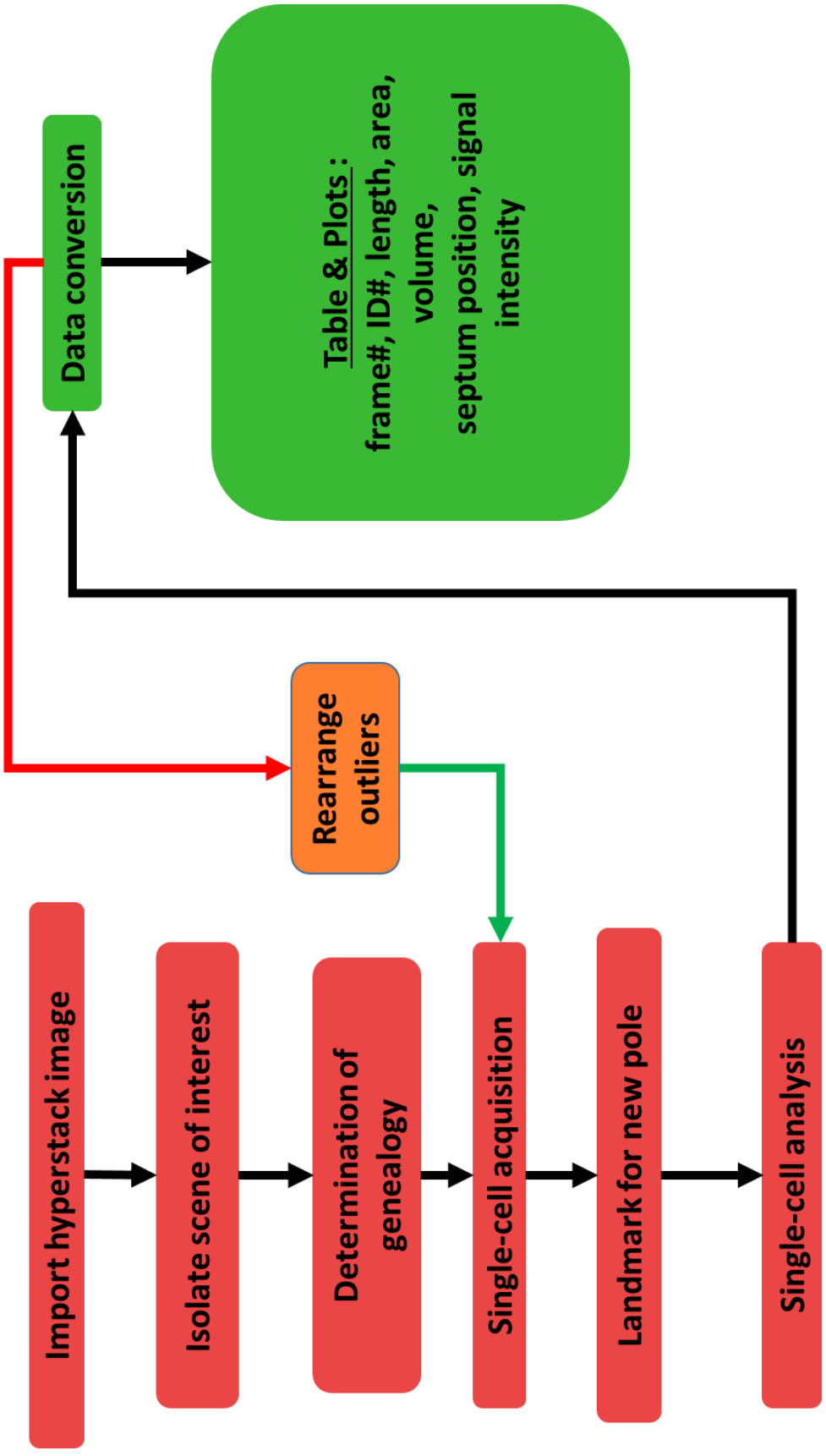
Morpholyzer Generation Tracker

V 1.0

User Manual

Content

Program flow chart



Import and pre-processing

A multichannel time-lapse image is opened in FIJI using the Bio-Formats importer **(FIJI → Plugins → Bio-Formats → Bio-Formats Importer)** (Fig. 1).

