Measurement of Multiple Cardiac Performance Endpoints in Daphnia and Zebrafish by Kymograph

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Introduction of kymograph

- A 2D plot of time versus space created from time-lapse images (videos)
- Display actual velocity of the time-lapse
- Usually used for microtubule movement, heartbeat, etc (observing motility)

The analysis pipeline of this study

Image process

Video tapping **@ 200 fps** for heartbeat

Video format conversion to **avi format**

Video stabilization using **Image Stabilizer** plugin (optional)

Information extraction

Use Kymograph plugin in ImageJ to analyze **cardiac rhythm**

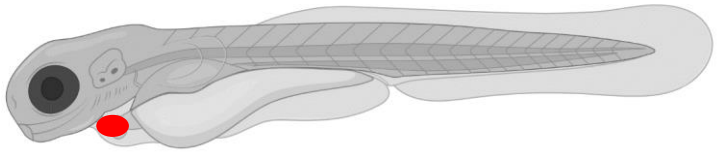
Result plotting, smoothening, and interval detection in ImageJ using **BAR Plugin**

Data calculation using **Microsoft Excel 2016**

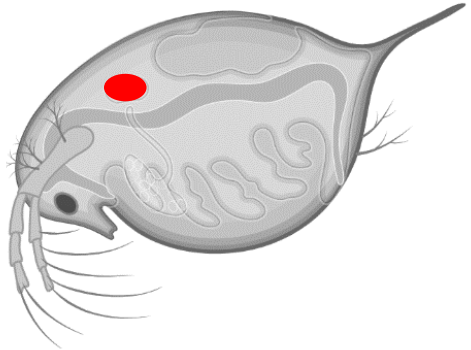
Obtainable Parameters

- **Heart rate**
- **Heartbeat interval**
- **Fractional Shortening**
- **Ejection Fraction**
- **Stroke Volume**
- **Cardiac Output**
- **Heartbeat regularity**

Zebrafish



Daphnia

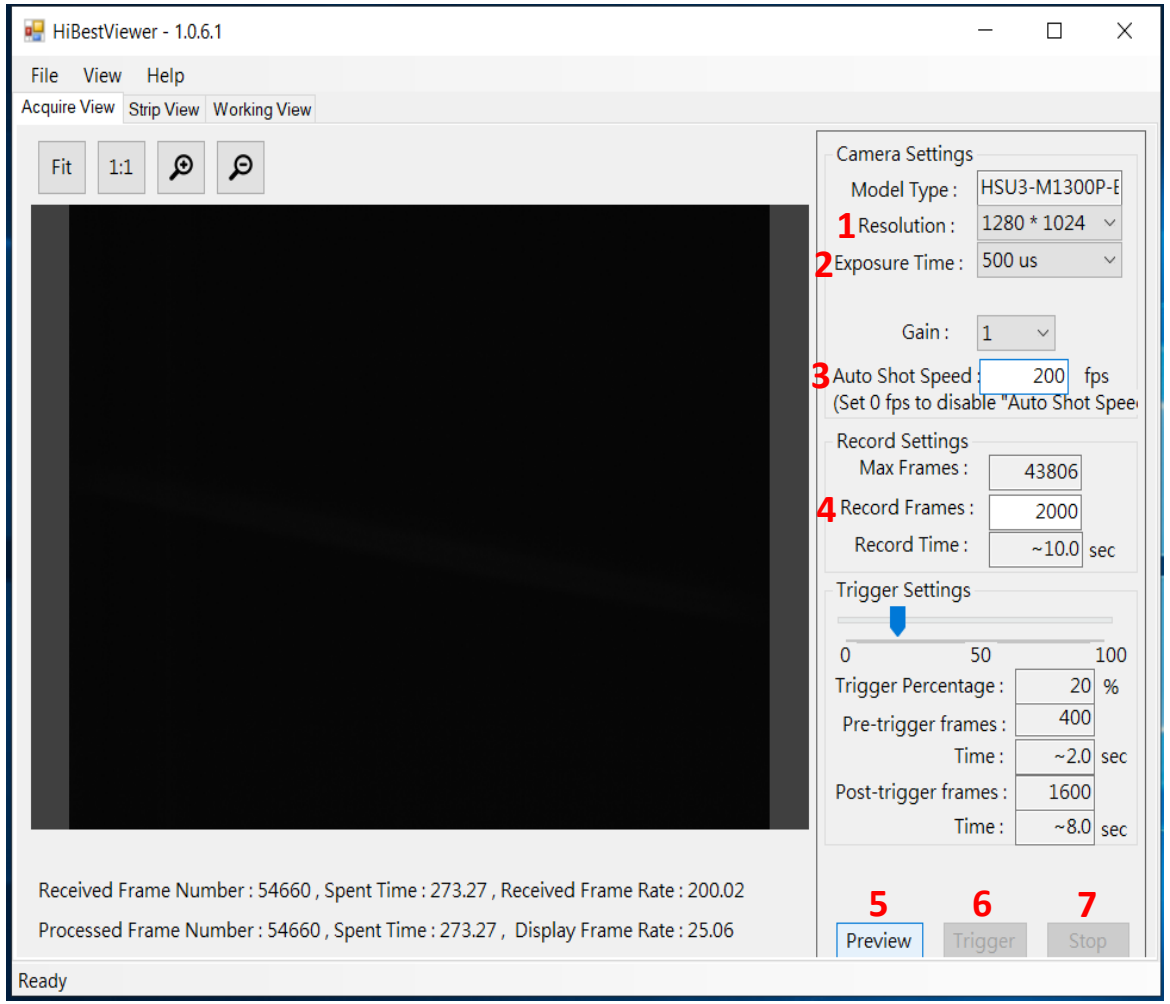


Video recording

- Video recording was done using high speed CCD
- Video recording that can be used for kymograph can be obtained from inverted microscope using HiBestViewer Application (AZ Instrument, Taiwan)

Video Recording using HiBestViewer Application

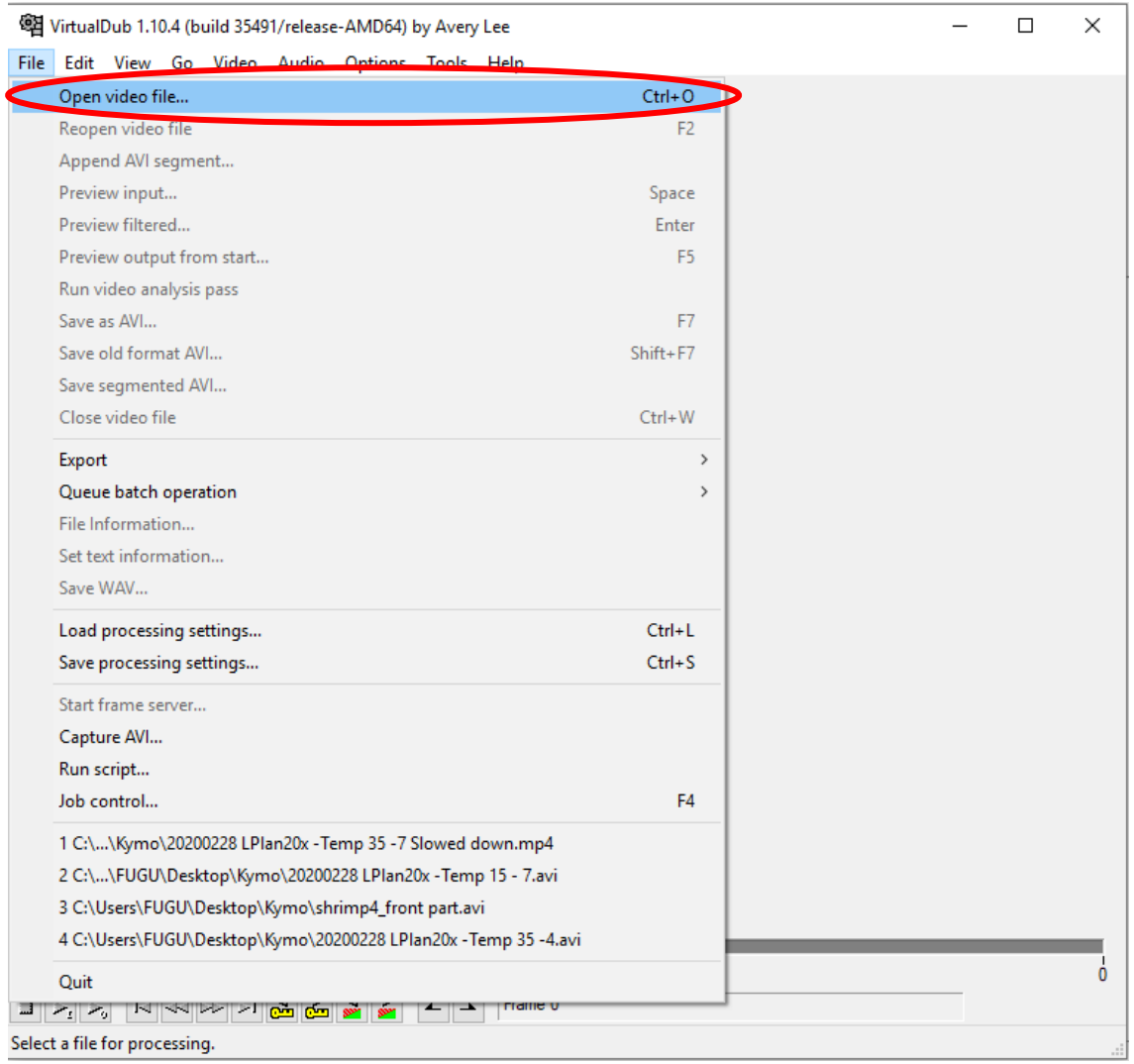
(AZ Instrument, Taiwan)

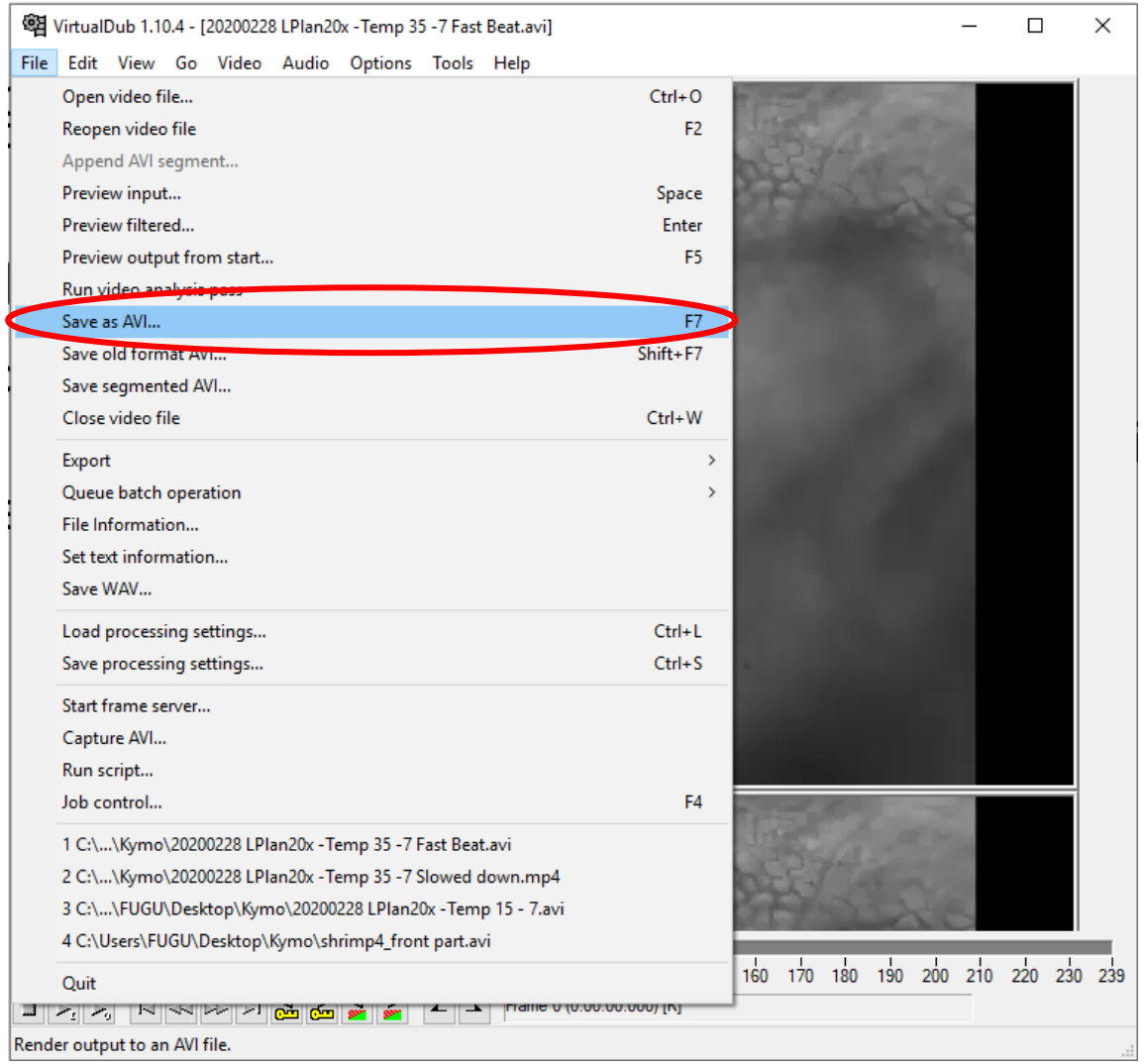


1. **Resolution :** Size of the video taken with a linear correlation to sharpness and clarity.
2. **Exposure time :** duration of time in which the sensor within the camera exposed to light
3. **Auto Shot Speed :** number of frames taken in one second for the recording (fps)
4. **Record Frame :** Number of frame set manually by the user to determine the recording length. The final duration of the video is presented in **Record Time**. This number cannot exceed the **Max Frames** or **0**.
5. **Preview :** Start viewing the current condition of the object of interest under the microscope.
6. **Trigger :** Start recording after preview
7. **Stop :** Stop the recording or the viewing.

