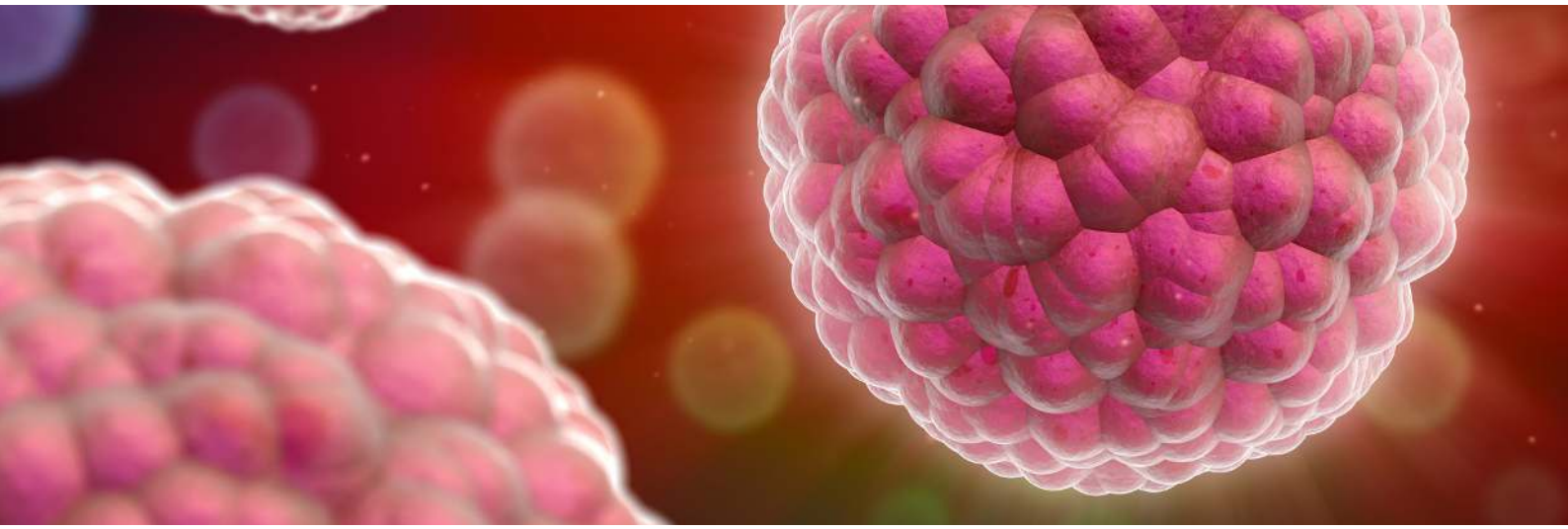
APPLICATION NOTE

CYTENA
A BICO COMPANY

High-throughput monitoring of drug effects on single-tumor spheroids

Juliane Strietz, PhD; Yong Chen, MS

CYTENA, Freiburg, Germany



Abstract

Cancer therapeutics discovery and development is a long and challenging process that requires reliable and relevant disease models to evaluate the efficacy of new drugs. 2D cancer cell culture models are easy-to-use and cost-efficient options but often lack the physiological relevance. 3D cell culture models have shown more promise in mimicking the tumor microenvironment in terms of nutrient and oxygen gradients as well as cell-cell and cell-matrix interactions. One of these models is the single-tumor spheroid, which is a spherical cancer cell aggregate that self-assembles in a scaffold free microenvironment. In this application note, we demonstrated how CYTENA's live cell imaging system, the CELLCYTE X™, can be used to monitor single-tumor spheroid growth and health and how it facilitates the comprehensive evaluation of cytotoxic and cytostatic drug effects.

