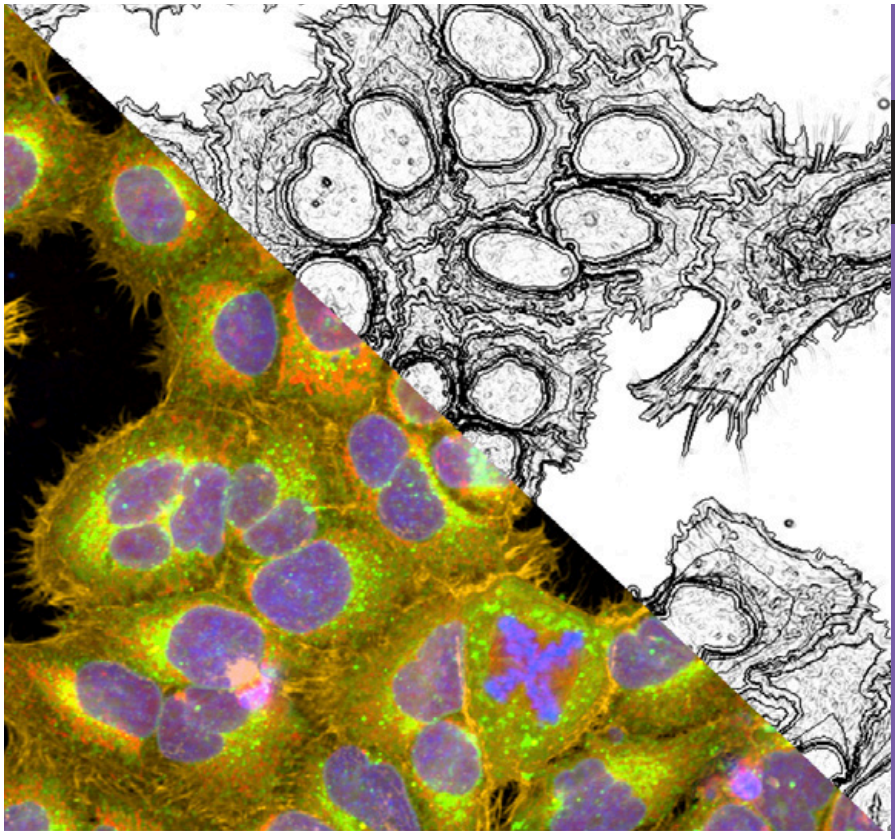

APPLICATION NOTE

High-Content Screening

Key Features:

- Introduction to the PhenoVue® Cell Painting kit containing all required dyes
- How to set up optimal imaging parameters and the effects of different acquisition modes on compound clustering
- How to easily analyze data using a cell painting specific building block
- How to visualize multidimensional data using PerkinElmer Signals™ Screening

Cell Painting for Phenotypic Screening

Cell Painting is a powerful method which combines cell and computational biology to describe the behavior of cells phenotypically following treatment with chemical or genetic perturbagens. Since the first publications describing the technique, Cell Painting has been rapidly adopted for phenotypic drug discovery and basic research.[1,2] Implementing the assay, however, comes with some challenges, ranging from the choice of cell model, appropriate labeling reagents, and optimizing instrumentation for detection, to making sense of up to thousands of features that are generated during data analysis.[3] Here we describe how a cell painting assay can be set up on the Opera Phenix® Plus high-content screening system.

We describe the PhenoVue cell painting kit containing all the necessary reagents, based on the "original" cell painting assay, to label cells following treatment with a perturbagen.[2] Furthermore, we show the effect of different acquisition modalities on compound clustering and introduce a cell painting specific building block available for Harmony® high-content analysis software that allows you to extract more than 5700 features. Finally, we show how PerkinElmer Signals Screening data management and analysis platform helps to visualize highly dimensional data.

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General Workflow of the Cell Painting Assay

In the first step, cells are plated into microtiter plates and treated with compound for several hours or days. The cell painting assay multiplexes six fluorescent dyes to label as many cellular compartments as possible. All six of these dyes are available in the PhenoVue cell painting kit making it easy to stain the cells after compound treatment. After staining and fixation, the cells are imaged on an Opera Phenix Plus high-content screening system. For image analysis, a cell painting specific building block is available for Harmony image analysis software to extract morphological features for phenotypic profiling. Finally, a computational secondary analysis is applied using PerkinElmer Signals Screening data analysis platform (Figure 1).

