Trypanosoma Segmentation Workflow

In preparation for the data analysis, the colonies are isolated, segmented and aligned across the time series. For this, we have created a FIJI script that automatically performs all of the necessary steps.

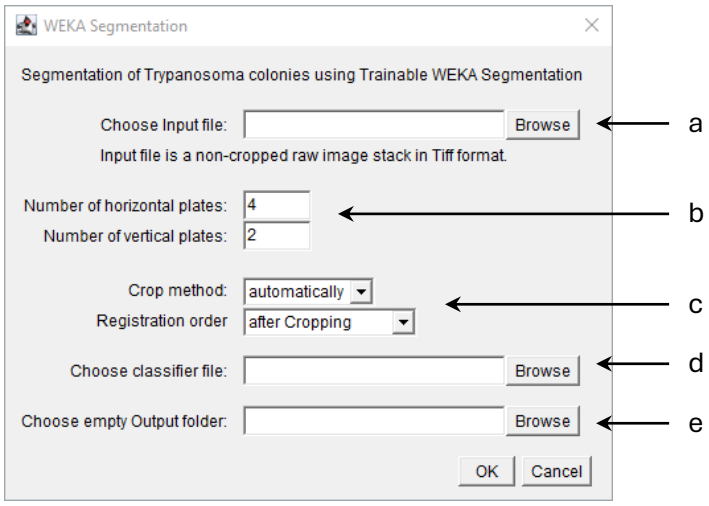
Before running the script for the first time:

Before running the script for the first time, MorphoLibJ and StackReg need to be installed in FIJI. Both are required for some functions that are utilized in the script.

1. Open FIJI.
2. In the menu bar, click “Help>Update...”. This updates FIJI, as well as the already installed packages.
3. After FIJI has been updated, the ImageJ Updater will open. In this window, click “Manage Update Sites”.
4. Another window will open that shows a list of all packages that can be installed directly in FIJI. In this list, tick the “IJPB-Plugins” (to install MorphoLibJ) and “BIG-EPFL” (to install StackReg) update sites. Click “Apply and Close”, then “Apply Changes” in the ImageJ Updater window.
5. The packages will install now. After closing and restarting FIJI, the packages will be functional.

Running the segmentation script:

1. Open FIJI.
2. In the menu bar, click “Plugins>Macros>Run...”, then choose the file “segment-colonies.ijm” in the Fiji folder in the repository.
3. The following dialog box will open:



In this dialog box, all necessary settings can be chosen.

a. As the **input file**, choose a raw image stack of the Trypanosoma colonies in Tiff format (to create your own data for the tutorial notebook use : **sample_image.tif**).
b. The number of **horizontal and vertical plates** is already set correctly for the sample image. To process an image with a different number of plates, this must be changed to match.
c. The **crop and registration method** should not be altered yet.

d. Choose the **classifier file**. This contains the trained segmentation algorithm for the Trainable WEKA Segmentation.
e. As the **output folder**, choose an empty folder. If the folder does not exist, it will be created by the script.

By clicking "OK", the script is started.
As a side note, file paths that include some symbols, like ä, ö, ü, might cause some errors and should be avoided, if possible. This can however be circumvented by running the script from the macro editor.

