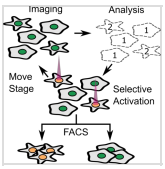

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Visual Cell Sorting

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1 *Works for me* This protocol is published without a DOI.

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ABSTRACT

Microscopy is a powerful tool for characterizing complex cellular phenotypes, but linking these phenotypes to genotype or RNA expression at scale remains challenging. Here, we present Visual Cell Sorting, a method that physically separates hundreds of thousands of live cells based on their visual phenotype. Automated imaging and phenotypic analysis directs selective illumination of Dendra2, a photoconvertible fluorescent protein expressed in live cells; these photoactivated cells are then isolated using fluorescence-activated cell sorting. First, we use Visual Cell Sorting to assess hundreds of nuclear localization sequence variants in a pooled format, identifying variants that improve nuclear localization and enabling annotation of nuclear localization sequences in thousands of human proteins. Second, we recover cells that retain normal nuclear morphologies after paclitaxel treatment, then derive their single cell transcriptomes to identify pathways associated with paclitaxel resistance in cancers. Unlike alternative methods, Visual Cell Sorting depends on inexpensive reagents and commercially available hardware. As such, it can be readily deployed to uncover the relationships between visual cellular phenotypes and internal states, including genotypes and gene expression programs.

PROTOCOL CITATION

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MATERIALS TEXT

A Lecia DMi8 Inverted Microscope was outfitted with Adaptive Focus; an Incubator i8 chamber with PeCon TempController 2000-1 and Oko CO_2regulator set to 5%; a 6-line Lumencor Spectra X Light Engine LED; Semrock multi-band dichroic filters (Spectra Services, Ontario, NY; cat. no. LED-DA-FI-TR-Cy5-4X-A-000, LED-CFP/YFP/mCherry-3X-A-000); BrightLine bandpass emissions filters for DAPI (433/24 nm), GFP (520/35 nm), RFP (600/37 nm), and NIR (680/22 nm); a 20X 0.8 NA apochromatic objective; and a Mosaic3 Digital Micromirror Device affixed to a Mosaic SS 405 nm/1.1 W laser and mapped to an Ixon 888 Ultra EMCCD monochrome camera. The microscope and digital micromirror device were controlled with the Metamorph Advanced Image Acquisition software package (v7.10.1.161; Molecular Devices, San Jose, CA).The image size was ~560 x 495 µm. Image bit depth ranged from 12-16 bits, depending on the brightness of cells in the field of view.

Cells were plated and imaged on glass-bottom, black-walled plates (CellVis, Mountain View, CA; P06-1.5H-N, P24-1.5H-N, P96-1.5H-N) in phenol-red free media at 5% CO_2 and 37 °C using the 20X 0.8 NA objective. ~50-100 cells were imaged per field of view. To image unactivated Dendra2, 474/24 nm excitation and 482/25 nm emission filters were used. To image activated Dendra2, 554/23 nm excitation and 600/37 nm emission filters were used. To image miRFP, 635/18 nm excitation and 680/22 nm emission filters were used. Prior to imaging, the Auto Focus Control system was activated. Metamorph's Plate Acquisition module was used to collect images and run Metamorph jounals that analyzed cells and directed their selective photoactivation by the digital micromirror device. For more information about the Metamorph journals used to image and activate cells, see the Appendix PDF.

SAFETY WARNINGS

Laser safety training strongly recommended

Microscope-based imaging, analysis, and activation

1 Prepare cells for imaging

1.1 24 to 48 hours before imaging, plate cells onto 6-well glass bottom, black walled plates at a density of 50,000 to 200,000 cells per well.

1.2 Wash cells with 1X DPBS and add complete media without phenol red.

2 Verify imaging conditions, analysis pipeline, and cellular phenotypes

2.1 Turn on Auto Focus Control.

2.2 Using the Well Plate Acquire dialog box, image ~25-100 sites of experimental conditions (and controls, if applicable).

These specific images will not be used for activation; rather, this analysis serves to ensure that the phenotypes match what one would expect.

2.3 Initialize a log file to collect phenotypic data.

2.4 Using the Journal > Loop > Loop Through Images in Directory command, run the analysis journal on the images to collect the desired phenotypic information.

The journal must include a "Integrated Morphometry – Measure" or a "Region Measurements" command to add phenotypic information for each cell to the log file.

