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Corresponding Author:	Sherry Shiying Aw Institute of Molecular and Cell Biology Singapore, Singapore SINGAPORE
Corresponding Author's Institution:	Institute of Molecular and Cell Biology
Corresponding Author E-Mail:	syaw@imcb.a-star.edu.sg
Order of Authors:	Sherry Shiying Aw
	Animesh Banerjee
	Shuang Wu
	Cheng Li
Additional Information:	
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TITLE:

Fully Automated Leg Movement Tracking in Freely Moving Insects Using Feature Learning Leg Segmentation and Tracking (FLLIT)

AUTHORS AND AFFILIATIONS

Animesh Banerjee[1], Shuang Wu[2], Cheng Lib[3], Sherry Shiying Awa*

[1]Institute of Molecular and Cell Biology, Agency for Science, Technology and Research, Singapore
[2]Bioinformatics Institute, Agency for Science, Technology and Research, Singapore
[3]Department of Electrical and Computer Engineering, University of Alberta, Edmonton, Alberta, Canada

Correspondence:
Sherry Shiying Aw (syaw@imcb.a-star.edu.sg)

KEYWORDS:

Drosophila, insect, machine-learning, tracking, gait analysis, neurodegeneration

SUMMARY

We describe detailed protocols for using FLLIT, a fully automated machine learning method for leg claw movement tracking in freely moving *Drosophila melanogaster* and other insects. These protocols can be used to quantitatively measure subtle walking gait movements in wild type flies, mutant flies and fly models of neurodegeneration.

ABSTRACT

The *Drosophila* model has been invaluable for the study of neurological function and for understanding the molecular and cellular mechanisms that underlie neurodegeneration. While fly techniques for the manipulation and study of neuronal subsets have grown increasingly sophisticated, the richness of the resultant behavioral phenotypes has not been captured at a similar detail. To be able to study subtle fly leg movements for comparison amongst mutants requires the ability to automatically measure and quantify high-speed and rapid leg movements. Hence, we developed a machine-learning algorithm for automated leg claw tracking in freely walking flies, Feature Learning-based Limb segmentation and Tracking (FLLIT). Unlike most deep learning methods, FLLIT is fully automated and generates its own training sets without a need for user annotation, using morphological parameters built into the learning algorithm. This article describes an in depth protocol for carrying out gait analysis using FLLIT. It details the procedures for camera setup, arena construction, video recording, leg segmentation and leg claw tracking. It also gives an overview of the data produced by FLLIT, which includes raw tracked body and leg positions in every video frame, 20 gait parameters, 5 plots and a tracked video. To demonstrate the use of FLLIT, we quantify relevant diseased gait parameters in a fly model of Spinocerebellar ataxia 3.

INTRODUCTION

In the last few decades, neurodegenerative diseases and movement disorders have grown more

prevalent in our aging populations. Although our understanding of many neurodegenerative diseases has advanced at the molecular and cellular level, fundamental features of the affected neuronal circuitry underlying disease remain poorly understood. Recently developed behavioral tracking tools[1-4] now allow us to study movement abnormalities in animal disease models in order to identify molecular, cellular and circuit dysregulation underlying disease.

Molecular pathways involved in many neurodegenerative diseases are conserved in the fruit fly *Drosophila melanogaster*, and *Drosophila* disease models have helped to elucidate fundamental mechanisms underlying neurodegeneration[5,6]. We recently showed that fly models of Parkinson's Disease (PD) and Spinocerebellar ataxia 3 (SCA3) exhibit distinct, conserved gait signatures that resemble those of the respective human diseases[1], demonstrating that the fly model can be used to understand circuit mechanisms underlying movement dysfunction in specific movement disorders. The rich and continually growing arsenal of tools in the fly model for targeted manipulation and visualization of neurons at the single gene and single cell level[7-10] makes the fly an ideal model one to probe the relationship between disease pathways, neuronal circuitry and behavioral phenotypic manifestation in vivo. To enable precise, automated insect gait analysis, we recently developed a machine learning method, **F**eature **L**earning-based **LI**mb segmentation and **T**racking (FLLIT)[1].

FLLIT consists of a fully automated multi-stage algorithm that first segments the leg pixels, which are subsequently used to locate and track the corresponding leg claws. FLLIT employs a boosting algorithm for segmentation, in contrast to deep learning algorithms used in recent work[2,3]. There are some similarities with convolutional neural networks in that for both frameworks, feature extraction is done automatically through learning convolutional kernels. The first step in FLLIT involves using morphological operations (edge and skeletonization) to automatically generate positive (pixels on the legs) and negative (background or pixels on the fly body) training samples with high confidence. Hence, FLLIT is fully automated and does not require user annotated training samples. Using the above training samples, a classifier is then trained in the framework of a boosting algorithm. An ensemble of weak classifiers is iteratively learnt, with each consisting a set of convolutional kernels for feature extraction and a decision tree. The final learnt classifier is then used for leg segmentation and is able to better discern difficult regions/hard samples better than morphological operations, producing an overall much more accurate segmentation for tracking[1]. From the segmented legs, we locate the tips and track them using the Hungarian algorithm: by matching tips across frames such that the sum of the distance moved by each tip is minimized. FLLIT can handle occlusion cases by remembering the last seen location (in fly centered coordinates) so that a leg tip is recovered once it is no longer under occlusion.

We previously showed that FLLIT can automatically and accurately track leg movements and analyze gait in an unmarked, freely moving fly or spider from high-speed video[1]; FLLIT should hence be broadly applicable for arthropod leg tracking. By extracting machine learning training sets using morphological parameters, FLLIT automatically trains itself to segment and track insect legs without the need for laborious manual annotation, which is required for most deep learning methods. FLLIT is hence fully automated. After leg segmentation and tracking, FLLIT automatically produces raw tracked body and leg positions in every video frame, 20 gait parameters, 5 plots

and a tracked video for gait analysis and visualization of gait movements. This protocol provides a step-by-step guide to using FLLIT.

PROTOCOL:

1. System setup

1.1 Ensure that the recording station has a high-speed camera and a stage over it to hold the arena chamber (**Figure 1**). Adjust the camera to record at a minimum of 250 frames per second (fps), with an accordingly fast shutter speed (in this case, recording is performed at 1000 fps with a shutter speed of 1 ms).

NOTE: Check that the video is suitable for tracking by ensuring that there is minimal or no motion blur of moving legs in all frames. If the moving leg is so blurred that a human annotator cannot track it, then the camera recording speed and/or shutter speed must be increased.

1.2 Place the infrared LED lights at the top of the stage with a diffuser (translucent sheet) between the camera and sample (**Figure 1A,B**).

1.3 Make the recording chamber by cutting a 1.6 mm thick acrylic sheet. In this experiment, use a field-of-view of 11 mm x 11 mm. Place the chamber between two glass slides (**Figure 1C**).

2. Preparation of flies for recording

2.1 Transfer the flies to be recorded into a new food vial 24 h prior to recording.

NOTE: Do not use CO_2 (usually used to anesthetize the flies upon first collection) on the flies less than 24 h before recording.

2.2 About 40 min before recording, transfer the flies into empty vials and keep on ice for 5-7 min.

2.3 In the meantime, wipe the arena and clear glass slides with water and a wipe.

NOTE: Do not use ethanol to clean the chambers and the slides.

