NYONE® Scientific and YT-SOFTWARE® reliably image and analyze spheroids grown in ultra-low attachment plates

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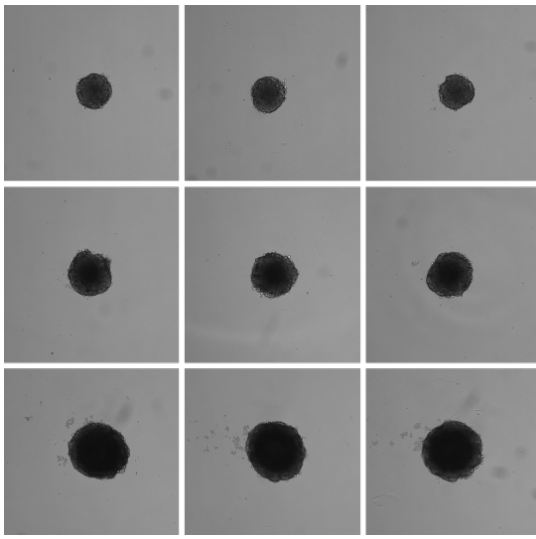
ABSTRACT

Three-dimensional (3D) cellular models provide a physiologically more relevant context than two-dimensional 2D cell culture models. One widely used 3D model is the multicellular spheroid model. Spheroids can be generated with different methods. One of them is seeding cells into ultra-low attachment (ULA) plates with a round or v-shaped bottom. However, due to this bottom, automated microscopy as used in high-throughput or high-content screening can be challenging. In this application note, we describe monitoring of tumor spheroid growth in 96 well ULA plates with our imager NYONE® Scientific and its YT-SOFTWARE®. Using YT-SOFTWARE®'s Spheroid Quantification application, we were able to image and analyze different cell lines seeded in various cell numbers into plates from four manufacturers. Monitoring of the development of each spheroid over time was enabled by the automatic generation of time charts and galleries. Thus, spheroids grown in ULA plates and analyzed with our imagers provide an interesting tool for high-throughput drug screening.



KEYWORDS: 3D, SPHEROID, DRUG SCREENING, HIGH THROUPUT SCREENING, CANCER RESEARCH

IMAGE AND EVALUATE MORE SPHEROIDS IN LESS TIME

Throughput & Convenience
- Image spheroid formation over time in a high-throughput manner (~1,5 min for 96 wells)
- Use ULA plates from the manufacturer of your choice
- Seed cells, start automation, walk away, get results (with our automation system)

Resolution Proves
- Observe the morphology of your spheroids in high-quality pictures
- Automatically analyze the average spheroid diameter to compare different conditions
- Get a quick overview with time charts, heat maps, or spheroid galleries

Master your challenges
- Keep your spheroids intact with gentle plate movements (harmonic motion)
- Benefit from our experience and get a lot of helpful tips from our customer support

INTRODUCTION

Tissues have a complex structure that is not accurately represented in traditional two-dimensional cell culture models. As a result, three-dimensional cell culture models, such as spheroids, have gained increasing attention in recent years [1]. Spheroids are spherical structures formed when cells are cultured in a non-adherent manner, allowing them to aggregate due to their tendency to adhere to each other. They can be created from a variety of cell types and mimic the microenvironment of various tissue types [2]. Multicellular spheroids contain multiple cell types and are organized in a way that mimics the 3D organization of tissues in the body. As a result, multicellular spheroids have been shown to be a useful model for studying a variety of biological processes and have been used in a wide range of applications, including the screening of potential drug candidates and the study of drug delivery. Classical spheroid models are typically generated by growing cells on agarose, which prevents attachment to the surface of the culture dish. However, these models have limitations because the cells randomly assemble into spheroids of varying sizes that float on the surface in different focal planes, making them difficult to investigate. In order to use spheroids as a model for drug screening, it is necessary to generate spheroids of uniform and reproducible size [3] and ensure they are in the same focal plane if imaging is the chosen method of analysis. Ultra-low attachment plates with U- or V-shaped bottoms have been developed to achieve this, but imaging can be challenging due to the round bottom. In this study, we evaluated the suitability of ultra-low attachment U-bottom plates from different manufacturers for imaging with our NYONE® imager in order to enable high-throughput analysis of tumor spheroid characteristics.

