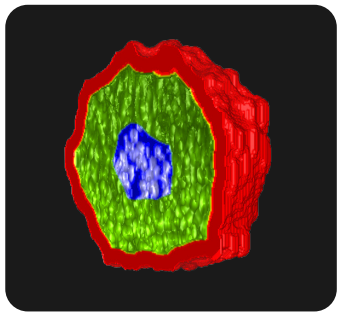
3D volumetric and zonal analysis of solid spheroids.

We address:

- How to reduce image acquisition time and data volume
- How to analyse fully and partially imaged spheroids in 3D
- How to define zones and quantify spatial differences within spheroids

Introduction



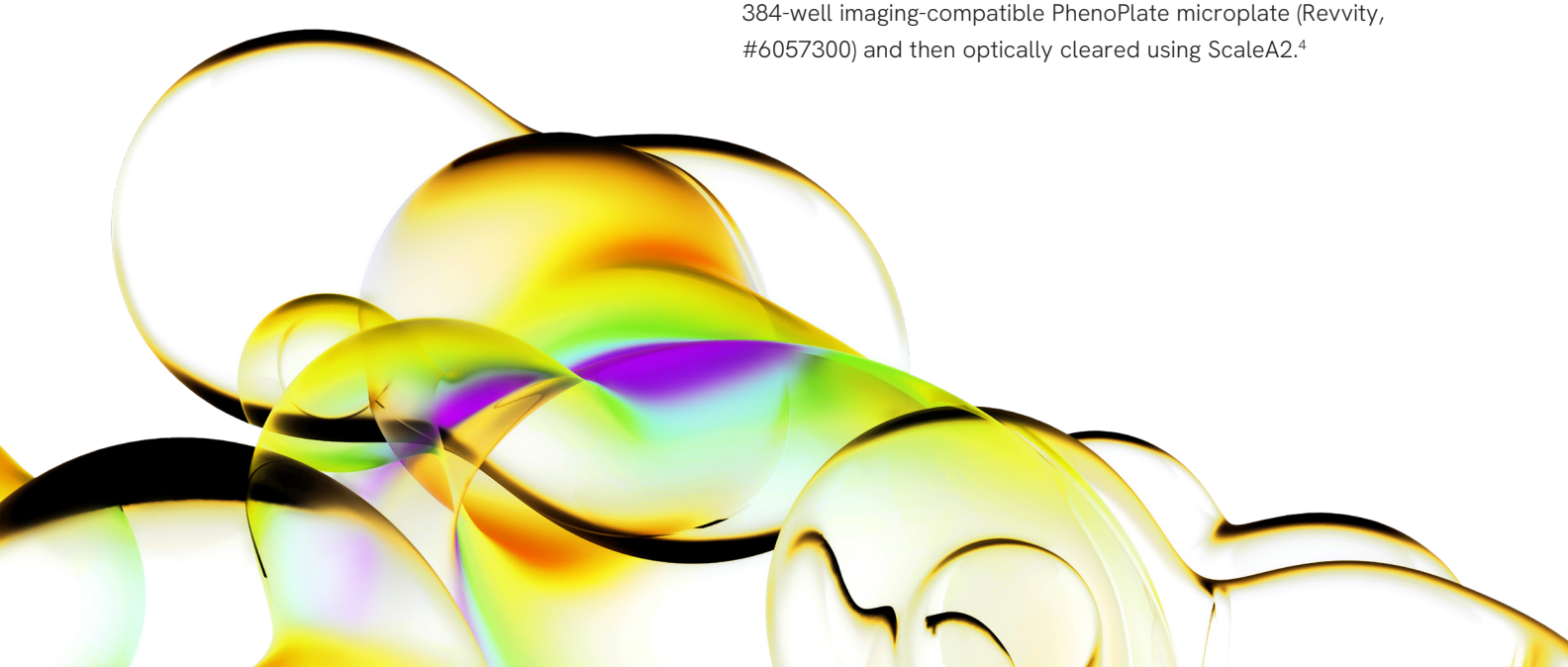
Multicellular 3D "oids" (tumoroids, spheroids, organoids) have the potential to better predict the effects of drug candidates during preclinical screening. However, compared to 2D cell monolayers, assays using 3D model systems are more challenging.[1]

In this technical note we describe how to image and analyze solid spheroids in 3D using the Opera Phenix Plus™ and Operetta® CLS™ high-content screening systems and Harmony® imaging and analysis software.

