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In vitro macrophage phagocytosis assay

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Summary

The key roles of macrophages in atherosclerosis include the phagocytosis of apoptotic and necrotic cells and cell debris, whose accumulation in atherosclerotic lesions exacerbates inflammation and promotes plaque vulnerability. Evidence is accumulating that macrophage phagocytic functions peak at early stages of atherosclerosis, and that the reduced phagocytosis at late stages of disease leads to the generation of necrotic cores and a defective resolution of inflammation, which in turn promotes plaque rupture, thrombus formation and life-threatening acute ischemic events (myocardial infarction and stroke). The impaired resolution of inflammation in advanced lesions featuring loss of macrophage phagocytic activity may be in part due to an imbalance between M1 and M2 subsets of polarized macrophages. A better understanding of the mechanisms that regulate macrophage phagocytic activity in the context of atherosclerosis may therefore help identify novel therapeutic targets. This chapter presents a protocol for establishing primary mouse macrophage cultures, a method for polarizing macrophages to the M1 and M2 states, and a method for the *in vitro* study of macrophage phagocytosis of IgG-opsonized or IgM/complement-component-3-opsonized erythrocytes.

Key Words. Phagocytosis, Macrophage, Opsonization, IgG, IgM, Complement Component 3, Red Blood Cells.

1. Introduction

Atherosclerosis is the main feature of atherothrombotic vascular disease, the leading cause of death in developed countries which may soon acquire this status worldwide ***(1)***. Atherogenesis is initiated by the accumulation of lipoproteins in the subendothelial space of the arterial wall followed by the activation of endothelial cells ***(2, 3)***. Activated endothelium secrets chemokines that attract monocytes, which once within the lesion differentiate into macrophages upon exposure to macrophage colony–stimulating factor (M-CSF) ***(4, 5)***. Once differentiated, lesional macrophages begin to ingest deposited lipoproteins through the scavenger receptor pathway ***(6)***. This process drives the transformation of macrophages into foam cells, which augment the inflammatory response and thus promote the recruitment of more monocytes and other inflammatory cells. Cytotoxic substances produced by foam cells cause the death of lesion-resident endothelial and smooth muscle cells ***(7, 8)***, producing enlarged lesions that contain dead cells, cell debris, lipids and extracellular matrix. A major function of macrophages is to ingest these components through phagocytosis. Macrophage phagocytic activity is high in the initial stages of atherosclerosis, but in advanced plaques it is defective and macrophages fail to engulf dead cells and cell debris, compromising the resolution of inflammation. Necrotic cores then accumulate and generate life-threatening vulnerable plaques that can easily undergo rupture, causing thrombus formation and myocardial infarction or stroke.

Phagocytosis is a receptor-mediated process by which cells ingest large particles (≥ 0.5 μm). Since macrophages can encounter a great variety of particles, they express many receptors that can mediate phagocytosis. Moreover, some molecules, such as antibodies and complement components (e.g. complement component 3, C3),

facilitate phagocytosis by tagging particles for recognition, in a process called opsonization. The two best-studied phagocytic receptors are Fcγ receptor (FcγR) and C3 receptor (CR3), which recognize IgG and C3-opsonized particles, respectively. In this chapter we describe a protocol for the *in vitro* study of these two phagocytotic routes using macrophage primary cultures. Because an imbalance between pro-inflammatory M1 and anti-inflammatory M2 macrophages in large lesions is thought to contribute to impaired resolution of inflammation ***(9),*** we describe an optional step for the polarization of macrophages to the M1 (classical) and M2 (alternative activation) phenotypes. The workflow for the complete protocol is shown in **Figure 1**.

[Figure 1 near here]

2. Materials

2.1. Establishing macrophage primary culture

1. Mice.

2. 70% ethanol in distilled H_2O.

