

# Image Analysis Practical Course using **QuPath** **(CMU&HPU)**



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# Getting Started

## Image Analysis Workflow in QuPath

1. **Import images** into a project.
2. **Navigate & adjust** brightness, contrast, or color vectors.
3. **Annotate regions** manually or automatically.
4. **Segment cells/tissues** using cell detection or pixel classification.
5. **Analyze data** and extract results.
6. **Export images & numerical data** for reporting.

# General Information

## 1. QuPath Project

In QuPath, a **project** is essentially a container that organizes and manages your imaging data and associated analyses. It provides a structured workspace to store and process images, annotations, and results. A project can include various elements such as:

- **Images:** Digital slides or microscopy images you import into the project. It can open .lif, .czi and .tif, among other formats.
- **Annotation objects:** These are manually or automatically defined regions or structures within the images, such as tissues, cells, or specific areas of interest.
- **Detection objects:** These are automatically generated objects (normally cells) that allow for further analysis associated with them, such as cell classification, subcellular detection, spatial analysis, etc.
- **Classifications:** The categorization or segmentation of areas or objects in the image based on specific features (like tissue type, cellular phenotype, etc.).
- **Results:** Quantitative or qualitative data generated from image analysis, like cell counts, intensity measurements, or other derived data points.
- **Scripts:** You can also include custom scripts in QuPath to automate analyses or customize workflows.

Creating a project in QuPath helps to keep all the components associated with a specific research task organized and ensures that images and results can be easily tracked and

processed. When you open a project, it automatically loads the data and settings associated with that specific analysis.

## 2. Qupath Objects

One of the most important concepts in QuPath is what is called **objects**. You can think of an object informally as something in an image that QuPath can identify, classify and measure. There are **two main kinds** of objects:

- **Annotations:** Objects that you usually create yourself, by drawing on the image. They can also be automatically generated, after a pixel classification step. They can contain other objects (annotations and detections) inside.
- **Detections:** Objects that QuPath usually creates for you, e.g. by detecting cells (where each cell is an object). These can be generated with QuPath cell detection tool or with artificial intelligence extensions.

## 3. QuPath thresholds

When we detect objects or when we classify objects, we are classifying pixels or objects respectively according to a chosen threshold. This threshold is an intensity value above or below which the pixels or the objects are assigned a class (e.g. class tissue, class CD3+, etc.). As we will see during the practice, when we are detecting for example the tissue in a brightfield image, we are working with the channel Average channels, i.e. the intensity of the pixels in this channel is the average from the 3 channels a brightfield image has (RGB). In this type of image, what we want to detect is pixels less bright (pixels with a lower value) than the background. So in this scenario, we normally assign a class to pixels that are below the threshold value, and leave unclassified the pixels above that value. When we deconvolve the channels from a brightfield image and we perform cell detection in a deconvolved channel, the situation is the opposite: higher intensity (higher pixel values) means that we have our staining there, and the background is less intense (lower pixel values). So we normally assign a class to pixels (or objects) that are above the threshold value, and leave unclassified the pixels below that value. This last scenario is what always happens in a fluorescence image: we are interested in the most intense pixels (or objects), and the background should be less intense (lower pixel values).

#### 4. QuPath navigation toolbar

QuPath provides you with multiple features and buttons in its navigation bar, giving you great flexibility in carrying out your analysis.

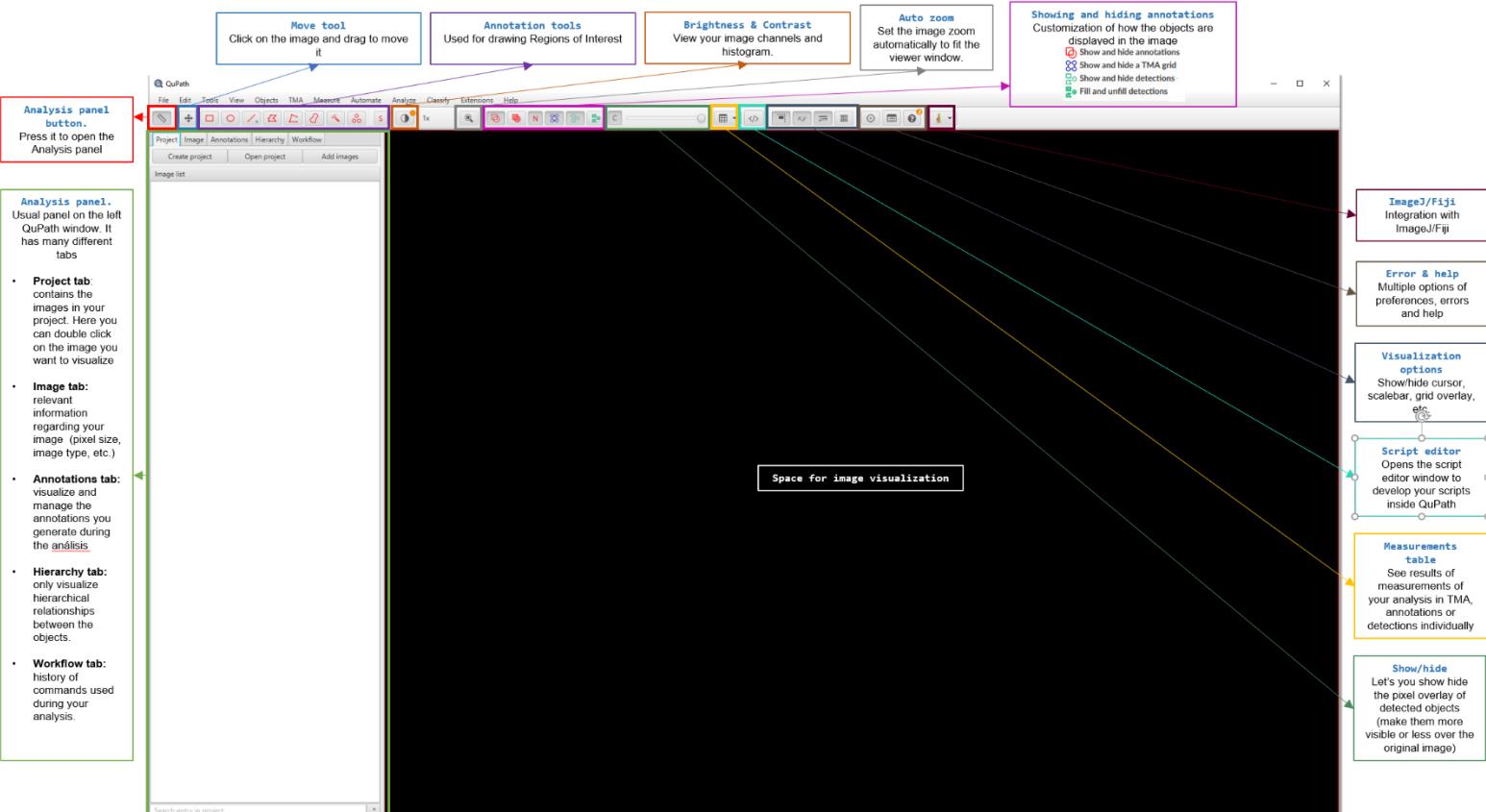


Figure 1. QuPath User Interface description.

As *Figure 1* shows, QuPath has many buttons. Among them, many customizable visualization options become really interesting, especially when your collection of objects grow on the image and it may start to become cluttered or confusing.

It is also interesting to emphasize the special importance of what *Figure 1* refers to as **Analysis panel**. This part of the QuPath's interface is where most of the relevant information is concentrated. It specifically has a series of very useful tabs for image analysis:

- **Project tab:** shows the images contained in the QuPath project. By default, the first image on the list will be automatically displayed.
- **Image tab.** Here, you will visualize relevant information regarding your image, such as **Pixel type** (bit depth) or **pixel width** and **pixel height** (pixel size), among other parameters. These parameters will be relevant for future decisions along the analysis workflow.

**TIP:** You can double-click on either ‘Pixel width’ or ‘Pixel height’ to enter a pixel size if your image is uncalibrated and you know the calibration, or if you want to change image calibration. You can also set the pixel size based on a specific region of your image. To do so, draw an annotation on your image and select it, double-click on either ‘Pixel width’ or ‘Pixel height’ under the **Image tab** to type its size in micron squared.

\*\*\*For image measurements you can also draw a line annotation (if you want to make it perfectly horizontal or vertical, hold down the Shift key) and you will find the length in micron information under **Image Tab**.

- **Annotations tab.** Here, you can visualize and manage the annotations you generate during the analysis (not detections, these are visualized from Hierarchy tab). You can create and assign classes to existing annotations from here. You can also visualize generated data belonging to annotations, such as annotation area, generated measurements inside annotations, etc.
  - **Changing Colors & Properties:** Clicking on an annotation inside the annotation list is a way to select an annotation (you can also double-click inside an annotation on the image to do so). But you can also set the properties of your annotations here if you like. One way is to select from a predefined list of classes on the right and choose **Set class**. Alternatively, right-click on any listed annotation and choose **Set properties** to choose any arbitrary name or color.

**Tip:** you can click on the three dots next to Auto-set to Add a new class, selecting that option from the list.

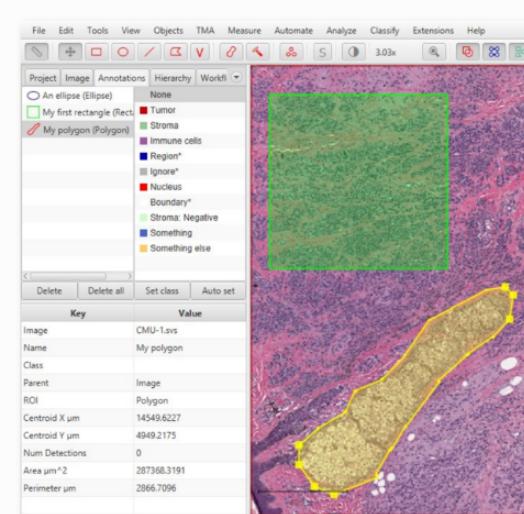
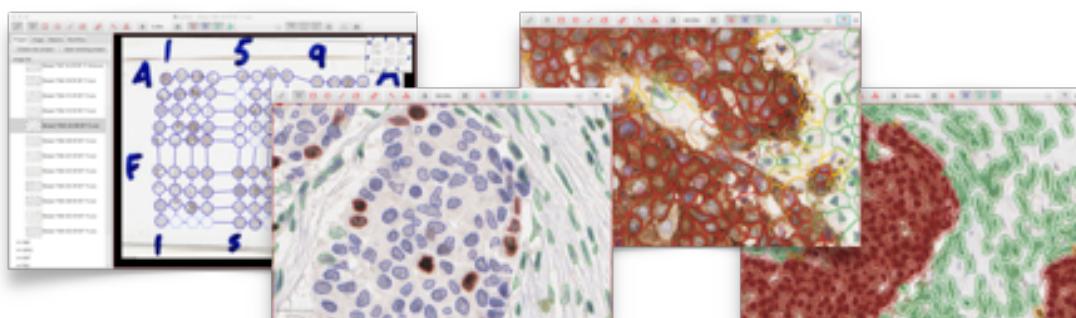


Figure 2. The Annotations tab.

- **Hierarchy tab.** You can see hierarchical relationships between the objects that are generated during your analysis. You cannot edit objects from here, it is just for visualization. You will see annotations and detections, and their hierarchy, inside this tab.
- **Workflow tab.** You will see a history of the commands you have been using during your analysis inside the actual image. This is helpful to wrap up everything you have done into a script, a necessary step to run a batch analysis for all your images. We will come later to this part.

## Part 1. Brightfield Image Analysis Pipeline



### Exercise 1. Positive and Negative Cells

- DATA ▶ Lung tissue stained with CD8 (Magenta) and CD3 (Teal)

- **GOAL ▶ T-Cell analysis (CD8+ and CD3+)**

## 1. Create a Project

To start our analysis, it is necessary to create a QuPath project (check section 1 in General Information for further details). To do so, follow these steps:

- Create an **empty folder** with the name `QuPath_project` inside `\Desktop\QuPath_course\Positive & Negative cells Analysis` to create your project location. All automatically generated files will be stored here, following QuPath architecture.

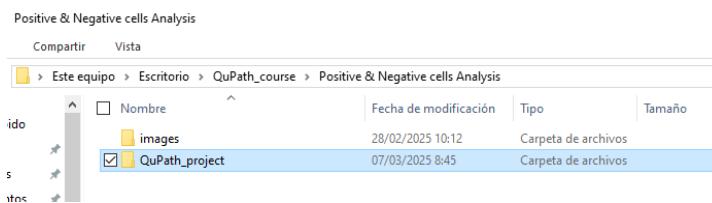


Figure 3. Empty folder creation for the QuPath project.

- Open the QuPath application
- Go to the Project tab

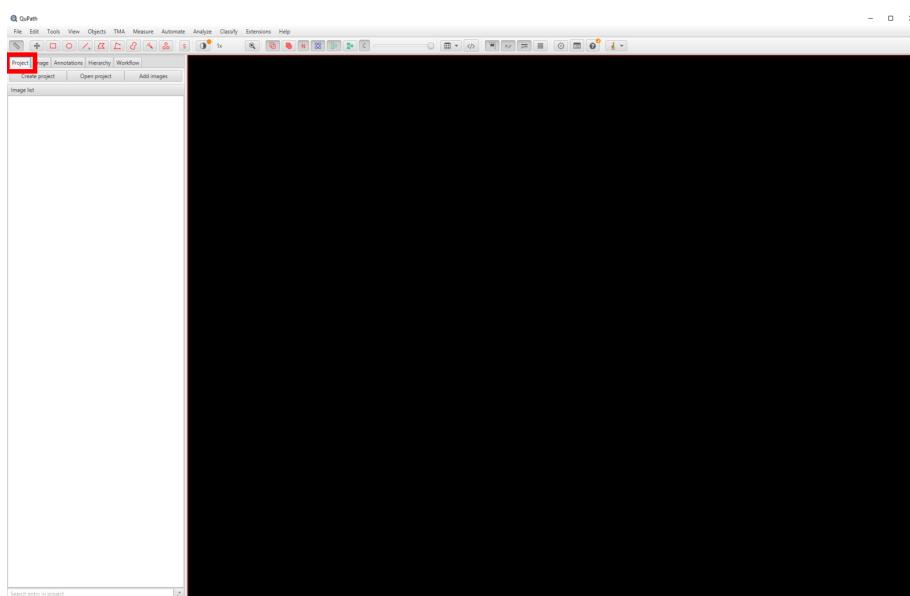


Figure 4. QuPath project tab (rounded in red)

- Click **Create a project**



Figure 5. Create project button inside the Project tab.

- When the pop-up window appears, browse and choose the empty folder that you have just created (Desktop\QuPath\_course\Positive & Negative cells Analysis\QuPath\_project) and click **Select folder**.

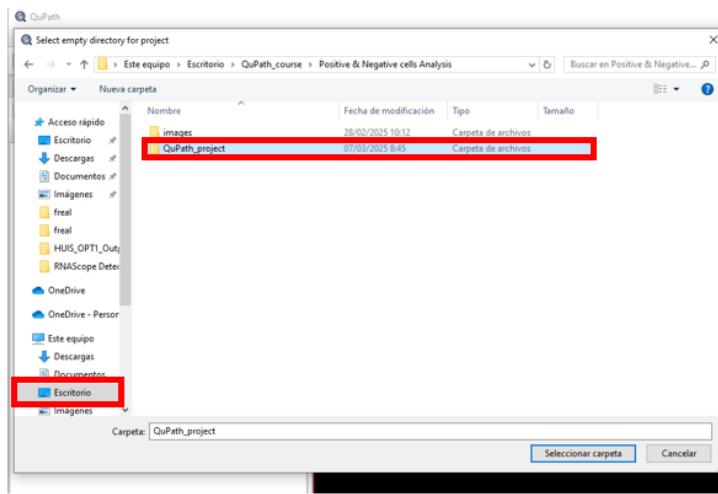


Figure 6. Selecting the empty folder to create the QuPath project.

- Congrats! Now you have your first QuPath project created:

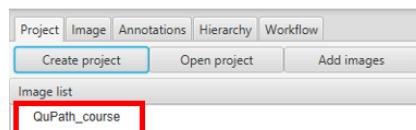


Figure 7. Created QuPath project.

**Note:** When creating a project, QuPath will automatically generate all files within your project folder. These files follow a typical QuPath architecture, needed for the software to interpret your project. Among all of them, the [project.qpproj](#) file is the most important file so QuPath can reopen your project later.

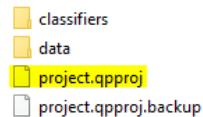


Figure 8. Typical QuPath project architecture.

## 1. Importing Images into a Project

Once the project is created, it is time to import the images we want to analyze. There are two ways to add images to a QuPath project:

### A) Using Add images

- Go to **Project** → **Add images** → **Choose files** and browse the image file (**Desktop\QuPath\_course\Positive & Negative cells Analysis\images\CD8A(M)+CD3(T)\_lung.czi**)



Figure 9. Importing images to the project using "Add images" button.

- Select the images and click **Open** → **Import**

**Note:** For Zeiss scan images (.czi), if there is more than one scene (i.e., pieces of tissue), the software will automatically divide your original image into different images corresponding to your scenes.

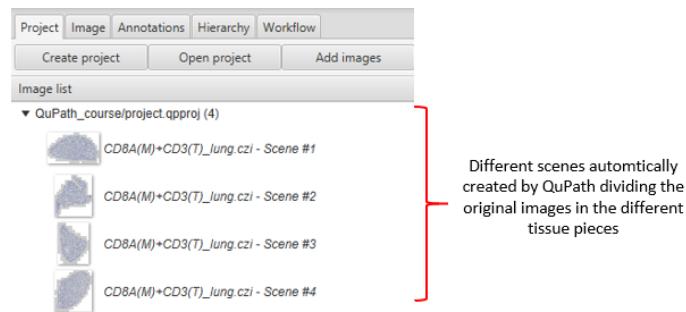


Figure 10. importing Zeiss scan images with scenes creation.

- Double click on the 4<sup>th</sup> image (**CD8A(M)+CD3(T)\_lung.czi - Scene #4**)
- Select *Brightfield Other* under **Set image type** in the prompt window and click **Apply**.

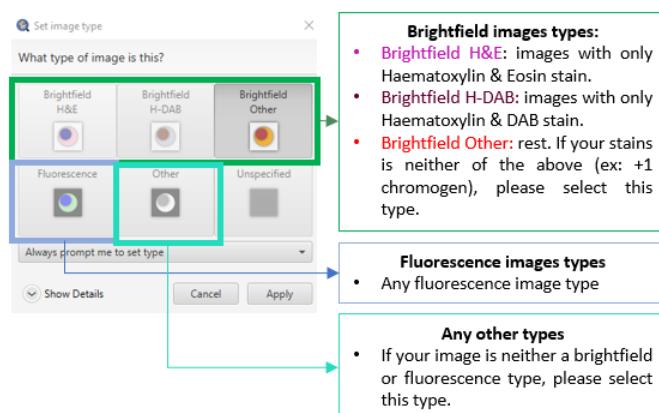


Figure 11. Image types in QuPath.

**Note:** It is **very important** to define the image type correctly as it will have an impact on further segmentation for analysis.

## B) Drag and drop

- Simply select all the files you want to import (in our case they are stored in **Desktop\QuPath\_course\Positive & Negative cells Analysis\images\CD8A(M)+CD3(T)\_lung.czi**) and drag image files into the QuPath workspace.
- Select *Brightfield Other* under **Set image type** in the prompt window and click **Apply**.
- QuPath will prompt you to confirm adding them to the project.

**Note:** In this case it is still very **important** to define the image type correctly as mentioned before.

**Tip (not necessary for this exercise):** The **images need to always be at the same location**.

QuPath does not save the images in the project folder, instead it links the project data to the image location. If you change the image location and try to open the project you would see a prompt asking you to enter the image new location (if images are not being found by the software, they will appear in red). Sometimes, QuPath will be able to find the new location, in which case, the new path will appear to the right of the Original URI; otherwise, you can simply click on **Search...** → browse the new location and click **Open** → **Check** that all errors are solved (images are found again) → click on **Apply changes**.

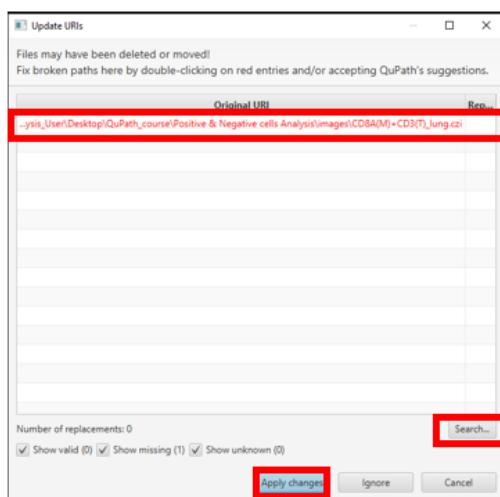


Figure 12. Updating image path after changed to a new location.

After having imported the images and selected the one we want to base our analysis on, you can now **navigate** through your images and review them. There are multiple ways of navigating in your image (check section 3 in General Information for further details)

- **Zoom in/out:** Scroll the mouse wheel over the image or use + and - keys.
- **Pan:** Click and drag on the image using the mouse.
- **Change brightness & contrast:** Click  (Brightness & contrast)
- **View metadata:** Click **Image** to check the image properties (bit depth, pixel format, physical dimensions and pixel size).

**Notice** the difference between brightfield and fluorescence images:

**Brightfield image example**

Name	Value
Name	CD8A(M)+CD3(T)_lung.czi - Scene #4
URI	file:/C/Users/Analysis_User/Desktop/Q...
Pixel type	uint8 (rgb)
Magnification	20.0
Width	33515 px (5774.65 µm)
Height	37964 px (6541.22 µm)
Dimensions (CZT)	3 x 1 x 1
Pixel width	0.1723 µm
Pixel height	0.1723 µm
Uncompressed size	3.6 GB
Server type	Bio-Formats
Pyramid	1 2 4 8 16 32 64
Metadata changed	No
Image type	Brightfield (other)
Stain 1	Hematoxylin: 0.651 0.701 0.29
Stain 2	DAB: 0.269 0.568 0.778
Stain 3	Residual: 0.633 -0.713 0.302
Background	255 255 255

**Fluorescence image example**

Name	Value
Name	endothelial_analysis.czi - 1892_CD31...
URI	file:/C/Users/Analysis_User/Desktop/Q...
Pixel type	uint8
Magnification	Unknown
Width	14020 px (9113.00 µm)
Height	9405 px (6113.25 µm)
Dimensions (CZT)	3 x 1 x 1
Pixel width	0.6500 µm
Pixel height	0.6500 µm
Uncompressed size	377.2 MB
Server type	Generated pyramid (Bio-Formats)
Pyramid	1 4 16
Metadata changed	No
Image type	Fluorescence

All image metadata
All image metadata

Pixel depth
Pixel depth

Image size in pixels and microns
Image size in pixels and microns

Pixel size
Pixel size

Image type (previously selected when imported the image)
Image type (previously selected when imported the image)

Image stains vectors (**only visible in Brightfield images**). They act as channels in fluorescence
Notice we do not have stain vectors like in Brightfield. To access the image channels, press on the (Brightness & contrast) button

Figure 13. Metadata difference between brightfield and fluorescence images.

## 2. Estimate Stain Vectors

When working with **brightfield images**, before analysis, you need to [Estimate stain vectors](#) for color deconvolution and distinguish colors in H&E, DAB, or multiplex staining from RGB images.

