High throughput cancer spheroid 3D fluorescence imaging and morphology/viability analysis

Light-Sheet Microplate Cytometer

CYTOQUBE™

Cell-based drug discovery assays are traditionally done using two-dimensional (2D) cultured cells. While three-dimensional (3D) cultures provide a more biologically relevant environment, the data acquisition is slow. For example, capturing in-focus images from 3D samples requires stepping acquisition through multiple Z-planes for every XY position. Thus, scan times for three fluorophore experiments in 96 well plates can readily exceed 60 minutes. To overcome this time barrier, we developed the CYTOQUBE® Light-Sheet Microplate Cytometer. The CYTOQUBE uses light-sheet optics and intelligent software to speed up data acquisition in 3D. Here we highlight the advantages of light-sheet acquisition and how the CYTOQUBE implements this technology to make 3D cultures a realistic paradigm for drug discovery.

Key benefits

Whole well 3D fluorescence imaging

Capture 3D fluorescence images of all spheroids across the entire well.

Individual spheroid analysis

Measure the morphology and fluorescence intensity of individual spheroids from 3D fluorescence images.

High throughput/Parallel processing of scan and analysis

Measure 3D fluorescence images across the entire well plate in as little as 21 minutes (three wavelengths, sample depth of 200 µm to 400 µm).

Introduction

In drug discovery, understanding spheroid morphology and viability is crucial as they provide insights into the effects of drug candidates on cellular health and function. Spheroids are three-dimensional cell cultures that better mimic the in vivo environment compared to traditional two-dimensional cell cultures. They offer a more accurate representation of how cells behave in the human body, making them invaluable for preclinical testing.

Spheroid morphology refers to the shape, structure, and size of these 3D cell aggregates, which can indicate changes due to drug treatment. Spheroid viability measures the proportion of live, healthy cells within the spheroid, reflecting the cytotoxic effects of compounds.

In this experiment, we utilized the CYTOQUBE to perform high-throughput 3D fluorescence imaging and analysis of 3D spheroid cultures. We used two compounds that impact cell survival and morphology, the PI3 kinase inhibitor LY294002 and Cdc25 phosphatase inhibitor Menadione. LY294002 inhibits the PI3K-Akt pathway, thereby suppressing cell migration and invasion and reducing overall cell viability. Menadione is known to induces oxidative stress by generating reactive oxygen species (ROS) within cells. This oxidative stress inhibits cell proliferation, induce apoptosis, and alters spheroid morphology. To assess cell viability and morphology, we again chose well-established fluorescent labels to identify nuclei (Hoechst 33342), live cells (Calcein AM), and dead cells (PI, Propidium Iodide).

By capturing 3D fluorescence images, we can accurately assess both the morphology and viability of individual spheroids. Our goal is to demonstrate that image data acquired with the CYTOQUBE provides meaningful morphology and viability data that are comparable to results in 2D drug discovery assays in both information content and speed of acquisition.

