SOP for analysis of ciliary structure and fluorescence with CiliaQ

0. Get started

Information

The CiliaQ workflow has been developed in the research group *Biophysical Imaging* at the *Institute of Innate Immunity*, Bonn, Germany (http://www.iii.uni-bonn.de/en/wachten_lab/). Before using CiliaQ, we recommend reading the CiliaQ publication.

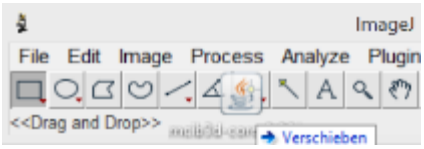
Please cite CiliaQ when presenting results obtained with CiliaQ:

> Jan N. Hansen, Sebastian Rassmann, Birthe Stueven, Nathalie Jurisch-Yaksi, Dagmar Wachten. CiliaQ: a simple, open-source software for automated quantification of ciliary morphology and fluorescence in 2D, 3D, and 4D images. Eur. Phys. J. E 44, 18 (2021). https://doi.org/10.1140/epje/s10189-021-00031-y

For news on CiliaQ and updates of the plugins or the user guide, please check the CiliaQ GitHub page: https://github.com/hansenjn/CiliaQ/. Alternatively, and for any other inquiries, feel free to contact jan.hansen@uni-bonn.de or dwachten@uni-bonn.de.

Installations

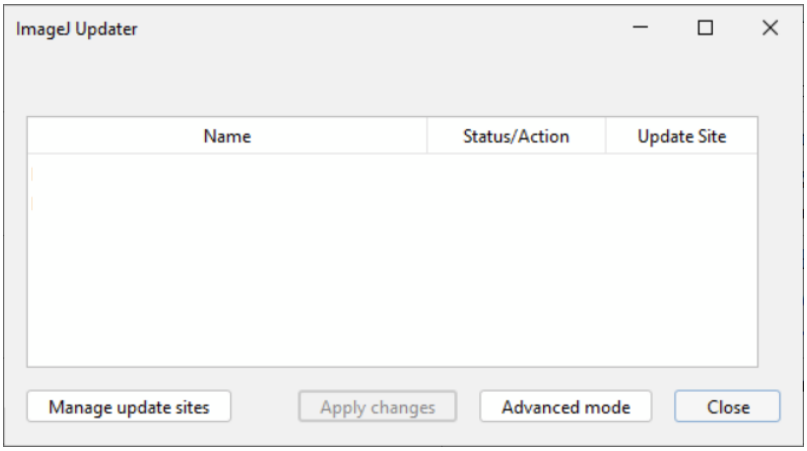
Install FIJI (https://fiji.sc/#download) and open FIJI. Download the latest releases of the following ImageJ plugins and install them into you FIJI distribution by dragging and dropping the .jar files into the status bar of the FIJI window; If a dialog pops up, press save and restart FIJI.



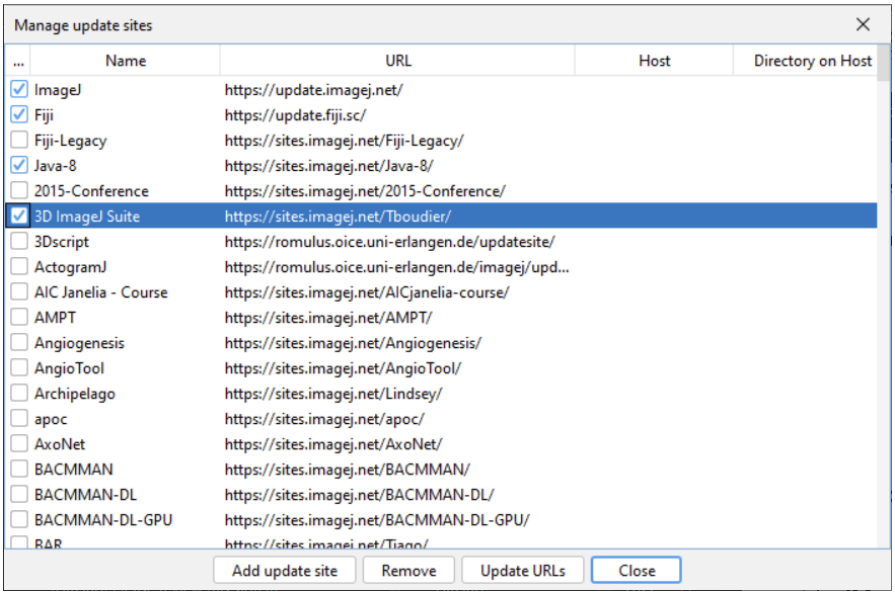
- CiliaQ_-… -SNAPSHOT.jar (https://github.com/hansenjn/CiliaQ/releases)
- CiliaQ_Preparator_JNH-…-SNAPSHOT.jar (https://github.com/hansenjn/CiliaQ_Preparator/releases)
    - Note: To use the CANNY 3D method in CiliaQ_Preparator the installation of the 3D ImageJ Suite's is required (see below)
- CiliaQ_Editor_JNH-…-SNAPSHOT.jar (https://github.com/hansenjn/CiliaQ_Editor/releases)

Note: Some methods in CiliaQ Preparator (Hysteresis threshold and CANNY 3D) requires the installation of the 3D ImageJ Suite to your FIJI/ImageJ:

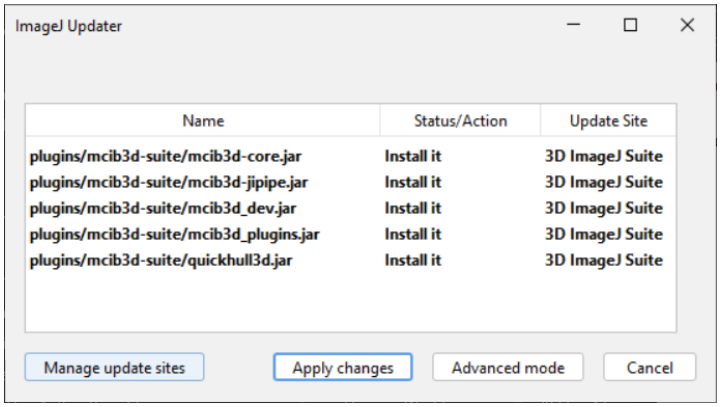
- Go to the menu entry Help > Update in your FIJI. This will show a dialog with a status bar that loads the following dialog.



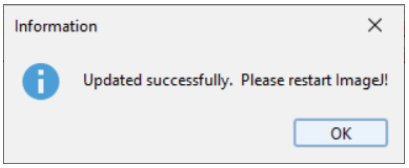
- Click "Manage Update Sites". The following window opens. Tick "3D ImageJ Suite" and press "Close".



- Now, in the original update dialog you will see new modules added for installation. Click apply changes.



- The installation process may take some time, depending on the download speed. The installation process terminates with the following dialog:



- Press OK, close FIJI and restart FIJI. To check that the 3D suite is installed, verify that the menu entry Plugins > 3DSuite is available in FIJI / ImageJ.

Workflow overview

CiliaQ follows a four-step workflow based on three ImageJ plugins and one R script:

1. Automatically preprocess images with CiliaQ Preparator to segment the channel that labels cilia into cilia voxels and background voxels.
2. Check for correct segmentation and optionally, if required, edit the segmentation manually using CiliaQ Editor.
3. Automatically quantify cilia with CiliaQ: Connects adjacent cilia voxels to cilia objects, quantifies cilia objects, filters out noise using a size threshold.
4. Convolve analysis data from many analyzed images and explore the data set in R using an R-markdown template (https://github.com/sRassmann/ciliaQ-output-joinR).

1. Segmenting cilia and background using CiliaQ Preparator

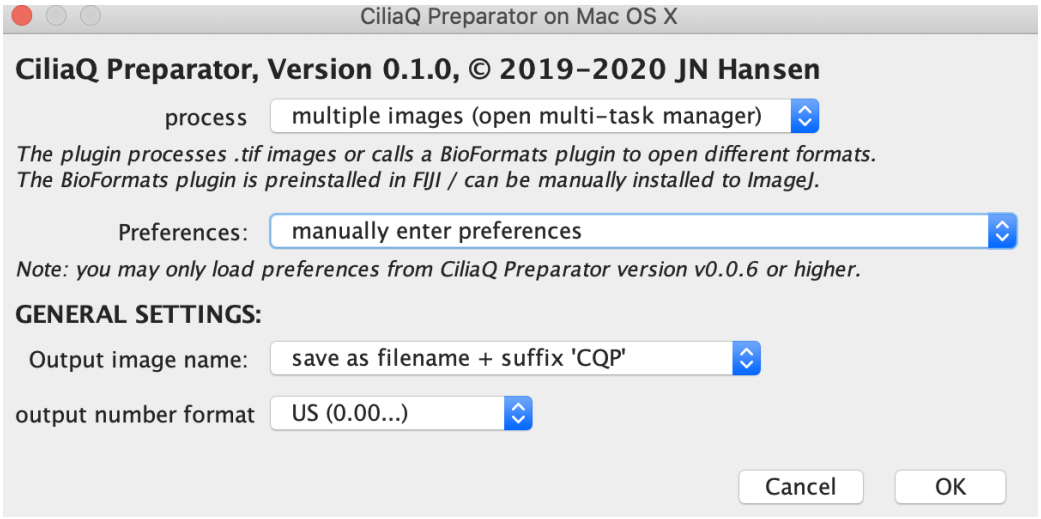
For segmenting the channel that allows to separate the object of interest (i.e. cilia) from background, use the ImageJ plugin *CiliaQ Preparator*. Process the image for ciliary reconstruction using CiliaQ Preparator (**PLUGINS -> CILIAQ -> CILIAQ PREPARATOR V…**).

Input files

The plugin processes multi-channel .tif files or raw microscopy files. For the latter, a plugin called BioFormats (which allows to open microscopy files) is required; in FIJI, this plugin is preinstalled.