2.4 Prepare the recording chamber. Fix one of the microscopic glass slides below the chamber with tape.

2.5 When the flies have been anesthetized on ice, transfer one fly in each chamber using a clean brush.

NOTE: Both male and female flies can be used in this setup, and, as far as possible, flies of both sexes should be analyzed to avoid sex-specific bias.

2.6 Secure the chamber with another microscopic glass slide with tape (**Figure 1C**).

2.7 Keep the chambered flies at room temperature for 15 - 20 min for acclimatization.

3. Generation of videos for FLLIT analysis

NOTE: This step is specific to the video camera used. In this case, a commercially available video camera is used (see **Table of Materials**).

3.1 Turn on the power source. Wait for the green LED for power and the orange LED for the ethernet interface connection to stabilize. Switch on the power for the infrared LED. Ensure that the voltage remains at 12.5 V.

3.2 Open the **Viewer** application on the connected computer system.

3.3 Change the recording frame rate to 1000 fps. Set the shutter speed to 1/1000 s (1 ms).

3.4 Place the chamber with the fly on the recording arena and select the **LIVE** button. Ensure that the camera is focused on the leg tips when the fly is walking upright on the floor of the chamber; the leg tips should be in sharp focus.

3.5 Click **Record** (**Figure 2**).

3.6 Record the fly walking, ensuring that:
The fly walked in a relatively straight trajectory without touching the edge of the arena.
The fly walked at least three strides per leg.
The fly does not pause during the walk.
The distance walked is equivalent to at least one body length.

NOTE: Having the background subtracted cleanly is critical for accurate segmentation. The automated background subtraction algorithm employed by FLLIT requires that the imaged fly moves at least one body length in distance.

3.7 Click **Rec Done** to stop recording (**Figure 2**).

3.8 Crop the video to ensure that the recording encompasses only a straight walk of the fly (as described in Step 3.6).

3.9 Click **Save** (**Figure 2**). Save the files in '.mraw' or '.tiff' format in the respective folders.

NOTE: '.mraw' format gives greater flexibility to change the file name (if required) and for storage of videos compared to '.tiff' file format.

4. Installation of FLLIT program

NOTE: Up-to-date instructions can be found at: https://github.com/BII-wushuang/FLLIT/blob/master/Compiled/Readme.pdf

4.1 Download FLLIT on any operating system

4.1.1 Download FLLIT from the following Github link: https://github.com/BII-wushuang/FLLIT/archive/master.zip. Extract the contents of the zip file.

4.1.2 Download sample datasets from the following Google Drive link: https://bit.ly/2EibvNY. Create a folder **Data** under FLLIT-master/Compiled and place dataset folders in this Data directory.

4.2 Install FLLIT In Ubuntu

4.2.1 Navigate to the FLLIT/Compiled directory.

4.2.2 Right-click and select **Open in Termina**l.

4.2.3 Issue the following command to download and install the MATLAB runtime libraries to $HOME/MCR:
bash MCR_2016.sh

4.2.4 After installation of the MATLAB runtime libraries has been completed, issue the following command to ensure that executable rights are accorded to FLLIT:
chmod +x FLLIT

4.2.5 Open a terminal in the FLLIT/Compiled directory and issue the following command to execute FLLIT:
bash run_FLLIT.sh $HOME/MCR/v901

4.3 Install FLLIT in Windows

4.3.1 For 7 and 10th Home Edition, install Docker Toolbox at:
(https://github.com/docker/toolbox/releases/download/v19.03.1/DockerToolbox-19.03.1.exe).

4.3.2 For Windows 10 Pro or Enterprise Edition, install Docker Desktop for Windows at:
(https://download.docker.com/win/stable/Docker\%20Desktop\%20Installer.exe).

4.3.3 To enable execution of GUI applications in a Docker container on Windows, first install VcXSrV (https://sourceforge.net/projects/vcxsrv). On starting VcXsrv, configure the settings as in **Figure S1**.

NOTE: Make sure Docker and VcXsrv are running before starting FLLIT.

4.3.4 Double click FLLIT.bat to run FLLIT.

NOTE: When executing for the first time, it will take some time to pull the Docker image from Docker Hub.

4.4 Install FLLIT in MacOS

4.4.1 Download Docker Desktop for MacOS at
https://download.docker.com/mac/stable/Docker.dmg

4.4.2 Install socat by opening a terminal and issuing the following command:
brew install socat

4.4.3 Start socat with:
socat TCP-LISTEN:6000,reuseaddr,fork UNIX-CLIENT:\"$DISPLAY\" & disown

4.4.4 Install XQuartz (https://www.xquartz.org) to enable execution of GUI applications in a Docker container on MacOS. Start XQuartz and change the preferences by checking the **Allow connections from network clients** in the Security tab as shown in **Figure S2**.

NOTE: Make sure that Docker, socat and XQuartz are all running before starting FLLIT.

4.4.5 Open a terminal in the FLLIT/Compiled directory and execute FLLIT with the following command:
bash FLLIT_Mac.sh

NOTE: When executing for the first time, it will take some time to pull the Docker image from Docker Hub.

5. Running FLLIT for automated leg tracking

5.1 Segmentation

5.1.1 Convert the video into individual TIFF files and copy into the FLLIT data folder.

5.1.2 Run FLLIT (In Ubuntu, right click to open FLLIT in Terminal).

5.1.3 Select the folder containing the frame-by-frame TIFF images of the video to be tracked and click the **Add** button.

5.1.4 In the pop-up window choose **0** for carrying out leg segmentation only, or **1** to include leg tracking with leg segmentation.

5.1.5 Click **Done** to initiate segmentation and tracking of the selected video.

5.2 Tracking

5.2.1 To check the accuracy of tracking and carry out error corrections (if any), click on **Select Data Folder**. Select the folder to be tracked and click **Open**.

5.2.2 Click on **View Tracking**.

NOTE: Check that **Viewer Mode** remains selected while viewing the tracked leg positions. Otherwise, any previous corrections made will be over-ridden.

5.2.3 Check labeling for all the legs in the first frame

NOTE: Since the leg labels are placed according to the position on the screen, if the fly is walking upright, the fly's RIGHT side is labelled as L1 (fore-leg), L2 (mid-leg), L3 (hind-leg) and the fly's LEFT side is labelled as R1 (fore-leg), R2 (mid-leg), R3 (hind-leg), respectively (**Figure 3**). If the fly is walking upside, down the leg labels will be correctly annotated.

5.2.4 If a leg is wrongly labeled and a correction is required, click **Pause viewing**, followed by **Adjust Prediction** (**Figure 3**).

5.2.5 From the right panel headed **Leg to Adjust**, select the leg that requires correction.

5.2.6 Double click on the correct position for this leg in the image window, click on **Save**, and then **Exit**. To go to the previous frame or subsequent frame, click **Pause viewing** followed by the forward and backward **<I** and **I>** buttons, respectively (**Figure 3**).

5.2.7 To correct any incorrectly tracked videos, open the Data folder of the video to be retracked and select **Manually Initiate Tracking**.

5.2.8 Click the **Tracking** button, which will then change its label to **Initial**.

5.2.9 Click **Adjust Prediction** and correct the leg labels by double-clicking each leg tip and then assigning it with the correct leg label. Click **Save and Exit**.

