Cell painting: Your questions answered

Cell painting is a powerful high-throughput high-content screening and image analysis method to phenotypically characterize a cell's response to a perturbagen (compound, drug, or gene). Unknown perturbagens are screened against known reference control treatments with defined mechanisms of action (MOAs). This provides a roadmap to annotate data using a machine learning algorithm to reduce the dimensionality of multivariant high-content data and to identify morphological profiling relationships with similarity clusters that are visualized using self-organizing maps.

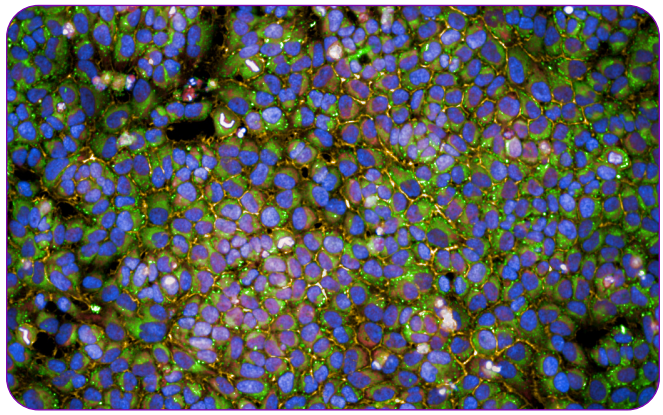
Since the first publications describing the technique, it has been rapidly adopted for phenotypic drug discovery and basic research. However, implementing the assay can present challenges, such as the choice of cell model, appropriate labeling reagents, optimizing instrumentation for detection, and data analysis approaches to make sense of the thousands of features that can be generated during data analysis.

Here, we provide answers to key questions from scientists and researchers to help you gain the most from cell painting assays.

Cell models

Q **What cell model should I use for cell painting?**

A Several studies report the use of the U-2 OS cell line, which is now widely considered the gold standard for the cell painting assay.[1,2,3,4,5] For best success, it is recommended to start with U-2 OS cells as described in the Bray et al. (2016) paper.[1]



Q **What other cell models work with cell painting?**

A In principle, most adherent cell types are suitable for cell painting, but the main reason for choosing a cell model is a biological question and whether the cell model is amenable for high-content imaging. Once the workflow process is validated in a U-2 OS cell model, adoption and transition to other cell models is more likely to succeed.

There is a wider variety of cell models being investigated by other groups outside of the U-2 OS cell line. For example, Willis et al. (2020) used the cell painting assay to characterize the phenotypic effects of reference compounds across six human-derived cell lines: U-2 OS, MCF7, HepG2, A549, HTB-9, and ARPE-19.[6] The authors concluded that the cell painting approach yielded similar biological activity profiles among morphologically diverse human-derived cell lines. This required the optimization of image acquisition and machine learning procedures for each cell type but did not necessitate cell type–specific optimization of the staining step.

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In another study, Way and co-authors successfully described the cell painting approach combined with stably expressing Cas9 A549, ES2, and HCC44 cells in CRISPR knockout experiments.[7] More recently, Jordi Carreras-Puigvert's research group applied the cell painting phenotypic approach on COVID-19-infected MRC-5 human lung fibroblasts.[8] This proof-of-concept study established antiviral compounds capable of reversing the morphological profile of the infected host towards a non-infected state and opens up new avenues for antiviral drug discovery.

Q **Can 3D cell models work with cell painting?**

A Yes. The first published evidence of 3D cell painting was reported by Inventia Life Science Operations Pty Ltd, Alexandria, Australia.[9] They describe a workflow for cell painting-based phenotypic screening using bioprinted U-87MG glioblastoma 3D cell cultures and the Operetta® CLS™ high-content analysis system.

Q **Will suspension cells work with cell painting?**

A Suspension cell models such as primary peripheral blood lymphocytes (PBLs) or Jurkat and HL-60 immortalized cell lines are not commonly used in the classical cell painting assay because of the large nucleus and small cytoplasmic area for morphological spatial measurements. A modified version of available cell painting bioprobes may be selected in a focused panel to address a biological question in a suspension cell model system.

