APPLICATION NOTE

Phenotypic analysis of CRISPR-Cas9 cell-cycle knockouts using cell painting.

Key features

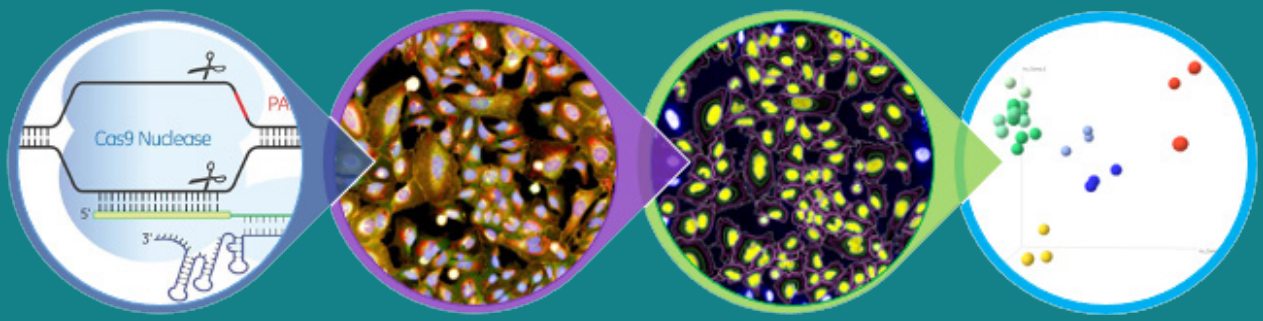
- Workflow for an arrayed CRISPR knockout screen using Cas9 stable cells and synthetic single guide RNAs
- Phenotypic analysis of gene knockouts with the PhenoVue™ cell painting JUMP kit
- One step solution for cell painting feature extraction in Harmony® image analysis software
- Phenotypic clustering using Principal Component Analysis and Self-Organizing Maps

Introduction

Gene editing using CRISPR (clustered regularly interspaced short palindromic repeats) technology has revolutionized the field of functional genomics by enabling site-specific permanent DNA edits (knockouts or knock-ins). With CRISPR-Cas9, gene editing has become much easier, providing researchers a tool to investigate gene functions in almost any cell type and with high confidence.[1, 2]

Cell painting is a phenotypic high-content assay for multiplexed cell analysis that labels various cell organelles and provides unbiased morphological profiling of chemical or genetic modifications. Combining CRISPR gene editing and cell painting provides researchers with the ability to correlate genotypes with phenotypes and holds the promise to help understand gene functions in more detail and at greater scale. Using cell painting, hundreds or even thousands of phenotypic features are extracted per cell. These feature sets or morphological profiles are then correlated between wildtype and knockout conditions to enable the identification of genes contributing to a disease or encoding a certain cell function or pathway. However, this generates vast amounts of raw image data and feature data that need to be handled and interpreted to be able to discover the final insights.[3-7]

For research use only. Not for use in diagnostic procedures.



Here, we present a harmonized workflow for morphological profiling of CRISPR knockouts using the cell painting assay (Figure 1). In Revvity's stably Cas9-expressing cells, four cell cycle regulating genes were knocked out using Edit-R™ CRISPR synthetic single guide RNAs, followed by labeling the cells with the PhenoVue™ cell painting JUMP kit. Finally, cells were imaged and analyzed on the Opera Phenix® Plus high-content screening system with Harmony® software and phenotypes clustered with Signals VitroVivo™ analysis software.

