Image analysis

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1 Introduction

We use `FIJI` to detect pupae, and a custom `FIJI` macro to measure pixel intensity inside the pupae over time. Before eclosion, flies in pupal cases are (or more acurately, they become) pigmented. After eclosion the pupal case is empty and becomes semi-transparent. Eclosion events will be detected based on the change in pixel intensity between time points. Here, I describe how to use `FIJI` and custom macros to analyse images.

2 Overview

1. Pre-process images
2. Identify pupae in one image ("reference") and save the position and shape of them as ROIs (Regions Of Interest).
3. Measure pixel intensity in ROIs over all time points.

3 Pre-requisites

- Install `FIJI` and `EclosionMonitorJ` macros on your computer (`fiji.pdf`).
- Have time-lapse images taken by the recording box in one folder (`eclosion_monitor.pdf`).

4 Pre-process images

We will apply a macro (`PrepJpg`) to pre-process all images. `FIJI` sometimes crashes while doing this if we have too many images with large size. To avoid this, we first resize all images.

4.1 Resize images

1. In a `UNIX`-based system (e.g. MPI Bio server using `PuTTY` from a `Windows` machine), navigate to your directory where you have a folder containing the raw images using `cd` command.

```
cd ~/projects/eclosion/rec_016_plate
ls
```

```
rec_016_2025-11-10-15-29-30
```

In this example, I am in directory `~/projects/eclosion/rec_016_plate`, within which there is a subdirectory `rec_016_2025-11-10-15-29-30`, where raw images are stored.

2. Make a folder for resized images.

```
mkdir resized
```

3. Run `convert` command to resize the image for each of all jpeg files in the directory.

```
for file in rec_016_2025-11-10-15-29-30/*jpeg
do
    echo $file
    prefix=`echo $file | sed 's@rec_016_2025-11-10-15-29-30/@@;s@.jpeg@@'`
    echo $prefix
    convert $file -resize 50% resized/${prefix}_resized.jpg
done
```

4. Once done, resized images are in the `resized` folder.

4.2 Run `PrepJpeg`

1. Make a new folder "for_analysis" to put pre-processed images.

```
mkdir for_analysis
```

2. Open `FIJI`.

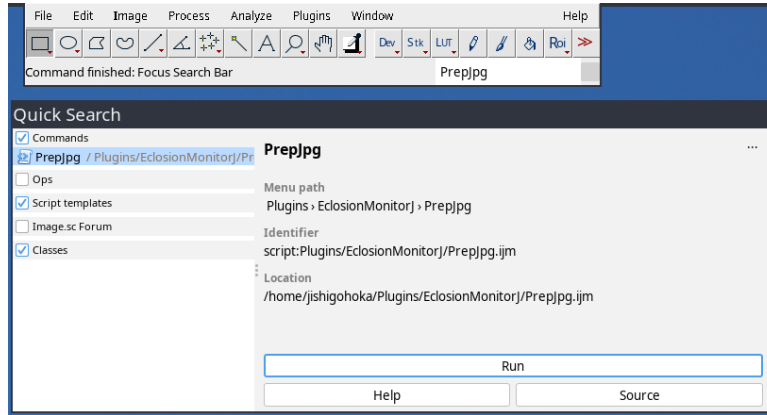3. In the search bar (or `Ctrl+L`) search "PrepJpg" (Fig. 1). Click "Run".

Figure 1: `PrepJpg`

4. A window "Choose Input Directory" will pop up. Select the `resized` folder and click "Select".

5. A new window asking you to specify the index of first JPG file will pop up.

   - If you want to pre-process all images in the `resized` folder, type 0.
   - If you want to pre-process images taken in a specific time period, identify the 0-based index of the first JPG (based on the dictionary order) and type this index in the box.

   Click "OK".

6. A new window asking you to specify the index of the last JPG file will pop up.

   - If you want to pre-process all images in the `resized` folder, type the number of JPG files - 1 (99 if there are 100 files).
   - If you want to pre-process images taken in a specific time period, identify the 0-based index of the last JPG and type this index in the box.

