

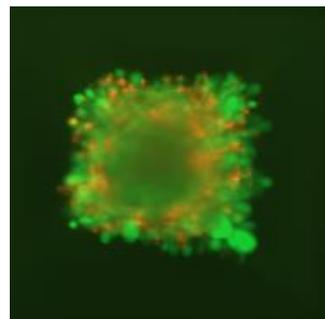
# Analysis of tumor spheroids growing in Kugelmeiers sphericalplates 5D using NYONE® and YT-software®

PHILIPP, L.-M.<sup>1,2</sup>, KRICHEL, T.<sup>1</sup>, CHRISTMANN, T.<sup>1</sup>, GEISEN, R.<sup>1</sup>,  
WERDELMANN, B.<sup>1</sup>, SEBENS., S.<sup>2</sup> & PIRSCH, M.<sup>1</sup>

[1] SYNENTEC GmbH ELMSHORN, GERMANY [2] UKSH KIEL (INSTITUT F. EXP. TUMORFORSCHUNG), GERMANY

## Abstract

Over the last years, it became obvious that the complex nature of cancer is not reflected in the widely used two-dimensional (2D) monolayer cell culture systems. Therefore, three-dimensional (3D) cell culture models became increasingly popular. One of these models is based on the formation of multicellular tumor spheroids. Spheroids strikingly mirror the 3D cellular context of *in vivo* tumors. In order to become an alternative to 2D systems, it must be possible to generate 3D spheroids with a homogenous size to obtain comparable and reproducible results. To solve this challenge, Kugelmeiers developed the sphericalplate 5D. This plate contains a patented microstructure (US Pat. 8,911,690 B2) enabling scientists to grow 9000 single spheroids or cell aggregates within only one 24-well plate. In this application note, we introduce a method for imaging multicellular tumor spheroids grown in the sphericalplates 5D using a NYONE® Scientific SC4 Cell Imager and SYNENTEC's image analysis YT-software®. For this purpose, two different cell lines, HepG2 and HCT116, were used. First of all, handling of the sphericalplates was established. After that, the formation of spheroids was imaged over five days. Lastly, the spheroids were treated with TRAIL or paclitaxel and the drug's effects were visualized and evaluated by Live/Dead-staining using Calcein AM and Propidium iodide.



Application Note  
AN-B241-XIX-01

**KEYWORDS:** fluorescence microscopy, Kugelmeiers, spheroid, 3D cell culture, sphericalplate 5D, HepG2, HCT116, TRAIL, paclitaxel, Calcein AM, Propidium iodide

## Introduction

The complex nature of cancer cells has challenged researchers studying cancer for decades. This complexity is not reflected in the conventional two-dimensional (2D) monolayer cell culture models. Cells cultured in 2D can differ in terms of both physiology and cellular responses compared to cells growing *in vivo*. Therefore, three-dimensional (3D) cell culture models have gained interest in recent years. In these models, cells do not grow as one layer of cells but form a self-organized complex three-dimensional structure. This structure provides a more accurate representation of tumor physiology as occurring in cancer diseases [4]. Spheroid cultures have already been established from cancers including glioma, breast, colon, ovary and prostate cancers, whose biological and biochemical characteristics have been investigated in the last years. Tumor-derived spheroids may prove to be instrumental in a high throughput screening platform for the investigation of cancer stem cells, or for the work with cancer stem cell-related tumor cells found in the circulatory system or body fluids [2]. Thus, the investigation of tumor-derived spheroids may provide invaluable clues to fight cancer [2]. However, in classical spheroid models, where cells are grown on agarose to prevent attachment, they randomly assemble into spheroids of various sizes and are therefore difficult to investigate. In order to analyze and evaluate these spheroids and the effects of potential drugs, it is necessary to generate spheroids of homogenous and reproducible size.

One tool to produce such spheroids is the sphericalplate 5D invented by Kugelmeiers (fig. 1). This 24-well plate contains 750 round-bottomed microwells per well with a high-end ultra-low attachment nanocoating to allow ideal cell aggregation and prevent settling of cells in other areas than the microcavities.

than the microwells. The close proximity of the microcavities within the wells enables efficient cell-cell communication in 3D cell culture. The plate is made of COC (cycloolefin-copolymer) for optimal imaging properties. Kugelmeiers sphericalplate 5D is a platform which scientists can use to generate uniform, size-controlled

cell clusters (spheroids) needed for scalable, reliable and safe application of cell therapy or other applications like assays that require a high amount of spheroid samples produced in a small space. Besides this, it can be used for organoid formation, 3D bioprinting, cancer research and drug screening. A great asset regarding the handling of the plates is the possibility to change the cell culture media or treatments without disrupting the cells or spheroids, what makes them easy to use, even for long term experiments.

A major challenge in the fight against cancer is the screening for new anti-cancer drugs. Many promising drugs in preclinical trials fail to be successful in the clinic [1,6]. One explanation might be the use of 2D cell culture models in many screening approaches. The use of 3D models is a novel and encouraging approach to drug screening. The most important group of drugs in oncology is chemotherapeutics, e.g. paclitaxel which is an anti-proliferative agent used to treat a number of cancers.

A more specialized drug is the cytokine tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). TRAIL was shown to selectively trigger cancer cell death by binding to death

receptors and was thus evaluated in preclinical trials.

Furthermore, TRAIL was initially reported to kill tumor cells more readily than normal cells, suggesting that this cytokine could be of great interest in cancer treatment [3].

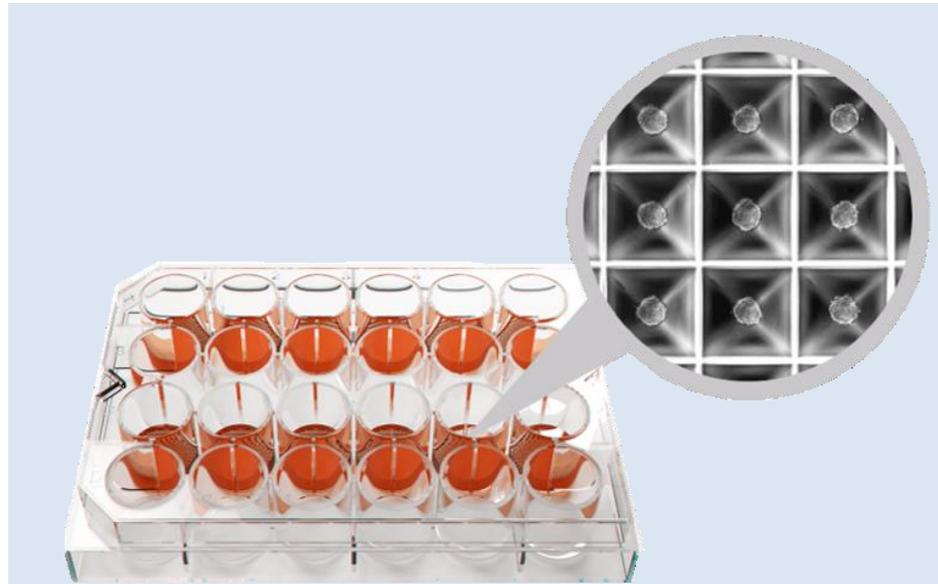


Fig. 1: Image of the Kugelmeiers 5D sphericalplate. The image shows the sphericalplate 5D and a close up image of the microwells containing spheroids.

