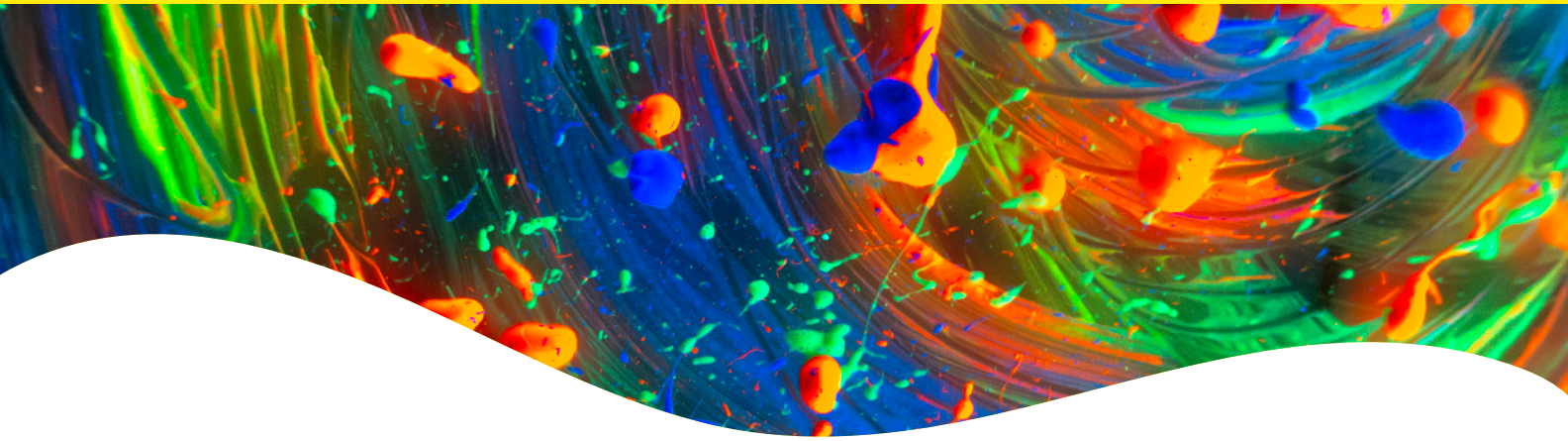
PhenoVue Live Cell Painting Kits (For 2 x 96-well plates and 10 x 96 well plates)



Overview

Cell Painting is a powerful phenotypic high-content screening (HCS) approach that integrates cell biology with advanced image analysis to capture and quantify cellular responses to a wide range of perturbagens. In this technique, multiple cellular compartments are "painted" using fluorescent probes, enabling simultaneous profiling of diverse phenotypic features. This provides deep insights into the effects of small molecules, biologics, or genetic perturbation.

The PhenoVue™ Live cell painting kit facilitates multiplexed labeling of live cells using four verified, fluorescent probes staining the nucleus, mitochondria, microtubules, and intracellular vesicles. For use in live-cell imaging workflows, the kit supports time-lapse acquisition to monitor phenotypic changes over time in response to chemical or genetic treatments.

The probes are compatible with standard cell culture conditions and have been optimized to have low impact on cell viability and proliferation, promoting reliable results even during extended imaging. By streamlining the staining workflow, the PhenoVue Live cell painting kit reduces hands-on time and limits experimental variability ultimately facilitating clear, reproducible data.

Product information

Product name	Part no.	Number of vials per unit	Shipping conditions
PhenoVue Live cell painting kit – 2 x 96 wells*	PLIVCP14	3	Dry ice
PhenoVue Live cell painting Kit – 10 x 96 wells**	PLIVCP15	3	Dry ice

*PhenoVue Live cell painting kit PLIVCP14 provides sufficient reagents to stain 2 x 96-well plates or 1 x 384-well plate, following the recommended concentrations and volumes.

**PhenoVue Live cell painting kit PLIVCP15 provides sufficient reagents to stain 10 x 96-well plates or 5 x 384-well plates, following the recommended concentrations and volumes.

Part no.	Kit contents	Format	Quantity	Storage
PLIVCP14	PhenoVue Hoechst 33342 nuclear stain	Liquid (H_2O)	1 vial (70 µL, 10000x)	2-8 °C or below. Protect from light
	PhenoVue 488 Live cell painting stain	Lyophilized	1 vial	2-8 °C or below. Protect from light.
	PhenoVue 555/647 Live cell painting stains mix	Liquid (DMSO)	1 vial (24 µL, 1000x)	-16 °C or below. Protect from light.
PLIVCP15	PhenoVue Hoechst 33342 nuclear stain	Liquid (H_2O)	1 vial (70 µL, 10000x)	2-8 °C or below. Protect from light
	PhenoVue 488 Live cell painting stain	Lyophilized	1 vial	2-8 °C or below. Protect from light.
	PhenoVue 555/647 Live cell painting stains mix	Liquid (DMSO)	1 vial (120 µL, 1000x)	-16 °C or below. Protect from light.

