Shedding New Light On **MICROSCOPY**

APPLICATION NOTE

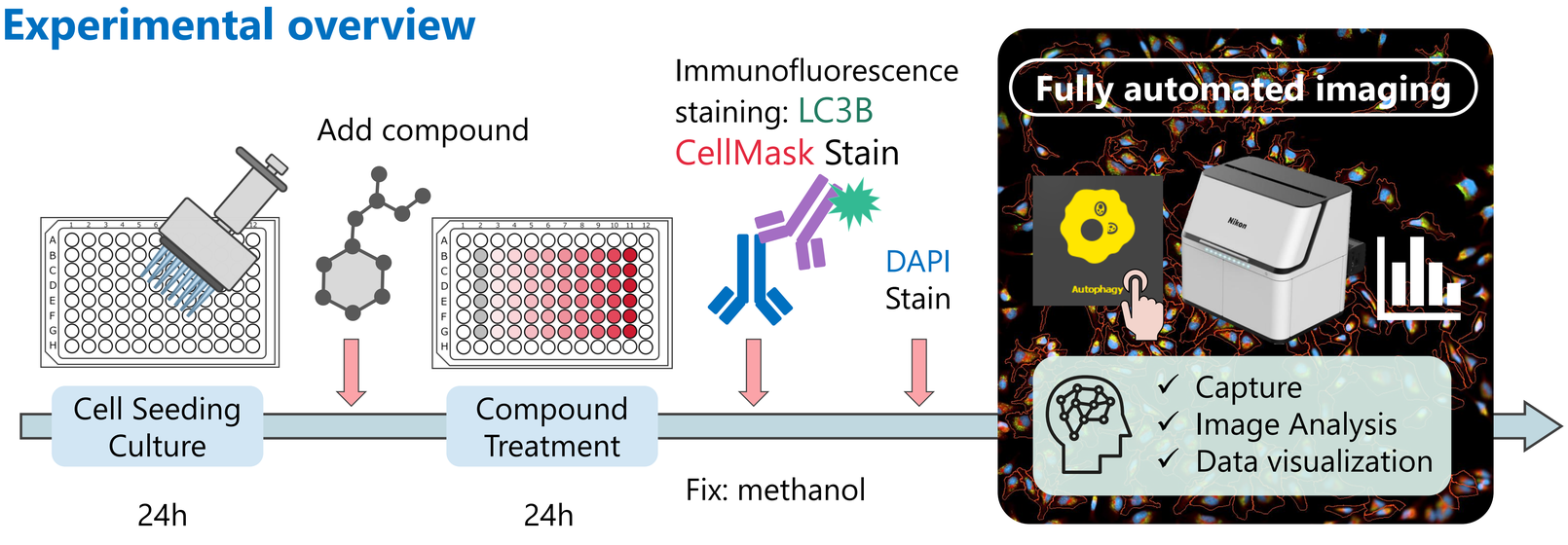
Smart Imaging System ECLIPSE Ji
Imaging Software NIS-Elements SE
Autophagy (Option)

Quantitative analysis of autophagy activity using AI-driven fully automated ECLIPSE Ji

ECLIPSE Ji with Smart Experiment software enables seamless operation from image acquisition to analysis and graph creation. Pre-trained Artificial Intelligence (AI) and pre-defined imaging procedures automatically optimize image acquisition and analysis condition settings, providing visualized data and EC_{50} information with simple operation. Autophagy is a system that maintains cells in a normal state by degrading intracellular proteins and organelles by lysosomes. The maintenance of homeostasis by autophagy has been shown to be involved in the prevention and control of various diseases such as cancer, neurological diseases, diabetes, heart failure, and aging, and this research field is attracting attention in the pharmaceutical and cosmetic industries. This application note introduces an example of using the Autophagy module of Smart Experiment to quantify the activity of drug-induced autophagy by measuring the fluorescence intensity of LC3B-derived immunofluorescence stained autophagosomes that have accumulated in cells.

