Interpreting Image-based Profiles using Morpheus and Single-Cell Visualization

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ABSTRACT

Image-based profiling quantitatively assesses the effects of perturbations on cells by capturing a breadth of changes via microscopy. Here we provide two complementary protocols to help explore and interpret data from image-based profiling experiments. In the first protocol, we examine the similarity among perturbed cell samples using data from compounds that cluster by their mechanism of action (MOAs). The protocol includes steps to examine feature-driving differences between samples using Morpheus software, and how to visualize correlations between features and treatments to create interpretable heatmaps. In the second protocol, we show how to interactively explore images together with the numerical data, while we provide scripts to create visualizations of representative single cells and image sites to understand how changes in features are reflected in the images. Together, these two tutorials help biologists and researchers interpret their image-based data to speed up research.

Basic Protocol 1: (Exploratory analysis using Morpheus software)

Basic Protocol 2: (Image and single-cell visualization following profile interpretation)

KEYWORDS

profiling; single-cell visualization; Morpheus; morphological analysis; high-dimensional data

INTRODUCTION

Automated microscopy allows biologists to acquire thousands of images from cells perturbed with drugs, small interfering RNA (siRNA), CRISPR-Cas9, and more. In a typical quantitative microscopy experiment, biologists select fluorescent biomarkers, such as antibodies or dyes for specific proteins or cell compartments, and measure only the features they hypothesize will be perturbed in their experiment. By contrast, in image-based profiling, the aim is to let the cells speak for themselves. Diverse stains are used, as in the Cell Painting assay, which stains eight components of the cells (Bray et al., 2016; Cimini et al., 2022). Then, image analysis software segments the cells and measures all possible morphological features from the single cells. The collection of features for a cell is called a “profile,” and typically a thousand or more features are measured per cell. It is then possible to analyze whether features are modified in a treated sample of cells compared to controls. Afterwards, samples can be grouped into clusters based on their image-based profiles (Figure 1). However, the biological meaning behind clusters is difficult to interpret because there are thousands of features in the profile. This leads to a common bottleneck: given a sample or cluster of samples, how do you interpret what a given profile means biologically?

Here, we present two basic protocols: Exploratory analysis using the Morpheus software (Basic Protocol 1) and image and single-cell visualization following profile interpretation (Basic Protocol 2). In Basic Protocol 1, we show researchers how to explore the overall large-scale associations of the data (after feature extraction and cleaning) using Morpheus software. There, the data can be grouped in different ways, revealing how features and samples are correlated. Exploring the data is essential to gain insights into the biological interpretation of the profiles. Then, in Basic Protocol 2, the goal is to help biologists create intuitions about the differences between treatments by examining example cells. This notebook contains Python scripts to help researchers crop representative or random single cells from each treatment and group the cropped images based on correlations of interest. In addition, representative images of each sample can be retrieved to understand how the cells are distributed across representative fields of view (*e.g.,* those captured from different sites (locations) within a sample well). In the Understanding Results section, we provide insights on how visualizing example cells from the samples and linking them to the correlations between the samples will provide the biologist with extensive information to formulate new hypotheses and interpretations from their data.

The two basic protocols yield a similarity matrix, hierarchical clustering for the samples, and representative example cells from their data. These outputs can be easily used for reports and publications. For the input data for both protocols, we use a dataset of images processed by CellProfiler, to identify cells and extract features (Stirling et al., 2021) and pycytominer, to normalize and aggregate single-cell profiles into population-averaged profiles (Way et al., 2022a). Extensive documentation is available online on feature extraction with CellProfiler (https://github.com/CellProfiler/tutorials), and for data aggregation, normalization, and feature selection with pycytominer (https://github.com/cytomining/pipeline-examples). In addition, we provide an example dataset in our GitHub repository, including CSV spreadsheets to be processed on Morpheus (https://github.com/ciminilab/2022_Garcia-Fossa_submitted). In our example dataset, each compound is annotated with its mechanism of action (MOA). However, these protocols can be used without having the MOA for every compound in their dataset, and instead comparing treated cells with the negative and/or positive controls, or comparing multiple perturbed samples with each other.

BASIC PROTOCOL 1

Exploratory analysis using Morpheus software

The main goal of this tutorial is to examine the correlations between the samples to check for their replicability, to explore the correlations among them, to discern how the features drive the differences between samples or groups, and to enable the interpretation of the biology behind the data.

After cell treatment, imaging and feature extraction, some profiles are dramatic in only one or a few features and the feature names have obvious meanings (nucleus area or integrated intensity of the mitochondria channel in the cytoplasm, which corresponds to the total amount of staining in that channel); in these cases, looking at the feature names will help to discern their connection to the biological meaning. However, some individual features have meanings that are more difficult to translate into plain language. Furthermore, the challenge is even greater to interpret a collection of feature names that all contribute strongly to a more complex morphological phenotype. For example, a collection of features from a channel stained for actin and wheat germ agglutinin together with DNA granularity was particularly important to predict 70 specific cell health phenotypes from Cell Painting data (Way et al., 2021a). Even phenotypes that are visually obvious and distinctive by eye, such as cells stalled in a particular stage of the cell cycle, are often difficult to predict just by examining a list of distinctive features; the problem is even more acute for samples without a visual discernible phenotype yet quite distinguishable using image metrics.

To help us in the exploration and interpretation process, we often use Morpheus (Morpheus), a web-based software used for versatile matrix visualization, analysis, clustering, filtering, and displaying charts. Originally designed to explore mRNA profiling data, the tool can be readily used by those without extensive computational or statistical experience. Morpheus can import matrix files from many formats (CSV, GCT, GMT, text file) and then explore the data as a matrix of colors in an interactive way. More information can be found in Morpheus' documentation (https://software.broadinstitute.org/morpheus/documentation.html) and we created guided tutorials for the COBA YouTube channel.

During this tutorial, the researcher will start by examining how similar is each sample to the other samples using per-well similarity matrices, sorting the data in a way that allows for interpretation. We provide a sample data set in which drugs with known mechansims of action (MOAs) have been added at various dose points prior to Cell Painting. To observe how MOAs are grouped, and if technical artifacts such as batch or plate-layout effects are playing a role in the distribution of the groups, we use hierarchical clustering. In the end, the researcher will be able to identify if drugs with similar MOAs have similar morphological profiles and if different MOAs are correlated based on their morphological profile. The researcher will also learn how to determine what features drive the differences between the groups. We emphasize that this is just one of the data-exploration approaches that can be used to interpret image-based profiles.

Materials

Data and Jupyter Notebooks (Kluyver et al., 2016) are available in our GitHub repo (https://github.com/ciminilab/2022_Garcia-Fossa_submitted). The data is in a GCT format, a tab-separated value table containing the extracted features aggregated by well in a Cell Painting assay. In this assay, 1,571 compounds were tested across 6 doses in A549 cells (Way et al., 2022b).

We randomly selected a plate map from this experiment (C-7161-01-LM6-011 plate map), and then downloaded the CSV (comma-separated value) files for five of its replicate plates (SQ00015195, SQ00015218, SQ00015219, SQ00015220, SQ00015221), from the cpg0004-lincs dataset(Way et al. 2022) available from the Cell Painting Gallery on the Registry of Open Data on AWS (cellpainting-gallery). Then, we added annotations to the data (labels for each MOA, compound, and concentration), and normalized the features to the negative control (DMSO) in a Jupyter Notebook (Kluyver et al., 2016) using the pandas library (Reback et al., 2020) and pycytominer (Way et al., 2022a). Then, we performed feature selection to exclude features with low variance (frequency cut = 0.05), high correlation to another feature in the profile (threshold of 0.9), features that have more than 5% of NA (not available) values, excluded blocklisted features, and removed outliers by excluding a feature if its minimum or maximum absolute value is greater than the threshold (threshold = 500). These steps are available in basic_protocol_1/notebooks folder using the Data_preparation.ipynb notebook in our GitHub repository (https://github.com/ciminilab/2022_Garcia-Fossa_submitted/tree/main/basic_protocol_1/notebooks). We opened the CSV file obtained using Data_preparation.ipynb in Morpheus and clicked on Tools > Transpose, allowing the CSV table to be better visualized in Morpheus. For the researcher to apply the protocol to their own data rather than our example dataset, we recommend using CellProfiler (Stirling et al., 2021) to extract features and pycytominer (Way et al., 2022a) for data preparation.

We calculated the average precision based on https://github.com/niranjchandrasekaran/profiling-workflow-demo/blob/master/analysis/0.calculate-ap.ipynb to enable us to remove weak profiles before analysis; no such profiles were found for this data set. To reproduce our results, follow the instructions for creating an environment at https://github.com/niranjchandrasekaran/profiling-workflow-demo, and use our notebook WeakProfiles_Replicability.ipynb available on our GitHub repository at https://github.com/ciminilab/2022_Garcia-Fossa_submitted/tree/main/basic_protocol_1/notebooks to calculate the replicability between the samples in our Morpheus_Example_FeatureSelected.csv dataset. See the Critical Parameters section for more information about removing weak profiles.