Storage and stability

- For convenience, store the kit at ≤ -16 °C. However, each reagent can be stored separately between ≤ -16 °C to 2-8 °C, as indicated in the table above. Avoid repeated freeze / thaw cycles. After reconstitution, aliquoted reagents must be stored at -16 °C or below.
- Allow the reagents to warm up to room temperature for 30 min before opening the vials and reconstitution. Aliquoted reagents must be stored at -16 °C or below and are stable for 6 months.
- The stability of these products is guaranteed until the expiration date provided in the certificate of analysis, when stored as recommended and protected from light.

Safety information

Chemical reagents are potentially harmful, please refer to the Safety Data Sheet (SDS) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Spectral properties

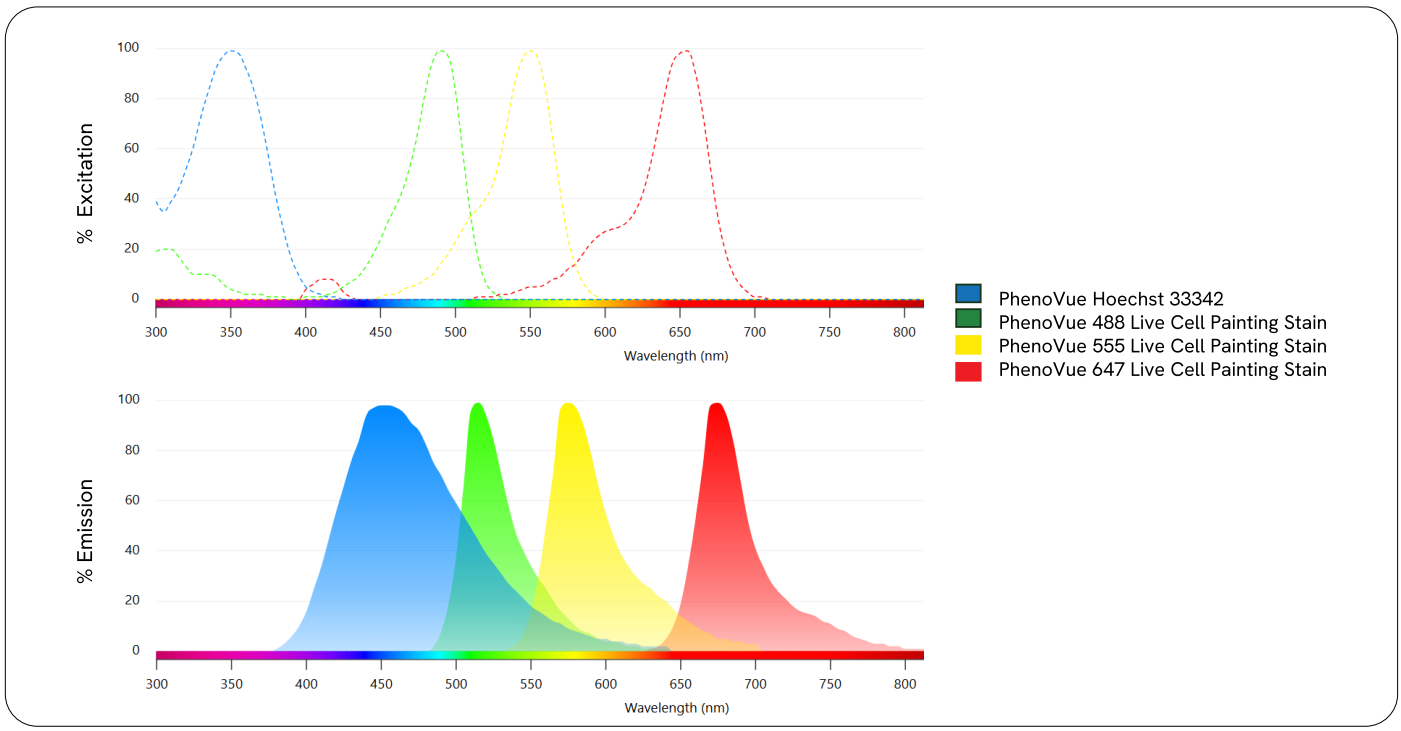


Figure 1: Excitation and emission spectra for the PhenoVue Live cell painting kit components.

Other materials and reagents required

Reagents or consumables	Usage
Cell culture medium	Diluent for PhenoVue stains and compounds.
Distilled H_2O	Reconstitution of PhenoVue 488 Live cell painting stain
PhenoPlate™ 96 or 384-well microplates	Cell plating, stimulation, staining and imaging

Reagent reconstitution and preparation of staining solution

1. **Prepare stock solution** of PhenoVue stains as described in the table below.

