**TECHNICAL NOTE** 



# Using CellProfiler to Analyze RNAscope<sup>®</sup> and BaseScope<sup>™</sup> 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<sup>®</sup> or BaseScope<sup>™</sup> 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<sup>®</sup> 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

| CellProfiler   | - 🗆 X  | Welcome to CellProfile  | r  |   |  | - 🗆 X  |
|--|--|---|--|---|--|--|
| <pre>ront search path ['C:\UserC\use</pre> | ata\llocal\lTemp\\\MEI85882\\wpl=data\\fonts\\tff", 'C:\Users\\ajolly ∧<br>a\\fonts\\afm", 'C:\Users\\ajolly\\AppData\\Local\\Temp\\_MEI8582\\m<br>y   | CellProfiler is automate<br>See a pipeline<br>Load an exam<br>Build your ow | Welcon<br>d image analysis softw<br>in action<br>ble pipeline, then click on<br>n pipeline   | me to CellPro<br>rare to measure biologica<br>on the "Analyze Images"   | filer!<br>I phenotypes in image<br>button.   | es.  |
| C Image:<br>C Mage:<br>C Metadata<br>C NamesAndTypes<br>C Groups   | To begin creating your project, use the images module to compile a list of files and/or folders that a<br>analyze. You can also specify a set of nules to include only the desired files in your selected folders. | 1: Start<br>2: Adjust<br>3: Test<br>4:<br>Analyze<br>Need more hel          | Download a pipeline<br>> Pipeline from File.<br>Use the Input modul<br>modules to identify i<br>Click the "Start Test<br>settings on a few im<br>Click the "Analyze In<br>P? | template from our websi<br>Run it, then modify it<br>es to <u>select</u> and <u>configur</u><br>mage features, make <u>ms</u><br>Mode <sup>+</sup> button to step th<br>ages.<br>mages <sup>*</sup> button to <u>proces</u> ; | te of examples. Load<br>to suit your assay.<br>a your images for ana<br><u>asurements</u> and <u>exp</u><br>rough the pipeline and<br>a all of your images w | it with <i>File &gt; Import</i><br>alysis. Add Analysis<br>att results.<br>d <u>check</u> the module<br>ith your pipeline. |
|  | Show files excluded by filters   | In-App H  | Manual   | Tutorials/Demos   | Q&A Forum  |  |
| View output settings ? Adjust modules: + + ^ v   | Filter images? Images only v<br>Apply filters to the file list Apply filters to the file list  | Click here to stop displa<br>Welcome Screen at any                          | of In-App help<br>of In-App help<br>nying this page when Ce<br>r time.   | guidance to image<br>analysis<br>ellProfiler starts. This page  | question<br>online<br>le can be accessed fr  | rom Help > Show  |
| Start Test Mode Analyze Images   |  |   |  |   |  |  |

**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

| -   |  |
|---|--|
| To begin creating your project, use the Images module to compile a list of files and/or folders that you want to analyze. You can also specify a set of rules to include only the desired files in your selected folders. | ¢  |
| Drop files and folders here   |  |
| Show files excluded by filters Filter images only Apply filters to the file list Apply filters to the file list   | ?  |
|   |  |
|   |  |
|   | To begin creating your project, use the Images module to compile a list of files and/or folders that you want to analyze. You can also specify a set of rules to include only the desired files in your selected folders.  DDDDDD files and folders have been been been been been been been be |



**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. MaskÓbjects
  - 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

| CellProfiler 3.1.8  |  |   |  | - 6 X |
|---|--|---|--|-------|
| File Edit Test Data Tools Window Help   |  |   |  |       |
| C Cellipeder 1.12<br>File End Tet Lab Tools Window Help<br>E Image<br>E Hanges<br>E NamesAndTypes<br>E Coopt<br>● O UnmixColors | Select the input color image None S<br>Hame the output image Hernatorylin<br>Stan ACC S<br>Add another stain | Add modules –<br>Sector UmmuCators –<br>Module Categories Allege –<br>Table Pacetary –<br>Color Solver –<br>Color | Search<br>tionApply<br>torrCalculate<br>resafeatures |       |
|   |  |   |  |       |
| View output settings  |  |   |  |       |
| ? Adjust modules: + - ^ v   |  |   |  |       |
| Start Test Mode Analyze Images  |  |   |  |       |

