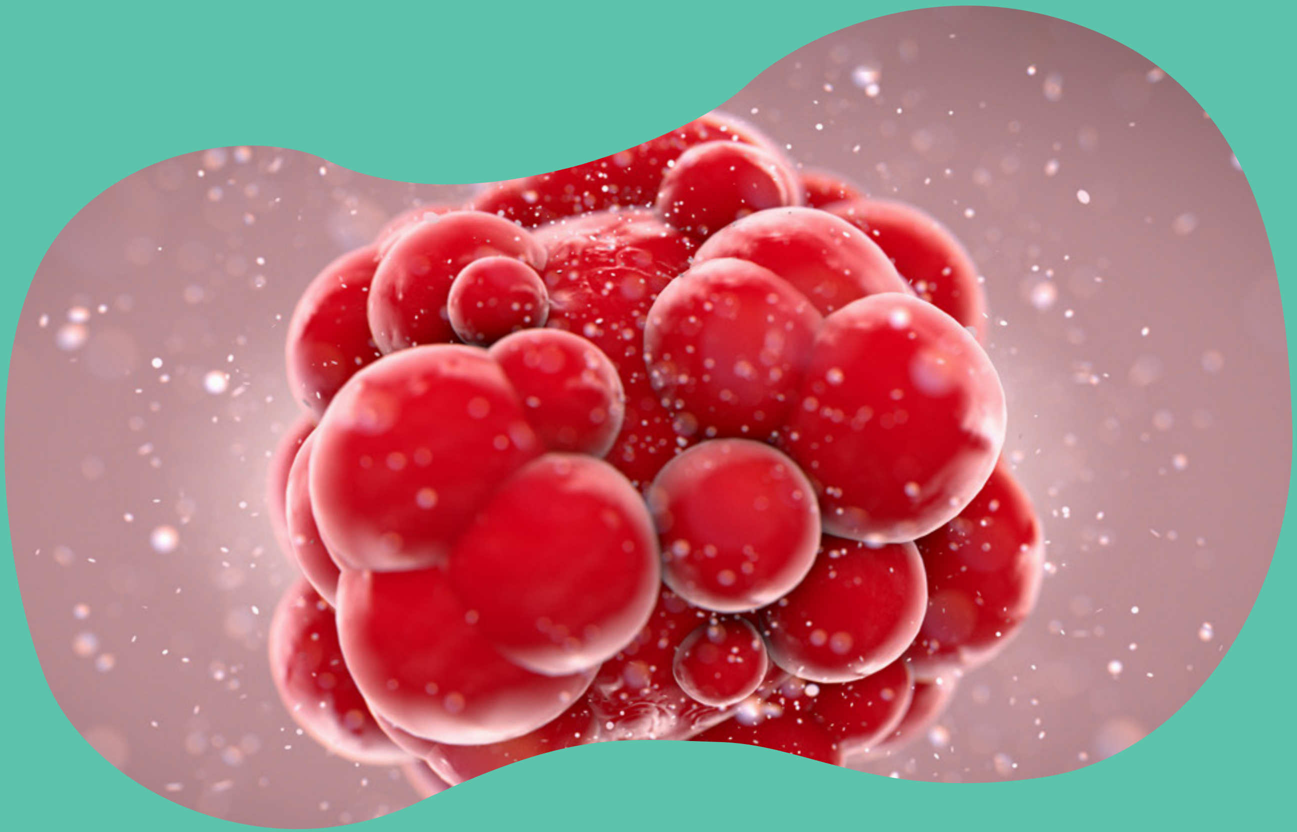
WHITE PAPER

High Content Screening in Three Dimensions

Signals Image Artist



revvity signals

Accelerating 3D Image Analysis and Multiparametric Data Analysis

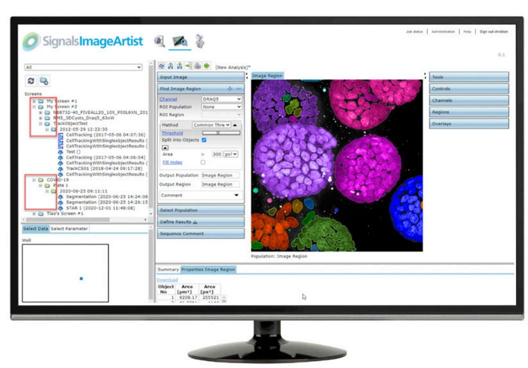
Researchers are increasingly looking to 3D cell cultures, microtissues, and organoids to bridge the gap between 2D cell cultures and in vivo animal models. 3D models provide more physiologically relevant conditions than biochemical assays and 2D cell cultures, as they more closely mimic the microenvironments, cell-to-cell interactions, and biological processes that occur in vivo. Plus, they show a greater degree of morphological and functional differentiation. Even though 3D assays are more challenging than conventional 2D assays, they are now an essential part of modern discovery processes.

