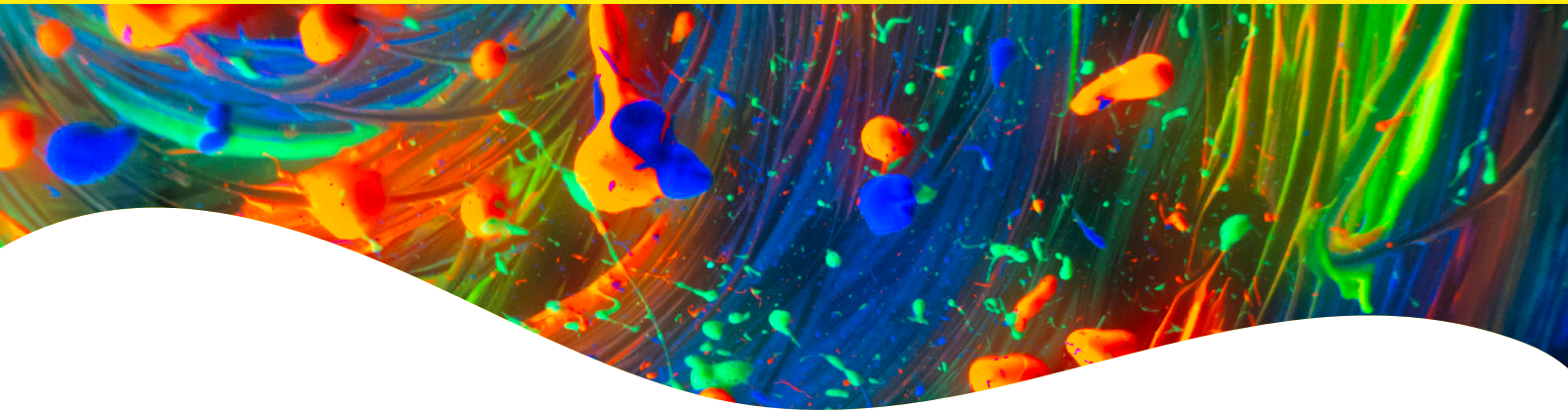
PhenoVue Cell Painting Target Add-on Kits 400LS anti-Mouse / anti-Rabbit - 1 x 384-well



Overview

Cell painting is a powerful phenotypic high-content imaging approach that combines cell biology, multiplexed fluorescent staining, automated microscopy and image analysis to characterize cellular responses to perturbagens such as compounds, drugs, genes or other test articles.

PhenoVue™ cell painting Target Add-on Kits support extending cell painting workflows by enabling the integration of a customer-selected mouse or rabbit primary antibody into a multiparametric phenotypic profiling assay. The selected primary antibody is detected using PhenoVue Fluor 400LS goat anti-mouse or anti-rabbit secondary antibody, providing an additional target-specific imaging layer alongside core cell painting markers.

This workflow allows users to combine broad phenotypic profiling with target-associated information in the same well and the same imaging experiment. The kit is particularly useful when users want to enrich cell painting assays with a protein marker relevant to their biological question, such as a transcription factor, signaling protein, lineage marker, structural marker or disease-associated target.

The kit does not include a primary antibody. Users should select and optimize the appropriate mouse or rabbit primary antibody depending on the target of interest, cell model and assay conditions.

Compared with the PhenoVue cell painting Kits, the PhenoVue cell painting Target Add-on Kits workflow uses an enhanced staining configuration adapted for antibody-based multiplexing. The PhenoVue 512 nucleic acid stain is removed, and dye concentrations are adjusted to support integration of the PhenoVue Fluor 400LS antibody detection channel.

The PhenoVue cell painting Target Add-on Kits enable users to:

- Integrate a customer-selected mouse primary antibody into cell painting workflows
- Add target-specific imaging information to multiparametric phenotypic profiling
- Improve characterization of compound-induced cellular states
- Support phenotypic clustering and mechanism-of-action studies
- Monitor selected protein markers in the context of broader cellular morphology
- Adapt cell painting workflows to specific biological applications and cell models

Product information

Product name	Part no.	Number of vials per unit	Shipping conditions
PhenoVue cell painting Target Add-on Kit anti-mouse 400LS - 1x384well*	PINGM4001	7	Dry ice
PhenoVue cell painting Target Add-on Kit anti-rabbit 400LS - 1x384well*	PINGRB4001	7	Dry ice

*PhenoVue cell painting Target Add-on Kits PINGM4001 and PINGR4001 provide sufficient reagents to stain 2 x 96-well plates or 1 x 384-well plate, following the recommended concentrations and volumes.

Kit contents	Format	Packaging*	Storage
PhenoVue Hoechst 33342 Nuclear Stain	Liquid (H_2O)	1 vial (70 µL - 20,000X)	2-8 °C or below. Protect from light
PhenoVue Fluor 555 - WGA	Lyophilized	1 vial of 0.02 mg	2-8 °C or below. Protect from light.
PhenoVue Fluor 488 ConcanavalinA	Lyophilized	1 vial of 1 mg	2-8 °C or below. Protect from light.
PhenoVue Fluor 568 Phalloidin	Desiccated	1 vial of 0.4 nmol	-16 °C or below. Protect from light.
PhenoVue 641 Mitochondrial Stain	Desiccated	1 vial of 22 µg	-16 °C or below. Protect from light.
PhenoVue Fluor 400LS - Goat anti-Mouse antibody Highly Cross-adsorbed Or PhenoVue Fluor 400LS - Goat anti-Rabbit antibody Highly Cross-adsorbed	Liquid	1 vial of 100 µL (100X)	-16 °C or below. Protect from light.
PhenoVue dye diluent A (5X)	Liquid (HBSS + 5%BSA)	1 vial of 8 mL (5X)	2-8 °C or below. Protect from light.

