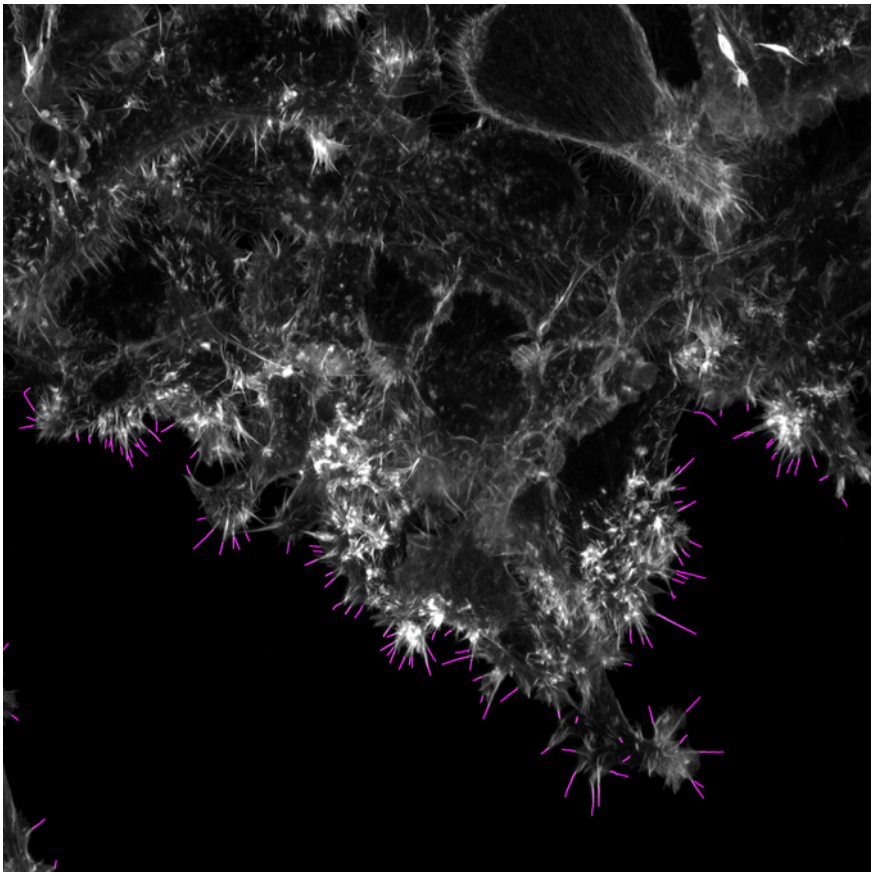
FiloQuant manual V1.0

Table of Contents



1) FiloQuant aims and distribution license

FiloQuant is a user friendly and modifiable tool for automated detection and quantification of filopodia properties such as length and density. FiloQuant is currently limited to the detection and quantification of filopodia and other finger-like protrusions that extend out from the cell edge and is not applicable to study filopodia localized at cell-cell junctions, or for detection of dorsal and ventral filopodia. FiloQuant currently works only on 2D images and does not yet support 3D analyses.

We developed FiloQuant as a plugin for the freely available, and popular, ImageJ with inter operating systems compatibility. FiloQuant was designed with four goals in mind:

First, we aimed to make this software as easy to use as possible. To this end we created versions of FiloQuant containing step-by-step user validation of the various processing stages to help users to achieve optimal settings for filopodia detection.

Second, this software was designed to simplify and speed up the analysis of filopodia properties. To this end semi-automated as well as a fully-automated versions of FiloQuant are provided. Using the semi-automated version of FiloQuant, users can analyze rapidly a large number of images while keeping control over the settings used to analyze each image and modify these settings on the fly to improve the accuracy of detection. Using the automated version of FiloQuant, users can choose the settings for analyzing a large number of images at once (batch analysis). This latter version of FiloQuant is especially useful for screening purposes or to analyse filopodia properties and dynamics from live-cell imaging data.

Third, we aimed to make FiloQuant as broadly usable/flexible as possible, with no limitation in terms of cell geometry or imaging modality. We successfully used FiloQuant with images (acquired on different microscopes) of cells migrating collectively or as single cells in various environments including 2D fibronectin and 3D cell-derived matrices. In addition, we tested the ability of FiloQuant to detect filopodia in neurons, which have a more complex morphology.

Finally, although this software was found to effectively identify filopodia in many different types of images, we appreciate that others might require extra functionalities or the ability to included FiloQuant within larger analysis routines. To facilitate easy modification of FiloQuant, we wrote this software using the simple ImageJ macro language that we also fully annotated, which can therefore be edited with limited coding knowledge.