Challenges of running high-content assays with 3D cell models include:

- Generation of high-quality images from large, thick cell samples
- Long imaging and image analysis times
- Analyzing responses of complex models in three dimensions
- Having to transfer data between software packages to enable 3D analysis
- Management of the huge volumes of data from 3D cell screens

To overcome these challenges Revvity offers solutions such as PreciScan, high NA water immersion objectives, Synchrony Optics and simultaneous image acquisition on multiple cameras. With Harmony®, Revvity Signals™ Image Artist and Revvity Signals™ VitroVivo we also provide software to perform intuitive image segmentation and rendering in 2D or 3D, image analysis for creating real multiparametric data sets, data visualization and automated statistical analysis.

Despite all these improvements it's still uncommon for higher throughput screening campaigns to be run with 3D cell models. This paper provides a recommended workflow for 3D HCS analysis, with a focus on reducing the 3D image analysis time by utilizing High Performance Computing (HPC) on premise or on a cloud-based server cluster.



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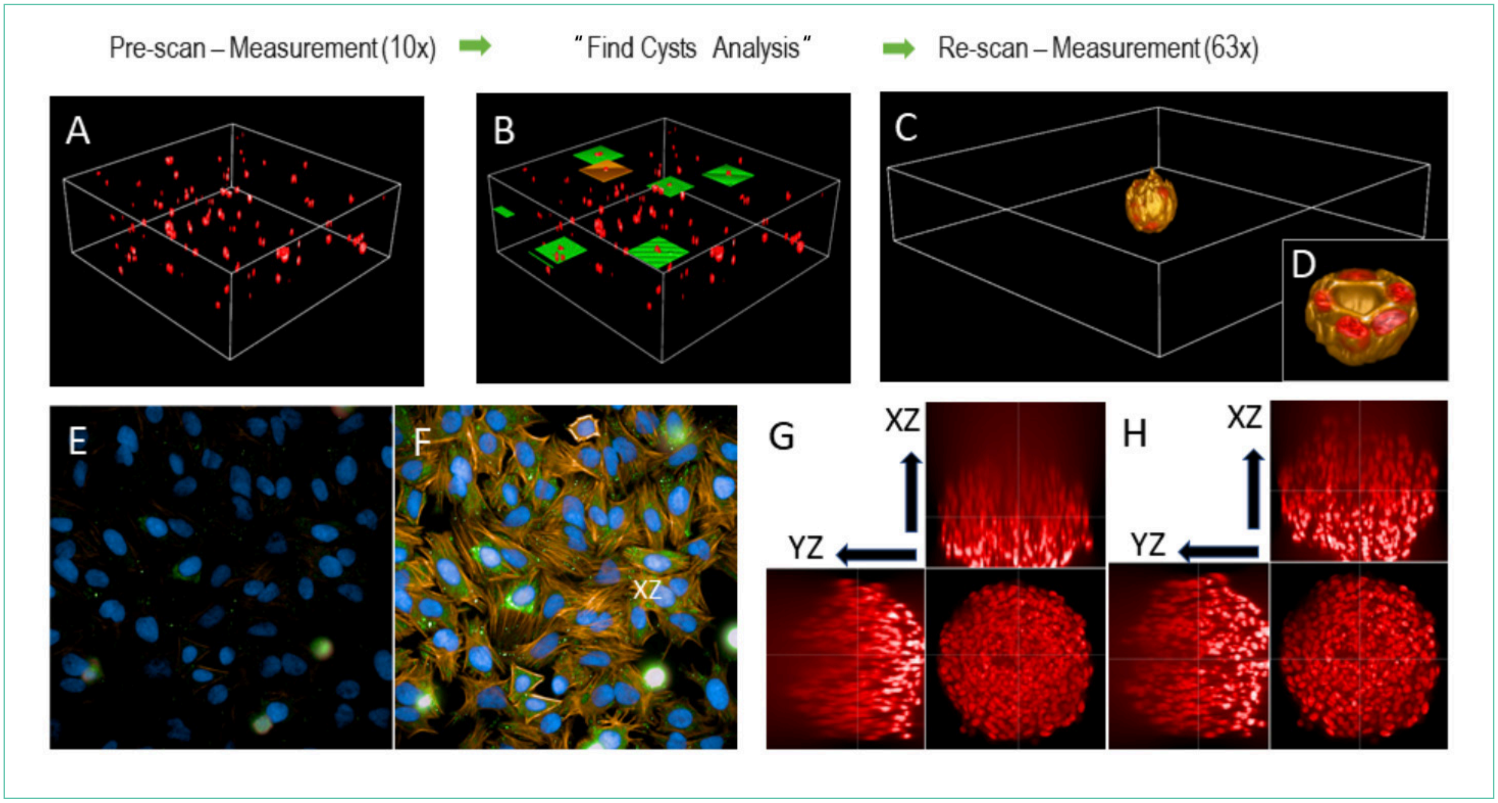