Keywords: autophagy, autophagosome, drug discovery, cosmetic development, automatic setting, EC_{50}, dose-response curve

Experimental overview



- **Key features**
  - ✓ Fully automated from image acquisition to analysis and graphing
  - ✓ Quantify autophagosomes
  - ✓ Easily quantify drug response
  - ✓ Automatically create dose-response curve
  - ✓ Automatically calculate EC_{50} /IC_{50}
  - ✓ Automatically calculate Z'-factor

(1) HeLa cells were seeded in 96-well plates and cultured for 24 hours. (2) The test substance Chloroquine was diluted to 10 different concentrations and added to each well and treated for 24 hours. (3) Cells were fixed with methanol. Membrane permeabilization was performed with 0.1% Triton X-100. (4) Immunofluorescence staining was done with Anti-LC3B antibody (primary) and Goat Anti-Rabbit IgG H&L (Alexa Fluor® 488)(secondary). Plasma membranes were stained with CellMask™ Deep Red and nuclei with DAPI. (5) The well plate was placed on ECLIPSE Ji and image acquisition and analysis was run automatically by selecting the Autophagy icon.

Detection region	Fluorescence label	Ex/Em (nm)
Nucleus of all cells	DAPI	345/455
Cell region	CellMask™ Deep Red	649/666
Autophagosomes (LC3B)	Anti-LC3B antibody (primary), Goat Anti-Rabbit IgG H&L (Alexa Fluor® 488) (secondary)	495/519
Magnification	**Field of view**	
20X	0.88 x 0.88 mm	

Table. 1: Detection regions, fluorescence labels, and image acquisition conditions