Q **Can I use cell painting in live-cell imaging?**

A Yes. The traditional cell painting assay design described by Bray et al. (2016)[1] uses a few fluorescent probes that are amendable for live-cell imaging. Careful selection of these probes and others can be used but it is important to understand their limitation including stability, cytotoxicity, and overall use. The use of many commercially available fluorescent probes for live-cell imaging is limited from hours to a few days. It is recommended to include brightfield imaging to supplement any fluorescent channels captured.

Q **Can I use genetically engineered cells in the cell painting assay?**

A Yes. Genetically engineered cells can be advantageous in the cell painting assay design to lessen the need for bioprobe reagents if tagged with a fluorescent protein. In this scenario, you cannot use all six of the PhenoVue™ cell painting probes because of the spectral emission overlap from fluorescent mutants with PhenoVue cell painting markers. Additionally, another advantage of fluorescent proteins is the capability to measure live-cell kinetic measurements over time.

Q **Are there any cell models that do not work with cell painting?**

A Not all cell model systems are amenable to HCS imaging due to several challenges arising from cell attachment, cell aggregate formation, poor biological response *in vitro*, etc. It is recommended to use the U-2 OS cell model as the standard when testing unknown or unpublished cell models. Careful consideration of the microplate surface coating and the bioprobe concentration will likely be required during assay development optimization. Additionally, be aware that the choice of reference control compounds for training in the cell painting process may not behave the same in all cell types.

Perturbagens and treatment

Q **What perturbagens can I use in the cell painting assay?**

A A perturbagen is a substance that perturbs or disrupts normal cellular function, the cell's phenotype, or morphology. Environmental chemical toxicants, drugs, chemicals, gene manipulation, or even growth factors in media are all considered perturbagens that can be investigated by the cell painting assay. This can include Revvity's druggable synthetic CRISPR genome library, siRNA, mRNA, and other genes to knockdown, edit, express, or modulate genes.

Labeling cells with cell painting reagents

Q **What microplates do you recommend for cell painting?**

A The choice of microplates or vessels used in experimental assays is based on supporting the cell biology, addressing the experimental design requirements such as throughput needs, and whether they are amenable for robotics and detection by instrumentation.[10] Revvity PhenoPlate™ microplates were designed for automated cellular imaging assays.

They offer excellent flatness and optical clarity, providing superior image quality and good cell adherence for the cell painting assay. The PhenoPlate's unique skirt-less design provides compatibility to image all well positions with water immersion objective lenses on the Operetta CLS and Opera Phenix® Plus high-content imaging systems.

Q **What dyes do you recommend for cell painting?**

A It is recommended that researchers follow the protocols described in the Bray et al. (2016) publication[1] or published by the JUMP consortium.[11] Revvity PhenoVue™ cell painting kits (two versions available) have been designed to match the experimental conditions described. By following the kit protocols, you will be able to achieve optimal performance. For alternative cell models, you may need to re-optimize the conditions and dye concentrations.

The PhenoVue cell painting kit is validated on a 2D fixed cell model, U-2 OS, as described in the Bray et al. (2016) publication.[1] Some components, such as the PhenoVue Fluor 568 - Phalloidin, are not suitable for live cell imaging. This is because they rely on staining intracellular structures (e.g., actin filaments, ER, and Golgi) and this requires cell fixation and permeabilization, which is not compatible with live cells. If the biology you want to study requires live cell probes, then a specific assay would need to be developed and validated.

Even though cell painting was originally designed with six different bioprobe markers, cell painting and other HCS imaging assays should be designed with the appropriate biomarkers to address the biological questions being asked. Using this strategy, one or more of the reagents in the cell painting panel can be replaced with other PhenoVue or BioLegend reagents or similar. For example, a primary antibody with an associated secondary antibody for indirect measurement of a protein can be applied in the experimental design.[8]

What is the JUMP-CP consortium?

