeating the procedure on a new wafer. When curing a drop of PDMS on a corner of the silicon wafer, polymerized PDMS should peel off easily. Otherwise, the silane solution has been oxidized and should be replaced with a new one. Alternatively, PDMS might have been cured in the oven too long. Curing for more than 2 h at 70 C might trigger the binding of the PDMS to the wafer (Théry and Piel, 2009).

### Problem 2

Laminin solution does not spread as a flat layer on the bacterial dish (step 15 in “before you begin”).

### Potential solution

Increasing the volume of DPBS (and thus the volume of laminin) will ease the covering of the surface (step 15 in “before you begin”).

### Problem 3

hGPCs adhere to the cell culture plate despite being cultured in non-adherent conditions (step 9).

### Potential solution

This is dependent on the cell line. One of our cell lines (NNI-24) tends to attach to the dish at the beginning of the culture and grow more slowly than the others. After 5 days of culture, spheres are small and attached to the dish. In this case, we just add 80  $\mu$ L of B27 mix to the dish and wait another 5 days of culture. In general, after 5 days, spheres have detached and are ready for trituration.

### Problem 4

Micro-contact printing didn't work and cells do not adhere or adhere but no pattern is observable (step 25f).

### Potential solution

It means either that not enough pressure was applied to the stamp, or that the coating of the PDMS dried out before blowing it off. We recommend applying pressure uniformly with tweezers and not letting the PDMS dry out. On the other hand, applying an exaggerated pressure causes sagging in the PDMS and thus the whole stamp adheres to the substrate. This will result in the deposition of proteins outside of the PDMS features. Moreover, the stamp must not be moved while leaning. The whole procedure has to be carried out with precision and requires the least unwanted movement during PDMS handling. We recommend mixing fluorescent BSA with the laminin to be able to visualize the pattern after stamping.

### Problem 5

The exported .csv file containing the tracks shows “C1”, “C2”, etc. headings instead of “X”, “Y”, “Velocity”, etc. (step 27e).

### Potential solution

This is most likely due to a bug in the manual tracking software. To avoid this, we recommend hitting “Delete all tracks” and closing the result tab after saving. In any case, if such an error occurs, the results can be used and headings can be manually replaced by copy-paste from an uncorrupted file.

### Problem 6

When Excel recognizes numbers as strings, they are adjusted to the left-hand side in each cell, instead of the right-hand side. This happens with decimal numbers (step 28c).

### Potential solution

To overcome this, the user must change the decimal separator in the system settings and use the dot. In Windows, go under "Excel options > Advanced > Editing options > Use system separators" and replace it with the dot. In MacOS, select "System Preferences" in the status bar, then "Language & region > Advanced" and select the dot as a decimal separator. Alternatively, one can use the "find and replace" function in Excel (Control + H) and replace commas with dots (or vice-versa) in the Excel file.

## RESOURCE AVAILABILITY

### Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Pascale Monzo ([pascale.monzo@ifom.eu](mailto:pascale.monzo@ifom.eu)).

### Materials availability

This study did not generate new unique reagents.

### Data and code availability

Raw data are available upon request from the [lead contact](#), Pascale Monzo ([pascale.monzo@ifom.eu](mailto:pascale.monzo@ifom.eu)). The codes utilized in this protocol are published and available at <https://www.nature.com/articles/nprot.2014.131#Sec28>.

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## AUTHOR CONTRIBUTIONS

M.C. and P.M. wrote the manuscript. M.C., T.D., and P.M. performed the experiments and analyzed the data. N.C.G. supervised the work, reviewed the manuscript, and provided funding support.

## DECLARATION OF INTERESTS

The authors declare no competing interests.

