

# Workshop structure

## 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 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

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## Part 5:

### **Deconstruction**

# Overview

## Table output of the Fiji plugin:

| 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  |

## Tasks & Questions:

>> clean-up & filtering

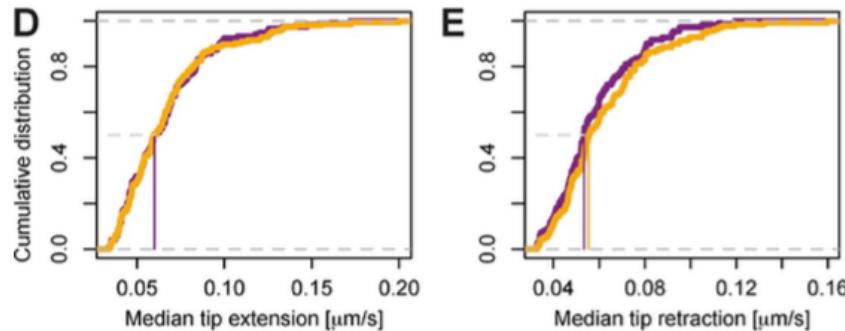
>> time series properties (tip persistence, time extending/retracting, etc.)

## Analyses:

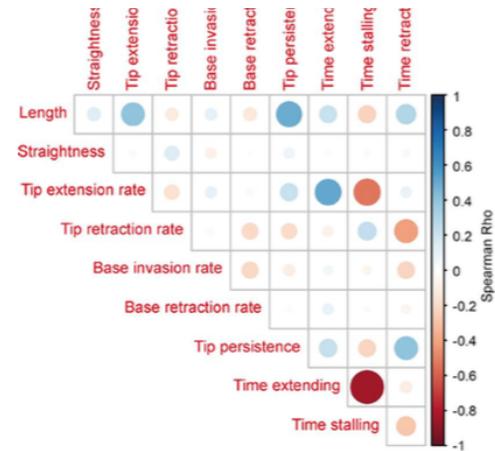
1. Do properties differ between different data sets (conditions)?
2. Do different properties correlate with each other? (e.g. faster extending --> longer)
3. Increase in fluorescence before the formation of a new filopodium?
4. Relationship between tip fluorescence and tip extension?

# Overview

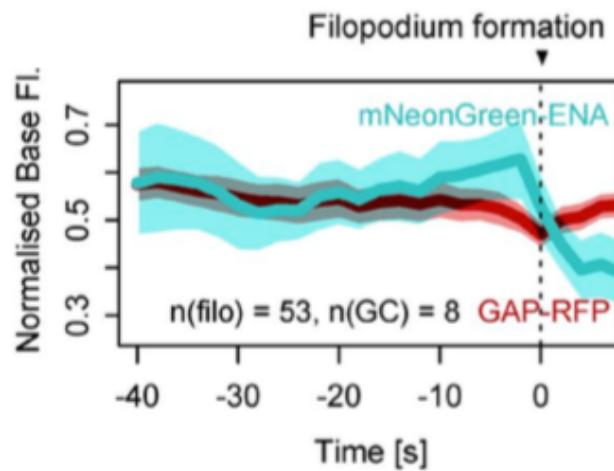
## a. Phenotype comparison



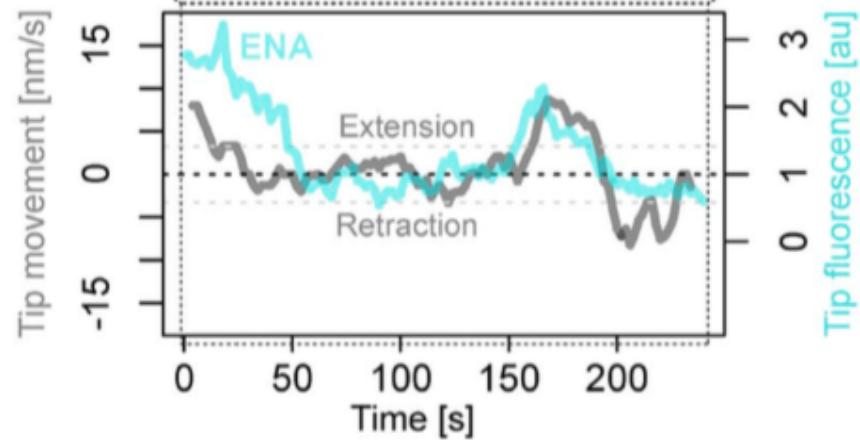
## b. Property correlations



## c. Base fluorescence & initiation



## d. Tip fluorescence and movement



# Overview

## a. Phenotype comparison

| s11_NeonCyto_GC4x_2.tif Filopodia |       |         |         |         |               |               |               |                 |
|-----------------------------------|-------|---------|---------|---------|---------------|---------------|---------------|-----------------|
| File                              | Edit  | Font    | T (0)   | dT (0)  | Base Mean (0) | Body Mean (0) | Proj Mean (0) | Tip Th Mean (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        |                 |

>> Import data from multiple tables

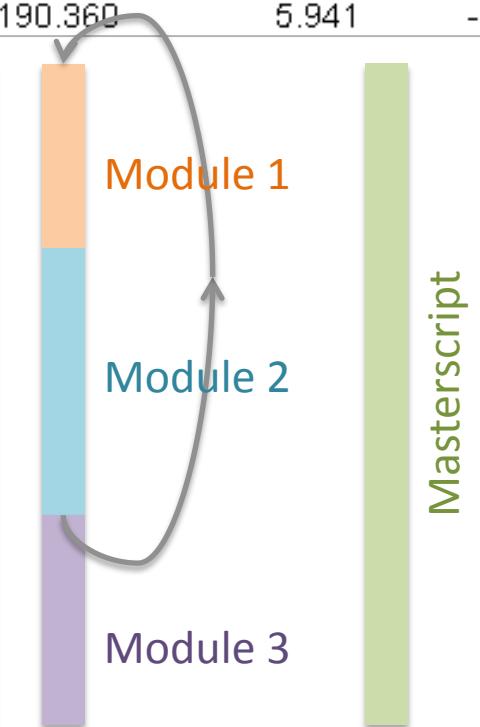
>> Filtering & Smoothing of movement metrics

>> Computation of new metrics per each time series:

- summary statistics (e.g. max length, med. ext. rate)
- autocorrelation (persistence)
- initial movement (at start of lifetime)

>> Statistical comparison between conditions (folders)

>> Visualisation



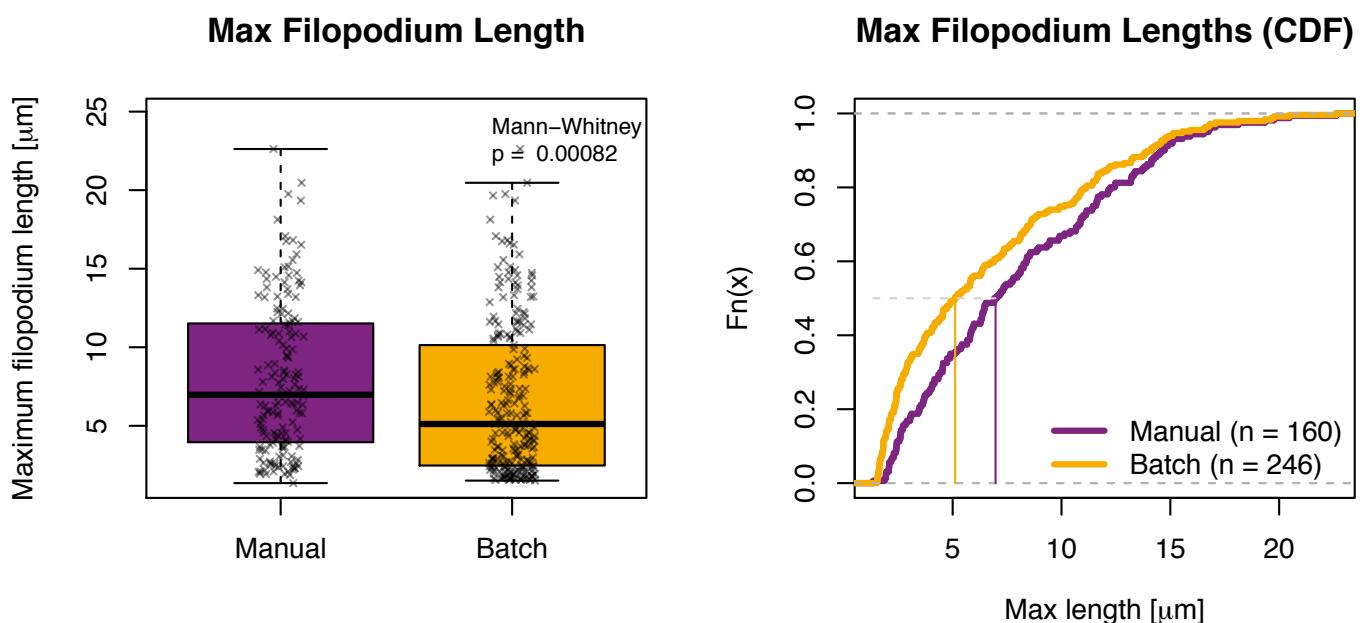
# Overview

## a. Phenotype comparison

Sample input:

| s11_NeonCyto_GC4x_2.tif Filopodia |       |         |         |         |               |               |               |
|-----------------------------------|-------|---------|---------|---------|---------------|---------------|---------------|
| File                              | Edit  | Font    | T (0)   | dT (0)  | Base Mean (0) | Body Mean (0) | Proj Mean (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        |

Sample output:



## 3a: Phenotype comparison (scripts overview)

### R script: MODULE 1 - Data import and basic processing

- User input section: timepoints per second, pixel width, file location, normalization setting etc.
- Import:
  - all “Filopodia” tables, “Coordinates” and “Bodies” tables
  - option for subsetting tables by name (e.g. “CTRL” in filename)
  - code patch to keep track of filopodium identity in R scripts
- Background correction (for fluorescence measurements; calls a separate script) & Fluorescence normalization [see later, parts 3c and 3d]
- Cleanup: remove outliers (DCTM outside of 0.5<sup>th</sup>-99.5<sup>th</sup> percentile)
- Processing: smoothen the measurements of movement (rolling mean)

```
MovingAverage <- function(x, w = 5) {filter(x, rep(1/w, w), sides = 2)}
```

## 3a: Phenotype comparison (scripts overview)

### R script: MODULE 2 - Computation of time-series properties

- Set thresholds for “extension”, “retraction” and “stalling” (default: 1 pxw/timepoint)
- Compute properties over timecourse:
  - max length over timecourse
  - median tip extension rate *while extending*
  - median tip retraction rate *while retracting*
  - similarly, median base invasion and base retraction rate
  - tip persistence (= root of autocorrelation function); key R function: `acf()`
- Separate newly forming vs pre-existing filopodia
- For analysing the behaviour of newly forming filopodia, compute:
  - initial rate of tip extension (set n timepoints counting as ‘initial’; default = 10)
- Compute new property: straightness (plus cleanup)
- Assign “tip states”: “extending”, “retracting”, “stalling”, and compute total proportion of time in each state

## 3a: Phenotype comparison (scripts overview)

### R script: MODULE 3 - Statistics & visualisations

- Imports graphing templates (a dependency script) containing code for:
  - statistical comparisons
  - visualisations (box plots and CDF curves)
- Plots visualisations comparing each of the measured properties across two datasets
- Exports a statistical summary for two datasets
- Currently only supports 2 datasets at a time (**Deconstruction?**)

## 3b: Correlations

### 2. Relationship between properties

**Goal 1:** Understanding relationships between morphodynamic properties

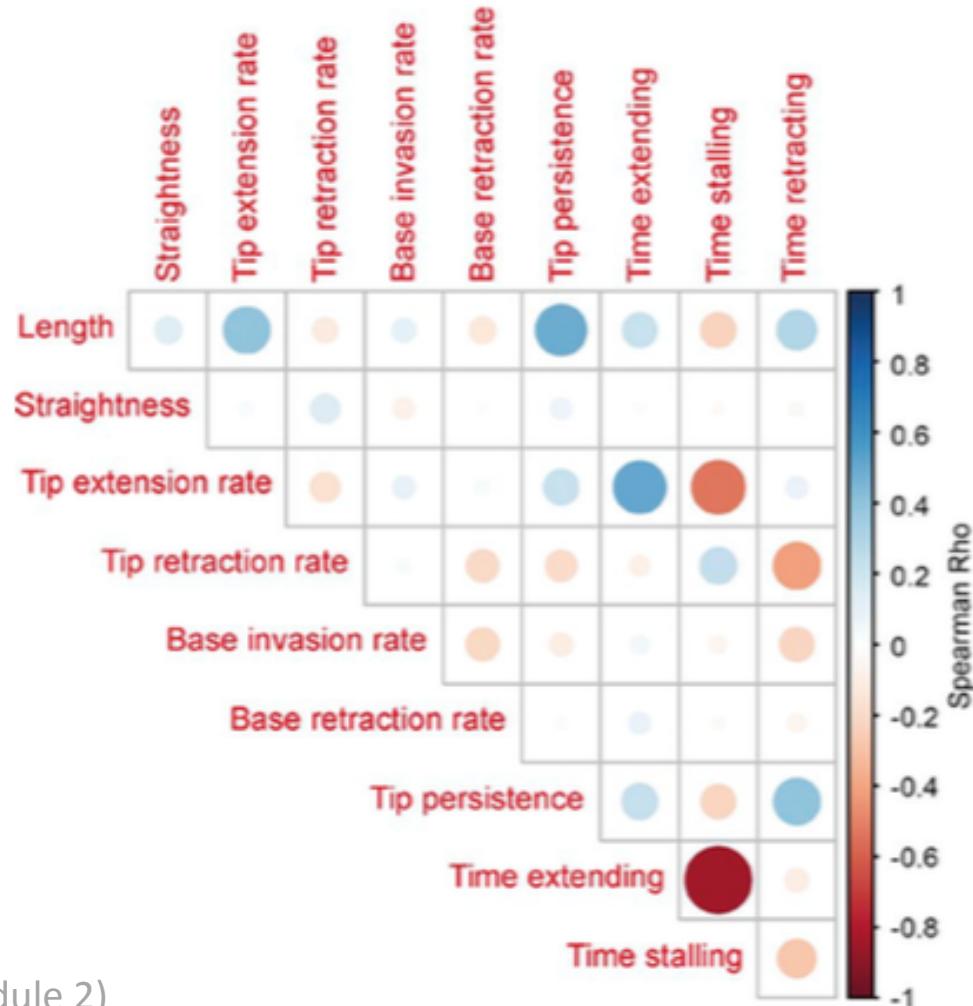
e.g. 'Do filopodia that start their life with a fast extension rate reach greater lengths?'  
or 'Which property correlates most strongly with max length?'  
or 'Is everything just noise?'

