Protocol for Puncta, Edge Intensity, Area and Shape Measurements

This document contains protocols for image analysis of cells in CellProfiler, including object identification, background correction, puncta analysis, integrated edge intensity, and area and shape measurements. The basic object identification is a part of the pipeline for each analysis, then the specific additional modules required are detailed under the headings for each task. The important settings used in each module are specified below, but these were optimized for a particular set of images and may need fine tuning for different image sets. Any settings not specified were left at their default value. These protocols were developed using images of human podocytes, but the basic approaches can be adapted to numerous other applications (e.g., analysis of EMT markers).
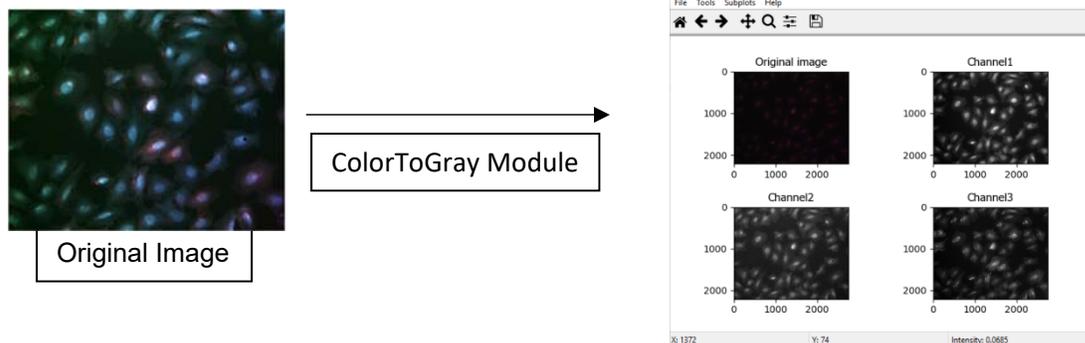
Basic Object Identification

NamesAndTypes:

- If using .czi images, image type must be set to 'Color image'.

ColorToGray:

- If using .czi images, conversion method must be set to 'Split', then the correct number of image channels must be input.

IdentifyPrimaryObjects:

- For typical immunofluorescence images, the nuclei will be used to identify the cell. The size of the nuclei depends on the cell type and image magnification. In this example, the images were taken at 20x, so a pixel size of 80-175 was found to work best.
    - For the Lazzara Lab objects, the below values are a good starting point:
        - 10X: 10-75 pixels
        - 20X: 80-175 pixels
        - 63X: 250-500 pixels
- The thresholding strategy determines the method CellProfiler uses to determine the intensity level to distinguish objects from background. A 'Global' strategy determines a single threshold value across the whole image, whereas the 'Adaptive' strategy determines a threshold at each pixel based on a window of pixels surrounding it. The 'Adaptive' strategy is more robust but more computationally expensive, and is best used for images with inconsistent background intensity, for images with more constant backgrounds 'Global' is as effective and runs more quickly.

- The threshold correction factor can be adjusted to fine tune the calculated thresholding from above, by multiplying the threshold by the value you set. If a pipeline consistently produces images with unidentified nuclei in the background, a threshold correcting factor less than one can be used. If a pipeline often identifies bright regions of the background as objects, a threshold correction factor greater than one can make the threshold more stringent.

Original Image → IdentifyPrimaryObjects Module
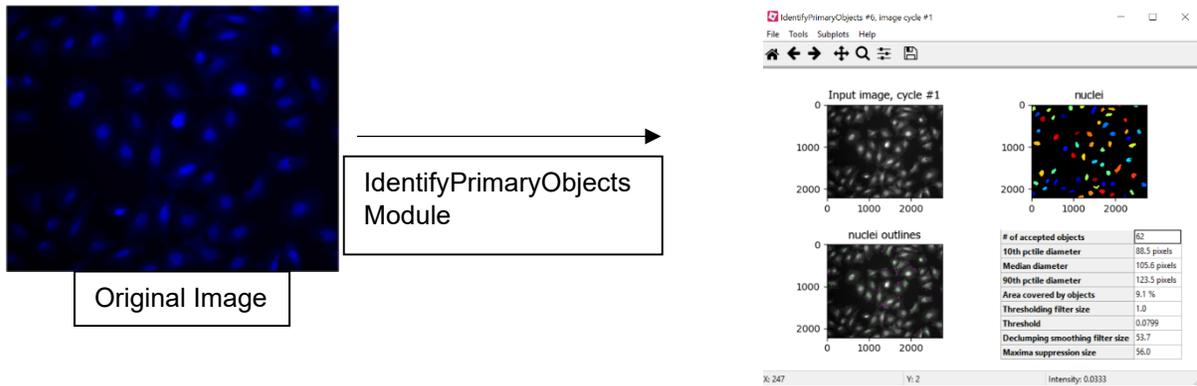
IdentifySecondaryObjects:

Once the nuclei of all the cells in an image are identified as primary objects, CellProfiler can identify the cell bodies surrounding each nucleus as secondary objects.

- The Threshold Smoothing Scale is used to reduce jaggedness and possible holes in identified objects. If the images being analyzed have minimal noise, have already been smoothed using another program like ImageJ, or are binary, smoothing is not required. If the objects in the pipeline have unusually jagged edges, smoothing may be helpful. The scale is based on a Gaussian distribution, such that a '0' for this scale indicates no smoothing, and a 1.3488 is a smoothing with sigma 1. More information on this scale can be found by selecting the '?' box next to this parameter in CellProfiler.

Original Image → IdentifySecondaryObjects Module
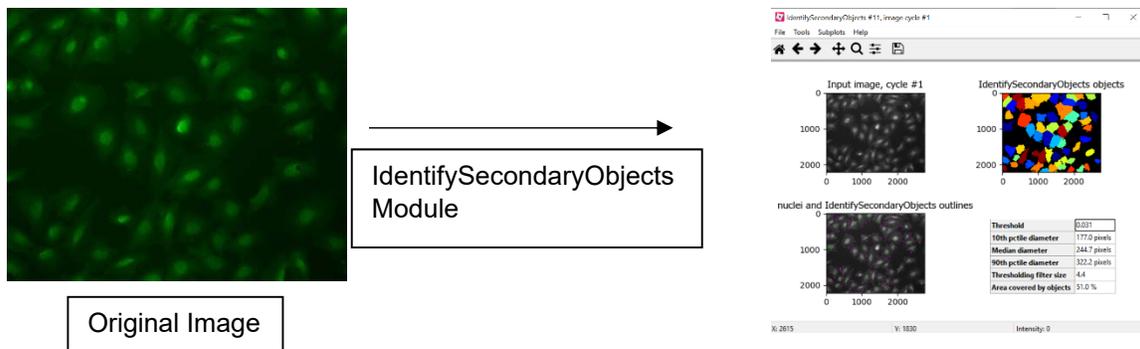
NOTE: A high Threshold Smoothing Scale was used, so much of the detail in the cell borders is minimized.
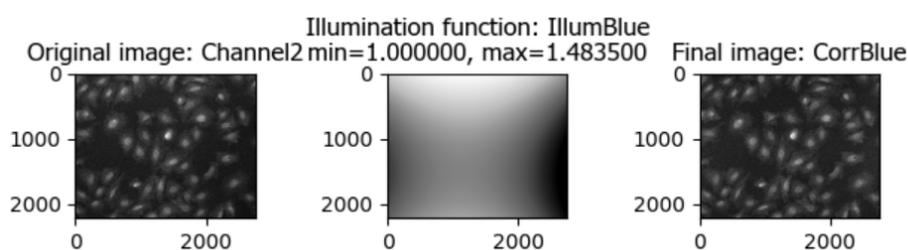
Background Correction

Background correction in CellProfiler is performed by calculating a function for background correction in one module, then applying that function to an image in another module.

CorrectIlluminationCalculate:

- The best Illumination Function to use is dependent on the features of the image. A detailed guide for choosing this is available by clicking the '?' box next to this setting in CellProfiler.
- The best Smoothing method is heavily influenced by the selected Illumination method. If using the 'Regular' Illumination method, the 'Fit Polynomial' Smoothing method works best.