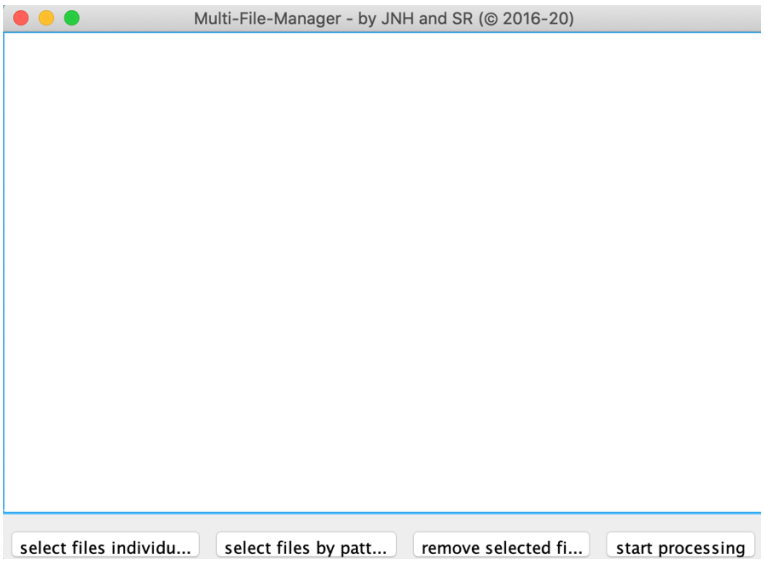
Settings

Upon launching the plugin, the following dialog pops up:



The **process** setting offers three possibilities to load data into the plugin and process these as a batch

- Multiple images: a dialog will open after pressing ok (see below). Here you can generate a list of images to be processed. Add files to the list (select files individually) and press start processing. Alternatively, files can be added to the list based on their file names (select files by pattern)



- Active image in FIJI: the currently front-most image opened in FIJI will be processed
- All images open in FIJI: all images that are open in FIJI will be processed

Note that the software can process only saved images – every image to be processed needs to be saved on the hard disk before it can be processed. This is required because CiliaQ_Preparator needs a path where it shall store the output image and metadata. Only saved images can provide that path in FIJI. If you process the image in FIJI and subject an unsaved image to CiliaQ_Preparator, CiliaQ_Preparator produces errors.

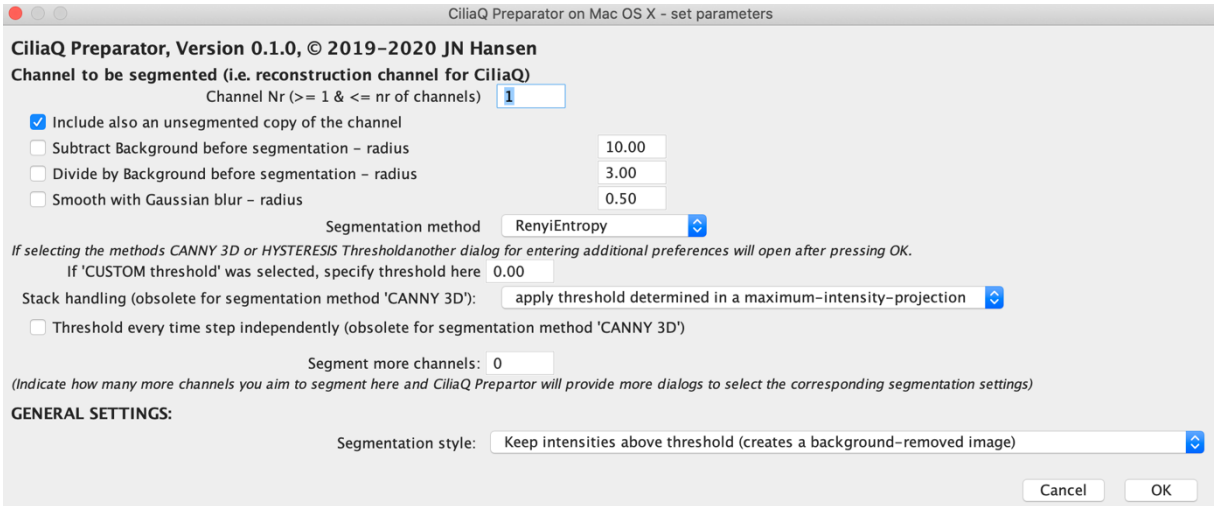
Preferences can be either manually entered or loaded from the meta-data file generated by a previous CiliaQ Preparator processing (ending "…_CQP.txt").

If manually enter preferences is selected, further dialogs are displayed to enter the preferences.

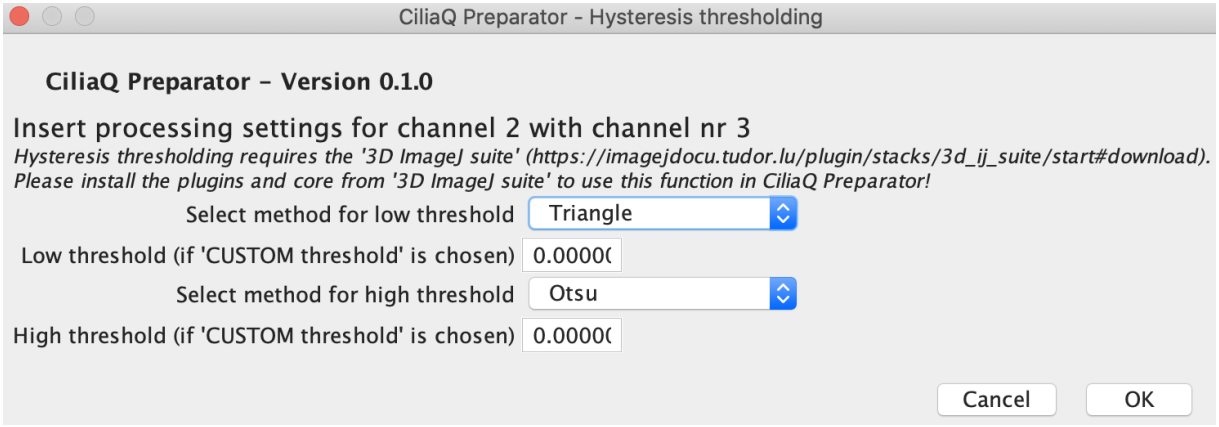
If load preferences from existing CiliaQ Preparator metadata file is selected, a dialog will open that requests you to select a CiliaQ Preparator metadata file to import settings – no settings need to be manually entered.

Manually enter preferences

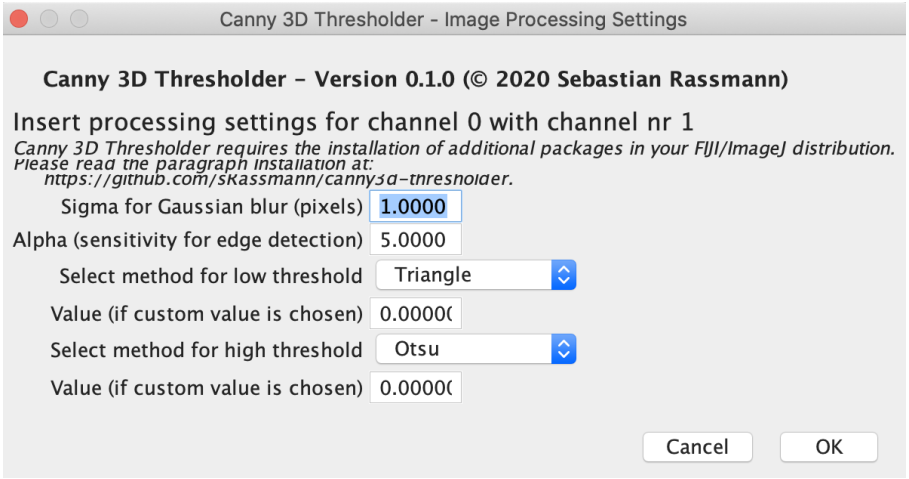
The first dialog allows to enter preferences to segment a channel. Set the parameters for the main channel that you want to segment to separate cilia objects from the background – usually the default settings work well. However, you may adapt them to improve better detection results.



- **Channel Nr**: the number of the channel in the image stack that needs to be processed (e.g. first channel = 1, second channel = 2, etc.)´
- **Include also an unsegmented copy of the channel**: If you select this option, the channel is duplicated before segmentation. In the output file you will find two versions of the channel, one segmented version and next to it the raw unsegmented version. Selecting this option is required if a later-on segmentation correction with CiliaQ Editor is planned.
- CiliaQ Preparator offers three functions to remove the background from the image, normalize intensities, and reduce noise. This can improve the performance of image segmentation later. Try these options if segmentation does not perform well for optimization.
    - **Subtract Background**: a function that subtracts the local background and thereby, can improve the contrast between background an cilia.
    - **Divide by Background**: a function that divides the image by a blurred copy of the image. Allows to normalize to local intensity levels. Can improve the segmentation of images where the general intensity levels in the channel largely vary across the image and/or where intensity levels largely vary within cilia.
    - **Gaussian Blur**: Can reduce detection / camera noise in the image and smooth the edges of cilia.
- **Segmentation method**: Here you can either select one of ImageJ's common intensity threshold algorithms (such as ReniyEntropy, Huang, Triangle, Li, Huang …), a Hysteresis threshold, or the method "CANNY 3D".
    - ImageJ's threshold algorithms separate the cilia objects from the background based on the histogram of the image.
    - A hysteresis threshold uses two thresholds to segment the image into cilia objects and background - one low threshold and one high threshold (see also: https://imagejdocu.tudor.lu/plugin/segmentation/hysteresis_thresholding/start).
        - To specify the low and high threshold, CiliaQ Preparator will show an additional dialog - the low and high threshold can be determined using ImageJ's threshold algorithms.
        - How the hysteresis threshold works: First, the image is segmented into three groups of voxels: (1) voxels with an intensity below the low threshold, (2) voxels with an intensity equal to or above the high threshold, (3) voxels with an intensity equal to or above the low threshold and below the high threshold. Second, based on these three groups, the image is further segmented into fore- and background as follows: voxels from group (1) will be considered background; voxels from group (2) will be considered foreground ("cilia voxels"); voxels from group (3) will be considered foreground if they connect to voxels from group (2) and considered background if they do not.



- CANNY 3D performs an edge detection algorithm (Sobel filtering), combined to a hysteresis threshold and a hole-filling-algorithm. In detail, the following steps are performed to segment foreground from background:
  1. The image stack is smoothed with a 2D Gaussian blur filter to supress random noise (the z dimension is assumed to have a lower resolution and a blur effect due to confocal imaging. Thus a 2D filtering is sufficient and no 3D filtering is required).
  2. Edges are detected using a 3D Sobel kernel.
  3. A 3D Hysterisis Threshold is performed: All voxels above a defined high threshold are kept, voxel below the defined low threshold are neglected, voxels in between the low and high threshold are only kept if they are connected to voxels above the high threshold. High and low thresholds can be defined using either custom values or can be calculated using ImageJ's thresholding methods based on the histogram of the whole stack.
  4. Holes encapsulated in all dimensions are filled.
- If CANNY 3D is selected, another dialog will open (after finishing this dialog) that allows to adapt the settings for the Canny 3D threshold method.



- **Stack handling** -> For detecting cilia, it is recommended to select the option “apply threshold in maximum intensity projection”. Thereby, the relationship of background and foreground voxels is more equal than in a whole stack histogram (other option).
- **Threshold every time step independently**: This should be selected for time-lapse images, if bleaching is observed or if the intensity in the channel changes over time.
- **Segment more channels**: For specialized applications, CiliaQ Preparator allows to segment additional channels in the image – this is however not necessary for common applications. If

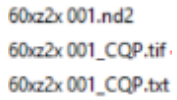
you wish to segment additional channels, select how many here and CIliaQ Preparator will show additional dialogs to set the preferences for additional channels.

- **GENERAL SETTINGS**: If selected in the option **Segmentation Style**, the segmented channel image is background-removed: intensities of cilia voxels will be kept in the output image, background voxels are reduced to an intensity value of 0. For CiliaQ analysis, this is recommended, as it allows to retrieve intensities for the reconstruction channel.

