STAR Protocols

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Protocol for *ex vivo* time lapse imaging of *Drosophila melanogaster* cytonemes

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Highlights

This protocol enables imaging of air sac primordium cytonemes dynamics for 4–5 h

ImageJ/FIJI Cytoneme Analysis tools provided for measuring cytoneme dynamics

RStudios pipeline provided for data processing of cytoneme dynamics measurements

This protocol describes how to image time and spatially resolved time lapses of Drosophila air sac primordium (ASP) cytonemes in *ex vivo* cultures of wing imaginal discs. It describes how to manually measure the length of cytonemes using custom-made FIJI/ImageJ tools, and to analyze data using a R/R-Studios pipeline. It can also be used for studies of cell division, organelle localization, and protein trafficking as well as other cellular materials that can be fluorescently tagged and imaged with minimal phototoxicity.

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Protocol for *ex vivo* time lapse imaging of *Drosophila melanogaster* cytonemes

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SUMMARY

This protocol describes how to image time and spatially resolved time lapses of Drosophila air sac primordium (ASP) cytonemes in *ex vivo* cultures of wing imaginal discs. It describes how to manually measure the length of cytonemes using custom-made FIJI/ImageJ tools, and to analyze data using R/R-Studios pipeline. It can also be used for studies of cell division, organelle localization, and protein trafficking as well as other cellular materials that can be fluorescently tagged and imaged with minimal phototoxicity.

BEFORE YOU BEGIN

3D printing ring device

⊙ Timing: 1–2 h

1. 3D print the inner and outer rings (Figures 1A and 1B) with Polylactic Acid (PLA) filaments. with a 3D Printer using the .stl files available for download. Troubleshooting 1

   Note: Recommended printers based on our experience: Ultimaker 3, LulzBot Mini 3D Printer).

2. Assemble the rings together with a 13 mm diameter hydrophilic Membrane Filter (Millipore HAWP01300)
   a. Assembly order from bottom up: inner ring, membrane, outer ring (Figure 1B).
   b. Push the rings tight to stretch the membrane.
   c. After the ring is assembled, use a sterile pipette tip to apply force in the middle of the membrane to release the hard stretching tension and form a small concave cavity for the wing disc (Figure 1C).
   d. Use one membrane assembly per culture.

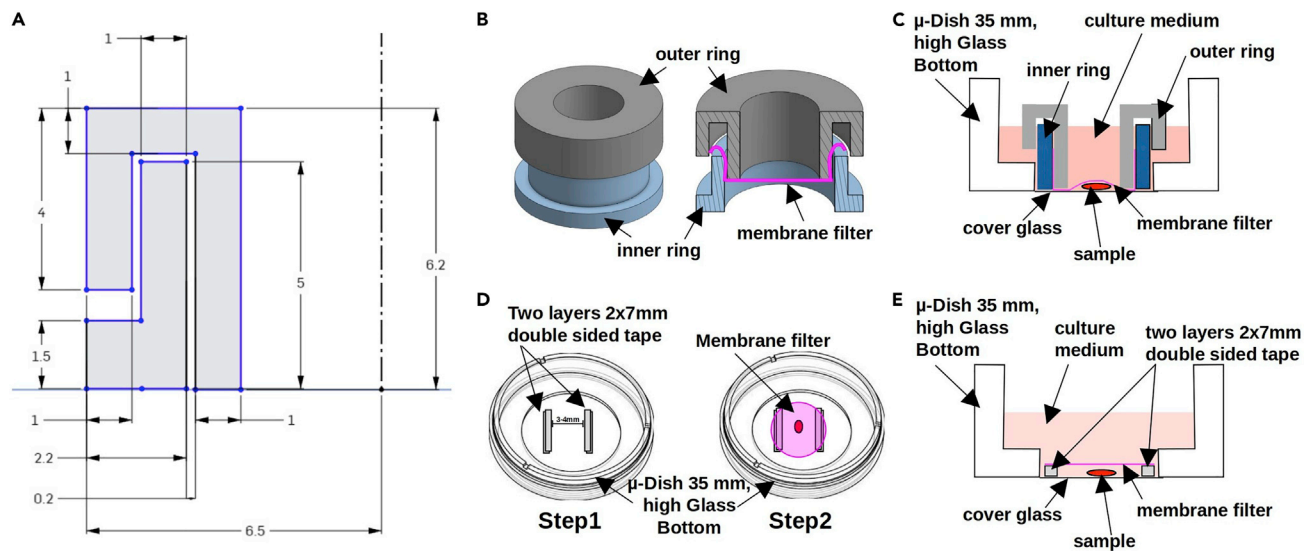   *Note:* After use, remove membrane, rinse rings with 70% ethanol, store in 70% ethanol.

   Alternatives: a small disc of approximately 13 mm diameter cut from larger hydrophilic membrane filter.

Alternative method to printing 3D rings

⊙ Timing: 5 min

3. Cut 4 pieces of double-sided tape, each approximately 2 × 7 mm.

Figure 1. 3D printed rings set up for wing imaginal disc *ex vivo* culture
(A and B) The 3D inner and outer rings were drawn (A - measurements in mm) and designed using Onshape (B).
(C) After assembly of the rings with filter membrane the ring in placed on top of the wing disc sample inside the culture dish and media is added (C).
(D and E) Alternatively double-sided tape can be used as spacer for the filter membrane (D) and assembled over the cover glass in the bottom of the dish with the sample (E), according to steps 1–6 in step-by-step method details.

