Chapter 20

Manufacturing a Bone Marrow-On-A-Chip Using Maskless Photolithography

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Abstract

The bone marrow (BM) is a complex microenvironment in which hematopoietic stem and progenitor cells (HSPCs) interact with multiple cell types that regulate their quiescence, growth, and differentiation. These cells constitute local niches where HSPCs are confined and subjected to specific set of physical and biochemical cues. Endothelial cells forming the walls of blood capillaries have been shown to establish a vascular niche, whereas osteoblasts lying along the bone matrix organize the endosteal niche with distinct and specific impact on HSPC fate. The observation of the interaction of HSPCs with niche cells, and the investigation of its impact on HSPCs behavior in vivo is hindered by the opacity of the bone matrix. Therefore, various experimental strategies have been devised to reconstitute in vitro the interaction of HSPCs with distinct sets of BM-derived cells. In this chapter, we present a method to manufacture a pseudo BM-on-a-chip with separated compartments mimicking the vascular and the endosteal niches. Such a configuration with connected but distant compartments allowed the investigation of the specific contribution of each niche to the regulation of HSPC behavior. We describe the microfabrication of the chip with a maskless photolithography method that allows the iterative improvement of the geometric design of the chip in order to optimize the adaptation of the multicellular architecture to the specific aim of the study. We also describe the loading and culture of the various cell types in each compartment.

Key words Bone marrow-on-a-chip, Maskless photolithography, Microfabrication, Hematopoietic stem cells, 3D cell culture, Hydrogel, Organ-on-a-chip

1 Introduction

Hematopoietic stem and progenitor cells (HSPCs) are essential to maintain hematopoietic lineages homeostasis throughout life [1]. HSPCs home within the bone marrow (BM), where they interact with complex multicellular microenvironments that include HSC progenies and multiple non-hematopoietic cell types. These microenvironments, or niches, are potent regulators of HSPC proliferation and differentiation through the action of multiple diffusible factors, physical cues, or direct cell–cell interactions between HSPCs and the niche cells. It is generally admitted

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that the major—and functionally distinct—niches for HSCs are on the first hand the sinusoidal and endothelial niches vascularizing the marrow and, on the other hand the peripheric endosteal niche in close contact with rigid bone wall [2, 3]. Finally, alterations of the niches components, or bidirectional interactions between leukemic cells and their niches, are now recognized as playing a critical role in the initiation and development of hematological malignancies [4, 5].

The molecular and cellular mechanisms at play when HSPCs interact with their complex environment, as well as the impact on HSPC behavior, and the implications for HSPC maintenance and expansion are still poorly understood, in physiological and malignant contexts. Investigating such interactions in vivo is indeed a real challenge. First, imaging the processes at play is obviously tremendously complicated [6, 7]. Most of the studies have been conducted in mouse by characterizing the impact of genetic ablation or amplification of specific stromal cell populations on HSPCs and on their regenerative capacities assessed by transplantation experiments [8–10]. However, these seminal works did not allow deciphering the cellular mechanisms at play.

In order to circumvent these limitations, we and others have developed an in vitro system based on microfabrication technologies to reconstitute some aspects of the physiological microenvironments and investigate HSPC interactions with stromal cells of the niche ranging from minimalistic models [11] to self-organizing organoids [12].

Our BM-on-a-chip [13] was first inspired by the pioneering work of Li Jeon and coll., who described the set-up for the microchannel geometry and the culture conditions necessary for inducing endothelial cells self-organization into hollow and perfusable three-dimensional (3D) networks [14]. From this first design, we used maskless photolithography with a digital micromirror (DMD)-based device (Fig. 1) to prototype different evolutions of the chip. We just had to modify our virtual mask (8-bit image) to test a new design. First, we added a channel to create an endosteal niche (Fig. 2a, channel 2), separated from the vascular niche (Fig. 2a, channel 4) by a channel allowing the loading of HSPCs (Fig. 2a, channel 3). This niche was made of osteoblasts cultured in a 3D matrix made of collagen-I and fibrin to model a minimal version of the endosteal niche [15]. We added two big channels for medium change (Fig. 2a, channels 1 and 6), and finally another channel for the cytokine-secreting fibroblasts necessary for the vascular network formation (Fig. 2a, channel 5) that were embedded in a fibrin hydrogel. All the channels of the chip were separated by pillars which allow compartmentalization but also nutrient diffusion and cell migration (Fig. 2a, b), inspired by those developed by Li Jeon and colleagues [14]. For the endosteal niche, we had to

