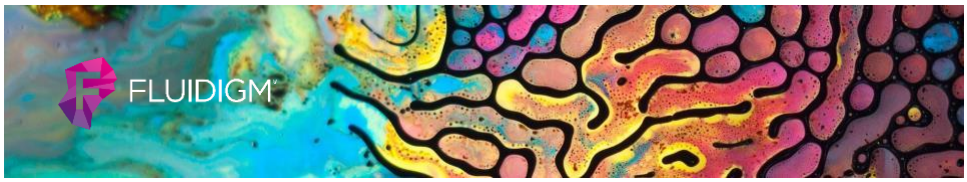
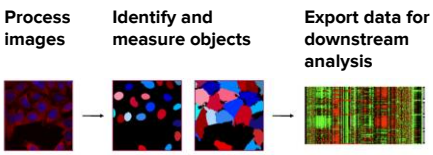
CellProfiler 3.0.0 Workflow for Hyperion Imaging System Data



CellProfiler introduction

Introduction to CellProfiler

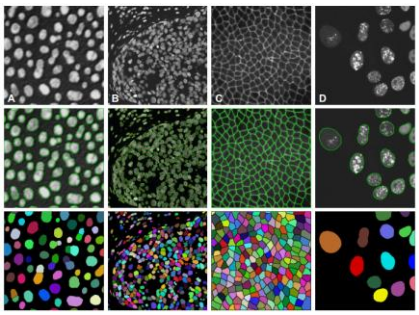
CellProfiler™
cell image analysis software



- Available from cellprofiler.org
- Developed by Carpenter Lab (Broad Institute and MIT)
- Free, open-source software based in Python™.
- Customer support freely available on the website and forum (forum.cellprofiler.org)
- Software available for Windows®, Mac®, Linux

Identifying objects

Cell segmentation



Identifying cells in an image (cell segmentation) is essential for quantitative single-cell biology in imaging.

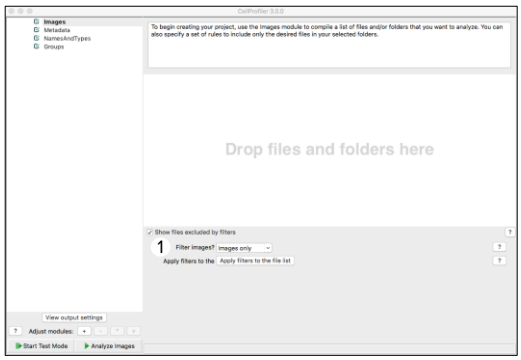
We need to convert pixel data to significant single-cell data.

Meijering, E. "Cell segmentation: 50 years down the road." *IEEE Signal Processing Magazine* 29 (2012): 140–145

CellProfiler basic concepts

CellProfiler basic concepts
Uploading images

Original images (single-file TIFFs) can be dragged and dropped to add them to the CellProfiler project.

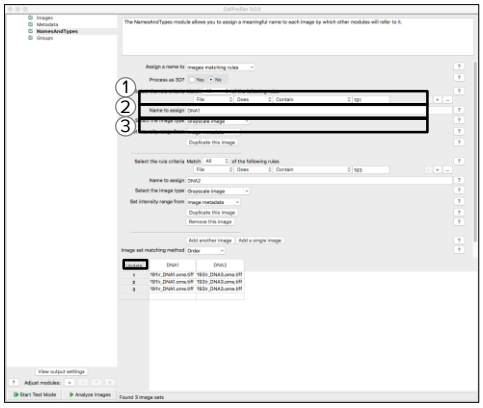


① Allows you to select subsets of images and files based on certain filters

CellProfiler basic concepts

Assigning names

Each uploaded image must be assigned a name for CellProfiler to recognize it.



① Search for keywords within filename (use the metal tag ie 191Ir).

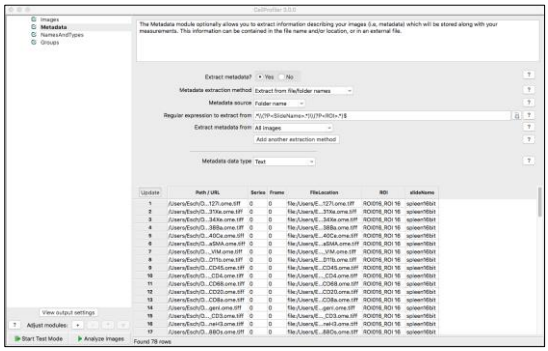
② Assign name to file.

③ Choose type of image (color or grayscale).

Click on update to be sure that name has been correctly assigned.

CellProfiler basic concepts

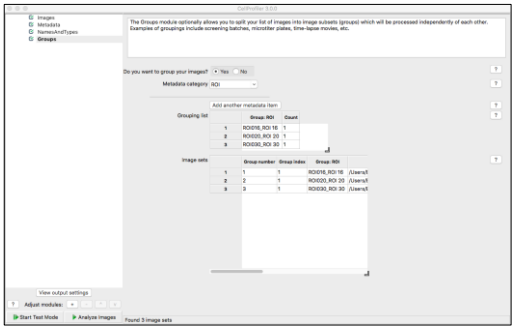
Metadata



Allows you to extract information describing your images directly from the filename or information embedded in picture

Useful for batch processing and for assigning names to images

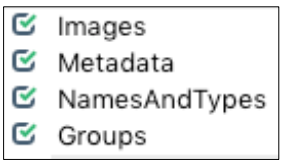
CellProfiler basic concepts
Groups



Allows for batch processing of different image sets

Different images can be processed automatically and separately using the same CellProfiler Pipeline.

