Article Title Cryogel-based injectable 3D microcarrier co-culture for maintenance of intact hematopoietic niches

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ABSTRACT:

Although hematopoietic stem cell (HSC) transplantation can restore functional hematopoiesis upon immune or chemotherapy-induced bone marrow (BM) failure, complications often arise during recovery leading to up to 25% transplant-related mortality in treated patients. In hematopoietic homeostasis and regeneration, HSCs derive the entirety of cellular blood components within the bone marrow, but one of the challenges in studying hematopoiesis remains to successfully mimic the relationship between the stroma and hematopoietic stem and progenitor cells (HSPCs).

This study and described protocols propose an advantageous method to culture and assess stromal hematopoietic support in 3D, representing a simplified *in vitro* model of the bone marrow niche which can be transplanted *in vivo* as an injectable. Using OP9 BM-derived stromal cells (BMSCs) and cKit+, Sca-1+, and Lin- (KLS+) HSPCs, we co-cultured these components on collagen-coated carboxymethylcellulose scaffolds for two weeks in the absence of cytokines, and established a methodology for *in vivo* subcutaneous transplantation. With this model we were able to detect early signs of extramedullary hematopoiesis. Benefits to this work can be useful in 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.

Basic Protocol 1: **Isolating HSPCs from mice**
Basic Protocol 2: **Co-seeding HSPCs with 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 colony forming unit assays**
Basic Protocol 5: **Scaffold drying device for transplantation preparation**
Basic Protocol 6: **Scaffold injection and subcutaneous transplantation**

KEYWORDS:
Hematopoietic, scaffold, biomaterial, stem cell niche, cryogel, injectable, tissue engineering, bone marrow

INTRODUCTION:

Bone marrow (BM) failure, whether secondary to chemotherapy in cancer patients or secondary 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 hematopoietic stem cell niches that allow expansion of repopulating hematopoietic stem cells is of intense interest for HSC regenerative therapies, as outlined by the American Society of Hematology (ASH) in the most recent research agenda (Bianco, Riminucci, Gronthos, & Robey, 2001; Morrison & Scadden, 2014; Mullighan, 2018).

HSCs are the primary population of cells that derive the entirety of cellular blood components through hematopoiesis. They reside in the bone marrow and give rise to myeloid, erythrocytic, megakaryocytic and lymphoid progenitors. *In vivo*, the hematopoietic stem cell 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. Previous models of the BM, including organ-on-a-chip microfluidic platforms and ossicle-based scaffolds, have demonstrated multi-cellular 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; Torisawa 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 OP9 BM-derived stromal cells (BMSCs) *in vitro* (**Figure 1**). Using collagen-coated carboxymethylcellulose cryogel scaffolds (CCMs) with the capacity to covalently interlock, we co-cultured HSPCs and BMSCs for up to two weeks in CCM micro-scaffolds. Then we performed *in vivo* subcutaneous injection through a process that triggers particle interlocking. Through this work, we have developed methodologies to support hematopoietic cells in 3D cultures without exogenous cytokines, image 3D co-seeded scaffolds over time, and inject these cellularized scaffolds subcutaneously. We show maintenance of hematopoiesis after 12 day *in vitro* co-culture, and robust cellularization of implants with evidence of *in situ* hematopoiesis over 12 weeks post-implantation.

In this *Current Protocols* paper, we expand upon our recent work (Tavakol et al., 2019). We

discuss in six basic protocols the (1) isolation of murine HSPCs, (2) seeding of HSPCs and BMSCs *in vitro*, (3) the maintenance and imaging of these co-cultured scaffolds, (4) end-point analysis of our 3D culture systems, (5) preparation of cellularized 3D scaffolds to fit injectable parameters, and (6) *in vivo* transplantation subcutaneously in immunodeficient mice.

STRATEGIC PLANNING

This work is based upon our previously-published paper by Tavakol et al., in *Biomaterials* (2019). Many of the methodologies and figures described here are an elaboration of this work.

In this protocol paper, 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 lab and have been carefully maintained at sub-confluency in our laboratory since 2013. DsRed HSPCs were harvested from endogenously-labeled C57Bl/6 mice. The methodologies described here are not limited to these cell types – any form of BMSCs and HSPCs may be tested in the forthcoming protocols, as the fluorescent labeling was only essential in characterization and imaging of the scaffolds over time. There is potential to use these methodologies to study human hematopoiesis as well.

The fabrication of the collagen-coated CCM scaffolds is described by Tavakol et al(Tavakol et al., 2019). The underlying collagen adsorption and immobilization to carboxymethylcellulose scaffolds has been studied in depth (https://onlinelibrary.wiley.com/doi/abs/10.1002/admt.201700340), whereas the rheological and *in-vivo* tissue reconstruction properties are part of manuscript available as preprint at present (https://www.biorxiv.org/content/10.1101/2020.01.30.926931v1).

BASIC PROTOCOL 1: ISOLATING HEMATOPOIETIC STEM AND PROGENITORS FROM MICE

Introductory paragraph:

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 are lysed and the cells can then be stained with antibodies for HSPC isolation based on fluorescent-activated cell sorting (FACS). The cells are then sorted into medium based on surface-marker expression and stored on ice. Typically, one mouse will yield 60,000 HSPCs.