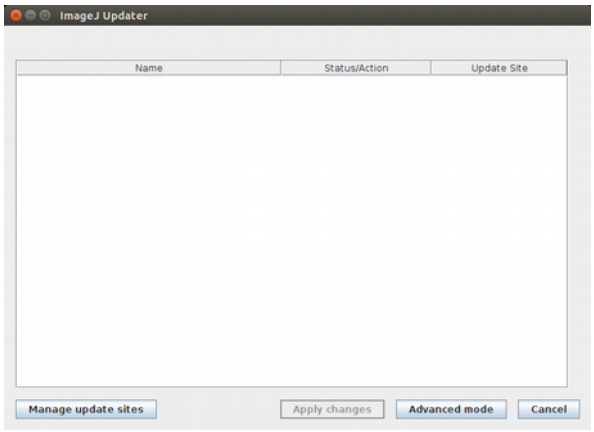
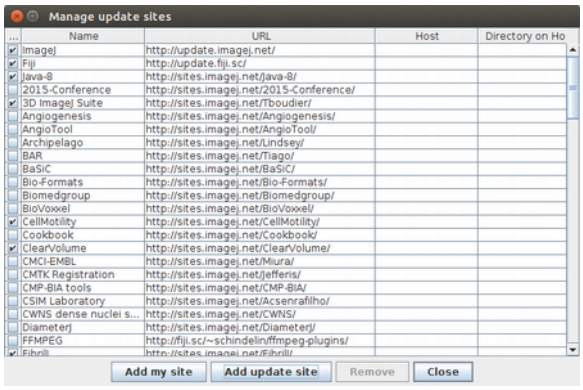
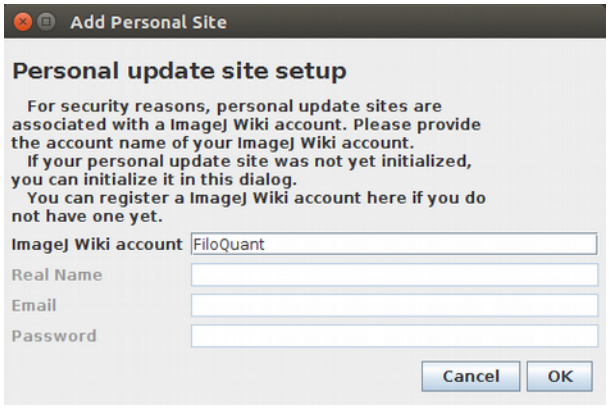
FiloQuant was created by Guillaume Jacquemet and Alexandre Carisey. This program is **free software**; you can redistribute it and/or modify it under the terms of the **GNU General Public License** as published by the Free Software Foundation (http://www.gnu.org/licenses/gpl.txt).

Please email Guillaume Jacquemet at guillaume.jacquemet@utu.fi for questions or to request new features.

2) Installation

We recommend the Fiji distribution of ImageJ (https://fiji.sc/) for use with FiloQuant as Fiji already contains all the necessary dependencies required by FiloQuant. To run FiloQuant in ImageJ, users need to install the following dependencies: Enhanced Local Contrast (CLAHE.class; http://imagej.net/Enhance_Local_Contrast_(CLAHE)), Skeletonize3D.jar (http://imagej.net/Skeletonize3D), AnalyzeSkeleton.jar (http://imagej.net/AnalyzeSkeleton) and Temporal-Color Code (http://imagej.net/Temporal-Color_Code).

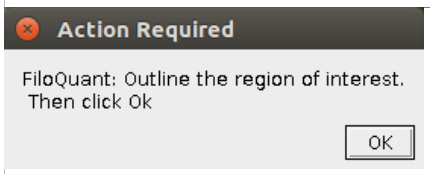
Installation of FiloQuant can be easily done in Fiji using the FiloQuant ImageJ update site:

1) In Fiji, click on “Help → Update”	
2) then “Manage update sites”	
3) and “add my site”	
4) In the field “ImageJ Wiki account” input: “FiloQuant” then click “OK”.	
Close the “Manage update sites” window and, in the ImageJ Updater window, click on “Apply changes”. The three versions of FiloQuant can then be found under “plugin → FiloQuant”.	

3) FiloQuant, step-by-step instructions (single images)

The single image or semi-automated version of FiloQuant contains step-by-step user validation of the various processing steps to help users achieve optimal settings for filopodia detection. Below you will find detailed instructions related to the various steps.

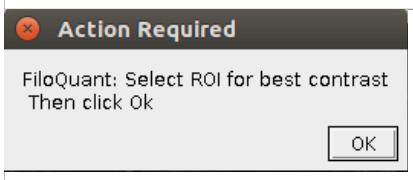
1: Choose the region of interest to analyze



Outline the region of interest (ROI) to be analyzed by drawing a square/other shape around the area on the initial image, then click OK.