#### Figure 6. Module help page

| C Hepformodule, "UnmixColors" – & X   |
|---|
| UnmixColors   |
| UnmixColors creates separate images per dye stain for histologically stained images.  |
| This modele creates separate images from a color image stained with light-abourbing dyse. Dyes are assumed to abourb an amount of light in the red, green and blue channels that increases proportionally in each channel with increasing amounts of tains the hue does not shift with increasing staining. The module separates have channels that increases proportionally in each channel with increasing gravitation and the properties of tains the hue does not shift with increasing staining. The module separates have channels that increases proportionally in each channel with increasing amounts of tains the hue does not shift with increasing staining. The module separates have channels that allows you to calibrate using two images stained with a single dye each. Some commonly known stains must be specified by the individual dye components. For example:<br>Green as Mathews Accurations + Orange G<br>Green as Methylene Blue or Easin<br>• Marson Tichtome. Methyl Me + Pinceau-Fuchsin  |
| If there are non-stained cells/components that you also want to separate by color, choose the stain that most closely resembles the color you want, or enter a custom value. Please note that if you are looking to simply split a color image into red, green and blue components, use the ColorToGray module rather than UnmixColors.   |
| Supports 20?         Supports 30?         Respects masks?           YES         NO         NO   |
| Technical notes   |
| This code is adapted from the ImageJ plugin, Colour_Deconstruction_jura written by A.C. Ruiflok, whose paper forms the basis for this code.   |
| References  |
| Ruitlok AC, Johnston DA (2001) "Quantification of histochemical staining by color decomolution." Analytical & Quantitative Cytology & Histology. 23: 291-299.   |
| See also ColorToGray.   |
| Settings:   |
| Select the input color image  |
| Consection on the histological states color image loaded or measured by some more module  |
|   |
| Name the output image   |
| Use this setting to name one of the images produced by the module for a particular stain. The image can be used in subsequent modules in the pipeline.  |
| Stain   |
| Use this setting to choose the absorbance values for a particular stain.  |
| The stains are:   |
| Stain Color Specific to   |
| AEC (3Amin-9-ethylcarbazole) Peroxidiase  |
| Acidan bus Polien tubes   |
| Azoamino Piasma   |
| DAD Berevisemen with the second |



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                                  | Rawdata                    | Hematoxylin         |
| (see Figure 8)                               |                            | RedISH              |
|  |                            | Exclusion           |
| Smooth<br>(see Figure 9)                     | Hematoxylin                | FilteredHematoxylin |
| IdentifyPrimaryObjects<br>(see Figure 10)    | FilteredHematoxylin        | Nuclei              |
| EnhanceOrSuppressFeatures<br>(see Figure 11) | RedISH                     | FilteredRNA         |
| IdentifyPrimaryObjects<br>(see Figure 12)    | FilteredRNA                | RNA                 |
| IdentifySecondaryObjects<br>(see Figure 13)  | FilteredHematoxylin        | Cells               |
|  | Nuclei                     |                     |
| MaskObjects                                  | RNA (Objects to be masked) | MaskedRNA           |
| (see Figure 14)                              | Cells (Masking objects)    |                     |
| RelateObjects                                | Cells (Parent objects)     | RelatedRNA          |
| (see Figure 15)                              | MaskedRNA (Child objects)  |                     |
| MeasureObjectSizeShape<br>(see Figure 16)    | RelatedRNA                 | N/A                 |
| DisplayHistogram                             | Cells                      | N/A                 |
| (see Figure 17)                              | 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<sup>®</sup> or BaseScope<sup>M</sup> 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

| File Edit Test Data Tools Window Help  |  |  |     |
|--|--|--|-----|
| Images     Matadata     Name:AndTypes     Groups     Groups     G' UmmicColors     G' UmmicColors     G' Government of the second |  |  | ^   |
|  | Select the input color image<br>Name the output image<br>Stain | Rawdata v (from NamesAndTypes)<br>Hematoxylin<br>Hematoxylin v | ?]? |
| <ul> <li>⑦ ☑ DisplayHistogram</li> <li>⑦ ☑ ExportToSpreadsheet</li> </ul>  | Name the output image<br>Stain                                 | RedISH V   | ?   |
|  | Red absorbance<br>Green absorbance                             | 1  | ?   |
|  | Blue absorbance  | 1  | ?   |
|  | Estimate absorbance from image                                 | Estimate Remove this image                                     | ?   |
|  | Name the output image  | Exclusion  | ?   |
|  | Stain<br>Red absorbance  | Custom v<br>1  | ?   |
|  | Green absorbance   | 1  | ?   |
|  | Blue absorbance  | 1<br>Fdimate   | ?   |
| View output settings   |  | Remove this image  | ?   |
| ? Adjust modules: + - ^ v  |  | Add another stain  | ?   |

