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Detecting Hematopoietic Stem Cell Proliferation using BrdU Incorporation

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Abstract

Cellular quiescence is a key component of hematopoietic stem cell (HSC) homeostasis; therefore a reliable method to measure HSC cell division is critical in many studies. However, measuring the proliferation rate of largely quiescent and rare populations of cells can be challenging. Bromodeoxyuridine (BrdU) incorporation into replicating DNA is a commonly used and highly reproducible method to detect cell division history. Here, we describe a protocol for BrdU incorporation analysis in hematopoietic stem and progenitor cells that can provide a sensitive measure of cell division even in rare cell populations. In combination with flow cytometry, this method can be generalized to analyze other cell populations and other tissues as identified by cell surface markers.

Keywords

BrdU; Flow Cytometry; Proliferation; Hematopoietic Stem Cells; Cell Cycle

1. Introduction

There are many methods available to study the proliferative status of cells, including measuring DNA synthesis, metabolic activity, cell division, or the presence of proteins associated with proliferation, such as Ki67. Each of these techniques has inherent advantages and disadvantages, and investigators should carefully consider which is most appropriate to answer a particular scientific question (1–3).

As stem cells are relatively few in number and mostly quiescent, many commonly used methods of detection may not be sensitive enough to accurately identify their proliferation. Also, since stem cells are not easy to maintain *in vitro* and often behave differently in culture, it is beneficial to assess them in an *in vivo* setting. Methods to detect the presence of proteins associated with proliferation, such as Ki67, PCNA, or MCM-2, provide a snapshot of a given cell population at the time of assay (4–7). However, these proteins are expressed in S, G1, and G2/M phases of the cell cycle and so mark loss of quiescence as opposed to proliferation per se. In addition, quantifying the results of staining for markers such as Ki67 can often be subjective (8). Another method is to measure the metabolic activity of cells

with the use of tetrazolium salts, such as MTT, XTT, or WST-1 (9–12). These assays can be read using a spectrophotometer and are easily quantifiable, but they may be less accurate, can be toxic and require *in vitro* culture of the cells. A third common method to assess proliferation is tracking cell division with a dye such as CFSE (carboxyfluorescein diacetate succinimidyl ester), which readily diffuses into cells and covalently binds to intracellular amines (13–16). The dye is split evenly between daughter cells upon division which allows for the tracking of subsequent cell divisions. This method has the advantage of allowing cell tracking over time, but is subject to staining efficiency and to follow the stained cells *in vivo* they must be transplanted back into an animal model.

Unlike the above methods, DNA intercalating agents can be injected directly into animals to allow for direct tracking of cells over time in their native *in vivo* state. Synthetic thymidine analogs, such as BrdU and EdU, which can incorporate into newly synthesized DNA during the S phase of the cell cycle, are a common method for directly tracking DNA replication (17–19). In order to identify incorporated BrdU, DNA must first be denatured so antibodies can gain access (20). EdU however, uses Click-iT technology, which allows EdU analogs to be fluorescently tagged with the addition of a small dye-labeled azide that is able to access DNA without denaturation (21). Therefore, if DNA structure is important for other assays, it could be advantageous to use EdU staining over BrdU despite its higher cost. One potential disadvantage of both methods is that the analogs themselves can damage cells and cause mutations, making downstream, long-term experiments problematic (22, 23). Despite this caveat, these techniques are especially useful for cells types that are not highly proliferative as incorporation can be tracked *in vivo* over the course of several days. In addition, BrdU and EdU incorporation assays can be used in conjunction other techniques such as flow cytometry or immunohistochemistry (24–27), making it feasible to analyze a large number of cells and cell types.

Here we describe a protocol to identify proliferating hematopoietic stem cells (HSCs) using BrdU incorporation and subsequent analysis using flow cytometry. Briefly, whole bone marrow is isolated from mice following BrdU injection. Bone marrow cells are then stained for HSC surface markers, after which they are fixed and permeabilized. DNA is then denatured to allow BrdU antibodies access to the incorporated analogs. Finally flow cytometry is used to identify BrdU positive HSCs. In addition to this protocol, we provide a method by which rare cell populations can be sorted prior to fixation in order to allow for co-staining protocols that may be disrupted by fixation, such as the use of Hoechst dye for the identification of HSC side population cells (28). While we focus on a method to identify proliferation in HSCs, this technique is applicable to many other rare cell populations and largely quiescent cell types.

