Assisted Segmentation of Pancreatic Islets and Quantification (ASPIQ)

This is a user guideline for the ImageJ/Fiji ASPIQ Macro scripts, providing a semi-automated approach with an automated pancreatic islet segmentation of immunohistochemically stained images combined with a manual breakpoint and user confirmation or adjustment.

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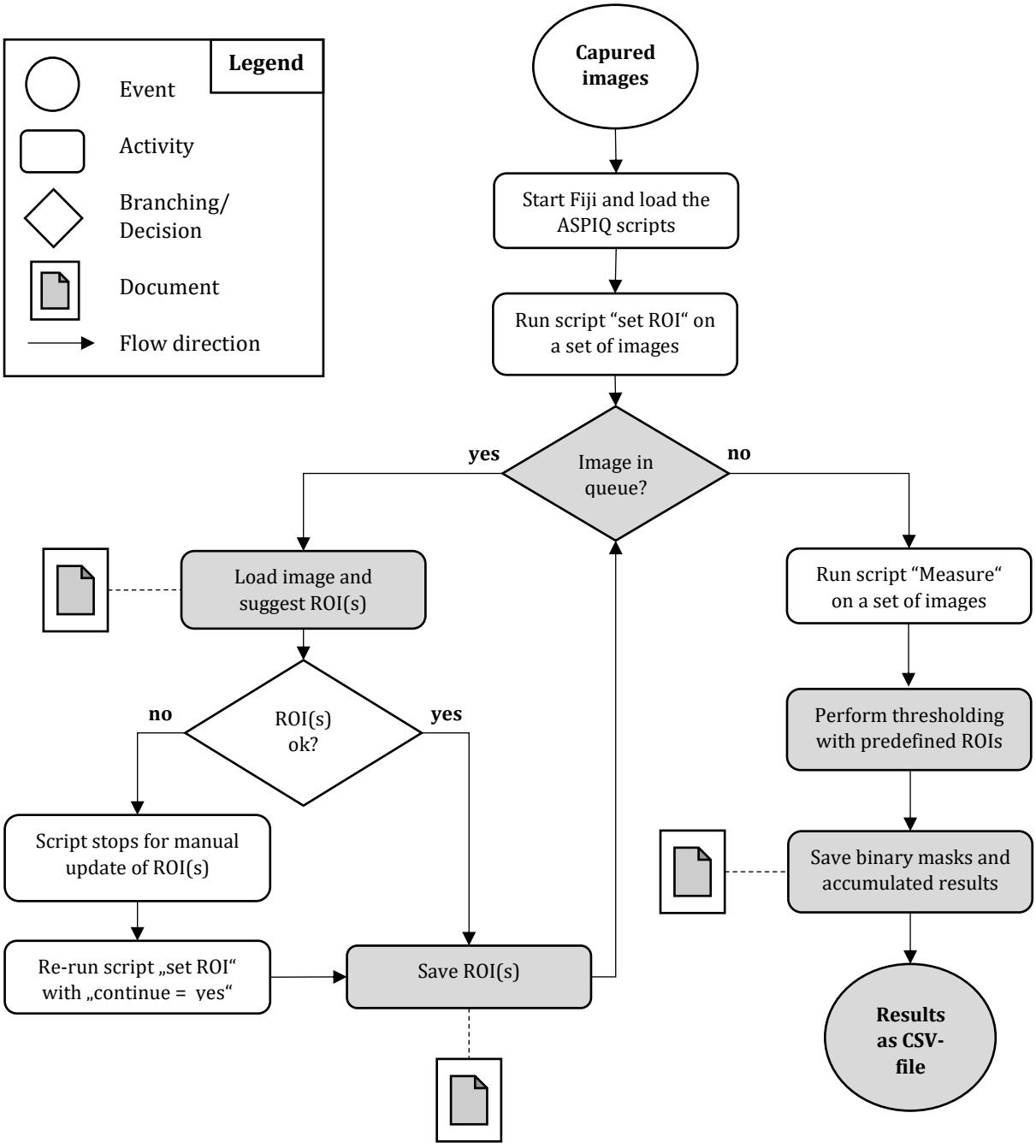


