MethodsX 7 (2020) 101027

Contents lists available at ScienceDirect

MethodsX

journal homepage: www.elsevier.com/locate/mex

Protocol Article

Optimized protocol for the preparation of single cells from cutaneous wounds for flow cytometric cell sorting and analysis of macrophages

Sajina Shakya[a,b], Kewal Asosingh[c,d], Judith A. Mack[a], Edward V. Maytin[a,e,*]

[a] Department of Biomedical Engineering, Lerner Research Institute, Cleveland Clinic, Cleveland, Ohio, USA
[b] Department of Chemical and Biomedical Engineering, Cleveland State University, Cleveland, Ohio, USA
[c] Flow Cytometry Core, Lerner Research Institute, Cleveland Clinic, Cleveland, Ohio, USA
[d] Department of Inflammation and Immunity, Cleveland Clinic, Cleveland, Ohio, USA
[e] Department of Dermatology, Dermatology and Plastic Surgery Institute, Cleveland Clinic, Cleveland, Ohio, USA

A B S T R A C T

The incidence of chronic, non-healing skin wounds is accelerating, largely due to the epidemic of obesity-related Type 2 diabetes. Abnormal inflammation in wounds contributes to delayed healing. During wound repair, blood monocytes are recruited into the wound bed where they differentiate into macrophages that secrete cytokines and regulate subsequent repair events. Because the study of wound macrophages via immunohistochemistry is often unsatisfactory due to nonspecific antibody staining, the ability to isolate and analyze single cells is important for determining the phenotypes of the wound macrophages. In this article, we have expanded upon a protocol originally described by Wilson et al, 2002 [1], and optimized it for isolation of large numbers of viable macrophages from murine skin wounds that are suitable for flow cytometric cell sorting or analysis. Several parameters were found to be critical for improved macrophage yields, including: (1) The proper amount of starting material (skin tissue); (2) The optimal time for addition of Brefeldin A during enzymatic digestion; (3) Revamped guidelines for centrifugation to maximize cell pellet recovery. This optimized protocol could be further modified to perform cell sorting and flow-based immunophenotyping of any cell type involved in wound healing and inflammation.

* Corresponding author at: Department of Biomedical Engineering, Lerner Research Institute, Cleveland Clinic, Mailstop ND-20, 9500 Euclid Avenue, Cleveland, Ohio 44195, USA.
E-mail address: maytine@ccf.org (E.V. Maytin).

https://doi.org/10.1016/j.mex.2020.101027

ARTICLE INFO
Method name:
Keywords: Flow cytometry, Skin wounds, Inflammation
Article history: Received 26 February 2020; Accepted 5 August 2020; Available online 8 August 2020

Specifications table

Subject Area	Medicine and Dentistry
More specific subject area	Inflammation and Wound healing
Protocol name	Isolation of macrophages from skin wounds
Reagents/tools	Instrument used for flow sorting: BD FACSAria II sorter and BD FACSDiva software (BD Biosciences) Instrument used for flow cytometry analysis: BD LSR Fortessa cell analyzer, and BD FACSDiva and FlowJo software (BD Biosciences)
Experimental design	Optimized technique to isolate living macrophages from cutaneous wounds in mice via digestion of the tissue with dispase and collagenase, and to analyze these cells via flow cytometry.
Trial registration	*N/A*
Ethics	The experiments described in this article were performed with mice under a protocol approved by the Institutional Animal Care and Use Committee (IACUC) of the Cleveland Clinic.
Value of the Protocol	*1. The protocol presents a detailed method to isolate large numbers of viable macrophages from murine skin wounds* *2. The separated, single cells can either be collected by flow sorting, or phenotypically characterized by flow cytometric analysis* *3. The experimental conditions have been optimized to maximize cell yield.*

Method

Introduction

Skin wound healing is an elegant and complicated biological process [2] in which macrophages play an integral part by clearing dead neutrophils and releasing specific cytokines that regulate the differentiation program of skin fibroblasts [3,4]. Macrophages are broadly categorized into two functional phenotypes: classically activated/pro-inflammatory (M1) and alternatively activated/ anti-inflammatory (M2) [5–7]. During the progression of wound repair, the relative balance between these two types of macrophages appears to be critical for proper and timely healing, and an abnormal ratio of M1 to M2 macrophages can contribute to wound healing failure in chronic diabetic wounds [8,9]. Thus, an optimized technique to isolate intact macrophages from wounds for flow-based analyses would be of great value for obtaining a better understanding of macrophage differentiation behavior in normal *versus* chronic wounds.

Here we present an optimized version of a method described by Wilson et al. [1] to prepare single cell suspensions from murine skin wounds for flow cytometry. Although flow cytometry is a very well-known technique for analyzing leukocytes from blood, the challenge posed by a dense, fibrous tissue such as skin is to isolate sufficient numbers of undamaged viable leukocytes (in our case macrophages) for analysis. Empirically we found that seemingly small protocol details, such as the type of centrifugation tube employed, can be critical to obtaining viable cells in high enough numbers to allow successful flow analysis, flow sorting, and downstream applications. The protocol we provide here has been optimized for isolation of individual cells from full-thickness cutaneous wounds, followed by staining with the hematopoietic cell marker CD45 and the macrophage pan-marker F4/80 [10–12] which also stains skin resident Langerhans cells [13]. Depending upon the study aim, if a distinction between the two cell populations is critical, markers like CD64 and MerTK may be better choices [14,15] to use instead or in tandem with F4/80. The stained cells can then be subsequently analyzed by two different approaches, each based upon the principle of flow cytometry. In one case, macrophages can be collected and pooled for RNA analysis; which we refer to as 'cell sorting'. Alternatively, macrophages can be analyzed by flow cytometric analysis of surface and cytoplasmic markers; we will call this 'immunophenotying'. While many procedural steps are

common to the two applications, cell sorting requires a different final series of steps than does flow cytometric analysis for immunophenotyping. For this reason, the final part of the procedure description ("Step 4"), has been divided into two sections, Step 4a (cell sorting) and Step 4b (flow cytometric immunophenotyping).

Method details

Step 1: Wounding and Wound Collection

Materials and reagents

- Dulbecco's Phosphate Buffered Saline (PBS, 1X) (Thermo Fisher Scientific, Cat# 14190250, Waltham, MA, USA)
- Ethanol, Absolute (Pharmco-Aaper, Cat# 111000200, Brookfield, CT, USA)
- Ketamine and xylazine for anesthesia (This will vary, depending upon your institution's animal protocol. At our institution, we use Ketamine at 100 mg/kg and Xylazine at 10-15 mg/kg body weight in sterile-filtered water, delivered intraperitoneally.)
- Forceps (Roboz, Cat# RS-5130, Gaithersburg, MD, USA)
- Hair clipper (Wahl Clipper Corp., Model# 9962, Sterling, IL, USA)
- Skin biopsy punch, disposable 5mm (Acuderm Inc., Cat# P550, Fort Lauderdale, FL, USA)
- Heat lamp or Slide warmer

Step 1 Procedure

Notes:

a) C57BL/6J mice were obtained from JAX Laboratories (Bar Harbor, ME) and maintained per guidelines of the American Association for Accreditation of Laboratory Animal Care. All procedures were approved by our hospital's Institutional Animal Care and Use Committee (IACUC).
b) Male and female mice, 8-10 weeks of age, were used for wounding.

Shaving:

- Put mice under light anesthesia and shave the fur from two-thirds of the dorsal skin, from below ear-level to the hind legs. Allow the mice to recover from anesthesia in a warm environment, either on a slide warmer or under a heat lamp.

