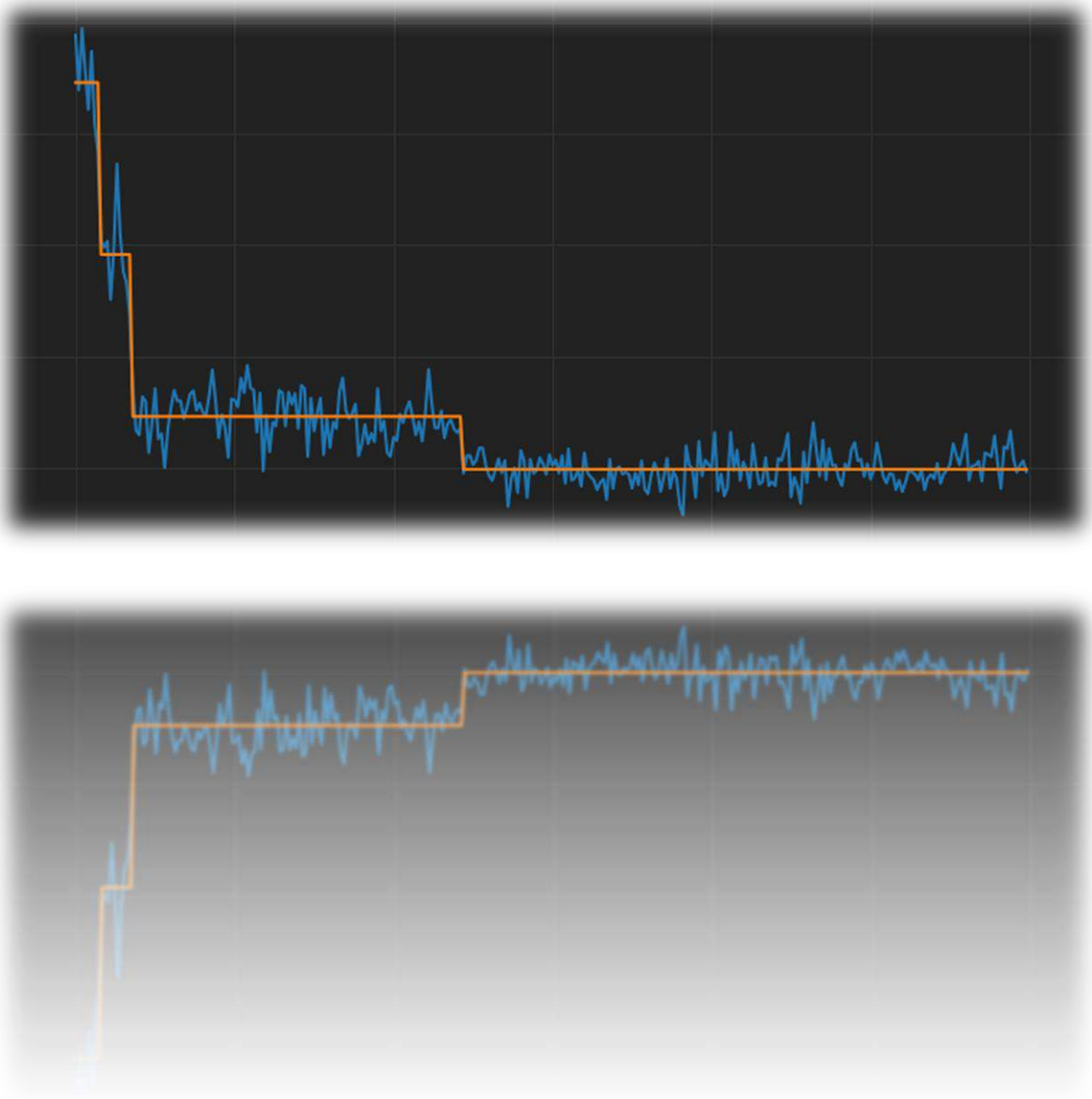
FluoroTensor v6.6.8r

User Guide



Contents

Chapter 1 – Getting Started

1.1 Prerequisites

FluoroTensor was developed in the Leicester Institute of Structural and Chemical Biology for studying stoichiometries of regulatory pre-mRNA splicing complexes.

Due to the *ad hoc* nature of FluoroTensor v6.6.8r there are a number of requirements that need to be met for it to be of optimal use. The recommended system requirements are shown below.

Recommended System Requirements

OS:	Windows 10 64 bit
CPU:	8th GEN intel i5-8500 / AMD Ryzen 5 3600XT or newer
RAM:	16GB DDR4 / 32GB DDR4 (recommended for large datasets.)
GPU:	(not required) / NVIDIA GTX 1070 (minimum for model training of datasets larger than 1M traces in reasonable time.)
Storage:	Minimum 1TB permanent storage for single molecule data (recommend SSD. Use permanent storage for fastest data load times instead of networked storage where possible.)
Software:	Microsoft Excel or equivalent program (such as open office) that can read .xlsx files and contains a numerical solver must be installed.
Monitor:	Minimum 1080p (1920x1080) display resolution.

In addition, there are requirements for data types, and limitations on the kind of analysed data that can be exported. FluoroTensor has been made available under the Creative Commons NonCommercial (CCNC) licence and as such, modifications and improvements to allow the program to perform outside the scope of its conception by non-commercial users are encouraged.

Colocalization Analysis

The colocalization and step detection suite allows accurate stoichiometry detection of up to 4 bleaching steps. The requirements of this component of the program are listed below:

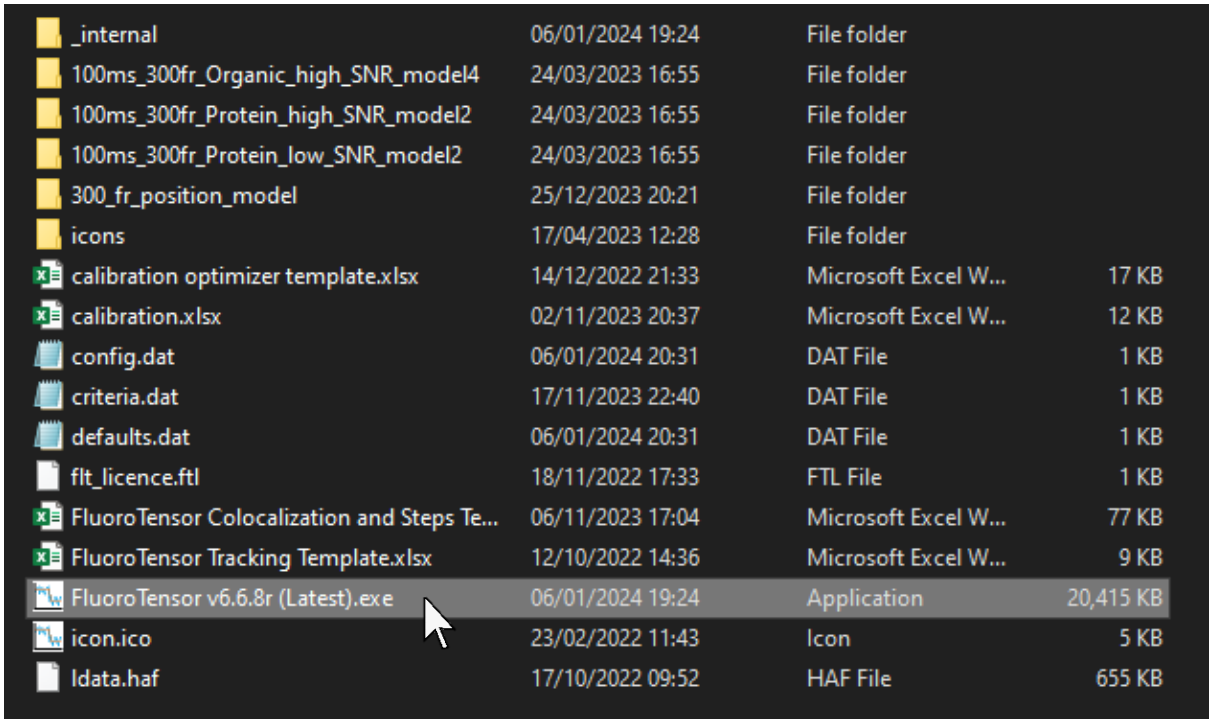
- Raw data for each colour channel must be contained within a single file in TIF (.tif or .tiff) format with consecutive frames in a 3D stack.
- Movies should be in 16-bit format such that the maximum intensity possible is $6.5x10^4$
- TIF files should be ordered by sequential illumination of each fluorophore delineated by the frame index rather than separated into higher dimensional channels. For example, a three-colour movie should be around 900 frames long with each channel consisting of 300 frames.
- The x, y resolution of TIF movies should be exactly 256x256 or exactly 512x512 pixels.
- The number of time points in each channel should be within 20% of the neural network domain. By default, this is 300 frames with the supplied models.
- Fluorophores in use should have similar stokes shift and emission wavelength difference to Cy5-mCherry-mEGFP and be loaded into the program in descending wavelength order such that the longest wavelength is the marker with respect to which colocalization is calculated (left channel in the display) and the shortest is in the right-hand channel (marked 488nm).
- The standard deviation in x and y of fluorescent signals in movies should be between 0.8 and 3 pixels (FWHM between 1.9 and 7.0 pixels) for optimal results.

Tracking

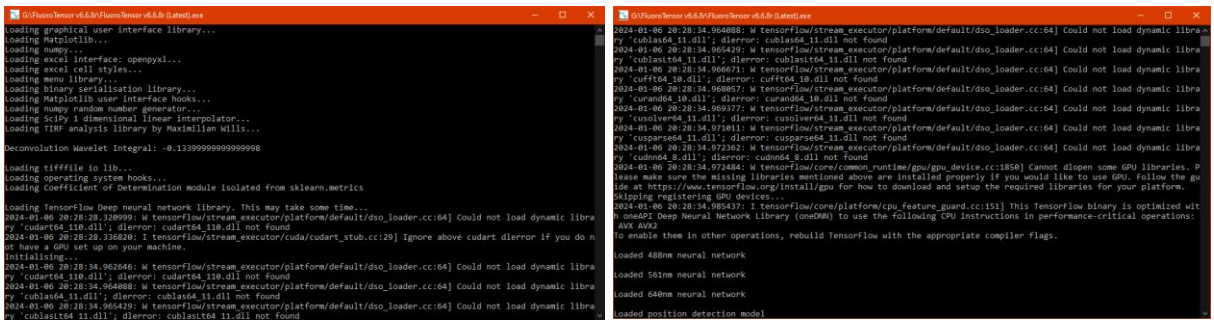
- Movies must be in TIF (.tif or .tiff) format as a 3D stack.
- Movies Should be any resolution less than or equal to 512x512.
- Channels should be split into separate TIF files.
- The standard deviation in x and y of fluorescent signals in movies should be between 0.7 and 3.5 pixels (FWHM between 1.6 and 8.2 pixels) for optimal results.

1.2 Running the Program from PyInstaller Executable (Recommended)

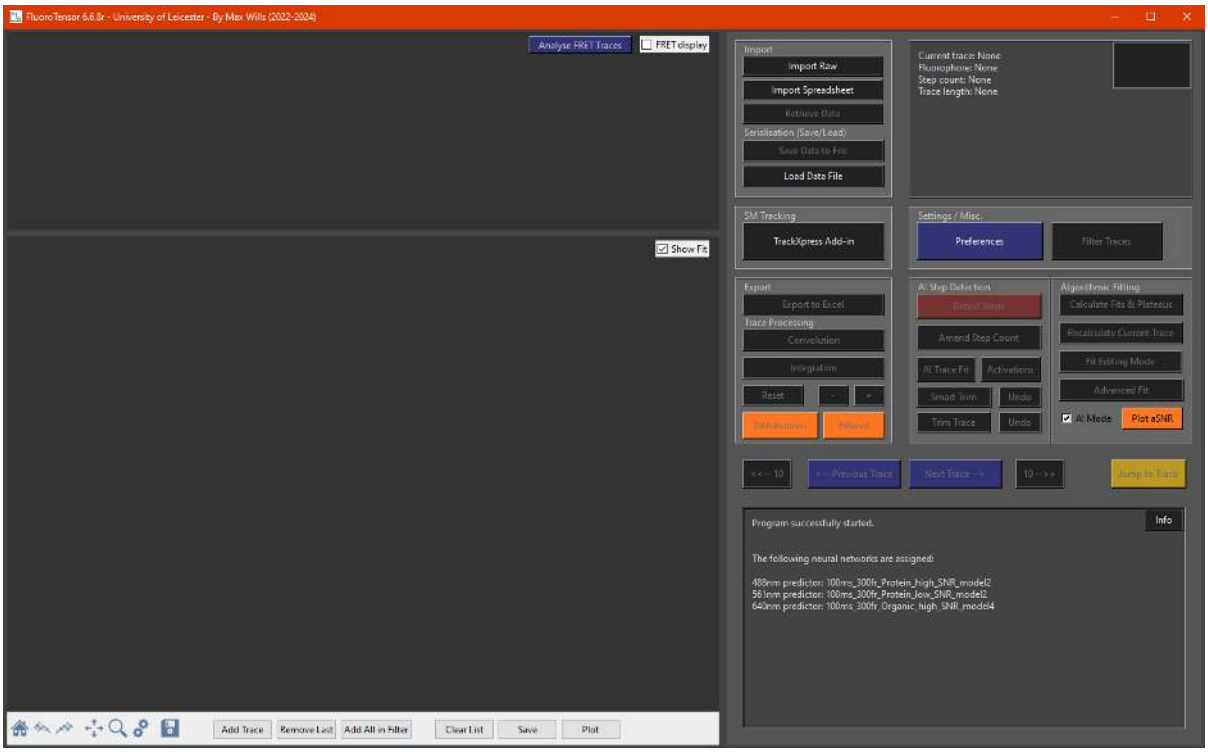
To run FluoroTensor from the executable (.exe) file on windows, download the zipped folder from https://github.com/LISCB/FluoroTensor. This folder contains everything you need to use FluoroTensor, export data and optimise calibration parameters providing you have met the prerequisites.