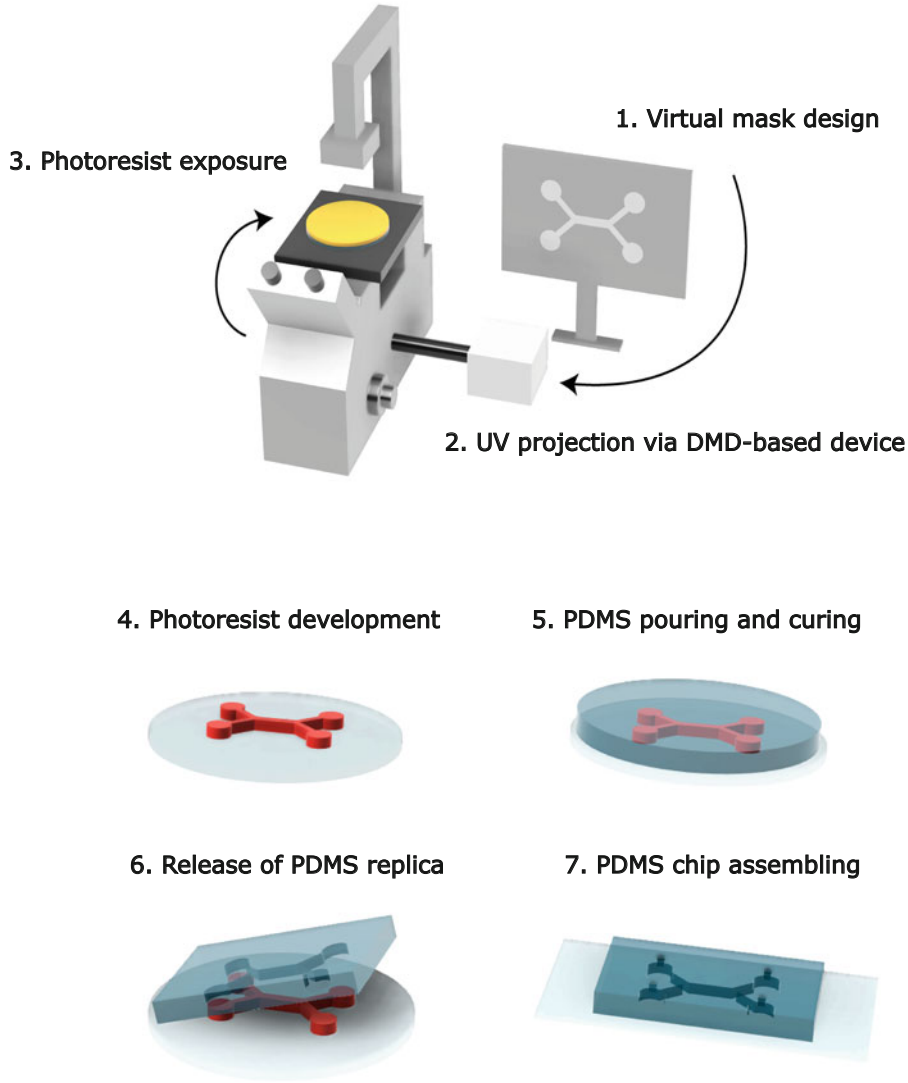

Fig. 1 Fabrication of the PDMS chip using maskless photolithography. A virtual mask is designed (1) and the corresponding UV image is projected via a DMD-based device (2) onto a photoresist (3). The exposed photoresist is then developed in a solvent, washed and baked, creating a master mold (4). Some PDMS is poured and cured onto the mold (5). The PDMS layer is then removed (6), punched to create inlets and bonded to a glass slide (7)

make another improvement. Indeed, the osteoblast imposed high constraints on the gel and made it collapse. To prevent this, we added some anchorage pillars in the channel, allowing the gel to resist cells' constraints (Fig. 2a, channel 2).

The endothelial cells and the HSPCs were cultured in the same hydrogel made of collagen-I and fibrin as for the osteoblasts. To confirm that this hydrogel was compatible with the self-organization of the endothelial cells into a hollow network, we