Wounding:

- Inject an approved dose of anesthetic and disinfect the shaved skin area with 70% ethanol.
- To make full thickness excisional wounds, pinch the dorsal shaved skin at midline to make a large fold. Lay the mouse down on its side on the surgical table, and pull the folded skin far away from the body (see Fig. 1a, b). Press the skin biopsy punch through the folded skin to create two clean wounds, located about a centimeter (1 cm) below the ear and 1 cm apart. Similarly, with a new biopsy punch, make a second set of wounds 1-2 cm caudal to the first set (Fig. 1c).
- Let the mice recover in a warm environment (under a heat lamp or on a slide warmer in a cage). After wounding, house each mouse in individual cages and monitor them until collection day.

Wound collection:

- At the chosen day post-wounding, under deep general anesthesia, harvest the entire wound area plus ~2 mm rim of surrounding unwounded skin, down to the subcutaneous fat layer. Store the collected tissue on wet ice during this process, in a labeled container such as the lid of a 60 mm tissue culture plate.
- Once all the wounds are collected, euthanize the mouse.

Optimization Item 1:

Through trial and error, we defined the amount of wound tissue that yields enough cells to perform successful cell sorting or flow cytometric immunophenotyping of macrophages. We found

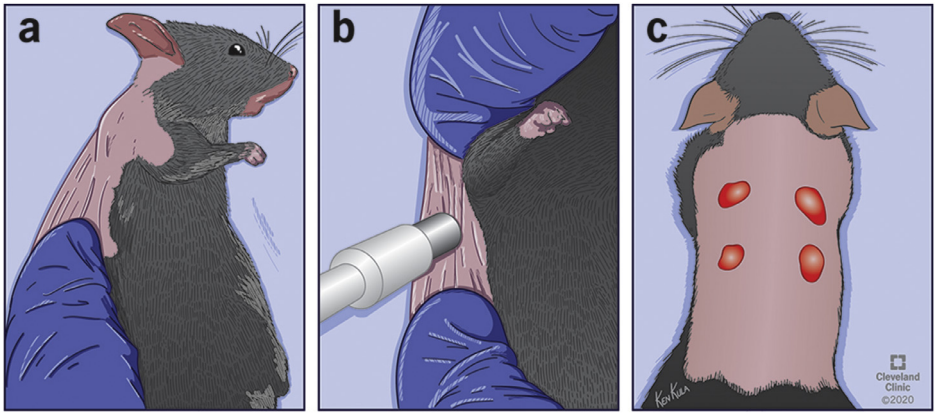


Fig. 1. Illustration of how 4 nearly-identical wounds are made on the back of a mouse. (***a***), The fully-anesthetized mouse is laid on its side and the skin is gently extended away from the body using a thumb and forefinger (shown in blue gloves). (***b***), A 5-mm biopsy punch is placed against the skin, and slightly rotated back and forth with pressure until it passes entirely through the double fold of skin. This creates two circular wounds. In a similar way, two more wounds are made 1-2 cm posterior to the first set of wounds. (**c**) Appearance of 4 wounds created on the dorsum of the mouse. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

that using only 2 wounds per mouse often gave too low a yield. Ultimately, pooling eight wounds per sample (obtained from 2 mice, with 4 wounds per mouse—See Fig. 1) consistently provided enough starting material for experiments.

Step 2: Tissue Digestion and Single Cell Preparation

Materials and reagents

- Collagenase, Type 1 (Worthington, Cat# LS004196, Lakewood, NJ, USA)
- Dispase II (Roche Diagnostics, Cat# 04942078001, Indianapolis, IN, USA)
- DNase I (Worthington, Cat# LS002060)
- Dulbecco's Phosphate Buffered Saline (PBS, 1X) (Thermo Fisher Scientific, Cat# 14190250)
- EDTA disodium salt (Thermo Fisher Scientific, Cat# BP120)
- Fetal Bovine Serum (FBS), Heat-inactivated (Gibco™, Thermo Fisher Scientific, Cat# 10082139)
- Fixation/Permeabilization Solution Kit with BD GolgiPlug™ (BD Biosciences Cat# 555028, San Jose, CA, USA); GolgiPlug™ contains Brefeldin A. *NOTE: This kit is not required if doing cell sorting only.*
- *Flow Buffer* (3% FBS and 0.1 mM EDTA in PBS)
- G418 Sulfate antibiotic (Corning, Cat# 61-234-RG, Corning, NY, USA)
- Hank's buffered salt solution (HBSS, 1X) (Thermo Fisher Scientific, Cat# 14175079)
- Trypan Blue solution (Lonza, Cat# 17-942E, Walkersville, MD, USA)
- Falcon® 40-µm cell strainer (Corning, Cat# 352340)
- Falcon® 15 ml polypropylene conical tube (Corning, Cat# 352097)
- Falcon® 50 ml polypropylene conical tube (Corning, Cat# 352098)
- Forceps (Roboz, Cat# RS-5130)
- Hemacytometer (Bright-Line™, Sigma-Aldrich, Cat# Z359629, St. Louis, MO, USA)
- Kimwipes™ (Kimberly-Clark, Cat# 34120, Milsons Point, NSW, Australia)
- Scissors (V. Mueller® Iris scissors, CareFusion, Cat# OP5526, McGaw Park, IL, USA)
- Syringe filter, Fisherbrand® 25 mm, 0.2-µm (Thermo Fisher Scientific, Cat# 09-719C)
- Syringe, BD 60 ml (Becton Dickinson, Cat# 309653, Franklin Lakes, NJ, USA)
- Centrifuge (5810 R, Eppendorf, Hauppauge, NY, USA)
- Incubator shaker (C24, New Brunswick Scientific, Edison, NJ, USA)

Step 2 Procedure

Notes:

a) To have enough cells to process samples for cell sorting or immunophenotyping, wounds from 2 mice are pooled for each sample (total of 8 wounds per sample).
b) Keep the tissue pieces and the cells on wet ice (or at 4°C) at all times, unless otherwise stated.
c) Prepare 500 ml of Flow Buffer and store it at 4°C (or on ice) at all times. This buffer will be used for all washing and incubation steps unless otherwise mentioned.
d) Throughout the protocol, it is important to use polypropylene conical tubes (and not polystyrene tubes or dishes) to avoid loss of cells that adhere to the plastic.
e) While some protocols suggest a combined dispase and collagenase digestion step for 1-2 h at 37°C, we found that for skin wounds, a longer protocol with separate dispase digestion (at 4°C overnight) and collagenase digestion (at 37°C for 2 h) steps results in better cell viability and yield.
f) For flow cytometric immunophenotyping, remember to include Brefeldin A (or Monensin) whenever intracellular cytokine staining is involved to inhibit extracellular transport. Here, we used GolgiPlug™ which contains Brefeldin A to hinder transport of the TNFα cytokine; this helps in signal enhancement.

Dispase digestion:

- Weigh the wound tissue, then disinfect the tissue with a quick rinse in 70% ethanol followed by a rinse in 1X PBS. Dab the tissue with a Kim-wipe to remove remnants of PBS. On average, four 7-day wounds pooled from one mouse weighs about 175 mg.
- Prepare *dispase digestion buffer* in HBSS by adding Dispase II at 1 mg/ml, G418 at 10 mg/ml, and FBS to a concentration of 3%. Filter using a 0.2-µm syringe filter and a 60 ml syringe. A Stericup vacuum filtration system with a 0.22 µm pore size (Millipore, Cat# S2GPU02RE, Burlington, MA, USA) can be used for processing a bigger volume. Prepare this buffer fresh every time.
- Each milligram of wound tissue will require 20 µl of the dispase digestion buffer. For each sample (8 wounds), aliquot the required amount of dispase digestion buffer into a 50 ml polypropylene conical tube on wet ice. We use 50 ml conical tubes, even though the total volume is generally less than 10 ml because it makes it easier to remove tissue pieces for the next step.
- For each sample, mince tissues into 2-3 mm^2 pieces using forceps and a scalpel, and pool them in a 50 ml tube with dispase buffer. Do this on wet ice. Incubate at 4°C overnight (no shaking required).
- The following step is only for flow cytometric immunophenotyping when any of the chosen cell markers is an intracellular cytokine. For the last two hours of dispase digestion, add BD GolgiPlug™ from the Fixation/Permeabilization solution kit to each sample, at a concentration of 1 µl/ml of digestion buffer.

