TECHNICAL NOTE

CYTENA
A BICO COMPANY

Monitoring of multi-spheroid growth and health

Juliane Strietz, PhD; Yong Chen, MS
CYTENA, Freiburg, Germany

Introduction

3D cell culture models are promising approaches in cancer research and drug development. These models more closely mimic tumor tissue compared to 2D cell culture models, which have been traditionally used but are considered oversimplified models. 3D cell culture models are characterized by complex cellular organization and interactions and, therefore, bridge the gap between simple 2D cell culture and animal models.[1,2]

One of the best characterized and most frequently used models is the tumor spheroid model. Tumor spheroids are spherical aggregates of cancer cells that self-assemble in a scaffold-free microenvironment. Various methods have been established to generate tumor spheroids, such as the pellet culture method that generates single spheroids and the liquid overlay method that generates multiple spheroids within a culture dish.[1,3]

In this technical note, we explain how CYTENA's live cell imaging system, the CELLCYTE X™, can be used to analyze the growth and health of multi-spheroid cultures that were generated with the liquid overlay method on Matrigel-coated plates for the evaluation of drug efficacy.

Material and methods

Cell culture

MDA-MB-231 GFP cells were cultured in Ham's F-12 medium (Thermo Fisher) supplemented with 10% fetal bovine serum (FBS) (Sigma) and 1% penicillin/streptomycin (P/S) (Thermo Fisher) at 37°C and 5% CO_2. They were passaged twice per week and seeded for the different studies as described below.

Multi-spheroid assembly

To generate multi-spheroids, wells of a 96-well flat bottom microtiter plate (Corning, #3595) were coated with 40 µL 50% Matrigel (Corning). The Matrigel was allowed to solidify for 30 minutes at 37°C. Afterwards, MDA-MB-231 GFP cells were detached, counted and seeded with 2,000 cells in 110 µL per well to reach a total volume of 150 µL per well. The cells were allowed to settle for 30 minutes at room temperature to ensure a homogeneous distribution of cells across the well. Subsequently, the microtiter plate was placed in the CELLCYTE X, which was housed in an incubator. To minimize condensation, the plate was equilibrated to 37°C for 30 minutes before the first scan was started. The multi-spheroid growth was monitored every 3 hours for 168 hours using 4X magnification and the spheroid scan module. The total spheroid area and total intensity of fluorescence were quantified using the total spheroid area recipe in CELLCYTE Studio, the system's acquisition and analysis software.

Multi-spheroid health study

MDA-MB-231 GFP multi-spheroids were seeded as described above. Multi-spheroid assembly was monitored for 72 hours before treatment with DMSO or 0.5 µM staurosporine (SSP). At the day of treatment start, C.LIVE Tox Red (CYTENA) was added at a final concentration of 500 nM together with the indicated treatments in 50 µL per well. To avoid disturbing the assembled multi-spheroids, the liquid was carefully released against the walls of the wells. The multi-spheroid health was monitored every 3 hours for additional 96 hours using 4X magnification and the spheroid scan module. The total spheroid area and total intensity of GFP and C.LIVE Tox Red fluorescence were automatically quantified using the total spheroid area recipe in CELLCYTE Studio.

Results and discussion

Generation and monitoring of multi-spheroid cultures

Seeding of multi-spheroid cultures

To generate multi-spheroid cultures with the liquid overlay method, the wells of a 96-well flat bottom plate are coated with Matrigel and cells of interest are seeded in culture medium on top of this layer. The basement membrane extract Matrigel prevents the seeded cells from adhering to the plate and promotes aggregation into multiple tumor spheroids. Here, MDA-MB-231 GFP cells were seeded and subsequently, the multi-spheroids treated with DMSO or staurosporine and stained with C.LIVE Tox Red to detect dead cells.

Scan setup

To scan multi-spheroid cultures, the spheroid module is used in CELLCYTE Studio. A scanning interval of 3 to 6 hours is recommended due to slow spheroid assembly and growth. Since spheroid assembly may take up to 72 hours, a scanning period of at least 72 hours is recommended. Here, MDA-MB-231 GFP multi-spheroids were scanned every 3 hours for 168 hours.

Exposure times for the fluorescence channels depend on the cell line and reagents used. Spheroids consist of multiple cells in close proximity often resulting in enhanced fluorescence signals compared to 2D cultures. Therefore, it is recommended to choose lower exposure times to avoid overexposure at later time points. For MDA-MB-231 GFP multi-spheroids stained with C.LIVE Tox Red, exposure times of 30 ms and 100 ms were used for the green and the red channel, respectively.