Other materials and reagents not provided

Reagents or consumables	Usage
Primary antibody	Immunostaining of the protein of interest
HBSS Buffer	Washing buffer and dilution of Triton X-100
Paraformaldehyde (PFA), methanol free	Fixation buffer
Triton X-100	Permeabilization buffer
DMSO	Reconstitution of PhenoVue 641 Mitochondrial Stain and PhenoVue Fluor 568 - Phalloidin
PhenoPlate™ 384-well microplates *	Cell plating, stimulation, staining and imaging
Aluminium single-tab foil	Plate sealing to protect fluorescent probes from light
Distilled H_2O	Reconstitution of Reagents

*This protocol can be adapted using PhenoPlate 96-well microplates. See Protocol Section for details. View our full range of high-quality imaging microplates at Revvity.com

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.
- After thawing, the PhenoVue Dye Diluent A (5x) may contain some aggregates which will not impair the product and image quality. If aggregate removal is preferred, the PhenoVue Dye Diluent A (5x) can be filtered (0.22 µm filter) prior to dilution. The resulting PhenoVue Dye Diluent A 1X must be stored at 2-8 °C for no more than 2 days.
- 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 supported until the expiration date provided in the certificate of analysis, when stored as provided 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

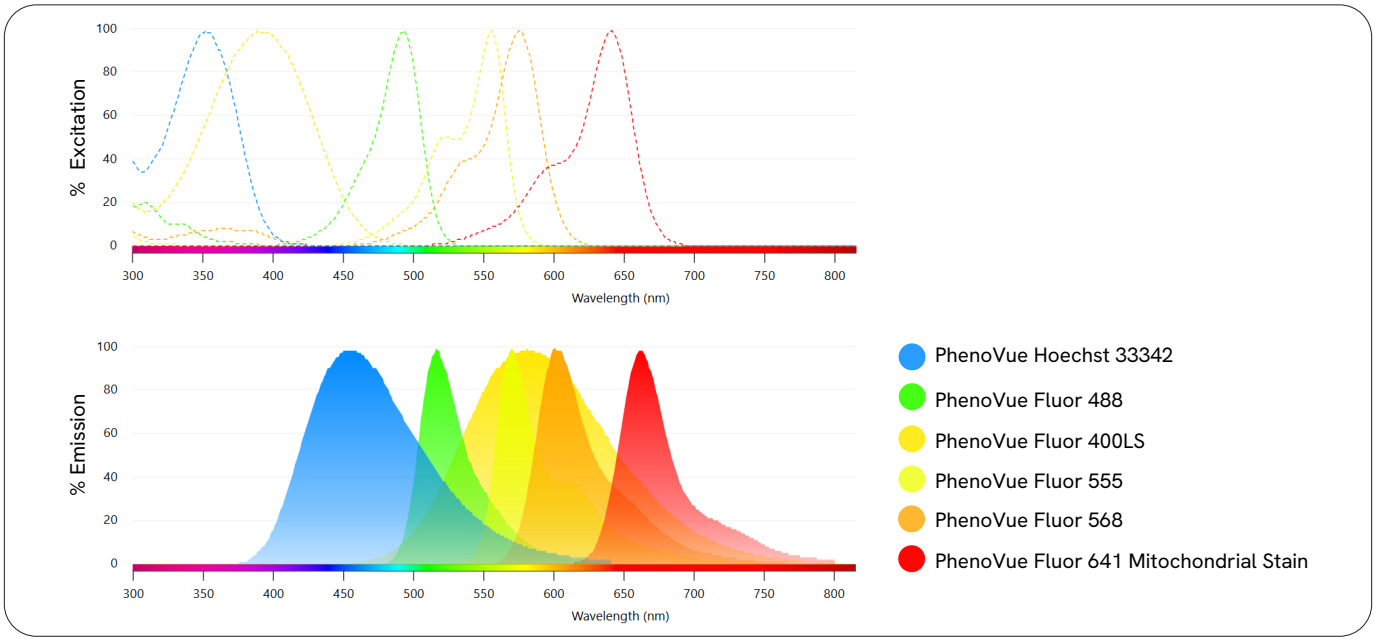


Figure 1: Excitation and emission spectrum associated with the PhenoVue cell painting Target Add-on Kit components.

Principle of the assay

The PhenoVue cell painting Target Add-on workflow combines organelle and morphology-based cell painting stains with an additional antibody-based detection channel.

Live cells are first stained with the mitochondrial stain. Cells are then fixed, permeabilized and incubated with a customer-selected mouse primary antibody targeting the protein of interest. After washing, cells are stained with the cell painting dye mix together with PhenoVue Fluor 400LS goat anti-mouse or anti-rabbit secondary antibody.

This approach enables simultaneous acquisition of core cell painting phenotypic features and a target-associated 400LS signal in the same cells.

Reagent preparation

Prepare the following staining solutions.

- **Staining Solution 1**

  Mitochondrial staining solution containing PhenoVue 641 Mitochondrial Stain.

- **Staining Solution 2**

  Primary antibody staining solution containing the user-selected mouse or rabbit primary antibody diluted in PhenoVue dye diluent A 1X.

  The optimal primary antibody concentration must be determined by the user. A preliminary primary antibody dose-response experiment is suggested.

- **Staining Solution 3**

  Cell painting Target Add-on staining solution containing:

  PhenoVue Fluor 555 WGA

  PhenoVue Fluor 488 Concanavalin A

  PhenoVue Fluor 568 Phalloidin

  PhenoVue Hoechst 33342 Nuclear Stain

  PhenoVue Fluor 400LS Goat anti-Mouse or anti-Rabbit Antibody

  Protect all stock and staining solutions from light.

