Chapter 7

Fabrication of PEG Hydrogel Microwell Arrays for High-Throughput Single Stem Cell Culture and Analysis

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Abstract

Microwell arrays are cell culture and imaging platforms to assess cells at a single cell level and in high-throughput. They allow the spatial confinement of single cells in microfabricated cavities on a substrate and thus the continuous long-term observation of single cells and their progeny. The recent development of microwell arrays from soft, biomimetic hydrogels further increases the physiological relevance of these platforms, as it substantially enhances stem cell survival and the efficiency of self-renewal or differentiation. This protocol describes the microfabrication of such hydrogel microwell arrays, as well as the cell handling and imaging.

Key words: Stem cells, Single cell analysis, Poly(ethylene glycol), PEG, Hydrogel, Time-lapse microscopy, Soft lithography, Microcontact printing

1. Introduction

Stem cells are inherently heterogeneous cell populations that call for high-throughput single cell analyses. Although single cell transplantation experiments (1), fluorescence-activated cell sorting (FACS) (2) or reporter gene expression dynamics (3) were successfully utilized to document behavioral variations within stem cell populations, most current in vitro methodologies fail to shed light on important long-term behaviors such as cell proliferation or differentiation because of difficulties in tracking single cells in vitro.

Microwell arrays are emerging cell culture and imaging platforms with a microtopography to capture cells. They are produced by microfabrication processes such as photolithography or soft lithography and consist of miniature cavities with diameters and depths of tens to hundreds of microns. Depending on the diameter

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of the microwells, microwell arrays can comprise several hundreds to thousands of microwells per square centimeter arranged on a regular grid. Cells can then be seeded onto these arrays and will sediment randomly onto the bottom of the microwells (4). Because microwell arrays are typically made of a cell-nonadhesive substrate such as agarose (5), poly(ethylene glycol) (PEG) hydrogels (6–8), or poly(dimethylsiloxane) (PDMS) (9, 10), cells cannot overcome the topographical barrier and therefore they remain trapped inside the microwells. Moreover, the high density of single cells in microwell arrays significantly improves the throughput of imaging-based single-cell analyses compared to standard multi-well plates (11).

Not surprisingly, microwell arrays have, for example, been successfully used to study in a high-throughput manner the proliferation rates of single adult rat hippocampal progenitor cells (12), neural stem cells (NSCs) (13), or hematopoietic stem cell (HSCs) (7, 9). The long-term microscopy combined with image analysis and retrospective cell fate analysis allowed for example to identify differences in proliferation rates of long-term HSCs and multipotent progenitors or to screen for effects of various growth factors on stem cell function (7). Of note, the applicability of microwells is not restricted to proliferation analyses of stem cells but can also be used for high-throughput image cytometry (14) or the screening of single hybridoma cells (15). Another important application of microwell arrays is the generation of multicellular spheroids such as embryonic bodies (EBs) (6, 16), neurospheres (NS) (13) or cancer spheroids (5). Compared to classical methods to produce multicelluar spheroids, i.e., hanging drops or scraping of monolayers (in the case of EBs), microwell arrays not only enhance the throughput, but also yield more homogeneous, size-controlled cell aggregates or prevent merging of individual, clonally derived spheroids.

A wide range of methods and materials can be used for the fabrication of microwell arrays. The first generation of microwell arrays were made by photolithography, exposing photoresists on glass with UV through photomasks, and thus offered relatively limited possibilities to biofunctionalize the microwell arrays (12). Subsequently, soft lithography and micromolding approaches were developed whereby a negative stamp (i.e., a stamp with micropillars) is first molded against PDMS or PEG hydrogels. Upon complete crosslinking, these substrates irreversibly replicate the features of the microstamp that can be removed. These techniques not only allowed to micropattern soft hydrogels, that were shown to enhance stem cell culture (7, 13), but also to locally functionalize microwells with biomolecules by integrating microcontact printing (μCP) approaches into the microwell array fabrication (7, 10). Notably, the local tethering of microwells such as cell-adhesive proteins or growth factors (7), extends the advantages of microcontact printed substrates to clinically important, nonadherent stem cells, such as

NSCs (13) or HSCs which would not be possible on flat, purely microcontact-printed cell culture substrates (17).