Access to the internet to use Morpheus at https://software.broadinstitute.org/morpheus/.

Protocol steps

1. Clone the GitHub repository into your computer or download the repository using https://minhaskamal.github.io/DownGit/#/home?url=https://github.com/ciminilab/2022_Garcia-Fossa_submitted, to have the dataset for this protocol.
2. Access the website https://software.broadinstitute.org/morpheus/ and click on “Select File” on the main screen, and select the Morpheus_Example_FeatureSelected.gct file you downloaded from

GitHub. On the current tab, you will see a heatmap. Notice all the Columns displayed for Compound, Concentration, etc.

If using your own data or the provided GCT file instead of the example json file (which bypasses this step), click on Options (gear symbol), select the Annotations tab, and change Column annotations. Deselect id and select Compound, Concentration, MOA, Wells, and Plate. This will enable the visualization of metadata information within each column.

3. Click on Options (gear symbol) -> Annotations -> Columns annotations and click All to select all columns. Right-click on the column labels (Compound, Concentration, MOA, Wells, and Plate) and enable "Show color" for all the columns, to color-code the columns.

You can avoid these steps by using the Morpheus_Example_FeatureSelected.json file instead.

4. Click on Options (gear symbol) -> Color Scheme, and de-select the Relative color scheme. Change the minimum to -1000 and the maximum to 1000. Try also with -100 and 100. While the Relative color scheme converts values to colors based on each feature's minimum and maximum values (making every row range from blue to red based on their own min and max), overriding and changing the color scheme to these new values allows seeing raw feature's values distributed within this new feature range. In this way, extreme feature values became visible.

Setting the relative color scheme in this way highlights outlier features (with higher or lower values when compared to the other features). Trying with different minimum and maximum values will highlight features within different ranges. This is just a quality control step to guarantee that feature selection was performed correctly.

5. Close the Option window and then click the zoom tool and select “Fit To Window”.
6. Use the mouse pointer to scroll throughout the rows’ names in the right corner of the screen, highlighting the features’ names. Where you see any values colored in red or blue - these are the unusual features that have high or low values when compared to the rest of the features.

Notice that if feature selection was performed, features that have points with abnormal distance from other values in the distribution will often be removed during this process. Removing outlier features is recommended because they could mean errors during image acquisition like artifacts, which could affect post-analysis (e.g., interfering with logistic regressions, or other learning methods). But remove them carefully, and try to understand why they appeared. We give more details about feature selection in the Critical Parameters section, but the pycytominer tool (Way et al., 2022a) has a function to drop outliers based on their absolute minimum and maximum values, allowing the change of the outlier cutoff value.

7. Open Options (gear symbol) -> Color Scheme, and select "Relative color scheme" to use the minimum and maximum values in each row to convert values to colors.

Note that the results will be quite different if you use the minimum and maximum values in each column.

8. Select “Tools > Similarity Matrix > Pearson correlation” on the Rows. This will calculate the correlation between the wells for all pairs of features in the dataset and generate a similarity matrix for them. Click on Options (gear symbol), Display, and select Link rows and columns.
9. Create a Hierarchical Clustering by selecting Tools -> Hierarchical Clustering. In “Metric”, select “Matrix vales (for a precomputed similarity matrix)”. Change “Cluster” to Rows and Columns. Click

OK. This step will group the features depending on how similar their profiles are using the correlation metric you have chosen.

Steps 8 and 9 will create correlations between the features, and cluster them by their similarity. The square blocks of red color along the diagonal denote high correlation, meaning the features in those rows and columns look similar to each other. Scroll down and look for the red squares, checking their names. You can see interesting clusters when looking for different feature groups: for example, check "Nuclei_AreaShape_Extent" (next to the Help menu, in the white box, type id:Nuclei_AreaShape_Extent and hit Enter. Click on the arrows next to it to redirect to the feature, and see the name highlighted). "Nuclei_AreaShape_Extent" and "Nuclei_AreaShape_Solidity", both related to how circular or protruded an object is and thus unsurprisingly tightly correlated, are grouped right next to Cells and Nuclei Intensity in the endoplasmic reticulum (ER), which would not be expected a priori. Since nuclei and ER are physically linked, we could hypothesize how these features' correlations could translate into biology. Seeing that a couple of unexpected features group together might point at interesting unexpected biology.

10. Go back to the first tab "Morpheus_Example_FeatureSelected" and select "Tools > Similarity Matrix > Pearson correlation" on the Columns. This will calculate the correlation between features for all pairs of samples in the dataset and generate a similarity matrix for them.

Pearson's coefficient is one of the many ways to calculate the correlation. There are other available methods in Morpheus (e.g., Spearman, Kendall, Cosine, etc). We give more details about Pearson's coefficient in the Background section below.

11. Click on Options (gear symbol), Display, and select Link rows and columns. Linking rows and columns helps navigate the large matrix, showing the respective correlations.
12. While holding the shift key, click on the MOA, Compound, and Concentration columns (in this order) to sort them by value. This step will display the samples in order, based on those categories of metadata (rather than based on the profile similarity itself). The scientist should focus on the MOAs and the different compounds inside each MOA - can you see if compounds belonging to the same MOA have a similar morphological profile (Figure 2)?

Square blocks of red color along the diagonal denote high correlation, meaning the compounds in those rows and columns look similar to each other. Going all the way towards the end of the dataset, on the RNA polymerase inhibitor MOA, you can see how the two compounds of this MOA (favipiravir and PSI-6130) have a similar morphological profile by looking at the higher correlations between them. At the top left of the heatmap, the user should check the adrenergic receptor antagonist MOA and the compounds that define this MOA. Can you see how there is not much correlation between the different compounds? Each compound, even belonging to the same MOA, seems to have a different morphological profile.

13. Having the same configuration as the step before (columns sorted by MOA > Compound > Concentration), continue to explore the similarity matrix and observe: are there different MOAs that have similar morphological profiles?

Go to the microtubule inhibitor and microtubule stabilizing agent MOAs (Figure 2)*. See how there are large squares colored in red when comparing the correlation coefficient for both groups, meaning these two MOA are closely correlated, and they produce similar morphological profiles. These phenotypes are indistinguishable in this cell type, at this time point, and with the stains used. Look for more unusual correlations.*

14. Sort the collapsed similarity matrix by MOA and by Plate by holding the shift key. Zoom out (pressing the minus - key) to have a broader view of the matrix.
15. Roll over to the DMSO MOA (the negative control in this dataset).

Observe how DMSO samples are distributed and do not have universally high correlations with each other. Some are correlated by Well position. This indicates that we performed plate normalization with DMSO as the baseline (meaning DMSO is not a phenotype in this dataset and all DMSO profiles should have feature values of zero, apart from technical noise). For this reason, some of the DMSO samples in similar positions (same Wells but in different plates) have some correlation, because the Well position within the plate is one of the strongest technical artifacts in cell-based experiments. Normalization performed in relation to the negative control of your plate is the morest reliable way to ensure that you can compare your samples even if they come from different plates, acquired on different days. Yet we can see some correlations between DMSO samples that are related to DMSO samples positioning within the plates, highlighting the importance of having the controls scattered to avoid plate position effects. We give more background about normalization in the Critical Parameters section below.

16. Create a Hierarchical Clustering by selecting Tools -> Hierarchical Clustering. In “Metric”, select “Matrix values (from a precomputed similarity matrix). Change “Cluster” to Rows and Columns. Click OK. This step will group the samples depending on how similar their profiles are (using the correlation metric you have chosen). The scientist can identify different groups and try to make sense of the groups.

In this step, observe that the samples do not primarily cluster based on their location on particular plates. Instead, plates are usually spread evenly inside the grouped samples, meaning there’s no strong plate effect. Normalization is important to achieve that, and more information is available in the Critical Parameters section below.

17. Use the Zoom out (minus key) to have a broader view of the clustering. Scroll through and find large squares of red color in the matrix, and observe which MOAs are clustering.

The displayed color labels of the columns are useful to find patterns in this broader view. Two groups (proteasome inhibitor and NFkb pathway inhibitor|proteasome inhibitor) are clustered together, meaning these two MOAs have similar morphological profiles.

18. Return to the tab containing the feature value rather than the similarity matrices. Go to Tools -> Marker Selection. Choose the T-test as the metric, MOA as the field, class A as DMSO, and class B as the tubulin polymerization inhibitor. Leave the default values for “Number of Markers” and “Permutations”. This step reveals what features are driving the differences between these two groups (Figure 3).

The “Number of Markers” will depend on the number of features you have and want to use for this analysis. “Permutations” will depend on the number of samples in each class. For more information about Marker Selection, see the Understanding Results section and (Gould et al. 2006).

