Label-free, real-time live-cell assays for spheroids: IncuCyte® bright-field analysis

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Introduction

A growing body of evidence suggests that more relevant and translational observations can be made with micro-tissues and organoids compared to 2D monolayer cell models. This is most notable in the cancer biology and hepatotoxicity field. For example, tumor spheroids exhibit more relevant morphology and increased cell survival compared to 2D cultures and have a hypoxic core. Current methods for assessing the growth and shrinkage of tumor spheroids are limited by one or more of the following: (1) assay workflows that are time-consuming, expensive or laborious, (2) a requirement to label the cells (e.g. a fluorescent probe) which may perturb the biology and may not be amenable to primary tissue, (3) single time-point readouts that do not report the full timecourse, (4) indirect readouts (e.g. ATP) that may overlook valuable morphological insight and/or mis-report cell growth.

In this application note we describe methods and validation data for miniaturized (96/384-well) live-cell tumor spheroid assays that are based on non-invasive bright-field image analysis performed with the IncuCyte® S3 Spheroid software module. Tumor spheroids are formed in ultra-low attachment (ULA) plates and monitored for size and morphology for up to 2 weeks. These assays are flexible, simple to run and provide automated and direct measures of tumor size in real-time.

Materials & Methods

Protocol

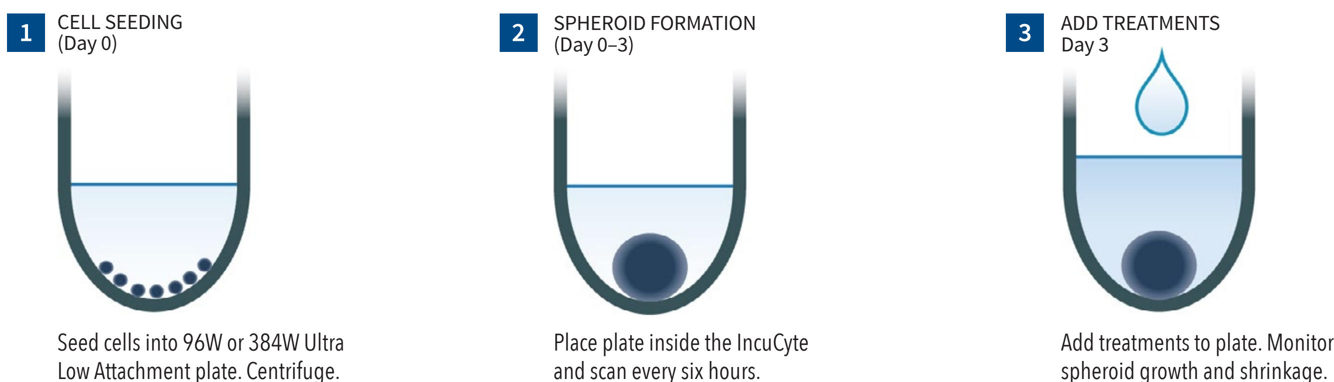

Figure 1. Assay Workflow

1. Cells of interest are harvested, counted and plated into ULA round-bottom 96- or 384-well plates at desired densities (in 100 or 50 µL per well for 96- or 384-well plate respectively). Plates are centrifuged (150xg, 10 min).
2. Spheroid formation is monitored to desired size (e.g. 200 – 500 µm) with 6-hour bright-field and HD phase-contrast image scans (either 4x or 10x magnification) using IncuCyte® S3 Spheroid software module.
3. Compounds are added (100 µL at 2x, or 25 µL at 3x final assay concentration (FAC) per well for 96- or 384-well plate respectively).
4. The spheroid growth and shrinkage assay is initiated and monitored in IncuCyte (6-hour repeat scanning, up to 2 weeks). Tumor size is reported in real-time based on bright-field image analysis and compared to fluorescence analysis of IncuCyte® NucLight Red label.

IncuCyte NucLight Red Cellular Reagents (MDA-MB-231-NR, EssenBio #4487; A549-NR #4491; HT-1080-NR, #4485 and SKOV3-NR, not commercially available) were grown to confluence in 75 cm2 tissue culture treated flasks and seeded into 96-well (Corning #7007) or 384-well (S-Bio #MS-9384UZ) ULA round-bottom plates such that by 72-hours, spheroids formed with desired size (e.g., 200-500 μM). Plates were centrifuged (150xg, 10 minutes) at room temperature. MDA-MB-231 cells required the addition of a basement membrane extract, Matrigel® (Corning #356234) at 2.5% v/v, to promote tight spheroid formation. All cells were cultured in F-12K supplemented with 10% FBS, 1% Pen/Strep, 1% glutamax and 0.5 μg/mL puromycin. All cell culture reagents were obtained from Life Technologies unless otherwise noted. Spheroid formation was monitored in an IncuCyte® live-cell analysis system over a 72-hour period at 6 hour intervals.

Validation Data

Spheroid Morphology

To first illustrate the value of the IncuCyte imaging approach, human lung (A549) and breast (MDA-MB-231 in Matrigel®) tumor cell lines were seeded at 5,000 or 2,500 cells per well respectively in 96-well ULA plates (Corning). High definition (HD)-phase and bright-field (BF) images were taken at 6-hour intervals (Figure 2). We observed clear morphological differences – A549 cells formed larger loose aggregates while MDA-MB231 spheroids were small and compact. These properties are consistent with those previously described elsewhere.

