A Cellular Imaging and Machine Learning Approach to Drug Discovery

Introduction

The paradigm of drug discovery is now shifting, following the once-declining number of new molecular entity (NME) registrations – as expressed in Eroom's law which states that over 60 years, irrespective of the advancement of technologies, it had become much more expensive and much slower to bring new therapeutics to market.[1] Better stratification of human diseases, intelligent experimental design using more predictive model systems for compound screening and profiling, and the aid of improved computational biology are helping to circumvent Eroom's law, as observed from the past decade of FDA NME approvals.[2] It is also the reason why computational and digital biology are being heavily explored and invested-in by new start-up companies, industry, academia, and government. The observation by Swinney that first-in-class-drugs were frequently discovered by phenotypic approaches may explain why phenotypic drug discovery is gaining traction over traditional target-based screening approaches.[3]

Cell Painting is considered a phenotypic screening method and a powerful application of high-content screening technology which combines cell and computational biology to elucidate the behavior of cells under the influence of perturbagens, such as chemical compounds, drugs, genes, or other entities affecting the cell.

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Defining Cell Painting

The first iteration of Cell Painting, without naming it as such, was published in a 2013 paper by Gustafsdottir and colleagues at the Broad Institute of Harvard and MIT.[4] The assay was then slightly modified into its current configuration by Bray and colleagues, who coined the term "Cell Painting", in a 2016 publication that describes the process in detail.[5] Cell Painting is not only being used to identify potential new therapeutics and assess human gene function [6] but has also been applied to the assessment of environmental toxicants by Nyffeler and colleagues at the US Environmental Protection Agency (EPA).[7]

Cell Painting is a multistep process in which cells are "painted" by labelling different cellular compartments with different fluorescent bioprobes to quantitatively profile multiple phenotypic parameters as a means to better understand the effects of chemical compounds, drugs, genes, or other test articles.

The process is dependent on the use of known reference compounds to "train" image analysis algorithms to generate multiparametric data that is subsequently filtered using machine learning approaches to reduce the overall complexity of the multidimensional data.