Materials:

- Endogenously labeled mouse (in our case, B6.Cg-Tg(CAG-DsRed*MST)Nagy/J maintained in-house since 2013, referred to as "DsRed" , available at www.jax.org, strain number 006051)
- Wild type control mouse (for negative control staining)
- FACS buffer (see recipe in Reagents and Solutions)
- Mortar and pestle
- 70 µm cell strainer (Corning)
- Red blood cell lysis buffer (420301, BioLegend)
- Lineage depletion kit (558451, BD Pharmingen)
- AutoMACS (Miltenyi Biotec)
  If no availability of AutoMACS, depletion can be performed with manual midi LD columns (Miltenyi Biotec) according to manufacturer's recommendations.
- Blocking solution (hIgG, 5 µg/mL FACS buffer) (I4506-10 MG, Sigma Aldrich)
- KLS sorting antibody cocktail (final dilution noted in parenthesis)

    - Streptavidin-PO (1/200)
    - c-Kit PE-Cy7 (1/200)
    - Sca-1 APC (1/100)
- DAPI (1/5000)
- FACS machine (Aria Fusion in this case)
- Hematopoietic Culture Media (HCM) (see recipe in Reagents and Solutions)
- Ice bucket
- Centrifuge
- 15- and 50- mL tubes (BD Falcon)
- Vortex

1. Isolate all tibiae, femurs, and pelvic bones from endogenously-labelled (DsRed) mice and clean off soft tissue with sterile tissues.
    - Store in 1x PBS on ice after isolation in sterile conditions.
    - Typically two to three adult male DsRed^{+} mice (aged 8–12 weeks) need to be euthanized to collect approximately 200,000 KLS+ cells in suspension.
2. Rinse bones by shaking tube to further remove remaining soft tissue from the bones in solution. Repeat twice.
3. Once rinsed with PBS, 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 and to prevent difficulty in collecting cells.
4. Filter cell suspension through 70µm cell strainer into 50ml falcon tube with a 10ml pipette.
5. Rinse bone pieces with FACS buffer and the aid of the 10 ml pipette, collect and filter the cell suspension, and continue to crush and repeat until no remaining large chunks of bone are visible and the crushed bone debris is white. Using a centrifuge, spin the cell suspension for 10min at 300g and 4°C.
6. Carefully discard supernatant from spin down cell suspension, and gently resuspend pellet in ice-cold 5ml 1x RBC lysis buffer per mouse for 30 seconds.
    Prepare 1x RBC lysis buffer from 10x stock solution, and filter through 0.2µm strainer prior to use.
7. Stop RBC lysis incubation with 20ml FACS buffer and using a centrifuge, spin the solution for 5min at 300g and 4°C.
8. Discard supernatant and resuspend in 1ml FACS buffer. Count cells at this step for yield calculations in case of low output.
    Note: if there is a sticky membrane pellet after this process, filter again or remove this debris with a pipette tip.
9. Add 50µl lineage antibody cocktail (Lineage depletion kit) per mouse and incubate the cell suspension for 20min on ice.
10. Wash by adding an additional 20ml FACS buffer and using the centrifuge, spin the suspension for 5min at 300g and 4°C.
11. Carefully discard the supernatant and resuspend the pellet in 1ml FACS buffer.
12. Take 50µl of WT sample for Lin+ single color control (SCC) and add 50µl FACS buffer, keep on ice.
13. Add 50µl magnetic beads per mouse to WT (control) and DsRed samples (labeled cells of interest). Incubate these solutions for 15min on ice.
14. Start AutoMACS and run *Rinse* program.
15. Wash each tube of cell suspension with 20ml FACS buffer and spin for 5min at 300g and 4°C.
16. Discard supernatant and resuspend cell suspension in 3ml FACS buffer.
17. Filter through a 70µm filter into 15ml falcon and bring to AutoMACS with collection tubes for positive and negative fractions.

18. Run *Deplete + QRinse* on AutoMACS machine.
19. Take the Lin- fractions and spin for 5min at 300g and 4°C.
    Note: count at this step; save the Lin+ fraction in the case that the yield is low and another round of AutoMACs sorting may be needed.
20. Discard supernatant and resuspend the Lin- fraction as the purified DsRed sample in 100µl and WT sample in 400µl blocking solution, and add 100µl blocking solution to Lin+ SCC – incubate for 15min on ice.
21. Divide WT sample into four Eppendorf tubes with 100µl each, and add 100µl FACS buffer to 200µl volume; keep two Eppendorf tubes on ice for SCC for unstained cells, and DAPI-stained cells.
22. Add 96µl FACS buffer to DsRed sample, stain sample with antibody solution, and each single-colored control. Incubate these solutions for 1 hour on ice.
23. Wash each individual Eppendorf tube with 1ml FACS buffer and spin for 5min at 300g and 4°C.
24. Resuspend Ds-Red Lin- sample in 1.5ml. For optimal sorting conditions, count cells and dilute if necessary, to a maximum cell concentration of 1-2x10^7/cells per ml. Resuspend DAPI-SCC in 200µl DAPI solution (1:5000) and WT SCC in 200µl FACS buffer.
25. Filter with 85µm mesh and bring to sort into 15ml falcon with 2ml HCM. Invert the tube prior to sorting to ensure that walls are wet with HCM, which increases the viability of sorted droplets that may touch the tube walls.
26. Once the sort is completed, perform a purity control by mixing the sorting tube three times by inversion, taking 10uL of the final solution, mixing with 90uL of 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 (purity = 100* number of events in KLS gate/ total number of events within the DAPI- FSC/SSC live cell gate).
    Note: Once KLS cells are sorted, keep HSPCs on ice for up to maximum three hours until co-seeding with scaffolds and BMSCs (Basic Protocol 2).