Introduction

2D cancer cell culture models are often the model of choice in cancer research because they are easy-to-use and cost-efficient tools for high-throughput drug screening and toxicity studies. However, in the last decade, multiple studies have suggested that 2D cancer cell culture models are most likely oversimplified representations of tumors and do not fully recapitulate the complexity of the cellular organization and interactions that are seen in tumor tissues of both *in vivo* animal models and human patients.[1]

3D cell culture models are promising alternatives that bridge the gap between simple 2D cell culture and animal models. They more closely mimic the tumor microenvironment in terms of nutrient and oxygen gradients as well as cell-cell and cell-matrix interactions.[1,2]

Researchers have established a variety of 3D cell culture models[2]; among them, the multicellular tumor spheroid model is one of the best characterized and most frequently used. Multicellular tumor spheroids are usually spherical aggregates of cancer cells that self-assemble in a scaffold-free microenvironment. Methods to generate these spheroids for drug development purposes in an efficient and rapid way include the hanging drop method generating single spheroids in a liquid drop, the liquid overlay method generating multiple spheroids within a culture dish and the pellet culture method that generates single spheroids in the culture dish.[2–4]

In this application note, we used CYTENA's CELLCYTE X live cell imaging system to monitor the self-assembly of tumor spheroids that were generated with the pellet culture method in u-bottom 96-well ultra-low attachment (ULA) microtiter plates. We further discuss how the CELLCYTE X can be used to evaluate the cytotoxic effect of drugs on tumor spheroids and how the different analysis metrics allow the dissection of different drug mechanisms of action in greater detail.

Material and methods

Cell culture

HT-29, PANC-1 GFP and MDA-MB-231 GFP cells were cultured in sterile-filtered 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.

Assembly of single-tumor spheroids

To generate single-tumor spheroids, the pellet culture method was used.[2,3] For that, HT-29, PANC-1 GFP or MDA-MB-231 GFP cells were detached, counted and seeded with the indicated cell number into a u-bottom 96-well ULA microtiter plate (faCellitate BIOFLOAT or Nexcelom^{3D}) in a total volume of 200 µL per well. To collect the cells in the middle of the well and facilitate spheroid assembly, the plate was centrifuged at 250 RCF for 8 minutes and afterwards handled with care to avoid disturbing the cells. Subsequently, the plate was placed in the CELLCYTE X inside the incubator. To minimize condensation, the plate was equilibrated to 37°C for 30 minutes before the first scan was started. The spheroid growth was monitored every 2 hours for 168 hours using 4X

magnification with the Spheroid Module. The total spheroid area and total intensity of fluorescence were automatically quantified using the total spheroid area recipe in CELLCYTE Studio, the system's acquisition and analysis software.

Drug treatment of single-tumor spheroids

MDA-MB-231 GFP single-tumor spheroids were seeded as described above using 5k cells in 150 µL medium per well. Spheroid assembly was monitored for 48 hours before treatment with either staurosporine (SSP), camptothecin (CPT) or etoposide (ETO). At the day of treatment start, the indicated drugs at the indicated final concentrations, along with C.LIVE Tox Red (CYTENA) at a final concentration of 500 nM, were carefully added in 50 µL per well to not disturb the assembled spheroids. Spheroid health was monitored every 3 hours for an additional 48 hours or 120 hours (depending on the drugs) using 4X magnification with the Spheroid 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

High-throughput monitoring of single-tumor spheroids

While 3D cell culture models such as single-tumor spheroids are relevant tools used in the drug discovery process, imaging and analysis of tumor spheroids can be challenging at times. Plate readers that facilitate high-throughput analysis of spheroids often use end-point assays or do not provide the optimal spheroid growth conditions for measurements of in-between time points. Live cell imaging addresses some of these challenges but relies on a robust autofocus that can handle growing spheroids with morphology changes over time.

In the following study, we used the widely accepted pellet culture method to rapidly generate tumor spheroids of HT-29, a colorectal cancer cell line, and PANC-1 GFP, a green fluorescent protein-expressing pancreatic cancer cell line. The cells were seeded with increasing seeding densities in ULA u-bottom microtiter plates, where cell attachment was prevented by the coating on the plate and only one spheroid per well could be formed due to the u-bottom shape of the wells. The spheroids were subsequently imaged with the CELLCYTE X live cell imaging system over a span of 168 hours **(Figure 1)**.