4. Combine two-layers of tape and space each tape sandwich 3–4 mm apart on the glass surface of a 35 mm Glass Button µ-Dish (Ibidi, Cat#: 81158) (Figure 1D-Step 1)

   Note: the sample will be placed in the space between pieces of tape, followed by membrane placed on top of sample and secured to the assembly by the tape (Figure 1D-Step 2)

Drosophila genetics and selection

⏲ Timing: 2 weeks

5. Normal Drosophila husbandry.
   a. For observation of ASP cytonemes, stocks contain either *btl*-Gal4 UAS-CD4:GFP or *btl*-LHG LexOp-CD4:GFP
   b. For studies of protein overexpression or RNAi knock-down, lines contain either *btl*-Gal4 UAS-CD4:GFP or *ap*-Gal4 UAS-CD4:GFP.
6. Wing discs are dissected from late stage (wandering) third instar larvae.

Media preparation alternatives

⏲ Timing: 30–60 min

7. Medium #1: Grace's medium (Gibco; Cat#:12300027) supplemented with 5 mM BisTris (Dye et al., 2017)
   a. Dissolve media powder designated for 1 liter in approximately 750 mL water.
   b. Add 1.05 g BisTris (Sigma; Cat#: 9754-25G) and stir to dissolve.
   c. Slowly adjust the pH to 6.6–6.7.

   Note: slow addition avoids formation of precipitate.

   d. Bring volume to 1 L
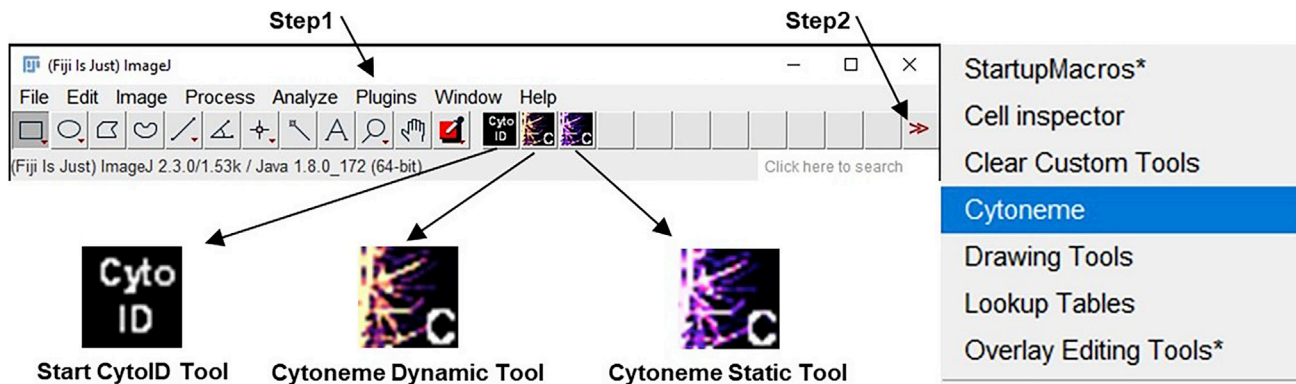
Figure 2. Installation of cytoneme analysis tool into FIJI
Installation of the tool into FIJI by click "Plugin>Install…" then choosing the "Cytoneme.ijm" file inside the "Cytoneme-Analysis-main" folder (step 1). Activation of Cytoneme Analysis Tool in FIJI tool bar by clicking in "More tools>Cytotneme" (step 2) according to steps 9–11 in before you begin.

   e. Filter media through a 0.22 μm filter (Millipore; Cat#: S2GVU05RE) in a cell culture hood.
   f. Supplement with 2%–5% FBS.

   Note: Media can be stored at 4°C for no longer than a month. Penicillin (64.7 mg/mL) and Streptomycin (100 mg/mL) may be added.

   g. For use, place an adequate volume in a test tube and bring to room temperature.

8. Medium #2: Ready to use Schneider's media (Gibco; Cat#:21720024) supplemented with 2%–5% FBS
   a. Open new bottle of Schneider's media in a cell culture hood, add 2%–5% FBS.

   Note: Media can be stored at 4°C for no longer than a month. Penicillin (64.7 mg/mL) and Streptomycin (100 mg/mL) may be added.

   b. For use, place an adequate volume in a test tube and bring to room temperature.

Installation of FIJI/ImageJ Cytoneme Analysis Tool

⊙ Timing: 5 min

9. Download, Install FIJI from: https://imagej.net/downloads
10. Download the Cytoneme Analysis Tool file from the GitHub repository
   a. Open GitHub link: https://github.com/gbarbosabio/Cytoneme_dynamics
   b. Click the green "Code" button and click the "Download ZIP" button.
   c. Download compressed file: "Cytoneme_dynamics-main". Decompress downloaded file.
   d. Open FIJI, click, Plugin>Install… (Figure 2 – step 1)
      i. Find the decompressed folder "Cytoneme_dynamics-main".
      ii. Click file "Cytoneme.ijm" to Open.
      iii. In the window "Save Plugin, Macro or Script", move up one level to the folder "Fiji.App", click on the folder "macros", then "toolsets", and finally click "Save". To complete installation, close FIJI and reopen.
11. To activate the Cytoneme analysis tools in the toolbar, click the button: "More Tools" ">>" and "Cytonemes" (Figure 2 – step 2). Three new buttons should appear on the FIJI Tools Bar: "Start CytoID Tool", "Cytoneme Dynamic Tool" and "Cytoneme Static Tool" (Figure 2).

