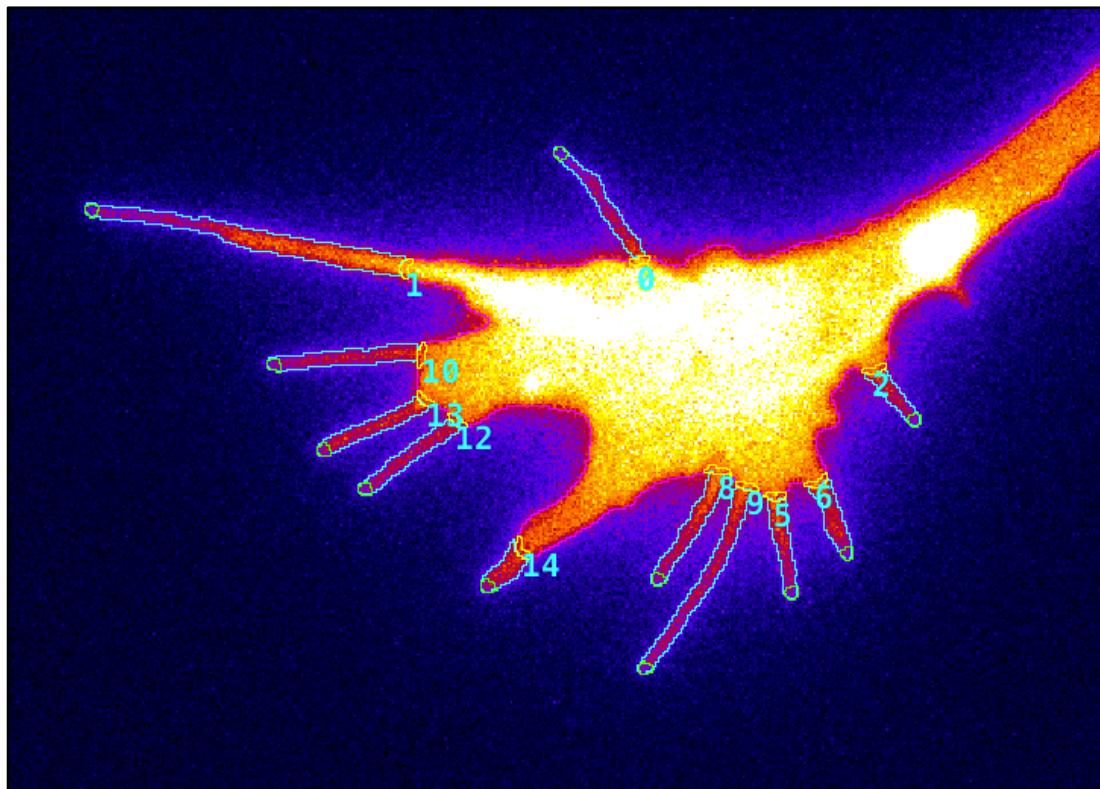


Filopodyan: User Guide



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1. Requirements and plugin installation

Filopodyan is a Fiji plugin for the analysis of thin protrusions (filopodia) in fluorescence microscopy timelapse images.

The Filopodyan plugin code is freely available at:

<https://github.com/gurdon-institute/Filopodyan/tree/master/Filopodyan>

Filopodyan was developed and extensively tested with Fiji Life-Line version June 2014, freely available for download from <https://imagej.net/Fiji/Downloads>.

With our latest release, we recommend using the latest version of Fiji (currently 2.0.0-rc-59/1.51n Java 1.8.0_66 [64-bit]).

Instructions for installation:

PC:

- download the plugin as a .jar file from the public Gurdon Institute github repository ([Filopodyan.jar](#))
- copy the downloaded Filopodyan.jar file into the Fiji plugins folder
- after relaunching Fiji, “Filopodyan” will appear in the “Plugins” menu

Mac:

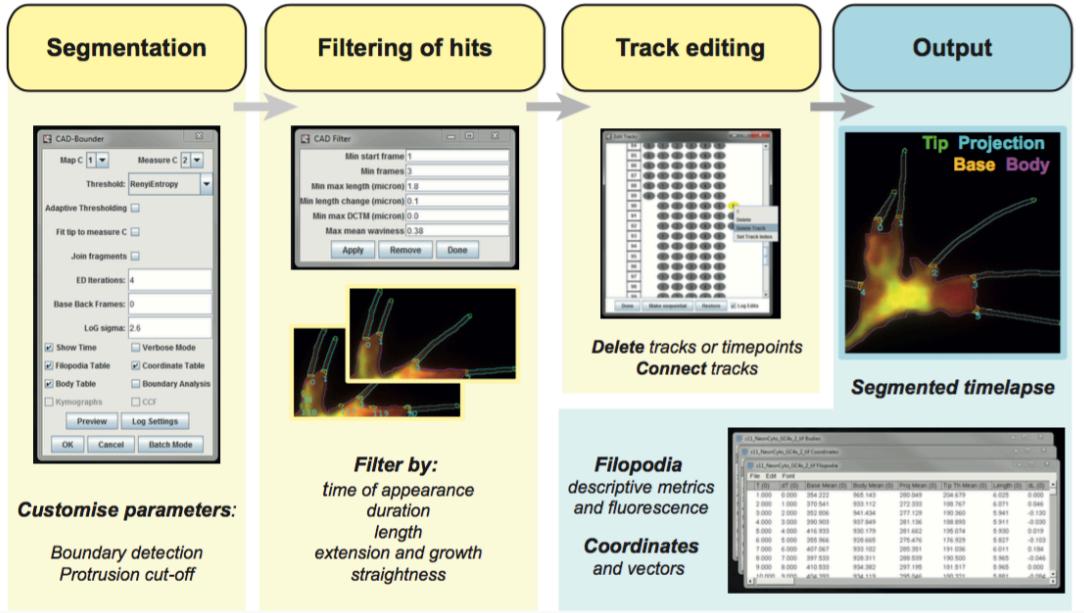
- download the plugin as a .jar file ([Filopodyan.jar](#))
- launch Fiji, go to Plugins > Install plugin, and select the downloaded file
- after relaunching Fiji, “Filopodyan” will appear in the “Plugins” menu

Test file:

A test file, ‘growth-cone-test-file.tif’, is provided in the github repository, alongside an associated text file detailing a set of settings suitable for segmenting this image.