Unlike fluorescence images (where each channel corresponds to a marker) brightfield images are coded in three channels (Red, Green, Blue or RGB) and all stains are a mixture of these three channels. Therefore, the original image does not have the stains separated and it is **necessary** to perform such separation to segment structures of interest during our analysis (e.g., to segment cell nuclei only in hematoxylin staining). After the deconvolution, you will have created different channels, one for each stain being able to see your results like in *Figure 14*:

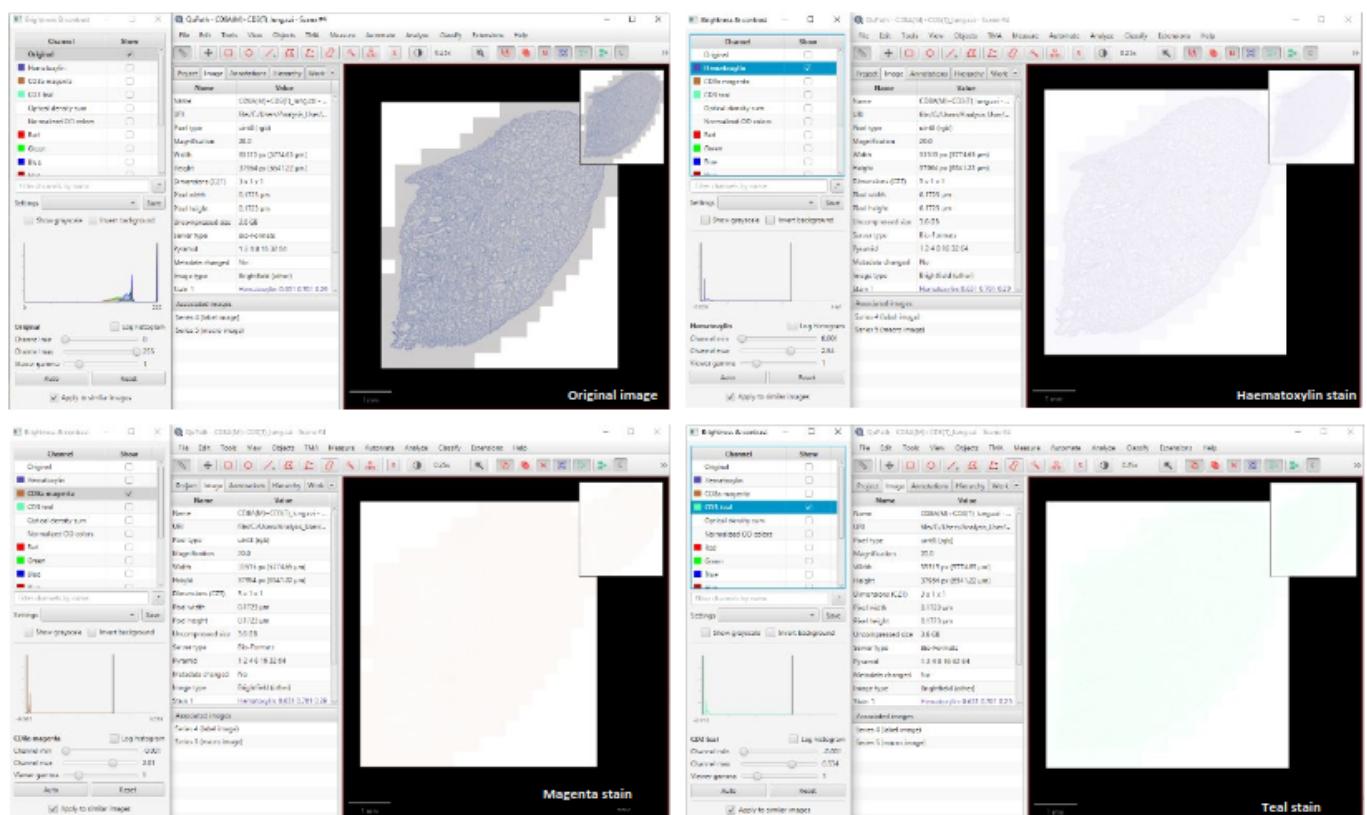


Figure 14. Result of estimate stain vectors.

**Note:** Fundamentally, color deconvolution digitally separates **up to three stains** from an RGB image. Therefore, if only two stains are present, a third ‘residual’ stain is auto generated. In our image (Figure 14) we have three stains (Hematoxylin, Magenta and Teal), so no residual is created.

The estimation of stain vectors may be done by two different ways:

### 1. Automatically by Analyze → Estimate stain vectors

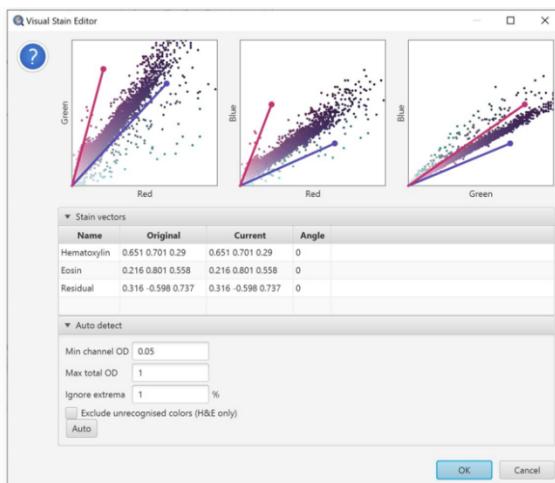


Figure 15. Automatic estimate stain vectors.

- Then click **Auto** → **wait until vectors update** → **OK**
- In the next window, again click in **OK** without changing the name.

**Note:** automatically creating your stain vectors is **not always perfect**. We personally **recommend** doing this process **manually** to secure later segmentation steps on the different resulting channels. Furthermore, automatically estimating stain vectors should **only be done when having 2 colors (Brightfield H&E or Brightfield H-DAB type of image, NOT with Brightfield Other image type)**. QuPath will let you do it at the beginning, but when naming the Residual channel, it will fail.

**2. Manually** by select the **Image tab** → **Navigate to an area where you can identify the stain of interest in a representative way (with variation in its intensity)**

- Press  and draw a small, squared annotation that includes a small region of a pure staining (only one staining should be present).

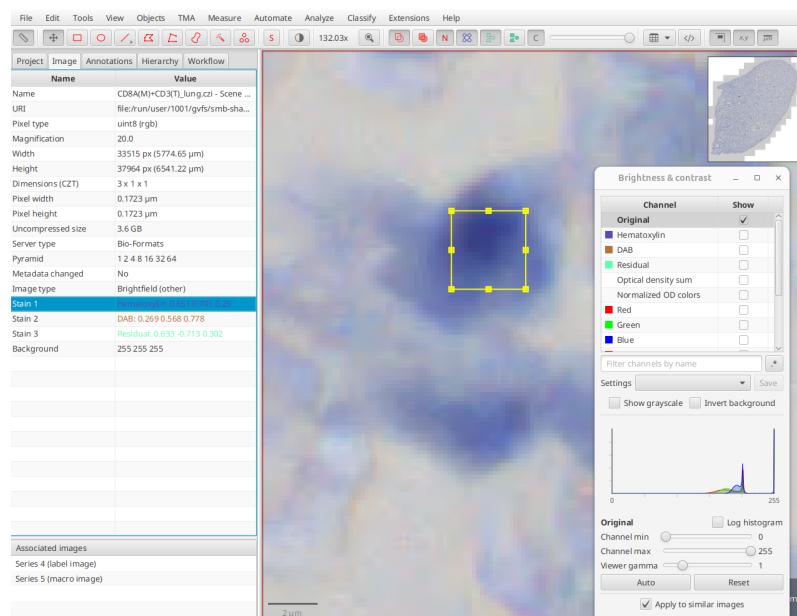


Figure 16. Draw an annotation for estimate stain vectors.

**Note:** it's **important** that you base your annotations on the **original image**. Do **not** use the other possible channels to see where to place the squared annotation! Make sure that you only have the  → **Original Channel** selected during the deconvolution implementation.

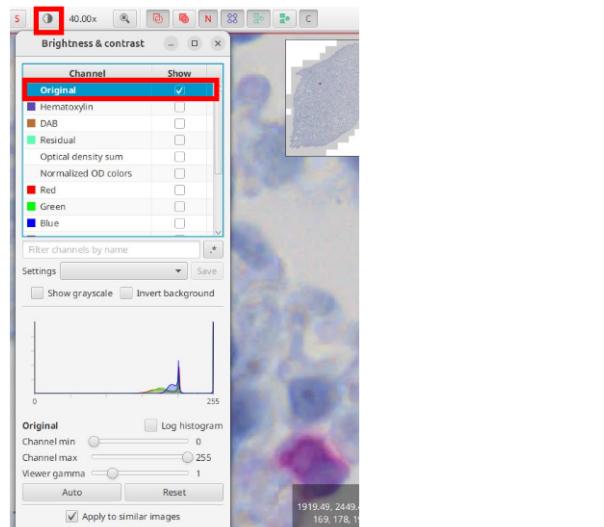


Figure 17. Estimate stain vectors step 1: Viewing only original channel for deconvolution.

- Under the Image tab, double click in the appropriate **Stain** → **press Yes** in the pop-up window → **Name your stain (Both the collective and the individual name)** → **Accept**

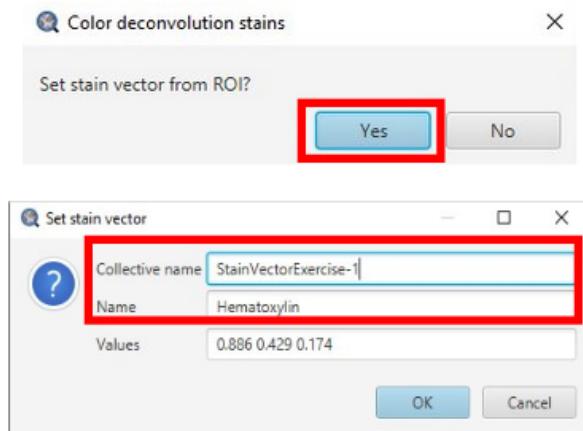


Figure 18. Estimate stain vectors step 2: Naming the stain. **Don't try to enter these same values.**

- You will need to **repeat** this action for **each stain** in the image.

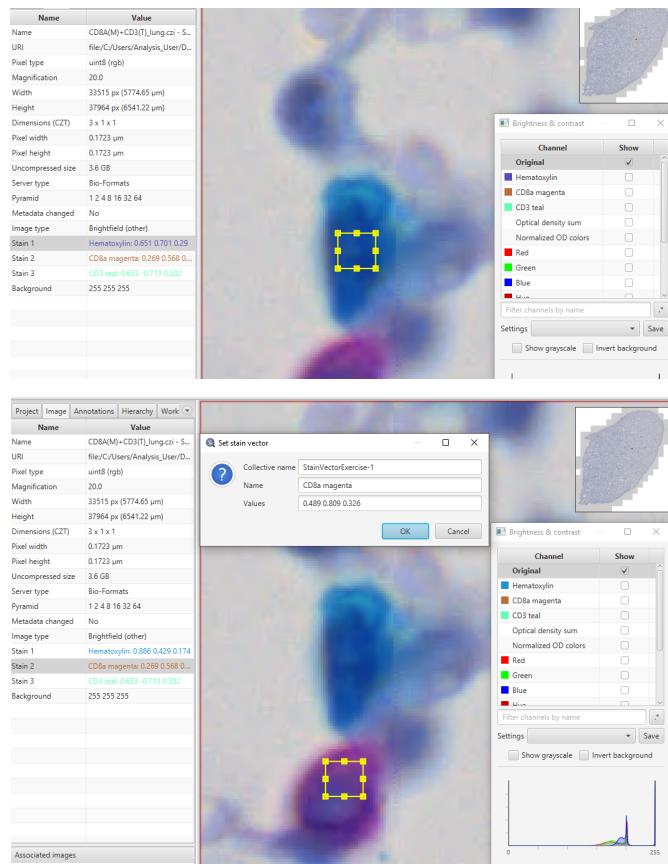


Figure 19. Estimate stain vectors step 3: Repeat for each stain.

Once the process has been completed for all your stains, new channels will have been created for each of the stains you have deconvoluted. We **truly recommend checking your resulting channels** as it will affect your analysis segmentations and results! One way to check how the deconvolution has gone is to click on → **click on your different channels and check the results by visualization.**

**Note:** It is possible that Hematoxylin deconvolution may not be the best in this exercise. It is completely normal, since Teal and Hematoxylin staining are not very well separated, so it is difficult to have pure staining for Hematoxylin. We will see how to overcome this problem later.

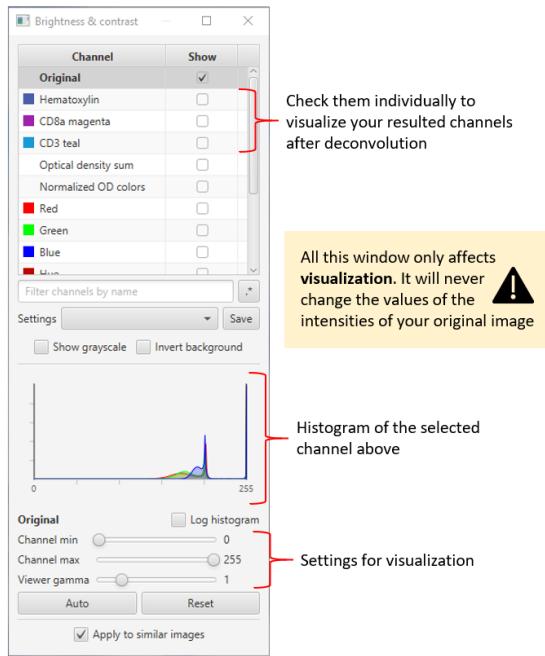


Figure 20. Brightness & contrast window.

### 3. Tissue Detection

In QuPath, there is often a manual and an automatic way of doing things. In tissue detection, both ways are possible:

#### 1. Manually by selecting [Annotation tools](#)



You can use the drawing tools (Rectangle, Polygon, Brush, etc.) from the **toolbar** (*section 3 in General Information*) to outline the tissue areas.

- Click on
- Start painting over your tissue until it is completely selected with the annotation

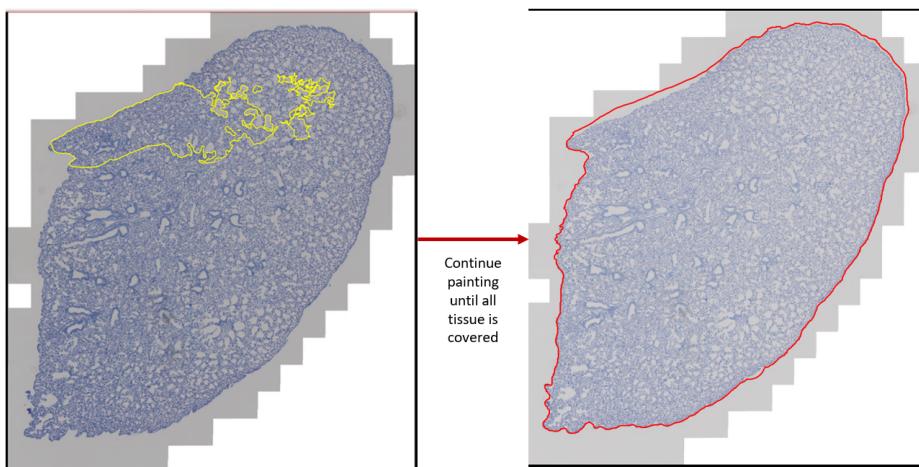


Figure 21. Manual tissue detection with drawing annotation tools.

**Note:** Notice how in Figure 21, when the annotation is selected, it is displayed in yellow. On the other hand, when you don't select it is red (if it is unclassified, otherwise it will be the color of the class). If you want to select it again, double click on it on the image or select it in the Annotation tab.

## 2. Automatically by using threshold

### 2.1. Create a Thresher

- Create a new class “**Tissue**”. If this class already exists, you can ignore this step. Go to the **Annotation Tab** → click on next to Auto Set → Add/Remove → Add class → name it “**Tissue**” → OK

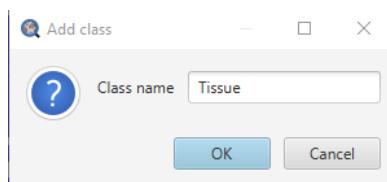


Figure 22. New annotation class creation.

You should now have a new annotation class created

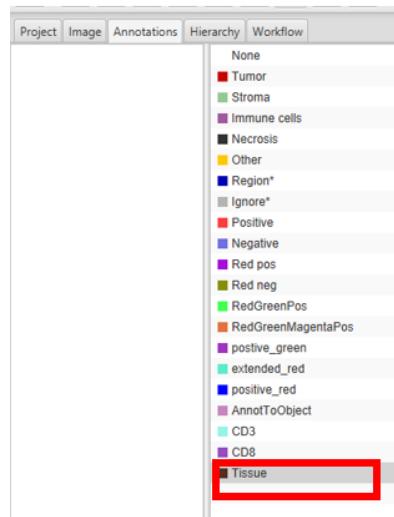


Figure 23. Resulted new annotation class.

- Go to **Classify** → **Pixel classification** → **Create thresher**  
This tool allows you to classify pixels according to a chosen intensity threshold, letting you to measure areas of interest or create objects based on intensity.
- Once the window appears you need to set multiple parameters. By hovering the mouse over any of the parameters you can see a short description of what each one does.

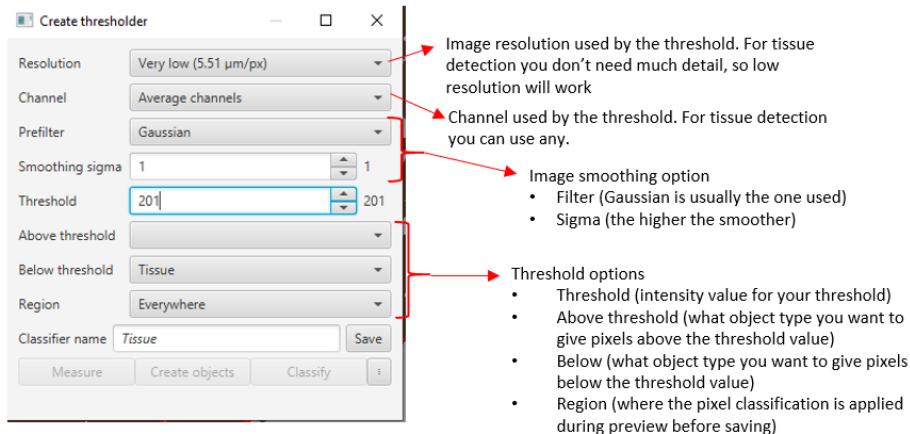


Figure 24. Create Thresholder parameters.

**Note:** keep in mind that as we have done the estimation of stain vectors manually, the **thresholds** set in the screenshots could **not be the best in your case**, but they can serve you as a **reference**. This only affects the threshold values, not the rest of the parameters, meaning that you should set all the parameters to the same values of the screenshots except the threshold value.

In order to determine the intensity value of the threshold (set in Figure 24 as 201) you need to access the channel in which you are setting the threshold and see the pixel intensity value. For that click on  and select the channel of interest, then by placing the mouse over the image you will be able to see the intensity value in the lower right of the image. If you are displaying Original Image, you will see the intensity for each of the three components of the image (RGB). If you wish to decide a threshold on the Average channels (as displayed on Figure 24), then you have to calculate the average of the three RGB values displayed on the image when you hover the mouse over a pixel.

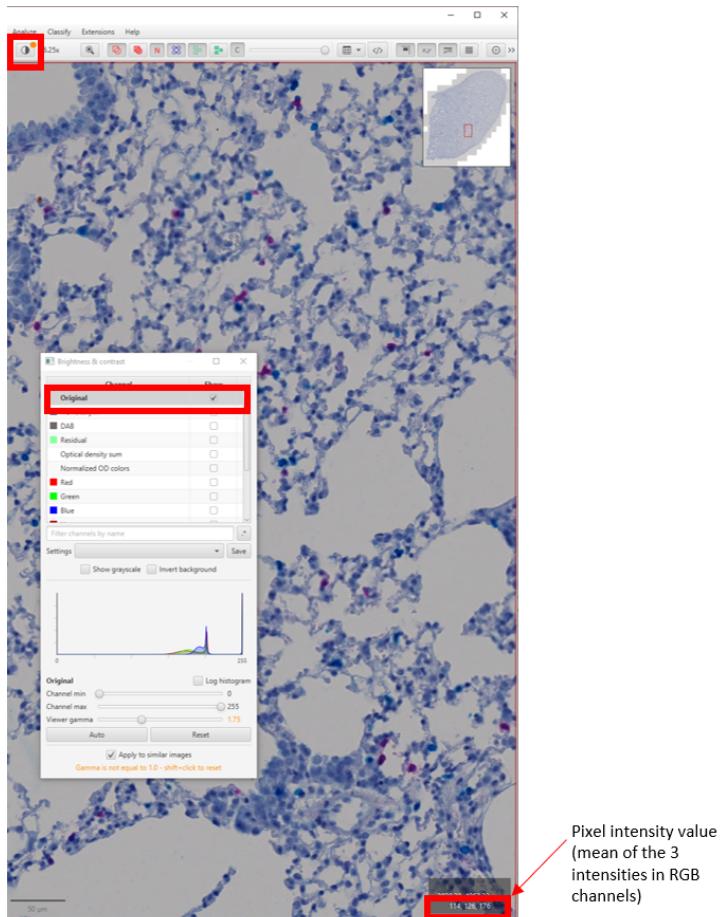


Figure 25. Access the pixel intensity value in an image.

In our case, according to *Figure 24* all pixels with an intensity less than 201 (average of RGB) will be classified as **Tissue** class and those with an intensity value greater than 201 will be classified as nothing.

**Note:** This is how we work with non-deconvolved (original) brightfield images, since the staining will always be less bright than the background. As we will see later, when we deconvolve the channels and we perform detection in an individual deconvolved channel, we want to apply the threshold to classify pixels with higher intensities than a desired value, not lower.

Therefore, we obtain the following result:

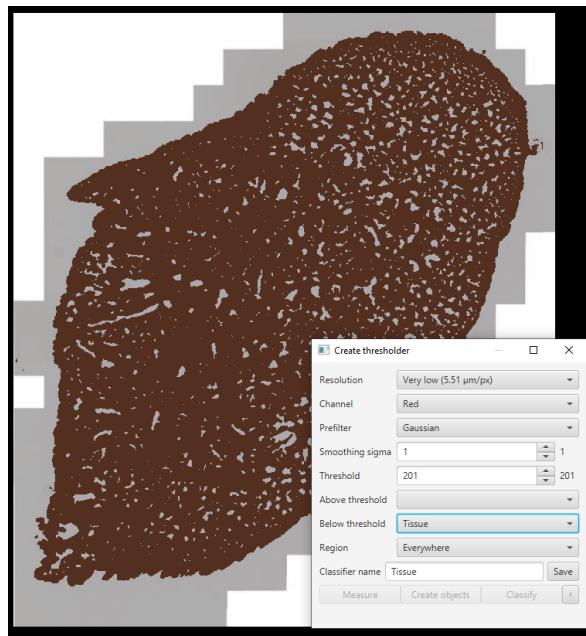


Figure 26. Resulted pixel classification after creating the threshold.

- After having copied the values in Figure 24 click on **Save**.
- **Do not close the Create thresholder window yet!**

## 2.2. Create Objects as annotations

- Now, let's create an annotation object for the tissue segmentation based on the thresholding classification we've just done.
- On the Create thresholder window click on **Create Objects**

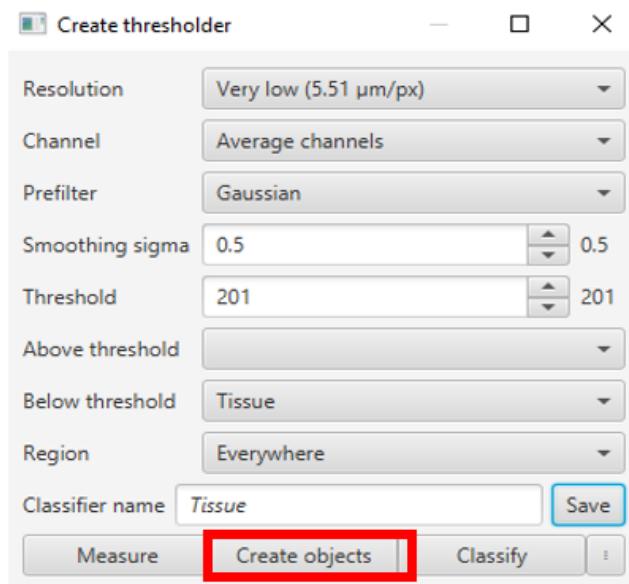


Figure 27. Create objects button.

- In the next pop-up window select a parent object. QuPath creates a hierarchical tree with your objects. The parent object refers to which object you want to set as a parent of the object you are now creating. In our case, we want the tissue object to belong to the full image as we do not have any annotations yet. To do so, select Full image and click **OK**.



Figure 28. Choose parent object in the object creation.

