

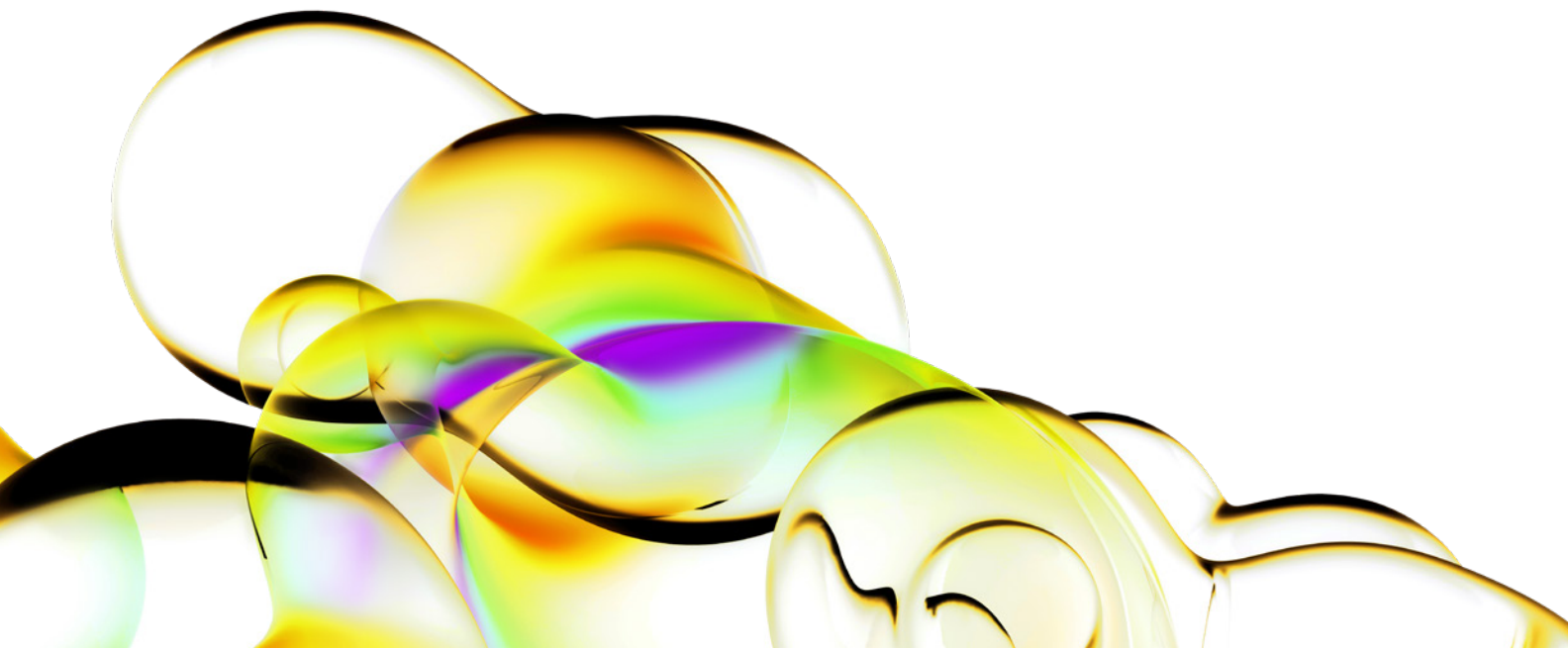
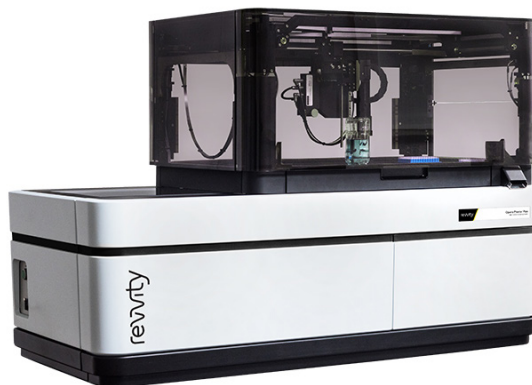
Opera Phenix Plus high-content screening system technical performance: Improved 3D imaging.

Spinning disk confocal microscopy is a common tool for live cell microscopy and reduces background fluorescence from out of focus planes. However, for 3D imaging, confocal image quality can be limited by a phenomenon known as 'pinhole crosstalk' [Shimozawa et al., 2013]. Out of focus light and scattered emission bleeds through neighboring pinholes and increases the background haze. This can lead to blurred images in conventional spinning disk systems. The confocal scanning unit of the Opera Phenix® Plus high-content screening system provides an increased distance between pinholes to reduce the pinhole crosstalk (Figure 1). This leads to improved 3D image quality.