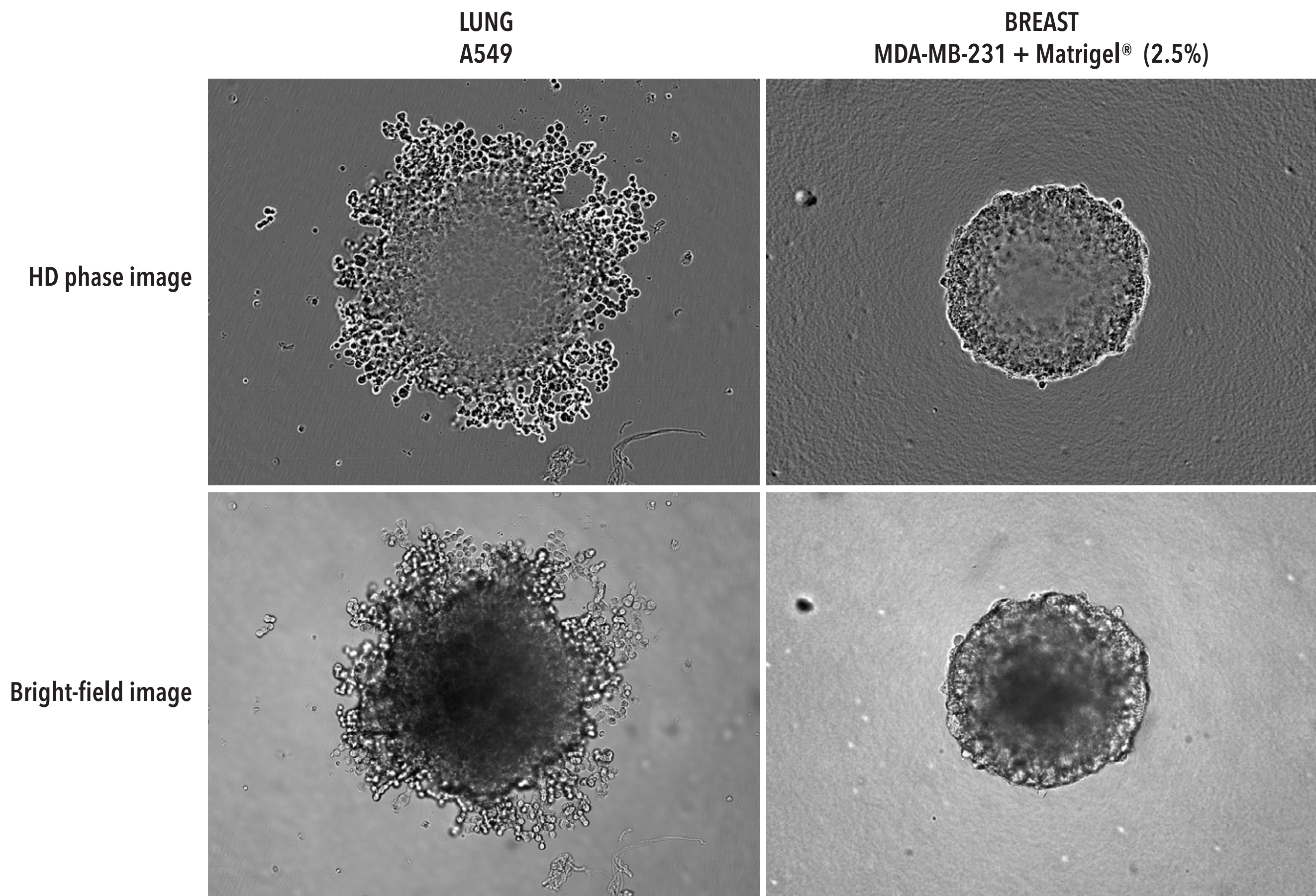

Figure 2. Visualizing different spheroid morphologies.
High quality HD phase and corresponding BF images of spheroids formed from A549 and MDA-MB-231 cells (2,500 or 5000 cells per well respectively), 72-hours post seeding. Visualization of detailed phenotypic variation is observed in HD phase images. A549 cells present a loose aggregate morphology compared to the compact spheroid formed by MDA-MB-231 cells. Compaction of MDA-MB-231 aggregates into spheroids was achieved by the addition of 2.5% v/v Matrigel® post centrifugation. All images captured at 10x magnification.

IncuCyte® BF size analysis

The size of tumor spheroids was measured using an automated software algorithm that masked the largest BF object in the field of view. To validate the BF masking approach, assay data was compared to a fluorescent labeling approach and an ATP-endpoint viability assay.

A549 cells stably expressing red fluorescent protein (IncuCyte NucLight Red Cellular reagent, cat # 4491), were seeded at different densities (625, 2,500 or 7,500 cell per well) in ULA round-bottomed plates to yield spheroids of differing sizes. BF and fluorescent images were acquired, masked and analysed using IncuCyte. Comparisons of BF and fluorescent segmentation revealed very similar spheroid sizes, supported by visual inspection of the images and masks (figure 3). Measured spheroid area was proportional to the initial cell seeding density.

