Using CellProfiler to Analyze RNAscope® and BaseScope™ Data

Introduction

This Technical Note provides guidelines for performing quantitative image analysis with CellProfiler on *in situ*-hybridized (ISH) slides that have been processed using the RNAscope® or BaseScope™ assays. You can use CellProfiler to analyze singleplex, duplex, and fluorescent multiplex images. Download CellProfiler from **https://cellprofiler.org**.

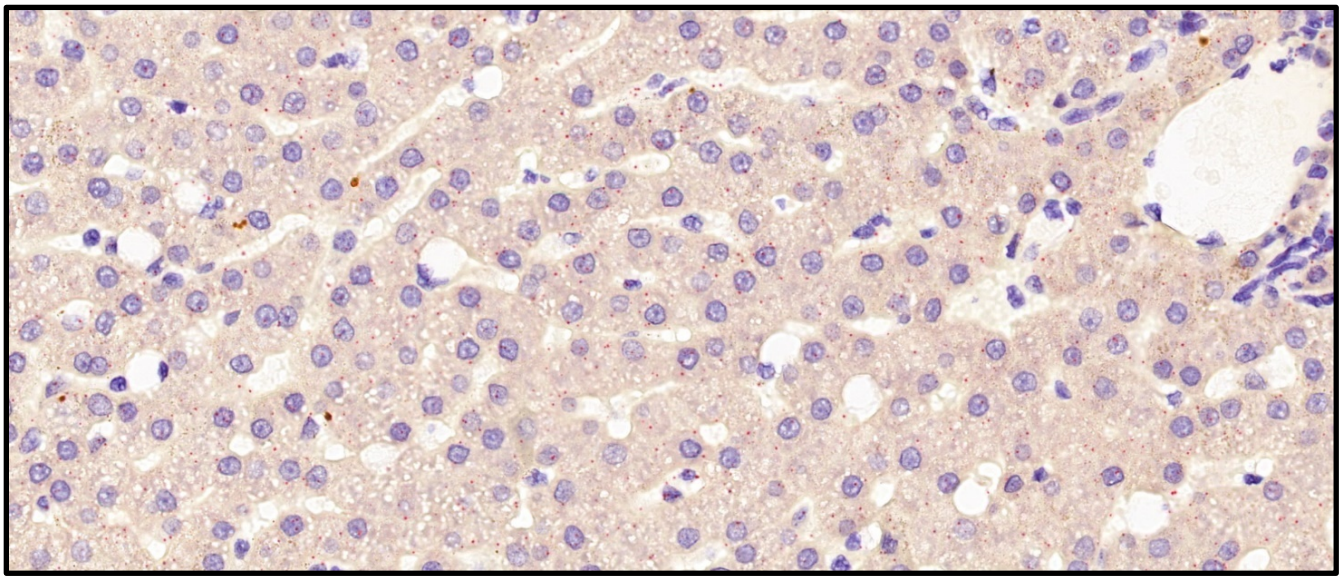
Apply the CellProfiler analysis workflow demonstrated in this Technical Note into larger workflows to analyze large images.

Note: CellProfiler has limited capability with handling large, high-resolution images. We recommend using a field of view limited to a maximum number of two gigapixels in (x,y) to count cells and punctate dots present within cell boundaries. To perform whole slide image (WSI) analysis using your CellProfiler pipeline, Glencoe sells a plugin to allow you to interface CellProfiler with the open source platform QuPath. The plugin works through the OMERO + platform. Information on this plugin can be found by contacting Glencoe through their webpage: **https://www.glencoesoftware.com/contact/**. Other open source options to perform whole slide image (WSI) analysis include QuPath, Orbit, and SlideToolkit.

Workflow

Below is an example of a chromogenic RNAscope® image stained with one probe in red. We will use this image to demonstrate image analysis using CellProfiler.

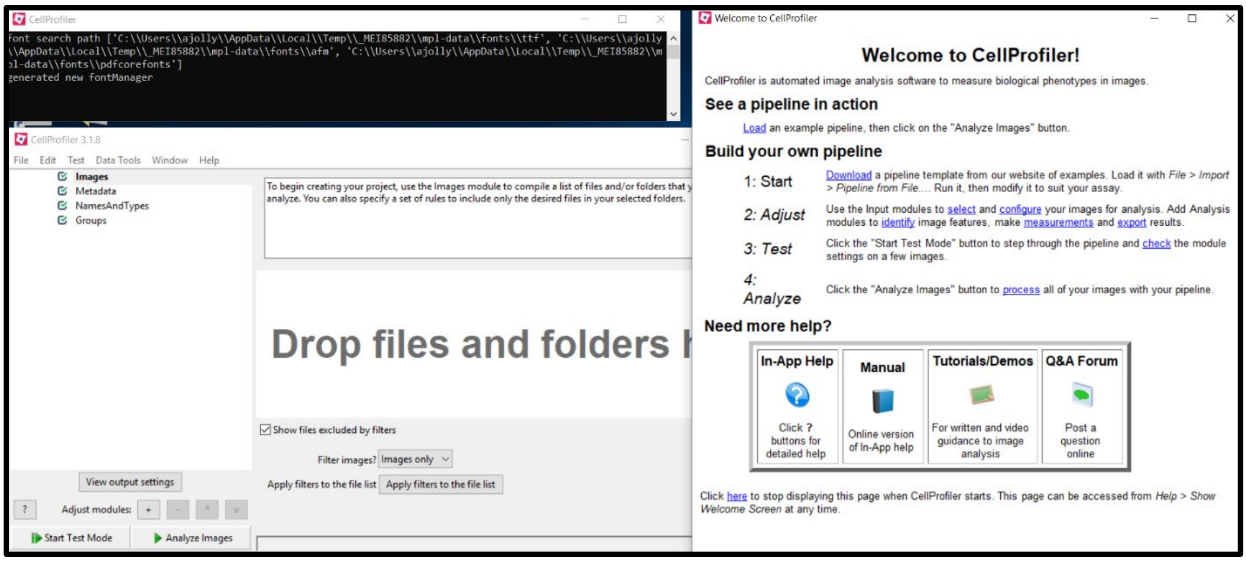
Figure 1. Singleplex image example



Part 1: Build the Pipeline Workflow

1. Open CellProfiler. The primary user interface pre-loads with the first four modules of a pipeline (left panel):
   - Images
   - Metadata
   - NamesAndTypes
   - Groups

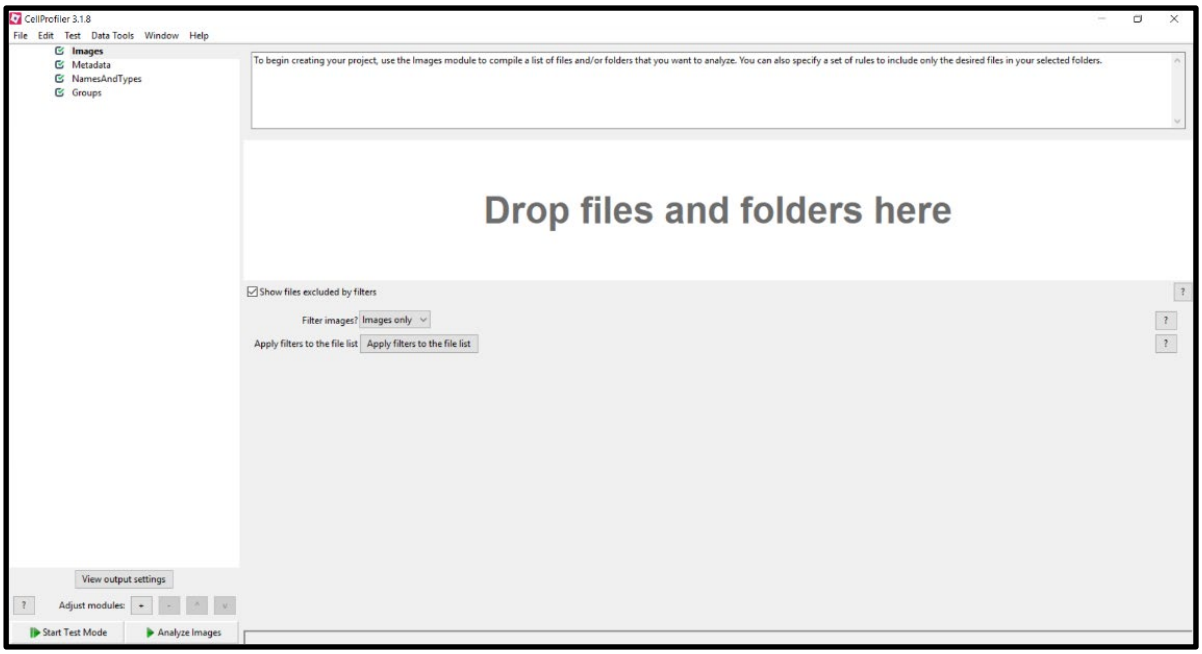
Figure 2. CellProfiler interface



Note: CellProfiler opens in three separate windows (see Figure 2), including a welcome page that includes many helpful links. Explore the help available in the manuals, tutorials and demos, and participate in the Q&A forum to answer specific questions.