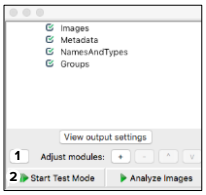
CellProfiler basic concepts
Overview

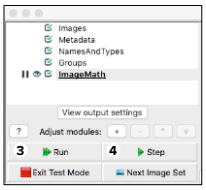


Module	Action
Images	Load single-channel TIFFs
Metadata	Unique identifiers given to individual file types
NamesAndTypes	Define individual channels
Groups	Group ROIs from different runs

CellProfiler Pipeline

Adding modules





① A Pipeline is a sequential set of image analysis/measurement and processing modules. Each module is how we give CellProfiler instructions (one at a time) in order to process, analyze and export any images and data.

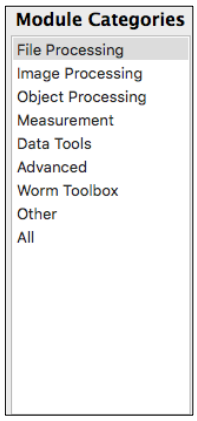
② Test mode allows you to run a Pipeline without generating export files.

③ Run button goes through all steps in a Pipeline.

④ Step button runs only the selected module.

CellProfiler Pipeline

Different module types



① Necessary for visual assessment of segmentation (**ImageMath**)

② Defines cell compartments and performs actual segmentation (**IdentifyPrimary/Secondary/TertiaryObjects**)

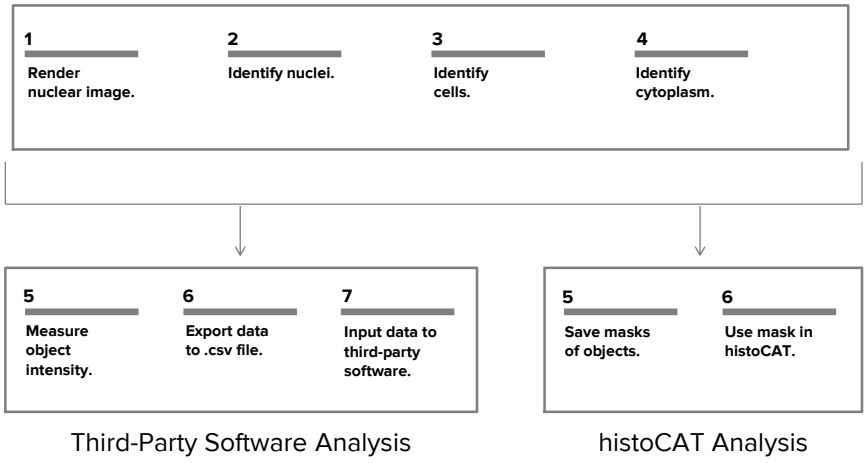
③ Required for extraction of signal intensities from previously identified cell compartments (**MeasureObjectIntensity**)

④ Saves output files of extracted signal intensities and any images generated during pipeline (**ExportToSpreadsheet, SaveImages**)

CellProfiler Pipeline overview for Hyperion Imaging System data

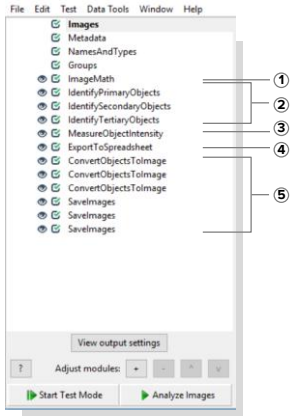
CellProfiler Pipeline

Hyperion™ analysis Pipeline



CellProfiler Pipeline

Hyperion analysis Pipeline



① Allows for proper visualization of nuclei using original 16/32-bit TIFF images (iridium intercalator)

② Identifies in order nuclei (Primary Objects), total cells (Secondary Objects) and cytoplasm (Tertiary Objects)

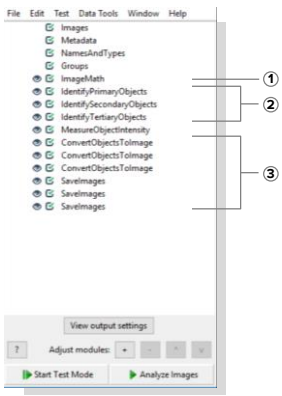
③ Measures signal for each defined cell compartment for all channels of interest

④ Exports .csv file containing all selected measurements

⑤ Masks for identified objects of interest can be saved for implementation with histoCAT.

CellProfiler Pipeline

histoCAT-only Pipeline



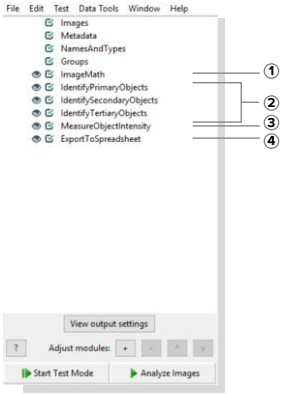
① Allows for proper visualization of nuclei using original 16/32-bit TIFF images (iridium intercalator)

② Identifies in order nuclei (Primary Objects), total cells (Secondary Objects) and cytoplasm (Tertiary Objects)

③ Masks for identified objects of interest can be saved for implementation with histoCAT.