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

| File Edit | Test Data Tools Window Help |   |     |
|-----------|-----------------------------|---|-----|
| C         | Images                      |   | 100 |
| C         | Metadata                    |   | A   |
| C         | NamesAndTypes               |   |     |
| S         | Groups                      |   |     |
| • 5       | UnmixColors                 |   |     |
| • 5       | Smooth                      |   | ~   |
| • •       | IdentifyPrimaryObjects      |   |     |
| • 5       | EnhanceOrSuppressFeatures   | Select the input image Hampton fin up (from Hampton 2017 #05) | 2   |
| • •       | IdentifyPrimaryObjects      | select the input image Hematoxy in v (non oniniccolor =05)    |     |
| • •       | IdentifySecondaryObjects    | Name the output image FilteredHematoxylin                     | ?   |
|           | MaskObjects                 |   |     |
| • 5       | RelateObjects               | Select smoothing method Circular Average Filter 🗸 🗸           | ?   |
| • 5       | MeasureObjectSizeShape      |   |     |
| • •       | DisplayHistogram            | Calculate artifact diameter automatically?  West No.          | 1   |
| • 5       | ExportToSpreadsheet         |   |     |

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.



| File Edit Test Data Tools Window Help  |  |   |     |
|--|--|---|-----|
| 당 Images<br>당 Metadata<br>당 NamesAndTypes  | identifies nuclei  |   | ^   |
| <ul> <li>Groups</li> <li>G UnmixColors</li> <li>G Smooth</li> </ul>                            |  |   | ~   |
| IdentifyPrimaryObjects     EnhanceOrSuppressFeatures     IdentifyPrimaryObjects                | Name the primary objects to be identified                                | Nuclei  | ?   |
| <ul> <li>IdentifySecondaryObjects</li> <li>MaskObjects</li> </ul>                              | Typical diameter of objects, in pixel units (Min, Max)                   | 10 100  | ?   |
| <ul> <li>RelateObjects</li> <li>MeasureObjectSizeShape</li> <li>Disclored listeness</li> </ul> | Discard objects outside the diameter range                               | ? ⊛Yes ⊖No                                    | r   |
| <ul> <li>ExportToSpreadsheet</li> </ul>  | Discard objects touching the border of the image                         | ? ⊖ Yes ⊛ No                                  | ?   |
|  | Threshold strategy   | Global v                                      | ?   |
|  | Thresholding method  | Otsu v  | ?   |
|  | iwo-class or three-class thresholding:<br>Threshold smoothing scale      | 0   | ?   |
|  | Threshold correction factor  | 1   | ?   |
|  | Lower and upper bounds on threshold                                      |   | ?   |
|  | Method to distinguish clumped objects                                    | Shape 🗸                                       | ?   |
|  | Automatically calculate size of smoothing filter for declumping          |   | ?   |
|  | Automatically calculate size of smoothing lines for declamping.          | © Yes () No                                   | 2   |
|  | Automatically calculate minimum allowed distance between local maximal   | ? O Yes   No                                  |     |
|  | Suppress local maxima that are closer than this minimum allowed distance | [1  | ?   |
| View output settings   | Speed up by using lower-resolution image to find local maximal           | ? ® Yes () No                                 |     |
| ? Adjust modules: + - ^ v  | Fill holes in identified objects   | After both thresholding and declumping $\sim$ | ?   |
|  | rlandling of objects if excessive number of objects identified           | Continue                                      | 1 1 |

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

| File Edit | Test Data Tools Window Help |  |   |
|-----------|-----------------------------|--|---|
| G         | / Images                    |  |   |
| G         | Metadata                    | enhances KNA signal  | ^ |
| 6         | NamesAndTypes               |  |   |
| G         | Groups                      |  |   |
| •         | UnmixColors                 |  |   |
| 00        | Smooth                      |  | ~ |
| •         | IdentifyPrimaryObjects      |  |   |
| • 6       | EnhanceOrSuppressFeatures   | Select the input image Red(SH v (from UpmicColors #05)   | 2 |
| 0         | IdentifyPrimaryObjects      | inclusion in the industrial state of the industrial st |   |
| • 6       | IdentifySecondaryObjects    | Name the output image FilteredRNA  | ? |
| 0         | MaskObjects                 |  |   |
| • 6       | RelateObjects               | Select the operation Enhance 🗸   | ? |
| •         | MeasureObjectSizeShape      | English Snarklar v   | 2 |
| •         | DisplayHistogram            | reactive type spectrum +   | 4 |
| •         | ExportToSpreadsheet         | Feature size 20  | ? |
|           |                             | Speed and accuracy Slow $\sim$   | ? |

