SARTORIUS

Protocol

Incucyte® NETosis Assay

For the Quantification of Programmed Neutrophil Death

This protocol describes a solution for the measurement of neutrophil programmed cell death known as NETosis. This method utilizes the Incucyte® Cytotox Green Dye and the Incucyte® Live-Cell Analysis System for image-based fluorescent measurements of neutrophil extracellular traps (NETs).

Required Materials

- Incucyte® Cytotox Green Dye (Cat. No. 4633)
- Incucyte® Imagelock 96-Well Plate (Cat. No. 4379)
- Low auto-fluorescence media (such as F12K)
- PMA or other stimulant

Optional:

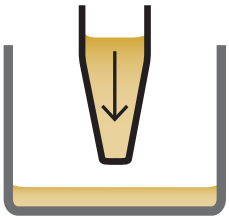
- Fibronectin 1 mg/mL stock (Sigma Cat. No. F1141)

General Guidelines

- 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.

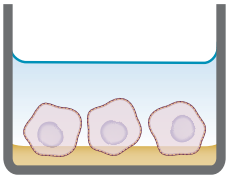
Quick Guide

1. Coat wells (optional)



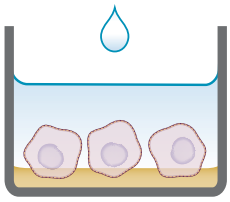
Coat wells of a 96-well plate (50 µL/well) with appropriate matrix.

2. Plate cells



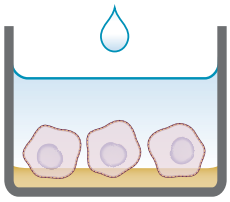
Seed cells in presence of Incucyte® Cytotox Green Dye (100 µL/well, 20,000 cells).

3. Add inhibitors (optional)



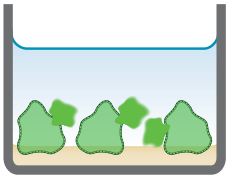
Add NETosis inhibitors (50 µL/well).

4. Induce NETosis



Induce NET release with stimulation of choice (e.g., 100nM PMA).

5. Live-cell imaging



Place in the Incucyte® Live-Cell Analysis System and capture images every 5–10 minutes for a minimum of six hours using Phase and Green channels. Analyze using integrated software.

Protocol

Coat Imagelock Plate (Optional)

Depending on cell line used, coat the Imagelock Plate with relevant coating matric according to manufacturer's recommendation (refer to Fibronectin coating procedure below)

1.1 Dilute fibronectin stock (1 mg/mL) to 0.1 µg/ml in PBS (without Mg_{2+}/Ca_{2+}).
1.2 Add 50 µL per well in cell culture hood, ensuring each well is covered.
1.3 Leave for 1 hour at room temperature.
1.4 Aspirate to remove solution.

Plate Cells

2.1 Dilute Cytotox Green Dye to a final assay concentration of 250 nM in low fluorescence media without serum (e.g., F12K).
2.2 Prepare neutrophil stock of 200,000 cells/mL in media containing Cytotox Green Dye.
2.3 Seed neutrophils (100 µL per well, 20,000 cells per well).
2.4 Allow cells to settle at room temperature for 15 minutes in cell culture hood. Alternatively, spin plate for 1 minute at 50X g.
2.5 Place into Incucyte® Live-Cell Analysis System and allow to warm to 37° C for 30 minutes before performing a "Scan Once Now" to assess cell viability.

NOTE: A single scan is recommended prior to the addition of NETosis inhibitors to assess a baseline of cell viability.

a. Objective: 20X (recommended)
b. Channel Selection: Phase Contrast and Green
c. Scan type: Standard (with lock)
d. Choose vessel type: 96-well Essen Imagelock

NOTE: Edit scan pattern to choose wells to be scanned. More than one image per well may be taken but this will increase the time taken per scan.

