Shedding New Light On **MICROSCOPY**

APPLICATION NOTE

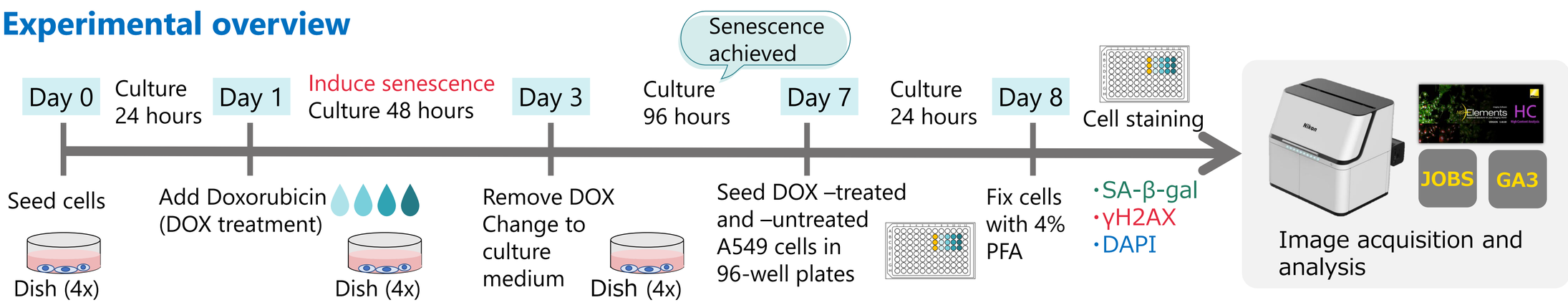
Digital inverted microscope ECLIPSE Ji
Imaging software NIS-Elements AR
Optional module NIS-A Bundle JOBS W/HC/RDB/GA3

Label-free quantitative analysis of cellular senescence and high-content imaging of granules

Have you ever struggled with coordinating multiple fluorescent dyes in a multiplexed staining experiment? Has your experiment ever been limited by the need for a cytoplasmic dye to identify cellular regions? High-content imaging with multi-target fluorescent staining provides a wealth of biologically meaningful information such as protein localization, expression levels, and morphological changes in parallel. However, detection of different fluorophores in the same fluorescence channel due to spectral crosstalk hinders quantitative image analysis. Volume Contrast (VC) is a label-free technique to identify cellular regions from brightfield images alone, bypassing cytoplasmic staining and reducing potential crosstalk. Accurate, quantitative image analysis methods enable robust measurements of cellular drug responses to achieve reliable, high-quality drug efficacy data. In this application note, we introduce an example of quantifying cellular senescence by multiplexed fluorescent staining of A549 cells with SA-β-gal (senescence marker), γH2AX (DNA damage), and DAPI (nuclei), with cell regions identified from VC images.

Keywords: Cellular senescence, SA-β-gal, DNA damage, γH2AX, granule analysis, drug discovery, high-content imaging, Volume contrast, EDF

Experimental overview



(1) A459 cells (2x10e7 cells/dish) were seeded in four 100mm dishes and cultured for 24 hours. (2) Media was changed to include test substance Doxorubicin (DOX) adjusted to 50, 100, 200, 400 nmol/l followed by a 48 hour culture period. (3) Doxorubicin-containing medium was removed and replaced with untreated culture medium, followed by a 96 hour culture period. (4) DOX-untreated and DOX-treated A549 cells (1x10e4cells/well) were seeded in 3 wells each of a 96-well plate and cultured for 24 hours. (5) Cells were fixed with 4% PFA. (6) SA-β-gal (Green) and γH2AX (Deep Red) were stained with the specified kits. Nuclei were stained with DAPI. (7) The well plate was placed on the ECLIPSE Ji digital inverted microscope and images were acquired using the HCA Fixed template protocol within NIS-Elements AR (HCA/JOBS) (Table 1). (8) Images were analyzed with a custom General Analysis 3 recipe (NIS-Elements AR). (9) Cellular data was exported to CSV and analyzed using Microsoft Excel®.