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: | Identif | yPrimar | yOb | jects | (ISH) |
|--------|-----|------------------|---------|---------|-----|-------|-------|
|--------|-----|------------------|---------|---------|-----|-------|-------|

| File Edit Test Data Tools Window Help                               |   |   |     |
|---|---|---|-----|
| E Images<br>E Metadata<br>E NamesAndTypes                           | NA signal   |   | ^   |
| © ☞ UnmixColors     © ☞ Smooth     © ☞ IdentifyPrimaryObjects       |   |   | v   |
| C EnhanceOrSuppressFeatures     C IdentifyPrimaryObjects            | Name the primary objects to be identified                       | RNA                                       | ? ^ |
| IdentifySecondaryObjects     MaskObjects                            | Typical diameter of objects, in pixel units (Min, Max)          | 1 10                                      | ?   |
| C RelateObjects     C MeasureObjectSizeShape     C DisplayHistogram | Discard objects outside the diameter range?                     | ● Yes ○ No                                |     |
| <ul> <li>♥ ☑ ExportToSpreadsheet</li> </ul>                         | Discard objects touching the border of the image?               | ● Yes ○ No                                | ?   |
|   | Threshold strategy  | Adaptive ~                                | ?   |
|   | Thresholding method<br>Two-class or three-class thresholding?   | Otsu V<br>Two classes V                   | ?   |
|   | Threshold smoothing scale                                       | 0   | ?   |
|   | Threshold correction factor                                     | 1.0                                       | ?   |
|   | Lower and upper bounds on threshold                             | 20  | ?   |
|   | Method to distinguish clumped objects                           | Intensity ~                               | ?   |
|   | Method to draw dividing lines between clumped objects           | Intensity $\sim$                          | ?   |
| At  | utomatically calculate size of smoothing filter for declumping? | ⊛ Yes ⊖ No                                | ?   |
| Automatically   | y calculate minimum allowed distance between local maxima?      |   | ?   |
| View output settings Sp   | eed up by using lower-resolution image to find local maxima?    | ⊛ Ves ⊖ No                                | ?   |
| ? Adjust modules: + - ^ v   | Fill holes in identified objects?                               | After both thresholding and declumping $$ | ?   |
|   | Handling of objects if excessive number of objects identified   | Continue ~                                | ? ~ |

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)

| File Edit | Test Data Tools Window Help |  |   |   |
|-----------|-----------------------------|--|---|---|
| C         | Images                      | A REAL PROPERTY AND A REAL |   |   |
| C         | Metadata                    | increases diameter of nuclei to cells, in order to account for   | somatic localization  | ^ |
| C         | NamesAndTypes               |  |   |   |
| C         | Groups                      |  |   |   |
| • 5       | UnmixColors                 |  |   |   |
| • 5       | Smooth                      |  |   | v |
| • 5       | IdentifyPrimaryObjects      |  |   |   |
| • 6       | EnhanceOrSuppressFeatures   | Select the input image   | FilteredHematondin v (from Smooth #06)  | 2 |
| • 5       | IdentifyPrimaryObjects      |  | (initial end of the second of |   |
| • 5       | IdentifySecondaryObjects    | Select the input objects   | Nuclei ~ (from IdentifyPrimaryObjects #07)  | ? |
| • 5       | MaskObjects                 |  |   |   |
| • 5       | RelateObjects               | Name the objects to be identified  | Cells   | ? |
| • 5       | MeasureObjectSizeShape      |  | Distance M.   |   |
| • 6       | DisplayHistogram            | Select the method to identify the secondary objects  | Distance - IN   |   |
| • 6       | ExportToSpreadsheet         | Number of pixels by which to expand the primary objects  | 50  | ? |
|           |                             |  |   |   |
|           |                             | Fill holes in identified objects?  | OV- ON-   | ? |
|           |                             | in nois in dentiled objects  | © res UNo   |   |
|           |                             |  |   | 2 |
|           |                             | Discard secondary objects touching the border of the image?  | ○Yes ◉No  | • |
|           |                             |  |   |   |

