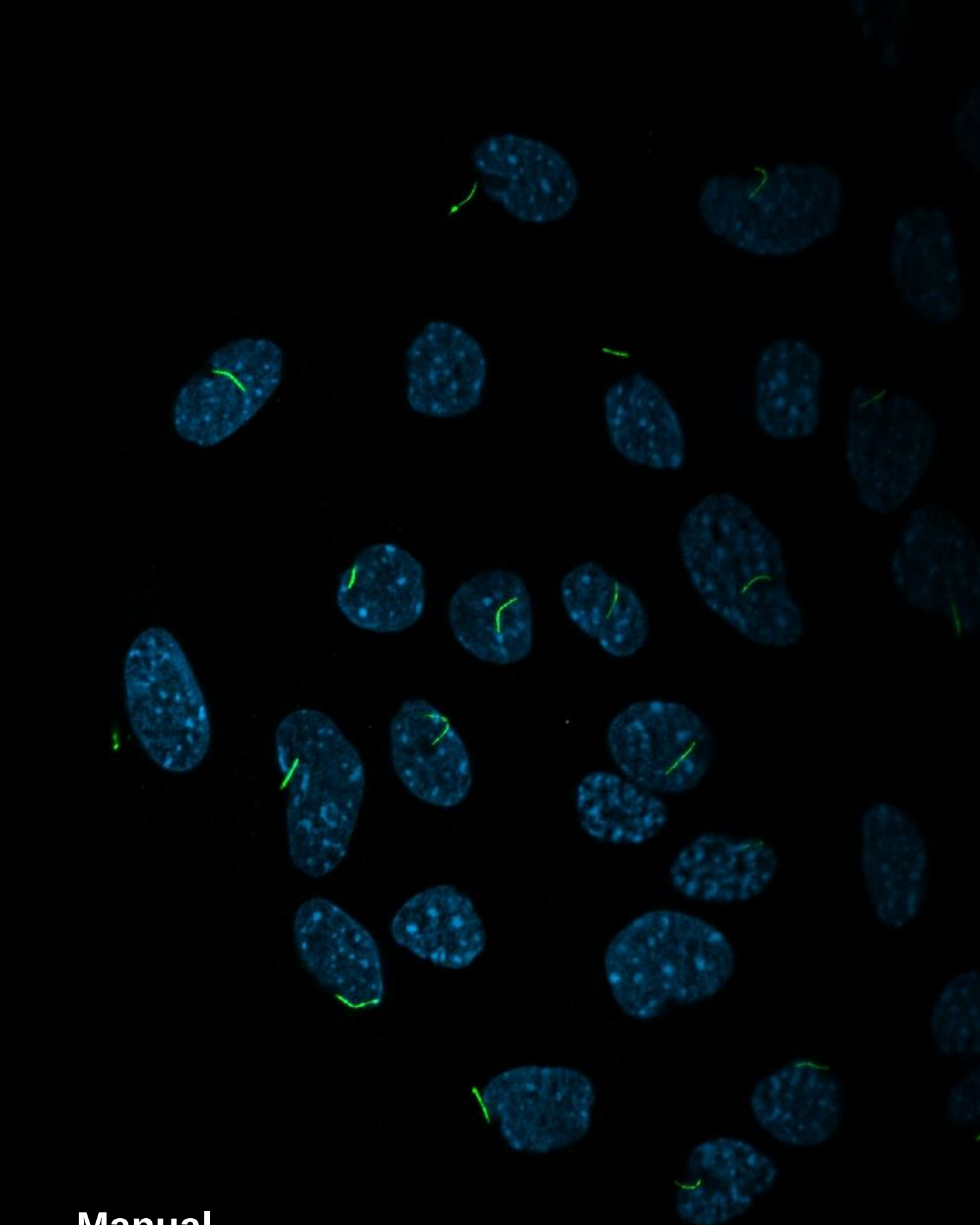

Manual
Automated Cilia Measuring

Author: Cenna van Manen
Based on manual written by Ideke Lamers on 2014-10-31
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Date: 04-2019

CONTENT

DISCLAIMER

INTRODUCTION

This document describes how to perform automated measuring of cilia length. It describes a small pipeline of 5 steps that can be followed to measure cilia lengths in a large number of microscopic images. In this manual ZEN software is used to acquire the images, however the pipeline can be adjusted for other imaging platforms and software. Before imaging larger sets of material to analyze with this pipeline there are a few things to keep in mind.

BEFORE IMAGING

To optimize your analysis there are a few things to keep in mind before starting the microscopic imaging. First, the quality of your imaging determines the quality of your analysis. The FIJI macros use several filters to optimize analysis, but these can not increase the quality of your images. It is important that you optimize your ciliary and nuclear staining for your specific tissue or cell type before imaging larger sets of material for automated analysis. In addition, make sure that your ciliary antibody or marker shows a whole cilium without any interruptions. Otherwise, the macro will measure the interrupted cilium as multiple short cilia instead of one long cilium.

Second, when you are optimizing your exposure settings during imaging it is advisable to slightly over expose your ciliary channel. The reason for this is the same as for the picking of the ciliary antibody or marker, that the macro might interpret one long cilium as several short cilia. Slightly over exposing the ciliary channel will help circumvent breaking of the cilia during automated analysis. Remember that these do not have to be the pretty images you would in presentations, but they rather have the serve the purpose of correct automated analysis.

Third, in some tissues or cell types the cilia occur in one microscopic plane, while in others you have to scroll through your slides to see all the cilia and nuclei. In case of the later, a Z- stacks is required to make sure you analyze all the cilia in one microscopic view and circumvent an analysis bias. The optimal distance between the stack images has to be determined per material type, so that all the cilia and nuclei are imaged. Nonetheless, if you can image all the cilia and nuclei in a single microscopic plane it is not advised to make stacks, due to the extra time and data storage that is required. For the same reason it is not recommended to use a very small distance between the stack images when making Z-stacks.

Last, this pipeline only allows for measuring of cilia that are recognized as having a line structure. Cilia that occur as dots will be recognized as background and will not be measured as being cilia. This is independent of the size of the dot. Images that do not contain any object that is recognized by the macro in either the nuclei or the cilia channel cannot be analyzed either.

THE PIPELINE

The ciliary measuring pipeline is divided in five steps. In the first step the ZEN imaging software is used to create the desired input for FIJI automated analysis. FIJI (FIJI is just imageJ) is a open source software package designed for image analysis in biological research. The pipeline contains three different macros for FIJI for your analysis.

In the second step of the pipeline FIJI is used to split the microscopic channels. Furthermore, the user has the option to merge the microscopic planes of Z-stacks, if these were used for imaging. This macro creates the input for the third and fourth step.

In the third step a second FIJI macro is used to measure the length of the cilia. Measuring of the cilia can be performed by a set of user defined variables to allow optimal analysis of the microscopic images. It is advised to also count the number of nuclei in each image in step four. This step allows you to automatically count the number of cells in your sample by counting the number of cells positive for a certain nuclear marker. These data can be used to either calculate the percentage of ciliated cells for cells that only have a primary cilium or the number of cilia per cell for multiciliated cells.

The last step is combining the acquired data, data imaging and data analysis to allow you to answer your research question. Since the method of data analysis is highly dependent on the research question this chapter only contains some descriptions and tips for your own analysis.

The five different steps in the pipeline can be followed as described above, or they can be used individually for other purposes or to personalize the pipeline. Therefore, the required input and acquired output for each step are described in each chapter.

REQUIRED SOFTWARE

This pipeline is based on the free to download software packages of ZEN and FIJI. The installers of these packages can be downloaded from the website of the distributor. The version of the software that was used to set up this pipeline is given below. It is possible that the use of a newer or older version results in errors or incorrect analysis. If step 1 of the pipeline is skipped it is no longer required to install the ZEN software. For the last step of the analysis it is possible to use the supplemented python script. This will require an additional installation of Python.

Zen

For setting up the analysis pipeline the 2.3 lite version of the Blue edition for Windows was used. The installer can be requested at the Carl Zeiss Microscopy website https://www.zeiss.com/microscopy/int/downloads/zen.html. If you fill in the 'Download Zen Lite' request form on the right and fill in your email address the downloader will be send to you. If you filled in 'Academic' as 'Customer Area' the software will remain free to use after installation. During installation you can choose to only install the 'Blue edition', since this is the program that will be used to create your image output at step 1.

FIJI

For setting up the analysis pipeline the 1.52e version for 64-bit Windows of FIJI was used. This software package of ImageJ can be downloaded at the FIJI website https://imagej.net/Fiji/Downloads. A specific version of the software package can be requested from the authors if that is required for analysis.

Python

For the script that is provided Python 2.7.14 was used. The python installer can be downloaded at https://www.python.org/download/releases/2.7/.

CHAPTER 1. - APOTOME RAW CONVERTS

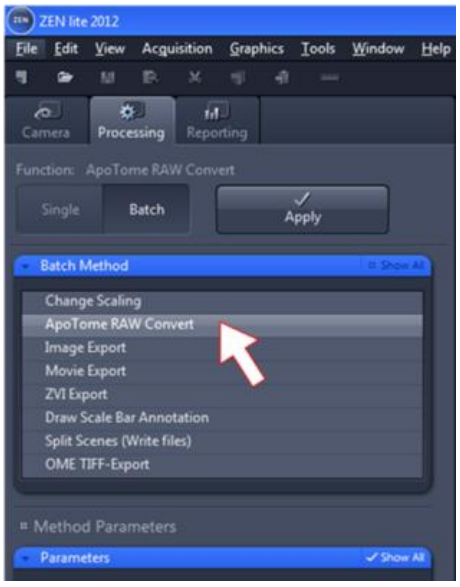
The first step in the pipeline is to convert the acquired images so that they can be used for the automated analysis with FIJI. This step is only required if the images were acquired using the ZEN software and the ApoTome. If this is not the case, proceed to chapter 2.

