Fiji Lab Walkthrough

The Fiji Community

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CONTENTS

Information courtesy of Helen Wilson, Michael Nelson, Edward Evans, Ellen Dobson, Curtis Reuden, as well as well as many others involved in ImageJ/FIJI development and production of educational materials, walkthroughs, and demonstrations.

CHAPTER

ONE

INSTALLING IMAGEJ/FIJI

1.1 Download FIJI

FIJI has pre-installed plugins and is recommended for this lab. Download it at https://imagej.net/software/fiji/downloads. Look for the zip folder and extract the contents to the desired location on your computer. Keeping it in "Program Files" is generally not recommended to avoid access/security issues.

1.2 Installation

We're going to run the new Jaunch launcher, because it runs faster and allows running without security popups. If it doesn't show up within two minutes of running, then run the backup launcher.

1.2.1 Windows

- Right-click on `fiji-win64.zip` and choose **Extract All...** to `Downloads\` (avoid placing it in `Program Files`).
- Open the `Fiji64.app` folder.
- **New launcher**: Run `Fiji-windows-x64.exe` in that folder.
- Running for the first time may take about 60 seconds.
- **Backup launcher**: Run `ImageJ-win64.exe` in that folder.

1.2.2 Linux

- Unzip `fiji-linux64.zip`:

```
unzip fiji-linux64.zip
cd Fiji.app/
```

- **New launcher**:

```
./fiji-linux-x64
```

- **Backup launcher**:

```
./ImageJ-linux64
```

1.2.3 macOS

- Double-click `fiji-macos.zip` to extract.
- (Optional: copy `Fiji.app` to Desktop.)
- Right-click on `Fiji.app`, choose **Show Package Contents**.
- You will see a folder containing another `Fiji` icon inside it.
  Double-click the **second** `Fiji` icon to launch.
- If it doesn't work, see backup instructions below.

Backup Instructions for macOS

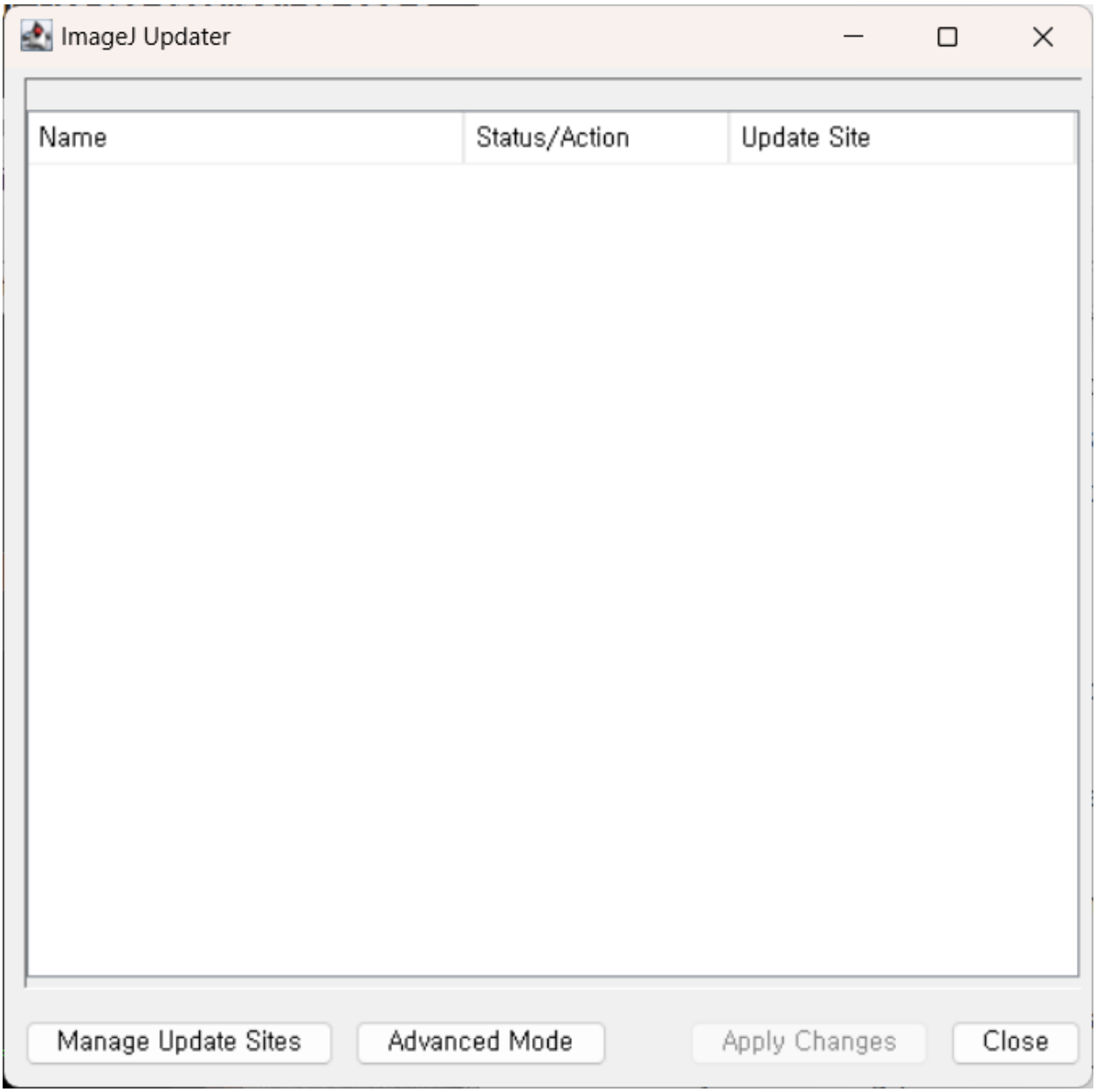
- Double-click `fiji-macos.zip` to extract.
- (Optional: copy `Fiji.app` to Desktop.)
- Try opening `Fiji.app`. This will likely be rejected by macOS for security reasons.
- Open **Apple Menu > System Settings**.
- Search for **Gatekeeper**.
- You should see the text
  "Fiji.app was blocked…" - click **Open Anyway**.
- Enter your password or use your fingerprint if prompted.
- Click **Open**.
  After this, you should be able to open `Fiji.app` directly.

1.2.4 A Side Note on Updating

When using FIJI, it is recommended to use **Help > Update** rather than **Help > Update ImageJ**.
Update ImageJ will specifically update only the base ImageJ package within FIJI, not the associated plugins.
This makes it more likely that you will run into version issues and other update problems.
Generally, updating when prompted upon opening FIJI will also help prevent issues.

1.3 Install Plugins with Update Sites

1. After running **Help > Update**, there will be an option to **Manage Update Sites**.
   This provides access to common plugins that are compatible with FIJI. Use the checkbox to add a plugin, then click **Apply and Close**.



2. Click **Apply Changes**, then restart FIJI.
3. You should now be able to search for and run the plugin.

1.4 Downloading a Practice Dataset

The complete list of files may be downloaded at the following links:

Google Drive: https://drive.google.com/drive/folders/1lgn-S5fZZZX0mwou23S_f6d3dG2Di64g?usp=drive_link

This manual: https://brp-optics.github.io/fiji_lab_walkthrough/installation.html

I also have a USB drive with all the resources on it, should anyone not have internet access.

1.5 Some usability options

I like to go into the menus and set the main FIJI window to be always on top, and the search tool to be Ctrl-L rather than **L**:

- Main window always on top: **Edit > Options > Appearance > IJ window always on top**
  - Note that one can "surface" the main FIJI window from any Fiji window by pressing **Enter**
- Change search tool: **Edit > Options > Search bar … > Pressing L focuses the search bar**

CHAPTER

TWO

COMMON KEYBOARD SHORTCUTS

Shortcut	Action
`+`	Zoom in
`-`	Zoom out
`Ctrl/Command + L`	Focus search bar
`Ctrl/Command + S`	Save
(No shortcut)	Save As
`Ctrl/Command + Shift + C`	Adjust Brightness and Contrast
`Ctrl/Command + Shift + Z`	Color channel control
`Ctrl/Command + Z`	Undo
`Ctrl + H` (Windows) `H` (Mac)	Open Histogram
`Ctrl/Command + K`	Plot / Line Profile
`Ctrl/Command + M`	Measure
`Ctrl/Command + Shift + T`	Threshold
`Shift`	Draw straight lines
`Alt + Arrow Keys`	Change current selection size
`T`	Open ROI (Region of Interest) Manager and add current selection as ROI
`Ctrl/Command + Shift + W`	Close all windows

2.1 Also Helpful

- **Window > Tile**
  Montages all open images/windows.
- **Image > Transform**
  Flip or rotate images.
- **Help > Update**
  Update FIJI.
- **Enter** (when focused on an image)
  Surfaces the ImageJ main toolbar window.

CHAPTER

THREE

GETTING STARTED, OPENING AN IMAGE

The easiest way to open a data set is to **drag and drop** it from its folder onto the Fiji main window. However, some file types need to be specifically imported. To import a sequence of images that are saved within a folder, you can use the **File > Import > Image Sequence** option to open them as a stack or as separate images.

Additionally, using a **Virtual Stack** in the **Bio-Formats Importer** may be useful if trying to view large data files. This makes it easier to get a quick look at data when there is limited RAM. However, if you need to perform an image operation, such as background subtraction, then more memory will be required as the data needs to be loaded.

Note

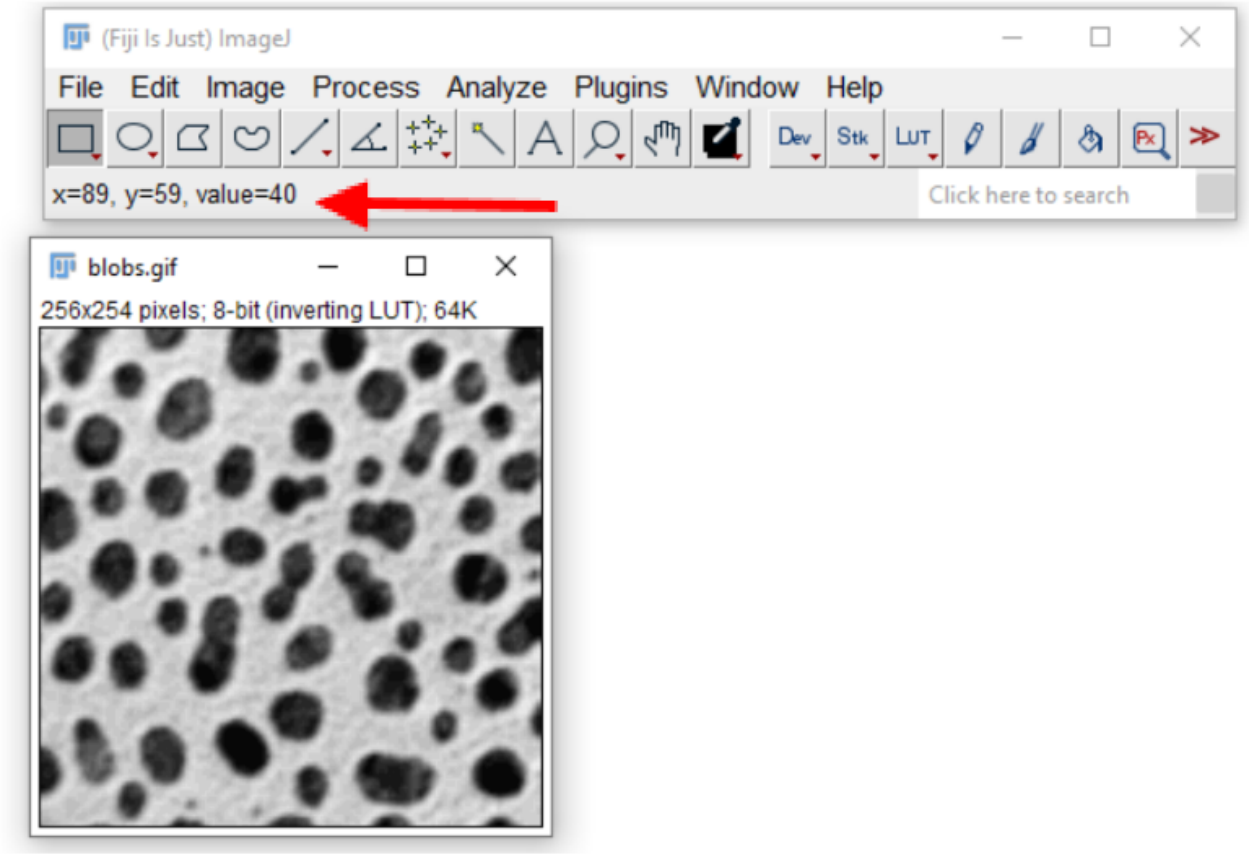
Pro tip: Press `Ctrl + L` (Windows) or `Command + L` (Mac) to jump straight to the search bar.
This can access nearly any menu command or plugin, usually faster than using the menus!

3.1 Starting with a basic image

Open “blobs.gif”, via **File > Open Samples > Blobs**, or **Ctrl + Shift + B**

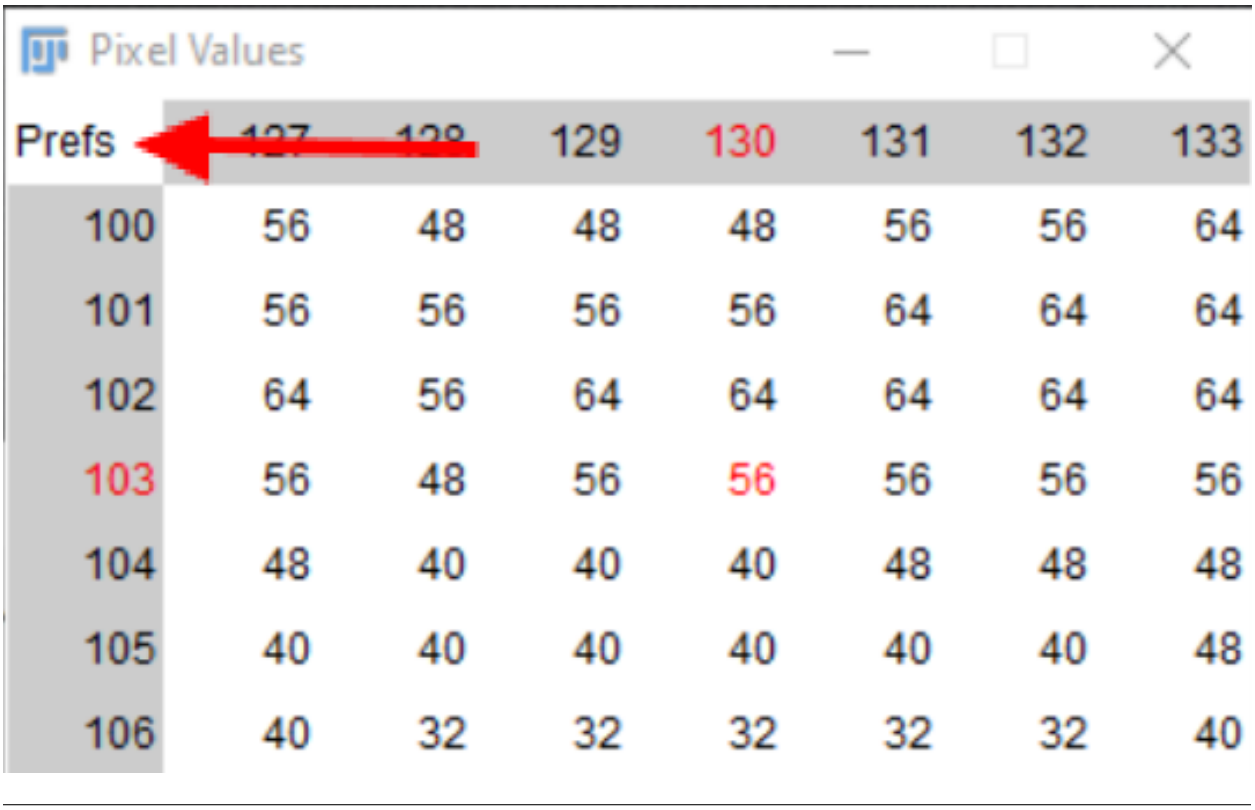
3.2 Inspecting Images

1. Hover over an area in the image.
   In the status bar of Fiji, you should see the pixel you are on, with XY coordinates, and its associated value.
   - In an RGB type image, Fiji will also tell you the associated color values for the red, green, and blue channels.



2. To see a larger area, use the **Pixel Inspection Tool**:
    - In the Fiji window, click the Pixel Inspection icon.
    - *If the icon is not present, you can use the double red arrows to add the icon, by opening the list and selecting* ***Pixel Inspector****.*

- This brings up a window of pixel values that can be moved around the image.
  To adjust the parameters of this window, click **Prefs**.
  This can be used to adjust the size of the window.



3.3 Inverting the Background

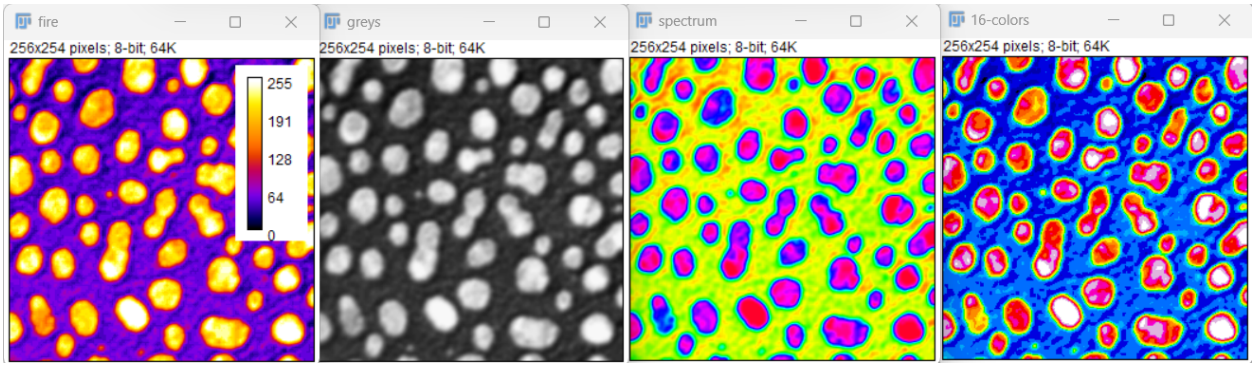
- Sometimes the background may be white instead of black, depending on what you are interested in within the image.
- The color scheme can be inverted by selecting **LUT > Invert LUT**.

  Note: if you use **Edit > Invert**, this will invert the actual image and change the pixel values themselves, which may not represent the original data.

3.4 Changing the Color Scheme (LUT)

A LookUp Table (LUT) controls the colormap of the images. This can easily be changed using the **LUT** button in the toolbar. Try a few LUTs and see how the information in the background and foreground changes.

1. Using **Image > Colors > Display LUTs** will display different types of LUTs.
2. It is also helpful to include a scale/calibration bar when changing LUTs.
   This can be added with **Analyze > Tools > Calibration Bar**.



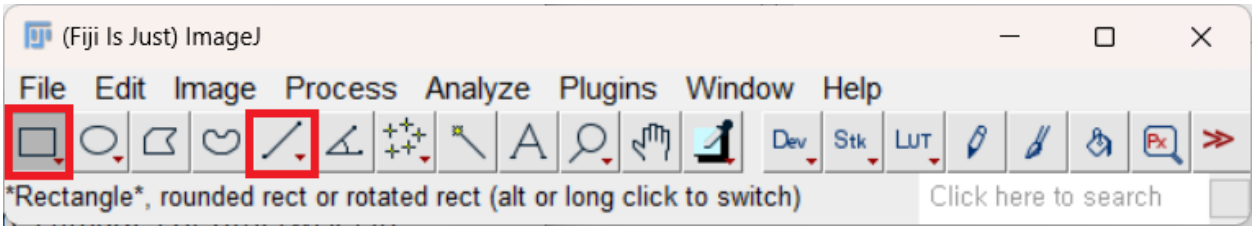
3.4.1 Colorblindness Consideration

To test how different images may appear to those with colorblindness, you can use the **Simulate Color Blindness** plugin if you have an RGB image.
`mpl-viridis` can be a good option as it is designed to be *perceptually uniform* (see https://imagej.net/imaging/visualization).

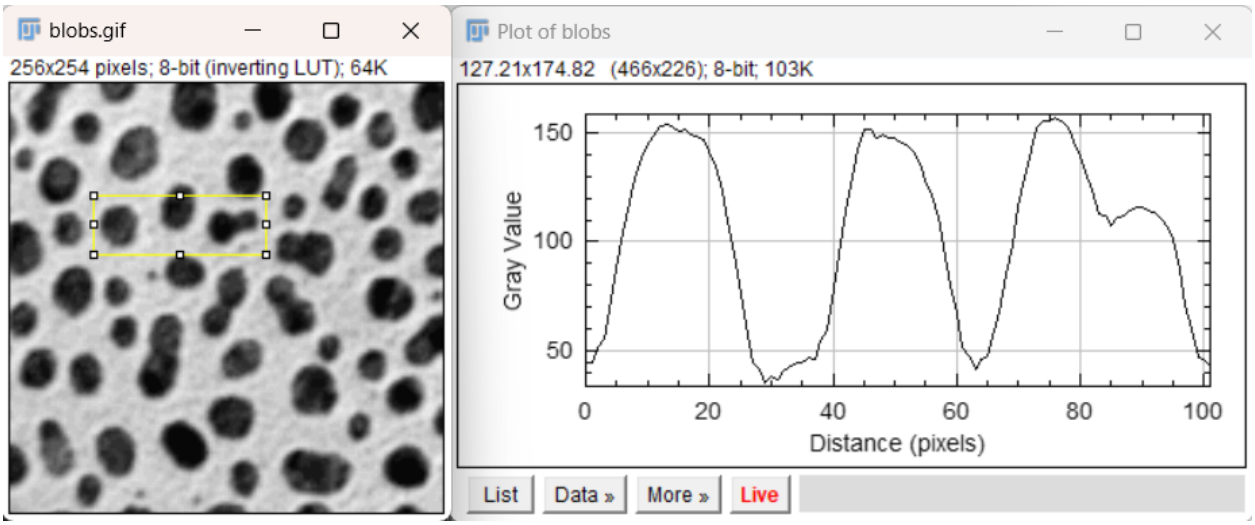
As a separate note, it may also be helpful to test any publication figures (images or otherwise) in grayscale, to see what color information may be lost if the paper is printed in grayscale. In many cases, it may be more practical to develop images/figures in grayscale, especially for presentations, and it is still a valid representation of the data.

3.5 Plot/Line Plot

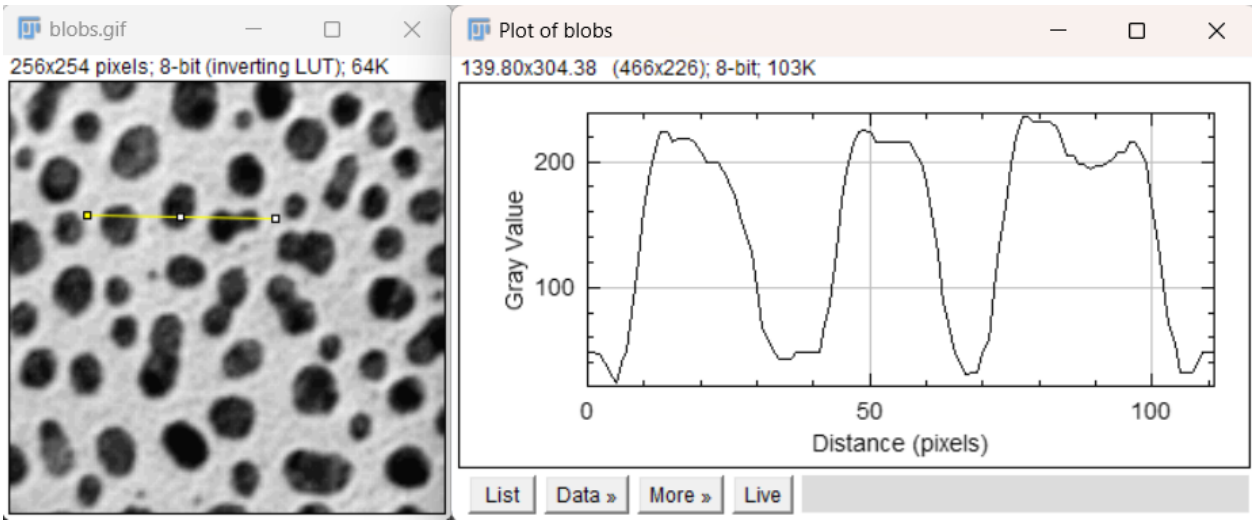
1. To see graphically how the pixel values vary across the image we can use the plot tool.
    - This can be very useful for determining how much noise is in the background of an image, or for finding the width of an object, such as a point spread function.
2. Draw a line or rectangle across the image, using the tools from the toolbar:



3. Press **Ctrl + K** to display the profile along the line.
    - The **Live** button in the plot window can be used to create an active plot that changes when the selection in the image is changed.
4. Here we can clearly see the increase in signal intensity across the three selected blobs.
   Since this is a rectangular selection, the values are averaged across the height of the rectangle.



5. In comparison, the plot of a single line shows similar intensity changes when carefully drawn through the three blobs, but the plot is noisier and more sensitive to where the line is placed.



3.6 Histograms

What is the distribution of pixel values in *blobs*? This is often one of the first things we want to know about an image (for example, to see if image intensities use the full range of the detector, or if they oversaturate the detector). Press **Ctrl + H** to open a histogram on *blobs* or on the sample image **boats**. Why might the histogram not be continuous on *blobs*? Is boats properly exposed?

