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Characterization of neutrophils and macrophages from *ex vivo* cultured murine bone marrow for morphologic maturation and functional responses by imaging flow cytometry

Margery G. H. Pelletier^a, Klaudia Szymczak^a, Anna M. Barbeau^a, Gianna N. Prata, Kevin S. O'Fallon^b, and Peter Gaines^{a,*}

^aDepartment of Biological Sciences, Biomedical Engineering and Biotechnology Program, University of Massachusetts Lowell, Lowell, MA

^bIntegrative Physiology Laboratory, Combat Feeding Directorate, U.S. Army Natick Soldier RDEC, Natick, MA

Abstract

Neutrophils and macrophages differentiate from common myeloid progenitors in the bone marrow, where they undergo nuclear morphologic changes during maturation. During this process, both cell types acquire critical innate immune functions that include phagocytosis of pathogens, and for neutrophils the release of nuclear material called nuclear extracellular traps (NETs). Primary cells used to study these functions are typically purified from mature mouse tissues, but bone marrow-derived *ex vivo* cultures provide more abundant numbers of progenitors and functionally mature cells. Routine analyses of these cells use conventional microscopy and flow cytometry, which present limitations; microscopy is laborious and subjective, whereas flow cytometry lacks spatial resolution. Here we describe methods to generate enriched populations of neutrophils or macrophages from cryopreserved mouse bone marrow cultured *ex vivo*, and to use imaging flow cytometry that combines the resolution of microscopy with flow cytometry to analyze cells for morphologic features, phagocytosis, and NETosis.

Keywords

Myeloid; Phagocytosis; NETosis; fluorescence microscopy; nuclear decondensation; cell morphology

*Corresponding Author: Peter Gaines, Department of Biological Sciences, University of Massachusetts Lowell, One University Avenue, Lowell, MA 01854, USA. Phone: 978-934-2894, Fax: 978-934-3044, peter_gaines@uml.edu.

Declaration

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1. Introduction

Neutrophils and macrophages are myeloid cells essential to innate immunity that differentiate from hematopoietic stem cells in the bone marrow. During their differentiation, these professional phagocytes acquire specialized functions, including the capacity to recognize, bind and internalize pathogens leading to the formation of intracellular phagosomes. The activated cells then destroy ingested pathogens by producing an arsenal of antimicrobial proteases, reactive oxygen species (ROS), and nitric oxide (NO) [1]. Both cell types also undergo nuclear morphologic changes during early maturation: nuclei of early neutrophil progenitors (e.g. myelocytes) and monocytes condense while forming indented/kidney-like structures [2, 3]. Myelocyte nuclei then continue to condense during late stages of maturation to form segmented or lobulated structures connected by thin strands of chromatin in mature, polymorphonuclear neutrophils (PMNs) [4]. By comparison, monocytes further mature into macrophages that form small, spherical nuclei as their nuclear-to-cytoplasmic (N/C) ratios dramatically decrease. Nuclear condensation and lobulation are architectural features thought to increase nuclear flexibility, which facilitates the capacity of neutrophils and perhaps monocytes to escape capillary beds (extravasation) and migrate between or even through endothelial cells or fibroblasts toward infected tissues [5–7]. Neutrophils are the first responders to acute infections and injuries, primarily operating in a “seek and destroy” fashion supported by the release of ROS and proteolytic enzymes. Neutrophils also produce critical pro-inflammatory cytokines that increase vascular permeability and promote capillary leakage, thereby facilitating the recruitment of more circulating neutrophils along with circulating monocytes. A unique feature of neutrophils is that activated cells can release a web of chromatin termed neutrophil extracellular traps (NETs) that help capture pathogens and expose them to localized, high concentrations of antimicrobial agents contained within the NETs. Recruited monocytes and differentiated macrophages release additional pro-inflammatory cytokines as well as digest microbes, dead neutrophils, and cellular debris within damaged tissue. Macrophages also produce anti-inflammatory cytokines that stimulate wound healing and tissue repair, along with presenting antigens that activate lymphocytes (e.g. T-cells). Combined, these myeloid cells create a partnership that provides an impressive defense mechanism to protect mammals from a plethora of potentially harmful pathogens including bacteria and fungi, while also promoting tissue remodeling and repair. Evidence for their importance to human biology is easily found in the symptoms of diseases that diminish their production and/or functionality, including inflammatory diseases, leukemias, or neutropenias. However, when functional activities of these cells are not properly regulated, they can be destructive. For instance, secretions produced by dysfunctional monocytes have been associated with rheumatoid arthritis and liver fibrosis [8, 9]. Production of ROS, NO, and/or pro-inflammatory cytokines by aberrantly active neutrophils or macrophages contribute to disorders that include autoimmune diseases, myocardial ischemia/reperfusion injury, and atherosclerosis [10, 11]. Interestingly, NETs produced by neutrophils have now been linked to pathological venous thrombosis, vasculitis, and autoimmune diseases [12, 13]. By studying the maturation and functional activities of mouse myeloid lineages that accurately reflect those of humans, we may better understand normal vs. aberrant human innate immunity and inflammatory responses, or how abnormal responses affect wound repair.

Mouse myeloid cells are obtained routinely from select genetic strains, including wild-type or those genetically altered to model human diseases that affect innate immunity and/or inflammatory responses. Terminally differentiated neutrophils can be selected from mixed populations present in peripheral blood or bone marrow [14, 15], whereas mature macrophages can be obtained from a peritoneal lavage following an infection response, directly from alveolar tissue, or as monocytes from bone marrow [16]. However, these sources present experimental limitations: 1) the harvested mature cells can be inadvertently stimulated either during the isolation process or have already been stimulated in the case of peritoneal macrophages [17], 2) fully matured cells cannot be used as models of myeloid differentiation, and 3) only limited numbers of cells can be isolated per mouse. Because of these issues, researchers have relied on immortalized cell lines as models of myelopoiesis for two general reasons: cultures can be easily expanded into large populations required for many experiments, and certain immature lines are inducible and allow for studies at progressive stages of differentiation. Nonetheless, as with primary cells, immortalized models also present certain limitations. Several neutrophil models have been well characterized, including the EML/EPRO and MPRO models, plus the SCF ER-Hoxb8 cell line, each modified to express either a dominant negative RAR α or an estrogen receptor-regulated Hoxb8 transcription factor, respectively [18–21]. Although mature cells derived from each line express protein profiles observed in mature primary neutrophils and display several characteristic functional responses ([7, 21], and unpublished observations), each model was genetically modified and the long-term culture required to generate each line may alter immune functions. Two immortalized murine monocyte/macrophage models are commonly used, RAW264.7 and J774A.1 cells, but these too are genetically altered (by the Abelson Murine Leukemia Virus or sarcoma-inducing mutations, respectively), plus are factor-independent and at advanced stages of differentiation. An alternative monocyte-like line is available that is blocked at an earlier developmental stage, GM-CSF ER-Hoxb8 cells, however these cells have not been thoroughly characterized and expression of the ER-Hoxb8 fusion protein has the potential to restrict normal functional responses of differentiated cells [18]. One method to circumvent the limitations of using myeloid cells isolated from mature tissues or derived from immortalized cell lines is to generate genetically unaltered myeloid progenitors directly from hematopoietic stem cells (HSCs) in mouse bone marrow, and then induce the progenitors into mature neutrophils or macrophages. Extracted whole bone marrow can be effectively depleted of lineage committed cells, and cultured *ex vivo* to yield an expanded population of HSCs, thereby minimizing experimental cost as well as reducing the number of mice required per assay [9, 22, 23]. The expanded HSC population can then be induced into common myeloid progenitors (CMPs) and myeloblasts with continued exponential cell population expansion, providing cells for analyses at an early stage of myelopoiesis. Throughout this process the cells can be genetically manipulated with viral vectors, producing progenitors that contain modified gene expression profiles potentially reflecting those associated with human diseases or developmental disorders [24]. Subsequently, expanded progenitor populations can be induced with different combinations of cytokines to drive terminal differentiation, yielding highly enriched populations of unstimulated neutrophils or macrophages [16, 22]. The resulting lineages generally exhibit more robust and consistent functional responses, particularly phagocytosis, when compared

to corresponding mature cells derived either from primary tissues or immortalized cell lines [17].

Accurate and quantitative assessment of the characteristic cellular features and functional responses of either wild-type or genetically modified (e.g. gene knockout or overexpression) progenitors and their mature counterparts are critical to understanding how genetic disorders lead to disease symptoms. Preliminary assessments typically use qualitative evaluation of cell morphologies by manual inspection of differentially stained cells with conventional microscopy. This technique allows for the identification of changes in nuclear structure (e.g. lobulation in neutrophils and decreased N/C ratios plus increased cell size in macrophages) and cytoplasmic features such as accumulation of intracellular vesicles and granules. Changes in the expression profile of lineage-specific cell surface markers can then be quantitatively measured by traditional flow cytometry, for either neutrophil (Gr-1 and Mac-1) or macrophage (F4/80 and Mac-1) differentiation. Analyses continue with evaluation of key functional responses, including phagocytosis or NETosis, both typically using fluorescence microscopy. For example, cells that have engulfed opsonized, fluorescence-labeled particles (e.g. bacteria or zymosan) are visually inspected for internalized fluorescence signals. Fluorescence microscopy also is used to detect changes of cellular components that are hallmarks of NETosis, such as nuclear translocation of cytoplasmic azurophilic granule proteins [e.g. myeloperoxidase (MPO) or neutrophil elastase (NE)], diffuse DNA staining indicating nuclear decondensation, or increased nuclear immunostaining with citrullinated Histone-H3. Labeling of cell surface markers such as Mac-1 is then used to detect membrane blebbing events prior to the release of nuclear material, aka NETs. These processes are key indicators of “suicidal” NETosis, but whole cell imaging also can detect the more recently termed “vital” NETosis. In the latter process, nuclei also become less lobular as DNA diffuses into the cytoplasm, but cell lysis does not occur; rather, the nuclear material is simply released into an extracellular vesicle leaving behind an intact cytoplasm [13, 25].

The above-described tools for assessing myeloid cell morphologic maturation or functional responses have been optimized and extensively performed, but each has limitations. Traditional flow cytometry allows for multiplex analyses on a single cell plus statistical measurements from high throughput assessment of thousands of cells, but the processing used to identify positive cells lacks a spatial analysis component that can cause misclassification or misinterpretation of the results from different datasets. This limitation can be particularly problematic for certain phagocytosis studies; although analyses of phagocytosed particles can be semi-quantitatively assessed by flow cytometry, the capacity of the technology to discern internalized vs. surface-bound fluorescent particles is limited, even when quenching reagents are utilized [26–28]. The recent advent of pHrodo particles has improved accuracy of phagocytosis measurements, since the particles only fluoresce when in an acidic environment as caused by phagosome-lysosome fusion. However, activation may be incomplete or aberrant in cells with disorders affecting lysosome formation, and the efficiency of the process (e.g. number of particles engulfed per cell, known as the phagocytic index), cannot be quantified. Traditional flow cytometry also provides the ability to gate subpopulations in heterogeneous samples, such as mixtures of mature cells extracted from peripheral blood or bone marrow, but visual inspection of

fluorescence signals in these subpopulations is not possible. On the other hand, direct observation by fluorescence microscopy provides a high level of resolution that can be important for these assessments, in particular quantifying internalized particles during phagocytosis. Regarding NETosis, this process has typically been analyzed by traditional fluorescence microscopy, which allows the researcher to differentiate between neutrophils undergoing nuclear decondensation vs. those retaining polymorphonuclear structure, observe nuclear localization of granule proteins, and identify changes in cell membrane shape during chromatin extrusion. Unfortunately, the process is subjective, time consuming, and yields low numbers of analyzed cells per sample. Processing of the cells in preparation for microscopy can be problematic, in particular attachment of cells to slides or coverslips since this affects their 3D structure and morphologic characteristics.