Figure 1. PreciScan functionality (A-C) on Revvity Operetta CLS and Opera Phenix systems. First run creates an image stack with low magnification (A) where an analysis automatically identifies objects of interest in x, y and z (B). The selected objects are then imaged with higher magnification and in z- dimension, resulting in a high-resolution image stack which can be rendered into a 3D image (C, D cyst cut-open). Water immersion objectives 40x, 1.1 NA (F) are at least 4x more sensitive than corresponding air objectives (E shows image acquired with 40x, 0.75 air objective with identical exposure time and excitation power). Using water immersion objectives (H) also reduces distortions in z compared to air objectives (G) which allows a more accurate 3D rendering and image analysis.

Generate High Quality Images in 3D

The outcome of a high content screening campaign depends heavily on the image quality. If images can't resolve the differentiating structures of interest, it's very unlikely you will derive any useful separation/classification later on. When you add a z-axis as the third dimension to your images, it becomes more difficult to resolve structures.

Revvity's Opera Phenix™ and Operetta CLS™ high-content systems have been designed with 3D models in mind. Confocal spinning disks and water immersion objectives enable you to image stacks with improved signal-to-noise and high x,y,z resolution. The spinning disk rejects out-of-focus light and water immersion lenses can image deeper into a 3D sample.

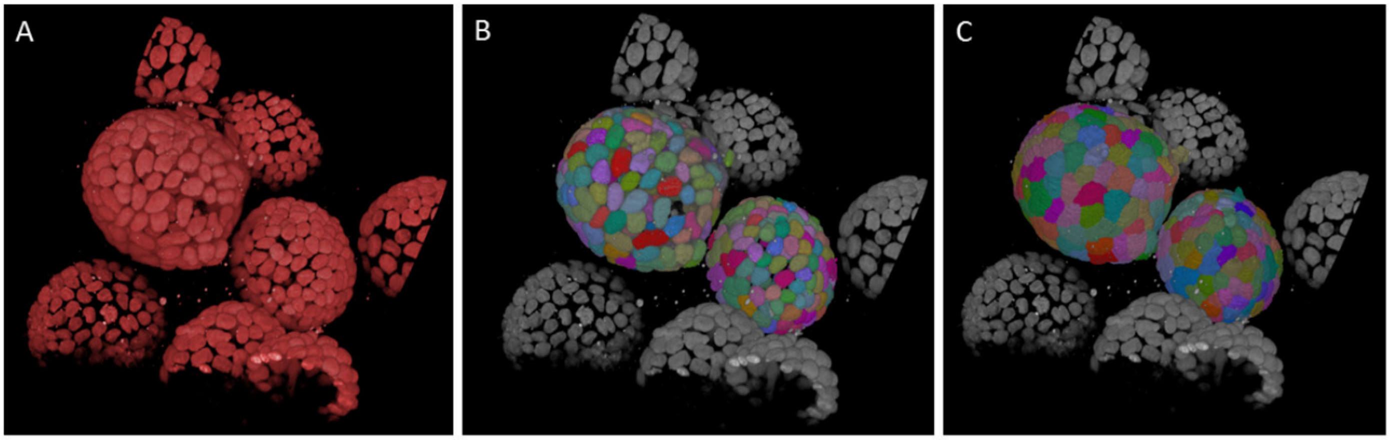
Water immersion objectives allow higher numerical apertures than air objectives, so they capture up to four times more light thereby enhancing imaging speed and also helping to minimize photo damage.