5.2.10 Click on **Resume** to initiate tracking.

5.3 Data processing and video generation

5.3.1 Click on **Data Process**. In the popup window, type in the number of frames per second (fps) at which the videos were recorded (e.g., 1,000 fps).

5.3.2 Use the following equation to calculate the actual field of view of the captured video so that gait parameters can be measured in millimeters:

$$\text{Actual Field of View} = \frac{\text{Actual Chamber size} * \text{Width of the image frame on screen}}{\text{Width of the chamber in the image frame on screen}}$$

NOTE: For example, if the actual chamber size is 7 mm, the width of the image frame is 137 mm, the width of the chamber in the image frame on the computer screen is 81 mm, and the width of the field of view was 11.83 mm (**Figure S3**).

5.3.3 To see the tracking results, go to the Tracking folder under the Results folder.

5.3.4 To generate a video of the tracked fly, select **Make video**. The video will be saved in the same Results folder as that of the original video data analyzed.

NOTE: The Start (first) and End (last) frames of the video can be selected.

5.4 Normalization to body length of each fly.

NOTE: As each fly may be slightly different in size, certain gait parameters should be normalized to the body length of each fly to facilitate comparisons (e.g., stride length may be longer in larger flies, and shorter in smaller flies).

5.4.1 Open three still images from the video of each fly (usually first, middle and last frames) using image software.

5.4.2 Magnify each image frame to 800% and label the anterior-most pixel of the head and posterior-most pixel of the abdomen at the midline using a bright color (e.g., yellow).

5.4.3 Open the labeled images in ImageJ.

5.4.4 Use **Set Scale** to input the scale accordingly: Set Distance in pixels: 512; Known distance: Actual Field of view (mm) as measured in step 5.3.2.; Unit of length: mm.

5.4.5 Draw a straight line between the labelled head and abdomen tip pixels to obtain the body length.

5.4.6 Open **Set Scale** again to obtain the value in a known distance, which is the body length in mm.

5.4.7 Take an average of the length determined in each of the three images to obtain the average body size in mm.

REPRESENTATIVE RESULTS

Following leg segmentation, tracking and data processing, FLLIT automatically generates raw data for the positions of the body and each leg claw, 20 gait parameters, 5 plots and a tracked video (**Table 1**).

Here, we demonstrate these analyses using a fly model of Spinocerebellar ataxia 3 (SCA3). The pan-neuronal driver *Elav-GAL4* was used to drive either the full-length wildtype human SCA3 with 27 glutamines in the polyQ tract (UAS-SCA3-flQ27), or a full-length mutant human SCA3 with 84 glutamines in the polyQ tract (UAS-SCA3-flQ84)[11]. SCA3 is typified by an ataxic gait with body veering, erratic foot placement and short, lurching steps[12,13] (**Table 2**). To characterize gait of mutant SCA3 flies and investigate whether they display a similar gait to that of human patients, we analyzed relevant gait parameters generated by FLLIT, namely: Number of body turns, footprint regularity, leg domain overlap and sizes, and leg stride lengths (**Table 2**).

We found that SCA3-Q84 flies exhibited more turns (**Figure 4A,A'**), erratic foot placement as exhibited by low footprint regularity (enlarged standard deviations of the AEP[14]) (**Figure 4B**), increased leg domain overlap (**Figure 4C-D**), enlarged leg domains in length and area (**Figure 4E,F**), and decreased stride length (**Figure 4G**).

FLLIT also generates a video showing the tracked fly and legs in the arena-centered and body-centered views, body trajectory and heading direction, and vertical and lateral displacements of each leg (**Figure 5**). The tracked videos allow side-by-side comparison of leg movements in different flies. Representative videos of *Elav-GAL4>SCA3-flQ27* (**Video 1**) and *Elav-GAL4>SCA3-flQ84* (**Video 2**) flies demonstrate that compared to *Elav-GAL4>UAS-SCA3-flQ27* flies (**Figure 5A**), *Elav-GAL4>UAS-SCA3-flQ84* flies (**Figure 5B**) exhibit irregular, intersecting leg domains of different sizes, indicative of a lurching, ataxic gait.

FIGURE AND TABLE LEGENDS

Figure 1. Setup of the recording station and arena. Recordings from the (**A**) front and (**B**) side views. (**C**) An example of an arena used for making fly recordings for FLLIT tracking.

Figure 2: View of the active window during fly gait recording using a dual head camera, which allows simultaneous recording of two flies.

Figure 3: Active FLLIT window showing the button panel and labelled legs after segmentation and tracking.

Figure 4: Representative FLLIT-generated data for relevant gait parameters of flies expressing wildtype (SCA3-flQ27) vs. mutant (SCA3-flQ84) SCA3. (**A**) Number of turns in the body trajectory. (**B**) Mid-leg footprint regularity normalized to body length. (**C-C'**) Traversed leg domains of each leg. (**D**) Domain overlap between legs. (**E**) Mid-leg domain length normalized to body length. (**F**) Mid-leg domain area normalized to body length^2. (**G**) Mid-leg stride length normalized to body length.

Figure 5: Snapshot of representative FLLIT-generated videos. (**A**) *Elav-GAL4>UAS-SCA3-flQ27* and (**B**) *Elav-GAL4>UAS-SCA3-flQ84* flies.

Video 1: Representative video of a fly expressing pan-neuronal wild-type human full-length SCA3 (*Elav-GAL4>UAS-SCA3-flQ27*).

Video 2: Representative video of a fly expressing pan-neuronal mutant human full-length SCA3 (*Elav-GAL4>UAS-SCA3-flQ84*).

Supplemental Figure 1: Configurations for VcXSrv.

Supplemental Figure 2: Configuration for Xquartz.

Supplemental Figure 3: Image labelled with the dimensions needed for calculating Field of view.

Table 1: Gait parameters generated by FLLIT.

Table 2: Table showing hallmark SCA3 gait features in human patients with their corresponding FLLIT parameters and output files.

DISCUSSION

In this manuscript, we describe in detail the steps involved in using FLLIT, an automated machine-learning program[1], to analyze gait in freely walking *Drosophila*. After tracking and data analysis, FLLIT automatically generates raw data for the positional information of the body and leg claws, producing twenty body and gait features as well as a video of the tracked fly to enable gait visualization.

There are now a number of methods for leg movement tracking of *Drosophila* and other animals[1-4,14-16], giving researchers a wide range of options depending on the goals of the experiment. Some of these are foot printing-based approaches, which are highly accurate but which report only claw contact points with the detection surface[4,14]. On the other hand, recent deep learning approaches[2,3,16] are highly versatile, allowing analysis of behaviors that require tracking of leg joints and other body parts in any animal, with the caveat that the algorithms need to first be trained with user annotated datasets. A third type of approach uses morphology or image-contrast-based methods[1,15,17] to find the outline of each leg to identify claw positions. In general, these methods deal poorly with behaviors where the legs cross over (e.g. during grooming). FLLIT combines the second and third approaches, using morphological parameters to train a boosting algorithm for leg segmentation. This allows FLLIT to bypass the laborious task of user annotation to generate the training dataset, while enhancing accuracy using machine learning. Future improvements to FLLIT will have to deal with instances where legs cross over, to allow for analysis of more complex behaviors.