Video conversion using VirtualDub

.mp4 video must be converted to .avi to be recognized by ImageJ



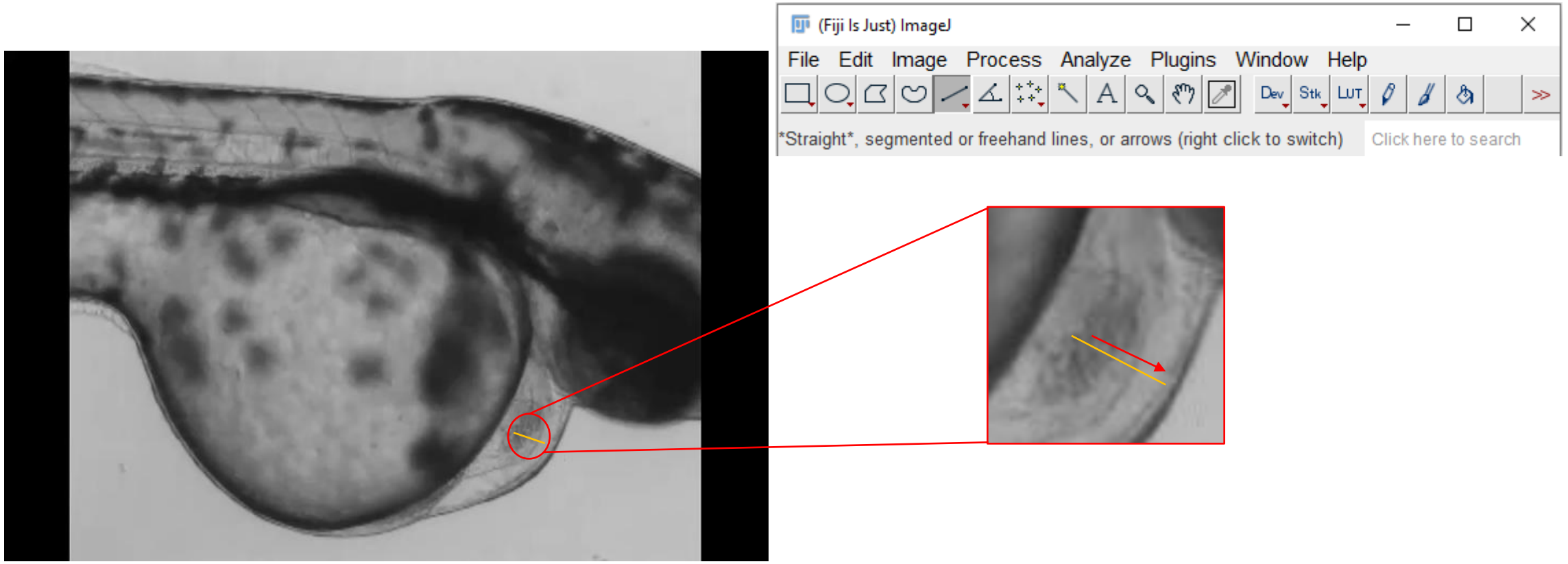


Outline

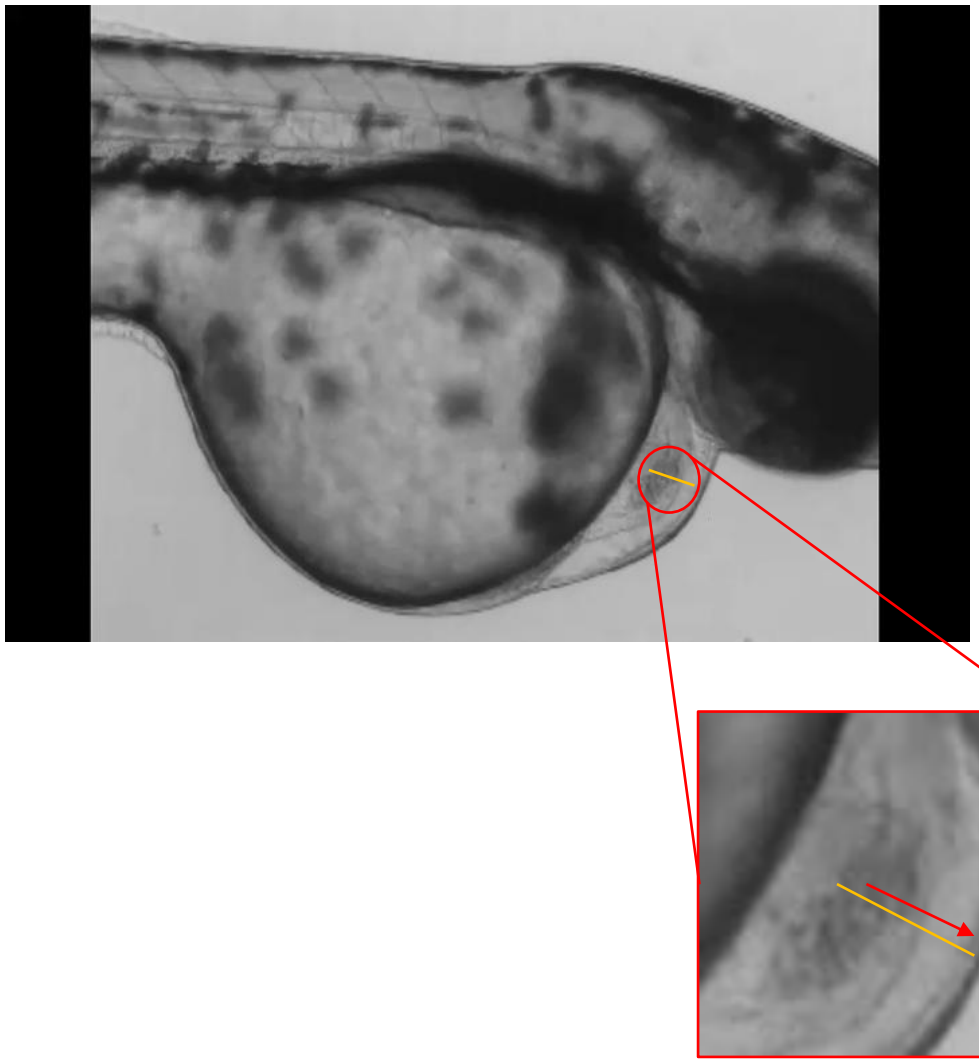
- Kymograph generation using ImageJ  and Zebrafish Heartbeat
- Image Stabilizer (Optional)
- Data Collection using **BAR Plugin**
- Data Calculation in Microsoft Excel
- Included macro

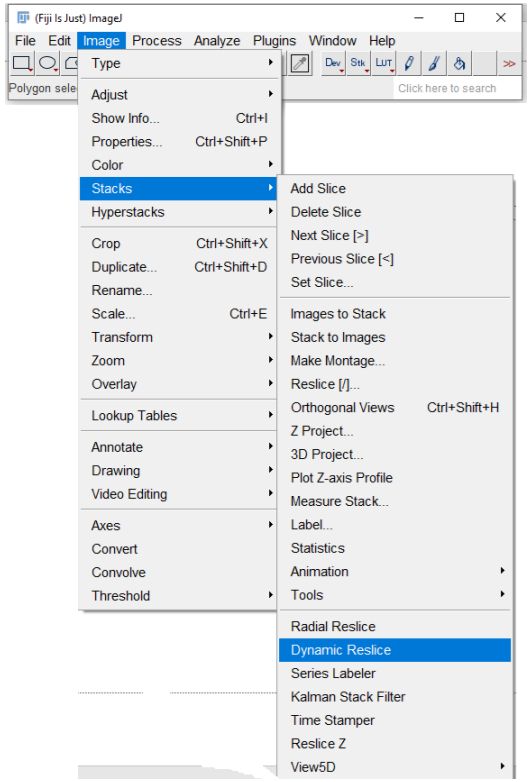
Kymograph creation

Manually determine and draw a line from the center to the outermost location of Region of Interest (ROI).
(We use zebrafish heart as the ROI for this example)

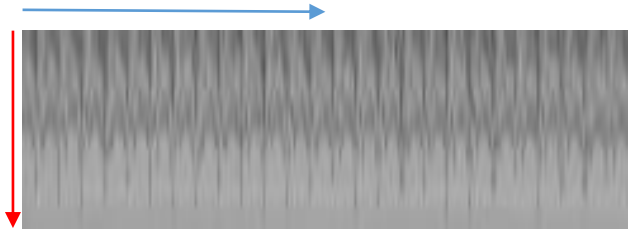


- Use Dynamic Reslice *(Image > Stacks > Dynamic Reslice > Rotate 90 degrees)* to create Kymograph





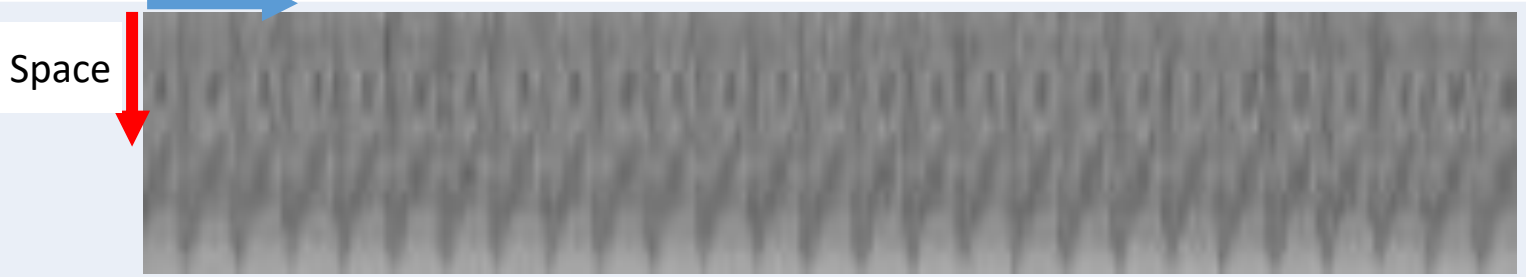
Resulting Kymograph



Time (Frame)

