**EyeHex: a toolbox for ommatidia segmentation**

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# Introduction

EyeHex is a MATLAB toolbox for ommatidia segmentation from 2D images of Drosophila eyes. The toolbox contains multiple Graphical User Interfaces allowing users to perform (1) manual ommatidia segmentation (for generation of training data for the external machine learning module), (2) automatic mapping ommatidia to a hexagonal grid, and (3) manual verification/correction of auto-segmented ommatidia.

The toolbox is to be used in combination with the machine learning module from WEKA-trainable segmentation plug-in [1] (included in Fiji toolbox [2]) to preprocess input 2D images.

# Software requirements

MATLAB (tested with version 2016b or later)

Fiji toolbox (download from https://fiji.sc/)

# Installation

1. Download and extract zip file or clone from github.

2. Place all the input images (e.g. img\*.tif) of the fly eyes into data/raw/ folder.

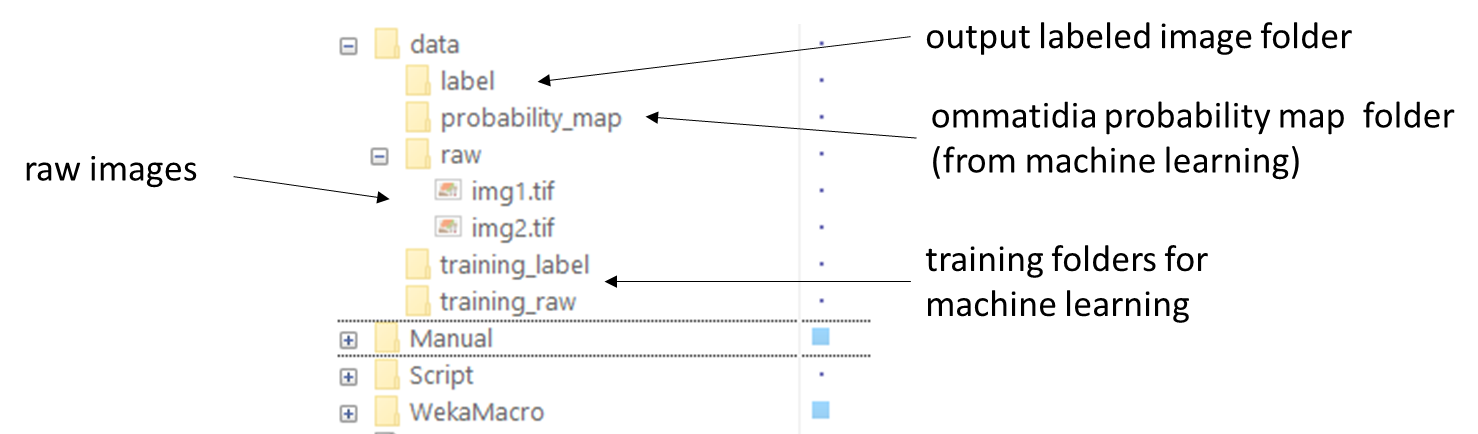


Figure 1. Examples of the data/ folder structure, with two raw images. Image img1.tif is used here to generate training data. The ommatidia segmentation is then applied to img2.tif file.

Additionally, you can add the raw stacks (in case of multi-focus images) to the data/raw\_stack/ folder to help with the visualization process if needed.

# Preparing training data

This step will help users generate training data for the machine learning module. It needs to be done once whenever a new type of image (from e.g. electron microscope, brightfield microscope…) or a drastic change in microscopy settings is introduced.

1. Browse to Script/ folder via MATLAB.

2. Type MAIN\_manual\_segmentation(input\_file) in MATLAB command window to run the manual segmentation GUI. The input\_file is the full name of the input image (e.g. ‘img1.tif’) in string format.

3. To add ommatidia, press **A** and manually clicking on the center of a few connected ommatidia. The first 3 facets must be adjacent (e.g. forming a triangle) on the hexagonal grid.

Press **Enter** to register the input. From this group of ommatidia (called a “patch”), a hexagonal grid of ommatidia will be automatically spawned and visualized on the GUI.