2. Materials

* These items are optional; see associated notes for details

2.1 BrdU Administration

1. Insulin syringes: 29G $\times$ ½

2. BrdU solution: 10 mg/ml BrdU solution diluted in 1X Dulbecco's phosphate buffered saline (DPBS).

2.2 Bone marrow isolation

1. HBSS+: 500 ml hank's balanced salt solution without calcium or magnesium (HBSS), 10 ml fetal bovine serum (FBS), 5 ml 1M N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (HEPES)
2. Needles: 27G $\times$ 1/2 and 18G $\times$ 1 1/2
3. Leur-Lok Syringes: 5 ml and 10 ml
4. 15 cm tissue culture dish
5. 40 μ m cell strainers
6. * RBC lysis buffer: 9 ml 0.16M sodium chloride (NH₄Cl), 1 ml 0.17M Tris-HCl pH 7.65; see Note 7
7. * Mortar and pestle; see Note 5

2.3 Enrichment of Progenitors

1. CD117 (cKit) MicroBeads, mouse (Miltenyi-Biotec)
2. AutoMACS Pro Separator (Miltenyi-Biotec); see Note 9
3. * Running buffer: 1X DPBS, 0.5% BSA (bovine serum albumin), 2mM EDTA; filter sterilized; see Note 9
4. * MACS Columns (Miltenyi-Biotec); see Note 9
5. * MACS Separators (Miltenyi-Biotec); see Note 9

2.4 Hematopoietic Stem Cell Staining

1. * Antibodies for lineage markers (Gr1, B220, Mac1, CD4, CD8, Ter119), cKit, Sca1, CD150, CD48, and CD34, see Note 10 for more staining information

⁷Strictly speaking, RBC lysis is not required. Whole bone marrow will be enriched for cKit⁺ cells in the next step and so red blood cells will be removed at this point. However, removal of RBCs before enrichment greatly reduces the number of total cells, thus allowing for the use of fewer cKit beads for enrichment.

⁵If multiple mice will be combined to form a single group for analysis then crushing may be preferred over flushing to extract whole bone marrow from the bones. If many bones are pooled, crushing can generate a higher bone marrow yield and is often quicker than flushing. However, it is important to note that over-crushing can result in cell shearing and produce small fragments of bone, both of which can decrease the overall yield. For crushing, bones should be cleaned of extraneous muscle and fat. This can be accomplished using a scalpel or razor blade to scrape the tissue from the outside of the bones. Cleaned bones are then crushed in approximately 5–10mL of cold HBSS+ using a mortar and pestle. Bones should only be crushed until large bone fragments are removed. After a minimal amount of crushing, the HBSS+ should be pipetted up and down to break up bone marrow clumps and filtered through a 40 μ m cell strainer. This process is then repeated one or two more times until the majority of the bone marrow is released from the bones. After crushing the bones a maximum of three times, the bones should then be repeatedly washed with additional HBSS+ to release the last of the bone marrow. After bone marrow has been filtered, continue on the step 6 of section 3.2.

⁹As an alternative to an automated separation machine, manual columns can also be used for enrichment. Miltenyi Biotec MACS Columns used with MACS Separators work well for this. The specific column used will depend on the number of labeled cells. Briefly, cells are resuspended in running buffer and applied to a pre-washed column housed in a magnetic separator. The column is then washed repeatedly, removed from the magnet, and then cells are eluted with the addition of running buffer. The resulting enriched cells are then ready for staining. See Miltenyi Biotec's website for more detailed instructions.

¹⁰There are many potential staining schemes for hematopoietic stem and progenitor cell staining, for more detailed information see (31, 32).