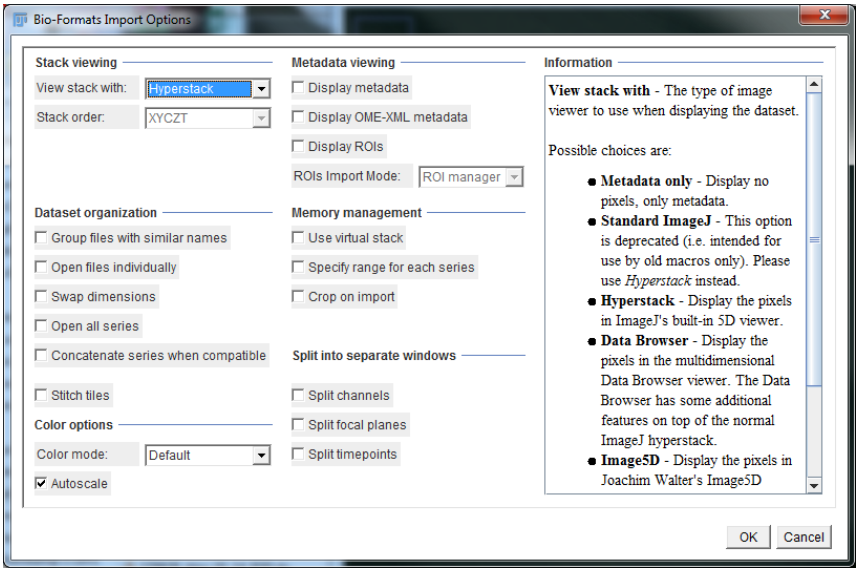


Fig. 1: Bio-Formats dialogue with correct settings

From the initial time-lapse image the scenes of interest are extracted by using the rectangle selection tool **(FIJI→ Rectangle**  **)**. A selection is drawn that includes the first cell in the starting frame, plus, the all the progeny in the desired final frame (Fig 2, upper panel). Note that the last frame should contain the end of the latest cell-cycle of interest. Using this selection, the function **(FIJI→ right click on image → Duplicate)** can be used to crop the scene in size and length (Fig. 2, lower panel).

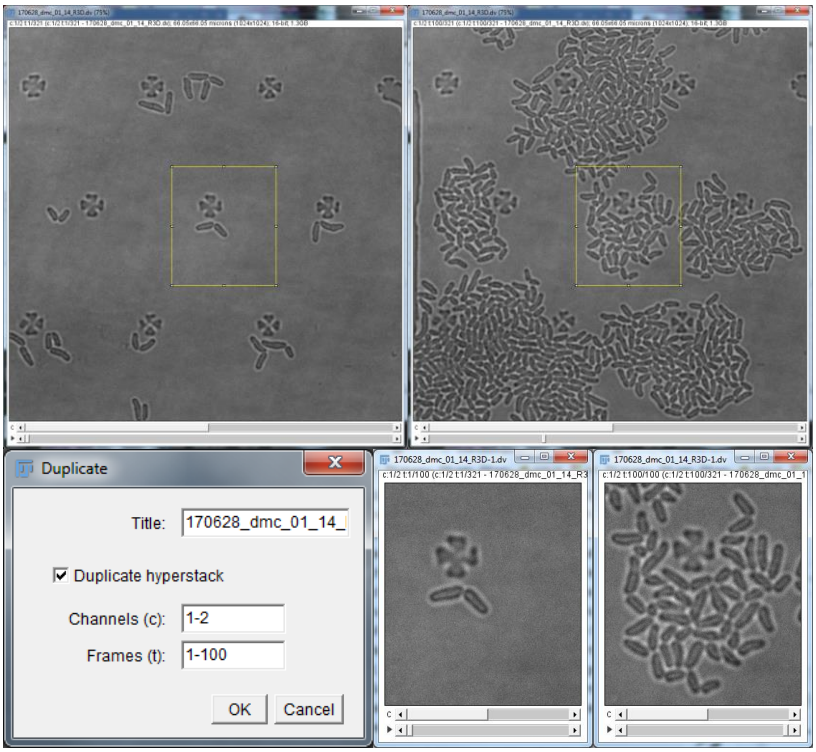


Fig. 2: Example of extracting a growing colony from a larger raw-image

Determination of genealogy and formatting

The individual pedigree of the video-clip has to be determined, by following the convention of a template dendrogram (→ Appendix A).

The point selection tool  is used to select the new born cells in the video. The single points are added to the ROI-manager by pressing ***'t'***.

Note that ideally only one cell should be followed from the beginning on. If two cells in the initial frame are obviously siblings, the numbering can start with 002 and 003. In case the cell in the initial frame does not show the state right after birth, it could be excluded. Cells that do not divide before the video stops should be excluded.