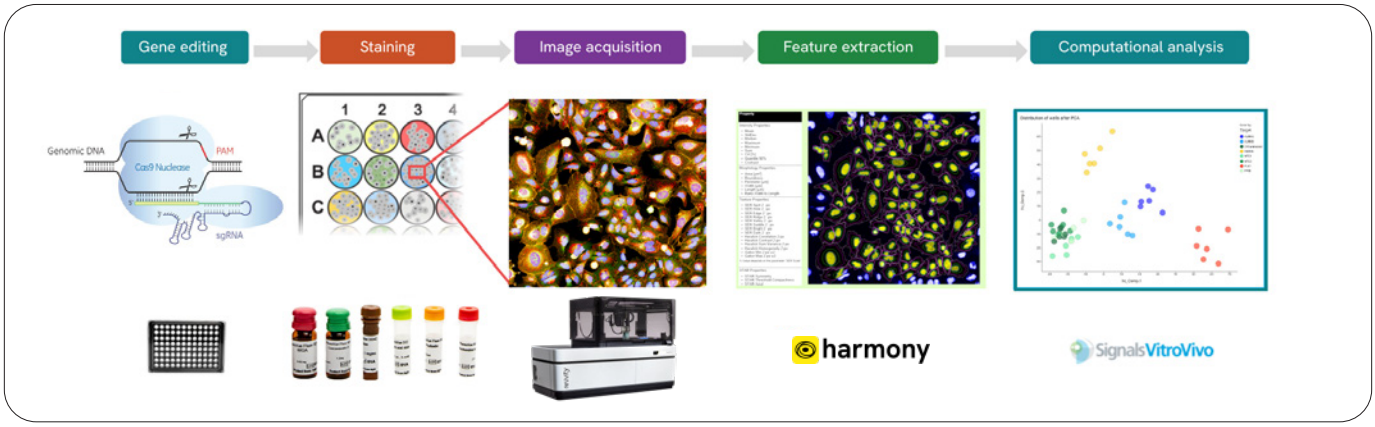


Figure 1: Harmonized workflow for arrayed CRISPR knockout screening using cell painting. U2OS cells stably expressing Cas9 were seeded into PhenoPlate™ 96 well microplates and four different cell cycle regulating genes (Aurora kinase A and B, geminin, and polo-like kinase 1) were knocked out using algorithm-designed Edit-R synthetic single guide RNAs. Cells were stained with the PhenoVue cell painting JUMP kit which comprises six fluorescent probes for labelling eight cell components (DNA, cytoplasmic RNA, nucleoli, actin, Golgi apparatus, plasma membrane, endoplasmic reticulum, and mitochondria). Imaging was performed on the Opera Phenix Plus high-content screening system. Images were then analyzed with Harmony high-content analysis software using a cell painting analysis building block and phenotypes clustered with the High Content Profiler app of Signals VitroVivo.

Materials and methods

Table 1: List of materials, instruments, and software used in phenotypic knockout assay.

Cells	U2OS CAG-Cas9 (chicken beta actin hybrid promoter driving CRISPR associated protein 9) stable cell line (generated with lentiviral particles, Revvity # VCAS10129)
Growth medium	DMEM high glucose (Cytiva # SH30081.FS) 10% Fetal bovine serum (Cytiva # SH30285.02) L-Glutamine (Gibco #25030081)
CRISPR-Cas9 knockout	DharmaFECT™ 4 transfection reagent (Revvity # T-2004-03) Dharmacon Edit-R synthetic single guide RNAs (sgRNAs) targeting cell cycle genes or controls • Lethal control 1 (targeting multiple repeat regions in the genome) (Revvity # U-008000-01-05) • Lethal control 2 (targeting multiple repeat regions in the genome) (Revvity # U-008000-02-05) • Non-targeting negative control 1 (Revvity # U-009501-01-05) • Non-targeting negative control 2 (Revvity # U-009502-01-05) • Negative control PPIB (Peptidyl-prolyl cis-trans isomerase B) (Revvity # UK-009050-01-05) • Aurora kinase A (Revvity # SQ-003545-01-0002) • Aurora kinase B (Revvity # SQ-003326-01-0002) • Geminin (Revvity # SQ-003270-01-0002) • Polo-like kinase 1 (Revvity # SQ-003290-01-0002)
Microplates and sealing	PhenoPlate 96-well (Revvity # 6055300) Pierce heat seal (4titude # 4ti-0531)
Cell painting kit	PhenoVue cell painting JUMP kit - 1 x 384 wells (Revvity # PING21)
Imaging instrument	Opera Phenix Plus high content screening system (Revvity # HH14001000)
Secondary analysis	High Content Profiler App in Signals VitroVivo (Revvity # INF02033-B)

