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. © 2018 ISEH – Society for Hematology and Stem Cells. Published by Elsevier Inc. All rights reserved.

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.
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 R7217 [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 (αITGA6) – 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 R7217 [113812], Biolegend
- CD117-APC Cy7: Clone 2B8 [105826], Biolegend
- FceRIα-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.
Results and Discussion

Preparation of HPCs

We examined erythromyeloid fate potentials of flow cytometrically sorted murine HPCs. To prepare HPCs, BM was harvested from the femurs and tibias of 1 to 10 mice. HPCs were enriched from BM cells by negatively selecting lineage (Lin)-positive cells using biotinylated antibodies against Ter119, CD19, CD8, CD4, Ly-6G/Ly-6C, and CD11b, with a magnetic bead approach (EasySep, StemCell Technologies). Some of the optimization steps below were conducted directly on Lin– BM cells, without additional flow cytometric sorting. For most of our work, Lin– BM cells were labeled with additional surface markers, including Kit, CD150, CD105, CD41, CD49f, CD55, and CD71. These cell surface markers were used to sort HPC subpopulations with putative potential for the erythroid (E), granulocytic (G) monocytic (M), megakaryocytic (Meg), and basophilic (Ba) lineages. The cell fate potential(s) of each HPC subpopulation was examined at the single-cell level using single-cell index sorting followed by single-cell culture.

Optimization of the single-cell culture protocol (Fig. 1) is considered under each of four headings: sort parameters; culture media; culture length and feeding; and optimization of cell surface marker-based cell fate readout.

Flow cytometric sort parameters: Wider nozzle and low pressure are essential when sorting specific cell types

We used a BD FACSAria II to sort a single cell into each well of 96-well plates. Plates were prepared as discussed below, each well containing 150 to 200 μL of culture medium. Three elements were optimized: the viability of the sorted cells prior to, during, and following the sort; cell purity; and delivery of precisely one cell into each well. For the latter, we aligned the deflection stream to deliver drops so that each landed precisely at the center of each well. We tested this by first running a plate with the cover in place, ascertaining the presence of a single droplet above the center of each well. We confirmed alignment before running each plate in any given experiment. Alignment is especially important when using a wide nozzle, where the droplet stream may be less stable.

The viability of cells prior to the sort was optimized by minimizing the time between cell harvesting and the sort and by keeping the cells cold (4°C). Adding DAPI to the staining medium allowed the exclusion of nonviable (DAPI+) cells from the sort population. Sorting purities for bulk cell populations were assessed immediately after the sort in the presence of DAPI and indicated any loss of viability during the sort. To maximize cell viability following the sort, we kept the plates on ice, returning them to the 37°C/5% CO2 incubator as soon as possible.

Flow cytometric sorting involves a large and rapid pressure drop when cells traverse the nozzle. In our initial sorts, we used a 100-μm nozzle at 20 psi. Two weeks following the sort, only 10% of wells (two plates: 7 of 96 wells and 13 of 96 wells) contained >10 viable cells per well. A control plate into which we sorted 10 cells into each well contained viable cells in essentially all wells (Fig. 2A). Sorting multiple cells per well may alter the microenvironment in ways that promote cell viability. Alternatively, or in addition, the effects of high-pressure sorting on cell viability may not be apparent when cells are sorted in bulk but becomes critical during single-cell sorting. Of note, the fraction of cells able to survive and divide following the sort may be lower than the fraction...
measured as “viable” by the criterion of membrane impermeability (using DAPI or trypan blue).

The use of a wider, 130-µm nozzle at 12 psi resulted in a striking improvement in cell survival, with 90% of wells containing >10 viable, dividing cells (Fig. 2A) (p < 0.0001, two-tailed Mann–Whitney test). High sorting pressure was previously reported to damage sperm [19], but, to our knowledge, there are no published studies that have directly assessed the effects of sorting pressure and nozzle size on the viability of other cell types. In general, it is difficult to test extensively the separate effects of nozzle size and sorting pressure on cell viability, because each nozzle size requires a relatively narrow range of optimal sorting pressures. Nevertheless, we were able to compare two sorting pressures with the 100-µm nozzle: either 20 psi, used routinely with this nozzle, or 14 psi (Supplementary Fig. E1, online only, available at www.exphem.org). Starting with pre-sort Lin– BM cell samples of similar viability, there was clear improvement in cell viability immediately following the sort, when sorting pressure was lower (14 psi). Of interest, high sort pressure was particularly deleterious to late erythroid progenitors (CFU-e), while having little effect on myeloid progenitors (Kit+ Lin–CD55+) and only a moderate effect on early erythroid progenitors (BFU-e) (Supplementary Fig. E1 and data not shown). The sensitivity to sort pressure is therefore cell type specific.

