APPLICATION NOTE

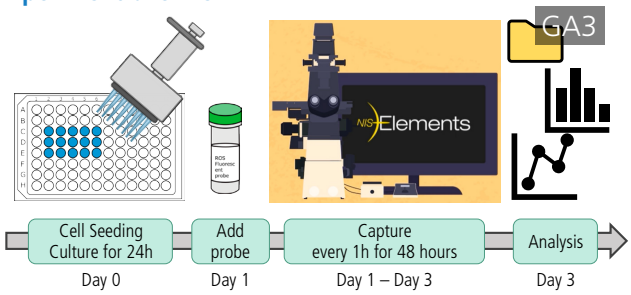
Volume Contrast, the analysis software of NIS-Elements

High-content live cell imaging of ROS production in activated macrophages

Immune cells play a role in maintaining a normal body by eliminating foreign substances through the inflammatory response. On the other hand, excessive inflammation can cause autoimmune diseases, allergies, diabetes, and Alzheimer's disease. Monocytes stimulated by foreign substances produce reactive oxygen species (ROS) in mitochondria, differentiate into activated macrophages, and decompose foreign substances. ROS have important functions as signaling molecules, but can also be toxic byproducts that damage the DNA within cells. Fluorescent excitation also produces ROS, causing phototoxicity issues that damage living cells. This application note introduces low-phototoxicity, high-content live-cell imaging for quantitative analysis of ROS by detecting ROS in activated macrophages using weak excitation light and a highly sensitive monochrome camera and performing label-free counting on cells within the field of view.

Keyword: inflammation, ROS, macrophages, Label-free, Live-cell high-content imaging, Image analysis

Experiment overview



1) Seed J774.1 cells in a 96-well polystyrene microplate (TC-treated) at a density of 2 x 10e5 cells/ml and culture for 24 hours at 37˚C in a 5% CO_2 incubator.
2) Remove the medium, wash the cells twice with HBSS, add Photo-oxidation Resistant DCFH-DA Dye Working Solution, and incubate for 30 minutes in an incubator.
3) Remove working solution and wash cells once with HBSS.
4) Add LPS diluted in phenol red-free DMEM medium at concentrations of 0 ng, 125 ng, 250 ng, 500 ng, and 1000 ng/mL to 3 wells each.
5) Place the well plate on a Ti2-E inverted microscope equipped with a stage-top incubator, and acquire time-lapse images using the HCA Live template in NIS-Elements HC (Table 1).
6) Create an image analysis recipe in General Analysis 3 and export the analysis data to CSV. Analyze the data in Microsoft Excel®.

Table 1: Imaging and analysis conditions

	Brightfield	ROS (Fluorescence: green)
Light source	Diascopic LED	D-LEDI (Power: 10%)
Ex/Em (nm)	—	475/535 nm
Exposure time	800 µs	80 ms
Mag, FOV	Mag: 20X, FOV: 0.89 x 0.89 mm	
Z stack	3 planes (0.8 µm x 3 steps)	
Multi-point	3 points/well	
Time-lapse	Interval 1 hour, duration 48 hours	
Mask	VC Cell Mask	ROS Mask

◆ Observation devices

- Microscope: Ti2-E
- Monochrome camera: DS-Qi2
- Fluorescent LED illumination system: D-LEDI
- Objective: CFI Plan Apochromat Lambda D 20X (NA 0.8)
- Stage top incubator

◆ Image analysis software/option modules

- NIS-Elements HC
- General Analysis 3 (GA3)
- NIS-A EDF module
- Volume Contrast (VC)

◆ Cells/Reagents/Materials

- Mouse macrophage-like cell line (JCRB J774.1)
- ROS Assay Kit -Photo-oxidation Resistant DCFH-DA- solution (DOJINDO LABORATORIES, R253)
- Lipopolysaccharide, from E.coli O111 (LPS) (FUJIFILM Wako Pure Chemical Corporation, Cat#125-05181)
- CELL CULTURE MICROPLATE, 96 WELL (Greiner Bio-One, 655090)

