Cryogel-based Injectable 3D Microcarrier Co-culture for Support of Hematopoietic Progenitor Niches

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Although hematopoietic stem cell (HSC) transplantation can restore functional hematopoiesis upon immune or chemotherapy-induced bone marrow failure, complications often arise during recovery, leading to up to 25% transplant-related mortality in treated patients. In hematopoietic homeostasis and regeneration, HSCs in the bone marrow give rise to the entirety of cellular blood components. One of the challenges in studying hematopoiesis is the ability to successfully mimic the relationship between the stroma and hematopoietic stem and progenitor cells (HSPCs). This study and the described protocols propose an advantageous method for culturing and assessing stromal hematopoietic support in three dimensions, representing a simplified in vitro model of the bone marrow niche that can be transplanted in vivo by injection. By co-culturing OP9 bone marrow–derived stromal cells (BMSCs) and cKit+ Sca-1+ Lin– (KLS+) HSPCs on collagen-coated carboxymethylcellulose scaffolds for 2 weeks in the absence of cytokines, we established a methodology for in vivo subcutaneous transplantation. With this model we were able to detect early signs of extramedullary hematopoiesis. This work can be useful for studying various stromal cell populations in co-culture, as well as simple transfer by injection of these scaffolds in vivo for heterotopic regeneration of the marrow microenvironment. © 2021 The Authors. Current Protocols published by Wiley Periodicals LLC.

Basic Protocol 1: Isolation of HSPCs from mice
Basic Protocol 2: Co-seeding of HSPCs and BMSCs on collagen-coated CCMs
Basic Protocol 3: Maintenance, real-time imaging, and analysis of co-seeded scaffolds
Basic Protocol 4: End-point analysis of co-seeded scaffolds using flow cytometry and CFU assays
INTRODUCTION

Bone marrow (BM) failure, whether secondary to chemotherapy in cancer patients or to inherited or immune-mediated bone marrow failure syndromes, is accompanied by massive remodeling of the BM microenvironment, which typically entails a reversible adipocytic conversion of the marrow (Tratwal et al., 2020). Although hematopoietic stem cell (HSC) transplantation can restore functional hematopoiesis, complications often arise during the first few weeks of recovery, leading to up to 25% mortality in treated patients (Gooley et al., 2010; Jenq & van den Brink, 2010). Identifying putative supportive stromal cells and engineering artificial and functional HSC niches that allow expansion of repopulating HSCs is thus of intense interest for HSC regenerative therapies, as outlined by the American Society of Hematology (ASH) in its most recent research agenda (Bianco, Riminucci, Gronthos, & Robey, 2001; Morrison & Scadden, 2014; Mullighan, 2018).

HSCs are the primary population of cells that give rise to the entirety of cellular blood components through hematopoiesis. They reside in the BM and give rise to myeloid, erythrocytic, megakaryocytic, and lymphoid progenitors. In vivo, the HSC niche within the BM modulates both the self-renewal and differentiation capacity of HSCs (Pinho & Frenette, 2019).

In vitro engineered models aim to recapitulate the physiological and pathophysiological functions of organ systems in the body, while balancing throughput and complexity (Tavakol, Fleischer, & Vunjak-Novakovic, 2021). Previous models of BM, including organ-on-a-chip microfluidic platforms and ossicle-based scaffolds, have demonstrated multicellular complexity, but have limited ease of use (Bourgine et al., 2018; Chou et al., 2020; Ferreira et al., 2012; Raic, Rödling, Kalbacher, & Lee-Thedieck, 2014; Torsiawa et al., 2014). Many of these existing models are xenogeneic, or require complex hematopoietic cytokines and even daily intraperitoneal injections for extended periods of time to enhance ossification (Reinisch et al., 2016). Moreover, transplantable systems often require non-standardized extracellular matrix components (e.g., Matrigel).

We have developed a co-culture system to study interactions between murine HSPCs and murine OP9 bone marrow–derived stromal cells (BMSCs) in vitro (Fig. 1). Using collagen-coated carboxymethylcellulose cryogel microscaffolds (CCMs) with the capacity to covalently interlock, we co-cultured HSPCs and BMSCs for up to 2 weeks. We then performed in vivo subcutaneous injection in mice through a process that triggers particle interlocking. Through this work, we have developed methodologies that support hematopoietic cells in 3D cultures without exogenous cytokines, allow imaging of 3D
co-seeded scaffolds over time, and generate cellularized scaffolds that can be injected subcutaneously. We show maintenance of hematopoiesis after 12 days of in vitro co-culture and robust cellularization of implants with evidence of in situ hematopoiesis 12 weeks post-implantation.

In this article, we expand upon our recent work in Tavakol et al. (2020). We present in detailed steps for the isolation of murine HSPCs (see Basic Protocol 1), seeding of HSPCs and BMSCs in vitro (see Basic Protocol 2), maintenance and imaging of the co-cultured scaffolds (see Basic Protocol 3), end-point analysis of the 3D culture system (see Basic Protocol 4), preparation of cellularized 3D scaffolds to fit injection parameters (see Basic Protocol 5), and in vivo subcutaneous transplantation in immunodeficient mice (see Basic Protocol 6).

STRATEGIC PLANNING

Many of the methodologies and figures described here are an elaboration of the work in Tavakol et al. (2020). Here, we describe the use of two specific cell populations from murine origin: GFP OP9 BMSCs and DsRed HSPCs. The BMSCs were provided by a collaborating laboratory and have been carefully maintained at subconfluency in our laboratory since 2013. The HSPCs were harvested from endogenously labeled C57Bl/6 mice (specifically, B6.Cg-Tg(CAG-DsRed*MST)Nagy/J), also maintained in-house since 2013. These DsRed mice are commercially available (Jackson Laboratory, strain 006051). Additional experiments made use of tdTomato+ KLS+ cells from mTmG reporter mice (Jackson Laboratory, strain 007676). The methodologies described here are not limited to these cell types; any BMSCs and HSPCs may be tested in these protocols, as the fluorescent labeling was only essential in characterization and imaging of the scaffolds over time. There is also potential to use these methodologies to study human hematopoiesis.

Fabrication of the CCM scaffolds is described in Tavakol et al. (2020). The underlying collagen adsorption and immobilization to carboxymethylcellulose scaffolds has been studied in depth (Serex et al., 2018), whereas the rheological and in vivo tissue reconstruction properties are part of a manuscript currently available as a preprint (Béduer, Bonini, Verheyen, Burch, & Braschler, 2020).
**NOTE:** All protocols involving live animals must be reviewed and approved by an Institutional Animal Care and Use Committee (IACUC) and must conform to government regulations for the care and use of laboratory animals.

**NOTE:** All solutions and equipment coming into contact with cells must be sterile, and proper sterile technique should be used accordingly.

### ISOLATION OF HSPCs FROM MICE

To isolate hematopoietic stem and progenitor cells from mice, the long bones are extracted and thoroughly cleaned of all soft tissue. The bones are crushed with a mortar and pestle to release the hematopoietic cells, and the cell suspension is filtered to remove debris. Red blood cells (RBCs) are lysed, and the remaining cells are stained with antibodies for HSPC isolation based on fluorescence-activated cell sorting (FACS). The cells are sorted into medium based on surface marker expression and stored on ice. Typically, one mouse will yield 60,000 cKit⁺ Sca-1⁺ Lin⁻ (i.e., KLS⁺) HSPCs.