Output

CiliaQ Preparator saves the output image for a given input image as a multi-channel Tiff-stack in the directory where the input image was located. The new file contains the original image's name and the additional ending "_CQP.tif".

In addition a metadata file with the ending "_CQP.txt" is saved.



The metadata file describes the input settings. Most importantly it also describes which channel in the output image corresponds to which channel in the input image:

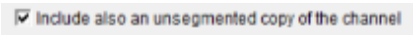
```
Channels in output image:
Channel 1:      previous channel 1 (segmented)
Channel 2:      previous channel 1
Channel 3:      previous channel 2 (segmented)
Channel 4:      previous channel 2
Channel 5:      previous channel 3 (segmented)
Channel 6:      previous channel 3
Channel 7:      previous channel 4
```

This information is however also stored in the .tif stack itself. Each slice image is labeled with the information from the raw image and whether it was segmented (labels of segmented images contain the prefix "segm: ").

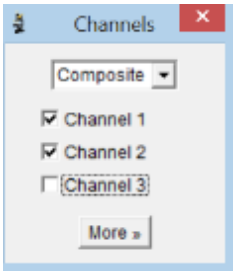
When you open an image in FIJI/ImageJ you find the slice image labels on the top of the image - example for the slice label of a segmented stack image:

At the front, the channel in the output image is marked ("c:5/7": channel number 5 of 7), it follows the shown z slice ("z1/12": slice 1 of 12) and then in brackets the image label, where it is written that the shown image was segmented ("segm") and the original channel / slice that the shown image was derived from ("c:3/4 z:1/12").

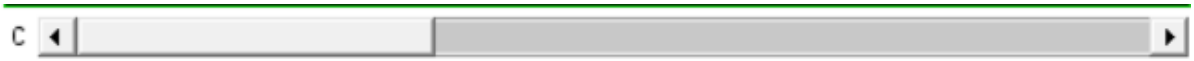
2. Scrutinizing and eventually correcting segmentation using CiliaQ Editor

When in CiliaQ Preparator the option 

was checked, the segmentation success can be easily scrutinized. Drag and Drop the output file with the ending "_CQP" into FIJI, open the **IMAGE > COLOR > CHANNELS TOOL**, select – if not already selected – "Composite" and select the channels that correspond to segmented and unsegmented channel of cilia labeling.



In the Channel bar below the image, you can select the corresponding channel, whose appearance in the image you may edit as follows.



Via **MORE > …** you can change the color of the channels. To easily judge on the segmentation success you may e.g. select "cyan" for the segmented channel and "red" for the unsegmented channel. Thereby, the detected cilia voxels will appear white in the image, while non-detected voxels appear red.

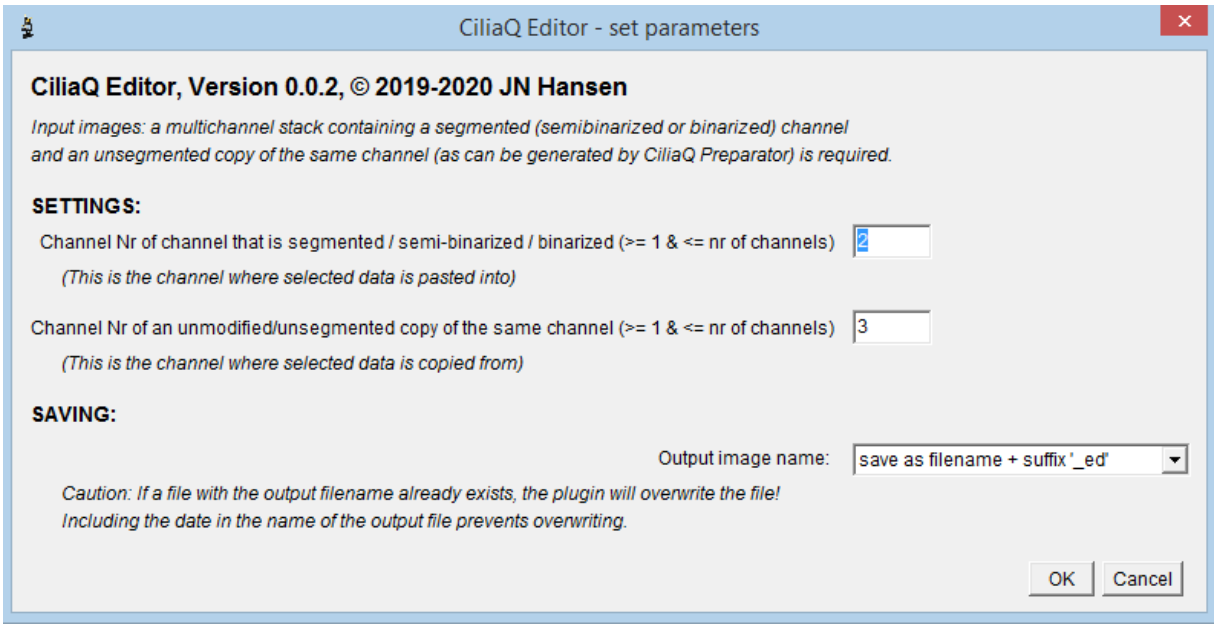
Using the **IMAGE > ADJUST > BRIGHTNESS/CONTRAST** tool, you may adapt the visualization of the channel images in FIJI to better judge. In the segmented channel, drag down the Maximum to a very low value to clearly see which voxels are detected.

Although, when using high quality images, CiliaQ detects most/all cilia correctly, segmentation errors occur. These occur e.g. under the following circumstances:

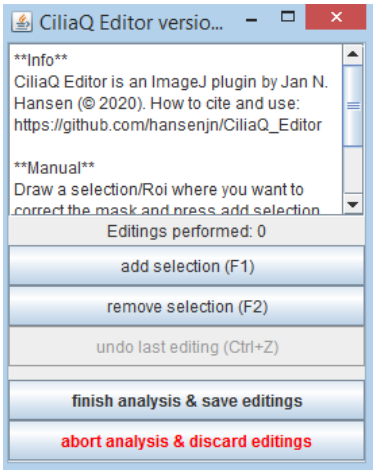
- Some cilia are fragmented – exemplary causes
  - The labeling used for segmentation was unequal along the cilium, faint positions did not pass the segmentation threshold.
- Many/Most cilia are not detected or only very sparsely detected - exemplary causes:
  - There was a very bright particle in the labeling channel, e.g. antibody aggregate, auto-fluorescent dirt particle, etc.
    - Repeat imaging, image a position that does not contain e.g. dirt particles.
  - The segmentation method is inappropriate / the staining is too weak
    - Trying different segmentation methods or, in case CANNY3D was used, adjusting the sensitivity / threshold settings might help.
  - Imaging quality is bad - the signal to noise ratio in the image is low
    - Improving imaging might help.
  - Unequal illumination – some parts are brighter / some parts are darker
    - Can be corrected when using the subtract background method in CiliaQ Preprator

If these errors occur only rarely or if you cannot provide a better imaging / segmentation, you may correct them manually. To this end, open the image to be corrected (if not already open) in FIJI and launch CiliaQ_Editor via **PLUGINS -> CILIAQ -> CILIAQ EDITOR V…**.

Next, indicate which channel in the opened image corresponds to the segmented and which channel corresponds to the unsegmented channel of cilia labeling.

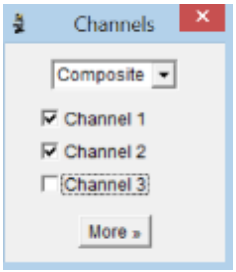


A dialog emerges:

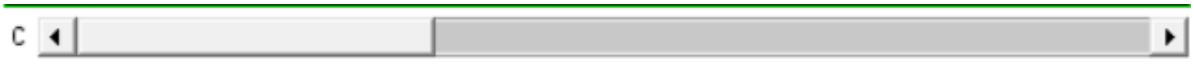


Note: for better visualization of the segmentation success, you may adapt the display of the image:

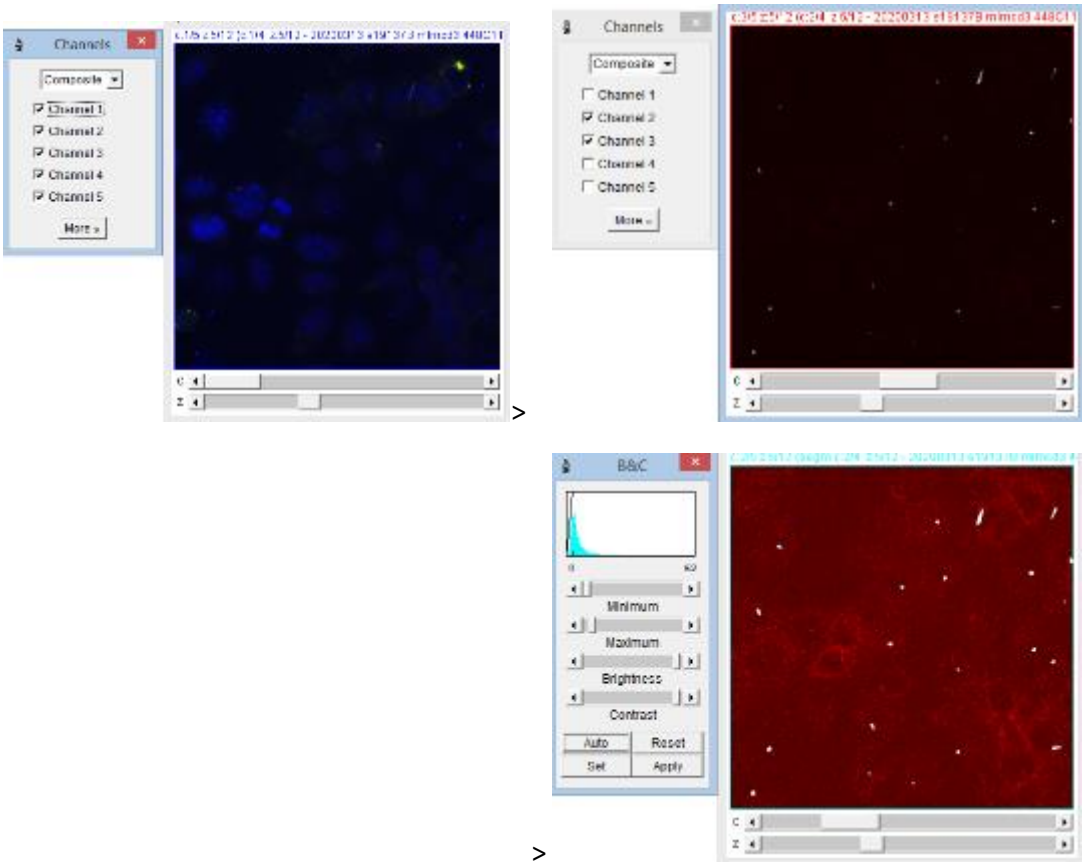
- Open the IMAGE > COLOR > CHANNELS TOOL, select – if not already selected – "Composite" and select the channels that correspond to segmented and unsegmented channel of cilia labeling.