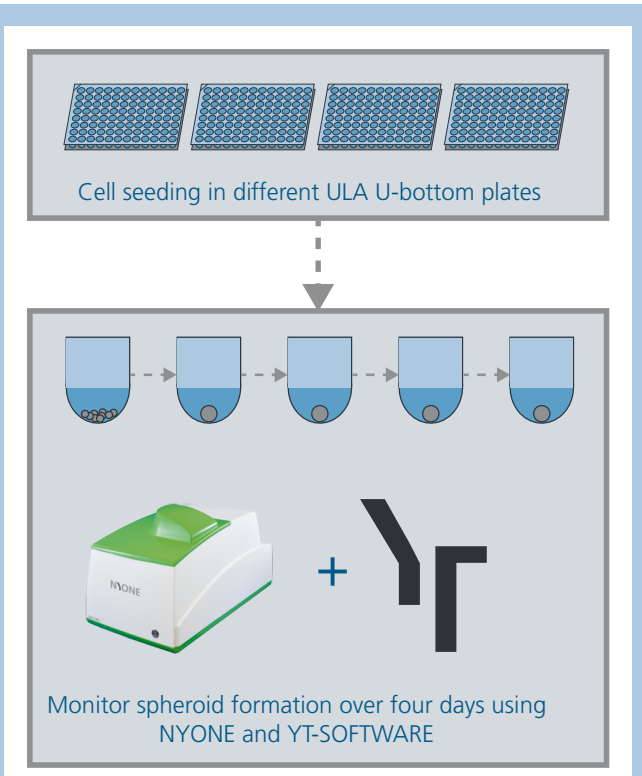


FIG. 1. WORKFLOW FOR FORMATION AND LIVE/DEAD ASSAY OF SPHEROIDS

Cells were seeded (1,250 cells/well, 5,000 cells/well and 20,000 cells/well) into ultra-low attachment (ULA) 96 well U-bottom plates. Spheroid formation was imaged with NYONE® Scientific and analyzed with YT-SOFTWARE®.

MATERIAL

- Panc1 cells (PANC-1, Panc-1, PANC.1; cell line isolated from metastasis of a pancreatic ductal adenocarcinoma)
- Panc89 cells (T3M-4, Panc-89, PANC-89; cell line from metastasis in the lymph node of a pancreatic ductal adenocarcinoma)
- A549 cells (A-549, A 549; cell line from lung adenocarcinoma)
- HCT116 cells (HCT 116, HCT-116; cell line from colon carcinoma)
- RPMI-1640 medium (e.g. PAN Biotech) supplemented with 10 % (v/v) FCS, 1 % (v/v) L-glutamine, 1 % (v/v) sodium pyruvate
- Trypsin 0.05 %/ EDTA 0.02 % (e.g. PAN Biotech)
- 96 well ultra-low attachment plates (see Tab. 1)

TAB. 1: OVERVIEW OF ULTRA-LOW ATTACHMENT PLATES USED IN THIS NOTE

Name	Manufacturer	Catalog Number
Costar®, Ultra-Low Attachment Microplate	Corning	7007
BIOFLOAT™ 96-Well plates	faCellitate	F202001
CELLSTAR®, CELL-REPELLENT SURFACE	Greiner	650970
Nunclon™ Sphera™, Sphera-Treated	Thermo Scientific™ Nunclon™ (called 'Nunc' here)	174925

METHODS

Cell culture and cell counting

We routinely cultured cancer cells in RPMI 1640 medium containing FCS (see above) using standardized cell culture conditions (37 °C, 5 % CO_2, humidified atmosphere). Before seeding cells for experiments, we trypsinized the cells and counted them using SYNENTEC's **Trypan Blue** application.

Spheroid formation

To form spheroids, we counted the cells and seeded them into five wells of ULA U-bottom 96 well plates (Table 1) at the following densities: 1,250 cells/well, 5,000 cells/well, and 20,000 cells/well. After allowing the cells to settle at the bottom of the wells for four hours without centrifugation, we imaged them for the first time (Fig. 1).

