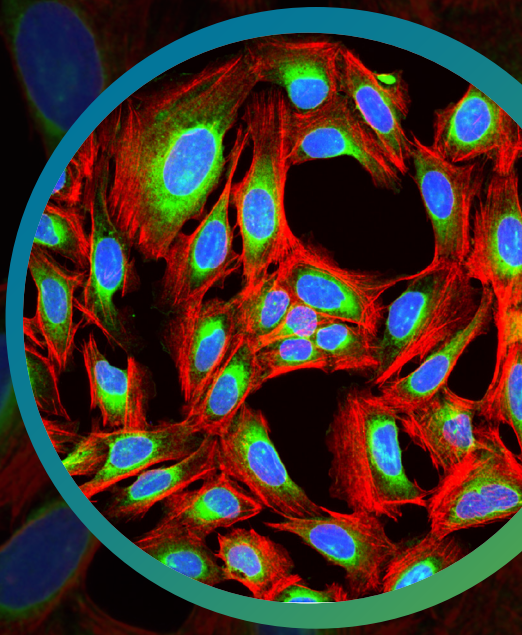
APPLICATION NOTE

High-content phenotypic profiling using the Cell Painting assay

Angeline Lim, PhD | Applications Scientist | Molecular Devices
Christopher Nishioka | Field Applications Scientist | Molecular Devices
Misha Bashkurov | Product Owner | Molecular Devices



Introduction

High-content phenotypic profiling is increasingly popular in research areas that span from gene function studies, drug discovery, and toxicology. The strength in this approach lies in the unbiased, multidimensional information captured at single cell resolution which enables individual cell states to be analyzed, profiled, and compared to population-level data. This profiling approach is in contrast to the more common phenotypic screens where only a few parameters are selected a priori, typically due to significance in a pathway of interest[1]. As a result, most of the biologically relevant changes caused by experimental treatments are often disregarded in conventional phenotypic screens.

The phenotypic profile of a cell is made up of hundreds to thousands of quantifiable features that are derived from the cell state. These features can include information extracted from biomarker expression, texture, and distribution. Often, these markers are specific to organelles and give information about their structure, spatial organization, and relationships to other sub-cellular structures. The assortment of information from all cells allows for an unbiased approach in studying effects of chemicals, small molecules, or genetic perturbations. Compounds with similar mechanisms of action (MOA) often lead to similar changes in cell morphology. Thus, comparisons between phenotypic profiles can provide insights into MOA for novel compounds[3]. Similarly, genetic perturbations in the same pathway often lead to similar phenotypic profiles, which suggests that phenotypic profiling can be used in high-throughput functional genomics studies[7].

Benefits

- Simplify your Cell Painting assay workflow using the ImageXpress Micro Confocal system
- Carry out unbiased image analysis using a robust software with machine learning capabilities
- Rapidly analyze large multidimensional datasets with a user friendly, web-based platform

 |

The Cell Painting assay, often used in phenotypic profiling, uses up to six fluorescent dyes to label and visualize a variety of subcellular structures at the single cell level. The aim of this assay is to visualize as much of the cell as possible in order to construct a representative image of cell state. The standard set of dyes for the Cell Painting assay labels the nuclei, endoplasmic reticulum, actin, Golgi apparatus, RNA (and nucleoli), as well as mitochondria. High-content cellular imaging systems equipped with suitable filter sets are used to rapidly acquire images of the fluorescently-labeled cells. This is followed by automated image analysis to identify, extract, and measure specific cellular features. The resulting set of measurements comprise the phenotypic profile, which can be further analyzed and used for hit picking or cluster analysis (Figure 1).

Here, we show a high-content phenotypic profiling workflow based on the Cell Painting protocol by Gustafsdottir et al[3]. This workflow utilizes easy to use tools without sacrificing quality – image segmentation and measurements are performed within IN Carta™ Image Analysis Software and data analysis in HC StratoMiner™. Within IN Carta software, the image analysis routine can be adjusted to achieve robust detection of cells and organelles. The deep learning semantic segmentation module (SINAP) can be used to improve detection of challenging features. Pre-trained deep learning models are available for the detection of nuclei or cells. In addition, users can train their own models based on their specific object of interest with their own dataset. HC StratoMineR is a web-based tool developed for processing large multi-dimensional datasets. The platform features a guided step-by-step workflow for high-content data analysis. Because HC StratoMiner functions as a web-based tool accessible through a web browser, users do not need additional computational resources that is often required to process large datasets. Using this workflow, we find that cells treated with the same compound show similar phenotypic profiles. Hierarchical clustering analysis grouped highly toxic compounds such as paclitaxel and rotenone together. Chloroquine and tetrandrine, both of which affect autophagy,[2,4] were also found in the same cluster. These results demonstrate that the proposed workflow is a user-friendly and robust approach for performing high-content phenotypic profiling.