An ATP end-point viability assay (CellTiter-Glo 3D, Promega # G9681) was performed at the end of the experiment (180-hours post spheroid formation), according to manufacturer's instructions. After 5 minutes of incubation, spheroids were pipette mixed, transferred into flat-bottomed, solid white plates (Corning #3917), then incubated for an additional 25 min prior to luminescence measurement on a CLARIOstar microplate reader (BMG) to generate ATP relative luminescent units (RLU) values. As expected, the luminescence signal increased with seeding cell number, however the fold increase was smaller than that observed for spheroid area (Figure 3). This may reflect changes in cell metabolism upon spheroid formation, particularly once the spheroid becomes large and develops a hypoxic core.

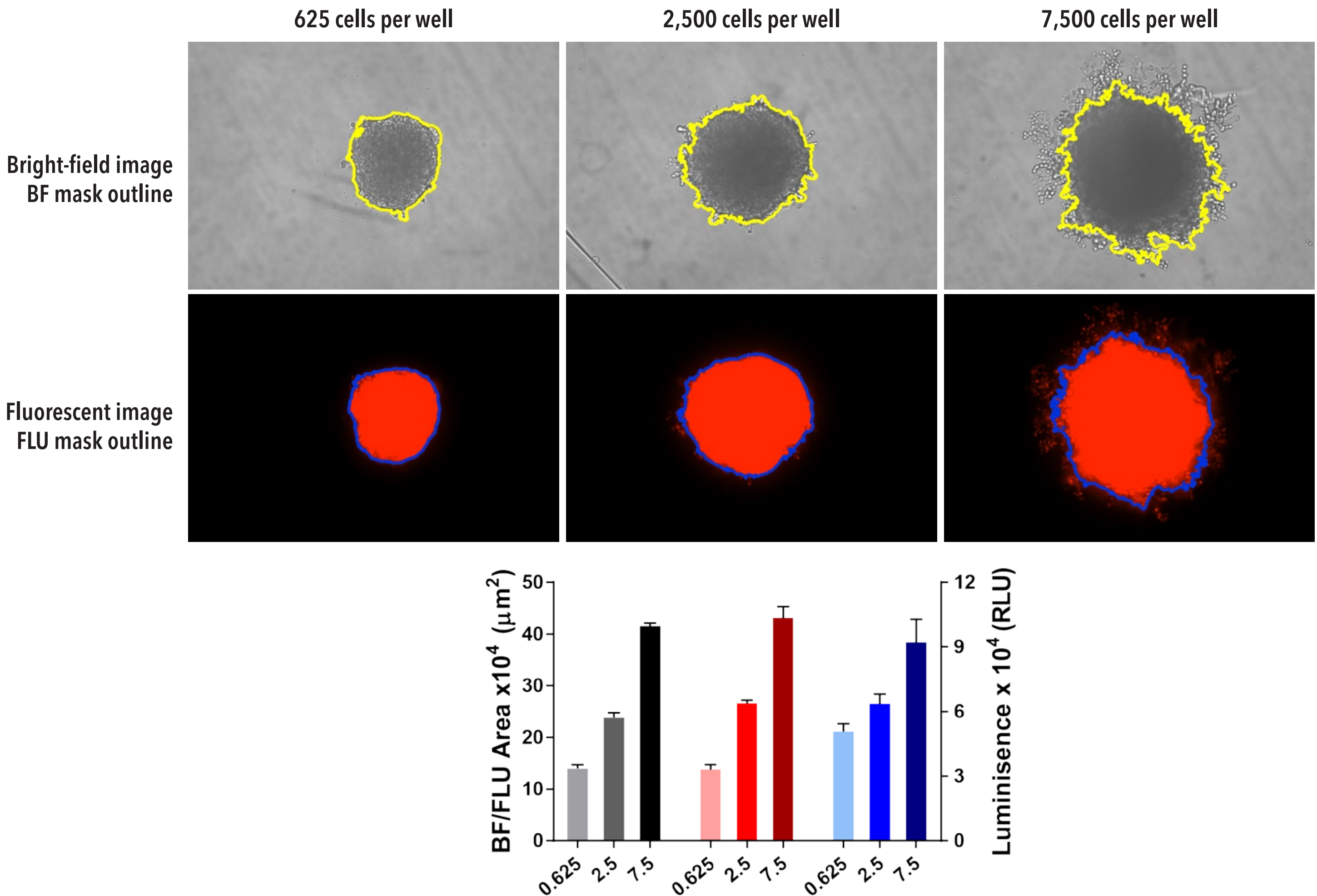

Figure 3. Fluorescent and [ATP] readouts support BF segmentation. Seeding density dependent spheroid growth of A549 NucLight Red cells. A549 NucLight cells were plated at a density of 625, 2,500 or 7,500 cells per well in ULA round-bottom 96- well plates. Spheroids were allowed to form followed by media replenishment 72-hours post seeding. BF and FLU images show segmentation mask of representative wells at 120-hours post-media replenishment. The bar graph demonstrates the correlation of the Largest BF and fluorescence Object area (μm2) as well as the [ATP] (Relative luminescent units, RLU) end-point analysis with increasing cell seeding density at 180-hurs. Data were collected over a 180-hour period at 6-hour intervals. All images captured at 10x magnification. Each data point represents mean ± SEM, n=4.