The ***input*** of this step consists of the .CZI images that were acquired using the Zen software in combination with the ApoTome. When the ApoTome is used the microscope takes several images for each plane and each channel across a preset grit. This allows for the software to distinguish background from signal using complex calculations. In the original .CZI files these calculations are not yet applied, however if you open your image in ZEN it will do the calculations and show the image with calculations applied. These calculations result in a more crisp image, however it is important to keep in mind that these images show an altered representation of the true image. In order to use these calculated images in FIJI the calculations need to be incorporated into the .CZI file. This is what is called a ApoTome RAW conversion. The ***output*** of this step are ApoTome RAW convert files with a .CZI extension that can be used as input for the FIJI macro described in chapter 2. The ApoTome RAW convert files can be obtained applying the following steps:

1. Open the Blue edition of the Zen software, go to the tab 'Processing' and select 'Batch'.

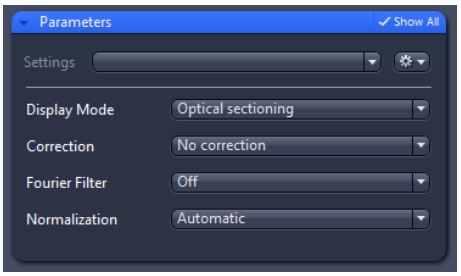
2. Click on 'add' and open all the .CZI files you want to process.

3. Select all the files in the list and select 'ApoTome RAW convert'.

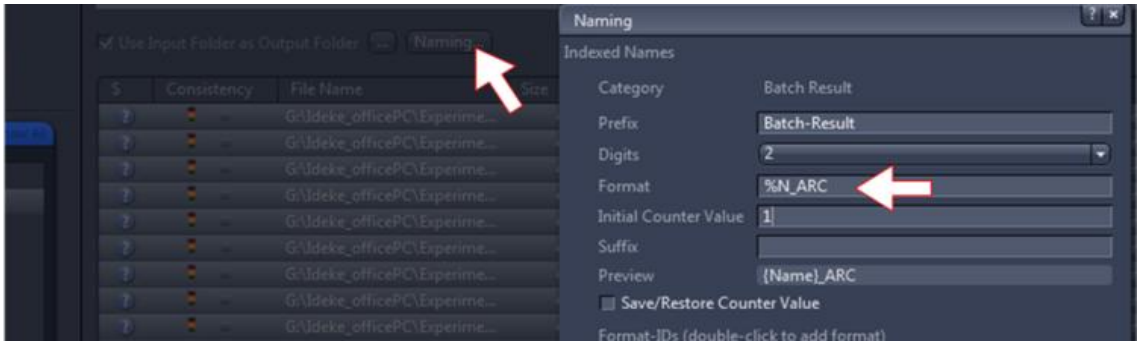


4. Select the following options on the left side:
   - Display mode: Optical sectioning
     This option determines which calculation will be performed. 'Conventional fluorescence' will show the image as if no ApoTome was used, 'Raw data' is without ApoTome calculations.
   - Correction: No correction
     This option can be changed if bleaching occurred in the image, to correct for the bleaching effect. The option 'Phase Errors' can be used if stripes occur in the picture. This is sometimes seen when the signal of one of the channels is too low for correct calculations. For more information see supplemental information.
   - Fourier filter: Off
     This filter can be altered to correct the background noise based on the Fourier Transform principle. This function is most applicable to Z-stacks.
   - Normalization: Automatic
     Set this option to 'Clip' to set the channel data as implicated in the saved images.

   If you do not see all these options, make sure the box 'Paramaters', 'Show All' is checked.

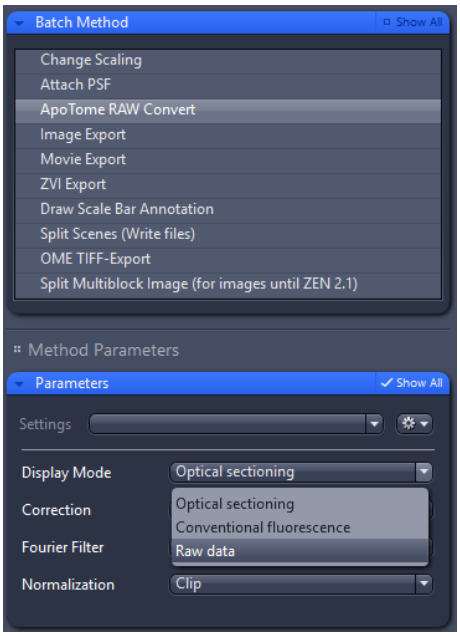


5. Make sure you do not overwrite the original files, by choosing one of the following options:
   - Uncheck the box 'Use Input Folder as Ouput Folder' to save the ApoTome convert files into a new folder, which can be chosen by clicking on '…'.
   - Rename the obtained .CZI files by clicking on the tab 'Naming…', for instance change the name for all files to: **'ARC_%N'** (places 'ARC_' in front of every new file, easy to separate them) can be done as indicated below.



6. Click ‘Apply’ to process all the selected images. The blue bar on the bottom will indicate the progress. The new files are .CZI files on which the ApoTome calculations have been applied, so that the images can be used by FIJI for the analysis.

Of note, it is also possible to use the true image instead of the calculated image for your analysis, even when you already used the ApoTome to make the images. This can be done by choosing the option ‘Conventional fluorescence’ at ‘Display mode’ in step 4. It is also possible to export the original photo by selecting ‘Raw data’, however mind that this image will contain the ApoTome shutter stripes.



CHAPTER 2 – PREPARING IMAGING FOR ANALYSIS

In order to analyze the images in step 3 and 4 the acquired images need to be adapted in several ways. This adaption is done using the 'Channel splitter' macro in FIJI. This macro has three functions. The first function of the macro is to split the channels per image. The macro will make a new folder per channel to store the new images.

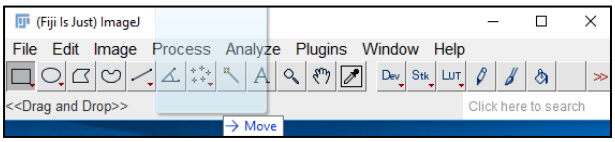
The second function of the macro is to convert the Z-stacks into single-plane image. This conversion uses the highest grey value of each pixel across the Z-stack and converts the data into a single-plane image. If the .CZI images are normal microscopic images, this function will not be skipped.

The third function is that the macro will convert the .CZI files into .tif files. This conversion can also be done using ZEN, but this is not recommended. For more information on the ZEN conversion, see the supplemental information.

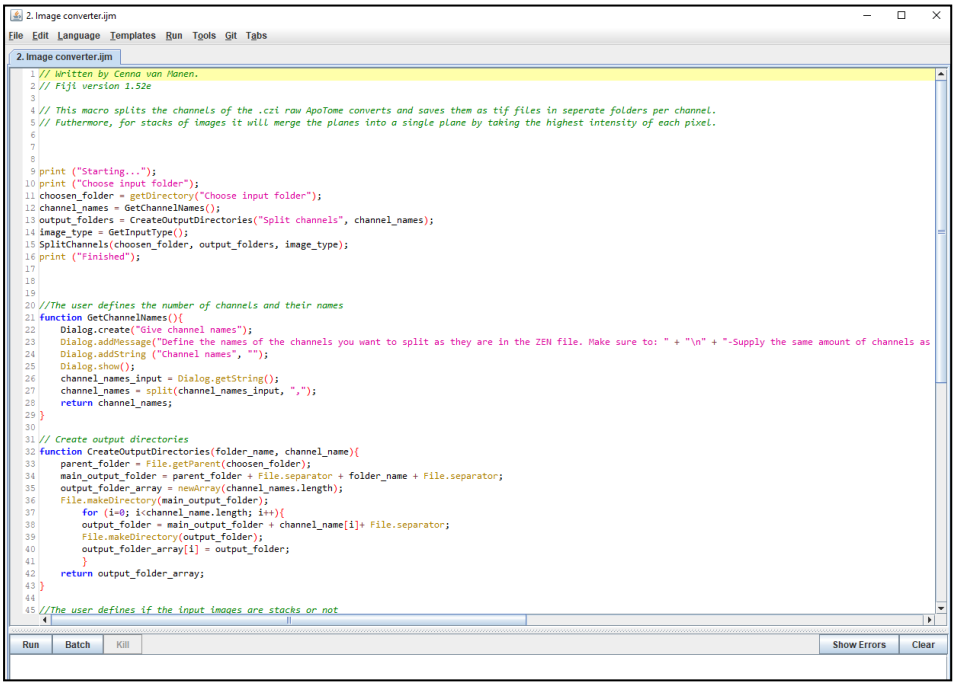
The ***input*** of this step consist of the .CZI files that are obtained in chapter 1. It is also possible to directly use the .CZI files that were obtained during imaging at the microscope, but only in case the ApoTome was not used during imaging. The ***output*** of this step will be a new folder that contains all the . tif files per channel. Convert the .CZI files by applying the following steps:

1. Start the FIJI software. A small window '(Fiji IS Just) ImageJ' will appear on your screen.

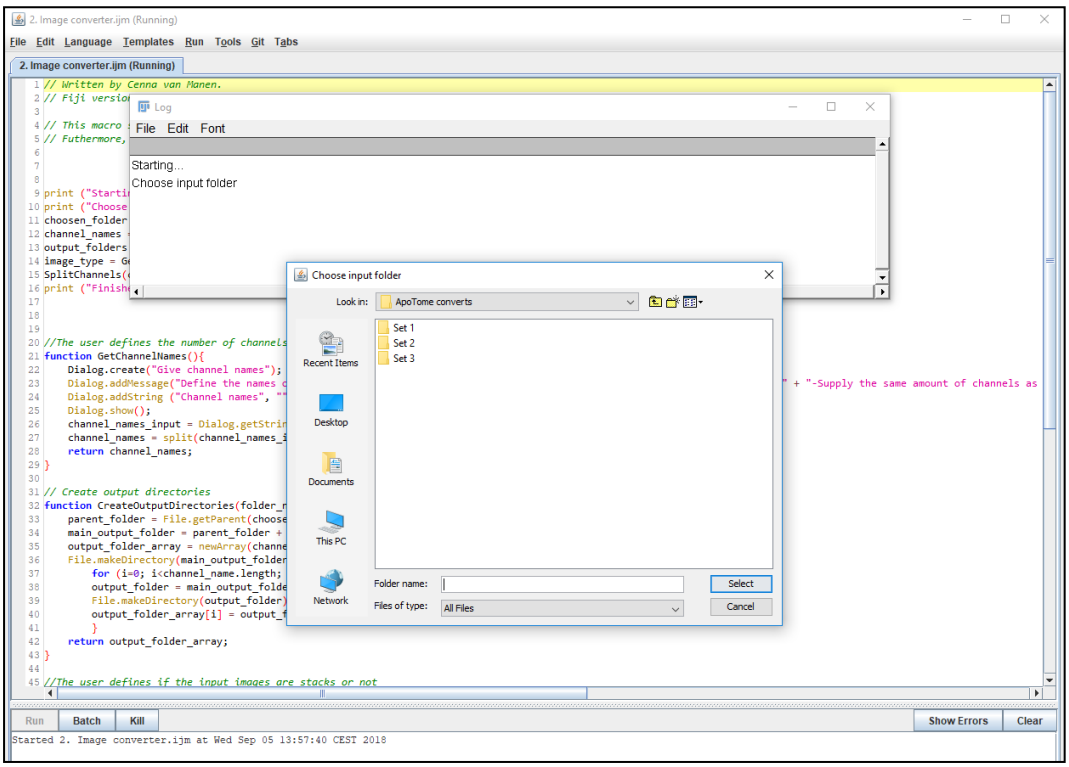
2. Open the 'Channel splitter.ijm' macro in the 'Automated Cilia Measuring' folder by simply dragging and dropping it into the lower gray bar.



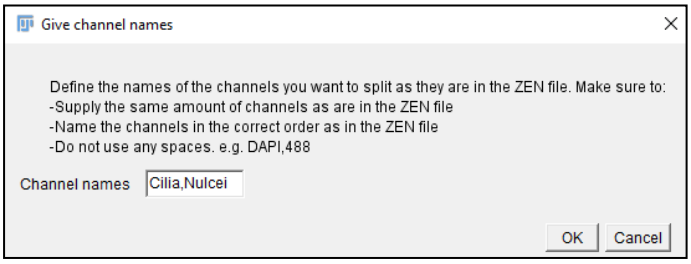
3. A new window will appear that contains the macro. The upper lines in green provide some information for the user. The rest of the window contains the macro.



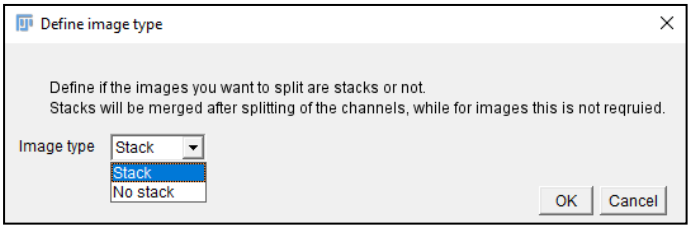
4. Click the ‘Run’ button on the left bottom of the macro window and two new windows will appear. The first window is the ‘Log’ window in the back with which you can’t interact. This window will show you the progress of the conversion. The second window is the ‘Choose input folder’ window. In the second window, select your folder with Raw .CZI files or ApoTome RAW convert .CZI files and click the button ‘select’.



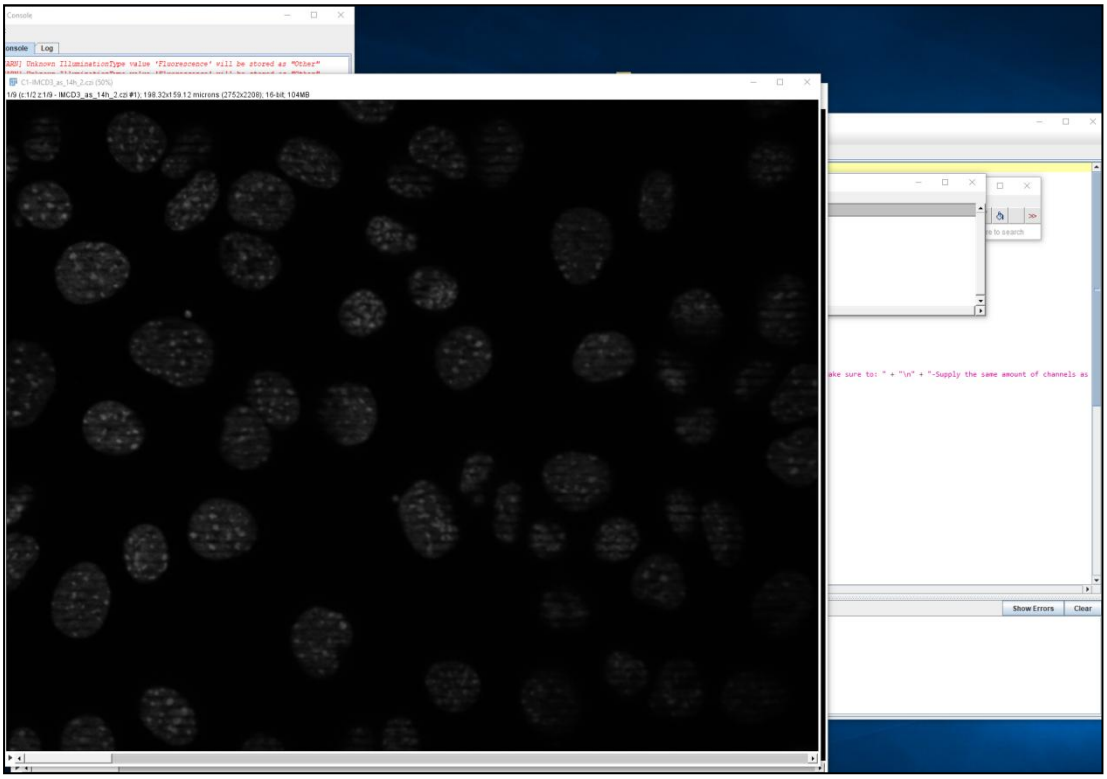
5. A new window ‘Give channel names’ will appear. Type the channel names as indicated in the window and click ‘Ok’. In the example below we name our channels Cilia and Nuclei.



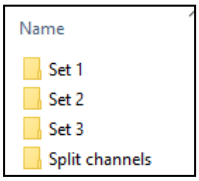
6. In the next window indicate if the images in the folder are Z-stacks (‘Stack’) or normal (single-plane) microscopic images (‘No stack’) and click ‘Ok’. It is not advised to have both Z-stacks and normal image for one analysis, but if you do make sure that they are in separate folders and run the macro for each folder.

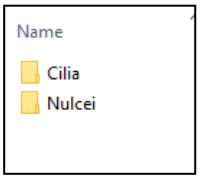


7. The macro will now start the conversion. The images will one by one pop up on the screen, be converted and saved again as .tif files. Make sure you **do not click** any of the FIJI windows during the macro run, since this will interfere with the conversion. During the run a 'Console' window with red next might appear in the back, but this can be ignored. When the conversion is completed, most windows will close and the 'Log' window will show 'Finished'.



8. The output of the conversion are .tif files that are saved into a newly created folder called 'Split channels'. This folder can be found next to the folder you choose as input. The 'Split channels folder will contain one folder per channel, as defined by the user in step 5.





CHAPTER 3. - MEASURE CILIA LENGTH

The next step in the pipeline is to measure the length of the cilia. To do this, first, the filter settings will have to be determined. The cilia measuring macro uses three types of filters to optimize automated analysis.

The first filter is the 'Min/max filter' that is used to exclude background signal. The second filter is the 'Grey value filter', which is used to define the grey value you consider as being true signal. The last filter is a 'Gaussian blur filter'. This filter helps the software to recognize a cilium with minor interruptions in the signal as one long cilium, instead of several smaller cilia. This method is similar to slightly over exposing your cilia during imaging and might therefore not be required if the cilia are imaged correctly. Not all filter have to be used for analysis, but the macro requires at least one filter to be used to distinguish the cilia. This could either be the min/max or grey value filter.

In order to get an idea about the optimal filter settings continue to 'Define filter settings'. If the filter settings are already determined, go directly to 'Automated cilia measuring'. The ***input*** of this analysis consist of a folder with single-plane, single-channel .tif images of the ciliary channel (*e.g.* ARL13B, acytylated tubulin or IFT88 channel). The ***output*** consist of a folder with different types of files as described in step 8 of the 'Automated cilia measure' analysis.