Space

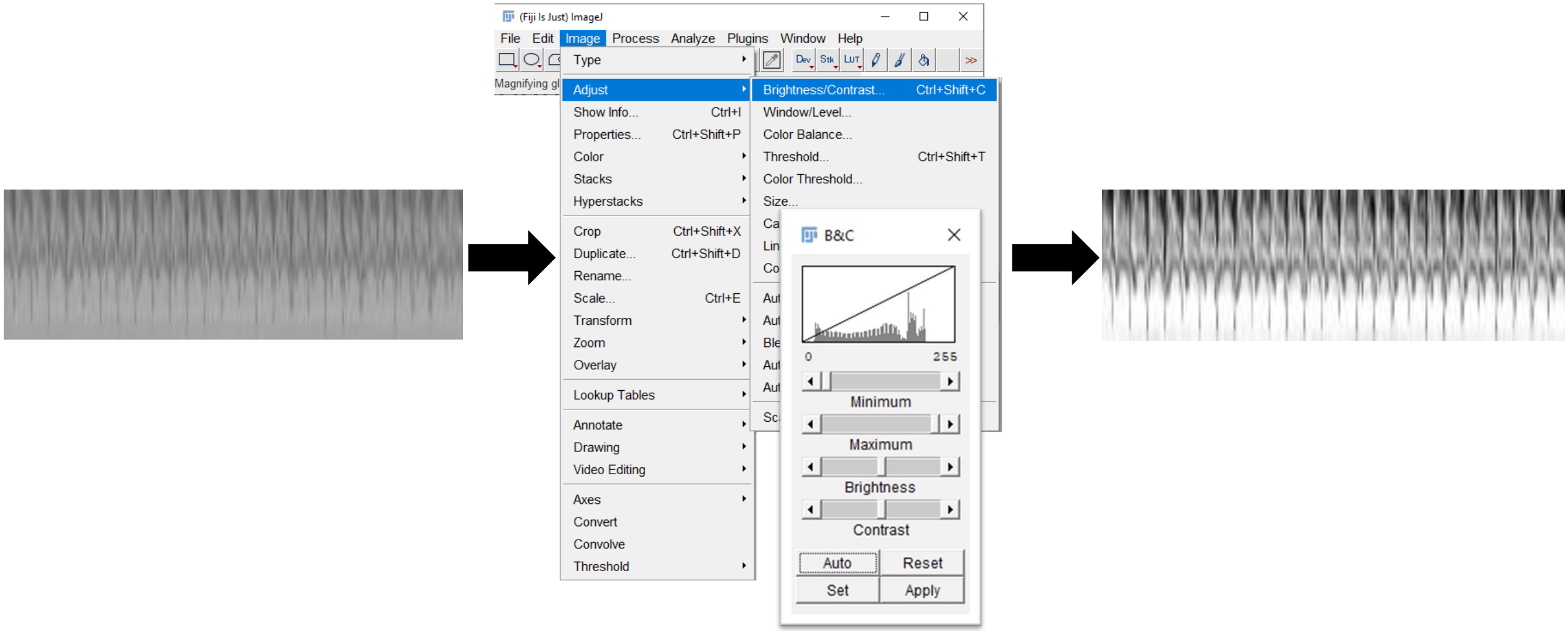
Result example

Location	Kymograph
Atrium	
Ventricle	

Time: Frame of the Video
Space: Distance of the Line

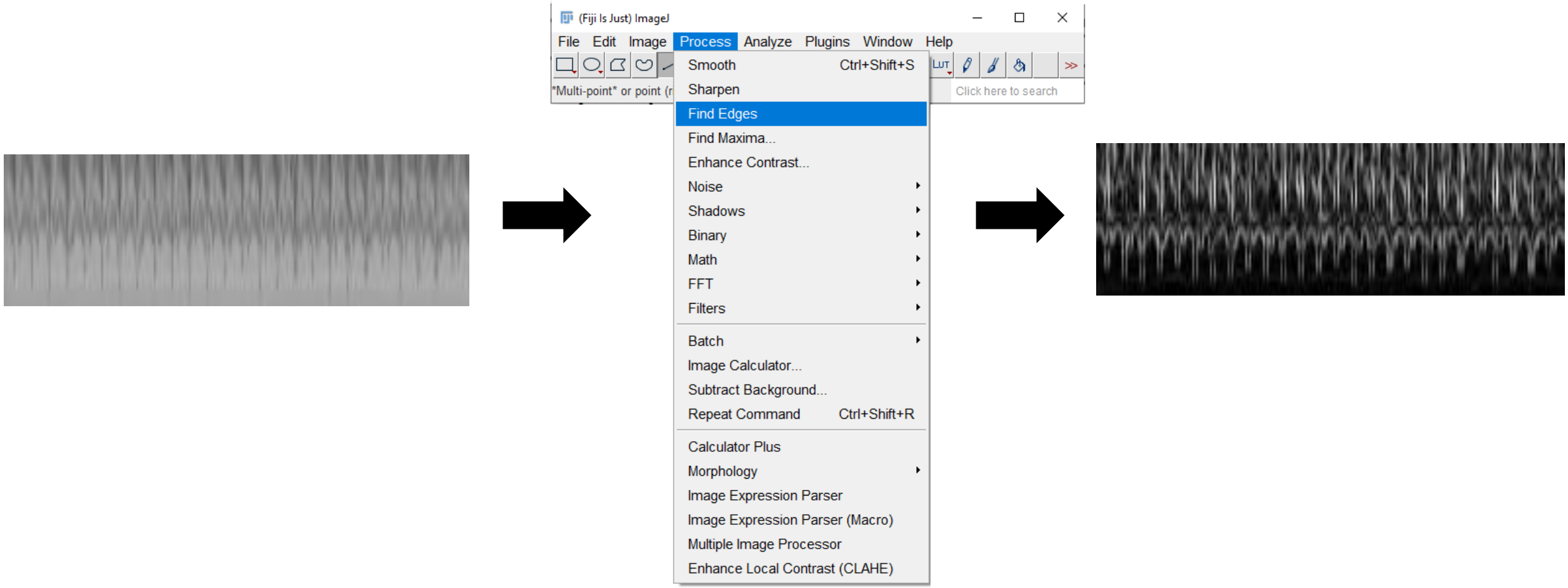
Editing

Resulting kymograph image contrast can be edited by using Brightness/Contrast (*Image > Adjust > Brightness/Contrast*)

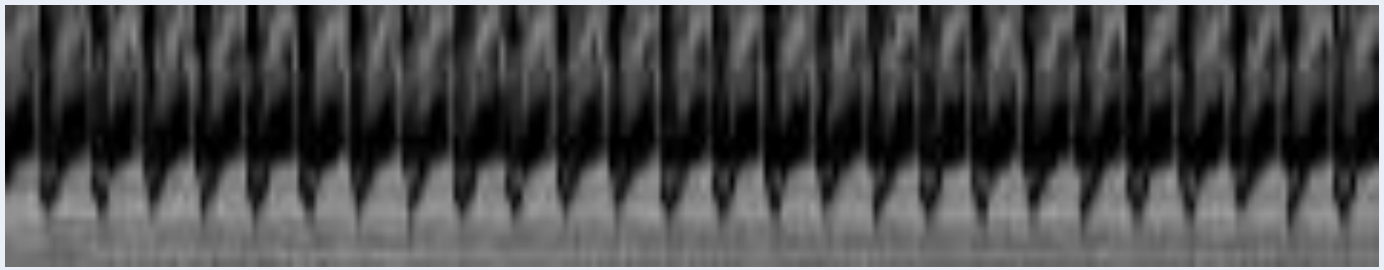


Editing cont.

Or by using Find Edges *(Process > Find Edges)*



Edited Result example

Location	Kymograph
Atrium	
Ventricle	

Time: Frame of the Video
Space: Distance of the Line

Image Stabilizer

- Image Stabilizer plugin is used for stabilizing ROI movement in the video.
- This process is optional as not all video has moving ROI that might interfere with the data collection.
- The Image was processed using Lucas-Kanade Algorithm
- The plugin can be downloaded from http://www.cs.cmu.edu/~kangli/code/Image_Stabilizer.html[1]

[1]K. Li, "The image stabilizer plugin for ImageJ," http://www.cs.cmu.edu/~kangli/code/Image_Stabilizer.html, February, 2008.

Image Stabilizer

- Open the video in ImageJ without ticking the Virtual Stack box in order to keep the video for use.

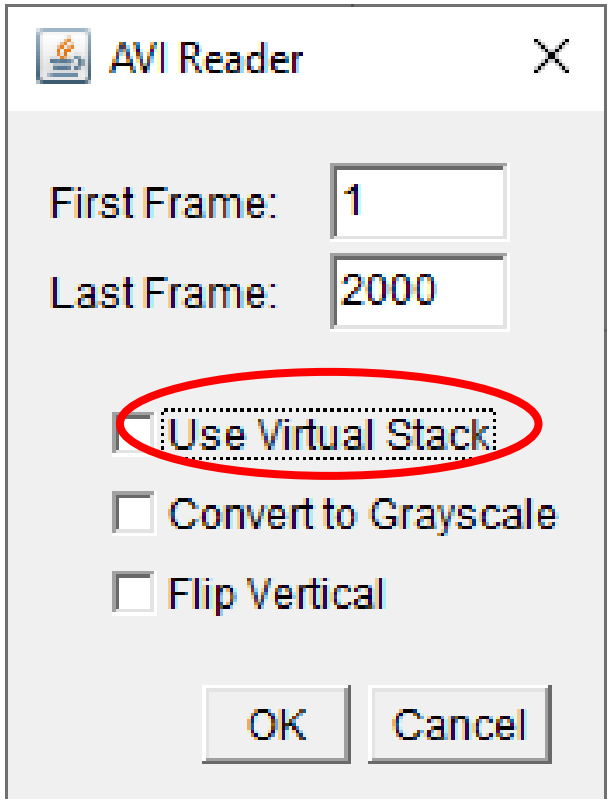


Image Stabilizer

- Run the Image Stabilizer Plugin (Plugins > Image Stabilizer).
- Move the video slider to the middle of the video duration, because the algorithm will stabilize the video using the selected frame as a template.

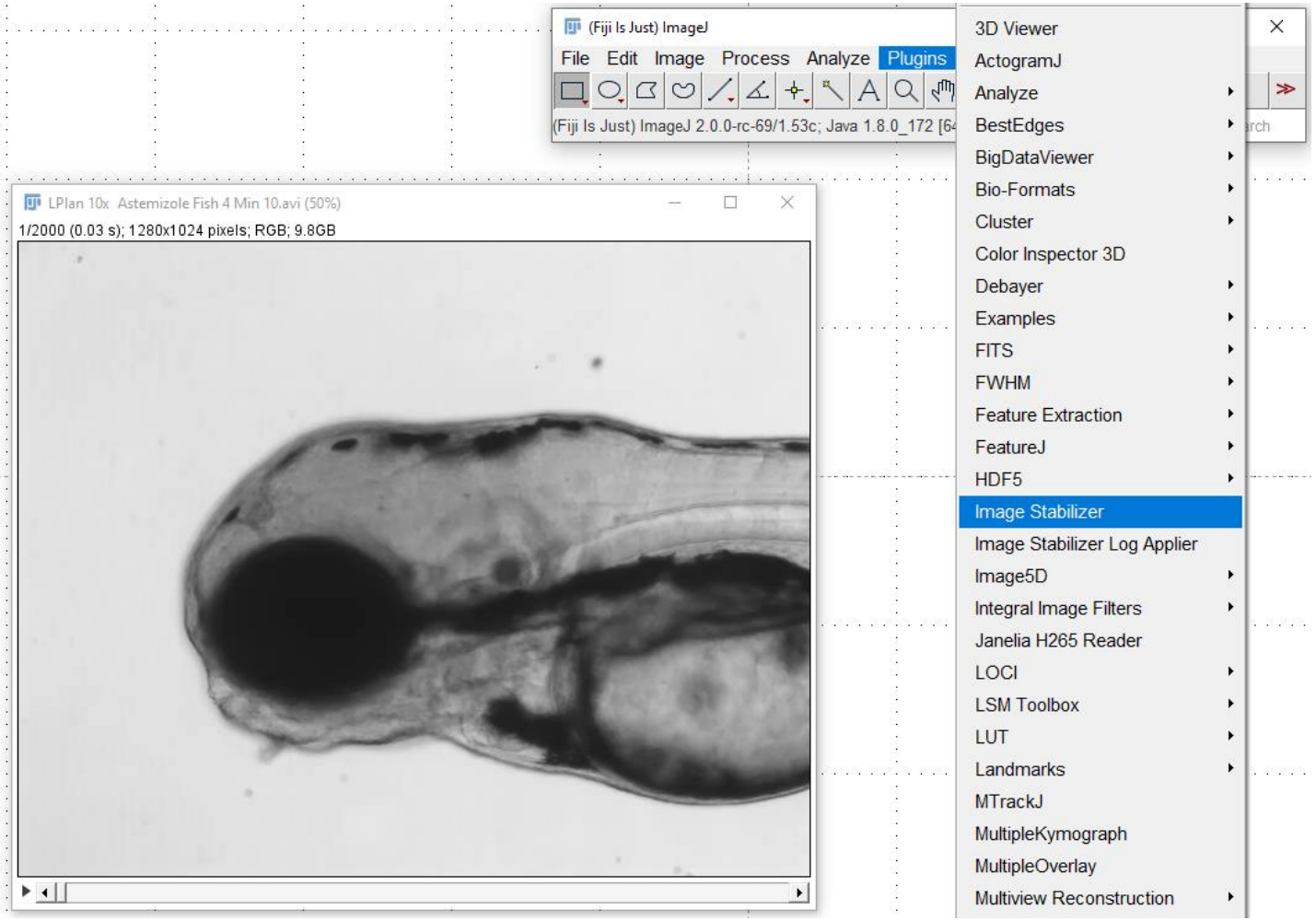
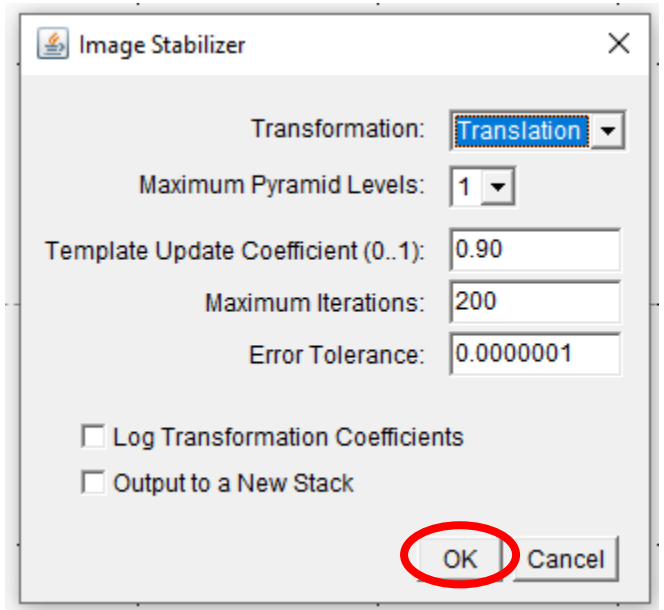


Image Stabilizer

- Popup window will open for Image Stabilizer settings. However we usually just left the settings as is, then click OK.
- Then wait for the stabilizing process to finish.
- Stabilizing process can be repeated until desired condition is reached.



