Video Article

# Isolation, Purification and Labeling of Mouse Bone Marrow Neutrophils for Functional Studies and Adoptive Transfer Experiments

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## Abstract

Neutrophils are critical effector cells of the innate immune system. They are rapidly recruited at sites of acute inflammation and exert protective or pathogenic effects depending on the inflammatory milieu. Nonetheless, despite the indispensable role of neutrophils in immunity, detailed understanding of the molecular factors that mediate neutrophils' effector and immunopathogenic effects in different infectious diseases and inflammatory conditions is still lacking, partly because of their short half life, the difficulties with handling of these cells and the lack of reliable experimental protocols for obtaining sufficient numbers of neutrophils for downstream functional studies and adoptive transfer experiments. Therefore, simple, fast, economical and reliable methods are highly desirable for harvesting sufficient numbers of mouse neutrophils for assessing functions such as phagocytosis, killing, cytokine production, degranulation and trafficking. To that end, we present a reproducible density gradient centrifugation-based protocol, which can be adapted in any laboratory to isolate large numbers of neutrophils from the bone marrow of mice with high purity and viability. Moreover, we present a simple protocol that uses CellTracker dyes to label the isolated neutrophils, which can then be adoptively transferred into recipient mice and tracked in several tissues for at least 4 hr post-transfer using flow cytometry. Using this approach, differential labeling of neutrophils from wild-type and gene-deficient mice with different CellTracker dyes can be successfully employed to perform competitive repopulation studies for evaluating the direct role of specific genes in trafficking of neutrophils from the blood into target tissues *in vivo*.

## Video Link

The video component of this article can be found at http://www.jove.com/video/50586/

## Introduction

Neutrophils are the most abundant leukocytes in humans. They are the main cellular component of the innate immune system and act as a first line of defense against invading microorganisms. Patients with acquired neutropenia and primary immunodeficiencies that affect neutrophil numbers and/or function develop life-threatening invasive bacterial and fungal infections, highlighting the importance of these cells in host defense [1]. Immune recognition of invading pathogens at the infection site by their cognate pattern-recognition receptors results in the induction of an orchestrated innate immune response, which leads to secretion of chemoattractants that generate a chemotactic gradient capable of recruiting neutrophils from the bloodstream into the inflamed tissue [2]. After neutrophils enter the infection site, they become activated, which leads to cytokine and chemokine production, pathogen uptake, and killing via oxidative and non-oxidative mechanisms [3]. Besides their well-recognized role in innate immunity, neutrophils have also been recently shown to play important roles as initiators of effective adaptive immune responses [4]. On the other hand, apart from their protective roles in immunity, neutrophils may also mediate tissue injury and immunopathology due to excessive accumulation and/or activation at sites of inflammation, as shown in a variety of infectious and autoimmune diseases [5-7].

Despite the indispensable role of neutrophils in mounting effective innate immune responses and their pleiotropic effector functions in several infectious diseases and inflammatory conditions, technical difficulties with handling these cells and lack of reliable experimental protocols has hindered research with neutrophils over the past decades. Therefore, use of reproducible assays for isolation of neutrophils should facilitate further research on neutrophil-mediated immunological functions *ex vivo* and *in vivo*. To date, several methods have been described for the isolation of neutrophils such as density gradient centrifugation of human blood and mouse blood or bone marrow [8,9], positive or negative immunomagnetic enrichment of neutrophils from mouse blood or bone marrow [10,11], and harvesting of neutrophils from the peritoneal cavity of mice following intraperitoneal injection of thioglycollate or other inflammatory agents [12]. Although neutrophils can be easily isolated in large numbers from human blood, this method is suboptimal in mice due to the limited volume of mouse blood that precludes isolation of sufficient neutrophils for functional studies or adoptive transfer experiments [13]. In addition, although the yield of thioglycollate-elicited cells from the peritoneal cavity is greater compared to that of mouse blood, the purity of neutrophils in the inflammatory peritoneal lavage varies between 60-90%, and the isolated neutrophils exhibit an activated phenotype. Thus, the cells collected using this method can only be used for performing

 

functional studies of activated but not of unstimulated neutrophils, as the mouse peritoneal cavity has few neutrophils at the steady state [12]. Instead, the bone marrow is a convenient reservoir for harvesting large numbers of either unstimulated or activated neutrophils [11,14], which can then be used for downstream functional studies such as phagocytosis, killing and degranulation, or for adoptive transfer into recipient mice.