Growth and shrinkage over time

Changes in the size of MDA-MB-231, HT-1080 and A549 tumor spheroids were monitored over time, in the absence and presence of the cytotoxic drug, camptothecin (CMP; Figure 4). For all three cell types, the control spheroids increased markedly over the 10-day period (2-8 fold) – the largest size increase was observed with MDA-MB-231 in Matrigel®. CMP inhibited spheroid growth, and in the case of HT-1080 cells an overall reduction in spheroid size compared to the t= zero control was observed.

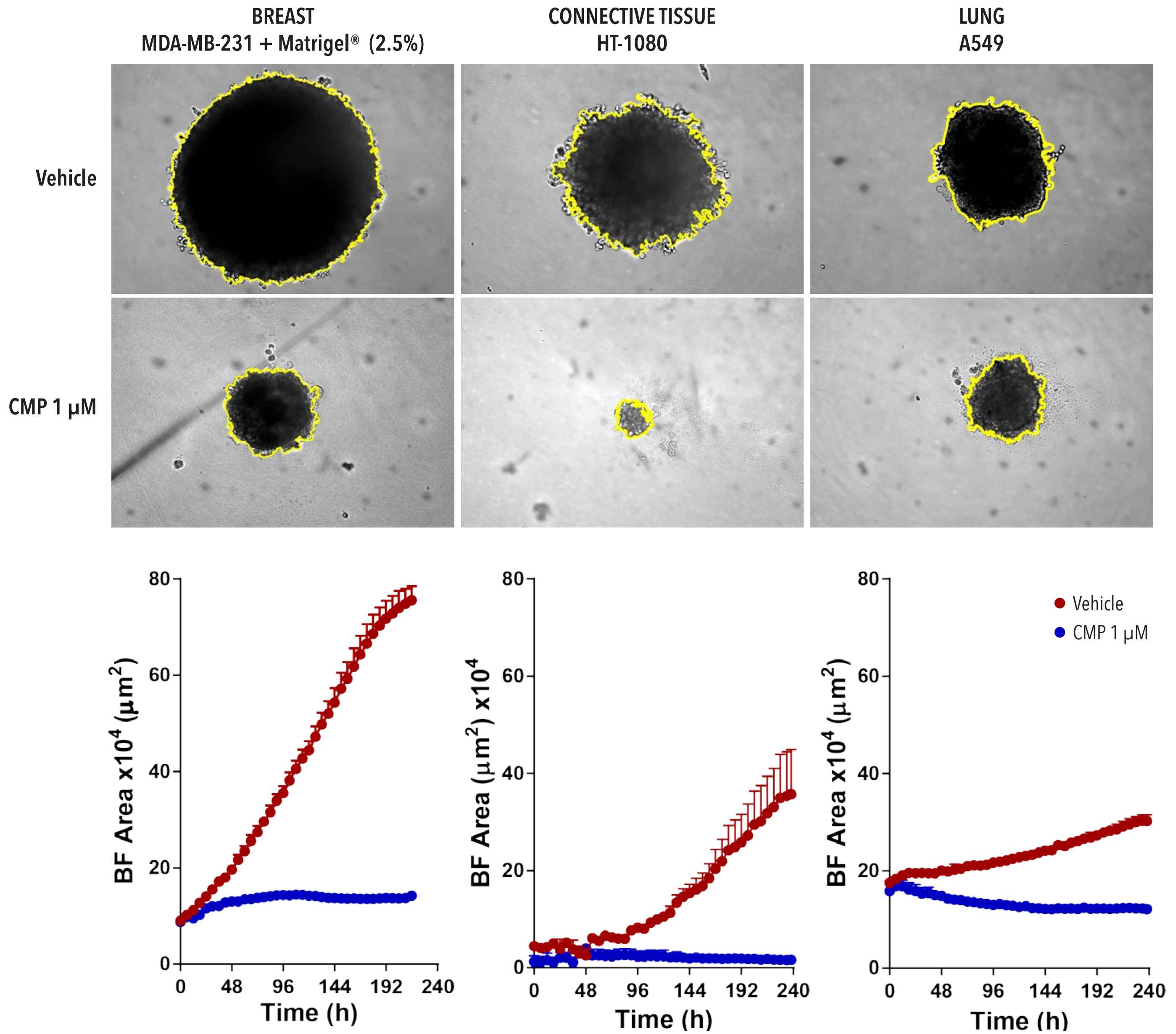

Figure 4. BF analysis enables accurate kinetic quantification of spheroids.
The differential pharmacological effect of 1 µM CMP on growth of MDA-MB-231, HT-1080 and A549 cells in a spheroid assay. Cells were grown in ULA round-bottom 96- well plates (2,500 cells per well) for 72-hours and treatment with ± 1 µM CMP followed. Segmented BF images compare treated vs. un-treated conditions at 240-hours. Time-courses illustrate the specific cell type-dependent kinetic profile of spheroid growth and shrinkage. The graphs display the Largest BF Object area (µm2) (y-axis) over the course of a 240-hour assay (x-axis) at 6-hour intervals. All images captured at 10x magnification. Each data point represents mean ±SEM, n=4.

