Methods in Molecular Biology
DOI 10.1007/7651_2025_628

Microwells as Minimalistic Niches to Study Heterotypic Interactions of Stromal and Hematopoietic Stem Cells

Adrian Candelas, Thomas Bessy, Benoit Vianay, Manuel Théry, and Stephane Brunet

Abstract

Hematopoietic stem and progenitor cells (HSPCs) can migrate and reside within the bone marrow in distinct microenvironments or niches. The niches organize around specific stromal cells, such as endothelial cells at the capillary or sinusoid walls, and osteoblasts along the bone matrix. Within each niche, a specific combination of external cues, including secreted and diffusible factors, cell-matrix, and cell-cell interactions, controls HSPCs behavior and fate. Deciphering the interplay between HSPCs and stromal cells of the niches is challenging: in vivo, it is hindered by the opacity of the bone matrix; in vitro, classical co-culture models only poorly recapitulate essential features of the physiological niches. The difficulty is moreover amplified by the exceptional migration capacity of HSPCs.

In this chapter, we present a method to overcome these limitations by producing arrays of microwells designed to mimic bone marrow niches in a functional manner. These "microniches" promote a long-term interaction between the HSPC and a stromal cell of interest. We describe their microfabrication based on a maskless photolithography method allowing the production of arrays of microwells with reproducible volume and geometry, and the iterative improvement of the geometric design of the wells. We describe the loading and culture of stromal cells with HSPCs. We discuss the potentiality of microwells, in basic and applied research, as a platform to investigate molecular mechanisms involved in direct cell-cell interactions and local effects of diffusible factors, for any adherent and non-adherent cells of interest.

Key words Hematopoietic stem and progenitor cells, High-resolution imaging, Hydrogel, Live imaging, Microfabrication artificial biomimetic niche, Microwell, Stem cell niche

1 Introduction

Adult stem cells (SCs) function and homeostasis are controlled to a large extent by extrinsic cues provided by the stem cell niches. These cues can induce cell-intrinsic regulatory networks and modulate gene expression programs, or drive asymmetric cell divisions that generate daughter cells with distinct cell fates [1, 2]. Paradigmatic of adult SC, hematopoietic stem and progenitor cells (HSPC) can self-renew and differentiate into lineage-committed daughter cells that will give rise to all short-lived cell types of the hematopoietic system [3]. HSPCs reside within the bone marrow (BM) in distinct niches: mainly the vascular niche, located deep in the BM,

enriched in sinusoidal capillaries, and the endosteal niche, positioned at the interface of the BM and the bone [4]. Strikingly, HSPCs share with their progenies a migratory phenotype which appears to be unique for adult stem cells. This migration capacity underlies the ability for HSPC to move from one niche to another and exchange between BM and blood [5]. Within each niche, HSPC behavior and fate are controlled by a specific combination of external cues that include diffusible factors produced by the stromal cells of the niche, and direct interactions with these cells [6, 7]. Altered interactions between the HSPC and the niche components can drive the emergence of malignant cells in the bone marrow and allow their overgrowth at the expense of HSPC homeostasis, leading to severe blood malignancies [8, 9].

Deciphering the interplay between the HSPC and stromal cells of the niches remains challenging due to limitations of in vivo and classical in vitro co-cultures models, amplified by the migration capacity of HSPCs. The in vivo approaches that have been developed so far, mostly on mouse models, allow analyses at a cell population level. They do not allow investigations at a single-cell level with adequate timescale dynamics [10, 11]. On the other side, classical in vitro co-cultures have been used [12]. They only poorly recapitulate essential features of the physiological niches, such as the confinement encountered by HSPCs, or prolonged interactions with the stromal cells of the niches. To overcome these limitations, various systems of intermediate complexity have been proposed, ranging from microwell arrays [13] to elaborate bone marrow-on-a-chip [14, 15]. Based on microfabrication technology, we have developed arrays of polyacrylamide (PAA) microwells to mimic BM niches in a minimalistic yet functional manner. Each microwell allows spatial confinement in a controlled and reproducible volume for the co-culture of a single HSPC and a stromal cell of interest (e.g., osteoblast or endothelial cell). This way, it favors stable heterotypic interactions.

The fabrication of these microwells arrays relies on standard photolithography techniques to produce a SU-8 mold with a chrome mask. This mask is designed in order to produce microwells of controlled dimensions and geometry (*see* Fig. 1a). This master mold is employed to create sequentially primary and secondary PDMS molds (Fig. 1b). The secondary mold features an array of pillars, used as a negative of the PAA gel to shape the dimensions and geometry of the polyacrylamide (PAA) microwells. A PAA solution is flowed by capillarity between the PDMS stamp and the coverslip. UV-induced polymerization of PAA leads to the assembly of an array of microwells formed by an anti-adherent polyacrylamide wall and an adhesive glass bottom (Fig. 2a, b). This configuration restricts cell adhesion to the microwell bottom, preventing cells escape and promoting cell-cell interactions within the well. Specific coating of the well bottom with adhesion molecules of