BASIC PROTOCOL 2: CO-SEEDING HEMATOPOIETIC STEM AND PROGENITOR CELLS WITH MESENCHYMAL STROMAL CELLS ON COLLAGEN-COATED CARBOXYMETHYLCELLULOSE MICROPARTICLES

Introductory paragraph:
Freshly-sorted HSPCs and trypsinized OP9s are counted and the appropriate ratio of cells are mixed. The CCM scaffolds are dried as a small clump within a 70 µm cell strainer, sitting on a SteriCup 500 mL media filtration device, and then carefully placed in an ultra-low adherent 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 one hour, after which additional media is added and the plate is placed back in the incubator.

Materials:
- KLS+ Sorted HSPCs (see Basic Protocol 1)
- Scaffolds (13.5 mg/mL CCM scaffolds in PBS is used here; see (Tavakol et al., 2019) for synthesis details).
- SteriCup filtration system (Millipore, C3240)
- 6-well ultra-low adhesion plate (Corning, CLS347)
- OP9 cells were donated from the Daley laboratory (McKinney-Freeman, Naveiras, & Daley, 2008), who received them directly from the Nakano laboratory (Nakano, Kodama, & Honjo, 1994).
    OP9 cells are also available at ATCC (www.atcc.org, CRL-2749); this source has not been tested for hematopoietic support in our laboratory.
- T150 cell culture flasks (Corning, CLS430825)
- BMSC media (see recipe in Reagents and Solutions)

- Co-Culture Media (see recipe in Reagents and Solutions)
- 0.05% Trypsin-EDTA (25300054, Life technologies)
- FACS buffer (see recipe in Reagents and Solutions)
- 2-, 5-, 10-, and 25-mL serological pipettes
- 1 mL sterile pipette tip
- 70 µm cell strainer (Greiner, *542040*)
- Cell counter slide
- Ice
- Centrifuge
- 15- and 50- mL tubes (BD Falcon)
- Vortex
- Incubator at 37°C, 5% CO_2

Preparation of OP9 BMSCs

1. Expand OP9 BMSCs at 70-80% confluency with typically 1:3 passages every 3-4 days for 1-2 weeks prior to use in T150 cell culture flasks with BMSC media. The same principle can be applied to other marrow stromal cell lines or primary cells from bone marrow.

   Note: Sub-confluency (plating at 30-50% confluency and splitting 1:3 or 1:4 when reaching 70-80% confluency) 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.
2. For co-seeding identify a T150 flask of 70-80% confluent BMSCs, remove BMSC medium, wash once with PBS, remove PBS and trypsinize with 3ml 0.05% Trypsin-EDTA solution for 2-3min at 37°C, 5% CO_2.
3. Tap the T150 flask firmly in the tissue culture hood and stop cell trypsinization with 4ml BMSC medium. Gently wash the bottom of the flask with BMSC medium to remove loosely attached cells with the aid of a 10 ml pipette and transfer to a 15ml Falcon tube.
4. Spin at 300g for 5min and 4°C.
5. Carefully discard supernatant, resuspend pellet in 500µl BMSC medium and count with Trypan Blue using a cell counter.
6. Adjust volume to concentration of $5x10^6$ cells/mL.

Preparation and co-seeding of CCM scaffolds

7. Stock collagen-coated CCM scaffolds at 13.5mg/ml in PBS at 4°C. Dry 370 µL of the collagen-coated CCM scaffolds on a 70 µm cell strainer in the top chamber of a SteriCup filtration system.

   Note 1: The CCM scaffold could be transferred into a cell strainer to facilitate its recovery and avoiding excessive loss of material. The CCM must touch the SteriCup filter to sufficiently dry.

   Note 2: After coating with collagen, the CCM scaffolds may stick to the wall of the pipette. As a result, a significant loss of CCM scaffolds during the transfer may occur. To avoid this, 1 mL of PBS could be prepared in advance in a 15 mL falcon tube. The experimenter could then pipette 1 mL of PBS to rinse the pipette and proceed directly to step 8.

8. Wash sequentially the CCM scaffolds with 1 mL of PBS and then 1 mL of medium. Dry the CCM scaffolds carefully (still placed within the strainer on the SteriCup filtration system, as in Step 7).

Note: the CCM scaffolds should be as dry as possible in order to achieve a homogenous and optimal cell seeding in step 10.

9. With a 2mL serological pipette or a cell scraper, scratch the CCM off the cell strainer and place it into a single well of a 6-well ultra-low attachment plate forming a viscous cluster or "bubble" in the center of the well. Repeat this process for the desired number of wells. This procedure is described below for 370 µL of CCM scaffolds.

10. Confirm cell counts for KLS+ sorted HSPC fraction and collected BMSCs, and adjust to a 1:10 and 1:100 ratio of HSPCs:BMSCs. In this case, we used for each well 45,000 HSPCs to 450,000 BMSCs for a 1:10 ratio and 4,500 HSPCs to 450,000 BMSCs for a 1:100 ratio.
    Note: Keep the cell suspension fraction of both cell types together and adjust to a volume of approximately 100 µL of total volume for optimal cell seeding, in co-culture medium. The volume used to resuspend the cell pellet is of particular importance. It should not exceed 50% of the initial volume of the stock (13.5 mg/mL). On the one hand, excessive dilution of the cell suspension will lead to a significant loss of cells, since excess of fluid will leak out of the CCM scaffolds. On the other, too concentrated a cell suspension leads to a highly heterogenous seeding and might results in a loss of cell viability during the incubation time in step 11.

11. Add total cell suspension to each dried CCM scaffold bubble in the 6-well ultra-low attachment plate. Place the seeded scaffolds in the incubator for 1 hour at 37°C, 5% CO_2.

