Chapter #

Measuring Apoptotic Cell Engulfment (Efferocytosis) Efficiency

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Abstract

Efferocytosis is the process of recognising and removing dead and dying cells, performed by a variety of phagocytic cells including macrophages. It has recently been shown that Liver X Receptor (LXR) signalling in macrophages regulates the expression of important efferocytosis receptors, bridging and signalling molecules. Here we describe a sensitive yet robust efferocytosis assay, optimised to measure bone marrow derived macrophage (BMDM) apoptotic cell engulfment capability. This assay can be applied to genetically or pharmacologically altered BMDMs.

Key words

Nuclear receptors, efferocytosis, apoptosis, macrophages, BMDM, Jurkats, engulfment.

1. Introduction

Efferocytosis is the term given to the process of the recognising, engulfing and processing dead and dying cells ***(1)*** which is performed by a variety of phagocytes including macrophages ***(2)***. Macrophages are a heterogeneous population of phagocytotic leukocytes ***(3)*** which have high efferocytotic capability ***(4)***, shown to engulf dead and dying cells in healthy tissues with high cell turnover such as the bone marrow, spleen and thymus ***(5)***. Macrophages also perform their efferocytic duties in atherosclerosis ***(6)*** – the leading cause of cardiovascular disease ***(7)*** – where macrophage efferocytosis capacity in atherosclerotic plaques has been linked to the progression of necrotic cores through, among others, the tyrosine kinase receptor Mer (MerTK) ***(6)***. Macrophages express nuclear receptors, several of which have been linked to efferocytosis activity including PPARγ, PPARδ, RXRα ***(8)*** and Liver X Receptors (LXRs) ***(9)***. LXRs regulate the expression of the important efferocytosis receptor MerTK ***(10)***, and several other bridging and signalling molecules ***(11)*** directly involved in efferocytosis.

This protocol is based upon original methodology described by Marissa Nadolski and Ed Thorp ***(12)***, and has been optimised to provide a simple yet robust assay for measuring efferocytosis efficiency in a relatively small number of cells. This is an ideal feature of an assay, when the subject is a precious commodity such as *primary* bone marrow derived macrophages from a rare genetically modified mouse ***(13)***, with which you may also want to perform additional experiments with. In this chapter the utilisation of 8 well chamber slides is described, which allows for multiple conditions to be tested such as various time points and a range of agonists and concentrations. After addition of Jurkat cells undergoing apoptosis, the BMDMs are fixed, imaged, and the efficiency of efferocytosis is quantified using freely available software.

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2. Materials

Prepare all solutions using ultrapure water and analytical grade reagents. Prepare and store all reagents at 4 °C or -20 °C as indicated (unless otherwise noted). Follow all local waste disposal regulations when disposing of waste materials.

2.1 Jurkat cell culture

1. Glutamine: 200nM
2. Gentamycin: 10 mg/mL
3. Complete RPMI media: (RPMI, 10 % FBS, 1 % glutamine, gentamycin 20 µg/mL).
4. Jurkat cells (*see* **Note 1 and 2**).
5. T25cm^2 filter cap flasks
6. Sterile polystyrene pipettes (5 mL, 10 mL, 25 mL).
7. 70 % ethanol.
8. Cell incubator with programmable control of CO_2 and temperature.

2.2. Bone marrow derived macrophage cell culture

1. Murine bone marrow derived macrophages (*see* **Note 3**).
2. Cell scrapers.
3. Sterile 8 well culture slides.
4. Cell incubator with programmable control of CO_2 and temperature.

2.3 Preparation of apoptotic cells

1. Complete RPMI media: (RPMI, 10 % FBS, 1 % glutamine, gentamycin 20 µg/mL).
2. 10 cm sterile TC dish.
3. Calcein AM (1 mg/mL in DMSO) store at -20°C.
4. Warmed PBS 1X 37 ºC.
5. UV irradiator.

6. Sterile polystyrene pipettes (5 mL, 10 mL, 25 mL).
7. 70 % ethanol.
8. Cell incubator with programmable control of CO_2 and temperature.
9. 1 % paraformaldehyde.

2.4 Efferocytosis assay

1. Complete DMEM: (DMEM, 10 % FBS, 1 % glutamine, gentamycin 20 µg/mL).
2. Apoptosing Jurkat cell solution (from step 2.3).
3. BMDM cultured on 8 well culture slides
4. Ice cold PBS 1X
5. 1 % paraformaldehyde
6. 70 % ethanol.
7. Single channel manual pipettes (1 mL, 200 µL),
8. Cell incubator with programmable control of CO_2 and temperature.

