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".

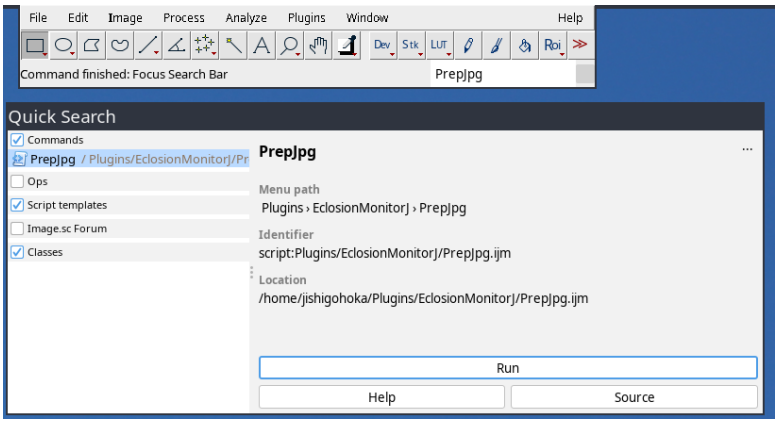

Figure 1: `PrepJpg`

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).

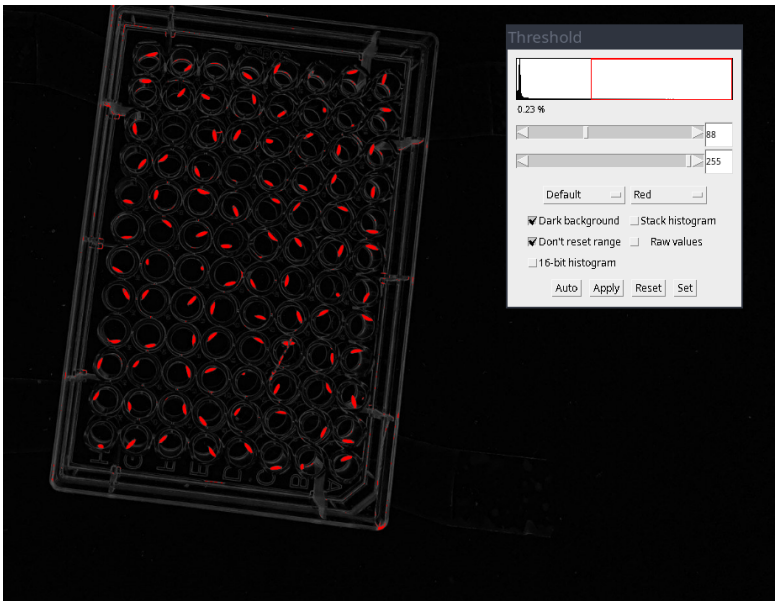

Figure 2: Threshold.

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”.

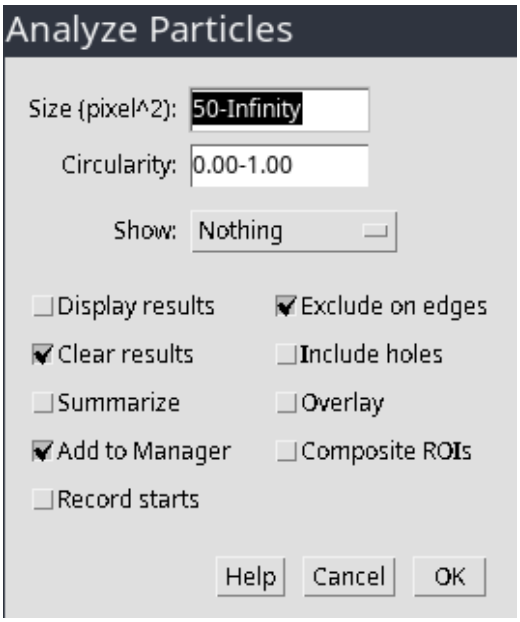

Figure 3: Analyze Particles.

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.