CellProfiler Pipeline

Third-Party Software Pipeline



① Allows for proper visualization of nuclei using original 16/32-bit TIFF images (iridium intercalator)

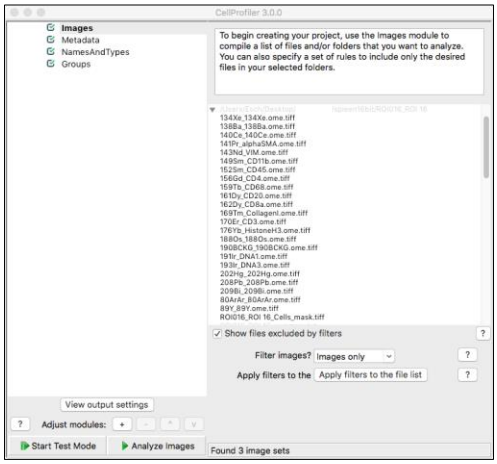
② Identifies in order nuclei (Primary Objects), total cells (Secondary Objects) and cytoplasm (Tertiary Objects)

③ Measures signal for each defined cell compartment for all channels of interest

④ Exports .csv file containing all selected measurements

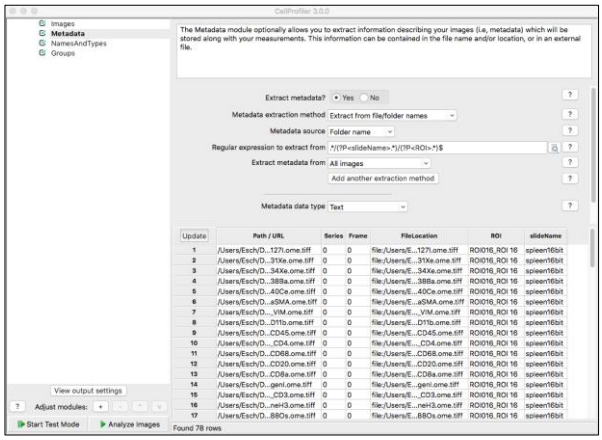
CellProfiler Pipeline

Step 1
Input Images



1. Click **Images** in the top-left pane.
2. Drag image folders to the right-hand pane.
3. If folders contain any other files, select **Images Only** from the drop-down menu and click **Apply Filters.**

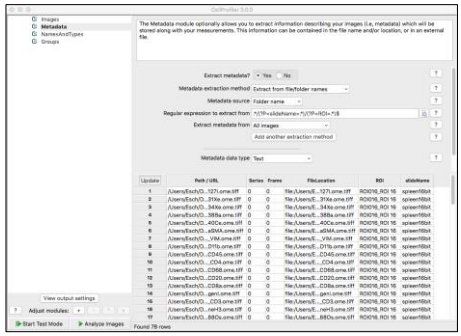
Step 2
Metadata



Purpose

Allows you to extract information describing your images directly from a filename or information embedded in a picture. Useful for batch processing and for assigning names to images.

Step 2
Metadata



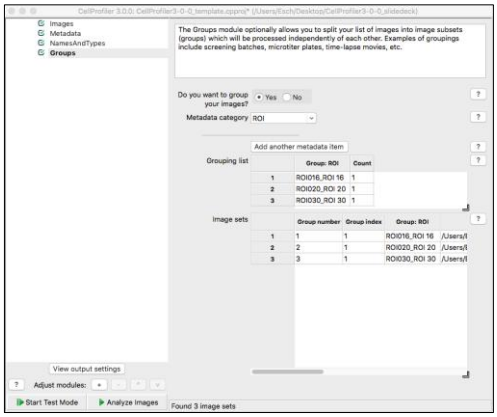
1. Click **Metadata** in the top-left pane.
2. Select **Yes** for metadata extraction, then choose **Extract from file/folder names**.
3. Select **Folder name** for metadata source.
4. Input the following under Regular Expression:
   .*\\(?P<SlideName>.*)\\(?P<ROI>.*)$
   If using a Mac, use the following Folder name regex:
   .*/(?P<SlideName>.*)/(?P<ROI>.*)$
5. Click **Update**.
6. All images are classified by a ROI and SlideName, as shown in figure.

Step 3
Assign NamesAndTypes

1. Click **NamesAndTypes** in the top-left pane.
2. Select **Images Matching Rules** from the drop-down menu.
3. In the field following **File Does Contain**, enter a unique identifier for each channel of interest to distinguish it from other channels.
4. After creating a rule, assign a name to the channel. The channel name must begin with a letter, not a number (typically, assign it the name of the antibody).
5. Click **Add Another Image** to create the next unique identifier using the same procedure.
6. Click **Update** to populate the table at bottom right.

Note: If third-party software analysis is the endpoint (i.e. CSV file format), each channel needs to be assigned a unique name / identifier.