## REFERENCES

- Azioune, A., Carpi, N., Tseng, Q., Thery, M., and Piel, M. (2010). Protein micropatterns: a direct printing protocol using deep UVs. *Methods Cell Biol.* *97*, 133–146.
- Azioune, A., Storch, M., Bornens, M., Thery, M., and Piel, M. (2009). Simple and rapid process for single cell micro-patterning. *Lab Chip* *9*, 1640–1642.
- Bellail, A.C., Hunter, S.B., Brat, D.J., Tan, C., and Van Meir, E.G. (2004). Microregional extracellular matrix heterogeneity in brain modulates glioma cell invasion. *Int. J. Biochem. Cell Biol.* *36*, 1046–1069.
- Bellon, G., Caulet, T., Cam, Y., Pluot, M., Poulin, G., Pytlinska, M., and Bernard, M.H. (1985). Immunohistochemical localisation of macromolecules of the basement membrane and extracellular matrix of human gliomas and meningiomas. *Acta Neuropathol.* *66*, 245–252.
- Charles, N.A., Holland, E.C., Gilbertson, R., Glass, R., and Kettenmann, H. (2012). The brain tumor microenvironment. *Glia* *60*, 502–514.
- Chen, C., Mrksich, M., Huang, S., Whitesides, G., and Ingber, D. (1997). Geometric control of cell life and death. *Science* *276*, 1425–1428.
- Chen, C.S., Mrksich, M., Huang, S., Whitesides, G.M., and Ingber, D.E. (1998). Micropatterned surfaces for control of cell shape, position, and function. *Biotechnol. Prog.* *14*, 356–363.
- Chong, Y.K., Sandanaraj, E., Koh, L.W., Thangaveloo, M., Tan, M.S., Koh, G.R., Toh, T.B., Lim, G.G., Holbrook, J.D., Kon, O.L., et al. (2016). ST3GAL1-associated transcriptomic program in glioblastoma tumor growth, invasion, and prognosis. *J. Natl. Cancer Inst.* *108*, djv326.
- Craighead, H.G., James, C.D., and Turner, A.M.P. (2001). Chemical and topographical patterning for directed cell attachment. *Curr. Opin. Solid State Mater. Sci.* *5*, 177–184.
- Csucs, G., Michel, R., Lussi, J.W., Textor, M., and Danuser, G. (2003). Microcontact printing of novel co-polymers in combination with proteins for cell-biological applications. *Biomaterials* *24*, 1713–1720.
- Cuddapah, V.A., Robel, S., Watkins, S., and Sontheimer, H. (2014). A neurocentric perspective on glioma invasion. *Nat. Rev. Neurosci.* *15*, 455–465.
- Elkin, B.S., Azeloglu, E.U., Costa, K.D., and Morrison, B.M., III (2007). Mechanical heterogeneity of the rat Hippocampus measured by atomic force microscope indentation. *J. Neurotrauma* *24*, 812–822.
- Fink, J., Thery, M., Azioune, A., Dupont, R., Chatelain, F., Bornens, M., and Piel, M. (2007). Comparative study and improvement of current cell micro-patterning techniques. *Lab. Chip* *7*, 672–680.
- Franze, K. (2013). The mechanical control of nervous system development. *Development* *140*, 3069–3077.
- Giese, A., and Westphal, M. (1996). Glioma invasion in the central nervous system. *Neurosurgery* *39*, 235–252.

