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An Assay to Determine Phagocytosis of Apoptotic Cells by Cardiac Macrophages and Cardiac Myofibroblasts

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[Abstract] In myocardial infarction (MI), a number of cardiomyocytes undergo apoptosis. These apoptotic cardiomyocytes are promptly engulfed by phagocytes. If the dead cells are not engulfed, their noxious contents are released outside, resulting in induction of inflammation. Therefore, the removal of these dead cells is necessary. However, the contribution of each phagocyte type to the removal of apoptotic cells in infarcted hearts remains unresolved. Here, we describe an *in vitro* protocol for a phagocytosis assay to compare the engulfment ability of cardiac macrophages and cardiac myofibroblasts.
Keywords: Phagocytosis assay, Myofibroblast, Engulfment, Apoptosis, Myocardial infarction

[Background] It has long been believed that the apoptotic cells generated in failed hearts are eliminated by cardiac macrophages. However, we found that cardiac myofibroblasts, which are responsible for tissue fibrosis, also have the ability to engulf apoptotic cells after MI (Nakaya *et al*., 2017). The discovery prompted us to compare the engulfment ability of cardiac macrophages and cardiac myofibroblasts. Herein, we provide a detailed protocol for an *in vitro* phagocytosis assay to evaluate the extent of phagocytic engulfment.

Materials and Reagents

1. Pipette tips, 1,000 µl (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 2179-HR)
2. Pipette tips, 200 µl (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 2069-HR)
3. Pipette tips, 20 µl (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 2149P-HR)
4. Pipette tips, 10 µl (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 2140-HR)
5. Surgical tape (3M, catalog number: 1527-0)
6. 8-0 braided silk (NATSUME SEISAKUSHO, catalog number: M6-80B2)
7. 5-0 braided silk (NATSUME SEISAKUSHO, catalog number: ER12-50B1)
8. 10 ml syringe (TERUMO, catalog number: SS-10ESZ)
9. 23-gauge needle (TERUMO, catalog number: NN-2332R)
10. 50 ml tube (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 339652)
11. 6 cm dish (Corning, catalog number: 430589)

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12. Surgical lancet (Akiyama Medical MFG, catalog number: FB10)
13. 70 μm EASYstrainer™ (Greiner Bio One International, catalog number: 542070)
14. 10 cm non-treated dish (Corning, catalog number: 430591)
15. 15 ml tube (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 339650)
16. 8-well slide chamber (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 154534)
17. Frosted glass slides (Matsunami Glass, catalog number: S2112)
18. Cover glass (Matsunami Glass, catalog number: C024601)
19. 40 μm EASYstrainer™ (Greiner Bio One International, catalog number: 542040)
20. Aluminum foil
21. 1.5 ml tube (BMBio, catalog number: BM-15)
22. 0.22 μm Minisart® filter (Sartorius, catalog number: 16534-K)
23. Wild type C57BL/6JJmsSlc mouse (Japan SLC)
24. Pentobarbital (Somnopentyl) (Kyoritsu Seiyaku, catalog number: SOM02-YA1312)
25. Water (NACALAI TESQUE, catalog number: 06442-95)
26. Phosphate buffered saline (PBS) (NACALAI TESQUE, catalog number: 14249-95)
27. Red blood cell (RBC) lysis buffer (Roche Diagnostics, catalog number: 11814389001)
28. Trypsin/Ethylenediaminetetraacetic acid (EDTA) (NACALAI TESQUE, catalog number: 35554-64)
29. Fixable Viability Dye eFluor™ 780 (Thermo Fisher Scientific, eBioscience™, catalog number: 65-0865-14)
30. Sevoflurane (Wako Pure Chemical Industries, catalog number: 193-17791)
31. Paraformaldehyde (PFA) (NACALAI TESQUE, catalog number: 26126-25)
32. 4',6-Diamidino-2-phenylindole (DAPI) (Dojindo, catalog number: 340-07971)
33. FluorSave™ (Merck, catalog number: 345789)
34. Bovine serum albumin (BSA) (Sigma-Aldrich, catalog number: A2153-100G)
35. Trypsin (Sigma-Aldrich, catalog number: T4799-5G)
36. Collagenase A (Roche Diagnostics, catalog number: 10103586001)
37. Serum-free DMEM (NACALAI TESQUE, catalog number: 08458-16)
38. Fatal bovine serum (FBS) (Thermo Fisher Scientific, Gibco™, catalog number: 10437028)
39. Penicillin-streptomycin (NACALAI TESQUE, catalog number: 09367-34)
40. Dispase (Roche Diagnostics, catalog number: 04942078001)
41. EDTA·2Na (Dojindo, catalog number: 345-01865)
42. FITC-conjugated anti-Ly6C antibody (BioLegend, catalog number: 128006)
43. PE-conjugated anti-Ly6G antibody (BioLegend, catalog number: 127607)
44. APC-conjugated anti-F4/80 antibody (BioLegend, catalog number: 123116)
45. PerCP/Cy5.5-conjugated anti-CD11b antibody (BioLegend, catalog number: 101230)
46. Dimethyl sulfoxide (DMSO) (Sigma-Aldrich, catalog number: D2650)
47. CellTracker™ Green 5-chloromethylfluorescein diacetate (CMFDA) dye (Thermo Fisher Scientific, Invitrogen™, catalog number: C7025)