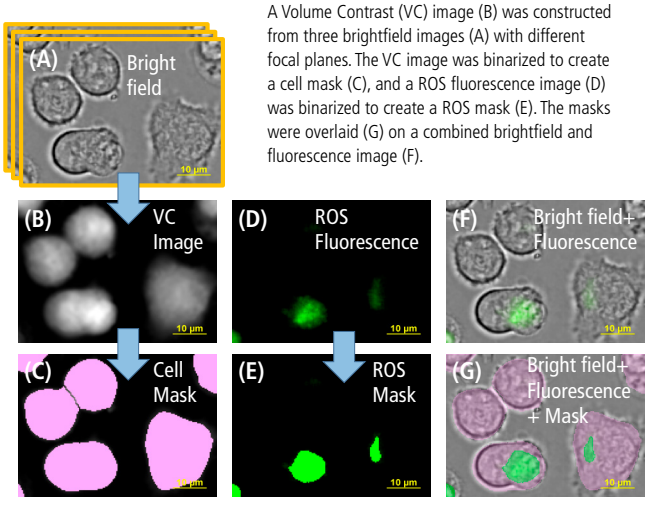


Fig.1: Creation of cell masks and ROS masks
A Volume Contrast (VC) image (B) was constructed from three brightfield images (A) with different focal planes. The VC image was binarized to create a cell mask (C), and a ROS fluorescence image (D) was binarized to create a ROS mask (E). The masks were overlaid (G) on a combined brightfield and fluorescence image (F).

Label-free cell counting using VC images

Counting cells by nuclear staining with Hoechst causes cell damage in live-cell imaging due to dye toxicity and the phototoxicity of fluorescent excitation. Brightfield imaging with diascopic light is superior because it does not cause phototoxicity to living cells (Fig. 2 (A)). However, the contrast between the background and cell regions in brightfield images is low, making it difficult to count the number of cells by binarizing the cell regions (Fig. 2 (B)). On the other hand, VC imaging can construct a fluorescence-like phase distribution image from three brightfield images with different focal planes, making it easy to distinguish between the background and the cell regions (Fig. 2 (C)). This makes it possible to binarize the cell regions from the VC image, create a cell mask, and count the number of cells in a field of view (Fig. 2 (D)).

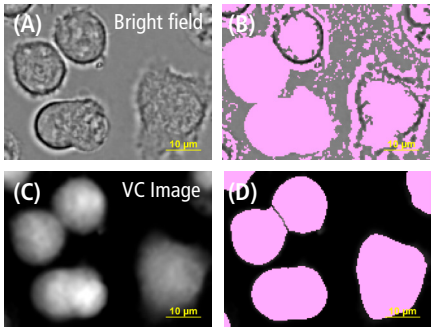


Fig 2. : Binarization
(A): Brightfield image
(B): Mask image created by binarizing the brightfield image by an intensity value.
(C): Volume Contrast (VC) image.
(D): Mask image created by binarizing the VC image by an intensity value.

Activation of macrophages with LPS

Lipopolysaccharide (LPS), a bacterial cell wall polysaccharide, binds to Toll-like receptor 4 on the cell surface of macrophages, and activates macrophages to produce the proinflammatory cytokines IL-6 and TNF-α, triggering an inflammatory response. In addition, LPS-stimulated macrophages exhibit a proinflammatory morphology (M1 macrophages) and produce ROS in mitochondria to phagocytose and decompose pathogens and dead cells. Since an excessive inflammatory response causes the onset of many diseases, anti-inflammatory drugs are being screened using ROS as an indicator. In this experiment, we analyzed the inflammatory response induced by stimulation of macrophages with LPS using ROS as an index.

Advantages of high-content live cell imaging

LPS stimulation increased the cell area per cell (Fig. 3 (D, F, H)). The number of cells increased in control wells without LPS (Fig. 3 (A, B, E, G)). On the other hand, in wells with higher LPS concentrations of 500 ng/mL or higher, the number of cells did not increase until 24 hours after the start of imaging (Fig. 3 (E)). Observation of time-lapse images revealed an increase in cell number due to cell division and a decrease in cell number due to phagocytosis of dead cells by macrophages (Fig. 4). Thus, time-lapse images provide information about real cell changes that cannot be obtained from numerical data alone. High-content live cell imaging provides a wealth of information about changes in cell morphology, behavior, and cell-to-cell communication.

