Application Note
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Detection of Necrotic Cores in Tumor Spheroids Using a Live/Dead Assay and NYONE® Scientific

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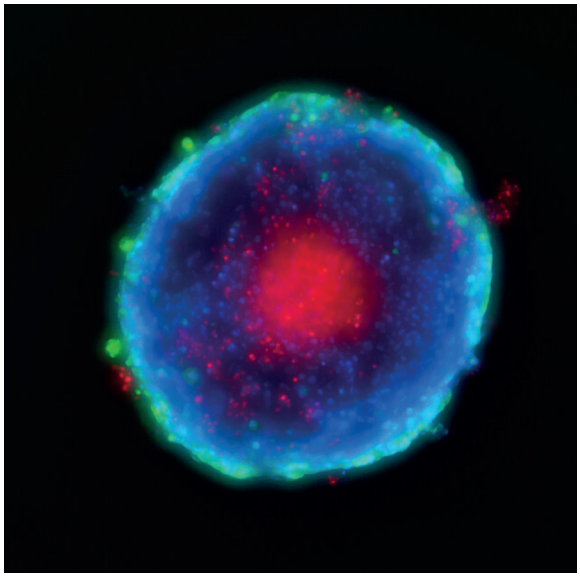
ABSTRACT

Three-dimensional (3D) cell culture models, such as spheroids, are a valuable tool for mimicking the physiology of tissues and tumors in vitro. One key aspect of spheroid physiology is their size. As spheroids grow larger, the complete penetration of nutrients and oxygen into the core and removal of metabolites becomes increasingly restricted, resulting in gradients and necrotic or hypoxic zones within the interior. This is similar to what is observed in tumors *in vivo*. For this reason, larger spheroids are often preferred as tumor models *in vitro*. However, necrotic cores can be undesirable for other applications where high cell viability is needed. Therefore, it is important to understand the properties of spheroids in order to select the appropriate model for a given application. In this study, we analyzed the presence of necrotic cores in tumor spheroids generated from colorectal cancer cells (HCT116) using a combination of a Live/Dead assay and high-content imaging. Our results showed that only spheroids with a diameter larger than 700 micrometers showed a prominent necrotic core, in line with previously published literature. Additionally, our assay design and imaging methods are simple, cost-effective and suitable for automation and high-throughput screening.



KEYWORDS: 3D, SPHEROID, DRUG SCREENING, HIGH THROUPUT SCREENING, LIVE-DEAD ASSAY, CANCER RESEARCH

IMAGE AND EVALUATE MORE SPHEROIDS IN LESS TIME

- Stain without washing steps
- Image spheroids directly in ULA plates without transfer or embedding
- Keep your spheroids intact with slow harmonic motion of the plate
- Increase throughput by fast measurement (~2,5 min for a full 96-well plate with four channels)
- Capture the whole spheroid with just one image
- Automatically analyze the average spheroid diameter and fluorescence intensity

INTRODUCTION

During drug screening, many promising candidates fail in early clinical phases. Therefore, cell models mimicking human physiology and disease as closely as possible are urgently needed [1]-[3]. The use of 3D cell models, specifically multicellular spheroids, is one option. Spheroids can recreate *in vivo* environments *in vitro* and simulate tumor characteristics such as angiogenesis and invasion. However, cell lines differ in their ability to form spheroids of a certain size. Moreover, seeding density and the duration of cultivation also

influence the size and thus the diameter of spheroids, which is an important parameter as it determines the viability of the cells in the core. In larger spheroids, the transport of oxygen and nutrients to the core of the spheroid is limited and, conversely, the removal of waste and decomposition products is reduced, so that the cells in this area become apoptotic or necrotic [4]-[8]. It is important to know the diameter from which a necrotic core starts to be present, as this also has an influence on the transport of drugs or penetration of fluorescent dyes into the core [1], [4], [8]. Spheroids above a certain diameter develop a necrotic core surrounded by a zone of hypoxia/senescence/quiescence while proliferation only takes place in the outer area of the spheroid. As a similar zonal architecture has also been described for solid tumors, these zones are important for mimicking a tumor microenvironment. In most models, a necrotic core is formed above a diameter of 500 - 600 µm [4], [5], [9]. In contrast, spheroids for other applications such as the transplantation of insulin-producing beta cells for diabetes treatment should not have a necrotic core as high cell viability is needed [10,11].
In this study, we aimed to analyze the presence of necrotic cores in tumor spheroids using the colon carcinoma cell line HCT116 and demonstrate the relationship between cell number, spheroid size, and necrotic core.