Detection region	Fluorescence label	Ex/Em (nm)
Nuclei	DAPI	345/455
SA-β-gal (senescence marker)	Cellular Senescence Detection Kit - SPiDER-βGal	500-540/ 530-570
γH2AX (DNA damage)	DNA Damage Detection Kit - γH2AX - Deep Red	646/668
Cell region	None (Construct VC image from bright-field image)	Bright-field
Magnification	**Field of view (FOV)**	
20X	0.88 x 0.88 mm / image	
# of points	**Z stacks**	
2points/well	1.825µm x 3 steps (Range:3.75µm)	

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

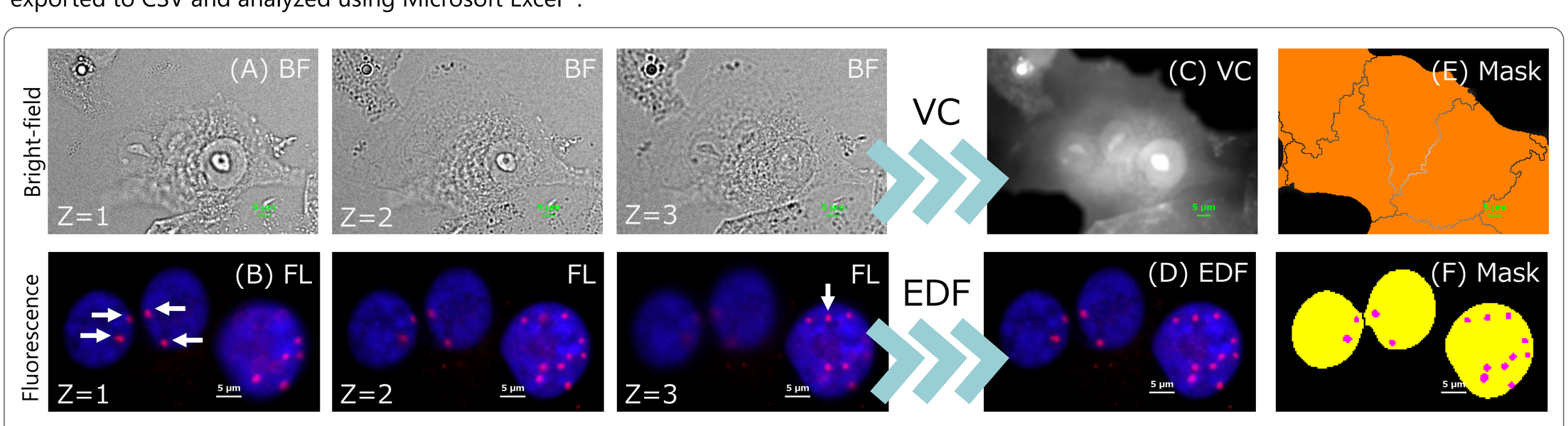


Fig. 1: Image analysis methods, Volume Contrast and EDF images
(A) Bright field image, (B) Fluorescence image, (C) Volume Contrast (VC) image, (D) Extended Depth of Focus (EDF) image, (E) Orange: cell mask, (F) yellow: nucleus mask, magenta: granule (γH2AX) mask, scale bar: 5µm. VC imaging is a technology that can construct a phase distribution image like a fluorescence image from three bright field images with different focal planes. This allows cell masks to be created with only brightfield imaging (E). One EDF image (D) can be constructed from Z-stacked images. Although the granules (white arrows) are not located in the same focal plane, one EDF image (D) can be generated in which all granules are in focus. Nuclear masks and granule (γH2AX) masks encompassing the full cell volume can be generated from EDF images (F).