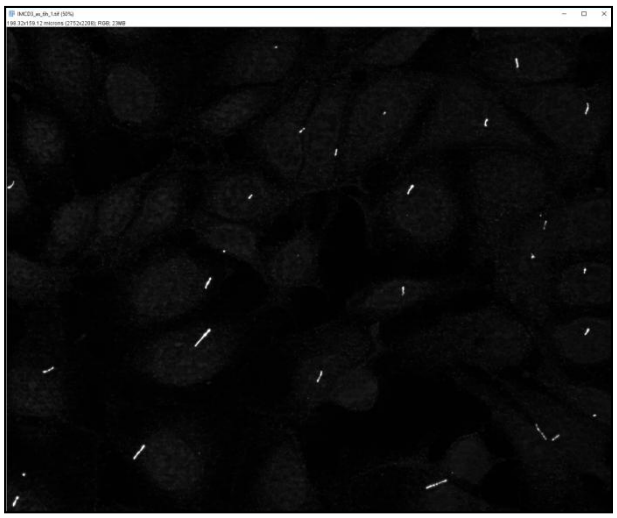
DEFINING FILTER SETTINGS

In order to get an idea about the optimal filter settings it is easiest to analyze the settings for one or a few representable images by hand, by following these steps:

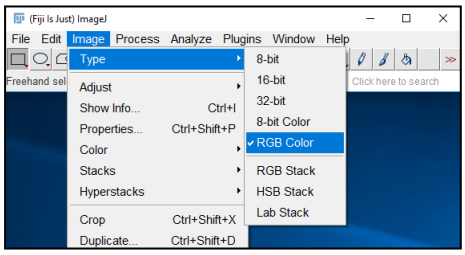
1. Open a .tif image which contains the cilia channel in FIJI by dragging and dropping, as described in chapter 2.

2. The minimal threshold value of the 'Min/max filter' can be determined by:

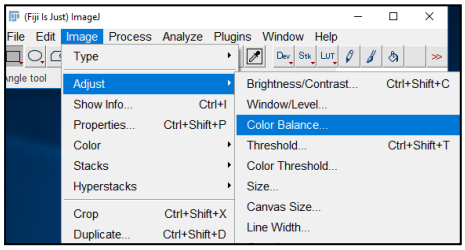
    a. Select the image by clicking in the upper grey bar of the image.



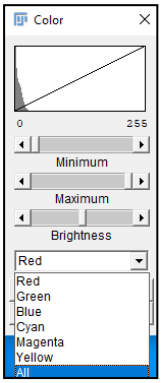
b. Change the image type by clicking Image > Type > RGB Color



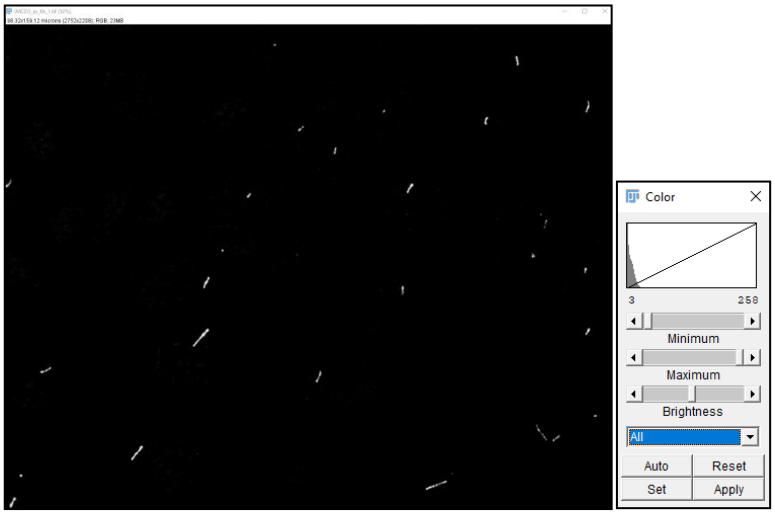
c. Open the filter window by Image > Adjust > Color Balance…



d. Click the dropdown menu behind ‘Red’ and select ‘All’



e. Now adjust the ‘Brightness’ level to the left to determine the optimal value that excludes background signal. The minimal threshold value for this filter can be found on the left below the graph. In this example the value would be 3.

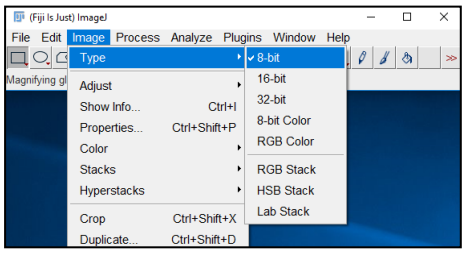


f. Click ‘Apply’ and the chosen setting will be permanently applied to the image. Make sure to not override the original image or to save the image under a new name.

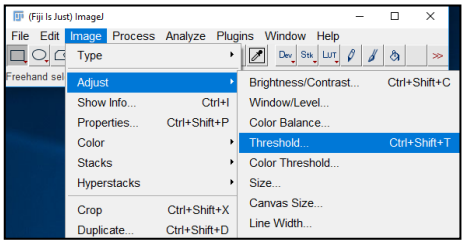
3. Determine the minimal threshold value of the ‘Greyvalue’ filter by:

a. Make sure the same image as in step 2 is selected with the applied min/ max filter.

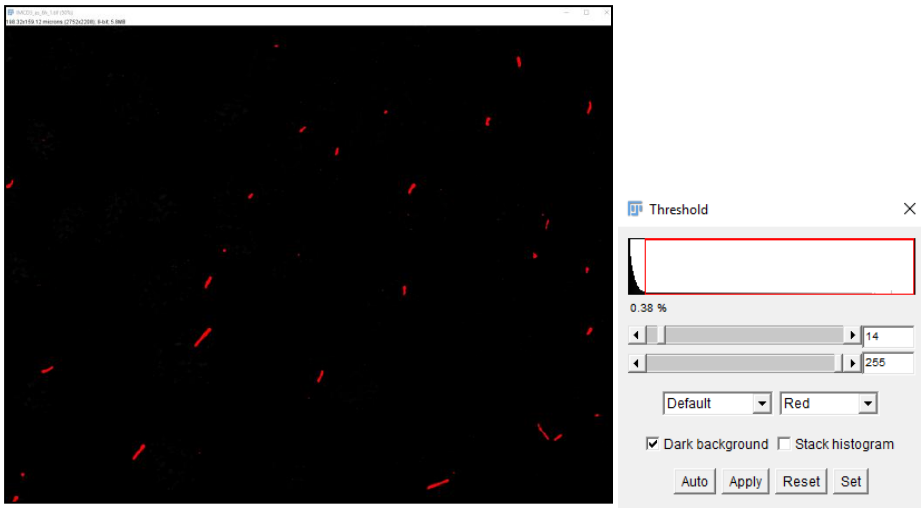
b. Change the image type by clicking Image > Type > 8-bit



c. Open the filter window by Image > Adjust > Threshold…



d. Make sure the box ‘Dark background’ is ticked and move the upper slider to the right to determine the optimal value so that all the cilia are all marked red, but there are no dots in the background. The optimal value should be just behind the downwards slope in the graph. The minimal threshold value for this filter can be found on the right side of the slider. In this example the value would be 14.

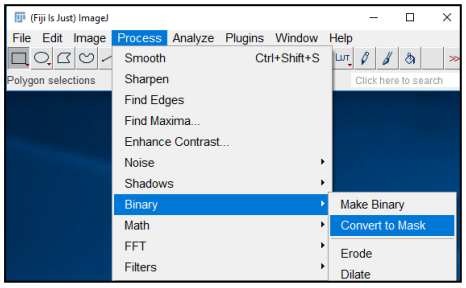


e. Click ‘Apply’. The background of the image will now turn white and the structures that will be recognized as being cilia will turn black.

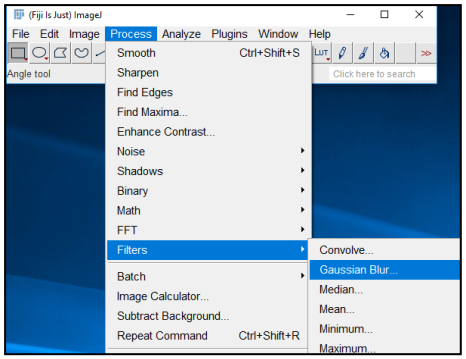
4. The last filter value is the ‘Gaussian blur’ sigma value.

a. Make sure the same image as in step 3 is selected with the applied grey value filter.