MATERIAL

- HCT116 cells (cell line from colorectal 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 with U-bottom (e.g. Nunc, Greiner Bio-One, FaCellitate, Corning)
- Hoechst 33342 (5 mg/mL, e.g. Invitrogen Cat. No. H1399)
- Propidium iodide (1 mg/mL, e.g. Invitrogen Cat. No. P3566)
- Calcein-AM (1 mM, e.g. Biolegend Cat. No. 425201)

METHODS

Cell culture and cell counting

We routinely cultured the 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 and counted them using SYNENTEC's **Trypan Blue** application.

Formation of spheroids

For spheroid formation, we counted the cells and seeded 200 µl cell suspension into ultra-low attachment (ULA) 96 well U-bottom plates (1,250 cells/well, 5,000 cells/well and 20,000 cells/well, 5 wells per condition). We let the cells settle to the bottom without centrifugation.

Live/Dead assay

We prepared an 11 x staining solution by diluting 770 µL propidium iodide (PI), 77 µL Calcein-AM and 19,25 µL Hoechst 33342 in standard cell culture medium (fill up to 3500 µL) (Tab. 1). Four days after cell seeding, we added 20 µL of the staining solution to each well and incubated the cells for 3 hours at 37 °C. We imaged the cells without any further washing steps.

Imaging and image processing

We imaged the cells with the 4 x objective of NYONE® Scientific capturing brightfield and the three fluorescent channels described in table 2 (Tab. 2). One image was acquired to image the whole well. We imaged the spheroids with six different focus offsets per channel to capture the spheroid in focus regardless of the size (see technical note for details). However, for the image analysis with the **Spheroid Quantification (2F)** application of YT-SOFTWARE®, only one focal plane per size was used.

Data evaluation

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

TAB. 1: LIVE/DEAD ASSAY STAINING CONCENTRATION AND DILUTION

Live/Dead Assay	Stock Concentration	Final Concentration	Dilution for 11x Solution
Hoechst 33342	5 mg/mL	2.5 µg/mL	1: 181,8
Calcein-AM	1 mM	2 µM	1: 45,45
Propidium iodide (PI)	1 mg/mL	20 µg/mL	1: 4,545

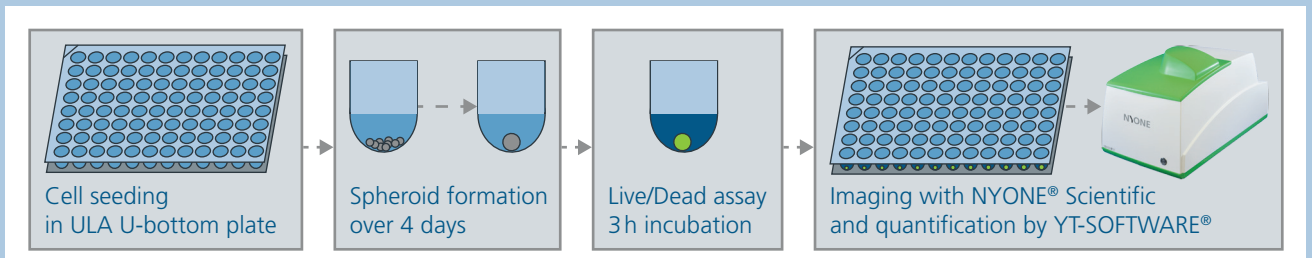


FIG. 1. WORKFLOW FOR SPHEROID FORMATION AND LIVE/DEAD ASSAY

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 and spheroids formed over 4 days. Afterwards, the spheroids were stained with propidium iodide (PI, red) for dead cells, Calcein-AM (green) for metabolically active cells and Hoechst 33342 for the nuclei. Then, the spheroids were imaged in the U-bottom plate with NYONE® Scientific and analyzed with YT-SOFTWARE®.

RESULTS AND DISCUSSION

To analyze the presence of a necrotic core in relationship with the spheroid diameter, we seeded 1,250, 5,000 or 20,000 HCT116 cells per well into ULA plates (Fig. 1). After spheroid formation, we stained them with Calcein-AM (green) for living/metabolically active cells, propidium iodide (PI, red) for dead cells, and with Hoechst 33342 (blue) for all cell nuclei. Spheroids stained with this Live/Dead assay were then imaged with NYONE® Scientific and analyzed using the **Spheroid Quantification (2F)** application of YT-SOFTWARE®. The scanning times were very fast. When only one focal offset was used and autofocos set to "never", a full 96-well plate was scanned in approximately 2,5 min. Time increased to 6,5 min with five offsets per channel and up to 17,5min for five offsets and "each image".