This chapter describes (a) the fabrication by micromolding of PEG microwell array using a PDMS stamp, (b) the functionalization of the microwells with collagen, and (c) how to image by microscopy single cells on the PEG microwell array. The protocol is based on the photopolymerization of acrylated PEG, a robust and simple method to make PEG hydrogels from commercially available precursor materials circumventing time-consuming chemical synthesis (7, 18, 19). The protocol also describes how to reliably integrate these PEG microwell arrays into multi-chambered microscopy slides which enables live-cell imaging using time-lapse microscopy and allows to perform multiple experiments in parallel.

2. Materials

2.1. Microfabrication of PDMS Master

1. 4" ⟨100⟩ silicon test wafers single-side polished (Siltronix, France).
2. GM1070 SU8 Photoresist (Gersteltec, Switherland) (light sensitive).
3. 1-Methoxy-2-propyl acetate (PMA, ACS grade).
4. Chrome Blank 5 in. soda lime mask (SLM) (Nanofilm, Westlake Village, USA) (light sensitive).
5. Clean room facility equipped for writing masks and for photolithography with the following equipment: a plasma oven (Tepla 300, PVA TePla, Germany), mask writer (Heidelberg DWL200, Heidelberg Instruments GmbH, Germany), mask developer (Suess DV10), mask aligner (Suess MA6, both Suess MicroTec AG, Germany), wet benches for chrome etching and resist stripping, spin coater (Sawatec LSM200), and programmable hot plates (Sawatec HP401Z, both Sawatec AG, Principality of Liechtenstein).
6. Mask design program, e.g., CleWin 4.0 (PhoeniX Software, The Netherlands).

2.2. Fabrication of the PDMS Stamp

1. Poly(dimethyl siloxane) elastomer kit (Sylgard1 184 Silicone Elastomer Kit, Dow Corning).
2. Flat-bottom glass beaker with an inner diameter of 11–12 cm (to host a 4-in. wafer).
3. Scalpel, ideally with a crescent-shaped scalpel blade (#12).

2.3. Functionalization of Glass Slides

1. Isopropyl alcohol (ACS grade).
2. Acetone (ACS grade).

3. Heptane (ACS grade).
4. Standard microscopy glass slides.
5. Plasma cleaner femto (Diener electronic GmbH+Co, Germany).
6. 3-(Trichlorosilyl)propyl methacrylate (TPM, Sigma-Aldrich, Switzerland).
7. Carbon tetrachloride (ACS grade).

2.4. Inking of the Stamp

1. Acrylamide/Bis-acrylamide, 30% solution, 37.5:1 ratio, electrophoresis grade.
2. *N*,*N*,*N*,*N*′-Tetramethylethylenediamine (TEMED).
3. 1 mg/ml Ammonium persulfate solution (APS) in ddH_2O (stored at –20°C).
4. 1.50 M Tris–HCl, pH 8.8.
5. 10 mM Hydrochloric acid (HCl).
6. Bovine collagen solution 3 mg/ml (Sigma-Aldrich, Switzerland).
7. 1× Phosphate-buffered saline (PBS, to store the remaining acrylamide gel).

2.5. Fabrication of the PEG Microwell Arrays

1. 20% (w/v) Poly(ethylene glycol) diacrylate average Mn 2000 (Sigma-Aldrich, Switzerland) in PBS (see Note 1) with 2% (v/v) 2-hydroxy-2-methyl propiophenone (Sigma-Aldrich, Switzerland) (store solution at 4°C and protected from light).
2. Ultraviolet (UV) lamp HBO 100 (Zeiss, Germany).
3. Elastosil E 41 (one-component silicon rubber, Wacker Chemie, Switzerland).
4. LabTek II 8 chamber slides (Nunc, Switzerland).
5. Oriented Polyester, Plastic Shim Stock, 0.004 in. Thick 5 in.×20 in., Color Tan (SmallParts Inc., USA).
6. 1× PBS (to store microwell arrays).

2.6. Cell Seeding and Time-Lapse Imaging

1. Clear polyolefin, advanced optical sealing tape for microscopy (Nunc, Switzerland).
2. Zeiss Observer Z1 (Zeiss, Germany), equipped with an motorized stage (Ludl, Austria) and an environmental chamber (Life Imaging Services, Switzerland).
3. Temperature controller “Brick” (Life Imaging Services, Switzerland).
4. CO_2 and humidifier “Cube” (Life Imaging Services, Switzerland).
5. MetaMorph 7.5 image acquisition software (Visitron, Germany).