- In the Channel bar below the image, you can select the corresponding channel, whose appearance in the image you may edit as follows.

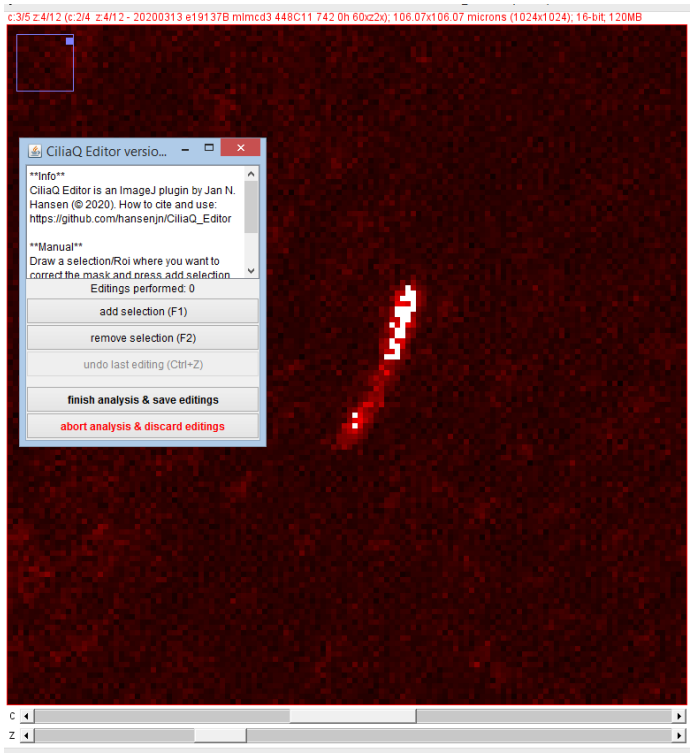


- Via **MORE > …** you can change the color of the channels. To easily judge on the segmentation success you may e.g. select “cyan” for the segmented channel and “red” for the unsegmented channel. Thereby, the detected cilia voxels will appear white in the image, while non-detected voxels appear red.
- Using the **IMAGE > ADJUST > BRIGHTNESS/CONTRAST** tool, you may adapt the visualization of the channel images in FIJI to better judge. In the segmented channel, drag down the Maximum to a very low value to clearly see which voxels are detected.

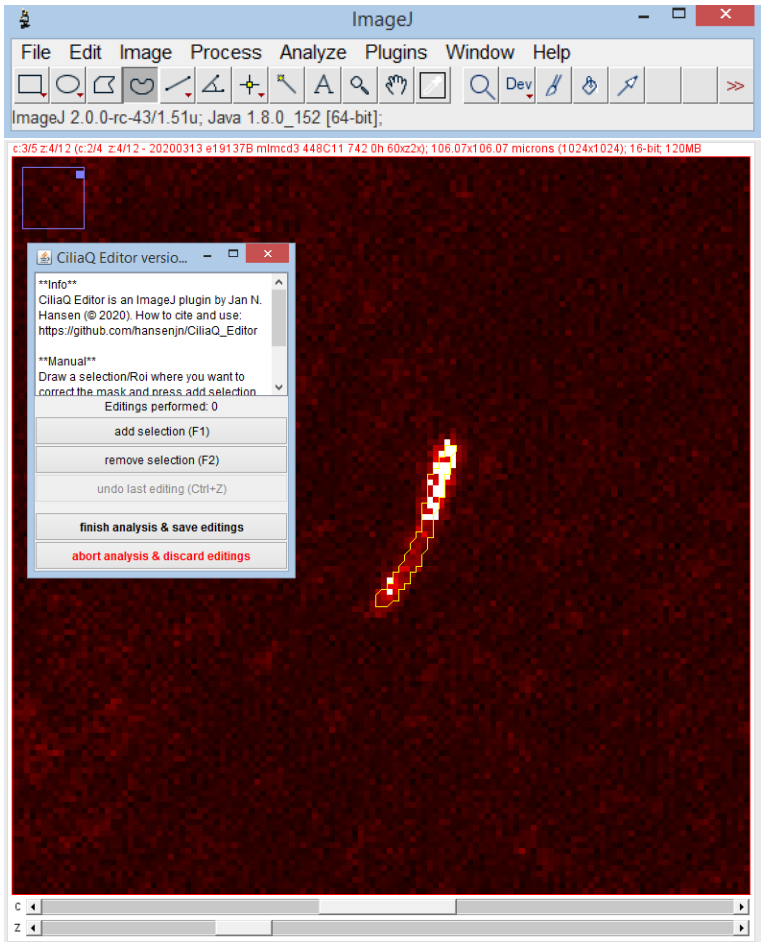


Now search for segmentation errors (move along the z axis – to use the scroll wheel of the mouse to move the z axis press “alt” on the keyboard while scrolling):

- Screen the image for cilia that have been incompletely detected, e.g.:

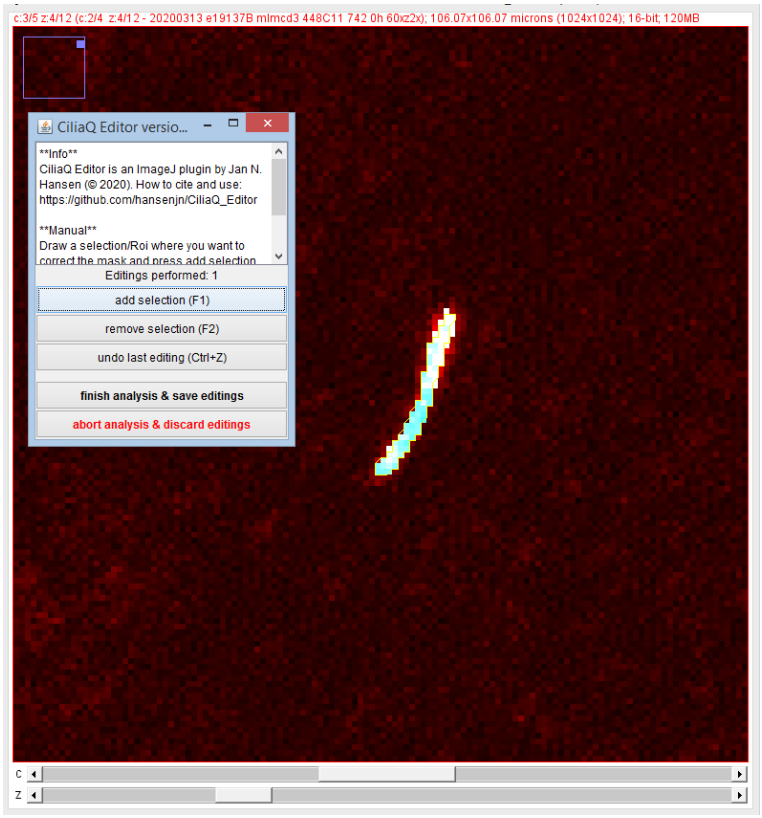


- By drawing a “Freehand” ROI, encircle the areas that belong to the cilium but that have not been detected.

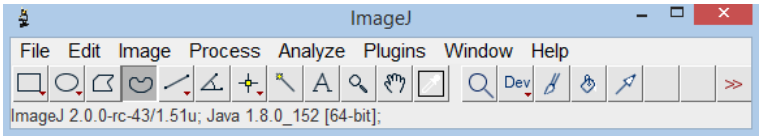


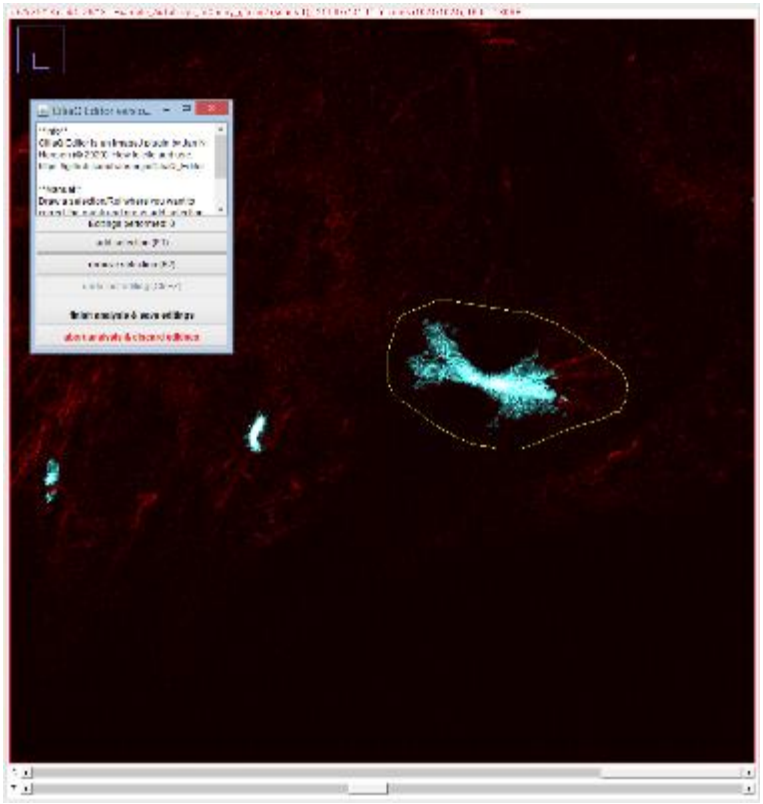
- Add the region to the segmented channel: Either click on “add selection” in the CiliaQ Editor dialog or alternatively, while the image is front-most on your screen,

you can press “F1” on your keyboard. For all voxels within the ROI, CiliaQ Editor will transfer the intensity values from the unsegmented to the segmented channel:

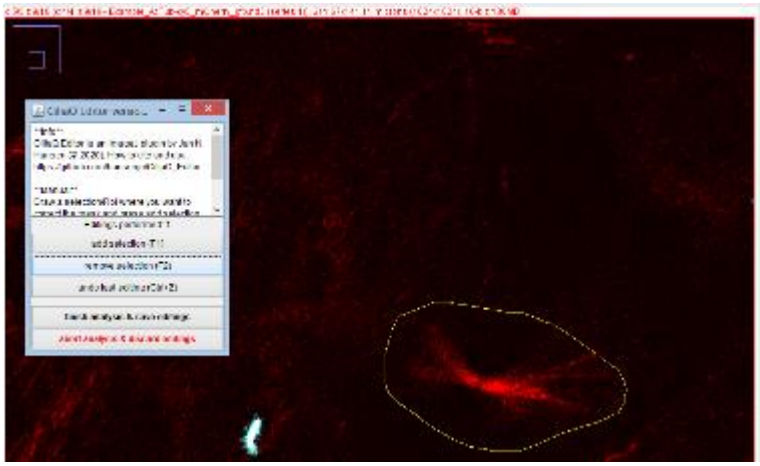


- Screen the image for falsely-as-cilia-detected objects, e.g. a spindle apparatus when detecting cilia using an acetylated tubulin labeling:
    - By drawing a “Freehand” ROI encircle the areas that belong to the cilium but have not been detected.

