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APPLICATION NOTE

Combining high-content imaging and DRUG-seq to improve Mechanism-of-Action profiling.

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Introduction

Drug discovery strategies are increasingly shifting from targeted assays that measure a small number of predefined endpoints toward broad, high-dimensional approaches that capture cellular responses at multiple biological levels. These approaches support improved hit prioritisation and early mechanism-of-action (MoA) insight.

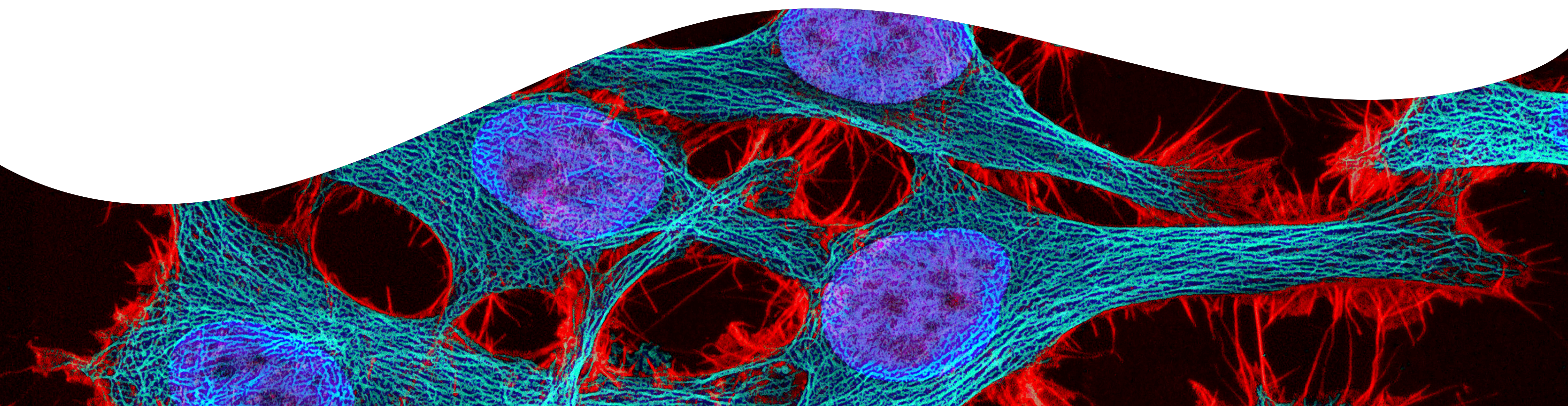
This shift is further driven by the growing use of artificial intelligence (AI), which is increasing demand for well-annotated, high-dimensional datasets that capture how compounds perturb cellular systems and enable translation of these effects into mechanistic understanding.

Morphological profiling approaches such as cell painting provide scalable, high-content insight into compound-induced cellular changes, enabling unbiased characterisation across large compound sets. In parallel, transcriptomic profiling captures gene expression changes that reflect underlying biological pathways and mechanisms.

While both approaches provide valuable but distinct views of cellular response, no single method captures both morphological phenotype and molecular mechanism at screening scale.

Conventional RNA sequencing has limited application in screening due to cost, operational complexity, and cell number requirements, particularly in multi-well formats such as 384-well plates. Recent advances in high-throughput transcriptomics, such as DRUG-seq, overcome these limitations, enabling transcriptomic profiling in a screening-compatible format[1].

In this application note, we demonstrate how cell painting and DRUG-seq can be used in combination to profile compound activity, highlighting how these approaches support mechanism-of-action analysis and generate richer datasets suitable for AI-driven drug discovery.



Materials and methods

Cell culture and compound treatment

Experiments were conducted by the Revvity Preclinical Screening Services (Cambridge, UK). Three cell lines (DLD-1, U2OS, and SW837) were seeded at optimised densities into 384 well optically clear PhenoVue™ Plates (Revvity) for cell painting or CulturPlates (Revvity) for DRUG-seq. Cells were incubated for 24 hours at 37°C, 5% CO_2 in a humidified atmosphere.

Cells were then treated with compounds using Flexdrop™ non-contact dispenser (Revvity) and incubated for another 4 h or 24 hours. A total of 126 compounds were used, 118 test compounds randomized across plates and 8 control compounds in all plates. Controls were added at a single dose (5 μM), while test compounds were added at two different concentrations that were selected based on published compound activity.

Three replicates per randomized test compounds were used.