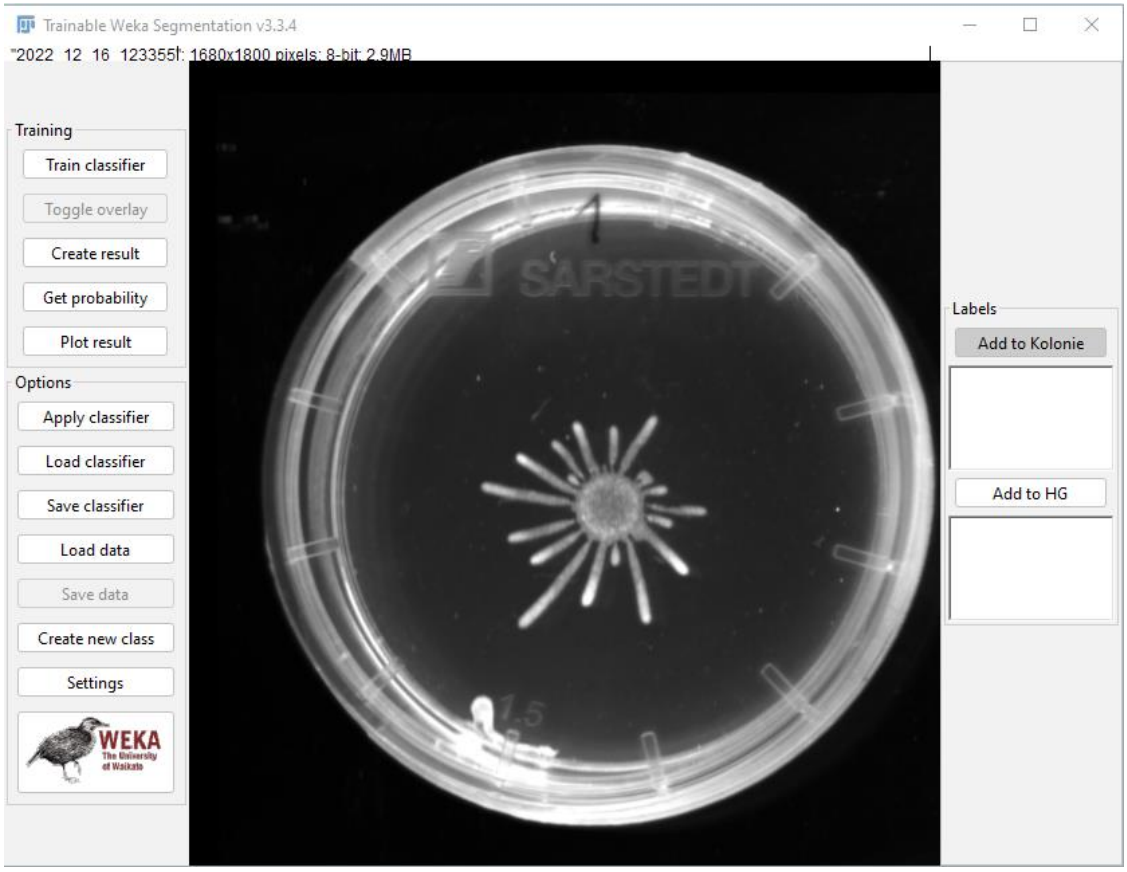
4. The script now processes the input image. A Log window will open that shows the progress of the image processing.

   The individual plates are first **cropped** from the original image by sectioning it into equal parts based on the number of horizontal and vertical plates that was specified in the dialog window.

   The images in the resulting stacks are now **aligned** using the Rigid Body transformation type in the StackReg plugin.

   Then, the stacks are **split** into their time stamps. A **Gaussian blur** with a σ value of 1.5 is applied to avoid fragmentation of the colony during segmentation.

   During the **WEKA segmentation**, the classifier is applied to the individual images. Depending on the processing power of the computer, this might take some time (in our experience up to 15 minutes, though typically much less). It might seem like the script is stuck on the WEKA segmentation window (seen below), but as long as the segmented images open and close, and the WEKA segmentation writes some output into the Log window from time to time, the segmentation is still running.



After the segmentation is completed, the images are **postprocessed**: The edge of the plate and any small artefacts and holes are removed. Lastly, the individual images are recombined into stacks.

The "results" folder contains the the final output data. These are used for data analysis.

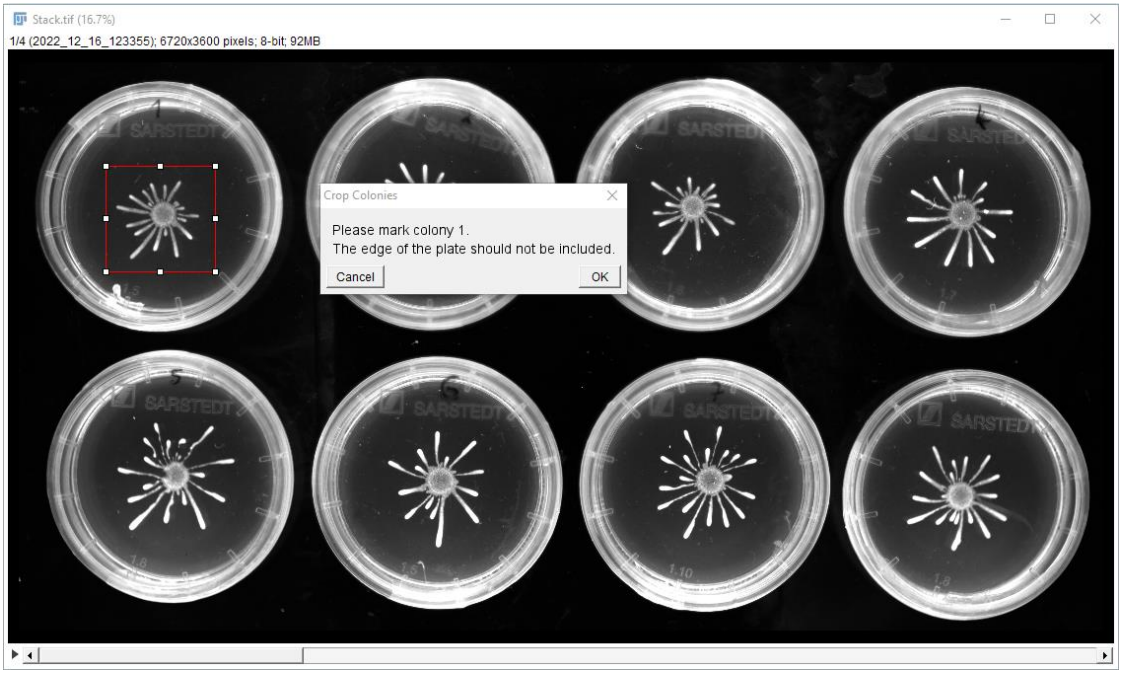
The "temp" folder has subfolders that contain different intermediary images that are needed by the script and may be useful for debugging purposes. They can be deleted, once the script is finished.

