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About

The software and analysis workflow are part of the following publication:

Hansen, J.N.; Rassmann, S.; Jikeli, J.F.; Wachten, D. *SpermQ*–A Simple Analysis Software to Comprehensively Study Flagellar Beating and Sperm Steering. *Cells* **2019**, *8*, 10.

Link: https://www.mdpi.com/2073-4409/8/1/10

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Source code

The source code for the ImageJ plugins and Java software developed for SpermQ analysis is accessible on GitHub (see also installation instructions below):

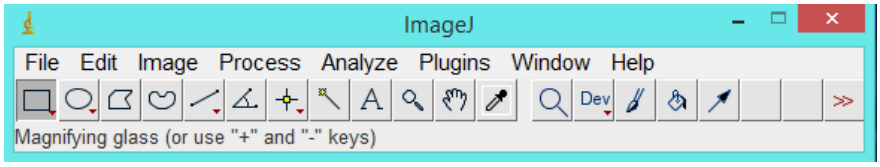
https://github.com/hansenjn/SpermQ

https://github.com/hansenjn/SpermQ_Preparator

https://github.com/IIIImaging/SpermQ_Evaluator

Step 1 – Install ImageJ and SpermQ plugins

- Download ImageJ from the official webpage: https://imagej.nih.gov/ij/download.html
- The download file will be a zip-archive – extract it and place it somewhere on your drive
- Open the extracted folder and launch **ImageJ.exe** in the folder **ImageJ**
- This is how an open ImageJ looks like:



- Download the .jar files from the latest release of SpermQ at https://github.com/hansenjn/SpermQ/releases and SpermQ Preparator at https://github.com/hansenjn/SpermQ_Preparator/releases:
  - SpermQPreparator_JNH-....jar
  - SpermQ_ -....jar
- Drag and drop the plugins (**.jar**-files) into the status bar of ImageJ (this is were "Magnifying glass …" is written in the picture above) to install them and press **save** in the two dialogs that will open one after the other. This will store the plugins in the ImageJ folder. CAUTION: Do not save the two jars into subfolders of the "plugins" folder but directly into plugins.
- Close ImageJ and open it again to complete the installation process.

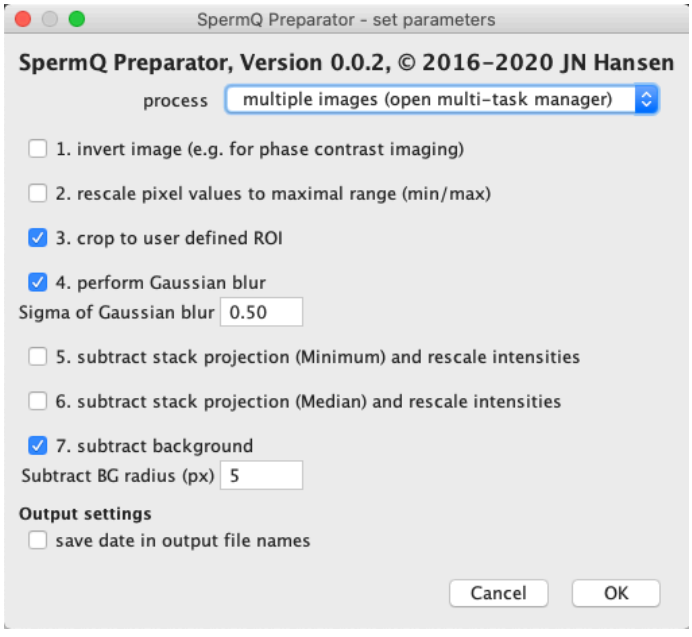
Special notes for applying SpermQ under Mac OS

In Mac OS, some more steps are necessary to allow SpermQ performance.

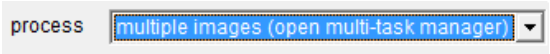
- Relocate the ImageJ icon (= ImageJ directory) to the desktop in order to avoid "Path Randomization" performed by the Mac OS, which impairs ImageJ to find the plugin file.
- Go into the jar files and click "get info" and switch from "private" to "write&read."
- Specify to use Java as a default app for the two jar files.

