

Workshop structure (ref)

Part 1

Introduction

What are filopodia and how have they been studied

Part 2

Intro to Filopodyan workflow (Fiji)

A guided walk through the plugin for segmentation & tracking, test using a simple demo file

Part 3

Intro to Filopodyan workflow (R)

Phenotype comparison; correlations between properties; filopodium initiation; tip elongation

Part 4

a. Analysis: Phenotype comparison

b. Analysis: Fluorescence & tip movement

Batch processing (Fiji) and downstream analysis
Tip fitting, direction-corrected tip movement, cross-correlation analysis

Part 5:

Deconstruction

Filopodyan: Filopodia Dynamics Analysis

1. Introduction to the plugin (from a user's point of view)
 - segmentation, tracking, filtering, editing and output
2. Demonstration on a simple sample file
3. Code structure overview
4. Various possible definitions for length, movement, etc.
5. Discussion on manual curation of segmentations

Installation

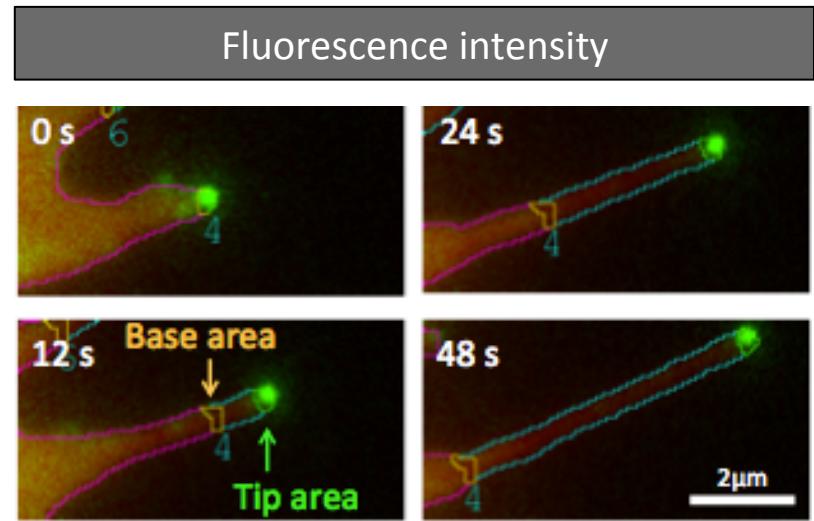
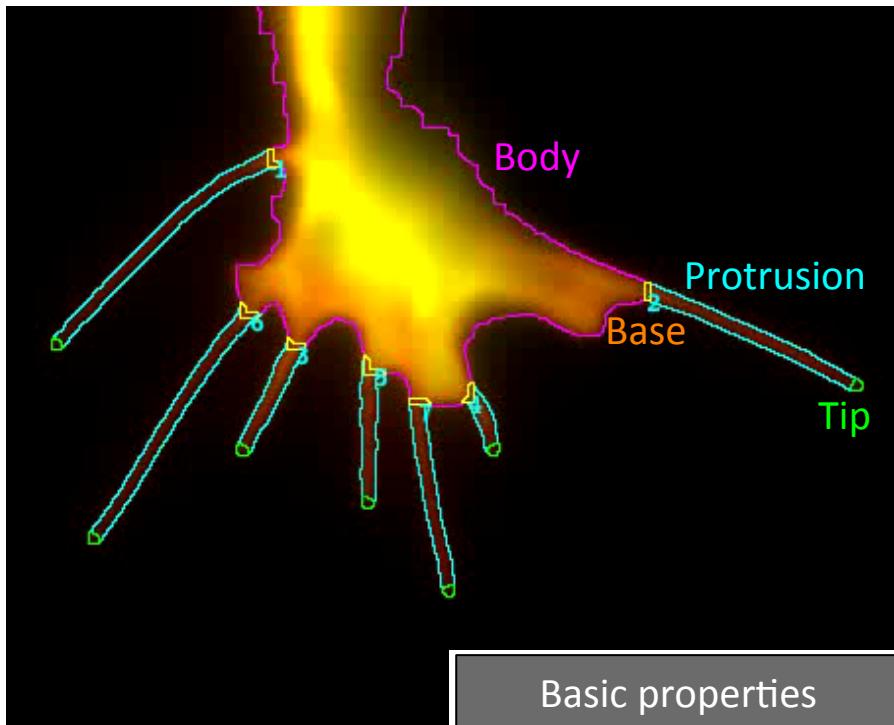
(1: easier to deconstruct, labile to Fiji/Java updates etc.)

1. Latest version of Fiji
2. Install the plugin as per instructions
3. Data:
 - Essential.zip (small) – sufficient to follow the workshop
 - Extended.zip - datasets for extended analysis etc.

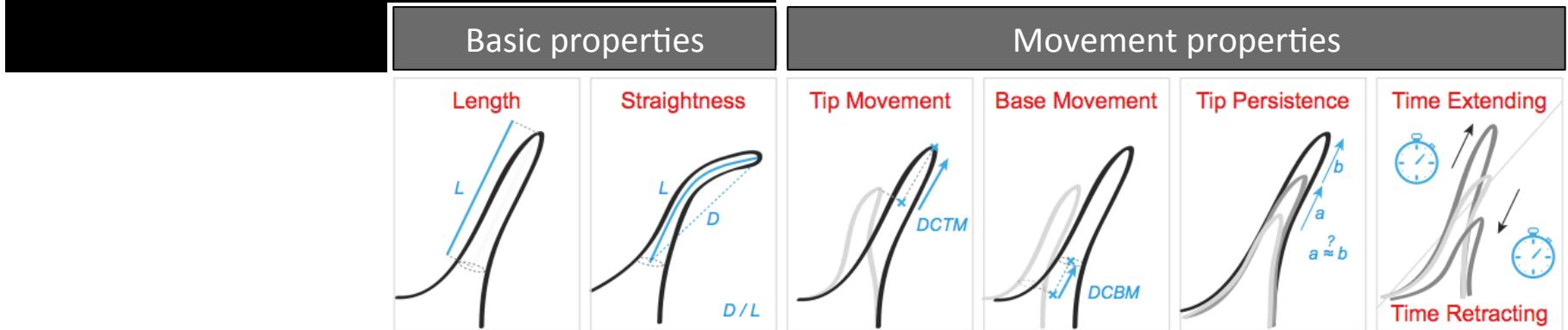
Installation (2: Robust)

1. Download Lifeline Fiji June 2014
(name it Fiji_lifeline14 before installation so it can coexist with latest version)
2. Insert CAD-Bounder_.jar into plugins folder
3. Data:
 - Essential.zip (small) – sufficient to follow the workshop
 - Extended.zip - datasets for extended analysis etc.

Filopodyan: Filopodia Dynamics Analysis

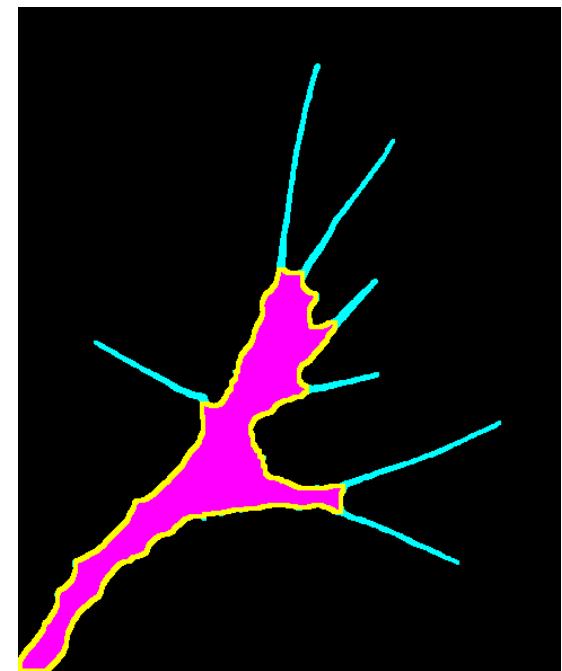
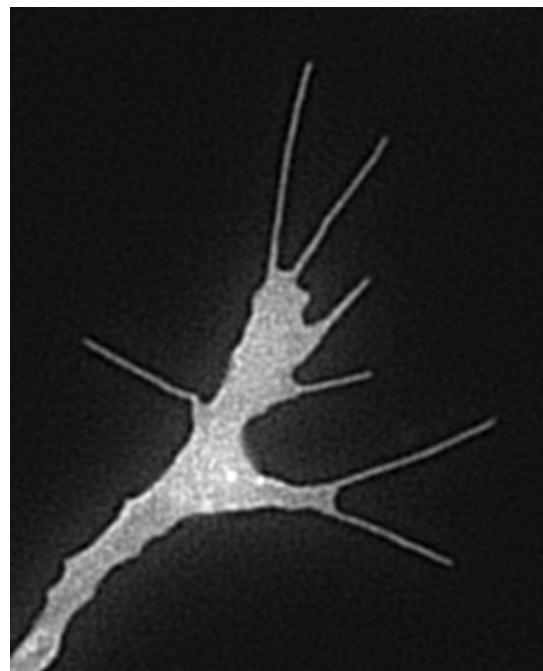
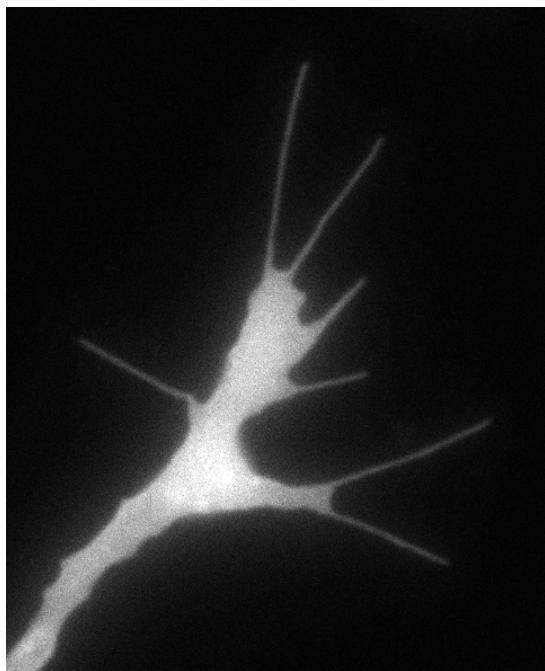


Tracking and analysis of filopodium dynamics:



Filopodyan: 1. Segmentation

Segmentation

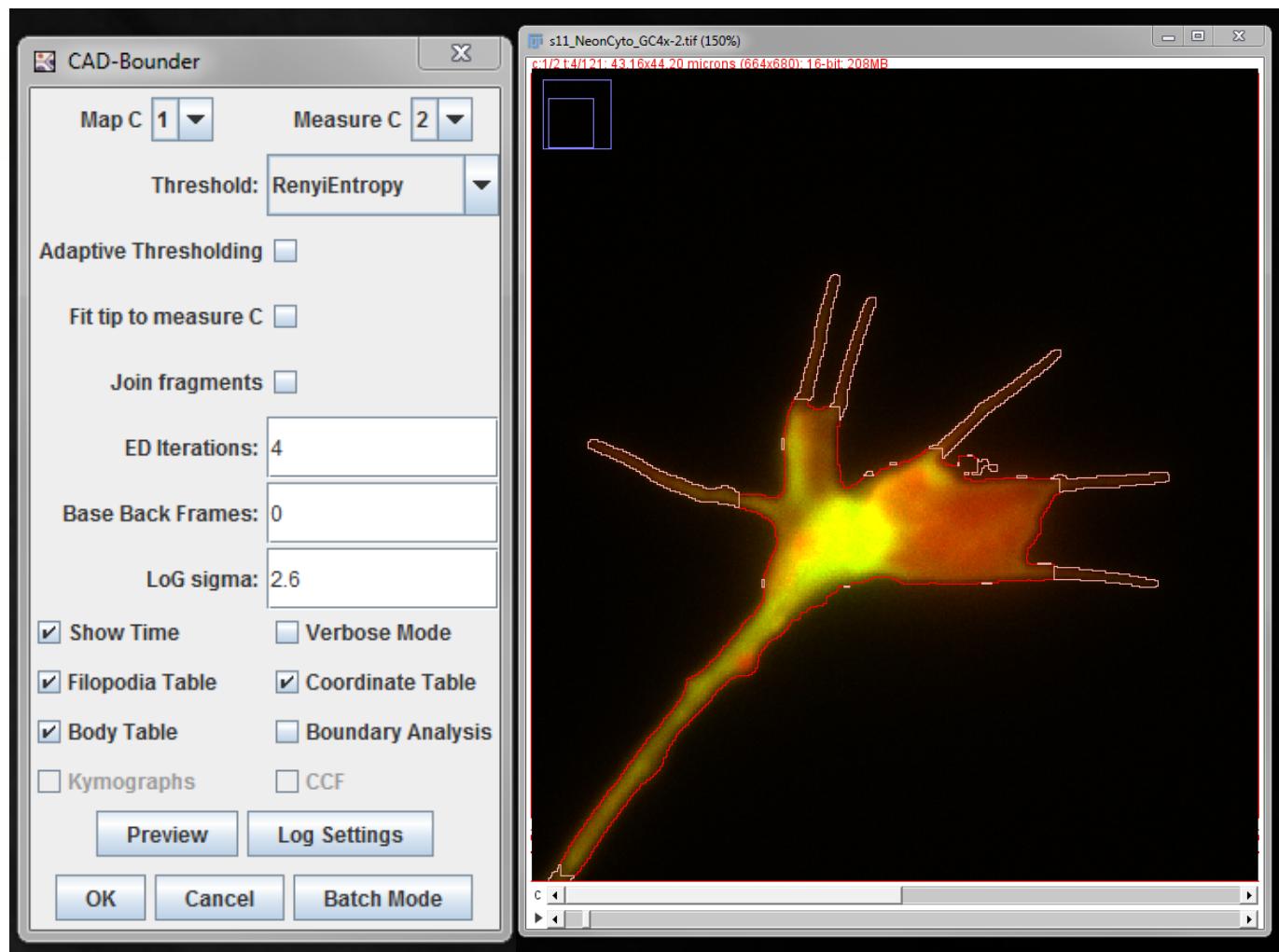


Filopodyan: 1. Segmentation

Segmentation

Customise parameters for:

- boundary detection
- protrusion cut-off



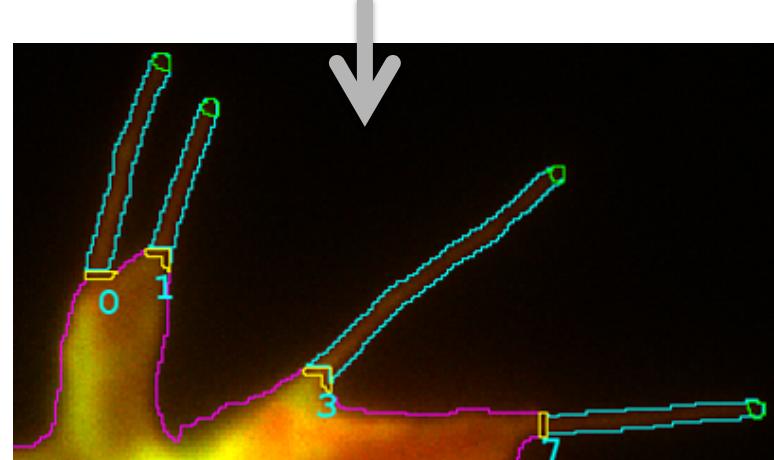
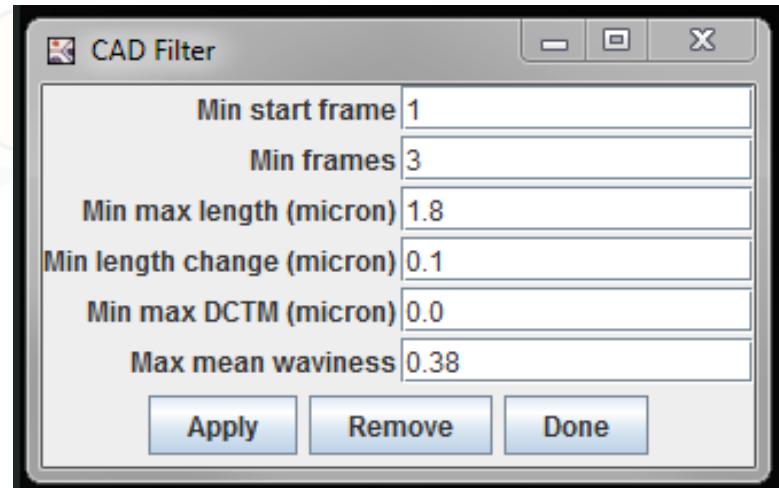
Filopodyan: 2. Object filtering

Segmentation

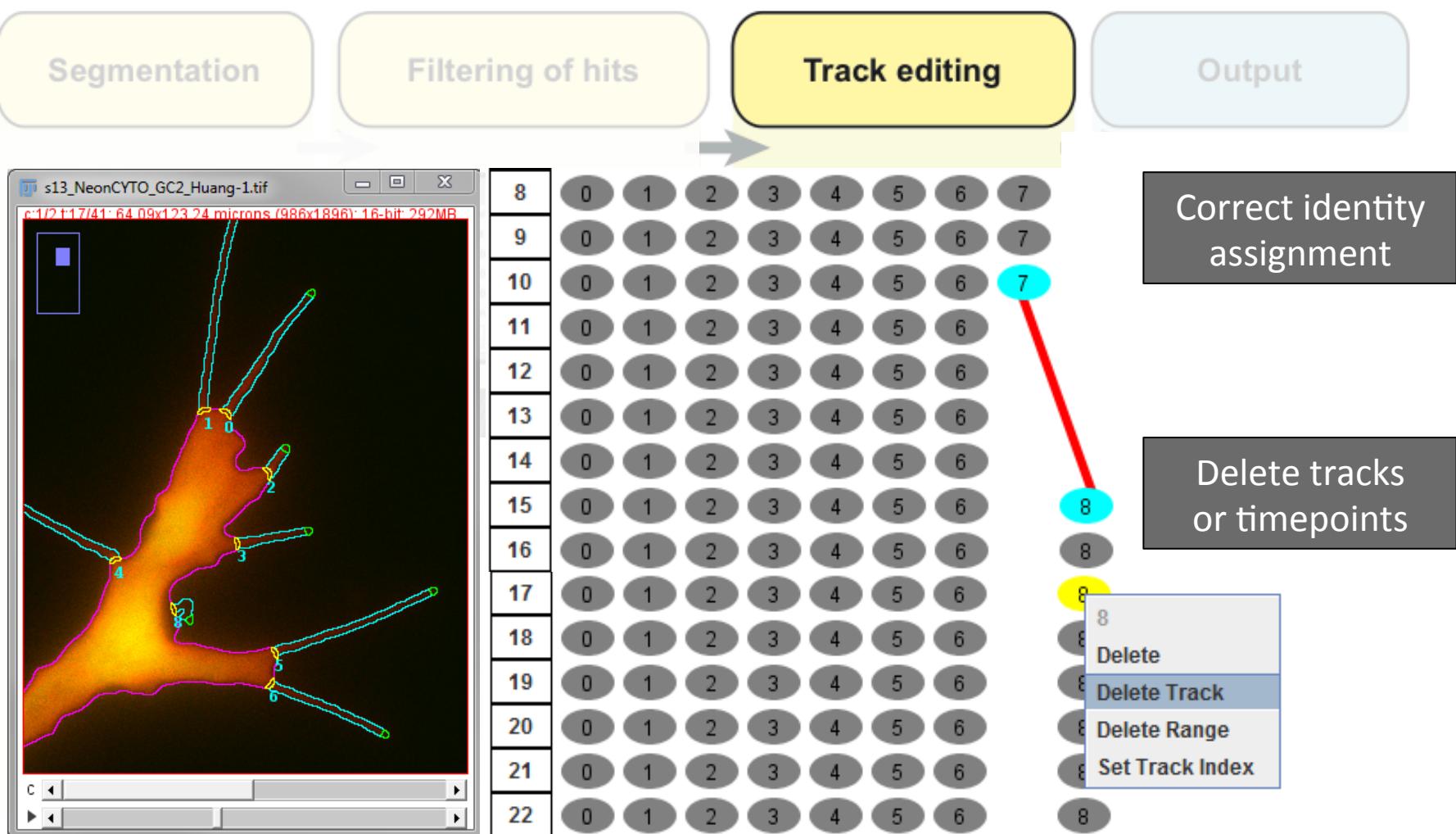
Filtering of hits

Filtering criteria:

- length
- straightness
- duration
- tip movement
- straightness/waviness
- time of appearance