3.6.1 Live views

Just like on the plot profile window, we can press the "live" button to get a continuously-updating histogram window. The histogram will update based on the selected region of interest.

3.7 Opening and viewing a Z-Stack

A stack is a 3D image. Usually the third dimension is z, the third spatial dimension.

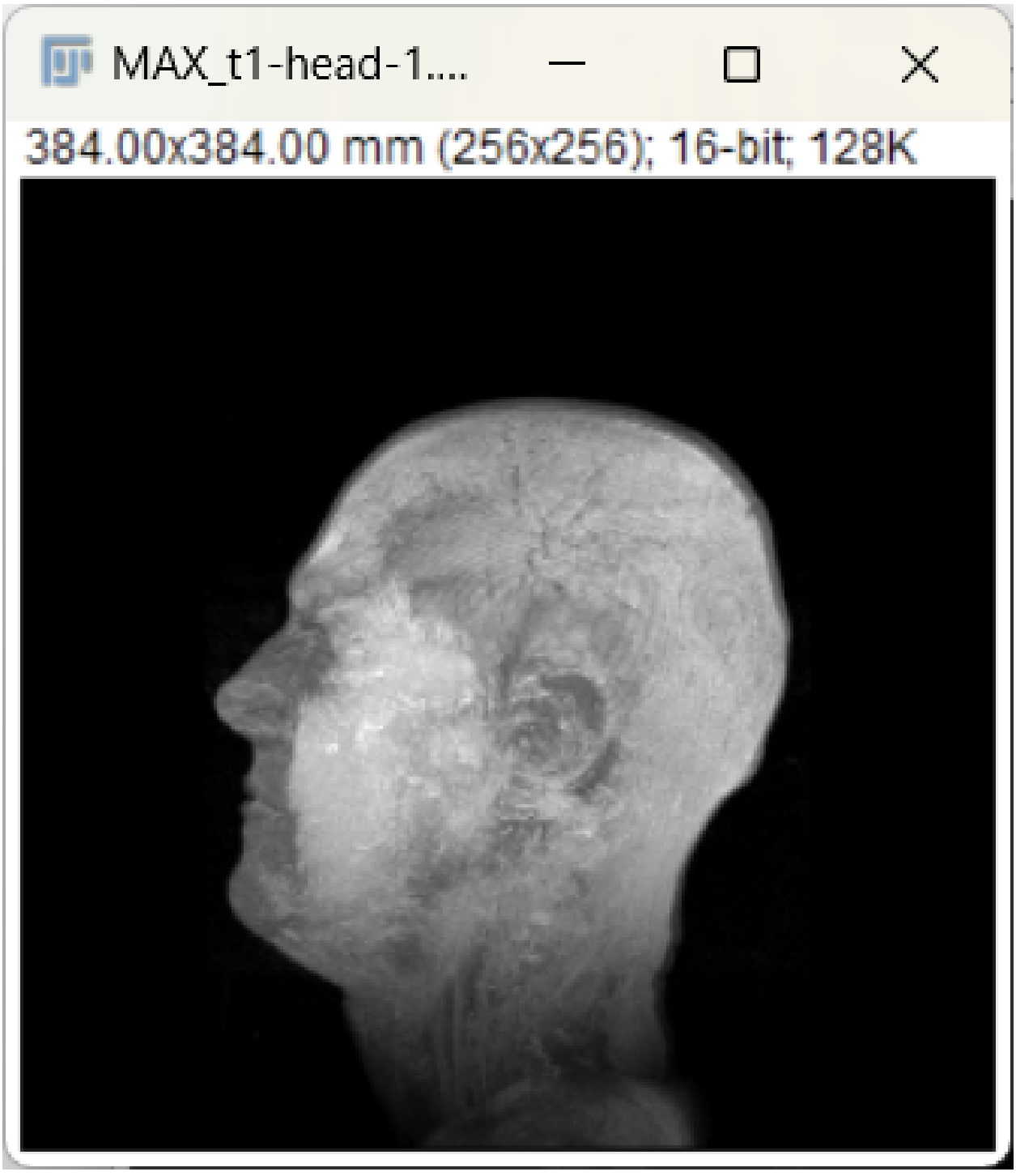
1. Open "t1-head.tif", via **File > Open Samples > T1 Head (16-bits)**.
2. Use the bar to scroll through the stack of images
3. We can also display the orthogonal view by using **Image > Stacks > Orthogonal Views** or **Ctrl/Command + Shift + H**

- The yellow crosshairs can be used to change the display for each orthogonal view, but one has to close the orthogonal view to select images.

4. To select from an orthogonal view, try **reslice**, in **Image > Stacks > Reslice** or press **/ (slash)** when the image is in focus. Suggested reslice parameters: 1.5 mm, start at top, avoid interpolation.
5. One can also run **3D Viewer** in **Plugins > 3D Viewer** to see an interactive rendering of the data.

3.8 3D Volume and Projections

1. Open `t1-head.tif`, **File > Open Samples > T1 Head (16 bits)**
2. To display the 3D Volume, use **Image > Stack > 3D Projection > Click ok** In the 3D projection dialogue box, there are various options for projection including axis of rotation and setting the slice spacing (step size) which may be useful for known parameters.



3. Additionally, the original image stack may be projected along one axis using **Image > Stacks > Z Project**. It is important to note that the information in the projection is highly dependent on the display parameter. A few examples are shown below (left to right: single slice of the stack, average projection, median projection, max projection).

3.9 Hyperstacks

Hyperstacks are multidimensional data, such as a multichannel timeseries or z-stacks.

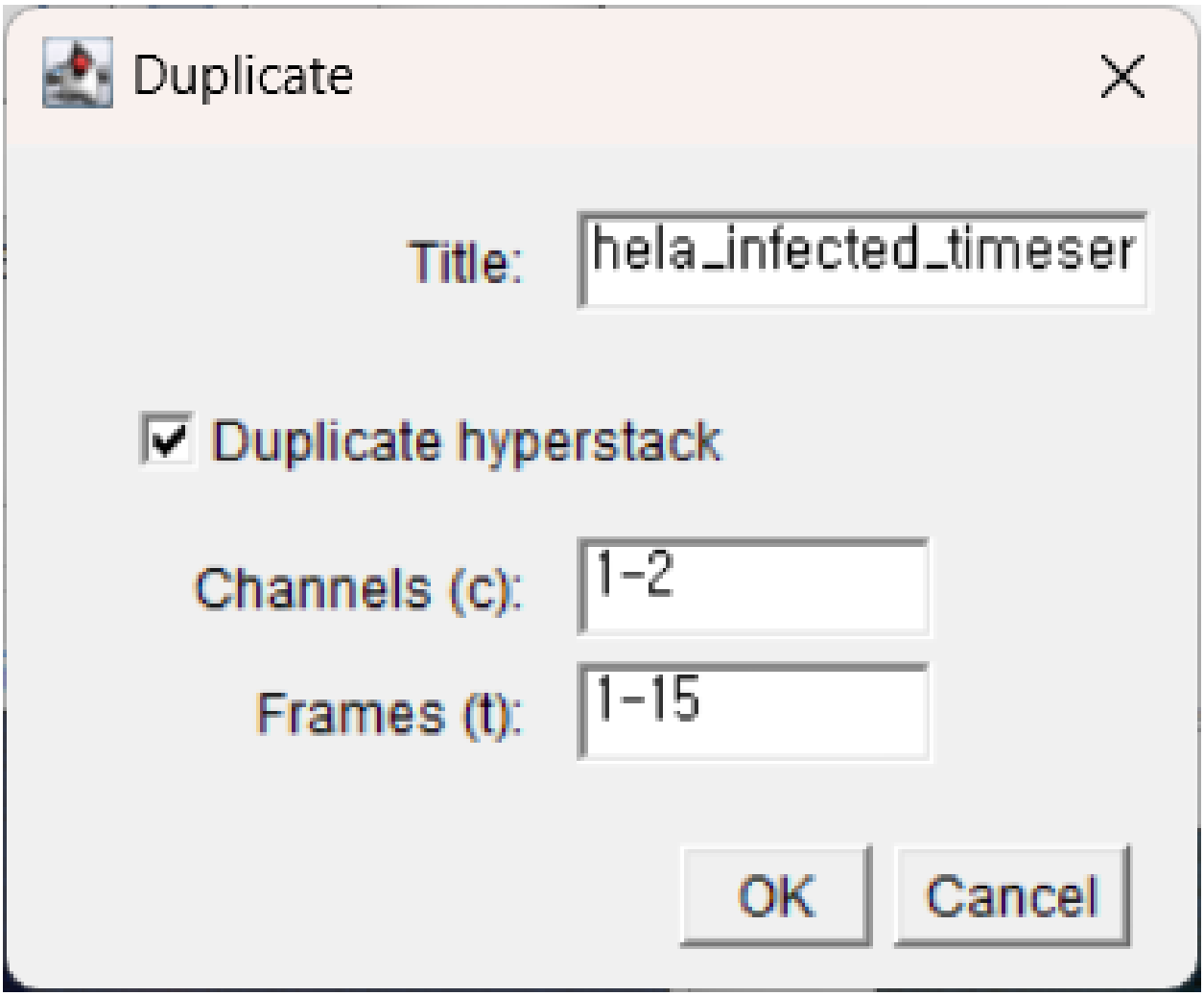
1. Start by opening the "hela_infected_timeseries.tif" image. This is available for download at: https://media.imagej.net/workshops/data/3d/

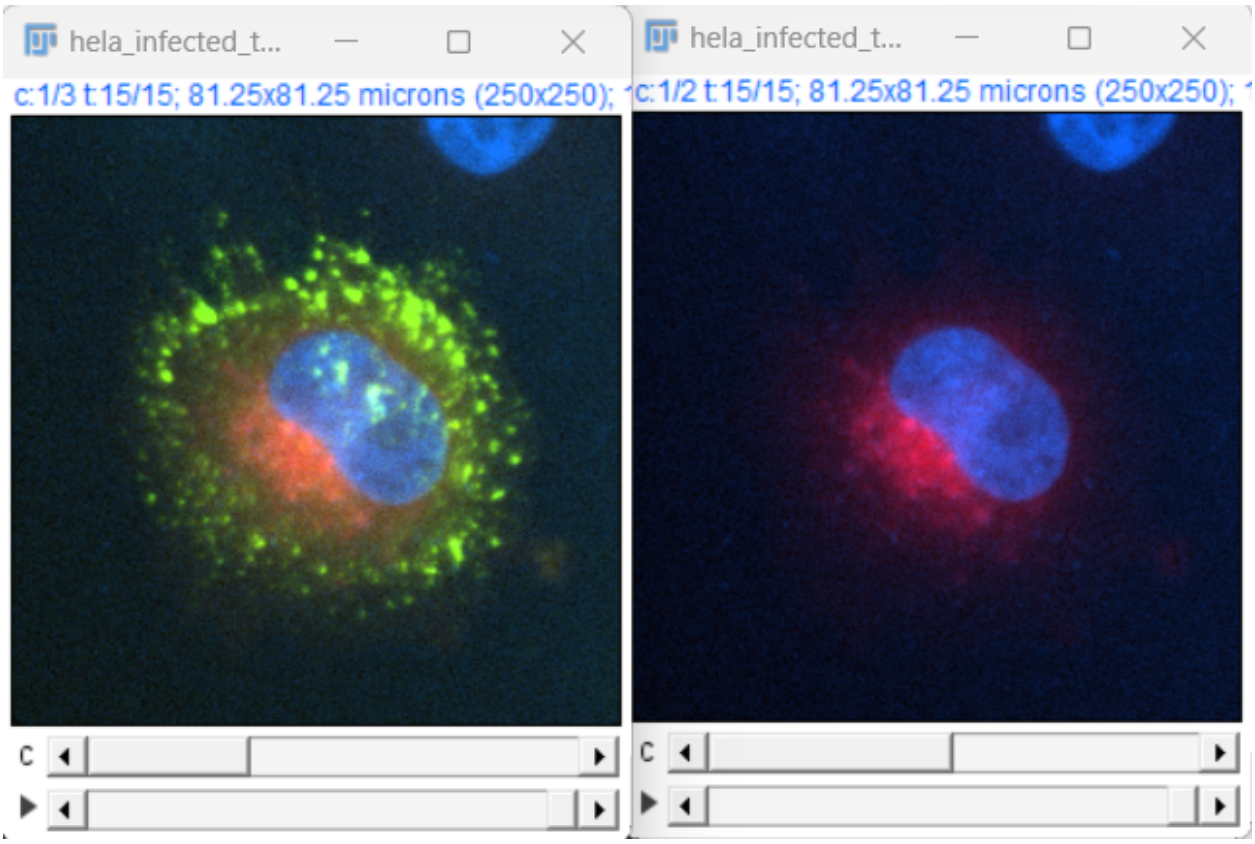
- If internet access is unavailable, the `Mitosis (5D stack)` sample from **File > Open Samples** works, too.

3.9.1 Duplicate A Channel Or Image

The "duplicate" tool allows us to work on a copy of the data (so we don't accidentally overwrite our original data), and it also allows us to work with a subset of large datasets.

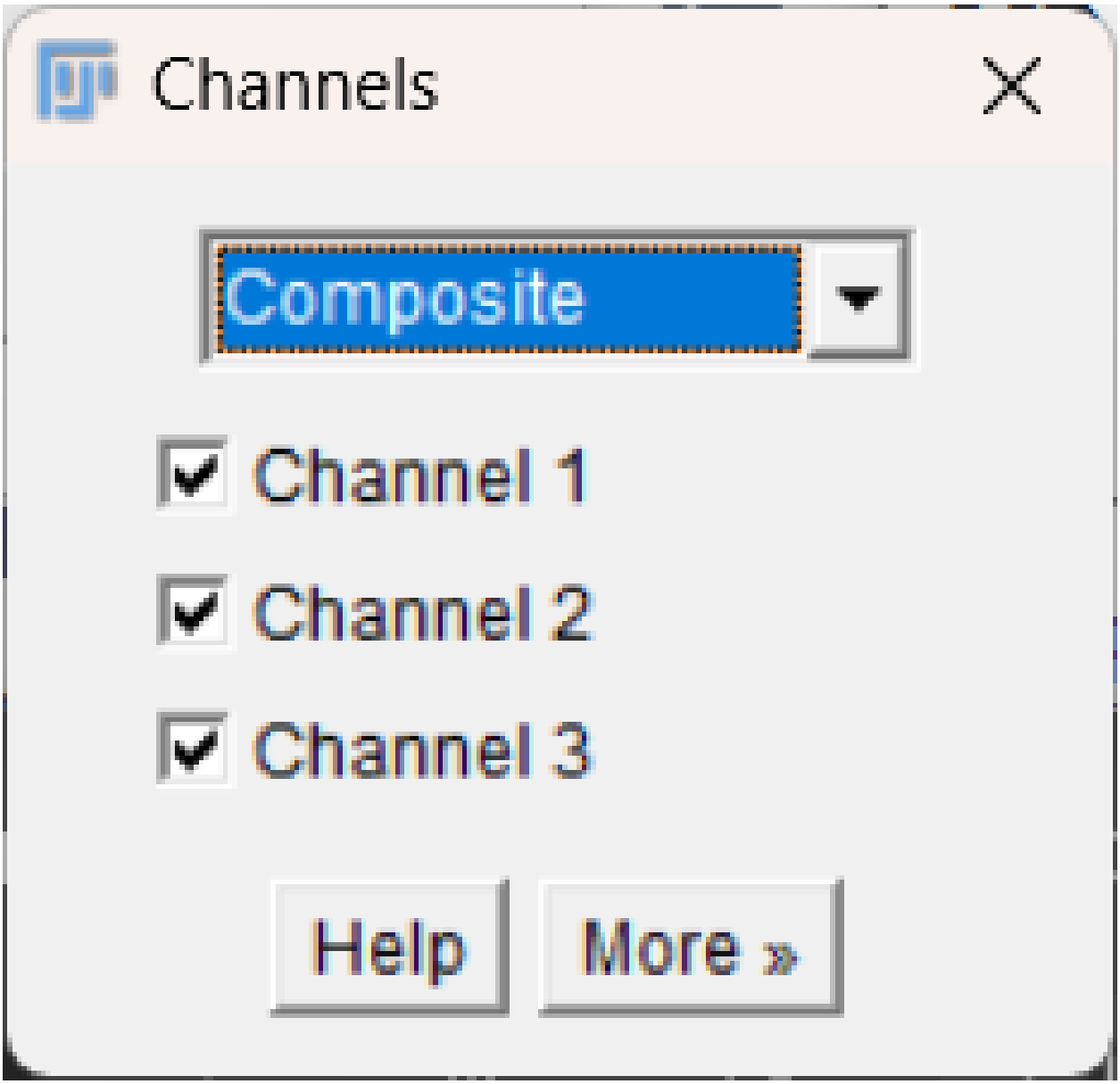
- Use **Image > Duplicate** to bring up the duplication options window. This can also be done with **Ctrl + Shift + D** or **Right Click > Duplicate**. Here, we can specify which RGB channels and timepoints we want to separate. If we use 1-2 in the channels menu, it should produce an image of just the red and green channels. The duplicate hyperstack box should be checked. (If you are using the mitosis image, try choosing just one value for the color channel.)


Duplicate
Title:
hela_infected_timeser
Duplicate hyperstack
Channels (c):
1-2
Frames (t):
1-15
OK
Cancel



3.10 Changing Channel Colors

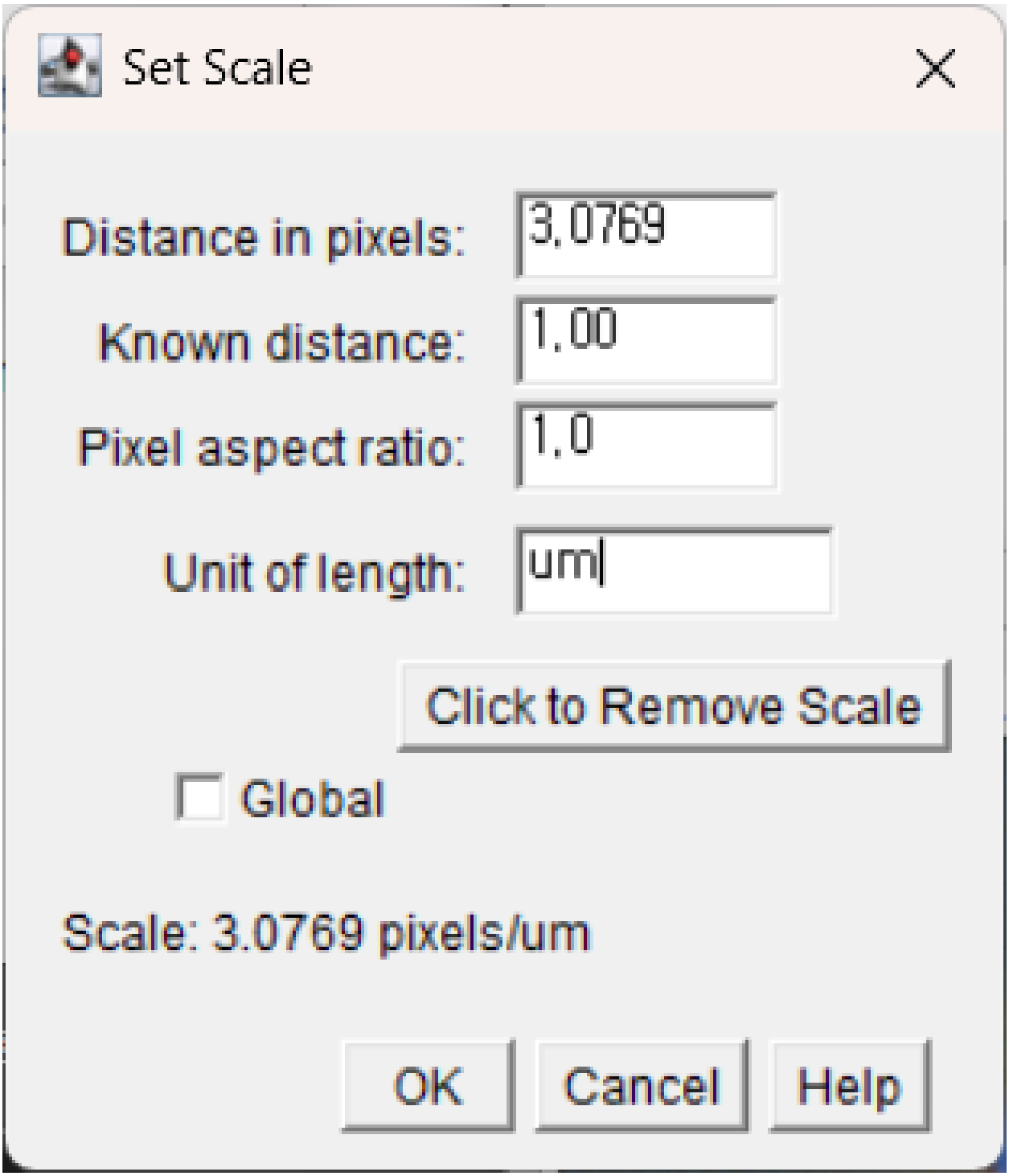
1. To change the display color of channels, use **Image > Color > Channels Tool** or **Ctrl + Shift + Z**.



Note: The “more” option can be used to apply colors to the selected channels.

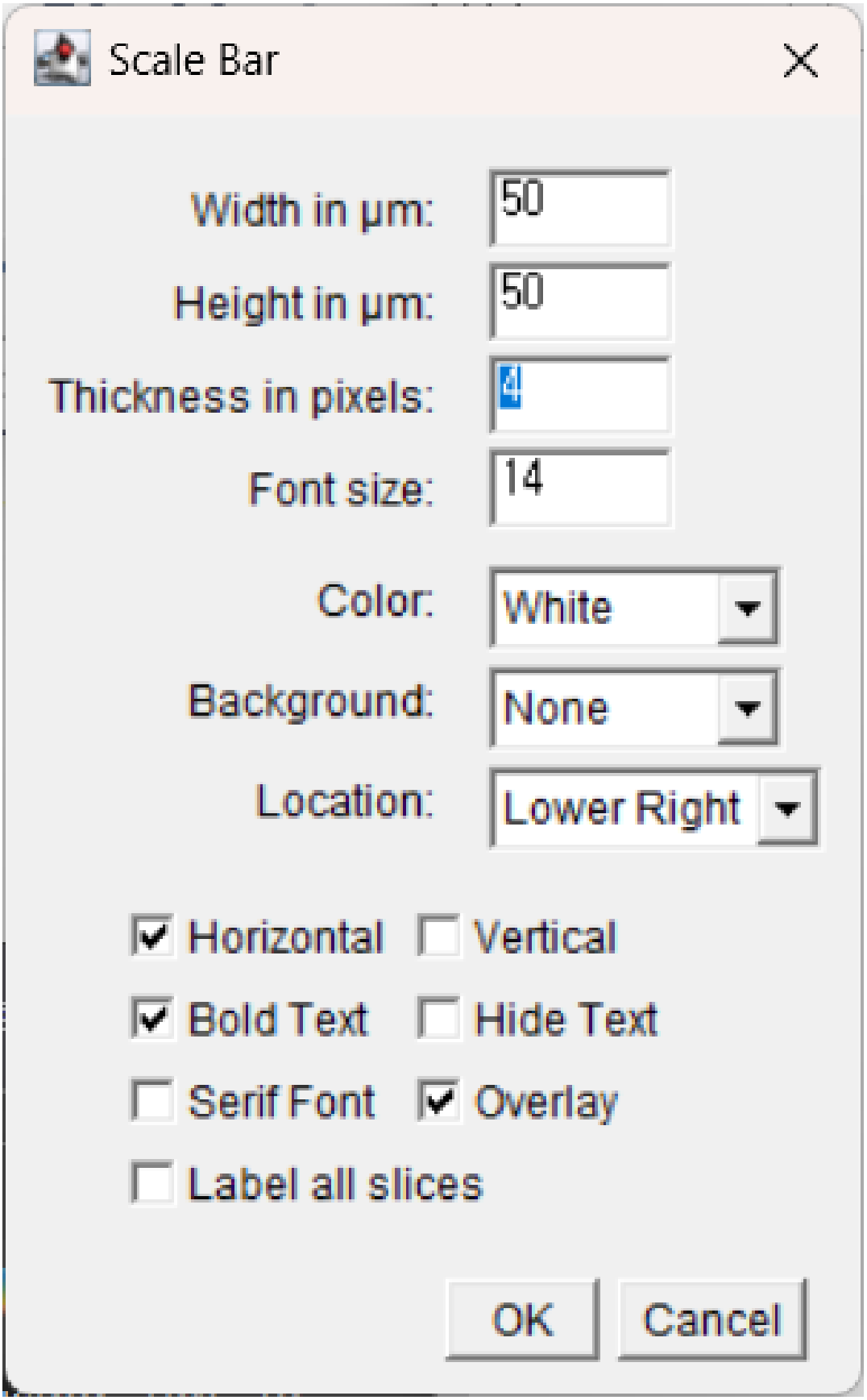
3.11 Set Scale and Scale Bars

1. If the size per pixel is known, such as from a microscope calibration, this can be used to change the image dimensions from pixels to the known field of view.
2. Use **Analyze > Set Scale** to enter the known distance.
    - For example, an image with a pixel size of `0.73 pixels/micron` would be entered as shown, using “um” or “micron”.