Imaging flow cytometry technology has integrated the spatial resolution provided by fluorescence microscopy with the high throughput format and quantitative analysis of flow cytometry, essentially providing a high throughput microscope. The multispectral imaging system utilizes various imaging modalities to capture up to 12 images per event during acquisition using brightfield, darkfield (side scatter (SSC)) or fluorescence modes. The acquisition dataset can then be analyzed with the imaging software for traditional flow cytometry readouts (e.g. intensity) as well as advanced image analysis capabilities. Unique labeling or morphologic features of the cell can therefore be detected, including fluorescence intensities produced by markers of a specific organelle (in particular the nucleus), overall cell shape by staining of the plasma membrane, or size of the cell. Labeled, individual events in a complex population then can be gated for specific properties and quickly inspected for visual confirmation of appropriate gating parameters, allowing the researcher to select “masking” features most appropriate to the different morphologic or fluorescence features being sought. For example, using a customized masking strategy, cell area from the masked bright field image can be compared to the masked nuclear area to determine the N/C ratio, and stained nuclei can be masked to identify nuclear condensation or lobularity, each important features when analyzing maturing, terminally differentiated, or functionally active neutrophils and/or macrophages. Conventional flow cytometry does not allow for visual inspection or image analysis, and this certainly is not feasible in high numbers with manual microscopy. Moreover, the capacity for the researcher to verify that proper gating and masking techniques were used by visually inspecting the events in each subpopulation for key nuclear or cytoplasmic features, and the ability to discover changes in cell features or expression localization in a population, is unparalleled by any other technology available to date.

Here we detail the optimized methodologies for cryogenic storage, recovery and *ex vivo* culture of mouse bone marrow to generate an expanded population of myeloid progenitors. The expanded population contains primarily cells with morphologic features consistent with CMPs and myeloblasts, which are then stimulated with a lineage-inducing cytokine cocktail that yields either fully mature neutrophils or macrophages. Steps for generating Wright-Giemsa stained cells to examine nuclear morphologic features for both lineages by light microscopy are presented, followed by methods to assess characteristic cell surface marker expression and N/C ratios by imaging flow cytometry. The presented results from these combined techniques illustrate how quantitative measurements can be obtained from cells at

progressive stages of differentiation for each myeloid lineage. Methods to identify two key functional responses by mature neutrophils and macrophages that are well-suited for imaging flow cytometry then are described: phagocytosis of internalized, fluorescently-labeled *E. coli* particles by both lineages, and the complex process of NETosis produced by mature neutrophils stimulated with inducing agents (phorbol myristate acetate (PMA), lipopolysaccharides (LPS), a calcium ionophore (A23187)), and *E. coli* bioparticles. Included with each imaging flow cytometry methodology are recommendations for data acquisition parameters provided by the INSPIRE software, plus the gating and masking strategies provided by the IDEAS software during data analyses, each applicable to either the FlowSight or ImageStream instruments (all provided by Amnis, Millipore Corp). By distinguishing distinct cellular or nuclear morphologic features of progenitors, neutrophils, or macrophages within heterogeneous groups of cells, combined with quantitative analyses of each cell's positivity for functional responses, applications of the described methodologies demonstrate the power of this emerging technology for basic research on the differentiation and function of professional phagocytes. The described methods are applicable to a broad range of research areas in academic, clinical or commercial laboratories aimed at characterizing human conditions that affect phagocytes, such as leukemias, neutropenias or aberrant inflammatory diseases.

2. Isolation of bone marrow cells for cryopreservation, thawing, and *ex vivo* culture

2.1 Results and Discussion

Bone marrow can be harvested from any mouse strain, most often C57BL/6 due to genetic consistency between these inbred mice. In this study, male C57BL/6 were used along with CD1 retired breeders, a cost effective alternative for preliminary studies since the mice tend to be larger allowing for ease of extraction and produce higher cell yields. Detailed steps for bone marrow extraction, red blood cell (RBC) lysis, and lineage depletion were previously published [22], and therefore are only briefly reviewed to lead the researcher into preparing the cells for cryopreservation or direct *ex vivo* culture. The steps for cryopreservation and thawing were adapted from previous methods designed for freezing macrophages [29], with modifications applied for freezing freshly extracted whole bone marrow. The *ex vivo* culture of lineage-depleted early hematopoietic progenitors begins with 3 days of expansion in media supplemented with SCF and IL-3, which supports both cell proliferation and maturation toward a population of cells resembling mostly CMPs or myeloblasts (collectively termed CMPs hereafter). The expanded cells are then divided into different conditions depending on which mature cell type is desired (see Figure 1 for an illustration of the entire process). A regimen of G-CSF plus SCF/IL-3 induces differentiation into promyelocytes followed by terminal neutrophil differentiation in G-CSF alone. A regimen of GM-CSF plus M-CSF yields primarily monocytes that are then terminally differentiated into macrophages with just M-CSF. Recommendations are provided for timing of passages to maintain optimal cell densities throughout each procedure, and typical yields at most steps are indicated. Samples can be easily removed from cultured cells to assess nuclear maturation by differential staining with Wright-Giemsa, or imaging flow cytometry to

quantitatively measure labeling of cell surface markers and N/C ratios indicative of each mature cell type, as described in the subsequent sections.

2.2 Methodology

2.2.1 Bone marrow extraction and preparation for cell cryopreservation—

Specific steps for preparing femurs (and tibias if desired) of recently euthanized mice* are previously described [22], which lead into the steps detailed below. If cells are not being prepared for cryopreservation, the bone marrow is resuspended in the cell staining buffer for lineage depletion (see [22] for details) and induced as described for each lineage in 2.2.4 and 2.2.5. The steps listed apply to a single mouse, but they can be easily adapted to parallel extractions from multiple mice by simply submersing the cleaned femurs in media and flushing the bone marrow out in rapid succession, so that all bone marrow cells can be processed together.

**Note: CD1 retired breeder or C57BL/6 male (8 weeks or older) mice used for the described procedures and results were purchased from Charles River Laboratories (Wilmington, MA) and housed prior to euthanasia at the UMass Lowell animal facilities under protocols approved by the UMass Lowell Institutional Animal Care and Use Committee.*

1. After flushing the marrow from the bone cavity with Iscove's Modified Dulbecco's medium (IMDM; Hyclone, SH30228) supplemented with 2% heat-inactivated fetal bovine serum (FBS; Gibco, 10437-036, *qualified is acceptable*), process the cell pellet in RBC lysis buffer (Sigma-Aldrich, R7757) according to the manufacturer's instructions, and centrifuge ($250 \times g$, 10 min).

Caution: Carefully monitor the time the cells are exposed to the RBC lysis buffer; avoid prolonged incubation by preparing a tube prior to starting the procedure with the required volume needed to dilute the RBC lysis buffer salt concentration and prevent damaging the stem cells (e.g. 1 min incubation in 1 ml RBC lysis buffer, and immediately transfer into 10 ml IMDM).

2. Decant supernatant and resuspend the cell pellet in 10 ml D-PBS (Hyclone, SH30028.02) and repeat centrifugation.

Caution: If the cell pellet is pink in color, then repeat the RBC lysis treatment.

3. Decant supernatant and resuspend the pellet in 10 ml D-PBS; take an aliquot and perform a cell count (typical yield $\sim 10^7$ cells per mouse).
4. Centrifuge ($250 \times g$, 10 min) and resuspend the pellet at 10^7 cells/ml Freezing Media [90% Certified FBS (Gibco, 16000044) and 10% DMSO (Sigma-Aldrich, D26650)].

Note: The quality of FBS can be critical when culturing certain myeloid progenitors (see [18]), therefore certified is recommended for each of these steps, whereas qualified is acceptable for all other steps.

5. Dispense 1 ml cell suspension into each cryovial, and transfer to a Nunc cooler for overnight storage at -80°C .
6. Transfer the stock cryovials to liquid nitrogen (LN_2) for long-term storage.

2.2.2 Thawing of cryopreserved bone marrow

1. Prepare basal hematopoietic progenitor (or simply “progenitor”) growth media (adapted from media used to *ex vivo* culture mouse HSCs [23]; IMDM with Certified FBS (15%, Gibco, 16000044), L-glutamine (2mM, Hyclone, SH30034.01), penicillin (5 U/ml) / streptomycin sulfate (5 $\mu\text{g/ml}$; Hyclone, SV30010) supplement with SCF (150 ng/ml, 250-03), IL-3 (30 ng/ml, 213-13), and IL-6 (30 ng/ml, 216-16) (recombinant murine cytokines from Peprotech, Rocky Hill, NJ), and allow the prepared media to pre-equilibrate using 10 ml per cryovial divided into two wells of a 6-well tissue culture plate (Corning, Corning, NY, 3516) and 5 ml* per cryovial in a conical centrifuge tube with the cap loosened, for 1 h at 37°C with 5% CO_2 .

**Note: It is not necessary or cost effective to add cytokines to the media contained in centrifuge tube for harvesting the thawed cells, therefore use of progenitor growth media alone is sufficient.*

2. Remove cryovial(s) from LN_2 and transfer to 37°C water bath for ~2–3 min.

Note: Visually confirm that the media is completely thawed.

3. Transfer the cryovial contents to the centrifuge tube with the pre-equilibrated progenitor growth media and centrifuge ($250 \times g$, 5 min).
4. Decant supernatant and gently resuspend pellet in the pre-equilibrated progenitor growth media with cytokines from the 6-well plate; return evenly suspended cells to the same wells and culture at 37°C with 5% CO_2 for 24 h.
5. Harvest cells by gently pipetting to disperse non-adherent cells without dislodging the contaminating adherent cells, transfer cell suspension to a tube, and centrifuge ($250 \times g$, 5 min).
6. Decant supernatant and proceed with the Lineage Depletion Protocol ([22], BD Biosciences, San Jose, CA; 558451).

Note: The typical yield per cryovial is $\sim 3 \times 10^6$ cells, but cell counts should be performed in preparation for the lineage depletion protocol as described. Once complete, lineage depletion via the BD IMag system typically produces $\sim 1 \times 10^6$ Lin-negative (Lin^-) cells from the combined yields of 3 cryovials of frozen bone marrow or 1×10^7 freshly harvested bone marrow cells.

2.2.3 Ex vivo culture into myeloid progenitors—This procedure begins by culturing the lineage-depleted (e.g. Lin⁻) cells with cytokines (SCF/IL-3) added to basic hematopoietic lineage (or simply “lineage”) growth media (adapted from media used to culture mouse immortalized cell lines, [7]), which supports the expansion of myeloid progenitors after three days of culture. Most cells in the expanded population will retain high N/C ratios but also exhibit low-level Mac-1 expression, both typical characteristics of CMPs (**D3 CMP**, see Figure 2A for representative pictures). All cells are collected, washed in D-PBS and then resuspended in basic lineage growth media supplemented with the appropriate cytokine combination to induce either neutrophil or macrophage differentiation.

1. Day 0: Plate 2×10^5 Lin⁻ cells/ml lineage growth medium (IMDM with L-glutamine plus horse serum (20%, HS, Gibco, 16050114), and penicillin (5 U/ml) / streptomycin sulfate (5 µg/ml)) supplemented with SCF (50 ng/ml) and IL-3 (50 ng/ml) in a 6-well tissue culture treated plate and incubate at 37°C with 5% CO₂.
2. Day 2: The cell density will increase by ~ 3-fold; expand the population by passaging into new wells without dislodging contaminating adherent cells, and supplement with fresh lineage growth media containing SCF/IL-3 while maintaining a final concentration of $2\text{--}3 \times 10^5$ cells/ml.
3. Day 3: Collect cells (the total population will have approximately doubled), centrifuge ($250 \times g$, 5 min), and resuspend the pellet in 5 ml D-PBS. Repeat centrifugation and resuspend cells in lineage growth medium in preparation for culture into either neutrophils or macrophages.

2.2.4 Neutrophil differentiation—The D3 CMPs in lineage growth media are initially induced with G-CSF/SCF/IL-3 for 2 days to generate a population of maturing neutrophils, many of which exhibit characteristics of myeloblasts, promyelocytes, or myelocytes, along with some terminally differentiated PMNs (**D5 Pro-PMN**). The cells are then collected, washed and resuspended in lineage growth media with G-CSF alone for two additional days to yield a population highly enriched in PMNs (**D7 PMN**, Fig. 1).

1. Day 3: Prepare the washed D3 CMPs at 2×10^5 cells/ml in new wells using lineage growth media supplemented with SCF (50 ng/ml), IL-3 (50 ng/ml), and G-CSF (50 ng/ml, Peprotech, 250-05).
2. Day 4: Following 24 h of culture, expand the population by doubling the media and passing the cells to new wells in the 6-well plate.
3. Day 5: Collect all cells (D5 pro-PMNs) by centrifugation at $250 \times g$ for 5 min and resuspend the cell pellet in lineage growth media with 50 ng/ml G-CSF only.
4. Day 6–7: Continue to monitor cell expansion for an additional 2 days, passaging as necessary to maintain densities no higher than 1×10^6 cells/ml, although most cells will be terminally differentiated, non-proliferating neutrophils (D7 PMN).