- Gladson, C.L. (1999). The extracellular matrix of gliomas: modulation of cell function. *J. Neuropathol. Exp. Neurol.* *58*, 1029–1040.
- Gorelik, R., and Gautreau, A. (2014). Quantitative and unbiased analysis of directional persistence in cell migration. *Nat. Protoc.* *9*, 1931–1943.
- Gritti, A., Parati, E.A., Cova, L., Frolichsthal, P., Galli, R., Wanke, E., Faravelli, L., Morassutti, D.J., Roisen, F., Nickel, D.D., et al. (1996). Multipotential stem cells from the adult mouse brain proliferate and self-renew in response to basic fibroblast growth factor. *J. Neurosci.* *16*, 1091–1100.
- Guetta-Terrier, C., Monzo, P., Zhu, J., Long, H., Venkatraman, L., Zhou, Y., Wang, P., Chew, S.Y., Mogilner, A., Ladoux, B., et al. (2015). Protrusive waves guide 3D cell migration along nanofibers. *J. Cell Biol.* *211*, 683–701.
- Hennig, K., Wang, I., Moreau, P., Valon, L., DeBeco, S., Coppey, M., Miroshnikova, Y.A., Albiges-Rizo, C., Favard, C., Voituriez, R., et al. (2020). Stick-slip dynamics of cell adhesion triggers spontaneous symmetry breaking and directional migration of mesenchymal cells on one-dimensional lines. *Sci. Adv.* *6*, eaau5670.
- Hrapko, M., van Dommelen, J.A., Peters, G.W., and Wismans, J.S. (2008). The influence of test conditions on characterization of the mechanical properties of brain tissue. *J. Biomech. Eng.* *130*, 031003–031010.
- Kawaguchi, K., Kageyama, R., and Sano, M. (2017). Topological defects control collective dynamics in neural progenitor cell cultures. *Nature* *545*, 327–331.
- Knott, J.C., Mahesparan, R., Garcia-Cabrera, I., Bolge Tysnes, B., Edvardsen, K., Ness, G.O., Mork, S., Lund-Johansen, M., and Bjerkvig, R. (1998). Stimulation of extracellular matrix components in the normal brain by invading glioma cells. *Int. J. Cancer* *75*, 864–872.
- McComb, R.D., and Bigner, D.D. (1985). Immunolocalization of laminin in neoplasms of the central and peripheral nervous systems. *J. Neuropathol. Exp. Neurol.* *44*, 242–253.
- Monzo, P., Chong, Y.K., Guetta-Terrier, C., Krishnasamy, A., Sathe, S.R., Yim, E.K., Ng, W.H., Ang, B.T., Tang, C., Ladoux, B., et al. (2016). Mechanical confinement triggers glioma linear migration dependent on formin, FHOD3. *Mol. Biol. Cell* *27*, 1246–1261.
- Monzo, P., Crestani, M., Chong, Y.K., Ghisleni, A., Hennig, K., Li, Q., Kakogiannos, N., Giannotta, M., Richichi, C., Dini, T., et al. (2021a). Adaptive mechanoproperties mediated by the formin FMN1 characterize glioblastoma fitness for invasion. *Dev. Cell* *56*, 2841–2855.e8.
- Monzo, P., Crestani, M., and Gauthier, N.C. (2021b). In vitro Mechanobiology of glioma: mimicking the brain blood vessels and white matter tracts invasion paths. In *Brain Tumors*, G. Seano, ed. (Springer US), pp. 159–196.
- Paulus, W., Roggendorf, W., and Schuppan, D. (1988). Immunohistochemical investigation of collagen subtypes in human glioblastomas. *Virchows Arch. A Pathol. Anat. Histopathol.* *413*, 325–332.
- Polleux, F., and Ghosh, A. (2002). The slice overlay assay: a versatile tool to study the influence of extracellular signals on neuronal development. *Sci. STKE* *2002*, p19.
- Ron, J.E., Monzo, P., Gauthier, N.C., Voituriez, R., and Gov, N.S. (2020). One-dimensional cell motility patterns. *Phys. Rev. Res.* *2*, 033237.
- Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., et al. (2012). Fiji: an open-source platform for biological-image analysis. *Nat. Methods* *9*, 676–682.
- Seed, B. (2000). Silanizing glassware. *Curr. Protoc. Cell Biol.* *8*, A.3E.1–A.3E.2.
- Singhvi, R., Kumar, A., Lopez, G.P., Stephanopoulos, G.N., Wang, D.I., Whitesides, G.M., and Ingber, D.E. (1994). Engineering cell shape and function. *Science* *264*, 696–698.
- Stringer, B.W., Day, B.W., D'Souza, R.C.J., Jamieson, P.R., Ensbey, K.S., Bruce, Z.C., Lim, Y.C., Goasdoue, K., Offenhauser, C., Akgul, S., et al. (2019). A reference collection of patient-derived cell line and xenograft models of proneural, classical and mesenchymal glioblastoma. *Sci. Rep.* *9*, 4902.
- Tan, M.S.Y., Sandanaraj, E., Chong, Y.K., Lim, S.W., Koh, L.W.H., Ng, W.H., Tan, N.S., Tan, P., Ang, B.T., and Tang, C. (2019). A STAT3-based gene signature stratifies glioma patients for targeted therapy. *Nat. Commun.* *10*, 3601.
- Théry, M., and Piel, M. (2009). Adhesive micropatterns for cells: a microcontact printing protocol. *Cold Spring Harb. Protoc.* *2009*, pdb.prot5255.
- Xia, Y., and Whitesides, G.M. (1998). Soft lithography. *Annu. Rev. Mater. Sci.* *28*, 153–184.