SARTORIUS

Protocol

Non-Apoptotic Cell Phagocytosis (Antibody-Dependent Cellular Phagocytosis) Assay for Incucyte®

For the Quantification of Antibody-Dependent Cellular Phagocytosis of Non-apoptotic cells

This protocol provides an overview of the phagocytosis of antibody-treated target cells by macrophages, referred to as antibody-dependent cellular phagocytosis (ADCP). It combines the pHrodo® Cell Labeling Kit for Incucyte® with the Incucyte® Live-Cell Analysis System. For the measurement of the phagocytosis of apoptotic cells (efferocytosis) refer to the Incucyte® pHrodo® Cell Labeling Kit Product Guide.

Required Materials

- pHrodo® Red Cell Labeling Kit for Incucyte® Phagocytosis Assays (Sartorius, Cat. No. 4649) Or
- pHrodo® Orange Cell Labeling Kit for Incucyte® Phagocytosis Assays (Sartorius, Cat. No. 4766)
- Target cells of interest
- Target cell culture media
- Effector cells of interest
- Effector cell culture media
- 96-well microplate (e.g., Corning® Cat. No. 3595)
- Monoclonal antibody and appropriate isotype control
- Citrate buffer, pH 4.0

General Guidelines

- Pre-warm pHrodo® Wash and Labeling Buffers (Components C and D) to 37°C in water bath.
- Following effector cell seeding, place plates at ambient temperature for 30 minutes to ensure homogenous cell settling.
- Remove bubbles from all wells by gently squeezing a wash bottle (containing 70-100 % ethanol with the inner straw removed) to blow vapor over the surface of each well.
- After placing the plate in the Incucyte® Live-Cell Analysis System, acquire scans immediately.

Initial Optimization Protocols

Effector Cell Seeding Density Optimization

For optimal assay results, conduct preliminary cell proliferation experiments to determine the seeding density of the effector cells which will result in 10-20% confluence 24 hours after plating. We have found that $1x10^4$ effector cells per well is a reasonable starting point to reach ~20% cell confluency and recommend optimizing above and below that density (e.g., 5 - 20 x 10^3 cells/well).

Target Cell Labeling Optimization

Target cells must be efficiently labeled in order to detect phagocytic events. The pHrodo® Labeling Dye concentration needs to be optimized to ensure that non-engulfed target cells have little or no fluorescence and engulfed cells have a higher fluorescence. Additionally, an analysis definition is required that can segment the high fluorescence of engulfed cells but does not segment any minimal fluorescence of non-engulfed cells. For target cell labeling optimization as per the protocol below, we recommend performing a serial dilution of the pHrodo® Cell Labeling Dye for Incucyte® in DMSO and labeling your target cells. Labeled target cells can then be added to media (pH 7.4) or citrate-based buffer (pH 4.0), which is used to mimic internalization into a low pH environment.

1. Pre-warm pHrodo® Wash and Labeling Buffers (Components C and D) to 37°C in water bath.
2. Harvest target cells and transfer into a 50 mL centrifuge tube. Centrifuge for 7 minutes at 1000 rpm.
3. Aspirate supernatant and resuspend cell pellet with 50 mL pHrodo® Wash Buffer (Component C).
4. Harvest cells by centrifugation for 7 minutes at 1000 rpm. Aspirate pHrodo® Wash Buffer and resuspend cell pellet in pHrodo® Labeling Buffer (Component D) to a density of $1x10^6$ cells/mL. Separate the suspension into 8 aliquots of 1 mL.
5. Solubilize the pHrodo® Cell Labeling Dye for Incucyte® (Component A) by adding 100 µL of DMSO (Component B) to create a stock concentration of 1 mg/mL.
6. Perform a 7-point, two-fold serial dilution of the pHrodo® Cell Labeling Dye for Incucyte® in DMSO.
   a. For cultured cell lines, generate a concentration range between 50 µg/mL and 0.8 µg/mL