#### Materials

- Endogenously labeled mice (see Strategic Planning)
- Wild-type control mouse
- Phosphate-buffered saline (PBS)
- FACS buffer (see recipe)
- 1× RBC lysis buffer (BioLegend, 420301), ice cold
- Lineage depletion kit (BD Pharmingen, 558451), including lineage cocktail and magnetic bead suspension
- Blocking solution (see recipe)
- Anti-streptavidin-PO (Invitrogen, S32365)
- Anti-cKit PE-Cy7 (BioLegend, 105814)
- Anti-Sca-1 APC (BioLegend, 10812)
- DAPI (Applichem, A4099.005)
- Hematopoietic culture medium (HCM; see recipe)
- Mortar and pestle
- 10-ml pipette
- 70-μm cell strainers (Corning, cat. no. CLS431751)
- 15- and 50-ml Falcon tubes
- 0.2-μm strainer
- Cell counter or hemocytometer
- AutoMACS magnetic cell separator (Miltenyi Biotec)
- 1.5-ml microcentrifuge tubes
- 0.85-μm mesh strainers
- FACS machine (e.g., Aria Fusion)

Additional reagents and equipment for euthanasia and bone removal

**NOTE:** If an AutoMACS instrument is not available, depletion can be performed using manual midi LD columns (Miltenyi Biotec) according to manufacturer’s recommendations.

#### Prepare hematopoietic cell suspension

1. Isolate all tibiae, femurs, and pelvic bones from two to three endogenously labeled (DsRed) mice and remove soft tissue using sterile lab tissues.

   *Typically, two to three adult male DsRed⁺ mice aged 8-12 weeks are needed to collect ~200,000 KLS⁺ cells in suspension.*
2. Place bones from all mice in a single tube containing PBS on ice.
3. Shake tube to rinse the bones and further remove any soft tissue. Repeat.
4. Crush bones with a mortar and pestle in a small amount of FACS buffer (enough to cover the bones).
   
   Add FACS buffer slowly to prevent foam formation (due to FBS), which will make it difficult to collect the cells.

5. Using a 10-ml pipette, filter the suspension through a 70-μm cell strainer into a 50-ml Falcon tube.
6. Rinse the bone pieces with FACS buffer (2 ml) and continue to crush them and filter the suspension, repeating until no large bone chunks are visible and the crushed bone debris is white.
7. Centrifuge the suspension 10 min at 300 × g, 4°C.
8. Carefully discard the supernatant and gently resuspend the pellet in 5 ml ice-cold RBC lysis buffer per mouse for 30 s.

   Prepare RBC lysis buffer from 10× stock solution and filter through a 0.2-μm strainer prior to use.
9. Stop lysis by adding 20 ml FACS buffer and centrifuging 5 min at 300 × g, 4°C.
10. Discard supernatant and resuspend in 1 ml FACS buffer.

   If there is a sticky membrane pellet after this process, filter again or remove this debris with a pipette tip.
11. Count cells for yield calculations in case of low output.

**Stain and sort cells**
12. Add 50 μl lineage antibody cocktail per mouse and incubate for 20 min on ice.
13. Wash by adding 20 ml FACS buffer and centrifuging 5 min at 300 × g, 4°C.
14. Carefully discard the supernatant, resuspend the pellet in 1 ml FACS buffer, and keep on ice.
15. Take a 50 μl WT sample for a Lin⁺ single-color control (SCC), add 50 μl FACS buffer, and keep on ice.
16. Add 50 μl magnetic bead suspension per mouse to the WT (control) and DsRed (labeled cells of interest) samples. Incubate 15 min on ice.
17. Start AutoMACS instrument and run the Rinse program.
18. Wash each suspension with 20 ml FACS buffer and centrifuge 5 min at 300 × g, 4°C.
19. Discard supernatant and resuspend suspension in 3 ml FACS buffer.
20. Pass through a 70-μm cell strainer into a 15-ml Falcon tube and bring to the AutoMACS along with collection tubes (i.e., 15-ml Falcon tubes) for positive and negative fractions.
21. Run Deplete + QRinse on the AutoMACS.
22. Take the Lin⁻ fractions and centrifuge 5 min at 300 × g, 4°C. Count cells.

   Save the Lin⁺ fraction in case the yield is low and another round of AutoMACS sorting is needed.
Figure 2  Flow cytometry gating scheme. (A,B) Gating strategy for cKit⁺ Sca-1⁺ Lin⁻ (KLS⁺) DAPI⁻ tdTomato⁺ HSPCs. The KLS gate is generously set to sort for progenitor cells. A more restrictive gate set on Sca-1 bright can be set if interested in enriching for more primitive progenitors and stem cells, potentially in combination with CD48, CD150, and CD34 as in Wilson et al. (2008). Nearly all live cells should be Lin⁻, because Lin⁺ cells have been magnetically depleted. Note that, in a raw total bone marrow sample, the Lin⁻ fraction makes up ~12% to 17% and that the Lin gate can be easily set based on the back-gating of KLS cells in the Lin gate. (C) From cell isolation to in vitro flow cytometric analysis logic. Microcarrier 3D co-cultures performed in the absence (conditioned medium and serum only) or presence of a cytokine cocktail consisting of 100 ng/ml SCF, 100 ng/ml FLT3L, 50 ng/ml TPO, 10 μg/ml LDL (Stem Cell Technologies, cat. nos. 78062.1, 78009.1, 78210, and 02698). (D-F) Gating strategy of in vitro expanded HSPCs. Plots are from the cytokine condition. (D) BrightCount beads (beads gate) allow for an absolute cell count. Live cells are gated on PI⁻, of which 99.9% are tdTomato⁻ GFP⁻. The majority of cells recovered are CD45⁺ Ter119⁻ hematopoietic cells. (E) The mature lineage antibody panel reveals a minority Ter119⁺ erythroid cells and B220⁺ B lymphoid cells, and a majority of B220⁻ Gr1⁺ CD11b⁺ myeloid cells. (F) The CD45⁺ Ter119⁺ population is gated to indicate cKit⁺ Lin⁻ cells, which are enriched in CXCR4, as expected for HSPCs. (G) Example of cell numbers generated from 3D niches with and without exogenous cytokines. (H) Maintenance of CXCR4 expression on cKit⁺ progenitors isolated from 3D co-cultures. *P < 0.05, **P < 0.01 by Student’s t test.
23. Discard supernatant and resuspend the Lin\textsuperscript{−} fraction as the purified DsRed sample in 100 µl blocking solution and the WT sample in 400 µl blocking solution. Also add 100 µl blocking solution to Lin\textsuperscript{+} SCC. Incubate for 15 min on ice.

24. Divide the WT sample into four 1.5-ml microcentrifuge tubes on ice (100 µl suspension each). Add 100 µl FACS buffer to each tube. Keep two on ice for SCC samples, one unstained and one DAPI-stained.

25. Stain the DsRed tube with KLS antibody cocktail using the following final concentrations, and stain the WT tubes using each single-color control. Incubate these solutions 1 hr on ice.