12. Once scaffolds have incubated, add 3 mL of co-culture medium (see recipe in Reagents and Solutions) and place in incubator overnight at 37°C, 5% CO_2.

BASIC PROTOCOL 3: MAINTENANCE, REAL-TIME IMAGING, AND IMAGING ANALYSIS OF CO-SEEDED SCAFFOLDS

Introductory paragraph:
As many CCMs were in each well, it was easy to collect a small sample (a few scaffolds, for example) for serial imaging over time. Hoechst (traditionally a live nuclear stain) also stained the scaffolds, as well as endogenously labeled BMSCs (GFP) and HSPCs (DsRed) made imaging useful for looking at proliferation of cells over time. End-point imaging with additional markers via fluorescent antibody staining was not performed, though could be performed in future work if the cell populations used in the study are not endogenously labeled prior to the start of the experiment.

Materials:
- 6-well ultra-low adhesion plate (Corning, CLS347)
- Co-Culture Media (see recipe in Reagents and Solutions)
- 2-, 5-, 10-, and 25-mL serological pipettes
- 1 mL sterile pipette tip
- Zeiss LSM 700 Inverted Confocal Microscope
- Deep cavity glass microscope slide
- Fiji/ImageJ (NIH)
- Hoechst 33258 (0.1 µg/mL)
- Incubator at 37°C, 5% CO_2
- Co-seeded scaffolds (see Basic Protocol 2)

Fabrication of the custom observation chamber for imaging:

1. A piece of polypropylene sheet (height 2.5mm) was cut using a laser cutter at the following dimension: 75mm x 25mm for the slide and 40mm x 14mm for the central chamber. Alternatively, observation chambers can be 3D printed using the 3D printing maps provided with this protocol.
2. A coverslip was then glued one side using silicone glue (Figure 3).

Maintenance and Imaging:

1. Maintain co-seeded CCM scaffolds in co-culture medium at 37°C, 5% CO_2 for two weeks, with addition of 3mL media at one-week post-seeding.
   If keeping CCM scaffolds in culture longer, we recommend doing half-medium changes to prevent aspiration of detached/suspension HSPCs.
2. At days 1, 4, 7, and 11, take a small volume (~100 µL) of suspended CCMs and transfer to a custom observation chamber (produced in lab; see Figure 3).
   To avoid loss of viability due to handling, the tip of the 200 µl tips can be cut with sterile scissors.
3. Supplement this small volume with either 100 µL of 1:1000 dilution of Hoechst 33258 (if staining the scaffold as well) or 100 µL of FACS Buffer.
   The cells used in these studies were endogenously labeled with GFP (MSCs) and DsRed (HSPCs). For other cells in future studies, it would be advantageous to label cells (e.g. transient labelling with cell painters) prior to use in in vitro studies. For Hoechst staining, keep samples stained for 15-20 minutes and wash before going to imaging.
4. Image co-seeded CCM scaffolds within 1 hour of transferring into the custom observation chamber.

Imaging Analysis:

5. Using Fiji/ImageJ's analysis tools, for each image, separate each fluorescent channel and create a compiled volume-rendered image for each cell type (HSPCs or BMSCs).
   Images used for quantification were composed of a 25-z-stacked, volume-rendered image.
6. Threshold each channel using Li algorithm ("image>threshold>Li algorithm")
   If necessary, convert the image to 8-bit.
   In order to ensure a reproducible thresholding, the rendering should closely overlap with the signal from the original channel. Therefore, a comparison before/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, Li algorithm was selected, and a first thresholding is performed by hitting the "Auto" button. Then, using the "Dark background" option (check the corresponding box), the upper 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 whereas highly exposed images usually require sliding the upper cursor to the right to decrease the background signal.
   For quantitative analysis (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 with the same settings.

7. Analyze the fluorescent area corresponding to each channel by using "Analyze>Analyze Particle area"

Note: The following parameters were used in routine: "Size" ranges from 4-Infinity (to remove non-specific fluorescent background), "Circularity" 0-1 (default). Additionally, "Display result", "Exclude on edges" were checked.
If necessary, the option "Includes holes" can be used for the CCM scaffold area quantification in order to increase the accuracy of the measurements.
Scaffold areas could also be quantified manually using the "Freehand selections" tool.

8. Calculate the fluorescent "total area" for each fluorescent label (HSPCs and BMSCs) as well as the "total scaffold area" for each image by summing all the "Area" values on Excel.

9. Calculate the percentage of the total particle fluorescent area for each fluorescent label (HSPCs or BMSCs):
   (HSPCs or BMSCs "total area")/("total scaffold area") x 100

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

BASIC PROTOCOL 4: END-POINT ANALYSIS OF CO-SEEDED SCAFFOLDS USING FLOW CYTOMETRY AND COLONY FORMING UNIT ASSAYS

Introductory paragraph:
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 Methocult™ medium. After one week the CFUs can be 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).

