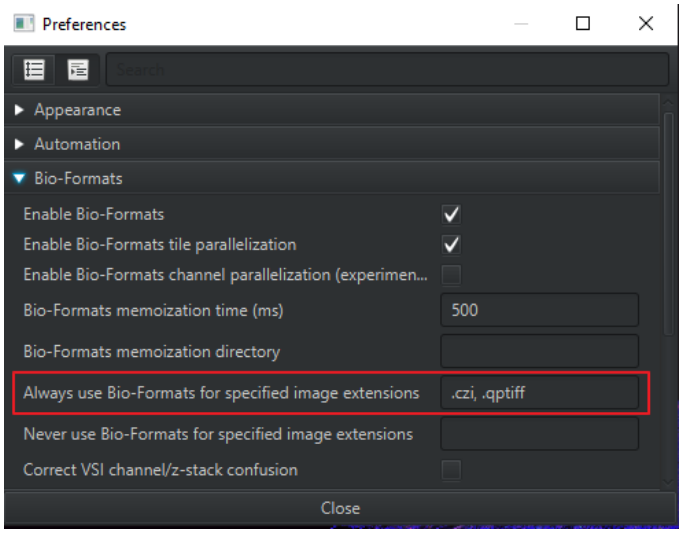
Using QuPath to analyze RNAscope™, BaseScope™ and miRNAscope™ images

Introduction

QuPath, short for Quantitative Pathology, is a free open source bioimage analysis software for whole slide images developed by Bankhead et al. at the University of Edinburg. This guide introduces the basic workflow for quantifying images from chromogenic RNAscope™ assays (Cat. No. 322300, 322350, 322430, 322100, 322150, 322440, 323200, 323250, and 323300), fluorescent RNAscope™ assays (Cat. No. 320850, 323100, 322800, and 324100), chromogenic BaseScope™ assays (Cat. No. 323900, 323600, 323700, and 323800), and chromogenic miRNAscope™ assays (Cat. No. 324500 and 324600) using QuPath.

Installing the software

1. Use the following link to download the latest, stable version of the QuPath software: **https://qupath.github.io/**. The latest version is v0.2.3 as of this writing.
2. For versions 0.2.0 and earlier, install the QuPath Bio-Formats extension **https://github.com/qupath/qupath-bioformats-extension** to enable opening of additional file formats, such as .qptiff from Polaris and .czi from Zeiss.
3. Under **Preferences,** adjust the following:



Notes:

- For quantification, you should use at least 40X magnification to acquire all RNAscope™, BaseScope™, and miRNAscope™ images or whole slide scans. Use uncompressed images for analysis.

- Some of the modules used in these instructions are still experimental, such as the subcellular detection module. This technical note provides a general workflow for the most recent stable release, as well as workarounds to analyze ISH images using current modules. As the software develops, these modules will be updated.

- We recommend viewing QuPath's documentation and introductory video tutorials (**https://www.youtube.com/c/QuPath/playlists**) to familiarize yourself with the software.

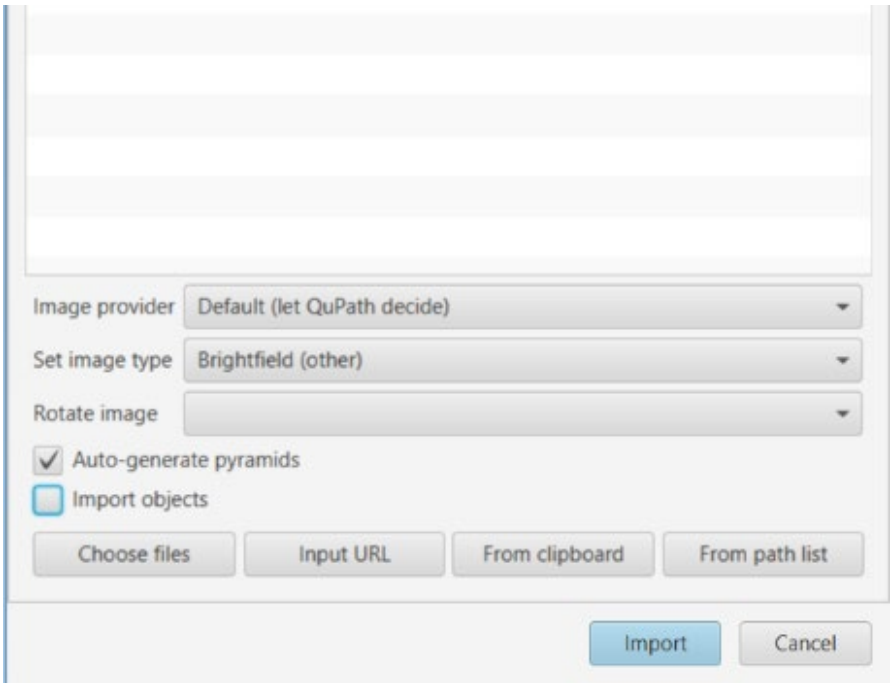
Chromogenic RNAscope™ and BaseScope™ image analysis

Singleplex chromogenic images

The following steps describe a general workflow for analyzing singleplex chromogenic RNAscope™ or BaseScope™ images. We recommend creating a project for your images before you begin the analysis so you can save the analysis settings and annotations for all the images in the project to the project folder.

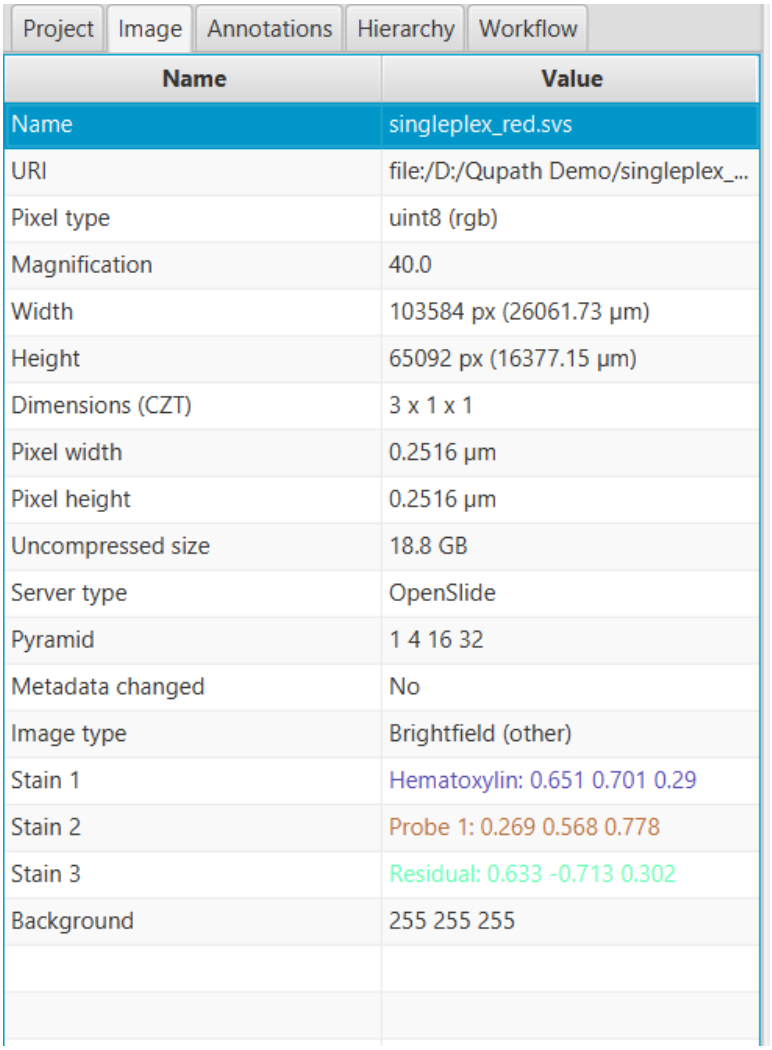
Image tab changes

1. To create a project, go to **File** → **project** → **create new project** and select a folder to save this project.
2. Import your images using **File** → **open** or by dragging the files onto QuPath's main window. You can see the list of your images under the project tab.
3. Set the image type to Brightfield (other) as shown in the following figure.



4. Double click an image in your list to open it in the viewer.
5. To change the stain name based on your probe, go to the **Image** tab.

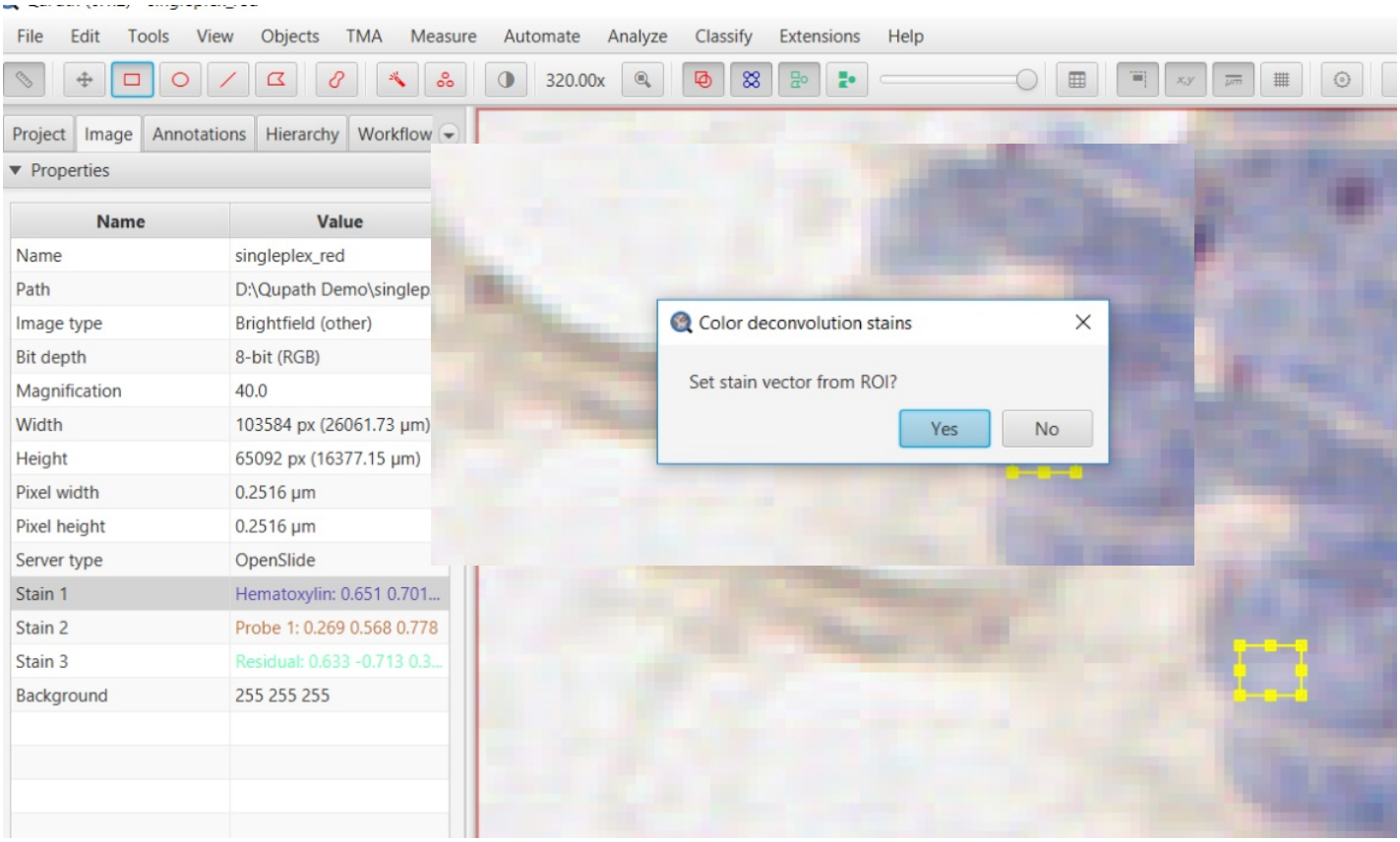
6. Find the stain name field in the list of image properties, and double-click the field to change the name. See the following figure for an example.



