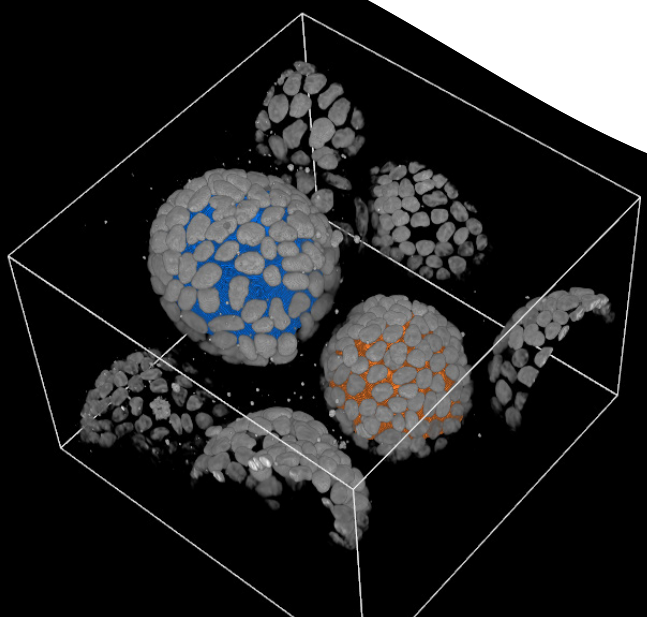
3D volumetric analysis of luminal spaces inside cysts or organoids.

We address:

- How to reduce the loss of image resolution as you image deeper into the sample
- How to enable 3D segmentation and analysis for your 3D high-content assays
- How to increase throughput for 3D imaging through shorter exposure times
- How to eliminate long and tedious data transfer steps



Introduction

High-content assays using 3D objects such as cysts or organoids can be challenging from the perspectives of both image acquisition and image analysis.

In this technical note we describe how to image and analyze epithelial Madin-Darby canine kidney (MDCK) cysts in 3D on the Operetta® CLS™ high-content analysis system.

3D assay development and imaging

Preparation of MDCK cysts

Once epithelial cells such as MDCK cells come into contact with extracellular matrix (ECM), they begin to form 3-dimensional structures in a process called cystogenesis. Spherical cysts generate with a central hollow lumen lined by a polarized monolayer of cells. MDCK cysts recapitulate *in vivo* processes of epithelial tissues and are a valuable, extensively studied model system.[1]

To grow MDCK cysts, we optimized the protocol previously described[2] by plating MDCK cells in 2 % Geltrex-enriched (Geltrex™, Thermofisher, # A1413202) growth medium directly into an ultra-low attachment (ULA) coated PhenoPlate 384-well microplate (Revvity, # 6057802). By omitting the commonly used ECM cushion (i.e. well bottom precoating), the distance of 3D objects to the objective lens was minimized. After 10 days, cells were stained with 4 µM DRAQ5™ (Biostatus, #DR50200) and then fixed using 3.7 % formaldehyde.

3D imaging

MDCK cysts were imaged on an Operetta CLS high-content analysis system, equipped with water immersion objectives, in confocal mode. To compare 3D image quality with air vs. water immersion objectives, the same MDCK cyst was imaged, using either a 40x hNA (high numerical aperture, NA 0.75) or a 40x water immersion objective (NA 1.1). A z-stack of 301 planes with a distance of 0.5 µm over a total distance of 150 µm was acquired. Compared to air objectives, images acquired with the water immersion objective show much better resolution especially the z-direction (Fig 1). This results in improved nuclei segmentation which leads to detection of twice as many nuclei from the same cyst compared to the air objective. With water immersion objectives, the refractive index of the immersion water matches the refractive index of the sample, resulting in less light diffraction and fewer optical aberrations. Additionally, the water immersion objective has a higher numerical aperture than the air objective and subsequently more excitation light is delivered into, and more emitted fluorescent light collected from, the sample.[3]