   Note: Suggested concentration range is based on data using the Incucyte® S3 and SX5 Live-Cell Analysis Systems. For cells extracted from blood or tissue, a higher concentration range may be required.
7. Add 10 µL of each concentration of labeling dye, or 10 µL DMSO for control, to 1 mL cell suspension i.e., a 1:100 dilution, which will provide a final assay concentration range of
   a. 0.5 µg/mL to 8 ng/mL
8. Incubate for 1 hour at 37 °C.
9. Harvest cells by centrifugation for 7 minutes at 1000 rpm.
10. Aspirate supernatant and wash cell pellet with 1 mL complete media (cell type appropriate). Harvest cells by centrifugation for 7 minutes at 1000 rpm, aspirate supernatant and resuspend in 1 mL complete media.
11. Prepare a citrate-based buffer solution at pH 4.0. For each dilution of pHrodo® labeled cells, prepare a micro-centrifuge tube containing 400 µL of buffer, and add 40 µL of labeled cells. Mix by trituration.
12. Prepare media control conditions. For each dilution of

		Media (pH 7.4)			Buffer (pH 4.0)								
[pHrodo®]	All	1	2	3	4	5	6	7	8	9	10	11	12
	A	pHrodo® Dye 500 ng/ml Target Cell (1) 10K/well Media pH 7.4			pHrodo® Dye 500 ng/ml Target Cell (1) 10K/well Buffer pH 4.0								
	B	pHrodo® Dye 250 ng/ml Target Cell (1) 10K/well Media pH 7.4			pHrodo® Dye 250 ng/ml Target Cell (1) 10K/well Buffer pH 4.0								
	C	pHrodo® Dye 125 ng/ml Target Cell (1) 10K/well Media pH 7.4			pHrodo® Dye 125 ng/ml Target Cell (1) 10K/well Buffer pH 4.0								
	D	pHrodo® Dye 62.5 ng/ml Target Cell (1) 10K/well Media pH 7.4			pHrodo® Dye 62.5 ng/ml Target Cell (1) 10K/well Buffer pH 4.0								
	E	pHrodo® Dye 31.25 ng/ml Target Cell (1) 10K/well Media pH 7.4			pHrodo® Dye 31.25 ng/ml Target Cell (1) 10K/well Buffer pH 4.0								
	F	pHrodo® Dye 15.63 ng/ml Target Cell (1) 10K/well Media pH 7.4			pHrodo® Dye 15.63 ng/ml Target Cell (1) 10K/well Buffer pH 4.0								
	G	pHrodo® Dye 7.81 ng/ml Target Cell (1) 10K/well Media pH 7.4			pHrodo® Dye 7.81 ng/ml Target Cell (1) 10K/well Buffer pH 4.0								
Vehicle	H	Target Cell (1) 10K/well Media pH 7.4			Target Cell (1) 10K/well Buffer pH 4.0								

Figure 1
Target Cell Labeling Optimization
Note. Example Plate Map for testing pHrodo® labeling of target cultured cell lines.

pHrodo® labeled cells, prepare a micro-centrifuge tube containing 400 µL complete media, and add 40 µL of labeled cells. Mix by trituration.

13. Per each cell dilution, aliquot 100 µL to three wells of a 96-well plate and allow the cells to settle at ambient temperature for 30 minutes.
14. Place plate into the Incucyte® Live-Cell Analysis System and perform a scan on demand using Phase and Red or Orange fluorescence channels, depending on reagent used.

Analysis Guidelines

Refer to the Data Acquisition and Analysis Section for more detailed information on setting up an Analysis Definition.

1. Create an Analysis Definition using representative images for both media and buffer conditions. Choose a threshold in which fluorescent objects are masked in the images for the buffer conditions but not masked in the images for media conditions at the lowest pHrodo® Labeling Dye concentrations (e.g., 0.5 - 2 RCU or OCU).

   Note: There may be some masking of fluorescent objects in the media condition for the highest pHrodo® Labeling Dye concentrations.
2. Plot the User Defined metric "Red (or Orange) Mean Intensity Object Average (RCU or OCU)" and select a pHrodo® Labeling Dye concentration that has a large difference in fluorescent intensity between media and buffer conditions, ensuring the media condition exhibits minimal fluorescence and that the cells can easily be masked in the buffer condition.