96-well spheroid growth and shrinkage assay

To illustrate the amenability of our approach to drug toxicity testing, a pharmacological study was performed in A549 and SKOV-3 cancer cell lines. Both cell types were grown in ULA round-bottom 96-well plates for 72-hours and then treated with test compound. The effect of CMP (0.457 nM – 1 µM) on A549 cells was investigated (Figure 5). Spheroid growth and shrinkage was quantified on the IncuCyte by evaluating changes in spheroid area, using the Largest BF Object Area (µm²) metric. Here we show a concentration dependent effect of CMP on spheroid shrinkage in A549 spheroids.

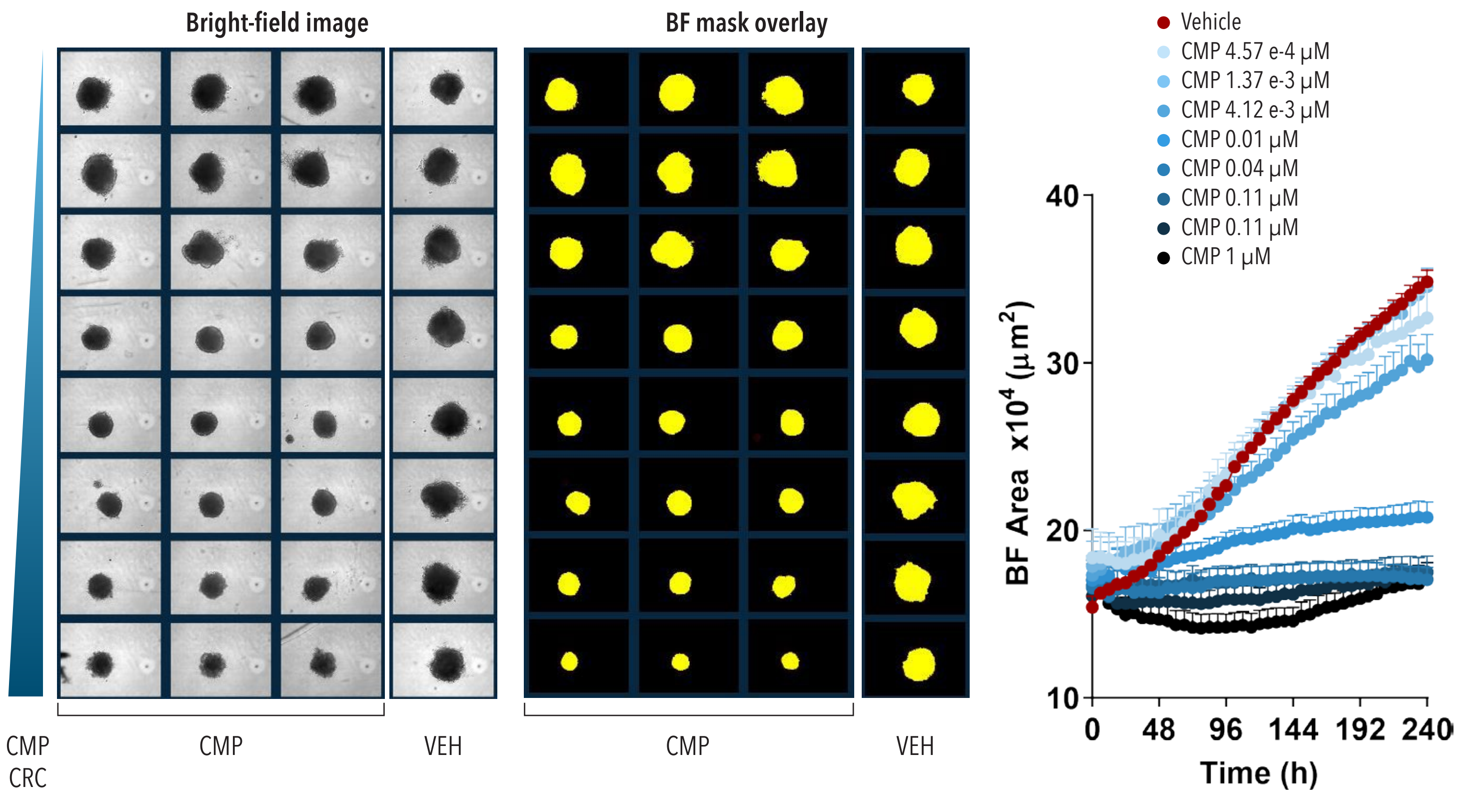

Figure 5 Quantification Spheroid Growth and Shrinkage.
Effect of CMP on growth of A549 cells in a spheroid assay. A549 cells were plated at a density of 5,000 cells per well and spheroid allowed to form (72-hours). Serial dilutions of CMP were then added to the cells and the kinetics of spheroid growth and shrinkage were measured. Images represent the BF view and segmentation at t=240-hours. Time-course plot shows the Largest BF Object Area metric (µm2) over time for all wells. Data were collected over a 240-hour period at 6-hour intervals, all images captured at 10x magnification. Each data point represents mean ±SEM, n=3.

In the second example, SKOV-3 cells were treated with the cytotoxic compound CMP, the apoptotic compound cisplatin (CIS) or the chemotherapeutic drug oxaliplatin (OXA) (Figure 6). The data shows a concentration-dependent inhibitory growth effect for all compounds, and illustrates how compound potencies can be directly compared within the same assay.