   Click "OK".

7. A new window "Choose Output Directory" will pop up. Select the `for_analysis` folder and click "Select".

8. `PrepJpg` will start running. It will make each image into 8-bit (i.e. 256 shades of gray), invert it (dark background with white pupae), and perform background subtraction (does not matter much for 96-well plates).

9. Once complete, a message window will pop up ("PrepJpg is completed") Pre-processed images are in the `for_analysis` folder.

4.3 Remove dark images

The recording box has two LED strips: white and red. White light is for "day" and is on 7:20 - 19:20 (UTC + 1). Red light for "night" and is on 19:20 - 7:20 (UTC + 1). These two LEDs are controlled by two separate analog timer switches, and the transition between the day and night is not perfect. In some boxes, there are periods of a few minutes when both lights are off, and when an image is taken during this period, completely dark image is recorded. This will affect later analysis.

To resolve this, manually remove dark images. In the `for_analysis` folder, check all images taken at 07:20 and 19:20, and if they are black, delete them.

5 Prepare reference

1. Make a new directory `ref`.

   ```
   mkdir ref
   ```

2. In the `for_analysis` folder, select one image before the first eclosion as a reference. Night time shortly before the first eclosion is recommended.

3. Copy this image to the `ref` folder.

   ```
   cp for_analysis/2025_11_10_15_30_resized.jpg ref/
   ```

4. Open this image in `FIJI`. (`Ctrl+O`).

5. To adjust threshold, press `Ctrl+Shift+T` (or Image > Adjust > Threshold. . .). Drag the top slider (low threshold) so that pupae are recognisable (Fig. 2).
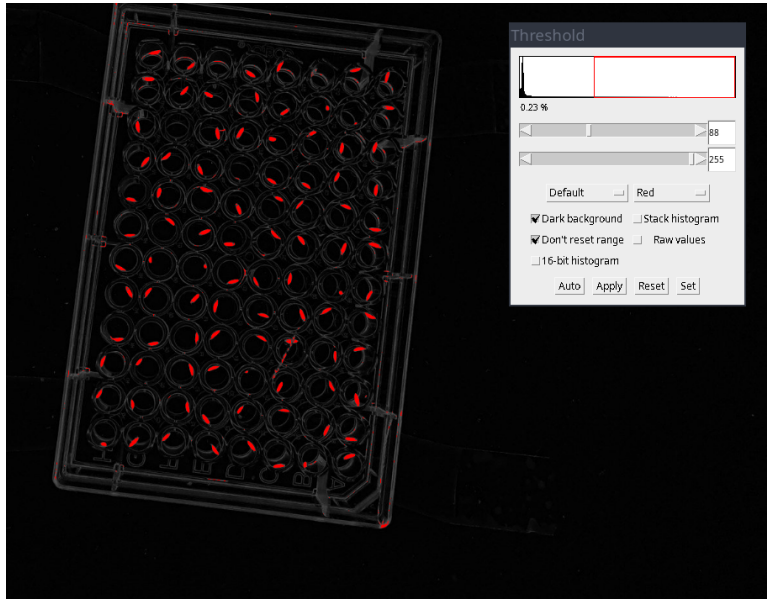
Figure 2: Threshold.

