APPLICATION NOTE

Enhancing cell painting with a target-specific imaging layer for phenotypic profiling.

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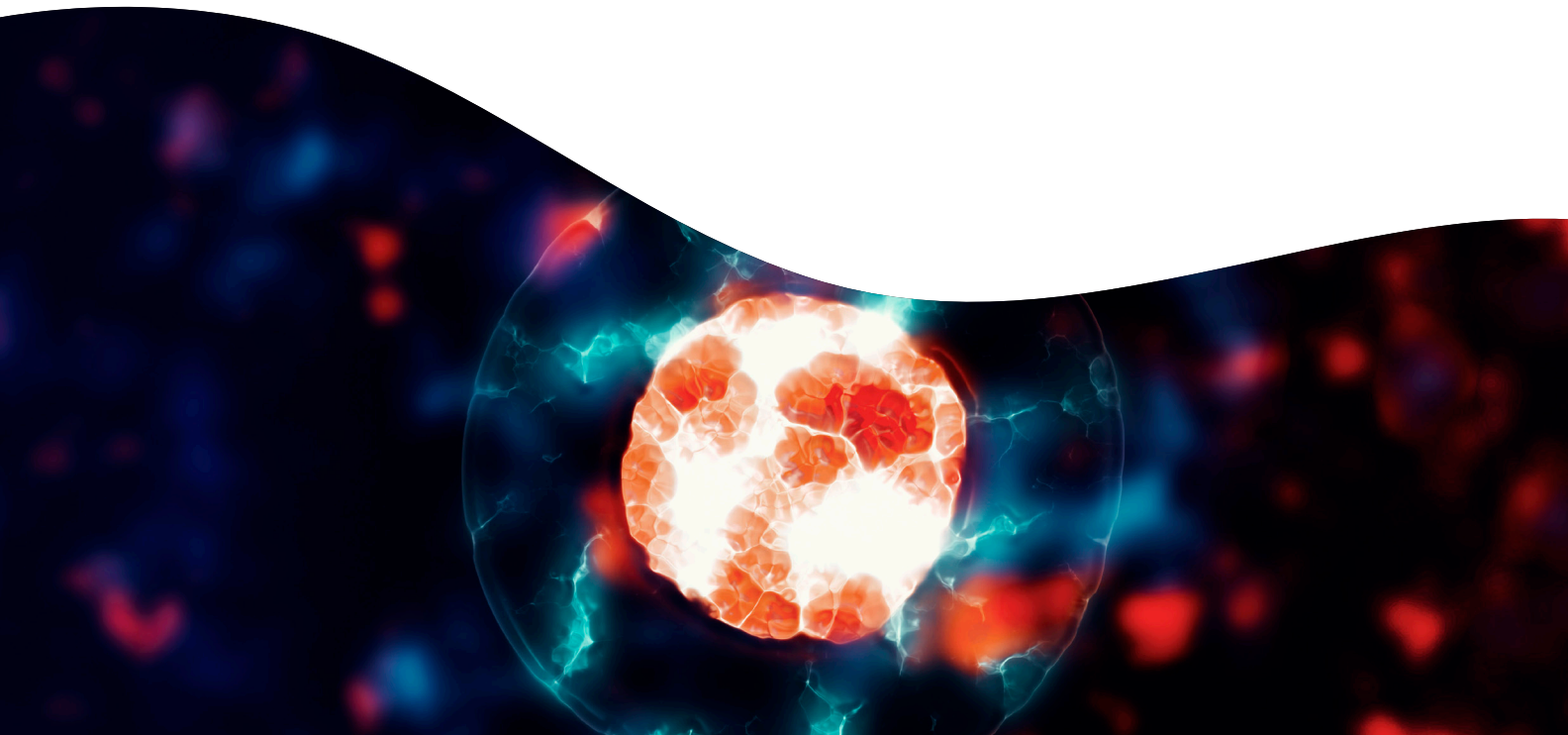
Introduction

Cell painting is a high-content imaging approach that uses multiplexed fluorescent staining and automated image analysis to describe cellular states through thousands of morphological, intensity, texture, and spatial features. Since its introduction, the assay has become a widely used strategy for phenotypic profiling, mechanism-of-action studies, compound clustering, and image-based drug discovery. Large-scale initiatives such as the JUMP cell painting project further highlight the value of morphology-based profiling as a scalable framework for connecting chemical and genetic perturbations to cellular phenotypes.

However, conventional cell painting is intentionally target-agnostic. This is one of its major strengths, because it allows unbiased detection of cellular responses across organelles and compartments. It can also be a limitation in biological contexts where direct information on a specific protein of interest is needed to interpret the phenotype. This is especially relevant for targeted protein degradation, where a compound may rapidly reduce the abundance of the target protein before the cell has developed a measurable morphological response.

In this case, a purely phenotypic assay can underestimate early target engagement or fail to distinguish compounds that act through the intended mechanism from compounds that only induce downstream or unrelated morphology changes.



The PhenoVue™ Cell Painting Target Add-on workflow was developed to help bridge this gap. It enables users to incorporate a customer-selected mouse or rabbit primary antibody into a cell painting experiment and detect the target with PhenoVue Fluor 400LS goat anti-mouse or anti-rabbit secondary antibody. The result is a single-well, same-cell imaging workflow that combines conventional cell painting morphology with a target-associated fluorescent signal.