Herein we describe a simple and fast (~ 2 hr) protocol, which provides a high yield (~6-12 × $10^6$ neutrophils/uninfected mouse, or up to 30-40 × $10^6$ neutrophils/infected mouse) of pure (80-95%) neutrophils with >95% viability from the bone marrow. This method uses commercially available Histopaque, which are density gradient cell separation media consisting of Ficoll and sodium diatrizoate, to separate neutrophils from the bone marrow of mice. This method yields significantly larger numbers of neutrophils per mouse compared to blood or peritoneal cavity, it can be used to collect neutrophils from mice both at steady state or after infection, and it is easier to layer compared to the density gradient centrifugation method that uses discontinuous Percoll gradients consisting of 55%/65%/75% Percoll in PBS [9]. In addition, the time and resources required to collect pure neutrophils are significantly decreased compared to neutrophil isolation using Fluorescence-Activated Cell Sorting. Also, because this method does not involve an immunomagnetic enrichment step, it is more cost-effective, and it avoids the exposure of cells to the magnetic column and antibodies, thus decreasing the likelihood of neutrophil activation.

In addition to performing functional studies of isolated neutrophils *ex vivo* and adoptive transfer of cells into recipient mice, this protocol also describes a method for labeling of isolated neutrophils using different CellTracker dyes. Differential labeling of neutrophils from mice of various genetic backgrounds can be adapted in competitive repopulation studies for tracking the transferred neutrophils in tissues of recipient mice using flow cytometry, which can provide mechanistic insight on the direct role of specific genes in trafficking of neutrophils from the blood into target inflamed organs [6].

## Protocol

# 1. Isolation of Mouse Bone Marrow Cells

1. Euthanize mice using the institution's animal care committee-approved protocol and spray the animal surface with 70% ethanol.
2. Make an incision of the skin in the mid-abdomen and remove the skin from the distal part of the mouse including the skin covering the lower extremities.
3. Cut off the muscles from the lower extremities using scissors and carefully dislocate the acetabulum from the hip joint, while avoiding breaking the femur head.
4. Remove the remaining muscles from the femur and tibia using a scalpel and scissors and separate the femur from the tibia at the knee joint exercising care to not break the bone ends. Place the bones in a Petri dish containing ice-cold RPMI 1640 1X supplemented with 10% FBS and 1% Penicillin/streptomycin.
5. Proceed to the following steps under a tissue culture hood. Take extra precaution to maintain strict sterile techniques to avoid neutrophil activation.
6. Rinse each bone with 70% ethanol (within a Petri dish) followed by three subsequent washes in ice-cold sterile PBS (within Petri dishes) to rinse off the ethanol from the surface of the bones.
7. Inside a clean sterile Petri dish, cut off the epiphyses of the bones and keep them aside.
8. Use a 25-gauge needle and a 12 cc syringe filled with RPMI supplemented with 10% FBS and 2 mM EDTA, and flush the bone marrow cells from both ends of the bone shafts onto a 50 ml screw top Falcon tube fitted with a 100 µm filter. In order to efficiently remove all cells, scrape the inner surface of the bones using the 25-gauge needle.

NOTE: Blanching of bones indicates that the cells have been sufficiently scraped.

NOTE: Use approximately 10 ml of media to flush a femur/tibia pair. Adding EDTA to the medium is essential to prevent clumping of the cells.

9. Cut the bone epiphyses in small 0.5-1 $mm^3$ pieces with a scalpel and smash them through the 100 µm filter using the back end of a 2.5 ml Eppendorf Combitip Plus Biopur pipette tip.
10. Centrifuge at 1,400 rpm for 7 min at 4 °C.
11. Lyse the red blood cells by resuspending the cell pellet in 20 ml of 0.2% NaCl for approximately 20 sec followed by addition of 20 ml of 1.6% NaCl. (Critical: Do not exceed 20-30 sec of hypotonic lysis to avoid bone marrow cell death. The use of hypotonic NaCl for lysis is recommended over ACK lysing buffer because the latter has the potential to activate the neutrophils).
12. Centrifuge for 7 min at 1,400 rpm at 4 °C to collect the cells.
13. Wash cells with RPMI 1640 1X supplemented with 10% FBS and 2 mM EDTA and centrifuge as in step 1.12.
14. The yield of bone marrow cells using this method is approximately 60-80 million per uninfected 8-12 week-old C57BL/6 mouse.