CorrectIlluminationApply:

- The input image should be the same input image the illumination function was calculated from (the same input image from the CorrectIlluminationCalculate Module).
- Select Illumination Function should be the output image from the CorrectIlluminationApply Module.
- The best setting for selecting how the illumination function is supplied is dependent on how the illumination function was calculated in the previous module. If 'Background' was used, select 'Subtract' here, and if 'Regular' was used, select 'Divide' here.
- Note: At this point, the background corrected image can be used to identify objects and/or measure cell features. For example, if you were measuring cell intensity, you can use the background corrected images as the input images for Secondary Object Identification, and could use either the background corrected images OR the original unadjusted images for the intensity measurement. CellProfiler performs modules from the top of the pipeline down, so make sure background correction modules are above any modules you plan to use background correction for.

NOTE: The images used for this analysis did not need much background correction, but the corrected images have sharper borders that help object identification.

Puncta Analysis

IdentifyPrimaryObjects:

The individual puncta will be identified as primary objects, and then related to the cells that contain them in a later module.

- Pixel size for CD16 puncta at 20X worked best at 5-20. The default settings for the rest of this module tend to work, as puncta are typically very bright compared to the background.

- To avoid confusion with the 'nuclei' primary objects, name the objects 'puncta'.
- If CellProfiler fails to identify large regions of puncta, it may be because they are too close and clumped to be individually distinguished. If the goal is to get an accurate count of puncta there is no easy fix, but by increasing the upper bound of pixel size can be used to identify these clumps for intensity measurements.
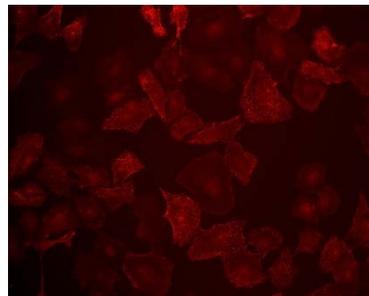
Original Image

IdentifyPrimaryObjects Module
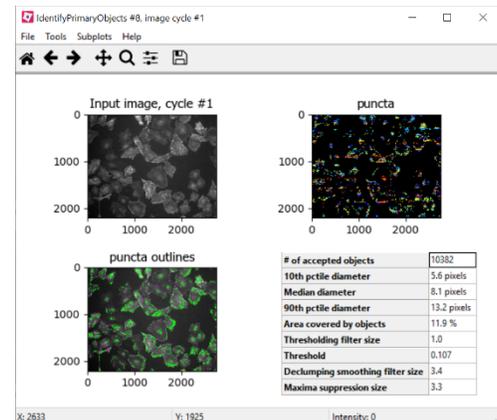
MeasureObjectIntensity:

- 'Select an Image to Measure' should be set to the puncta image channel, and 'Select objects to measure' should be set to the primary objects that identify the puncta (The 'puncta' objects from the previous module).

RelateObjects:

- This module assigns puncta to the cell that contains them.
- Parent Objects should be set to the secondary objects that identify the cells.
- Child objects should be the puncta.
- Depending on the analysis, it could be helpful to select 'Calculate parent means for all child measurements'. This is deselected by default, and gives the option to output data on averages of the puncta measurements per cell.

ExportToSpreadsheet:

- If it is known what measurements are needed for analysis, choose 'Yes' for 'Select the measurements to export'. This keeps spreadsheets from being unnecessarily large and complicated. For this analysis, it is important to select 'puncta' -> 'Intensity' -> Desired Intensity Metric, as well as 'SecondaryObject' -> 'Children'.

Integrated Edge Intensity

MeasureObjectIntensity:

- This module can be used once the secondary object has already been identified. 'Select an Image to Measure' should be set to the desired channel, and 'Select objects to measure' should be set to the secondary objects that identify the cells.

ExportToSpreadsheet:

- Important to export 'SecondaryObjects' -> 'Intensity'

Area, Shape Factor, and Circularity Index

MeasureObjectSizeShape:

- This module can be used once the secondary object has already been identified. 'Select objects to measure' should be set to the secondary objects that identify the cells.

ExportToSpreadsheet:

- Export 'SecondaryObjects' -> 'AreaShape'. From here, Area and Perimeter are the only required features but for different analyses different features could be useful. From the area and perimeter, shape factor and circularity index can be calculated.