This application note describes the principle and characterization of the PhenoVue Cell Painting Target Add-on approach, with a focus on the value of the 400LS channel for five-plex imaging and on a BRD4 degrader case study. The data help illustrate how a target-specific layer can support mechanism-aware phenotypic profiling, particularly at early timepoints when target modulation is detectable but broad morphological divergence remains limited.

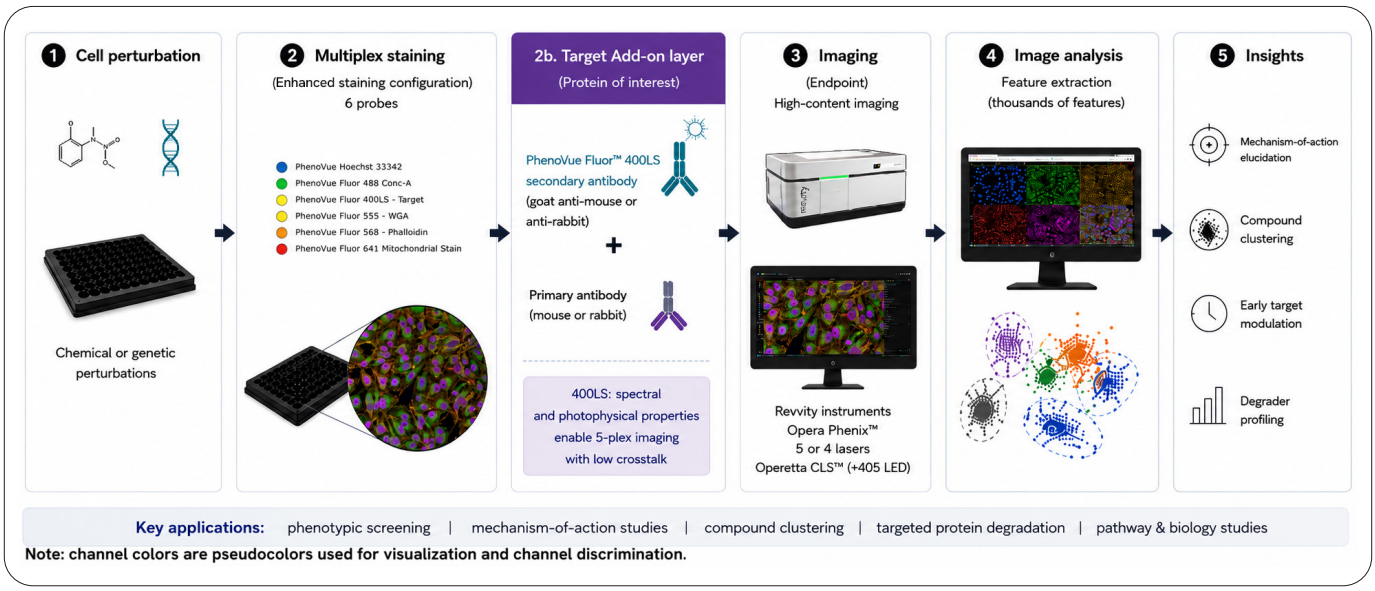


Figure 1: Principle of the PhenoVue Cell Painting Target Add-on workflow. Conventional cell painting provides a multiparametric phenotypic profile across major cellular compartments. The Target Add-on workflow adds a user-selected protein-of-interest readout detected with PhenoVue Fluor 400LS secondary antibody, enabling simultaneous assessment of morphology and target modulation in the same cells.

A PhenoVue Fluor 400LS-enabled strategy for 5-plex cell painting

The central technical requirement for this workflow is to add a target-specific channel without compromising the core information content of cell painting. The PhenoVue Cell Painting Target Add-on configuration was therefore improved around PhenoVue Fluor 400LS secondary antibodies, whose spectral properties support integration of an additional antibody-based readout into a multiplexed cell painting assay.

In the improved workflow, the standard cell painting staining configuration is adapted to accommodate the 400LS channel. The PhenoVue 512 nucleic acid stain is removed to reduce spectral interference, and the Hoechst 33342 concentration is decreased to maintain nuclear segmentation while minimizing crosstalk into the 400LS detection channel. The resulting five-channel configuration enables detection of nuclei, ER, actin/Golgi/plasma membrane, mitochondria, and the protein of interest in the same imaging experiment with minimal crosstalk.

The workflow was evaluated on Revvity high-content imaging systems. Compatibility was verified with Opera Phenix™ Plus with five lasers and Operetta CLS™ systems equipped with a 405 nm LED. Compatibility with Opera Phenix Plus with four lasers was predicted from the available acquisition configuration. Using Operetta CLS systems lacking a 405 nm LED is not recommended because reduced excitation suitability and increased crosstalk may impair discrimination of the 400LS signal.

Importantly, the workflow is not designed to replace conventional cell painting. Instead, it preserves the overall phenotypic profiling strategy while allowing users to enrich the assay with a biologically relevant protein marker. This can be particularly useful when the biological question requires both broad cell-state information and direct monitoring of a selected target.