One option to evaluate the effect of drugs on cells or spheroids is a Live/Dead assay using Calcein AM and Propidium iodide (PI). Calcein AM is a cell-permeable dye that can be used to determine cell viability. In living cells, the non-fluorescent Calcein AM converts to (green-) fluorescent Calcein after hydrolysis by intracellular esterases [5,7]. PI is a red-fluorescent nuclear stain commonly used to detect dead cells in a population as it does not enter intact viable cells [5,7].

In this application note, the growth of spheroids from different cell lines (HepG2, HCT116) in Kugelmeiers sphericalplates 5D was imaged to show the method's capabilities

to generate a high amount of spheroids in a homogenous size and morphology to be used in downstream applications. Moreover, the morphology of generated spheroids after treatment with paclitaxel or TRAIL was analyzed using a Live/Dead assay with Calcein AM/PI.

Working with Kugelmeiers sphericalplates 5D in combination with NYONE<sup>®</sup> is a practical and easy to handle method for spheroid-forming experiments. The SBS format of Kugelmeiers sphericalplate 5D and the high throughput imaging capabilities of NYONE<sup>®</sup> and CELLAVISTA<sup>®</sup> are a combination that can potentially be integrated in ultra-high-throughput systems and automated environments.

## Material & Methods

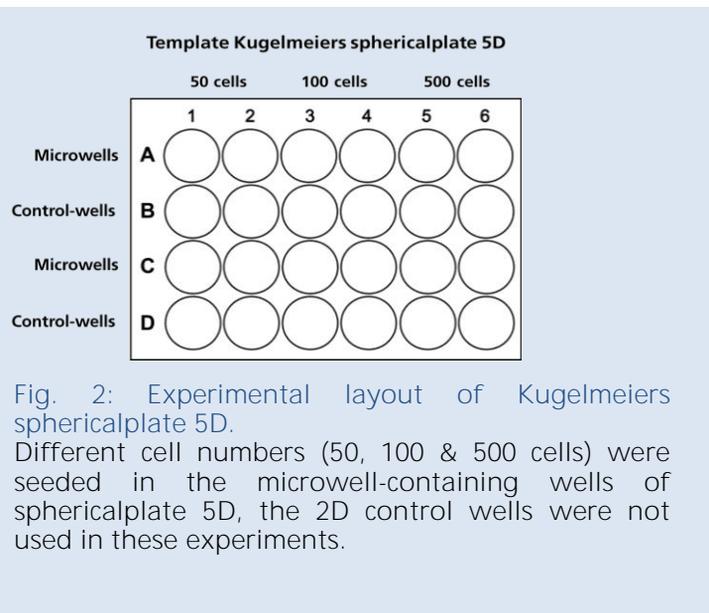
### Material:

- HCT116 (adherent monolayer-forming cells; human colon carcinoma cells)
- HepG2 (adherent monolayer-forming cells; human liver carcinoma cells)
- RPMI 1640 with 10 % FBS, 1 % sodium pyruvate, 1 % L-glutamine
- Phosphate buffered saline (PBS)
- Trypsin 1 % in PBS
- Paclitaxel 1  $\mu\text{M}$  in  $\text{H}_2\text{O}$
- Calcein AM (BioLegend cat. # 425201)
- Propidium iodide (BioLegend cat. # 421301)
- Recombinant human sTRAIL/ Apo2L 100 ng (Peprotech cat. # 310-04)
- Trypan Blue 0.02 % stock solution
- Transparent 96-well plate (e.g. Corning cat. # 3690)
- Kugelmeiers sphericalplate 5D
- One of SYNENTEC's imaging systems (here: NYONE<sup>®</sup> Imaging System)
- SYNENTEC's YT-software<sup>®</sup>

### Methods:

#### Counting and seeding of cells

The cells were counted via NYONE's cell viability assay using SYNENTEC's Trypan blue application (TN-B004-XV-17) and the required amount of cells per milliliter was calculated based on the results. Before seeding of cells into the sphericalplate it is important to pipette 0.5-1 ml cell culture medium to the plate and to centrifuge it with 500 x g for 3 min to avoid trapping of air bubbles in the microwells. A cell concentration of  $3.75 \cdot 10^4$  cells (50 cells/ microwell),  $7.5 \cdot 10^4$  cells (100 cells/ microwell) and  $3.75 \cdot 10^5$  cells (500 cells/ microwell) was seeded per well containing the microwell inlay (fig. 2). The plate was moved gently to homogeneously distribute the cells before they were incubated for 24 h to allow cell sedimentation prior to the first measurement.



## Brightfield imaging and analysis of spheroids

For brightfield imaging and quantification of spheroids, SYNENTEC's *Single Cell Cloning* application of YT-software® was chosen. To achieve optimal results during brightfield measurements of spheroids with NYONE® the settings of the imaging parameters in tab. 1 are recommended.

The plates were imaged using the "Very Gentle" Mode of NYONE® Scientific to cause little to no movement of media during the measurement. Each measurement was conducted in only 11 minutes.

