Real-time visualization and quantification of Neutrophil Extracellular Traps

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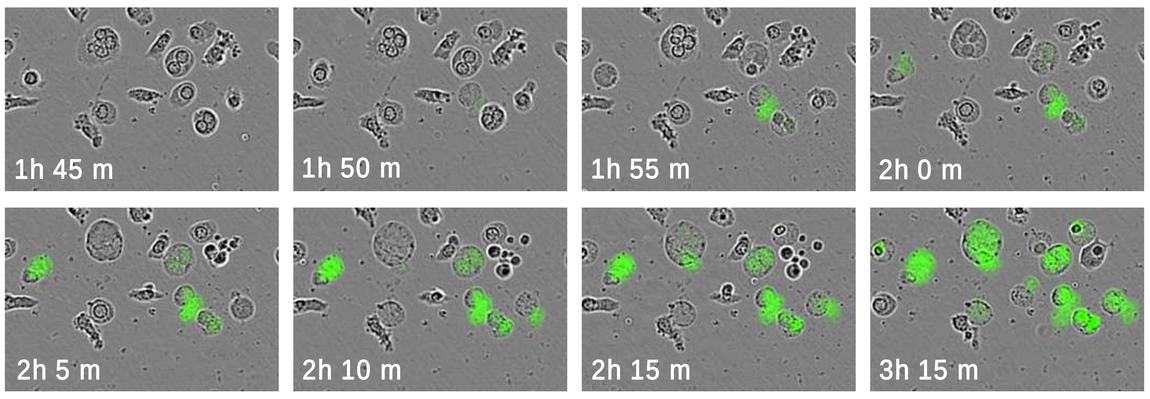
Summary & Impact

- Expulsion of extracellular traps is a defense mechanism utilised by neutrophils upon contact with microbes.
- Extracellular Traps are formed of DNA and antimicrobial proteins including myeloperoxidase (MPO) and neutrophil elatase (NE).
- Here we describe a simple kinetic live-cell imaging approach using a combination of phase and fluorescence imaging to visualise stages of neutrophil extracellular trap (NET) formation.
- Phase analysis enables monitoring of changes in cellular morphology and nuclear decondensation.
- IncuCyte Cytotox reagent fluoresces upon binding DNA, allowing NET release to be observed in real-time.
- Other reagents can be multiplexed with Cytotox and enable visualisation of cellular events including ROS formation, externalisation of phosphatidylserine and Caspase activation.
- These assays are flexible, simple and provide automated and direct measures of NET formation in real-time.

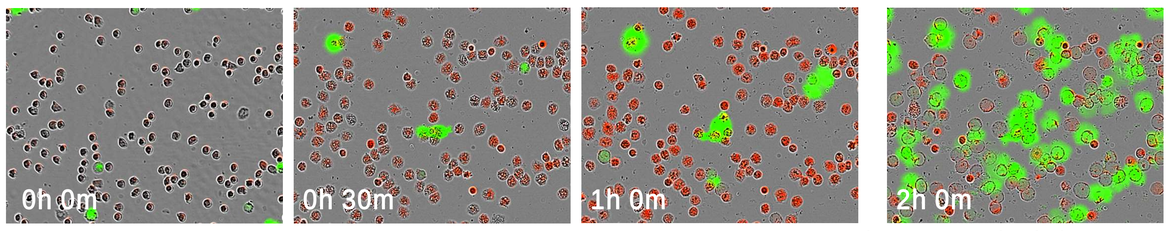
Live-cell visualisation of NETosis



- HL-60 cells were differentiated to neutrophil-type using DMSO (1.25% v/v) and ATRA (0.1 µM). The resulting multinuclear dHL-60 cells were stimulated using PMA.
- Nuclei begin to decondense ~2 hours post stimulation. As the cytoplasm mixes with karyoplasm the nuclei are no longer visible in Phase images.
- The nuclear contents are moved to the plasma membrane and released. External DNA binds to Cytotox reagent and fluorescence enhancement (green) is observed.
- Green fluorescence begins to appear ~2h post stimulation.