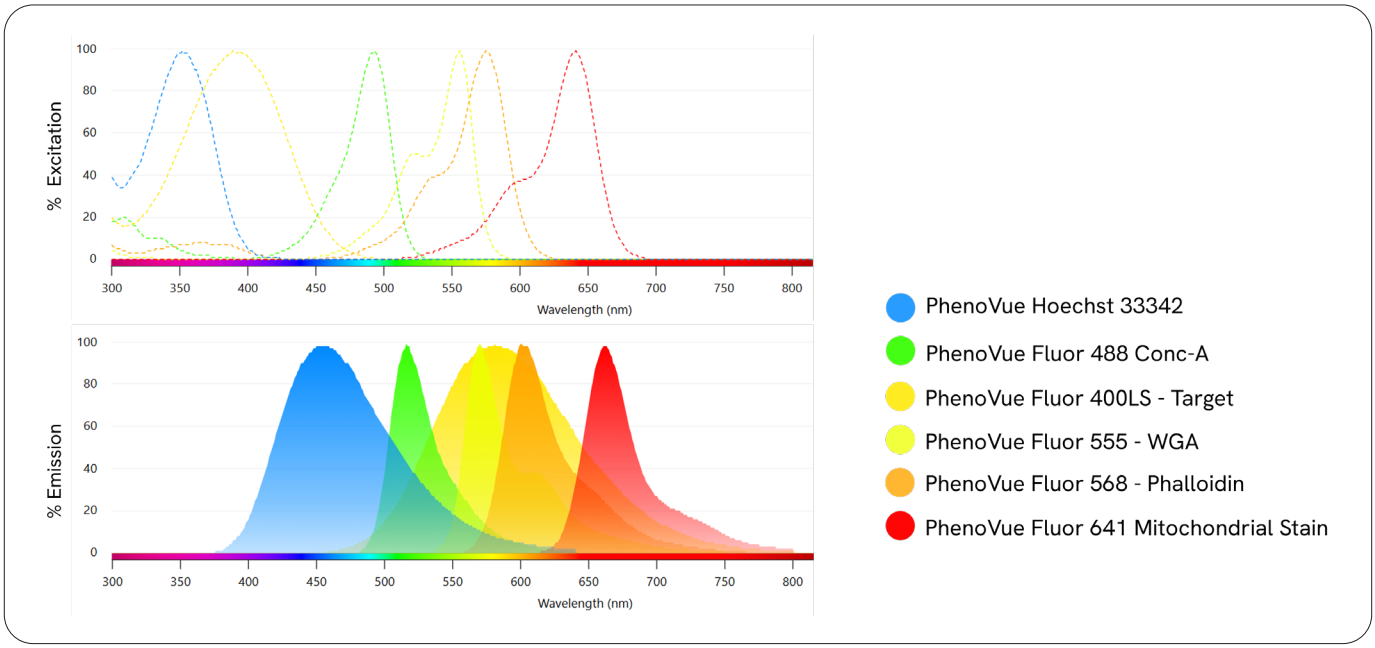


Figure 2: Five-channel imaging configuration enabled by PhenoVue Fluor 400LS. The improved workflow combines PhenoVue Hoechst 33342 nuclear staining, PhenoVue Fluor 488 Concanavalin A for ER-associated features, PhenoVue Fluor 555 WGA for Golgi and plasma membrane-associated features, PhenoVue Fluor 568 Phalloidin for F-actin/cytoskeleton staining, PhenoVue 641 Mitochondrial Stain, and PhenoVue Fluor 400LS antibody-based detection of the protein of interest. The PhenoVue 512 nucleic acid stain is omitted to support spectral separation of the 400LS target channel.

For visualization purposes, the 400LS target channel may be displayed using a violet pseudocolor to distinguish it from yellow/orange Cell Painting signals; this display color does not reflect the spectral emission maximum of the dye.

Evaluation strategy across target classes and cellular compartments

To challenge the workflow across different biological contexts, three targets were selected based on their subcellular localization, relative abundance, and relevance to drug discovery. EGFR was used as a membrane-associated receptor model in A431 cells. p62/SQSTM1 was selected as a cytoplasmic autophagy-related protein in HeLa cells. BRD4 was selected as a low-abundance nuclear target in HeLa cells and as a relevant model for targeted protein degradation studies.

This target panel was intentionally diverse. EGFR represents a high-expression membrane protein and a comparatively favorable detection context. p62/SQSTM1 provides a cytoplasmic marker associated with autophagy biology. BRD4 provides a more stringent evaluation case because it is nuclear, relatively low in abundance, and mechanistically relevant to BET inhibitor and PROTAC pharmacology.

Detection of BRD4 in a multiplexed cell painting context therefore demonstrates the sensitivity and spectral performance of the approach (figure 3).

Primary antibody titration was included as a critical step (figure 4). Because the primary antibody is selected by the user and is not provided in the kit, its optimal concentration depends on target abundance, antibody affinity, cell model, fixation and permeabilization conditions, and expected subcellular localization. In the experiments, concentration-dependent 400LS signals were observed, supporting selection of working antibody concentrations that balance signal strength, specificity, and antibody consumption.

Target	Cell model	Localization	Evaluation rationale
EGFR	A431	Membrane-associated receptor	High-expression oncology and PROTAC-relevant target; favorable detection model.
p62/SQSTM1	HeLa	Cytoplasmic /autophagy-related	Intermediate challenge; relevant to autophagy, oncology, inflammation, and neurobiology.
BRD4	HeLa	Nuclear/nucleoplasmic	Low-abundance target; stringent model for target-specific detection and TPD-relevant pharmacology.