3D spheroid preparation and imaging

Spheroid preparation using CellCarrier Spheroid ULA 96-microplates

HeLa cells were seeded at three different cell numbers (1.25E3, 2.5E3 and 5E3) into CellCarrier Spheroid ULA 96-well microplates (Revvity, #6055330).[2] After 48 hours, spheroids were fixed with 3.7% formaldehyde and nuclei were stained with DRAQ5™. Mitotic cells were labeled using an anti-phospho-histone H3 antibody (Sigma, #H9908) combined with an Alexa 546 secondary antibody (Life Technologies, #A11081) as described previously.[3] For a fast workflow, spheroids can be imaged within 96-well round bottom ULA microplates, using long working distance objectives. However, for high resolution imaging, spheroids were transferred into a 384-well imaging-compatible PhenoPlate microplate (Revvity, #6057300) and then optically cleared using ScaleA2.[4]



PreciScan reduces image acquisition time and data volume

All spheroids were imaged using the "PreciScan" feature of Harmony software as described in detail previously.[5] PreciScan is an intelligent acquisition routine which identifies the x/y position of an object of interest in a well. Using a low magnification pre-scan, a simple online analysis and a high magnification re-scan, only the objects of interest are targeted for high resolution imaging. The re-scan may contain z-stacks and/or a time series. PreciScan is an ideal tool to save measurement and analysis time as well as data storage space (e.g. 25-fold less space and time for a 384-well plate spheroid assay using the 20x objective, and 100-fold less with the 40x objective) and is available on both the Operetta CLS and Opera Phenix systems.

Optimizing imaging depth with water immersion objectives and clearing

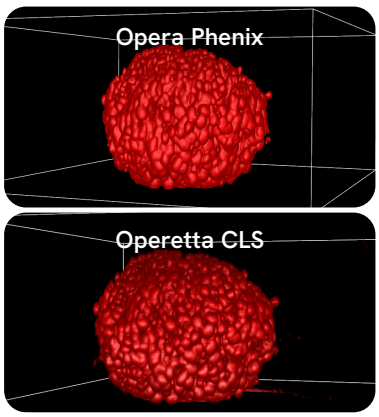
Spheroids were imaged in confocal mode on an Opera Phenix system equipped with four cameras, laser-based excitation, microlens-enhanced spinning disk and water immersion objectives or on an Operetta CLS system with one camera, LED-based excitation, single spinning disk and water immersion objectives. Images acquired using water immersion objectives, are of much higher quality, especially in z-direction.[6,7] Additionally, optical clearing greatly enhances imaging depth. Optical clearing homogenizes refractive indices within the sample and reduces light scattering and optical aberrations. The use of longer wavelength dyes, whenever feasible, also decreases light scattering and increases light penetration into the 3D sample. As a result, imaging depth and signal detection are improved.

3D analysis strategy for fully imaged spheroids

Harmony software (and later versions) contains tools for 3D visualization, as well as extensive 3D segmentation and quantification capabilities. Here we show how to quantify morphological parameters of a solid spheroid, e.g. 3D volume, sphericity or diameter, alongside cell-based properties such as number and sphericity of nuclei. In addition, we show how to subdivide spheroids into concentric zones (outer ring to inner core) to quantify spatial differences within spheroids such as the distribution of mitotic cells (Figure 1).

1. Create 3D or XYZ view

- Visualize 3D samples either in the XYZ- or the 3D view
- Explore samples in 3D - rotate, zoom, pan or shift
- Create movie exports - 3D rotation or plane movie through all stack planes



2. Find Spheroid and Find Nuclei

- Find the whole spheroid using the *Find Image Region* building block
- Apply a local threshold to compensate for light attenuation in z
- Choose from different *Find Nuclei* methods - all dedicated to segment nuclei in 3D

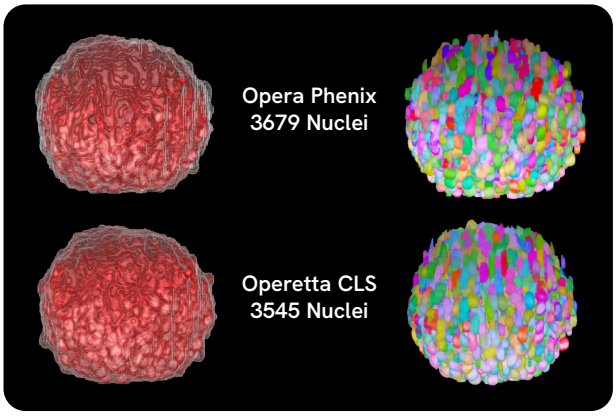


Figure 1. 3D analysis strategy for fully imaged spheroids using Harmony software. Morphological properties of the whole spheroid, as well as cell-based quantifications in concentric zones of the spheroid, are possible. Spheroids were labeled with DRAQ5™ (red), a pHH3 antibody (orange) and cleared with ScaleA2 for five days. Image stacks were recorded confocally using a 20x water immersion objective (numerical aperture 1.0) and 1 µm-z-stacks covering an overall height of 300 µm on the Opera Phenix or the Operetta CLS system. While the Opera Phenix provides the highest 3D image quality, the Operetta CLS performs almost equally well with respect to imaging depth und nuclei detection for the HeLa spheroids tested. Step 4 and step 5 are shown with Opera Phenix images only, but Operetta CLS images are comparable.

