Phenotype discrimination using the PhenoVue cell painting and multi-organelle staining kits.

Key points

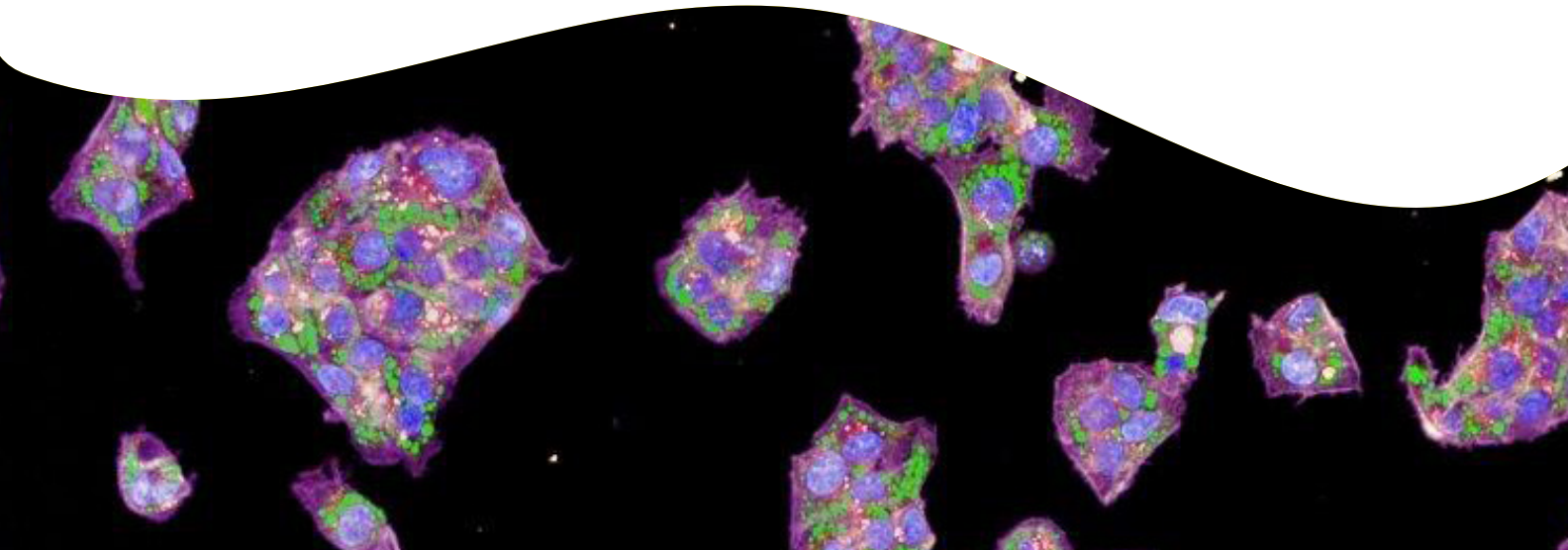
- Labeling different combinations of cellular components can be advantageous depending on experimental aims
- Phenotypes were succesfully differentiated by both kits
- Both kits are compatible with the cell painting building block in Harmony™ software for feature extraction

Introduction

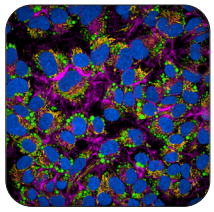
Cell painting has gained significant interest in recent years as it allows researchers to capture a comprehensive picture of cellular responses to various perturbations. The cell painting assay uses six stains to label DNA, cytoplasmic RNA, nucleoli, actin, Golgi apparatus, plasma membrane, endoplasmic reticulum and mitochondria. However, other combinations of "paints" or dyes are also possible, enabling the visualization of slightly different cellular components and processes depending on research needs. One such example is the PhenoVue™ multi-organelle staining kit. This kit allows staining of DNA, lipid droplets, actin, mitochondria and lysosomes. With its lysosomal and lipid droplet labels this kit is tailored towards studying cellular metabolism or disorders associated with dysfunctional lysosomes or lipid homeostasis (see info box).