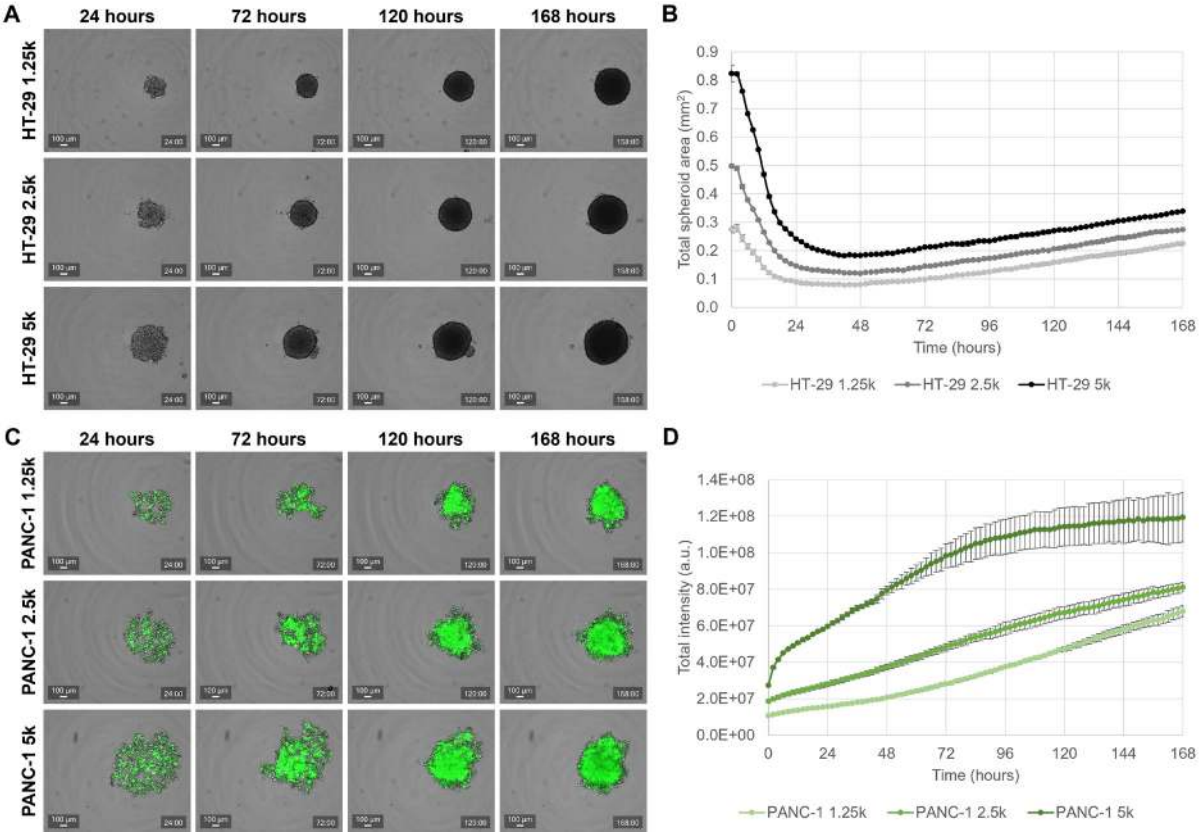


Figure 1: *Morphology and size of tumor spheroids of different cell lines and seeding densities. (A) HT-29 and (C) PANC-1 GFP cells were seeded in 96-well ultra-low attachment plates and spheroid assembly was monitored for 168 hours at 4X magnification and with the spheroid scan module. Spheroid growth was quantified as (B) total spheroid area or (D) total intensity of green fluorescence using CELLCYTE Studio. Depicted are mean ±SEM, n = 3.*

HT-29 cells grew into compact tumor spheroids over the time course of 168 hours and as expected, higher seeding densities resulted in spheroids of bigger size **(Figure 1A)**. Brightfield images were analyzed using the total spheroid area recipe in CELLCYTE Studio and the total spheroid area was quantified **(Figure 1B)**. The total spheroid area showed highest values at 0 hours directly after seeding and reached lowest values between 24 hours and 48 hours. This is in accordance with the protocol used. At 0 hours after centrifugation during seeding, HT-29 cells were collected as sheets of cells in the wells of the ULA u-bottom plate resulting in a high spheroid area value. The cells contracted over the following 48 hours into compact spheroids with a smaller spheroid area. Afterwards, the spheroid area increased again due to cell proliferation and spheroid growth.