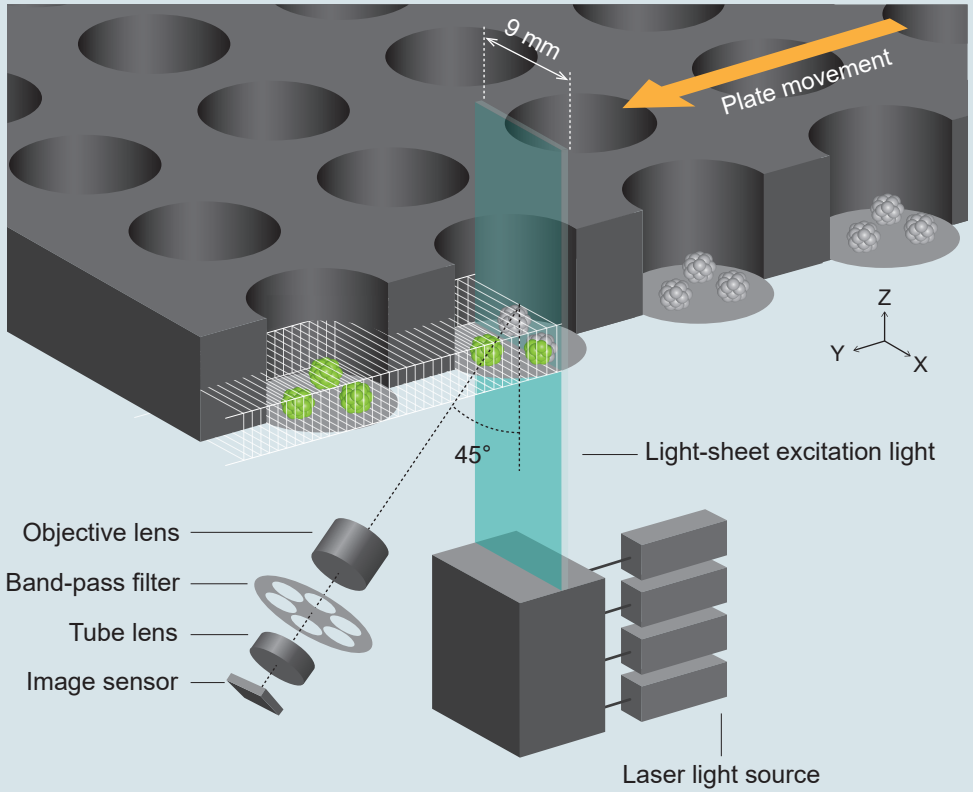
Light-sheet enables high throughput

What is light-sheet imaging?

Light-sheet fluorescence microscopy is an optical imaging technique with many spatio-temporal benefits that are achieved through a combination of special illumination and detection optics. A sheet of illumination light is created and scanned through the sample. Unlike a scanning laser point source or traditional widefield fluorescence, light-sheet provides fast, gentle, optical sectioning reducing phototoxicity, photobleaching, and 3D acquisition times. The exact configuration of the light-sheet system is specific to the application.

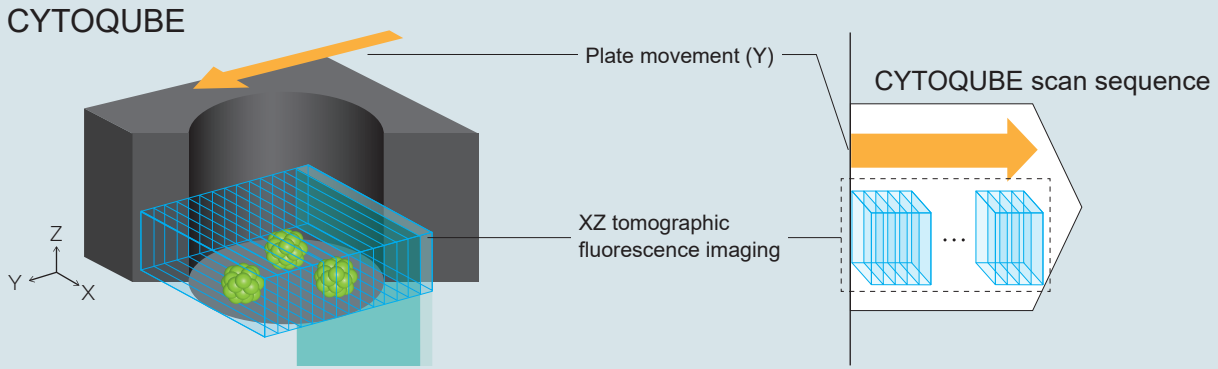
How does the CYTOQUBE use light-sheet?

The CYTOQUBE is specifically designed to quickly assay living 3D cell cultures in 96, 384 or 1536 well plates. In the CYTOQUBE, illumination optics create a light-sheet the width of a single 96 well. This orthogonally oriented sheet of light passes through the sample, illuminating the XZ plane. As the plate is scanned through the light-sheet excitation light, the camera captures XZ images of all the cells in the well, while our CYTOQUBE software automatically integrates these frames into a 3D tomographic image of each well.