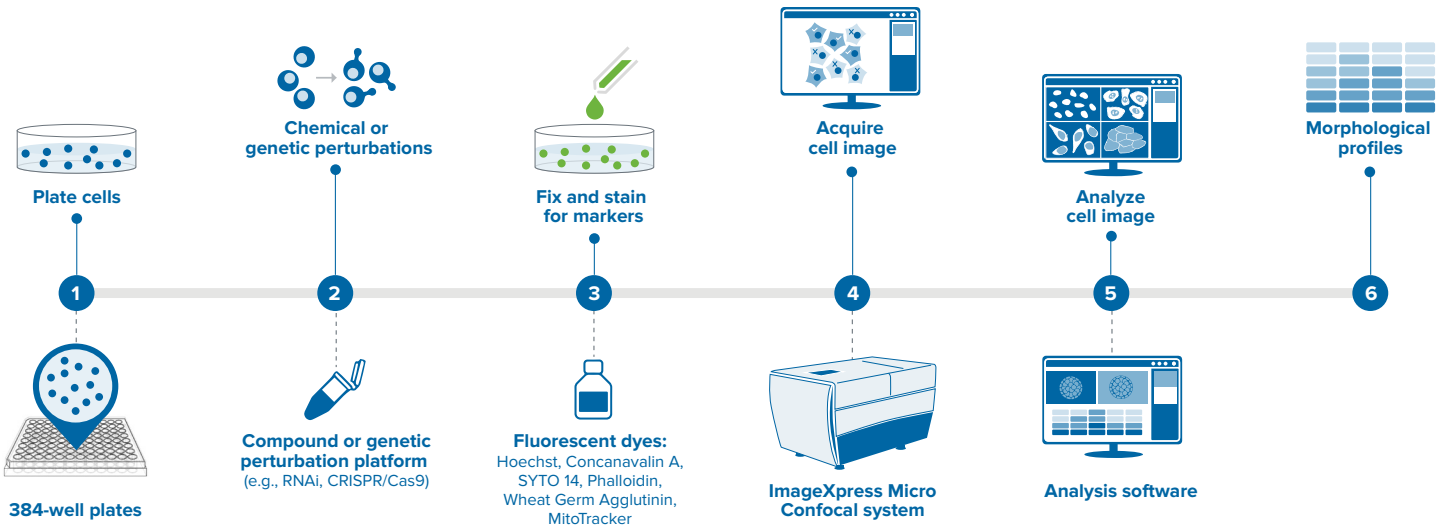


Figure 1. *Typical Cell Painting assay workflow. The Cell Painting assay is a morphological profiling tool that generates multiparametric profiles at single cell resolution. It is highly versatile and can be adapted to different cell lines. A standard workflow will include seeding cells at suitable density followed by the desired treatment or perturbation. Next, various cellular structures are stained with the appropriate dyes and imaged using a high-content imaging system. Image analysis provides hundreds to thousands of measurements which form the cells' phenotypic/morphological profile.*

Methods

Cell culture

U2OS cell line (ATCC) was passaged and maintained according to the manufacturer's recommendations. The Cell Painting assay was performed according to Bray et al[1]. Briefly, U2OS cells were seeded in Greiner 384-well µClear plates at 2000 cells per well in a total of 40 µL of McCoy media (supplemented with 10% FBS). Cells were incubated at 37°C for 24 hours before compound treatment.

The culture medium was replaced with 2% (vol/vol) FBS in McCoy 24 hours after seeding and before compounds were added. The following 11 compounds were used here: Ca-074-Me, CCCP, chloroquine (Enzo), cytochalasin D, etoposide (Calbiochem), latrunculin B, rapamycin (Sigma), rotenone (Enzo), staurosporine, paclitaxel, and tetrandrine (unless indicated, all compounds were purchased from SeleckChem). Compounds were tested in quadruplicate wells in a seven point, 1:3 dilution series. DMSO controls, negative, and positive controls were included in the same plate. Cells were incubated with the compounds for 24 hours.