Optimization Item 2:

For samples and controls intended for flow cytometric immunophenotyping, we add the GolgiPlug™ (containing Brefeldin A) 2 h before the end of the dispase digestion step. This helps to retain secretory cytokines (here, TNFα) inside the Golgi body within cells, while preventing cell death that results from longer term exposure to the GolgiPlug™.

Collagenase digestion:

- Prepare *collagenase digestion buffer* in HBSS by adding Collagenase I, 1 mg/ml; G418, 5 mg/ml; DNase I, 75 U/ml. Filter using a 0.2-µm syringe filter and a 60 ml syringe. Again, a Stericup vacuum filtration system with a 0.22 µm pore size can be used for processing a bigger volume. Prepare this buffer fresh every time. It can be prepared a day earlier and stored at 4°C.
- If doing flow cytometric immunophenotyping of an intracellular cytokine marker, add BD GolgiPlug™ from the Fixation/Permeabilization solution kit (1 µl/ml) to the filtered collagenase digestion buffer mixture from above.
- Each milligram of wound tissue requires 80 µl of the collagenase digestion buffer. For each sample, aliquot the required amount of the collagenase digestion buffer into a labeled 50 ml conical tube at room temperature. Using forceps, transfer tissue pieces from the dispase to the collagenase digestion buffer. Incubate for 2 h at 37°C with gentle shaking (95 rpm) in an incubator shaker. Save the remaining dispase buffer, which will contain a population of cells released from the first digestion step, on ice.

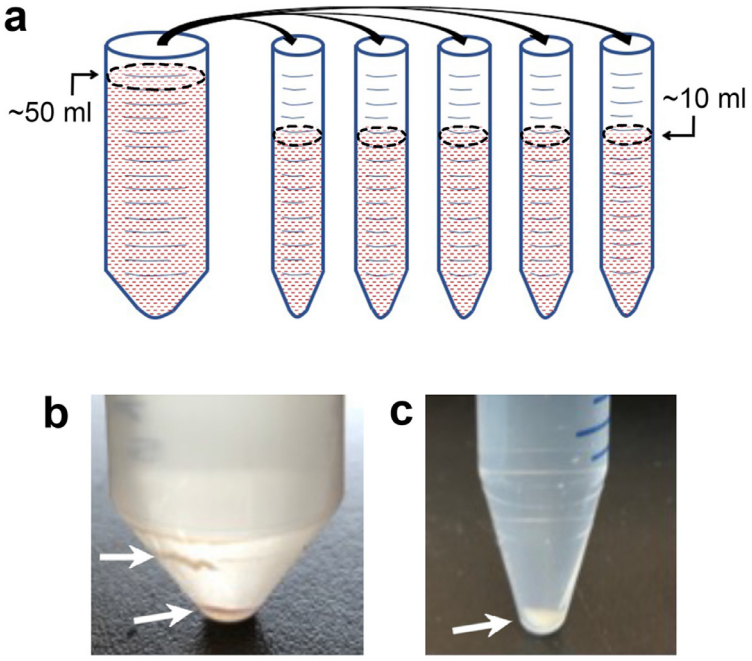


Fig. 2. Use of appropriate tube sizes for centrifugation is critical to keep the cells intact and to form a proper cell pellet. (a), Schematic showing the transfer of the filtered cell suspension from one 50 ml conical tube to multiple 15 ml conical tubes for centrifugation, as described in the text. (b), Centrifugation of a large volume in a 50 ml conical tube results in a poorly-formed pellet or smear. (c), Use of a 15 ml conical tube yields a clean, properly formed cell pellet.

- After incubation, combine the cells and tissues from the dispase and collagenase digestions and filter through a 40 µm cell strainer into one or more 50 ml conical tubes, as required. Use 4-5 ml of Flow Buffer to rinse both digestion tubes and pass this through the cell strainer.
- For centrifugation, divide the filtered cell suspension into 15 ml Falcon tubes, with ~10 ml volume in each (Figs. 2–4). Each sample may require 5 or more tubes. The rationale here is that under the required centrifugation conditions (see below), cells fail to form nice pellets in 50 ml tubes when large volumes are used, and many cells are lost in the process.

Optimization Item 3:

Single cell suspensions obtained after dispase and collagenase digestions are transferred to 15 ml tubes for better centrifugation. As shown in Fig. 2, the cells do not form a proper pellet when centrifuged with large volumes in 50 ml conical tubes, but when redistributed into 15 ml tubes and centrifuged, the cells form a nice pellet as desired. This along with Optimization Item 4 (below) resulted in better and cleaner yields. For example, from the same number of wounds, we obtained ~6.7 million cells vs ~3.2 million cells (>50% increase) after the two modifications.

- Centrifuge at 350 x g for 10 min at 4°C.
- Being careful not to disturb the cell pellet, remove the supernatant using a vacuum aspirator or pipettor, leaving about 1 ml above the cell pellet.
- Add 3 ml of Flow Buffer to the cell pellet and resuspend using a 10 ml pipet. Centrifuge at 350 x g for 10 min at 4 °C.
- Again, remove the supernatant with a pipettor or vacuum aspirator, leaving about 500 µl of supernatant.
- Resuspend the cell pellets in 1 ml of Flow Buffer per tube, then combine the cell suspensions into a single 15 ml conical tube and pipet to mix well. Count live cells with a hemacytometer.

Assessment of viability of the single cell suspension:

Combine 10 µl of a well-mixed cell suspension and 10 µl of Trypan Blue solution. Pipette 10 µl of this mix onto a hemacytometer. Incubate for 2 min and count the unstained (viable) cells. We typically obtain an average of 10 million live cells per sample (8 wounds) with >97% viability.

Note: It is normal to see some debris along with single cells in the mixture under the microscope.

Step 3: Cell Staining with Fluorophore Markers for Flow Sorting or Flow Cytometric Immunophenotyping

For cell sorting (where subsets of cells are collected), or flow cytometric immunophenotyping (in which cells are only analyzed but not sorted), the procedure described in Step 3 is the same for both approaches. After that, in Step 4 the procedures diverge. Step 4a is unique to cell sorting of macrophages, while Step 4b contains instructions designed for flow cytometric immunophenotyping.