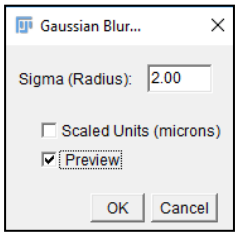
b. Make a mask of the image by clicking Process > Binary > Convert to Mask



c. Open the filter window by clicking Process > Filters > Gaussian Blur…



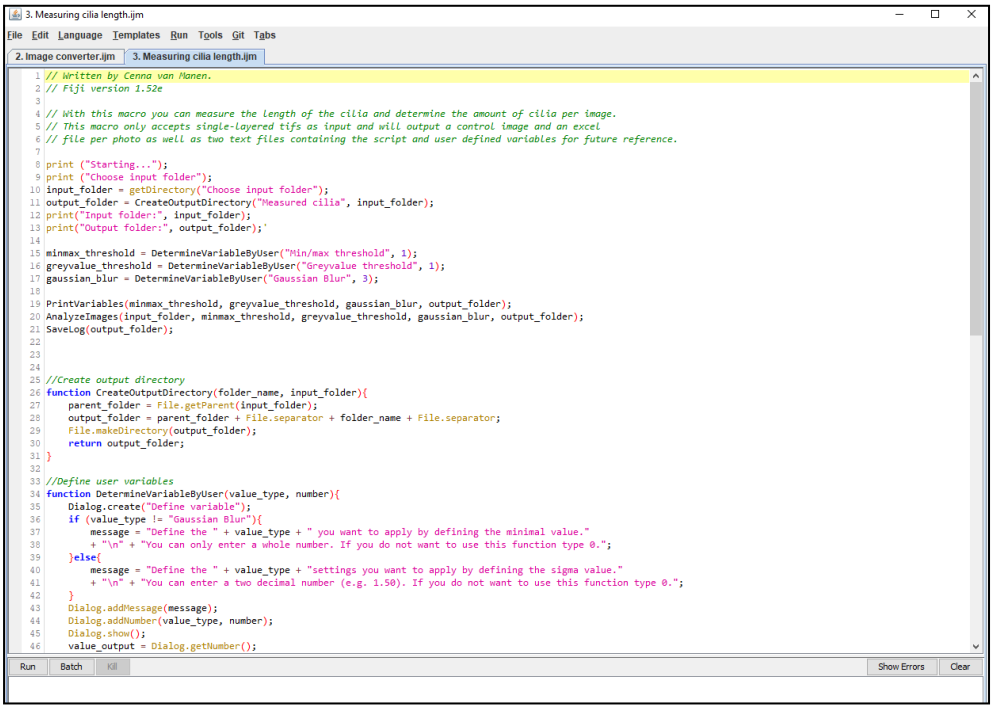
d. Make sure the ‘Preview’ box is checked and alter the sigma value to determine the optimal value so that none of the cilia are interrupted. This value is usually between 1 and 4. In this example the value would be 2.00.



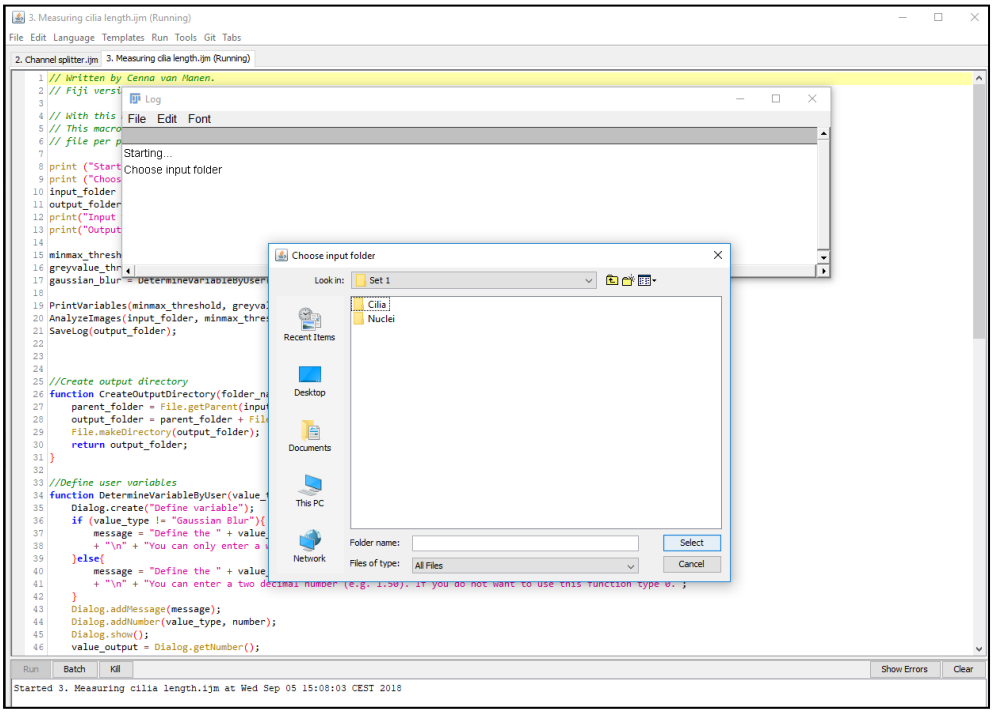
Remember these values for when you run the cilia measuring macro in FIJI. To test if these filter settings indeed allow for correct analysis a test folder can be made with a few representable images. This step is optional, but will validate the chosen filter settings. This analysis can be done by measuring the cilia of these images by hand and compare these values to the outcome of the automated analysis. How to measure cilia by hand using FIJI is described in the supplementary information.

AUTOMATED CILIA MEASURING

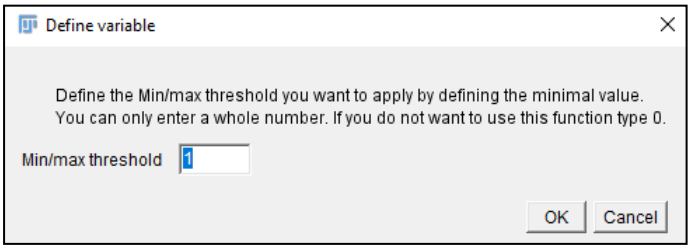
1. Start up the FIJI software and open the ‘Measuring cilia length’ macro by drag and drop as described in chapter 2. If you did not close the first macro, the second macro will appear in the same window, in a new tab, at the top of the window.



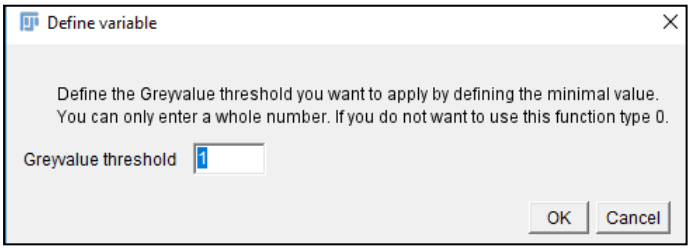
2. Click the ‘Run’ button on and two new windows will appear. The first window is the ‘Log’ window, which will show you the progress of the conversion. A copy of the complete log will be saved together with your results at the end of the run. The second window is the ‘Choose input folder’ window. In the second window, select your folder with the converted .tif files of the cilia channel and click the button ‘select’.



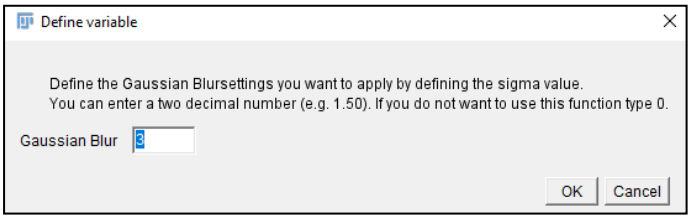
3. A new window ‘Define variable’ will appear in which you can define the first variable. In this window the ‘Minimal’ value of the ‘Min/max threshold’ can be implied as determine in step 2 of ‘Defining filter settings’. If this threshold is not used, type ‘0’, as indicated. Click ‘Ok’.



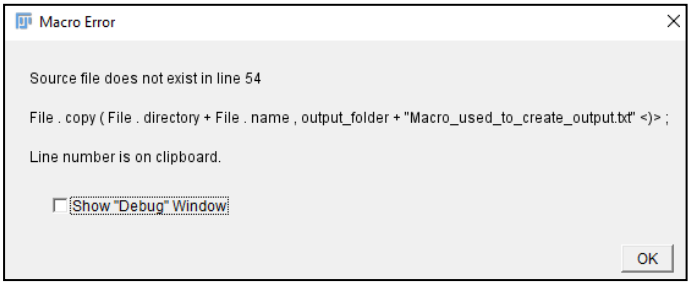
4. In the next window the ‘Minimal value’ for the ‘Greyvalue threshold’ is given as indicated. This value is determined in step 3 of ‘Defining filter settings’.



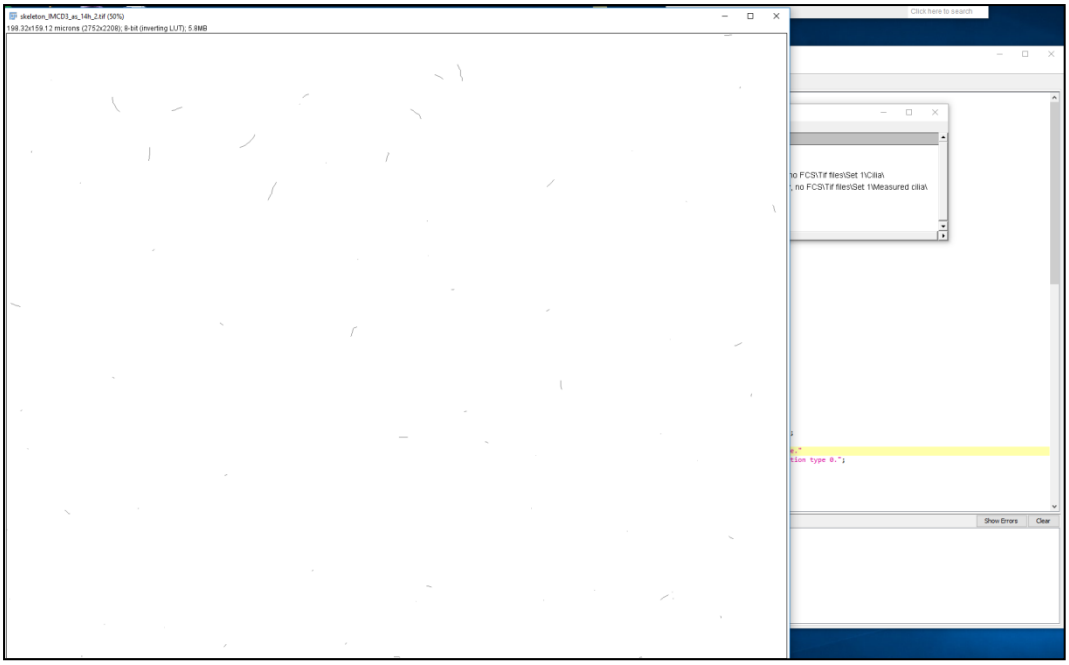
5. In the last window the ‘Sigma value’ of the ‘Gaussian blur filter’ is given as indicated. This value is determined in step 4 of ‘Defining filter settings’.