Materials*:*
- Co-Culture Media (see recipe in Reagents and Solutions)
- 0.05% Trypsin-EDTA (25300054, Life technologies)
- FACS buffer (see recipe in Reagents and Solutions)
- Collagenase I (17100-017, ThermoFisher Scientific)
- 2-, 5-, 10-, and 25-mL serological pipettes
- BrightCount contain 1000 beads/µl [Invitrogen, REF: C36950, LOT: 1850623)
- DAPI solution
- BD LSR II SORP flow cytometer
- 6-well ultra-low adhesion plate (Corning, CLS347)
- Methylcellulose (M3434, STEMCELL Technologies)
- STEMvision machine (STEMCELL Technologies)
  Note: Alternatively, counting can be performed with a bight field microscope equipped with 4x and 10x objectives and manual scoring performed according to manufacturer's instructions (https://cdn.stemcell.com/media/files/manual/MA28405-Mouse_Colony_Forming_Unit_Assays_Using_MethoCult.pdf)
- 16-Gauge Blunt-End Needles (STEMCELL Technologies, 28110)
- 3 cc Syringes (STEMCELL Technologies, 28240)
- SmartDish 6-well, meniscus free plates (STEMCELL Technologies, 27371)
- Penicillin/Streptavidin (P/S, 15140122, Thermo Fisher Scientific)
- Blocking solution
- 1 mL sterile pipette tip
- 70 µm cell strainer (Corning)

- Cell counter slide
- Ice bucket
- Centrifuge
- 15- and 50- mL tubes (BD Falcon)
- Vortex
- Incubator at 37°C, 5% CO_2

Collagenase I digestion of scaffolds:

1. Remove carefully (most of) the medium from cell culture well and collect this cell suspension ("non-adherent" fraction) on ice separate from the rest of the isolation.
2. Gently wash the CCM particles with serum-free medium twice.
3. Remove the excess medium and add 1mL of diluted collagenase I (0.04%) per well (24-well plate) or 2 mL (6-well plate) for 25 min at 37°C, 5% CO_2.
4. Inactivate CCM digestion with 2-4 mL of co-culture medium.
5. Pipette up and down with a 2- or 5- mL serological pipette for 1-2 minutes.
6. Use a cell strainer (100 µm) to isolate the cells from the particles, and repeat the washing process twice more.
7. Collect the cell suspension in a 50 ml falcon tube. Discard the cell strainer and residual CCM particles.
8. Pipette up and down to obtain a single-cell suspension.
9. Spin down cell suspension ("non-adherent") and collagenase-digested ("adherent") conditions at 300g for 10 min at 4°C.
10. Resuspend pellet and transfer to Eppendorf tube for total of 500 µL of cell suspension in FACS buffer.
11. Split each condition/Eppendorf tube for CFU and flow cytometry analysis.

    Note: CFU and flow cytometry allocations should be optimized for each experimental set up. It is safe to split CFU and flow cytometry fractions approximately 1:10. Suspension 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.
    Plating conditions should be optimized for an ideal output of 60 colonies/plate. The assay is not linear when more than 100 colonies grow per plate. Discrimination of separate colonies actually becomes impossible if more than approximately 150/plate (see manufacturer's recommendations at https://cdn.stemcell.com/media/files/manual/MA28405-Mouse_Colony_Forming_Unit_Assays_Using_MethoCult.pdf)

Flow cytometry staining:

12. Resuspend cell pellet (~20µl) with 75µl blocking solution, and transfer into Eppendorf tubes for 10 min at room temperature.
13. Add 5µl Lineage cocktail (1:20 dilution) and incubate for 20min on ice.
14. Wash with 1ml FACS buffer and filter with 85µm mesh.
15. Spin at 1300rpm, 10min, 4°C, and aspirate supernatant.
16. Cells were then incubated with the antibody cocktail described in Table 1 for 45 min on ice.

    Note: in these experiments, 10µl of BrightCount beads were added for each condition for counting cell number on the flow cytometry analysis machine.
17. Add 200 µL of DAPI solution, and acquire using a flow cytometry analysis machine.
18. Example analysis and gating of samples are shown in **Figure 2**.

Table 1. Example flow cytometry staining for bulk hematopoietic progenitor quantification (CD45+Lin-cKit+Sca1+/-). Exploration of murine lineage commitment can be determined with alternative combinations of surface markers including CD45 pan-hematopoietic market for better separation from the stromal component (e.g. granulocytic: Gr1, monocytic: Mac1/F4-80, megakaryocytic: CD48/CD41, erythroid Ter119/CD71, lymphoid: IL7R/B220).

Ab x Scaffold	Fluorophore	1 in	2x Ab titration	1:10 Ab dilution	For 35 samples (30µl Ab mix + 10µl beads per sample)
Lin	PO	1:	200		5
cKit	PE-Cy7	1:	200		5
Sca1	BV711	1:	50		21
CD45	AF700	1:	100		11
				BD-buffer:	**1,008 µl**

Colony forming unit assay:

19. Thaw methylcellulose the day prior to use, and add 1% P/S to the bottle.
    It is recommended to shake this 100 mL bottle vigorously, let the bottle sit to remove bubbles, and aliquot each section at 2.3 mL per 15-mL falcon tube and store long-term at -20°C and at 4°C for short term (few hours).
20. For each methylcellulose aliquot, add the appropriate volume of cell suspension for approximately 100 KLS+ cells/6-well plate.
    Note: in our experimental set up, 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 5-10x dilutions can be performed for CFU assays when the concentration of progenitors is unknown.
21. Using a blunt-end needle, mix cell suspension and methylcellulose, let sit for five minutes to allow bubbles rise to the surface, and slowly collect remaining methylcellulose on the bottom of the Falcon tube. Then, carefully add 1.1 mL of this solution into each well of a meniscus-free 6-well plate.
22. Add each plate to a humifying chamber, and follow instructions as provided by Stem Cell Technologies. Leave plates in an incubator for 7- and 14- days without manipulation.
23. Image colonies using Stem Cell Technology's STEMvision machine (or score live with a brightfield microscope), following all protocols for imaging and analysis as listed by the manufacturer.

