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High Throughput Single-Cell Fate Potential Assay of Murine Hematopoietic Progenitors *in Vitro*

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Abstract

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, that measures their differentiation into any of 5 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.

Keywords

hematopoiesis; erythropoiesis; cell fate; single cell cultures

Introduction

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 were reported [7]. However, the influence of various assay parameters on assay

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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, while unlikely to recreate *in vivo* conditions, nevertheless 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). While we provide a set of conditions that successfully promote differentiation of murine HPCs into 5 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 8–12 weeks old adult BALB/cJ male or female mice (Jackson Laboratories, Maine, USA).

Cell preparation

Femurs and tibiae 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); 1mM EDTA). Harvested BM cells were lineage-depleted using the Mouse Streptavidin RapidSpheres Isolation Kit (STEMCELL Technologies [Cat# 19860A]), with the following biotinylated antibodies:

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 Bioscience)

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], Biolgened)

CD41-BV605 (Clone MWReg30 [#133921], Biolegend)

CD49f (=itga6) – BV421 (Clone GoH3 [#313624], Biolegend)

Following washes, cells were re-suspended in 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 130µ nozzle. Cells were cultured for 3 to 10 days, in IMDM+ 20% FBS, with the following added growth factors:

SCF (50 ng/mL): Recombinant Murine SCF, #250-03 Peprotech

IL-3 (10 ng/mL): Recombinant Murine IL-3, #213-13 Peprotech

IL-6 (10 ng/mL): Recombinant Murine IL-6, #216-16 Peprotech

EPO (2U/mL): PROCRIT® (epoetin alfa), #606-10-971-8

IL-11 (50 ng/mL): Recombinant Murine IL-11, #220-11 Peprotech

IL-5 (10ng/mL) : Recombinant Murine IL-5, #215-15 Peprotech

TPO (50 ng/mL): Recombinant Murine TPO, #315-14 Peprotech

G-CSF (15 ng/mL): Recombinant Murine G-CSF, #250-05 Peprotech

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 days 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).

Concentration for each antibody batch was first optimized with appropriate titrations, to minimize non-specific 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 erythro-myeloid fate potentials of flow-cytometrically sorted murine HPCs. To prepare HPCs, bone marrow (BM) was harvested from the femurs and tibiae of 1 to 10 mice. HPCs were enriched from BM cells by negatively selecting lineage (Lin)⁺ 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 the majority of our work, Lin⁺ BM cells were labeled with additional surface markers, including Kit, CD150, CD105, CD41, CD49f, CD55, 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. 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 the four headings below: 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 FACS Aria II to sort a single cell into each well of 96 well plates. Plates were prepared as discussed in the sections below, each well containing 150 to 200 μ l of culture medium. Three elements were optimized: the viability of the sorted cells prior to, during, and following the sort; Cell purities; and delivery of precisely one cell into each well. For the latter, we aligned the deflection stream to deliver drops so that each lands precisely at the center of each well, testing 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). DAPI added to the staining medium allowed exclusion of non-viable (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 was possible.

Flow cytometric sorting involves a large and rapid pressure drop when cells traverse the nozzle. In our initial sorts we used a 100 µ nozzle at 20 psi. Two weeks following the sort, only 10% of wells (2 plates: 7 wells of 96 and 13 wells of 96) contained >10 viable cells per well. A control plate where we sorted 10 cells into each well contained viable cells in essentially all wells (Fig 2A). Sorting multiple cells per well may alter the micro-environment 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 µ 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 directly assess 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, since each nozzle size requires a relatively narrow range of optimal sorting pressures. Nevertheless, we were able to compare two sorting pressures with the 100 µ nozzle, either 20 psi, used routinely with this nozzle, or 14 psi (Supplementary Fig. 1). 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. 1 and Data not shown). The sensitivity to sort pressure is therefore cell-type specific.

Culture conditions I: well shape, media, and well coating all impact 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⁺CD49^{low}CD105⁺,) or single myeloid-biased multipotential progenitors (MPPs) (Kit⁺CD55⁺CD49^{high}CD150^{high}, [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 Methods). Unlike recent single-cell cultures of human progenitors [7], we did not use stromal cell lines. We screened three types of base media for their ability to support growth (the same cytokine cocktail was added to all these media): Iscove's Modified Dulbecco's Medium (IMDM, Gibco, #12440-053) with added 20% fetal bovine serum (FBS, Hyclone GE Healthcare, #SH30910.03); a serum free medium, StemSpan (StemCell technologies, #09650), and StemSpan with added 5% FBS. 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 hr), or collagen solution (containing 95–98% type I and 2–5% type III bovine collagen; StemCell technologies; # 04902, at room temperature for 1 hr). We found that, while 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 on the one hand, 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 re-feeding cultures and of culture length