FLLIT is robust to slight changes in illumination, recording resolution and frame speed[1]. However,

frame speed of recorded videos should not fall below 250 fps, and FLLIT runs optimally for videos recorded at 1000 fps. If there is motion blur in the images, such that it is challenging for a human annotator to identify the leg position, FLLIT will not be able to accurately identify leg tips in those frames. In the light of this, it is essential that the camera be focused sharply on the leg tips. To prevent segmentation artifacts, the arena should be cleaned thoroughly, and should not be moved during the recording. For accurate background subtraction and clean segmentation, the fly should move at least one body length during the recording, without pausing. After automatic segmentation and tracking the labeling of the all legs must be checked. If the fly gait is not tracked or tracked wrongly, the file should be retracked manually using the **Manually Initiate Tracking** option (step 5.2.7 – 5.2.10).

Neurodegenerative diseases and movement disorders are increasingly prevalent in our aging societies. Fly models of neurodegeneration have been studied for more than 2 decades, during which advances have been made regarding the molecular and cellular aspects of disease pathophysiology. However, specific behavioral consequences of disease have been technically difficult to assess. For example, while reports of trembling movements in the fly have been made[18,19], these had not been quantitatively studied until recently[1]. The climbing assay has been a useful and quantitative, yet relatively coarse measure[6]. This technical deficit has similarly hampered high-resolution movement analysis in other animal models. The advent of new tools for behavioral analysis, hence, has promise to rejuvenate the field of movement disorders to enable researchers to study how molecular and cellular mechanisms of neuromuscular diseases lead to specific behavioral outcomes in animal models. In this paper and in our previous work[1], we showed using FLLIT that fly models of SCA3 exhibit a hyperkinetic ataxic gait, while PD fly models exhibit a hypokinetic rigid gait, recapitulating movement hallmarks of the respective human diseases[1]. Gait analysis also enabled us to identify distinct neuronal populations underlying specific movement dysfunctions. Going forward, detailed movement analysis, combined with the powerful imaging and functional tools available in the fly, will allow us to gain novel insight into mechanisms of locomotor dysfunction, illuminating our understanding of neurodegenerative diseases with respect to circuit mechanisms.

FLLIT should be widely applicable to study gait in other small arthropods, as it was previously demonstrated to be highly accurate for tracking spider leg movements[1]. While we focus here on the use of detailed movement phenotyping for quantifying pathogenic gait and its underlying circuitry, advances in movement tracking have already revolutionized, and will have continuing impact on, the understanding of normal walking coordination and gait and its underlying circuits, especially in myriad different branches of the evolutionary tree.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