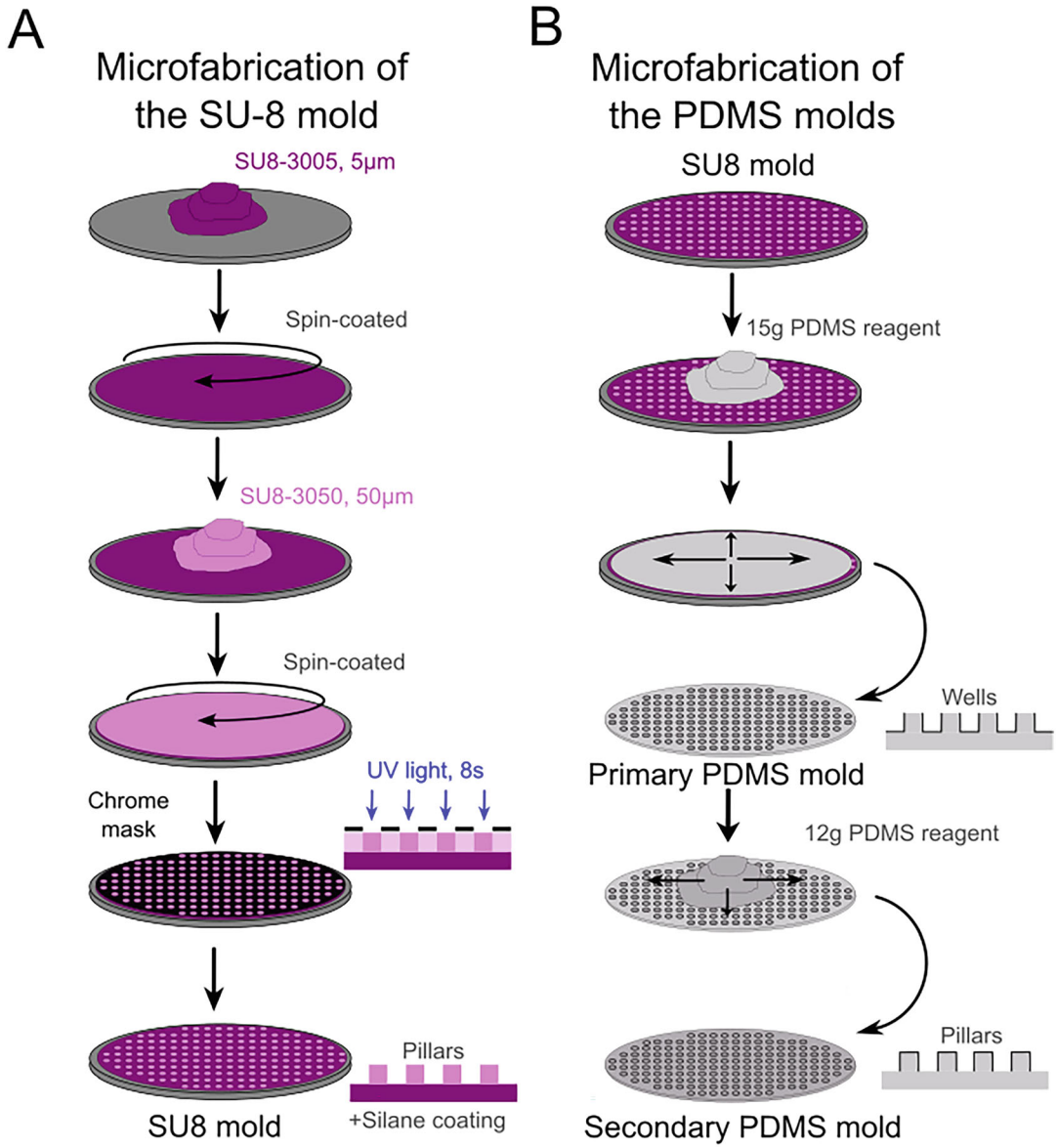

Fig. 1 Scheme of master mold fabrication. (**a**) Microfabrication of the SU-8 master mold. A double-layer coating of SU-8 is performed. A chrome mask with circular patterns is used to create SU-8 pillars. (**b**) Microfabrication of the primary and secondary PDMS molds. The primary mold is generated as a negative of the SU-8 master one, resulting in a PDMS structure with wells. The secondary mold is then created as a negative replica of the primary mold, producing multiple PDMS pillars designed to shape the polyacrylamide microwells

interest can be performed to favor cells seeding and spreading (Fig. 2c). Glass bottom enables high-quality imaging of the microwell content and quantitative analyses using immunostaining (Fig. 2d, e) or live-imaging approaches (Fig. 3). The number of microwells contained in an array is compatible with high-