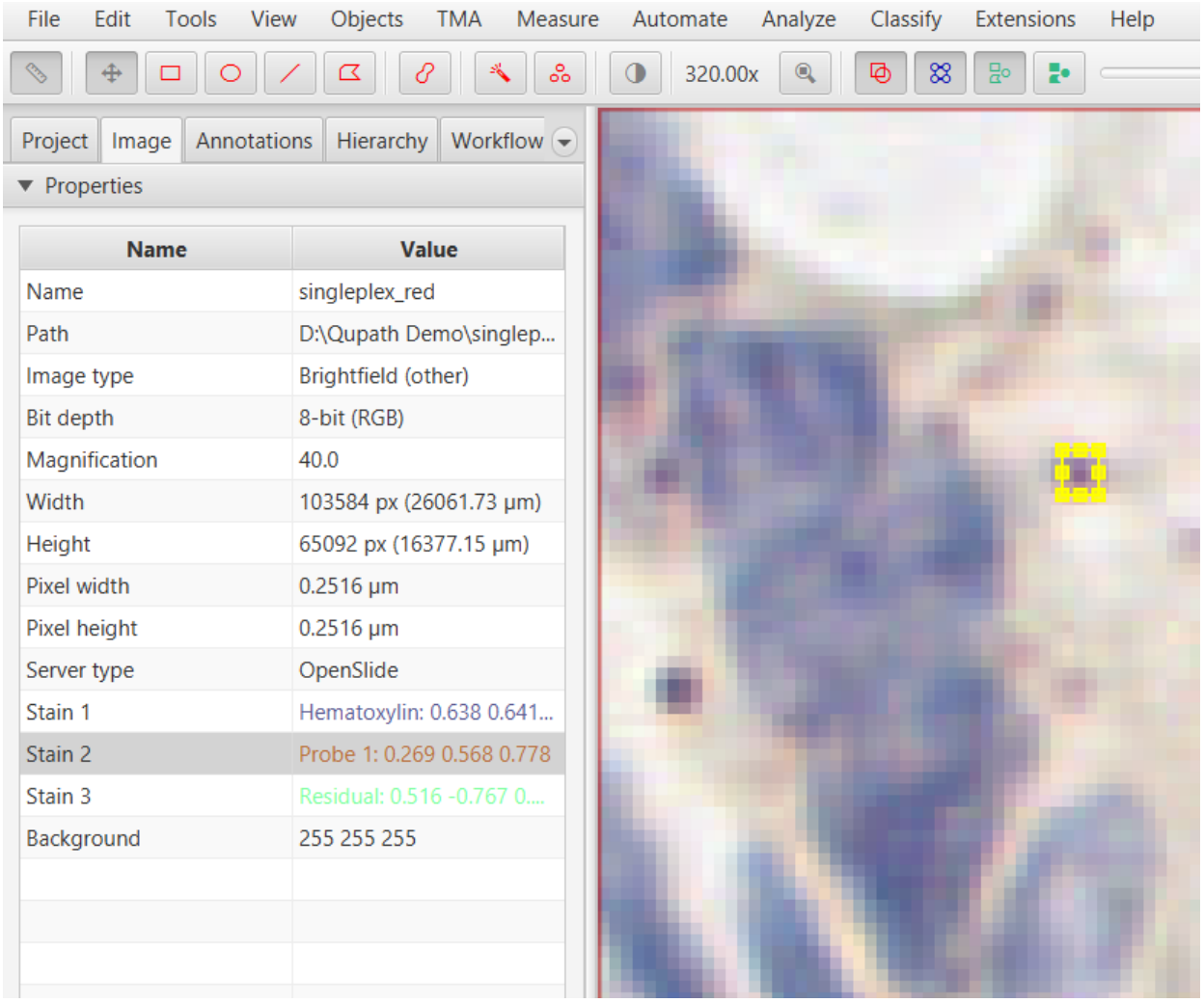
Stain vector selection

To analyze the image, perform a color deconvolution to separate the nuclei and the probe stain, then use the results to segment the nuclei and probe. Carefully select stain values as this step directly impacts the segmentation step.

1. To select the stains, use the **Rectangle** annotation tool to select an example Hematoxylin stain in your image. Make sure the chosen area does not contain any probe signal.
2. To set the average color of the selected area to the "Stain 1" color vector, double click on the **Stain 1** field, and click **OK** to confirm.

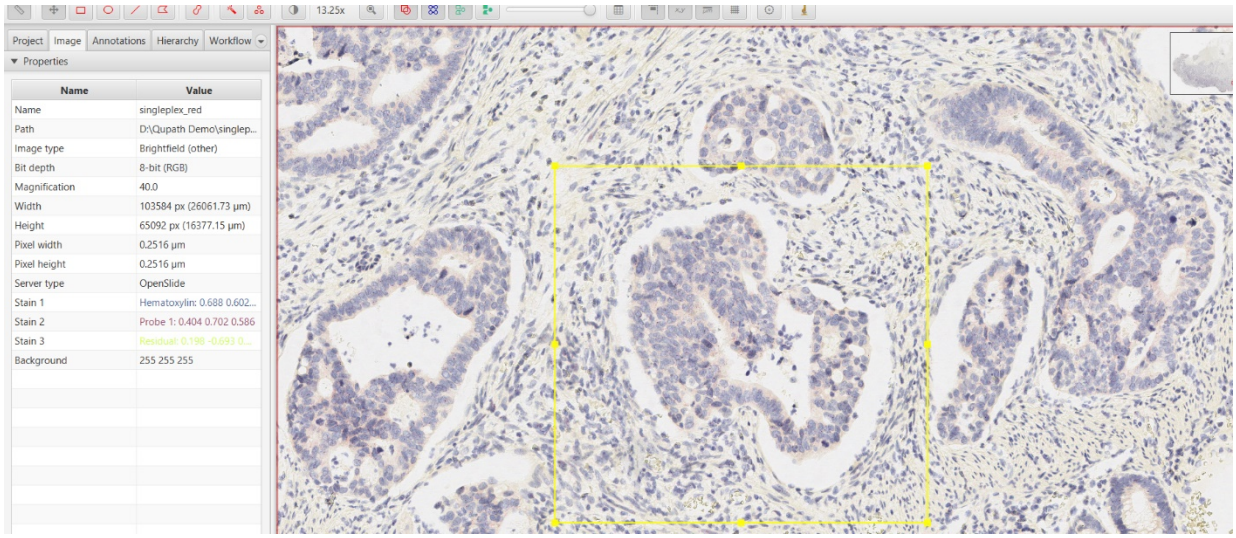


3. Repeat steps 1 and 2 to select the probe stain. Find an area where the hematoxylin signal does not blend with the probe stain.



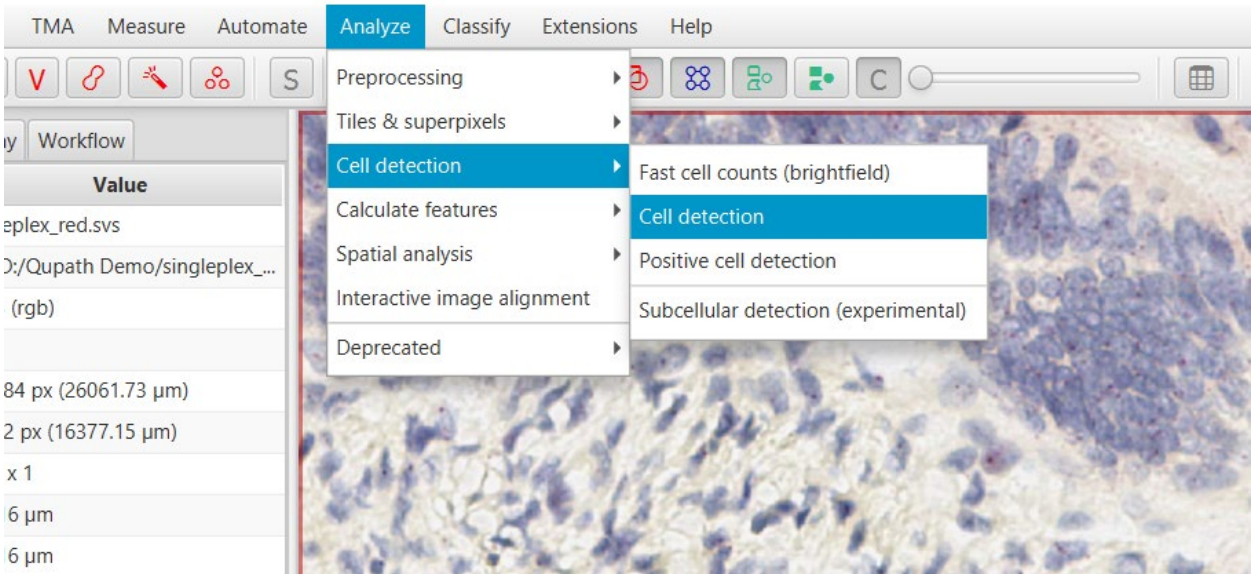
Note: You can also add stain values manually if they were previously recorded for similar images. Double-click the stain field and enter the values.

4. To perform the next steps, QuPath requires you to select a region using any of its annotation tools (see the following figure as an example). To analyze the whole slide, you must annotate the whole tissue.