3. Dissection material: tweezers, scissors and sterile scalpel blades.

4. Conical sterile polypropylene centrifuge tubes (15 mL).

5. Phosphate buffered saline (PBS): 1.54 mM KH_2PO_4, 155.17 mM NaCl and 2.71 mM Na_2HPO_4, pH 7.4, sterilized by autoclaving.

6. Roswell Park Memorial Institute (RPMI) 1640 medium with antibiotics: 100 units/mL penicillin and 100 µg/mL streptomycin (see **Note 1**).

7. Cell culture dishes (60 mm and 100 mm).

8. Paper towels.

9. Syringes (10 mL) and needles (25G).

10. Distilled H_2O.

11. Heat-inactivated (30 minutes at 56ºC) fetal bovine serum (FBS).

12. Differentiation medium: RPMI 1640 supplemented with 10% heat-inactivated FBS, 20% L-cell conditioned medium (see **Note 2**), 2 mM L-glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin.

2.2. Seeding macrophages on coverslips

1. 24-well cell culture plates.

2. Round coverslips (matching the well size, e.g. 12 mm for 24-well plates).

3. PBS (as in section 2.1., item 5).

4. Differentiation medium (as in section 2.1., item 12).

5. Sterile cell scrapers.

6. Conical sterile polypropylene centrifuge tubes (50 mL).

7. Cell counting chamber.

2.3. Macrophage polarization (optional)

1. M1 polarization medium: RPMI 1640 supplemented with 10% heat-inactivated FBS (see section 2.1., item 11), 2 mM L-glutamine, 100 units/mL penicillin, 100 µg/mL streptomycin, 100 ng/mL lipopolysaccharide (LPS), and 10 ng/mL interferon γ (INFγ).

2. M2 polarization medium: RPMI 1640 supplemented with 10% heat-inactivated FBS (see section 2.1., item 11), 2 mM L-glutamine, 100 units/mL penicillin, 100 µg/mL streptomycin, and 10 ng/mL interleukin 4 (IL-4).

3. Differentiation medium (as in section 2.1., item 12).

4. PBS (see section 2.1., item 5).

5. Permanent marker

2.4. Phagocytosis assay

1. PBS (see section 2.1., item 5).

2. Starvation medium: RPMI 1640 medium containing 0.1% heat-inactivated FBS (as in section 2.1., item 11), 2 mM L-glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin.

3. Sheep red blood cells (RBCs) in Alsever's solution (see **Note 3**) (e.g., Cat. No. SR0053, Oxoid).

4. Polypropylene microcentrifuge tubes (1.5 mL).

5. 20 mM glucose in HBSS (see **Note 4**).

6. Rabbit IgM fraction anti-sheep RBCs (e.g., Cat. No. CL9000-M, Cedarlane) (see **Note 5**).

7. Rabbit IgG fraction anti-sheep RBCs (e.g., Cat. No. 55806, Cappel) (see **Note 5**).

8. Complement component 5 (C5)-deficient serum (e.g., Cat. No. C1163, Sigma) (see **Note 6**).

9. Conical sterile polypropylene centrifuge tubes (50 mL).

10. Ice.

2.5. Cell fixing and immunostaining

1. PBS (as in section 2.1., item 5).

2. 4% formaldehyde in PBS (for 500 mL: in a fume hood heat 400 mL of PBS to aprox. 60°C on a hot plate with magnetic stirrer, add 20 g of paraformaldehyde and incubate with mixing, add dropwise 1 M NaOH until all powder is dissolved, fill up with PBS up to 500 mL, cool the solution, filter and adjust pH to 7.4, use fresh or freeze at -20°C).

3. 0.5% (v/v) Triton X-100 in PBS.

4. 0.1% (v/v) Triton X-100 in PBS.

5. Blocking solution: PBS supplemented with 1% (w/v) bovine serum albumin (BSA) and 0.1% (v/v) Triton X-100.

6. Anti-rabbit secondary antibody conjugated with a fluorochrome of choice (e.g. FITC, AlexaFluor488, etc.).

7. Mounting medium of choice containing diamidino-2-phenylindole (DAPI; e.g. ProLong Gold Antifade Mountant with DAPI, Molecular Probes, see **Note 7**).