Subsequently, a list of cell-IDs is generated that get subject to the Single Cell Acquisition in a later step. Please also note that the ID# is eventually printed in the upper right corner of the point selection. If you desire a centered numbering within the cell, this should be considered. Un-centered numbers do not affect the performance (Fig. 3 A & 4).

After all cells are assigned correctly, the FIJI-macro **MoGenFormat.ijm** is executed.

For the option **'automatic numbering'** (Fig. 3 D), the order of the ROIs should strictly follow the convention of the dendrogram in a consecutive way. If two cells are present in the first frame, the option **'automatic start at:'** should be set to **2**.

For the option **'take ROI names'** (Fig. 3 D), the individual ROIs have to be renamed using (**FIJI → ROImanager → Rename)**. Here the order of the ROIs is not important. The correct format for the numbering is three digits with zero padding, e.g. 023.

If a 100x objective was used for imaging, the 2.5x scale-up should be performed in order to enhance the precision of the cell outline.

This process creates the initial experiment-folder, named after the original scene `C:\Users\Public\Documents\MorpholyzerGentrack\yourfilename\` including the subfolders `\0riginals`, `\SingleCell\0riginal` and `\R0Is`. The list of the division events, containing information about the frames, coordinates and ID#s will be saved as `\0riginals\Cell-ID#_ yourfilename.zip`.

Further processing requires a particular formatting of the image. Running the macro also results in a copy of the original image that additionally contains two copies of the contrast channel. The first channel now contains the cell-ID numbers, the second channel is a thresholded copy, the third channel contains an unmodified copy of the contrast image and the fourth channel the original fluorescence signal (Fig. 4 B). In case more than one fluorescent channel was used for imaging, the further channels are appended.

The copped, original scene (2 channels, multiple frames) image is saved in the folder `\0riginals` with the suffix `_0RI.` If the check-box for scale-up is ticked, a copy of the processed but unscaled scene (4 channels) is saved with the suffix `_PRE` and the processed and scaled image (4 channels) is saved with the suffix `_INI`. If the check-box is not ticked, the processed but unscaled image (4 channels) is saved

with the suffix `_INI`. After saving, all images are closed and the `_INI` is opened automatically for further processing. This might take a moment.

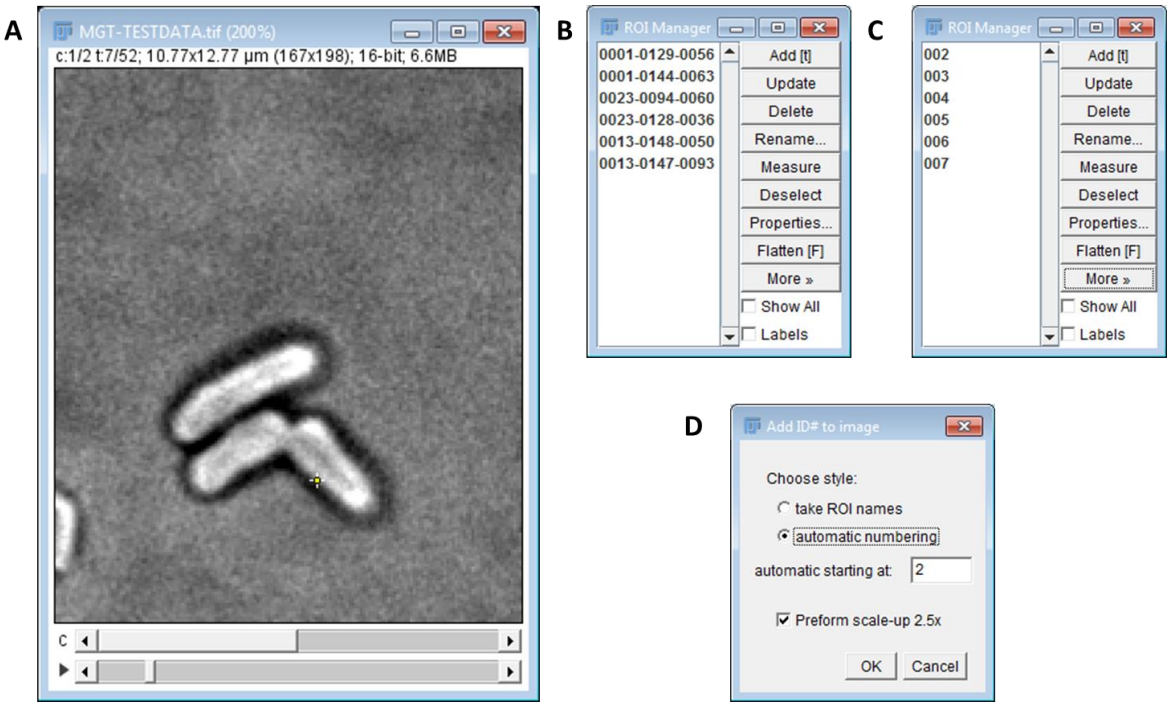


Fig. 3: (A) Select new-born cell with point selection tool. (B) List of raw selection (in correct order for autonaming). (C) Renamed list after macro run. (D) Dialog for preferences

