

Standardized Tabas Laboratory *In-Vitro* Efferocytosis Engulfment Assay

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I: Macrophage Efferocytosis Assay.

II: Preparation of Apoptotic Cells.

III: Enumeration of Efferocytosis Engulfment.

IV: Media compositions.

I. Efferocytosis Assay:

Day 1: Plate elicited macrophages onto 24-well tissue culture-treated plate in MF media (containing 20% L-cell conditioned medium) for a desired confluency on the day of assay at ~90%. 2 hrs post plating, rinse non-adherent cells away and reconstitute wells with MF medium.

Day 2: Reconstitute cells in fresh MF medium. Primary macrophages should be left to differentiate in L-cell conditioned media for a minimum of 48 hrs.

Day 3: If experimental conditions require overnight treatment, do so on day 3 and perform remainder of assay on day 4. In absence of overnight treatment, the assay may be performed on day 3 or 4, being sure to keep timing consistent for any future experiments. At this point the macrophages can be subjected to drug or other treatment according to the individual experiment—it is best to do this in MF medium without LCM if this incubation is < 24 h (omitting the LCM may help decrease long-term experimental variability, because different preps of LCM can lead to subtle variations in macrophage physiology).

Day 3 or 4:

1. Prepare Calcein AM labeled ACs as described below.
2. Remove the media from the macrophages and overlay ACs in 500 μ l media, swirling the plate briefly to ensure even coverage.
 - a. It is advised to initially perform the efferocytosis assays at a 1:1 ratio of macrophage:ACs, but varying this ratio is a common step to adjust the assay to suit individual needs.
 - b. This ratio can be confirmed on a fluorescent microscope.
 - c. At ~90% confluency, a 24-well-plate well contains ~ 250,000 macrophages.
3. Incubate cells at 37°C 20-45 minutes, checking level of engulfment on fluorescent scope.

Proper duration of efferocytosis assay can vary depending on the batch of primary macrophages and level of efferocytosis desired. It is recommended to include several time points in the assay. If assaying for enhancement of efferocytosis, shorter times

- are preferable, i.e., when the basal efferocytosis is ~10%. If assaying for inhibition of efferocytosis, longer times are better, i.e., when the basal efferocytosis is ~ 30%.
4. Remove media/ACs and rinse cells twice with ICE COLD 1X PBS.
 5. Check wash efficiency under a fluorescent microscope.
 6. Keep rinsing (may need to forcibly agitate plates) until ACs bound to the plates or to the outside of macrophages are rinsed away. Monitor rinsing efficiency at scope, being careful to not excessively rinse away macrophages themselves.
 7. Fix cells in 1% paraformaldehyde (PFA), adding just enough to cover the cells (~300 μ l)
 - a. Stock is 16% PF, dilute 1:15 in 1X PBS.
[must use 1X PBS diluted from 10X PBS].
 - b. Higher concentrations of PFA can cause increased instances of “ghosting” where green fluorescence is not a focused point but spread throughout the macrophage.
 8. Incubate in 1% PFA ~ 9min, rinse 2X with 1X PBS and add 500 μ l 1X PBS to each well.

II. Generating Apoptotic Jurkat Cells:

1. Spin down Jurkat cells at 1700 RPM (~500g) 5 min.
2. Resuspend pellet in fresh Jt media (media composition listed below).
3. Count cells on hemocytometer.
Plate 2×10^7 cells per petri dish (100mm diameter) in 10 ml Jt media. Prepare extra plates in case additional cells are needed.
4. Do not expose the cells to prolonged visible light for remainder of protocol.
5. Add 10 μ l Calcein AM to the cells (vehicle is DMSO so do not use at more than 1,000X).
Calcein AM (Invitrogen C3099) is a viability dye that only fluoresces in living cells and thus marks the cells that were alive before apoptosis inducement.
6. Pipet up and down to ensure proper mixing of the dye and incubate at 37°C for 1.5-2 h.
To monitor labeling, look at the cells under a fluorescence microscope or look for a green cell pellet.
7. Collect cells into a conical tube; wash the dish with 5 ml Jt media to collect residual cells.
8. Pellet cells 1700 RPM 5 min.
9. Wash 3 x 5 ml 1X PBS, carefully removing as much supernatant as possible each time to remove all unincorporated dye.

10. Resuspend the pellet in 10 ml Jt media and plate onto a bacterial Petri dish.
 11. UV (254 nm) irradiation: EL Series Ultraviolet Hand Lamps (attached to a lamp stand) from UVP (Upland, CA)—Model UVS-18 assembly 8W bulb (95-0200-01) 115V/60Hz/0.16Amp.
 - a. Remove dish lid, place dish ~5 inches from UV source, expose to UV light 5 min
 - b. For additional exposure the UV light of the tissue culture hood may also be on.
 - c. To ensure homogenous exposure “swirl” the plate every 60-90 s.
 - d. For multiple dishes irradiate one at a time (dishes should wait their turn at 37°C).
- Incubate the irradiated cells at 37°C until ~ 50% of the cells are visibly blebbing (1-2 h). The time at which blebs appear may vary depending on Jurkat cell passage number.
12. Collect cells 1700 RPM 5 min and resuspend in 40 ml MF media without LCM.
 13. Pipet up and down to achieve a homogenous and non-aggregated mixture for overlay.
 14. To achieve ~ 1:1 macrophage:AC ratio, use 0.5 ml of ACs (see below).

III. Taking Images & Quantitation:

1. Plates can be stored at 4°C for several days but images are best if taken same day.
2. To determine which regions of the well to enumerate:
 - a. Avoid areas at well centers or near edges as apoptotic cells can aggregate in these regions.
 - b. Scan the well at low magnification, getting a sense of the high and low spots, which are to be avoided.
 - c. Identify regions that represent the average engulfment, these are the areas where efferocytosis will fall in the linear part of the curve if the entire well were enumerated.
3. Increase the magnification to 30-40X and take images of 6-8 fields using both the green fluorescence and bright field.
 - a. The bright field should be as dim as possible while still allowing for identification of individual macrophages [this will ensure that all green fluorescence is accounted for].
 - b. The green fluorescence can weaken over time. Therefore, it may be necessary to capture the green and bright field images separately and merge them in Photoshop.

4. To quantify:
 - a. Count macrophages that have internalized green apoptotic bodies. These green apoptotic bodies are typically $>3 \mu\text{m}$ structures or *clusters* of smaller green dots. We will occasionally find macrophages with one or a few *isolated* green "specks" (less than $3 \mu\text{m}$)—we do not count this as positive.
 - b. Next count the total number of macrophages in the field.
 - c. Divide the number of macrophages with engulfed ACs by the total number of macrophages $\times 100 = \% \text{ efferocytosis}$.
 - d. For statistical significance one should count more than 500 total macrophage and perform assay in triplicate.

IV. Media:

Jurkat (Jt) Media

1. RPMI 1640 1X (10-040-CV): 450 ml
2. Penicillin/streptomycin: 5 ml
3. Glutamine: 5 ml
4. FBS (bought heat inactivated): 50 ml
5. Thaw 37°C , combine, filter

Macrophage (MF) Media

1. 1X DMEM high glucose (4.5 g/L): 350 ml
2. Penicillin/streptomycin: 5 ml
3. Glutamine: 5 ml
4. FBS (bought heat inactivated): 50 ml
5. L cell medium (contains M-CSF): $\sim 90 \text{ ml}$
6. Thaw 37°C , combine, filter