- On the next prompt specify the following values in Figure 29:

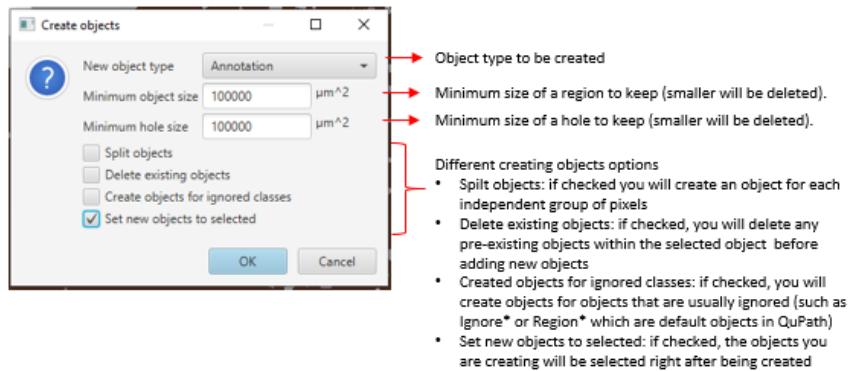


Figure 29. Create objects parameters for tissue detection.

**Note:** in the *Minimum object size* and *Minimum hole size* parameters, notice how we have specified very high values in our case. By doing so, we are making sure to only have into account a big object (the tissue) and no detecting the small holes seen after the thresholding live visualization in Figure 26 (acting as a kind of fill holes option).

- Click **OK** and close the Create thresholder window.
- Notice how as a result the tissue is now surrounded by a **yellow** region, which is the object we have just created. You can see the object list on the **Annotation tab**. By having checked the **Set new objects to be selected** in Figure 29, it is selected by default after having created it (when an object is selected it appears in **yellow**). Now we have created our first object and we have detected the tissue! You can also access the object properties and measurements through the **Annotation tab**.

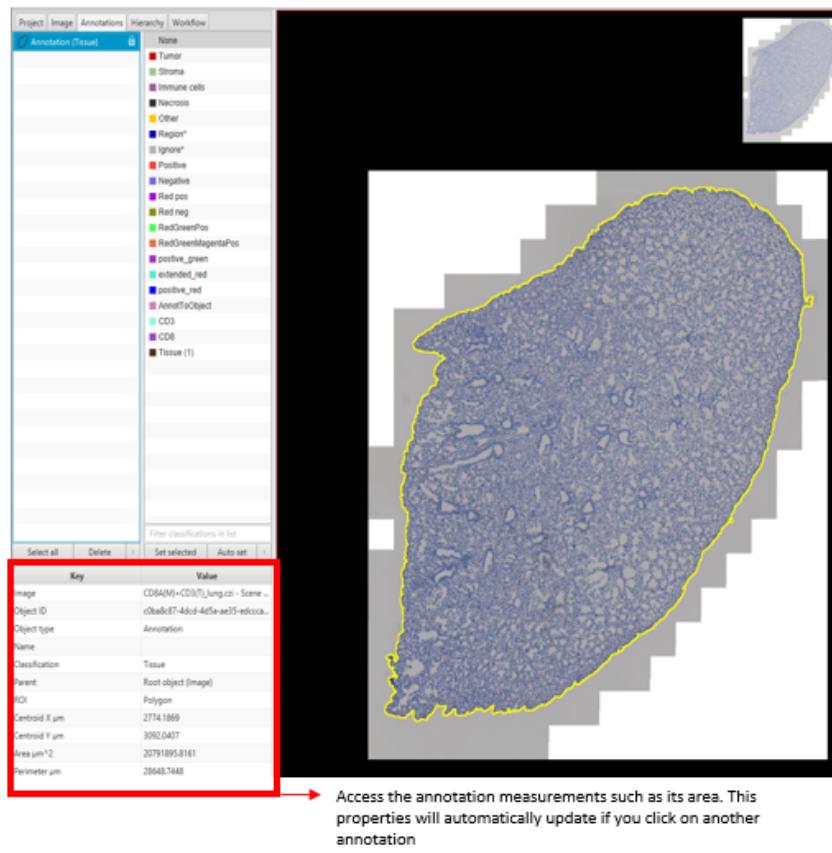


Figure 30. Result of creating an object. Visualizing object properties and measurements.

## 4. Cell Detection

For cell detection, QuPath offers different alternatives for achieving the segmentation of individual cells. One of the **alternatives** allows you to **simply detect** the cells (section 1), while the other one goes further and not only allows you to **detect individual cells, but also to classify them** (section 2):

### 1. Basic Cell Detection (just for detection, will not classify)

#### 1.0. Measure the nuclei size

- Click on the **brush annotation tool** ( ) and zoom in a nuclei. Draw the shape of various nucleus of different sizes.

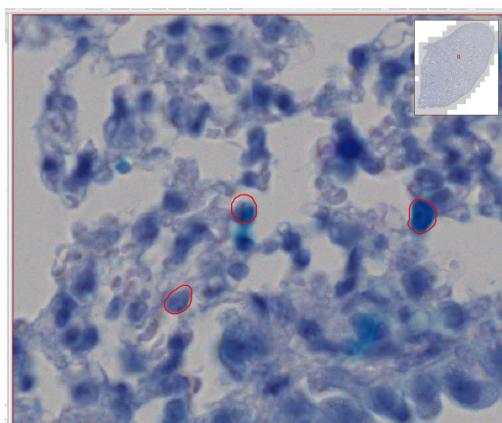


Figure 31. Drawing nuclei with the brush annotation tool.

- Go to the **Annotation tab** and select one of them (annotation contours are in **yellow** if selected). Look to the measurements of the annotation and search for the **Area  $\mu\text{m}^2$**  value. Check **all** of the annotations you have drawn just in the previous step and **remember** the minimum and maximum size between them.

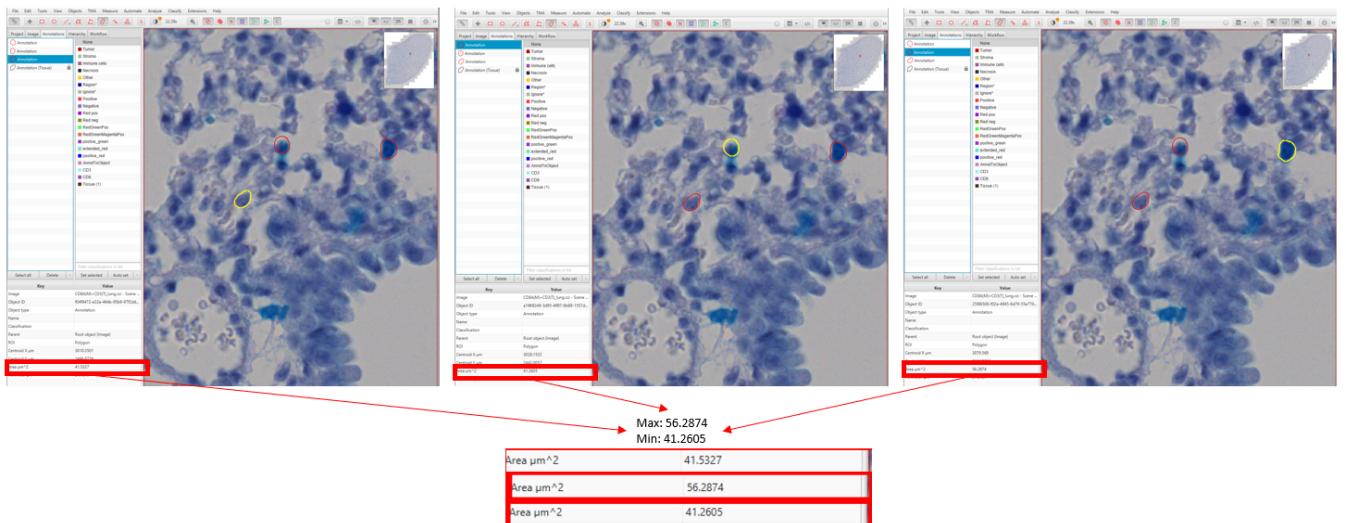


Figure 32. Check maximum and minimum area of cells.

- Select the nuclei annotations and delete them.

#### 1.1. Run the Cell Detection

- Create a small square annotation for testing the cell detection (use the button).

**Note:** When doing your analysis, it is important to always test your detection in small regions before running them in the entire image. This helps to save time and computational resources. We will use the small annotation to optimize the parameters used for cell detection and after being happy with the results, you can run it in the entire image.

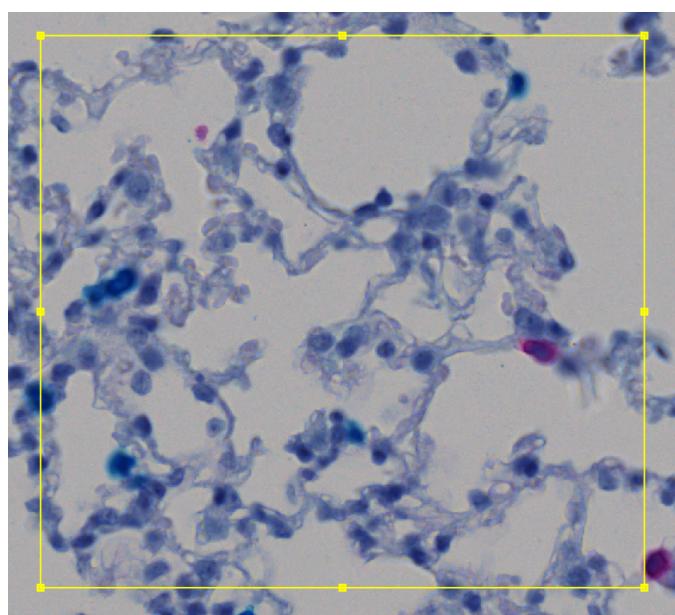


Figure 33. Squared annotation for testing cell detection.

- Make sure the annotation is selected (in yellow)
- Go to **Analyze** → **Cell detection** → **Cell Detection**. The detection window will open

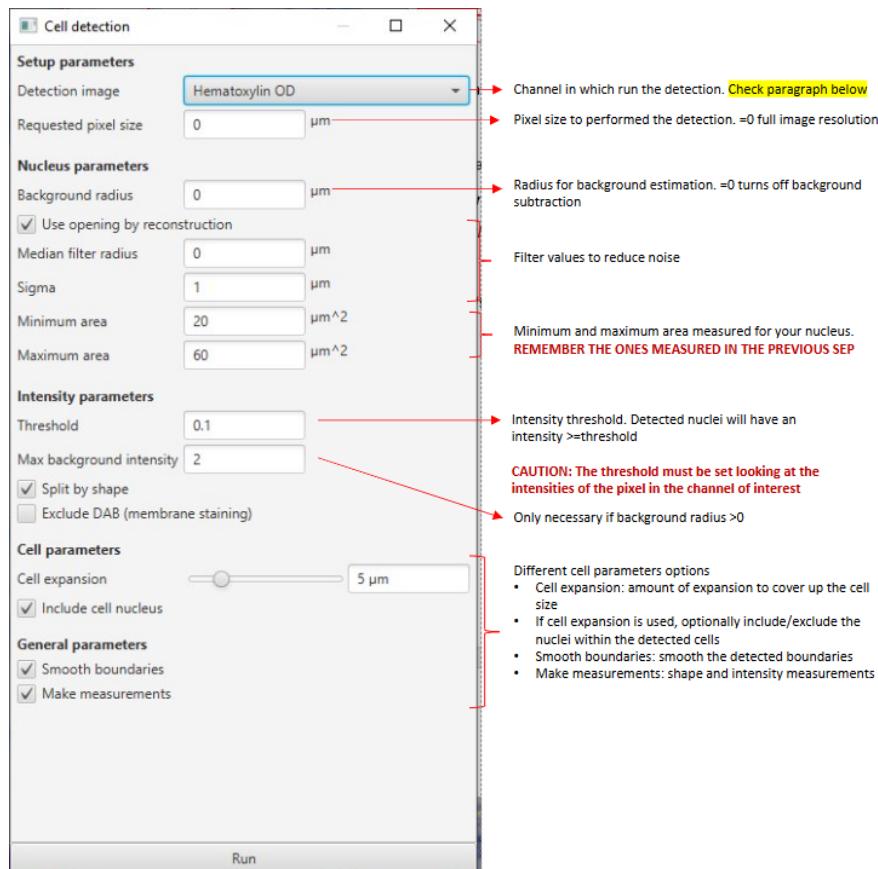


Figure 34. Cell detection parameters.

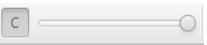
- Try to enter the values in Figure 34 as a first try (it won't be perfect as the threshold required for correct segmentation depends on the stain deconvolution done by each person, but it is a good starting point) and click **Run**. If you are not happy with the result, adjust the threshold parameter to your specific case (look at the **notes** below). You have to click on **Run** each time you change a parameter to display the results. It can help to take screenshots to compare before and after changing a parameter. You can also create more than one annotation and run cell detection with different parameters on each annotation. If the last strategy is used, it is crucial to select the correct annotation before clicking on Run. **If cell detection is not working very well** using Hematoxilin channel, it can be because Hematoxilin and Teal stainings are not very well separated (deconvolved) as explained before. You can use **Optical density sum** instead and see how it looks. You will have to tune threshold to get an optimal detection, but you can keep all the other parameters shown in Figure 34.

**Note 1:** Pay special attention to how the hematoxylin channel has been selected in Figure 34. Since you want to segment the nuclei in this stain, to determine the threshold it is

essential that you click on  and select *Hematoxilin channel*. And then you can hover the mouse over several areas of different intensities that you want to detect as nuclei. Look at the intensity value we set in Figure 34 and try different thresholds until you are happy with the result.

**Note 2:** When we deconvolve the channels and we perform detection in an individual deconvolved channel, we want to apply the threshold to classify pixels with higher intensities than a desired value, not lower. That's why we are setting a threshold value above which all pixels will be classified as positive, and when a group of positive pixels fulfill the detection algorithm criteria (such as maximum and minimum area), they become part of an object (a cell in this particular case).

- Use visualization tools to check the results

- Use  to show/hide the detection overlay
- Use  to move to different stains or the original image
- Click on **View → Cell Display → Nuclei only** to visualize only the nuclei

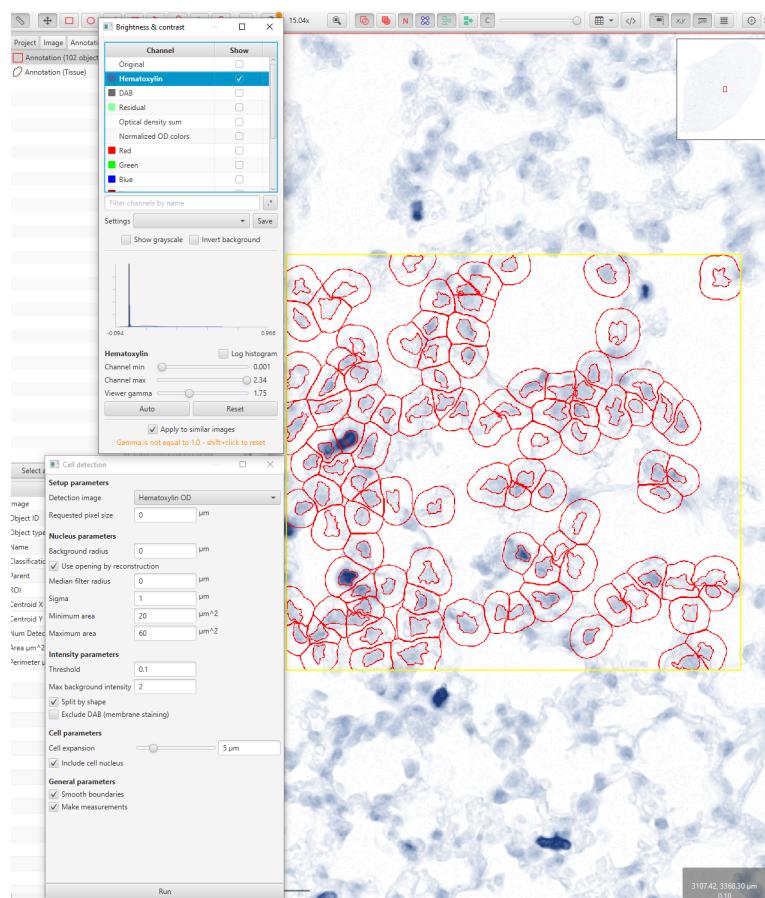


Figure 35. Result of cell detection.

- Once you are happy with the detection in the small testing annotation, delete the annotation and the detections inside, **but do not close the Cell Detection window yet!** For that:
  - Select the annotation (should be in **yellow**). **Be careful not to select the tissue annotation we created on section 3!!**
  - Press the Supr button in your keyboard
  - In the next **Delete objects** pop-up window click on **Yes** in order to delete the annotation (testing)

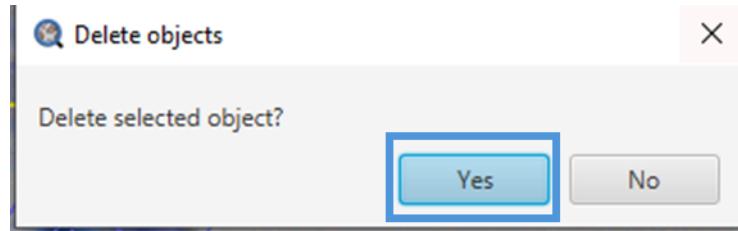


Figure 36. Deleting objects 1.

- Then another **Delete objects** pop-up window will appear click on **No** in order to delete the detection (cells)

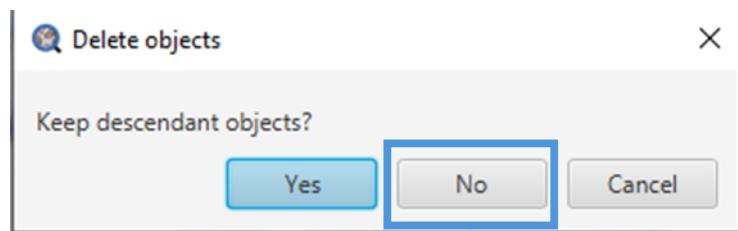


Figure 37. Deleting objects 2.

Tip: Another way of deleting your objects is to click on **Objects → Delete...** and then click on one of the options depending on what you want to delete. In this case, **Delete all objects** will do the job, since this command will delete all annotations and cells (detections).

- Select the tissue annotation previously created (in **yellow**)
- Click on **Run** on the **Cell detection** with your parameters.

## 2. Positive Cell Detection (Detection AND Classification)

This approach classifies the cells according to a **staining** in addition to **detecting** the cells. Therefore, if your analysis required just a simple cell detection, the above approach 1 would have been sufficient. However, in this particular case we want to classify the cells by positivity, so **Positive Cell Detection** tool gives us everything in approach 1, plus a classification of the detected cells. Consider that this only allows to classify detected cells according to **one staining**, if you have more than one, another approach is required. We will see how to classify cells according to multiple stainings in the fluorescence part

(exercise 4). In this exercise, we will see how to create cells and classify them according ONLY to CD8 marker (magenta stain).

## 2.0. Delete the results of the previous Cell Detection (go to **Objects → Delete → Delete all detections**).

### 2.1. Measure the nuclei size

- You should do the same nuclei measurement done for the basic Cell Detection. As you have already done it, you already have these values!

### 2.2. Run the Positive Cell Detection

- Select the square annotation we created before. If you deleted it, create a small square annotation for testing the positive cell detection (use the  button).
- Make sure the annotation is selected (in yellow)
- Go to **Analyze → Cell detection → Positive Cell Detection**. The detection window will open:

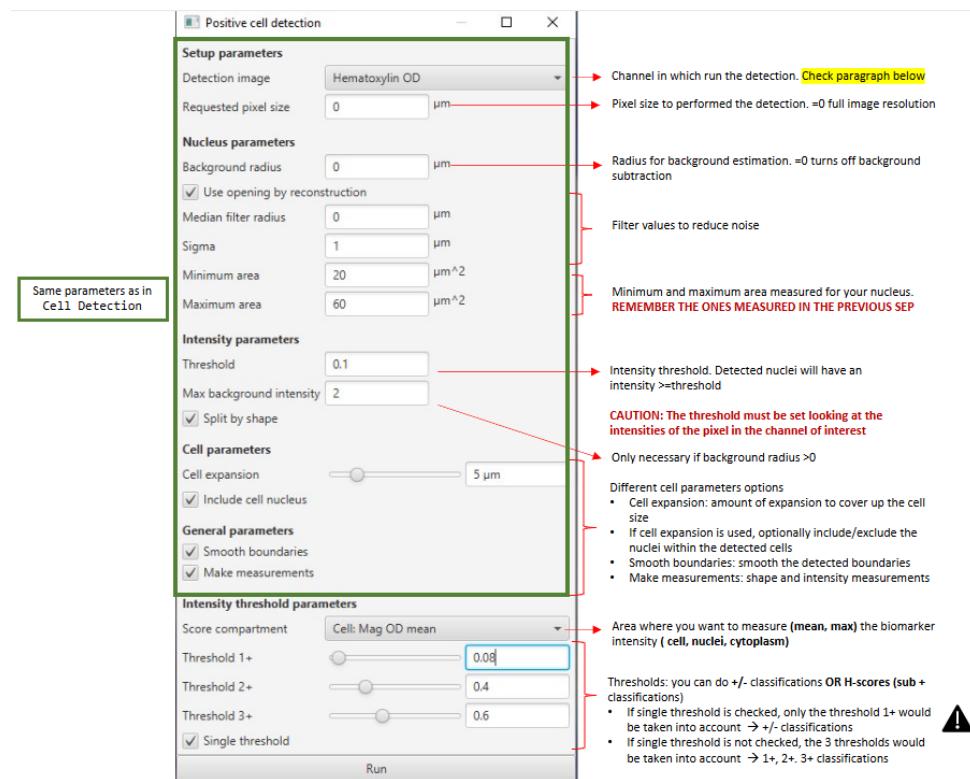


Figure 38. Positive Cell Detection parameters.

- Try to enter the values in Figure 38 as a first try (it won't be perfect as the segmentation depends on the stain deconvolution done by each user, but it is a good starting point) and click **Run**. If you are not happy with the result, adjust the threshold parameter to your specific case. You have to click on **Run** each time you change a parameter to display the results. It can help to take screenshots to compare before and after changing a parameter. You can also create more than one annotation and run cell detection with different parameters on each annotation. If the last strategy is used, it is crucial to select the correct annotation before clicking on Run. **If cell detection is**

not working very well using Hematoxilin channel, it can be because Hematoxilin and Teal stainings are not very well separated (deconvolved) as explained before. You can use **Optical density sum** instead and see how it looks. You will have to tune threshold to get an optimal detection, but you can keep all the other parameters shown in Figure 38.

**Tip:** If after the first try you do not find the right threshold value to classify cells according to what you see on the image, click on a detected cell (until it appears in yellow). In the **Annotation Tab** you can see the intensity value in each of the score compartment mentioned in Figure 38, which you could directly use in the threshold 1+ parameter:

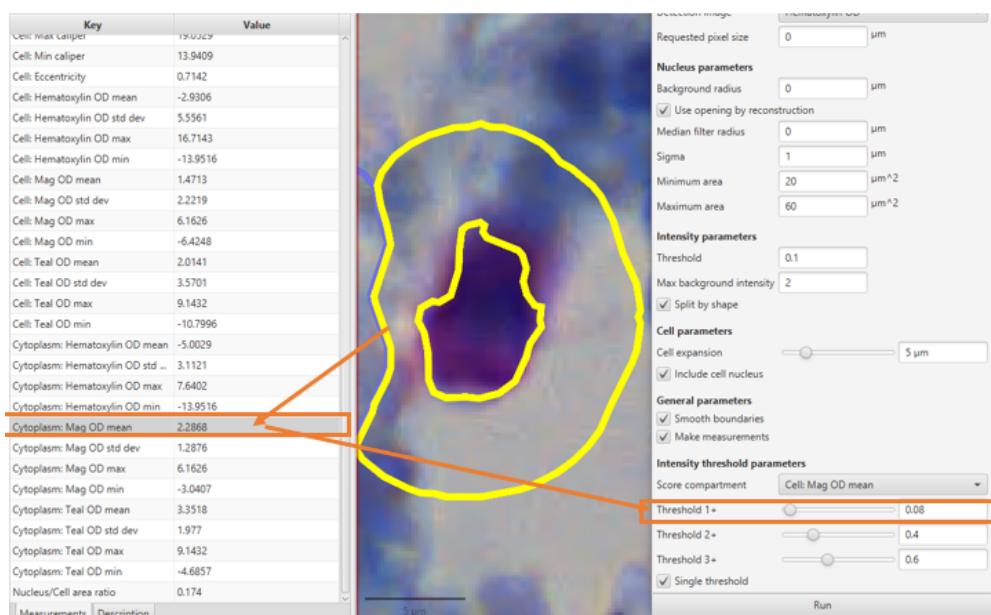


Figure 39. Tip to determine your threshold in Positive Cell Detection.