Step 4
Groups

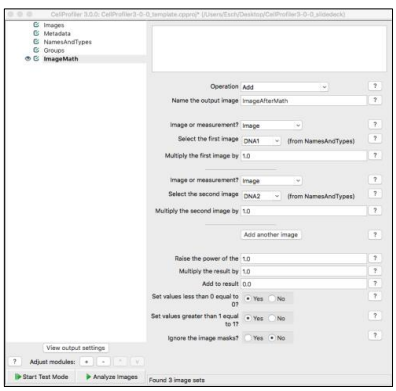


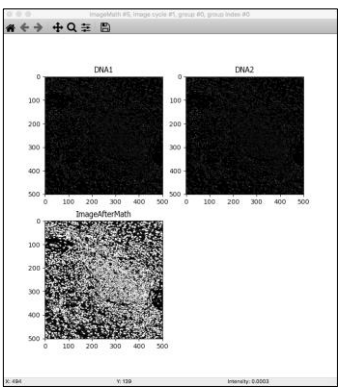
1. Click **Groups** in the top-left pane.
2. Select **Yes** for grouping images.
3. For Metadata category, select **ROI** from the drop-down menu.
4. It may be necessary to click on another module, then return to **Groups** for the table to populate.

Step 5
ImageMath

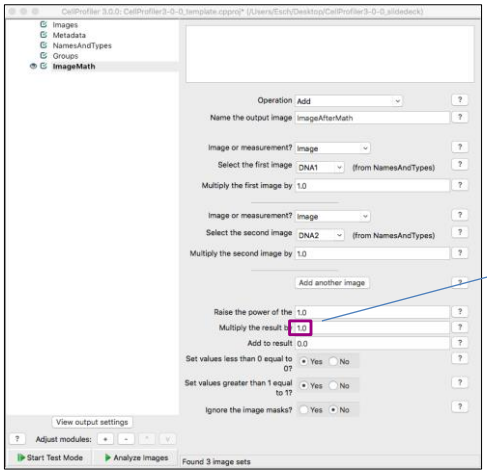
Purpose

To visually identify nuclear stain, which is necessary for assessment of proper segmentation



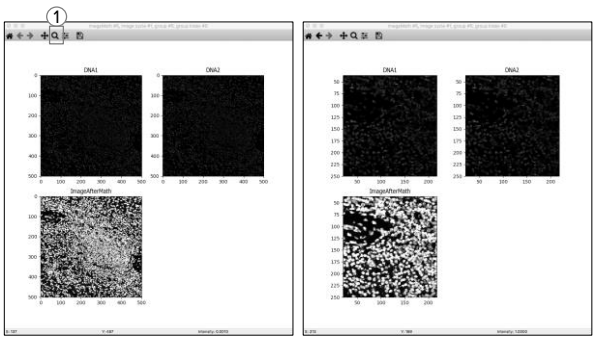


Step 5
ImageMath



1. Click + next to Adjust Modules at lower-left.
2. Select **Image Processing** from the left-hand menu.
3. Select **ImageMath** from the right-hand menu.
4. Click **Add To Pipeline**.
5. Rename the output image if desired.
6. Select images for 191-Ir and 193-Ir (here called DNA1 and DNA2 in NamesAndTypes).
7. Enter a value in the **Multiply the result by** field. For 16-bit images, 50 is a good starting value. For 32-bit images, 0.001 is a good starting value. (For the 16-bit images in this example, 200 was a good multiplier value).
8. Click **Start Test Mode** at lower-left, then click **Step** to visualize output.
9. If rendering is sub-optimal, adjust the value in the **Multiply the result by** field and click **Step** in Test Mode.

Step 5
ImageMath



Visual confirmation of ImageMath can be done post-processing.

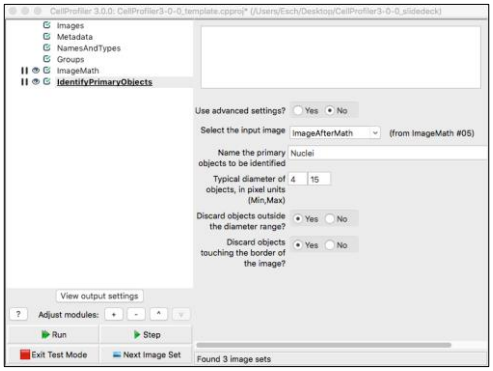
When **Step** is clicked in Test Mode, a pop-up window displays the output of the current module.

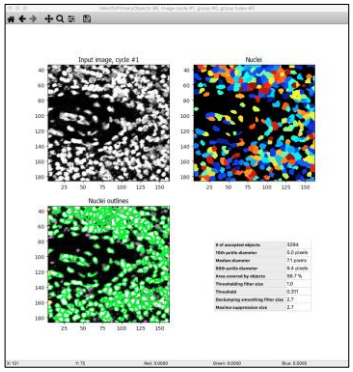
To better assess rendering at the single-cell level, click the **Zoom** button (at top, third from right), then click and drag on any image to select an area for zoom.

Step 6
Identify Primary Objects

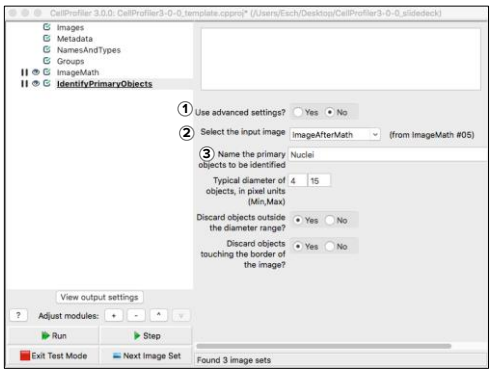
Purpose

To identify primary object of interest (nuclei) to start cell segmentation





Step 6
Identify Primary Objects



Purpose

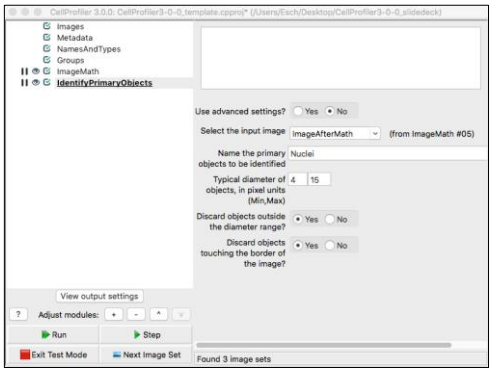
To identify primary object of interest (nuclei) to start cell segmentation

① Select input image (ImageMath-rendered nuclei).

