

Introduction

Cell Painting is an established technique for image-based cell phenotyping with increasing adoption in high-throughput research programs spanning drug discovery, toxicology, and functional genomics. Automation of sample preparation and imaging is critical to conduct Cell Painting assays at scale. Here we present a streamlined three-instrument solution that automates the most recent Cell Painting assay protocol from cell seeding to imaging. We provide a technical overview of bulk liquid handling instrument configuration across multiple steps of the Cell Painting protocol and demonstrate cellular staining, distribution, and retention to ensure sample consistency and reproducibility. We introduce the Agilent BioTek Cytation C10 confocal imaging reader for automated multichannel image acquisition required for the conventional Cell Painting cellular stain combination. Multichannel images were analyzed using an existing open-source CellProfiler software pipeline, demonstrating proof-of-concept compatibility with the growing ecosystem of open-source Cell Painting data analysis tools that leverage traditional and deep-learning solutions. Importantly, this automated platform supports the Cell Painting protocol while remaining flexible to accommodate customized workflows using novel stain and imaging combinations, providing additional value for targeted investigations inspired by the cell painting approach.

Experimental

Cell culture and reagents

Reagents were sourced from Sigma, unless noted. A549 and U2-OS cell lines were acquired from ATCC and cultured in ADMEM media with 10% FBS. Cell Painting reagents were purchased from Thermo Fisher Scientific (part numbers indicated in Table 1).

Cell seeding and treatment

Cells were seeded according to the recommendations in the updated protocol for Cell Painting.¹ A volume of 30 μ L of media containing 85,000 cells/mL was dispensed in 384-well microplates (Agilent part number 204628-100) with the Agilent BioTek 406 FX washer dispenser. After 24 hours of incubation, 10 μ L of media containing DMSO was dispensed to all wells with the Agilent BioTek 406 FX, representing typical treatment vehicle concentration (0.1% DMSO). For test compound addition, the Agilent Bravo automated liquid handling platform performs pipette-based transfers, including plate stamping from compound source plates to live-cell assay plates.

Cell Painting sample processing

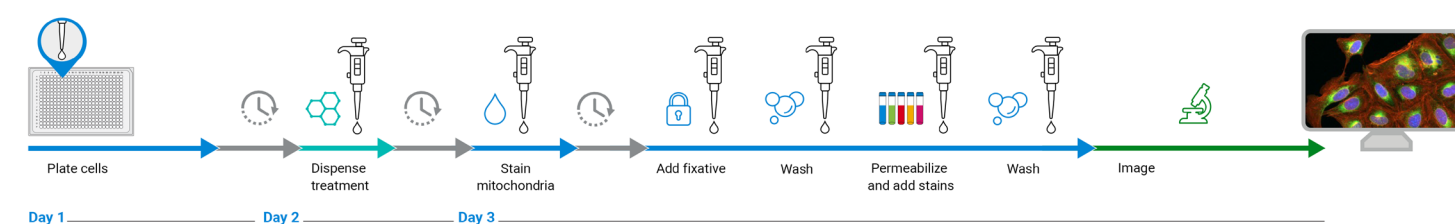
After 24 hours of control treatment, 20 μ L of live-cell mitochondrial dye was dispensed per well with the BioTek 406 FX washer dispenser. Following 30 minutes of incubation, 20 μ L of paraformaldehyde was dispensed with the BioTek 406 FX. Following 20 minutes of room temperature incubation, plates were washed with 80 μ L wash buffer per well, for four cycles with BioTek 406 FX. Wash protocols were optimized to aspirate to the minimal residual volume that maintained cell monolayer coverage, ensuring both washing efficiency and sample retention. Remaining Cell Painting dyes (Table 1) in permeabilization buffer were dispensed at 20 μ L per well with the BioTek 406 FX. After 30 minutes of staining at room temperature, plates received a final wash with the BioTek 406 FX for four cycles, including a final per-well fill volume of 80 μ L for storage and imaging.