19. Sort the p-value column by right-clicking on the p-value column. Explore the names of the features that determine the difference between DMSO and tubulin polymerization inhibitors. If you have a large number of features with p-value 0.00, these will continue to be sorted alphabetically and not by strength; in this case, you can sort to find the highest and lowest T test values, which should represent the strongest features.

The features that differentiate between DMSO and tubulin polymerization inhibitors belong to all three cell compartments: Cells, Nuclei, and Cytoplasm, (where the Cytoplasm of each cell is defined as the region identified as Cell, excluding the region identified as the nucleus. Texture and granularity (both measures of whether the stain is smooth or not) are frequent features altered by this perturbation. Tubulin is not stained in this assay but since tubulin interacts with actin, it seems reasonable that the profile would be altered in Phalloidin (F actin) staining. Another frequent feature group is Nuclei_StdIntensityEdge, the standard deviation in intensity at the edge in several channels; this likely indicates differences in the variation in staining of various organelles just outside the nuclei.

20. Go back to the similarity matrix and go to File > Save Dataset, and write a name for your File, click Ok to save as GCT version 1.3, and save the table, allowing it to be opened again in Morpheus when needed.

BASIC PROTOCOL 2

Image and single-cell visualization following profile interpretation

With large datasets, it often becomes challenging to retrieve images of sites or single cells for visualization to perform quality control, validate a pipeline, and, most importantly, interpret any morphological changes detected in the profiles explored during the quantitate data analysis and exploration (visualized with heatmaps, UMAPs, etc). Along with visualizing sample and feature correlations as in Basic Protocol 1, it is also important to think biologically about organelle distribution, general morphology such as cell and nuclei shape, and intensities of each stain. Connecting the numbers (Pearson coefficients, T-tests, morphological feature values in the profiles, etc.), with how the cells look in the images can help the researcher to decipher a complex profile.

In Basic Protocol 2, we describe how to use a script we created to retrieve random or representative images from the dataset and plot them together, allowing the scientist to choose which samples to observe and how to group and display them. While random images are often helpful, especially in cases of high heterogeneity it can be helpful to computationally determine which cells' phenotypes are the most *representative* in your sample, and compare them to control cells. This is not a trivial step but can sometimes provide critical insight into the morphological change. In this protocol, we use Jupyter Notebook to derive representative cells by performing a clustering analysis on the morphological space of the population of single cells and sampling from the subpopulation closest to the center of the sample(s) of interest. This notebook can also be used to compute similarity matrices as in Morpheus; while for large-scale experiments we recommend examining the whole experiment as in Basic Protocol 1, once a few treatments of interest are identifies the ability to calculate similarity in the notebook allows users to, for example, visualize single cells and ordering the images by most correlated to the negative control.

From the Jupyter Notebook, the scientist will obtain representative or random image sites and single cells, enabling comparison of the images with the correlation coefficient values obtained in the similarity matrix. By establishing the relationship between the images and the heatmaps, the user can start hypothesizing about the biological processes and morphological profiles that are significant, which could lead to more specific biological questions and assays. As in Basic Protocol 1, we provide some hints and interpretations for each step, but the major discussions of biological interpretations are presented in the Understanding Results section below.

Materials:

This protocol assumes the use of a web browser to run Google Colab. Open our Google Colab notebook (link https://github.com/ciminilab/2022_Garcia-Fossa_submitted/blob/main/basic_protocol_2/notebook/Basic_Protocol_2.ipynb) and create a copy on your own Google Drive, enabling you to run Basic Protocol 2. To adapt this protocol to their own data, users should either download the Jupyter Notebook to their local computer and install the requirements based on the requirements.txt file or use Google Colab and mount their Google Drive (https://colab.research.google.com/notebooks/io.ipynb) to enable access to data they have stored within their Google Drive. In both cases, the scientist must adapt the pathnames and filenames within Section 2 of the Notebook to point to their dataset.

Our dataset table is in a CSV format, a comma-separated value table containing the extracted features for single cells in a Cell Painting assay. In this assay, 1,571 compounds were tested across 6 doses in A549 cells (Way et al., 2022b). Notice that we use the same dataset from Basic Protocol 1, but here we require information about single cells, and each row of the table must have cell features, and X and Y locations within the image, to enable single-cell image retrieval. We also provide all the images of where these single cells are located. For this purpose, we selected only a subset of samples within the dataset to allow the user to explore the data even having low computer memory available. We also performed normalization and feature selection with this dataset using pycytominer (Way et al., 2022a). The Jupyter Notebooks required to create this dataset from publicly available data sets (1_Samples_retrieval.ipynb and 2_Generate_Profiles.ipynb) are available on our GitHub under the basic_protocol_2/notebook folder.

The Jupyter Notebook functions were written using Python 3.9 (Van Rossum and Drake, 2009). Data processing was performed using pycytominer (Way et al., 2022a) tools for normalization, feature selection, and data annotation. Check pycytominer documentation (https://pycytominer.readthedocs.io/en/latest/) for details on how to change parameters and inputs depending on your dataset.

The GitHub repository contains the following files relevant to Basic Protocol 2:

- util folder with .py files containing functions written to be used on this notebook. These functions are installed onto the notebook using pip install and then imported from utils.correlations import *;
- basic_protocol_2/Images folder, which contains the subset of images downloaded from https://github.com/broadinstitute/cellpainting-gallery. We provide PNG images that were compressed from the original TIFF images; PNG is a lossless format that requires less storage space.
- basic_protocol_2/data folder, which contains the "BasicProtocols2_Example.zip" with a CSV file. To use this notebook with their data, the user could extract the features using CellProfiler (Stirling et al., 2021), and export the information to a spreadsheet, which can be read in the Jupyter Notebook. Or, if using a database file, the scientist could transform it into a CSV file using our available Samples_retrieval.ipynb Jupyter Notebook. The notebook will perform annotation, normalization, and feature selection if you have not already run those steps; they can also be by passed if these steps have already been done (such as by notebook 2_Generate_Profiles.ipynb)

Protocol steps

1. Open the Google Colab notebook "Basic Protocol 2_Visualize cells and images.ipynb" available in the link on our GitHub page (https://github.com/ciminilab/2022_Garcia-Fossa_submitted/blob/main/basic_protocol_2/notebook/Basic_Protocol_2.ipynb). Be sure to access the notebook from our GitHub repository, allowing you to check for any recent updates.
2. Click the Copy to Drive button, and the notebook will be available on your Google Drive in the Colab Notebooks folder.

This step allows the researcher to have their copy of the notebook, and if they wish, perform any modifications to run the notebook using their own data.

3. Run the first three cells in the notebook Section 1 - Import Libraries by clicking on the start button at the top left (or hit Ctrl + Enter). The first line will clone the GitHub repository and install the functions, the second line will install the required libraries to run this notebook (this process takes ~5 min to run), and finally, import the libraries to allow their use inside the notebook. Run the lines of code in the order that they appear in the notebook.

The Python packages required to run this notebook are also available on GitHub under the requirements.txt file. This file can be used to install packages via pip or to generate an environment using Anaconda or miniconda to run this Jupyter Notebook locally.

4. Run only the first cell inside Section 2 - Define Inputs. This will define the inputs required for running the cells in the notebook. The script requires the filename and pathname to access the CSV table and read it as a DataFrame. It also needs the pathname for the images directory.

The pathnames are all based on the ones available in the GitHub repository for this project. If you clone the repository in the first step, there is no need to change these inputs. To run this notebook with new data, mount the notebook inside Google Drive and provide the inputs for the variables (running the second code cell inside Section 2 instead of the first cell).

5. Run the cells inside Section 3a, which will import the dataset and perform annotation, normalization, and feature selection. The table contains all the features measured for every single cell, and also metadata information about the mechanism of action (MOA) of each compound, compound names, and concentrations tested. For more informaiton about feature selection, see the Critical Parameters section.

If your data set has already been annotated, normalized, and feature selection, skip section 3A and proceed directly to section 3B to load it in with no adjustments. If running section 3A with your own data, you will need to have already annotated your data with Metadata (such as in CellProfiler's Metadata module) or provide a table here that provides the ability to map the measurement data to treatment metadata. If you want to normalize the whole plate rather than a negative control, remove the "samples" parameter from the "normalize" funciton in the last cell of section 3.a.3..

6. Run the first three cells in Section 4 (through cell 4.1.1) and choose "Metadata_Compound_Concentration" for this demonstration. These options were generated based on the name of the columns with the "Metadata_" prefix. This choice will impact the information visualized on the plots for the next steps. If the choice is "Metadata_Compound_Concentration", then you will see values such as DMSO 0.0, etc. When using new data, the researcher should add the "Metadata_" prefix to any such columns before loading it into the notebook, as it will appear under this dropdown and be used for aggregation (Figure 4 A).

We use dropdown interaction to allow the researcher different choices based on the DataFrame. This is because they might be interested in looking at the data from a mechanism of action perspective, or based on the compound's names. Be aware that if using new data, the tables must have columns containing metadata information with the "Metadata_" prefix.