2.5 Slide preparation

1. 8 well culture slide removal tool (*see* **Note 4**)
2. Fluoromount aqueous mounting media (Sigma).
3. 22 x 50 mm glass cover slips
4. Nail varnish.

2.6 Image acquisition

1. Fluorescent microscope with 20x magnification and GFP filter (excitation wavelength 450-490 nm, emission wavelength 515-565 nm) and digital camera (*see* **Note 5**).
2. Image acquisition software (*see* **Note 6**).

2.7 Efferocytosis quantification

1. ImageJ image analysis software with grid overlay plugin (https://imagej.nih.gov/ij/download.html).

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3. Methods

3.1 Jurkat cell culture

This is your source of apoptotic cells.

1. Grow Jurkat cells in complete RPMI in T25cm^2 filter cap flasks. Count daily with a hemocytometer maintaining cell density at between $2x10^5$ and $1x10^6$ cells/mL (*see* **Note 7**).
2. Grow sufficient number of cells as required for the number of experiments you are performing.

3.2 BMDM cell culture

1. When BMDM reached 90 % confluency (*see* **Note 8**), scrape the cells from the TC dish and pipette up and down several times in a 10 mL pipette to homogenise the cells into a single cell suspension.
2. Count BMDMs on a hemocytometer
3. Centrifuge cells at 500 *xg* for 10 min.
4. Resuspend cell pellet in complete DMEM to a final concentration of $2.5 x10^5$ cells/mL.
5. Seed re-suspended BMDM in onto 8 well slides, 250 µL/well (*see* **Note 9 and Fig. 1**).
6. Incubate overnight at 37°C, 5 % CO_2.

3.3. Preparation of apoptotic Jurkat cells

1. Count Jurkat cells using a hemocytometer.
2. Centrifuge cells at 500 *xg* for 5 min.
3. Resuspend pellet at 2 x 10^6 cells/mL and plate 20 x10^6 cells (10 mL) onto a 10 cm TC dish.
4. Add 10 uL of Calcein AM solution to the dish and mix thoroughly by gently pipetting up and down several times using a 10 mL pipette.
5. Incubate for approximately 2 hours at 37ºC 5 % CO_2 (*see* **Note 10 and Note 11**).
6. Collect cells in 15 mL conical tube.
7. Wash dish with 3 mL of Jurkat complete media and add to the conical tube of Jurkat cells.
8. Centrifuge at 500 *xg* for 5 min (*see* **Note 12** and **Fig. 2**).
9. Wash cells by removing the supernatant, resuspending the cells pellet in 5 mL of warmed PBS 1X and centrifuging at 500 *xg* for 5 min.
10. Repeat step 9 twice more for a total of three PBS washes.
11. Resuspend the cell pellet in 10 mL of warmed (37ºC) Jurkat complete media and plate onto a 10 cm TC dish.
12. Irradiate cells for 5 minutes (*see* **Note 13 and 14**). Swirl dish every 60 seconds to ensure homogenous exposure of cells to UV light source.
13. Incubate the irradiated cells at 37ºC for approximately 2 hours (*see* **Note 15**) until approximately 50 % of the cells are visibly blebbing (**Fig. 3**).
14. Collect the cells into a 15 mL conical tube.
15. Centrifuge at 500 *xg* for 5 min. Resuspend the apoptotic cells at 2.5x10^5 cells/mL in complete DMEM media.

3.4. Efferocytosis assay

1. Remove complete DMEM media from chamber well slides with BMDM.
2. Add apoptotic Jurkats in a 1:1 ratio to the BMDM (250 μL).

3. Incubate BMDM and apoptotic Jurkats together for various periods of time ranging from 0 to 90 min (*see* **Note 16**).
4. Remove media, and immediately wash cells with 250 µL ice cold PBS.
5. Remove PBS and repeat ice cold PBS washes twice more for a total of 3 washes (*see* **Note 17**).
6. Add 250 uL cold 1 % PFA solution and incubate at 4°C in the dark for 10 min.
7. Repeat one more PBS wash step.
8. Add 250 µL PBS to cover each well. Slides can now be stored wet overnight at 4 °C in the dark or proceed immediately to slide preparation.

3.5 Slide preparation

1. Flick off PBS from the 8 well slides.
2. Remove the 8 well plastic frame using the supplied removal device in accordance with manufacturer's instructions.
3. Add 3-4 drops of mounting media along the length of the slide.
4. Carefully place glass coverslip onto the slide, taking care not to introduce bubbles (*see* **Note 18**).
5. Gently brush a small amount of nail varnish on to the edges of the slide to seal it (*see* **Note 19**).
6. Allow varnished slides to dry in the dark at room temperature for a minimum of 1 hour (*see* **Note 20**).