Try to add patches both at the middle and near the edges of the eye to get the most inclusive training data.

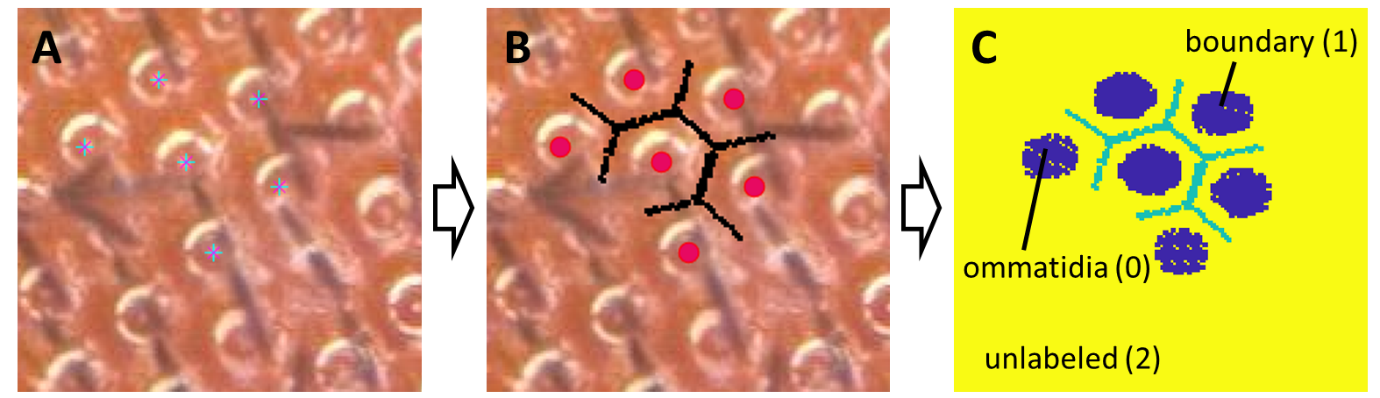


Figure 2. Manual segmentation for the training data. From a few manually labeled ommatidia (A), a hexagonal patch is spawned, with the ommatidia centers (red solid circles) and boundaries (black) visualized in panel B. A labeled image (C) is then exported, containing information on the ommatidia region (pixel value 0), the boundary regions (pixel value 1) and non-labeled region (pixel value 2). The example image (img1.tif) is taken from a brightfield microscope.

4. In case of bad grid spawning, press **R** and remove the patch with the freehand tool.

5. Define the inside region of the eye (hotkey **I**), outside region of the eye (hotkey **O**) or remove defined inside/outside region (hotkey **P**).

You can press **M** to toggle the overlay of inside/outside regions and ommatidia marks over the original image.

6. Press **Ctrl + E** to export the segmentation data to the training folders. This data includes the raw image, stored in data/training\_label/, and the label image, stored in data/training\_label/ folder. The label ommatidia image will have pixel value of 0 (ommatidia region), 1 (boundary region) and 2 (unlabeled region). The label eye image will have pixel value of 0 (eye region), 1 (non-eye region) and 2 (unlabeled region).

Note:

-Press **F1** to access all the hotkeys (zoom in/out, save/load progress).

-Use mouse right-click to zoom in and navigate around different image region.

-No need to segment all ommatidia. The machine learning module should work even with less than 50 ommatidia segmented.

-You can save/load the segmentation progress with **Ctrl + H** (to save progress) and **Ctrl + L** (to load progress).

# Automated preprocessing with machine learning

This step allows to generate the probability image of ommatidia region, in contrast to the boundary region, based on the trained classifier using the data from the previous step. Here, a macro for Fiji is provided to easily load the training data and apply the classifier to the all eye images.

1. Start Fiji and run the macro TrainClassifier\_gui.bsh in WekaMacro/ folder (by dragging the file to Fiji interface and press F5).

2. A user interface will appear, prompting you to select the path to directories of the data/ folder in the EyeHex toolbox.