Figure 1: ASPIQ Workflow Overview

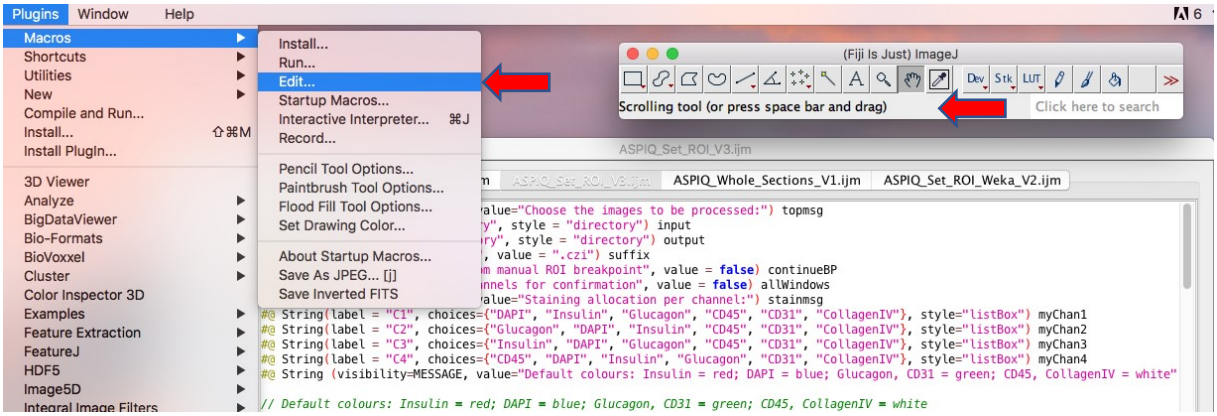
Flowchart in Business Process Modelling Notation (BPMN) with starting point at captured images and ending with measurement results stored as csv-file. The first part of the process is the ROI definition which is repeated as long as images are in the queue without ROIs. The user is confirming the proposed ROIs or adjusting them manually. After the definition of the ROIs, the measurement script is executed on a set of images as a final step for quantification of the underlying data. Automated process steps are grey-colored.

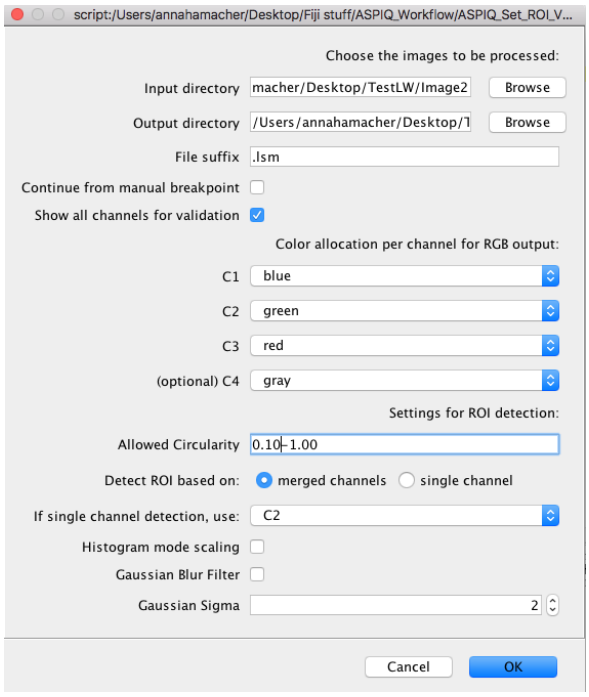
Prerequisites:

- The scripts were developed for immunohistochemically stained pancreas sections of mice. The scripts should also work for other species, but this needs further testing.
- The scripts were tested with images being stained for: DAPI, glucagon, insulin, CD45, collagen IV, CD31, caspase-3.
- Apotome phased images might need some preprocessing if Fiji struggles at stitching the tiles on image import with the bioformats-importer.
- Images to be processed (input) must be located in a different directory than the result directory (output). Recommendation is to create a copy of your original files.
- Try to avoid special characters or spaces in file names.
- The scripts have been tested successfully on WinOS and MacOS.