BASIC PROTOCOL 5: SCAFFOLD DRYING DEVICE FOR SYRINGE PREPARATION

Introductory paragraph:

In order to subcutaneously transplant the co-cultured scaffolds, a controlled-pressure, cell-protecting drying device is necessary. Instructions are provided for the construction of the drying device from common laboratory materials or, alternatively, through the 3D printing maps provided. Subsequent instructions are provided in Basic Protocol 6 for the injection of the co-seeded scaffolds. **Video 1** visually shows both drying the scaffolds and injection *in vivo*.

Materials:

All plastic consumables used in this part should be made from polypropylene. This allows for the assembling of 2 plastic parts together using hot air gun/soldering iron. Additionally, it makes the device and columns suitable for sterilization by autoclaving

- 15 mL conical tubes (PP, BD Falcon)
- 1000 µL tips (Axygen (PP, 7.9cm))
- 20 µL tips (PP, MultiGuard, Sorenson, Bioscience Inc.)
- 1000 µL pipette tip box (PP, Axygen scientific)
- Commercial/craft felt material (Coop, Switzerland, 82%viscose, 18% polypropylene, autoclavable)
- 50 mL conical tube (PP, BD Falcon)
- 5 mL syringes (PP, BD sciences)
- Hot air gun
- Soldering iron
- Metal saw or knife

Loading column fabrication:

1. Cut the falcon 15 ml in 2 parts: bottom part at the 3 mL line and top part at the 12 mL line using a saw or a sharp knife. Discard the middle section.
2. Assemble the 2 parts by melting a rim of plastic with a hot air gun, followed by rapidly pressing the two parts together.

   Note: The 2 pieces should be tightly assembled in order to prevent any leaks. The tightness can be tested by filling up the column with water. If any air bubbles appear, discard the column and repeat step 1.

3. Cut the very bottom part (conic part) of the falcon tube. The diameter of the hole should be the same than the top part of the 1000 mL tip (typically, 1.9cm outer diameter).
4. Assemble the tip and the falcon tube, again by melting a rim of plastic on both the tip and the shortened Falcon tube with a hot air gun followed by pressing.

   Note: Tightness can be checked as mentioned previously in the note of Step 2.
5. Cut the bottom part of the tip (remove between 1.2-1.5 cm). Outer diameter 0.4 cm. Average total height size of the loading column 11.5 cm.

Transfer tip fabrication:

6. Cut the top section of the 20 µL tip at 1.2 cm from the top (larger opening).
7. Cut the bottom part at 2.3 cm from the bottom (the narrowest part), below the filter.

Fabrication of column holder:

8. Cut the top section of the 5 mL syringe at 2 cm from the top (part with the handles).
9. Assemble one handle of the syringe with the top part of a 1000 µL tips.
10. Repeat 8 and 9 up to 4 times on the same tips.

    Note: The holder can also be made from the top "shelf" of the empty 1000 µL box by simply assembling 4x 1000 µL tips at each corner of the shelf. Each hole could then be used as a holder for the columns.

    Note: Adapt the height of the holder by stacking several 1000 µL together.

Fabrication of drying box:

11. Make a hole in the top "shelf" of the empty 1000 µL pipette box at the size of a 50 ml falcon tube.
12. Insert the falcon tube and melt it with a soldering iron in order to attach it to the box.
13. Make a hole in the falcon tube at the desired height (here 2 cm) from the top part.

    This will define the hydrostatic pressure at 200 Pa (1 cmH_2O = 98.07 Pa), which is suitable for cell viability and to give the adequate consistency of the CCM scaffolds for injection.

14. Cut a cleaning cloth or felt material into a rectangle of 8 x 7 cm and with a protruding tail of at least 6 cm longer on one side.
15. Attach pieces together as seen in **Figures 4A-D and 5A-E.**
16. Test for leakage before autoclaving. Autoclave and use under the hood as indicated in the next section.

BASIC PROTOCOL 6: SCAFFOLD INJECTION AND SUBCUTANEOUS TRANSPLANTATION

All experimental procedures were approved by the Animal Care and Use Committee of the Canton of Vaud (ACUC, Vaud, Switzerland). All animals were hosted in the EPFL facilities and were kept under a controlled 12 h light/dark cycle and at constant room temperature 22 ± 2 °C. Note that NSG mice are used in our protocol because OP9 BMSCs are derived from a mixed background (Nakano et al., 1994). **Video 1** demonstrates the process of preparing scaffolds for delicate drying, loading the syringe, and injecting the subcutaneous space of a NSG mouse; Injected scaffolds were maintained for up to 12 weeks *in vivo*.

Materials:

- Co-Culture Media (see recipe in Reagents and Solutions)
- Prepared co-cultured CCMs (see Basic Protocols 1-4)
- PBS
- Custom drying device (see Basic Protocol 5)
- 20G flexible catheter (BD Biosciences 381703)
- 1 mL syringe
- Betadine (Mundipharma Medical Company)
- Isoflurane USP-PPC (Animalcare Ltd) and compatible anesthetic gas-air mixer
- Ophthalmic liquid gel (Viscotears, Alcon)
- Hair shaver
- Heating pad
- 8-16 week old NOD SCID-γ (NSG 5557, Jackson laboratories) immunodeficient female mice
- Antibiotic solution (see recipe in Reagents and Solutions)

Mouse pre-transplantation preparation

1. Autoclave all supplies for implantation, including drying device and suture supplies.
2. Weigh each mouse individually for post-surgery monitoring, and induce anesthesia with 4.0% Isoflurane.
3. Turn on heating pad and place mice on top of it while mouse stays anesthetized at 1.5-2% maintenance Isoflurane.
4. Shave the back of the mouse and add Bétadine at the injection site to help keep the mouse's injection site as sterile as possible.
5. Add ophthalmic liquid solution to the eyes of each mouse.