Cell culture and CRISPR-Cas9 knockout

Adherent U2OS (osteosarcoma) cells were transduced with Edit-R lentiviral Cas9 nuclease expression vector to generate a cell line stably expressing Cas9 (CRISPR associated protein 9) under a CAG (chicken beta actin hybrid) promoter. Cells were positively selected with blasticidin to generate a pool of U2OS CAG-Cas9 cells. Next, cells were plated at 1250 cells/well in 100 µL growth medium into two PhenoPlate-96-well microplates. The following day, cells were reversely transfected with synthetic Edit-R sgRNAs (25 nM) targeting four cell cycle regulating genes (Aurora kinase A and B, Geminin, Polo-like kinase1 (PLK1)) as well as control targets (negative and lethal controls) using 0.03 µL DharmaFECT 4 transfection reagent per well. To efficiently knockout the four cell cycle target genes, three sgRNAs targeting the same gene were pooled per well. CRISPR editing efficiency was assessed by T7E1 endonuclease assay.

Cell painting

Cell painting was performed according to instructions provided with the PhenoVue cell painting JUMP kit and described by Cimini et al.[4] In brief, live cells were stained with 500 nM PhenoVue 641 mitochondrial stain, followed by fixation with 4% PFA (paraformaldehyde). After washing, a mix of 0.1% Triton X-100, 1.5 µg/mL PhenoVue Fluor 555 – WGA, 5 µg/mL PhenoVue Fluor 488 - Concanavalin A, 8.25 nM PhenoVue Fluor 568 – Phalloidin, 1.62 µM PhenoVue Hoechst 33342 nuclear stain, and 6 µM PhenoVue 512 nucleic acid stain was applied to the cells. Eight cell components (DNA, cytoplasmic RNA, nucleoli, actin, Golgi apparatus, plasma membrane, endoplasmic reticulum, and mitochondria) are stained with six dyes. Finally, plates were heat sealed and stored at 4 °C until measurement.

Image acquisition

IImages were acquired on the Opera Phenix Plus high-content screening system in confocal mode using a 20x water immersion objective. Three fields of view were measured per well, each with a 1 µm-spaced stack of 4 planes. The six cell painting stains were measured in five channels (Figure 3).

Feature extraction and secondary analysis

To extract phenotypic features (such as morphology, texture, intensity), *Maximum Intensity Projection* images of 4 planes were analyzed in Harmony software using the dedicated *Calculate Cell Painting Properties* building block in standard setting (Figure 2).

Downstream analysis of morphological profiles was done by importing feature data into the High Content Profiler app of Signals VitroVivo. A reduction of dimensionality was achieved by analyzing only a subset of features (1924 features). Phenotypic clustering was performed with a Principal Component Analysis (PCA) and a Sammon Connections Network.