3. Methods

The procedure to culture single stem cells on micromolded PEG microwell arrays includes three main steps. First, a PDMS microstamp needs to be replicated from a microfabricated master via soft lithography (Fig. 1a). This master is produced by photolithography

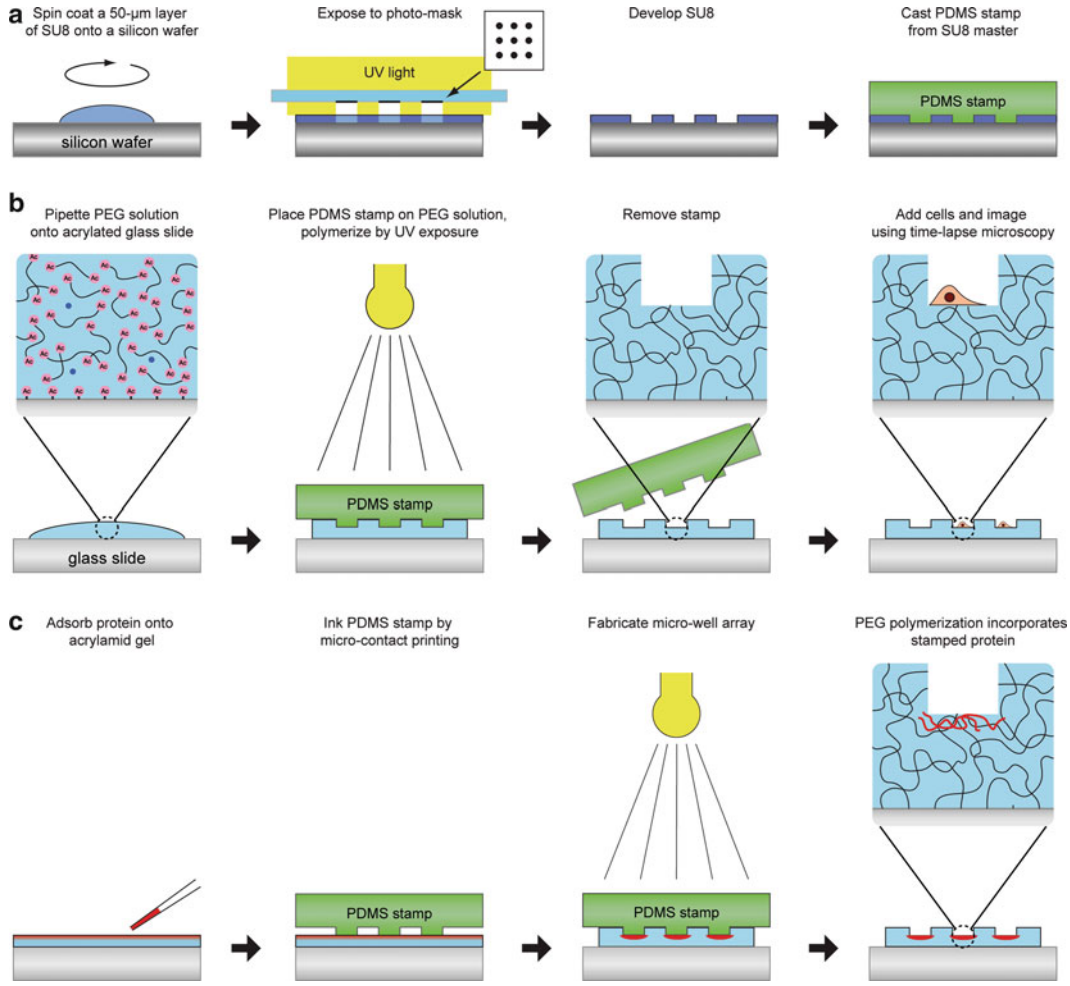

Fig. 1. The fabrication of microwell arrays and their functionalization with proteins. (**a**) Fabrication of the PDMS stamp. First, a SU8 master is produced by spin coating a 100-µm layer of SU8 onto a silicon wafer that is then exposed to a photomask. After development of the SU8 structures, a PDMS stamp can be molded from the SU8 and then be used for the fabrication of PEG microwell arrays (**b**). Therefore, a drop of PEG-diacrylate with a photoinitiator is applied onto an acrylated glass slide, covered with the PDMS stamp and exposed to UV light. The UV exposure triggers the polymerization of the PEG, its covalent attachment to the glass surface. The irreversible incorporation of the shape of the PDMS stamp finally allows the removal of the PDMS stamp and the seeding of single cells onto the microwell arrays. (**c**) This process also allows the functionalization of the bottom of microwells with adhesive proteins such as collagen by integrating a microcontact printing step. First, collagen is adsorbed onto an acrylamide gel from where it is transferred onto the PDMS stamp. During the micromolding of PEG microwell arrays with this inked PDMS stamp, the collagen is transferred into the PEG gel and locally polymerizes due to the change of pH.

