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Delineating stages of erythropoiesis using imaging flow cytometry

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ABSTRACT

Adult humans need to make 2.5 million red blood cells (RBCs) every second to maintain a steady state level of 25 trillion circulating RBCs. Understanding normal erythropoiesis as well as diseases that afflict the erythron, such as genetic anemias, hyperproliferative disorders, and myelodysplastic syndromes, requires a robust method to delineate erythropoietic intermediates. In order to apply the power of flow cytometry to these studies, challenges of limited immunophenotypic markers, incorporation of significant changes in morphology, and maturational changes that occur along a continuum need to be met. Imaging flow cytometry (IFC) provides a solution to address these challenges. Integration of changes in immunophenotype, loss of RNA (ribosomes), and enucleation, with morphological characteristics of cell and nuclear size, can be used to delineate erythroblasts that correlate with classical histological classifications. A protocol is described that demonstrates the basic approaches of staining panel selection, mask generation and selection of features to best sequentially refine erythroid intermediates and remove contaminating cells with overlapping immunophenotype. Ultimately erythroid cells in the murine bone marrow are divided into seven sub-populations using IFC including four erythroblasts (pro-, basophilic, polychromatophilic and orthochromatic), the pyrenocyte, which contains the eliminated nucleus, the enucleated reticulocyte and the mature RBC.

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1. Introduction

Erythropoiesis is an incredibly robust process, producing 2.5 million red blood cells (RBCs) per second at steady state [1]. Delineating intermediates of erythropoiesis is critical for the diagnosis and study of diseases such as hereditary anemias, hyperproliferative disorders, and myelodysplastic syndromes. Additionally, quantifying erythropoietic intermediates will be an important tool as attempts to make ex vivo RBCs for transfusion struggle to mimic the productivity of the in vivo system. Utilization of flow cytometric analytical techniques in studying erythropoiesis have been hampered by paucity of markers that correlate with the morphological changes that historically define precursors. Additionally, characteristics of erythroid maturation occur along a continuum, without the step-wise changes in phenotype that have made immune cells so amenable to flow cytometry. Many cell types share with erythroid cells the challenges of limited cell markers, morphologic classification and relevant changes along a continuum. Approaches need to be developed that extend the benefit of the quantitative power of flow cytometry and functional assays to these systems without losing connectivity to extant data based on morphology. Imaging flow cytometry (IFC) is one such approach because it can overlay morphometric and fluorescent marker data on the same cell. Here we utilize IFC to correlate changes in cell and nuclear size with cell surface immunophenotype, cell cycle status, and ribosome content during erythroid maturation in the murine marrow. A gating protocol is presented that ranks erythroid intermediates by maturation state and then subdivides them to correlate with erythroblast stages classically defined by morphologic examination. These studies also present examples of combining masking approaches with a variety of intensity measurements that can be applied to many biological systems.

2. Materials and methods

2.1. Cell isolation

Murine (CD-1, Charles River) femoral marrow was flushed as previously described [2] and filtered through a 40-μm cell strainer (BD Biosciences).

2.2. Cell staining

For live cell staining, 10^7 marrow cells were blocked in 10% rat whole serum (Invitrogen) prepared in PB2 (Dulbecco's

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phosphate-buffered saline and 0.1% glucose, Invitrogen; 0.3% bovine serum albumin, Gemini BioProducts) for 15 min on ice and stained in 5% rat whole serum with 1:200 dilution of antibodies for 20 min on ice. Antibodies used in different panels in figures were PE-anti-mouse CD71 (clone R17217) from eBioscience; AF488-Ter119 (clone TER-119) from BioLegend; PECF594 CD117 (kit; clone 2B8) and EF450 CD44 (clone IM7) from eBioscience; and APC-Ter119 (clone TER-119) from BD Biosciences. Stained cells were washed in PB2 and spun at 300 g for 5 min and resuspended in 60 μl of PB2. 2 μg/ml Thiazole orange (TO) (Sigma-Aldrich/EMDmillipore) was included during antibody staining where specified. For DNA staining either 50 μM Vybrant Violet (VV) (Thermo Fisher Scientific) was used during antibody staining or cells were brought to 2.5 μM DRAQ5 (eBioscience) or 1 μg/ml 14′, 6-Diamidino-2-Phenylindole, Dihydrochloride (DAPI) (Thermo Fisher Scientific) just before acquisition. Cell cycle analysis was carried out on marrow cells treated with bromodeoxyuridine (BrdU) [3] for 90 min and detected according to manufacturers instructions using a FITC BrdU flow kit (BD Biosciences). Initial testing of staining panels included FMO negative controls which contain all the stains except one, to determine what the background staining is in that channel. These controls can also be included for critical or faint stains. The addition of morphometric information on staining pattern also helps to distinguish true signal from artifact.