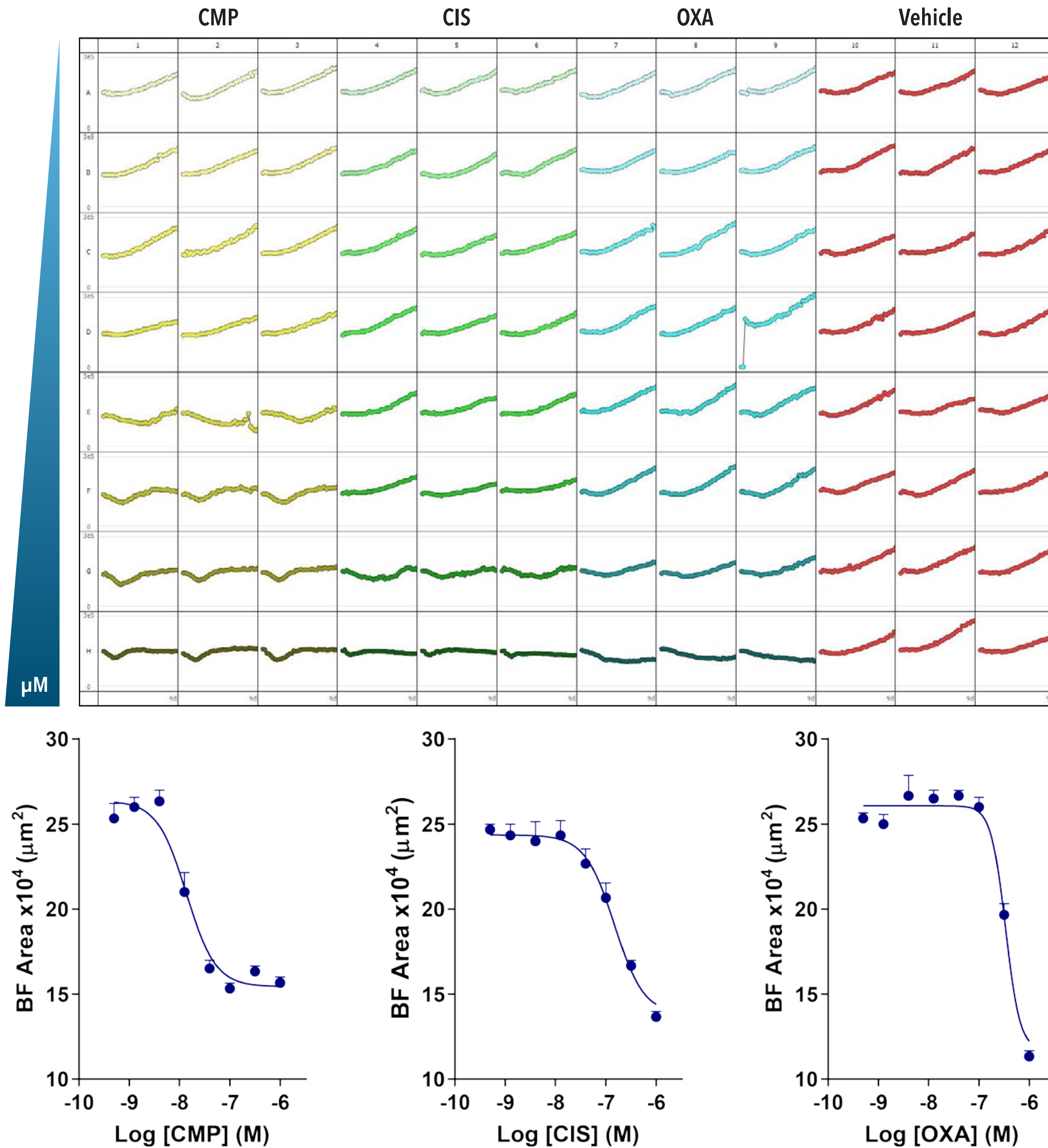

Figure 6. Effect of CMP, CIS and OXA on growth of SKOV3 cells in a spheroid assay.
SKOV3 cells were plated at a density of 5,000 cells per well and spheroid allowed to form (72-hours). Cells were then treated with serial compound dilutions and kinetics of spheroid growth and shrinkage were obtained. Plate-view shows the individual well Largest BF area (µm2) over time. Concentration response curves represent the Largest BF area (µm²) at 204-hours post-treatment. Data were collected over 240-hour period at 6-hour intervals. Each data point represents mean ±SEM, n=8.

Intra- and inter-plate reproducibility

A series of independent experiments using A549 cells and ± 1 µM CMP were performed to assess reproducibility and precision of the IncuCyte® BF analysis tool (10x magnification, Figure 7). Once spheroids had formed (72-hours), growth was monitored and analyzed for a further 180-hours. Variability plot analysis and microplate overview image shows the individual well Largest BF Object area (µm2) values and spheroid BF segmentation respectively. Highly consistent BF segmentation, with reproducible well-to-well kinetic measurements (average CV values of less than 10%) were observed. Inter-plate variability was assessed in 10 individual experiments. The bar graph shows the Largest BF Object area (µm²) for both treatment groups across all experiments.