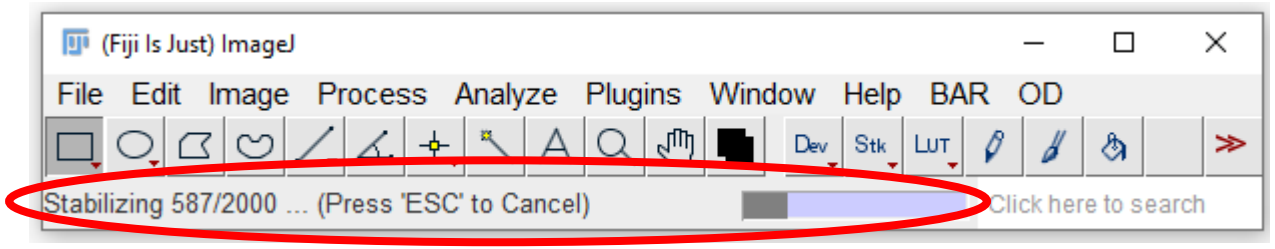
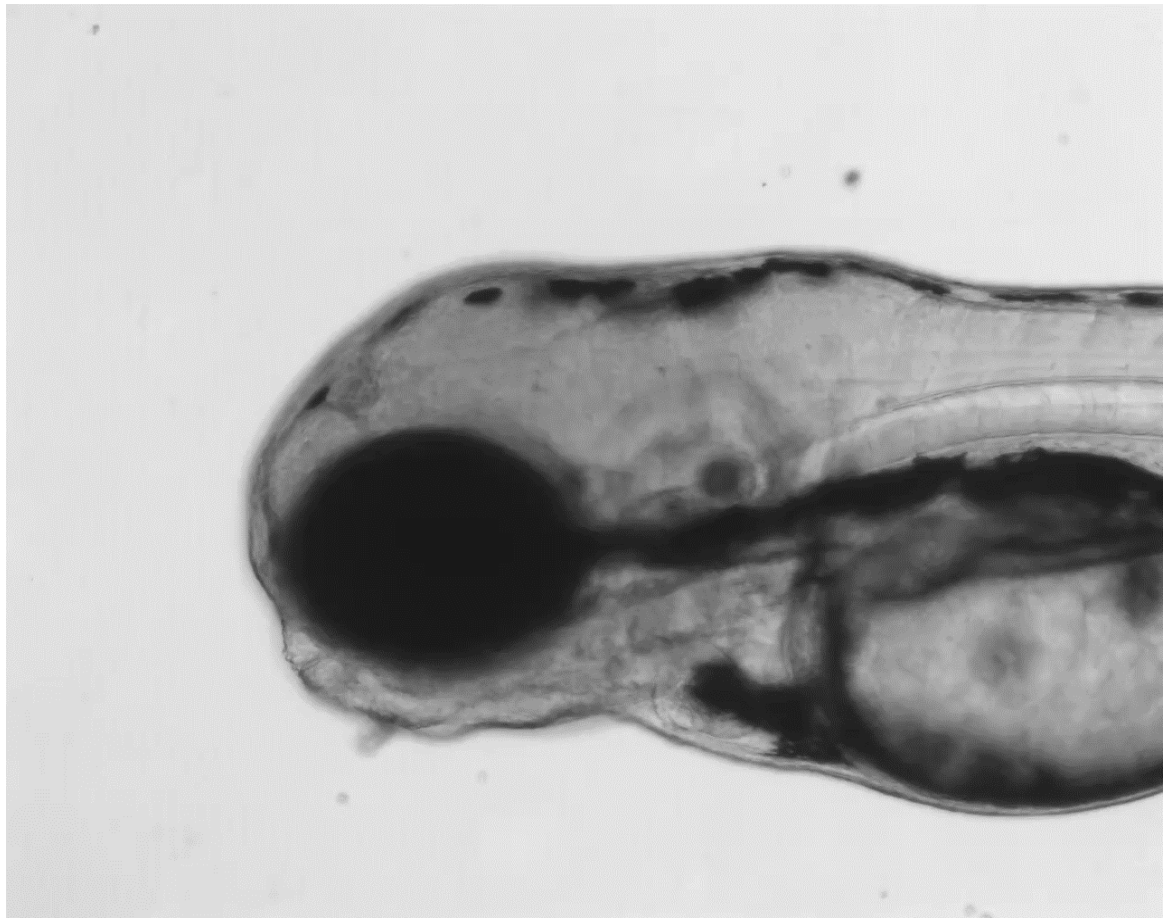
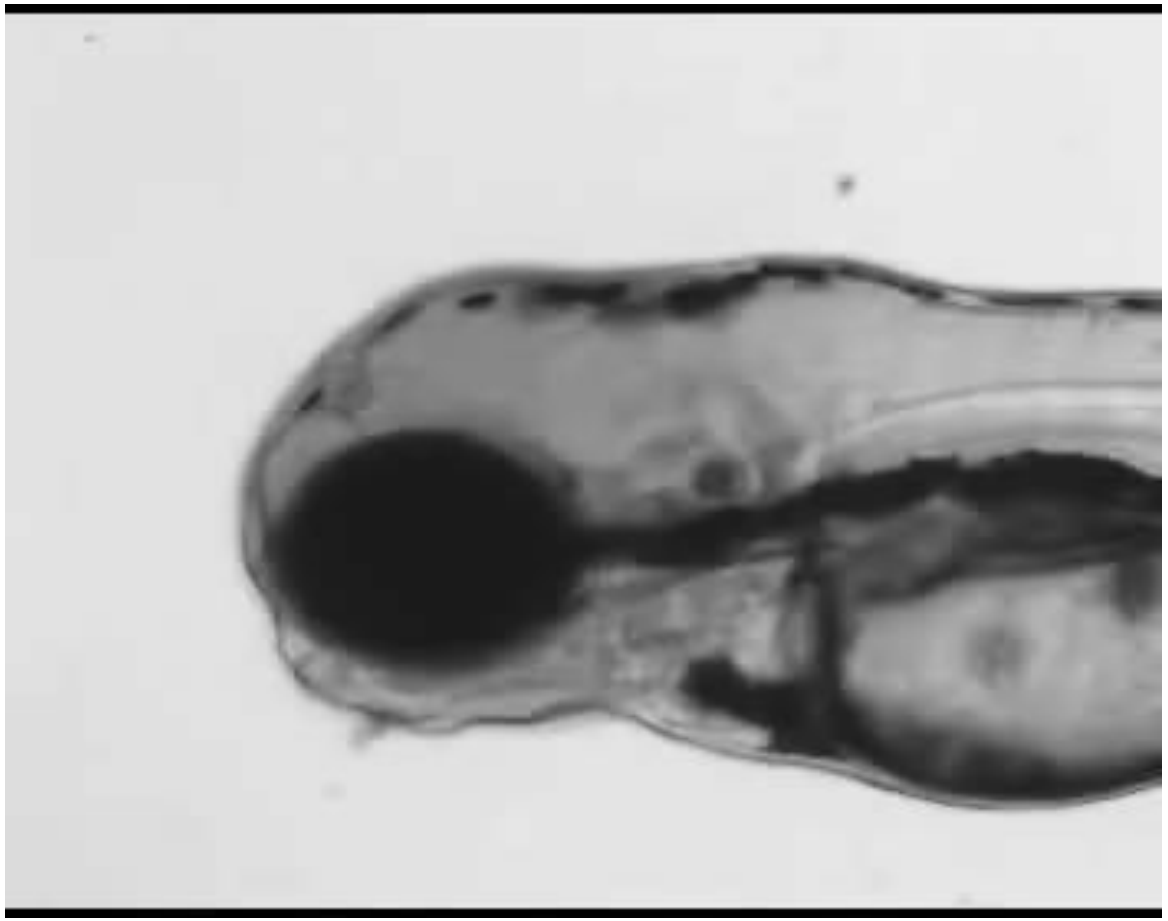


Image Stabilizer
Result

Before

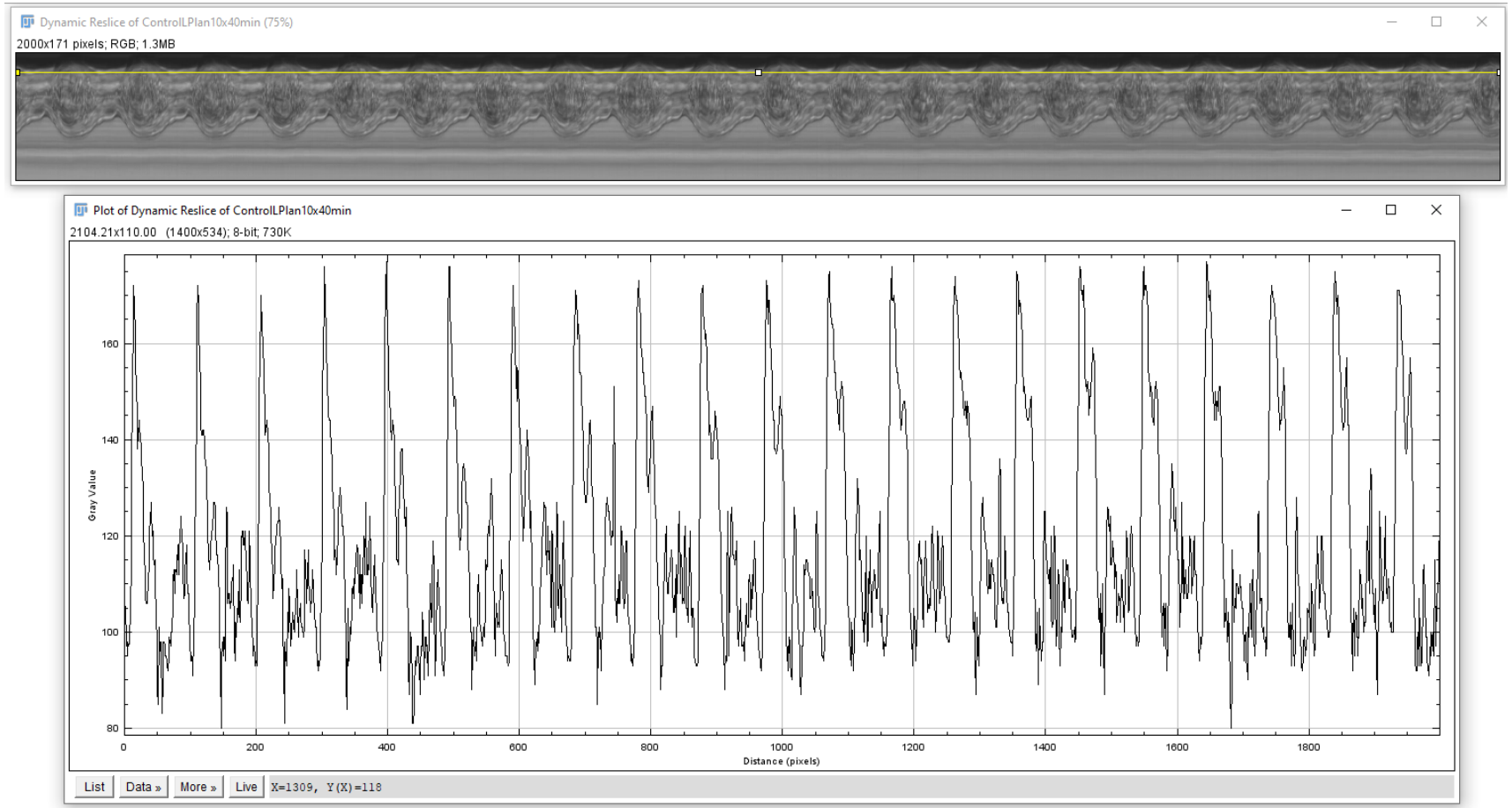


After



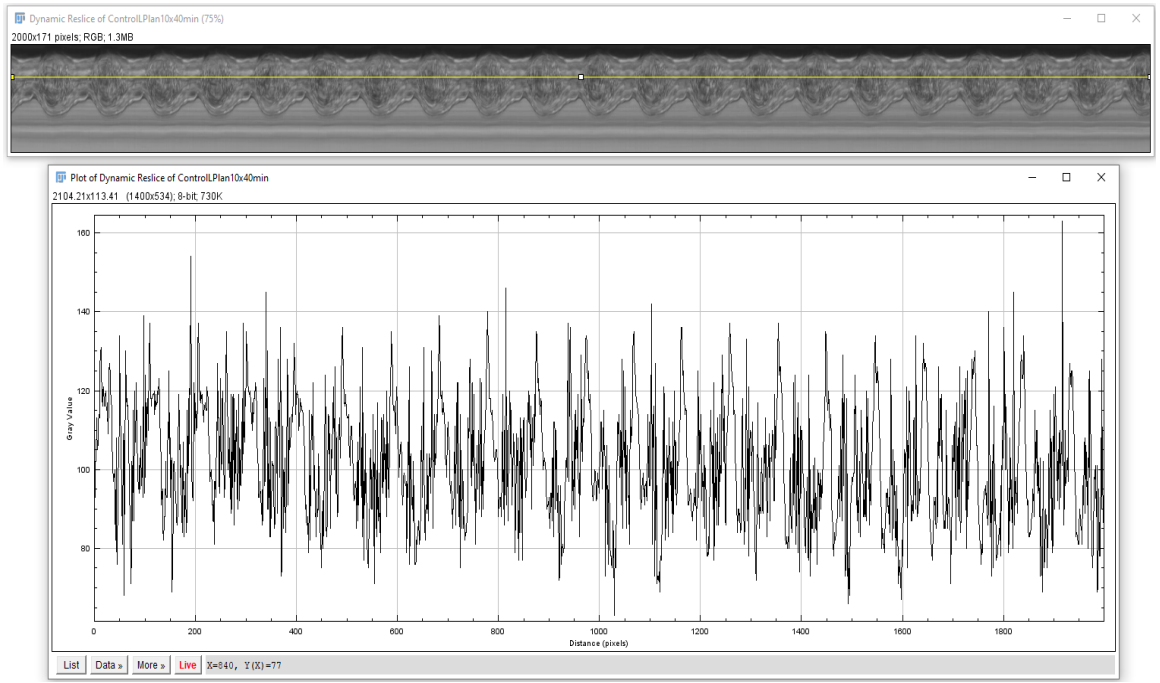
Data Collection Using BAR Plugin

We can obtain ROI data peaks from the kymograph by making a line followed by the Plot Profile Command **(Ctrl + K)**