- 1:200 streptavidin-PO
- 1:200 cKit PE-Cy7
- 1:100 Sca-1 APC

26. Wash each microcentrifuge tube with 1 ml FACS buffer and centrifuge 5 min at 300 \( \times g \), 4°C.

27. Resuspend the DsRed Lin\textsuperscript{−} samples in 1.5 ml FACS buffer. For optimal sorting, count cells and dilute, if necessary, to a maximum of 1–2 \( \times 10^7 \) cells/ml.

28. Resuspend the DAPI-SCC sample in 200 µl DAPI (1:5000) and the WT SCC samples in 200 µl FACS buffer.

29. Filter all samples through 85-µm mesh strainers into 15-ml Falcon tubes containing 2 ml HCM. Invert tubes prior to sorting to ensure that walls are wet with HCM.

   This increases the viability of sorted droplets that may touch the tube walls.

30. Once the sort is completed, perform a purity control by mixing the sorting tube three times by inversion, then mixing 10 µl of the final solution with 90 µl HCM and reanalyzing the total volume of the suspension in the sorter.

   A total of 500-1000 events should be detected within the KLS gate at >90% purity, where purity is 100 times the number of events in KLS gate divided by the total number of events in the DAPI-FSC/SSC live cell gate. An example of KLS gating during the sort and associated back-gating is shown in Figure 2.

   Sorted KLS\textsuperscript{+} cells can be kept on ice for up to 3 hr until co-seeding with scaffolds and BMSCs (see Basic Protocol 2).

**CO-SEEDING OF HSPCs AND BMSCs ON COLLAGEN-COATED CARBOXYMETHYLCELLULOSE MICROPARTICLES**

Freshly sorted HSPCs and trypsinized OP9 cells are counted and the appropriate ratio of cells is mixed. The CCM scaffolds are dried as a small clump in a 70-µm cell strainer on a SteriCup 500-ml media filtration device, and then carefully placed in an ultra-low-adhesion 6-well plate. The cell suspension is then seeded onto the CCM scaffolds, which are rehydrated through this process. The plate is placed in the incubator for 1 hr, after which additional medium is added and the plate is returned to the incubator.

OP9 BMSCs were donated by the Daley laboratory (McKinney-Freeman, Naveiras, & Daley, 2008), who received them from the Nakano laboratory (Nakano, Kodama, & Honjo, 1994) and lentivirally transduced them for GFP expression as described in Tavakol et al. (2020). Both the parental and GFP\textsuperscript{+} lines were authenticated by the ATCC on June 24, 2021, by short tandem repeat (STR) analysis, indicating an exact match of the ATCC reference OP9 cell line (CRL-2749). OP9 cells are available from the ATCC (CRL-2749), but this source has not been tested for hematopoietic support in our laboratory.
For preparation of stock 13.5 mg/ml collagen-coated CCM scaffolds, see Tavakol et al. (2020). This procedure is described for 370 μl (5 mg) of CCM scaffolds.

**Materials**

- OP9 BMSCs (see protocol introduction)
- BMSC medium (see recipe)
- Phosphate-buffered saline (PBS)
- 0.05% (w/v) trypsin-EDTA (Life Technologies, 25300054)
- Trypan blue
- 13.5 mg/ml collagen-coated CCM scaffolds in PBS (Tavakol et al., 2020), stored at 4°C
- Sorted KLS+ HSPCs (see Basic Protocol 1)
- Co-culture medium (see recipe)

**Prepare OP9 BMSCs**

1. Expand OP9 BMSCs in BMSC medium in T150 flasks at 70%-80% confluency, typically using 1:3 passages every 3-4 days for 1-2 weeks.

   *Subconfluency (plating at 30%-50% confluency and splitting 1:3 or 1:4 when 70%-80% confluency is reached) is critical to maintaining an undifferentiated BMSC culture with predictable hematopoietic support and differentiation capacities. We recommend creating stringent parental/stock and working cell cryobanks such that thawed cell batches are never expanded for more than 4-6 weeks in vitro prior to plating at confluency for a co-culture experiment. After thawing, always plan at least one “recovery” passage prior to co-culture experiments. The experiments described use OP9-GFP cells at passage 12. The same principle can be applied to other marrow stromal cell lines or primary cells from bone marrow.*

2. Identify a flask of 70%-80% confluent BMSCs for co-seeding. Remove BMSC medium and wash once with PBS.

3. Remove PBS and trypsinize with 3 ml of 0.05% trypsin-EDTA for 2-3 min at 37°C, 5% CO₂.

4. Tap the flask firmly in the tissue culture hood and stop trypsinization by adding 4 ml BMSC medium.

5. Gently wash the bottom of the flask with BMSC medium using a 10-ml pipette to remove loosely attached cells. Transfer suspension to a 15-ml Falcon tube.

6. Centrifuge 5 min at 300 × g, 4°C.

7. Carefully discard supernatant, resuspend pellet in 500 μl BMSC medium, and count viable cells using trypan blue and a cell counter.

8. Adjust concentration to 5 × 10⁶ cells/ml.

**Prepare and seed CCM scaffolds**

9. Dry 370 μl collagen-coated CCM scaffolds in a 70-μm cell strainer in the top chamber of a SteriCup filtration system.
The CCM scaffolds can be transferred to a cell strainer to facilitate recovery and avoid excessive loss of material. The cell strainer must touch the SteriCup filter directly for the CCMs to dry sufficiently.

Collagen-coated CCM scaffolds may stick to the pipette wall, leading to a significant loss of scaffolds during transfer. To avoid this, rinse the pipette briefly by pipetting 1 ml PBS up and down before proceeding to the next step.

10. With scaffolds still in the strainer, wash sequentially with 1 ml PBS and then 1 ml medium. Dry scaffolds carefully each time.

The scaffolds should be as dry as possible to achieve a homogenous and optimal cell seeding in step 12.

11. Using a 2-ml serological pipette or a cell scraper, scratch the CCM off the strainer and place in a single well of a 6-well ultra-low-adhesion plate, forming a viscous cluster or “bubble” in the center of the well. Repeat for the desired number of wells.

12. Confirm cell counts for BMSCs and sorted KLS HSPCs. Prepare mixed suspensions at 1:10 and 1:100 ratios of HSPCs to BMSCs. For optimal seeding, prepare mixtures in a total volume of ~100 μl in co-culture medium.

In this case, we used 45,000 HSPCs and 450,000 BMSCs for a 1:10 ratio and 4,500 HSPCs to 450,000 BMSCs for a 1:100 ratio.

The volume used to resuspend the mixed suspension is of particular importance: it should not exceed 50% of the initial volume of the scaffold stock (13.5 mg/ml). On the one hand, excessive dilution of the cell suspension will lead to a significant loss of cells, as excess fluid will leak out of the CCM scaffolds. On the other hand, a cell suspension that is too concentrated leads to a highly heterogenous seeding and may result in a loss of cell viability during incubation in the next step.

13. Add 100 μl mixed cell suspension to each dried CCM scaffold bubble in the 6-well plate and incubate for 1 hr at 37°C, 5% CO₂.

