TECHNICAL NOTE

Opera Phenix Plus high-content screening system technical performance: Crosstalk suppression.

Authors

Angelika Foitzik
Karin Böttcher
Alexander Schreiner

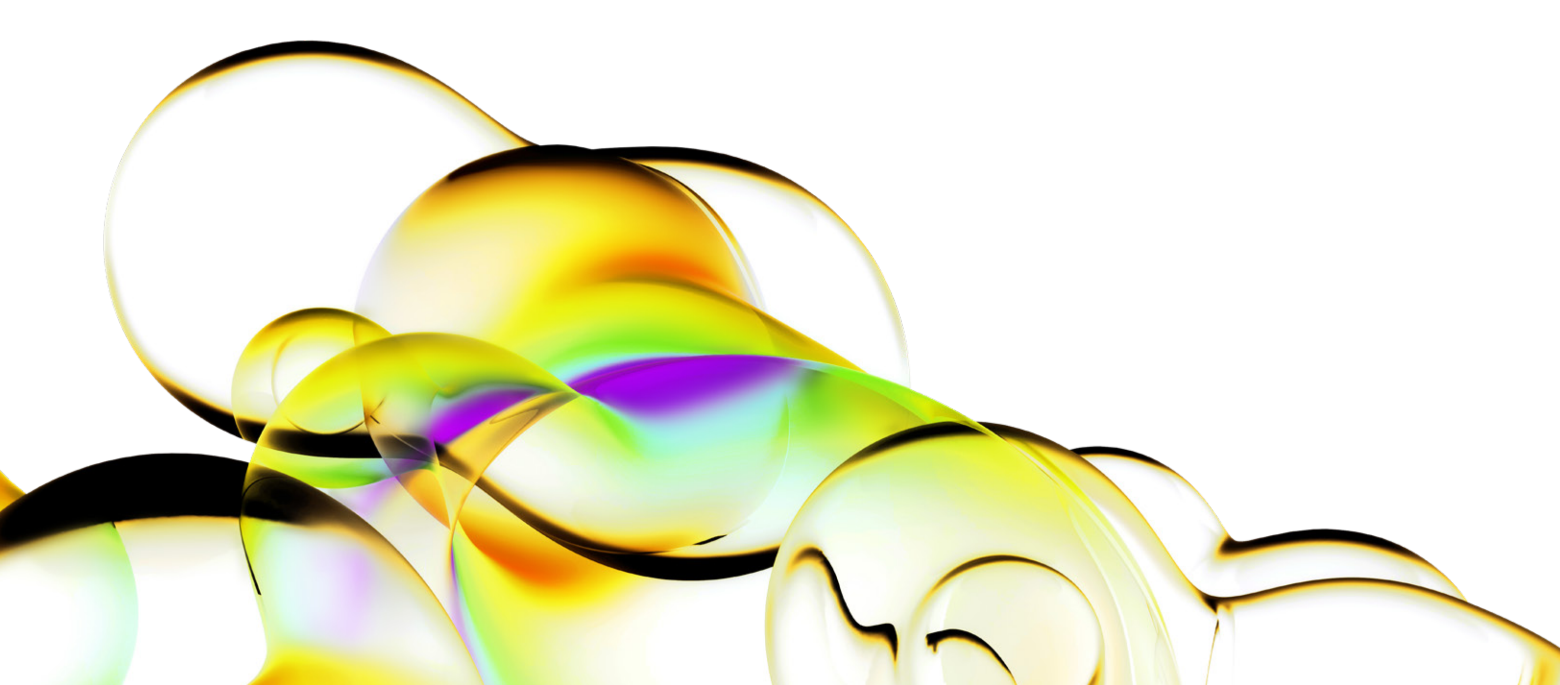
Revvity, Inc.

Crosstalk suppression

Simultaneous multi-color imaging is a technology commonly used to increase the speed of high-content screening systems. However, one of the main problems arising from this approach is spectral crosstalk which can limit assay sensitivity. To overcome this challenge, we have developed an innovative optical concept for the Opera Phenix® Plus high-content screening system, known as Synchrony Optics™ (Fig. 1).