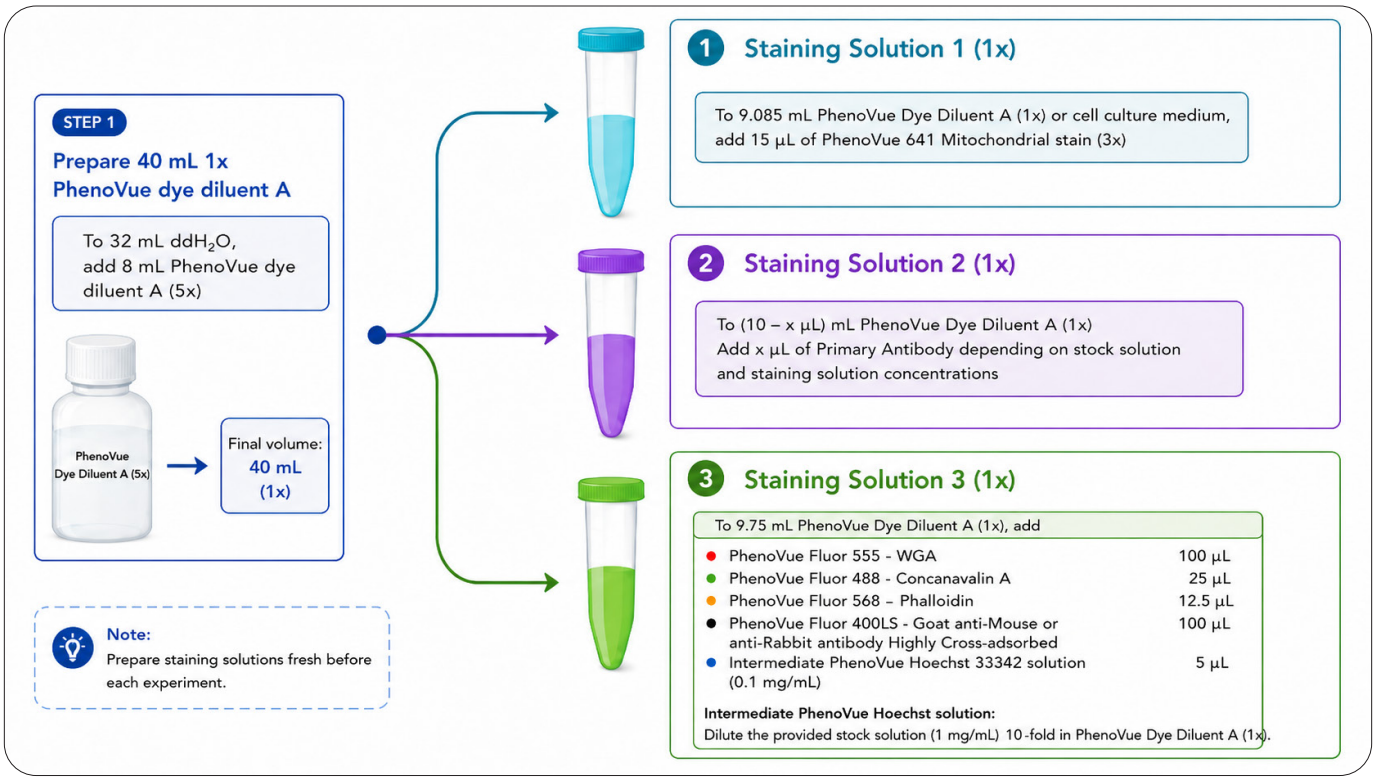
	Reagent name	1. Reconstitution/preparation of stock solution	2. Reconstitution/preparation of staining solutions	Final concentration of reagents per well
	PhenoVue dye diluent A (5x)	Dilute 5 times in distilled H_2O to give a 1x ready to use buffer.	Ready to use for dilution of other reagents.	HBSS + 1% BSA (1x)
Staining Solution 1	PhenoVue 641 Mitochondrial Stain	Reconstitute with 40 µL DMSO to give a 1 mM (2 000x) stock solution.	Dilute stock solution 666 times in PhenoVue dye diluent A (1x) or cell culture medium to give a 1500 nM (3x) staining solution.	500 nM (272 µg/mL)
Staining Solution 2	Primary Antibody* (Mouse or Rabbit species)	/	Dilute to the desired staining concentration (1x) in PhenoVue dye diluent A (1x) *	1x
Staining Solution 3	PhenoVue Fluor 555 - WGA	Reconstitute with 130 µL dH_2O to give a 0.15 mg/mL (100x) stock solution.	Dilute stock solution 100 times in PhenoVue dye diluent A (1x) to give a 1.5 µg/mL staining solution.	43.7 nM (1.5 µg/mL)
	PhenoVue Fluor 488 - Concanavalin A	Reconstitute (each vial) with 500 µL dH_2O to give a 2 mg/mL (400x) stock solution.	Dilute stock solution 400 times in PhenoVue dye diluent A (1x) to give a 5 µg/mL staining solution.	48 nM (5 µg/mL)
	PhenoVue Fluor 568 - Phalloidin	Reconstitute with 60 µL DMSO to give a 6.6 µM (800x) stock solution.	Dilute stock solution 800 times in PhenoVue dye diluent A (1x) to give a 8.25 nM staining solution.	8.25 nM (12 ng/mL)
	PhenoVue Hoechst 33342 nuclear stain	Ready to use stock solution at 1 mg/mL (20,000x).	Dilute stock solution 20,000 times in PhenoVue dye diluent A (1x) to give a 50 ng/mL staining solution.	81 nM (50 ng/mL)
	PhenoVue Fluor 400LS - Goat anti-Mouse or Goat anti-Rabbit antibody Highly Cross-adsorbed	Ready to use stock solution at 100x	Dilute stock solution 100 times in PhenoVue dye diluent A (1x) to give a 1x staining solution.	1x

* The optimal primary antibody concentration depends on the selected antibody, target abundance, and experimental conditions. A preliminary antibody titration experiment is suggested to determine the optimal working concentration.