7. Run the cell in Section 4.2 to choose all the compounds available on the dataset to visualize. This step will select all the compounds in the dataset.

*To select just a few compounds of interest to be visualized, run Section 4.3. This piece of code will create an interactive checkbox with the compound names for you to choose only a few options (*Figure 4 A).

8. Run the cells in Section 5 to generate and graph the correlation between the compounds. Choose a column to be the labels for the correlation matrix using the dropdown. Then, we use pycytominer (Way et al., 2022a) to return a per-well aggregated DataFrame, and a correlation matrix is generated. There is an option to export the matrix as an image, type the name, and press "Enter/return".

In Section 5, the function that applies pycytominer operations aggregates the data and then performs a Pearson correlation analysis in the dataset. If the user wants to visualize the matrix with different labels, they can choose a different column and rerun the notebook from that cell onward; their dataset will the be re-aggregated and a new correlation matrix calculated based on the new column.

9. Run the three cells in section 5.1 to insert the correlation values calculated in the previous step inside the initial DataFrame as a new column. This function will get the chosen compound and find the correlation

values for every other compound related to the first. Choose “DMSO 0.0” for comparison because for this dataset, the aim is to evaluate what compounds have morphological profiles more similar to the control.

Choose whichever compound to have as a point of reference to be added to the DataFrame. This choice will depend on their biological question.

10. Run all of the cells inside section 5.2, and choose “DMSO 0.0”. This choice reflects the biological question of what compounds are closely correlated to the negative control (DMSO, in our case). However, this is a dynamic Jupyter Notebook where the user could be interested in other compounds or MOAs.
11. In Section 6 - Run the first cell of the notebook choose the method to select the cells from the DataFrame. You can choose the “random” method to select random samples for each treatment/group you have, orr choose “representative” method to select the most representative cell within each subgroup. Many cells in this section rely on correlation to the reference compound selected in section 5.1; if you want to change reference compounds, rerun those cells before returning to section 6 and running all cells there.

Choosing the representative method uses the KMeans algorithm with the scikit-learn package (Pedregosa et al., 2011) to cluster data and find the most representative cell (the cell(s) closest to the mean of the subgroup) within each subgroup. The random method will instead return a random sample of one cell for each subgroup (Reback et al., 2020). The representative method allows you to evaluate average change, while the random method is often helpful for quality control to check for out-of-focus or unusual cells.

12. Run the next cell and select how many cells you would like to display from each subgroup and whether or not you would like the images shown in order of subgroup correlation to the reference compound. .

*Answering "Yes" to “Would you like to use the correlations to order your image plot?” will order the dataset based on the correlation values to the reference compound selected in step 9, starting at 1.0 and descending; answering “No” will keep the DataFrame in the original order. The second question is about how many cells (c) to plot for each group. The generated image will have (c * the number of subgroups) rows. Looking at one cell per subgroup creates a compact visualization, especially for many subgroups; looking at several per subgroup can increase confidence in the overall visual appearance of each subgroup, especially when displaying random cells. .*

13. Choose whether each image should be rescaled to the minimum and maximum before being displayed, or whether raw intensity values should be plotted instead. Raw intensities are typically more comparable across conditions (see below for caveats), but may be harder to see when the signal is dim and thus may require external rescaling after saving.

While in general raw images are more comparable than individually rescaled images, caution should be taken especially in comparing images from treatments imaged on different plates or different plate batches. Each plate is stained, imaged, and has feature normalization run independently; plates from different batches may additionally have other differences such as e.g. individual reagent lots used. Thus, a treatment that induces "2x negative-control-mean-intensity" in channel X from Plate 1 may be overall dimmer in raw pixel intensity values than a different treatment that induces "0.5x negative-control-mean-intensity" in channel X from Plate 2 if the plate means intensities in channel X are quite different. Any conclusions drawn based on looking at images should be subsequently checked against normalized feature data. .

14. Plot the selected single cells in random order by running the first cell of section 6.1. This step allows a first view of the cells without the labels, so the researcher can explore the images before knowing to which group the cells belong. Once you have explored the data, run the rest of the cells in section 6.1 to append labels to see if your hypotheses were correct, to create an un-shuffled version of the image, and to save the image to disk.

Looking at cells without labels allows the researcher to formulate new hypotheses without bias about what they believe each treatment "should look like". Parameters to examine might include the organelles’ distribution within the *cells; how the mitochondria, endoplasmic reticulum, or the Golgi apparatus are organized; changes in overall intensity of individual stains;, or overall cell structure changes. This can be quite valuable for unbiased hypothesis generation!*

15. Run section 6.2 to display the full images from which the single cell crops have been pulled (Figure 5 B). Looking at the entire field of view (FOV) may provide insights into additional biological aspects.

Looking at whole images allows w more holistic view of the overall cell shape and size distributions present in the images (e.g., how were the cells affected by the treatment: do they look larger or smaller when compared to the negative control? Does their shape change, are they more elongated, or rounder?). It also allows examination of cell density (e.g., how are the cells distributed within the FOV? Can you see more cells in the FOV between perturbations and the control, meaning the perturbation might induce some proliferative signaling pathway? Or are there fewer cells in the FOV, meaning the perturbation might reduce cell viability?).

COMMENTARY

Background Information

Image-based profiling typically starts with using fluorescent markers to stain different targets and/or compartments of the cell. In our example data for both protocols, we used Cell Painting data. Cell Painting is a morphological profiling assay that multiplexes six fluorescent dyes, imaged in five channels, to reveal eight relevant cellular components. The experiment's aim was to characterize chemical perturbations in cells by measuring the morphological changes after cells were exposed. Briefly, cells were plated in multiwell plates, perturbed with the treatments to be tested, then stained, fixed, and imaged on a high-throughput microscope. Images were acquired for DNA, RNA, endoplasmic reticulum, mitochondria, and AGP (actin, Golgi, and plasma membrane).

Software such as CellProfiler (Stirling et al., 2021) makes it easy to obtain and extract information from these images, extracting thousands of morphological features distributed into categories relating to the compartment measured (Nucleus, Cell, Cytoplasm) and types of metrics (measurements of size, shape, texture, intensity, granularity, and more) to produce a feature profile that enables the detection of subtle phenotypes. To facilitate understanding of the features, CellProfiler feature name outputs are organized as follows: *[Compartment]_[FeatureGroup]_[Feature]_[Channel]_[Parameters]*. Not all features have Channel information - for example, shape features relate only to the outlines of the chosen cellular compartments. From a Cell Painting assay, the Nuclei are identified by the DNA channel, the Cells by the RNA or AGP channel, and the Cytoplasm is the Cell, excluding the Nuclei object. FeatureGroups are associated with the measurements made on the Compartments (e.g., AreaShape, Intensity, Texture, Granularity, and more). To understand how each module works to extract information from the images, check the latest documentation available for CellProfiler (https://broad.io/cellprofilermanual). You can check a list of all the features extracted from one particular analysis of a Cell Painting assay at https://github.com/carpenterlab/2022_Cimini_NatureProtocols/blob/main/CellProfiler_features.csv , but note that the names of the features will vary based on the parameters used to analyze the assay.

The essential steps after extraction of the features are aggregation, normalization, and feature selection. These are the steps we describe in our Jupyter Notebooks using pycytominer. Then, profiles of cells treated with different experimental perturbations are compared to identify the phenotypic impact of chemical or genetic perturbations, grouping compounds and/or genes into functional pathways, and identifying signatures of disease. We demonstrate these last two steps using Morpheus software and scripts on Jupyter Notebooks in the Basic Protocols above.

Pearson correlation coefficient

Understanding the correlation coefficients calculated for the samples on both protocols is important for this protocol paper. A Pearson correlation coefficient is a way to represent the measurement of similarity, where it measures the strength of the linear relationship between two variables (in our case, the relationship between two wells across a large set of features or for two features across a large set of wells). A Pearson coefficient of 1 means a perfect positive correlation, 0 means no correlation was found, and -1 represents a perfect negative correlation (Pearson and Galton, 1895). A similarity matrix is a way to assess the covariance in features between all pairs of columns or rows. In each square of the matrix, a Pearson correlation coefficient was calculated for all features in the data set between each pair of samples. The squares at the intersection of those two samples are set as the value of that correlation coefficient, and so on for each pair of wells. This allows us to see at a high level how similar the overall phenotype is between any pairs of samples in our experiment, and therefore how phenotypically distinct our treatments are.

Critical Parameters

We reiterate that normalizing the features is fundamental before executing the steps in this paper. Normalization is usually performed on all of the features to fix range issues and allow comparison between the features (Caicedo et al., 2017). Normalization is also recommended to increase the signal-to-noise ratio (Chandrasekaran et al., 2021). A normalization performed on a plate level is recommended because this also corrects to some degree for plate-to-plate batch effects. Where sufficient negative controls exist, we recommend normalizing the features to the negative control. Check the profiling recipe for more information about how to process single-cell morphological profiles and details on how to normalize Cell Painting data for more information.

