SCIENTIFIC POSTER

# Image analysis strategies for 3D cell painting assays featuring primary human hepatocyte spheroids.


## Authors

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## Overview

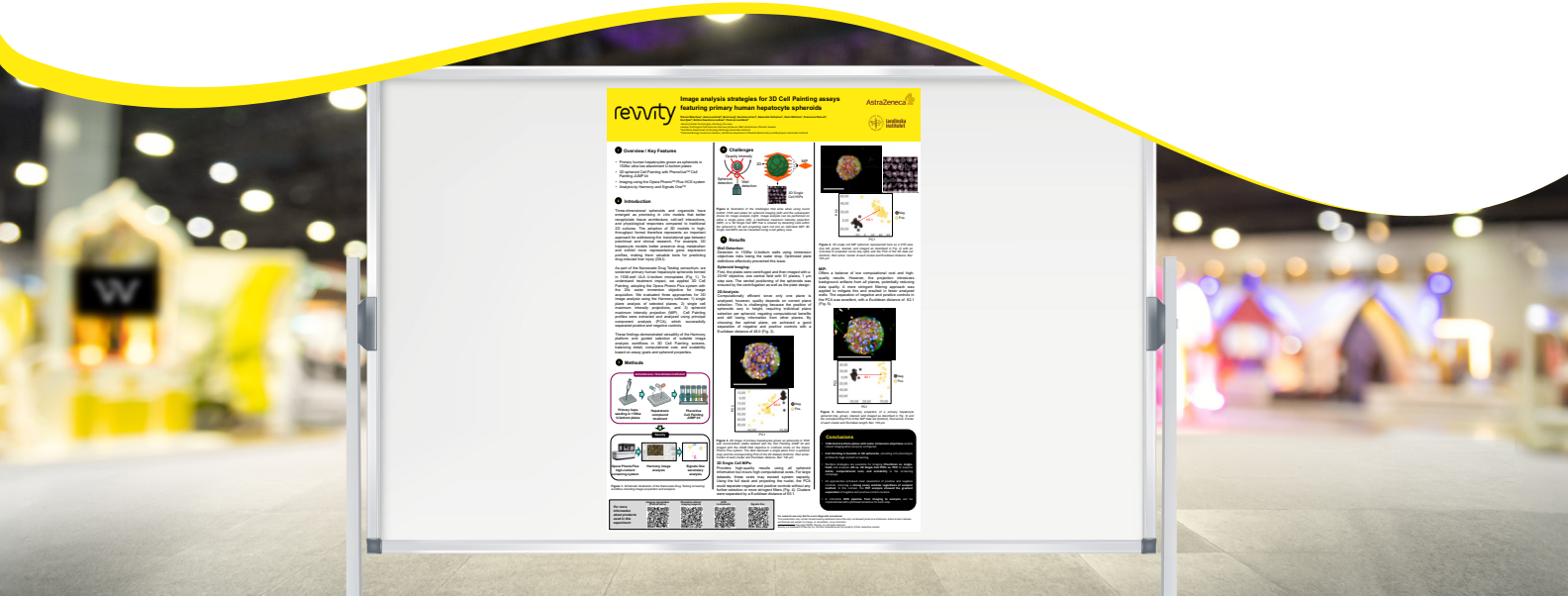
- Primary human hepatocytes grown as spheroids in 1536w ultra-low attachment U-bottom plates
- 3D spheroid cell painting with PhenoVue™ Cell Painting JUMP kit
- Imaging using the Opera Phenix™ Plus HCS system
- Analysis by Harmony™ high-content imaging and analysis software and Signals One™

### Introduction

Three-dimensional spheroids and organoids have emerged as promising *in vitro* models that better recapitulate tissue architecture, cell-cell interactions, and physiological responses compared to traditional 2D cultures. The adoption of 3D models in high-throughput format therefore represents an important approach for addressing the translational gap between preclinical and clinical research.

For example, 3D hepatocyte models better preserve drug metabolism and exhibit more representative gene expression profiles, making them valuable tools for predicting drug-induced liver injury (DILI).

As part of the Nanoscale Drug Testing consortium, we screened primary human hepatocyte spheroids formed in 1536-well ULA U-bottom microplates (Fig. 1). To understand treatment impact, we applied 3D cell painting, adopting the Opera Phenix Plus system with the 20x water immersion objective for image acquisition.