If you want to use the whole image, just click “OK”

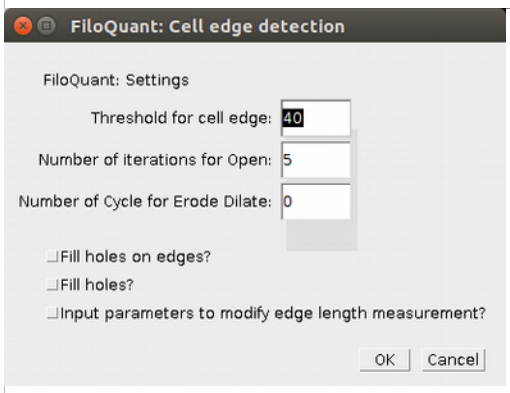
2: Brightness / Contrast adjustment



To calculate optimal contrast settings, you can select an ROI by drawing a square and then click OK.

Alternatively, to use the whole image for auto contrast calculations, just click “OK”

3: Parameters to detect the cell edge



Input the parameters that FiloQuant will use to generate the filopodia-free cell edge:

- **Threshold for cell edge**: intensity value to be used for the intensity based thresholding (input range:1-255).

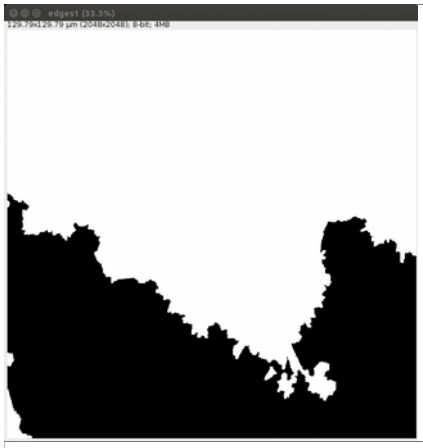
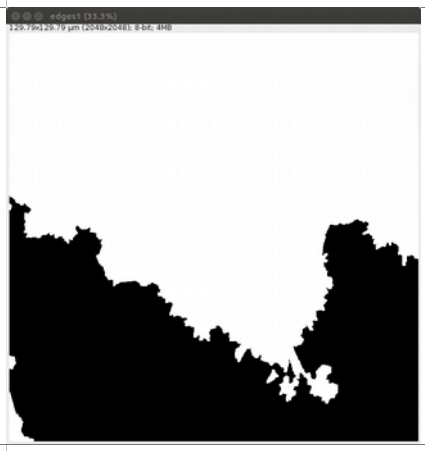
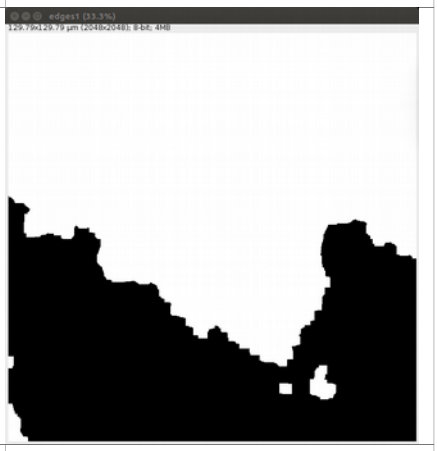
- **Number of iterations for Open**: Open is the process used by FiloQuant to shave off the filopodia. Input here the number of times you want to perform this operation (input range: 1-100).

- **Number of cycles for Erode Dilate***: Alternative method to shave off filopodia. Input here the number of time you want to perform this operation (input range: 1-100). *We recommend to use Open first.

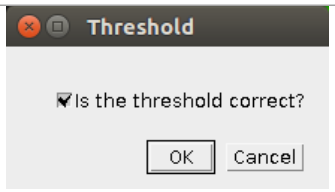
Fill holes on edges? Tick this box if you want FiloQuant to fill the holes at the image boundary

Fill holes? Tick this box if you want FiloQuant to fill the hole in the middle of the image.

Input the parameters to modify edge length measurement? Tick this box if you want to control how the edges are measured.

		
“Fill holes on edges?” box ticked	**No holes filled**	**“Fill holes?” box ticked**
		
Number of iterations for Open = 0	**Number of iterations for Open = 5**	**Number of iterations for Open = 20**

4: Validation of filopodia-free cell edge detection

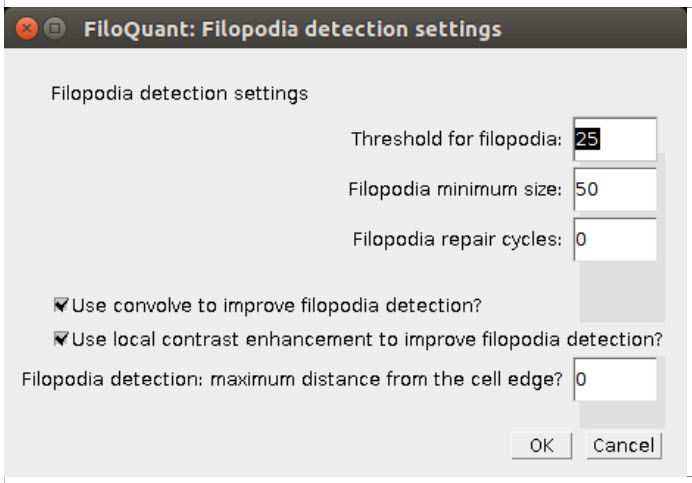


Is the threshold correct?