Step 2 – Prepare your data using SpermQ preparator

- Launch Plugins > JNH > Prepare Data for SpermQ



- This tool allows you to crop individual sperm from the prepared images and preprocess them in order to improve the sperm-to-background relationship.
    - Inverting the intensities in the image allows to obtain an image where the sperm is bright (hight intensity) and the background is dark (low intensity) – this is necessary to be applied to data from e.g. phase-contrast or brightfield microscopy, where the flagellum appears dark and the background appears light, before SpermQ analysis.
    - Rescaling intensities to the maximal range optimizes the intensity range for threshold calculation in SpermQ (see also the subsection "An additional step between SpermQ Preparator and SpermQ" below).
    - Cropping to a user-defined ROI allows you to reduce the size of the image that is used to form a threshold between background and sperm. In the reconstruction step every particle in the image influences the segmentation of the sperm from background. Reducing the image only to the region that is relevant for a sperm excludes other sperm from the analysis (avoiding confusion in the software) and dirt particles.
    - A Gaussian blur will decrease the noise level.
    - Subtract stack projection (Minimum or Median) and rescale intensities – This allows you to remove non-moving particles, background, or dead sperm from the image. The software projects the entire time series to one image in which each pixel has the lowest or median intensity measured at the pixel position across the time span. Only non-moving particles will remain in the projection image. Subtracting this projection from the original time series will thereby remove dead sperm or non-moving dirt particles from the image. After subtraction, intensities are again scaled to cover the full range of possible intensities.
    - Subtract background – This is another measure to homogenize the brightness across all positions in the image, yielding a better threshold calculation and reconstruction. Setting this to low radii also allows to equalize the intensity difference between head and flagellum, rendering the threshold calculation and reconstruction by the software more precise.
- Variable settings:



    - This setting allows you to select how to load the input images into the software.

    - By default, the software overwrites output files of previous analyses performed on the same image. If you do not want that – i.e. because you are selecting multiple sperm from one image – please select this setting! If selected, output files will be labeled in the name with a unique time stamp ("yyyymmdd_hh:mm:ss")

- When pressing ***OK***, the software will offer in the next step for each input image a maximum projection of the image. This allows you to set a ROI where the cell that you want to analyze is localized. Leave space (as a rough estimate: a third of the length of the flagellum) around the sperm cell so that the software can perform the correction of the flagellar track using normal lines (see original publication).
- Note that for each loaded image you can only crop one sperm – **If you want to crop multiple sperm from one image, you need to load the image multiple times** and select the "save

date in output file names" option to avoid that multiple processing of one image overrides already output files**.**

- Finally, SpermQ Preparator will process all images and produce output files that are saved at the location where the original loaded input images were located. Output files receive an additional file ending "_SpQP" and the date (if the last checkbox in the dialog was selected).
- The following output files are produced:
  - <input image name>_SpQP_<eventually date>.tif
    - Output image as a ".tif" stack-image – can be opened with ImageJ and needs to be loaded into SpermQ for analysis (see next chapter)

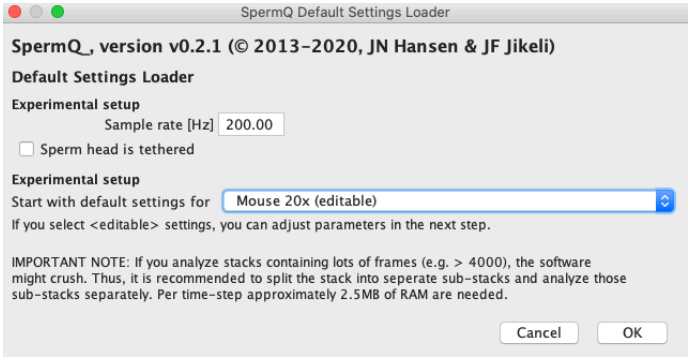
  - <input image name>_SpQP<eventually date>m.txt
    - A text file documenting the settings applied – can be opened with txt-file editor.
  - <input image name>_SpQP<eventually date>_roi.
    - The ROI that was set manually for this image and used to crop it.
    - This file can be opened using ImageJ.

How to find the best SpermQ Preparator settings for your data set?

Please read the chapter *How to optimize the SpermQ workflow for your data* set? at the end of this document.