The same was observed for PANC-1 GFP cells, although this cell line formed spheroids with a less compact morphology **(Figure 1C)**. This cell line stably expressed a GFP, which was alternatively used for the measurement of spheroid growth. For that, the total intensity of green fluorescence was quantified using the total spheroid area recipe **(Figure 1D)**. With this approach, the spheroid size and growth correlated with the total cell number reflected by low total fluorescence values at 0 hours that increased over a period of 168 hours as the spheroids assembled and grew. Again, higher seeding densities resulted in bigger spheroids with higher total fluorescence intensities.

Taken together, the CELLCYTE X live cell imaging system enabled the reliable long term monitoring of scaffold-free tumor spheroid growth, allowing in-plate comparison of experimental conditions in a high-throughput manner. The spheroid growth could be

evaluated using multiple readouts such as total spheroid area or total fluorescence intensity if the cell line of interest expresses a fluorescent protein. As such, the CELLCYTE X supports multi-metric quality control applications that evaluate spheroid size and morphology for further downstream applications such as drug screening.

Health of single-tumor spheroids as readout for drug efficacy

High-throughput screening of different cell culture models is required during drug candidate identification and validation to speed up the process and provide enough data points for comprehensive drug evaluation. Live cell imaging is a promising approach to address this requirement. However, time-consuming manual data point collection and inconsistent growth conditions outside of the incubator during the data collection process pose challenges. Here, we have addressed these challenges with the CELLCYTE X's live cell imaging and demonstrated how spheroid health can be used as a readout for drug efficacy.