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48. Poly-L-lysine solution (Sigma-Aldrich, catalog number: P4707)
49. Dexamethasone (Sigma-Aldrich, catalog number: D1756-25MG)
50. Collagenase A solution (see Recipes)
51. Culture medium (see Recipes)
52. Dispase solution (see Recipes)
53. FACS buffer (see Recipes)
54. Primary antibodies (see Recipes)
55. 8-well slide chamber coated with poly-L-lysine (see Recipes)
56. 10 mM CMFDA dye (see Recipes)
57. 10 mM dexamethasone (see Recipes)

Equipment

1. Pipettes 1,000 µl (Gilson, catalog number: F123602)
2. Pipettes 200 µl (Gilson, catalog number: F123601)
3. Pipettes 20 µl (Gilson, catalog number: F123600)
4. Pipettes 2 µl (Gilson, catalog number: F144801)
5. Respirator (Shinano Manufacturing, catalog number: SN-480-7X2T)
6. Optical microscope (Olympus, model: SZX7)
7. Surgical tools such as tweezers and small scissors (tools can be purchased from NATSUME SEISAKUSHO and MEISTER)
8. Clean bench (Panasonic Healthcare, model: MCV-B131S)
9. Water bath (TAITEC, model: Personal-11)
10. Centrifuge (TOMY SEIKO, model: LC-200)
11. CO_2 incubator (SANYO, model: MCO-18AIC)
12. Cell sorter (BD, BD Bioscience, model: FACSARIA III)
13. Fluorescence microscope (KEYENCE, model: BZ-9000)

Software

1. BZ-II image analysis application (KEYENCE CORPORATION)

Procedure

Procedures A and B are also applicable to phagocytosis assay of necroptotic cells by cardiac myofibroblasts (Horii *et al.*, 2017).

A. Establishment of an MI mouse model by surgical operation
   1. Prepare an 8- to 10-week-old male wild type mouse of the strain, C57BL/6J.
      Note: Male mice are chosen to be subjected to MI operation to avoid the risk of acquiring

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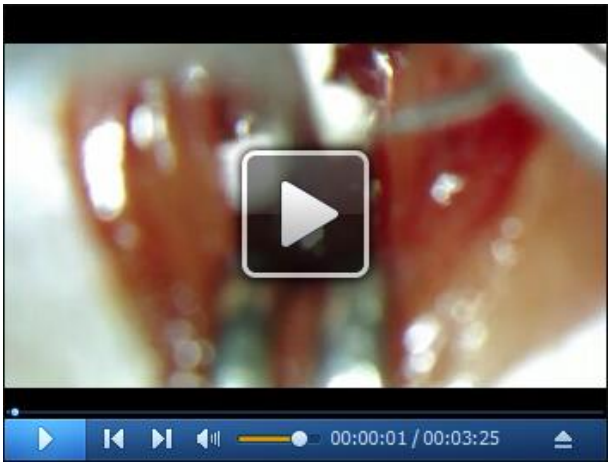
variable data due to estrous cycle of female mice.