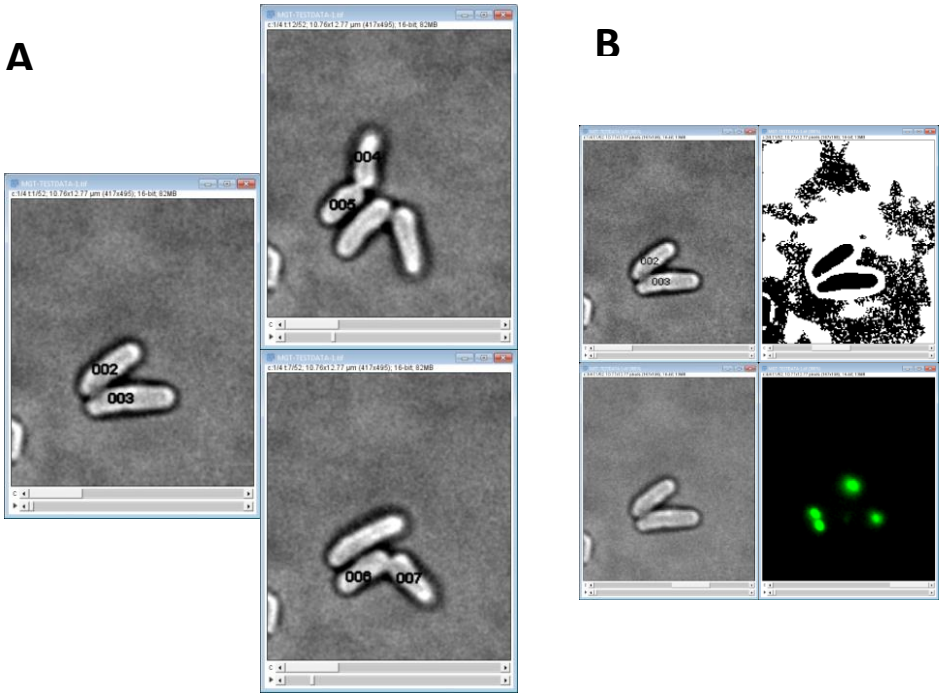


Fig. 4: (A) Cell-ID#s are printed into the first frame of new-born cells (B) After running the macro the image contains the numbering (Ch1), a thresholded copy (Ch2), the original contrast image (Ch3) and the fluorescent signal (Ch4).

ingle Cell acquisition

For each cell-ID#, the whole cell cycle has to be captured from birth until division. In an iterative fashion the cells' outlines are stored in the ROI-manager and processed. For creating the selections various approaches could be followed:

- The manual approach is the slowest, but most robust and precise method. By using the **(Fiji→Selection brush tool**  **)** each cell-outline is manually selected. With a double-click on the icon, the tool-diameter can be set to an appropriate value. By pressing **'t'** the selection is added to ROI manager. The shortcuts **(FIJI→ Shift + < + Alt)** and **(FIJI→ < + Alt)** can be used to skip between the frames.

- If the quality of the image allows, the thresholded image in channel two could be used to acquire the outlines with the **(FIJI→ Wand tool**  **).** The threshold method that works best could be determined by **(FIJI → Image → Adjust → Auto threshold)** on a mono-dimensional copy of the contrast image. The option **'Try all'** results in a collage of all possible variants. For segmentation water shedding could be applied.

- Machine learning aided segmentation (not validated yet)

After the whole cell cycle of a particular cell-ID# is captured in the ROI-manager (Fig. 5 A, C), the FIJI-macro **MoGenTrack.ijm** is executed. A dialogue demands for an ID# that should be entered in the correct format (Fig 5 B, e.g.: 002). The process saves a list of the created ROIs, using the ID# as suffix `_xxx` in the filename, as `\ROIs\RoiSet_yourfilename_xxx.zip`. The single ROIs in the list are renamed according to the ID# and the frame# introducing the identifier format `TixxxTfxxxT`. To keep track of the overall process the cell outlines are drawn into the first channel and a copy of the processed original image is saved as `\0riginals\yourfilename_xxx.tif`. The ROI-manager can be closed and the outlines of the next Cell-ID on the list can be captured.