Filopodyan: 3. Track editing



Filopodyan: 4. Output

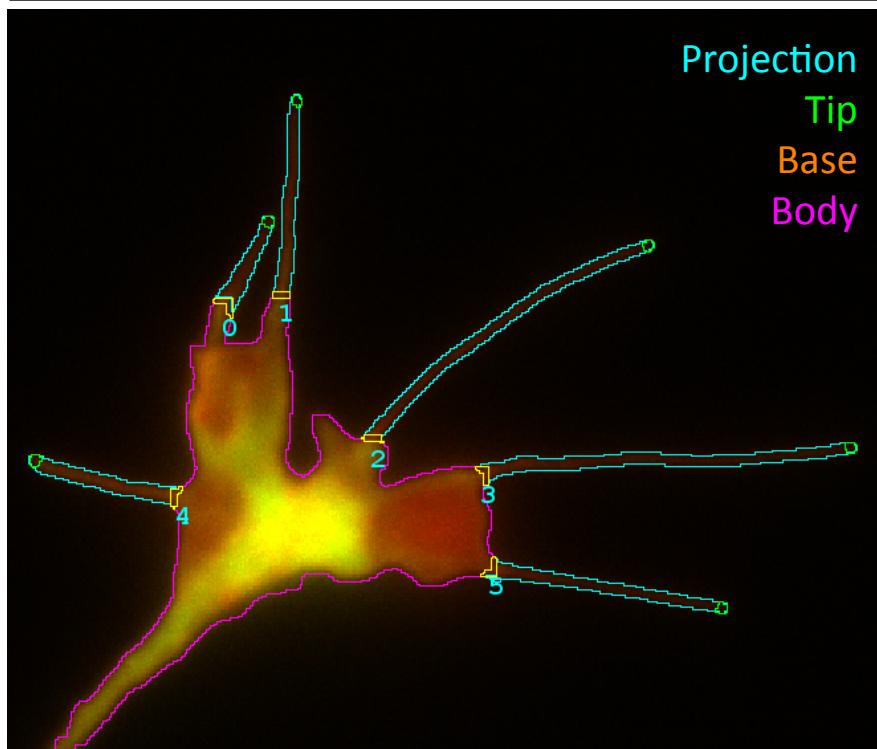
Segmentation

Filtering of hits

Track editing

Output

Segmented timelapse



Measurements

Length	Fluorescence (Tip)
ΔLength	Fluorescence (Base)
Tip movement (vec)	Fluorescence (Body)
Base movement (vec)	Fluoresc. (Projection)
Tip XY	
Base XY	

s11_NeonCyto_GC4x_2.tif Filopodia								
T (0)	dT (0)	Base Mean (0)	Body Mean (0)	Proj Mean (0)	Tip Th Mean (0)	Length (0)	dL (0)	
1.000	0.000	354.222	965.143	280.049	204.679	6.025	0.000	
2.000	1.000	370.541	933.112	272.333	188.767	6.071	0.046	
3.000	2.000	352.806	941.434	277.129	190.360	5.941	-0.130	
4.000	3.000	390.903	937.849	281.136	188.893	5.911	-0.030	
5.000	4.000	416.933	930.179	281.662	195.074	5.930	0.019	
6.000	5.000	355.966	928.665	275.476	176.929	5.827	-0.103	
7.000	6.000	407.067	933.102	285.351	191.036	6.011	0.184	
8.000	7.000	397.533	928.311	288.539	190.500	5.965	-0.046	
9.000	8.000	410.533	934.382	297.195	181.517	5.965	0.000	
10.000	9.000	404.393	934.119	295.046	180.321	5.881	-0.084	

Interactive demonstration

Sample file: growth-cone-test-file.tif

Filopodyan user guide: <https://github.com/gurdon-institute/Filopodyan/blob/master/Filopodyan%20User%20Guide.pdf>

1. Start Fiji

2. Open sample file

tips:

Renyi or Huang

LoG between 2 and 5

ED between 2 and 6

3. Launch Filopodyan

4. Segment and track filopodia:

(segmentation) set suitable segmentation parameters (with the help of Preview)

(filtering) set filtering parameters (hit Apply to preview)

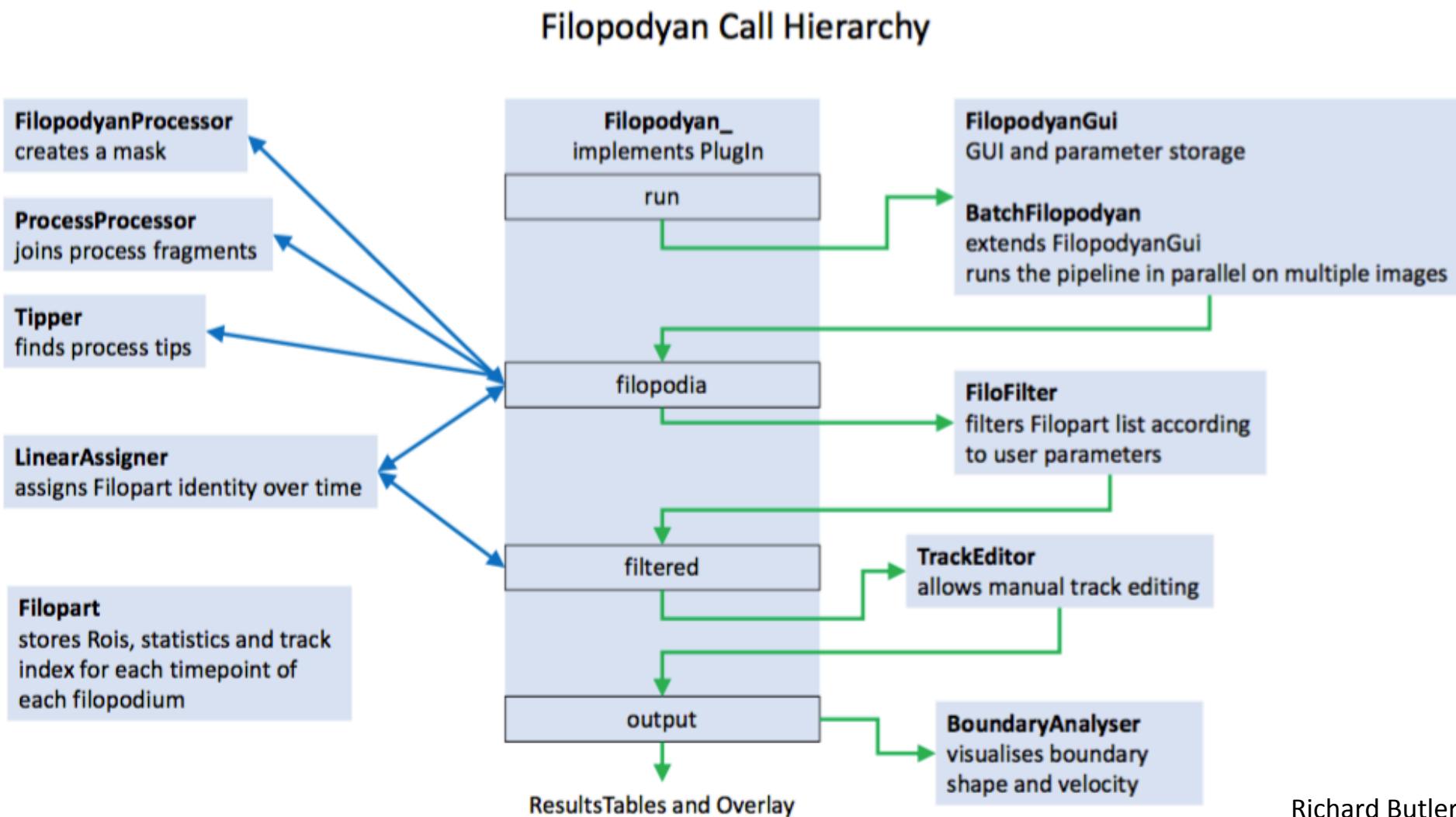
(manual editing) explore possibilities to delete tracks or timepoints