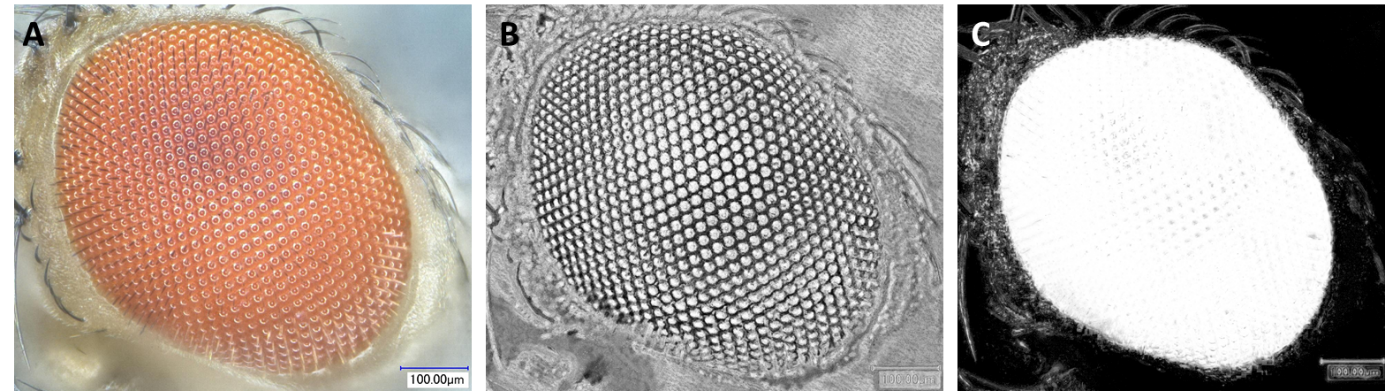


Figure 3. Automated preprocessing with WEKA-Fiji tool: The trained classifier will convert the raw image (A) in data/raw/ folder to the ommatidia probability map (B) and the eye probability map (C) and save the result to data/label/ folder.

The WEKA plug-in in Fiji will train the classifier with the training data (in data/training\_raw/ and data/training\_label/) and apply the classifier to all images in data/raw/ folder. For each image, a tiff file containing the probability map of the ommatidia region is created and saved to data/probability\_map/ folder.

Note:

-The machine learning model can be changed by modifying the TrainClassifier\_gui.bsh file. The default model is set to Fast Random Forest, with 100 trees. However, changing the model (e.g. normal Random Forest, Deep Neural) or increasing the number of trees are found to have little effects on the classifier accuracy.

-The training and classifying process, depending on your computer, takes 2-5 minutes per image.

-From the probability map, you can see which region of the eye is badly segmented, usually due to the lack of training data. Go back to the Manual Segmentation step to focus more on these regions.

# Hexagonal grid expansion

This step generates a hexagonal grid of ommatidia from the learned ommatidia probability map. This grid is spawned from the first 3 user-prompted adjacent ommatidia, which forms the origin and the axes for the grid. This grid will attempt to expand from this origin to detect as many ommatidia as possible (up to 1200).

1. Browse to Script/ folder via MATLAB.

2. Type MAIN\_expand\_hexagon(input\_file) in MATLAB command window. The input\_file is the full name of the input image (e.g. ‘img2.tif’).

3. The ommatidia probability map of the input image will be displayed. Click on the center of three adjacent ommatidia, preferable in the middle of the eye. Press **Enter**.

The program will spawn the hexagonal grid from the three ommatidia. The spawning process will be displayed in real time in the same figure panel and also saved into Script/tmp/ folder.