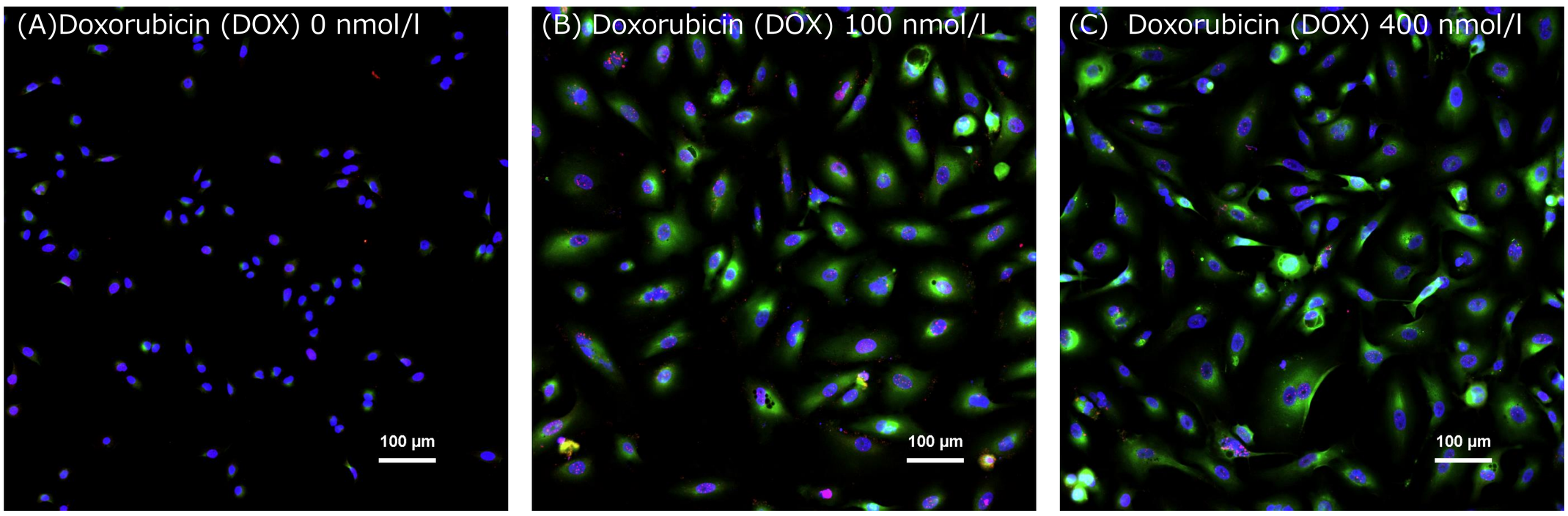
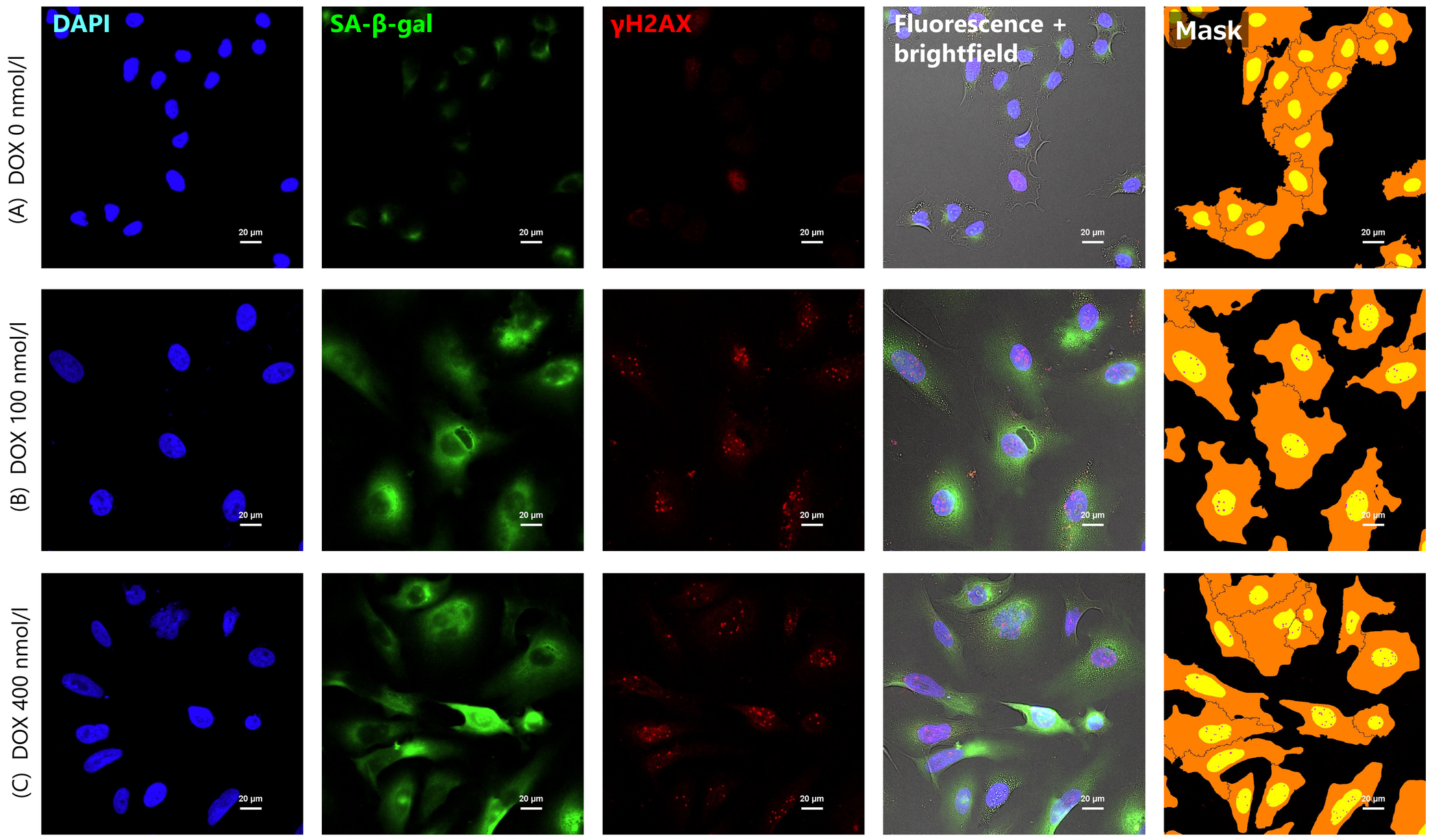