Opera Phenix Synchrony optics feature a microlens-enhanced dual-disk design with a pinhole distance optimized for thick samples such as 3D cell models, and dual-field excitation that

separates excitation of neighboring spectral channels in space and time. This design minimizes crosstalk when performing simultaneous multicolor measurements with up to four cameras. The result is fast, multichannel acquisition of high quality 3D images.

However, acquiring images beyond x and y dimension increases the data volume dramatically. Imaging 30 wells of a 384-well plate with 20 fields, 4 channels and 60 z steps can easily reach more than 300 GB. To reduce the amount of data, it is preferable to acquire high-resolution data only from the regions or objects of interest.

The PreciScan plug-in for Harmony software enables intelligent image acquisition for accurate targeting of objects of interest, such as spheroids, stem cell colonies, or rare cell phenotypes, in x, y and z dimensions, The software delivers a fully automated, integrated workflow of low-magnification prescans, image analysis, and higher magnification rescans to reduce acquisition times and data volume and ultimately speed up analysis significantly.



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Extract Detailed Morphologic Information from 3D Samples

Once a z stack of images is acquired, there are different ways to analyze it. The simplest and probably most well-established method is to analyze each image separately. Another widely used approach is the so called Maximum Projection. Here, all selected planes are reduced to a single image and each pixel gets the maximum value across the stack planes. The rationale is that the "in focus" pixels should have the highest intensities across the z-stack and therefore create a new image with reduced background signal. However, for colocalization studies and thicker objects this method is not applicable.

Revvity Harmony software enables 3D visualization and analysis, via an intuitive approach, combined in one software package. The algorithms used for the different tasks are packaged into so-called Building Blocks, which can be used as is or tuned through a choice of parameters. To analyze samples in three dimensions the 2D z-stack images are combined into a real 3D object (Figure 2A), which can subsequently be analyzed by chaining together different Building Blocks to form an analysis sequence. A typical analysis sequence starts with segmentation Building Blocks to identify 3D objects such as nuclei (Figure 2B) and cytoplasm (Figure 2C), followed by Building Blocks that calculate a variety of parameters related to intensity values, texture and morphology for each object. During this analysis the cell or other object is translated into numbers which describe the phenotype of the segmented objects and defined populations.

Managing and Analyzing the Huge Amounts of Data

Image analysis requires a lot of computing power and, as a result, it is typically performed on high-powered workstations or servers. However, if you run complex analysis on a large number of plates, or on a 3D dataset, even those powerful workstations will reach their limits. Fortunately, Signals Image Artist, Revvity's centralized image storage and analysis system, can be run on High Performance Computing (HPC) systems. HPC employs parallel processing for running computer-intensive application programs, like image analysis, quickly.

With Signals Image Artist on HPC you can run analysis sequences defined in Harmony on an extremely powerful computer cluster in parallel, meaning each node of the cluster analyzes one well at the same time. Additionally, those clusters are scalable to fit your requirements and can be completely cloud-based. Figure 3 shows an example of reduction in execution time when using cluster computing. Running a 3D analysis on a standard Harmony workstation equipped with 16 cores and 64 GB RAM took 126 min. The same analysis on a cluster on the Amazon® cloud using either 10 or 20 nodes, each with 8 cores and 61 GB Ram, took 88 or 54 minutes respectively. The analysis time was cut by nearly 60% on this small 3D dataset (30 well, four channels, 20 fields, 60 z-slices) and would be even more efficient when running larger screening campaigns.

Also, new storage capacity of standard hard discs is increasing almost as quickly as prices are decreasing. Revvity's Signals Image Artist platform for image storage and analysis is, for example, offered on-premise but also research organizations can implement and manage Signals Image Artist as a completely cloud-based solution.

The second advantage of Signals Image Artist is the option to run it in the cloud. Vendors like Amazon® Web Services offer a variety of configurations to meet a wide range of requirements for storage or numbers of nodes. With this approach you completely eliminate the need for on-premise installations, hardware maintenance, backups, and hardware upgrades.