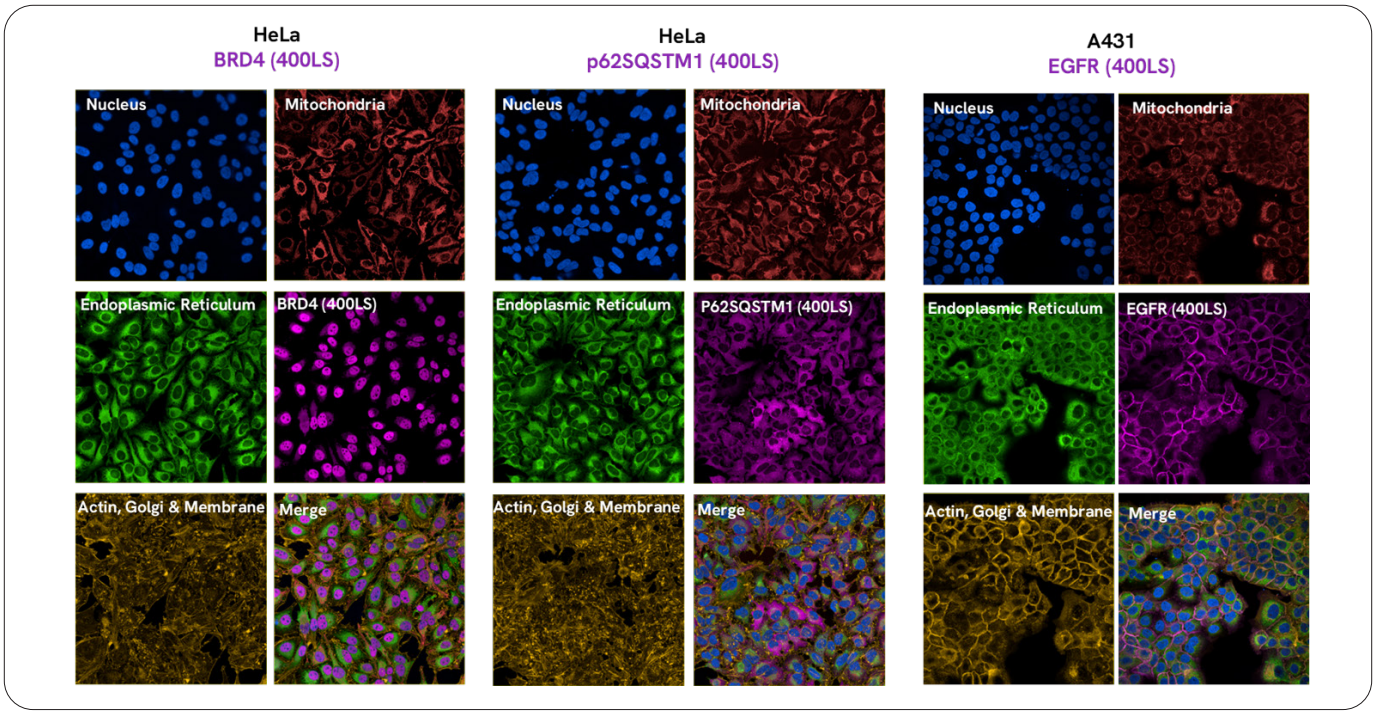


Figure 3: Compatibility of the PhenoVue cell painting Target Add-on workflow with membrane-associated, cytoplasmic, and nuclear targets. Representative images show integration of the 400LS target channel with cell painting markers for EGFR in A431 cells, p62/SQSTM1 in HeLa cells, and BRD4 in HeLa cells.