Within this folder are the Excel templates, settings, neural networks, libraries, and the executable. Running the executable (FluoroTensor v6.6.8r (Latest).exe), will open a terminal window in which debugging information is displayed. The first output in this console will be libraries being loaded. The string of TensorFlow errors can be safely ignored since they are concerned with GPU acceleration which is not required for FluoroTensor to run efficiently:

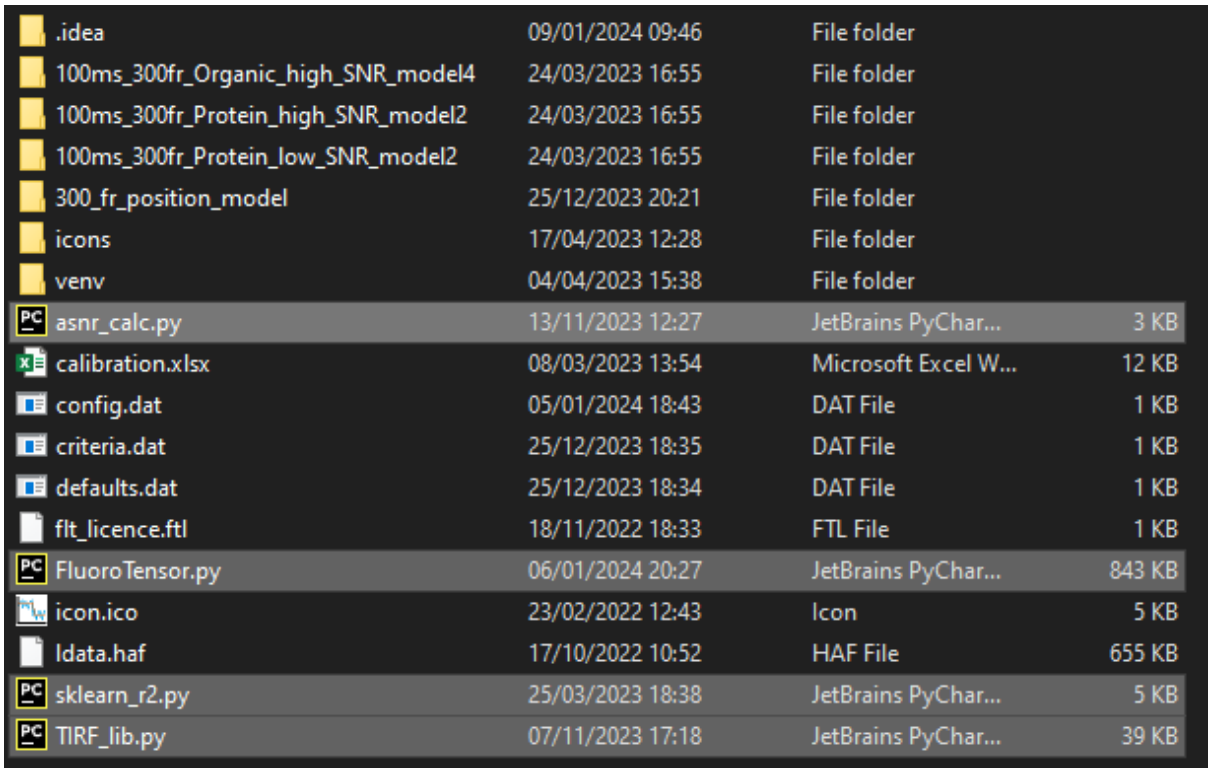


After TensorFlow is loaded, the console will display progress as the neural network models are loaded. Immediately following this, the main GUI window will appear:



1.3 Running the Program from Code

If you want to make modifications to the source code or if you're happier running the program without an executable or on a different platform, you'll first need to create a virtual environment using an IDE such as PyCharm. Python version should be 3.8 or 3.9. In the project folder you'll need the following python files from https://github.com/LISCB/FluoroTensor:



In addition, you'll need the other files and folders for settings, neural networks, icons, etc., as shown above. The .idea and venv folders are part of the virtual environment and should be created automatically when creating a new project. Once this is done, the following command will need to be executed in the project terminal to install the required python packages:

```
pip install tkscrolledframe==1.0.4
pip install openpyxl==3.1.2
pip install numpy==1.26.2
pip install matplotlib==3.4.0
pip install scipy==1.11.4
pip install easygui==0.98.2
```

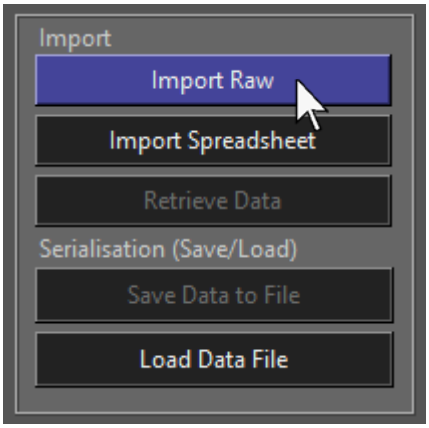
```
pip install scikit-learn==1.3.2
pip install tifffile==2023.9.26
pip install opencv-python==4.8.1.78
pip install tensorflow==2.7.1
pip install pillow==9.5.0
```

After all packages are installed, you can now run FluoroTensor from the IDE within the virtual environment; the debugging output that would normally be shown in the terminal window when running the .exe will now appear in the IDE 'Run' console output. After a short time, after all the components are loaded, the main window will appear in much the same way.

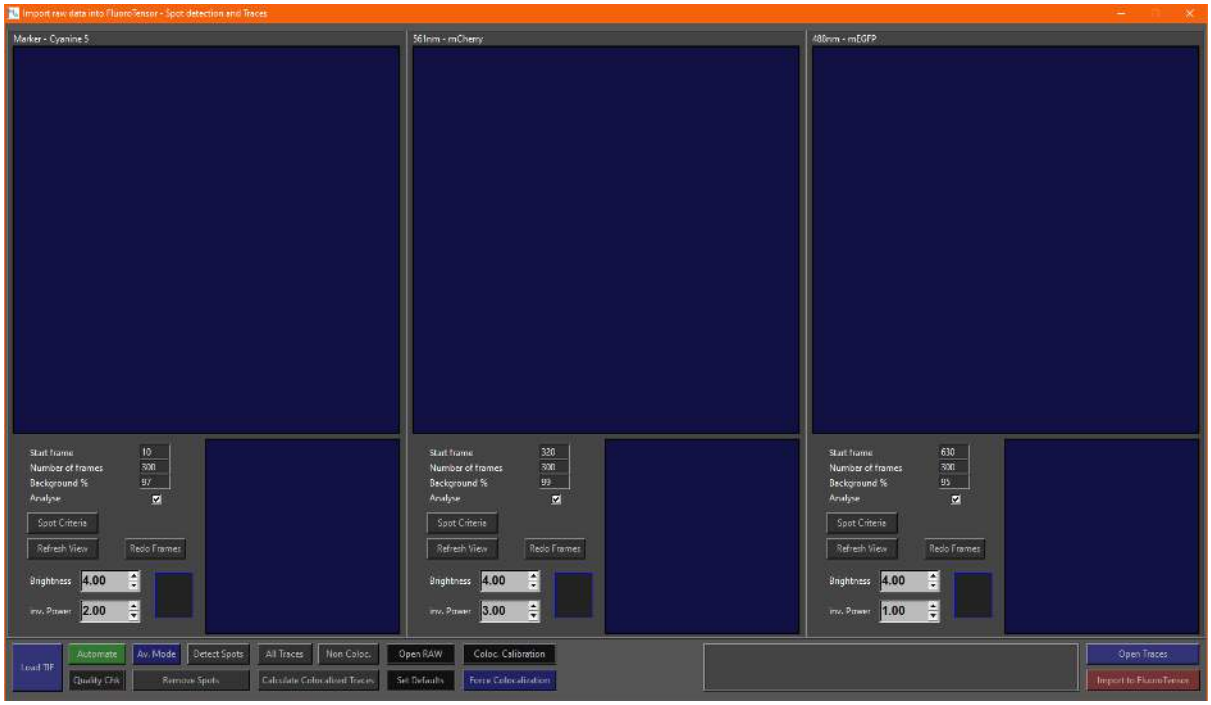
Chapter 2 – Image Analysis and Colocalization

2.1 Opening The Raw Data Analysis Window

Navigate to the ‘Import’ widget at the top right of the main window and click on the ‘Import Raw’ button:

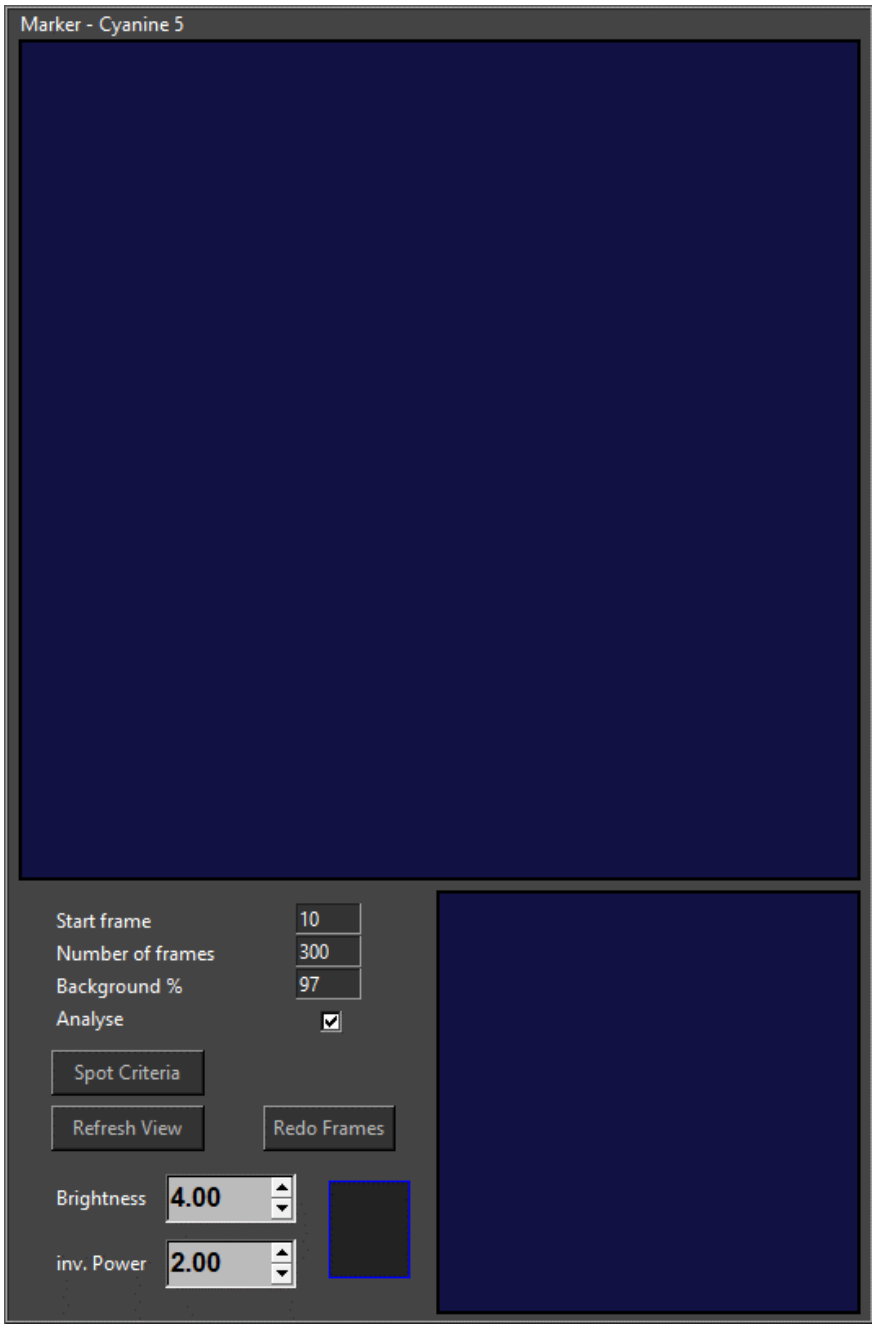


This will open a new window with an interface for analysing raw .tif files. The interface is split into three channels, each with its own controls and settings and a toolbar at the bottom:



2.2 An Overview of the Raw Data Analysis Interface

Each channel has an upper canvas, the larger one, which will display the enhanced version of the averaged frames + maximum projection and a smaller, lower canvas which displays the unenhanced image.

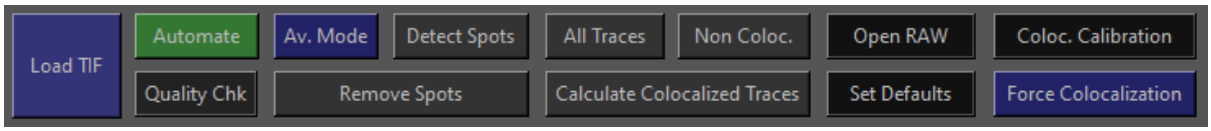


Each channel also has some additional controls. The input boxes control which frames of the raw data constitute a specific channel and the percentile of background intensity that should be removed when enhancing the image. If the 'Analyse' checkbox is ticked, the channel is active and will be displayed and analysed, otherwise it will be ignored and show the default

blue canvas to show that it is inactive. The spot criteria button in each channel opens a window which allow the parameters for spot detection to be adjusted. 'Refresh View' redraws the enhanced canvas to effect changes made to the brightness or background removal. 'Redo Frames' recalculates the average and maximum projection and re-enhances the image followed by refreshing the canvas if changes have been made to the frames of a channel.