2. To anesthetize the mouse, administer pentobarbital (50 mg pentobarbital/kg body weight of mouse) (see Recipes) via an intraperitoneal injection.
3. Fix the mouse on its back on the heating pad with surgical tape.
4. Perform artificial respiration using a respirator (volume of air for respiration: 0.5 cc, respiratory frequency: 120 bpm).
5. Under an optical microscope, surgically open the chest and expose the heart using sterilized surgical tools.
6. Perform permanent occlusion of the left coronary artery using 8-0 braided silk (see Video 1).
7. Close the chest using 5-0 braided silk.
8. After the operation, give the mouse appropriate treatment and keep it warm until recovery.

Video 1. How to establish an MI mouse model



B. Isolation of cardiac myofibroblasts from the MI mouse model

1. Euthanize two mice after 3 days of the MI operation by administering pentobarbital (150 mg pentobarbital/kg body weight of mouse) via an intraperitoneal injection.
2. Open the chest using sterilized scissors to expose the heart.
3. Cut the right atrium and prick the left ventricle with a 10 ml syringe containing 10 ml of ice-cold PBS and mounted with a 23-gauge needle.
4. Perfuse the heart with 10 ml of ice-cold PBS.
5. Collect the heart and remove the atria.
6. Put the heart in a 50 ml tube containing 10 ml of ice-cold PBS for storage, while other mice are being sacrificed.
7. Discard the supernatant and add 5 ml of ice-cold PBS.
8. Transfer the heart along with PBS into a 6 cm dish on a clean bench.
   Note: From this step, the experiment should be performed on a clean bench.
9. Cut each heart into 15 small pieces (2-3 mm^2) using two surgical lancets, while keeping them on ice.

10. Discard PBS using a pipette.
    Note: Please be careful not to discard heart pieces.
11. To wash the heart pieces, sprinkle 5 ml of PBS on them.
12. Repeat steps B10-B11.
13. Transfer the heart pieces into a 50 ml tube and remove PBS.
14. Add 4 ml of collagenase A solution (see Recipes) to the tube.
15. Incubate the tube, containing the mixture of the minced hearts and collagenase A solution, in a water bath at 37 °C, while shaking at 120 rpm for 10 min.
16. Discard the supernatant, which contains numerous hematopoietic cells.
17. Repeat steps B14-B16 once again.
18. Add 4 ml of collagenase A solution to the tube, which now contains the residual material.
19. Shake the tube, containing the mixture of the minced hearts and collagenase A solution, in a water bath at 120 rpm and 37 °C for 10 min.
20. Pass the cell suspension, which contains isolated cells, through a 70 μm EASYstrainer™ into a new 50 ml tube.
21. Centrifuge the collected cell suspension at 300 *x g* for 5 min at room temperature and discard the supernatant.
22. Add 1 ml of the culture medium (see Recipes) to the cell pellet and keep it on ice.
23. Repeat steps B18-B22 eight times in total.
24. After eight agitations, combine all the cell suspensions (1 ml x 8) into a new 50 ml tube.
25. Centrifuge the cell suspension at 300 *x g* for 5 min and discard the supernatant after centrifugation.
26. Suspend the cell pellet in 1 ml of RBC lysis buffer, and incubate for 1 min at room temperature.
27. Add 9 ml of the culture medium to the cell suspension.
28. Centrifuge the cell suspension at 300 *x g* for 5 min, and discard the supernatant after centrifugation.
29. Suspend the cell pellet in 10 ml of the culture medium.
30. Plate this cell suspension on a 10 cm non-treated dish and incubate overnight in a 5% CO_2 incubator at 37 °C.
31. After overnight incubation, aspirate the culture medium and add 10 ml of fresh culture medium.
32. Culture the isolated cardiac cells for more than 6 days.
    Note: Myofibroblasts attach themselves to the plate; unattached contaminating cells, including hematopoietic cells, are removed by changing the culture medium. When the myofibroblasts reach pre-confluence, passage them.
33. One day before conducting the *in vitro* phagocytosis assay, discard the culture medium from the dish and wash the dish twice with 10 ml of PBS.
34. Add 1 ml of trypsin/EDTA and incubate at 37 °C for 1 min.
35. Add 9 ml of the culture medium and transfer the cell suspension into a 15 ml tube.
36. Centrifuge the cell suspension at 300 *x g* for 5 min and discard the supernatant after