Fig. 2: Fluorescence merged image of A549 cells

Blue: DAPI (nuclei), Green: SA-β-gal (senescence marker), Magenta: γH2AX (DNA damage), Doxorubicin (DOX) treatment: (A) 0 nmol/l, (B) 100 nmol/l, (C) 400 nmol/l. Scale bar: 100µm. Doxorubicin treatment increased the fluorescence intensity signal of SA-β-gal (green), confirming cell senescence.



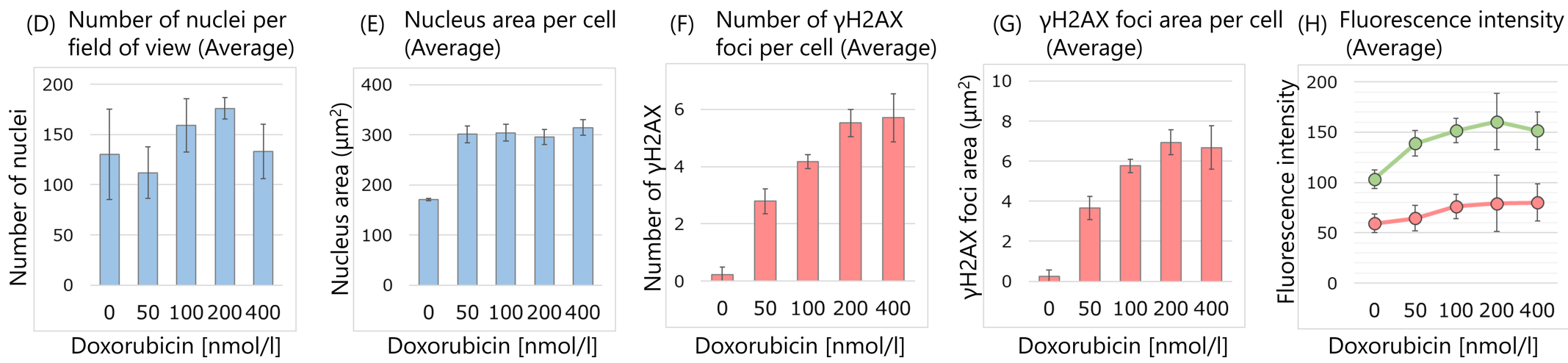
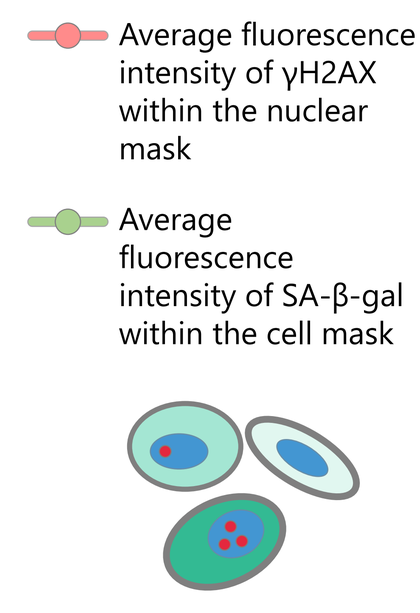