In collaboration with the Institute of Bioorganic Chemistry (IBCH) in Poland, we analyzed the phenotypes induced by a set of 15 reference compounds using both the PhenoVue cell painting kit and the PhenoVue multi-organelle staining kit to compare phenotypic discrimination results of the two assays. Among the compounds are well-known agents that affect various organelles and serve as reference standards for cell painting, compounds that impact lipid homeostasis, such as oleic acid, and six drug-induced liver injury (DILI) compounds with varying DILI scores (DILIrank database).

Our results indicate that both kits successfully differentiated the phenotypes induced by the compounds; however, the multi-organelle kit showed better separation indicated by greater Euclidian distances for certain compounds, suggesting a slightly more sensitive detection of subtle phenotypic changes at lower concentrations.



The **PhenoVue Multi-organelle Staining Kit** includes five probes to visualize nuclei, mitochondria, lysosomes, actin, and lipid droplets with good spectral separation and reduced spectral overlap.

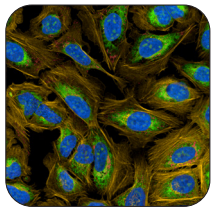


The kit does not rely on living cells which might be beneficial in high-throughput applications.

Moreover, the long Stokes shift of PhenoVue Fluor 400LS Phalloidin conjugate enables actin filaments to be distinguished from mitochondria which are stained with a PhenoVue Fluor 555.

All five probes are measured separately in 5 channels.

The **PhenoVue Cell Painting JUMP Kit** includes six dyes to label 8 cell compartments: DNA, cytoplasmic RNA, nucleoli, actin, Golgi apparatus, plasma membrane, endoplasmic reticulum, and mitochondria.



The kit provides a standardized ready-to-use protocol, with the first staining step applied to live cells (PhenoVue 641 mitochondrial stain). After fixation, all other dyes are applied to the cells.

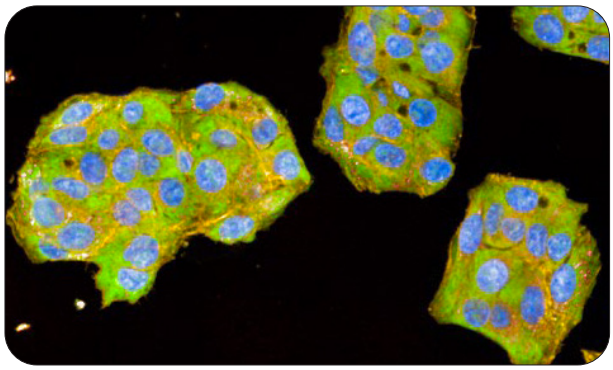
Image acquisition spectrally combines two stainings in one channel: WGA and phalloidin, staining plasma membrane, Golgi apparatus and actin. Six probes are therefore measured in five channels.

Results and discussion

Figure 1 shows example images of HepG2 control cells after staining them with either the PhenoVue cell painting JUMP kit or the PhenoVue multi-organelle staining kit. Both kits result in high quality, brightly stained cell compartments and organelles, which are subsequently phenotypically analyzed using the cell painting building block followed by a Principal Component Analysis (PCA).

Cell painting DMSO control image

DNA, RNA/nucleoi, Endoplasmic reticulum, Golgi/plasma membrane/actin, Mitochondria



Multi-organelle DMSO control image

DNA, Lipid droplets, Mitochondria, Lysosomes, Actin

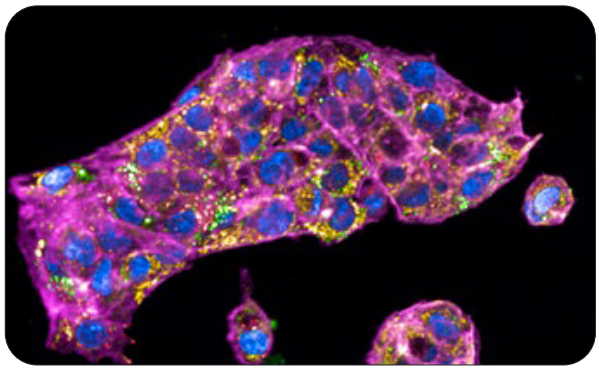


Figure 1: Example images of control HepG2 cells labeled with either the PhenoVue Cell Painting JUMP or the Multi-organelle kit.