Part 1: ROI Definition of Pancreatic Islets

There are several ways to run Macro scripts in Fiji. Without installing them and setting a shortcut, the quickest two ways are 1. to open them via Plugins > Macros > Edit or 2. drag&drop them into the Fiji menu bar. In the Macro Editor the "set ROI"-script can be easily re-run by using Cmd+R, which will be most likely necessary due to manual adjustment of single ROIs. Make always sure to use the latest versions of the scripts.



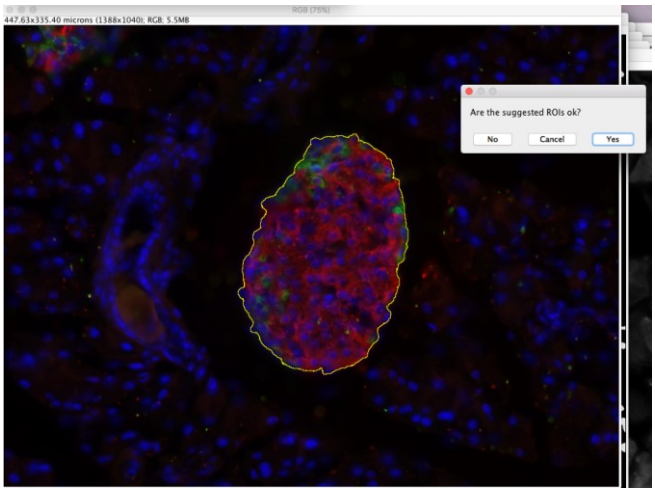


When **running the "set ROI"-script for the first time,** it is necessary to define several parameters. In general, script parameters are automatically stored and reloaded in case of re-running a script.

- Define the *input and output directory* (need to be separate directories). The script is only processing images inside the input directory (subdirectories are ignored).
- The "*continue from breakpoint*"-toggle must be off, when starting on a new set of images.
- The *color allocation* is applied on the automatically stored RGB overlay image. The original image files stay untouched.
- The adjustment of the allowed *circularity* can be necessary in case of ill, deformed type 1 diabetes islets (lowering of minimum value).

- As a standard, the ROI detection algorithm is executed on a merged RGB image of all available channels. For other use cases it can be limited to a single channel.
- By default, *Histogram mode scaling* is not necessary. It can improve the results of the ROI detection in case of channels with much background noise of stained structures, that are available everywhere in the image (e.g. CD45, collagen IV). It sharpens the contrast by removing pixels in 8bit mode between histogram min up to histogram mode.
- By default, *Gaussian Blur Filter* is not necessary. It can improve the results of the ROI detection significantly in case of weak stainings or single channel ROI detection.

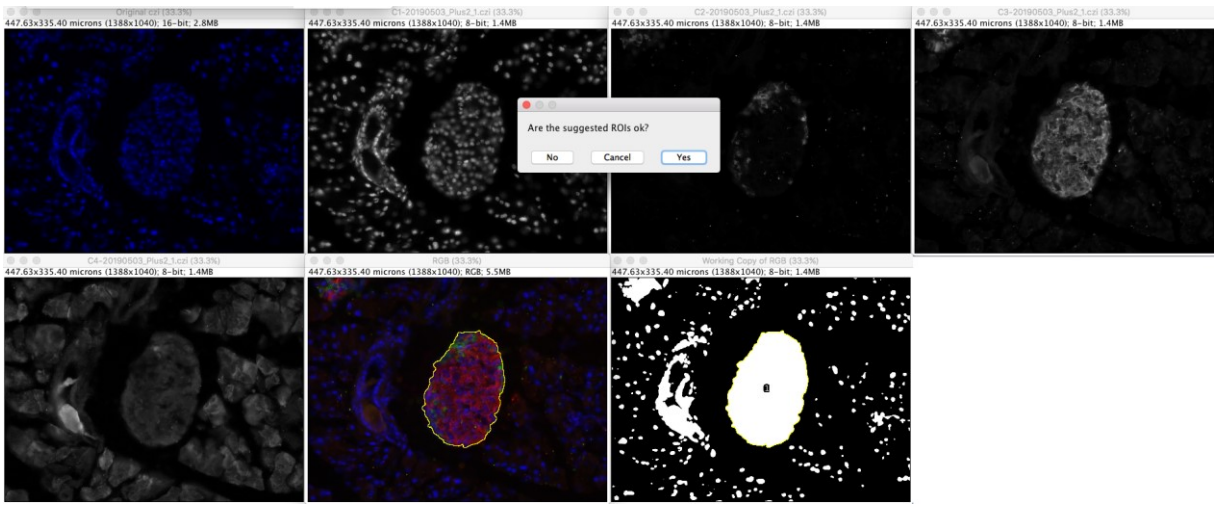
After starting the script, it is **loading the first image** placed in the input directory and **suggesting the detected ROI(s) to the user**. If you left the "*Show all channels for confirmation*" option untoggled during script startup, then you will get one big merged picture to confirm the correctness of the identified ROI:



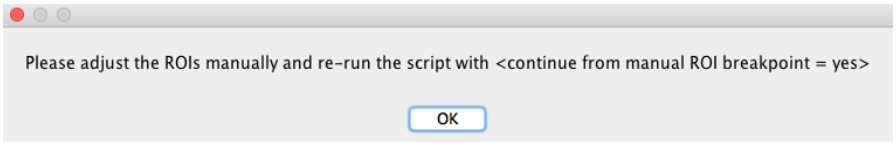
Two main actions are now possible:

1. **Yes**: the image is stored together with the ROI(s) to a separate, image specific directory inside the output directory and the next image from the input queue is loaded.
2. **No/Cancel**: the script stops and the ROI(s) can be adjusted and updated manually.

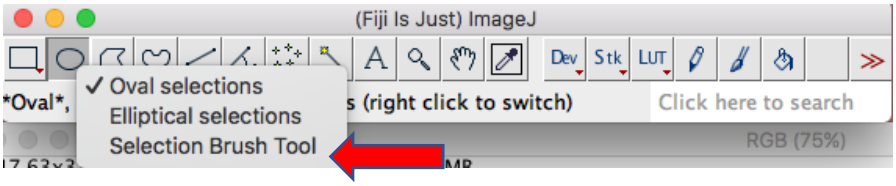
If you activated "*Show all channels for confirmation*" during script startup, then you will see multiple windows arranged on your screen for the confirmation including all single channels and created masks:

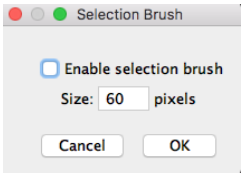


If you confirm the ROI(s), the script will save them and proceed with the next image in the queue. If you decide to adjust the ROI(s) manually, another infobox will pop up:



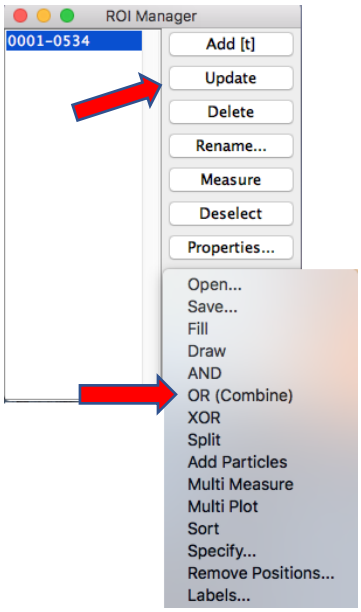
For adjustment of the suggested ROI(s), I recommend the "Selection Brush Tool" with a size of 10-100 pixels (depending on the needed modification). "Enable selection brush" must be activated to use this functionality.