In data normalized to the negative controls, negative control samples (or samples with otherwise weak phenotypes) will show limited similarity to one another and thus will show minimal clustering after step 16 (hierarchical clustering). Somewhat unintuitively, this means that these samples will be spread across the entire data set post-clustering, and it is therefore expected to sometimes see one or a small number of "random" negative controls or weak perturbations clustering with a strong, consistent perturbations; this should not be taken as a sign that the strong perturbation in question is weak or similar to negative controls. One should always check whether a phenotype wants to explore further via the latter half of Basic Protocol 1 and in Basic Protocol 2 shows strong similarity between replicates pre- or post-clustering; if not, it is unsuitable for further analysis.

Proper reduction of the feature space is also an essential step to perform before analyzing new data data in our protocols; this step will be automatically performed when following the profiling recipe (Chandrasekaran et al.). If performing these steps on your own, a common starting point is looking for correlated features: when two features are too correlated, only one should be kept for further analysis. Since Pearson correlations are sensitive to large absolute feature values, we also recommend screening for unusual feature values; we provide guidance on performing this in Morpheus in Basic Protocol 1 steps 3-6. Some feature reduction algorithms, such as support vector machine, give weights for each feature and remove the ones with fewer weights (Caicedo et al., 2017). We typically perform feature reduction in pycytominer (Way et al., 2022a) , which provides six options for reducing the feature space: removing based on variance threshold (removing features that have relatively few unique feature values and/or a single value that is far more common than the rest of the feature values), correlation threshold (removing features that are highly correlated to other features and thus redundant), drop NA columns (removing features where a large number of values are missing), drop outliers (removing features with aberrantly large absolute values), remove noise (removing features which tend to have a high variance across replicates), and blocklisting (removing features thought to not typically add useful biological information to Cell Painting profiles) (Way, 2019). Many of these feature removal methods have tunable parameters which ultimately guide the fraction of features removed; as such, it is critically important to check that the threshold values are appropriate for your data and to adjust them when necessary.

Profiles should be assessed for their quality before data interpretation, to remove treatments with no apparent phenotype and, in some applications, to exclude compounds that are too toxic to the cells

(Rezvani et al., 2022). One method to perform profile quality assessment is to measure the precision with which one can retrieve correctly retrieve replicate wells. This approach was used in the example data we provide to check for replicability of the profiles; more details are provided in (Way et al., 2021b).

Troubleshooting:

See Table 1 for problems, possible causes, and solutions.

Understanding Results

When analyzing results, you may find a profile of interest shows a dramatic difference from controls or other samples based on only a small number of similarly-named features (such as a large number of features that fall within the nucleus or many changes in the texture of a particular stain), and the feature names have obvious meanings (*e.g.,* nucleus area or integrated intensity of the mitochondria channel in the cytoplasm). In this scenario, interpretation may be straightforward, though you may need to look up the meaning of the feature names in the CellProfiler manual to understand them better and discern their connection to the biological meaning. Some caution is warranted here; for example, DNA-damaging drugs could affect actin features, because F-actin plays a role in DNA repair. Damage induced to the DNA induces nuclear actin formation (Belin et al., 2015), and these nuclear actin structures play a role in double-stranded-break (DSB) repair, such as recruitment of proteins to enable repair of the heterochromatin through homologous recombination, and assisting on DSB movement in euchromatin repair (Caridi et al., 2019). There may not be a straight line from a feature name to the biological function because cells are deeply interconnected systems, and changes that start in a single genetic pathway can ripple throughout other pathways in the cell. Nevertheless, feature names can often create insights.

Instead of a few, easily interpretable features, you may find there are many dominant features in the profile and their collective meaning is not obvious. In such cases, an expert might be able to stare at the list and derive some meaning. For example, an expert might realize that many different stains showing increased correlation may actually be related to a decreased cell size (because in a cell with less spread, organelles are more likely to overlap one another on the x-y plane. If you've looked at your feature list but need some backup, consider sharing your data on forum.image.sc to get some experts to weigh in. An example of this can be found in the morphological profile induced by the microtubule inhibitor (cabazitaxel) and microtubule stabilizing agent (ixabepilone) in this data set. To understand what are the features that differentiate between our negative control (DMSO) and the microtubule perturbations, we performed marker selection using a T-test. Marker selection comes from genome analysis, but it could be defined also as a feature selection. The model takes the features belonging to two classes as input, and a T-test is calculated to assess marker features that discriminate between the two classes (DMSO vs Microtubule) (Gould et al., 2006). Our results show that many important features (Figure 6 A) belong to Granularity and Texture feature groups across a number of different stains, which makes sense in the context of induction of massive cytoskeletal disruption. Since microtubule dispruption perturbs cell division, the presence of "Nuclei_AreaShape_FormFactor" (a measure of shape uniformity inwhich linear and/or irregular shapes have values near 0 and a perfect circle is 1) helps indidate that we are looking not just at cytoskeletal disruption generally, but specifically disruption of the microtubules. This result highlights that the aggregate of different features is important for connecting profiles to perturbations.

Examining example images directly alongside the list of important features can also help decipher a complex profile. An example where looking at features and images could help uncover the biological meaning of an event is during an assay to identify cells in different phases of the cell cycle, using fluorescent markers such as DAPI to measure DNA content (Ferro et al., 2017). Based on significant changes in the feature space where the minor axis of the Nuclei and Cell area are low, and DNA staining intensity is high, the researcher could look at single cells and realize this feature changes relates to cells that actually are going through metaphase. Basic Protocol 2 facilitates displaying single cells and images, which can otherwise be challenging to locate and access in large-scale experiments. In our example of microtubule-related drugs the representative images, we observe that both drugs interfere with the cell cycle to produce similar morphologies, disrupting the overall appearance of every channel. As seen in Figure 6B, both treatments induce multinucleation (Figure 6 B, DNA column), as has been previously described for microtubule inhibitors (Azarenko et al., 2014); disruption of the cell cycle is also likely apparent in the lower overall cell count in the treated vs control cells (Figure 6C). The Golgi localization and distribution are visually quite distinct as compared to DMSO (Figure 6 B, AGP column), which could be related to microtubules function in vesicular trafficking and their role in modelling the shape of organelles, including Golgi (Fourriere et al. 2020; Thyberg and Moskalewski 1985). We can therefore relate these morphological features and observations to the mechanism of actions of these drugs, providing a useful pattern to follow for investigators examining their own data and formulating their hypotheses. Sometimes, though the most important differences are not human visible; image-based profiling approaches have sometimes outperformed human expert image analysis for precisely such reasons (Gibson et al. 2015; Zhou et al. 2021).

Finally, we should note that in some situations, following the procedures provided still does not allow you to make much headway in truly understanding the induced phenotype. If so, one can still use profile data in other ways, such as by simply using the profile as a signature of the sample and and trying to use drugs to revert this disease phenotype to a healthy-associated phenotype. If one has access to computational experts, one can also try to query their data against publicly available data sets (Rohban et al. 2022), though these approaches are currently still experimental. The interpretation of complex profiles is a challenge but when successful can propel research in new directions to uncover exciting new mechanisms.

Time Considerations

For **Basic Protocol 1**, supposing that the tables were pre-processed for normalization and feature selection before input into Morpheus, the total time to explore the data is approximately 1 hour. **Basic Protocol 2** could take up to 2.5-3 hours if running the protocol with different settings and taking time to evaluate the images and create hypotheses.

CONFLICT OF INTEREST STATEMENT:

SS and AEC serve as scientific advisors for companies that use image-based profiling and Cell Painting (AEC: Recursion, SS: Waypoint Bio, Dewpoint Therapeutics) and receive honoraria for occasional talks at pharmaceutical and biotechnology companies.

DATA AVAILABILITY STATEMENT:

The data that support the protocol are openly available at https://github.com/ciminilab/2022_Garcia-Fossa_submitted.git

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INTERNET RESOURCES:

https://forum.image.sc Forum for image analysis questions and discussions. \

https://cellprofiler.org/ Download and learn how to use CellProfiler.

https://github.com/CellProfiler/tutorials Beginners and advanced tutorials for CellProfiler.

https://www.youtube.com/c/COBACenterforOpenBioimageAnalysis/videos Video tutorials for CellProfiler, Morpheus, and other many tools on the Center for Open Bioimage Analysis (COBA) channel.

https://github.com/cytomining/pycytominer Data processing functions for profiling perturbations. More information on how to use pycytominer, documentation, and workflows.