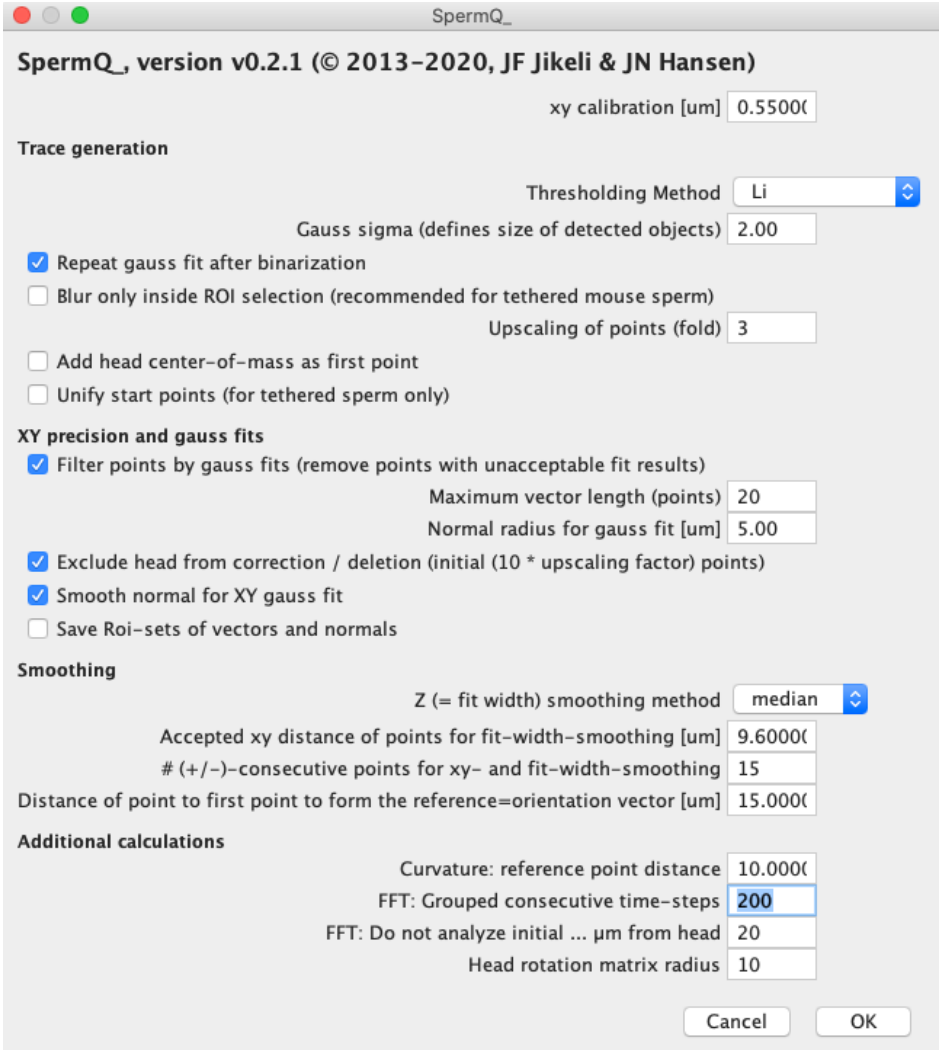
Step 3 – SpermQ analysis

- Open ImageJ and launch Plugins > JNH > Analysis > SpermQ Tracking
- A first dialog pops up where you need to indicate the Sample rate (Hz), whether sperm were tethered at the head or not, and where you can select default settings for different magnifications and species.

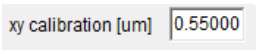


- If you selected an "(editable)" default setting, a dialog shows up that allows to tune the individual settings that determine reconstruction and further analysis.

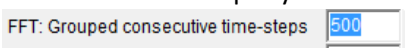
EXAMPLARY DIALOG:



- For a detailed description of every single parameter please see the Materials & Methods part of the original publication (https://www.mdpi.com/2073-4409/8/1/10).
- Note, however, that you should always adapt the following parameters to the data you load:

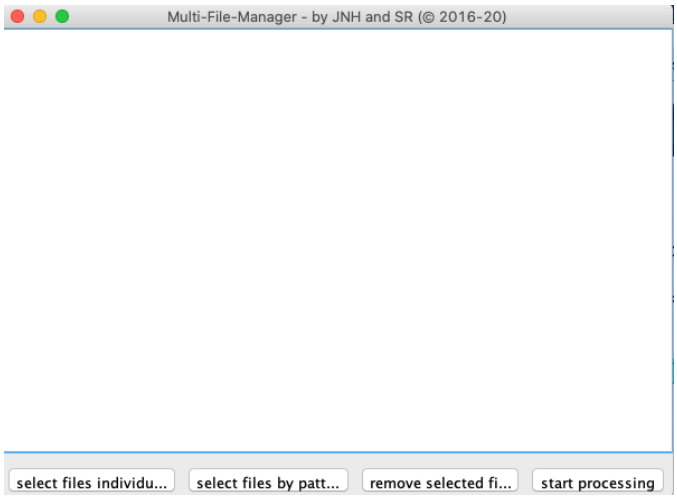


  - This parameter is given by your imaging setup and should be adapted accordingly.
  - Note that other parameters ("Gauss sigma", "Maximum vector length", "# (+(-)-consecutive points for xy- and fit-width smoothing", "Head rotation matrix radius") are given in the unit *points*. If you largely change the xy calibration in a default setting (e.g. from 0.55 to 0.3 µm), you should also rescale these parameters accordingly to the change in calibration (e.g. when changing from an xy calibration of 0.55 to 0.3 µm you should multiply these parameters by 0.55/0.3 = 1.83).