   Note: By counting the number of phase and fluorescent objects, a percentage of labeled cells may also be obtained for each concentration of dye. A percentage of labeled cells can also be obtained using the Incucyte® Cell-by-Cell Analysis Software Module (Cat. No. 9600-0031).

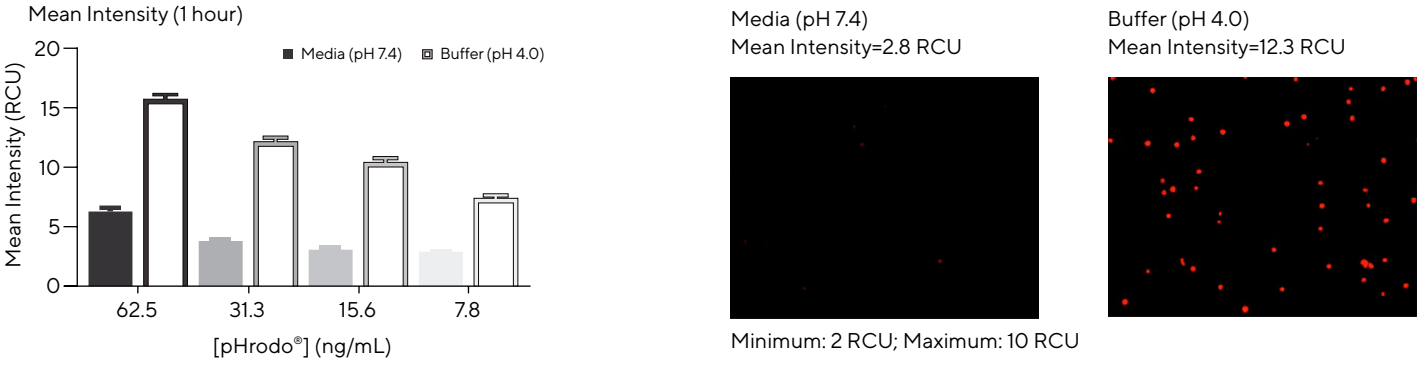


Figure 2

Target Cell Labeling Optimization Example Analysis

Note. A serial dilution of pHrodo® Red Cell Labeling Dye for Incucyte® (500 ng/mL – 7.8 ng/mL) was performed as per the optimization protocol using non-adherent Ramos cells. An Analysis Definition was applied using Top Hat Segmentation with a Threshold of 2 RCU. The Red Mean Intensity Object Average (RCU) was plotted for all concentrations. Graph shows Mean Intensity for the four lowest concentrations at 1 hour with media conditions in closed bars and buffer conditions in open bars. The pHrodo® Dye concentration of 15.6 ng/mL was selected based on a low level of fluorescence in media and a large difference in mean fluorescent intensity between media and buffer conditions. Red fluorescence images with user defined min – max fluorescence for 15.6 ng/mL illustrate difference in fluorescence for each condition.

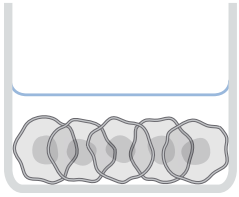
Phagocytosis of Non-Apoptotic Cells (Antibody-Dependent Cellular Phagocytosis) Protocol

The protocol described is applicable for the measurement of antibody-dependent phagocytosis (ADCP) of non-adherent target cells. If adherent target cells are being used, we recommend first seeding a mixture of labeled target cells and antibody treatments and then following incubation seeding effector cells on top.

Important: Prior to initiating the assay, it is recommended that your experimental design includes replicate wells of each condition being tested (e.g., labeled target cells alone as well as target:effector co-cultures at each ratio ± antibody, isotype, or vehicle controls) in order to determine the assay signal window. For initial optimization experiments, we recommend also including labeled apoptotic cells as positive apoptotic control to confirm effector cell is phagocytic.