The use of a red nuclear dye such as DRAQ5™ improves penetration depth and signal detection, as longer wavelength light is scattered less.[4]

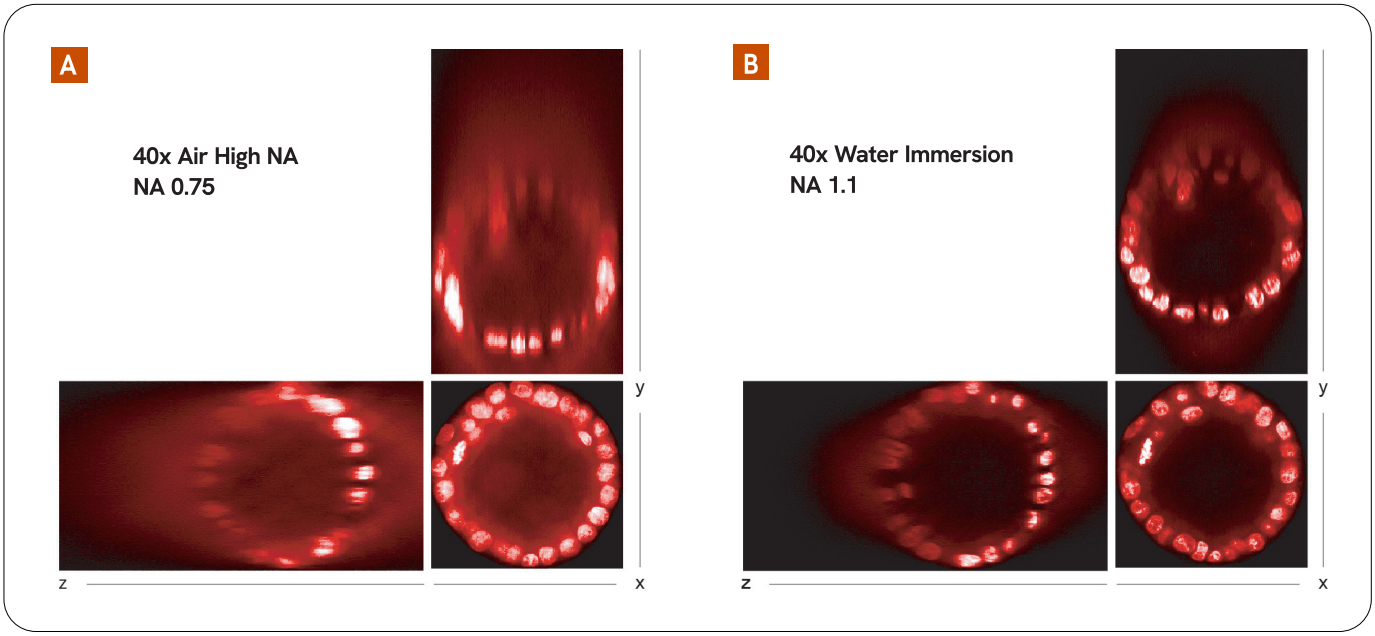


Figure 1 A-B. Water immersion objectives improve 3D image quality and nuclei segmentation results.

A/B: XYZ view of 301 planes covering 150 µm of the same MDCK cyst imaged on the Operetta CLS high-content analysis system in confocal mode using either a 40x high NA air objective (A) or a 40x water immersion objective (B). Imaging conditions were adjusted to yield comparable intensities of DRAQ5™-stained nuclei. Image display settings are identical. The 3D image quality is dramatically improved with the water immersion objectives. Acquisition times are also improved, being almost four times shorter with the water immersion lens.