2.2.5 Macrophage differentiation—Day 3 CMPs are initially cultured in GM-CSF plus M-CSF for 2 days, at which point cell morphologies predominantly resemble monocytes (**D5 pro-MΦ**), followed by continuous culture in M-CSF alone to produce advanced stage monocytes or macrophages after 2 days (**D7 MΦ**), or a homogeneous population of macrophages after 5 days (**D10 MΦ**, Fig. 1).

1. Day 3: Passage the washed D3 CMPs into new wells in lineage growth media supplemented with GM-CSF (50 ng/ml, 315-03) and M-CSF (50 ng/ml, 315-02) (both cytokines from Peprotech), at a final concentration of 2×10^5 cells/ml.
2. Day 4: Following 24 h of culture, expand the population by doubling the media and passing the cells to new wells in the 6-well plate.
3. Day 5: Collect all cells (D5 pro-MΦ) in a conical tube, centrifuge ($250 \times g$, 5 min) and resuspend the cell pellet in lineage growth media with M-CSF (50 ng/ml) at 2×10^5 cells/ml. Passage the cells to petri plates (Fisherbrand, FB0875713A), as this will reduce the adherence of maturing monocytes and macrophages, minimizing cell damage during harvesting with a cell lifter before analyses and/or passage.
4. Day 7: Cells should expand to $\sim 6 \times 10^5$ cells/ml and can be harvested (D7 MΦ), or maintain culture in M-CSF if fully mature macrophages are required (see below).
 - Harvesting cells: Incubate plate on ice for 10 min, gently scrape the cells using a cell lifter (Celltreat, Shirley, MA, 229305), and transfer cell suspension to a pre-chilled centrifuge tube keeping cells on ice until analysis.

Note: Before removing the cell suspension, visually inspect the plate to confirm that the cells have been dislodged from the petri plate and repeat scraping if necessary. It is very important to work swiftly but attempt to prevent any unnecessary disturbance to the cells that might cause premature activation.
 - Sustained culture: Carefully add 1–2 ml lineage growth media with M-CSF (50 ng/ml), taking care not to disturb adherent cells (maturing macrophages), in order to replenish depleted cytokines and maintain an approximate cell concentration of $5\text{--}8 \times 10^5$ cells/ml.
5. Day 10: Incubate cultures for an additional 3 days for a highly enriched population of fully mature macrophages (D10 MΦ). Final yield will be $\sim 8 \times 10^5$ cells/ml, which can be harvested by incubating cells on ice for 10 min and then gently dislodging cells with a cell lifter as described above.

3. Assessment of stained nuclear morphologies, cell surface marker protein expression, and N/C ratios to classify lineage maturation

3.1 Results and Discussion

Ex vivo cultured D3 CMPs in the two lineage-specific regimens (detailed in Section 2.2.4 and 2.2.5, plus Fig. 1) should be assessed by observing the distinct morphologic changes in overall cell size and nuclear structure as the cells differentiate, as well as specific changes in cell surface protein expression patterns. This can be accomplished using a combination of the procedures described below, typical results of which are presented here. First, cytocentrifuged cells were stained with Wright-Giemsa for visual inspection, which revealed changes in nuclear morphologies as the D3 CMPs differentiated into neutrophils or macrophages (representative examples are presented in Fig. 2A). The majority of D3 CMPs exhibited characteristics of early myeloid progenitors with large nuclei and small cytoplasmic volumes (Fig. 2A, left panel, open block arrows). When progenitors were cultured for 2 additional days to induce neutrophil differentiation, the number of identifiable progenitors decreased while cells with characteristic lobulated nuclei became apparent (Fig. 2A, D5 PMN, closed block arrows). However, there was a considerable subpopulation of immature cells that indicated the cultures required additional time for cells to mature; this finding illustrated the importance of periodic assessment during cell maturation. After 2 more days of culture, a highly enriched population of morphologically mature, lobulated neutrophils were observed (Fig. 2A, D7 PMN). By comparison, staining of the CMPs after 2 days of culture to induce macrophage differentiation revealed cell morphologies with monocyte features, including increased cytoplasmic volumes and condensed, non-circular or kidney-shaped nuclei localized to the center of the cells (arrows in D5 pro-M Φ , Fig. 2A, lower panel). By D7 of culture, monocytes were still observed but many cells displayed condensed nuclei localized to the outer periphery of the expanding cytoplasm as well as large cytoplasmic vacuoles, all features of terminal macrophage differentiation (Fig. 2A, D7 M Φ , arrows). As was determined with neutrophil inductions, additional maturation time was necessary and after 3 more days of culture, cytopsin images showed a nearly homogenous population of morphologically mature macrophages (D10 M Φ). The nuclear and overall cell morphologic changes were quantitated by manual assessment of multiple inductions, which led to the graphically presented data in Fig. 2B at progressive stages of neutrophil or macrophage maturation. Consistent with the stained images, a majority of the D3 CMPs were classified as progenitors (>80%), but these numbers steadily diminished in either lineage induction regimen. In contrast, the population cultured to induce neutrophil maturation were visually identified as >75% PMNs (Fig. 2B, upper graphs), and >95% of the macrophage-induced population were categorized as morphologically mature macrophages (Fig. 2B, lower panels).

In the second procedure, imaging flow cytometry was used to assess cell surface marker expression profiles of the distinct populations depicted in Fig. 2, the results of which correlated with the visual assessments of Wright-Giemsa-stained cells. This protocol details the use of three fluorescence-labeled antibodies (Abs) against cell surface markers that are characteristic of either neutrophils or macrophages: Mac-1 (CD11b/CD18), expressed by both cell types; Gr-1 (here the Ab used detects Ly-6C/G), expressed at low levels by

monocytes, essentially absent in macrophages, but expressed at high levels in neutrophils; and F4/80, expressed at high levels by macrophages. The results of these procedures demonstrate that D3 CMPs exhibit lower levels of expression for each marker as compared to the differentiated cells, although there is a significant number of cells that express both Gr-1 and Mac-1 indicating residual neutrophils and macrophages following lineage depletion (Figure 3A). By comparison, the terminally differentiated neutrophils (D7 PMN) exhibit Mac-1^{high} and Gr-1^{high} expression, but low levels of F4/80. By comparison, D5 pro-MΦ also exhibit Mac-1^{high} and F4/80^{low} expression, but only moderate levels of Gr-1, consistent with a population of monocytes (previously shown to express Ly-6C [16]). Culture of these cells for 3 or 5 more days total in M-CSF (D7 and D10 MΦ) produces populations that are predominantly Mac-1^{high} and F4/80^{high}, but Gr-1^{low}. Pictures of each cell type obtained from images at 60X magnification provide individual assessment of expression levels for each marker (Fig. 3B, images from 20X magnification can be found in Supplemental Fig. 1A).

Lastly, morphometric analysis was performed on the spatial image data collected with the imaging flow cytometer to evaluate N/C ratios using the IDEAS software with customized masking and feature applications. This analysis tool allows the researcher to analyze heterogeneous populations such as those obtained during the early stages of differentiation (e.g. D3 CMP, D5 pro-PMN or D5 pro-MΦ) or whole blood samples, and identify the specific subset or cell type of interest based on the characteristic changes in N/C ratio. In-focus single cell events were selected for experimental analysis using the gating strategy depicted in Figure 4A. With these required parameters, the focus value for both brightfield and Ch07 (DNA stain) were assessed for each event as the initial gate criteria (In Focus, Fig. 4A, left panel); the In Focus subpopulation then was used to identify events with a specific aspect ratio and area for brightfield (Single BF, Fig. 4A, center panel) and then finally Ch07 (Single BF+Nuc, Fig. 4A, right panel). The initial analysis used the default masks in the IDEAS software to calculate the N/C ratio, but upon visual inspection it was clear that the default mask was not detecting the proper area of the brightfield image or the nuclear DNA stain. Therefore, to ensure the highest level of accuracy, multiple custom masks were created for both the brightfield channel as well as for DNA dye intensity, and then the area feature was applied to each masked region. The corresponding area is listed for each example event in blue for the brightfield mask comparative analysis (Fig. 4B) and in yellow for the DNA area evaluation (Fig. 4C). Because of the range of the area feature values, careful consideration was taken to select the representative mask for the final N/C ratio calculation. The results from these analyses provide histograms that indicate how N/C ratios change with the differentiating neutrophils vs. macrophages (Fig. 4D). The population induced with G-CSF (D7 PMN) produced a small increase in N/C ratios as compared to the D3 CMPs, since the lobulated nuclei of mouse neutrophils occupies most of the cellular compartment and there is no increase in cytoplasmic area. In contrast, M-CSF cultured macrophages (D10 MΦ) exhibited significantly decreased N/C ratios, consistent with their characteristic compact, spherical nuclei and significantly increased cytoplasmic volumes (see images for representative cells in Fig. 4D). Importantly, similar results were observed at either magnification for these analyses (see Supplemental Fig. 1B for graphed data from both 20X and 60X analyses). The comprehensive morphometric analyses outlined here for

conventional microscopy with differential cell staining, combined with detection of dynamic cell surface marker protein expression changes as well as the quantitative assessment of N/C ratios with imaging flow cytometry, provides a convenient means to quantitatively measure overall changes as each lineage matures. This framework can also be applied using different cell morphologies, markers or ratios to identify mixed hematopoietic lineages in alternative heterogeneous populations.

3.2 Methodology

During the culture of each cell type, small samples can be cytocentrifuged for Wright-Giemsa staining and observed under brightfield microscopy, and/or immunolabeled with fluorophore-conjugated antibodies against lineage-specific cell surface markers for detection with imaging flow cytometry. The first method is subjective and only a limited number of cells can be quickly assessed, but provides initial qualitative assessment of immature vs. mature cells based on nuclear morphology and cytoplasmic features (Fig. 2A–B). Imaging flow cytometry applied to the second method allows for quantitative assessment of lineage-specific marker expression plus analyses of individual cells for distribution of the markers as compared to nuclear staining (e.g. DAPI, Fig. 3). The analysis can be multiplexed with the addition of a cell membrane impermeable DNA dye (i.e. Sytox-Green) as a measure of cell viability (data not shown). Finally, methods to measure changes in N/C ratios of macrophages are presented including described gating strategies and a customized masking approach to determine cytoplasmic or nuclear area. The steps of each described method have been optimized for staining consistency between cell types and efficient labeling to provide reproducible signal intensities, plus notes or comments have been included describing caveats or the advantages of alternative approaches.

3.2.1 Cell cytocentrifugation with Wright-Giemsa staining for assessment of nuclear morphology

1. Harvest $\sim 1 \times 10^5$ cells and centrifuge ($1,400 \times g$, 5 min).
2. Decant supernatant and resuspend cell pellet evenly in 300 μ l PBS with 0.1% bovine serum albumin (BSA).
3. Transfer to the cytocentrifuge filter slide apparatus (e.g. Shandon Cytospin, Thermo Scientific, Waltham, MA; or Hettich Cyto System, Beverly, MA) and centrifuge ($55 \times g$, 5 min).
4. Remove and disassemble the cytospin apparatus, then allow slide to air dry for ~ 5 min.
5. Place slide in glass coplin jar with Wright stain (Sigma-Aldrich, WX16) for 3 min.
6. Transfer slide to Sorenson's phosphate buffer (pH 6.2; containing 80% (v/v) potassium phosphate (monobasic, 67 mM) and 20% (v/v) sodium phosphate (dibasic, 67 mM)), for 5 min.
7. Submerge slide in Giemsa stain (Sigma-Aldrich, GS500) for precisely 15 s.

8. Rinse slide in distilled, deionized water by gently dipping into two separate coplin jars, and allow the slide to air dry.
9. Add Permount (Fisher Scientific, SP15) over cells on the slide, carefully apply a 22×22 mm glass coverslip, and gently press out air bubbles.
10. Allow the mounting reagent to cure overnight on a flat surface.
11. Cytospins were visually inspected with an oil emersion 60× objective and images were collected with an Olympus camera (Olympus, WA).
12. To evaluate the distribution of cell types, 300 cells for each differentiation time point were manually assessed for morphologic features from three separate sets of inductions. The data can be compiled and presented for comparisons in bar graph form with associated p values (Excel, Richmond, WA) using an unpaired two tailed student t test with equal variance.

3.2.2 Live cell immunolabeling

1. Prestaining DNA: Adjust the volume so that the cell concentration in each well is $\sim 5 \times 10^5$ cells/ml. Add 120 μ l/ml NucBlue (Molecular Probes, Probes, Eugene, OR; R37605) directly to the cultured cells. Incubate the cells in tissue culture plates for 30 min at 37°C with 5% CO₂.