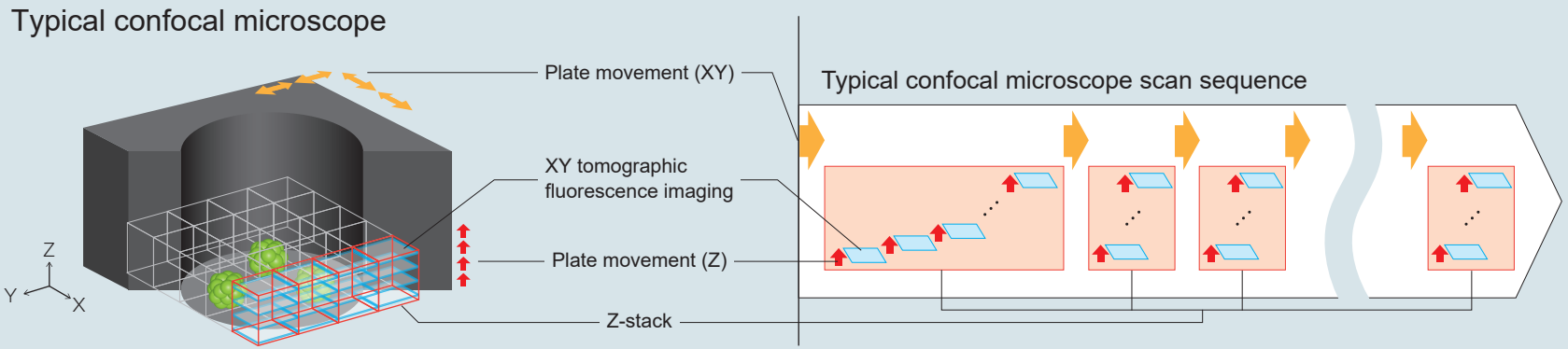
How does the CYTOQUBE workflow compare to traditional plate scanners using laser confocal?

In the CYTOQUBE, the combination of fast image acquisition and high-quality image data is achieved through an elegant and easy to use light-sheet microscope with advanced image processing capabilities. These advantages are readily observed when comparing the CYTOQUBE's acquisition workflow to traditional confocal systems. In the CYTOQUBE, each well is scanned once to acquire a single-wavelength fluorescent 3D image. In traditional laser scanning confocal systems, the acquisition time is increased since the point illumination of the laser must first be scanned in X-Y and then repeated at various intervals in Z. This workflow is repeated for each wavelength to be imaged, resulting in significantly longer time-to-answer.



CYTOQUBE
- Plate movement: a single-axis scan
- Acquire images while moving
- XZ tomographic fluorescence imaging

Typical confocal microscope
- Plate movement: 3-axis scan
- Repeat movement and image acquisition
- XY tomographic fluorescence imaging



Method

Spheroids cultured in spheroid culture plates were suspended in medium containing 2.5 % Matrigel, and seeded into 96 well plates (40 to 60 spheroids/well). Reagents were then added to the spheroids to a final concentration of 50 µmol/L or 100 µmol/L.

The spheroids were cultured at 37 °C; a fluorescent dye was added after three washing operations at 0 hour and 24 hours after the addition of the reagent; and staining was performed at 37 °C for 45 minutes. After staining, scanning and analysis were performed in the CYTOQUBE without a cell washing procedure.