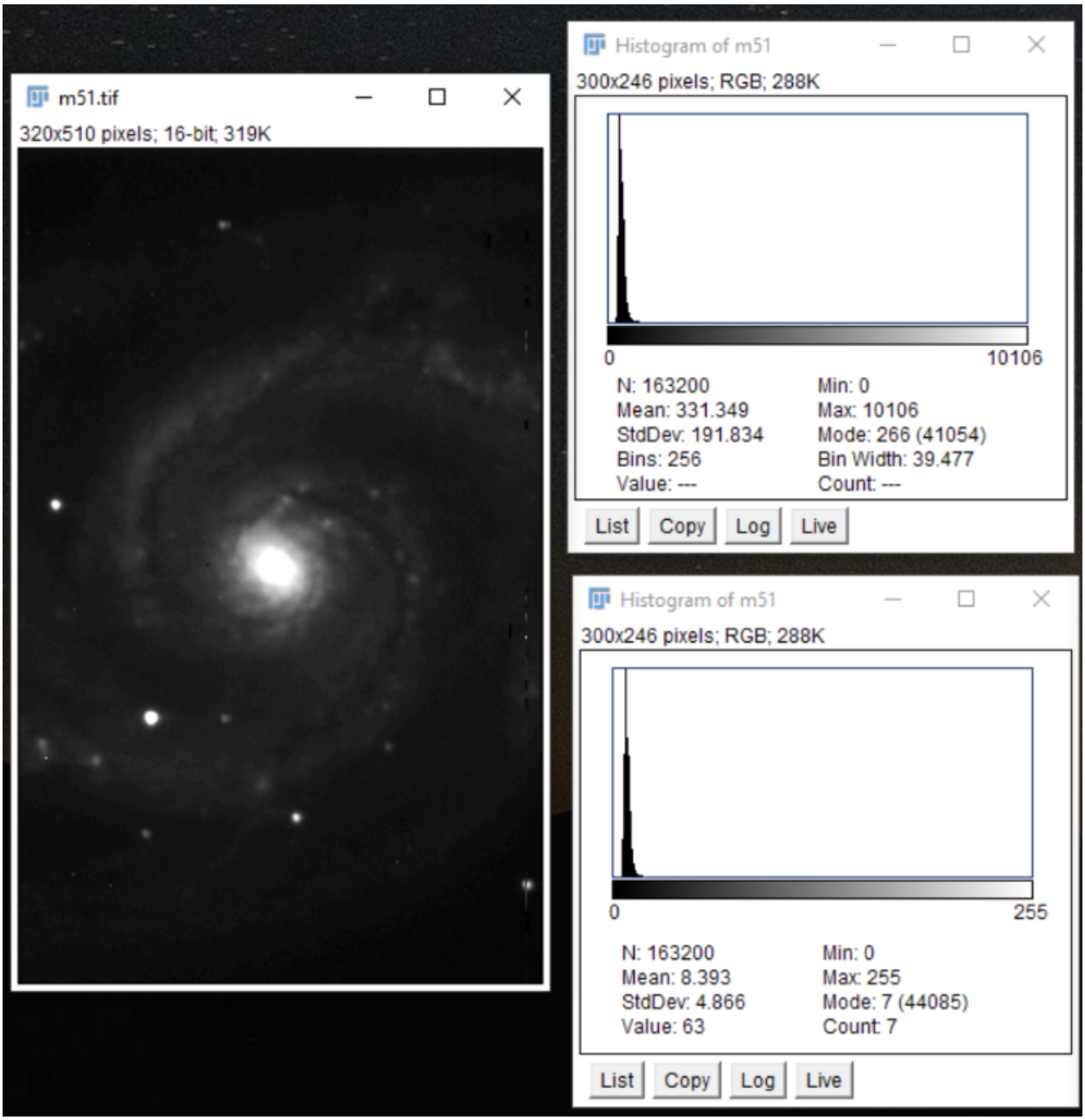
- Further info for setting the scale based on a measurement in an image can be found here: https://serc.carleton.edu/eyesinthesky2/week2/get_to_know_imagej.html

3. To add a scale bar, use **Analyze > Tools > Scale Bar**.


Scale Bar
Width in µm:
50
Height in µm:
50
Thickness in pixels:
4
Font size:
14
Color:
White
Background:
None
Location:
Lower Right
Horizontal
Vertical
Bold Text
Hide Text
Serif Font
Overlay
Label all slices
OK
Cancel

3.12 Bit Depth and Image Type

1. Open the `m51.tif` image, **Open > Open Samples > M51 Galaxy (16 Bits)**.
2. Adjust the contrast so we can see the image a bit better. **Image > Adjust > Brightness and Contrast**.
3. Make a histogram of the image, **Ctrl + H**, and notice the scale of the histogram and the mean value. Keep this window open. 16-bit images have a maximum value of 65,536.
4. Convert the 16 bit image to an 8 bit image, **Image > Type > 8 bit**. a. 8-bit images have a maximum of 256 values. This means the data resolution and range is lower compared to a higher bit depth, so the data is compressed.
5. Make another histogram and keep the window open. How did the values change?



3.13 Changing Image Types, cautiously.

Some operations - especially image math operations - only work on images of a certain type, and sometimes we desire images of a certain type. For example, if we want to multiply two 8-bit images, it would be prudent to first convert them to 16-bit images so the output is to a 16-bit image and does not overflow.

However, changing the image type through **Image > Type** can result in the loss of information as we have seen. Not all image types can be converted back to the original. Be careful.

On an unrelated note, the following error is likely to display if you are not able to complete a type conversion.


Converter
Supported Conversions:
8-bit -> 16-bit*
8-bit -> 32-bit*
8-bit -> RGB Color*
16-bit -> 8-bit*
16-bit -> 32-bit*
16-bit -> RGB Color*
32-bit -> 8-bit*
32-bit -> 16-bit
32-bit -> RGB Color*
8-bit Color -> 8-bit (grayscale)*
8-bit Color -> RGB Color
RGB -> 8-bit (grayscale)*
RGB -> 8-bit Color*
RGB -> RGB Stack*
RGB -> HSB Stack*
RGB -> Lab Stack
RGB Stack -> RGB Color
HSB Stack -> RGB Color
* works with stacks
OK

3.14 Adjusting Brightness and Contrast

- Changing the contrast in an image can be a good visualization tool. Using **Ctrl + Shift + C** or **Image > Adjust > Brightness and Contrast** will display the B&C menu. Using Auto or the B&C sliders changes the look up table (LUT), which does not change the pixel values. This is good for visualization, but **using the Apply button will change the pixel values, which generally should not be done.**

3.15 (Optional) Accessing Image Metadata

- Open our favorite image, "blobs": **Ctrl + Shift + B**
- Press **Ctrl + i** (**Image > Info**) to see the image metadata.
- Press **Shift + p** (**Image > Properties**) for an older tool which allows one to see and set some image (and stack) properties.

CHAPTER

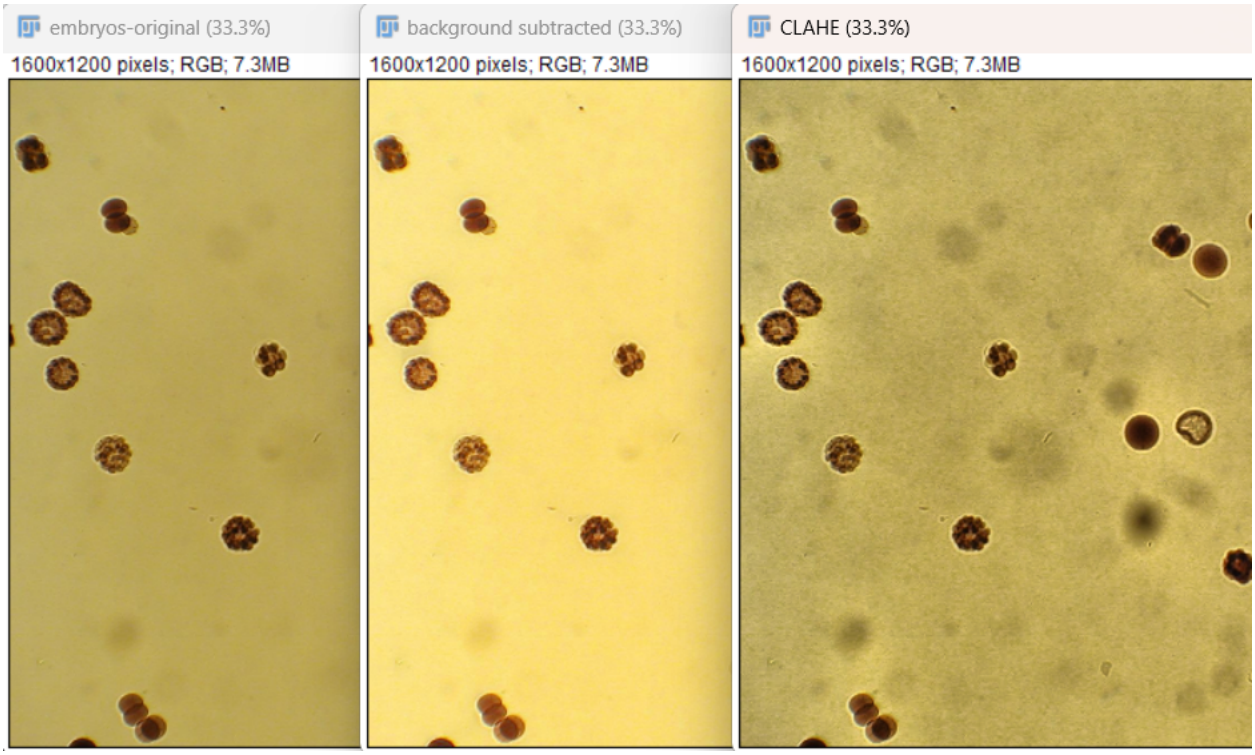
FOUR

BASIC SEGMENTATION

4.1 (Optional) Background Subtraction

For this section, we demonstrate with the **embryos** sample, from **File > Open Sample > Embryos**.

Oftentimes the background illumination is not uniform, and this interferes with intensity-based thresholding. Fiji includes multiple methods to perform background subtraction in ImageJ, including **Process > Subtract Background**, **Mean/Median Background Subtraction**, or **Gaussian Blur Subtraction**. If you are not planning a quantitative analysis, **Enhance Local Contrast** (**CLAHE**, Contrast Limited Adaptive Histogram Equalization) may also be worth a try.



Left to right: original, background subtracted, with CLAHE.

However, any background subtraction used to process images should be carefully considered in relation to the original source of noise, how the image information is used, and any further processing or quantification of the image.

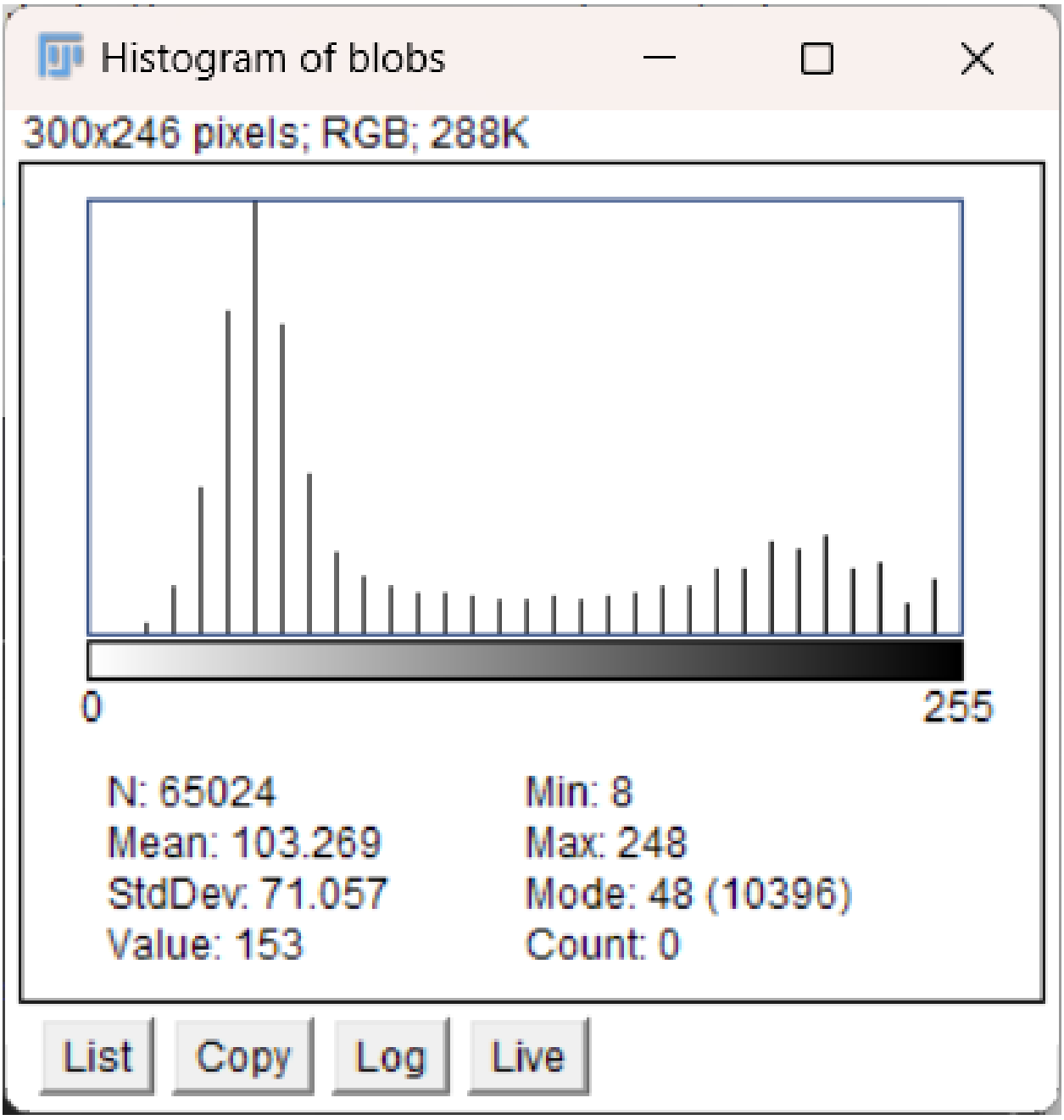
In some cases the Subtract Background plugin, or other methods, can also introduce artifacts that are not present in the original image.

Some Image.sc forum discussion here:
https://forum.image.sc/t/consensus-on-subtract-background-built-in-or-other/7061

4.2 Segmentation / Thresholding

For this section we will open up blobs again: **Ctrl-Shift-B**

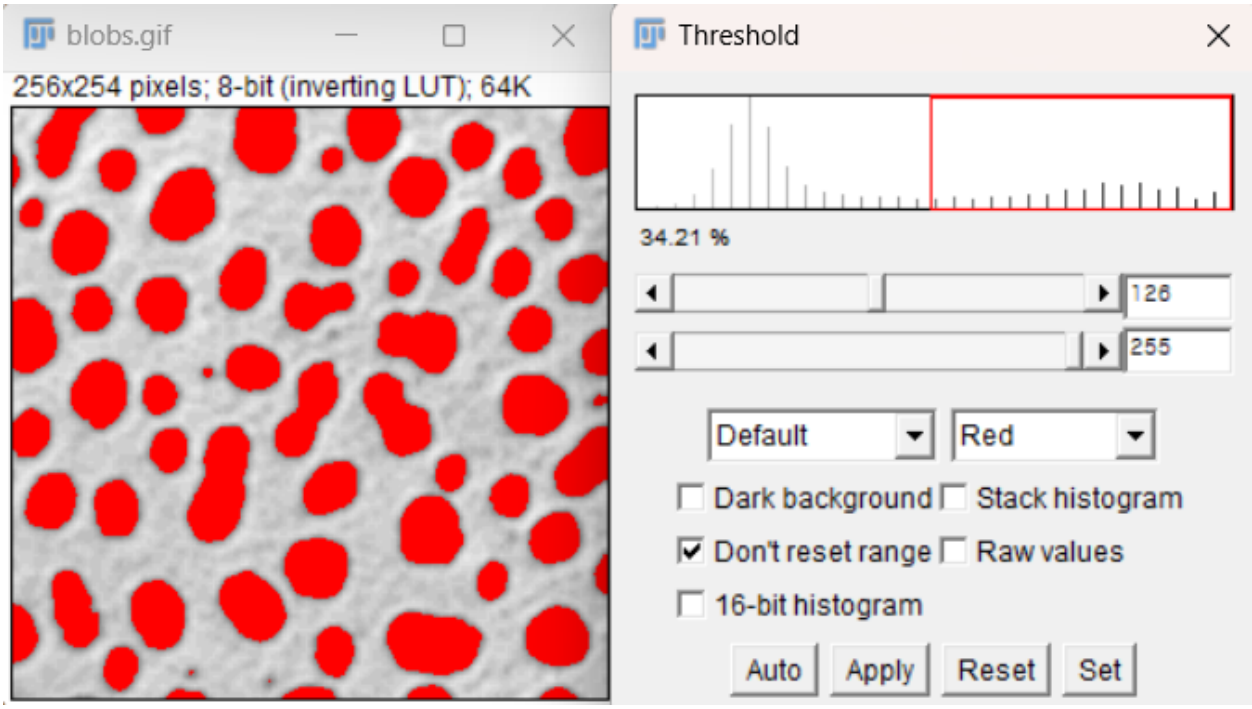
1. If we want to segment out the blobs we are interested in, one possible method is to use an intensity threshold. First, let's look at the histogram of the intensity values using **Ctrl-H**.



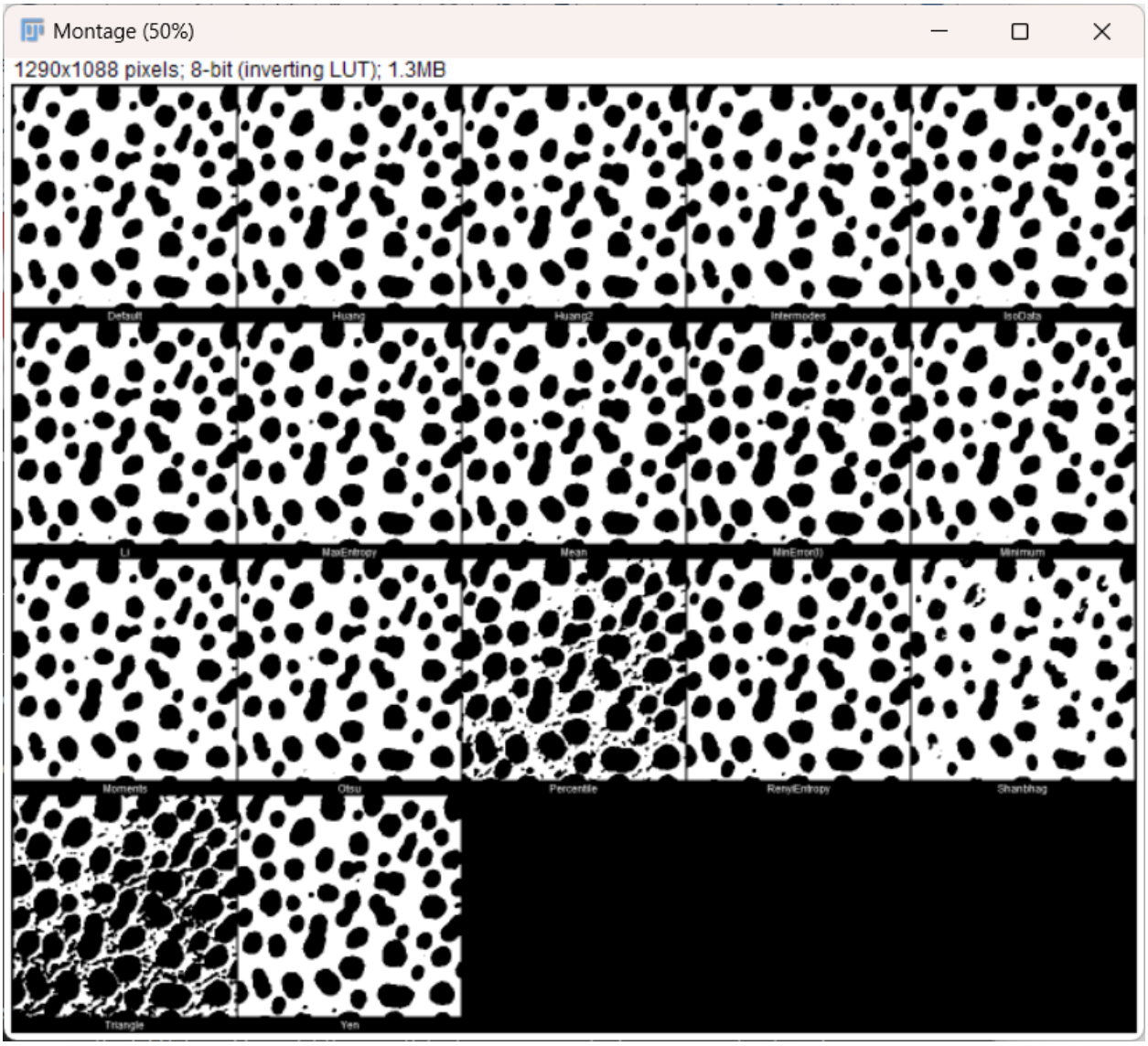
We can see that most of the background pixels have an intensity value around 50, whereas there is another grouping of pixel intensities around 210, which is likely the areas we are interested in because this image has a white background.

Notice that the values are stretched outwards; this indicates that the original range was likely very small or somehow adjusted.

2. Use **Image > Adjust > Threshold** to see the automatic threshold applied based on the original histogram.
   This seems to segment the blobs pretty well. You can change the slider bars to see how the segmentation changes as the threshold moves.
   When ready, click **Apply** to create the binarized mask. Notice that the blobs now have a value of `255`, and the background has been set to `0`. This represents a **permanent change** in the image values, so going forward, most quantified analysis involving pixel values must be performed by applying the segmentation as a mask on the original image (see [Masks] section).

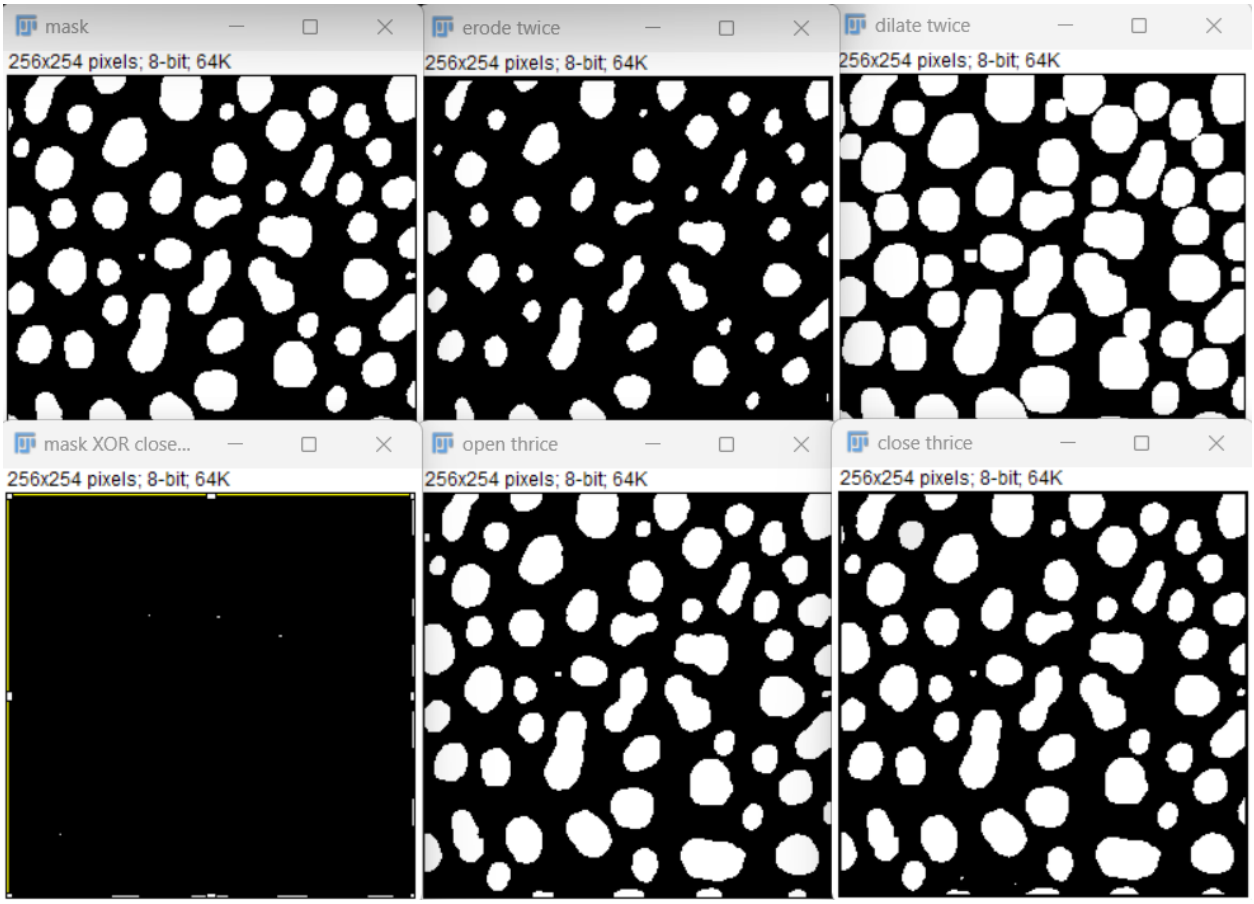


- There is also an **Auto Threshold** option under **Image > Adjust > Auto Threshold**, that you can use to compare different methods of thresholding. It is easiest to see the comparisons using the **Try All** method.


