

Video Article

Identification of a Murine Erythroblast Subpopulation Enriched in Enucleating Events by Multi-spectral Imaging Flow Cytometry

Diamantis G. Konstantinidis¹, Suvarnamala Pushkaran¹, Katie Giger¹, Stefanos Manganaris², Yi Zheng¹, Theodosia A. Kalfa¹

¹Cancer and Blood Diseases Institute, Cincinnati Children's Hospital Medical Center, University of Cincinnati College of Medicine

²IBM

Correspondence to: Theodosia A. Kalfa at Theodosia.Kalfa@cchmc.org

URL: <http://www.jove.com/video/50990>

DOI: [doi:10.3791/50990](https://doi.org/10.3791/50990)

Keywords: Basic Protocol, Issue 88, Erythropoiesis, Erythroblast enucleation, Reticulocyte, Multi-Spectral Imaging Flow Cytometry, FACS, Multiparameter high-speed cell imaging in flow, Aspect ratio, Delta centroid XY

Date Published: 6/6/2014

Citation: Konstantinidis, D.G., Pushkaran, S., Giger, K., Manganaris, S., Zheng, Y., Kalfa, T.A. Identification of a Murine Erythroblast Subpopulation Enriched in Enucleating Events by Multi-spectral Imaging Flow Cytometry. *J. Vis. Exp.* (88), e50990, doi:10.3791/50990 (2014).

Abstract

Erythropoiesis in mammals concludes with the dramatic process of enucleation that results in reticulocyte formation. The mechanism of enucleation has not yet been fully elucidated. A common problem encountered when studying the localization of key proteins and structures within enucleating erythroblasts by microscopy is the difficulty to observe a sufficient number of cells undergoing enucleation. We have developed a novel analysis protocol using multiparameter high-speed cell imaging in flow (Multi-Spectral Imaging Flow Cytometry), a method that combines immunofluorescent microscopy with flow cytometry, in order to identify efficiently a significant number of enucleating events, that allows to obtain measurements and perform statistical analysis.

We first describe here two *in vitro* erythropoiesis culture methods used in order to synchronize murine erythroblasts and increase the probability of capturing enucleation at the time of evaluation. Then, we describe in detail the staining of erythroblasts after fixation and permeabilization in order to study the localization of intracellular proteins or lipid rafts during enucleation by multi-spectral imaging flow cytometry. Along with size and DNA/Ter119 staining which are used to identify the orthochromatic erythroblasts, we utilize the parameters "aspect ratio" of a cell in the bright-field channel that aids in the recognition of elongated cells and "delta centroid XY Ter119/Draq5" that allows the identification of cellular events in which the center of Ter119 staining (nascent reticulocyte) is far apart from the center of Draq5 staining (nucleus undergoing extrusion), thus indicating a cell about to enucleate. The subset of the orthochromatic erythroblast population with high delta centroid and low aspect ratio is highly enriched in enucleating cells.

Video Link

The video component of this article can be found at <http://www.jove.com/video/50990/>

Introduction

Terminal differentiation within the erythroid lineage in mammals concludes with the dramatic process of enucleation, through which the orthochromatic erythroblast expels its membrane-encased nucleus (pyrenocyte)¹, generating a reticulocyte². The exact mechanism of this process, which is also the rate-limiting step of successful, large-scale, production of red blood cells *in vitro*, is not yet fully elucidated. The localization of key proteins and structures within enucleating erythroblasts relies on the use of fluorescent and electron microscopy³⁻⁵. This tedious process typically results in the identification of a limited number of enucleation events and does not always allow meaningful statistical analysis. Expanding on a method of erythroblast identification described previously by McGrath *et al.*⁶, we have developed a novel approach of identifying and studying enucleation events by Multi-Spectral Imaging Flow Cytometry (multiparameter high-speed cell imaging in flow, a method that combines fluorescent microscopy with flow cytometry)⁷, which can provide a sufficient number of observations to obtain measurements and perform statistical analysis.

Here, we describe first two *in vitro* erythropoiesis culture methods used in order to synchronize erythroblasts and increase the probability of capturing enucleation at the time of evaluation. Then we describe in detail the staining of erythroblasts after fixation and permeabilization in order to study the localization of intracellular proteins or lipid rafts during enucleation by multi-spectral imaging flow cytometry.

Samples are run on an imaging flow cytometer and the collected cells are gated appropriately to identify orthochromatic erythroblasts⁶. Orthochromatic erythroblasts are then analyzed based on their aspect ratio, as measured in brightfield imaging, versus their value for the parameter delta centroid XY Ter119-DNA, which is defined as the distance between the centers of the areas stained for Ter119 and DNA, respectively. The population of cells with low aspect ratio and high delta centroid XY Ter119/DNA is highly enriched in enucleating cells. Using wild-type (WT) erythroblasts versus erythroblasts with Mx-Cre mediated conditional deletion of Rac1 on Rac2^{-/-} or combined Rac2^{-/-}; Rac3^{-/-} genetic background and this novel analysis protocol of multi-spectral imaging flow cytometry, we recently demonstrated that enucleation

resembles asymmetric cytokinesis and that the formation of an actomyosin ring regulated in part by Rac GTPases is important for enucleation progression⁷.

Protocol

1. Long-term *In vitro* Erythropoiesis Culture (*Ex vivo* Erythroid Differentiation Culture Protocol by Giarratana *et al.*⁸, Modified and Adapted for Mouse Cells)

This is a 3-step long-term *in vitro* erythropoiesis protocol. In the first step (days 0-4) 2×10^5 cells/ml are placed in erythroblast growth medium supplemented with stem cell factor (SCF), interleukin-3 (IL-3), and erythropoietin (Epo). In the second step (days 5-6), cells are resuspended at 2×10^5 cells/ml and co-cultured on adherent stroma cells (MS5) in fresh erythroblast growth medium supplemented only with Epo. In the third step (days 7-9), cells are cultured on a layer of MS-5 cells in fresh erythroblast growth medium without cytokines up to enucleation (**Figure 1A**).