Material and Methods

Cell Culture, Compound Treatment and Staining

Here we used the PhenoVue cell painting kit which contains all six dyes needed to perform a cell painting assay. The dyes in the kit perform equally as well as those in the original publication.[2] HeLa cells were plated at 1.5E4 cells per well in 100 µl growth medium in a CellCarrier™ Ultra 96-well plate. After 24 hours incubation, cells were treated for two days with 100 µl of a 2x concentrated solution of either compounds or respective DMSO concentrations (triplicates per compound). Then the cell culture medium was removed and 50 µL of PhenoVue 641 Mitochondrial stain solution was added to each well. Plates were incubated for 30 minutes at 37 °C, 5% CO_2 and cells then fixed by adding 15 µl of 16 % PFA (3.7 % final). Plates were incubated in the dark at room temperature for 20 minutes and then washed once with 100 µl of HBSS. Cells were permeabilized by adding 50 µL of HBSS + 0.1 % TritonX-100 (vol/vol). Plates were incubated in the dark at room temperature for 15 minutes and washed twice with 100 µl of HBSS. Subsequently the wash solution was replaced by 50 µl of mixed staining solution in PhenoVue Dye Diluent A (final dye concentrations are provided in Table 1). Plates were incubated in the dark at room temperature for 30 minutes and then washed three times with 100 µl of HBSS. Please refer to Table 1 for all materials used in this application.

The compounds were chosen for this demonstration because they are known to affect the cellular compartments stained by the individual cell painting dyes.[3,4,5] Fenbendazole treatment leads to giant multinucleated cells, Tetrandrine to abundant endoplasmic reticulum, Etoposide to large nucleoli, CA-074Me affects abundance of Golgi, Berberine chloride causes redistribution of mitochondria and Cytochalasin D disrupts the actin cytoskeleton.

Table 1. Materials.

Cells	HeLa (CLS #300194)
Growth Medium	Complemented MEM (MEM alpha, SVF10%, 2 mM HEPES, 1% Penicillin/Streptomycin)
Cell Treatment	0.25 µM Fenbendazole (Sigma #35032)
	8 µM Tetrandrine (SantaCruzBiotech #sc-201492)
	10 µM Etoposide (Tocris #1226)
	10 µM CA-074Me (SantaCruzBiotech #sc-214647)
	20 µM Berberine chloride (Sigma #PHR1502)
	2 µM Cytochalasin D (Sigma #C8273)
PhenoVue Cell Painting Kit	PerkinElmer # PING12
Final Concentrations of Cell Painting Dyes	5 µg/ml PhenoVue Hoechst 33342 Nuclear Stain
	100 µg/ml and 2.5 µg/ml PhenoVue Fluor 488 - Concanavalin A
	3 µM PhenoVue 512 Nucleic Acid Stain (comparable to SYTO™ 14)
	1.5 µg/ml PhenoVue Fluor 555 - WGA
	33 nM PhenoVue Fluor 568 - Phalloidin
	500 nM PhenoVue 641 Mitochondrial Stain (comparable to MitoTracker Deep Red FM)
Microplate	CellCarrier-96 Ultra, tissue culture treated (PerkinElmer #6055302)
Imaging Instrument	Opera Phenix Plus High-Content Screening System (PerkinElmer #HH14000000)

Image Acquisition

Images were acquired with different parameters, i.e. objective, confocal or non-confocal mode, binning 1 or binning 2 and different emission filter combinations to investigate the impact of these parameters on the clustering of cellular phenotypes. With these combinations it is possible to address the question of whether increased resolution aids phenotypic clustering or if the use of a lower magnification and lower resolution will impair clustering. While higher resolution enables more specific quantification of smaller structures, it decreases throughput and statistical power for profile generation. Acquiring more fields of view can improve statistics but will increase the time for both image acquisition and computational processing.