Montage (50%)
1290x1088 pixels; 8-bit (inverting LUT); 1.3MB
Default
Huang
Huang2
Intermodes
IsoData
Li
MaxEntropy
Mean
MinError(I)
Minimum
Moments
Otsu
Percentile
RenyiEntropy
Shanbhag
Triangle
Yen

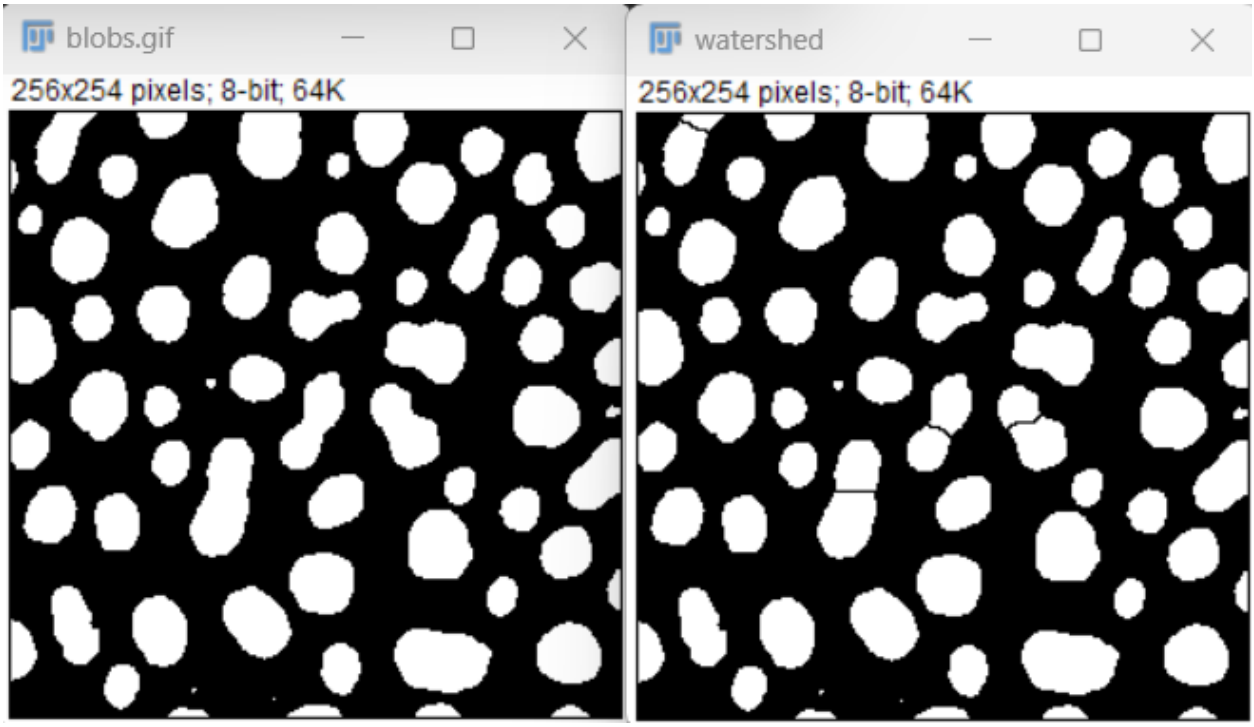
4.3 Morphological filtering

Often the mask of the particles will pick up a lot of noise, and it is useful to filter them. The morphological operations **Erode** and **Dilate** from the menu **Process > Binary** can be combined in sequence to remove noise. When Erode is run first, the sequence is called **Open**, and removes small objects and speckle noise. When Dilate is run first, the sequence is called **Close** and fills small holes or gaps between mask objects.



4.4 Splitting chunks by watershed

Often two smooth convex objects (cells!) will be touching and thereby detected as part of the same mask element. **Watershed segmentation** or **Process > Binary > Watershed** splits masks by drawing a narrow channel at narrow bottlenecks. This is useful for separating cells that were identified as part of the same mask.



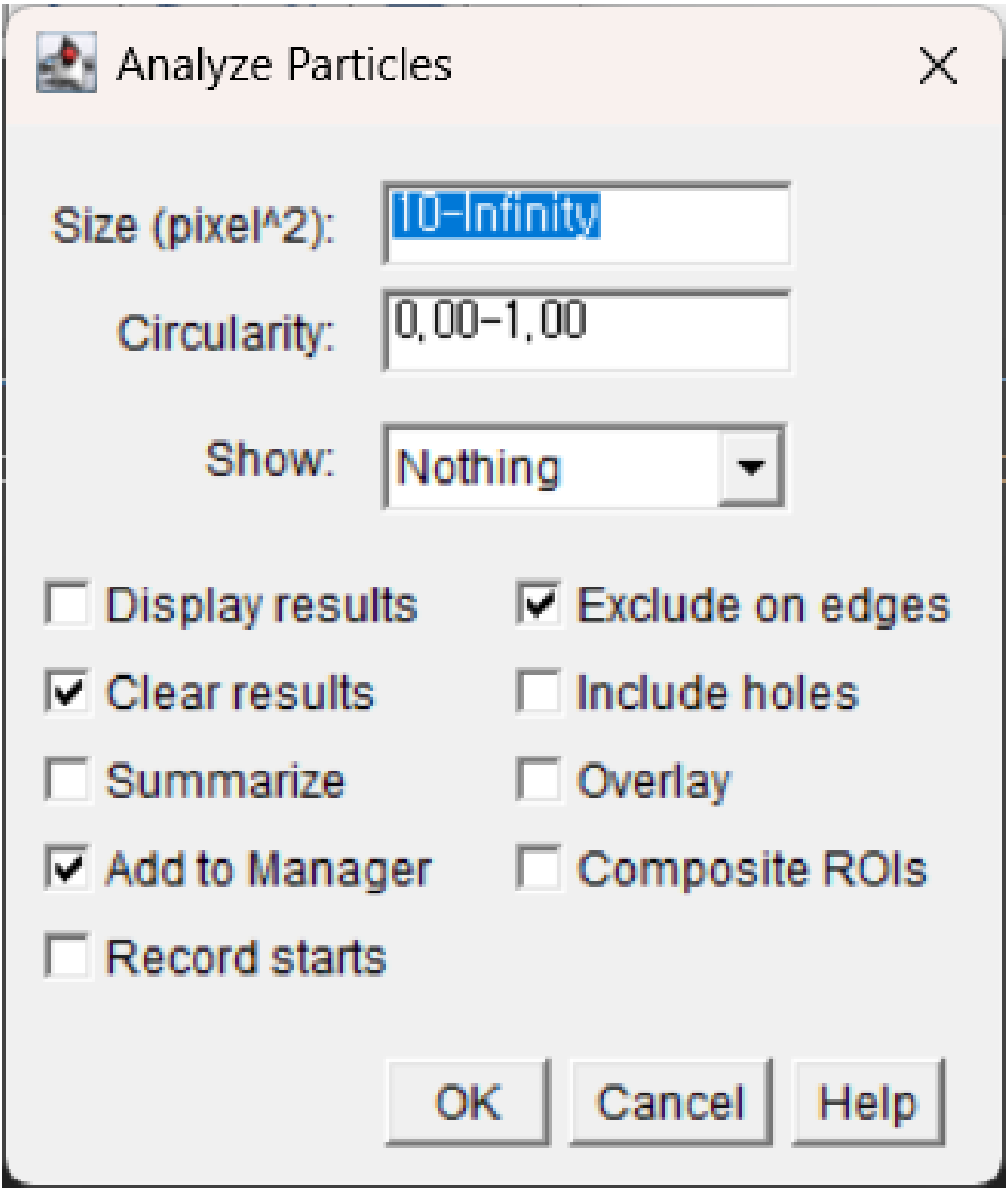
4.5 Adding Selections to ROI Manager

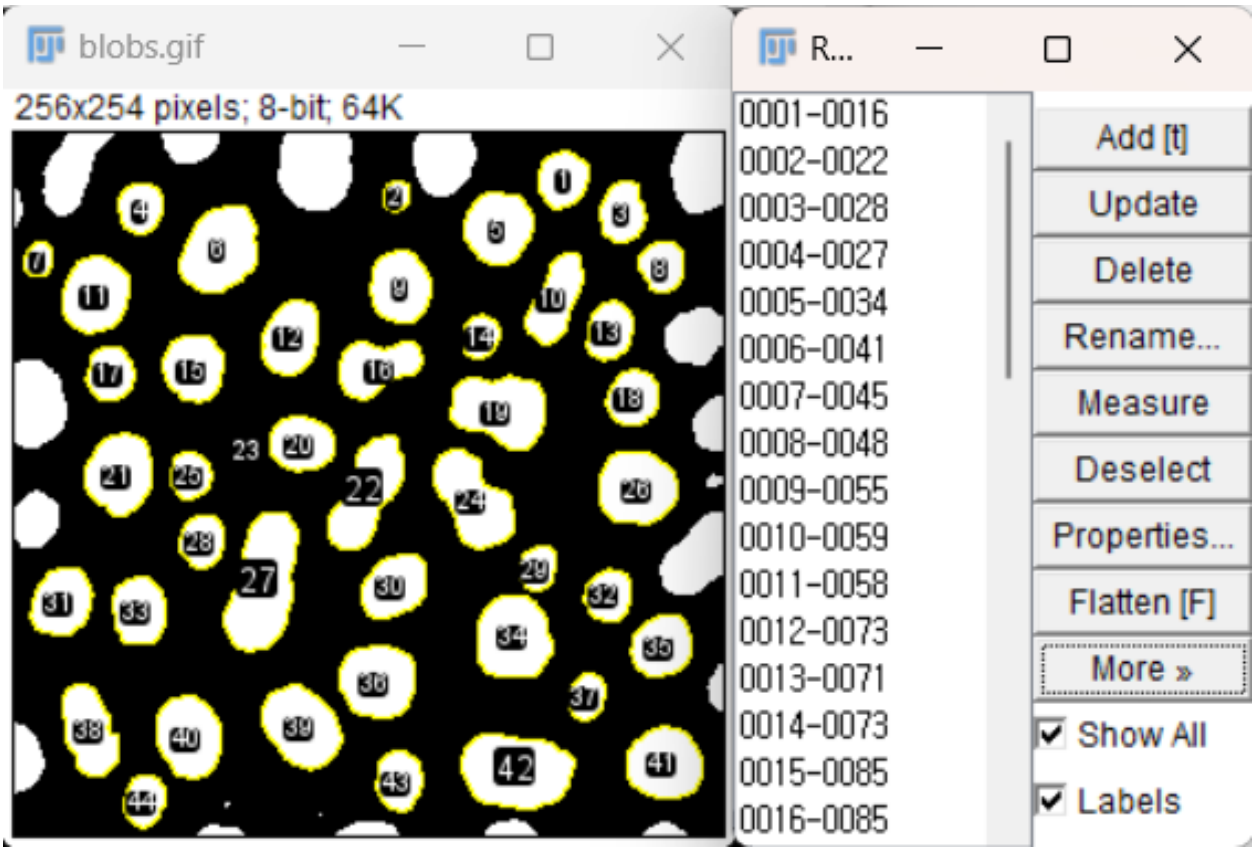
1. Press `T` to open the ROI manager.
2. Use **Edit > Selection > Create Selection** to select all of the blobs based on the current threshold.
3. In the ROI Manager window, use **More > Split** to divide the selection into multiple ROIs.
    - You can click on the regions to see the specific blob it corresponds to.
    - If needed, the selected regions can be saved in a file and reopened later using **More > Save**.
    - One can also add selections to the ROI manager manually, if there are only a few objects to segment or if thresholding segmentation does not work for your sample.

4.6 Analyze Particles

The **Analyze Particles** function can be useful for applying size exclusion or removing any blobs that are below our desired size threshold.
For example, we can see some smaller red circles in the image that may represent noise rather than a full blob we are interested in.
This can be especially useful if we have a biological problem, such as segmenting cells, where we know the cells must be above a certain size.

1. Open the ROI manager by pressing `T`.
    - If there are previous ROI selections that you would like to remove:
        - Click on the first one, hold `Shift`, then click on the last one.
        - Press `Delete` to clear them.
2. Use **Analyze > Analyze Particles** to open the function window.
    - **Size** can be used to determine what blobs are included. For example, specify `"30-Infinity"` to remove smaller blobs.
        - For many biological applications, it's best to know the size of the pixels to understand how much area is excluded, and whether it corresponds to the expected size based on the experiment.
    - Ensure the **Add to Manager** box is selected to add the ROIs to the ROI manager.
    - For the **Show** dropdown, select **Masks** to display the following:


Analyze Particles
Size (pixel^2):
0-Infinity
Circularity:
0,00-1,00
Show:
Nothing
Display results
Exclude on edges
Clear results
Include holes
Summarize
Overlay
Add to Manager
Composite ROIs
Record starts
OK
Cancel
Help

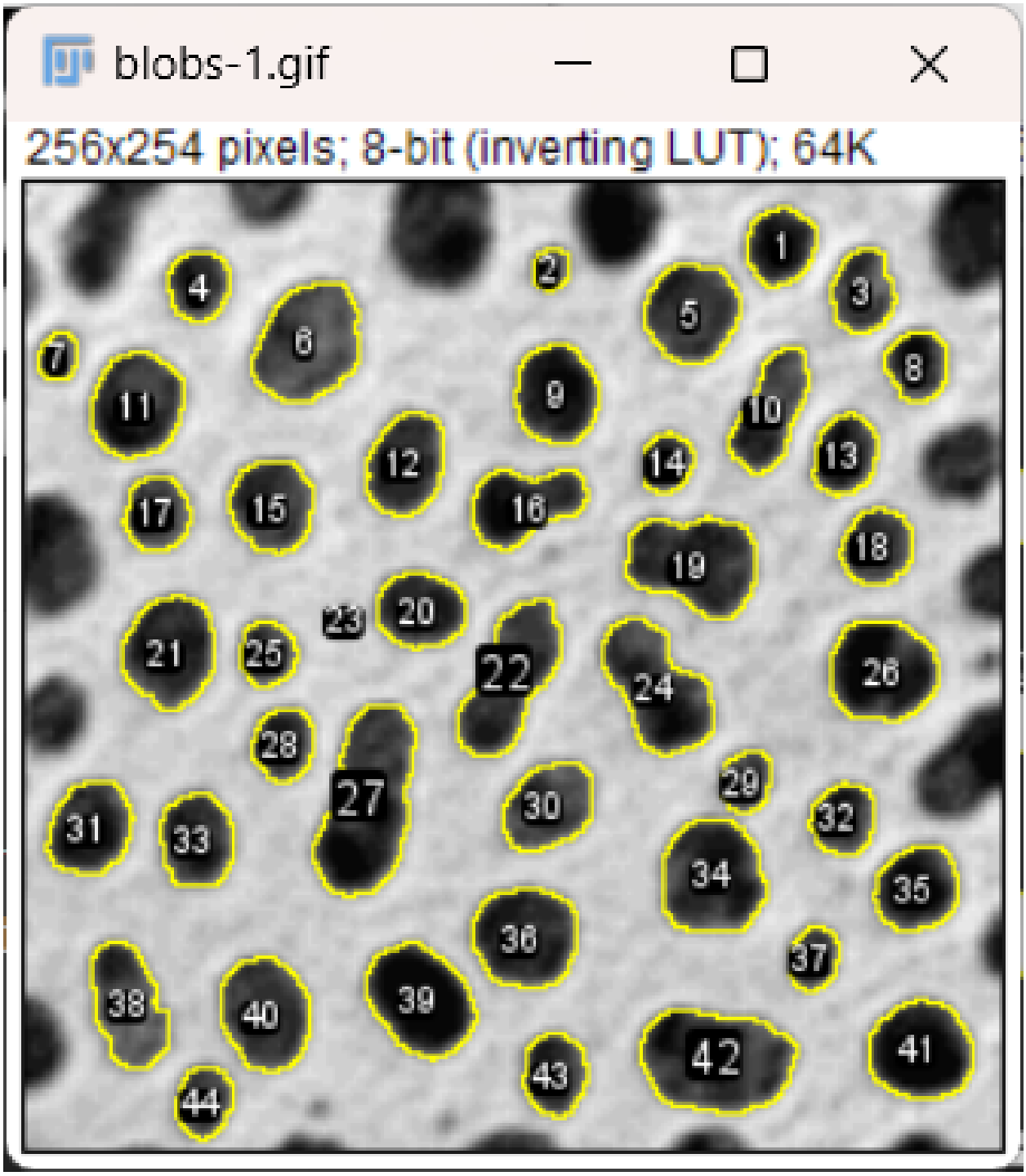



```
> Notice that some of the blobs that are present in the binarized image, like the␣
↪one next to number 29, are not included in the mask.
```

4.7 Masks for Measurement

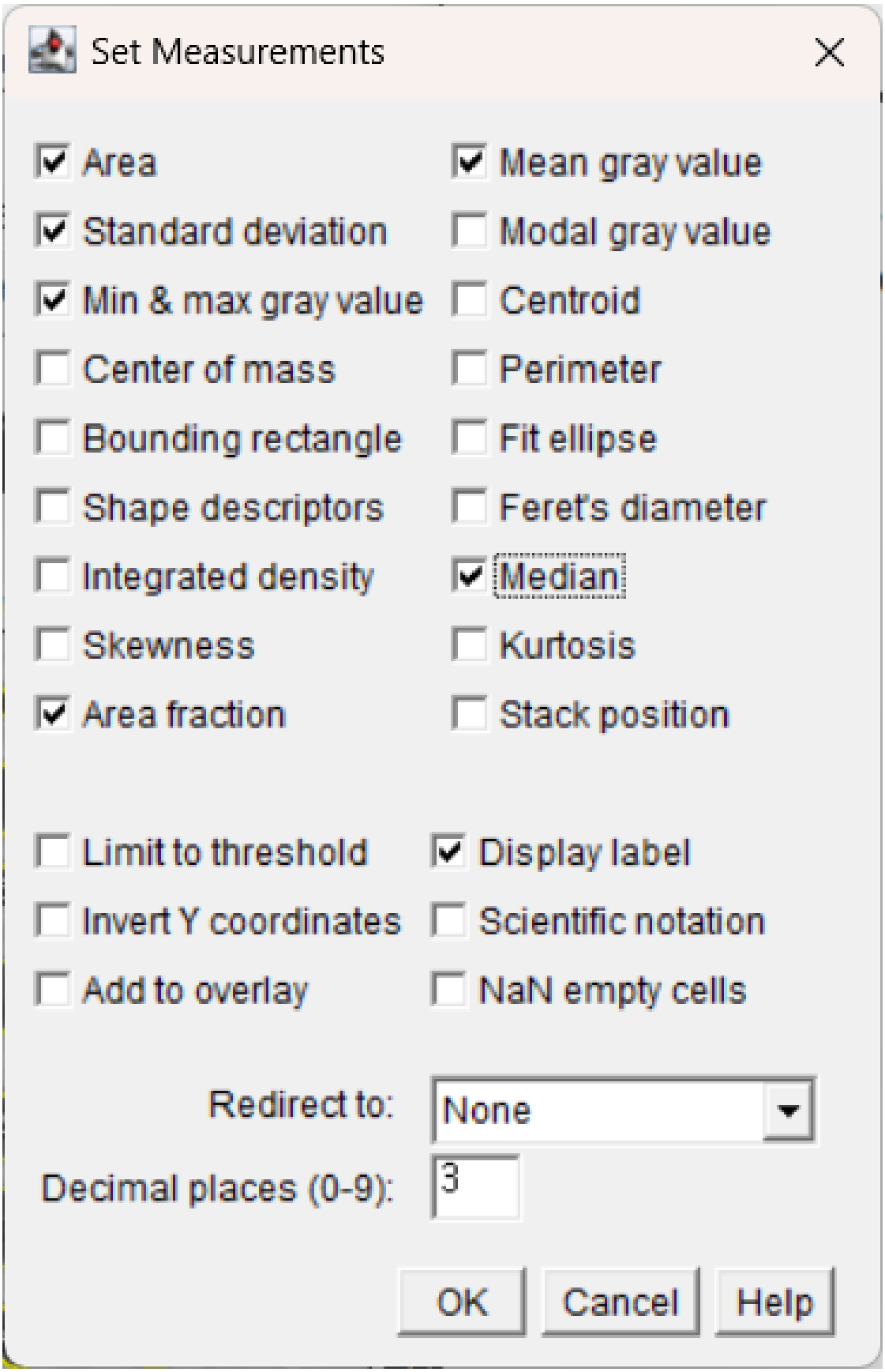
1. Once you have a mask or the desired image, use **Edit > Selection > Create Selection** to add the regions to the ROI manager.
2. After selection, you can also use **Edit > Selection > Create Mask** to make a mask of the desired areas if the image is not already binarized.
3. To copy the ROI selection onto the original image:
   - Click on the mask while the selection is open.
   - Then click on the original image and press **Shift + E** or use **Edit > Selection > Restore Selection**.

4.8



4.9 Making Measurements

1. To set the desired measurements to be collected, use **Analyze > Set Measurements**. This window will determine what results are displayed or saved from the image.


Set Measurements
Area
Mean gray value
Standard deviation
Modal gray value
Min & max gray value
Centroid
Center of mass
Perimeter
Bounding rectangle
Fit ellipse
Shape descriptors
Feret's diameter
Integrated density
Median
Skewness
Kurtosis
Area fraction
Stack position
Limit to threshold
Display label
Invert Y coordinates
Scientific notation
Add to overlay
NaN empty cells
Redirect to:
None
Decimal places (0-9):
3
OK
Cancel
Help

2. To create the measurements:
    - Use **Analyze > Measure** or **Ctrl + M**, while the desired image is active.
    - This may produce one measurement for the whole image if an ROI is not active.
    - To measure all ROIs at once, use **Measure** in the ROI Manager.
        - The checkboxes for **Show All** and **Labels** can be useful to see what regions are being measured.
    - If you want, use **Edit > Selection > Make Inverse**.
        - This will now select the entire background.
        - You can measure again to get the value for the background.

CHAPTER

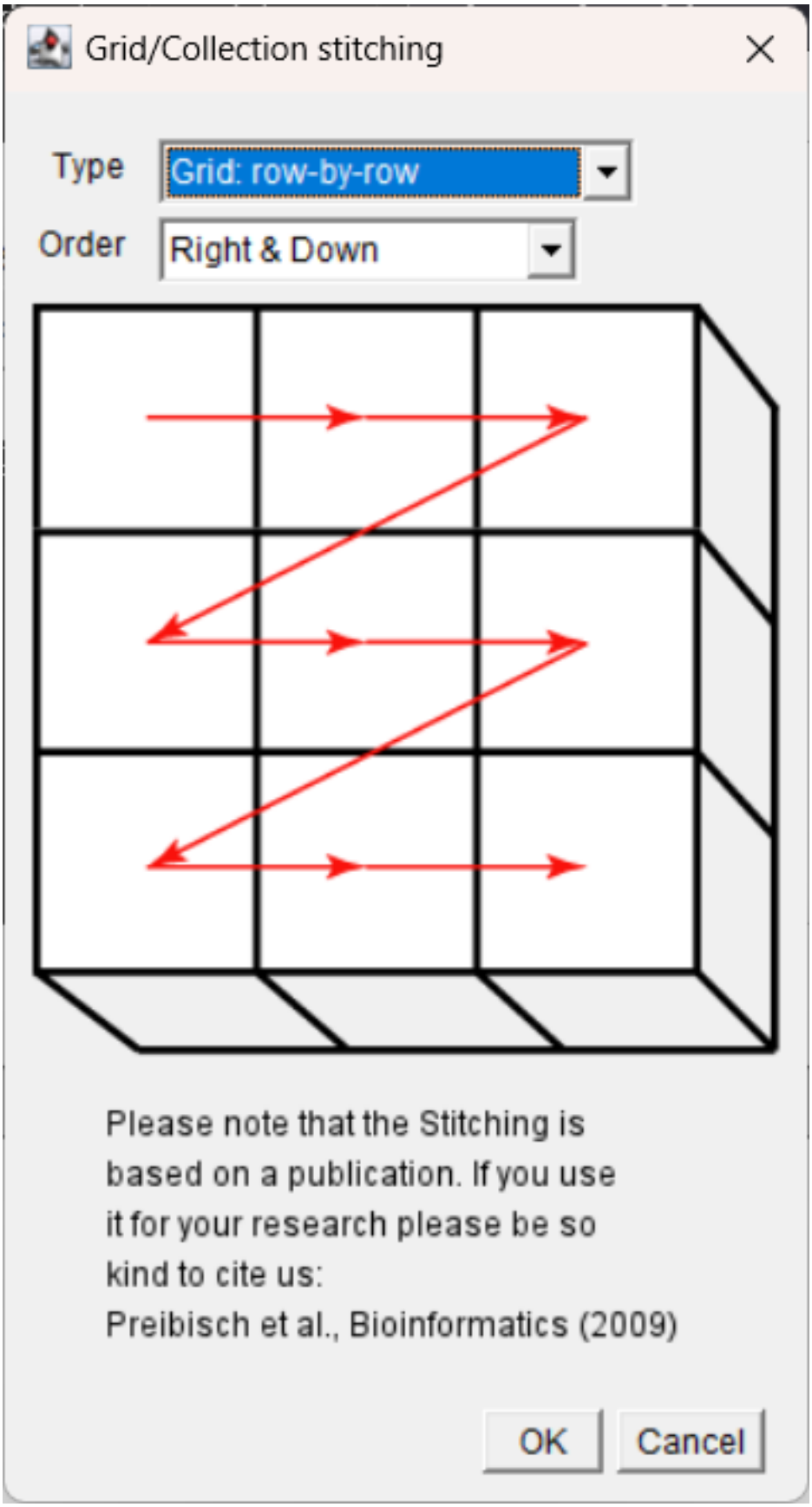
FIVE

BASIC REGISTRATION

5.1 Stitching Images

There are multiple ways to stitch images using the **Grid/Collection Stitching** plugin, including with metadata or file position.