FIGURES:

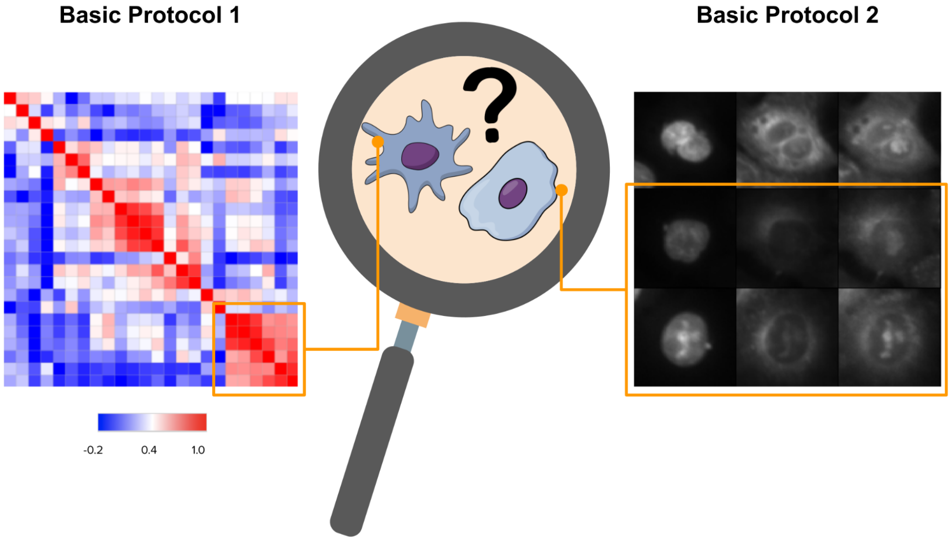
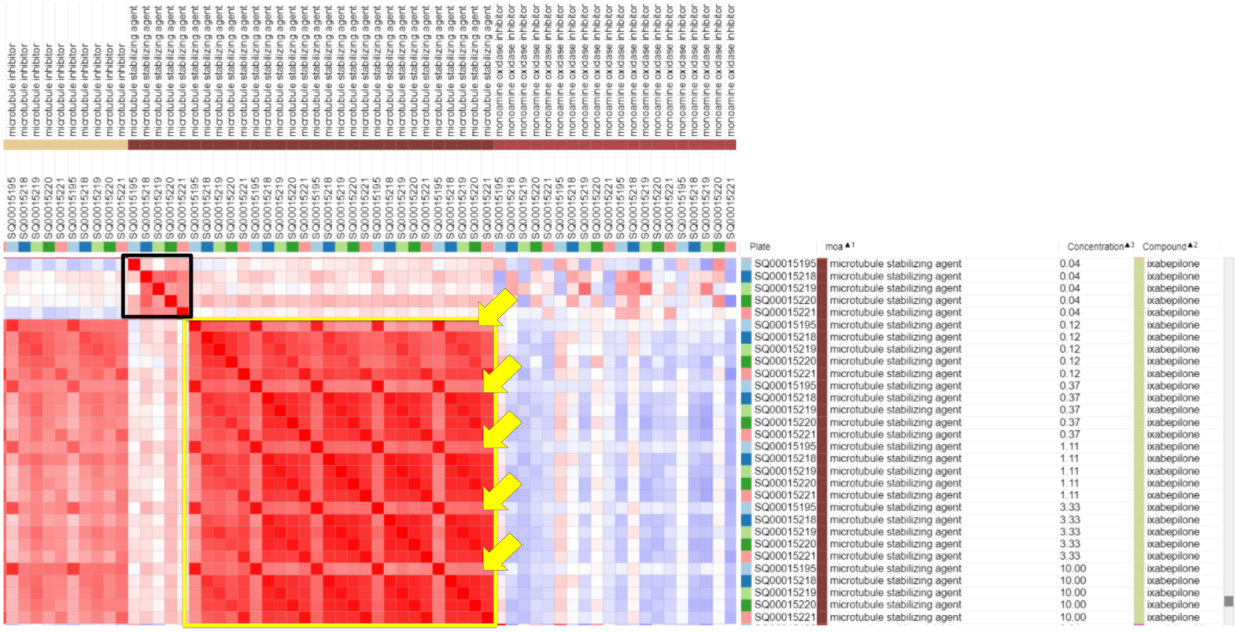


Figure 1 - In Basic Protocol 1, based on sample clustering, biologists can understand the underlying morphology that makes certain samples cluster in a certain way. In Basic Protocol 2, biologists can examine representative cells from each sample.

Microtubule inhibitor versus Microtubule stabilizing agent


microtubule inhibitor SQ00015195
microtubule inhibitor SQ00015218
microtubule inhibitor SQ00015219
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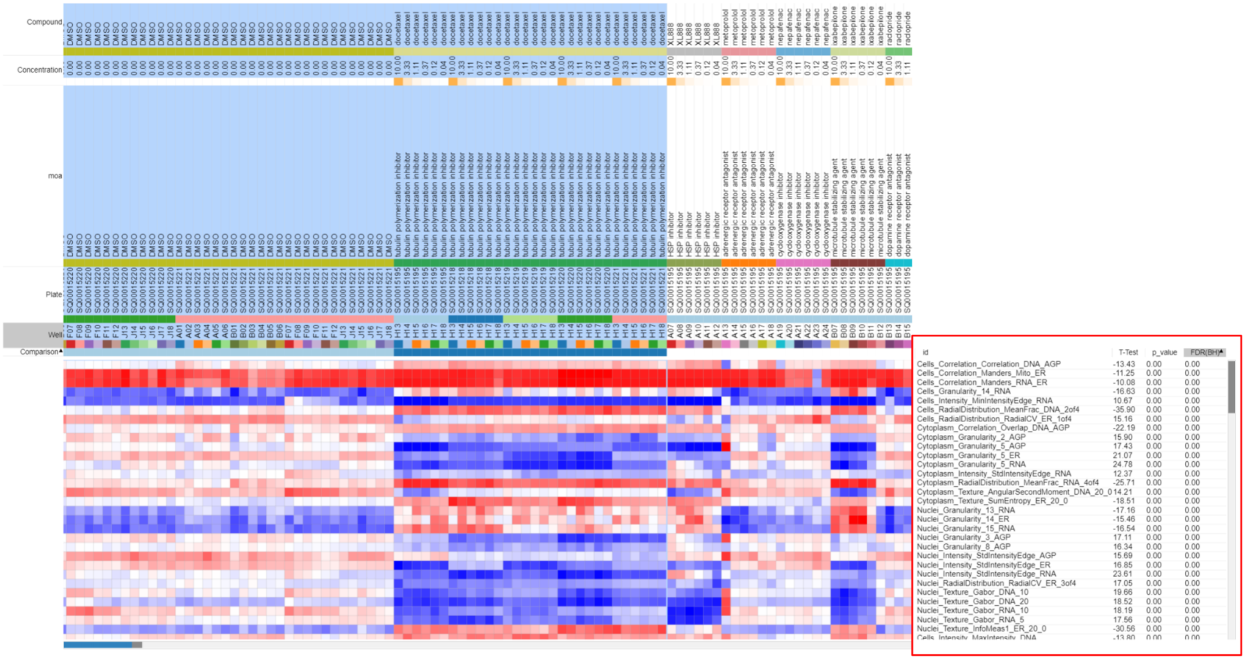
monoamine oxidase inhibitor SQ00015219
monoamine oxidase inhibitor SQ00015220
monoamine oxidase inhibitor SQ00015221

Plate	moa ▲1	Concentration ▲3	Compound ▲2
SQ00015195	microtubule stabilizing agent	0.04	ixabepilone
SQ00015218	microtubule stabilizing agent	0.04	ixabepilone
SQ00015219	microtubule stabilizing agent	0.04	ixabepilone
SQ00015220	microtubule stabilizing agent	0.04	ixabepilone
SQ00015221	microtubule stabilizing agent	0.04	ixabepilone
SQ00015195	microtubule stabilizing agent	0.12	ixabepilone
SQ00015218	microtubule stabilizing agent	0.12	ixabepilone
SQ00015219	microtubule stabilizing agent	0.12	ixabepilone
SQ00015220	microtubule stabilizing agent	0.12	ixabepilone
SQ00015221	microtubule stabilizing agent	0.12	ixabepilone
SQ00015195	microtubule stabilizing agent	0.37	ixabepilone
SQ00015218	microtubule stabilizing agent	0.37	ixabepilone
SQ00015219	microtubule stabilizing agent	0.37	ixabepilone
SQ00015220	microtubule stabilizing agent	0.37	ixabepilone
SQ00015221	microtubule stabilizing agent	0.37	ixabepilone
SQ00015195	microtubule stabilizing agent	1.11	ixabepilone
SQ00015218	microtubule stabilizing agent	1.11	ixabepilone
SQ00015219	microtubule stabilizing agent	1.11	ixabepilone
SQ00015220	microtubule stabilizing agent	1.11	ixabepilone
SQ00015221	microtubule stabilizing agent	1.11	ixabepilone
SQ00015195	microtubule stabilizing agent	3.33	ixabepilone
SQ00015218	microtubule stabilizing agent	3.33	ixabepilone
SQ00015219	microtubule stabilizing agent	3.33	ixabepilone
SQ00015220	microtubule stabilizing agent	3.33	ixabepilone
SQ00015221	microtubule stabilizing agent	3.33	ixabepilone
SQ00015195	microtubule stabilizing agent	10.00	ixabepilone
SQ00015218	microtubule stabilizing agent	10.00	ixabepilone
SQ00015219	microtubule stabilizing agent	10.00	ixabepilone
SQ00015220	microtubule stabilizing agent	10.00	ixabepilone
SQ00015221	microtubule stabilizing agent	10.00	ixabepilone