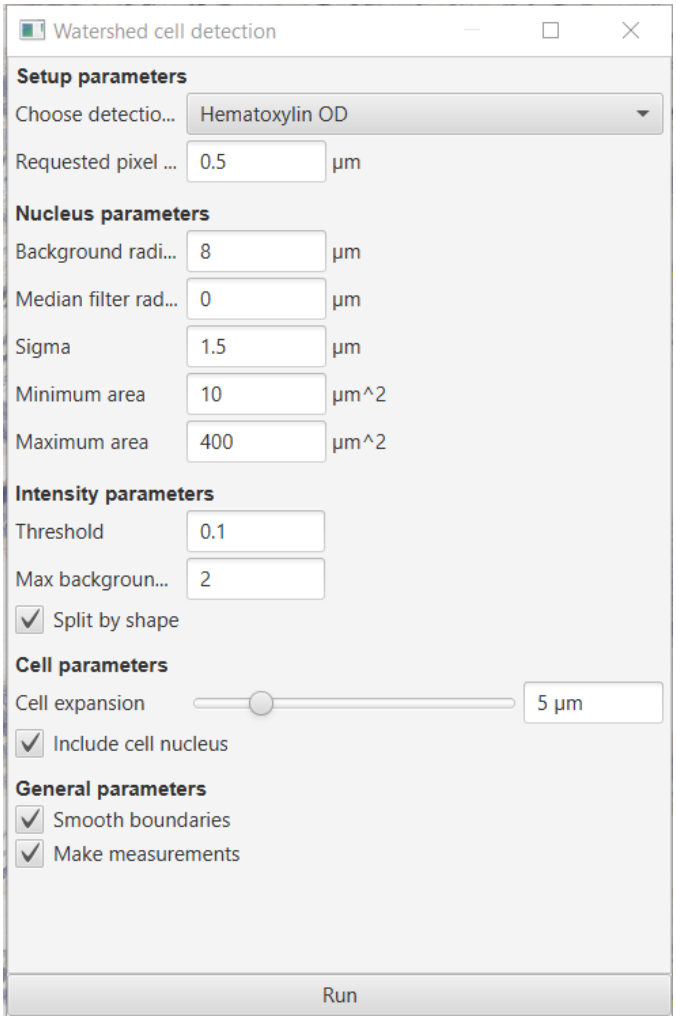


Cell segmentation

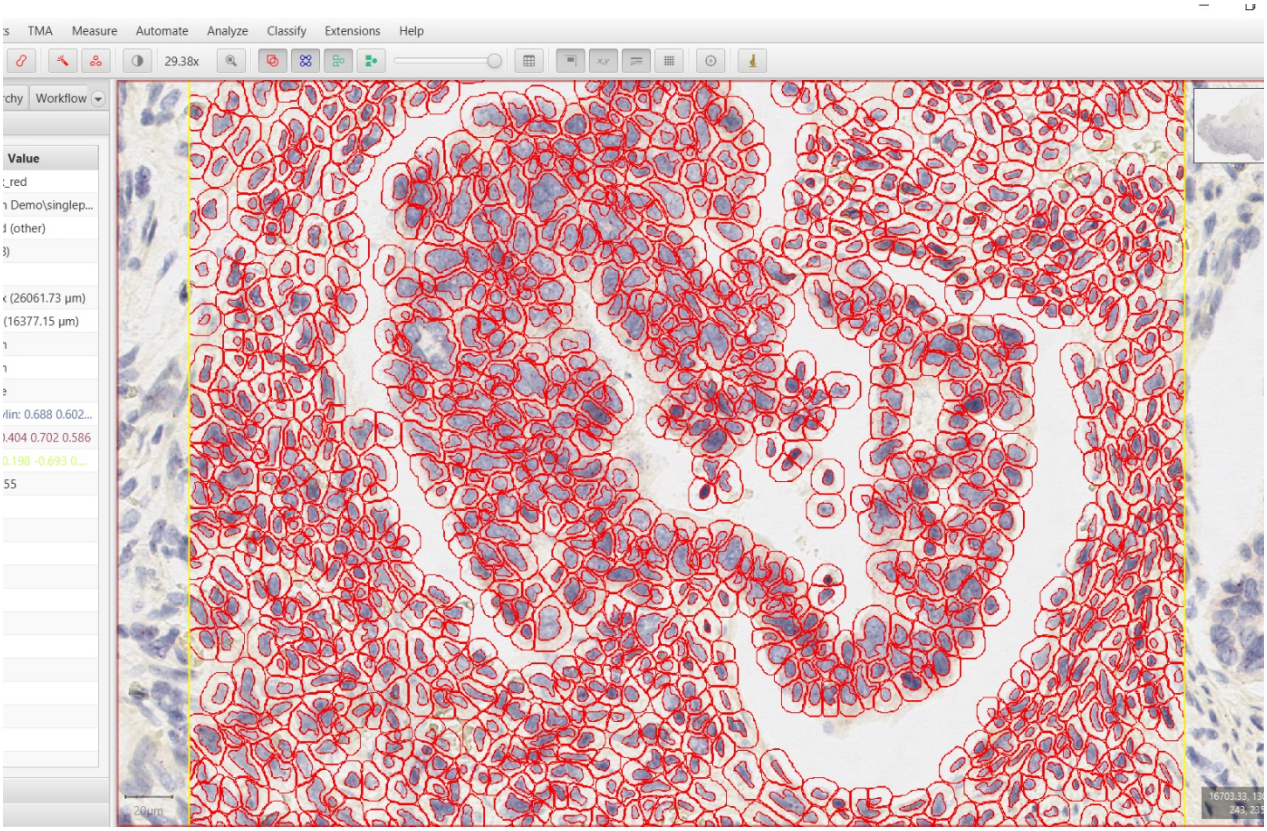
1. To perform cell segmentation, go to **Analyze** → **Cell detection** → **Cell detection**.



2. Select the default settings as shown in the following figure.



3. Visually inspect the results of the segmentation



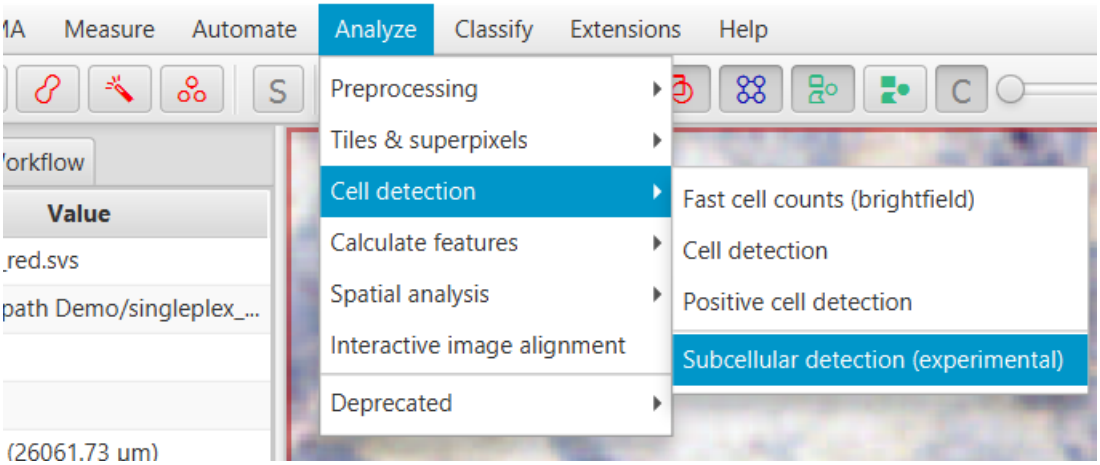
4. To improve results, do the following:

   - To remove noise, select a median filter option.
   - To capture more faintly stained nuclei, increase the background radius parameter.
   - To adjust the resolution at which the images are being processed, change the requested pixel size parameter to match the native image resolution.

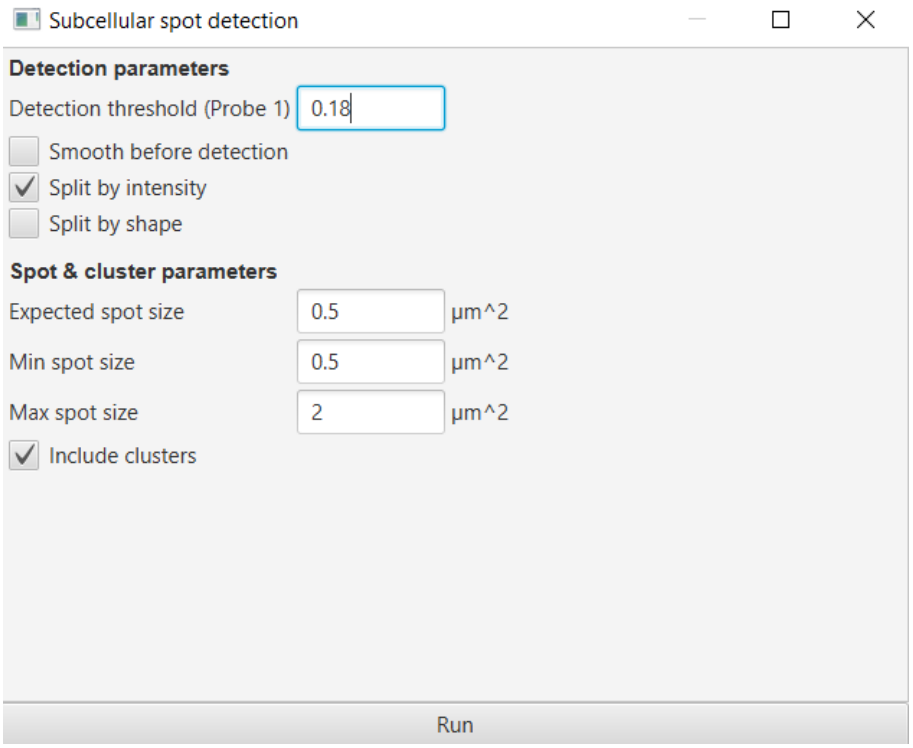
   A detailed explanation of these parameters can be found in this video.

Probe detection

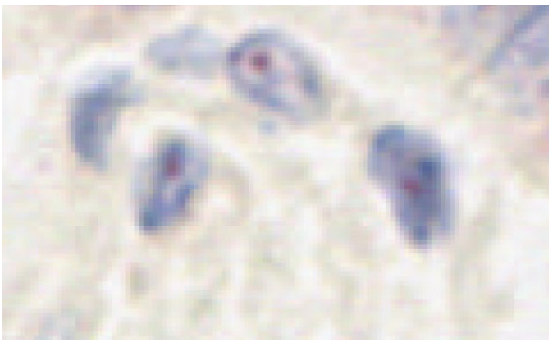
1. For probe detection, go to **Analyze → Cell detection → Subcellular detection (experimental)**.

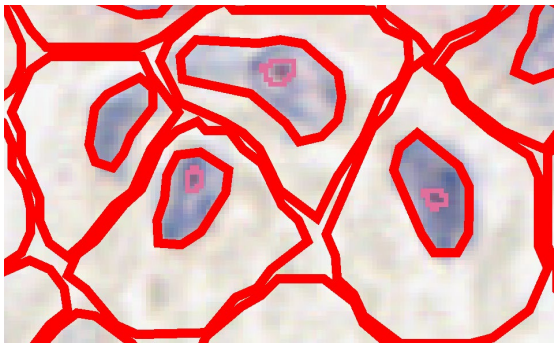


2. Adjust the detection threshold interactively. Start with one value and inspect the results; then, adjust until all the probe dots are detected. See the following for example parameters.



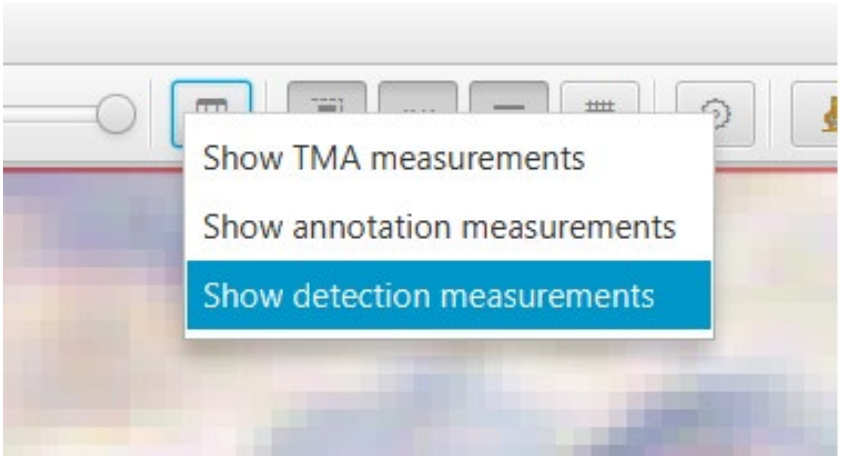
A zoomed example of probe detection (pink circle) is shown in the following images.