1. Open the **Grid/Collection Stitching** plugin. I find the easiest way to find it is to search for it: **Ctrl-L > Grid/Collection**. It is also at **Plugins › Stitching › Grid/Collection stitching**. In this example, use **Grid: snake by rows** with the order of **Right & Down**.


Grid/Collection stitching
Type
Grid: row-by-row
Order
Right & Down
Please note that the Stitching is based on a publication. If you use it for your research please be so kind to cite us:
Preibisch et al., Bioinformatics (2009)
OK
Cancel

2. In the dialogue box, specify the following parameters:
    - Grid size: **`x = 3, y = 3`**
    - Tile overlap [%]: `0`
      (This is a known value — the image we are stitching was part of a larger image. In an experimental context, this may be part of the image acquisition settings.)
    - First file index: `1`
    - Directory: point to the `Leaf_stitch` folder
    - File name: `leaf-{i}.tif`
        - `{i}` specifies where in the filename to iterate through values.
        - If this is written as `{ii}`, there will be an error because the first file is `1`, not `01`.
    - Uncheck the **Compute overlap** box, since the overlap value is known.
    - You may get a warning about no overlap being found. Press 'ok' to bypass it.

3. Also try re-running the plugin with an overlap value of 10%.
    - Notice how there are errors near the borders of the leaf and on the ruler (especially the faded “9” value), but errors at the center of the leaf may be harder to spot.
    - This is why knowing the expected overlap value is important, as spotting errors in experimental data may be difficult.
    - In many cases, using stitching information from the image metadata is more helpful, because the position

information comes from the microscope.

CHAPTER

SIX

SCRIPTING

Scripts can be generated in a variety of ways, including the **Macro Recorder**.
For functions included in the ImageJ Macro language, please see:
https://imagej.net/ij/developer/macro/functions.html

More general scripting info:
https://imagej.net/scripting/

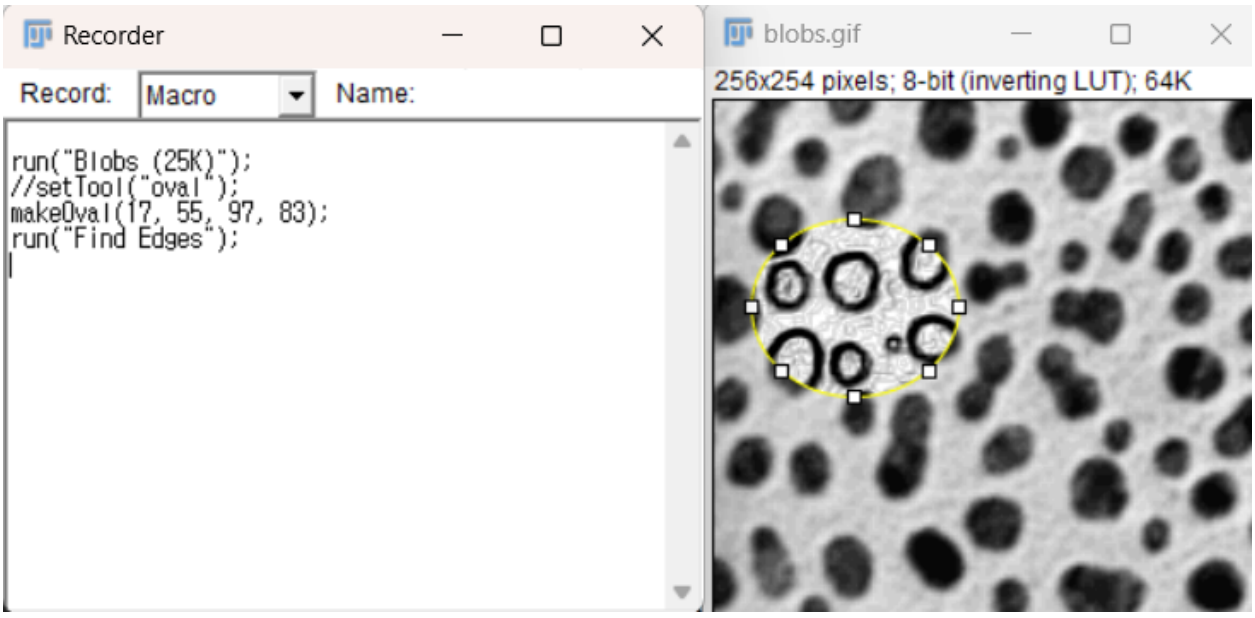
6.1 Macro Recorder

https://imagej.net/scripting/macro

1. Use **Plugins > Macros > Record** (**Ctrl-U**) to start recording simple steps and begin generating code. Note that when clicking around in FIJI, you may generate unnecessary or overly specific commands, which means macros generated through the recorder will typically require some editing before use on other images.

For example:

- Open **Blobs** via **File > Open Samples > Blobs**
- Draw a circle or rectangle using the tools
- Run **Process > Find Edges**

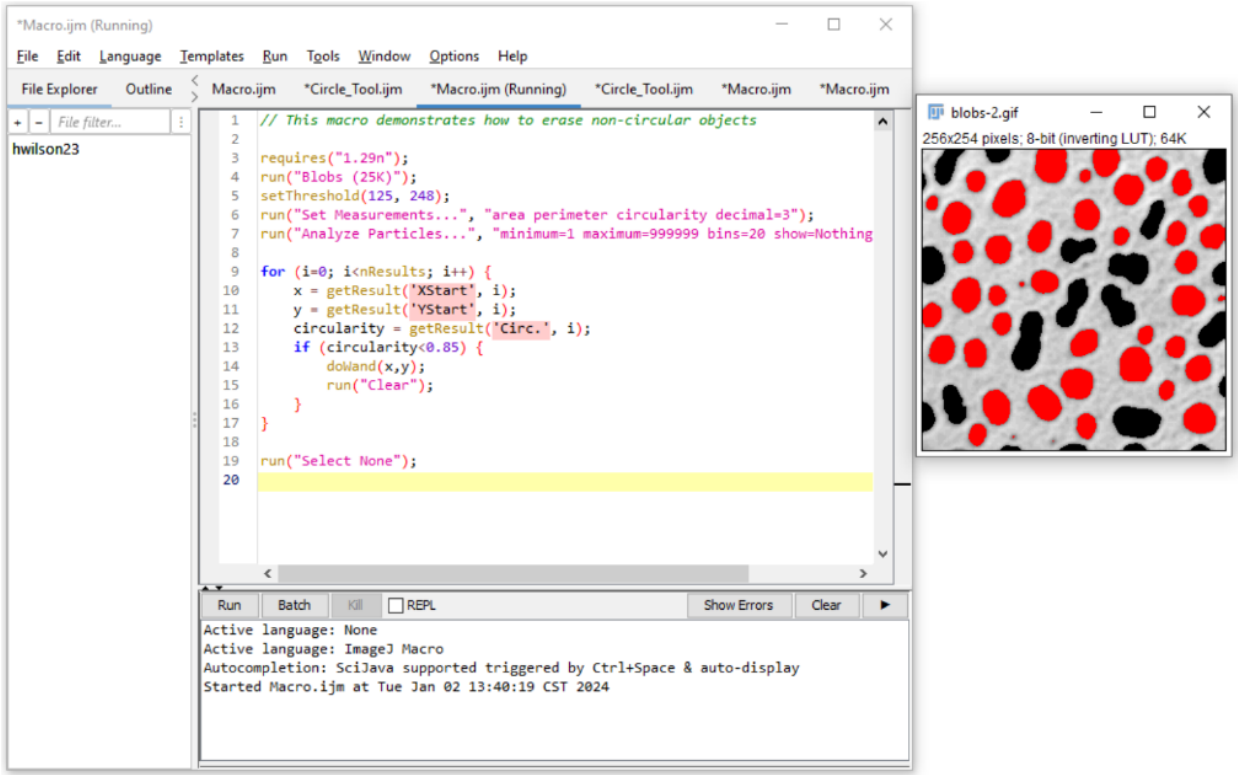


- Press **Create** in the Macro Recorder to save the macro to disk and open it in the Editor.
- Close the blobs image and press "run" in the Editor to run the macro, which repeats the analysis verbatim.

More example scripts:
https://imagej.net/ij/macros/

6.1.1 Why does the recorder not show a response for all plugins?

Not every developer has made the plugins compatible with the macro recorder, which may cause various bugs or a lack of response from the recorder. In some cases, a script may be used to generate similar functionality, but other times this may just be a limitation of the plugin. The Image.sc forum would be a good resource for help if you are able to provide enough information about the error and the analysis goals.

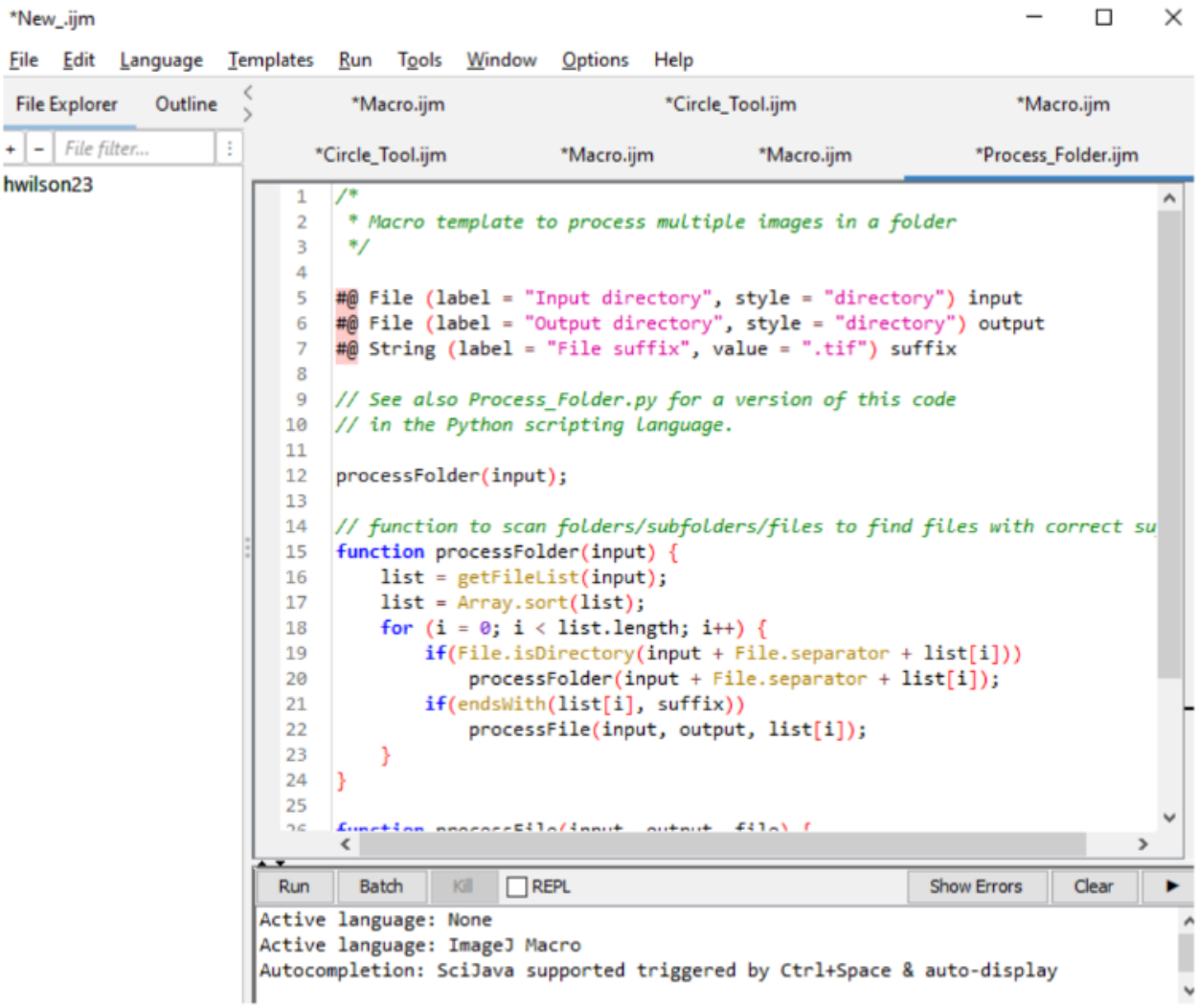


6.2

6.3 Batch Processing

1. Batch processing can be done directly via the macro script editor using a provided template: https://imagej.net/scripting/batch#option-2—script-template
2. Open the script editor: **Plugins > New > Macro**
3. The template can be accessed in the script editor by using **Templates > ImageJ 1.x > Batch > Process Folder (ImageJ Macro)**

This template contains an existing loop to process each file in the folder using the “processFolder” and “processFile” functions. It requires the user to specify the input and output directory as well as the file information.



Here is a sample "processFile()" function to apply the above oval selection and edge finding macro to every file in the directory:

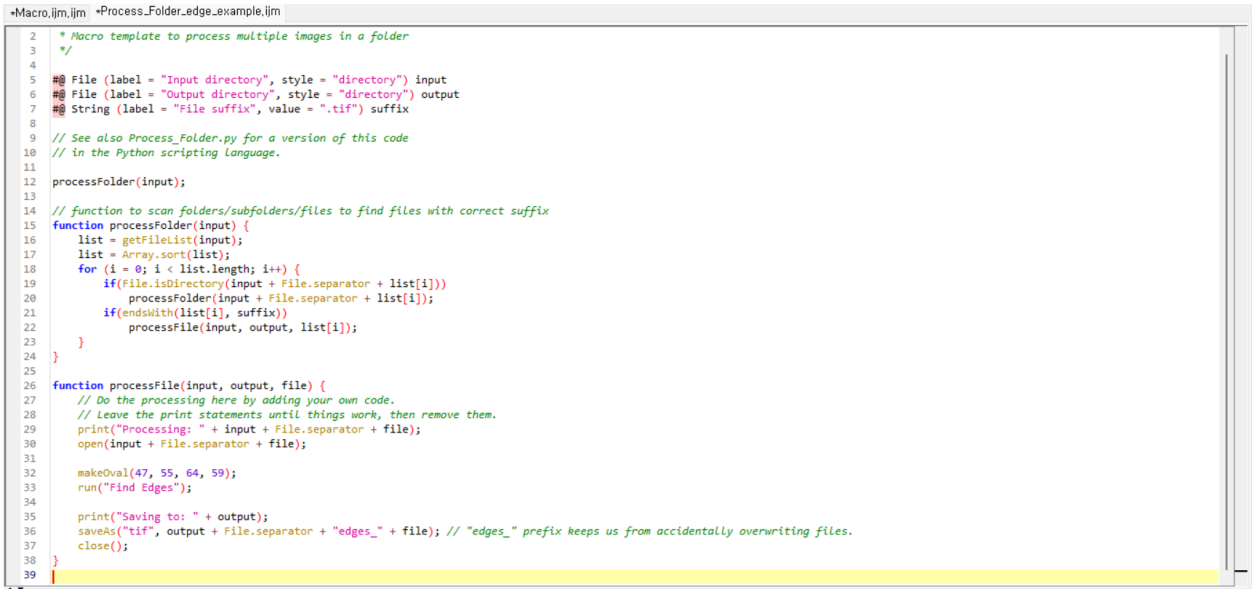
```
function processFile(input, output, file) {
        // Do the processing here by adding your own code.
        // Leave the print statements until things work, then remove them.
        print("Processing: " + input + File.separator + file);
        open(input + File.separator + file);

        makeOval(47, 55, 64, 59);
        run("Find Edges");

        print("Saving to: " + output);
        saveAs("Tiff", output + File.separator + "edges_" + file);
        close(); //Close the file after processing it.
}
```

- When you are inserting the macro recorder script into the bottom function, remember to open and save the file using the **`open()`** and **`saveAs()`** functions.
- The benefits of this is that the files can be more specifically iterated if needed with minor adjustments to the code. For example, try changing `i++` to `i = i + 2` to process every other file.

- It is good practice to use different “input” and “output” directories, to keep from overwriting original data.



- When the macro runs properly, you can comment out the print statements with `//`.
- Batch scripts run much faster when the image is not displayed on screen.
- To prevent newly opened images from being displayed during a batch script, one can start the script with the command:

```
setBatchMode(true);    // Don't show newly opened images
```

Ending the batch script with the following command will close all the images that were opened in the background except the active image:

```
setBatchMode(false); // Return to regular image opening behavior.
```

One can also use

```
setBatchMode("show"); // Show a single image
setBatchMode("exit and display");
```

Note

Note: the most common causes of scripting errors are failure to end a line with a semicolon and failure to include `open()` or `save()` functions for the images.

CHAPTER

SEVEN

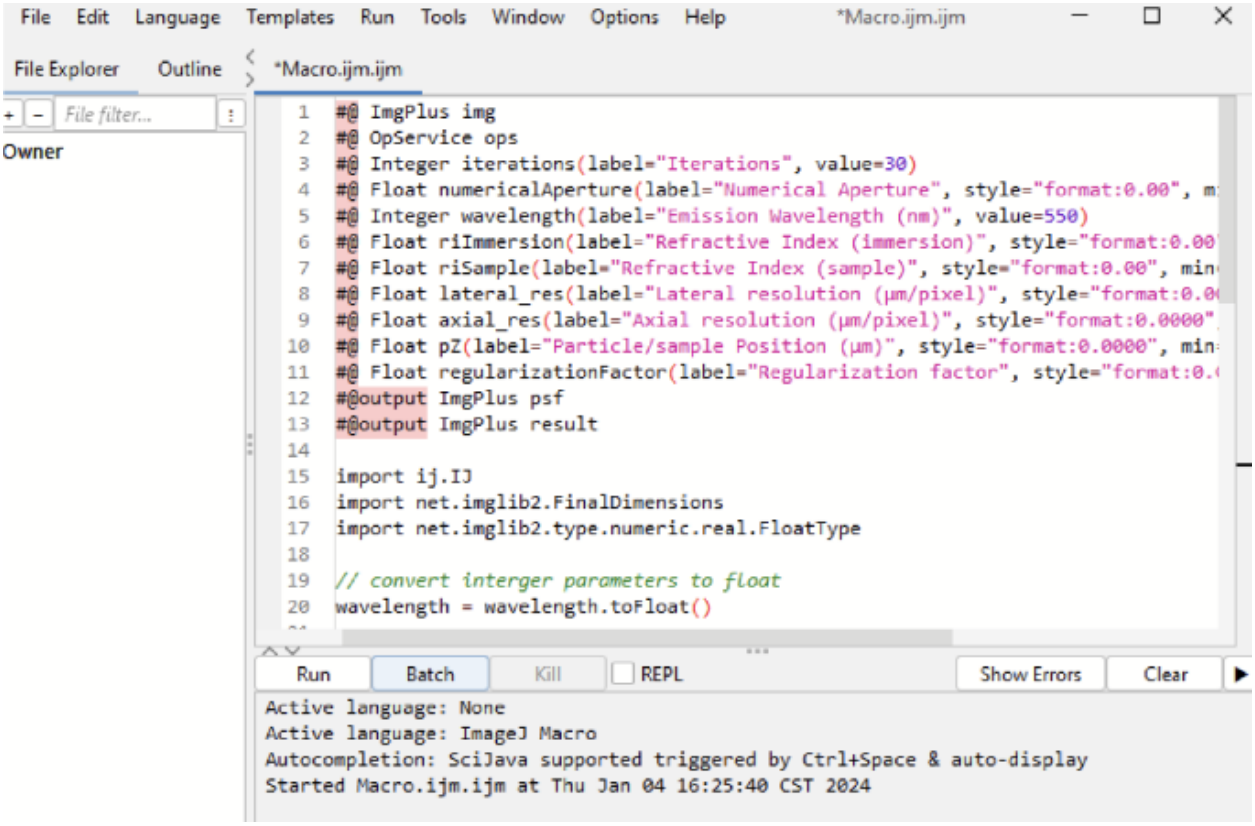
DECONVOLUTION

Deconvolution is a method of denoising a microscopy image using information about the optical setup. In this case, we estimate a point spread function for the deconvolution using the user input parameters of numerical aperture, resolution, emission wavelength, and refractive index.

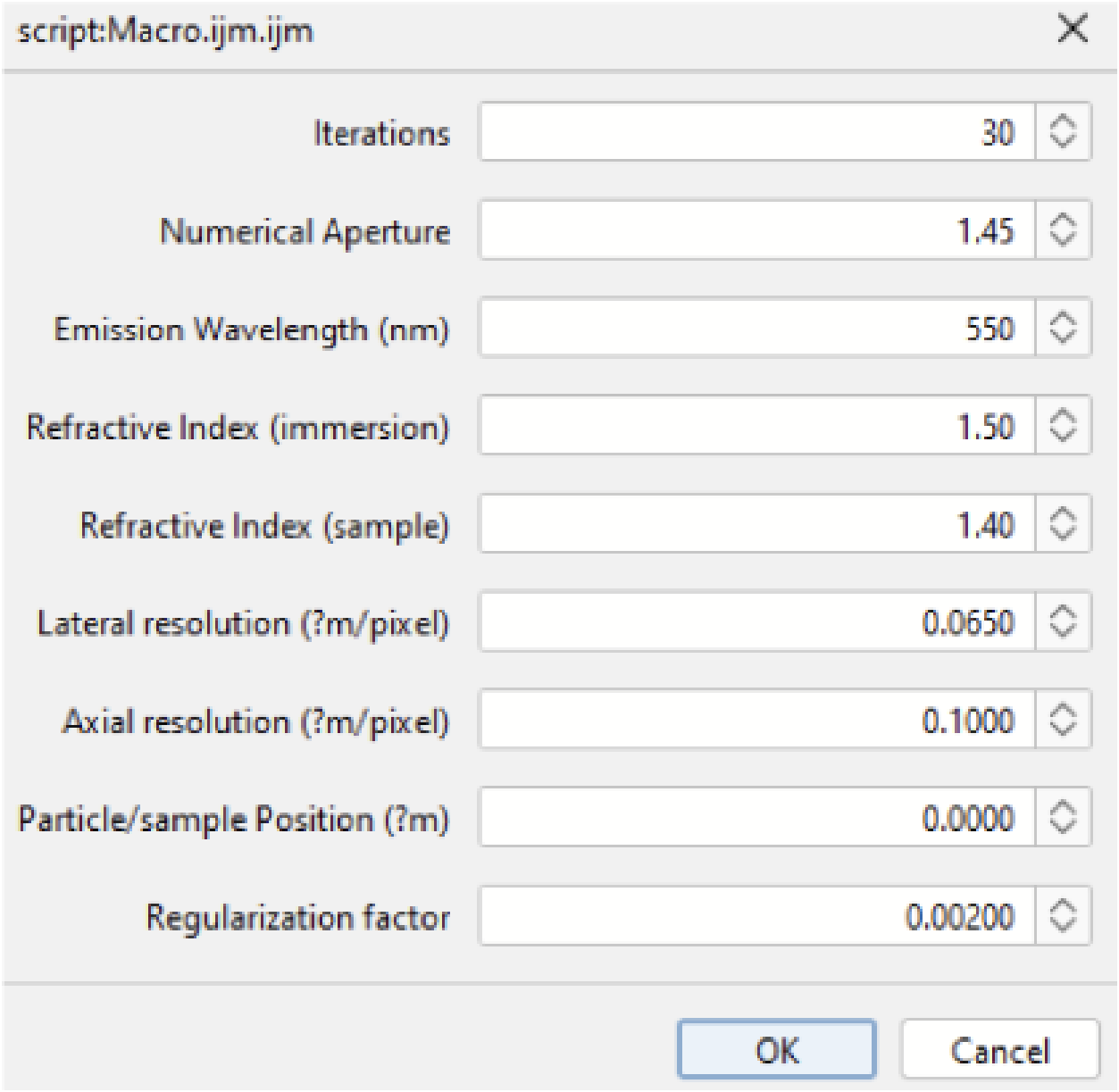
A Fiji macro developed by **Ed Evans** is available here:
https://github.com/elevans/fiji-scripts/blob/main/imagej2/deconvolution/decon.groovy

More background on the algorithm (Richardson-Lucy + Total Variation Regularization):
https://doi.org/10.1002/jemt.20294

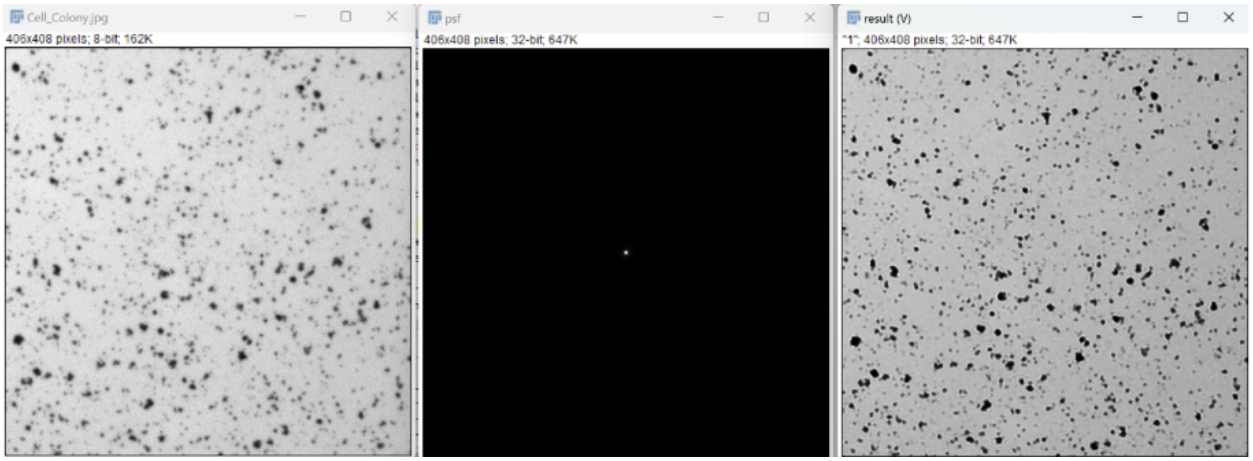
1. Copy-paste the code into the script editor, or open the `.groovy` file. **Change the script language to Groovy**, then run the script on the selected image.



