Supplementary protocol for article:

A simple ImageJ-based method to measure cardiac rhythm in Zebrafish embryos

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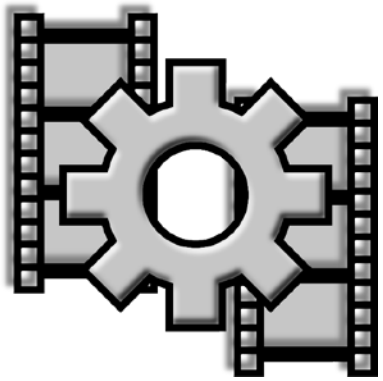
Required Software

adobe premiere

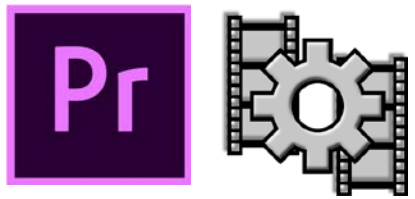


VirtualDub

ImageJ

Excel

Cardiac rhythm analysis pipeline



Video recording @ frame rate of 60 fps

↓

Convert video into image sequence frame-by-frame

↓

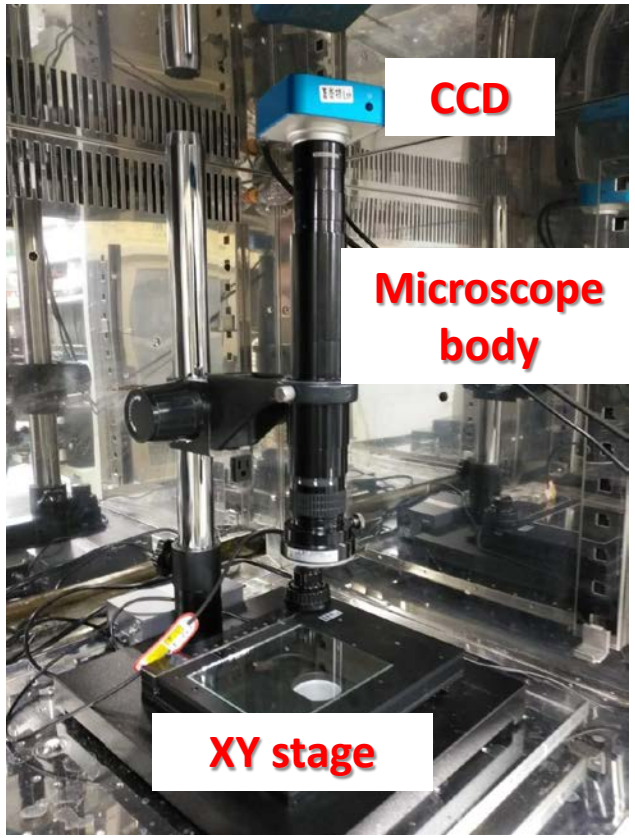
Extract the dynamic pixel changes from images

↓

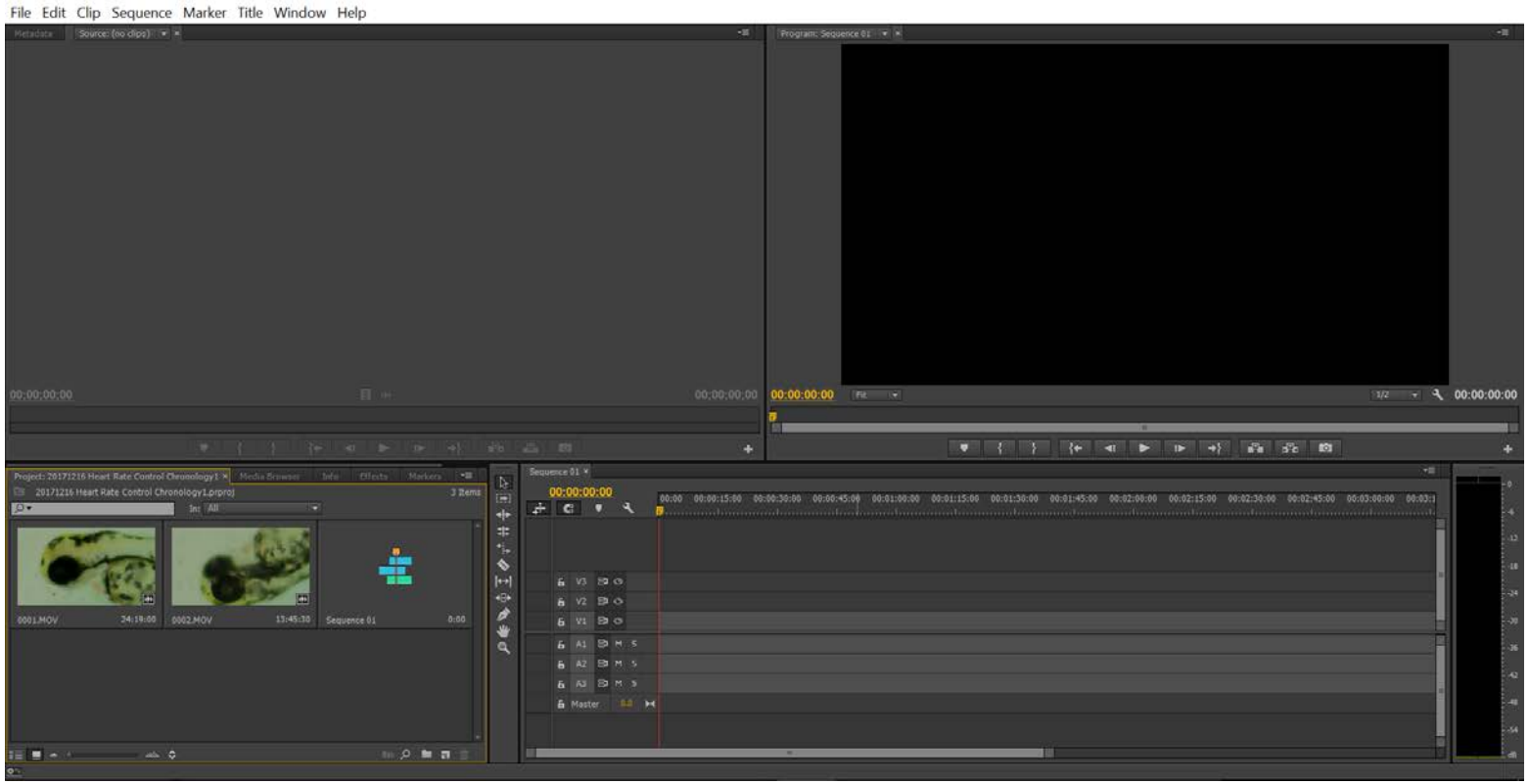
Calculate heartbeat frequency by Short-time Fourier transform

↓

Perform statistic analysis

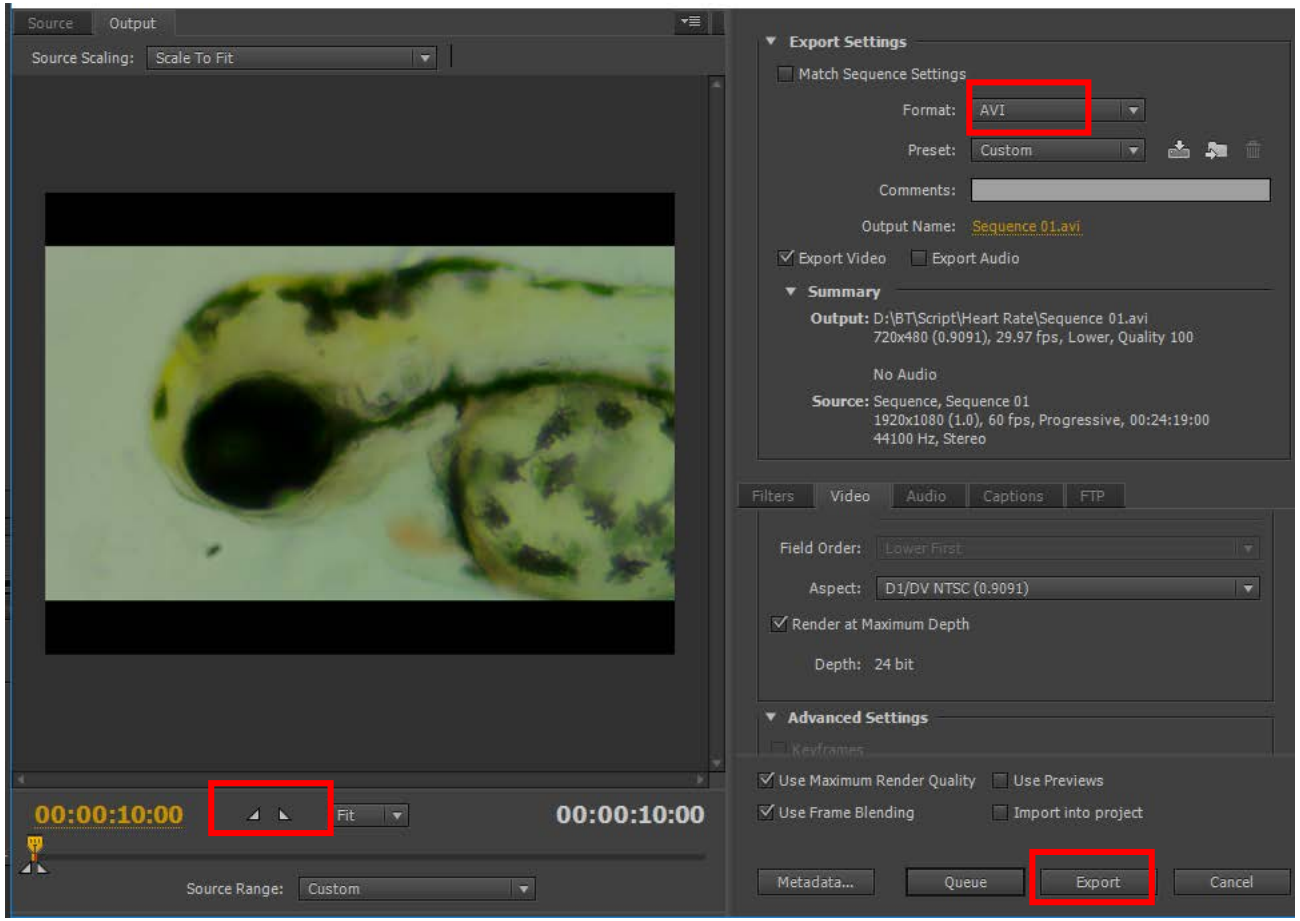


Checking Interesting Time Point



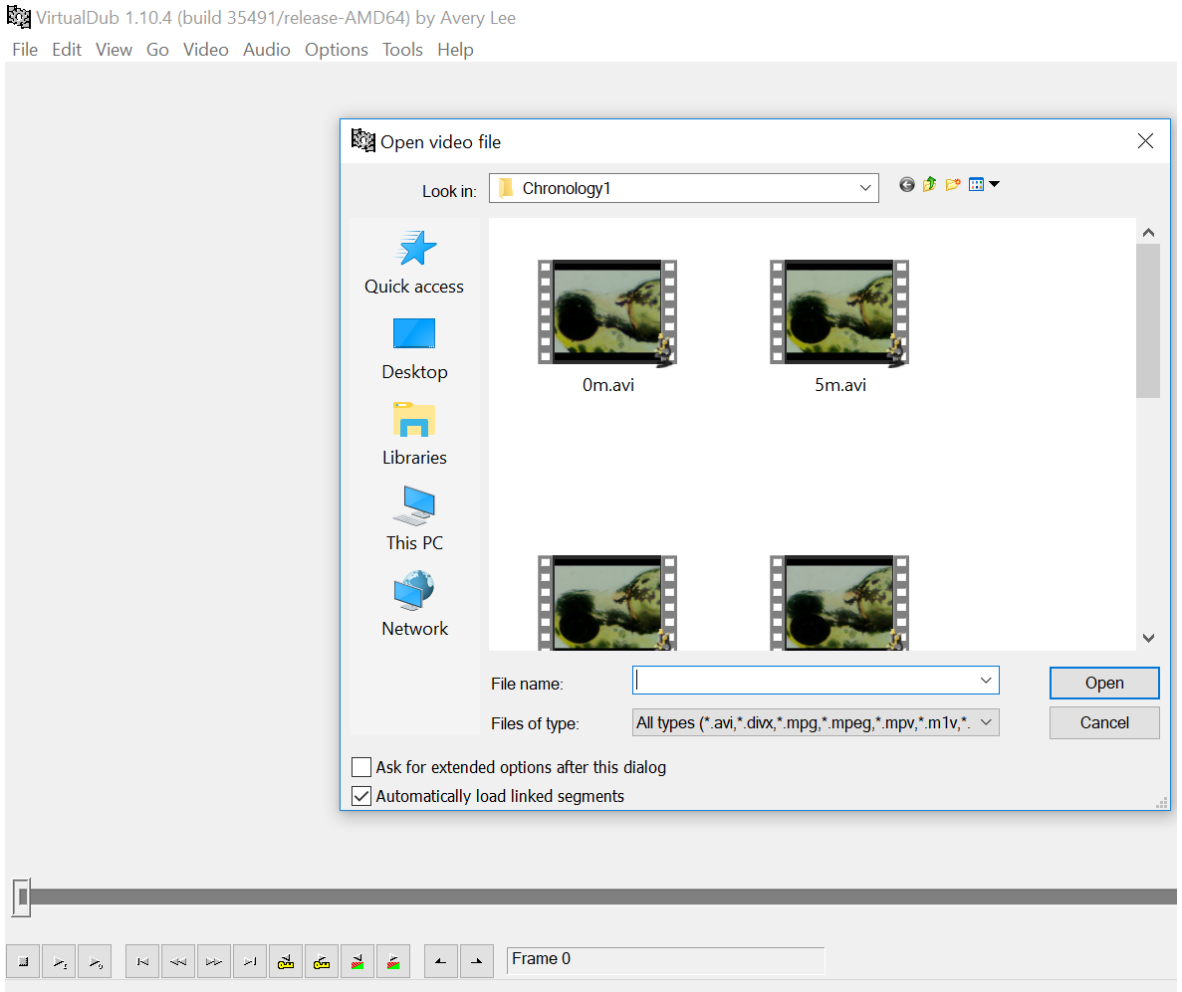
- Drag Video File to Adobe Premiere
- Click **Ctrl+N** to make sequence
- Check interesting time point