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

| File Edit Test Data Tools Window Help  |  |   |
|--|--|---|
| G Images<br>G Metadata<br>G Name£AndTypes<br>G Groups<br>⊕ G UnmicColors<br>⊕ G Smooth   | masks RNA that lays in cells   | < v                                     |
| C IdentifyPirmayObjects     G EnhanceOfcoppresentation     G EnhanceOfcoppresentation     G IdentifyPirmayObjects     G IdentifyPirmayObjects     G IdentifyPirmayObjects     G RelateObjects     G RelateObjects     G RelateObjects     G DisplayHistogram     G ExportToSpreadsheet | Select objects to be masked RNA v (from identifyPrimaryObjects #09) Name the masked objects Mask using a region defined by other objects or by binary image? Mask using a region defined by other objects or by binary image? Mask using a region defined by other objects or by binary image? Mask using a region defined by other objects or by binary image? Mask using a region defined by other objects or by binary image? Mask using a region defined by other objects or by binary image? Mask using a region defined by other objects or by binary image? Mask using a region defined by other objects or by binary image? Mask using a region defined by other objects of by binary image? Mask using a region defined by other objects the are partially masked Keep V Mask using a region defined by other objects the are partially masked Keep V Mask using a region defined by other objects the are partially masked Keep V Mask using a region defined by other objects the are partially masked Keep V Mask using a region defined by other objects the are partially masked Keep V Mask using a region defined by other objects the are partially masked Keep V Mask using a region defined by other objects the are partially masked Keep V Mask using a region defined by other objects the are partially masked Keep V Mask using a region defined by other objects the are partially masked Keep V Mask using a region defined by other objects the are partially masked Keep V Mask using a region defined by other objects the are partially masked Keep V Mask using a region defined by other objects the are partially masked Keep V Mask using a region defined by other objects the are partially masked Keep V Mask using a region defined by other objects the are partially masked Keep V Mask using a region defined by other objects the are partially masked Keep V Mask using a region defined by other objects the are partially masked Keep V Mask using a region defined by other objects the are partially masked Keep V Mask using a region defined by other objects Keep V Mask usi | 2 |

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

Figure 15. Module settings: RelateObjects

| File Edit Test Data Tools Window Help   |  |   |
|---|--|---|
| 🕑 Images<br>🕑 Metadata  | relates RNA signal to individual ccells                              | ^ |
| NamesAndTypes   |  |   |
| C UnmixColors   |  |   |
| Smooth     Smooth     Gettingerman@biactr   |  | Ŷ |
| C EnhanceOrSuppressFeatures     C IdentifyPrimaryObjects  | Parent objects Cells v (from IdentifySecondaryObjects #10)           | ? |
| <ul> <li>IdentifySecondaryObjects</li> <li>MaskObjects</li> </ul>                                   | Child objects MaskedRNA 🗸 (from MaskObjects #11)                     | ? |
| C RelateObjects   | Name the output object RelatedRNA                                    | ? |
| <ul> <li>C MeasureObjectSizeShape</li> <li>DisplayHistogram</li> <li>ExportToSpreadsheet</li> </ul> | Calculate per-parent means for all child measurements? (a) Yes () No | ? |
| - Andrewski - Carlos - Al   | Calculate child-parent distances? Both $\sim$                        | ? |
|   | Calculate distances to other parents? O Yes                          | ? |

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

| File Edit | Test Data Tools Window Help  |  |     |
|-----------|--|--|-----|
| C         | images in the second seco | in a second bit of DMA data                                  | 1.1 |
| C         | Metadata   | measures size of NNA dots                                    | ^   |
| C         | NamesAndTypes  |  |     |
| C         | Groups   |  |     |
| • •       | UnmixColors  |  |     |
| • 6       | Smooth   |  | × . |
| • 6       | IdentifyPrimaryObjects   |  |     |
| 00        | EnhanceOrSuppressFeatures  | Called Stricts to an annual (for a Radat Object #17)         | 2   |
| 00        | IdentifyPrimaryObjects   | Select objects to measure Relatedriva (from relatedrive +12) |     |
| • 6       | IdentifySecondaryObjects   | Add another object   | 2   |
| • 6       | MaskObjects  |  |     |
| 00        | RelateObjects  |  |     |
|           | MeasureObjectSizeShape   |  | 2   |
|           | DisplayHistogram   | Calculate the Zernike features? 🔿 Yes 💿 No                   |     |
| • 6       | ExportToSpreadsheet  |  |     |