Culture conditions I: Well shape, media, and well coating all affect outcome

We tested the effect of well shape on the yield of viable cells following culture, comparing round-bottom with flat-bottom wells. We sorted single erythroid progenitors (EPs) (Kit+CD55+CD49flCD105+) or single myeloid-multipotent progenitors (MPPs) (Kit+CD55+CD49fhiCD150fhi [20]) into single wells of either shape. In both cases, there was a clear advantage to round-bottom wells following 8 to 10 days of culture (Fig. 2B) (p < 0.0001 and p = 0.0008 for EPs and MPPs, respectively, two-tailed Mann–Whitney test).

To support the growth of myeloid lineages, we cultured the cells in the presence of a variety of growth factors (listed in the Methods). Unlike recent single-cell cultures of human progenitors [7], we did not use stromal cell lines. We screened three types of base medium for their ability to support growth (the same cytokine cocktail was added to all these media): IMDM (Gibco, Catalog No. 12440-053) to which 20% FBS had been added (HyClone GE Healthcare, Catalog No. SH30910.03); a serum-free medium, StemSpan (StemCell Technologies, Catalog No. 09650), and StemSpan to which 5% FBS had been added. We also tested the effect of coating the culture wells prior to the sort with extracellular matrix proteins, either fibronectin (Gibco; 50 µg/mL at room temperature for 1 hour) or collagen solution (containing 95%–98% type I and 2%–5% type III bovine collagen; StemCell Technologies, Catalog No. 04902) at room temperature for 1 hour. We found that although all media and coating combinations supported at least some growth of all the myeloid lineages we examined, there were no universal conditions that optimized growth for all lineages (Fig. 3). Broadly, there appeared to be a split between G/M lineages, which grew best in IMDM/20% FCS and collagen-coated wells, and E/Meg/Ba lineages, which grew best in StemSpan in the absence of well coating or in the presence of fibronectin. Consequently, the frequency of cells from any particular lineage in the culture well varied widely. We conclude that the most reliable readout of single-cell fate potential assays is binary, that is, the presence or absence of a given lineage. The absolute frequency of the various lineages in a given well is highly dependent on the chosen culture conditions.

Culture conditions II: Effect of refeeding cultures and of culture length

We asked what the optimal duration of culture would be prior to assessing fate potentials of the single-cell progenitors. This parameter is expected to be highly dependent on the type of progenitor. We examined EP and MPP single-cell cultures on days 4, 8, and 10 following plating. Indeed, we found that, on average, EPs peaked in growth on day 8 (Fig. 4A, top), whereas MPPs had a more complex response (Fig. 4A, bottom). The majority of MPP wells contained similar cell numbers on days 4 and 8, decreasing by day 10. A minority of wells (~7%), characterized by a particularly high proliferative response, contained their highest cell numbers on day 10 (Fig. 4A, bottom). As might be expected, this heterogeneity derives from the likely heterogeneity of progenitors, which differ in both developmental stage and proliferative potential. The majority of progenitors reach their proliferative peak earlier, suggesting they are more mature. This outcome highlights the need to examine single-cell cultures at multiple time points to gain a complete assessment of progenitor potentials and differentiation stage.

We initiated all cultures with the following recombinant murine growth factors added to the medium: SCF (50 ng/mL), IL-3 (10 ng/mL), IL-6 (10 ng/mL), IL-5 (10 ng/mL), IL-11 (50 ng/mL), TPO (50 ng/mL), G-CSF (15 ng/mL), and GM-CSF (15 ng/mL), as well as human recombinant EPO (2 U/mL). We asked whether cells cultured for periods of 8 to 10 days would benefit from refeeding with fresh growth factors. We refed the cultures by adding to the wells 50 µL of medium containing all the above growth factors at 3× concentration. Cultures were refed on day 4 and analyzed on day 8; cultures that were analyzed on day 10 were refed twice, on days 4 and 8. We found that this clearly benefited EPs, which reached a peak on day 8 with a higher number of cells per well (Fig. 4B, top). However, refeeding the cultures did not prolong the life of the culture, so that the loss of viable cells seen by day 10 took place in both refed cultures and controls (Fig. 4B, bottom). This suggests that growth factors promote cell viability in differentiating cells but, once the cells are differentiated, have no further activity on mature cells,
in agreement with the known effects of EPO on EPs and mature erythroblasts [21].