Add NETosis Inhibitor (Optional)

3.1. Make up at 4X final assay concentration of NETosis inhibitor and warm solutions to 37° C prior to addition to Imagelock Plate containing cells.
3.2. Add 50 µL NETosis inhibitor per well and allow treatment to incubate with cells for 30–60 minutes.
3.3. Another scan on demand may be performed to assess effect of treatment on cells.

Induce NETosis

4.1. Prepare PMA or other NETosis stimulant in low fluorescence media containing 250 nM Cytotox Green Dye at final assay concentration 100 nM.
   a. If cells have been pre-treated with inhibitor, add PMA in 50 µL per well (4X final assay concentration) backspace.
   b. If cells have not been pre-treated add PMA in 50 µL per well (3X final assay concentration).

4.2. Allow solutions to warm to 37° C before addition to cell plate backspace.

Live-Cell Imaging

5.1. Place plate into Incucyte® Live-Cell Analysis System and commence scheduled scans.
   a. Objective: 20X (recommended)
   b. Channel Selection: Phase Contrast and Green
   c. Scan type: Standard (with lock)
   d. Choose vessel type: 96-well Essen Imagelock

Scan Schedule: 5 or 10 minute intervals for minimum of 6 hours.

Analysis

NETosis will begin approximately 2 hours after stimulation with PMA After 6 hours, choose representative images from PMA-stimulated and vehicle control wells to create a processing definition. Vehicle wells are likely to contain a small number of dead cells (small round objects) which can be excluded from the analysis using area and eccentricity filters. Use TopHat to subtract background, choosing an appropriate radius (i.e., slightly larger than the radius of the object to be masked).