NET formation in primary human Neutrophils

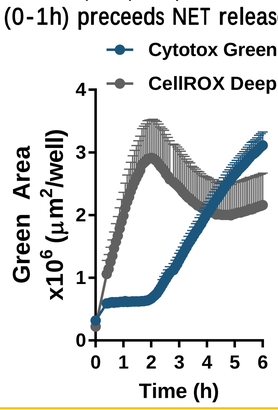
Primary human neutrophils were extracted from blood and seeded in serum-free media into an IncuCyte Imagelock 96-well plate, in the presence of reagents (IncuCyte Cytotox, 250 nM and CellROX Deep Red, 5 µM). Cells were then stimulated with PMA (100 nM) and placed into IncuCyte S3 and HD Phase and fluorescence images were acquired.

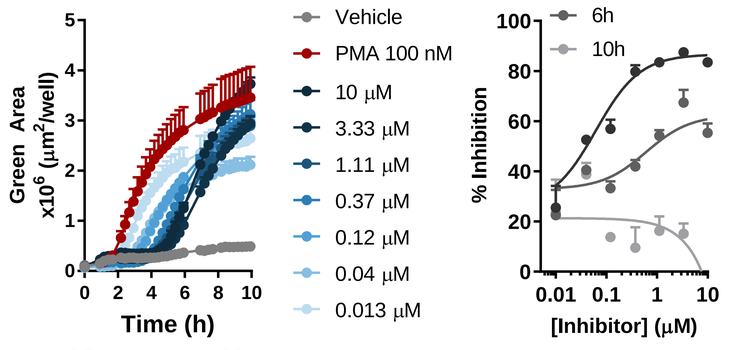


- Morphology changes are observed as cells flatten and become adherent. Reactive oxygen species (ROS) begin to form – this is visualised using CellROX Deep Red reagent (red fluorescence)
- 1h post stimulation, ROS peaks and intracellular membranes begin to permeablise.
- >2 hours post-stimulation, many nuclei are no longer visible in phase and nuclear contents are expelled into extracellular space (green fluorescence).

PMA-induced NETosis is ROS-dependent

Overlay of ROS (grey) and NET release (blue) response: **ROS burst (0-1h) preceeds NET release (>2h)**

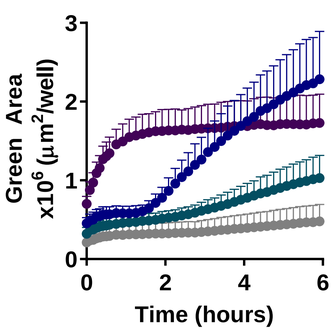




- Left: DPI inhibits NADPH oxidase activation (respiratory burst) for up to 6h and therefore NET release is inhibited.
- Right: PMA-induced NETosis is inhibited in the presence of DPI up to 6h.

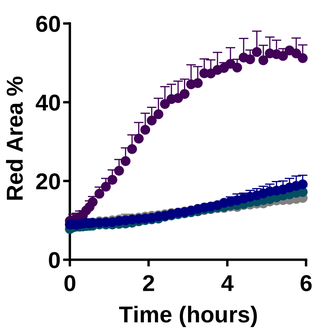
NETosis mechanisms can be differentiated

Cytotox reagent: DNA



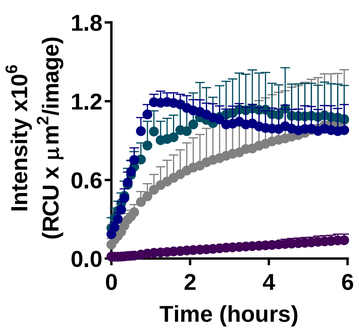
Ionomycin (purple) induces rapid formation of large NETs; while PMA (blue) induces NET formation approx. 2h post-stimulation. CMP (green) induces apoptosis and dead cells are small objects.

Annexin V: externalised PS



Ionomycin-treated cells (purple) externalise PS rapidly (within 4h) however in this time frame PMA and CMP treatment show lower, slower increases in PS externalisation (a marker of early apoptosis).

