IncuCyte™ Phagocytosis of Cells and Efferocytosis Assay General Protocol

Phagocytosis is a specific form of endocytosis by which cells internalize solid matter, thus eliminating cellular debris and pathogens. While most cells are capable of phagocytosis, it is the phagocytes of the immune system, including macrophages, neutrophils and immature dendritic cells that specialize in this process. In these cells, phagocytosis is a mechanism by which micro-organisms can be contained, killed and processed for antigen presentation and represents a vital facet of the innate immune response to pathogens, and plays an essential role in initiating the adaptive immune response.

Engulfment of cells is a specialized form of phagocytosis occurring when cells either display an 'eat me' signal or when a 'don't eat me' signal is inhibited. Clearance of dying cells by macrophage engulfment is known as efferocytosis and is a key function of inflammation. The process of efferocytosis begins with the exposure of the phosphatidylserine (PS) on the surface of dying cells. PS acts as an "eat-me" signal which triggers target engagement and the effector cell membrane extends around the dying cell, eventually enveloping it to form a discrete phagosome. This vesicle matures and acidifies to form a phagolysosome, in which the contents degrade. Attenuation of the 'don't eat me' enables the engulfment and clearance of healthy living cells, offering opportunities for therapeutics, notably in the immuno-oncology field.

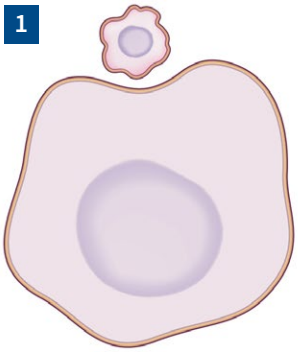
Common methods used to quantify phagocytosis and efferocytosis are often end-point and require numerous wash/quench steps and cell lifting (e.g., flow cytometry). In addition, these approaches have the added complication of the need to eliminate or quench the probe to remove interference from target cells which have adhered to, but not been engulfed by effector cells. More recently the introduction of pH-sensitive probes, such as pHrodo® conjugated reagents (IncuCyte™ pHrodo® Bioparticles® for Phagocytosis) enable accurate detection of cell engulfment as the probe transitions from a low fluorescent state at neutral pH outside the phagocyte, to highly fluorescent within the acidic environment. Thus washing or quenching steps are no longer required, resulting in simplified protocols.

We have developed a new image-based methodology that combines the IncuCyte™ pHrodo® Red Cell Labeling Kit and image-based fluorescent measurements, enabling simple mix and read protocols suitable for screening.

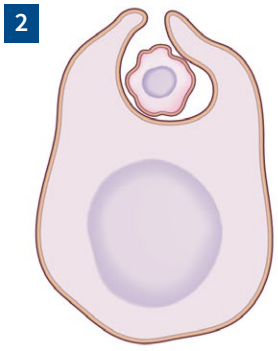
The assay enables:

1) Real-time visualization and fully automated analysis of efferocytosis.
2) A simple three step protocol devoid of wash steps or stopping of efferocytosis.
3) High sensitivity, requiring low cell numbers.

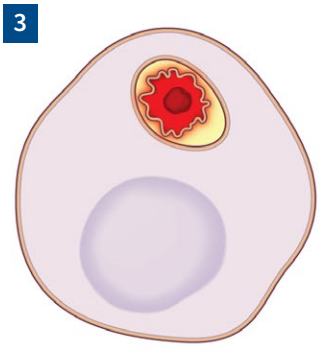
IncuCyte Phagocytosis of Cells Assay Concept. Phagocytes (± modulating treatments) are combined with IncuCyte pHrodo Red labeled apoptotic cells while being imaged within your incubator using the IncuCyte® ZOOM live-cell imaging system. As the IncuCyte pHrodo Red labeled cells reach the acidic phagosome, the fluorescence intensity of the fluorescent label is greatly enhanced, enabling efferocytosis to be quantified directly and in real-time.



pHrodo Bioparticles Added to Phagocytes
Little or no pHrodo fluorescence while apoptotic cells remain in the pH 7.4 extracellular environment.



Phagocytosis Initiated Following Receptor Activation
Formation of the phagocytic cup.



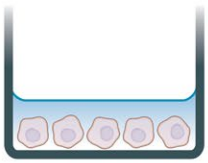
Formation of the Phagosome
Engulfment of pHrodo® labeled cells by pinching off. The acidic environment of the phagosome (pH 4.5-5.5) leads to increased pHrodo® fluorescence.

Phagocytosis of Cells Overview

This protocol provides an overview of the IncuCyte™ Phagocytosis Assay methodology. It is compatible with the IncuCyte® ZOOM instrument using your choice of phagocyte and target cells in combination with IncuCyte™ pHrodo® Labeling Kit.

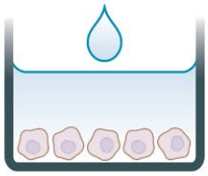
Note that these conditions have been optimized using J774A.1 macrophage cell line (effector cells) and Jurkats (target cells), however the methodology can be adapted to accommodate any phagocyte or target cell.

1 SEED EFFECTOR CELLS



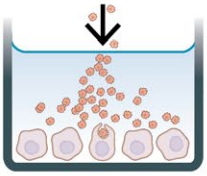
Seed phagocytic cells (e.g. J774A.1, 1-10K/well) in 96-well plates. Culture overnight (50 µL/well).

2 APPLY TREATMENTS



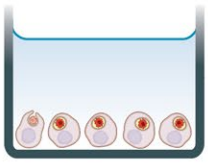
Treat effector cells with compounds prior to phagocytosis (0.5-24 h pre-treatment, 25 µL/well).

3 ADD INCUCYTE PHRODO LABELED TARGET CELLS



Add IncuCyte pHrodo-labeled target cells to treated wells (10 µg/well, 25 µL/well).

4 LIVE CELL FLUORESCENT IMAGING



Capture images every 10-30 mins (20x or 10x) in an IncuCyte ZOOM system for 2 - 48 hours. Analyze using integrated software.

General Protocol

Day 0

1) Seed phagocytic cells (50 µL per well) at an appropriate density into a 96-well flat bottom plate (Corning, 3595) such that by day 1 the cell confluence is approximately 10 - 20%. The seeding density will need to be optimized for cell type used; however we have found that 1 x10^3 to 1 x10^4 cells per well are reasonable starting points.
   a. Phagocyte cell adherence and growth can be monitored by recording phase images using the IncuCyte ZOOM live cell imaging device and confluence algorithm.

Day 1

1) Once the effector cells have reached appropriate confluence remove the cell plate from the incubator and add desired treatments. The volumes/dilutions may be varied; however we recommended 25 µL, prepared at 4x final assay concentration.
   a. Incubate the treatments for the desired duration.
2) After incubation with the treatments, add the IncuCyte pHrodo labeled target cells of your choice to the plate; we recommend a target to effector cell ratio of at least 10:1.
   a. Remove bubbles at the liquid surface by gently squeezing a wash bottle (containing 100% ethanol with the inner straw removed) to blow vapor over the surface of each well.
3) Image the plate in the IncuCyte ZOOM instrument with a 20x or 10x objective using the Standard Scan Type. We recommend 2 images per well, and scanning of phase and fluorescence every 15 minutes for minimum of 4 hours, and up to 48 hours.