**Goal 2:** Understanding relationships between morphodynamic properties & fluorescence measurements

e.g. 'Do filopodia with higher base fluorescence before formation reach longer lengths?'

Script tasks:

- >> Pre-processing (like Module 1 + Module 2)
- >> Compute correlations
- >> Visualise



JELLY BEANS  
CAUSE ACNE!

SCIENTISTS!  
INVESTIGATE!

BUT WE'RE  
PLAYING  
MINECRAFT!  
...FINE.



WE FOUND NO  
LINK BETWEEN  
PURPLE JELLY  
BEANS AND ACNE  
( $P > 0.05$ ).



WE FOUND NO  
LINK BETWEEN  
BROWN JELLY  
BEANS AND ACNE  
( $P > 0.05$ ).



WE FOUND NO  
LINK BETWEEN  
PINK JELLY  
BEANS AND ACNE  
( $P > 0.05$ ).



WE FOUND NO  
LINK BETWEEN  
BLUE JELLY  
BEANS AND ACNE  
( $P > 0.05$ ).



WE FOUND NO  
LINK BETWEEN  
TEAL JELLY  
BEANS AND ACNE  
( $P > 0.05$ ).



WE FOUND NO  
LINK BETWEEN  
SALMON JELLY  
BEANS AND ACNE  
( $P > 0.05$ ).



WE FOUND NO  
LINK BETWEEN  
RED JELLY  
BEANS AND ACNE  
( $P > 0.05$ ).



WE FOUND NO  
LINK BETWEEN  
TURQUOISE JELLY  
BEANS AND ACNE  
( $P > 0.05$ ).



WE FOUND NO  
LINK BETWEEN  
MAGENTA JELLY  
BEANS AND ACNE  
( $P > 0.05$ ).



WE FOUND NO  
LINK BETWEEN  
YELLOW JELLY  
BEANS AND ACNE  
( $P > 0.05$ ).



WE FOUND NO  
LINK BETWEEN  
GREY JELLY  
BEANS AND ACNE  
( $P > 0.05$ ).



WE FOUND NO  
LINK BETWEEN  
TAN JELLY  
BEANS AND ACNE  
( $P > 0.05$ ).



WE FOUND NO  
LINK BETWEEN  
CYAN JELLY  
BEANS AND ACNE  
( $P > 0.05$ ).



WE FOUND A  
LINK BETWEEN  
GREEN JELLY  
BEANS AND ACNE  
( $P < 0.05$ ).



WE FOUND NO  
LINK BETWEEN  
BEIGE JELLY  
BEANS AND ACNE  
( $P > 0.05$ ).



WE FOUND NO  
LINK BETWEEN  
LILAC JELLY  
BEANS AND ACNE  
( $P > 0.05$ ).



WE FOUND NO  
LINK BETWEEN  
BLACK JELLY  
BEANS AND ACNE  
( $P > 0.05$ ).



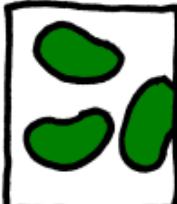
WE FOUND NO  
LINK BETWEEN  
PEACH JELLY  
BEANS AND ACNE  
( $P > 0.05$ ).



News  
GREEN JELLY  
BEANS LINKED  
TO ACNE!

95% CONFIDENCE

ONLY 5% CHANCE  
OF COINCIDENCE!



SCIENTISTS...

## 3b: Correlations (scripts overview)

### R scripts: Correlations\_DataInput and Correlations

- FilopodyanR\_DataInput.R:

Import, processing, computation of new properties (equivalent to Modules 1 & 2)

+ new features:

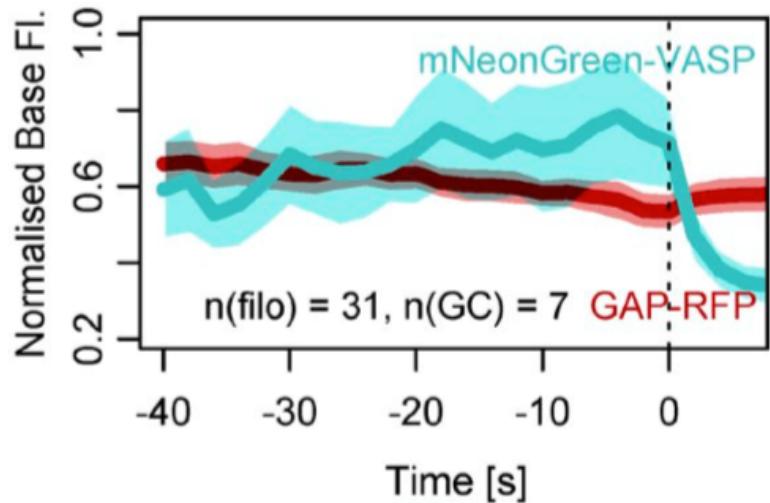
- ‘initial’ movement properties for newly forming filopodia (e.g. first 10 t)
- ‘post-10’ properties (to avoid self-correlation)

- FilopodyanR\_Correlations.R:

- Organises the features data into one data frame (df.sel.filo.metrics)
- Computes correlation matrix - cor(df..., method = “Spearman”)
- Visualises correlation matrix - corrplot() (from corrplot package)
- Outputs:
  - visualisation of the correlation matrix
  - .csv table of correlation coefficients and p-values (adjusted!)
  - XY scatterplots for selected pairs of properties

## 3c: Base Fluorescence

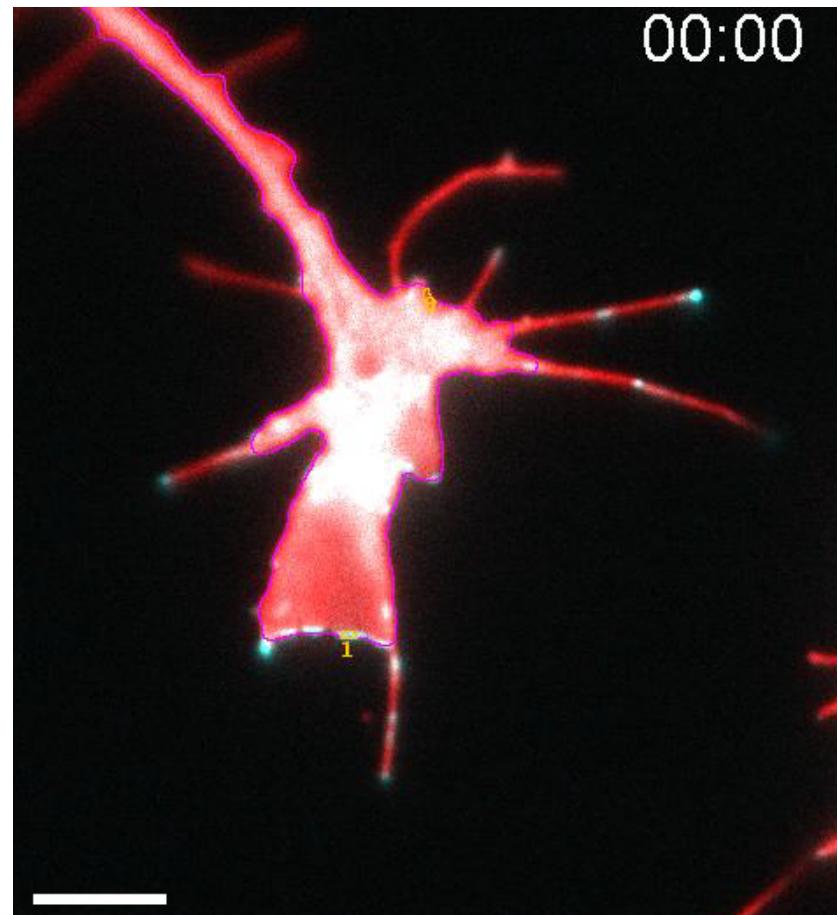
### 3. Base fluorescence before filopodium emergence



Script tasks:

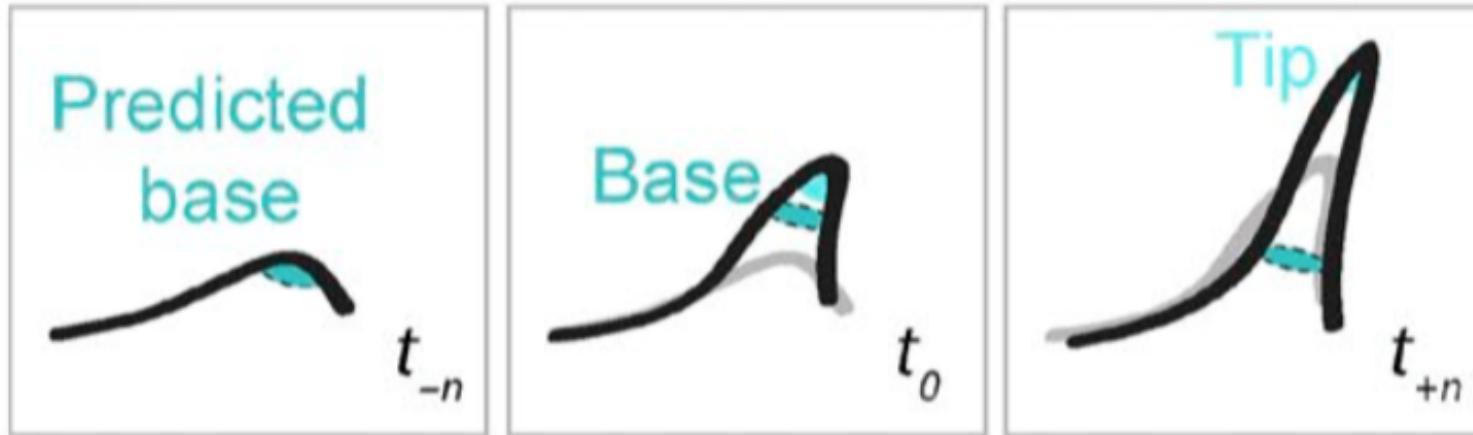
```
>> Background subtraction  
>> Normalisation  
>> Computation (Mean +- CI)  
>> Visualisation
```

} 2 channels



# Key definitions

## 'Predicted' base



position in timepoints  $n < 0$  is based on Euclidean proximity to the position at  $t_{n+1}$ .

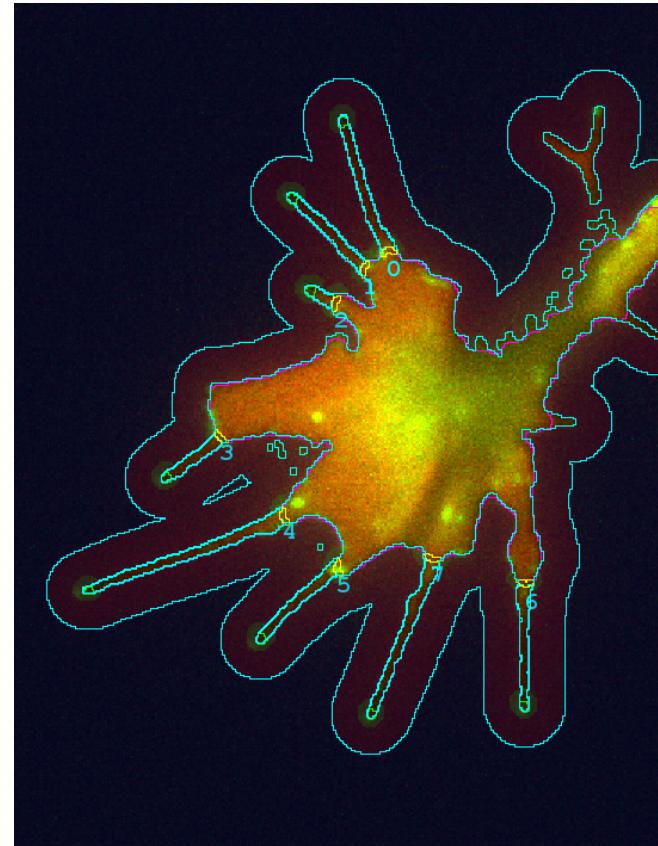
## 'Background'

several methods of measurement...

