More content from high-content screening: Analyzing spheroids in 3D.

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1 Introduction

When developing a HCS assay, a critical aspect is the choice of cellular model. Multicellular 3D models (e.g., spheroids, organoids) have the potential to better predict the effects of drug candidates *in vivo.* However, they tend to make all steps of the workflow more complex compared to 2D cultures.

Generating large numbers of uniform spheroids at high quality for screening is one of the challenges. Using spheroids and cysts as examples, we show how to grow these models effectively using ULA coated U-bottom plates or low concentration gels. Careful selection of dyes and clearing strategies can improve image quality while targeted imaging of spheroids helps to shorten imaging time and minimize data volume. However, effective analysis of spheroids is still a major bottleneck. Dedicated high-content software tools can help users to explore 3D data sets and quantify in 3D changes such as volume, shape and number of nuclei per spheroid to better understand drug effects.

2 Essentials for 3D applications: Harmony software and water immersion objectives

High quality images are a prerequisite for successful 3D image analysis. To show the importance of water immersion objectives to acquisition of the best possible image data, Madin-Darby canine kidney (MDCK) cell derived cysts were imaged using either a high NA air objective or a water immersion objective. The same sample was imaged on an Opera Phenix™ high-content screening system and an Operetta® CLS™ high-content analysis system. The Opera Phenix system features laser-based excitation and a microlens enhanced spinning disc while the Operetta CLS is equipped with a unique LED light source (8 LEDs) and a single spinning disc. This allows us to dissect out the influence of excitation source, spinning disc architecture, z-sampling rate and imaging objective on data quality for 3D analysis.

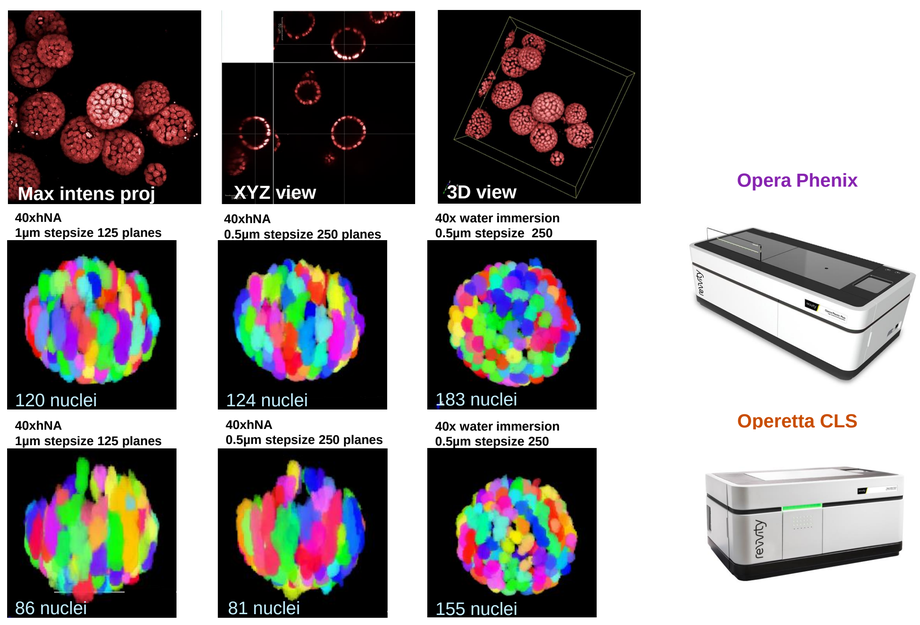
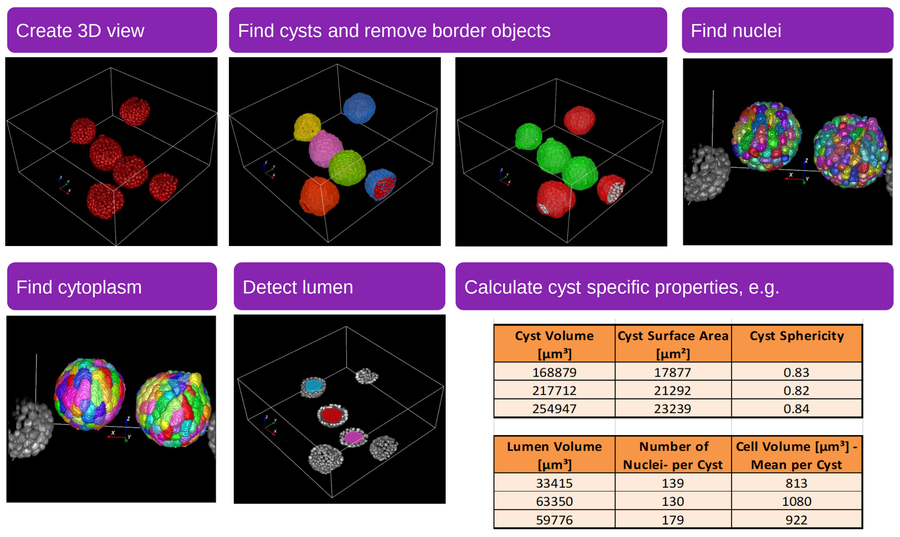