Staining

Live cells were stained with MitoTracker DeepRed (500 nM) for 30 min in the dark at 37°C, then fixed with PFA (3.2% vol/vol) for 20 min. Cells were washed and then permeabilized with triton-100 (0.1%) at room temperature for 20 min. Staining solution was prepared at the following concentrations: 5 µL/mL phalloidin, 100 µg/mL concanavalin A, 5 µg/mL Hoechst, 1.5 µg/mL WGA and 3 µM SYTO 14 dye in blocking solution (1X HBSS and 1% wt/vol BSA). Cells were washed and incubated with the staining solution for 30 min at room temperature. Staining solution was removed and cells were washed three times and then sealed with adhesive foil. All wash steps were performed with 1X HBSS.

Image acquisition

Images were acquired using the ImageXpress® Micro Confocal High-Content Imaging System (Molecular Devices) using the 20X Plan Apo objective, confocal pinhole size at 60 µm. The following filters were used (ex/em): DAPI 377/447, FITC 475/536, TRITC 543/593, TexasRed 560/624, Cy5 631/692. Four field of views were imaged per well. A small Z-stack of three images were acquired with best focus projection option used to account for plate flatness issues which may compromise image focus.

Feature extraction

Image analysis was carried out using IN Carta software. The image segmentation protocol was set up as follows: Hoechst-stained nuclei were segmented as a primary target using the custom segmentation (pre-trained Nuclei model). Objects touching edges were excluded. Cells were segmented within TRITC channel using the Robust option. Three additional organelle classes were segmented with the indicated option: Mitochondria (networks), actin (fibers) and endoplasmic reticulum (networks). A total of two hundred eighty measurements per cell were selected as an output.

Data analysis

After feature extraction was completed, cell level data was exported into csv format and uploaded into HC StratomineR (CoreLife Analytics) along with a text file that contains metadata with compound information. The plate map was defined within the StratoMineR interface. Using the Quality Control tab, outlier wells were removed from analysis (wells with less than 50 cells were removed). Data transformation was performed as recommended followed by feature scaling. Principal component analysis (PCA) was used for data reduction. The resulting 15 components were used to calculate the distance score which is defined as a measure of phenotypic effect of the treatment on the cells in those wells[6]. The distance score can then be used in hit selection and hierarchical clustering.

Results

Cells were treated with 11 compounds, some of which have been used in previous Cell Painting studies as reference compounds[5]. Following compound treatment, cells were stained for mitochondria, actin, the Golgi apparatus, nucleoli (RNA particles), endoplasmic reticulum (ER), and the nuclei (Figure 2).

IN Carta software was used to extract all the various cellular features from each detected cell. The software has A. an intuitive user interface B. provides measurements that are important for phenotypic profiling including intensity, texture, shape, spatial coordination (such as spatial relationships between organelles), and co-localization C. robust feature identification with the deep learning semantic segmentation module (SINAP) that allows for unbiased feature segmentation. These models can be further trained on images based on user specific objects of interest.

The SINAP module was used in the analysis protocol to improve nuclei identification (Figure 3). The built-in nuclei model was trained on over 1000 images. Training set included nuclei acquired using imaging modalities (fluorescence and brightfield) at different magnifications and stained with different dyes (DAPI, Hoechst, Hematoxylin/Eosin), therefore making it suitable for use on a large variety of images. During image analysis set up, we observe that the nuclei model gives improved nuclei detection and was able to accurately split touching nuclei. The SYTO14 stain was used to define the cytoplasm using the 'Robust' segmentation method. The ER, mitochondria, and actin were also segmented and analyzed. In addition, overall intensity measurements were taken from all channels in both the whole cell, nuclei and cytoplasm.

The analyzed features can be easily reviewed within IN Carta software. Options such as heatmap display and histograms are available for general data exploration. Cell-by-cell or summary data can be exported as a csv file for downstream data mining and analysis (Figure 4).