# Background measurements in Filopodyan

Measurements available in Filopodyan:

- Base fluorescence (Base F) Fluorescence
- Tip fluorescence (Tip F)
- Projection (filopodium) fluorescence (Proj F)
- Body fluorescence (Body F)
- Frame background Background
- Boundary background
- Local base background
- Local tip background



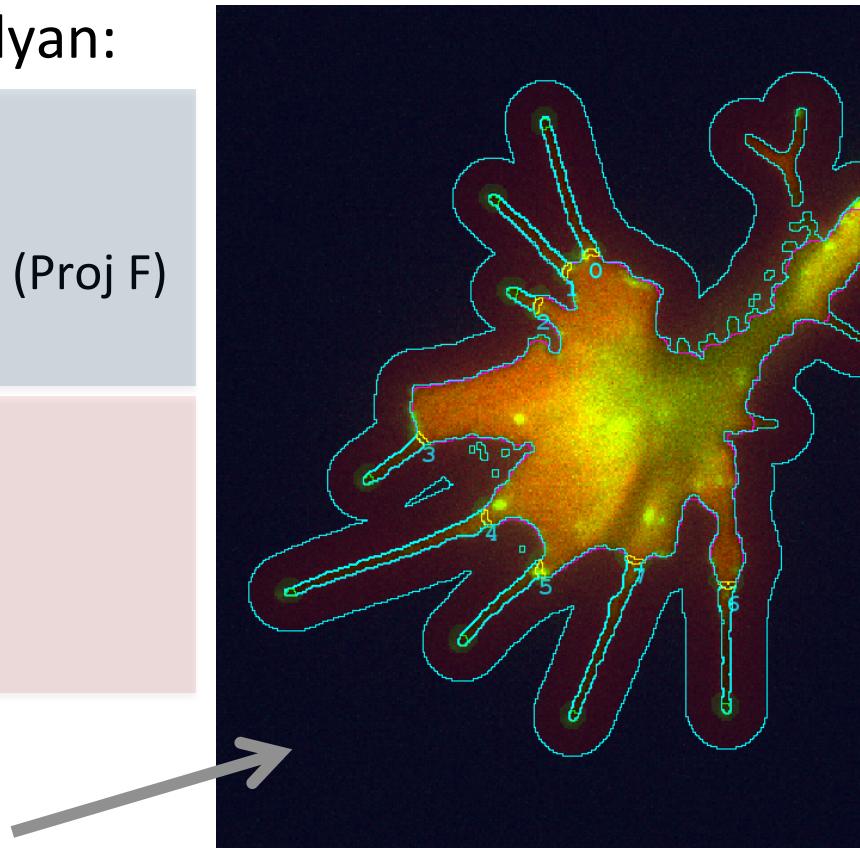
# Background measurements in Filopodyan

Measurements available in Filopodyan:

- Base fluorescence (Base F)
- Tip fluorescence (Tip F)
- Projection (filopodium) fluorescence (Proj F)
- Body fluorescence (Body F)
- Frame background
- Boundary background
- Local base background
- Local tip background

Frame background:

Inverse of the signal ROI, after thresholding step for initial object detection (i.e. the field of view devoid of objects as bright as the cell body)



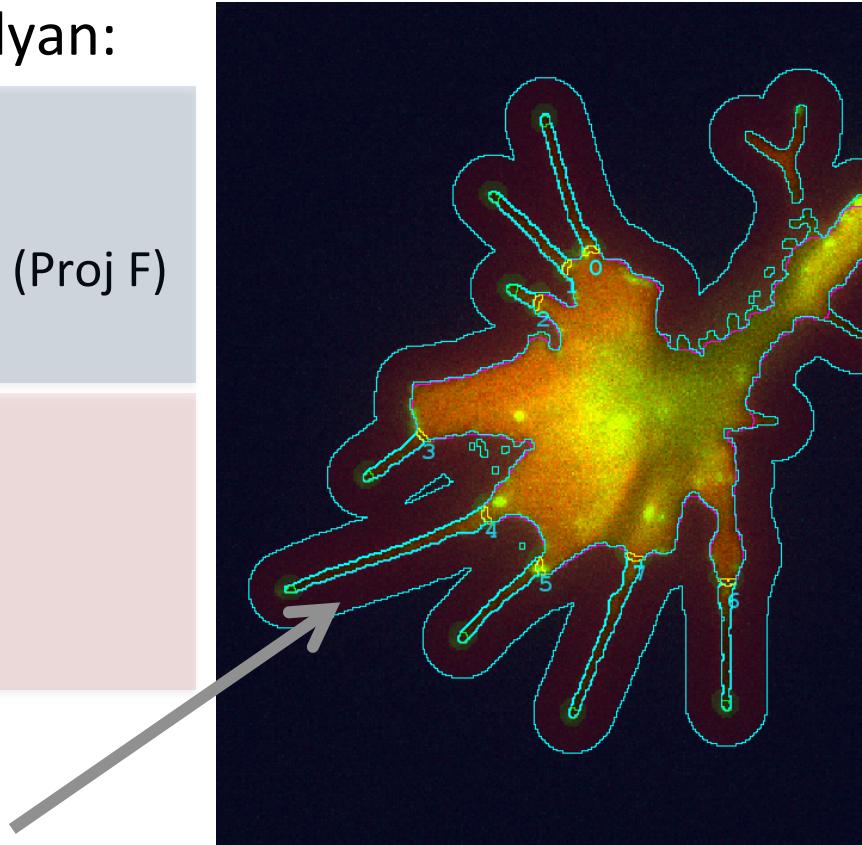
# Background measurements in Filopodyan

Measurements available in Filopodyan:

- Base fluorescence (Base F)
  - Tip fluorescence (Tip F)
  - Projection (filopodium) fluorescence (Proj F)
  - Body fluorescence (Body F)
- 
- Frame background
  - Boundary background
  - Local base background
  - Local tip background

Boundary background:

ROI within a fixed distance from the cell boundary (signal ROI enlarged by fixed distance, minus signal ROI)



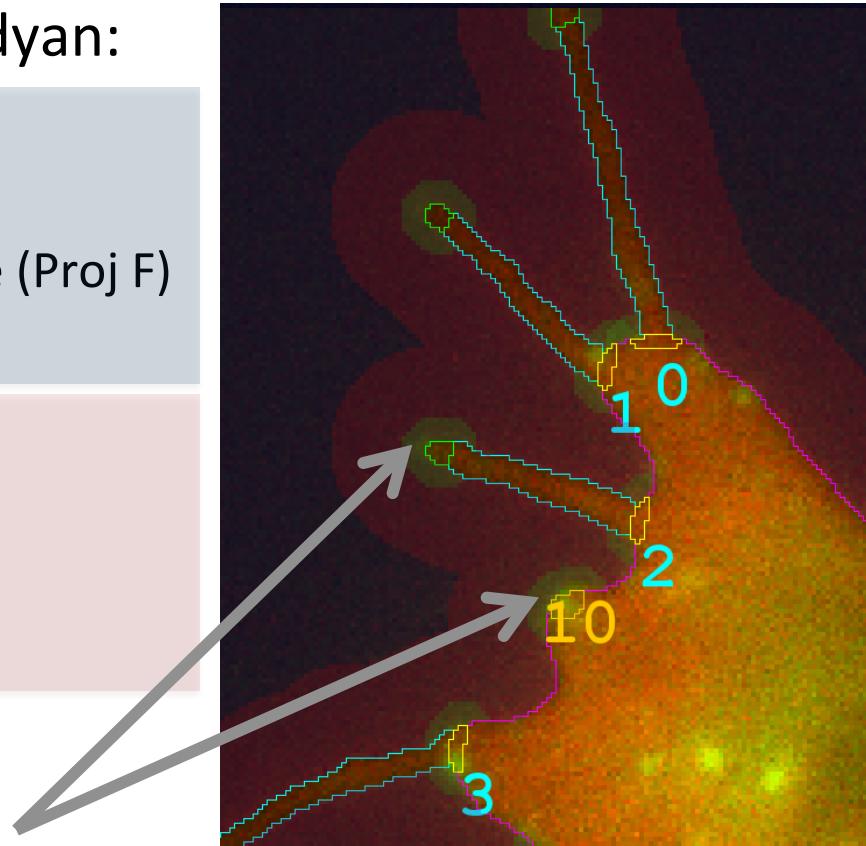
# Background measurements in Filopodyan

Measurements available in Filopodyan:

- Base fluorescence (Base F)
- Tip fluorescence (Tip F)
- Projection (filopodium) fluorescence (Proj F)
- Body fluorescence (Body F)
- Frame background
- Boundary background
- Local base background
- Local tip background

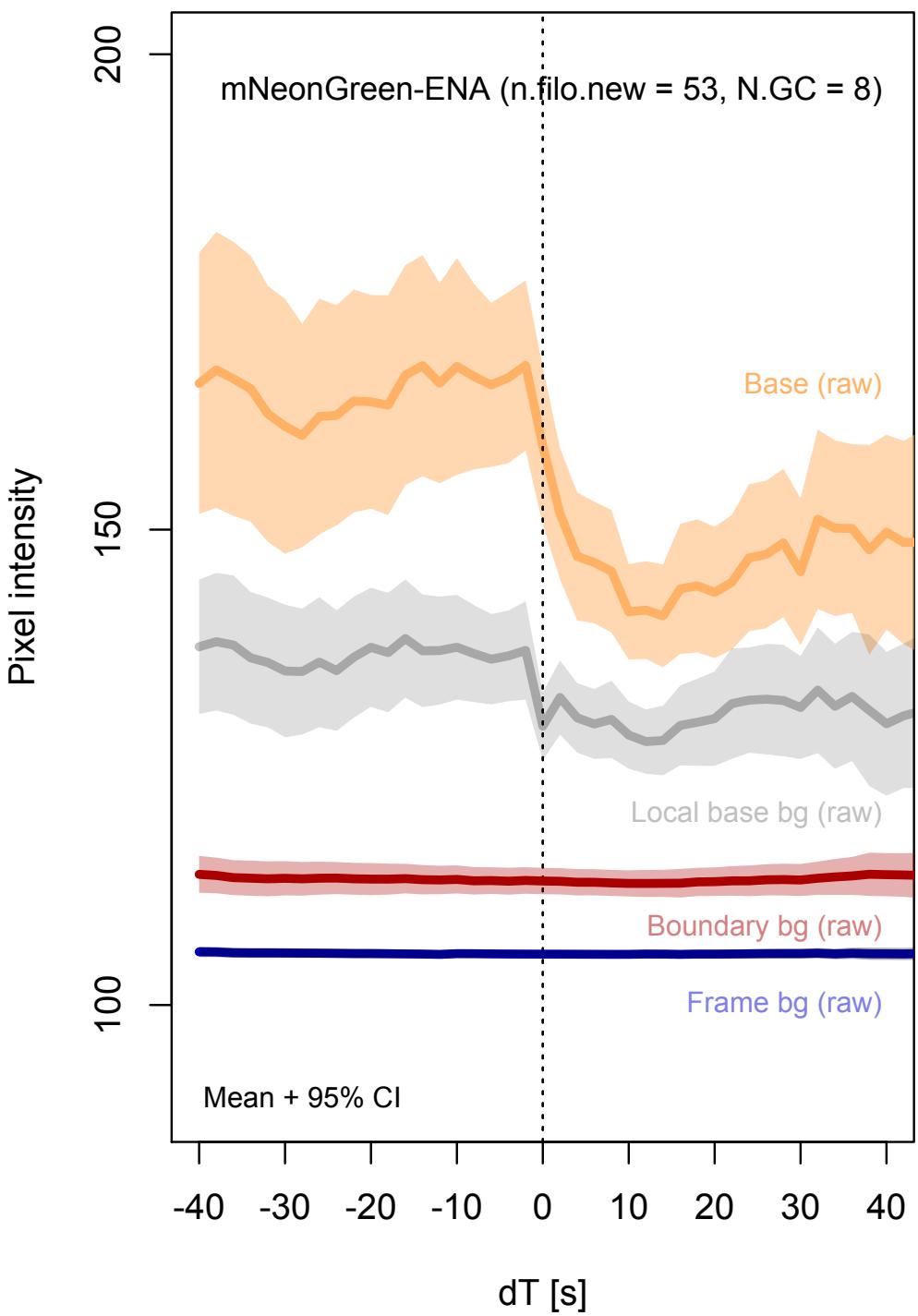
Local base/tip background:

Defined area within specified distance from the base/tip coordinates, not overlapping with the body area.



## Applying to an example dataset:

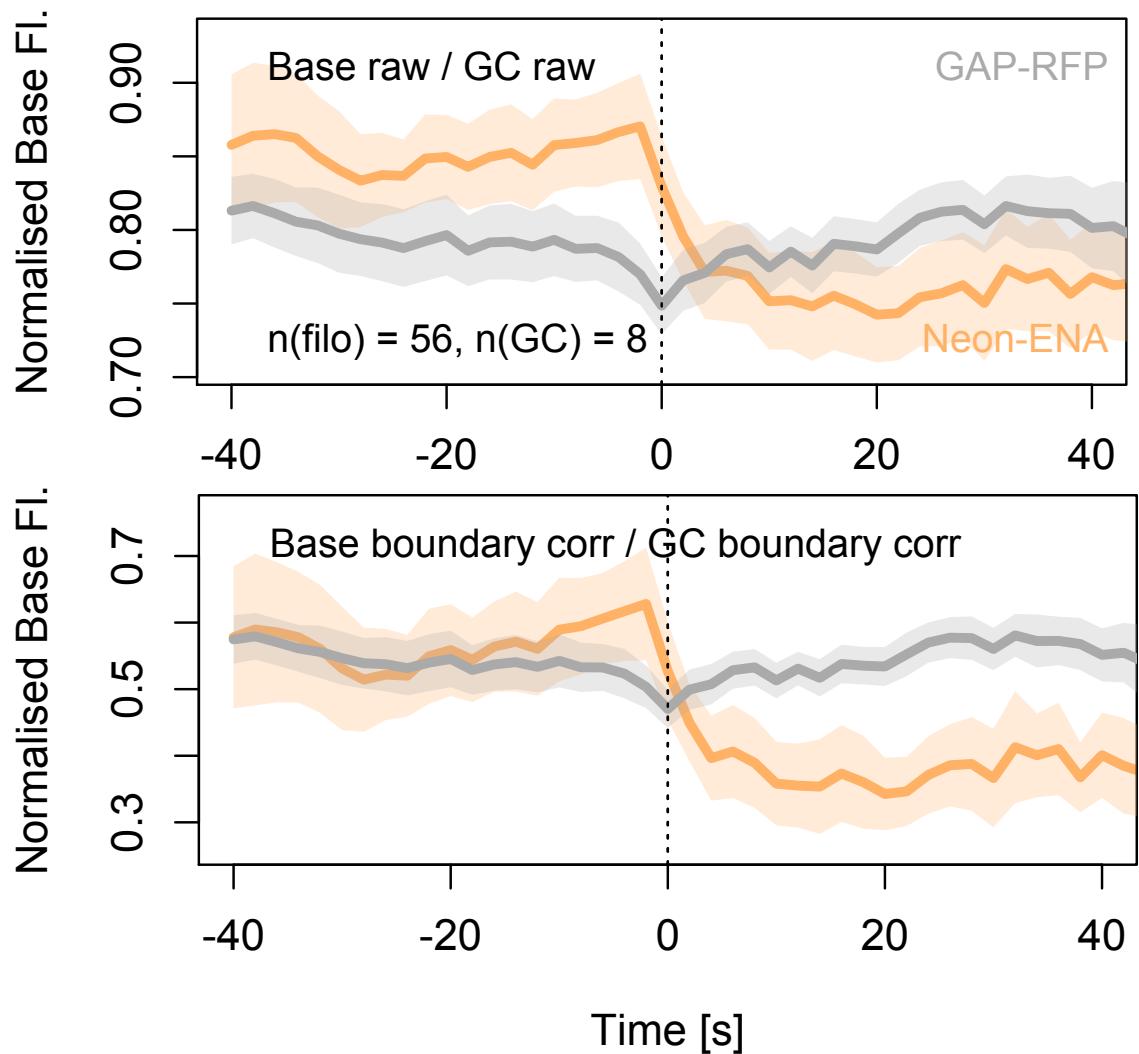
- Base fluorescence (Base F)
- Tip fluorescence (Tip F)
- Projection fluoresc. (Proj F)
- Body fluorescence (Body F)
- Frame background
- Boundary background
- Local base background
- Local tip background