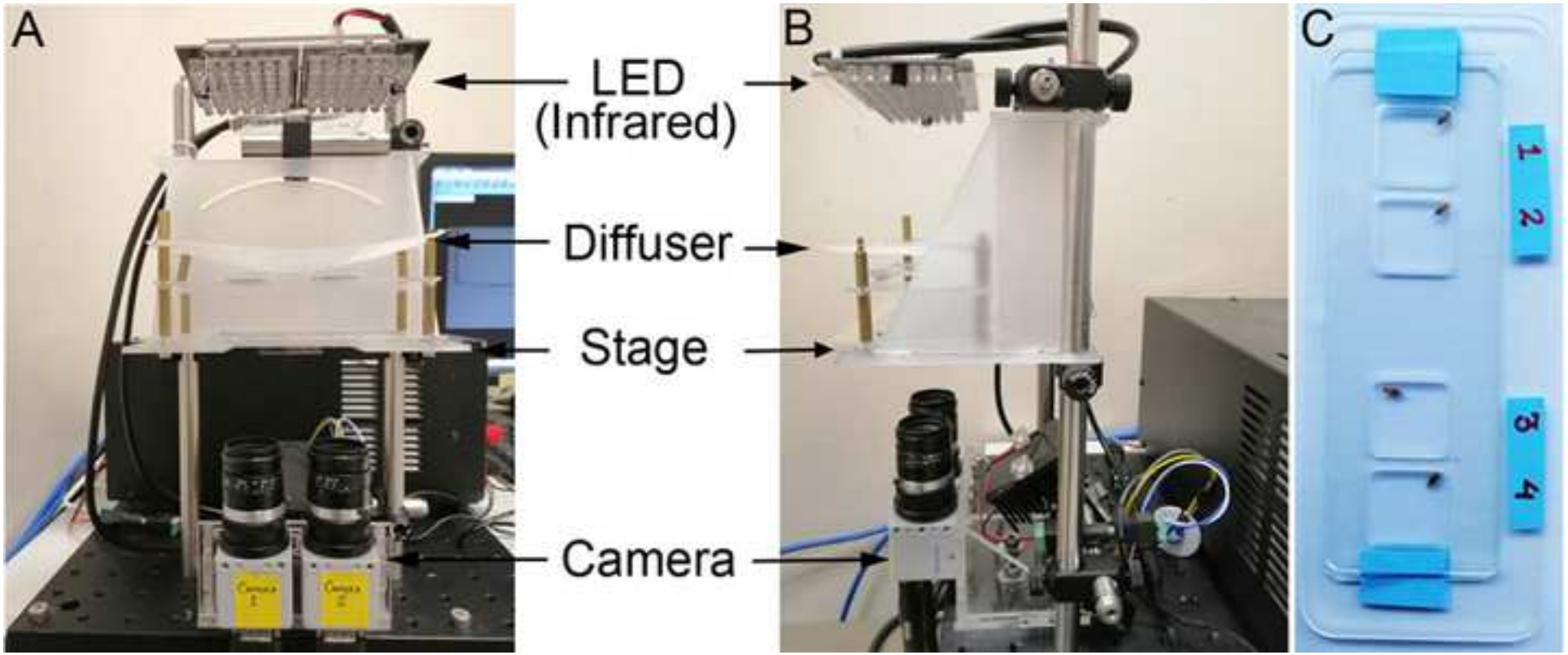


Figure 2

Click here to access/download;Figure;Figure 2 PFV SCREEN SHOT2.tif

Figure 3

Click here to access/download;Figure;Figure 3 Tracked fly in FLLIT.tif

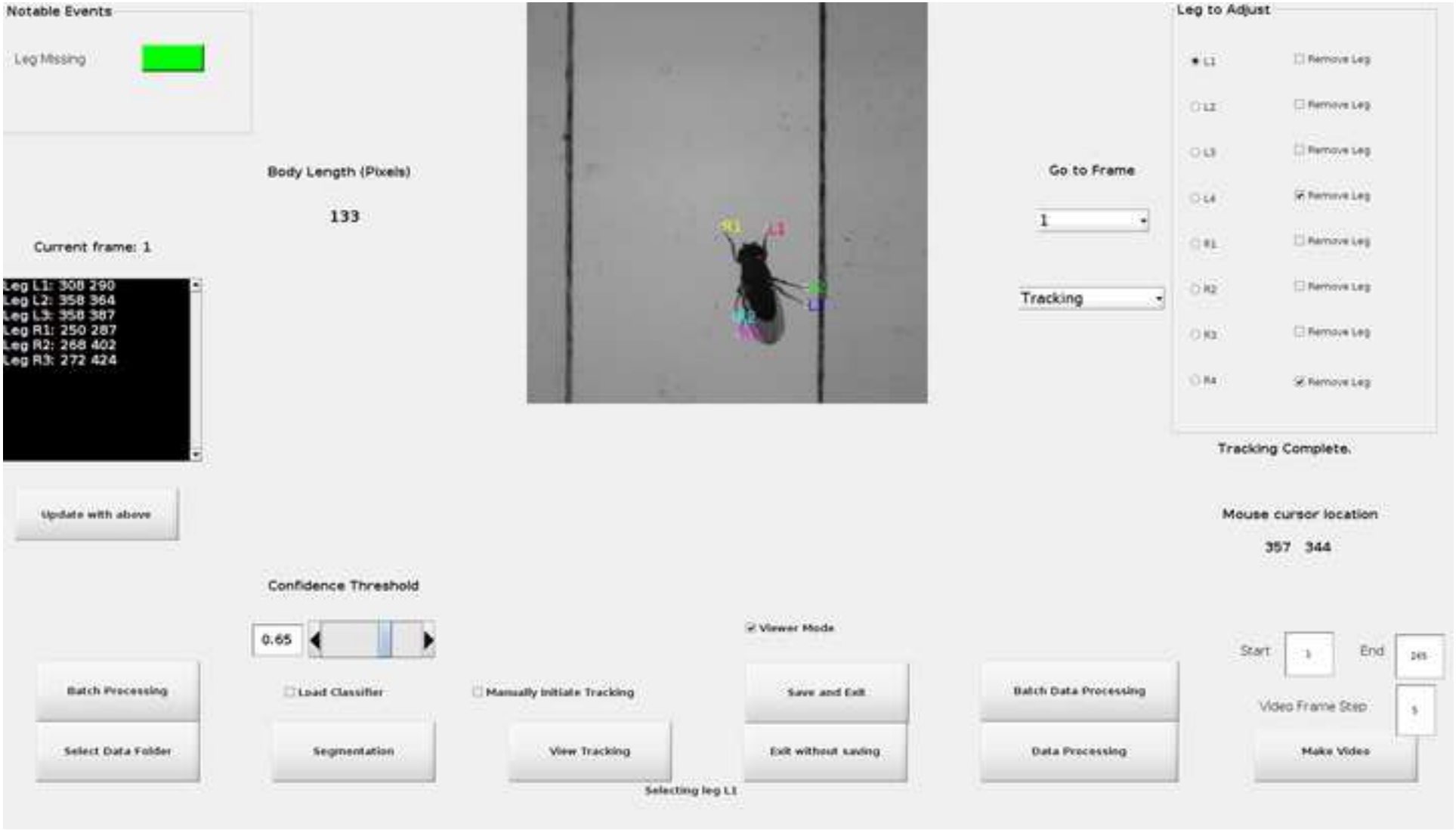


Figure 4

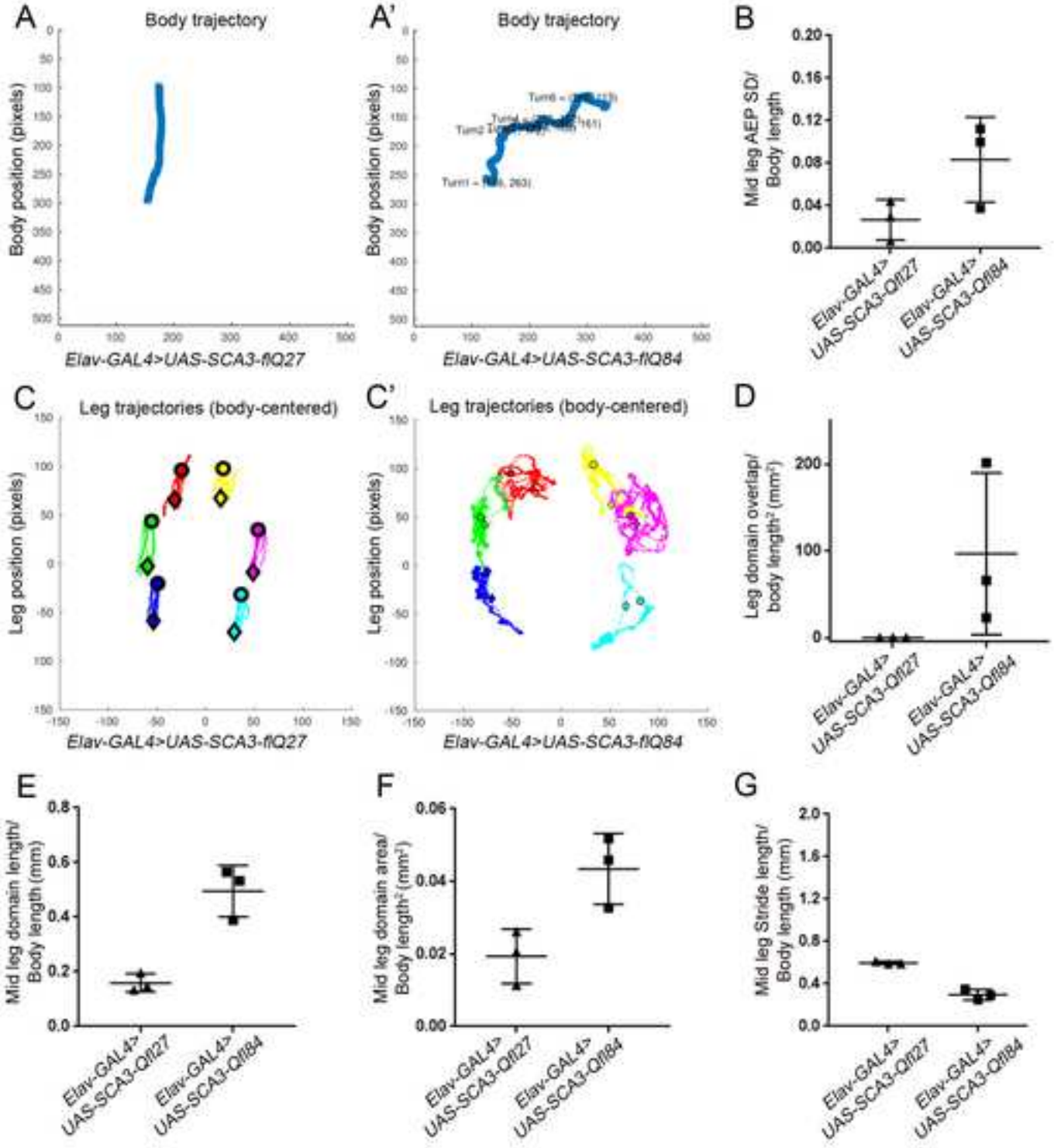
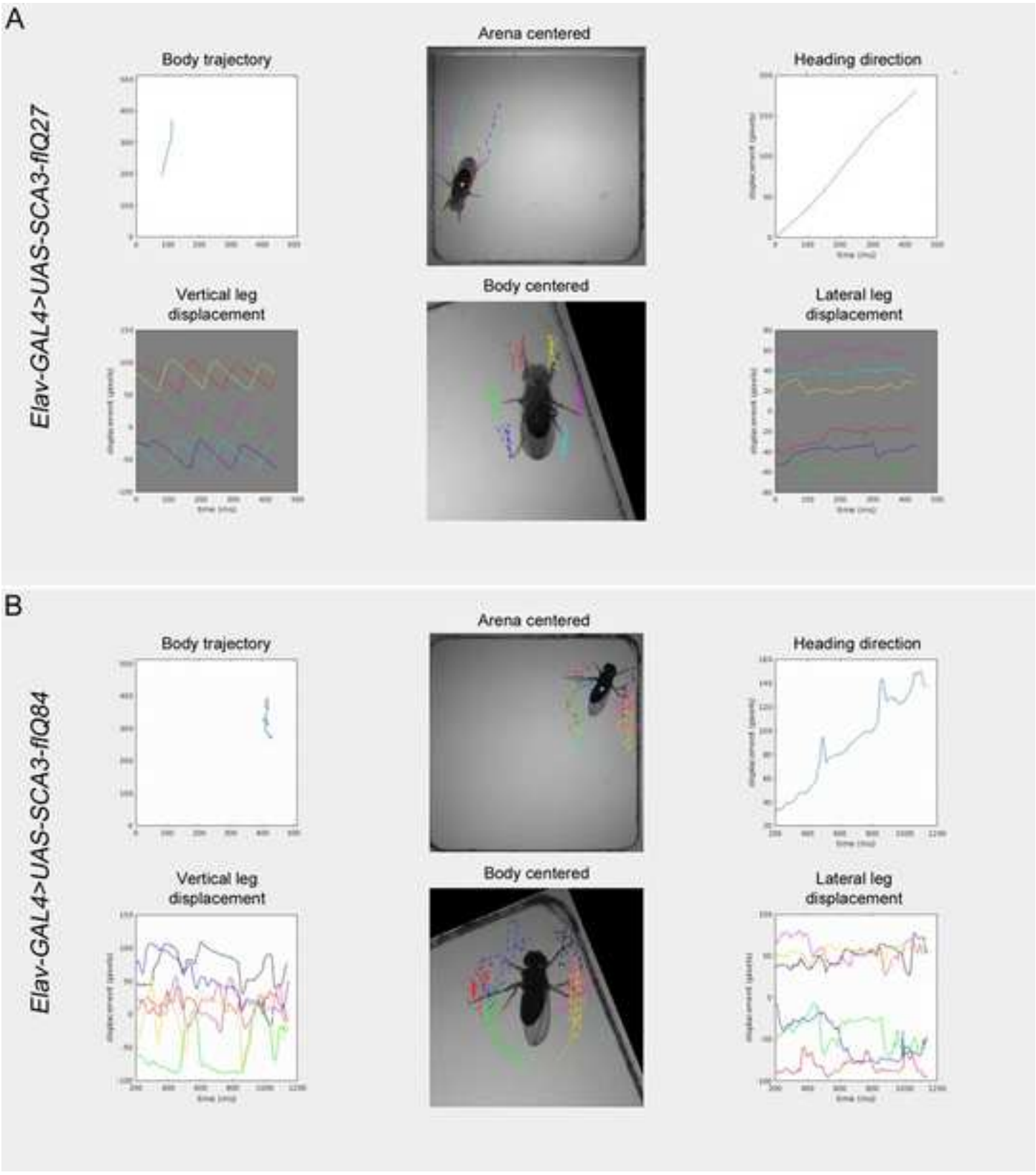