The Opera Phenix Plus system can be equipped with up to four large field of view (FOV) sCMOS cameras to maximize throughput and Synchrony Optics enable parallel acquisition of up to four spectrally adjacent channels at the same time with minimal crosstalk.

For research use only. Not for use in diagnostic procedures.



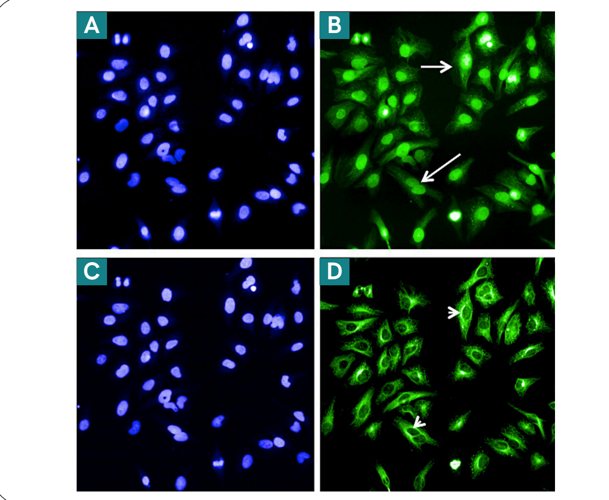


Figure 1: **Sequential acquisition is one of the main strategies used to overcome spectral crosstalk on many high-content screening systems**. A and C: nuclei stained with Hoechst in blue, B and D: tubulin fibers labelled with Alexa 488 in green. The emission spectrum of Hoechst strongly overlaps with that of Alexa 488. Hence, one can see nuclei as spectral crosstalk in the green channel when these two channels are acquired simultaneously (B, arrows). Only when the acquisition is done sequentially for Hoechst and Alexa 488, nuclei are no longer visible in the green channel (D, arrowheads).

The concept behind Synchrony Optics

Synchrony Optics separate adjacent fluorescence excitation wavelengths and the respective emissions during simultaneous confocal acquisition, thereby minimizing spectral crosstalk. Figure 2 shows the confocal light path of Synchrony Optics. Excitation lasers are placed into two groups so that spectrally neighboring laser lines – which usually cause the most crosstalk – are in different groups. In this example, we focus on the 405 nm and 488 nm laser for the excitation of Hoechst together with GFP. The Hoechst emission spectrum broadly overlaps with that of GFP. Thus, measuring these dyes simultaneously leads to a high amount of spectral bleed-through. To overcome this, light from each laser is focused onto two opposing quadrants of the microlens-enhanced spinning disk. This creates a non-overlapping pattern of excitation spots which are focused onto the sample. The emitted fluorescence is then collected and split according to its wavelength at the combination dichroic mirror. Light from the fluorophore with the shorter emission wavelength (yellow, from Hoechst in the example) will then pass through the lower quadrant where it is reflected towards camera 1.

Light from the other fluorophore (here red, from GFP) passes straight through the combination dichroic mirror and then enters the upper quadrant before reaching camera 2. Any longer wavelength component of Hoechst emission that passes the combination dichroic mirror is blocked at the spinning disk due to the non-overlapping pinhole pattern. In this way, Synchrony Optics can reduce spectral crosstalk to a large extent. Besides confocal imaging, the Opera Phenix Plus system can acquire images also in non-confocal mode. However, the crosstalk reduction is only effective in confocal mode, as it relies on the presence of the spinning disk in the light path.