Materials and reagents

The following four antibodies:

⇒ FITC-conjugated rat anti-mouse CD45 antibody, Clone 30-F11 (BioLegend, Cat# 103107, San Diego, CA)
⇒ PE/Cy5-conjugated rat anti-mouse F4/80 antibody, Clone BM8 (BioLegend, Cat# 123111)
⇒ Brilliant Violet 650™-conjugated rat anti-mouse TNFα antibody, Clone MP6-XT22 (BioLegend, Cat# 506333)
⇒ PE-conjugated polyclonal sheep anti-Arginase-1 antibody (R&D Systems, Cat# IC5868P, Minneapolis, MN, USA)

- AbC™ Total Antibody Compensation Bead Kit (Thermo Fisher Scientific, Cat# A10497)
- Fixation/Permeabilization Solution Kit with BD GolgiPlug™ (BD Biosciences Cat# 555028); *this kit is not required if doing cell sorting only.*
- LIVE/DEAD™ Fixable Blue Dead Cell Stain Kit (Thermo Fisher Scientific, Cat# L23105)
- Mouse BD Fc Block™, Purified Rat Anti-Mouse CD16/CD32 (BD Biosciences, Cat# 553141)
- TRIzol™ Reagent (Thermo Fisher Scientific, Cat# 15596018)
- 1.7 ml microcentrifuge tubes (Denville, Cat# C2170, Saint-Laurent, QC, Canada)
- 2 ml microcentrifuge tubes (USA Scientific, Cat# 1620-2700, Ocala, FL, USA)
- CellTrics® filters, 30 µm (Sysmex Partec GMBH, Cat# 04-004-2326, Görlitz, Germany)
- Fisherbrand® serological pipets (Thermo Fisher Scientific, 5 ml, Cat# 13-678-11D, and 10 ml, Cat# 13-678-11E)
- Centrifuge 5415 D (Eppendorf) [store in a cold-room, at 4 °C, for cold centrifugation]

Optimization Item 4:

We found that one can minimize loss of cells due to the washing and pipetting steps by limiting the total number of centrifugations. Thus, we perform only 2 instead of 3 washes between each step. This does not diminish the ultimate outcome.

Step 3 Common parameters, and set up of Controls

Notes:

a) During these experiments it is important to focus on living cells, not cells that have died during processing because the latter could provide spurious results. To distinguish between these two populations, Live/Dead dye is added as an important gating step. Treat the cells as gently as possible while mixing to avoid damaging them. Use 5- or 10- ml serological pipets to mix the cells in the conical tubes, or 1 ml pipettes in microcentrifuge tubes. Do not vortex live cells.
b) *Centrifugation parameters* to use when preparing controls and samples: for 1.7 ml microcentrifuge tubes, 400 x g for 5.5 min at 4°C.; for 15 ml conical tubes, 350 x g for 10 min at 4°C.
c) *Working concentrations for the antibodies* used in this protocol are based upon optimization done for our particular tissue/cell type (refer to Supplemental Table 1 for an example). Optimization is performed by testing with at least 6 serial dilutions, starting with a concentration recommended in the antibody datasheet, and tested by flow cytometry. The

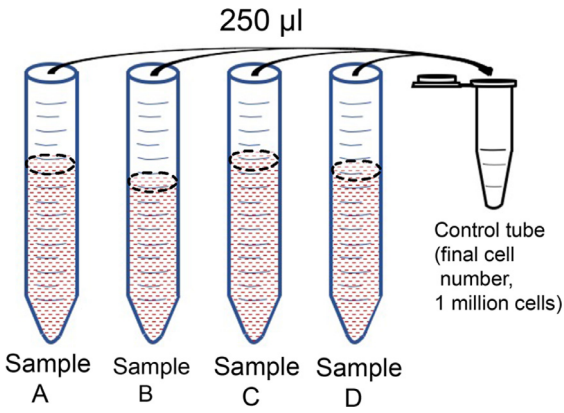


Fig. 3. Schematic showing preparation of control tubes. To prepare a control tube, take an aliquot of cell suspension from each sample and mix these in the 1.7 ml tube as shown; refer to Note (d).

working concentrations for the antibodies used here are as follows: anti-CD45 at 1:200, anti-F4/80 at 1:80, anti-Arginase-1 antibody at 1:10 and anti-TNFα antibody at 1:40. (To see the results from the titration experiments that informed our selection of the working dilution to use for each of the above antibodies, *see* Supplemental Fig. 1.)

d) *It is critical to have a proper set of controls* for successful flow cytometry. These controls may contain either cells, or fluorescent beads ("Compensation control beads," see **note f**). Control tubes and experimental tubes should be treated in the same way, with the same number of washes and centrifugations. To prepare control tubes that contain cells, take equal aliquots of cell suspension from each sample to achieve a final control sample with 1 million cells. For example, if you have 4 samples, each resuspended at 1 million cells/ml, then take 250 µl from each sample tube and put in a 1.7 ml microcentrifuge tube (Fig. 3).

e) For sorting of macrophages, a list of controls that are required is shown in Fig. 4. For flow cytometric immunophenotyping of macrophages (to analyze M1 versus M2 polarization), a list of controls is provided in Fig. 5.

f) COMPENSATION CONTROLS are single-color controls used to compute a compensation matrix that can account for spectral spillover. The AbC^{TM} Compensation beads were used to capture antibodies for antibody compensation controls for both applications, except for the case of Arginase-1 antibody. Our Arginase-1 was a sheep primary antibody and was not compatible with the AbC^{TM} beads kit, so we used cells instead.

g) Compensation beads were labeled as outlined in the kit manual.

h) For flow cytometric immunophenotyping, we use a solution containing 4.2% paraformaldehyde for fixation and a buffer containing saponin for permeabilization (for intracellular staining) from the BD Fixation/Permeabilization Solution Kit. However if desired, other reagents containing the fixative or permeabilizing agent can be used.

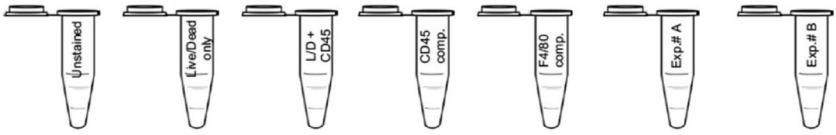
i) While we recommend an antibody concentration for a fixed number of cells (below) based upon trial runs, it is important to note that saturating antibody titers are not dependent on the number of cells, as long as the cell number does not change more than approximately 10-fold. In this protocol, we have used 1 million cells per sample. If the antibodies we used had been titered to saturation for the 1 million cells, then the same concentration could have been used for 100,000 cells or 10 million cells. The number of cells per tube could have been changed as needed, keeping in mind that the ratio between the number of cells and antibody concentration is kept constant across the samples.

Step 3 Procedures common to Cell sorting and to Flow cytometric immunophenotyping

Staining with Live/Dead^{TM} blue:

a **Chart of All Tubes Required for Cell Sorting**

Tube:	Control Tube #1	Control Tube #2	Control Tube #3	Control Tube #4	Control Tube #5	Experiment Tube #A	Experiment Tube #B
Contents:	Cells	Cells	Cells	Compen-sation beads	Compen-sation beads	Cells	Cells
Antibody mix:	No Antibody	No Antibody	CD45	CD45	F4/80	CD45 F4/80	CD45 F4/80
Live/Dead blue:	No	Yes	Yes	No	No	Yes	Yes



b **Plan for Preparation of the Control Tubes for Cell Sorting**

Tube #	Fluorophore tagged markers			Tube Label	Purpose
	Live/Dead™ blue	CD45	F4/80		
1				Unstained	Compensation
2	✓			Live/Dead only	Compensation
3	✓	✓		L/D + CD45	FMO *
4		✓		CD45 Comp.	Compensation
5			✓	F4/80 Comp.	Compensation

✓ = Marker added into tube

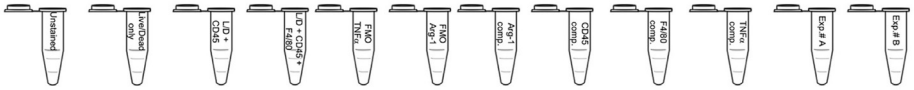
* = FMO, Fluorescence minus one

Fig. 4. Preparation of controls required for cell sorting of macrophages (a, b). For further explanation of these controls, refer to Note (d).