- A dialog will prompt for the experimental parameters. Enter the known parameters from the experiment (or for the demo use the default values).



Example: Try with **Cell Colony** (FIJI Sample) and default parameters to see a demonstration.
You'll notice sharper edges and reduced background noise — even though the parameters may not match the real imaging conditions.

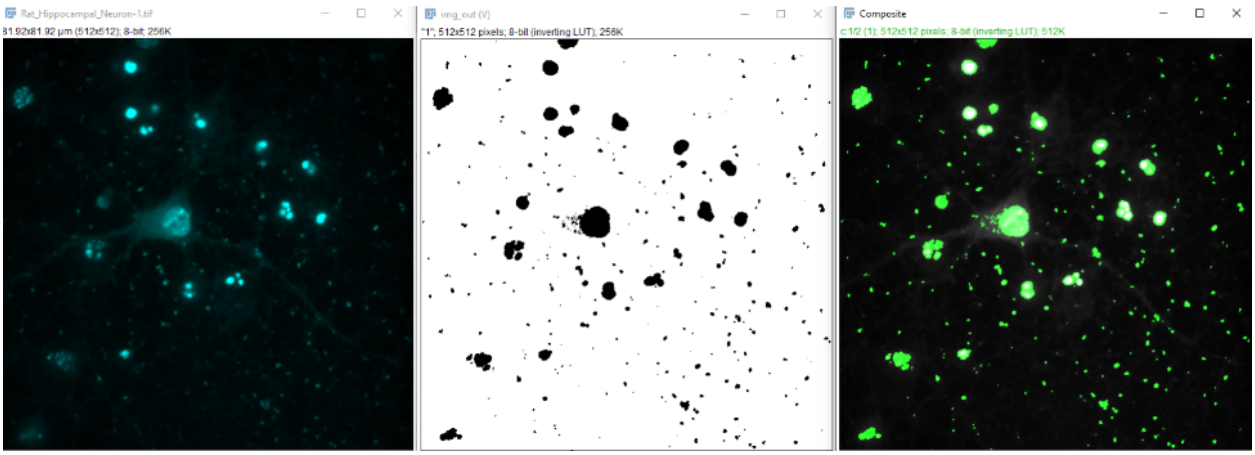

Cell_Colony.jpg
406x408 pixels; 8-bit; 162K
psf
406x408 pixels; 32-bit; 647K
result (V)
"1"; 406x408 pixels; 32-bit; 647K

CHAPTER

EIGHT

BACKGROUND SUBTRACTION – GAUSSIAN FILTERS

Performing background subtraction with a Gaussian filter can be an effective method of resolving objects from a noisy background. More info here: https://bioimagebook.github.io/chapters/2-processing/4-filters/filters.html#gaussian-filters



Background Subtraction - Gaussian filters Performing background subtraction with a gaussian filter can be an effective method of resolving objects from a noisy background. More info here: https://bioimagebook.github.io/chapters/2-processing/4-filters/filters.html#gaussian-filters

1. Use **File > Open Samples > Neuron (5 channel)** to open the starting image. We only want one channel to work with, so right click and **duplicate channel 4**.

- Try a variety of thresholds on this image, including the triangle method. Here we will try to improve this threshold by separating out the smaller point-like structures.

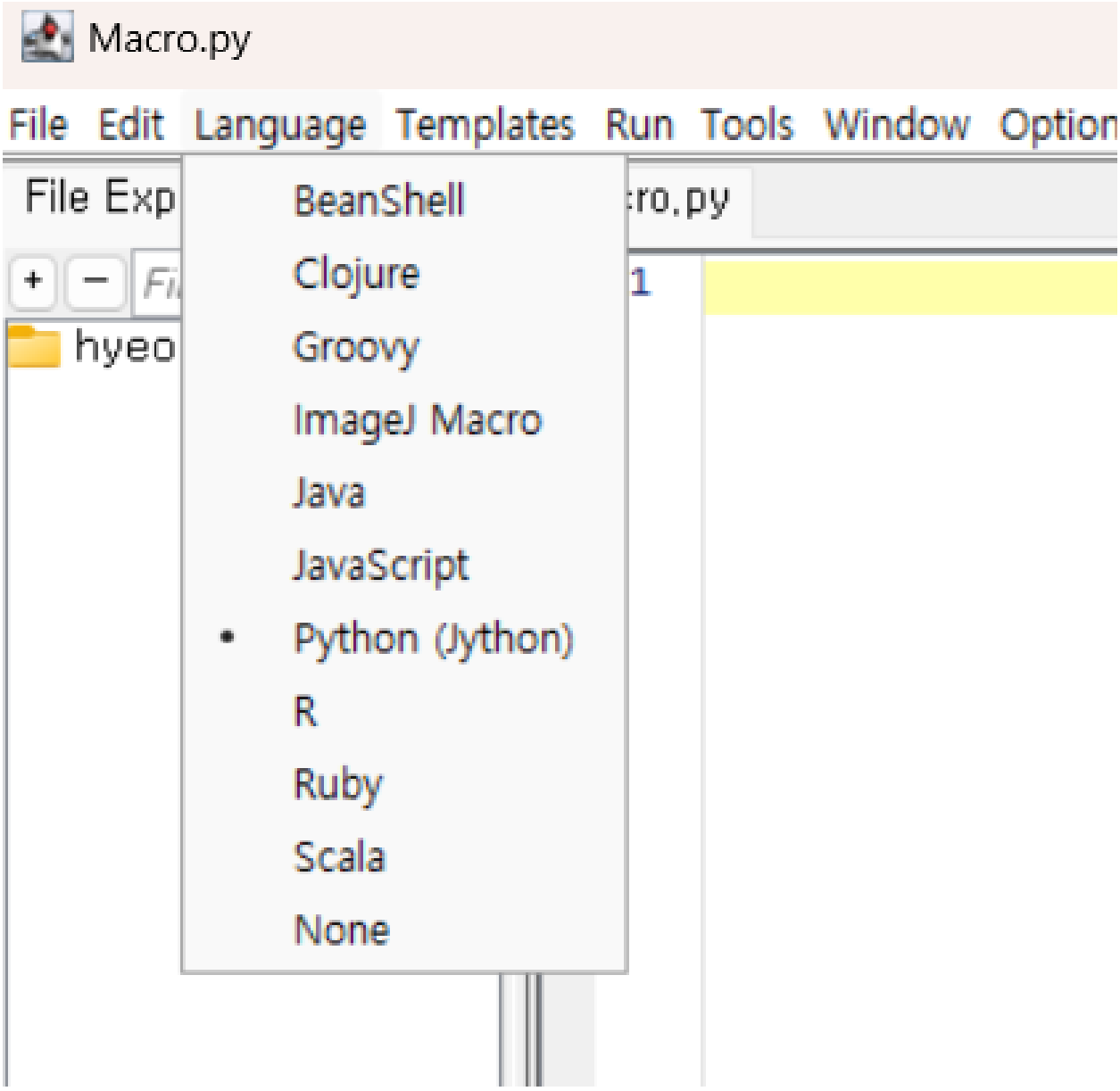
2. Open the gaussian subtraction script found here, developed by Ed Evans:

   https://github.com/elevans/fiji-scripts/blob/main/imagej2/filters/ijo_gaussian_subtraction.py

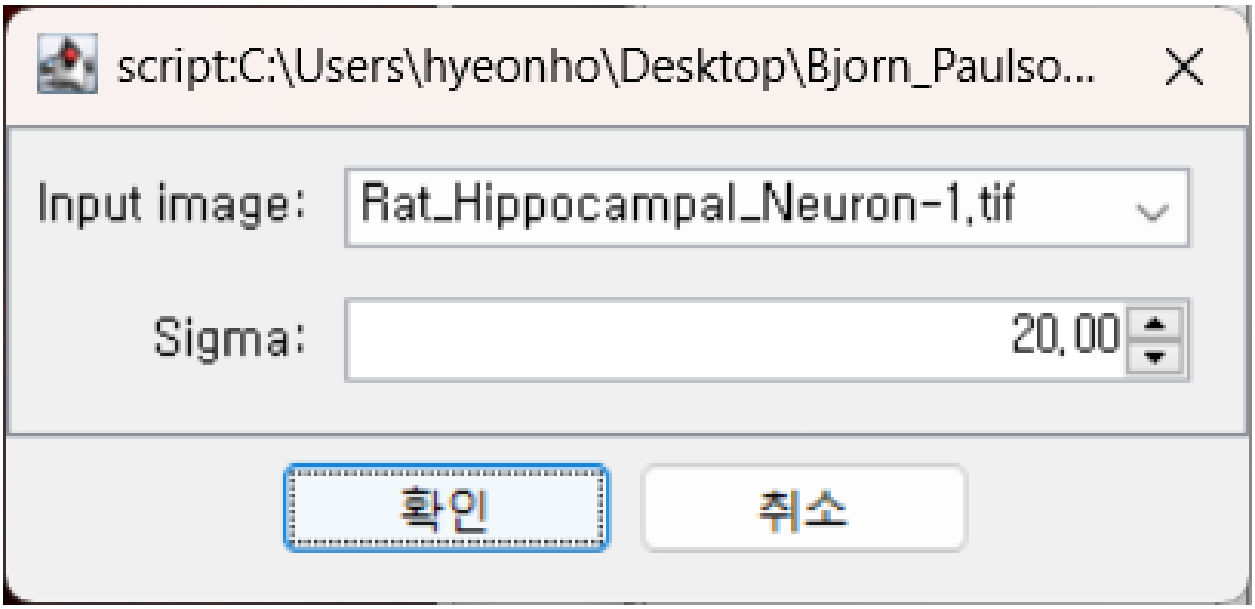
(Download raw file in GitHub.)

Open this in Fiji to open the Macro Editor, or open the Macro Editor via **Plugins > New > Macro**

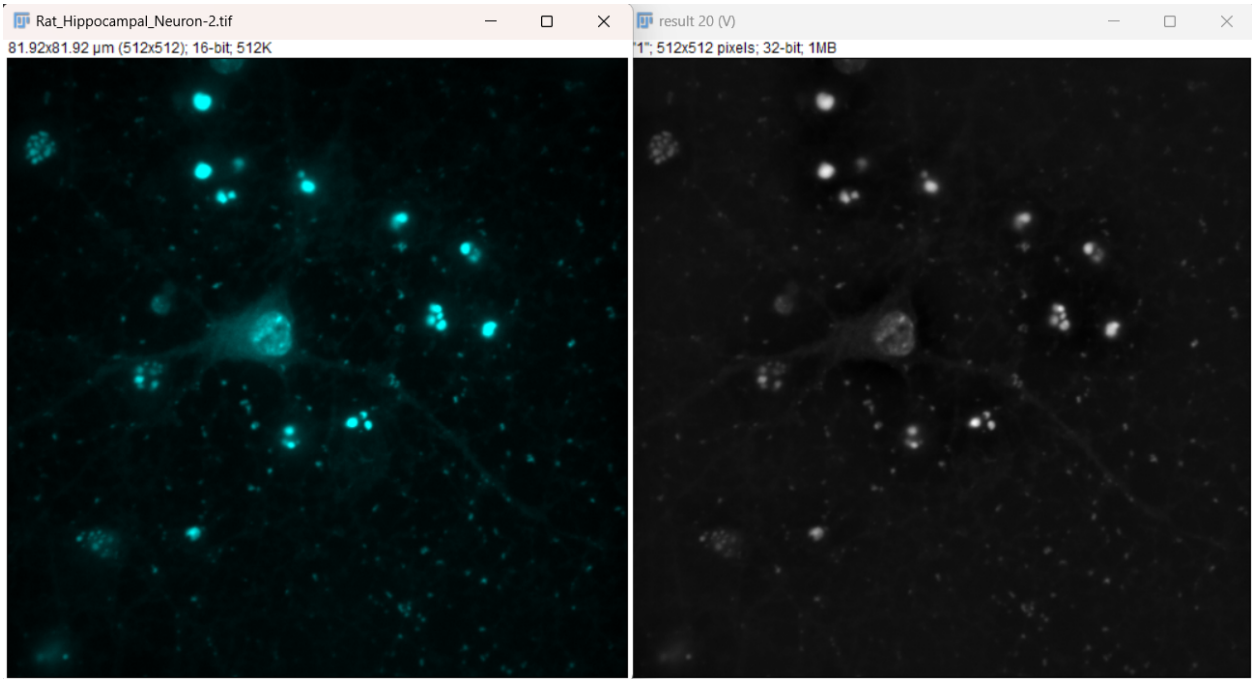
This script is written in python, so be sure to change the macro language to python before running, otherwise an error will be produced:



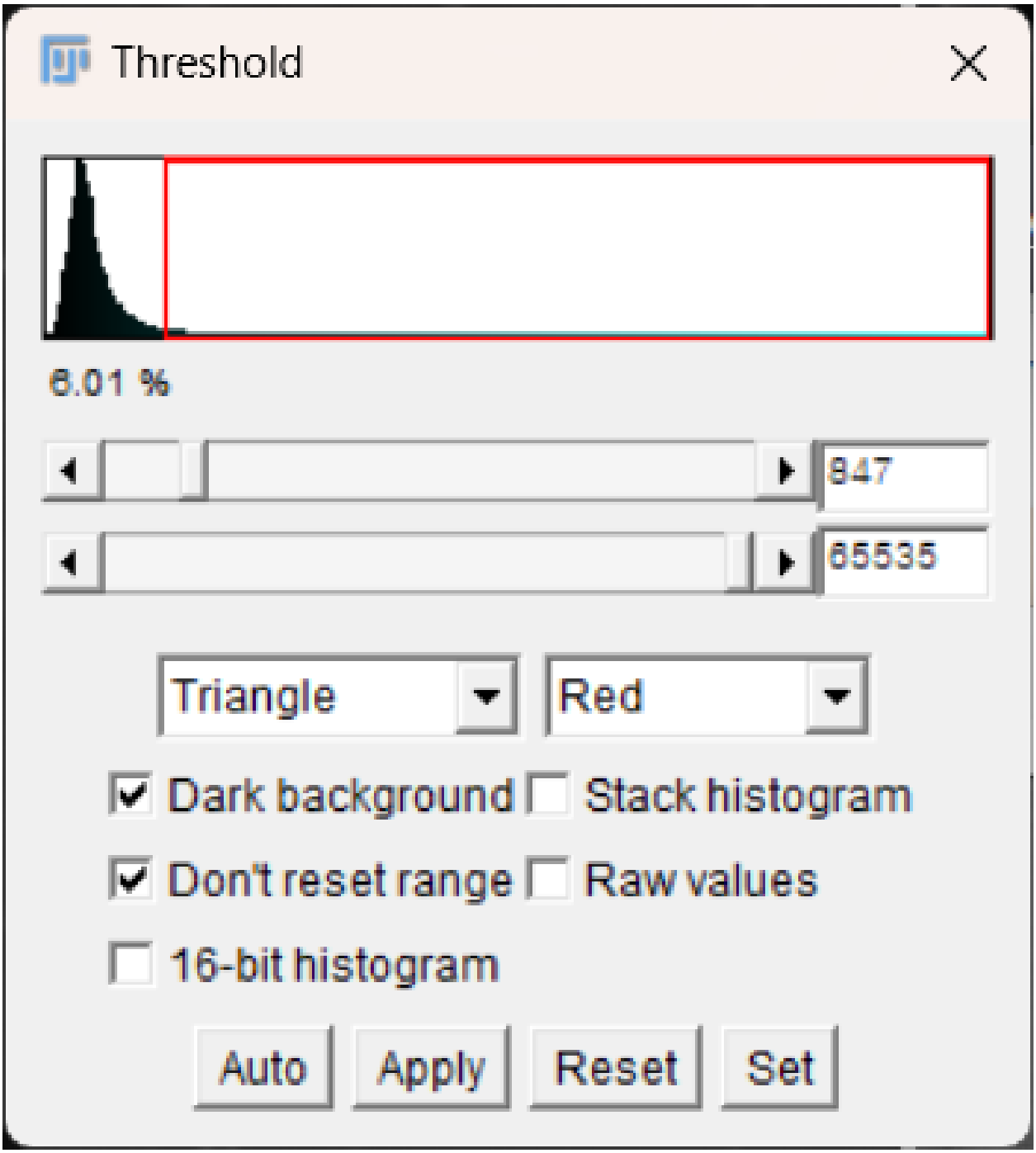
3. With the single channel image highlighted, run the script. You should be prompted to input a sigma value for the gaussian filter. Larger values will blur out larger and larger objects. For this example, use 20 and then use 1. Feel free to try a variety of values to see how the results change.

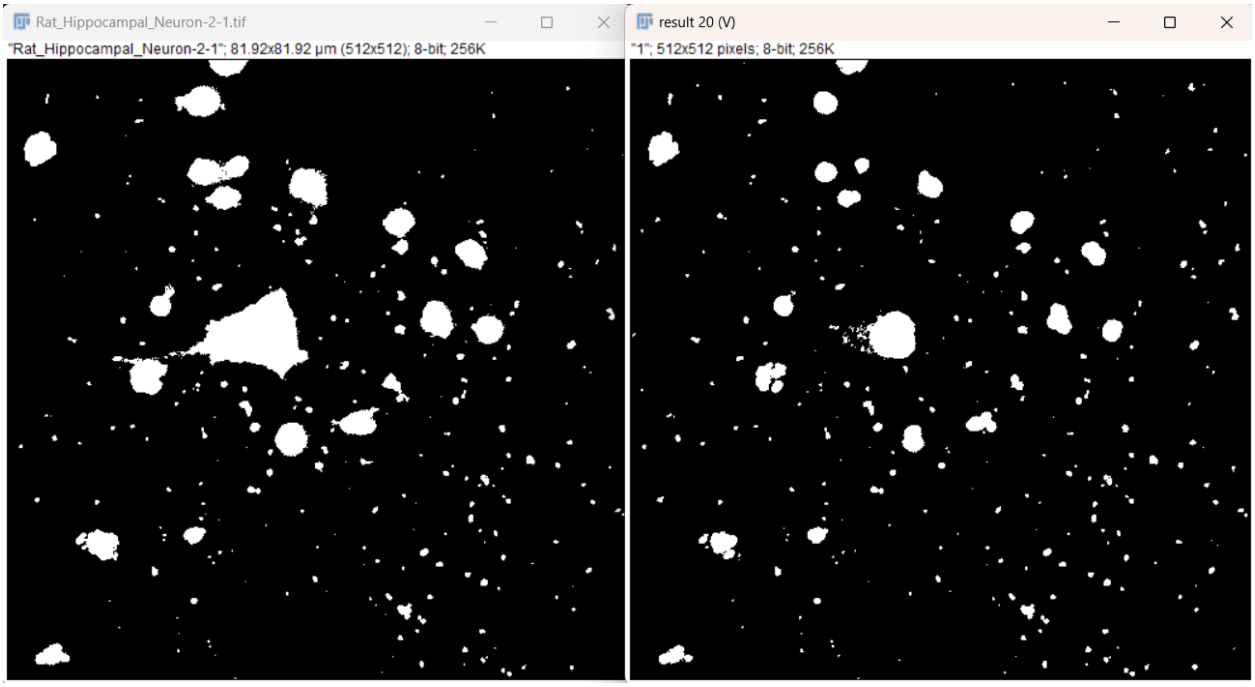


4. After running the script, we now see that some structures are highlighted a bit differently in result (V) (notice how the cell body is harder to see)
    - Some structures are highlighted differently.
    - The cell body may be less visible, while smaller axons or dots stand out more.



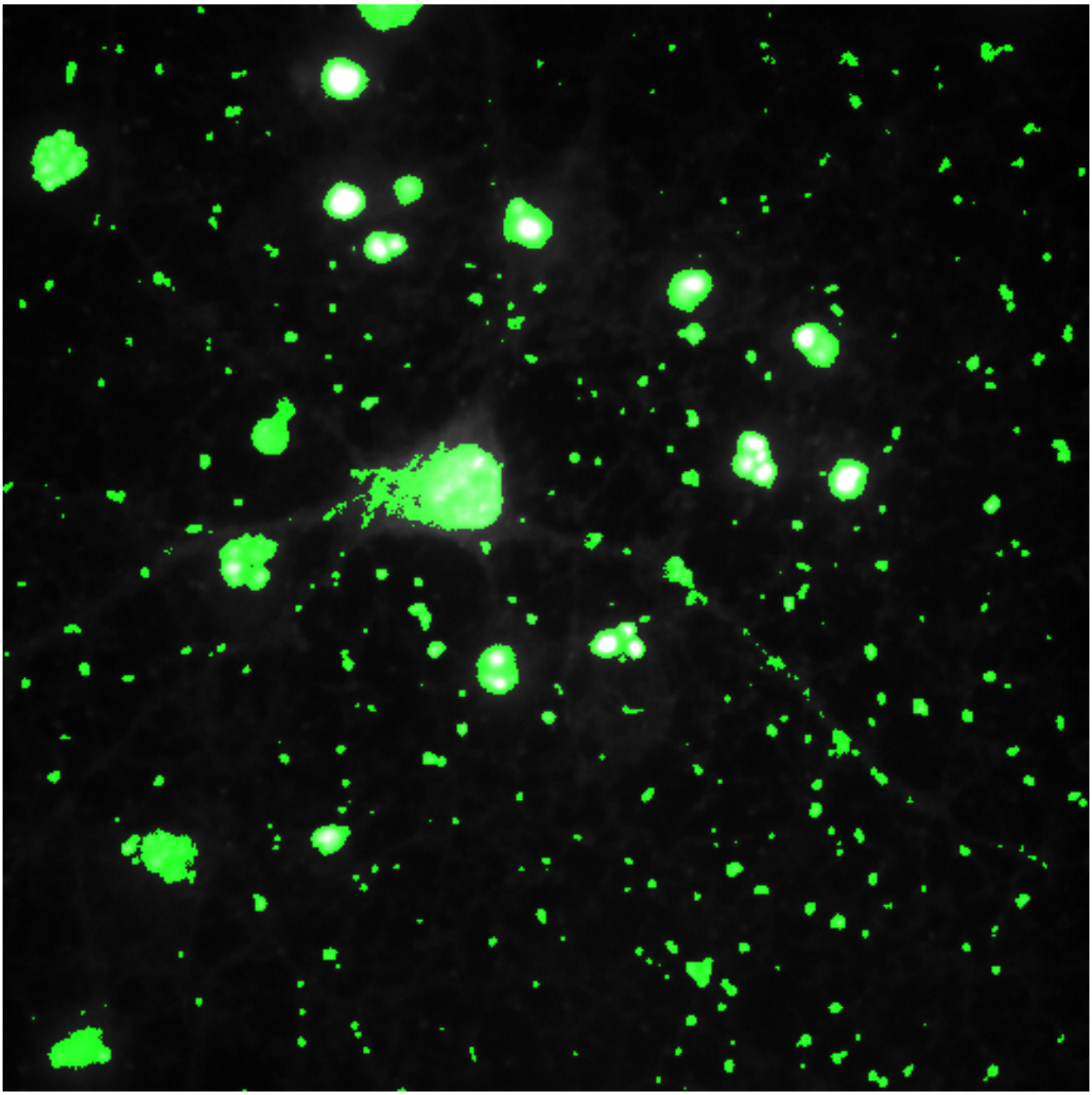
5. After running the script, we now see that some structures are highlighted a bit differently in `result (V)` (notice how the cell body is harder to see):


Threshold
6.01 %
847
65535
Triangle
Red
Dark background
Stack histogram
Don't reset range
Raw values
16-bit histogram
Auto
Apply
Reset
Set



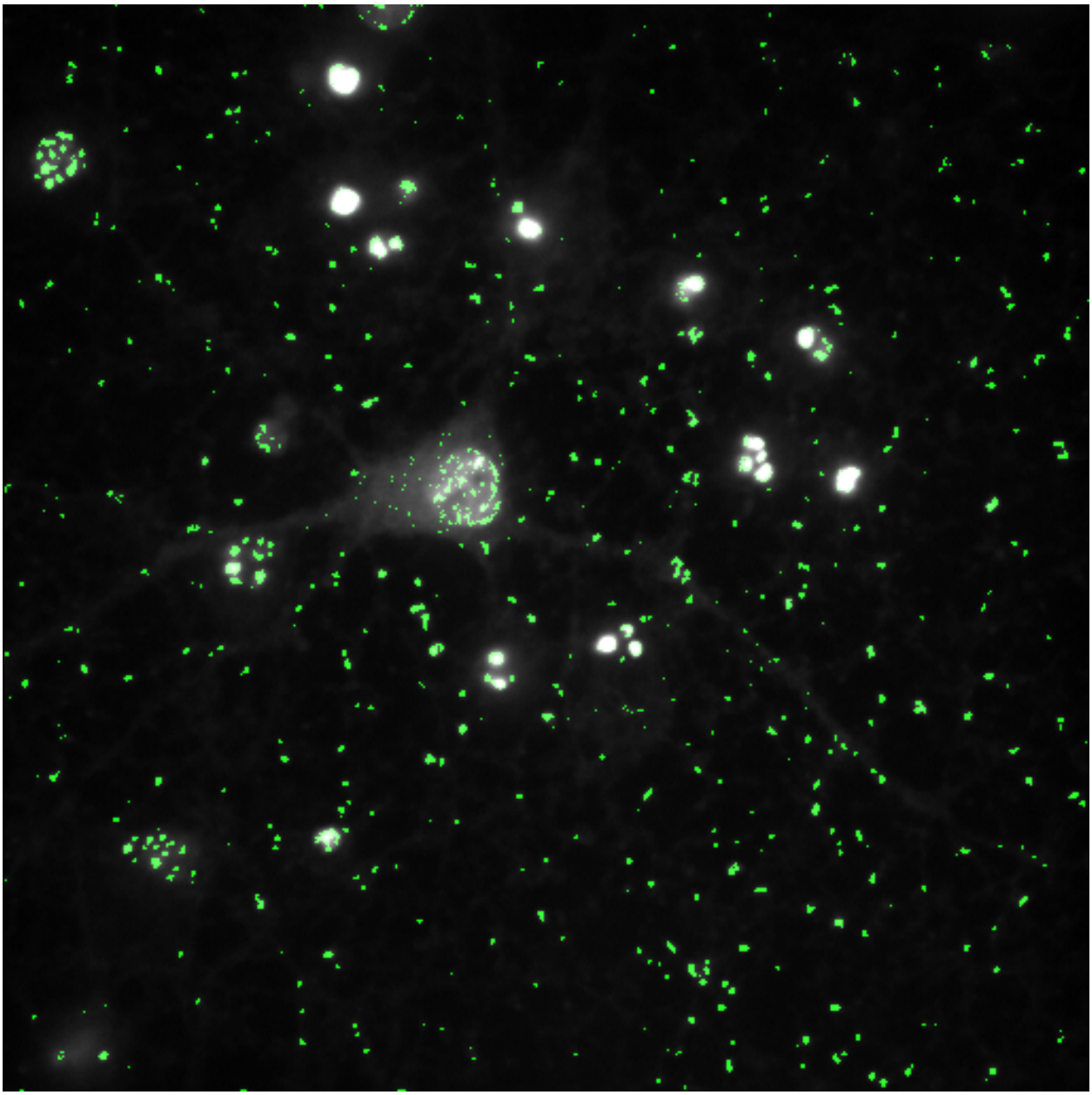
6. If we decide this mask is sufficient for our data, we can then apply the mask to the output image and merge the display. a. With the output image still selected, choose **Apply** on the threshold window, while making sure the dark background box is selected. It then will ask you to convert the output to an 8-bit mask. i. The areas of interest should now have values of 255 while the background has a value of `0`. If this is inverted, it is likely that the dark background box in the threshold window was not selected, you can simply use **Edit > Invert** to switch the values. b. To overlay the mask on the original image, we can make a composite. This requires that the original image is also converted to 8-bit for display. Use **Image > Type > 8-bit**

c. To make the composite, use **Image > Color > Merge Channels**. Here, I will set the original channel duplicate to C4 (gray) and the mask to C2 (green). This should procedure the following composite image, that can be used for display:



d. As an additional display, we could use **Edit > Selection > Create Selection** on the mask image, then **Edit > Selection > Restore Selection** on the original image to get outlines of the mask.