The Cell Painting assay (left) is based on PhenoVue Hoechst 33342 (DNA, Nucleus), PhenoVue 512 Nucleic Acid Stain (RNA-Nucleoli), PhenoVue Fluor 488 Concavalin A (Endoplasmic Reticulum), PhenoVue Fluor 555 WGA (Golgi and Plasma Membrane), PhenoVue Fluor 568 Phalloidin (F-actin) and PhenoVue 641 Mitochondrial Stain (Mitochondria).

The multi-organelle assay (right) is based on PhenoVue Hoechst 33342 (DNA, Nucleus), PhenoVue 493 Lipid Stain (Lipid Droplets), PhenoVue Fluor 400LS Phalloidin (F-actin), anti-HSP60 antibody/PhenoVue Fluor 555 (Mitochondria), anti-LAMP1 antibody/PhenoVue Fluor 647 (Lysosomes).

Images were acquired in confocal mode on the Opera Phenix system using a 20x water immersion objective. Each image shows one field of view in a maximum intensity projection of four planes, with 1 µm distance between planes.

Drug induced phenotypes for the two assays were then compared using Signals Research Suite™ High Content Profiler and phenotypes were visualized as a Principal Component Analysis (PCA) for all compounds and concentrations (Figure 2). Both assays showed dose-dependent clustering of the different compounds.