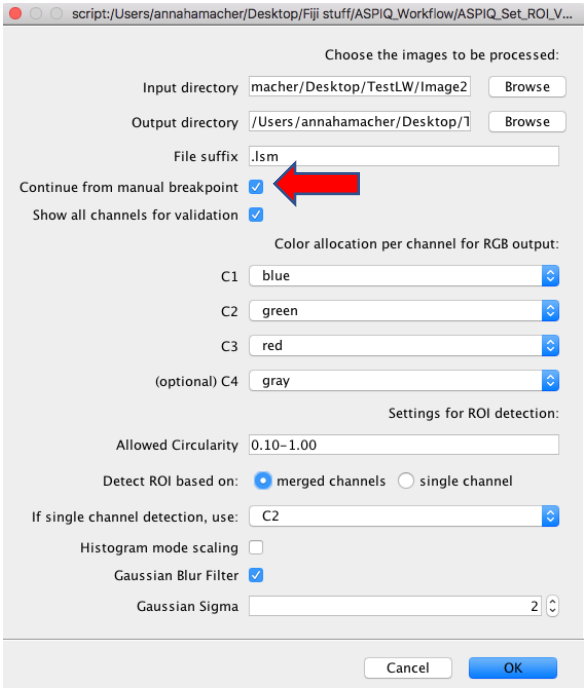


- Holding **Shift** forces the Selection Brush Tool to add pixels to the selection
- Holding **Alt** forces the Selection Brush Tool to subtract pixels from the selection

If the algorithm detects more than one ROI, although there is only one real ROI in the image which was fragmented, you can for example mark all ROIs in the ROI manager and use the function "OR (Combine)" with subsequent "Update". Then you can delete all ROI entries except the first.

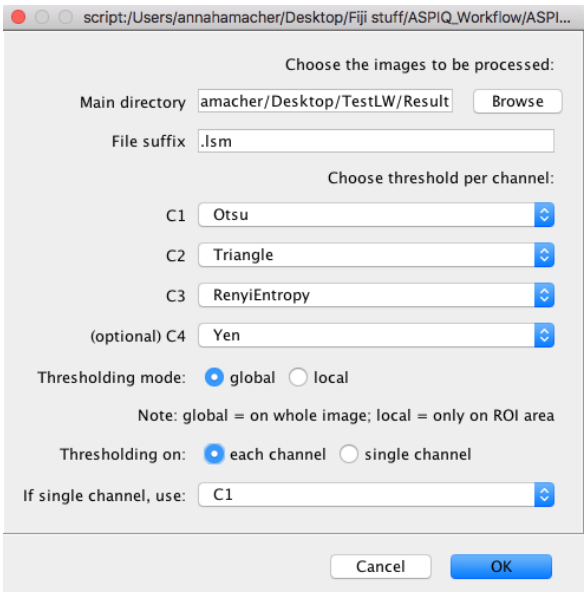


In general, make sure to select the ROI in the ROI manager before any modification and to click "Update" when you are done. Verify the modified ROI on the "RGB"-image and "Deselect"/"Delete" any other ROI(s) if they are still visible (toggle "Show All" off and on, if needed)**.** Afterwards re-run the "set ROI"-script and make sure, that you activate the "continue from breakpoint" option. The other settings will be still present from the former run and do not need to be changed.