6. Click "Apply"

7. Save the thresholded image in `ref`. Click File > Save as, then save it as e.g. `2025_11_10_15_30_resized_threshold.jpg`

6 Detect pupae

1. In `FIJI`, open the threshold-adjusted reference image.

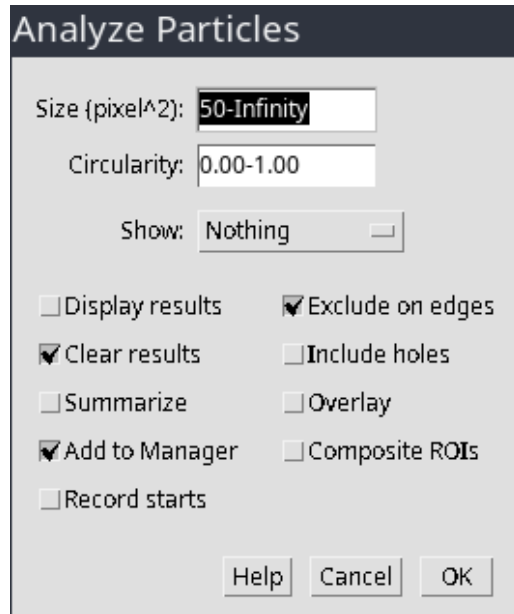2. To detect particles, Click Analyse > Analyze Particles. . . Set the values as in Fig. 3, then click "OK".

Figure 3: Analyze Particles.

3. ROI manager window will pop up and detected particles will be indicated as ROIs on the image (Fig. 4). They do not have to be perfect but have to be good enough.

- If there are too many small errors, close ROI manager (without saving), and run "Analyze Particles" again with a larger minimum value of the size parameter.
- If there are too many pupae undetected, eclose ROI manager (without saving), and run "Analyze Particles" again with a smaller minimum value of the size parameter.
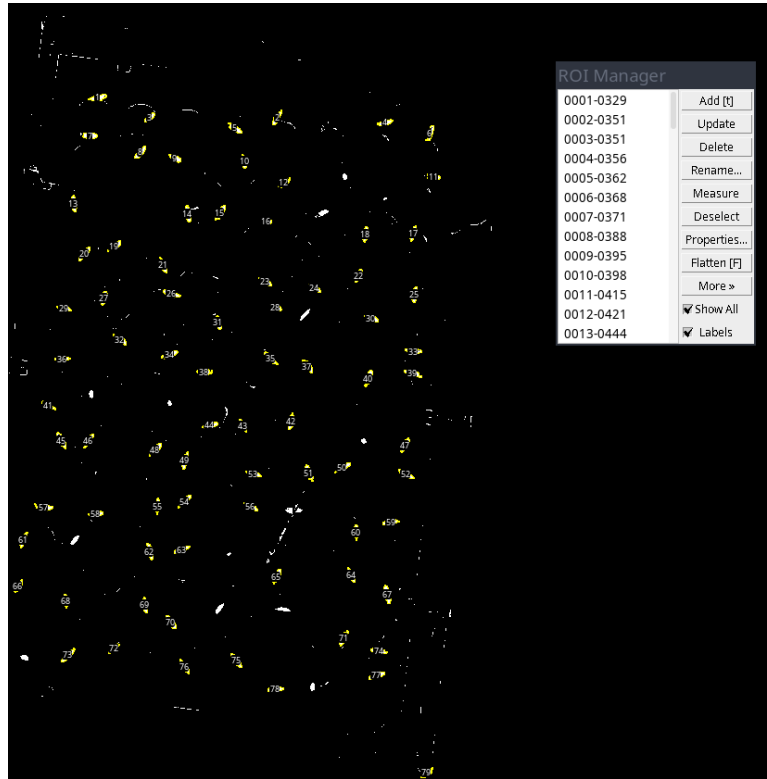
Figure 4: Pupae detected by the particle analyzer.

4. Save ROIs from the ROI Manager window by clicking More > Save As.

5. Close ROI Manager and the threshold-adjusted image.

6. Still in `FIJI`, open non-threshold-adjusted reference image and the ROI set.

   - In ROI Manager, make sure that both "Show All" and "Labels" are ticked.

7. Maually add and delete ROIs.

   - To add a ROI, use the freehand selection tool (Fig. 5), then press `T`.
   - To delete a ROI, click on the label of the ROI, and press `del`.