CYTOQUBE scan settings

Sample depth		200 µm to 400 µm
Background subtraction		ON
Sensitivity	Hoechst 33342	13 to 16
	Calcein AM	5
	PI	12 to 17

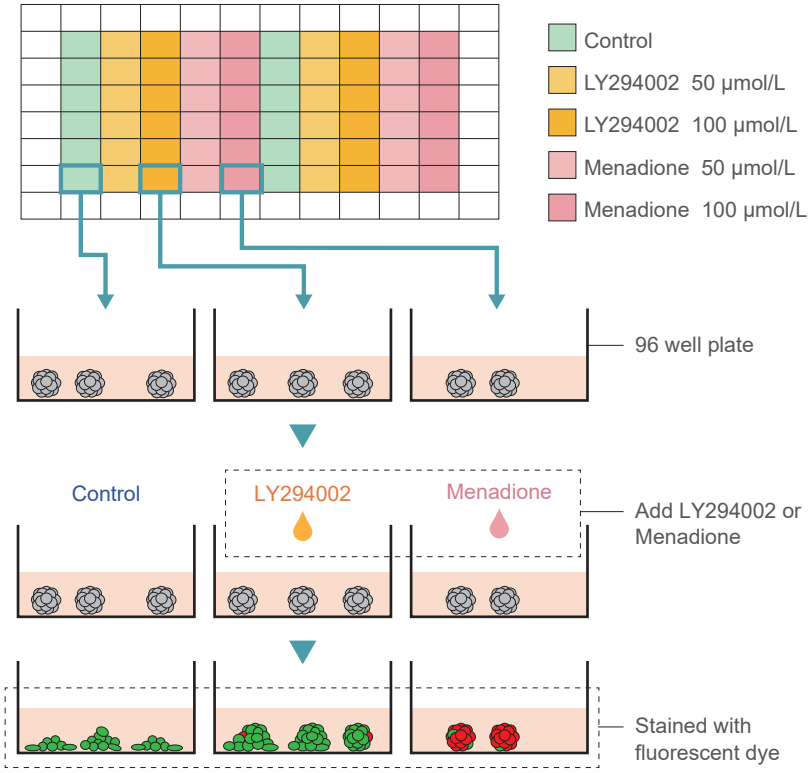
Approximately 10 minutes are required to scan (3 colors) and analyze 3D fluorescence images of 10 columns of a 96 well microplate.

Cells: Human glioblastoma-derived U-87 MG cells

Reagents: PI3 kinase inhibitor LY294002
Cdc25 phosphatase inhibitor Menadione

Fluorescent dyes: 5 µg/mL Hoechst 33342
0.125 µmol/L Calcein AM
4 µg/mL Propidium Iodide (PI)

96 well microplate



Acquired images

Figure 1 displays a list of maximum projection images of 3D fluorescence images captured across the entire well. Even while scanning a microplate, it is possible to sequentially inspect fluorescence images from the wells that have been scanned.