At a high-level, the process of Cell Painting incorporates high-throughput robotics and automation to seed cells and deliver compounds to microplates, the use of multiple fluorescent bioprobes to tag cell compartments, acquisition of images, image analysis, and machine and deep learning data analysis tools to enrich and summarize findings of complex multidimensional data. The highly-dimensional data is displayed using tools such as PCA (Principal Component Analysis) or t-SNE (t-Distributed Stochastic Neighbor Embedding) that allow for clustering of similar phenotypic profiles that may "suggest" a mechanism or mode of action (MOA) for unknown compounds by comparison with reference compounds of known MOA.
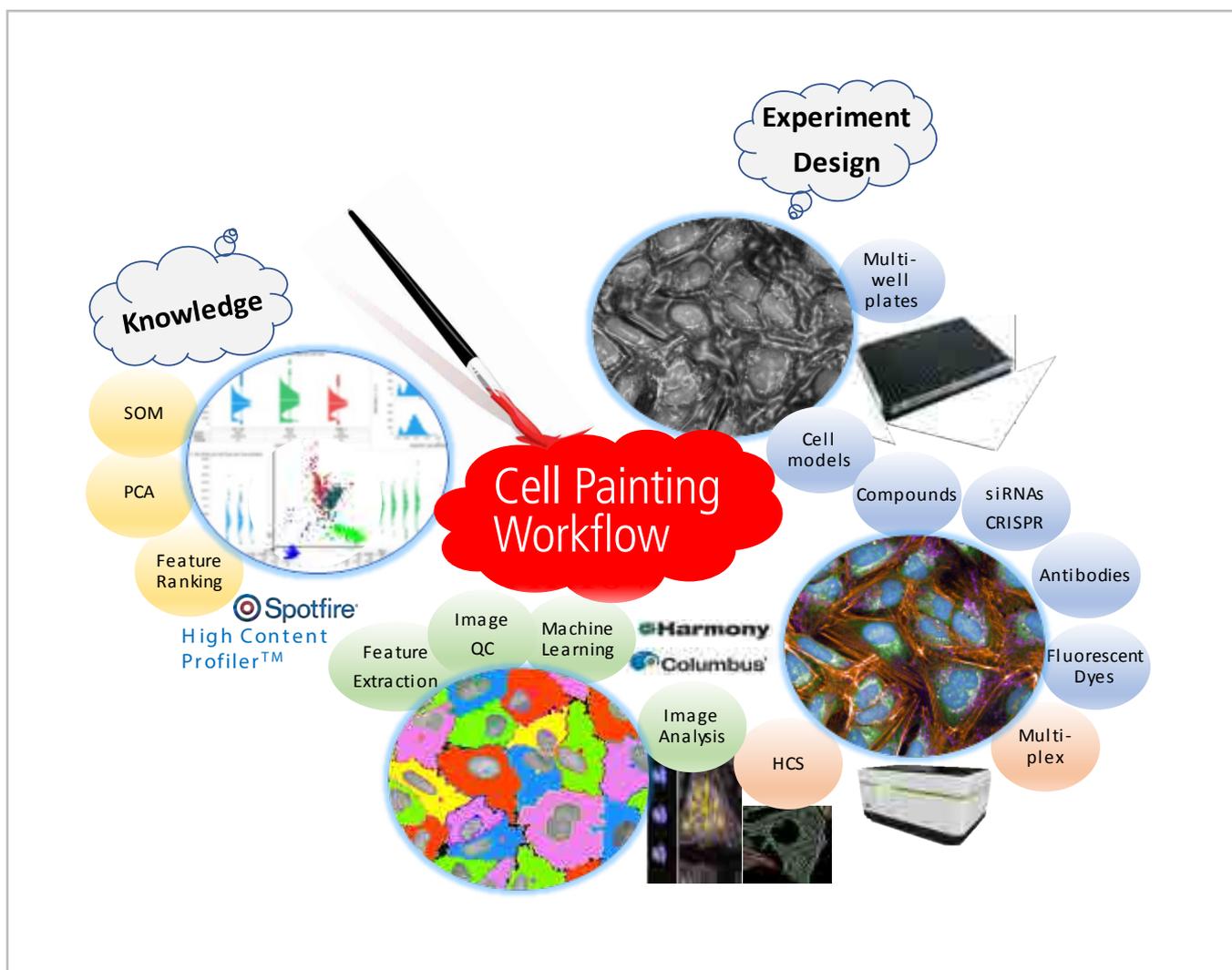

Figure 1. Successful Cell Painting requires careful consideration of experimental design, reagents, consumables, imaging hardware, and imaging and analysis software.

Cell Models

In the publications by Gustafsdottir and Bray, U-2 OS (human bone osteosarcoma epithelial) cells were the cell line of choice for the Cell Painting assay. U-2 OS cells are highly desirable for this application, since they are available commercially and are compatible with high-content imaging approaches. A U-2 OS cell displays a "fried-egg"-like morphology with a slightly raised nucleus and a large flat cytoplasmic area. This is a unique characteristic of U-2 OS cells and since, under normal conditions, the cells do not typically aggregate with one another, the nuclear and cytoplasmic markers can be readily identified and robustly segmented in high-content image analysis.

More than 13 different cell types are cited in the literature in Cell Painting applications including the commonly-known HCS-compatible cells: A549, HEK-293, HeLa, HepG2, HTB-9, MCF-7, SH-SY5Y, and the mouse cell line NIH-3T3. In addition, primary human cells, cell lines that closely resemble the disease of interest, stem cells and co-culture models such as HepatoPac® are being evaluated for Cell Painting. More recently Willis and colleagues characterized the Cell Painting phenotypic outcomes across 6 different cell lines that includes A549, ARPE-19, HepG2, HTB-9, MCF7, and U-2 OS.[8]

For Cell Painting, the chosen cells are typically seeded into microplate wells (96-, 384- or 1536-well plates) and incubated (37 °C, 5 % CO_2, > 90 % RH) overnight (18 - 24 hours) at a cell density pre-determined during the assay development process. The cells are then treated with the desired test article (chemical compound, drug, siRNA, protein, etc.) plus known reference compounds for subsequent processing and analysis. Cells are typically treated for 24 - 96 hours, however, this may vary from minutes in a fast-kinetic assay to several days in longitudinal studies.