14. Add 3 ml co-culture medium and incubate overnight at 37°C, 5% CO₂.

Cytokines may be added to culture medium to increase hematopoietic progenitor expansion and/or differentiation (for example, see Fig. 2).

MAINTENANCE, REAL-TIME IMAGING, AND ANALYSIS OF CO-SEEDED SCAFFOLDS

As many CCMs are in each well, it is easy to collect a small sample (e.g., a few scaffolds) for serial imaging over time. Hoechst stain, traditionally a live nuclear stain, also stains the scaffolds. Combined with the endogenous cell labels (GFP for BMSCs and DsRed for HSPCs), this makes imaging useful for looking at proliferation of cells over time. End-point imaging with additional markers via fluorescent antibody staining was not performed, though it could be performed if the cell populations are not endogenously labeled prior to the start of the experiment.

Materials

- Silicone glue
- Co-seeded scaffolds (see Basic Protocol 2)
- Co-culture medium (see recipe)
- 0.1 μg/ml Hoechst 33258
- FACS buffer (see recipe)
- 2.5-mm-thick polypropylene sheet
- Laser cutter
- Coverslips
- Incubator at 37°C, 5% CO₂
200-μl pipette tips with tip cut off
Zeiss LSM 700 Inverted Confocal Microscope
Fiji/ImageJ software (NIH)

Fabricate custom observation chambers
1. Cut pieces of 2.5-mm-thick polypropylene sheet with the following dimensions using a laser cutter:
   - 75 × 25 mm for the slide
   - 40 × 14 mm for the central chamber
2. Adhere a coverslip to one side using silicone glue to form the bottom of the chamber (Fig. 3).
   Alternatively, chambers can be 3D printed using the 3D printing map provided (see Supplementary Information) or deep-cavity glass microscope slides can be used.

Maintain and image co-cultures
3. Maintain co-seeded scaffolds in co-culture medium for 2 weeks at 37°C, 5% CO₂.
   Add 3 ml medium at 1 week post-seeding.
   If keeping in culture longer, we recommend half-medium changes to prevent aspiration of any detached HSBCs.
4. On days 1, 4, 7, and 11, take a small volume of suspended CCMs (~100 μl) and transfer to an observation chamber.
   To avoid loss of viability due to handling, use a 200-μl tip with the tip cut off using sterile scissors.
5. Supplement this volume with 100 μl of 1:1000 Hoechst 33258 (if staining the scaffold) or 100 μl FACS buffer. For Hoechst staining, incubate 15-20 min and wash with PBS just before imaging.
   If cells are not endogenously labeled, it would be advantageous to label them (e.g., transient labeling with cell painters) prior to use in in vitro studies.
6. Image co-seeded scaffolds within 1 hr of transferring to the chamber. When ready to image, apply a coverslip on top of the sample, being careful to avoid air bubbles.

Analyze images
7. Using Fiji/ImageJ analysis tools, for each image, separate each fluorescent channel and create a compiled volume-rendered image for each cell type (HSPCs and BMSCs).
   Images used for quantification are composed of a 25-z-stacked, volume-rendered image.
8. Threshold each channel using the Li algorithm (“image > threshold > Li algorithm”). If necessary, convert the image to 8-bit.

   *In order to ensure reproducible thresholding, the rendering should closely overlap with the signal from the original channel. Therefore, a comparison before and after thresholding is often required.*

   *When necessary, the rendering is manually and slightly adapted. To this end, the original channel is selected. In the thresholding window, the Li algorithm is selected and a first thresholding is performed by clicking the “Auto” button. Then, using the “Dark background” option (check the corresponding box), the upper cursor is used to fine-tune the rendering.*

   *Most often, a more realistic rendering may be achieved for low-contrast images by sliding the upper cursor to the left in order to increase the thresholding area. In contrast, highly exposed images usually require sliding the upper cursor to the right to decrease the background signal.*

   *For quantitative analysis (e.g., time-course analysis or comparisons of two or more simultaneous conditions), do not adjust thresholding manually. Find the most representative settings and acquire all conditions using the same settings.*

9. Analyze the fluorescent area corresponding to each channel using “Analyze Particle area” and the following parameters:

   - Size range: 4 to infinity (to remove nonspecific fluorescence background)
   - Circularty: 0-1 (default)
   - Display result: checked
   - Exclude on edges checked

   *If necessary, use the option “Includes holes” for scaffold area quantification to increase the accuracy of the measurements. Scaffold areas can also be quantified manually using the “Freehand selections” tool.*

10. Calculate the fluorescent “total area” for each label (i.e., GFP and DsRed for HSPCs and BMSCs) as well as the “total scaffold area” for each image by summing all the “Area” values in Excel.

11. Calculate the percentage of the total particle fluorescent area for each fluorescent label (HSPCs or BMSCs):

    \[
    \% \text{ total particle fluorescent area} = \frac{(\text{HSPC or BMSC “total area”})}{(\text{“total scaffold area”})} \times 100
    \]

12. Calculate this value for each label and create a ratio for HSPC to BMSC relative fluorescent areas for each image over time.

**END-POINT ANALYSIS OF CO-SEEDED SCAFFOLDS USING FLOW CYTOMETRY AND CFU ASSAYS**

To assess hematopoietic expansion, cells are isolated from the co-culture and prepared for flow cytometric analysis as a single-cell suspension. In parallel, to assess hematopoietic progenitor function, cells are seeded for hematopoietic colony-forming unit (CFU) assays in semi-solid methylcellulose-based medium. After 1 week, CFUs are counted and categorized according to size and complexity. Colony assays to specifically assay long-term hematopoietic stem cell activity (LT-HSC) are described elsewhere (Kerenyi, 2014).

The protocol below uses MethoCult methylcellulose-based medium from Stemcell Technologies and their STEMvision colony-counting instrument. Alternatively, counting can be performed using a brightfield microscope equipped with 4× and 10× objectives and manual scoring according to manufacturer’s instructions.
Materials

- Co-seeded scaffolds (see Basic Protocol 2)
- Serum-free medium: HCM (see recipe) without FBS
- 0.04% (w/v) collagenase I (Thermo Fisher Scientific, 17100-017) in Hanks’ balanced salt solution (HBSS)
- Co-culture medium (see recipe)
- FACS buffer (see recipe)
- Blocking solution (see recipe)
- Lineage cocktail from lineage depletion kit (BD Pharmingen, 558451)
- Flow cytometry antibody cocktail (see Table 1)
- BrightCount beads (1000 beads/μl, Invitrogen, C36950)
- DAPI (Applichem, A4099.005)
- MethoCult medium (Stemcell Technologies, M3434)
- Penicillin/Streptavidin (Thermo Fisher Scientific, 15140122)

- 2- or 5-ml serological pipettes
- 70-μm cell strainer (Corning, CLS431751)
- 50-ml Falcon tubes
- 1.5-ml microcentrifuge tubes
- 85-μm mesh filter
- Flow cytometer (e.g., BD Biosciences LSR II SORP or Beckman Coulter CytoFLEX LX)
- 16-G blunt-end needles and 3-cc syringes (Stemcell Technologies, 28110 and 28240)
- 6-well meniscus-free plates (e.g., Stemcell Technologies SmartDish, 27371)
- Humidifying chamber
- Incubator at 37°C, 5% CO₂
- STEMvision colony counter (Stemcell Technologies)