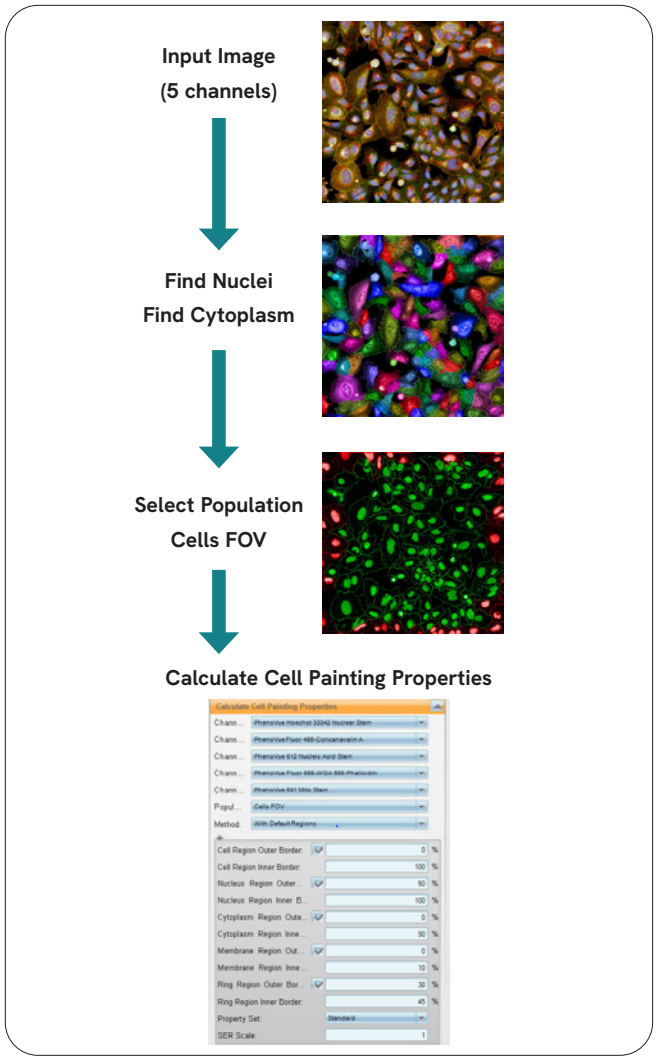


Figure 2: Feature extraction in Harmony software using the dedicated *Calculate Cell Painting Properties* building block. In total, only four building blocks are needed to extract up to 5931 cell painting properties per cell. The Cell Painting building block automatically creates different cell regions and calculates the corresponding morphology, intensity, and texture features within them.

Results and discussion

All knockouts of the four cell cycle regulators strongly affect the cell number. Compared to the control, the knockouts show up to 10x less cells per well, meaning that control wells are much denser, and cells have less space. This leads to much smaller cells in the control, while knockout cells are larger (Figure 3).

While many phenotypical changes remain invisible to the human eye, changes in cell proliferation are the most apparent difference after treatment (Figure 4).

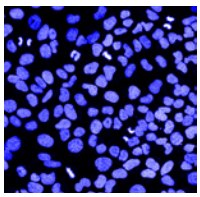
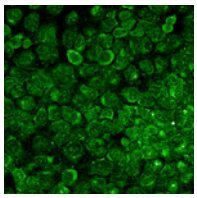
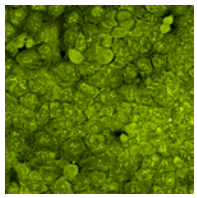
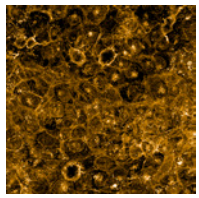
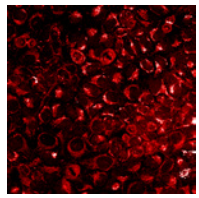
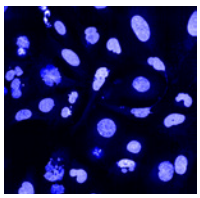
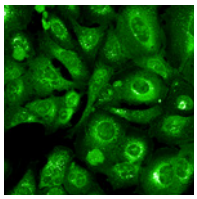
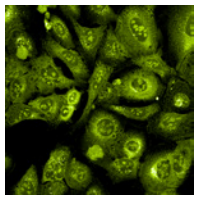
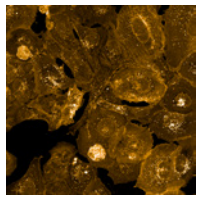
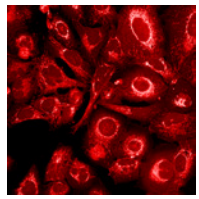
Cell painting Dyes and targets	PhenoVue Hoechst 33342 Nuclear stain	PhenoVue Fluor 488 concanavalin A Endoplasmatic reticulum	PhenoVue 512 nucleic acid Nucleoli-RNA	PhenoVue fluor 555-WGA + PhenoVue Fluor 568-Phalloidin Plasma membrane, Golgi app., actin	PhenoVue 641 mitochondrial stain Mitochondria
Untreated control					
CRISPR knockout Aurora kinase A					
Opera Phenix Plus	Ex 375 nm Em 435-480 nm	Ex 488 nm Em 500-530 nm	Ex 488 nm Em 515-550 nm	Ex 561 nm Em 570-630 nm	Ex 640 nm Em 650-760 nm

Figure 3: Knockout of Aurora kinase A inhibits cell proliferation visualized in a cell painting assay. The six cell painting dyes were acquired with the indicated excitation and emission settings in confocal mode on the Opera Phenix Plus system using a 40x water immersion objective (here, for visualization reasons a higher magnification then in the assay run was used). Each image shows one field of view in a maximum intensity projection of four planes, with distance between planes being 1 µm.