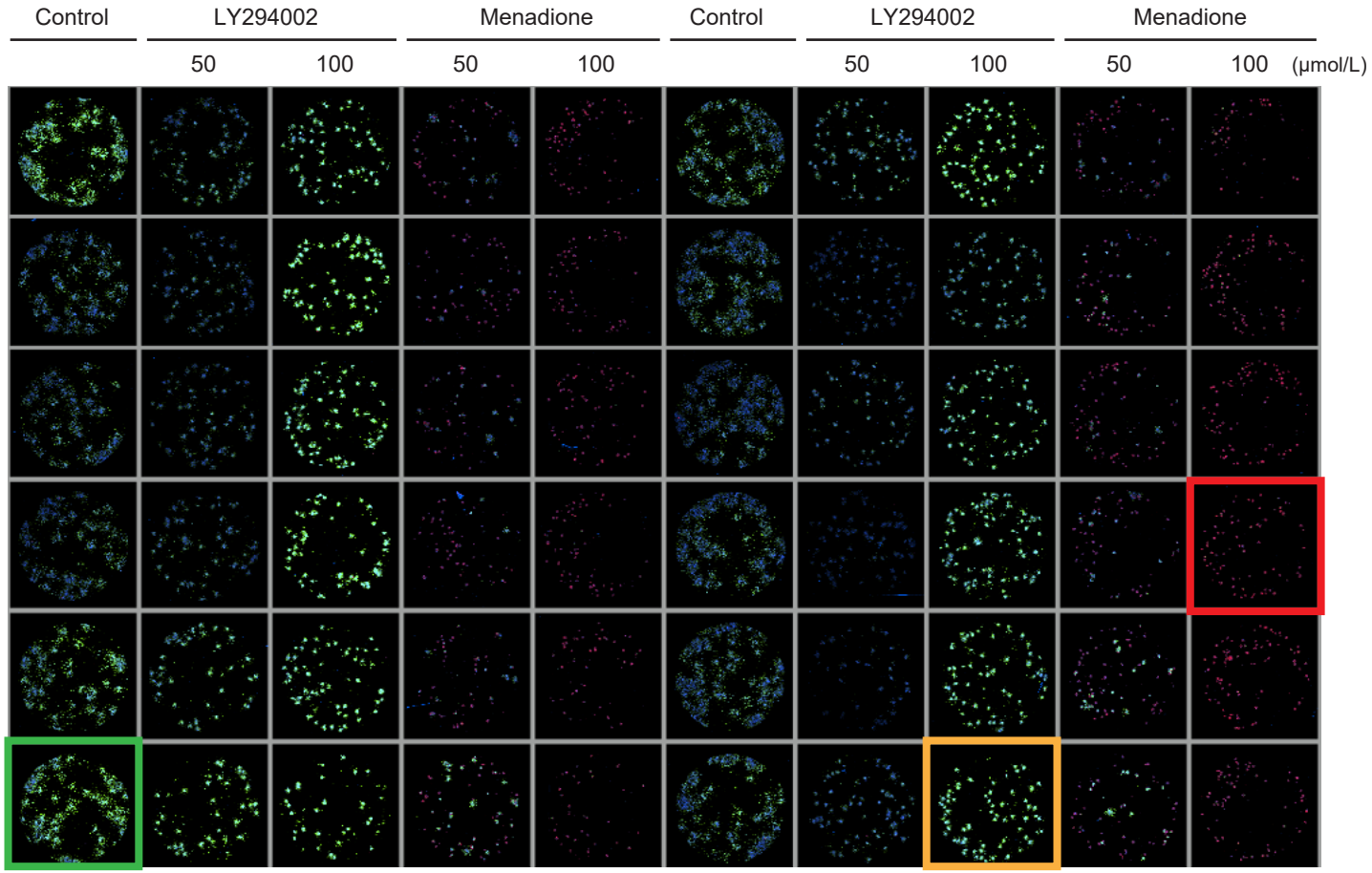


Fig. 1: Fluorescence image of a 96 well microplate 24 hours after adding LY294002 or Menadione (maximum projection image of 3D image)
Please see the next page for the 3D and projection images of the images within the frames.

