Efferocytosis Assay Detailed Protocol

The following protocol is a detailed worked example designed to enable the user to run a successful IncuCyte™ Phagocytosis of Cells Efferocytosis assay without the need for seeding validation or cell labeling optimization studies. It specifically describes the use of IncuCyte® ZOOM for monitoring apoptotic cell uptake in a single 96-well plate. In this example, effector cells \((J774A.1)\) are treated with several concentrations of an inhibitor of phagocytosis, and the resulting effect on engulfment of target cells (IncuCyte™ pHrodo® Red labeled Jurkat) is measured.

**Materials**
- IncuCyte™ pHrodo® Red Cell Labeling Kit for Phagocytosis
- Cell culture media \((J774A.1, \text{DMEM +10\% FBS}; \text{Jurkat, RPMI +10\% FBS})\)
- Growing stocks \(J774A.1\) wild-type (effector cells)
- Growing stocks Jurkat wild-type (target cells)
- Phagocytosis inhibitor (Lantruculin A, Cytochalasin D) or effector cell treatment of choice
- 96-well microplate (Corning® 3595)
- Cell scraper
- D-PBS (-/- Ca\(^{2+}\), Mg\(^{2+}\))
- Centrifuge and 50 ml centrifuge tubes

**Detailed Demonstration Protocol**

**Day 0**

**Effector cell: seeding**
1) Remove a freshly confluent (~80\%) T75 flask of \(J774A.1\) cells from the tissue culture incubator and place within a sterile cell culture hood.
2) Remove media and wash cells with D-PBS (10 mL).
3) Add 10 mL fresh cell culture media to flask and detach cells by scraping.
4) Perform a cell count (e.g. trypan blue staining + haemocytometer).
5) Dilute the cell suspension to \(2 \times 10^5\) cells/mL in culture media.
6) Using a multi-channel pipette seed cells (50 \(\mu\)L/well, i.e. \(1 \times 10^4\) cells per well) into chosen wells of 96-well microplate. Gently tap the plate to disperse cells evenly.
   a. Exclude effector cells from a small number of wells, this will serve as a negative control in order to observe any change in intensity of labelled Jurkats in the absence of effector cells.
7) Remove bubbles from all wells by gently squeezing a wash bottle (containing 100\% ethanol with the inner straw removed) to blow vapor over the surface of each well. Keep the tip of the wash bottle approximately 5 cm from the media surface.
8) Allow the cells to settle in the hood for 30 minutes before placing in incubator (37\%/5\% CO\(_2\)) overnight.

**Target cell: induction of apoptosis**
1) Remove a confluent T175 flask of Jurkat cells from the tissue culture incubator and place within a sterile cell culture hood.
2) Perform a cell count (trypan blue staining + haemocytometer). Transfer the cell suspension to 50 mL centrifuge tubes and centrifuge for 7 minutes at 1000 rpm. Resuspend cells in fresh media to a cell count of \(1 \times 10^6\) cells/mL (generate 1 x T175 flasks, 50 mL per flask).
3) To the flask add camptothecin to induce apoptosis. Final concentration should be 10 \(\mu\)M (using a 10 mM stock solution, add 50 \(\mu\)L to 50 mL cells).
Day 1

1) In the IncuCyte® ZOOM software, schedule 24 hour repeat scans every 15 minutes.
   a. Objective: Ensure desired objective is installed (10x or 20x).
   b. Vessel Type: Select Corning® 3595 (96-well microtitre plate).
   c. Channel selection: Select ‘Phase’ and ‘Red’ (800 ms acquisition time) channels.
   d. Scan mode: Select ‘Standard’ scan type and set ‘Scan Pattern’ to 1 image per well.
2) Solubilize IncuCyte pHrodo Red Cell Labeling Dye by addition of 100 µl DMSO. This will generate a stock solution of 1 mg/mL concentration.

**Effector cells: Treatment**

3) Prepare solutions of inhibitor in J774A.1 media to 4x the desired final assay concentration (FAC).
4) For concentration response curves, perform 1:3 serial dilution in media.
5) Add the inhibitor and vehicle control (25 µL per well, 4xFAC) to the cell plate. Replace cells in cell culture incubator and leave for 30 minutes.