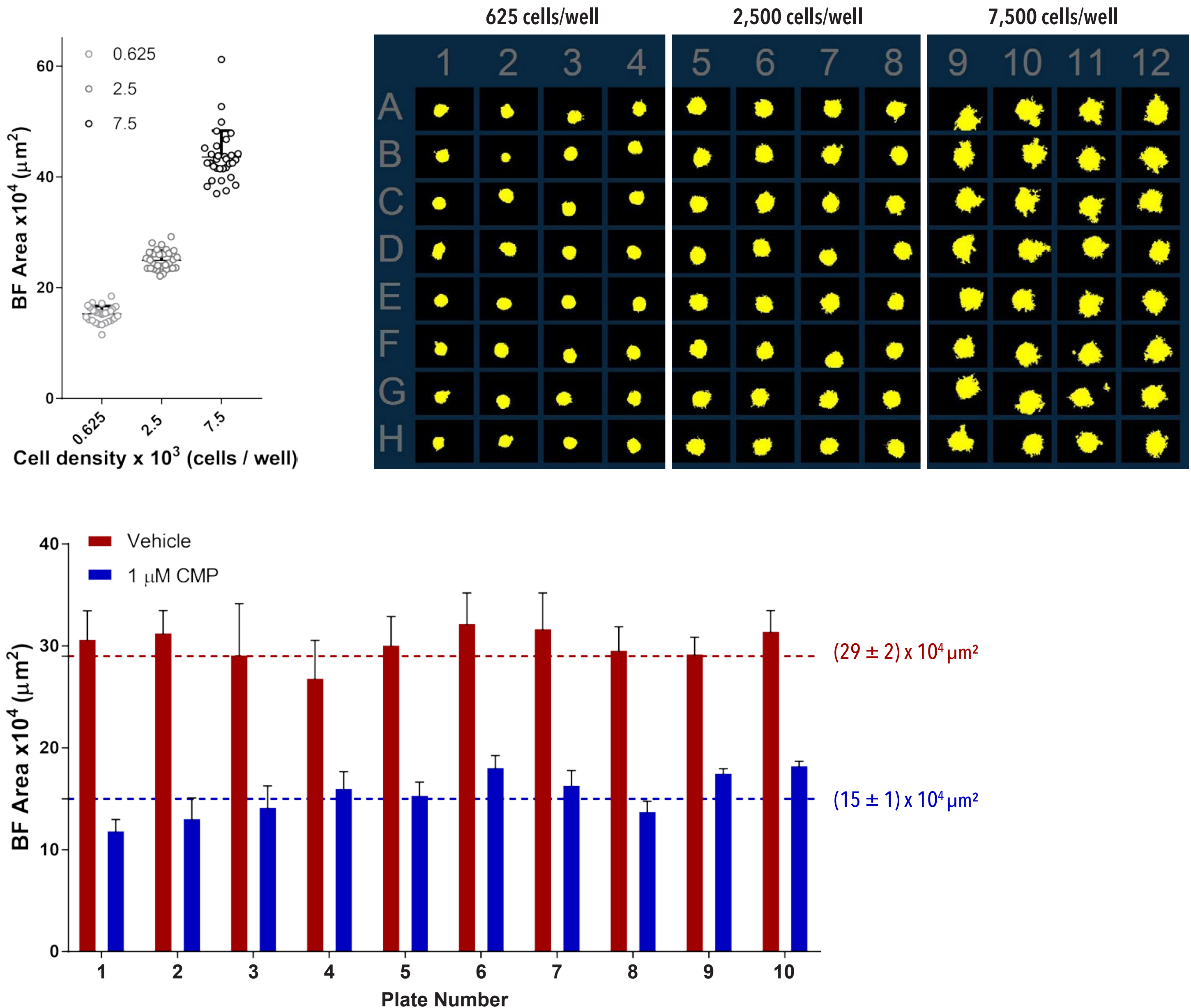

Figure 7. Precision and reproducibility of spheroid growth and shrinkage.
Growth and shrinkage of A549 cells in a spheroid assay. For intra-plate reproducibility assessment, cells were plated at a density of 625, 2,500 or 7,500 cells per well and spheroids allowed to form for 72-hours. Variability plot analysis shows the largest BF area of individual wells at 180-hours post spheroid formation. Microplate overview image shows BF segmentation at 120-hours post spheroid formation. Data is expressed as mean ± SD (n=32). For inter-plate variability studies, cells were plated at a density of 2,500 cells per well, spheroid allowed to form for 72 - hours then treated with ±1 µM camptothecin. Bar graph analysis shows the Largest BF Object area (µm2) for both treatment groups. Data were collected over a 180-hour period at 6-hour intervals. All images captured at 10x magnification. Each data point represents mean ±SD, n=3-24, 10 plates.

Miniaturizing to 384-well format

To maximize data output, the spheroid growth and shrinkage assay was miniaturized to 384-well format. A549, HT-1080 and SKOV-3 cell types at four cell densities were plated in 384-well ULA round-bottom plates for 72-hours. Spheroid BF area was quantified on the IncuCyte system using the Largest BF Object Area (µm²) metric. Data demonstrates the cell area dependence with seeding density and differential growth profile across the cell types (Figure 8).