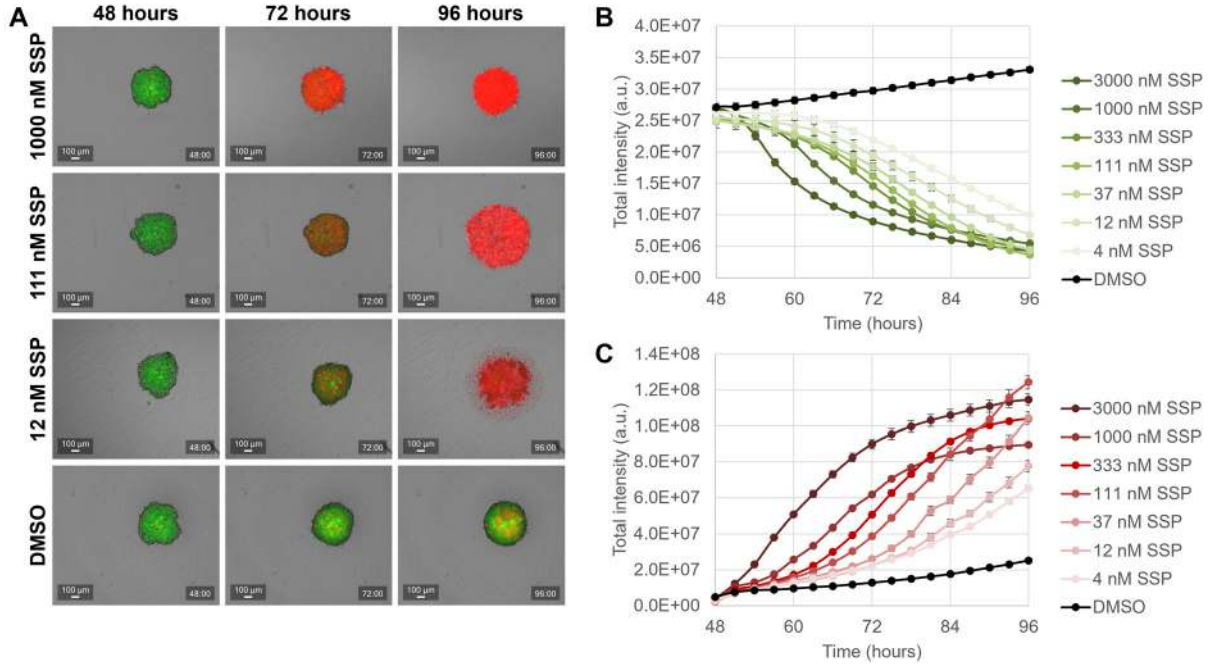


Figure 2: *: Health of tumor spheroids after drug treatment. (A) MDA-MB-231 GFP cells were seeded in 96-well ultra-low attachment plates and spheroid formation was allowed for 48 hours. After that, C.LIVE Tox Red and staurosporine (SSP) were added and the spheroid health was monitored for additional 48 hours at 4X magnification and with the spheroid scan module. Spheroid health was quantified as (B) total intensity of green fluorescence and (C) total intensity of red fluorescence using CELLCYTE Studio. Depicted are mean ±SEM, n = 6.*

For that, we seeded MDA-MB-231 GFP breast cancer cells in 96-well ULA plates and allowed spheroid assembly for 48 hours. Subsequently, the spheroids were treated with increasing concentrations of the protein kinase inhibitor SSP, and the drug effect was assessed by simultaneously monitoring the cell health and cell death in the spheroids for an additional 48 hours **(Figure 2A)**. Spheroid health was evaluated using the green fluorescence of GFP as an indicator for living healthy cells **(Figure 2B)**. Cell death in the spheroids was assessed using CYTENA's C.LIVE Tox Red reagent, which can only enter dead cells where it binds to DNA and emits red fluorescence **(Figure 2C)**.

Control spheroids that were treated with the vehicle DMSO showed an increase in total green fluorescence, indicating good spheroid health and steady growth. Simultaneously, low values of total red fluorescence over time indicated only minimal cell death. In contrast, spheroids treated with increasing concentrations of SSP showed a drop in total green fluorescence in a dose-dependent manner. Spheroids that were treated with higher concentrations of SSP showed a higher and faster reduction in green fluorescence, indicating the loss of living healthy cells. This was confirmed by the simultaneous dose-dependent increase of total red fluorescence. There, spheroids that were treated with higher concentrations of SSP showed a faster and higher increase in red fluorescence, indicating more cell death in these spheroids.

In this study, the CELLCYTE X imaging system was used to evaluate the cytotoxic effect of a specific drug on cancer cells in a high-throughput manner. Placement inside the incubator and automated data point collection ensured the undisturbed course of the study. It further enabled the continuous documentation of the impairment of the treated spheroids in real time taking in consideration various spheroid health aspects in parallel. The multi-metric analysis of the spheroids enabled simultaneous assessment of both live and dead cells. These complementing analysis metrics provided extra confirmation of the cytotoxic drug effect observed and therefore facilitated the comprehensive assessment of the drug efficacy.

Evaluation of cytotoxic and cytostatic drug effects on tumor spheroids

The assessment of anti-cancer drug efficacy is sometimes further complicated by the different effects drugs may have on cancer cells. Cytotoxic drugs usually induce apoptosis in cancer cells that leads to cell death, whereas cytostatic drugs do not kill cancer cells but instead stop cell proliferation. Conventional chemotherapeutic drugs are commonly described as cytotoxic drugs, but observations have been made that intermediate drug concentrations in some cases also lead to cytostatic effects.[5,6]

To evaluate the effect of two different anti-cancer drugs, spheroids were generated from MDA-MB-231 GFP cells over 48 hours. Subsequently, the spheroids were either left untreated (DMSO) or treated with 3 µM CPT or 3 µM ETO for an additional 120 hours **(Figure 3A)**. CPT and ETO are both topoisomerase inhibitors that cause DNA damage and therefore eventually should lead to apoptosis in the cells. To compare the effect both drugs have on spheroid size, live cells and dead cells, the total spheroid area in the brightfield channel **(Figure 3B)**, the total intensity of GFP in the green fluorescence channel **(Figure 3C)** and the total intensity of C.LIVE Tox Red in the red fluorescence channel **(Figure 3D)** were analyzed, respectively.