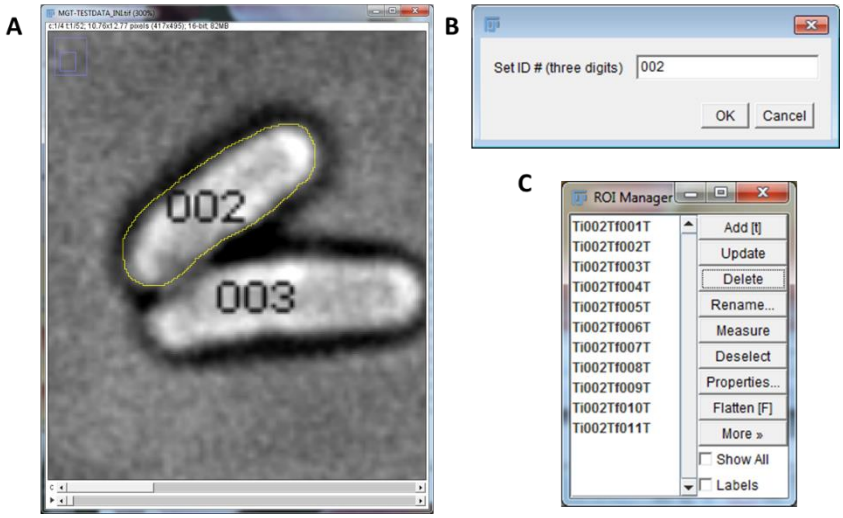


Fig 5: (A) Example of a cell-outline selection (B) User-dialogue demanding for cell-ID# (C) List of cell-outline-ROIs renamed after executing the macro

Landmark for the new pole

After the last cell cycle of interest is captured, the FIJI-macro **MoLandNewPole.ijm** should be executed. The appearing dialogue contains a check-box that should be ticked when the macro executed for the first time (Fig. 6 A). This turns the second channel black and paints the captured cell silhouettes in white (Fig. 6 B, C). The output of this step could be used as a quality check.

In order to keep track of the cell orientation with respect to the old and new cell pole a manual step is necessary. Using the second channel together with the paintbrush tool **(FIJI→Paint brush tool**  **)**, a black dot has to be drawn into the third of the cells that reflects the new pole (Fig. 6 D).

After all new cell-poles are assigned, the FIJI-macro **MoLandNewPole.ijm** has to be executed again. This time without ticking the check-box. This process exports a copy of the cropped out ROIs' bounding rectangle as multidimensional *.tif together with the frame number and the cell-ID in the filename (Fig. 6 E) to the location `\SingleCell\0riginal`. The algorithm draws the cell outline-in white into the exported copy and in black into the first channel of the hyperstack image, in order to avoid a conflict with already processed neighbors. The processed original-image is saved with the suffix `_P0L` in the folder `\0riginals`.