6. Press ‘Ok’. It is possible that the macro will give the following error. If so, close the macro window and reopen again as described in step 1.



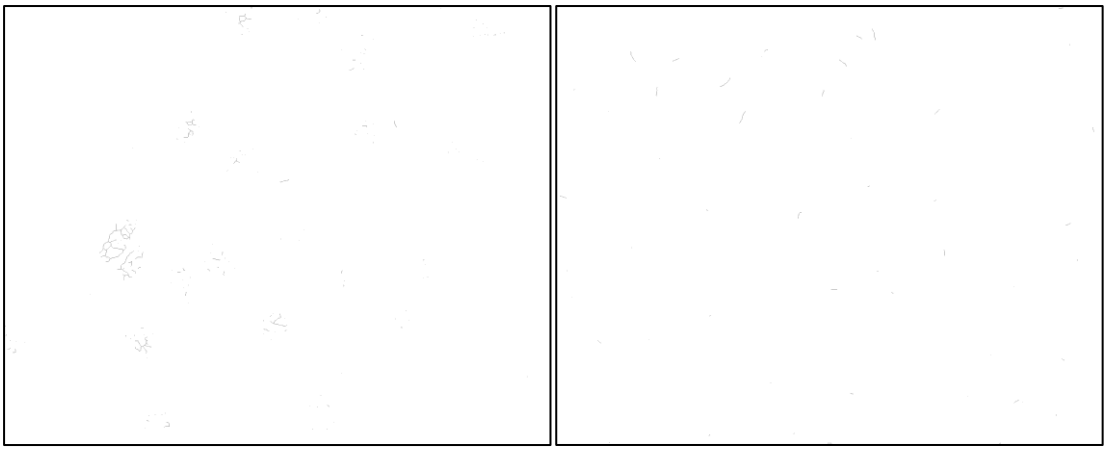
7. If there was no error the macro will start the measuring of the cilia and the images that are analyzed will pop up on the screen. Again, make sure you **do not click** any of the FIJI windows during the macro run, since this will interfere with the analysis.



8. While the windows are popping up during the run you can assess the quality of the analysis. This can also be done after the run by going through the skeleton files in the ‘Measured cilia’ folder, see step 8. If the thresholds are not set correctly, the macro will either stop the analysis and give an error or it will not be able to distinguish the cilia correctly.

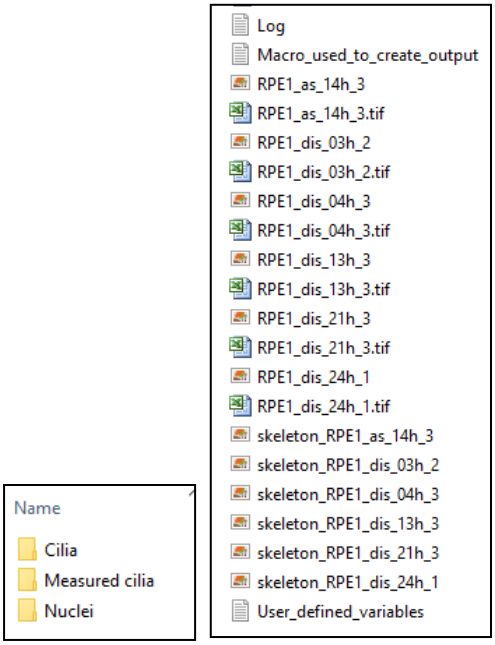
   When the macro gives an error, in most cases the variables are set too high and no cilia could be observed. If the macro can’t distinguish the cilia from each other, in most cases the variables are set too low. When the thresholds are set correctly you expect an image with properly separated cilia.

Incorrect threshold settings **Correct threshold settings**



9. When the macro run is finished, all windows will close automatically, except for the macro window. The results of your run are saved in a separate folder called ‘Measured cilia’ that can be found next to the chosen folder with cilia .tif images.

   The ‘Measured cilia’ folder will contain an excel and two .tif files per image that was analyzed. The excel file contains the lengths of all the cilia that were measured in that image. The .tif files contain the mask created by FIJI and the skeleton image, which can be used to assess the quality of the analysis. The output folder also contains three text files with a copy of the complete log of the run, a copy of the complete macro that was used for the run and a summary of the variable values that were defined by the user at the start of the run. These files might be useful in the future to see which variable were used, but might also be requested when publishing data that was obtained using this macro.



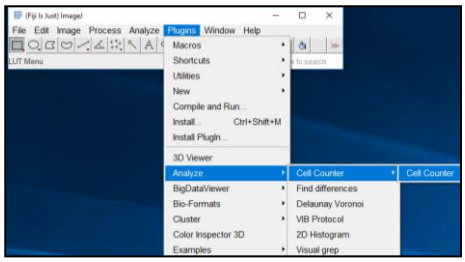
Of note, if your images do not contain anything that is recognized as a cilium, it will not be able to finish the macro run and give an error, see step 7. These images will have to be removed from the analysis before starting a macro run or, preferably, avoid inclusion of such image in the analysis completely.

CHAPTER 4. - COUNT NUCLEI

The next step is to count the number of nuclei per image. This FIJI macro is based on DAPI stained nuclei, but other nuclear or cell markers are also expected to function properly, however this is not tested. For proper analysis, the nuclei counting macro uses two types of filters that were also used to measure the cilia length; the 'Grey value filter' and the 'Gaussian blur filter'. Determine the optimal filter settings for the nuclei images in the same manner as for the cilia measuring script, see step 1, 3 and 4 of the 'Define filter settings' section in the previous chapter.

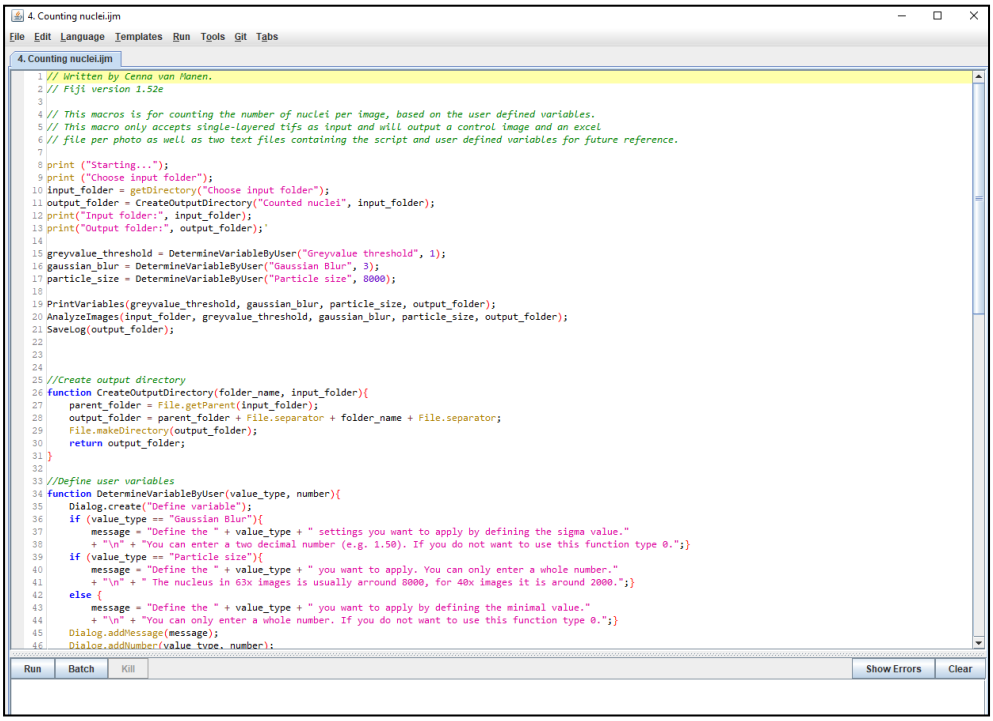
The third value to determine is the particle size you want to count as a nucleus. For 64x images the size of a nucleus is usually larger than 8000 particles. For 40x times images it will be larger than 2000 particles. Keep in mind that the size will depend on the cell type and cell cycle stage that you are analyzing. Furthermore, some nuclei along the border are counted, while other will not.

To test if the filter settings are correct a test folder can be made with a few representable images as described for the cilia measuring script. In this the nuclei can be counted for instance with the ROI manager, as described for the cilia measurements in the supplemental information, or by using the Cell Counter Plugin. Due to the many options in FIJI many other counting methods are available, but these will not be described here.