Figure 1: *Harmony® high-content analysis software allows acquisition, visualization and image analysis in one comprehensive software solution.*

MDCK cells were seeded in 2 % Geltrex-enriched (ThermoFisher) phenol red-free growth medium into ULA (ultra-low attachment) coated PhenoPlate™ 384-well microplates (Revvity). Cysts were grown for 10 days inside a cell incubator with medium refreshing on days 4 and 8. On day 10, live cysts were stained with 4 µM DRAQ5 (Revvity) for 2 h and then fixed with 3.7 % formaldehyde. Image acquisition was done on either Opera Phenix or Operetta CLS system using either a 40xhNA air (NA0.75) or 40x water immersion (NA1.1) objective.

(A) The images show the maximum intensity projection, an XYZ view and a 3D view of the data set acquired on the Opera Phenix system using the 40x water immersion objective (left to right).

(B) To study the difference in image quality for 3D analysis, the number of nuclei was analyzed. 3D nuclei segmentation could not be improved by a higher resolved z-stack for the high NA air objective but greatly improved when using the water immersion objective, which detects up to 1.5x times more nuclei than air objectives on the Opera Phenix and 2x times more on the Operetta CLS system. For this relatively transparent sample, water immersion has a greater effect on nuclei detection than spinning disc geometry or excitation light source.



Cyst Volume [µm³]	Cyst Surface Area [µm²]	Cyst Sphericity
168879	17877	0.83
217712	21292	0.82
254947	23239	0.84

Lumen Volume [µm³]	Number of Nuclei- per Cyst	Cell Volume [µm³] - Mean per Cyst
33415	139	813
63350	130	1080
59776	179	922

Figure 2*: Harmony software allows the quantitative analysis of 3D data sets including hollow spaces inside objects.*

Quantitative 3D analysis including hollow spaces inside objects using Harmony: Images were acquired on the Operetta CLS using a 40x water immersion objective. Individual cysts as well as their nuclei and cytoplasm were segmented in 3D after excluding border objects. Also, the lumen was identified. Properties can be calculated for the whole cyst or individual cells (examples in the table).

3 Uniform spheroid formation using CellCarrier Spheroid ULA 96 microplates

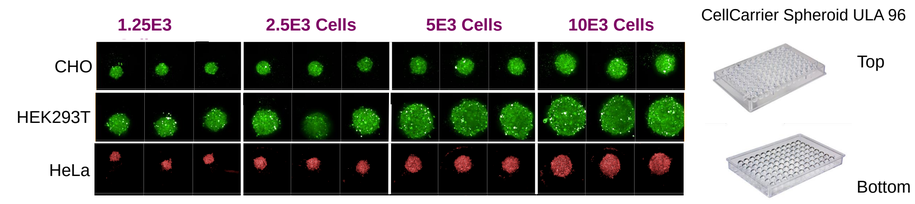


Figure 3: *Formation of uniform spheroids using Revvity CellCarrier Spheroid ULA 96 round bottom microplates. Increasing numbers of CMFDA-pre-stained CHO (upper panel) and HEK293T (middle panel) cells as well as DRAQ5-pre-stained HeLa cells were seeded into the microplate and incubated for 2 days. Cells form uniformly shaped spheroids of increasing size. Images were acquired on an Opera Phenix system using a 20x air LWD objective lens in widefield mode (maximum intensity projection from 30 planes, 0 to 58 µm, distance 2 µm).*

4 Optical clearing improves imaging depth

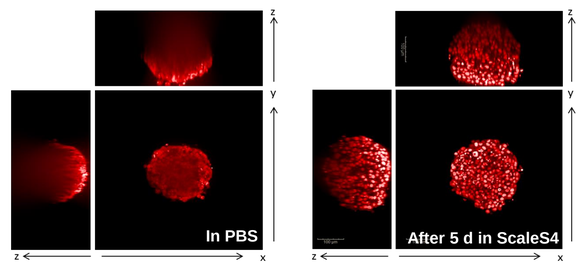


Figure 4: *Optical clearing improves imaging depth. The same HeLa spheroid was fixed, stained with DRAQ5 and imaged before (left, in PBS) and after (right) clearing (5 d in ScaleS4). 301 planes at 1 µm distance were acquired on an Opera Phenix in confocal mode using a 20x water immersion objective. The overall z-height of the spheroid is 260 µm.*