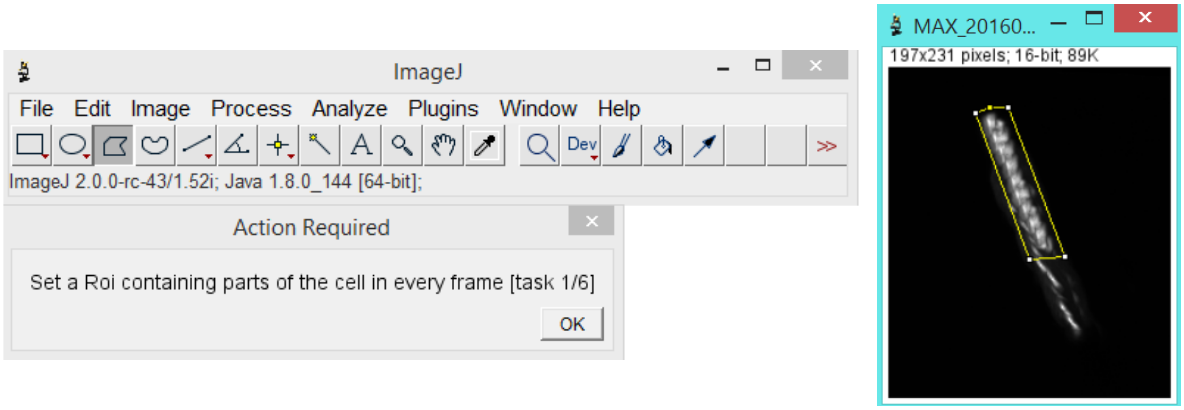


  - Unless you are studying changes in the beat frequency over time, this parameter should be set to at least the number of frames of the analyzed time series. You may just set it to a much-to-high value (e.g. 100000000; then, SpermQ will readjust it for each analyzed time series to the corresponding number of frames.

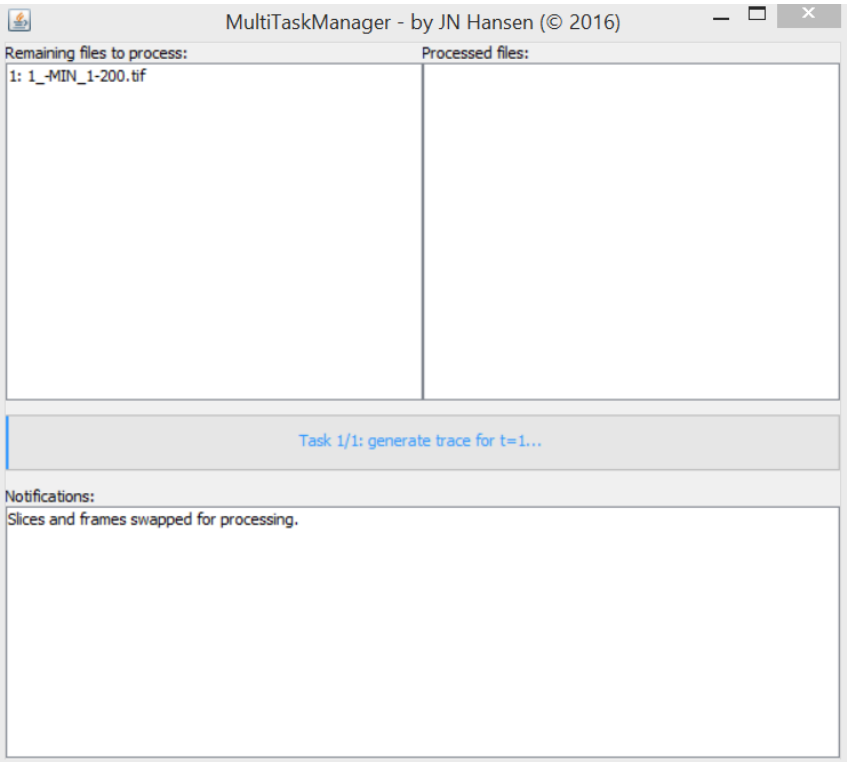
- After pressing **OK**, SpermQ offers a dialog to load image sequences that shall be processed. Press **add file(s)** and import all cropped time-series (see chapter 2) that shall be analyzed.



- After pressing **start processing**, SpermQ will offer a time projection for each time series to be analyzed and ask you to set a ROI. The ROI should contain at least a tiny part of the flagellum in each time point. Just set a ROI around the projected head positions. Setting this ROI allows SpermQ to neglect dirt particles or other sperm that are depicted in the time series.



- Once a ROI is set for every loaded time series, SpermQ will analyze one time series after another. The status of the analysis is visualized in the *MultiTaskManager* Window. Wait until everything is finished.