Tab. 1: Optical and processing settings for *Single Cell Cloning* application with brightfield

Parameter	Settings
Auto focus	Each well
Intensity	100 %
Exposure time [ms]	> 5
Object min. compactness Object max. compactness	0.1 1.0
Object min. size [ $\mu\text{m}^2$ ] Object max. size [ $\mu\text{m}^2$ ]	3000 Maximum
Object min. longishness Object max. longishness	50 100
Object min. intensity Object max. intensity	0 255
Colony min. area [ $\mu\text{m}^2$ ]	2000
Colony min. quality [%]	60
Multiple cell layers expected	✓
Internal 2 x 2 binning	✓

## Treatment of spheroids

After seeding cells to Kugelmeiers spherical plates 5D, the spheroids grew to a required size of 150-200  $\mu\text{M}$ , monitored by NYONE® and subsequently treatment was started. In these experiments, HepG2 cells were treated with 1  $\mu\text{M}$  paclitaxel for 24-48 hours and HCT116 cells were treated with 100 ng/ml TRAIL for 24 h. The concentration of reagents was calculated for 2250  $\mu\text{l}$  solution per well, because 250  $\mu\text{l}$  of a prepared stock solution was added to the wells containing 2 ml cell culture medium.

## Live/Dead-staining with Calcein and Propidium iodide

Stock-solutions of the dyes were prepared in advance and 1 ml of the cell culture medium was discarded from every well very carefully. Pipetting and discarding the medium can cause turbulences within the wells and thus removal of spheroids from the microwells. For Live/Dead staining, stock solution of Calcein AM (1 mM) was diluted 1:500 to reach a concentration of 2  $\mu\text{M}$  in the staining solution. PI stock solution (0.5 mg/ml) was diluted 1:50 for a concentration of 10  $\mu\text{g/ml}$ . 250  $\mu\text{l}$  of staining solution was added to each well containing 1.25 ml of medium. Thus, the final concentration of Calcein AM was 0.33  $\mu\text{M}$  and of PI 1.67  $\mu\text{g/ml}$  (calculated for 15 wells: 6 x microwell (2x), 2x control (2x) = 14 wells + 1 excess).

Measurement of Live/Dead-staining using NYONE<sup>®</sup> Scientific and YT-software<sup>®</sup>

After an incubation time of 60-120 min (recommended), the fluorescence signals were imaged in the *Confluence (2F)* application of NYONE<sup>®</sup> with the settings displayed in tab. 2+3.

To avoid bleaching during optical setup it is recommended to use the "Snapshot" function rather than "Liveview".

To check the optical settings like exposure and focus offset in every channel, taking a "Snapshot" (function is available next to the "Liveview"-button) is recommended.

Settings for image processing (tab.3) can be stored in templates, alongside all other measurement parameters. Generating templates in YT-software is key to automation and reproducible result generation. Image processing was conducted using *Confluence 2F* (tab. 3).

The image processing operator is optimized to detect cell covered area in BF and FL.

Tab. 2: Excitation and emission settings for the Live/Dead-staining

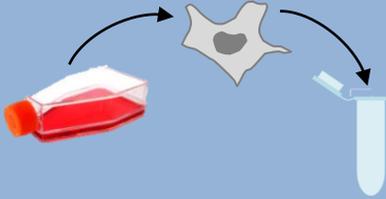
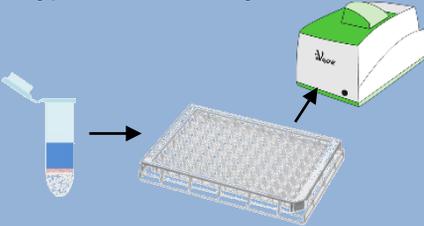
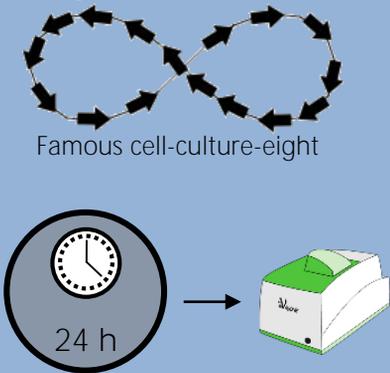
Live/Dead-staining	Excitation	Emission
Calcein	494 nm (Blue)	517 nm (Green)
Propidium iodide	535 nm (Lime)	617 nm (Red-LP)

Tab. 3: Optical and processing settings for the measurement of Live/Dead-staining using the *Confluence (2F)* application

Parameter	Settings
Auto focus	Each well
Intensity	100 %
Exposure time [ms]	> 10
Expected Hole Max. Intensity	35
Object min. size [ $\mu\text{m}^2$ ] Object max. size [ $\mu\text{m}^2$ ]	3000 maximum
Object min. compactness Object max. compactness	0.1 1.0
Object min. longishness Object max. longishness	50 100
Object min. intensity Object max. intensity	0 255
Object min. contrast Object max. contrast	0 1
Object min. std. dev. Object max. std. dev.	0 255
Object min. roughness Object max. roughness	0 500
Multiple cell layers expected	✓
Internal 2 x 2 binning	✓

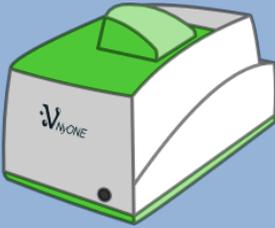
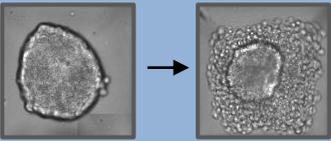
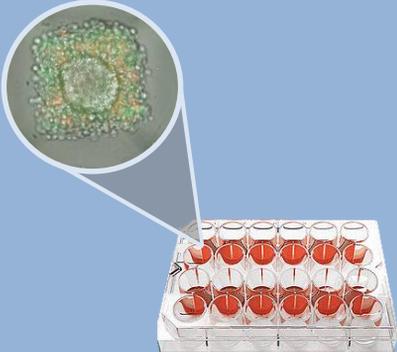
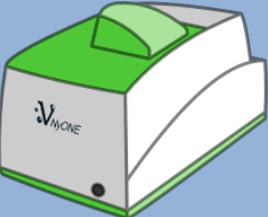
## Experimental workflow