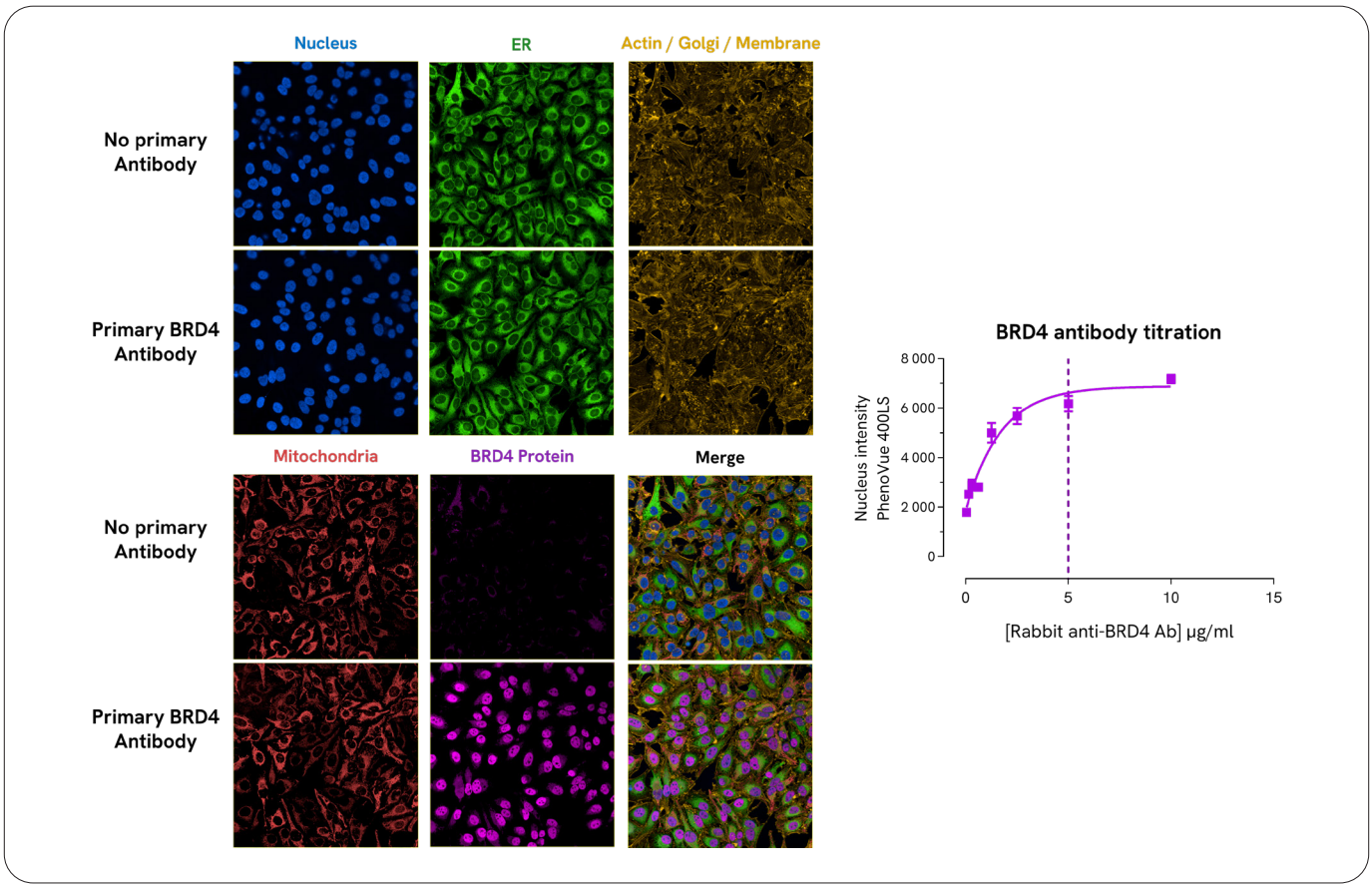


Figure 4: Detection of BRD4 in a multiplexed cell painting workflow. HeLa cells were stained with an anti-BRD4 primary antibody followed by PhenoVue Fluor 400LS secondary antibody together with cell painting markers. Representative images and antibody titration curves show concentration-dependent BRD4-associated 400LS signal and successful discrimination of the nuclear target channel from cell painting channels.

BRD4 case study: adding target modulation to phenotypic profiling

BRD4 was used as the main case study because it represents a biologically relevant and technically demanding target. BRD4 is a bromodomain and extra-terminal family protein involved in transcriptional regulation, and it has been extensively explored in oncology through both inhibitor and degrader strategies. BRD4-targeting PROTACs are particularly useful for demonstrating the value of a target-specific cell painting layer, because degradation of the BRD4 protein can occur before large-scale morphological remodeling becomes evident.

HeLa cells were treated with reference phenotypic compounds and BRD4-related compounds, including BRD4-targeting PROTACs and the BET inhibitor JQ1. The Target Add-on workflow was compared with conventional PhenoVue cell painting. Multiparametric image features were extracted and analyzed using dimensionality reduction and Euclidean distance to vehicle controls. The purpose of the comparison was not only to determine whether the new workflow could preserve conventional phenotypic clustering, but also to test whether the target layer provided additional information when BRD4 modulation occurred in the absence of a strong morphology shift.

At 24 hours, both the conventional cell painting workflow (data not shown) and the Target Add-on workflow (figure 5) discriminated pronounced phenotypic perturbations. Reference compounds such as cytochalasin D (F-actin depolymerizing agent) and etoposide (Topoisomerase II inhibitor) generated clear phenotypic separation, and BRD4 degraders also induced concentration-dependent changes. These results indicate that the enhanced Target Add-on configuration can help preserve the core phenotypic profiling capability of conventional cell painting while adding a BRD4-associated readout.

A direct comparison of Euclidean distances to vehicle controls further showed that the BRD4 Target Add-on workflow can selectively enrich phenotypic discrimination for biologically relevant conditions (figure 6). In the 24-hour dataset, stronger separation was observed for some specific conditions following incorporation of the BRD4-associated imaging layer. These conditions are related to BET degraders (ZXH-3-26, MZP-54, and dBET6) which induce BRD4 degradation, a feature that was checked in the additional 400LS channel (data not shown). When used at low concentrations, only the BRD4 Target Add-on workflow allows discrimination from the vehicle. These conditions induce BRD4 degradation without significant phenotype modulation explaining why the standard cell painting workflow cannot discriminate them. In contrast, other reference compounds which modulate cell phenotype like cytochalasinD, etoposide and tetrandrine which does not directly interact and impact BRD4, showed limited or non-significant enhancement with the BRD4 Target Add-on workflow under the tested conditions. This pattern supports a cautious conclusion: the Target Add-on workflow should not be positioned as universally increasing all cell painting distances, but rather can enrich interpretation when the added target channel captures biologically relevant modulation.

A notable result was observed at the earlier 2-hour timepoint (figure 6). At 2 hours, BRD4-targeting PROTACs reduced BRD4-associated 400LS signal, but conventional cell painting features alone showed limited separation from vehicle. In contrast, the Target Add-on workflow improved discrimination of BRD4 degraders, supporting the interpretation that target degradation was already detectable before a robust phenotypic response had developed. This temporal separation between target modulation and downstream morphology is where the Target Add-on strategy may provide significant biological value.