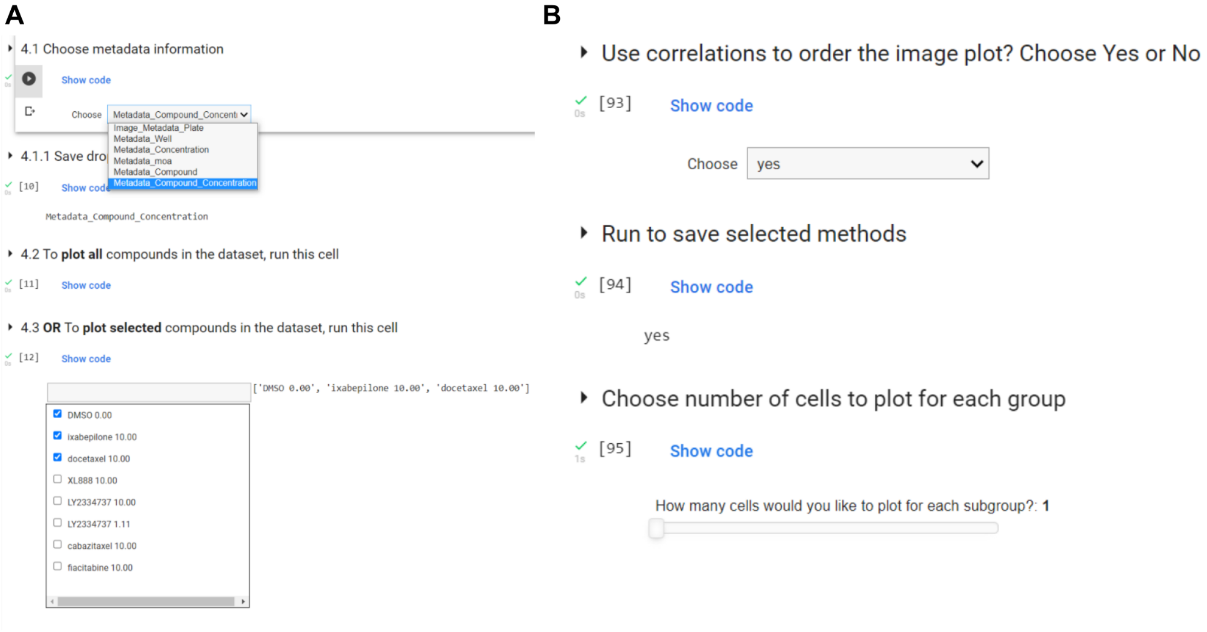
Figure 2 - Similarity matrix generated in Morpheus. Columns were sorted by MOA, Compound, and then Concentration. The yellow box emphasizes the highly correlated samples in the MOA class *Microtubule stabilizing agent*, and how these correlations are high across the five plates where replicate samples are located, and across many doses for the same compound. The recurring pattern within this block (highlighted by the yellow arrows) indicates that one of the five replicates shows a somewhat different profile than the other four, indicating a possible batch of plates with a technical anomaly. The effective concentration of a drug is highlighted by the lowest dose of ixabepilone clustering together (black box) but having weak correlations with the highest doses of ixabepilone.

T-test: DMSO versus tubulin polymerization inhibitor



id	T-Test	p_value	FDR(BH)
Cells_Correlation_Correlation_DNA_AGP	-13.43	0.00	0.00
Cells_Correlation_Manders_Mito_ER	-11.25	0.00	0.00
Cells_Correlation_Manders_RNA_ER	-10.08	0.00	0.00
Cells_Granularity_14_RNA	-16.63	0.00	0.00
Cells_Intensity_MinIntensityEdge_RNA	10.67	0.00	0.00
Cells_RadialDistribution_MeanFrac_DNA_2of4	-35.90	0.00	0.00
Cells_RadialDistribution_RadialCV_ER_1of4	15.16	0.00	0.00
Cytoplasm_Correlation_Overlap_DNA_AGP	-22.19	0.00	0.00
Cytoplasm_Granularity_2_AGP	15.90	0.00	0.00
Cytoplasm_Granularity_5_AGP	17.43	0.00	0.00
Cytoplasm_Granularity_5_ER	21.07	0.00	0.00
Cytoplasm_Granularity_5_RNA	24.78	0.00	0.00
Cytoplasm_Intensity_StdIntensityEdge_RNA	12.37	0.00	0.00
Cytoplasm_RadialDistribution_MeanFrac_RNA_4of4	-25.71	0.00	0.00
Cytoplasm_Texture_AngularSecondMoment_DNA_20_0	14.21	0.00	0.00
Cytoplasm_Texture_SumEntropy_ER_20_0	-18.51	0.00	0.00
Nuclei_Granularity_13_RNA	-17.16	0.00	0.00
Nuclei_Granularity_14_ER	-15.46	0.00	0.00
Nuclei_Granularity_15_RNA	-16.54	0.00	0.00
Nuclei_Granularity_3_AGP	17.11	0.00	0.00
Nuclei_Granularity_8_AGP	16.34	0.00	0.00
Nuclei_Intensity_StdIntensityEdge_AGP	15.69	0.00	0.00
Nuclei_Intensity_StdIntensityEdge_ER	16.85	0.00	0.00
Nuclei_Intensity_StdIntensityEdge_RNA	23.61	0.00	0.00
Nuclei_RadialDistribution_RadialCV_ER_3of4	17.05	0.00	0.00
Nuclei_Texture_Gabor_DNA_10	19.66	0.00	0.00
Nuclei_Texture_Gabor_DNA_20	18.52	0.00	0.00
Nuclei_Texture_Gabor_RNA_10	18.19	0.00	0.00
Nuclei_Texture_Gabor_RNA_5	17.56	0.00	0.00
Nuclei_Texture_InfoMeas1_ER_20_0	-30.56	0.00	0.00
Cells_Intensity_MaxIntensity_DNA	-13.80	0.00	0.00

Figure 3 - Features that are driving the differences between the groups. A T-test was performed on DMSO versus tubulin polymerization inhibitor classes using the Marker Selection tool in Morpheus in Step 20. Features that differentiate between DMSO and a tubulin polymerization inhibitor are highlighted using a red box.



4.1 Choose metadata information

Show code

Choose Metadata_Compound_Concentration

- Image_Metadata_Plate
- Metadata_Well
- Metadata_Concentration
- Metadata_moa
- Metadata_Compound
- Metadata_Compound_Concentration

4.1.1 Save dro

[10] Show code

```
Metadata_Compound_Concentration
```

4.2 To **plot all** compounds in the dataset, run this cell

[11] Show code

4.3 **OR** To **plot selected** compounds in the dataset, run this cell

[12] Show code

```
['DMSO 0.00', 'ixabepilone 10.00', 'docetaxel 10.00']
```

☑ DMSO 0.00
☑ ixabepilone 10.00
☑ docetaxel 10.00
☐ XL888 10.00
☐ LY2334737 10.00
☐ LY2334737 1.11
☐ cabazitaxel 10.00
☐ fiacitabine 10.00

Use correlations to order the image plot? Choose Yes or No

[93] Show code

Choose yes

Run to save selected methods

[94] Show code

```
yes
```

Choose number of cells to plot for each group

[95] Show code

How many cells would you like to plot for each subgroup?: **1**

Figure 4 - User's interactions with the Jupyter Notebook. (A) Demonstration of the dropdown options, and choice box to choose only a subset of the compounds (step 6 of this protocol). To use new data, add the "Metadata_" prefix to the label columns. (B) More examples of interaction through dropdowns and sliders to choose the number of cells to plot.