All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Cincinnati Children's Hospital Medical Center.

1. Harvest of bones and isolation of low-density bone marrow cells
 1. Add 2 ml sterile IMDM containing 2% fetal bovine serum (FBS) in a 15-ml conical tube and keep on ice.
 2. Euthanize a 2-6 month old wild type C57/BL6 mouse (along with or without genetically-targeted mouse of interest) following institution-approved protocol (e.g. CO₂ inhalation, followed by cervical dislocation).
 3. Isolate pelvic bones, femurs, and tibiae of both legs using forceps and scalpel, add them to the tube containing IMDM+2% FBS and keep on ice.
 4. Add 1 ml IMDM+2% FBS in a sterile flow-cytometry tube and flush bones using forceps and a tuberculin syringe with a 25-G x 5/8" needle. Flush IMDM+2% FBS through the bones a few times gently (by aspirating ~500 μ l from the cell suspension and flushing it again through the bone), and collect the bone marrow cells into the flow-cytometry tube. Flushing is complete when bones appear white.
 5. Filter cell-suspension through a 40- μ m cell strainer set on top of a 50-ml conical tube. Wash cell strainer with IMDM+2% FBS and using the same medium adjust final volume of cell suspension to 5 ml.
 6. Prepare low-density bone marrow cells (LDBM) by density gradient centrifugation: carefully layer the 5 ml of cell-suspension on 5 ml of density gradient cell separation medium 1.083 g/ml in a 15-ml tube and spin at 750 x g for 25 min at room temperature (RT) with no brake/low acceleration.
 7. Transfer supernatant (everything up to about the 2-ml mark of the 15-ml tube in order to acquire buffy coat) to a new 15-ml tube and perform one more wash with IMDM+2% FBS at 525 x g for 5 min at RT.
 8. Aspirate supernatant and lyse any remaining red blood cells (RBC) by suspending the pellet in 3 ml RBC lysis buffer for 5 min at RT. If a greater purity of erythroblasts is required at the final step (e.g. for biochemical studies), purify LDBM cells further at this step to Lin^{neg} cells by magnetic separation.
2. *Ex vivo* erythroid differentiation culture protocol starting from LDBM or Lin^{neg} cells (**Figure 1A**)
 1. Add 7 ml IMDM+2% FBS, spin at 525 x g for 5 min at RT and resuspend the pelleted cells in 2 ml erythroblast growth medium (EGM), consisting of:
 - a. StemPro-34 medium, containing
 - b. 2.6% StemPro-34 medium supplement,
 - c. 20% BIT-9500,
 - d. 900 ng/ml ferrous sulfate,
 - e. 90 ng/ml ferric nitrate,
 - f. 100 units/ml penicillin/streptomycin, 2 mM L-glutamine
 - g. 10^{-6} M hydrocortisone
 - h. and freshly added cytokines:
 - i) 100 ng/ml SCF,
 - ii) 5 ng/ml IL-3, and
 - iii) 3 IU/ml Epo.
 2. Count the cells using an automated cell counter or manually at a microscope using a hemocytometer.
 3. Plate in a 6-well cell-culture plate at a concentration of 5×10^5 cells/well to a final volume of 2.5 ml in EGM (2×10^5 LDBM cells/ml) and incubate at 37 °C (this is considered as day #0 of culture).
 4. Change medium by aspirating 1.5 ml of the supernatant, spinning at 525 x g for 5 min at RT, resuspending in 1.5 ml of fresh medium containing all 3 cytokines and adding back to the wells every day on days #2, 3, 4, to optimize proliferative phase. On day 3, remove all cells, count and split into appropriate number of wells in order to sustain well cell concentrations of $\sim 2 \times 10^5$ cells/ml. Maintain culture at 37 °C/5% CO₂.
 5. On day 4, plate MS5 cells (murine stromal cell line) to wells of a new cell-culture 6-well plate. The MS5 cell-culture medium is α -MEM containing 20% FBS, 100 units/ml penicillin/streptomycin, and 2 mM L-glutamine.
 6. On day 5, count number of cells (now significantly enriched in erythroblasts) in each well of the original culture plate using an automated cell counter or manually at a microscope using a hemocytometer.
 7. Lift all cells from each well and transfer to 15-ml conical tubes, spin down at 525 x g for 5 min at RT, aspirate supernatant and dissolve pellets with fresh EGM containing only Epo (3 IU/ml) to a concentration of 2×10^5 cells/ml.
 8. Aspirate supernatant from the wells where MS-5 cells were plated the previous day (targeted to 70-80% confluency at the time of co-culture) and add the erythroid cells in these wells in EGM containing only Epo (although not their medium of choice, MS-5 cells survive well in EGM for several days).
 9. Change medium adding fresh EPO on day #6.

10. Change medium on day #7, using EGM with no added cytokines. On days 7 through 9, test samples for enucleation with flow cytometry after staining with anti-Ter119 and Syto-16 daily and proceed to staining samples for Multi-Spectral Imaging Flow Cytometry (as detailed in section 3).

Note: Before plating and on day 2, 4, 6, and 7 of culture, monitor cultured cells for erythroblast enrichment and differentiation with flow cytometry by assessing surface markers CD44 and Ter119 vs size (FSC).⁹ Alternatively CD71, Ter119, and FSC can also be used^{10,11}.