2. Select the **Images** module, then drag the image you want to analyze into the right panel while the module is active (see Figure 3).

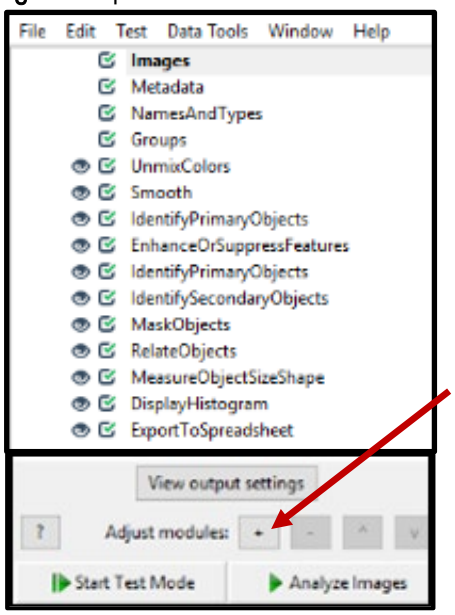
Figure 3. Loading images



Note: For best results, use images that are uncompressed .tif files no larger than 2 gigapixels in (x,y). 40x magnification is required.

3. Click the + button (see Figure 4) to add a new module to your pipeline in the following order. Make sure to save your pipeline file frequently.
   a. UnmixColors
   b. Smooth
   c. IdentifyPrimaryObjects
   d. EnhanceOrSuppressFeatures
   e. IdentifyPrimaryObjects
   f. IdentifySecondaryObjects
   g. MaskObjects
   h. RelateObjects
   i. MeasureObjectSizeShape
   j. DisplayHistogram
   k. ExportToSpreadsheet

Figure 4. Pipeline overview



4. To find a module, use the Search box or select the correct category under **Module Categories** to choose the module from the drop-down menu. For example, the UnmixColors module is listed under **Image Processing** (see Figure 5).
5. Add each module to your pipeline by clicking **+ Add to Pipeline**.

Note: Each module is accompanied by a Help page that includes module definitions, citations, and resources (see Figure 6). To locate the Help page for any given module, click the **? Module Help** button (see Figure 5).

Figure 5. Finding modules

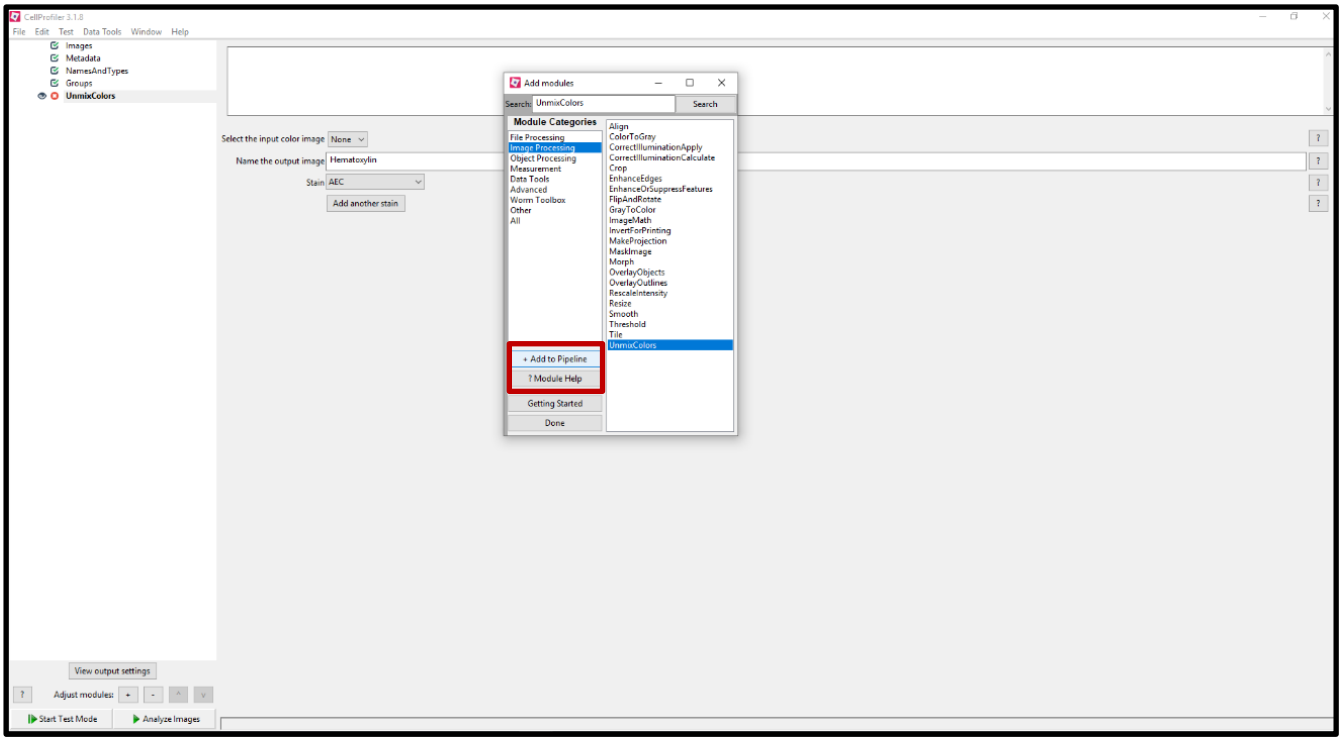
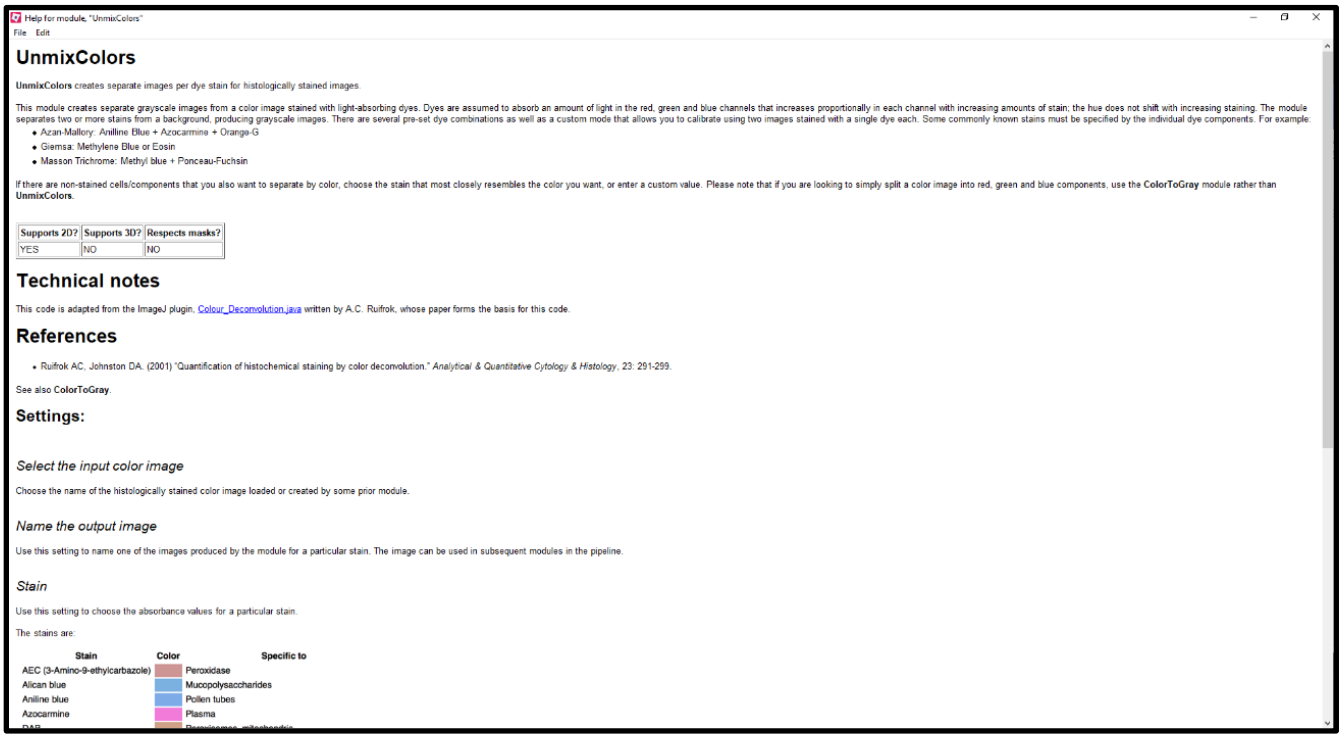


Figure 6. Module help page



6. Put the modules together by entering the input file names according to the following table. When all the names are entered correctly, a green checkmark will appear to the left of the module.