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centrifugation.

37. Add 10 ml of the culture medium and suspend the cell pellet.
38. Count the cell number and adjust it to 1 x 10^5 cells/ml by adding the culture medium.
    Note: Approximately 2.5 x 10^5 myofibroblasts can be obtained from one mouse.
39. Add 200 µl of this cell suspension to an 8-well slide chamber coated with poly-L-lysine (2 x 10^4 cells/well).
40. Incubate in a 5% CO_2 incubator at 37 °C overnight.

C. Isolation of cardiac macrophages from the MI mouse models

1. Collect the hearts from the MI mouse models after 3 days of the operation and cut the hearts into small pieces.
   Note: This procedure is the same as that described in steps B1-B10. The experiment should be performed using a clean bench from this step.
2. Add 10 ml of dispase solution (see Recipes).
3. Incubate the mixture of the minced hearts and dispase solution in a water bath at 37 °C for 10 min, while shaking at 180 rpm.
4. Pass the cell suspension through a 70 µm EASYstrainer™ and collect it in a new 50 ml tube.
5. Centrifuge the cell suspension at 300 *x g* for 3 min at room temperature and remove it after centrifugation.
6. Suspend the cell pellet in 2 ml of the culture medium.
7. Repeat steps C2-C6 thrice.
8. Combine the three cell suspensions (2 ml x 3) in a 50 ml tube.
9. Centrifuge the cell suspension at 300 *x g* for 3 min and discard the supernatant after centrifugation.
10. Suspend the cell pellet in 1 ml of Red blood cell lysis buffer and incubate for 1 min at room temperature.
11. Add 9 ml of FACS buffer (see Recipes).
12. Centrifuge the cell suspension at 300 *x g* for 3 min at room temperature and discard the supernatant after centrifugation.
13. Add 1 ml of FACS buffer.
14. Add 1 µl of the Fixable Viability Dye eFluor™ 780 and incubate on ice for 30 min.
15. Centrifuge the cell suspension at 300 *x g* for 3 min at 4 °C and discard the supernatant after centrifugation.
16. Add 100 µl of primary antibodies (see Recipes) diluted with FACS buffer and incubate on ice for 30 min in a dark environment.
17. Add 300 µl of FACS buffer.
18. Centrifuge the cell suspension at 300 *x g* for 5 min at 4 °C and discard the supernatant after centrifugation.
19. Add 400 µl of FACS buffer.

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20. Centrifuge the cell suspension at 300 *x g* for 5 min at 4 °C and discard the supernatant after centrifugation.
21. Add 400 µl of FACS buffer.
22. Sort the cardiac macrophages using FACSAria III.

    Note: Cardiac macrophages are identified as Ly6G$^-$/CD11b$^+$/F4/80^{hi}/Ly6C^{lo} (Figure 1).