Note: Addition of the NucBlue prior to staining for cell surface markers is most important for cells that require scraping during harvesting, (i.e. D7 MΦ and D10 MΦ, or D7 PMN). For progenitors, the cells can be stained after addition of the antibodies, as noted in Step 10 below. NucBlue is provided in a dropper format by the manufacturer, but for accuracy and scalability, drops have converted to metric volumes.

2. Harvest 1×10^6 cells/condition, centrifuge ($250 \times g$, 5 min), decant supernatant, and gently resuspend the cell pellet in 5 ml of ice-cold D-PBS.
3. Repeat centrifugation and resuspend pellet in 100 μ l Wash Buffer (D-PBS with 2% FBS and 0.1% sodium azide) per condition, and transfer to pre-cooled microcentrifuge tubes on ice.

Note: Rapid pipetting can activate macrophages or neutrophils, which may increase cell death. Precautions should be taken to minimize disturbance to the cells and keep samples on ice whenever possible.

4. Add 1 μ l of Fc Block and incubate for 15 min on ice.

Note: Omit step for the unstained control or DNA dye samples.

Caution: Protect the samples from light exposure (e.g. ice bucket with a solid lid) for the remaining steps, however, light-block microfuge tubes are not recommended as this restricts the ability to visualize the pellet after centrifugation resulting in cell loss.

5. Add fluorescence-conjugated primary antibodies (typically using 4 µg/ml), or the appropriate corresponding isotype control (see Table. 1).
6. Gently flick tubes to mix and incubate the samples for 45 min on ice.
7. Centrifuge cells ($1,400 \times g$, 5 min, 4°C) and decant supernatant while avoiding the pellet.
8. Resuspend cells in 400 µl of ice-cold Wash Buffer (D-PBS with 2% FBS) and centrifuge ($1,400 \times g$, 5 min, 4°C).

Note: Add secondary fluorescence-conjugated antibody if required for detection from unconjugated primary.
9. Decant supernatant and resuspend the pellet in 75 µl Analysis Buffer (D-PBS with 2% FBS).

Note: Sodium azide should be avoided for the final step to limit cell death.
10. The cells can be labeled with NucBlue at this stage if desired by adding 8 µl and incubating for 10 min at 37°C.
11. For cell viability assessment: add Sytox-Green (Molecular Probes, S7020) directly to the labeled cells at a final concentration of 20 nM and incubate for 5 min at room temperature.
12. Process samples and acquire images with a calibrated imaging flow cytometer using the parameters detailed below.

3.2.3 Imaging flow cytometry—Proper set-up for the acquisition of events is outlined here for both the FlowSight and the ImageStream imaging flow cytometers. A template should be created in the INSPIRE software and applied to all subsequently acquired samples to allow for direct data comparison post-analysis. Many of the described parameters are specific to the experimental design but the theory can be applied to other types of assays. Optimizing the laser power settings using highest intensity samples to prevent collecting events that are beyond the detectable intensity range is essential to collecting reliable data, along with titration of the antibodies for each cell type. Here, the laser power settings were optimized for the fluorophore configuration based on the Raw Max Pixel value for each channel preventing saturated events. Gating should be used during acquisition to prevent collection of unnecessary events (e.g. debris), which leads to large raw image files (.rif) with a low percentage of useable events.

The majority of the images presented in this study were obtained using a calibrated ImageStreamX MarkII (MilliporeSigma, Billerica MA) equipped with two cameras plus 405 nm, 488 nm, 642 nm, and 785 nm lasers, and imaged with the 60X objective for enhanced visualization of cellular components. Results were also obtained with a FlowSight (MilliporeSigma) equipped with the same laser configuration and a 20X objective for image acquisition. The INSPIRE software was used with minor modifications from the FlowSight procedure due to operational differences between the instruments (i.e. laser powers and gating parameters with the enhanced resolution of the 60X objective).

3.2.3.1 FlowSight and ImageStream acquisition parameters

1. Set the flow rate to slow speed for high resolution imaging.
2. Load multi-fluorophore labeled samples to determine optimal laser power settings based on Raw Max Pixel (RMP) intensity values of the channel corresponding to each fluorophore used.
3. Lasers and power settings used to obtain the results presented were as listed below in Table 2.

Note: It is important to keep the 405 nm laser power low when using DAPI or Hoechst as it has a broad emission spectrum and can overpower intensity signals in other channels, making compensation difficult or inaccurate. Titer the amount added to the analysis buffer if needed.

4. Acquisition Gates: Area of the default mask (M01) vs. the Aspect Ratio_M01 was used as the initial gate to ensure that single cell area events were collected.

Note: Additional gates for intensity can be added for sample collection of a certain subset population and is dependent on the experimental design.

5. Data Acquisition: Greater than 5,000 events in the single cell acquisition gate were collected for each experimental sample. Single fluorophore-labeled samples for each fluorophore were processed with the same laser power parameters as the experimental samples, however the brightfield and SSC (785 nm) lasers were turned off during acquisition and 1,000 events were collected.

3.2.3.2 Imaging flow data analysis with IDEAS software: A compensation matrix file was created post acquisition using the IDEAS compensation wizard. The compensation matrix file (.ctm) was applied to the raw dataset (.rif) for each experimental sample for initial analysis.

3.2.3.2.1 Gating strategy: In this procedure, the desired subpopulation of single cell events that are in-focus and exhibit selected characteristics (i.e. area of fluorescence detected is within the cell or nucleus) have been selected using a customized gating strategy. The features and masks provided by the software have been altered or additional masks were created to better represent the events. Represented in this work is the standard format of “Feature_Mask Name(X, X)”, which is the feature applied to the mask listed. The applied parameters begin by using the Gradient RMS feature value for both brightfield (Ch01) and DNA stain (Ch07) to identify and gate (In Focus Ch1&7) the in-focus events (see Fig. 4A, left panel). The in focus Ch1&7 subpopulation was then assessed and gated (Single BF) to identify events within a specified area and aspect ratio value using the brightfield images (Ch01, Fig. 4A, middle panel). This second gate will exclude aggregated debris events as well as events with very low area, which typically appear to be debris or Speed Bead images. The Single BF population was processed again with area vs. aspect ratio parameters, however this analysis utilized nuclear intensity (Ch07) so as to select for single nuclei events

(Single BF+Nuc). The resulting population Single BF+Nuc was then used to process the experimental analysis including N/C ratio values, or intensity changes during differentiation of antibody-fluorophore detection for cell surface marker proteins (the results of which were shown in Fig. 3).

Note: G-RMS is typically used as a measure of focus, which can be problematic since subset populations can have a very different GRMS value (apoptotic or macrophages) indicating a coincidental phenotype. Therefore, it is critical to review the included events as well as the excluded events to ensure that the user is not inadvertently selecting for a subset population when attempting to analyze overall population characteristics.

3.2.3.2.2 Masking and feature selection: The advantage of imaging flow cytometry is the ability of the researcher to visually identify and verify key experimental features in a gated population of interest. By using the customized masking approach to maximize the power of this technology, one must identify the most important feature being sought from the experiment being conducted, and determine the key characteristic that would differentiate the desired subpopulation from all of the events collected in the sample. The selection of the appropriate mask that accurately represents or identifies that key characteristic is absolutely crucial for the accuracy of the subsequent feature value calculations. Therefore, several customized masks were created using a variety of parameters as shown in Fig. 4B, with careful investigation to eliminate masks that are too tight (i.e. they do not encompass the whole object; Fig. 4B, Adaptive Erode(M01, Ch01, 80) and Erode(M01, Ch01, 6), or Fig. 4C, Threshold(M07, Ch07, 60) and Intensity(M07, Ch07, >200)) or are too wide and therefore include background pixels (Fig. 4B, System(M01, Ch01, 5) and M01/Morphology, or Fig. 4C, Intensity(M07, Ch07, >80), M07/Morphology)). Using the average measurements from multiple masks increases the accuracy of the feature values allowing for reliable quantitative data.

To estimate the N/C ratios for cells in each myeloid lineage, the average value for brightfield area was measured using three customized masks that were selected after visual inspection to determine that the masks captured an accurate representation of the overall cell morphology (here for the brightfield images in (System(M01, Ch01, 95), Object(M01, Ch01, Tight), and (AdaptiveErode(M01, Ch01, 95), Eq. 1 and Fig. 4B). The average area of the DNA intensity region was selected in the same way for nuclear dye intensity of Ch7 images (Morphology(M07, Ch07), Threshold(M07, Ch07, 70), and System(M07, Ch07, 90); Eq. 2 and Fig. 4C). Using these masks, a combined feature was created in the Feature Manager using the average nuclear area divided by the average cytoplasmic area (area of the cell reduced by the area of the nucleus (Eq. 3)), thereby calculating the “per cell” N/C ratio value as well as the mean N/C ratio for the analyzed populations (Fig. 4D).

$$\text{Avg. Area}_{01} = \frac{(\text{Area}_{\text{System(M01, Ch01, 95)}} + \text{Area}_{\text{Object(M01, Ch01, Tight)}} + \text{Area}_{\text{AdaptiveErode(M01, Ch01, 95)}})}{3}$$

(1)

$$\text{Avg. Area}_{07} = \frac{(\text{Area}_{\text{Morphology}}(\text{M07}, \text{Ch07}) + \text{Area}_{\text{Threshold}}(\text{M07}, \text{Ch07}, 70) + \text{Area}_{\text{System}}(\text{M07}, \text{Ch07}, 90))}{3} \quad (2)$$

$$N/C = \frac{\text{Avg. Area}_{07}}{(\text{Avg. Area}_{01} - \text{Avg. Area}_{07})} \quad (3)$$

4. Analysis of phagocytosis using imaging flow cytometry

4.1 Results and Discussion

Analysis of phagocytosis has become a critical technique to assess the capacity of neutrophils and macrophages to fight bacterial infections. During this process, the cells bind pathogens coated with opsonin proteins via cell surface receptors (e.g. Fc receptors) and activate intracellular cytoskeletal mechanisms (e.g. f-actin polymerization) that drive pathogen engulfment and phagosome formation [30]. Such analyses are particularly important to identify the mechanism that certain pathogens use to elude innate immune responses. *Leishmania* is a protozoan with the ability to survive in neutrophils or macrophages after ingestion, resulting in Leishmaniasis that can lead to cutaneous diseases (nonhealing ulcers) or visceral diseases causing damage to the spleen, liver, or bone marrow [31]. Certain bacteria also are able to evade destruction after engulfment; *Anaplasma phagocytophilum* can survive in neutrophil phagosomes by inhibiting NADPH oxidase activity, whereas *Mycobacterium tuberculosis* inhibits phagosome maturation by blocking signals required for phagosome lysosome fusion, thereby impeding delivery of bactericidal proteases within the phagolysosome [32–35]. Thus, rapid and quantitative measurement of the phagocytes' capacity to internalize pathogens and form intracellular phagosomes is important in both clinical research and for the assessment of novel drugs designed to overcome the capacity of these pathogens to evade destruction after engulfment.

Described here are computational image analysis methods that utilized the *ex vivo* cultured neutrophils and macrophages to evaluate their capacity to engulf and internalize bioparticles using imaging flow cytometry. The bioparticles are actually *E. coli*-coated latex beads that are labeled with pH-activated fluorophores that fluoresce in the acidic environment of the phagolysosome. Therefore, use of these particles: a) eliminated the need to use quenching reagents to discern surface-bound vs. internalized particles, and b) allowed the image analysis software to quantitatively measure the number of phagolysosomes or individual bioparticles (as is possible with the 60X ImageStream) within the cell. The percentages of cells with internalized bioparticles, detected as intracellular “spots” (% phagocytic positive) and the average number of spots per cell (the phagocytic index as a measure of spot counts by the IDEAS software) are then quantified using the gating and masking strategies as depicted in Figure 5A, and described below under data acquisition and analysis.