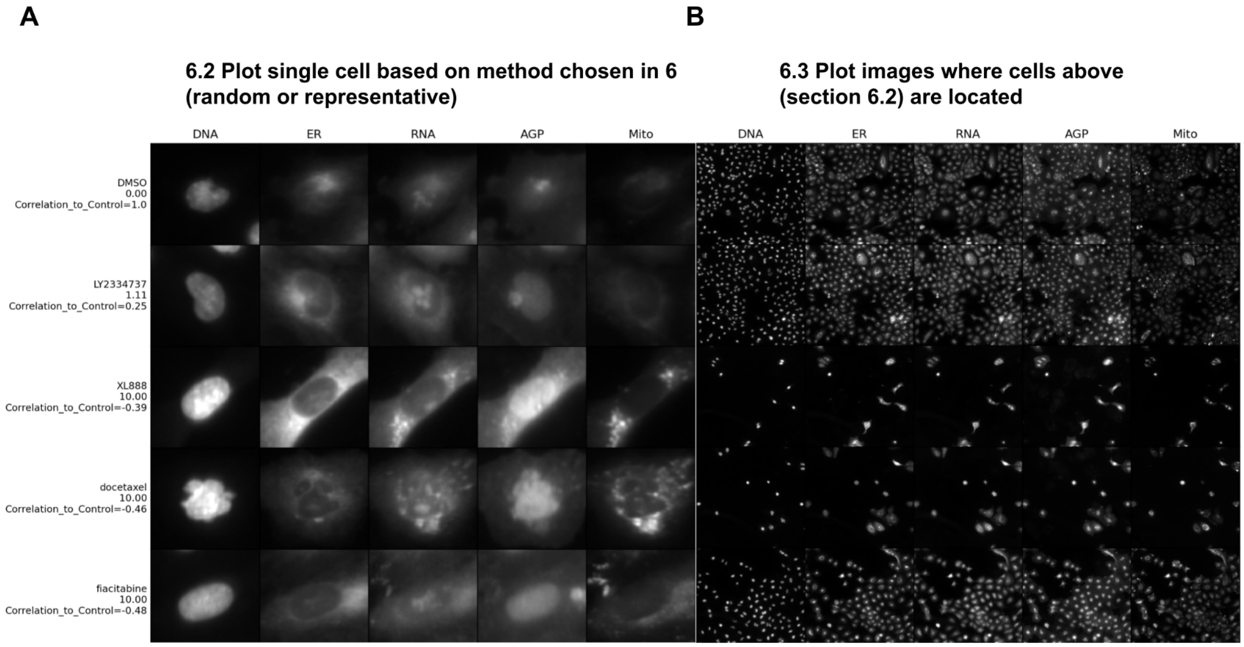
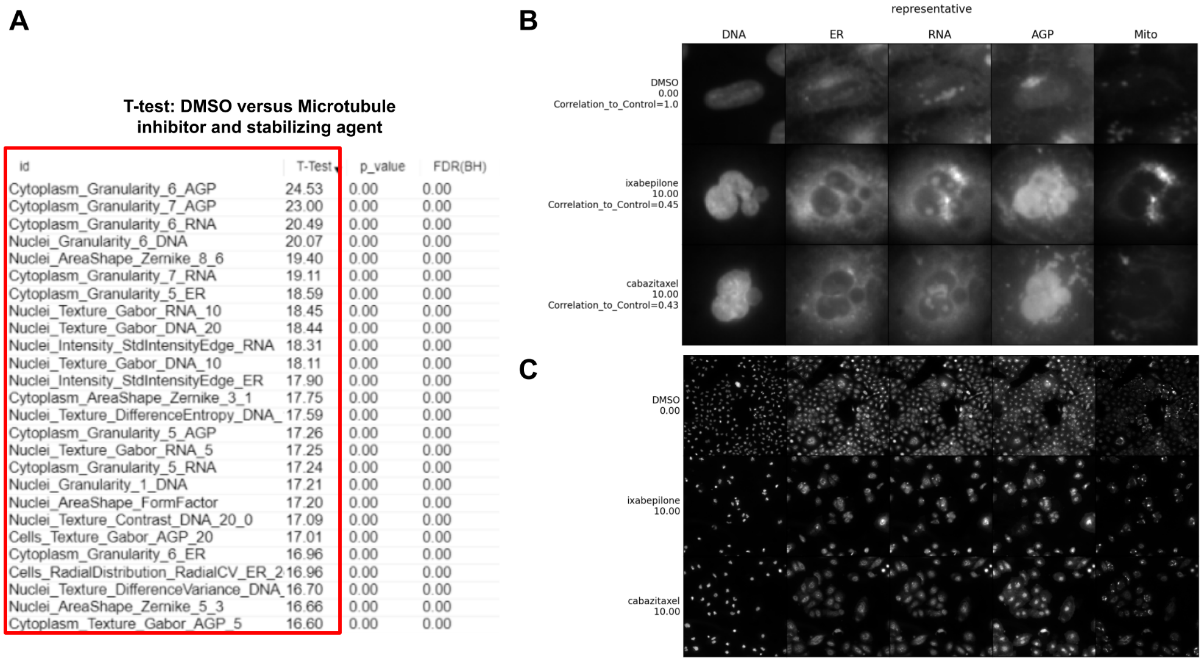

A
B
6.2 Plot single cell based on method chosen in 6 (random or representative)
6.3 Plot images where cells above (section 6.2) are located
DNA
ER
RNA
AGP
Mito
DNA
ER
RNA
AGP
Mito
DMSO
0.00
Correlation_to_Control=1.0
LY2334737
1.11
Correlation_to_Control=0.25
XL888
10.00
Correlation_to_Control=-0.39
docetaxel
10.00
Correlation_to_Control=-0.46
fiacitabine
10.00
Correlation_to_Control=-0.48

Figure 5 - Steps to plot single cells and representative images in order of correlation values. Both images were plotted with rescaled intensity, using the representative method, 1 cell per subgroup, and ordered top to bottom by the correlation values. On the Y-axis we have the compound's names and concentrations in μM, and the X-axis above has the names of stained structures, showing the different fluorescence channels available in this experiment (DNA, ER for endoplasmic reticulum, RNA, AGP for actin, Golgi and plasma membrane, and Mito for mitochondria). (A) Shows a single representative cell for each group in this dataset and (B) shows the field of view where each representative cell is located.



T-test: DMSO versus Microtubule inhibitor and stabilizing agent

id	T-Test	p_value	FDR(BH)
Cytoplasm_Granularity_6_AGP	24.53	0.00	0.00
Cytoplasm_Granularity_7_AGP	23.00	0.00	0.00
Cytoplasm_Granularity_6_RNA	20.49	0.00	0.00
Nuclei_Granularity_6_DNA	20.07	0.00	0.00
Nuclei_AreaShape_Zernike_8_6	19.40	0.00	0.00
Cytoplasm_Granularity_7_RNA	19.11	0.00	0.00
Cytoplasm_Granularity_5_ER	18.59	0.00	0.00
Nuclei_Texture_Gabor_RNA_10	18.45	0.00	0.00
Nuclei_Texture_Gabor_DNA_20	18.44	0.00	0.00
Nuclei_Intensity_StdIntensityEdge_RNA	18.31	0.00	0.00
Nuclei_Texture_Gabor_DNA_10	18.11	0.00	0.00
Nuclei_Intensity_StdIntensityEdge_ER	17.90	0.00	0.00
Cytoplasm_AreaShape_Zernike_3_1	17.75	0.00	0.00
Nuclei_Texture_DifferenceEntropy_DNA_	17.59	0.00	0.00
Cytoplasm_Granularity_5_AGP	17.26	0.00	0.00
Nuclei_Texture_Gabor_RNA_5	17.25	0.00	0.00
Cytoplasm_Granularity_5_RNA	17.24	0.00	0.00
Nuclei_Granularity_1_DNA	17.21	0.00	0.00
Nuclei_AreaShape_FormFactor	17.20	0.00	0.00
Nuclei_Texture_Contrast_DNA_20_0	17.09	0.00	0.00
Cells_Texture_Gabor_AGP_20	17.01	0.00	0.00
Cytoplasm_Granularity_6_ER	16.96	0.00	0.00
Cells_RadialDistribution_RadialCV_ER_2	16.96	0.00	0.00
Nuclei_Texture_DifferenceVariance_DNA_	16.70	0.00	0.00
Nuclei_AreaShape_Zernike_5_3	16.66	0.00	0.00
Cytoplasm_Texture_Gabor_AGP_5	16.60	0.00	0.00

Figure 6 - Interpretation of data using our Basic Protocols 1 and 2. (A) A marker selection was performed to test what are the features that differentiate DMSO vs Microtubule inhibitors (cabazitaxel) and Microtubule stabilizing agents (ixabepilone). The red box highlights the features. (B) Single cells are cropped based on an algorithm to retrieve representative cells. (C) Field-of-view where representative single cells are located. DNA, ER = endoplasmic reticulum, RNA, AGP = Actin, Golgi and Plasma membrane, and Mito = Mitochondria.

TABLES:

Table 1. Troubleshooting Guide for Basic Protocols 1 and 2.

Problem	Possible Cause	Solution
All/almost all samples have a correlation value close to 1 (Morpheus after generating Similarity Matrix)	Features are not normalized	Check if the data were normalized, meaning all features must be in a range from 0 - 1
Cells on Google Colab notebook can't run	Notebook was not copied to user's Google Drive	Add a copy of the Notebook to your own Google Drive by clicking on "Copy to Drive"
UserWarning: KMeans is known to have a memory leak on Windows with MKL (Math Kernel Library) when there are less chunks than available threads. You can avoid it by setting the environment variable OMP_NUM_THREADS=2	Memory leak	Set the environment variable OMP_NUM_THREADS = x, being x the value specified on your error output. Follow the solution in this thread on stack_over_flow