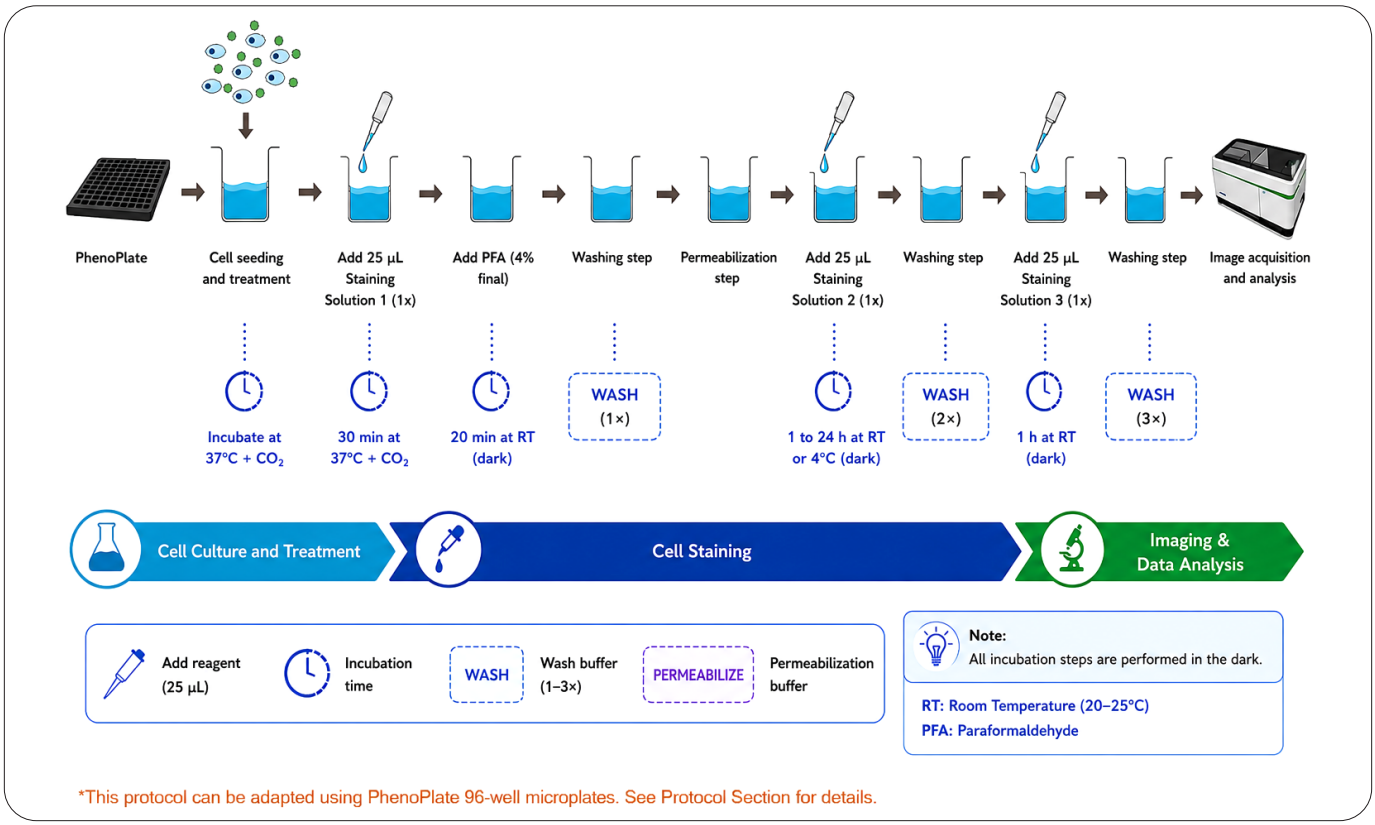
Aliquot stock solutions and store at -20 °C for up to 6 months. Avoid repeated freeze-thaw cycles. Discard the staining solution after use; do not reuse or store for future experiments.

Example preparation of Staining Solutions

The following example describes the preparation of 10 mL Staining Solutions, sufficient for 1 x 384-well plate.



Experimental workflow



Protocol for 384-well imaging plates

This protocol can be adapted to 96-well PhenoPlate microplates by adjusting cell seeding density and reagent volumes.

1. **Dispense** 40 µL of cells per well into PhenoPlate 384-well microplates and incubate overnight at 37 °C, 5% CO_2.
2. **Add compounds** or perturbagens and incubate at 37 °C, 5% CO_2. Typical compound treatment time is 24 to 48 hours, depending on the biological question and cell model.

   Note: Adjust the cell suspension volume according to the compound volume added, to reach a final volume of **40 µL per well** for cells plus compounds.
3. **Add 25 µL of Staining Solution 1.**
4. **Incubate for 30 minutes** at 37 °C, 5% CO_2, protected from light.
5. **Add 25 µL of 16% methanol-free paraformaldehyde** to reach 4% final concentration.
6. **Incubate for 20 minutes** at room temperature, protected from light.
7. **Wash** once with 60 µL of HBSS.
8. **Remove** HBSS.
9. **Add 25 µL of permeabilization solution** containing HBSS with 0.1 to 0.5% Triton X-100.
10. **Incubate for 15 minutes** at room temperature, protected from light.
11. **Remove permeabilization solution.**
12. **Add 25 µL of Staining Solution 2** containing the selected mouse or rabbit primary antibody.
13. **Incubate for 1 to 24 hours** at room temperature or 4 °C, protected from light. The optimal incubation time and temperature depend on the selected antibody and target protein.
14. **Wash** twice with 60 µL of HBSS.
15. **Remove HBSS.**
16. **Add 25 µL of Staining Solution 3.**
17. **Incubate for 60 minutes** at room temperature, protected from light.
18. **Wash** three times with 60 µL of HBSS.
19. **Do not remove the final 60 µL of HBSS.**

Note: HBSS can be supplemented with 0.05% sodium azide if image acquisition is not performed immediately.

20. **Seal the plate** and store at 4 °C protected from light until image acquisition.
21. **Image acquisition:** Refer to the following section for recommended microscope settings
22. **Image Analysis:** When using Revvity's high-content imaging instruments, refer to the dedicated section below.

Tips and guidance

- **Optimal primary antibody concentration** depends on the selected antibody, target abundance, localization, cell type and fixation/permeabilization conditions. A preliminary dose-response experiment is suggested to select a concentration that provides specific signal while minimizing background and antibody consumption.
- **Percentage of Triton X-100** may be adjusted between 0.1% and 0.5% depending on your primary antibody and protein target of interest. Nuclear targets may require stronger permeabilization, while cytoplasmic or membrane-associated targets may require milder conditions.
- **Hoechst concentration** is enhanced to support nuclei segmentation while minimizing crosstalk into the PhenoVue Fluor 400LS channel.

  Note: This concentration has been improved for most applications but may be slightly adjusted if needed, depending on the cell model and imaging instrument used.
- **PhenoVue 512 nucleic acid stain** is not included in this workflow to support compatibility with the additional 400LS antibody detection channel.

Guidance on acquisition settings

The PhenoVue cell painting Target Add-on Kit enables multiplexed imaging across five fluorescent channels:

- Hoechst nuclear stain
- PhenoVue Fluor 400LS secondary antibody channel
- PhenoVue Fluor 488 Concanavalin A
- PhenoVue Fluor 555 WGA / PhenoVue Fluor 568 Phalloidin
- PhenoVue 641 Mitochondrial Stain

Images can be acquired using Revvity high-content imaging systems, including Opera Phenix™ Plus and Operetta CLS systems, provided that the appropriate excitation and emission channels are available.

Sequential acquisition of the Hoechst and PhenoVue Fluor 555/568 channels is suggested to minimize crosstalk from the PhenoVue Fluor 400LS signal into the 555/568 channel.