2.5 Carrier Cell Isolation

1. Murine splenocytes
2. Anti-mouse B220-Biotin
3. Anti-Biotin MicroBeads (Miltenyi-Biotec)

2.6 Cell Sorting

1. FBS (fetal bovine serum)
2. 1.5 ml tubes
3. FACS tubes

2.7 BrdU Staining

1. FITC BrdU Flow Kit (BD Biosciences); contents included in kit: Fluorochrome-conjugated anti-BrdU Antibody, BD Cytotfix/Cytoperm Buffer, BD Perm/Wash™ Buffer (10X), BD Cytoperm Permeabilization Buffer Plus, 7-AAD, BrdU (10 mg/mL), and DNase. * Components of this kit can be purchased separately from other sources.

3. Methods

All centrifugation steps should be carried out at 4°C

3.1 BrdU Administration

1. Weigh mice to be BrdU injected.
2. Prepare 1mL insulin syringes with BrdU solution at 1mg BrdU/6g of body weight. Stock BrdU solution is 10mg/mL so 100µl/6g of body weight is required (see Note 1 for example).
3. Inject the prepared BrdU solution IP (intraperitoneal) 24–72hrs before time of sacrifice. As an alternative to IP injection, BrdU may also be administered in the drinking water (see Note 2 for more information). The time of BrdU exposure must be tailored to the cell population being studied. The more quiescent the population, the more time is required to gain measurable incorporation (see Note 3 for timing suggestions).

¹For an average mouse of 25g, 416.7µl of BrdU solution would be needed (100µl/6g of body weight).

²For longer labeling studies BrdU can also be administered in the drinking water either as an alternative to IP injections or in conjunction. BrdU drinking water (1mg/mL) should be protected from light and replaced daily. 1% glucose can also be added to improve palatability. Care should be taken if periods of longer than one week of administration are used as BrdU can become toxic to animals and may adversely affect results (29, 30).

³The length of BrdU treatment before time of sacrifice should be varied depending on the cell population under investigation. For rapidly dividing cells, a shorter period of BrdU incorporation should be used to achieve data in dynamic range of the assay. 24hrs of BrdU incorporation will result in approximately 2–5% of LT-HSCs (Lineage[−] cKit⁺ Sca1⁺ CD150⁺ CD48[−] CD34[−]) being labeled. If having a higher percentage of HSCs labeled is desirable, the length of time can be extended from 24hrs to 48 or 72hrs, resulting in approximately 7–15% of LT-HSCs being labeled at baseline.

3.2 Bone Marrow Isolation

1. Sacrifice mice using approved procedure.
2. Remove 6 bones per mouse. Collect two tibiae, two femurs, and two pelvic bones. Six bones may not be required if cells are not going to be sorted (see Note 4 for more details).
3. Flush bone marrow from bones using HBSS+. Use a 5mL syringe with a 27G needle to flush each bone with 5ml of HBSS+ into a 15 cm culture dish. Pool bone marrow from all 6 bones. If several mice will be combined into a single group for analysis then crushing the bones may be more advantageous; see Note 5 for details.
4. Disassociate bone marrow clumps. Use a 10ml syringe with an 18G needle to draw up HBSS+ containing bone marrow from previous step. Repeatedly draw up and expel bone marrow with the bevel against the bottom of the culture plate until no more visible clumps are remaining, avoiding aeration of the solution. For normal bone marrow with will be approximately 5–6 repetitions (see Note 6).
5. Filter the bone marrow. Use a 40µm cell stainer to filter the bone marrow to remove any clumps that may remain.
6. Remove red blood cells from the bone marrow using RBC lysis buffer. This lysis step is optional (see Note 7 for details). Centrifuge filtered bone marrow at 400g for 6mins. Remove supernatant and resuspend cell pellet in 2ml of RBC lysis buffer. Incubate at room temperature for 20mins.
7. Wash cells to remove RBC lysis buffer. Add 10mL of HBSS+ to cells in RBC lysis buffer and centrifuge at 400g for 6mins. Remove supernatant.