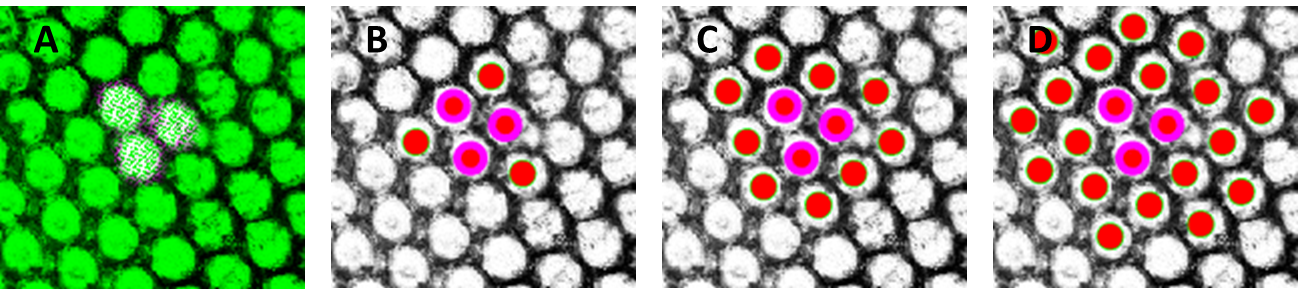


Figure 4. Expansion of hexagonal grid from (A) three manually added adjacent ommatidia. The grid is then expanded automatically layer by layer of ommatidia (B-D) and eventually covers the whole eye.

# Manual correction

As ommatidia at the eye’s edges are heavily tilted, it is difficult for the machine learning module to recognize them properly. Also, non-eye region is not defined, leading to over-spawning of ommatidia during the automatic hexagonal grid expansion. Therefore, a final manual correction is required.

1. Browse to Script/ folder via MATLAB

2. Type MAIN\_manual\_correction(input\_file) in MATLAB command window to run the manual correction GUI. The input\_file is the full name of the input image (e.g. ‘img2.tif’)