Tab. 4: Overview of experimental steps

Step	Procedure	Information
<p>Cell preparation</p> 	<p>Remove medium from the cells, wash with 5 ml PBS and detach cells via incubation with 5 ml Trypsin (5 min, 37 °C).</p> <p>Stop trypsin reaction by adding 5 ml full cell culture medium (containing FBS).</p>	<p>Step is necessary for adherent cell lines.</p> <p>To prevent cell re-attaching during the following steps, place remaining cells in culture flask or 15 ml centrifugation tube on a rocker in the incubator or softly shake it from time to time.</p>
<p>Trypan Blue viability cell count</p> 	<p>Dilute 25 µL cells with 225 µL PBS (1:10 dilution) and add 250 µL Trypan Blue stock solution [0.02 %] (1:20 final cell dilution).</p> <p>Measure plate with NYONE®-Trypan Blue Viability Application.</p>	<p>For detailed information please refer to SYNENTEC's Technical Note for Trypan Blue measurements (TN-B004-XV-17).</p>
<p>Preparing Kugelmeiers sphericalplate 5D</p> 	<p>Add 0.5-1 ml cell culture medium to the wells containing the microwell inlays and centrifuge the plate with 500 x g, 3 min.</p>	<p>Step is necessary to avoid air bubbles in the microwells of the Kugelmeiers plates, which could prevent the cells from settling into the microwells.</p>
<p>Seeding cells</p>  <p>Famous cell-culture-eight</p> <p>24 h</p>	<p>Dilute cell suspension to a required concentration of cells/ 1 ml (recommended 50-500 cells/ microwell), pipette cell suspension into the wells and fill empty wells with PBS or medium to avoid evaporating, shake plate gently.</p> <p>Let the plates sit for 24 h to get the cells sedimented before the first measurement (do NOT centrifuge).</p>	<p>Cell suspension should not exceed the volume of 1 ml, because the wells already contain 1ml medium from the preparing step.</p> <p>Move the plate gently to homogeneously distribute the cells by forming the „cell-culture-eight“ (see picture).</p> <p>After sedimentation of the seeded cells for 24 h, measure the plate with the <i>Single Cell Cloning</i> application and the brightfield channel of the YT-software®.</p>

## Experimental workflow

Tab. 4.1: Overview of experimental steps

Step	Procedure	Information
<p>Daily measurement</p> 	<p>For the daily measurement, open the YT-software®, go to <i>Single Cell Cloning</i> and choose „open experiment“ to open your saved template.</p>	<p>To evaluate e.g. the colony count, the area of colonies, growth curves, growing of spheroids, to create a gallery for selected spheroids over time or histograms it is necessary to measure at regular intervals.</p> <p>Be very careful carrying the plate around to avoid turbulences in the microwells.</p>
<p>Spheroid treatment</p> 	<p>Add 250 µl of reagents to the wells.</p> <ul style="list-style-type: none"> <li>• HepG2: 1 µM paclitaxel (final concentration)</li> <li>• HCT116: 100 ng/ ml TRAIL (final concentration).</li> </ul>	<p>To avoid mixing up the spheroids in the microwells, be very careful adding the treatments.</p>
<p>Live/Dead-staining</p> 	<p>The Live/Dead-staining is carried out by Calcein (live) and Propidium iodide (dead) dye.</p> <p>Discard 1 ml of cell culture medium from the wells (1.25 ml remain) and replace it with 250 µl staining solution (45 µl Calcein AM + 450 µl PI + 3255 µl medium) for a final concentration of</p> <ul style="list-style-type: none"> <li>• Calcein AM 0.33 µM</li> <li>• PI 1.67 µg/ ml</li> </ul> <p>≅ final volume 1.5 ml</p> <p>→ Incubation time 60-120 min</p>	<p>To avoid mixing up the spheroids in the microwells, be very careful discarding the medium and replacing it with the staining solution.</p>
<p>Measurement of FL signals</p> 	<p>The fluorescent signals of the Live/Dead-staining are imaged with the optical settings and processing of the <i>Confluence (2F)</i> application of the YT-software®,</p>	<p>For the measurement it is not necessary to discard or to change the medium.</p>

## Results

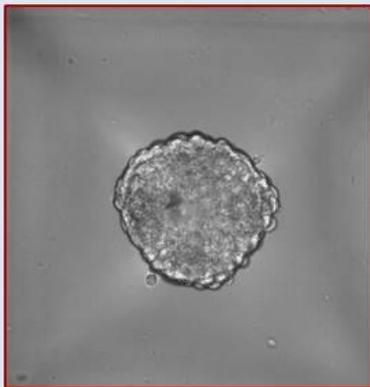
1 NYONE® can image and analyze the growth of spheroids in the Kugelmeiers plates in a user-friendly and automatable manner

Measurements of Kugelmeiers spherical plates 5D and evaluation of spheroids in BF were carried out using SYNENTEC's *Single Cell Cloning* application of YT-software®. The application was originally developed to detect and analyze clones derived from single cells over time. However, it is also capable to detect spheroids non-invasively.

Image processing is used to detect cell-covered area in the high-resolution images. The objects detected and labelled by image processing are called "blobs". The image processing settings can be adjusted by visually inspecting of results during experimental setup. These very stringent and accurate settings will be stored in templates for further use and automatability.

Very important for the analysis of the spheroids is the confluence and the detected size of the "blobs". Based on these results, YT-software® calculates the area of colonies (spheroids). After processing the imaged wells, the image processing overlay shows the confluence (yellow image detection) and the calculated "colony" (blue image detection, figure 3) of the spheroid. In principle, it is possible to evaluate every single spheroid of each microwell. Every spheroid can be represented in a pictures series by the gallery function of YT-software®. These show the development over time (fig. 4) and can potentially be used to confirm morphological changes.

### Before processing



### After processing

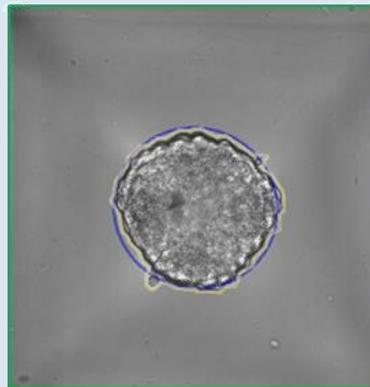


Fig. 3: SYNENTEC's image analysis detects spheroids. Brightfield image of spheroids from cancer cells. *Single Cell Cloning* application in YT-software® recognizes confluence (yellow line) and calculates a „blob“ (blue line) representing the spheroid's area.