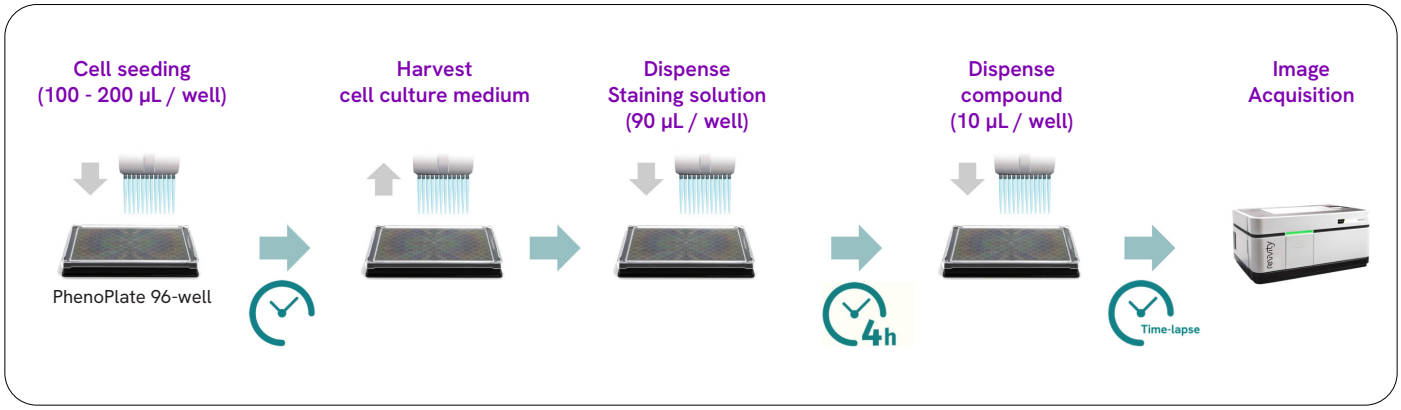
Reagents	PhenoVue Live Cell Painting Kit 2 x 96-well (PLIVCP14)	PhenoVue Live Cell Painting Kit 10 x 96-well (PLIVCP15)
PhenoVue 488 live cell painting stain	Reconstitute with 120 µL dH_2O to give a 200x stock solution.	Reconstitute with 600 µL dH_2O to give a 200x stock solution.
PhenoVue Hoechst 33342 nuclear stain	Ready to use stock solution at 1 mg/mL (10000x).	Ready to use stock solution at 1 mg/mL (10000x).
PhenoVue 555/647 live cell painting stains mix	Ready to use stock solution (1000x).	Ready to use stock solution (1000x).

Aliquot stock solutions and store at -20 °C for up to 6 months. Avoid repeated freeze-thaw cycles.

2. **Prepare staining solution in cell culture medium that is intended to be used on live cells and includes:**

	Reagents	2. Preparation of staining solution * (90µL/96 well)	For 1 mL staining solution
Staining solution	PhenoVue Hoechst 33342 nuclear stain	Step 1. Intermediate solution: Dilute the stock solution 100 times in cell culture medium. Step 2. Final dilution: Dilute the intermediate solution 90 times in cell culture medium (1.1x)	11 µL (final dilution - step 2)
	PhenoVue 488 live cell painting stain	Dilute stock solution 180 times in cell culture medium to give a 1.1x staining solution.	5.5 µL
	PhenoVue 555/647 live cell painting stains mix	Dilute stock solution 900 times in cell culture medium to give a 1.1x staining solution.	1.1 µL

Experimental workflow



Protocol for 96-well imaging plate

384-well microplate may also be used; in that case, adjust the cell seeding density appropriately and reduce all reagent volumes two-fold.

Cell Culture

Seed cells in PhenoPlate 96-well imaging microplates* or any other suitable cell culture vessel. Incubate under appropriate conditions (typically 37 °C, 5% CO_2) until cells reach 50–70% confluency.

Live-Cell Imaging Protocol

1. **Remove** the culture medium by gentle aspiration.
2. **Add** 90 µL of staining solution per well and incubate for 4 hours at 37 °C, 5% CO_2.
3. **Add** 10 µL of compound solution and continue incubation at 37 °C, 5% CO_2.

Note: If using a different volume for compound addition, adjust the staining solution volume accordingly to reach a total of 100 µL per well. Ensure that the final concentration of the staining solution is maintained at 1x.

Image acquisition

Transfer the microplates to the imaging system and initiate image acquisition (e.g., for time-lapse imaging). Refer to the following section for recommended microscope settings.

Image Analysis

When using Revvity's high-content imaging instruments, refer to the dedicated section below.