Fig. 3: Representative images and analysis results

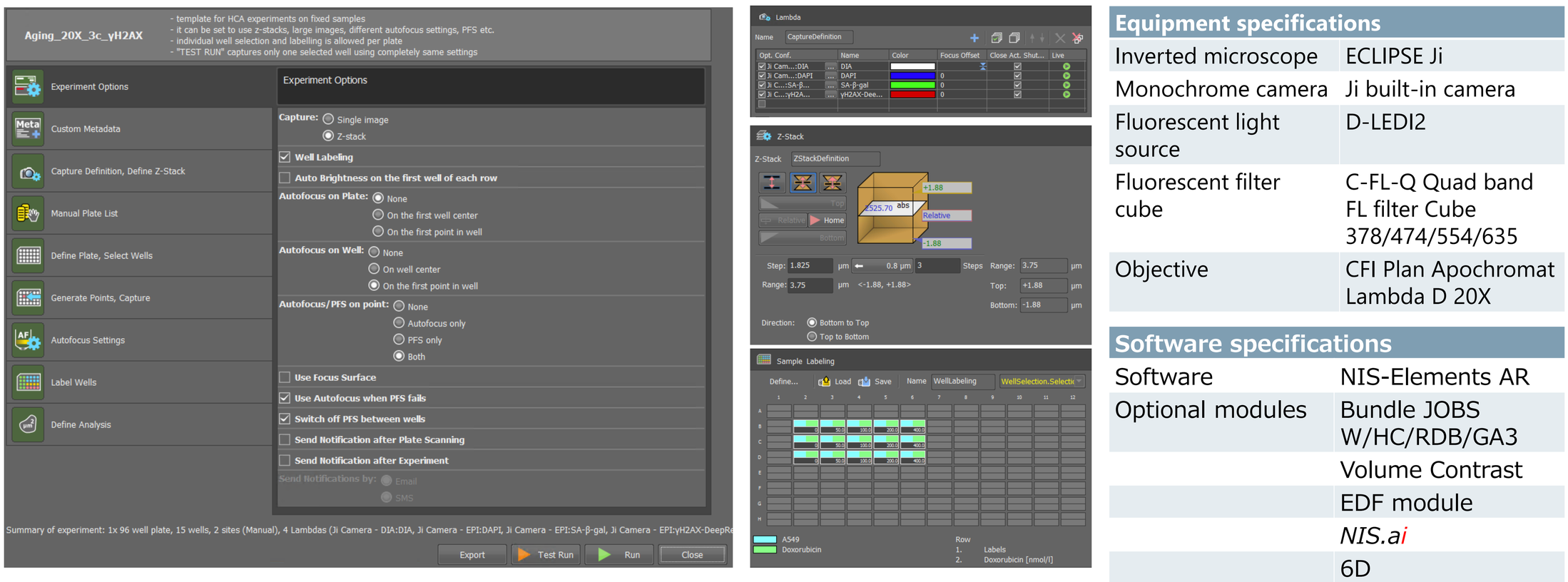
(A, B, C) Enlarged image of Figure 2, scale bar: 20 µm. Mask image on rightmost column: A nucleus mask (yellow) was created by binarizing the nucleus region of the fluorescence image detected with DAPI. The immunofluorescently stained γH2AX (red) granules in the nucleus mask region were binarized to create a γH2AX granule mask (magenta). The label-free VC image was constructed from the brightfield image to generate the cell mask (orange). Number of nuclei per FOV (D), average nucleus area (E), number of γH2AX foci per cell (F), average area of γH2AX foci per cell (G), average fluorescence intensity of γH2AX within the nucleus mask (H, pink line), The average fluorescence intensity (H, green line) of SA-β-gal within the cell mask was also measured. The area of nuclei increased approximately 1.8 times with Doxorubicin treatment. The number and area of γH2AX foci significantly increased at Doxorubicin concentrations above 200 nmol/l. On the other hand, the average fluorescence intensity per cell of SA-β-gal (senescence marker) was maximal at 200 nmol/l and still slightly elevated at 400 nmol/l. From these results, cell senescence in A549 cells was significantly confirmed at Doxorubicin concentrations of 200 nmol/l or higher.