The Joint Undertaking in Morphological Profiling Cell Painting (JUMP-CP) consortium is a group of academic, biotechnology, pharmaceutical, and commercial partners building tools, resources, and a massive public dataset for the scientific community. Revvity is proud to be a supporting partner of the JUMP-CP consortium offering products and services including microplates, Revvity CRISPR reagents, and PhenoVue cell painting kits and labeling reagents. For more information, please visit: https://jump-cellpainting.broadinstitute.org/

Image acquisition

Q **What are the key requirements of imaging instrumentation for cell painting assays?**

A The detection of individual labeled cells with the six different PhenoVue cell painting reagents requires a high-throughput microscopy system to spatially measure each probe's intensity and morphology. The Opera Phenix Plus is the instrument of choice for many high-content imaging practitioners to quickly capture cell painting images. The Opera Phenix Plus is a laser-based high-content screening system designed with optical filters for measuring the six different PhenoVue cell painting probes in four or five different channels in widefield or confocal mode. The latest Operetta CLS high-content imaging system (HH16000020, released Oct. 2021) is equipped with eight LEDs uniquely designed with modified excitation and emission spectra for the cell painting assay.

Q **Is brightfield imaging possible?**

A Yes, transmission light images can be captured on both the Operetta CLS and Opera Phenix Plus systems.

Q **Which magnification should be used?**

A The choice of magnification will depend on the biological question being asked and the ability to separate the different morphological phenotypes. In principle, different magnifications are possible, but high-content imaging practitioners need to balance the resolution of biological details and acceptable screening time. The 20x water objective lens is recommended as a good starting point to balance cellular details and measurement time.

Q How many z-planes should I capture?

A Z-planes or z-slice images are an option with both the Operetta CLS and Opera Phenix Plus systems for all independent fluorescent and brightfield channels. For every z-slice image captured, the duration of the image acquisition process is lengthened. Deciding when to add a z-stack to the image acquisition process will depend on the cell model, the spatial location of the cell painting probes within the cell, and the experimental design question, such as how much detailed resolution is needed. Using the water immersion objective lenses at 20x, 40x, and 63x, the minimal z-slice intervals are 0.8, 0.5, and 0.5 microns, respectively. For challenging complex cell model systems and 3D cell models, z-slice stacks are of utmost importance to ensure image quality for image analysis segmentation. To facilitate the image acquisition process, we recommend using PreciScan™ intelligent acquisition, which is available as a plug-in for the Harmony® high-content analysis software.

Image analysis

Q What is an HCS feature?

A A feature in HCS is defined as a piece of digital information with details about the image properties generated from the image analysis segmentation process at the pixel level. Features can include individual pixels, region of interest, or measurements of the properties such as size, shape, intensity, position, and texture. HCS features are generated during the image analysis process and, depending on the complexity of the experiment, there can be as many as hundreds to thousands of individual features.

Q How many cell objects should I count for a robust assay?

A This is a statistical question and the answer has a large variance based on the signal to noise ratio (SNR) of the feature measured, the mean, and standard deviation (SD) of each independent feature. Because the cell painting assay is dependent on all features with unknown SNR, mean, and SD, for a good robust statistical outcome it is recommended to use more than 200 cellular objects - the more the better.

Q How do I deal with cellular autofluorescence and artifacts?

A To mitigate autofluorescence and artifacts in HCS, there are strategies to consider such as identifying an artifact as an outlier using image analysis, i.e., fluorescent intensity above normal threshold. Additionally, it is known that with highly metabolic cells, such as primary hepatocytes, autofluorescence is inherent and notable at around 500 nm. To help overcome this, we recommend using red-shifted probes or identifying quenchers that can help reduce the signal during assay development. However, be aware this modification can confound the cell painting probe selection.

Q What image analysis segmentation features should I measure?