Imaging and analysis

Plates were imaged on the Agilent BioTek Cytation C10 confocal imaging reader with a 20x magnification air objective (0.45 NA) using the specified combination of channels outlined in Table 1. Multichannel images were automatically collected at four sites per well, resulting in the evaluation of ~1,500 cells per well. To evaluate cell dispersal and retention, cell counts were analyzed with Agilent BioTek Gen5 software. CellProfiler open-source software (v. 4.2.6) was used to analyze cell painting images using available image analysis pipelines (https://github.com/carpenter-singh-lab/2023_Cimini_NatureProtocols/tree/main/pipelines). Example pipeline single-cell analysis results were aggregated at the well level and visualized with GraphPad Prism software.

Automation solutions supporting a Cell Painting assay workflow

Conventional Cell Painting assay workflow



Agilent automation-enhanced Cell Painting assay workflow



Figure 1. Conventional Cell Painting assay workflow from cell seeding to image collection, and the proposed Agilent automation solutions supporting the various steps of the process.

Results and Discussion

Automated liquid handling validation for Cell Painting sample preparation

Cell Painting protocol step and instrument feature association

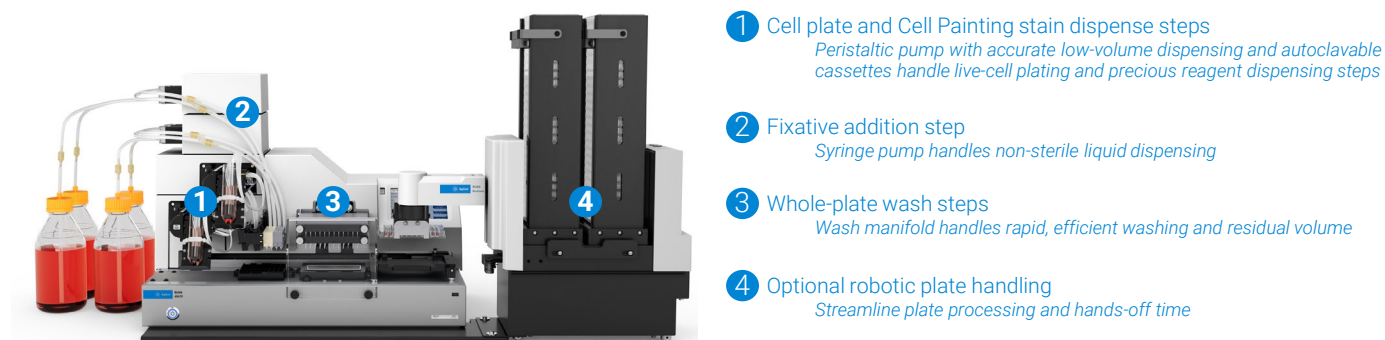


Figure 2. Association of Cell Painting assay sample preparation steps and the associated Agilent BioTek 406 FX washer dispenser features supporting each step. Diagram numbering corresponds to instrument configured with 1) dual peristaltic pump dispensing heads 2) dual syringe pumps 3) wash manifold and 4) optional; the Agilent BioTek BioStack microplate stacker for automated plate delivery.