Figure 5

Click here to access/download;Figure;Figure 5 Video snapshot.jpg



Video 1

Click here to access/download

Video or Animated Figure

Video 1 Elav-GAL4_SCA3-flQ27_22d.mp4

Video 2

Click here to access/download

Video or Animated Figure

Video 2 Elav-GAL4_SCA3-flQ84_22d.mp4

Category	Parameters
Raw data	Body position
	Body trajectory
	Arena-centred leg claw positions
	Body-centred leg claw positions
Body motion	Body length (mm)
	Instantaneous body velocity (mm/s)
	Turning points of the body trajectory
Individual stride parameters	Stride duration (ms)
	Stride period (ms)
	Stride displacement (mm)
	Stride path covered (mm)
	Anterior extreme position (mm)
	Posterior extreme position (mm)
	Stride amplitude (mm)
	Stance linearity (mm)
	Stride stretch (mm)
Leg motion	Leg speed (mm/s)
	Gait index
	Movement percentage
	Mean stride period (ms)
	Footprint regularity (mm)
	Leg trajectory domain area (mm^2)
	Length and width of the leg trajectory domain (mm)
	Leg domain intersection/overlap (mm^2)
	Stance width (mm)

Description
Positional coordinates of the body centroid in each frame
Angle of rotation of the body axis in degrees (relative to the y-axis)
Positional coordinates of each leg claw in each frame based on arena coordinates
Positional coordinates of each leg claw in each frame based on arena coordinates
Length of the sample animal estimated in each frame (anterior-most position on head to posterior-most position on the wings)
Instantaneous velocity of the body (centroid) in the sample animal
To locate the turning points, the trajectory is reduced to a piecewise-linear curve using the Dougl asâASPeucker algorithm, following which a turning event is identified as involving an angle > 50 deg between two neighbouring linear segments constituting the simplified trajectory
The duration of a stride event
The duration from one stride event to the next
Displacement of the leg claw during a stride event
Total path covered by the leg claw during a stride event
Landing position (relative to the body) of a leg claw at the end of a stride event
Take-off position (relative to the body) of a leg claw at the start of a stride event
Displacement along the direction of motion for a stride event
Defined as the deviation of the stride path from a curve smoothed over (at 20ms intervals) the corresponding anterior
Distance of the leg claw position from the body centre in the middle of a stride event
The instantaneous speed of each leg
This measures the type of gait coordination exhibited by the (six-legged) sample animal during its motion. A gait index of 1 corresponds to a tripod gait, _1 corresponds to a tetrapod gait while 0 constitutes an non-canonical gait.
Percentage of the time that a leg is in motion
Average duration from one stride event to the next
Measured as the standard deviations of the posterior and
anterior extreme positions of a leg
The area of the minimal convex hull that contains the entire leg trajectory in the body-centred frame of reference
Obtained via the maximum projected distance of the claw positions onto the major (domain length) and minor (domain width) principal axes
The intersection/overlap between each possible
Average of the distance between the AEP and PEP of the left and middle legs

File/Plot (if applicable)
First two columns of CoM.csv
Third column of CoM.csv
trajectory.csv
norm_trajectory.csv
bodylength.csv
BodyVelocity.csv; BodyVelocity.pdf
BodyTrajectory.pdf
StrideParameters.csv
LegSpeed.csv; Gait.pdf
GaitIndex.csv; GaitIndex.pdf
LegParameters.csv
LegParameters.csv
LegParameters.csv
LegParameters.csv; LegDomain.pdf
LegParameters.csv
LegDomainOverlap.csv
StanceWidth.csv

		Gait fe
Gait features of Spinocerebellar ataxia 3 (SCA3)	Veering	Erratic foot placement and leg crossing over
Measurement Parameter	Number of body turn events	Footprint regularity
FLLIT File	BodyTrajectory.pdf	LegParameters.csv

eature	
Lurching steps	Short strides
Size of leg domains, degree of domain overlap	Stride length
LegDomainOverlap.csv	StrideParameters.csv

Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials.xlsx

Name of Material / Equipment	Company
Acrylic Sheets	Dama
Clear Glass slides	Biomedia
High speed camera	Photron
Infra Red LED	Any - Generic from hardware store
Kimwipe	Kimberly Clark

Catalog Number
1.6 mm thickness, Clear sheets
BMH 880101
Fastcam MC2.1
940nm Infrared Light Board
34155-01LS

Country
Singapore, Singapore
Singapore, Singapore
Tokyo, Japan
Singapore, Singapore
Irving, Texas, USA

Comments / Description

A shutter speed of 1 msec or faster is ideal to reduce motion blur of captured images

Editorial comments:
Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version. Please use American English throughout.