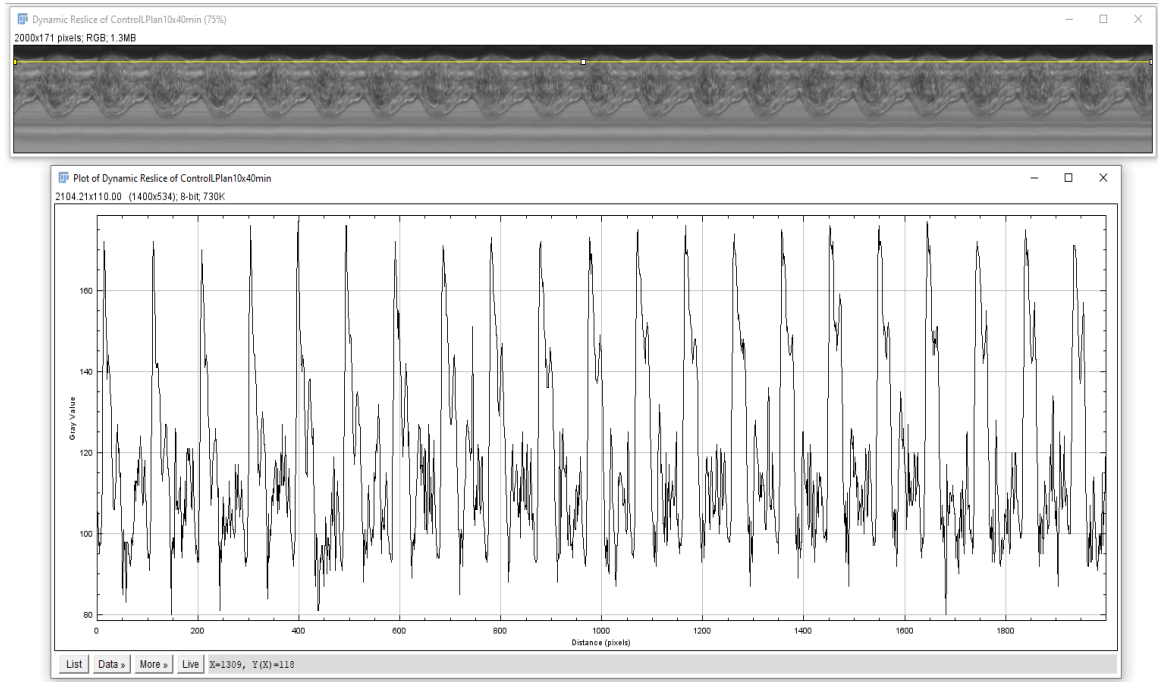


Caution

- Specified line is subjective, it might show unsuitable result



Not Suitable

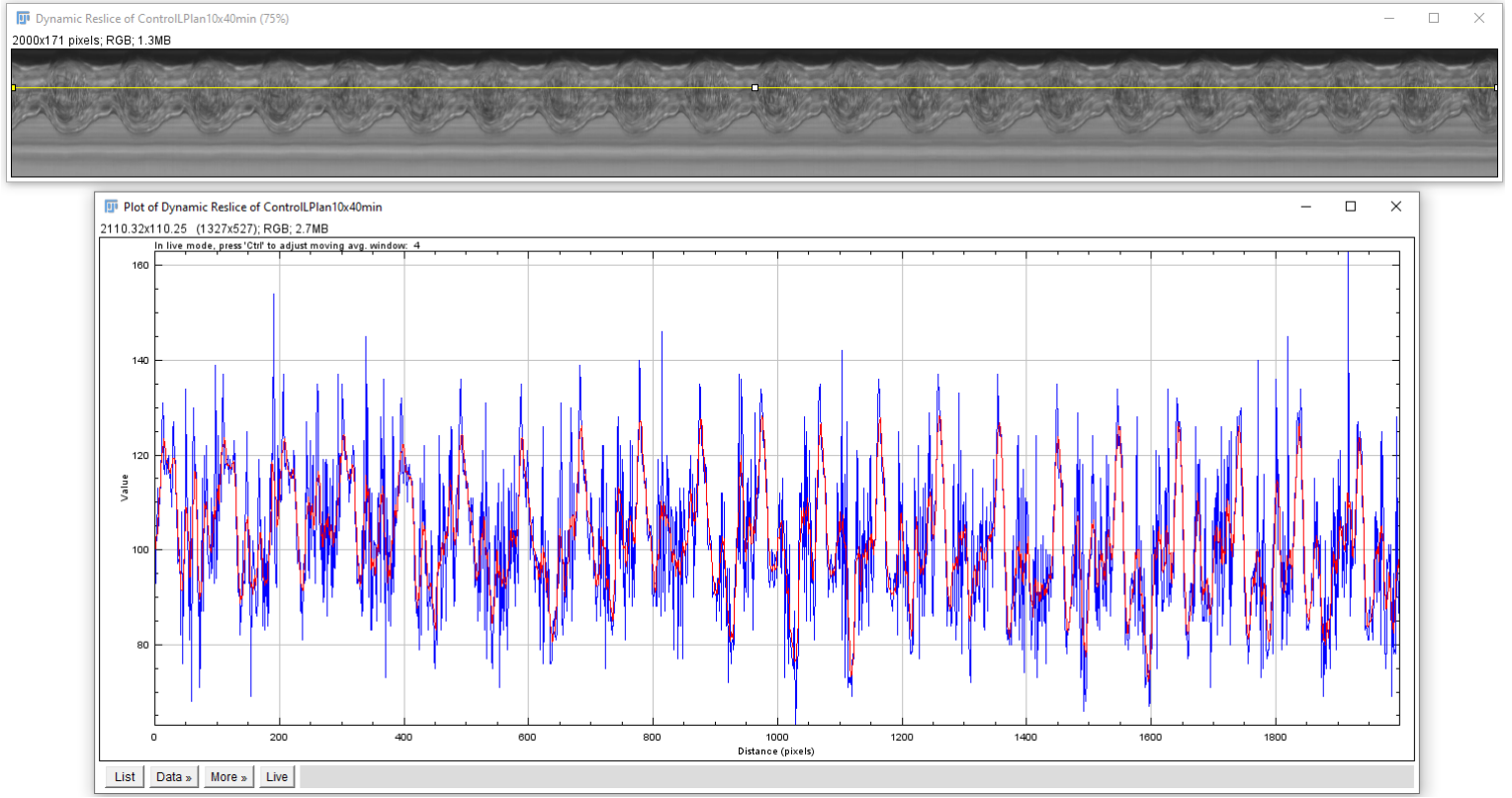


Suitable

Data Collection Using BAR Plugin

Smoothed Plot

- The unsuitable reading can be adjusted by using **Smoothed Plot Profile tool** (BAR > Analysis > Smoothed Plot Profile)