IMPORTANT NOTE: Use of this kit on Operetta CLS systems without the 405 nm LED is not recommended.

HCS instruments		PhenoVue Hoechst 33342	PhenoVue 400LS	PhenoVue 488	PhenoVue 555/568	PhenoVue 641
Opera Phenix Plus 5 lasers	Excitation laser (nm)	375	425	488	561	640
	Emission filters (nm)	435-480	570-630	500-550	570-630	650-760
Opera Phenix Plus 4 lasers	Excitation laser (nm)	405	405	488	561	640
	Emission filters (nm)	435-480	570-630	500-550	570-630	650-760
Operetta CLS 8 LED (1600)	Excitation LED (filters) (nm)	370 (355-385)	405 (390-420)	475 (460-490)	550 (530-560)	630 (615-645)
	Emission filters (nm)	430-500	570-650	500-550	570-650	655-760

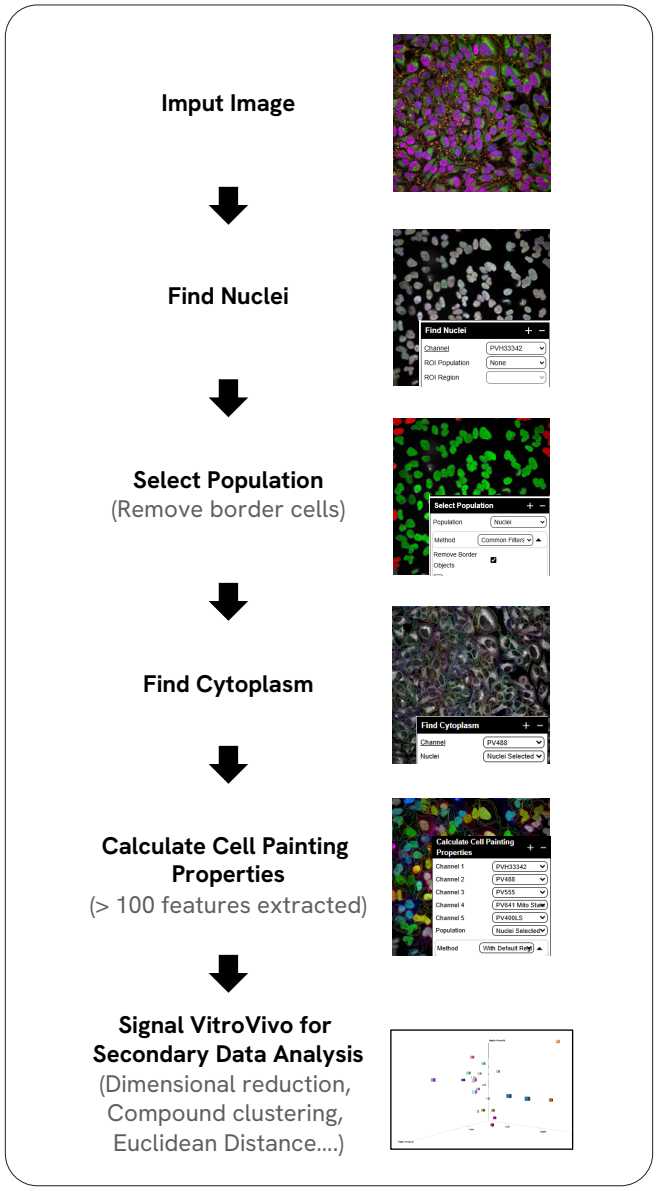
Guidance on image analysis using Revvity's Harmony or Signals Image Artist (SImA) software

For Revvity high-content imaging systems, image analysis can be performed using Harmony or Signals Image Artist software.

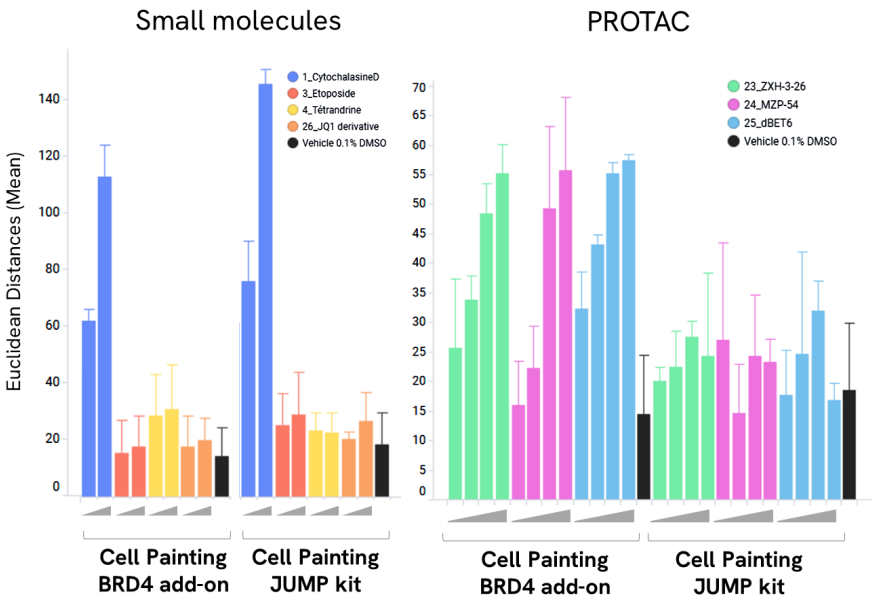
Suggested analysis sequence:

1. Apply flatfield correction.
2. Segment nuclei using the Hoechst channel.
3. Remove border nuclei using population selection filters.
4. Segment cytoplasm using the PhenoVue Fluor 488 channel and the selected nuclei population.
5. Calculate cell painting properties using all acquired channels.
6. Extract target-associated intensity and texture features from the PhenoVue Fluor 400LS channel when relevant.
7. Perform secondary data analysis, including dimensionality reduction, clustering or Euclidean distance analysis, depending on the experimental objective.

   For external analysis workflows, CellProfiler or Python-based software approaches may also be used.