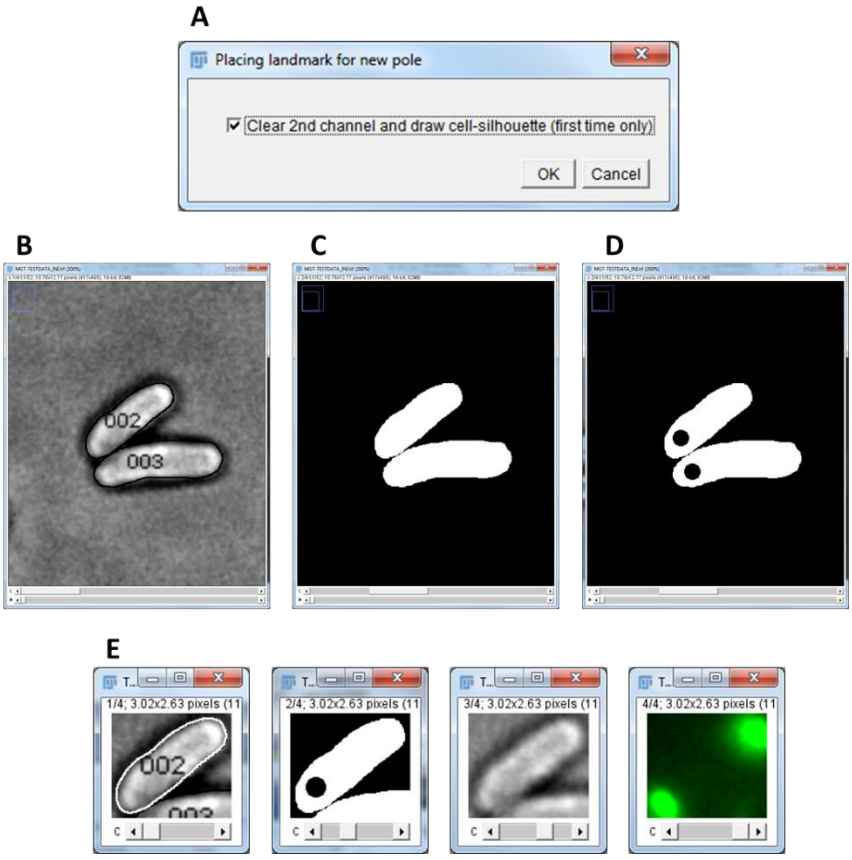


Fig. 6: (A) User-dialogue with check-box that should only be ticked, when executed for the first time (B) First channel contains cell-outlines in black (C) Second channel after the execution of the macro with ticked check-box (D) Second channel after placing landmark close to the new cell-pole (E) Exported single-cell image containing the cell-outlines in white (Ch1), the landmark (Ch2) and the original image information (Ch3&4).

Single cells analysis

The generated single-cell images are further analyzed by running the macro **MoSingleCell.ijm**. After execution, the starting-point for cell detection has to be chosen (Fig. 7 A). The default (x = 0; y = 0) starts the detection in the upper-left corner. If the automatic centerline construction fails, a different option might work. In some rare cases the process fails despite all options. For further information on that, see the chapter → **Quality Check and Rearrange Outliers**.

A user-dialogue appears that demands for or the location of the initial experiment folder (`C:\Users\Public\Documents\MorpholyzerGentrack\yourfilename\`).

This process creates additional subfolders for each channel, plus, `\0riginalExecuted`, `\Export`, `\R0I` and `\Sink` in the folder `\SingleCell`.

In a first place, the algorithm opens a file and finds the white outline, determines the pole-to-pole axis via the longest line and turns the cell into an upright position (Fig. 7 B). From the upper pole the mid-cell line is constructed following the cell shape. Along that line, perpendicular lines are constructed reaching towards the borders of the outline (slices). This collection of ROIs serves as a mask for various line-scans on all other channels. The results of each channel are saved in the corresponding folder `\SingleCell\Channel x` sorted by cell-ID, frame and channel. Additionally, a copy of the rotated image and the constructed ROIs are saved in the folders `\0riginalExecuted` and `\R0I`. For all channels and respective overlays, a *.jpg file, containing the out- and center-line, is exported to `\Export` (Fig. 7 C, Appendix B). Besides frame# and ID#, the filename of these also contain the cells' length, area and a measure of the appearance of a septal signal from the centerline scan of the fluorescent image. This measure is relative to the upper pole.

After successful processing, the current file is moved from `\0riginal` to `\Sink`. Once all files are processed, the program stops automatically. An appearing user-dialogue demands for the names of the experiment and each channel.

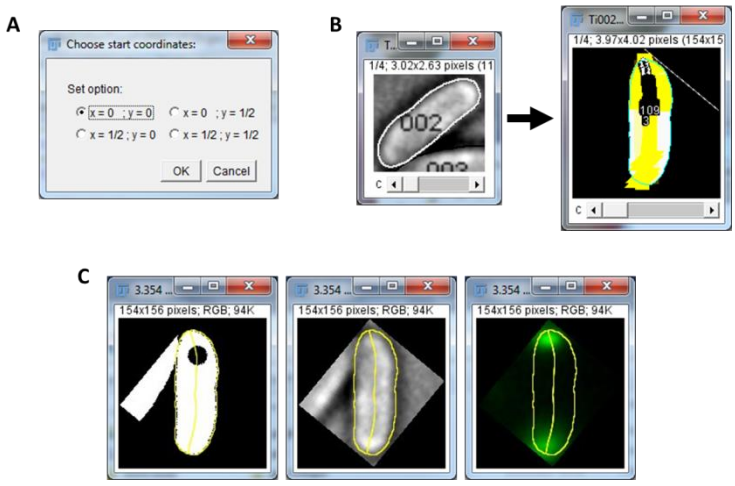


*Fig. 7: (B) Process of turning a cell upright and constructing a mask (C) Exported *.jpg-files.*

Quality Check and Rearrange Outliers

After the single cell analysis has finished, the generated data should be checked for consistency. Therefore, the exported *.jpgs files at `\SingleCell\Export` can be used. By using the preview mode of the windows file- explorer, all processed cells could be scanned quickly (Fig. 8).

