APPLICATION NOTE

AX R Confocal Microscope

Automatic spheroid detection and drug efficacy analysis using AX R confocal microscope

In drug development, two-dimensionally cultured monolayer cells have been used to evaluate the efficacy of a drug, measure effective concentration, and analyze mechanisms of action, through the use of cells. However, the use of two-dimensional (2D) cells for these purposes is problematic in that the cells show a different drug response or resistance to that of the living organism, so correct evaluation is difficult. On the other hand, cellular aggregates such as spheroids are known to exhibit reactions similar to those of the organism by forming the three-dimensional (3D) structure (reference 1). For this reason, the use of spheroids for pharmacological and toxin evaluation has increased in recent years. In addition, with spheroids, characteristics and physicochemical environments vary depending on the differences in shape and structure of the outer and inner layers (reference 2), so information on positional relationships in the three-dimensional structure is also important to analyze drug efficacy. Consequently, the acquisition of clear 3D images, high throughput for imaging a large number of samples, and analysis of 3D images are essential for effective and efficient analysis of spheroids using a microscope.

The AX R confocal microscope can acquire clear 3D images having a Z sectioning effect. In addition, use of the JOBs function of NIS-Elements imaging software can achieve seamless imaging, and use of the GA3 function can provide complex 3D quantitative analysis. This application note introduces a case example from acquisition to analysis of 3D images by exposing the staurosporine, which is known to have an anticancer effect on spheroids.

Keywords: AX R, NIS-Elements, 3D analysis, spheroid, toxicity evaluation

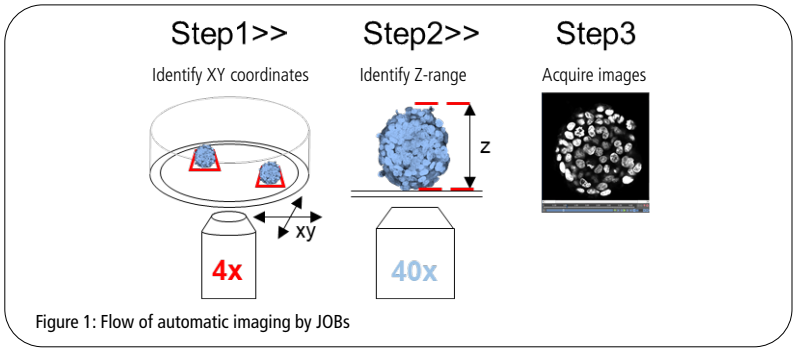

Figure 1: Flow of automatic imaging by JOBs

Materials and methods

Spheroids were formed by seeding 50 HT29 cells derived from human colon adenocarcinoma in a round-bottom 96-well plate and incubating for 3 days. The formed spheroids were transferred to a flat-bottom 96-well plate and exposed to staurosporine for 24 hours under multiple concentration conditions. Following this, nuclei and apoptotic cells were stained and images were acquired using a microscope.

An Eclipse Ti2-E inverted microscope equipped with an AX R confocal microscope system was used for imaging, and NIS-Elements AR v5.42.00 was used for microscope control and image analysis. To streamline imaging, the XY coordinates of the spheroids in each well were identified with the JOBs function of NIS-Elements using a low-magnification (4x) objective. At that coordinate, the objective was switched to the imaging magnification (40x) and the Z-range (where the fluorescent signal of the cell nuclei within a spheroid was detected) was automatically identified. 3D images of spheroids were acquired at these XY coordinates and within this Z-range. Object detection of all cell nuclei within the spheroids and fluorescence intensity measurement was performed by the GA3 function of NIS-Elements using the acquired images.

Results

Clear cell nuclear images of 3D spheroids could be acquired for each well, and individual nuclei could be isolated and detected by the GA3 function (Fig. 2).

The decrease in spheroid diameter with increasing staurosporine concentration and increase in apoptotic cells visualized in yellow were confirmed in the images acquired at different concentrations of staurosporine that were overlaid with the fluorescence images of all cell nuclei (red) and apoptosis-induced cells (green).

In addition, the detailed 3D quantitative analysis using the GA3 function showed that the cells for which apoptosis was induced increased and the cell density decreased at the concentration 0.25 µM or more (Fig. 4 (A-C)). The analysis also showed that at concentrations of 1 µM and above, the ratio of apoptotic cells decreased, as did the number of cells (Fig. 4 (D)).