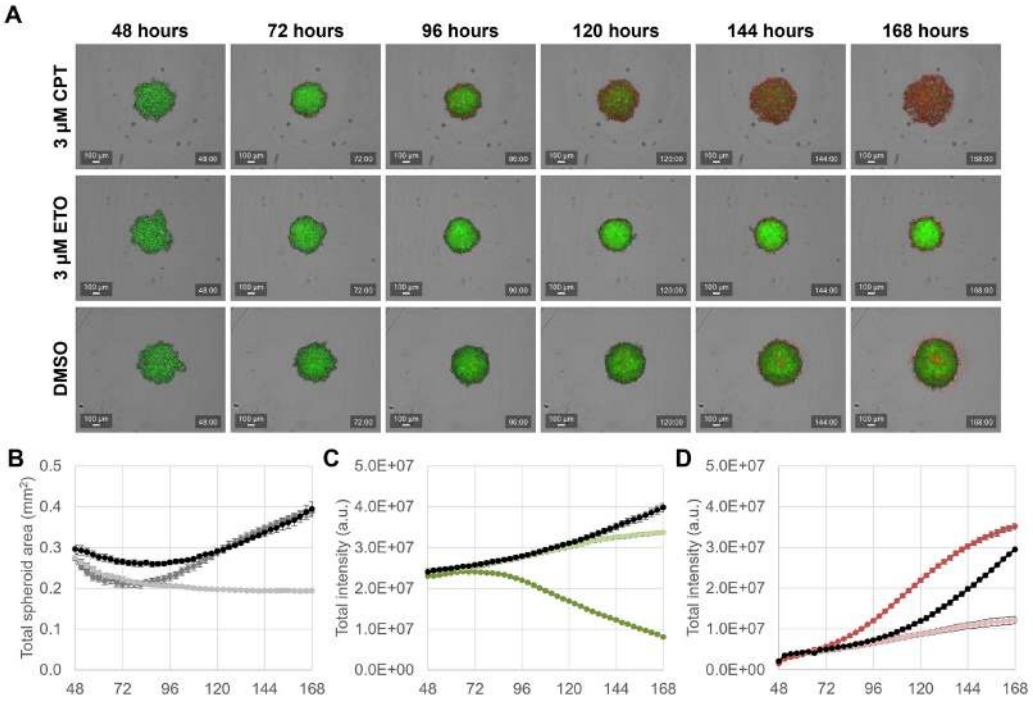


Figure 3: *Cytotoxic and cytostatic effects of anti-cancer drugs. (A) MDA-MB-231 GFP cells were seeded in 96-well ultra-low attachment plates and spheroid formation was allowed for 48 hours. After that, C.LIVE Tox Red and camptothecin (CPT) or etoposide (ETO) were added and the spheroid health was monitored for additional 120 hours at 4X magnification and with the spheroid scan module. Spheroid health was evaluated by (B) total spheroid area, (C) total intensity of green fluorescence and (D) total intensity of red fluorescence using CELLCYTE Studio. Depicted are mean ±SEM, n = 6.*

Spheroids that were exposed to the vehicle DMSO showed a constant increase in total spheroid area and total intensity of green fluorescence, indicating unimpaired spheroid growth through cell proliferation. The total intensity of red fluorescence, which marks dead cells stained by C.LIVE Tox Red, increased slowly over time. This was in accordance with literature stating that healthy spheroids with larger diameter show apoptosis induction in the spheroid core most likely because of nutrient deprivation.[6]