# 2. Separation of Neutrophils by Density Gradient Centrifugation

1. Count the bone marrow cells and resuspend in 1 ml of ice-cold sterile PBS.
2. Add 3 ml of Histopaque 1119 (density, 1.119 g/ml) in a 15-ml conical tube.
3. Overlay 3 ml of Histopaque 1077 (density, 1.077 g/ml) on the 3 ml of Histopaque 1119.

NOTE: Histopaque 1119 and Histopaque 1077 should be warmed to 18-26 °C before use.

Critical Step: Prepare gradients immediately before use as preparing the gradient in advance will result in diffusion between the two layers and suboptimal neutrophil purity and recovery.

Critical Step: Overlaying Histopaque 1077 over 1119 needs to be done slowly in order to avoid mixing the two densities, which will preclude cell separation during centrifugation.

4. Overlay the bone marrow cell suspension on top of the Histopaque 1077.

Critical Step: Overlaying the bone marrow cell suspension over Histopaque 1077 needs to be done slowly in order to avoid disturbing the interface between the cells and Histopaque 1077.

NOTE: Resuspending bone marrow cells from an uninfected mouse in 1 ml of PBS yields neutrophil purity of >90%. However, pooling many bone marrow samples compromises neutrophil purity; for example, resuspending 300 x $10^6$ cells in 3 ml PBS reduces neutrophil purity from >90% to ~80%. Therefore, investigators should perform pilot experiments to identify the ideal cell count/volume conditions for their specific experiments

5. Centrifuge for 30 min at 2,000 rpm at 25 °C without brake.

NOTE: Centrifugation of the gradient at room temperature is critical and essential for effective separation of the neutrophils.

6. Collect the neutrophils at the interface of the Histopaque 1119 and Histopaque 1077 layers.
7. Wash the collected neutrophils twice with RPMI 1640 1X supplemented with 10% FBS and 1% Penicillin/streptomycin and centrifuge at 1,400 rpm for 7 min at 4 °C.
8. Count the neutrophils and determine their viability.

NOTE: Neutrophils are typically >95% viable and >90% pure as determined by FACS analysis. The typical yield of neutrophils from the bone marrow (*i.e.* 2 femur and 2 tibia bones) of an uninfected 8-12 week-old C57BL/6 mouse is ~6-12 million cells. This number is substantially greater when neutrophils are harvested from bone marrow of infected animals. Hence, ~30-40 million neutrophils/mouse were recovered when *Candida*-infected mice were used for cell harvesting [6].

## 3. Labeling of Neutrophils Using CellTracker Dyes

1. Resuspend the neutrophils at 5 x $10^6$ cells/ml in PBS prewarmed at 37 °C.
2. Add a CellTracker dye at a final concentration of 5 µM.

NOTE: CellTracker Green (CMFDA (5-Chloromethylfluorescein Diacetate) and CellTracker Orange (CMTMR (5-(and-6)-4-Chloromethyl Benzoyl Amino Tetramethylrhodamine) was used in this protocol to differentially label neutrophils from wild-type and gene-deficient mice.

NOTE: Prepare a stock solution of 10 mM of CellTracker Green and CellTracker Orange, aliquot, and store at -80 °C until the day of the experiment.

3. Incubate neutrophils with 5 µM of the corresponding CellTracker dye for 10 min at 37 °C in a shaking water bath in the dark.
4. Wash cells twice with ice-cold RPMI 1640 1X supplemented with 10% FBS and 1% Penicillin/streptomycin.

NOTE: Efficient washing of the cells after the labeling step with the CellTracker dye is essential to avoid dye cross-contamination before mixing differentially-labeled neutrophil populations for downstream competitive repopulation studies.