Summary

- ✓ A549 cells had increased nucleus area and number of γH2AX granules in a Doxorubicin dose-dependent manner.
- ✓ Doxorubicin treatment induced cell senescence, indicated by elevated fluorescence intensity of γH2AX in the nucleus mask and SA-β-gal in the cellular mask.
- ✓ High-content imaging can detect morphological changes in cells and protein localization, such as nucleus enlargement and the number of granules in the nucleus region, leading to deeper biological insights.
- ✓ Label-free cell masks could be generated from Volume Contrast images without using fluorescence wavelengths, resulting in analysis with minimal crosstalk.
- ✓ One EDF image was generated in which all granules were in focus, even if the granules were not located in the same focal plane, enabling highly accurate quantitative analysis.
- ✓ Using NIS-Elements HC's wizard-based image acquisition template, images could be easily acquired even for highly customizable experiments.
- ✓ The fluorochromes used in this experiment were highly specific and the signal intensity was strong, resulting in reliable, high-quality data.
- ✓ Since SPiDER-βGal can stain SA-β-gal in living cells, it is a useful readout for optimizing experimental conditions, such as determining the time required for drug treatment to induce senescence.

Imaging equipment and software configuration



Equipment specifications	
Inverted microscope	ECLIPSE Ji
Monochrome camera	Ji built-in camera
Fluorescent light source	D-LEDI2
Fluorescent filter cube	C-FL-Q Quad band FL filter Cube 378/474/554/635
Objective	CFI Plan Apochromat Lambda D 20X

Software specifications	
Software	NIS-Elements AR
Optional modules	Bundle JOBS W/HC/RDB/GA3
	Volume Contrast
	EDF module
	NIS.ai
	6D

Fig. 4: HCA Fixed image acquisition template for NIS-Elements AR (HCA/JOBS)
NIS-Elements AR (HCA/JOBS) guides you through setting acquisition conditions using wizard-style image acquisition templates. Images can be acquired easily and with a high flexibility. The seamless workflow from image acquisition to analysis streamlines your experiments.

Materials and reagents

Cell Culture	
Cell Line	A549 Cell
Growth medium	DMEM
Culture vessel	1) SANPLATEC CORP #26502, NEST 100 mm Dish for Cell Culture 2) Ibidi #89626, µ-Plate 96 Well Square
Test substance	
Compound	Doxorubicin
Test concentration	0, 50, 100, 200, 400 nmol/l
Plate map example	1 2 3 4 5 6 7 8 9 10 11 12 A B 0 50 100 200 400 C 0 50 100 200 400 D 0 50 100 200 400 E F G H

Reagents		
Product name	Product number	Supplier
-Cellstain®- DAPI solution	D523	Dojindo Laboratories
Cellular Senescence Detection Kit - SPiDER-βGal	SG03	Dojindo Laboratories
DNA Damage Detection Kit - γH2AX - Deep Red	G267	Dojindo Laboratories

Cell staining protocol

■ SA-β-gal staining

1) Remove the cell culture supernatant, add 4% PFA solution (100 µl/well) to each well, and incubate at room temperature for 3 minutes.
2) Remove the PFA solution and wash the cells three times with PBS (100 µl/well).
3) Add SPiDER-βGal working solution (100 µl/well) to the wells and incubate at 37°C for 30 minutes. *Refer to "Assay for fixed cells" in the SG03 Instruction Manual. https://www.dojindo.com/manual/SG03/
4) After incubation, wash the cells twice with PBS (100 µl/well).