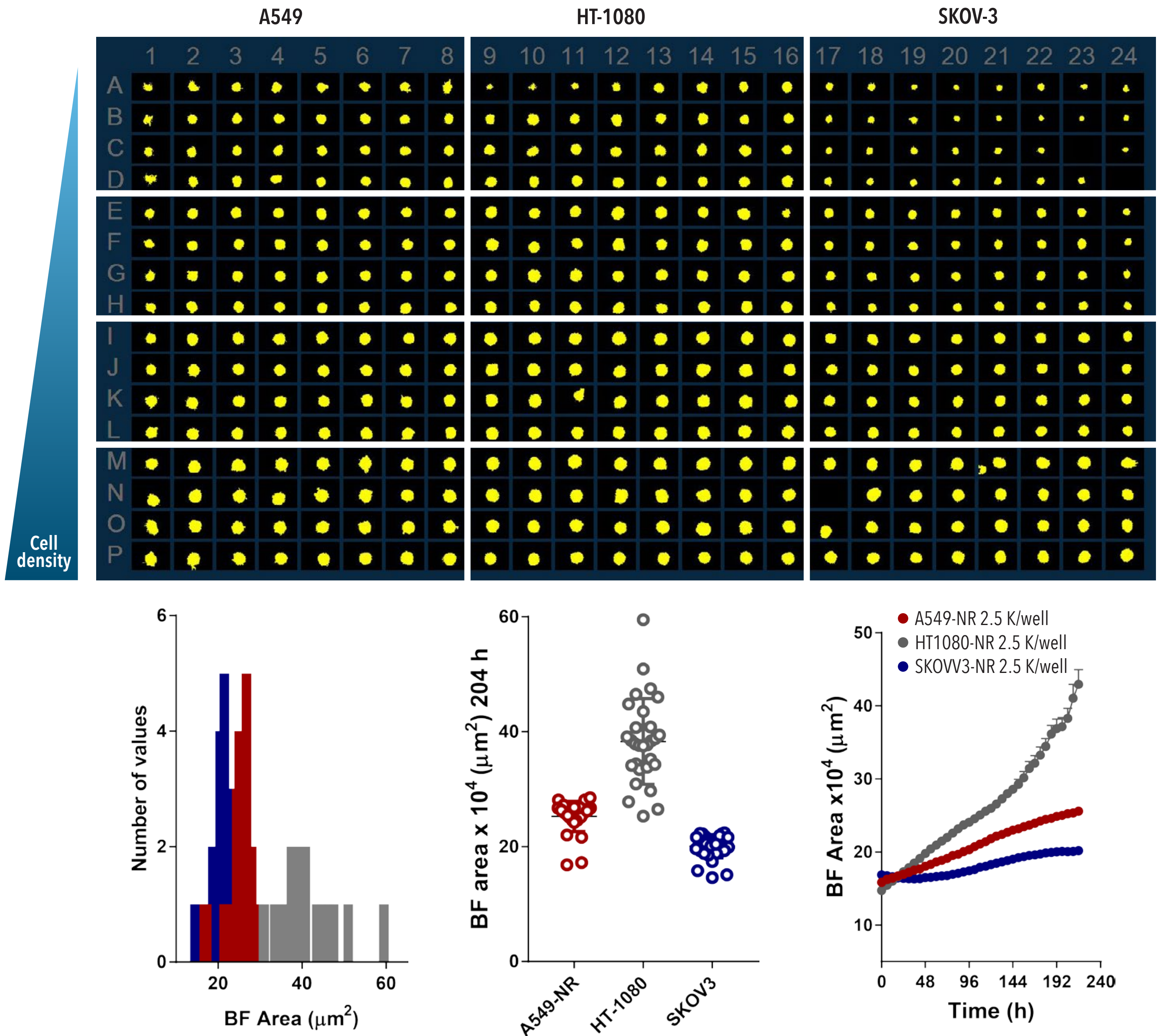

Figure 8 Miniaturizing Spheroid Growth and Shrinkage assay for assay optimization.
Comparison of temporal growth profiles of A549, HT-1080 and SKOV-3 cells in a miniaturized spheroid assay. All cells seeded at a density ranging from 310 to 7,500 cells per well plated in a ULA round-bottom 384-well plate. Media was replenished 72-hours post seeding. Microplate overview image shows BF segmentation mask at 204-hours post-media replenishment. Histogram compares the distribution frequency of the BF area (µm²) across all cell types plated at 2,500 cells per well at this time-point. Variability plot analysis shows the largest BF area of individual wells at 204-hours. Time-course plots represent the differential temporal profile of the Largest BF Object Area metric (µm²) across the cell types. Data were collected over a 204-hour period at 6-hour intervals, all images captured at 10x magnification. Each data point represents mean ±SEM, n=32.

Conclusions

We have demonstrated that the IncuCyte® Live-Cell Analysis System enables the study of spheroid analysis in real-time. Brightfield in combination with phase contrast imaging allows for label free study of spheroid morphology, growth and shrinkage in 96- and 384-assay formats. IncuCyte® HD phase images facilitate comprehensive visualization of spheroid morphological features (shape, size) and intercellular compaction (loose aggregates vs. compact spheroids.) characteristic for each cell type. Phase contrast images provide qualitative information while BF provides the means for objective spheroid kinetic quantification and cell dependent growth rate profile assessment for a diverse type of spheroid.

With no need for a predefined end-point selection, a highly consistent BF segmentation, and reproducible well-to-well kinetic data, the IncuCyte shows amenability to the pharmacological study of growth and shrinkage of spheroids.

References

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