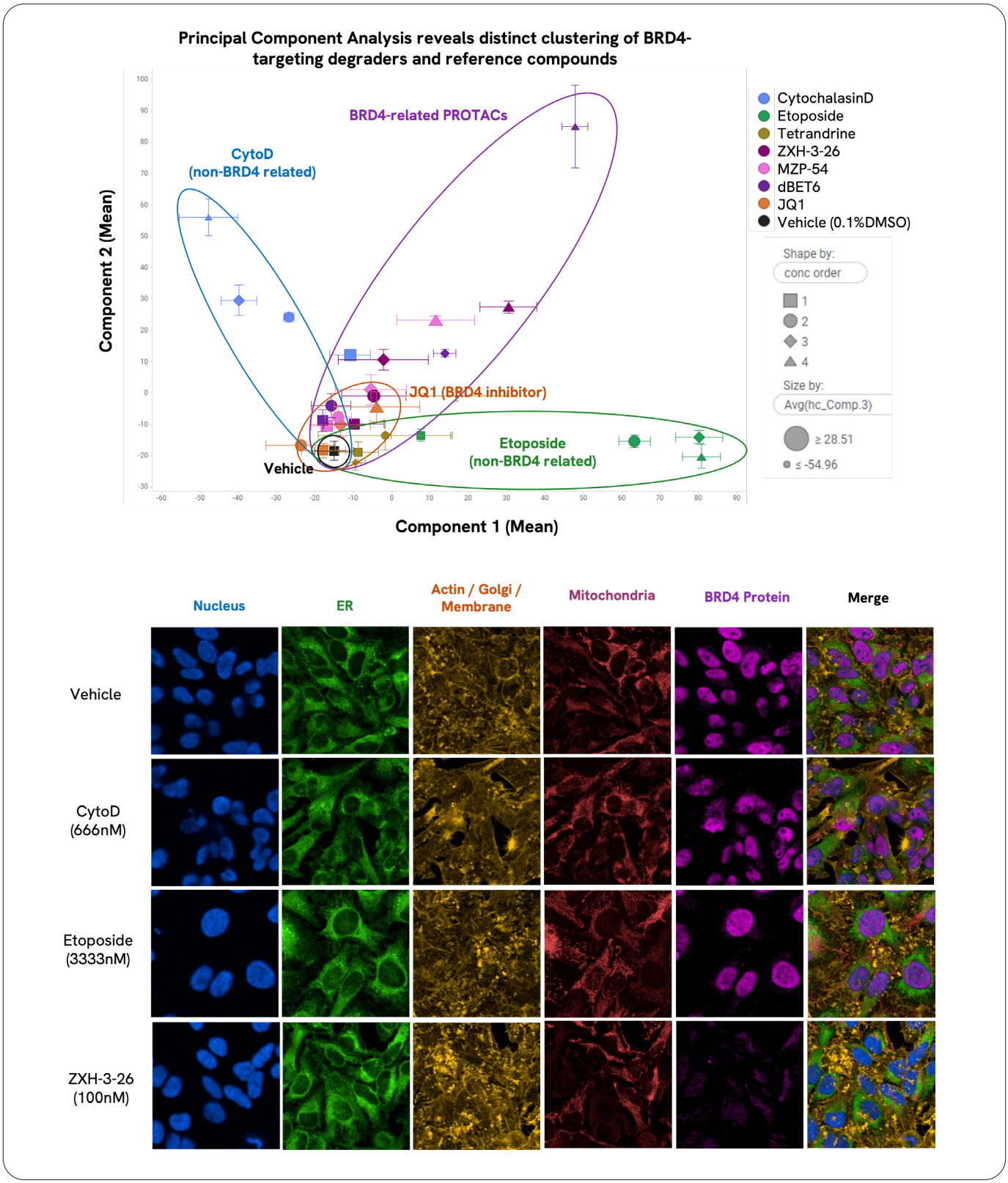
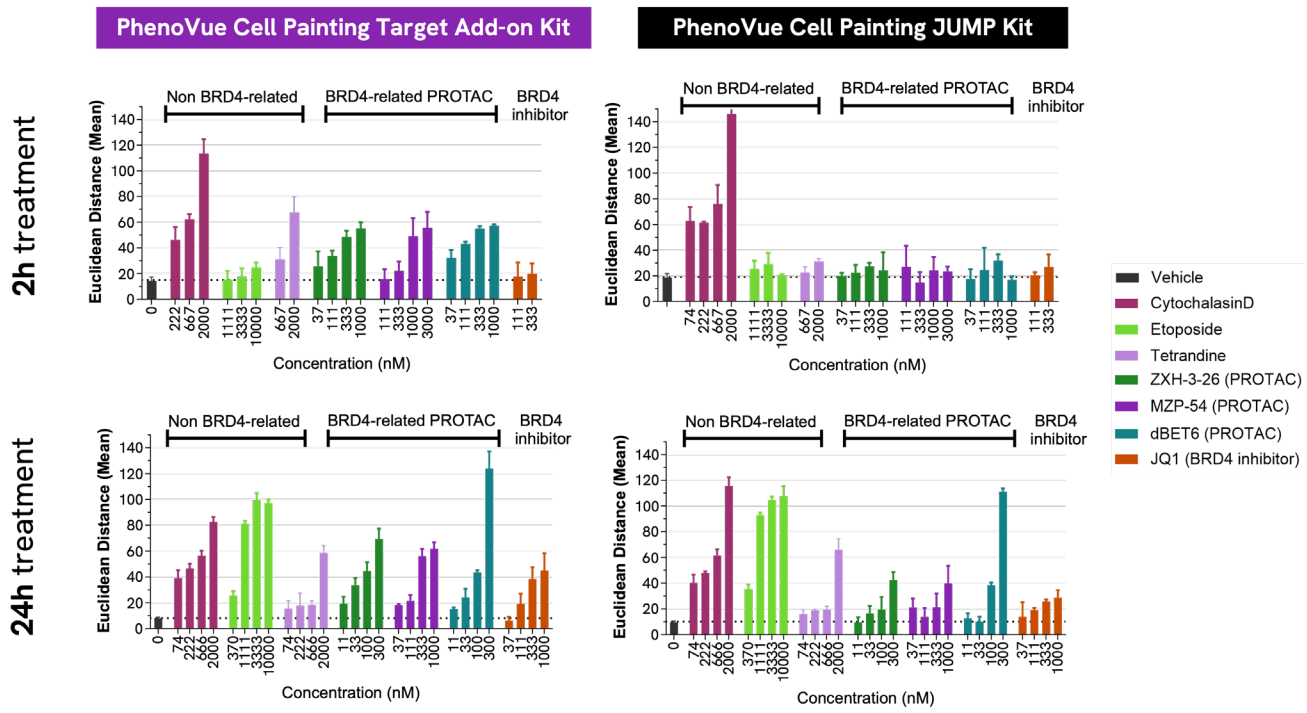