8. Microscope glass slides (e.g. 76 x 26 mm).

9. Fluorescence or confocal microscope.

3. Methods

3.1. Establishing macrophage primary culture

1. Euthanize a mouse, from which the lower limbs will be obtained (see **Note 8**).

2. Spray the mouse with 70% ethanol to disinfect.

3. Using tweezers and scissors remove the skin from both legs (see **Note 9**).

4. Cut away each leg at the hip joint (where the head of the femur meets the pelvis), and place them in a 15 mL tube containing PBS.

5. In a flow cabinet, transfer the legs to a paper towel (see **Note 10**) and remove muscles with a sterile blade to obtain clean femurs and tibias.

6. Put the bones on a 60 mm dish containing RPMI medium with antibiotics.

7. Hold one bone with sterile tweezers and cut both joints with sterile scissors to gain access to the bone marrow (see **Note 11**).

8. Aspirate RPMI medium with antibiotics into a 10 mL syringe and fit a 25G needle.

9. Introduce the needle into the bone lumen and flush out the bone marrow into a new 60 mm dish until the bone color turns white.

10. Aspirate the medium containing the bone marrow using the same syringe and pass through the needle to disaggregate cells.

11. Repeat steps 7–10 for all the bones.

12. Transfer the cell suspension from the dish to a 15 mL tube and centrifuge at 300 *x g* for 5 minutes at room temperature.

13. Discard the supernatant and add 1 mL of distilled H_2O to lyse RBCs. Pipette up and down for 10 seconds and add PBS to 15 mL.

14. Centrifuge at 300 *x g* for 5 minutes at room temperature. Repeat the lysis step if after centrifugation the pellet still contains RBCs.

15. Discard the supernatant and resuspend the pellet in 45 mL differentiation medium (see **Note 12**).

16. Plate the cells on four 100 mm dishes (10 mL of cell suspension per dish).

17. Incubate at 37°C in a 5% CO_2 atmosphere.

18. After 48h (day 2) of culture add 10 mL of fresh differentiation medium (see **Note 13**) to each plate. At this point some attached cells can be observed (see **Figure 2**, top).

19. After further 48h (day 4) of culture, discard the medium and add 10 mL of fresh differentiation medium to each plate.

20. After a further 48h (day 6) of culture, discard the medium and add 10 mL of fresh differentiation medium to each plate. At this point the attached cells usually reach confluence (see **Figure 2**, bottom).

21. After 7 days in culture, bone marrow procursors are considered fully differentiated to macrophages (M0 state) and are ready for experiments.

[Figure 2 near here]

3.2. Seeding macrophages on coverslips

1. In the flow cabinet, prepare a 24-well plate (see **Note 14**).

2. Put a glass coverslip in each well and expose the plate to UV light for 20 minutes in order to sterilize it.

3. Aspirate medium from one 100 mm dish containing a confluent monolayer of primary macrophages (see **Note 15**) and wash twice with PBS to remove dead and unattached cells.

4. Add fresh differentiation medium and gently scrape off the macrophages with a cell scraper (see **Note 16**).

5. Transfer the medium containing cells to a 50 mL tube and centrifuge at 260 *x g* for 5 minutes at room temperature.

6. Discard the supernatant and resuspend the cell pellet in differentiation medium.

7. Count the cells in a cell counting chamber (see **Note 17**).

8. Prepare a suspension containing 5 x 10^4 cells per mL of differentiation medium (e.g. for one 24-well plate prepare around 1.25 x 10^6 cells in 25 mL).

9. Transfer 1 mL of the cell suspension (around 5 x 10^4 cells, see **Note 18**) into each well containing a coverslip (prepared in step 2) and briefly shake plates to distribute the cells evenly.