5. Inspect output:

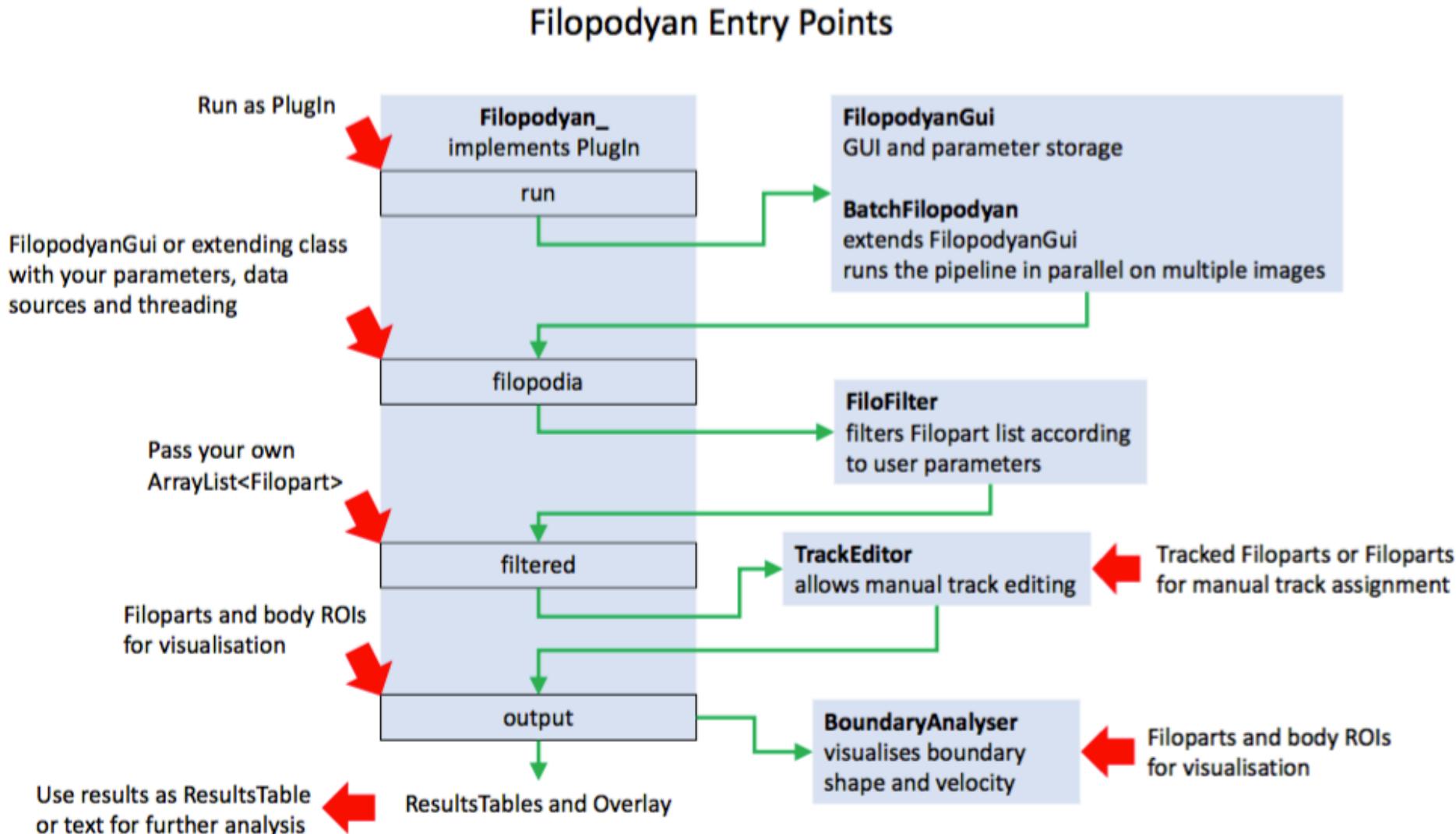
- image with ROI overlays

- data tables

Structure of the code - overview



Structure of the code – entry points



Segmentation strategy

Key Class:
FilopodyanProcessor.java



Thresholding + Erosion-dilation

Chosen because worked well and was relatively easy to code and process

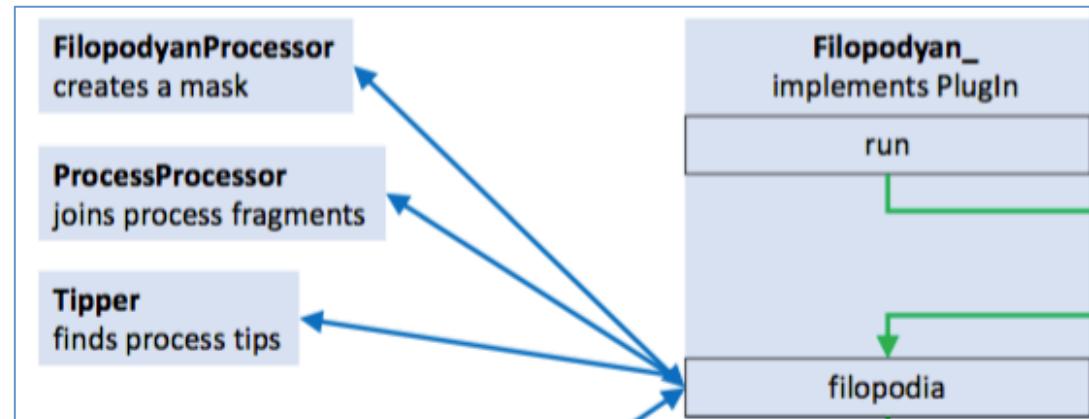
Limitations: dense filopodia (nearby neighbours); filopodia crossing

other possibilities (thresholding):

- adaptive thresholding (implemented)

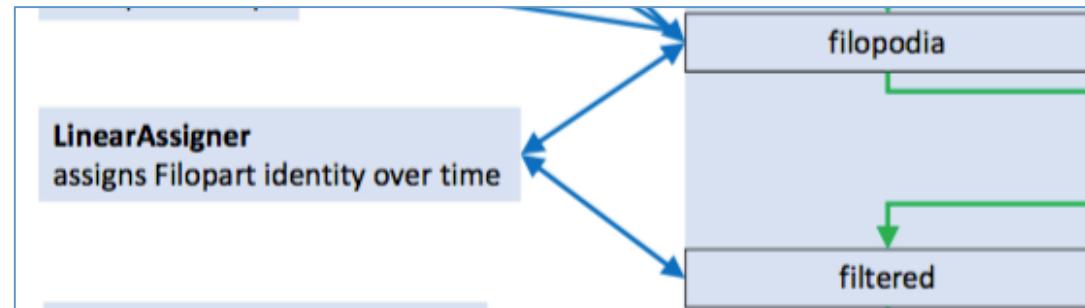
other possibilities (filopodia detection):

- edge detection (e.g. used by FiloQuant)
- tubularity



Segmentation strategy

Key Class:
LinearAssigner.java



Identity assignment:

Costing function with a rapid one-step Hungarian linear assignment algorithm

$$cost = \frac{dist(base_1, base_2) + dist(tip_1, tip_2)}{\sqrt{overlap\ area}} \delta T.$$

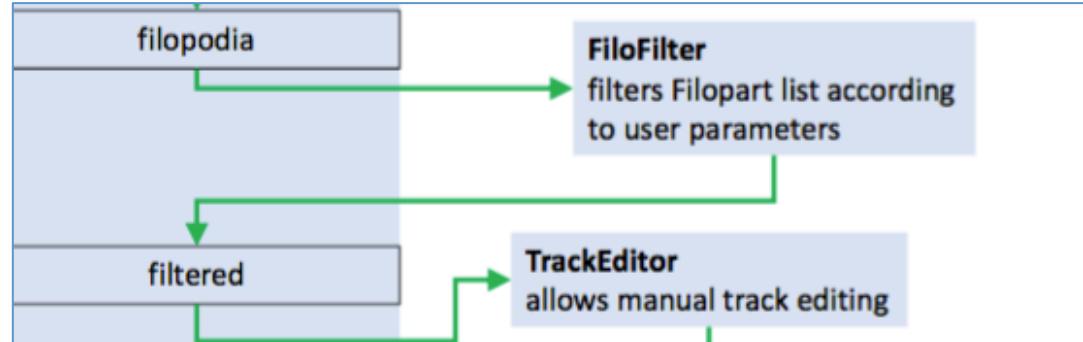
Empirically adjusted (not accessible to the user during an analysis session)

other possibilities:

- TrackMate?

Object filtering

Key Class:
FiloFilter.java

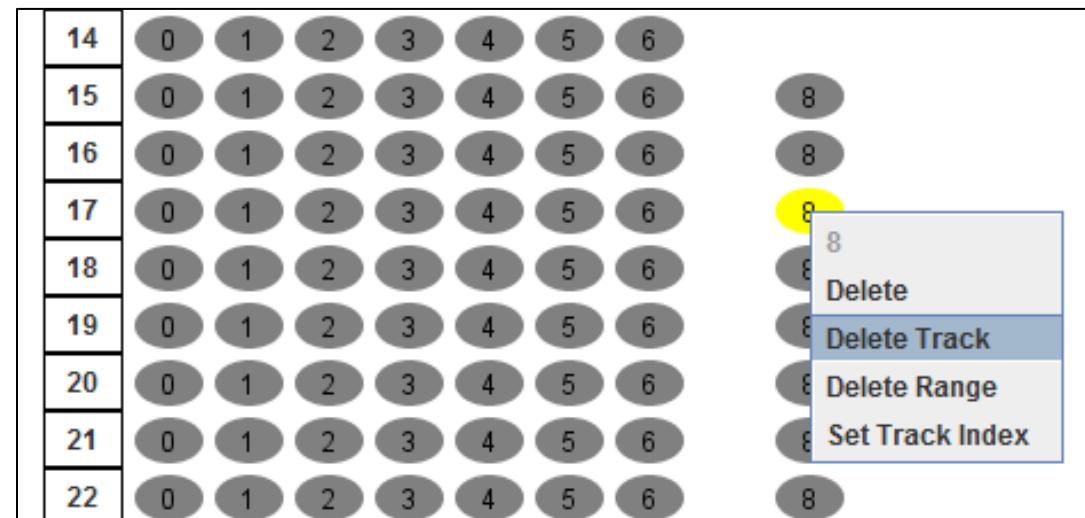
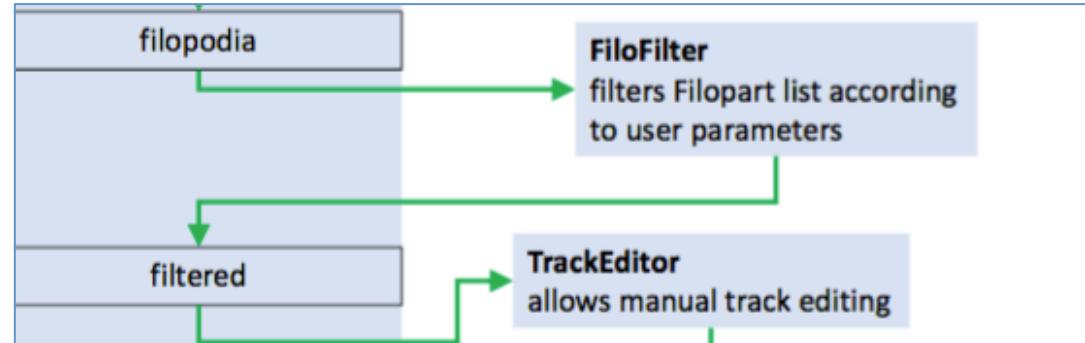


**Parameters for filtering
& user-adjustable filter thresholds:**

min start frame –	Remove pre-existing filopodia
min number of frames –	Remove short-lived structures / noise
min max length –	Remove structures that never reach a defined threshold length
min max dL –	Remove static structures that never change in length beyond threshold
min max DCTM –	Remove structures with static tips which never move beyond threshold
min max DCBM –	Remove structures with static bases
max mean waviness –	Remove non-straight structures / noise

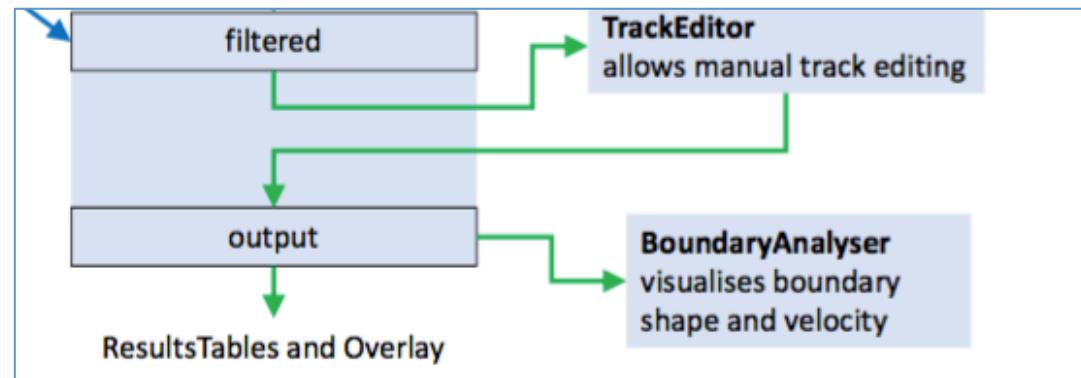
Manual editing of tracks

Key Class:
TrackEditor.java



Measurements & Output

Key Classes:
ResultsTables and Overlay



Output tables:

Filopodia – filopodia properties (length, movement) and fluorescence (tip, base, body, local background fluorescence) for each timepoint

Coordinates – coordinates, vector measurements for each timepoint

Bodies – body fluorescence and global background fluorescence for each timepoint

Measurements

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 $\ [\text{tip}(t-1), \text{tip}(t)]\ : [\text{base}, \text{tip}]$ in Coordinates table)
DCBM	Direction-corrected base movement (identical to $\ [\text{base}(t-1), \text{base}(t)]\ : [\text{base}, \text{tip}]$ in Coordinates table)

Measurements

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

Definitions: what is “length”?