Table 1. Singleplex chromogenic workflow

Module	Input name(s)	Output name(s)
Images	N/A	N/A
Metadata	N/A	N/A
NamesAndTypes	All images (color image)	Rawdata
Groups	N/A	N/A
UnmixColors (see Figure 8)	Rawdata	Hematoxylin
		RedISH
		Exclusion
Smooth (see Figure 9)	Hematoxylin	FilteredHematoxylin
IdentifyPrimaryObjects (see Figure 10)	FilteredHematoxylin	Nuclei
EnhanceOrSuppressFeatures (see Figure 11)	RedISH	FilteredRNA
IdentifyPrimaryObjects (see Figure 12)	FilteredRNA	RNA
IdentifySecondaryObjects (see Figure 13)	FilteredHematoxylin	Cells
	Nuclei	
MaskObjects (see Figure 14)	RNA (Objects to be masked)	MaskedRNA
	Cells (Masking objects)	
RelateObjects (see Figure 15)	Cells (Parent objects)	RelatedRNA
	MaskedRNA (Child objects)	
MeasureObjectSizeShape (see Figure 16)	RelatedRNA	N/A
DisplayHistogram (see Figure 17)	Cells	N/A
	Children	
	MaskedRNA_Count	
ExportToSpreadsheet	N/A	N/A

7. Visually inspect all of the modules. If an error message appears, hover over the error to identify the problem.

Part 2: Adjusting the Modules in Test Mode

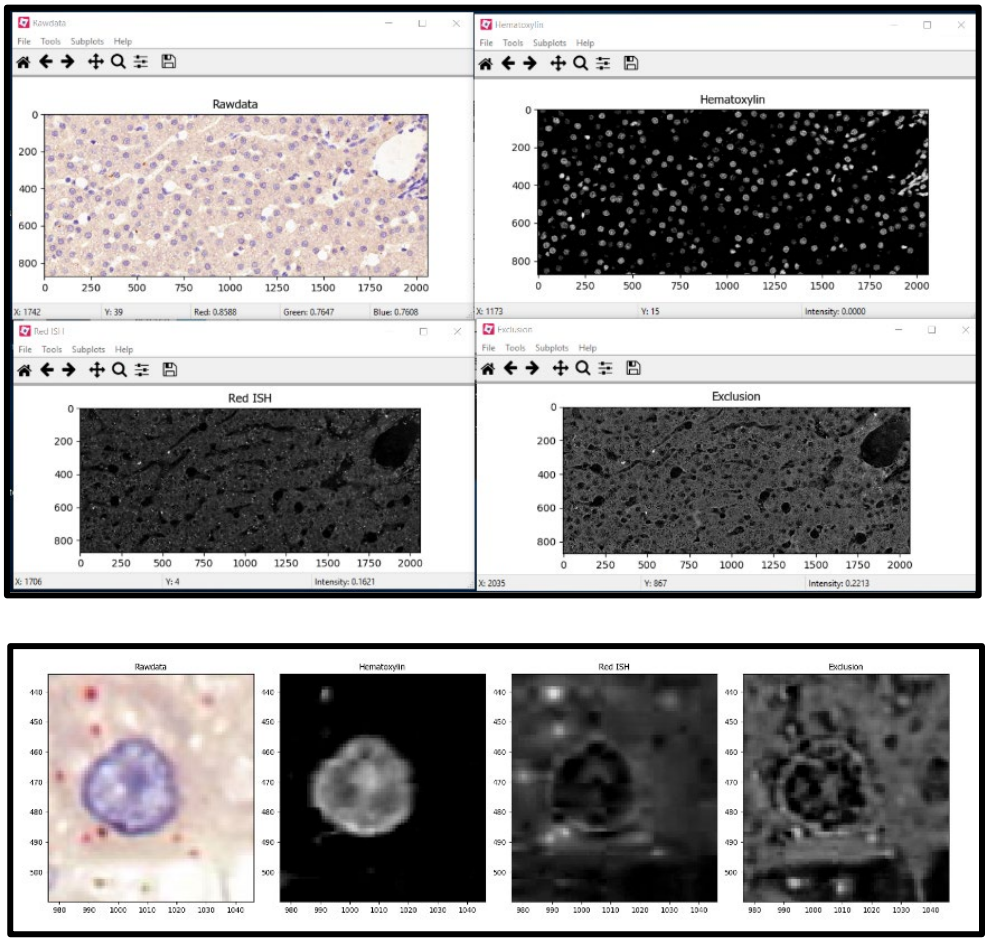
1. Select the **NamesAndTypes** and set it to **color image.**
2. Select the module and drag a test image into the **Images** module.
3. Click **Start Test Mode**.

Note: Using the test mode interface, adjust the parameters within each module to optimize and tailor the analysis to your unique image. We recommend testing one module at a time.

4. Select the **Step** button to move from one module to the next, or select the **Run** button to run all of the analysis modules. Output (image) windows will display with the results of each module beginning with the UnmixColors module.

Note: The output windows will only appear if the eye icon to the left of the module is open (click on the eye to open it). See Figure 7 for an example of test mode output windows.

Figure 7. Test mode output windows: UnmixColors

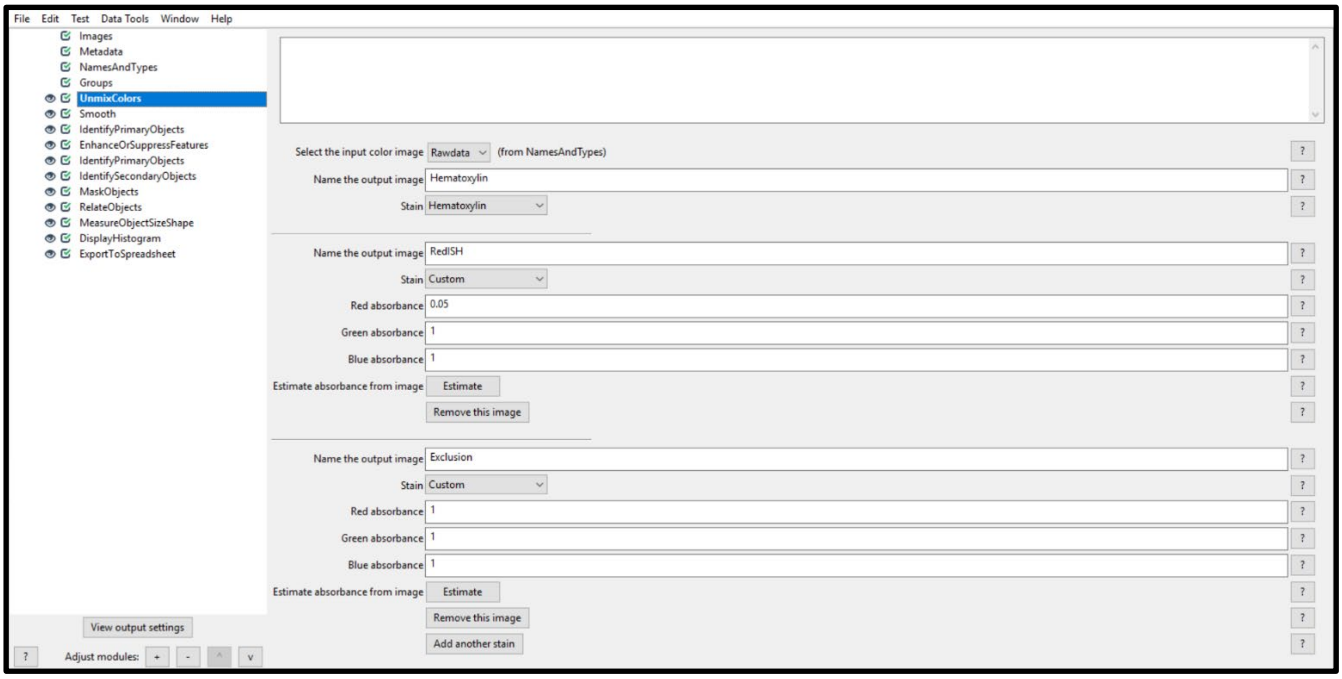


Note: When reviewing the output images of each module using Test Mode, pay close attention to the output of the initial UnmixColors module and the two IdentifyPrimaryObjects modules. These three modules are the most important steps in the workflow and may require significant adjustments to tailor them to each unique image. Recommended settings for each of these modules are provided, but the exact settings will depend on the image. We recommend exploring the different settings within each module and referring to the help menus provided by CellProfiler.

5. Select the **UnmixColors** module, and choose the settings from the drop-down menus. Alternatively, you can use the actual images and create crops of each relevant color (each cropped image should only contain the color of interest), or enter the (R,G,B) values directly.