- If you are happy with the threshold, click OK

- If you are not happy with the threshold, untick the box and click OK. You will then be able to remodify the parameters to detect the filopodia-free cell edge.
Repeat until you are satisfied with the parameters

5: Parameters to detect filopodia



- **Threshold for filopodia**: intensity value to be used for the intensity based thresholding (input range: 1-255).

- **Filopodia minimum size:** Input the minimum size (in pixels) of structures to be considered further for analysis. This concerns only the filopodia that are not found to be connected to the cell body.

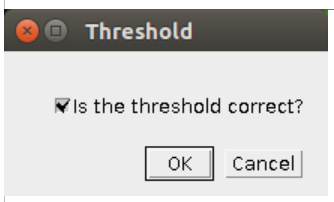
- **Filopodia repair cycles**: Input the number of times you want to perform the "close" operation to try to restore broken filopodia. We recommend 0 or 1. Inputting 0 will disable the option.

- **Use convolve to improve filopodia detection?** Tick this box if you want to use a standard convolution kernel to help with filopodia detection. We recommend this option as we found it to be very powerful in extracting faint filopodia.

- **Use local contrast enhancement to improve filopodia detection?** Tick this box if you want to use this option to improve the detection of faint filopodia. This option needs to be disabled if the image is noisy.

- **Filopodia detection: maximum distance from the cell edge?** Input the maximum distance (in pixels) that filopodia are allowed to be from the cell edge to be considered further for analysis. Inputting 0 will disable this option.

6: Validation of filopodia detection

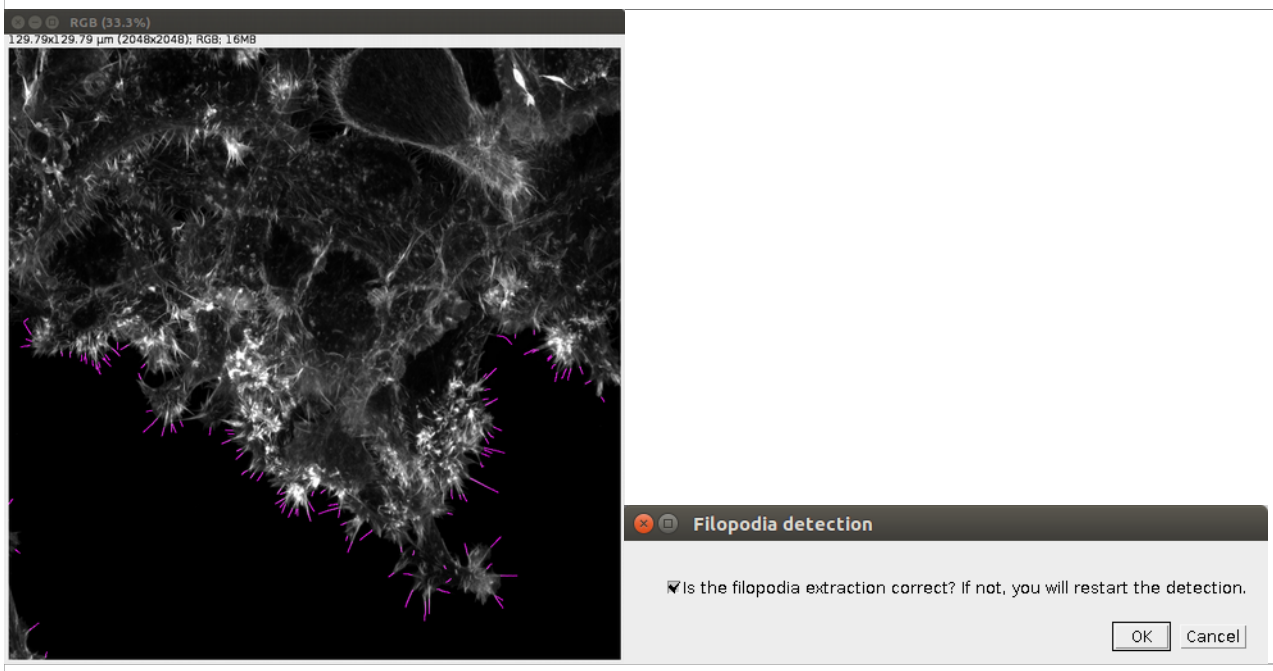


Is the threshold correct?

- If you are happy with the threshold, click OK

- If you are not happy with the threshold, untick the box and click OK. You will then be able to remodify the parameters for filopodia detection. Repeat until you are satisfied with the parameters.
Note: filopodia filtering in function of size happens at a later stage and cannot be validated at this step.