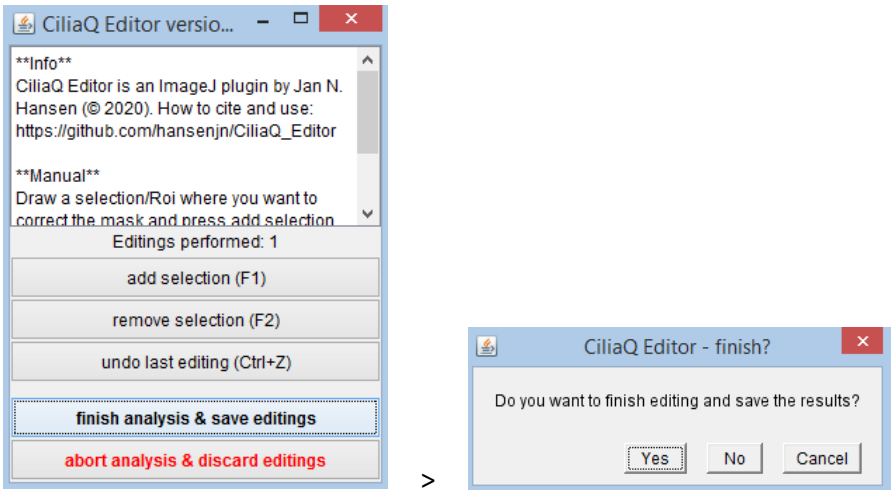




- Add the region to the segmented channel: Either click on “remove selection” in the CiliaQ Editor dialog or alternatively, while the image is upmost on your screen, you can press “F2” on your keyboard. CiliaQ Editor will set all voxels within the ROI in the segmented channel to zero:



When you have corrected the segmentation, click “finish analysis & save editings” and confirm the emerging dialog:



CiliaQ Editor will save the edited image under the image's name plus the suffix "_ed.tif" in the directory from where the image was loaded before CiliaQ Editor processing. In addition, a folder named by the image's name plus the suffix "_ed" is saved in that directory.

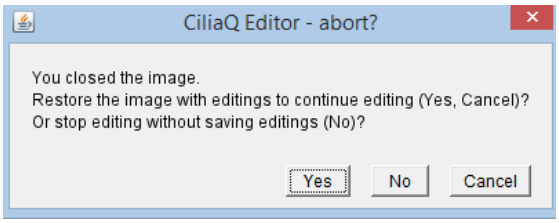
- The image is next subjected to CiliaQ analysis
- As a documentation of the editing process, the folder contains the ROIs that have been used for editing in CiliaQ Editor.

Extra notes:

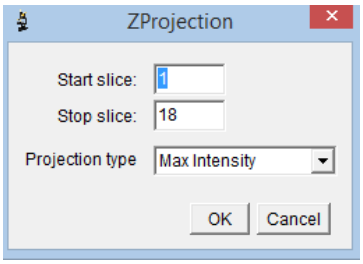
- Clicking "Undo last editing" in the CiliaQ Editor dialog or pressing "Ctrl" and "z" on the keyboard while the edited image is upmost on the screen allows to undo editing operations, operation by operation.
    - Note: there is no "redo"! Once you "undo" there is no way to get back the selection that you "undid".
- CiliaQ Editor adds / removes voxels only in the slice that is selected when pressing "add…" or "remove…". If an object is falsely detected in multiple slices, you need to correct it in multiple slices. Make sure that you are in the right slice when editing the image.
- CiliaQ Editor always transfers voxels from the unsegmented to the segmented channel or removes them from the segmented channel (according to the channel numbers that are initially set before editing). The editing is independent of which channel C is currently selected in the image by the channel bar when pressing "add…", "remove…", or "undo…".

- When closing the image or the CiliaQ Editor dialog before performing any editings, the dialog or the image just closes. If you aim to still edit the image, you need to relaunch CiliaQ Editor, if only the dialog has been closed, or reopen the image and relaunch CiliaQ Editor, if the image has been closed.
- After editing using CiliaQ Editor:
    - If you close the CiliaQ Editor dialog, CiliaQ Editor will ask you whether this was on intention and if you really do not want to save the edited image – if you unintentionally closed the dialog, you may continue editing by clicking "No" or "Cancel".

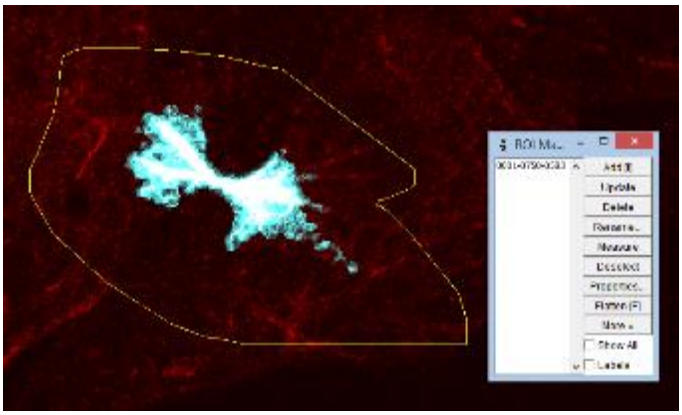
- If you close the edited image, CiliaQ Editor will ask you whether this was on intention – if not, you may restore the edited image and can continue editing or save it before finishing.



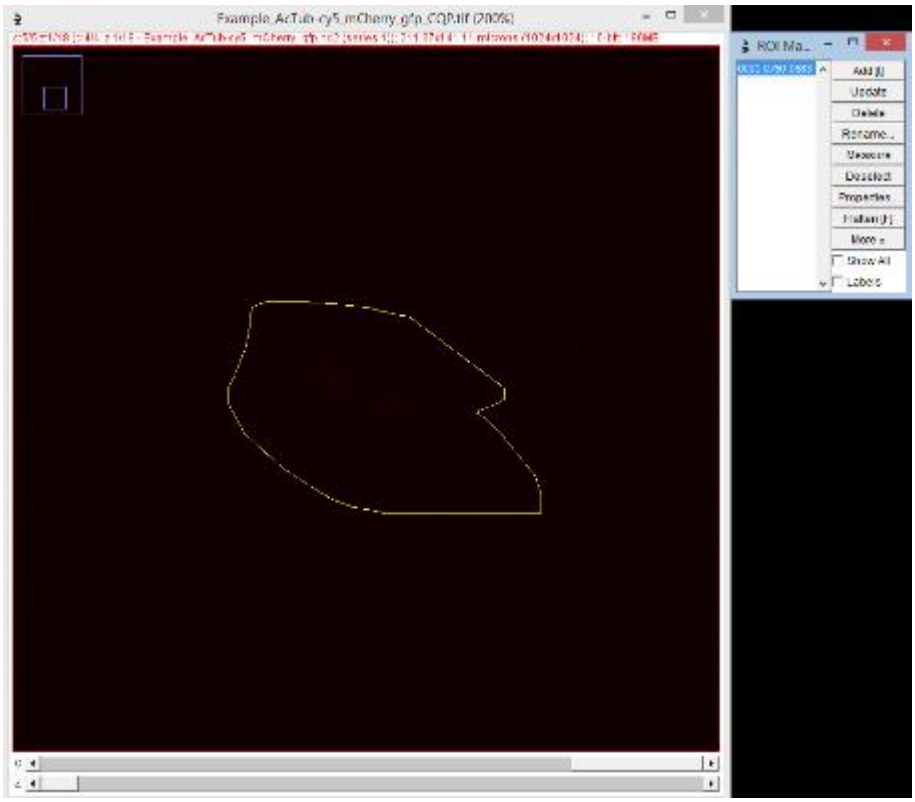
- When analyzing 3D images, it may help to also show a maximum projection of the image to discover incorrectly detected cilia / non-cilia objects. To show a maximum projection, go to **IMAGE > STACKS > Z PROJECT…**, select Projection type "Max Intensity" and click OK.



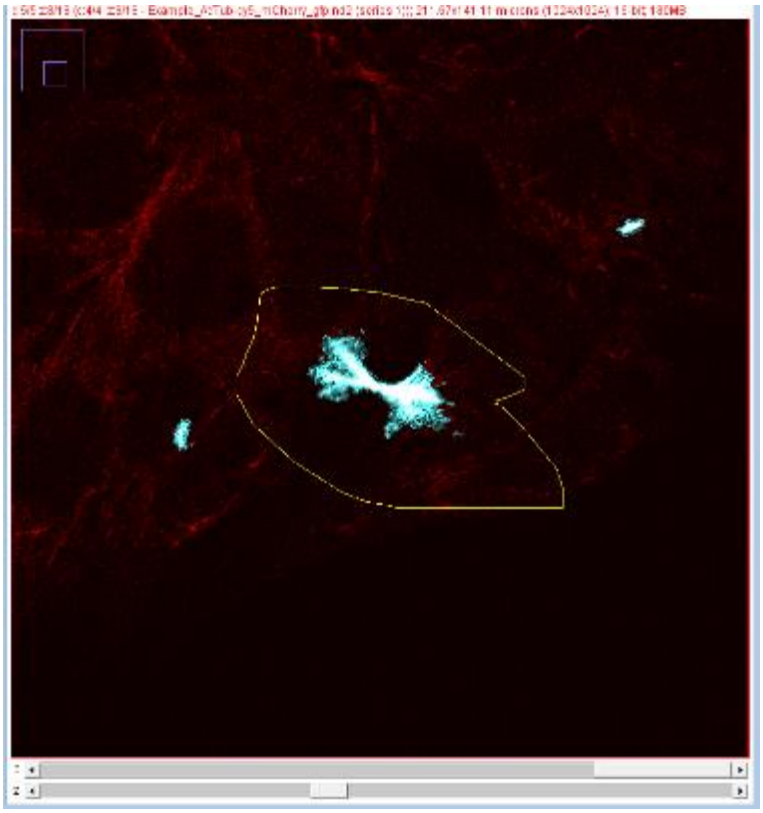
  - Next a maximum projection of the image will pop up.
  - Note that CiliaQ Editor only allows to add or remove regions in the original image. You cannot use the maximum projection for editing, if you transfer ROIs from the maximum projection to the edited image:
    - Press t on the key board to transfer the ROI to the ROI manager