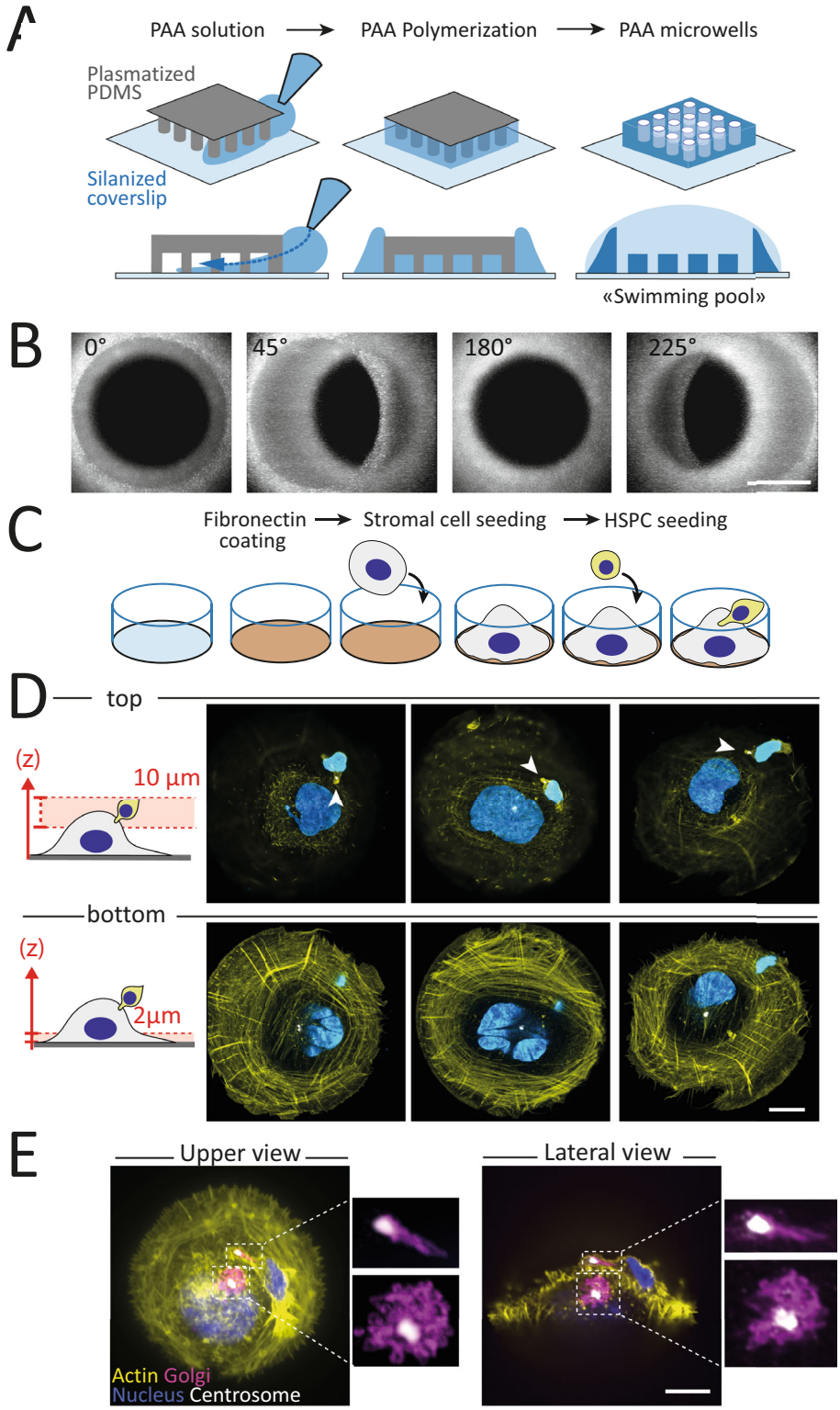

Fig. 2 From microwell fabrication to immunostaining in microwells. (**a**) Schematic representation of polyacrylamide microwells fabrication using a secondary PDMS stamp as a negative mold. (**b**) 3D-reconstructed representative rotational views of a microwell. 0° correspond to the bottom view of the well. PAA is labeled

throughput analyses. We have validated this system by demonstrating that human HSPCs can interact with specific stromal cells. These interactions induce HSPC morphological and functional polarization [16] and trigger HSPC asymmetric division, boosting eventually the siblings' heterogeneity [17].

Microwells, as a minimalistic niche model, represent a powerful platform to investigate molecular mechanisms involved in direct cell-cell interactions of any adherent and non-adherent cells of interest, as well as local effects of diffusible factors, confined in the microwells. They support mechanistic investigations at cellular levels: long-term live-cell video-microscopy, fixation, immunolabeling, and high-resolution imaging. Manipulation of cellular elements, perfusion of diffusible components, akin conditioned media or pharmacological drugs, can be developed. This underscores the system's broad utility in both basic and applied research.

2 Materials

2.1 Microfabrication

Solutions must be prepared and treatments must be performed in a microfabrication clean room. Alternatively, a laminar flow hood can be used to avoid the presence of dust.

1. Silicon wafer.
2. SU-8 3005 and SU-8 3050 negative photoresists.
3. Spin coater.
4. Chrome mask.
5. SU8 developer.

Fig. 2 (continued) with dextran-488 dissolved in the PAA solution prior to polymerization. Scale bar: 25 μm. (**c**) Schematic representation of the sequential cell seeding: stromal or feeder cell (in gray) is loaded first, followed by HSPC (in yellow) in a fibronectin (in brown)-coated microwell. (**d**) Representative confocal images of HSPCs interacting with osteoblasts seeded in microwells. Upper panel: as depicted in red in the scheme, sum slices projection of 2 μm wide Z-stack confocal images at the bottom of the microwell is presented for three different microwells. The intracellular architecture of the osteoblast adhering at the bottom can be visualized. The centrosome (in white) is positioned at the cell center and apposed to the nucleus (in cyan). The actin network (in yellow) is composed of radial and circular actin bundles. Lower panel: as depicted in red in the scheme, sum slices projection of 10 μm wide Z-stack confocal images encompassing the HSPC atop the osteoblast cell body is presented for three different microwells. Actin (in yellow) delineates the HSPC cortex. Upon interaction with osteoblasts, HSPCs become polarized: they form an elongated structure named "magnupodium" toward the site of contact (marked by an arrow) with the osteoblast. The centrosome of the HSPC (in white) is located at the tip of the magnupodium. Scale bar: 10 μm. (**e**) Representative confocal images of a human HSPC interacting with an endothelial cell seeded within a microwell. Upper and lateral views of the projection are presented in the left and right panels, respectively. The cell nuclei appear in blue, the Golgi apparatus in magenta, Actin in yellow, and the Centrosome in white. Scale bar: 10 μm. For each view, insets in dashed lines represent zoomed images of the Golgi, tightly associated with the centrosome, in both feeder cell and the HSPC