Cell painting

Staining was performed following PhenoVue™ Cell Painting JUMP kit recommendations (Revvity). Following staining, cells were washed a final three times with PBS/0.1% Triton X-100 before the assay plates were sealed and stored at 4°C until imaging.

Imaging was conducted using the Opera Phenix™ high-content imager for the appropriate colour channels.

DRUG-seq workflow

MERCURIUS™ DRUG-seq libraries were prepared according to the manufacturer's instructions (Alithea Genomics). Final libraries were quantified using a Qubit® fluorometer (Thermo Fisher Scientific) and loaded on an Element Biosciences® AVITI™ platform using 2×150 bp paired-end sequencing, aiming for 2M reads/sample.

Data analysis

Raw fluorescence images acquired on the Opera Phenix system were corrected for channel-specific illumination effects, followed by per-channel intensity normalisation. Images were rescaled using percentile-based intensity bounds and then standardised relative to DMSO control wells, enabling treatment-induced changes to be expressed as deviations from the DMSO-treated control population.

Features were extracted with machine learning pipeline as described previously in Blanck, 2025[2].

For DRUG-seq analysis, gene expression signatures were generated from normalised expression values. Biological replicates were averaged by condition, and expression profiles were standardised per gene across conditions to account for baseline expression differences.

Dimensionality reduction methods, including PCA, UMAP and t-SNE, were used to visualise global and local relationships between treatment profiles, while hierarchical clustering was applied to identify groups of compounds with similar transcriptional or phenotypic responses.

For differential expression analysis, raw DRUG-seq counts were normalised using TMM and transformed to $\log_2$CPM with edgeR. Plate of origin was included as a covariate to account for batch effects, and linear modelling with empirical Bayes moderation was performed using limma.

Compound-specific contrasts were generated against a common reference, and the resulting moderated t-statistics and $\log_2$ fold-changes were used as continuous transcriptional signatures for downstream comparison with cell painting profiles.

Results

DRUG-seq enables pathway-level clustering of compound activity

SW837 harbors KRAS G12C, an activating oncogenic KRAS mutation that drives RAS–MAPK pathway signaling while retaining nucleotide cycling, making it a relevant model for evaluating MAPK pathway perturbation.

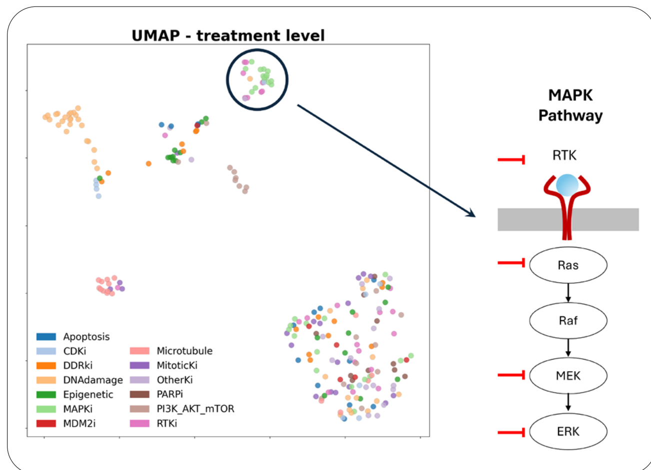


Figure 1: DRUG-seq enables pathway-level clustering of compound activity. UMAP analysis of the 126-compound library in SW837 cell line shows that the MAPK inhibitors form a distinct cluster (black circle) irrespective of the pathway node targeted (red arrows). Notably inhibitors of the receptor tyrosine kinase (RTK) also appear included in this cluster.

Unsupervised analysis of the profiles of the 126 compounds tested revealed that compounds targeting the MAPK pathway formed a distinct cluster. Notably, compounds acting at different nodes within the pathway generated highly similar transcriptional responses, indicating that transcriptional signatures capture shared pathway-level responses despite differences in target location (Figure 1).

DRUG-seq captures mutation-selective pathway responses

To further assess the ability of DRUG-seq to resolve biologically meaningful differences in compound activity, a focused analysis of RAS pathway inhibitors was performed using cell lines harbouring KRAS G12C (SW837) or G13D mutations (DLD-1). Although both mutations activate RAS signaling, only G12C creates the cysteine handle targeted by covalent inhibitors; G13D lacks this residue and remains pharmacologically distinct. Cells were treated for 4 hours to capture early transcriptional responses.

Consistent with expected biology, KRAS G12C inhibitors selectively modulated ERK pathway gene expression in G12C mutant cells, with minimal effects observed in G13D cells (Figure 2). In contrast, a MEK inhibitor (Trametinib) and a pan KRAS inhibitor (BI 2865) broadly inhibited ERK pathway gene expression across both cell lines, reflecting activity downstream of, or across, RAS signalling.