2. Structure of Filopodyan: overview

Filopodyan has a modular structure as illustrated by the flowchart:



In step 1 of the analysis (**Segmentation**), the user selects appropriate parameters for image segmentation, aided by the Preview function, and selects the desired output.

In step 2 (**Filtering**), candidate structures are filtered through a filter that rejects false positive hits based on user-defined thresholds for parameters relating to length, shape, time in existence and movement of tracked structures.

In step 3 (**Track editing**), user has the option to remove structures from the reconstruction (either in single timepoints or across the entire timelapse) or to reassign their identity, as is deemed necessary.

Finally, user is presented with the **output**: an image file containing an overlay visualizing the segmentation across the timelapse, and data tables containing information on the parameters for each of the reconstructed structures.

3. Step-by-step instructions for use

3.0. Considerations for imaging

3.0.1. Morphology marker

Filopodyan was developed using a membrane-localising fluorescent protein (GAP-RFP) to visualize cell morphology. It is possible to use other markers (cytoplasmic or cytoskeletal fluorescent proteins) but the quality of resulting reconstructions is likely to suffer. It is currently not possible to use brightfield images with Filopodyan.

3.0.2. Camera pixel size

Smaller pixel size allows for better reconstructions. We obtained best results at 65 nm per pixel or below. Larger pixel size precludes separating structures near to each other.

3.0.3. Out-of-focus light

For best results, minimize out-of-focus light. On a TIRF/HILO imaging setup, this can be achieved by optimizing the angle of illumination; on a line-scanning confocal microscope, by reducing pinhole size.

3.1. Segmentation

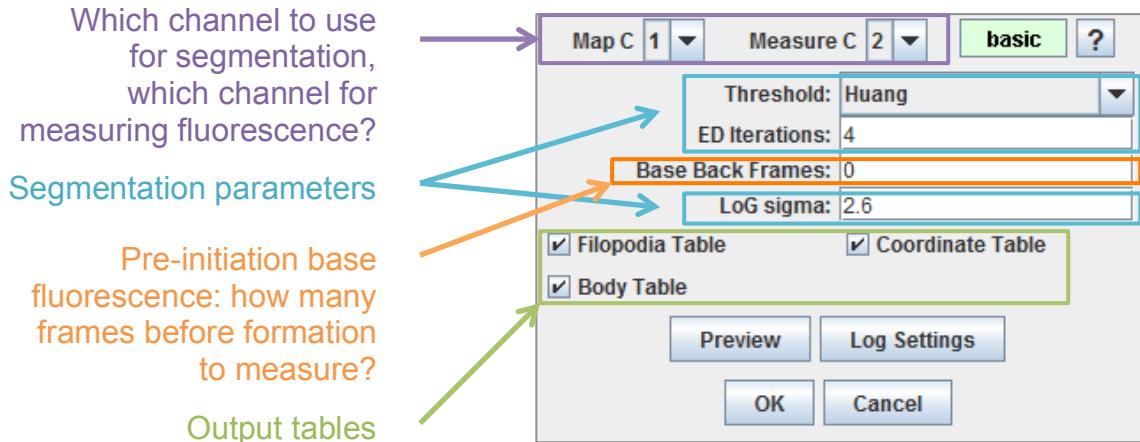
3.1.1. Import image sequence into Fiji as a calibrated .tiff file.

Crop the image so as to remove areas containing confounding sources of signal (e.g. other cells visible in the field of view). Avoid overcropping – the presence of a larger area containing background may be helpful with some settings.

3.1.2. Open Filopodyan plugin.

Menu location: *Plugins > Filopodyan*. The image file to be analysed needs to be the selected active window when opening the plugin. If no active image window is open when Filopodyan is initiated, it launches in Batch mode.

3.1.3. Set “Map C” as the channel used for mapping the cell morphology, and “Measure C” as the channel used for fluorescence measurements.



The graphical user interface (GUI) of Filopodyan (in basic mode).

3.1.4. Identify suitable **parameters for boundary mapping**, using the Preview function to visualize results on a single timepoint.

Parameters to alter at this stage include:

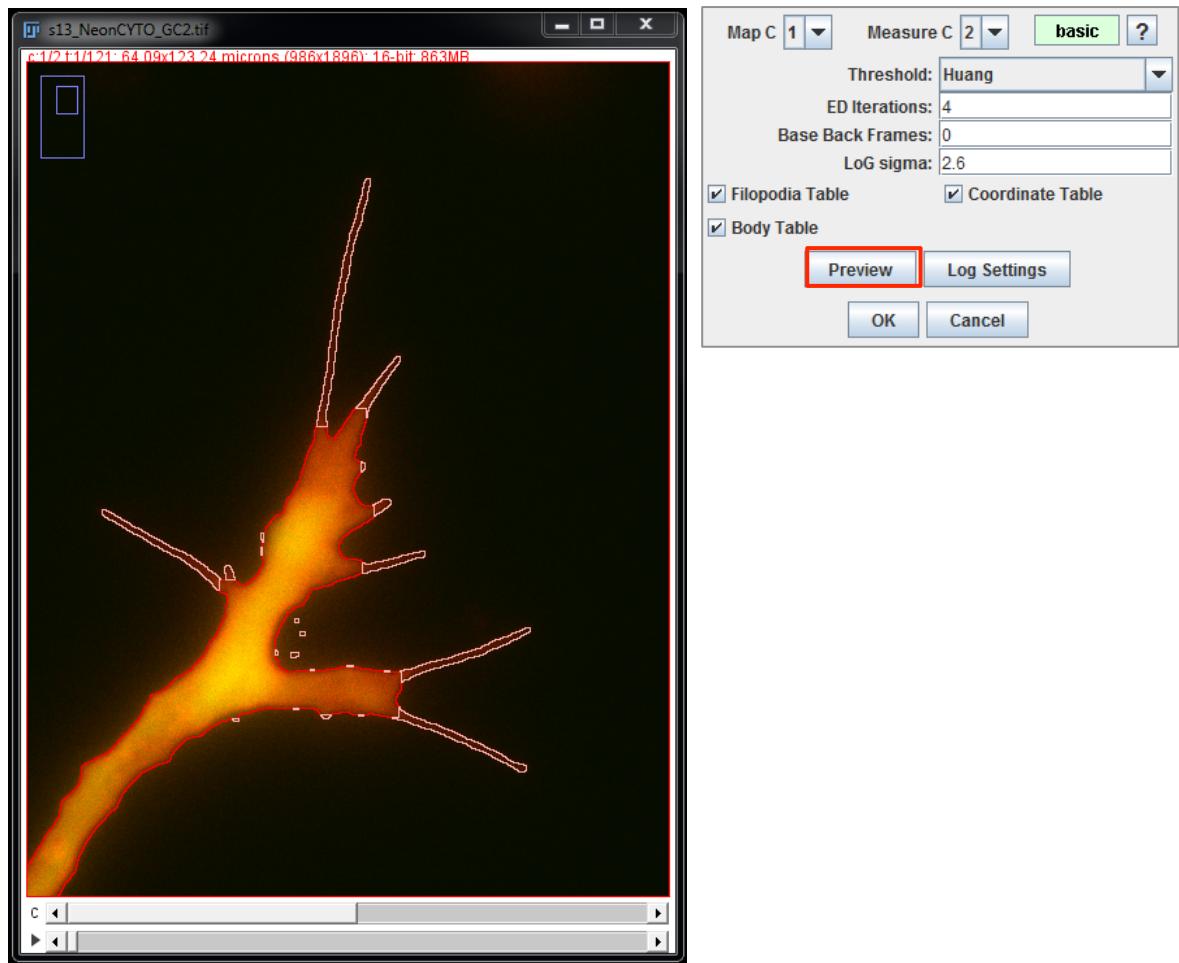
A. Thresholding method

Different thresholding methods perform differently on datasets with different signal-to-noise ratio (SNR) and uniformity of signal. As a rule of thumb, we found Renyi Entropy and Huang to be most valuable for accurate reconstructions in images with a good SNR, and the more permissive Triangle method tended to be more valuable at images with low SNR where Renyi and Huang failed to produce accurate segmentation.

B. LoG Sigma

Higher LoG is better for larger/thicker structures; lower LoG is better for smaller/thinner structures. In our experience, values between 2.6 and 4.1 have been most useful (at 65 nm/pixel).

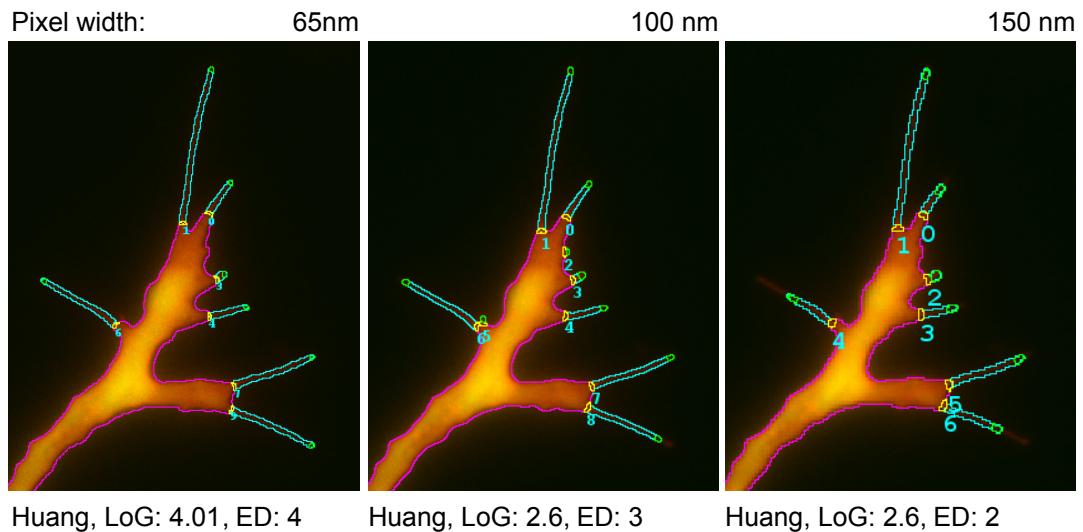
Preview various combinations of the thresholding method and the value LoG sigma on a selected image slice. When finding a combination that seems suitable, preview the combination on other slices within the stack, tweaking the parameters until satisfied with the position of the mapped cell boundary.



Filopodyan interactive Preview window displays a rapid estimate of the reconstruction with given parameters for the currently displayed timepoint.

3.1.5. Identify suitable parameters for protrusion detection (ED Iterations), using Preview button to visualize results in a selected timepoint.

Larger ED number – cut-off occurs closer to cell centre (larger protrusions); smaller ED number – cut-off occurs more distally.

