**Analysis of the orientation of head and thorax basal network of actin fibers**

Requirements

- Install **Fiji/ImageJ** (this pipeline was tested using ImageJ 1.51h, Java 1.8.0\_66 (64bit), website: <http://imagej.nih.gov/ij>)

- Install **Matlab** (this pipeline was tested using 2018b Matlab version

1. Preprocessing and input data

Input data is a series of hyperstacks*(x,y,z)* images of the head-neck-thorax region of pupae stained with Phalloidin (actin reporter).

*Preprocessing for each hyperstack:*

* The apical plane is automatically detected on the hyperstack (see the code for detection of the apical plane and projection of the data). Using the position of the apical plane, a projection for the apical signal and a projection of the basal signal are generated.
* Overall noise is subtracted from the apical and basal projections using Fiji plugin “Subtract background” (Rolling Ball Radius = 100 pixels).
* Using Fiji apical and basal projections are concatenated, rotated so that head is up and thorax is down, and cropped for a box of 1142X1142pixels (1 pixel = 0.108µm) centered on the neck region.

*Architecture of the input data:* Each animal corresponds to a folder named Pupa1 (Pupa2, … or Pupa(n)). Each folder contains a file called ‘Image.tif’: this file is the concatenation of the apical and the basal signal for the given animal (see the examples in ‘Data\Test\_segmentation’ folder).

1. Correction of the global signal intensity

In Fiji, run ‘**A\_CorrectAverageIntensity.ijm**’.

Indicate the path ‘Data\Test\_segmentation’. In this folder, 2 examples of data are shown (a control animal and a *Dg* mutant).

‘A\_CorrectAverageIntensity.ijm’ will assist the user in estimating the global signal intensity using apical signal, and will accordingly correct the basal signal.

*Why correcting basal signal for global signal intensity?*

Phalloidin stainings show a sample-to-sample variability in global intensity. As the analysis requires the use of a threshold value for segmenting actin fibers (we will use a single threshold value for all conditions to get an unbiased segmentation of the fibers), the variability in global staining intensity needs to be corrected for before applying the threshold. Thus, apical signal is used to estimate the global staining intensity of the sample. This value is then used to correct for the intensity of the basal signal.

1. Automatic segmentation of the basal network

In Fiji, run ‘**B\_SegmentBasalNetwork.ijm**’.

Indicate the path ‘Data\Test\_segmentation’.

This code will automatically segment the basal network fibers. To do so, a set of filters are used to enhance signal-to-noise ratio, and a fixed threshold value is used to binarize the image (the same value is used for all animals and conditions).

1. Get the position of the head-thorax boundary

In Fiji, run ‘**C\_SegmentApicalHeadThoraxBoundary.ijm**’.

Indicate the path ‘Data\Test\_segmentation’.

This code assists the user in segmenting the apical position of the head-thorax boundary. The position of this landmark will be used to define a ROI corresponding to the neck, and a ROI corresponding to the thorax (the drawn head-thorax boundary and these 2 ROIs are saved in ‘HeadThoraxROIs.zip’).

1. Get the position of the head-thorax boundary

In Fiji, run ‘**D\_AnalyzeSkeleton.ijm**’.

Indicate the path ‘Data\Test\_segmentation’.

Pipeline of the code.

* The segmented basal network is cut into small linear fragments by keeping the junctions and removing the vertices of the skeleton.
* The orientation and the length (approximated as the length of the diagonal of the bounding rectangle of the fragment) of each kept “junction” are quantified. These measurements are done and saved for the neck and for the anterior thorax region, using the ROIs obtained after running ‘C\_SegmentApicalHeadThoraxBoundary.ijm’ (outputs: ‘AnglesHead.csv’, ‘AnglesThorax.csv’, ‘LengthsHead.csv’ and ‘LengthsThorax.csv’). As it is very error-prone to measure the orientation of very small junctions, junctions with a length inferior to 5X0.108µm are excluded from the analysis.
* A map showing the measured junctions color coded for their orientation is generated (output: ‘AngleMap.tif’).

1. Average and plot the data

In Matlab, open ‘**E\_GatherAndPlotAngles.m**’. In the parameter section of the code, indicate the path ‘Data\Values’. Add ‘Functions’ folder to Matlab path.

This code will gather the data of angles and length of junctions for the neck and thorax regions, for all the animals of each condition.). Average orientation in the neck and in the thorax is calculated and plotted for each animal, pooled by condition.

Remark: The measured orientation is calculated so as to vary from 0° (parallel to M-L axis) to 90° (perpendicular to M-L axis). With this linear data, classical averaging can be used to calculate an average orientation (no need to use a method of averaging specific for circular data like for angles varying from 0 to 360°).