Export Video File



- Click Ctrl+M to render video
- Select AVI video format
- Set in and out point to determine video length and point
- Click export to render the video

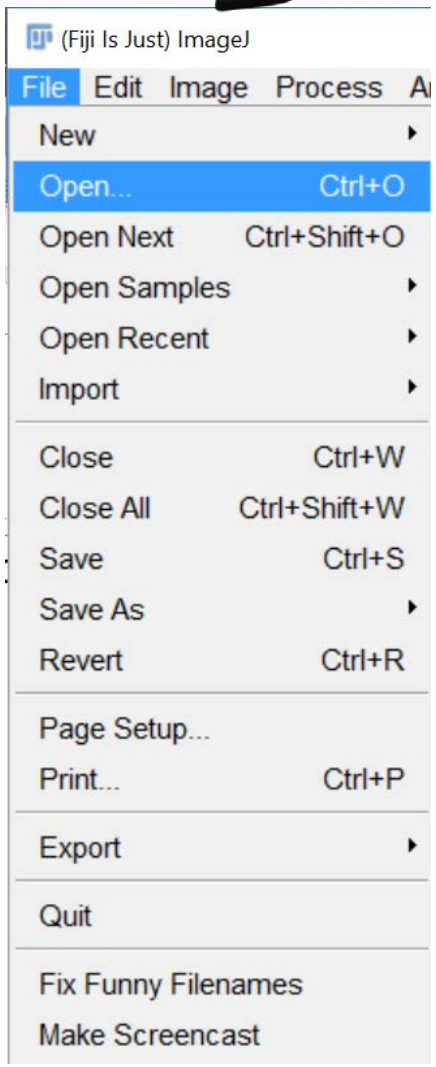
Convert to Uncompressed Format in VirtualDub

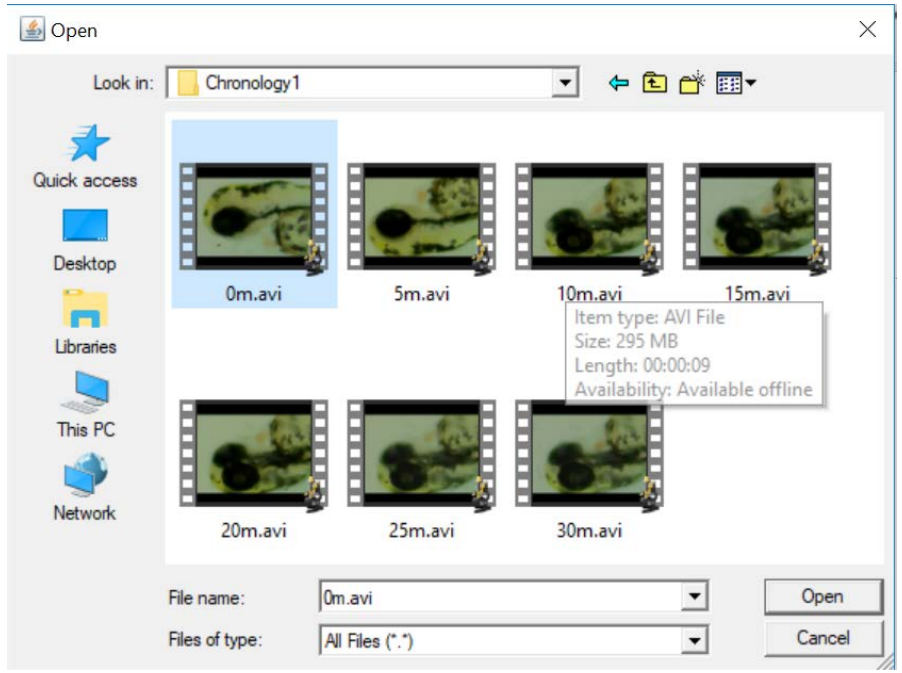


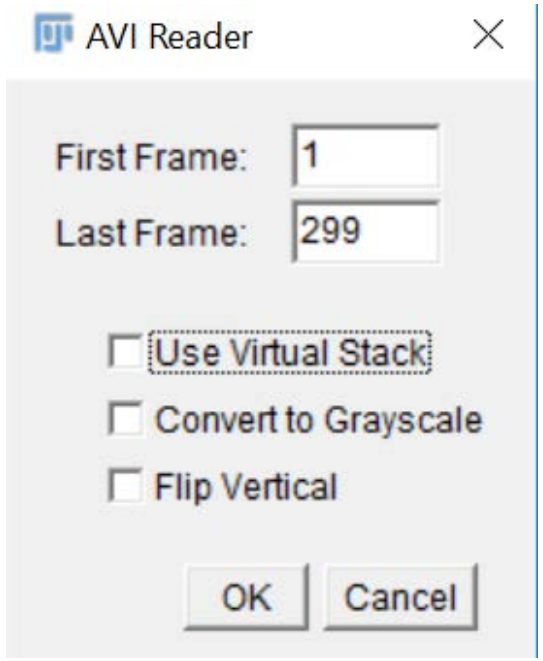
- Click Ctrl+O to select the video file
- Click F7 function key to convert the file format to uncompressed AVI

Open the Video File with ImageJ

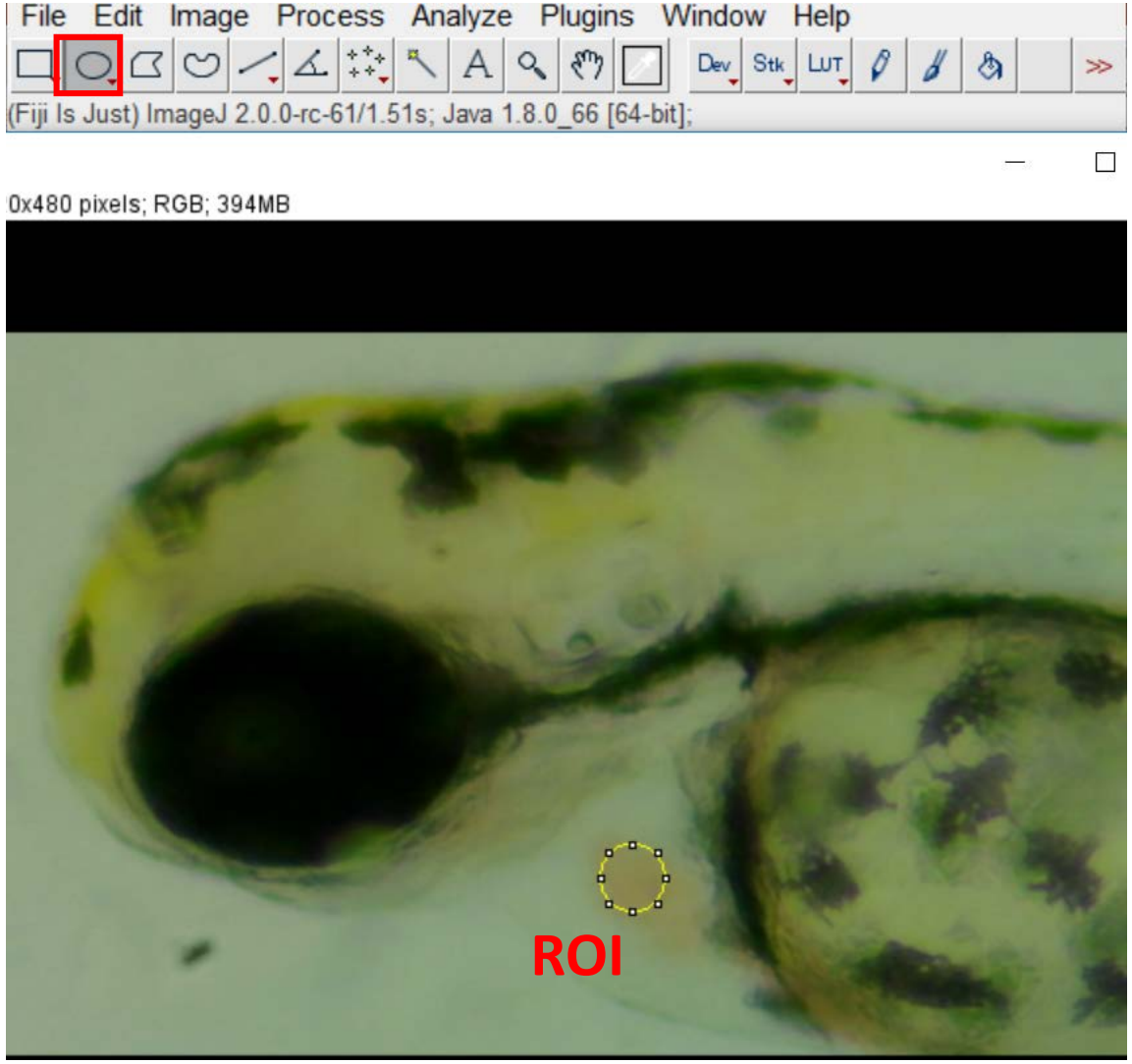
- Click File + Open to Select Video File
- Select the VirtualDub converted video
- In AVI Reader of ImageJ, choose the frames selected and uncheck all the boxes





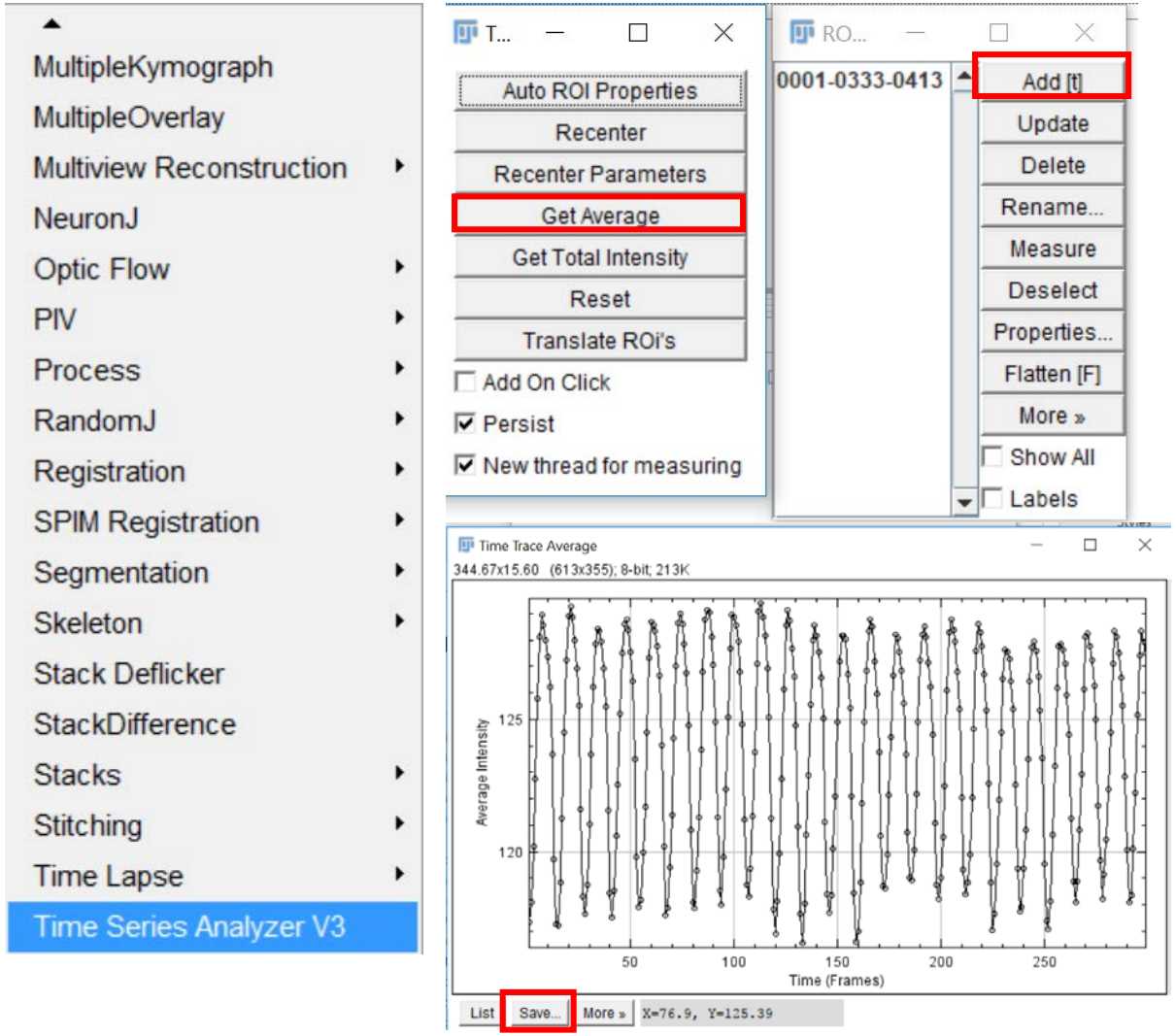


Select Region of Interesting (ROI) in ImageJ



- Use *Oval* brush selection to choose ROI in specific heart area (atrium or ventricle regions)
- The ROI can be specified in atrium, ventricle or the entire heart region to measure the beating rate