# Background-corrected vs raw data - comparison

## Analysis with or without bg-correction

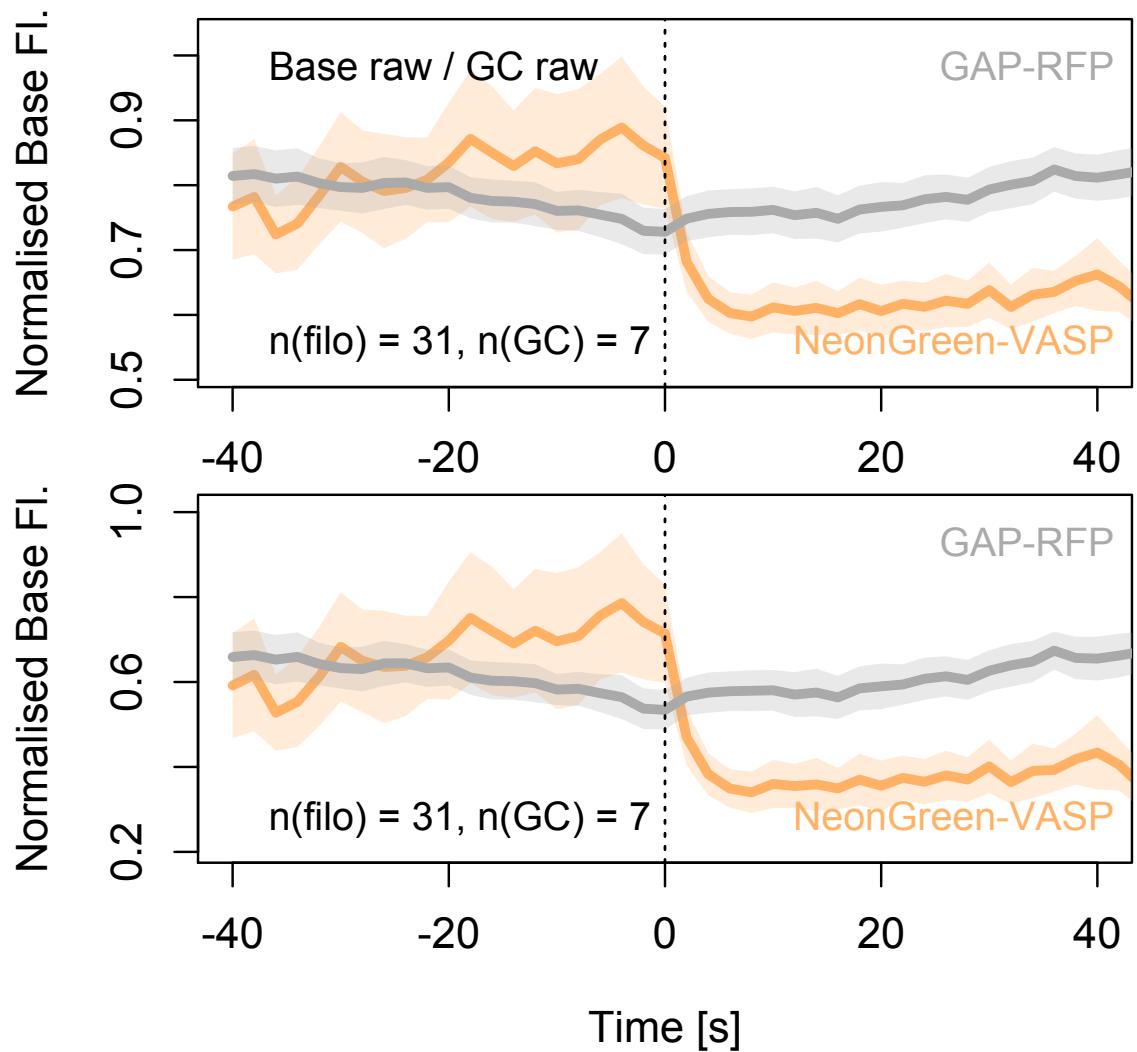
example dataset 1



# Background-corrected vs raw data - comparison

## Analysis with or without bg-correction

example dataset 2

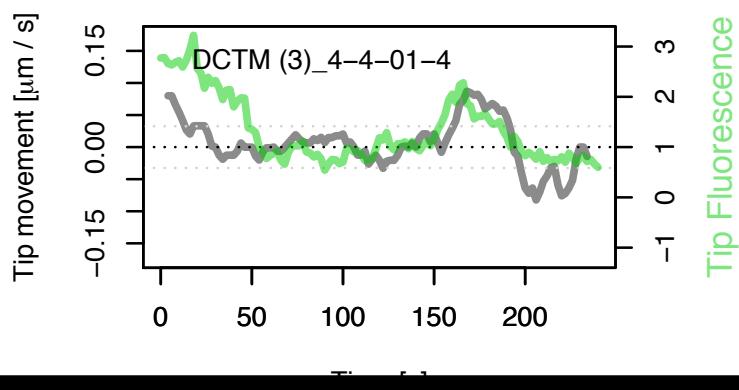
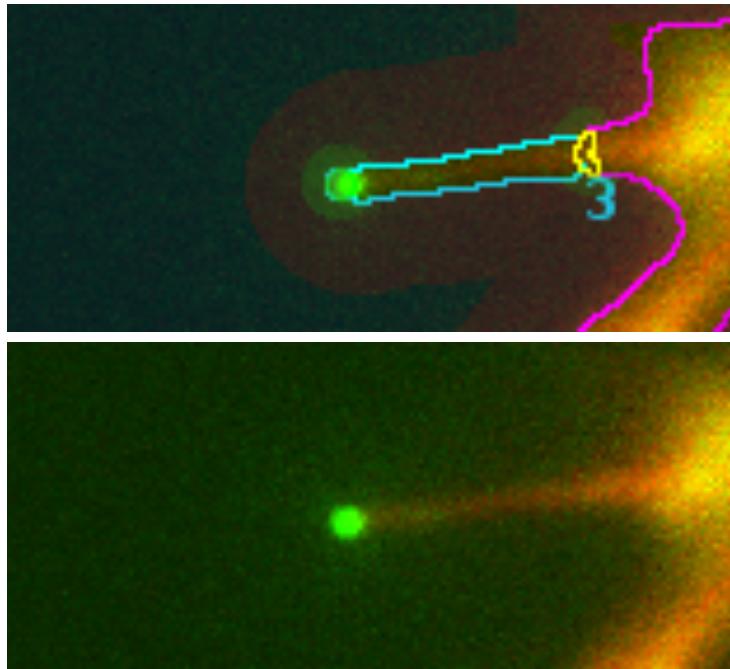


### 3c: Base Fluorescence (scripts overview)

#### R scripts: Masterscript (Modules 1 & 2, including BgCorrection); BaseF

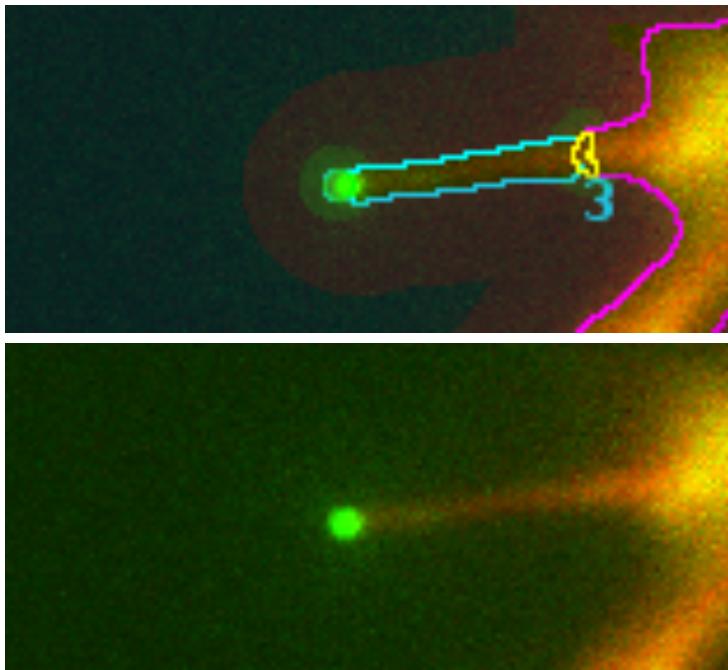
- Masterscript, Modules 1 & 2:
  - as in phenotype comparison (except source BgCorrection script)
- FilopodyanR Module 1-2\_BgCorrection.R:
  - takes value of bg.corr.setting (defined in parent script), e.g. one of ‘none’, ‘local’, ‘boundary’, ‘frame’
  - a complicated patch to keep F and Bg measurements in the same time frame
  - creates data frames with Bg-corrected measurements – xxx.corrected
- FilopodyanR BaseF.R:
  - computes mean and CI for Bg-corrected base F measurements for both folders
  - visualises mean base F +- CI for both folders

### 3d: Tip Fluorescence and Tip Movement

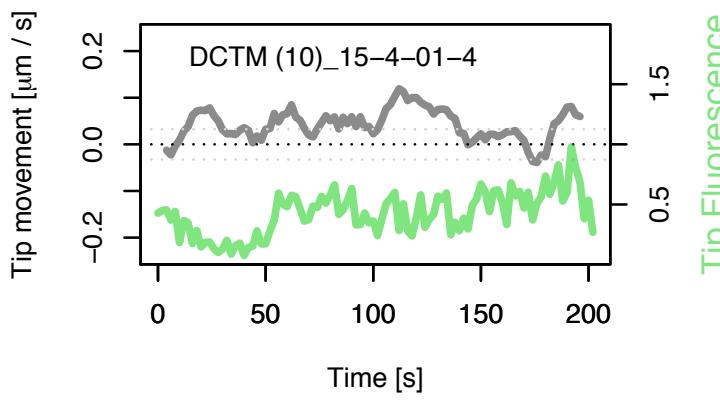
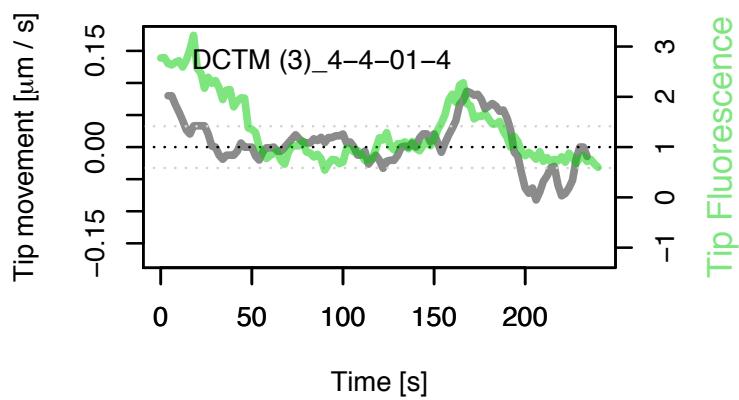
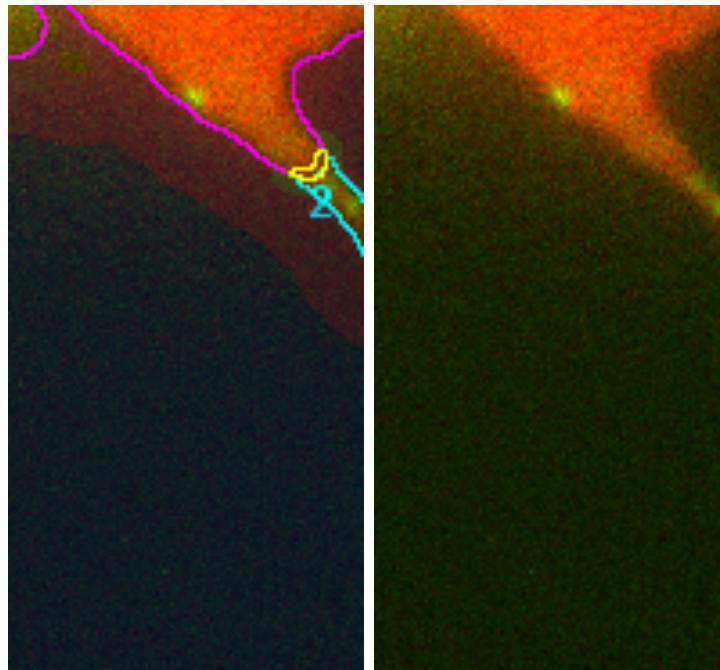


# Fluorescence and movement – Diverse relationships

Positively correlating example:

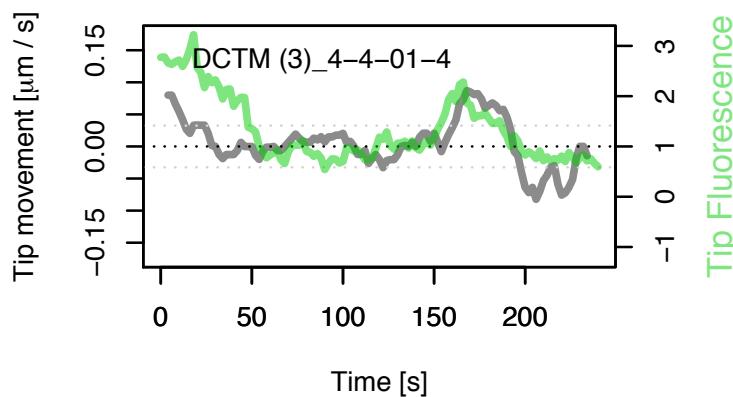
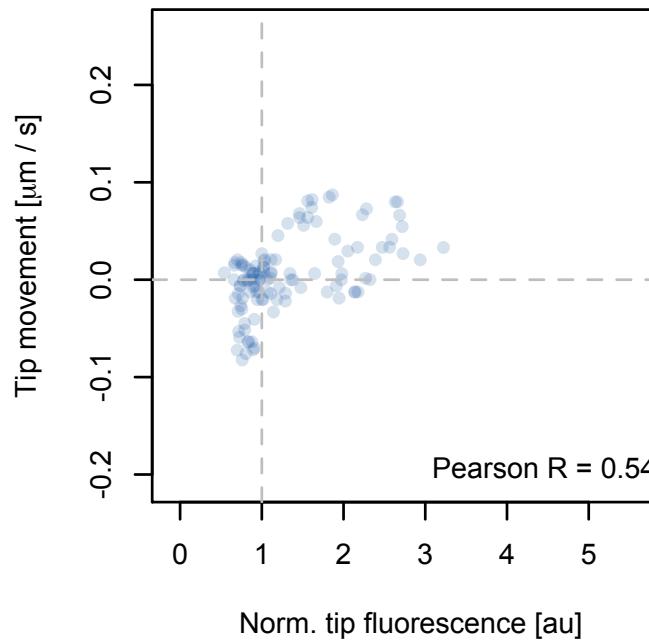


Non-correlating example:

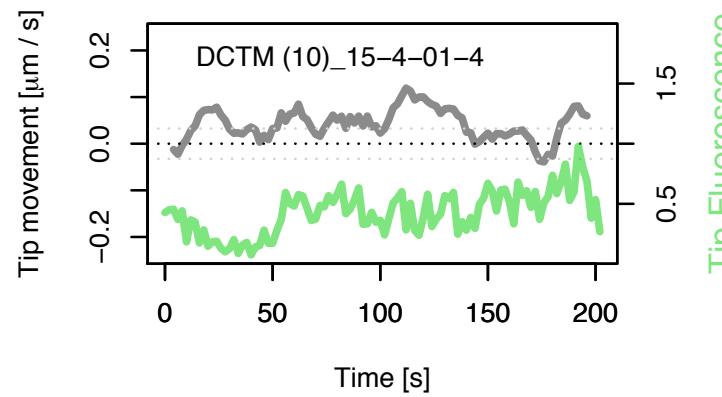
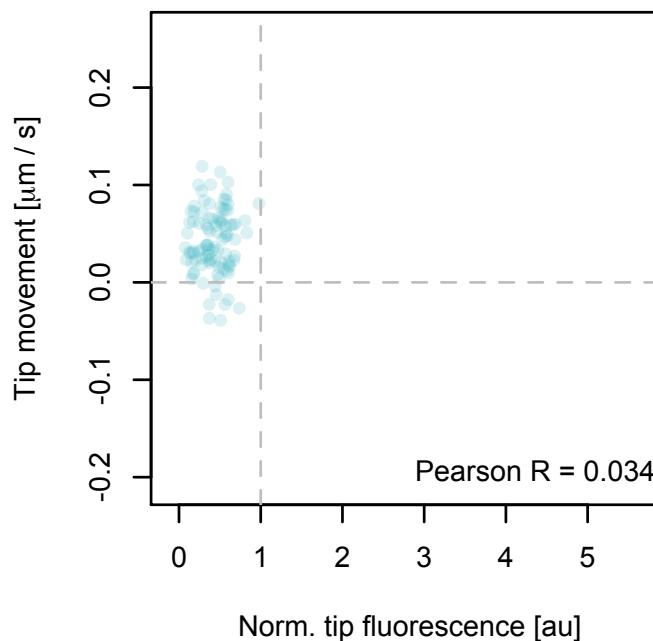


# Fluorescence and movement – Diverse relationships

Positively correlating example:

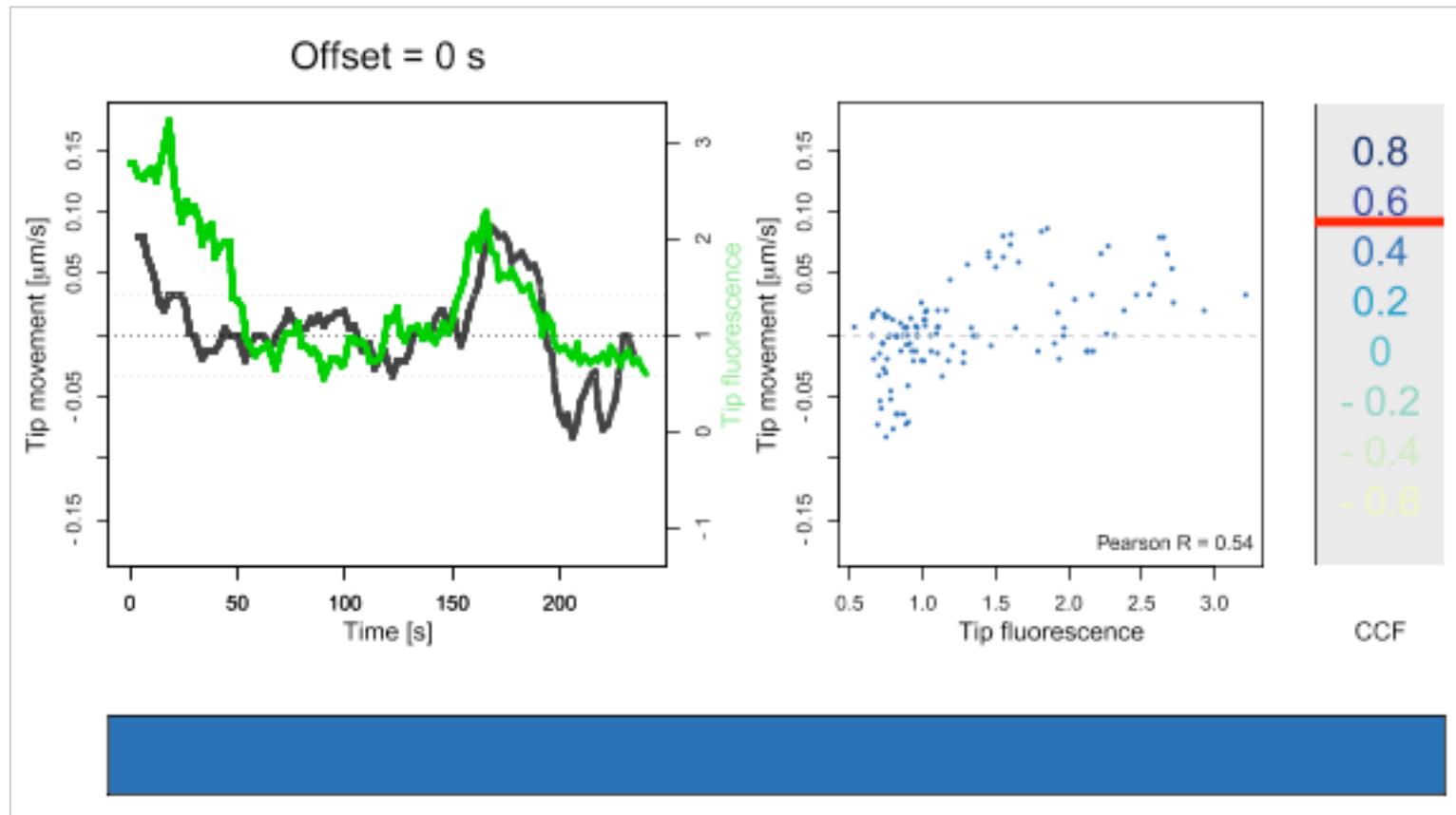


Non-correlating example:

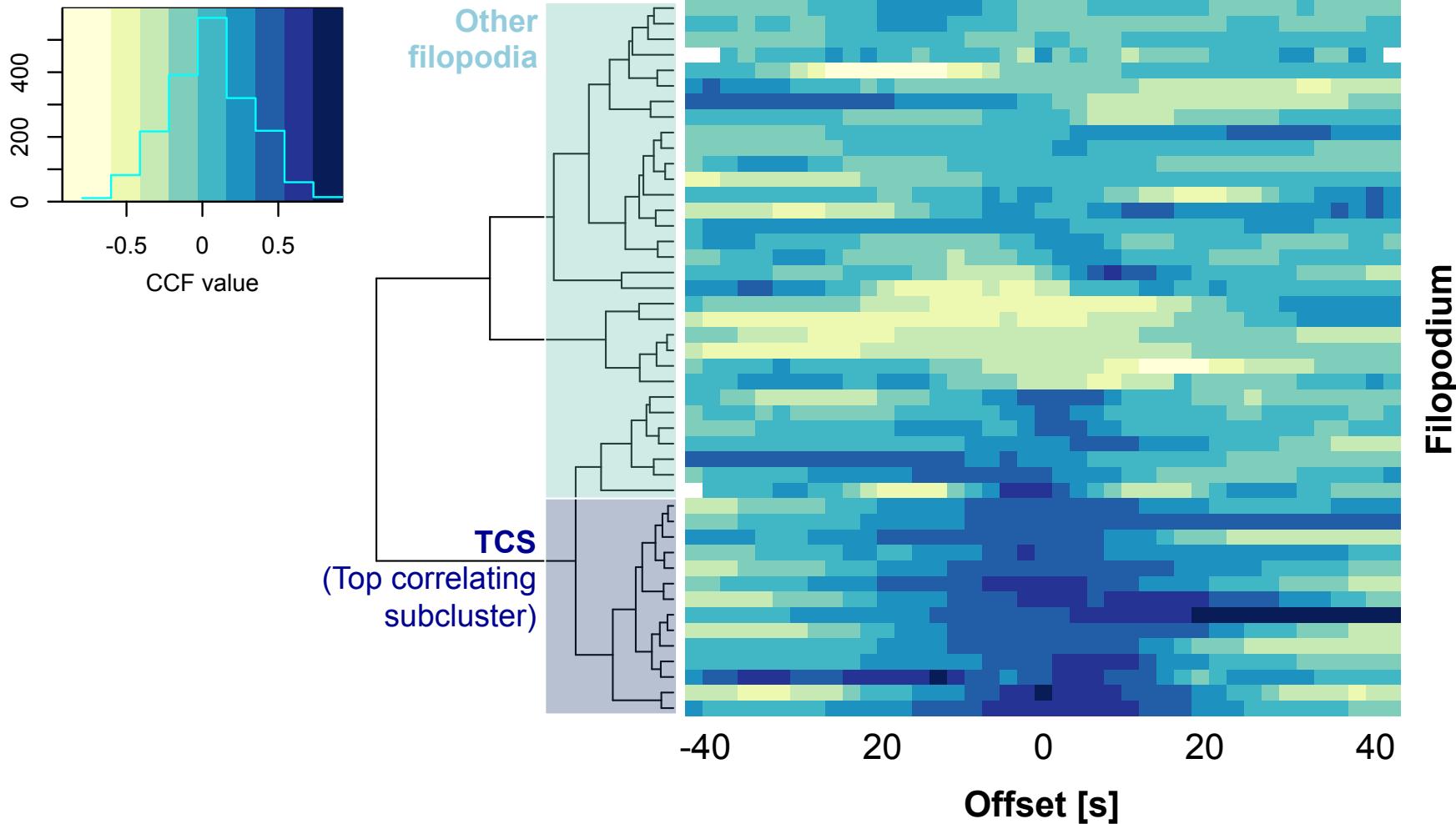


# Fluoerscence and movement - Timing of effect

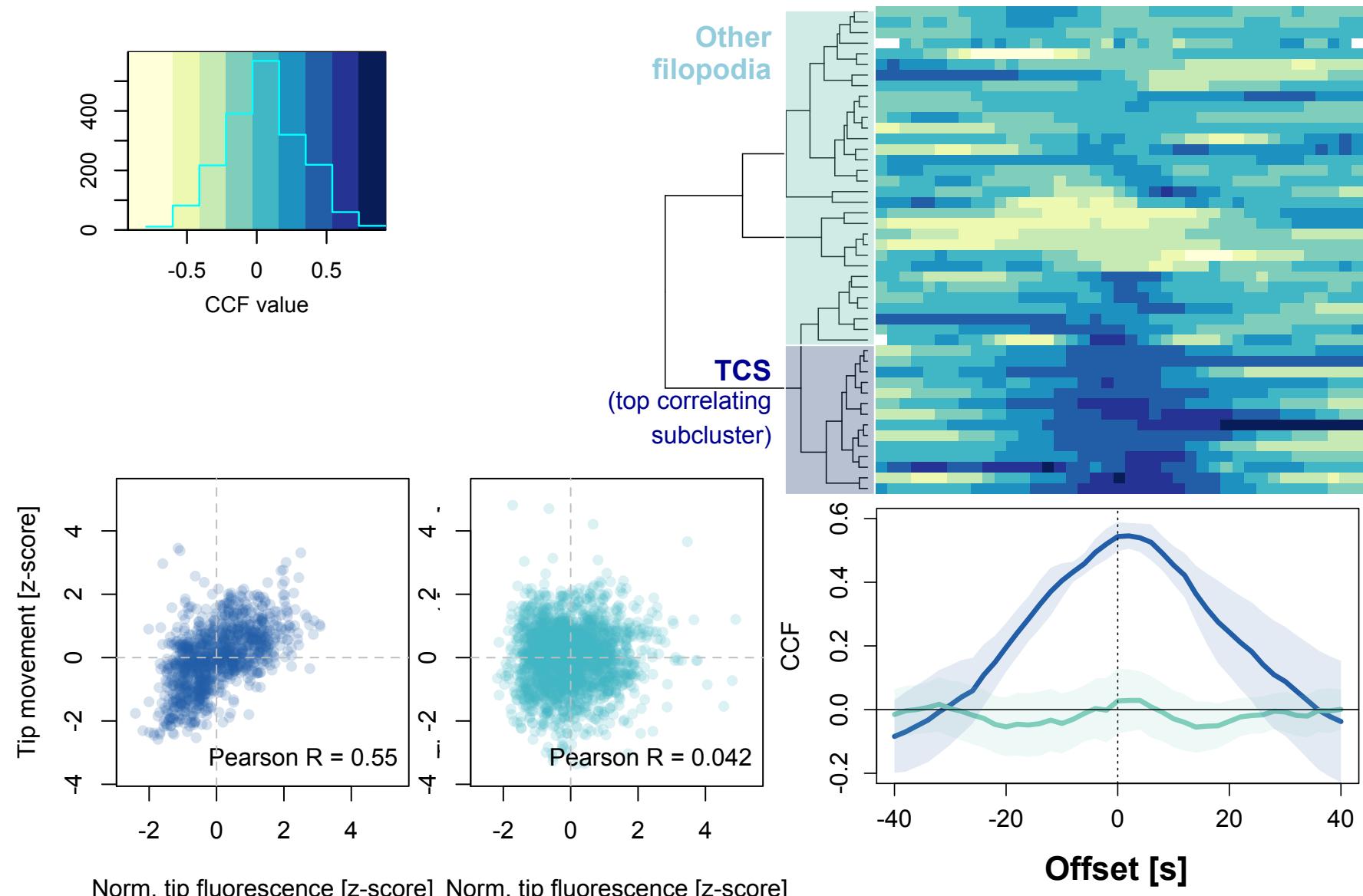
Can the relationship be delayed? (Cross-correlation function)