Definition *based on perimeter* of the filopodium;

$$\text{Length} \approx \text{perimeter} / 2$$

Problem:

overestimated because of: base width, curvature at the tip
error can be (partially?) corrected

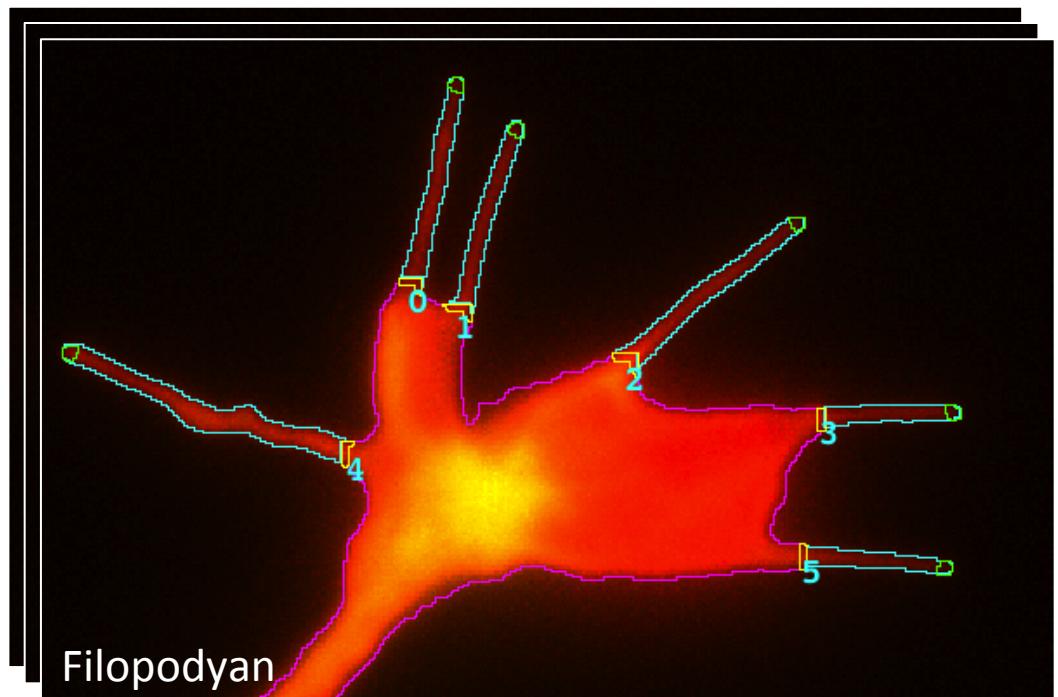
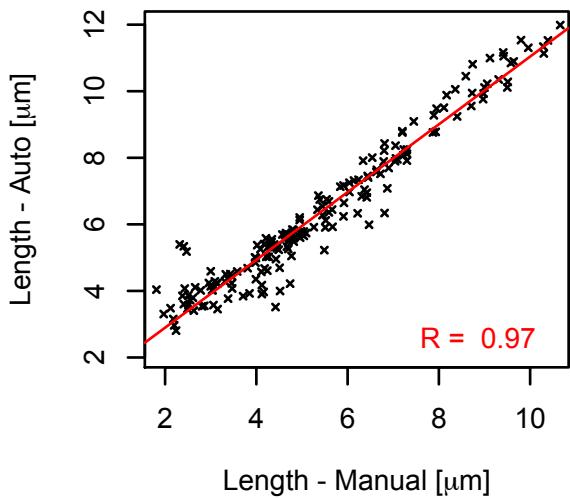


Alternatives:

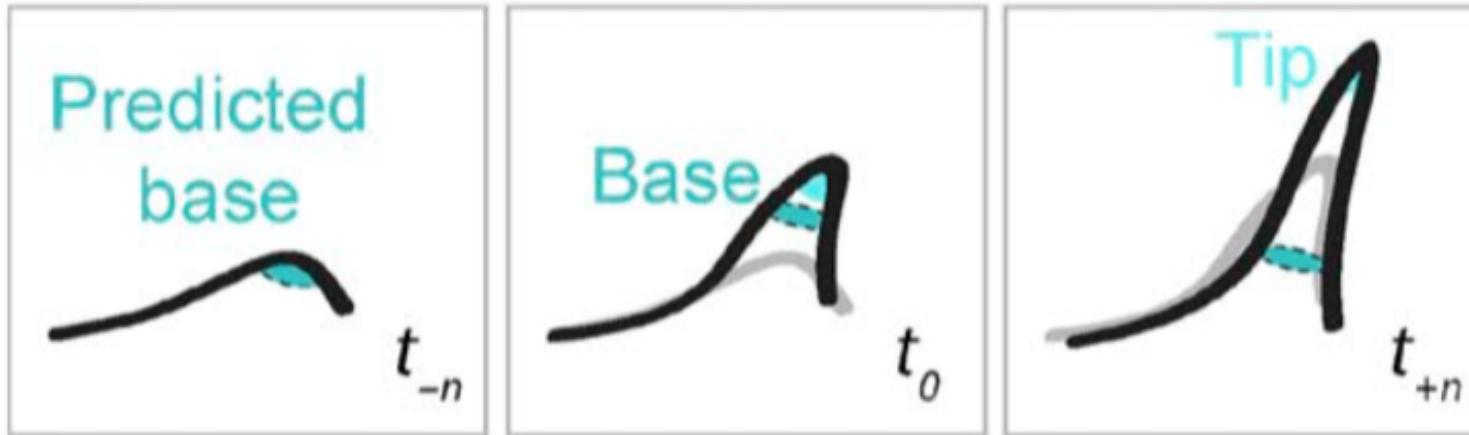
- tip-to-base Euclidean distance;
 - rejected – required for measuring straightness
- skeletonization;
 - rejected by Richard for reasons of computational implementation

Validation of reconstruction of filopodia with Filopodyan

Are reconstructed lengths equivalent to manual reconstruction by a human?



Definitions: What is “base”? “predicted base”? “tip”?



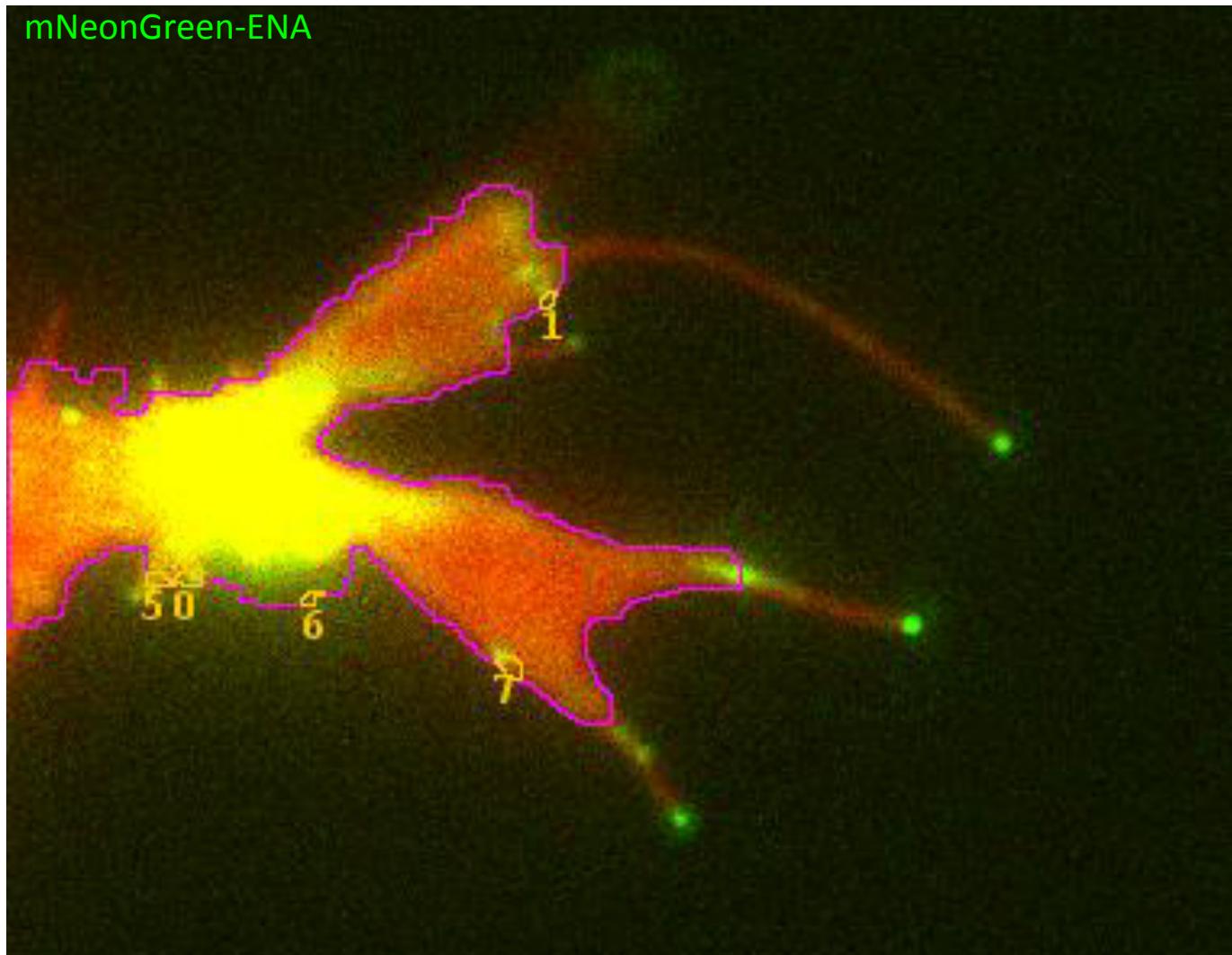
Base: definition based on point of contact between Body and Filopodium

Predicted base: based on Euclidean proximity to the base position at t_0 .