Image analysis

To analyze the growth and health of multi-spheroid cultures in CELLCYTE Studio, the total spheroid area recipe is used to mask objects of interest in the brightfield and fluorescence channels (**Figure 1**). The following recommendations for the individual analysis parameters can be used as an orientation:

Analysis of spheroid growth in brightfield channel

Contrast Sensitivity (a.u.): intermediate value (e.g., 55), sensitive enough to detect spheroids in both late and early stages, but not too sensitive to detect possible background noise from the coating of the plate

Smoothing (a.u.) and Filled Hole Size (µm²): high values (e.g., 4.5 and 2000, respectively) to ensure complete masking of detected spheroids despite contrast differences within the spheroids

Minimum Object Size (µm²): low value (e.g., 40) to ensure detection of early stage spheroids

Analysis of spheroid health using GFP expression in green channel

Contrast Sensitivity (a.u.): high value (e.g., 70), sensitive enough to detect spheroids not only in late but also in early stages

Smoothing (a.u.): intermediate, leave at default value of 2

Filled Hole Size (µm²): high value (e.g., 1000) to ensure complete masking of detected spheroids despite contrast differences within the spheroids

Minimum Object Size (µm²): low value (e.g., 40) to ensure detection of early stage spheroids

Analysis of spheroid health using C.LIVE Tox Red in red channel

Contrast Sensitivity (a.u.): high value (e.g., 80), sensitive enough to detect early onset of cell death in all spheroid stages

Smoothing (a.u.): intermediate, leave at default value of 2

Filled Hole Size (µm²): low value (e.g., 50) to ensure that only red cells are masked and no masking artifacts are generated

Minimum Object Size (µm²): low value (e.g., 50) to ensure masking of individual dead cells in the spheroids

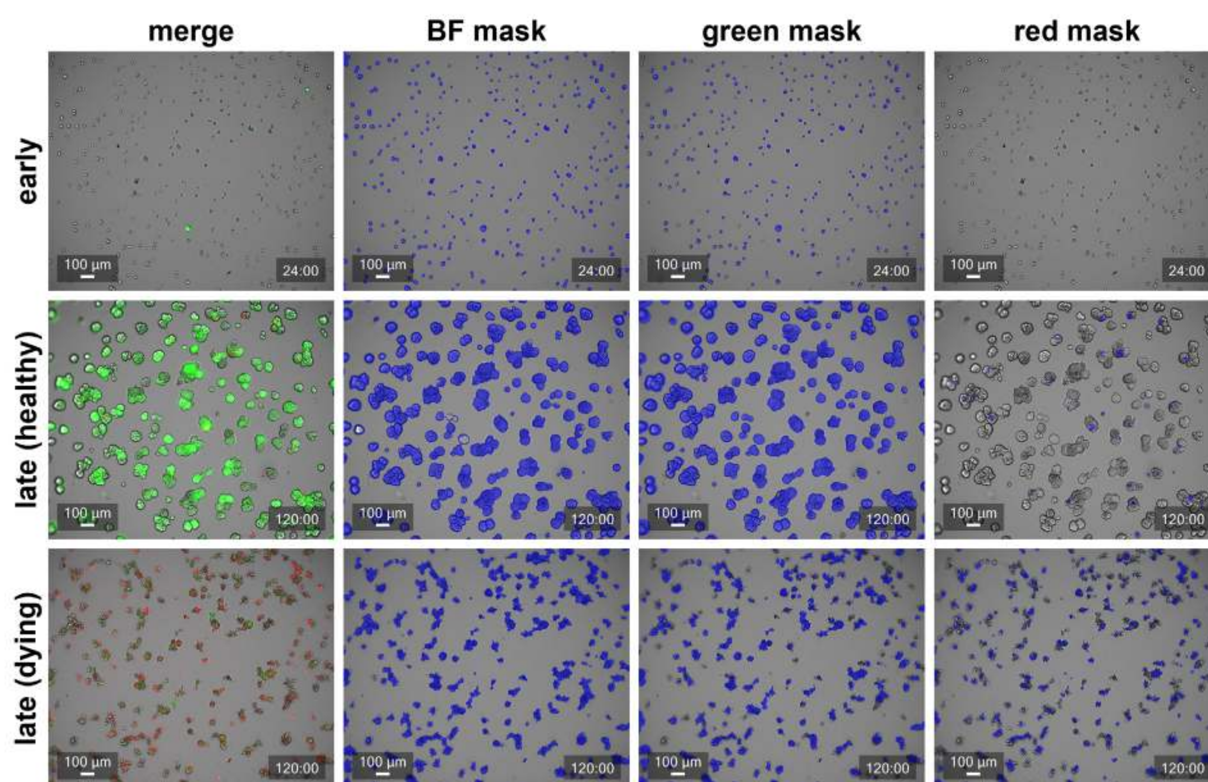


Figure 1: *Exemplary masks (in blue) for early and late spheroid stages as well as healthy and dying spheroids analyzed in different channels. MDA-MB-231 GFP spheroids were masked for spheroid area in the brightfield (BF) channel, for GFP expression in the green channel and for C.LIVE Tox Red staining in the red channel.*