# Fluorescence and movement – Clustering according to relationship



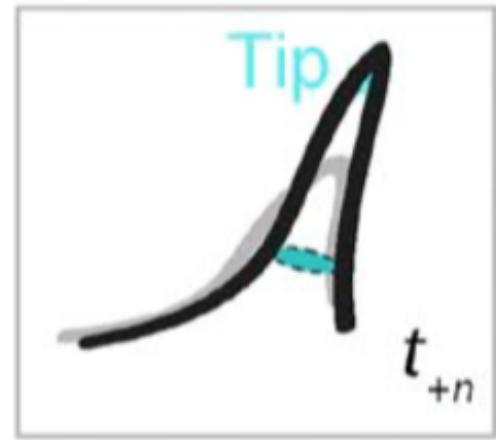
# Fluorescence and movement – Clustering according to relationship



# Key definitions

## Tip position:

Part of the filopodium most distant from the body



## Tip fluorescence:

'Tip thresholded fluorescence' (to reduce amount of background measured as part of the ROI):

Otsu split of the tip ROI

## Tip movement:

'Direction-corrected tip movement' [DCTM]

In R, filtered with a 5-timepoint rolling mean filter ('fdctm') to minimise noise

### 3d: Tip movement and fluorescence (scripts overview)

R scripts: CCF, subcluster analysis;

- **FilopodyanR CCF.R:**
  - Import of data (.Rdata; saved downstream of masterscript)
  - z-score normalisation of tip f. and movement measurement - scale()
  - Plot XY correlation for all timepoints over entire dataset
  - Compute cross-correlation function (CCF) for each filopodium - ccf()
  - Weighting according to the length of timecourse
  - Visualisation of CCF values per filopodium (heatmap)
- **FilopodyanR CCF\_subcluster-analysis:**
  - Split filopodia into subclusters according to their CCF:  
TCS: top (positively) correlating subcluster filopodia,  
nonTCS: non-correlating filopodia
  - (NB requires manual input of the size of top correlating subcluster, section 3)
  - Identify representative filopodia for each subcluster
  - Plot XY correlation for individual filopodia (within each subcluster)
  - Plot line time course for individual filopodia
  - Phenotype analysis comparing the properties of filopodia subclusters

## 3d: Tip movement and fluorescence (scripts overview)

### R scripts: CCF Randomisations, MarkovChains

- **FilopodyanR CCF\_Randomisations.R:**
  - Goal: Statistical significance of the observed relationship for subclusters
- **FilopodyanR MarkovChains.R:**
  - Goal: Statistical significance of the observed relationship for individual filopodia

I USED TO THINK  
CORRELATION IMPLIED  
CAUSATION.



THEN I TOOK A  
STATISTICS CLASS.  
NOW I DON'T.



SOUNDS LIKE THE  
CLASS HELPED.

WELL, MAYBE.



# Fluorescence and movement – Statistical likelihood (1)

Is the value of cross-correlation higher-than-random?

(A) *Randomisations*

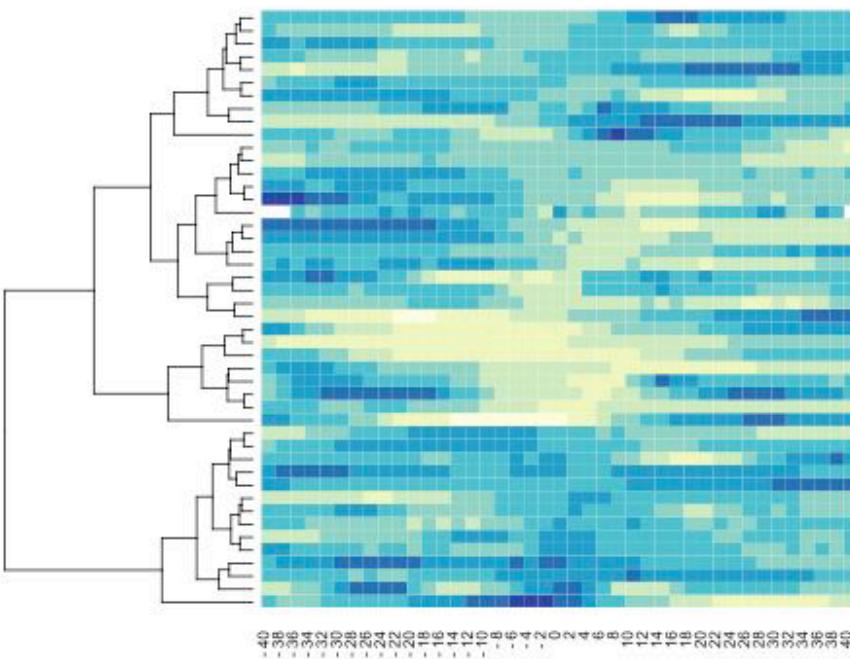
*What is the chance that a **randomised dataset** resulted in a  
positively correlating subcluster of the **same size**  
with the **same (or higher) mean CCF**?*

# Fluorescence and movement – Statistical likelihood (1)

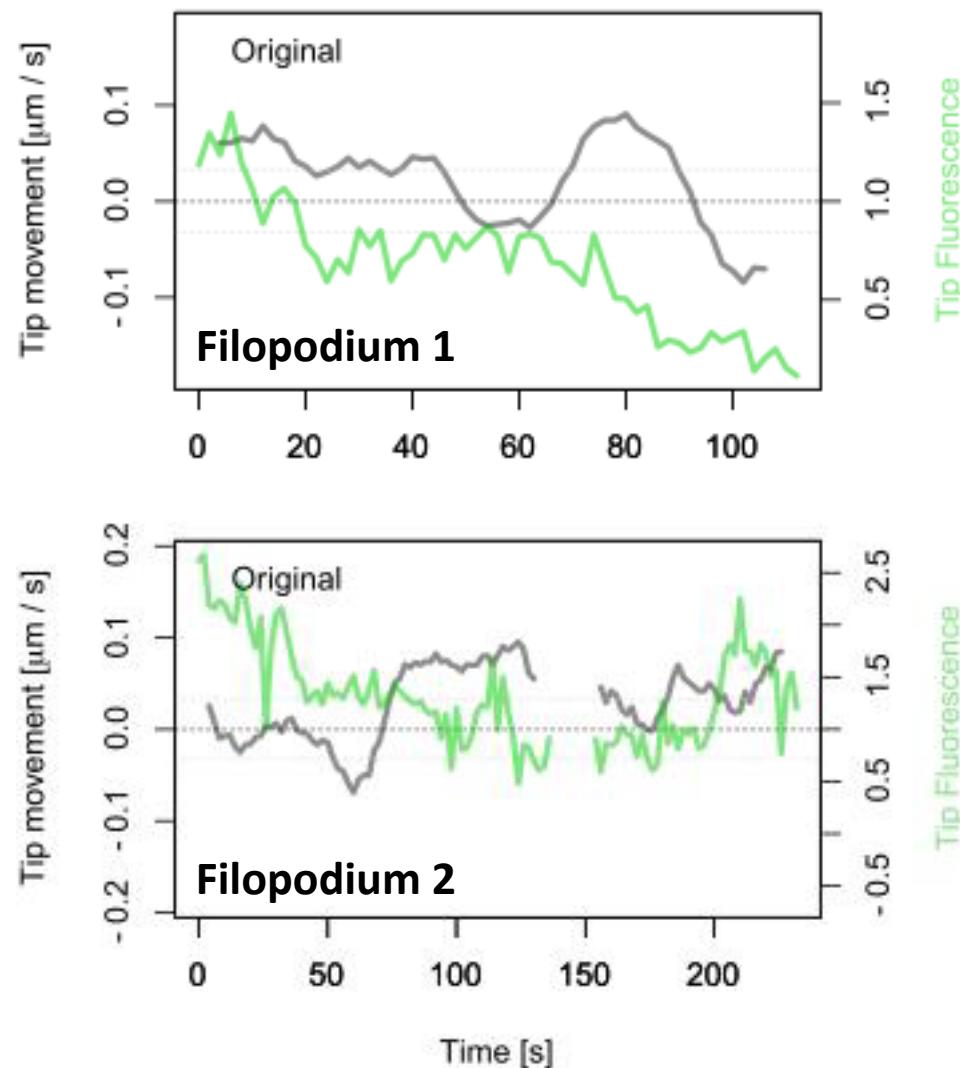
For each dataset of randomised filopodia:

- >> recalculate cross-correlation
- >> recluster

Sample of clustering on randomised filopodia:



Randomised filopodia sequences:

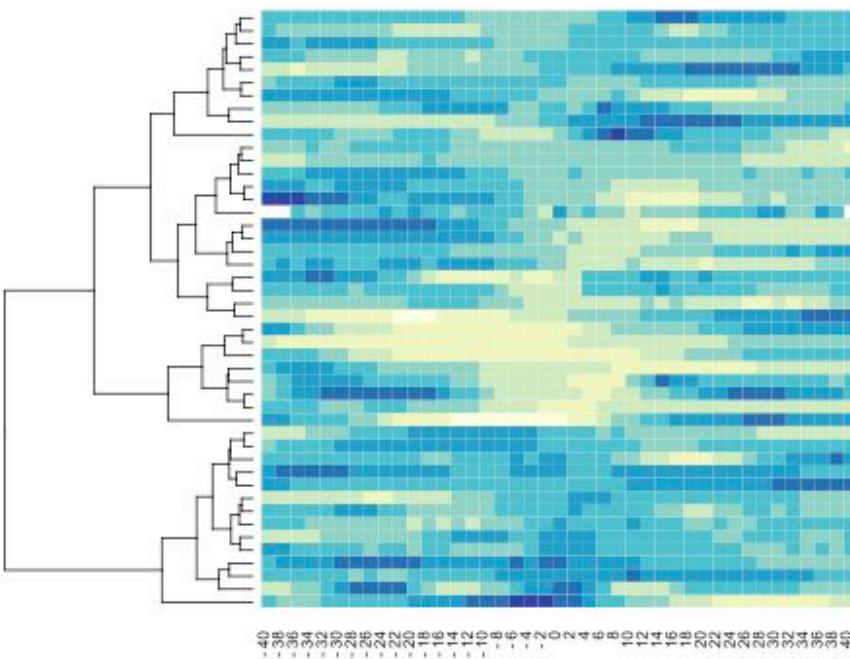


# Fluorescence and movement – Statistical likelihood (1)

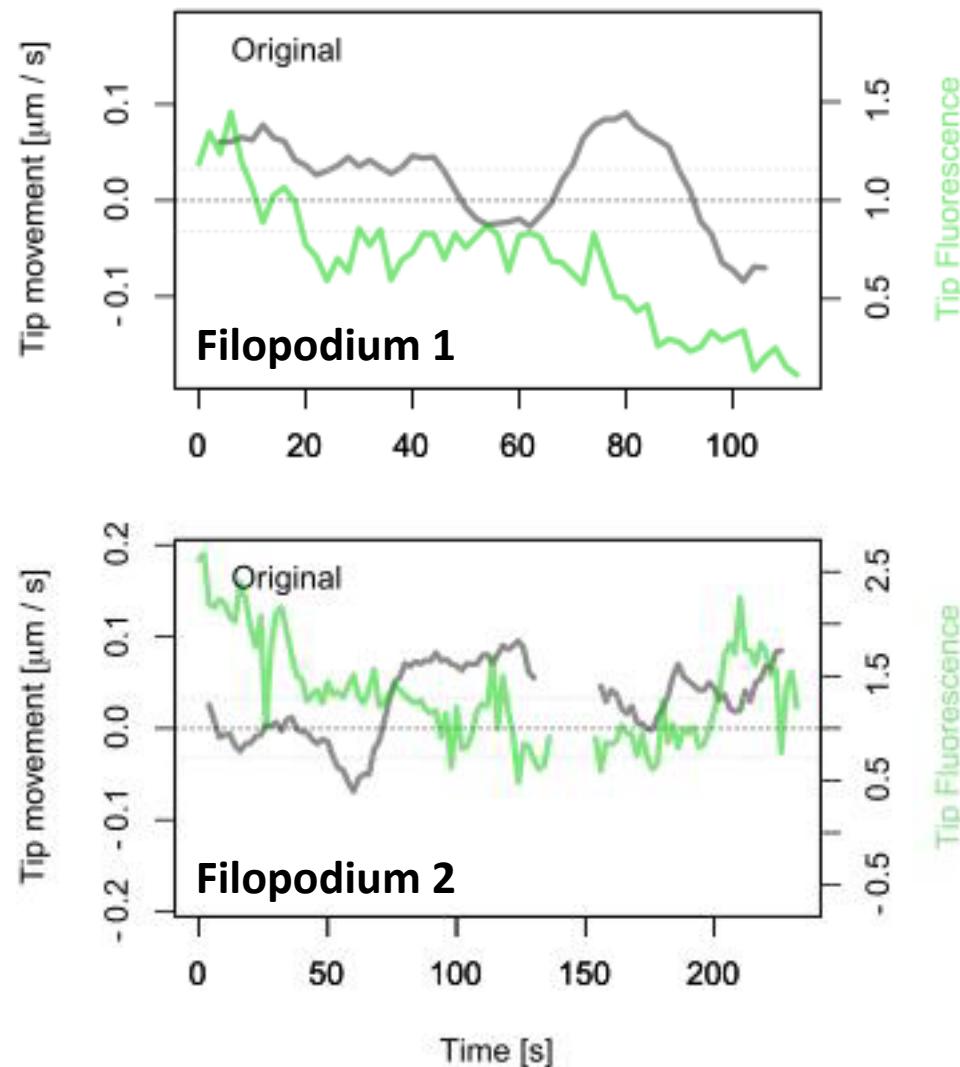
For each dataset of randomised filopodia:

- >> recalculate cross-correlation
- >> recluster

Sample of clustering on randomised filopodia:

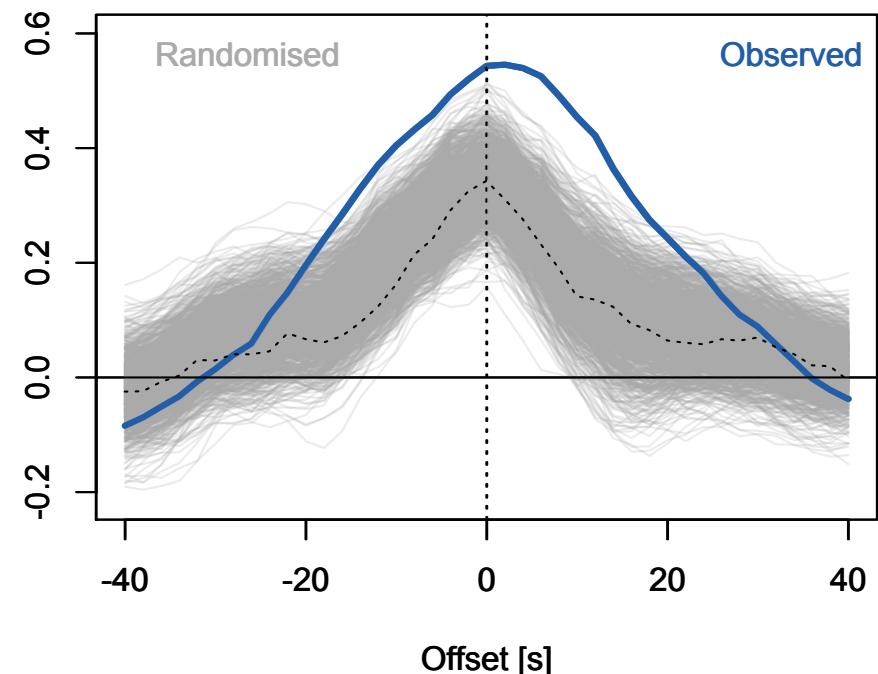
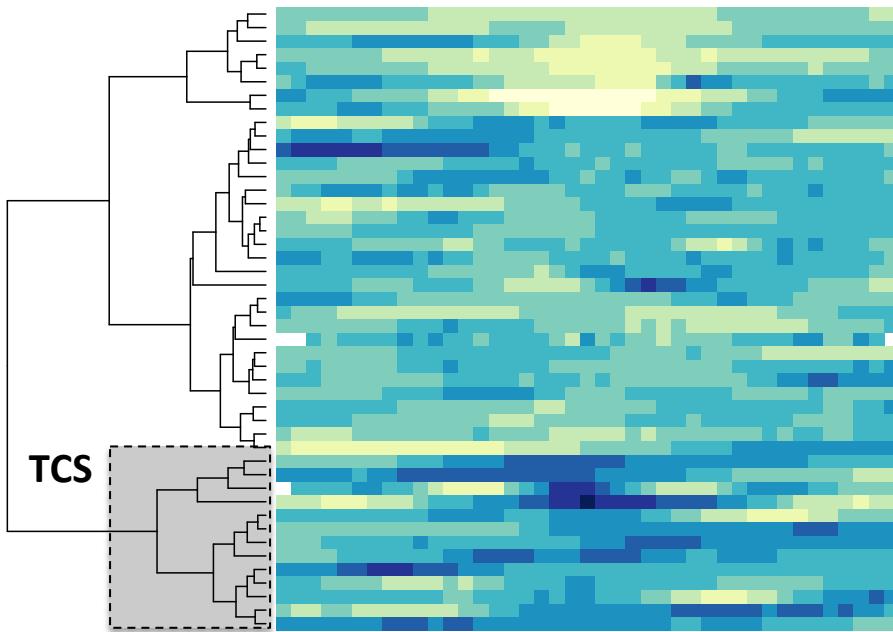


Randomised filopodia sequences:



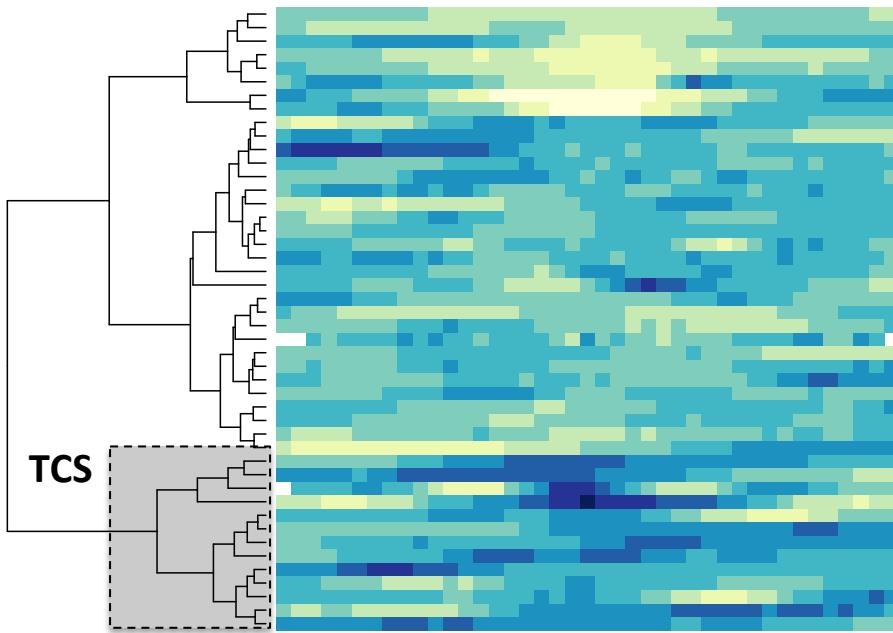
# Fluorescence and movement – Statistical likelihood (1)

Example (representative) randomisation:

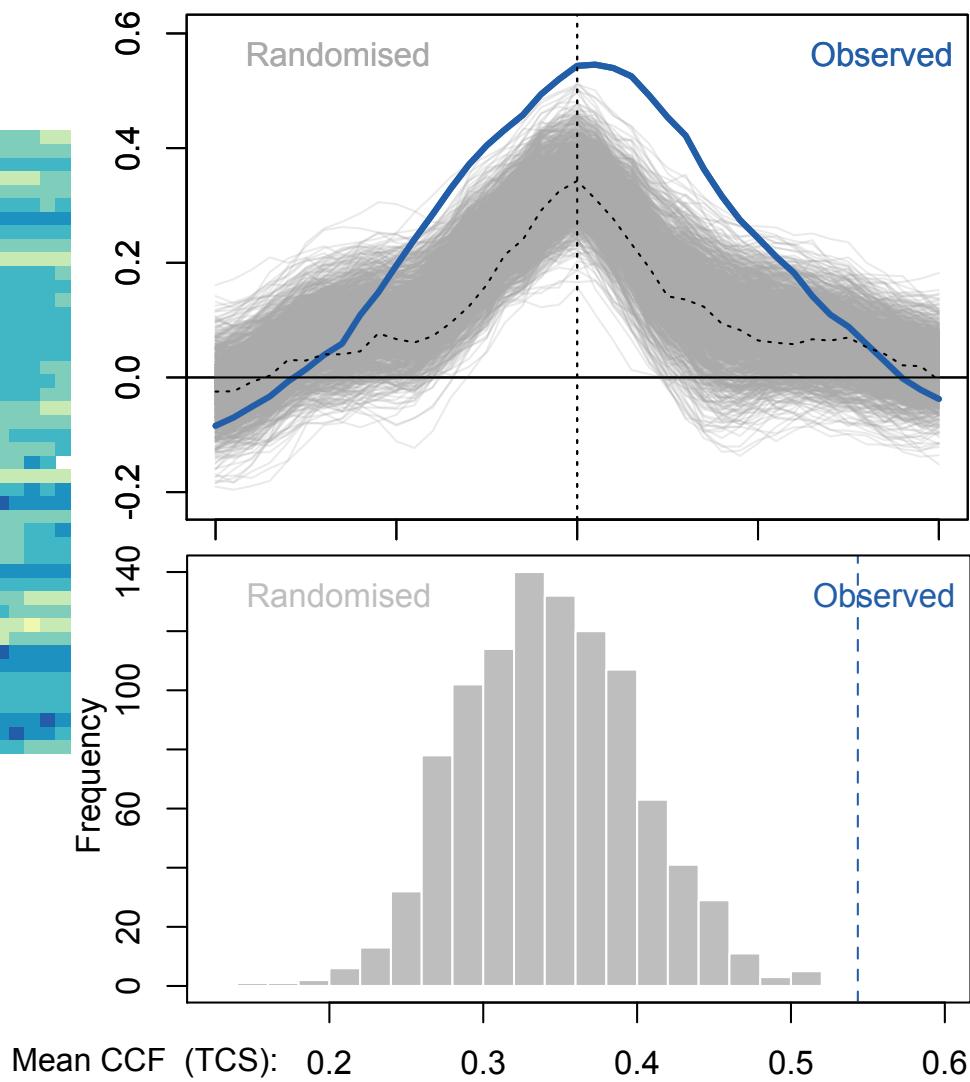


# Fluorescence and movement – Statistical likelihood (1)

Example (representative) randomisation:



→ There is less than 1 in 1000 probability ( $P < 0.001$ ) for a chance observation of a positively correlating subcluster of ~14 filopodia with mean CCF as seen in the observed data set.



# Fluorescence and movement – Statistical likelihood (2)

Is the value of cross-correlation higher-than-random?

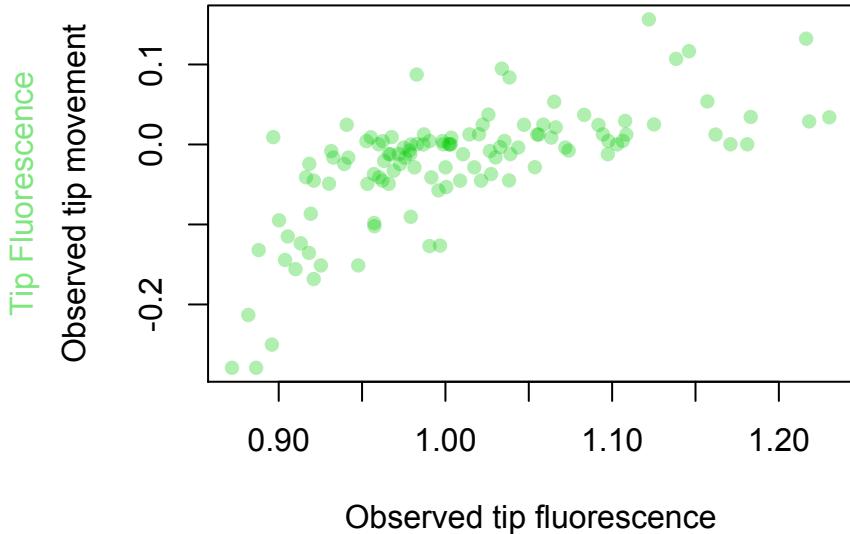
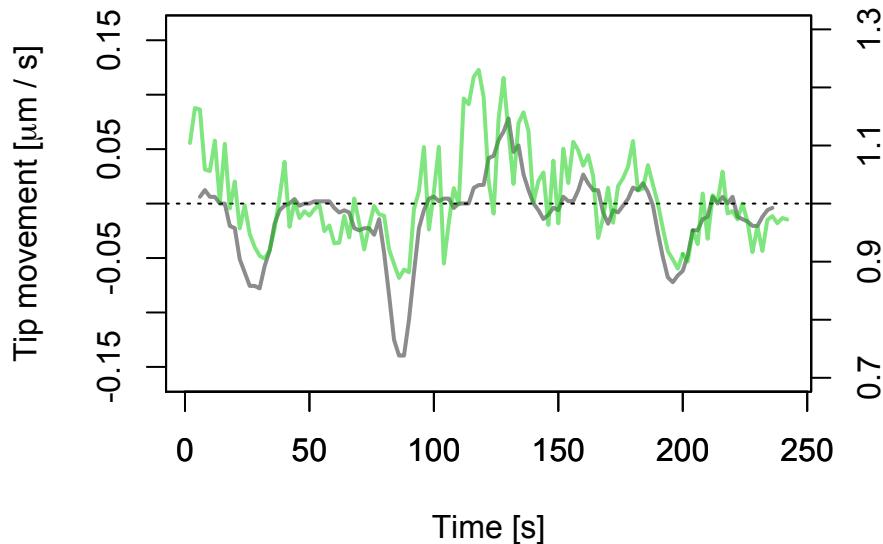
(B) *Markov Chains*

*What is the chance that measurements from a filopodium with **uncoupled fluorescence and movement** resulted in a positive correlation of the **same magnitude** as in the observed filopodium?*

# Fluorescence and movement – Statistical likelihood (2)

## ORIGINAL DATA

(example filopodium f0)



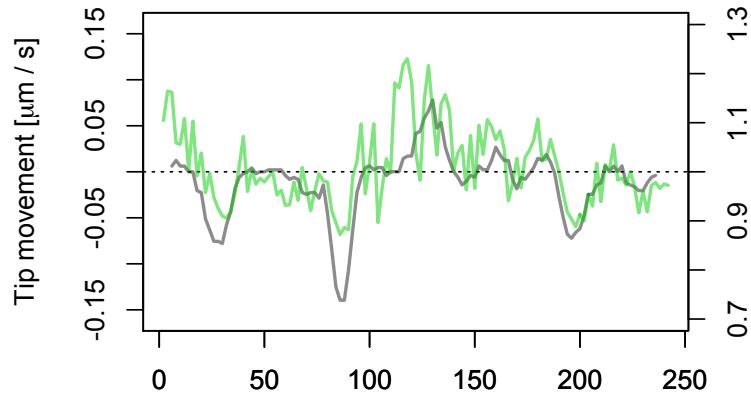
How likely is it that these two plots show the observed correlation by chance?