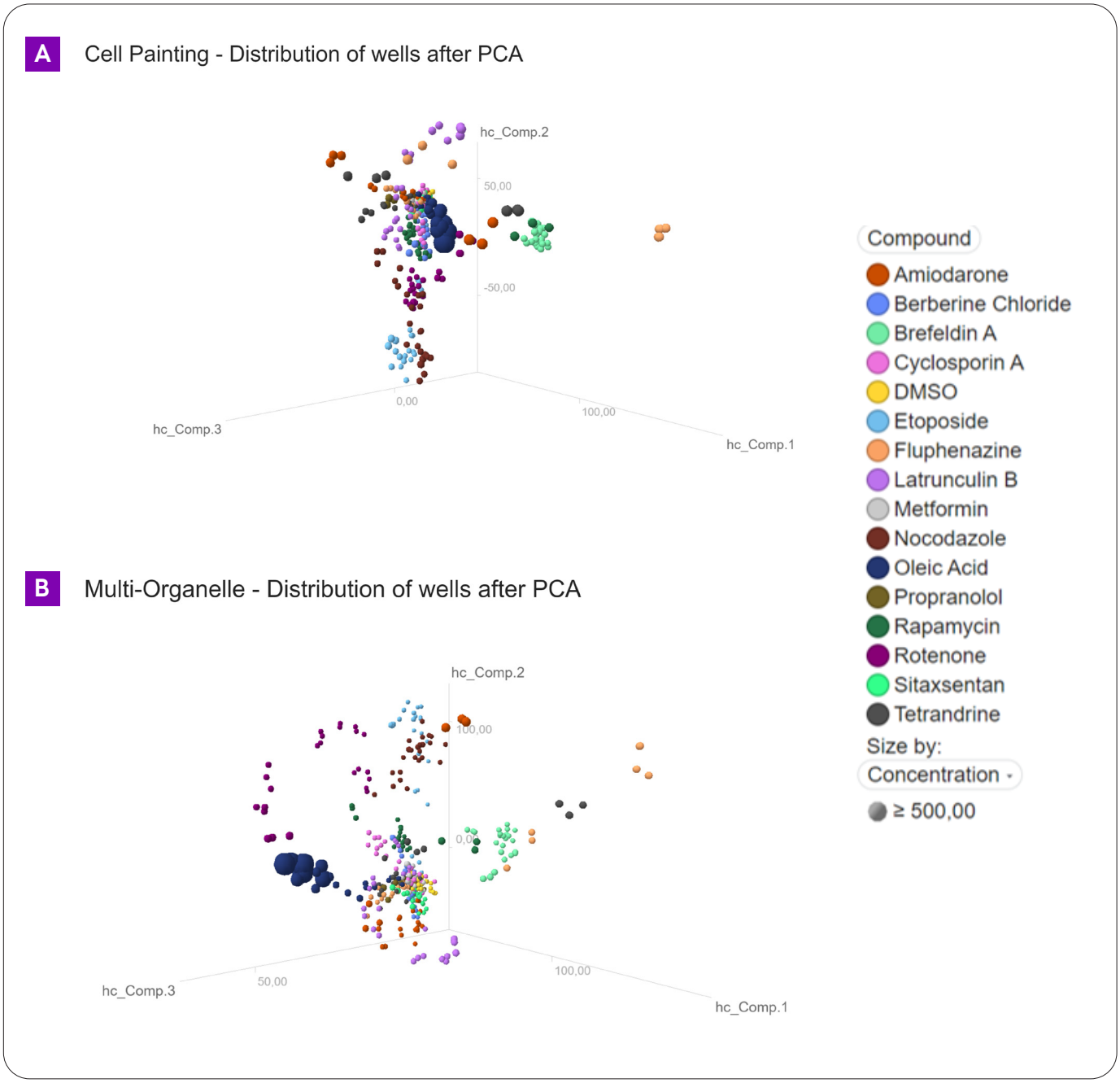


Figure 2: Clustering of individual wells /treatments after dimensionality reduction by Principal Component Analysis.
(A) Cell painting kit-stained wells and (B) multi-organelle kit-stained wells show a dose dependent clustering of the different treatments.

For further analysis an independent 2-dimensional PCA was calculated separately for each compound together with the DMSO control wells. Out of these individual PCAs the geometrical center for each concentration (3 wells) was determined and the Euclidian distance to the geometrical center of the DMSO control wells was calculated (example shown for amiodarone in figure 3).

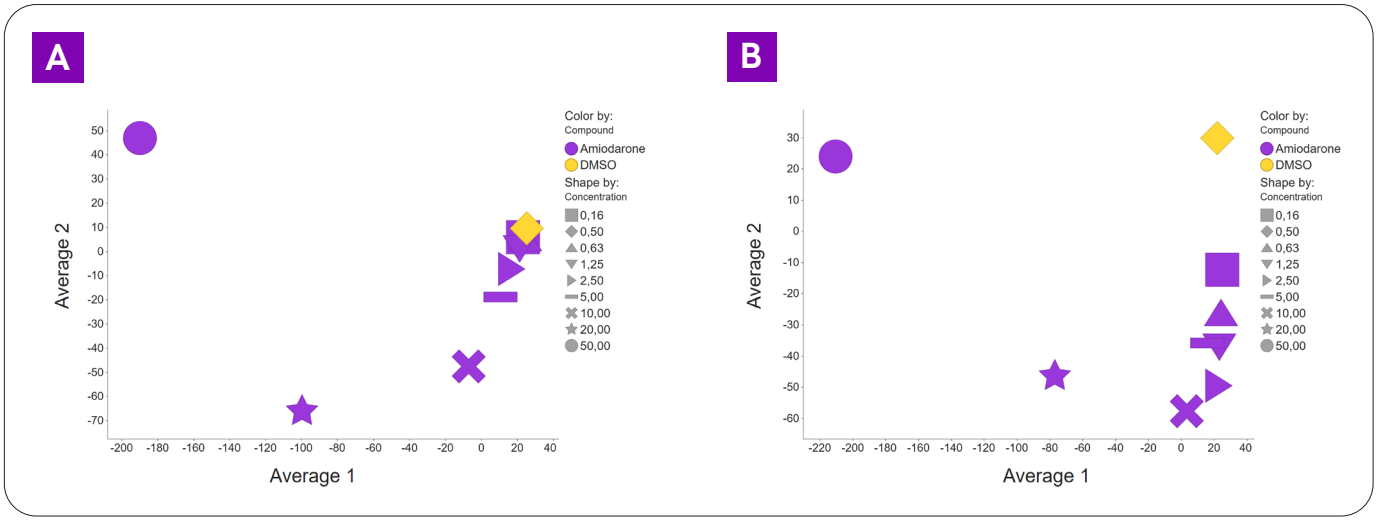


Figure 3: Principal Component Analysis of amiodarone dose response and DMSO.

PCA for the amiodarone dose response with DMSO control wells derived from (A) cell painting kit-stained wells and (B) multi-organelle kit-stained wells. The average from triplicates of each concentration were determined by calculating the geometrical center. The DMSO control wells are colored yellow and the amiodarone wells purple. Each concentration is indicated by a different shape.