Use Time Series Analyzer V3 Plugin



- Choose Time Series Analyzer V3 plugin
- Click “Add” Button to choose ROI
- Click “Get Average” to get the peak and data
- Click “Save” in the peak window to keep the data for further analysis
- After this analysis, the **dynamic pixel change** can be extracted

Combine the data in Microsoft Excel

	A	B	C	D	E	F	G	H
1	Time	A0m	A5m	A10m	A15m	A20m	A25m	A30m
2	0.034483	132.5756	121.4167	123.9664	122.8646	125.3867	123.1311	122.1574
3	0.068966	128.7311	122.8802	123.4888	122.1146	129.4767	122.9726	121.9028
4	0.103448	127.563	122.1198	121.5336	120.2188	130.7767	122.1037	123.6852
5	0.137931	125.5168	122.375	120.1716	119.0156	131.5533	121.2896	128.8981
6	0.172414	124.3739	125.1667	120.3134	120.401	131.0167	121.247	129.9722
7	0.206897	124.1513	128.7031	122.0821	121.0365	129.0267	122.9085	131.5417
8	0.241379	124.1807	125.9323	127.0597	122.0052	126.82	123.8781	129.9769
9	0.275862	123.958	123.9063	128.5858	124.3073	125.2167	125.1189	128.0509
10	0.310345	123.5168	124.1719	130.2798	125.1406	124.35	125.064	125.0926
11	0.344828	124.4118	123.724	130.7948	126.8594	122.7067	124.3598	124.0509
12	0.37931	126.4958	122.8646	129.2761	126.0052	122.0633	123.0732	123.1204
13	0.413793	127.6597	122.5781	125.6381	123.2135	121.5433	122.8323	122.3426
14	0.448276	129.521	122.2083	124.8396	123.3802	121.1567	122.9634	122.1019
15	0.482759	131.6765	120.2292	124.944	123.6563	124.1267	122.6707	121.2176
16	0.517241	131.1513	122.0781	124.7351	123.4792	129.56	122.5884	121.2222
17	0.551724	128.3571	122.724	123.5485	122.4688	130.1433	122.439	123.9722
18	0.586207	127.1218	122.6615	122.7612	121.6146	131.5367	121.7134	129.2593
19	0.62069	126.4706	125.4844	121.1903	119.8854	130.96	121.2409	130.1667
20	0.655172	125.3698	129.099	123.0336	121.8698	129.2133	121.8445	131.4306
21	0.689655	124.4664	125.9948	126.7873	122.151	128.1967	123.2622	129.1481
22	0.724138	123.6261	124.6875	128.806	124.5521	125.8767	125.0549	126.0833

Atrium | Atrium Peak | Ventricle | Ventricle Peak

- Make 4 sheets in Excel:

Atrium and Ventricle (from Plugin). Atrium Peak and Ventricle Peak sheets for further analysis

- Time obtained from frame/29

(the video recording speed is 60 fps, but after video output by Adobe Premiere the speed reduce to 29 fps)

Input The Data Into Origin 9.1

	A(X)	B(Y)	C(Y)	D(Y)	E(Y)	F(Y)	G(Y)	H(Y)
Long Name								
Units								
Comments								
F(x)	Time	A0m	A5m	A10m	A15m	A20m	A25m	A30m
1	0.03448	117.945	125.6875	123.8114	129.58713	128.93047	132.49193	131.32558
2	0.06897	117.985	124.28438	123.11404	129.23106	128.23178	132.46774	131.59302
3	0.10345	117.705	123.25938	122.82895	128.87122	127.28146	132.66936	131.41861
4	0.13793	116.99	122.6375	122.46491	128.35606	126.27152	132.5242	131.84883
5	0.17241	117.82	122.65938	122.30264	127.28409	125.30132	132.57259	132.15117
6	0.2069	122.01	122.3	121.83334	125.98106	124.93378	132.06451	132.32558
7	0.24138	124.96	122.0125	124.42105	125.5303	124.2649	131.54033	132.08139
8	0.27586	125.595	123.91563	128.38597	124.67424	124.15232	130.85484	131.63954
9	0.31034	125.495	127.95	128.24562	123.88636	124.98676	129.79839	131.23256
10	0.34483	124.975	128.11563	128.26315	123.44697	129.96358	128.96774	130.37209
11	0.37931	124.545	128.24063	128.17105	124.61364	130.37418	127.77419	129.31395
12	0.41379	123.485	127.90625	127.57456	129.77652	130.75497	127.22581	128.44186
13	0.44828	121.5	127.61875	126.16228	129.73485	130.44371	127.02419	128.63954
14	0.48276	120.405	126.99062	125.11842	129.49243	130.00331	127.03226	132.03488
15	0.51724	118.97	125.84375	124.0307	128.97728	129.70198	129.68549	131.51163
16	0.55172	118.45	124.93437	122.71053	128.75	128.56291	131.58871	132.01163
17	0.58621	118.615	124.36875	122.20175	127.84849	127.38742	131.95967	131.94186
18	0.62069	117.65	124.11875	121.4079	127.26894	126.41722	131.51613	132.11627
19	0.65517	118.66	123.50625	120.36404	125.76515	126.03642	131.89516	132.61627
20	0.68966	122.85	122.93125	120.35526	124.95454	125.46027	131.10484	132.54651
21	0.72414	125.17	122.79688	127.16228	124.43561	125.00662	130.20161	132.6628
22	0.75862	125.54	127.28125	128.58333	123.27273	124.27483	129.32259	132.34883
23	0.7931	125.515	128.59688	128.75	122.64015	126.82119	128.32259	131.89536
24	0.82759	125.13	128.51875	128.49562	122.43182	131.04967	127.26613	130.90698
25	0.86207	124.515	128.55313	128.19298	127.06061	131.02649	126.45968	129.32558
26	0.89655	122.95	128.19063	127.64035	128.92802	130.75829	126.18549	129.30232

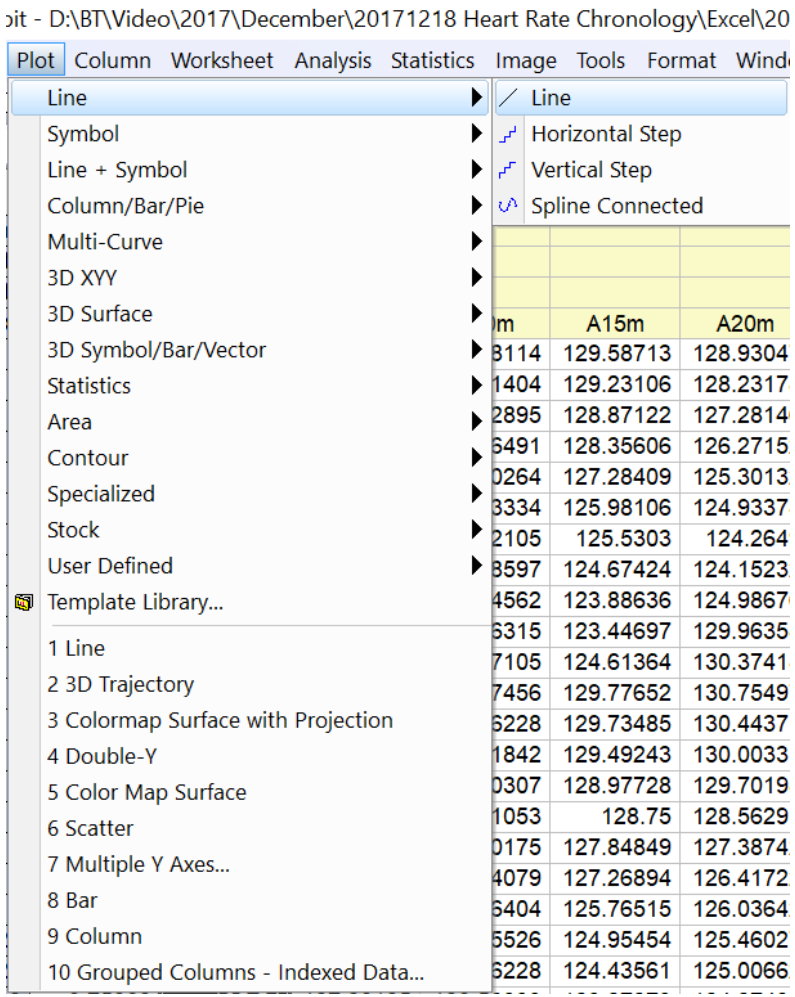
Sheet1

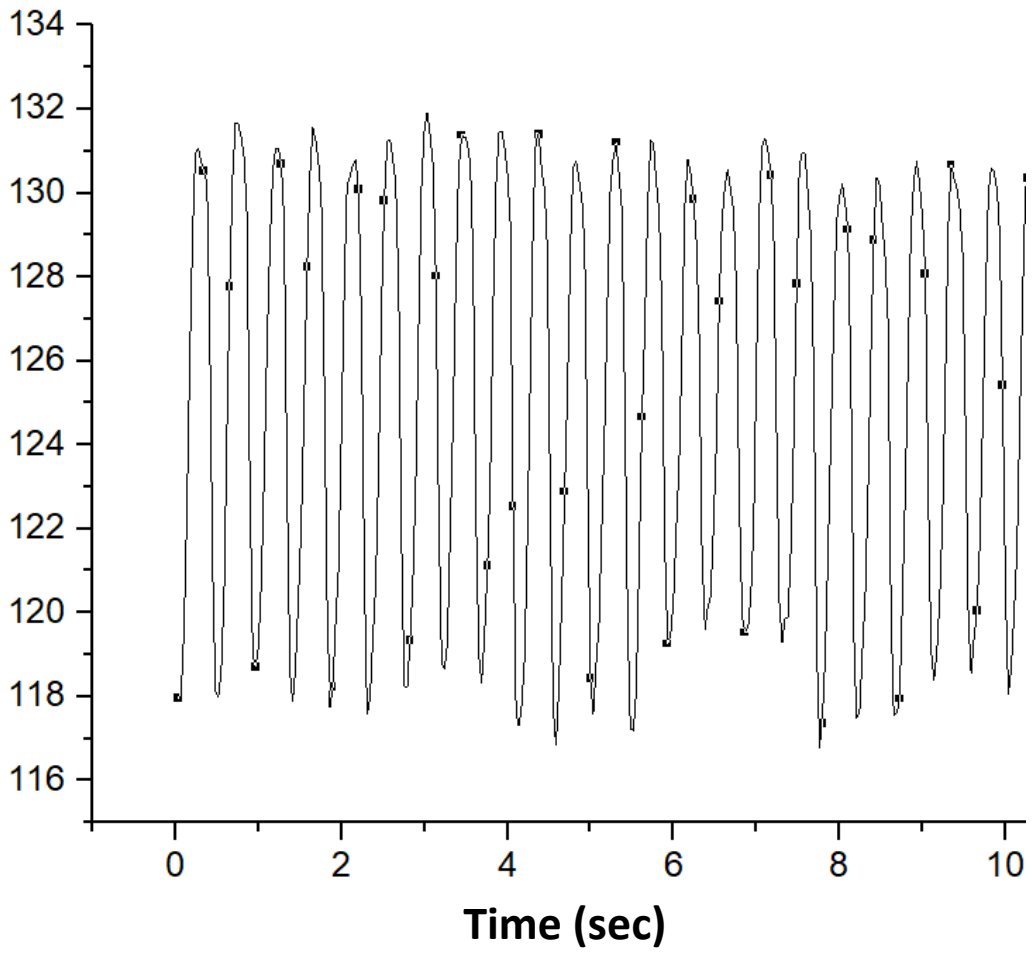
- The data matrix is directly copy from Excel and paste to Origin 9.1
- In this case, we perform time chronology analysis of zebrafish embryo heartbeat for 30 min, and calculate the bmp (beat per min) at 0, 5, 10, 15, 20, 25 and 30 min time points.