The paper has been thoroughly proofread.

2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points.

This formatting has been done.

3. Please provide an email address for each author.

The email addresses of the authors are as follow:

Animesh Banerjee: banerjeea@imcb.a-star.edu.sg
Shuang Wu: wushuang@bii.a-star.edu.sg
Cheng Li: chengli@bii.a-star.edu.sg
Sherry Aw: syaw@imcb.a-star.edu.sg

4. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.For example: Photron 85 FASTCAM MC2, Windows 7 and Windows 10 Home Edition, XQuartz, Kimwipe, Microsoft paint, etc.

We have removed all commercial language in the text with the following exception: For different Windows versions the command to install FLLIT will differ (Lines 192 - 208). This is an important instruction and is required for proper installation. Therefore, we have kept the version of Windows used and the corresponding installation instructions in the text.

5. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, alphabets or dashes.

This has been fixed.

6. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.”

All the text in the protocol were checked and changed to imperative tense.

7. In the JoVE Protocol format, “Notes” should be concise and used sparingly. Only one note can follow one step. These should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step.

This has been fixed.

8. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.

This has been fixed.

9. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.

This has been fixed.

10. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed?

This has been fixed.

11. 2.1 note: When do you use CO2? Not mentioned in the step above. Is there any sex specific bias to be taken into account?

CO_2 is usually only used to anesthesize flies upon first collection, and used prior to recording. Instead, ice is used to anesthetise flies prior to recording. Both male and female flies can be used in our set up, and, as far as possible, flies of both sexes should be analysed to avoid sex-specific bias. This is now mentioned in the text in Step 2.5.

12. 3, 5: Please include how individual steps are performed. Please include all the button clicks in the software, knob turns etc. For example, Click “Save” to save the files

This has been fixed.

13. Please do not box the commands. These can be italicized instead.

This has been fixed.

14. 5.3.6: Please make actions steps in imperative tense or move this part to the results.

This has been made into action steps in imperative tense.

15. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

We have now highlighted 2.75 pages or less of the Protocol (including headings and spacing) that identify the essential steps of the protocol for the video.

16. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

No figures were reused in this manuscript. The tables were edited and reformatted to reflect slight updates made to FLLIT since it was published.

17. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
b) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique

We have now revised the Discussion substantially to address these 5 points.

18. Please do not abbreviate the journal titles in the references section.

The output style that we used in this manuscript is the JoVe Endnote output style, which we downloaded from www.jove.com/files/jove.ens. Please advise which other Endnote style we should use and where to find it?

19. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an .xls/.xlsx file.

This has been revised. Some items were generic items from the hardware store with no company or catalog number, and hence only the description and technical specifications of the item are given.

Reviewers' comments:

Reviewer #1:

Aw and colleagues present a methods paper showing how to use an automated software suite for limb tip tracking in Drosophila. This includes setting up the rig for making the measurements and using a software suite to extract a set of parameters about the leg positions in each frame. The manuscript clearly explains how the authors obtained and analyzed data using this software suite.

Thank you for your kind comments.

Major:

I think this paper would benefit from an expanded discussion of the different published methods for limb and posture tracking in Drosophila. The authors cite LEAP and deeplabcut, as well as a recent 3d tracking paper. Another recent paper used a purely linear model with a training set to extract limb tip positions (DeAngelis et al., eLife, 2019). Since this paper is primarily about applying a method, readers would benefit from understanding the advantages and drawbacks of each of these methods, as they have been applied, relative to this particular one. For instance, all the others require annotation. The linear one, like this one, works best with walking flies and fails to track limbs in grooming flies. LEAP and deeplabcut in principle can track legs during all sorts of behaviors, as could the 3d tracker.

We have now give an overview (in the Discussion) of the different published methods for limb and posture tracking in *Drosophila*.

Figure 4 A,A',C,C' require units for axes, or possibly a scale bar instead.

Axes have been added.

The application focus here is clearly on the authors' primary research interest. I wonder if they couldn't devote a little more space to the other applications of this sort of behavioral measurement: dissecting walking coordination more generally and its underlying circuits. That was the focus of most of the other related methods for limb tracking.

We now discuss this in the final paragraph of the Discussion.

Somewhere, it would also be useful to summarize some of the technical details of this algorithm. For instance, how does it find limb tips to generate its annotation? What kind of deep learning algorithm is it using?

FLLIT employs a boosting algorithm instead of a deep learning algorithm. There are some similarities with Convolutional Neural Networks in that for both frameworks, feature extraction is done automatically through learning convolutional kernels. We now summarise the technical details of this algorithm in the Introduction, in lines 69 – 85.

Minor:

Photron camera reference should list company location city, country; same with other companies listed in the materials table.

These information are now included in the materials list. Some items were generic items from the hardware store with no company or catalog number, and hence only the description and technical specifications are given.

In my PDF, the screenshot figures were heavily distorted by compression artifacts, which should be corrected in any final version.

There is a link in the top right corner of each PDF page containing each figure, that when clicked will allow the download of that figure in the correct resolution. The figures downloaded from these links will be the figures published in the final version.

I was unable to open the *.mp4 supp movies on my mac, running OSX 10.13.6 with quicktime up to date (or so I believe).

We have chosen MP4 as it is generally the most universal video format. However Quicktime apparently only plays MP4 files in certain compression formats. Could we kindly recommend that you download and try this VLC, a video player for mac able to play a wide range of video formats? Here is the free download link: http://www.videolan.org/vlc/download-macosx.html?

Reviewer #2:

Manuscript Summary:
The manuscript contains the instructions to use a software for leg tracking in freely moving insects, including a description of the setup needed to collect the data. The protocol seems straightforward and well described.

Thank you for your kind comments.

Major Concerns:
In my opinion, the instructions to download and install the software are not adequate for most scientists. The main method proposed is to clone a git repository. To do this, the authors give a command that only work for people who have git installed in their computer, which I think is a minority (it did not work for me). Cloning the git repo is recommended because of the size, but I think this will be an unnecessary bottleneck for most users. It would be better to create a small distribution that can be downloaded from github more easily, and perhaps add the example dataset somewhere else.