Results

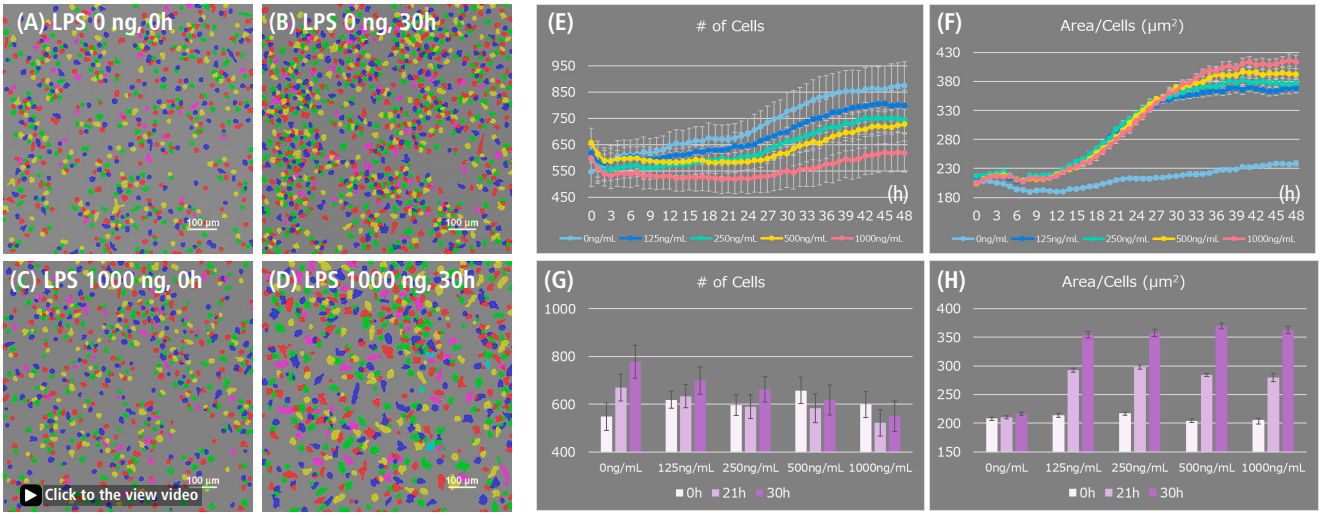


Fig. 3: Changes in number of cells and cell area per cell after addition of LPS
(A-D): Cell masks overlaid on the brightfield image, Scale bar: 100 µm, (A, B): LPS 0 ng/mL, (C, D): LPS 1000 ng/mL, 0h (A, C) and 30h (B, D) after the start of imaging, (E-F): Changes in number of cells and cell area per cell at 1-hour intervals, (G-H): Changes in number of cells and cell area per cell at 0h, 21h, and 30h after the start of imaging