Make Graphic by using Origin 9.1

	A(X)	B(Y)
Long Name		
Units		
Comments		
F(x)	Time	A0m
1	0.03448	117.945
2	0.06897	117.985
3	0.10345	117.705
4	0.13793	116.99
5	0.17241	117.82
6	0.2069	122.01
7	0.24138	124.96
8	0.27586	125.595
9	0.31034	125.495
10	0.34483	124.975
11	0.37931	124.545
12	0.41379	123.485
13	0.44828	121.5
14	0.48276	120.405
15	0.51724	118.97
16	0.55172	118.45
17	0.58621	118.615
18	0.62069	117.65
19	0.65517	118.66
20	0.68966	122.85
21	0.72414	125.17
22	0.75862	125.54
23	0.7931	125.515
24	0.82759	125.13
25	0.86207	124.515
26	0.89655	122.95

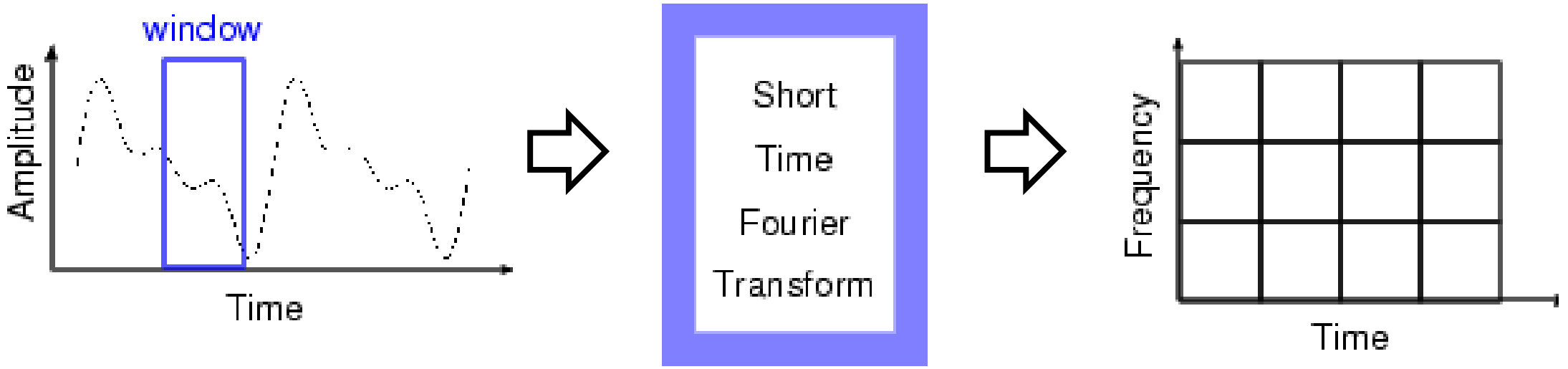
Sheet1



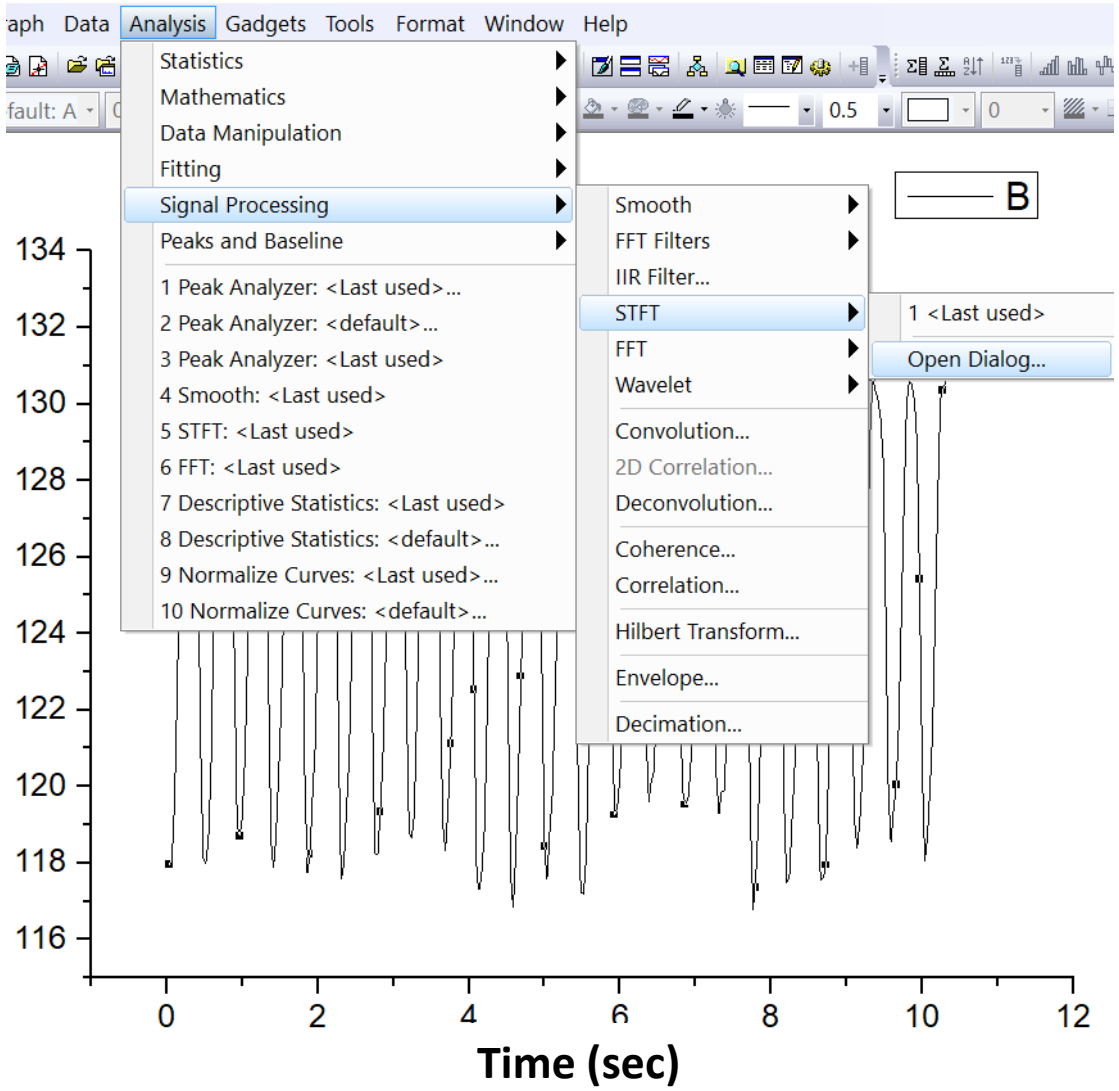


ORIGIN 9.1
Data Analysis and Graphing Software

Perform Short-time Fourier transform (STFT) to get heart beat frequency over time changes

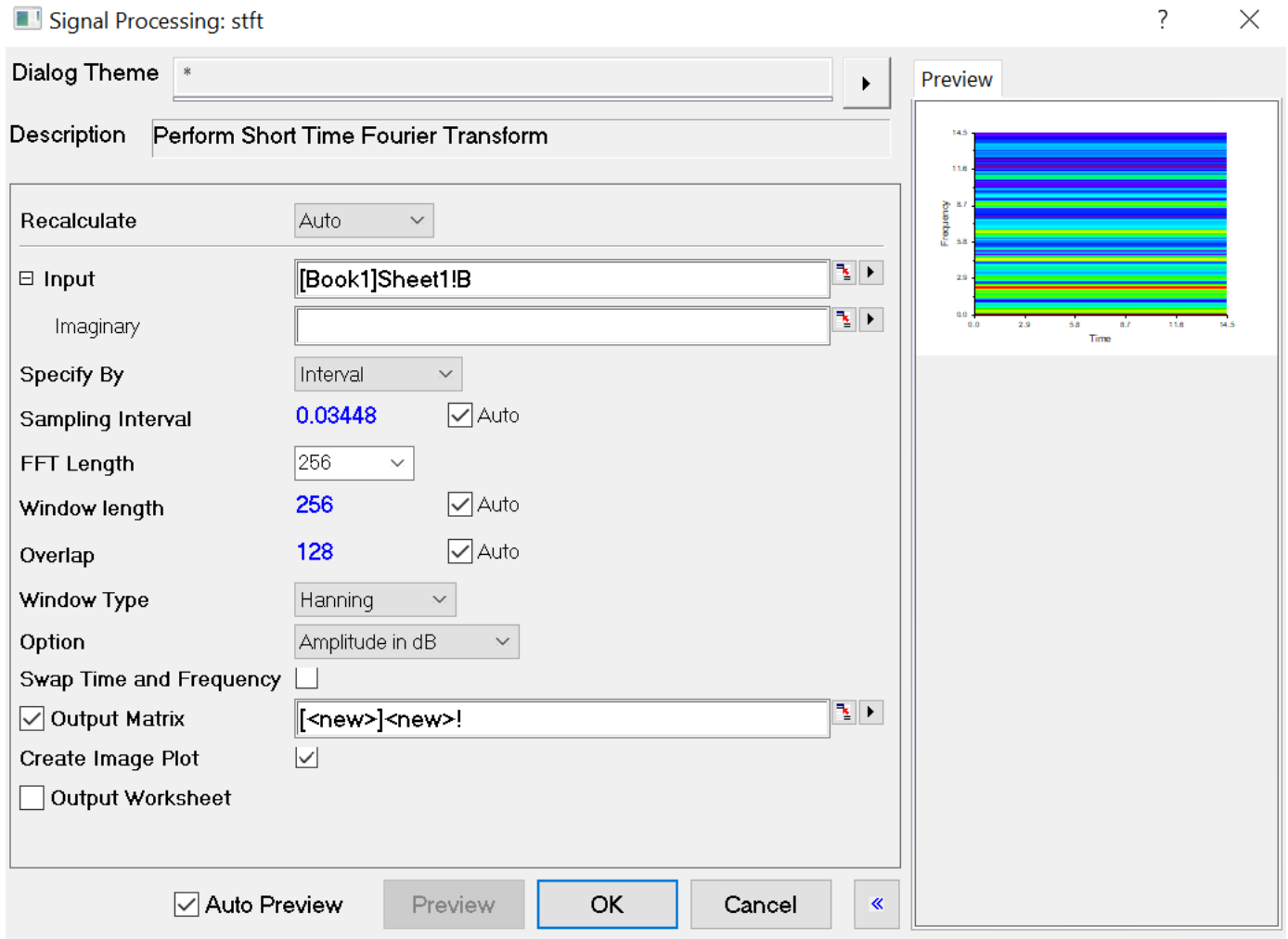


Perform STFT by using Origin 9.1 software



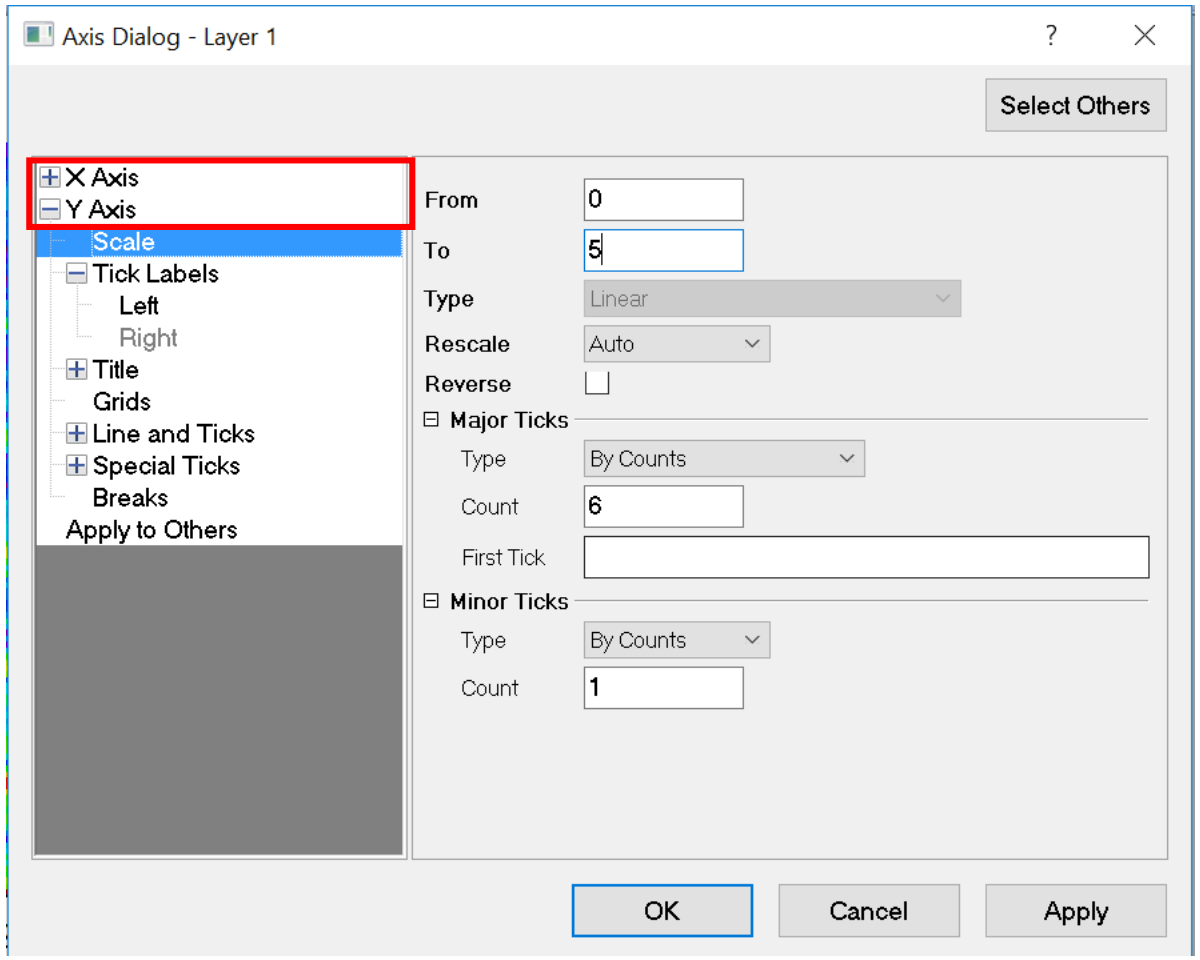
- Click Analysis -> Signal Processing -> STFT -> Open Dialog (for new setup) or <Last used> for previous setup