7
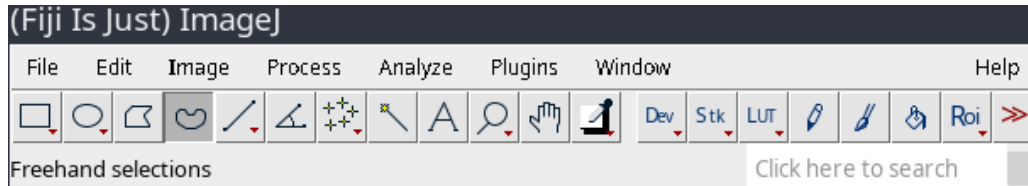
Figure 5: Freehand selection tool.

8. Save manually corrected ROIs from ROI Manager window by clicking More > Save As.

9. Close ROI Manager and image.

7 Measure pupae

1. In `FIJI`, search for the `MeasurePupae` macro in the search bar, and click "Run".
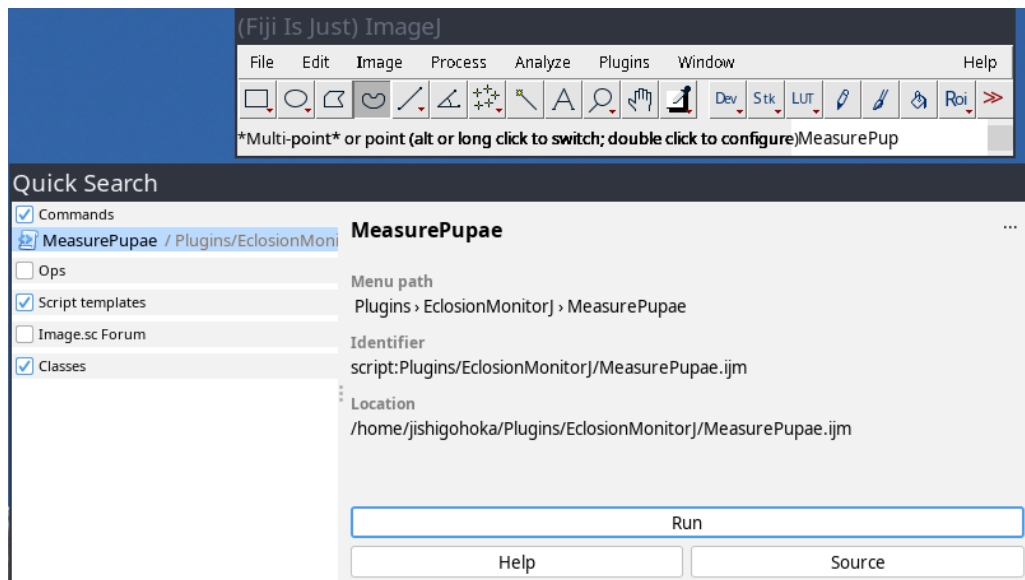
Figure 6: `MeasurePupae` macro.

2. A new window "Choose folder" will pop up. Choose the `for_analysis` folder where all processed images are stored, and click "Select".