5. Lastly, the quality of the segmentation and registration results should be controlled.
   The segmentation quality can be assessed by simply comparing the output data with the raw image. The colony should be recognized entirely, and no artifacts (e. g. the edge of the plate) should be visible.
   The registration quality can best be assessed by creating Z projections of the segmented stacks. We have created a second macro script to quickly create such projections. For this, run the script by clicking "Plugins>Macros>Run..." and choosing "z-project-colonies.ijm". When the dialog box asks to, open the desired colony stack(s). A Z projection will be calculated and saved into a "z-projections" folder in the output folder. In this projection, the correct alignment of the different time points can be assessed easily.

Improving Segmentation and Registration Quality:

While the standard settings were found to achieve good segmentation and registration results in most cases, this can vary depending on the shape of the colony and the contrast of the raw image. Here are some common problems and how they might be solved.

- **Instead of the colonies, part of the edge of the plate or another artifact is visible in the segmented image**:
  The segmentation script should be run again, this time choosing "manually" as the crop method. Using this option, the individual colonies are not separated by slicing the raw image automatically. Instead, the script will ask the user to manually mark the colonies, like shown in the image below.



After marking the first colony, press “OK” in the Crop Colonies window, then repeat for all remaining colonies.
Notably, the edge of the plates should ideally not be included in the marked rectangle.

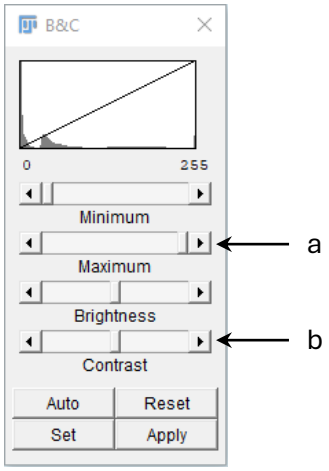
- **Some finger structures are missing**:
  This might sometimes be solved by setting a higher σ value for the Gaussian blur:
  To open the script in the macro editor, click “Plugins>Macros>Edit…” and choose “segment-colonies.ijm”. In lines 11– 24, the default values that are displayed in the dialog window can be changed. To set a different σ value for the Gaussian blur, simply change the number value in line 23:

```
////////////////////////////////////////////////////////////////////////////////
//////////////////////
// -> the default entries in the dialog box can be changed here:
// file paths that include ä, ö, ü can cause errors during saving, these should be
avoided

// -> path to the Input file:
defaultinput = relative + "sample-image.tif";
// -> path to the classifier file:
defaultclassifier = relative + "classifier.model";
// -> path to the Output folder:
defaultoutput = relative + "output\\";

// -> sigma parameter for Gaussian Blur (does not need to be changed usually):
sigma = 1.5;
////////////////////////////////////////////////////////////////////////////////
//////////////////////
```

  The script can be run directly from the macro editor by clicking “Run” at the bottom left of the window.

  If this approach does not yield better segmentation results, manually removing the artifacts from the colony images in the “split seg” temporary file folder and recombining the colony stacks could be an option, though potentially time consuming for larger numbers of colonies.

- **Parts of the colony with lower contrast are not recognized in the segmented image**:
  The brightness and contrast of the raw image can be adjusted before running the script on it. To do this, click “Image>Adjust>Brightness/Contrast”. A window will open, where the brightness maximum can be lowered (a) and/or the contrast can be increased (b). The darker parts of the colonies should thereby be made brighter, while keeping the brightness of the middle of the plate low. The settings can be confirmed by clicking “Apply”. Save the image and run the script using this preprocessed image as input.



- **Failed alignment of the colonies in the stack**:

Choosing the manual crop method (as seen above) was found to achieve better alignment in some cases, where the edge of the plate disturbs the registration process.
Setting the registration order to “after segmentation” can also improve the alignment. This can also be used in combination with the manual crop method.
For some colonies, acceptable registration results were found not to be achievable. These colonies are omitted in data analysis.