7: Validation of filopodia detection



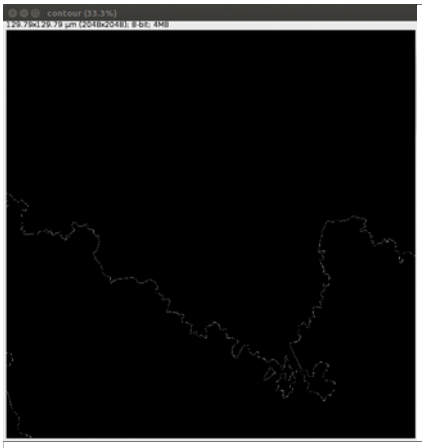
- If you are happy with the filopodia detection (in purple), click OK.

- If you are not happy with the filopodia detection, untick the box and click OK. You will be able to restart the analyses again.
Repeat until you are satisfied with the parameters.

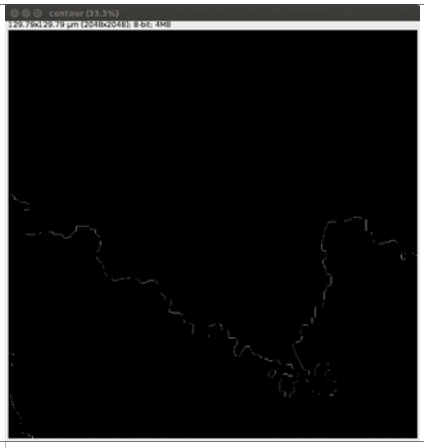
8: Parameters used to measure the length of the cell edge

Note: this window becomes available only if the box "**Input the parameters to modify edge length measurement?**" is ticked (in step 3). Otherwise default parameters are used.
The following options can be used to smooth the edges before measurements are made.

	Number of iterations for Close: Input here the number of times you want to perform this operation (input range: 1-100). **Number of iterations for Erode**: Input here the number of times you want to perform this operation (input range: 1-100). **Number of iterations for Dilate**: Input here the number of times you want to perform this operation (input range: 1-100).



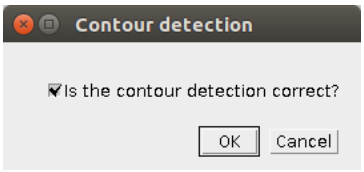
Values : 0, 0, 0



Values: 10, 10, 10

Values, 50, 50, 50

9: Validation of contour detection



Is the contour detection correct?

- If you are happy with the contour, click OK

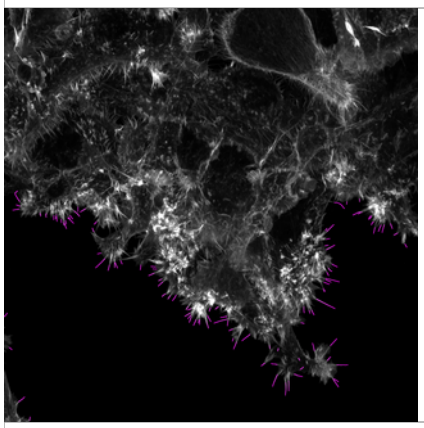
- If you are not happy with the contour, untick the box and click OK. You will then be able to remodify the parameters for contour detection.
Repeat until you are satisfied with the parameters.

10: Results

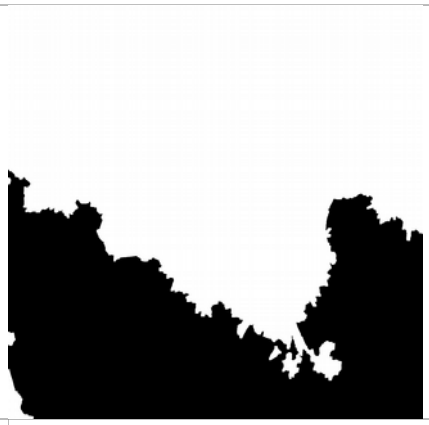
- The result can be found in the same location as the original image

- Result includes:

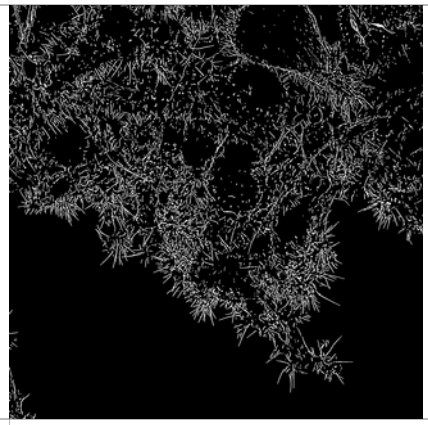
- a results.csv file containing the length of all the detected filopodia in one column and the length of all the detected edges in another column.
- a settings.csv file containing all the settings used for the analysis of this particular image. This file can be found in the folder “intermediate files”.
- The following images:



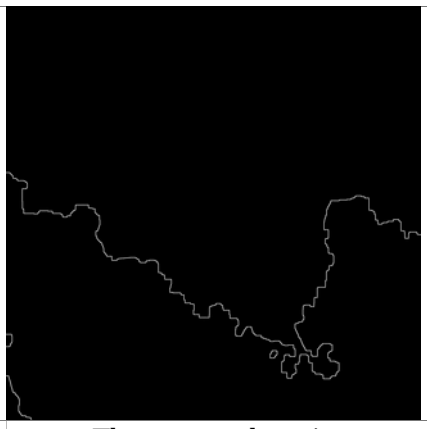
The result image. Detected filopodia are in purple.