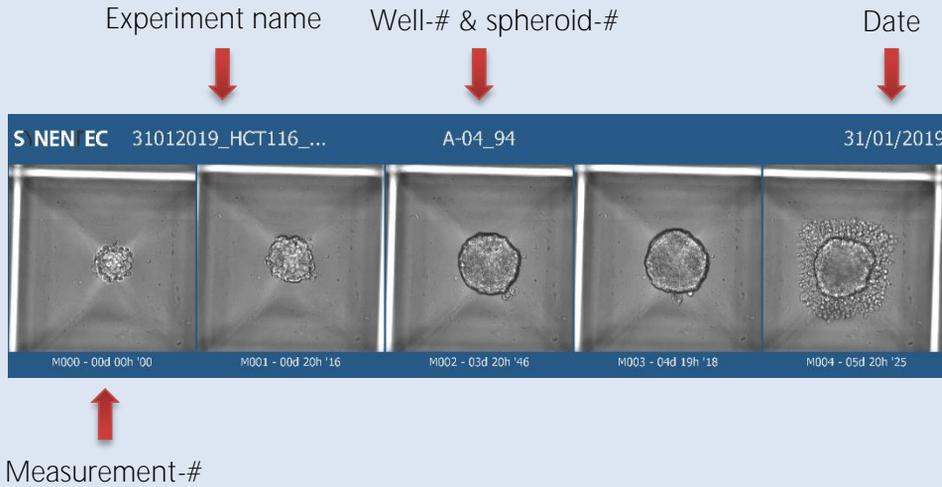


Fig. 4: SYNENTEC's clone gallery shows the development of single spheroids over time. Brightfield images of spheroids from HCT116 cells. The figure was exported using the gallery function. Additionally to the images of each measurement, it contains information about the experiment name, well and spheroid number as well as measurement dates.

24 h hours after seeding, the plates were imaged using SYNENTEC's *Single Cell Cloning* application of YT-software® to capture M000 (the first measurement). The spheroid growth over time can easily be analyzed and documented in the brightfield channel of NYONE®. Figures 5A and 5B show spheroid formation of HepG2 and HCT116 cell lines over time.

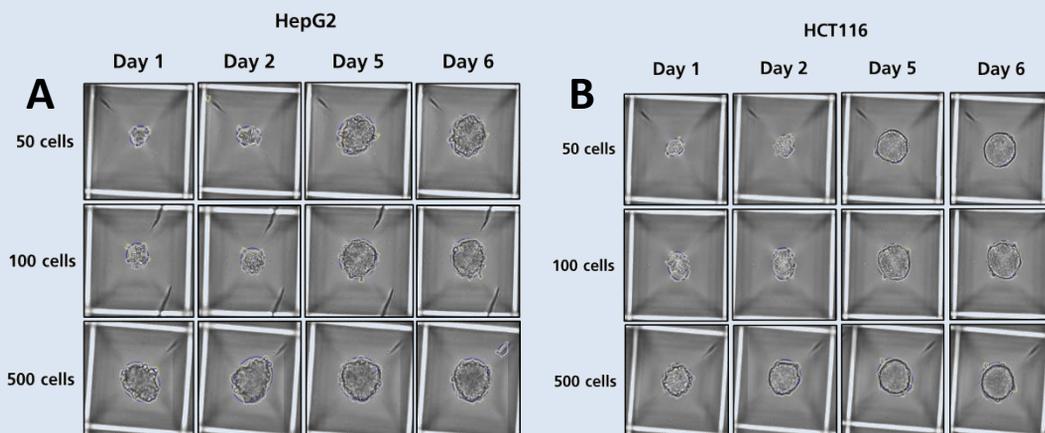


Fig. 5: Measurement and analysis of spheroids over time at different days after seeding. 50, 100 or 500 HepG2 (A) or HCT116 (B) cells were seeded per microwell of sphericalplate 5D. Brightfield images were taken on different days after seeding and analyzed by YT-software® (yellow: cell confluence; blue: "colony" ). High-resolution optics enable automated detection of spheroids as early as 24 h after seeding.

As seen in fig. 6A, HepG2 cells produced spheroidal aggregates, whereas HCT116 cells formed perfectly spherical and compact spheroids (figure 6B).

Interestingly, regardless of inoculation density, the spheroids had nearly the same size and shape after five days of growth (figures 6A + 6B). Due to the design of sphericalplates 5D spheroid size is limited to approximately 400-450  $\mu\text{M}$ .

When the spheroids reach this threshold, proliferation seems to rest although the spheroids could become increasingly dense rather than expanding. This is also shown by growth curves of the spheroids. Figure 6A shows the layout of a 24-well plate representing Kugelmeiers sphericalplate 5D. Row A and C are contain microwell inlays, row B and D are control wells which were not imaged in this experiment, but can be used as a 2D control. The spheroids in the columns seeded with 50 and 100 cells (column 1-4) increase their size, while column 5 and 6, seeded with 500 cells, are stagnating over time. These results are even more obvious in graphs generated from the exported data for the area of colonies from the YT-software<sup>®</sup> (fig. 6 B). While the area of colonies clearly differs in the beginning, according to the seeded cell density, it reaches a peak value for all three densities after five days.