Figure 5: Compound-induced phenotype clustering on BRD4 with 24h compound treatment. Features extracted from images were further analyzed with dimensional reduction using Principal Component Analysis. Component 1 (x-axis), component 2 (y-axis) and component 3 (dot size) were plotted and displayed on the graph (left panel). Depending on their mechanism of action and concentration, the various compounds induce distinct phenotypes that cluster away from the vehicle condition. Some images from the 3 distinct phenotypes are displayed (right panel) with vehicle (cell culture medium + 0.1%DMSO), cytochalasinD (F-actin depolymerizing agent = loss of actin filament with bundles in the 555 channel), etoposide (Topoisomerase II inhibitor, DNA damage inducer which display nuclear size increase in the Hoechst channel) and ZXH-3-26 (BRD4 PROTAC inducing degradation of BRD4 protein = loss of BRD4 signal in the 400LS channel).



2h

Ordinary one-way ANOVA Dunnett's multiple comparisons test	Target Add-on kit	Standard Cell Painting Kit
Reference compounds		
CytochalasineD (0.074µM) vs Vehicle	****	****
CytochalasineD (0,222µM) vs Vehicle	****	****
CytochalasineD (0.6µM) vs Vehicle	****	****
CytochalasineD (2µM) vs Vehicle	****	****
Etoposide (1.1µM) vs Vehicle	ns	ns
Etoposide (3.3µM) vs Vehicle	ns	ns
Etoposide (10µM) vs Vehicle	ns	ns
Tetrandrine (666nM) vs Vehicle	ns	ns
Tetrandrine (2000nM) vs Vehicle	****	ns
BRD4 targeting compounds (Inhibitor & degraders)		
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

24h

Ordinary one-way ANOVA Dunnett's multiple comparisons test	Target Add-on kit	Standard Cell Painting Kit
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	*	****
Etoposide (1.1µM) vs Vehicle	****	****
Etoposide (3.3µM) vs Vehicle	****	****
Etoposide (10µM) vs Vehicle	****	****
Tetrandrine (74nM) vs Vehicle	ns	ns
Tetrandrine (222nM) 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	***	**
JQ1 (1000nM) vs Vehicle	****	***
ZXH-3-26 (11nM) vs Vehicle	ns	ns
ZXH-3-26 (33nM) vs Vehicle	*	ns
ZXH-3-26 (100nM) vs Vehicle	****	ns
ZXH-3-26 (300nM) vs Vehicle	****	****
MZP-54 (37nM) vs Vehicle	ns	ns
MZP-54 (111nM) vs Vehicle	ns	ns
MZP-54 (3333nM) vs Vehicle	****	ns
MZP-54 (1000nM) vs Vehicle	****	****
dBET6 (11nM) vs Vehicle	ns	ns
dBET6 (33nM) vs Vehicle	**	ns
dBET6 (100nM) vs Vehicle	****	****
dBET6 (300nM) vs Vehicle	****	****

Figure 6: Target-specific BRD4 imaging may improve early discrimination of BRD4-targeting degraders. HeLa cells were treated with reference compounds, BRD4-targeting PROTACs, or JQ1 for 2 hours or 24 hours and analyzed using conventional cell painting or the Target Add-on workflow. At 2 hours, BRD4 degraders show stronger separation when the 400LS BRD4 target channel is included, consistent with early target degradation preceding broad phenotypic divergence. At 24 hours, both workflows detect stronger compound-induced phenotypic changes while the Target Add-on workflow tends to enhance discrimination of some subtle PROTAC effect at low concentrations.

Interpretation: where the Target Add-on workflow adds impactful value

The evaluation data support a clear positioning: the Target Add-on workflow is valuable when the biological effect of interest is modulation, target engagement, or target loss, and when this effect may not immediately translate into a strong morphological phenotype. This situation is common in targeted protein degradation, transcriptional regulation, pathway biology, and early timepoint pharmacology.

For TPD applications, a degrader may rapidly lower the abundance of the protein of interest while downstream cellular effects emerge later. A conventional cell painting assay can capture those later downstream effects, but it does not directly report whether the target was degraded. Conversely, a target-only assay may quantify degradation but does not reveal the cellular state associated with target loss. The Target Add-on workflow helps address both limitations by combining target-associated signal and multiparametric morphology in the same cells.