Note: For a complete video tutorial, see Method.Video.S1 at README page of the GitHub repository.

R/RStudios analysis pipeline preparation

○ Timing: 5 min

12. Open R link: https://cran.r-project.org/mirrors.html.
13. Download R one of the repository institutional links and install
14. Download RStudios from: https://www.rstudio.com/ and install
15. Open RStudios, go to File>Open File
    a. Find the decompressed folder "Cytoneme_dynamics-main"
    b. Open file "Cytoneme_filipodia_dynamics_pipeline_ALLinONE"

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Deposited data		
Wild Type_Sample_1 timelapse max z-projection	This paper	https://github.com/gbarbosabio/Cytoneme_dynamics/tree/main/Image_sample
"Wild type_Sample_1" data frame	This paper	https://github.com/gbarbosabio/Cytoneme_dynamics/tree/main/Experiment_data_sample/WT
"Wild type_Sample_2" data frame	This paper	https://github.com/gbarbosabio/Cytoneme_dynamics/tree/main/Experiment_data_sample/WT
"apGAL4-botvRNAi_Sample_1" data frame	This paper	https://github.com/gbarbosabio/Cytoneme_dynamics/tree/main/Experiment_data_sample/ApGAL4-botvRNAi
"apGAL4-botvRNAi_Sample_2" data frame	This paper	https://github.com/gbarbosabio/Cytoneme_dynamics/tree/main/Experiment_data_sample/ApGAL4-botvRNAi
Experimental models: Organisms/strains		
btl-Gal4, UAS-CD4:GFP/CyO,wp	Roy et al. (2014)	N/A
ap-Gal4/CyO,wp; btl-LHG,LexOp-CD4:GFP/TM6b	Roy et al. (2014)	N/A
UAS-botvRNAi	Bloomington Drosophila Stock Center	BDSC#: 61257
Software and algorithms		
FIJI	Schindelin et al. (2012)	https://imagej.net/downloads
Cytoneme analysis tool	This paper	https://github.com/gbarbosabio/Cytoneme_dynamics
R	Bunn and Korpela (2000)	https://cran.r-project.org/mirrors.html
RStudios	N/A	https://www.rstudio.com/
ggplot	Wickham (2009)	Install with RStudio
"Cytoneme_filipodia_dynamics_pipeline_ALLinONE"	This paper	https://github.com/gbarbosabio/Cytoneme_dynamics
Other		
Ultimaker 3 3D Printer	Ultimaker	N/A
13 mm diameter, MCE membrane, hydrophilic	MilliporeSigma	HAWP01300
μ-Dish 35 mm, high Glass Button	Ibidi	Cat.No:81158
Grace's Insect Medium, unsupplemented, powder	Gibco – Thermo Fisher Scientific	Cat.No:12300027
Stericup Quick Release Durapore 0.22 μm PVDF 500mL	MilliporeSigma	Cat#:S2GVU05RE
FBS	UCSF cell culture	Cat#:97068-085
Schneider's medium	Gibco – Thermo Fisher Scientific	Cat.No:21720024
BisTris	Sigma-Aldrich	Cat#: 9754-25G
Cell Strainer	VWR	Cat#: 76327-098
Dissecting forceps	VWR	Cat#: 82027-408
Confocal Laser Scanning Microscope FV3000	Olympus	N/A

STEP-BY-STEP METHOD DETAILS
Wing imaginal disc *ex vivo* culture

🕐 Timing: 15–30 min

With these steps you will dissect and mount the sample in the culture dish for imaging.

Note: Practice the dissection if you are not familiar with wing disc dissection for ASP observation - cytonemes are dynamic and sensitive to perturbation.

1. Clean all dissection tools with 70% ethanol and allow ethanol to fully evaporate.

2. Prepare the culture dish to receive the sample (Figure 1).

   Note: Keep the dish covered with a lid when possible.

   a. For a 3D ring with membrane option, assemble on a clean surface.
   b. For options with membrane and tape spacers, prepare the tape spacers attached to the glass of the 35 mm μ-Dish (Figure 1D – step 1) and have the membrane ready for placement.

3. Prepare wandering stage larvae for dissection
   a. Select larvae of the desired genotype.
   b. Place larvae in a cell strainer (VWR; Cat#: 76327-098), rinse with 70% ethanol, rinse with sterile water.

4. Transfer larvae to a dissection dish containing sterile culture media, dissect under a stereoscope (Figure 3A).
   a. Use two fine forceps (VWR; Cat#: 82027-408) to cut larvae in half, preserving the anterior which contains the mouth hocks (Figure 3A – step 1).
   b. Grab the mouth hooks with one forceps and gently push the larval body wall with the second forceps towards the mouth hooks, flipping the cuticle inside-out (Figure 3A – step 2).
   c. Carefully remove fat body, gut, ventral nerve cord (VNC), leaving imaginal discs and trachea (Figure 3A – step 3).
   d. Place 20 μL of media in the middle of the μ-Dish and transfer the cleaned sample to it (Figure 3B).
   e. Dissect the two wing discs from the carcass, while preserving as much attached trachea as possible.