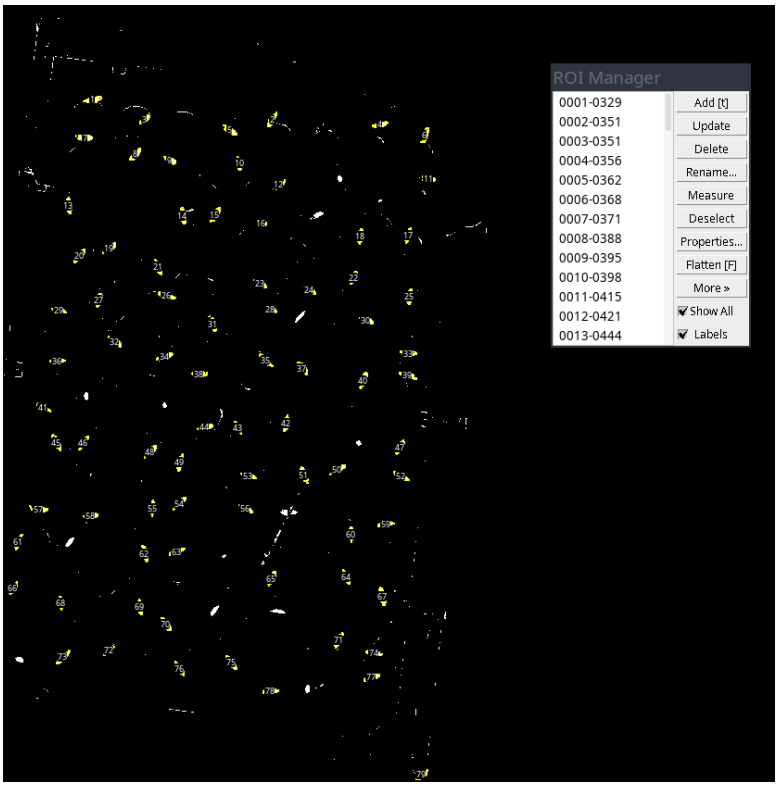

Figure 4: Pupae detected by the particle analyzer.

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` .

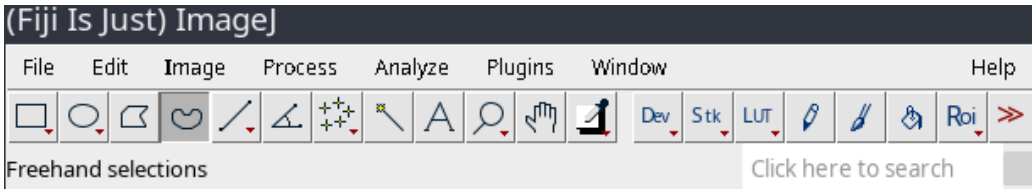

Figure 5: Freehand selection tool.

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".