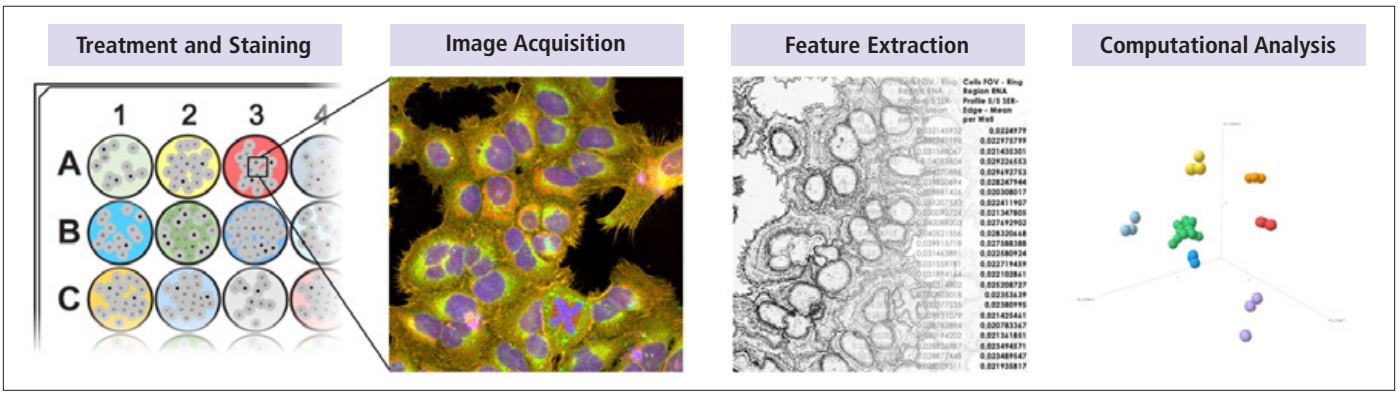

Figure 1. Workflow of the Cell Painting assay. Cells are plated into microtiter plates and treated with compounds. After a treatment of several hours or days, cells are stained with a set of fluorescent dyes using the PhenoVue cell painting kit. Images are acquired on the Opera Phenix Plus high-content screening system. Phenotypic features are extracted from images using a cell painting specific building block available for the Harmony imaging and analysis software. Cell features are then analyzed using computational models, e.g. principal component analysis in PerkinElmer Signals Screening analysis platform, to differentiate clusters of cellular phenotypes.

Another important aspect is spectral crosstalk. Using six dyes in one experiment will lead to spectral crosstalk between certain channels. The selection of emission filters is crucial to maximize fluorescence intensity and preferably decrease spectral crosstalk. In this assay PhenoVue Fluor 488 - Concanavalin A and PhenoVue 512 Nucleic acid stain with nearby excitation and emission spectra will be the dyes with the most spectral crosstalk raising the question of whether the amount of spectral crosstalk would influence the clustering of cellular phenotypes.

Therefore, plates were imaged using different emission filter settings for the separation of the PhenoVue Fluor 488 - Concanavalin A and PhenoVue 512 Nucleic acid stain channels. PhenoVue 568 - Phalloidin and PhenoVue 555 - WGA were always imaged in the same channel in accordance with Bray et al.[2] Please refer to Table 2 for an overview of image acquisition modalities used in this cell painting application. Images of cells stained with single dyes and complete cell painting mix are shown in Figure 2.

Table 2. Image acquisition options.

Channel	Excitation [nm]	Emission [nm]	Optical Mode	Objectives	Binning	Fields	z-planes
DNA (PhenoVue Hoechst 33342 Nuclear Stain)	375	435-480	Confocal Non-confocal	10x 20x Water Immersion 40x Water Immersion	1 and 2	(10x) 3 (20x) 3 (40x) 6	3
Endoplasmic Reticulum (ER) (PhenoVue Fluor 488 - Concanavalin A)	488	500-530 500-550					
RNA (PhenoVue 512 Nucleic Acid stain)	488	500-550 515-550 570-630					
WGA/Phalloidin (WGP) (PhenoVue Fluor 568 - Phalloidin and PhenoVue Fluor 555 - WGA)	561	570-630					
Mitochondria (PhenoVue 641 Mitochondrial Stain)	640	650-760					