## 4. Adoptive Transfer of Neutrophils in Mice and Analysis of Transferred Neutrophils Using Flow Cytometry

1. Resuspend neutrophils in ice-cold PBS at a concentration of 25 x $10^6$ cells/ml and inject 200 µl of the suspension into the lateral tail vein so that 5 x $10^6$ neutrophils are transferred per mouse. For competitive repopulation neutrophil studies, mix wild-type and gene-deficient neutrophils at a 1:1 ratio and inject a total of 5 x $10^6$ neutrophils per mouse as above.

NOTE: At least up to 10 x $10^6$ neutrophils may be adoptively transferred per mouse without obvious immediate toxicity to the animals.

2. At different times following adoptive transfer (*e.g.* 1, 2, 3 or 4 hr post-transfer), euthanize mice and harvest blood, and/or bone marrow and/or other target organ(s) of interest.
3. Prepare single cell suspensions from these tissues for quantitative and qualitative analysis of adoptively transferred labeled neutrophils using published protocols [6,15].
4. Following live/dead viability staining and Fc blockade, label cells with CD45 (clone 30-F11), Ly6G (clone 1A8) and CD11b (clone M1/70) and gate on live $CD45^+$ $Ly6G^+$ $CD11b^+$ neutrophils. Neutrophils in this gate include native neutrophils of the recipient mouse as well as the adoptively transferred labeled neutrophils, which are $FITC^+$ (if labeled with CellTracker Green) or $PE^+$ (if labeled with Cell Tracker Orange).

NOTE: Neutrophils may be tracked in blood, bone marrow and kidney of *Candida*-infected mice for at least 4 hr post-transfer.

NOTE: Fixation with 2% paraformaldehyde in PBS does not adversely affect the mean fluorescence intensity of the neutrophils labeled with CellTracker Green or CellTracker Orange for at least 48 hr.

## Representative Results

This protocol is optimized for the harvest of bone marrow cells from mice and the subsequent separation of neutrophils from these cells by density gradient centrifugation using commercially available Histopaque cell separation media. Neutrophils isolated using this method can be used for a variety of downstream functional studies *ex vivo* and for adoptive transfer experiments in recipient mice.

The typical yield of collection of bone marrow cells from both femurs and tibia per uninfected 8-12 week-old C57BL/6 mouse is ~60-80 million cells with a viability of ~94-98%. Downstream density gradient centrifugation of these cells using the Histopaque density separation media typically yields ~6-12 million neutrophils per uninfected mouse. Neutrophils are 80-95% pure and >95% viable, as verified by flow cytometry (**Figure 1**). Although it is possible to pool bone marrow cells from several animals before the density gradient centrifugation step, pooling cells slightly decreases the purity of the isolated neutrophils. For example, while overlaying 60-80 million bone marrow cells from one uninfected mouse in 1-3 ml of PBS yields >90% pure neutrophils, the cell purity decreases to ~80% when ~300 million bone marrow cells are pooled together in 3 ml of PBS and overlaid.

In addition, this protocol also describes the steps for subsequent labeling of neutrophils with CellTracker dyes, which allows for tracking of the adoptively transferred cells into recipient mice using flow cytometry. Labeling of neutrophils with 5 μM of CMFDA or CMTMR does not appear to affect neutrophil survival as labeled neutrophils remain >95% viable (data not shown). Labeled neutrophils may be tracked into various mouse tissues for at least 4 hr after adoptive transfer using flow cytometry by gating on live $CD45^+$ $Ly6G^+$ $CD11b^+$ cells (**Figure 2**). The use of differential labeling dyes in wild-type versus gene-deficient neutrophils in competitive repopulation studies allows for assessment of the direct role of a specific gene (*e.g.* chemoattractant receptor [6]) in trafficking of neutrophils from the blood into a given target organ.