2. Fast Enucleation Assay, According to the Protocol Described by Yoshida *et al.*¹² with Modifications (Figure 1B)

1. Stress erythropoiesis induction and stroma cell preparation for *in vitro* erythropoiesis culture
 1. Anesthetize a 2-6 month old wild type C57/BL6 mouse per IACUC guidelines (e.g. with isoflurane solution). Ensure that the mouse has been adequately anesthetized by checking for absence of reflexive responses to a gentle hind-paw pinch and for regular respirations during the procedure. Use vet ophthalmic ointment on eyes to prevent dryness while under anesthesia.
 2. Induce stress erythropoiesis via tail bleeding to a final volume of 500 μ l. Using an insulin syringe, inject equal volume of normal saline intraperitoneally to assure fluid resuscitation of the animal (hold animal in Trendelenburg position before injecting and inject in the lower abdomen so as not to damage internal organs).
 3. Do not leave the mouse unattended until it has regained motor control as indicated by the animal starting to move around the cage and being able to stand and walk without falling. The phlebotomized mouse is placed in a cage without other mice.
 4. After 2 days, plate MS-5 cells in wells of a 24-well cell-culture plate. Incubate MS-5 cells at 37 °C/5% CO₂ in MS5 cell medium (a-MEM containing 20% FBS, 100 units/ml penicillin/streptomycin, and 2 mM L-glutamine) with the goal to be 70-80% confluent in plate wells after 48 hr.
2. Harvest of spleen and processing of splenocytes
 1. Four days (96 hr) after stress erythropoiesis induction, euthanize previously bled mouse through IACUC-approved protocol (e.g. CO₂ inhalation followed by cervical dislocation).
 2. Harvest spleen and put it in a 15-ml conical tube containing IMDM 2% FBS and keep on ice.
 3. Back in the laboratory, in the tissue culture hood under sterile conditions, invert spleen-containing tube on a 40- μ m cell strainer set on top of a 50-ml tube. Crush spleen using the plunger of a 5-ml plastic syringe.
 4. Wash cell strainer with IMDM+2% FBS and using the same medium, adjust the final volume of cell suspension to 5 ml.
 5. Carefully layer the 5 ml splenocyte suspension on 5 ml density gradient cell separation medium 1.083 g/ml in a 15-ml tube and spin at 750 x g for 25 min at RT with no brake/low acceleration.
 6. Transfer supernatant (solution down to about the 2-ml mark of the 15-ml tube in order to acquire buffy coat) to a new 15-ml tube and perform one more wash with IMDM+2% FBS at 525 x g for 5 min at RT.
 7. Aspirate supernatant and lyse red blood cells by suspending the pellet in 3 ml RBC lysis buffer for 5 min at RT.
 8. Add 7 ml IMDM+2% FBS to wash cells and to dilute and neutralize the RBC lysis buffer and spin at 525 x g for 5 min at 4 °C.
 9. Aspirate supernatant and suspend pelleted cells in 2 ml of erythroblast growth medium (EGM).
3. Culture of the isolated low-density splenocytes, enriched in erythroblasts, on plastic (first stage of fast *in vitro* erythropoiesis culture)
 1. Count cells on an automated cell counter or manually by a hemocytometer. Usual number of cells isolated per spleen at this stage is $\sim 15 \times 10^6$.
 2. Suspend cells further in EGM containing the cytokines (at final concentrations): SCF 50 ng/ml, IL-3 5 ng/ml, and Epo 2 U/ml.
 3. Plate $1-5 \times 10^6$ cells in a final volume of 1 ml/well (same number of cells per well depending on total number of cells) of a 24-well cell-culture plate and incubate O/N at 37 °C/5% CO₂.
4. Culture of erythroblasts on MS5 cells (second stage of fast *in vitro* erythropoiesis culture)
 1. Aspirate supernatant from the plastic well and lift the cells (highly enriched in erythroblasts) by adding 2 ml of cold 10 mM EDTA in PBS for 5 min, on ice to each well.
 2. Put cells in fresh tubes, wash once in EGM (without cytokines) and resuspend in the same.
 3. Plate $5 \times 10^5-1 \times 10^6$ cells in a 1-2 ml volume to each MS5 cell-coated well of a 24-well plate. If pharmacological inhibitors are being used in the experiment, add them at appropriate concentrations now.
 4. Incubate for 6 to 8 hr (time guided by microscopic observation of approximately 30%-40% enucleation in the untreated WT sample). The binding of erythroblasts to MS-5 cells accelerates their enucleation.
 5. Lift cells from each well by adding 2 ml of cold PBS + 10 mM EDTA, for 5 min, on ice. Along with the erythroblasts, MS-5 cells will also be collected but these can later be easily excluded during flow cytometric analysis as FSC^{hi} Ter119⁻ cells.
 6. At this stage, cells can be fixed and stained for analysis by Multi-Spectral Imaging Flow Cytometry.

3. Staining of Erythroblasts for Localization of Intracellular Proteins or Lipid Rafts During Enucleation by Multi-spectral Imaging Flow Cytometry

1. Wash cells in PBS, spin 525 x g for 5 min at RT and aspirate supernatant.
2. Fix cells by resuspending cell pellets in 500 μ l of 3.7% formaldehyde in PBS for 15 min (fixation time may vary depending on the antigen being probed) and pipetting gently.
3. Transfer to 1.5-ml plastic centrifuge tubes and incubate for 15 min at RT.
4. Spin on a bench microcentrifuge 2,000 x g for 20 sec, aspirate supernatant and perform one wash by adding 500 μ l PBS to each tube and pipetting gently.