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

| File Edit | Test Data Tools Window Help |  |  |         |
|-----------|-----------------------------|--|--|---------|
| C         | Images                      |  |  |         |
| C         | Metadata                    |  |  | <u></u> |
| C         | NamesAndTypes               |  |  |         |
| C         | Groups                      |  |  |         |
| • 6       | UnmixColors                 |  |  |         |
| • 6       | Smooth                      |  |  | v       |
| • 5       | IdentifyPrimaryObjects      |  |  |         |
| • 6       | EnhanceOrSuppressFeatures   | Select the object whose measurements will be displayed | Calle v (from IdentifyGerondan/Objects #10)  | 2       |
| • 5       | IdentifyPrimaryObjects      | Select the object most measurements will be displayed  | Cens · (non rochary contary co | 10.00   |
| • 6       | IdentifySecondaryObjects    | (  | Category: Children V   | ?       |
| • 5       | MaskObjects                 | Select the object measurement to plot                  |  |         |
| • 5       | RelateObjects               |  | MaskedRNA_Count V  |         |
| • 6       | MeasureObjectSizeShape      | Number of bins   | 100  | 2       |
| • 6       | DisplayHistogram            |  |  |         |
| • 5       | ExportToSpreadsheet         | How should the X-axis be scaled?                       | linear v   | ?       |
|           |                             | How should the Y-axis be scaled?                       | linear 🗸   | ?       |
|           |                             | Enter a title for the plot if derived                  |  | 2       |
|           |                             | Line a due for the plot, it desired                    |  |         |
|           |                             | Specify min/max bounds for the X-axis?                 | ○Yes ●No   | ?       |

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.



 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           | Classify the MaskedGreenRNA_Count separately from the               |
| (see Figure 20)           | Maskeakina_Count  |



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



| File Edit 1 | Test Data Tools Window Help |   |  |  |
|-------------|-----------------------------|---|--|--|
| C           | Images                      | ·   |  |  |
| S           | Metadata                    |   |  | ^.   |
| C           | NamesAndTypes               |   |  |  |
| C           | Groups                      |   |  |  |
|             | UnmixColors                 |   |  |  |
|             | Smooth                      |   |  | ~  |
| ØG          | IdentifyPrimaryObjects      |   |  |  |
|             | EnhanceOrSuppressFeatures   |   |  | · ·  |
|             | IdentifyPrimaryObjects      | Make each classification decision on how many measurements? | Single measurement V   | 1  |
|             | EnhanceOrSunnressEeatures   | Calact the object to be cherified                           | C.B. (Kenne Identife Secondar Objects #17)   | 2  |
| 08          | IdentifyPrimaryObjects      | Select the object to be classified                          | Cells * (Infinite Reling Secondary Objects = 12)   |  |
|             | Identify Secondary Objects  |   | Category: Children V   | ?  |
| 0.0         | MaskObjects                 | Select the measurement to classify by                       |  |  |
|             | <b>BelateObjects</b>        |   | MaskedGreenRNA_Count ~   |  |
| 05          | MaskObjects                 | Select hin spacing  | Evenly snared bins   | 2  |
|             | RelateObjects               | select on specing   | creatly appear on a  |  |
|             | MeasureObjectSizeShape      | Number of bins  | 3  | ?  |
|             | ClassifyObjects             |   |  | line of the second seco |
|             | DisplayHistogram            | Lower threshold   |  | ?  |
|             | DisplayHistogram            |   |  | 2  |
|             | ExportToSpreadsheet         | Use a bin for objects below the threshold?                  | ○Yes   | <u> </u>   |
|             |                             |   |  | -  |
|             |                             | Upper threshold   | 1.0  | ?  |
|             |                             |   |  |  |
|             |                             | Use a bin for objects above the threshold?                  | ⊖Yes ® No  | f  |
|             |                             |   |  |  |
|             |                             |   |  | ?  |
|             |                             | Give each bin a name:                                       | () Yes ● No  |  |
|             |                             |   |  | 1.00   |
|             |                             | Retain an image of the classified objects?                  | O Ves @ No   | 1  |
|             |                             |   |  |  |
|             |                             |   |  |  |
|             |                             | Colored de la biorde de la colored de                       | C # (for the state of the state of the state state   | 2  |
|             |                             | Select the object to be classified                          | Cells (from identifySecondaryObjects +12)  |  |
|             |                             |   | Category: Children V   | ?  |
|             |                             | Select the measurement to classify by                       | Manual in the second seco |  |
|             |                             |   | MaskedRNA_Count ~  |  |
|             | View output settings        | Select bin spacing  | Evenly spaced bins v   | ?  |
|             |                             |   |  | - Internet   |
| ? A         | djust modules: + - ^ v      | Number of bins  | 3  | ?  |
|             |                             | I owner three hold  | 0.0  | 2 4  |
| Start 1     | Test Mode Analyze Images    | Found 1 image sets  |  |  |