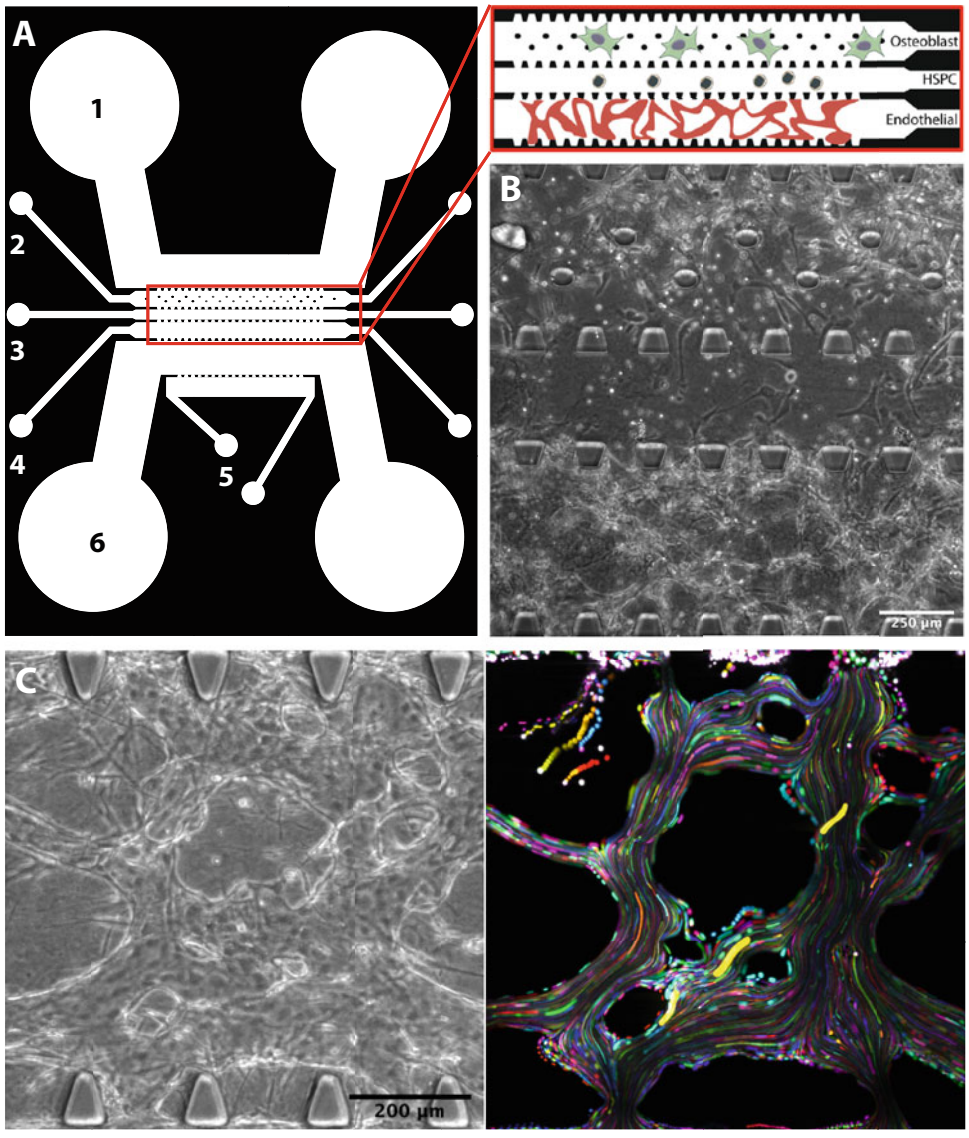

Fig. 2 BM-on-a-chip allowing the interaction of HSPCs with endosteal and vascular compartments in 3D hydrogels. (**a**) Illustration of the virtual mask (PDF file) loaded on the software of the DMD-based device. The chip comprises channels for medium circulation (no. 1 and no. 6), an endosteal compartment (no. 2), a HSPC injection channel (no. 3), a vascular compartment (no. 4), and a channel for cytokine-secreting fibroblasts (no. 5). The inset on the right (red rectangle) describes the organization of the three central channels. (**b**) Image of the three central channels of the chip in phase contrast (average projection of 5 z-slices with 5 µm spacing). (**c**) (left) Transmitted light image of the vascular network and (right) corresponding image of fluorescent beads perfused in the hollow structures formed by the HUVECs (projection of 300 time points separated by 0.33 s, made using the Fiji plugin "Temporal-Color Code"). Images are taken after 6 days of culture