3. A new window "Select ROI set" will pop up. Choose the manually corrected ROI set, and click "Open".

4. Analysis will start, with a new "Results" window open (Fig. 7). Wait until it is done.

	Label	Area	Mean	StdDev	Mode	Min	Max	Median	
33145	2025_11_13_02_40_resized.jpg:0738-1469	81	91.062	27.826	110	21	135	97	
33146	2025_11_13_02_40_resized.jpg:0730-1402	73	107.000	39.423	127	15	158	125	
33147	2025_11_13_02_40_resized.jpg:0884-1267	46	107.435	43.777	134	12	161	123	
33148	2025_11_13_02_40_resized.jpg:0808-1113	54	110.833	48.371	140	0	163	127	
33149	2025_11_13_02_40_resized.jpg:0774-1081	67	105.313	30.070	100	33	149	119	
33150	2025_11_13_02_40_resized.jpg:0788-1453	79	101.937	47.388	138	5	163	123	
33151	2025_11_13_02_40_resized.jpg:0856-1053	49	94.898	35.854	111	8	148	105	
33152	2025_11_13_02_40_resized.jpg:0735-1365	87	95.414	27.520	112	19	128	106	
33153	2025_11_13_02_40_resized.jpg:0573-1267	86	106.698	34.095	138	18	154	119	
33154	2025_11_13_02_40_resized.jpg:0424-1336	53	92.189	33.044	118	0	127	102	
33155	2025_11_13_02_40_resized.jpg:0897-1217	86	112.058	35.162	135	0	155	128	
33156	2025_11_13_02_40_resized.jpg:0736-1305	63	86.667	35.598	108	0	123	104	
33157	2025_11_13_02_40_resized.jpg:0535-1370	62	103.419	35.726	126	8	155	121	
33158	2025_11_13_02_40_resized.jpg:0701-1098	73	94.507	27.769	105	15	135	100	
33159	2025_11_13_02_40_resized.jpg:0927-1438	59	88.949	38.313	108	6	137	108	
33160	2025_11_13_02_40_resized.jpg:0613-1099	37	104.081	34.458	123	7	155	109	
33161	2025_11_13_02_40_resized.jpg:0512-1208	79	105.190	34.639	134	9	150	120	
33162	2025_11_13_02_40_resized.jpg:0771-1302	65	88.169	34.349	116	13	138	99	
33163	2025_11_13_02_40_resized.jpg:0662-1083	61	103.131	39.995	105	0	149	121	
33164	2025_11_13_02_40_resized.jpg:0818-1227	62	103.790	29.653	126	31	139	117	
33165	2025_11_13_02_40_resized.jpg:0591-1162	69	112.406	32.233	131	27	160	122	
33166	2025_11_13_02_40_resized.jpg:0938-1287	79	121.835	38.302	140	17	167	139	
33167	2025_11_13_02_40_resized.jpg:0611-1322	69	98.159	31.761	102	4	140	105	

Figure 7: `MeasurePupae` running.

5. Once completed, a new window saying "Measuring Pupae completed" will pop up.

6. Confirm that results.csv has been created in `for_analysis` folder (Fig. 8).

Figure 8: results.csv

8 Prepare the position information of pupae

We will need to match the ROIs representing pupae to the well of the plate.

8.1 Plate

1. In FIJI, open the reference image.

2. Using the multi-point tool, mark the centre of each well following A1, B1, C1, . . . , H11, H12 (Fig. 9).
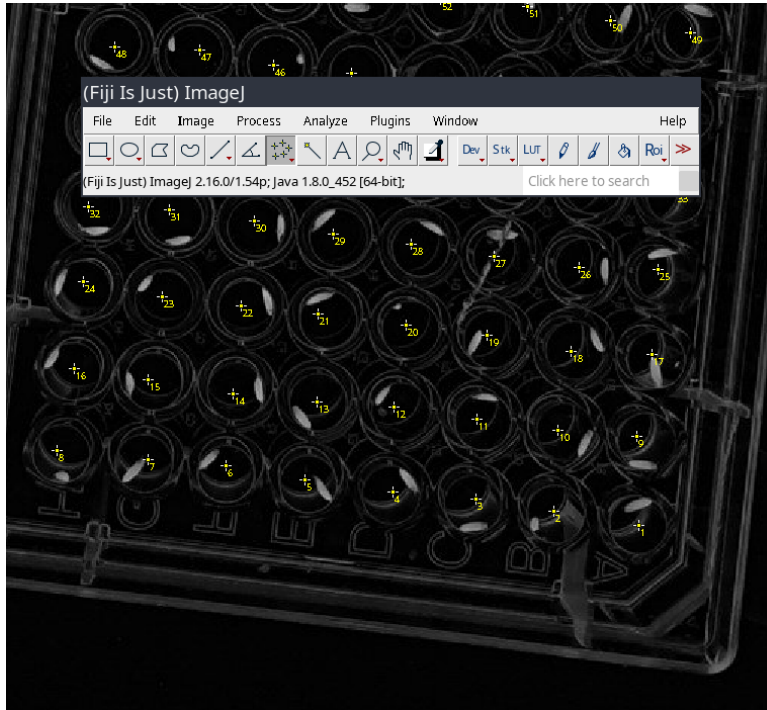
Figure 9: Multi-point tool to mark wells.