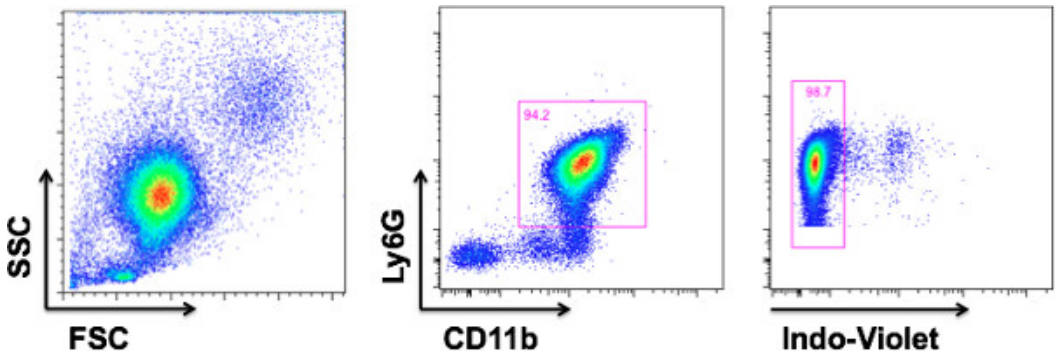


**Figure 1. Representative FACS plot of isolated neutrophils from bone marrow cells of an uninfected C57BL/6 mouse using the density gradient centrifugation protocol.** The left panel demonstrates the initial gating used for the bone marrow cells collected from the interface of Histopaque 1119 and Histopaque 1077 using forward and side scatter (FSC/SSC). As shown, neutrophils collected from the interface are >90% pure (middle panel) and >95% viable (right panel).

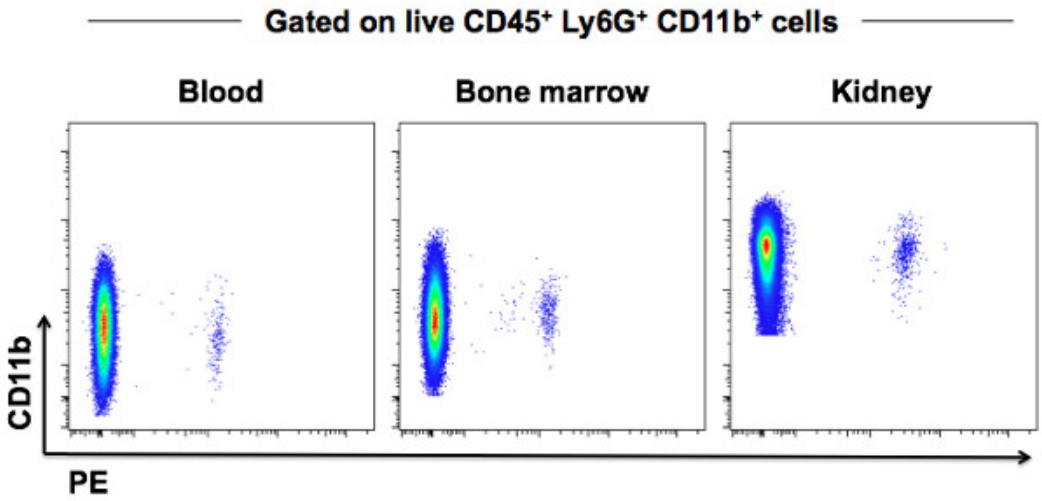


**Figure 2. Representative FACS plot of adoptively transferred CMTMR-labeled neutrophils harvested from blood, bone marrow and kidneys of a recipient *Candida*-infected C57BL/6 mouse 4 hr following cell transfer.** The adoptively transferred CMTMR-labeled neutrophils are PE-positive and can be differentiated from the native neutrophils of the recipient mouse, which are PE-negative. Note that expression of CD11b is greater in neutrophils harvested from the kidney compared to neutrophils harvested from blood and bone marrow. The increase in CD11b expression in kidney neutrophils is consistent with previous results [15] and likely reflects activation of the cells upon entry in the kidney, which is the main target organ in the mouse model of systemic candidiasis.

## Discussion

Herein we present a reliable, simple, fast and economical protocol for isolation of large numbers of neutrophils from the bone marrow of mice with high purity and viability using a density gradient centrifugation approach. When this protocol is performed correctly, ~6-12 × $10^6$ neutrophils can be recovered from an uninfected mouse and as many as ~30-40 ×$10^6$ neutrophils may be isolated from a mouse after infection [6]. The isolated neutrophils are 80-95% pure and >95% viable.