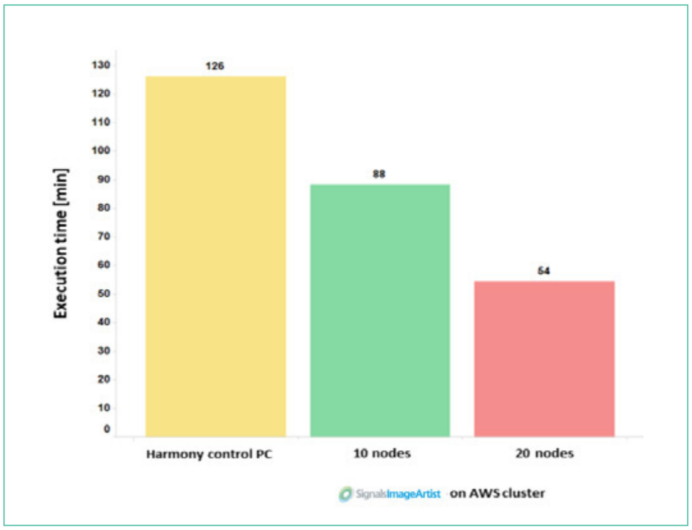


Figure 3. Execution time comparison of a 3D image analysis sequence between a standard Harmony PC with 16 cores and 64GB RAM and two different AWS-hosted HPC consisting of either 10 or 20 r4.2 xlarge nodes, each with eight cores and 61GB RAM. Demo data set contained 30 wells with 20 different fields and 60 z-slices, four channels which summed up to 210 GB of raw images.

Multiparametric Data Analysis

Because different phenotypes may potentially still be hidden within the multiparametric result table coming from the image analysis, additional tools are needed to uncover interesting data points. Revvity's Signals VitroVivo statistical tools are packaged into an easy-to-use workflow powered by TIBCO Spotfire®. Based on the TIBCO Spotfire® backbone there are pre-built apps for executing screening-specific tasks with just one click.

Once you describe the data structure in a short dialog, the entire analysis is run automatically. Using individual tabs you walk through QC, outlier detection, plate views, and normalization steps to get a quick overview of the results. Algorithms like PCA

(Figure 4 A) and t-SNE reduce the dimensionality of your data set and create powerful 2D or 3D plots to identify different phenotypes. Unsupervised machine learning, based on a self-organizing map (SOM), helps group the results into different classes based on phenotypic similarity, even if no controls are available. With given controls, feature selection and classification based on selectable algorithms are automatically performed by the automated workflow of the HCP app. Finally, a hit list is created by using only those features for classification that were chosen by the feature selection algorithm. When a concentration series tested, a curve fit for selected treatments can be done. Following this automated workflow allows end-users without a bioinformatics background to create a clean data table with outliers, artefacts and other unwanted signals removed. All data points can be related to the underlying raw image stored in Signals Image Artist but available in Signals VitroVivo as a label or tooltip (Figure 4B).