Representative Data in Green Channel

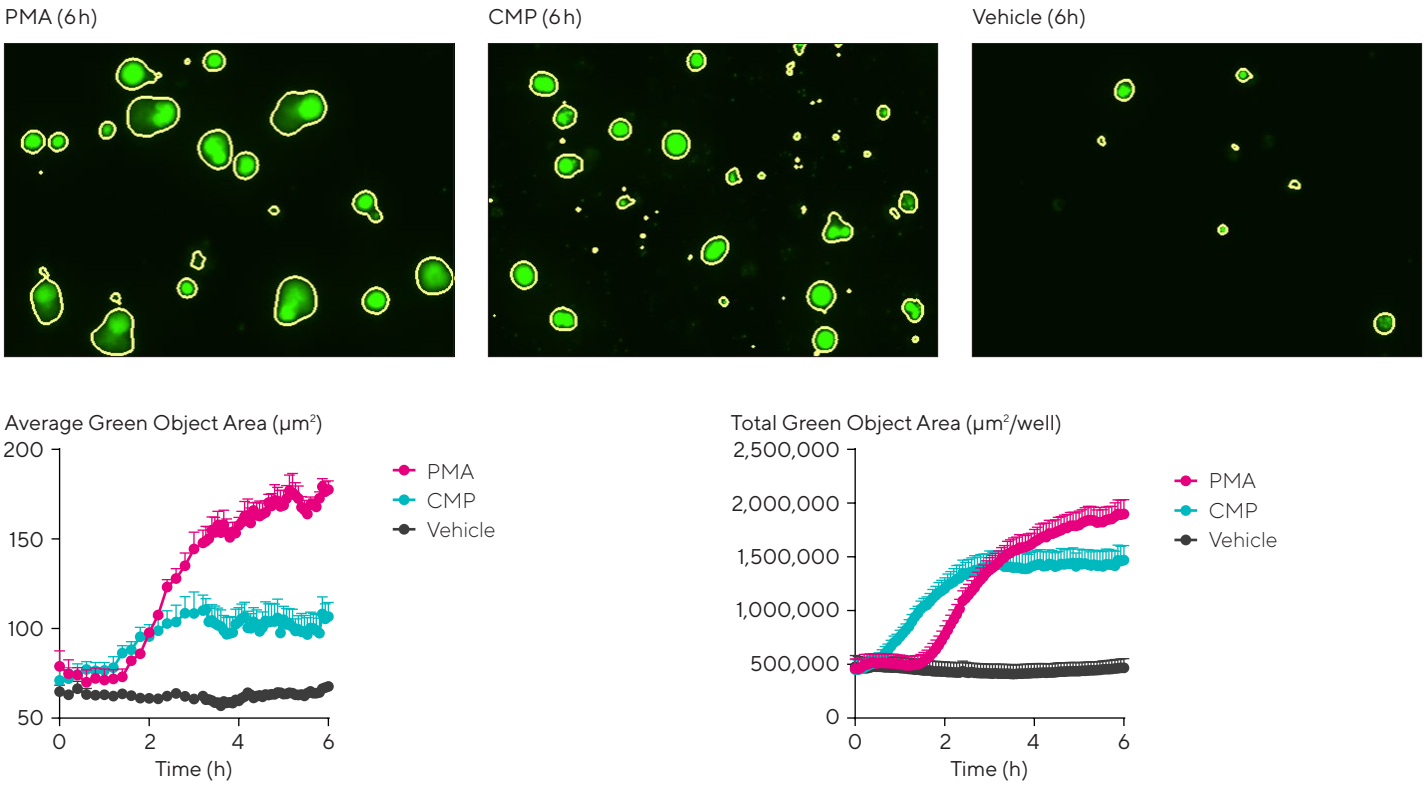


Figure 1: Cytotox Green Dye is used to visualize NET release (extracellular DNA, PMA treatment) and cell death (intracellular DNA, CMP treatment). Fluorescent masking incorporates green objects of all sizes (no area filter). PMA-stimulated cells yield a larger average object area than apoptotic cells (CMP-treated). NET release begins approximately 2 h post stimulation. Images shown at 6 h post treatment (100 nM PMA, 10 µM CMP and 1% DMSO respectively).

To demonstrate both NETosis and apoptosis, PMA (100 nM) was used to induce NETosis while CMP (camptothecin, 10 µM) was used to induce apoptosis. Yellow outline shows the green fluorescence mask (in Figure 1, no area or eccentricity filters have been applied). The average area of a green object (graph on left) shows that PMA induces large green DNA clouds (NETosis) while CMP permeabilizes the cell membrane allowing Cytotox Green to bind nuclear DNA—yielding small round objects (nuclei) which fluoresce green.

By excluding smaller objects (dead cells), a NETosis signal can be cleanly separated from the apoptosis signal. To generate Figure 2, a minimum area of 300 µm² was set in the processing definition which excluded apoptotic cells.

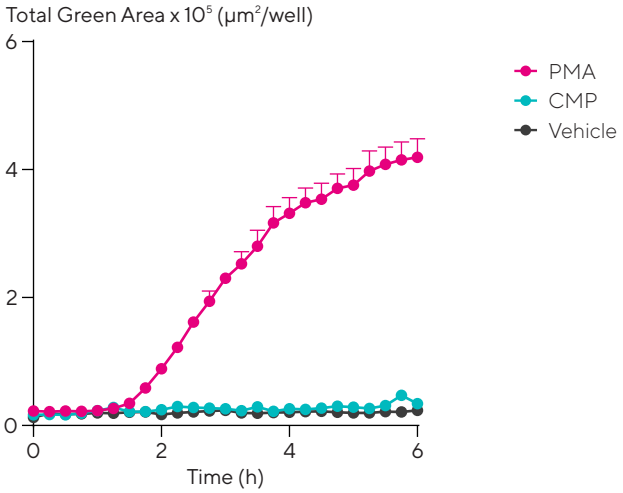


Figure 2: A NETosis signal can be generated by excluding dead cells. By filtering out green objects < 300 µm², apoptotic cells are excluded from the mask. Only the largest objects (NETs) remain masked.

Find more information at www.sartorius.com/incucyte

Sales and Service Contacts

For further contacts, visit www.sartorius.com

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