Application of the methods to analyze neutrophils at progressive stages of differentiation revealed increase percentage of phagocytic-positive (pHrodo⁺) cells, with D7 PMN exhibiting the highest percentage of cells gated as positive for the bioparticles (>65% after 45 min and >80% after 2 h, Figure 6A, left graphs). The phagocytic indices also changed

between the D5 and D7 PMN cells, however the magnitude of change depended on the imaging apparatus: the lower 20X image resolution provided by the FlowSight identified an average of 2.6 spots per cell, whereas the higher 60X resolution from the ImageStream increased detection to >13 spots per cell (Fig. 6A, right graphs). The distributions of the phagocytic indices between individual cells detected by each device were also quantitatively different, however the distributions for percentages of positive cells were similar (Fig. 6B, left and center panels). Importantly, manual inspection and enumeration of visible spots of the 60X ImageStream images provided phagocytic indices similar to those provided by the IDEAS analysis of the 20X images, but both were less than those provided by the IDEAS analysis of the 60X images (Fig. 6B, right panel). Finally, images of the positive cells reveal the locations of the spots within each cell at either magnification, which can be compared to the nuclear staining with NucBlue (Fig. 6C and Supplemental Fig. 2, top panels).

Macrophage phagocytosis was also quantitatively assessed using the same described methods as for neutrophils. As shown in Figure 7A, analyses at selected stages of maturation identified an increase in both the phagocytic capacity and the phagocytic index as the cells mature into functional macrophages. The D5 pro-M Φ population that contained both monocytes and immature macrophages displayed the lowest phagocytosis percentage of pHrodo⁺ cells (~40%) as compared the more mature cells in the D7 M Φ population (55%) and the D10 M Φ culture comprised almost entirely of terminally differentiated macrophages (~90%). These results confirmed the gain of function provided by further culturing of the differentiating macrophages and the utility of this functional marker for evaluating macrophage maturation. In addition, the mature macrophages produced a higher average phagocytic index as compared to the less mature cells (>5 vs. 2 spots per cell, respectively). As expected due to the increase resolution of the 60X magnification images, the highest average phagocytic index values were exhibited by the D10 M Φ (~19). The disparity between the phagocytic index estimations with the same software potentially is due to the 20X counting of phagolysosomes as compared to the 60X magnification, which is able to detect individual bioparticles. Similar to the observations of phagocytosis by neutrophils, the distribution patterns of macrophages with each phagocytic index were different between data sets obtained at 20X vs. 60X, but the overall pattern correlated well with the manual counts, again providing further confidence in the power of the masking strategies to quantitatively measure phagocytosis (Fig. 7B). Images of the cells with different numbers of pHrodo⁺ spots reveal the distribution of phagosomes within each cell, along with the locations of their nuclei via NucBlue staining (Fig. 7C and Supplemental Fig. 2, lower panels).

4.2 Methodology

The method begins with sonicating the fluorescence-labeled bioparticles (data shown for *E. coli*, but *S. aureus* and Zymosan are also available from Molecular Probes) just prior to use to ensure sufficient dissociation. The particles are then opsonized with fresh serum (or frozen serum from freshly acquired blood) so that the particles will attach to Fc receptors on the phagocyte surface. Serum obtained directly from sacrificed mice provides better opsonization than commercially available reconstituted serum from powder, which from our experience yields poor engulfment ([21] and unpublished observations). The opsonized particles then are added to the cells along with more mouse serum as a non-specific blocking

agent. The cells are simultaneously stained with NucBlue, a cell-permeable analog of Hoechst used to stain DNA in live cells. After incubation with gentle agitation, the cells are analyzed for spots of green fluorescence, each of which indicate either individual phagolysosomes or even individual bacterial clusters within the vesicles, depending on the imaging apparatus used and magnification (e.g. 20X vs. 60X).

4.2.1 Mouse serum collection

1. Immediately prior to collection, euthanize mouse by CO₂ inhalation and collect blood from a cardiac bleed using a 25-gauge needle.
2. Transfer blood to a microcentrifuge tube and incubate at room temperature for at least 15 min.
3. Vortex samples for 15–30 s to disperse any clots and centrifuge ($2,000 \times g$, 10 min, 4°C).

Note: Typical yield per mouse is 300–500 µl cleared serum.
4. The serum should be divided into single use aliquots and stored at –80°C.

4.2.2 Opsonization of bioparticles

1. Resuspend lyophilized pHrodo-Green *E. coli* bioparticles (Molecular Probes, P35366) at 10 mg/ml in Hanks balanced saline solution (HBSS, Gibco, 14175079) plus FBS (10%) and sodium azide (2mM).
2. Vortex the bioparticles (3×15 s), and then sonicate on ice (3×20 s, 35% power at 70 Watts, Branson Sonifier Cell Disruptor 200 (Branson Ultrasonics, Danbury, CT)).

Note: The bioparticle suspension can be stored at 4°C in a light-blocking vial prior to use.
3. Transfer an appropriate volume of bioparticles to a new tube planning for 10 µl per analysis, and then add twice this volume of mouse serum (e.g. 20 µl per analysis).
4. Incubate bioparticle/serum mixture for 1 h at 37°C protected from light (a hybridization oven with gentle rocking works well).
5. Add 500 µl of ice-cold HBSS and sonicate each sample on ice (20 s at 35% power, 70 Watts).
6. Centrifuge ($1,400 \times g$, 15 min, 4°C) and carefully decant supernatant avoiding the cell pellet.
7. Resuspend the cell pellet in 500 µl ice cold HBSS.
8. Repeat centrifugation, decant supernatant and resuspend the pellet in 10 µl of ice-cold HBSS per assay.

4.2.3 Macrophage or neutrophil stimulation with *E. coli* bioparticles

1. Collect 1×10^6 cells per assay by centrifugation ($250 \times g$, 5 min).
2. Resuspend the cell pellet in 5 ml ice-cold HBSS and centrifuge ($250 \times g$, 5 min).
3. Decant supernatant and resuspend in 100 μ l of ice-cold HBSS per assay, and keep cells on ice.
4. Add the following to a polystyrene snap-cap tube (15×60 mm, Falcon, 352063):
 - 710 μ l ice-cold HBSS
 - 100 μ l freshly prepared mouse serum
 - 100 μ l of washed cells
 - 80 μ l of NucBlue (equivalent to 2 drops/ml)
 - 10 μ l of opsonized bioparticles (always add last)
5. Secure lids to snap cap tubes, parafilm the caps to prevent leakage, and coat the inside surface by tilting the tubes several times.

Note: In our experience flip-top microfuge tubes do not provide the necessary agitation required to evenly coat the cells with bioparticles.
6. Gently rock tubes (45 min, 37°C) while maintaining the control tubes on ice.
7. Gently transfer cells to a pre-cooled 1.5 ml microcentrifuge tube and centrifuge ($1,400 \times g$, 10 min).
8. Decant supernatant and gently resuspend cells in 35 μ l of HBSS plus 2% FBS.
9. Store samples on ice for immediate processing with imaging flow cytometer.

4.2.4 Imaging flow cytometry—Similar to the procedure described in Section 3 above the samples were processed by imaging flow cytometry with minor modifications noted below to accommodate changes to fluorophore panel design.

4.2.4.1 FlowSight and ImageStream acquisition parameters

1. Flow rate was set to slow speed for high resolution imaging.
2. Laser power settings were determined as previously described.
3. Lasers and power settings were set according to Table 3:

Table 3

Laser wavelengths and power settings for phagocytosis analysis

Excitation Laser (nm)	Power Setting (mW)	Fluorophore-Ab	Emission Channel
		Bright Field	1 & 9
405	5.0	NucBlue	7
488	30.0	pHrodo-Green Bioparticles	2
642	off	n/a	
785	3.86 (FlowSight) 2.0 (ImageStream)	SSC (side scatter)	6 (FlowSight) 12 (ImageStream)

4. Acquisition Gates: Settings were as described in the previous section except that two gates were created. The first gate excluded the events deemed as debris (Area_M01 <50 and Aspect Ratio <0.25) during collection to reduce the overall file size and speed of process data post-acquisition. The second gate ensured that single cell area events were counted during the acquisition (Area_M01:125–300 and Aspect Ratio >0.45).

Note: Due to the nature of the phagocytosis assay there is a significant amount of debris as the non-engulfed bioparticles in the sample are detectable by the instrument and therefore captured as an event.

5. Data Acquisition parameters were the same as described above.

4.2.4.2 Data analysis with IDEAS software: The compensation matrix file was created and applied to the raw dataset for each experimental sample as previously described.

4.2.4.3 Gating strategy: The initial gates that were described in the previous protocol were used to select events that are in-focus (In Focus Ch1&7) and Single BF events based on area and aspect ratio comparison. The third scatterplot (Area_Morphology(M07, Ch07) vs. Shape Ratio_Morphology(M07, Ch07)) measured area compared to the value for Shape Ratio (SR), which was gated for single nuclei (Single BF+SR_Nuc). The change to the Shape Ratio feature was necessary as the aspect ratio did not reliably gate for single nuclei events as in the previously described assay upon visual inspection, most likely due to the change in the nuclear morphology particularly with the PMN cultures (see Section 6 for a further explanation). The Single BF+SR_Nuc events were plotted on a histogram for intensity_MC_Ch2, and the threshold level for expression was set at 2.5e4. The events with a value greater than the threshold were gated as positive signal (pHrodo⁺) and those lower than the threshold were gated as negative signal (pHrodo⁻). The percent phagocytosis was calculated based on the percentage of events in either gate, and the pHrodo⁺ events were analyzed further to determine the phagocytic index using the masking strategy outlined in the next section.

4.2.4.4 Masking and feature selection: To quantify the value for each phagocytic index (i.e. phagocytosed bioparticles), we utilized the capabilities of the imaging flow analysis software to count the spots of intensity emitted by the pHrodo-*E. coli* bioparticles. The

IDEAS default settings did not provide the level of accuracy that was needed for reporting the data (see Fig. 5B for example images under Default). Therefore, we applied several masks and then selected the three peak masks (Peak (M02, Ch2, Bright 15, 22.5, and 30, Fig. 5B) that accurately detected the spots of intensity in Ch02. The spot count feature was added for each of the selected masks for both the _4 and _8 pixel connectivity options. A combined feature was created to calculate the average spot count value (Avg.Spot Count) to determine the phagocytic index for the pHrodo⁺ population.

$$\text{Avg. Spot Count} = \frac{(\text{SpotCount} - \text{Peak (M02, Ch2, Bright 15)}) + \text{Peak (M02, Ch2, Bright 22.5)} + \text{Peak (M02, Ch2, Bright 30)}}{3}$$

(4)

5. Analysis of NETosis formation by mature neutrophils using imaging flow cytometry

5.1 Results and Discussion

The release of NETs by activated neutrophils has been recognized as an important innate immune response that facilitates the capture and destruction of pathogens. The process begins with decondensation of chromatin caused by the actions of granule proteins (NE and MPO) that translocate into the nucleus and enzymatically degrade the histones [36]. Chromatin unraveling also depends on signaling by the Raf-MEK-ERK pathway that triggers activation of NADPH oxidases; the resulting oxidants may contribute to chromatin and histone degradation [37]. The granule enzymes remain attached to the unraveling chromatin as the nucleus swells, the nuclear envelope then dismantles and the nuclear material mobilizes into the cytoplasm. NETosis is typically stimulated by PMA, which is a rapid activator of NADPH oxidases and therefore tests whether NETosis mediated by this complex is abnormal, as is found in patients with chronic granulomatous disease who lack any component of the NADPH oxidase complex [38]. After invading the cytoplasm, the nuclear material along with attached granule proteins can be ejected through a rupturing cell membrane, providing localized high concentrations of antimicrobial agents that trap and possibly kill bacteria. This suicidal form of NETosis causes complete cell lysis and therefore is similar to apoptotic or necrotic cell death. Suicidal NETosis is unique, however, in that it is not affected by inhibitors of caspases or programmed necrosis mediators, respectively, but does appear to involve cellular autophagy [39]. Alternatively, vital NETosis involves membrane “blebbing” and the nuclear web is instead released via vesicular exportation, leaving behind anuclear cytoplasts that have been shown to exhibit chemotaxis and phagocytosis of live bacteria [13, 25]. Interestingly, vital NETosis may be mediated by entirely different mechanisms: typically stimulated by live bacteria (e.g. *S. aureus* or *E. coli*) or the gram-negative bacterial component, LPS, this form of NETosis is regulated by TLR2 and TLR4 from platelets along with complement [40, 41]. A third stimulant, the calcium ionophore A23187, induces rapid NETosis by activating calcium-dependent potassium channels along with multiple signaling pathways including ERK, Akt, and those stimulated

by NADPH-dependent pathways [42]. The combined activities of these pathways may explain why this stimulus produces faster and more complete NETosis as compared to PMA or LPS. Both types of NETosis cause neutrophils to undergo dramatic changes to their lobulated nuclear structure, membrane blebbing and/or cell lysis.