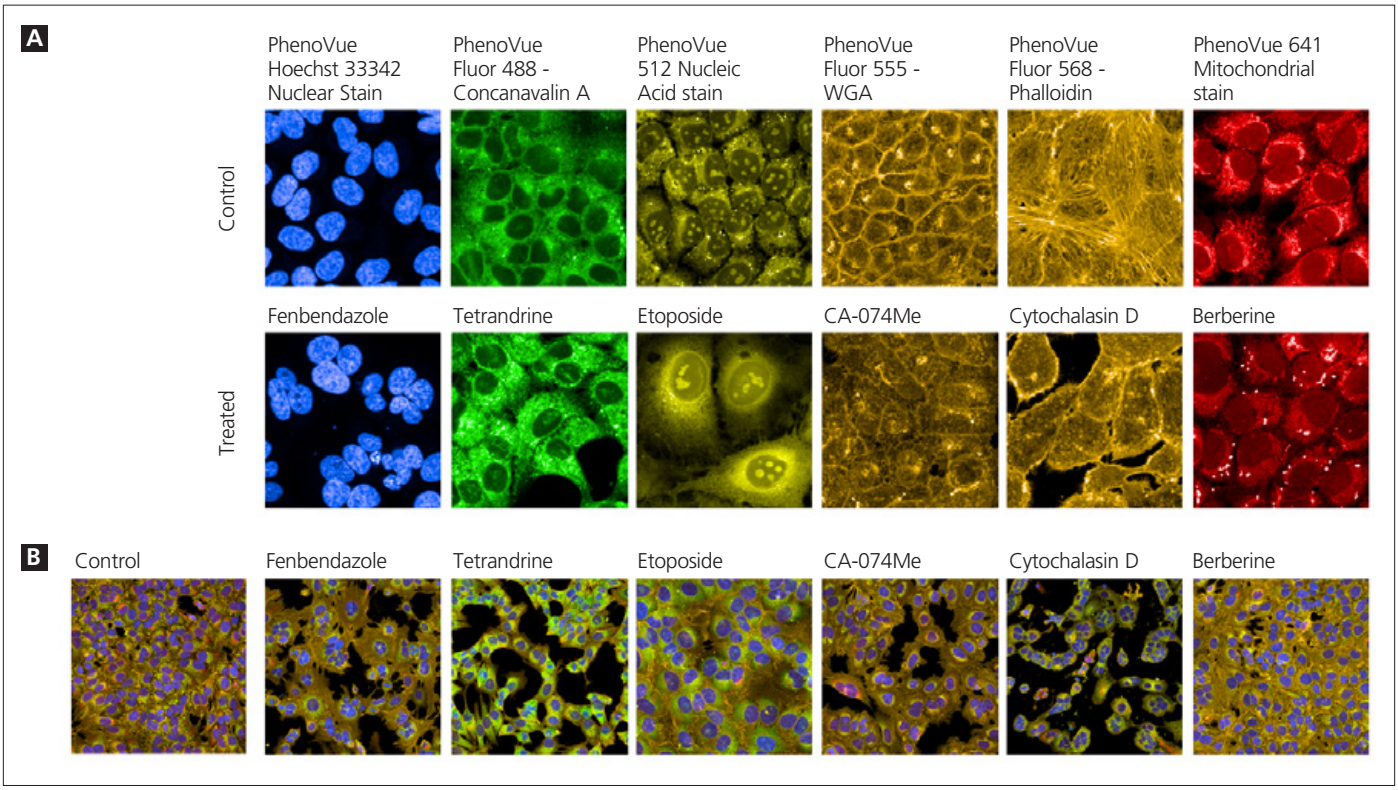

Figure 2. Representative images of cell painting staining patterns. (A) HeLa cells labeled individually with PhenoVue Hoechst 33342 Nuclear stain, PhenoVue Fluor 488 - Concanavalin A, PhenoVue 512 Nucleic acid stain, PhenoVue Fluor 568 - Phalloidin, PhenoVue Fluor 555 - WGA and PhenoVue 641 Mitochondrial stain either untreated (upper panel) or after treatment (lower panel). Fenbendazole treatment leads to giant multinucleated cells, Tetrandrine to abundant endoplasmic reticulum, Etoposide to large nucleoli, CA-074Me affects abundance of golgi, Cytochalasin D lead to disruption of the actin cytoskeleton and Berberine chloride to redistribution of mitochondria. Cropped images were acquired with the 40x water immersion objective. (B) Composite images of HeLa cells stained with all cell painting kit dyes. Left image shows staining pattern of untreated cells. The images to the right show staining patterns after indicated treatment. All images were acquired on the Opera Phenix Plus high-content screening system using a 40x water immersion objective.

Analysis

Images were analyzed within Harmony image and analysis software using a cell painting specific building block. This building block allows the extraction of more than 5700 cell properties. This reduces the time taken to set up an analysis sequence substantially as the whole analysis sequence consists of only four building blocks (Figure 3).

For further analysis, we extracted 3105 properties per object and used the mean of each property per well for further analysis. For visualization and clustering of the phenotypes a principle component analysis was done using PerkinElmer Signals Screening analysis platform. For each compound, 3 wells were treated. It is expected that replicates cluster together but separate from other treatment groups. We tested the impact of the following image acquisition settings on phenotypic clustering:

Confocal vs. Non-confocal Acquisition

To test the influence of confocal vs. non-confocal acquisition on phenotypic clustering, images were acquired with the same objective and filter settings either using or not using the confocal spinning disc. As shown in Figure 4, clustering of the triplicates is not influenced by this. This might be different if the specimen were to have a higher background fluorescence than these monolayer cultures. In this case, a confocal acquisition would increase the signal to background ratio and may aid clustering/separation.

Binning 1 vs. Binning 2