We asked what would be the optimal duration of culture, 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 panel), whereas MPPs had a more complex response (Fig. 4A, lower panel). 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, lower panel). This heterogeneity is as might be expected 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 for examining single cell cultures at multiple time points in order 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 media: Stem Cell Factor (SCF, 50 ng/ml), Interleukin 3 (IL-3, 10 ng/ml), Interleukin 6 (IL-6, 10 ng/ml), Interleukin 5 (IL-5, 10 ng/ml), Interleukin 11 (IL-11, 50 ng/ml), Thrombopoietin (Tpo, 50 ng/ml), G-CSF (15 ng/ml) and GM-CSF (15, ng/ml); as well as human recombinant Erythropoietin (Epo, 2 U/ml). We asked whether cells cultured for periods of 8 to 10 days would benefit from re-feeding with fresh growth factors. We re-fed the cultures by adding to the wells 50 µl of media containing all the above growth factors at 3× concentration. Cultures were re-fed on day 4, and analyzed on day 8; cultures that were analyzed on day 10 were re-fed 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 panel). However, re-feeding 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 re-fed cultures and in controls (Fig. 4B, lower panel). This suggests that growth factors promote cell viability in differentiating cells but, once differentiated, have no further activity on mature cells, in agreement with known effects of Epo on EPs and on 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 High Throughput Sampler (HTS) attachment.

As is the case for all antibody –based flow cytometric analysis, we identified cells that are positive for each antibody signal by first filtering for aggregate-free, viable cells, as illustrated in Supplementary Figure 2A. The precise gates that determine location 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 Figure 2B).

All antibody binding consists of both specific and non-specific 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, # 011-000-003), which will compete for non-specific 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 is necessary following antibody incubation. During a wash, antibodies are less likely to dissociate from high-affinity, specific binding sites than from non-specific 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, following the addition of washing medium to the wells and centrifugation, plates were rapidly inverted and the media 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 following either one or three washes, finding that the loss due to three washes was sufficiently small (Supplementary Fig. 3A).

An 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). We show the corresponding contour plots for these in Supplementary Figure 3B. At very low cell number, we noted some systematic trends, such as an increase in the fraction of apparent CD41⁺ cells, or 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 µg/ ml and 0.156 µg/ ml for the CD41 and Gr1 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 relatively stably maintained for CD41 and Ter119.

While 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 5 antibodies following either 3, 7 or 10 days of culture is shown in Figure 6. Ideally, FACS 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 of pressure sensitivity is not clear, the rapid pressure changes experienced by cells during the sort will likely generate mechanical stress, which is may impact the cytoskeleton, alter membrane conductance and stimulate intracellular signaling pathways that may lead to apoptosis [22].

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

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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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 media, culture well shape and coating; **2=** sort pressure and nozzle size; **3=** culture parameters including media, culture duration, growth factor re-feeding; **4=** antibody binding assay, optimizing antibody concentrations at low cell number conditions.

Figure 2. Optimization of sort parameters

A Left: Sorting pressure and nozzle size have a substantial impact on the viability of sorted cells. Single cells sorted with either a 130 μ /12 psi or a 100 μ /20 psi nozzle. For comparison, we also sorted 10 cells per well with the 100 μ /20 psi nozzle. **Left:** wells are plotted in decreasing order of cell number per well. **Right:** summary statistics for the data shown in the left panel. Box extends from the 25th to 75th percentiles, whiskers extend from 5th to 95% percentiles, center value is the median, outliers are shown. P value is for a two-tailed Mann-Whitney test.

B Well shape: round bottom wells promote cell viability and higher cell numbers. EPs and MPPs were cultured for 8 and 10 days, respectively, into either flat bottom or round bottom wells. Left panels show well data, right panels show corresponding summary statistics, see details in 'A'.

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Figure 3. Effect of culture media and well coating on lineage growth
A, B Lin⁻ BM cells were placed in culture wells, which were either uncoated, or coated with either fibronectin or glycogen. A cocktail of cytokines was added to one of three media preparations, as indicated. Cells were cultured for 5 days and were 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 of the cell surface markers (A), and their relative frequency within each culture (B), for each of the nine sets of conditions.

Figure 4. Effect of culture duration and of re-feeding with cytokines

A Single EPs or MPPs were sorted into wells and cultured for either 4, 8 or 10 days. Cultures were re-fed with fresh growth factors on days 4 and 8. The number of viable cells per well are shown. Data is 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 re-fed with fresh growth factors on days 4 (for cultures examined on day 8), or on days 4 and 8 (for cultures that were examine on day 10). Data is 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.

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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. They were incubated with a range of concentrations of antibodies directed at Gr1, Ter119 and CD41, and assayed by for antibody binding by flow cytometry. Shown are the median fluorescence intensity (MFI), the sensitivity index (SI), and positive cells for each antibody binding. In 'A', these parameters are plotted against decreasing cell number/well; in 'B', they are plotted against decreasing antibody concentration. All cell populations are subsets of the viable cell gate as shown in Supplementary Figure 2. The SI was calculated as (MFI of

positive population – MFI of negative) / (Robust SD of negative population. population)
[23]. Corresponding contour plots for these data are shown in Supplementary Figure 3B.

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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 following 3, 7 or 10 days of culture. Each row shows cell-surface marker expression for cells from a single progenitor in a single well.