High compound concentrations typically produce pronounced phenotypes making them easier to dinstinguish. To assess assay performance, we focused on the respective 4 lower concentrations in the dose response and calculated Euclidian distances to the DMSO controls (Fig 4). Euclidian distance, a widely used metric, quantifies the degree of phenotypic separation in the PCA. For many compounds the separation efficiency between the cell painting and the multi-organelle assays were comparable. However for some compounds such as amiodarone, berberine chloride, oleic acid, Propranolol and sitaxsentan the multi-organelle assay exhibited greater Euclidian distances, indicating better phenotypic separation compared to the cell painting assay. Interestingly, from these 5 compounds, 3 compounds are causing DILI (amiodarone, propranolol and sitaxsentan) and 1 compound disturbs lipid homeostasis (oleic acid).

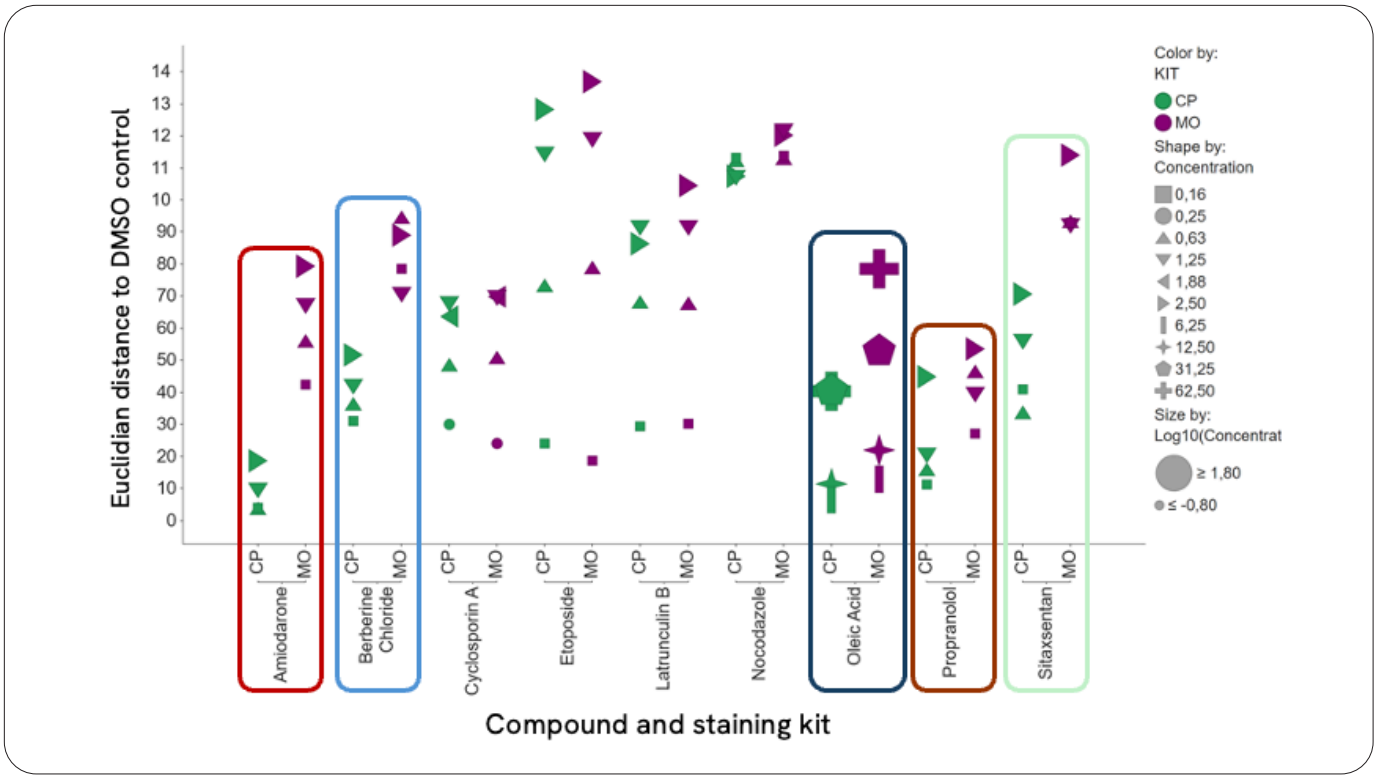


Figure 4: The multi-organelle assay (MO) shows higher Euclidian distances for some compounds compared to the cell painting assay (CP). The Euclidian distance to the DMSO control was calculated for the lowest four concentrations of the dose response to focus on the more subtle phenotypic changes. Many of the reference compounds show similar Euclidian distances regardless of which of the two assays is used. Amiodarone, berberine chloride, oleic acid, propranolol and sitaxsentan induced phenotypes however are better separated by the multi-organelle assay.