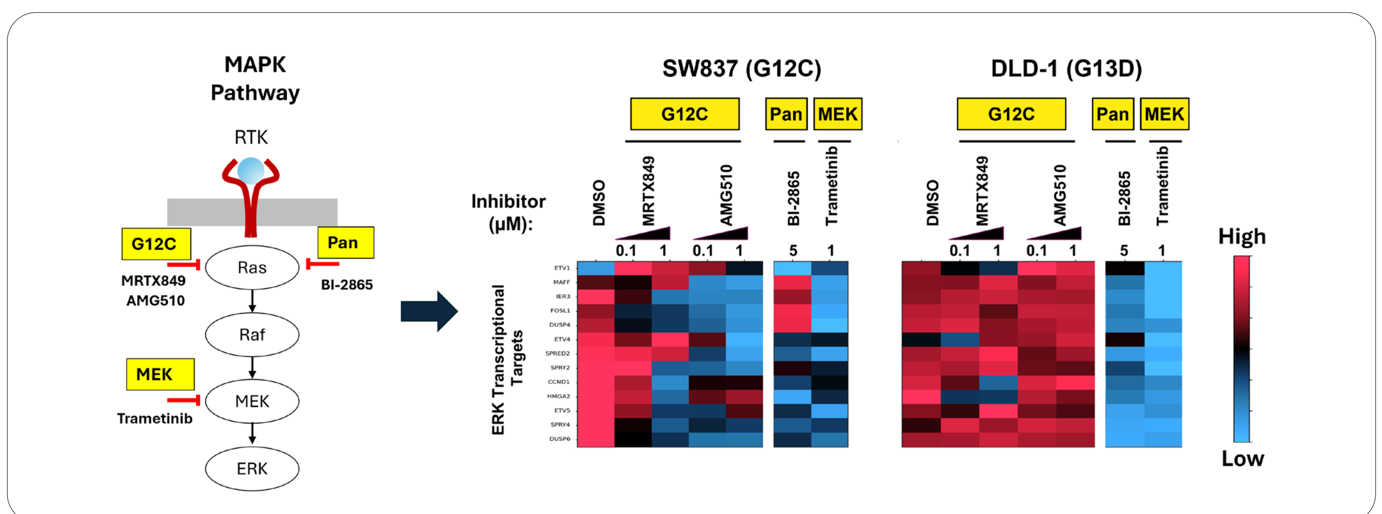


Figure 2: DRUG-seq captures mutation-selective pathway responses. Analysis of ERK target transcriptional responses in SW837 and DLD-1 cell lines, treated with different RAS pathway inhibitors. G12C inhibitors downregulate ERK target expression in SW837 cells but show minimal effects in DLD-1. In contrast, MEK or pan-KRAS inhibitors downregulate ERK target expression in both cell lines.

Interestingly, while BI 2865 inhibited the majority of ERK pathway genes in both models, a subset of genes showed reduced sensitivity in the G12C mutant background, suggesting subtle differences in pathway modulation between mutant contexts.

These results demonstrate that DRUG-seq can resolve mutation-selective responses and capture acute pathway modulation. Importantly, this analysis shows that targeted gene and pathway-level insights can be extracted directly from transcriptomic datasets.

This capability provides a scalable complement to targeted assays such as qPCR, enabling simultaneous measurement of specific pathway markers alongside broader transcriptional changes within a single experiment.

Complementary insights from cell painting and DRUG-seq profiling

To compare morphological and transcriptomic profiling directly, a subset of approximately 30 compounds, including multiple concentrations for some compounds, was analysed using both cell painting and DRUG-seq. Both approaches captured biologically meaningful patterns of compound activity, but differed in how individual mechanism classes were resolved (Figure 3).

