APPLICATION NOTE

Acquire and analyze images of FUCCI spheroids on the SpectraMax MiniMax cytometer

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Benefits

- **Monitor cell cycle progression in living cells via cellular imaging**
- **Easily analyze multiple parameters of spheroids**
- **Design custom spheroid analysis in SoftMax Pro Software**

Introduction

Spheroids are small three-dimensional (3D) cellular microenvironments grown using a variety of specialized culture methods such as low-adhesion microplates. This 3D cell culture confers a high degree of clinical and biological relevance to *in vitro* models and is now widely used in high-throughput screening (HTS) and advanced cell culture to study compound toxicology, or major diseases such as cancer. Multicellular tumor spheroids (MCTS), a model that mimics the organization of a tumor, is used frequently to bridge the gap between traditional 2D environments, such as a cell monolayer in a microplate, and animal models. It is widely accepted that 3D techniques more accurately replicate cancer cell biology *in vivo* and are useful for evaluating new anti-cancer strategies[1].

FUCCI (fluorescent ubiquitination-based cell-cycle indicator) spheroids have been developed to study cancer cell cycle progression as they allow the identification of cells in various phases of the cell cycle. The FUCCI technology is based on the overexpression of two modified cell cycle-dependent proteins, geminin and Cdt1, each respectively fused to a green fluorophore (AmCyan for geminin) and a red fluorophore (mCherry for Cdt1). Cdt1 and geminin levels fluctuate differentially throughout the cell cycle: Cdt1 levels peak in G1 phase; while geminin levels rise in late S, G2 and M phase. This results in the nucleus of FUCCI-expressing cells appearing red in G1 phase and green in late S, G2 and M phase (Figure 1)[2,3,4].

The SpectraMax® i3x Multi-Mode Microplate Reader with the SpectraMax® MiniMax™ 300 Imaging Cytometer is equipped with transmitted light (TL), green (Ex/Em: 460/541) and red (Ex/Em: 625/713) fluorescence channels. The MiniMax cytometer with SoftMax® Pro Software can be used to image and analyze a variety of cells. Here, we demonstrate how the MiniMax cytometer can easily acquire and analyze images of FUCCI spheroids.

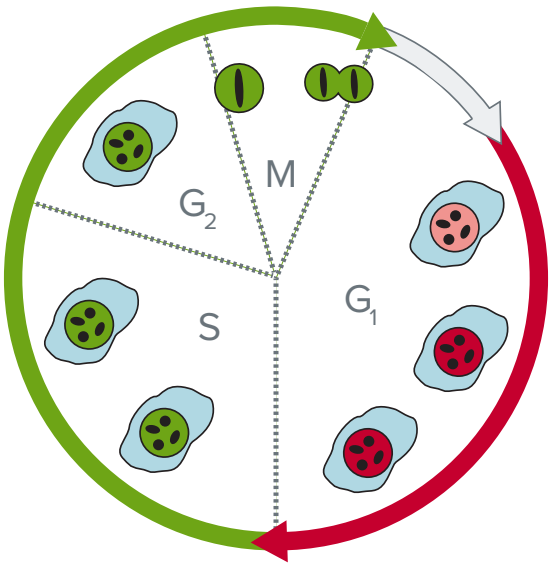

Figure 1. FUCCI cell cycle analysis. mCherry-Cdt and AmCyan-geminin fluorescently labeled cell cycle proteins are expressed or degraded during different phases of the cell cycle such that cells appear red during G1 phase and green during S, G2, and M phases. Cellular imaging can be used to monitor the cell cycle under various experimental conditions.