4.5 Image acquisition

1. At the microscope, image slides at 20x magnification using the GFP filter in a darkened room.

2. Acquire a minimum of 3 images per well to ensure fair representation of the sample (*see* **Note 21**).

4.6 Efferocytosis quantification

1. Open image using ImageJ software on appropriate computer (*see* **Note 22**).
2. Divide images into 9 equal squares with grid overlay plugin as shown in **Fig. 4**.
3. Count the total number of cells (both the bright and dark adherent BMDMs) in a grid square as shown in **Fig. 4**.
4. Count the number of (1) bright fluorescent cells (which have engulfed labelled Jurkats) in the same grid square as shown in **Fig. 5a and b**.
5. Count the number of BMDM cells with attached Jurkats (2) in the same grid square as shown in **Fig. 5a and b**.
6. Calculate the proportion of total cells performing efferocytosis (efferocytosis efficiency) as % of total cells by using the following equation:
   (Total number of cells / (Number of bright cells + number of cells with attached Jurkats)) x 100.
7. Repeat for the remaining 4 grids as shown in **Fig. 4**.
8. Calculate the mean efferocytosis efficiency (%) for this image by adding the five resulting percentages together and dividing by 5.
9. Repeat this for each of the 3 images you have for each well (*see* **Note 23**).

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4. Notes

1. Jurkats are an immortalised T lymphocyte cell line which are widely available commercially. Follow supplier's instructions for storage and revival.
2. We have also applied this same apoptosis protocol to BMDMs to investigate the phagocytotic effects of BMDM capability on macrophages (BMDM), in addition to the Jurkat T cell model.
3. BMDM are a common resource. For isolation and culture and storage of BMDM see reference 13: 'Isolation, Culture and Polarisation of Murine Bone Marrow and Peritoneal

Macrophages. Pineda-Torra I, Gage M, Juan A, Pello OM. Methods Mol Biol. 2015;1339:101-9.

4. The 8 well culture slide removal tool should be supplied by the manufacturer with the 8 well culture slides.
5. We use a Zeiss Axio Vert.A1 fluorescent microscope with a Zeiss Axiocam 503 mono digital camera with Zen Pro acquisition software.
6. We use the freely available Zen lite v2.3 software for viewing our acquired images on different hardware which is available from www.zeiss.com/microscopy/int/products/microscope-sofware/zen-lite.
7. Jurkats were counted by staining with Trypan Blue; 1 in 2 dilution. Using the culture conditions described, Jurkat cells were diluted in fresh media 1 in 5, every 2 days to maintain a maximum cell density of $1x10^6$ cells/mL in T25 flasks standing upright.
8. Using our complete DMEM growth media, this was typically on day 5.
9. Pharmacological treatment can be applied at this point (for example an LXR agonist).
10. This incubation is to ensure maximum incorporation of Calcein AM dye.
11. Avoid bright light when using the Calcein AM (for example by switching off tissue culture hood light and covering cells in aluminium foil during transit) to preserve fluorescence.
12. Cell pellet should appear green (Fig 2), demonstrating incorporation of dye into living cells.
13. For irradiation we use a Hug Flight UV steriliser (model HF-15151-E). If using different equipment please follow your local health and safety regulations for UV exposure protection.
14. Remove lid of TC dish so cells get full UV exposure.
15. Check cells every 30 minutes with a light microscope for blebbing, as the rate of apoptosis induction may depend on the power of your UV lamp and distance of cells from UV source.
16. Efferocytosis efficiency may be influenced by your mutation/experimental condition – we have found it best to try a range of time points to establish 20-30 % baseline

efferocytosis, and saturation at approximately 80 % -which may not necessarily be solely measuring first-time uptake events.

17. We have found that if you are using multiple conditions and/or timepoints it helps to have more than one experimentalist on hand to ensure the incubations and washes are performed consistently in a timely fashion.

18. Using forceps, place coverslip gently from one end so that any bubbles that may form can travel through the mounting fluid, escaping to the edge and don't become trapped.

19. Take care not to move the glass cover slip when applying the varnish, as not to dislodge the fixed cells.

20. For best results we image our slides within 24 hrs of fixation.

21. See **Fig. 1** for our typical slide layout and depiction of image acquisition.

22. There are other commercially available software packages for image analysis which may also be able to quantify your efferocytosis images. However, ImageJ is freely available and ensures maximum usability and audience for this protocol.

23. Our image analysis is performed blindly by our investigators, and the data is grouped after efferocytosis efficiency has been analysed for each image.

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