2.5 Save the imaging conditions used for the Well Plate Acquire dialog box as a state file.

2.6 Close the log file. Check the distribution of phenotypes in experimental conditions and controls by running custom software (e.g. Python script) with the log file as input.

3 **Load the site map.** As of Metamorph v7.10.1.161, this must be done by.

3.1 Closing Metamorph

3.2 Replacing the *htacquir.cfg* file in the Metamorph application Groups > Metamoprh directory with an *htacquir.cfg* file that contains the site map. *htacquir.cfg* files that contain various site maps for 6- and 24- well plates used in our experiments can be found on the GitHub repository under the Metamorph directory.

3.3 Reopening Metamorph and reloading the saved state file (load everything except for site map settings).

> *In Metamorph v7.10.1.161,the site map can be contaminated by extra sites in the top left corner after this operation. Check the "Sites" tab of the Well Plate Acquire dialog box and remove any extra sites by left clicking.*

4 **Center the well**

4.1 Move the objective to the approximate center of well A1.

4.2 Under the Well Plate Acquire "Plate" tab, select "Set A1 Center ..." > "Set A1 Center to Current".

4.3 Under the "Sites" tab, move the objective to the top center site by right clicking.

4.4 Using the eyepiece and brightfield illumination settings, check whether the objective is centered at the top of the well. If not, manually change the A1 center settings (measured in microns) to move it in the desired direction.

4.5 Repeat steps (d) and (e) until the top center site of the site map is centered on the top.

4.6 Re-check that cells are in focus and that Auto Focus Control in "on". Auto Focus Control can be turned off by the objective moving too far from the plate and hitting the plate holder.

5 Make final preparations to start imaging, analysis, and activation

5.1 Select the wells to be subject to Visual Cell Sorting under the “Plates” tab by left-clicking

5.2 Select appropriate journals to be run at the Start of Plate, After Imaging, and End of Plate under the “Journals” tab

The “Start of Plate” journals (labelled “startup.jnl” in the GitHub) serve to add a delay to imaging, if necessary; set the 405 nm pulsetimes for the activations; set any phenotypic threshold values (e.g. NC ratios) for activation; etc.

The “After Imaging” journals contain analysis and activation scripts that are performed after each image is taken

The “End of Plate” journals turn off the laser to increase its lifetime

5.3 OPTIONAL: Re-align the digital micromirror device.

Under Devices > Mosaic Targeted Illumination, click “Update Settings” in the Configuration tab. Follow the instructions to re-calibrate the device.

5.4 OPTIONAL: Run the experiment without the laser on to check that the correct cells are being identified and activated.

In the Well Plate Acquire dialog box, hit “Acquire”

6 Start the experiment

6.1 Turn on the laser

6.2 Hit “Acquire” to begin acquisition, analysis, and activation.

7 Prepare cells for fluorescence-activated cell sorting (FACS)

7.1 Trypsinize cells and resuspend in DPBS supplemented with 1-2% FBS or BSA.

8 Gate and sort populations

8.1 Make a gate for live cells using a SSC-A vs. FSC-A plot.

8.2 Within the live cell gate, make a gate for single cells using a FSC-W vs. FSC-A plot.

8.3 Within the single cell gate, make a gate for Dendra2-positive cells using a FITC-A histogram plot.

In some clonally derived lines, Dendra2 expression will silence over the course of weeks to months. If Dendra2-negative cells exceed 10%, we recommend resorting the population or returning to a lower passage stock.

8.4 Create an activated (PE-YG-A) vs. unactivated (FITC-A) Dendra2 scatter plot. Draw gates for the activated populations of interest.

Activated populations will appear as diagonal clouds with higher PE-YG-A signals than a negative control.

8.5 Create a ratio (PE-YG-A / FITC-A) histogram. Show the activated populations of interest (defined in Step 8.4) within the ratio histogram. Create sorting gates for each population.

Activated populations will appear as diagonal clouds with higher PE-YG-A signals than a negative control.

8.6 Sort populations of activated cells according to the gates set on the ratio histogram plot.

9 Spin cells for 5 minutes at 300-500xg, then plate cells in warm, complete media.

Data analysis

Analyze data using FlowCytometryTools (v0.5.0) in Python (v3.6.5) or flowCore (v1.11.20) in R (v3.6.0).

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