   Note: Preparations should include dorsal trunk (DT) and lateral trunk (LT) anterior and posterior to wing disc, as well as the stalk (most dorsal tip) of the wing disc. Haltere and leg discs linked to the wing disc may be included.

   f. Place the wing disc with the side attached to trachea (columnar epithelial cells) oriented down, in contact with the glass surface. Troubleshooting 2
   g. Discard other larval material.

5. Carefully place the pre-assembled 3D ring on top of the 20 μL drop of medium OR place the membrane over the drop and attach to the double-sided tape spacers. Troubleshooting 3
6. Carefully add 2 mL of media to the μ-Dish and close the lid (Figure 1C).

   ⚠ CRITICAL: The ASP is sensitive to touch and turbulence; it is important to dissect the wing disc quickly without touching or pinching it. Multiple discs may be added to the same dish to increase the chance of having a properly oriented, undamaged sample.
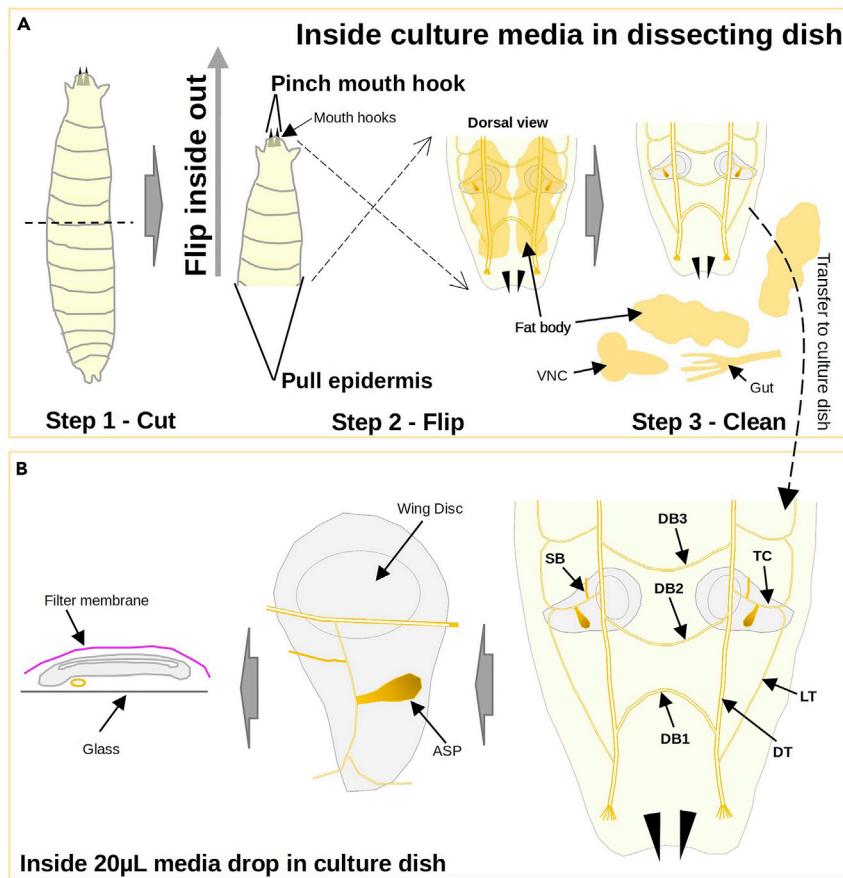
Figure 3. Dissection scheme of the wing imaginal disc for ASP cytoneme visualization *ex vivo*
(A) The clean larvae are placed in a dissecting dish containing media and cut in half then the cuticle is flipped inside-out and finally the sample is cleaned by removing unwanted tissues (A). The sample is transferred to a 20 μL drop of media in the culture dish where the indicated tracheas are cut (dashed lines) and the wing imaginal disc is released. (B) Then place the tissue with the trachea facing the glass bottom, and the filter membrane will gently keep the tissue in place (B) according to steps 1–6 in step-by-step method details.

Imaging cytonemes with a confocal microscope

⏱ Timing: 1–5 h

In this step, images will be captured with a point scanning or spinning disc confocal microscope. To observe cytoneme dynamics for up to 4–5 h, the microscope must be equipped for fast acquisition and low laser intensity to minimize phototoxicity. Troubleshooting 4

7. Place the 35 mm μ-Dish containing the sample on the stage of an inverted microscope
8. Locate the wing disc and choose the appropriate objective.
   a. In this protocol we report images made with an Olympus FV3000 inverted point scanning confocal microscope using the oil immersion objective UPLFLN40XO.

   Note: This protocol is suitable for a variety of inverted confocal microscopes and objectives.

9. Select appropriate laser (i.e.: 488 nm for GFP excitation).
10. Use low laser power to minimize phototoxicity

11. Use maximum detection voltage gain such that structures of ASP cells remain detectable, and background levels are not excessive.
12. Adjust zoom if necessary.
13. Choose the lowest pixel resolution that allows visualization of cytonemes (i.e.: 0.200 μm/pixel).
14. Set z-stack acquisition to image the entire depth of ASP.
15. Crop to area of interest
    a. Include ample space around ASP because the tissue may move during imaging and cytonemes will extend at least 40 μm from the ASP surface

    Note: Acquisition time for each z-stack should be less than one minute to resolve cytoneme movement (i.e.: 60 stacks with 0.5 μm spacing imaged at 1 stack/min.). Size of cropped area or depth of the z-stack can be reduced if acquisition takes longer than one minute.