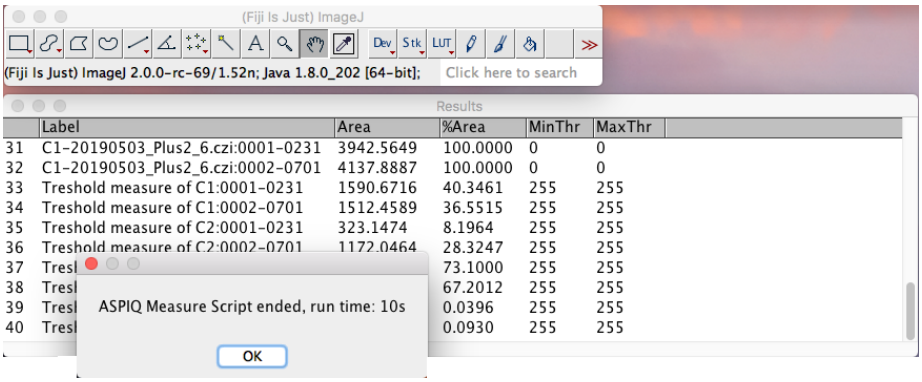


Part 2: Thresholding and Measurement of Pancreatic Islets

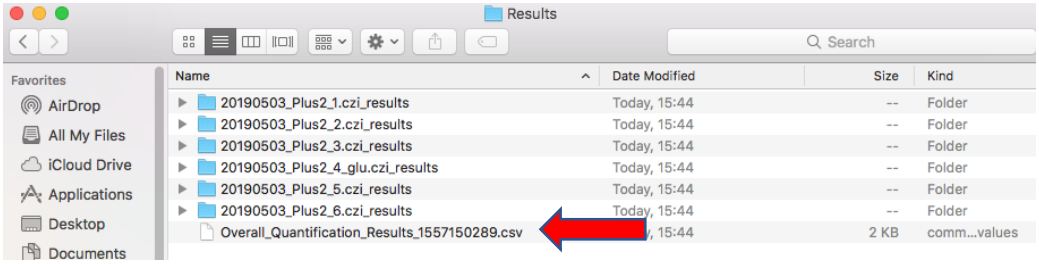
When the definition of the ROIs is done for each image, the **"Measure"-script** can be run to complete the quantification of the underlying data. For this, you need to define the directory, where the images and the ROIs are stored (the former result/output directory) and the threshold allocation per channel (dropdown-menu). The thresholds need to be defined by the user, so make sure that you look at least at 5-10 images of your whole set to validate which threshold fits best for which staining/channel. By default, *global thresholding* is performed, but can be changed to *local thresholding* if needed. If thresholding is not needed to be processed on all channels, it can be limited to one specific channel as well.



The script proceeds without any further user interaction and gives a notification with the duration, when it's done:

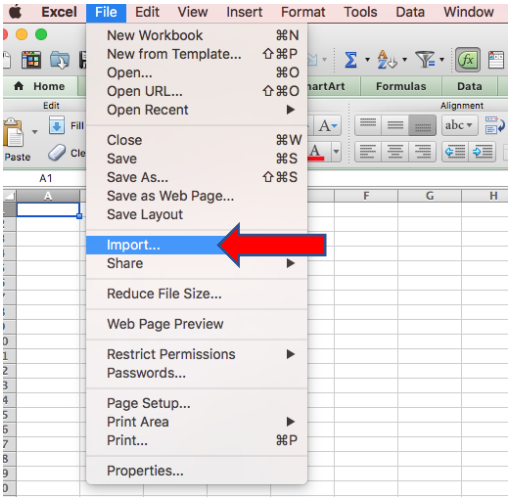


The ROIs per image are always listed first, showing the total area of each ROI. The label includes the filename, so that the assignment of ROIs and image files is assured. After the total area of each ROI, the threshold of the single channels (1-4) are measured and listed with Area and %Area. The results are stored in one common CSV-file inside the defined result directory. The name of the CSV-file includes a timestamp, so it is possible to re-run the script without overwriting existing data (e.g. to run it multiple times with different thresholds).

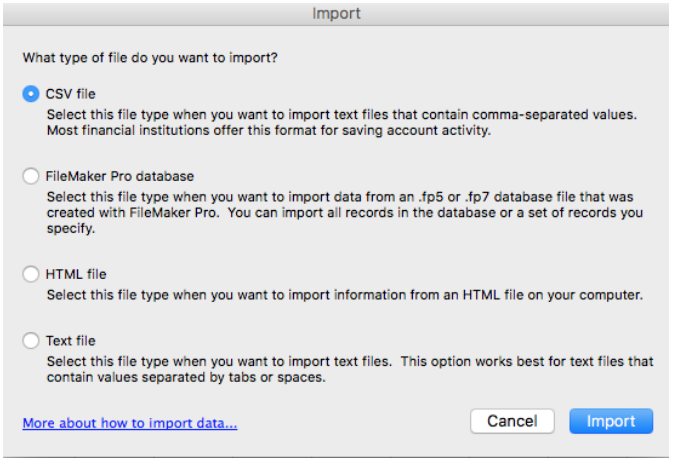


<u>Recommendation:</u> pick at least several random images and verify the threshold masks, which are stored in the image specific directory, to be sure that the thresholding was executed correctly.