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Opera Phenix Plus high-content screening system.



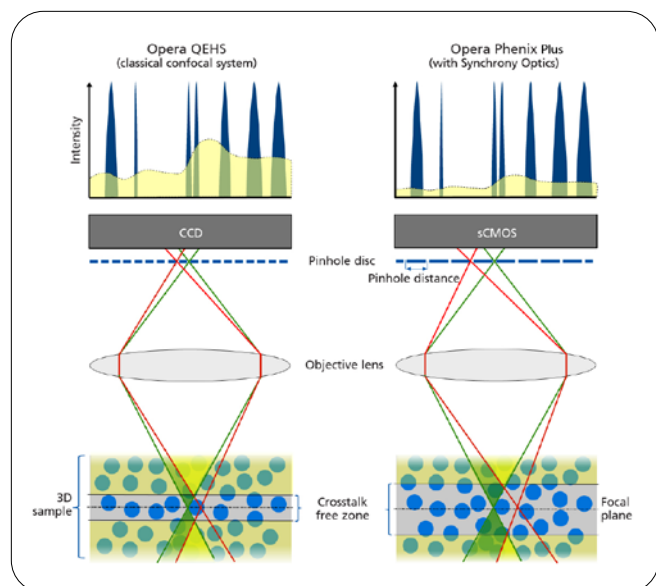


Figure 1: Increasing pinhole distances decreases pinhole crosstalk. Excitation light (shown in green) coming through one pinhole is focused into the focal plane and the resulting emission light (green shaded areas) is collected through exactly the same pinhole and detected on the sCMOS chip. A neighboring pinhole (red light path) can collect out of focus emission light indicated by the yellow shaded areas resulting in increased image background. Between the two yellow zones is the crosstalk free zone where no out of focus light is collected by adjacent pinholes. The thickness of the crosstalk free zone depends on the distance between pinholes. As seen from the light paths in the left panel (Opera QEHS), a small distance between pinholes results in a thin crosstalk free layer with increased image background intensity. Increasing the pinhole-to-pinhole distance (right panel, Opera Phenix Plus) dramatically decreases background haze due to an increase in the crosstalk free zone.

For analysis, intensity profile lines at exactly the same positions in the image stacks were found and matched against each other for quantitative comparison (Figure 2). The analysis clearly demonstrates that the Opera Phenix Plus images provide improved signal to noise ratios compared to the Opera QEHS images, which dramatically improves contrast.

Additionally, image stacks from both measurements were analyzed with Harmony® high-content imaging and analysis software to compare texture properties as well as the detection and segmentation of nuclei (Figure 3). For each image stack, the same image analysis sequence was used. First, the microtissue regions were found using the building block *Find Image Region* and then the building block *Find Nuclei* was used to segment it into nuclei. Subsequently, the texture property SER Edge (3px) in the center of the microtissue region for each plane was calculated using the building blocks *Select Region* and *Calculate Texture Properties*. The evaluation of both measurements revealed that the texture values on Opera Phenix Plus images are consistently higher than on Opera QEHS images throughout the whole image stack. The nuclei segmentation is also improved. In previous studies, we correlated texture values with the ability to segment nuclei and determined a texture value of 0.006 to be the lower limit [Letzsch et al., 2014]. Below 0.006 an accurate segmentation of nuclei is not possible. The Opera QEHS image stack reaches this threshold at a z height of 45 µm. In contrast to that, this texture threshold is extended to a z height of 75 µm in the Opera Phenix Plus image stack.

Application

A 3D microtissue stained with Hoechst was imaged either with an Opera Quadruple Enhanced High Sensitivity (QEHS) or an Opera Phenix Plus system to characterize the image quality of a thick specimen (300 µm in diameter). The Opera QEHS is equipped with a traditional confocal scanning unit (CSU10) comprising a pinhole-to-pinhole distance of 250 µm. By comparison, the confocal scanning unit of the Opera Phenix Plus system provides a pinhole-to-pinhole distance of 550 µm. On each instrument, a 100 µm confocal image stack of 100 planes of the identical microtissue was acquired using a 40x water objective (Opera QEHS: Olympus 40x water NA 1.15; Opera Phenix Plus: Zeiss 40x water NA 1.1).