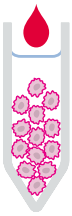
Quick Guide

1. Seed Effector Cells



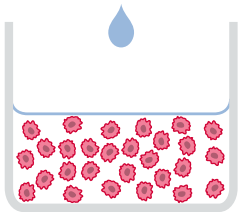
Seed phagocytic effector cells (50 µL/well). Culture overnight.

2. Label Target Cells



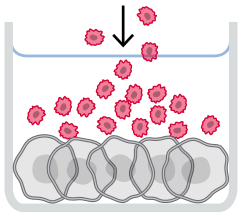
Label target cells with pHrodo® Labeling Dye for Incucyte®.

3. Treat Target Cells



In compound plate, combine Ab treatments and labeled target cells (2xFAC). Incubate for one hour.

4. Add Target Cells



Add pHrodo® for Incucyte® labeled target cells to the effector cells (50 µL/well).

Day 0

Seed Effector Cells

Note: This protocol is applicable for non-adherent target cells, see paragraph above for adherent target cell recommendations.

1. Harvest effector cells and determine cell concentration (e.g., Trypan blue + hemocytometer).
   Note: Grow enough effector cells in advance to accommodate the different cell densities required to set up the assay (e.g., 1 x 10^6 total cells for seeding 1 x 10^4 effector cells/well).
2. Prepare cell seeding stock in culture media to achieve 10-20% confluence after 24 hours.
   Note: The seeding density will need to be optimized for each cell type used per the preliminary optimization protocol.
3. Using a multi-channel pipette, seed effector cells (50 µL per well) into a 96-well microplate.
4. Remove bubbles from all wells by gently squeezing a wash bottle (containing 70-100% ethanol with the inner straw removed) to blow vapor over the surface of each well.
5. Allow the cells to settle on a level surface for 30 minutes, then incubate overnight at 37°C with 5% CO_2.

Day 1

Label Target Cells With pHrodo® Cell Labeling Kit for Incucyte®

Note: Grow enough target cells in advance to accommodate the different cell densities required to set up the assay. We recommend testing target-to-effector cell ratios at a range between 10:1 to 1:1.

1. Pre-warm pHrodo® Wash and Labeling Buffers (Components C and D) to 37°C in water bath.
2. Harvest non-adherent non-apoptotic (healthy) target cells and transfer into a 50 mL centrifuge tube. Centrifuge for 7 minutes at 1000 rpm.
3. Aspirate supernatant and resuspend cell pellet with 50 mL pHrodo® Wash Buffer (Component C). Gently mix cells by trituration and determine cell count using a hemocytometer.
4. Harvest cells by centrifugation for 7 minutes at 1000 rpm. Aspirate pHrodo® Wash Buffer and resuspend cell pellet in pHrodo® Labeling Buffer (Component D) to a density of 1x10^6 cells/mL.
5. Reconstitute pHrodo® Cell Labeling Dye for Incucyte® (Component A) in 100 µL of DMSO (Component B) to create a stock concentration of 1 mg/mL.
6. Add the solubilized pHrodo® Cell Labeling Dye to the target cell suspension at the concentration determined during optimization (refer to Target Cell Labeling Optimization under General Guidelines). Incubate the centrifuge tube containing cells for 1 hour at 37°C.
7. Remove excess pHrodo® Labeling Dye from cells:
   a. Centrifuge the cell labeling dye suspension at 1000 rpm for 7 minutes. Aspirate off supernatant and resuspend apoptotic target cells in 50 mL of target cell media.
   b. Harvest cells by centrifugation for 7 minutes at 1000 rpm. Aspirate supernatant and resuspend apoptotic target cells in effector cell media to yield a cell density of 1 x 10^7 cells/mL.
      Note: For optimal viability, it is important to be gentle with non-apoptotic cells during labeling.

Treat Target Cells

1. Using target cell media, prepare 2x the final desired concentration of antibody, isotype control, and vehicle control in a separate 96-well compound plate.
2. Add pHrodo® labeled target cells at 2x the final desired density to the treatment plate, gently mix by trituration, and incubate for 0.5 – 1 hour at 37°C.