A Feedback from HCS practitioners suggests the nuclear texture feature as one of the most important measurements to discern variability in phenotypic morphology. However, a single feature is not a multivariant analysis approach and does not account for other subtle cellular changes that are measured during image analysis in a cell painting assay. With the cell painting assay, there is an option to measure tens, hundreds, or thousands of features. Deciding which single feature to target is not a recommended strategy, rather, take a holistic approach and allow the algorithms to cluster MOA similarities.

Q Do I need to segment all the compartments during image analysis, in particular nucleoli?

A The advanced morphology features extracted (such as STAR morphology in Harmony® or Signals Image Artist™ software) analyze how intensities, as well as textures, are distributed within a segmented region. Nuclei are easily segmented and hence the use of STAR method-based features will describe how the PhenoVue 512 nucleic acid stain is distributed within the nucleus based on the intensity as well as texture. Therefore, segmentation of the nucleoli is not required.

Data analysis and mechanism of action studies

Q What are the options for secondary data analysis?

A The secondary data analysis following the image analysis and feature extraction process is subjective offering many statistical approaches. These options include the use of a simple z-score to rank the HCS feature data with the most statistical relevance compared to the entire data list or to employ a more comprehensive approach to take full advantage of the multivariant analysis methods such as principal component analysis (PCA) or t-SNE. The large HCS image feature datasets are best handled by tools to compute this type of data, typically outside of MS Excel. We recommend using Revvity's Signals VitroVivo™ platform for secondary analysis that provides straightforward methods to easily import, annotate, and calculate multivariant HCS data directly from Signals Image Artist software platform or from raw data.

Q Can I use multidimensional data for the cell painting assay?

A Yes. The data generated during the cell painting process is multidimensional and can be amplified by using additional secondary tools such as artificial intelligence (AI) or machine learning (ML) during the data analysis process. Reducing the features and dimensionality of the data is critical for a more effective work stream.

Q How do I create morphological profiles for each small molecule to consider readout distributions across different cells and cellular subpopulations?

A You can create morphological profiles at the single-cell level, which allows you to address complex biological questions about the subpopulation identification and responses. A critical bottleneck is analysis time and generation of large datasets for secondary analysis. If you are using a co-culture model, then this may be necessary if the whole well response is not modeling the disease state in question. In monocultures with inherent heterogenicity, the whole well is typically used.

Q How do I decide which HCS features to measure with PCA secondary analysis?

A You don't need to. The algorithm and statistical tools will provide guidance for the most critical key features to include in the analysis. The z-score ranking is useful in seeing the most robust features, but this can include hundreds to thousands of features. Deciding on the most important is not practical nor is it recommended for cell painting assays.

Q How many features are enough or too much to model MOA prediction?

A To begin, we recommend capturing and analyzing all measured HCS features, perhaps thousands, to determine outcomes. Then go back and reduce features to determine the sensitivity, robustness, and variability of the outcome. In practice, most researchers are using between 100 to 300 features, but this will vary depending on the experimental design.

Q Can I use cell painting to predict the MOA of novel compounds using a library of compounds with known MOAs?

A Absolutely, this is the power and design of the cell painting assay. You can fingerprint the known reference control perturbagens with known MOAs with the unknowns to determine an expected MOA that may have a similar on-target effect or even off-target effects in follow-up assays. With all phenotype and morphological profiling assays, follow-up experiments are required to validate the predicted clustered annotated MOA from the cell painting assay.

Q Can I use the cell painting approach to measure targets?

A The probes used in the PhenoVue cell painting kit to phenotypically profile the morphological characteristics of cells can be applied in biomarker discovery or validating a target prior to screening or in confirmation secondary screening assays. The reference control compounds used in the cell painting approach to cluster MOAs can be used to make assumptions on the target, but predicting the target is a more compelling research endeavor that would require validation.

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Revvity provides solutions that span the whole cell painting workflow.
To find out more, visit: www.revvity.com

You can also find a wealth of guidance on cell painting on the JUMP-Cell Painting Consortium website.

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