10. Incubate cells at 37°C in a 5% CO_2 atmosphere for a few hours (or overnight) to allow cells to attach.

11. If phagocytic activity is to be examined in polarized macrophages, proceed as indicated in section 3.3. For phagocytic assays with M0 macrophages, go directly to section 3.4.

3.3. Macrophage polarization (optional)

1. Prepare polarization medium for macrophages (1 mL for each well of a 24 well plate) just before use (see **Note 19**).

2. Remove medium from all wells (from step 10 in the previous section) and wash them once with PBS.

3. Add the appropriate polarization (M1 and M2) or differentiation (M0) medium to each well. Identify wells on the plate cover using a marker.

4. Incubate at 37°C in a 5% CO_2 atmosphere for 16–24h.

3.4. Phagocytosis assay

1. Wash the macrophages once with PBS and incubate with starvation medium for 2 hours (see **Note 20**) at 37°C in a 5% CO_2 atmosphere.

2. Mix sheep RBC solution by flipping the tube, and transfer an appropriate volume (depending on the design of your experiment) to two 1.5 mL tubes, one for IgM opsonization and another for IgG opsonization. Use 0.5 μL RBC solution for each well of a 24-well plate. Remember to leave some control wells free of RBCs.

3. Wash RBCs by adding 1 mL of 20 mM glucose in HBSS, centrifuge at 1500 *x g* for 4 minutes at room temperature, and discard the supernatant.

4. Repeat step 3 (see **Note 21**).

5. Resuspend each RBC pellet in 0.25 mL of 20 mM glucose in HBSS.

6. Prepare the IgM and IgG solutions: add 3 μL of anti-sheep RBC IgM or 0.1 μL of anti-sheep RBC IgG per μL of RBC solution used in step 2 to 0.25 mL of 20 mM glucose in HBSS.

7. Mix RBC solutions (obtained in step 5) with IgG or IgM solution (obtained in step 6).

8. Incubate for 30–60 minutes at room temperature with gentle mixing to enable opsonization (see **Note 22**).

9. Centrifuge at 1500 *x g* for 4 minutes at room temperature, discard the supernatant and resuspend each pellet in 1 mL of 20 mM glucose in HBSS (see **Note 23**).

10. Centrifuge at 1500 *x g* for 4 minutes at room temperature, and discard the supernatant. Store the pellet of IgG-opsonized RBCs at 4°C while performing steps 11–13 with the IgM-opsonized RBCs.

11. Mix 20 μL of C5-deficient serum (containing C3) with 180 μL of 20 mM glucose in HBSS to obtain 200 μL of diluted C5-deficient serum.

12. Resuspend IgM-opsonized RBC pellet in 200 μL of diluted C5-deficient serum (containing C3) and incubate at 37°C for 30 minutes with mixing.

13. Add 1 mL of 20 mM glucose in HBSS to the same tube (IgM/RBCs sample), centrifuge at 1500 *x g* for 4 minutes at room temperature, and discard the supernatant.

14. Resuspend in 50 mL tubes the complexes containing RBC/IgG (obtained in step 10) or RBC/IgM/C3 (obtained in step 13), using an appropriate volume of starvation medium (calculate 1 mL of starvation medium for each well).

15. Aspirate the starvation medium from plates containing cultured macrophages (prepared in section 3.4, step 1) and add 1 mL per well of medium containing RBC/IgG or RBC/IgM/C3 complexes (see **Note 24**). Incubate 15 minutes at 37°C in 5% CO_2 atmosphere. Remember to leave some wells without RBCs.

16. Place the plates on ice and wash wells 2–3 times with 1–2 mL PBS to arrest the phagocytosis process. Proceed directly to step 1 in section 3.5.

3.5. Cell fixing and immunostaining

1. Fix the macrophages (from step 16 in section 3.4) by incubating with 4% formaldehyde (in a fume hood) for 10–15 minutes at room temperature (see **Note 25**).