Assay validation



Ordinary one-way ANOVA Dunnett's multiple comparisons test	PhenoVue Cell Painting BRD4 add-on Kit	PhenoVue Cell Painting JUMP Kit
CytochalasineD (0.6µM) vs Vehicle	****	****
CytochalasineD (2µM) vs Vehicle	****	****
Etoposide (1.1µM) vs Vehicle	ns	ns
Etoposide (3.3µM) vs Vehicle	ns	ns
Tetrandrine (74nM) vs Vehicle	ns	ns
Tetrandrine (666nM) vs Vehicle	ns	ns
JQ1 (111nM) vs Vehicle	ns	ns
JQ1 (333nM) vs Vehicle	ns	ns
ZXH-3-26 (37nM) vs Vehicle	ns	ns
ZXH-3-26 (111nM) vs Vehicle	ns	ns
ZXH-3-26 (333nM) vs Vehicle	***	ns
ZXH-3-26 (1000nM) vs Vehicle	****	ns
MZP-54 (111nM) vs Vehicle	ns	ns
MZP-54 (333nM) vs Vehicle	ns	ns
MZP-54 (1000nM) vs Vehicle	***	ns
MZP-54 (3000nM) vs Vehicle	****	ns
dBET6 (37nM) vs Vehicle	ns	ns
dBET6 (111nM) vs Vehicle	**	ns
dBET6 (333nM) vs Vehicle	****	ns
dBET6 (1000nM) vs Vehicle	****	ns

Figure 2: Integration of a target-specific antibody layer into cell painting workflows enhances phenotypic discrimination.

The cell painting JUMP assay was compared with an enhanced target-specific imaging workflow using the PhenoVue cell painting BRD4 Add-on Kit. HeLa cells were seeded at 20,000 cells per well in PhenoPlate 384-well microplates and incubated overnight at 37 °C with 5% CO_2 prior to compound treatment. Cells were then treated with the indicated compounds for 2 hours before fixation and staining.

In this example, cells were stained using the PhenoVue cell painting Add-on reagents together with an anti-BRD4 primary antibody followed by detection with a PhenoVue Fluor 400LS secondary antibody, enabling incorporation of an additional BRD4-associated imaging layer into the cell painting workflow. Compared to the original cell painting JUMP assay, the PhenoVue cell painting Add-on workflow uses an enhanced dye composition and staining conditions compatible with antibody-based multiplexing. Importantly, the add-on kit does not include a primary antibody and supports flexible integration of customer-selected mouse or rabbit primary antibodies into cell painting assays.

Images were acquired using the Opera Phenix™ High-Content Screening System with a 20X water immersion objective under standardized acquisition settings across all experimental conditions. Multiparametric image analysis and Euclidean distance calculations were subsequently performed to compare phenotypic separation between conventional cell painting and the BRD4 add-on workflow.

Enhanced phenotypic separation was observed for several compounds following incorporation of the BRD4-associated imaging layer, including cytochalasin D and multiple BET degraders such as ZXH-3-26, MZP-54, and dBET6. In contrast, etoposide, tetrandrine, and JQ1 showed limited or non-significant enhancement under the tested conditions. These results demonstrate that incorporation of a target-specific antibody layer into cell painting workflows can enrich phenotypic information content and improve discrimination of compound-induced cellular states.

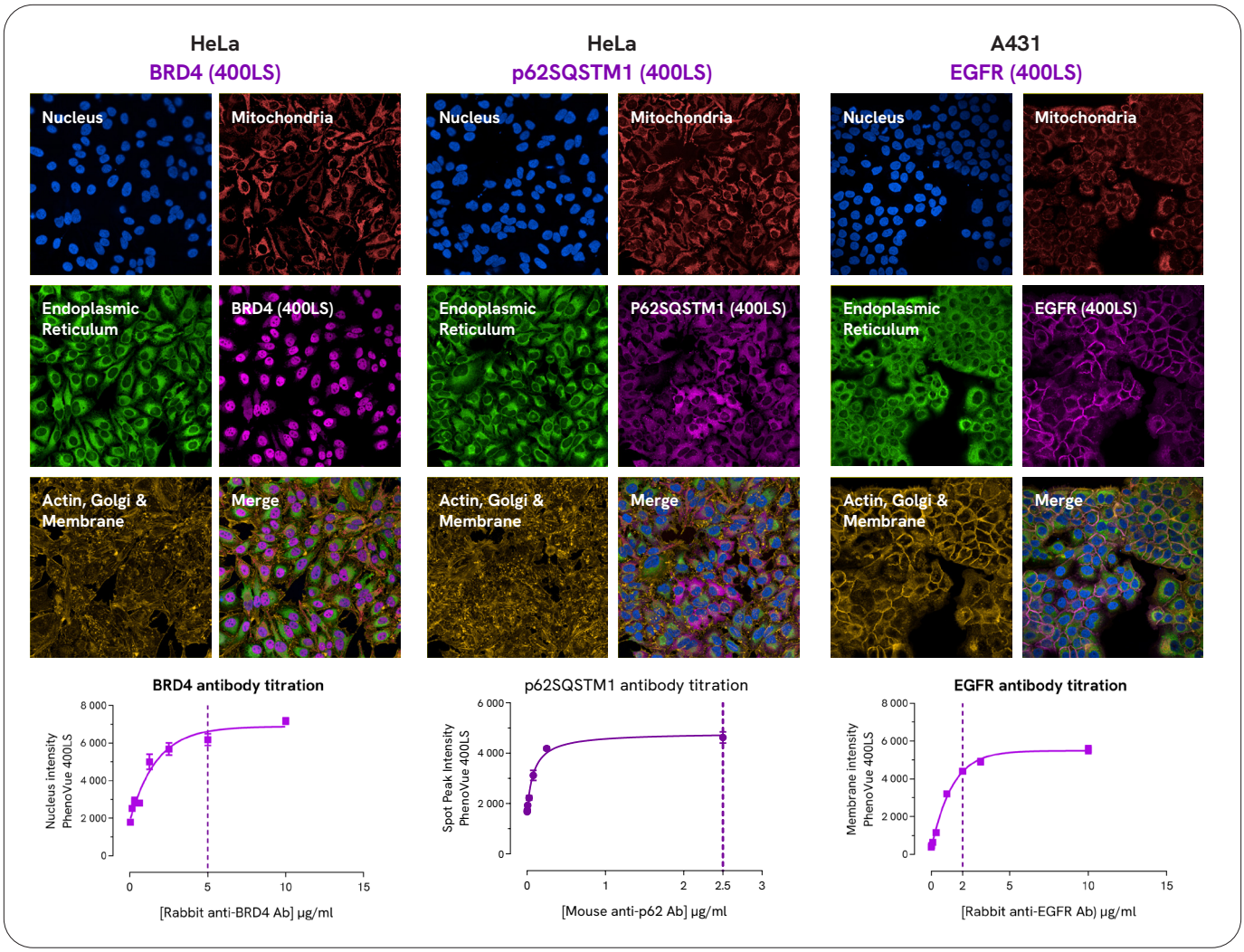


Figure 3: Compatibility of the PhenoVue cell painting Target Add-on workflow with nuclear, cytoplasmic, and membrane-associated targets

Primary antibody dose-response experiments were first performed to determine the optimal primary antibody concentration for target-specific multiplexed cell painting workflows. HeLa or A431 cells were seeded in PhenoPlate microplates and incubated overnight at 37 °C with 5% CO_2 prior to fixation, permeabilization, and staining using the PhenoVue cell painting Target Add-on workflow.