Perform STFT by using Origin 9.1 software



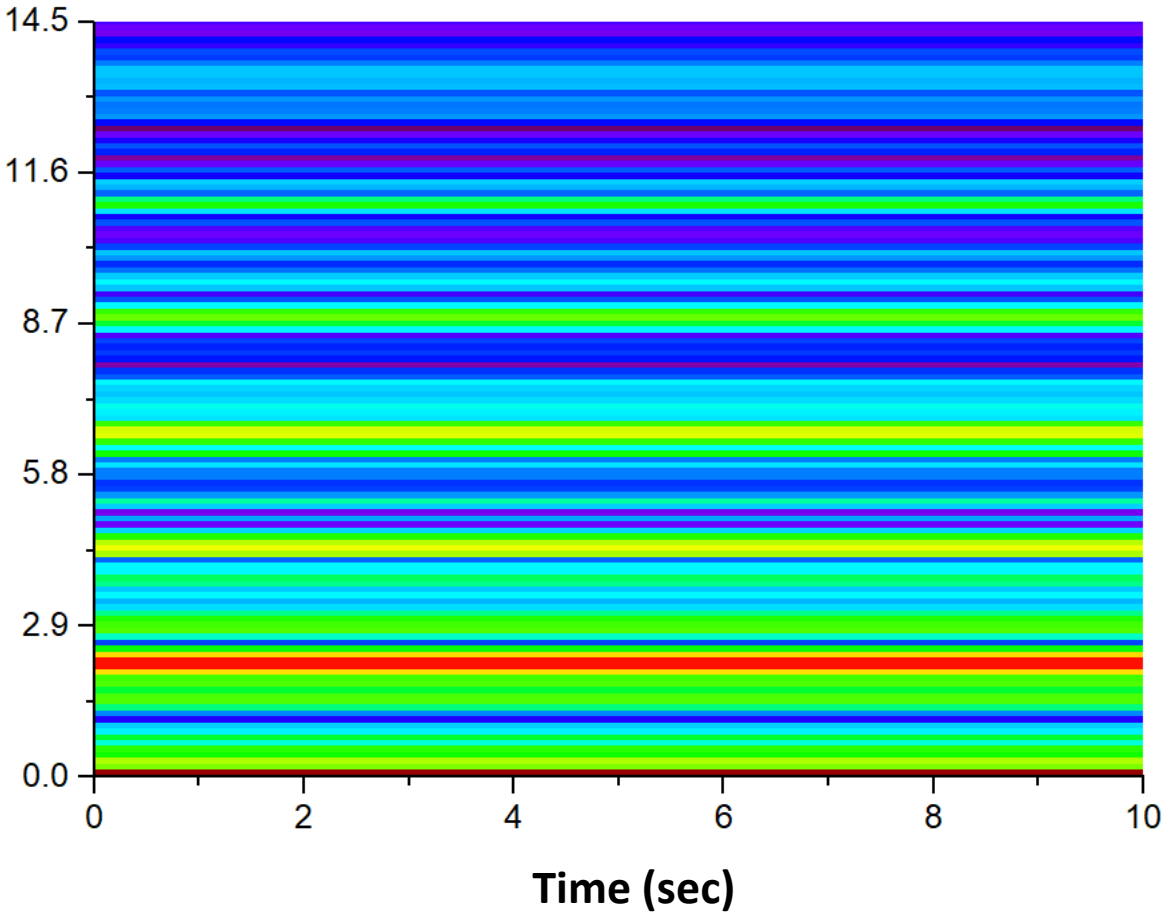
- Change the setup based on the need
- On this experiment, no need to change the parameter (we use the default setting)

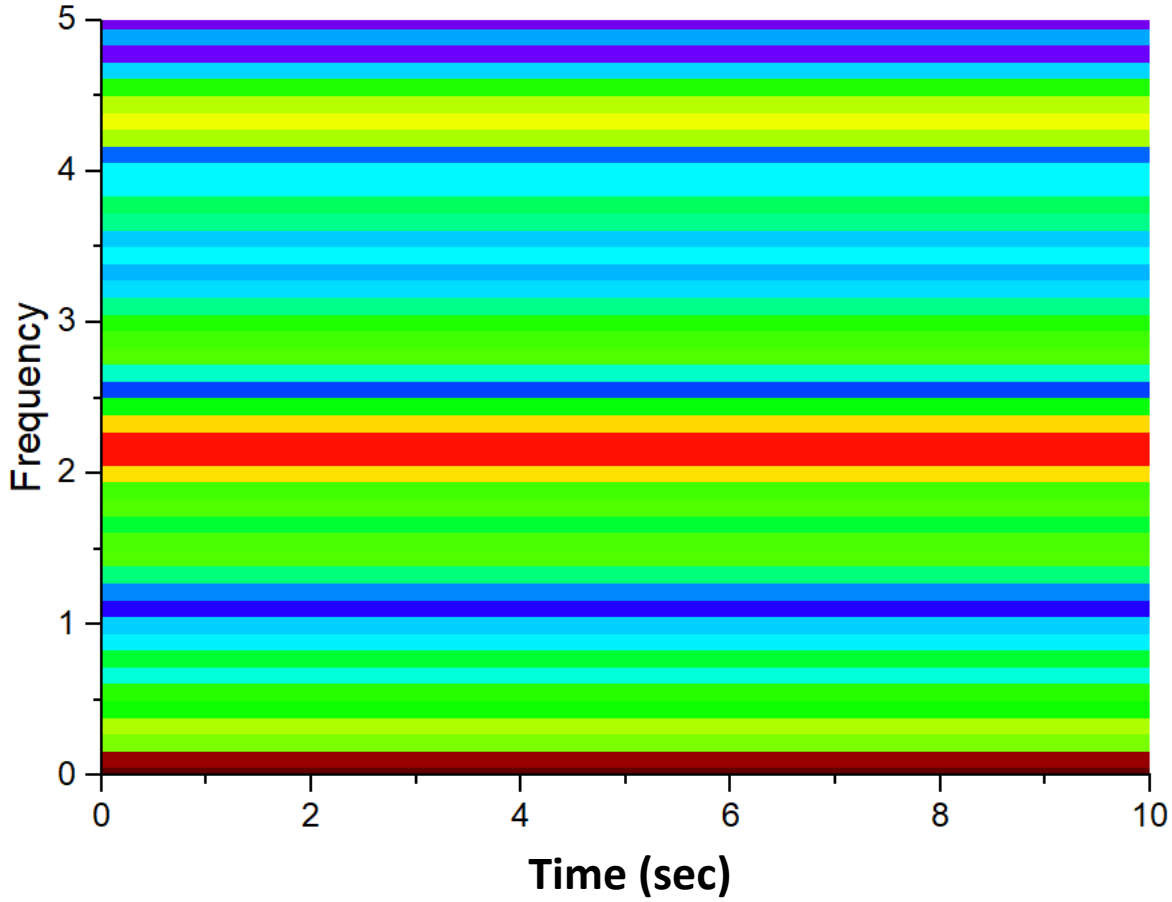
Change Scale in STFT Result



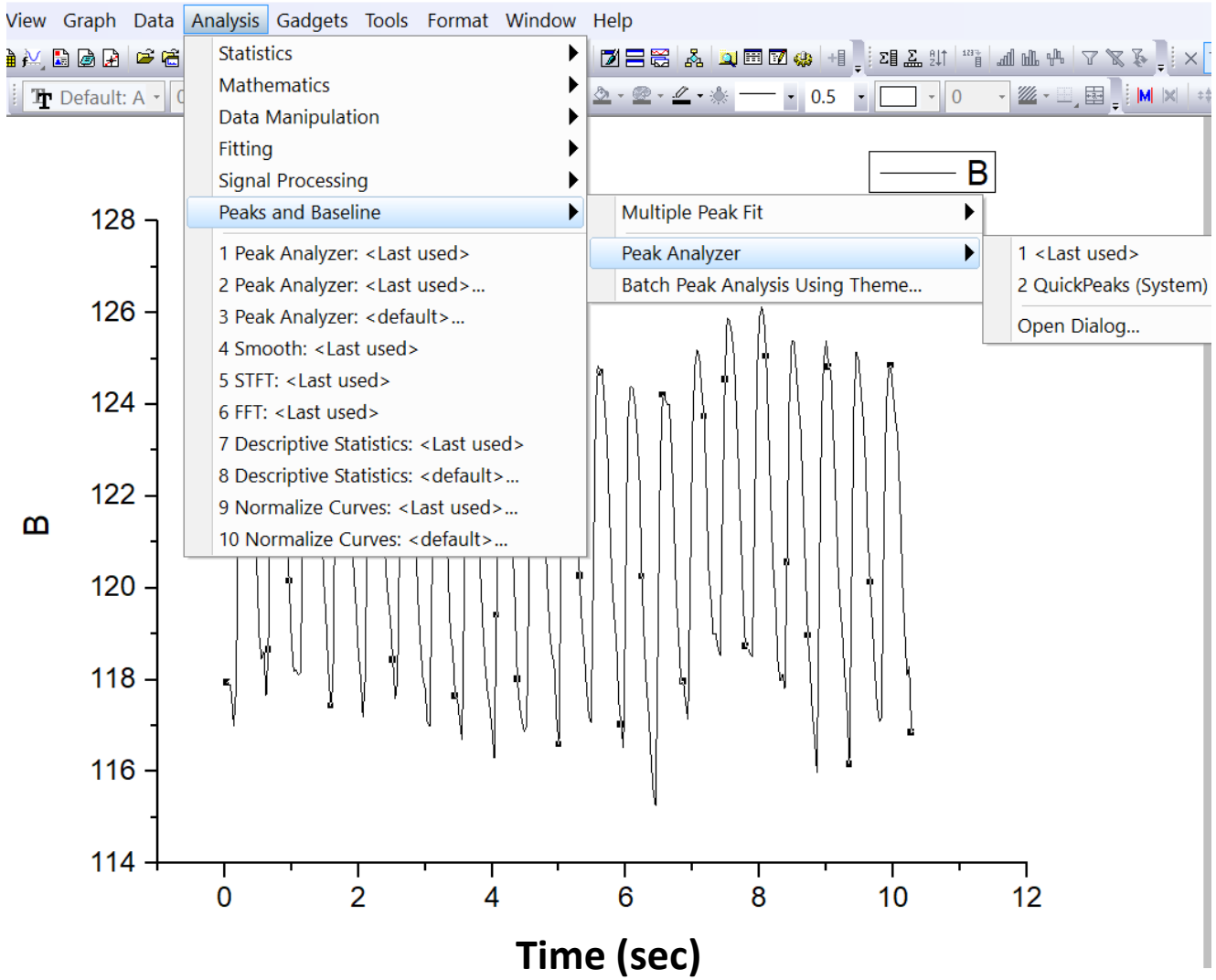
- When the scale range is too wide, it can be adjusted
- To change the X and Y scale double click on the graphic result