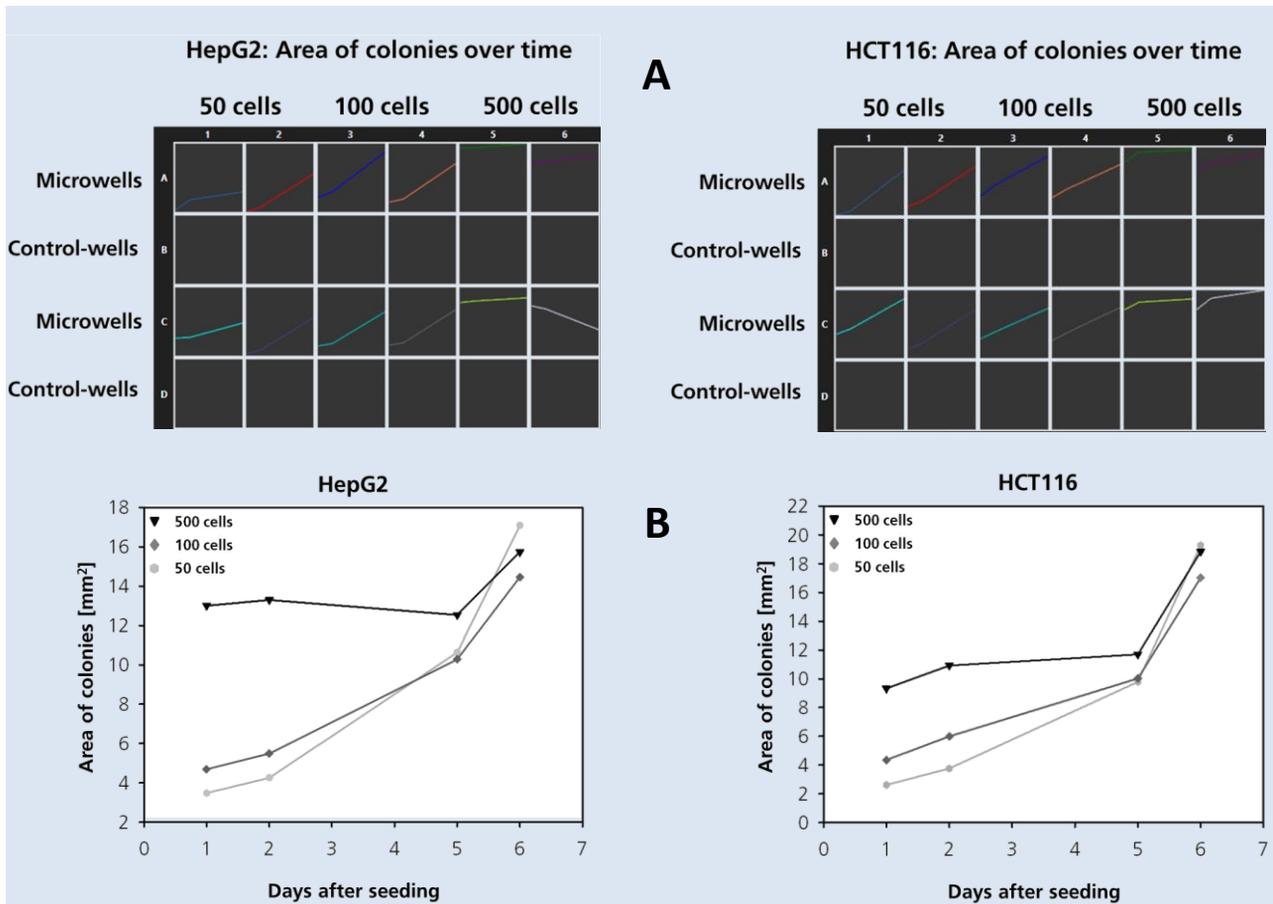


Fig. 6: The development of the spheroids over time can be visualized in growth curves.

A) The growth of the spheroids was monitored by NYONE<sup>®</sup>. The area of colonies was used to generate growth curves directly in YT-software<sup>®</sup>.

B) The data was exported and used to create growth curves using SigmaPlot. Each dot represents the mean of duplicate wells from two independent experiments.

## 2 Spheroids can change their morphology upon treatment with therapeutic agents

To show that spheroids react to drugs, two different treatments corresponding to the cell lines nature were chosen (fig. 7). HepG2 cells are known to be sensitive to paclitaxel in 2D cultures (personal communication with Nina Hedemann). Thus, they were treated with 1  $\mu\text{M}$  paclitaxel for 48 h. HCT116, on the other hand, are responsive to TRAIL treatment in 2D cultures (personal communication with Ania Trauzold). Therefore, HCT116 cells were treated with 100 ng/ml recombinant human sTRAIL/ Apo2L (TRAIL) for 24 h.

Brightfield analysis of HepG2 cells did not display a strong reaction to treatment. Only some cells appeared at the edge of the spheroids so that they had a rougher morphology. On the other hand, HCT116 cells showed a very clear reaction to treatment with TRAIL. A large cell layer of cells surrounding the spheroid formed and additionally the spheroids became visibly smaller, probably a result of dead cells detached from the aggregate.

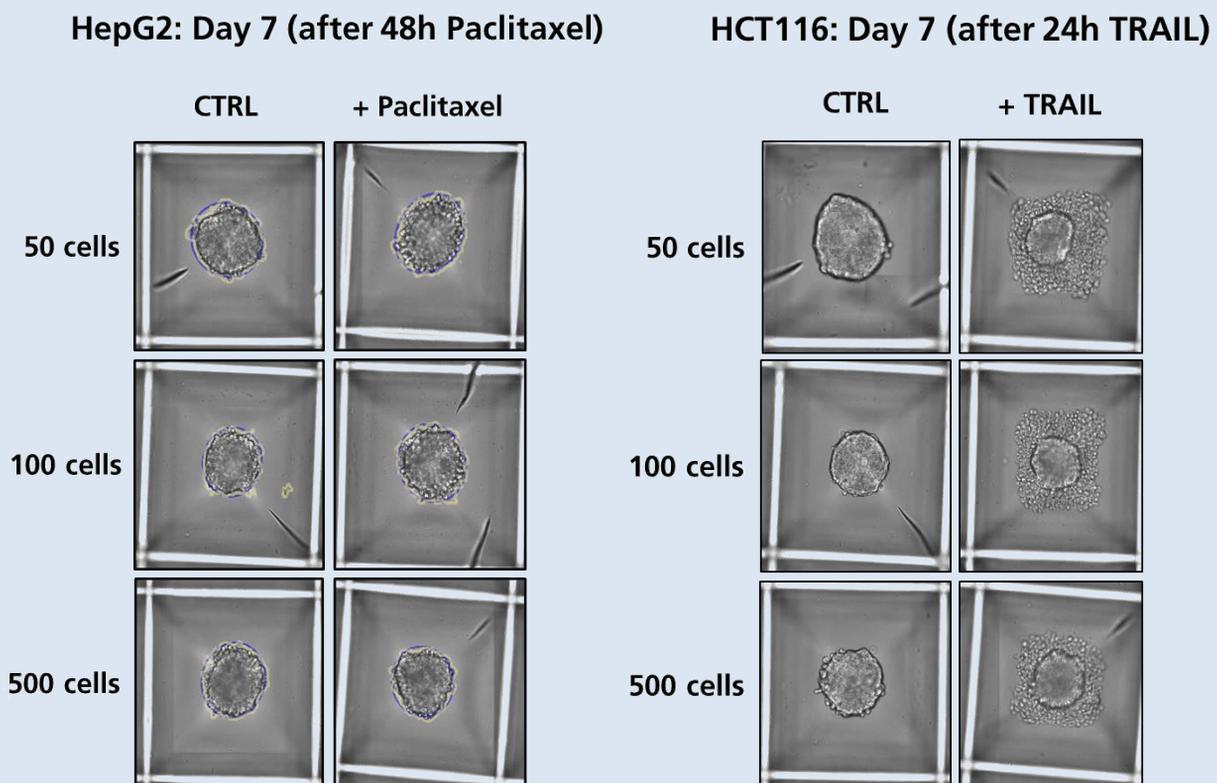


Fig. 7: NYONE<sup>®</sup> could monitor morphological changes after treatment with paclitaxel or TRAIL.