To test the influence of binning on phenotypic clustering, images were acquired with the same objective and filter set either in confocal or non-confocal mode with binning 1 or 2. As shown in Figure 4, binning had no effect on phenotypic clustering in neither confocal nor non-confocal mode.

Different Emission Filter to Separate ER and RNA Channel

Another question is whether spectral crosstalk would influence the clustering. The two dyes with the most spectral overlap are PhenoVue Fluor 488 - Concanavalin A and PhenoVue 512 Nucleic acid stain. They were acquired in either separate channels or with no spectral separation in one channel. As can be seen in Figure 4, the spectral separation of these two dyes had no influence on phenotypic clustering of the triplicates.

Another option to decrease spectral crosstalk is to titrate the respective dyes relative to each other. We found that the concentration of PhenoVue Fluor 488 - Concanavalin A suggested in the original cell painting assay paper[2] is too high for the sensitive Opera Phenix Plus system. We were able to decrease the PhenoVue Fluor 488 - Concanavalin A concentration by a factor of 40. Consequently, the spectral crosstalk into the PhenoVue 512 Nucleic acid stain channel is significantly decreased (Figure 5A). The decrease of spectral crosstalk leads to more pronounced nucleoli in the PhenoVue 512 Nucleic acid stain channel. To test if this would affect phenotypic clustering, we treated the cells with Etoposide because it alters the nucleoli morphology. Cells treated with Etoposide were stained with a cell painting dye mix containing either 100 µg/ml or 2.5 µg/ml PhenoVue Fluor 488 - Concanavalin A and acquired with the same settings. As expected from the previous findings this had no effect on the separation and clustering (Figure 5B).

Different Objectives, i.e. 10x, 20x Water Immersion and 40x Water Immersion

To compare the phenotypic clustering of datasets acquired with different objectives, images were acquired with the same settings apart from the objective. The results show that for clustering of this small chemical space, the choice of objective had no effect. This is in line with the observation that binning had no effect on phenotypic clustering as well (Figure 4). Binning does not affect the optical resolution but the digital resolution is decreased by a factor of 2 when switching from binning 1 to binning 2. Likewise, the optical resolution is decreased by a factor of 2 when switching from 40x to 20x and from 20x to 10x. The optical resolution is likely to have a much greater influence if the chemical space being tested, and therefore the resulting cellular phenotypes, was more complex.