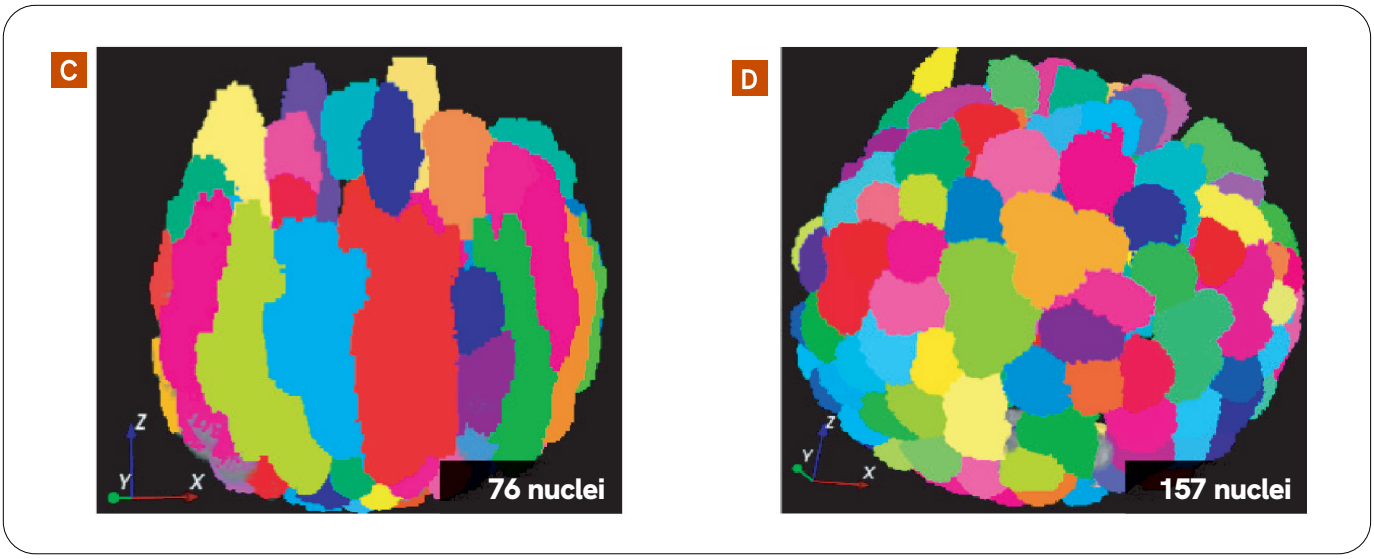
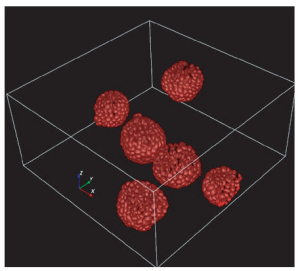


Figure 1 C-D. Water immersion objectives improve 3D image quality and nuclei segmentation results.

Water immersion objectives improve nuclei segmentation by a factor of two, compared to air objectives. Only 76 nuclei could be detected with the air objective, while the water immersion objective detected 157 nuclei from the same cyst. With the air objective, the distortion in Z caused several nuclei to be fused incorrectly and counted as one.

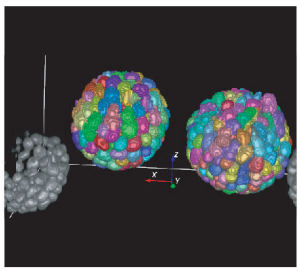
3D volumetric analysis strategy and visualization tools

Using Harmony® 4.8 high-content imaging and analysis software, we created a simple strategy for 3D analysis of spherical cysts and their hollow spaces (Fig 2). A similar strategy can be applied for cylindrically-shaped tubules or organoids with luminal spaces.



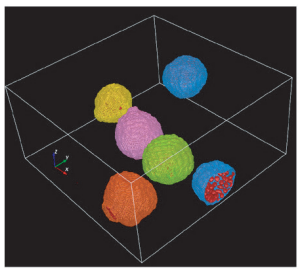
1 Create 3D view

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



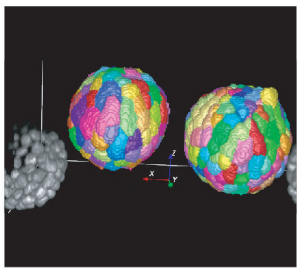
4 Find nuclei

- Choose from four different methods - all dedicated to segment nuclei in 3D
- Here we used *Method C*