In rare cases, the construction of the centerline fails without interrupting the SingleCellAnalysis-process. This leads to outliers in the data set, which show an aberrant length measure (Fig 8 C). It is recommended to sort the files by name. Thereby most of the outliers are placed at the beginning and at the end of the file list respectively. The files in between should also be checked. It is also recommended to check the completeness of the landmarks in `\Export\Channel 2` (Fig 8 A).

To rearrange the identified outliers, the folder `\Polish` has to be created manually. Any *.jpg- files of a rejected cell could be copied into that folder. The R macro **MGT_delete.R** should be executed. This process selectively deletes all files in the data-set connected to the particular identifier TixxxTfxxxT and moves the original *.tif-file back from `\SingleCell\Sink` to `\SingleCell\0riginal`.

The files that are now present in the folder `\SingleCell\0riginal` could be reopened in FIJI. The contrast chancel in should be copied to the fist channel by (Channel 3 → ctrl+a and ctrl+c ; Channel 1 ctrl+v). Now the **(Fiji→Selection brush tool**  **)** could be used to reset the outline. Running the FIJI-macro **MoReDo.ijm** saves the rearranged image with the new outline by overwriting.

After all outliers are rearraged, the SingleCellAnalysis (**MoSingleCell.ijm**) could be executed again.

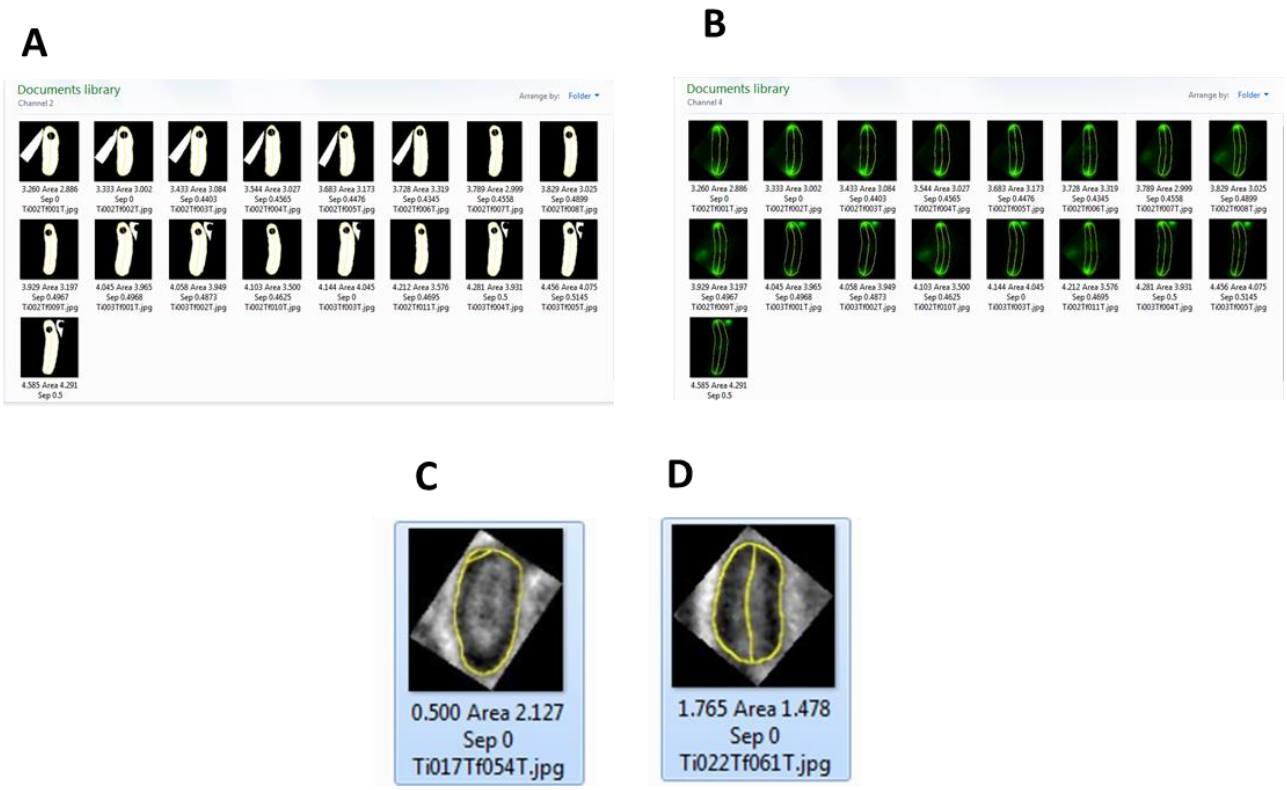


*Fig. 8: (A+B) Thumbnails of exported *.jpg files in windows explorer. (C)example of failed center-line construction (D))example of correct center-line construction*

Data conversion

To finalize the analysis of the obtained and rearranged scans, the R-macro **MGT_Heatwave.R** is executed. The appearing dialogue asks for the root-folder of the experiment with the prepared data (`C:\Users\Public\Documents\MorpholyzerGentrack\yourfilename\`).