Drug, Compound or Perturbagen Dosing

Cell Painting requires a subset of known reference compounds to be used as a "training set" to teach the machine learning algorithms how to classify perturbagen responses during secondary data analysis and is highly dependent on the modification by the reference control compounds of the bioprobe markers used in the experiment. Therefore, determination of the effects of the known reference compounds in advance of the screen is of utmost importance.

The number and diversity of compounds in the screening library, or other materials used to perturb the cells, is a critical consideration. A high number of test articles alone may not provide the desired outcome, rather the choice of a diverse spatial bioactivity compound set should improve the algorithms for clustering and identifying compound classes.

Since the Cell Painting assay is considered a phenotypic assay, identification of reference compounds that selectively target and modify the response detected by the bioprobe markers is highly desirable and compound selection will determine the overall strength of the model to cluster perturbagen responses appropriately. While some compounds directly target only a single cell compartment, e.g. the topoisomerase-II inhibitors etoposide and doxorubicin, or the mitochondrial uncoupler, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone (FCCP), other compounds such as staurosporine, which is considered a non-specific broad-base kinase inhibitor, have a direct impact on several cellular functions and compartments, e.g. nuclear morphology, cell cycle, nucleic acids, cell health, mitochondria and cytoskeleton structure, dependent on time and dose.[9] These types of reference tool compounds are useful for training the algorithms to evaluate unknowns in a screening campaign.

There are several methods available to deliver compounds to cells via automated, robotic liquid handling devices and can include delivery by pipette, pin-tools, or even acoustic "echo" dispensing. Critical considerations in compound dosing include the stability and solubility of each compound in its final concentration.

Selecting Cell Paints

Following treatment with perturbagens, the cells are "painted", i.e. labeled or stained, with the chosen bioprobes. In the early Cell Painting papers (Gustafsdottir and Bray), dyes were specifically chosen to minimize the cost of labelling while profiling as many cellular characteristics as possible. This raises the question of whether these commonly used, commercially-available dyes, probes, and other markers are representative of the biology or disease. If the HCS feature measurements do not adequately cover the full extent of the predicted disease-model outcomes, then the selection of the multiplex probe set in the experimental design may require modification and replacement with new probes to properly capture the full extent of the biology. In target-based screening, probes are intended to directly visualize the target or its immediate effects but, in phenotypic drug discovery, choosing bioprobes to maximize coverage of the range of characteristics may be enough for training the machine learning algorithms for the desired outcomes.

In the current Cell Painting experimental design, 6 fluorescent probes are used to target specific cell compartments to determine protein expression or signaling pathways, to identify organelles and their function, or identify whole-cell morphology (Figure 2). In their efforts to minimize the cost and time for higher throughput screens, the bioprobe panels described by Gustafsdottir and Bray do not include antibody markers. However, this should not deter HCS imaging practitioners from implementing antibody labeling approaches in a Cell Painting panel of their own for a more in-depth assessment of expression of biological proteins, or pathways, not covered by the previously published bioprobe panels.