Studies of NETosis have primarily relied on analyses of stimulated cells by confocal fluorescence microscopy, using three different types of labels that detect changes to either nuclear or cellular structures: DNA labeling dyes, granule protein markers, or membrane markers (see [43–46] for examples). We therefore begin this section by presenting the methodologies for performing confocal fluorescence microscopy on the *ex vivo* differentiated neutrophils stimulated by PMA, LPS, or A23187 and analyzed for changes in protein expression and DNA localization. First, a DNA dye that emits fluorescence (DAPI) was used to reveal nuclear morphologic changes in stimulated cells, including decondensed DNA and increased circularity as nuclear membrane integrity is compromised during early stages of NETosis. As shown in Figure 8, DAPI identified the lobulated or donut-shaped nuclei in unstimulated D7 PMNs, with regions of punctate DNA staining indicative of condensed chromatin (Fig. 8B). By comparison, cells stimulated with the calcium ionophore exhibited diffuse, evenly stained nuclei that indicated spreading of chromatin into cytoplasmic regions, providing evidence of nuclear decondensation. Similar results were observed in a subset of the cells stimulated with either PMA or LPS (note enlarged image inserts provided for each type in Fig. 8B). Importantly, the extent of DNA decondensation produced in the *ex vivo* differentiated neutrophils was similar to that observed in human neutrophils: A23187 produced significantly more cells with diffuse nuclear staining during the 1 h stimulation as compared to those stimulated with PMA [44]. Second, fluorescence-labeled antibodies that detect the enzymes associated with nuclear degradation (NE and MPO) were used to identify accumulation of the enzymes in nuclei of cells undergoing NETosis. This change was observed within the D7 PMNs; MPO was detected primarily in cytoplasmic regions, whereas expression resided throughout the diffuse nuclei in stimulated cells (see unstimulated vs. stimulated panels in Fig. 8A). Third, release of NETs was visualized with DNA dyes by focusing on regions adjacent to the cells with ruptured membranes, where webs of DNA can be observed spreading from the cells undergoing late-stage NETosis. Extracellular DNA also was observed outside of multiple PMNs stimulated with each inducing agent, as depicted in Fig. 8B (PMA or A23187 stimulated), although more was identified outside of the A23187-stimulated cells as compare to those stimulated with PMA. The results of these methods demonstrate the utility of this traditional assessment tool but also allows for comparing such results to those obtained by imaging flow cytometry described in the second methodology.

Use of imaging flow cytometry to assess NETosis provides quantitative measurements that overcome the qualitative limitation of conventional fluorescence microscopy for assessing this complex process. Here we provide the steps of this methodology to assess NETosis of our *ex vivo* differentiated neutrophils, the designs of which were guided in part by similar studies recently reported for detecting NETosis by human peripheral blood neutrophils [25]. The described methods utilize three key parameters to measure cellular changes: 1) nuclear compactness as a measure of chromatin decondensation; 2) nuclear circularity as a measure of lobulation that reverses while decondensation is occurring, again during early NETosis;

and 3) overall cell shape, or circularity, as a measure of cell lysis or membrane blebbing that increases during late stage NETosis. The masking selection and gating strategies provided by the IDEAS software are described for both imaging flow cytometers. The provided strategies allow the user to designate each parameter and then apply the parameters to the whole population of cells, thereby providing for quantitative measurements of different subpopulations representing distinct stages of NETosis. The capacity to use these strategies to direct the data acquisition software toward selecting subpopulations of neutrophils with features that match the hallmarks of NETosis, combined with the capacity to mask such features and then visually confirm that the masking and gating strategies identify the correct feature, provide a very powerful tool to quickly generate data on the numbers of neutrophils undergoing NETosis, and even the types of NETosis that are occurring, (e.g. early vs. late, suicidal vs. vital).

The *ex vivo* cultured D7 PMNs were stimulated with the 4 inducers of NETosis, stained with Hoechst to detect DNA, and images were acquired by imaging flow cytometry. The data was assessed as described below and illustrated in Fig. 9A, using comparisons of cell morphologies provided by brightfield imaging to nuclear features provided by DNA stains. Application of this strategy revealed four quadrants of events, each containing cells with different features of NETosis (Fig. 9B, the example scatter plot was generated from LPS-stimulated cells, whereas the images derived from each quadrant show representative cells stimulated with each agent). Quadrant 1 (Q1) identified cells within the higher range of overall cell circularity but a low nuclear circularity value, consistent with unstimulated PMNs with lobulated nuclear structure. Quadrant 2 (Q2) identifies cells at an early stage NETosis in which their overall shapes were still circular (therefore maintaining a high circularity score), however nuclear circularity changed (e.g. increased Y-axis values). These results were as expected, because loss of nuclear membrane integrity associated with DNA decondensation and diffusion caused increased nuclear circularity values as the DNA migrated into cytoplasmic regions (see picture inserts of A23187-stimulated cells showing diffuse nuclear staining, Fig. 9B). Many cells in Quadrant 3 (Q3) exhibited decreased cell circularity with maintained high values for nuclear circularity, indicating the formation of irregular, blebbed membrane structures together with decondensed DNA, potentially capturing cells at a stage just before NET release. The fourth quadrant (Q4) identifies cells at the late stages of NETosis, in which cellular as well as nuclear circularity have diminished. For our continued analysis of NETosis, the gating strategy parameters were applied to analyzed cells stimulated with each condition, and the corresponding scatter plots with delineated quadrants plus percentage of events in each quadrant are shown in Fig. 9C.

To allow for easy comparisons of the percent cells detected in each of the 4 quadrants shown in Fig. 9C, values were assembled into the graphs shown in Figure 10. The graphs reveal that unstimulated cells primarily resided in Q1, whereas fewer stimulated cells resided in this quadrant, with lowest numbers observed for A23187 stimulated cells. Interesting, most of the stimulated cells resided in the three quadrants that indicate the progressive stages of NET release (Q2–4). The calcium ionophore stimulated cells exhibited the highest percentage undergoing NETosis as indicated by Q2 and Q3, consistent with previous studies indicating faster NET release compared to the other stimuli [44]. We suspect that the lower percentage of events produced by A23187 in Q4 is because most cells at this late stage of

NETosis have ruptured, consistent with the abundant amounts of cell debris that were detected during data acquisition. Also of note is that PMA and LPS stimulation produced the highest numbers of cells in late stage NETosis (Q4). As an additional qualitative measure of NETosis, we also immunolabeled the cells for Mac-1 to characterize cell membrane features, and for MPO to identify its localization in the stimulated cells (Fig. 10B). Importantly, the images captured from representative samples of cells in each quadrant are consistent with our designations of cell features in each quadrant (see Fig. 9B). For example, many cells in Q2 and Q3 showed MPO localization to their nuclei and changes to their membrane morphologies as identified by Mac-1 expression (particularly noticeable in cells within Q3). Cells in Q4 showed both loss of nuclear circularity as the nuclear material is observed being ejected from the cell along with abnormal overall cell shape.

5.2 Methodology

5.2.1 Neutrophil stimulation for microscopy

1. Collect 1×10^5 total cells per assay/condition and centrifuge ($250 \times g$, 5 min).
2. Decant supernatant and resuspend cells in 5 ml of ice-cold HBSS.
3. Centrifuge the cells ($250 \times g$, 5 min).
4. Decant supernatant and resuspend cells in 200 μ l of ice-cold IMDM without phenol red (Gibco, 21056-023) per assay.
5. Transfer 200 μ l of cells to each well on 8-well Lab-Tek Chamber Slides (Nunc, 154534).
6. Add the stimulating reagent for each sample:
 - a. 20 nM PMA (Fisher Scientific, BP685) in DMSO (Sigma-Aldrich, D26650)
 - b. 1 μ g/ml LPS (Sigma-Aldrich, L6529) in D-PBS
 - c. 25 μ M Calcium Ionophore (A23187, Fisher Scientific, BP595) in DMSO
 - d. D-PBS (unstimulated control)
7. Incubate for 1 h at 37 °C with 5% CO₂.
8. Add 200 μ l of Fixation Buffer (4% methanol-free paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, 15710) in PBS) and incubate at room temperature for 15 min.
9. Gently remove supernatant and add new 200 μ l of Fixation Buffer.
10. Incubate at room temperature for 30 min.
11. Add 200 μ l of Wash/Blocking Buffer (0.5% BSA in Tris-buffered saline (TBS)) and slowly remove supernatant.
12. Repeat previous wash step.

13. Add 200 μ l of Wash/Blocking Buffer and store in a 150 mm tissue culture dish (Falcon, 353025) at 4 C, or proceed with staining.
14. Add 200 μ l Perm/Wash Buffer (0.1% TritonX-100 (Sigma-Aldrich, T8532) in Wash/Blocking Buffer) and incubate at room temperature for 15 min.
15. Add 200 μ l of Wash/Blocking Buffer and remove supernatant.
16. Repeat previous wash step.
17. Add 200 μ l of Wash/Blocking Buffer and incubate the slide at 37°C for 30 min for blocking.
18. Add FITC-conjugated anti-MPO antibody (1:50; Abcam, Cambridge, MA; ab90812) and PE-conjugated anti-Mac-1 antibody (1:100; BD, 557397), or isotype controls (e.g., Abcam, ab91356 and BD, 553989, respectively).
19. Incubate at room temperature for 1 h.
20. Add 200 μ l Wash Buffer and remove supernatant.
21. Repeat previous wash step.
22. Disassemble the chambers and let the slide air dry.
23. Add ProLong Gold with DAPI (1 drop/well; Molecular Probes, P36935) and apply a glass cover slip (25 $\times$ 60 mm).
24. Press to remove bubbles and allow to dry in dark at room temperature for 24 h before visualization.

5.2.2 Imaging of NETosis with confocal microscopy—Confocal images were obtained on a Zeiss LSM 710 upright confocal microscope with a C-Apochromat 40X/1.2 W Korr UV-VIS-IR water objective. All image acquisition configurations were set to 16 bit and a 1AU pinhole. The samples were excited using a 405, 488 and 514 lasers for DAPI (DNA), FITC (MPO), and Sytox-Green (DNA), respectively (see Table 4). Laser power and gain intensity optimization for Fig. 8 were achieved by acquiring single channel images and utilizing the channel range indicator function within the Zeiss 2009 confocal imaging software. Final images presented were scaled to fit using Image J software. All large fields of view were equally scaled at 813 $\times$ 813 pixel dimensions while the respective zoomed-in fields of view were cropped from the original at 213 $\times$ 213 pixel dimensions.

5.2.3 Neutrophil stimulation for imaging flow cytometry

1. Collect $1.5\text{--}2 \times 10^6$ cells per assay/condition and centrifuge ($250 \times g$, 5 min).
2. Decant supernatant and resuspend cells in 5 ml of ice-cold HBSS.
3. Centrifuge ($250 \times g$, 5 min).
4. Decant supernatant and resuspend cells in 100 μ l of ice-cold IMDM without phenol red (Gibco, 21056-023) per assay.

5. Transfer 100 μ l of cells to each microcentrifuge tube and add the stimulating reagent:
 - 20 nM PMA (Fisher Scientific, BP685) in DMSO (Sigma-Aldrich, D26650)
 - 1 μ g/ml LPS (Sigma, L6529) in PBS
 - 25 μ M Calcium Ionophore (A23187, Fisher Scientific, BP595) in DMSO
 - D-PBS (control)
6. Incubate for 1 h at 37°C with 5% CO₂ keeping the tubes open.
7. Add 300 μ l of Fixation Buffer (4% paraformaldehyde in PBS) and incubate at room temperature for 15 min.
8. Wash by adding 1000 μ l Wash Buffer (2% FBS in PBS) and centrifuge (250 $\times$ g, 5 min).
9. Decant supernatant and wash again with 500 μ l Wash Buffer.
10. Decant supernatant and resuspend in 100 μ l Wash Buffer.
11. If performing immunolabeling of Mac-1: add Fc Block (anti-mouse CD16/CD21, 1:100; BD 553141) and incubate 15 min on ice for blocking.
12. Decant supernatant and resuspend in 100 μ l Wash Buffer.
13. If labeling MPO and Mac-1, add FITC-conjugated anti-MPO antibody (1:50; Abcam, ab90812) and PE-conjugated anti-Mac-1 antibody (1:100; BD, 557397), or appropriate isotype control (Abcam, ab91356 and BD, 553989, respectively).
14. Incubate for 1 h at room temperature.
15. Wash by adding 1000 μ l Wash Buffer and centrifuge (250 $\times$ g, 5 min)
16. Decant supernatant and wash again with 500 μ l Wash Buffer.
17. Resuspend immunolabeled cell pellet in 50 μ l Wash Buffer.