Materials

- SpectraMax i3x Multi-Mode Microplate Reader (Molecular Devices cat. #i3x)
- SpectraMax MiniMax 300 Imaging Cytometer (Molecular Devices cat. #5024062)
- Corning 96-well ultra-low attachment U-bottom plates (Corning cat. #7007)
- A375S FUCCI cells (kindly provided by Institute of Cancer Research, London)
- DMEM/F12 medium (Invitrogen cat. #11320-033)
- EGF (Invitrogen cat. #PHG0311)
- B27 Supplement (Invitrogen cat #17504001)

Methods

Spheroid generation and culture

Cells expressing FUCCI reporters mCherry-Cdt1 and AmCyan-geminin were generated at the Institute of Cancer Research (ICR), according to the procedure outlined by Dufau et al.[1] A375S FUCCI spheroids were prepared as follows. 1000 cells per well in DMEM/F12 supplemented with EGF (20 ng/mL) and B27 (1x) were seeded in Corning 96-well ultra-low attachment U-bottom plates. Plates were centrifuged (6 min, 800 g) and then incubated at 37°C with 5% CO_2. Finally, spheroids were washed three times with media containing 10% FCS to remove EGF and B27 and cultured in this media for 1 to 6 days.

Data analysis

The MiniMax cytometer's transmitted light channel gave us best results from an out-of-focus image. A new custom data analysis was developed in SoftMax Pro "Field Analysis" to identify cell-confluent regions. An object classification was applied to discriminate between the spheroid and other objects such as debris and individual cells, which were then excluded from the analysis (Figure 2, A and E). Finally, a region of interest was selected to exclude unwanted objects from the image. In the green or red fluorescence channel, spheroids were easily identified in "Object Count" using size and intensity over background (Figure 2, B-D, F-G). SoftMax Pro Software was then used to calculate the "% Area coverage" of the image and the "Object Area" (in μm^2) of the spheroid. Acquisition and analysis settings are shown in Table 1.

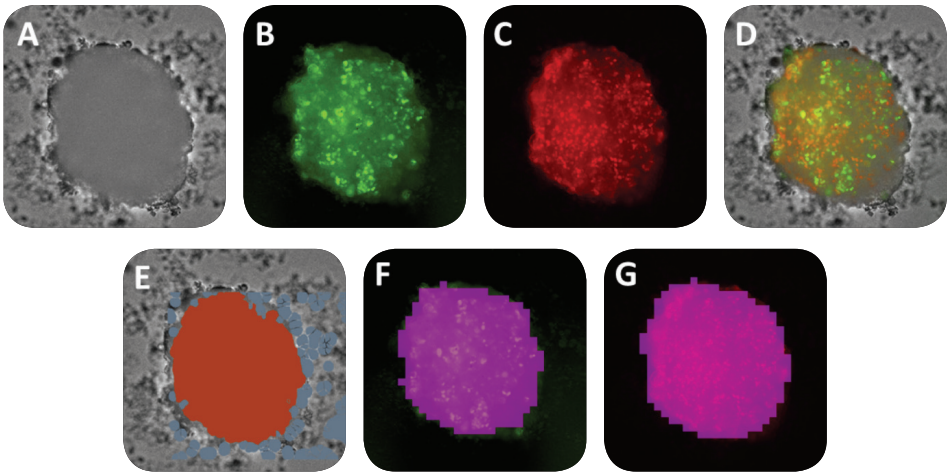

Figure 2. Images of a FUCCI spheroid. A: transmitted light; **B:** green fluorescence; **C:** red fluorescence; **D:** composite image with transmitted light, green and red images overlaid; **E:** transmitted light image with spheroid identified as a red overlay by SoftMax Pro Software, and other objects, such as debris and individual cells, identified in gray by the software and excluded from analysis. **F:** green fluorescent image with identified region (object) shown as a purple overlay. **G:** red fluorescent image with the object overlay. Areas identified by the software and shown as overlays were used in image analysis.

	Transmitted light	Green fluorescence (541 nm)	Red fluorescence (647 nm)
Focus adjustment	1000–1200 µm	1000–1200 µm	1000–1200 µm
Exposure	6 ms	8 ms	2000 ms
Data analysis setting	Field Analysis: custom analysis with classification and region of interest selected	Object Count with size and intensity over background Size: 380-2000 µm Background: 380	Object Count with size and intensity over background Size: 350-1200 µm Background: 380

Table 1. Acquisition and analysis settings for FUCCI spheroids. These values are indicated for the plate used here and for these spheroids. Other plates and spheroids may require different adjustments.