We evaluated three approaches for 3D image analysis using the Harmony software: 1) single plane analysis of selected planes, 2) single cell maximum intensity projections, and 3) spheroid maximum intensity projection (MIP). Cell painting profiles were extracted and analyzed using principal component analysis (PCA), which successfully separated positive and negative controls.

These findings demonstrated versatility of the Harmony platform and guided selection of suitable image analysis workflows in 3D cell painting screens, balancing detail, computational cost, and scalability based on assay goals and spheroid properties.

## Methods

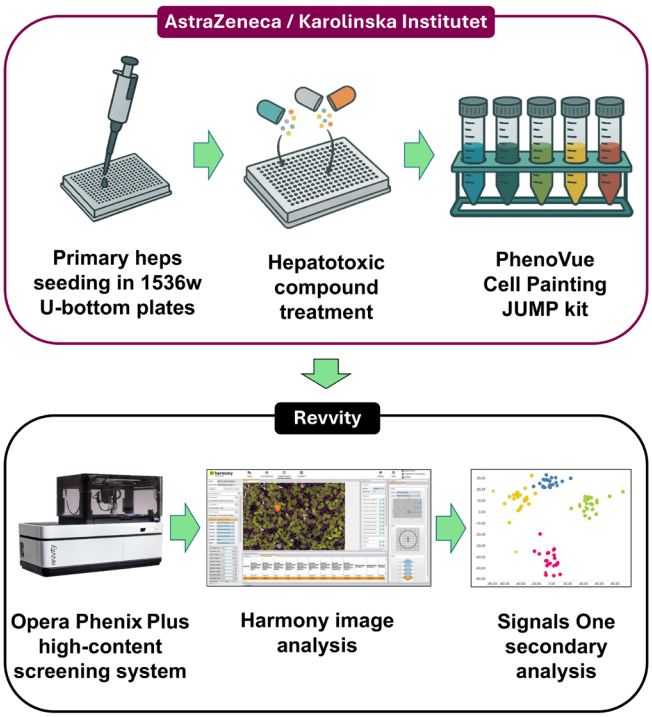



Figure 1: Schematic illustration of the nanoscale drug testing screening workflow including image acquisition and analysis.

## Challenges

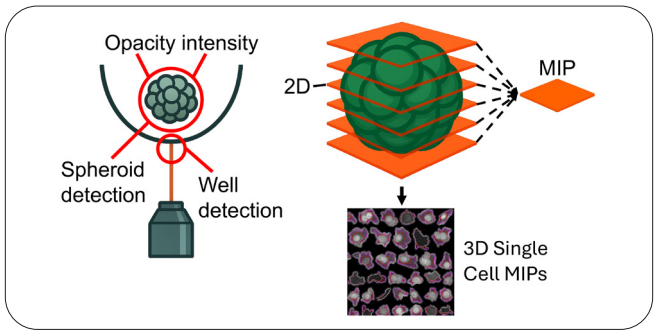



Figure 2: Illustration of the challenges that arise when using round bottom 1536 well plates for spheroid imaging (left) and the subsequent choice for image analysis (right). Image analysis can be performed on either a single plane (2D), a traditional maximum intensity projection (MIP), or a 3D single cell MIP that is created by detecting cells within the spheroid in 3D and projecting each cell into an individual MIP. 3D single cell MIPs can be visualized using a cell gallery view.

## Results

### Well detection

Detection in 1536w U-bottom wells using immersion objectives risks losing the water drop. Optimized plate definitions effectively prevented this issue.

### Spheroid imaging

First, the plates were centrifuged and then imaged with a 20×W objective, one central field with 51 planes, 1 µm step size. The central positioning of the spheroids was ensured by the centrifugation as well as the plate design.

### 2D analysis

Computationally efficient since only one plane is analyzed; however, quality depends on correct plane selection. This is challenging because the position of spheroids vary in height, requiring individual plane selection per spheroid, negating computational benefits and still losing information from other planes. By choosing the optimal plane, we achieved a good separation of negative and positive controls with a Euclidean distance of 46.0 (Fig. 3).