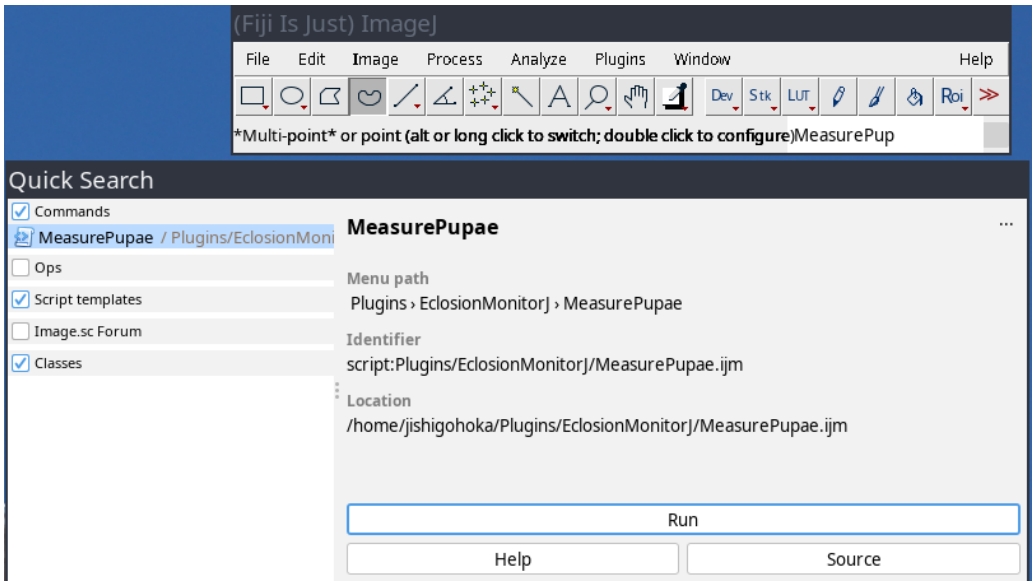

Figure 6: `MeasurePupae` macro.

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.

Results

File Edit Font Results

	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).

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	A	B	C	D	E	F	G	H	I
1		Label	Area	Mean	StdDev	Mode	Min	Max	Median
2	1	2025_11_10_15_30_resized.jpg:0361-1384	69	60.174	25.273	71	0	97	68
3	2	2025_11_10_15_30_resized.jpg:0455-1269	90	104.133	41.196	0	0	163	118
4	3	2025_11_10_15_30_resized.jpg:0634-1429	89	102.247	30.163	105	0	148	110
5	4	2025_11_10_15_30_resized.jpg:0372-1129	82	96.72	40.863	132	0	145	110
6	5	2025_11_10_15_30_resized.jpg:0547-1144	86	106.988	48.002	0	0	186	121
7	6	2025_11_10_15_30_resized.jpg:0479-1477	70	92.971	25.799	103	3	121	103
8	7	2025_11_10_15_30_resized.jpg:0723-1231	87	125.092	39.128	149	10	167	142
9	8	2025_11_10_15_30_resized.jpg:0445-1112	84	117.167	35.382	124	0	158	127
10	9	2025_11_10_15_30_resized.jpg:0331-1139	90	91.989	37.422	109	0	145	106
11	10	2025_11_10_15_30_resized.jpg:0544-1479	82	106.598	32.121	105	0	143	115
12	11	2025_11_10_15_30_resized.jpg:0629-1476	94	92.17	48.076	128	0	155	116
13	12	2025_11_10_15_30_resized.jpg:0369-1497	75	87.453	31.956	94	0	134	96
14	13	2025_11_10_15_30_resized.jpg:0932-1105	93	114.935	32.74	115	12	163	125
15	14	2025_11_10_15_30_resized.jpg:0780-1136	70	121.171	37.889	144	14	172	134
16	15	2025_11_10_15_30_resized.jpg:0924-1155	65	113.292	35.285	124	0	163	124
17	16	2025_11_10_15_30_resized.jpg:0608-1215	92	104.565	46.614	0	0	162	123
18	17	2025_11_10_15_30_resized.jpg:0491-1157	83	105.205	52.983	0	0	164	127
19	18	2025_11_10_15_30_resized.jpg:0702-1425	38	84.895	55.809	4	0	162	108
20	19	2025_11_10_15_30_resized.jpg:0465-1319	59	98.932	51.926	0	0	161	126
21	20	2025_11_10_15_30_resized.jpg:0627-1252	87	98.851	49.227	0	0	153	118
22	21	2025_11_10_15_30_resized.jpg:0849-1329	65	105.2	44.024	142	0	173	117
23	22	2025_11_10_15_30_resized.jpg:0353-1331	77	98.208	40.781	115	0	145	115

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).