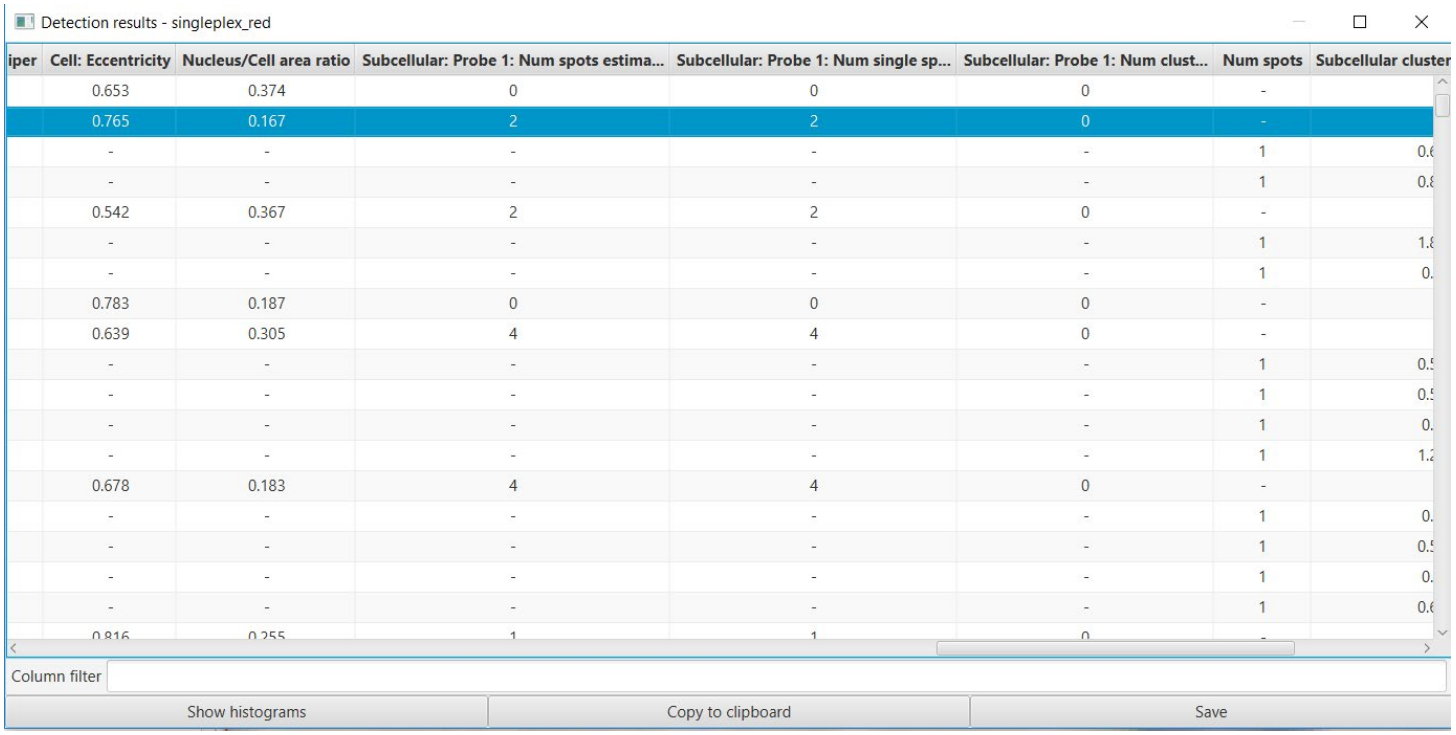


QC probe detection results

1. During the process of optimizing the probe detection parameters, inspect the results of the detection by using **Show detection measurements**.



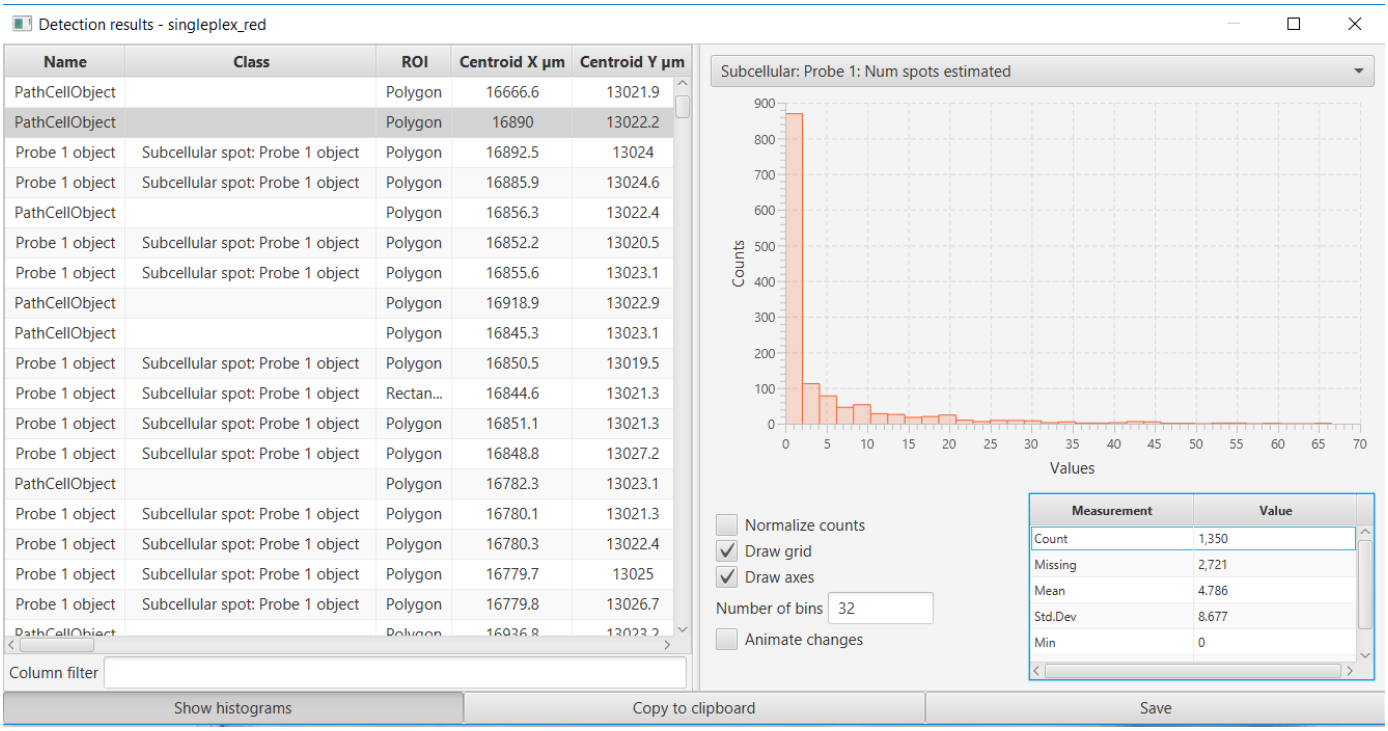
A table of cell by cell measurements results, including the number of probe copies detected within each cell. For an example, see the following highlighted measurement for a cell with two probe copies.



2. Double-click on a measurement in the table to highlight the linked cell in the image.
3. Compare the number of detected probes in the table to the number of copies seen visually in the cell and change the parameters until they match.

Measurements

The cell measurement table allows you to view the measurements as histograms for the whole region (see the following example).



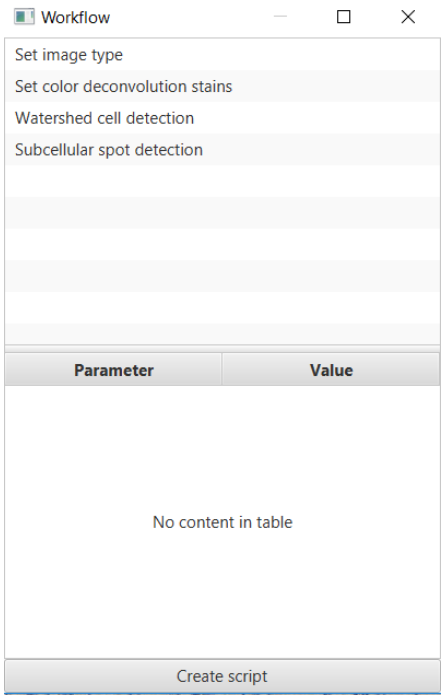
You can export the results by selecting the table and copying to other data analysis tools, such as an Excel spreadsheet, for further analysis.

Scripting the workflow

In QuPath, you can convert your workflow into a script. This allows you to easily apply the same workflow to other images in your project or other regions of interest within the same image.

1. To create a script, choose **Create a workflow** from the workflow tab. A window appears with a list of processing steps. QuPath records all optimization steps as separate steps in the workflow.

2. For each processing step, keep the parameter that gives the best results and delete the others. The workflow should look like the following figure.



3. Click on **Create script**, and save the results using **File → save**.
4. (Optional) To visually classify the cells based on expression levels, add **setCellIntensityClassifications("Subcellular: Probe 1: Num spots estimated", 1, 4, 10)** to the end of the script. This will classify the cell into three colors/classes. The numbers represent expression levels of 1+, 4+ and 10+ probe copies per cell
5. Apply the script to other regions using **Automate → show script editor → Run**.

Duplex chromogenic images

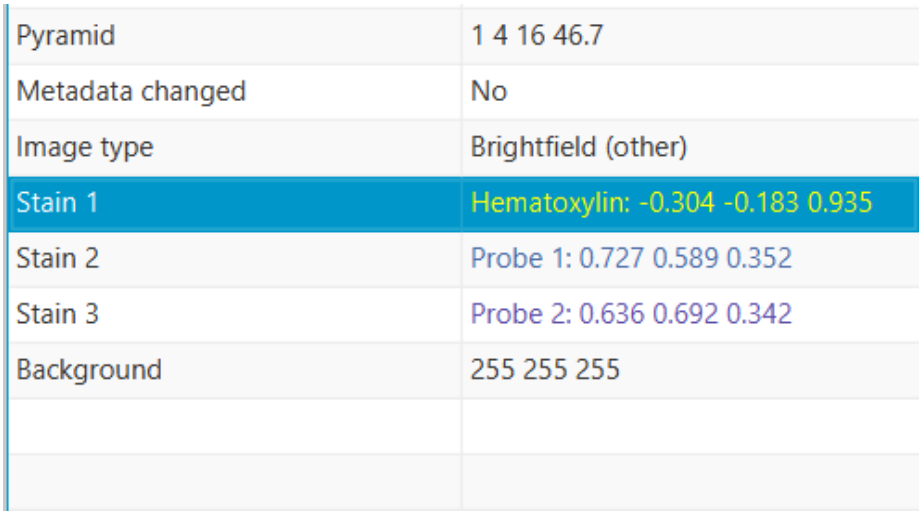
The workflow for analyzing duplex images follows the same basic steps for analyzing singleplex chromogenic images. The changes that need to be considered at each step are explained.

Stain vector selection

1. After selecting the stain vector for the first probe, follow the same steps to select a stain vector for the second probe.