The brightness control can be adjusted by the arrows or by typing in a number and pressing refresh. In addition, the brightness of a channel can be incremented or decremented in steps of 0.5 using the middle mouse scroll wheel. 'inv. Power' can be largely ignored and is calculated automatically by the program when a file is loaded or the frames are changed. This setting affects background uniformity and will be explained later on. The small box to the right of these settings displays a histogram of intensities from an intermediate stage of enhancement.

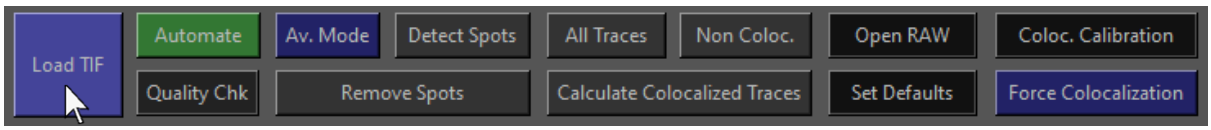
Below the three channels is the toolbar. On the left are the controls used for loading and analysing data:



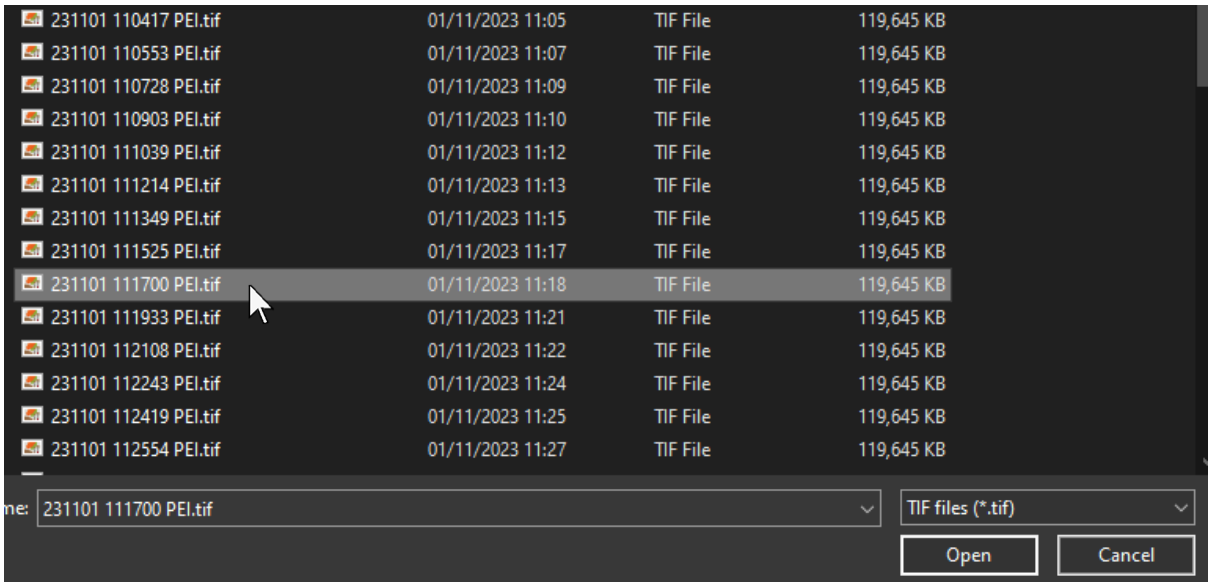
On the right is an information box which displays the filename and number of frames, to the right of which are buttons for showing traces and importing the dataset back into the main window of the program for further analysis once the raw data has been analysed. The space on the left is reserved for a status label and progress bar for tasks in progress:

2.3 Loading a Tiff file

To load a file, press the 'Load TIF' button in the lower left corner of the toolbar:

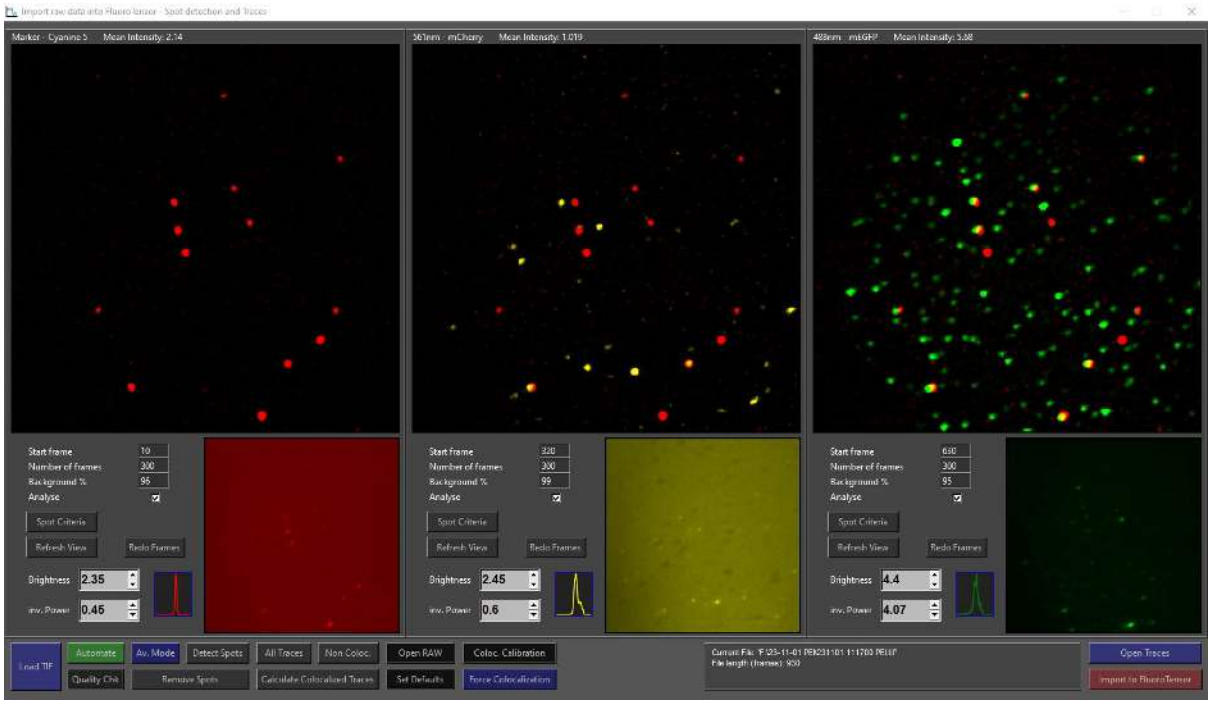


This will open a file dialogue box from which you can select a .tif file and open it:



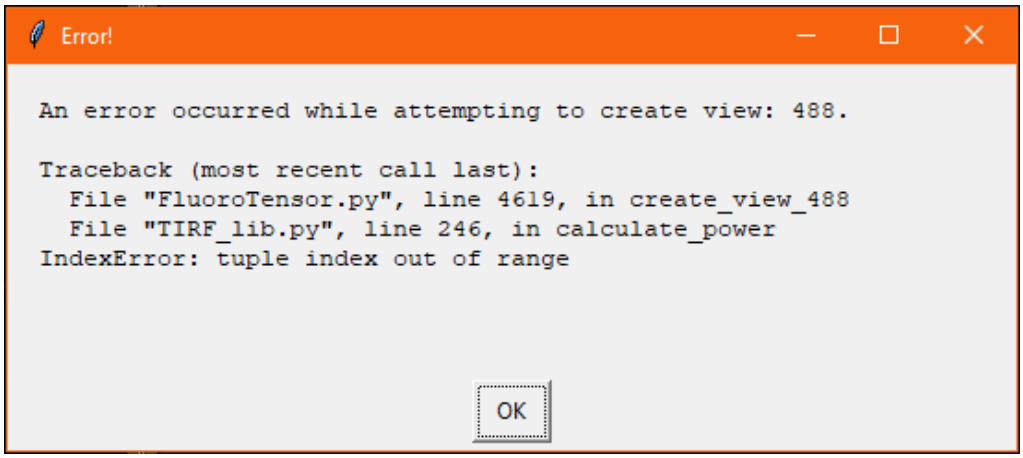
A progress bar will be displayed in the toolbar and the GUI will stop accepting input until the file has been loaded. For large files or from slow storage media such as USB sticks or network drives the window may become unresponsive for a short period of time.

Once the file has been successfully opened, each channel will render the enhanced views automatically. By default, the marker channel will be superimposed upon the secondary channels so colocalised spots can be identified easily by the user.

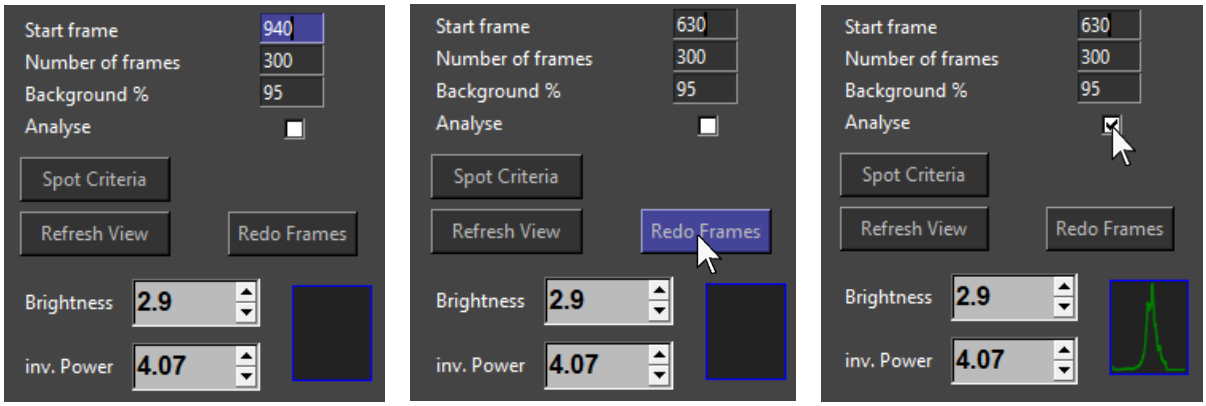


The Marker channel is shown in red, the 561nm channel is shown in yellow and the 488nm channel is shown in green.

In the event that the frames are set incorrectly, for example beyond the length of the file, an error might appear when the file is loaded:

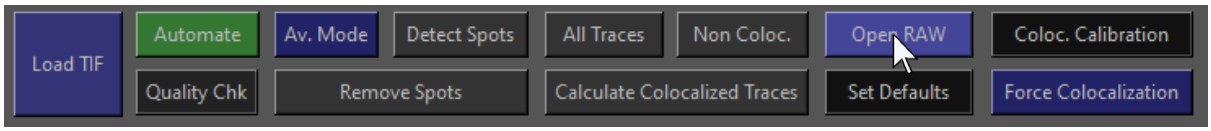


This will cause the offending channel to become inactive. In order to proceed, simply set the frames to be within the bounds of the file correctly, press ‘Redo Frames’ and then tick the analyse checkbox. Depending on the file, it may be necessary to press ‘Redo Frames’ an additional time to ensure the ‘inv. Power’ property is calculated correctly for the channel.

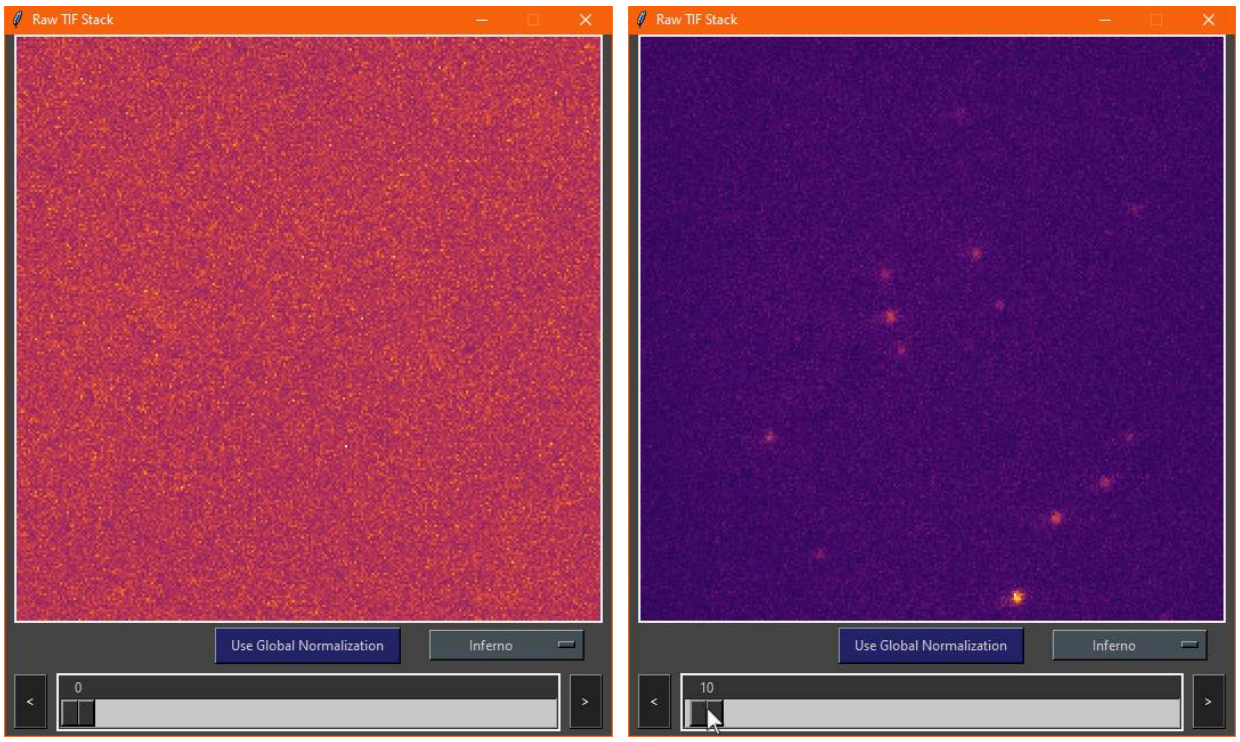


2.4 Viewing Raw Data

If your microscopy set up does not have an automated acquisition software that times the laser shutters with the camera, you will likely need to look through the raw data to find the points at which the colour channels switch. To do this, click on 'Open RAW' in the toolbar:

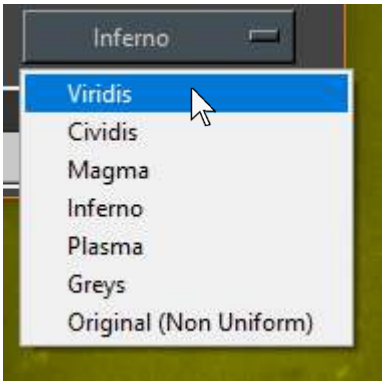


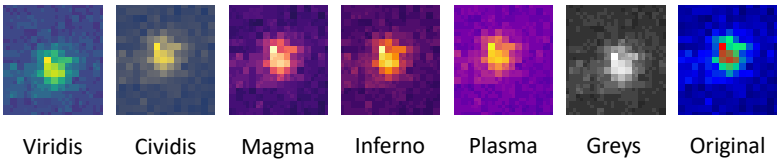
This will open a pop-up window which displays the raw single molecule movie 1 frame at a time. Use the slider to scroll through frames. Alternatively, to go frame by frame use the arrows at left and right of the slider.