	Object size (μm^2)	% Area covered
Transmitted light	242609	5.5
Green fluorescence	329500	7.5
Red fluorescence	329400	7.5

Table 2. Data analysis for FUCCI spheroids. Object area in μm^2 and percentage of the area covered per image were calculated in SoftMax Pro Software.

Results

The acquisition and analysis settings in SoftMax Pro Software allow rapid and easy identification of a single spheroid per well in transmitted light, and in both green and red fluorescence channels. SoftMax Pro can calculate the percentage of the area covered, and the object area in μm^2, in all channels (Figure 2, Table 2). Best results were obtained in the green or red fluorescence channels.

For the FUCCI spheroids, the red images displayed a smooth, rounded spheroid while the green image showed small outgrowth at the surface confirming that cells inside the spheroid were red in phase G1, while the cells at the surface were green and preparing to enter the M phase (Figure 2) of the cell cycle.

The software can identify both small and substantial changes in the size and shape (roundness parameter) of the spheroids and can therefore be used to investigate the effect of various treatments, e.g. possible anticancer drugs, on cell proliferation (Figure 3). The optical configuration of the green and red fluorescence illumination in the MiniMax generates 2D spheroid images with a 3D appearance.

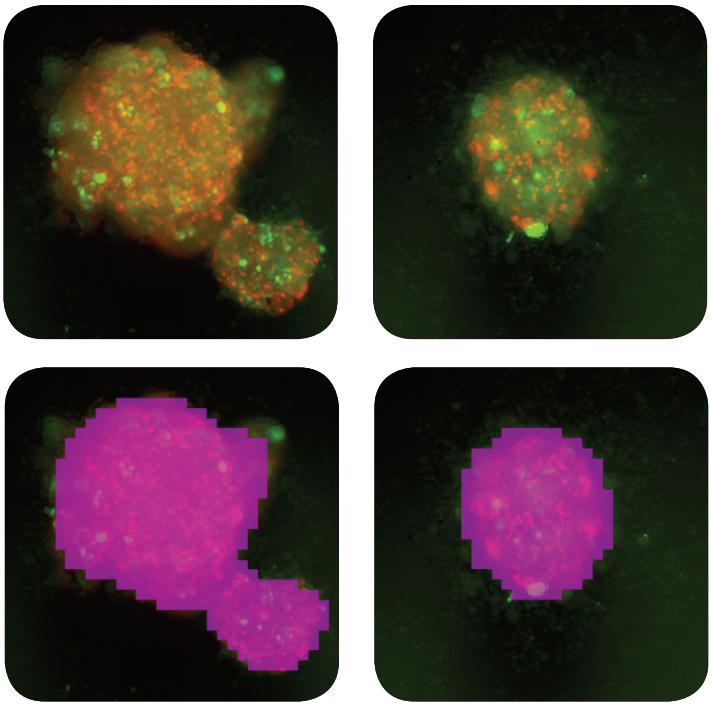

Figure 3. Images of FUCCI spheroids in different sizes and shapes. Composite images (top) with the identified region shown with a purple overlay (bottom) from the red channel. Red channel data analysis: left spheroid size of 387000 μm^2 and roundness parameter of 0.43; right spheroid size of 184500 μm^2 and roundness parameter of 0.64.

Conclusion

The MiniMax cytometer with integrated data analysis provided by SoftMax Pro Software was used to successfully image and analyze FUCCI spheroids in transmitted light, and in both green and red fluorescence channels. Spheroids can be evaluated in several ways, including area covered in the image, object size, and object shape, offering researchers a range of multi-parametric cell-based analyses.

The SpectraMax i3x reader with MiniMax cytometer seamlessly combines the benefits of imaging with the ease of use of a microplate reader. With a simple workflow from data collection to analysis, SoftMax Pro Software completes this total solution for FUCCI spheroid analysis.

References

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