Individual z-planes vs. Maximum Intensity Projection

Sometimes cells within a well are not all at the same focal height. To test if z-stacks are always required to compensate for this, a z-stack of three planes with 1 µm distance was acquired and either individual planes or a maximum intensity projection image (MIP) was analyzed. Again, for this data set there was no influence on phenotypic clustering (Figure 4).

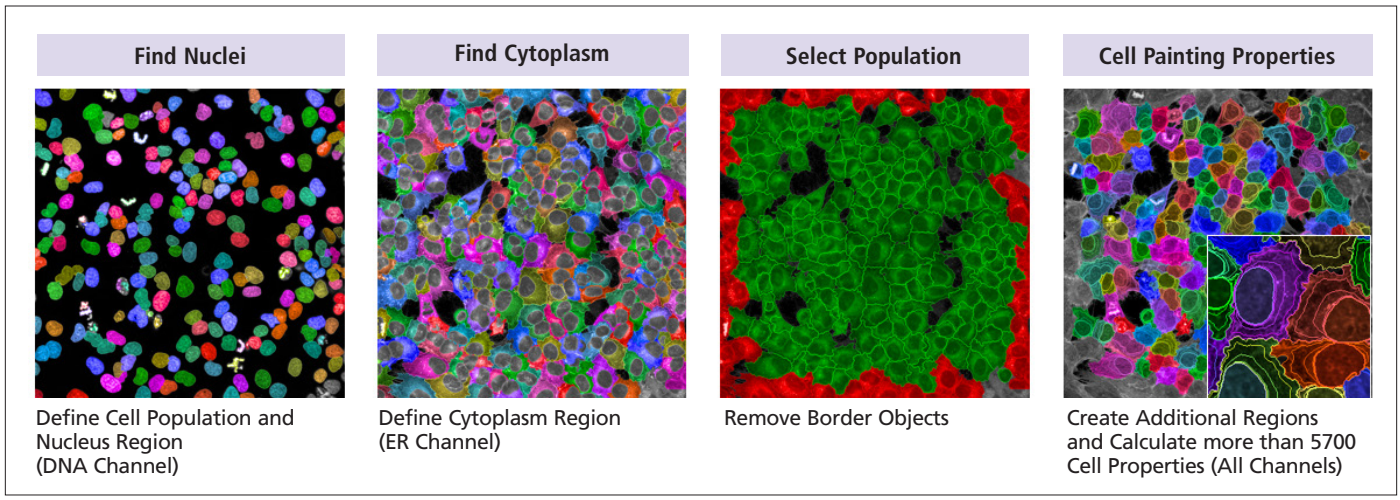

Figure 3. The image analysis sequence in Harmony software using the cell painting specific building block requires only four building blocks to extract more than 5700 cell properties.