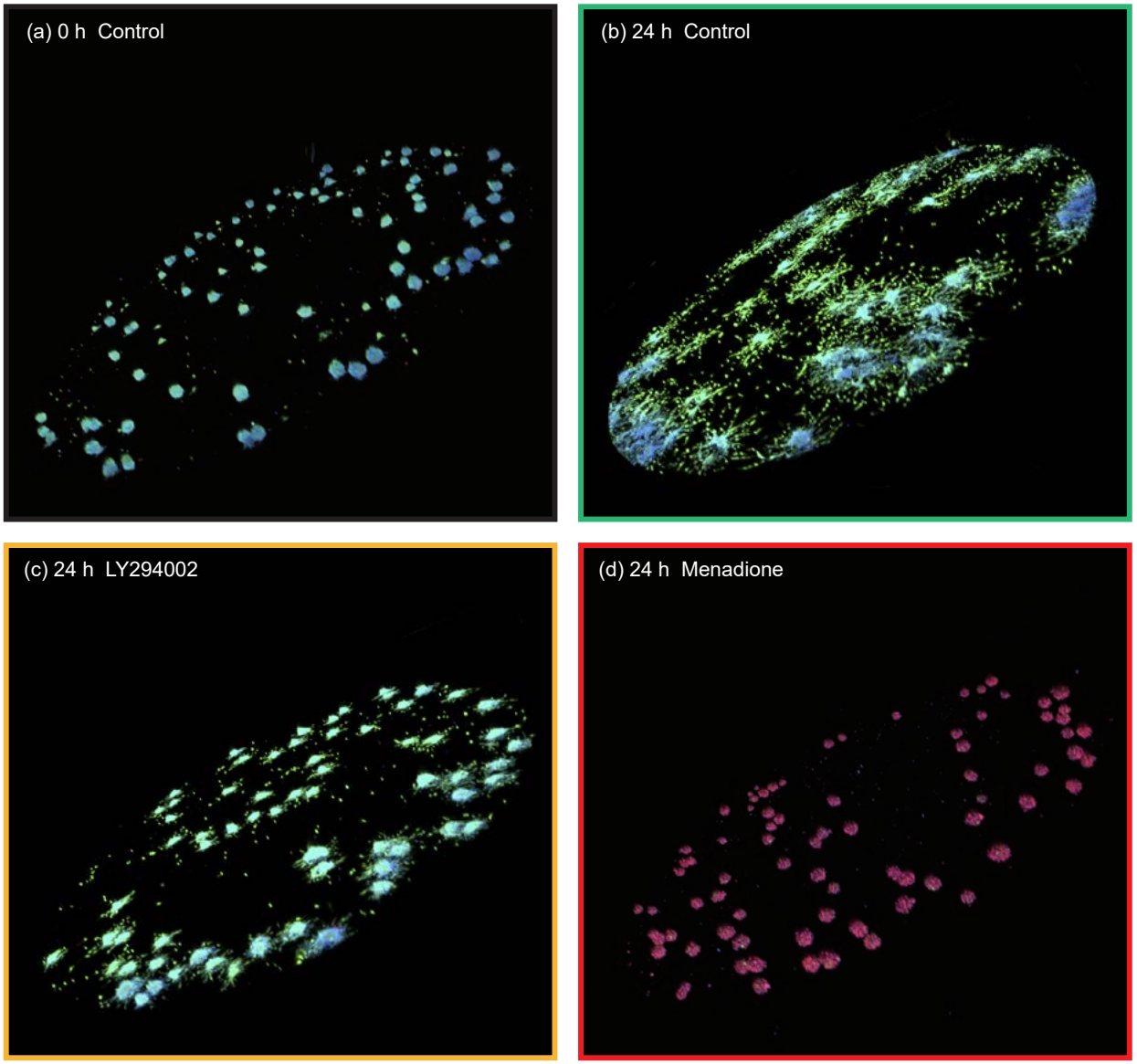


Fig. 2: Representative 3D fluorescence images showing control at 0 hour (a), control at 24 hours (b), 100 µmol/L LY294002 at 24 hours (c), and 100 µmol/L Menadione at 24 hours (d).

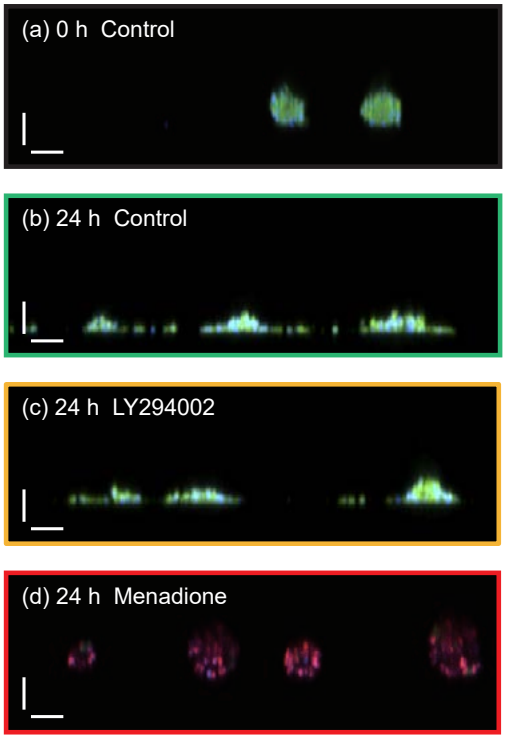


Fig. 3: XZ plane projection image (scale bar = 100 µm)