5 PreciScan acquisition saves time and data

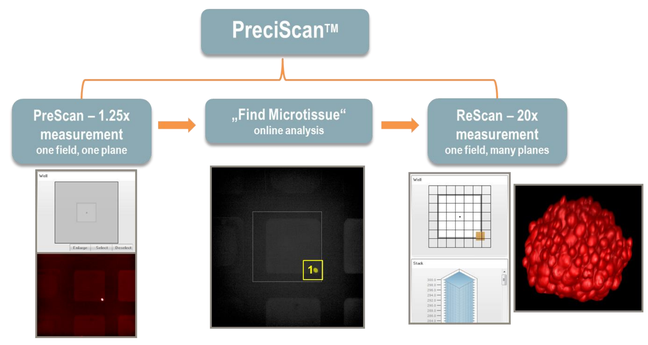


Figure 5*: PreciScan is an intelligent fully automated 3-step acquisition routine identifying the x/y position of an object or rare event in a well on the basis of a low magnification "PreScan" measurement. Based on evaluating the PreScan with a simple online analysis, only this part within the well (or only wells containing objects at all) will be targeted for a higher resolution acquisition (e.g., fine-tuned 20x stack enabling 3D analysis). PreciScan saves significant measurement and analysis time as well as data storage space.*

6 Analysis of incompletely imaged spheroids

Spheroids may not always be completely optically clear, e.g., if the spheroid size is too large. To establish spheroids of different sizes, we seeded 1.25k, 2.5k or 5k cells and stained them with DRAQ5 and an anti phospho-Histone-3 antibody. After clearing, they were imaged using a 20x water immersion objective on an Opera Phenix system and analyzed using Harmony software.

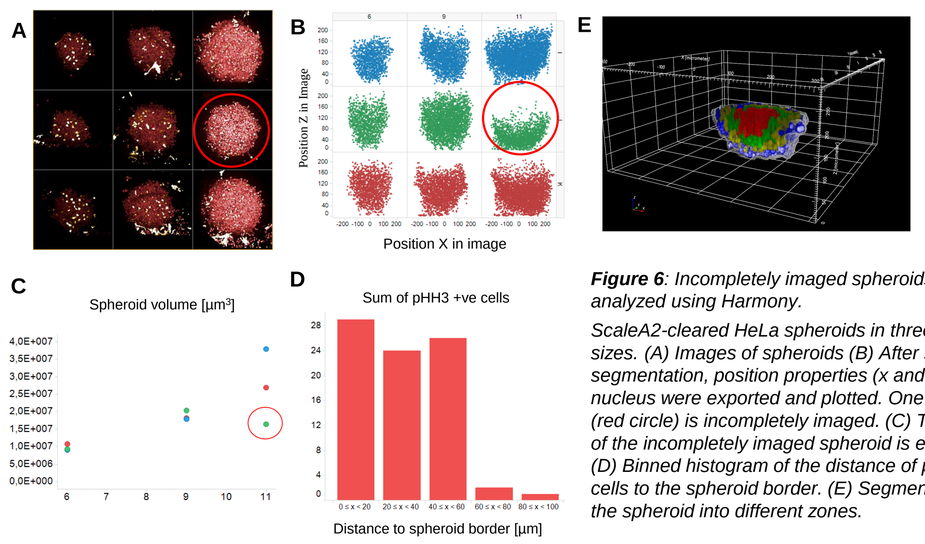


Figure 6*: Incompletely imaged spheroids can be analyzed using Harmony.*

ScaleA2-cleared HeLa spheroids in three different sizes. (A) Images of spheroids (B) After single cell segmentation, position properties (x and z) of each nucleus were exported and plotted. One spheroid (red circle) is incompletely imaged. (C) The volume of the incompletely imaged spheroid is encircled. (D) Binned histogram of the distance of pHH3+ve cells to the spheroid border. (E) Segmentation of the spheroid into different zones.

7 Summary

In 3D imaging, extracting meaningful data is more difficult compared to 2D imaging. Often, 3D objects are not completely imaged through, and biological readouts remain incomplete. Here we have shown that:

- High NA water immersion objectives substantially improve 3D image quality and nuclei segmentation when compared directly to air objectives
- Harmony software a offers a wide range of 3D analysis and visualization tools
- Harmony enables the analysis of large 3D objects including hollow spaces as well as single cells
- The PreciScan tool increases acquisition speed and decreases data volume

References

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