Digest scaffolds

1. Carefully collect most of the medium from the co-culture wells and set aside on ice as the non-adherent fraction.
2. Gently wash CCM particles twice with serum-free medium.
3. Remove excess medium and add 2 ml of 0.04% collagenase I per well of the 6-well plate. Incubate 25 min at 37°C, 5% CO₂.
4. Inactivate collagenase by adding 4 ml co-culture medium using a 2- or 5-ml serological pipette and pipetting up and down for 1-2 min.
5. Pass through a 70-μm cell strainer into a 50-ml Falcon to isolate cells from particles.
6. Repeat steps 4-5 twice more, then discard strainer and residual CCM particles.
7. Pipette sample up and down to obtain a single-cell suspension (the collagenase-digested adherent fraction).
8. Centrifuge non-adherent and adherent fractions for 10 min at 300 × g, 4°C.
9. Resuspend pellets for a total of 500 μl cell suspension in FACS buffer.
10. Split each fraction to two microcentrifuge tubes for CFU and flow cytometry analysis.

CFU and flow cytometry allocations should be optimized for each experimental setup. It is safe to split the samples ~1:10 for CFU and flow cytometry, respectively. Suspension
Table 1  Example Flow Cytometry Panels for Bulk Hematopoietic Progenitor Quantification (CD45+ cKit+ Sca-1+ Lin−)∗

| Panel          | Antibody          | Clone   | Isotype     | Conjugation | Dilution | Supplier, cat. no.  |
|----------------|-------------------|---------|-------------|-------------|----------|---------------------|
| HSPC sorting   | cKit (CD117)      | 2B8     | Rat IgG2b   | PE-Cy7      | 1:100    | BioLegend, 105814   |
|                | Sca-1 (Ly-6A/E)   | E13-161.7 | APC       | 1:50       | BioLegend, 122508 |
|                | Lineage cocktail  |         | Biotin     | 1:20       | BD Biosciences, 51-9000794 |
|                | Streptavidin      |         | Pacific Orange | 1:100    | Life Technologies, S32365 |
|                | DAPI              |         | UV         | 1:5000     | Axon Lab, A4099.0005 |
| HSPC panel     | cKit (CD117)      | 2B8     | Rat IgG2b   | PE-Cy7      | 1:100    | BioLegend, 105814   |
|                | Sca-1 (Ly-6A/E)   | E13-161.7 | APC       | 1:50       | BioLegend, 122508 |
|                | Lineage cocktail  |         | Biotin     | 1:20       | BD Biosciences, 51-9000794 |
|                | Streptavidin      |         | Pacific Orange | 1:100    | Life Technologies, S32365 |
|                | CD184 (CXCR4)     | 2B11    | PerCP-eFluor710 | 1:25  | Invitrogen, 46-9991-82 |
| PI panel       | pan-CD45          | 30-F11  | Rat IgG2b   | Alexa Fluor 700 | 1:50  | BioLegend, 1013128 |
|                | Ter119            | TER-119 | Rat     | BUV395      | 1:200    | BD Biosciences, 563827 |
|                | B220 (CD45R)      | RA3-6B2 | PE-Cy5     | 1:25       | BioLegend, 103210 |
|                | Gr1 (Ly-6G/Ly-6C) | RB6-8C5 | APC        | 1:250      | BioLegend, 108412 |
|                | CD11b             | M1/70   | APCeFluor780 | 1:500  | Invitrogen, 47-0112-82 |
|                | CD11b             | M1/70   | UV         | 1:5000     |          |

∗Exploration of murine lineage commitment can be determined with alternative combinations of surface markers including CD45 pan-hematopoietic marker for better separation from stromal components (e.g., granulocytic: Gr1; monocytic: CD11b; erythroid: Ter119/CD71; lymphoid: B220/CD3).

(nonadherent) fractions can contain a highly variable number of HSPCs depending of time of culture, so a lower allocation or a set of dilutions may be appropriate.

**Stain for flow cytometry**

11. Centrifuge cytometry aliquots (50 μl at a 1:10 allocation) at 300 × g and resuspend pellets (∼20 μl) with 75 μl blocking solution. Transfer to fresh microcentrifuge tubes and incubate 10 min at room temperature.

12. Add 5 μl lineage cocktail and incubate 20 min on ice.

13. Wash with 1 ml FACS buffer and pass through a 85-μm mesh filter.

14. Centrifuge 10 min at 300 × g, 4°C, and aspirate supernatant.

15. Incubate with the antibody cocktail in Table 1 for 45 min on ice. To count cell numbers, include 10 μl BrightCount beads.

16. Add 200 μl DAPI and incubate ∼10 min before acquiring using a flow cytometer.

An example of analysis and gating is shown in Figure 2.

**Perform CFU assay**

17. Thaw a 100-ml bottle of MethoCult medium the day prior to use and add 1% penicillin/streptomycin directly to the bottle. Shake vigorously, let sit to allow bubbles to settle, and dispense 2.3-ml aliquots in 15-m1 Falcon tubes.
18. To each MethoCult aliquot, add the appropriate volume of cell suspension to give ~100 KLS± cells per 6-well plate.

Plating conditions should be optimized for an ideal output of 60 colonies/plate. The assay is not linear with more than 100 colonies/plate, and discrimination of separate colonies becomes impossible with more than ~150 colonies/plate (see manufacturer’s recommendations at https://cdn.stemcell.com/media/files/manual/MA28405-Mouse_Colony_Forming_Unit_Assays_Using_MethoCult.pdf).

We back-calculated the actual number of seeded cells based on fractions of culture that were left for CFU analysis. It is highly recommended to optimize CFU cell density prior to experimentation. Alternatively, serial 5-10× dilutions can be performed for CFU assays when the concentration of progenitors is unknown.

19. Mix cells and MethoCult using a 16-G blunt-end needle and let sit for 5 min to allow bubbles to rise to the surface.

20. Slowly collect cell/methylcellulose suspension from below the bubbles and carefully add 1.1 ml to each well of a meniscus-free 6-well plate.

21. Place each plate in a humidifying chamber and leave in an incubator for 7 and 14 days without manipulation.

22. Image colonies using the STEMvision instrument (or score live using a brightfield microscope) according to manufacturer’s protocols for imaging and analysis.

TRANSPLANTATION OF SCAFFOLDS BY SUBCUTANEOUS INJECTION

To perform in vivo experiments, co-culture scaffolds are injected subcutaneously in anesthetized mice. Prior to injection, the scaffolds must be gently packed (concentrated). To achieve this, a controlled-pressure, cell-protecting drying device was developed (Fig. 4). The scaffold suspension is loaded into the column of the drying device and controlled capillary action is used to gently pack the scaffolds. The steps below describe drying of the scaffolds and injection into mice. For preparation of the drying device, see the Support Protocol that follows. Video 1 demonstrates the process of loading scaffolds on the column for delicate drying, loading the syringe with scaffolds in the transfer tip, and performing the subcutaneous injection. In our experiments, injected scaffolds were maintained for up to 12 weeks in vivo.