- For each sample from Step 2 (in 15 ml tubes), add Flow Buffer to adjust the cell count to 1 million cells/ml.
- Prepare a 1 ml aliquot for the unstained control (Tube# 1) into a labeled 1.7 ml microcentrifuge tube (as outlined in Note c) and treat this tube similarly to the rest of the cells. Remember to centrifuge/wash control tubes the same number of times as the other tubes for the entire procedure. If doing flow cytometry analysis, separate an additional aliquot for an Arginase-1 compensation control (Tube# 7 in Fig. 5).

a **Chart of All Tubes Required for Flow Cytometric Immunophenotyping**

Tube:	Control Tube # 1	Control Tube # 2	Control Tube # 3	Control Tube # 4	Control Tube # 5	Control Tube # 6	Control Tube # 7	Control Tube # 8	Control Tube # 9	Control Tube # 10	Experimental Tube A	Experimental Tube B
Contains:	Cells	Cells	Cells	Cells	Cells	Cells	Cells	Compensation beads	Compensation beads	Compensation beads	Cells	Cells
Antibody mix:	No Antibody	No Antibody	CD45	CD45 F4/80	CD45 F4/80 Arg-1	CD45 F4/80 TNFα	Arg-1	CD45	F4/80	TNFα	CD45 F4/80 Arg-1 TNFα	CD45 F4/80 Arg-1 TNFα
Live/Dead Blue:	No	Yes	Yes	Yes	Yes	Yes	No	No	No	Yes	Yes	Yes



b **Plan for Preparation of the Control Tubes for Flow Cytometric Immunophenotyping**

Tube #	Fluorophore tagged markers					Tube Label	Purpose
	Live/Dead™ blue	CD45	F4/80	Arg-1	TNFα		
1						Unstained	Compensation
2	✓					Live/Dead only	Compensation
3	✓	✓				L/D + CD45	Gating Control
4	✓	✓	✓			L/D + CD45 + F4/80	Gating Control
5	✓	✓	✓	✓		FMO TNFα	FMO
6	✓	✓	✓		✓	FMO Arg-1	FMO
7**				✓		Arg-1 Comp.	Compensation
8*		✓				CD45 Comp.	Compensation
9*			✓			F4/80 Comp.	Compensation
10*					✓	TNFα Comp.	Compensation

Footnotes: ✓ = Marker added into tube
* = Compensation control with AbC™ compensation beads
** = Compensation control with cells (unique to Arg-1)
FMO = Fluorescence minus one

Fig. 5. Preparation of controls required for flow cytometric immunophenotyping of macrophages (a, b). For further explanation of controls, refer to Note (d), above

- Stain cell samples in 15 ml tubes for dead cells using the LIVE/DEAD™ Fixable Blue Dead Cell Stain Kit as recommended in the user guide. Remember to bring the vial of DMSO and a tube of reactive dye from the kit to room temperature before using. Add 1 µl of reconstituted fluorescent reactive dye per 1 mL of cell suspension.
- Mix well using a 10 ml pipet and incubate on ice for 30 min in the dark.
- After incubation, add 5 mL of Flow Buffer to each sample and centrifuge. Remember to centrifuge control Tube# 1 (and Tube# 7 for flow cytometry analysis) as well, to assure all tubes receive the same treatment.
- Remove the supernatant as before, leaving about 500 µl. Resuspend the pellet in 6 ml of Flow Buffer (and the control pellet in 1 ml of Flow Buffer) and centrifuge one more time.
- Remove supernatants from the 15 ml tubes as before, leaving about 250 µl. (Resuspend the control pellets in 1 ml of Flow Buffer and place on ice).

Blocking Fc receptors and staining for fluorophore-tagged (extracellular) antibody markers:

- Resuspend cell pellets in the 15 ml tubes at a concentration of 1 million cells per 50 µl of Flow Buffer.
- Separate out a 50 µl total aliquot of cells for the Live/Dead only control (Tube# 2) into a labeled 1.7 ml microcentrifuge tube. As explained in Note (c) above, the control is prepared by mixing aliquots of cell suspension from all samples [that is, if you have 4 samples, take 12.5 µl from each sample to prepare a control tube]. Add 50 µl of Flow Buffer to bring the volume to a total of 100 µl.
- To the remaining cells in the 15 ml tubes, add 1 µg (= 2 µl) of Mouse BD Fc Block™ per 50 µl of cell suspension and incubate for 5 min on ice in the dark.
- During this incubation, prepare the cell surface antibody mix as follows: (1) For Live/Dead + CD45 control (control Tube# 3), prepare 50 µl of anti-CD45 antibody at 1:100 in Flow Buffer. (2) For the remaining cells in 15 ml tubes, prepare a cocktail of anti-CD45 antibody at 1:100 and anti-F4/80 antibody at 1:40 in Flow Buffer. Prepare 50 µl of this mix per 1 million cells. Calculate the volume of the antibody cocktail as follows:

Total volume of antibody cocktail = 50 µl x total number of cells per million in combined samples.

For example, if Sample A has 5 million cells and Sample B has 8 million cells, the total volume of antibody cocktail is: 50 x (5+8) = 650 µl.

- After incubation with Fc Block, separate an aliquot of 50 µl of cell suspension for the Live/Dead + CD45 control (Tube# 3) into a labeled 1.7 ml microcentrifuge tube by again mixing aliquots from all samples (as outlined in Note d). Do not wash Fc Block away prior to adding the antibody cocktail.
- Add the antibody mix prepared as above into control Tube# 3 and the 15 ml tubes and incubate for 20 min on ice in the dark.

NOTE: If you are doing cell sorting of macrophages, then continue on to Step 4a and refer to Fig. 4 for the appropriate tubes needed for controls and experiments.

NOTE: If you are doing flow cytometric immunophenotyping of macrophages, skip to Step 4b and refer to Fig. 5 for the appropriate tubes needed for controls and experiments.

Step 4a Procedure for cell sorting of macrophages

During the incubation, transfer the remaining cells from the 15 ml tubes into as many microcentrifuge tubes (1.7 ml or 2 ml) for experimental samples, irrespective of the cell number per sample tube. See Fig. 4 for a description of the tubes needed for all applicable conditions by the end of this process.

- After incubation, add 500 µl of Flow Buffer to all the microcentrifuge tubes, then centrifuge.
- Remove the supernatant using a 1 ml pipette, leaving about 50 µl. Resuspend the cell pellet in 1 ml of Flow Buffer and centrifuge once more.

- Remove the supernatant carefully using a 1 ml pipette, leaving about 50 µl. Resuspend the pellet to a concentration of 1 million cells per 100 µl of Flow Buffer and store in the cold for cell sorting. Plan to sort these cells as soon as possible after staining.

Collection of cells after sorting:

Flow sorting was done using a BD FACS Aria II sorter and BD FACS Diva software (BD Biosciences) with technical assistance from personnel at the Cleveland Clinic LRI Flow Cytometry Core. Cells were sorted using a 100 µm nozzle size at a low sheath pressure of 20 psi.

- Label 2 ml microcentrifuge tubes or 15 ml conical tubes as required to collect the desired cell populations. For each experimental sample, we collected two subsets of cell populations for RNA isolation, as follows:
  (1) $CD45^+$ (CD45 only positive) cells for control.
  (2) $CD45^+$ $F4/80^+$ (CD45 and F4/80 double positive) macrophages.
- Add 500 µl of TRIzol™ reagent into the labeled collection tubes before loading them on to the sorter.
- Right before sorting, filter the cell samples (controls and experimentals) through CellTrics® 30 µm filters to remove any clumps.
- After sorting, calculate the volume of cell suspension sorted into each tube using a 1 ml pipette and add more TRIzol to bring the ratio of sort volume and TRIzol to at least 1:3. Pipette up and down multiple times to lyse the cells and homogenize well.
- Store the homogenized sample on ice. When ready to isolate RNA, incubate the homogenized sample for 5 min at room temperature and proceed with RNA isolation following standard protocol.