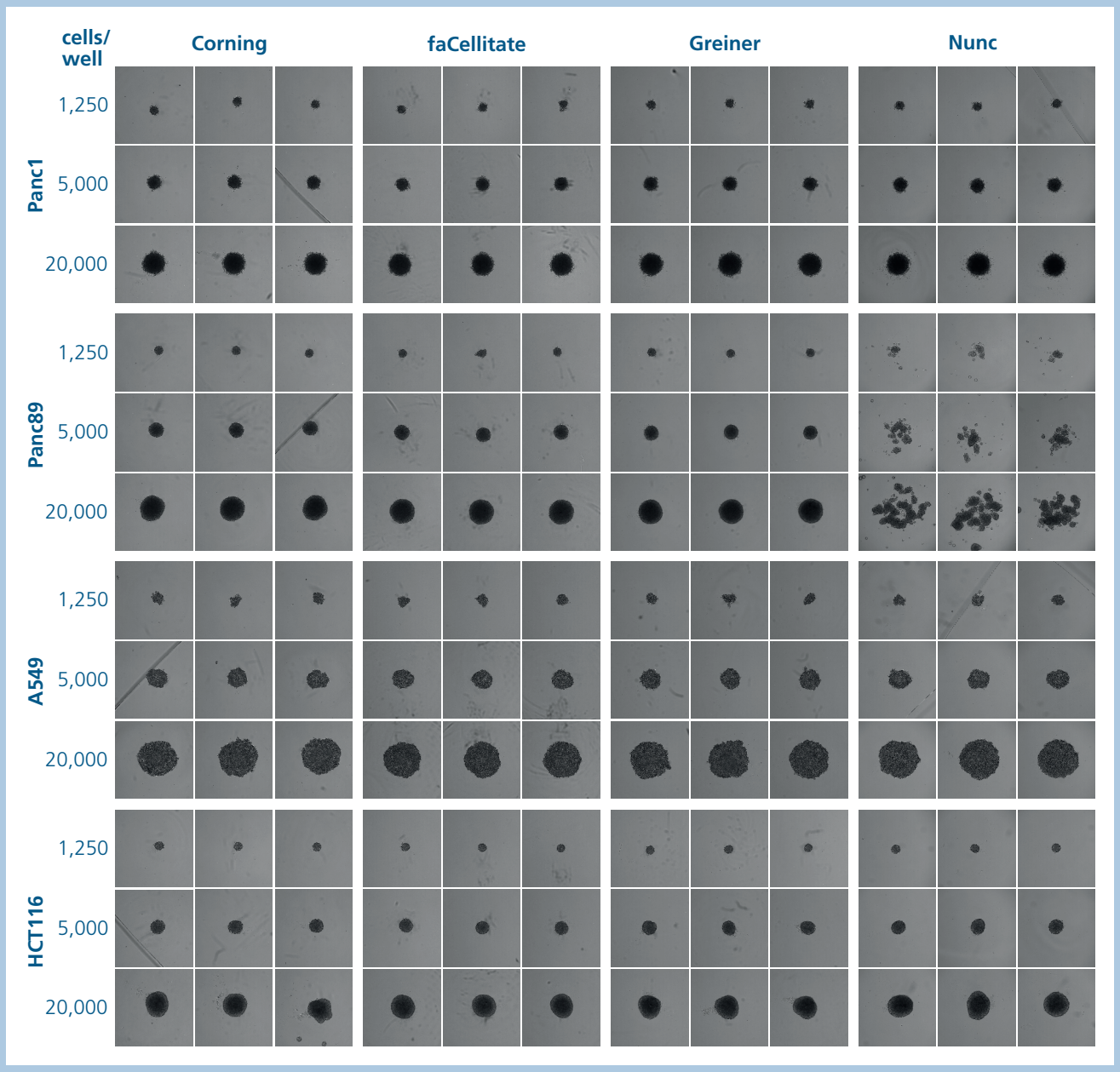


FIG. 2. SPHEROID FORMATION AFTER 24 HOURS

We seeded 1250, 5000, and 20000 Panc1, Panc89, A549, or HCT116 cells per well into ultra-low attachment (ULA) plates from Corning, faCellitate, Greiner Bio-One, or Nunc. After 24 hours, we monitored spheroid formation using the 4 x objective of NYONE® Scientific.

Imaging and Image Analysis

We imaged the spheroids using the 4 x objective of NYONE® Scientific during formation. We acquired one image in the center of each well, using "very gentle" plate movement to prevent agitation of the spheroids (see technical note for more details). We imaged the spheroids with different focus offsets in a single measurement, as the optimal focal plane may vary between cell lines. However, for image analysis with the **Spheroid Quantification** application of YT-SOFTWARE®, we used only one focal plane.