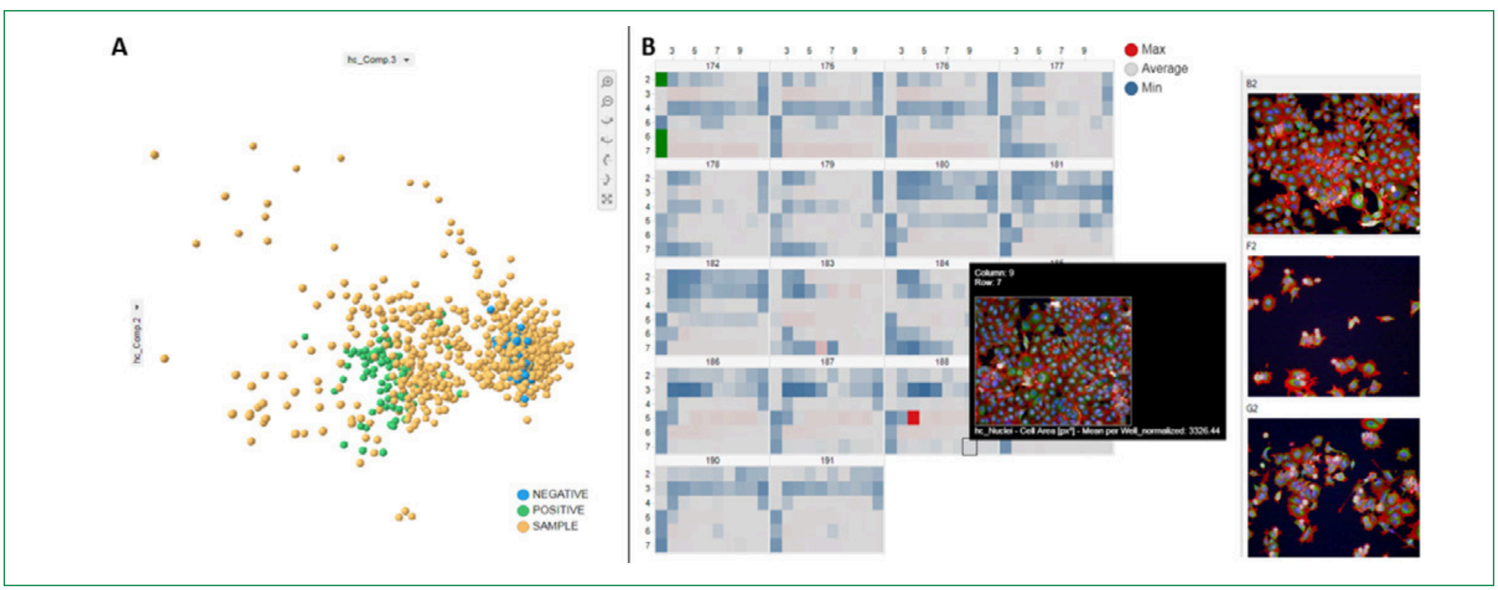
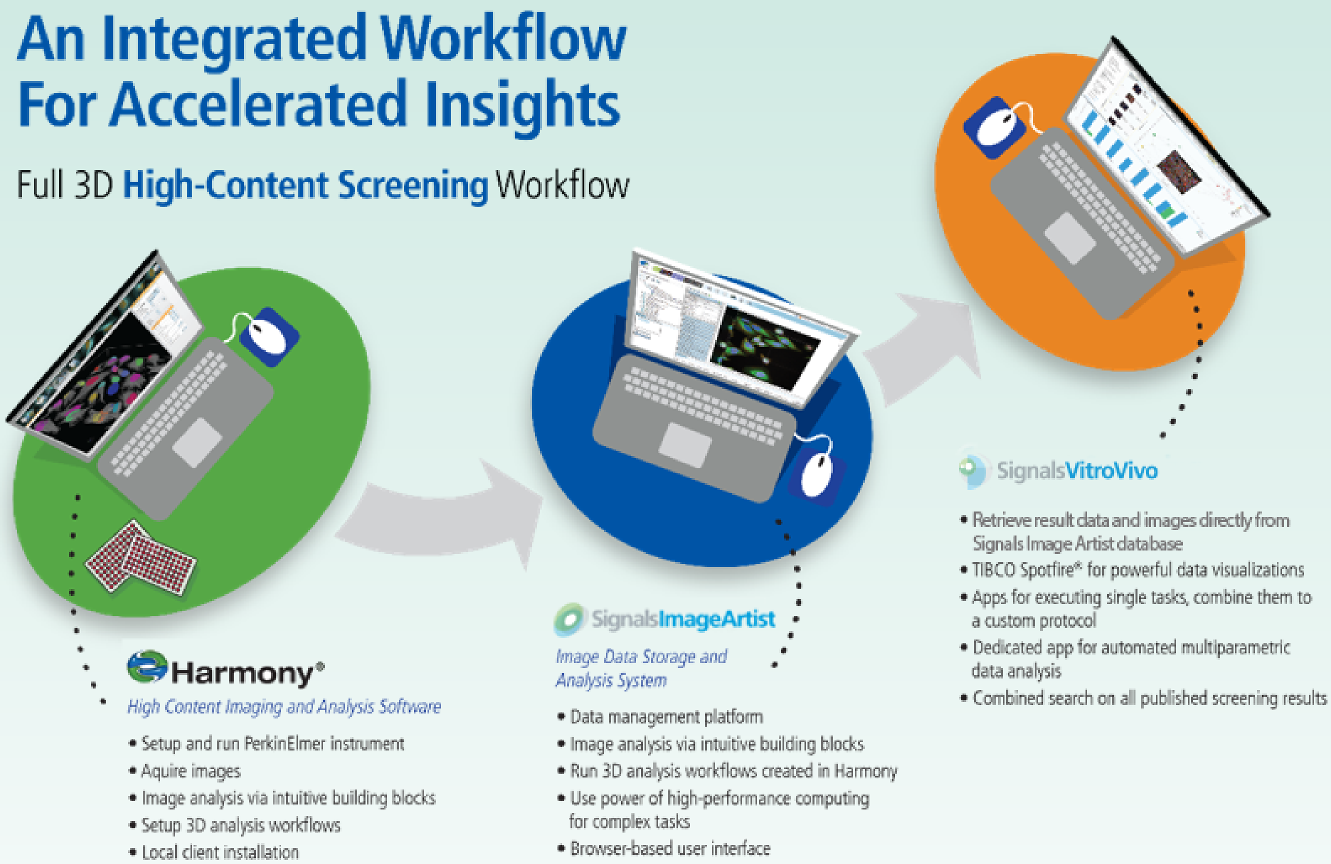