When using software other than Revvity's, refer to Cimini et al., Nature Protocols (2023), for a detailed data reduction workflow.

Tips

- Do not remove the culture medium containing PhenoVue live cell painting stains (no-wash protocol), as washing steps reduce fluorescence signal intensity.
- PhenoVue live cell painting stains are cell-permeable and designed for use with live cells. These dyes are not fixable and are therefore incompatible with fixed-cell protocols.
- Reagent concentrations have been carefully optimized to achieve high signal intensity while minimizing cytotoxicity. Increasing concentrations beyond recommended levels may result in cytotoxic effects.
- The PhenoVue 488/555/647 live cell painting stains have been validated to have no adverse effects on cell viability or proliferation.
- PhenoVue Hoechst 33342 nuclear stain is used at a low concentration (100 ng/mL) to minimize its impact on cell proliferation. While it does not induce cytotoxicity or block cell division, it may slightly slow proliferation depending on the cell type.
- Due to the dynamic of the live-cell painting approach, signal intensity and patterns may evolve over time. Therefore, it is essential to compare compound-induced phenotypes to vehicle controls at each time point. Additionally, when working with multiple plates, vehicle controls must be included on every plate to ensure consistency and reliable comparisons.
- We recommend using confocal acquisition mode to achieve superior image quality, as it provides a higher signal-to-background ratio compared to non-confocal mode.

Recommendations for acquisition settings

The PhenoVue live cell painting kit enables simultaneous multiplexing of four fluorescent channels. To ensure optimal signal intensity and image quality on Revvity high-content screening systems, we recommend the following acquisition settings, depending on the instrument used:

HCS instruments		PhenoVue Hoechst 33342	PhenoVue 488	PhenoVue 555	PhenoVue 647
Opera Phenix™ Plus 5 lasers	Excitation laser (nm)	375	488	561	640
	Emission filters (nm)	435-480	500-550	570-630	650-760
Opera Phenix Plus 4 lasers	Excitation laser (nm)	405	488	561	640
	Emission filters (nm)	435-480	500-550	570-630	650-760
Operetta CLS™ 4 or 8 LED	Excitation LED (filters) (nm)	370 (355-385)	475 (460-490)	550 (530-560)	630 (615-645)
	Emission filters (nm)	430-500	500-550	570-650	655-760

Confocal mode is strongly recommended, as it significantly improves image quality by enhancing the signal-to-background ratio.

Given the dynamic nature of live cells, we also recommend acquiring two z-planes per field and applying a maximum intensity projection.

Recommendations for Image Analysis using Revvity's Harmony or Signals Image Artist (SImA)

1. Input Image

Acquire 2 z-planes and apply a "maximum projection" to better account for cellular variability and morphology over time.

Set Flatfield correction to "Basic" or "Advanced".

2. Find Nuclei

Use the PhenoVue Hoechst channel for nuclei segmentation.

3. Select Population to remove border nuclei

Apply the "select population" building block on the nuclei population with common filters method and remove border objects to get the nuclei selected.

4. Calculate image to merge the 4 channels

Use the "calculate image" building block to merge the 4 channels to prepare image for cytoplasm segmentation. Use the formula "A+B+C+D" with channels A, B, C, D which are respectively PhenoVue Hoechst, PhenoVue 488, PhenoVue 555 and PhenoVue 647.

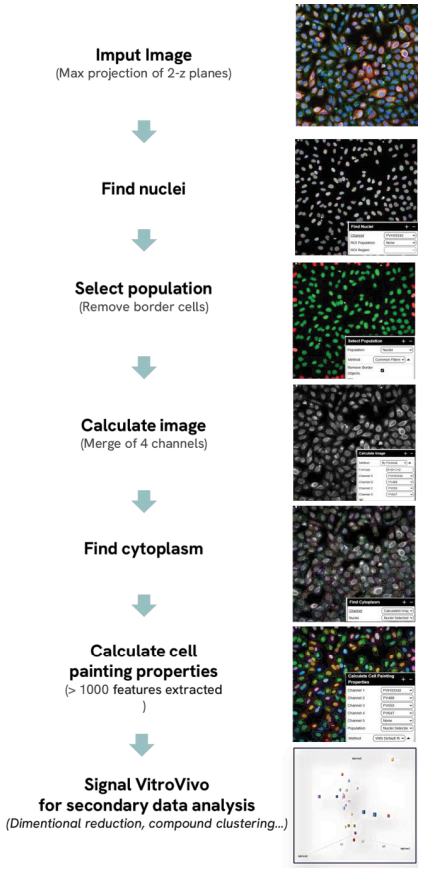
5. Find cytoplasm

Apply the "find cytoplasm building block" on the calculated image (channel) and the nuclei selected (with removed border objects).