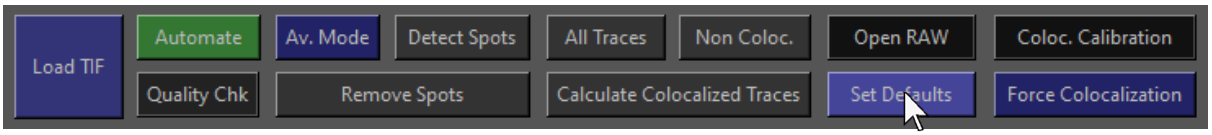
Global normalization normalizes the current frame to the brightest frame in the channel, rather than by the brightest pixel in the frame. This can help reduce the flicker when scrolling through based on variable noise levels and signal intensities.

The colour map can be changed using the dropdown menu. The default is 'Inferno' which is shown in the illustrations above. Six perceptually uniform colour maps are available. The last colour map which was originally implemented is not recommended since it is not perceptually uniform and over-represents contrast at the colour boundaries.

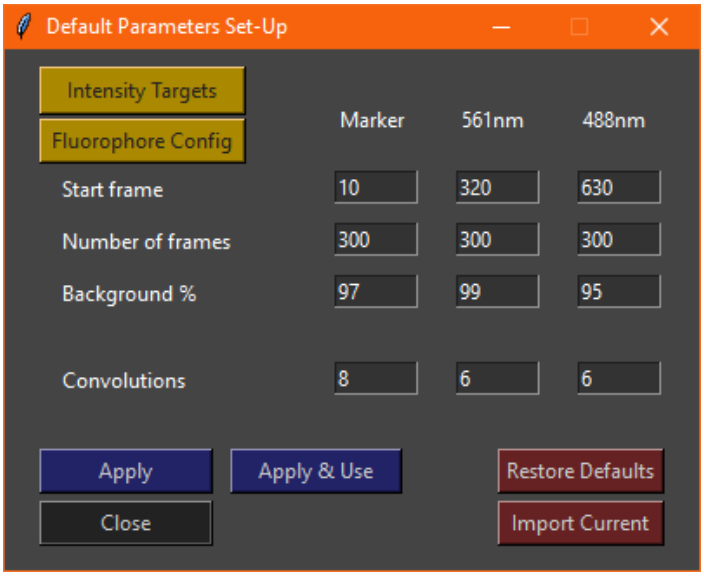




The default frames can be set from the toolbar if you expect the frames for each channel to be the same from file to file as is usually the case with automated acquisition software that has shutter control. This will be useful later for automation. Click the 'Set Defaults' button in the toolbar to do so:



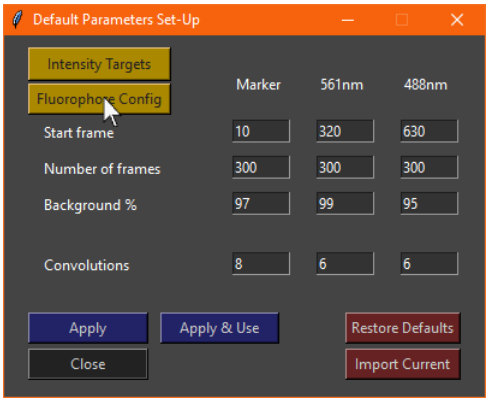
A popup will then show up where you can edit the default frames and background to subtract, assuming relative uniformity across the dataset:

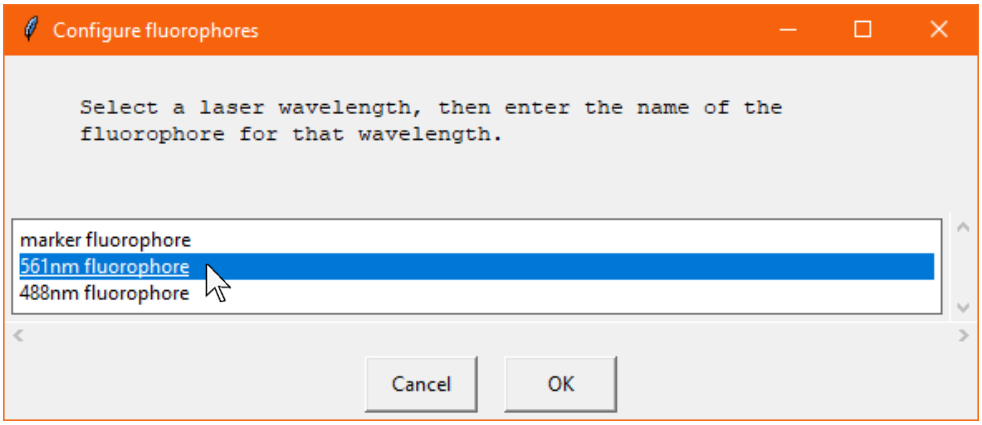


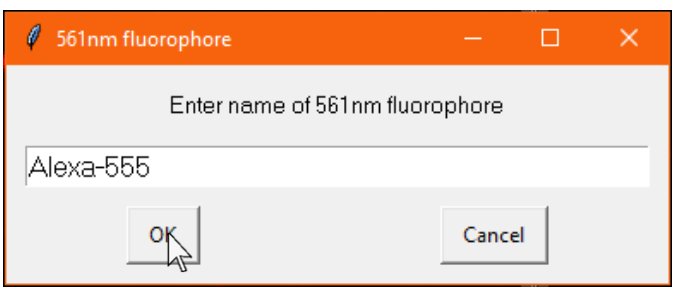
'Restore Defaults' will reset the frames to factory defaults. These values are saved even when the program is closed. Thus, the next time FluoroTensor is launched for raw data analysis, the frames in each channel will be set according to the default values set here.

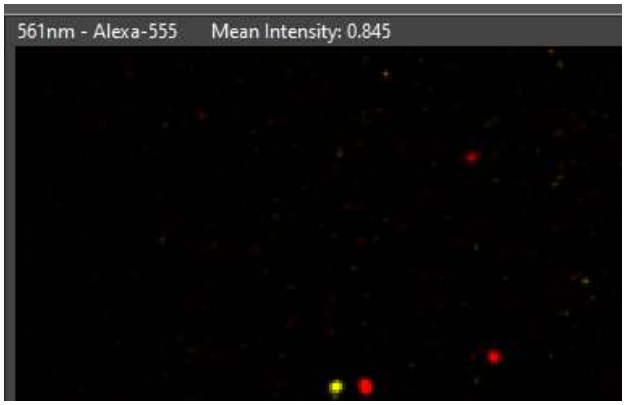
2.5 Configuring Fluorophore Names

Fluorophores can be renamed by pressing 'Fluorophore Config' in the default settings window. This will bring up a listbox where you can select which channel to configure. Select a channel, press OK and then type the name of the fluorophore:





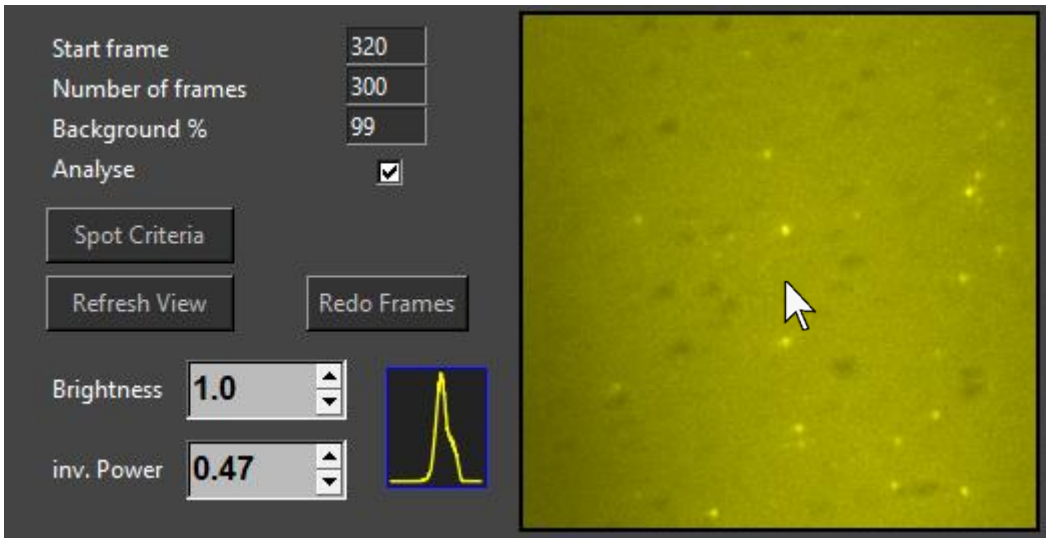




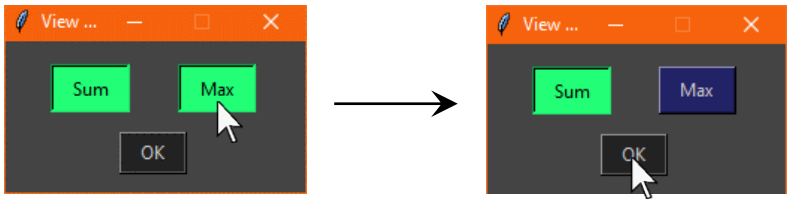
After refreshing the channel, the correct fluorophore name will now be displayed at the top.

2.6 Disabling Sum or Maximum Projection

Sometimes you may wish to only use the sum or maximum projection rather than the default which is to use both. To do this, left click on the unenhanced smaller canvas in the relevant channel:

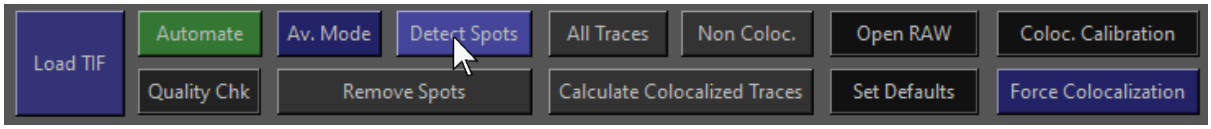


Then click the mode you want to toggle off from the options in the pop-up window and press ok. The program will then re-enhance the image using the remaining mode:

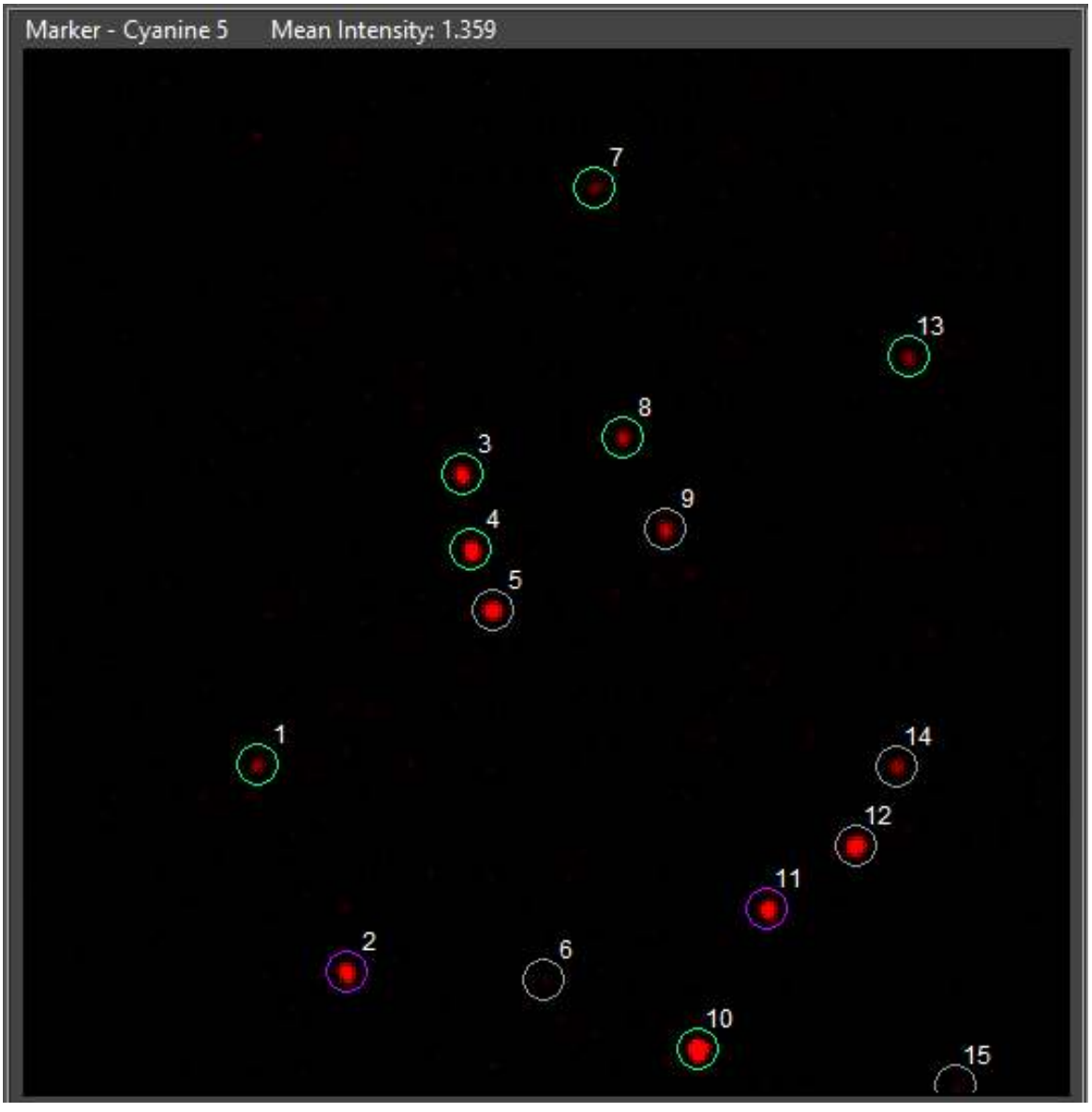


2.7 Detecting Fluorescent Foci

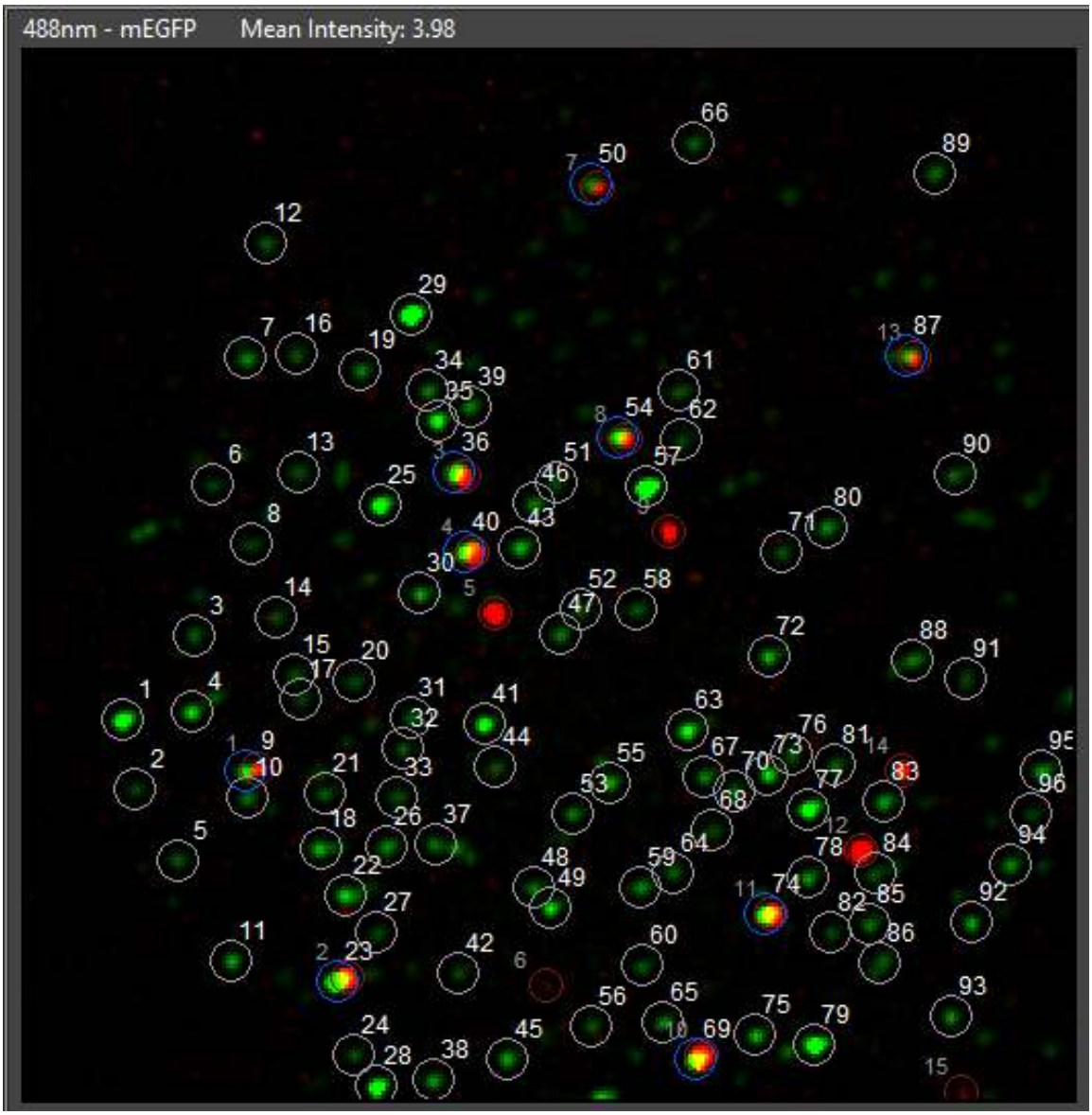
To detect fluorescent signals of single molecules, press the 'Detect Spots' button in the toolbar:



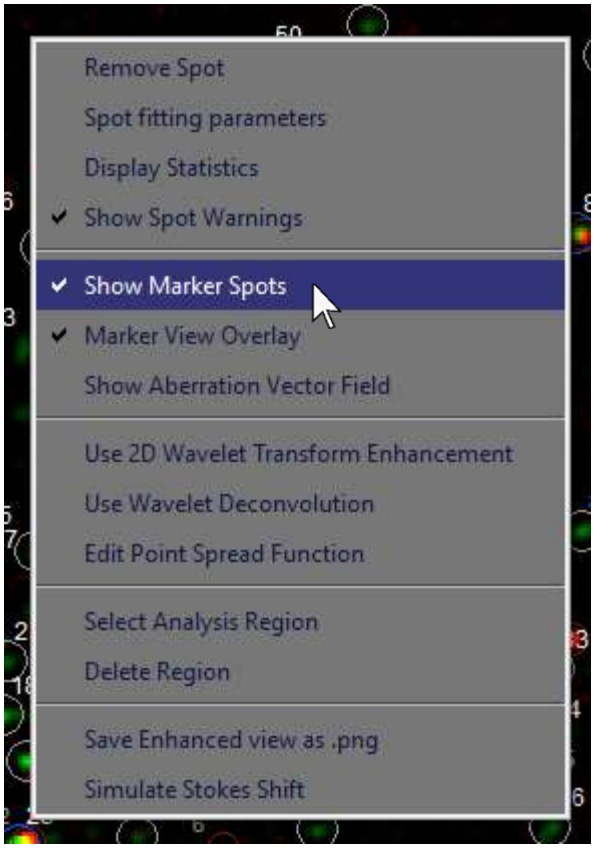
Spots that have been detected will be circled and numbered. The circles in the marker channel are colour coded based on colocalization. White spots are not colocalized, orange spots are colocalized with a corresponding spot in the 561nm channel, green spots are colocalized with a corresponding spot in the 488nm channel and purple spots are colocalized with corresponding spots in both channels:

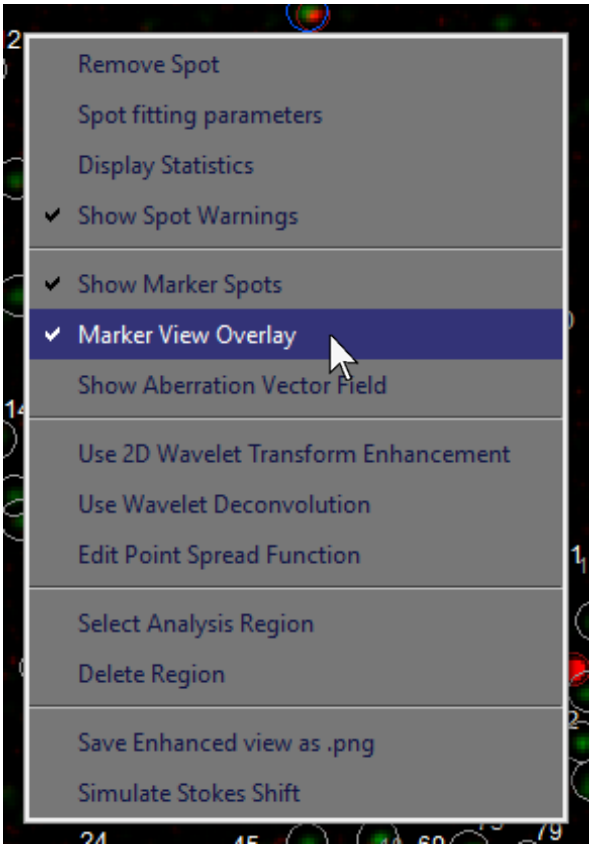


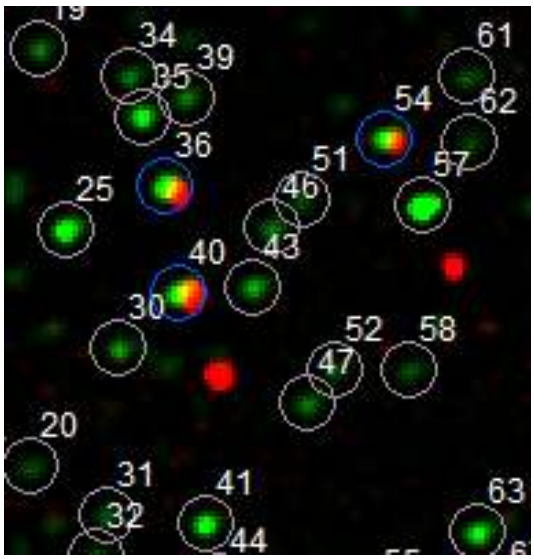
The spots detected in the secondary channels are also circled and colour coded but in a slightly different way:

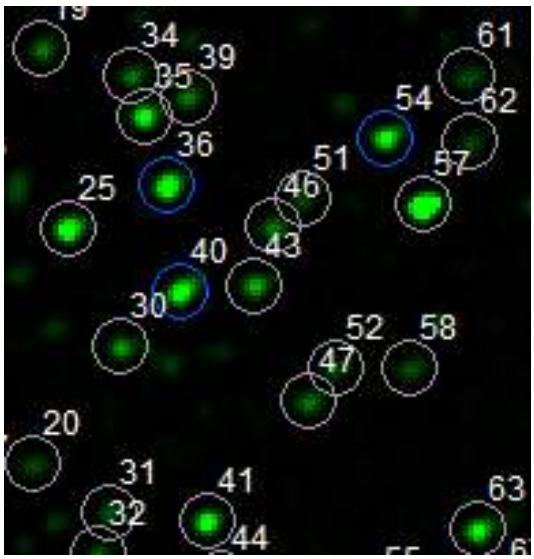


Secondary channel spots which are colocalized with markers are circled in blue and non-colocalized spots are circled in white. By default, the marker spots are circled in a smaller, fainter red circle and the channel is superimposed. This can be disabled by right clicking on the view and navigating to the ‘Show Marker Spots’ and ‘Marker View Overlay’ toggles in the context menu:

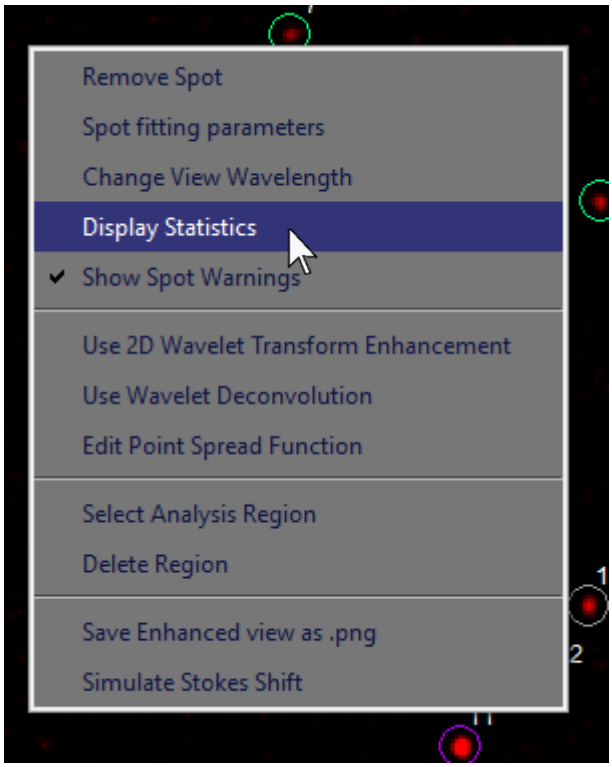




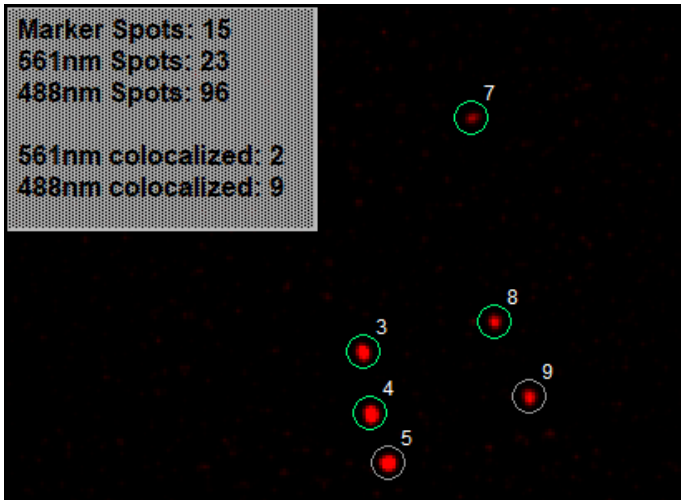


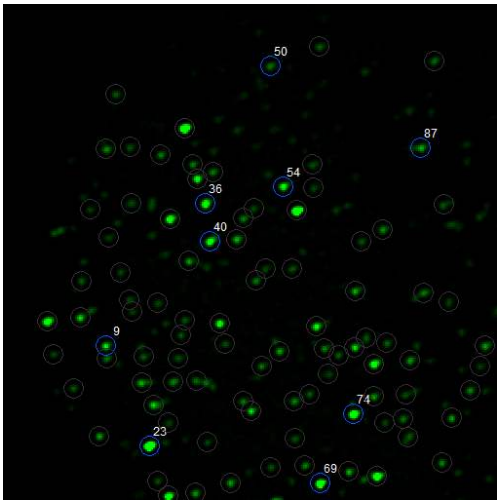


The number of spots detected and colocalized can be shown. This function also darkens non-colocalized spots so that colocalized spots can be identified more clearly by eye. To do this, right click on any of the three channel's canvases and click 'Display Statistics':