**Note:** Pay special attention to how the hematoxylin channel has been selected in Figure 38 for nuclei segmentation, but how the magenta stain has been selected for the CD8+ cells.

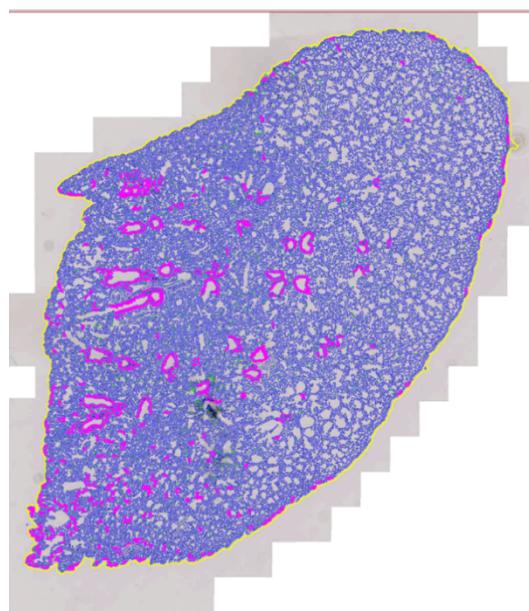
**Note:** It is also important to see the Intensity threshold parameters in Figure 38 to see the **different types of classifications** the Positive Cell Classification lets you do. One of the intensities classifications are **1+, 2+, 3+**, which are used to indicate weakly, moderately and strongly positive staining - used to dynamically calculate H-scores. H-scores are calculated by estimating (or otherwise determining) the percentage of cells in each category (Negative, 1+, 2+, 3+) and multiplying this by the number associated with the category (0, 1, 2, or 3) - then summing the results.

The results then range from 0–300, where 0 indicates ‘all cells are negative’, and 300 indicates ‘all cells are strongly positive’. This only applies if you unselect “Single threshold” option; otherwise, only Threshold 1+ applies and the cells will be classified into just positive or negative classes.

- Use visualization tools to check the results

- Use to show/hide the detection overlay
- Use to move to different stains or the original image
- Click on **View → Cell Display → Nuclei only** to visualize only the nuclei

This is the best you can get with Cell detection based on binary **threshold**. It is far from optimal as you can see, but it is faster and might be enough depending on your analysis needs. Other detection techniques based in Deep Learning give us better results (we'll see an example with fluorescence later!)



*Figure 40. Positive Cell Detection result.*

## 5. Review and Export Results

There are multiples ways of exporting your analysis results in QuPath. You can export the results of your analysis from a **single image** or for **different images** stored in a project.

**Note:** In our case, and since we're only working with the Scene 4 image, **we'll only do the exportation for one image**, but take into account that you could also export results across multiple images using the **Measure → Measurement Exporter (DO NOT EXPORT DATA USING THIS APPROACH NOW)**:

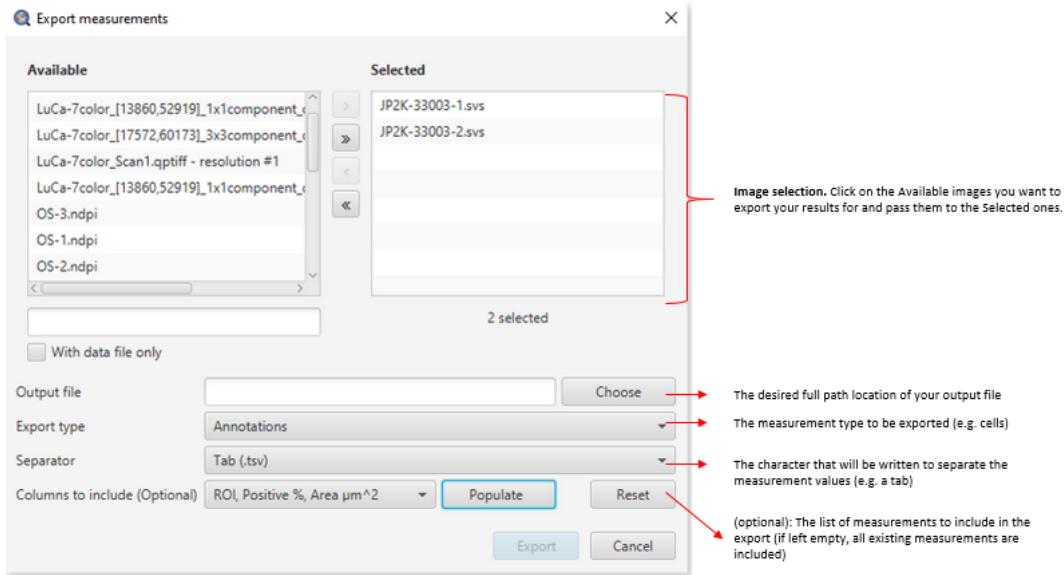


Figure 41. the Measurement Exporter.

In our case, **let's do it with a single image** (our Scene 4 image we have been working on). For a single image, you could also export the results regarding the **annotations** (the tissue annotation in our case) or the detections (individual cells in our case). For this positive & negative exercise, we are going to export the results regarding the annotations in our image:

- Click on: → Show annotation measurements.

Thumbnail	Image	Object type	Name	Classification	Parent	ROI	Centroid X µm	Centroid Y µm	Num Detections	Num Subcellular clusters: krt5 object	Num Subcellular spot: krt5 object	Area µm^2	Perimeter µm	Object ID
	Mn-KRT5_RNAScope-DAB+CK5-IHC-PURPLE_M_TA-CK5.czi - Scene #14	Annotation			Root object (image)	Rectangle	1793.9	2475.2	0	0	0	0.193	1.758	3556ea5c-777a-4fcd-8c3-d6763e97e30
	Mn-KRT5_RNAScope-DAB+CK5-IHC-PURPLE_M_TA-CK5.czi - Scene #14	Annotation			Root object (image)	Rectangle	1884.8	2744.9	3643	948	2159	79354.2	1139	10bda286-f57b-4946-b8c7-04c35838d4e
	Mn-KRT5_RNAScope-DAB+CK5-IHC-PURPLE_M_TA-CK5.czi - Scene #14	Annotation			Root object (image)	Rectangle	1967.5	2895.5	0	0	0	0.193	1.758	c3b066d-b872-417e-b031-20571cf849df

Figure 42. Show annotation measurements

**Tip:** In the resulted table, each thumbnail is an object (annotation or detection depending on what you have selected) and by clicking it or in the table you can review each object in the original image **interactively**. You can also click on the image to check the value on the measurement table.

- You can then save your measurement by pressing **Save** and choosing an appropriate name for your output .txt file.

**Note:** you could also click on the **Copy to clipboard** option, open Excel and paste your results!

**Tip:** by pressing **Ctrl+L**, you will open the *Command List* window, where can search for any QuPath command, and it is a fast way to use a command instead of searching for it navigating through all the menus. Keep this in mind for future uses. For example, press **Ctrl+L** → write **show annotation measurements on the search tab of the command**

Command	Menu Path	Keys	Help
Duplicate selected annotations	Objects → Annotations...	Shift+D	?
Expand annotations	Objects → Annotations...		?
Fill annotations	View	Shift+F	?
Fill holes	Objects → Annotations...		?
Insert into hierarchy	Objects → Annotations...	Ctrl+S...	?
Make inverse	Objects → Annotations...		?
Merge selected	Objects → Annotations...		?
Remove fragments & holes	Objects → Annotations...		?
Resolve hierarchy	Objects → Annotations...	Ctrl+S...	?
Select annotations	Objects → Select...	Ctrl+Alt...	?
Show annotation grid view	Measure → Grid views...		?
Show annotation measurements	Measure		?
Show annotation names	View	N	?
Show annotations	View	A	?
Signed distance to annotations 2D annotation	Analyze → Spatial analysis		?

Figure 43. Command list window.

**Note:** Up to this point we have based the analysis on a single image. Normally, it is of interest to perform the analysis on a batch of images for which QuPath automatically saves the workflow followed during your analysis, so that you can later apply the entire developed workflow to your batch of images. We will see how to do it in the fluorescence exercise 3.

## 6. Save your work

When you're done, it is important to save your project and images you've been working with. To do so:

- **File ▶ Save** or by responding positively to any saving prompts whenever you try to open another image or to quit.

This will save a. qpdata file, the file format for storing objects and other image-related data.

**Note that** this does not actually store the image itself (which may be huge), but rather only a link to it.

If you then want to reopen your QuPath project, you can simply drag the project.qpproj file onto the QuPath viewer!

## Exercise 2. RNAScope Detection

- DATA ▶ Bladder tissue stained with CK5 RNA (DAB) & CK5 protein (purple)
- GOAL ▶ Quantify CK5 RNA expression

### 1. Create a Project

- Create an empty folder with the name `QuPath_project` inside `\Desktop\QuPath_course\ RNAScope Detection` in order to create your project location. All automatically generated files will be stored here, following QuPath architecture.



- Open the QuPath application
- Go to the Project tab
- Click **Create a project**
- When the pop-up window appears, browse and choose the empty folder that you have just created (`Desktop\QuPath_course\Positive & Negative cells Analysis\QuPath_project`) and click **Select folder**
- Go to **Project → Add images → Choose files** and browse the image file (`\Desktop\QuPath_course\ RNAScope Detection \images\ Mn-KRT5_RNAScope.czi`)
- Select the images and click **Open → Import**
- Double click on `Mn-KRT5_RNAScope.czi` to open it.
- Select *Brightfield Other* under **Set image type** in the prompt window and click **Apply**.

### 2. Estimate Stain Vectors

For simplicity, in this exercise we're going to do the stain vector estimation **automatically**, but keep in mind what was mentioned in Exercise 1 about how **automatically creating your stain vectors is not always perfect** and that it is not a good practice to do it with images of type *Brightfield Other*. **Again, this is for simplicity.**

- Go to **Analyze → Estimate stain vectors**
- Then click **Auto → OK**
- In the next window, name your stain vectors as **Exercise\_2** and click in **OK**.

**Note:** As we have done the automatic vector estimation, QuPath has detected only 2 stains Hematoxylin and DAB (it is not able to detect 3 stains so, as mentioned in Exercise 1, it does not work with Brightfield Other images). As for this use case the magenta staining is not necessary for the RNAscope detection, we are going to ignore it.

But keep in mind that what QuPath has automatically called Residual channel (Figure 44) should be the Magenta channel. If we wanted to use this channel, we would need to do the manual vector estimation.

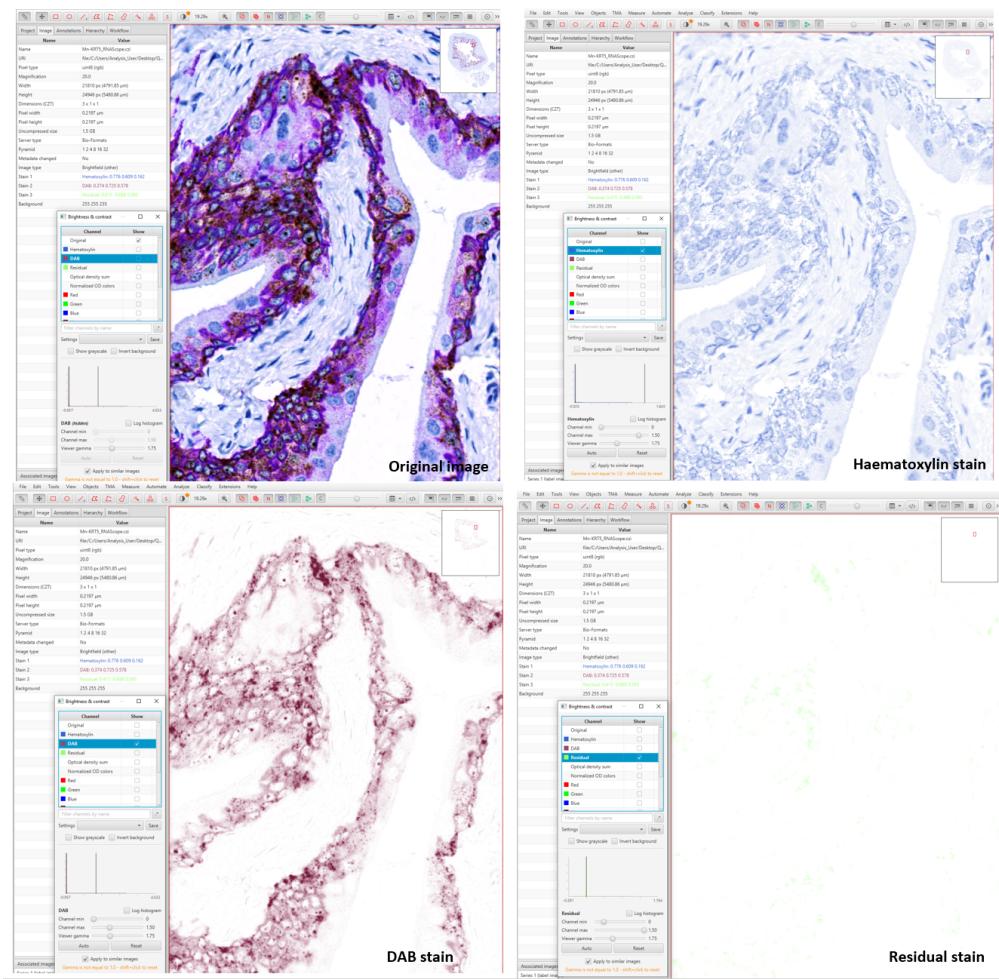


Figure 44. Automatic estimate stain vectors for exercise 2.

### 3. Tissue Detection

As in the previous exercise 1, tissue detection can be done manually or automatically with pixel classification. We're going to do an automatic detection as it is faster and more precise. In this case, the image is a bladder so we're going to detect the hole tissue and the urothelium.

#### 1. Tissue segmentation by using threshold

##### 1.1. Create a Thresher

- Create a new class “**Tissue**”. If this class already exists, you can ignore this step. Go to the **Annotation Tab** → click on  next to **Auto Set** → **Add/Remove** → **Add class** → **name it “Tissue”** → **OK**
  - If you get an error message saying that it already exist, then forget about this step.
- Go to **Classify** → **Pixel classification** → **Create threshold**  
 This tool allows you to classify pixels according to a chosen intensity threshold, allowing you to measure areas of interest or create objects based on intensity.
- Once the window appears enter the following values:

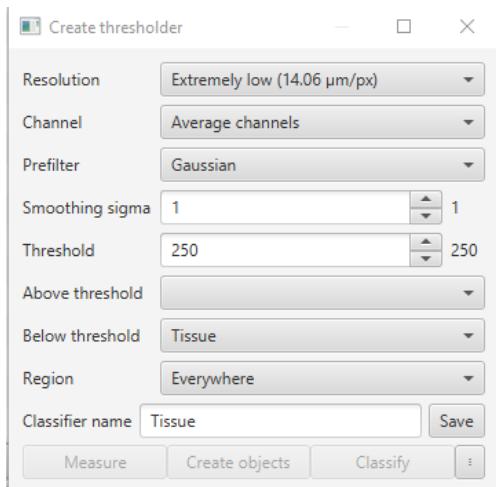


Figure 45. Create threshold for bladder tissue.

**Note:** Notice how in this exercise, we are able to give the values directly as we've done the automatic estimation of stain vectors, so hopefully QuPath does it similar for all of us and we can share the same values for thresholds and pixel intensity in the different channels.

- After having copied the values in Figure 45 click on **Save**.
- **Do not close the Create threshold window yet!**

## 1.2. Create Objects as annotations

- Now, let's create an annotation object for the tissue segmentation based on the thresholding classification we've just done.
- On the **Create threshold** window, click on **Create Objects**
- In the next pop-up window, select **Full image** and click **OK**.
- On the next prompt specify the following values:

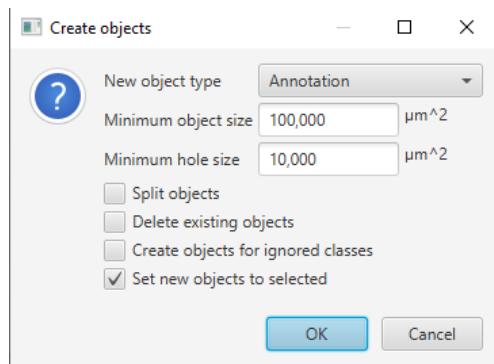


Figure 46. Create objects for bladder tissue.

- Click **OK** and close the Create thresholder window.
- Notice how as a result the tissue is now surrounded by a **yellow** region, which is the object we have just created. You can see the object list on the **Annotation tab**. Now we have created our object and we have detected the tissue!

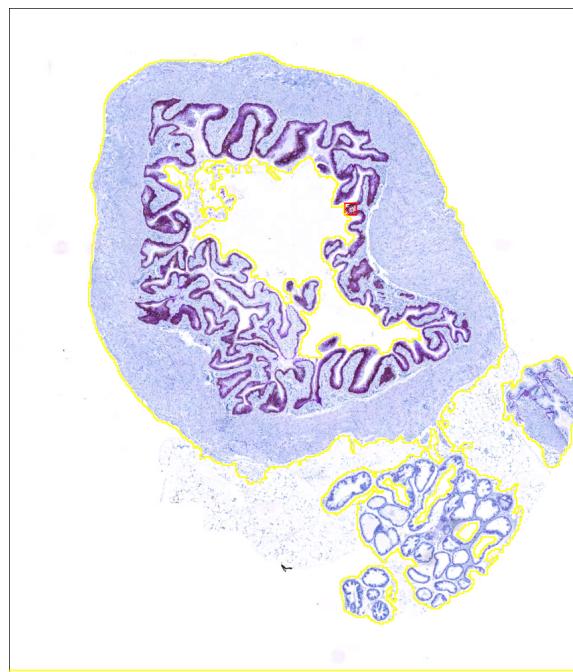


Figure 47. Tissue detection for bladder tissue.

## 2. Urothelium segmentation by using threshold

### 2.1. Create a Thresholder

- Create a new class “**Urothelium**”. If this class already exists, you can ignore this step. Go to the **Annotation Tab** → click on next to **Auto Set** → **Add/Remove** → **Add class** → name it “**Urothelium**” → **OK**
- Go to **Classify** → **Pixel classification** → **Create thresholder**
- Once the window appears enter the following values:

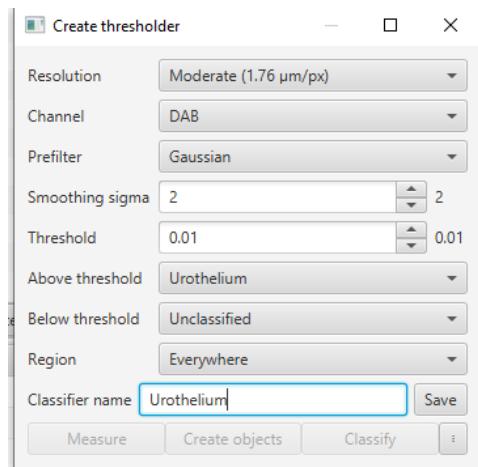


Figure 48. Create thresholder for urothelium tissue.

**Note:** Notice how in this exercise we are able to give the values directly as we have done the automatic estimation of stain vectors, so hopefully QuPath does it similar for all of us and we can share the same values for thresholds and pixel intensity in the different channels.

- After having copied the values in Figure 48, click on **Save**.
- **Do not close the Create thresholder window yet!**

## 2.2. Create Objects as annotations

- Now, let's create an annotation object for the tissue segmentation based on the thresholding classification we've just done.
- Make sure you have the Tissue annotation selected.
- On the **Create thresholder** window, click on **Create Objects**
- In the next pop-up window select **Current selection** and click **OK**. This will create the object inside the Tissue annotation as it is selected.
- On the next prompt specify the following values:

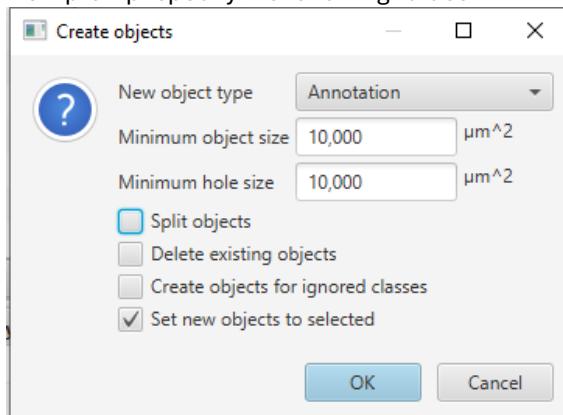


Figure 49. Create objects for urothelium tissue.

- Click **OK** and close the Create thresholder window.

- Notice how as a result the urothelium region is now surrounded by a **yellow** region, which is the object we have just created. You can see the object list on the **Annotation tab**. Now we have created our second object by detecting the urothelium!

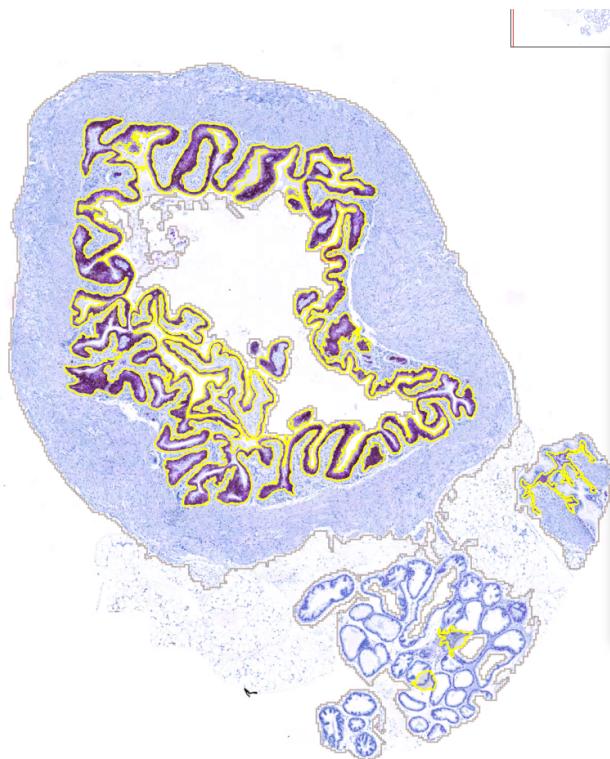


Figure 50. Tissue detection for urothelium tissue.

### 3. Edit segmentation

As mentioned previously, the detections based in thresholds are **not always perfect**. Sometimes, we cannot find the perfect threshold value, so it is worth it to do a postprocessing manual step. In our case, we have detected also prostate, which we don't want for this analysis. For that, we want to delete the prostate tissue and keep an annotation that just includes bladder. Follow the next steps to achieve this:

- Select the **Tissue** annotation (it has to be in **yellow**) and unlock it (right click on the **Tissue** annotation inside the **Annotations tab** → **Unlock**).

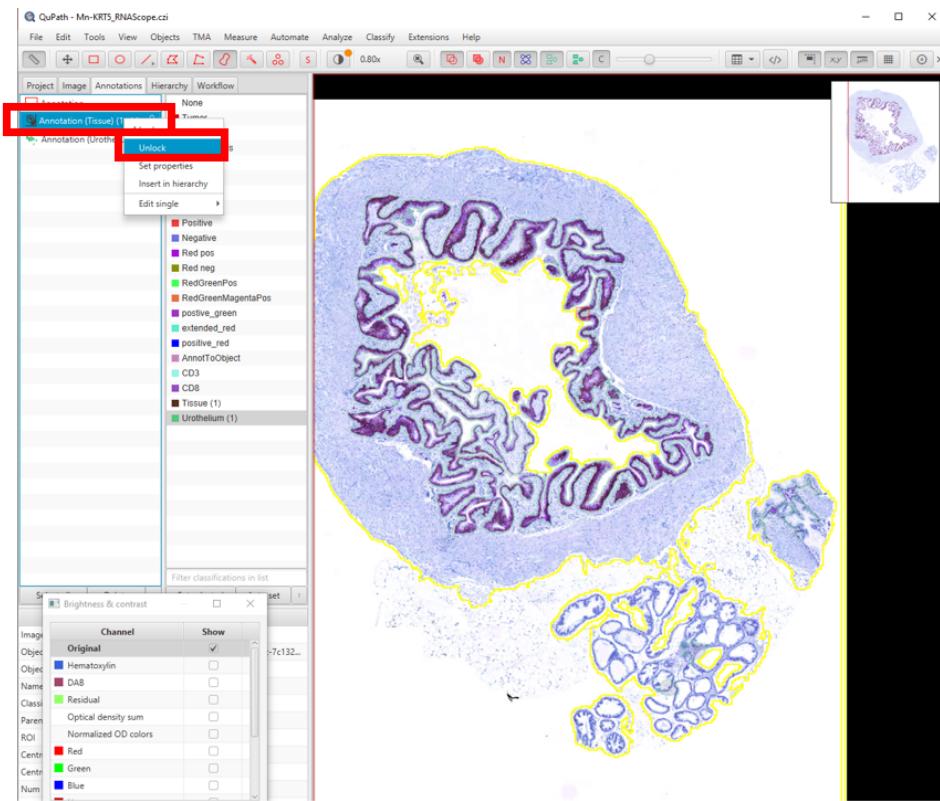


Figure 51. Select and unlock annotation.