   For duplex analysis, focus on finding the optimal separation between the two probes. You can ignore the nuclear stain vector selection or assign it to a color that is completely different from the colors assigned to the probe stains, as long as separation of the two probes have been optimized.

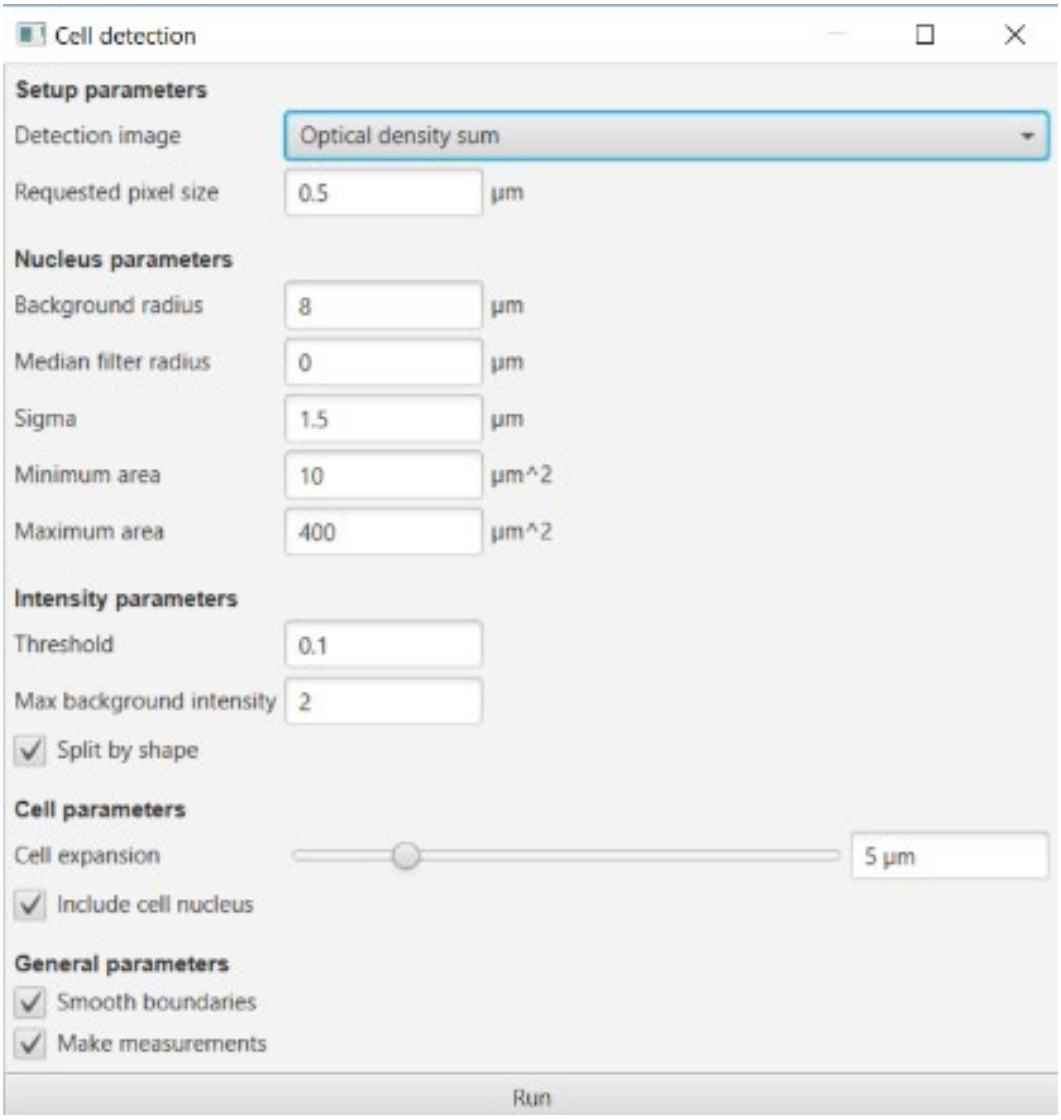
Note: This is an optional choice. However, QuPath will be more efficient at separating the probes from each other if the nuclear stain vector is completely different from the two probes.



Pyramid	1 4 16 46.7
Metadata changed	No
Image type	Brightfield (other)
Stain 1	Hematoxylin: -0.304 -0.183 0.935
Stain 2	Probe 1: 0.727 0.589 0.352
Stain 3	Probe 2: 0.636 0.692 0.342
Background	255 255 255

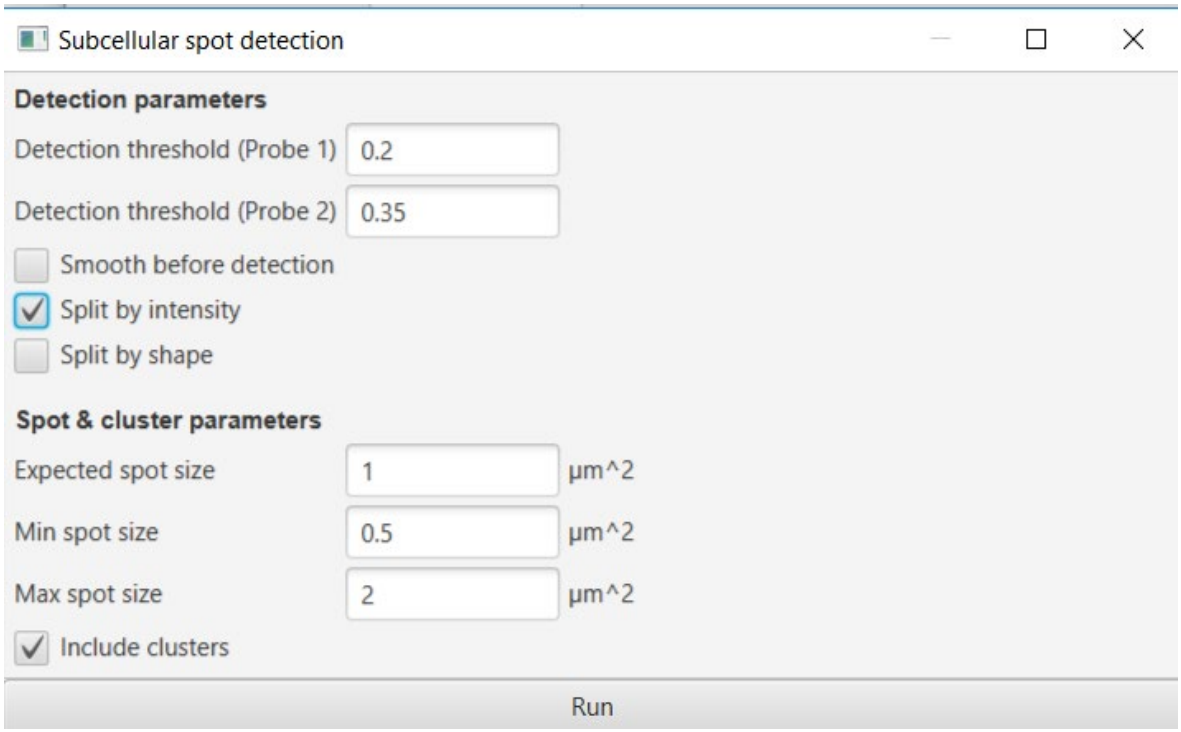
Cell segmentation

1. Use **Optical density sum** to segment the nuclei. You can adjust other parameters to optimize the segmentation as previously described for singleplex chromogenic images and as shown in the following figure:



Probe detection

1. If needed, adjust the threshold parameter for the second probe in the subcellular cell detection module. See ***Cell segmentation*** on page 6 to change the two values interactively until both probes are detected appropriately.

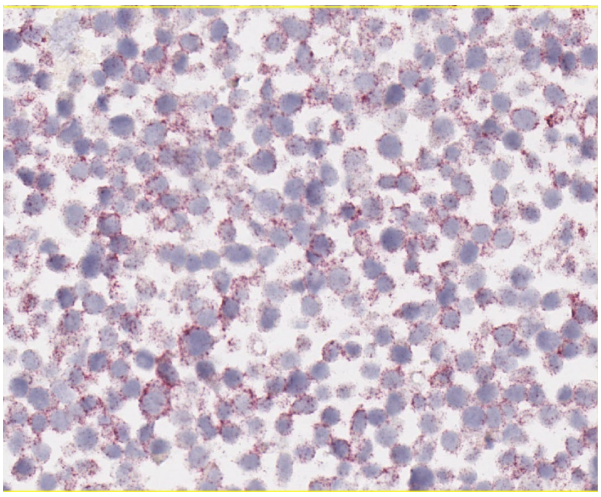


Measurements

For duplex analysis, the measurement table contains extra columns for probe 2 results.

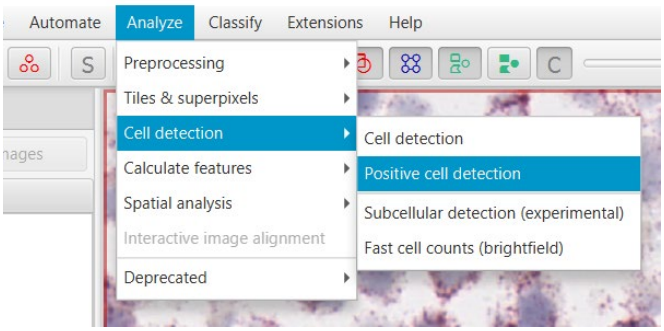
Chromogenic miRNAscope™ image analysis

Expression in the miRNAscope™ assay is typically in the form of small puncta that can form relatively big clusters (see the following image). You can use the alternative mean optical density method to relatively quantify miRNAscope™ assay samples.



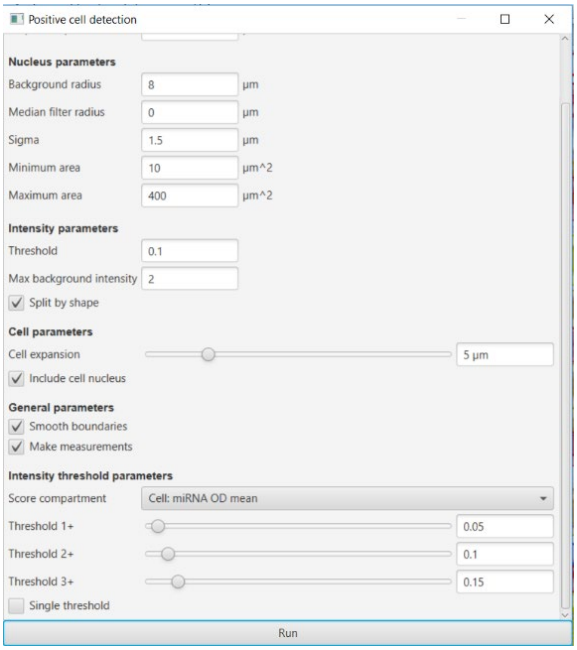
Step summary

1. Follow the color deconvolution steps used for the singleplex assay to select stains for the nuclei and probe of interest.
2. Use the positive cell detection module to show cell classification based on expression level. This module directly visualizes expressions of different levels and can also export the class of each cell based on expression level.



Note: If you only want to export the mean optical density value per cell, you can use the typical cell detection module described previously.

3. Use the positive cell detection module. This module simultaneously allows the segmentation of cells and the ability to classify the cells based on the mean optical density of the signal of interest.