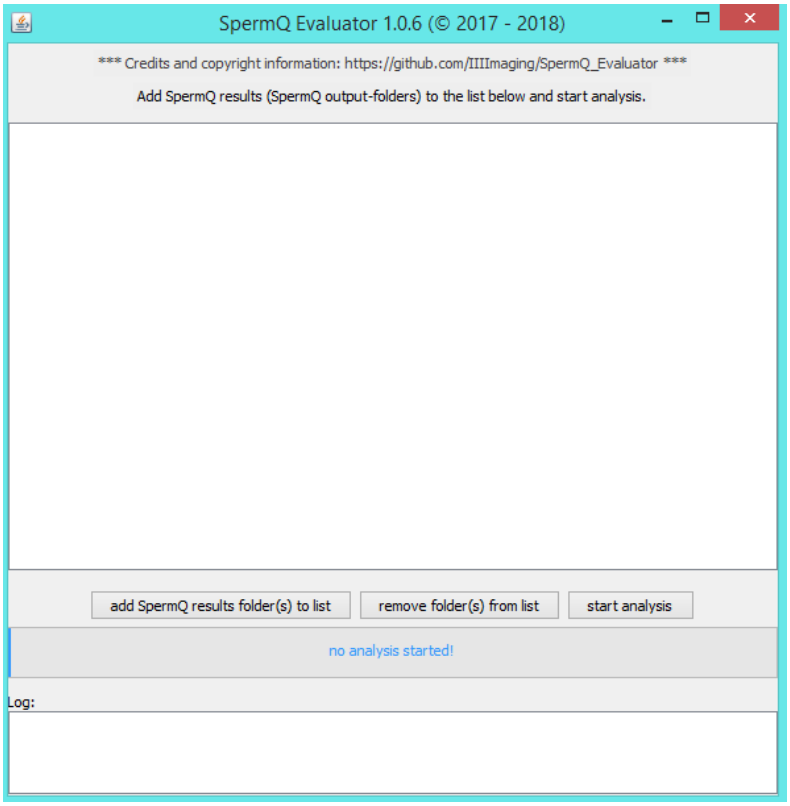
- Finally, SpermQ produces output folders with all results, which are saved at the location where the original loaded input images were located. Output folders have an additional folder-name ending: _spq_<date>.
- The output folder contains many files, among which are:
    - **Kymograph images** (as well as tables as .txt-files) for each flagellar parameter and its FFT-analysis – they are in the 16-bit range, so can only be correctly visualized in ImageJ, but you may export them from ImageJ as .png files for visualization in other software.
    - A **results.txt** file where the settings of the analysis and the results for the head position, head angle in space and sperm rolling are listed.
    - Images showing the **tracked sperm flagellum**
        a. <filename>_spq_<date>_ti.tif: shows a copy of the input time-series where only the tracked flagellar points are printed.
        b. <filename>_spq_<date>_ti_zC_median.tif: same as a, but where each flagellar point is intensity-coded with the flagellar width (= z position)
        c. <filename>_spq_<date>_oriZCmedian: flagellar track over time in the coordinate system defined by the head-midpiece-axis
- The easiest way to analyze and convolve results from multiple sperm is to use the SpermQ_Evaluator tool (see next chapter).

How to find the best SpermQ settings for your data set?

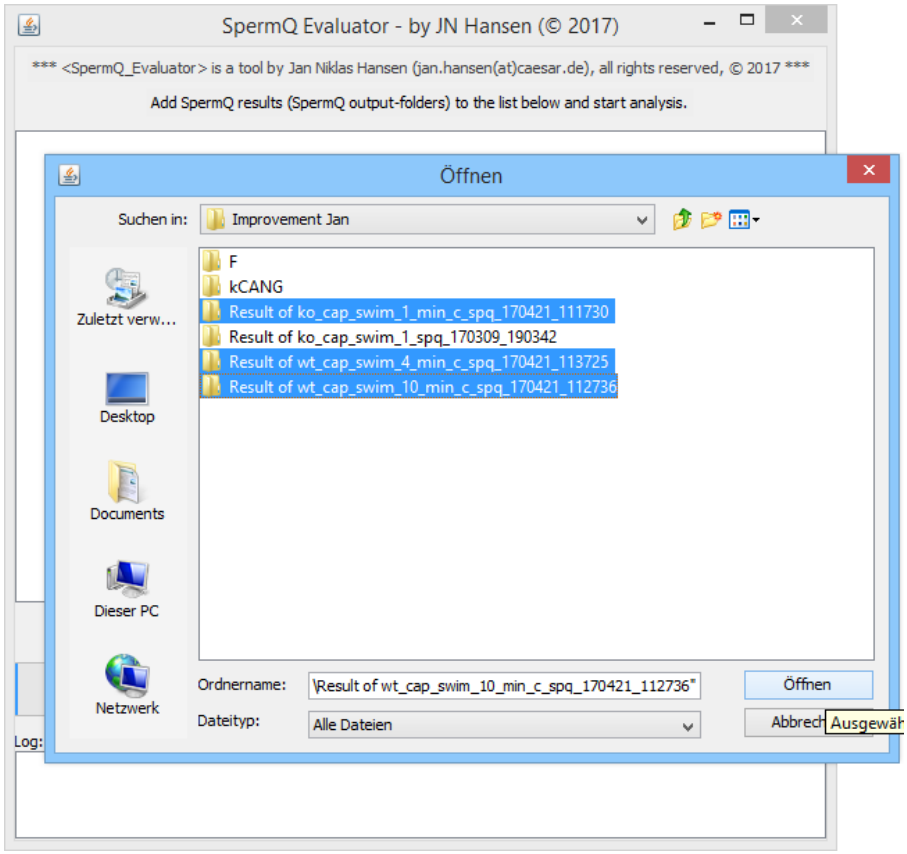
Please read the chapter *How to optimize the SpermQ workflow for your data* set? at the end of this document.