Cell staining, distribution, and retention analysis

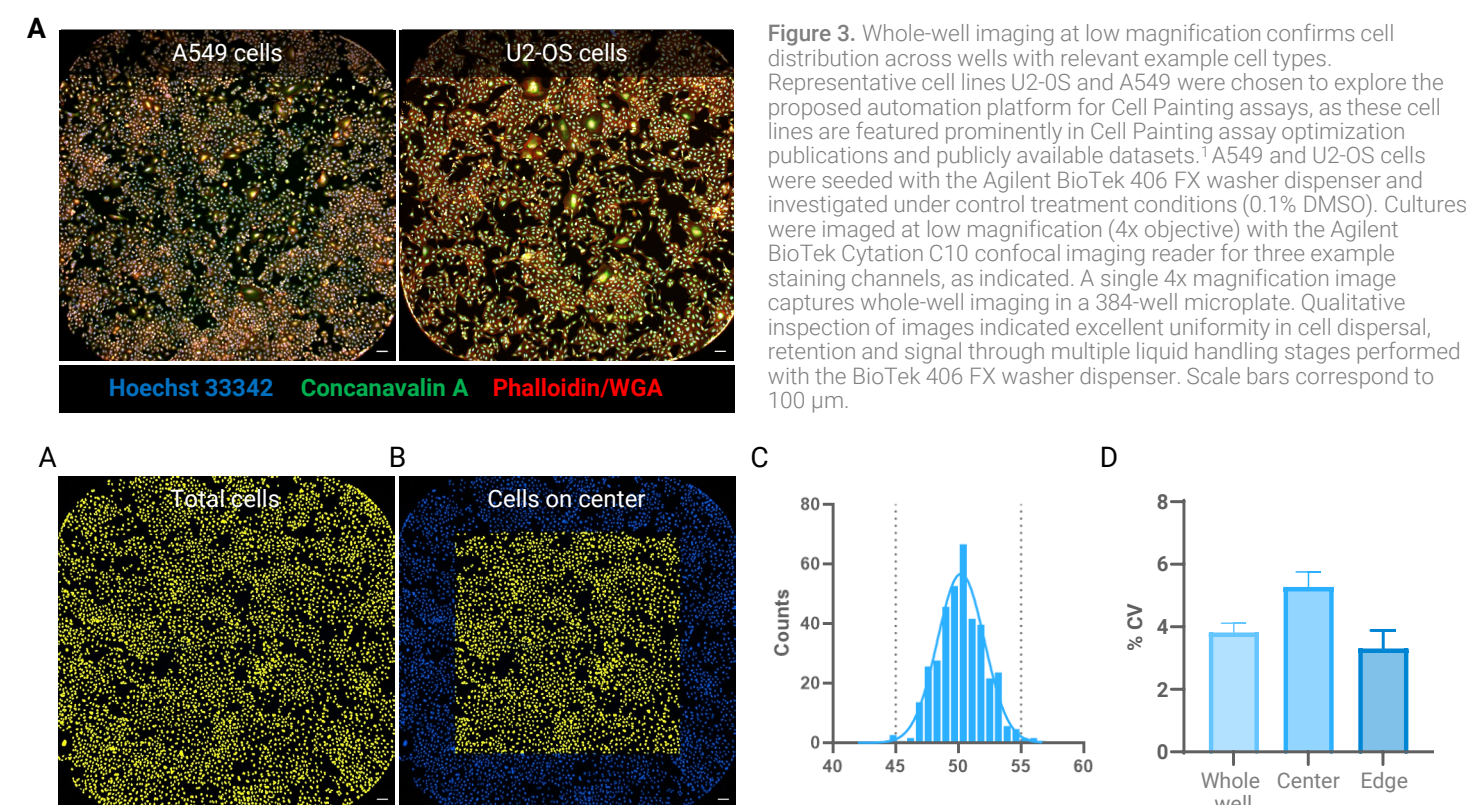


Figure 3. Whole-well imaging at low magnification confirms cell distribution across wells with relevant example cell types. Representative cell lines U2-OS and A549 were chosen to explore the proposed automation platform for Cell Painting assays, as these cell lines are featured prominently in Cell Painting assay optimization publications and publicly available datasets.¹ A549 and U2-OS cells were seeded with the Agilent BioTek 406 FX washer dispenser and investigated under control treatment conditions (0.1% DMSO). Cultures were imaged at low magnification (4x objective) with the Agilent BioTek Cytation C10 confocal imaging reader for three example staining channels, as indicated. A single 4x magnification image captures whole-well imaging in a 384-well microplate. Qualitative inspection of images indicated excellent uniformity in cell dispersal, retention and signal through multiple liquid handling stages performed with the BioTek 406 FX washer dispenser. Scale bars correspond to 100 μ m.