2.3. Acquisition of imaging flow cytometry data

Cells were acquired on an ImageStreamX imaging flow cytometer (Amnis/EMD Millipore, Seattle, WA) using INSPIRE (version 4.1) software with lasers set to maximum values that did not cause saturation in the brightest stains. Typical laser settings for live cells were 90 mW 405 nm, 100 mW 488 nm, 20 mW 658 nm and fixed cells 75 mW 405 nm, 40 mW 488 nm, 120 mW 658 nm. Stains were read in the following channels: Brightfield channels 1 (488 nm laser – 467.5/75 nm) and 9 (594 nm laser – 577.5/35 nm), TO, AF488 and FITC channel 2 (488 nm laser – 532.5/55 nm), PE channel 3 (488 nm laser – 577.5/35 nm), PECF594 channel 4 (488 nm laser – 627.5/65 nm), EF450, VV, and DAPI channel 7 (405 nm laser – 467.5/75 nm), DRAQ5 and APC channel 11 (658 nm laser – 700/80 nm).

2.4. Analysis of imaging flow cytometry data

Data were analyzed using IDEAS (version 6.2, Amnis/EMDmillipore) software and its compensation wizard and gated as described in the Results section. The masks and features used are presented in Table 1.

3. Results

3.1. Morphometric and immunophenotypic changes during erythropoiesis

Erythropoiesis follows the general pattern of differentiation where hematopoietic stem cells mature into progressively restricted progenitors, which then differentiate into precursors that accumulate products associated with specialized functions as they gain distinctive morphologic features (Fig. 1A). There are two unipotential erythroid progenitors; the burst forming unit-erythroid (BFU-E) and its descendent, the colony forming unit-erythroid (CFU-E). Recently, significant improvements defining the cell surface immunophenotype of these progenitors in human and mouse systems have been published [4–6]. The CFU-E divides to form the most immature erythroid precursor, the proerythroblast, which already expresses many erythroid-specific genes [7,8] including glycophorin A, that can be recognized by CD235a and Ter119 in human and mouse, respectively [9]. Proerythroblasts are large cells with a high nuclear to cytoplasmic ratio and very basophilic cytoplasm when stained with a Romanowsky-based protocol (eg., Wright, Giemsa). As erythroblasts continue to divide, they progressively lose RNA content and accumulate hemoglobin and the resultant change in cytoplasmic staining is reflected in their nomenclature from basophilic to polychromatophilic and finally "orthochromatic" or "normoblasts" indicating cells with the "correct" color. Late stage murine erythroblasts, unlike their human counterparts, do not gain a pink cytoplasm, although the decrease in basophilic staining is still quite evident [10] (Fig. 1A). Concurrent with these cytoplasmic changes, the total cell volume decreases and the nucleus condenses. Finally, orthochromatic cells undergo a unique division creating an enucleate reticulocyte with trace basophilic elements and a short-lived nucleated cell, the pyrenocyte. While pyrenocytes have sometimes been referred to by the misnomer "free-nuclei", they actually contain a small amount of cytoplasm and, importantly, a cell membrane which is involved in signaling macrophages for consumption and useful for immunophenotyping [11,12]. Reticulocytes continue to mature in the marrow before exiting to complete their maturation to RBCs in the circulation.

A number of fluorescent stains that change during murine erythropoiesis are available for use in IFC (Fig. 1B). Erythroid-specific Ter119 (or CD235a in human) can be used to gate erythroid cells, although Ter119 total intensity is relatively constant after the proerythroblast stage. However, cells appear to become brighter (Fig. 1B) in Ter119 signal during maturation because there is a higher density of the antibodies on smaller cells. For example, the polychromatic and orthochromatic erythroblasts imaged in Fig. 1B have the same value for total Ter119 intensity. In IFC, this

Table 1
Masks and features used in analysis of erythropoiesis by IFC.

Default	Mask default name	Features default name	Axis label
Yes	MC	Intensity_MC_DRAQ5 Intensity_MC_CD71 Intensity_MC_Kit	Intensity_DRAQ5 Intensity_CD71 Intensity_Kit
Yes	M01	Gradient RMS_M01_BF	Gradient RMS_M01_BF
Yes	M02	Median Pixel_M02_Ter119	Median Pixel_M02_Ter119
No	Morphology(M11,DRAQ)	Area_Morphology(M11,DRAQ)	Area_Nucleus
No	Morphology(M02,Ter119)	Median Pixel_ Morphology(M02,Ter119)_Ter119	Median Pixel_Morph_Ter119
No	AdaptiveErode(M01,BF,81)		
No	Morphology(M03,CD71)		
No	Morphology(M02,Ter119)Or AdaptiveErode(M01,BF,81)Or Morphology(M03,CD71)	Area_(Default mask name) Aspect Ratio_(Default mask name)	Area_Cytoplasm Aspect Ratio_Cytoplasm

MC = combined mask, M01-M11 = MChannel#, BF = brightfield.

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