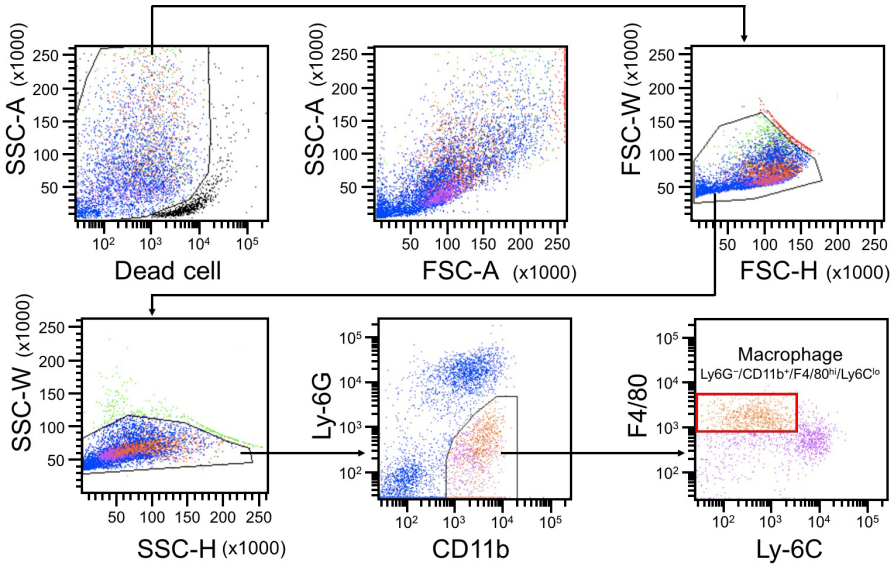

Figure 1. The flow gating strategy for sorting of cardiac macrophages. Cardiac cells isolated from the MI mouse models were labeled with eFluor™ 780 viability dye and antibodies against Ly6G, CD11b, F4/80 and Ly6C. First, living cells were gated by eliminating dead cells stained with eFluor™ 780 viability dye. After that, Ly6G$^-$/CD11b$^+$/F4/80^{hi}/Ly6C^{lo} cells (red gate) amongst living cells were sorted as cardiac macrophages by FACSAria III.

23. Centrifuge the sorted cell suspension at 300 *x g* for 5 min at 4 °C and discard the supernatant after centrifugation.
24. Add 1 ml of the culture medium and count the cell number.

    Note: Approximately 1 x 10^5 macrophages can be obtained from eight mice.
25. Adjust the cell number to 2.5 x 10^5 cells/ml by adding the culture medium.
26. Add 200 µl of this cell suspension to an 8-well slide chamber coated with poly-L-lysine (5 x 10^4 cells/well, see Recipes).

    Note: Macrophages are smaller than myofibroblasts. To equalize the confluence of macrophages and myofibroblasts, the number of macrophages plated on the 8-well slide chamber is 2.5 times that of the myofibroblasts.
27. Incubate in a CO_2 incubator overnight.

D. Obtaining apoptotic thymocytes

1. Euthanize two 4- to 8-week-old female mice by administering sevoflurane.

   Note: Usually, thymocytes of female mice are used for preparation of apoptotic cells owing to

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the following two reasons: (1) A large number of thymocytes can be obtained because the size of female thymus is bigger than that of male thymus. (2) Most thymocytes are small and uniform in size, whereas other cells such as splenocytes vary in size. Small apoptotic cells are more prone to be engulfed and the uniformity of the cell size aids in ensuring reproducibility of the experimental conditions of phagocytosis.