in a clean room facility and results in a PDMS stamp consisting of an array of micropillars. In a second step, this PDMS stamp, which optionally can be coated with a protein of interest, is used to micromold PEG-diacrylate (Fig. 1b). To do so, the PEG precursor is mixed with a photoinitiator and applied onto an acryl-functionalized glass slide. After placing the PDMS stamp onto the PEG precursor, the sandwich is exposed to ultraviolet (UV) light at 350 nm. This leads to the free-radical polymerization of the acrylate groups of PEG and to the covalent attachment of the hydrogel to the acrylated glass slide, such that the PDMS stamp can be removed and the PEG retains its molded shape. Thanks to multiwell chamber that is glued onto the glass slide, cells can then be seeded onto these microwell arrays and can be imaged via time-lapse microscopy.

Because trapping of single cells on microwell arrays is a stochastic process, the number of cells per microwell depends on the density of microwells on the array and the cell seeding density. For efficient single cell analyses, these two parameters should match, but lower number of cells may also be seeded (as it may be the case for rare cells) or much higher numbers allowing the formation of multicellular spheroids (6).

As mentioned above, micromolding of microwell arrays allows the patterning of the bottom of the microwells with a protein of interest, such as the key extracellular matrix (ECM) component collagen, by expanding the method to integrate a microcontact printing step (Fig. 1c). In this optional treatment, collagen is adsorbed onto an acrylamide gel and the PDMS mold is then incubated on this acrylamide gel such that the collagen is transferred just to the top of the micropillars. During the subsequent micromolding of the PEG microwell array, the collagen polymerizes due to the change of pH and will form an interpenetrating network with the PEG gel.

3.1. Microfabrication of SU8 Master

1. Design a photomask using the mask writing program CleWin. Create arrays of circles with a diameter of 150 μm (see Notes 2 and 3). The arrays should measure about 4 × 5 mm (see Note 4). Repeat the designs with an offset of 2–3 mm.
2. Convert the file using the Heidelberg conversion software. Invert the design in the conversion settings (see Notes 5 and 6).
3. In the clean room facility, write the photomask, develop it with a mask developer, etch it in a chrome etch bath and strip the remaining photoresist from the mask.
4. Immediately prior use, clean silicon wafers in an oxygen plasma for 7 min at 500 W.
5. Pour a drop with a diameter of 3–4 cm of SU8 GM10170 onto the wafer and spin-coat it at 1,700 rpm for 40 s to obtain a 50-μm layer of SU8 (see Note 7).

6. Prebake the wafer on programmable a hotplate 30 min at 130°C, ramping the temperature at 4°C/min (see Note 8).
7. Align the photomask and the wafer using a mask aligner, and expose the wafer with 400 mJ/cm^2 with UV light (290–390 nm). Exact exposure dose depends on the mask design and may need to be determined experimentally.
8. After exposure, bake the wafer on a programmable hot plate for 40 min at 100°C (ramp 4°C/min).
9. Develop the wafer for 2–3 min in PMA under slight agitation, wash in a second PMA bath for another 2 min, and then rinse twice in IPA. Let dry under a fume hood.

3.2. Fabrication of the PDMS Stamp

1. Prepare 20–30 g of PDMS by mixing prepolymer and curing agent at ratio 10:1 (w/w) in a plastic cup. Mix rigorously and degas until all air bubbles are removed.
2. Place wafer in a glass container lined with a double layer of thin foil, pour PDMS onto the wafer to a thickness of 3–4 mm. Work in a clean and dust-free environment.
3. Bake the PDMS replica at 80°C for >4 h, remove the wafer from the oven, and allow to cool down.
4. Cut the border of the PDMS with a scalpel blade, carefully peel off the PDMS from the wafer and then cut the individual arrays slightly larger than an array using a scalpel. Store unused stamps in a clean Petri dish, protected from dust (see Note 9).