#### 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.





|                              |  | File Edit Test Data Tools Window Help |
|------------------------------|--|---------------------------------------|
|                              |  | 🕑 Images                              |
| Download pip                 | Metadata   |                                       |
|                              | NamesAnd Types   |                                       |
| https://cellpro              | https://cellprofiler.org/examples/published_pipelines  |                                       |
|                              |  | IdentifyPrimaryObjects                |
| <ul> <li>BaseSco</li> </ul>  | pe quantification.cppipe   | GentifyPrimaryObjects                 |
|                              |  | IdentifyPrimaryObjects                |
| – Multiple                   | ex RNAScope quantification.cppipe  | 🔿 🖸 Measureimageintensity             |
| •                            |  | IdentifyPrimaryObjects                |
|                              |  | GentifyPrimaryObjects                 |
|                              |  | GentifyPrimaryObjects                 |
|                              |  | GentifyPrimaryObjects                 |
| Erben L, He M-X, Laer        | remans A, Park E, Buonanno A (2017). QA Novel Ultrasensitive In Situ   | GentifySecondaryObjects               |
| Hybridization Approa         | ch to Detect Short Sequences and Splice Variants with Cellular Resolution. Mol   | C RelateObjects                       |
| Neuropiol / doi PMIC         | D: 29264769  | RelateObjects                         |
|                              |  | KelateObjects     FilterObjects       |
| [Download] (CellProfil       | ler version 2.2.0)   | G FilterObjects                       |
|                              |  | FilterObjects     FilterObjects       |
| [Download] (CellProfil       |  | G PalateObjects                       |
|                              |  | RelateObjects                         |
| CellProfiler                 | search   | RelateObjects                         |
| cell image analysis software |  | C RelateObjects     EilterObjects     |
|                              |  | FilterObjects                         |
| Download                     | Published pipelines  | FilterObjects                         |
| Help                         | CellProfiler enables reproducible research because a saved pipeline includes all the modules and   | FilterObjects                         |
| i i cip                      | settings. Here, we provide links to pipelines used in some published studies, as well as the version of  | RelateObjects                         |
| Getting started              | CellProfiler used to create them. You can instead download the most current version of CellProfiler;   | RelateObjects                         |
| Examples                     | older pipelines should automatically update to the current version when loaded.  | RelateObjects                         |
|                              | If you used CellProfiler in a publication and would like to post your pipelines here please contact us   | RelateObjects                         |
| Tutorials                    | we would be happy to host them. Please remember to give CellProfiler the proper citation in your   | MaskObjects                           |
| Forum                        | paper.   | MaskObjects     MaskObjects           |
|                              | Discussion in the transfer of the state of t |                                       |
| Blog                         | Please note that each download link contains a compressed ZIP file with the following:   |                                       |
| Custom support               | The CellProfiler pipelines.  |                                       |
|                              | Supplemental notes describing the pipelines.   |                                       |
| Educational modules          |  |                                       |
| Cite us                      | List of publications   | ч. — <u>-</u>                         |
|                              | Jones TR, Carpenter AE, Lamprecht MR, Moffat J, Silver SJ, Grenier JK, Castoreno AB, Eggert US, Root   | View output settings                  |
| About                        | DE, Golland P, Sabatini DM (2008). Scoring diverse cellular morphologies in image-based screens  |                                       |
| CellProfiler Analyst         | with iterative feedback and machine learning. PNAS 106(6):1826–1831 / doi. PMID: 19188593. PMCID:<br>PMC2634799  | ? Adjust modules: + - ^ v             |
| Download                     | [Download]   | Start Test Mode Analyze Images        |

 Table 4. Multiplex fluorescent assay CellProfiler analysis workflow

| Module                        | Input Name(s)  | Output Name(s)          |
|-------------------------------|--|-------------------------|
| Images (see Figure 22)        | <b>Note:</b> Load single images for each<br>channel. Include C1 through C4 in<br>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                 |  |
| MeasurelmageIntensity     | OrigNuclei/ NucleiArea            | N/A                       |  |
| <b>č</b> <i>i</i>         | 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                       |  |
| MegsureObjectIntensity    | OriaRed (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