Spheroids were generated from HepG2 and HCT116 cells. On day 5 or 6, the cells were treated with 1  $\mu\text{M}$  paclitaxel (HepG2) or 100 ng/ml TRAIL (HCT116). On day 7, the plates were imaged in NYONE<sup>®</sup>. (CTRL= Control, untreated cells)

### 3 Cell death of spheroids can be analyzed by Live/Dead-analysis through Calcein and Propidium iodide staining

To analyze cell death of spheroids, we used a Calcein/PI staining. During protocol development, cells were repeatedly imaged every 30 minutes to evaluate the right time frame for both dyes to exhibit maximum FL intensity. An incubation time of 90 minutes (at 37 °C) showed optimal signal intensities in our setting. Figure 8 shows the staining of HepG2 cells on day 7 of spheroid formation and after 48 h treatment with paclitaxel. The staining

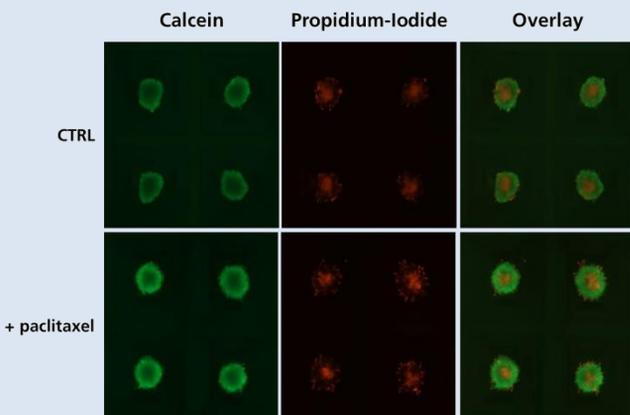
intensity of Calcein is stronger at the periphery of the aggregates. This is not surprising as the periphery is assumed to be the proliferating area of the spheroids and may thus contain more viable cells. However, it cannot be ruled out that the dye does not penetrate completely into the spheroids/aggregates or that the excitation or emitted light is weakened by other cells in the three-dimensional structure. On the other hand, the core of the spheroids shows no Calcein fluorescence, but a clear PI-staining, showing an accumulation of dead cells inside. These results underline the

hypothesis that some spheroids are composed of a necrotic core and a proliferating outer region. After treatment with paclitaxel more dead cells were detected around the aggregate.

Figure 6 shows the staining of HCT116 cells on day 7 of spheroid formation and after 24 h treatment with TRAIL. The pictures of the untreated cells (CTRL) solely show Calcein-stained, viable cells. No PI-stained, dead cells could be detected. Most likely, these spheroids did not contain a necrotic core. However, it may be the case that HCT116 cells form firmer and more impermeable, impenetrable spheroids, so that the dye could not enter the core of the aggregate.

After treatment with TRAIL, however, a strong PI signal could be detected. This signal was mainly found outside of the original spheroid.

HepG2 (Day 7) after 48h paclitaxel + Live/Dead-staining



HCT116 (Day 7) after 24h TRAIL + Live/Dead-staining

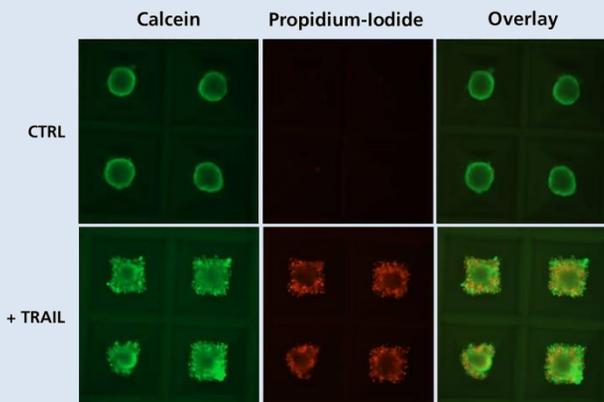


Fig. 8: The effect of drugs can be analyzed by a Live/Dead assay using Calcein AM and Propidium-iodide.

Spheroids were generated from HepG2 or HCT116 cells and grown for five or six days. Subsequently, they were treated with 1 μM paclitaxel (HepG2) or 100 ng/ml TRAIL (HCT116). On day 7, all spheroids were stained with Calcein AM (stains living cells) and Propidium-iodide (stains dead cells) for 90 min. After incubation, they were imaged in NYONE®. Four representative spheroids are shown. (CTRL= Control, untreated cells)

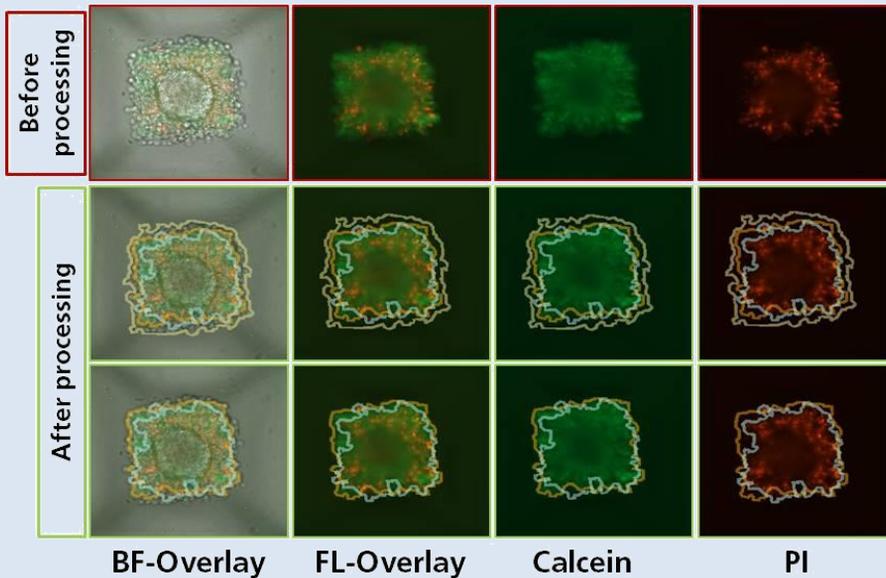


Fig. 7: Live/Dead assay analysis in *Confluence (2F)* wizard of YT - software®.

Brightfield (BF) and fluorescent (FL) images of spheroids from HCT116 cells after FL staining. The upper pictures show raw images. The second and third row show graphic overlays based on results from BF (yellow), Calcein (orange) and PI (blue) image processing.