To demonstrate compatibility with different target localizations, cells were stained using anti-BRD4, anti-p62/SQSTM1, or anti-EGFR primary antibodies followed by detection with PhenoVue Fluor 400LS secondary antibodies. BRD4 was used as an example of a nuclear target, p62/SQSTM1 as an example of a cytoplasmic and autophagy-associated target, and EGFR as an example of a membrane-associated target.

Representative images demonstrate successful integration of the target-specific antibody layer together with conventional cell painting channels, including nuclei, mitochondria, endoplasmic reticulum, actin, Golgi and plasma membrane staining. The multiplexed workflow preserved overall cellular morphology, staining quality and signal separation across all tested conditions.

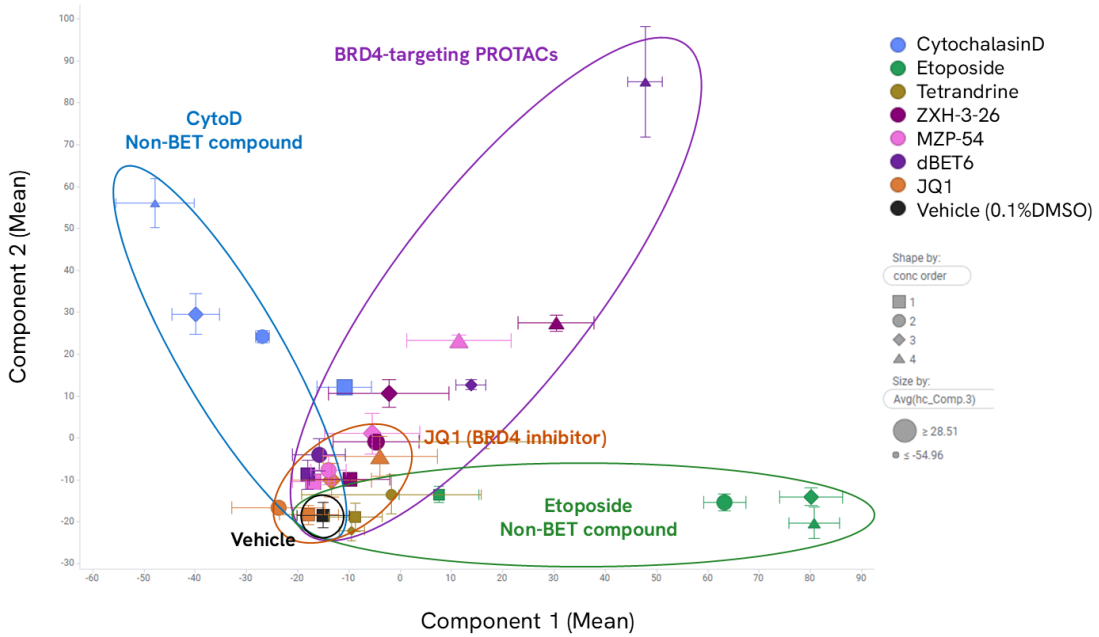
Primary antibody titration curves demonstrated concentration-dependent increases in PhenoVue Fluor 400LS signal followed by signal plateau stabilization, enabling identification of optimal working concentrations while minimizing background signal and antibody consumption. Dashed lines indicate the selected primary antibody concentrations used for subsequent experiments.

Images were acquired using the Opera Phenix high-content screening system with a 40X water immersion objective under standardized acquisition settings.

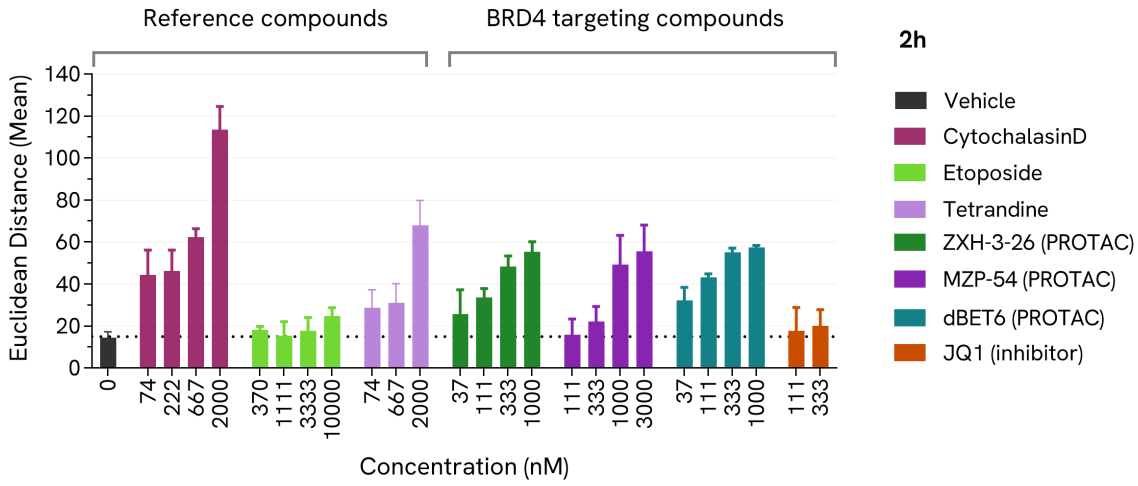
These results demonstrate that the PhenoVue cell painting Target Add-on workflow is compatible with multiple target classes and subcellular localizations while maintaining robust multiplexed cell painting image quality and target-specific signal detection.