    - Click on the edited image and next click on the ROI in the ROI Manager

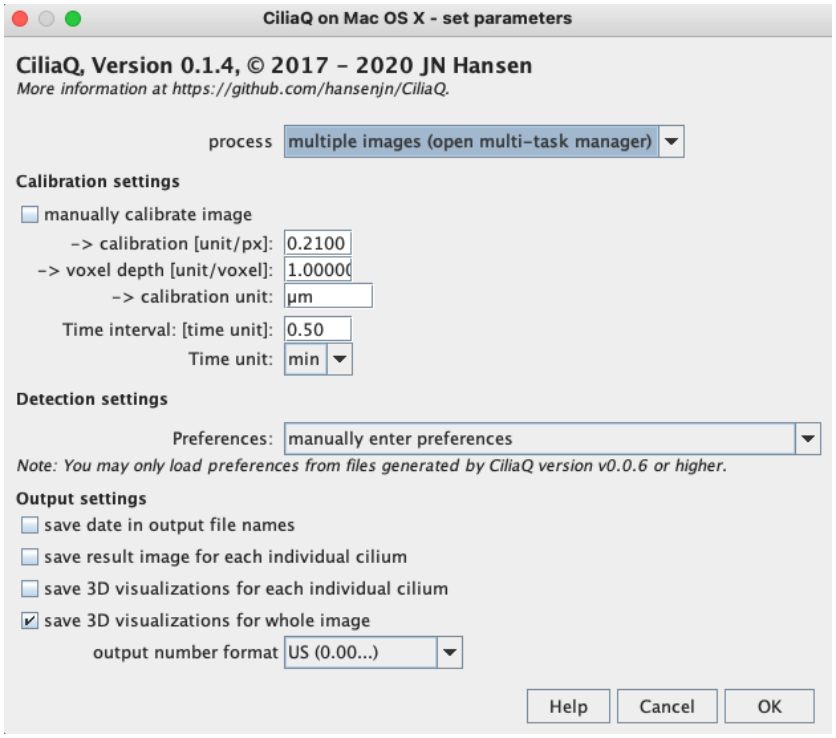


- The ROI is shown in the edited image, but, automatically, the slice bar “Z” will move to slice 1.
- Now, adjust the slice bar to the slice, where you aim to remove / add the selection and add / remove the selection.

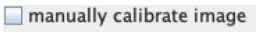


3. Quantify cilia unsing CiliaQ

Analyze the "_CQP.tif" image (from step 1) or, if the CQP image has been edited, the "_CQP.tif" image (from step 2) with CiliaQ: Launch CiliaQ at **PLUGINS -> CILIAQ -> CILIAQ**. A first dialog pops up for adjusting more general settings (NEW in version v0.1.4 – simplified settings dialog):



General settings:

- **Process**: See notes on the process settings for CiliaQ Preparator (section 1).
- **Calibration information**: If the image does not contain any calibration information (you can check the calibration information for an opened active image in FIJI via **IMAGE > PROPERTIES**), check the 

check box and enter the calibration information for all images to be analyzed.

Detection settings:

- The settings for cilium detection will be entered in the next step either by
  - Calling a second dialog:
    

  - Loading the detection settings from a previous CiliaQ analysis by opening an existing CiliaQ output file ("…_CQ<evtl date>.txt"):
    

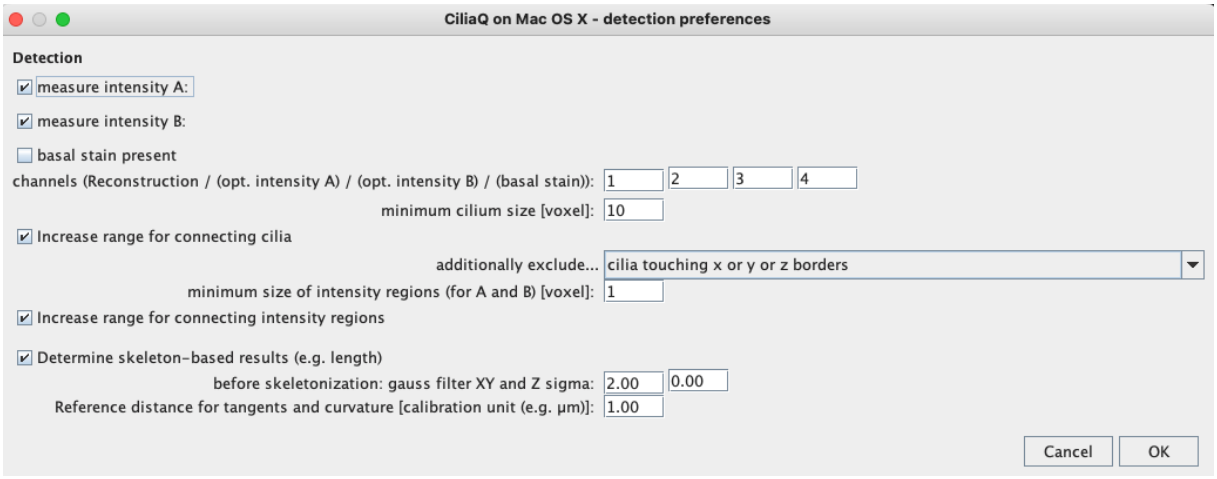
Output settings:

- Modifying the **output settings** allows to retrieve more output files or to add a data suffix in the output file names.
- Please read the section "Output settings and CiliaQ output files" for information on adjusting these settings.
- Note: 3D visualizations can slow down the analysis pipeline because their generation is time-consuming.

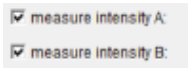
Manually entering detection settings by calling a second dialog:

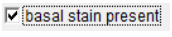
When 

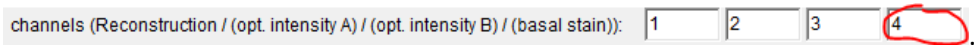
has been selected in the previous dialog, another dialog will be shown where you can modify the detection settings:

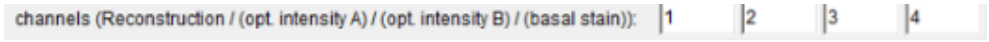


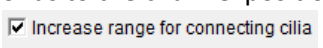
- **Intensity quantifications**: Set here whether you want to quantify any intensity values or cilia coverage in additional channels of the multi-channel image:



- **Basal stain**: If a channel in your image provides a basal stain, e.g. using gamma-Tubulin or centriole markers, check 

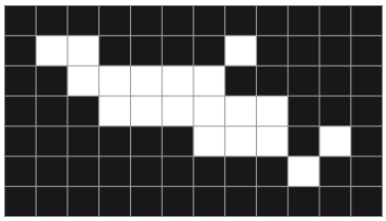
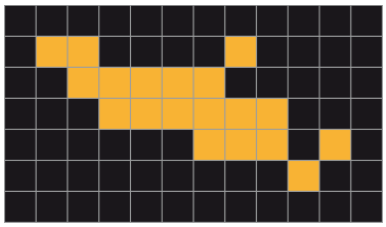
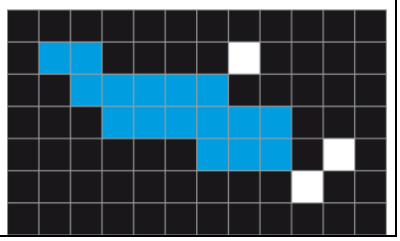
and specify the channel number here (red circle):


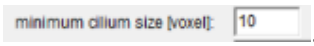
. CiliaQ will then use the intensity information from the basal stain to orient the output intensity profiles from base (length position 0, higher intensity in basal stain) to tip (max length position, lower intensity in basal stain).
- **Channel specification**: Specify here what can be found in which channel of the image:
  - 

    - The setting shown in this image means that
      - the first channel is the segmented channel containing cilia labeling and serves for reconstructing the cilia
      - the second channel will be quantified for intensities and referred to in the output files as intensity “A”
      - the third channel will be quantified for intensities and referred to in the output files as intensity “B”
      - NOTE: here the channel position in the “_CQP.tif” or “CQP_ed.tif” image need to be specified – do not specify the position here that corresponds to the channel position in the raw microscopy file!
- **Object detection / connection:** 

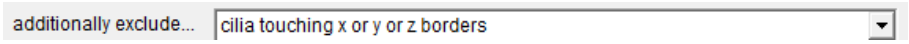
determines which mode is used to connect adjacent voxels to form a cilium object.
  - When the box is checked, CiliaQ will connect a voxel in the image to all direct (blue voxels in image below) and diagonally adjacent voxels (orange voxels in image below) to form a cilium object. If the box is not checked, diagonally adjacent voxels (orange in image below) will not be connected but only directly adjacent voxels (blue).

- The “increase range” function is helpful when the image features a bad signal-to-noise-ratio or when cilia are incompletely labeled and contain small gaps in labeling. However, the “increase range” function also features the disadvantage that noise voxels can be added to cilium objects and can cause erroneous length detection.
- Example:

Segmented channel (white: positive, “ciliary” voxels, black: background voxels)	Detected cilium object when “increase range” option is selected.	Detected cilium object when “increase range” option is not selected.
		

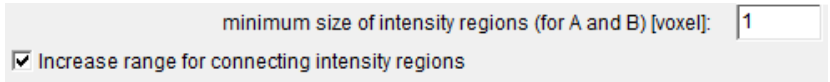
- **Noise filtering**: Estimate the minimum size of a cilium: 

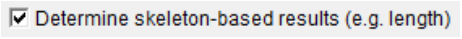
. All cilium objects containing less voxels than the minimum specified here will be considered as noise and removed from the image.
- **Removing incomplete objects at the image borders**: CiliaQ allows to exclude objects that do not lie completely within the imaged field of view. Objects contacting the image borders in x, y, and z are automatically removed if the following option is set as follows:



  - If you analyze a 2D image, change the exclude option to

- **Segmented intensity channels**: CiliaQ allows to analyze channels as “intensity A” or “intensity B” channels that are segmented like the reconstruction channel. This allows to determine the overlap of the ciliary 3D object with objects in these segmented intensity channels. To this end, CiliaQ also offers to perform noise removal and specify whether obejcts detected in additional channels than the reconstruction channel shall be detected with or without the “increase range” function:

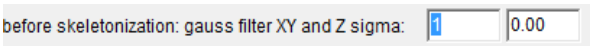


  - Leave the ”minimum size of intensity regions (for A and B)” at 1 if you do not provide segmented intensity channels.
- **Skeleton-based results**: If you select 

, the cilium will be skeletonized by CiliaQ to determine the cilium length and to determine intensity profiles along the central track (“skeleton”) of the cilium.
  - Before skeletonization, a Gauss filter is applied to smoothen the surface of the ciliary object and avoid that the skeleton gets side branches or additional kinks. This

settings needs to be adapted when length estimations do not properly work (e.g. skeletons show side kinks or >1 branches, see output parameters).

- Usually, a Gauss filter is applied only in XY and at sigma values of 1 or 2. Make sure you use the identical sigma value for all data from the same dataset – total length values might slightly vary among results from different sigma settings.