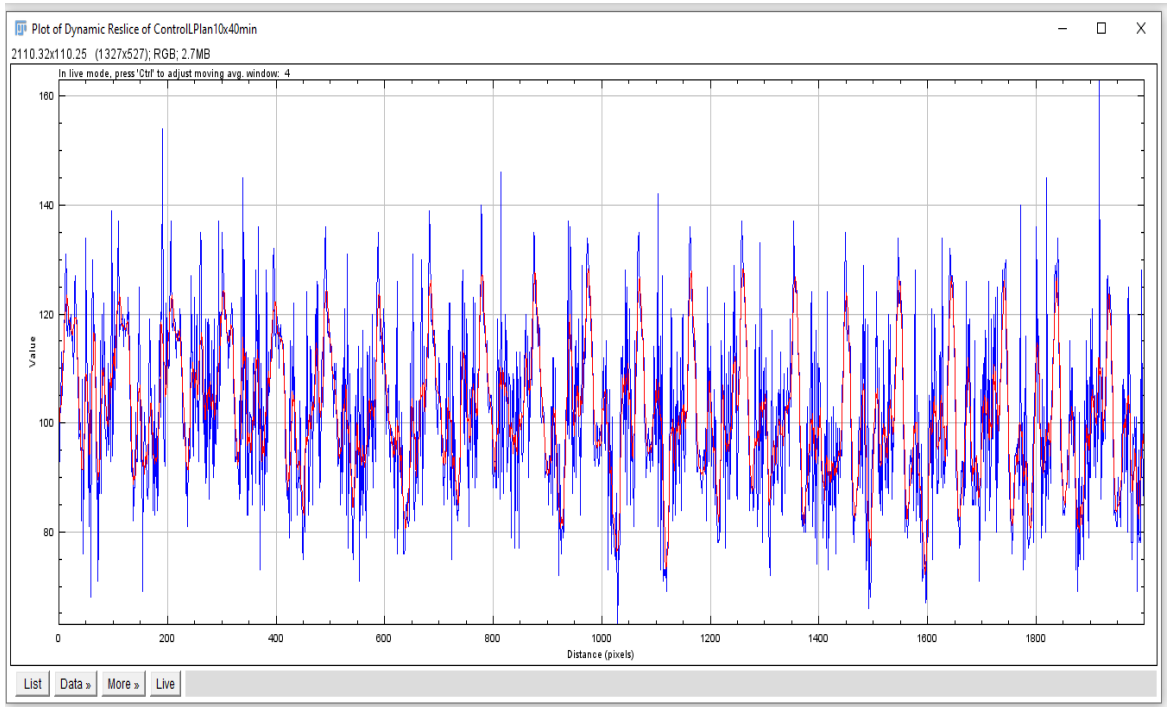
Blue Line = Raw Plot

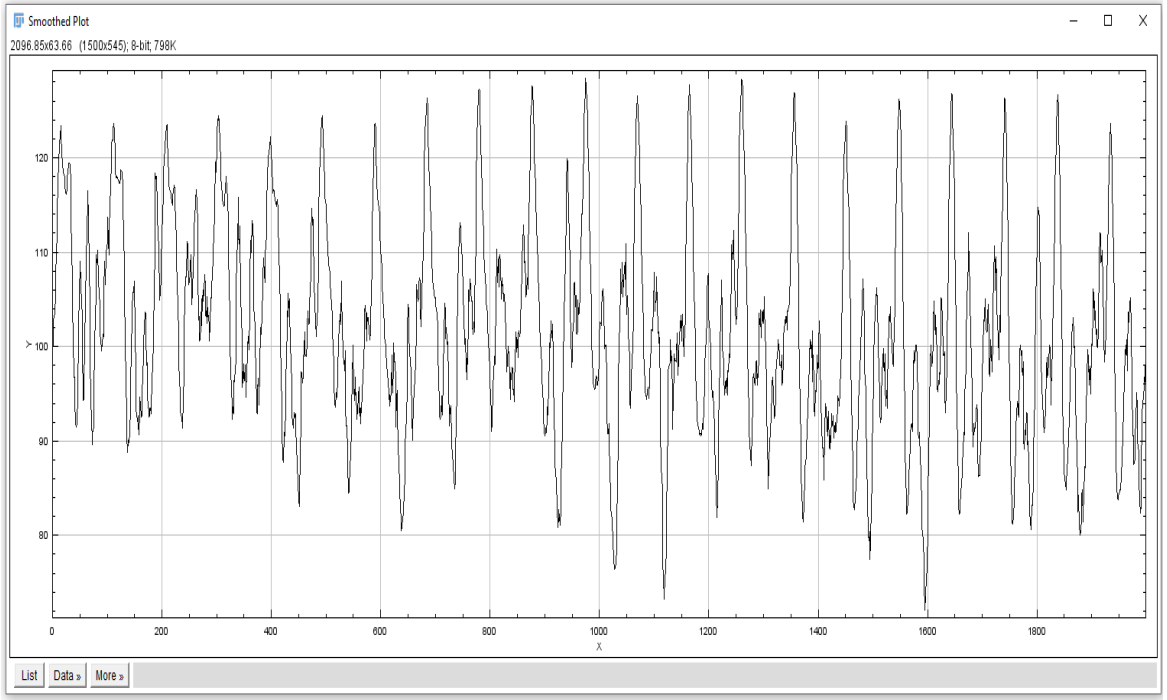
Red Line = Smoothed Plot Profile

Data Collection Using BAR Plugin

Smoothed Plot

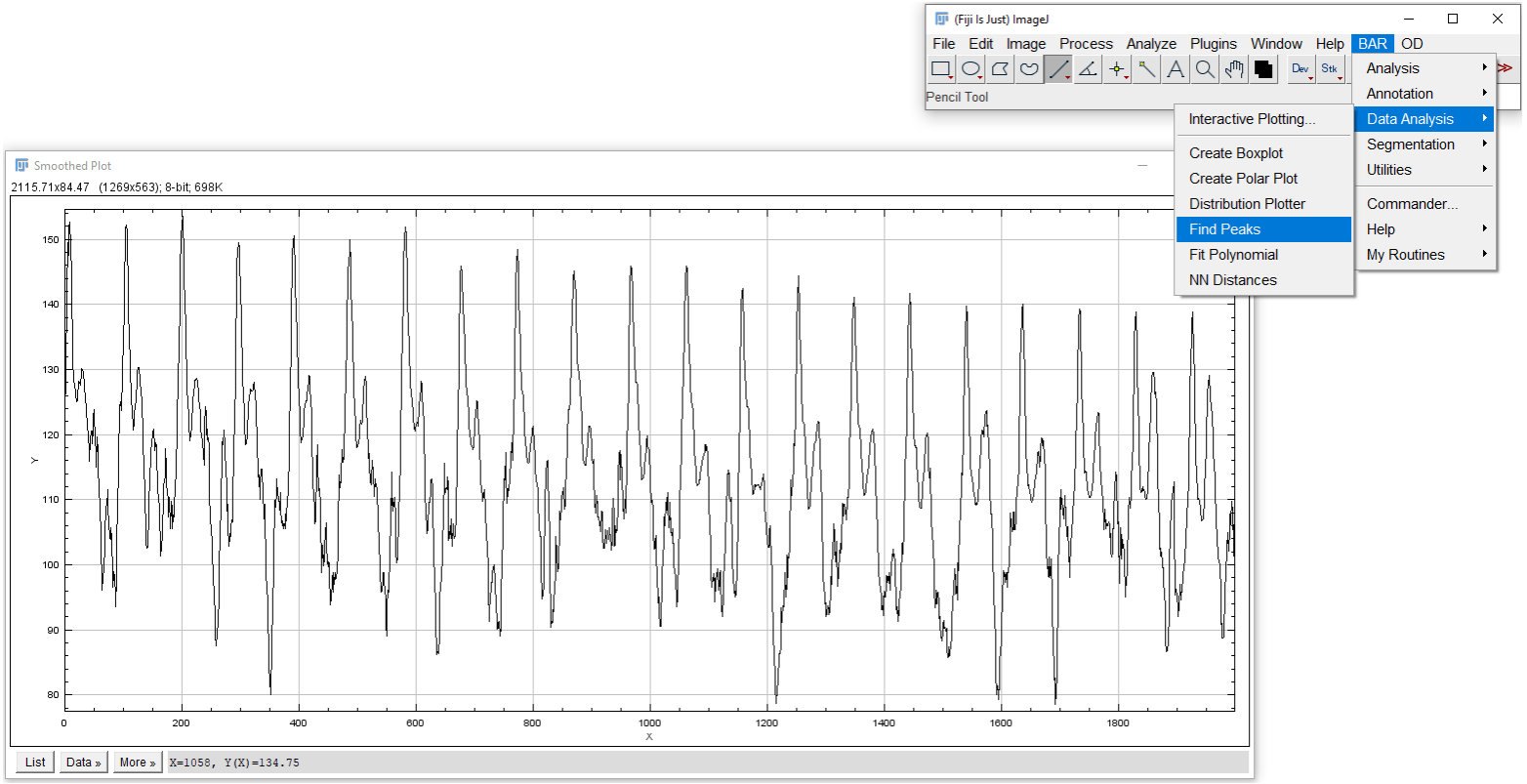
- To use the Smoothed Plot for the next step, we made a macro to create a new standalone plot based on the smoothed plot, otherwise BAR Plugin will rather use the Raw Plot for Detection.





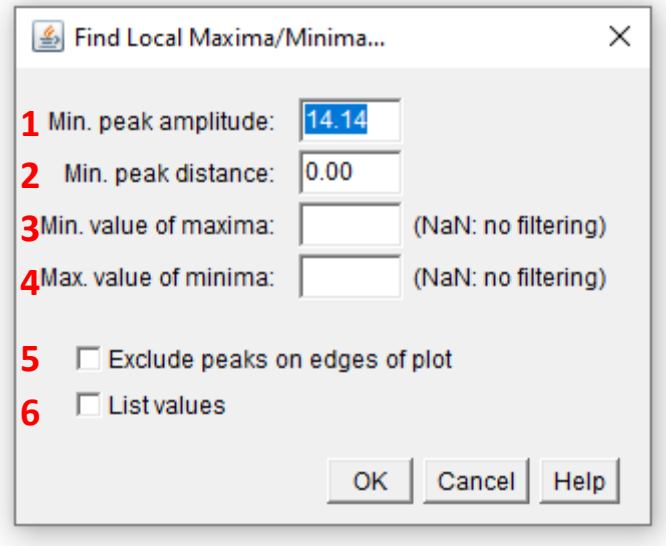
Data Collection Using BAR Plugin

- To obtain the Peak data, we use the Find Peaks tool (BAR > Data Analysis > Find Peaks) provided by BAR Plugin.



Data Collection Using BAR Plugin

- Afterwards, a window will pop up. This window is used to set the parameter for the peaks.



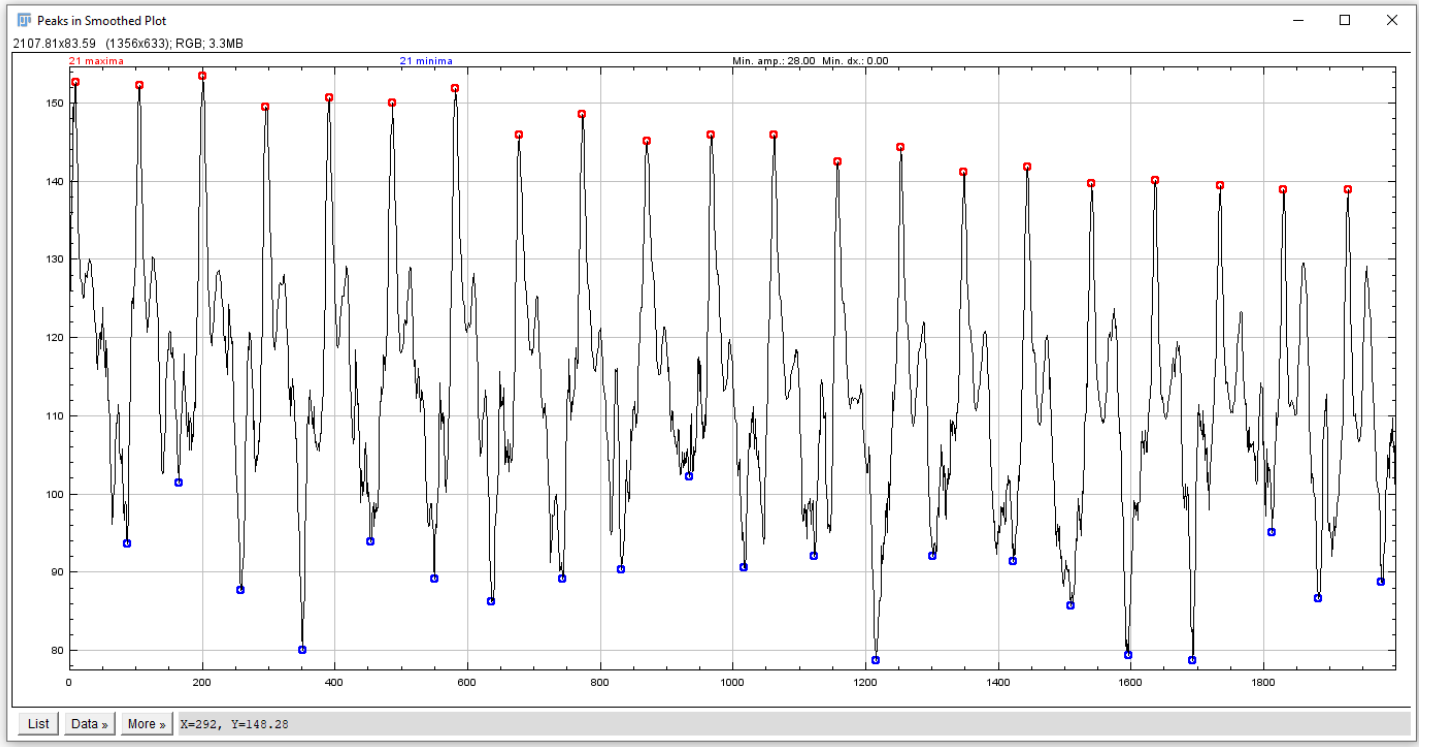
Min peak amplitude value is set automatically by the plugin at first. For zebrafish however, we usually adjust it to **double** or **triple** the automatic value, as there is a lot of short peaks.

1. **Min. peak amplitude :** Minimal amplitude of the peak to be considered as a peak, otherwise it will not be recorded
2. **Min. peak distance :** Minimal distance between peaks to be considered as peaks
3. **Min. value of the maxima :** Minimal value of the peak to be considered as the maxima
4. **Max. value of the minima :** Maximal value of the peak to be considered as the minima
5. **Exclude peaks on edges of the plot :** Exclude the peaks on the edges as it might be premature, thus messing up with the data.
6. **List values :** Automatically list all the data.

We usually adjust the **Min. peak amplitude** and check the box for the **exclude peaks** and **list values**, while leaving other options empty unless there are some necessary alteration. Then press **OK**

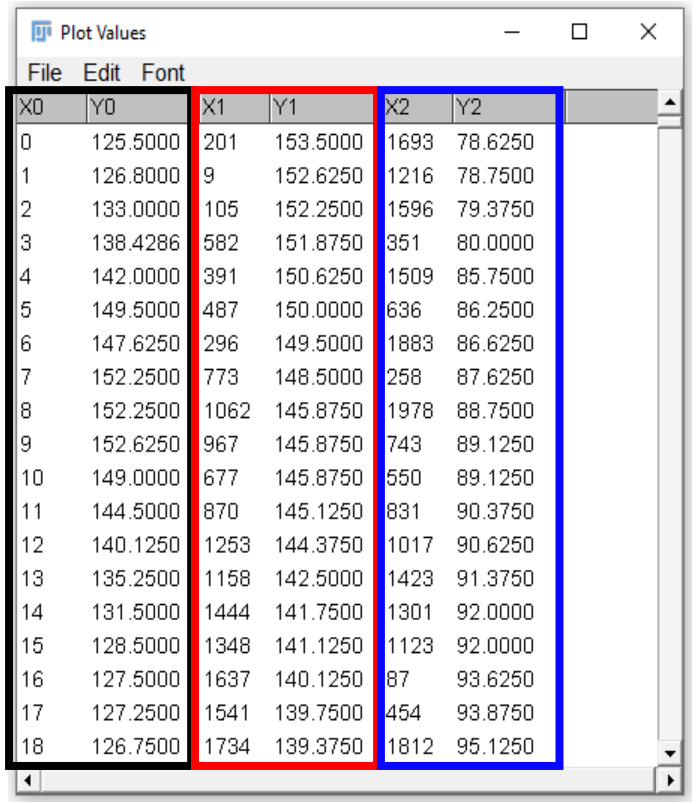
Data Collection Using BAR Plugin

- Final result of the BAR Plugin will mark the maxima and minima of the plot. However, as can be seen below, either the maxima or minima will have a fuzzy result, therefore only the maxima can be used for this example



Data Collection Using BAR Plugin

- Then we get the values from the plot.



X0	Y0	X1	Y1	X2	Y2
0	125.5000	201	153.5000	1693	78.6250
1	126.8000	9	152.6250	1216	78.7500
2	133.0000	105	152.2500	1596	79.3750
3	138.4286	582	151.8750	351	80.0000
4	142.0000	391	150.6250	1509	85.7500
5	149.5000	487	150.0000	636	86.2500
6	147.6250	296	149.5000	1883	86.6250
7	152.2500	773	148.5000	258	87.6250
8	152.2500	1062	145.8750	1978	88.7500
9	152.6250	967	145.8750	743	89.1250
10	149.0000	677	145.8750	550	89.1250
11	144.5000	870	145.1250	831	90.3750
12	140.1250	1253	144.3750	1017	90.6250
13	135.2500	1158	142.5000	1423	91.3750
14	131.5000	1444	141.7500	1301	92.0000
15	128.5000	1348	141.1250	1123	92.0000
16	127.5000	1637	140.1250	87	93.6250
17	127.2500	1541	139.7500	454	93.8750
18	126.7500	1734	139.3750	1812	95.1250

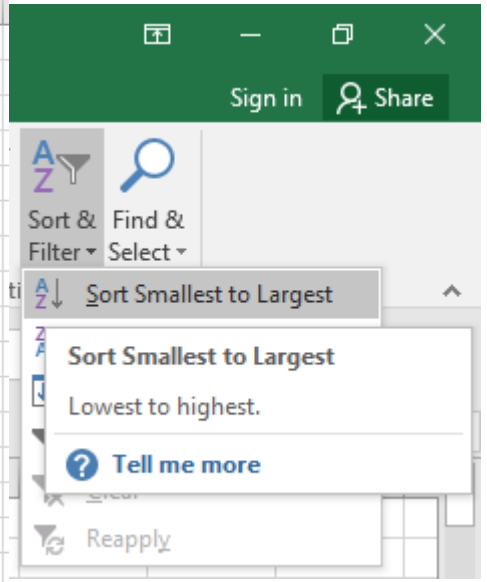
- **Black rectangle** represents the value (Y0) on each points of time (X0) within the plot
- **Red rectangle** represents the time where the **maxima** happens (X1) and the value of the **maxima** (Y1)
- **Blue rectangle** represents the time where **minima** happens (X2) and the value of the **minima** (Y2)

The value is sorted according to X0.
To calculate time difference between beats, we need to sort the data according to X1 or X2 using Microsoft excel. Copy the data using Ctrl + A then Ctrl + C.