This combined readout can support several experimental decisions. It can help distinguish compounds that modulate the intended target from compounds that induce unrelated morphology. It can identify early target modulation before downstream phenotypic remodeling. It can help compare degraders and inhibitors that engage the same pathway but generate distinct cellular states. It can also support subpopulation analysis, for example comparing target-positive and target-negative cells within the same well when target expression is heterogeneous.

The Target Add-on workflow does not universally improve all cell painting clustering results. Rather, it adds a mechanistic layer that can enhance interpretation and improve discrimination when target modulation is biologically relevant and not fully captured by morphology alone.

Application areas

- Targeted protein degradation: simultaneous monitoring of target abundance, degradation kinetics, and downstream phenotypic consequences.
- Mechanism-aware compound profiling: combining unbiased morphology with a pathway or target marker selected by the user.
- Early pharmacology: detecting target modulation before broad phenotypic remodeling becomes apparent.
- Subpopulation analysis: comparing cellular phenotypes in target-positive versus target-negative cells, or in cells with different target expression levels.
- Cell model adaptation: adding structural or lineage markers to support segmentation or phenotypic interpretation in complex cell models.

Materials and methods

Cells were seeded in PhenoPlate microplates and incubated overnight at 37 °C with 5% CO_2 before compound treatment or staining.

Staining, imaging and analysis process were performed according to the PhenoVue Cell Painting Target Add-on Kit (PINGM4001 or PINGRB4001) or PhenoVue Cell Painting JUMP Kit (PING21) suggestions.

For the BRD4 case study, HeLa cells were treated with reference phenotypic compounds, BRD4-targeting PROTACs, or the BET inhibitor JQ1 for either 2 hours or 24 hours. After treatment, live cells were incubated with PhenoVue 641 Mitochondrial Stain, fixed with methanol-free paraformaldehyde, washed, and permeabilized using HBSS containing 0.5% Triton X-100. Permeabilization conditions were adjusted according to target localization and antibody performance.

Cells were then incubated with the selected mouse or rabbit primary antibody directed against the protein of interest. The PhenoVue Cell Painting Target Add-on Kit does not include a primary antibody; users should select and optimize the primary antibody according to their biological model. Preliminary primary antibody titration is suggested to identify a concentration that provides enhanced target-associated signal while limiting background staining and antibody consumption.

After primary antibody incubation and washing, cells were stained with the Cell Painting Target Add-on staining solution containing PhenoVue Hoechst 33342 Nuclear Stain, PhenoVue Fluor 488 Concanavalin A, PhenoVue Fluor 555 WGA, PhenoVue Fluor 568 Phalloidin, and PhenoVue Fluor 400LS goat anti-mouse or anti-rabbit secondary antibody. The staining configuration omits PhenoVue 512 nucleic acid stain and uses a reduced Hoechst concentration to support spectral compatibility with the 400LS target channel.

Images were acquired on Revvity high-content imaging systems using compatible excitation and emission settings for five-channel imaging. For image analysis workflows, flatfield correction was applied before segmentation. Nuclei were segmented using the Hoechst channel, cytoplasm was segmented using 488 channel, and cell painting features were calculated across acquired channels. Target-associated intensity and texture features were extracted from the PhenoVue Fluor 400LS channel when relevant. Secondary analysis including dimensionality reduction, compound clustering, and Euclidean distance analysis relative to vehicle-treated controls was performed on Signals™ VitroVivo software (Spotfire, Revvity).

Channel/readout	Marker or reagent	Purpose
Nucleus	PhenoVue Hoechst 33342	Nuclear segmentation and nuclear morphology.
Target channel	PhenoVue Fluor 400LS secondary antibody	Detection of user-selected protein of interest.
ER-associated features	PhenoVue Fluor 488 Concanavalin A	Organelle and texture features contributing to cell painting profiles.
Actin/Golgi/plasma membrane	PhenoVue Fluor 568 Phalloidin and PhenoVue Fluor 555 WGA	Cytoskeletal and membrane-associated morphology.
Mitochondria	PhenoVue 641 Mitochondrial Stain	Mitochondrial morphology and intensity features.

Conclusion

The PhenoVue cell painting Target Add-on workflow helps extend conventional Cell Painting by enabling users to integrate a protein-of-interest readout into the same multiplexed high-content imaging experiment. By leveraging the PhenoVue Fluor 400LS antibody detection channel, the workflow supports five-channel imaging while preserving the multiparametric nature of cell painting.

Evaluation across EGFR, p62/SQSTM1, and BRD4 demonstrates that the approach is compatible with targets located at the membrane, in the cytoplasm, and in the nucleus. The BRD4 degrader case study illustrates a compelling use case: early target modulation can be detected before broad morphological divergence is captured by conventional cell painting alone. This may allow users to connect target engagement or degradation with phenotypic consequences in a single assay.

Overall, the PhenoVue Cell Painting Target Add-on workflow helps provide a practical route toward mechanism-aware phenotypic profiling. It is particularly well suited for applications where unbiased morphology needs to be interpreted alongside a selected biological marker, including targeted protein degradation, pathway modulation, compound mechanism-of-action studies, and cell-state stratification.

Key points

- Adds a user-selected target-specific imaging layer to Cell Painting workflows.
- Combines phenotypic profiling and target-associated information in the same cells.
- Supports five-channel imaging enabled by PhenoVue Fluor 400LS secondary antibodies.
- Helps reveal early target modulation when morphology alone remains limited.
- Compatible with multiple target localizations, including membrane-associated, cytoplasmic, and nuclear proteins.

References

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