- NEW in version v0.1.4: **Tangent and curvature calculation:** From CiliaQ version v0.1.4 on, CiliaQ calculates and outputs the tangent vector and the local curvature for each ciliary point. This requires to specify how these are calculated.
  - For each ciliary skeleton point, the tangent is formed as the vector from the point at a specific arc length distance upstream on the cilium to the point at a specific arc length distance downstream on the cilium. Next, the tangent vector is normalized to a length of unity.
  - This specific distance of the point for which the tangent vector is calculated to the points used to calculate the tangent vector is set here:

  - From the tangent vectors, the local curvature is determined by retrieving the normalized tangent vectors of the specific upstream point P1 and downstream point P2 (tangents T1 and T2, respectively) and calculating the curvature according to the geometric curvature equation:
    - (T2-T1) / 2 / (arclength distance P1 to P2 on the cilium)
  - Finally, the curvature is signed by the sign of the cross product of T1 and T2.
  - **Note: The parameters tangent vector and curvature are relevant only for specific applications in the physics community – so if you apply CiliaQ for standard applications leave the setting at the default value.**

Loading the detection settings from a previous CiliaQ analysis (NEW in version v0.1.4):

When 

has been selected in the previous dialog, a dialog will be shown where you need to open a "...CQ.txt" or "...CQ_<date>.txt file from a previous CiliaQ analysis. You can import settings from files generated with CiliaQ version v0.0.6 or higher.

If you open a file from a CiliaQ analysis with CiliaQ versions before v0.1.4, you are requested by another dialog to add the missing parameter for **tangent and curvature calculation** (see previous section) **(Note: The parameters tangent vector and curvature are relevant only for specific applications in the physics community – so if you apply CiliaQ for standard applications leave the setting at the default value).**

Output settings and CiliaQ output files

All output files from CiliaQ for a given input image are saved in the directory of the input image and contain the name of the analyzed input image plus the suffix "_CQ" and, eventually, extra-suffixes depending on the type of output file.

CiliaQ always outputs the following files:

- Filename suffix _CQ.txt: A tab-delimited text file containing the analysis settings and results
- Filename suffix _CQ_RP.tif: A copy of the input image after noise filtering the reconstruction (and if applicable the intensity region) channel(s). This image also contains labels of the detected cilia objects (labels correspond to the column ID in the output text files).

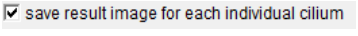
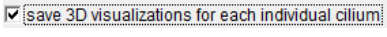
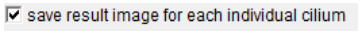
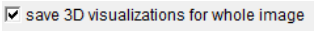
- Filename suffix _CQ_SKL.tif: An Image (stack) showing the detected skeletons, labelled with the analysis IDs of the respective cilium objects.
- Filename suffix _CQs.txt: A tab-delimited text file containing only the results lines from the file with suffix _CQ.txt, without table caption. Retrieve the caption for that table from the table in the file with suffix _CQ.txt.
- NEW in version v0.1.4: Filename suffix _CQl.txt: A tab-delimited text file containing a table with all ciliary skeleton points for all different cilia. For each point several parameters are output:

Directory	File name	ID	Point-ID	T [frames]	X [micron]	Y [micron]	Z [micron]	Arc length [micron]	Tangent vector X [micron]	Tangent vector Y [micron]	Tangent vector Z [micron]	Curvature [micron^(-1)]	Intensity A	Intensity B	Intensity A (normalized to reconstruction channel)	Intensity B (normalized to reconstruction channel)
/Users/jar	Test_CQP.tif	1	1	0	4.955924	17.990682	1.811333	0	1	0	0	0	1317.658085	960.437812	0	0
/Users/jar	Test_CQP.tif	1	2	0	5.023813	17.990682	1.811333	0.067889	1	0	0	0	1262.324752	1011.881669	0	0
/Users/jar	Test_CQP.tif	1	3	0	5.091703	17.990682	1.811333	0.135779	0.777632	0	-0.628719	0	1206.991418	1063.325525	0	0
/Users/jar	Test_CQP.tif	1	4	0	5.159592	17.990682	1.646667	0.313891	0.38116	0	-0.924509	0	1292.992678	1134.43819	0	0
/Users/jar	Test_CQP.tif	2	1	0	10.930188	42.091408	2.140667	0	0.707107	0.707107	0	0	2264.937771	1700.916978	0	0
/Users/jar	Test_CQP.tif	2	2	0	10.998078	42.159297	2.140667	0.09601	0.707107	0.707107	0	0.891457	3011.020785	2068.003541	0	0
/Users/jar	Test_CQP.tif	2	3	0	11.065967	42.227187	2.140667	0.19202	0.707107	0.707107	0	0.891457	3182.137939	1981.458393	0	0
/Users/jar	Test_CQP.tif	2	4	0	11.133856	42.295076	2.140667	0.28803	0.613869	0.613869	-0.496316	1.301903	3448.144003	2059.135501	0	0
/Users/jar	Test_CQP.tif	2	5	0	11.201746	42.362965	1.976	0.478642	0.356165	0.356165	-0.863883	1.357777	3808.951876	2300.968143	0	0
/Users/jar	Test_CQP.tif	3	1	0	12.831091	11.676971	1.811333	0	0.894427	-0.447214	0	-2.380543	1170.328826	887.666445	0	0
/Users/jar	Test_CQP.tif	3	2	0	12.89898	11.676971	1.811333	0.067889	0.894427	-0.447214	0	-2.380543	1133.88516	1030.555692	0	0
/Users/jar	Test_CQP.tif	3	3	0	12.966869	11.609082	1.811333	0.163899	0.777632	0	-0.628719	-1.828857	1181.663733	1159.333825	0	0
/Users/jar	Test_CQP.tif	3	4	0	13.034759	11.676971	1.646667	0.354512	0.356165	0.356165	-0.863883	-1.573416	1193.665954	1236.336568	0	0
/Users/jar	Test_CQP.tif	4	1	0	14.460435	26.680522	2.140667	0				0	1371.989595	837.991569	0	0

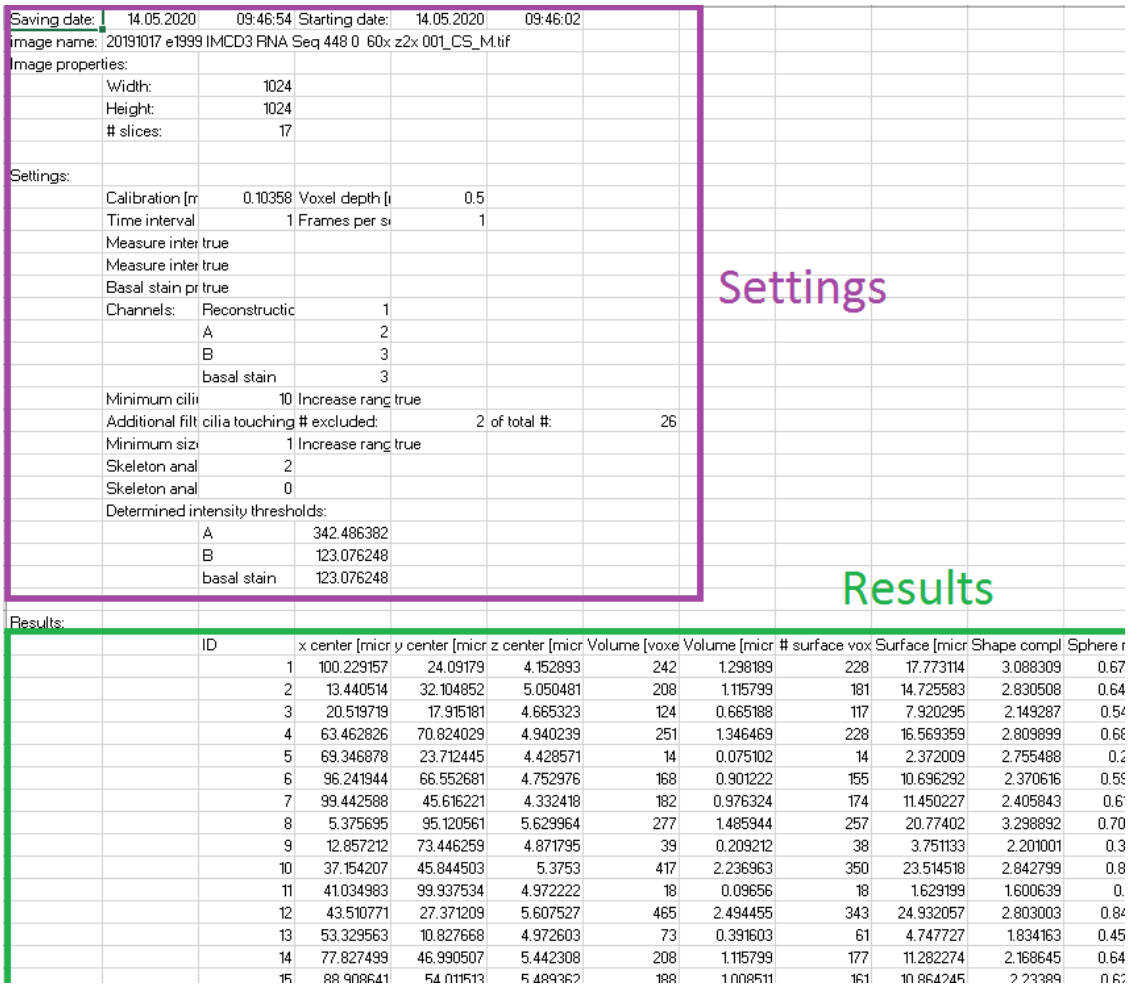
  - The table is formatted in a way that it can be readily imported into R and used for plotting in R, e.g. with ggplot2.

Modifying the **output settings** allows to retrieve more output files or a data suffix in the output file names:

- **Save date**: Saving the date in the output file names after the "_CQ"-suffix avoids that a CiliaQ analyses overwrites already existing CiliaQ results files from a different CiliaQ analysis on the same image. For example, when you try different CiliaQ settings on the same image and want to compare the results obtained by different settings you should include the date in output file names to avoid overwriting.

- **Individual cilium images**: A folder, containing stack images for each detected cilium is output (if  is selected) and 3D visualizations (if  is selected), is saved. The folder name corresponds to the name of the analyzed image name plus suffix "_CQ".
  - Note: if you analyze time-lapse images, check  because otherwise you will not obtain text files containing time-resolved results for individual cilia. The general results file (with ending "_CQ.txt" only contains time-averages of the results).
- **3D visualization for whole image**: If  is selected, 3D visualization of the noise-filtered reconstruction channel (filename suffix _CQ_RP_3D.tif) and of the detected skeletons (filename suffix _CQ_SKL_3D.tif) are output.