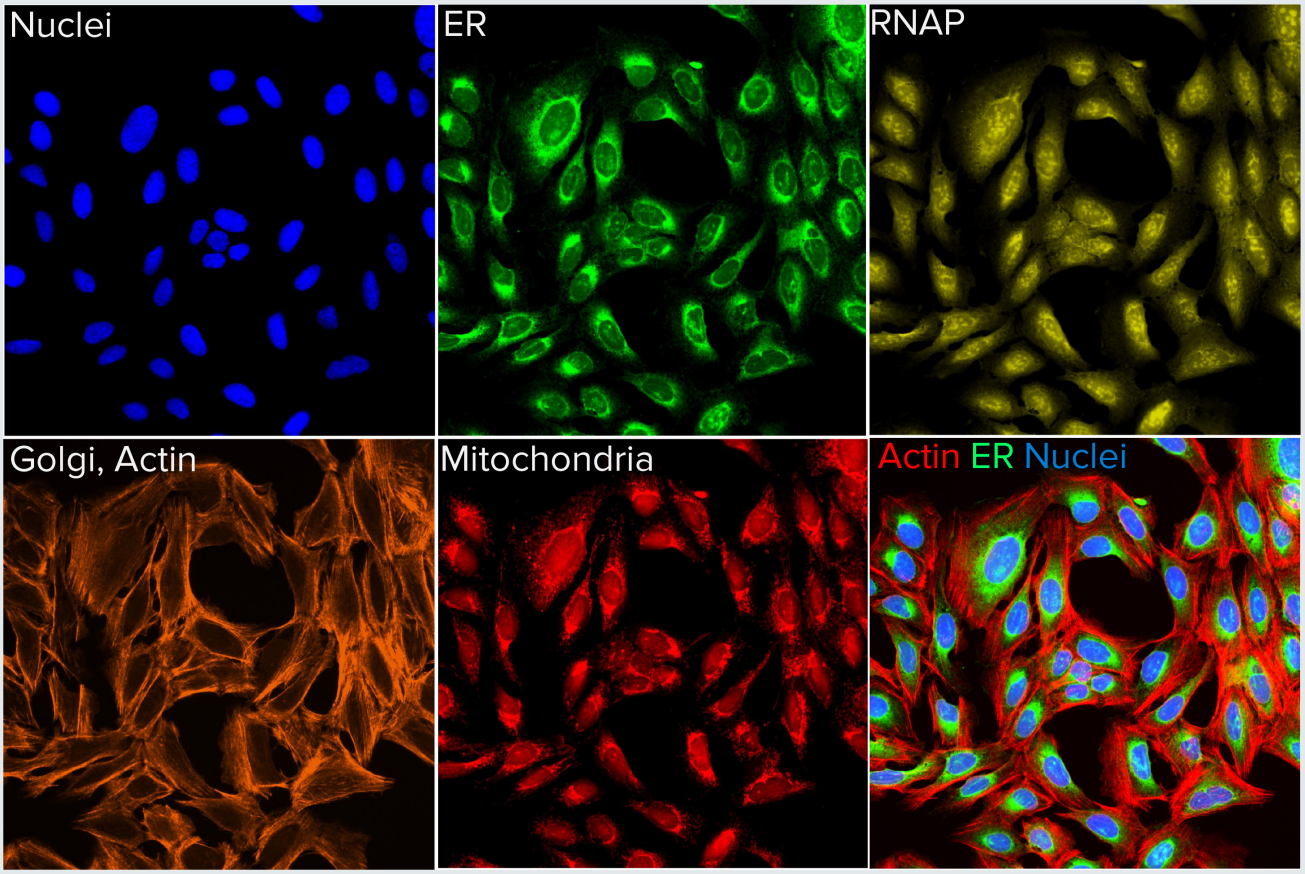


Figure 2. *Cell Painting assay. Cells were compound treated, stained, then imaged using the ImageXpress Micro Confocal system. Example images of each acquired channel from a control well is shown. The last panel shows a composite image consisting of actin, ER (endoplasmic reticulum), and nuclei staining.*

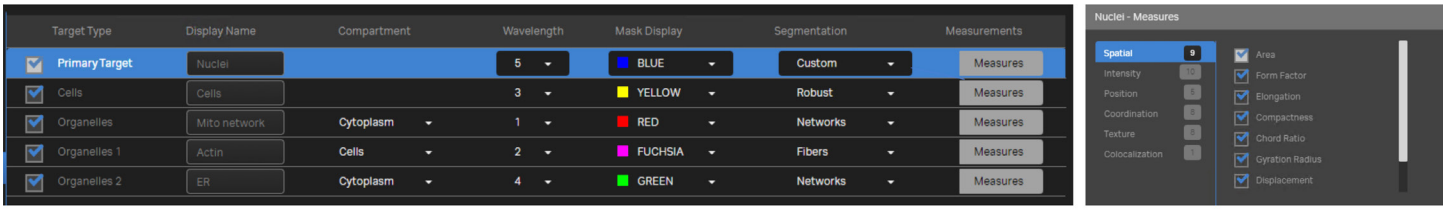
 |

Data analysis

Measurements obtained from IN Carta software were exported and then uploaded into HC StratoMiner for further data analysis. Here, we used HC StratoMineR (a web-based high-content dataset tool), due to its ease-of-use, where the guided platform allows non data-science experts to navigate the data analysis workflow. Additionally, because HC StratoMineR is a web-based tool, there is no need for the end user to configure and set up additional computational resources required for analysis of large and complex datasets.