   Example: Figure 8 displays the UnmixColors settings applied to the test image in Figure 1. For a singleplex red RNAscope® or BaseScope™ assay, start with the **Hematoxylin** color palette provided in the CellProfiler drop-down menu, set the red ISH color to **Custom** with (R,G,B) to (0.05,1,1), and set the Exclusion channel to **Custom** with (R,G,B) to (1,1,1). The settings entered in the exclusion channel will eliminate black pixels from the analysis.

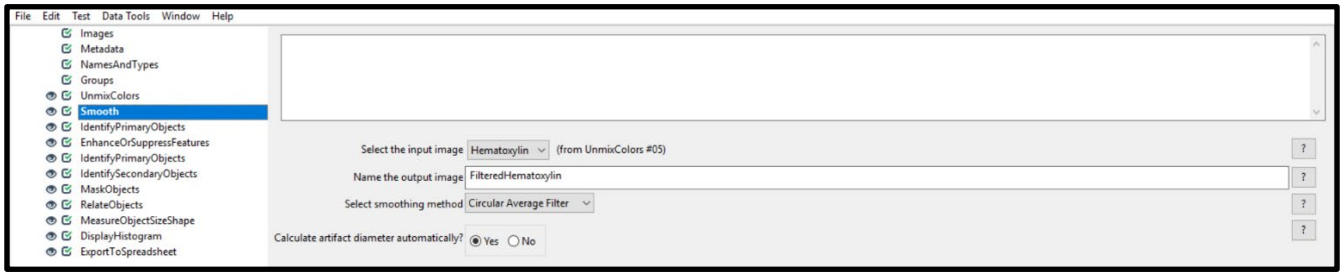
Figure 8. Module settings: UnmixColors



6. Select the **Smooth** module, and choose the settings from the drop-down menus. The best smoothing method depends on the unique attributes of your particular image.

   Example: Figure 9 displays the setting applied to smooth the nuclear image in Figure 1 (Circular Average Filter). Depending on the image attributes, the **Gaussian Filter** setting can also be used. Some nuclear images are best analyzed using the Suppress Features function within the **EnhanceOrSuppressFeatures** module, as an alternative to the **Smooth** module. For this example image, the **Circular Average Filter** gave the best results.

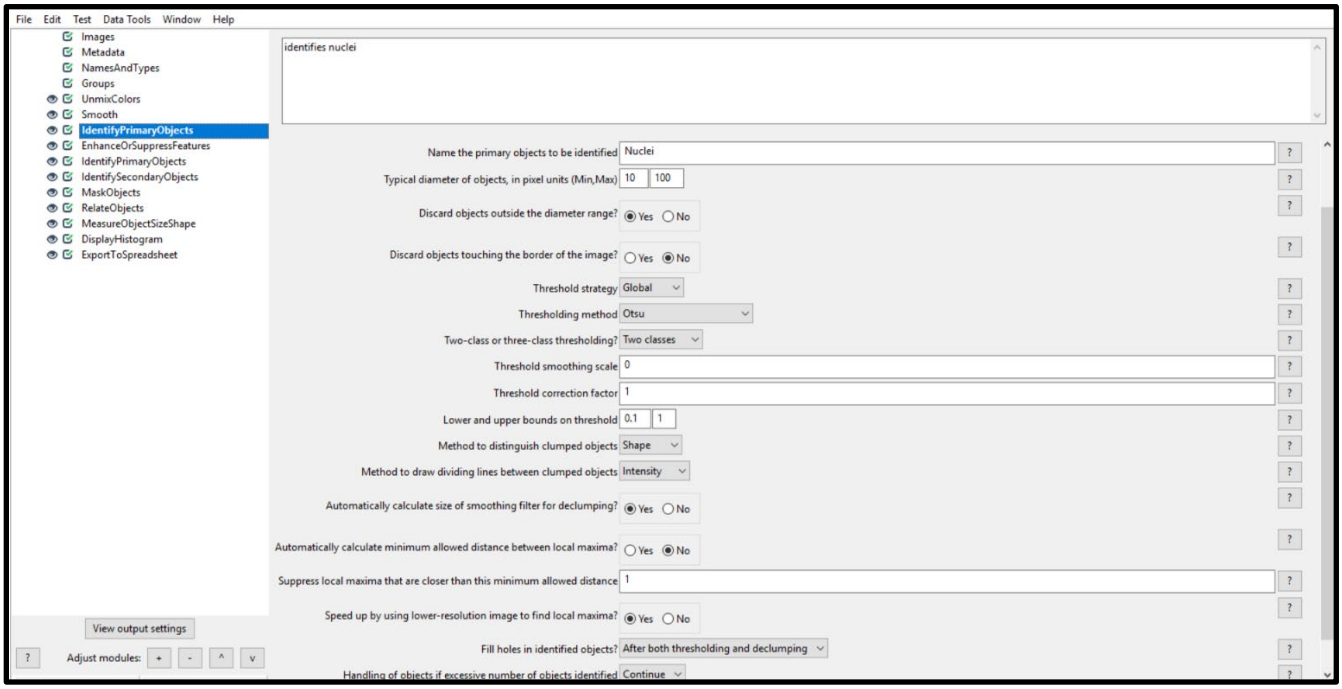
Figure 9. Module settings: Smooth



7. Select the **IdentifyPrimaryObjects** module, and choose the settings from the drop-down menus. To view advanced settings, select **yes** next to **Use advanced settings?**.

Example: Figure 10 displays the recommended settings for this module. Start with setting the (Min,Max) object diameter to (10,100) and discard objects outside of the diameter range. Set the threshold strategy and method to **Global** and **Otsu**, and use two-class thresholding. Use **Shape** to distinguish clumped objects and **Intensity** to draw dividing lines between objects. We recommend filling holes in identified objects after both thresholding and declumping.

Figure 10. Module settings: IdentifyPrimaryObjects (nuclei)



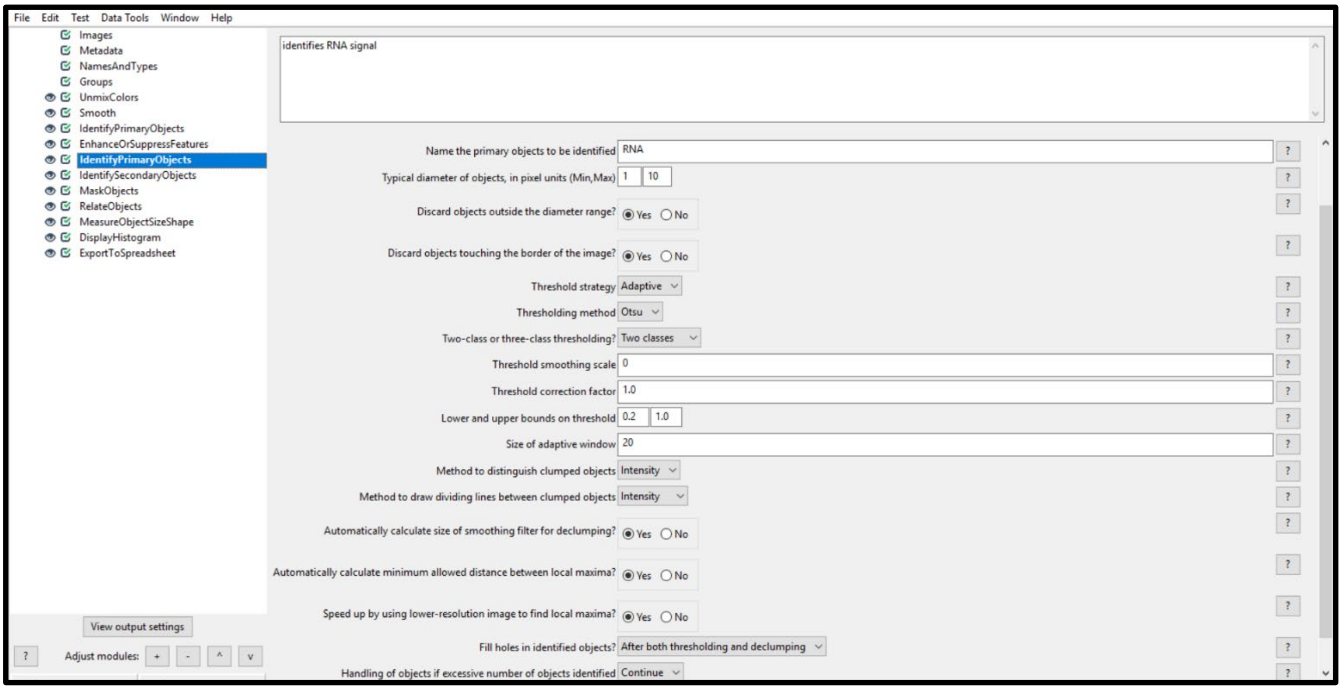
8. Select the **EnhanceOrSuppressFeatures** module, and choose the settings from the drop-down menus. See Figure 11 for example settings (ISH).