The cell edge



The image used to detect filopodia

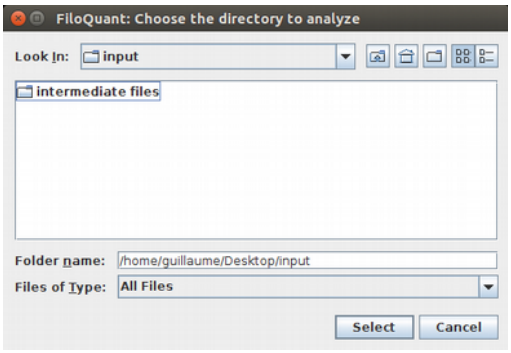
		
The detected filopodia	The contour detection	

Note: The initial thresholding of the cell edge can sometimes be challenging. It is possible during step 1 and 2 to manually outline the cell using the ImageJ freehand tool and then use the “fill” function.

4) FiloQuant, semi-automated analysis

Using the semi-automated version of FiloQuant, users can analyze rapidly a large number of images while keeping control over the settings used to analyze each image and modify these settings on the fly to improve the accuracy of detection.

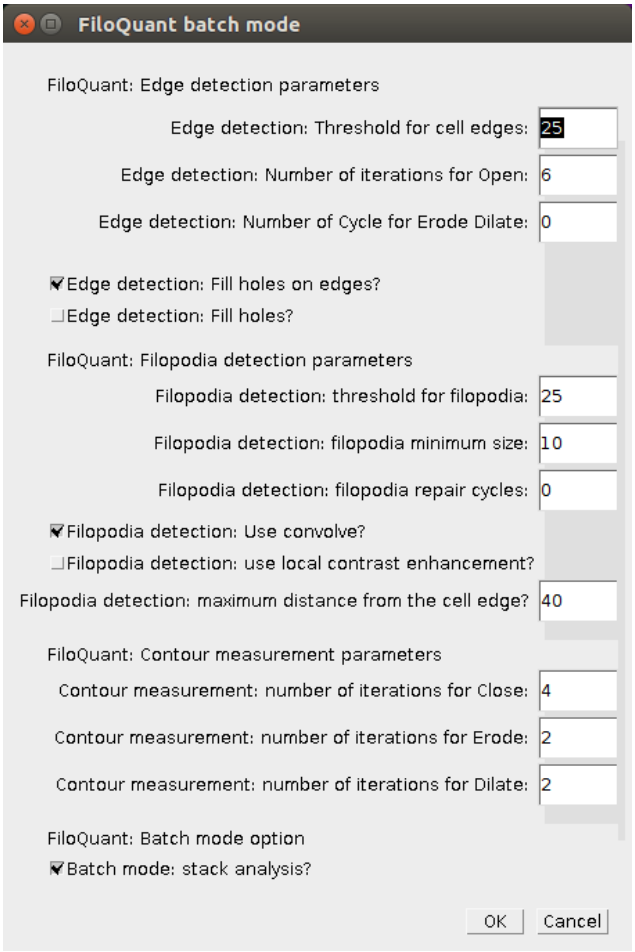
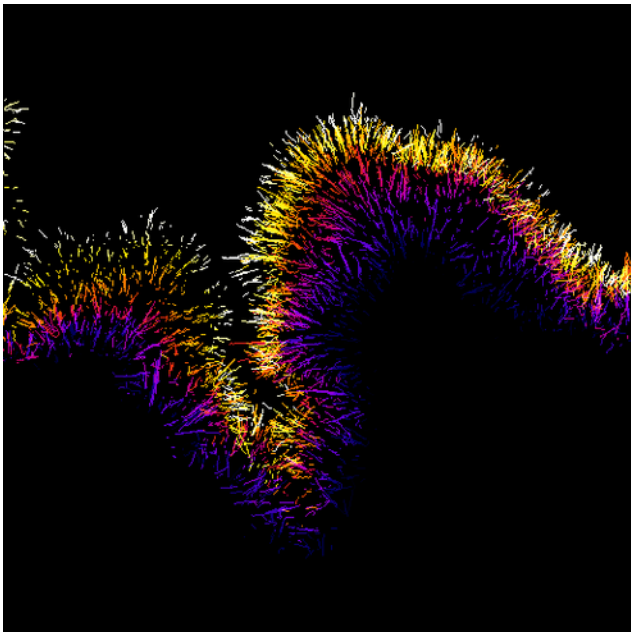
The analysis process is very similar to the one described for the single image analysis except that the user is prompted to choose the location of the folder containing the images to analyse and a folder where the results can be saved.

<table>
<tr><td colspan="2">1) Choose the input folder</td></tr>
<tr><td> 
</td><td>Choose the folder containing the images to be analysed.