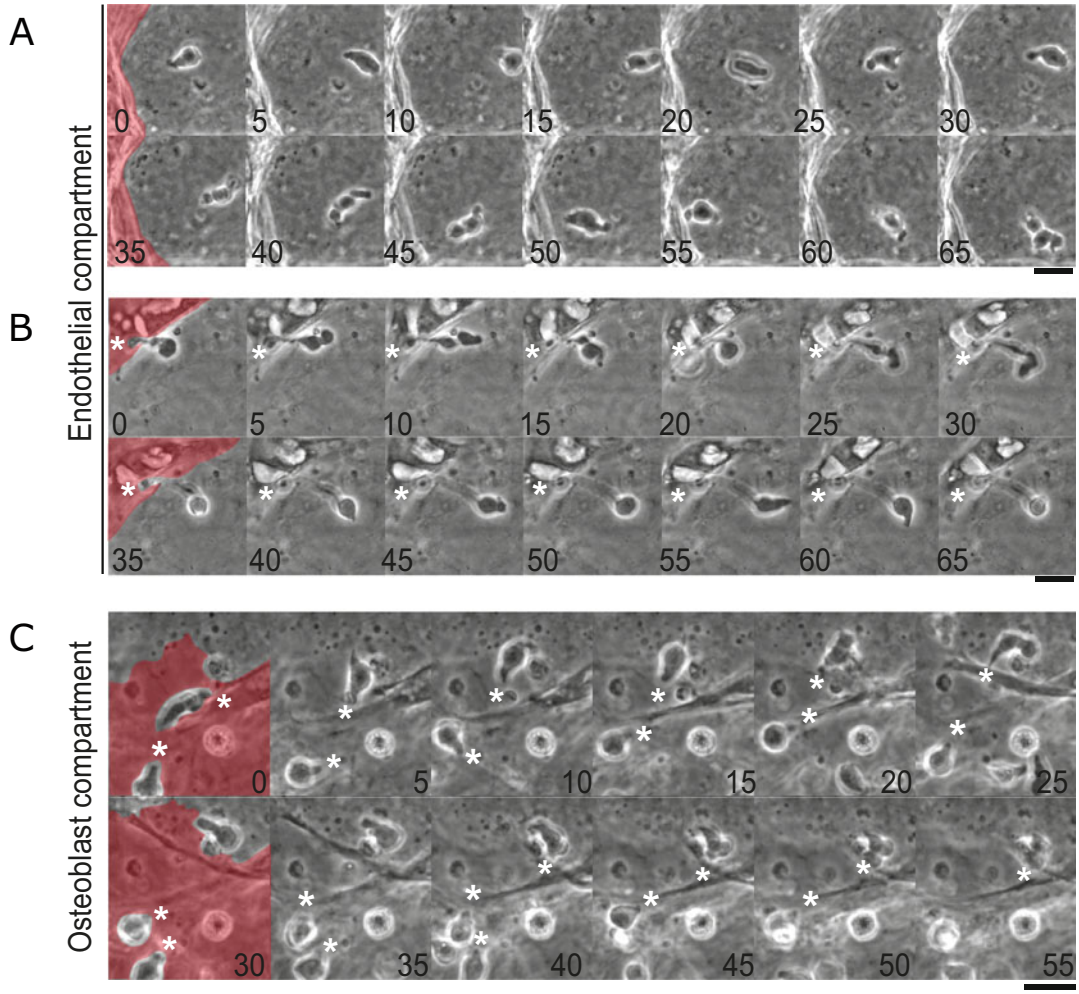

Fig. 3 Live-cell imaging of individual HSPCs in the BM-on-a-chip compartments. (**a**) Gallery of transmitted light images of a HSPC freely migrating in the hydrogel within the vascular compartment. Nearby endothelial cell appears salmon-colored in the left images of the gallery. Time points (in minutes) are indicated in the bottom right corners of each frame. Scale bar: 10 μm. (**b**) Gallery of transmitted light images of a HSPC interacting with an endothelial cell within the vascular compartment. The endothelial cell appears salmon-colored in the left images. The point of contact is highlighted with a white asterisk. Time points (in minutes) are indicated in the bottom left corners of each frame. Scale bar: 10 μm. (**c**) Gallery of transmitted light images of a HSPC interacting with an osteoblast within the endosteal compartment. The osteoblast is salmon-colored in the left images. 2 points of contact are highlighted with white asterisks. Time points (in minutes) are indicated in the bottom right corners of each frame. Scale bar: 10 μm

perfused fluorescent beads in the chip and observed their trajectory by fluorescence microscopy (Fig. 2c).

As illustrated in Fig. 3, individual HSPC behavior can indeed be investigated using live-cell imaging in each compartment of the BM-on-a-chip. Some HSPCs are found migrating freely in the hydrogel despite the proximity of stromal cells (Fig. 3a). Others

can undergo heterotypic interactions with endothelial cells or osteoblasts, in the vascular and endosteal compartments respectively (Fig. 3b, c). In both cases, interacting HSPCs exhibit an elongated shape with a thin stalk at the point of contact with the stromal cell. Such interactions are stable in time (Fig. 3b, c). Our BM-on-a-chip appears therefore as a powerful system to (1) dissect the cellular and molecular mechanisms at play in these heterotypic interactions and (2) analyze the impact of such interactions on HSPCs fate.

Importantly, our model is compatible with chemical fixation, immunolabeling and high magnification imaging.

2 Materials

2.1 Microfabrication

1. Silicon wafer.
2. SU-8 3005 and SU-8 3050 negative photoresists (MicroChem Inc.).
3. Spin Coater.
4. Propylene glycol monomethyl ether acetate (PGMEA).
5. Hot plate.
6. Isopropyl alcohol.
7. Deionized (DI) water.
8. Acetone.
9. Polydimethylsiloxane (PDMS).
10. Silane: Trichloro(1H,1H,2H,2H-perfluoro-octyl)silane.
11. DMD-based photolithography device (e.g., PRIMO device from Alvéole, France).
12. UV lamp, 365 nm.
13. Lab oven.
14. Glass desiccator and vacuum pump.
15. Plasma cleaner.
16. Microfabrication tools: tweezers, scalpel, 1.5 mm and 5 mm punchers, Scotch tape.
17. Ultraclean glass coverslips 75 × 25 mm.

2.2 Hydrogels

1. Phosphate-buffered saline 1×, without calcium chloride and without magnesium chloride.
2. Phosphate-buffered saline 10× with calcium chloride and magnesium chloride.
3. 0.2 N Sodium hydroxide solution.
4. pH paper.

5. 0.4 μm sterile filters.
6. 5 mg/ml Rat tail Collagen-I solution.
7. 10 mg/ml Fibrinogen solution, (*see* **Note 1**).
8. 100 U/ml Thrombin solution (*see* **Note 2**).

