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METHODS AND TECHNIQUES

High-throughput single-cell fate potential assay of murine hematopoietic progenitors in vitro

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The advent of single-cell transcriptomics has led to the proposal of a number of novel high-resolution models for the hematopoietic system. Testing the predictions generated by such models requires cell fate potential assays of matching, single-cell resolution. Here we detail the development of an in vitro high-throughput single-cell culture assay using flow cytometrically sorted single murine bone marrow progenitors, which measures their differentiation into any of five myeloid lineages. We identify critical parameters for single-cell culture outcome, including the choice of sorter nozzle size and pressure, culture media, and the coating of culture dishes with extracellular matrix proteins. Further, we find that accurate assay readout requires the titration of antibodies specifically for their use under low-cell-number conditions. Our approach may be used as a template for the development of single-cell fate potential assays for a variety of blood cell progenitors.

Recent single-cell transcriptomic studies have led to the proposal of new models for the hematopoietic hierarchy [1–10]. Testing these models requires matching cell transcriptional state with functional cell fate potential at the single-cell level. Older studies have reported "low-throughput" single-cell differentiation assays in vitro, where outcome is measured using colony formation and morphological criteria [11–13]. Recently, tracking of single hematopoietic stem cell differentiation by in vitro imaging has also been described [14,15], and index sorting was used to link single-cell transcriptomics with single-cell fate potential assays including single-cell transplantation [16,17]. Single-cell cultures using human progenitors have been reported [7]. However, the influence of various assay parameters on assay efficiency and outcome have not been detailed. To our knowledge, no high-throughput assays have been developed for primary murine progenitors.

Ultimately, cell fate potential in vivo would be the most definitive and relevant measure. Indeed, in vivo clonal studies with single transplantable hematopoietic stem cells have established their heterogeneity [18]. However, transplantation assays that test single-cell fate potential in vivo are currently limited to cells with substantial proliferative output. Single-cell in vitro cultures, although unlikely to recreate in vivo conditions, provide a flexible setting in which to manipulate extracellular conditions and measure their effects on fate outcomes. Further, they can be scaled up for analysis of thousands of individual cells with relative ease.

Below we describe the development of a single-cell culture assay for murine hematopoietic progenitor cells (HPCs). We examined the effects of a number of key parameters during flow cytometric cell sorting, cell culture, and flow cytometric readout of differentiation outcome (Fig. 1). Although we provide a set of conditions that successfully promote differentiation of murine HPCs into five cell fates, what follows is also a template that can be adapted for the detection of other differentiation outcomes from narrower or broader sets of progenitors.

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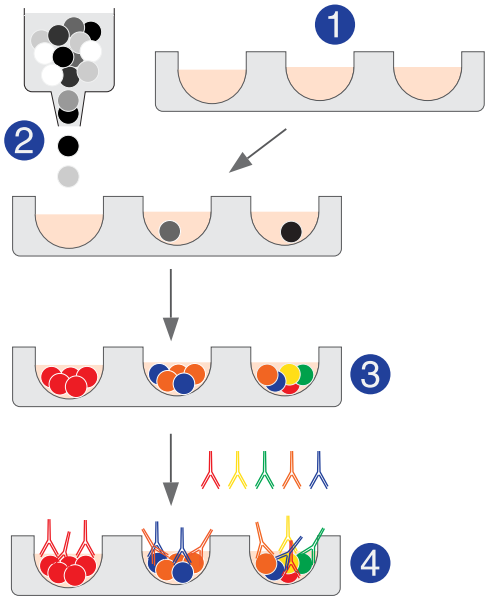

Figure 1. Optimization of a single-cell culture assay for murine hematopoietic progenitors. A cartoon depicting the parameters optimized in the development of the single-cell culture assay: **1** = culture medium, culture well shape, and coating; **2** = sort pressure and nozzle size; **3** = culture parameters including medium, culture duration, and growth factor refeeding; **4** = antibody binding assay, optimizing antibody concentrations under low-cell-number conditions.

Methods

Mice

Bone marrow (BM) was harvested from 8- to 12-week-old adult BALB/cJ male or female mice (Jackson Laboratory, Bar Harbor, ME).