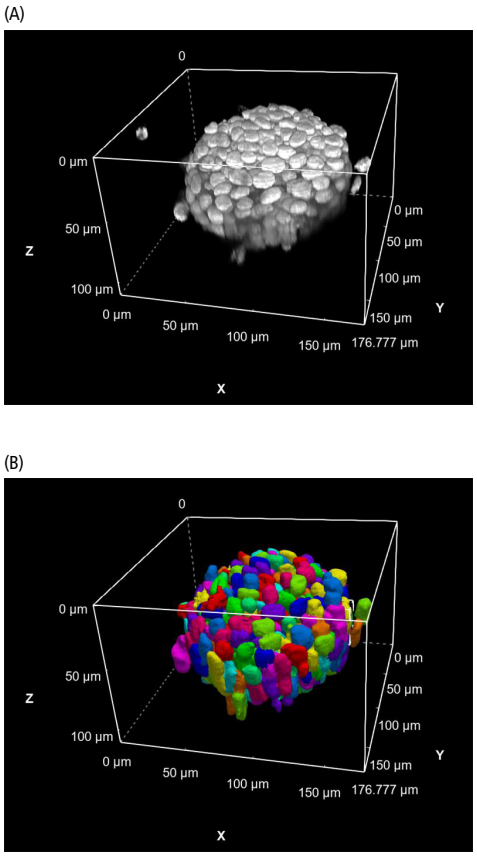

Figure 2: Images of cell nucleus of a spheroid acquired using AX R
CFI Plan Apochromat Lambda 40XC objective was used.
(A) 3D fluorescence image
(B) Cell nucleus objects detected using the GA3 function of NIS-Elements

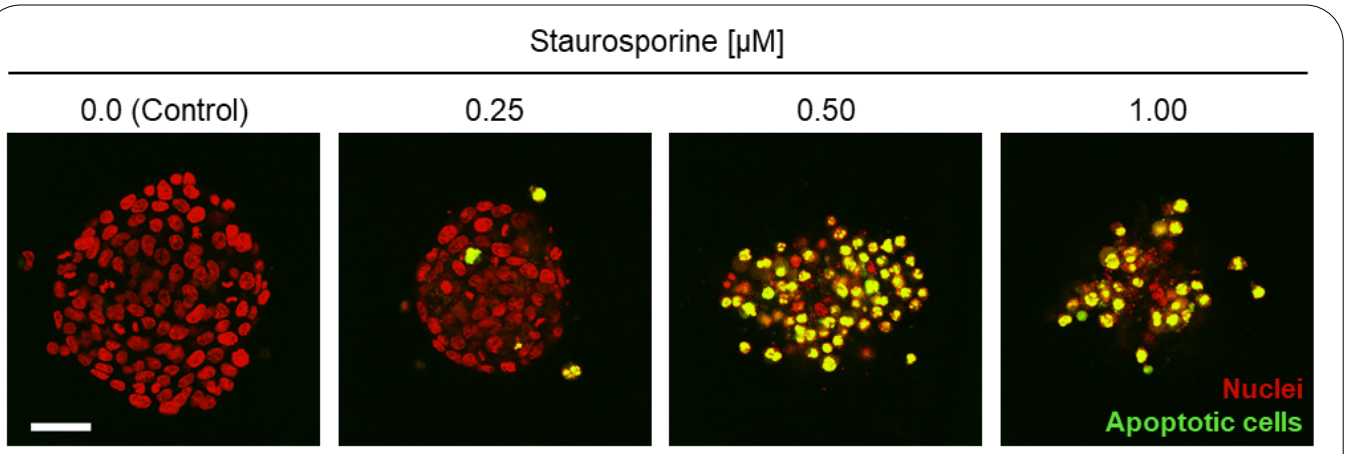

Figure 3: Confocal images of a spheroid under different exposure conditions of staurosporine
Cell nuclei are labeled in red and apoptosis-induced cells in green. Scale bar: 50 μm