HCT116 cells formed compact and rather sharply defined spheroids (Fig. 2). The compactness of the spheroids was also observable in brightfield. Due to the lack of transmitted light, dark spheroids with high contrast to the background were visible. The Live/Dead assay revealed, that the cells in the core of the spheroids were PI positive only at the cell number of 20,000.

In contrast to Calcein-AM and Hoechst 33342, PI could penetrate verifiable into the core of the spheroids. Interestingly, Calcein-AM was only visible in the outer area of the spheroids also in the smaller spheroids. It can be argued that only there the cells were viable and wonder why the smaller spheroids lack a PI-positive core. However, since Hoechst was also only visible at the outer area, the effect appeared probably not due to viability but to a slow penetration of the dyes into these compact spheroids. Therefore, the Calcein-AM signal can rather be used for assesment of the architecture of the spheroids (loose or compact) than about the viability of the cells in the different zones. Ham et al. already described that due to the close cell-cell contact the spheroids are so compact that only a slow penetration takes place. Therefore, they should be incubated longer than 2D cell layers, nevertheless a good staining might still not be achieved [4]. Mittler et al. also claim that the slow transfer of fluorescent dyes into the spheroid leads to uneven staining and to a dye gradient, so that the outer zone is stronger colored compared to the core [1].

To quantify the fluorescence signals, we used the **Spheroid Quantification (2F)** application of YT-SOFTWARE® (Fig. 3). This application recognized the cell areas (light-yellow marked), detected the spheroids (circle), and determined the average spheroid diameter. In addition, YT-SOFTWARE® analyzed the fluorescence of the spheroids and then labeled them with differently colored circles. Thus, Calcein-AM-positive spheroids were marked with a yellow circle and the spheroids that were also PI-positive were marked with a red circle (Fig. 3 A). In addition to determining whether the spheroid is Calcein-AM- and/or PI-positive, the average fluorescence intensity BC (background corrected) was also determined (see technical note for a detailed description) (Fig. 3 B).

TAB. 2: IMAGING SETTINGS FOR LIVE/DEAD ASSAY

Live/Dead-staining	Excitation LED [nm]	Emission Filter [nm]
Hoechst 33342	UV (377/50)	Blue (452/45)
Calcein-AM	Blue (475/28)	Green (530/43)
Propidium iodide (PI)	Lime (562/40)	Red-LP (593-LP)

We plotted the spheroid diameter, Calcein-AM as well as PI intensity into one graph to nicely visualize all parameters at once. Firstly, the analysis showed that higher cell numbers lead to larger spheroids (400 µm for 1,250, 530 µm for 5,000, 700 µm for 20,000). Secondly, a higher Calcein-AM intensity was detected for small spheroids than for large spheroids (50 for 1,250, 45 for 5,000, 31 for 20,000). Thirdly, the PI signal increased with the size of the spheroids corresponding to the known observations about the development of a necrotic core in spheroids with larger diameters (6.3 for 1,250, 8.6 for 5,000, 28 for 20,000) [12].
In summary, the data are in line with literature and demonstrate the capability of using our imager NYONE® Scientific for high-content screening of a 3D spheroid model.

CONCLUSION

SYNENTEC's cell imager NYONE® Scientific enables fast imaging of the stained spheroids by taking one image per well with a 4 x magnification objective that captures the whole spheroid. The **Spheroid Quantification (2F)** application of YT-SOFTWARE® automatically analyzes the spheroids stained with a Live/Dead assay, directly determines the spheroid diameter and quantifies their average fluorescence intensity. Thus, the Live/Dead assay can be used to analyze at which spheroid size a necrotic core is present and at which size the supply to the core is still sufficient. Hence, this can be used to determine the optimal cell number and characterize spheroids of different cell types. The assay is rapid, reliable, straightforward, and suitable for high-throughput screening.