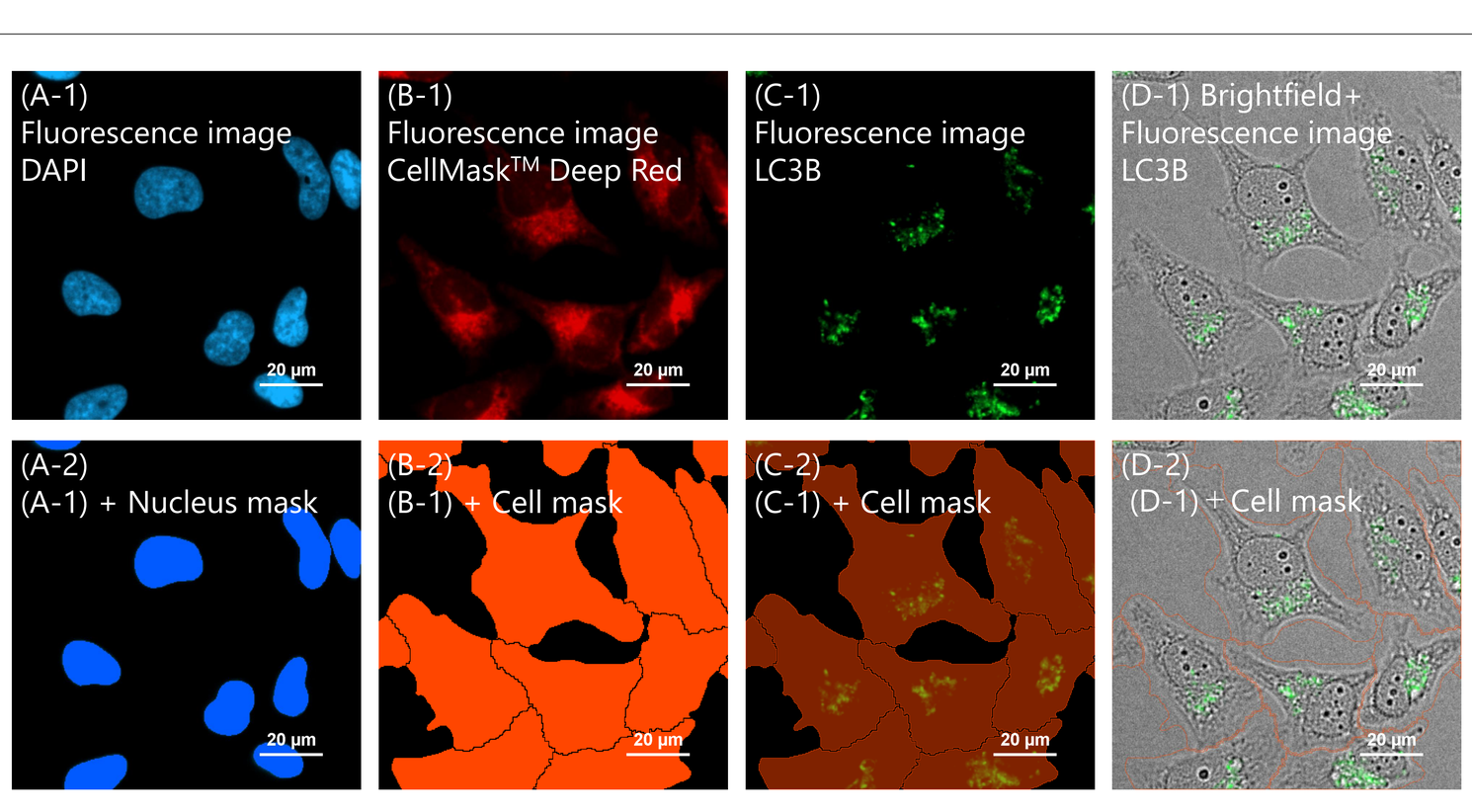


Fig. 1: Binarization and fluorescence intensity measurement of autophagosomes
Nucleus region (A-1) detected by DAPI is binarized to create a nucleus mask (A-2). A cell mask was created (B-2) using a fluorescence image (B-1) stained with CellMask™ Deep Red. Autophagosomes are quantified by measuring the intensity of immunofluorescence-stained LC3B-derived fluorescence in the region of the cell mask (C-1).
Scale bar: 20 µm