Images need to be in a .tiff or .tif format</td></tr>
<tr><td colspan="2">2) Choose the output folder</td></tr>
<tr><td></td><td>Choose the folder where you want to save the results</td></tr>
<tr><td colspan="2">3) Perform analyses as described for the single image above</td></tr>
<tr><td colspan="2">4) Results can be found in the output folder</td></tr>
</table>

5) FiloQuant automated analysis, live-cell imaging and filopodia tracking

Using the automated version of FiloQuant, users can choose the settings for analyzing a large number of images at once (batch analysis). This latter version of FiloQuant is especially useful for screening purposes and/or to analyse filopodia properties and dynamics from live-cell imaging data.

1) Choose the input folder	Choose the folder containing the images and/or stacks to be analyzed. **- CRITICAL: Make sure that there are no spaces in the file names or in the file paths** **- Images need to be in a .tiff or .tif format**
2) Choose the output folder	Choose the folder where you want to save the results
3) Input all the parameters to be used	
	 Example of a time projection of detected filopodia created by FiloQuant when the “Batch mode: stack analysis” option is enabled.
4) Results can be found in the output folder	

Parameters that FiloQuant will use to generate the filopodia-free cell edge:

- **Threshold for cell edge**: intensity value to be used for the intensity based thresholding (input range:1-255).
- **Number of iterations for Open**: Open is the process used by FiloQuant to shave off the filopodia. Input here the number of times you want to perform this operation (input range: 1-100).
- **Number of cycle for Erode Dilate***: Alternative method to shave off filopodia. Input here the number of time you want to perform this operation (input range: 1-100). *We recommend to use Open first.
- **Fill holes on edges?** Tick this box if you want FiloQuant to fill the holes at the image boundary
- **Fill holes?** Tick this box if you want FiloQuant to fill the hole in the middle of the image.
- **Input the parameters to modify edge length measurement?** Tick this box if you want to control how the edges are measured.

Parameters to detect filopodia:

- **Threshold for filopodia**: intensity value to be used for the intensity based thresholding (input range: 1-255).
- **Filopodia minimum size:** Input the minimum size (in pixels) of structures to be considered further for analysis.
- **Filopodia repair cycles**: Input the number of times you want to perform the "close" operation to try to restore broken filopodia. We recommend 0 or 1. Inputting 0 will disable the option.
- **Use convolve to improve filopodia detection?** Tick this box if you want to use a standard convolution kernel to help with filopodia detection. We recommend this option as we found it to be very powerful in extracting faint filopodia.
- **Use local contrast enhancement to improve filopodia detection?** Tick this box if you want to use this option to improve the detection of faint filopodia. This option needs to be disabled if the image is noisy.
- **Filopodia detection: maximum distance from the cell edge?** Input the maximum distance (in pixels) that filopodia are allowed to be from the cell edge to be considered further for analysis. Inputting 0 will disable this option.

Contour measurement:

- **Number of iterations for Close**: Input here the number of times you want to perform this operation (input range: 1-100).
- **Number of iterations for Erode**: Input here the number of times you want to perform this operation (input range: 1-100).
- **Number of iterations for Dilate**: Input here the number of times you want to perform this operation (input range: 1-100).

Batch Mode:

- **Batch mode: stack analysis?** Tick this box if the folder you want to analyse contains stacks of images rather than single images, e.g. live-cell imaging data. This option will:

  - organise the result data differently (more suitable for stacks)
  - generate a time projection of the detected filopodia
  - generate a tracking file of the detected filopodia that can be further analyzed using automated tracking software such as TrackMate.

6) Results, known issues and troubleshooting:

The output table contains:

- x, y coordinates of each detected filopodia (in the unit of the image calibration)
- length of the detected filopodia (in the unit of the image calibration, as indicated in the heading)
- length of the detected edge (in the unit of the image calibration, as indicated in the heading)

The filopodia are measured and counted using the AnalyzeSkeleton algorithms (Arganda-Carreras et al., 2010).

Known issues:

(I) Broken filopodia phenotype

When the labelling intensity of a filopodium is extremely weak it result in difficulties to threshold the full length of the protrusion. This may lead to the detection of multiple fragmented filopodia instead of a single protrusion. This will artificially increase the number of filopodia detected by FiloQuant and result in FiloQuant underestimating the true length of the filopodium. As the coordinates of each filopodium counted / measured are also provided by the software, users can re-evaluate the data and surmise if a filopodium has been incorrectly measured.

The broken filopodia phenotype can be improved by tuning the " **Threshold for filopodia"** parameter or enabling **" Use convolve to improve filopodia detection"** or **" Use local contrast enhancement to improve filopodia detection".** The **"Filopodia repair cycles"** may also fix filopodia that are broken (only the filopodia broken by one or two pixels).

However, the best way to resolve the "broken filopodia" issue is to acquire images with higher signal / noise ratio (see below for advice).