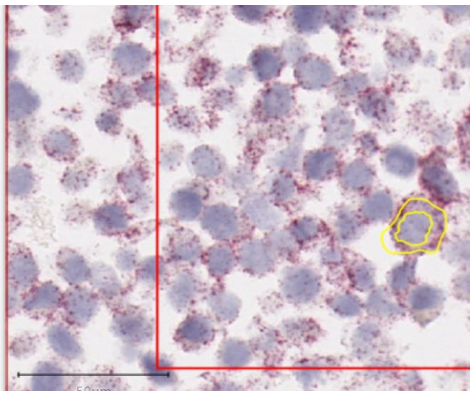
4. Adjust the cell segmentation parameters using the guidelines in the chromogenic analysis section. Within the same window, choose **Cell: probe_name OD mean** as the scoring method under **Intensity threshold parameters**. Probe name is miRNA in the preceding example.
5. The module allows you to set three threshold values that classify cells into four classes based on expression level. The threshold values are chosen experimentally and need to be adjusted for the expression levels in your images. To relatively compare results, keep the threshold values consistent across all images within an experiment.

The values chosen in the preceding example will classify the cells into 4 classes:

- Negative if less than 0.05
- 1+ if >0.05 and <0.1
- 2+ if >0.1 and <0.15
- 3+ if >0.15

The following cell is classified in the 3+ class with a mean optical density of 0.243 (greater than a threshold of 0.15):

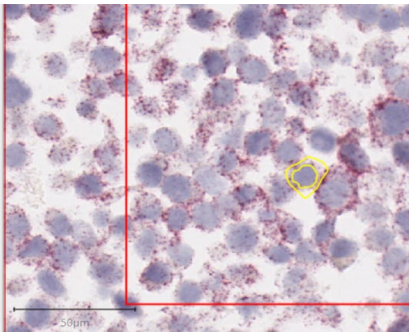
Key	Value
Image	Hela-miR21.svs
Name	3+
Class	3+
Parent	PathAnnotationObject
ROI	Polygon
Centroid X µm	2515.4269
Centroid Y µm	1816.7435
Nucleus: Area	108
Nucleus: Perimeter	40.4309
Nucleus: Circularity	0.8302
Nucleus: Max caliper	13.9362
Nucleus: Min caliper	10.503
Nucleus: Eccentricity	0.564
Nucleus: Hematoxylin OD m...	0.1399



Cell: Min caliper	18.3911
Cell: Eccentricity	0.6245
Cell: Hematoxylin OD mean	0.0142
Cell: Hematoxylin OD std dev	0.1405
Cell: Hematoxylin OD max	0.3306
Cell: Hematoxylin OD min	-0.638
Cell: miRNA OD mean	0.243
Cell: miRNA OD std dev	0.2023
Cell: miRNA OD max	1.3058
Cell: miRNA OD min	-0.0263
Cytoplasm: Hematoxylin OD ...	-0.0529
Cytoplasm: Hematoxylin OD ...	0.1258

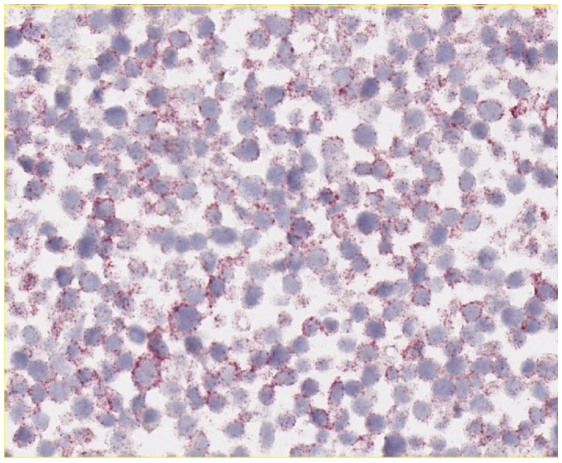
The following cell is classified in the 2+ class with a mean optical density of 0.148 (greater than a threshold of 0.1 and less than a threshold of 0.15):

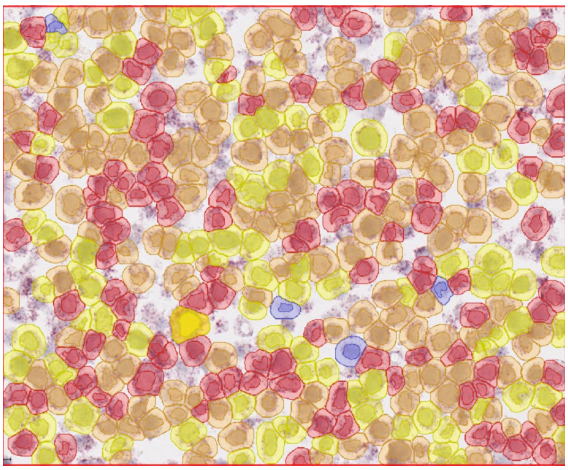
Key	Value
Image	Hela-miR21.svs
Name	2+
Class	2+
Parent	PathAnnotationObject
ROI	Polygon
Centroid X µm	2503.6001
Centroid Y µm	1808.5943
Nucleus: Area	78
Nucleus: Perimeter	33.1644
Nucleus: Circularity	0.8912
Nucleus: Max caliper	10.881
Nucleus: Min caliper	9.8292
Nucleus: Eccentricity	0.2793
Nucleus: Hematoxylin OD m...	0.2212



Cell: Max caliper	18.7821
Cell: Min caliper	14.8492
Cell: Eccentricity	0.546
Cell: Hematoxylin OD mean	0.0615
Cell: Hematoxylin OD std dev	0.1602
Cell: Hematoxylin OD max	0.5156
Cell: Hematoxylin OD min	-0.4846
Cell: miRNA OD mean	0.1484
Cell: miRNA OD std dev	0.2023
Cell: miRNA OD max	0.8602
Cell: miRNA OD min	-0.0529
Cytoplasm: Hematoxylin OD ...	-0.0413
Cytoplasm: Hematoxylin OD ...	0.1135
Cytoplasm: Hematoxylin OD ...	0.5156

5. Run the positive cell detection module and try different threshold values. The following figures display the output for the threshold values previously described. Cells within the same class display the same color.





6. Use the detection measurement table to export the results as *probe* mean OD per cell. These results can be plotted as histograms and compared across samples. You can also export the class of each cell, allowing you to count the cells within each class. The results can be compared across images based on the percentage of cells within each class.

Fluorescent RNAscope™ image analysis

Before analyzing your image, ensure that the staining is within the linear range based on exposure time (for example, signal should not be oversaturated). To compare staining intensity between samples, we recommend using the same exposure time on all slides.

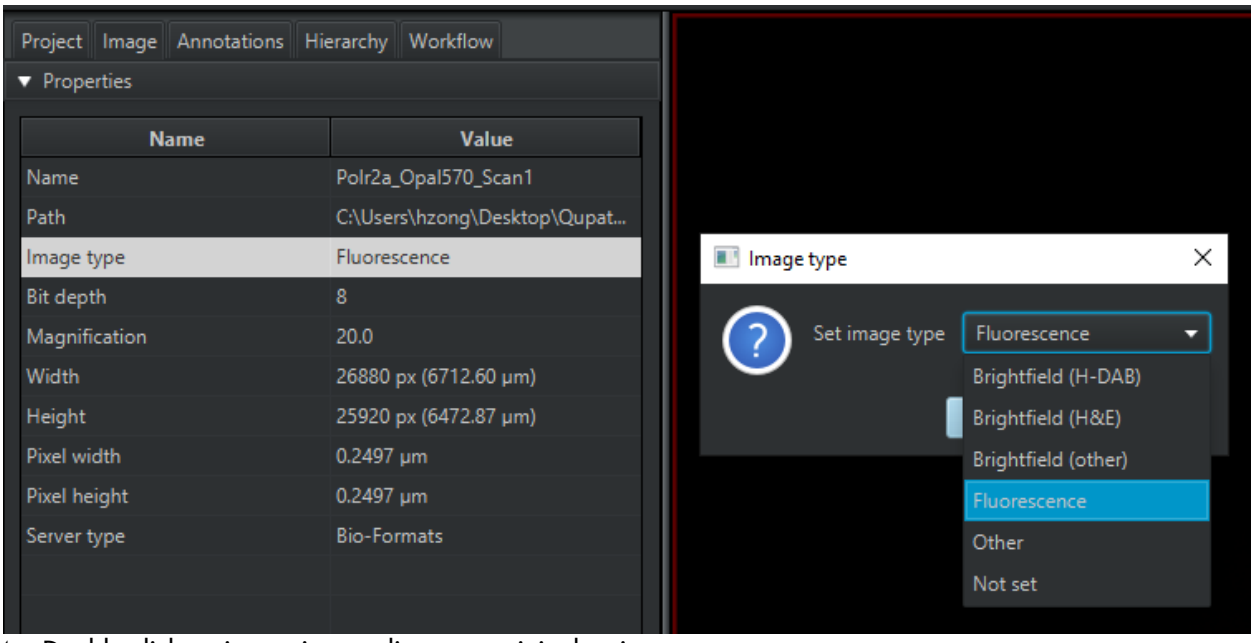
Note: Maximum projection images may work better than single plane images for dot detection.

The following steps describe a general workflow to analyze fluorescent RNAscope™ images. We recommend creating a project for your images before you begin the analysis so you can save the analysis settings and annotations for all the images in the project to the project folder.

Create a project and import images

1. To create a project, go to **File → project → create new project** and select a folder to save this project.
2. Import your images using **File → open** or by dragging the files onto QuPath's main window. You can see the list of your images under the project tab.

3. During the import process, QuPath should automatically set the image type as **Fluorescent**. If not, double-click on the **Image type** tab, and set Image type to **Fluorescence** in the drop-down menu.

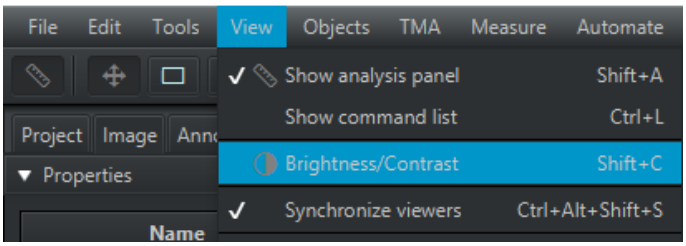


4. Double-click an image in your list to open it in the viewer.
5. Click on the **Image** tab to find a list of the image properties.