Results

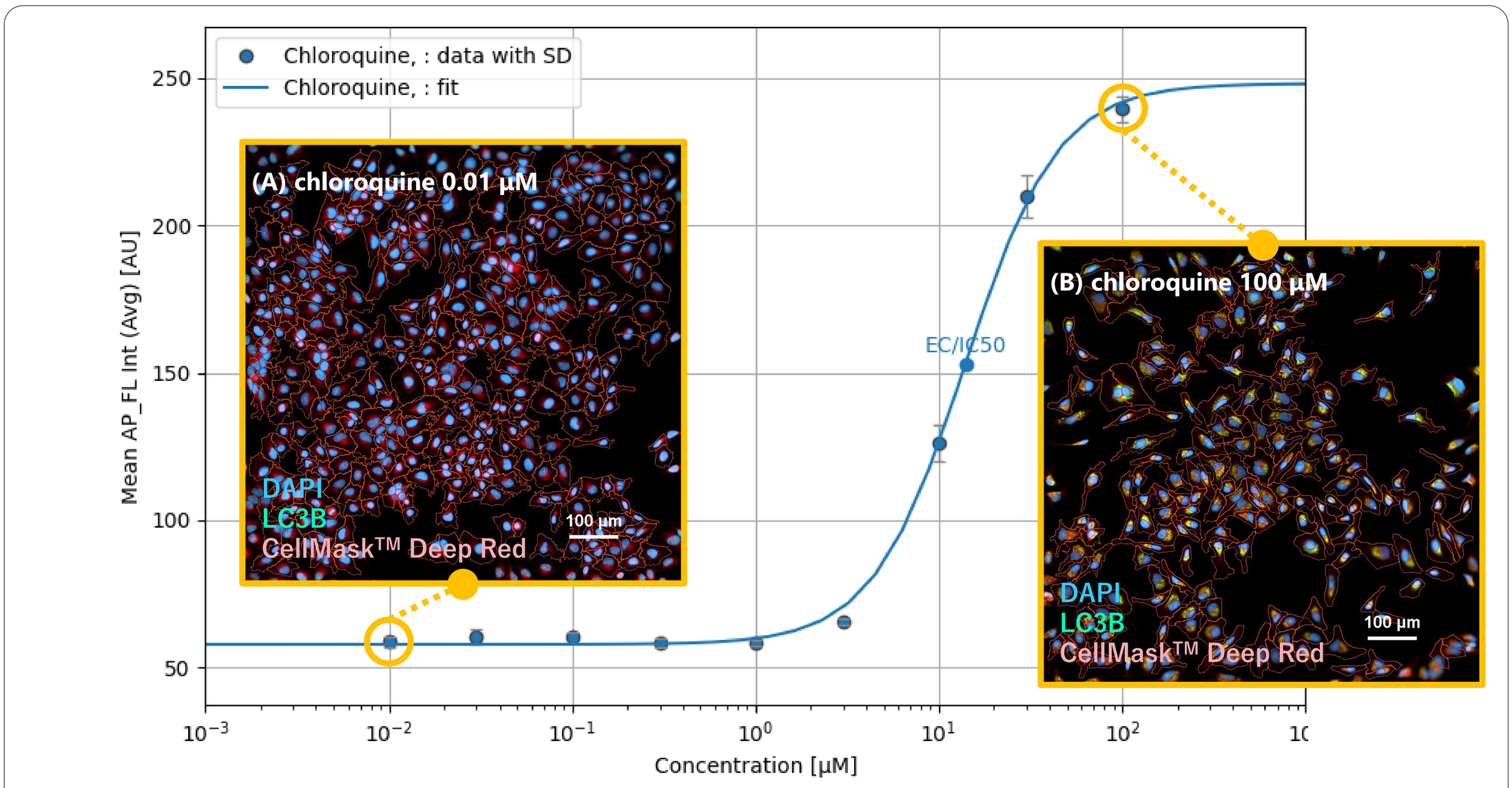


Fig. 2: Images and dose-response curve of HeLa cells treated with chloroquine for 24 hours.
(A) chloroquine 0.01 µM, (B) chloroquine 100 µM, (A, B) fluorescence image with mask outline overlaid on fluorescence image; Fluorescence image: DAPI (blue), LC3B immunofluorescence stain (green), CellMask™ Deep Red (red); Masks: nuclear mask (blue), cell mask (red); Scale bar: 100 µm. A dose-response curve showing the results of a chloroquine dose-dependent increase in the mean fluorescence intensity of LC3B (autophagosome). X-axis: drug concentration (log), Y-axis: Y-axis: mean fluorescence intensity of autophagosome in the cell masked region, EC_{50} = 13.958µM, Z'-factor = 0.5347

Summary

- ✓ The mean fluorescence intensity derived from LC3B (autophagosome) in the cell mask region detected by fluorescence could be measured.
- ✓ The dose-dependent increase in LC3B-derived fluorescence intensity with chloroquine could be confirmed.
- ✓ Smart Experiment can be run fully automatically from image acquisition to analysis and graph creation.
- ✓ A dose-response curve is automatically created and the IC_{50} can be calculated.
- ✓ This procedure was divided into a few simple steps: place the well plate on Ji, select the Autophagy icon, and input the sample information. Under the conditions of this experiment, it took approximately 20 minutes from the start of imaging to the graph display.
- ✓ A pretrained "CellFinder.ai" finds the optimal focal plane - there is no need to set tedious autofocus.
- ✓ Researchers can concentrate on other research activities by leaving tedious tasks to AI.

Sample preparation protocol

1) HeLa cells are seeded in a 96-well plate at a density of 4.5x10e3 cells/well and culture for 24 hours at 37℃ in a 5% CO_2 incubator.
2) Chloroquine is diluted with medium to concentrations of 0, 0.01, 0.03, 0.1, 0.3, 1, 3, 10, 30, and 100 µM, and each concentration of medium is added to each of the 6 wells. Cells are treated with chloroquine for 24 hours at 37° C in a 5% CO_2 incubator.
3) Cells are washed once with PBS.
4) Ice-cold methanol is added to the wells and left at 4C for 5 minutes to fix the cells.
5) Cells are washed 3 times with PBS.
6) 0.1% Triton X-100 is added in PBS to the wells and left at room temperature for 5 minutes for membrane permeabilization.
7) Cells are washed once with PBS.
8) Add blocking solution (1% BSA, 10% normal goat serum, 0.3 M glycine, 0.1% Tween-20 in PBS) to the wells and leave at room temperature for 1 hour.
9) Cells are washed with PBS.
10) Add the primary antibody (1:1000) diluted in blocking solution to the wells and leave at room temperature for 1 hour.
11) Wash cells 3 times with PBS.
12) Add secondary antibody (1:1000) diluted with blocking solution and CellMask™ Deep Red (5 µg/ml) to the wells and leave at room temperature for 1 hour.
13) Wash cells 3 times with PBS.
14) DAPI (2 µg/ml) is added to the wells and leave at room temperature for 5 minutes.
15) Wash cells 3 times with PBS.