16. Set total acquisition time for total period of viewing.

    Note: The total acquisition time interval must be greater than or equal to the sum of all z-stack acquisition times.

EXPECTED OUTCOMES

A successful experiment will generate multiple z-stacks (either single channel or multi-channel) for several time points. The expected result is a set of images of the ASP with many cytonemes extending and retracting (troubleshooting 5). One or more cell divisions (troubleshooting 6) are likely and are an indication of tissue viability (Methods video S1).

QUANTIFICATION AND STATISTICAL ANALYSIS
Cytoneme length measurement using FIJI/ImageJ Cytoneme Analysis Tool

© Timing: 4 h

This application was created to measure cytoneme lengths in a semi-automated way. Length measurements are obtained from traces drawn over the cytonemes on max z-projections images.

   Note: For a complete video tutorial of the following steps, see Method.Video.S2 at README page of the GitHub repository

1. Open image in FIJI with File>Open…
2. Create a max z-projection of the image with Image>Stacks>Z Project…
3. Choose the Start and Final Z Slice and select Max Projection in Projection Type.
   a. Adjust with Image>Adjust>Brightness and Contrast pulldown. Adjust Image>Lookup Table, to select grayscale and invert background to white.
   b. Save max intensity z-projection file
   c. Close original Z-Stack file
4. Activate Cytoneme Analysis Tool in the tool bar (see step 13).
5. Click the first button "Start CytoID Tool", click the image.
6. The "Welcome…" window will open and the following information must be completed and/or confirmed
   a. "Group name" – the name of the experimental group
   b. "Sample number" – choose a number to identify the sample (e.g.,: 1, 2, 3, …)
   c. "Time Calibration" –the number of HOURS, MIN or SEC per frame in the time lapse
      i. Tip: time units cannot be mixed; designate as decimal fractions (e.g., 1.5 min or 90 s)
   d. "Pixel calibration" - designate how many units each pixel represents in the image.
   e. "Pixel unit" – choose the space unit used in the image ("cm", "mm", "μm', "nm").

    i. To use "pixel" as the unit, choose 1 for "Pixel calibration".

7. Press the "OK" button, three windows will appear:
   a. "ImageTitle_data" – a file containing the image title and "_data" that will remain open and store all measurements in a data frame containing the CytonemeID ("ID"), x and y position of the base of the cytoneme ("Xi" and "Yi"), x and y position of the tip of the cytoneme ("Xf" and "Yf"), calibrated time of the frame ("Time"), and length of the cytoneme ("Length").
   b. "CytonemeID" – this text window contains a random cytoneme ID number for the cytoneme being measured. The first cytoneme ID number is preset.
   c. "Measuring" – This window contains a brief explanation of options and an "OK" button

⚠ CRITICAL: Push "OK" only after cytoneme measurements are complete.

8. In the toolbar click the second button: "Cytoneme dynamics tool"
9. Scroll through the time lapse image using the scroll bar and choose the initial time frame containing the cytoneme to be measured and "draw a line" over the cytoneme by clicking and holding in the cytoneme base and dragging until the tip.
   a. The user may want to zoom in the image to facilitate the drawing and analysis.
   b. For cytonemes that are not extended at t = 0, click the spot where the cytoneme will start extending in the time frame immediately before the frame in which it appears.

⚠ CRITICAL: The accuracy of the traces is key for reliable measurements.

10. Once the mouse click is released, the next time frame will appear and the measured data will be annotated in the data frame.
11. Repeat this until the cytoneme lengths have been measured in all the time frames in which it appears.
12. After completing length measurements for a cytoneme, push the letter "c" on the keyboard and a new random "CytonemeID" will appear.
13. Repeat steps 9–12 for as many cytonemes you desire to measure.

Note: The cytoneme order is not relevant in this analysis.

Note: You may use the ROI Manager window to verify if a given cytoneme has been measured.

14. With length measurements complete, push the button "OK" in the "Measuring" window. All windows will close and the data frame file (Table 1) named with the "Group name" and "Sample number" will be saved in the folder with the max intensity z-projection image.

⚠ CRITICAL: Maintain the name configuration for the data frame file.

15. MEASUREMENT ERROR CORRECTION: if a measurement was incorrect, select all lines of the data frame with error and delete, then scroll back to the desired time point and resume measuring.
16. UNWANTED CLOSE: if the "CytonemeID" is closed prematurely, the Cytoneme Analysis Tool is stopped. To resume, push the key "c" on the keyboard and a new random ID will be generated. Troubleshooting 7

Note: All the measurements made with the incomplete entered ID must be deleted because the ID number will change for subsequent measurements. Delete all lines in the measurements table for the cytoneme and repeat all measurements.

Data organization for processing

⏱ Timing: 5 min

Table 1. Example of first 20 rows of raw data measured in "Wild type_Sample_1"

ID	Xi	Yi	Xf	Yf	Time	Length
12928	215	256	215	256	8.4	0
12928	215	257	197	264	9.45	4.3899
12928	215	255	200	259	10.5	3.5461
12928	215	258	192	259	11.55	4.7736
12928	211	260	179	261	12.6	6.4472
12928	215	259	173	260	13.65	8.4008
12928	211	260	173	261	14.7	7.7571
12928	210	260	183	261	15.75	5.6175
12928	212	262	193	262	16.8	4.0604
12928	209	260	209	260	17.85	0
10461	207	247	207	247	11.55	0
10461	206	251	203	254	12.6	1.1934
10461	205	249	190	266	13.65	5.6657
10461	204	251	183	280	14.7	9.0129
10461	204	251	184	287	15.75	9.8568
10461	206	253	190	278	16.8	7.2228
10461	205	257	199	265	17.85	2.4351
10461	205	256	204	257	18.9	0.3978
27596	237	276	237	276	1.05	0

These instructions are for the downstream data processing using RStudio. Organize the files into folders exactly as instructed below.