Figure 11. Module settings: EnhanceOrSuppressFeatures

9. Select the **IdentifyPrimaryObjects** module, and choose the settings from the drop-down menus.

Example: Figure 12 displays the recommended settings for this module (ISH channel). Name the primary object to be identified (in this case **RNA**). Start with setting the (Min,Max) object diameter to (1,10) and discard objects outside of the diameter range. Set the threshold strategy and method to **Adaptive** and **Otsu**, and use two-class thresholding. As a starting point we recommend using the (Min,Max) object diameter (1,10) and to discard objects outside of the diameter range. Use **Intensity** to distinguish clumped objects and to draw dividing lines between objects. We recommend filling holes in identified objects after both thresholding and declumping.

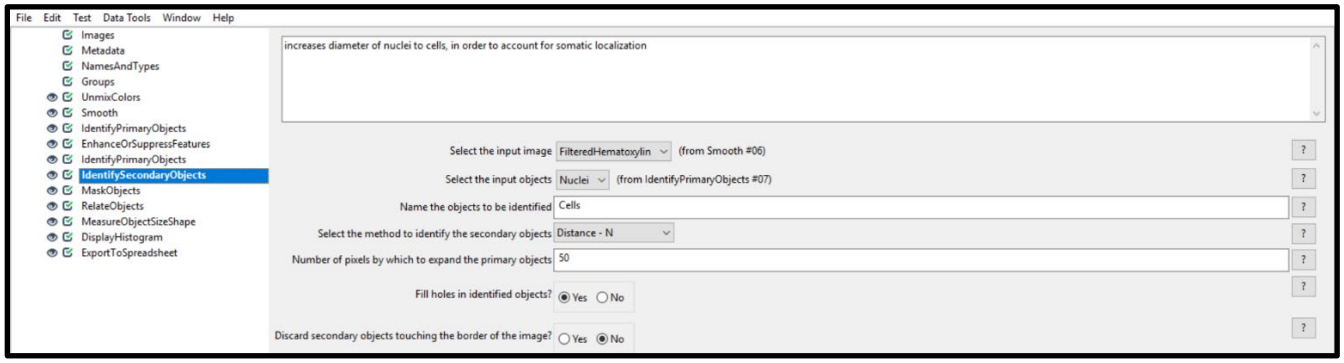
Figure 12. Module settings: IdentifyPrimaryObjects (ISH)



10. Select the **IdentifySecondaryObjects** module, and choose the settings from the drop-down menus.

    Example/Recommendations: Figure 13 displays the recommended settings for this module. Make sure to set the method to identify secondary objects to **Distance-N**. For our sample image, we used a value of 50 pixels to propagate the cell cytoplasm from the nuclear outlines.

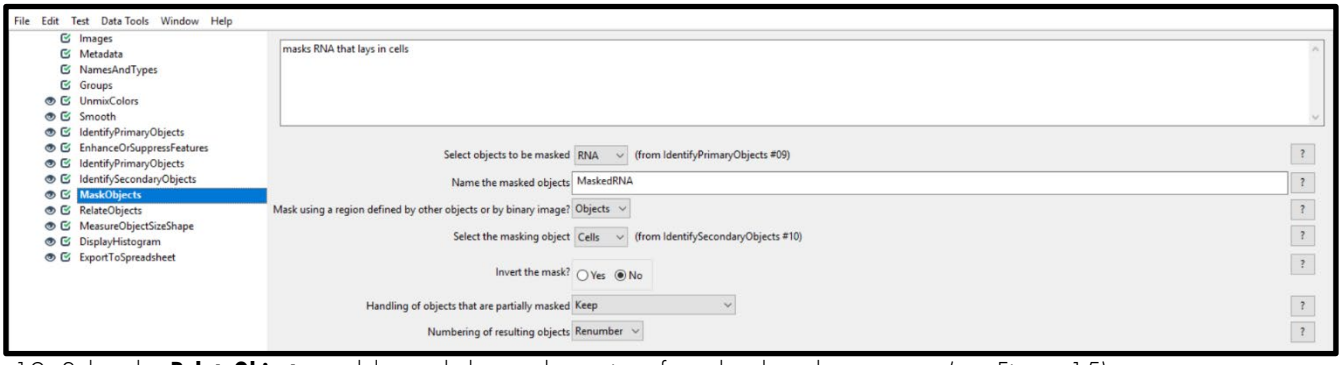
Figure 13. Module settings: IdentifySecondaryObjects (cells)



11. Select the **MaskObjects** module, and choose the settings from the drop-down menus.

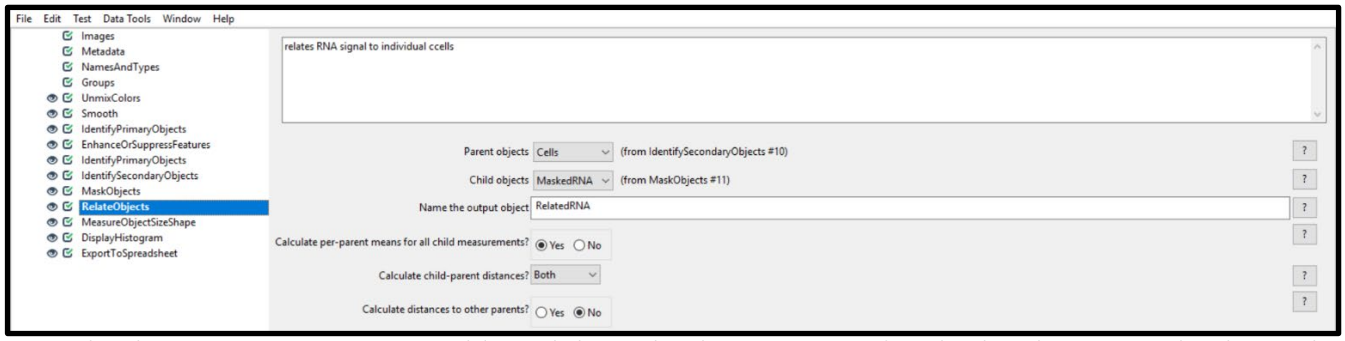
    Example/Recommendations: Figure 14 displays the recommended settings for this module. You must select **RNA** for the objects to be masked if your primary object identified is RNA (see Step 8). Select **Cells** for the masking object.

Figure 14. Module settings: MaskObject



12. Select the **RelateObjects** module, and choose the settings from the drop-down menus (see Figure 15).

Figure 15. Module settings: RelateObjects



13. Select the **MeasureObjectSizeShape** module, and choose the object to measure from the drop-down menu. The object is the output of the RelateObjects module (see Figure 16).

Figure 16. Module settings: MeasureObjectSizeShape

14. Select the **DisplayHistogram** module, and choose the settings from the drop-down menus (see Figure 17). Start with the default number of bins (**100**) to quickly visualize the cell population distribution and adjust from there.

Figure 17. Module settings: DisplayHistogram

15. Adjust the **ExportToSpreadsheet** settings to suit your requirements.

Part 3: Interpreting the Data

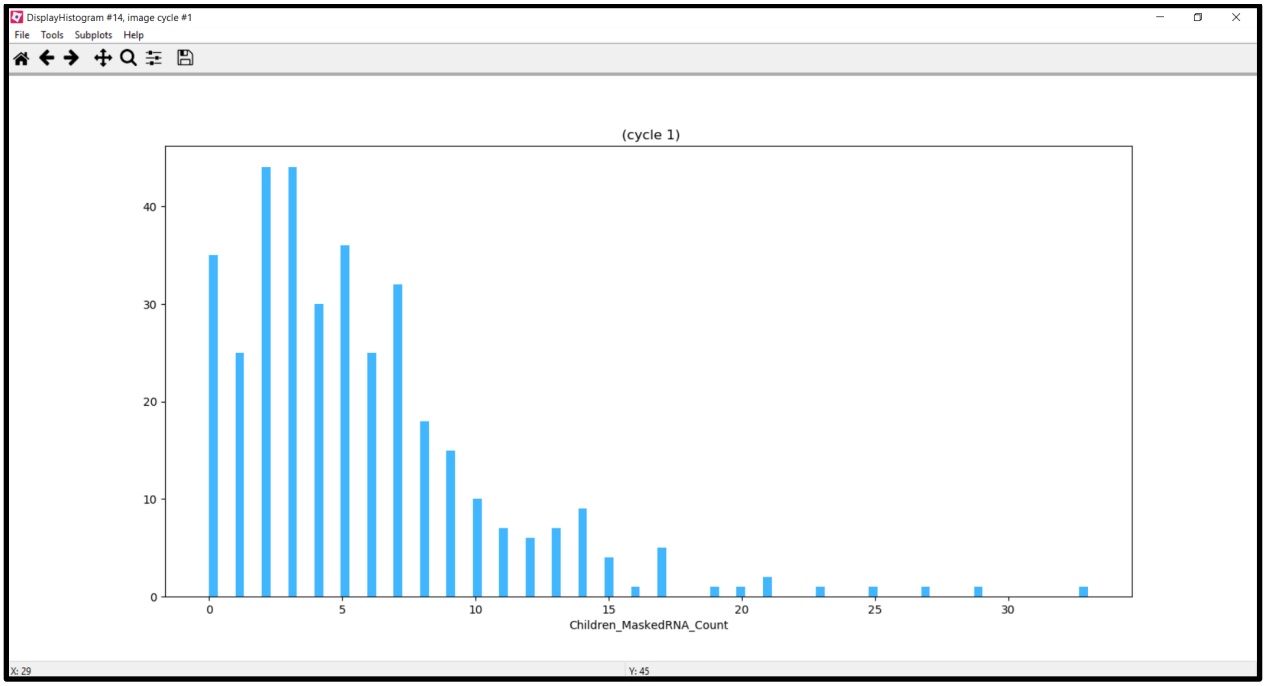
The CellProfiler workflow includes a histogram showing the dots per cell identified using the pipeline. Figure 18 displays the histogram of the data taken from the example in Figure 1.