Data Calculation in Microsoft Excel

Sort the maxima **(X1)** data from the lowest to highest, then copy the values for calculation in the next slide.

	A	B	C	D	E	F
1	X0	Y0	X1	Y1	X2	Y2
2	0	125.5	201	153.5	1693	78.625
3	1	126.8	9	152.625	1216	78.75
4	2	133	105	152.25	1596	79.375
5	3	138.4286	582	151.875	351	80
6	4	142	391	150.625	1509	85.75
7	5	149.5	487	150	636	86.25
8	6	147.625	296	149.5	1883	86.625
9	7	152.25	773	148.5	258	87.625
10	8	152.25	1062	145.875	1978	88.75
11	9	152.625	967	145.875	743	89.125
12	10	149	677	145.875	550	89.125
13	11	144.5	870	145.125	831	90.375
14	12	140.125	1253	144.375	1017	90.625
15	13	135.25	1158	142.5	1423	91.375
16	14	131.5	1444	141.75	1301	92
17	15	128.5	1348	141.125	1123	92
18	16	127.5	1637	140.125	87	93.625
19	17	127.25	1541	139.75	454	93.875
20	18	126.75	1734	139.375	1812	95.125
21	19	126	1927	138.875	164	101.375
22	20	125	1830	138.875	934	102.25
23	21	125.25				



X0	Y0	X1	Y1	X2	Y2
1	126.8	9	152.625	1216	78.75
2	133	105	152.25	1596	79.375
0	125.5	201	153.5	1693	78.625
6	147.625	296	149.5	1883	86.625
4	142	391	150.625	1509	85.75
5	149.5	487	150	636	86.25
3	138.4286	582	151.875	351	80
10	149	677	145.875	550	89.125
7	152.25	773	148.5	258	87.625
11	144.5	870	145.125	831	90.375
9	152.625	967	145.875	743	89.125
8	152.25	1062	145.875	1978	88.75
13	135.25	1158	142.5	1423	91.375
12	140.125	1253	144.375	1017	90.625
15	128.5	1348	141.125	1123	92
14	131.5	1444	141.75	1301	92
17	127.25	1541	139.75	454	93.875
16	127.5	1637	140.125	87	93.625
18	126.75	1734	139.375	1812	95.125
20	125	1830	138.875	934	102.25
19	126	1927	138.875	164	101.375

Data Calculation in Microsoft Excel

	A	B	C	D	E	F	G	H	I	J
1	Atrium	Ventricle	Atrium Interval	Ventricle Interval	A-V Interval	V-A Interval	Atrium heart rate	Ventricle heart rate	Average A-V Interval	Average V-A Interval
2	66	105	0.465	0.48	0.195	0.27	125.5506608	125.1372119	0.20210526	0.27578947
3	159	201	0.49	0.475	0.21	0.28				
4	257	296	0.47	0.475	0.195	0.275				
5	351	391	0.485	0.48	0.2	0.285				
6	448	487	0.485	0.475	0.195	0.29				
7	545	582	0.46	0.475	0.185	0.275				
8	637	677	0.475	0.48	0.2	0.275				
9	732	773	0.495	0.485	0.205	0.29				
10	831	870	0.485	0.485	0.195	0.29				
11	928	967	0.465	0.475	0.195	0.27				
12	1021	1062	0.485	0.48	0.205	0.28				
13	1118	1158	0.475	0.475	0.2	0.275				
14	1213	1253	0.47	0.475	0.2	0.27				
15	1307	1348	0.47	0.48	0.205	0.265				
16	1401	1444	0.49	0.485	0.215	0.275				
17	1499	1541	0.485	0.48	0.21	0.275				
18	1596	1637	0.475	0.485	0.205	0.27				
19	1691	1734	0.485	0.48	0.215	0.27				
20	1788	1830	0.47	0.485	0.21	0.26				
21	1882	1927								

A. Peaks obtained in Atrium

B. Peaks obtained in Ventricle

C and D. Interval between peaks in Atrium and Ventricle respectively in seconds

Equation : $Peak_{n+1}$-$Peak_n$/200 (video is 200 fps)

E. Interval from Atrium peaks to Ventricle peaks

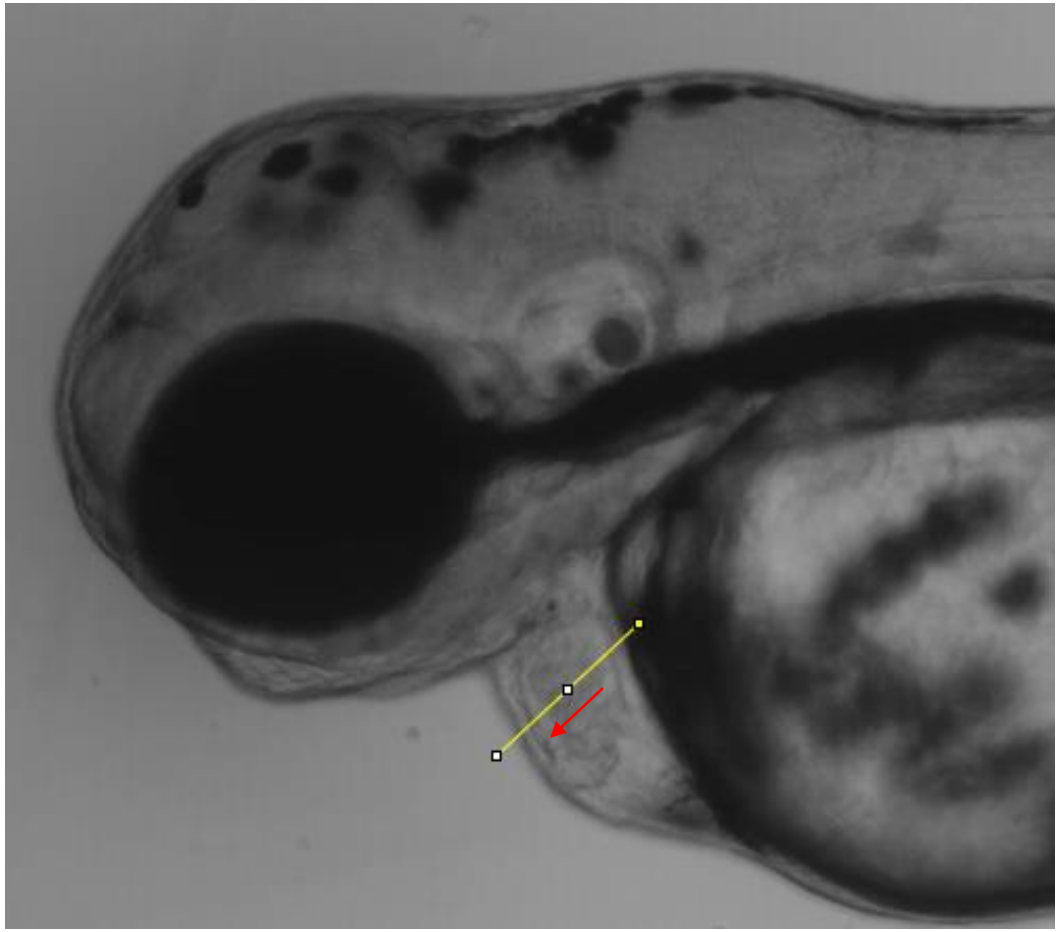
F. Interval from Ventricle peaks to Atrium peaks

E and F are used for determining pumping regularity between chamber

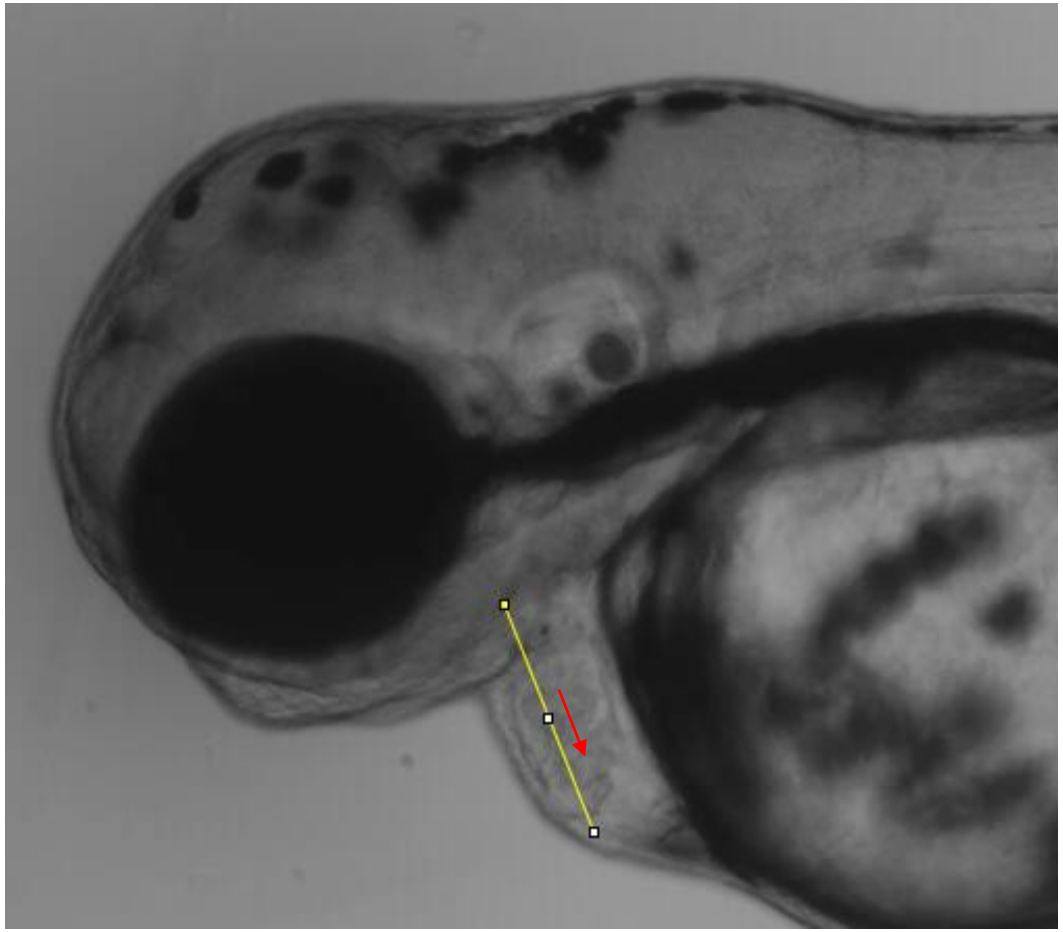
Other Data Collection

- Collection of data according to below slides to calculate ejection fraction, shortening fraction, stroke volume, and cardiac output