3.3. Acrylation of Glass Slides

1. Clean glass slides first with detergent, then wash with ddH_2O, acetone and isopropyl alcohol. Dry with air gun or in fume hood.
2. Plasma clean glass slides in an oxygen plasma at ~200 mTorr, 100 W for 5 min.
3. In the meantime, prepare a 1-mM solution of TPM (3-(trichlorosilylpropyl) methacrylate) in a 4:1 mixture of heptane and carbon tetrachloride.
4. Immediately after plasma cleaning, bath the glass slides in the TPM solution for 5 min.
5. After the treatment, wash slides with heptane, acetone, and water, dry with air gun or in fume hood.
6. Acrylated slides can be stored at 4°C until further use.

3.4. Functionalization of PDMS Stamp (Optional)

1. Cast a 0.8-mm thick acrylamide gel made of 4.4 ml acrylamide/bis-acrylamide, 2.5 ml Tris–HCl pH 8.8, 3 ml ddH_2O, 100 µl APS, and 10 µl TEMED using a standard gel casting system.
2. After polymerization, disassemble the gel casting system in a sterile hood. Cut a 2 × 4 cm piece from acrylamide gel, transfer

it onto a clean Petri dish and add 2–3 ml of 10 mM HCl for 20 min (see Note 10). Remaining gels can be stored in PBS at 4°C for up to 2 weeks.

3. Remove buffer with a pipette without touching the gel surface. Then slightly tilt the Petri dish to carefully adsorb the remaining liquid droplets with a kimwipe from the border of the acrylamide gel.
4. Add 100 µl of the collagen solution (3 mg/ml) equally onto the acrylamide gel, distribute homogeneously on the hydrogel with a pipette tip.
5. Let the collagen adsorb onto the acrylamide by evaporation until there are no visible droplets on the acrylamide gel left.
6. Place eight of the cut PDMS stamps on the collagen soaked acrylamide gel with the pillars. Mind the orientation of the PDMS, the micropillars of the stamp have to be in contact with the gel. Incubate for 30 min in the closed Petri dish.

3.5. Fabrication of the PEG Microwell Arrays

1. Apply some Elastil E 41 glue onto a piece of Parafilm or tin foil. Fold Parafilm and distribute the silicone glue homogeneously until a thin film is obtained.
2. Remove the chamber from the LabTek Chamber slide with the provided tools, remove remaining adhesive from the chamber walls and briefly put the chamber walls on the silicone glue.
3. Place the LabTek chamber on the acrylated glass slide, press firmly to remove potential air bubbles in the glue and to ensure complete sealing of the chamber, but avoid lateral movement of the chamber to keep the glass at the bottom of the wells clean from glue. Let the rubber glue cure at room temperature for >2 h.
4. Cut small pieces (2 × 5 mm) from the 100-m plastic spacer and place them on the bottom of the chambered glass slide such that they can hold the PDMS stamp.
5. Place 20–30 µl of the PEG precursors solution into all wells without introducing air bubbles to the PEG solution.
6. Gently place the (inked) PDMS stamp onto the PEG drop. Do not press strongly to avoid squeezing out the PEG.
7. Expose the sample for 60 s to an UV source at 100 mW/cm^2 (at 350 nm). Exact exposure times may be determined experimentally.
8. Carefully remove the PDMS stamp with tweezers and add PBS onto each microwell.
9. Sterilize the hydrogel microwell array twice with UV in a cell culture hood.
10. Store the arrays at 4°C in a Petri dish sealed with Parafilm for >12 h to allow the hydrogel to swell. They can be stored under these conditions for up to a week.

3.6. Cell Seeding and Time-Lapse Imaging

1. Preheat the incubator of the microscope and the sample holder to 37°C for 2–4 h.
2. Wash arrays 3× with medium for 30 min.
3. Prepare your cells according to your protocols.
4. To seed cells homogeneously on the microwell array, remove the medium (see Note 11) and immediately seed cells onto the array (see Note 12). Cover with a precut sealing tape.
5. Carefully place sample on the microscope. Cover sample with the environmental chamber and turn on CO_2 and humidity controller (see Note 13). Let equilibrate the sample for >1 h.
6. After thermal equilibration, acquire positions of single cells using the "multi-dimensional acquisition" application of MetaMorph.
7. Set illumination and time-lapse settings and launch the time-lapse experiment (see Note 14).