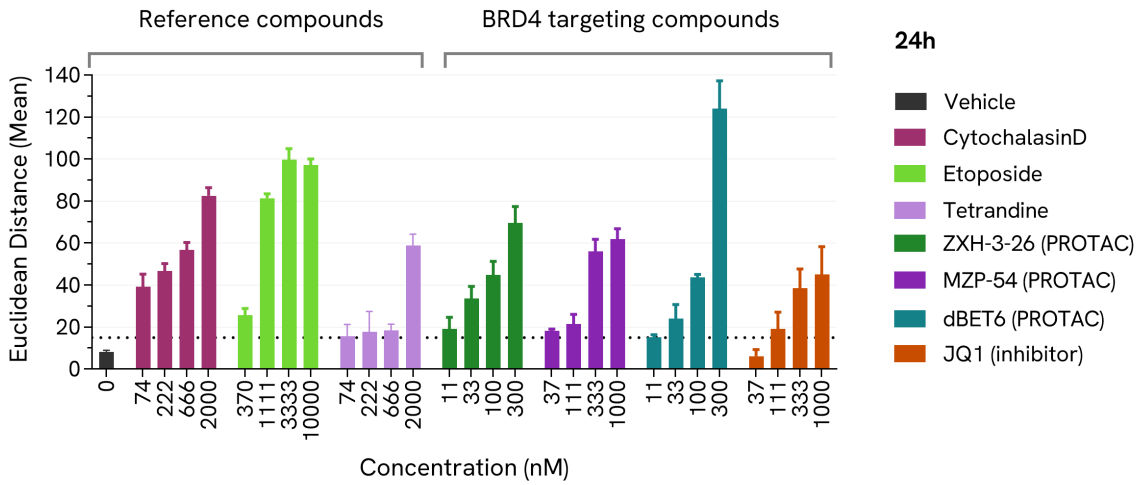
A

Principal Component Analysis reveals distinct clustering of BRD4-targeting degraders and reference compounds



B





	Ordinary one-way ANOVA Dunnett's multiple comparisons test	2h	24h
Reference compounds	CytochalasineD (0.074µM) vs Vehicle	****	****
	CytochalasineD (0,222µM) vs Vehicle	****	****
	CytochalasineD (0.6µM) vs Vehicle	****	****
	CytochalasineD (2µM) vs Vehicle	****	****
	Etoposide (0.37µM) vs Vehicle	ns	*
	Etoposide (1.1µM) vs Vehicle	ns	****
	Etoposide (3.3µM) vs Vehicle	ns	****
	Etoposide (10µM) vs Vehicle	ns	****
	Tetrandrine (74nM) vs Vehicle	ns	ns
	Tetrandrine (666nM) vs Vehicle	ns	ns
	Tetrandrine (2000nM) vs Vehicle	****	****
BRD4 targeting compounds (Inhibitor & degraders)	JQ1 (37nM) vs Vehicle	ns	ns
	JQ1 (111nM) vs Vehicle	ns	ns
	JQ1 (333nM) vs Vehicle	ns	***
	JQ1 (1000nM) vs Vehicle	ns	****
	ZXH-3-26 (37nM) vs Vehicle	ns	ns
	ZXH-3-26 (111nM) vs Vehicle	ns	*
	ZXH-3-26 (333nM) vs Vehicle	****	****
	ZXH-3-26 (1000nM) vs Vehicle	****	****
	MZP-54 (111nM) vs Vehicle	ns	ns
	MZP-54 (333nM) vs Vehicle	ns	ns
	MZP-54 (1000nM) vs Vehicle	****	****
	MZP-54 (3000nM) vs Vehicle	****	****
	dBET6 (37nM) vs Vehicle	ns	ns
	dBET6 (111nM) vs Vehicle	*	**
	dBET6 (333nM) vs Vehicle	****	****
	dBET6 (1000nM) vs Vehicle	****	****

Figure 4: Multiparametric phenotypic profiling reveals distinct clustering and phenotypic separation of BRD4-targeting degraders and reference compounds

HeLa cells were seeded at 20,000 cells per well in PhenoPlate 384-well microplates and incubated overnight at 37 °C with 5% CO_2 prior to compound treatment. Cells were then treated for either 2 hours or 24 hours with reference compounds, including cytochalasin D, etoposide and tetrandrine, or BRD4-targeting compounds including the BET inhibitor JQ1 and the BRD4 degraders ZXH-3-26, MZP-54 and dBET6. Following treatment, cells were stained using the PhenoVue cell painting Target Add-on workflow with an anti-BRD4 primary antibody and a PhenoVue Fluor 400LS secondary antibody. Images were acquired using the Opera Phenix High-Content Screening System under standardized acquisition conditions, followed by multiparametric image analysis.

(A) Principal Component Analysis (PCA) performed at 24 hours revealed distinct clustering patterns between vehicle-treated cells, reference compounds and BRD4-targeting degraders. BRD4-targeting PROTACs generated concentration-dependent phenotypic trajectories that clearly separated from vehicle-treated cells and clustered independently from non-BET reference compounds. In contrast, the BRD4 inhibitor JQ1 induced a more limited phenotypic shift and remained closer to the vehicle cluster under the tested conditions. Cytochalasin D and etoposide produced distinct phenotypic signatures consistent with their unrelated mechanisms of action, demonstrating the ability of the workflow to discriminate diverse cellular perturbations within multidimensional feature space.

(B) Euclidean distance analysis relative to vehicle-treated controls demonstrated concentration-dependent phenotypic separation induced by multiple compounds at both 2 hours and 24 hours. Cytochalasin D produced strong phenotypic separation at both timepoints, while etoposide induced a more pronounced response at 24 hours compared with 2 hours. Among BRD4-targeting compounds, degraders including ZXH-3-26, MZP-54 and dBET6 generated robust concentration-dependent phenotypic divergence, particularly after 24 hours of treatment. In contrast, JQ1 induced comparatively weaker phenotypic separation, despite targeting the same BRD4 pathway. Statistical analysis was performed using ordinary one-way ANOVA followed by Dunnett's multiple comparisons test relative to vehicle-treated controls.

Together, these results demonstrate that integration of a target-specific BRD4 imaging layer into the PhenoVue cell painting workflow enables robust discrimination of compound-induced cellular states and supports multidimensional phenotypic characterization of targeted protein degraders and mechanistically distinct reference compounds.

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