3. To save the points

   1. Click Analayze > Tools > ROI Manager
   2. Click Add in ROI manager. The added ROI contains all points you added.
   3. Click in ROI Manager, More > Save... and save the points.

4. To measure the coordinate of these 96 points,

   1. Click Analyze > Set Measurements...
   2. Tick "centroid" and "Display label" (Fig. 10), and click OK.
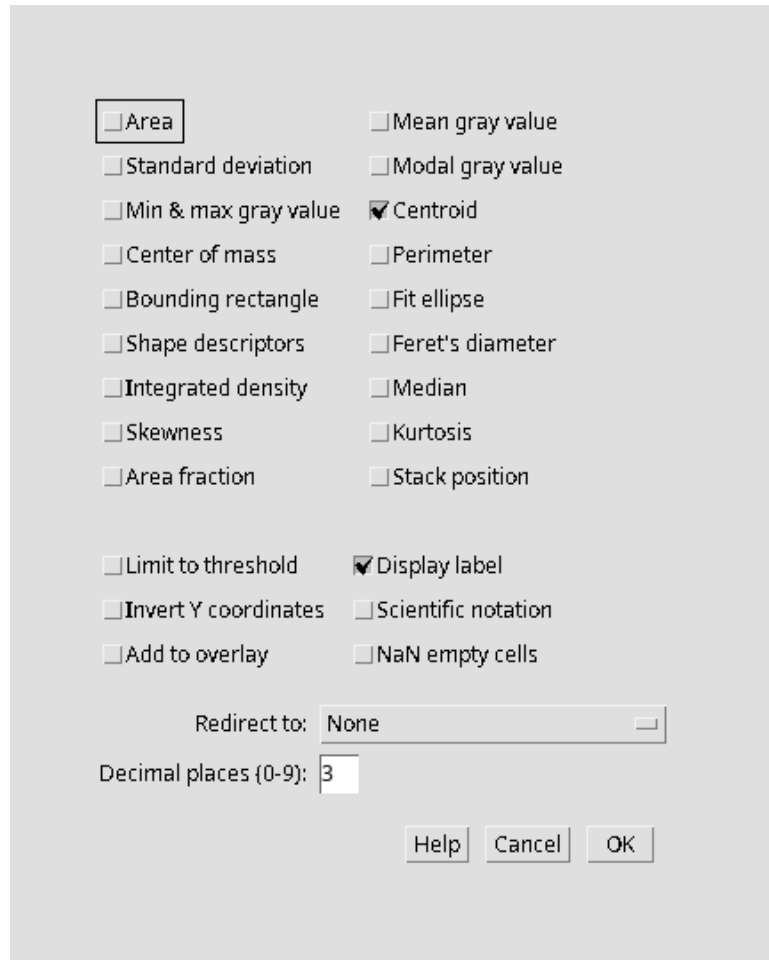
Figure 10: Set Measurements...

1. Measure the coordinates by clicking Analyze > Measure.
2. A Results window will open (Fig. 11)

Figure 11: Results window

1. Click File > Save As... then save it as a csv.

8.2 Pupae

1. In `FIJI`, open the reference image and the ROI set for pupae.
2. To measure the coordinate of pupae
    1. Click Analyze > Set Measurements...
    2. Tick "centroid" and "Display label", and click OK.
    3. Measure the coordinates by clicking Analyze > Measure.
    4. A Results window will open
    5. Click File > Save As... then save it as a csv.

9 What's next?

- `eclosion_detection.pdf`