Principal component analysis (generalized weighted least squares) was used to reduce the 280 measurements into 15 components. Hierarchical clustering was then carried out, and the relationship between each well represented in a dendogram (Figure 5). Cells treated with the same compounds tend to be clustered together. For example, cluster 9 is made up of only staurosporine treated cells. Compounds known to have similar cellular effects were also clustered together. Cytochalasin D and latrunculin B, both actin polymerization inhibitors were found in cluster 6. Tetrandrine and chloroquine, both known to affect the autophagy pathway, were also clustered together (cluster 5). These results demonstrate that the proposed workflow is a feasible, and easy to implement approach for performing high-content phenotypic profiling.

A



B

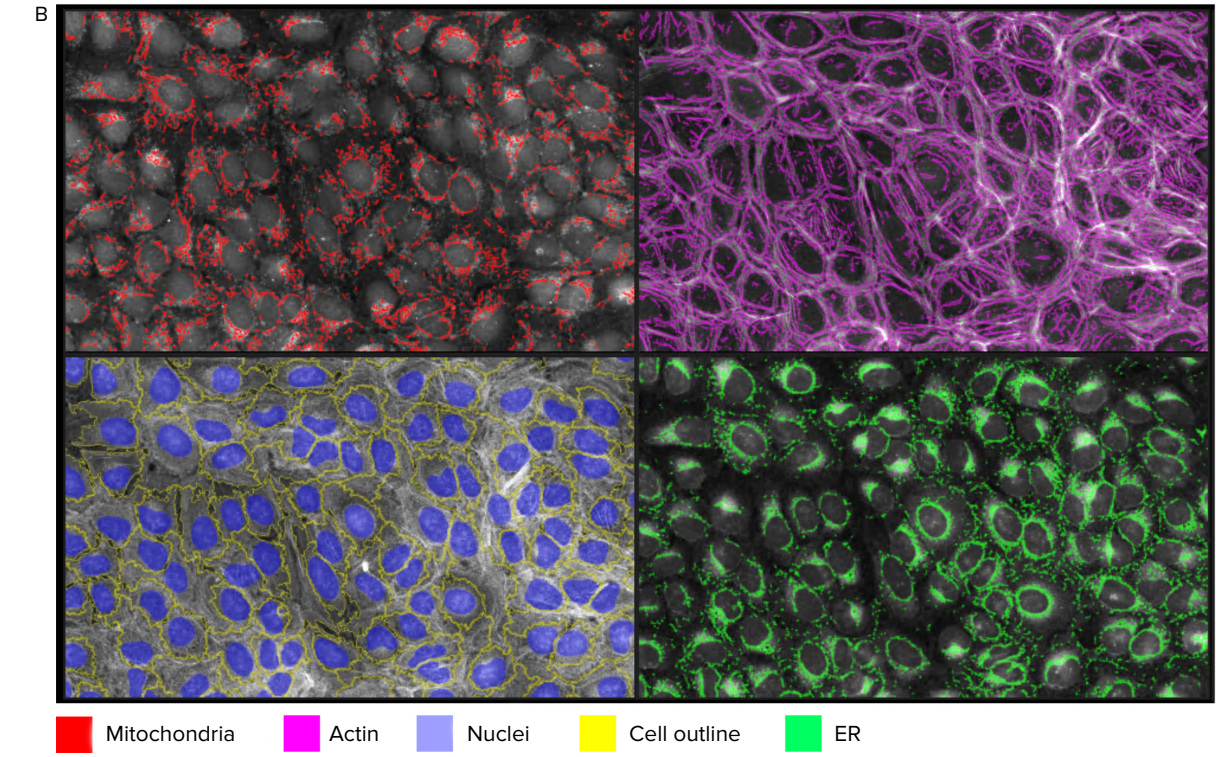
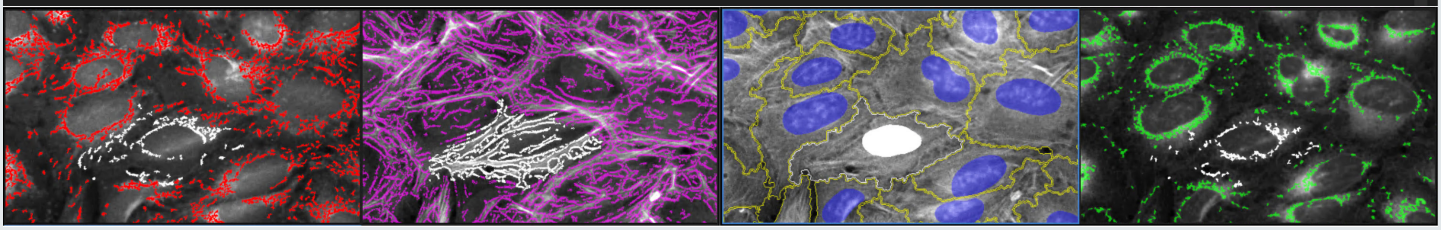