NSG mice are used for this protocol because OP9 BMSCs are derived from a mixed background (Nakano et al., 1994). All experimental procedures were approved by the Animal Care and Use Committee of the Canton of Vaud (ACUC, Vaud, Switzerland, license VD3629). All animals were housed in the EPFL or UNIL-CLE Conventional Animal Facilities (Epalinges, Switzerland) and were kept under a controlled 12-hr light/dark cycle at constant room temperature 22° ± 2°C.

Materials

- 8- to 16-week-old NOD SCID-γ immunodeficient female mice (NSG 5557, Jackson Laboratories)
- Isoflurane USP-PPC (Animalcare) and compatible gas-air mixer
- Betadine (Mundipharma Medical)
- Carprofen (Rimadyl, Pfizer)
- Ophthalmic liquid gel (Viscotears, Alcon)
- Co-cultured CCM scaffolds (see Basic Protocols 2-3)
- Phosphate-buffered saline (PBS)
Antibiotic drinking solution (see recipe)
Animal scale
Heating pad
Hair shaver
15-ml Falcon tube
Custom drying device (see Support Protocol; Fig. 4)
1-ml syringe
20-G flexible catheter (BD Biosciences, 381703)
Tools for euthanizing mice and isolating implants

Additional reagents and equipment for paraffin embedding, staining, and other analyses

Prepare for transplantation
1. Autoclave all supplies for implantation, including the drying device.
2. Weigh mouse for post-surgery monitoring, then induce anesthesia with 4.0% isoflurane.
3. Place mouse on a warm heating pad and maintain anesthesia at 1.5%-2% isoflurane.
4. Shave the back of the mouse and apply Betadine at the injection sites to keep them as sterile as possible.

   Two injections should be made in each mouse to provide a scaffold injection and a contralateral control.

5. Administer pre-operative carprofen (5 mg/ml) as an analgesic.
6. Apply ophthalmic liquid solution to both eyes.

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**Figure 4** Drying device to protect cells and scaffolds during implantation. (A) Overview of drying scheme from cell suspension (2) to collection of particles (3) to insertion on syringe (4). (B) Drying device using repurposed 1000-μl pipette tip box. (C) Metrics for determining pressure drop for cells and scaffolds. By equilibrating the CCM and medium to a predefined hydrostatic pressure (ΔP, typically on the order of 0.2 kPa or ~2 cm water column), CCM scaffolds can be dehydrated to help pack the scaffolds prior to injection. For more information, see Support Protocol. (D) Loading column and transfer tip dimensions. (E) Scaffold polymer concentration increases according to the negative pressure applied by the device. Figure adapted in part from Tavakol et al. (2020) with permission.
**Pack scaffolds on drying device (see Video 1)**

7. Prepare scaffolds in a 15-ml conical tube from *in vitro* culture. Typically, for each condition, a total of ~3-6 ml seeded scaffolds and associated medium are loaded into the column for drying. Although the video shows scaffolds being applied directly from the 6-well plate, it is best to transfer them to a tube before transporting them to the animal facility.

   If two people are performing surgery, this procedure can be done while the mouse is being anesthetized.

8. Add PBS to the capillary conductors of the drying device to moisturize the device prior to use.

9. Transfer scaffolds (in solution) to the loading column of the drying device and allow to settle in the transfer tip. Collect ~50 μl seeded scaffolds per injection.

   *It may take 1-2 min for the scaffolds to settle. During this process, the column should be stabilized as much as possible to prevent scaffold loss during preparation.*

**Perform subcutaneous injection (see Video 1)**

10. Load a sterile 1-ml syringe with 0.1 ml coated scaffold without cells and follow with 0.1 ml air to help injection fluidity.

11. Insert a 20-G flexible catheter (with needle) into the mouse’s skin so the needle is ~2 cm deep (subcutaneous), then remove the needle, keeping the catheter on place.

   *Catheters that are not equipped with needles can also be used for this procedure. To do so, first make a hole in the mouse’s skin using an 18-G needle and insert the needle ~2 cm deep. Then insert the catheter and proceed to the next step.*

12. Connect the scaffold-filled transfer tip (step 9) to the syringe (step 10).

13. Slowly push the scaffolds into the subcutaneous implantation site. After implantation, slowly remove the catheter.
Figure 5  Subcutaneous injection of co-cultured CCM scaffolds. (A-C) Gross images of implant site on day of implant and 12 weeks post-injection, showing outside the skin (B) and underneath the skin with visible vasculature integration (C). (D-K) Hematoxylin and eosin (H&E) staining and immunohistochemistry (IHC) of scaffolds at 1.5 and 12 weeks post-implantation with visible maintenance of tdTomato/DsRed\(^+\) hematopoietic cells (D,F,H) and GFP\(^+\) stromal cells (J), as well as integration of CD31\(^+\) endothelial cells at both time points in the OP9/KLS\(^+\) co-culture groups (E,G,I). Dotted line indicates boundary between implanted scaffold and subcutaneous tissue.

This step must be performed gently to make sure all particles remain inside the subcutaneous pocket and do not leave the injection site. No sutures are required because CCM particles covalently interlock during the high-density injection procedure. An organized, malleable CCM “bubble” should become apparent immediately after injection (Fig. 3E).

The transfer tip should not be reused for a second injection.

14. Return animal to its cage as it starts to wake up.

The entire injection procedure takes \(\sim 20\) min for two injections on the same mouse.

15. Provide animal with antibiotic drinking solution for the duration of the study.

Sacrifice and sample harvest

16. At the end point (in our case, 12 weeks), euthanize mouse and shave its back to better visualize the implantation site (Fig. 5A-C).

17. Isolate implants (which should be visible by eye) and surrounding tissue. Fix overnight in 4% paraformaldehyde.

To minimize damage to the implant, we recommend isolating and fixing the implant with surrounding skin and only then, if desired, carefully dissecting the fixed implant prior to embedding.

18. Perform paraffin embedding or other tissue processing as desired, including any immunohistological staining or analysis (see examples in Fig. 5D-K).
PREPARATION OF CUSTOM SCAFFOLD DRYING DEVICE

Instructions are provided here for constructing the device from common laboratory materials. The assembled device can be seen in Figure 4 and Video 1. Alternatively, the device can be assembled from 3D-printed parts made using the 3D printing maps provided (see Supplementary Information).

All plastic consumables used should be made from polypropylene, as this allows assembly using a standard crafting hot glue gun and soldering iron. Additionally, it makes the device and columns suitable for sterilization by autoclaving.

Materials
- 15- and 50-ml Falcon tubes
- Metal saw or sharp knife
- 1000-μl pipette tip (Axygen, 7.9 cm)
- 20-μl barrier pipette tip (MultiGuard, Sorenson BioScience)
- 5-ml syringe (BD Biosciences)
- 1000-μl pipette tip box (Axygen Scientific)
- Soldering iron
- Hot glue gun (traditionally with ethylene vinyl acetate polymer glue)
- Commercial or craft felt material, autoclavable (e.g., 82% viscose, 18% polypropylene, Coop, Switzerland)

Fabricate loading column
1. Cut a 15-ml Falcon tube in three parts by cutting at the 3-ml and 12-ml lines using a saw or a sharp knife. Discard the middle section.
2. Assemble the bottom and top pieces by melting the rim of both pieces with a hot glue gun and rapidly pressing them together.