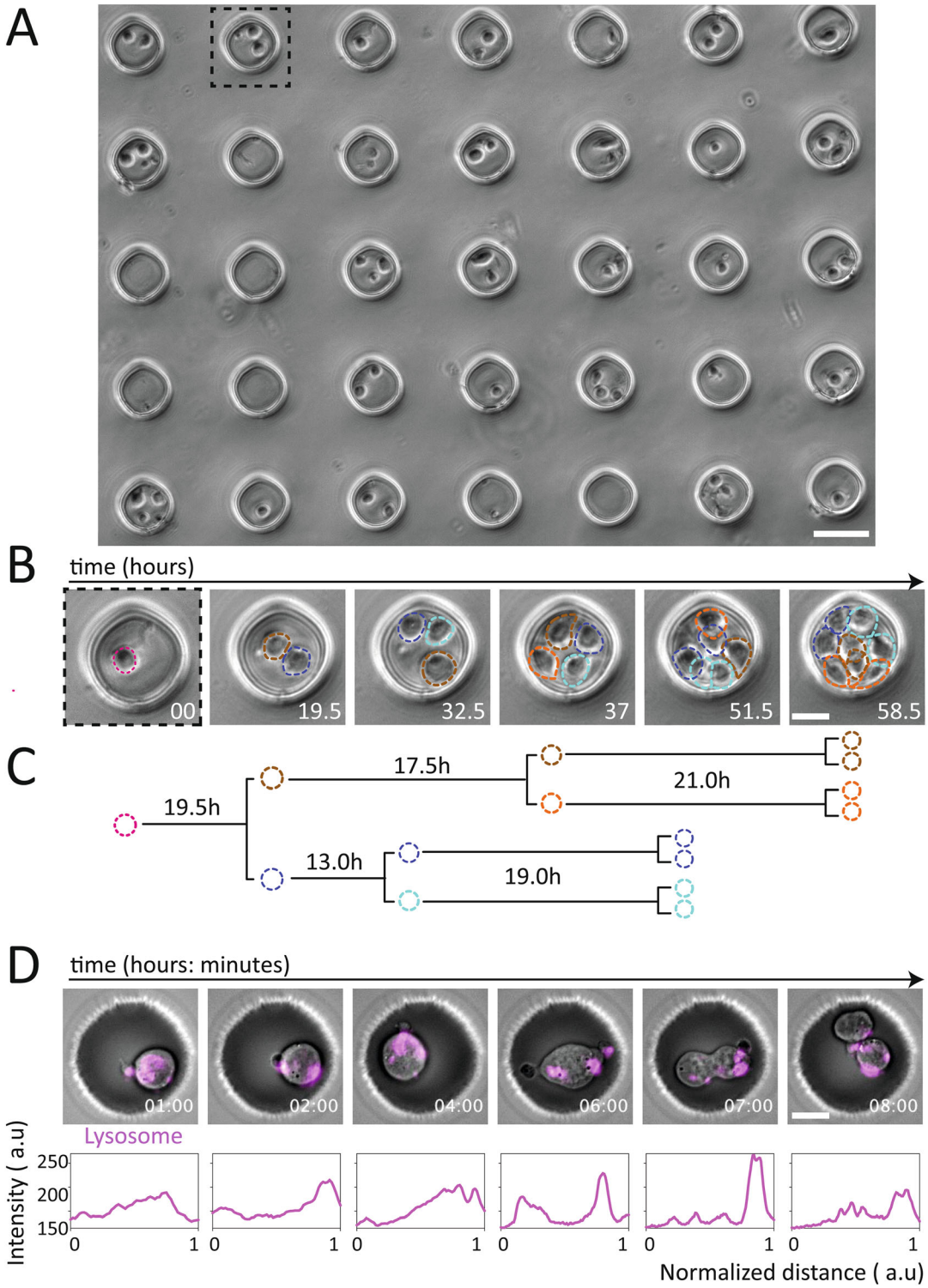

Fig. 3 Live cell imaging in microwells. (**a**) Representative phase-contrast image of a PAA stamp showing 50 μm-large microwells seeded with HSPCs only. Scale bar: 50 μm. (**b**) Time-lapse image sequence using transmitted light of the microwell dashed-lined in A. Time is indicated in hours. Three successive cell divisions can be tracked. Mother and progeny cells are color-coded using dashed lines. Scale bar: 20 μm. (**c**)

6. Isopropyl alcohol.
7. Silane: Trichloro(1H,1H,2H,2H-perfluorooctyl)silane.
8. UV lamp, 365 nm.
9. Air blow gun.
10. Hot plate or lab oven.
11. Tweezers.
12. Rocking platform.
13. Polydimethylsiloxane (PDMS).
14. Glass desiccator and vacuum pump.

2.2 Polyacrylamide Hydrogels

Solutions must be prepared and treatments must be performed in a microfabrication room or under a laminar flow hood.

1. Acetone.
2. Isopropyl alcohol.
3. Ethanol 96%.
4. Acetic acid.
5. 3-(trimethoxysilyl)propyl methacrylate.
6. 2-hydroxy-2-methylpropiophenone.
7. 37.5/1 acrylamide/bisacrylamide solution.
8. Squared 20 × 20 mm or circular 25 mm diameter coverslips.
9. PDMS stamps from the secondary PDMS mold.
10. Teflon racks for coverslips.
11. Sonicator.
12. Air blow gun.
13. Plasma reactor.
14. N,N,N′,N′-Tetramethylethylenediamine (TEMED).
15. Ammonium PerSulfate (APS) 10% W/V.
16. Tweezers.
17. Ultrapure de-ionized water (dH20).
18. Phosphate-buffered saline (PBS) 1x, without calcium chloride and without magnesium chloride.
19. Fibronectin (1 mg/mL) in PBS 1x.

Fig. 3 (continued) Corresponding lineage tree showing cell cycle durations (in hours). (**d**) Upper row: representative transmitted light and fluorescence time-lapse image sequence of a microwell containing a HSPC undergoing cell division. The segregation of lysosomes, labeled with LysoBrite (in magenta) in the daughter cells, can be assessed. Time is indicated in hours:minutes. Scale bar: 10 μm. Lower row: Line scan quantification of the lysosome signal intensity (in arbitrary units: a.u) along the long cell axis is presented

2.3 Cells, Culture Media, and Fixation

1. Human umbilical vein endothelial cells (HUVEC-XL; 191,027, Lonza) (*see* **Note 1**).
2. Human osteoblast cell line (hFOB; CRL-11372, ATCC).
3. Normal foreskin fibroblasts (Bj; CRL-2522, ATCC).
4. Human hematopoietic stem and progenitor cells (HSPCs), referred as human CD34+ cells, purified from cord blood as previously described in [16]. Cells are thawed and kept in suspension overnight at 37 °C, the day before use.
5. HUVEC medium containing EGM-2 MV Microvascular Endothelial Cell Growth Medium-2 BulletKit (Lonza CC-3203). Stored at 4 °C and warmed at 37 °C before use.
6. hFOB and Bj medium containing DMEM-F12 medium supplemented with 10% fetal bovine serum (FBS) and antibiotics/antimycotics (100 U/mL Penicillin, 100 μg/mL Streptomycin, and 0.25 μg/mL Fungizone). Stored at 4 °C and warmed at 37 °C before use.
7. HSPC medium containing IMDM medium supplemented with 10% FBS, 100 ng/mL SCF, 10 ng/mL G-CSF, 20 ng/mL Il-3, and antibiotics/antimycotics (100 U/mL Penicillin, 100 μg/mL Streptomycin, and 0.25 μg/mL Fungizone, adapted from [18]). Store at 4 °C and warm at 37 °C before use.
8. HSPC medium containing IMDM medium without red phenol supplemented with 10% FBS, 100 ng/mL SCF, 10 ng/mL G-CSF, 20 ng/mL IL-3, and antibiotics/antimycotics (100 U/mL Penicillin, 100 μg/mL Streptomycin, and 0.25 μg/mL Fungizone) is used for live imaging. Stored at 4 °C and warmed at 37 °C before use.

3 Methods

All the fabrication process must be conducted in a microfabrication clean room. Alternatively, a laminar flow cabinet can be used to avoid the presence of dust.

3.1 Master Mold Fabrication

The silicon wafer with the microstructures is fabricated following manufacturer datasheet and using standard photolithography techniques. It is further used as a master mold for microwells fabrication (*see* **Note 2**, Fig. 1a). The different steps of baking can be performed either in a hot plate under a chemical hood or in a lab oven.