2. Rinse twice with PBS (see **Note 26**).

3. Permeabilize the cells with 0.5% Triton X-100 in PBS for 10 minutes at room temperature.

4. Wash three times with 0.1% Triton X-100 in PBS for 5 minutes.

5. To block non-specific binding of the antibodies, incubate coverslips with blocking solution for 30 minutes at room temperature.

6. Prepare a dilution of the antibody conjugated to a fluorochrome in blocking solution; allow around 150–200 μl per coverslip (see **Note 27**).

7. Remove the blocking solution by holding each coverslip on its edge with forceps and touching a paper towel.

8. Immediately incubate the coverslips with diluted antibody for 1 hour at room temperature in a humidified chamber in the dark.

9. Aspirate the antibody solution and wash coverslips 3 times with 0.1% Triton X-100 in PBS, then once with PBS (5 minutes each wash).

10. Using tweezers, take each coverslip and remove liquid by touching the corner of a paper towel. Invert the coverslip on a drop of mounting medium (with DAPI) placed on a glass slide. Mount 2–3 coverslips per glass slide.

11. Allow the slide to dry in the dark over night at 4°C or proceed directly to the next step, depending on the mounting medium used (see supplier instructions).

12. Under a fluorescence or confocal microscope, count the number of phagocytized RBCs in individual macrophages.

13. Calculate the phagocytic index, which is usually given as the average number of erythrocytes per 100 macrophages. You can also determine the percentage of macrophages that engulfed at least one erythrocyte.

4. Notes

1. Medium can also contain L-glutamine and heat-inactivated fetal bovine serum (medium composition is not crucial at this point). Instead of medium, sterile PBS can be used.

2. L-cell conditioned medium (LCM) is a source of macrophage colony-stimulating factor (M-CSF) which drives differentiation of progenitor cells to macrophages. Differentiation medium can be supplemented with 100 U/mL recombinant M-CSF instead of 20% LCM. This increases the cost of the experiment but gives more reliable and reproducible results.

3. Alsever's solution is an isotonic, balanced salt solution widely used as a blood preservative, permitting prolonged storage under refrigeration and preserving the antigenic properties of erythrocytes. Sheep RBCs in Alsever's solution are available normally in 20 mL vials and have quite short expiry date (around 2–3 months), so before each experiment make sure that the reagent has not expired.

4. Alternatively, glucose can be dissolved in PBS.

5. Many suppliers do not give the exact concentrations of the IgG and IgM fractions, so the amount of antibodies used for opsonization normally requires optimization.

6. Complement C5-deficient serum contains C3, which binds to IgM antibodies on the RBC surface and can then be recognized by CR3 (also called Mac-1) on macrophages. Serum used for the experiment should lack C5 to avoid activation of the alternative complement pathway, which would result in erythrocyte lysis!

7. It is also possible to use mounting medium without nuclear stain and perform an additional staining step (after step 9 in section 3.5) with DAPI or Hoechst.

8. The protocol description is for one mouse. Adjust amounts and volumes to the number of mice required for your experiment.

9. Femurs and tibias are the richest source of murine bone marrow. If the number of mice is limited, extract additional marrow from the upper limbs.

10. To minimize the risk of contamination you can incubate the legs in 70% ethanol for 1 minute and wash once with PBS just before removing the muscles.

11. In case of tibia it is necessary to cut away almost half of the bone (from the side where the foot and the leg meet). To avoid contamination after opening a bone, do not put it down until the bone marrow is flushed out.

12. Bone marrow progenitors have a high proliferation potential and normally one animal is enough to yield sufficient macrophages for a phagocytosis assay. For all experiments maintain similar seeding density, but if counting cells bear in mind that bone marrow consists of different progenitors, and macrophage precursors are just a fraction of this. We typically resuspend the bone marrow in 45 mL medium to seed on four 100 mm dishes, but the volume can differ depending on the experiment.