   *The two pieces should be assembled tightly to prevent any leaks. The tightness can be tested by filling the column with water. If any air bubbles appear, discard the column and begin again.*
3. Cut off the very bottom conical part of the tube so that the diameter of the hole is the same as the top of a 1000-μl pipette tip (typically, 1.9 cm o.d.).
4. Attach a 1000-μl tip to the column by melting the rim of both pieces with the hot glue gun and pressing together tightly.

   *Tightness can be checked as above.*
5. Cut off the bottom of the tip to remove 1.2-1.5 cm (0.4 cm o.d.).

   *The average total height of the loading column is 11.5 cm.*

Fabricate transfer tip
6. Cut the top section of a 20-μl barrier pipette tip at 1.2 cm from the top (larger opening).
7. Cut off the bottom section (the narrowest part) at 2.3 cm from the tip, below the filter. Attach the two pieces using the hot glue gun to make the transfer tip.

Fabricate column holder
8. Cut a 5-ml syringe at 2 cm from the top and keep the top section (the part with handles).
9. Attach one handle of the syringe to the top part of a 1000-μl tip.
10. Repeat steps 8 and 9 up to four times on the same tip to allow multiple dried scaffolds to be prepared at once.
A holder can also be made from the top shelf of an empty 1000-μl tip box by simply assembling four 1000-μl tips at each corner of the shelf. Each hole can then be used as a holder for the columns.

Adapt the height of the holder by stacking several 1000-μl tips together.

**Fabricate drying box**

11. Cut a 50-ml Falcon tube at 6 cm from the tip and keep the bottom piece to serve as the waste reservoir (recipient; Fig. 4).

12. In the top shelf of an empty 1000-μl pipette tip box, make a hole with same diameter as the 50-ml Falcon tube.

13. Insert the recipient in the hole and attach by melting with a soldering iron.

14. Make a hole in the Falcon tube 2 cm from the top (Fig. 4B,C).

   *This will define the hydrostatic pressure at 200 Pa (1 cmH₂O = 98.07 Pa), which is suitable for cell viability and gives adequate consistency of CCM scaffolds for injection.*

15. Cut a cleaning cloth or felt material into an 8 × 7–cm rectangle with a protruding tail of at least 6 cm on one side to serve as the capillary conductor (Fig. 4A,C).

16. Assemble pieces as shown in Figure 4 and test for leakage.

17. Autoclave and use under the hood as described (see Basic Protocol 5).

**REAGENTS AND SOLUTIONS**

**Antibiotic drinking solution**

- 225 ml drinking water
- 25 ml co-amoxiclav (final 10% v/v)
- 300 μl of 10% Baytril
- Two bags (250 mg each) Dafalgan/paracetamol

Prepare fresh each week in a 250-ml red/photoprotective water bottles

**Blocking solution**

- FACS buffer (see recipe)
- 5 μg/ml hIgG (I4506, Sigma-Aldrich)

Prepare fresh and store on ice

**BMSC medium**

- Alpha-minimum essential medium (a-MEM) + Glutamax (Thermo Fisher Scientific, 32561)
- 10% (v/v) fetal bovine serum (FBS, Gibco, 10270-106)
- 1% penicillin/streptavidin (Thermo Fisher Scientific, 15140122)

Store up to 1 month at 4°C

**Co-culture medium**

First, prepare conditioned HCM by culturing confluent BMSCs in HCM (see recipe) for 48 hr. Collect conditioned medium, filter with a 0.2-μm filter, and store up to 2 months at −20°C.

Prepare co-culture medium by mixing conditioned medium with an equal volume of fresh HCM. Store up to 1 month at 4°C protected from light.

**FACS buffer**

- Phosphate-buffered saline (PBS)
- 1 mM EDTA (Thermo Fisher Scientific, 15575020)
2% (v/v) fetal bovine serum  
Store up to 1 month at 4°C

**Hematopoietic culture medium (HCM)**

IMDM + Glutamax (25 mM HEPES)  
10% (v/v) fetal bovine serum (FBS, Gibco, 10270-106)  
1% penicillin/streptomycin (Thermo Fisher Scientific, 15140122)  
Store up to 1 month at 4°C

**COMMENTARY**

**Background Information**

Since the first *in vitro* description of mixed bone marrow stromal cultures with hematopoietic output in 2D conditions (Dexter, Wright, Krizsa, & Lajtha, 1977), more refined methods have demonstrated the capacity for *limited* hematopoietic expansion *in vitro* in highly controlled conditions, often stroma-free, and with potential use for HSPC expansion in clinical transplantation (Bujko, Kucia, Ratajczak, & Ratajczak, 2019; Derakhshani et al., 2019). These methods, however, do not allow the study of HSPC interactions with the niche that regulates their behavior in homeostatic conditions. This microenvironment-mediated regulation is thought to be critical in early stages of hematological neoplastic transformation, such as in myelodysplastic syndromes (reviewed in Le, Andreeff, & Battula, 2018). Moreover, it is thought that microenvironment-directed pharmacological modulation could constitute a novel approach for treatment of hematological diseases (Behrmann, Wellbrock, & Fiedler, 2020). For these reasons, the need for “engineering artificial and functional hematopoietic stem cell niches” has been identified as one of the top priorities for hematological research by the 2018 roadmap of the American Society of Hematology (Mullighan, 2018). Methods for heterotopic implantation of bone marrow ossicles to model hematopoietic niches and associated hematological malignancies have been extensively reviewed elsewhere in recent years (Abarrategi et al., 2018; Dupard, Grigoryan, Farhat, Coutu, & Bourgine, 2020), and novel approaches are rapidly emerging (Shah et al., 2019; Tavakol et al., 2020). Here, we present in detail the method for a scalable approach to maintain a minimal hematopoietic niche *in vitro* whose intact cellular interactions can be transferred *in vivo* for creation of highly vascularized subcutaneous nodules with persistence of *in situ* hematopoiesis in the absence of ossification. Advantages of our method as compared to current standards are ease of use, scalability, remarkable vascularization upon *in vivo* transfer, and the potential for clinical-grade manufacturing of the proposed scaffold.

**Critical Parameters and Troubleshooting**

Critical parameters that influence this protocol are the source of HSPCs and BMSCs, as well as the choice of recipients for *in vivo* transplantation. In particular, the minimalistic culture protocol in absence of exogenous cytokines presented here depends on the utilization of a highly supportive marrow stromal line. Complementation with exogenous cytokines is definitely possible, but requires optimization depending on the purpose of the experimental approach. Readouts should be also adapted to the particular application. In our example, progenitor readouts were used (CFU assays and CD45+ cKit+ Lin– quantification by flow cytometry), as our interest was in short-term hematopoietic outputs. Readouts of stem cell function will require sequential CFU assays *in vitro* (e.g., serial CFU-IC or LT-HSC CFU assays) and long-term multi-lineage repopulation assays *in vivo*. Additionally, lineage-specific panels and appropriate histological readouts need to be tailored to the specific experimental question being addressed.

For troubleshooting guidelines, see Table 2.