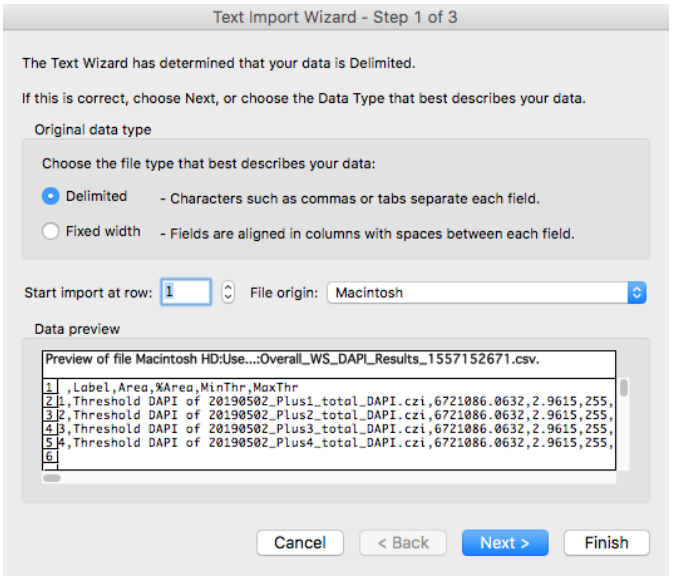
I also recommend to import the CSV-file inside Excel rather than just clicking on it, to make sure, that the "." and "," setting inside numbers is correctly translated.



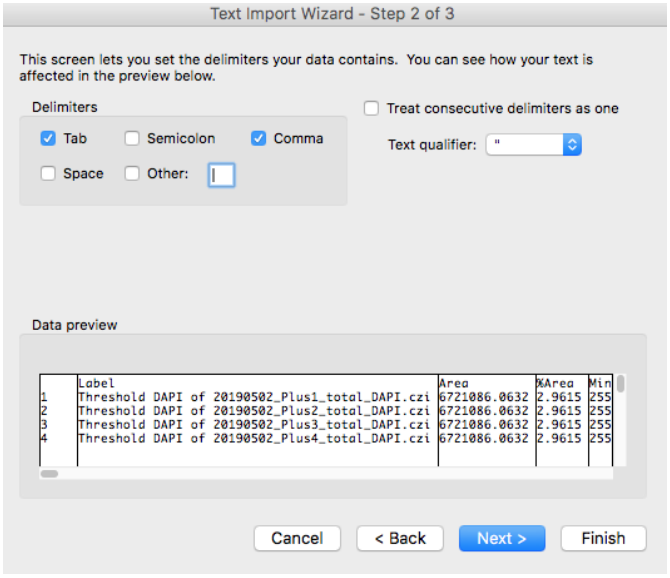
Then follow the menu and make sure the correct options are set:



Choose CSV file

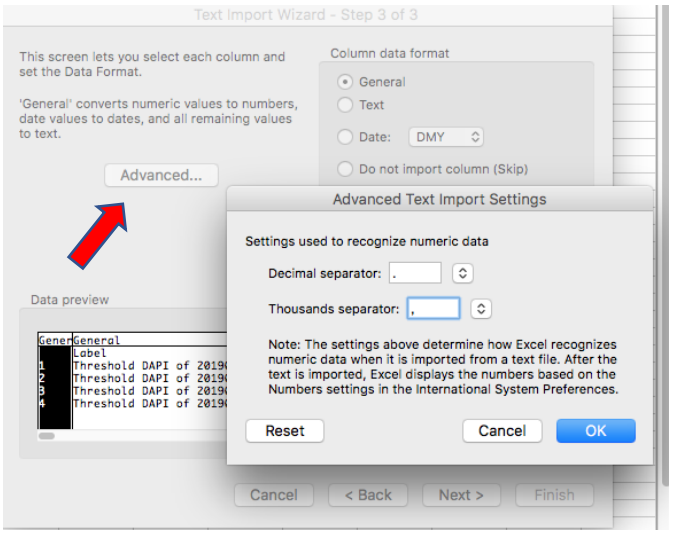


Pick Delimited



Tab & Comma, Other can be set to ":"