Set image display

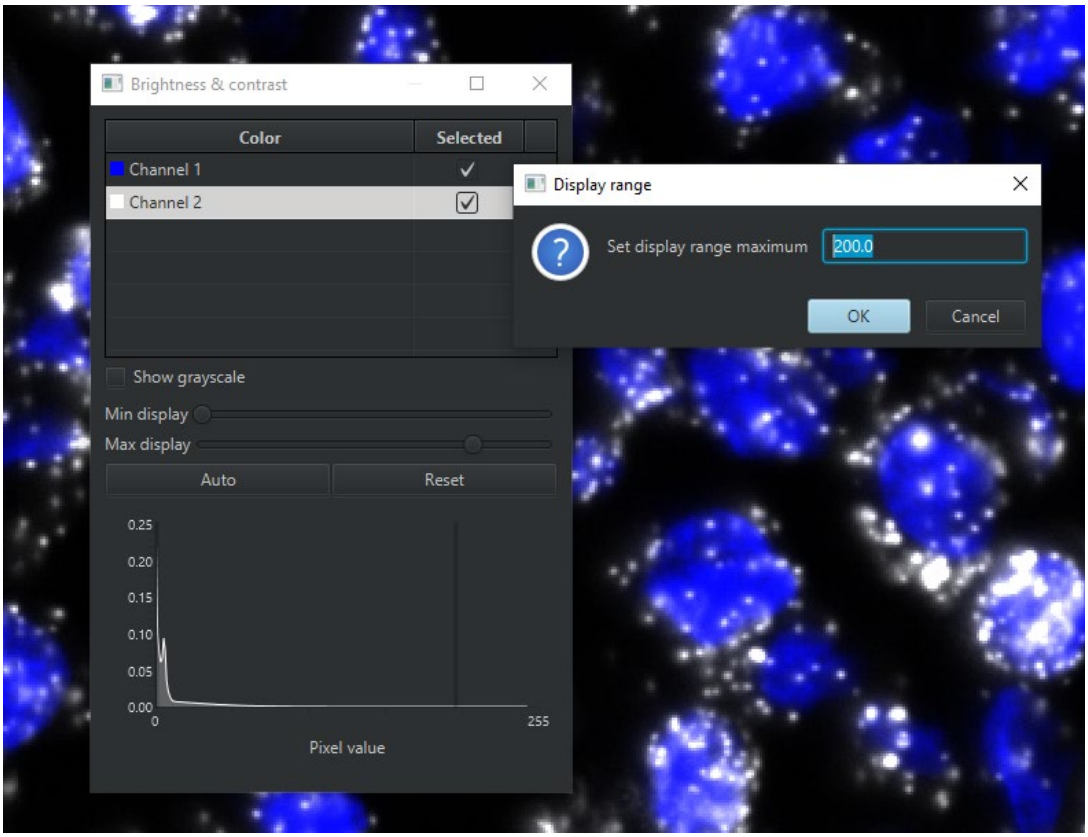
QuPath automatically sets the fluorescent image display as "auto", which can be misleading when comparing signal intensity among different images.

1. To reset the image display, go to **View → Brightness/Contrast (Shift+C)** or click on the  icon on the tab panel.

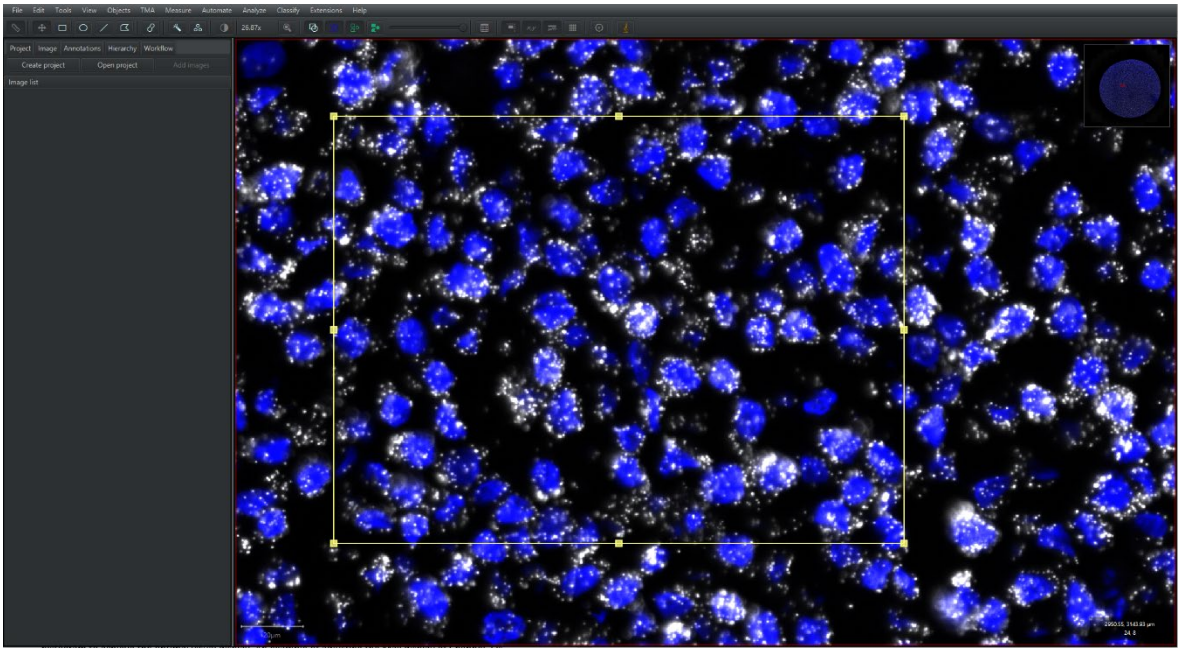


2. Highlight the channel you want to adjust by clicking on the channel.

3. To adjust the Minimum and Maximum display, double-click on either **Min display** or **Max display** to manually input a number or drag the slide bar in the histogram to achieve the optimal visual display. See the following figure for an example.

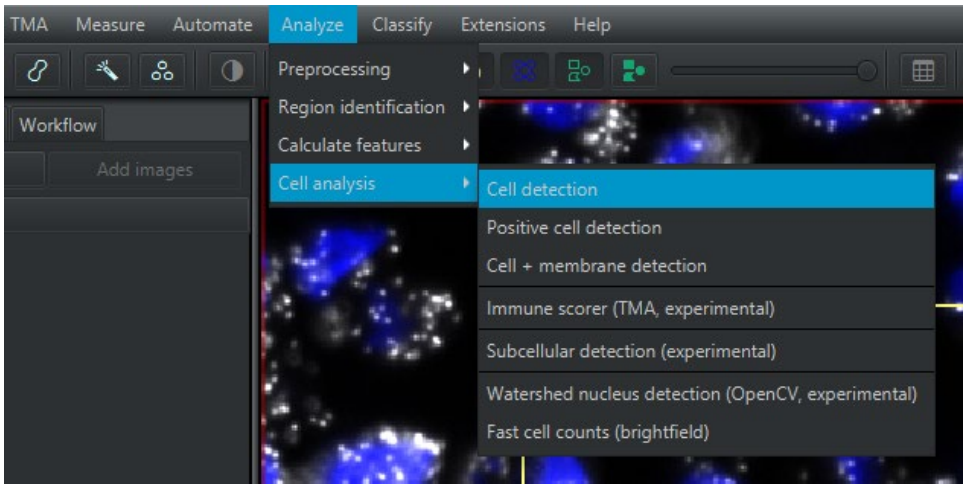


4. To perform the next steps, use a QuPath annotation tool to select a region. To analyze the whole slide, select the whole tissue.



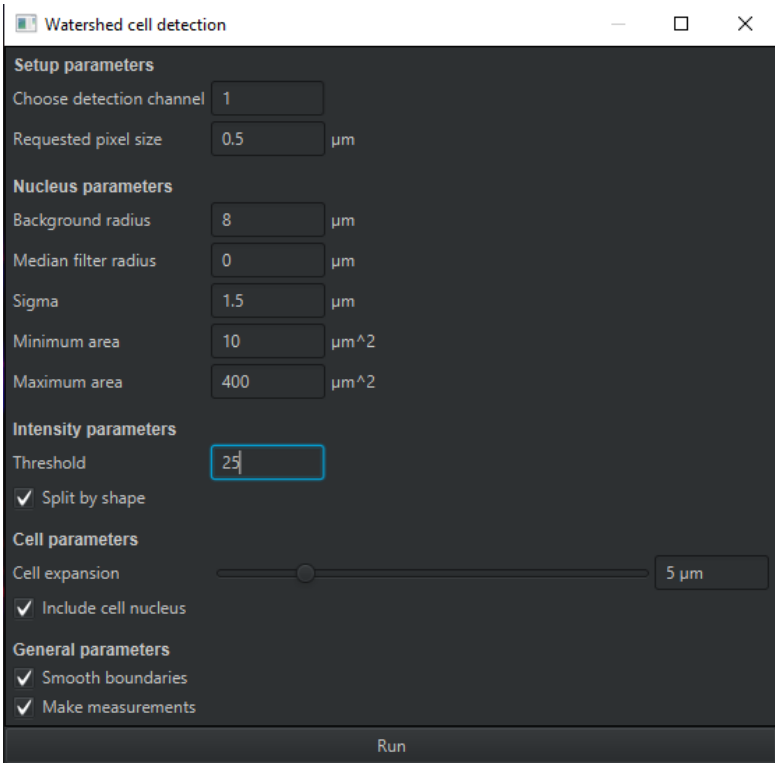
Cell segmentation

1. To perform cell segmentation, go to **Analyze → Cell detection → Cell detection**.

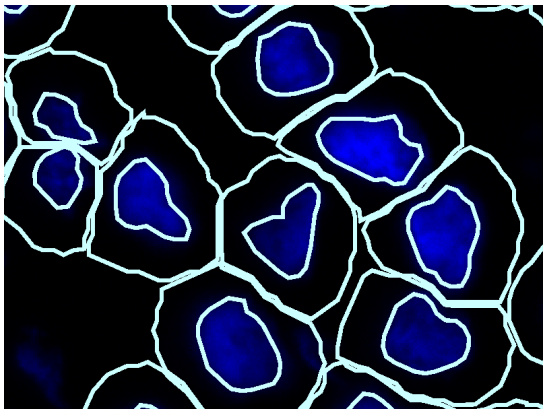


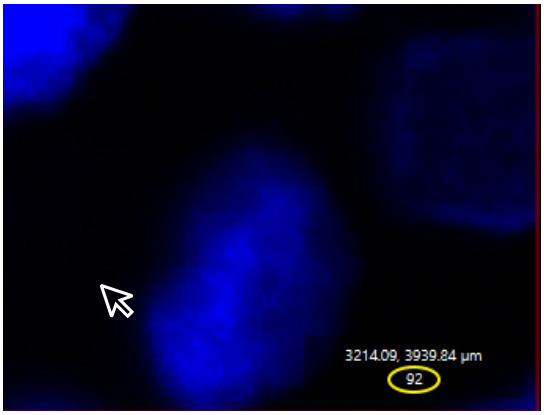
2. Adjust the threshold under **Intensity Parameters** to maximize the detection results. Watershed cell detection uses the intensity threshold to identify cell nuclei.
3. Start with the default settings as shown in the following figure.

4. Choose a detection channel for nucleus detection. In the following example, DAPI staining is in channel 1.



5. Click **Run**, and visually inspect the results of the cell detection. An example of good detection is shown in the following image.