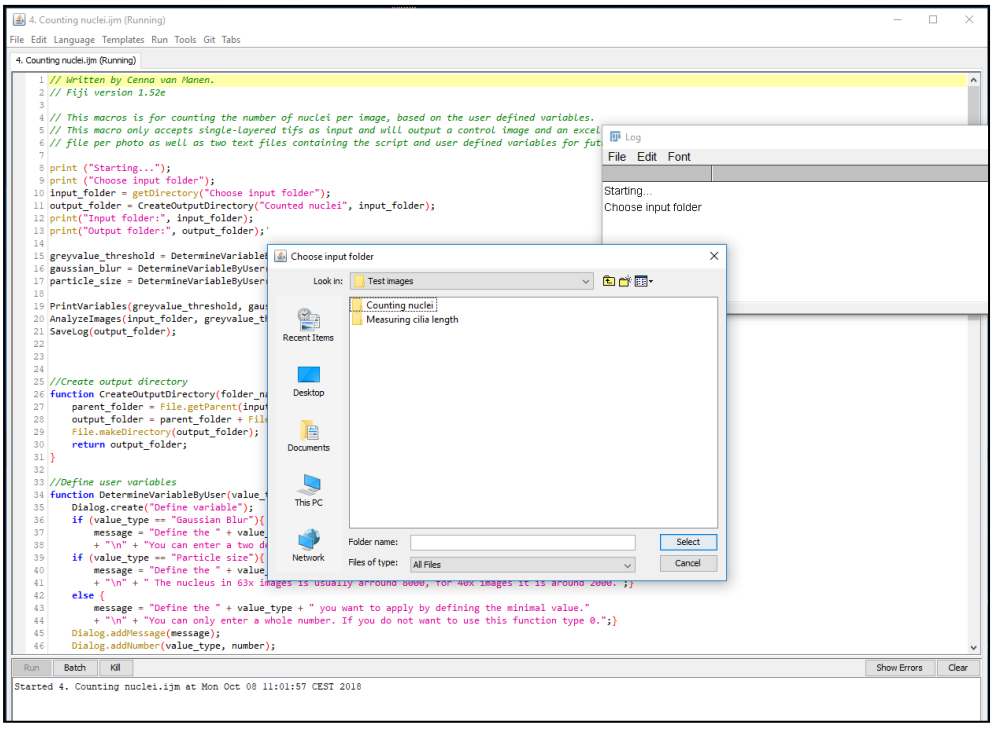


AUTOMATED NUCLEI COUNTING

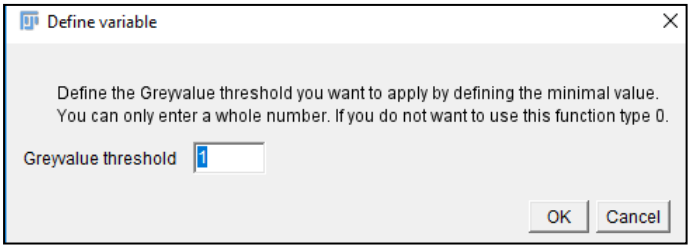
1. Start up the FIJI software and open the 'Counting nuclei' macro by drag and drop as described in chapter 2.



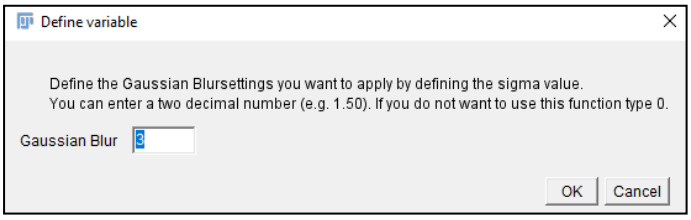
2. Click the ‘Run’ button on and two new windows will appear. In the ‘Choose input folder’ window, select your folder with the converted .tif files of the nuclear channel and click the button ‘select’. As with the cilia measuring script, a copy of the complete log will be saved together with your results at the end of the run.



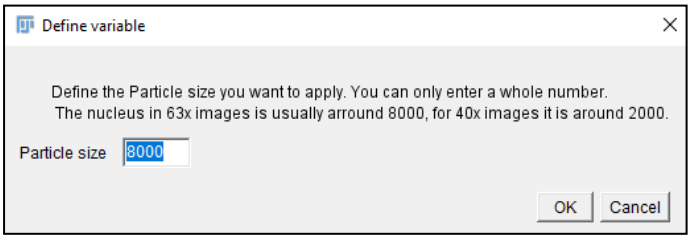
3. In the next window the ‘Minimal value’ for the ‘Greyvalue threshold’ is given as indicated.



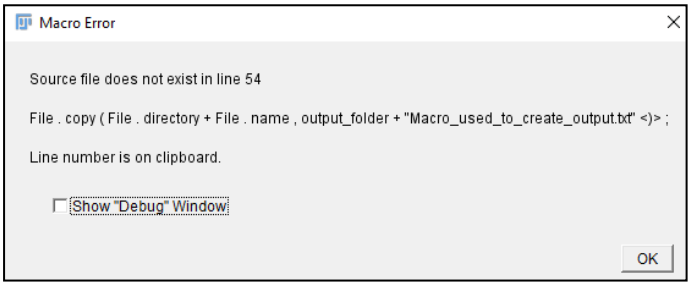
4. Next, the ‘Sigma value’ of the ‘Gaussian blur filter’ is given as indicated.



5. In the last window the particle size is indicated. This can be any whole number >0.



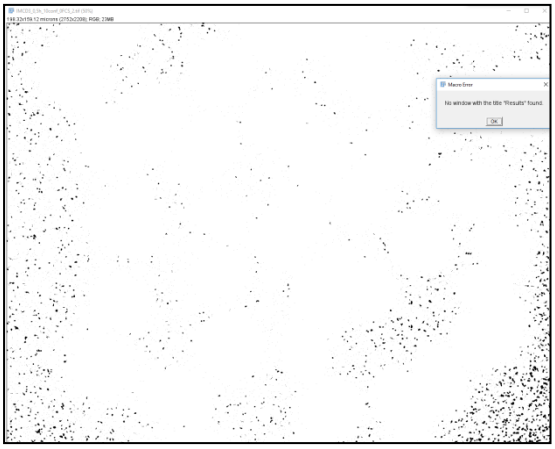
6. If the following error occurs, close the macro window and reopen again as in step 1.

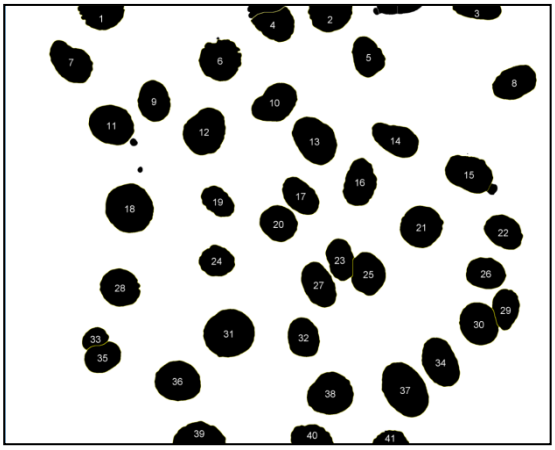


7. If there was no error the macro will start counting the nuclei. Again, make sure you **do not click** any of the FIJI windows during the macro run. During the run you can assess the quality of the analysis or by going through the.tif files in the 'Counted nulcei' folder after the run.

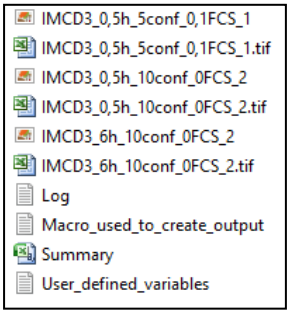
   If the thresholds are not set correctly, the macro will either stop the analysis and give an error or it will not be able to distinguish the nuclei correctly. When the macro gives an error 'No window with the title "Results" found', in most cases the variables are set too low and the nuclei could not be distinguished from each other.

Incorrect threshold settings **Correct threshold settings**





8. When the macro run is finished, the results are saved in the folder 'Counted nuclei', which can be found next to the chosen folder with cilia .tif images. The folder will contain an excel and a .tif file per image that was analyzed. The .tif file contains the mask created by FIJI with the numbers of the nuclei. Furthermore, the folder also contains an excel file 'summary' in which the nuclei per image can be found for the whole set of analyzed images. Last, the output folder also contains three text files as with the cilia measuring macro.