② Name primary objects Nuclei if desired.

③ Adjust lower and upper limits of the nuclei diameter range.

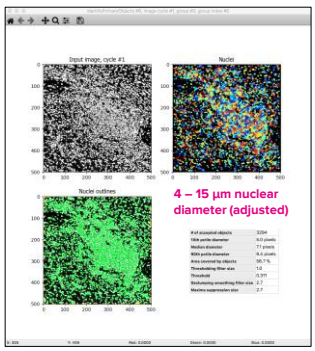
Step 6
Identify Primary Objects

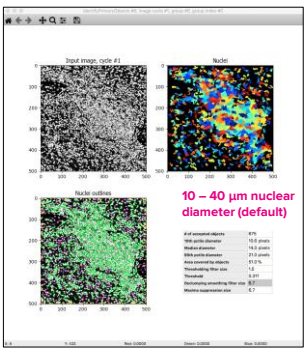


1. Click + next to Adjust Modules at lower-left.
2. Select **Object Processing** from the left-hand menu.
3. Select **IdentifyPrimaryObjects** from the right-hand menu.
4. Click **Add To Pipeline**.
5. Select the ImageAfterMath input image created in the **ImageMath** module.
6. In Test Mode, click **Step** to visualize output. Use the **Zoom** button to assess segmentation at the single-cell level.
7. If nuclear segmentation is sub-optimal, adjust the minimum and maximum values for the object diameter. For spleen samples, 4–15 µm is an appropriate range. Click **Step** in Test Mode.
8. Other parameters may need to be optimized for different tissue types. Click **?** for more information about individual module settings.

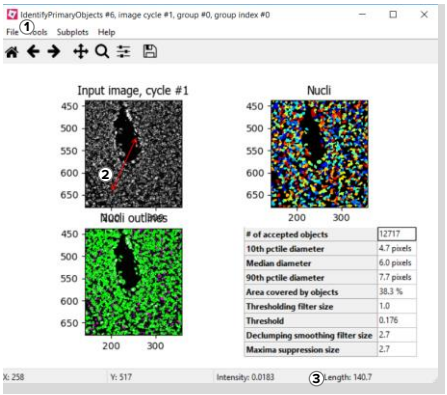
Step 6
Identify Primary Objects

- Visual confirmation of segmentation is key.
- All objects identified by green outlines are positively identified objects.
- All objects identified by pink outlines have been discarded because they are noncompliant with input parameters.





Step 6
Identify Primary Objects



Average object size (useful for measuring typical object size) can be identified by using the Measure Length tool.

Zoom in to area of interest.

① Click **Tools.**

Click **Measure Length.**

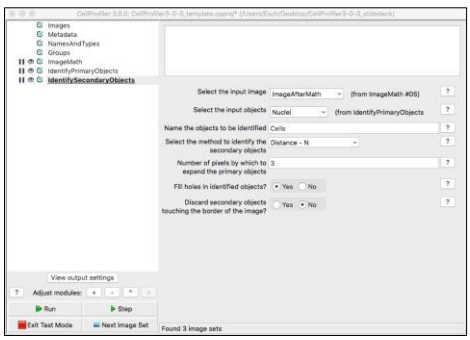
② Click and drag the cursor to frame the object. A red line displays drawn length

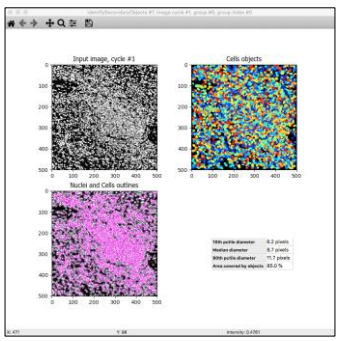
③ The length is displayed in the lower right-hand corner.

Step 7
Identify Secondary Objects

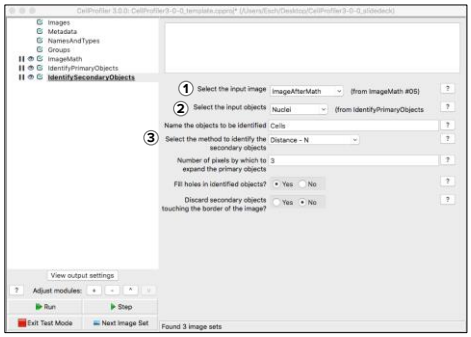
Purpose

To identify single-cell boundaries by using the nucleus as the input parameter





Step 7
Identify Secondary Objects



Purpose

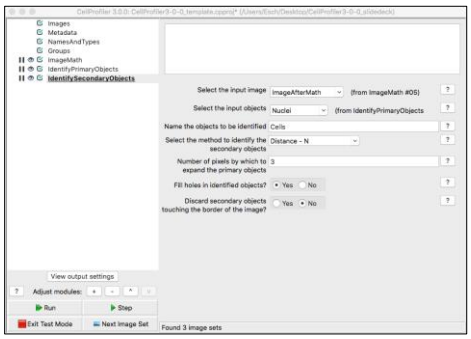
To identify single-cell boundaries by using the nucleus as the input parameter

① Select input image for analysis (ImageMath-rendered nuclei)

② Select Primary Objects (Nuclei).