3. Calculate 3D properties of spheroid and nuclei

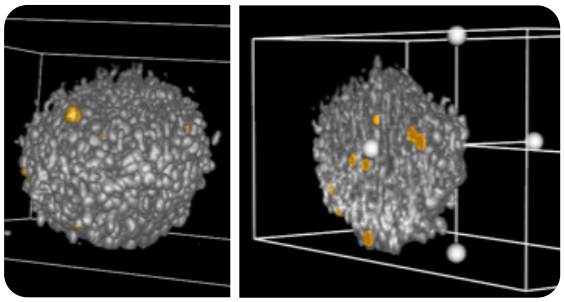
- Analyze morphological 3D properties for the spheroid and segmented cells using *Calculate Morphology Properties*
- 3D readouts are easily obtained for fully imaged spheroids while only partially captured objects require a more sophisticated analysis (see Figure 2)

Spheroid volume [µm^3]	Sphericity [-]	Footprint area [µm^2]	Spheroid height [µm]
15590200	0.77	80314	286

Spheroid cross section area [µm^2]	# of nuclei per spheroid	Sphericity of nuclei	Volume of nuclei [µm^3]
79057	3679	0.71	1075

4. Find mitotic cells

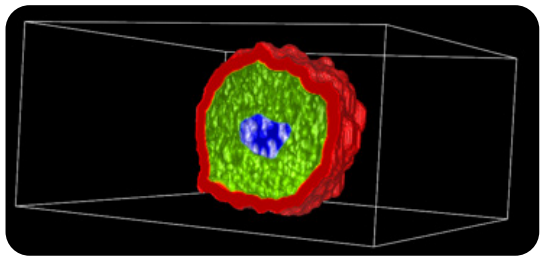
- Use a *Find Nuclei* building block to identify mitotic, pHH3 positive cells based on a local threshold
- Create cut-open views with the *Clip Box* tool to explore objects from inside (right side)



Cut Open View using *Clip Box* Tool

5. Find Spheroid and Find Nuclei

- Calculate the shortest distance of each nucleus to the spheroid border
- Use *Select Region* and *Select Population* to classify nuclei into zones. Freely adjust width of zones.
- Analyze number of mitotic cells per zone and spatial differences for various readouts such as cell morphology



Cut Open View using *Clip Box* Tool

Figure 1. (continued)

Critical aspects when analyzing partially imaged spheroids

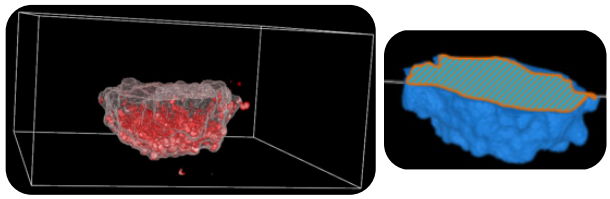
Depending on the size and density of spheroids, it will often not be possible to image them in their entirety, even after optical clearing. Here, in Figure 2, we show how to adapt the image analysis strategy shown in Figure 1 to partially imaged spherical objects. The analysis sequence for partially imaged spheroids was then applied to spheroids with different initial seeding densities (Figure 3).

Calculate the visible fraction of the spheroid [%]

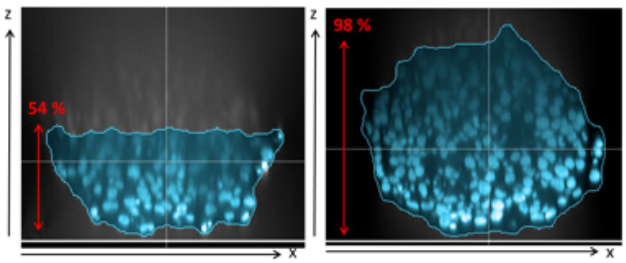
- Identify the partially imaged object with *Find Image Region*
- Use *Calculate Morphology Properties* to determine the Maximum *Cross Section Area* [µm^2] (area of the largest possible cross section in xy) and the *Maximum Thickness* [µm]
- Assuming the object to be spherical, the spheroid diameter can be calculated from the *Maximum Cross Section Area* using *Calculate Properties*