3.3 Enrichment of Progenitors

1. Incubate with cKit beads. Resuspend the cell pellet in 800µl of HBSS+ and add 200µl of cKit beads. The amount of cKit beads used should be adjusted for the total cell number (see Note 8 for details). Mix well and incubate at 4°C for 30mins. Add 10mL of HBSS+ to cells and centrifuge at 400g for 6mins. Remove supernatant.
2. Resuspend cell pellet in 5mL of HBSS+ and filter cells through a 40µm cell strainer into a new tube.
3. Magnetic bead enrichment to isolate cKit⁺ cells. Use a positive selection program (poseld2) on an AutoMACS machine to enrich for cKit-tagged cells.

⁴The collection of six bones per mouse is recommended if the enriched progenitor cells will be sorted for a more rare HSC population. If however, the cKit-enriched progenitor population will be directly analyzed by flow cytometry without prior sorting, collecting four bones should provide adequate cell numbers. An estimate of the number of bones required can be determined assuming that each bone will yield between 10–20 million whole marrow cells (this number can be further increased with proficient crushing). LT-HSCs are approximately 0.01% of the bone marrow cells so on average one bone would give about 1000–2000 HSCs (although the achieved number will be lower than this due to loss at each staining step).

⁶Be careful not to draw up the bone marrow with the syringe too many times as this will shear the cells.

⁸The volume of cKit beads used will vary depending on the total cell number. 200µl of beads should be used for every 1×10⁸ cells. The appropriate volume of beads should be diluted 1:5 in HBSS+.

The negative fraction after enrichment can be discarded or kept for other experiments. Manual isolation columns can also be used here in place of an AutoMACS machine (see Note 9).

4. Centrifuge the positive fraction (cKit⁺ cells) at 400g for 6mins and discard supernatant.

3.4 Hematopoietic Stem Cell Staining

1. Stain bone marrow for HSCs. For staining of long-term HSCs (LT-HSCs) the antibody cocktail should contain lineage markers (Gr1, B220, Mac1, CD4, CD8, Ter119), cKit, Sca1, CD150, CD48, and CD34 (see Note 10 for more staining information). Make an antibody cocktail with antibodies at a 1:100 dilution in HBSS+, with the exception of CD34 which should be used at a 1:50 dilution. Resuspend cell pellets in 100µl of antibody cocktail. Incubate in the dark at 4°C for 15mins (see Note 11). Wash cells with 2mL of HBSS+. Centrifuge at 400g for 6mins and discard the supernatant.

* If analysis of LT-HSCs using cell surface markers (Lineage⁻ cKit⁺ Sca1⁺ CD150⁺ CD48⁻ CD34⁻) is sufficient, skip directly to section 3.7 for fixation and BrdU staining. If however, the staining methods used will be disrupted by fixation, then sorting of HSCs before fixation may be required (such as using Hoechst staining for side population identification (28)). For sorting proceed to section 3.5 and 3.6.

3.5 Carrier Cell Isolation

Due to the low number of HSCs after sorting, it is recommended to use carrier cells during the subsequent fixation and staining steps to minimize loss of HSCs.

1. Isolate spleen cells. Remove spleen from a control mouse and gently crush in HBBS+ to release cells from the capsule. Use a 5ml syringe with an 18G needle to draw up HBSS+ containing splenocytes. Repeatly draw up and expel cells until no more visible clumps are remaining.
2. Filter the splenocytes. Use a 40µm cell stainer to filter the cells to remove any clumps that may remain. Centrifuge splenocytes at 400g for 6mins and discard supernatant.
3. Enrich B220⁺ B cells. Incubate splenocytes with B220-biotin. Resuspend cell pellet in 100µl of HBSS+ with 1µl of B220-biotin antibody. Incubate in the dark for 15mins at 4°C. Wash with 1mL HBSS+. Centrifuge at 400g for 6mins and discard supernatant.
5. Incubate splenocytes with biotin beads. Resuspend the cell pellet in 800µl of HBSS+ and add 200µl of biotin beads. Mix well and incubate at 4°C for 30mins. Add 10mL of HBSS+ to cells and centrifuge at 400g for 6mins. Remove supernatant.

¹¹Make sure cells are kept covered from this point forward to avoid bleaching of the fluorophores.