Data evaluation

We exported the data of the processed experiments from YT-SOFTWARE® and subsequently analyzed them with the statistics software GraphPad Prism.

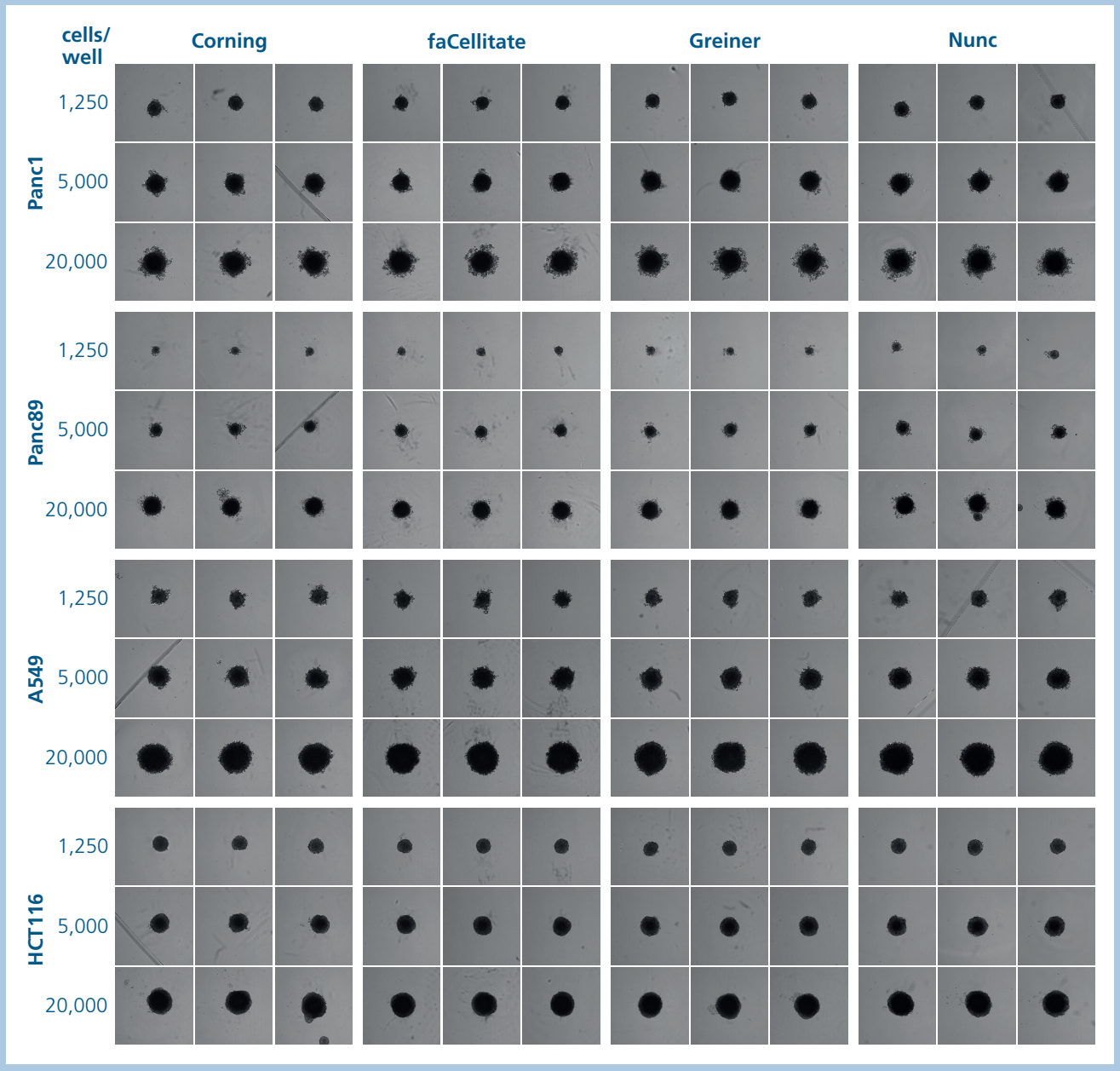


FIG. 3. SPHEROID FORMATION AFTER 96 HOURS

We seeded 1250, 5000, and 20000 Panc1, Panc89, A549, or HCT116 cells per well into ultra-low attachment (ULA) plates from Corning, faCellitate, Greiner Bio-One, or Nunc. After 96 hours, we monitored spheroid formation using the 4x objective of NYONE® Scientific.