6. Calculate Cell Painting Properties

Apply the "calculate cell painting properties" building block. Input all 4 channels and on the nuclei selected population.

This generates multi-parametric data for profiling.



Tips

Merging 4 channels helps maintain robust segmentation, especially with perturbagens affecting specific channels.

- Image acquisition tip: Use max projection from 2 planes to better capture dynamic cell morphology.
- For external tools (e.g., CellProfiler, Python), refer to Cimini et al., Nature Protocols (2023)
- For secondary data analysis such as dimensional reduction and compound clustering, refer to Cimini et al., Nature Protocols (2023).

Assay Validation

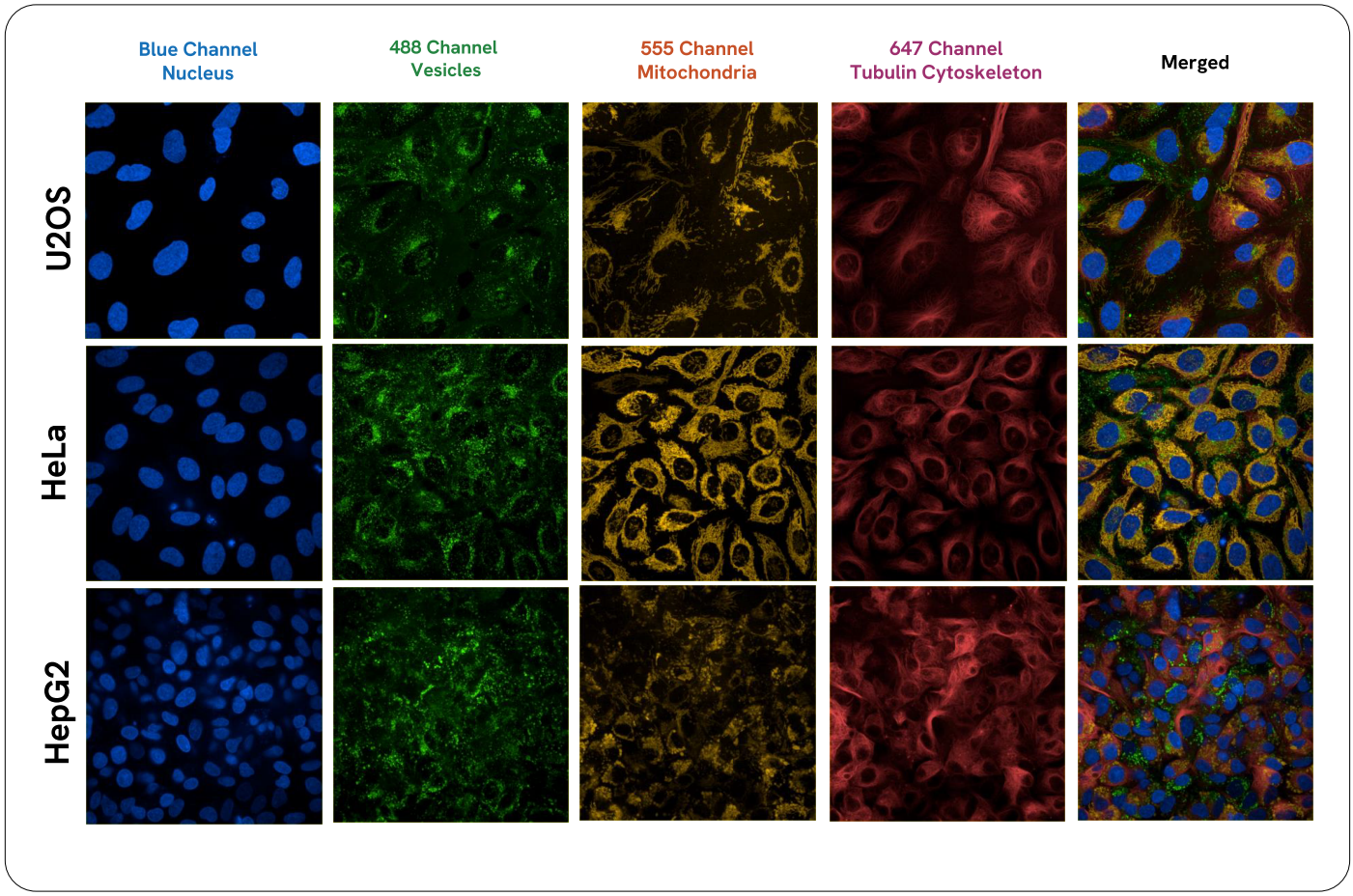


Figure 1: PhenoVue live cell painting staining: U2OS, HeLa, and HepG2 cells were seeded in PhenoPlate 96-well imaging microplates and incubated overnight at 37 °C with 5% CO_2. Cells were then stained with PhenoVue live cell painting stains in their respective complete culture media containing 10% FBS (U2OS: McCoy's 5A; HeLa: MEM; HepG2: DMEM), without any washing steps.