2.3 Cells and Culture Media

1. Human umbilical vein endothelial cells (HUVECs) (*see* **Note 3**).
2. Human osteoblast cell line (hFOB).
3. Mitotically inactivated normal human lung fibroblast cells (NHLF) (*see* **Note 4**).
4. HSPCs, purified from cord blood as previously described in [16] (*see* **Note 5**). Cells are thawed and kept in suspension overnight at 37 °C, the day before the loading in the chip.
5. HUVEC medium containing EGM-2 MV Microvascular Endothelial Cell Growth Medium-2 BulletKit (Lonza CC-3203). Store at 4 °C and warm at 37 °C before use.
6. hFOB 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®). Store at 4 °C and warm at 37 °C before use.
7. NHLF cells medium containing FGM-2 Fibroblast Growth Medium-2 BulletKit (Lonza, CC-3132). Store at 4 °C and warm at 37 °C before use.
8. 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 [17, 18]). Store at 4 °C and warm at 37 °C before use. In the chip, use the medium without cytokines.

3 Methods

3.1 Virtual Mask Design

The virtual mask is an 8-bit image (Fig. 2a) and can be drawn with an open-source software like Inkscape (https://inkscape.org/). The design of the chip might need to be optimized to meet your specific needs. The advantage of maskless photolithography is that you do not need to go through the entire process of the actual fabrication of a genuine photomask mask to test a new version of your PDMS chip, you just need to change the design of your virtual mask and expose a new layer of photoresist. This makes prototyping easy and fast.

1. The global size of the virtual mask (*see* Fig. 2a for the shape) is 11.5 × 14 mm (width × length).
2. The endosteal, vascular and NHLFs compartments must be at least 500 μm wide, and the HSPC compartment at least 300 μm wide.
3. Separate the channels with separation pillars: they measure 90 × 70 μm (length × width, *see* Fig. 2a for the shape) and are spaced by 70 μm (*see* **Note 6**).
4. Add some anchorage pillars (50 μm wide, 150 μm spacing) in the endosteal compartment (Fig. 2a, channel no. 2) to prevent the hydrogel from collapsing due to the constraints imposed by the cells (*see* **Note 7**).

3.2 Master Mold Fabrication

All the fabrication process (Fig. 1) must be performed in a clean room or at least in a laminar flow cabinet to prevent the presence of dust.

1. Grip layer (*see* **Note 8**): spin coat a 5 μm thick layer of SU-8 3005 resist onto the silicon wafer (refer to your manufacturer's protocol). You can also do it onto a glass slide (*see* **Note 9**).
2. Soft bake: ramp slowly from 65 °C to 95 °C for 2 min then bake it at 95 °C for 3 min.
3. Expose the whole SU-8 to UV light at 200 mJ/cm^2 using a UV lamp.
4. Post-Exposure bake: ramp slowly from 65 °C to 95 °C for 2 min then bake it at 95 °C for 2 min (this fully polymerized layer will make strong anchor for the structures on its top).
5. Spin coat a 75 μm thick layer of SU-8 3050 resist onto the grip layer of SU-8 (refer to your manufacturer's protocol).
6. Soft bake: ramp slowly from 65 °C to 95 °C for 5 min, then bake it at 95 °C for 35 min.
7. Spin coat a second 75 μm thick layer of SU-8 3050 resist onto the previous one to finally obtain a 150 μm thick layer of uncured SU-8.
8. Soft bake: ramp slowly from 65 °C to 95 °C for 5 min then bake it at 95 °C for 35 min.
9. UV exposure: place the SU-8 coated wafer onto the microscope holder with the SU-8 facing the objective (*see* **Note 10**) and make sure you focus on the SU-8 surface (*see* **Note 11**). Load your virtual mask image onto the dedicated software and project it with UV light (375 nm) using the DMD-based device, at 32 mJ/mm^2. Your system needs a "stitching mode" to create smooth junctions between the DMD images (*see* **Note 12**).

10. Post exposure bake: ramp slowly from 65 °C to 95 °C for 2 min then bake it at 95 °C for 8 min.
11. Development: this step must be performed under a chemical hood. Put some SU-8 developer (PGMEA) in a beaker, place the wafer (SU-8 facing up) in it and let it develop with gentle agitation for 30 min. After 20 min of development, change the developer with some fresh one for the remaining 10 min. At the end of the development time, only the exposed areas will remain. Using a wash bottle, wash the sample a first time with clean PGMEA, then with isopropyl alcohol until all the non-cured resist has been removed (*see* **Note 13**).
12. Dry it with air flow. You have obtained what is thereafter referred to as the master mold.
13. Hard bake: to solidify the master mold and make it more durable, bake it for 2 h at 150 °C.
14. Silanization: this surface treatment step is necessary to further prevent the PDMS from attaching to the master mold. In a glass desiccator, put the master mold. Place a centrifuge tube's cap in the desiccator and add 50 µl of silane in it. Apply a 10 mBar vacuum, wait for its stabilization then close hermetically the desiccator for 1 h (*see* **Note 14**).
15. Bake the silanized master mold for 2 h at 120 °C.