An extension of the Cell Painting assay by Nyffeler and colleagues used duplicate microplates with the supplementary probes Hoechst 33342 and propidium iodide as a means of identifying cytotoxic compounds in addition to the classical Cell Painting measurements to gain further insight into the biology. The caveat with duplicate microplates with the same compound treatment is that cell-to-cell measurements cannot be directly correlated, but whole-well means of the cell population response are retained.
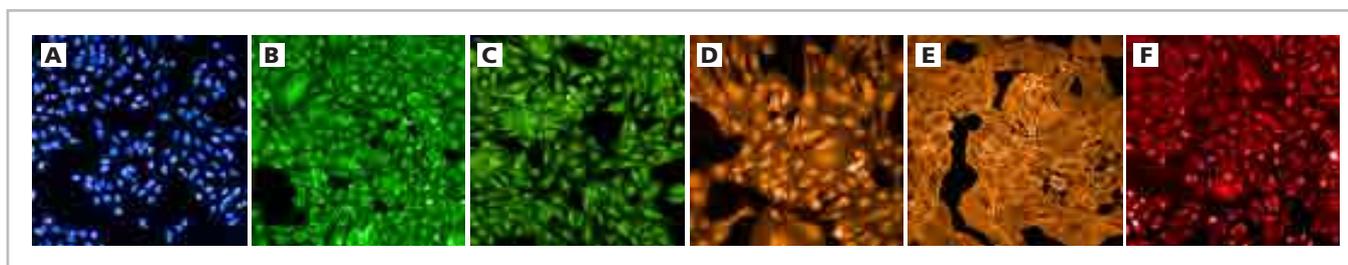
Figure 2. Cell Painting bioprobe markers in U-2 OS cells seeded in CellCarrier-384 Ultra microplates, images captured with 20xW objective lens on the Opera Phenix® high-content screening system. (A) Nucleus, Hoechst 33342; (B) Endoplasmic reticulum, Concanavalin A - AlexaFluor™488; (C) Nucleoli and cytoplasmic RNA, SYTO™14; (D) Golgi and plasma membrane, Wheat Germ Agglutinin - AlexaFluor™555; (E) Actin, phallodin - AlexaFluor™568; (F) Mitochondria, MitoTracker™ Deep Red.

Perhaps the next generation of Cell Painting probes and approach is being introduced by Kang and colleagues, termed "Fluopack" and comprising a panel of 44 different fluorescent bioprobe markers weighted towards covering biological phenotypes in this order: lipid function, ion concentrations, organelle morphology, pH sensors, ROS, mitochondria function, cell death, and drug conjugates.[10] In the study, both wildtype parental cells and CRISPR knockout cells are seeded in the same microplate with only one bioprobe per well and imaged with a 60x objective lens. The advantage of this method is a broader coverage of biological activity, and a reduction of interference and spectral crosstalk from multiple fluorescent probes during image acquisition; the disadvantage is the requirement for additional plates or wells to cover all probes used and loss of cell-to-cell measurements. Kang, et al., claim to have expanded from 44 up to 170 different fluorescent markers in the panel, thus increasing the coverage of biological response per compound treatment.

Bringing Cell Paints to Life Through Image Acquisition

Images of labelled cells are generated with a high-throughput microscopy system (HCS imager) which independently captures each bioprobe. Image acquisition is either conducted sequentially per fluorescent probe with different exposure times or simultaneously if the HCS instrument is equipped with multiple camera detectors. While there are 6 fluorescent probes with significant spectral overlap in the Cell Painting assay, only 4 or 5 different fluorescent channels are used to detect the fluorescent signals due to the optical filter configuration on most HCS imagers. To overcome this dilemma, the spatial localization in x, y and z dimensions of each bioprobe inside the cell is used to separate the endpoint measurement when two fluorescent probes with similar spectra properties are in close proximity to one another, for example, SYTO™ 14, a nuclei acid dye which binds and localizes to the nucleus and Concanavalin A conjugated to Alexa Fluor™ 488 (ConA-AF488) which localizes to the cytoplasm and membrane. Modifications of the Cell Painting probes are being implemented in laboratories in efforts to best characterize a disease state or are tailored for detection on an HCS imaging device.

One of the biggest questions from HCS scientists is how to handle the narrow separation of the spectral properties of multiple fluorescent bioprobes, particularly two pairs, SYTO™ 14 and ConA-AF488 or WGA-Alexa Fluor™ 555 and F-actin Phalloidin-Alexa Fluor™ 568. (Figure 3).