1. Spin-coat a 5 μm thick SU-8 3005 resin layer onto a silicon wafer (*see* **Note 3**).
2. Bake the coated wafer on a hot plate at 95 °C for 2 min (Soft bake).

3. Expose the first resin layer to UV light (23 mW/cm^2) for 5 s.
4. Bake the wafer on a hot plate at 95 °C for 2 min (Post-Exposure Bake).
5. Spin-coat a 50 μm layer of SU-8 3050 resin over the first polymerized layer (*see* **Note 4**).
6. Bake the wafer on a hot plate at 95 °C for 15 min.
7. Place the wafer and a chrome mask with transparent disks of desired diameters in contact mode.
8. Expose the second resin layer to UV light (23 mW/cm^2) for 8 s.
9. Bake post-exposure the wafer on a hot plate at 95 °C for 5 min.
10. Immerse the wafer in SU-8 developer for 8 min under gentle agitation to remove unexposed resin.
11. Rinse the wafer thoroughly with isopropanol.
12. Dry the wafer using an air blow gun.
13. Hard bake the wafer with microstructures at 150 °C for 2 h.
14. Gas-phase silanization: This surface treatment step is necessary to further prevent the PDMS from attaching to the SU-8 mold. In a glass desiccator, put the master mold. Place a centrifuge tube's cap in the desiccator and add 10 μL of silane in it. Apply vacuum at 10 mba, wait for its stabilization for 5 min, close hermetically the desiccator for 1 h.
15. Bake the silanized master mold overnight at 150 °C to complete the silanization process.

3.2 Primary and Secondary PDMS Mold Fabrication

To create the final PDMS mold (secondary mold or negative mold), a two-step process is required. First, a primary PDMS mold is produced from the SU-8 master mold. This primary mold features wells and serves as an intermediate step. The primary mold can be stored for months in a sealed glass Petri dish, ensuring its long-term usability. Using this stored primary mold, the secondary negative mold is then fabricated, with its surface presenting pillars that will shape the microwells in the hydrogel (Fig. 1b).

1. Prepare around 15 g of PDMS reagent by mixing the silicone elastomer base and the curing agent at 10:1 ratio (wt./wt.) in a disposable weighing boat. Mix manually and gently for 3 min with a large plastic pipette to obtain a homogenous PDMS content.
2. Degas the PDMS mixture in a vacuum chamber for 1 h to remove air bubbles (*see* **Note 5**).
3. Clean thoroughly the surface of the SU8 mold using an air blow gun.

4. Place the SU-8 mold in a plastic dish and pour the degassed PDMS mixture onto the center of the SU-8 mold. Spread the PDMS evenly to cover the mold. The PDMS layer should approximatively be 3–4 mm thick (*see* **Note 6**).
5. Bake the SU-8 mold loaded with PDMS at 70 °C for 1 h on a hotplate under a chemical hood or in an oven to cure the PDMS.
6. Peel off the cured PDMS mold from the SU8 mold using tweezers (*see* **Note 7**) and place the primary PDMS mold, wells facing up, on a clean glass support.
7. Perform a gas-phase silanization of the primary PDMS mold for 1 h, as previously described (see Subheading 3.1, **step 14**). This surface treatment step is mandatory to further prevent the secondary PDMS mold from attaching to the primary PDMS mold.
8. Bake the silanized primary PDMS mold overnight at 80 °C. The primary PDMS mold is now ready for further use. This is the positive mold to generate the secondary PDMS mold. The primary mold can be stored at room temperature for 3 months.
9. Prepare around 12 g of PDMS reagent, as described in **step 1**.
10. Degas the PDMS mixture in a vacuum chamber for 1 h to remove air bubbles (*see* **Note 5**).
11. Clean thoroughly the surface of the PDMS primary mold using an air blow gun.
12. Pour the degassed PDMS mixture onto the center of the primary PDMS mold. Spread the PDMS evenly to cover the mold (*see* **Note 6**).
13. Bake the primary PDMS mold with the new PDMS mixture at 70 °C for 1 h on a hotplate under a chemical hood or in an oven to cure the PDMS mixture.
14. Peel off the secondary PDMS mold from the primary one using tweezers (*see* **Note 7**). Place the secondary PDMS mold, pillars facing up, on a clean plastic support. The secondary PDMS mold can be stored at room temperature for a month.

3.3 Coverslip Preparation

The coating of the coverslips with silane groups enables chemical bonding of the glass with the polyacrylamide through the methacrylate groups. This is crucial process for a proper attachment of the hydrogel to the glass coverslip (*see* **Note 8**). It ensures the stability of the hydrogel during long-term live imaging or in immunostaining experiments.

1. Place coverslips in a rack, where the maximum of the coverslip surface is exposed.

2. Sonicate the coverslip rack in acetone for 15 min at room temperature.
3. Sonicate the rack in isopropanol for 15 min at room temperature.
4. Dry the coverslips thoroughly using an air blow gun. Cover the coverslip racks with a clean glass container to protect them from dust.
5. Prepare the silanization solution: mix 50 mL of ethanol, 0.5 mL of acetic acid, and 1 mL of 3- (trimethoxysilyl)propyl methacrylate. Use a total volume of 150 mL for 40 coverslips (*see* **Note 9**).
6. Place the cleaned and dried coverslip racks inside the plasma reactor. Run a long plasma treatment (5 min full treatment): the parameters will depend on the efficiency of the plasma reactor (*see* **Note 10**).
7. Just before the end of the plasma treatment, pour the silanization solution into a clean glass container with a broad surface to allow even spreading over the coverslips.
8. Immerse the coverslips in the silanization solution immediately after plasma treatment. Incubate the coverslips for 10 min on a rocking platform (*see* **Note 11**). Make sure all the coverslips are completely immersed.
9. Transfer the silanized coverslips to a glass Petri dish containing ethanol for an initial wash.
10. Dip each coverslip individually into a beaker of ethanol for 10 times using a tweezer and dry the coverslip thoroughly with an air blow gun.
11. Place the coverslips in flat glass surface avoiding their overlapping.
12. Bake the coverslips at 120 °C for 1 h in an oven or a hot plate. Coverslips are now ready to be used for the microwells fabrication. Silanized coverslips can be stored in an empty coverslip box or in a rack covered with parafilm for weeks.