Other Data Collection



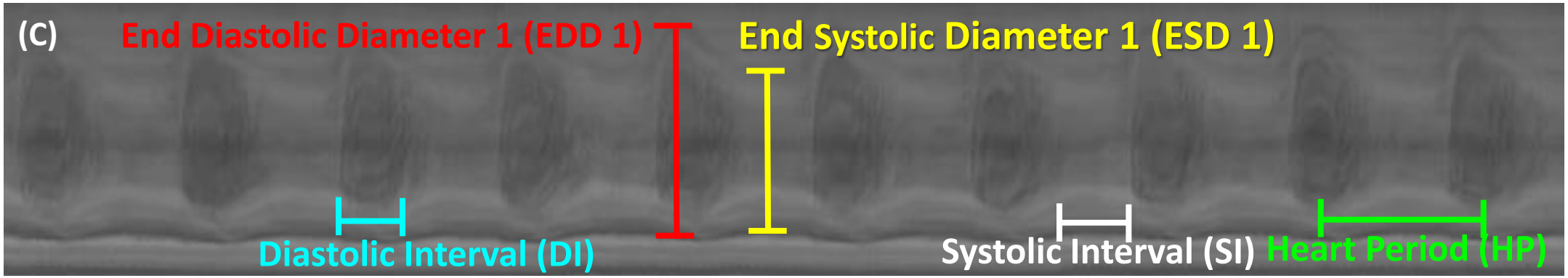
Ventricular short axis



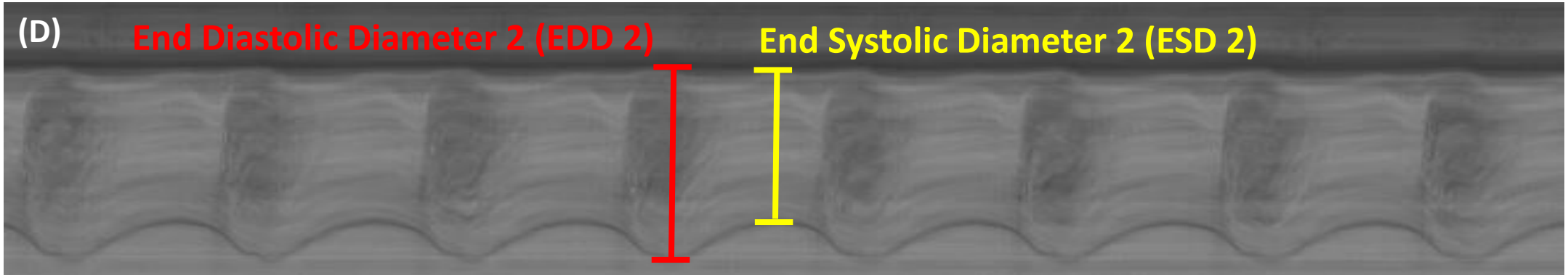
Ventricular long axis

Other Data Collection

Ventricular short axis Kymograph



Ventricular long axis Kymograph

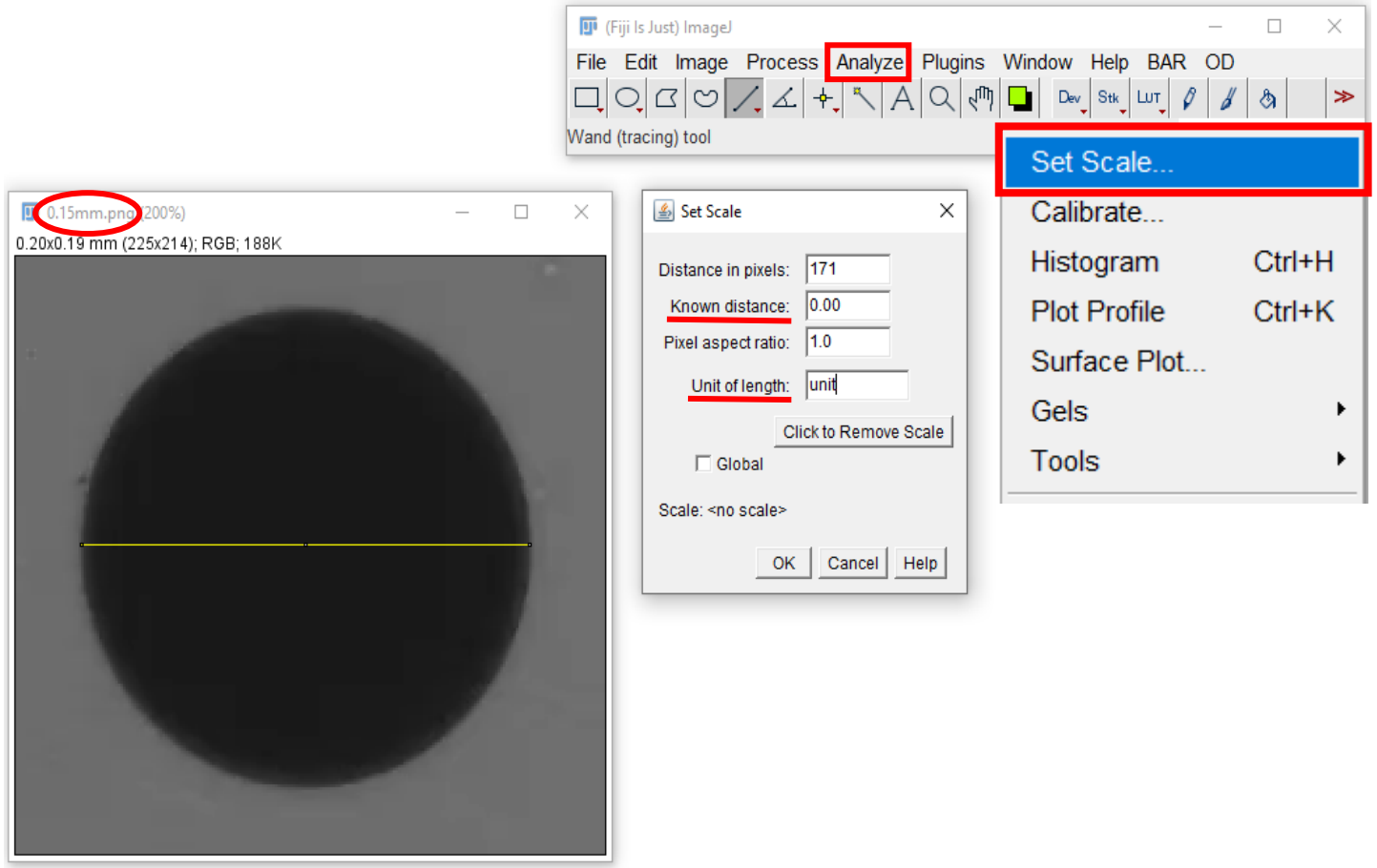


Other Data Collection

- End Diastolic Diameter (EDD) and End Systolic Diameter (ESD) data (1 & 2) can be obtained by manually measuring the length of the heart when it is at the biggest and the smallest on both the short- and long-axis. In addition, it is necessary to set the scale to obtain the data in desired unit using calibration scale.
- Diastolic interval (DI), Systolic interval (SI), and Heart period (HP) can be obtained by measuring the heart when they are full of blood, empty of blood, and the interval between beat respectively. This measurement can be done by measuring horizontally such as depicted in the last slide. The unit for this measurement is frame and can be converted to seconds or minutes according to the refresh rate of the recording equipment.

Set Scale

- This step is necessary to automatically convert length unit from pixel to mm or cm during measurement process
- The Set Scale menu can be accessed from Analyze > Set Scale
- The calibration scale is a circular shape object with diameter of 0.15 mm
- The scale can be set by changing the Known distance and Unit of length in Set Scale menu according to the calibration scale



Other Data Collection

From the data collected in previous slides, we are able to calculate data as presented below

A	B	C	D	E	F	G	H	I	J	K	L
EDV		ESV		EDV (μm^3)	ESV (μm^3)	Stroke Volume (μm^3/beat)	Stroke Volume (pL/beat)	Heart rate (BPM)	Cardiac Output	Ejection Fraction (%)	Shortening Fraction (%)
Long Axis (μm)	Short Axis (μm)	Long Axis (μm)	Short Axis (μm)								
171.5039578	93.22779244	48.37291117	121.3720317	1,435,066.72	372,921.88	1,062,144.83	1,062.14	125.14	132,913.84	74.01	29.23

A, B, C, and D are data collected from slide **31** and **32**

E and F are **End Diastolic Volume** and **End Systolic Volume** respectively with the equation :

$$\frac{1}{6}\ x\ \pi\ x\ Long\ Axis\ x\ Short\ Axis^2$$

Assuming the heart is spheroid in shape with missing axis having the same length as the short axis.

G and H are Stroke Volume = EDV – ESV

I is the Heart rate calculated using Microsoft excel

J is the Cardiac Output = Stroke Volume x Heart rate, as it shows the volume of blood pumped within a minute

K Ejection Fraction is percent heart volume changes during diastole and systole

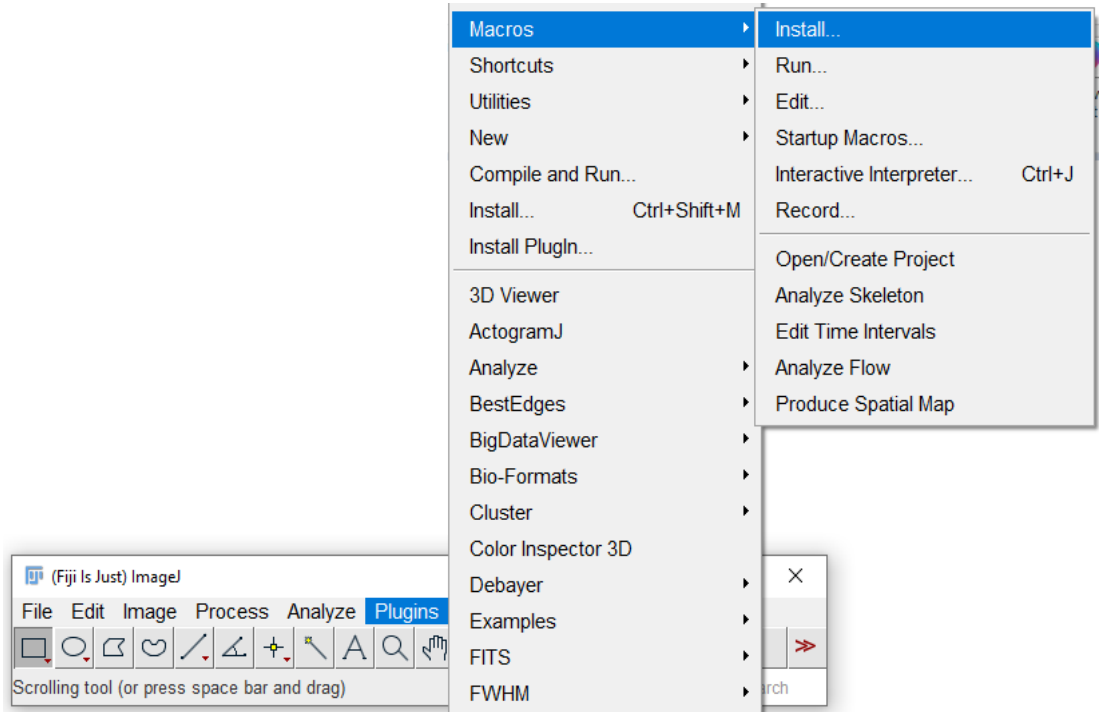
$$Ejection\ Fraction = \frac{EDV - ESV}{EDV}\ x\ 100\%$$

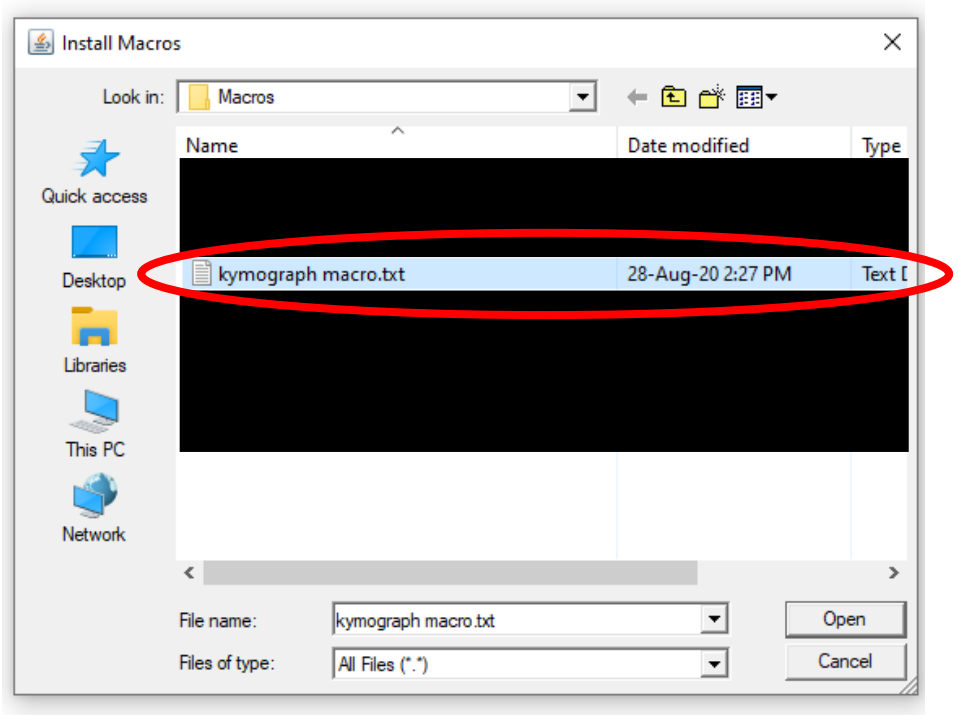
L Shortening Fraction is percent heart short axis changes during diastole and systole according to

$$Ejection\ Fraction = \frac{EDV\ Short\ Axis\ - ESV\ Short\ Axis}{EDV\ Short\ Axis}\ x\ 100\%$$

Installation of ImageJ macro

- Add the macro to the ImageJ > Plugin > Macros folder.
- Open ImageJ then install the macro (Plugins > Macros > Install…) by locating the macro in previous Macros folder.





Macro

Our macro was made to help users in obtaining the data.

These are the command strings contained in the **kymograph macro.txt**

The first macro is to make kymograph using ImageJ **Dynamic Reslice** and **rotating** the kymograph by **90 degrees**. This macro is bound to **F2** key on user's keyboard, which can be modified by changing it to the user's preference.

macro "Make Kymograph [**F2**]" {

run("
Dynamic Reslice", "**rotate**");
}

Macro

The second macro is to enable users' to use smoothed plot by creating a new plot based on the plot made by the BAR Plugin. This macro is bound to F3 key

```
macro "Smoothed Plot [F3]" {
run("Smoothed Plot Profile", "window=4 pixelunits=true");
waitForUser("Adjust ROI location on Kymograph, Select Plot Window, Then Click OK");
Plot.showValues("Plot Values");
  x1 = Table.getColumn("X0");
  Y1 = Table.getColumn("Y1");
  xValues = x1;
  yValues = Y1;
      Plot.create("Smoothed Plot", "X", "Y", xValues, yValues);
      Plot.setLineWidth(1);
      Plot.show();
}
```

Macro

The third macro is to run Find Peaks, bound to F4 key.

```
macro  "Find Peaks [F4]" {

        run("Find Peaks");
}
```

Macro

The last macro is to close all windows made during data gathering in ImageJ, bound to F5 key.

```
macro "Close All Windows [F5]" {
    while (nImages>0) {
        selectImage(nImages);
        close();
    }
```