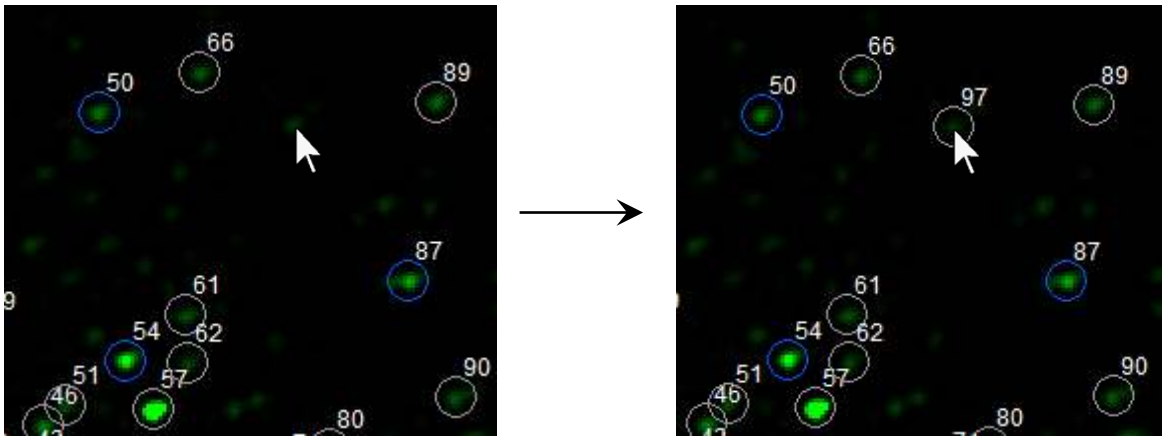
The spot statistics will then be displayed in the upper left corner of the marker channel canvas:



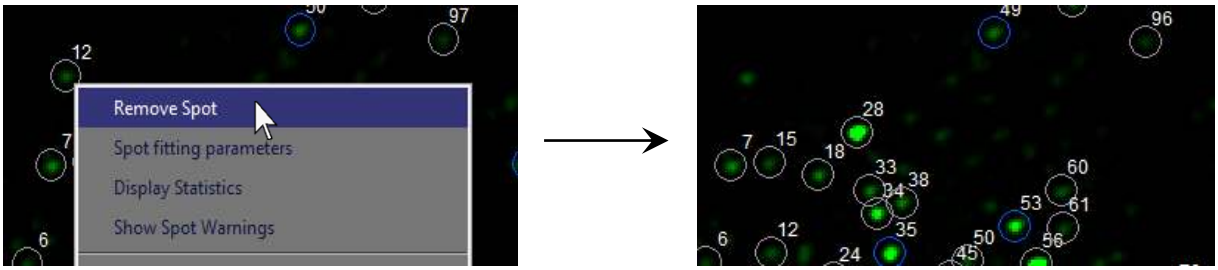


2.8 Manually Adding and Removing Fluorescent Foci

Spots can also be manually added and removed if necessary. To add a spot manually, left click within 5 pixels of the centre of the spot in the enhanced canvas:

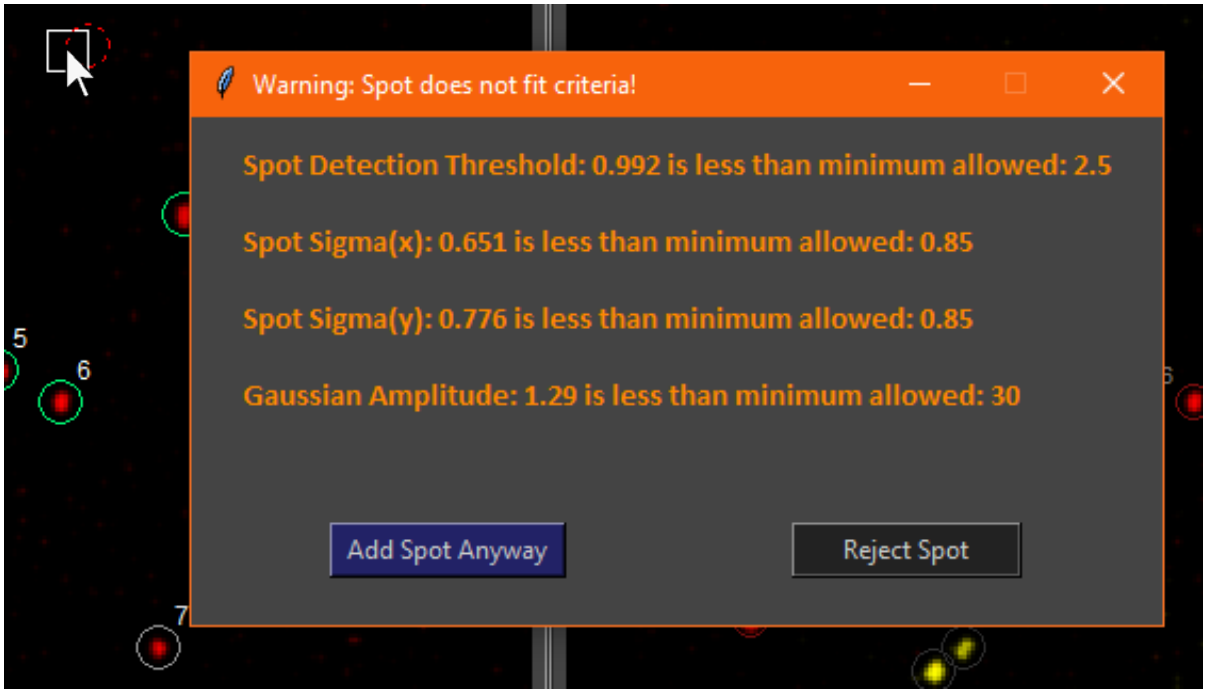


Any spot, whether detected automatically or added manually can be removed. To do this, simply right click near the spot and select 'Remove Spot' at the top of the context menu. Make sure to click precisely as the nearest spot to where the cursor was when the button was clicked will be removed:

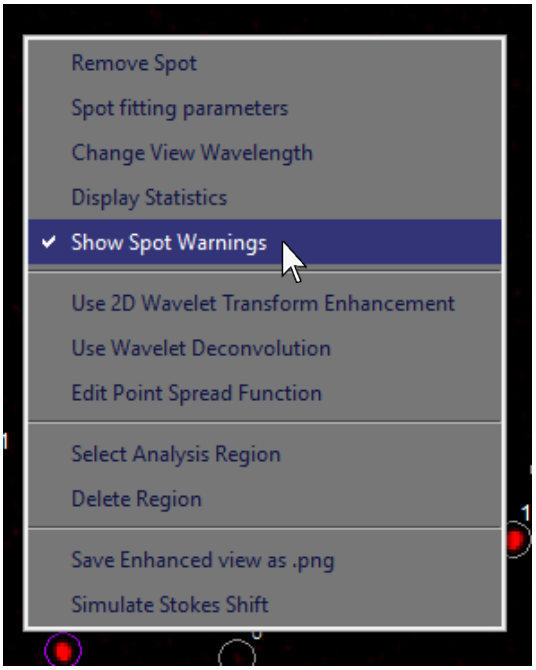


Note that spots will be renumbered. Any spot with a higher index than the removed spot will have its own index reduced by one to compensate for the removed spot.

If a spot is added that does not fall within the parameters of the spot criteria, a popup window will appear, warning the user that the selected spot is invalid. The spot can then be forced, or the user can cancel adding the spot at this point:

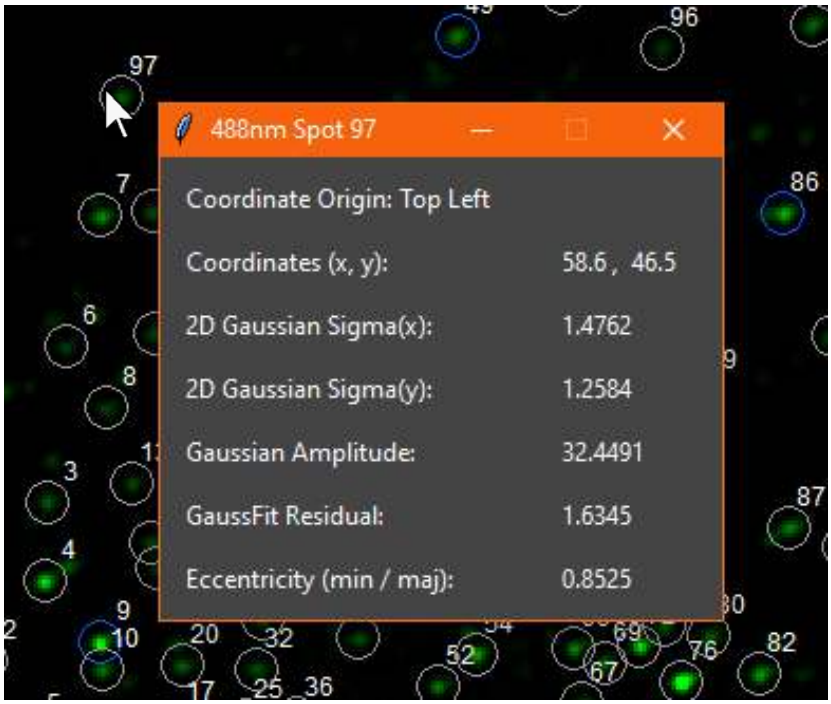


If the quality of spots is not important in the context of a particular experiment, and the user wishes to bypass this warning, it can be disabled by right clicking in the canvas of any of the channels and toggling 'Show Spot Warnings':



2.9 Finding Spot Fitting Parameters

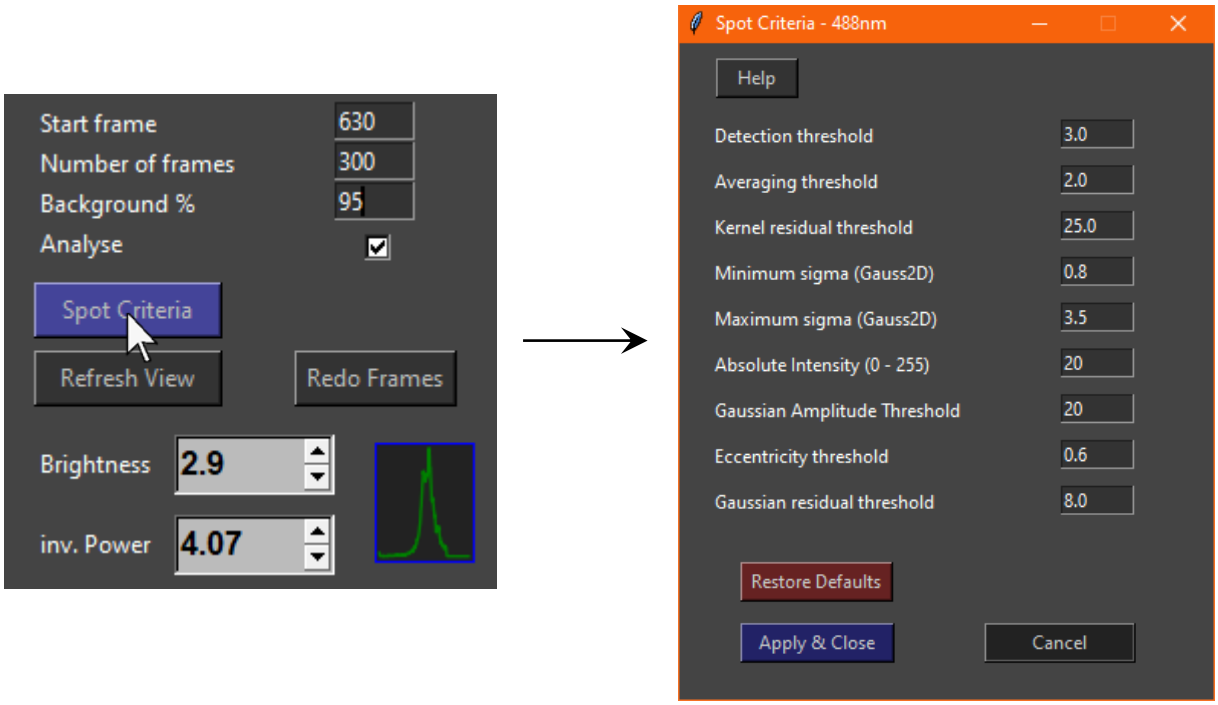
To find the parameters of the fit of a spot, right click it and select ‘Spot fitting parameters’ from the context menu. A small popup window will appear next to the spot, displaying these parameters:



This information can also be found in the excel template when the analysed data is exported later after the traces are analysed.