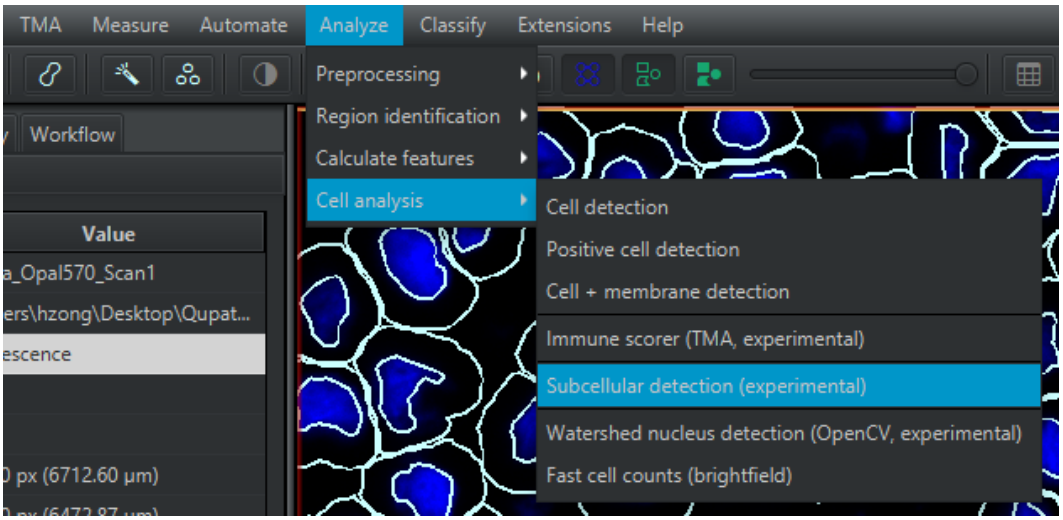




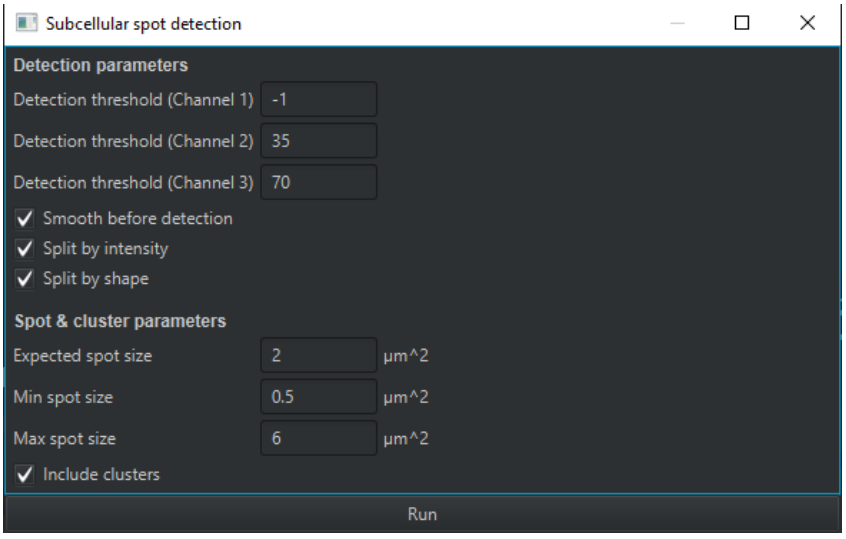
6. For suboptimal results, first adjust the **Threshold** value under **Intensity parameters**. If the value is too high, nuclei are not detected. If the value is too low, more false positives are detected.
   a. To check the intensity values in the DAPI channel, hover the cursor over the nucleus to display the pixel values on the bottom right of the viewer.
   b. Move the cursor over the nuclear boundary to obtain an estimate to use for the **Threshold** value.
7. If adjusting the Threshold value does not work, adjust other parameters as follows:
   - To remove noise, increase the median filter radius.
   - To capture more faintly stained nuclei, increase the background radius parameter.

Probe detection

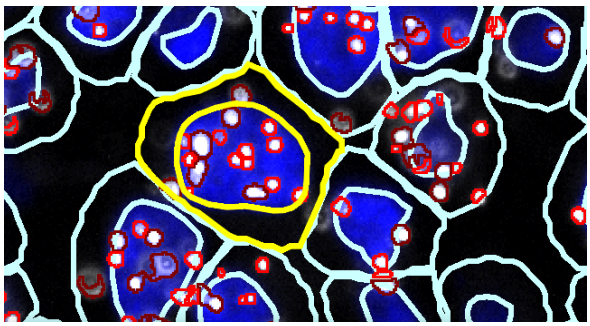
1. For probe detection, use the subcellular detection module. Go to **Analyze → Cell detection → Subcellular detection (experimental)**.



2. Adjust the **Detection threshold** of all the channels so that detection occurs interactively. In the following example, the detection thresholds of channel 2 and channel 3 are 35 and 70, respectively.
   a. Leave the **Detection threshold** of the channel(s) not to be detected, such as the DAPI channel, as **-1**.
   b. To estimate the pixel intensity, move the cursor over the dots and note the number displayed at the bottom right of the viewer.
   c. Start with one value and inspect the results, then adjust until most probe dots are detected.

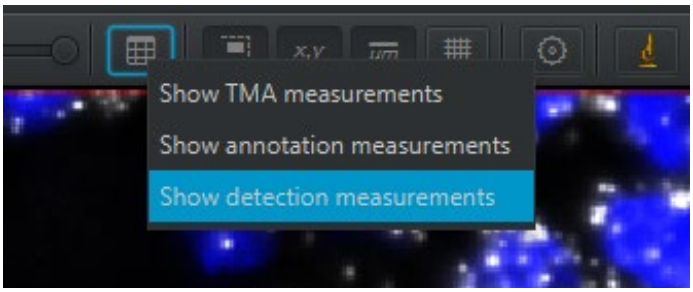


A zoomed example of probe detection (red circles) is shown in the following image. Only one channel is shown.

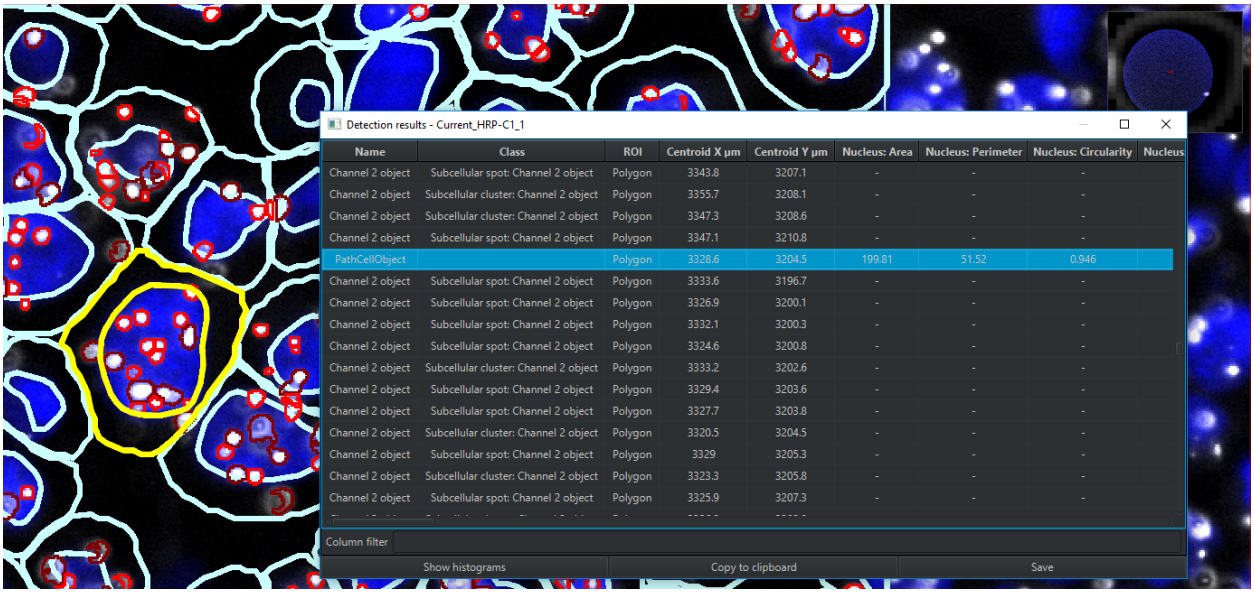


QC probe detection results

1. During the process of optimizing the probe detection parameters, you can inspect the detection results detection by reviewing the measurements using **Show detection measurements**.



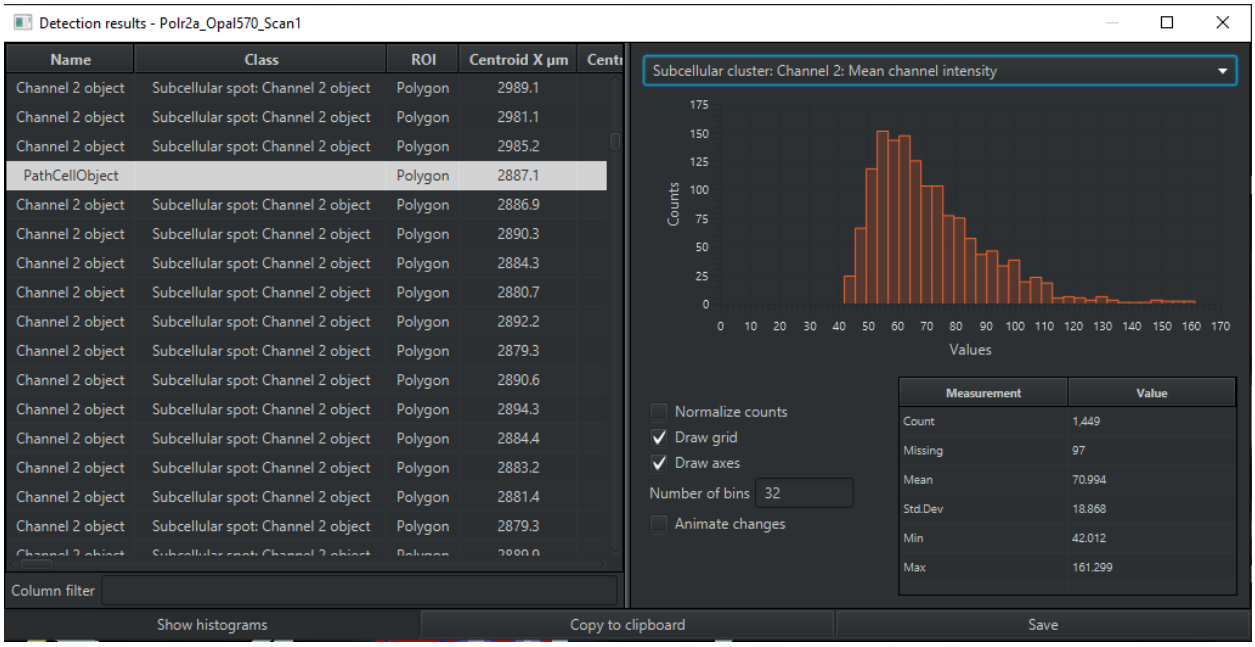
A table of cell by cell measurements results, including the number of probe copies detected within each cell. For an example, see the following highlighted measurement for a cell with 17 probe copies.



2. Double-click on a measurement in the table to highlight the linked cell in the image.
3. Compare the number of detected probes in the table to the number of copies seen visually in the cell and change the parameters until they match.

Measurements

The cell measurement table allows you to view the measurements as histograms for the whole region (see the following example). You can export the results by selecting the table and copying to other data analysis tools, such as an Excel spreadsheet, for further analysis.



To script the workflow, see **Scripting the workflow** on page 12. You can batch export annotation measurements in a project using the script here.

References

1. Bankhead, P. et al. **QuPath: Open source software for digital pathology image analysis.** *Scientific Reports* (2017). https://doi.org/10.1038/s41598-017-17204-5
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3. Qupath Youtube channel https://www.youtube.com/c/QuPath/playlists

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