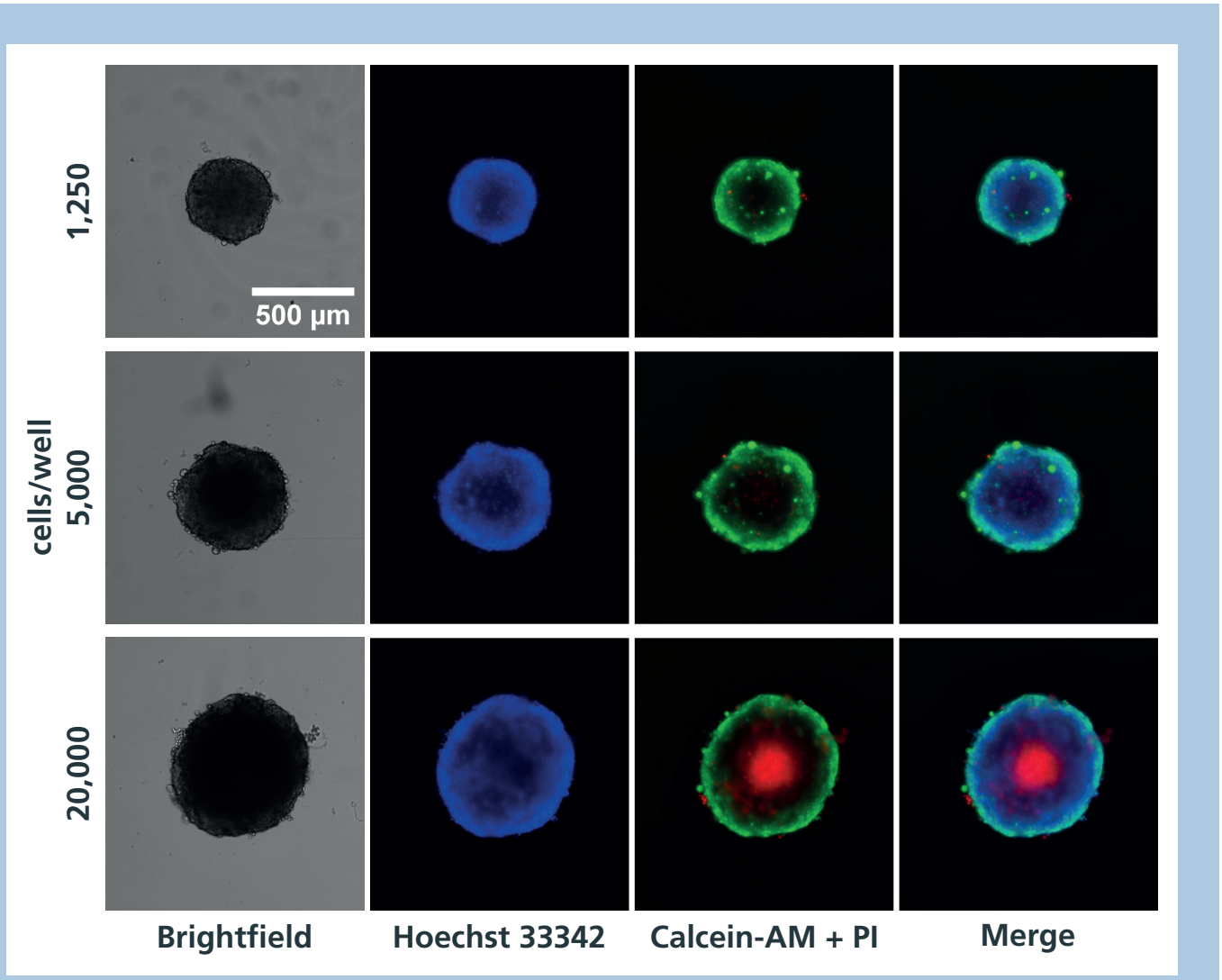


FIG. 2. AFTER STAINING WITH A LIVE/DEAD ASSAY, LARGE HCT116 SPHEROIDS SHOWED A NECROTIC CORE

Spheroids of HCT116 cells were generated with 1,250 cells/well, 5,000 cells/well and 20,000 cells/well over 4 days. Then the spheroids were stained with propidium iodide (PI, red) for dead cells, Calcein-AM (green) for metabolically active cells and Hoechst 33342 for the nuclei. Scale bar: 500 µm.