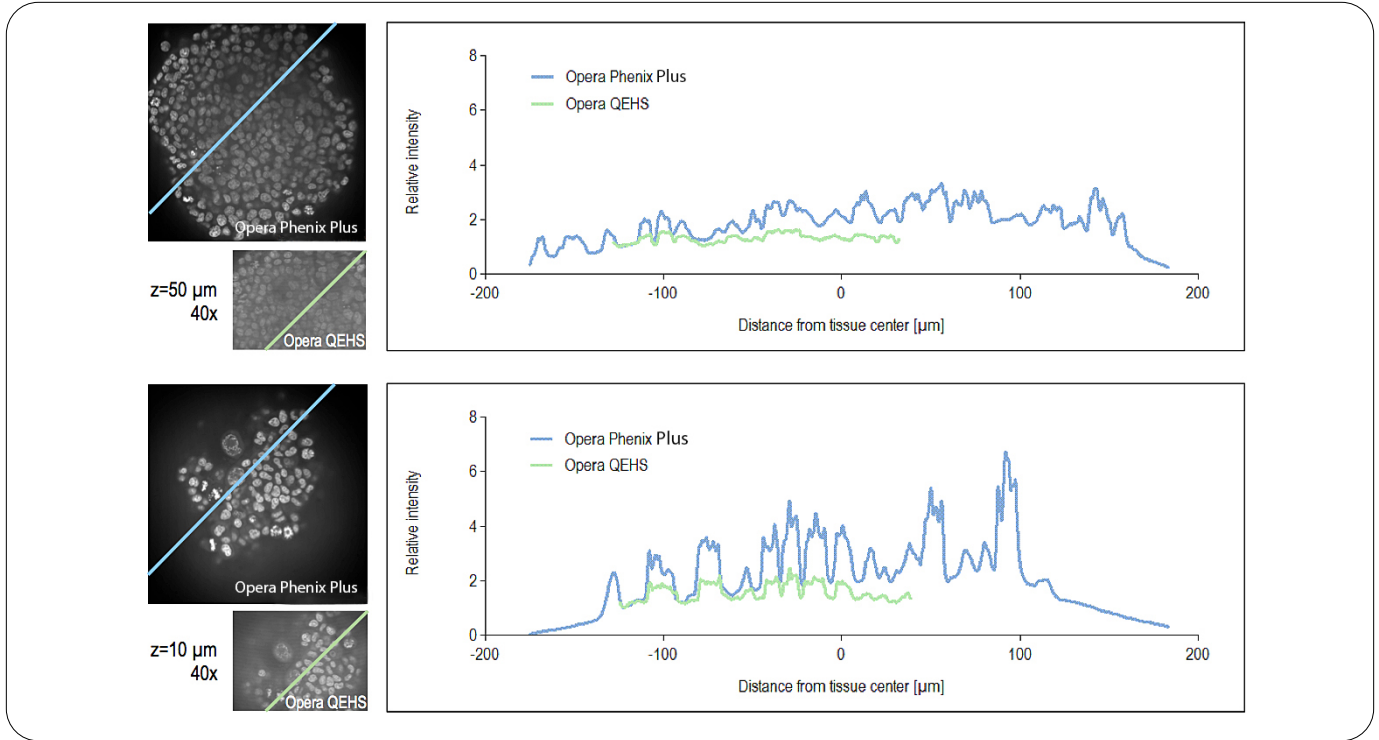


Figure 2: **Images acquired with the Opera Phenix Plus system display an increased signal to noise ratio.** In the line profiles shown, each spike corresponds to one nucleus. Opera Phenix Plus provides a better image contrast as the differences between the peaks and the valleys within the line profile (blue) are larger. This can be observed in both analyzed z heights at 10 μm (lower panel) and 50 μm (upper panel). Moreover, the comparison of both line profiles reveals the differences in the field of view. As seen on the left side, the image field of the Opera Phenix Plus is 3 times larger than images from the Opera QEHS.