Changed Scale STFT Result



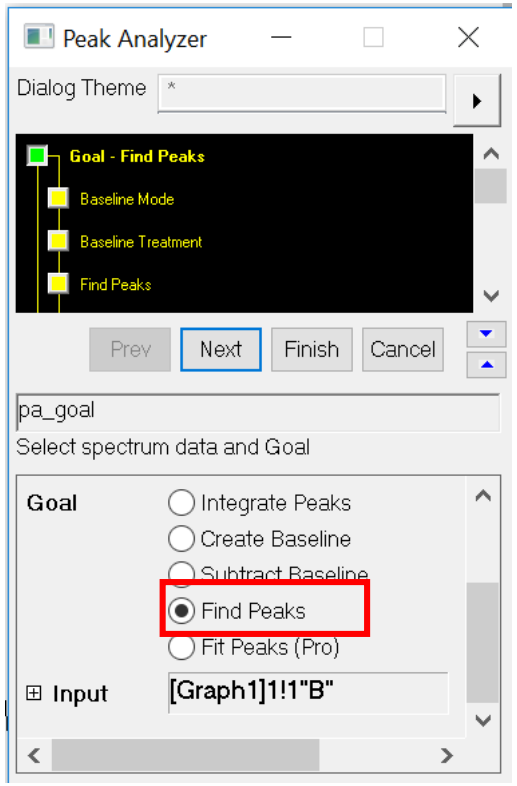


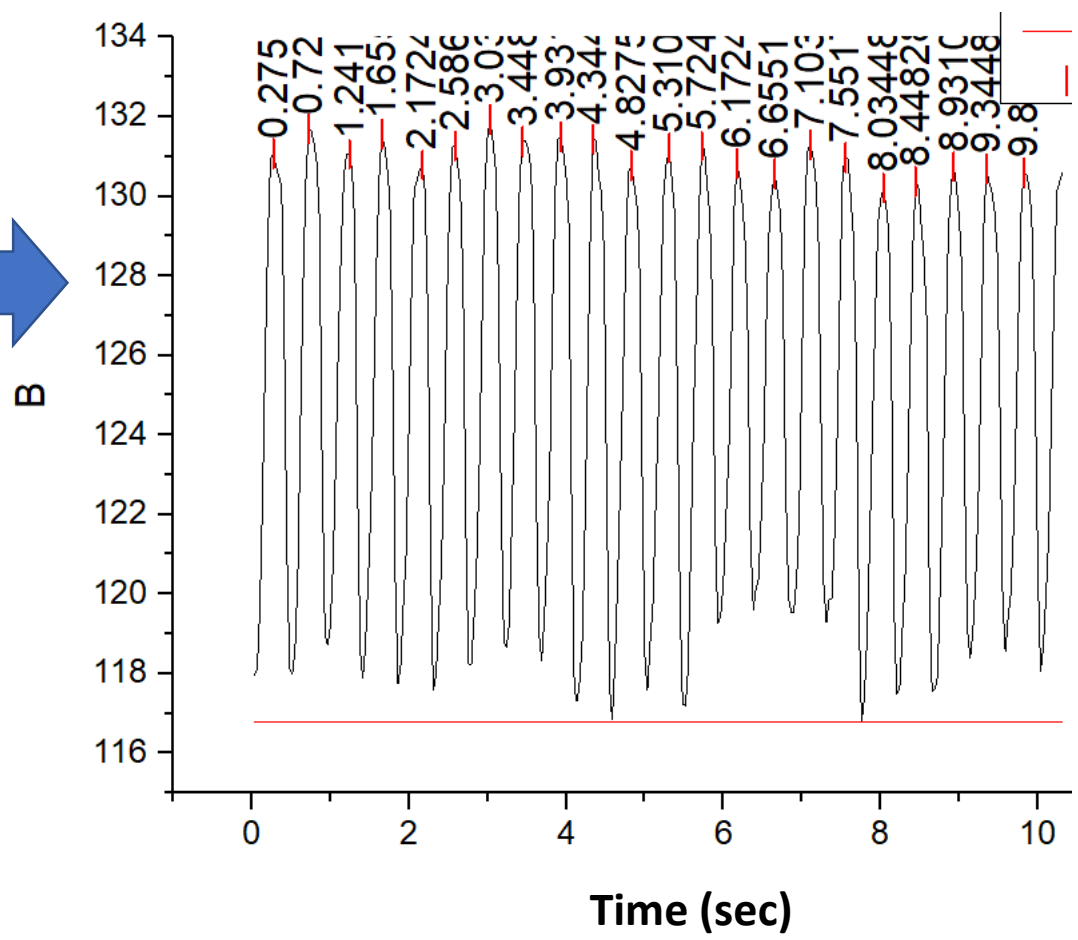
Use Peak Analyzer to detect the peak interval



- After select graphic, in tool box, use Analysis -> Peaks and Baseline -> Peak Analyzer -> Open Dialog (for new setup) or Last used (for previous setup)

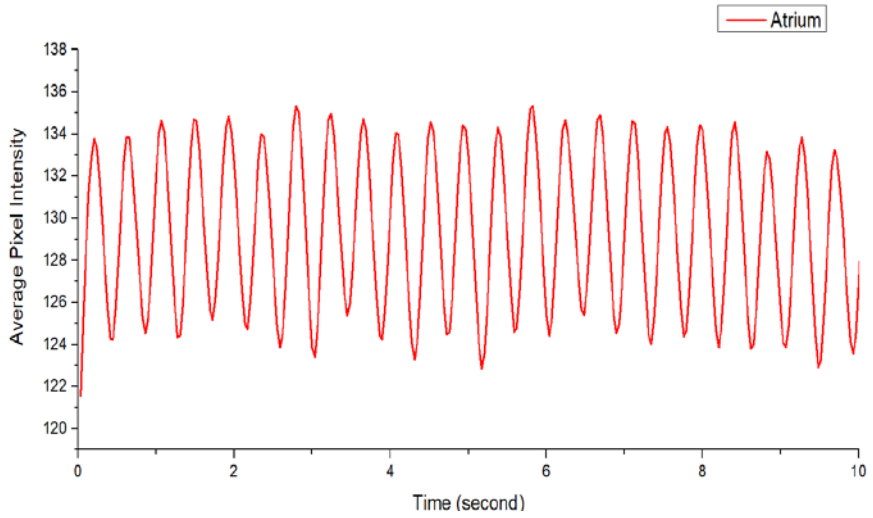
Setting up Peak Analyzer

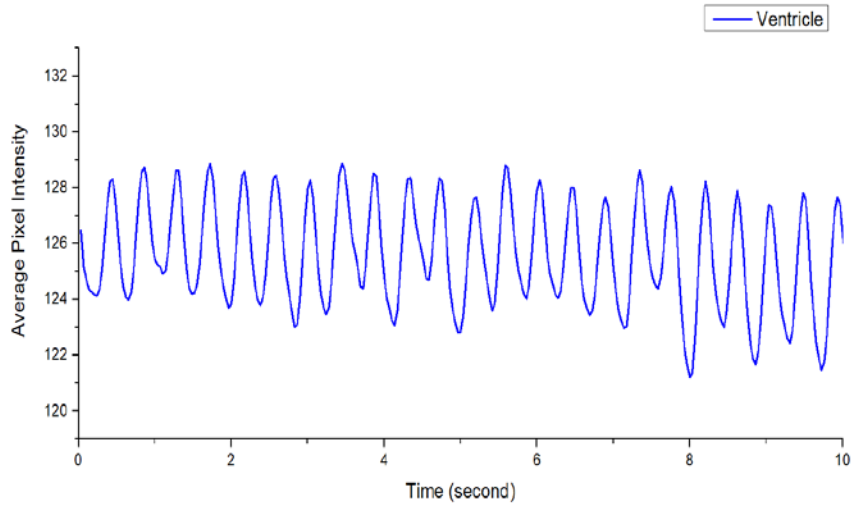


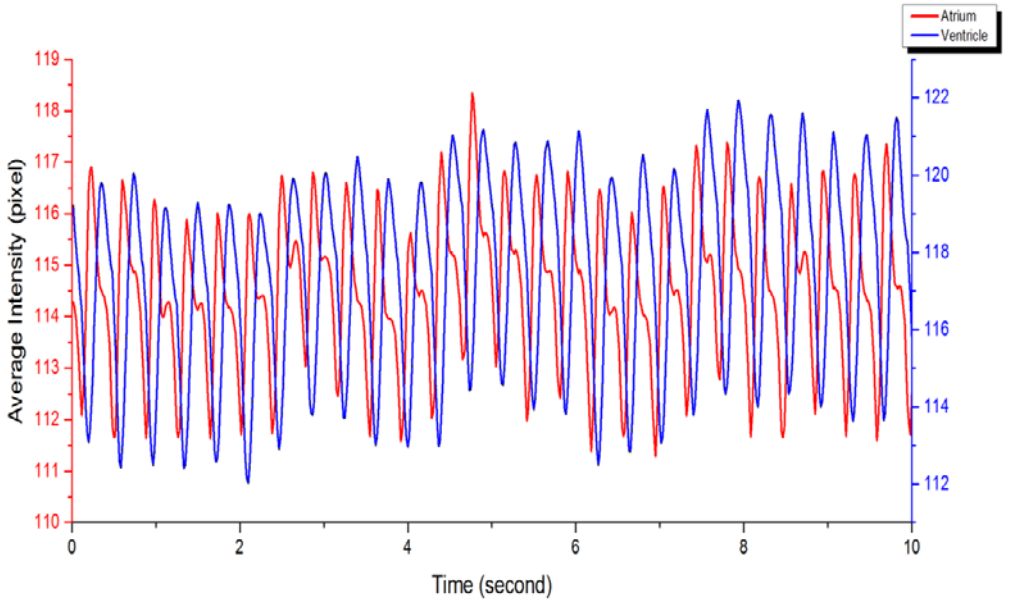


- To obtain time and intensity of each peak, we use “Find Peaks” function

Merge atrium and ventricle rhythm







Copy the Data for Further Analysis

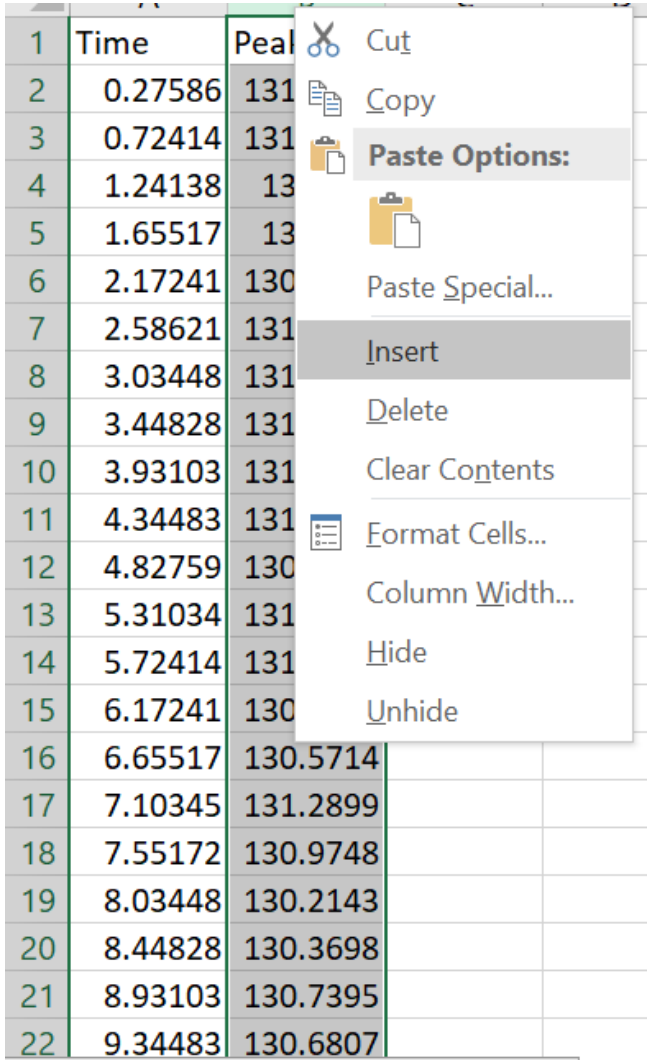
	pcx(X)	pcy(Y)
Long Name	X	Y
Units		
Comments	Peak Centers of B	Peak Centers of B
F(x)		
1	0.27586	131.0714
2	0.72414	131.6807
3	1.24138	131.063
4	1.65517	131.563
5	2.17241	130.7899
6	2.58621	131.2689
7	3.03448	131.9286
8	3.44828	131.3782
9	3.93103	131.4874
10	4.34483	131.4328
11	4.82759	130.7731
12	5.31034	131.2143
13	5.72414	131.2605
14	6.17241	130.8151
15	6.65517	130.5714
16	7.10345	131.2899
17	7.55172	130.9748
18	8.03448	130.2143
19	8.44828	130.3698
20	8.93103	130.7395
21	9.34483	130.6807
22	9.82759	130.5966
23		
24		
25		

Sheet1 Baseline_Data1 **Peak_Centers**

	Time	PeakA0m
2	0.27586	131.0714
3	0.72414	131.6807
4	1.24138	131.063
5	1.65517	131.563
6	2.17241	130.7899
7	2.58621	131.2689
8	3.03448	131.9286
9	3.44828	131.3782
10	3.93103	131.4874
11	4.34483	131.4328
12	4.82759	130.7731
13	5.31034	131.2143
14	5.72414	131.2605
15	6.17241	130.8151
16	6.65517	130.5714
17	7.10345	131.2899
18	7.55172	130.9748
19	8.03448	130.2143
20	8.44828	130.3698
21	8.93103	130.7395
22	9.34483	130.6807

- Minimize the graphic and open the sheet
- Change the window into Peak_Centers
- Copy X and Y data and paste into Excel.
- X as the peak time and Y as peak intensity