If we use a sigma value of 1 and repeat the process, we can see there a further differences in the cell body and other structures, although this seems to also pick up more noise:



CHAPTER

NINE

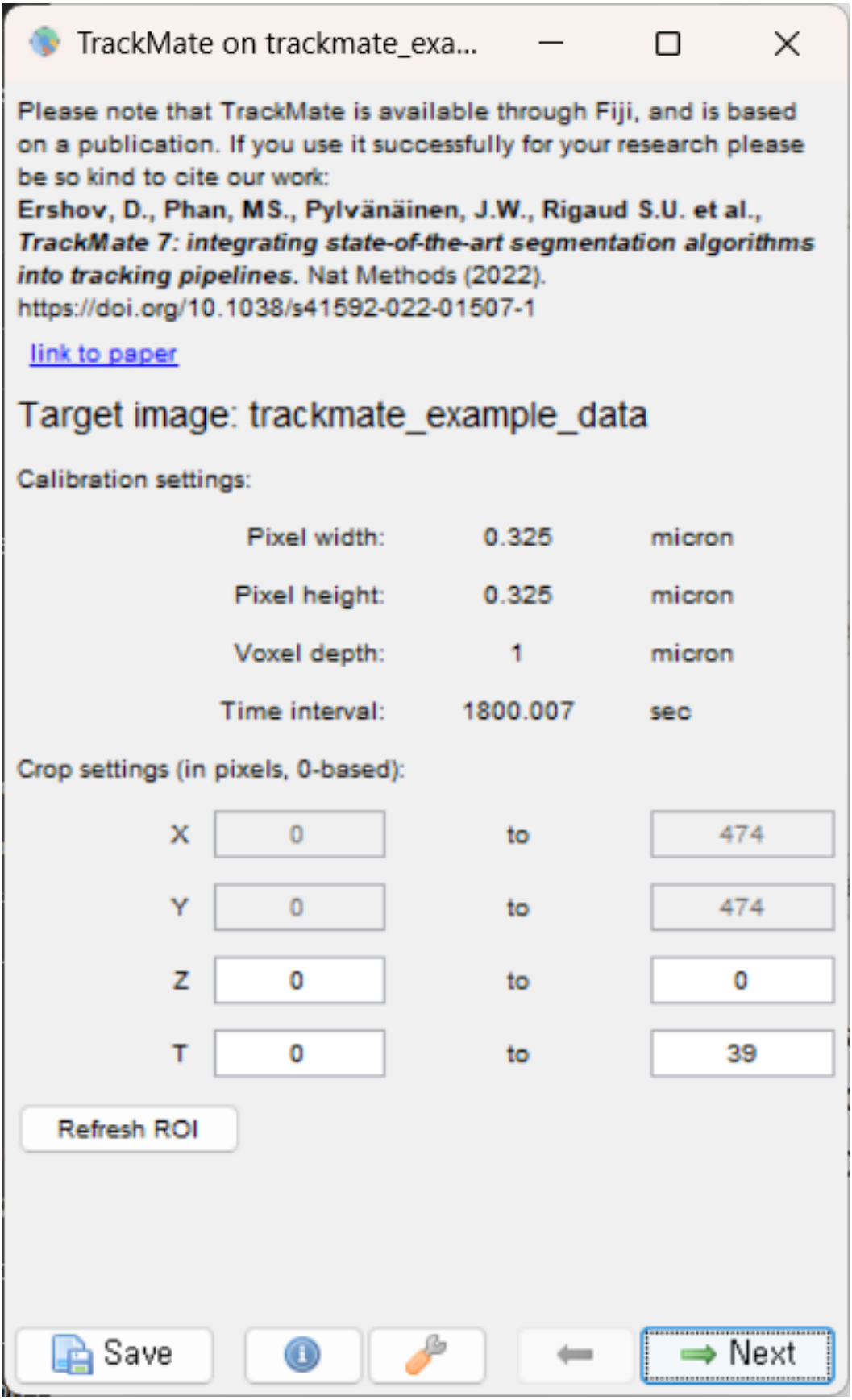
TRACKMATE – EXAMPLE FROM DOCUMENTATION

Ershov, D., Phan, M.-S., Pylvänäinen, J. W., Rigaud, S. U., Le Blanc, L., Charles-Orszag, A., … Tinevez, J.-Y. (2022). TrackMate 7: integrating state-of-the-art segmentation algorithms into tracking pipelines. *Nature Methods, 19*(7), 829–832. doi:10.1038/s41592-022-01507-1

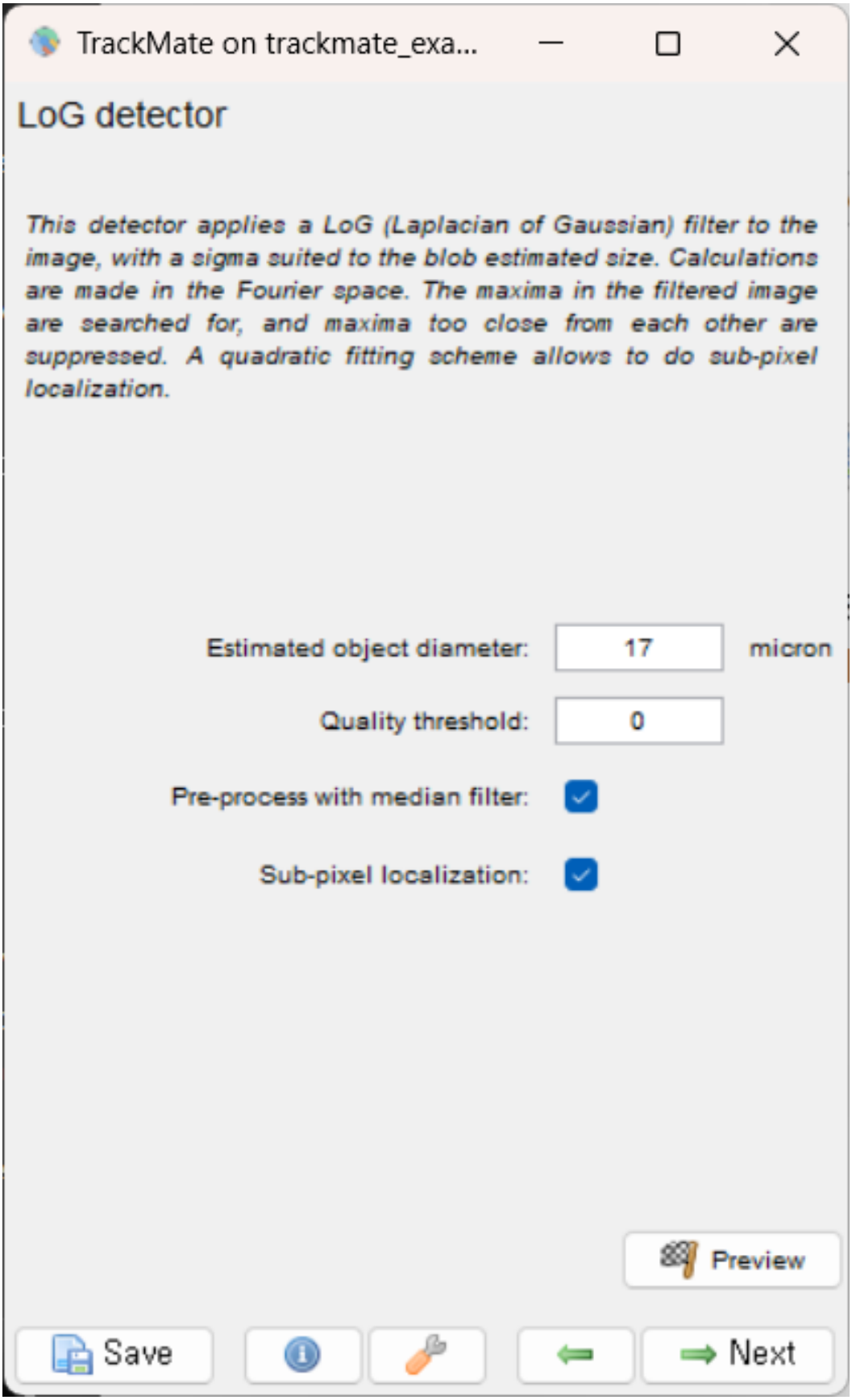
TrackMate documentation and tutorials:
https://imagej.net/plugins/trackmate/
TrackMate manual:
https://imagej.net/media/plugins/trackmate/trackmate-manual.pdf

The following demo can be accessed here: https://napari.imagej.net/en/latest/examples/trackmate.html

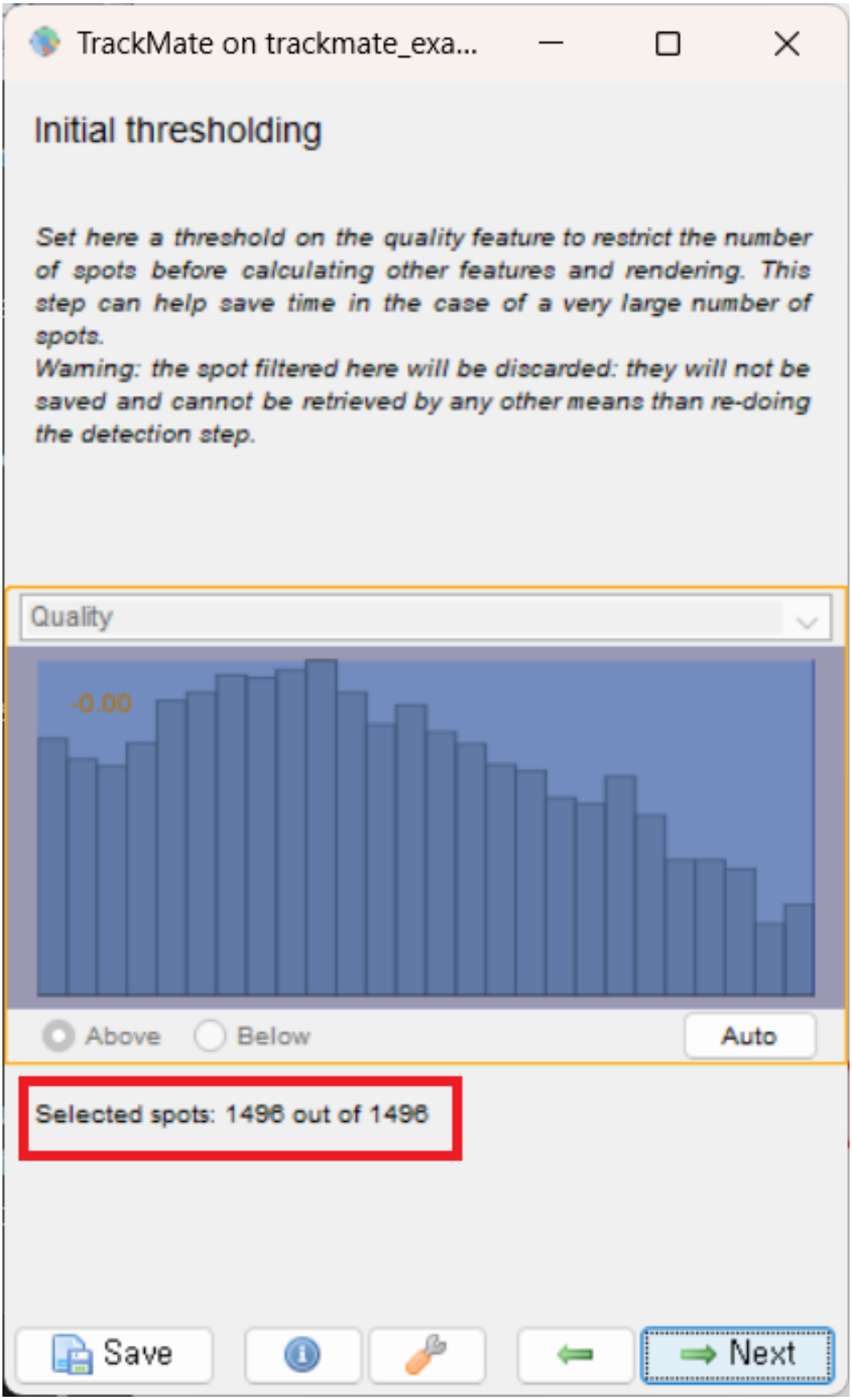
1. Open the `trackmate_example_data.tif`.
2. Run the TrackMate plugin (**Plugins > Tracking > TrackMate**). Select "Next" as we don't need to make any changes. The settings in this window allow you to crop the original data as needed.


TrackMate on trackmate_exa...
Please note that TrackMate is available through Fiji, and is based on a publication. If you use it successfully for your research please be so kind to cite our work:
Ershov, D., Phan, MS., Pylvänäinen, J.W., Rigaud S.U. et al., TrackMate 7: integrating state-of-the-art segmentation algorithms into tracking pipelines. Nat Methods (2022).
https://doi.org/10.1038/s41592-022-01507-1
link to paper
Target image: trackmate_example_data
Calibration settings:
Pixel width: 0.325 micron
Pixel height: 0.325 micron
Voxel depth: 1 micron
Time interval: 1800.007 sec
Crop settings (in pixels, 0-based):
X 0 to 474
Y 0 to 474
Z 0 to 0
T 0 to 39
Refresh ROI
Save
Next

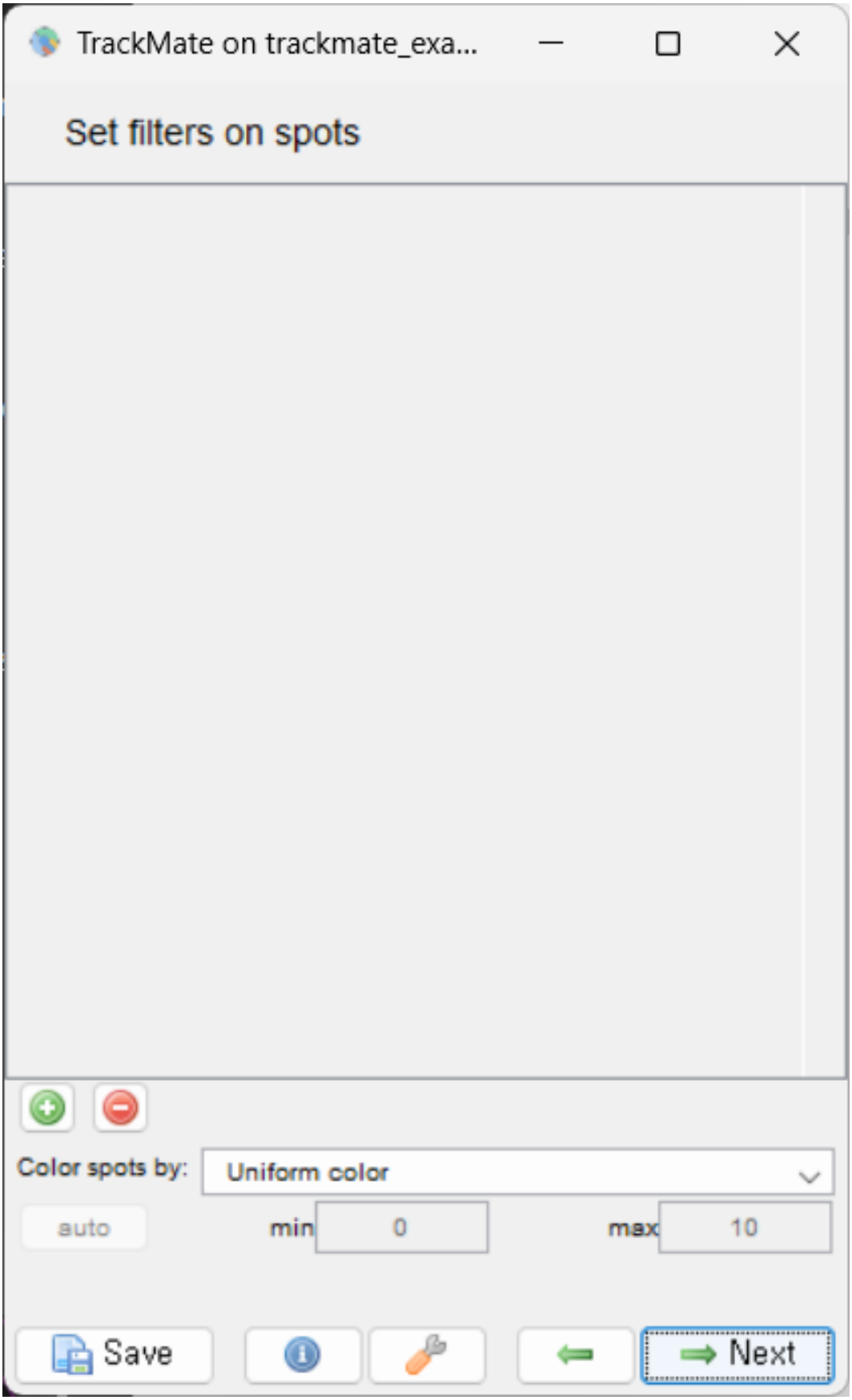
3. Use the Laplacian of Gaussian (LoG) detector. The plugin provides various algorithms to help process the image, also including the difference of gaussian and hessian detectors.
4. Enter 17 as the estimated object diameter and 0 as a quality threshold, “Next.”


TrackMate on trackmate_exa...
LoG detector
This detector applies a LoG (Laplacian of Gaussian) filter to the image, with a sigma suited to the blob estimated size. Calculations are made in the Fourier space. The maxima in the filtered image are searched for, and maxima too close from each other are suppressed. A quadratic fitting scheme allows to do sub-pixel localization.
Estimated object diameter:
17
micron
Quality threshold:
0
Pre-process with median filter:
Sub-pixel localization:
Preview
Save
Next

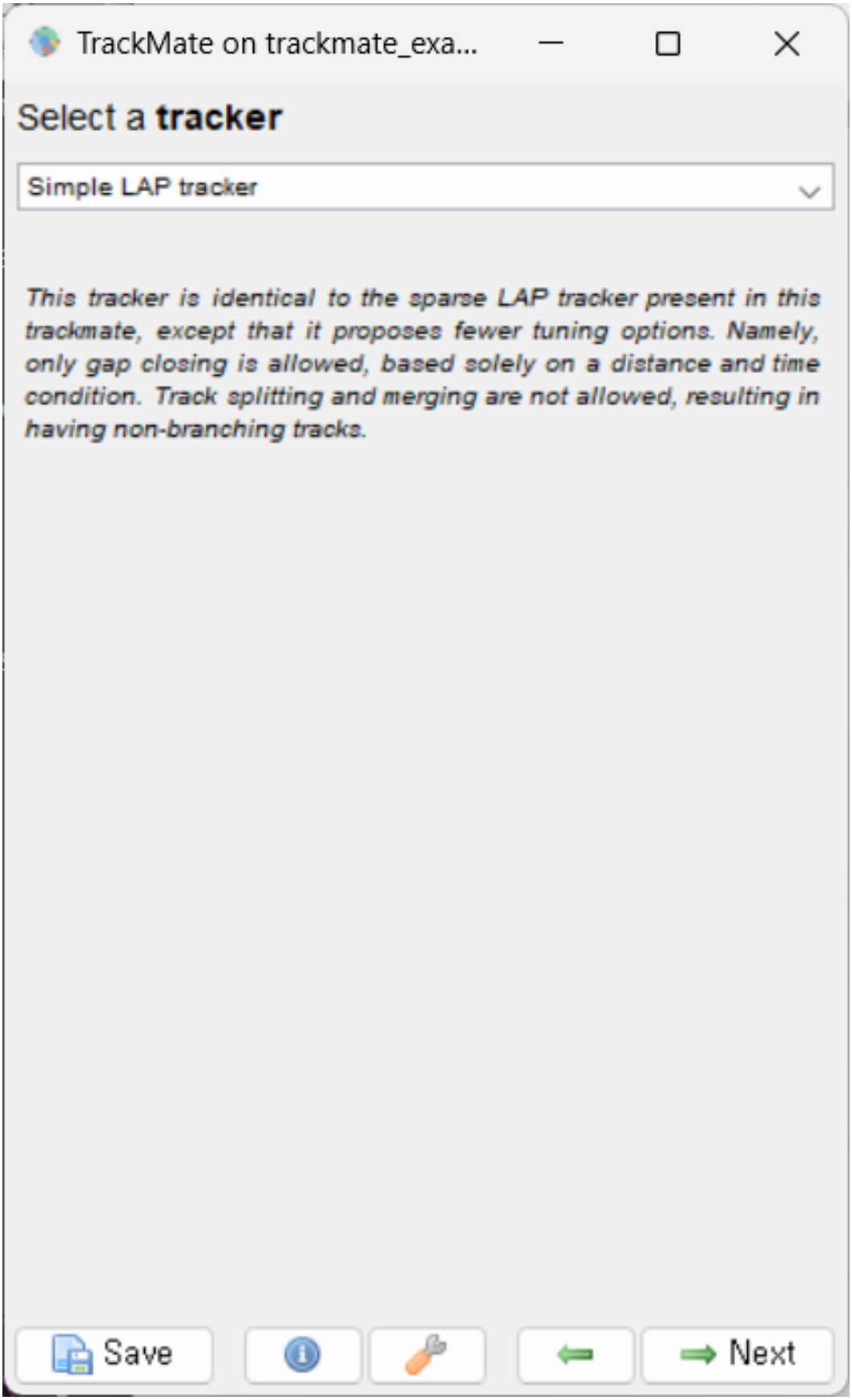
5. The next page provides a summary. Select “Next.”
6. For Initial Thresholding, verify you see 1496 spots selected, and select “Next.”