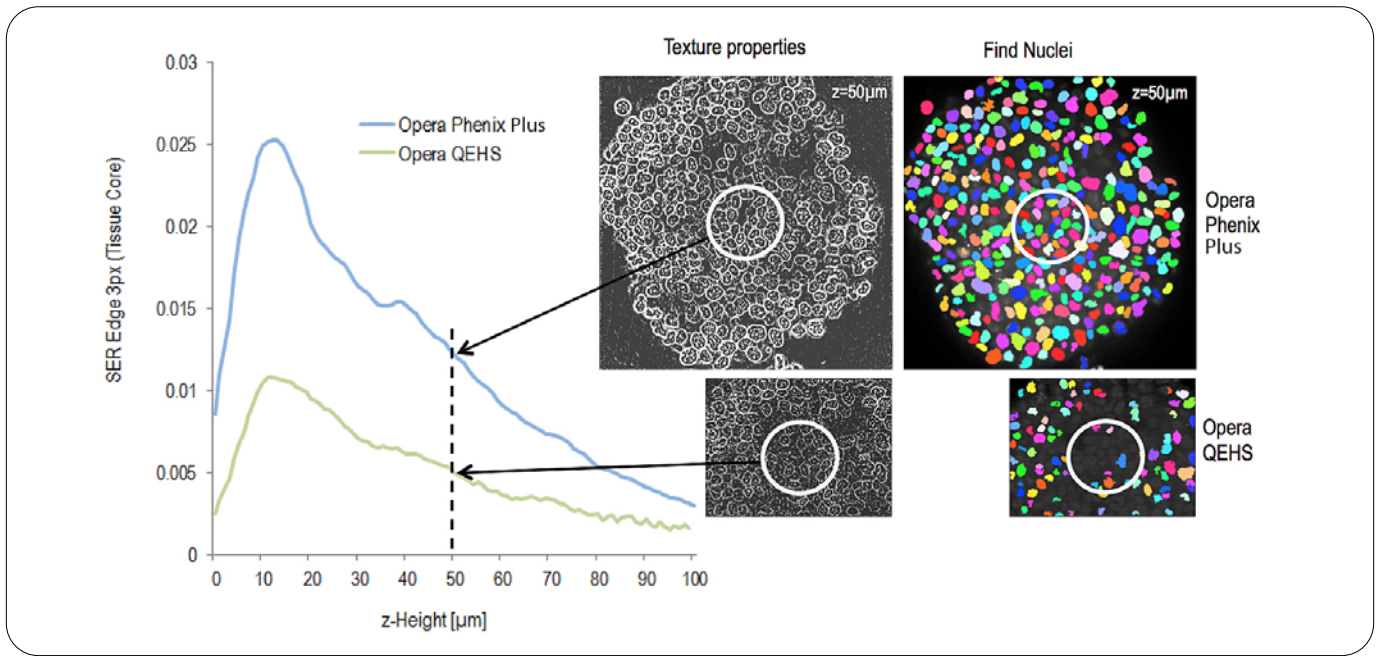


Figure 3: **Opera Phenix Plus images of a 3D microtissue show improved nuclei segmentation and higher texture values compared to the Opera QEHS.** The texture in the center of the 3D tissue, depicted by the white circle, was plotted against the z height. The texture measured on the Opera Phenix Plus system is consistently higher than on the Opera QEHS. In previous studies, we determined a texture value of 0.006 to be the lower limit for an accurate segmentation of nuclei. The Opera QEHS reaches this threshold at a lower z height compared to the Opera Phenix Plus and consequently, nuclei at higher planes can still be segmented on the Opera Phenix Plus but not on the Opera QEHS, as depicted by the images on the right.

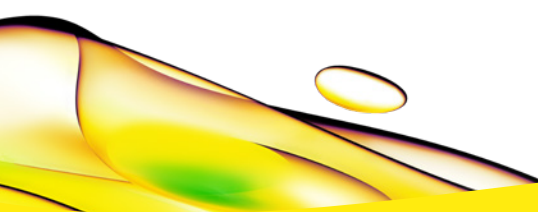
Conclusions

Today's high-content screening applications are becoming increasingly complex, as more physiologically-relevant cell models such as microtissues are being used instead of cell monolayers. To allow detailed phenotypic analysis of thick specimens without compromising resolution, throughput or sensitivity, state-of-the-art imaging technology is required. The Opera Phenix Plus high-content screening system provides a confocal scanning unit with optimized pinhole to pinhole distance for reduced spatial crosstalk in thick specimens. In addition, the use of water immersion objectives facilitates higher resolution and increased sensitivity especially deeper inside tissues. Furthermore, Synchrony™ Optics enable effective z-sectioning and simultaneous acquisition of up to four channels with minimal crosstalk, driving exceptional 3D imaging at high speed. For analysis, the Opera Phenix Plus system is provided with the Harmony high-content imaging and analysis software. It includes the xyz viewer for improved visualization of 3D data and Z-mapping for improved 3D analysis. Based on the maximum intensity projection, Z-mapping creates a new image which preserves the z information of each pixel.

References

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Letzsch, S. and Boettcher, K. (2014): Imaging Microtissue Cores using the Opera High Content Screening System. *Revvity Application Note*



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