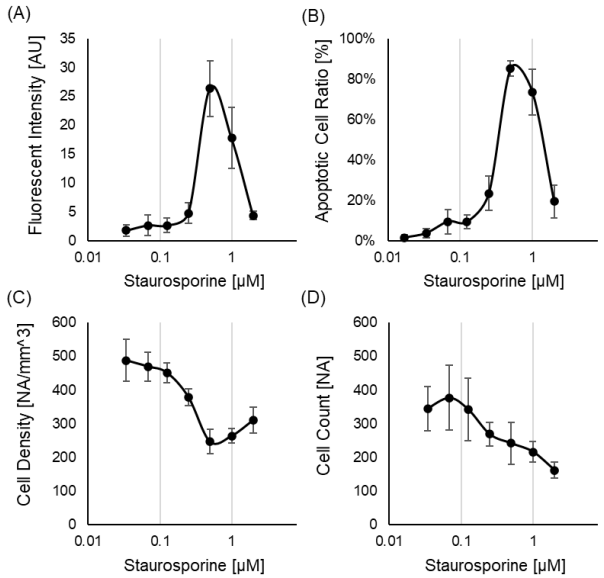

Figure 4: 3D quantitative analysis results of spheroids at each concentration point of staurosporine
All values are average ±standard deviation. n=6 at each concentration point
(A) Fluorescence intensity of apoptotic cell markers in spheroids
(B) Ratio of apoptotic cells to number of all cells in spheroids
(C) Volume of spheroids extracted based on fluorescence of cell nuclei
(D) Number of cell nuclei included with spheroids

Discussions / Summary

The nucleus of each spheroid could be clearly detected by the GA3 function using the images acquired by the AX R confocal microscope. This enables various quantitative analyses according to the purpose, such as volume and short/long diameter in addition to fluorescence intensity and cell count. The combination of AX R and JOBs has enabled fully automated acquisition of an entire well plate from the start to completion of imaging. In addition, this automation can avoid the possibility of overlooking some of the spheroids, even if there are multiple spheroids in a well. Although manual detection or image acquisition of the entire vessel area has been conventionally required, throughput can now be considerably improved.

This time, the apoptosis-inducing effect due to staurosporine and the decrease in cell density could be quantitatively indicated. Figure 4 (A-B) shows concentration dependent changes, suggesting that an accurate response was obtained, in the same way as with many compounds. In addition, it is considered that the increase in dead cells due to excessive exposure to staurosporine at the concentration of 1 μM or more was confirmed as a decrease in apoptotic cells and the total number of cells. On the other hand, there was no tendency suggesting that there was a difference in the effect between the outer layer and inner layer of the spheroid. Since the ratio of apoptotic cells increased significantly between the concentration range of 0.25 μM to 0.50 μM, quantitative analysis at concentration points in this range may clearly reveal tendency. One of the benefits of having detailed quantification results is the ability to gain insights like these and expand on them with your next experiment.

Using 3D samples such as spheroids and organoids is expected to expand in drug discovery research for cancer therapeutic drugs, etc. from the viewpoint of resolving problems caused by using 2D cultured cells and reducing the number of animal experiments (reference 3). It is expected that the efficient acquisition of high-resolution 3D images using the AX R confocal microscope and JOBs, which can construct a flexible imaging sequence, and various image analyses using GA3, will make a considerable contribution to drug discovery research.

References

1. B. Calpe and W. J. Kovacs, "High-throughput screening in multicellular spheroids for target discovery in the tumor microenvironment". *Expert Opinion on Drug Discovery*. Vol. 15(8). (2020).
2. Y. Feng and R. M. Eglen, "Three-dimensional cell cultures in drug discovery and development". *SLAS Discovery*. Vol. 22 (5). (2017).
3. V. Brancato et al., "Could 3D models of cancer enhance drug screening?". *Biomaterials*. Vol. 232. (2020).

Product information

AX R Confocal Microscope

Supports high-speed, high-resolution, large field-of-view confocal imaging, with reduced phototoxicity to living cells and photobleaching.

- High speed: Up to 720 fps (resonant at 2048 x 16 pixels)
- High resolution: Up to 8K (galvano)/2K (resonant)
- High throughput: Ultra-wide FOV of 25 mm

NIS-Elements Imaging Software

This is a software platform that can perform integrated control of the microscope and peripheral equipment, acquisition of images in up to 6 dimensions, data management, and image processing / analysis. Tools that can customize the automation of imaging workflow are mounted to support complicated experiment systems and advanced analysis.