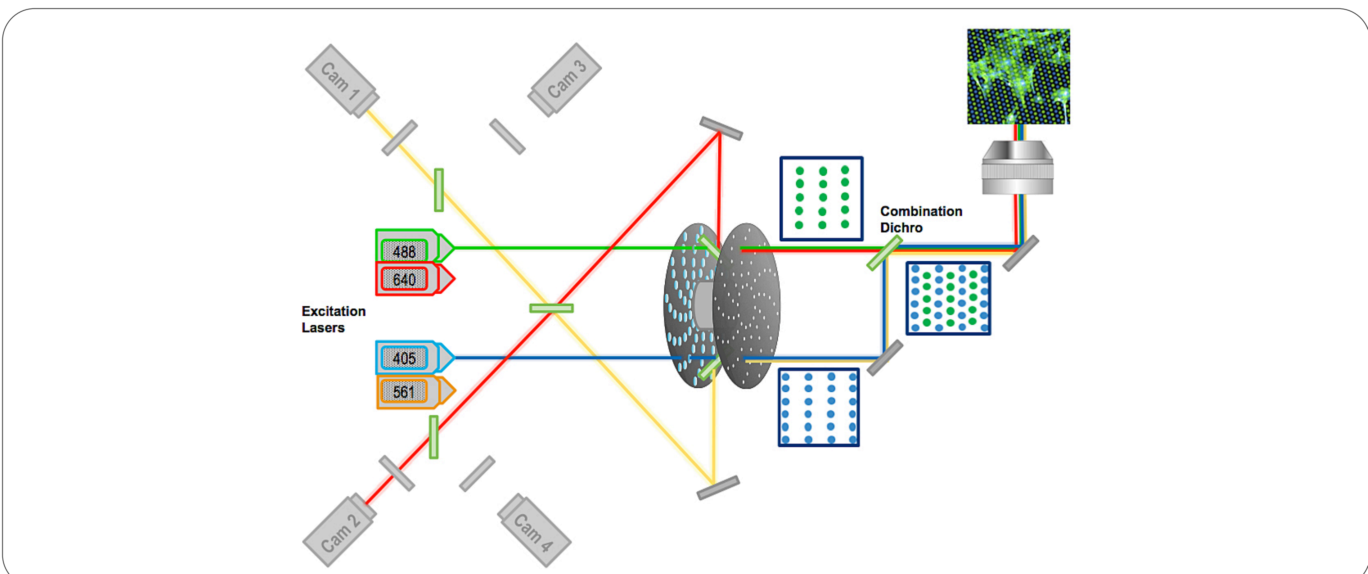


Figure 2: **Synchrony Optics – the novel confocal light path of the Opera Phenix High-Content Screening System.** The separation of excitation wavelengths in time and space is achieved by projecting non-overlapping excitation pinhole patterns onto the sample. Excitation lasers are placed into two groups so that adjacent laser lines are in different groups. The 405 nm (blue) and 488 nm (green) lasers are focused through different quadrants of the spinning discs. Hence, the resulting emission light can only pass through the pinhole disc in the respective quadrant.

Significant improvements in image visualization and assay statistics

To compare the effect of spectral crosstalk between systems with either a classical spinning disk confocal setup or Synchrony Optics, a crosstalk sensitive, 2-color, epigenetic assay for Histone de-acetylase (HDAC) inhibitors using Hoechst to stain DNA and FITC-labeled antibodies to detect acetylated Histone H3 in the nucleus, was recorded. The channels were measured either sequentially or simultaneously on both an Opera QEHS (Quadruple Excitation High Sensitvity) with a Yokogawa CSU10 confocal head and on an Opera Phenix Plus with Synchrony Optics (Fig. 3). When the two channels were acquired in simultaneous mode on the classical confocal system, the Opera QEHS, crosstalk caused Hoechst signal to be recorded on top of the FITC signal, and consequently negative control samples showed high signal levels. On instruments such as Opera QEHS such spectral crosstalk can only be avoided by measuring sequentially. As expected with this setting, the negative control shows no signal. In contrast, the images from Opera Phenix Plus show no difference regardless of whether they were acquired in sequential or simultaneous mode. Synchrony Optics is effectively suppressing the spectral crosstalk from Hoechst into the FITC channel.