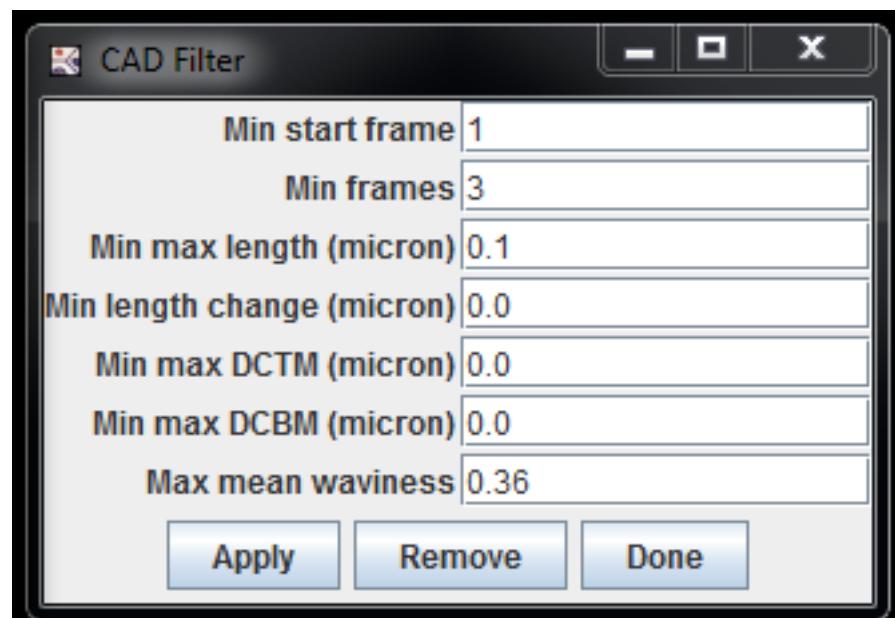


Initial guidelines for segmentation parameters depending on image pixel size. Smaller pixel dimensions are recommended; at pixel widths >100 nm image resampling may be required.

3.1.6. When satisfied with the combination of parameters for boundary mapping and protrusion detection, apply the parameters to image stack (“OK”).

3.2. Automated filtering

3.2.1. Using the CAD Filter GUI window, set the parameter thresholds used to filter candidate hits.



The graphical user interface for the CAD Filter component of the Filopodyan plugin.

Parameters used for filtering candidate hits:

min start frame

sets the minimum starting frame of the candidate structure

min start frame = 1 keeps all candidates

min start frame = 2 keeps only newly formed structures that do not exist in frame one

min frames

sets the minimum number of frames that a structure needs to exist in order to pass the filter

min frames = 1 keeps all candidates

min frames = 3 keeps those candidates whose tracks span 3 or more timepoints

min max length

sets the length that needs to be reached by a tracked structure over the course of its tracked lifetime in order to pass the filter

min max length = 0 keeps all candidates

min max length = 2 keeps those candidates whose max length over time exceeds 2 μm

(NB: correct image calibration is required)

min max dL

sets the minimal change in length between timepoints that a candidate structure is required to attain during its tracked lifetime in order to pass the filter.

min max dL = 0 keeps all candidates

min max dL = 0.1 rejects all candidates whose change in length never exceeds 0.1 μm

min max DCTM

sets the minimal direction-corrected tip movement between timepoints that a candidate structure is required to attain during its tracked lifetime in order to pass the filter

min max DCTM = 0 keeps all candidates

min max DCTM = 0.1 rejects all candidates whose tip movement never exceeds 0.1 μm throughout their tracked lifetime

min max DCBM

sets the minimal direction-corrected base movement between timepoints that a candidate structure is required to attain during its tracked lifetime in order to pass the filter

min max DCBM = 0 keeps all candidates

min max DCBM = 0.1 rejects all candidates whose base movement never exceeds 0.1 μm throughout their tracked lifetime

max mean waviness

sets the maximum of the mean waviness that a candidate structure can possess across its tracked lifetime in order to pass the filter. (waviness = 1 – straightness)

max mean waviness = 1 keeps all candidates (true? check this!)
max mean waviness = 0.4 rejects all candidates whose mean waviness over time exceeds 0.4 (i.e. whose mean straightness over time is below 0.6)

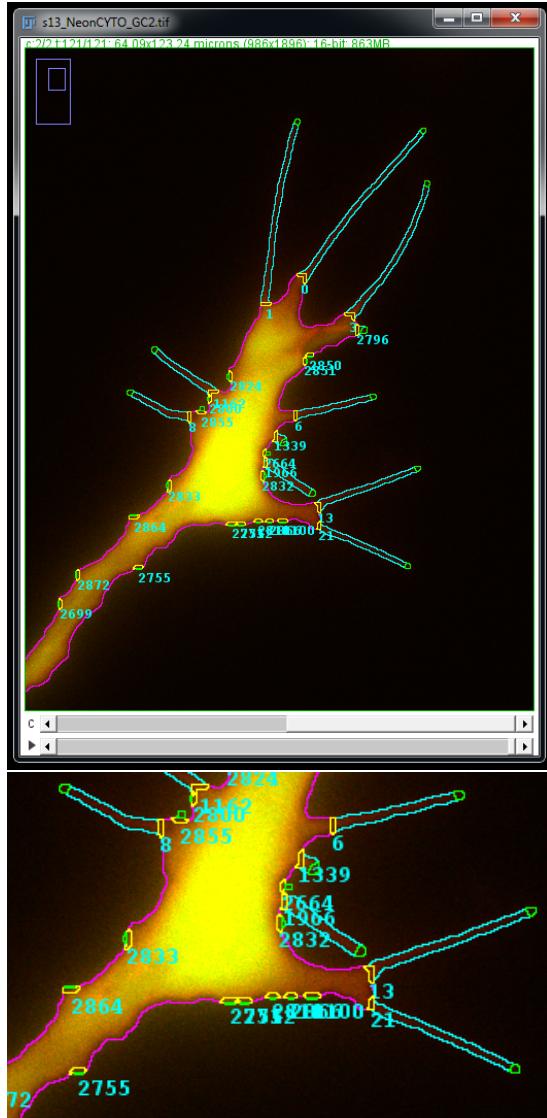
We found the following thresholds useful for candidate filtering in published datasets of growth cone filopodia acquired at the time resolution of 2 s:

min start frame = 1
min frames = 3
min max length = 1.8
min max dL = 0.1
min max DCTM = 0.0
min max DCBM = 0.0
max mean waviness = 0.36