3.3 Microfluidic Chip Molding and Assembling

1. In a disposable weighing boat, prepare 10 g of fresh PDMS by mixing the silicone elastomer base and the curing agent at a 9:1 ratio (wt./wt.): 9 g of silicone and 1 g of curing agent. Mix vigorously.
2. Place the weighing boat in a glass desiccator and degas the PDMS mix until all air bubbles have been removed.
3. Place the master mold in a plastic dish and pour the PDMS mixture on it. The PDMS layer must be approximatively 3–4 mm thick. Degas in a glass desiccator to remove the remaining bubbles.
4. Bake in a lab oven at 70 °C for 1 h to cure the PDMS.
5. Carefully peel-off the PDMS from the master mold (*see* **Note 15**).
6. Cut the PDMS at the desired shape around the structures. Do not cut too close to the structures to prevent damages nor too far to be able to further bond it to the glass coverslip. You will obtain what is thereafter referred to as the PDMS chip.
7. Create the inlets of the channels: place the PDMS chip on a surface with the structures facing up and remove a cylinder of PDMS at the circular openings area using a 5 mm puncher for the medium reservoirs (Fig. 2a, channels 1 and 6) and a

1.5 mm puncher for the cell loading inlets (Fig. 2a, channels 2, 3, 4, and 5). The 1.5 mm holes are made to fill the channels using a 20 µl pipette tip.

8. The plasma bonding step enables to permanently bond the PDMS chip to a glass coverslip: place a clean glass coverslip and the PDMS chip (with the structures facing up) in a plasma reactor and plasma treat for 30 s to 3 min, depending on your plasma reactor. Place the PDMS chip on the treated surface of the glass coverslip. The bonding is covalent and very strong, you will have only one chance to place it correctly so do it very carefully. Once placed, press gently on the PDMS chip using tweezers to make sure all the chip is correctly bonded (*see* **Note 16**).
9. Your PDMS chip is now finished and is ready to be used the next day (*see* **Note 17**).

3.4 Hydrogel Loading with Cells

HUVECs, hFOBs, and HSPCs are all encapsulated in the same hydrogel, a mixture of collagen-I and fibrin (formed by the cleavage of fibrinogen by thrombin), hereafter referred to as Coll/fib hydrogel. NHLFs are encapsulated in fibrin only (*see* **Note 18**). Until cell loading, all the following steps need to be performed on ice to prevent gelation of collagen-I.

1. On ice, in a prechilled microtube, prepare 50 µl of 4 mg/ml collagen-I solution at neutral pH with 4.44 µl of 10× PBS, 4.37 µl of 0.2 N NaOH, 1.19 µl of 1× PBS, and 40 µl of 5 mg/ml rat tail collagen-I stock solution (*see* **Note 19**).
2. In another prechilled microtube, add the fibrinogen: mix 37.5 µl of the 4 mg/ml collagen-I solution prepared in **step 1** with 18.75 µl of 10 mg/ml fibrinogen and 18.75 µl of 1× PBS.
3. In another microtube, prepare a 20 µl solution of thrombin at 5 U/ml by mixing 1 µl of 100 U/ml thrombin stock solution in 19 µl of 1× PBS. Dispose 4 µl of this thrombin solution in 3 other prechilled microtubes.
4. Prepare the HUVECs (for the vascular compartment) and the hFOBs (for the endosteal compartment). Detach the cells, centrifuge, and count them, then put 2×10^5 HUVECs in a 1.5 ml centrifuge tube and 1.5×10^5 hFOBs in another 1.5 ml centrifuge tube (*see* **Note 20**). Use the dedicated medium for each cell line (*see* section Materials 2.2).
5. Centrifuge at 350 × *g* for 5 min at room temperature.
6. Now focus only on the HUVECs to finish preparing them (**steps 7** to **10**).
7. Remove the supernatants using a P1000 pipette but let approximately 10 µl. After a few seconds (this will allow the remaining

medium to flow from the wall down to the bottom of the tube), remove the rest of the supernatant using a P20 pipette so that only the "dry" pellet remains.