5. Spin on a bench microcentrifuge 2,000 x g for 20 sec, aspirate supernatant and keep tubes on ice for at least 15 min. Permeabilization steps 3.6-3.9 should be done quickly and efficiently, and following spinning/aspiration at RT, cell pellets should immediately be put back on ice, in order for cells to better retain their integrity.
6. Take acetone solutions out from -20 °C freezer and put on ice. Permeabilize cells by resuspending cell pellets first in 500 µl ice-cold 50% acetone (1:1 with dH₂O) and pipetting gently.
7. Spin on bench microcentrifuge 2,000 x g for 20 sec, aspirate supernatant and resuspend cell pellets in 500 µl ice-cold 100% acetone by pipetting gently.
8. Spin on bench microcentrifuge 2,000 x g for 20 sec, aspirate supernatant and resuspend cell pellets once more in 500 µl ice-cold 50% acetone by pipetting gently.
9. Spin on bench microcentrifuge 2,000 x g for 20 sec, aspirate supernatant and wash cells once in cold FACS buffer (PBS + 0.5% BSA) by pipetting gently.
10. Prepare labeling cocktail with antibodies or markers for the molecules of interest: 0.1 U/100 µl AF488-phalloidin for F-actin staining and 1 µl/100 µl Ter119-PECy7 for erythroid cell staining. Alternative or additional staining can also be done using AF-488-anti-β-tubulin antibody (1:50), AF-594-conjugated cholera toxin subunit B to label lipid rafts (1:200), anti-pMRLC (Ser19) primary antibody for the phosphorylated myosin regulatory light chain (1:50), followed by anti-rabbit AF-488-conjugated secondary antibody (1:400), and anti-γ-tubulin primary rabbit antibody (1:100) followed by anti-rabbit AF-555-conjugated secondary antibody (1:300).
11. Following supernatant aspiration, resuspend cell pellets in 100 µl of the marker cocktail, pipette gently and incubate for 30 min at RT.
12. Prepare FACS buffer containing 2.5 µM of the nuclear stain Draq5.
13. Wash cells in FACS buffer, spin down on bench microcentrifuge 2,000 x g for 20 sec, aspirate supernatant and resuspend in 60 µl FACS buffer containing Draq5.
14. Run samples on the imaging flow cytometer to collect at least 10,000 events per experiment, compensate the raw data files as previously published¹³, and analyze results as shown in **Figure 2**, using the analysis software specific to the imaging flow cytometer.

Representative Results

First, cells are analyzed based on their Brightfield Aspect Ratio (the ratio of the length of their minor versus their major axis) and their Brightfield Area (indicative of their size). Events with a Brightfield Area value lower than 20 and higher than 200 are mostly debris and cell aggregates, respectively, and are excluded from the analysis (**Figure 2A**). Single cells (gate "R1") are then analyzed based on their value for the Gradient RMS parameter, which indicates sharpness of image. Gate "R2" is created containing cells with Gradient RMS value more than the 50th percentile in order to select the images taken well in focus (**Figure 2B**). Cells are then gated based on their size as measured by their Brightfield Area, and their positivity for the erythroid marker Ter119 as measured by the Ter119 fluorescent stain-Mean Pixel parameter (gate "Ter119 positive", **Figure 2C**). Cells very low or very high for Ter119, are either non-erythroids or remaining cell aggregates, respectively, and are excluded from the analysis. In the next step, cells are selected based on their Draq5 Aspect Ratio Intensity (the ratio of the minor versus the major axis intensity of their nucleus) and the Intensity of Draq5 (**Figure 2D**). Draq5 negative cells (mostly enucleated cells, such as reticulocytes and RBCs), and cells with a low Draq5 Aspect Ratio (mostly doublets) are excluded from the analysis. Draq5 positive cells (gate "DNA positive", mostly erythroblasts at this point) are then analyzed based on their Ter119 Area, which indicates the size of the cell, and their Ter119 Mean Pixel/Area (density of Ter119 expression), which indicates the brightness of Ter119 staining. Orthochromatic erythroblasts (gate "OrthoE") are recognized as small, Ter119^{hi} cells (**Figure 2E**). Finally, a subpopulation of the orthochromatic erythroblasts highly enriched in enucleating cells is characterized by low Brightfield Aspect Ratio, which is a measurement of cell elongation, calculated by the ratio of minor axis/major axis of the cell image in the Brightfield channel M01, and by high Delta Centroid XY Ter119/Draq5, which is defined by the distance between the center of the incipient Ter119⁺-reticulocyte and the center of the Draq5⁺-nucleus (gate "enucleating cells" in **Figure 2F**).

Along with antibody against Ter119 and the DNA-stain Draq5, the cells have also been stained for filamentous actin (F-actin) with fluorescent phalloidin to evaluate localization of F-actin during erythroblast enucleation⁷. Of note, a progression of enucleation can be visualized in the fixed cells, as cells with a decreasing aspect ratio (*i.e.* increasingly elongated) and increasing delta centroid XY Ter119/Draq5 are observed (**Figure 3**). F-actin is observed to concentrate at the cleavage furrow during enucleation and then dissipate once the nucleus is extruded. Moreover, co-localization of actin and myosin at the cleavage furrow between incipient reticulocyte and nucleus can be demonstrated by multi-spectral imaging flow cytometry after co-staining of WT erythroblasts for pMRLC (phosphorylated myosin regulatory light chain) and F-actin⁷.

Other proteins and structures of interest can also be stained with appropriate antibodies or fluorescent markers to allow imaging studies of their role during enucleation. Polarized microtubule formation is visible in WT orthochromatic erythroblasts prior to enucleation but not in erythroblasts treated with colchicine (**Figure 4A**). Inhibition of tubulin polymerization by colchicine diminishes cell polarization, as demonstrated by measuring the parameter delta centroid XY BF/Draq5 between the center of the cell body seen in Brightfield channel and the center of the nuclear staining achieved with Draq5 (**Figure 4B**).

Utilizing WT or Rac-deficient (after genetic or pharmacologic manipulation) erythroblasts in multi-spectral imaging flow cytometry allowed imaging studies that demonstrate the role of Rac GTPases in enucleation. Rac GTPases regulate at least in part the formation of an actomyosin ring as well as the confluence of lipid rafts in the furrow between incipient reticulocyte and pyrenocyte⁷.