RESULTS AND DISCUSSION

1. NYONE® reliably images spheroid formation in ULA plates from different manufacturers in a high-throughput manner

In order to test the suitability of NYONE® for imaging ULA plates from different manufacturers, we examined the ability of NYONE® to image spheroids grown in these plates (see Fig. 1 and Tab. 1 for details). The results, shown in Fig. 2 and Fig. 3, demonstrate that NYONE® was able to image spheroids in all four plates with high quality and in a high throughput manner. Example scanning times ranged from 1:38 min for one focal plane and autofocus "never" to 3:31 min for five focal planes and autofocus "each image" (for 96 wells).

Additionally, we were interested in determining whether spheroid formation varied between the plates. To do this, we seeded three different cell numbers of the cancer cell lines Panc1, Panc89, A549, and HCT116 and observed their spheroid formation daily. Panc1, A549, and HCT116 formed uniform spheroids or aggregates that were similar in all four plates. However, in the Nunc plates, Panc89 cells did not form a single spheroid after 24 hours, but rather formed many small aggregates/spheroids (Fig. 2). This may be due to the fact that we did not centrifuge the plates, as suggested by the manufacturer. We chose not to centrifuge the plates in order to test the suitability of the plates for high-throughput applications, as not all liquid handling systems have a plate centrifuge. After 96 hours, a spheroid had formed in these plates, but was sometimes surrounded by smaller spheroids (Fig. 3). Therefore, if a plate centrifuge is not available in the laboratory, the Nunc plates may not be the best option for spheroid formation with some cell lines.

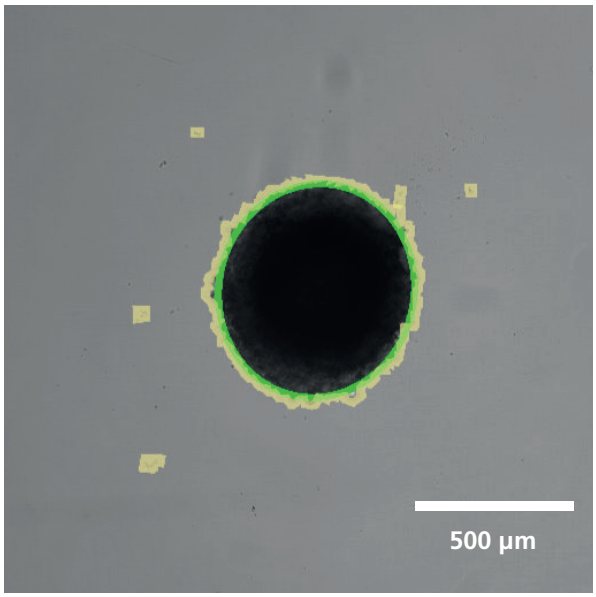


FIG. 4. IMAGE PROCESSING BY THE SPHEROID QUANTIFICATION APPLICATION OF YT-SOFWARE

The confluence is indicated by a yellow outline and the detected spheroid is marked by a green circle

Some of the plates from Corning and Nunc had scratches on the lower bottom, which did not impact spheroid formation, but did affect the quality of the images and could potentially interfere with image analysis.