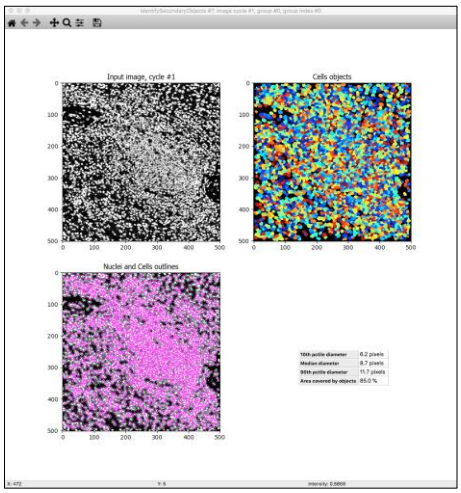
③ Select algorithms for identifying Secondary Objects.

Step 7
Identify Secondary Objects



1. Click + next to Adjust Modules at lower-left.
2. Select **Object Processing** from the left-hand menu.
3. Select **IdentifySecondaryObjects** from the right-hand menu.
4. Click **Add To Pipeline**.
5. In the first drop-down menu, select the ImageAfterMath image created in the ImageMath module.
6. In the second drop-down menu, select **Nuclei** as the input objects.
7. Choose a segmentation method from the fourth drop-down menu. Here, **Distance – N** was selected to draw annuli around segmented nuclei.
8. Enter a value for expanding Primary Objects. For closely packed spleen cells in germinal centers, a distance of ~3 pixels is sufficient.
9. In Test Mode, click **Step** to visualize output. Use the **Zoom** button to assess segmentation at the single-cell level.
10. If secondary object segmentation is sub-optimal, other parameters may need to be optimized. Likewise, different tissue types may require different segmentation strategies. Click **?** for more information about individual module settings.

Step 7
Identify Secondary Objects



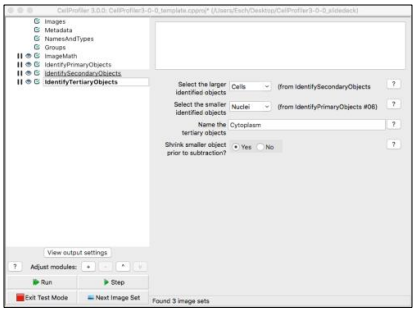
Visual confirmation of Secondary Object identification

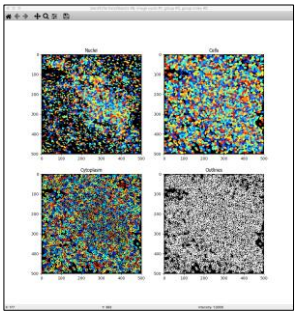
- Positively identified Secondary Objects (cells) are identified by a pink outline.
- Original Primary Objects nuclei can be visualized by doing the following:
  - Click on **Subplots.**
  - Click on **Nuclei and cell outlines.**
  - Click on **Nuclei.**
  - Click on **Overlay.**

Step 8
Identify Tertiary Objects

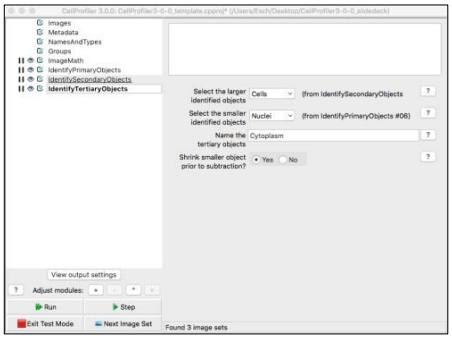
Purpose

To identify cytoplasm by using cell boundaries and nuclei as inputs





Step 8
Identify Tertiary Objects

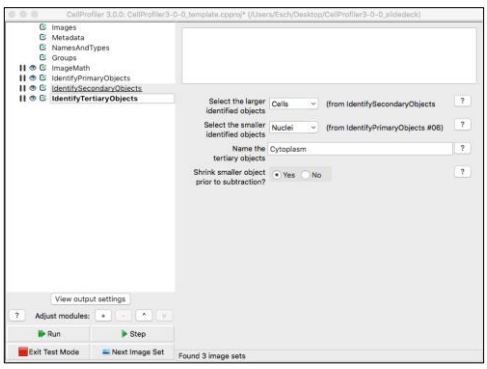


Purpose

To identify cytoplasm by using cell boundaries and nuclei as inputs

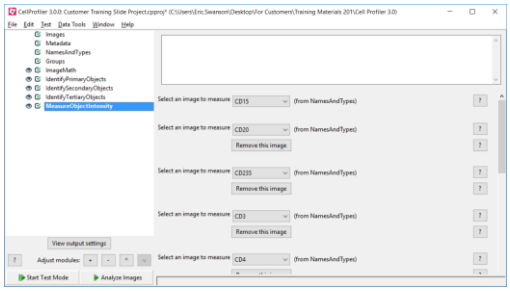
- Select larger (cell boundaries) and smaller (nuclei) objects