Figure 3. *Feature extraction in IN Carta software The analysis protocol was created in the IN Carta software to segment the various cellular structures. Here, we used the built-in nuclei model to achieve robust segmentation of nuclei across all treatments. Other cellular features such as the cytoplasmic compartment, actin filament network, ER, and mitochondria were also segmented. Measurements related to intensity, texture, colocalization and shape are selected during the analysis setup. We selected a total of 280 measurements for cells and subcellular structures in the protocol. A) A screen capture of the analysis protocol used. Output of the analysis is selected under "Measures". Shown here are some of the measurements available under the "Spatial" category. B) Example images with overlay of the feature mask in the IN Carta software.*

 |

A

#	Object ID	Row	Column	FOV	Nuclei Area wv5	Nuclei Form Fa...	Nuclei Elongati...	Nuclei Compa...	Nuclei Chord R...	Nuclei Gyratio...	Nuclei Displac...	Nuclei Diamet...	Nuc
104	103	C	9	2	446.35	0.79	0.72	1.22	0.44	9.29	0.70	32.33	
105	104	C	9	2	495.94	0.75	0.52	1.26	0.41	9.98	0.12	34.29	
106	105	C	9	2	317.44	0.86	0.62	1.13	0.54	7.54	0.70	25.14	
107	106	C	9	2	465.60	0.77	0.53	1.22	0.43	9.50	1.09	33.54	
108	107	C	9	2	365.86	0.77	0.97	1.24	0.42	8.49	0.37	29.18	



B

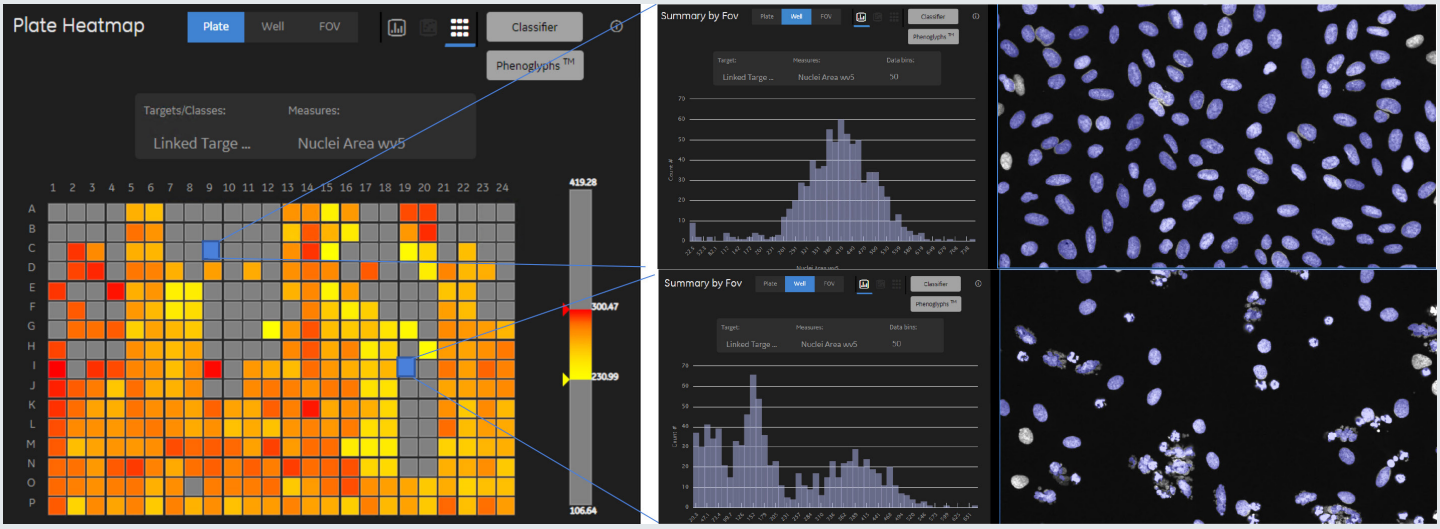


Figure 4. *Data view in IN Carta The software has various visualization tools for data exploration. A) Displayed measurements are linked to the object in the image and can be selected by clicking on a line in the measurement table or on the object/cell in the image view panel. The selected object here is shown in white overlay mask. B) Options such as heatmap display and histograms are available. The selected measurement can be viewed as a heat map across the entire plate. The heatmap scale can be adjusted so that only a selected range of values are displayed. Histogram display is also available for easy view of the distribution of the selected measurement. Measurements can be exported into csv format for further data analysis.*