Time-lapse imaging was performed over a 48-hour period using the Opera Phenix Plus high-content screening system, equipped with a 63x water immersion objective in confocal mode. Representative images captured at 4 hours (U2OS) and 24 hours (HeLa and HepG2) are shown.

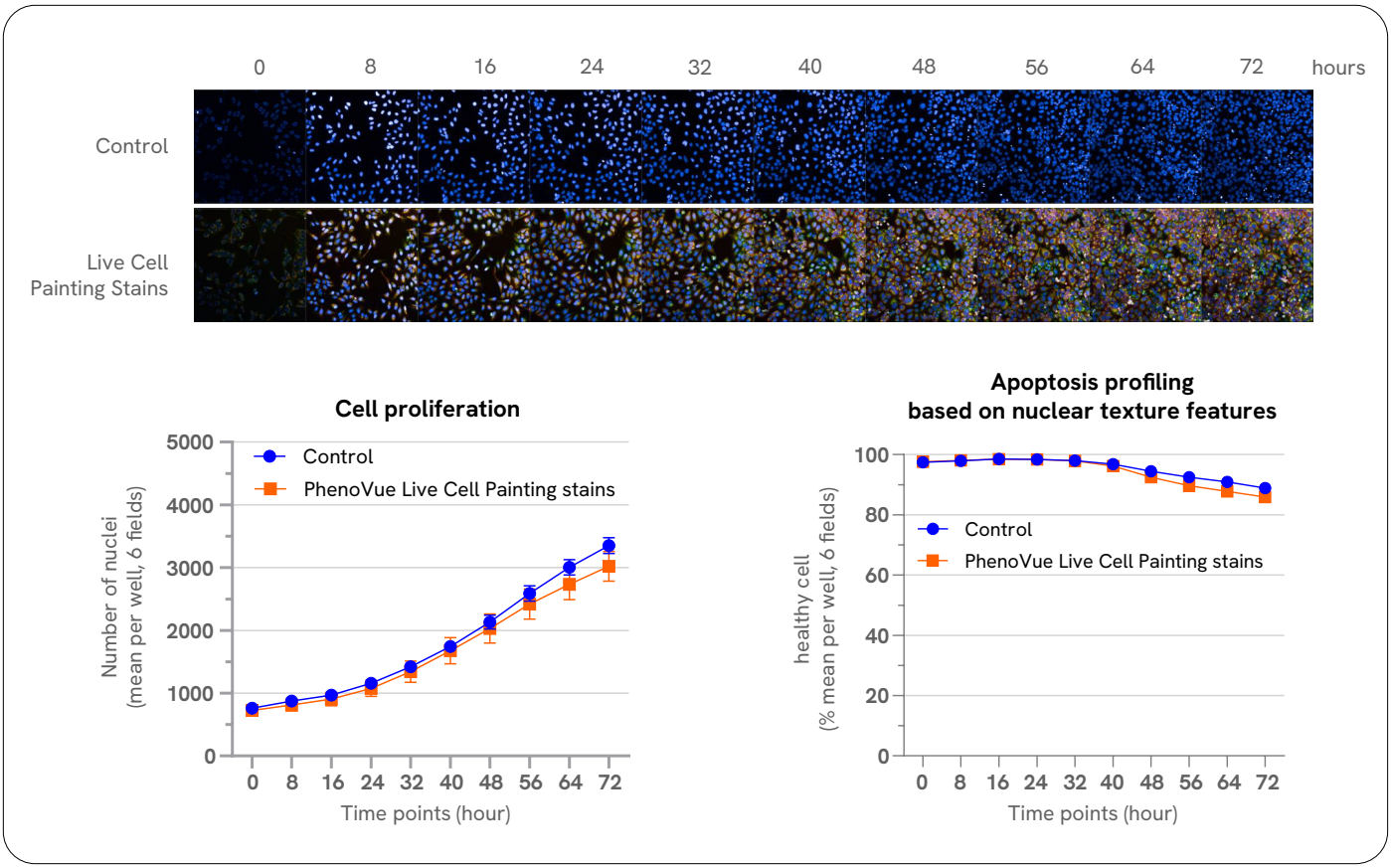


Figure 2: Cell proliferation and health.