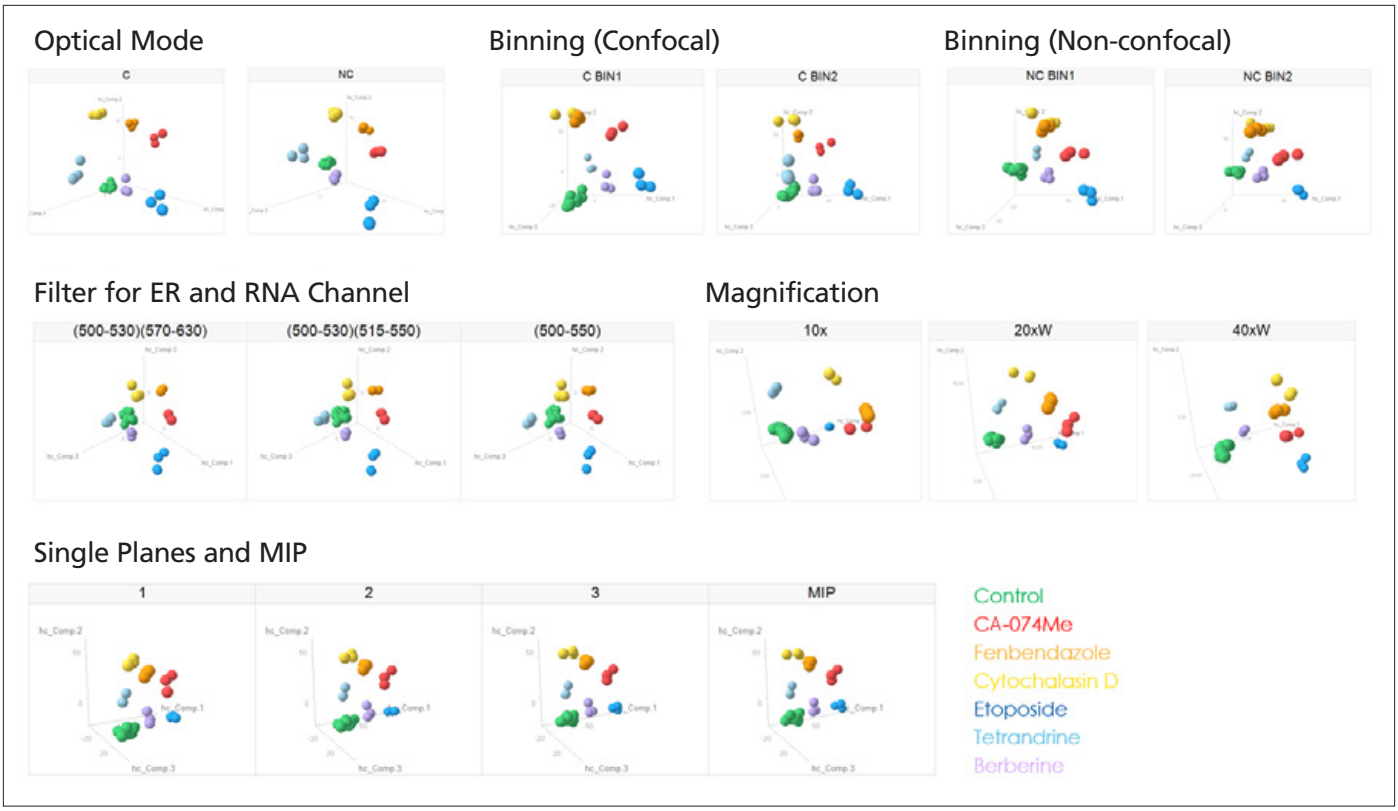

Figure 4. Principle component analysis to reduce dimensionality and visualize the data. Wells containing cells with similar phenotypes will cluster together. For each compound, three wells were treated and principal component analysis was performed using the PerkinElmer Signals Screening platform. None of the tested image acquisition setup alterations or analysis strategies had any clear impact on phenotypic clustering.