**Antibody staining for lineage readout: Antibody binding under low-cell-number conditions**

We measured the lineage composition in each well by staining the cells directly in the culture wells with a cocktail of antibodies and analyzing antibody binding using a flow cytometer (BD LSR II) with a HTS attachment.

As is the case for all antibody-based flow cytometric analyses, we identified cells that are positive for each antibody signal by first filtering for aggregate-free, viable cells, as illustrated in (Supplementary Fig. E2A, online only, available at www.exphem.org). The precise gates that determine locations of negative and positive events that pass these filters were deduced from a number of variables, including single color controls, contour plot pattern, and fluorescence-minus-one (FMO) controls, in which one antibody at a time was subtracted from the staining cocktail (Supplementary Fig. E2B).

Figure 3. Effect of culture medium and well coating on lineage growth. (A, B) Lin− BM cells were placed in culture wells, which were uncoated or coated with either fibronectin or glycogen. A cocktail of cytokines was added to one of three medium preparations, as indicated. Cells were cultured for 5 days and then assayed for their expression of the indicated cell surface markers by flow cytometry. Bar charts indicate the absolute number of cells staining positively with each cell surface marker (A) and their relative frequency within each culture (B), for each of the nine sets of conditions.

All antibody binding consists of both specific and nonspecific components. To maximize the specific component, and thereby increase the ratio of signal to noise, we supplemented the staining medium with 200 μg/mL rabbit IgG (Jackson Immuno Research, Catalog No. 011-000-003), which will compete for nonspecific antibody binding sites, as well as act as a blocking agent for IgG Fc receptors. We allowed cells to incubate with the antibody cocktail for 3 hours on ice. Staining in a low volume (40 μL) similarly improves binding by increasing the effective epitope concentration. Finally, a sufficient number of washes are necessary following antibody incubation. During a wash, antibodies are less likely to dissociate from high-affinity specific binding sites than from nonspecific low affinity sites, thereby resulting in an improved signal to noise. We were initially concerned, however, that excessive washing might lead to cell loss. We used a technique whereby, after the addition of washing medium to the wells and centrifugation, plates were rapidly inverted and the medium flicked off. Cells remained in the wells as a result of fluid surface tension, and the wash step
was repeated. With this approach, we compared the number of cells remaining in the wells after one wash with that for three washes, finding that the loss resulting from three washes was sufficiently small (Supplementary Fig. E3A, online only, available at www.exphem.org).

Appropriate antibody concentration is a key parameter that needs to be determined for any antibody binding assay; we found that this is especially important under conditions of low cell number. We titrated both cell number and antibody concentration, measuring the fraction (%) of antibody-bound cells for each of our assay antibodies, as well as the median fluorescence intensity (MFI) of the positive population and the sensitivity index (SI) (Fig. 5A, B). The corresponding contour plots for these are provided in (Supplementary Fig. E3B). At very low cell number, we noted some systematic trends, such as an increase in the fraction of apparent CD41^+ cells and a decrease in the fraction of apparent Gr1^+ cells (Fig. 5A). However, for each antibody, it is possible to select a concentration that largely avoids these systematic errors (2.5 and 0.156 μg/mL for the CD41 and Gr1

Figure 4. Effect of culture duration and refeeding with cytokines. (A) Single EPs or MPPs were sorted into wells and cultured for either 4, 8, or 10 days. Cultures were refed with fresh growth factors on days 4 and 8. The numbers of viable cells per well are shown. Data are shown either in order of decreasing cell number per well (left) or summary statistics (right). Box and whiskers as in Fig. 2A. (B) Single EPs were sorted into wells and cultured for 8 or 10 days. Cultures were all initiated with a full complement of growth factors. Indicated cultures, but not controls, were refed with fresh growth factors on day 4 (for cultures examined on day 8) or on days 4 and 8 (for cultures examined on day 10). Data are shown either in order of decreasing cell number per well (left) or summary statistics (right). Box and whiskers as in Fig. 2A. P Values are for a two-tailed Mann–Whitney test.
Figure 5. Optimization of antibody binding under low-cell-number conditions. (A, B) Fresh BM cells were placed in 96-well plates at increasing dilutions. These were incubated with a range of concentrations of antibodies directed at Gr1, Ter119, and CD41, and assayed for antibody binding by flow cytometry. Shown are the median fluorescence intensity (MFI), sensitivity index (SI), and positive cells for each antibody binding. In (A), these parameters are plotted against decreasing cell number/well, and in (B), against decreasing antibody concentration. All cell populations are subsets of the viable cell gate as illustrated in Supplementary Fig. E2. The SI was calculated as (MFI of positive population—MFI of negative)/(robust SD of negative population · population) [22]. Corresponding contour plots for these data are provided in Supplementary Fig. E3B.
Figure 6. Example of single-cell culture assays with murine HPCs. Single HPCs were sorted into individual wells and cultured as described in this article. Fate potentials of individual progenitors were assayed with the indicated antibodies after 3, 7, or 10 days of culture. Each row provides cell surface marker expression for cells from a single progenitor in a single well.
antibodies, respectively). We also noted that, in addition to systematic errors, readings at very low cell number are marked by increased fluctuations, as might be expected through stochastic fluctuations of any one given cell type in a small sample. The SI is maintained relatively stable for CD41 and Ter119.