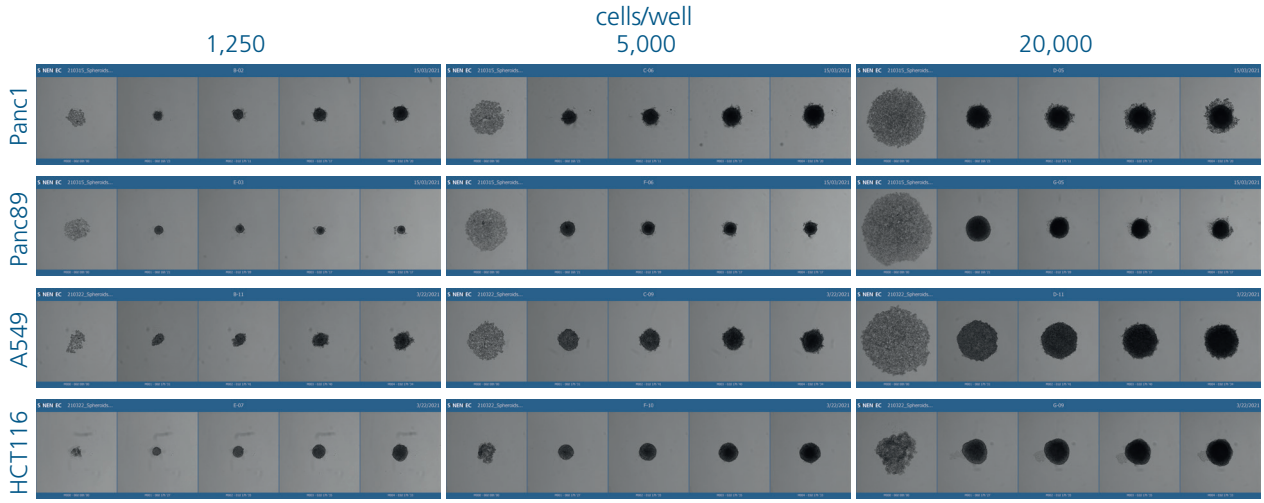


FIG. 5. SPHEROID FORMATION IN ULA PLATE FROM GREINER BIO-ONE

We seeded 1250, 5000, and 20000 Panc1, Panc89, A549, or HCT116 cells per well into ultra-low attachment (ULA) plates from Greiner Bio-One and monitored spheroid formation over time. YT-SOFTWARE® automatically generated galleries of the different time points, providing a quick overview.