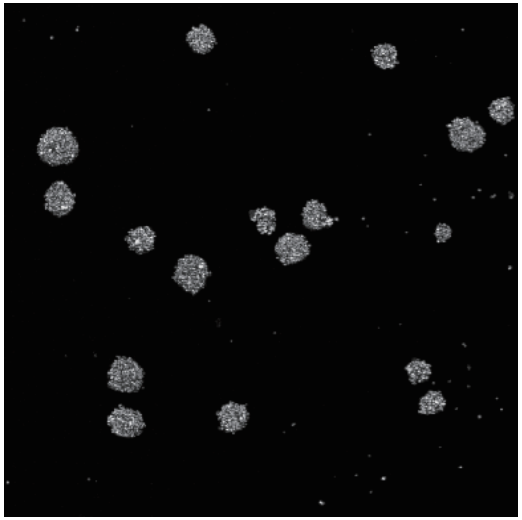
Fig. 1, 2, 3: Merging of Hoechst 33342 (blue), Calcein AM (green), and PI (red)

Recognition of spheroids

The maximum length (diameter) in the XY plane, maximum thickness in the XZ plane, and volume of individual spheroids were estimated in the Hoechst 33342-staining image. In each spheroid, the number of Calcein AM-staining voxels (the cells were alive in this region) and PI-staining voxels (the cells were dead in this region) were obtained in the Calcein AM and PI images, respectively.

$$\text{Live index} = \frac{\text{Number of Calcein AM-staining voxels}}{(\text{Number of Calcein AM-staining voxels}) + (\text{Number of Calcein PI-staining voxels})}$$

Fluorescence image (Hoechst 33342)



Recognize individual spheroids

In the 3D fluorescence image of Hoechst 33342, cell clusters with a volume above a threshold* are recognized as spheroids. *Threshold value = 300 000 μm^3

Morphology and viability analysis of individual spheroids

The scatter plot for the live index and the ratio of thickness to diameter of each spheroid is shown in Figure 4. Each dot represents each spheroid. The number of spheroids in a well was 58 (control, 0 h), 39 (control, 24 h), 51 (LY294002 100 µmol/L, 24 h), and 55 (Menadione 100 µmol/L, 24 h).
Figure 5 shows the results obtained from four plates (a total of 48 wells), with each point representing the average value of one well.

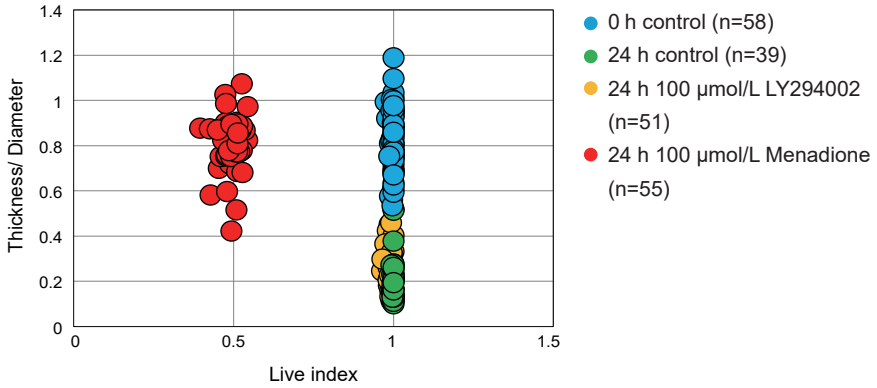


Fig. 4: Cell viability and morphological changes in spheroid (one well analysis)