- 6 Resuspend cell pellet in 5mL of HBSS+ and filter cells through a 40µm cell strainer into a new tube.
- 7 Magnetic bead enrichment to isolate B220⁺ cells. For AutoMACS use a positive selection program (poseld2) to enrich for B220-tagged cells. The negative fraction after enrichment can be discarded or kept for other experiments. Manual isolation columns can also be used here in place of an AutoMACS machine (see Note 9).
- 8 Centrifuge the positive fraction (B220⁺ cells) at 400g for 6mins and discard supernatant.
- 9 Stain B220⁺ cells. Resuspend cell pellet in 100µl of HBSS+ with 1µl of Strep antibody (see Note 12). Incubate in the dark for 15mins at 4°C. Wash with 1mL HBSS+. Centrifuge at 400g for 6mins and discard supernatant.

3.6 Cell Sorting

1. Prepare collection tubes. Add approximately 1mL of 100% FBS to a 1.5mL eppendorf tube (see Note 13). Invert tube 10 times to insure complete coating of the inside walls of the tube. Transfer FBS to a new tube and repeat until all collection tubes are coated. Use a vacuum to remove trace amounts of FBS remaining in tubes. Fill tubes with 1mL of HBSS+.
2. Sort B220⁺ splenocytes. Sort the B220⁺ splenocytes using FSC/SSC, singlet and live discrimination. Sort enough cells to allow for approximately 1×10⁵ B220⁺ cells per sample of sorted HSCs.
3. Sort HSCs. Sort Lineage⁻ cKit⁺ Sca1⁺ CD150⁺ CD48⁻ CD34⁻ HSCs using FSC/SSC, singlet and live discrimination. Each mouse should yield on average about 2000 LT-HSCs.
4. Re-stain B220⁺ cells (see Note 14). Centrifuge cells at 400g for 6mins and discard supernatant. Resuspend cell pellet in 100µl of HBSS+ with 1µl of B220 antibody. Incubate in the dark for 15mins at 4°C. Wash with 1mL HBSS+. Centrifuge at 400g for 6mins and discard supernatant.
5. Mix sorted HSCs and B220⁺ cells. Add approximately 1×10⁵ re-stained B220⁺ cells per sorted HSC sample. Mix well and centrifuge at 400g for 6mins. Discard supernatant and proceed to section 3.7 for fixation and BrdU staining.

3.7 BrdU Staining

1. Fix cells. Resuspend the cell pellet in 100µl of Cytotfix/Cytoperm Buffer. Incubate in the dark for 15mins at room temperature. Wash the cells with 1mL of

¹²Stain the splenocytes using a B220 antibody with a unique fluorophore that was not used for HSC staining. If an additional fluorophore is not available, use the same fluorophore as was used for the lineage markers in the HSC stain on bone marrow. Only lineage negative cells will be sorted from the bone marrow so the resulting cells should not contain this fluorophore.

¹³Pre-coating the collection tubes with FBS can significantly increase the survival of sorted cells as they are much less likely to stick to the walls of the tubes.

¹⁴The intensity of the B220 fluorophore will have diminished during the sorting process. It will be critical to have strong signal from the B220 cells in order to separate them from the HSCs during later flow analysis after BrdU staining.

1X Perm/Wash Buffer (see Note 15). Centrifuge at 300g for 6mins and discard supernatant (see Note 16). The protocol can be stopped here and stored overnight (see Note 17).

2. Permeabilize cells. Resuspend the cell pellet in 100µl of Cytoperm Permeabilization Buffer Plus. Incubate in the dark for 10mins on ice. Wash cells with 1mL of 1X Perm/Wash Buffer. Centrifuge at 300g for 6mins and discard supernatant.
3. Re-fix cells. Resuspend the cell pellet in 100µl of Cytofix/Cytoperm Buffer. Incubate in the dark for 5mins at room temperature. Wash the cells with 1mL of 1X Perm/Wash Buffer. Centrifuge at 300g for 6mins and discard supernatant.
4. DNase treat the cells. Resuspend the cell pellet in 100µl of 300µg/ml DNase (see Note 18). Incubate cells in the dark for 1hr at 37°C. Wash the cells with 1mL of 1X Perm/Wash Buffer. Centrifuge at 300g for 6mins and discard supernatant.
5. BrdU stain the cells. Resuspend the cell pellet in 50µl of BrdU antibody mix, which contains 1µl of BrdU antibody per 50µl of 1X Perm/Wash Buffer. If staining for other intracellular markers is desired, they can be included here in the BrdU antibody mix. Incubate cells in the dark for 20mins at room temperature. Wash the cells with 1mL of 1X Perm/Wash Buffer. Centrifuge at 300g for 6mins and discard supernatant.
6. Resuspend the cells in 500µl of HBSS+. Optional: To stain for total DNA content 20µl of 7-AAD can be added to the cell pellet before addition of HBSS+.