Cell preparation

Femurs and tibias were harvested immediately following euthanasia and placed in cold (4°C) "staining buffer" (phosphate-buffered saline [PBS] containing 0.2% bovine serum albumin [BSA] and 0.08% glucose). Bones were flushed using a 2-mL syringe with a 26-gauge needle and then crushed with a pestle and mortar to obtain any remaining cells. Flushing the bones is gentler than crushing, promoting cell viability; however, cell yield is lower, and some cell types may be protected from flushing in bone niches that are less accessible to flushing. For this reason, we first flush the bones, obtaining as many cells as possible, and then crush the bones to obtain any remaining cells. Harvested bone marrow cells were filtered through a 70-µm strainer and washed in cold Easy Sep buffer (PBS, 2% fetal bovine serum [FBS], 1 mmol/L EDTA).

Harvested BM cells were lineage-depleted using the Mouse Streptavidin RapidSpheres Isolation Kit (STEMCELL Technologies, Catalog No. 19860A), with the following biotinylated antibodies (with catalog numbers in square brackets):

- Anti-CD11b: Clone M1/70 [557395], BD Biosciences
- Anti-Ly-6G and Ly-6C: Clone RB6-8C5 [553125], BD Biosciences
- Anti-CD4: Clone RM4-5 [553045], BD Biosciences
- Anti-CD8a (Ly-2): Clone 53-6.7 [553029], BD Biosciences
- Anti-CD19: Clone 1D3 [553784], BD Biosciences
- Anti-TER119: Clone TER119 [553672], BD Biosciences

Single-cell liquid cultures of mouse BM progenitors

Lineage-depleted cells were then labeled with the following antibodies in the presence of 1% rat serum:

- Streptavidin Alexa Fluor 488: Molecular Probes, to mark lineage-positive cells
- CD117-APC Cy7: Clone 2B8 [105826], Biolegend
- TER119-BUV395: Clone TER-119 [563827], BD Biosciences
- CD71- PE Cy7: Clone RI7217 [113812], Biolegend
- CD55-AF647: Clone RIKO-3 [131806], Biolegend
- CD105-PE: Clone MJ7/18 [#120408], Biolegend
- CD150-BV650: Clone TC15-12F12.2 [115931], Biolegend
- CD41-BV605: Clone MWReg30 [133921], Biolegend
- CD49f (=itga6) – BV421: Clone GoH3 [313624], Biolegend

Following washes, cells were resuspended in 4′, 6-diamidino-2-phenylindole (DAPI)-containing buffer, and single cells were sorted from each of these gates into 96-well plates, retaining index-sorting parameter for each cell, using a BD FACSAria II with a 130-µm nozzle. Cells were cultured for 3 to 10 days in Iscove's modified Dulbecco's medium (IMDM) + 20% fetal bovine serum (FBS), with the following added growth factors:

- Stem cell factor (SCF, 50 ng/mL): Recombinant Murine SCF [250-03], Peprotech
- Interleukin (IL)-3 (10 ng/mL): Recombinant Murine IL-3 [213-13], Peprotech
- IL-6 (10 ng/mL): Recombinant Murine IL-6 [216-16], Peprotech
- Erythropoietin (EPO, 2 U/mL): PROCRIT (epoetin alfa) [606-10-971-8]
- IL-11 (50 ng/mL): Recombinant Murine IL-11 [11], Peprotech
- IL-5 (10 ng/mL): Recombinant Murine IL-5 [215-15], Peprotech
- Thrombopoietin (TPO, 50 ng/mL): Recombinant Murine TPO [14], Peprotech
- Granulocyte colony-stimulating factor (G-CSF, 15 ng/mL): Recombinant Murine G-CSF [250-05], Peprotech
- Granulocyte–macrophage CSF (GM-CSF, 15 ng/mL): Recombinant Murine GM-CSF [315-03], Peprotech

Fresh growth factors were added to the medium of each well on days 4 and 8. The clones in each well were labeled on day 3, 7, or 10 with the following cell surface markers for flow cytometric analysis:

- TER119-BV421: Clone TER-119 [116233], Biolegend
- CD71-PE Cy7: Clone RI7217 [113812], Biolegend
- CD117-APC Cy7: Clone 2B8 [105826], Biolegend
- FcεRIα-AF700: Clone MAR-1 [134323], Biolegend
- CD41-BV605: Clone MWReg30 [133921], Biolegend
- Cd11b-PE Cy5: Clone M1/70 [101209], Biolegend
- Ly 6G/C-FITC: Clone RB6-8C5 [553126], BD Biosciences

The concentration for each antibody batch was first optimized with appropriate titrations, to minimize nonspecific binding under conditions of low cell number. Clones were analyzed using the high-throughput sampler (HTS) attachment of the BD LSR II.