**Introduction**

To mimic the initial avascular stages of solid tumors in vivo, spheroids need to develop hypoxia and subsequent necrosis in their center. Indeed, due to the presence of quiescent cells, spheroids are more resistant to certain compounds compared to cells grown as 2D monolayers and therefore considered a more relevant model for drug screening. Widely used methods to obtain multicellular spheroids involve culturing malignant cells in anchorage-independent growth conditions, generating a single spheroid per well and have poor compatibility with high throughput and HCS approaches. We have developed a simple method to grow multiple homogenous spheroids per well in 96 well plate format. Spheroids are attached to the glass bottom and are directly compatible with conventional biochemical readouts as well as High Content Analysis without troublesome plate transfer. Our method is fully compatible with automation and allows spheroids to develop up to 600 µm in diameter with interwell CVs of 5% or less.

**Robust, homogenous and reproducible multiple spheroids growth and maturation**

![Image of spheroids growth and maturation](image_url)

Figure 3: Typical well in 24 well format 3D Brightfield acquisition HT-29 spheroids after 12 days in culture (Operetta, PerkinElmer). Multiple spheroids on same a level allow fast and robust image acquisition.

**Linear and homogenous growth**

![Linear and homogenous growth](image_url)

Figure 4: Control of growth over time. Growth kinetic of HT-29 spheroids.Spheroids are continuously growing for up to 30 days, reaching a size of 600µm.

**Methods**

**HCA compatibility facilitates characterization of MOA**

![Image of HCA compatibility](image_url)

Figure 5: Low coefficient of variation. Calculated CV (coefficient of variation) of measured spheroids diameter. Maximum observed CV’s are 5% between wells of a same plate as well as different plates, regardless of the age of spheroids.

**Characterization of drug MOA**

![Characterization of drug MOA](image_url)

Figure 7: Illustration of developments and image acquisition available with spheroids treated with different drugs in drug response. Calc: calreticulin. PI: Propidium iodide. BF: Brightfield

**Conclusion**

![Conclusion](image_url)

Figure 9: Validation of Oncosphere™ for image stored HCA and direct insight into spheroid response and compound MDA. Differences in local effects of a cytostatic versus a cytotoxic compound can be distinguished. Z’ is 0.5-0.5 for various parameters n=37 spheroids in 3 wells.

**Key advantages**

- New HCS solution with functional spheroids
- Robust and automated assay
- Mature spheroid model
- More spheroids per data point per screen with CV<5%
- Applicable to HCS IC50 curve determination
- Directly compatible with imaging and HCA

Altogether, the production method presented allows production of multiple spheroids per well which translates into high assay robustness. The full compatibility with High Content Analysis opens up new avenues toward the development of innovative and more pertinent readouts that will contribute to a higher definition of compound Mode Of Action.

**Improved robustness for 3D spheroid screening using High Content Analysis**

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