A

B

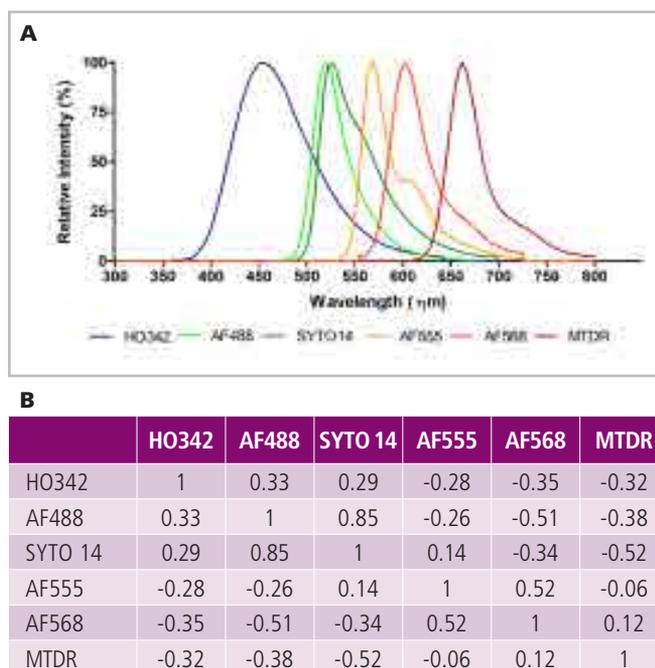|	HO342	AF488	SYTO 14	AF555	AF568	MTDR
HO342	1	0.33	0.29	-0.28	-0.35	-0.32
AF488	0.33	1	0.85	-0.26	-0.51	-0.38
SYTO 14	0.29	0.85	1	0.14	-0.34	-0.52
AF555	-0.28	-0.26	0.14	1	0.52	-0.06
AF568	-0.35	-0.51	-0.34	0.52	1	0.12
MTDR	-0.32	-0.38	-0.52	-0.06	0.12	1

Figure 3. (A) Emission wavelengths of fluorescent probes used in Cell Painting panel. Hoechst 33342 (HO342), Alexa Fluor™ 488 (AF488), SYTO™ 14, Alexa Fluor™ 555 (AF555), Alexa Fluor™ 568 (AF568) and MitoTracker™ Deep Red (MTDR). (B) Pearson correlation coefficient matrix showing the theoretical spectral similarities and differences of bioprobes used in Cell Painting assay: r=1.0 is perfect correlation; 0.5 shows 50% correlation; < 0 no correlation.

Painting by Numbers – Image Analysis Segmentation

In Cell Painting, image segmentation is used to identify unique properties of individual cells that result in feature measurements with an associated numerical value derived from the image pixel information.

These feature measurements at the single-cell level are averaged per well and include cell morphology such as size, shape and texture, intensity of the bioprobe marker or even dynamics e.g. cell motility or redistribution of proteins in live cells. A subset of these higher-level features can provide additional information depending on the algorithm that is employed. For example, the length or width of an object, length-width ratio of the cell object, how circular, compact, or amorphous the cell shape is, the granularity of the texture measurement, or the variability in intensity across an area, and numeration of object counts, spots, or other pixel regions making up an area, or even a Boolean calculation of one or more of the measured image properties and in some cases, population and subpopulations of the response

can be measured, which is especially useful in heterogeneous cell models or in mixed co-culture systems.

Multiplying the number of possible measurements from the images by the number of fluorescent biomarker probes per cellular compartment and any subpopulations quickly expands the list of potential features for analysis. In most cases, mean average or total well-level information from one or more fields is calculated from the single-cell level data.

A typical phenotypic screen in its simplest form creates a few dozen measurements with 10 - 15 of these features used in the analysis, however reporting the full extent of an image analysis algorithm output can easily be extended by multiple texture, morphology and other advanced image analyses, to generate hundreds or thousands of features in a Cell Painting assay.