CellROX: ROS



PMA (blue) stimulates rapid ROS production (0-1h) which preceeds NET release. Ionomycin (purple) releases NETs via a ROS-independent mechanism.

Continuous Live-Cell Analysis: Methodology

IncuCyte® S3 Live-Cell Analysis System

A flexible assay platform that sits inside a standard tissue culture incubator. IncuCyte automatically and continuously acquires and analyzes HD phase and fluorescent images of living cells cultured in microplates, dishes, or flasks.

IncuCyte® Software

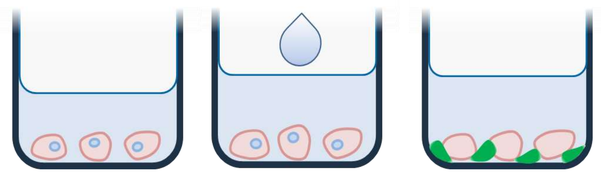
Fast, flexible, and powerful control hub for continuous live-cell analysis comprising image acquisition, processing, and date visualization.

IncuCyte® Reagents & Consumables

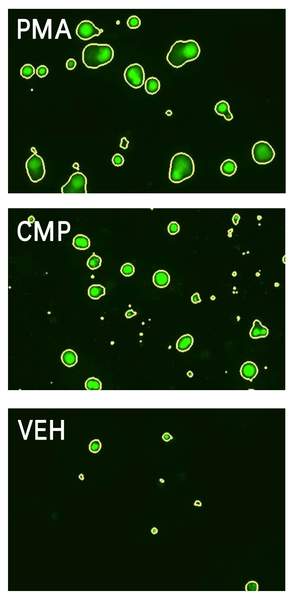
A suite of non-perturbing cell labeling and reporter reagents. Includes nuclear-targeted GFP and RFPs for cell counting, no-wash caspase 3/7 substrate for apoptosis, and cell kits for angiogenesis.

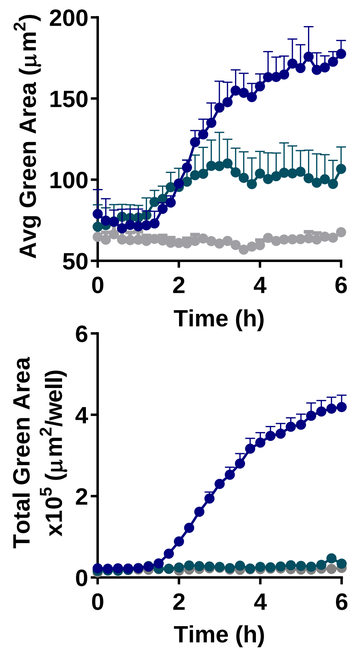
NETosis Assay Workflow

1. Seed cells in presence of reagents.
2. Add treatments e.g. inhibitor
3. Stimulate NET formation using chosen method (e.g. PMA, 100 nM)
4. Place into IncuCyte and rapidly scan phase & fluorescence channels.



Quantification of NETs by fluorescence masking



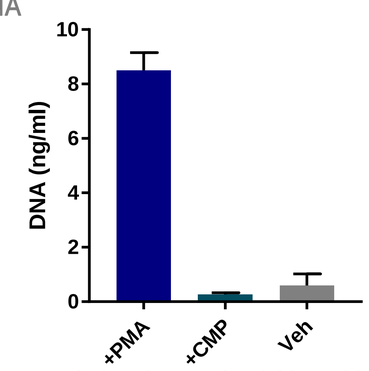


- PMA-stimulated cells generate NETS. These are visualised as large asymmetric clouds of extracellular DNA which fluoresce in the presence of IncuCyte Cytotox reagent. These objects have the largest Average Area (blue data, left).
- CMP-treated cells undergo apoptosis. Permeablisation of the plasma membrane enables nuclear staining resulting in small round objects (green data).
- Untreated (vehicle) cells in culture contain a small number of dead cells. Similar to apoptosis-induced but fewer in number (grey data).
- Fluorescent masking algorithms yield average fluorescent object size (top graph) which clearly separates NETosing (PMA-stimulated) and apoptotic (CMP-treated) cells. **NB Masked fluorescence area is outlined in yellow.**
- Filtering out smaller objects enables exclusion of dead cells from NETosis signal (bottom graph).