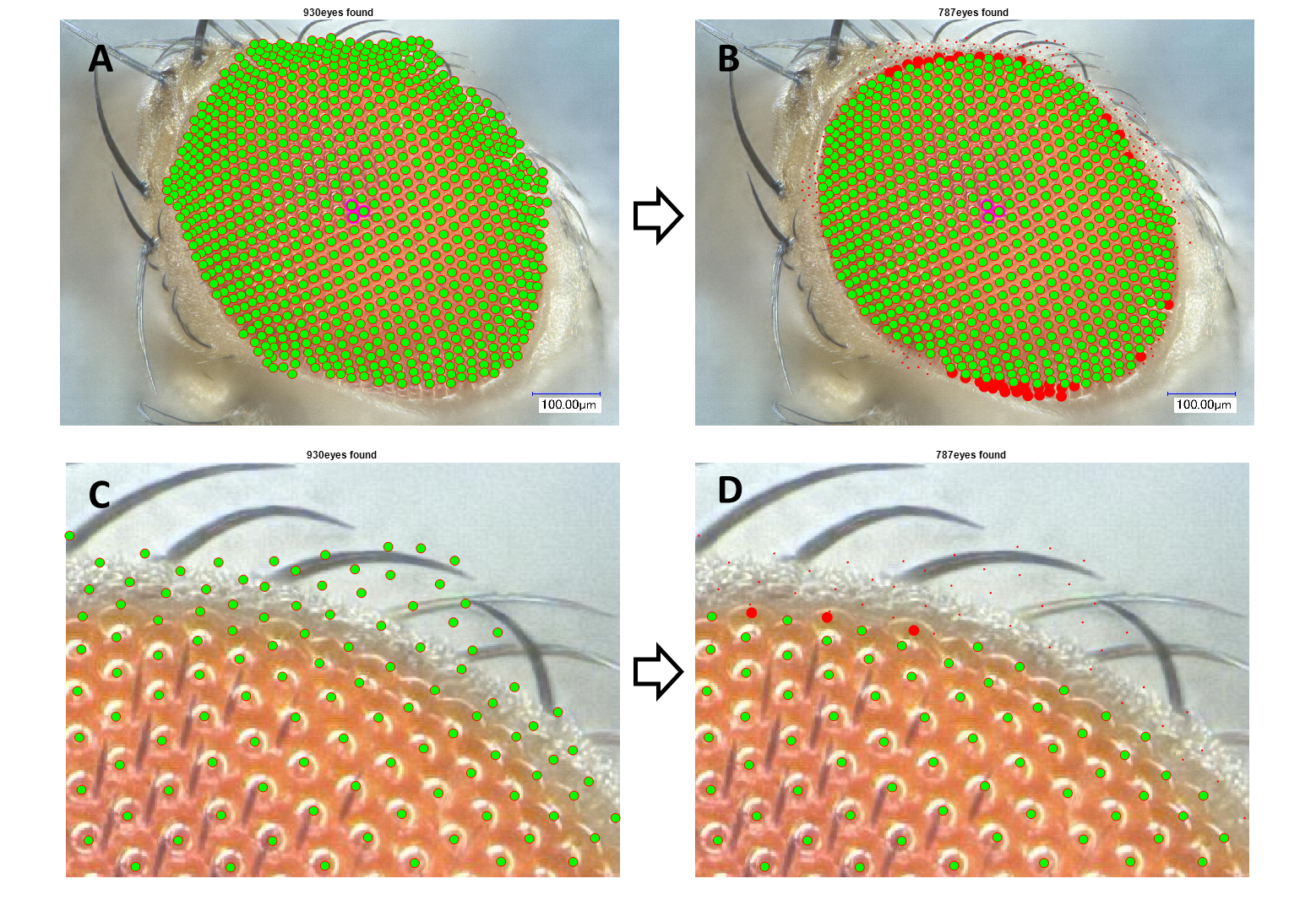


Figure 5. Manual correction after automatic ommatidia detection: (A,C) raw image overlaid with automatic ommatidia segmentation. (B-D) manually corrected ommatidia segmentation. Panel C-D is a zoom-in region of panel A-B. In (A-D), automatically detected ommatidia are shown as green circles, manually added ommatidia are shown as red circles and manually removed ommatidia are shown as red dots. The example image (img2.tif) is taken from a brightfield microscope.

The program will load the largest hexagonal grid spawned in the previous hexagonal grid expansion process. You can select a grid of specific size by pressing **Ctrl + Left/Right** arrow keys.

3. Add (press **A**) or remove (press **R**) ommatidia. You will focus mostly on the edges of the eye where errors might appear. You can save and load the correction progress by pressing **Ctrl + H** (to save progress) and **Ctrl + L** (to load progress). The ommatidia count will always be displayed on top of the image.

4. Press **Ctrl + E** to export the image label to data/label/ folder.

Note:

-Press F1 to access all the hotkeys (zoom in/out, save/load progress).

-Use right-click to zoom in and navigate around different image region.

-Press Q to zoom out to the original view.

# Exporting ommatidia’s coordinates and order of birth

Following the labeled image’s export, a new figure showing the original compound eye will appear. To rearrange detected ommatidia by the birth order of their respective progenitors (R8 specification), using the mouse, select two points on the newly created figure to specify the direction from the posterior to the anterior of the eye. The order of ommatidia progenitors’ birth will be calculated accordingly.

A Comma Separated Values (CSV) file specifying each ommatidium’s position in the Cartesian and Hexagonal coordinates and order of birth will be saved to data/csv/ folder.

# Label alignment

As the hexagonal grid expansion is performed based on the probability map, rather than the raw image, there might be some small misalignments between the exported label image and the raw image. If you want to know the exact ommatidia position, to extract features from individual ommatidia or to create new training data, you can manually realign the label image to match the raw image.

1. In the manual correction GUI, after the ommatidia has been manually segmented, press **Ctrl + I** to enter the alignment interface. The raw image overlaid with the label will be shown.

2. Select a few (~10) anchor points at the center and at the edges of the eye with hotkeys **A** (add) and **R** (remove).

3. Drag and drop the anchor points so that the boundary labels match with the boundary in the raw image.

4. Press **Enter**. The program will automatically generate the aligned labeled image and export it to data/label/ folder. You will also have an option to export it as training data for the machine learning module (similar to the first manual segmentation process).

# License:

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# Citing EyeHex:

Tran, Dostatni & Ramaekers, EyeHex toolbox for complete segmentation of ommatidia in fruit fly eyes, BioRXiv, 2024

# References:

[1] Arganda-Carreras, I.; Kaynig, V. & Rueden, C. et al. (2017), "Trainable Weka Segmentation: a machine learning tool for microscopy pixel classification.", Bioinformatics (Oxford Univ Press) 33 (15).

[2] Schindelin, J.; Arganda-Carreras, I. & Frise, E. et al. (2012), "Fiji: an open-source platform for biological-image analysis", Nature methods 9(7): 676-682.