Figure 4. Validation of liquid handling instrumentation through quantitative evaluation of center-to-edge cell distribution and retention. (A) The Agilent BioTek Gen5 software image analysis was used to establish total well cell count (yellow) from 4x magnification images using the nuclear stain of Cell Painting image set. (B) Cell counts were restricted to the center region of the well that was equal to 50% of the total well area (yellow area). The ratio of cell counts in the well center to the whole well was calculated and compared to the expected value of 50%. Scale bar in (A) and (B) corresponds to 100 μ m. (C) Frequency histogram analysis for a 384-well plate summarizing the per-well distribution of cells of center/total converges on the expected 50% value within a narrow (< 5%, dashed lines) error range, indicating cells were evenly distributed at the per-well level and did not demonstrate bias from edge effects or loss of retention from liquid handling steps. (D) Summary of the average per-plate coefficient of variation of the whole well, center, and edge cell distribution analysis indicated excellent performance (\leq 5% CV, N = 4 plates, n = 384 wells/plate) suggesting the Agilent BioTek 406 FX washer dispenser produced consistent well-level cell dispersal and retention for sample process automation in Cell Painting assays.

Automated multichannel imaging for conventional Cell Painting assay

Cell Painting imaging configuration on the Agilent BioTek Cytation C10 confocal imaging reader

Table 1. Summary of conventional stain set and corresponding image collection channels on the Agilent BioTek Cytation C10 confocal imaging reader.

Cell Painting Stains and Channels					
Cell Painting Stain Mix Component	Stain Source (Thermo Fisher Scientific)	Cellular Compartment/s	Channel	Mode	Well Image Positions
MitoTracker Deep Red	PN M22425	Mitochondria	Cy5	Confocal	
Alexa Fluor 568 Phalloidin, Alexa Fluor 555 WGA	PN A12380, PN W32464	F-actin cytoskeleton, Golgi, plasma membrane	TRITC	Confocal	
SYTO 14 Green	PN S7576	Nucleoli, cytoplasmic RNA	RFP	Widefield	
Alexa Fluor 488 Concanavalin A	PN C11252	Endoplasmic reticulum	GFP	Widefield	
Hoechst 33342	PN H3570	Nucleus	DAPI	Widefield	
Label-Free (No Stain)	N/A	Label-free cellular analysis	Brightfield	Widefield	