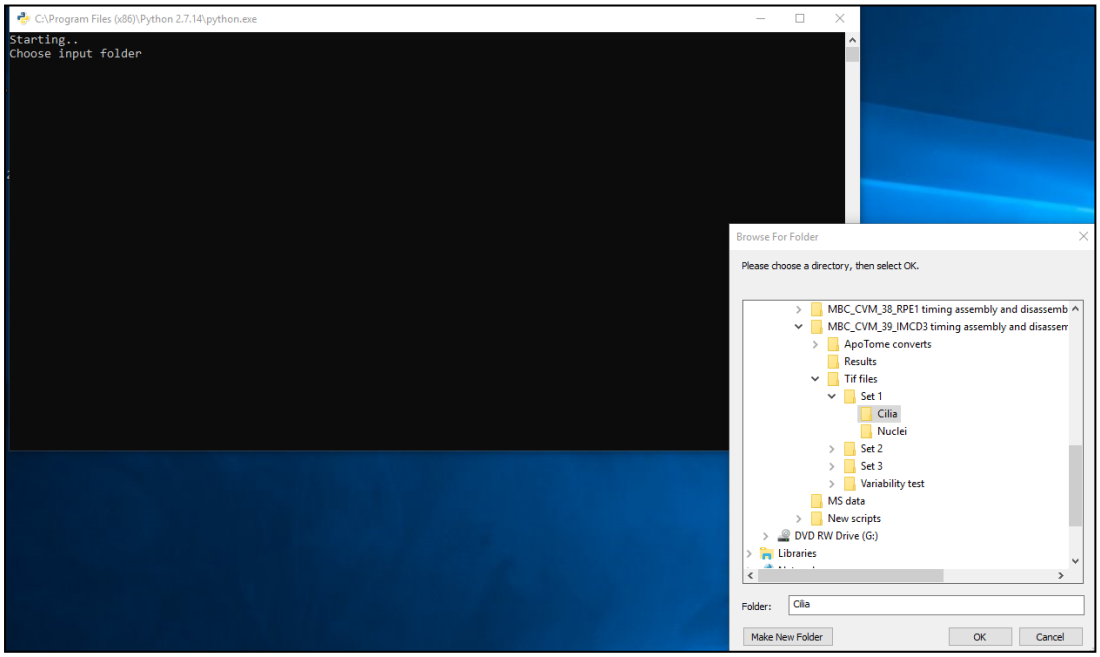
	A	B	C	D	E	F
1	Slice	Count	Total Area	Average S	%Area	Mean
2	IMCD3_0,5h_10conf_0FCS_2.tif	41	6194.542	151.086	19.63	255
3	IMCD3_0,5h_5conf_0,1FCS_1.tif	46	6257.494	136.032	19.83	255
4	IMCD3_6h_10conf_0FCS_2.tif	90	10842.347	120.471	34.359	255

CHAPTER 5. - DATA PROCESSING AND ANALYSIS

The last step of the analysis in processing the data. Since this is very dependent on the type of question you want to answer, we will not go into detail about the analysis. For smaller projects working with the excel will be sufficient to combine the nuclei counting and the cilia measurement data. For larger projects it might be advisable to use matlab or R to process and analyze your data.

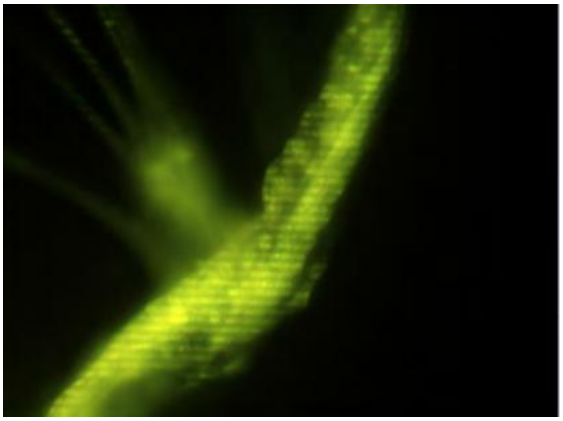
One small tool that might help combining your cilia measuring data is the 'Data sorter and combiner' python script. This script will combine all the excel files in the chosen cilia measurement folder into a single excel file. Furthermore, it will exclude all measurements with a length of 0.

When python is installed, you can simply double click the python file and two new windows will open. The black window is the log window, the other window allows you to select your cilia measurements input folder. When the script is done all windows will disappear and a new excel file 'Combined data' will appear in the chosen folder.



SUPPLEMENTAL INFORMATION

PHASE ERRORS



Phase errors can occur as a consequence of a low signal or image discrepancy when the ApoTome is used for imaging. The Zen software can somewhat correct for this during the ApoTome conversion calculations.

CONVERT .CZI FILES TO .TIF FILES WITH ZEN

This step is an optional step if you want to export your images to .tif files using the ZEN software instead of the FIJI macro described in chapter 2. Using ZEN instead of FIJI is however not recommended, since the current ZEN version does not allow for correct scaling and measuring of the cilia. If you do however want to use this method, you can either use the original .CZI files or the ApoTome Raw Convert .CZI files from step 1 as input.

1. If you skipped step 1, open the Zen software and go to the tab 'Processing' and select 'Batch'. Otherwise clear the list of selected files from step 1 by clicking on 'Remove All' at the bottom.

2. Click on 'add' and open all the .CZI files you want to process.

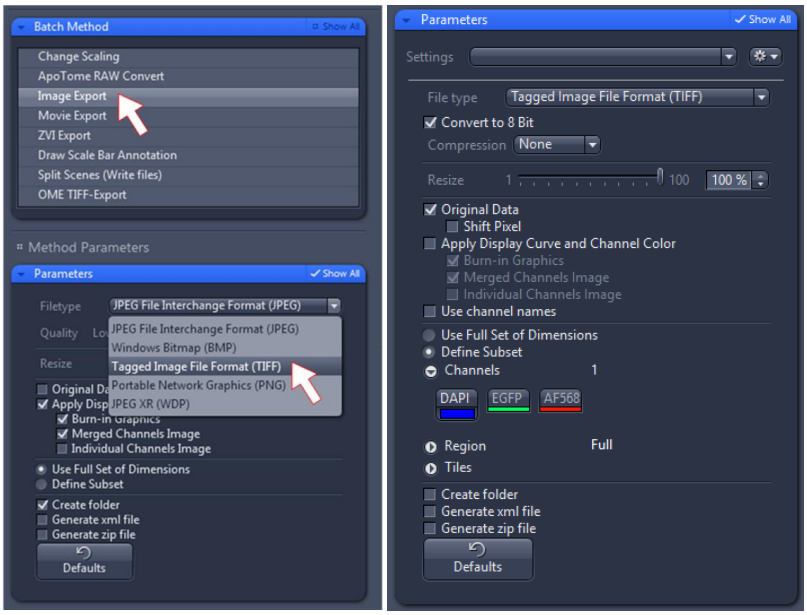
3. Select the upper file in the list and click on 'Image Export'. Here we will convert multi channel .CZI files into single channel .tif files. To do this choose the following options:
    a. File type: Tagged Image File Format (TIFF)
    b. Check the box 'Convert to 8 Bit'
    c. Compression: None
    d. Resize: 100%
    e. Check the box 'Original Data'

f. Uncheck the box ‘Shift Pixel’
g. Uncheck the box ‘Apply display Curve and Channel Color’
h. Check the box ‘Use channel names’
i. Click on ‘Define Subset’
j. Channels: 1 *Later on we will set this to 2, 3, etc to export all the other channels*
k. Region: Full
l. Tiles: Existing Tiles
m. Uncheck the box ‘Create folder’
n. Uncheck the box ‘Generate xml file’
o. Uncheck the box ‘Generate zip file’

If you analyzed a single channel per image, apply the same options as above, but make the following changes:

g. Uncheck the box ‘Use channel names’ *Mind that the channel name should be in the name of the saved file*
h. Click on ‘Use Full Set of Dimensions’ and proceed to step m.

If you do not see all these options, make sure the box ‘Paramaters’, ‘Show All’ is checked.



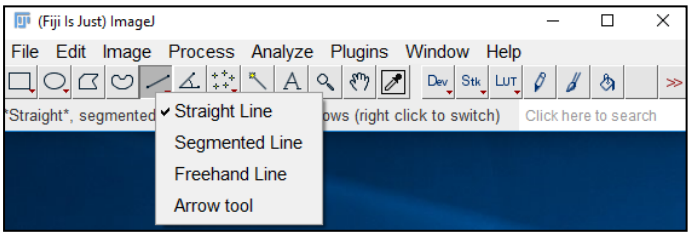
4. Click the button ‘Copy Parameters’ at the top of the screen and select all files. Click ‘Paste Parameters’ to apply the same export method to all the files.

5. Uncheck the box ‘Use Input Folder as Output Folder’ to save the .tif files into a new folder, which can be chosen by clicking on ‘…’.

6. Click ‘Apply’ to process all images. The blue bar on the bottom will indicate the progress.

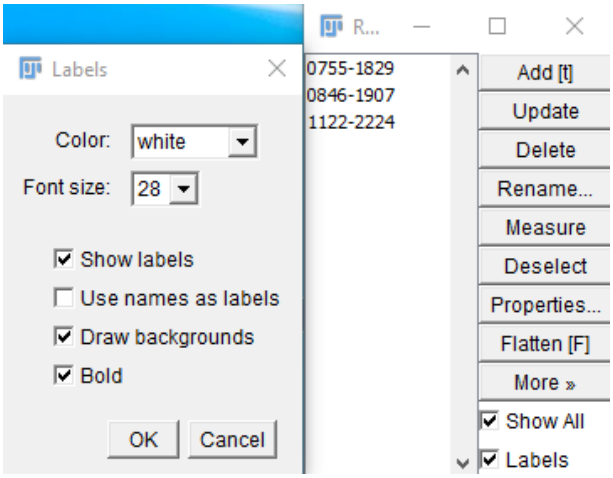
MEASURE CILIA BY HAND

1. In FIJI, choose ‘Segmented line’ by right clicking on the line option.



2. Open the ‘ROI manager’ under Analyze > Tools > ROI manager.

3. Add numbers to each of your measurements in the ROI manager by clicking more > labels…, select the following settings:
    a. Color: white
    b. Font size: 28
    c. Check the box ‘Show labels’
    d. Check the box ‘Draw backgrounds’
    e. Check the box ‘Bold’
    f. Make sure the box ‘Use names as labels’ is unchecked



4. Open the measurement screen by pressing ‘ctrl + m’

5. Double click to start drawing a line and single click along the whole cilium to measure it, finish the line by double clicking.

6. Press ‘m’ to measure the line and ‘t’ to register it in ROI manager, before drawing a new line.

7. When finished with the image do the following:
    a. Press ‘shift + F’ to flatten the image
    b. Save the image as tif file
    c. Save the ROI selection by pressing more > save…
    d. Save the results by clicking File > Save As