Use the histogram to examine the distribution of dots/cell, verify the mean dots/cell, and view the maximum and minimum dots/cell in your image. Make sure to spot-check the data and correlate the results with a visual inspection of your image. In the example, most cells have two to three dots/cell, which matches a scan of the raw image. To translate the dots/cell into a semi-quantitative score or H-score using the binning criteria established by Advanced Cell Diagnostics, refer to the data analysis guidelines provided on the ACD website.

A .csv file containing the per cell data for every cell is also exported in the final module. You can transform this information into our typical H-score output or your preferred data presentation format.

Note: The cells can be sorted according to expression level within the CellProfiler workflow using a series of ClassifyObjects modules, by specifying the binning criteria for each round of cell sorting.

Figure 18. Example histogram module output



Part 4: Adding Another Channel to the Chromogenic Assay Analysis Workflow

To analyze a duplex chromogenic assay, modify the singleplex chromogenic workflow by duplicating the initial ISH channel modules (EnhanceOrSuppressFeatures and IdentifyPrimaryObjects) and by modifying the UnmixColors module to accurately separate the channels. For each additional ISH channel, another set of MaskObjects and RelateObjects modules must be added to the workflow. To calculate the number of cells that are positive for both ISH targets (co-expressing cells), use the ClassifyObjects module. See Figure 19 and Table 2 for the workflow overview. Notes on each of the modules, especially on those modules that differ from the singleplex workflow, are given in Table 3. Figure 20 provides the details for the ClassifyObjects module.

Figure 19. Sample chromogenic duplex assay workflow

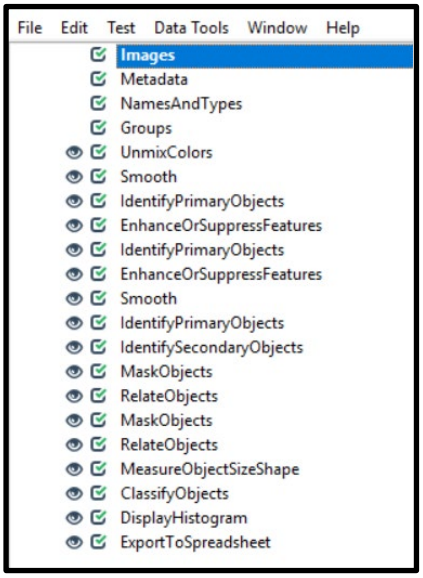


Table 2. Duplex chromogenic workflow

Module	Input Name(s)	Output Name(s)
Images	N/A	N/A
Metadata	N/A	N/A
NamesAndTypes	All images	Rawdata
Groups	N/A	N/A
UnmixColors	Rawdata	Hematoxylin
		RedISH
		Exclusion
		GreenISH
Smooth	Hematoxylin	FilteredHematoxylin
IdentifyPrimaryObjects	FilteredHematoxylin	Nuclei
EnhanceOrSuppressFeatures	RedISH	FilteredRNA
IdentifyPrimaryObjects	FilteredRNA	RNA
EnhanceOrSuppressFeatures	GreenISH	FilteredGreenISH
IdentifyPrimaryObjects	FilteredGreenISH	GreenRNA
IdentifySecondaryObjects	FilteredHematoxylin	Cells
	Nuclei	
MaskObjects	RNA (Objects to be masked)	MaskedRNA
	Cells (Masking objects)	
RelateObjects	Cells (Parent objects)	RelatedRNA
	MaskedRNA (Child objects)	

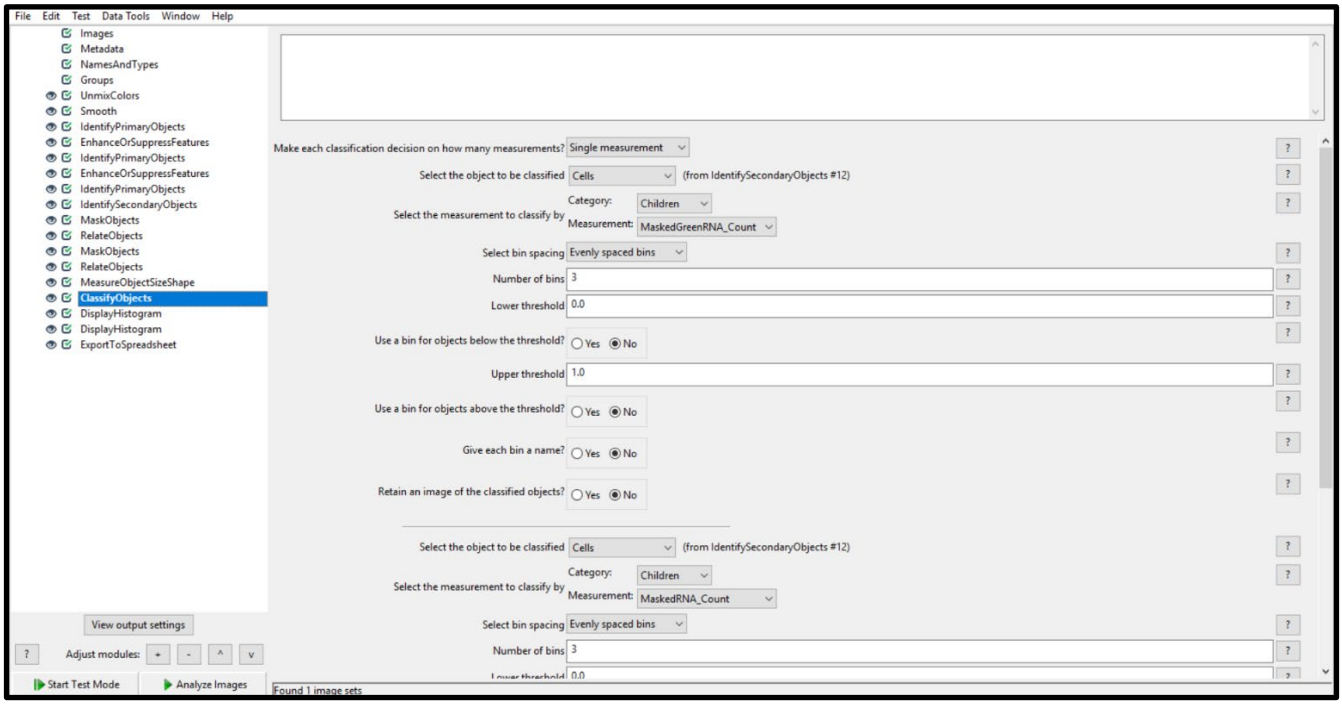
Module	Input Name(s)	Output Name(s)
MaskObjects	GreenRNA (Objects to be masked)	MaskedGreenRNA
	Cells (Masking objects)	
RelateObjects	Cells (Parent objects)	RelatedGreenRNA
	MaskedGreenRNA (Child objects)	
MeasureObjectSizeShape	RelatedRNA	N/A
	RelatedGreenRNA	
ClassifyObjects	Cells (object name)	NotExpressing
	Children	RedExpressing
	MaskedGreenRNA_Count	GreenExpressing
	Children	Coexpressing
	MaskedRNA_Count	CoexpressingCells
DisplayHistogram	Cells	MaskedRNA_Count
DisplayHistogram	Cells	MaskedGreenRNA_Count
ExportToSpreadsheet	N/A	N/A