Results and Discussion

Multichannel image acquisition across Cell Painting stains

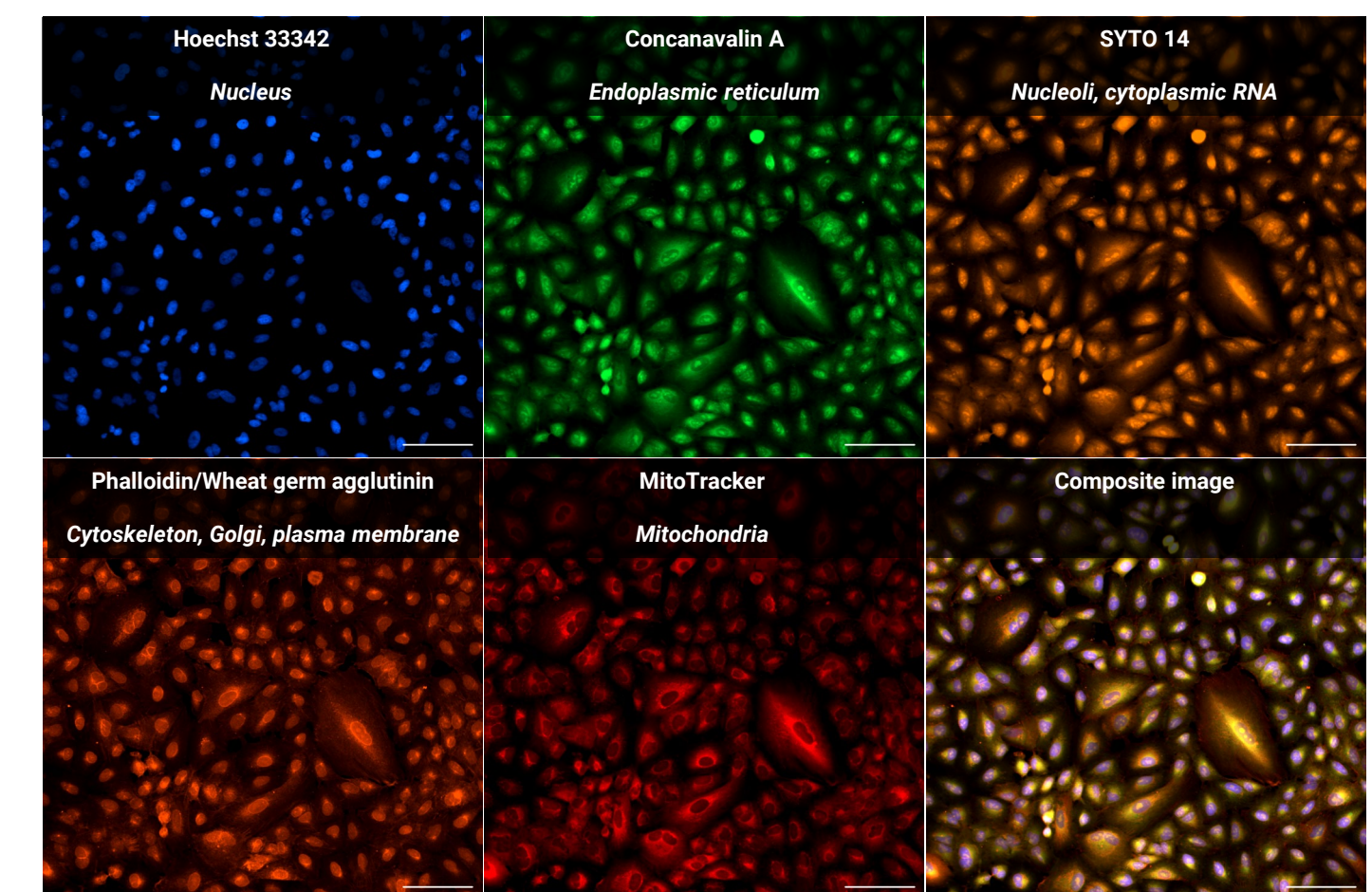
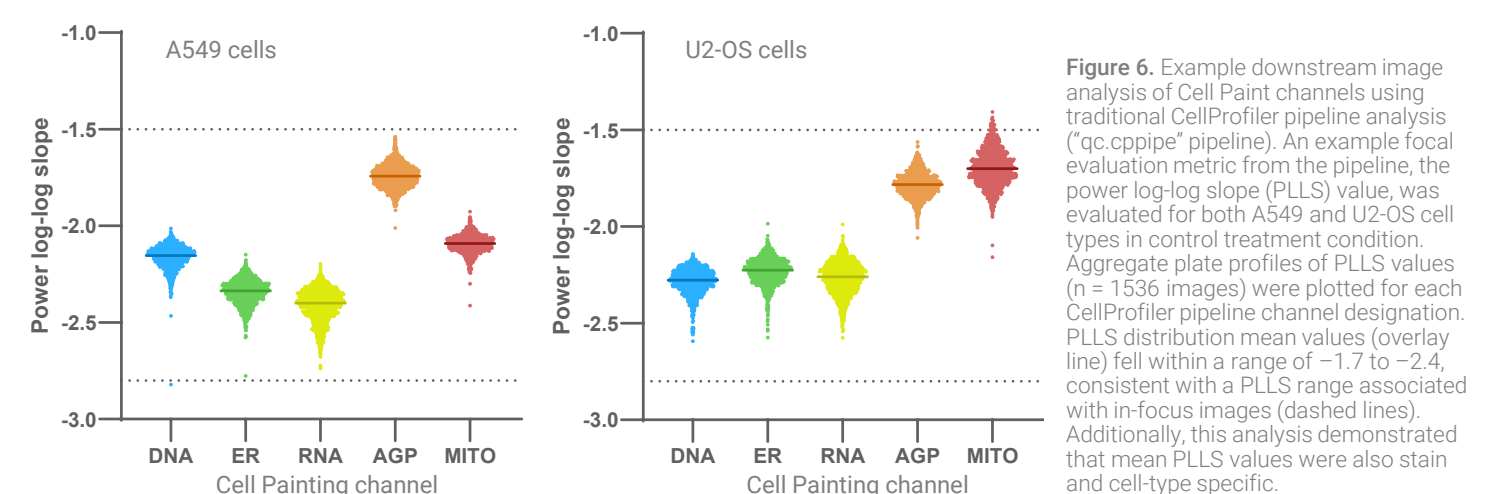