Step 4 – Analyze results using SpermQ Evaluator

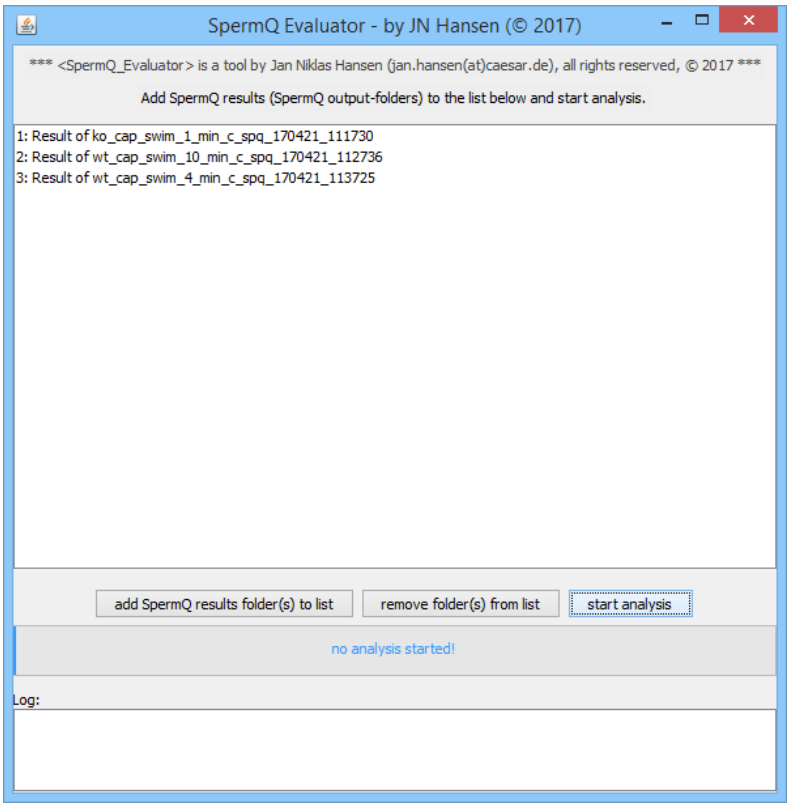
- SpermQ Evaluator collects and summarizes SpermQ results from many different analyzed images (e.g. a whole data-set).
- SpermQ_Evaluator is a pure java tool and will only run on your device if you have java installed (https://www.java.com/de/)
- Get the latest version of SpermQ Evaluator here (download the **.jar** file) https://github.com/IIIImaging/SpermQ_Evaluator/releases
- Launch SpermQ_Evaluator by double-click on the file.
- A window opens where you can load data sets:



- Load the SpermQ results folders into the Processing list by pressing **add…** . In the next step a dialog will be offered, where you can add the results folders that shall be analyzed:

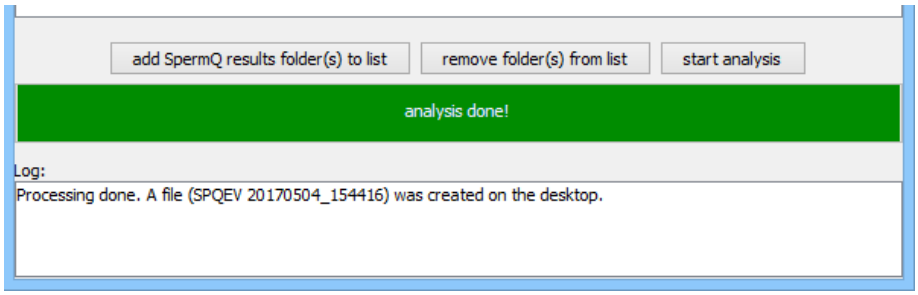


- Select the output folders of interest and press **Open** (or for German users: “Öffnen”).
- Next the list of data that you have selected is presented in the top list:



- You may eventually add more results folder(s) – to this end, press again **add…** and open files.
- When you have collected all the files to be analyzed in the top list, press start analysis.

- Analysis will take some time. The analysis progress is displayed in the bottom progress bar. If any errors occur during processing, this will be written in the “Log”-section of the main dialog. When SpermQ Evaluator has finished analysis, it will let you know by switching the bar color to green and notifying you in the “log” section of the main dialog:



- All analysis results are saved in a folder on the desktop (the folder name is written in the log section (see above)). Go to that folder and see the data.
- For each analyzed time series, SpermQ Evaluator produces a PDF-file displaying the most important information (see for example the Supplementary Figures in the original publication: https://www.mdpi.com/2073-4409/8/1/10).
- Additionally, the folder contains a lot of Tab-delimited txt-files, which you can directly copy and paste into an excel-sheet.
    - The file name of each txt-file indicates the results that it contains.