Note: Fixed samples can be stored at 4°C in Analysis Buffer (Wash Buffer supplemented with 0.01% sodium azide (Sigma-Aldrich, S2002)) for approximately one week.
18. Immediately prior to analysis, add nuclear (DNA) dyes: propidium iodide (5 μ g/ml; Invitrogen, V13242) and/or Hoechst (1:1500; Molecular Probes, H3570).
19. Incubate at room temperature for 10 min and process with the imaging flow cytometer.

5.2.3.1 FlowSight and ImageStream acquisition parameters: Data acquisition parameters for the INSPIRE software were described in previously detailed methods above with minor adjustments as required and noted below.

1. Flow rate was set to slow speed for high resolution imaging.
2. Laser power settings were optimized as described above.
3. Lasers and power settings used in this method are detailed in Table 5:

Table 5

Laser wavelengths and power settings for NETosis analysis

Excitation Laser (nm)	Power Setting (mW)	Fluorophore-Ab	Emission Channel
		Brightfield	1 & 9
405	5.0	Hoechst	7
488	30.0	FITC-MPO PE-Mac-1	2 3
642	off	n/a	
785	3.86 (FlowSight) 2.0 (ImageStream)	SSC (side scatter)	6 (FlowSight) 12 (ImageStream)

4. Gating and acquisition parameters were as previously described in Section 3.2.3.1.

5.2.3.2 Data analysis with IDEAS software: Compensated events were analyzed as previously described with the following modifications to the gating and masking strategies.

5.2.3.2.1 Gating strategy: Initial comparison of the acquired events began as previously described with plotting the GRMS 01 for BF focus but compared to area of BF (FocusBF +AreaBF) to select for in-focus events with a certain range in size, consistent with single cells (Fig. 9A, left panel, boxed region). The Focus1+AreaBF events were then analyzed using a histogram of the Spot Distance. Minimum feature values were calculated for the Morphology(M07, Ch07) mask, which allows for selecting events with a single nuclear spot (and therefore no distance, hence a value of zero, Fig. 9, second panel). Also included in this gating strategy are events with an irregular nuclear shape such as cells with nuclear material that is diffusing into membrane blebs or being released, thereby excluding true doublet events (see bar above spot distance peak labeled as “a”, indicating 0 to 1 for distance values within the gate). The third gating strategy uses the Morphology(M07, Ch07) mask to compare the feature values for the area and the mean pixel intensity, allowing the user to exclude cells that lack DNA (e.g. cytoplasts or aggregated debris) or have either a small or large area of DNA (Fig. 9A, third panel, the large square indicates the accepted events). Finally, the selected events were analyzed for shape morphology by plotting the value of the circularity feature with the BF mask (AdaptiveErode(M01, Ch01, 95)) compared to circularity of the nuclear mask (Morphology(M07, Ch07)). Gates (Quadrants) were set at 15 for BF circularity and 9 for nuclear circularity values (Fig. 9B).

5.2.3.3 Masking and feature selection: Features and masking were used in this method as previously described, with the addition of the circularity feature for the two masks used in this gating strategy, AdaptiveErode(M01, Ch01, 95) and Morphology(M07, Ch07).

6. Advanced image analysis of NETosis during neutrophil phagocytosis

6.1. Results and Discussion

Finally, we observed morphologic changes to the D7 PMN population that were stimulated with pHrodo-labeled *E. coli* bioparticles during the phagocytosis data analysis with the IDEAS software (see Fig. 6C). The D7 PMN dataset was processed using the same parameters as previously outlined for NETosis, however due to the increase in the frequency of unbound bioparticles, the masking strategy required augmentation as reviewed below (Fig. 11A). The circularity of the adjusted mask as compared to the nuclear circularity was plotted with the same gating parameters as NETosis described previously, the results of which revealed a distribution that most closely resembled cells stimulated with LPS for 1 h (Fig. 11B). These results demonstrate that *E. coli*-stimulated cells undergo significant levels of NETosis, with greater than 80% of the cells residing in Q3 and Q4. We also assessed the cells in each quadrant that were pHrodo⁺ and therefore contained engulfed bioparticles (Fig. 11C). Interestingly, cells in Q1 typically had low spot counts, whereas those in Q3 and Q4 exhibited high spot counts (Fig. 11C, note inserted spot count numbers in yellow text within each event). These results indicate that *E. coli* stimulates both phagocytosis and NETosis, or what we term “phagoNETosis”. It is important to note that the level of LPS theoretically encountered by the cells stimulated with the *E. coli* bioparticles is significantly higher than the purified LPS amounts used in our previous assays (Figs. 9 and 10), which may explain why more cells stimulated with the *E. coli* particles resided in Q3 and Q4 as compared to those stimulated with LPS (Fig. 11B) [47]. This identified assay has the potential for future applications for identifying neutrophil phagocytic responses that are aberrant due to blood-borne pathogens, such as those caused by certain bacteria (e.g. *A. phagocytophilum* or *M. tuberculosis*), infection-induced vasculitis now linked to NET production, or the pathogenesis of systemic lupus erythematosus that may be exacerbated by NETs [13].

6.2. Methodology

6.2.1. Gating strategy and feature/masking analysis with IDEAS software—

Initial analysis of the phagocytosis data set uses the NETosis parameters as outlined above. With an alternate sample composition, such as the free bioparticles debris, the masking approach can be modified to properly analyze the events. Use of the combined mask option allows for increased accuracy for detection of the image region without including the debris. This led to a false-positive enrichment of subpopulations in Q3 and Q4, and another, more complex masking approach had to be applied using a combined masking strategy. The IDEAS software modifies the initial mask based on a secondary set of masking criteria and accurately displayed a depiction of the image (Fig. 11A, Combined Mask 2: Range(LevelSet(AdaptiveErode(M01, Ch01, 95), Combined, 8), 100–5000, 0–1)).

7. Conclusions

The capacity of imaging flow cytometry to rapidly and accurately assess the maturation and function of neutrophils or macrophages in large populations is predicted to significantly advance current studies of mouse models aimed at understanding human diseases that affect myelopoiesis or innate immune responses. Moreover, this technology can identify nuclear and cytoplasmic changes to myeloid cells that otherwise would be overlooked by conventional techniques. For example, our analyses of neutrophils that were stimulated with *E. coli* bioparticles identified a population not only undergoing phagocytosis but also exhibiting signs of NETosis including membrane blebbing. Such discoveries will certainly continue as we apply the techniques described here to the comparative analyses of wild-type vs. mutant cells, in particular those that display aberrant nuclear maturation. The use of imaging flow cytometry for clinical applications is expanding, which will provide comprehensive information regarding abnormal nuclear features or functional responses of immune cells in patients with bone marrow pathologies. Aberrant nuclear morphology is a common feature of cancer cells, including hematopoietic malignancies such as myelodysplasias and acute myelogenous leukemia, thus rapid assessment of neutrophils may increase the chances of early detection [48–50]. Moreover, patients with myelodysplasias that present with neutrophils with abnormal nuclear lobulation, termed Pseudo-Pelger-Huët anomaly, has been identified as an indicator that the patient's disorder will progress to acute leukemia [51]. Additionally, there is now evidence that biomarkers or indicators of sepsis and potential for septic shock include abnormal morphology and chemotaxis of circulating neutrophils, therefore the ability to rapidly analyze blood samples for such anomalies in patients vulnerable to sepsis, such as burn patients, may expedite use of life-saving treatments [52]. As this technology continues to advance in both the rate of data acquisition, accuracy in identifying myeloid cell features and functions, and decreases cost, the applications of imaging flow cytometry will continue to expand into other fields of outside of myeloid biology by provide important applications in clinical settings.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- *Ex vivo* culture of cryopreserved mouse hematopoietic stem cells is described.
- Imaging flow cytometry identifies nuclear-to-cytoplasmic ratios in myeloid cells.
- Phagocytosis and phagocytic indices from neutrophils or macrophages are quantified.
- Quantitative assessments of NETosis are determined for stimulated neutrophils.
- Neutrophils stimulated with *E. coli* exhibit features of phagocytosis and NETosis.

Figure 1. Schematic overview of experimental design for isolation, culture, and differentiation of neutrophils and macrophages

(A) Shown is an illustration of the mouse bone marrow extraction, cell cryopreservation, thawing, recovery and lineage depletion. After thawing the bone marrow, the cells recovered for 24 h in progenitor growth media, then lineage depleted for enrichment of hematopoietic progenitor cells. (B) Depiction of the *ex vivo* culture process leading from lineage-depleted (Lin⁻) bone marrow cells to terminally differentiated neutrophils and macrophages. The Lin⁻ cells were cultured in lineage growth media with SCF/IL-3 for 3 days to generate progenitors. The derived common myeloid progenitors (D3 CMP) were then divided into two separate cultures, using the recommended cytokine cocktail in lineage growth media for inducing either neutrophil or macrophage differentiation for the indicated number of days.

Figure 2. Visual microscopic analysis of neutrophil vs. macrophage morphology

(A) Representative 60X oil emersion photomicrographs of Wright-Giemsa stained cytocentrifuged cells during the differentiation process revealed changes in nuclear and cytoplasmic features. The progenitors (D3 CMP) exhibit a high N/C ratio (examples indicated with open arrows), whereas neutrophils exhibit characteristic lobulated nuclei (block arrows) as compared to macrophages that exhibit increased cell sizes along with condensed, round nuclei (arrows in D5 pro-MΦ and D10 MΦ). The scale bar in each image indicates 20 μ m. (B) Using the cytocentrifuged stained cells from the above imaged slides, the *ex vivo* cultures were visually examined for morphologic features and categorized into a stage of differentiation for which the percentages were calculated for each timepoint indicated. Shown are graphs of cells identified with features consistent with progenitors (high N/C ratio), myelocyte/monocyte-like (mid-N/C ratio), macrophage-like (low N/C ratio) or neutrophil-like (lobulated nuclei), with average percentages $\pm$ standard deviations (SD) indicated from at least three independent inductions of thawed bone marrow cells. Indicated *p* values were calculated using an unpaired two-sample Student *t* Test assuming equal variances.

Figure 3. Analyses of immunolabeled cell surface marker protein expression profiles in populations of differentiating neutrophils and macrophages with imaging flow cytometry (A) Shown are overlaid histograms of intensity within the MC (master channel) mask for each of the corresponding channels, for each antibody fluorophore to evaluate the expression profiles for Gr-1, Mac-1, and F4/80 exhibited by the *ex vivo* cultured cells induced to either neutrophils (left panels) or macrophages (right panels). The patterns indicate typical changes as neutrophils show increased Gr-1 and Mac-1 expression but predominately lack F4/80, whereas macrophage induced populations exhibit loss of Gr-1 expression and increased Mac-1 and F4/80 expression. (B) Representative compensated images of D3 progenitors (CMPs), differentiating neutrophils (D5, D7 PMN) and macrophages (D5, D7, D10 MΦ) are shown to demonstrate the expression and localization of the three cell surface markers along with DNA staining to illustrate changes in nuclear shape. The scale bar indicates 7 μm, and yellow numbers represent calculated N/C ratio for the event; all images were acquired using the ImageStream instrument at 60X magnification and image display gains for each CSM channel (Ch02, Ch03, Ch11) were set to the same parameters with the IDEAS software to allow for visual comparison of intensity expression between events shown.