2.10 Modifying Spot Criteria

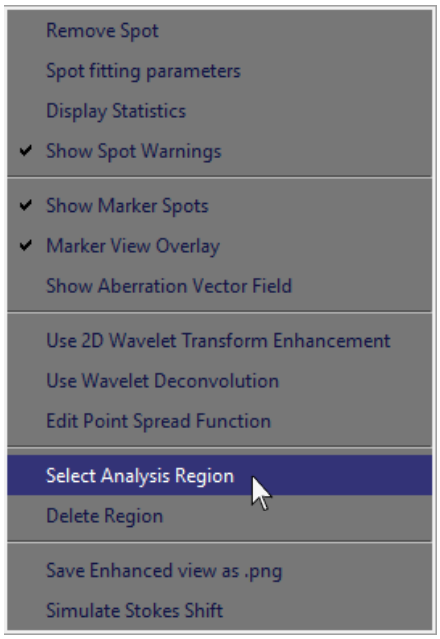
The criteria or thresholds for detecting spots are independent for each channel. Each channel has a button which will bring up a popup window in which the parameters can be edited. For example, if you find that the detection threshold is too low and prevents FluoroTensor from picking spots in the 488nm channel, click ‘Spot Criteria’ in the 488nm channel and reduce the threshold so the spots are picked correctly:



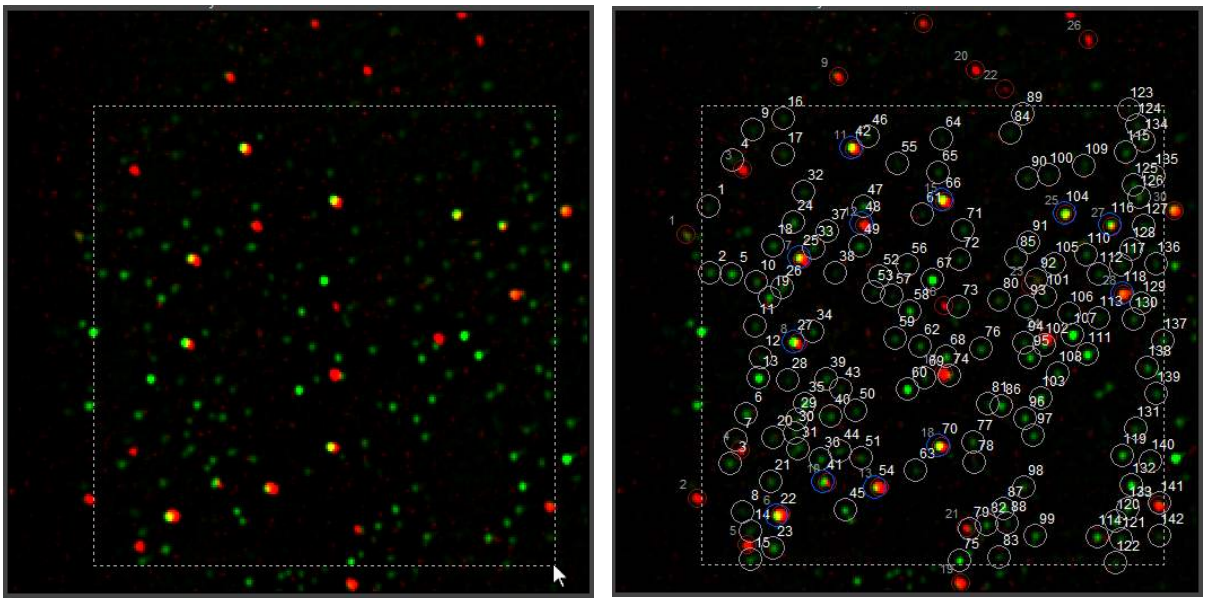
Pressing ‘Help’ will open a help window which describes in detail what each parameter does. These parameters are saved if the program is closed and restarted. The default values can be easily restored in the case that the parameters are detrimentally altered and the working parameters cannot be remembered.

2.11 Analysing a Custom Field of View

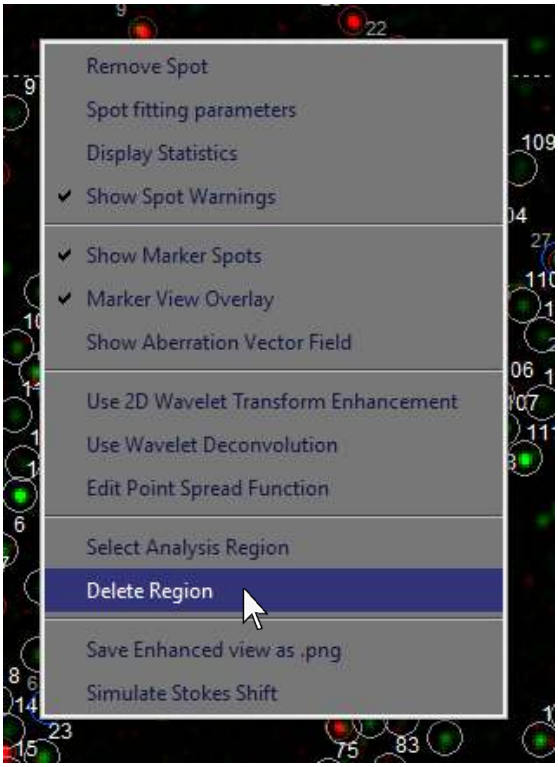
It may be necessary to only analyse a region within the field of view of a channel. Examples of such cases include poor focus or spherical aberration at the fringes of the image. To draw a custom region, right click within the canvas in the relevant channel and choose 'Select Analysis Region' from the context menu:



You can then draw a box in the region of the field of view that you wish to analyse. Spots in this channel will only be detected within this region:

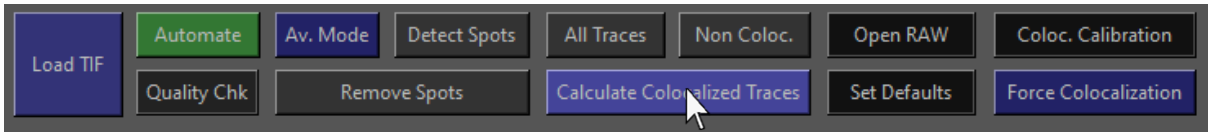


The region will stay even when a different file is loaded and can be used if the TIRF illumination field does not cover the full area from file to file. To remove the region and detect spots in the whole field of view, right click in the canvas of the relevant channel and choose ‘Delete Region’ from the context menu:

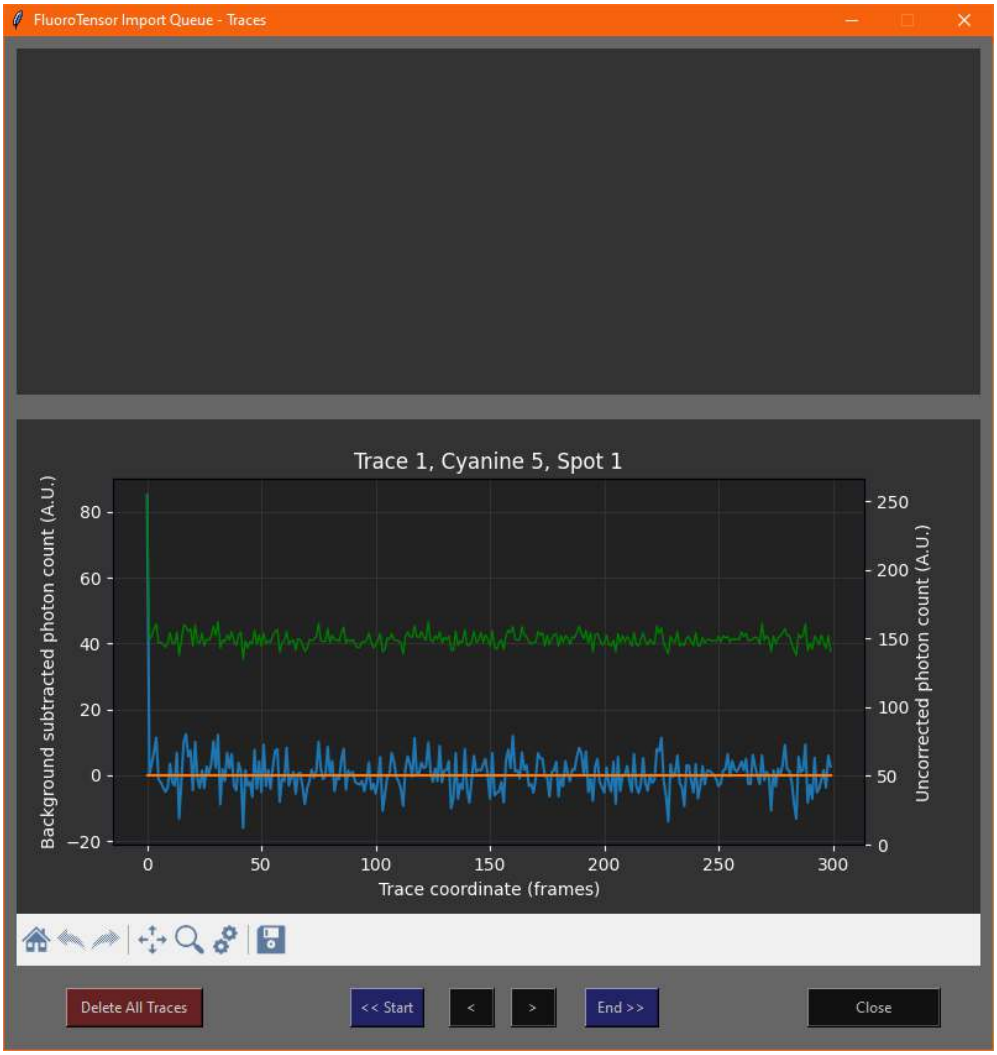


2.12 Calculating Colocalized Traces

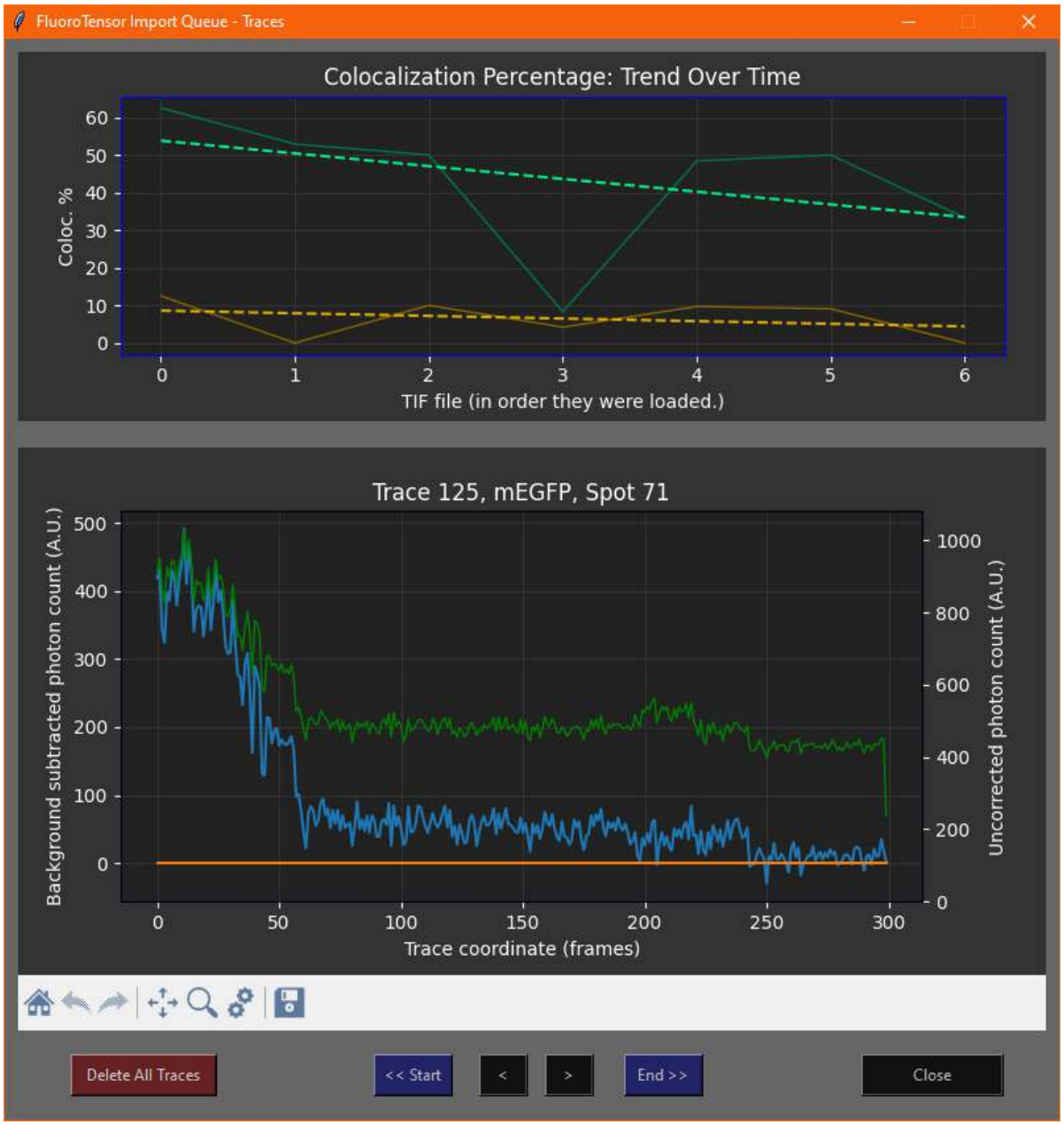
To calculate colocalized traces, click the 'Calculate Colocalized Traces' button on the toolbar. Doing this will calculate the traces for any colocalized spot. The order will be marker trace, then 488nm colocalized trace if present, then 561nm colocalized trace if present.