13. At this point it is better to add medium (instead of changing it) because there are still many unattached macrophage progenitors.

14. Although the protocol is given for 24-well plates, it can be adjusted for other formats. In any case, remember to dedicate some wells as technical controls for immunostaining, e.g. do not add RBCs to some wells to have negative controls (wells where no fluorescence should be detected). These wells will be a reference of unspecific fluorescence or background.

15. Instead of primary cell cultures, some established macrophage cell lines can be used, such as RAW264.7; however, the phagocytosis period needs to be extended, usually up to 30 minutes. The phagocytic activity of some cell lines might need to be augmented by stimulation with phorbol myristate acetate (PMA).

16. Alternatively, wash the cells twice with PBS and detach them with 2x or 4x trypsin. Bear in mind that macrophages are strongly attached to the plate surface so do not use 1x trypsin as it may be ineffective.

17. A Neubauer chamber can be used, or any other cell counting chamber available in your laboratory.

18. The number of macrophages seeded on each well can vary from 2.5 x 10^4 to 1.5 x 10^5 cells and may require some optimization depending on the cell type and mouse genotype. Seeding densities that are too high or too low can make it difficult to quantify RBC-to-macrophage ratios. If cells are too close to each other, it is difficult to assess which of the adjacent cells engulfed a particular erythrocyte. On the other hand, if cells are seeded too sparsely many photographs are needed to obtain sufficient numbers of cells for RBC/macrophage quantification.

19. Macrophage polarization is an optional step that depends on the experimental strategy. As an alternative, other stimuli or drugs can be applied before the phagocytosis assay.

20. It is also possible to starve macrophages in medium without FBS. The duration of starvation may be extended but the same duration must be maintained for all experiments.

21. The purpose of these washes is to eliminate broken cells from the RBC stock solution before using it. At the end of step 4 the supernatant should be free of signs of hemolysis. Two washes are normally enough.

22. If available, use a noria mixer as it will provide the best conditions for opsonization.

23. It normally takes a while to resuspend the pellet, but this is a sign the opsinization was successful.

24. At this point, it is possible to add a short centrifugation step (300 *x g* for 1 minute at room temperature) to synchronize the phagocytosis process.

25. In order to save time, perform steps 1–5 (section 3.5) directly on the 24-well plate containig coverslips (since volumes of the fixation, permeabilization and blocking solutions do not have to be exact).

26. Plates can be stored at 4°C for quite a long time before processing. It is important to fill up all the wells with PBS and wrap each plate with parafilm to avoid evaporation.

27. The antibody dilution may vary depending on the source, and normally needs to be optimized. Remember to use an antibody that recognizes antibodies involved in the opsonization process, e.g. if rabbit IgGs and IgMs were used to form complexes with RBCs, for the detection use a secondary antibody anti-rabbit IgG/IgM made in a different species, e.g. donkey or goat.

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FIGURE LEGENDS

Figure 1. Work flow of the phagocytosis assay with estimated times for each step. MΦ, macrophages; RBCs, red blood cells; C3, complement component 3.

Figure 2. Primary macrophage cell culture. Mouse bone marrow progenitors were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, 20% L-cell conditioned medium, 2 mM L-glutamine, 100 units/mL penicillin, and 100 µg/mL streptomycin. Pictures show representative cell cultures after 2 days (top) and 6 days (bottom).