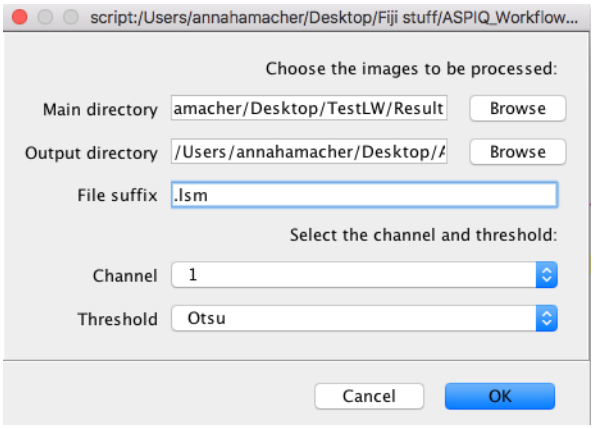
Be aware that in this case the column labels need to be shifted one column to the right beginning at "Area", since the ROI ID does not have a column label on its own.



Change "." and "," if necessary

Part 3: Whole Section Measurement (e.g. DAPI only)

A separate script is available to perform global thresholding on a single channel without ROI definition and therefore on the whole image. This is useful, for example, for quantification of the total DAPI area of a whole section. The script processes a set of images placed in the defined main directory and stores the results in an accumulated CSV-file, just like for the single pancreatic islets.

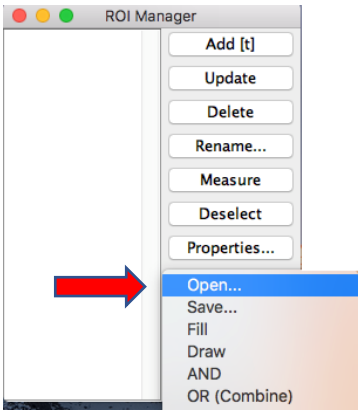


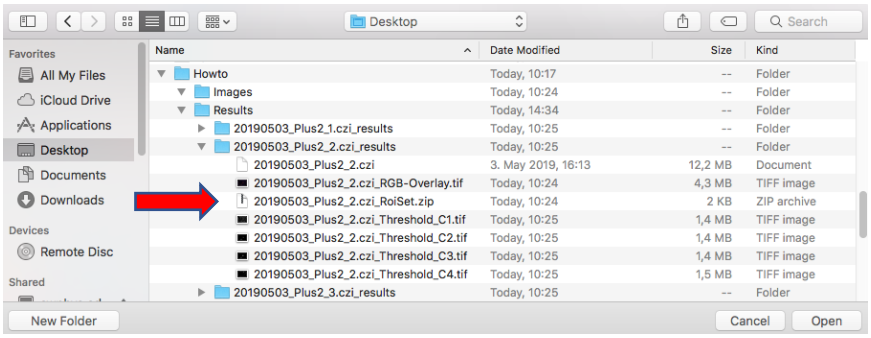
There is no user interaction of the script. The script will pop up with the total runtime after completion. The result file and threshold masks for verification are stored in the defined output directory. The original image files stay untouched in the main directory.

Part 4: FAQ

Q: How can I reload already defined ROI(s) to verify the thresholding manually?

A: If you, for example, want to do the thresholding manually to correct a value in the accumulated results sheet, you can open the specific image and import the defined ROI(s) by opening the image specific ROI.zip file or just drag&drop the zip-file into the Fiji menubar.





Don't forget to save the corrected threshold as tif-file in the image specific directory for later reference, when you are done.

Q: The islet didn't get properly detected, although its outline is (partly) visible in the RGB threshold (window with title "Working Copy of RGB"). How can I get the segment into the ROI Manager?

A: There are several possibilities to get the visible outline into the ROI manager:

1. If the object touches the **edges**, it is not detected by the macro. Re-run "Analyze" - "Analyze Particles" with parameter "Exclude on edges" off (the other parameters can remain untouched).
2. If the object has a lower **circularity** than defined at startup, you can re-run "Analyze" – "Analyze Particles" with lower circularity (the other parameters can remain untouched).
3. If the object is smaller than pre-defined, you can re-run "Analyze" – "Analyze Particles" with a lower **size** restriction (the other parameters can remain untouched).
4. If the area detected is too big and you want to get it divided, type "Watershed" in the menubar's quicksearch field and perform **watershed separation** on the threshold. Afterwards re-run "Analyze" – "Analyze Particles".