Figure 5. Example multichannel images of A549 cells stained with conventional Cell Painting stains and imaged on the Agilent BioTek Cytation C10 confocal imaging reader. Stain component identity and associated labeled subcellular compartments are indicated for each imaging channel. Individual image channels were acquired on the BioTek Cytation C10 with the channel settings as indicated in Table 1. At 20x magnification, qualitative inspection of images indicated expected subcellular compartmentation characteristics associated with individual stains and stain combinations. Scale bars correspond to 100 μ m.

Image compatibility in downstream analysis



Supports traditional and AI-based Cell Painting analysis open-source workflow

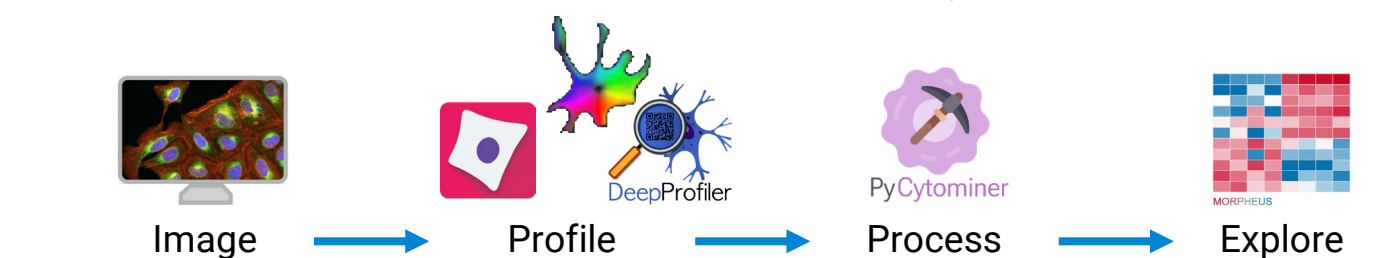


Figure 7. Example workflow highlighting traditional and AI/ML open-source toolkits specifically designed for scaled cellular phenotypic profiling with Cell Painting assay image data.

Conclusion

Agilent automated solutions provide an efficient instrumentation platform for Cell Painting from sample preparation to image acquisition. Cell seeding, bulk liquid dispensing, plate washing, drug dispensing, and automated fluorescence and brightfield imaging come together to deliver improved reproducibility and efficiency over standard approaches. Images are compatible with the evolving open-source software landscape for downstream segmentation and cell profiling analysis methods.

References

Cimini, B.A., Chandrasekaran, S.N., Kost-Alimova, M. *et al.* Optimizing the Cell Painting Assay for Image-Based Profiling. *Nat Protoc* 2023, 18, 1981-2013.