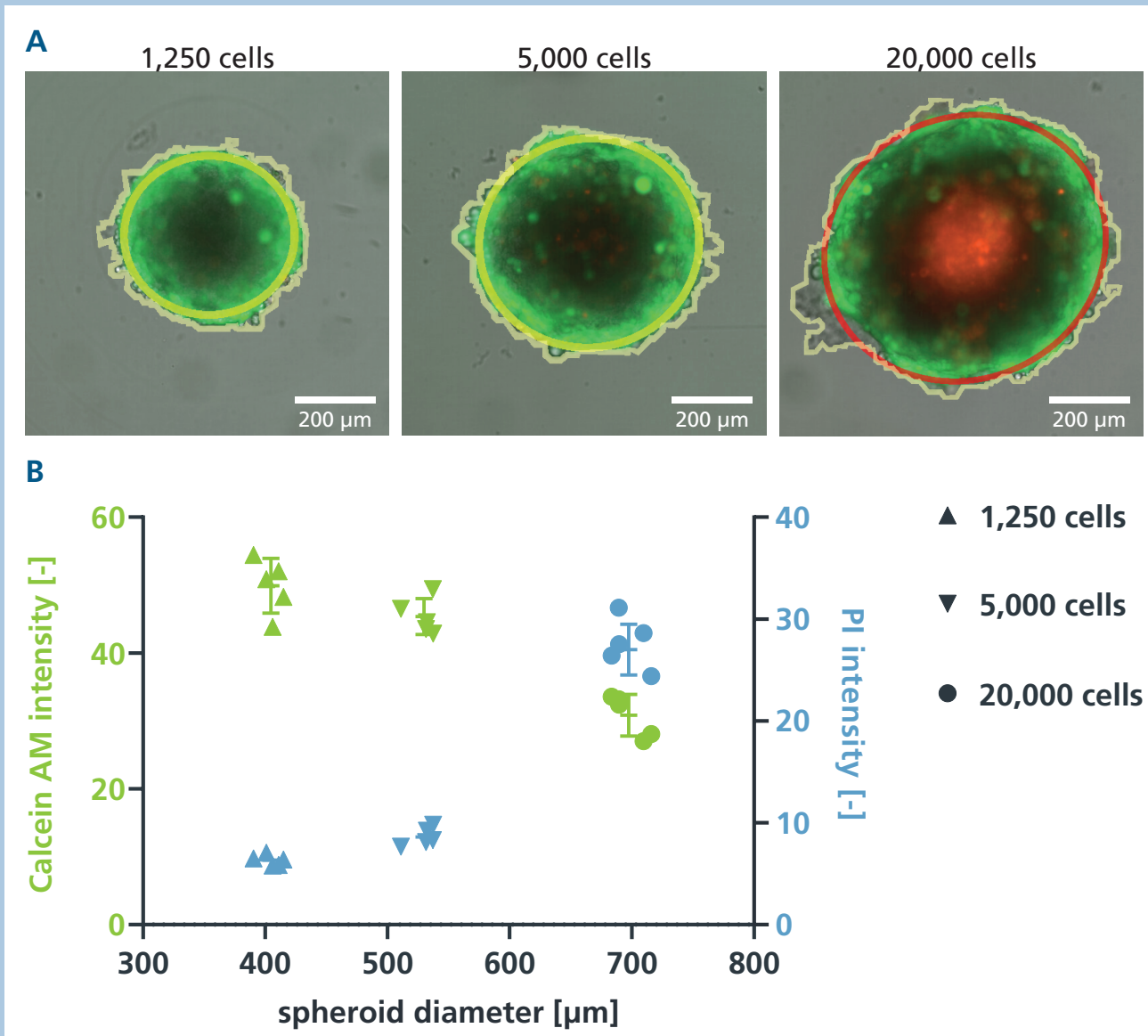


FIG. 3. SPHEROID DIAMETER AND FLUORESCENCE INTENSITIES WERE ANALYZED BY SPHEROID QUANTIFICATION 2F APPLICATION

The **Spheroid Quantification (2F)** application of YT-SOFTWARE® automatically analyzes the spheroids. The cells are encircled by a light-yellow line and the detected spheroid is marked with a circle. The application analyzes the average spheroid diameter as well as the intensity of the fluorescent signal within the spheroid. A) The small spheroids are only Calcein-AM-positive and therefore encircled orange, while the large spheroid is Calcein-AM- and PI-positive and therefore encircled in red. If the spheroid was only PI-positive, the ring would be light blue (not shown). B) The PI intensity increased with the spheroid diameter and cell number (average PI intensity background corrected). Correspondingly, the average Calcein-AM intensity decreased with the size of the spheroids (one experiment with 5 spheroids). Scale bar: 200 µm.

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Institut für Experimentelle Tumorforschung

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