Assay validation

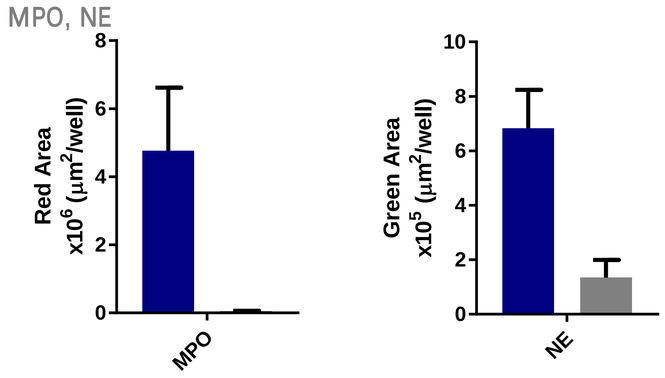
After kinetic 18h analysis, cells in 96-well microtitre plate were removed from IncuCyte. Supernatant samples were removed and cells were fixed and immunofluorescence staining of myeloperoxidase (MPO) and neutrophil elastase (NE) was carried out.

PicoGreen Assay quantifies cell-free DNA



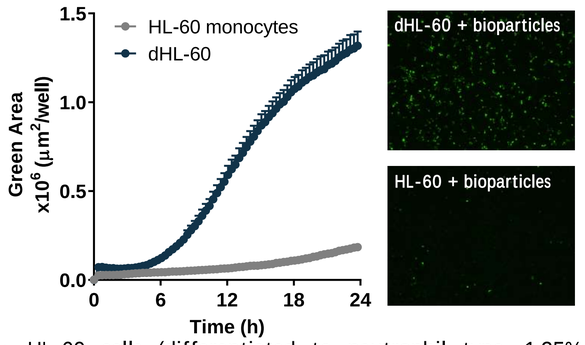
- PMA-induced NETosis yields a high concentration of cell-free DNA
- CMP-induced apoptosis is not detected by this method

Immunofluorescence confirms presence of extracellular MPO, NE

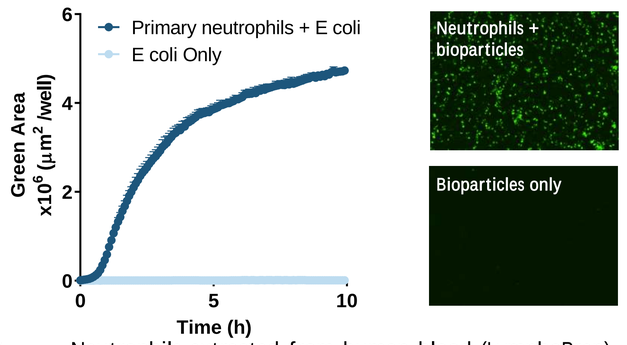


- Total fluorescence area observed after immunofluorescence with anti-MPO or anti-NE antibodies and appropriate conjugated secondary Ab
- PMA-treated cells release MPO and NE into extracellular space
- Untreated cells release no MPO, little NE

Neutrophils and neutrophil-like cells are phagocytic



- HL-60 cells (differentiated to neutrophil type, 1.25% DMSO, 0.1 µM ATRA, 5d) phagocytose IncuCyte pHrodo (Green) Bioparticles which undergo fluorescence enhancement upon engulfment into phagolysosome. Undifferentiated monocytes (grey) are non-phagocytic, fluorescence remains low. Images: t=24h, 0.5 – 4.0 GCU.



- Neutrophils extracted from human blood (LymphoPrep) were seeded into a 96-well microplate and IncuCyte pHrodo bioparticles were then added. Increased fluorescence is observed as the human neutrophils engulf the bioparticles (dark blue) while non-engulfed bioparticles have low fluorescence (pale blue).