17. Create a folder with the experiment title (i.e.: "Drug test")
18. Inside this folder create one folder for each experimental group and label each folder with the group name (i.e.: "Control", "Treatment1", "Treatment2", …)
19. Copy all data files generated with FIJI/ImageJ to the corresponding experimental group

△ CRITICAL: For optimal performance of the analysis routines, data sets must be organized exactly as indicated in "17, 18, and 19".
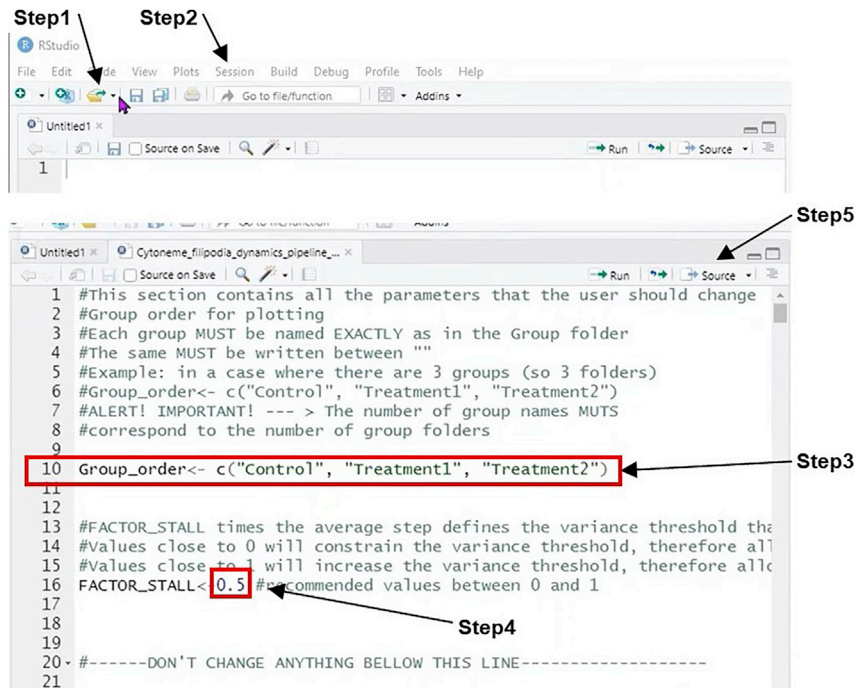
Data processing in RStudio

⏱ Timing: 10 min

These steps will guide the user to analyze all the cytoneme data of single or multiple groups.

Note: For a complete video tutorial of the following steps, see Method.Video.S3 at README page of the GitHub repository

20. Open the "Cytoneme_filipodia_dynamics_pipeline_ALLinONE" file in RStudio (Figure 4 – step 1).
21. Go to Session>Set Working Directory>Choose Directory…
22. Choose the experiment folder (i.e.: "Drug test") and click Open (Figure 4 – step 2).
23. List the desired group order for the graph analysis (Figure 4 – step 3).
    a. The group name must match the folder's name for each of the group
    b. The names should be within quotation marks.
    c. The names should be inside parentheses and followed by a comma.
    d. Example: Group_order<- c("Control", "Treatment1", "Treatment2")

Note: FACTOR_STALL can be changed by replacing the number "0.5" by any number higher than zero and smaller than 1. The product of FACTOR_STALL and the average of all step sizes

Figure 4. Data processing and graph plotting using R/R Studios
Open "Cytoneme_filipodia_dynamics_pipeline_ALLinONE" file downloaded from the GitHub repository (step 1). Choose the file directory that contains the data sets acquired in FIJI and organized in folders by group, Session>Set Working Directory>Choose Directory… (step 2). Indicate the groups in order of preference for analysis (step 3), if desired adjust the STALL_FACTOR (step 4) and click the Source button (step 5).

for a given cytoneme defines the limit of a step to be considered STALLING (i.e.: if the FACTOR_STALL is 0.5 and the average step size is 2 μm, then any step below 1 μm will be considered stalling) (Figure 4 – step 4).