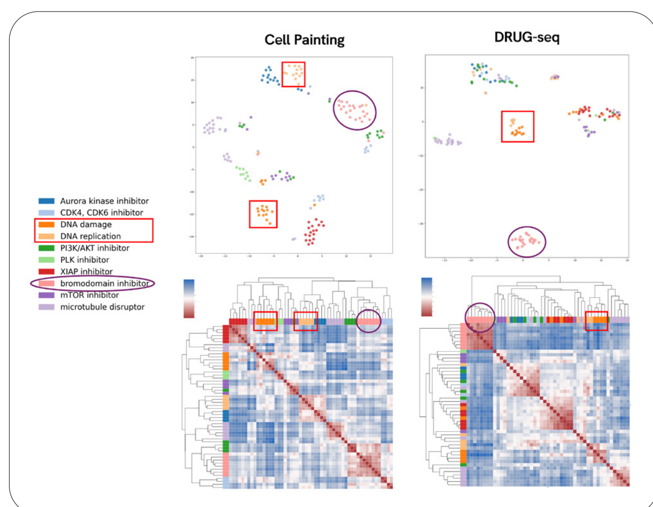


Figure 3: Complementary insights from cell painting and DRUG-seq profiling. t-SNE projections (top panels) and hierarchical clustering (bottom panels) of cell painting and DRUG-seq profiles for a subset of approximately 30 compounds in U2OS cells. For some compound classes such as DNA damage and DNA replication (red square) the separation with Cell Painting is clearer than with DRUG-seq. For others, such as bromodomain inhibitors (purple circle) DRUG-seq provides a better separation.

Cell Painting provided strong separation of several compound classes associated with mitotic and structural phenotypes, including Aurora kinase inhibitors, PLK inhibitors and microtubule disruptors. XIAP inhibitors also formed a clearer and more isolated cluster in the cell painting data than in the DRUG-seq data, indicating that this class is more strongly resolved by morphological profiling.

Notably, DNA damage and DNA replication compounds formed distinct clusters in the morphological data, indicating that cell painting can distinguish related but different phenotypic responses. In the matched subset, DNA damage and DNA replication compounds occupied a shared transcriptional neighbourhood in DRUG-seq, suggesting related pathway-level responses.

In the full 126-compound DRUG-seq dataset, these mechanisms show greater separation, indicating that transcriptomic resolution improves as compound diversity increases. Some classes, including bromodomain and mTOR inhibitors, showed coherent but less sharply defined grouping in both modalities, indicating detectable but moderate class separation.

Bromodomain inhibitors formed clusters in both assays but were more tightly grouped and more clearly separated from other mechanism classes in the DRUG-seq data, indicating a stronger transcriptional definition of this MoA.

Some classes, including mTOR inhibitors, showed coherent but less sharply defined grouping in both modalities, suggesting that both assays provide signal, but with only moderate class separation.

Together, these observations demonstrate that cell painting and DRUG-seq provide complementary views of compound activity, capturing different but biologically relevant aspects of response to perturbation.

Conclusion

The results show that DRUG-seq enables robust, screening-scale transcriptional profiling of compound activity, supporting pathway-level classification, mutation-selective response analysis and MoA interpretation.

In the 126-compound dataset, MAPK pathway inhibitors clustered together despite acting at different pathway nodes, highlighting the ability of DRUG-seq to capture shared biological response programmes across related mechanisms.

In the matched cell painting and DRUG-seq subset, the two modalities provided distinct but complementary views of compound activity.

Cell Painting gave strong phenotypic separation for morphology-rich mechanisms, including mitotic and structural perturbations and XIAP inhibition.

DRUG-seq added transcriptional context, resolving pathway-level relationships not apparent from morphology alone, including MAPK pathway responses, mutation-selective ERK modulation, and the shared transcriptional neighbourhood of DNA damage and DNA replication mechanisms.

Bromodomain inhibitors clustered in both assays, but the tighter DRUG-seq grouping provided stronger transcriptional definition of this MoA.

Together, these data show that combining cell painting and DRUG-seq provides orthogonal evidence for compound characterisation: Cell Painting strengthens phenotypic discrimination, while DRUG-seq strengthens pathway-level interpretation and helps clarify borderline or overlapping mechanism assignments.

These findings are consistent with previous comparisons of cell painting and transcriptional profiling, which demonstrate that the two modalities provide complementary views of compound-induced cellular responses[3].

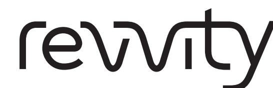
This complementarity supports more confident compound prioritisation and generates high-dimensional datasets well suited to downstream AI and machine learning applications, including compound classification, MoA prediction and prioritisation.

References

1. Ye, C., et al. (2018). DRUG-seq for miniaturized high-throughput transcriptome profiling in drug discovery. *Nat Commun*. 9(1):4307. doi: 10.1038/s41467-018-06500-x.

2. Blanck, M. (2025). "Machine-learning prediction of drug mechanism of action from high-content cell painting images".

3. Way, G.P., et al. (2022). Morphology and gene expression profiling provide complementary information for mapping cell state. *Cell Syst*. 13(11):911-923.e9. doi: 10.1016/j.cels.2022.10.001.



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