Every Picture Tells a Story – Data Analysis, Machine Learning and Visualization

HCS imaging scientists must determine which of the many generated features to use in the subsequent data analysis to report the findings. In the early days of HCS imaging twenty years ago, only 1 or 2 features were used to profile the responses to drugs or compounds, quite similar to HTS plate reader assays.[11] As the HCS community moves to analyze large multivariate data sets, they face the challenge of interpreting the full breadth of all the high content imaging data that is generated in the Cell Painting assay – which can equate to thousands of features. It is a daunting task for any human to fully comprehend dozens, let alone hundreds or even thousands, of independent HCS generated features, therefore the use of supplementary informatics tools is necessary to reduce this multifaceted information into something more practical and meaningful. A process referred to as "reduction in dimensionality" of HCS-generated features helps scientists with both the visualization and interpretation of the data.

A commonly-used approach to identify the most relevant features among thousands of measurements is determination of Z-score. This process alone provides a hierarchical filter of every feature based on signal and robustness providing rank order for analysis. Z-score filtering may be combined with another secondary analysis process called Principle Component Analysis (PCA) to analyze the data in a multifactorial matrix relationship format, otherwise known as multivariate analysis.

In the context of high-throughput microscopy and phenotypic screening, PCA was first described by Perlman and colleagues in 2004 as a method to analyze HCS data.[12] PCA in its simplest terms reduces the dimensionality of complex feature data by using eigenvectors in multiple matrix factorial dimensions to correlate similarities and their differences. This is visualized by showing compounds or perturbagen responses in 3D space as population clusters or clouds that have similarities within one another or are spatially distinct from other groupings, e.g. the DMSO vehicle control cluster will differ from a reference control compound. This provides scientists with a visual representation of the secondary data analysis that is helpful in predicting similar

mechanisms or mode of action (MoA) classes; the greater the distance apart, the greater the likelihood the perturbagen does not exhibit the same mode of action (MoA).

Other statistical measurements that scientists are exploring to interrogate the Cell Painting assay include K score, phi score, and standardized mean difference.[13] In addition, classification and segregation of active perturbagen responses from a screen may be done with advanced classification methods such as Random Forest and Mahalanobis Distance. Both methods use analysis and training to separate and correlate HCS multivariate analysis.

Self-organizing maps (SOM), sometimes referred to as Kohonen or Sammom connection networks, are unsupervised machine learning algorithms dependent on AI and convolutional or deep neural networks to cluster similarities in spatial nodes indicating the strength of the profile relationships. SOM's reduce high dimensional space to lower dimensional visualization using cluster nodes and are expressed as a two-dimensional projection. Another machine learning method that has been adopted to reduce nonlinear dimensionality of HCS multivariate analysis is t-distributed stochastic neighbor embedding (t-SNE). This method projects visualization of neighboring data in a 2D or 3D fashion similar to PCA, with nearest points with similarities and distant points unrelated, with a probability to predict MoA of compounds from screens based on classification and clustering with known reference compounds.[14]

There are many approaches to analyse post image segmentation or even deep learning segmentation-free multivariate data from a Cell Painting assay. The method chosen may result in different outcomes, therefore it is important to understand how the analysis is manipulated and the importance of validation for the entire workflow process.

Conclusion

The Cell Painting approach evokes new ideas and strategies for HCS practitioners to identify new drugs and to study mechanisms of action by leveraging machine learning to measure subtle phenotypic changes at the cellular level. The newer generation of Cell Painting fluorescent bioprobe panels offers great promise to better understand *in vitro* disease models that are essential to further reduce animal testing, streamline testing of chemical or biological entities for new therapeutics or investigate environmental toxicants. Still, as with other high-content screening assays, there is inherent bias built into the data from end-user input, and the use of supervised and unsupervised machine learning, AI, deep learning, CNN, or other computational processing needs to be carefully examined to reduce as much bias as possible. Cell Painting, although not a new approach to phenotypic screening in drug discovery, has invoked the use of several analytical tools to effectively analyze large amounts of extracted feature data generated from an HCS experiment, that may include artificial intelligence or machine learning guidance, to better understand, fingerprint, and interpret the data by classifying phenotypic profiles to determine MoA to identify new discoveries from unknown perturbagens.

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