- Zoom out of the image to erase easily (the eraser and brush size changes automatically when zooming over the image. If you have too much zoom, the eraser will be smaller)

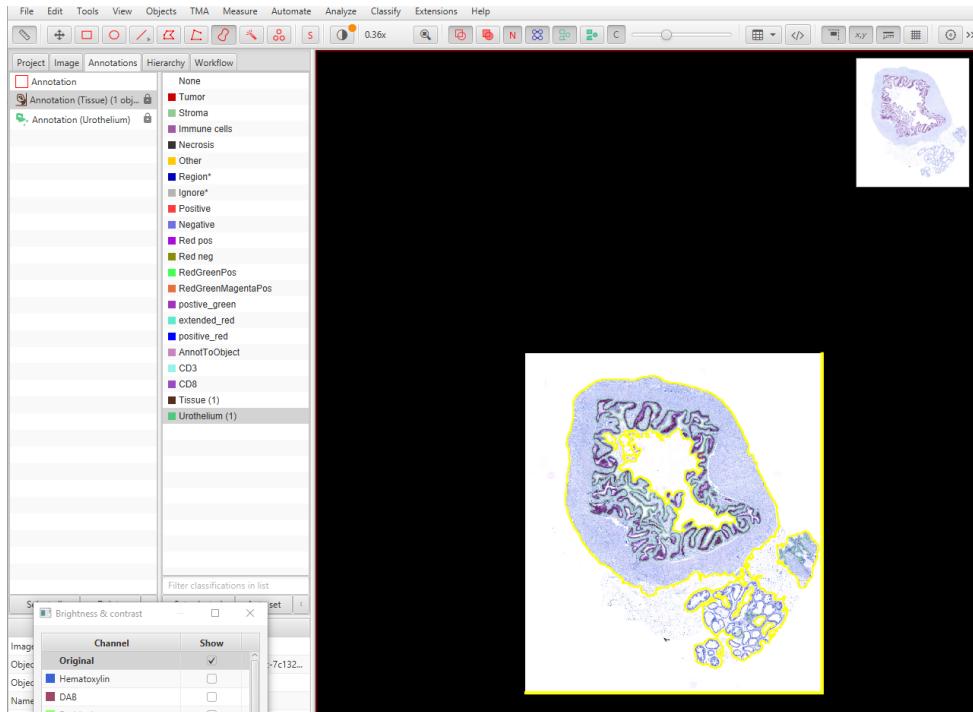


Figure 52. Zooming out the image for erasing.

- Click on  and press Alt simultaneously with left-click on mouse click out of the tissue to activate the eraser tool.
- Erase the incorrect detections until obtaining the correct detection. In this case, we will erase the prostate tissue (bottom right on the image). As a result, you must finish with a Tissue annotation that looks like the selected one (in yellow) in the following image:

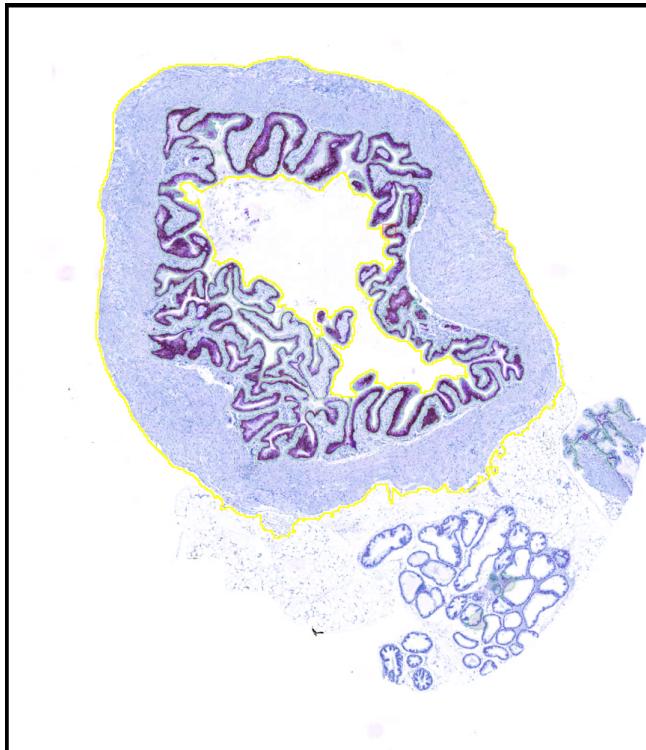


Figure 53. Tissue annotation after the manual correction.

- Now let's do the same for the Urothelium annotation. Select the Urothelium annotation (it has to be in yellow) and unlock it (right click on the Urothelium annotation inside the **Annotations tab**)
- Maintain the same zoom out of the image as before in order to erase easily
- Click on  and press Alt simultaneously with left-click on mouse click out of the tissue to activate the eraser tool.
- Erase the incorrect detections until obtaining the correct detection. Now, we will erase the same remaining prostate tissue (bottom right on the image). As a result, you must finish with a Urothelium annotation that looks like the selected one (in yellow) in the following image:

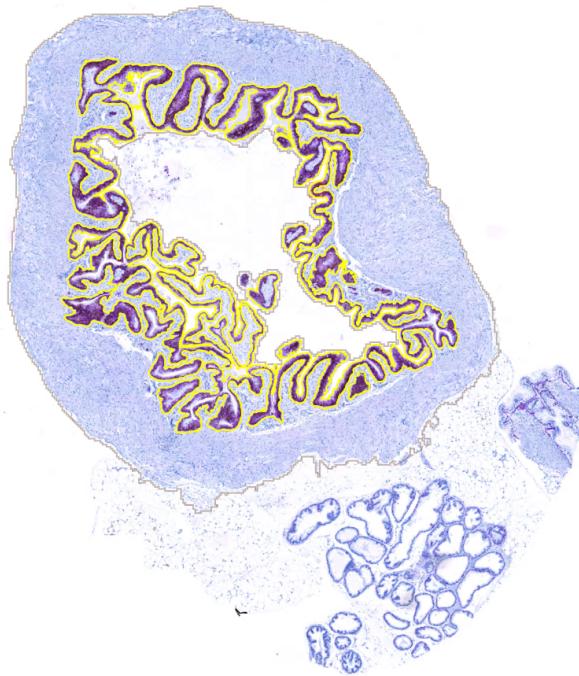


Figure 54. Urothelium annotation after manual correction.

- Select **Move tool**
- Lock both Tissue and Urothelium annotations again in the **Annotation tab**.

## 4. Cell Detection

For cell detection in this exercise, we do not want a classification like in exercise 1. Instead, we want to just detect the cells and afterwards detect the spots within them. Therefore, our cell detection will be done with the QuPath Cell Detection tool to **simply detect cells but NOT classify them**.

### 4.1. Measure the nuclei size

- Click on the **brush annotation tool** and zoom in a nuclei. Draw the shape of various nucleus of different sizes.
- Go to the **Annotation tab** and select one of them (annotation contours are in **yellow** if selected). Look to the measurements of the annotation and search for the **Area  $\mu m^2$**  value. Check **all** the annotations you have drawn just in the previous step and **remember** the minimum and maximum size among them.
- Select the nuclei annotations you have just drawn and delete them.

### 4.2. Run the Cell Detection

- Create a small square annotation for testing the cell detection (use the button) for testing
- Make sure the annotation is selected (in **yellow**)

- Go to **Analyze → Cell detection → Cell Detection**. The detection window will open, and you should enter the following values:

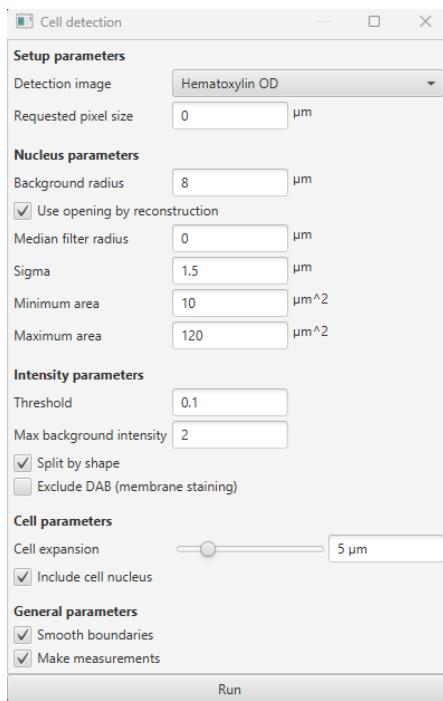


Figure 55. Cell detection parameters for exercise 2.

- Click on **Run**.
- Click on **View → Cell Display → Cell boundaries only** to visualize only the complete cell

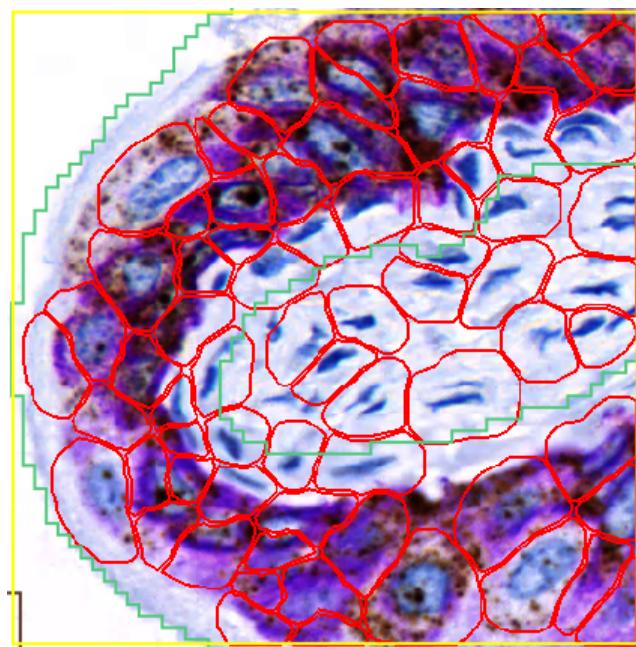


Figure 56. Results of cell detection in exercise 2.

- Once you are happy with the detection in the small testing annotation, delete the annotation and the detections within, **but do not close the Cell Detection window yet!** For that:
  - Select the annotation (should be in **yellow**). **Be careful not to select the tissue annotation we created on section 3!!**
  - Press the Supr button in your keyboard
  - In the next **Delete objects** pop-up window click on **Yes** in order to delete the annotation (testing)

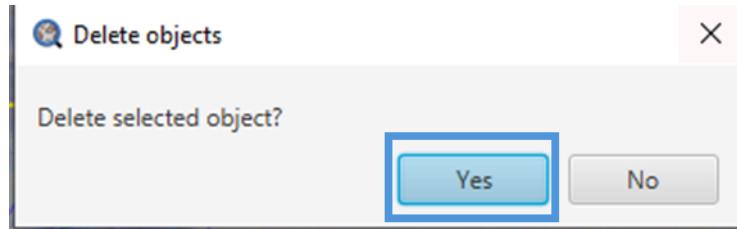


Figure 57. Deleting objects 1.

- Then another **Delete objects** pop-up window will appear click on **No** in order to delete the detection (cells)

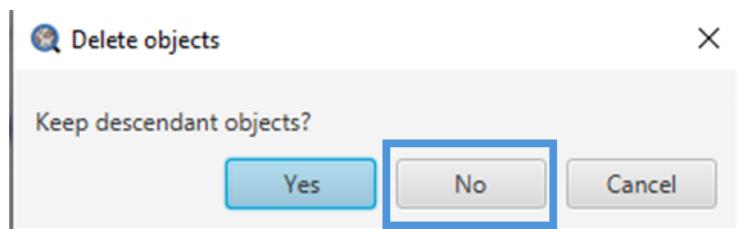


Figure 58. Deleting objects 2.

- Select the Urothelium annotation previously created (in **yellow**)
- Click on **Run** on the Cell detection.

## 5. Subcellular Spot Detection

After Cell Detection, in RNAScope analysis it is necessary to detect the small spots and bigger clusters of spots inside the cell. It is **very important** that the detections you have created **are Cells** and not just Detections. Cells are a special subtype of detection that must contain nuclei and cytoplasm. So, it is very important to do a cell expansion bigger than 0 um when doing Cell detection. Otherwise, QuPath will not be able to create subcellular detections (spots) inside our detections.

- Go to **Analyze→ Cell detection → Subcellular Detection (experimental) to detect the spots.**
- The detection window will open and you should enter the following values:

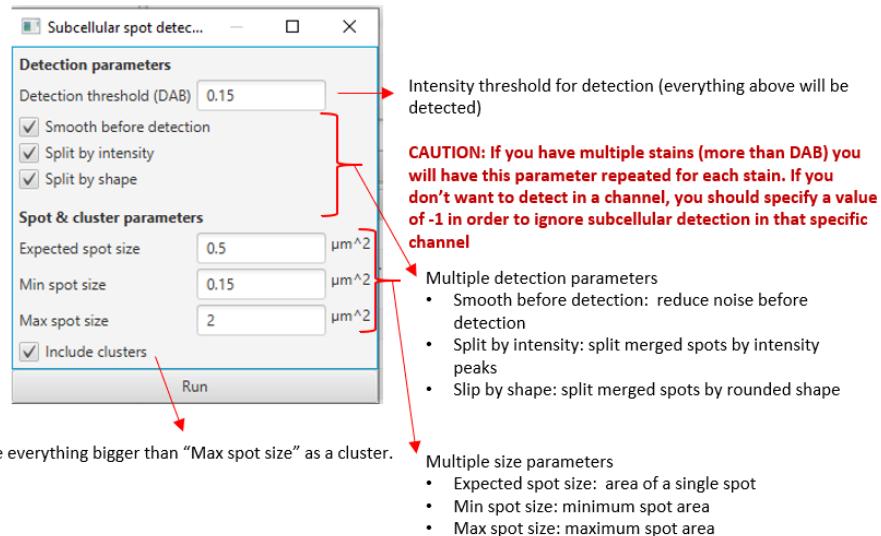


Figure 59. Subcellular spot detection parameters.

- Click on **Run** and review the results

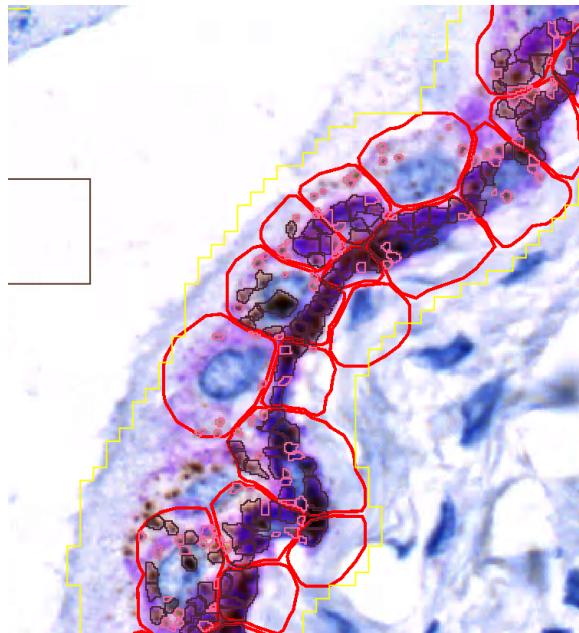


Figure 60. Spot detection results.

**Note:** Notice that by selecting the **Include cluster** option in the **subcellular spot detection** window, we have as a result the objects divided into 2 classes. The **pink class** corresponds to individual spots, while the **brown class** corresponds to clusters (clusters are detected spots with a size > max spot size, so they are classified as groups of spots, i.e. clusters).

## 6. Export Results

As mentioned in **exercise 1**, you can review and export the results in different ways. For RNAScope analysis, it is interesting to export the results regarding both each annotation (to have a count of all spots detected inside the urothelium) and each individual cell. For doing that

- Click on:  → **Show annotation measurements** to obtain the results regarding the annotations (Tissue and Urothelium)
- You can then save your measurement by pressing **Save** and choosing an appropriate name for your output .txt file.
- Click on:  → **Show detection measurements** to obtain the results regarding the detections (each object/cell)
- You can then save your measurement by pressing **Save** and choosing an appropriate name for your output .txt file.

**Note:** *you could also click on the Copy to clipboard option in both measurement tables, open Excel and paste your results! With this you could have all your results in just 1 file.*

## 7. Save your work

When you're done, it is important to save your project and images you've been working with. To do so:

- **File ▶ Save**, or by responding positively to any saving prompts whenever you try to open another image or to quit.

This will save a. qpdata file, the file format for storing objects and other image-related data.

**Note that** this does not actually store the image itself (which may be huge), but rather only a link to it. If you then wanted to reopen your QuPath project, you can simply drag the project.qpproj file onto the QuPath viewer!

## Part 2. Fluorescence Image Analysis Pipeline



## Exercise 3. Vessels and Pericytes Analysis

- **DATA** ▶ Breast tumor section stained with CD31 (red, channel 3), Pericytes (Green, channel 2) and nuclei (blue, channel 1)
- **GOAL** ▶ Analyze vascular density and quantify the pericyte area surrounding vessels.

### 1. Create a Project

- [Open QuPath](#).
- [Go to Project tab](#).
- Select [Create Project](#)
- Choose an empty folder to create your project location. All generated files will be stored here, following QuPath architecture.
- Drag and Drop “endothelial\_analysis.lif” to QuPath interface. The file is in the desktop, inside “QuPath\_course\endothelial\_analysis\images”.
- Select [Fluorescence](#) under “Set image type” in the prompt window. **Make sure that Auto-generate pyramids option is enabled**.
- Click [Import](#).
- Make sure that the first image is opened (endothelial\_analysis.lif - 1892\_CD31-NG2\_ICC). If not, [double click on it](#).

### 2. Viewing and Navigating

- You can navigate through the image in a very intuitive way: with the mouse wheel, you can zoom in or zoom out; by clicking and dragging inside the image, you can move inside the sample. Zoom in a particular area of interest. **Note that** you are visualizing a tissue with a blue staining for DAPI (nuclei), CD31 in red (endothelial cells) and NG2 in green (pericytes).
- **Channel Tool** by clicking  on the upper toolbar. Here, you can activate or deactivate channels from the visualization, and you can modify the channel histograms. **These changes will not affect analysis, it is just for visualization**. You can also edit the names and colors by double-clicking on the channel. **Do that according to the image below**.

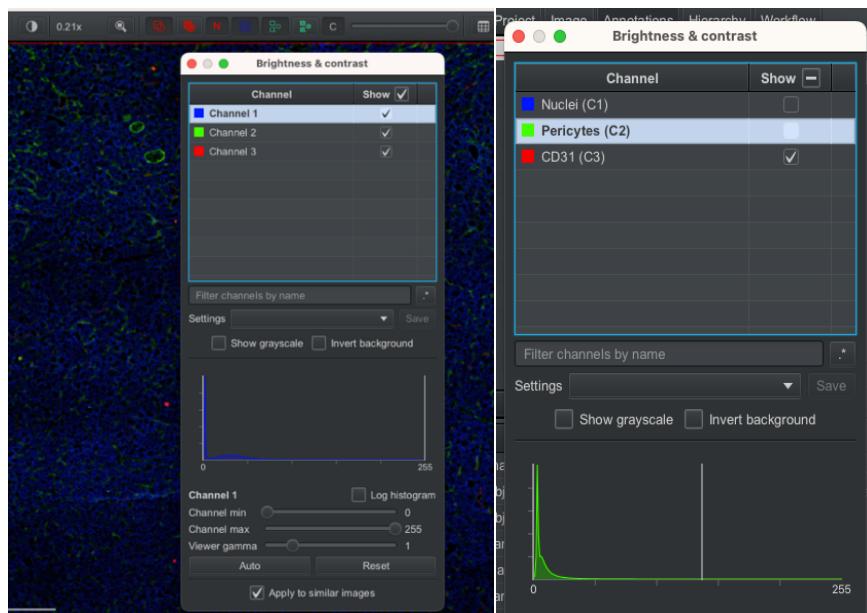


Figure 61. Channel tool and renaming channels

### 3. Annotations

- Create a new class and call it “**Test**”. If this class already exists, you can ignore this step. Go to the **Annotation Tab** → click on next to **Auto Set** → **Add/Remove** → **Add class** → **name it “Test”** → **OK**
- Go to **Annotation tab**. Select the red rectangle as an annotation tool to test the analysis.
- Draw a relatively small rectangle inside the image. This shape is editable by selecting the annotation under Annotations tab (it becomes yellow)
- **Set an annotation class:** With an annotation selected, you can click any of the classes on the right and click on **Set selected**; by doing this, your annotation will be classified to that class. This is very useful to filter your objects by class in complex analysis. Select “**Test**” class and click on **Set selected**.
- Double click on the image outside the annotation. Note that now, the annotation is not **yellow** anymore but the color of the assigned class. This will help later to **filter objects by classes**.
- **Select all the annotations** in the image. Go to **Objects > Select > Select annotations**.

**Note: Lock annotations:** If you don't want to edit the annotation by mistake, you can lock it. Just right-click the annotation on the list and select **Lock**. The annotation will have become non-editable, and you can't move it or modify it. You can edit the annotation again by selecting **unlock**. Annotations will become locked automatically when performing any analysis inside them.

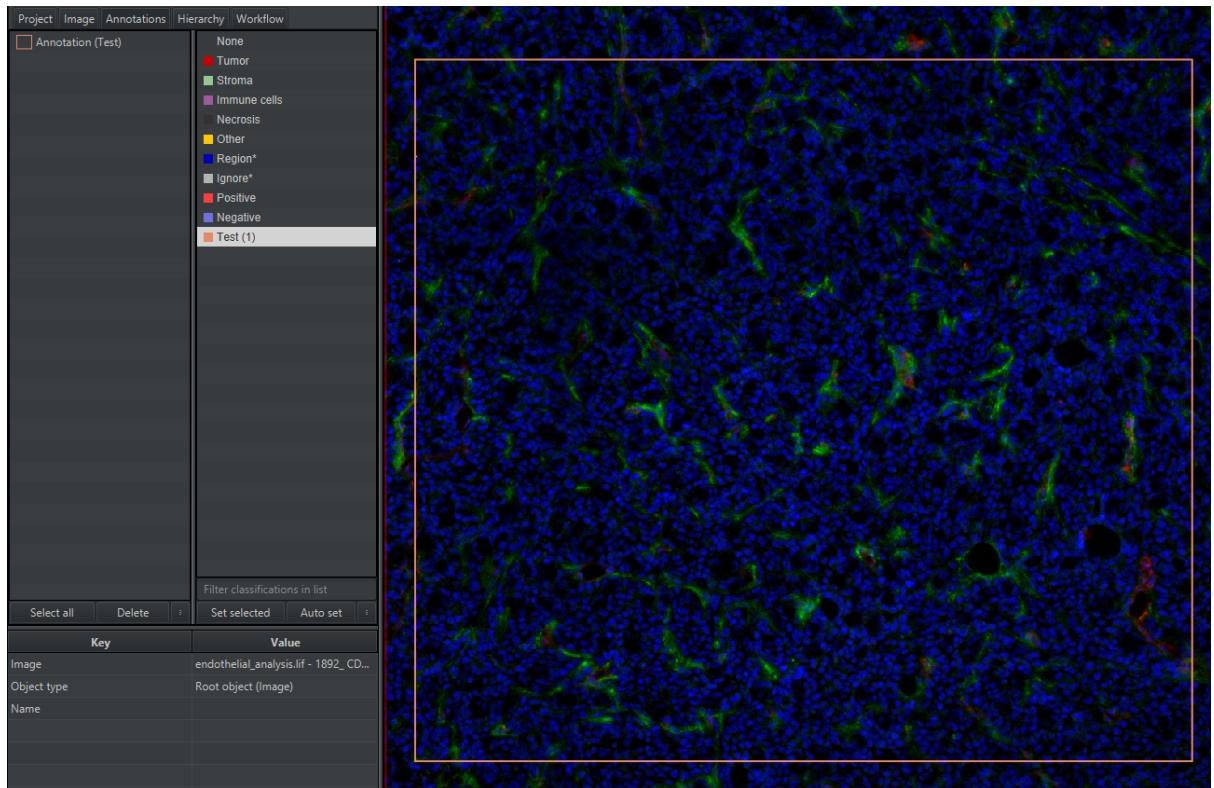


Figure 62. Creating and classifying an annotation

## 4. Vessel Detection (Create objects)

### 1. Create a Thresholder

With this tool, you can **classify pixels** according to a chosen threshold. This will allow you to measure areas or create objects according to your chosen threshold.