3.4 Microwells Fabrication on a Coverslip

The final step is the polymerization of the polyacrylamide (PAA) hydrogel containing the microwells. Using the secondary PDMS mold to shape the size and arrangement of the microwells, the PAA hydrogel is polymerized between the silanized coverslip and the PDMS mold. The microwells will present polyacrylamide and anti-adherent walls, preventing seeded cell escape, and a glass bottom, as an adherent surface, formed by the coverslip (Fig. 2a) (*see* **Note 12**).

1. Clean thoroughly the silanized coverslips in ethanol for at least 5 min (*see* **Note 13**).

2. Cut the secondary PDMS mold into around 3 × 4 cm rectangles stamps (*see* **Note 14**).
3. Prepare the working area: place a sheet of parafilm on a black background. Cover the setup with a clean glass dish to keep the working area dust-free.
4. Place the PDMS stamp into a glass dish cleaned previously with ethanol. Orient the stamps with the column structures (for microwells) facing up. Blow using an air blow gun to adsorb the PDMS stamp to the dish and clean the dust from the PDMS pillars (adhesive tape can also be used to remove dust from the top of the structures (*see* **Note 15**).
5. Prepare the PAA solution: mix a 20% (37.5/1 acrylamide/bisacrylamide) solution and 1% photoinitiator (2-hydroxy-2-methylpropiophenone) in dH_20 and vortex gently. One aliquot of polyacrylamide solution is prepared per round of microwells fabrication (*see* **Note 16**).
6. At this moment, all the reagents and devices must be ready for a fast and clean procedure. Program the UV machine (23 mJ/cm^2 for 5 min at 100% continuous UV exposure). Keep TEMED in the chemical hood and APS near to the plasma reactor. Arrange pipettes and tips close to the working area. Dry the coverslip cleaned on ethanol with the air blow gun. Place it in the designated working area, covering it with a glass dish for protection.
7. Introduce the PDMS stamps into the plasma reactor and start a soft plasmatization (no longer than 30 s. *see* **Note 17**).
8. Add 1:100 TEMED and 1:100 APS to the PAA solution during the plasma treatment (*see* **Note 18**).
9. Prepare the silanized coverslips and pick up the air blow gun for immediate use during the first seconds of vacuum removal.
10. Open the plasma reactor and quickly flip the PDMS stamp with the pillars contacting the coverslip, placing the stamp in the center of the coverslip. Use the air blow gun vertically to press the stamp firmly onto the coverslip to ensure proper attachment.
11. Mix the PAA solution gently and dispense a drop of 20 μL of PAA solution close to a side of the stamp, but avoiding to touch it in order to not disrupt PDMS attachment (*see* **Note 19**). The drop should finally touch the side and the PAA solution will flow rapidly by capillarity between the PDMS pillars and the silanized coverslip (Fig. 2a).
12. Immediately transfer the coverslip-PDMS-PAA assembly to the UV machine, keeping it horizontal at all times to avoid any tension on the glass that could disrupt the week PDMS-coverslip bond. Start the UV exposure. The PAA hydrogel will rapidly polymerize during this step.

13. Add dH_20 to the coverslip after UV treatment to keep the polymerized hydrogel hydrated. With the help of the tweezer tip, remove the PDMS stamp from the corner, avoiding to damage the hydrogel.
14. Keep the coverslip with the microwells in a plastic dish with dH_2O. Wash three times the microwells with dH_2O to remove free PAA radicals. Store them overnight at 4 °C.

3.5 Sterilization and Coating of the Microwell Bottom

Before cell culture, microwells must be sterilized to avoid contaminations. The microwell bottom can be coated with adhesion molecules of interest in order to facilitate the spreading and adhesion of the seeded cell. Treatments must be performed in a culture hood and using sterile or filtered reagents in all the steps of this section.

1. Wash extensively the microwells with dH_2O.
2. Add 3 mL of 70% ethanol to the microwells and expose it to the UV light from the culture hood for 1 h.
3. Wash the microwells twice with dH_2O and one with PBS 1x.
4. Under a culture hood, remove PBS as much as possible with a micropipette, without drying the microwells.
5. Add 50 μL of a Fibronectin solution (10 μg/mL) or of a solution of other adhesion molecule of interest that can react with methacrylate groups, and incubate it for 5 min at 37 °C.
6. Wash the microwells three times with PBS 1x. Microwells are now ready for cell seeding (Fig. 2b). They can be stored hydrated in a parafilm-sealed container for days at 4 °C.

3.6 Microwells Loading with Cells

This process must be performed in a culture hood and using sterile or filtered reagents. The goal is to have microwells containing a feeder cell adhered to the coverslip and a non-adherent HSPC on top of the feeder cell (Fig. 2c–e). We coined the external sides of the hygrogel "swimming pool" since they are used to hold a droplet of medium, allowing cells to concentrate on top of the microwells (Fig. 2a).

1. Use a pipette to carefully remove most of the PBS from the plate to the top of the microwells. Ensure the coverslip surface appears mostly dry while the hydrogel remains still wet.
2. Add 50 μL of cell medium on top of the hydrogel in the swimming pool (Fig. 2a) to cover it.
3. Prepare the individual feeder cells of interest (*see* Subheading 2.3) for loading in the microwells. Dissociate the cells in the culture flask using trypsin or an alternative method. Dissociation must be thoroughly conducted to be able to seed individual cells in the microwells.

