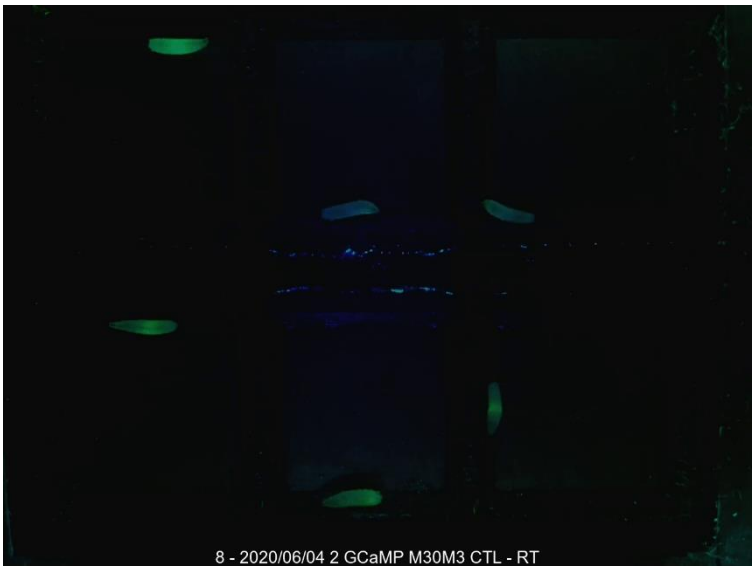
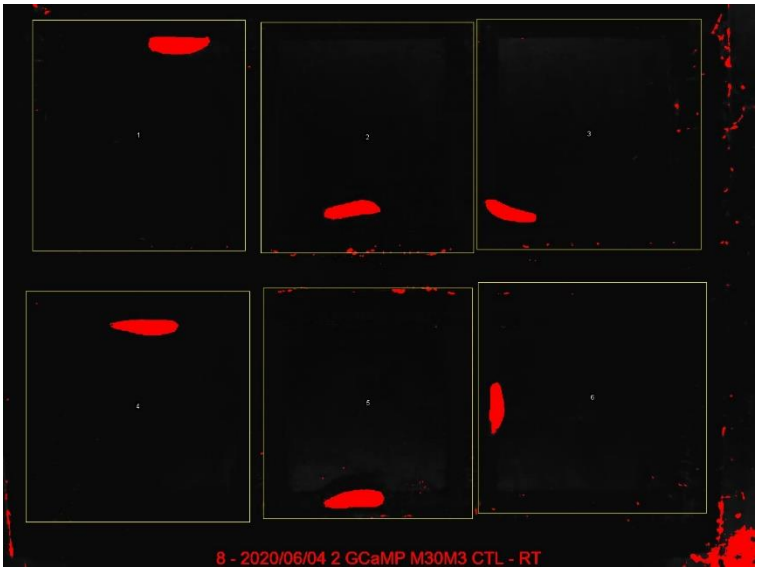
After following the installation instructions described in the README file, follow these steps to analyze larval behavior on the set of images in the folder "example":

1- Using ffmpeg, extract one image per second of your video in .bmp format and name them with sequential numbers. Or extract the example stills from the file “images extracted from video every 5 seconds.rar”.
2- In Fiji, run LTT(Larva Tracking Tool)>>GCAMP-larva.
3- Open one image of the series to set ROIs.
4- Outline one larva (or any other region of your interest). This is used to set the upper and lower size thresholds.
5- The image will be split into RGB channels, Red and Blue will be closed, and Green will be transformed into an 8-bit Grayscale image for further processing.
6- Adjust threshold values. To do so, DO NOT click the OK button. Go to Image>Adjust>Threshold and set values. DO NOT click any button in the Threshold window. Optimal threshold values will mark the whole larva in red while keeping the background in the original color. Once the threshold value has been set, click OK in the "Adjust Threshold" window.
7- Indicate the number of areas that contain individual larvae (6 in this example)
8- Select the input folder containing all the .bmp images to analyze.
9- Select the output folder. By default, it is the same as the input folder.
10- Select ROIs. Each time ROIs are selected, they are saved in a Roiset1.zip file that can be used to repeat the analysis in the same exact areas. In that case, the "Load saved Areas" box has to be checked (go to step 11). If not, leave the box unchecked and proceed to step 10.
11- Select the areas that contain the larvae. The areas can have any irregular shape, they do not need to be squared. That is useful to exclude overexposed background areas.
12- An image with labelled ROIs will be saved in the output folder.
13- Set measurements. Typical parameters are:
    a. -Area: will measure the area in pixels of the object detected within each ROI
    b. -Integrated density: will measure the brightness of the object detected within each ROI
    c. -Shape descriptors: will calculate the size in pixels of the major and minor axis.
    d. -Centroid: will provide the X and Y coordinates of the centroid, as defined in ImageJ.
14- One separate .txt file per ROI will be created, where values of each parameter obtained from each of the images will be appended.
15- Select the type of files to analyze (.bmp in this example)
16- The results will be saved in individual files for each ROI. A log window will display the total number of pictures analyzed and the average time per picture. Running the 55 images of this example takes 11 seconds (~200 ms per image) in a Windows 10 computer with Intel Core5 processor and 4 GB RAM. Tests done in Mac computers were much slower. See here for a discussion on this issue https://forum.image.sc/t/mac-java-speed-issues/304/5
17- Files with the data obtained for each ROI can be further analyzed using Excel or other software.