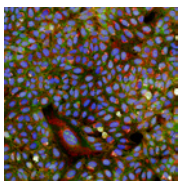
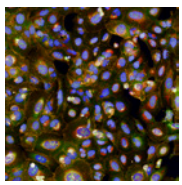
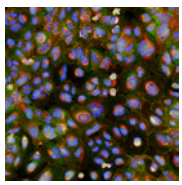
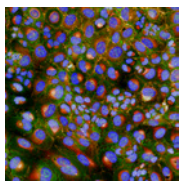
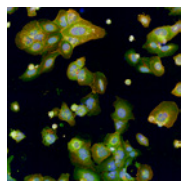
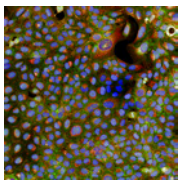
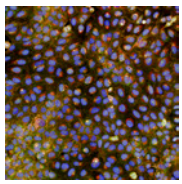
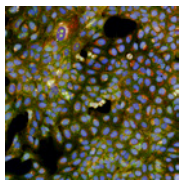
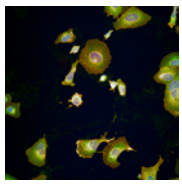
Untreated control	Aurora kinase A knockout	Aurora kinase B knockout	Geminin knockout	PLK 1 knockout
				
PPIB	NTC 1	NTC 2	Lethal 1	Lethal 2
				

Figure 4: CRISPR-Cas9 knockout of cell cycle regulators leads to distinct phenotypic changes in a cell painting assay. Knock out of Aurora Kinase A, B, Geminin and Polo-like Kinase 1 (PLK 1) has a clear effect on cell proliferation and results in reduced cell densities. Untreated control wells, non-targeting control wells (NTC, negative controls) 1 and 2, and PPIB knockout wells show the highest confluency. Lethal controls 1 and 2 have only a few cells left per well. They serve as quality control for CRISPR-Cas9 gene editing. Images were acquired on the Opera Phenix Plus system using a 20x water immersion objective. Each image shows one field of view in a maximum intensity projection of four planes.

The effect of the knockouts on cell cycle regulation can be assessed by comparing the average cell number per field of view (FOV) (Figure 5). While the controls (NTC 1 and 2, untreated and PPIB) have around 1000 cells per FOV, the average number of cells in cell cycle knockouts (AURK A and B, geminin, and Pololike kinase 1) is clearly reduced by up to a factor of 10.

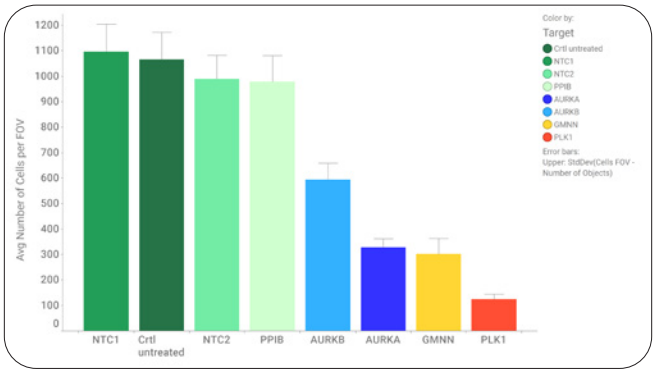


Figure 5: CRISPR-Cas9 knockout of cell cycle regulators leads to reduced average cell counts. While the four cell cycle genes have distinct functions within the cell cycle, all knockouts negatively affect cell proliferation (n=6 wells, 3 wells per plate).