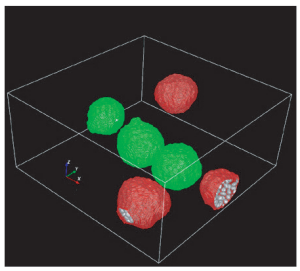
2 Find cysts

- Identify individual cysts using the *Find Image Region* building block
- Apply a local threshold to compensate for light attenuation in z



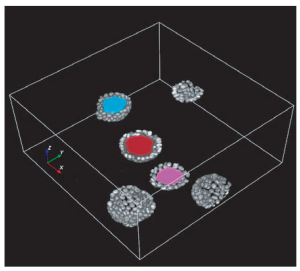
5 Find cytoplasm

- Choose from two methods for 3D segmentation of the cytoplasm
- Here we used *Method A*



3 Remove border objects

- Remove objects not completely covered using the building block *Select Population*



6 Find hollow space

- Create a region for the luminal space of the cysts with the building block *Select Region*
- Create cut-open views of the cysts to control results of the inner segmentation

Cyst centroid X in image [µm]	Cyst centroid Y in image [µm]	Cyst centroid Z in image [µm]
-117.0	13.1	47.0
86.5	-38.5	66.6
-1.7	-22.5	58.0

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

7 Calculate cyst specific properties

- Use *Calculate Morphology Properties* to calculate various morphological properties
- Calculate XYZ coordinates of cysts or cells within the cyst using *Calculate Position Properties*
- Calculate results as mean values per well or object-related for each cyst

Figure 2. Analysis strategy to calculate volumetric and positional properties for MDCK cysts.

Conclusions

3D assays are much more challenging than conventional 2D assays, but are becoming an essential part of the discovery process. One of the challenges is the production of high quality images from 3D cell models as, often, objects such as nuclei are distorted along the Z-axis and cannot be segmented properly. As shown in this technical note, water immersion objectives dramatically improve 3D image quality and detect twice as many nuclei as air objectives, when tested with identical objects (Fig 1).

However, up until now, even from high-quality 3D images, it has been difficult to extract information as suitable software for analysis of 3D high-content data has not been available.[5] Part of the problem is that 3D high-content data sets are extremely large. In the cyst example, every stack holds as much data as three 96-well plates if imaged with just one field per well. Furthermore, 3D image analysis software packages are often offered as separate packages as they were developed for single samples acquired on a classical microscope. Processing plate-based high-content data with such packages is very laborious and time-consuming, requiring considerable user interaction and an additional data transfer step. Harmony software is a single software package for 3D image acquisition, 3D visualization and 3D analysis, eliminating data transfer between acquisition and analysis.

Harmony software's 3D visualization and analysis tools have been developed for large 3D high-content data sets and allow volumetric analysis of 3D objects such as cysts, organoids or spheroids. Besides the morphology and positional properties shown here, Harmony allows calculation of additional 3D morphology, 3D intensity and 3D texture properties for detailed phenotypic characterization of 3D cell models. In addition, to avoid unnecessary data such as empty images, Harmony's PreciScan offers an automated workflow of low magnification pre-scan and high magnification re-scan for targeted imaging of spheroids or other rare events.

In summary, the Operetta CLS high-content analysis system equipped with water immersion objectives and Harmony software is able to overcome the most critical challenges with 3D assays and provides an ideal package for 3D imaging and 3D phenotypic characterization of more physiologically relevant cell culture models.

References

1. Elia, N., & Lippincott-Schwartz, J. (2009). Culturing MDCK Cells in Three Dimensions for Analyzing Intracellular Dynamics. Current Protocols in Cell Biology, 43(1), 4–22. https://doi. org/10.1002/0471143030.cb0422s43.
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Revvity, Inc.
940 Winter Street
Waltham, MA 02451 USA

(800) 762-4000
www.revvity.com