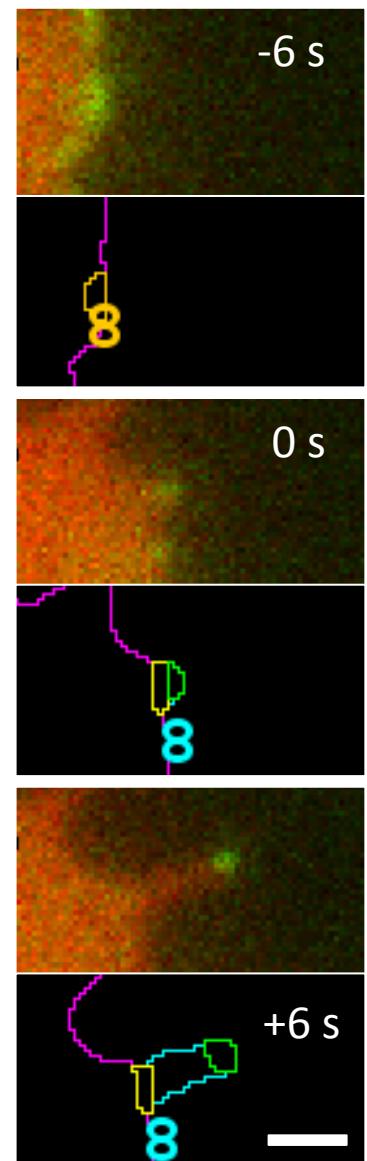
Tip: Point of maximum distance *from base*. (Limitation: bent filopodia.)

“Predicted base” positioning

mNeonGreen-ENA



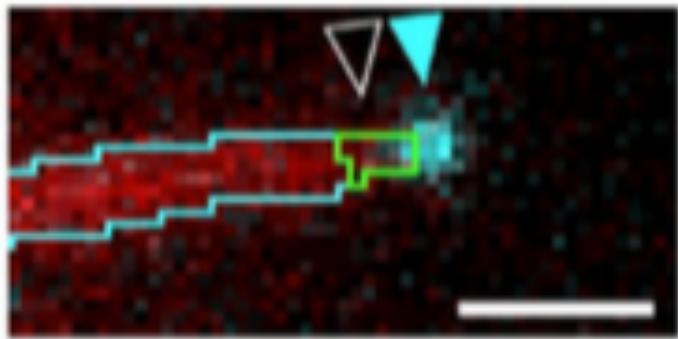
Segmented objects filtered for **newly forming filopodia**



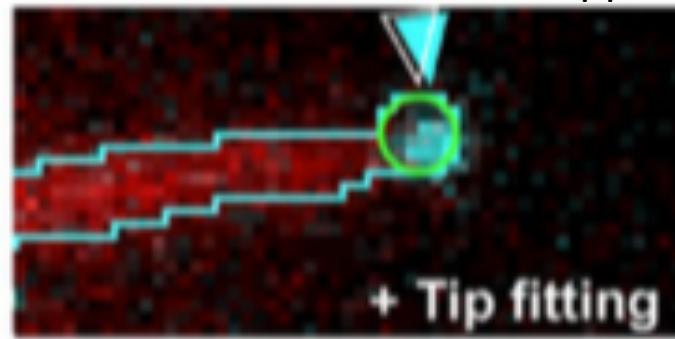
“Tip fitting”

Additional segmentation functionalities for more accurate tip identification:

Tip fitting:



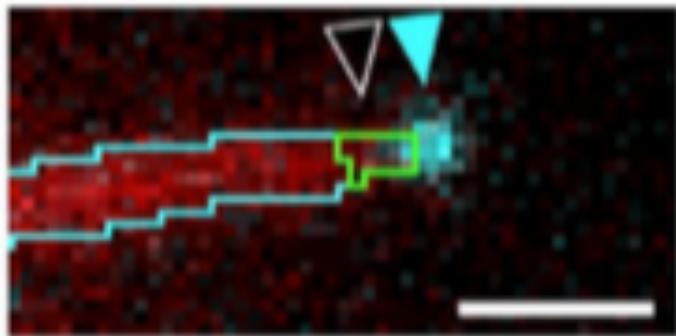
Class: Tipper



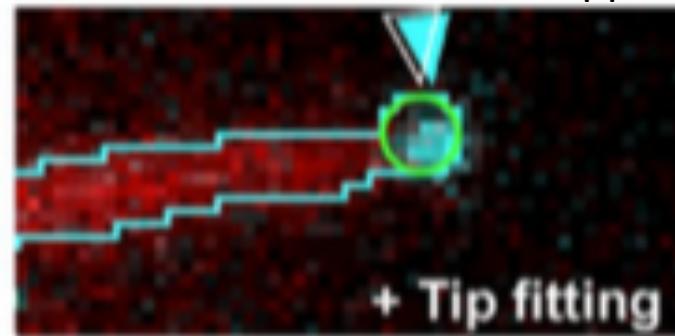
“Tip fitting”

Additional segmentation functionalities for more accurate tip identification:

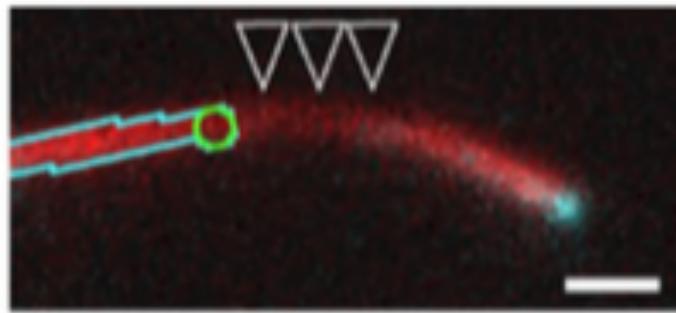
Tip fitting:



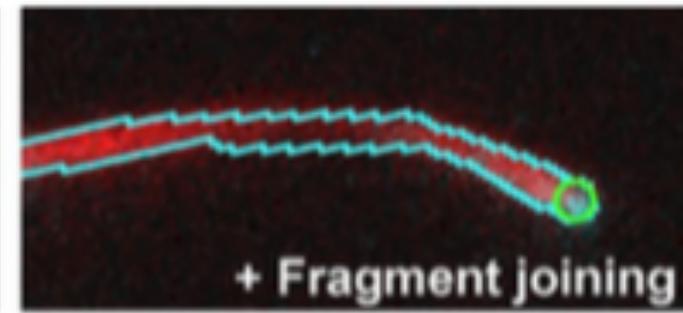
Class: Tipper



Fragment joining:



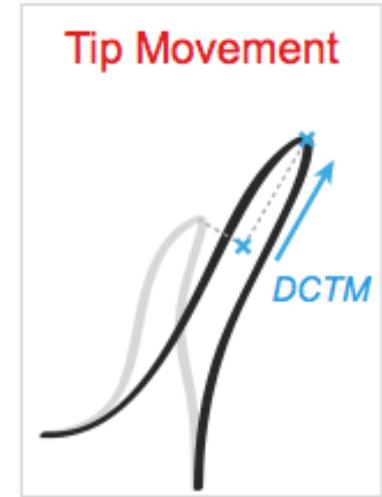
+ Fragment joining



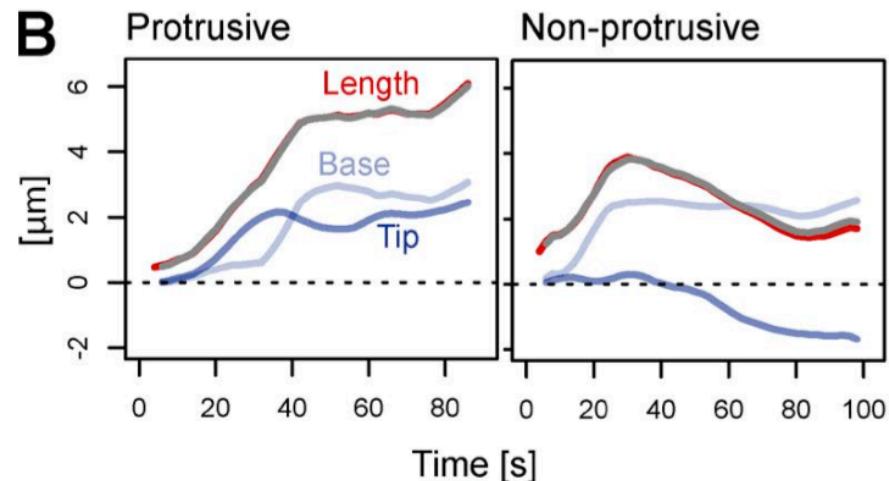
What is “tip movement”?

How do we get an adequate measure of tip extension?

