APPLICATION NOTE

**High content analysis (HCA) microscope system**

# Hepatotoxicity test of drug-induced lipidosis using high-content imaging

Drug-induced lipidosis is a cytotoxicity that causes inflammation and dysfunction due to the excessive accumulation of lipids in tissues and organs. Non-alcoholic steatohepatitis (NASH) also accumulates excess lipid droplets in the liver and progresses to cirrhosis and liver cancer. Therefore, lipid droplet analysis is being studied in a wide range of fields, including safety tests that screen for toxicity of drug candidate substances and the development of drugs and supplements that inhibit the accumulation of lipid droplets. In this application note, we introduce an example where accumulation of lipid droplets was analyzed by measuring the number, area and fluorescent intensity of lipid droplets from microscopic images using the lipid droplet staining fluorescent dye Lipi-Green and human hepatoma cell lines (HepG2 cells).

## Observation devices/Software

- Microscope: Ti2-E
- Monochrome camera: DS-Qi2
- Fluorescent LED illumination system: D-LEDI
- Objective: CFI Plan Apochromat Lambda D 20X

■ Image Analysis Software
- NIS-Elements AR

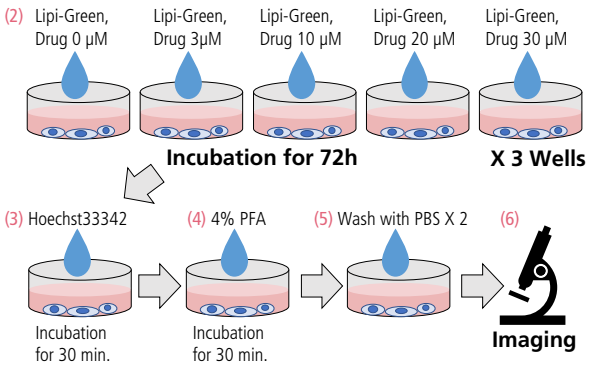
■ Option modules
- NIS-A Bundle HC W/RDB
- General Analysis 3 (GA3)
- NIS-A EDF module

## Cells/Reagents/Materials

- Human hepatoma cell line, HepG2 (JCRB Cell Bank, JCRB1054)
- Lipi-Green (Dojindo Laboratories, LD02)
- *-Cellstain*®-Hoechst 33342 solution (Dojindo Laboratories, H342)
- (±)-Propranolol hydrochloride (SIGMA-ALDRICH, P0884-1G)
- EZVIEW®CulturePlateLB (AGCTECHNO GLASS, 5866-096)
- Cellmatrix® Type I -C (Nitta Gelatin Inc.)

## Experiment overview

(1) Seed HepG2 cells at 10,000 cells/well on a collagen-coated 96-well glass bottom plate and culture at 37 ˚C for 24 hours in a 5% $CO_2$ incubator.
(2) After changing the medium, add Lipi-Green 0.5 µmol/l and 0 µM, 3 µM, 10 µM, 20 µM and 30 µM of propranolol in 3 wells each, and culture the cells at 37 ˚C for 72 hours in the 5% $CO_2$ incubator.
(3) 72 hours after drug addition, add Hoechst 33342 to the wells and incubate at 37 ˚C for 30 minutes in the 5% $CO_2$ incubator.
(4) Remove the supernatant, add 4% PFA to the wells and incubate for 30 minutes at room temperature.
(5) Remove the supernatant and wash the cells twice with PBS.
(6) Place the well plate of fixed cells on the microscope stage and acquire images using the JOB Wizard.
(7) Create an image analysis recipe with the GA3 module of NIS-Elements and export the data as a CSV file. Analyze the data using Microsoft Excel®.