NOTE: The samples in TRIzol can be frozen at -20 °C if necessary, for later RNA isolation. However, it is discouraged for this protocol as the number of sorted cells is limited. Please see the *Method Validation* section below for further details.

Step 4b Procedure for Flow Cytometric Immunophenotyping of macrophages

Tubes needed to accommodate all applicable conditions are shown in Fig. 5.
Fixation/Permeabilization:

- After incubation with the cell surface antibody mix in Step 3 above, add 2 ml of buffer to the 15 ml tubes and centrifuge.
- Remove the supernatant carefully using a 1 ml pipette, leaving about 100 µl. Resuspend in 3 ml of buffer and centrifuge again.
- Remove the supernatant and resuspend the cells at a concentration of 1 million cells per 100 µl of Flow Buffer.
- Add 250 µl of Fixation/Permeabilization solution per 1 million cells, mix by pipetting, and incubate for 20 min on ice in the dark.
- During the incubation, prepare 1 X Perm/Wash buffer from the 10X BD Perm/Wash buffer in the Fixation/Permeabilization Solution Kit as recommended.
- After incubation with the fixation/permeabilization buffer, add 500 µl of 1 X Perm/Wash buffer per 1 million cells, mix and centrifuge.
- Remove the supernatant using a 1 ml pipette, resuspend the cell pellet in 1 ml of BD Perm/Wash buffer and centrifuge again.
- Remove the supernatant and resuspend the cell pellets at 1 million cells per 50 µl of Flow Buffer.
- Prepare an aliquot of 50 µl of cell suspension for the Live/Dead + CD45 + F4/80 control (Tube# 4) as before.
- To the remaining sample tubes, add 1 µg (= 2 µl) of Mouse BD Fc Block™ per 50 µl of cell suspension and incubate for 5 min in the dark at room temperature.
- During this incubation, prepare the intracellular antibodies as follows: (also Refer to Fig. 5 for more information about the controls).
  (1) For the FMO TNFα control (Tube# 5), prepare 50 µl of anti-Arginase-1 antibody at 1:5 in Flow Buffer.

(2) For the FMO Arg-1 control (Tube# 6), prepare 50 µl of anti-TNFα antibody at 1:20 in Flow Buffer.
(3) For the Arg-1 comp. control (Tube# 7), prepare 50 µl of anti-Arginase-1 antibody at 1:5 in Flow Buffer.
(4) For the experimental tubes, prepare a cocktail containing anti-Arginase-1 antibody at 1:5, and anti-F4/80 antibody at 1:40 in Flow Buffer. Prepare 50 µl of this mix for as many experimental samples as required. This can be simply calculated as: (Number of samples) x 50 µl.

- After incubation with Fc Block, prepare aliquots of 50 µl of cell suspension (~1 million cells ideally) for the FMO control tubes (Tube# 5-6) in labeled 1.7 ml microcentrifuge tubes as before.
- From the remaining cells, prepare the experimental samples. Transfer one 50 µl aliquot (1 million cells) from one 15 ml tube to one 1.7 ml microcentrifuge tube. Note: It is important to have an equal number of cells in each experimental sample.

Staining intracellular markers for M1 and M2:

- Add 50 µl of intracellular antibody mix prepared as above to respective control tubes and experimental tubes. Incubate for 25 min in the dark at room temperature
- During this incubation, prepare the compensation controls with beads (Tube# 8, 9 and 10). Stain the beads as outlined in the kit manual.
- After the incubation, add 500 µl of Flow Buffer to all tubes and centrifuge @ 900 x g for 5.5 min at room temperature. Resuspend the cell pellets in 1 mL of buffer and centrifuge once more.
- Resuspend the pellets in 100 µl of Flow Buffer and store in the dark until ready for flow cytometry analysis.

Performance of Flow Cytometric Immunophenotyping:

- Flow cytometry was performed on a BD LSR Fortessa cell analyzer and BD FACS Diva software (BD Biosciences) at the Cleveland Clinic Lerner Research Institute. More details are provided below under '*Validation of flow cytometric immunophenotyping*'.

Method validation

Validation of cell sorting

Single cells were isolated from wounds as described. After staining, the cells were sorted, using a BD FACS Aria II sorter and BD FACS Diva software (BD Biosciences), with technical assistance from the Cleveland Clinic Flow Cytometry Core personnel. The gating strategy for cell sorting, as determined by the FMO control tubes and experimental samples, is depicted in Fig. 6 in which representative dot plots show the selection of the gate boundaries (Fig. 6**a–e**), and the final dot plot for the experimental sample (Fig. 6**f**). Two groups of CD45+ cells, one group being F4/80+ and the other F4/80-, were determined by the two right-hand quadrants in Fig. 6**f**. Both streams of cells were collected directly into TRIzol reagent. For the particular experiment illustrated (n= 10 samples, 8 wounds/ sample), the following average numbers of cells were obtained:

- 55,400 $CD45^+$ $F4/80^+$ double positive macrophages
- 85,900 $CD45^+$ single positive (F4/80 negative) cells

When ready for RNA isolation, the homogenized samples stored in ice (or thawed in ice for frozen samples) were incubated at room temperature for 5 min. RNA was isolated as per the standard TRIzol RNA isolation protocol. To enhance the amount of RNA obtained, Phase Lock Gel™ Heavy tubes (VWR, Cat# 10847-802, Radnor, PA, USA) were used for better separation of the aqueous layer from the interphase and organic phase during centrifugation. The RNA pellets obtained after isopropanol precipitation were washed twice with 75% ethanol and resuspended in RNase free water. Quantification was done using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific) and complementary DNA (cDNA) was prepared using 200 ng of RNA each.

To validate the specificity of the sort, we performed a quantitative PCR (qPCR) assay for the *Adgre1* (adhesion G protein-coupled receptor E1) gene that encodes for the F4/80 protein. TaqMan primers