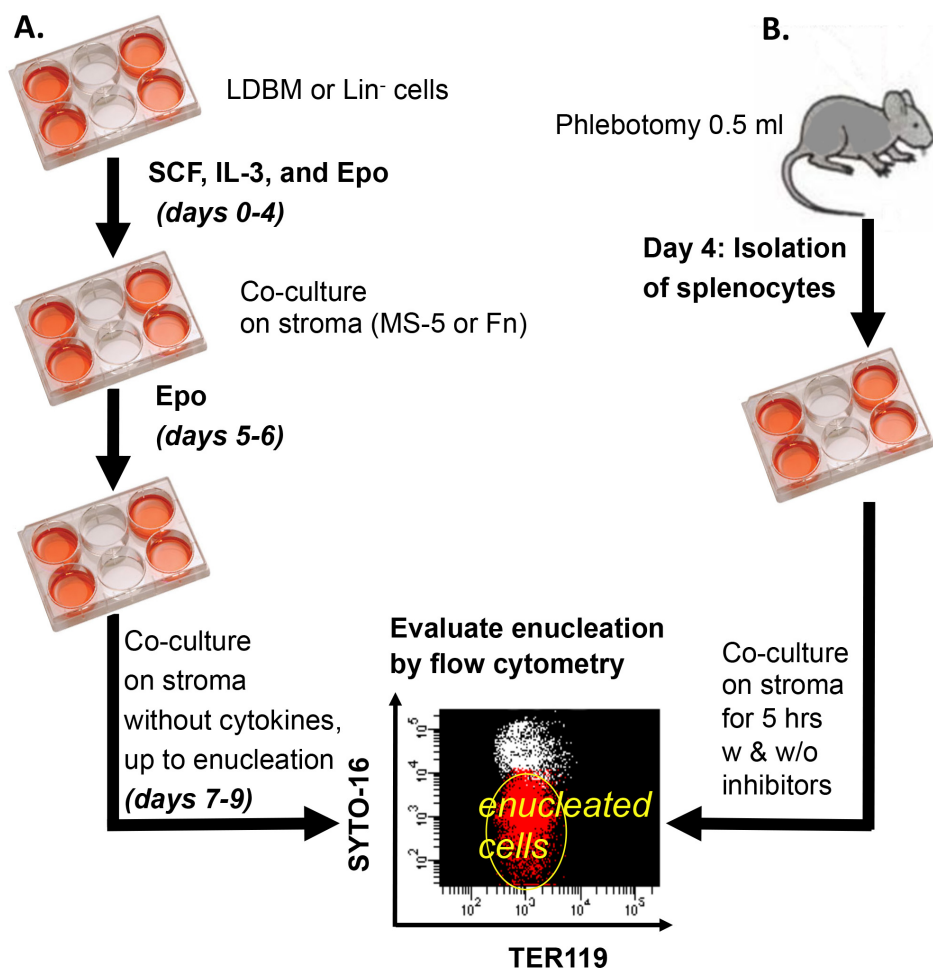


Figure 1. Schematic demonstration of the erythropoiesis *in vitro* protocols used in order to produce enucleating erythroblasts for studies. **A.** Long-term *in vitro* erythropoiesis culture initiated from LDBM or Lin⁻ cells. **B.** Fast enucleation assay initiated by splenocytes highly enriched in erythroblasts after stress erythropoiesis induction *in vivo* by phlebotomy. [Please click here to view a larger version of this figure.](#)