- Tip displacement? **Limitation:** Swinging and lateral movements.
- Length change? **Limitation:** Shifting of base position
- Vector measure of “direction-corrected tip movement”:
projection of the tip displacement vector onto the base-tip vector; preserves the component of tip displacement that is equivalent to the tip’s forward extension

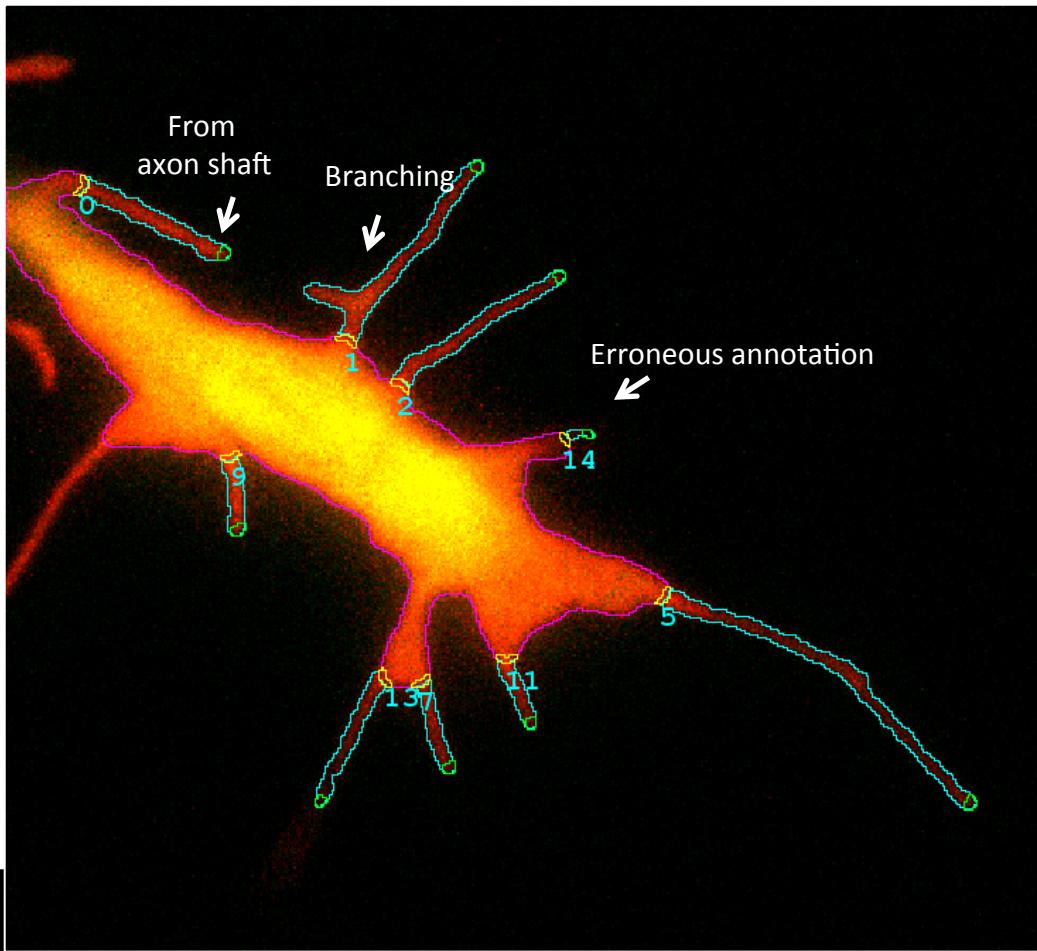


Advantage: measuring independently the contributions of tip movements (extension/retraction) and base movements (invasion/retraction) to the change in filopodium length



Automated filtering and manual correction

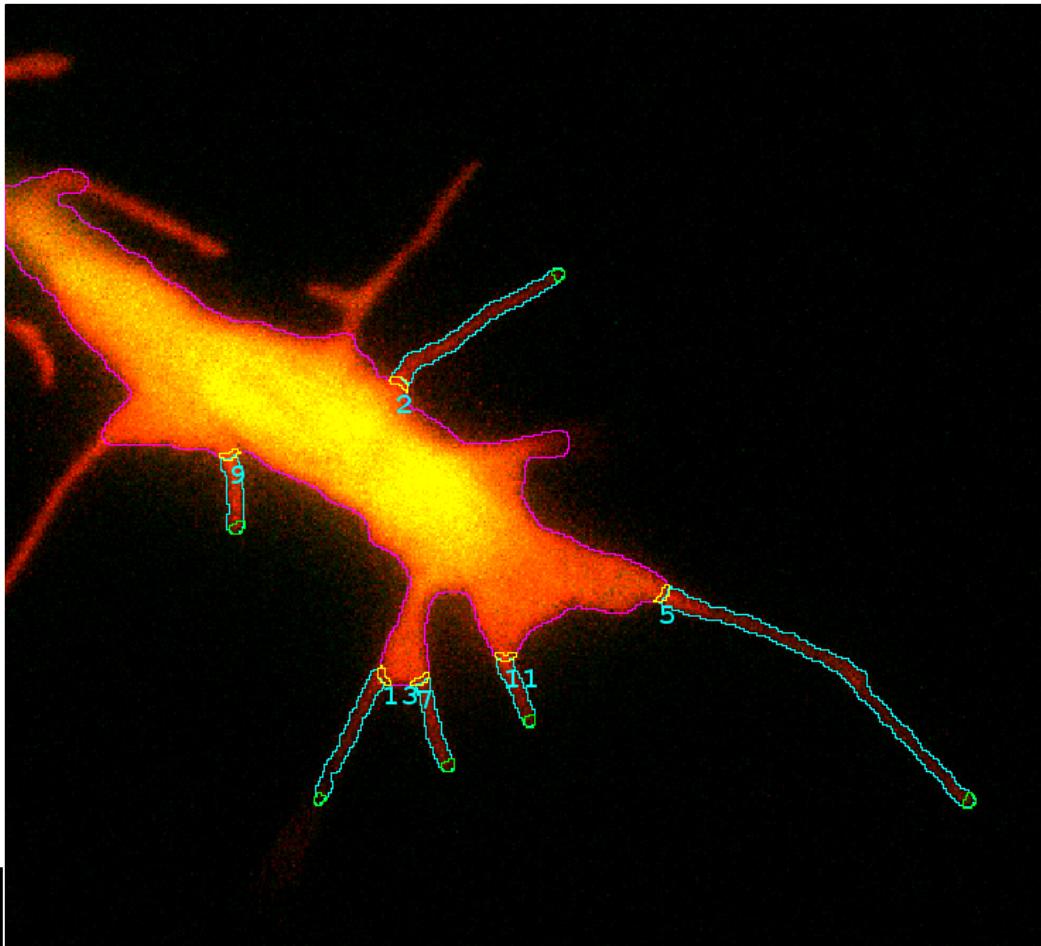
Fully automated reconstruction makes some mistakes. How much does it matter?



For 19 GCs:
15 min batch run

Automated filtering and manual correction

Fully automated reconstruction makes some mistakes. How much does it matter?



For 19 GCs:

6h manual work

- 8
- 8
- 8 Delete
- 8 Delete Track
- 8 Delete Range
- 8 Set Track Index
- 8

Automated filtering and manual correction

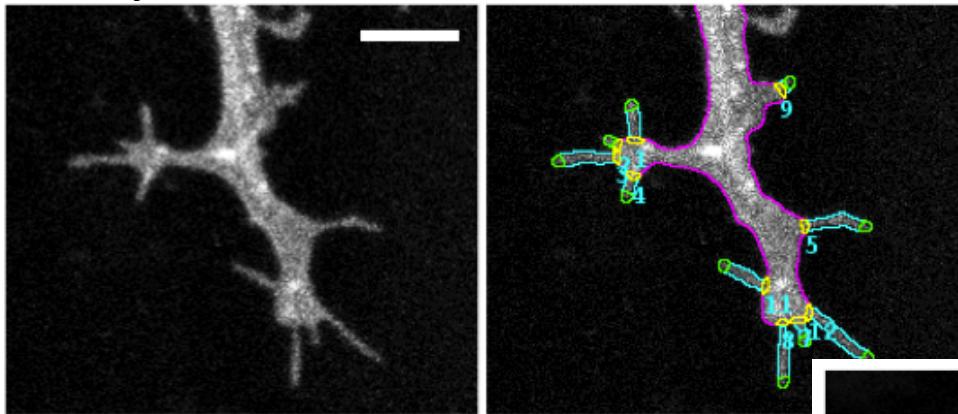
Fully automated reconstruction makes some mistakes. How much does it matter?

Workflow reproduction activity (part 4a):

Phenotype comparison between manually curated and batch-reconstructed files

Applicability to diverse cell types

Drosophila tracheal cells



Benjamin Richier

Tested across:

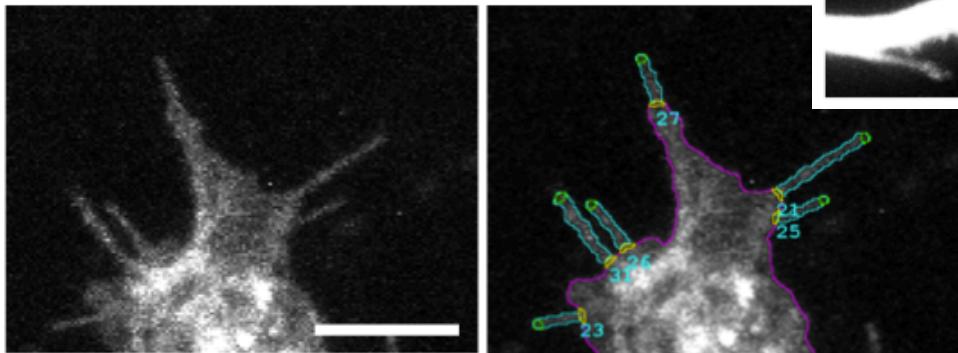
4 cell types

in vitro and *in vivo*

Drosophila, *Xenopus* and human cells

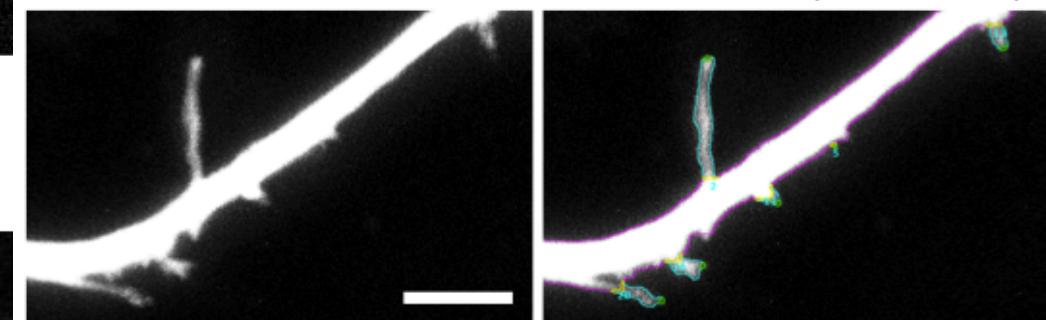
TIRF, line-scanning confocal, 2-photon

Drosophila dorsal closure epithelium



Benjamin Richier

Human cortical neurons (in culture)



Manuel Peter
(Livesey lab)