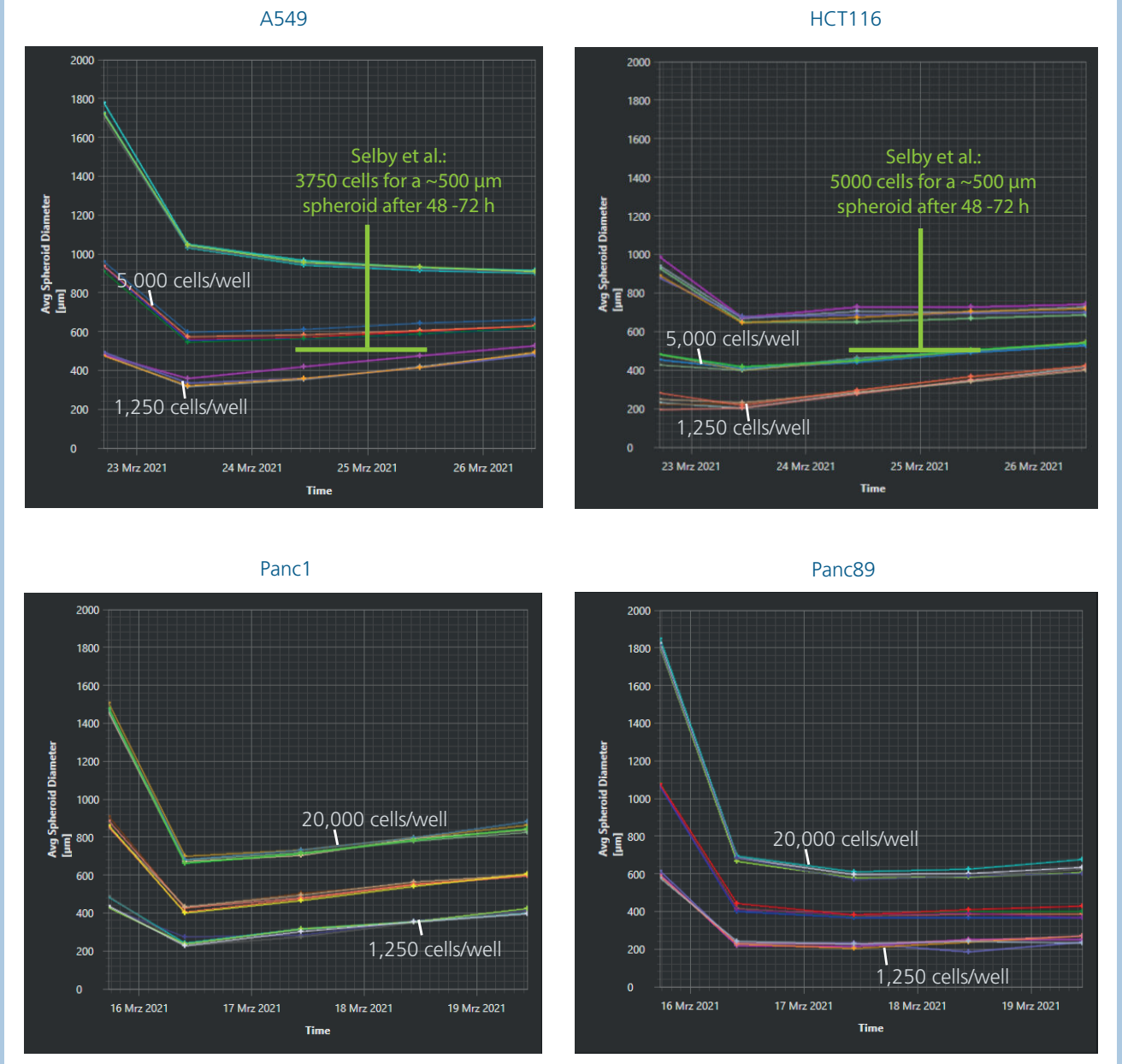


FIG. 6. AVERAGE SPHEROID DIAMETER OVER TIME

YT-SOFTWARE® was used to determine and depict the average spheroid diameter of Panc1, Panc89, A549, and HCT116 cell lines in Greiner Bio-One plate. The curve progression was determined based on the cell numbers used: 1,250 cells (bottom lines), 5,000 cells (middle lines), and 20,000 cells (upper lines). The experiment was performed with five technical replicates for each cell line and cell number.

2. Monitoring and quantification of spheroid formation

Spheroid formation was monitored over time by imaging after 4 h, 24 h, 48 h, 72 h, and 96 h with NYONE® Scientific. For higher convenience, users could also automatically image the spheroids at desired intervals using our recently launched automation system.

The **Spheroid Quantification** application of YT-SOFTWARE® can detect the cell confluence (yellow line) and spheroids (green circles) in images (Fig. 4). It also has the ability to automatically generate galleries of the detected spheroids, which can be used to visualize differences in spheroid formation between cell lines and the effect of seeding density (Fig. 5). In addition to galleries, time charts provide a quick overview of spheroid formation (Fig. 6). YT-SOFTWARE® calculates the average diameter and plots the results. The upper curve in Fig. 6 represents the data from the high cell numbers of 20,000, the middle curve represents the data from cell numbers of 5,000, and the lower curve represents the data from cell numbers