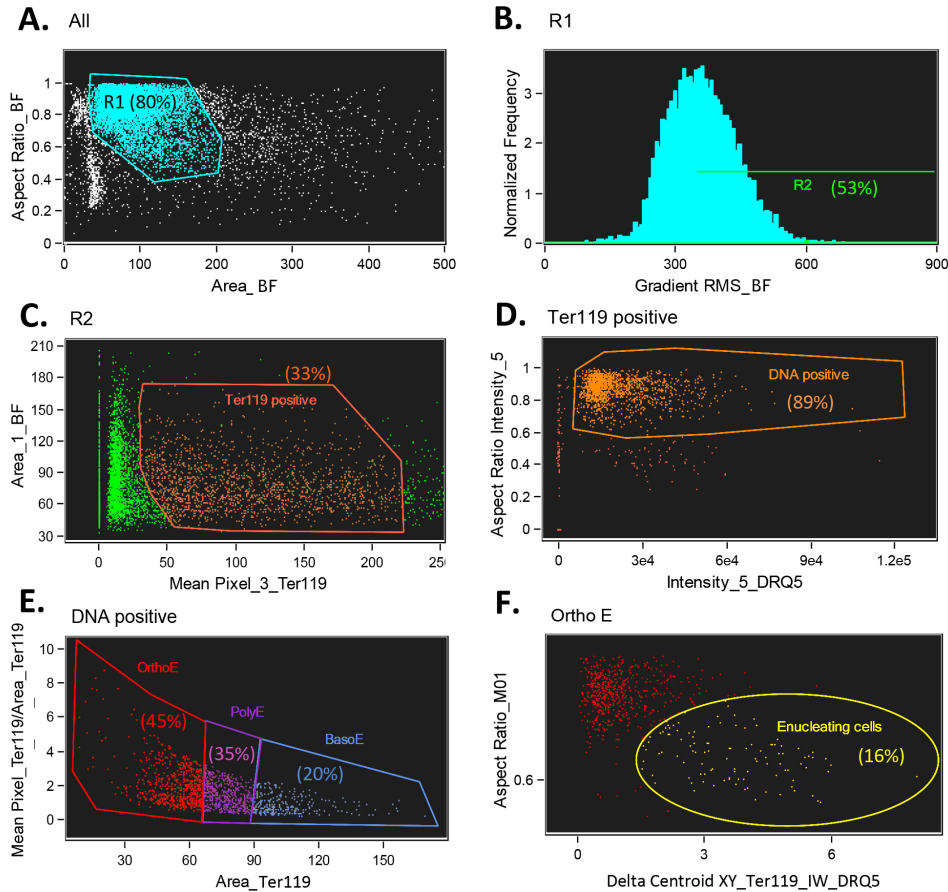


Figure 2. Analysis of data utilizing the analysis software specific to the imaging flow cytometer. Successive gating of the populations of interest is shown in panels A-F. The number in parenthesis indicates the approximate percent of the corresponding parent population, in this experiment. **A.** Initial gating of R1 population removes cell aggregates and cellular debris. **B.** Gate R2 out of R1 includes the cells that have been imaged clearly, excluding out of focus cells. **C.** Ter119-positive cells (out of R2) are selected, excluding cells that are either negative for Ter119 or are too intensely stained because of their presence in aggregates. **D.** DNA-positive cells (out of Ter119-positive cells) are selected, after plotting for Aspect Ratio Intensity versus intensity of the nuclear stain (here Draq5 read in channel 5). **E.** DNA- and Ter119-positive cells are gated into basophilic, polychromatophilic and orthochromatic erythroblasts based on their location in the Ter119 Mean Pixel versus Ter119 Area (here read in channel 3), as shown previously by McGrath *et al.*⁶. **F.** The enucleating cells are those cells out of the orthochromatic erythroblasts, which have low Aspect Ratio (a measurement of cell elongation in brightfield (BF) channel) and high Delta Centroid XY Ter119/Draq5 (distance between center of forming Ter119⁺-reticulocyte and center of nucleus). This research was originally published in Blood: Konstantinidis DG, Pushkaran S, *et al.* Signaling and cytoskeletal requirements in erythroblast enucleation. *Blood*. 2012;119(25):6118-6127 by the American Society of Hematology. [Please click here to view a larger version of this figure.](#)

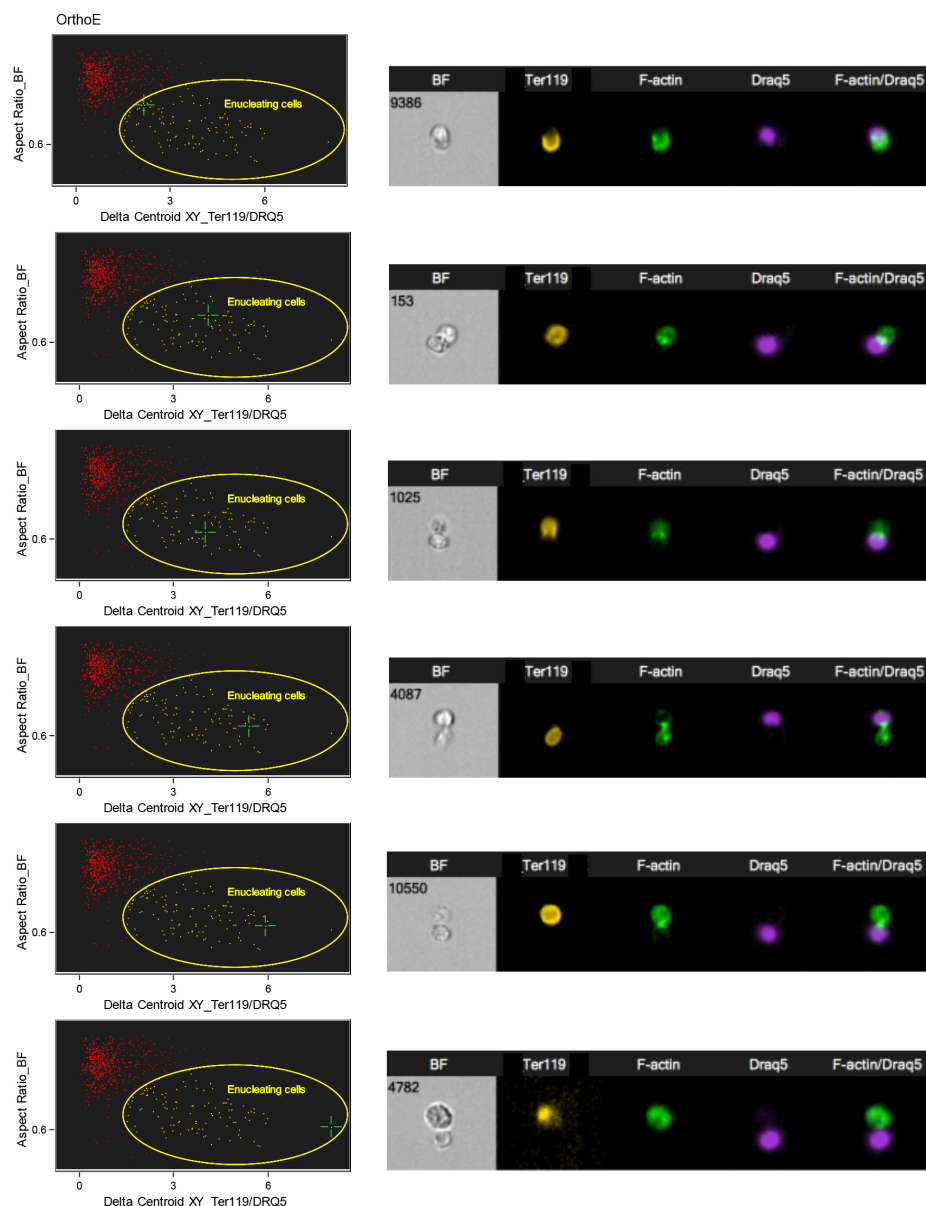


Figure 3. Representative images of enucleating erythroblasts with progressively increasing Delta Centroid XY Ter119/Draq5. WT mouse orthochromatic erythroblasts, stained with Ter119-PECy7, phalloidin-AF488, and Draq5, are gated per their aspect ratio and delta centroid XY Ter119/Draq5. Cells are shown fixed at different, successive stages of enucleation, with a progression that corresponds to decreasing aspect ratio and increasing delta centroid XY Ter119/Draq5 (green-cross within the yellow gate shows the position of the cell imaged on the right). In the cell images from top to bottom, F-actin can be observed during enucleation to concentrate at the cleavage furrow and then dissipate once the nucleus is extruded (as shown in cell #4782 at the lower image). [Please click here to view a larger version of this figure.](#)