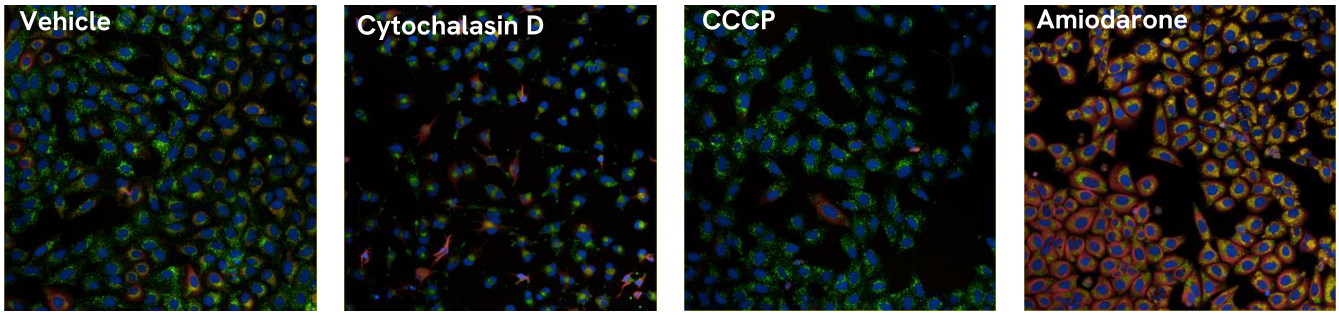
Figure 2 shows U2OS cells seeded in PhenoPlate 96-well microplates at a density of 8,000 cells/well and incubated overnight at 37 °C in a humidified atmosphere with 5% CO_2. Control (blue) corresponds to cells stained with 100 ng/mL PhenoVue Hoechst 33342 nuclear stain. Orange curve corresponds to cells incubated with the staining solution, directly in cell culture medium (no washing steps).

Cells were maintained at 37 °C, 5% CO_2, and imaged in time-lapse mode every 8 hours over a 72-hour period using the Operetta CLS high-content imaging system with a 20x water immersion objective in confocal mode.

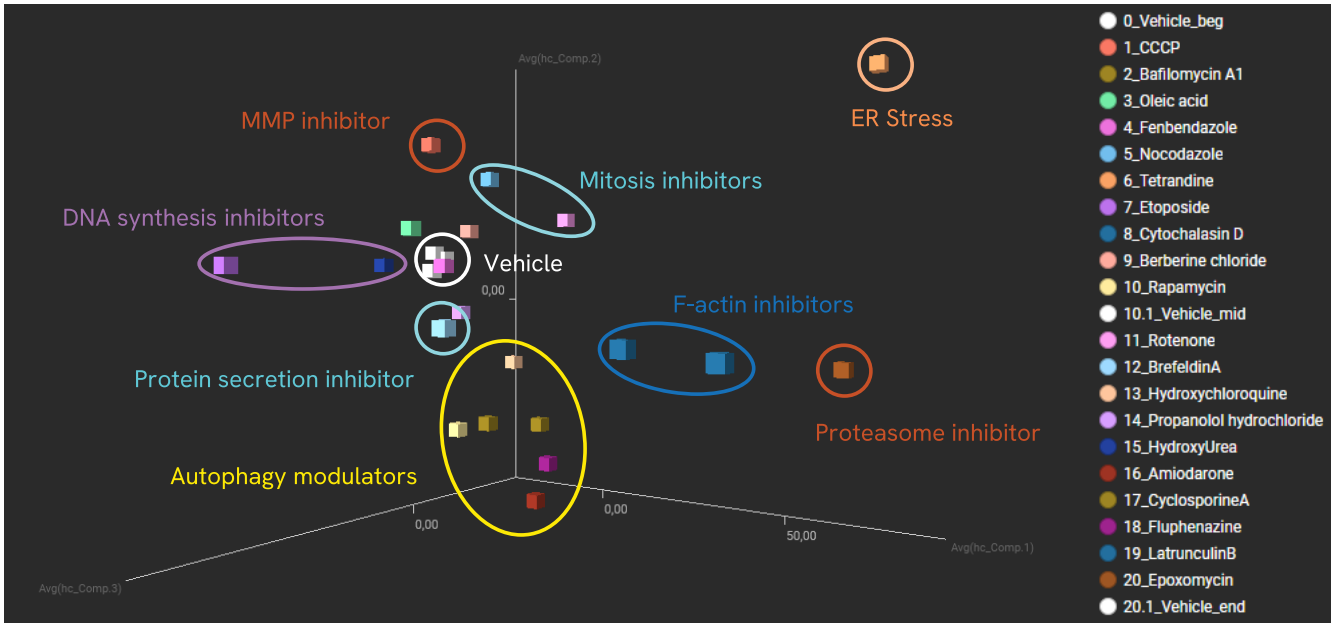
The images and corresponding proliferation curve demonstrate active cell proliferation with no significant difference between control and stained conditions, indicating that the PhenoVue live cell painting stains do not negatively impact cell proliferation.

In parallel, nuclear texture features from the same dataset were used to distinguish healthy cells from apoptotic ones. As shown in the graph, no significant increase in apoptosis was observed in cells treated with the PhenoVue 488/555/647 live cell painting stains compared to the control.