2. Open the chest of mice by using sterilized scissors and remove the thymus using sterilized tweezers.
3. Transfer the thymus into a 6 cm dish containing 4 ml of serum-free DMEM on ice.
4. Smash the thymus using sterilized, frosted glass slides and suspend the smashed thymus in 4 ml of serum-free DMEM.
5. Pass the thymocyte suspension through a 40 μm EASYstrainer^{TM} into a 50 ml tube.
6. Centrifuge the cell suspension at 300 *x g* for 5 min at room temperature and discard the supernatant after centrifugation.
7. Suspend the cell pellet in 1 ml of RBC lysis buffer and incubate for 1 min at room temperature.
8. Add 9 ml of serum-free DMEM.
9. Centrifuge the cell suspension at 300 *x g* for 5 min at room temperature and discard the supernatant after centrifugation.
10. Add 10 ml of serum-free DMEM and count the cell number.
11. Transfer 2 x 10^8 cells from the cell suspension into a 50 ml tube.
12. Add serum-free DMEM and adjust the cell number to 1 x 10^7 cells/ml.
13. Add 1/10,000 times the volume of the cell suspension of 10 mM CMFDA dye (see Recipes) (final concentration would be 1 μM) and incubate for 30 min at 37 °C in a CO_2 incubator, while blocking light by covering with an aluminum foil.
    Note: When the final volume of the cell suspension reaches 20 ml in step D12, add 2 μl of 10 mM CMFDA dye and adjust the final concentration to 1 μM.
14. Dispense 3 ml of the cell suspension into each 15 ml tube and add 12 ml of the culture medium to each tube.
    Note: To stop CMFDA labeling reaction, use the culture medium containing FBS.
15. Incubate on ice for 5 min.
16. Centrifuge the cell suspension at 300 *x g* for 5 min at room temperature and discard the supernatant after centrifugation.
17. Add 10 ml of the culture medium and combine all the cell suspensions in one 15 ml tube.
18. Repeat steps D16-D17 thrice.
19. Count the cell number.
20. Adjust the cell number to 1.2 x 10^7 cells/ml in one 15 ml tube.
21. Add 1/1,000 times the volume of the cell suspension of 10 mM dexamethasone (see Recipes) (final concentration would be 10 μM).
    Note: When final volume of the cell suspension reaches 10 ml in step D20, add 10 μl of 10 mM dexamethasone and adjust the final concentration to 10 μM.

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22. Incubate the cell suspension for 5 h at 37 °C in a CO_2 incubator, while blocking light by covering with an aluminum foil.
23. Centrifuge the cell suspension at 300 *x g* for 5 min at room temperature and discard the supernatant after centrifugation.
24. Add 10 ml of the culture medium.
25. Repeat steps D23-D24 thrice.
26. Count the cell number and adjust it to 1 x 10^6 cells/ml (for cardiac myofibroblasts) and 2.5 x 10^6 cells/ml (for cardiac macrophages).
    Note: Approximately 2.5 x 10^8 apoptotic thymocytes can be obtained from one mouse.

E. *In vitro* phagocytosis assay
1. Prepare the cardiac myofibroblasts and cardiac macrophages cultured on the 8-well slide chamber.
2. Discard the medium.
3. Add 200 µl of apoptotic thymocytes (2 x 10^5 cells/well for myofibroblasts and 5 x 10^5 cells/well for macrophages).
   Note: The number of engulfed apoptotic cells is ten times the number of phagocytes per well.
4. Incubate for 3 h at 37 °C in a CO_2 incubator.
5. Discard the supernatant and wash with 200 µl of PBS thrice by using a pipette.
   Note: Unengulfed apoptotic cells are attached to phagocytes and these cells are removed by washing with PBS.
6. To fix the cells, add 200 µl of 1% PFA/PBS solution and incubate for 15 min at room temperature.
7. Discard the supernatant and wash with 200 µl of PBS.
8. Discard the supernatant and remove the chamber wall from the glass slide.
9. Mount the slide with FluorSave™ reagent containing 0.1% DAPI and place a cover glass over the glass slide.
10. Observe under phase contrast and fluorescence microscopes.
    Note: Images are captured at 20x magnification by BZ-9000. Obtain phase contrast images as well as the images captured by using GFP-BP and DAPI-BP filters.