Add Target Cells to Effector Cells

1. Remove the effector cell plate from the incubator and add the target cell and antibody mixture to the cell plate (50 µL per well) using a multichannel pipette.
2. Remove bubbles and immediately place the microplate in the Incucyte® Live-Cell Analysis System (refer to Data Acquisition and Analysis section).

Data Acquisition and Analysis

Acquisition

Using Incucyte® integrated software, schedule repeat scanning for every 15-30 minutes, for up to 48 hours.

a. Scan type: Standard
b. Image Channels: select "Phase" and "Red" or "Orange".
c. Objective: 10X or 20X depending on cell types used.
d. Scan pattern: 2 – 4 images per well.

Analysis

To generate the metrics, the user must create a Basic Analyzer Analysis Definition suited to the cell type, assay conditions, and magnification selected.

1. To help choose a threshold that ensures fluorescence of engulfed target cells only is segmented, look at images of non-engulfed cells and adjust fluorescence values to minimize any fluorescence from these cells. Select an image from a well containing a high density of target cells but no effector cells during the peak assay response (e.g., 4 - 12 hours). Under Image Channels, expand the Red (or Orange) drop down and deselect the Autoscale option. Adjust the scale until no fluorescence is observed in the image and note the minimum and maximum values.
2. In the Analysis Definition:
   a. Select representative images for each condition being tested (e.g., target cells alone and target:effector co-cultures at each ratio).
   b. Deselect the Autoscale option and set the minimum and maximum values identified in Step 1.
   c. Set up the mask for the phase confluence measure with fluorescence channel turned off (optional).
   d. Turn the red (or orange) fluorescence channel on. To exclude background fluorescence from the mask, use the background subtraction feature in the Red (or Orange) 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.
      i. The radius chosen should reflect the size of the fluorescent object but contain enough background to reliably estimate background fluorescence in the image; 20 – 30 µm is often a useful starting point.
      ii. The threshold chosen will ensure that objects below a fluorescence threshold will not be masked. Choose a threshold in which red objects are masked in the image without effector cells but not masked in images without effector cells (e.g., 0.5 - 2 RCU or OCU).
3. Recommended Metrics for Phagocytosis of Cells: As effector cells engulf target cells, the area of fluorescence and intensity inside the effector cells increases. This can be reported in two ways:
1. Quantification of an increase in fluorescence area.
   a. Suggested metric: Total Red (or Orange) Object Area (µm²/image or µm²/well)
2. Quantification of increase in intensity integrated over the area of detectable fluorescence.
   a. Suggested metric: Total Red (or Orange) Object Integrated Intensity (RCU or OCU x µm²/image).
3. Individual cell identification can be enabled with the Incucyte® Cell-by-Cell Analysis Software Module (Cat. No. 9600-0031), providing cell density is optimized for accurate segmentation of cells. This enables the subsequent classification into subpopulations based on properties including fluorescence intensity, size and shape. For further details of this analysis module and its application see:

Incucyte® Cell-by-Cell Analysis Software | Sartorius

   a. Post-classification data can be displayed as either % of cells expressing fluorescence or mean intensity of positive fluorescent objects.

Related Products and Applications

In addition to the pHrodo® Cell Labeling Kit for Incucyte®, a comprehensive range of Bioparticles® for phagocytosis of bacterial Gram positive, Gram negative or yeast-derived pathogens by immune cells are available for use with the Incucyte® Live-Cell Analysis System.

Product	Cat. No.	Amount	Ex. Maxima	Em. Maxima
pHrodo® Red *E. coli* Bioparticles® for Incucyte®	4615	2 mg	560 nm	585 nm
pHrodo® Green *E. coli* Bioparticles® for Incucyte®	4616	2 mg	509 nm	533 nm
pHrodo® Red Zymosan Bioparticles® for Incucyte®	4617	1 mg	560 nm	586 nm
pHrodo® Green Zymosan Bioparticles® for Incucyte®	4618	1 mg	506 nm	533 nm
pHrodo® Red *S. aureus* Bioparticles® for Incucyte®	4619	2 mg	560 nm	585 nm
pHrodo® Green *S. aureus* Bioparticles® for Incucyte®	4620	2 mg	509 nm	533 nm

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