8. Resuspend the HUVECs in 16 µl of the collagen-I + fibrinogen solution previously prepared. Mix gently while avoiding bubbles.
9. Using a P20 pipette set on 20 µl, take the HUVECs suspension and add it into one of the 3 prechilled microtubes (previously prepared in **step 3**) containing 4 µl of thrombin (*see* **Note 21**). The HUVECs are now at 10^7 cells/ml in the Coll/fib hydrogel.
10. Mix efficiently, quickly but gently, still while avoiding bubbles and load slowly the suspension in the inlet of channel no. 4 (Fig. 2a) using a P20 pipette (*see* **Note 22**). It should not be necessary to use all the 20 µl (*see* **Note 23**).
11. Repeat the **steps 7–10** with the hFOBs pellet (cells will be finally at 7.5×10^6 cells/ml) except that loading of the cell suspension will be in channel no. 2 (Fig. 2a).
12. Incubate 30 min at 37 °C in humid atmosphere to limit evaporation.
13. In the meantime, thaw the mitotically inactivated NHLFs, resuspend them in some HUVEC medium, centrifuge and count them, then put 1×10^5 cells in a centrifuge tube. Follow the same procedure to "dry" the pellet as in **step 7**.
14. The NHLFs are not encapsulated in Coll/Fib but in fibrin (fibrinogen + thrombin) hydrogel. In a microtube, prepare a 20 µl solution of 5 mg/ml fibrinogen by mixing 10 µl of the 10 mg/ml fibrinogen stock solution with 10 µl of PBS.
15. In the last microtube prepared in **step 3** containing 4 µl of 5 U/ml thrombin, add 4 µl of PBS 1× and mix to obtain an 8 µl solution of 2.5 U/ml thrombin.
16. Resuspend the NHLFs in 12 µl of the 5 mg/ml fibrinogen solution prepared in **step 14** and mix gently.
17. Using a P20 pipette set on 20 µl, add the 12 µl of NHLFs suspension in the 8 µl of 2.5 U/ml thrombin solution prepared in **step 15** and mix gently. Cells are now at 5×10^6 cells/ml in a hydrogel of 1 U/ml thrombin and 3 mg/ml fibrinogen.
18. Load them gently in channel no. 5 (Fig. 2a).
19. Incubate 5 min at 37 °C in humid atmosphere to limit the evaporation.
20. Add some HUVEC medium in one of the two reservoirs of channel no. 6 (Fig. 2a) and hFOB medium in one of the two reservoirs of channel no. 1 (Fig. 2a). Media should fill the

channels and form droplets in the opposite reservoirs (*see* **Note 24**).

21. Place your PDMS chip in a plastic petri dish and place it in your incubator at 37 °C (*see* **Note 25**). Do not change the medium for the next 3 days. However, if the medium level decreases in the inlets, add a few microliters of fresh one.
22. HSPCs are loaded 72 h after HUVECs and hFOBs to allow a proper auto-organization in the chip. Do not forget to thaw and resuspend the HSPCs in their dedicated medium (IMDM supplemented with FBS, SCF, G-CSF, and IL-3) the day before their loading. HSPCs are embedded in Coll/Fib hydrogel following exactly the same procedure as for the HUVEC and hFOB cells, except that 10^5 cells (final density of 5×10^6 cells/ml in the chip) will be loaded in channel no. 3 (Fig. 2a) (*see* **Note 26**).
23. After HSPC loading, incubate at 37 °C.
24. Change the media in channels no. 1 and no. 6 (Fig. 2a) with HSPC medium, without SCF, G-CSF and IL-3.

4 Notes

1. Dissolve the fibrinogen lyophilized powder in 1× PBS at 37 °C under gentle agitation. Filter the solution using a 0.4 μm sterile filter and a syringe, aliquot and store at −20 °C.
2. Dissolve the thrombin lyophilized powder in a 0.1% BSA solution. Filter the solution using a 0.4 μm sterile filter and a syringe, aliquot and store at −20 °C.
3. Culture the HUVECs on 0.1% gelatin coated flasks. They must be harvested at 80% of confluency for the experiment. To do so, seed at 3.5×10^3 cells/cm^2 in a culture flask 72 h before the experiment. Use low-passage HUVECs for a better vascular network formation.
4. NHLFs secrete proangiogenic growth factors supporting endothelial cells morphogenesis [14]. In the chip, NHLFs tend to proliferate in the hydrogel having for consequence their escape from the hydrogel. Their proliferation is prevented by treating them with 10 μg/ml Mitomycin-c for 2 h. Freeze the inactivated NHLF in FGM-2 + 10% DMSO. Thaw them just before their loading in the PDMS chip.
5. HSPCs originate from human cord blood samples. Purify them using Ficoll™ density gradient, followed by MACS® enrichment using anti-CD34 antibody. Cryopreserve the HSPCs in FBS + 10% DMSO at 7×10^5 cell/ml.
6. These dimensions can be adapted but if the pillars are too close, the medium will not diffuse well into the compartments; if they

are too spaced, the hydrogels will leak from one channel to the other during their loading.