Figure 4. Gating and masking strategies with imaging flow cytometry for assessment of nuclear-to-cytoplasmic ratios during neutrophil or macrophage differentiation

(A) All events were initially plotted for the Gradient Raw Mean Squared (RMS) values for the customized brightfield mask (AdaptiveErode(M01, Ch01, 95)) vs. the nuclear stained images with the Morphology(M07, Ch07) mask. The initial gating parameter “Focus BF +Nuc” selected events for both the brightfield image and the nuclear DNA stain images that were in-focus (left panel; representative example images shown below demonstrate events that were out of focus (labeled as “a”), compared to the selected in-focus images (labeled as “b”). The gated FocusBF+Nuc events were then measured using the aspect ratio value compared to the area of the brightfield mask (AdaptiveErode(M01, Ch01, 95)) (center panel, inset image and equation describes the aspect ratio assessment criteria; representative images for “Single BF” events (c) and multiple or aggregated cells (d) are shown below). The gate was set for Single BF events with a high aspect ratio (>0.6) but low area (<600) to exclude doublets or multi-cell aggregates. The Single BF events were compared using the nuclear DNA stain mask (Morphology(M07, Ch07)) for the nuclear area vs. aspect ratio (right panel with images illustrating multiple nuclei (e) and single nucleus events (f)) and gated for “Single BF+Nuc” events. The resulting subpopulation of Single BF+Nuc events was used for the subsequent analysis of cell surface marker expression levels and N/C ratio assessment. (B, C) Examples of customized mask options (masked region displayed in teal

overlay) for either brightfield images (B) or nuclear intensity detection (C) are provided, with inserted blue or yellow values representative of the area value for each described mask, illustrating the necessity for careful mask selection that accurately represents the object image, particularly when evaluating subtle changes such as with N/C ratio calculations. Scale bar indicates 7 μm . (D) The N/C ratio value was calculated for the gated “Single BF +Nuc” subpopulation (described above) using a combined feature calculation of the average area from three brightfield masks or three Ch07 masks (calculated in Eq. 1–3, masked selected are labeled with asterisks in B and C). Using this method for evaluating the N/C ratio was determined for the *ex vivo* cultured cells at specific stages of differentiation (progenitors (D3 CMP), neutrophils (D5 and D7 MN), and macrophages (D5, D7, and D10 M Φ)). The histograms shown depict the distributions of N/C ratios for the indicated cell types, as calculated by the software. Representative images (brightfield, DNA stained, and merged) illustrating the change in N/C ratio as the D3 CMPs undergo differentiation to either neutrophils or macrophages are also shown. N/C ratio value is listed in yellow for each event. Scale bar indicates 7 μm .

Figure 5. Masking and gating strategies for analysis of phagocytosis by imaging flow cytometry (A) Depicted are three scatter plots with gated areas indicated for each selection strategy, in this case used to analyze neutrophils after phagocytosis of the pHrodo-Green *E. coli* bioparticles. All events were initially plotted for the Gradient Raw Mean Squared (RMS) values for the customized brightfield mask (AdaptiveErode(M01, Ch01, 95)) vs. the nuclear stained images with the Morphology(M07, Ch07) mask. The initial gating parameter “Focus BF+Nuc” selected in-focus events for both the brightfield and the nuclear DNA stained images (left scatter plot). The gated FocusBF+Nuc events were then measured using the aspect ratio value compared to the area of the brightfield mask (AdaptiveErode(M01, Ch01, 95)) (left-center panel), and the gate was set for “Single BF” events with a high aspect ratio (>0.6) but low area (<600) to exclude doublets or multi-cell aggregates. The Single BF events were compared using the nuclear DNA stain mask (Morphology(M07, Ch07)) for the nuclear area vs. shape ratio (right-center panel, inset image and equation describes the shape ratio (SR) assessment criteria) and gated for “Single BF+SR_Nuc” events. The resulting subpopulation of Single BF+SR_Nuc events was used for the subsequent analysis of channel 2 (Ch02) fluorescence intensity of the combined mask (MC), and the results were gated to determine the percentage of events positive for pHrodo-Green *E. coli* bioparticles (pHrodo⁺) or negative for particle engulfment pHrodo⁻, as shown in the representative histogram (right panel). (B) Shown are representative images of nine masks (masked region displayed in white (gray) overlay) that were used to calculate the spot count feature of the corresponding masked region (value indicated in yellow text of each image) on 4 different stimulated neutrophils. The first two masks are the default settings provided by the IDEAS software, which are either not sensitive enough or too sensitive. The evaluation of several masking options lead to optimal mask settings as depicted in the last three images. The final average spot count values (indicated in blue within the left most BF images) were calculated from the three optimal mask settings. The white bar in the lowest set of images indicates 7 μm .

Figure 6. Quantitative measurements of phagocytosis by neutrophils using imaging flow cytometry

(A) Neutrophils generated after 7 days of *ex vivo* culture of bone marrow progenitors were stimulated with pHrodo-Green *E. coli* bioparticles while also stained with NucBlue. Live cells were analyzed and images were acquired after either 45 min (at 37°C or 4°C) or for 2 h (at 37°C) of incubation. The percentages of cells positive (pHrodo⁺) for fluorescence particles (spots) were then calculated and reported as percent phagocytosis (left graph). The average number of fluorescence particles (spot count) per event was also calculated and graphed for D5 and D7 PMN stages of differentiation, and indicated as phagocytic index (right graph). Data was generated and graphed from 20X images (D5 and D7) vs. 60X images (D7 only); values inserted above each bar indicate actual average percentages or index values. (B) Distributions of spot counts for each analyzed event were graphed from either 20X or 60X images, with numbers of spots shown as Normalized Frequencies (left two graphs). Also displayed is the graph of the percentage distribution for the number of spots per cell generated by manually counting the 60X images (308 images assessed), of the same D7 PMNs processed by the imaging flow cytometer (right graph). Data shown are representative of at least 2 independent assays. (C) Images of representative cells with green fluorescence (phagocytosed bacteria, *E. coli*) vs. nuclear staining (red fluorescence, NucBlue) and merged images are shown for cells exhibiting 1, 3 or 15 spots. All images shown were obtained from an ImageStream with a 60X objective. The scale bars in the leftmost brightfield images indicate 7 μ m.

Figure 7. Quantitative measurements of phagocytosis by macrophages using imaging flow cytometry

(A) Macrophages generated by 5, 7 or 10 days of *ex vivo* culture of bone marrow progenitors were stimulated and stained as described for neutrophils, each for 45 min at 37°C or 4°C as a control. Shown are the percentages of phagocytic positive cells (left graph) or phagocytic indices (right graph) at each indicated stage of macrophage differentiation. (B) Distributions of spot counts for cells after 10 days of culture are shown as determined by imaging flow cytometry with 20X or 60X magnification (left two graphs), or using manual counts from the 60X images (right graph). (C) Images from macrophages that have engulfed *E. coli* particles are shown (1, 5, or 15 spots), including nuclear staining along with merged images; all images were obtained with the ImageStream at 60X magnification and scale bars indicate 7 μm.

Figure 8. Induction of early-stage NETosis in primary neutrophil cultures detected by confocal microscopy

(A) Representative images are shown of untreated vehicle controls (PBS, left column) and stimulated cultures (LPS, PMA, or A23187), incubated for 60 min. Cells were labeled with DAPI (DNA) and FITC-anti-myeloperoxidase (MPO) antibody to visualize MPO expression. Control cell images exhibit the typical lobular nuclear morphology, whereas numerous cells stimulated with LPS or PMA show nuclear decondensation. By comparison, most A23187-stimulated cells exhibit loss of donut-shaped, lobular nuclei. Most stimulated cells exhibited increased MPO expression, some of which was closely associated with nuclear regions stained with DAPI. (B) Images are shown of untreated vehicle controls (PBS, left column), and stimulated cultures incubated with PMA, or A23187 for 60 min, each stained with both DAPI and Sytox-Green. The control cells exhibit typical lobular nuclear morphology, whereas some of the stimulated cells show extracellular regions of DNA stain indicating NET release. All images were taken at 40X magnification, with scale bars (top left panels in A and B) indicating 20 μm. Inserts are shown as 200% zoom settings relative to the parent image.

Figure 9. Gating strategies for identifying cell images for quantitative measurements of NETosis

(A) Gates were applied to the cell populations stimulated with different NETosis inducers to exclude debris and multiple cell aggregates, each indicated by regions within a series of scatterplots. First, events in brightfield views were selected for in-focus cells (Area vs. gradient RMS) to exclude cell doublets and debris. The gated region selected for the next analysis is indicated (left panel). Only those events with spot distances between 0–1 were selected (as indicated with the horizontal line, middle panel), allowing for the selection of cells with either a single nuclear signal or an elongated signal that is attached (e.g. ejecting DNA), but exclude doublets (cells shown below histograms depicting nuclear features are from neutrophils with diffusing or ejecting DNA, or clearly a doublet of two cells). The selected cells were then gated for nuclear fluorescence intensity (Mean Pixel_Morphology, Y-axis) vs nuclear area (Area_Morphology, X-axis) (right panel). The events within the gated region indicated by the large rectangle in the last scatter plot were then further analyzed. (B) The cells produced by the first series of gating strategies then were analyzed for nuclear circularity (nuclear stain, Y-axis, labeled as Circularity_Morphology(M07,Ch07)) vs. cell circularity (brightfield, X-axis, labeled as Circularity_AdaptiveErode(M01,Ch01,95)), and four different quadrants were delineated within the scatterplots for quantitative assessments (lines depict each quadrant). The images shown were obtained from events in each quadrant of the analyzed cells stimulated with each agent, providing representative pictures of cells at each stage of NETosis as identified by each quadrant. (C) The gating strategy was applied to each of the populations of stimulated cells, and shown are the resulting scatter plots with depicted quadrants plus percentages of cells in each quadrant. All plots shown were generated with the data collected

with the ImageStream using a 60X objective with cells stimulated for 1 hour with each inducing agent to cause NETosis.

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Figure 10. Quantitative imaging of cell vs. nuclear circularity during NETosis identifies four distinct stages

Events from the four quadrants of the scatter plots were quantified and graphed, which indicates the percentages of cells that are at each stage of NETosis. Percentages shown are representative of three independent *ex vivo* cultures of derived neutrophils stimulated with each agent. Also shown are representative images of the cells stimulated with PMA and A23187 as acquired from the ImageStream, highlighting the changes in both nuclear and cell morphology as identified with DNA staining, brightfield imaging, and expression patterns of MPO, and Mac-1.

Figure 11. Analyses of phagocytosis with *E. coli* stimulation reveals NETosis by imaging flow cytometry

Images of neutrophils that were stimulated with pHrodo-Green *E. coli* bioparticles were analyzed for cellular and nuclear circularity using the IDEAS software. (A) Shown are examples of the masking strategy (masked region displayed in teal overlay) used to accurately determine the spot count. The first two sets of images (No mask and M01) are those of the cells with stained nuclei to show examples in the brightfield and the default mask, respectively, indicating that particles outside of the cells are detected (note masking beyond the cell membrane in those under M01). The masking strategy was then adjusted to capture images labeled “Combined Mask 1”, using the mask AdaptiveErode((M01, Ch01, 95), Combined, 8), and those labeled “Combined Mask 2”, using Range(LevelSet(AdaptiveErode(M01, Ch01, 95), Combined, 8), 1 00–5000, 0–1)). The last four images illustrate those used while modifying the masking strategy leading to the combined masks actually utilized. (B) Scatter plots for *E. coli* and LPS stimulated cells are shown with the quantitatively analyzed quadrants indicated within each plot (left panels), plus the resulting percentages of cells in each quadrant (right graphs). Cell circularity feature indicated in the X-axes utilized different masks for each stimuli, due to alternative masking required to accurately detect cell circularity when analyzing cells with *E. coli* bioparticles, which are not present in the LPS-stimulated cells. (C) Shown are images of cells representative of those found in each quadrant for NETosis analyses, each showing the numbers of spots identified in the images (yellow text). Each image identifies the fluorescence spots indicating phagocytosis, plus changes in nuclear and cell morphology (e.g. cells undergoing phagoNETosis).

Table 1

Example conjugated antibodies panel design

Antibody	Conjugate	Description, Cat. No.	Isotype Control	Manufacturer
Fc Block	n/a	CD16/CD32, 553141	n/a	BD Biosciences
Gr-1	FITC	Ly-6C/G, 553126	553988	BD Biosciences
Mac-1	PE	CD11b, 561689	553989	BD Biosciences
F4/80	AF647	MF48021	R2a21	Life Technologies

Table 2

Laser wavelengths and power settings for immunolabeling

Excitation Laser (nm)	Power Setting (mW)	Fluorophore-Ab	Emission Channel
		Bright Field	1 & 9
405	5.0	NucBlue	7
488	30.0	FITC-Gr-1 PE-Mac-1	2 3
642	75	AF647-F4/80	11
785	3.86 (FlowSight) 2.0 (ImageStream)	SSC (side scatter)	6 (FlowSight) 12 (ImageStream)

Table 4

Confocal microscopy settings

Fluorescence Dye/Antibody	Laser (λ)	Laser Power & Gain Intensity	Filter (λ)	Beam Splitter
DAPI: Nuclear DNA	405	7.0/600	410–498	405
FITC: MPO	488	1.0/800	493–552	488/561/633
APC:H3Cit	633	5.5/600	638–744	488/561/633
PE:Mac1	561	7.0/775	566–625	458/561
Sytox-Green: DNA	514	6.0/~500	519–676	458/514