- We are going to create a new class “**positive\_red**”. If this class already exists, you can ignore this step. Go to **Annotations tab > click on the three dots next to Auto set > select Add/Remove > Add class > name it “positive\_red”.**
- Make sure that the annotation that you classified as “Test” is selected.
- Go to **Classify > Pixel classification > Create thresholder**. A pop-up window will show.
- **Channel Tool:** Deactivate all the channels except CD31 (C3). Adjust the histogram to your preference to see vessels properly.
- **Parameter:** By hovering the mouse over any of the parameters to see a short description of what it does.

Let's see how this works, introduce the following parameters:

- Resolution: Full.

- Channel: CD31
- Prefilter: Gaussian, with a Smoothing sigma of 0.

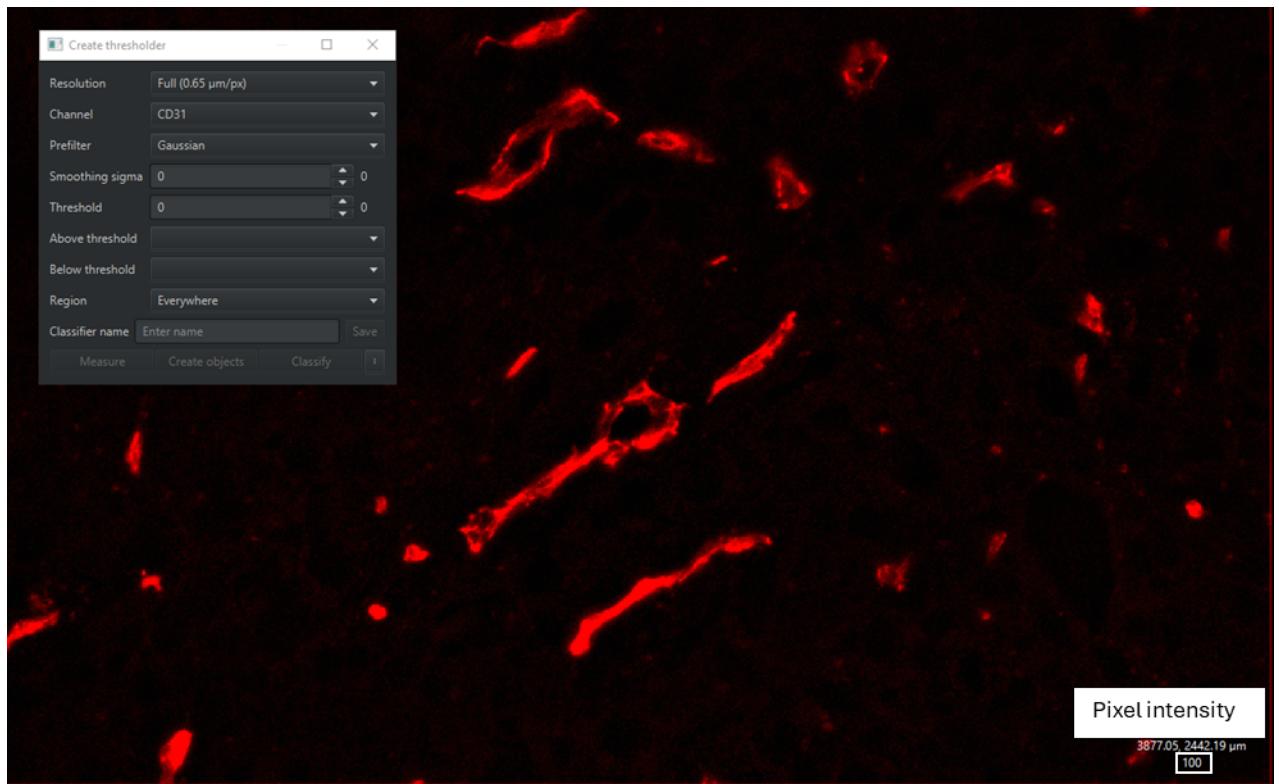


Figure 63. Opening thresholder and checking image intensity

- **Set the Threshold:** Make some zoom in into a vessel and **hover the mouse over the pixels** that constitute the vessel. **Look at the right-bottom part of the image** as shown on Figure 63. You will see the intensity values for the pixels you are selecting with the mouse. This will help you to have an idea of the threshold you can start with.  
**Note:** In fluorescence, your staining should be brighter than the background (more intense pixels, so higher values). Keep this in mind to understand how thresholds work. You will see practical examples of how different values of threshold work, until we reach the best threshold value.
    - **Very high threshold:** **Above threshold**, select class **positive\_red**. **Leave Below threshold empty** (or Unclassified) and Region on Everywhere (Region will only affect to the previsualization of the classifier). Enter 100 on the **Threshold** and **click on the image**. See how all the pixels that are brighter than 100 are classified as **positive\_red** class.
- Note that you are missing many regions that are vessels. This means that the threshold is too high.

- **Very high threshold:** Change the Threshold to 3 and click again on the image.

Note that you are now selecting the vessels plus a lot of background pixels, so this threshold is too low.

- **Best threshold:** Enter 12 and click on the image.

Now this looks much better. Some background pixels are selected, but we will discard them later with size filtering.

- **Fill the rest of the parameters according to the figure below.**

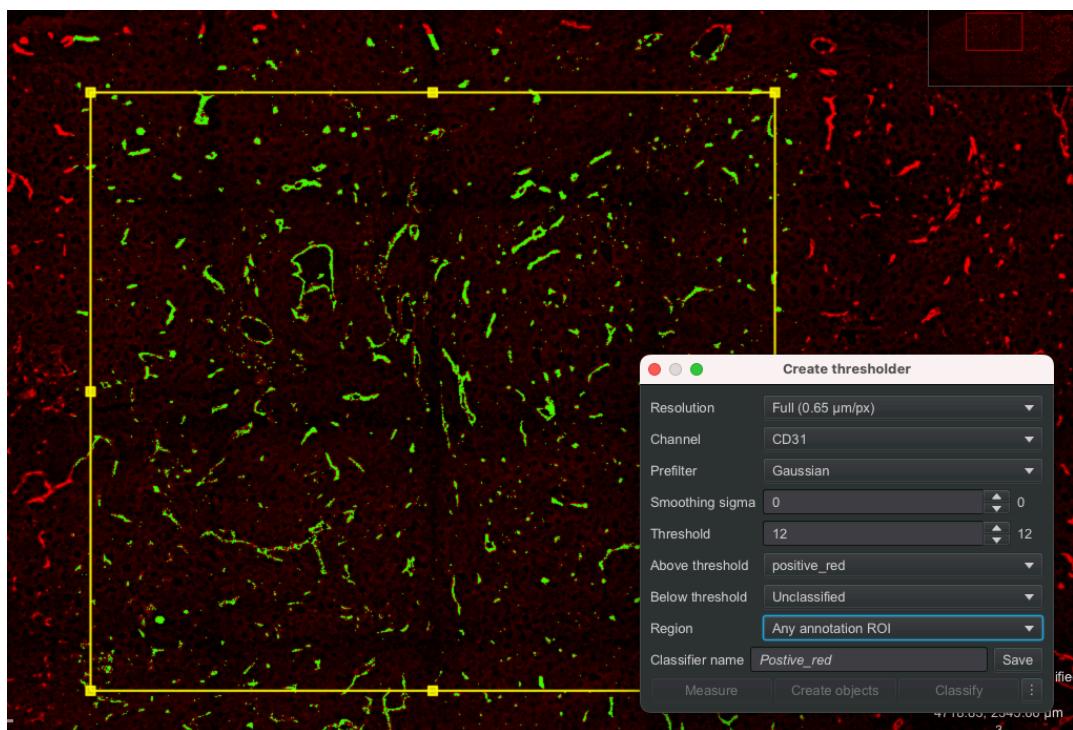


Figure 64. Configuring a pixel classifier

**\*\*Visualization Tools:** As shown previously, you can show or hide pixel masks by clicking the **C** on the upper toolbar. You can also adjust mask opacity with the bar on its right. This is useful to see if you are ok with the pixels that are being selected.



- **Save the Model:** Enter `model_red` in Classifier name box and click on **Save**.

## 2. Create Objects as Annotations

- Click on **Create objects**.
- **Choose parent object:** On next window, you have the following options to select:
  - Current selection (the annotation you previously selected)

- All annotations
- Full image

Select Current selection and click **OK**

**Note:** if you had not previously selected your annotation, you would only have All annotations and Full image. Creating and selecting annotations is very useful to process just the regions you want and not all of them.

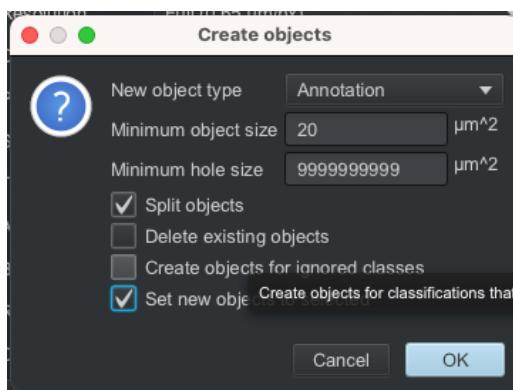


Figure 65. Entering sizes and other parameters for the creation of objects

- By choosing a **Minimun object size**, you will create objects only for groups of pixels with a minimum area of **20um<sup>2</sup>**, filtering out small detections.
- By entering a very big **Minimun hole size**, any holes will be ignored (or filled), since all the holes inside the created objects will be part of that same object. If you wanted to keep holes, you could enter a smaller value here, according to the size of the holes you want to keep.
- **Check Split objects** option to create an object for each independent group of pixels.
- **Check Set new objects to selected** to make sure that the objects we are generating are selected right after being created.
- Click **Ok & close** the Create Thresher window.

**Note:** the vessels are now surrounded by yellow regions; these are the objects that have just been created. You can see the object list on the left panel, under the Annotations tab. All of them, except the rectangle, are selected, since we marked that option in the Create objects window. **Keep all of them selected now.**

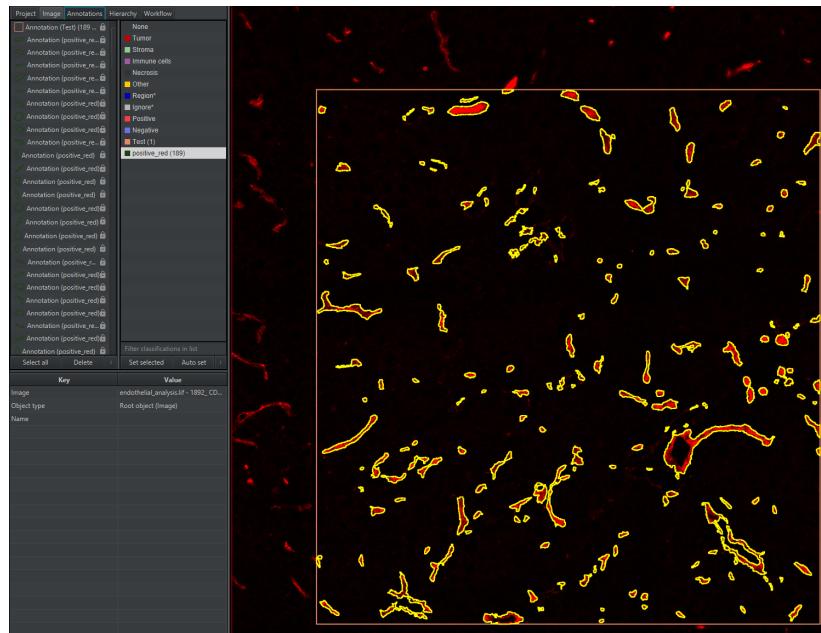


Figure 66. Annotations created with pixel classification tool. Notice that they are selected (yellow)

### 3. Expand Annotations

We perform this step because we have detected vessels using CD31 staining, but NG2 (pericytes) staining is surrounding CD31. So we need to expand CD31 annotations to be able to measure how much NG2 is around the vessels.

- Go to Objects > Annotations > Expand annotations

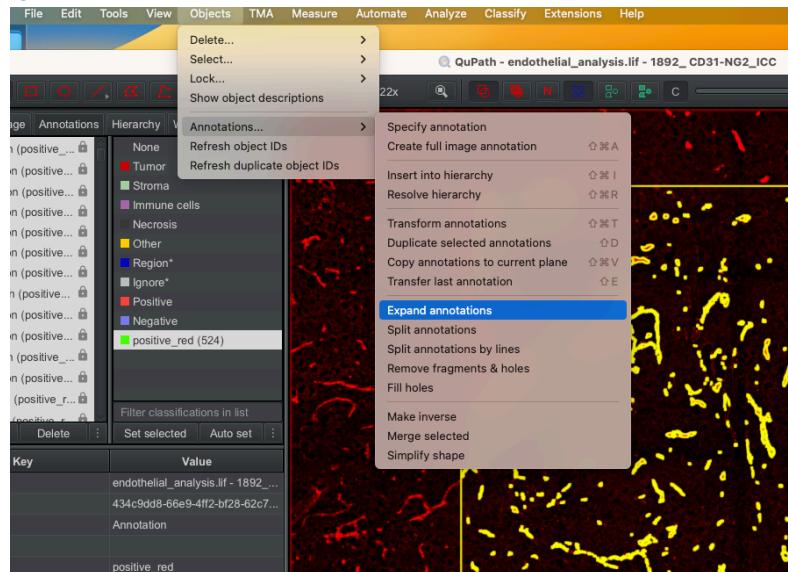


Figure 67. Expand annotations

- Choose an Expansion radius of 2 um.
- Leave Line cap on Round
- Set both Remove interior and Constrain to parent options unchecked.

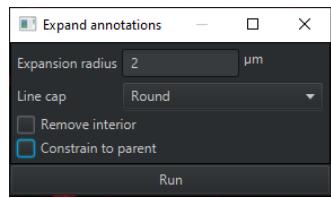


Figure 68. Expand annotations dialog window

- Click on **Run**.

**Be careful not to double click on the image!!!**

**Note:** you can see now the inner original annotations that you generated with pixel classifier, plus external green annotations surrounding them. You can still move around and zoom in/out, but **be careful not to double click on the image!**

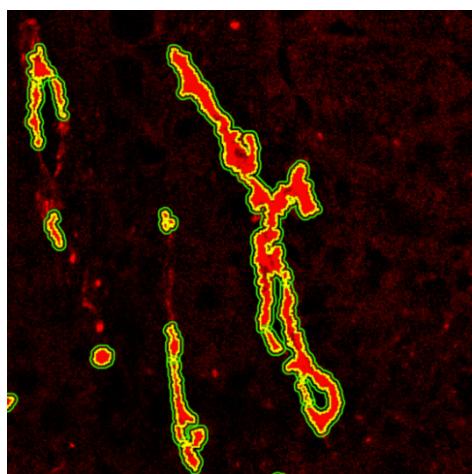


Figure 69. Original annotations (selected in **yellow**) and expanded annotations (displayed in **green**)

#### 4. Delete original annotations and keep expanded ones

- **Command List** (**Ctrl+L**): you can search for any command that QuPath has, and it is a fast way to use a command instead of searching for it navigating through all the menus.  
Keep this in mind for future uses.
- **Search for delete** in the Command list search box
- **Click on Delete selected objects**
- **Click on Yes.**

Now, you deleted the original annotations generated with pixel classifier, but you kept the expanded annotations around each one of them. We will see an alternative and more convenient way of doing this by scripting later.

## 5. Pericytes Detection within the Vessels

### 1. Create Thresholder

- **Channel Tool**  : **Uncheck CD31** (Channel 3) and **check Pericytes** (Channel 2). Adjust the histogram according to your preference, in order to see green staining properly.
- We are going to create a new class “**positive\_green**”. **If this class already exists, you can ignore this step.** Go to **Annotations tab > click on the three dots next to Auto set > select Add/Remove > Add class > name it “positive\_green”**.
- Go to Annotations tab and **right-click on positive\_red class**, and click **Select objects by classification** from the list
- Enter the keyboard command **Ctrl+L** and search for “**thresholder**”. Double click on **Create thresholder**. This is another way of accessing the creation of a pixel classifier by command search.
- Note:** In fluorescence, your staining should be brighter than the background (more intense pixels, so higher values). Keep this in mind to understand how thresholds work. This was explained with practical examples when creating thresholder for CD31 channel (red channel).
- Enter the following parameters:

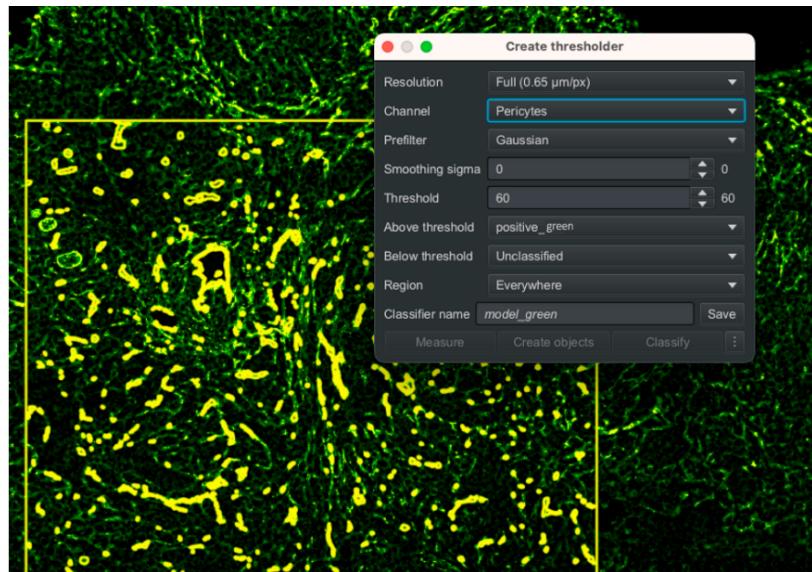


Figure 70. Pixel classifier configuration for green channel (pericytes)

**Note:**you can hide/show pixel masks with **C** on the upper toolbar to select the right parameters.

- Save the model: Enter “**model\_green**” inside Classifier name box. Click on **Save**. **Do not close thresholder window!!**

## 6. Area Measurements based on Pixel Classification

### 1. For Pericytes

- Make sure that the annotations are still selected (they must be displayed in yellow). If not, use **Select objects by classification** strategy, as explained before.
- Click on **Measure** inside Create thresholder window.
- On Select objects, choose Current selection
- Click **OK**
- Close thresholder
- Double-click inside any of the annotations

**Note:** below annotation list, you can see some data related to that annotation. The measurement we have just generated on the previous step is “*model\_green: positive\_green area  $\mu\text{m}^2$* ”.

Key	Value
Image	endothelial_analysis.tif - 1892...
Object ID	7e15a02c-bd21-4a11-9e22-d...
Object type	Annotation
Name	
Classification	positive_red
Parent	Root object (Image)
ROI	Geometry
Centroid X $\mu\text{m}$	3809.2469
Centroid Y $\mu\text{m}$	2202.876
model_green: positive_red ar...	9.7175
Area $\mu\text{m}^2$	85.7894
Perimeter $\mu\text{m}$	93.0015

Figure 71. Visualizing measurements of an annotation. We have generated green area measurement.

### 2. For Vessels

- Select all the “positive\_red” annotations as explained before (**Select objects by classification**).
- Go to **Classify > Pixel classification > Load pixel classifier**. This is a way of loading and using a pixel classifier that you have previously saved.
  - **Select model\_red**
  - **Click on Measure**
  - **Choose Current selection as Select objects**
  - Click **OK**
- **Close Load pixel classifier** window
- **Double-click inside one of the annotations**

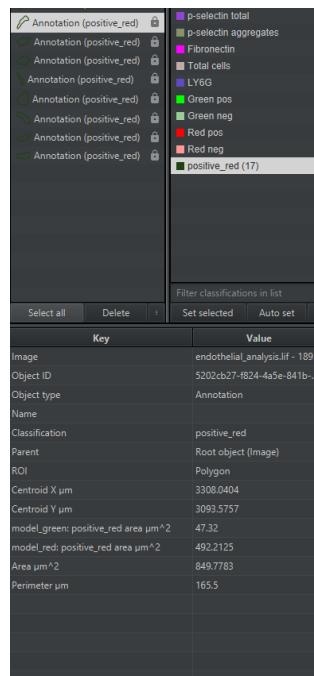


Figure 72. Visualizing measurements of an annotation. Now we have both green and red area measurements.

**Note:** We have just generated a measurement of the area of the red positive pixels within the vessels (red classifier that we used at the beginning to create objects from annotations). Now, we have measurements of red-positive (vessels) and green-positive (pericytes) areas for each annotation that we generated for each vessel.

**Calculate whole tissue area:** To calculate vascular density (vessel area normalized by tissue area) we need to measure the whole tissue area. We will not do this as part of this practice. But just for your information, we can manually annotate the tissue or use any of the channels to perform tissue detection using a Pixel Classification. This procedure was explained in [exercise 1, section 3](#). The only difference is that in this case, we select any of the channels to do the Pixel Classification instead of Average Channels. For this purpose, we have to use a very low threshold value (but being careful that it is not so low that we detect the whole image instead of just the tissue), very big Minimum size number and large Minimum hole size, to detect the whole tissue.

## 7. Review & Export Results

- Select **Measure > Show annotation measurements**. Or [click on the table icon as shown on the image below > Show annotation measurements](#). You will see all the annotations you have generated, and all the information associated with them, including green and red measurements we have just created.



Annotations: endothelial_analysis.tif - 1892_CD31-NG2_ICC									
Thumbnail	Image	Object type	Name	Classification	Parent	ROI	Centroid X µm	Centroid Y µm	model_green: positive_red area µm
	endothelial_analysis.tif - 1892_CD31-NG2_ICC	Annotation		positive_red	Root object (Image)	Geometry	3500.2	3371.9	21.55
	endothelial_analysis.tif - 1892_CD31-NG2_ICC	Annotation		positive_red	Root object (Image)	Geometry	3827.4	2576.9	31.26
	endothelial_analysis.tif - 1892_CD31-NG2_ICC	Annotation		positive_red	Root object (Image)	Geometry	3399.1	2258.9	2.112
	endothelial_analysis.tif - 1892_CD31-NG2_ICC	Annotation		positive_red	Root object (Image)	Geometry	4520.6	3504.1	0
	endothelial_analysis.tif - 1892_CD31-NG2_ICC	Annotation		positive_red	Root object (Image)	Geometry	4077	2935.9	2.957

Figure 73. Measurement table visualization.

- Measurement table: each thumbnail in the table is an object and by clicking it on the image or on the table you can review each object interactively.
- Export results: click on **Save**, to save a .txt file with all the data. This .txt file is tab separated and can be imported to Excel or any other data processing software, so you can further process your data there.