| File Edit                              | Test Data Tools Window Help   | _ |  |   |
|--|---|---|--|---|
| 22220000000000000000000000000000000000 | Images<br>Metadata<br>Netadata<br>NamesAndTypes<br>Groups<br>ColorToGray<br>ColorToGray<br>ColorToGray<br>ColorToGray<br>EnhanceOSuppressFeatures<br>EnhanceOSuppressFeatures<br>IdentifyPrimaryObjects<br>IdentifyPrimaryObjects<br>IdentifyPrimaryObjects<br>IdentifyPrimaryObjects<br>IdentifyPrimaryObjects<br>IdentifyPrimaryObjects<br>IdentifyPrimaryObjects<br>IdentifyPrimaryObjects<br>IdentifyPrimaryObjects<br>IdentifyPrimaryObjects | • | To begin creating your project, use the Images module to compile a list of files and/or folders that you want to analyze. You can also specify a set of rules to include only the desired files in your selected folders.  C/LUSers/ajolly/Desktop/CellProfiler Webinar/Test images C/L | < |
| 00000000000000000000000000000000000000 | IdentifyPrimaryObjects<br>IdentifyPrimaryObjects<br>IdentifySecondaryObjects<br>RelateObjects<br>RelateObjects  |   | Filter images? No filtering \vee   | ? |

#### Figure 23. Fluorescent assay NamesAndTypes module

|      | mages                     |  |        | _ |
|------|---------------------------|--|--------|---|
| R    | Metadata                  | The NamesAndTypes module allows you to assign a meaningful name to each image by which other modules will refer to it.   |        | ~ |
| G    | NamesAndTypes             | Files are loaded as single channel RGB files. DAPI is blue and named C1, the green channel files contain C2, the red channel files contain C3, and the white channel files contain C4. |        |   |
| C    | Groups                    |  |        |   |
|      | ColorToGray               |  |        |   |
|      | ColorToGray               |  |        | ~ |
|      | ColorToGray               |  |        | _ |
| • 5  | EnhanceOrSuppressFeatures | Automatic language matching rules of   | 2      | 1 |
| • 5  | EnhanceOrSuppressFeatures | Assign a name to images matching rules in  |        |   |
| • 5  | EnhanceOrSuppressFeatures |  | ?      |   |
| • 5  | IdentifyPrimaryObjects    | Process as 30? O Yes  No   |        |   |
| • 5  | IdentifyPrimaryObjects    |  | Trans. |   |
| • 5  | IdentifyPrimaryObjects    | Match All v of the following rules   | 3      |   |
| • 5  | IdentifyPrimaryObjects    | Select the rule oriteria File V Does V Contain V C1  | +      |   |
| • 5  | MeasureImageIntensity     |  |        |   |
| • 5  | IdentifyPrimaryObjects    | Name to assign these images C1   | ?      |   |
| • 5  | IdentifyPrimaryObjects    |  |        |   |
| • 5  | IdentifyPrimaryObjects    | Select the image type Color image v  | 1      |   |
| • 5  | IdentifyPrimaryObjects    | Set intensity ranne from Image bit-depth v   | 2      |   |
| • 5  | IdentifySecondaryObjects  | Set interary range norm  | 20100  |   |
| • 5  | RelateObjects             | Duplicate this image   | ?      |   |
| • 5  | RelateObjects             |  |        |   |
| • 5  | RelateObjects             |  |        |   |
| • 5  | FilterObjects             | Match All v of the following rules   | ?      |   |
| • 5  | FilterObjects             | Select the rule criteria File V Does V Contain V C2  | · • …  |   |
| • 5  | FilterObjects             |  |        |   |
| • 5  | RelateObjects             | Name to assign these images C2   | ?      |   |
| • 5  | RelateObjects             |  |        |   |
| • 5  | RelateObjects             | Select the image type Color image V  | ?      |   |
| • 5  | FilterObjects             | Cat interaction on the Image bit death   | 2      |   |
| • 5  | FilterObjects             | Set intensity range from intege on weight  |        |   |
| • 5  | FilterObjects             | Duplicate this image   | ?      |   |
| • 5  | FilterObjects             |  |        |   |
| • 5  | RelateObjects             | Remove this image  | 7      |   |
| • 5  | RelateObjects             |  |        |   |
| • 5  | RelateObjects             |  |        |   |
| • 5  | RelateObjects             | Match All v or the ronowing rules  | 1      |   |
| @ [4 | MaskOhiects *             | File v Does v Contain v C3   | · · ·  |   |
|      | View output settings      |  |        |   |
| ? A  | djust modules: + - ^ V    | Update   |        |   |

Figure 24. Fluorescent assay ColorToGray module



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