Cell staining protocol

■ DNA Damage Detection Kit - γH2AX - Deep Red

＊This is slightly different from the method described in the G267 instruction manual.

1) After SA-β-gal staining, add 0.1% Triton X-100/PBS solution (100 µl/well) and incubate at room temperature for 30 minutes.
2) Remove the supernatant and wash the cells twice with PBS (100 µl/well).
3) Add Blocking Solution (100 µl/well) and incubate for 20 minutes at room temperature.
4) Remove the supernatant and wash the cells twice with PBS (100 µl/well).
5) Add γH2AX staining solution (100 µl/well) and incubate overnight at 4 degrees.
6) Remove the supernatant and wash the cells twice with PBS (100 µl/well).
7) Add Secondary antibody staining solution (100µl/well) and incubate for 1 hour at room temperature.
8) Remove the supernatant and wash the cells twice with PBS (100 µl/well).

Acknowledgments

Sample provided by: Dojindo Laboratories, Co. Ltd.
We would like to express our sincere gratitude to everyone at Dojindo Laboratories for their cooperation in establishing the experimental conditions for senescence induction and the staining conditions protocol optimized for imaging.

Product information

Digital inverted microscope ECLIPSE Ji

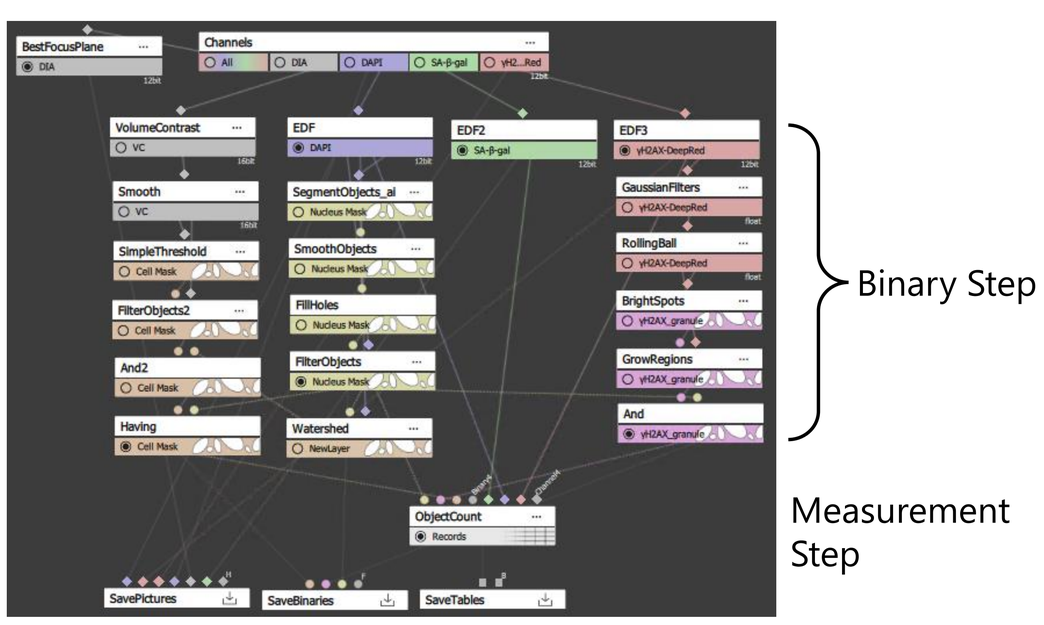
ECLIPSE Ji enables a high flexibility in experiments when used in combination with NIS-Elements AR. Flexible customization allows you to optimize your experiments to suit your individual needs.