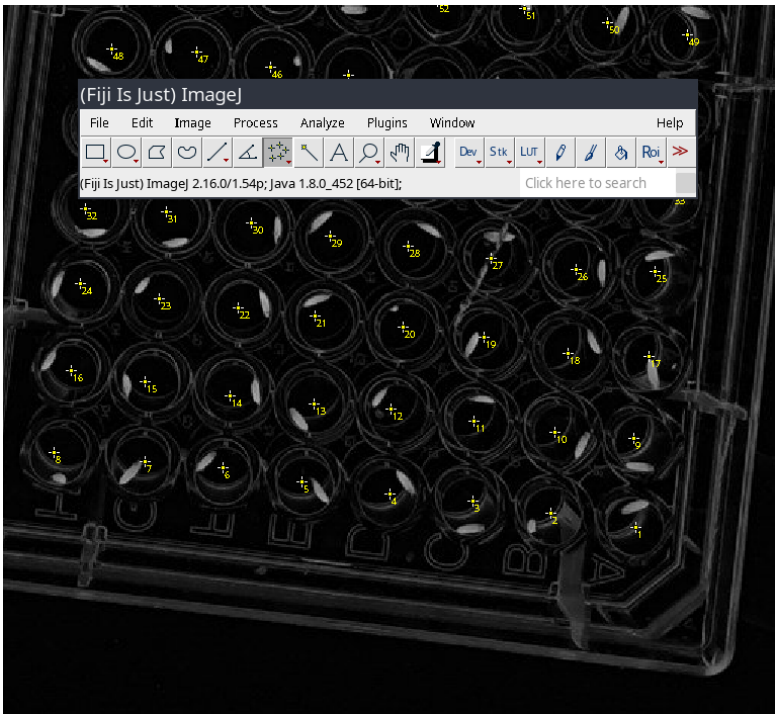


Figure 9: Multi-point tool to mark wells.

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.

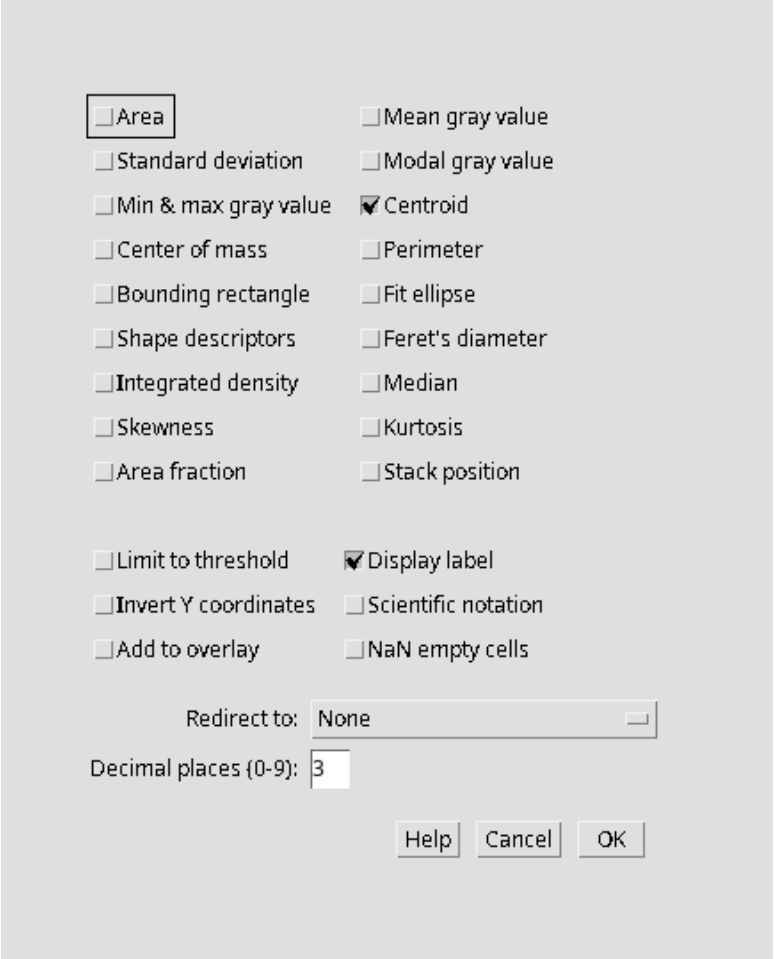
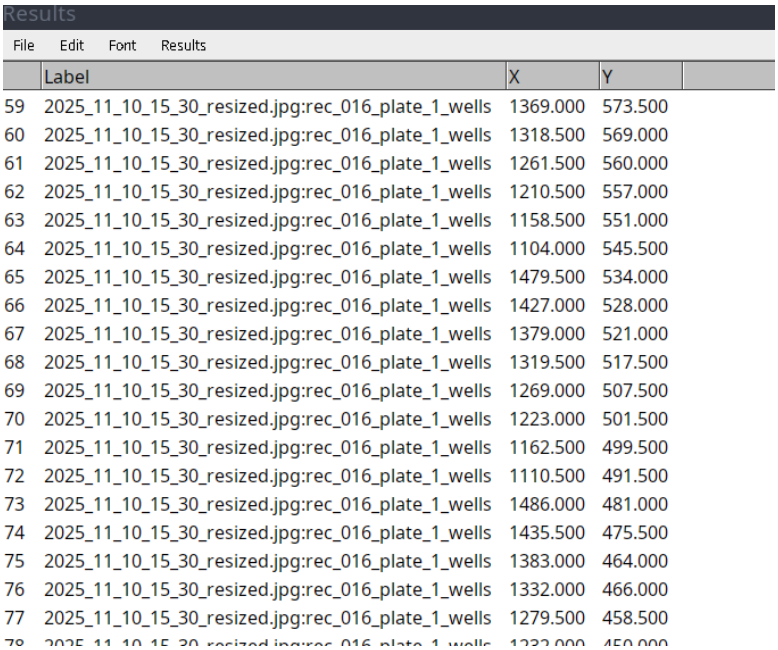


