# Protocol for Quantifying Protein Expression with Correlation Plots

This protocol was written for 4 channel .czi images with the goal of measuring nuclear HIF1alpha and cJun expression, but can be generalized to measuring any fluorescent signal. This protocol assumes the images used have a DAPI or equivalent nuclear stain channel. If the images used have been stained for a protein that can be used to identify the cell boundaries (ex. E-cadherin) the expression of proteins within the boundary of the whole cell can be quantified as well.

If two or more proteins are quantified in the same images, correlative plots can be produced to show the intensity of each protein signal per cell, like so:
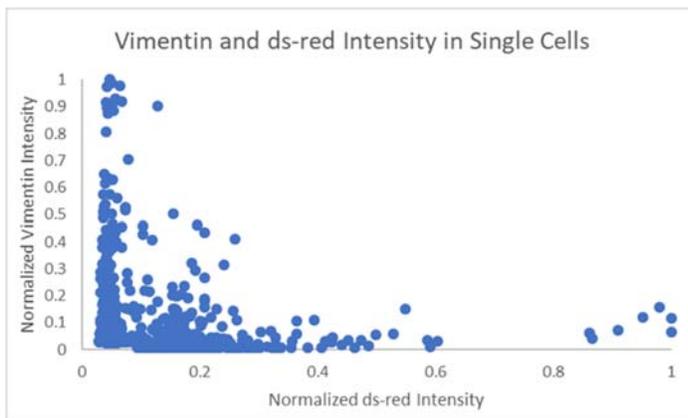
The important settings used in each module are described below, and any settings not mentioned were left at their default value.

Basic Object Identification

To quantify nuclear expression only primary object identification is needed, but a protocol for secondary object identification is included for cytosolic expression.

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.
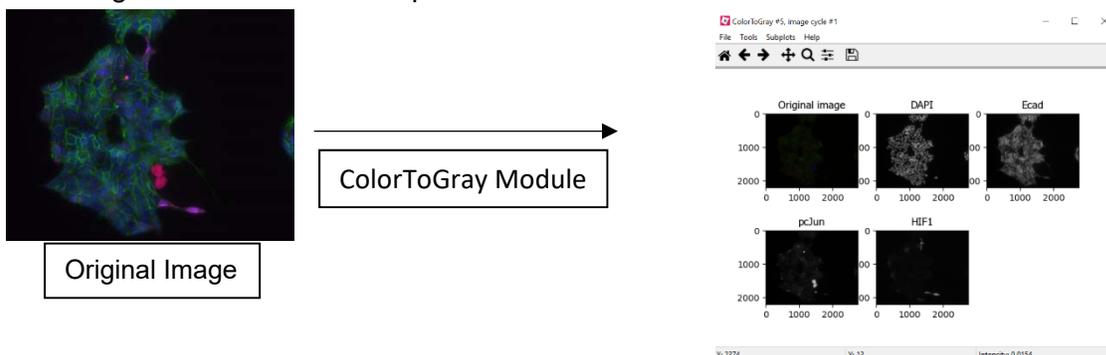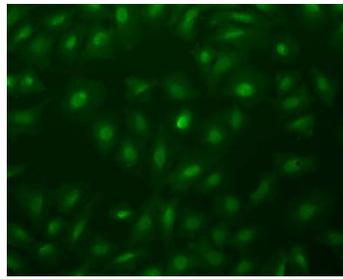
IdentifyPrimaryObjects:

- 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.
    - A starting place for each of the Lazzara Lab objectives:
        - 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
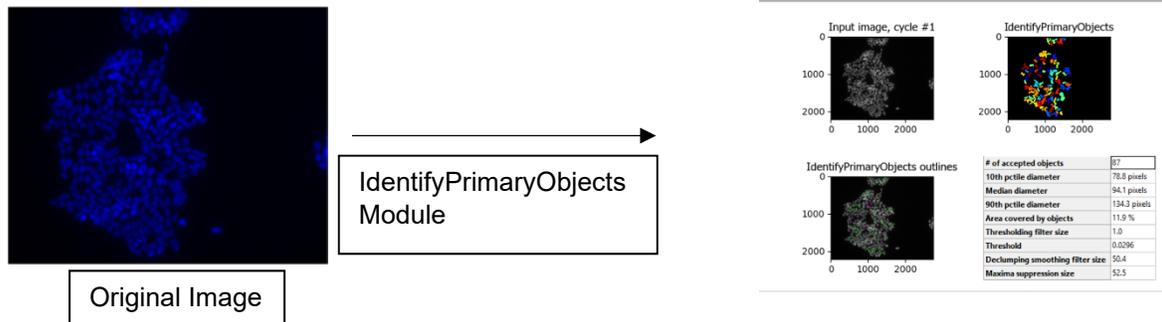
IdentifySecondaryObjects (For quantification of cytosolic expression):

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
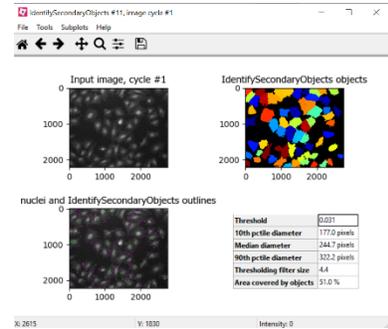
NOTE: A high Threshold Smoothing Scale was used, so much of the detail in the cell borders is minimized.

MeasureObjectIntensity:

- For each image channel being measured a new instance of the MeasureObjectIntensity module must be added.
- 'Select an Image to Measure' should be set to the measured protein image channel, and 'Select objects to measure' should be set to the primary objects if quantifying nuclear expression, or the secondary objects if quantifying cytosolic expression.

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 the secondary or primary object tab (dependent on area measured) then -> 'Intensity' -> Desired Intensity Metric.

Excel Sheet Output and Data Analysis:

- The output spreadsheet should look like the example to the right, where the first column is the image number, the second is the cell number, and the third and fourth are intensity measurements. Each row represents a single cell, so a scatterplot using column 3 and 4 as the axes would be a scatterplot of the expression of these proteins where each point represents a single cell.