**Target cells: Labeling**

6) Transfer apoptotic Jurkats into a 50 ml centrifuge tube and harvest by centrifugation (7 minutes, 1000 rpm).
7) Resuspend cell pellet with 50 mL IncuCyte pHrodo Red Cell Wash Buffer. Count the cells using a haemocytometer (note do not use Trypan blue as cells are apoptotic).
8) Harvest cells by centrifugation (7 minutes, 1000 rpm) and resuspend in IncuCyte pHrodo Red Cell Labeling Buffer to a density of 1 x10⁶ cells/ml.
9) Add IncuCyte pHrodo Red Cell Labeling Dye to a final concentration of 250 ng/mL (1 in 4000 dilution of stock).
10) Place the centrifuge tube containing cells and labeling solution into incubator for 1 hour.
11) Collect cells by centrifugation (7 minutes, 1000 rpm) and wash in 50 mL complete media; resuspend cells in media and centrifuge (7 minutes, 1000 rpm).
12) Resuspend cells in complete media (J774A.1 media) to 1 x10⁷ cells/mL (equivalent to 2.5 x10⁵ cells/well, 25x seeding density of J774A.1).

**Target + effector cells**

13) Immediately after Jurkat resuspension, remove the cell plate from the incubator and add the Jurkat cell suspension to the cell plate (25 µL per well) using a multichannel pipette.
14) Remove bubbles from all wells and place cells in IncuCyte ZOOM.
15) In the IncuCyte ZOOM set scans to commence immediately and monitor increase in fluorescence.

**Illustrative data**

**Figure 1:** Plot on right shows the increase in masked fluorescent object area (proportional to the number of phagosomes containing engulfed target cells). Fluorescence area and intensity increases where effector cells have been exposed to apoptotic target cells (positive control). In the absence of effector cells no increase in fluorescence is observed (negative control).
Figure 2: J774A.1 murine macrophage cells were seeded at 1.0 x 10^4 cells/well and incubated with 2.5 x 10^5 target cells/well (25:1 ratio). Exemplar data show blended phase and red fluorescent images (top row) at 0, 12 and 24 hour incubation times. Bottom row shows the masking of fluorescence (blue) area.

Briefly, the IncuCyte ZOOM software enables users to define a minimum fluorescence intensity threshold. Any fluorescent pixel which has an intensity above this threshold is considered to be above the signal-to-noise ratio and becomes ‘masked’. This masked area increases as more effector cells engulf target cells and become fluorescent. Hence the metric ‘Total fluorescent object area’ is a useful surrogate for counting of engulfed cells.

Figure 3: Microplate view showing overview of inhibitor screen. Row A contains highest concentration of inhibitor, decreasing 3-fold until row G. Positive control contains vehicle only (DMSO), with apoptotic Jurkats in the presence of J774A.1. Negative control contains apoptotic Jurkats only, in the absence of effector cells. Concentration response curves can be calculated from the exported data.
Fluorescence Metrics

As effector cells engulf apoptotic target cells, the area of fluorescence and intensity inside the effector cells increases. This can be reported in two ways: as an increase in fluorescence area (‘Total Object Area’); or increase in intensity, integrated over the area of detectable fluorescence (‘Total Integrated Intensity’).

To generate these metrics the user must create a Processing Definition suited to the cell type, assay conditions and magnification and then apply the Processing Definition to the data set as an Analysis Job.

1) Follow the instruction provided within the ‘Incucyte ZOOM® Fluorescent Processing Overview’ Technical Note to (1) create an appropriate image collection including the fluorescence channel with which to (2) create a Processing Definition to mask cells using fluorescent object analysis; (3) launch an Analysis Job to analyze images and produce metrics based on parameters contained within the saved Processing Definition.

2) To exclude background fluorescence from the mask, use the background subtraction feature in the Parameters drop-down menu. The feature ‘Top-Hat’ will subtract local background from brightly fluorescent objects within a given radius; this is a useful tool for analyzing objects which change in fluorescence intensity over time, as in this case.

   a. The radius chosen should reflect the size of the cells; in the illustrated data the radius chosen was 20 µm.
   b. The threshold chosen will ensure that objects below a fluorescence threshold will not be masked. To choose this threshold, examine the raw images of negative controls at a late time-point (where effector cells have engulfed target cells and become fluorescent). The threshold should exclude fluorescence from the negative control wells; in the illustrated data the threshold was 8 RCU.