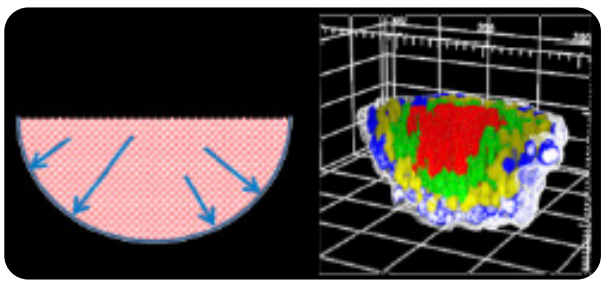
2*sqrt(*Max Cross Section Area* / 3.14)

- Calculate the fraction of spheroid imaged by dividing the visible height (i.e. *Maximum Thickness*) of the spheroid by the spheroid diameter
- Knowing the percentage of the imaged spheroid height is useful in many ways: e.g. to quantify the clearing success, to interpret results, as pre-selection quality criteria for differently sized spheroids, or to normalize results



$$\text{Fraction of spheroid imaged [\%]} = \frac{\text{Visible height [µm] x 100}}{\text{Spheroid diameter [µm]}}$$





How to define zones in partially imaged spheroids

- If the spheroid is only partially imaged, the upper border needs to be excluded from distance calculations and only the side and bottom borders selected. This can be done with a series of *Select Region* building blocks to create an open bowl region:
  1. Create a region covering the top border of the spheroid
  2. Subtract spheroid border region by the top border region using the *Restrict By Mask* function

Figure 2. Harmony software provides tools to analyze partially imaged spheroids. Knowing which fraction of the spheroid was imaged and how to define concentric zones even if the upper border is not visible are important prerequisites to robustly extract meaningful biological readouts from partially imaged spheroids. Spheroids were labeled with DRAQ5™ (red) and a pHH3 antibody (orange). To achieve partial clearing, spheroids were cleared with ScaleA2 for less than 1 hour. Image stacks were recorded confocally using a 20x water immersion objective (numerical aperture 1.0) and 300 planes with a distance of 1 µm on the Opera Phenix system.