3.8 Flow Cytometry

1. Single-color controls should be prepared for each fluorophore used to allow for correct voltage and compensation setup (see Note 19).
2. Gating strategies for flow cytometry will depend on whether cKit⁺ progenitors or sorted HSCs were used for BrdU staining. See **2a** for enriched progenitors and **2b** for sorted cells.
 - a. cKit⁺ progenitors. Cells should first be gated through FSC/SSC and singlet discriminations. As the number of BrdU positive LT-HSCs will be low it is helpful to set the gate for BrdU positive cells before gating for HSCs. This BrdU⁺ gate can then be applied to the final HSC gate. See Note 20 for more detail staining strategy.

¹⁵If using the Perm/Wash Buffer from the BD Biosciences BrdU Flow Kit, it is supplied at 10X and should be diluted 1:10 with deionized water prior to use. Unused diluted buffer can be stored at 4°C for future use.

¹⁶After fixation the cells will be fragile. In addition to the slower centrifugation speed (400g reduced to 300g), care should also be taken when resuspending cell pellets. Flick the tubes to resuspend; do not vortex.

¹⁷The protocol can be stopped here following the initial fixation (step 1 of section 3.7). After centrifugation, resuspend the cell pellet in HBSS+ and store overnight in the dark at 4°C.

¹⁸If using the DNase solution from the BD Biosciences BrdU Flow Kit, it is supplied at 1mg/ml and should be diluted with 1X DPBS immediately prior to use to make a working stock of 300µg/ml.

¹⁹As the cells being analyzed for flow cytometry have been fixed they will have very different FSC and SSC properties than live cells. For this reason if cells will be used for single color controls, it may be helpful to fix them as well to be able to more accurately set these parameters.

- b. Sorted LT-HSCs. Cells should first be gated through FSC/SSC and singlet discriminations. As the cells here are a mixture of HSCs and B220⁺ splenocytes, the splenocytes must be removed from further analysis. The B220 cells were re-stained after sorting and so should still retain strong signal and can easily be separated from the remaining HSCs. After removing the B220⁺ cells, the remaining HSCs can then be gated for BrdU positivity. See Figure 1 and Note 21 for more details.

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²⁰For long-term HSCs, cells should first be gated for Lineage[−] cells, then for cKit⁺ Sca1⁺. This double positive gate should then be gated for CD150⁺ CD48[−] cells. The final LT-HSC gate is CD34[−] cells. There will not be many cells left at this point, so it may be helpful to bring forward the CD48⁺ population from the previous window as a reference point for determining where the CD34 gate should be placed (The CD48⁺ cells should not be included in the gating strategy here, just used to help set the appropriate gate). Once the final Lin[−] cKit⁺ Sca1⁺ CD150⁺ CD48[−] CD34[−] gate is set, the BrdU⁺ gate that was set on the total cells can then be applied.

²¹After removing B220⁺ splenocytes, the remaining HSCs may not be easily identified by surface HSC markers as the signal intensity will have decreased during sorting. It may be helpful here to pick one positive HSC marker that has retained signal to separate the remaining cells from any contaminating debris or unstained cells. This entire population can then be gated for BrdU positive cells (see Figure 1).

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Figure 1.

Example flow plots of sorted HSCs after BrdU staining. The panel on the left depicts the separation of B cells (B220+ splenocytes) from sorted HSCs. In this example CD150 is used as a representative marker of the HSC population. The sorted HSC gate (shown in the right panel) is then gated for BrdU positive cells. The following markers are used in this example: B220 PE Cy5, CD150 PE and BrdU FITC.