Insert New Column in Excel



- Insert new column between time and peak to calculate time interval

Calculate Time Interval

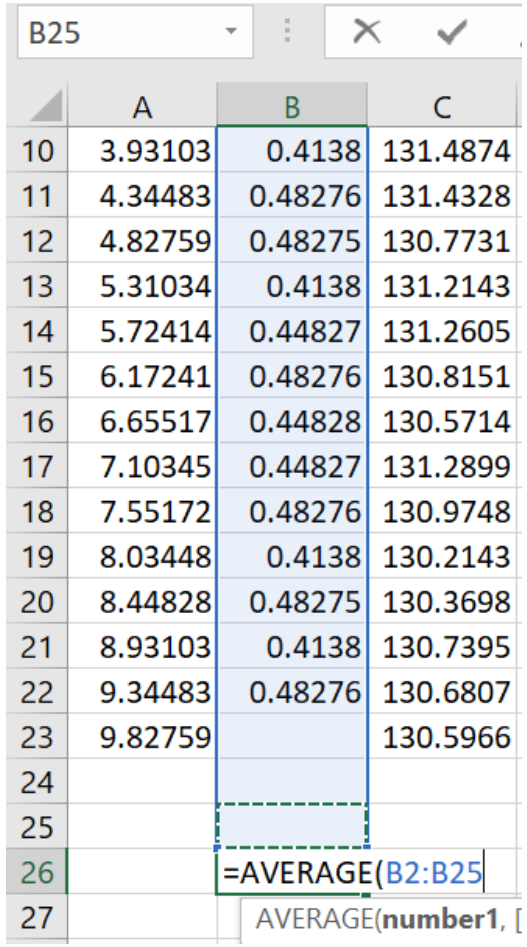
A2 | =A3-A2

	A	B	C	D	E
1	Time	Time Inter	PeakA0m		
2	0.27586	=A3-A2	131.0714		
3	0.72414		131.6807		
4	1.24138		131.063		
5	1.65517		131.563		
6	2.17241		130.7899		
7	2.58621		131.2689		
8	3.03448		131.9286		
9	3.44828		131.3782		
10	3.93103		131.4874		
11	4.34483		131.4328		
12	4.82759		130.7731		
13	5.31034		131.2143		
14	5.72414		131.2605		
15	6.17241		130.8151		
16	6.65517		130.5714		
17	7.10345		131.2899		
18	7.55172		130.9748		
19	8.03448		130.2143		
20	8.44828		130.3698		
21	8.93103		130.7395		
22	9.34483		130.6807		

	A	B	C
10	3.93103	0.4138	131.4874
11	4.34483	0.48276	131.4328
12	4.82759	0.48275	130.7731
13	5.31034	0.4138	131.2143
14	5.72414	0.44827	131.2605
15	6.17241	0.48276	130.8151
16	6.65517	0.44828	130.5714
17	7.10345	0.44827	131.2899
18	7.55172	0.48276	130.9748
19	8.03448	0.4138	130.2143
20	8.44828	0.48275	130.3698
21	8.93103	0.4138	130.7395
22	9.34483	0.48276	130.6807
23	9.82759	-9.82759	130.5966

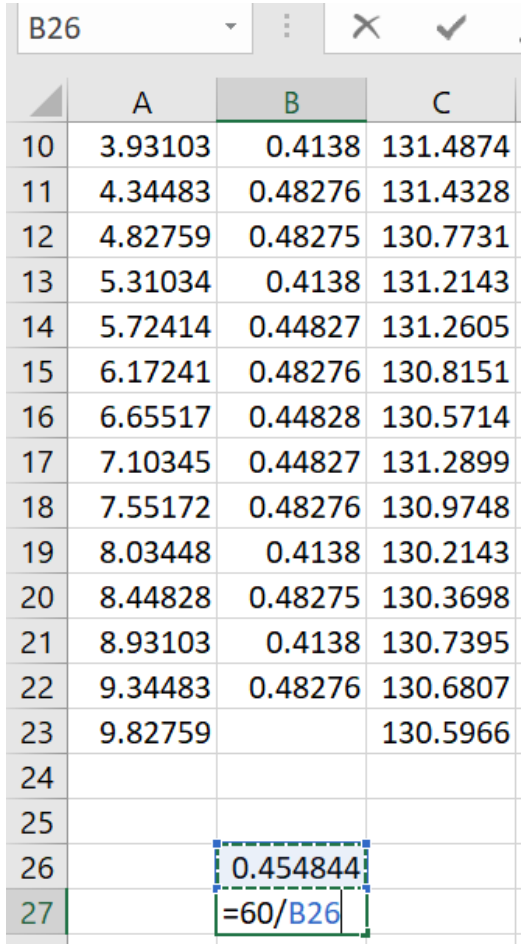
- In time interval column, use formula: =(A3-A2) or two time point that want to be analysed
- Drag down the formula and delete the last calculation as it will show false result

Calculate Time Interval and Beat per Minute



	A	B	C
10	3.93103	0.4138	131.4874
11	4.34483	0.48276	131.4328
12	4.82759	0.48275	130.7731
13	5.31034	0.4138	131.2143
14	5.72414	0.44827	131.2605
15	6.17241	0.48276	130.8151
16	6.65517	0.44828	130.5714
17	7.10345	0.44827	131.2899
18	7.55172	0.48276	130.9748
19	8.03448	0.4138	130.2143
20	8.44828	0.48275	130.3698
21	8.93103	0.4138	130.7395
22	9.34483	0.48276	130.6807
23	9.82759		130.5966
24			
25			
26		=AVERAGE(B2:B25	
27			

AVERAGE(**number1**, [



	A	B	C
10	3.93103	0.4138	131.4874
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14	5.72414	0.44827	131.2605
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20	8.44828	0.48275	130.3698
21	8.93103	0.4138	130.7395
22	9.34483	0.48276	130.6807
23	9.82759		130.5966
24			
25			
26		0.454844	
27		=60/B26	

- Calculate average time interval by using the formula: =AVERAGE(B2:B25) or the average of selected time interval
- Calculate the average heart rate (bpm) by using the formula: =60/average time interval

Combine all the data in Excel

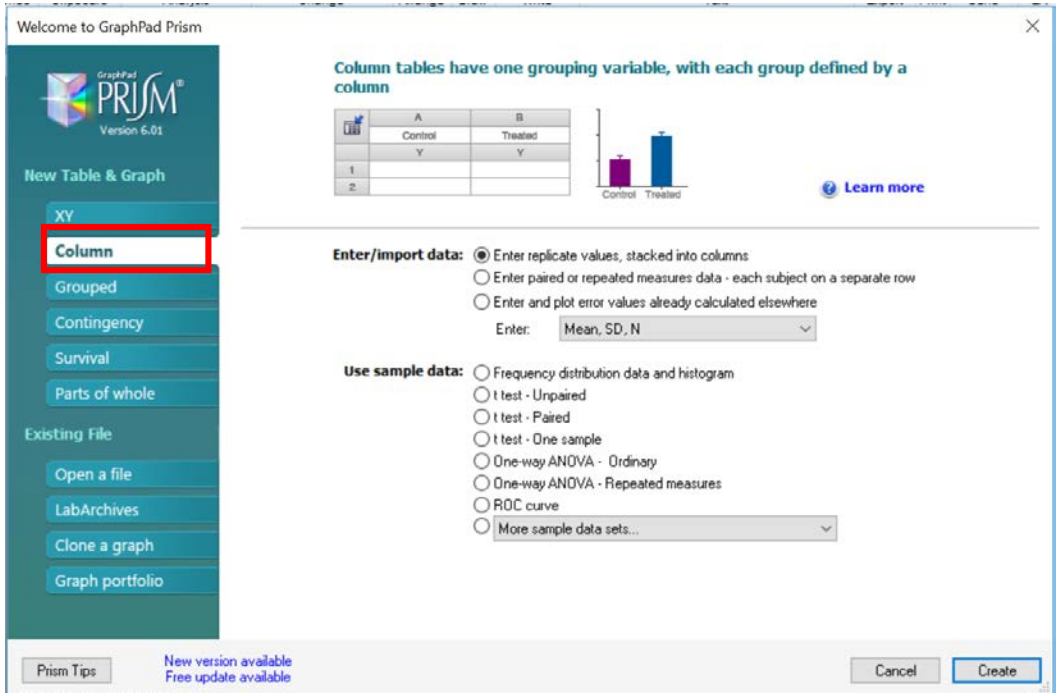
L2 =AVERAGE(B2:K2)

	A	B	C	D	E	F	G	H	I	J	K	L
1		Fish1	Fish2	Fish3	Fish4	Fish5	Fish6	Fish7	Fish8	Fish9	Fish10	Average
2	A0m	0.402299	0.422789	0.474549	0.424288	0.494828	0.467242	0.45156	0.416792	0.466338	0.536398	0.455708
3	A5m	0.422789	0.388966	0.472414	0.488203	0.482759	0.403735	0.505747	0.444992	0.488204	0.545639	0.464345
4	A10m	0.45156	0.440439	0.517242	0.55173	0.534483	0.41979	0.542146	0.495463	0.51341	0.555781	0.502204
5	A15m	0.491833	0.498276	0.545639	0.662561	0.509982	0.46798	0.577586	0.517242	0.545639	0.578094	0.539483
6	A20m	0.471264	0.553752	0.630541	0.650575	0.536398	0.499093	0.616092	0.559838	0.625287	0.655172	0.579801
7	A25m	0.477586	0.545639	0.634483	0.669951	0.577012	0.679045	0.625287	0.5625	0.616092	0.732759	0.612035
8	A30m	0.484574	0.545977	0.625287	0.627587	0.672414	0.606896	0.645321	0.565923	0.593103	0.836991	0.620407
9												
10	V0m	0.402759	0.422789	0.474549	0.421289	0.499093	0.467241	0.45156	0.416792	0.467242	0.535496	0.455881
11	V5m	0.424288	0.388965	0.471264	0.547667	0.509982	0.402299	0.501724	0.446634	0.486388	0.545639	0.472485
12	V10m	0.456486	0.438871	1.030651	0.58215	0.536398	0.418291	0.542146	0.992337	0.515326	0.557809	0.607047
13	V15m	0.605911	0.996552	1.091954	1.374384	0.543611	0.934866	1.155173	1.094828	1.090518	0.868965	0.975676
14	V20m	0	0	0	0	0	0	0	0	0	0	0
15	V25m	0	0	0	0	0	0	0	0	0	0	0
16	V30m	0	0	0	0	0	0	0	0	0	0	0
17												
18												
19												
20												
21												
22												

... 5uM Astemizole BPM Chronology **5uM Astemizole Time Interval** Control BPM Chronology C ...

- Combine all the data to make it easier to calculate the result

Select Table & Graph Format



- For endpoint analysis use “Column”
- For time chronology analysis use “Grouped”