Loading scaffold drying device (see Video 1 for detailed visuals)

6. Prepare scaffolds in a 15 mL conical tube from *in vitro* culture (typically a total of ~3-6 mL of seeded scaffolds and their associated media are loaded into a column for drying per condition)
   Note: if two people are performing surgery, this procedure is done while anesthetizing the mouse.
7. Using prepared device as described in Basic Protocol 5, add PBS to chamber components to moisturize device prior to use.

8. Transfer the scaffolds (in solution) through the loading column of the drying device and allow for the scaffold to settle to the tip of the transfer tip, to collect approximately 50 μL of cell seeded scaffolds for injection per site.

    Note: This process may take 1-2 minutes, as the scaffolds settle. During this process, keep the column and mouse stabilized as much as possible, to prevent losing scaffolds during the preparation.

Subcutaneous injection (see Video 1 for detailed visuals)

9. Prepare a sterile 1mL syringe by loading 0.1 mL of coated scaffold without cells and follow by 0.1 mL of air to help injection fluidity.
10. Insert the catheter (equipped with the needle) into the mouse's skin. Insert the needle about 2cm deep in the mouse (subcutaneous). Remove the needle but keep the catheter on place.

    Note: Catheters that are not equipped with needles can also be used for this procedure. To do so, prior to catheter introduction, make a hole in the mouse's skin using a 18G needle and insert the needle about 2cm deep in the mouse (subcutaneous). Then insert the catheter and proceed to step 11.
11. Connect the CCM scaffolds-filled transfer tip (step 8) to the syringe (step 9).
12. Slowly push the CCM scaffolds into the subcutaneous implantation site, and slowly remove the catheter post-implantation.

    This step must be performed gently, as to make sure all particles remain inside the subcutaneous pocket inside the mouse and do not leave the injection site. As the CCM particles covalently interlock during the high-density injection procedure, no sutures are required. The organized, malleable CCM "bubble" should become apparent immediately after injection, as shown in ***Figured 3E.*** *The transfer tip should not be reused for a second injection.*
13. Return the animal to its cage as it starts to wake up; the entirety of the injection procedure takes approximately 20 minutes for two injections on the same mouse.
14. Treat animal with an antibiotic solution in the drinking water for the duration of the study, to be made fresh once a week for 12 weeks.

Sacrifice and sample harvest

15. Euthanize mice at end-point (12 weeks in this case), and shave the back of the animal to better visualize the implantation site as shown in **Figure 4F-G.**
16. Isolate implants and surrounding tissue, which should be visible by eye, and fix in 4% paraformaldehyde overnight.

    Note: in order to minimize damage to the implant, we recommend isolating and fixating the implant with the surrounding skin and then, if desired, carefully dissecting the fixed implant prior to paraffin embedding.
17. Perform paraffin embedding or other tissue analysis as desired, including any immunohistological staining or analysis. Examples are provided in **Figure 4H.**

REAGENTS AND SOLUTIONS:

FACS solution:

1X PBS, 1 mM EDTA (15575020, Thermo Fisher Scientific), 2% (v/v) fetal bovine serum. Store at 4*C for up to a month.

Blocking solution:

5 μg/ml hIgG in FACS buffer solution. Make fresh and store on ice.

Antibiotic solution for mice:

Use 250mL water red/photoprotective bottles and add 250ml of drinking water, Co-amoxy mepha (10%(V/V) – 2.5 mL), 300 -µL Baytril (10%); and 2 bags (250 mg each) of Dafalgan/paracetamol.

BMSC media:
Alpha-minimum essential media (a-MEM) plus Glutamax (32561, ThermoFisher), 10% fetal bovine serum (FBS, 10270-106, GIBCO), and 1% Penicillin/Streptavidin (P/S, 15140122, Thermo Fisher Scientific). Store at 4*C for up to a month.

Hematopoietic culture media:
(IMDM + Glutamax 25 mM HEPES, 10% FBS, 1% P/S). Store at 4*C for up to a month.

Co-Culture media: store at 4*C for up to a month protected from light.
50% fresh hematopoietic culture media (IMDM + Glutamax 25 mM HEPES, 10% FBS, 1% P/S)
50% conditioned hematopoietic culture media (Conditioned media was obtained by culturing confluent BMSCs with hematopoietic culture media for two days (48 h), filtering with a 0.2 um filter, and freezing the media for no longer than two months at −20 °C.)

COMMENTARY

BACKGROUND INFORMATION:

Since the first *in vitro* description of mixed bone marrow stromal cultures with hematopoietic output in 2D conditions by Dexter et al. (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 for 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 the 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, Nature Biotechnology 2019; Tavakol et al. 2019). 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 the 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 the ease-of-use, the scalability, the remarkable vascularization upon *in vivo* transfer, and the potential for clinical grade manufacturing of the proposed scaffold.

CRITICAL PARAMETERS:

Critical parameters to influence the protocol are the source of HSPCs and BMSCs, as well as the choice of recipients in the case of *in vivo* transplantation. In particular, the minimalistic culture protocol in absence of exogenous cytokines presented here depends on the utilization of 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+Lin-cKit+ 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 addressed with our methodology.