Although the factors that result in these binding properties may be complex, the altered binding behavior of some antibody reagents at low cell concentrations suggests that antibody concentration needs to be carefully titrated and chosen so as to minimize the effect of cell number per well. We also found that titration of each antibody batch is advisable. An example of single-cell readouts from murine hematopoietic progenitors labeled with five antibodies after either 3, 7, or 10 days of culture is provided in Fig. 6. Ideally, fluorescence-activated cell sorting approaches can be complemented by orthogonal methods, such as imaging, to verify the identity of cells with a particular pattern of antibody binding.

Conclusion

We developed a high-throughput single-cell culture assay for murine HPCs based on flow cytometrically sorted cells and flow cytometric readout of lineage potential. We found that round-bottom wells, low sorting pressure, and a wide nozzle promote survival and higher cell number. Sensitivity to sort pressure was cell type specific, suggesting that disregarding this parameter may skew downstream analysis of hematopoietic progenitor populations. Although the precise mechanism underlying pressure sensitivity is not clear, the rapid pressure changes experienced by cells during the sort will likely generate mechanical stress, which may affect the cytoskeleton, alter membrane conductance, and stimulate intracellular signaling pathways that may lead to apoptosis [23].

Single-cell cultures need to be assessed for lineage differentiation at a number of time points because heterogeneous progenitors attain peak growth and maturation asynchronously. Further, culture medium and extracellular matrix protein coating of the wells differentially promote one lineage growth over another. Prolonged cultures benefit from refeeding the cells with fresh growth factors. Finally, lineage readout with the use of flow cytometry requires careful antibody titration because of the altered antibody binding properties under low cell number conditions.

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References

Supplementary data

Supplementary Figure E1. Effect of sort pressure on cell viability A, B BM cells were first enriched for Lin- cells using magnetic beads and an antibody cocktail directed at lineage + cells. The remaining cells were labeled with a cocktail of antibodies that isolated myeloid progenitors (Kit+ Lin- CD55-), BFU-e (Kit+CD55-CD105-CD49fCD41 CD150medCD71high) and early or late CFU-e progenitors (Kit+CD55-CD105-CD49fCD41 CD150medCD71med or CD71high) [20]. DAPI staining shows the fraction of all BM cells, and of Lin- BM cells that are viable prior to sorting. DAPI was added to post-sort samples and these were analyzed immediately following the sort. The viability of each cell type is shown, for two different sorts through a 100μ nozzle, either 20 psi (A), or 14 psi (B).
Supplementary Figure E2. Analytical flow chart for antibody binding data. A All cells are initially filtered through a “singlets” gate that excludes aggregates, using the height vs. area signals of the same parameter (e.g. side scatter or forward scatter), selecting cells within a diagonal gate (top left panel). Dead cells and debris are then excluded by gating on DAPI-negative cells, excluding low FSC events (top right panel). The filtered cells then used to establish gates for the positive signal from each antibody. These gates are established using a number of criteria, including fluorescence-minus-one (FMO) gates (B). B Example of FMO samples. Each sample is labeled with all but one antibodies (and also with DAPI; an FMO sample for DAPI is not shown here). The positive gate(s) for each antibody should contain no cells in its corresponding FMO sample.
Supplementary Figure E3. Antibody labeling under low cell number conditions. A Single cell cultures in multi-well plates were assayed for antibody binding, by incubating the cells with antibodies in the same wells. Data shows a comparison between 1 and 3 washes following cell incubation with antibodies, and before flow cytometric analysis. The loss in cells as a result of added washes is relatively small (median = 12 for 1 wash, 10 for 3 washes, \( p = 0.027 \), two-tailed Mann-Whitney test.). The same data is plotted either in decreasing order of cells/well, or as a box and whiskers plot, as in Fig. 2A. B Selected contour plots for data presented in Fig. 5.