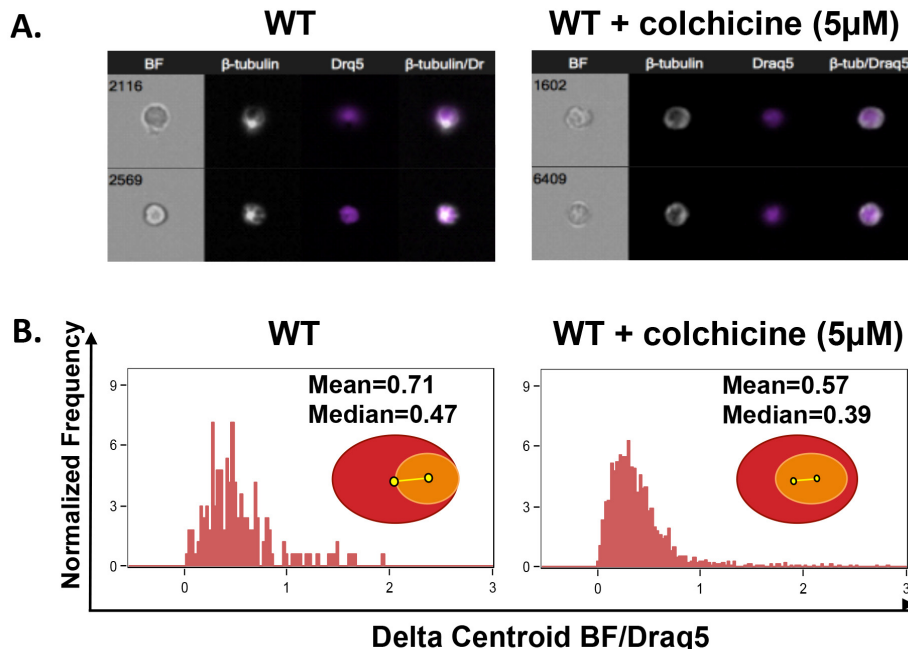


Figure 4. Formation of a unipolar microtubule assembly and polarization of orthochromatic erythroblasts precedes enucleation. A. Polarized microtubule formation is visible in control WT erythroblasts (stained with anti- β -tubulin–AlexaFluor-488 and the nuclear stain Draq5), whereas β -tubulin is diffusely stained in the erythroblasts incubated with colchicine (5 μ M) for 6 hr in the fast *in vitro* enucleation assay. **B.** Multi-spectral imaging flow cytometry can offer a quantitative evaluation of cell polarization by analysis of the distribution of the parameter Delta Centroid XY BF/Draq5, which measures the distance between the center of the cell body as seen in bright-field and the center of the nuclear staining achieved with Draq5 (schematic representation in the inset). Delta Centroid BF/Draq5 values of colchicine-treated WT orthochromatic erythroblasts are statistically significantly different than the control Delta Centroid BF/Draq5 values ($p < 0.001$). This research was originally published in Blood: Konstantinidis DG, Pushkaran S, *et al.* Signaling and cytoskeletal requirements in erythroblast enucleation. *Blood*. 2012;119(25):6118-6127 by the American Society of Hematology. [Please click here to view a larger version of this figure.](#)

Discussion

In recent years the study of erythroblast enucleation has gained increasing momentum since it is the step in *in vitro* erythropoiesis cultures that is most difficult to reproduce efficiently in order to achieve successful, large-scale production of red blood cells *ex vivo*. Up until recently, the study of erythroblast enucleation utilized mainly fluorescence microscopy and flow cytometry methods. Fluorescence microscopy methods, albeit helpful in identifying participating molecules, require days of microscopic observation to identify a small number of orthochromatic erythroblasts undergoing enucleation within hundreds of cells fixed at a particular point in time. Flow cytometry methods, on the other hand, are very helpful in evaluating the rate of enucleation in a culture, as well as the effects of pharmacologic or genetic manipulation of particular molecules on this process, but do not provide any data on the intracellular localization of these molecules.

Multi-spectral imaging flow cytometry combines the benefits of flow cytometry and immunofluorescence microscopy since it allows rapid acquisition of both flow cytometric and morphologic data on several thousands of cells. This is a significant advantage versus classic flow cytometry in erythropoiesis studies, since the different stages of erythroblast differentiation (proerythroblasts, basophilic, polychromatophilic, and orthochromatic) have been defined using morphological criteria⁶. However, multi-spectral imaging flow cytometry is optimal for visualization of cellular structures rather than relative quantitation comparisons in population numbers. For such comparisons, routine flow-cytometry that does not require the permeabilization step necessary for immunofluorescence of intracellular structures performs better. For example the relative percentage of BasoE:PolyE:OrthoE in **Figure 2E** does not correspond to the physiologic ratio of 2:4:8, since the mature erythroblasts are more sensitive to the permeabilization step and are preferentially lost during the staining process.

The imaging data can be processed in association with the flow cytometry data using the analysis software specific to the imaging flow cytometer, allowing the collection of cells with certain morphologic characteristics within gates. Approximately one hundred enucleating cells can be identified within a population of 10,000 erythroblasts with the analysis method described above in a fast and efficient manner⁷, allowing more meaningful observations and statistical evaluation.

Moreover, image processing is facilitated with the analysis software specific to the imaging flow cytometer allowing quantitative analysis of such characteristics as the Delta Centroid XY to study *e.g.* the relative position of the nucleus to the cytoplasm that can be used as a measure of polarization.

Critical steps within the protocol and troubleshooting

It is well known that even gentle pipetting can result in the separation of reticulocyte and pyrenocyte¹². This has the potential to severely limit the number of enucleation events imaged. As a result, care must be taken, particularly when lifting erythroblasts bound to MS5 cells in culture.

Fixation with formaldehyde solution can cause alterations to extracellular regions of surface markers resulting in decreased specific antibody binding and/or increased non-specific antibody binding. An advantage of the imaging flow cytometer is that surface staining is visualized and its quality can thus be evaluated. Dotted, instead of uniform, staining for abundant surface markers such as Ter119 indicates overfixation and should be tackled through a mix of lower formaldehyde concentration and shorter duration of fixation.

Following fixation, keeping cells on ice for at least 15 min is vital in order to prevent excess cell breakage during the permeabilization process due to temperature differences. Although acetone permeabilization maintains the fragile late erythroids better than detergent-mediated permeabilization, the step where 100% acetone is required will result in a noticeable, but not detrimental, loss of cells. At the end, after a wash with cold FACS buffer, cells are allowed at room temperature for the antibody incubation steps.