- In principle a file can start with the following short codes:

Begin of file	Parameter, for which results are collected in the file
cAng	Curvature angle
Curv	Curvature
HMaxI	Maximum intensity value in the head
HV	Velocity of the head center in space
List	Contains a List of all the data files analyzed in the current run
Log	Contains the content of the "log"-section created during SpermQ Evaluator analysis
Th2D	Head orientation angle theta
X	Coordinate of the flagellum on the head-midpiece axis
Y	Coordinate of the flagellum on the axis that is normal to the head-midpiece axis
Z	Gaussian curve width of the flagellum = relative parameter for the flagellar z position

- Next each file contains additional letter-codes in the name

Letter code	Indication
_f	Results for the frequency spectrum of the respective parameter
_f1	Primary-peak position in the frequency spectrum
_f2	Secondary-peak position in the frequency spectrum
_a1	Primary-peak value (amplitude) in the frequency spectrum
_a2	Secondary-peak value (amplitude) in the frequency spectrum
_com	Center-of-mass of the frequency spectrum
_avg	Average of all time-steps, where a result for the parameter was given
_medi	Median of all time-steps, where a result for the parameter was given
_min	Median of the 5 lowest Minima found over time
_max	Median of the 5 highest Maxima found over time
_ampl	Amplitude = Max - Min

- For example, look into an exemplary file, e.g. "cAng_ampl.txt" that contains the results for the amplitude of the curvature angle. Copy the file into an excel-sheet:

	A	B	C	D	E	F
1	This file was generated using SpermQ_Evaluator, a java application by Jan Niklas Hansen (© 2017) (all rights reserved,					
2	Date of proc	04.05.2017	15:50:09			
3						
4	This file contains the kymograph results for the parameter cAng:					
5	Results Amplitude (Max-Min):					
6	arc length	Result of ko_cap_swim_1	Result of wt_cap_swim_1(	Result of wt_cap_swim_4_min_c_spq_170421_113725		
7	0	2.466544	2.741214	2.917004		
8	0.6667	12.321732	14.461416	14.780035		
9	1.3334	22.393908	25.319151	26.033295		

- The header (rows 1-5) contains information about the software (row 1), analysis time (row 2), and the content of the file (row 4 -> curvature angle results; row 5 -> Amplitude was calculated over time).
- Row 6 contains the legend for the following data table. Each analyzed data-set is placed in a column (e.g. column B), while column A contains the respective arc-length of each datapoint.

Feeling puzzled by the load of parameters?

Compare these parameters between the different conditions in your data sets:

- **cAng_ampl**: Reveals the amplitude of the curvature angle will tell you the amplitude of the flagellar beat along the flagellum
- **cAng_medi**: Reveals the median curvature angle. The absolute values of the median can be used as index for the asymmetry of the flagellar beat
- **cAng_f_avg_f1**: Reveals the average primary beat frequency at each arc-length position
- **Th2D_f_f1**: Should reveal the primary general beat frequency of the flagellum
- **Y_ampl**: Reveals the amplitude of the flagellar beat normal to the head-midpiece-axis

How to optimize the SpermQ workflow for your data set?

Pre-processing of images (SpermQ Preparator)

The most important steps for successful detection of the sperm cell can also be manually tested in ImageJ. Perform these manual operations and improve them by trial and error.

The goal for preprocessing is to develop settings that allow to enhance the contrast of flagellum to background and to achieve an equal intensity of the background in the image. If achieved, the threshold algorithm in SpermQ can well separate the flagellum from the background.

To improve these settings, you can use a few frames of your stack showing an exemplary sperm cell of interest and try what happens when doing the very same as the SpermQ Preparator plugin in ImageJ. To create such a stack for testing, drag and drop an exemplary image into ImageJ and set a quadratic ROI around the region you want to use during testing. Go to Image > Duplicate in ImageJ and select the frames that you want to use (e.g. frames 1-20). Then afterwards you can try the following, analogously to SpermQ Preparator steps.

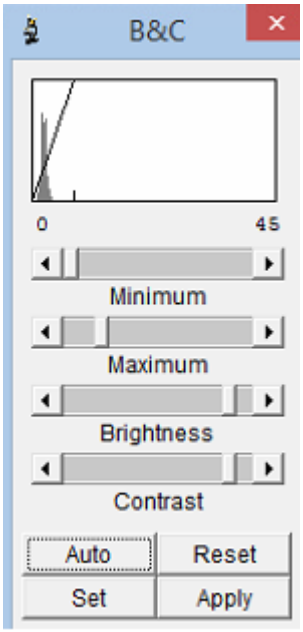
Crop to user defined ROI: Open the file in ImageJ, select a rectangular region of interest (“ROI”) and go to Image > Crop.

Perform Gaussian Blur in ImageJ (Process > FIlters > Gaussian Blur): modulate the sigma size here to reduce the noise in the image.