7. The size and spacing must be optimized so that the anchorage pillars stabilize the hydrogel without perturbing the its loading.
8. When peeling-off the PDMS layer from the SU-8 master mold, the SU-8 structures are submitted to high mechanical constraints. A first layer of SU-8 (5 μm thick) is spin coated and cured to prevent them from detaching from the substrate.
9. It can be quite complicated to control/monitor the UV exposure onto the SU-8 and to make sure the image is focused since the wafer is upside-down and not transparent. An alternative is to spin-coat the SU-8 on a glass slide so that it is easier to center the exposure on the SU-8 resist. Besides, a glass slide will fit on the microscope holder, which can be more complicated with a silicon wafer. Before spin coating a glass slide, wash it using acetone then isopropyl alcohol and bake it at 120 °C for 30 min.
10. Different objectives can be used to do maskless photolithography. In this case, since the desired structures are quite thick (150 μm), it is preferable to use an objective with a large depth of field (e.g., a 4× objective) so that the projected images are focused over a greater height (*see* **Note 11** to know how to focus).
11. Thanks to the autofluorescence of the SU-8, the UV image that is projected can be observed through the microscope to make sure of the focus. However, the area you will use for this purpose will be cured by the UV and thus cannot be used to project the chip design. To avoid wasting a too big area of your resist during this focusing step, define a "sacrificial" area. Make sure this sacrificial area is at least 1 cm far from the area you have chosen to be exposed to the virtual mask of the chip.
12. Stitching of DMD images. To make an image bigger than the size of a single DMD-image (a few hundreds of microns depending on the objective, for example around 2600 μm × 1600 μm for a 4× objective), the software must divide the virtual mask into subimages that will be sequentially projected. However, this process creates sharp junctions in the mold, which will lead the final PDMS chip to leak. To obtain smooth junctions between these subimages, PRIMO's dedicated software can add grey level gradients on the edges of each subimage, thus allowing the final structures to be smooth and the PDMS chip hermetically sealed onto the glass.
13. A visual control at the microscope must be performed after washing. If some photoresist remains in the non-exposed area, continue the development for a few minutes. Besides, a white film can remain after washing if the substrate has been underdeveloped. In this case, repeat the washing step: spray the

substrate with PGMEA to remove the film and then with isopropyl alcohol to remove the PGMEA.

14. Silanization can be performed in a plastic dish instead of a desiccator. Place the master mold in the dish, put the centrifuge tube's cap and add the 50 μl of silane in it. Seal the dish with parafilm and incubate for 1 h. The silanization can be done again after multiple uses of the master mold if the PDMS start to be hard to detach.
15. To peel-off the cured PDMS, use a scalpel and tweezers. First, cut the PDMS roughly around the structure's area. Remove the part of interest slowly using tweezers while making sure you do not degrade the mold. Afterward, cut the PDMS more precisely.
16. Some residual dust can remain on the PDMS chip and prevent efficient bonding. Before plasma treating it, use some 3 M Scotch tape to remove all the dust present on the surface that will be bonded to the glass. Use air flow or tape to remove the dust on the glass coverslip as well. Bond the PDMS chip to the glass directly after plasma treatment as the surface activation is stable for a few minutes only.
17. It is necessary to do the bonding the day before the experiment and to put the chip in the oven at 65 °C overnight so that the PDMS retrieves its hydrophobicity. Indeed, after the plasma treatment, the PDMS becomes hydrophilic, which can allow the hydrogel to go through the separation pillars and thus escape from its dedicated channel.
18. The volumes of most of the solutions prepared here are larger than necessary to account for volumetric errors in pipetting (e.g., Collagen-I solution is very viscous). Besides, this protocol is for one PDMS chip. Since some cells need to be thawed for the experiment, it can be more convenient to do the experiment on multiple PDMS chips in parallel. The protocol will thus need to be scaled up.
19. Collagen-I stock solution is at 17.5 mM in acetic acid (pH $\approx$ 3.8). For gelation, physiological pH and salt concentration are required and are tuned with the NaOH and the 10$\times$ PBS solutions, respectively. Always control the pH with the pH paper, 5 μl is sufficient.
20. Adapt the number of cells in each compartment if required.
21. The addition of the thrombin to the hydrogel mix makes it crosslink really fast. To make sure it remains liquid during its loading into the channels, add the cell suspension to the 4 μl thrombin solution at the last moment.
22. Cut the extremity of the 20 μl tip to enlarge its diameter. This will help to fit to the channel's inlet.

23. Once the solution reaches the outlet of the channel, stop applying pressure or the solution may leak in the other channels.
24. Due to the previous incubation times of the hydrogels in the absence of medium (for their polymerization), it is possible that they shrink a little bit. When loading the medium, some bubbles can thus form at the hydrogel/medium interface. If they are not too big, do not try to remove them, they will disappear after a few hours. If they are problematic or if the medium does not flow into the channel, gently apply pressure on the chip using a pipette tip to remove them.
25. Make sure that both inlets and outlets are filled with liquid and that no air bubble remains. Besides, to limit medium evaporation, create a humid atmosphere by placing caps of 15 ml conical tubes filled with sterile water in the petri dish.
26. In previous steps, nothing was loaded in the central channel (no. 3, (Fig. 2a)). Sometimes, this channel stays totally dry for days and sometimes it is full of medium. Based on our experience, it seems easier (and safer) to load HSPCs in the chip when the channel is filled with medium. If it is dry, fill the channel with PBS using a P20 pipette. Apply a Kimwipes on the opposite outlet in order to better "aspirate" the medium by capillarity.

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