### 3D single cell MIPs

Provides high-quality results using all spheroid information but incurs high computational costs. For large datasets, these costs may exceed system capacity. Using the full stack and projecting the nuclei, the PCA could separate negative and positive controls without any further selection or more stringent filters (Fig. 4). Clusters were separated by a Euclidean distance of 65.1.

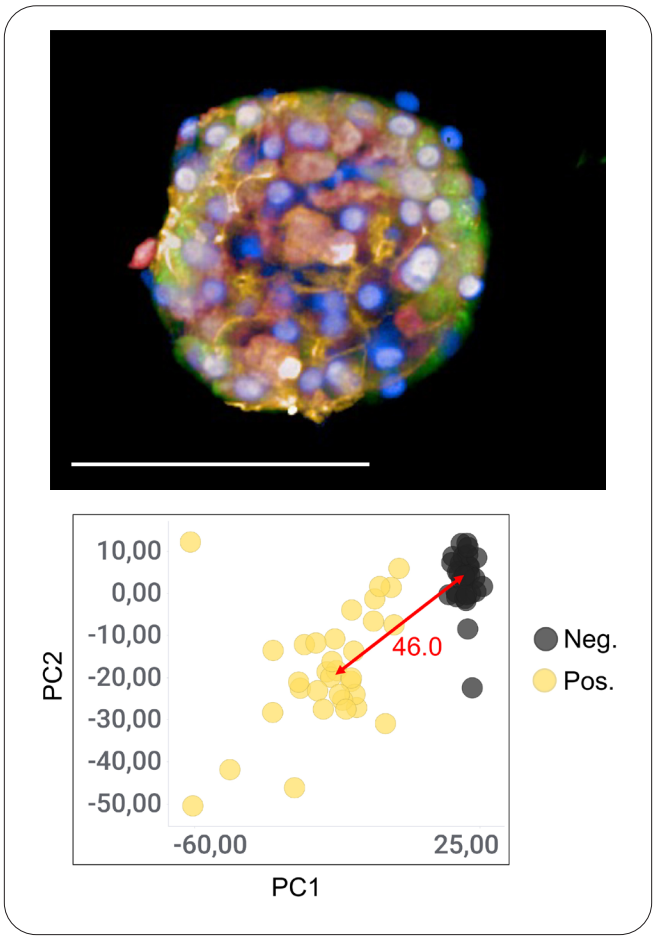



Figure 3: 2D image of primary hepatocytes grown as spheroids in 1536 well round-bottom plates labeled with the Cell Painting JUMP kit and imaged with the 20xW hNA objective in confocal mode on the Opera Phenix Plus system. The data represent a single plane from a spheroid (top) and the corresponding PCA of the 2D dataset (bottom). Red arrow: center of each cluster and Euclidean distance. Bar: 100 µm.