4. After concentrating the feeder cells in 1000 cells/μL by centrifugation, add 5–10 μL on the hydrogel in a zigzag pattern to ensure the coverage of whole surface of the hydrogel, and optimize the distribution of cells the microwells.
5. Check under the microscope the cell seeding efficiency. Transfer the plate to the incubator for 10 min. Use gentle manipulations to avoid displacing cells. Thermal currents will help the cells settle into the microwells.
6. Assess the presence of feeder cell in the microwells under the microscope after incubation (*see* **Note 20**).
7. Fill the plate with medium and gently resuspend with an inclined tip (you may remove cells from the wells when pipetting vertically) to remove the excess of cells outside the wells as well as those stuck in the corners of the swimming pool.
8. After 2 h, the cells should be fully spread within the microwells.
9. Repeat the protocol for HSPC seeding: seed up to 5000 HSPC using a 2-10 μL pipette in a zigzag pattern (*see* **Note 21**).
10. Carefully transfer it to the incubator. Wait for 10 min for the HSPCs to have sedimented into the microwells.
11. Assess the presence of cell doublets (feeder cell and HSPC) in the microwells under the microscope to evaluate the efficiency of your preparation.
12. Add supplemented IMDM medium afterward to maintain the cell culture (*see* **Note 22**).
13. Microwells are now loaded with cell doublets (Fig. 2) and ready for long-term cell culture and experimentation: immunostaining experiments can be performed at time points of interest (Fig. 2d, e, *see* **Note 23**) as well as short- or long-term live imaging (Fig. 3, *see* **Note 24**).

4 Notes

1. HUVEC-XL from Lonza were used from passage 2 to 5 for optimal cells expansion.
2. Microwell size should be decided at this step. Keep in mind the interest of designing a chrome mask with a range of microwell diameters. A 37 μm well diameter is suitable for analysis of HSPCs alone. A 50 μm well diameter is suitable to seed one to four HSPCs together with one feeder cell. 75 to 100 μm well diameters are optimal to track HSPCs during multiple generations as well as for fast live imaging: large adhesion area associates with flat feeder cells and reduce the required Z-stacks during image acquisition to track HSPC above the feeder cell.

3. When peeling off the PDMS layer from the SU-8 master mold, the SU-8 structures are subjected to significant mechanical stress. To prevent detachment of these structures from the substrate, an initial SU-8 layer (5 μm thick) is spin-coated and cured as a protective base layer.
4. The contact between the mask and the wafer may occasionally fail, mainly due to the formation of an edge bead upon spin-coating a thick layer of SU8. To solve this problem, swiftly shift the spinning speed of 10,000 rpm at the end of the spinning in order to expel the edge bead.
5. Bubbles must be completely removed before pouring PDMS onto the mold. Pour PDMS slowly and close to the mold surface to prevent bubbling.
6. In case of remaining bubbles in the PDMS mixture, degas the mixture until the bubbles disappear in a vacuum chamber.
7. Use a scalpel and tweezers to peel off the cured PDMS. First, cut the PDMS roughly around the structure's area. Remove the part of interest slowly, starting from the edges and using tweezers while making sure you do not damage the mold. Cut the PDMS precisely.
8. Proper silanization is crucial for creating a stable PAA hydrogel. It is essential for long-term imaging and immunofluorescence protocols. This requires high-quality reagents: store the silane coating reagent in the fridge, well-sealed, and covered with parafilm. Use racks to process multiple coverslips simultaneously. Failures are evident when polyacrylamide forms an extended thin layer rather than compact walls around the hydrogel.
9. Although the silanization solution can be storage in the fridge for a few weeks, preparing a fresh solution is recommended for optimal results.
10. The resistance of the glass surface may require long-term plasmatization. Testing can be performed by adding a 20 μL droplet of the polyacrylamide reagent used in the next section on the silanized coverslip. If the final silane treatment is correct, the droplet will not spread on the coverslip. On the contrary, if the treatment is not sufficient, the surface will remain hydrophilic allowing the droplet spreading and flattening.
11. At this moment, the racks are not anymore necessary. You can work with the coverslips individually.
12. Both glass coverslip and PDMS stamp (secondary mold) must be very clean. Dust presence between the PDMS mold and the glass prevents PAA seeping to the bottom and impairs cell attachment and protein coating.

13. A coverslip rack can be used to prepare several coverslips in a row.
14. Larger PDMS stamps create more microwells, but increase the risk of PAA seeping under PDMS pillars. Smaller stamps (~3 × 4 cm) are recommended to obtain 50 μm diameter wells.
15. Protect the clean PDMS stamp from dust before use.
16. Prepare an Eppendorf of 50 μL PAA mixture for each hydrogel polymerization reaction. Since the photoinitiator is viscous, its presence can be checked by observing the droplet clinging to the plastic surface. Then, mix thoroughly using a vortex.
17. Short-term plasmatization is required here. Its duration will depend on the efficiency of the plasma reactor used. Note that a harsh plasmatization can reduce the stiffness of the PDMS stamps. The goal of this plasmatization is to render the PDMS hydrophylic for the PAA solution, improve the capillarity effect, and thus optimize the filling of the space between PDMS pillars and the silanized coverslip.
18. Perform the following step quickly, as the attachment between the PDMS stamp and coverslip relies on weak forces, which diminish over time. Add TEMED at the bottom of the Eppendorf within the first 15 s of plasma treatment and APS at the top during the last 15 s. Due to oxygen sequestering within the PDMS, the TEMED/APS solution is not efficient to trigger PAA polymerization prior PDMS detachment. The addition of the photoinitiator with UV accelerates the polymerization process.
19. Add a slight excess of PAA solution to form a "swimming pool" around the PDMS stamp. This structure favors the retention of cells and reagents during incubation procedures (Fig. 2a).
20. In case of a low density of cells, more cells can be added on the microwells to increase the density at a satisfying level. In the case of an undesired high cell density, cells can be removed during the wash step, by applying a flow of culture medium using a pipette. When many cells entered already the microwells, flush culture medium on top of the target area. For feeder cells adhering artifactually to the top of the hydrogel, use trypsin to detach them and wash gently to remove any remaining debris. This can happen when using highly adherent feeder cells (e.g., fibroblasts).
21. In case of initial low cell density, adapt the concentration to 500 cells/μL; pipetting the cells with a 2 μL pipette. Adding sequentially 2 μL volumes increase the efficiency of the loading of the microwells.
22. Pre-warm all solutions to 37 °C and handle cells gently to maintain viability during preparation and staining.