24. Push the button "Source" (Figure 4 – step 5). Troubleshooting 8

Expected outcomes
This R script will process the data of each cytoneme in each sample of all groups. It will process the difference in length from one time point to the next (called "step") and from this information will compute a list of parameters for each cytoneme in a sample that is stored in the file "Cyt_Para_GroupName_SampleX":

Genotype – Group same

Sample – Sample Name

CytonemeID – randomized ID number for each cytoneme

Average_stepsize – the length average of all steps for a cytoneme

Ave_FWD_stepsize - the length average of all forward steps for a cytoneme

Ave_REV_stepsize - the length average of all reverse steps for a cytoneme

Total_Displacement- total displacement by the tip of the cytoneme

Displa_FWD - total displacement of forward direction by the tip of the cytoneme

Displa_REV - total displacement of reverse direction by the tip of the cytoneme

Displa_STALL - total displacement during stall by the tip of the cytoneme

Max_length – max length reached by each cytoneme

Stall_time – time spend in stall

Average_stall_speed – average speed in stall

Average_Speed – average speed of the whole movement of each cytoneme

FWD_Speed - average speed in forward

REV_Speed - average speed reverse

Lifetime – time of cytoneme persistence

After computing these parameters for each sample, a correlation plot between all numerical parameters is generated with the label "CorPlot_GroupName_SampleX" for each sample and for the group "CorPlot_combined_GroupName" (Figure 5B), then the cytoneme parameter files of all samples are combined into a single file "Cyt_Para_combined_GroupName". A file named "Cyt_Stats_GroupName_SampleX" contains descriptive statistics for each parameter (mean, median, standard deviation) of each sample in the group and a file "Cyt_Stats_combined_GroupName" with the descriptive statistics for all samples combined in the group. Finally, six graphs are generated showing (1) length variation, (2) step size, and (3) status (forward, stall or reverse) for each cytoneme in relation to the time each cytoneme is active, and "RelativeTime" graphs (4) length variation (Figure 5C), (5) step size (Figure 5D), and (6) status (Figure 5E) plotted with starting points for all cytonemes normalized to time = 0.

In the experiment folder (step 17), the file "Cyt_Para_combined_GroupName" for each group is saved and all data for the groups are combined in the file "Combine_ALLgroups". Several plots are generated in the folder "Graphs": dispersion plots of all numerical parameters compared to lifetime (Figure 5F), and total displacement and violin plots comparing all groups for each parameter (Figures 5G and 5H).

> *Optional:* The data in "Combine_ALLgroups" can be pasted into the web app "SuperPlotOf-Data" (https://huygens.science.uva.nl/SuperPlotsOfData/) for generation of plots comparing the cytoneme parameters between groups and replicates (Goedhart, 2021).

LIMITATIONS

Whereas conditions have been described that support wing disc development for at least 16 h during *ex vivo* culture (Aldaz et al., 2010), the method presented here for the wing disc-associated ASP supports ASP development for only 4–5 h. Nevertheless, the culture conditions represent a significant improvement over previous methods. Imaging is limited by the small diameter of cytonemes (~200 nm (Wood et al., 2021)) that requires bright fluorescent tags for detection with a confocal microscope and low laser intensity to minimize photobleaching and phototoxicity. Analysis of cytoneme lengths and dynamics must contend with the fact that cytonemes do not lie in a single optical plane. The method presented here measures the cytoneme length in 2D max intensity projection. It is semi-automated because fully automated analysis, such by FiloQuant (Jacquemet et al., 2017) or Filopodyan (Urbančič et al., 2017), is limited by the high density of cytonemes, the presence of
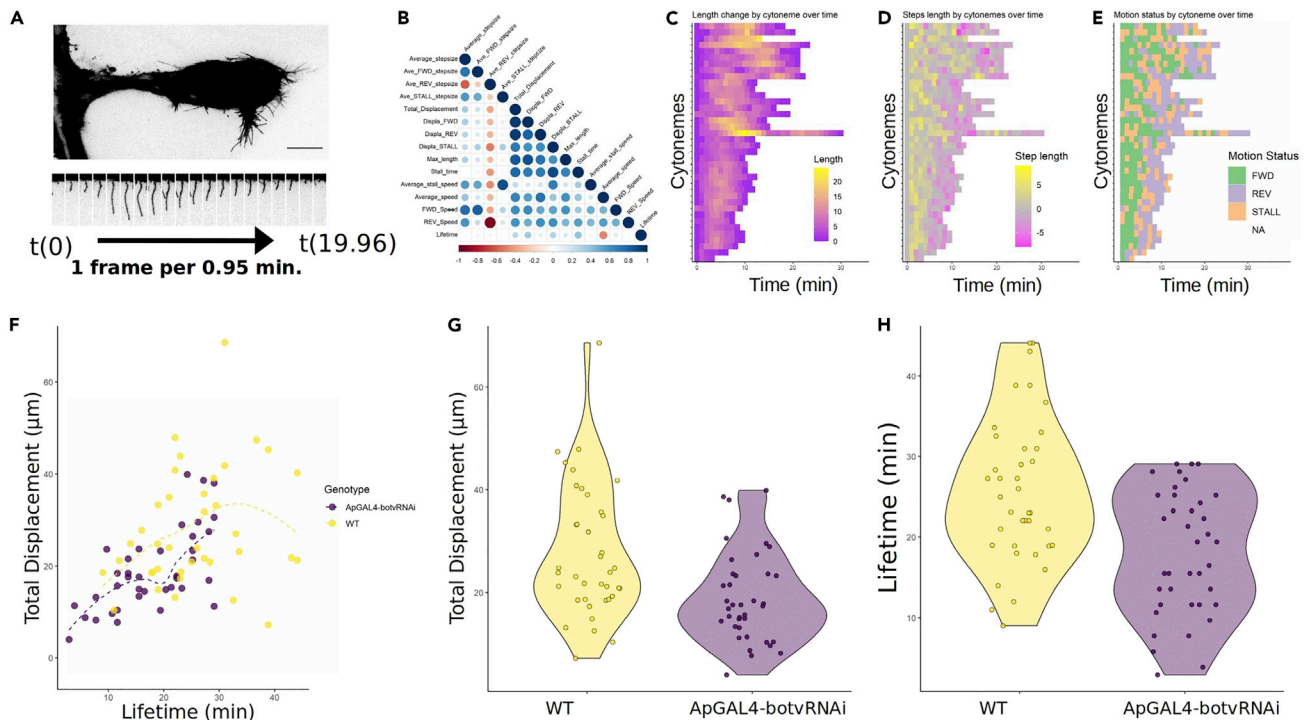
Figure 5. ASP cytoneme dynamics analysis comparing wild-type sample and knockdown of brother-of-toutvelu in the apterous domain of the wing disc