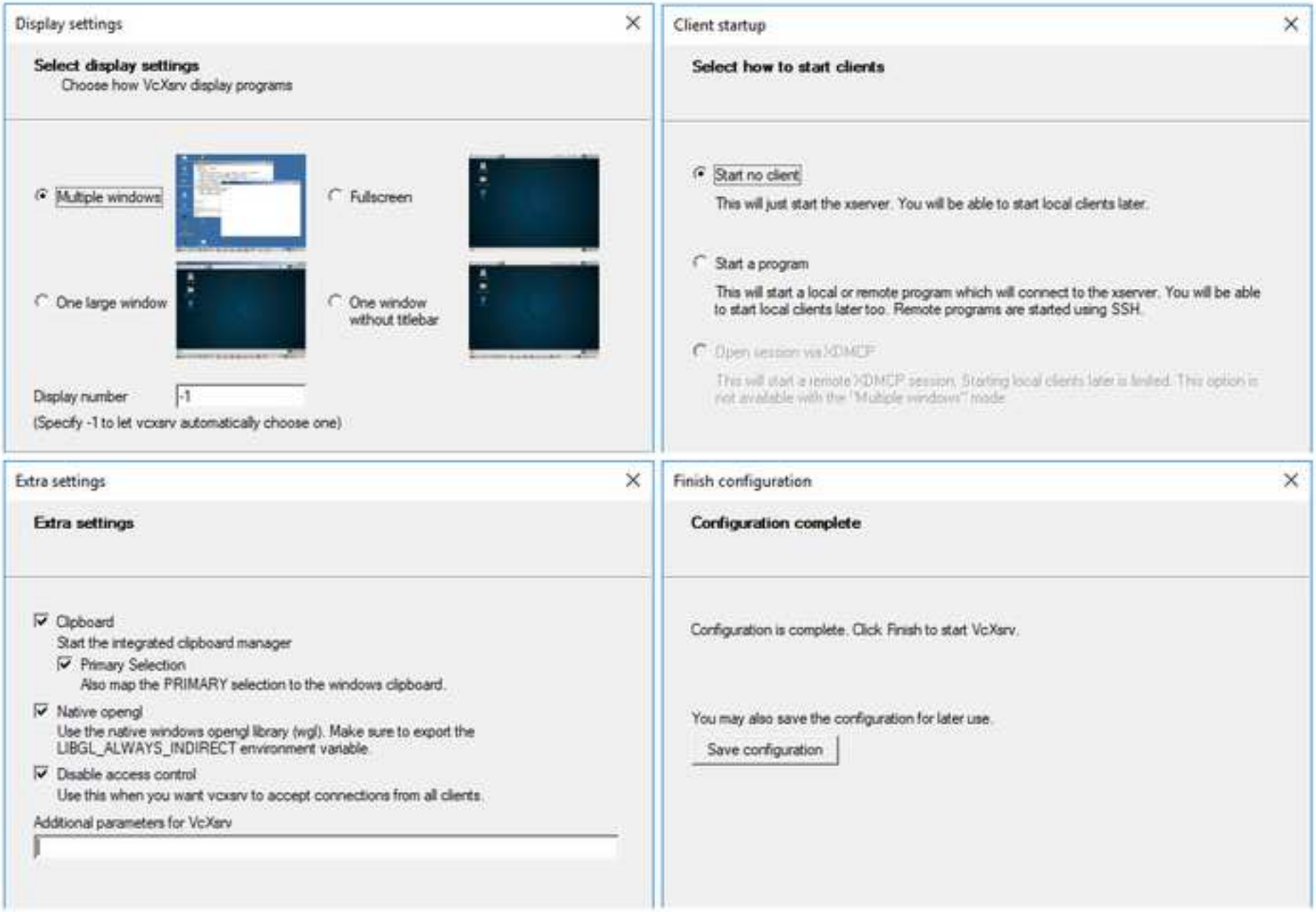
Thank you for your suggestion. We have now moved the example dataset to Google Drive and provide the download link. FLLIT itself can now be directly downloaded from Github without cloning the git repository.

Minor Concerns:
Line 97: The field of view is given in a strange way. Is this the uncertainty? Unless this uncertainty is an important detail, I think it would be better to simply write 11.5x11.5, or something like that.

We have now fixed this by using the standard in our lab, which is 11 x 11 cm.

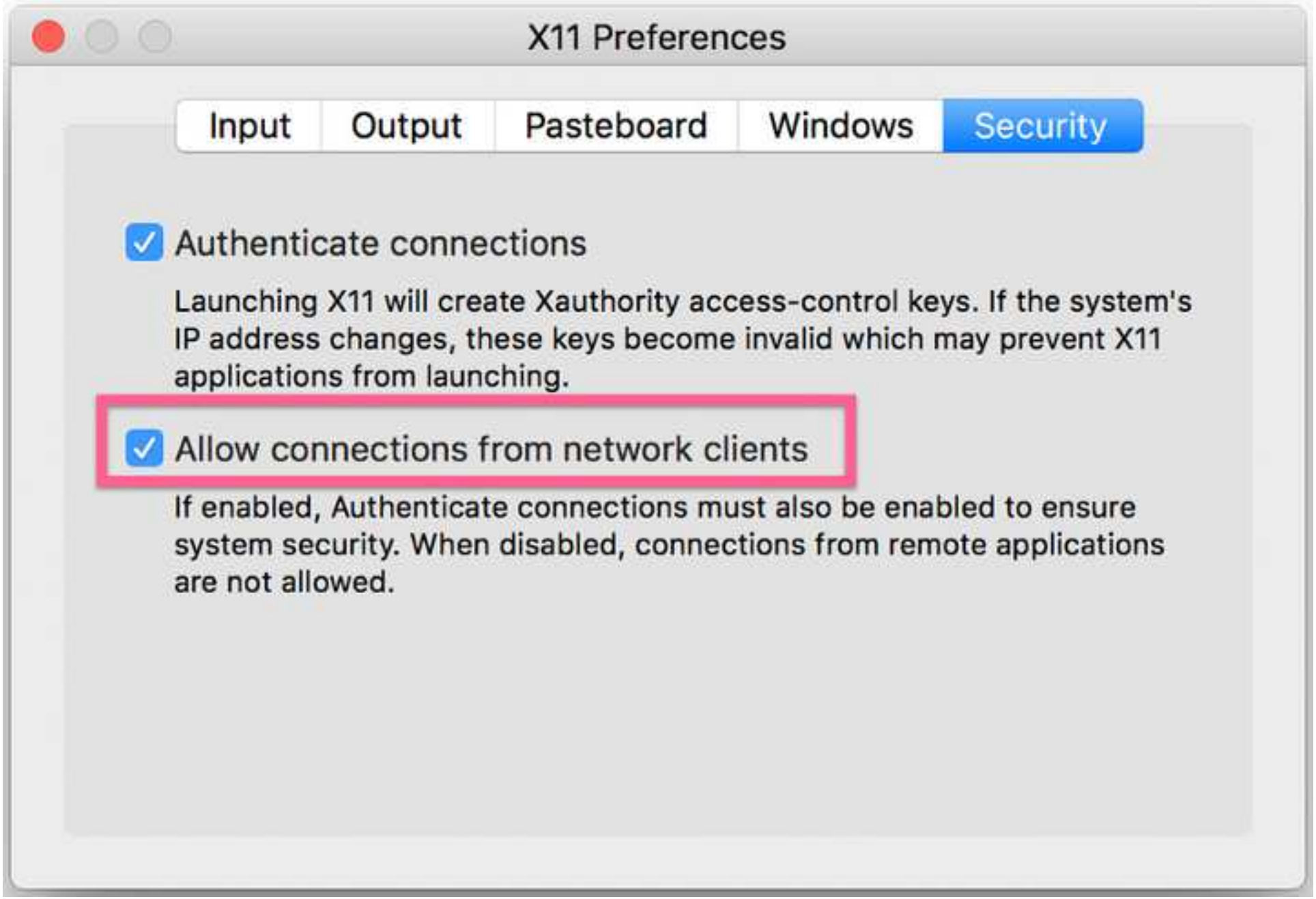
Supp Figure 1

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Supplemental Figure 1.jpg



Supp Figure 2

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Supplemental Figure 2.jpg



Supp Figure 3

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Supplemental Figure 3 To show FOV.tif