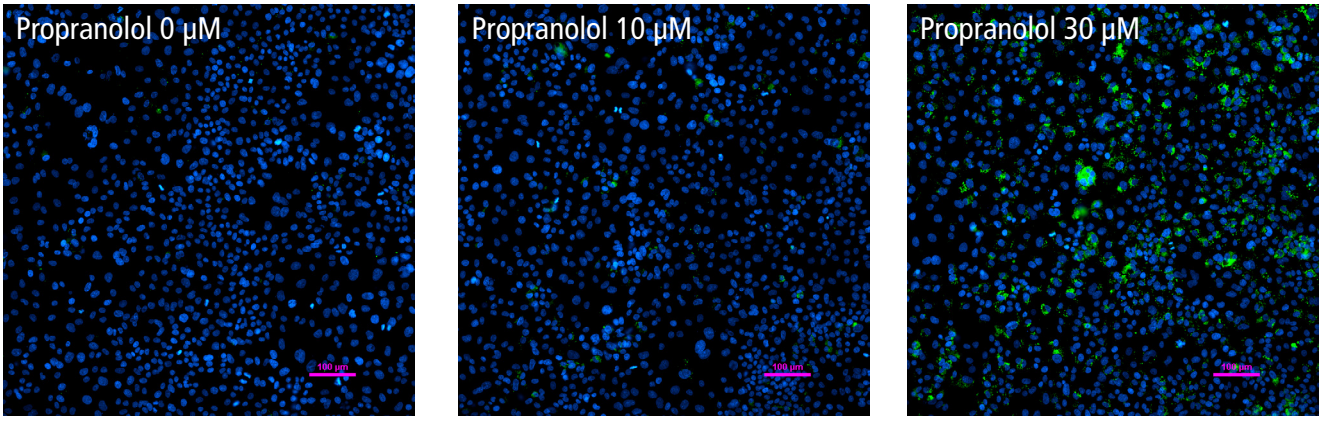




Fig.1 Merged fluorescent stained images of nuclei (blue: Hoechst 33342) and lipid droplets (green: Lipi-Green).
Propranolol concentration (from left): 0 µM, 10 µM, 30 µM
The images show that the lipid droplets increased depending on the propranolol dose.

Objective: 20X
Scale bar: 100 µm

## Image acquisition

Using a 20x objective and DS-Qi2 camera with a large FOV of 25 mm, a wide area of 0.89 mm x 0.89 mm (Fig.3 (A)) was acquired in each shot at 4 locations in each well (3.17 $mm^2$ in total). Z-stack images in a Z range of 6 µm (0.5 µm x 13 steps) were acquired with a brightfield and two wavelengths of fluorescence (blue and green) (Fig. 2. (A)).

| | Brightfield | Hoechst 33342 (fluorescence: blue) | Lipi-Green (fluorescence: green) |
|---|---|---|---|
| Light Source | Diascopic LED | D-LEDI (Power: 30%) | D-LEDI (Power: 35%) |
| Ex/Em (nm) | — | 385/460 nm | 475/535 nm |
| Exposure | 2 ms | 30 ms | 30 ms |

Table1. Image acquisition conditions

## Image analysis

Although the lipid droplets are not located within the same focal plane, by generating an EDF (Extended Depth of Focus) image, an image in which all the lipid droplets are focused can be obtained (Fig. 2 B). By binarizing this image, mask images of nuclei and lipid droplets can be created (Fig. 2 C). The number of cells was measured from the nucleus mask, and the number, area, and fluorescence intensity of lipid droplets per cell were measured from the lipid droplet mask (Fig. 4).

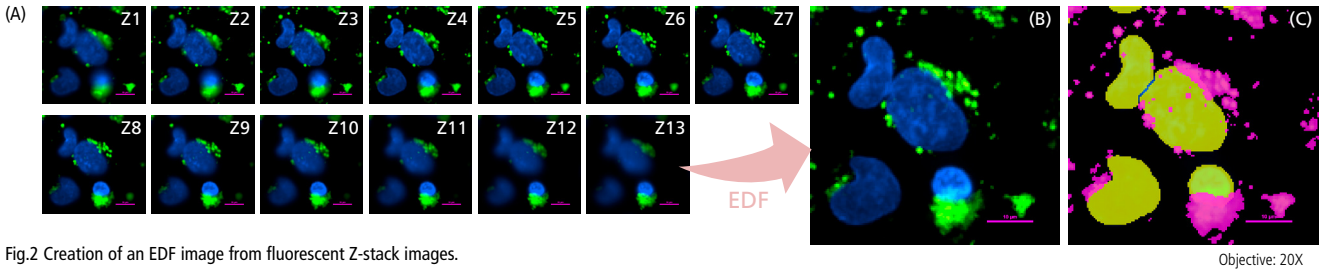
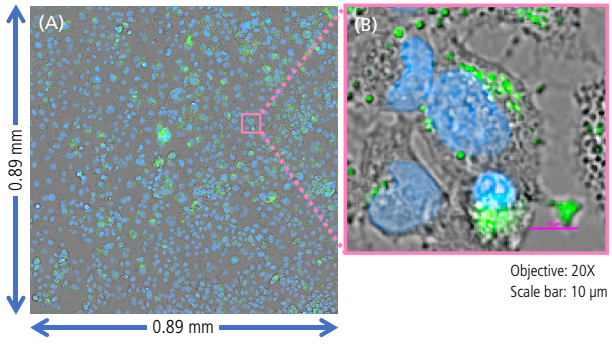