	Opera QEHS (classical confocal system)		Opera Phenix (with Synchrony Optics)	
	Sequential/ confocal	Simultaneous/ confocal	Sequential/ confocal	Simultaneous/ confocal
Positive control				
Negative control				

Figure 3: **Synchrony Optics reduce spectral crosstalk by up to 98% during simultaneous confocal measurements, as shown in this crosstalk-sensitive epigenetic assay**. As in this assay Hoechst stain and FITC signal are both located in the nucleus, bleed-through of Hoechst emission into the green channel is recorded on top of the actual signal. This effect becomes especially apparent in the negative control, which should show no signal. On classical confocal systems such as the Opera QEHS, the negative control is contaminated by Hoechst crosstalk in simultaneous mode. This is effectively suppressed by Synchrony Optics on the Opera Phenix Plus (compare the red boxed images). Also note the 3x larger field of view of the Opera Phenix Plus system.

When comparing the quality of the above mentioned epigenetic assay using the statistical parameter Z′, a dramatic Z′ improvement in the simultaneous confocal data obtained with Opera Phenix Plus becomes apparent (Fig. 4). On classical confocal systems, sequential acquisition is required to achieve a comparable Z′ value, at the cost of much prolonged acquisition speed.

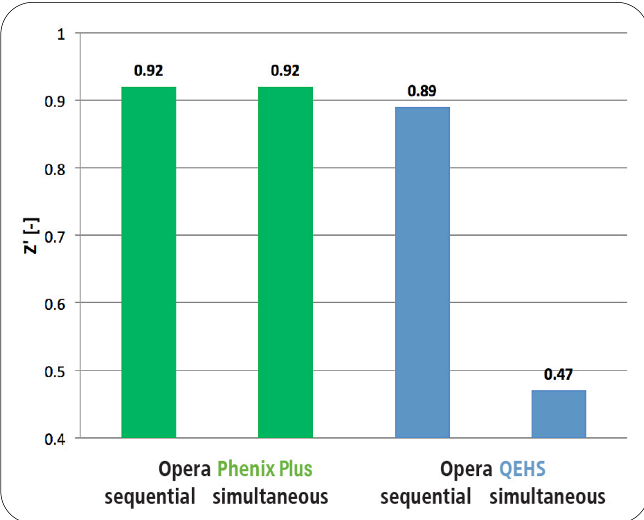
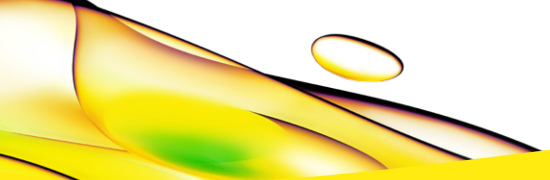
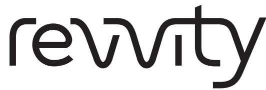


Figure 4: **Synchrony Optics improve the Z′ value of an epigenetic assay when measured in simultaneous mode.** Synchrony Optics on the Opera Phenix Plus system allow simultaneous acquisition with minimal crosstalk, yielding Z′ values comparable to sequential acquisition.

Conclusions

Today's high-content screening applications are becoming more complex, as more physiologically relevant cell models like live cells, co-cultures and 3D models are being used. To allow detailed phenotypic analysis of these cell models without compromising resolution, throughput or sensitivity, suitable imaging technologies are required. The Opera Phenix Plus high-content screening system combines a unique set of hardware and software features to allow improved imaging and analysis of today's highly demanding cell-based assays. Synchrony Optics, the proprietary optics of the Opera Phenix Plus, are capable of suppressing spectral crosstalk to an absolute minimum thereby significantly improving image quality and assay robustness for crosstalk-sensitive assays. Synchrony Optics are key to unleashing the full power of a multi camera system by enabling parallel acquisition of spectrally close fluorophores with minimal crosstalk.





Revvity, Inc.
940 Winter Street
Waltham, MA 02451 USA

(800) 762-4000
www.revvity.com

For a complete listing of our global offices, visit www.revvity.com
Copyright ©2023, Revvity, Inc. All rights reserved.

1160786_RB