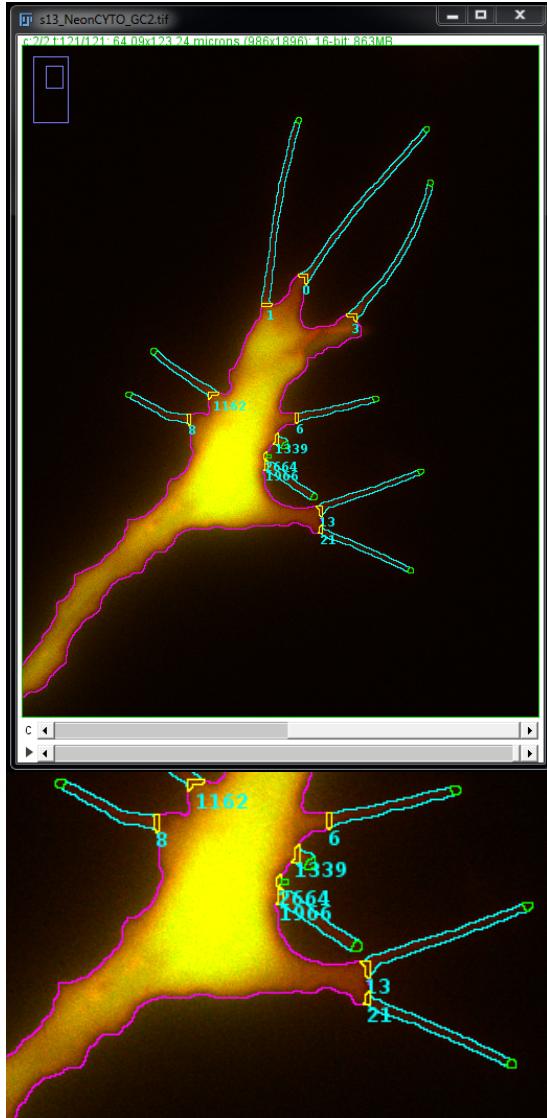
These values are provided for rough guidance only. Suitable or optimal parameters will vary greatly depending on the application.

3.2.2. Selecting “Apply” in the CAD GUI window will apply the selected thresholds and display an updated overlay showing only the candidates retained after the filter. “Remove” removes the filter and restores the original reconstruction from the initial segmentation, retaining all candidates. Toggle between “Apply” and “Remove”, trying different settings to see their effect on the resulting filtered image. Proceed with “Done” when happy with the settings.

Before filtering



After applying CAD Filter

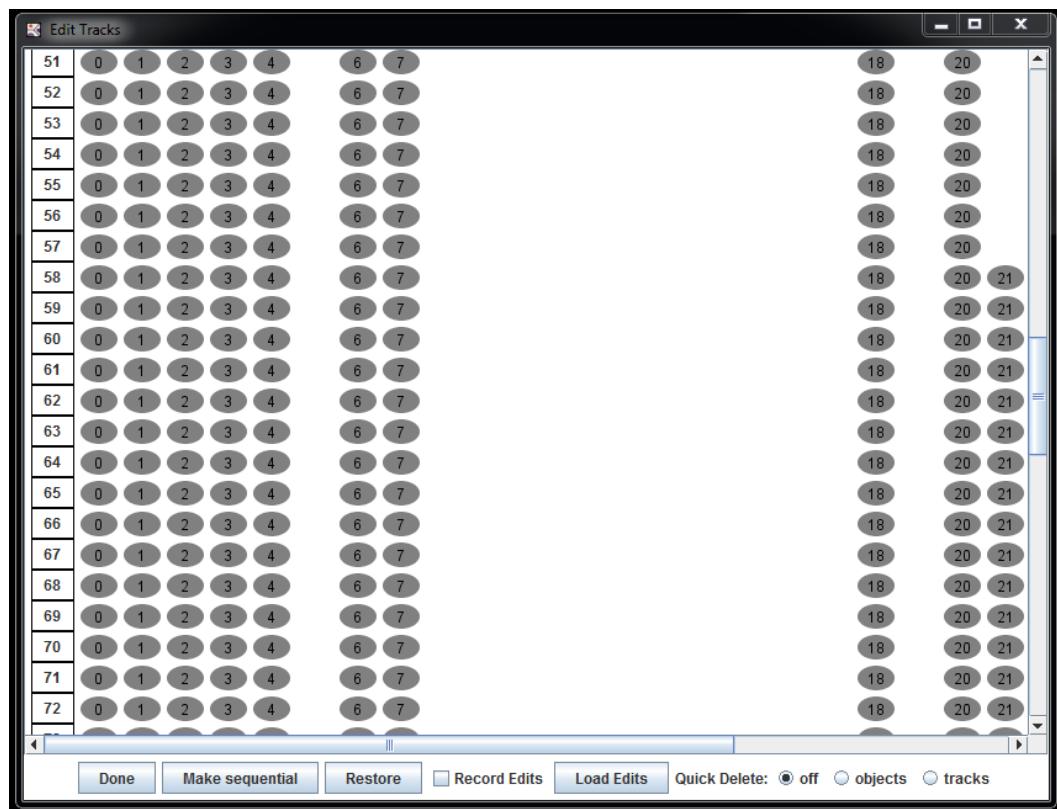


Applying automated candidate filtering using the set parameter thresholds removes many false positive hits.

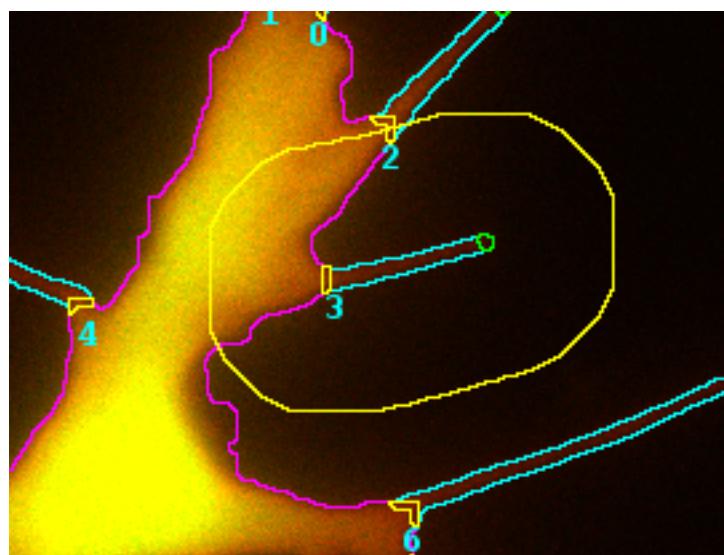
3.3. Manual editing

Upon completion of the customized automated filtering, the user may alter the reconstruction using the options provided in the Edit Tracks GUI.