The process runs a series of subscripts that eventually result in a table and various plots, reflecting the growth-characteristics of the analyzed colony. A detailed description of the process is found below:

The subscript Renamer renames the single-cell folders located at `\SingleCell\Channel x` according to the cell length.

The subscript Heatwave creates the folder structure `\Heatmaps\Channel x\DATA\SUM` + `\MAX`, `\MEAN`, `\MIN`. From the perpendicular line scans (Slices) the algorithm calculates four statistics (sum, mean, min and max), resulting in one dimensional arrays of a length proportional to the cell length.

The subscript MID uses the linearized landmark scans from `\Heatmaps\Channel 2\DATA\MEAN` to determine the orientation of the cell. If the black dot is localized in the upper half of the cell, the character **'Z'** will be added to the filename saved at `\Heatmaps\MID\`. This is further used to equilibrate the orientation.

The subscript FILSAV joins the information of cell ID, frame number, area and length in a structured way. Additionally it equilibrates the relative septum position according to the cell's orientation and calculates the approximate cell volume by rotating and summing up the slices.
In the folder `\yourfilename_ChronolistFILASV` individual plots for each cell are created. For all time points the lengths, areas and volumes are plotted next to each other.

The subscript signal35 extracts subcellular fluorescent profiles by using the linearized fluorescent scans in `\Heatmaps\Channel 4\DATA\MEAN`. Therefore, the actual length is divided by five. For each fifth the sum of the mean values is calculated and divided by the number of the slices. The orientation of the cells is equilibrated and the measures are stored in the code OxxxOIxxxMxxxNIxxxNxxx (OldPole, OldIntermediate, Midcell, NewIntermediate, New). The values of the three middle parts are summed up in a separate folder. The temporal fluorescent profile of single cells is visualized in plots saved at the created folders `\Heatmaps\3area_SignalMean`, `\3area_SignalPlotMEAN`, `\5area_SignalPlotMEAN`, `\5area_SignalMean`.

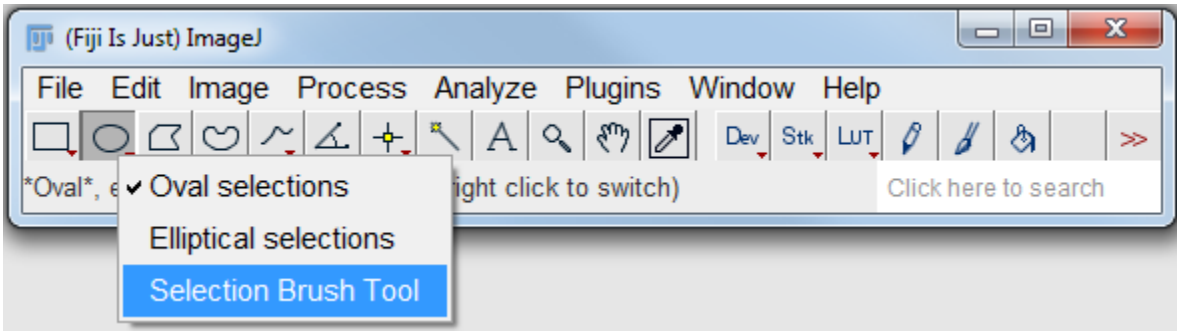
The subscript table results in the final output. In the root folder the file `yourfilename_ChronoTABLE.txt` is created. It contains a table with the headers "frame", "id", "length", "area", "volume", "septum_position", and "signal". The rows of the table are sorted by ID and frame.

Tips

Change threshold method in Line 59 method=Mean in **MoGenFormat.ijm**

To optimize the workflow, all scenes of interest could be cropped in one batch.

The selection brush tool is hidden behind **(FIJI→ right click on oval selection tool → selection brush tool)**



In case the shortcuts **(FIJI→ Shift + < + Alt)** and **(FIJI→ < + Alt)** for skipping the frames does not work probably, the Windows language selection shortcut might have to be turned off.

Appendix

Fig. A: Template Dendrogram to assign the cell ID#