FIGURE 1

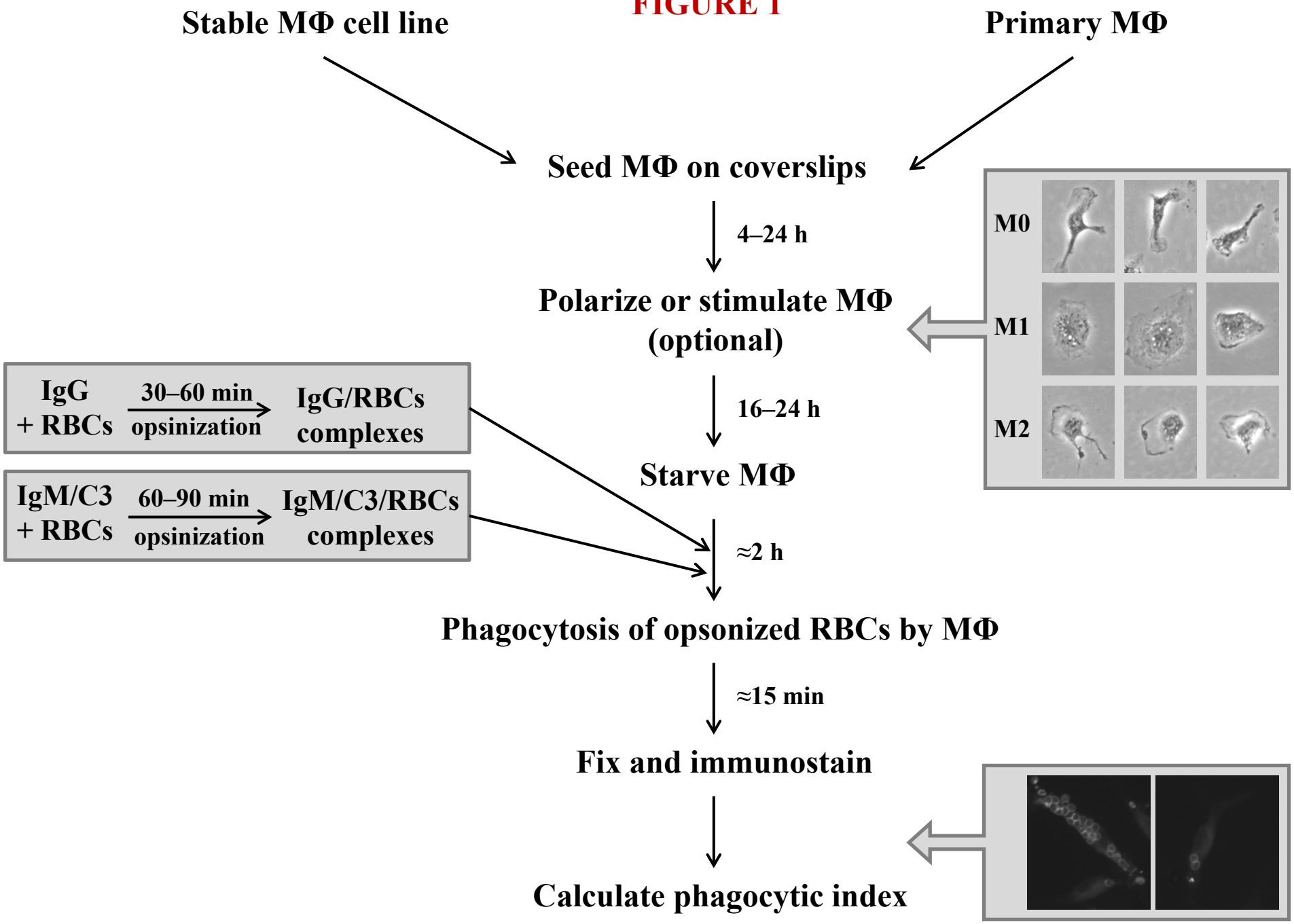
Stable MΦ cell line
Primary MΦ
Seed MΦ on coverslips
4–24 h
Polarize or stimulate MΦ (optional)
M0
M1
M2
16–24 h
IgG + RBCs
30–60 min opsinization
IgG/RBCs complexes
IgM/C3 + RBCs
60–90 min opsinization
IgM/C3/RBCs complexes
Starve MΦ
≈2 h
Phagocytosis of opsonized RBCs by MΦ
≈15 min
Fix and immunostain
Calculate phagocytic index

FIGURE 2