Conclusions

The cell painting assay is widely established and one key development has been the assembly of sets of reference compounds with known mechanism of action or known toxicity (Seal et al, 2024). However, cell painting has also been adapted by groups with alternative fluorescent dyes to facilitate visualization of other cellular components. For example, LipocyteProfiler is such a cell painting adaptation incorporating BODIPY to stain neutral lipids in addition to generic morphological features (Laber et al, 2023).

Here we present a cell painting adaptation assay, the multi-organelle assay that stains lipids and lysosomes in addition to some of the generic morphoplogical markers. As a proof of concept we compared the multi-organelle assay to the cell painting assay in HepG2 cells and found that both kits were able to discriminate phenotypes induced by 15 reference compounds. For some compounds, the multi-organelle assay showed higher Euclidian distances on the Principal Component Analysis and slightly improved phenotypic discrimination. The multi-organelle assay is compatible with the cell painting building block in Harmony image analysis software, which enables a convenient and fast extraction of up to 5930 features per cell. Taken together the multi-organelle assay provides robust morphological cell profiles that include lipid droplet and lysosome phenotypic traits to be able to analyze pertubations affecting, or disorders associated with these cellular components.

Materials and methods

Table 1: List of compounds

Compound Name	Details	Concentrations
Amiodarone (hydrochloride)	CAS 19774-82-4, Cayman Chemical #15213	0.15625 µM 0.625 µM 1.25 µM 2.5 µM 5 µM 10 µM 20 µM 50 µM
Sitaxsentan (sodium salt)	CAS 210421-74-2, Cayman Chemical #29244	
Propranolol (hydrochloride)	CAS 318-98-9, Cayman Chemical #23349	
Etoposide	CAS 33419-42-0, Cayman Chemical #12092	
Metformin (hydrochloride)	CAS 1115-70-4, Cayman Chemical #13118	
Berberine Chloride	CAS 633-65-8, Cayman Chemical #10006427	
Brefeldin A	CAS 20350-15-6, Cayman Chemical #11861	
Fluphenazine (hydrochloride)	CAS 146-56-5, Cayman Chemical #23555	
Latrunculin B	CAS 76343-94-7, Sigma-Aldrich #428020	
Nocodazole	CAS 31430-18-9, Cayman Chemical #13857	
Rapamycin	CAS 53123-88-9, Cayman Chemical #13346	
Rotenone	CAS 83-79-4, Sigma-Aldrich #557368	
Tetrandrine	CAS 518-34-3, Cayman Chemical #19874	
Oleic Acid-Albumin from Bovine Serum	Sigma-Aldrich #O3008	6.25 µM 12.5 µM 31.25 µM 62.5 µM 125 µM 250 µM 375 µM 500 µM
Cyclosporin A	CAS 59865-13-3, Cayman Chemical #12088	0.25 µM 0.625 µM 1.25 µM 1.875 µM 2.5 µM 5 µM 7.5 µM 10 µM
DMSO	CAS 67-68-5, Sigma-Aldrich #D8418-50ML	0.5%

Cells and compound treatment

HepG2 cells were plated at 3000 cells/well in 30µL growth medium into two PhenoPlate™ 384-well microplates and incubated overnight. Next, cells were treated with 8 different concentrations of 15 compounds (see table 1) in triplicates for 24h, followed by applying either the PhenoVue multi-organelle staining kit or the PhenoVue cell painting JUMP kit.