## Integration of the Pipeline for Vessels Detection into a Groovy Script for Batch Analysis

- Go to **Workflow tab** and see how, for example, **Select objects by classification** has appeared under **Command history**.
- Click on that and **note that** you can see in the lower table how this same operation is translated into a groovy command as **selectObjectByClassification("positive\_red")**
- You can easily and automatically generate code because QuPath will record and translate almost everything into code.
- Here, you can either select **Create workflow** or **Create script**. The first option allows you to choose which commands you want to keep before translating them into a script. The second one directly translates the whole list into a groovy script, and you can also edit from here to eliminate all operations that you do not need.
- Click on **Create workflow**. You can select one or more commands, right-click and select **Remove selected items** to eliminate all the commands you do not want into your code. Let's keep the ones below (so delete the rest by selecting them and removing them):
  - Select objects by class: **selectAnnotations();**

- Pixel classifier create annotations:  
`createAnnotationsFromPixelClassifier("modelRed", 20.0, 9.9999999E8, "SPLIT", "SELECT_NEW")`
- Expand annotations: `runPlugin('qupath.lib.plugins.objects.DilateAnnotationPlugin', {"radiusMicrons":2.0,"lineCap":"ROUND","removeInterior":false,"constrainToParent":false})`
- Delete selected objects: `clearSelectedObjects(true);`
- Delete selected objects: `clearSelectedObjects();`
- Select objects by classification:  
`selectObjectsByClassification("positive_red");`
- Pixel classifier measurements:  
`addPixelClassifierMeasurements("model_green", "model_green")`
- Pixel classifier measurements: `addPixelClassifierMeasurements("model_red", "model_red")`
- Save annotation measurements:  
`saveAnnotationMeasurements('/C:/Users/Analysis-User/Desktop/QuPath_course/endothelial_analysis/output/results.csv')`

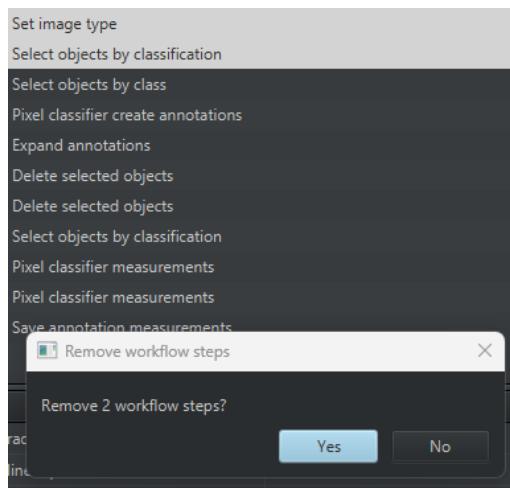


Figure 74. Removing commands from list before translating to script

- Click on **Create script**
- **If you are OK with your exporting path, skip this step.** Once you have the whole script, you must **enter the path** into the `saveAnnotationMeasurements`, with `''` and with `/` instead of `\` as separator.

For example: `saveAnnotationMeasurements(" /C:/Users/Analysis-User/Desktop/QuPath_course/endothelial_analysis/output ")`.

```

1 selectAnnotations();
2 createAnnotationsFromPixelClassifier("model_red", 20.0, 1.0E25, "SPLIT", "SELECT_NEW")
3 runPlugin('qupath.lib.plugins.objects.DilateAnnotationPlugin', {'radiusMicrons':2.0,'lineCap':'ROUND','removeInterior':false,'constrainToParent':false})
4 clearSelectedObjects();
5 clearSelectedObjects();
6 selectObjectsByClassification("positive_red");
7 addPixelClassifierMeasurements("model_green", "model_green")
8 addPixelClassifierMeasurements("model_red", "model_red")
9 saveAnnotationMeasurements('/C:/Users/Analysis-User/Desktop/QuPath_course/endothelial_analysis/output')

```

Figure 75. The script should look like this

- If your goal is to analyze the full image instead of just a defined region, you can go to **Objects > Annotations > Create full image annotation**. And then, this can be translated to code the same way by `createFullImageAnnotation(true)`. Go to **Workflow tab > right click on Create full image annotation > select Copy command**.
- Go to your script and add `//` to the beginning of the line `selectAnnotations()`. This will comment on that line of the script, and that line will do nothing when we run the script. This is a useful way to eliminate some actions for testing, and it is particularly useful to add explanations so that other people that may use your script, have some important information or instructions.
- Click enter to create a new line below this and paste the command you copied `createFullImageAnnotation(true)`.

```

1 //selectAnnotations();
2 createFullImageAnnotation(true);
3 createAnnotationsFromPixelClassifier("model_red", 20.0, 1.0E25, "SPLIT", "SELECT_NEW")
4 runPlugin('qupath.lib.plugins.objects.DilateAnnotationPlugin', {'radiusMicrons':2.0,'lineCap':'ROUND','removeInterior':false,'constrainToParent':false})
5 clearSelectedObjects();
6 clearSelectedObjects();
7 selectObjectsByClassification("positive_red");
8 addPixelClassifierMeasurements("model_green", "model_green")
9 addPixelClassifierMeasurements("model_red", "model_red")
10 saveAnnotationMeasurements('/C:/Users/Analysis-User/Desktop/QuPath_course/endothelial_analysis/output')

```

Figure 76. Final version of the script

- Save the groovy script with **File > Save**
- Now you can **save this groovy script in the script folder inside your QuPath project folder**.
- Once the script is ready, you could directly click on Run and it would run **JUST** for the image opened in the viewer. **DO NOT DO THIS STEP!!**
- Open command search window (**Ctrl+L**) and search for **Delete all objects. Double click** to apply it. This will delete all objects (annotations and detections, in case of having just annotations).
- Go to Project tab and **double click on the other image**. When asked about saving changes, click on **Yes**.
- Go to **Brightness & Contrast** tool and **set for the channels the exact same names you put in the other image**. This is very important, since the classifiers were created linked to a particular channel name. If you followed the guide, the names should be **Nuclei** for Channel 1, **Pericytes** for Channel 2 and **CD31** for Channel 3. There is a way to automatically change channel names and colors by script commands, but not by QuPath interface tough. As most of the things, you can search on Internet how to do it if you need it at some point.

- Right click on the image > select Multi-view... > Close viewer. When asked about saving changes, click on Yes.
- For running the script in batch mode for some or all the images you have inside your QuPath Project, you can click the three vertical dots and select Run for project.

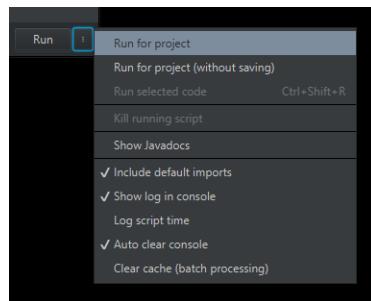


Figure 77. Run for project

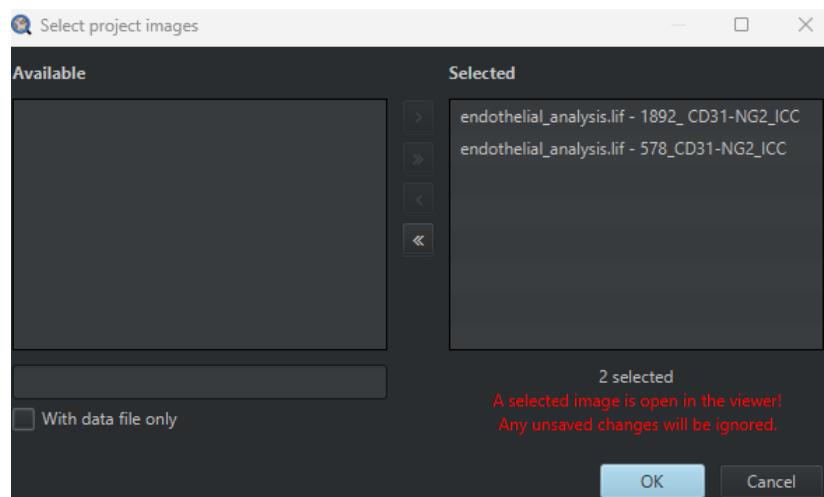


Figure 78. Select project images to be analyzed in batch

- In the window that has just opened, you must move to the right table the images that you want to analyze. And then click on OK and that's it, batch analysis will start.
- After processing is completed, you can open any image and check the results on it. You can see all the generated masks and measurements associated with them. And all the data is already exported to the directory you defined in the path.
- **\*\*ADDITIONAL STEP:** Let's see a more robust script: drag and drop the script named **script\_curso\_FINAL.groovy** (Desktop\QuPath\_course\endothelial\_analysis) to the Script Editor. We will explain to you why this script is more robust.

## Exercise 4. Cell Phenotyping and Spatial Analysis of Multiplexed Images

- **DATA** ▶ Highplex image with 11 cellular markers.
- **GOAL** ▶ Phenotype each cell type into different immune cell populations (we will work with CD3 and CD8a in this exercise) and study their spatial distribution by calculating their distances to the tumor border and among other immune cells. Visualization of complex spatial data with density maps.

### 1. Create a Project

- Open QuPath.
- Go to Project tab
- Drag and drop the “project.qpproj” file located in “QuPath\_course\Spatial Analysis\QuPath\_project” to QuPath main GUI (Graphical User Interphase)
- You will see an error window asking you to update the path to the images. In this case, QuPath should be able to automatically find the new path, so to the right of the red errors, you should see in white the updated path. [Click on Apply changes](#). In a case where QuPath was not able to do this automatically, you would have to click on Search and add the updated path to the folder with your images.

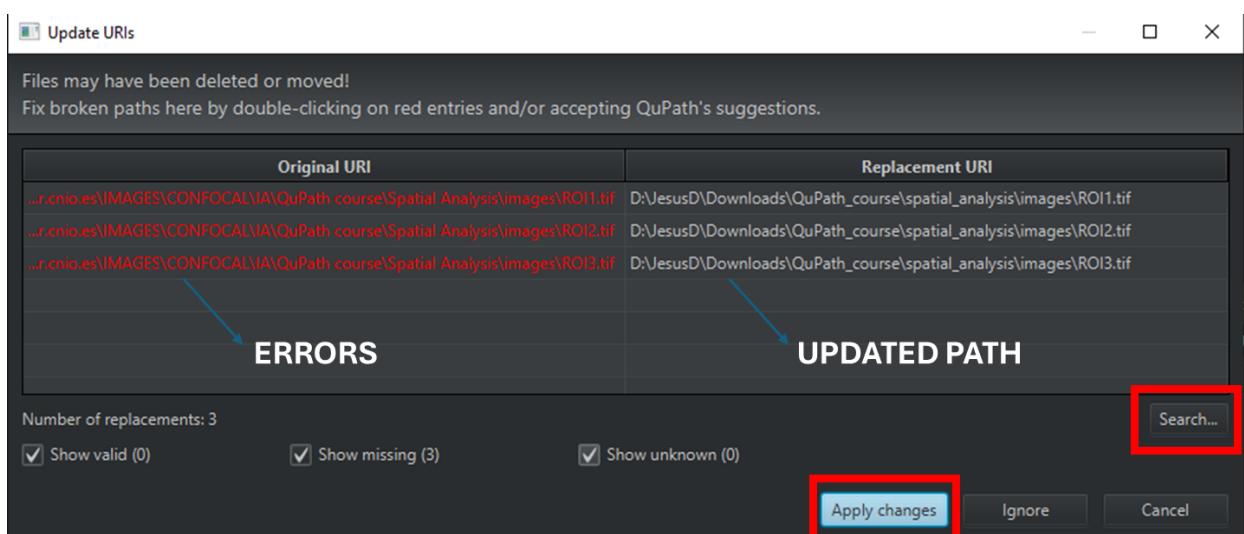


Figure 79. Updating path to the images

- Go to Project tab and make sure the thumbnail of the image you want to visualize is displayed; if so, you can double click on it and wait until it shows on the QuPath

viewer. By default, the first image on the list will be automatically displayed. **Double click on ROI2.tif** to open it.

## 2. Nuclei & Cell Detection

- Go to Brightness & Contrast and activate just DAPI channel.
- **Measure Nuclei Size:** Select Brush annotation tool and zoom in a nuclei. Draw the shape of a nuclei and make a filled shape.

**Note:** You can expand the annotation by drawing from the inside or **erode** it by holding Alt in the keyboard and drawing from the outside. If you want a **coarser brush**, zoom out; if you want a **finer brush**, zoom in. You can move around the image with the **paint brush** active by holding the spacebar and dragging on the image.

- **Draw some nuclei of different shapes and sizes.** Go to the annotation tab and **select one of them**. Look down to see area  $\mu\text{m}^2$  value. Check all of them. This gives you an idea of the mean, maximum and minimum size of the nuclei. **Remember** minimum and maximum size.
- **Select all the annotations and delete them.**
- Go to **Brightness and contrast** and **activate DAPI, CD3 and CD8a channels**.
- Create a full image annotation: **Ctrl+L and search for Create full image annotation**. In this case, the image is not so big so we can afford this for testing

**Note:** for larger images, it is better to create a small annotation for testing as previously done in exercise 3.

- Go to **Analyze > Cell detection > Cell detection**.
- Enter the values shown on the image below and click on **Run**. This is the best you can get with Cell detection based on threshold. It is far from optimal as you can see, but it is faster, and it may be enough depending on your needs.

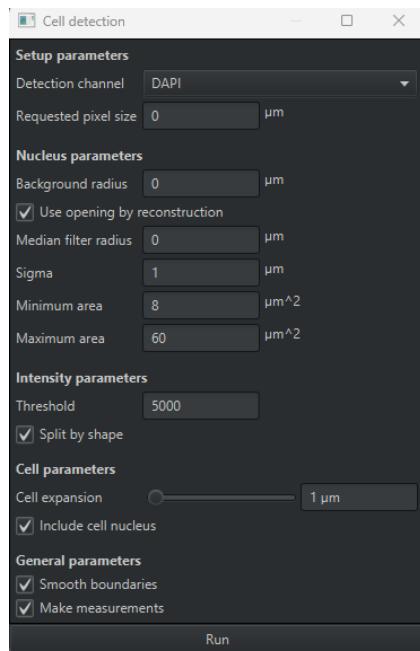


Figure 80. Parameters for cell detection in this exercise

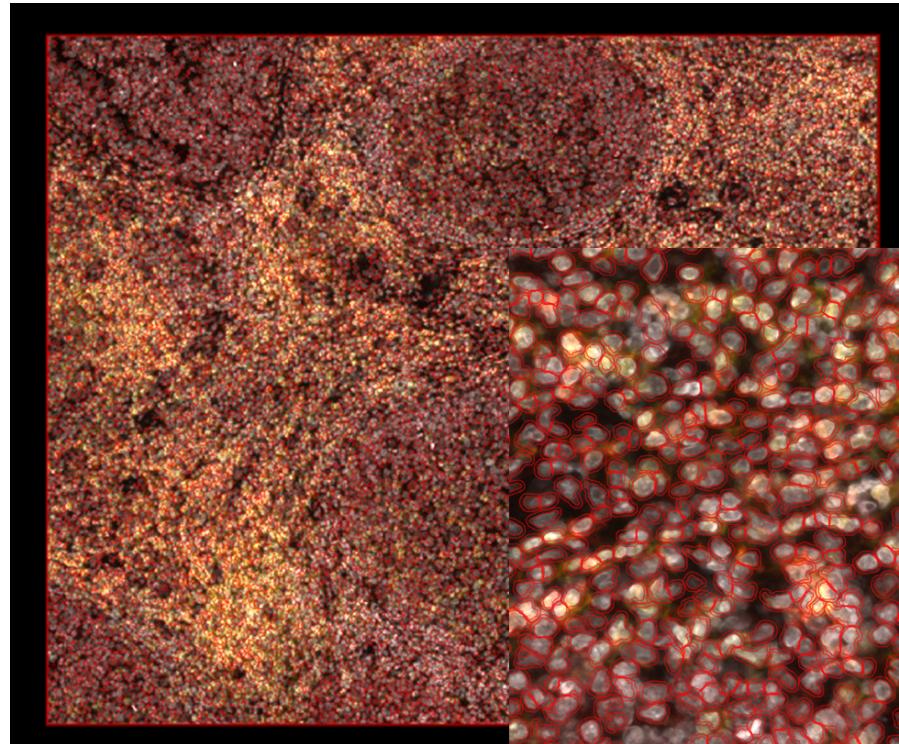


Figure 81. Masks of cells created after cell detection operation

### 3. Cell Phenotyping (Classify Cell Detections using object classifier)

- Go to Annotations panel, click on the three dots > Add/remove > Add class.

- (If these classes already exist, you can ignore this step) Create a CD8 and CD3 class.
- You can edit class color by double-clicking on it.

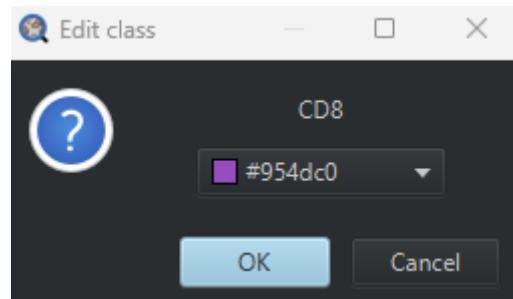


Figure 82. Changing class color

- Go to **Brightness and contrast** and activate just **CD3 channel**. **Adjust it** so you can see the staining properly.
- Now go to **Classify > Object classification > Create single measurement classifier**.
- **Create CD3 Cell Classifier:** Add the values you see in the screenshot below. You should see the cells that are being classified as CD3 class, according to the chosen threshold.

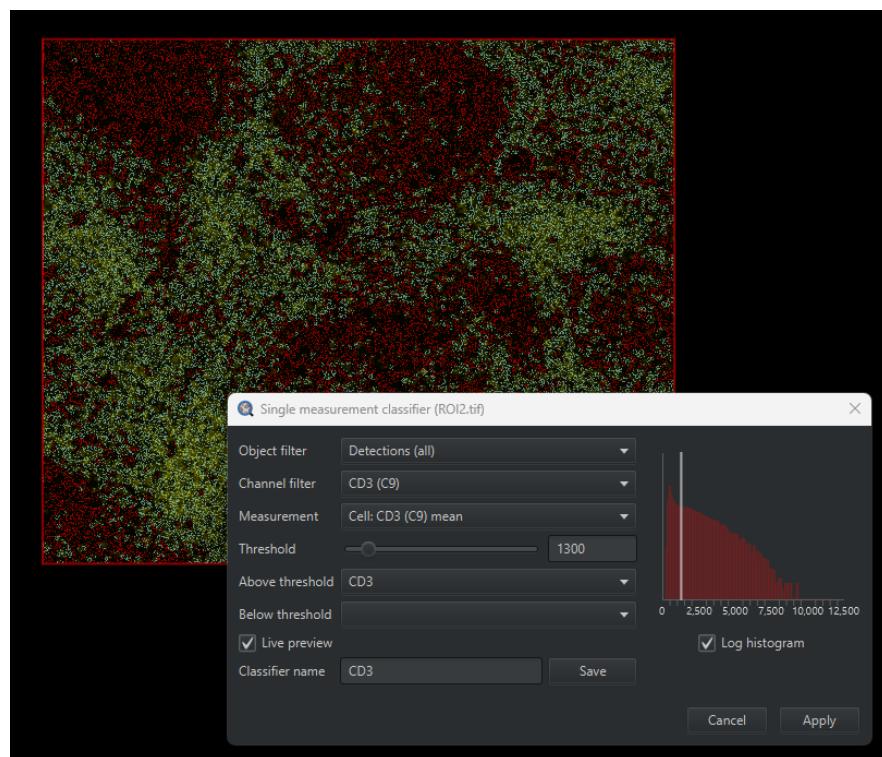


Figure 83. Object classifier configuration for CD3+ cells

- You can double-click any cell and check the intensity values (or any other calculated feature) in the lower part of Annotations tab. This is useful to know which value is best for cell classification.

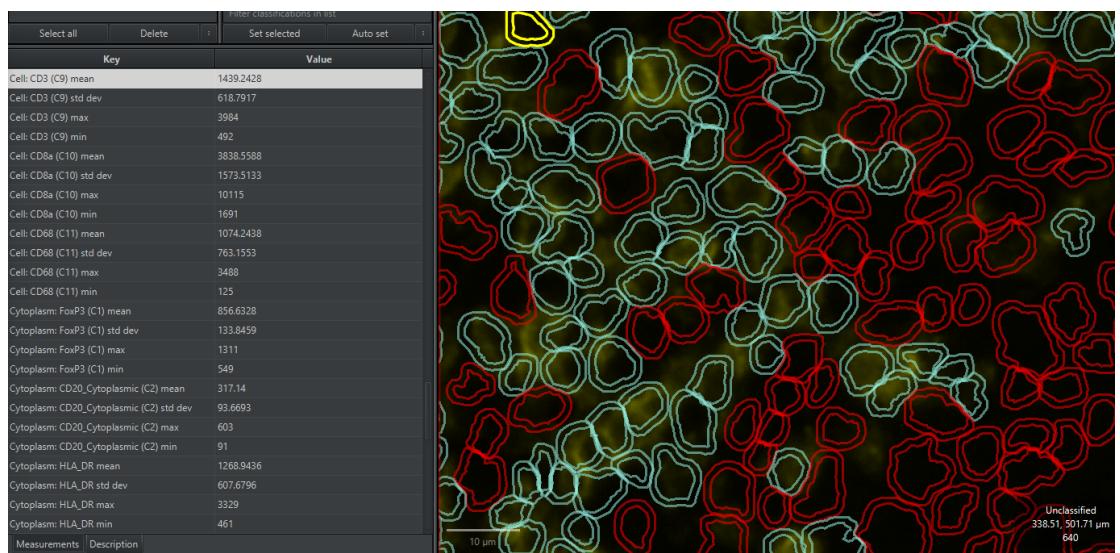


Figure 84. Visualizing measurements associated to a classified cell

- Click on **Save**, once you are happy with classification. You have just saved a **Cell Classifier**. We need to save it because we will combine it with another cell classifier and make a **Composite Classifier** for cell phenotyping.

**Note:** if you did not need to combine different cell classifiers, you would just click on **Apply**. That would run the classifier and save it. **Just bear in mind**, if you run another cell classifier after, the already-classified objects will be reclassified. To avoid that, we use the **Composite Classifier**.

- Go to Brightness and contrast and activate just CD8 channel. Adjust it so you can see the staining properly.
- **Create CD8 Cell Classifier:** Do the same as with CD3 classifier, but in this case, enter the following values:

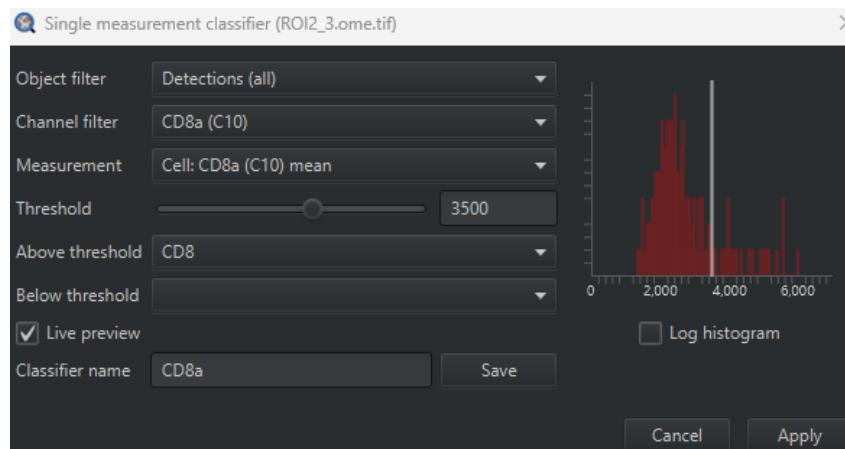


Figure 85. Object classifier configuration for CD8+ cells

- Enter **Classifier name** and click on **Save**.
- **Create Composite Classifier for CD3CD8 Cell Classifier:** Go to **Classify > Object classification > Create composite classifier.**
  - Use the arrows to **move the 2 classifiers to the right**. Enter **CD3CD8** in **Classifier name** and click on **Save & Apply**.

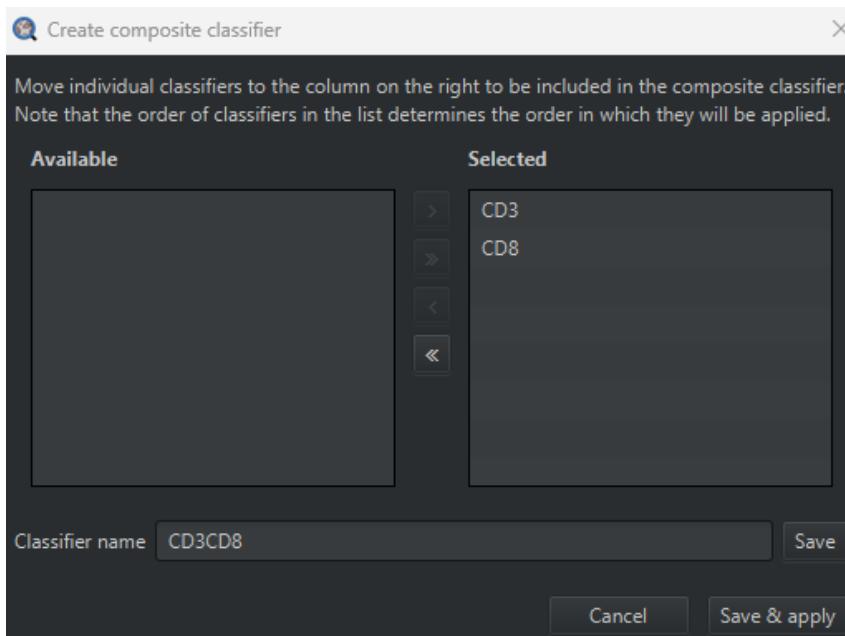
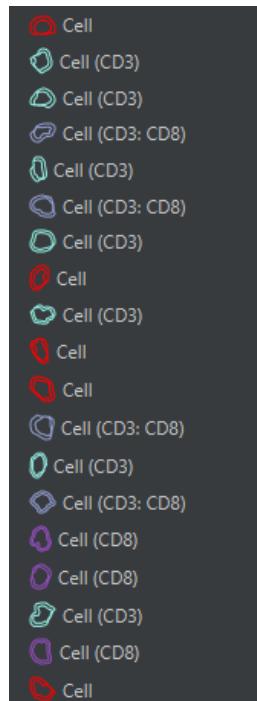


Figure 86. Choosing the single measurements classifiers used to create the composite classifier.