Some spheroids are very solid and impermeable/ impenetrable; others are more like loose aggregates, with the consequence of different penetrations of the dye depending on the spheroids. NYONE® Scientific SC4 and YT-software® are capable to not only detect and analyze the spheroids in BF. It is possible to process different FL signals in different channels' individually or in conjunction. The software recognizes the different fluorescent stains in the cells or spheroids, processes and evaluates them (fig. 9) across the channels using *Confluence (2F) image* processing.

Figure 10 shows the processed and evaluated data from untreated (CTRL) and treated (TRAIL) HCT116 cells (50 cells) after PI staining. Obviously, the PI-positive cells are increased after TRAIL treatment, indicating that more dead cells were present.

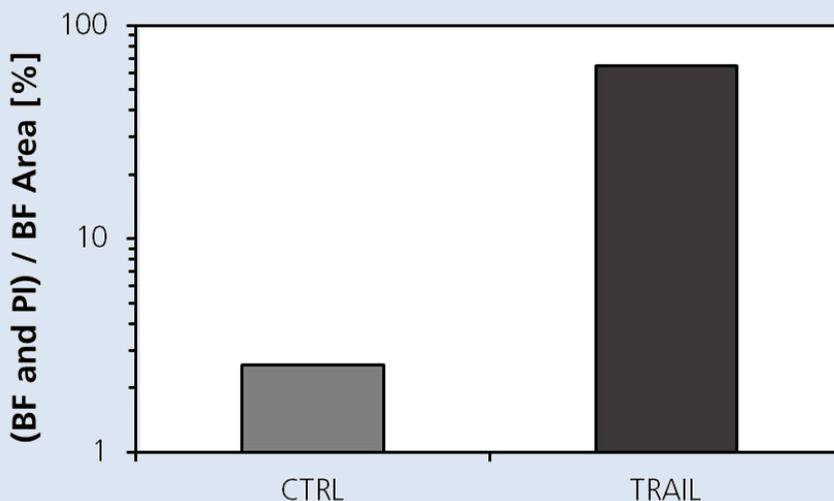


Fig. 10: TRAIL increased the size of the PI-positive area.

Spheroids generated from HCT116 cells were treated with 100 ng/ml of TRAIL for 24 h and stained with Calcein AM and PI. The fluorescence was imaged with NYONE® and the images analyzed with *Confluence (2F)* application. The data was exported and analyzed with SigmaPlot. Here, the percentage of the PI-positive area is shown for untreated (CTRL) or TRAIL-treated cells. The mean of duplicate wells is shown.

## Conclusion & Outlook

The experiments in Kugelmeiers sphericalplate 5D in combination with the NYONE<sup>®</sup> are a very potent and easy-to-use tool for working with spheroid systems. Depending on the amount of seeded cells, it is possible to grow a 3D spheroid cell culture model in a few days. The NYONE<sup>®</sup> and the YT-software<sup>®</sup> are very intuitive and easy to handle.

As soon as a template is created, starting the measurement only takes minutes, just like the data handling and export which is made very easy and automatically. Laser-based autofocusing is extremely precise and adjustable to ensure optimal imaging of samples. Image processing across every channel is a powerful and robust tool to generate meaningful data.

NYONE<sup>®</sup> Scientific SC4 uses highly sensitive 16 bit sCMOS technology at a high dynamic range and precision.

Future applications with Kugelmeiers sphericalplates 5D are planned as well as experiments with ULA (ultra-low attachment) plates for bigger spheroids, generated using 5D plates.

Kugelmeiers sphericalplates 5D in combinations with the NYONE<sup>®</sup> and the YT-software<sup>®</sup> is a space- and time-saving method and a very easy-to-use alternative to other spheroid forming methods like e.g. hanging drops technologies.

## SPHERICALPLATE 5D<sup>®</sup>

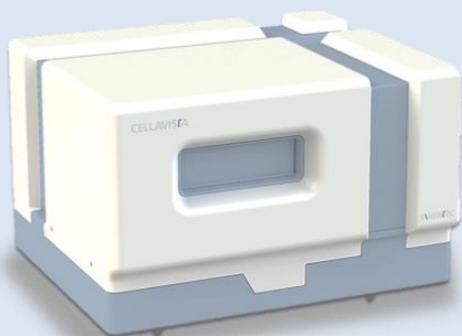
### 3D cell culture rEvolution<sup>®</sup>



### Acknowledgements

These experiments were carried out with kind support of Kugelmeiers AG, Zurich, Switzerland, who also provided sphericalplates 5D. Moreover, we thank Nina Hedemann and the group of Ania Trauzold for support in the establishment of the protocol.

All applications are compatible with all models of NYONE® and CELLAVISTA® and are readily available in YT-software®.



CELLAVISTA® 4



NYONE®

## References

- [1] Cook, N., Jodrell, D. I., & Tuveson, D. A. (2012). Predictive in vivo animal models and translation to clinical trials. *Drug Discovery Today*, 17(5–6), 253–260. <https://doi.org/10.1016/j.drudis.2012.02.003>
- [2] Ishiguro, T., Ohata, H., Sato, A., Yamawaki, K., Enomoto, T., & Okamoto, K. (2017). Tumor-derived spheroids: Relevance to cancer stem cells and clinical applications. *Cancer Science*, 108(3), 283–289. <https://doi.org/10.1111/cas.13155>
- [3] Lalaoui, N., Micheau, O., Mérino, D., Solary, E., & Morizot, A. (2007). TRAIL in cancer therapy: present and future challenges. *Expert Opinion on Therapeutic Targets*, 11(10), 1299–1314. <https://doi.org/10.1517/14728222.11.10.1299>
- [4] Mandavilli, B. S., Neeley, C., Bates, MK., & Persmark, M. (2015). Trends & Developments in BioProcess Technology. *BioProcessing Journal*, 14(3). ISSN 1538-8786
- [5] Mitlo, T., Hesley, J., Cromwell, E. F., Sirenko, O., Owens, W., & Luke, S. (2015). High-Content Assays for Characterizing the Viability and Morphology of 3D Cancer Spheroid Cultures. *ASSAY and Drug Development Technologies*, 13(7), 402–414. <https://doi.org/10.1089/adt.2015.655>
- [6] Takimoto, C. H. (2001). Why drugs fail: Of mice and men revisited. *Clinical Cancer Research*, 7(2), 229–230
- [7] Wang, S., Zhang, C., Li, Y., Li, P., Zhang, D., & Li, C. (2018). Anti-liver cancer effect and the mechanism of arsenic sulfide in vitro and in vivo. *Cancer Chemotherapy and Pharmacology*, 83(3), 519–530. <https://doi.org/10.1007/s00280-018-3755-9>