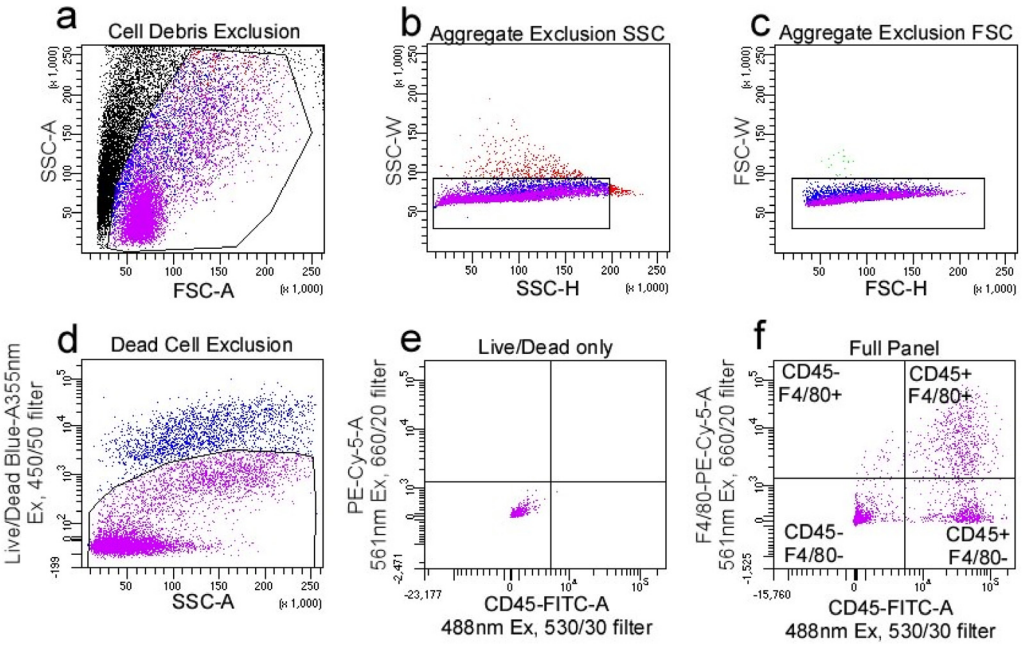


Fig. 6. Gating strategy and representative dot plots for cell sorting. Gates were set using control tubes #1-5 shown earlier in Fig. 4b. (a), Cell debris was excluded using forward- and side-scatter gates from the unstained control (Tube# 1). Aggregates were excluded using side-scatter (b) and forward-scatter (c) gating. (d), Dead cells were rejected by gating of the Live/Dead only control (Tube# 2). (e), Viable macrophages were selected by gating of the CD45+ and Live/Dead dye negative control (Tube# 3). (f), The full panel showing CD45+ F4/80+ ("double positive") cells in the upper right guardant. Control tubes #4 and #5 (images not shown) were used to compensate for spectral spillover between different fluorescence channels.

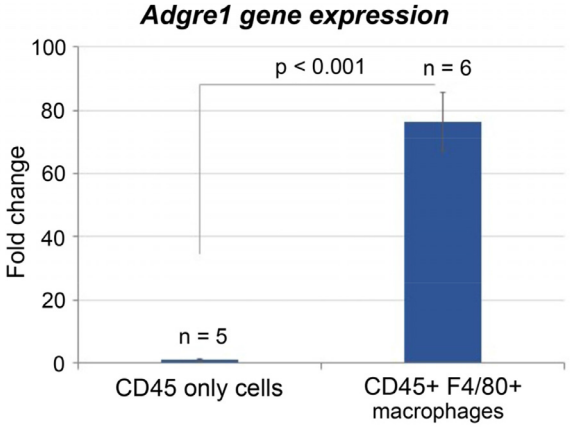


Fig. 7. Evaluation of Adgre1 expression for the two flow sorted cell populations. The gene expression level of macrophage specific marker protein F4/80 is at least 75-fold higher in the double positive macrophage cell population than in the CD45 only positive control cell population. Statistical analysis was performed using Student's t-test. *n*, number of samples.

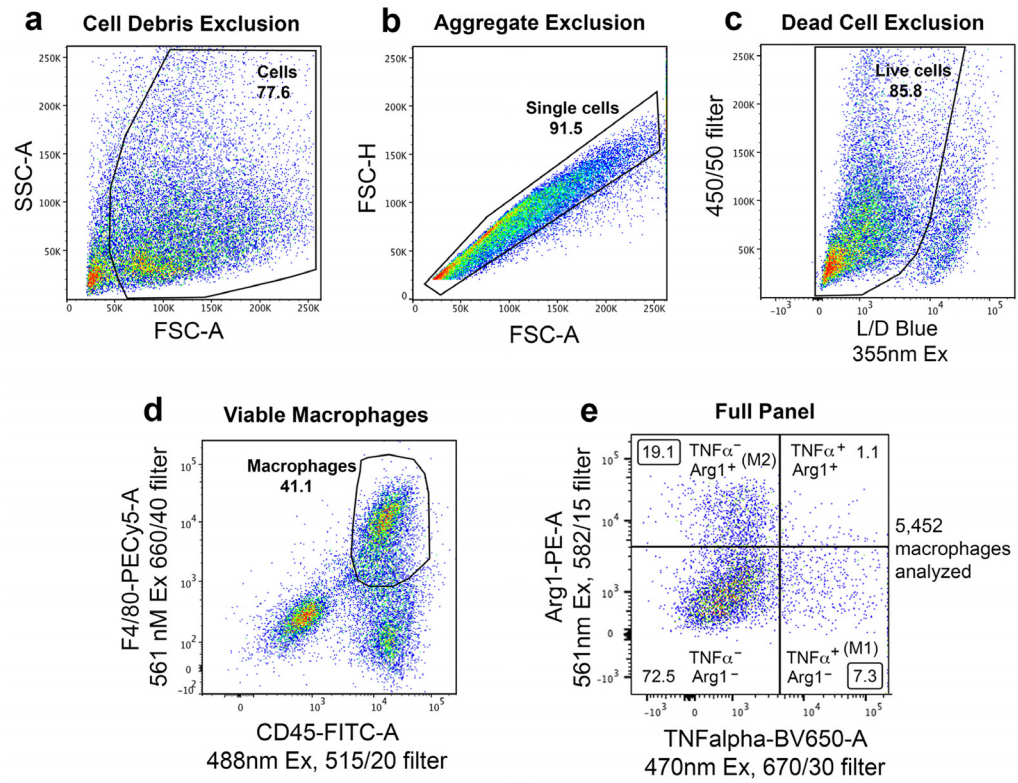


Fig. 8. Gating strategy for flow cytometric immunophenotyping with representative dot plots for the control and experimental samples. Macrophages were analyzed from wounds at 5 days post wounding. (a), The smaller cell fragments and debris are excluded from analysis using forward- and side scatter, FSC/SSC. (b), All non-single cells or cells clumps are gated out using FSC-A/FSC-H. (c), Staining with Live/Dead blue is used to exclude the positive (dead) cells, leaving only live cells for further analysis. (d), Cells double positive for CD45 and F4/80 are gated as macrophages for M1/M2 phenotype characterization. Numbers in graphs a-d represent the percentage of cells that passed the gating criteria. (e), The full panel shows a representative dot plot with the quadrants selected for the experiment; the percentage of cells in each quadrant is indicated. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

for *Adgre1* (Thermo Fisher Scientific, Cat# 4331182) along with 18S primers (Cat# 4333760F) for the endogenous control were used for this purpose. We evaluated 10 samples for the $CD45^+$ only positive group, among which half the samples had undetermined C_t values (cycle threshold) indicating that the target gene was too low to be detectable. The C_t values for the remaining 5 samples were analyzed in comparison to C_t values for the $CD45^+$ $F4/80^+$ double positive samples (n= 6) to calculate the relative expression of *Adgre1* gene in the two groups. Using the delta-delta C_t ($\Delta\Delta C_t$) method, we found that *Adgre1* gene expression was more than 75-fold higher (p-value < 0.001) in the double positive macrophage group, indicating a successful sort (Fig. 7).