Figure 10: Set Measurements...

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



Results

File Edit Font Results

	Label	X	Y
59	2025_11_10_15_30_resized.jpg:rec_016_plate_1_wells	1369.000	573.500
60	2025_11_10_15_30_resized.jpg:rec_016_plate_1_wells	1318.500	569.000
61	2025_11_10_15_30_resized.jpg:rec_016_plate_1_wells	1261.500	560.000
62	2025_11_10_15_30_resized.jpg:rec_016_plate_1_wells	1210.500	557.000
63	2025_11_10_15_30_resized.jpg:rec_016_plate_1_wells	1158.500	551.000
64	2025_11_10_15_30_resized.jpg:rec_016_plate_1_wells	1104.000	545.500
65	2025_11_10_15_30_resized.jpg:rec_016_plate_1_wells	1479.500	534.000
66	2025_11_10_15_30_resized.jpg:rec_016_plate_1_wells	1427.000	528.000
67	2025_11_10_15_30_resized.jpg:rec_016_plate_1_wells	1379.000	521.000
68	2025_11_10_15_30_resized.jpg:rec_016_plate_1_wells	1319.500	517.500
69	2025_11_10_15_30_resized.jpg:rec_016_plate_1_wells	1269.000	507.500
70	2025_11_10_15_30_resized.jpg:rec_016_plate_1_wells	1223.000	501.500
71	2025_11_10_15_30_resized.jpg:rec_016_plate_1_wells	1162.500	499.500
72	2025_11_10_15_30_resized.jpg:rec_016_plate_1_wells	1110.500	491.500
73	2025_11_10_15_30_resized.jpg:rec_016_plate_1_wells	1486.000	481.000
74	2025_11_10_15_30_resized.jpg:rec_016_plate_1_wells	1435.500	475.500
75	2025_11_10_15_30_resized.jpg:rec_016_plate_1_wells	1383.000	464.000
76	2025_11_10_15_30_resized.jpg:rec_016_plate_1_wells	1332.000	466.000
77	2025_11_10_15_30_resized.jpg:rec_016_plate_1_wells	1279.500	458.500

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`