 |

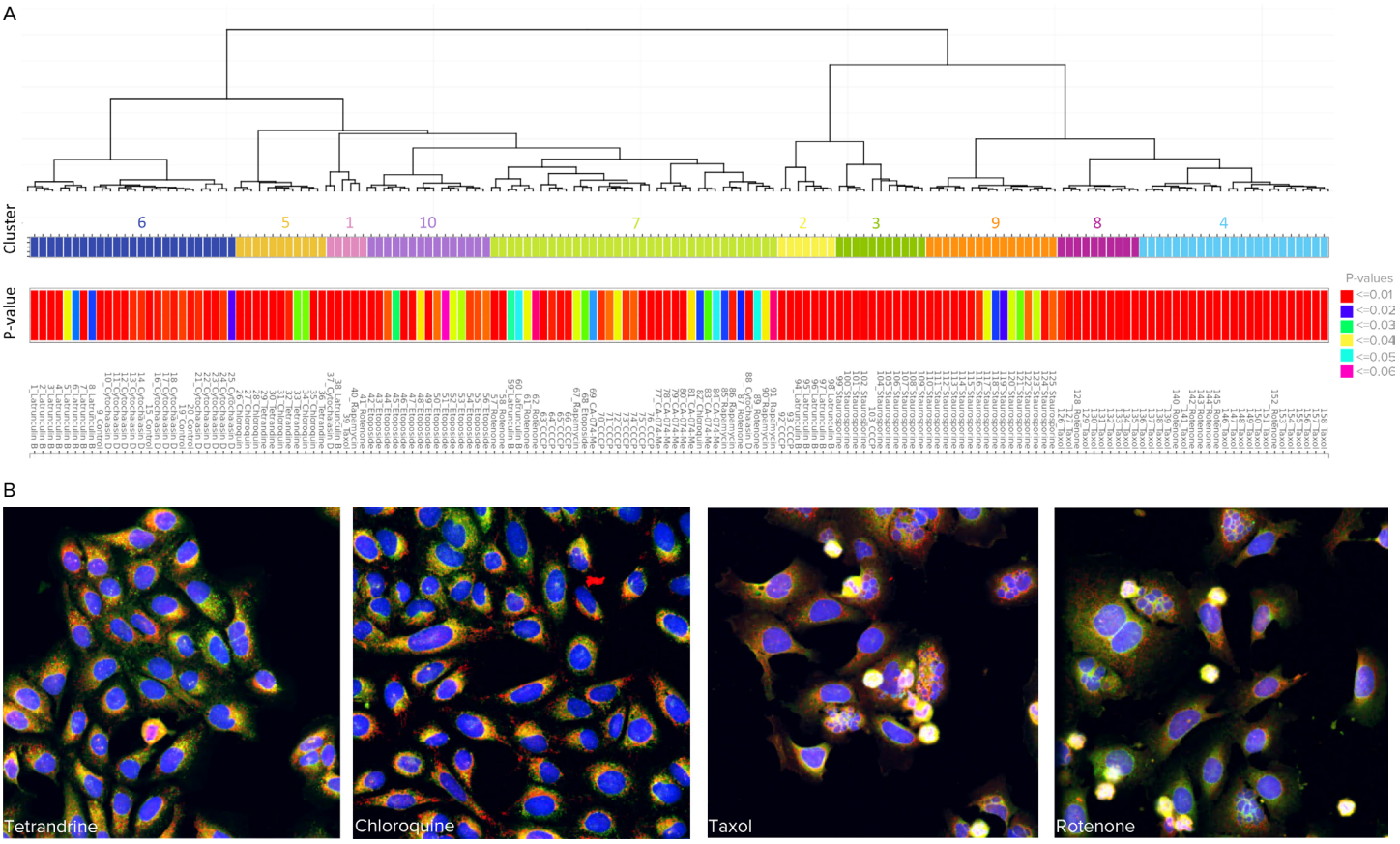


Figure 5. *Cluster analysis. A) A dendrogram that represents the hierarchical relationships is shown. Wells belonging to the same cluster (numbered) are represented by colored bars. P-values based on the distance score are shown for each well. B) Examples of compound treated cells belonging to the same clusters are shown. Cluster 5 consists of tetrandrine and chloroquine treated cells. Note the increased number of ER punctae in both wells. Cluster 4 consists of rotenone and paclitaxel treated cells. Note the presence of blebbing in some of the cells belonging in these wells, suggesting cytotoxic effects.*