Spheroids that were treated with CPT showed a reduction of the total spheroid area in the brightfield channel within the first 48 hours of treatment. However, afterwards, the total spheroid area increased again and reached values comparable to the DMSO-treated spheroids. This effect is caused by the physical disruption of the dying spheroids by the cytotoxic drug and has been described in literature before.[6,7] This observation suggests that the total spheroid area quantified from the brightfield channel alone might not always be a reliable readout for spheroid health, which is why the GFP fluorescence of living cells was quantified in parallel. A significant decrease of the total green fluorescence intensity was observed, suggesting a loss of living cells in the CPT-treated spheroids. This observation was further corroborated by the increase of total red fluorescence intensity of C.LIVE Tox Red stained cells, confirming the increase of dead cells in the spheroids and the cytotoxic effect of CPT on MDA MB 231 spheroids.

Interestingly, spheroids that were treated with ETO at the same concentration used for CPT showed a different effect even though both drugs have similar mechanisms of action on the molecular level. For the ETO-treated spheroids, after a slight reduction in the first 48 hours of treatment, the total spheroid area in the brightfield channel remained the same, suggesting that either the cell proliferation or the cell health was

impaired. However, the total intensity of the green GFP fluorescence was comparable to the untreated spheroids in the first 72 hours of treatment and stagnated after that time, indicating that the cells in the spheroids were still alive and that the treatment might actually have a cytostatic effect. This was further corroborated by the total intensity of the C.LIVE Tox Red fluorescence, which stayed at a minimum throughout the course of the treatment, indicating no drug-induced cell death and therefore no cytotoxic effect. Interestingly, the red fluorescence signal of the ETO-treated spheroids was even lower than that of the control spheroids, suggesting that either the smaller spheroid size led to less nutrient deprivation or that although the cells in the spheroid core were still alive, they were metabolically less active – an observation that has been described in literature before.[6]

Since the signs of drug efficacy may vary between different drugs,[5–7] finding a suitable combination of readouts that covers various mechanisms of drug action might be challenging. The CELLCYTE X enables the evaluation of multiple metrics in parallel such as total spheroid area for spheroid growth and the total intensity of fluorescence markers for live and dead cells. This simultaneous monitoring provides a clear distinction between cytotoxic and cytostatic drug effects observed. Since the latter has been discussed before to have implications for tumor drug resistance and development of more aggressive phenotypes,[6] it is of great importance during the drug discovery process to be able to make this distinction and evaluate the potential of a new drug with the best comprehensive understanding of the biological processes in mind.

Conclusion

The CELLCYTE X live cell imaging system offers real-time monitoring and analysis of the single-tumor spheroids for the drug discovery process. The studies in this application note demonstrated:

- The robust autofocus algorithm enabled consistent monitoring of scaffold-free tumor spheroid growth over long periods of time in a 96-well plate
- Onboarded analytic software provided measurements such as total area and intensity to facilitate a full assessment of various spheroid growth and health aspects for comprehensive evaluation of drug efficacy
- Clear distinctions between cytotoxic and cytostatic drug effects on the spheroid model were made possible through automatic brightfield and fluorescent image acquisitions and a multi-metrics analysis module

References

1. 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

2. 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

3. Ivascu A, Kubbies M. Rapid generation of single-tumor spheroids for high throughput cell function and toxicity analysis. *SLAS Discovery*. 2006; 11(8): 922-932. DOI: 10.1177/1087057106292763

4. Zanoni M, Piccinini F, Arienti C, et al. 3D tumor spheroid models for in vitro therapeutic screening: a systematic approach to enhance the biological relevance of data obtained. *Scientific Reports*. 2016; 6(1): 19103. DOI: 10.1038/srep19103

5. Rixe O, Fojo T. Is cell death a critical end point for anticancer therapies or is cytostasis sufficient? *Clinical Cancer Research*. 2007; 13(24): 7280-7287. DOI: 10.1158/1078-0432.CCR-07-2141

6. Mittler F, Obeïd P, Rulina AV, et al. High-content monitoring of drug effects in a 3D spheroid model. *Frontiers in Oncology*. 2017; 7: 293. DOI: 10.3389/fonc.2017.00293

7. 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 single-cell 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