Bone marrow is a suitable reservoir for obtaining mouse neutrophils for downstream functional studies and adoptive transfer experiments. First, it has been previously shown that bone marrow neutrophils are functionally similar to blood neutrophils in mice as cells from both compartments exhibit similar oxidative burst and degranulation [16]. Second, mouse bone marrow neutrophils have been shown to survive protracted periods of time in culture, significantly longer compared to mouse blood neutrophils [16]. Therefore, harvesting of bone marrow over blood neutrophils could allow for greater yield of cells when processed for functional assays *ex vivo*. Third, isolation of neutrophils from the bone marrow offers the advantage of recovering significantly larger numbers of neutrophils, which are sufficient for downstream adoptive transfer experiments, both at steady state and after infection. Instead, considerably fewer neutrophils can be recovered from mouse blood, even after infection, and not many neutrophils are present at steady state in the peritoneal cavity. Thus, thioglycollate or other agents are typically used to promote recruitment of activated neutrophils in the peritoneum; this methodology is hampered by the variable purity of neutrophils within the peritoneal acute inflammatory exudate (unpublished, data not shown) and the induction of an activated neutrophil phenotype, which is different from that of unstimulated neutrophils recovered from unmanipulated mice.

Several methods are available for isolation of neutrophils from bone marrow of mice. These include (a) density gradient centrifugation approaches such as the one we present here that uses Histopaque density separation media, and a similar methodology that uses discontinuous Percoll gradients [17], (b) positive immunomagnetic selection approaches, during which bone marrow cells are labeled with a neutrophil-specific antibody such as Ly6G, and neutrophils are then enriched by binding of the Ly6G-positive cells on a magnetic column [18], (c) negative selection immunomagnetic approaches, during which bone marrow cells are labeled with a cocktail of antibodies that bind on cells other than neutrophils (*i.e.* CD5 for T lymphocytes, CD45R/B220 for B lymphocytes, CD49b/DX5 for NK cells, CD117 for mast cells and hematopoietic stem cells, F4/80 for macrophages and Ter119 for erythrocytes) [11], and then neutrophils are enriched by elution as the antibody-negative fraction of cells that does not bind on the magnetic column, and (d) Fluorescence Activated Cell Sorting, during which bone marrow cells are labeled with antibodies that bind on neutrophils and other cells, and neutrophils are then separated using a Fluorescence Activated Cell Sorter as the cells that are positive for neutrophil markers such as the combination of Ly6G and CD11b.

Although both positive immunomagnetic selection approaches and Fluorescence Activated Cell Sorting provide very high yields and purities of mouse neutrophils, these methods have the disadvantage compared to our protocol that labeling agents bind on the surface of neutrophils, which could potentially alter their function. Positive immunomagnetic selection approaches have the additional limitation that antibody-labeled neutrophils bind on a magnetic column for separation, which may also affect cell function. Fluorescence Activated Cell Sorting has the additional drawback that significantly longer time is required for collection of the cells using a Fluorescence Activated Cell Sorter in a laboratory core facility, which may adversely affect survival of neutrophils due to their short half-life. Our method is comparable to the density gradient centrifugation method that uses discontinuous Percoll gradients, but layering the two Histopaque density separation media is technically much less challenging compared to layering the Percoll gradients consisting of 55%, 65% and 75% vol/vol in PBS, which often results in intermixing of the Percoll interfaces due to the small density differences between the three layers. With regard to negative immunomagnetic selection approaches, they

have also been reported to be reliable for isolation of large numbers of functionally competent neutrophils with high purity and viability from bone marrow of mice [11]. Their advantage over positive immunomagnetic selection approaches and Fluorescence Activated Cell Sorting is that neutrophils are not labeled and do not bind to the magnetic column, thus avoiding cell activation. Our protocol provides an alternative reliable method to negative immunomagnetic selection approaches for separation of mouse neutrophils from bone marrow, with the advantage that it can be adapted in any laboratory without the need for equipment required for immunomagnetic separation, and without the additional cost of antibodies that are used for labeling the non-neutrophil fraction of the bone marrow cells.