Step 8
Identify Tertiary Objects



1. Click + next to Adjust Modules at lower-left.
2. Select **Object Processing** from the left-hand menu.
3. Select **IdentifyTertiaryObjects** from the right-hand menu.
4. Click **Add To Pipeline**.
5. In the first dropdown menu, select **Cells**.
6. In the second dropdown menu, select **Nuclei**.
7. In Test Mode, click **Step** to visualize output. Use the **Zoom** button to assess segmentation at the single-cell level.
8. If tertiary object segmentation is sub-optimal, Primary and/or Secondary Objects would need modifications. Likewise, different tissue types may require different segmentation strategies. Click **?** for more information about individual module settings.

Step 9
Measure Object Intensity

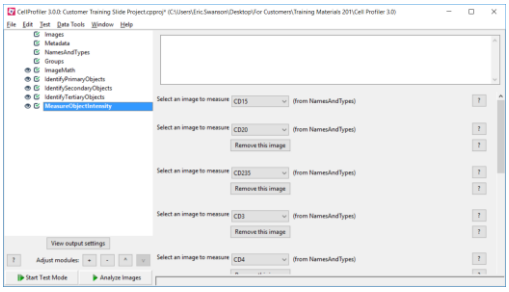


Purpose

To select intensity features for identified objects.

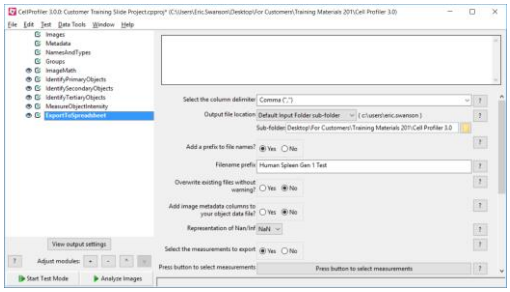
This allows extraction of intensity data from the raw data of each acquired channel from each identified (segmented) object.

Step 9
Measure Object Intensity



1. Click + next to Adjust Modules at lower-left.
2. Select **Measurement** from the left-hand menu.
3. Select **MeasureObjectIntensity** from the right-hand menu.
4. Click **Add To Pipeline**.
5. In the first drop-down menu, select a channel to be measured. **NOTE:** Do not select rendered images like **ImageAfterMath** for measurement.
6. Click **Add Another Image** and select the next channel. Repeat for all channels to be measured.
7. At the bottom, select objects for be measured from the drop-down menus. Here, channels are measured within each of the three compartments: Nuclei, Cells and Cytoplasm.

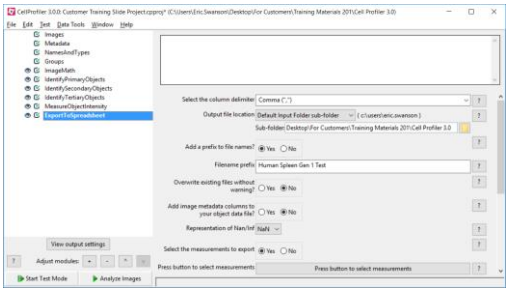
Step 10
Export To Spreadsheet



Purpose

Export all single-cell data from segmented objects into .csv files for processing with Cytobank, FlowJo.

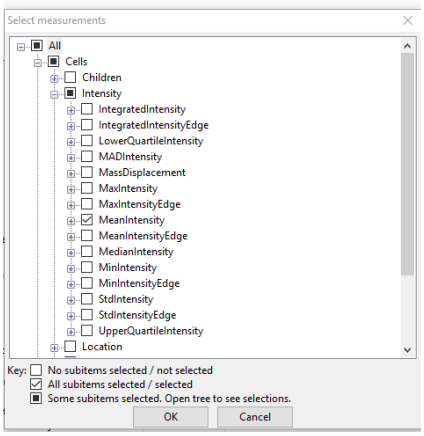
Step 10
Export To Spreadsheet



1. Click + next to Adjust Modules at lower-left.
2. Select **File Processing** from the left-hand menu.
3. Select **ExportToSpreadsheet** from the right-hand menu.
4. Click **Add To Pipeline**.
5. In the second and third drop-down menus, select the desired data output location. To add a filename prefix, select **Yes** and enter desired text in adjacent field.
6. The default option for Select Measurements to Export option is **No**, which exports all available measurements for all Objects. To create a custom export of desired measurements, choose **Yes**, then choose measurement options for each Object in the pop-up window. Note: Each ROI will be saved in the same .csv file.
7. Click **?** for more information about individual module settings.

Step 10

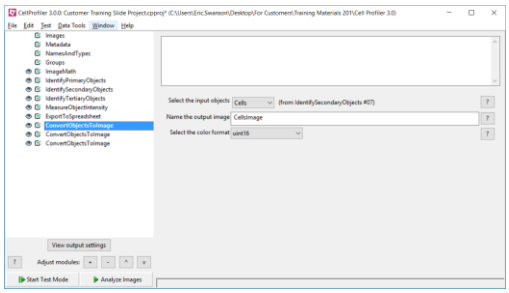
Export To Spreadsheet: custom measurements



1. To create a custom export of desired measurements, choose **Yes** for Select Measurements to Export, then choose measurement options for each Object in the pop-up window.
2. Sub-menus can be expanded by clicking squares to the left of measurement options.
3. Check boxes next to measurements to select. If directory box is checked, all sub-menu items are also selected.
4. It is recommended that MeanIntensity be selected for all channels of interest for each Object.
5. Click **OK** when all measurements have been selected.
6. Click **?** for more information about individual module settings.