4. Notes

1. The density of PEG is approximately 1.2 kg/l and needs to be compensated when preparing the PEG precursor solution.
2. There are two main aspects to be considered when designing the mask. First, the critical dimension (i.e., the most difficult feature to microfabricate) is not the size of the microwells, but the wall separating them. Walls with a large aspect ratio (i.e., very high, but thin PEG structures) are fragile and difficult to mold because the hydrophobicity of the PDMS hinders the filling of narrow gaps of the mold with the PEG precursor solution. In our experience, an aspect ratio of 1–2 ensures complete filling; thus, the microwells should not be more than twice as high as the offset between each microwell (8). Hydrogel pillars or closed structures where air cannot escape from the PDMS stamp during the molding are similarly difficult to produce.
3. Second, PEG hydrogels intrinsically absorb water after polymerization and swell. This swelling leads to an enlargement of PEG structures and accordingly to a diminishment of the diameter and the depth of the microwells. For this reason, the diameters of microwells should be designed approximately 50% larger than the intended size (depending on the wall thickness between the microwells and the PEG concentration).
4. Note that the size of a microwell array (4 × 5 mm) is designed to fit into a well in an 8-well LabTek chamber, but can be easily adjusted to other cell culture devices.

5. We routinely use a mask writer to fabricate glass photomasks with a maximal resolution of 1 μm. However, the fabrication of microwell arrays normally does not need a high spatial resolution and may also be done using transparencies with a high-quality laser printer as described previously (17). Skip steps 2 and 3 of this section in this case.
6. As the fabrication of microwells arrays comprises two molding steps (PDMS replica, PEG micromolding) and because each molding step inverts the topography, the wafer must have the same topography as the PEG structures, i.e., it must consist of SU8 microwell arrays (the PDMS stamp will then host arrays of micropillars and the PEG hydrogel contain the microwell arrays again). To obtain the right topography in SU8 (a negative photoresist that polymerizes upon UV-exposure), the area of the microwells (the dots on the mask design) must remain the chrome on the photomask. Our setup requires inverting the design during the conversion (black becomes white and vice versa). However, if transparencies are used, this may not be the case. In this case, ensure that the mask consists of arrays of dots to obtain PEG microwell arrays.
7. Other thicknesses can be achieved by changing the photoresist or the spinning rate. This may also require adjusting the baking and exposure times of the entire process. Check the manufacturer's homepage (www.gersteltec.ch) for additional details.
8. We prebake the wafers at higher temperatures than indicated by the manufacturer to decrease baking times.
9. PDMS is very hydrophobic and thus easily attracts dust. The best way to remove dust or PDMS cutting shred is by gently applying a tape (e.g., Magic tape from 3 M) on the PDMS. Dust will now stick to the tape and can easily be removed together with the tape. Also note that, although the cleaning of PDMS stamps was described previously (17), we do not recommend reusing PDMS stamps to avoid cross-contaminations.
10. This step is required to exchange buffer and prevent the gelation of the collagen on the acrylamide gel. It may also be possible to print other proteins, but for that the buffer need to be adjusted and protein initially functionalized with a heterofunctional acryl-PEG-NHS linker to covalently bind the protein to the hydrogel (20).
11. Work quickly to avoid the complete drying of the hydrogel, which can induce air bubbles on the surface of the gel.
12. The number of cells to be seeded depends on the density of microwells in the array their size and on your application. Because cell trapping in the microwells is random, the number of cells follows a Poisson distribution for large microwells. For single cell proliferation analyses one cell per 2–3 microwells

typically is sufficient to avoid too many microwells with no or more than one cell, but may also be lower for rare primary cells like hematopoietic stem cells.

13. Typically, air flow rates of 15–20 l/h and a humidity of 90–95% are sufficient to avoid evaporation of the sample. Also avoid wetting of the sealing tape, which will increase evaporation and the risk that the sample dries out. Also ensure that the temperature of the sample and the microscope is well equilibrated to avoid focus drifts during the time-lapse.
14. For slowly moving and proliferating cells, such as HSCs, time intervals of 4 h are sufficient to assess the proliferation kinetics of single cells, but may need to be higher for other cell types or other readouts. Do not frequently image cells in fluorescent channels to avoid phototoxicity. If a fluorescent readout is available, cell counting may be automated (14, 21). However, for brightfield images, automated cell counting is difficult and not reliable and thus is mostly done manually.

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