Evaluation of drug efficacy using multi-spheroid cultures

The health of multi-spheroid cultures can be analyzed to evaluate the efficacy of a drug of interest. As described above, MDA-MB-231 GFP multi-spheroids were generated and treated after 72 hours either with the vehicle DMSO or the inhibitor staurosporine in the presence of C.LIVE Tox Red, which stains dead cells (**Figure 2A**). The spheroid growth was analyzed as total spheroid area in the brightfield channel (**Figure 2B**). Simultaneously, the total intensity of GFP (**Figure 2C**) and C.LIVE Tox Red (**Figure 2D**) were quantified to evaluate live and dead cells, respectively.

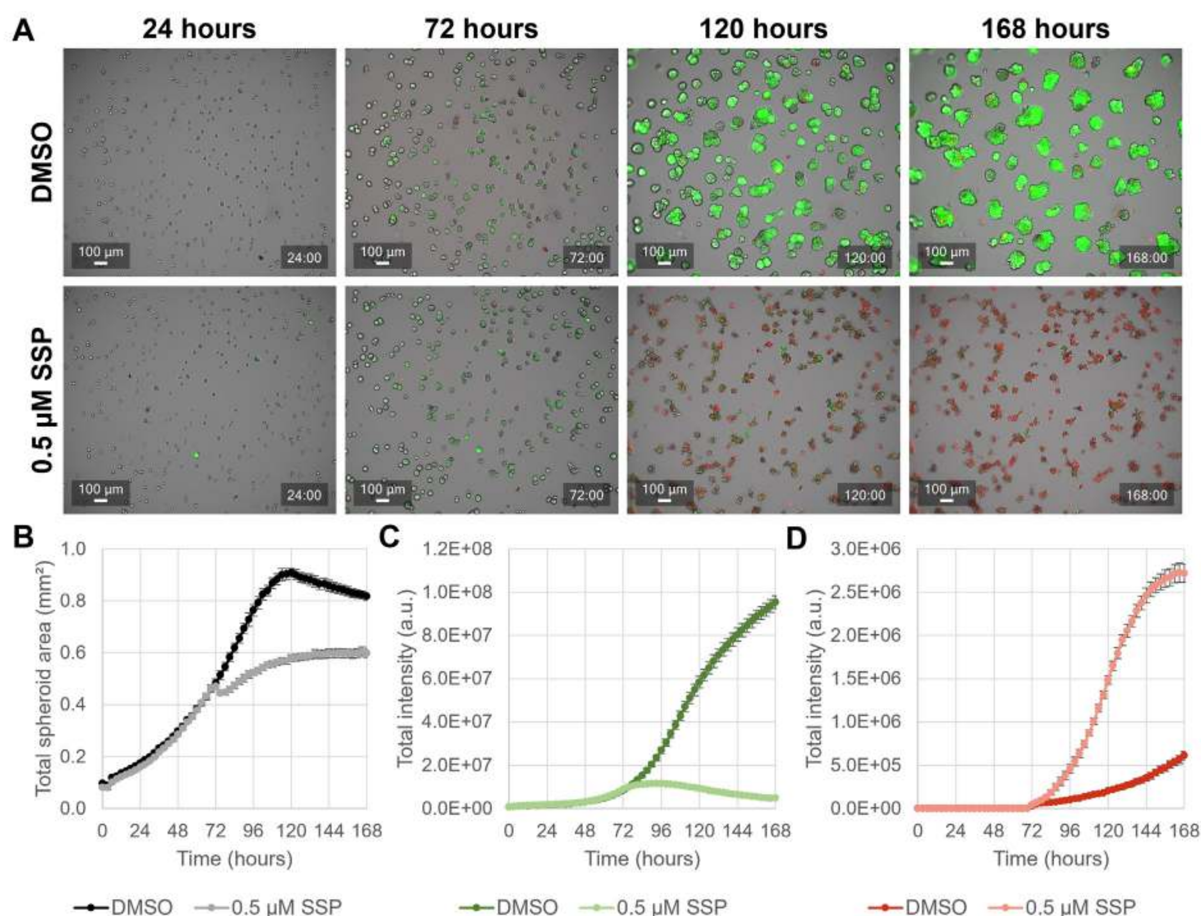


Figure 2: *Multi-spheroid cultures for evaluation of drug efficacy. (A) MDA-MB-231 GFP cells were seeded in 96-well plates coated with 50% Matrigel. Multi-spheroid assembly was monitored for 72 hours at 4X magnification and with the spheroid scan module. Afterwards, the spheroids were treated with vehicle (DMSO) or 0.5 µM staurosporine (SSP) and (B) the spheroid growth was quantified as total spheroid area using CELLCYTE Studio. Additionally, the spheroid health was monitored by quantifying (C) the total intensity of green GFP fluorescence or (D) the total intensity of C.LIVE Tox Red fluorescence. Depicted are mean ±SEM, n = 4.*

The assembly of multi-spheroids was monitored for 72 hours showing steadily increasing total spheroid area and total green fluorescence intensity before C.LIVE Tox Red and the treatments were added.

DMSO-treated multi-spheroid cultures continued to grow for the following 96 hours. The total spheroid area reached its highest value after 120 hours and showed a slight drop after that, which can be explained by the individual spheroids reaching a size where they collided with other spheroids, leading to fusion of spheroids. The health and growth of these spheroids with a smaller total spheroid area was confirmed by the total intensity of GFP fluorescence, which steadily increased over the time frame of 168 hours. Cell death as quantified by total intensity of C.LIVE Tox Red fluorescence stayed at a minimum and increased only slightly at later time points most likely due to apoptosis in the spheroid cores.

CYTENA A BICO COMPANY

Staurosporine-treated multi-spheroids showed a small drop in total spheroid area in the brightfield channel after treatment start and a slight area increase for the next 48 hours before it remained the same for the last 48 hours of the assay. The slight increase in area can be explained by the physical disruption of the affected spheroids as it has been described in literature before.[4,5] This observation was confirmed by a decrease of total green fluorescence, indicating a loss of living cells. Simultaneously, there was a significant increase in total red fluorescence intensity observed, indicating an increase of dead cells in the multi-spheroid culture.