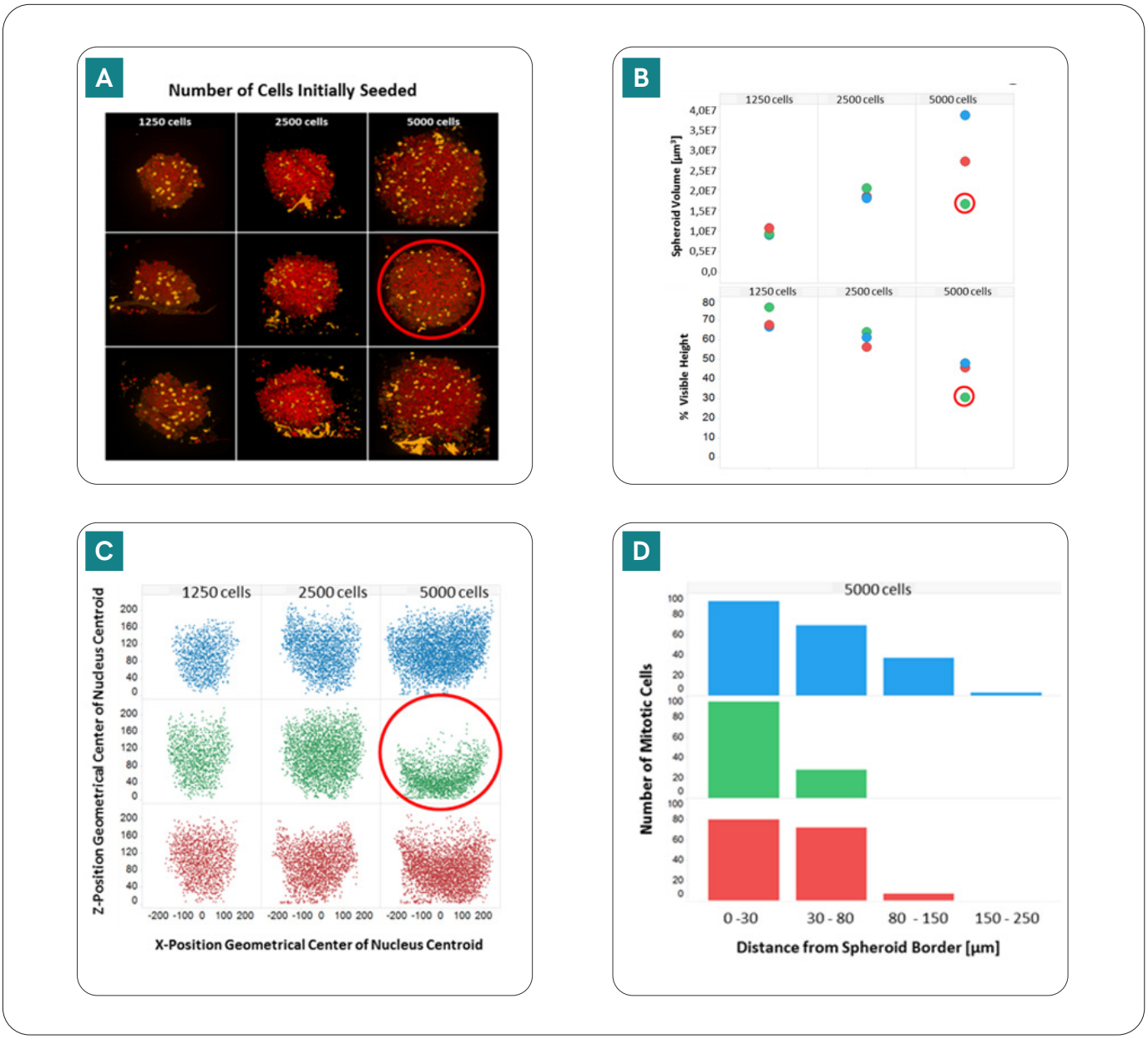


Figure 3. Partially imaged spheroids can be analyzed with Harmony software. Three different spheroid sizes, small, medium and large, were generated by initally seeding 1250, 2500 or 5000 cells per well. Spheroids were labeled with DRAQ5™ (red), a pHH3 antibody (orange) and cleared with Scale A2 for less than one hour.
Secondary analysis and visualizations (B, C, D) were done with TIBCO Spotfire®. (A) Maximum intensity projection images from stacks with 300 planes and 1 µm plane distance. Images were acquired on an Opera Phenix system using a 20x water immersion objective. In this visualization, differently sized spheroids appear to be very similar within each group. (B) The 3D volume analysis shows that small and medium sized spheroids are more uniformly sized while large spheroids are more heterogeneous. The fraction of the visible height of the spheroids identifies one spheroid to be less imaged-through (red circle). (C) Plotting the xz-positions of each nucleus of the spheroids is another way to create an imaging profile of the spheroids. The one outlier is apparently less effectively cleared by ScaleA2 (red circle). (D) Mitotic cells are predominantly located in the outer regions (0-30 µm and 30-80 µm). A histogram binned by the nuclei distance to the spheroid border is shown for the three large spheroids. Note, that the outlier spheroid in green contains mitotic cells only in the outer two regions (up to 80 µm), as the other regions were not visible.

Conclusions

Running successful high-content screens using 3D model systems requires careful attention to several critical aspects. Typical challenges for imaging and analysis are long imaging times, huge data volumes and issues with 3D image quality such as image distortion in z and light attenuation in z.

The Opera Phenix high-content screening system has been designed with 3D in mind, providing highest 3D image quality.[8] Water immersion lenses, a microlens-enhanced spinning disk and strong excitation lasers contribute to enhanced image quality, while four cameras and Synchrony Optics™ maintain sensitivity whilst increasing speed. However, despite being slower due to its single camera, the Operetta CLS performs exceptionally well for spheroids, due to its water immersion lenses and strong LEDs.

Moreover, the acquisition routine PreciScan, allows spheroids to become centered within the field of view, thereby avoiding empty images and unnecessary data.

Solid spheroids are often composed of different zones due to diffusion gradients of oxygen, nutrients, drugs or dyes and therefore it is crucial to be able to analyze these spatial differences within spheroids.[9] Here, we have presented 3D analysis strategies created with Harmony imaging and analysis software for both fully and partially imaged spheroids. The flexible building blocks of Harmony allow typical 3D morphology, intensity and texture readouts for spheroids, or cells within spheroids, as well as distance measurements and zone definitions.

While fully imaged 3D objects are relatively straightforward to analyze in 3D, a more sophisticated analysis strategy is needed for spheroids which are only partially imaged. To create correct 3D regions and to discriminate the outer shell from the inner core in partially imaged spheroids, it is, for example, important to provide flexible region definitions and to quantify the extent to which the spheroid was imaged.

Together, Revvity high-content screening systems and Harmony software enable highly-detailed, multiparametric phenotypic analysis of 3D cell models, helping to increase the physiological relevance of results and accelerate research.

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