Isolated neutrophils using the described density gradient centrifugation method can be used for a variety of downstream functional studies *ex vivo* such as phagocytosis, killing, chemotaxis, cytokine production, oxidative burst and degranulation assays using published protocols [6]. In addition, isolated neutrophils may be adoptively transferred into recipient infected mice to evaluate the role of neutrophil transfer on mouse survival and the effects of transferred neutrophils on immunological correlates such as cytokine production at sites of infection in recipient mice. To that end, Tosello Boari and colleagues adoptively transferred bone marrow-derived neutrophils into *Trypasonoma*-infected recipient mice, which down-regulated IFN-γ production in infected spleen and liver and resulted in improved survival in an IL-10 dependent manner [19].

Furthermore, the ability to label neutrophils with the CellTracker dyes CMFDA and CMTMR without apparent dye-induced decrease in cell viability provides an opportunity to differentially label neutrophils from mice of various genetic backgrounds and track the adoptively transferred labeled cells in different mouse target organs using flow cytometry or intravital microscopy. The CellTracker dyes are retained in viable neutrophils and do not diffuse to adjacent cells. In our studies we use a concentration of 5 μM for cell labeling and we are able to successfully track labeled neutrophils in blood, bone marrow and kidneys of *Candida*-infected recipient mice for at least 4 hr after neutrophil transfer [6]. Besides CellTracker Green and CellTracker Orange, other dyes including CellTracker Violet, CFSE and SNARF-1 have also been used successfully to label mouse bone marrow-derived neutrophils for adoptive transfer experiments [11,19,20]. In addition, the CellTracker Green and CellTracker Orange dyes have been effectively used at a similar concentration of 5 μM for labeling mouse splenic T cells for adoptive transfer and tracking experiments [21]. The ability to differentially label neutrophils with various CellTracker dyes allows for designing competitive repopulation experiments in which a 1:1 ratio of differentially labeled neutrophils from mice with different genetic backgrounds can be adoptively transferred into recipient mice with an aim to determine the direct role of specific genes in trafficking of neutrophils from the blood into inflamed tissues. We recently reported that the chemokine receptor Ccr1 mediates trafficking of neutrophils from the blood into *Candida*-infected kidneys late in the course of the infection, which results in excessive accumulation of neutrophils in the kidney, tissue injury and decreased survival [6].

As with any protocol, our method also has limitations. For example, despite the aforementioned advantages of harvesting neutrophils from bone marrow over blood, there are notable differences between neutrophils obtained from these two compartments, which depending on the downstream research application of the harvested cells could have an impact on the experimental results. Specifically, blood neutrophils are mature cells, whereas neutrophils from the bone marrow consist of three different subpopulations that range from immature promyelocytes/myelocytes to less immature metamyelocytes/band forms to mature neutrophils, as previously described [22]. Hence, the difference in cell maturity between bone marrow and blood neutrophils could result in variation in certain cell functional characteristics as it was previously reported for neutrophil responses to fMLF (N-formylmethionyl-leucyl-phenylalanine) and ingestion of particles [23]. Furthermore, because any neutrophil manipulation *ex vivo* could result in cell activation and apoptosis, caution is essential when handling the cells during gradient centrifugation. In addition, because the CellTracker dyes at concentrations greater than 5 μM could adversely affect neutrophil survival, this possibility should be tested in pilot experiments by individual investigators, depending on the downstream functional assay that will be tested.

In summary, we present a reliable and reproducible method that can be employed by any laboratory to collect and label large numbers of mouse neutrophils from the bone marrow. This approach can be used for a variety of neutrophil functional studies including *in vivo* adoptive transfer and tracking experiments by flow cytometry and intravital microscopy. The use of this and of other reliable protocols for mouse neutrophil purification, isolation, labeling and downstream adoptive transfer and tracking studies should deepen our understanding of neutrophil physiology at the molecular level.

## Disclosures

The authors declare that they have no competing interests.

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All mice were maintained at an American Association for the Accreditation of Laboratory Animal Care–accredited animal facility at the National Institute of Allergy and Infectious Diseases (NIAID) and housed in accordance with the procedures outlined in the Guide for the Care and Use of Laboratory Animals under the auspices of a protocol approved by the Animal Care and Use Committee of the NIAID.

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