TrackMate on trackmate_exa...
Initial thresholding
Set here a threshold on the quality feature to restrict the number of spots before calculating other features and rendering. This step can help save time in the case of a very large number of spots.
Warning: the spot filtered here will be discarded: they will not be saved and cannot be retrieved by any other means than re-doing the detection step.
Quality
-0.00
Above
Below
Auto
Selected spots: 1496 out of 1496
Save
Next

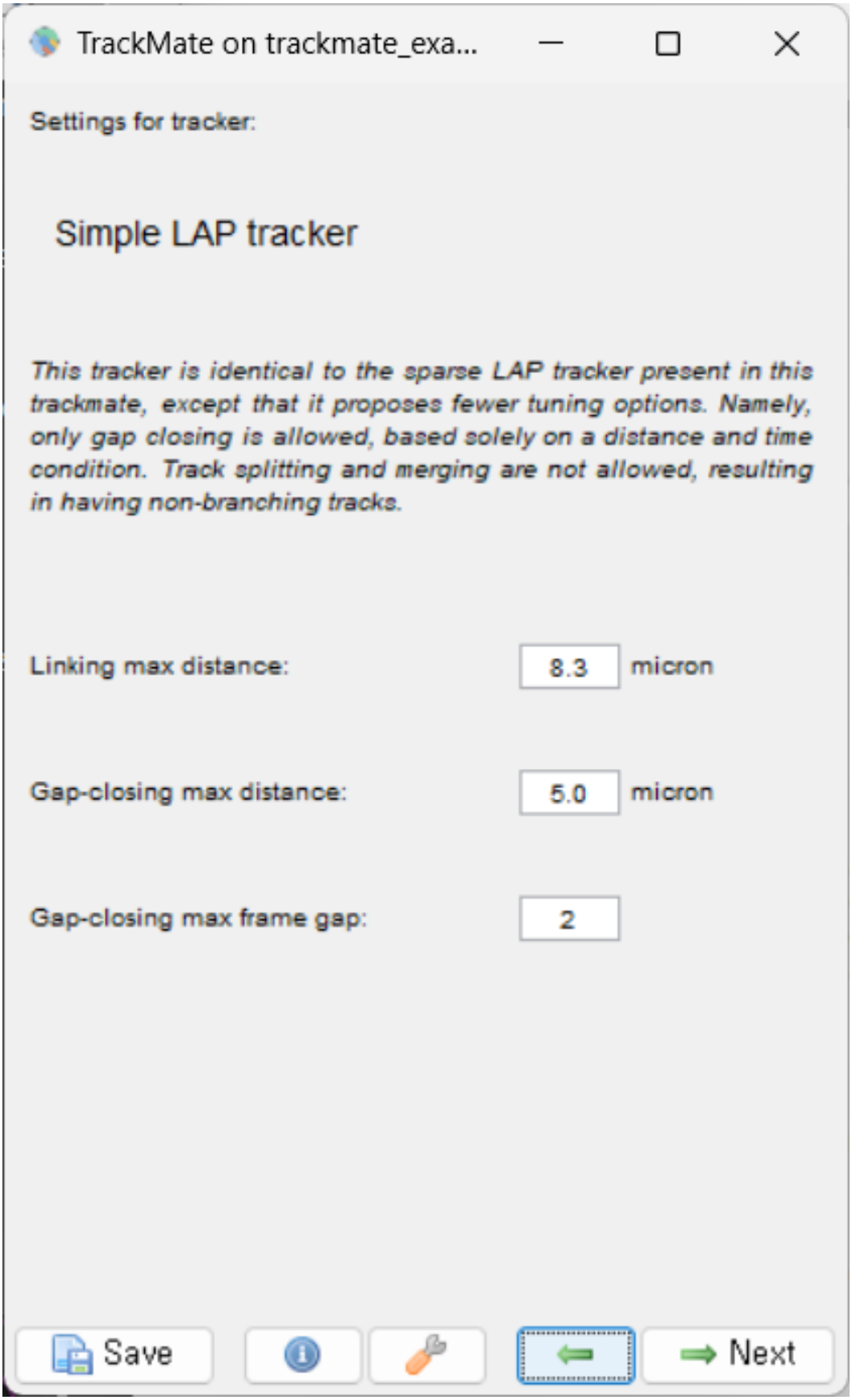
7. Select “Next” as we do not need a filter. If a filter was required, we could use the “Plus” button to add a filter and adjust the selection metric.


TrackMate on trackmate_exa...
Set filters on spots
Color spots by:
Uniform color
auto
min
0
max
10
Save
Next

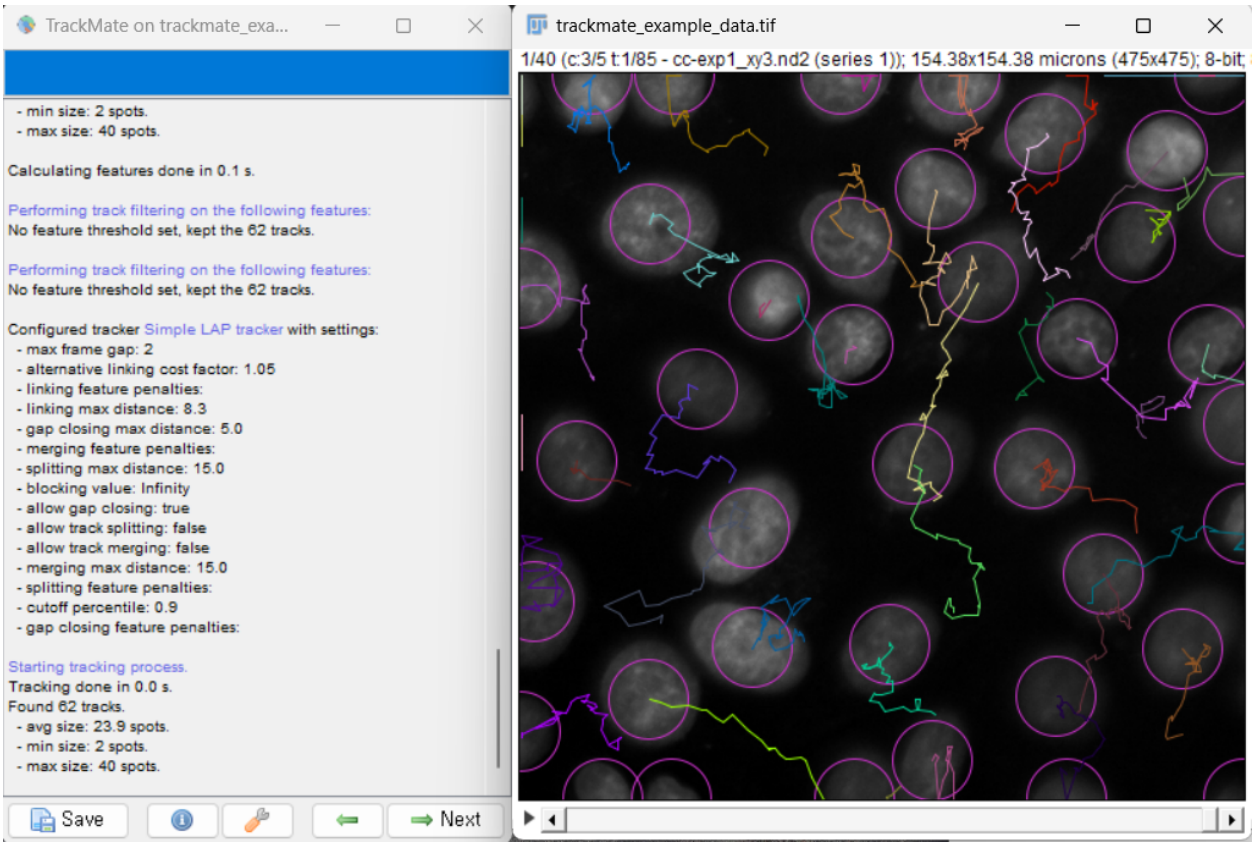
8. Use the “Simple LAP tracker”, then select “Next.” TrackMate also provides a variety of other methods.


TrackMate on trackmate_exa...
Select a tracker
Simple LAP tracker
This tracker is identical to the sparse LAP tracker present in this trackmate, except that it proposes fewer tuning options. Namely, only gap closing is allowed, based solely on a distance and time condition. Track splitting and merging are not allowed, resulting in having non-branching tracks.
Save
Next

9. Then we set the parameters for the tracker. The linking max distance will be 8.3 microns, the gap-closing max distance is 5 microns, and the gap-closing max frame gap is 2 micron. Select “Next.”


TrackMate on trackmate_exa...
Settings for tracker:
Simple LAP tracker
This tracker is identical to the sparse LAP tracker present in this trackmate, except that it proposes fewer tuning options. Namely, only gap closing is allowed, based solely on a distance and time condition. Track splitting and merging are not allowed, resulting in having non-branching tracks.
Linking max distance:
8.3
micron
Gap-closing max distance:
5.0
micron
Gap-closing max frame gap:
2
Save
Next

10. A summary page is displayed. You can scroll through the image and see the proposed tracks for each cell. Select "Next."
11. Select "Next" three more times as we will not filter any of the tracks and do not need to edit the display options.
12. Select "Execute" for the final image, which can be run over all of the frames.



CHAPTER

TEN

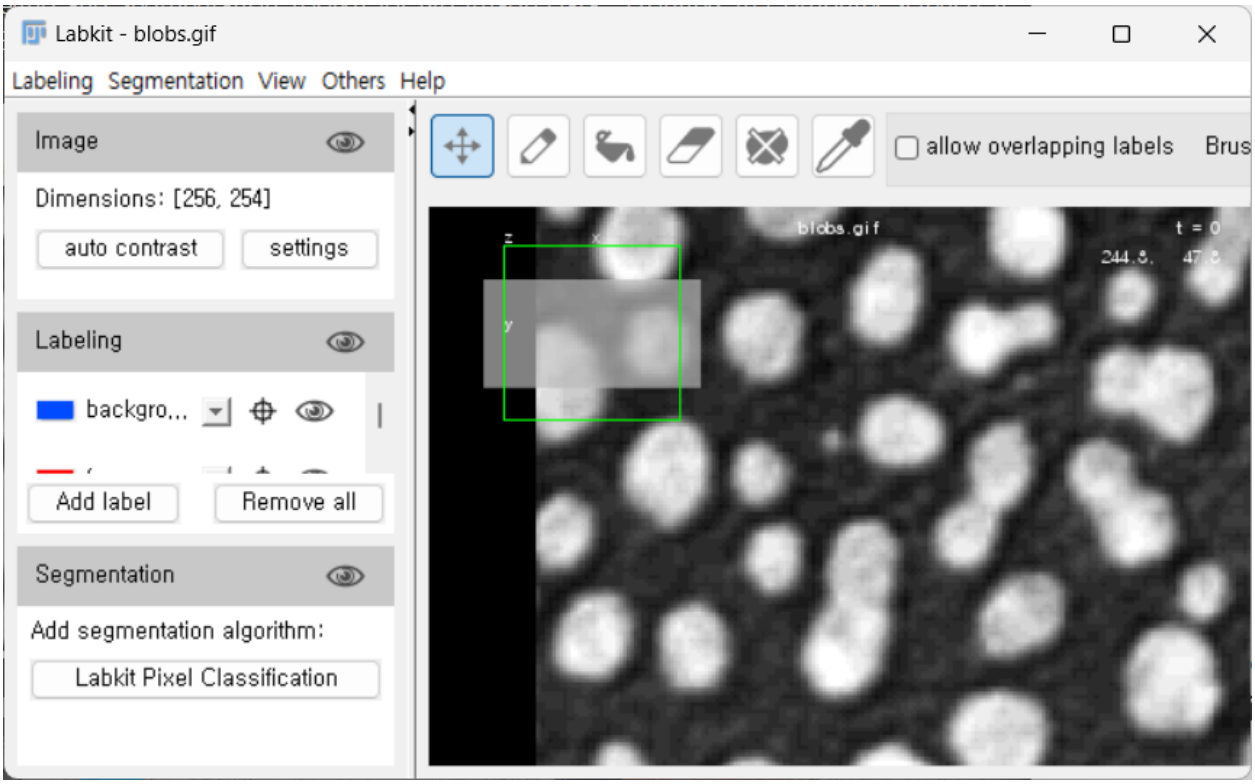
LABKIT SEGMENTATION

https://imagej.net/plugins/labkit/

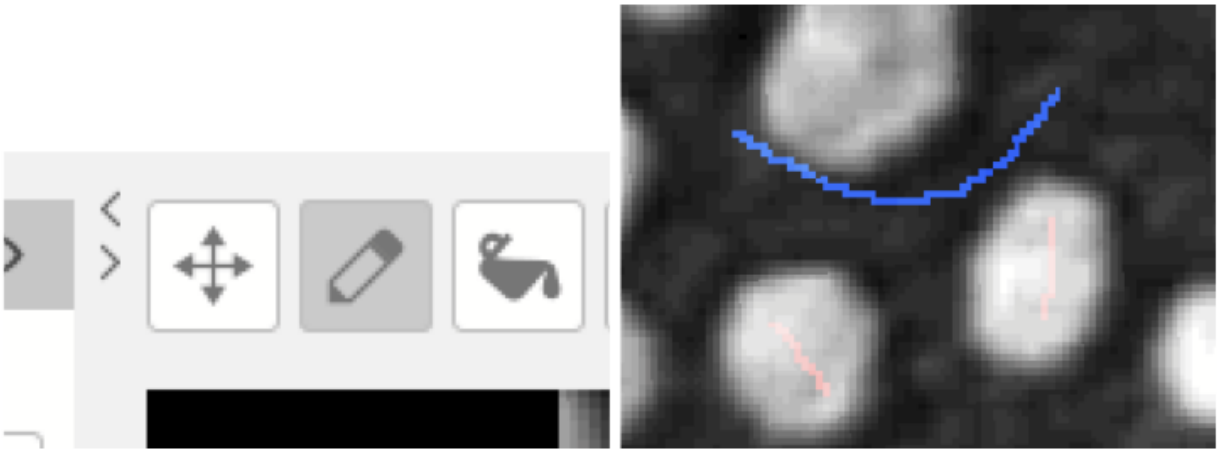
Arzt, M., Deschamps, J., Schmied, C., Pietzsch, T., Schmidt, D., Tomancak, P., … Jug, F. (2022). LABKIT: Labeling and Segmentation Toolkit for Big Image Data. *Frontiers in Computer Science, 4*. doi:10.3389/fcomp.2022.777728

Labkit uses a pixel classifier to create image segmentation. This requires labeling the foreground and background of an image.

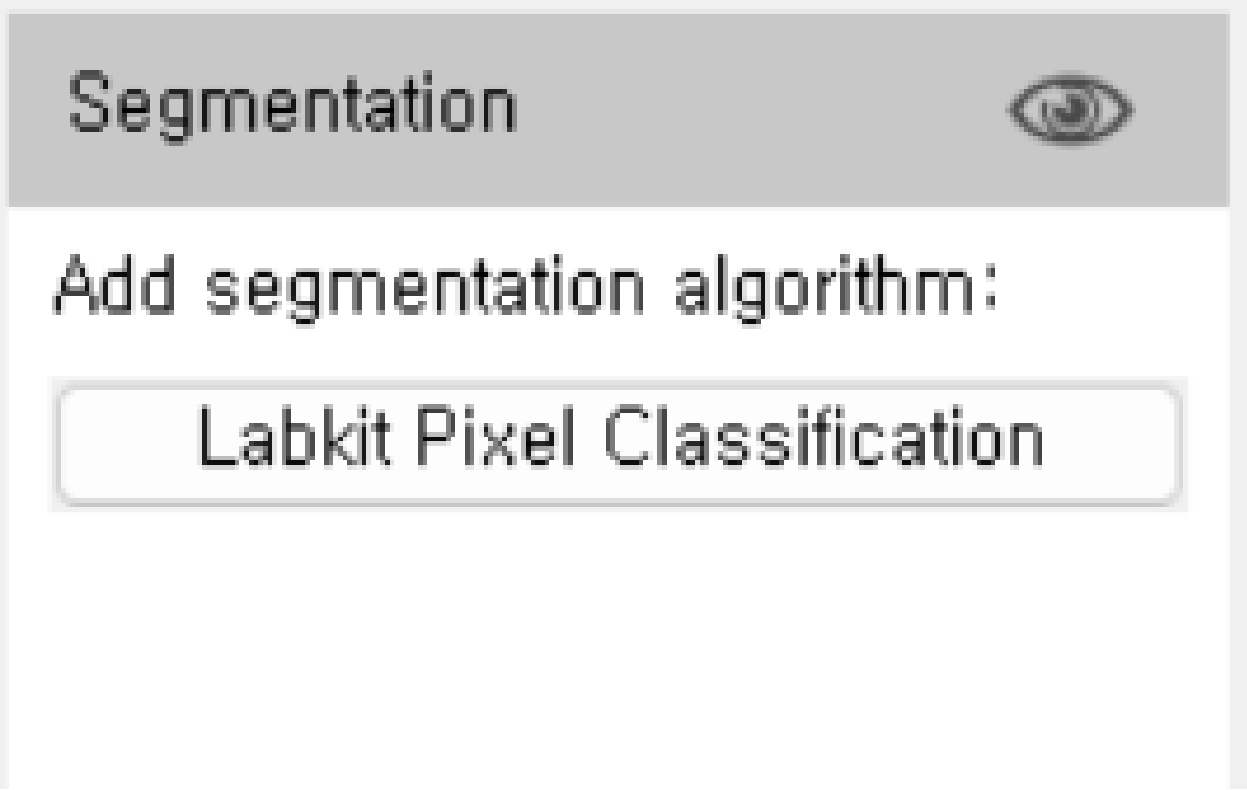
1. Open Blobs with **File > Open Samples > Blobs**. Then open Labkit using **Plugins > Labkit > Open Current Image with Labkit**.

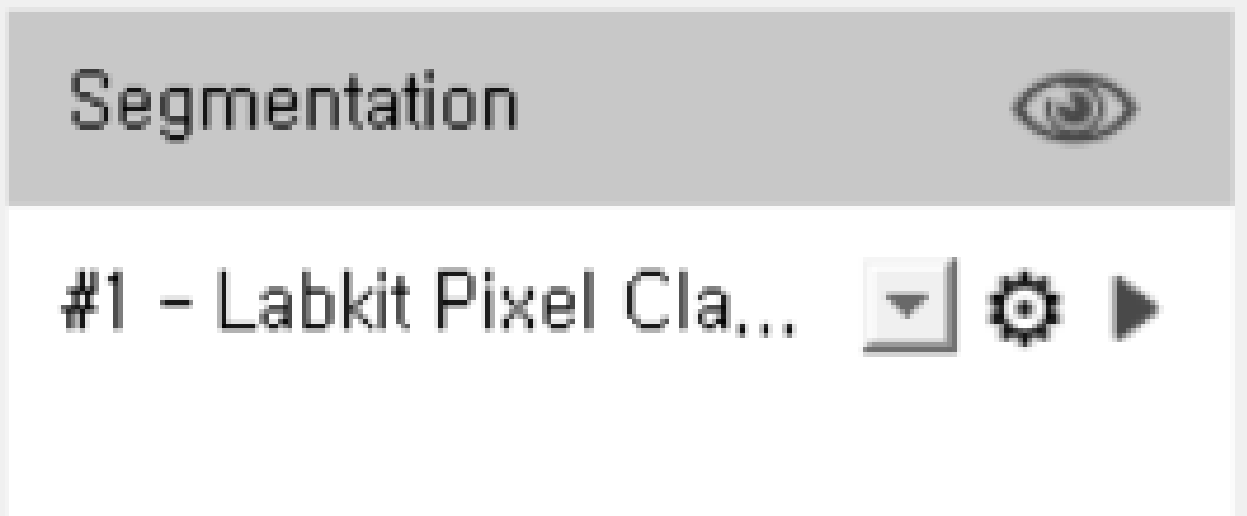


2. The Labkit window will automatically load the selected image. Next, use the pencil tool to draw a line on a few pixels that are background (blue) and foreground (red).

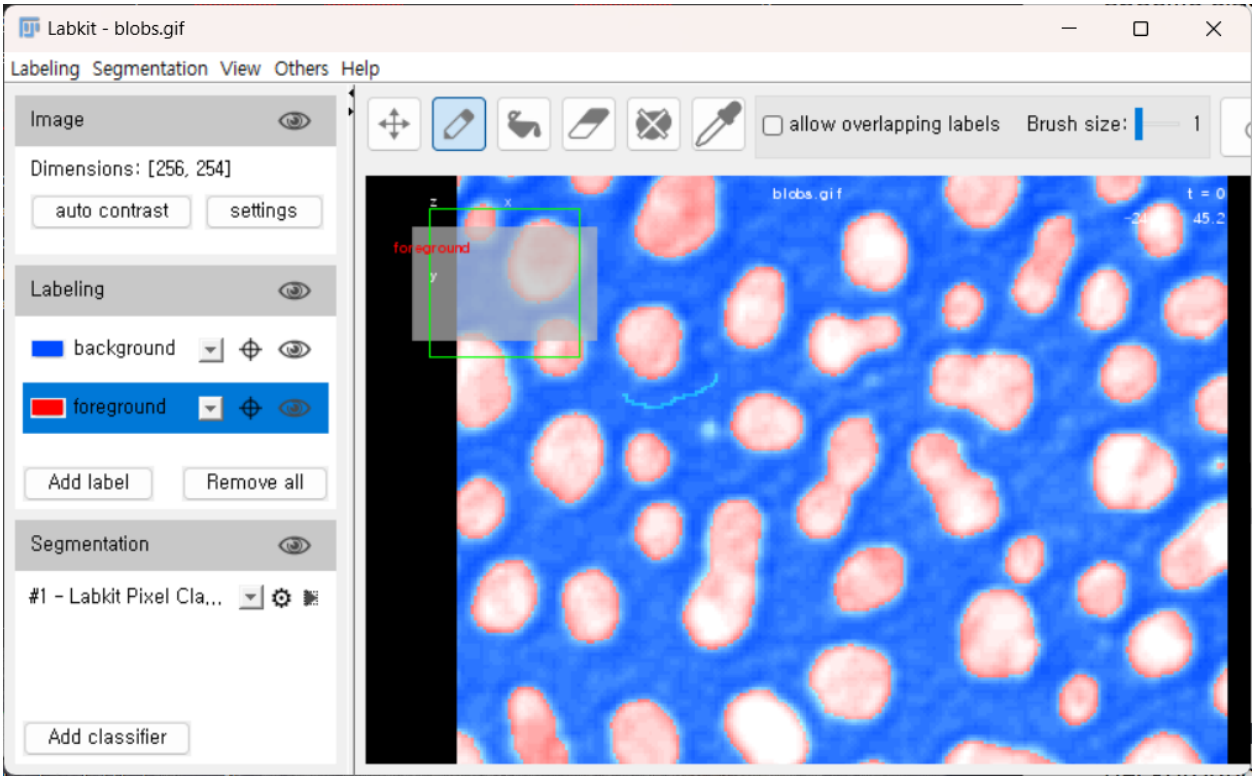


3. Now, we can run the pixel classifier to segment the image. Use the “play” button for the specific classifier within the segmentation part of the window.

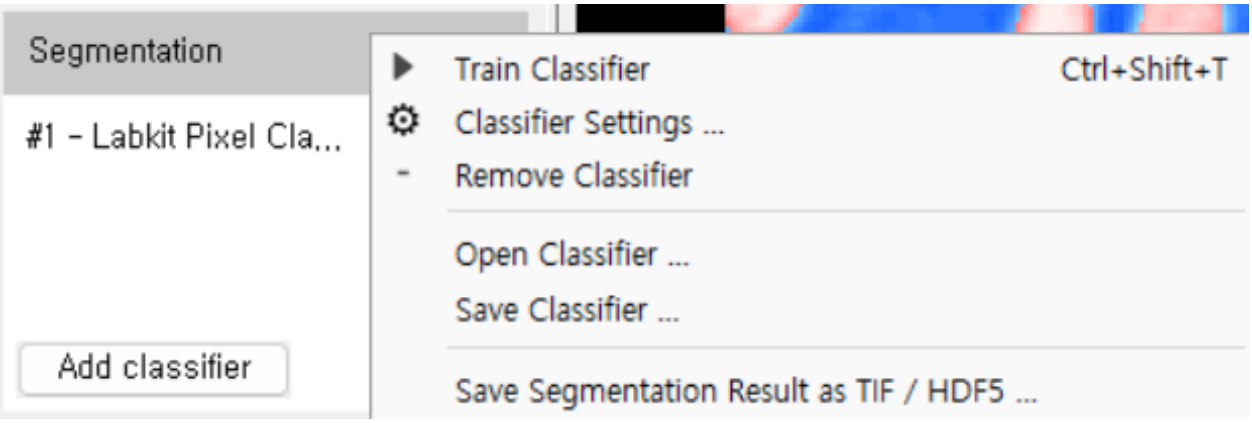




4. This should produce a classified image similar to the one shown below with blue as the background and red as the object of interest.



5. The classifier can be saved and applied to multiple images. Click on the settings button on the classifier to save either the classifier or the image results.


Segmentation
#1 - Labkit Pixel Cla...
Add classifier
Train Classifier
Ctrl+Shift+T
Classifier Settings ...
Remove Classifier
Open Classifier ...
Save Classifier ...
Save Segmentation Result as TIF / HDF5 ...

CHAPTER

ELEVEN

OTHER RESOURCES

- **Introduction to Bioimage Analysis (Pete Bankhead)**:
  https://bioimagebook.github.io/README.html
- **ImageJ User Guide (Ferreira and Rasband)**:
  https://imagej.net/ij/docs/guide/146.html
- **Introduction and Segmentation in FIJI (YouTube)**:
  https://www.youtube.com/watch?app=desktop&v=CZExS_mkGsQ
- **ImageJ Tutorials**:
  https://imagej.net/imaging/
- **COBA: Center for Open Bioimage Analysis (YouTube)**:
  https://www.youtube.com/@cobacenterforopenbioimagea1864/featured
- **Scientific Figure Making with Fiji and Inkscape (Jan Brocher)**:
  https://www.youtube.com/watch?v=F6ll37NOgXc
- **ImageJ Documentation: Built-in Macro Functions**:
  https://wsr.imagej.net/developer/macro/functions.html
- **ImageJ Macro Cheatsheet (Robert Haase)**:
  https://github.com/BiAPoL/imagej-macro-cheat-sheet/blob/master/ImageJ_macro_cheatsheet.pdf
- **ImageJ Documentation: ImageJ Ops –** allows one to use other libraries (ex. OpenCV) natively in ImageJ:
  https://imagej.net/libs/imagej-ops/
- **TrackMate Information**:
  https://imagej.net/plugins/trackmate/
- **StarDist Information**:
  https://github.com/stardist/stardist
- **Labkit Information**:
  https://github.com/juglab/labkit-ui
- **I2K Conference (YouTube)**:
  https://www.youtube.com/@I2KConference