Table 3. Notes on duplex chromogenic workflow and modules

Module	Notes
Images	N/A
Metadata	N/A
NamesAndTypes	Use 'color image'
Groups	N/A
UnmixColors	Add GreenISH with settings (1,0.05,1)
Smooth	Same settings as singleplex
IdentifyPrimaryObjects	Identify nuclei as in the singleplex chromogenic workflow
EnhanceOrSuppressFeatures	Enhance speckles feature size = 20
IdentifyPrimaryObjects	Min, Max (1,10)
EnhanceOrSuppressFeatures	Enhance speckles feature size = 20
IdentifyPrimaryObjects	Min, Max (1,4) Green ISH dots tend to be smaller than red ISH dots.
IdentifySecondaryObjects	Propagate cell boundaries as in the singleplex chromogenic workflow
MaskObjects	Same settings as singleplex
RelateObjects	Same settings as singleplex
MaskObjects	Add GreenRNA
RelateObjects	Add MaskedGreenRNA
MeasureObjectSizeShape	Measure RelatedRNA and RelatedGreen RNA features separately
ClassifyObjects (see Figure 20)	Classify the MaskedGreenRNA_Count separately from the MaskedRNA_Count

Module	Notes
DisplayHistogram	Display data from MaskedRNA_COunt
DisplayHistogram	Display data from MaskedGreenRNA_Count
ExportToSpreadsheet	Same settings as singleplex

Figure 20. ClassifyObjects module output



Part 5: Fluorescent Assay Analysis Workflow

To analyze a multiplex fluorescent assay, modify the duplex chromogenic workflow by eliminating the color deconvolution step and duplicating the ISH channel modules (EnhanceOrSuppressFeatures, IdentifyPrimaryObjects, MaskObjects, and RelateObjects) until the desired number of ISH channels have been included. To calculate the number of cells that are positive for two ISH targets (co-expressing cells), use the ClassifyObjects module or the FilterObjects module. A good starting point for a multiplex fluorescent assay CellProfiler workflow is the pipeline published by Erben et al. (2017). Download this pipeline from the CellProfiler website at **https://cellprofiler.org/examples/published_pipelines** (see Figure 21). Table 4 and Figures 22-24 contains the names and unique module details associated with this workflow.

Note: The Erben et al. analysis workflow focuses on the red FISH signal. If needed, adjust the workflow, including the names, to calculate your desired data output.

Figure 21. Sample fluorescent assay workflow from Erben et al (2017)

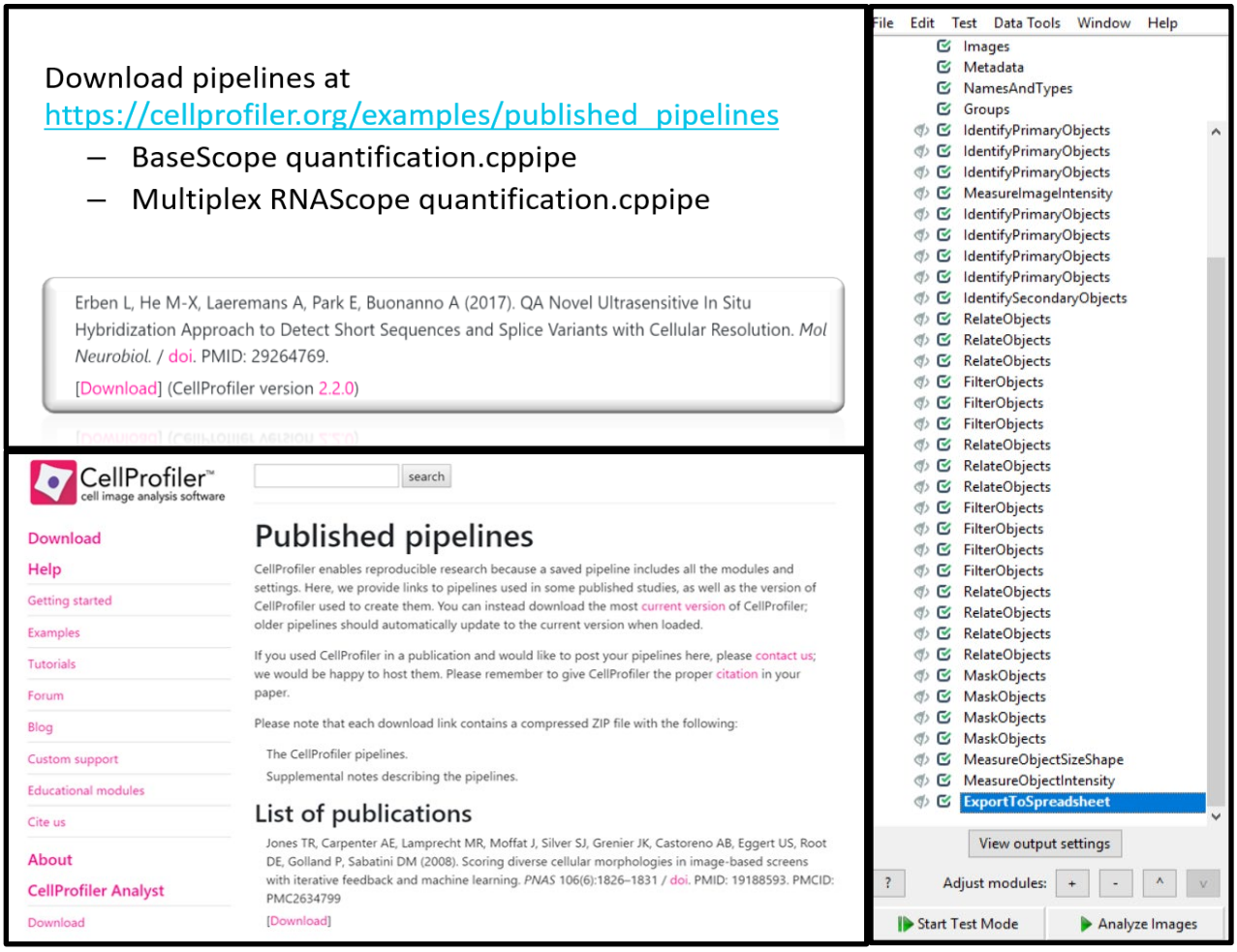


Table 4. Multiplex fluorescent assay CellProfiler analysis workflow

Module	Input Name(s)	Output Name(s)
Images (see Figure 22)	**Note:** Load single images for each channel. Include C1 through C4 in the file names.	N/A
Metadata	N/A	N/A
NamesAndTypes (see Figure 23)	C1	DAPI (color image)
	C2	Green (color image)
	C3	Red (color image)
	C4	White (Grayscale image)
Groups	N/A	N/A
ColorToGray (see Figure 24)	C1	OrigNuclei
	Split	
	RGB	
ColorToGray	C2	OrigGreen
	Split	
	RGB	
ColorToGray	C3	OrigRed
	Split	
	RGB	

Module	Input Name(s)	Output Name(s)
EnhanceOrSuppressFeatures	OrigGreen	EnhancedGreen
EnhanceOrSuppressFeatures	C4	EnhancedWhite
EnhanceOrSuppressFeatures	OrigRed	EnhancedRed
IdentifyPrimaryObjects	OrigNuclei	NucleiArea
IdentifyPrimaryObjects	OrigGreen	GreenArea
IdentifyPrimaryObjects	OrigRed	RedArea
IdentifyPrimaryObjects	C4	WhiteArea
MeasureImageIntensity	OrigNuclei/ NucleiArea	N/A
	C4/ WhiteArea	N/A
	OrigRed/ RedArea	N/A
	OrigGreen/ GreenArea	N/A
IdentifyPrimaryObjects	OrigNuclei	Nuclei
IdentifyPrimaryObjects	EnhancedGreen	Green
IdentifyPrimaryObjects	EnhancedRed	Red
IdentifyPrimaryObjects	EnhancedWhite	White
IdentifySecondaryObjects	OrigNuclei	Cells
	Nuclei	
RelateObjects	Cells (Parent objects)	RelateObjects
	Red (Child objects)	
RelateObjects	Cells (Parent objects)	RelateObjects
	Green (Child objects)	
RelateObjects	Cells (Parent objects)	RelateObjects
	White (Child objects)	
FilterObjects	Cells	RedCells
FilterObjects	Cells	WhiteCells
FilterObjects	Cells	GreenCells
RelateObjects	RedCells	RelateObjects
	Red	
RelateObjects	RedCells	RelateObjects
	Green	
RelateObjects	RedCells	RelateObjects
	White	
FilterObjects	RedCells	RedandGreenCells
FilterObjects	RedCells	RedandWhiteCells
FilterObjects	RedCells	RedandNoneCells
FilterObjects	RedCells	RedandBothCells
RelateObjects	RedandGreenCells (Parent objects)	RelateObjects
	Red (Child Objects)	
RelateObjects	RedandWhiteCells (Parent Objects)	RelateObjects
	White (Child Objects)	
RelateObjects	RedandNoneCells (Parent Objects)	RelateObjects
	Red (Child Objects)	
RelateObjects	RedandBothCells (Parent Objects)	RelateObjects
	Red (Child Objects)	
MaskObjects	Red	RedDotsonRedandGreenCells
MaskObjects	Red	RedDotsonRedandWhiteCells
MaskObjects	Red	RedDotsonRedandNoneCells
MaskObjects	Red	RedDotsonRedandBothCells
MeasureObjectSizeShape	Red	N/A
MeasureObjectIntensity	OrigRed (Image)	N/A
	Red (Objects)	