In the Edit Tracks GUI, each grey oval represents a single structure in a single timepoint, visually representing all reconstructed structures (as columns) across all timepoints (as rows).



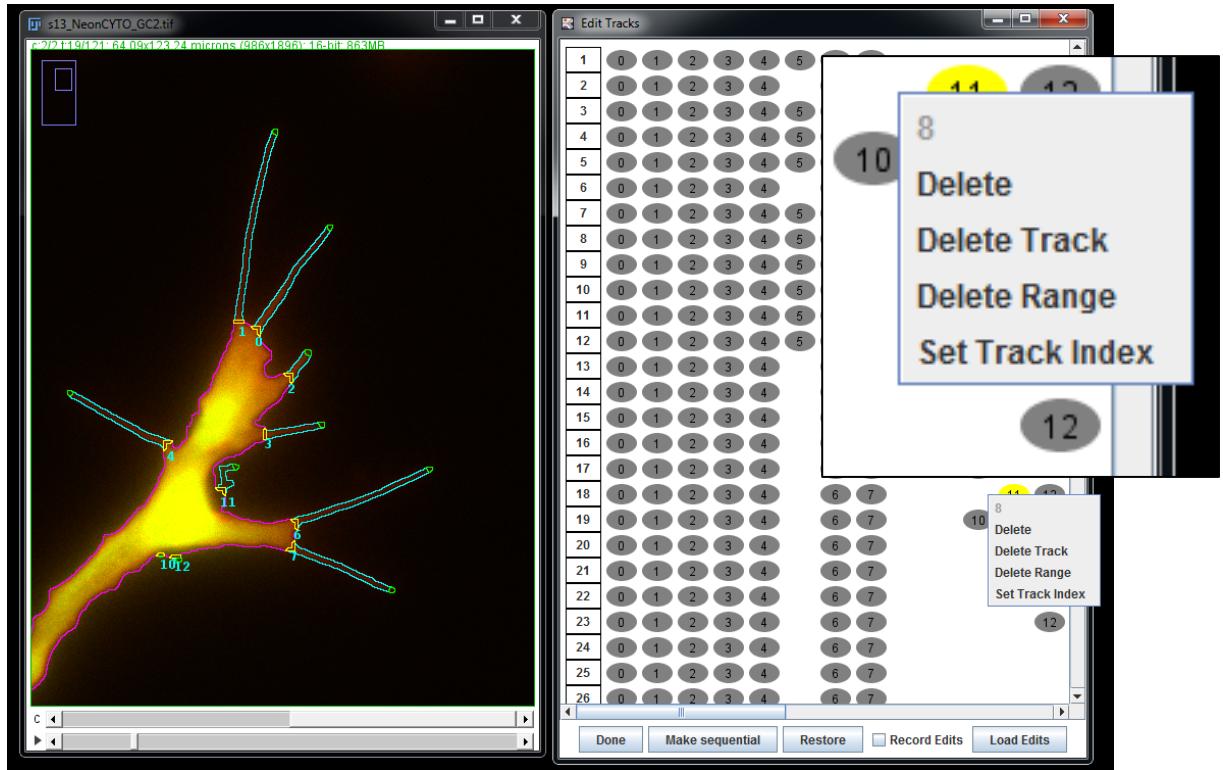
The GUI of the Edit Tracks module for manual editing of the reconstruction.



For ease of navigation, a single click on one of the grey ovals highlights the selected structure in the selected timepoint with an animated yellow circle.

3.3.1. Manually removing structures from reconstruction

Structures can be removed by user by right-clicking onto a grey oval within the Edit Tracks GUI and selecting from the provided options.



Direct manual editing of the reconstruction in the Edit Tracks module.

“Delete”: removes structure from the reconstruction only in the selected timepoint.

“Delete Track”: removes tracked structure from the reconstruction across the entire timelapse.

“Delete Range”: user may define the range of timepoints to delete.

“Set Track Index”: reassign identity of the selected track; see Section 3.3.2 (Manually reassigning identity) below.

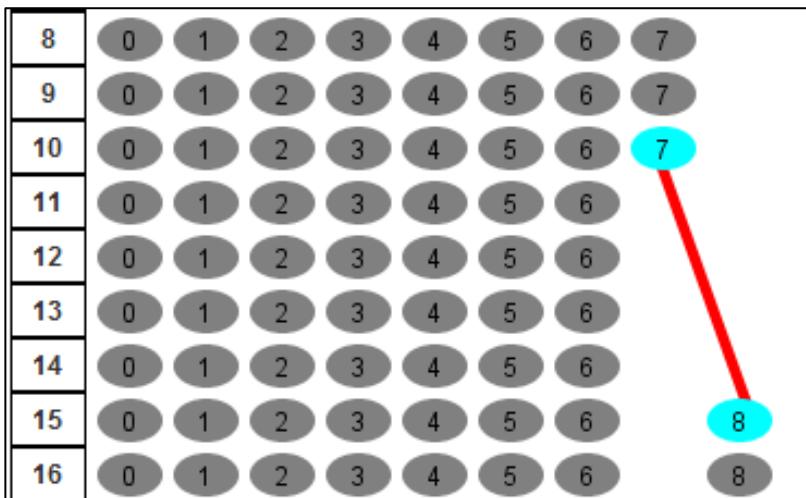
Single reconstructed structures and tracks can also be removed using shortcuts; see section 3.3.3 (Shortcuts) below.

3.3.2. Manually reassigning identity

Identity of tracked structures can be reassigned by the user. For instance, what a human experimenter might deem to be a single filopodium undergoing small movement over time might mistakenly be reconstructed by Filopodyan as two (or more) disconnected tracks. Such cases can be manually corrected, using one of the following options:

A) Direct connect within Edit Tracks GUI:

Dragging a line between two oval representations will join into a single track the reconstructed tracks that they correspond to.



Connecting tracks in the Edit Tracks GUI.

Restriction: if the tracks co-exist in any timepoints, this would lead to a duplicated structure in that timepoint. Those potential duplications need to be removed prior to joining tracks.