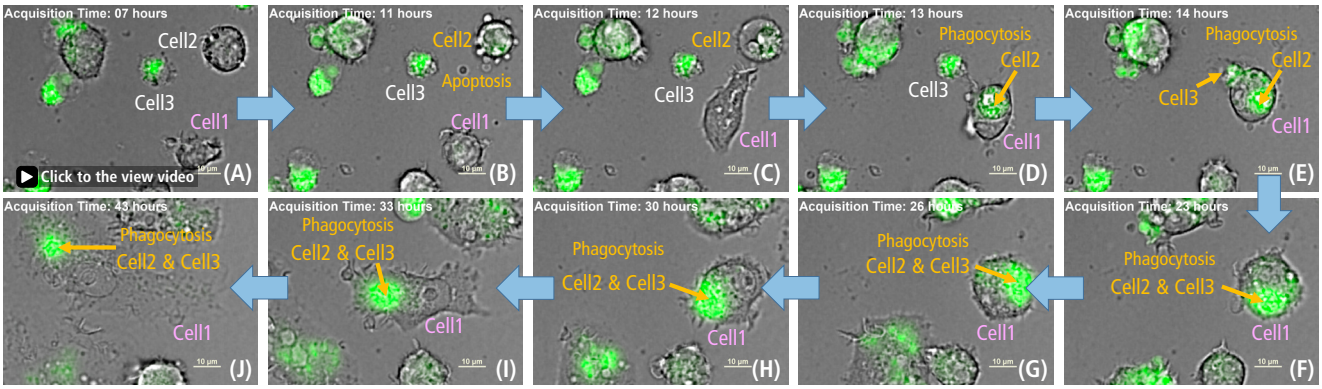


Fig. 4: Phagocytosis of dead cells by activated macrophages
(A)-(J): Time-lapse images of wells to which LPS 500 ng/mL was added (brightfield and fluorescence overlaid images), Green: ROS, Scale bar: 10 µm.
Cell2 underwent apoptosis (B) and exhibited dead cell morphology after 1 hour (C). Cell1 phagocytosed dead Cell2 and Cell3, resulting in increased ROS (C-F).

Results

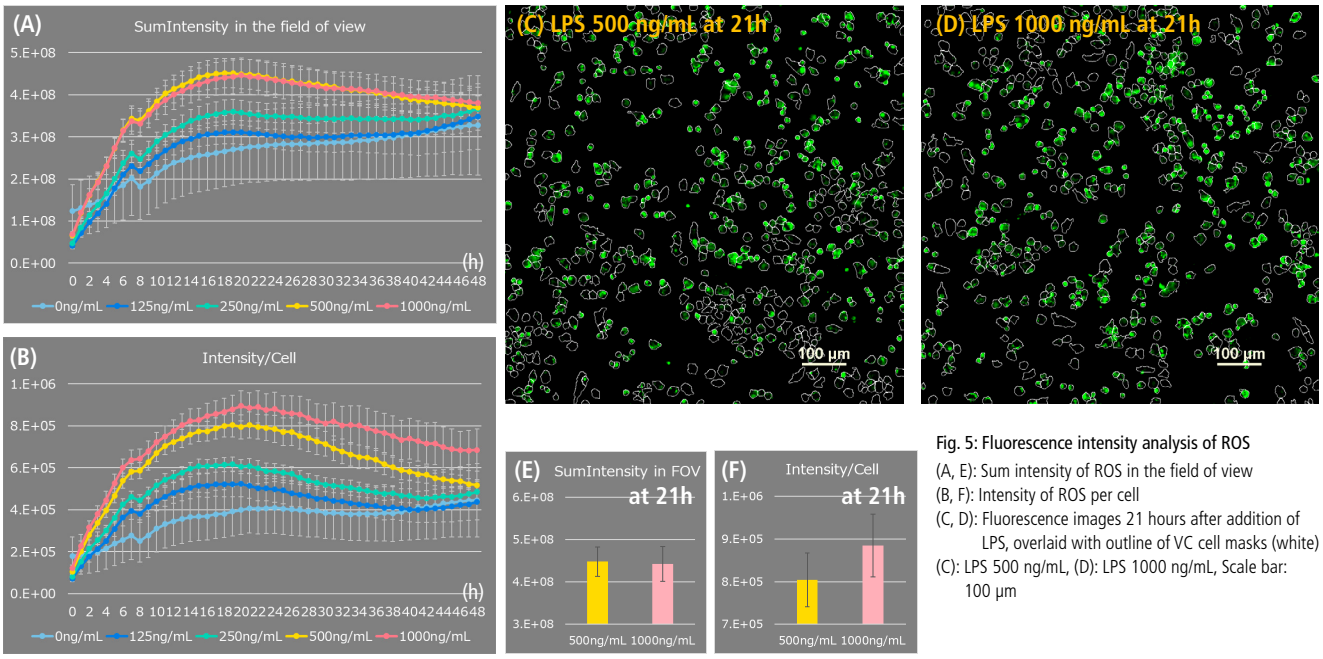


Fig. 5: Fluorescence intensity analysis of ROS
(A, E): Sum intensity of ROS in the field of view
(B, F): Intensity of ROS per cell
(C, D): Fluorescence images 21 hours after addition of LPS, overlaid with outline of VC cell masks (white)
(C): LPS 500 ng/mL, (D): LPS 1000 ng/mL, Scale bar: 100 µm