**Understanding Results**

In the protocols described here, the culture of HSPCs can be assessed through cocultures with marrow stromal cells in 3D, mimicking some of the basic interactions between these cells in mammalian BM. To that end, these culture methodologies can be applied towards facile transplantation of cocultured scaffolds *in vivo* for the purposes of BM regeneration post-radiation. Here, we outline the use of murine cell sources for 3D culture, including primary bone marrow–derived KLS+ HSPCs and the OP9 BMSC cell line.
In these protocols, we outline the appropriate metrics for culturing HSPCs in basal cytokine-free conditions. In these methods, the only exogenous cytokines provided to the co-culture system were from OP9 BMSC conditioned medium, which was only employed to increase the stromal cell supportive mechanisms of BMSCs towards the KLS+ cells during in vitro culture. Therefore, the results described here are only a subset of the envisioned results that may be generated from 3D co-culture systems. By using exogenous cytokines, HSPCs in co-culture can be directed towards myeloid and lymphoid differentiation lineages. For example, adding macrophage colony stimulating factor (M-CSF) would push cells into a myeloid/monocytic lineage in vitro. To that end, we show brief characterization of how low levels of cytokines may increase the total cell yield and progenitor/downstream cell population types (Fig. 2G). If using human hematopoietic cells, the context of a starting CD34+ progenitor population would alter the results described here; however, we believe these methodologies can be applied towards human CD34+ cells with the use of proper (equivalent) controls as compared to the KLS+ cells derived in this work. Many in vitro 3D co-culture models using human cord blood–derived CD34+ cells use exogenous cytokines such as FMS-like tyrosine kinase 3 ligand (FLT-3L), thrombopoietin (TPO), and stem cell factor (SCF) to increase the yield of HSPCs in culture. Our results demonstrate that a large percentage of progenitors were also found in suspension, against the hypothesis that the larger proportion of immature cells are attached to the BMSCs and scaffold. This finding may vary from group to group, as different 3D systems and in vitro models may affect HSPC attachment to the surrounding stroma. In future work, additional stromal cell types (adipocytes, endothelial cells, etc.) may be incorporated into the model, which will further influence the ability of such in vitro systems to support hematopoiesis, though advancements in effectively vascularizing tissues and in vitro models remain a challenge in the field (Fleischer, Tavakol, & Vunjak-Novakovic, 2020).

In our in vitro culture techniques, we provide appropriate characterization of structural and functional metrics to assess the efficiency of co-culture with BMSCs and HSPCs. Through serial imaging, we are able to understand the relative location of HSPCs to BMSCs using endogenously labeled cells, which is important in mimicking the role of BMSCs in providing the appropriate stromal support needed to maintain hematopoiesis. To functionally characterize the co-culture
system, we looked at fold expansion and percentage of immature to mature hematopoietic cells post-culture using flow cytometry. Similarly, flow cytometric analysis of 2-week 3D co-cultures revealed the presence of the main hematopoietic lineages with CD45^+ as a pan-hematopoietic marker (Fig. 2D-G).

To corroborate findings from flow cytometry, CFU assays provide an indication of the number of colonies that can be formed from a single HSPC. Further identification of colonies and their categorization can give insight into their multipotent potential, including CFU-GEMM (granulocyte, erythroid, monocyte, megakaryocyte), CFU-GM (granulocyte, monocyte), and BFU-E (erythroid) colonies.

For transplantation applications, our methodologies demonstrate ease of use and feasibility of collecting, drying, and injecting 3D scaffolds in vivo. Subcutaneous transplantation applications for hematopoietic transplant are of significant interest to clinicians, as an easier route of stem cell delivery may help optimize delivery of gene-edited HSCs or prevent adverse effects in HSC transplant patients. Scaffolds seeded with OP9 cells show little to no vascularization versus the readily vascularized (CD31^+ staining) of OP9/KLS co-seeded implants, which also show a significant presence of tdTomato^+ donor-derived cells as previously reported (Tavakol et al., 2020). Twelve weeks of implantation was originally chosen as the end point due to this classical time point for ossicle formation, but vascularization associated to hematopoietic cellularity can already be observed at 1.5 weeks post-implantation (Fig. 5). Our techniques can help give insight into both injection of 3D scaffolds and recovery of transplanted tissue after many months in vivo. We hope this work can inform future researchers to design multi-faceted tissue-engineered tools to model hematopoiesis in vitro (Basic Protocols 1-4) and regenerate bone marrow in vivo (Basic Protocol 5).

**Time Considerations**

**Isolation of HSPCs**

Approximately 1-2 hr are needed to euthanize animals, collect bones, and crush them for marrow isolation. Staining for MACS sorting requires ~1 hr, and MACS sorting requires ~15-30 min. Final staining and FACS requires another ~2-3 hr.

**Co-seeding HSPCs and BMSCs on CCMs**

Expansion of BMSCs requires over 1-2 weeks. Once they are ready, ~30 min are required to prepare BMSCs in suspension, ~30 min are required to prepare scaffolds for cell seeding, and ~1.5 hr are needed to incubate the cell suspensions and scaffolds. After additional medium is added, co-cultures are maintained for 2 weeks prior to evaluation and use for transplantation.

**Analysis of co-seeded scaffolds by flow cytometry and CFU assays**

To prepare the adherent fraction, ~1.5 hr is required for collagenase digestion, collection of the cell suspension, and washing. This may vary depending on number of samples. For flow cytometry, ~3 hr are required for blocking, staining, and running the samples. For CFU assays, ~2 hr are required to calculate the optimal cell seeding density, aliquot cell suspensions, and plate aliquots on 6-well plates. Two weeks after plating, manual or automatic scoring and then visual verification of colony assignation requires ~15 min per well of a 6-well plate. Serial imaging will require ~1-3 hours each day.

**Implantation**

It takes ~2 hrs to design the drying device. All materials should be autoclaved ~1 day prior to the experiment. On the day of implantation, ~15-20 min is needed to prepare each mouse, ~5-10 min are needed for scaffold drying and syringe preparation, and ~10-15 min are needed per mouse for injection. After the desired time in vivo (in our case, 12 weeks), ~15-20 min are needed per mouse for euthanasia and harvesting scaffolds.

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**Author Contributions**

D. N. Tavakol, F. Bonini, J. Tratwal, T. Braschler, and O. Naveiras contributed to the
design of the study. All authors contributed to the writing and proofing of the manuscript. D. N. Tavakol, F. Bonini, J. Tratwal, and J. Brefie-Guth performed and optimized the in vitro study. J. Brefie-Guth and F. Bonini prepared the biomaterial and optimized the collagen modification. F. Bonini designed the 3D maps. F. Bonini and T. Braschler designed the first version of the dehydration device, and D. N. Tavakol, J. Tratwal, M. Genta, J. Brefie-Guth helped with device optimization. J. Tratwal performed FACS and analyzed the results in Figure 2 together with O. Naveiras. J. Tratwal, D.N. Tavakol, M. Genta, and J. Brefie-Guth carried out the in vivo studies. J. Tratwal and F. Bonini analyzed the in vivo data.

Conflict of Interest
The authors declare no conflict of interest.

Data Availability Statement
Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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