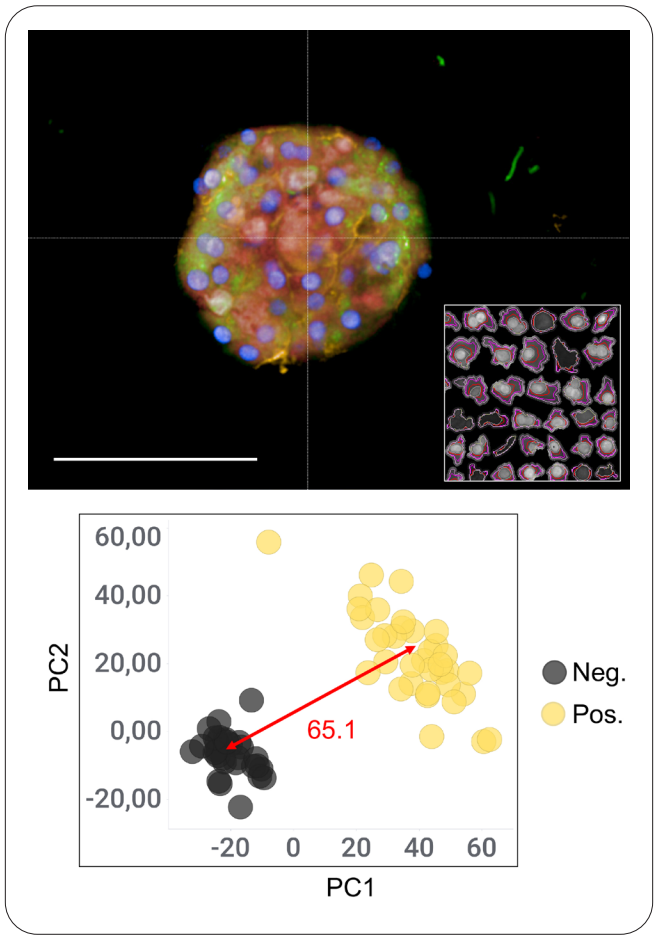



Figure 4: 3D single cell MIP spheroid, represented here as a XYZ-view (top image, grown, stained, and imaged as described in Fig. 3) with an overview of projected nuclei (top image, bottom right corner) and the PCA of the 3D data set (bottom image). Red arrow: center of each cluster and Euclidean distance. Bar: 100 µm.

**MIP**

Offers a balance of low computational cost and high-quality results. However, the projection introduces background artifacts from all planes, potentially reducing data quality. A more stringent filtering approach was applied to mitigate this and resulted in fewer analyzed wells. The separation of negative and positive controls in the PCA was excellent, with a Euclidean distance of 82.1 (Fig. 5).

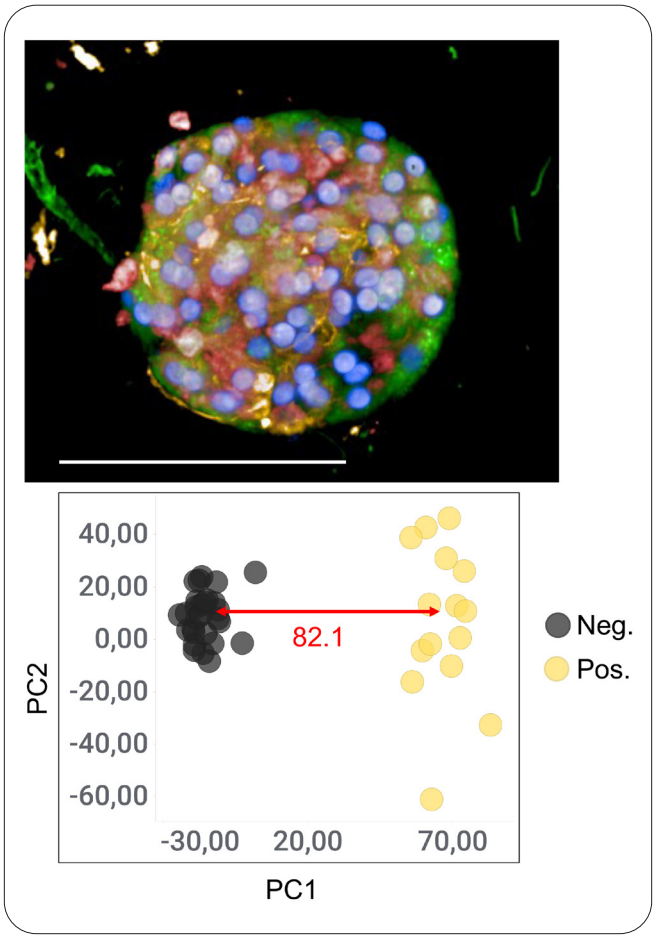



Figure 5: Maximum intensity projection of a primary hepatocyte spheroid (top, grown, stained, and imaged as described in Fig. 3) and the corresponding PCA of the MIP data set (bottom). Red arrow: center of each cluster and Euclidean length. Bar: 100 µm.

## Conclusions

- **1536 ULA U-bottom plates with water immersion objectives** enable robust imaging when properly configured.
- **Cell painting is feasible in 3D spheroids,** providing rich phenotypic profiles for high-content screening.
- Multiple strategies are available for imaging **(PreciScan vs. single-field)** and analysis **(2D vs. 3D single cell MIPs vs. MIP)** to balance **detail, computational cost, and scalability** in the screening campaign.
- All approaches achieved clear separation of positive and negative controls, ensuring a **strong assay window regardless of analysis method.** In this context, the **MIP analysis showed the greatest separation** of negative and positive control clusters.
- A complete **HCS pipeline from imaging to analysis** can be implemented with optimized solutions for each step.

**For more information about products used in this experiment:**

**Imaging microplates (PhenoPlate™)**

**PhenoVue cellular imaging reagents**

**HCS instruments**

**Signals One**