A



B



C

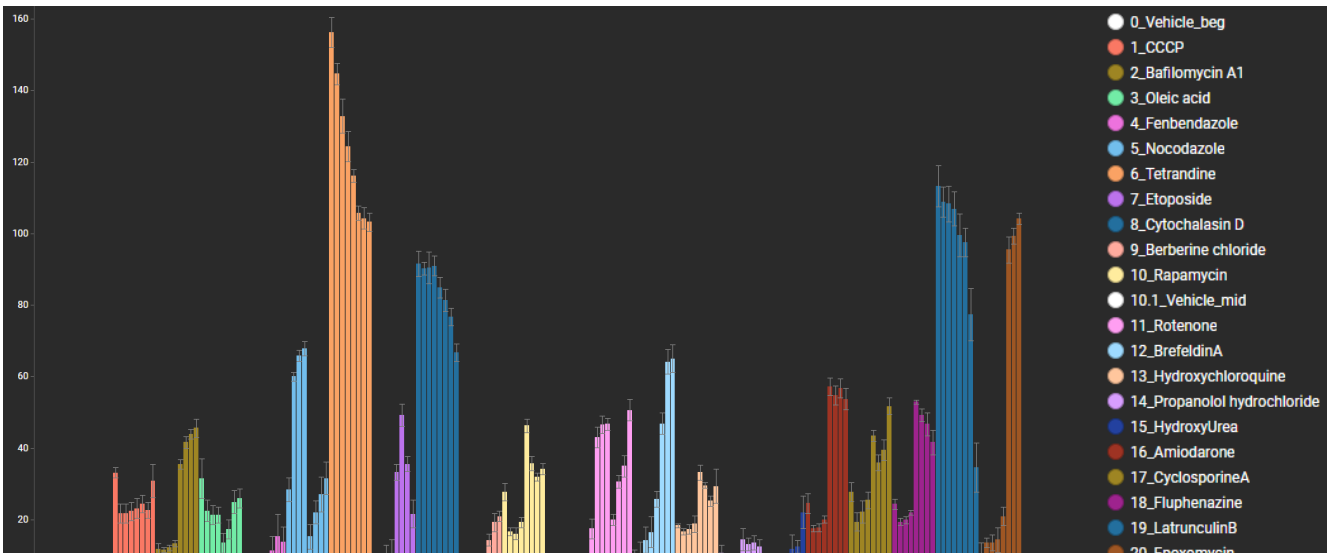


Figure 3: Compound-Induced Phenotypic Profiling

Figure 3 shows U2OS cells seeded in PhenoPlate 96-well microplates at a density of 10,000 cells/well and incubated overnight at 37 °C in a 5% CO_2 humidified incubator. Cells were then stained with the PhenoVue live cell painting stains mix in complete cell culture medium and incubated for 4 hours at 37 °C, 5% CO_2. Following staining, cells were treated with various compounds at 10 µM (except oleic acid at 250 µM) and maintained under standard culture conditions.

Time-lapse imaging was performed using the Opera Phenix Plus high-content imaging system (20x water immersion objective, confocal mode, 9 fields of view per well, 2 planes), at the following time points: 1 h, 2 h, 3 h, 4 h, 14 h, 24 h, 34 h, and 48 h post-treatment. To monitor potential staining drift during acquisition, vehicle controls were strategically distributed across the plate (beginning, middle, and end). These controls displayed high reproducibility, with no significant staining drift observed.

A. Representative images at 24 h are shown for vehicle control (0.1% DMSO), cytochalasin D (F-actin depolymerizing agent), CCCP (mitochondrial membrane potential inhibitor), and amiodarone (drug associated with severe DILI and autophagy modulation).

B. Image analysis was conducted on maximum projections using Harmony™ software. Secondary analysis was performed in SignalVitroVivo™ software via Principal Component Analysis (PCA), enabling dimensionality reduction and phenotypic clustering by compound and time point. A 3D PCA plot at 24 h illustrates distinct clustering of compound-treated cells relative to the vehicle, confirming the emergence of diverse phenotypic signatures.

C. To quantify phenotypic deviation from the control, Euclidean Distance (ED) was calculated for each compound relative to the median vehicle profile at each time point, as described by Caicedo et al. (Nat. Methods, 2017). The resulting kinetic profiles revealed distinct phenotypic profiles:

- Rapid, transient effects: cytochalasin D, latrunculin B, tetrandrine
- Gradual, sustained effects: berberine chloride, hydroxyurea, epoxomicin, bafilomycin A1
- Early, moderate and stable effects: CCCP
- Fluctuating effects over time: nocodazole, rotenone

This dynamic profiling highlights the utility of PhenoVue live cell painting in capturing diverse and time-resolved compound-induced phenotypes.