(A and B) Wild type ASP and one cytoneme extending and retracting cycle (A – scale bar: 20 μm). Correlation plot for all numerical parameters of cytoneme movement in wild type sample (B).

(C–E) Length variation (C), step size variation (D) and movement status (forward, stall or reverse) (E) for each cytoneme represented in each line over time (min). All initial time points are normalized to zero.

(F–H) Dispersion plot of total displacement (μm) over lifetime (min) for all cytonemes in wild type (yellow) and ApGAL4-botvRNAi (magenta) sample groups (F). Violin plot for total displacement (G) and lifetime (H) of the cytonemes of wild type (yellow) and ApGAL4-botvRNAi (magenta) sample groups.

All plots (B–H) were generated with data processing in RStudio on steps 20 to 24 in quantification and statistical analysis.

overlapping cytonemes during extension and retraction cycles and presence of branching cytonemes. AI approaches such as ones offered by ZeroCostDL4Mic (von Chamier et al., 2021), may be useful.

TROUBLESHOOTING

Problem 1
The inner and outer 3D printed ring may not fit together if printing is not sufficiently precise.

Potential solution
This may be corrected by printing the rings again with higher resolution, or by slightly smoothing the surface of the rings with a nail file. Caution is advised as the rings must fit tightly in order to stretch the filter membrane.

Problem 2
Air in the trachea complicates placement of the wing disc with trachea facing down on the glass.

Potential solution
Flip the wing disc to the desired position and immediately reduce the media volume. Volume should be small enough for surface tension to hold the tissue in place but not so small that the tissue dries.

Problem 3

It is possible that during placement of the ring or the membrane on top of the sample, the wing imaginal disc may flip or get twisted in the dish due to fluid movement.

Potential solution

Place more than one wing disc in the dish to increase the chance that a disc is in an optimal orientation for imaging.

Problem 4

The ASP is sensitive to damage, photobleaching and phototoxicity.

Potential solution

Cell damage may be caused by mishandling during dissection. Avoid touching or pinching the imaginal disc and ASP cells, or stretching the tissues. This is a critical part of the protocol. Practice, discard any unhealthy sample, and repeat until successful.

Photobleaching and phototoxicity are common issues when imaging live samples with visible light. The fluorescent tag must have high fluorescent yield so that low laser power is sufficient. Alternatively, multi-photon confocal microscope imaging might reduce light exposure.

Problem 5

The cytonemes stalled during the time course. If the ASP is pushed against the glass, even by pressure caused by the filter membrane, the cytonemes may adhere to the glass and not release. This may also create the artifactual appearance of many cytoneme.

Potential solution

To distinguish this artifact from normal cytonemes, look for extension/retraction movements that follow the curvature of the tissue and are not parallel to the glass. Be attentive during dissection and mounting the sample. When placing the ring on top of the sample make the movement as steady as possible. Avoid leaving the sample with minimal culture media by adding the 2 mL of media as soon as possible after placing the ring. If using the double-sided tape spacers, be careful when placing the membrane, making sure it is stretched between the two spacers, and with the forceps make sure the membrane attaches to the tape before adding the media. Add the 2 mL media carefully and slowly to avoid any pressure to the membrane or sideway ring movement.

Problem 6

There was no cell division during the time course.

Potential solution

Cell divisions in the ASP are infrequent and may not be observed in an imaging period of 4–5 h.

Problem 7

The data frame is a text editable window. It is possible that after attempting to delete a line the user may place the text cursor in the wrong place.

Potential solution

When deleting a data line in the ''_data'' window, make sure to place the text cursor in the end (right side of the length number) of the last measurement line. This will guarantee the following data point will be annotated in the next line.

Problem 8

If the user attempts a RStudios analysis run by clicking ''Source'' but an error occurs (title or group or order may be incorrect), some analysis files will be generated before the error stops the analysis.

Potential solution

Delete all files generated inside the experiment folder and inside any group folder before re-running the analysis in RStudios. Also repeat steps 22 and 23.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Thomas B. Kornberg (Tom.Kornberg@ucsf.edu)

Materials availability

The inner and outer rings were designed on Onshape and the .stl files for 3D printing of are available for download here: https://github.com/gbarbosabio/Culture_rings_3D.git

Data and code availability

The datasets/code generated during this study are available at GitHub: https://github.com/gbarbosabio/Cytoneme_dynamics, as well as at Zenodo (Barbosa and Kornberg, 2021).

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2022.101138.

AUTHOR CONTRIBUTIONS

G.O.B. and T.B.K. did the ring design, data analysis, and manuscript writing. G.O.B. did a 3D sketch of the ring, printed, created, and optimized the *ex vivo* protocol, created the FIJI tool, created RStudio's analysis pipeline, and did the experiments and the image analysis.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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