Data analysis

1. Capture three types of images (phase contrast images as well as images obtained using GFP-BP and DAPI-BP filters) from 12-15 randomly selected fields (Figure 1).
2. Merge the images using the BZ-II image analysis application.
3. Manually count the number of phagocytes and engulfed cells (CMFDA-positive and merged phagocytic cells).

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Note: In the phase contrast images, unengulfed cells can be observed in the form of light phase and blurry images, whereas engulfed cells can be observed in the form of dark phase images. Do not consider these light phase cells as engulfed cells.

4. Calculate the phagocytosis index (Figure 2).

   Note: Phagocytosis index is defined as the number of engulfed cells per phagocyte. First, count the number of nuclei of myofibroblasts or macrophages that are stained by DAPI (pink arrowheads) and then count the number of engulfed apoptotic cells labeled with CMFDA (yellow arrowheads). After the counting, phagocytosis index is calculated as the number of engulfed apoptotic cells divided by the number of nuclei of myofibroblasts or macrophages. For example, when the number of nuclei of myofibroblasts is 22 and the number of engulfed apoptotic thymocytes is 7, phagocytosis index is calculated as 7/22 = 0.32. Following the calculation, phagocytosis index is averaged over 12-15 randomly selected fields.

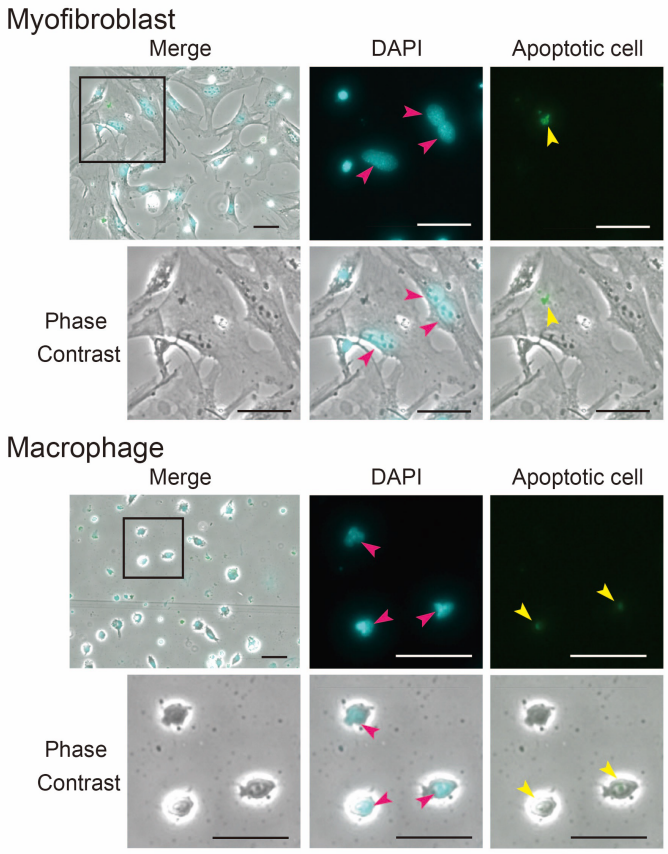

Figure 2. Cardiac myofibroblasts and macrophages engulf the apoptotic thymocytes. Cardiac myofibroblasts and macrophages were isolated from the MI mouse models 3 days after operation. The mouse apoptotic thymocytes are labeled with CMFDA (green). The pictures with arrowheads were rescaled to allow better visualization of phagocytosis. Count the number of phagocytes (pink arrowheads) and engulfed cells (yellow arrowheads) and calculate the phagocytic index. Scale bars = 50 μm.

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Notes

In our opinion, this protocol is applicable for measuring phagocytosis by other cell types as well. In fact, we measured phagocytosis of apoptotic thymocytes by NIH3T3 cells using the same protocol (Nakaya *et al.*, 2006).

It should be noted that the cell sizes of phagocytes and apoptotic cells have to be considered in phagocytosis assay. The size of apoptotic cells should be smaller than that of phagocytes.