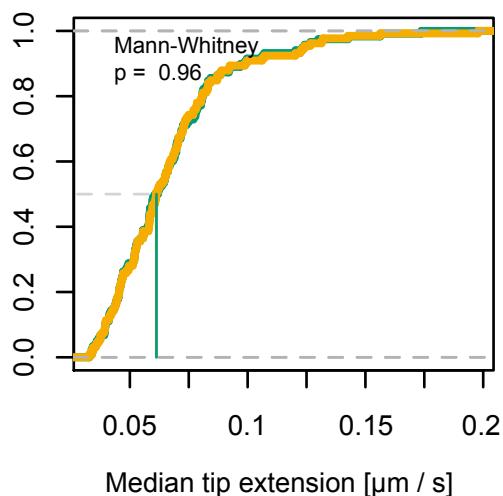
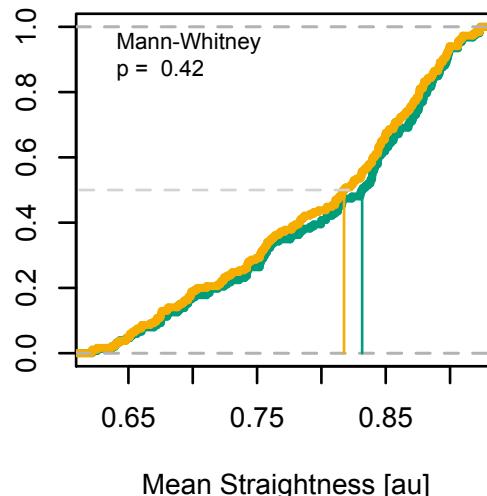
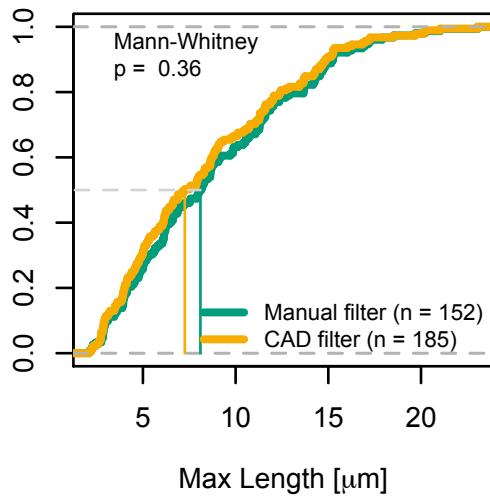
Validation of automated filtering

	CAD + Manual	CAD only
Processing time	6 h (incl. manual adjustments)	15 min (batch, unsupervised)
Length [µm]¹	8.7 ± 4.7 *	8.3 ± 4.7 *
Straightness [au]²	0.80 ± 0.081 *	0.80 ± 0.083 *
Tip extension rate [µm / s]³	0.07 ± 0.026 *	0.07 ± 0.027 *
Tip retraction rate [µm / s]³	-0.06 ± 0.019 *	-0.07 ± 0.022 *
Base invasion rate [µm / s]³	0.08 ± 0.041 *	0.08 ± 0.06 *
Base retraction rate [µm / s]³	-0.08 ± 0.061 *	-0.08 ± 0.11 *
Time tip extending (%)⁴	21 #	19 #
Time tip retracting (%)⁴	12 #	11 #
Time tip stalling (%)⁴	59 #	59 #
Tip persistence (%)⁵	7 #	6 #

¹ Max of lifetime ² Mean of lifetime
³ Median of lifetime ⁴ Proportion of lifetime
⁵ Root of autocorrelation of tip movement

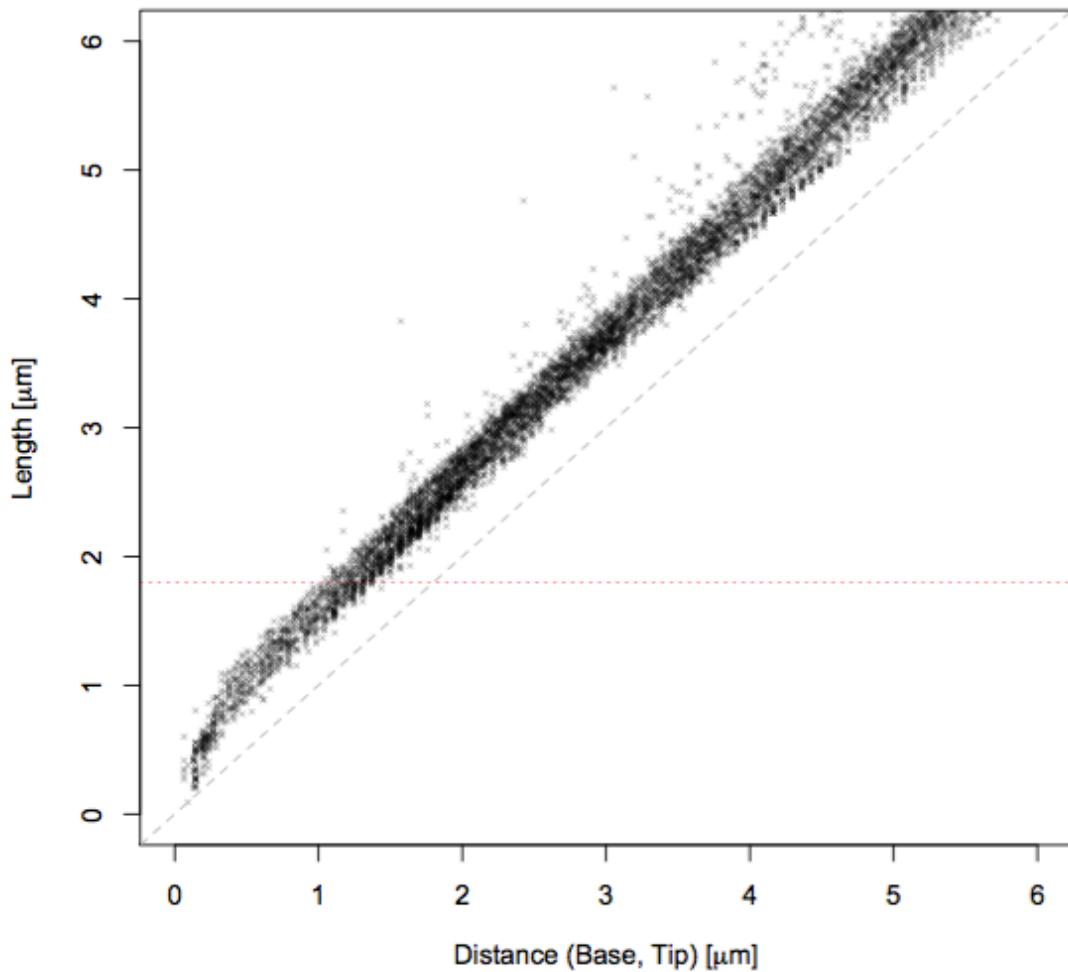
* Mean \pm SD
 # Median

Cumulative distribution



Length measurement correction

“Length” vs Tip-to-Base Distance



Length measurement correction

Defined as: $L = \text{Perimeter length} / 2 - EC \text{ (end-correction)} * SF \text{ (scaling factor)}$

$$EC = \frac{(\pi - 2)}{2} w$$
$$w = 2 \sqrt{2 \ln 2} \sigma px$$

EC: end correction

derived from a) tip correction = 1/4 circumference with radius = w/2
b) base correction = $\frac{1}{2} w$

w: width

calculated by formula for full width at half maximum, using:

σ = user-defined sigma for Laplacian-of-Gaussian

px = pixel size (in μm)

SF: scaling factor

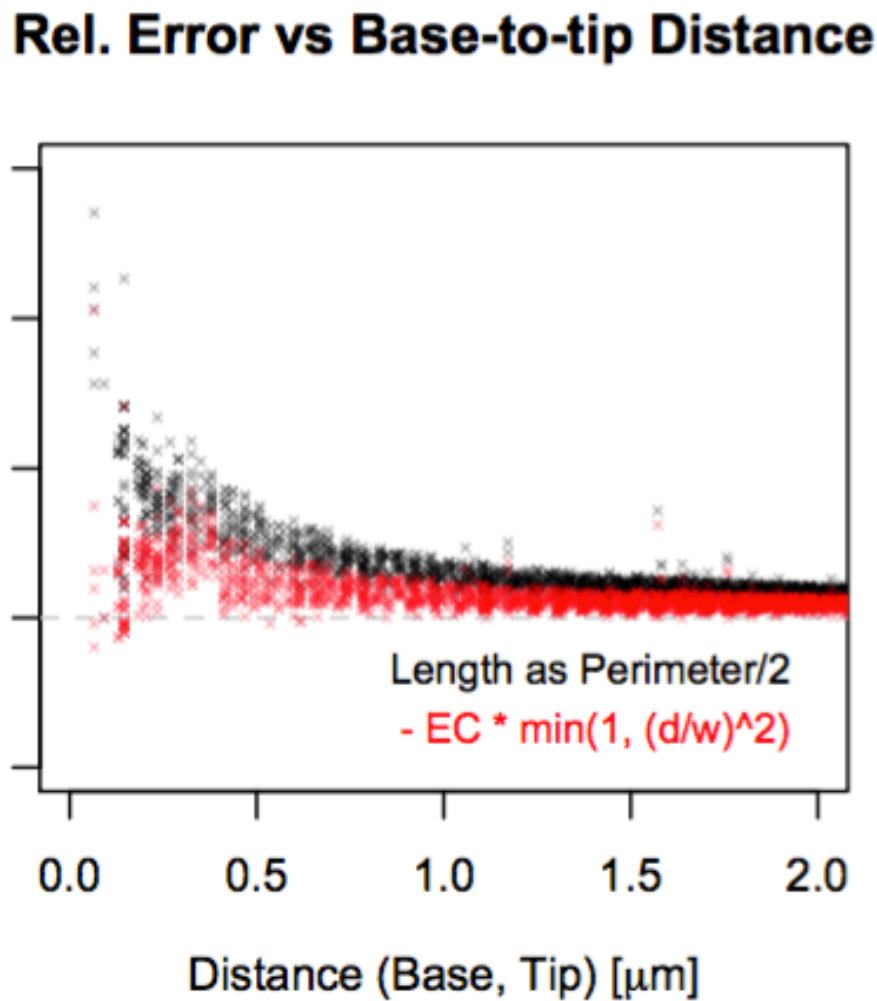
($SF = 1$ if $D > w$; $SF < 1$ if $D > w$)

D: Euclidean distance (Base, Tip)

$$SF = \min\left(\left(\frac{D}{w}\right)^2, 1\right)$$

Length measurement correction

Rel. Error (Length - Distance) / Distance)



Euclidean distance vs Corr. Length