Copy the results from Excel to GraphPad Prism

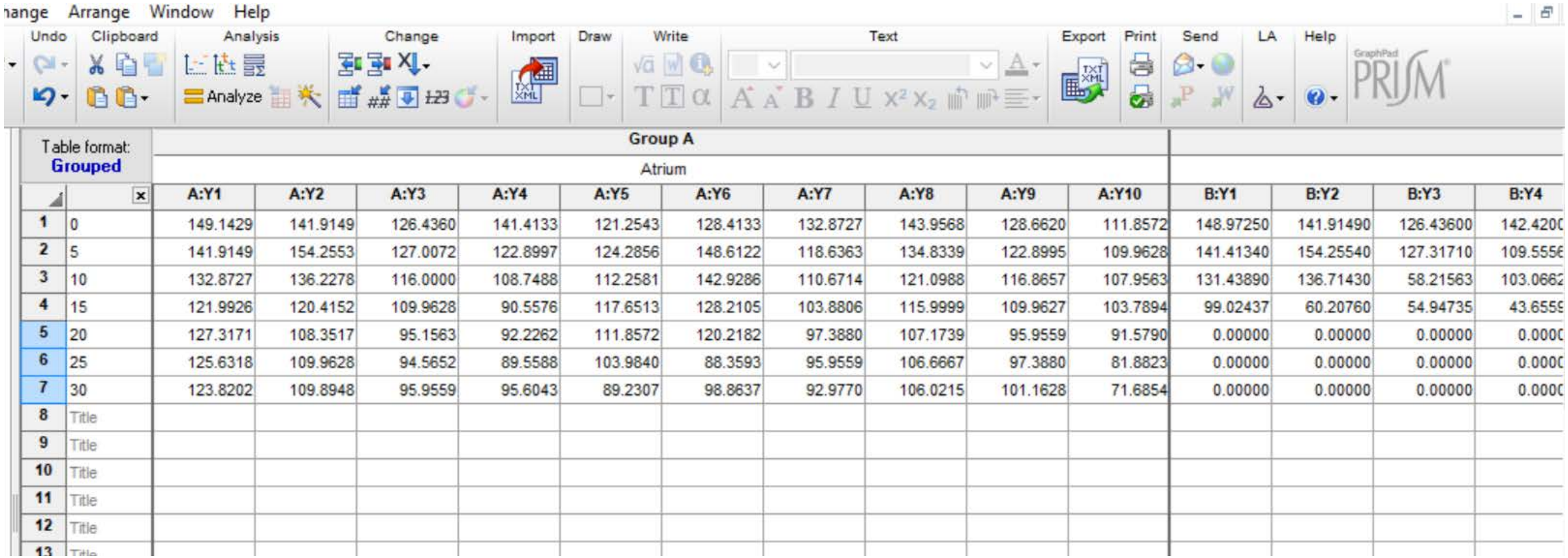


Table format: Grouped		Group A													
		Atrium													
		A:Y1	A:Y2	A:Y3	A:Y4	A:Y5	A:Y6	A:Y7	A:Y8	A:Y9	A:Y10	B:Y1	B:Y2	B:Y3	B:Y4
1	0	149.1429	141.9149	126.4360	141.4133	121.2543	128.4133	132.8727	143.9568	128.6620	111.8572	148.97250	141.91490	126.43600	142.4200
2	5	141.9149	154.2553	127.0072	122.8997	124.2856	148.6122	118.6363	134.8339	122.8995	109.9628	141.41340	154.25540	127.31710	109.5556
3	10	132.8727	136.2278	116.0000	108.7488	112.2581	142.9286	110.6714	121.0988	116.8657	107.9563	131.43890	136.71430	58.21563	103.0662
4	15	121.9926	120.4152	109.9628	90.5576	117.6513	128.2105	103.8806	115.9999	109.9627	103.7894	99.02437	60.20760	54.94735	43.6559
5	20	127.3171	108.3517	95.1563	92.2262	111.8572	120.2182	97.3880	107.1739	95.9559	91.5790	0.00000	0.00000	0.00000	0.0000
6	25	125.6318	109.9628	94.5652	89.5588	103.9840	88.3593	95.9559	106.6667	97.3880	81.8823	0.00000	0.00000	0.00000	0.0000
7	30	123.8202	109.8948	95.9559	95.6043	89.2307	98.8637	92.9770	106.0215	101.1628	71.6854	0.00000	0.00000	0.00000	0.0000
8	Title														
9	Title														
10	Title														
11	Title														
12	Title														
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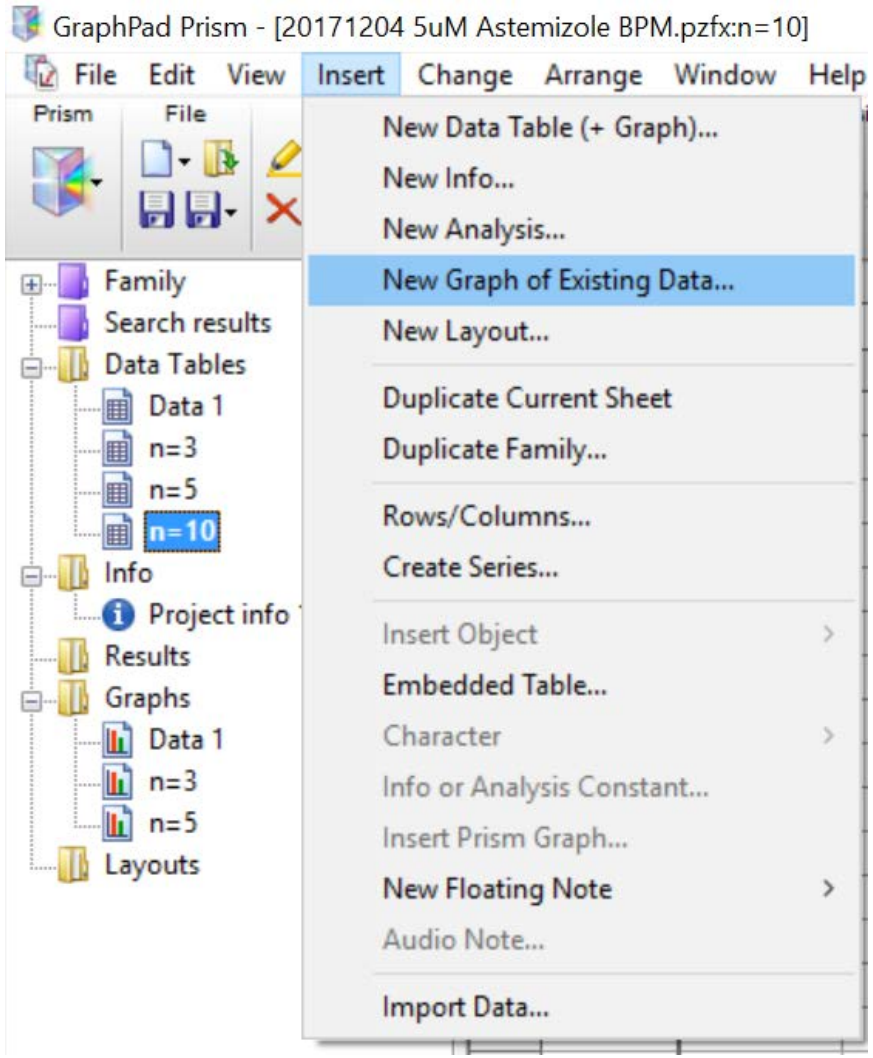
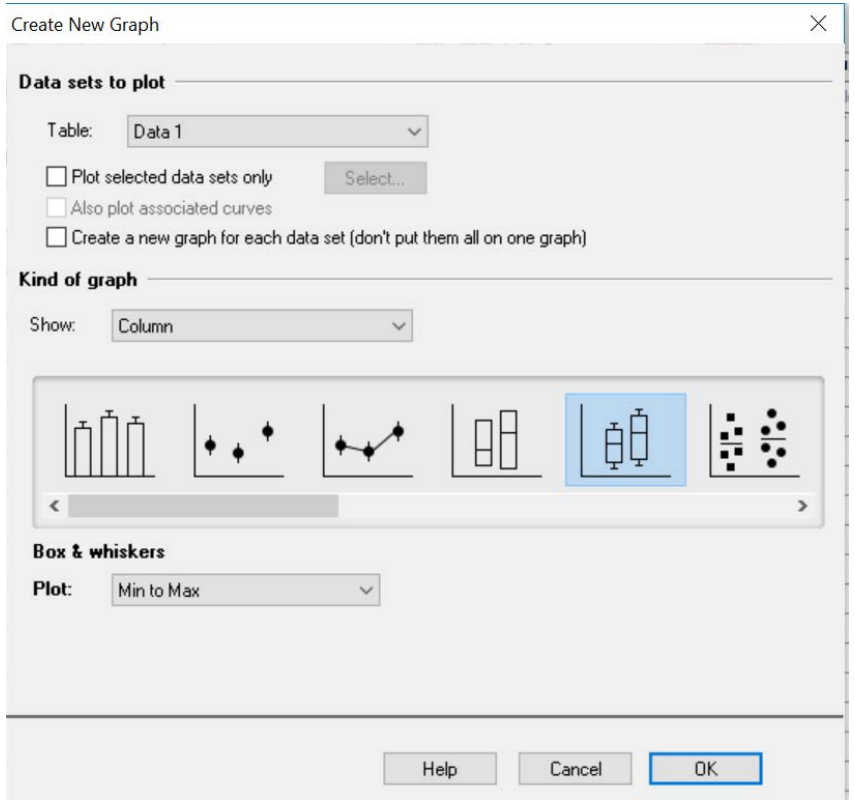
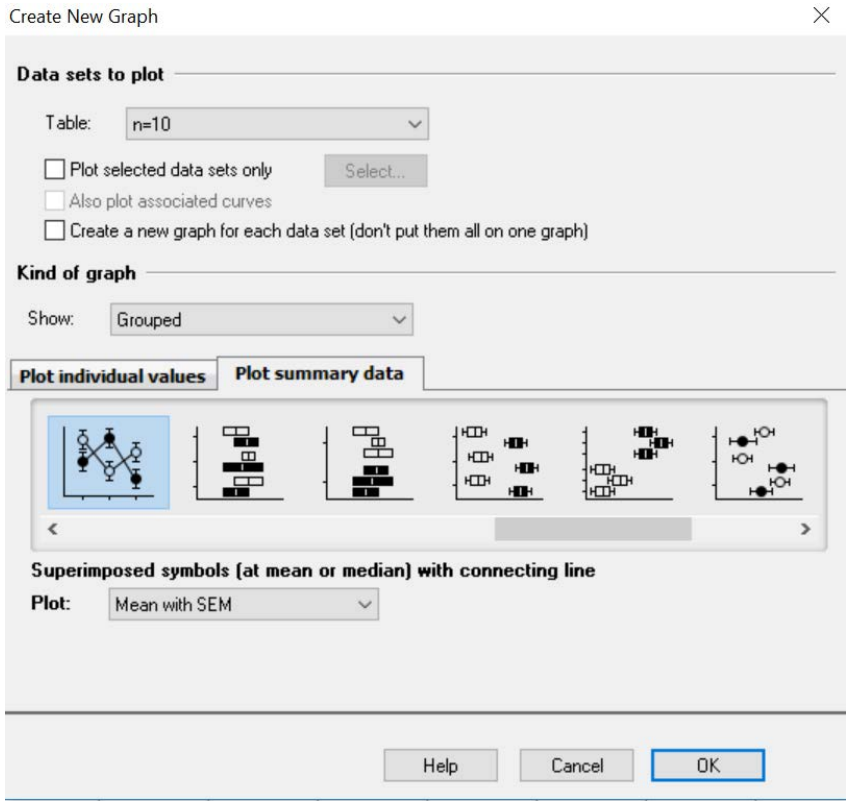
Create Graph from The Data



- Click Insert -> New Graph of Existing Data

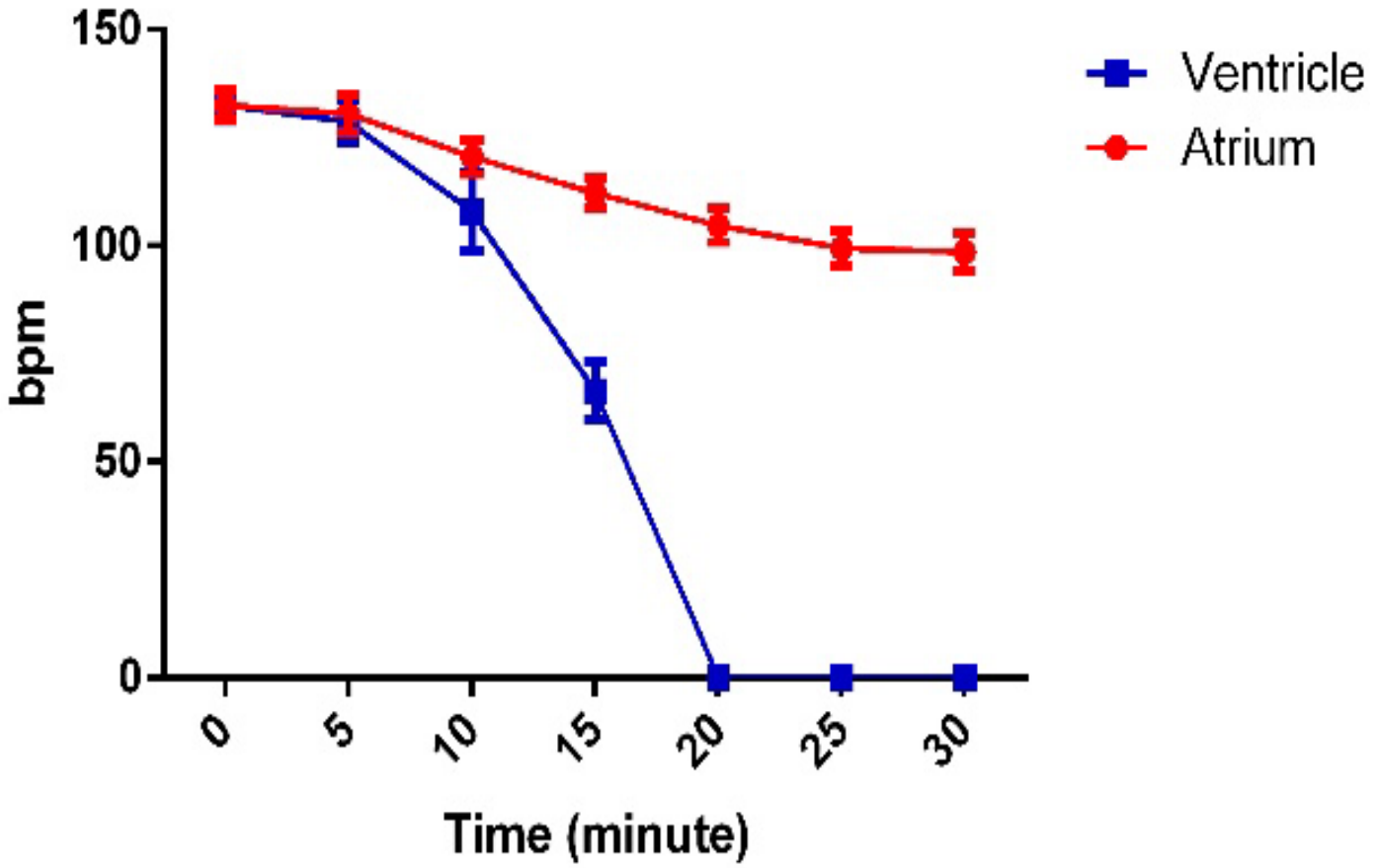
Select Plot Summary Data Type





- For endpoint analysis use min to max plot (left)
- For time chronology analysis use Mean with SEM (right)

Select Plot Summary Data Type



- This data is time chronology analysis of cardiac rhythm after 5uM astemizole treatment in zebrafish embryos aged at 72 hpf