>>

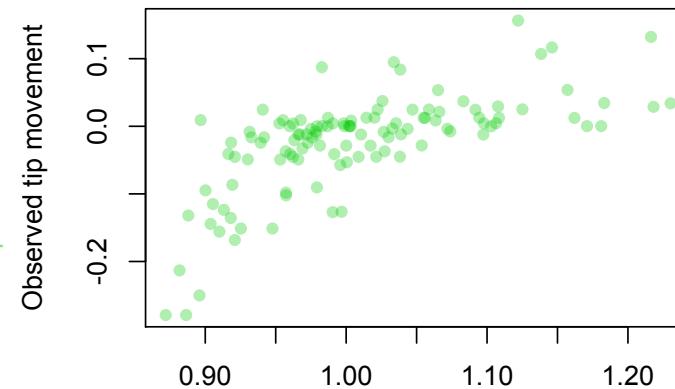
1. Assume the two curves are independent – extract properties of each
2. Run independent simulations for tip F and tip movement which preserve these properties and see how often the simulations yield a similar level of correlation

# Fluorescence and movement – Statistical likelihood (2)

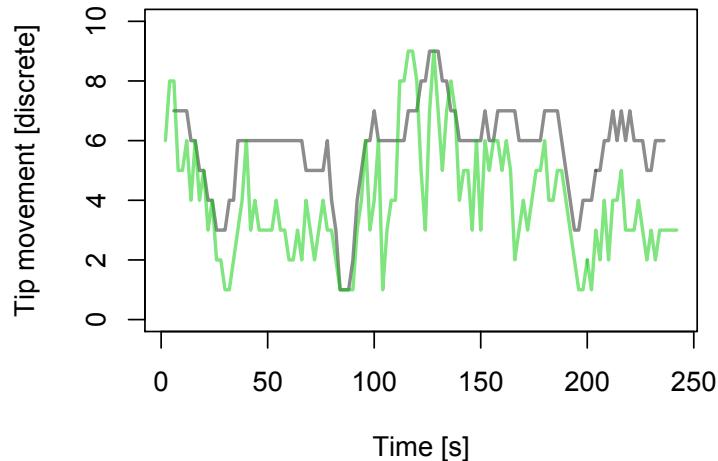
**ORIGINAL:** Timeline



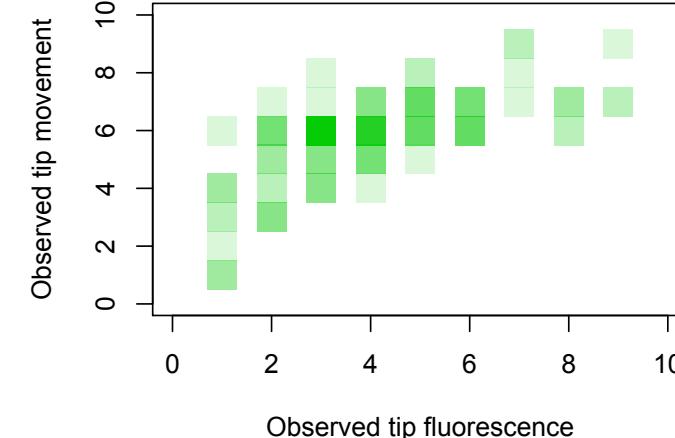
Correlation



**BINNED:**



Tip Fluorescence [disc.]

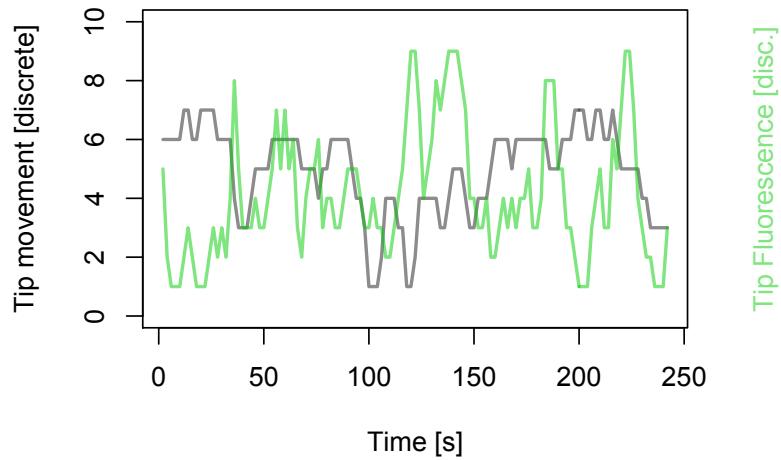


Observed tip fluorescence

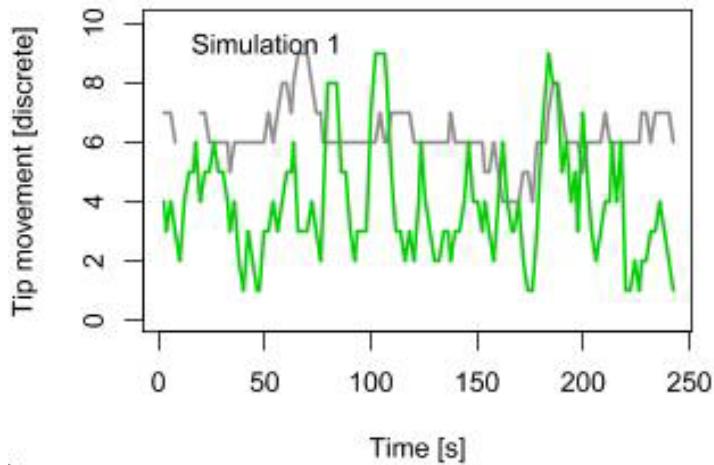
# Fluorescence and movement – Statistical likelihood (2)

## Simulation:

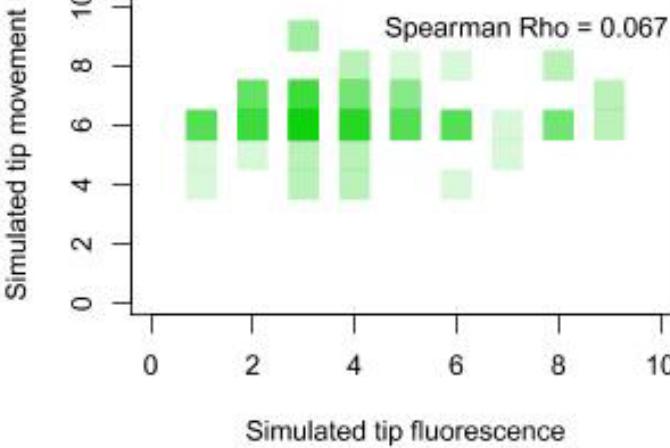
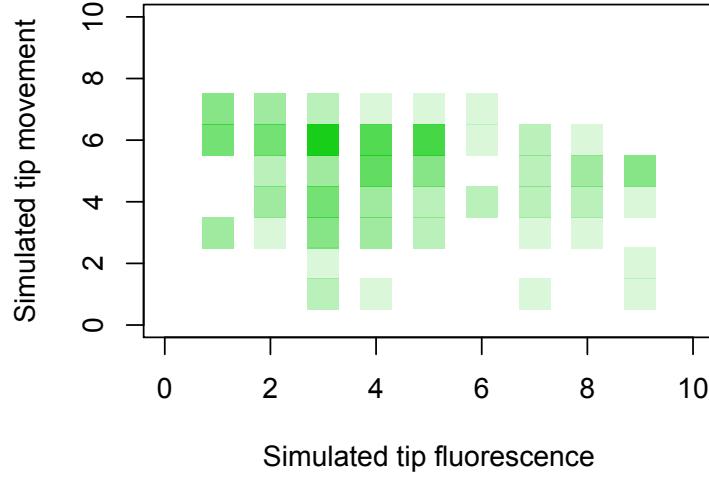
(example simulation for one filopodium):



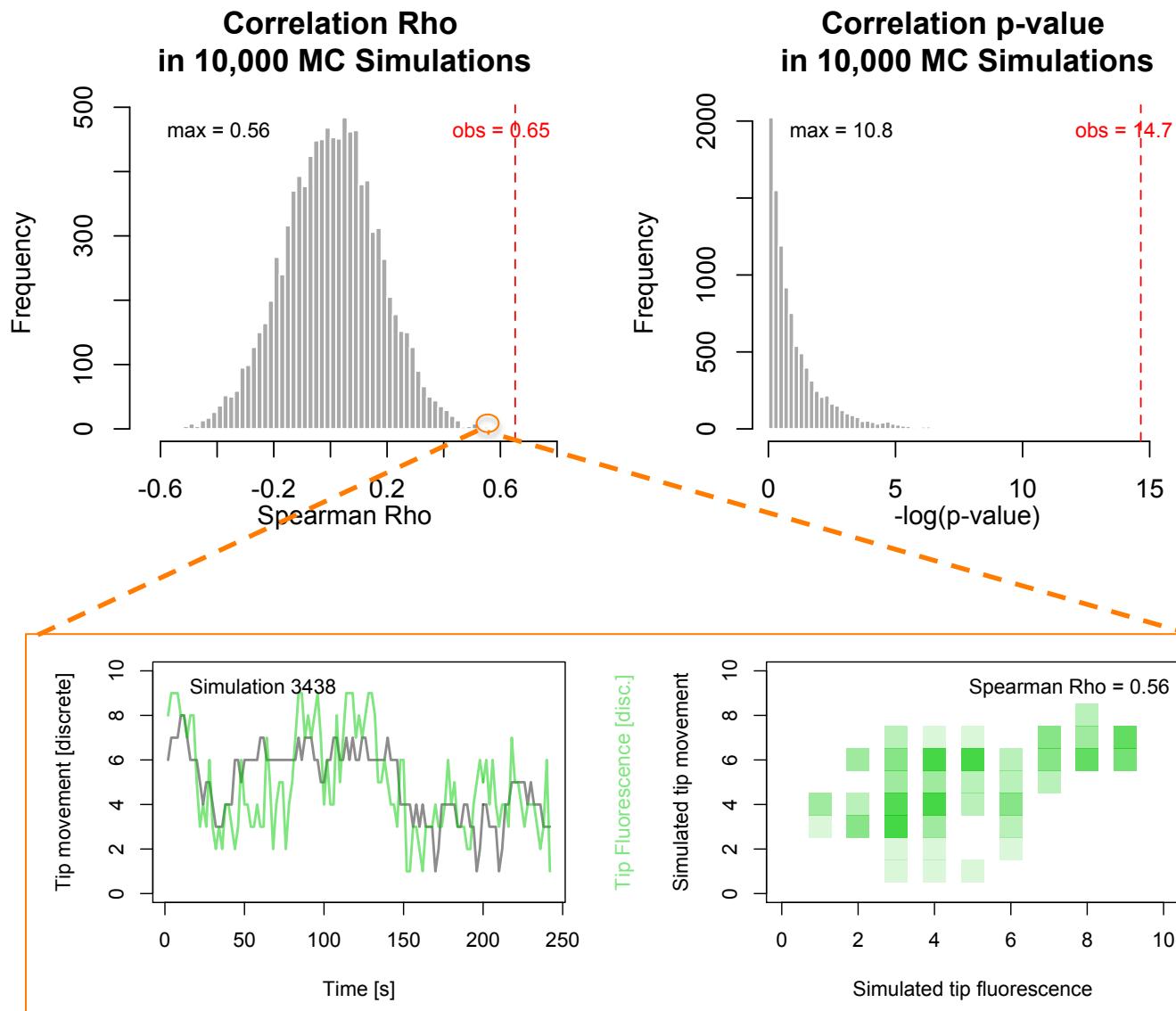
Tip Fluorescence [discr.]



Tip Fluorescence [discr.]



# Fluorescence and movement – Statistical likelihood (2)



# Summary

1. Output of the Filopodyan plugin requires diverse approaches to data analysis:

- data processing (filtering, cleaning, background-correction, ...)
- phenotype comparison (statistics / hypothesis testing)
- relationship between properties (correlations)
- fluorescence measurements over time (visualizing timelines and CI)

2. Analysis of relationship between fluorescence and movement:

- accounting for a **delayed effect** by use of cross-correlation function
- **diversity** in the relationship between tip movement and tip localisation
- **hierarchical clustering** for grouping filopodia with similar relationship
- assessing **significance**: randomisations and Markov Chains

# Weaknesses and lessons learnt from the R pipeline

## User friendliness and robustness

- **Complex modular structure**
  - would benefit from a more robust pipeline architecture
    - e.g. code from modules 1 & 2 exists as independent scripts to be sourced from masterscript (3a, 3c), and as parts of another script (e.g. DataInput in 3b) → versioning difficulties
  - modules reused for purposes of different analyses → versioning difficulties
- **Co-evolved with FIJI plugin**
  - Superfluous code (old fixes surviving as code fossils)
- **Ideally...**
  - a single integrated analysis package with a user-friendly R GUI (similar to the Fiji GUI) for multiple different types of analysis