Fig.2 Creation of an EDF image from fluorescent Z-stack images.
(A) Z-stack images: 0.5 µm x 13 Steps (Z range: 6 µm), blue: nuclei, green: lipid droplets (triglyceride)
(B) An EDF image was generated from Z stack images using the EDF module of NIS-Elements
(C) Mask image acquired by binarizing the EDF image using the General Analysis 3 module. Yellow: nucleus mask, pink: lipid droplets mask
Objective: 20X
Scale bar: 10 µm




Objective: 20X
Scale bar: 10 µm

Fig.3 Merged brightfield and fluorescence images (blue: nuclei, green: lipid droplets)
(A) Imaging range in one shot with DS-Qi2 camera and 20X objective
(B) Enlarged image of (A)

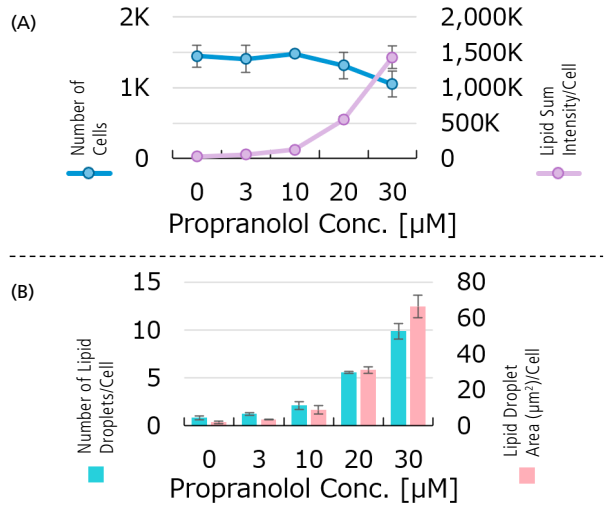



Fig.4 Analysis results at each concentration of propranolol
(A) Number of cells (blue, left Y-axis), total lipid intensity/cell (purple, right Y-axis)
(B) Number of lipid droplets/cell (green, left Y-axis), area of lipid droplets/cell (pink, right Y-axis)
At a concentration of 30 µM, the number of cells decreased and the fluorescence intensity of the lipid droplets increased significantly. The number and area of lipid droplets increased depending on the concentration of propranolol. n=3, mean (SD)

## Summary

- At a propranolol concentration of 20 µM or higher, lipid droplets were markedly formed and accumulation of lipids was induced by the drug (Fig.4).
- Even small lipid droplets with a diameter of 1 µm or less that are not located in the same focal plane can be quantitatively analyzed by constructing an EDF image from the Z stack images (Fig.2).
- The DS-Qi2 high-definition camera with a large field of view of 25 mm captures cell regions over a wide area in each shot, enabling quantitative analysis using highly reliable statistical data (Fig.1, Fig.3).


## Acknowledgments

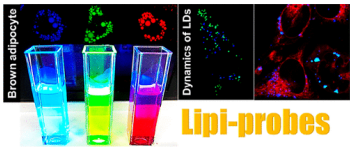
Nikon Corporation wishes to express its sincere gratitude to everyone at Dojindo Laboratories for their cooperation in establishing the lipid staining condition protocol using Lipi-Green.


## Product information

### Lipi-Green lipid droplet probes

**(Dojindo Laboratories, Co. Ltd.)** **https://dojindo.com/**

The Lipi series is low-molecular fluorescent reagent with high lipophilicity that enhances fluorescence in a hydrophobic environment. Lipid droplets in living cells and fixed cells can be clearly observed simply by adding a reagent.




### High Content Analysis (HCA) microscope system

The High Content Analysis (HCA) option of the NIS-Elements imaging software is installed in the Ti2-E microscope and camera combination. Quick and easy execution, from image acquisition to analysis, is possible with a single system.