Figure 4. Scatter plot of a PCA analysis (A) in Signals VitroVivo - different clusters stand for different phenotypic profiles. Each point represents results of a specific well after dimensionality reduction. Plate layout visualization (B) of a screening campaign the color of each well represents values of a selected parameter. On top of each well the original image can be rendered directly from Signals Image Artist for visual validation.

Image analysis workflow using Revvity's Harmony, Signals Image Artist, and Signals VitroVivo. Each product can be used separately, but for higher throughput, complex analysis, collaboration across different sites or cloud-based workflow we recommend combining them into a complete HCS workflow. This combination will also yield higher capacity on the instrument for additional screening runs when storage and analysis are swapped out to Signals Image Artist and Signals VitroVivo.



Conclusion

To run a higher-throughput, 3D phenotypic screening campaign, start with a high-end automated microscope - like the Operetta CLS or Opera Phenix system - to create the best quality images in all 3 dimensions. The acquired images are then processed with Harmony, the intuitive software for rendering 3D objects but also for powerful image analysis. You simply add different Signals Image Artist Building Blocks to create a complex analysis sequence containing flexible segmentation and calculation of hundreds of parameters for each identified object. When using 3D objects, all standard parameters and additional 3D specific parameters, like volume of an object, can be measured.

Since complex image analysis of large 3D datasets requires a lot of computing power, the Signals Image Artist image storage system, with its ability to run on High Performance Computing, is the best solution for large batch analysis. The option to run it completely in the cloud provides easy scalability in terms of storage and computing power. The resulting multiparametric data tables from image analysis are accessible directly out of Signals VitroVivo, Revvity's TIBCO Spotfire®-based analytical platform. Within Signals VitroVivo there is a dedicated HCS app for automated analysis applying different statistical algorithms. Signals VitroVivo also combines the statistical analysis results in graphs and tables with the underlying raw images rendered directly from

Summary

Like many in screening labs, you may struggle with two trends: an increasingly complex drug discovery process and an exponential growth in data. Older solutions fail to support these changes, but newer, integrated technology platforms do. Creating integrated HCS workflows in a single platform increases accuracy; reduces time and helps to identify the most appropriate hits or drug candidates. Investigators, lead and data scientists, lab managers and directors, chemists, and R&D scientists all rely on Revvity for automated solutions to simplify and accelerate their workflows for High Content Screening.

Harmony

Easily quantify complex cellular phenotypes with Harmony high-content analysis software. Harmony software is designed for Operetta CLS and Opera Phenix high-content screening instruments.

Signals Image Artist

Powerful image analysis capabilities with highly flexible and easy to use Signals Image Artist Building Blocks to analyze simple and complex phenotypes of cells with the added speed of cluster-based High-Performance Computing providing real time results.

Signals VitroVivo

Powered by TIBCO Spotfire® data visualization and analytics, Signals VitroVivo software platform enables you to:

- Visualize and analyze HCS, HTS and SPR data in a single, instrument-agnostic workflow to facilitate the identification of potential drug candidates
- Optimize a data processing workflow from data import, QC, to results, in a single session that reduces errors and aggravation when consolidating different files
- More efficiently manage and analyze complex datasets to discover additional insights

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