Step 11

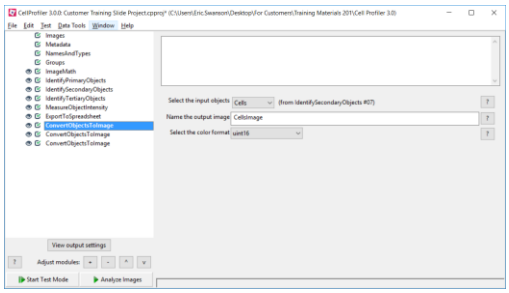
Convert Objects to Image



Purpose

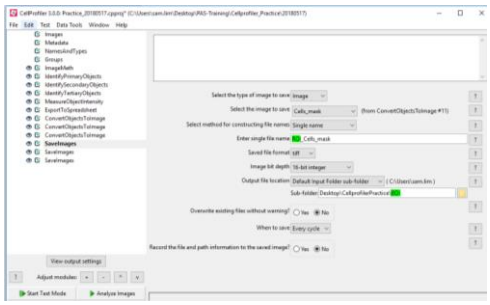
Save image of segmentation mask. This can be used as an input for tertiary analysis.

Step 11
Convert Objects to Image



1. Click + next to Adjust Modules at lower-left.
2. Select **Object Processing** from the left-hand menu.
3. Select **ConvertObjectstoImage** from the right-hand menu.
4. Click **Add To Pipeline**.
5. In the first dropdown menu, select **Cells**. Additional **ConvertObjectstoImage** modules can be added to create masks for other Objects like Nuclei and Cytoplasm.
6. Name the output image CellsImage.
7. Select uint16 from the dropdown menu.

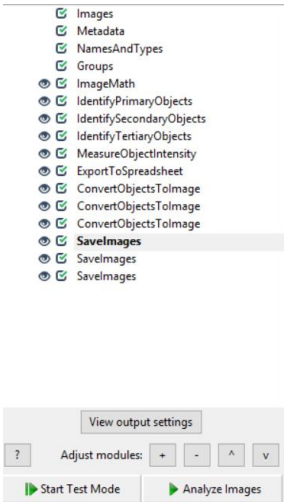
Step 12
Save Images



1. Click + next to Adjust Modules at lower-left.
2. Select **File Processing** from the left-hand menu.
3. Select **SaveImages** from the right-hand menu.
4. Click **Add To Pipeline**.
5. In the first drop-down menu, select **Image**
6. Choose the image generated from the **ConvertObjectstoImage** module in the second drop-down menu (here, **Cells_mask**).
7. Select a method for constructing filenames. For custom filenames, select **Single name** from the dropdown menu. Right-click within the **Enter single file name** field, right click, and select **ROI** from drop-down menu. **ROI** highlighted in green should appear. Type an underscore followed by the mask name (for example, ROI_Cells_mask).
8. Save file format as **tiff** and the image bit depth as **16-bit integer**.
9. Select **Default Input Folder sub-folder** from the dropdown menu, then in the Sub-folder field navigate to the parent folder containing the individual ROI folders containing single-channel images. At the end of the filepath, type a backslash (forward slash on Mac), then right-click and select **ROI** from the dropdown menu.
10. Create a new **SaveImages** module for each cellular compartment that requires a mask. Mask images will be saved as individual files.
11. Click on **?** for more information about individual module settings.

Step 12

Exit test mode, start analysis

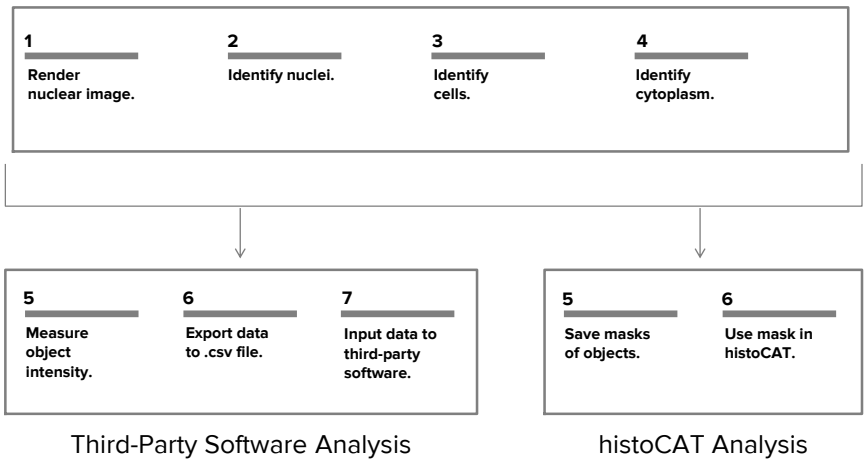


Purpose

All modules are run. This pipeline ends with the generation of object masks that can be used to identify single cells in histoCAT.

CellProfiler Pipeline

Hyperion™ analysis Pipeline



Third-Party Software Analysis

histoCAT Analysis

Simplify the complex quest to understand and apply biology.

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