- If you go to **Hierarchy**, you can open Annotation and see all the cells with all the possible combinations derived from your classifier: **no class (double negative)**, **CD3**, **CD8** and **CD3:CD8**



*Figure 87. Cell classifications after running the composite classifier.*

**Note:** You can load and apply your already saved classifiers by Classify > Object classification > Load object classifier. If you select one, your objects will be classified according to the selected one. **Don't do it now, since it will reclassify all the objects!!!!**

**Check & Edit the thresholds in the classifiers:** You can see the values stored inside an Object Classifier by dragging the classifier you want to check from the Windows explorer (*QuPath\_project\classifiers\object\_classifiers*) to QuPath interface. All classifiers are automatically stored inside the QuPath project. You can edit the values from here and click on File > Save to overwrite it. This is the only way to edit an already-created classifier. So, you can follow this strategy to edit the classifier, then load it and apply it to see how the changes are applied.

**Note:** Composite classifiers are not linked to the individual single measurement's classifiers. So, if you wish to modify a composite classifier, you should load and modify the composite classifier, not the individual ones.

#### 4. Tumor Detection (Creating a Pixel Classifier to detect Tumor)

- Create a Pixel Classifier for tumor region
    - Leave only Pan\_Citokeratin channel active using Brightness & Contrast tool.
    - Select the annotation of the full image.
    - Go to Classify > Pixel classification > Create threshold (or Ctrl+L)

- Enter the values shown below and click **Save**. **Do not close the thresherder window!**

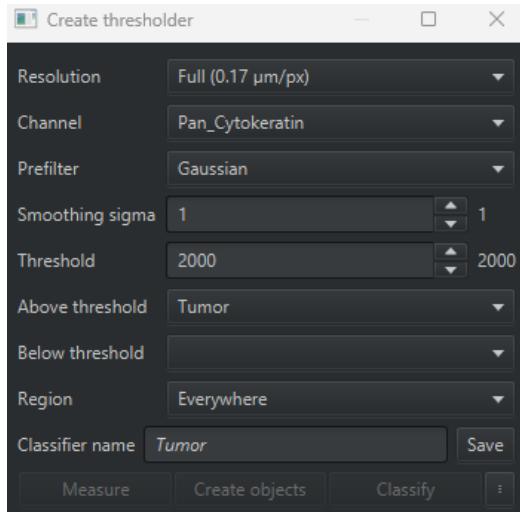


Figure 88. Pixel classifier configuration for tumor detection using Pan\_cytokeratin channel.

- Click on **Create objects**.
- Select **Current selection** and click on **Ok**.
- Enter the following values:**

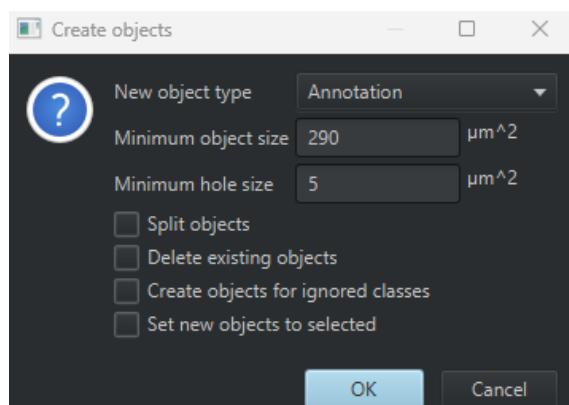


Figure 89. Create objects configuration for tumor detection.

- Close thresherder window.** Now you can see how the region for the tumor was created.

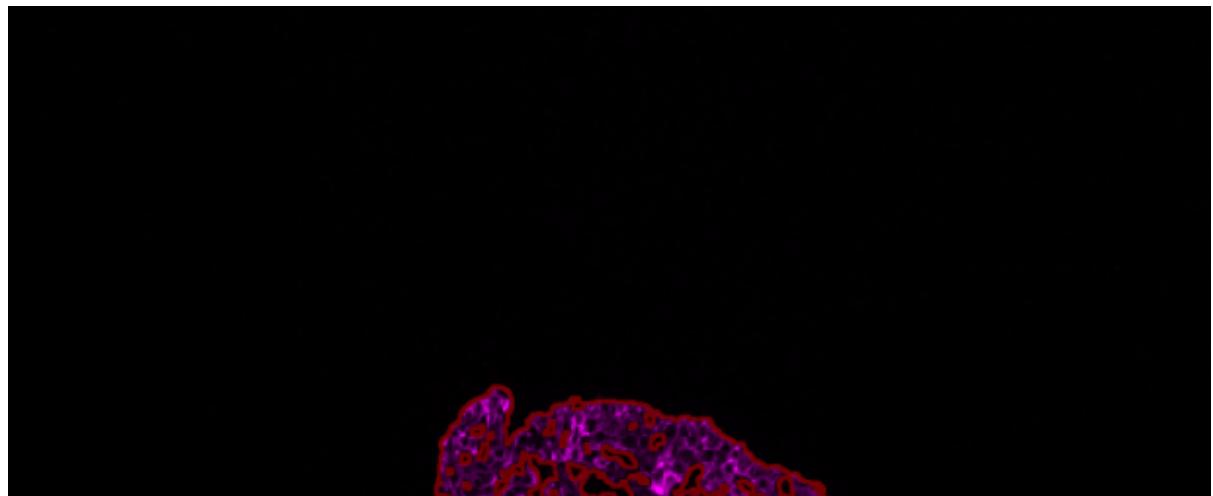


Figure 90. Tumor annotation after running pixel classifier.

## 5. Spatial Analysis: Calculating Cell Populations Distances to Tumor Border and Visualizing Density Maps

We can calculate distances from cells to annotations (tumor in this example), distances between cells and even cluster cells according to a chosen minimum distance. You can find all these tools under [Analyze > Spatial analysis](#). Everything is very well documented in [Help > Documentation](#).

### 1. Distance to the Tumor Border

- Go to [Analyze > Spatial analysis > Signed distance to annotation 2D](#)
- Click on [No](#) on next window

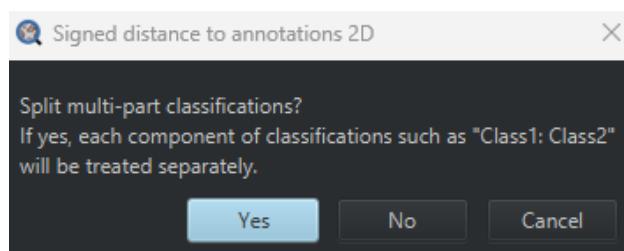


Figure 91. Do not split multi-part classification. We want to work with the compound classifications.

- [Go to Show detection measurements](#).



- The last column on the right shows the distance of every cell to the closest border of Tumor annotation. Since it is a signed distance, all the cells inside tumor will have a negative value. One of the first columns will show the cell classification. The results

provide therefore **the distance of every classified cell to its closest border of tumor annotation.**

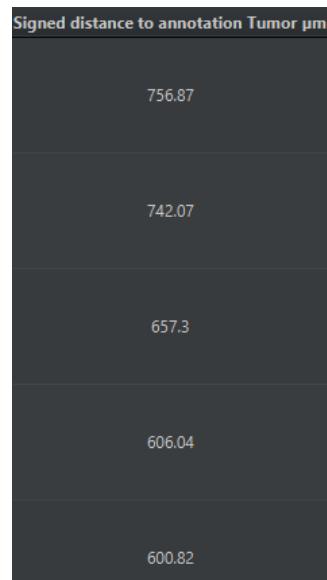


Figure 92. Distance of cells to tumor.

## 2. Distance to the Nearest Neighbor Cells

- Go to **Analyze > Spatial analysis > Detection centroid distances 2D**
- Click on **No** on next window. You have just calculated the distance of every cell to its nearest neighbor of all the other classes.
- **Go to Show detection measurements**



- You can visualize the results as explained before.

	Distance to detection CD3 $\mu\text{m}$	Distance to detection CD8 $\mu\text{m}$	Distance to detection CD3:CD8 $\mu\text{m}$
	60.32	50.92	115.03
	47.4	36.07	113.58
	0	70.24	140.51
	60.66	20.56	123.58
	67.86	13.85	118.44

Figure 93. Distance of each cell to its nearest neighbors of all the other classes.

### 3. Density Maps

- Go to **Analyze > Density maps > Create density map**. Select all detections and choose the Main class you want. You can adjust the Density radius to see a finer or coarser map. You can also select a Secondary class to visualize a heatmap of cells that are double-positive to your markers of interest. You can also customize appearance.
- You can save these values by entering a name in **Save map box** and clicking on **Save**.

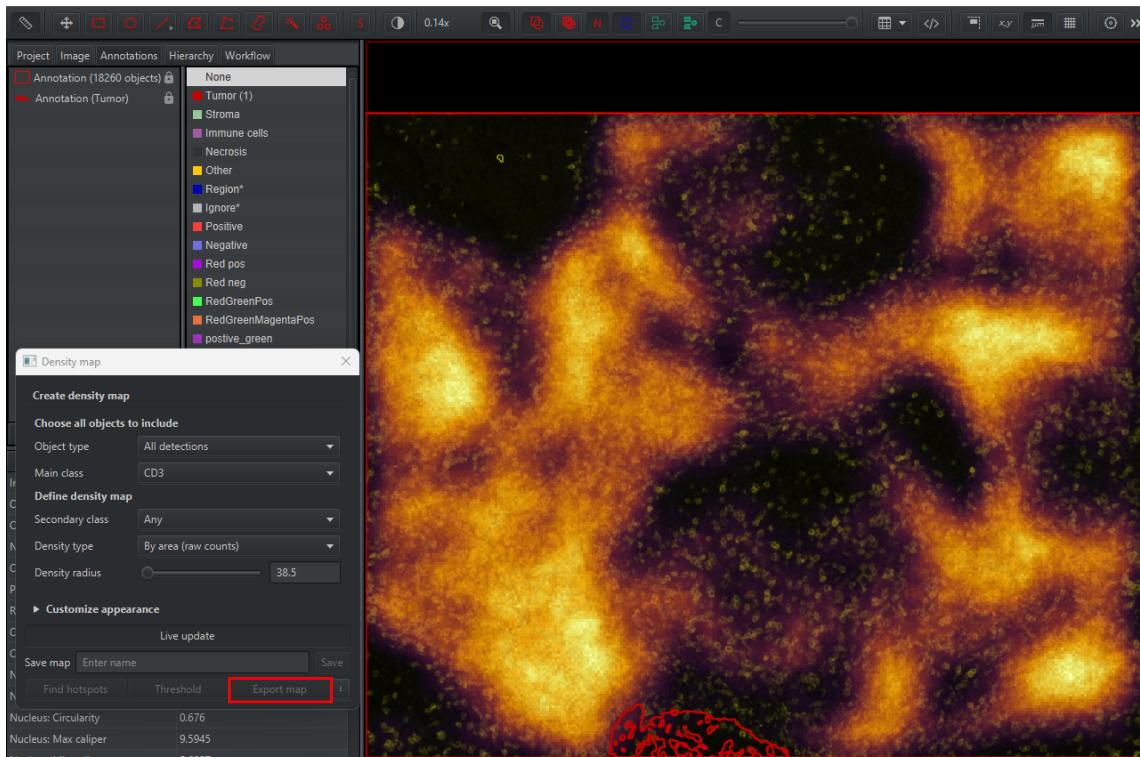


Figure 94. Example of a density map for CD3+ cells with a Density radius of 38.5.

- You can export this map by clicking on **Export map**.
- **EXTRA:** You can also find hotspots to create annotations based on the number of events that you chose in the selection of classes. You can decide how many groups of the chosen density must be together to be considered as Hotspot with **Min object count** value, and how many hotspots you want to create with **Num hotspots**. Click on **Apply** and then, you will see the annotations from Hotspots within the **Annotation tab**.

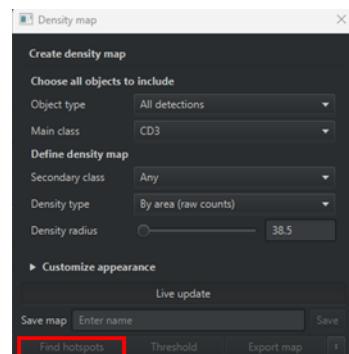


Figure 95. Find hotspots in Density map.



Figure 96. Find hotspots configuration.

- **EXTRA:** Click on **Threshold**, then enter a number and click on **Apply** to create annotations based on the density of your selected classes.

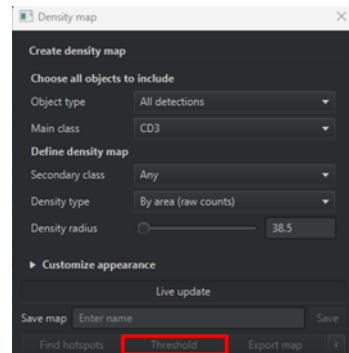


Figure 97. Create annotations based on a Threshold applied to the Density map.



Figure 98. How applying a Threshold to a Density map looks on live mode.

- Finally, to export all data, go to **Show annotation measurements** and click on **Save**

	ROI2.tif	Annotation	Hotspot 4	CD3	Annotation	Ellipse	296.31	703.29	94
	ROI2.tif	Annotation	Hotspot 2	CD3	Annotation	Ellipse	128.52	367.71	90
	ROI2.tif	Annotation		CD3	Annotation	Geometry	505.36	460.69	8177
	ROI2.tif	Annotation		CD3	Annotation	Geometry	528.82	456.79	1119
	ROI2.tif	Annotation	Hotspot 5	CD3	Annotation	Ellipse	217.77	763.98	87

- Do the same for detection measurements.

## 6. Advanced Exercise: Deep Learning-based Segmentation with Cellpose

**For this part, we will gather in front of a computer to see how this works, please do not do try as not all computers can do it.**

To access this feature, you must first install Cellpose by following the steps described in (<https://cellpose.readthedocs.io/en/latest/installation.html>) and in Cellpose-QuPath extension (<https://github.com/BIOP/qupath-extension-cellpose>).

**Note:** while installation is not covered here, CMU workstations Rosalind and Poch-Gascon are pre-configured for using these tools. If you would like to install them on your own computer, we are happy to assist you. **Keep in mind** that AI-based segmentation performs significantly better if your computer has a dedicated NVIDIA GPU (graphics card). While it's still possible to run the process without one, it may be considerably slower.

- Delete all objects by following “**Ctrl + L**” > **Delete all objects**
- Go to **Extensions > Cellpose > Cellpose detection** script template. This will open a groovy script to segment nuclei with DL.
- All the values are displayed in the image below, but we will cover them one by one in the text below.
- Go to **Image tab** and check **pixel width** and **pixel height** (they should be the same). **Enter 0.17 on pixelSize**. If the image was not calibrated, you would see Unknown in **pixel width** and **height**, and you would enter 1 as **pixelSize**.
- In **channels** you must choose the exact **name of the channel you want to use for cell segmentation**. In this case, we will let this as **DAPI**, since that is the name of the channel for nuclei in our image. You can check the name opening Brightness and contrast window and searching for it.
- **Measure nuclei diameter:** select line annotation tool and draw the diameter of some nuclei. Check the length of these annotations as explained previously. Since the image is calibrated, you will see this distance in microns, and **you must convert it to pixels by dividing this value by pixel size** (pixel width or pixel height), since cellpose needs the approximate diameter in **pixels**.
- Delete all annotations.
- Go to **diameter**, uncomment this line (delete the first **//**) and enter 35. 35 pixels is the approximate diameter in pixels of a cell in this image.
- Go to **cellExpansion** and enter a value of 5.0. This is also in pixels.

- Go to `measureIntensity` and uncomment it as shown before.

```

23 def pathModel = 'cyto3'
24 def cellpose = Cellpose2D.builder( pathModel )
25     .pixelSize( 0.17 ) // 
26     .channels( 'DAPI' )
27 //     .tempDirectory( new File( '/tmp' ) )
28 //     .preprocess( ImageOps.Filters.median )
29 //     .normalizePercentilesGlobal( 0.1, 99 )
30 //     .tileSize( 1024 ) //
31 //     .cellposeChannels( 1,2 ) //
32 //     .cellprobThreshold( 0.0 ) //
33 //     .flowThreshold( 0.4 ) //
34     .diameter( 35 ) // M
35 //     .useOmnipose() // L
36 //     .addParameter( "cluster" ) //
37 //     .addParameter( "save_flows" ) //
38 //     .addParameter( "anisotropy", "3" ) //
39     .cellExpansion( 1.0 ) // P
40 //     .cellConstrainScale( 1.5 ) //
41 //     .classify( "My Detections" ) //
42 //     .measureShape() // P
43     .measureIntensity() // Add
44 //     .createAnnotations() // N
45 //     .simplify( 0 ) //
46     .build()

```

Figure 99. The parameters of this script should look like this.

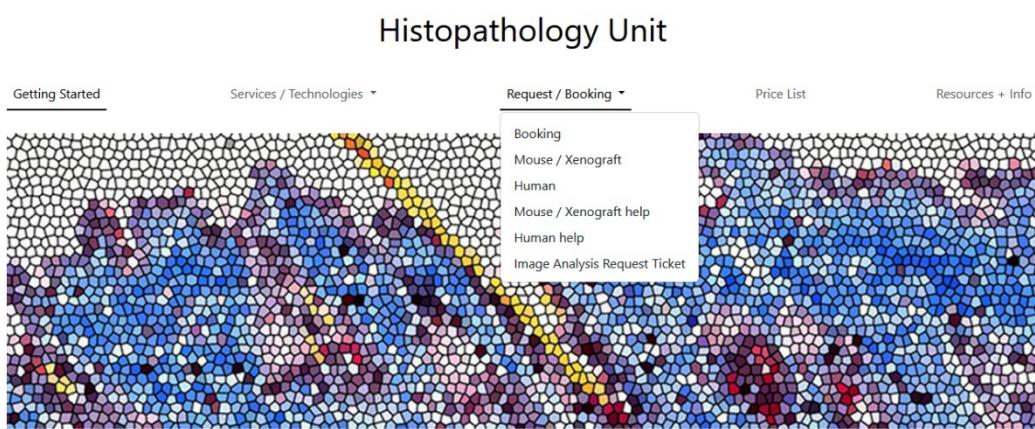
- **Draw** two square annotations close to each other and **select** one of them
- Click on **Run**.
- **Select** the other annotation and run a QuPath cell detection. You can easily re-run the one we ran previously going to the **Workflow tab**, looking for **Cell detection** command and **double-clicking** on that. This will open a **Cell detection** window with the parameters we used previously. You can click on **Run** and that will do the cell segmentation.
- Check the results with DAPI channel active and compare between both methods.
- The rest of the analysis (cell classification, distance analysis and density maps) is the same as explained above.

# Image Analysis Support Request

## For Brightfield Images (HPU)

### 1. Make an Image Analysis Request through the CNIO Intranet

- Enter the CNIO intranet and log in with your CNIO credentials. Once in click in **Core Units & Facilities > Histopathology > Request/Booking > Image Analysis Request Ticket**



### 2. Verify that you have access to the HPU Image Server in Bespin

- In the HPU we work with images located in the bespin server.
- Important Note:** it is not allowed to work with images stored on hard disks or even connect them to the workstations in the unit. Therefore, it is essential to save the images in the server, whether it is your group bespin folder or in the HPU Image Analysis Server.

Please, verify that you have access to: [\\bespin\Stage\Image\\_Analysis](\\bespin\Stage\Image_Analysis).

If you cannot access the Image\_Analysis folder request access to [histopatologia@cnio.es](mailto:histopatologia@cnio.es)

### 3. Book the desired Workstation for Image Analysis

- The workstations in the unit are computationally efficient and have installed multiple specific programs for image analysis. Therefore, it is useful to book them to do your analysis. For doing so, please enter in: **Core Units & Facilities > Histopathology > Request/Booking > Booking**

---

Histopathology Request

Requestor name Calvo de Mora Román, María	Requestor Group / Unit Microscopía Confocal	Requestor email MCALVO@CNOIO.ES
SAP Service Select a service	Units 0	Amount 0
Comments  		
<input type="button" value="Submit request"/>		

---

Once in the workstations:

- Log in with the analysis\_user:
  - **Workstation 1**
    - User: HP50061\analysis\_user
    - Password: cnio-HPU
  - **Workstation 2:**
    - User: HPW50062\analysis\_user
    - Password: cnio-HPU
- Once the session is open, access with your personal credentials to the bespin server where your images are located.

## For Fluorescence Images (CMU)

### 1. Request by LD Unidad Microscopía Confocal Email

[microscopia.confocal@cnio.es](mailto:microscopia.confocal@cnio.es) if meeting is required to:

- Help you to develop a comprehensive experimental design tailored to automated image analysis. It is essential to gather detailed information regarding **the experimental setup**, including **image acquisition** and the **analysis strategy**.
- To help you to define the **desired outputs** of the experiment.
- To decide whether it is best to **utilize commercial or open-source software** or **bespoke scripts** for data analysis.

### 2. Make a Biolimage Analysis Support Request through the Biolimage analysis Form

The link to the form will be sent by email after the meeting

The form consists of several input fields:

- User Name: Enter value here
- Email: Enter a name or email address
- Lab: Enter value here
- Image Analysis Options: Enter value here
- Channel-Marker Matching: Enter value here
- Image Analysis Description: Enter value here

- Fill the form with all the information requested. Please provide as much details as possible for us to provide you with the best image analysis strategy.
- Once the form is filled and submitted, your Image Analysis request will be added into the **Ticket System**

### 3. Verify that you have access to the CNIO Servers

- In the CMU we work with images located in the **Sacarino** or **Bespin** servers.
- CMU does not have now a specific server-point for user's image analysis.

Please to run your own analysis use your own Bespin or Sacarino for short temporal analysis.

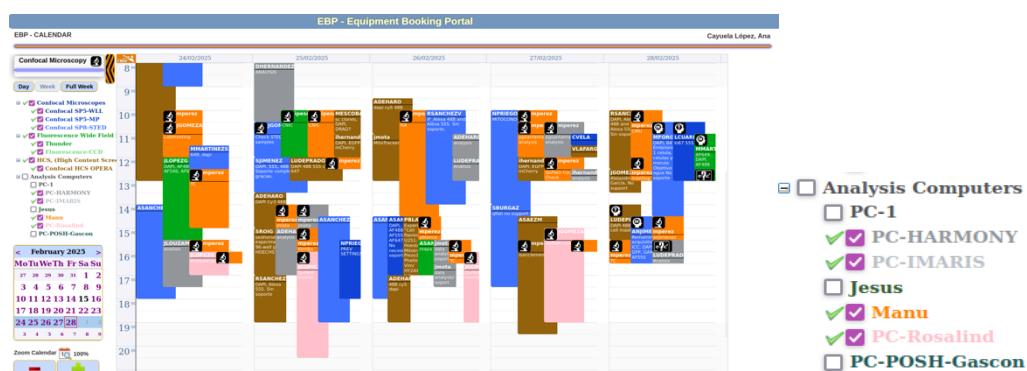
**Important Note:** it is not allowed to work with images stored on hard disks or even connect them to the workstations in the unit. Therefore, it is essential to save the images on the servers.

#### 4. Develop and image analysis pipeline in the specific software & Meeting for Reviewing

- Review results together and refine the pipeline, if needed.
- Your pipeline will be ready to use, and your reviews outputs delivered

#### 5. Book the desired Workstation for Image Analysis

- The workstations in the CMU are computationally efficient and have installed multiple specific programs for image analysis. Therefore, it is useful to book them to do your analysis. For doing so, please enter in: **Core Units & Facilities > Confocal Microscopy > Request/Booking > Booking**



- Log in with the `analysis_user` in all workstations **DO NOT USE YOUR PERSONAL CREDENTIAL**

User: `Analysis_user`

Password: `cnio-CMU`

##### ○ PC-Rosalind

- User: `AZW51689\analysis_user`
- Password: `cnio-CMU`

##### ○ PC-Poch-Gascon

- User: AZW52637\analysis\_user
- Password: cnio-CMU

Your images can be on your own Bespin server.