TROUBLESHOOTING:

Table 2. Troubleshooting

Step	Problem	Possible reason	Solution
Basic Protocol 1	Yield of KLS+ cells is low	Young mice were used, not enough marrow cavities were harvested	Sacrifice multiple mice in anticipation of low yields
Basic Protocol 2, Steps 10-12	Cells do not fully adhere during co-seeding on scaffolds	Seeding volume too high, cells disperse and do not attach	Decrease seeding volume to only cover scaffold particles Incubate cells in suspension on scaffolds for longer than 1 hour; make sure to hydrate the scaffold bubble with extra media to prevent drying of the scaffolds
Basic Protocol 4	Density of CFU images is too high to count	Too many HSPCs seeded in MethoCult at start of assay	Count KLS+ cell fraction prior to plating, and optimize ratios prior to experiment. Plate 5-10x serial dilutions if ration unknown. Save a fraction of HSPCs on ice while analyzing flow cytometry data on day of analysis and only plate once the expected progenitor enrichment is known by FACS
Basic Protocol 6	Co-seeded scaffolds are stuck in syringe during injection	Over-drying of scaffolds prior to injection	Take up some PBS into the syringe to moisturize it prior to injection; this should not affect the injection site other than to ensure full delivery of scaffolds

UNDERSTANDING RESULTS:

In the protocols we describe in this manuscript, the culture of hematopoietic stem and progenitor cells can be assessed through co-cultures with marrow stromal cells in 3D, mimicking some of the basic interactions between these cells in the mammalian bone marrow. To that end, these culture methodologies can be applied towards facile transplantation of co-cultured scaffolds *in vivo*, for the purposes of bone marrow 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 our described 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 the BMSCs towards the KLS+ cells during their *in vitro* culture periods. Therefore, the results described in this paper 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*. 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, like 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 our 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 remains 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. Though not described in this protocol, further staining may help identify downstream cell populations in the scaffolds over time. 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, further panel optimization may be performed to look at downstream cell populations throughout the *in vitro* culture periods. To corroborate findings in the flow cytometry, colony forming unit assays can provide an indication of the number of colonies able to be formed from one 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. Our techniques described 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 both model hematopoiesis *in vitro* (Basic Protocols 1-4) and regenerate the bone marrow *in vivo* (Basic Protocols 5-6).

TIME CONSIDERATIONS:

Basic Protocol 1

Steps 1 to 4: ~1-2 hrs are needed to euthanize animals, collect bones, and crush bones for marrow isolation.

Steps 5 to 17: ~1 hr is needed to stain cells for MACS sorting.
Steps 18 to 19: ~15-30 min are needed for MACS sorting.

Steps 20 t0 25: ~2-3 hours are needed for subsequent staining and FACS.

Basic Protocol 2

Step 1: Expansion of cells over 1-2 weeks.

Steps 2 to 6: ~30 min are required to prepare the BMSCs in suspension.

Steps 7 to 10: ~30 min are required to prepare scaffolds for cell seeding.

Steps 11 to 12: ~1.5 hr is needed to incubate cell suspension with scaffolds prior to adding further media.

Basic Protocol 4

Steps 1 to 11: ~1.5 hr is required for collagenase digestion, cell suspension collection, and washing prior to flow cytometry staining. This may vary depending on number of samples.

Steps 12 to 17: ~3 hrs are required for blocking, staining, and running samples at flow cytometer.

Steps 18 to 21: ~2 hrs are required for calculating cell seeding density, aliquoting cell suspensions, and plating CFU aliquots on 6-well plates.

Steps 22: 2 weeks post plating, either manually score live under microscope or image colonies and automatically score (StemCell Vision), then visually verify colony assignation (~15 min total per well of a 6-well plate).

Basic Protocol 6

Step 1: ~1 day prior to experiment, autoclave all materials for experiment.

Steps 2 to 5: ~15-20 min preparation time per mouse.

Steps 6 to 8: ~5-10 min for drying and syringe preparation.

Steps 9 to 13: ~10-15 min for injection per mouse.

Steps 14-17: ~15-20 min for euthanizing and scaffold harvest per mouse.

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FIGURE LEGENDS:

Figure 1. Overall approach to *in vitro* studies with 3D scaffolds with HSPC and BMSC co-cultures (Made with Biorender).

Figure 2. Example flow cytometry gating scheme for isolating (A) endogenously labeled DsRed HSPCs, (B) lineage-negative hematopoietic cells (CD45+), and (C) Sca-1-negative, cKit+positive cells subsequently.

Figure 3. Example imaging chamber for live 3D confocal analysis.

Figure 4. Injection of co-cultured CCM subcutaneously. (A) Once cells are added to the CCM scaffolds, the co-cultures can support growth for up to three months; in these experiments, CCMs supported cultures for up to two weeks. (B) By equilibrating the CCM and media to a predefined hydrostatic pressure level (ΔP), typically on the order of 0.2 kPa (ca. 2 cm water column), CCM

scaffolds can be dehydrated to help pack scaffolds prior to injection. More information in Basic Protocol 5. (C) An implantation catheter is used to help assist in subcutaneous injection. (D) The co-seeded CCM scaffolds are injected. (E-G) Gross images of scaffold implant site 12-weeks post-injection, both outside the skin (E-F) and underneath the skin with visible vasculature integration (G). (H) Example H&E and IHC staining of scaffolds with visible maintenance of GFP+ BMSCs (H), DsRed+ hematopoietic cells (J), and integration of CD31+ endothelial cells (I) 12-weeks post-implant. Schematics in Figure 4A-D were reproduced from Tavakol et al. (2020) with permission from Elsevier.

Figure 5. Drying device to protect cells and scaffolds during implantation. (A) Overview of drying scheme from cell suspension to collection of particles to insertion on syringe. (B) Visualization of drying device from repurposed 1000μL pipette tip box. (C) Schematic of box metrics for determining pressure drop for cells and scaffolds. (D) Drying column loading tip. (E) Scaffold polymer concentration increases according to the negative pressure applied by the device.

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