The imaging flow cytometer has a set rate of flow (cells/sec) depending on the lens used (rate is decreased as the magnification increases). Following final wash before measurement, it is recommended that cell pellets are resuspended in a small volume (50–60 μ l), in order to accelerate processing of the sample. For a large number of samples that require long duration of run (over 30 min), samples should be kept on ice.

The long-term enucleation assay offers the benefit of an expanded erythroid cell production in order to produce enough cells that can be collected at the enucleation stage for biochemical evaluation like immunoblotting and pull-downs. The fast enucleation assay gives the benefit of time requiring only 2 days to perform, although a mouse needs to be phlebotomized 4 days prior to the experiment. We have not observed a significant difference in enucleation efficiency between the two methods. Of note, flow cytometry evaluation, which does not require the permeabilization step necessary for immunofluorescence of intracellular structures, as described before⁷, is most appropriate for the quantitative evaluation of enucleation efficiency.

Limitations of the technique

The method described here utilizes induced stress erythropoiesis *in vivo* and culture in medium containing hydrocortisone *in vitro* in order to amplify erythroid populations and synchronize them at the stage of enucleation. Both of these conditions likely imitate erythroblast-enucleation under stress. In addition, hydrocortisone significantly increases erythroid yield and survival. To obtain similar yield of enucleation events from bone marrow cells derived from wild type mice with steady-state erythropoiesis and cultured without hydrocortisone (therefore avoiding synchronization), we would need to culture cells from multiple mice and run and process a lot more events through Multi-Spectral Imaging Flow Cytometry. Although multi-spectral imaging flow cytometry accelerates collection of morphological data immensely in comparison to immunofluorescence microscopy using magnification lens up to 60x, comparison of the observations obtained by imaging flow cytometer with classic, Z-stack, and confocal microscopy images is valuable to obtain better detail, since the objective lens in a microscope can provide magnification up to 100x and Z-stack images give a 3-dimensional impression of the structures, which is not attainable by imaging flow cytometer in the same cell. The relative high number of similar cells collected in a multi-spectral imaging flow cytometry experiment, at a random orientation, partially compensates this limitation allowing observations from a different view-point.

Disclosures

The authors declare no competing financial interests.

Acknowledgements

The authors thank the Research Flow Cytometry Core at Cincinnati Children's Hospital Research Foundation and Richard Demarco, Sherree Friend, and Scott Mordecai from the Amnis Corporation (part of EMD Millipore) for expert technical support. This work was supported by the National Institutes of Health grants K08HL088126 and R01HL116352 (T.A.K.) and P30 DK090971 (Y.Z.).

References

- McGrath, K. E., Kingsley, P. D., Koniski, A. D., Porter, R. L., Bushnell, T. P., & Palis, J. Enucleation of primitive erythroid cells generates a transient population of "pyrenocytes" in the mammalian fetus. *Blood*. **111**, 2409–2417, doi:10.1182/blood-2007-08-107581 (2008).
- Chasis, J. A., & Mohandas, N. Erythroblastic islands: niches for erythropoiesis. *Blood*. **112**, 470–478, doi:112/3/470 [pii] 10.1182/blood-2008-03-077883 (2008).
- Koury, S. T., Koury, M. J., & Bondurant, M. C. Cytoskeletal distribution and function during the maturation and enucleation of mammalian erythroblasts. *J Cell Biol*. **109**, 3005–3013 (1989).
- Ji, P., Jayapal, S. R., & Lodish, H. F. Enucleation of cultured mouse fetal erythroblasts requires Rac GTPases and mDia2. *Nat Cell Biol*. **10**, 314–321, doi:ncb1693 [pii] 10.1038/ncb1693 (2008).
- Keerthivasan, G., Small, S., Liu, H., Wickrema, A., & Crispino, J. D. Vesicle trafficking plays a novel role in erythroblast enucleation. *Blood*. **116**, 3331–3340, doi:10.1182/blood-2010-03-277426 (2010).
- McGrath, K. E., Bushnell, T. P., & Palis, J. Multispectral imaging of hematopoietic cells: where flow meets morphology. *J Immunol Methods*. **336**, 91–97, doi:S0022-1759(08)00141-5 [pii] 10.1016/j.jim.2008.04.012 (2008).
- Konstantinidis, D. G. *et al.* Signaling and cytoskeletal requirements in erythroblast enucleation. *Blood*. **119**, 6118–6127, doi:10.1182/blood-2011-09-379263 (2012).
- Giarratana, M. C. *et al.* Ex vivo generation of fully mature human red blood cells from hematopoietic stem cells. *Nat Biotechnol*. **23**, 69–74 (2005).
- Chen, K., Liu, J., Heck, S., Chasis, J. A., An, X., & Mohandas, N. Resolving the distinct stages in erythroid differentiation based on dynamic changes in membrane protein expression during erythropoiesis. *Proc Natl Acad Sci U S A*, doi:0909296106 [pii] 10.1073/pnas.0909296106 (2009).

10. Kalfa, T. A. *et al.* Rac1 and Rac2 GTPases are necessary for early erythropoietic expansion in the bone marrow but not in the spleen. *Haematologica*. **95**, 27-35, doi:95/1/27 [pii] 10.3324/haematol.2009.006239 (2010).
11. Koulis, M., Pop, R., Porpiglia, E., Shearstone, J. R., Hidalgo, D., & Socolovsky, M. Identification and analysis of mouse erythroid progenitors using the CD71/TER119 flow-cytometric assay. *J Vis Exp.*, doi:10.3791/2809 (2011).
12. Yoshida, H., Kawane, K., Koike, M., Mori, Y., Uchiyama, Y., & Nagata, S. Phosphatidylserine-dependent engulfment by macrophages of nuclei from erythroid precursor cells. *Nature*. **437**, 754-758 (2005).
13. Orlyn, W. E. *et al.* Sensitivity measurement and compensation in spectral imaging. *Cytometry A*. **69**, 852-862, doi:10.1002/cyto.a.20306 (2006).