Recipes

1. Pentobarbital (prepared fresh)
   a. Transfer 1 ml of pentobarbital from vial into a 15-ml tube
   b. Add 12 ml of sterile distilled water
2. Collagenase A solution (prepared fresh)
   a. Take 50 ml in PBS to a 50-ml tube
   b. Add:
      50 mg of BSA
      50 mg of trypsin
      50 mg of collagenase A
   c. Mix well, and incubate at room temperature
   d. Filter the solution through a 0.22-μm Minisart® filter
3. Culture medium (can be kept for 1 month at 4 °C)
   a. Remove 55 ml of serum-free DMEM from 500 ml of serum-free DMEM
   b. Add 50 ml of heat-inactivated FBS (30 min at 56 °C)
   c. Add 5 ml of penicillin-streptomycin
4. Dispase solution (50 ml) (prepared fresh)
   a. Take 125 mg of trypsin in a 50-ml tube
   b. Add:
      112 mg of dispase
      100 μl of 0.5 M EDTA·2Na
      Add PBS up to 50 ml
   c. Mix well and filter the solution through a 0.22-μm Minisart® filter
5. FACS buffer (prepared fresh)
   PBS supplemented with 2% FBS
6. Primary antibodies/FACS buffer (prepared fresh)
   a. Take 100 μl of FACS buffer in a 1.5-ml tube
   b. Add:
      1 μl of FITC-conjugated anti-Ly6C antibody (used at a dilution of 1:100)
      0.5 μl of PE-conjugated anti-Ly6G antibody (used at a dilution of 1:200)

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1 µl of APC-conjugated anti-F4/80 antibody (used at a dilution of 1:100)
0.5 µl of PerCP-Cy5.5-conjugated anti-CD11b antibody (used at a dilution of 1:200)

c. Mix well
d. Incubate on ice

7. 8-well slide chamber coated with poly-L-lysine (prepared fresh)
   a. Add 200 µl of poly-L-lysine solution to the 8-well slide chamber
   b. Incubate in a CO_2 incubator for 3 h
   c. Discard the poly-L-lysine solution
   d. Wash using PBS once
8. 10 mM CMFDA dye (can be kept for several months at -20 °C)
   Add 10.76 µl of DMSO to 50 µg of CMFDA dye
9. 10 mM Dexamethasone (can be kept for several months at -20 °C)
   Add 50 µl of DMSO to 164.5 µg of Dexamethasone

Acknowledgments

When using this protocol, please refer to M Nakaya *et al*. (2017) J Clin Invest. Funding was provided by the Ministry of Education, Culture, Sports, Science, and Technology of Japan (MEXT) [to M.N (17H03984)]; from Grant-in-Aid for Scientific Research on Innovative Areas (Homeostatic regulation by various types of cell death) from MEXT [to M.N (17H05510)]. All animal experiments were performed using approved protocols and in accordance with the guidelines of Kyushu University.

References

1. Horii, Y., Matsuda, S., Watari, K., Nagasaka, A., Kurose, H. and Nakaya, M. (2017). Phagocytosis assay of necroptotic cells by cardiac myofibroblasts. *Bio Protoc* 7(18): e2252.
2. Nakaya, M., Tanaka, M., Okabe, Y., Hanayama, R. and Nagata, S. (2006). Opposite effects of rho family GTPases on engulfment of apoptotic cells by macrophages. *J Biol Chem* 281(13): 8836-42.
3. Nakaya, M., Watari, K., Tajima, M., Nakaya, T., Matsuda, S., Ohara, H., Nishihara, H., Yamaguchi, H., Hashimoto, A., Nishida, M., Nagasaka, A., Horii, Y., Ono, H., Iribe, G., Inoue, R., Tsuda, M., Inoue, K., Tanaka, A., Kuroda, M., Nagata, S. and Kurose, H. (2017). Cardiac myofibroblast engulfment of dead cells facilitates recovery after myocardial infarction. *J Clin Invest* 127(1): 383-401.