Normalization of fluorescence intensity by cell number

Cell proliferation was suppressed in a LPS concentration-dependent manner (Fig. 3 (E)). Analysis of fluorescence intensity over the entire field of view showed the same fluorescence intensity for LPS at 500 ng/mL and 1000 ng/mL, but an accurate comparison cannot be made by simply analyzing the fluorescence intensity over the entire field of view, due to the difference in the number of cells (Fig. 5 (A, E)). When the number of cells in the entire field of view was measured and the ROS fluorescence intensity per cell was calculated, the fluorescence intensity of LPS at 1,000 ng/mL 21 hours after the start of imaging was approximately 1.1 times that of 500 ng/mL (Fig. 5 (B, F)). Label-free cell counts from Volume Contrast images allowed us to quantify changes in ROS per cell in activated macrophages over time.

Low phototoxicity using weak fluorescent excitation

ROS were detected with weak excitation light using a high-NA CFI Plan Apochromat Lambda D 20X objective (NA 0.8) and a DS-Qi2 high-sensitivity monochrome CMOS camera. This reduced phototoxicity allowed the cells to proliferate even up to 30 hours after the start of imaging (Fig. 3 (E, G)). In addition, ROS could be detected without a decrease in fluorescence intensity even at the 48-hour time point captured at 1-hour intervals (Fig. 5 (A, B)).

Analysis using time-lapse imaging

The timing of drug-induced changes cannot be predicted in advance. In automated time-lapse imaging, the captured time-lapse images can be used to learn when changes occur in the cell and how the cell behaves at that time.

Photo-oxidation Resistant ROS detection reagent

With conventional ROS detection reagents, highly reproducible experiments were difficult to carry out due to auto-oxidation caused by excitation light. The ROS detection reagent from DOJINDO LABORATORIES used in this experiment is a reagent whose dyes exhibit reduced auto-oxidation due to excitation light. Fluorescence was detected specifically in the area where ROS was produced in the cell, and background noise was low, enabling highly accurate analysis. In addition, the intracellular residence of the dye is high, and high-intensity fluorescence was detected even in images taken 48 hours after the start of imaging, enabling analysis of ROS over a long period of time.

Summary

- ✓ Label-free cell counting from Volume Contrast images allows quantitative analysis of live cells with reduced phototoxicity.
- ✓ By comparing fluorescence intensity per cell, small differences in drug efficacy can be quantified and analyzed with high accuracy.
- ✓ Analysis of ROS production by activated macrophages over time allows quantification of inflammation.
- ✓ Time-lapse imaging enables analysis of changes at multiple drug treatment times from a single well plate.
- ✓ This live cell assay can improve experimental efficiency and reduce costs because images are automatically captured after imaging begins.
- ✓ Live-cell high-content imaging can provide a wealth of information, including the actual changes and dynamics of cells, that cannot be obtained from numerical data alone.

Acknowledgments

We would like to express our sincere gratitude to everyone at Dojindo Laboratories for their cooperation in establishing the protocol for ROS staining conditions.

Product information

DOJINDO

Photo-oxidation Resistant ROS Detection Kit

(Dojindo Laboratories, Co. Ltd.) https://www.dojindo.co.jp/

This product is more sensitive than conventional DCFH-DA, and does not leak out of the cell because it covalently binds to the proteins after intracellular uptake. In addition, auto-oxidation by excitation light is suppressed, enabling ROS detection over time.

Nikon

Volume Contrast analysis module of NIS-Elements

This add-on module allows NIS-Elements imaging software to construct fluorescence-like phase distribution images from brightfield images captured at multiple z-depths. Label-free quantitative phase analysis is possible without the need for special optical accessories.