Conclusion

- The ImageXpress Micro Confocal system is suitable for imaging high-content, Cell Painting assays.
- The IN Carta software combines ease-of-use with advanced feature segmentation options (SINAP) to facilitate robust and unbiased identification of cellular features.
- The StratoMineR platform allows non-expert users to rapidly carry out phenotypic data analysis with its intuitive guided workflow.
- Overall, our results show the feasibility of using ImageXpress Micro Confocal system, IN Carta software, and StratoMineR for image-based profiling assays.

 |

References

1. Bray MA, Singh S, Han H, Davis CT, Borgeson B, Hartland C, Kost-Alimova M, Gustafsdottir SM, Gibson CC, Carpenter AE. Cell Painting, a high-content image-based assay for morphological profiling using multiplexed fluorescent dyes. *Nat Protoc.* 2016 Sep;11(9):1757-74. doi: 10.1038/nprot.2016.105. Epub 2016 Aug 25. PMID: 27560178; PMCID: PMC5223290.
2. Gong K, Chen C, Zhan Y, Chen Y, Huang Z, Li W. Autophagy-related gene 7 (ATG7) and reactive oxygen species/extracellular signal-regulated kinase regulate tetrandrine-induced autophagy in human hepatocellular carcinoma. *J Biol Chem.* 2012 Oct 12;287(42):35576-88. doi: 10.1074/jbc.M112.370585. Epub 2012 Aug 27. PMID: 22927446; PMCID: PMC3471698.
3. Gustafsdottir SM, Ljosa V, Sokolnicki KL, Anthony Wilson J, Walpita D, Kemp MM, Petri Seiler K, Carrel HA, Golub TR, Schreiber SL, Clemons PA, Carpenter AE, Shamji AF. Multiplex cytological profiling assay to measure diverse cellular states. *PLoS One.* 2013 Dec 2;8(12):e80999. doi: 10.1371/journal.pone.0080999. PMID: 24312513; PMCID: PMC3847047.
4. Mauthe M, Orhon I, Rocchi C, Zhou X, Luhr M, Hijlkema KJ, Coppes RP, Engedal N, Mari M, Reggiori F. Chloroquine inhibits autophagic flux by decreasing autophagosome-lysosome fusion. *Autophagy.* 2018;14(8):1435-1455. doi: 10.1080/15548627.2018.1474314. Epub 2018 Jul 20. PMID: 29940786; PMCID: PMC6103682.
5. Nyffeler J, Willis C, Lougee R, Richard A, Paul-Friedman K, Harrill JA. Bioactivity screening of environmental chemicals using imaging-based high-throughput phenotypic profiling. *Toxicol Appl Pharmacol.* 2020 Jan 15;389:114876. doi: 10.1016/j.taap.2019.114876. Epub 2019 Dec 30. PMID: 31899216.
6. Omta WA, van Heesbeen RG, Pagliero RJ, van der Velden LM, Lelieveld D, Nellen M, Kramer M, Yeong M, Saeidi AM, Medema RH, Spruit M, Brinkkemper S, Klumperman J, Egan DA. HC StratoMineR: A Web-Based Tool for the Rapid Analysis of High-Content Datasets. *Assay Drug Dev Technol.* 2016 Oct;14(8):439-452. doi: 10.1089/adt.2016.726. Epub 2016 Sep 16. PMID: 27636821.
7. Rohban MH, Singh S, Wu X, Berthet JB, Bray MA, Shrestha Y, Varelas X, Boehm JS, Carpenter AE. Systematic morphological profiling of human gene and allele function via Cell Painting. *Elife.* 2017 Mar 18;6:e24060. doi: 10.7554/eLife.24060. PMID: 28315521; PMCID: PMC5386591.

Contact Us

Phone: +1.800.635.5577
Web: www.moleculardevices.com
Email: info@moldev.com
Check our website for a current listing of worldwide distributors.

Regional Offices

USA and Canada	+1.800.635.5577	China (Beijing)	+86.10.6410.8669	Japan	+81.3.6362.9109
United Kingdom	+44.118.944.8000	China (Shanghai)	+86.21.3372.1088	South Korea	+82.2.3471.9531
Europe*	00800.665.32860	Hong Kong	+852.3971.3530		

**Austria, Belgium, Denmark, Finland, France, Germany, Ireland, Netherlands, Spain, Sweden and Switzerland*

1/21 2386A
Printed in USA