B) Manual identity reassignment

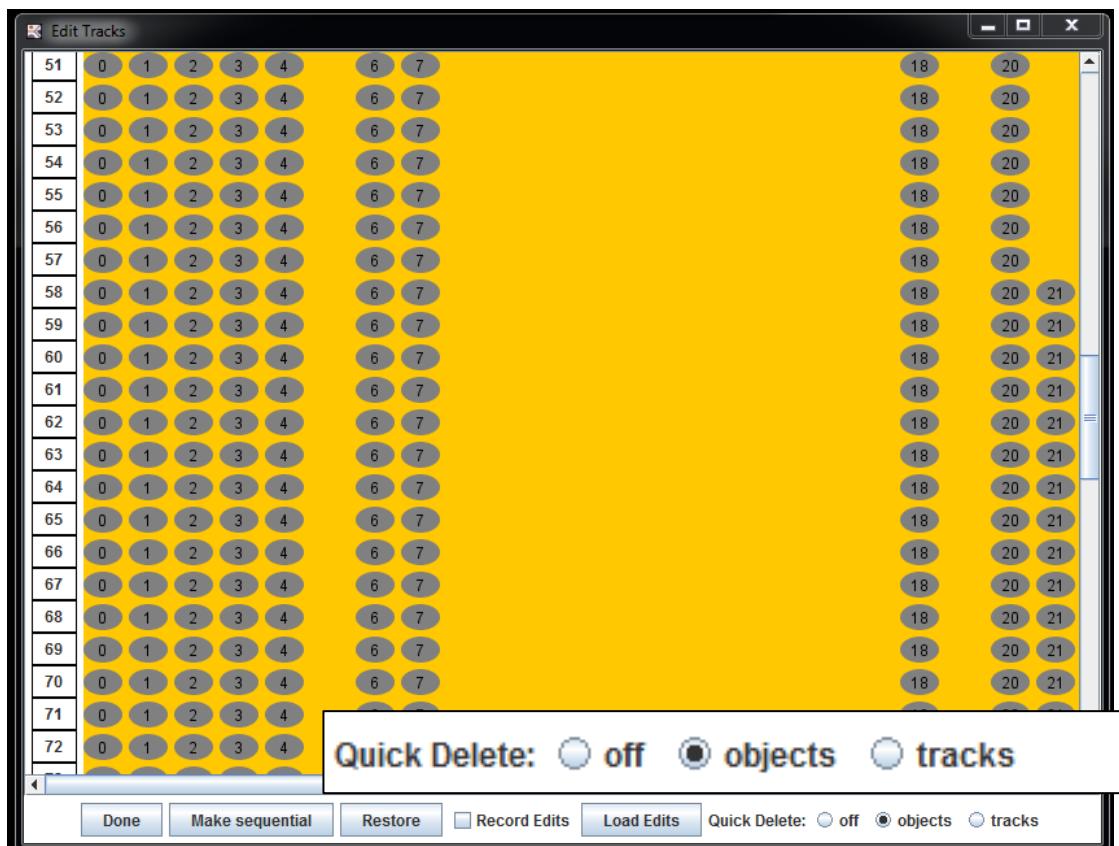
Right-clicking on an oval within the Edit Tracks GUI provides the option to “Set Track Index”, allowing the user to change the index (number) of the selected structure.

3.3.3. Shortcuts

The following shortcuts are provided for easier work with the Edit Track GUI.

“Quick Delete: objects”: right click on a grey oval deletes the structure from the reconstruction in one timepoint only.

“Quick Delete: tracks”: right click on a grey oval deletes the tracked structure from the reconstruction across the entire timelapse.



Shortcuts for quick deletion of objects/tracks in the Edit Tracks Window.

3.3.4. Other useful options in Edit Tracks window:

“Make sequential”: adjusts the indices (numbers) of tracks so that they form a continuous sequence with no gaps in numbering.

“Record Edits”: Keeps a log of all manual edits, to be saved in a separate text file.

“Load Edits”: Asks for a log of recorded edits to reapply the same edits on the same image (e.g. if reproducing a previous analysis).

“Record Edits” needs to be deselected when loading edits

“Restore”: Undoes all manual edits and restores the initial state of reconstruction as it was immediately after applying CAD Filter.

3.4. Plugin output

Note: For compatibility with downstream data processing scripts in R (available at <https://github.com/gurdon-institute/Filopodyan/tree/master/FilopodyanR>), save table files as .txt files in a tab delimited format.

3.4.1. Filopodia table

The Filopodia table provides basic information relating to shape, movement and fluorescence measurements for each reconstructed structure at every timepoint.

All rows are aligned according to the dT column (relative time in existence).

s13_NeonCYTO_GC2.tif Filopodia												
T (0)	dT (0)	Base Mean (0)	Body Mean (0)	Proj Mean (0)	Tip Mean (0)	Tip Th Mean (0)	Length (0)	dL (0)	DCTM (0)	DCBM (0)	T (1)	dT (1)
1.000	0.000	284.413	767.951	188.849	148.478	159.818	3.877	0.000	NaN	NaN	1.000	0.000
2.000	1.000	291.326	744.415	195.628	146.391	154.222	4.106	0.230	0.198	-0.090	2.000	1.000
3.000	2.000	279.167	741.381	188.256	140.708	148.833	4.133	0.027	0.181	0.053	3.000	2.000
4.000	3.000	287.619	738.152	201.848	152.143	164.944	4.761	0.628	0.037	-0.432	4.000	3.000
5.000	4.000	282.061	737.202	199.305	147.714	175.875	4.861	0.100	0.053	-0.181	5.000	4.000
6.000	5.000	290.111	744.525	203.663	154.478	168.462	5.110	0.249	0.000	-0.181	6.000	5.000
7.000	6.000	317.750	737.766	203.752	148.222	158.053	5.783	0.674	0.144	-0.469	7.000	6.000
8.000	7.000	289.595	742.972	198.568	146.240	155.737	5.691	-0.092	0.234	0.325	8.000	7.000
9.000	8.000	317.786	741.253	196.968	147.292	160.067	6.051	0.360	0.198	-0.181	9.000	8.000
10.000	9.000	312.978	740.138	195.014	149.913	155.889	6.292	0.241	0.325	0.234	10.000	9.000