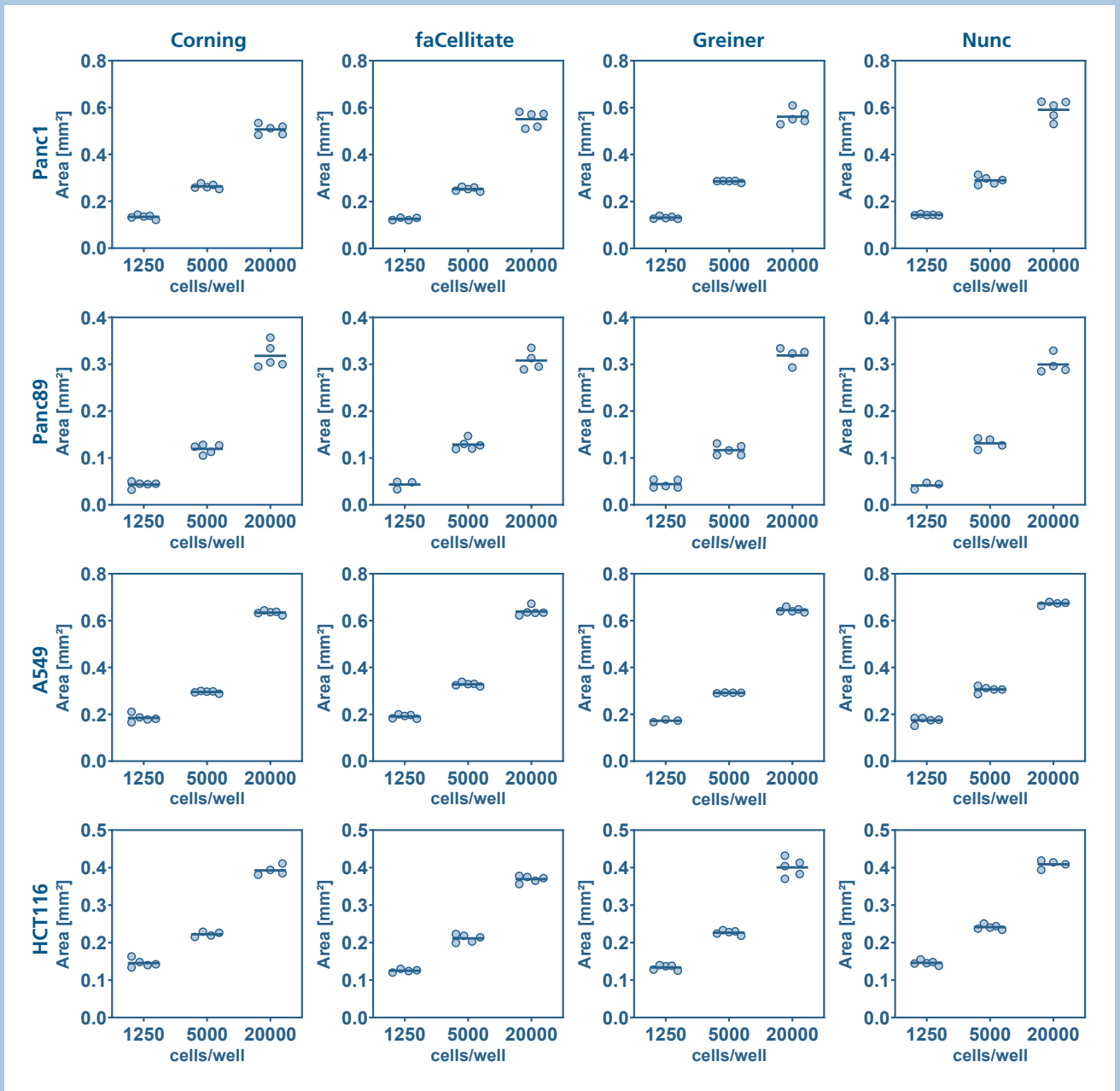


FIG. 7. SPHEROID AREA ANAYLSIS

The **Spheroid Quantification** application from YT-SOFTWARE® was used to determine the area of spheroids and these values were plotted against the seeding density.

of 1,250. The replicates were closely grouped demonstrating the robustness of the system. The average spheroid diameter of the loose cells decreases as they form compact spheroids/aggregates after 24 hours. Panc1 cells form compact aggregates that grow in size, while Panc89 cells form compact spheroids with a constant diameter. A549 cells form aggregates that become more compact over time at high cell numbers, but at lower cell numbers they grow in size. HCT116 cells form compact spheroids that increase in size over time. For screening oncology compounds, a diameter of 300-500 µm is typically desired [4]. Selby et al. tested seeding densitites of 60 cell lines (the so-called NCI60 panel) to generate such spheroids [4]. A549 and HCT116 cells are part of this panel and the cell numbers to reach ~500 µm spheroids after 48 to 72 h were 3750 and 5000, respectively [4]. These cell numbers fit perfectly to the sizes determined by our system (Fig. 6).

To better compare the different cell lines, seeding densities and

plates, we plotted the average spheroid area after 96 h for the different conditions into one graph (Fig. 7). Again, the replicates were closely grouped demonstrating the robustness of the assay. Moreover, cells formed spheroids of comparable size in all four plates and as expected, the area increased with increasing seeding density. The different cell lines formed spheroids of different sizes, with Panc89 cells forming the smallest spheroids, followed by HCT116 cells. Panc1 and A549 spheroids were larger, indicating that they are not as dense and compact as the other cell lines. Therefore, it is important to conduct preliminary experiments for each cell line to determine the optimal seeding density for forming spheroids of a certain size.

CONCLUSION

We demonstrated the ability of SYNENTEC's imagers to scan spheroids grown in ULA plates in a high-throughput manner. Moreover, our automation system allows for the convenient automatic imaging of spheroid growth over time. For a fast overview, the **Spheroid Quantification** application generates galleries, time charts, and heat maps, making the application a valuable tool for research and drug discovery.

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