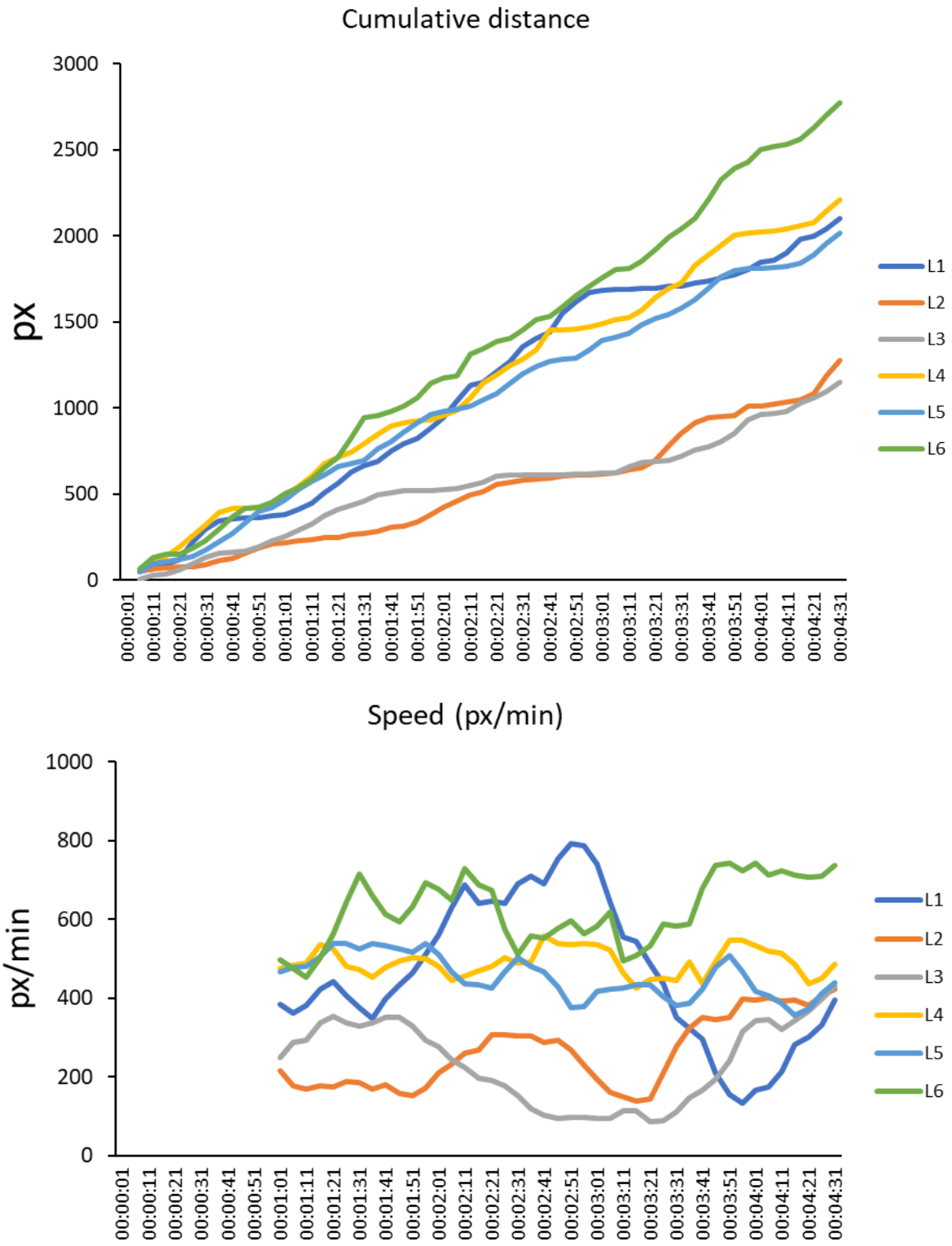
The files with the raw data and processed data are provided as .txt and analysis.xlsx files.



Original image. Larvae expressing muscle GCaMP are individually placed in the 6-well pupariating arena



Same image as above after extracting the green channel, setting threshold values and labelling ROIs.



Graphics showing the total distance travelled in 4.5 minutes and the speed of larvae. The speed is calculated as the sum of pixels travelled during the last minute and hence cannot be calculated until at least one minute has elapsed.