23. To ensure optimal fixation, immerse the hydrogel in the fixative solution of your choice adding it slowly to prevent the dissociation of cell-cell interactions in the microwells and cell loss from the microwells. Classical fixative solutions can be used, as Paraformaldehyde (PFA) 4% in PBS, or PFA 3% and glutaraldehyde 0.1% in Cytoskeleton Buffer (10 mM MES pH 6.1, 138 mM KCl, 3 mM MgCl, 2 mM EGTA, 10% sucrose (as used in Fig. 2d, e). During immunostaining procedure, maintain the hydrogel facing upward. The duration of antibody incubation in microwells is similar to the one used in classical fixation protocols. For efficient antibody staining and sample mounting, remove the "swimming pool" walls to have a thinner sample. Add montage medium (for instance Mowiol) to the hydrogel allowing covering the whole surface before flipping and mounting.
24. Live imaging can be performed using either an adapted magnetic imaging chamber, or alternatively by affixing the coverslip with microwells to the base of a P6 plate with custom-made holes. It is recommended to change the medium every 24–40 h, during the course of the experiment.

Acknowledgments

This work was supported by the European Research Council (Consolidator Grant 771599 (ICEBERG) to MT, by the Bettencourt-Schueller foundation and the Schlumberger foundation for education and research. AC received PhD fellowship from the Fondation pour la Recherche Médicale (Grant FDT20170437071).
The confocal images have been generated using a ZEISS LSM 800 confocal at the Technological Core Facility of the Saint-Louis Research Institute. The facility is supported by grants from Université Paris Cité, ITMO-Cancer of the French National Science Alliance (Aviesan), the Conseil Regional d'Ile-de-France (Cancéropôle), the National Cancer Research Institute (InCa), the Ministère de la Recherche, the Association Saint-Louis, and the Association Jean Bernard.

References

1. Chacón-Martínez, Carlos A, Koester J, Wickström SA (2018) Signaling in the stem cell niche: regulating cell fate, function and plasticity. Development (Cambridge) 145. https://doi.org/10.1242/DEV.165399
2. Post Y, Clevers H (2019) Defining adult stem cell function at its simplest: the ability to replace lost cells through mitosis. Cell Stem Cell 25:174–183. https://doi.org/10.1016/j.stem.2019.07.002
3. Buenrostro JD, Corces MR, Lareau CA, Wu B, Schep AN, Aryee MJ, Majeti R, Chang HY, Greenleaf WJ (2018) Integrated single-cell analysis maps the continuous regulatory landscape of human hematopoietic differentiation. Cell 173:1535–1548.e16. https://doi.org/10.1016/j.cell.2018.03.074

4. Wilson A, Trumpp A (2006) Bone-marrow haematopoietic-stem-cell niches. Nat Rev Immunol 6:93–106. https://doi.org/10.1038/nri1779
5. Mazo IB, Massberg S, von Andrian UH (2011) Hematopoietic stem and progenitor cell trafficking. Trends Immunol 32:493. https://doi.org/10.1016/j.it.2011.06.011
6. Crane GM, Jeffery E, Morrison SJ (2017) Adult haematopoietic stem cell niches. Nat Rev Immunol 17:573–590. https://doi.org/10.1038/nri.2017.53
7. Pinho S, Frenette PS (2019) Haematopoietic stem cell activity and interactions with the niche. Nat Rev Mol Cell Biol 20:303–320. https://doi.org/10.1038/s41580-019-0103-9
8. Verovskaya EV, Dellorusso PV, Passegué E (2019) Losing sense of self and surroundings: hematopoietic stem cell aging and leukemic transformation. Trends Mol Med 25:494–515. https://doi.org/10.1016/j.molmed.2019.04.006
9. Duarte D, Hawkins ED, Lo Celso C (2018) The interplay of leukemia cells and the bone marrow microenvironment. Blood 131:1507–1511. https://doi.org/10.1182/blood-2017-12-784132
10. Xie Y, Yin T, Wiegraebe W, He XC, Miller D, Stark D, Perko K, Alexander R, Schwartz J, Grindley JC et al (2009) Detection of functional haematopoietic stem cell niche using real-time imaging. Nature 457:97–101. https://doi.org/10.1038/nature07639
11. Christodoulou C, Spencer JA, Yeh SCA, Turcotte R, Kokkaliaris KD, Panero R, Ramos A, Guo G, Seyedhassantehrani N, Esipova TV et al (2020) Live-animal imaging of native haematopoietic stem and progenitor cells. Nature 578:278–283. https://doi.org/10.1038/s41586-020-1971-z
12. Gillette JM, Larochelle A, Dunbar CE, Lippincott-Schwartz J (2009) Intercellular transfer to signalling endosomes regulates an ex vivo bone marrow niche. Nat Cell Biol 11:303–311. https://doi.org/10.1038/ncb1838
13. Lutolf MP, Doyonnas R, Havenstrite K, Koleckar K, Blau HM (2009) Perturbation of single hematopoietic stem cell fates in artificial niches. Integr Biol (Camb) 1:59–69. https://doi.org/10.1039/b815718a
14. Chou DB, Frismantas V, Milton Y, David R, Pop-Damkov P, Ferguson D, MacDonald A, Vargel Bölükbaşı Ö, Joyce CE, Moreira Teixeira LS et al (2020) On-chip recapitulation of clinical bone marrow toxicities and patient-specific pathophysiology. Nat Biomed Eng 4:394. https://doi.org/10.1038/s41551-019-0495-z
15. Souquet B, Opitz MM, Vianay B, Brunet S, Théry M (2021) Manufacturing a bone marrow-on-a-chip using maskless photolithography. In: Espéli M, Balabanian K (eds) Methods in molecular biology. Humana, New York, pp 263–278. https://doi.org/10.1007/978-1-0716-1425-9_20
16. Bessy T, Candelas A, Souquet B, Saadallah K, Schaeffer A, Vianay B, Cuvelier D, Gobaa S, Nakid-Cordero C, Lion J et al (2021) Hematopoietic progenitors polarize in contact with bone marrow stromal cells in response to sdf1. J Cell Biol 220. https://doi.org/10.1083/jcb.202005085
17. Candelas A, Vianay B, Gelin M, Faivre L, Larghero J, Blanchoin L, Théry M, Brunet S (2024) Heterotypic interaction promotes asymmetric division of human hematopoietic progenitors. Development 151. https://doi.org/10.1242/dev.203088
18. Biedzinski S, Agsu G, Vianay B, Delord M, Blanchoin L, Larghero J, Faivre L, Théry M, Brunet S (2020) Microtubules control nuclear shape and gene expression during early stages of hematopoietic differentiation. EMBO J 39:e103957. https://doi.org/10.15252/embj.2019103957