Validation of flow cytometric immunophenotyping

Single cells were isolated from full thickness skin wounds and stained for cell surface markers followed by intracellular markers after fixation, as described earlier in Fig. 5. Samples were acquired using a BD LSR Fortessa cell analyzer and BD FACS Diva software (BD Biosciences) at the Cleveland Clinic LRI Flow Cytometry Core with assistance from the core personnel. Gating hierarchy was determined as per standard flow analysis using the unstained, live-dead blue, and fluorescence minus

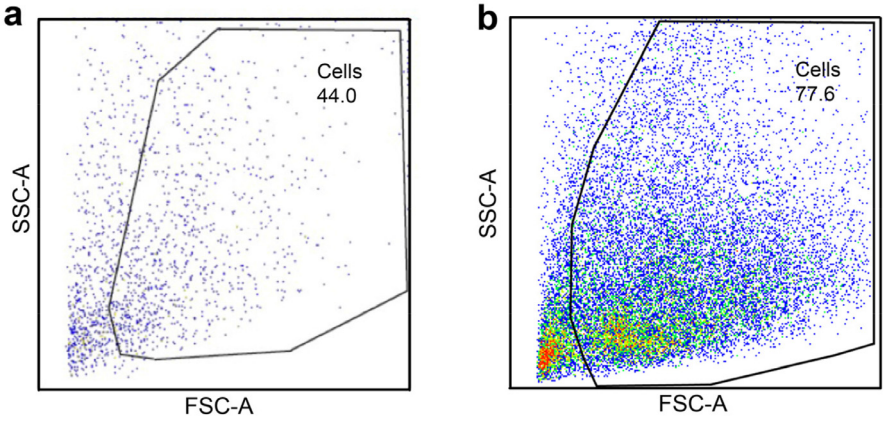


Fig. 9. Improvement in single cell yields due to optimization of the protocol for single cell isolation from skin wounds. Percentage of cells retained at the end of a typical flow cytometry run, before (a) and after (b) optimization. In this representative example, the total number of cells before optimization was 2,018 with a viability 81.9% (a). After optimization, the total number of cells was 22,614 with a viability of 85.8% (b).

one (FMO) controls. Data analysis was performed using FlowJo version 10.5.0. The gating hierarchy for flow analysis is shown in Fig. 8. Using this gating strategy, dead cells and debris were excluded along with cell clumps (Fig. 8**a–c**). For further analysis, only cells expressing a high intensity of $CD45^+$ $F4/80^+$ signals, which should be macrophages, were selected (Fig. 8**d**). Fig. 8**e** depicts a representative dot plot for the experimental sample after all the gating was completed. Each dot in these plots represents a macrophage which was further analyzed for the expression of the M1 marker, TNFα, and the M2 marker, Arg-1. Preliminary analysis of macrophage phenotypes in this particular experiment shows that most of the macrophages in the 5-day wounds analyzed here are either highly positive for Arg-1 (Arg-1^+) or double negative for both Arg-1 and TNFα (Arg-1^- TNFα^-). Only a small fraction of the cells are highly positive for TNFα (TNFα^+), and a negligible portion of the cells are highly double positive (Arg-1^+ TNFα^+). Hence, the majority of these macrophages are unpolarized (Arg-1^- TNFα^-), and amongst the polarized cells a larger proportion have an M2 phenotype (Arg-1^+ positive) as compared to an M1 phenotype (TNFα^+).

Summary

We have provided a detailed protocol for the isolation of single cells from cutaneous wounds, with an emphasis on analysis of macrophages using cell sorting and flow cytometric immunophenotyping. Four parameters in particular (highlighted in the boxed sections shown above) were discovered to make a significant difference in the cell yield; the overall improvement is illustrated in Fig. 9. With the changes made here, we believe that this technique should be helpful for the identification and functional assessment of specific cell populations extracted from wounds and inflamed skin. The method could also be extended to isolate other, different cell populations from wounds (such as neutrophils or lymphocytes) by employing markers specific to the cell type of interest.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was supported by the National Institutes of Health, NHLBI grant number 1P01-HL107147. *MethodsX* thanks the reviewers of this article for taking the time to provide valuable feedback. The authors thank Eric Schultz, Joseph Gerow and Jena Korecky in the LRI Flow Cytometry Core for technical assistance with instrument setup and cell sorting, and Ken Kula in the Center for Medical Art and Photography, for the drawing in Fig. 1.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.mex.2020.101027.

References

[1] L. Wilson, C. Fathke, F. Isik, Tissue dispersion and flow cytometry for the cellular analysis of wound healing, Biotechniques 32 (2002) 548–551.
[2] A.J. Singer, R.A. Clark, Cutaneous wound healing, N. Engl. J. Med. 341 (1999) 738–746.
[3] S.A. Eming, T. Krieg, J.M. Davidson, Inflammation in wound repair: molecular and cellular mechanisms, J. Invest. Dermatol. 127 (2007) 514–525.
[4] T.J. Koh, L.A. DiPietro, Inflammation and wound healing: the role of the macrophage, Expert Rev. Mol. Med. 13 (2011).
[5] F.O. Martinez, S. Gordon, The M1 and M2 paradigm of macrophage activation: time for reassessment, F1000Prime Rep. 6 (2014) 13.
[6] P.J. Murray, J.E. Allen, S.K. Biswas, E.A. Fisher, D.W. Gilroy, S. Goerdt, S. Gordon, J.A. Hamilton, L.B. Ivashkiv, T. Lawrence, M. Locati, A. Mantovani, F.O. Martinez, J.L. Mege, D.M. Mosser, G. Natoli, J.P. Saeij, J.L. Schultze, K.A. Shirey, A. Sica, J. Suttles, I. Udalova, J.A. van Ginderachter, S.N. Vogel, T.A Wynn, Macrophage activation and polarization: nomenclature and experimental guidelines, Immunity 41 (2014) 14–20.
[7] M. Hesketh, K.B. Sahin, Z.E. West, R.Z. Murray, Macrophage phenotypes regulate scar formation and chronic wound healing, Int. J. Mol. Sci. 18 (2017) pii: E1545, doi:10.3390/ijms18071545.
[8] P. Bannon, S. Wood, T. Restivo, L. Campbell, M.J. Hardman, K.A. Mace, Diabetes induces stable intrinsic changes to myeloid cells that contribute to chronic inflammation during wound healing in mice, Dis. Models Mech. 6 (2013) 1434–1447.
[9] B. Shook, E. Xiao, Y. Kumamoto, A. Iwasaki, V. Horsley, CD301b+ Macrophages are essential for effective skin wound healing, J. Invest. Dermatol. 136 (2016) 1885–1891.
[10] C. Wang, X. Yu, Q. Cao, Y. Wang, G. Zheng, T.K. Tan, H. Zhao, Y. Zhao, Y. Wang, D. Harris, Characterization of murine macrophages from bone marrow, spleen and peritoneum, BMC Immunol. 14 (2013) 6.
[11] Y. Lavin, A. Mortha, A. Rahman, M. Merad, Regulation of macrophage development and function in peripheral tissues, Nat. Rev. Immunol. 15 (2015) 731–744.
[12] S. Kaba, R. Nakamura, M. Yamashita, T. Katsuno, R. Suzuki, I. Tateya, Y. Kishimoto, K. Omori, Alterations in macrophage polarization in injured murine vocal folds, Laryngoscope 129 (2019) E135–E142.
[13] B. Malissen, S. Tamoutounour, S. Henri, The origins and functions of dendritic cells and macrophages in the skin, Nat. Rev. Immunol. 14 (2014) 417–428.
[14] S. Tamoutounour, M. Guilliams, F. Montanana Sanchis, H. Liu, D. Terhorst, C. Malosse, E. Pollet, L. Ardouin, H. Luche, C. Sanchez, M. Dalod, B. Malissen, S. Henri, Origins and functional specialization of macrophages and of conventional and monocyte-derived dendritic cells in mouse skin, Immunity 39 (2013) 925–938.
[15] S. Chakarov, H.Y. Lim, L. Tan, S.Y. Lim, P. See, J. Lum, X.M. Zhang, S. Foo, S. Nakamizo, K. Duan, W.T. Kong, R. Gentek, A. Balachander, D. Carbajo, C. Bleriot, B. Malleret, J.K.C. Tam, S. Baig, M. Shabeer, S.E.S. Toh, A. Schlitzer, A. Larbi, T. Marichal, B. Malissen, J. Chen, M. Poidinger, K. Kabashima, M. Bajenoff, L.G. Ng, V. Angeli, F. Ginhoux, Two distinct interstitial macrophage populations coexist across tissues in specific subtissular niches, Science 363 (2019) 1190 http://dx.doi.org/10.1126/science.aau0964.