Cell painting

From two plate copies, one plate was stained with the PhenoVue cell painting JUMP kit (Revvity # PING 21) according to the provided protocol. Initially, live cells were incubated with Staining Solution 1, containing 500 nM PhenoVue 641 mitochondrial stain, and then fixed with 16% paraformaldehyde (4% final concentration). The plate was then washed with 1x PBS (150 µl/well) using a plate washer

and stained with Staining Solution 2, which included 0.1% Triton X-100, 1.5 µg/ml PhenoVue Fluor 555 - WGA, 5 µg/ml PhenoVue Fluor 488 - Concanavalin A, 8.25 nM PhenoVue Fluor 568 - Phalloidin, 1.62 µM PhenoVue Hoechst 33342 nuclear stain, and 6 µM PhenoVue 512 nucleic acid stain. After incubation, the plate was washed with 1x PBS (180 µl/well) using a plate washer. Finally, 60 µl of PBS was added to all wells, and the plate was sealed.

Multi-organelle staining

The second plate was stained using PhenoVue multi-organelle staining kit (Revvity #PMOS11) according to the protocol provided. To reduce cell detachment, the initial step of washing live cells with PBS was omitted, and cells were fixed by adding 16% paraformaldehyde directly to the cell culture media (4% final concentration). The cells were then washed with 1x PBS (180 µl/well) using a plate washer, permeabilized with 0.1% Triton X-100 solution, washed again with 1x PBS (180 µl/well) using a plate washer, and saturated with PBS-1% BSA solution. After washing with 1x PBS (180µl/well) using a plate washer, Staining Solution 1, containing PhenoVue anti-LAMP1 antibody (1x) and PhenoVue anti-HSP60 antibody (1x), was added to the cells and incubated overnight at 4°C. The next day, the cells were washed (1x PBS, 180 µl/well, using a plate washer) and stained with Staining Solution 2, which consisted of 20 ng/mL PhenoVue Hoechst 33342 nuclear stain, 1x PhenoVue 493 lipid stain, 1x PhenoVue Fluor 400LS - Phalloidin, 1x PhenoVue Fluor 555 goat anti-mouse antibody highly cross-adsorbed, and 1x PhenoVue Fluor 647 goat anti-rat antibody highly cross-adsorbed. Finally, the plate was washed with 1x PBS (180µl/well) using a plate washer and finally 60 µl of PBS was added to all wells, and the plate was sealed.

Image acquisition

The image acquisition setting in terms of excitation and emission are given in table 2. Images were acquired on an Opera Phenix high-content screening system in confocal mode using a 20x water immersion objective. 9 fields of view were acquired per well, each with a 1µm-spaced stack of 4 planes.

Table 2: Excitation and emission settings used for cell painting or multi-organelle staining kit-stained plate.

Kit	Dye(s)	Excitation [nm]	Emission [nm]
Cell Painting	PhenoVue Hoechst 33342 nuclear stain	405	435-480
	PhenoVue Fluor 488 - Concanavalin A PhenoVue 512 nucleic acid stain	488	500-550
	PhenoVue Fluor 555 - WGA PhenoVue Fluor 568 - Phalloidin	561	570-630
	PhenoVue 641 mitochondrial stain	640	650-760
Multi-organelle	PhenoVue Hoechst 33342 nuclear stain	405	435-480
	PhenoVue Fluor 400LS - Phalloidin	405	570-630
	PhenoVue 493 lipid stain	488	500-550
	PhenoVue Fluor 555	561	570-630
	PhenoVue Fluor 647	640	650-760

Feature extraction and secondary analysis

To extract phenotypic features (such as morphology, texture, intensity) from different cell regions, Maximum Intensity Projection images were analyzed in Harmony high-content imaging and analysis software. The analysis involved segmenting the nuclei and cytoplasm, removing objects that touch the image borders, and extracting 5930 cellular features using the dedicated building block Calculate Cell Painting Properties in extensive setting.

Downstream analysis of morphological profiles was done by importing feature data into the High Content Profiler app of Signals Research Suite. A reduction of dimensionality was achieved with a Principal Component Analysis (PCA).

References

Seal, Srijit, et al. “A Decade in a Systematic Review: The Evolution and Impact of Cell Painting.” *ArXiv* (2024).

Laber, Samantha, et al. “Discovering cellular programs of intrinsic and extrinsic drivers of metabolic traits using Lipocyte Profiler.” *Cell Genomics* 3.7 (2023).

Application Note: Phenotypic analysis of CRISPR-Cas9 cell-cycle knockouts using cell painting (Revvity.com)

Application Note: Orthogonal validation of CRISPR-Cas9 and siRNA generated phenotypes using cell painting. (Revvity.com)

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