Materials and reagents

Cell Culture	
Cell Line	HeLa (RIKEN RCB0007)
Growth medium	MEM + 10%FBS + 1%Pc/Sm
Culture vessel	EZVIEW® Culture Plate B (Glass Bottom Plate) Microplate 96 well (AGC techno glass (IWAKI), 5866-096)
Test substance	
Compound	Chloroquine
Test concentration	Negative control: 0 µM Positive control (1): 10 µM Positive control (2): 30 µM To make a dose-response curve, design required concentration points as follows: Ex: (0) 0 µM, (1) 0.01 µM, (2) 0.03 µM, (3) 0.1 µM, (4) 0.3 µM, (5) 1 µM, (6) 3 µM, (7) 10 µM, (8) 30 µM, (9) 100 µM
Plate map example	

	1	2	3	4	5	6	7	8	9	10	11	12
A	b	b	b	b	b	b	b	b	b	b	b	b
B	b	(0)	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	b
C	b	(0)	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	b
D	b	(0)	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	b
E	b	(0)	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	b
F	b	(0)	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	b
G	b	(0)	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	b
H	b	b	b	b	b	b	b	b	b	b	b	b

“b” : blank well

Reagents		
Product name	Product number	Supplier
Chloroquine	QJ-5953	Combi-Blocks
Anti-LC3B antibody [EPR18709] - Autophagosome Marker (primary antibody)	ab192890	Abcam
Goat Anti-Rabbit IgG H&L (Alexa Fluor® 488) (secondary antibody)	ab150077	Abcam
DAPI Solution	62248	Thermo Fisher Scientific
CellMask™ Deep Red Plasma Membrane Stain	C10046	Thermo Fisher Scientific, Invitrogen™
MEM (Minimum Essential Medium)	11095080	Thermo Fisher Scientific
Fetal bovine serum (FBS)	10437028	Thermo Fisher Scientific
Penicillin-Streptomycin (Pc/Sm) (10,000 U/ml)	15140122	Thermo Fisher Scientific
Methanol	134-01833	Fujifilm-Wako
Dimethyl sulfoxide (DMSO)	276855	Sigma-Aldrich

☐ Compatible vessel*

- 24-well plate
- 96-well plate

＊Compatible with only glass bottom well plates.

Reference

Ferreira, PMP, et al., Chloroquine and hydroxychloroquine in antitumor therapies based on autophagy-related mechanisms. *Pharmacol Res* (2021)

Product information

Smart Imaging System ECLIPSE Ji

ECLIPSE Ji is an AI-Driven, fully automated imaging system. By using it in combination with NIS-Elements SE, image acquisition, analysis, and graph creation can be run seamlessly and automatically. It is equipped with "CellFinder.ai“, which uses AI to find the optimal focal plane in autofocus settings that normally require advanced human judgment. Various trained AIs are implemented in the image acquisition and analysis process. This greatly reduces the number of steps for setting and optimization and makes it easier for everyone to get results.

Imaging Software NIS-Elements SE Autophagy（Option）

- ✓ Fully automated from image acquisition to analysis and graph display.
- ✓ Autophagosome fluorescence intensity in the cell region detected by fluorescence can be analyzed easily and automatically.
- ✓ One-click reports can be created and output with PDF including images, analysis results, dose-response curves, and EC_{50} /IC_{50} calculation results.
- ✓ Cellular imaging and analysis with Ji is easier and more comfortable.