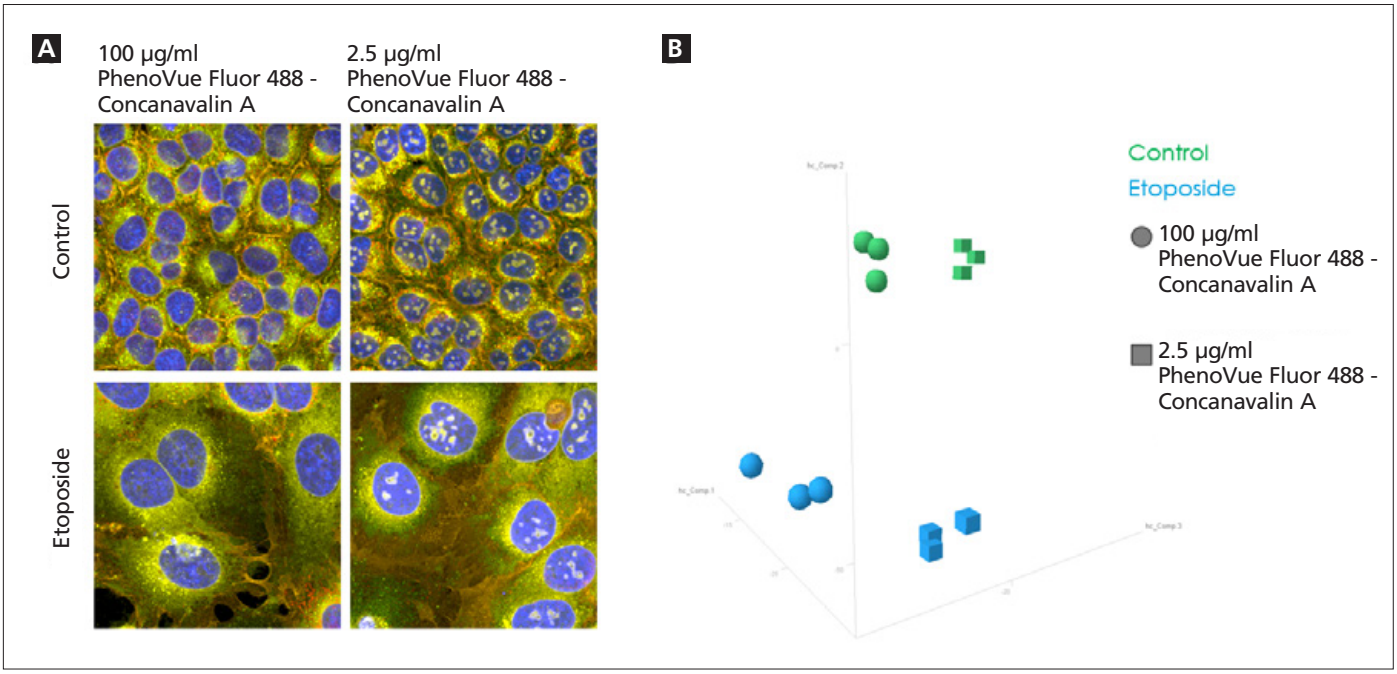

Figure 5. Opera Phenix Plus allows the concentration of the dyes to be decreased – most strikingly that of PhenoVue Fluor 488 - Concanavalin A by a factor of 40. (A) As a result, the nucleoli staining becomes more prominent. (B) However, this has no influence on phenotypic clustering as controls and treated wells are well separated and cluster together depending on the PhenoVue Fluor 488 - Concanavalin A concentration.

Conclusions

Phenotypic profiling or screening has become more than just a trend and is now a widely used approach in both drug discovery and basic research. One prominent example of a phenotypic assay is cell painting which uses different dyes to stain multiple cellular compartments simultaneously. Bray et al's cell painting assay[2] uses six different dyes acquired in 5 channels to stain the major cellular compartments. From these images hundreds to thousands of properties are extracted and used for phenotypic profiling. Here we have used the PerkinElmer PhenoVue cell painting kit, which includes comparable dyes, to perform this assay and tested the impact of different acquisition modalities on phenotypic clustering. We have found that phenotypic clustering is robust over a wide range of image acquisition settings. We have also found, due to the sensitivity of the Opera Phenix Plus system, that the concentration of PhenoVue Fluor 488 - Concanavalin A could be reduced by a factor of 40 and the concentration of the other dyes can also easily be reduced (data not shown). For phenotypic profiling assays one would expect that, when extracting large numbers of properties, a substantial amount will be highly redundant and that only a subset is needed to discriminate the phenotypes. However, at the time of image analysis it is not clear which features would be the ones with the highest variance, so they need to be extracted first and reduced later. Therefore, the important question is how to extract all of these parameters in an easy and straightforward way. Harmony software offers all tools needed for this and with the newly-introduced cell painting building block it is extremely convenient to set up the analysis sequence with as little as four building blocks. In this application note we have used only a small chemical space leading to a respectively small phenotypic space. Hence, it might be that for more complex screening approaches with thousands of compounds the resolution becomes of more importance in order to discriminate even more subtle phenotypic differences and perhaps the signal to background ratio would need to be improved if the cellular model suffers from background fluorescence (e.g. thicker specimens or lipid-rich cellular models). The Opera Phenix Plus system offers water immersion objectives that allow acquisition of images with higher resolution and also, due to the smaller depth of focus, less background fluorescence. If more background suppression is needed, the confocal spinning disc can also be used. The Opera Phenix Plus system provides all you need for high-quality image acquisition, and combined with Harmony software, the feature extraction is fully integrated into your workflow. Finally, for secondary analysis PerkinElmer Signals Screening can be used which offers tools for quality control, data visualization and hit detection.

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