Module	Input Name(s)	Output Name(s)
	RedDotsonRedandGreenCells (Objects)	
	RedDotsonRedandWhiteCells (Objects)	
	RedDotsonRedandNoneCells (Objects)	
	RedDotsonRedandBothCells (Objects)	
ExportToSpreadsheet	N/A	N/A

Figure 22. Fluorescent assay Images module

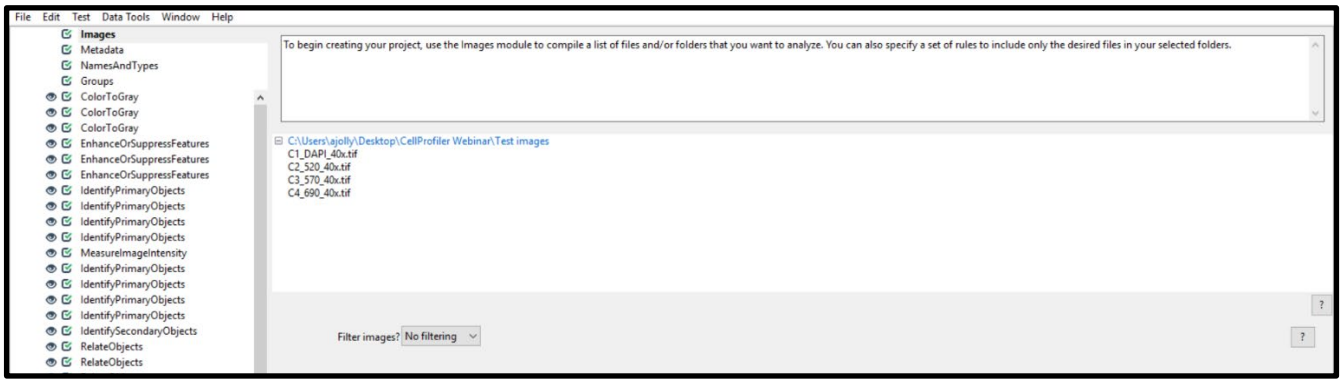


Figure 23. Fluorescent assay NamesAndTypes module

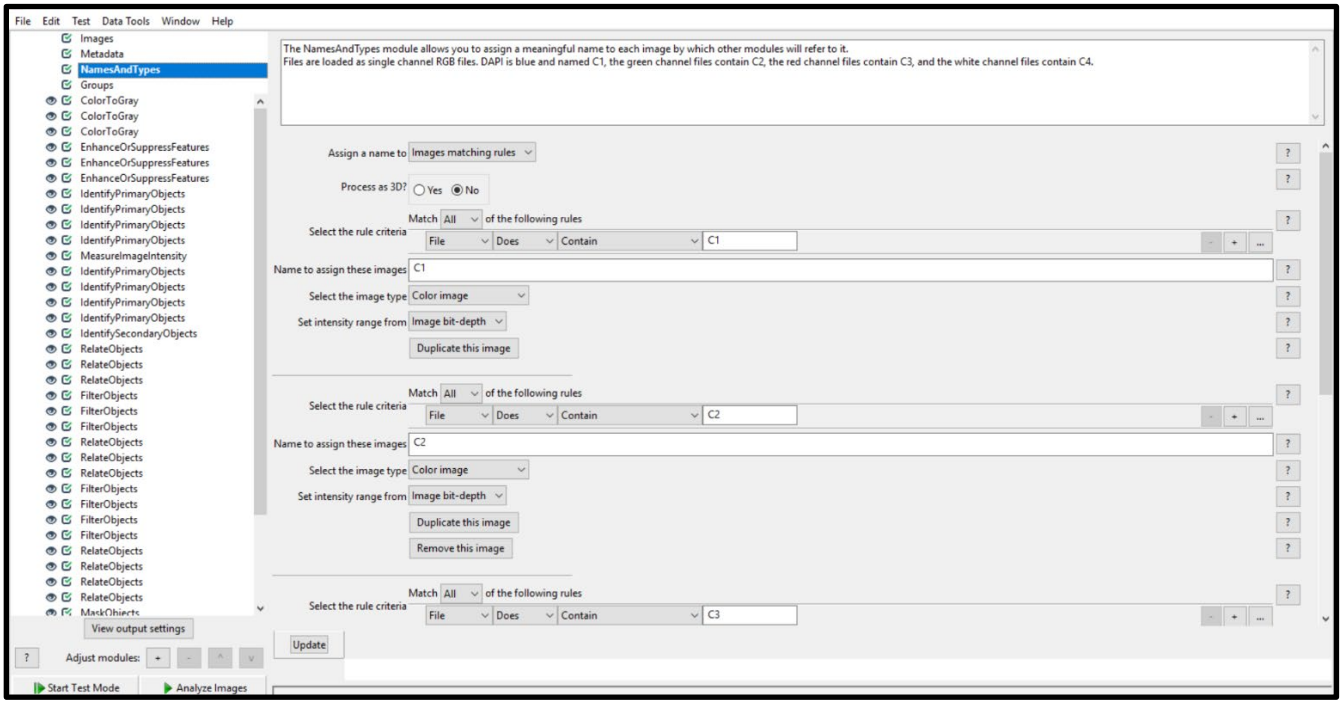
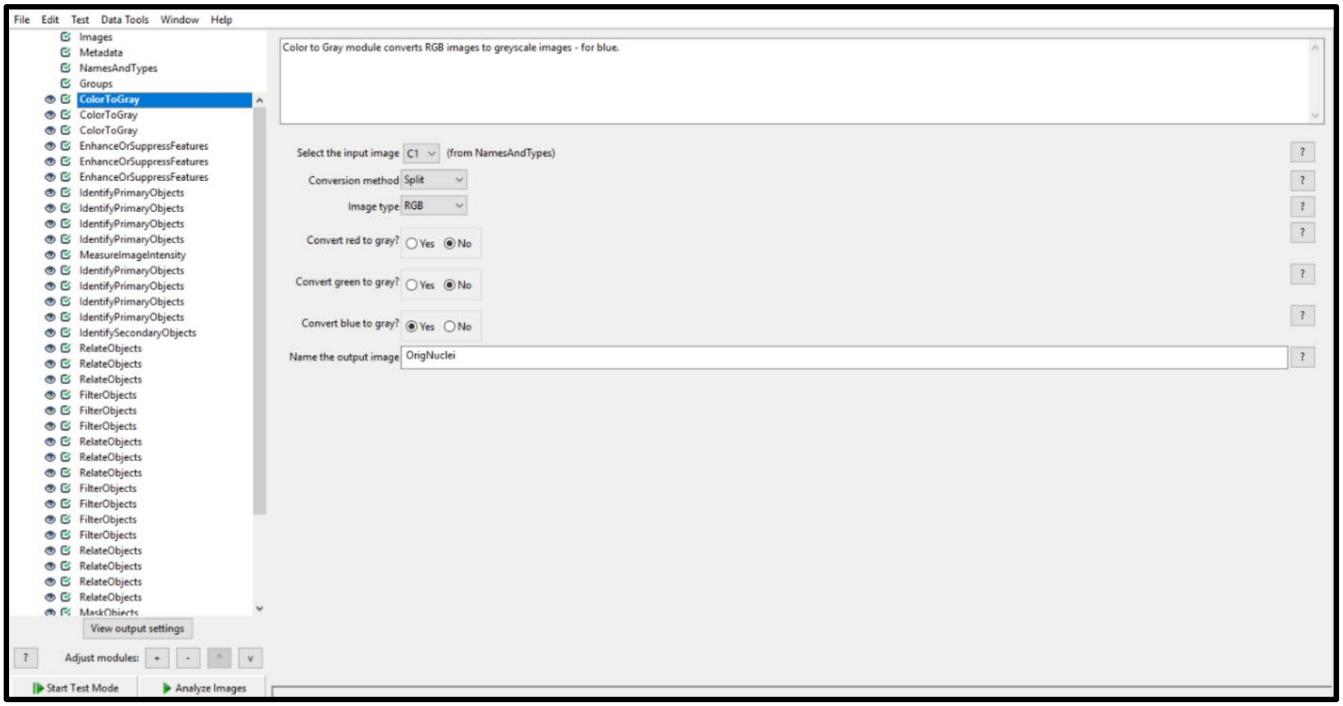


Figure 24. Fluorescent assay ColorToGray module



Headquarters
7707 Gateway Blvd Suite 200, Newark, CA 94560 Phone 1-510-576-8800 Toll Free 1-877-576-3636
For support, email **support.acd@bio-techne.com**
www.acdbio.com

a bio-techne brand