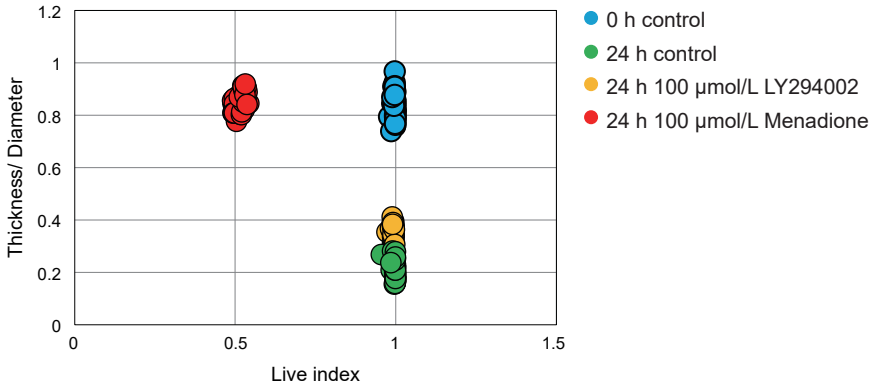


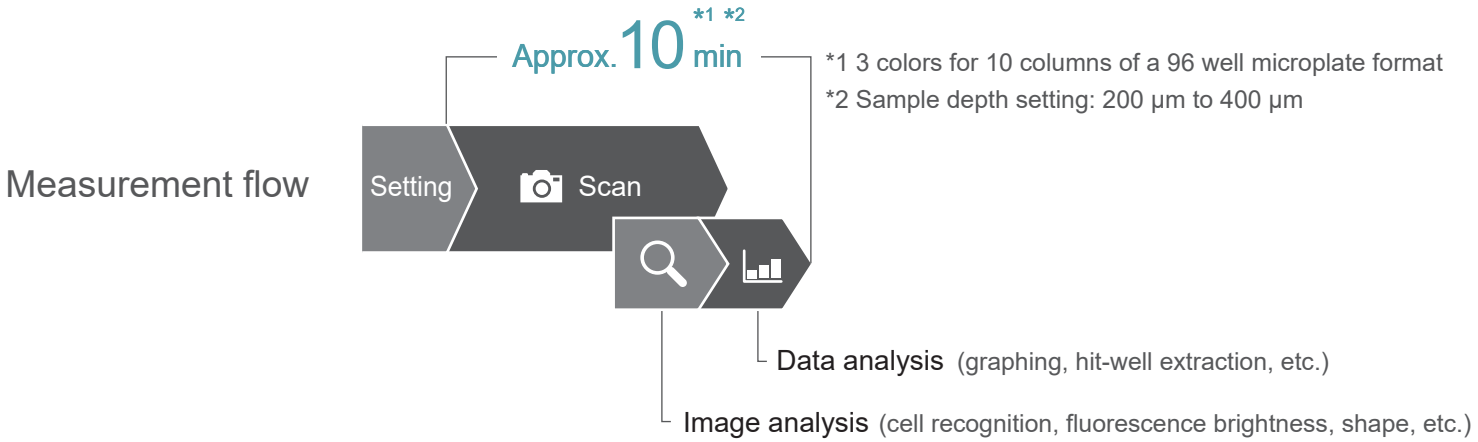
Fig. 5: Cell viability and morphological changes in spheroid (summary of 48 wells)

Results

At the start of the experiment (0 h), the spheroids in the control group were almost all spherical, and they appeared flattened after 24 hours. Additionally, it was demonstrated that most cells were still alive. After 24 hours of adding 100 µmol/L LY294002, the spheroid thickness slightly decreased, and most cells remained viable.
In contrast, after 24 hours of adding 100 µmol/L Menadione, the spheroids maintained a spherical shape similar to the control group before treatment, but approximately 40 % to 60 % of cells within the well were confirmed to be dead.

Conclusion

Using light-sheet technology, the CYTOQUBE acquires 3D fluorescence images of entire microplate wells with a single-axis scan. Using this technology, it is possible to rapidly obtain 3D fluorescence images of all spheroids of various sizes and shapes within a well. Additionally, quantitative analysis results of the morphology and fluorescence brightness values of each spheroid are displayed simultaneously with image acquisition. By using the CYTOQUBE, it is expected that drug discovery research using 3D cultured cells will be accelerated.



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