Test whether **subtracting a Minimum projection** removes differences in intensity level across the image; Create a Minimum intensity projection (Image > Stacks > Z Project and select minimum intensity will give you the projection) and subtract the projection from you tif stack (via Process > Image Calculator)

Subtract Background (Process > Subtract Background): modulate the pixel size here. A small pixel size can equalize intensity differences between head and tail of the sperm cell, which is beneficial during threshold calculation in SpermQ (see below)

After testing different conditions, look at the image and check whether you can well separate the cell from the background by eye (To better see that, enhance the brightness a bit (via Image > Adjust > Brightness/Contrast; set Minimum to 0 and drag down maximum until you can see the cell nicely):



Here you can for example see that at some positions the cell is hardly separable from background levels:

This will result in an incomplete detection in SpermQ and problems with the analysis. However by playing with the Gauss filter and subtracting the background this might be improvable.

In addition, during optimizing pre-processing settings for SpermQ evaluator, also check whether SpermQ may detect the trial picture by manually checking how SpermQ would perform. How to do this manually is explained below.

An additional step between SpermQ Preparator and SpermQ

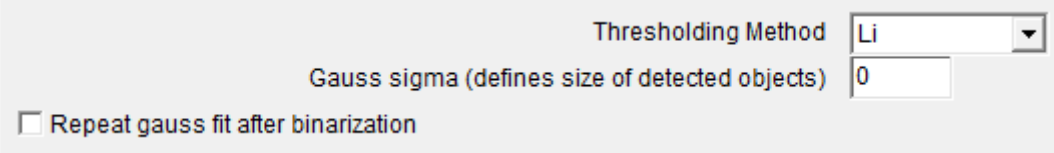
Because in many steps in SpermQ Evaluator intensity levels are subtracted, output images of SpermQ Evaluator may just cover small intensity numbers. However threshold algorithms may perform better, when the whole range of intensity levels is covered.

Try this step also in your analysis pipeline (it can also be automatically conducted by SpermQ Evaluator – see setting “2. rescale pixel values …”) and test whether it improves your data.

- Open the output SpQP.tif file (or the just use the manually generated test image during finding optimal settings), measure the intensities in the image (before first doing this it might be necessary to set your ImageJ as follows Analyze > Set Measurements.. > check Min and Max) via Analyze > Measure.
- Go to Image > Adjust > Brightness & Contrast and set what you measured as Min and Max in ImageJ.
- Press Apply and apply it to all stack images.

SpermQ settings

In the SpermQ settings you find the following lines

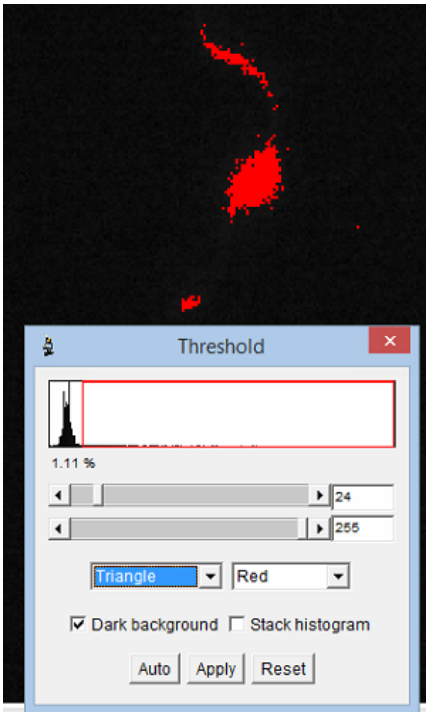


These are essential for the detection of the sperm cell by SpermQ. For detecting the flagellum SpermQ will do the following:

If "*Gauss sigma*" is set higher than 0: blur the frame image with the respective Gauss sigma (can be manually mimicked by Process > FIlters > Gaussian Blur)

Duplicate the frame image (Go to Image > Duplicate and uncheck "stack" option)

Thresholding Method: Use the duplicated frame image and go to Image > Adjust > Threshold. Here you can modulate the Thresholding method and see what is detected as flagellum in red (the threshold should be set accordingly to that hardly any background pixels are red but almost the whole flagellum is shown in red).



Thereby, you can try choosing the best method. In this example pics there are big gaps, this will not end up in a good reconstruction in SpermQ (SpermQ analysis will fail). After selecting the best possible option (most continuous flagellum), got to apply, which will create a binary image.

When you have small gaps these might be fillable by the next setting:

Repeat gauss fit after binarization; This option makes sense to be used / set when you need to fill small gaps, however this maybe not good to choose if your thresholding also reveals many background pixels as positive (red). If so these might be falsely fused to the flagellum). To mimic this option just do again Process > Filters > Gaussian Blur and set the same sigma as indicated at Gauss sigma.

After these steps SpermQ determines a rough flagellum using the skeleton method. You can just mimic that by selecting Plugins > Skeleton > Skeletonize in ImageJ. Thereby you see what is detected as a skeleton. Here you want to see a skeleton going through the entire flagellum; if this is the case your settings are good, if this does not work you should consider to adapt them to get the whole flagellum in one piece.

(In case of you see small side branches, don’t worry and just go on, they will be chopped off later by SpermQ)