To visualize morphologic profiles and cluster phenotypes based on their similarities or differences, a downstream analysis was done using the High Content Profiler app of Signals VitroVivo. To exclusively focus on phenotypes and not the cell number, the average cell count was excluded from further secondary analysis. Additionally, features from the membrane and ring region were excluded as they were not useful in this case (Figure 6).

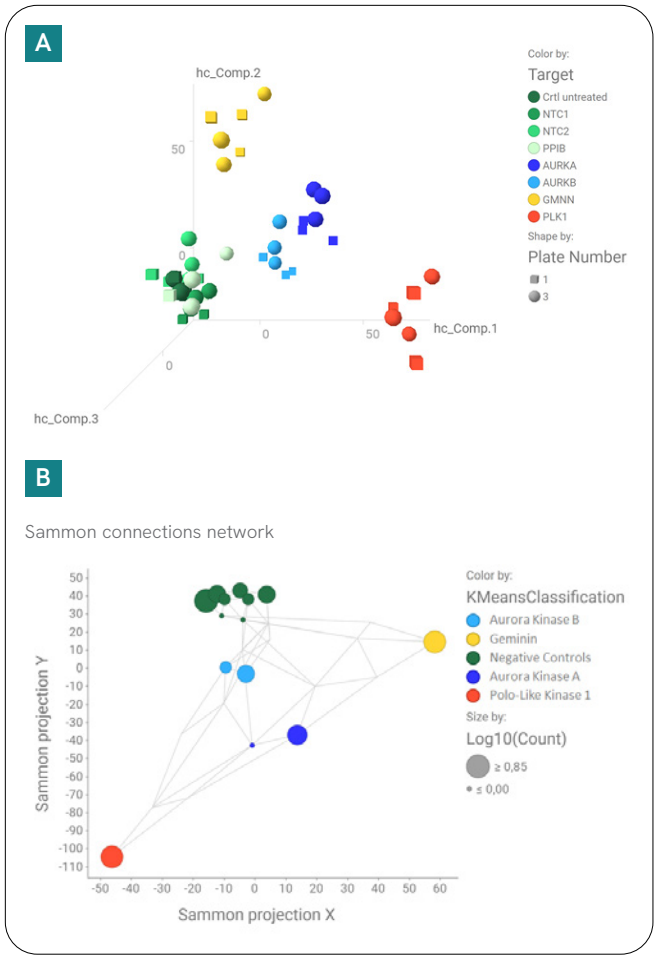


Figure 6: Phenotypic analysis of CRISPR-Cas9 cell cycle knockouts in Signals VitroVivo results in clearly separated knockout and control-specific clusters.
A: Principal Component Analysis (PCA): In PCA, the initial, large cell painting feature data set is reduced to a 3-dimensional coordinate system to describe variation between the knockout phenotypes using the first three principal components. Here, we can see target- and control-specific clusters from two independent plates, with data points shaped according to plate number.
B: Self-Organizing Map (SOM) and Sammon Connections Network (k-means clustering)[8]: In a Self-Organizing Map and the Sammon Connections Network an artificial neural network clusters similar phenotypes together. If two data points have extremely similar profiles, they will be clustered together in the same node. The size of the nodes is log10 of the number of wells grouped in that node. The farther away two nodes are, the more dissimilar they are to each other. AURK A and B knockouts show few wells with a slightly different phenotype, hence they were grouped into two nodes. The Sammon Connections Network plot is used to visualize the results of the SOM in a two-dimensional way.

Conclusions

Here, we have shown a workflow for phenotypic analysis of CRISPR-Cas9-mediated knockouts using the cell painting assay. Cell painting provides an unbiased method for morphological profiling and allows correlation of phenotypes of different cell cycle regulator gene knockouts and controls based on their similarities or differences.

Revvity offers and supports all tools and reagents required for a harmonized screening workflow from one source: Cas9-stable cell lines (or lentiviral particles to modify your own cell line), Edit-R synthetic sgRNAs, cell painting reagents, high-content imaging instruments and dedicated cell painting analysis and phenotypic clustering software tools.

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