The tab-delimited text files can be copy-pasted into excel for further analysis



Saving date:	14.05.2020	09:46:54	Starting date:	14.05.2020	09:46:02						
Image name:	20191017 e1999 IMCD3 RNA Seq 448 0 60x z2x 001_CS_M.tif										
Image properties:											
	Width:	1024									
	Height:	1024									
	# slices:	17									
Settings:											
	Calibration [m	0.10358	Voxel depth [	0.5							
	Time interval	1	Frames per s	1							
	Measure inter	true									
	Measure inter	true									
	Basal stain pr	true									
	Channels:	Reconstructic	1								
		A	2								
		B	3								
		basal stain	3								
	Minimum cili	10	Increase rang	true							
	Additional filt	cilia touching	# excluded:	2	of total #:	26					
	Minimum siz	1	Increase rang	true							
	Skeleton anal	2									
	Skeleton anal	0									
	Determined intensity thresholds:										
		A	342.486382								
		B	123.076248								
		basal stain	123.076248								
Results:											
		ID	x center [micr	y center [micr	z center [micr	Volume [voxe	Volume [micr	# surface vox	Surface [micr	Shape compl	Sphere r
		1	100.229157	24.09179	4.152893	242	1.298189	228	17.773114	3.088309	0.67
		2	13.440514	32.104852	5.050481	208	1.115799	181	14.725583	2.830508	0.64
		3	20.519719	17.915181	4.665323	124	0.665188	117	7.920295	2.149287	0.54
		4	63.462826	70.824029	4.940239	251	1.346469	228	16.569359	2.809899	0.68
		5	69.346878	23.712445	4.428571	14	0.075102	14	2.372009	2.755488	0.2
		6	96.241944	66.552681	4.752976	168	0.901222	155	10.696292	2.370616	0.59
		7	99.442588	45.616221	4.332418	182	0.976324	174	11.450227	2.405843	0.6
		8	5.375695	95.120561	5.629964	277	1.485944	257	20.77402	3.298892	0.70
		9	12.857212	73.446259	4.871795	39	0.209212	38	3.751133	2.201001	0.3
		10	37.154207	45.844503	5.3753	417	2.236963	350	23.514518	2.842799	0.8
		11	41.034983	99.937534	4.972222	18	0.09656	18	1.629199	1.600639	0.
		12	43.510771	27.371209	5.607527	465	2.494455	343	24.932057	2.803003	0.84
		13	53.329563	10.827668	4.972603	73	0.391603	61	4.747727	1.834163	0.45
		14	77.827499	46.990507	5.442308	208	1.115799	177	11.282274	2.168645	0.64
		15	88.908641	54.011513	5.489362	188	1.008511	161	10.864245	2.23389	0.62

Settings

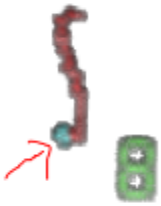
Results

Scrutinize results by eye

Load the output images into FIJI, check for falsely-detected cilia and remove the respective lines from your analysis results. This allows to eventually delete results in the analysis sheet that correspond to non-ciliary particles that were falsely detected. You can also check out the 3D representation to filter out falsely detected objects.

3D output of the detected skeletons (skeleton in red, IDs in green):

- The labeling of the skeleton shows which endpoint in intensity profiles corresponds to arc length 0. Arc length 0 is represented as a cyan dot. This allows to manually orient the profiles later (you might want to reverse some of them if the arc length = 0 point is positioned at the ciliary tip).



Description of output parameters

Please note that many output parameters are very technical and not relevant for the standard user. Important parameters are (if applicable):

- For measuring intensity levels in the cilium (choose the parameter that is most stable in your images):
    - Average intensity
    - Average intensity of the 10% of voxels with highest intensities
    - Average intensity on centerline
- Cilia length
- Cilia bending index
- Intensity profiles

A detailed description of all parameters follows here.

x, y, z center

- The center of the cilium in x, y, and z

Volume

- The ciliary volume is given in voxel and in units (i.e. micron³)

surface voxels

- Number of voxels that are located at the ciliary surface

Surface

- The surface of the ciliary object.
- Calculated by summing up the surfaces of all surface voxels
- The surface of a voxel is determined as the sum of
    - #sides without neighbor in x and y direction * voxel width * voxel depth
    - #sides without neighbor in z direction * voxel width * voxel width

Shape complexity index

- Is calculated by comparing the surface area of the cilium to the surface of a sphere containing the same volume as the cilium
- The smaller the more spherical the shape
- The higher the more aspherical the shape

Sphere radius

- Radius of a sphere containing the same volume as the cilium

Maximum span:

- THIS PARAMETER IS NOT YET IMPLEMENTED AND THUS DOES NOT PROVIDE RESULTS

A/B: Colocalized volume

- Only usable, if an intensity channel that has been binarized / semi-binarized / background-removed is provided
- Describes the overlap of the ciliary object with objects in the background-removed intensity channel
- Provided in units (e.g. micron³) and as percent of the individual ciliary volume

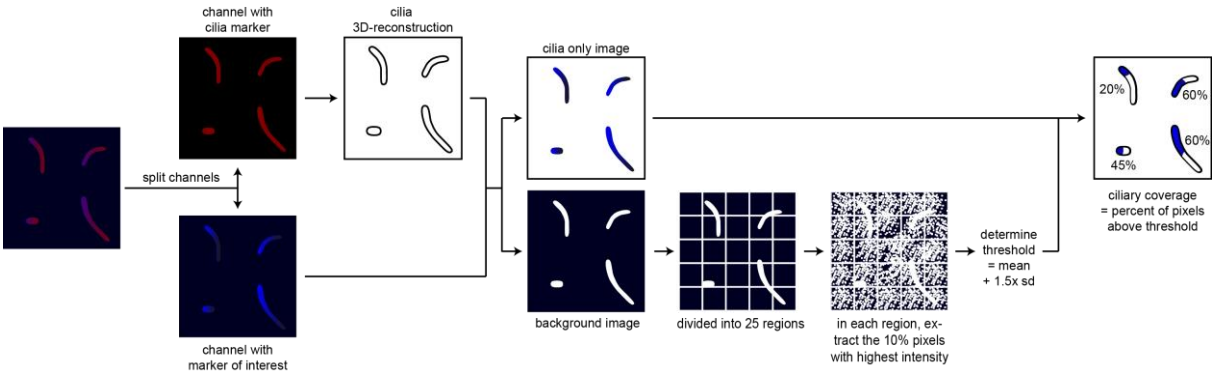
A/B: Colocalized compared to BG volume

- This parameter describes the ciliary enrichment of a stained or fluorescent protein compared to the expression level in the soma.

- A threshold is calculated as follows:
  - Divide the entire image into 25 cuboids (5x5 in xy)
  - Collect the 10% highest voxels from each cuboid
  - Threshold = Average + 1.5x standard deviation of all collected voxel intensities
  - The thresholds are individually determined for each image and are also output in the settings section:

Determined intensity thresholds:	
A	342.486382
B	123.076248

- The threshold is used to determine the % of ciliary voxels that lie above the threshold and thus, contain higher intensities than the soma intensity level.
- Schematic overview for this parameter:



Minimum, Maximum, Average, SD of intensity

- Provided for the reconstruction channel, the A, and the B intensity channel
- Quantifies the minimum, maximum, average, and the standard deviation (SD) of the intensities belonging to the ciliary object in the respective channel (reconstruction channel, channel A, channel B)

Average intensity of the 10% of voxels with highest intensities

- Provided for the reconstruction channel, channel A, and channel B
- The intensities of the voxels belonging to the cilium are sorted and only the 10% voxel with the highest intensities are extracted. The average of the extracted voxels is output.
- This parameter can be used to determine the absolute ciliary localization of a protein. It is less noise-sensitive than the parameter “Average intensity”, because falsely as ciliary voxels detected voxels do not count (they usually have a lower intensity than ciliary voxels and thus are not contained in the highest 10%).

Skeleton-based parameters

Skeleton-based parameters are only output if ☑ Determine skeleton-based results (e.g. length) was enabled.

of found skeletons (quality parameter)

- If above 1 the cilium might be incompletely or incorrectly detected > check by eye.
- If above 1 all skeleton-based parameters should might be incorrect > check by eye.

branches (quality parameter)

- If above 1, the skeleton could be incorrect and not span the entire cilium > check by eye.

Tree length [micron] (quality parameter)

- This parameter should not largely deviate from the next parameter cilia length. If it largely deviates, scrutinize the skeleton by eye to confirm whether it is really correct.

Cilia length [micron] (largest shortest path of largest skeleton)

- The length of the cilium.
- If cilia are extremely short and in turn, spherical, no skeleton might be determined and the output of ciliary parameters for these short cilia might be blank.
- If you nevertheless want to include the short cilia into your calculations, fill the blank "cilia length" values with the length of a voxel length and also add a voxel length to all output values for the "cilia length".

Orientation vector

- Given as x-, y-, and z-component of the vector from the first to the last skeleton point

Cilia Bending Index

- Arc length of the skeleton divided by the Euclidean distance from first to last skeleton point

Integrated A/B intensity

- The sum of all intensities in the intensity profile (see below)
- This parameter is less noise sensitive than the average parameters based on all intensities in the cilium, because the intensities in the intensity profile are independent of the width of the ciliary object and thus independent of cilium detection algorithms.

Average A/B intensity on center line

- The average of all intensities in the intensity profile (see below)
- This parameter is less noise sensitive than the average parameters based on all intensities in the cilium, because the intensities in the intensity profile are independent of the width of the ciliary object and thus independent of cilium detection algorithms.

A/B: Colocalized on centerline compared to BG volume

- This parameter describes the ciliary enrichment of a stained or fluorescent protein compared to the expression level in the soma
- A threshold is calculated as presented above
- The threshold is then used to determine the % of intensity values in the intensity profile (see below) that lie above the threshold and thus, contain higher intensities than soma intensity
- This parameter is less noise sensitive than the colocalized parameters based on all intensity in the cilium, because the intensities in the intensity profile are independent of the width of the ciliary object and thus independent of cilium detection algorithms.

Intensity profiles

- The profiles describe the intensity distribution along the ciliary center line (detected skeleton) for each channel (e.g. intensity A):

Profile A (arc length step: 0.103580)							
104.240825	122.716006	98.916934	108.310102	118.666667	91.161959	78.950129	81.65

- If you do not provide a basal stain but still want to assure that arc length 0 corresponds to the base, you need to investigate the skeletons by eye and eventually, inverse individual profiles manually (see above).

Arc length (NEW in version v0.1.4) – available only in the CQI.txt output file

- The arc length position of each point on the ciliary centerline (skeleton) is calculated as explained in https://www.mdpi.com/2073-4409/8/1/10.

Tangent vector (NEW in version v0.1.4) – available only in the CQl.txt output file

- For each ciliary skeleton point, the tangent vector is determined as the vector from the point at a specific arc length distance upstream on the cilium to the point at a specific arc length distance downstream on the cilium (the specific distance is set by the user, see above).
- The tangent vectors are normalized to a length of unity.

Curvature (NEW in version v0.1.4) – available only in the CQl.txt output file

- The curvature at a specific point on the cilium is determined based on the equation for the geometric curvature.
- More specifically, the curvature is determined from the normalized tangent vectors of an upstream point P1 and downstream point P2 (tangents T1 and T2, respectively) as:
    - (T2-T1) / 2 / (arclength distance P1 to P2 on the cilium)
- Finally, the curvature is signed by the sign of the cross product of T1 and T2.

Intensity threshold

- Documents the intensity thresholds determined for the channel A and B for determining the parameters “colocalized … compared to BG volume” for the respective image.

4. Explore data sets

To explore data sets analyzed with CiliaQ programmatically, have a look at the following R script: https://github.com/sRassmann/ciliaQ-output-joinR.