Imaging software NIS-Elements AR

NIS-Elements AR's HCA/JOBS (Bundle JOBS) provides complete solutions for high content imaging. Streamline your experiments with a seamless workflow from image acquisition to analysis.

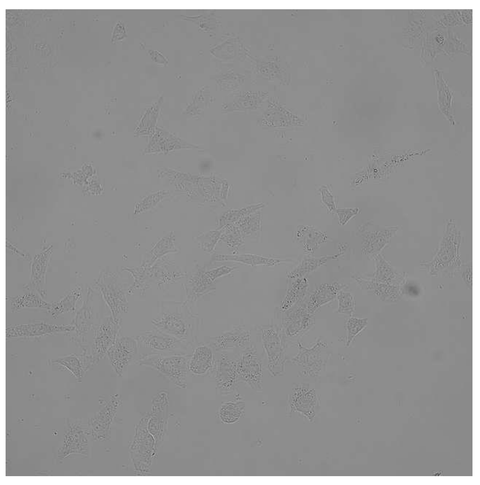
General Analysis 3, image analysis module

Flexible, customizable image analysis routines can be constructed within a graphical programming interface to obtain binary masks of cellular regions or features of interest and perform subsequent measurements.

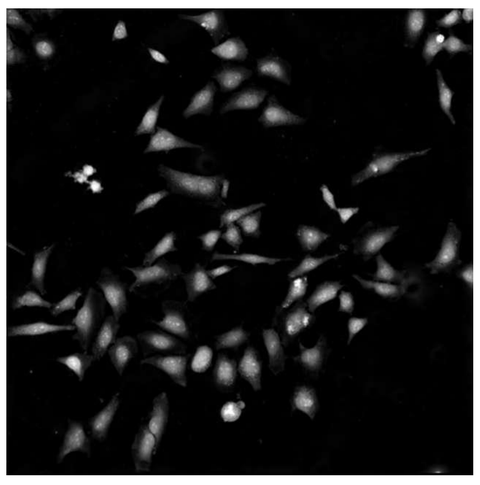


Volume Contrast (VC)

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.



Bright-field image



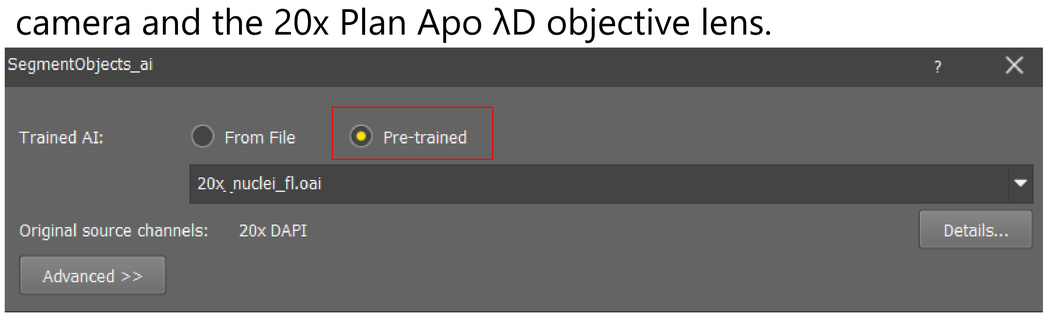
Volume Contrast image

Extended Depth of Focus (EDF)

EDF reconstruction produces one image that includes all in-focus features from an image set acquired across multiple z-depths. This technique is effective for quantitative analysis of images in which the depth of field is shallow and small structures are not located in the same focal plane.

NIS.ai (SegmentObject.ai)

SegmentObject.ai utilizes neural networks to learn classifications for targets that are difficult to extract using conventional binarization or image processing. Several pre-trained models*1 are available. Highly accurate nucleus segmentation was achieved in this experiment using one of these pre-trained models based on fluorescent images of DAPI acquired with ECLIPSE Ji's built-in camera and the 20x Plan Apo λD objective lens.



*1 NIS-Elements v6.01 or higher