Parameter measurements in the Filopodia table	
T	Timepoint within the timelapse (absolute time)
dT	Timepoint relative to first moment in existence
Base Mean	Mean pixel intensity of the base (or predicted base) area
Body Mean	Mean pixel intensity of the cell/growth cone body area
Proj Mean	Mean pixel intensity of the protrusion (projection) area
Tip Mean	Mean pixel intensity of the tip area
Tip Th Mean	Mean pixel intensity of Otsu-thresholded tip area
Length	Estimated protrusion length (half perimeter, corrected for base and curvature)
dL	Change in Length from preceding timepoint
DCTM	Direction-corrected tip movement (identical to [tip(t-1), tip(t)] : [base,tip] in Coordinates table)
DCBM	Direction-corrected base movement (identical to [base(t-1),base(t)] : [base,tip] in Coordinates table)

3.4.2. Coordinates table

The Coordinates table provides additional information on the coordinates and vectors calculated by CAD-Boudner for each reconstructed structure.

	T (0)	Base X (0)	Base Y (0)	Tip X (0)	Tip Y (0)	$\ [\text{body},\text{base}]\ (0)$	$\ [\text{base},\text{tip}]\ (0)$
1	0.000	40.885	33.280	42.705	30.615	32.461	3.227
2	1.000	40.820	33.345	42.770	30.420	32.397	3.515
3	2.000	40.820	33.280	42.900	30.290	32.397	3.642
4	3.000	40.625	33.670	42.965	30.290	32.086	4.111
5	4.000	40.495	33.800	42.965	30.225	31.995	4.345
6	5.000	40.365	33.930	42.965	30.225	31.866	4.526
7	6.000	40.105	34.320	43.030	30.095	31.293	5.139
8	7.000	40.300	34.060	43.160	29.900	31.656	5.048
9	8.000	40.170	34.190	43.225	29.705	31.513	5.427
10	9.000	40.300	33.995	43.420	29.445	31.852	5.517

Parameter measurements in the Coordinates table	
dT	Timepoint relative to moment of formation
Base X	X position of the base
Base Y	Y position of the base
Tip X	X position of the base
Tip Y	Y position of the base
$\ [\text{body},\text{base}]\ $	Euclidean distance from body centroid to base
$\ [\text{base},\text{tip}]\ $	Euclidean distance from base to tip
$\ [\text{base},\text{tip}]\ : [\text{body},\text{base}]$	The projection of the base-tip vector onto body-base vector
$[\text{base},\text{tip}] \cdot [\text{body},\text{base}]$	= “angle-corrected tip distance”; dot product of the (base,tip) and (body,base) vectors
$\Delta \ [\text{base},\text{tip}]\ : [\text{body},\text{base}]$	= “angle-corrected extension”; change in the projection of (base,tip) vector onto (body,body) vector
$\ [\text{tip}(t-1), \text{tip}(t)]\ : [\text{base},\text{tip}]$	= “direction-corrected tip movement”; the projection of Euclidean tip displacement onto the (base,tip) vector (= direction-corrected tip movement)
$\ [\text{base}(t-1), \text{base}(t)]\ : [\text{base},\text{tip}]$	= “direction-corrected base movement”; the projection of Euclidean base displacement onto the (base,tip) vector

As in Filopodia table, the index given in parentheses next to the parameter name means that the measurements in that column correspond to the filopodium with the number provided by that index. E.g. the column “Tip X (1)” provides tip positions over time (in rows) for filopodium number 1.

3.4.3. Bodies table

The Bodies table provides information on the coordinates of the body centroid and measurements relating to the body of the cell/growth cone analysed.

T	X	Y	Mean	StdDev
1.000	21.450	59.280	767.951	345.332
2.000	21.580	59.410	744.415	330.870
3.000	21.580	59.345	741.381	332.948
4.000	21.645	59.540	738.152	331.609
5.000	21.580	59.605	737.202	332.864
6.000	21.580	59.670	744.525	333.155
7.000	21.580	59.540	737.766	337.649
8.000	21.515	59.540	742.972	336.217
9.000	21.450	59.540	741.253	334.224
10.000	21.450	59.670	740.138	339.130
11.000	21.450	59.605	744.036	335.693
12.000	21.385	59.735	744.994	342.621
13.000	21.385	59.865	743.250	343.325

Parameter measurements in the Bodies table

T	Timepoint within the timelapse
X	X coordinate of body centroid
Y	Y coordinate of body centroid
Mean	Mean fluorescence intensity within the body ROI
StdDev	SD of fluorescence intensity within the body ROI

4. Advanced options

- Tip Fitting

If the tip fitting option is enabled, Filopodyan will use the signal in the channel defined as measurement channel (“Measure C”) to reposition the initially assigned tip positions so as to match the detected signal. This option is useful for quantifying the fluorescence of proteins of interest within filopodia tips. However, we warn against its use for phenotypic comparisons between conditions, especially if different proteins differ in the extent to which they localize to filopodia tips (e.g. GFP-VASP and GFP-cyto).

- Fragment Joining

With fragment joining option enabled, Filopodyan will join the area of strong signal intensity with the closest point on the detected object boundary. This is useful if filopodia are partially out of focus, or if poor signal to noise ratio obstructs successful reconstruction of filopodia length in their entirety.

- Adaptive Thresholding

At poor signal-to-noise ratio, adaptive thresholding is occasionally desirable and may perform better than global thresholding. Optimal thresholding method and parameters to use in conjunction with adaptive thresholding may differ substantially from those without adaptive thresholding, so an independent parameter optimization is recommended.

- Loading saved track edits

If reconstructed tracks are edited manually, these edits can be easily saved and reapplied if it is necessary to repeat the analysis. In order to do so, save the tracked edits tab in log window (as a text file); when repeating the analysis (on an identical image file with the same parameters), load edits by using “Load Edits” option in the “Edit Tracks” window, selecting the previously saved edits file.