(II) Very high density of filopodia

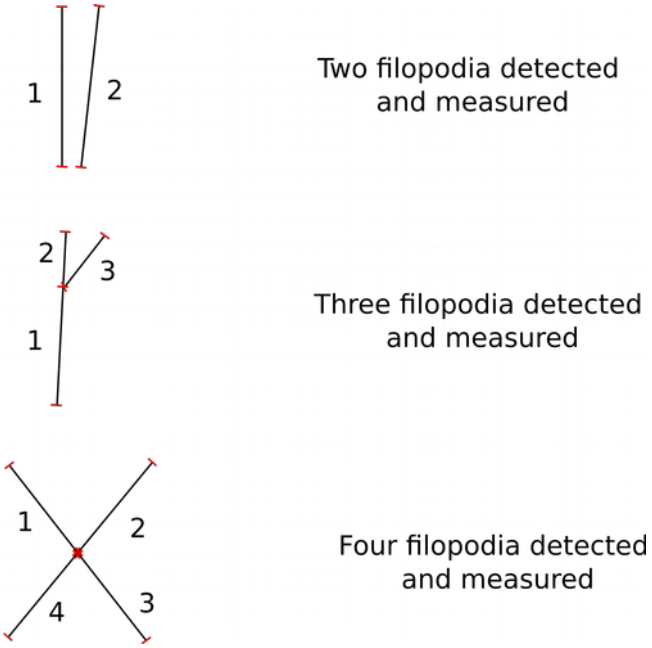
When the filopodia density is very high (maximal density depends on the imaging modality / resolution) two problems may arise:

- Individual filopodia can still be detected but can no longer be thresholded along their entire length; FiloQuant analyses will underestimate the filopodium's length.
- Individual filopodia can no longer be detected; FiloQuant will fail to count or detect filopodia properties.

High filopodia density may be resolved by improving the image resolution (see below for advice).

(III) Branching filopodia and crossing filopodia

FiloQuant can detect and quantify branching and crossing filopodia. However, in the present version of FiloQuant, each branch will be counted and measured as an independent filopodia (see below). This may result in an overestimation of filopodia numbers and an underestimation of true filopodia length. However, as the coordinates of each filopodia measured are also provided by the software, it is recommended to re-evaluate the data and to surmise if a filopodium has been incorrectly measured.



7) How to improve filopodia segmentation

FiloQuant will not work well on images with a low signal to noise ratio, or images taken with insufficient resolution to separate individual filopodia. This is due to the fact that filopodia will be very difficult to segment in these conditions. Below you will find a list of advice to improve image resolution / decrease image noise.

1. Tips to improve sample preparation

For fixed samples, in our hands, best results were obtained using the following protocol.

Cells were plated on high quality coverslip (#1.5) or high quality MatTek dishes (#1.5 or #1.7). Cells were fixed using 4% PFA for 10 min at room temperature. Cells were permeabilized using 0.25 % Triton for 3 min at room temperature and blocked with 1 M glycine for 30 min.

For short-term storage (weeks, recommended protocol):

Cells were incubated with Alexa Fluor 488 phalloidin at 4ºC (1/100 dilution in PBS) until imaging (minimum length of staining done overnight at 4ºC). Just before imaging, the sample was washed three times in PBS and mounted in vectashield media (or other soft mounting media).

For longer-term storage (months)

Cells were incubated overnight with Alexa Fluor 488 phalloidin at 4ºC (1/100 dilution in PBS) and washed three time with PBS. Coverslips were then mounted on slides using Mowiol (or other hard mounting media).

Live-cell imaging

For live cell imaging, we recommend the use of a bright green fluorescent protein (e.g. mEmerald, mClover3 or mNeonGreen) that is membrane targeted or tagged to an actin probe (e.g. Lifeact or Utrophin).

2. An example of ideal imaging set-up to improve filopodia detection:

The easiest way to improve filopodia segmentation by FiloQuant is to use high-end / super-resolution microscopes. However, this is also the less practical solution if such instruments are not available. Below is a list of imaging set-up we found to work best and to produce images that are efficiently segmented using FiloQuant.

(I) Structure illumination microscopy provides excellent image resolution and images acquired using this system work beautifully during subsequent segmentation of filopodia.

(II) Another option would be to acquire images using a microscope system (e.g. TIRF, spinning disk) equipped with high magnification and high NA objective (i.e. 100X) and low noise camera (Orca Flash 4 camera). The low noise camera generates ideal images for the efficient segmentation of filopodia.

(III) A third possibility would be to use an imaging system (e.g. TIRF, spinning disk) equipped with high magnification and high NA objective (i.e. 100X) and an EMCCD camera. If the image is too noisy and/or the resolution too low, following image acquisition, images can then be further processed using freely available software that improve image resolution and decrease noise. We found that the SRRF ImageJ plugin (Gustafsson et al., 2016) works very well for such purposes (https://bitbucket.org/rhenriqueslab/nanoj-srrf/wiki/Home).