Taken together, the CELLCYTE X can be used to reliably monitor the growth of multi-spheroid cultures generated by the liquid overlay method. Imaging of multiple channels at the same time allows multi-metric analysis and the comprehensive evaluation of spheroid health in drug screening assays.

References

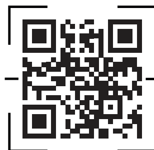
1. Gunti S, Hoke ATK, Vu KP, London NR. Organoid and spheroid tumor models: techniques and applications. Cancers (Basel). 2021;13(4): 874. DOI: 10.3390/cancers13040874

2. Vinci M, Gowan S, Boxall F, et al. Advances in establishment and analysis of three-dimensional tumor spheroid-based functional assays for target validation and drug evaluation. BMC Biology. 2012; 10(29). DOI: 10.1186/1741-7007-10-29

3. Costa EC, de Melo-Diogo D, Moreira AF, et al. Spheroids formation on non-adhesive surfaces by liquid overlay technique: considerations and practical approaches. Biotechnology Journal. 2018; 13(1): 1700417. DOI: 10.1002/biot.201700417

4. Mittler F, Obeïd P, Rulina AV, et al. High-content monitoring of drug effects in a 3D spheroid model. Frontiers in Oncology. 2017; 7. DOI: 10.3389/fonc.2017.00293

5. Walzl A, Unger C, Kramer N, et al. The resazurin reduction assay can distinguish cytotoxic from cytostatic compounds in spheroid screening assays. SLAS Discovery. 2014; 19(7): 1047-1059. DOI: 10.1177/1087057114532352



CYTENA, A BICO COMPANY

CYTENA spun off from the University of Freiburg, Germany, in 2014 with its patented singlecell dispensing technology. Today, as part of BICO, the world's leading bioconvergence company, CYTENA continues building on that groundbreaking technology to develop high-precision instruments for isolating, dispensing, imaging and handling biological cells. Its award-winning devices are manufactured in Germany and used at prestigious academic and pharmaceutical labs around the world to automate workflows in numerous application areas, including stable cell line development, single-cell omics, high-throughput screening and drug discovery. CYTENA's breakthrough innovations for the lab combine advanced automation, state-of-the-art software engineering and the latest insights in cell biology to maximize efficiencies in the life sciences and create the future of health. Learn more at cytena.com.

info@cytena.com | +49 761 21632 000 | www.cytena.com