Once the traces are calculated, a window will automatically pop up where you can click through the traces to look at them. The trace of the signal without background subtraction is shown in green using the right-hand axis. The trace of the background-subtracted signal is shown in blue and plotted on the left-hand axis:

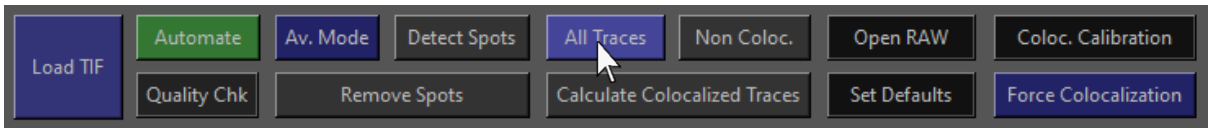


Once any traces have been calculated, the program will warn you if you try to calculate them again if they weren't manually deleted first. This is to prevent accidental duplication. FluoroTensor will also warn you if you have opened the same .tif file more than once in the same session. If multiple files are analysed, the traces from each file will be added to the overall dataset and the colocalization trend will be displayed in the upper plot of the trace window. This might be helpful to check if the sample is degrading over time during the course of the microscopy experiment.



2.13 Calculating All Traces

If the distribution of steps of all spots is of interest, not just colocalized spots, it is possible to calculate the traces of all spots in all channels. Do this by clicking 'All Traces' in the toolbar:

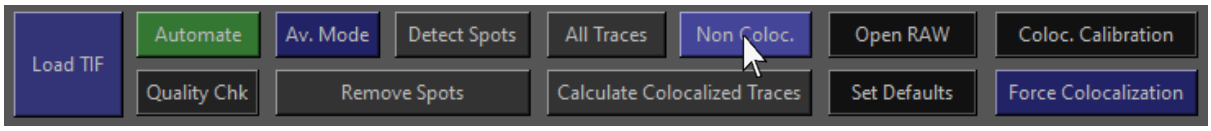


In this case, the traces of marker spots will be calculated first, then the 561nm channel, then the 488nm channel. In much the same way, once all traces have been calculated, the trace window will pop up and you can scroll through traces.

Of particular importance is that only one mode of trace calculations should be used at a time. For example, all files should be analysed for colocalization and the data processed and exported. Then for all traces, the process should be begun anew, this time calculating all traces. The data should then be exported to a separate file.

2.14 Calculating Non-Colocalized Traces

While it is recommended to perform negative controls experimentally for example in the absence of the component in the marker channel, preliminary information can be gathered by analysing non-colocalized spots. To do this, click 'Non Coloc.' In the toolbar:

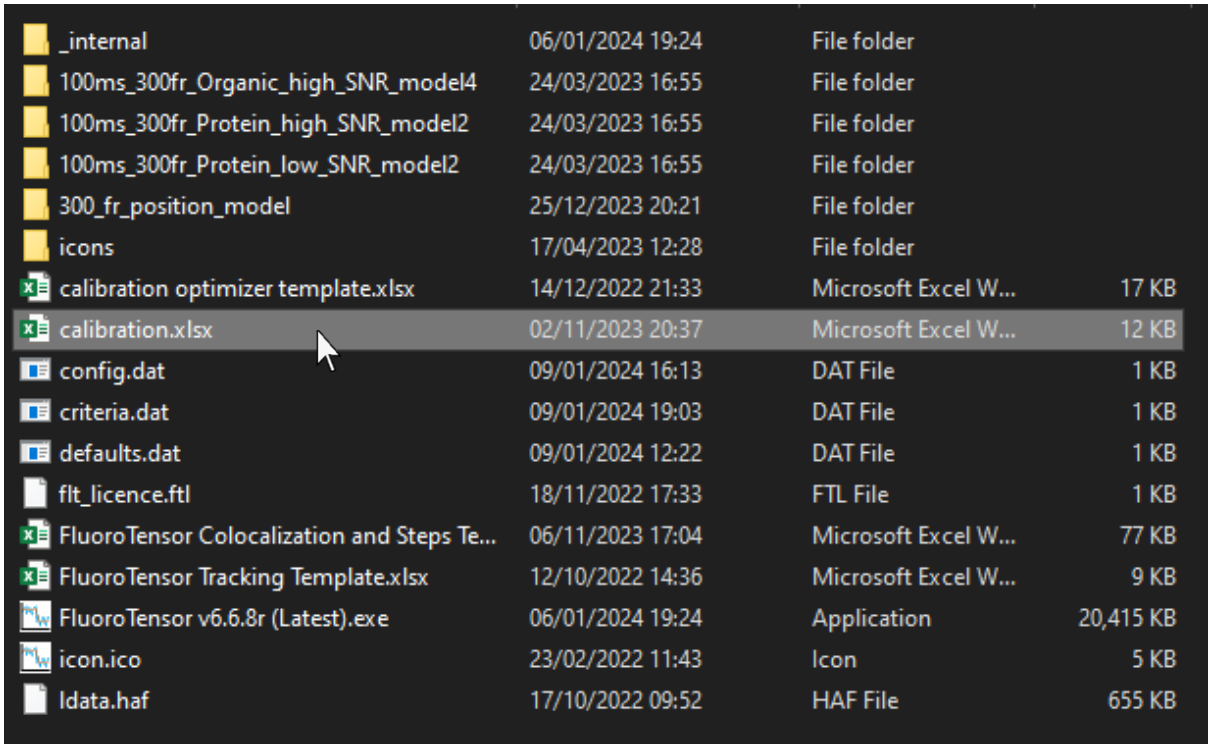


Once again, this should be used separately from other analyses and exported into a separate file. For a full analysis, you will end up with three exported Excel files, one for colocalized traces, one for all traces, and one for non-colocalized traces. More detail about full experimental analyses will be given in chapter 7.

Chapter 3 – Calibration

3.1 The Calibration File

The calibration file holds a record of the parameters used to correct for chromatic aberration by transforming the coordinates of secondary channel spots onto the markers. It is an Excel '.xlsx' file that can be found in the FluoroTensor folder. This file should not be renamed otherwise the program will be unable to locate it:



Open the file to view the history of calibration parameters:

	A	B	C	D	E	F	G	H	I	J	K	L	M	N
1	Calibration Date (YYYYMMDD)		20220324	20220527	20220815	20220818	20221212	20221213	20221219	20230117	20230203	20230217	20230301	20231102
2	FORCE CALIBRATION													
3	X_0		31.14	26.16	0	0	0	0	0	0	0	0	0	0
4	Y_0		1.11	0.5	2942.457	0	0	0	0	0	0	0	0	0
5	X_C		1089.61	1155.47	12891.46	0	202.57	165.155	242.535	292.999	270.194	448.28	279.272	387.47
6	Y_C		0	33.73	43.63	0	159.57	127.425	170.312	192.454	191.992	151.358	108.487	191.799
7	SF_x		1148.29	3984.77	282.34	10000	63.54	97.665	114.902	88.222	63.638	122.417	76.799	128.294
8	SF_y		212.3	878.31	4427.96	10000	68.84	87.25	100.941	81.119	91.064	102.274	69.06	104.801
9														
10	Colocalization Criterion		1.5	1.5	1.5	2	2	2	2	2	2	2	2	2

Calibration parameters are stored in columns in rows 1 through 10. Each calibration is dated by when the data it was produced from was collected. The date order is YYYYMMDD. X_o and Y_o should be set to zero as they are legacy parameters kept to prevent incompatibility with

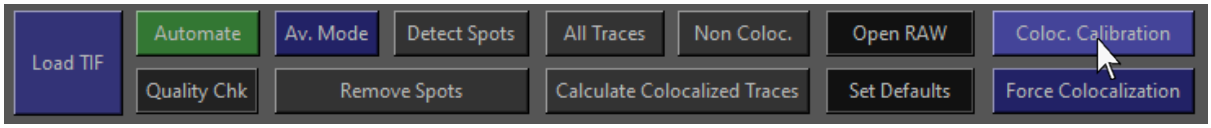
some of our other internally used programs. The colocalization criterion is the distance in pixels from the centre of a marker spot and secondary spot under which it qualifies as colocalized. This threshold should be set appropriately. When samples are dilute and the number of spots in each channel is 50-100, the random colocalization rate is 1-2% with a 2-pixel threshold.

The calibration method is suitable for systems where chromatic aberration can be solved with a linear approximation within the threshold distance. It is recommended where possible to record separate calibration movies regularly, if not on the same day the microscope is used. This can be done with small red/green fluorescent beads with similar Stokes shift to the fluorophores in use, or with a dual labelled single-molecule standard using the same fluorophores as in the experiment. A simple standard is a biotinylated dual-labelled oligo using BSA-streptavidin deposited directly onto a cleaned glass slide.

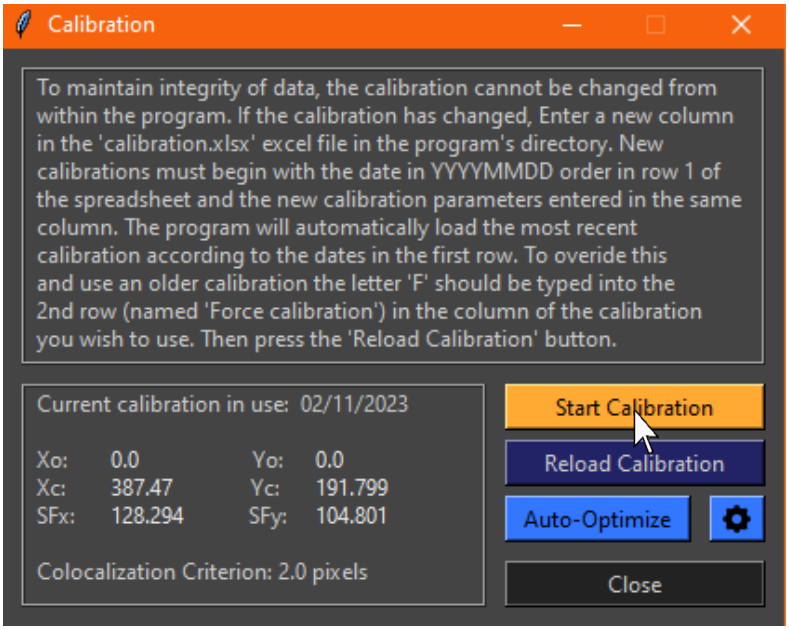
The parameters X_c, Y_c, SF_x, and SF_y are solved in an Excel sheet from the analysed calibration data. The following section will describe how this is done both for fluorescent beads and dye labelled single molecules.

3.2 Calibrating FluoroTensor with Fluorescent Beads

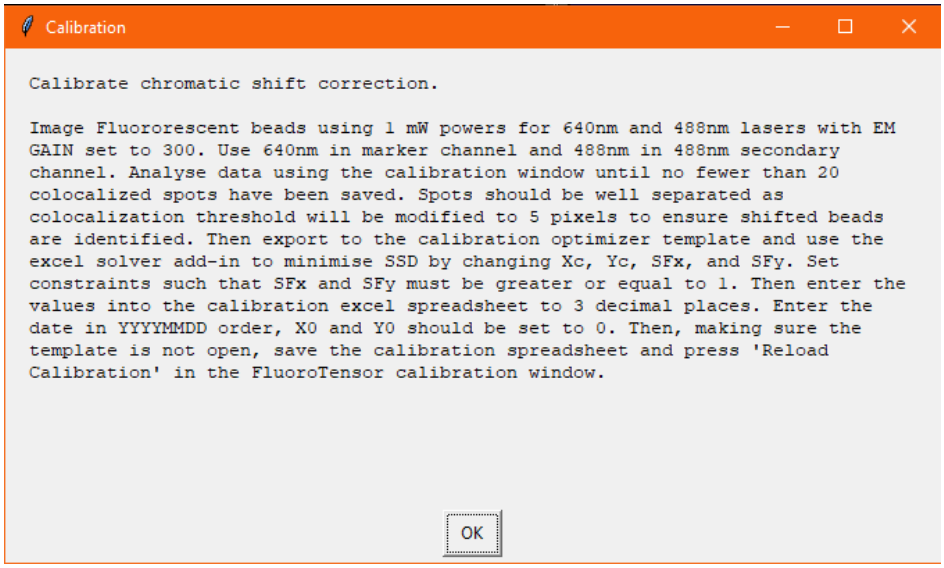
Open the calibration window by clicking ‘Coloc. Calibration’ on the toolbar:



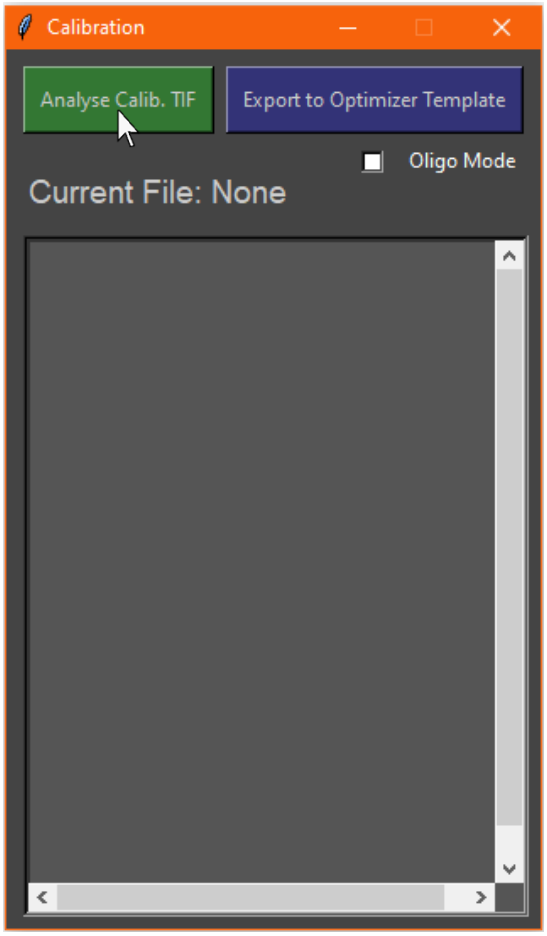
The window will show the current calibration and the date it was collected. The disclaimer can be ignored, but encourages the proper keeping of the calibration record. The reason being that old data may need to be reanalysed and if the setup or alignment of the microscope system has changed, and/or the calibration has changed, older records need to be maintained to correct the chromatic aberration from before changes to the system were made. The calibration popup window will look like this:



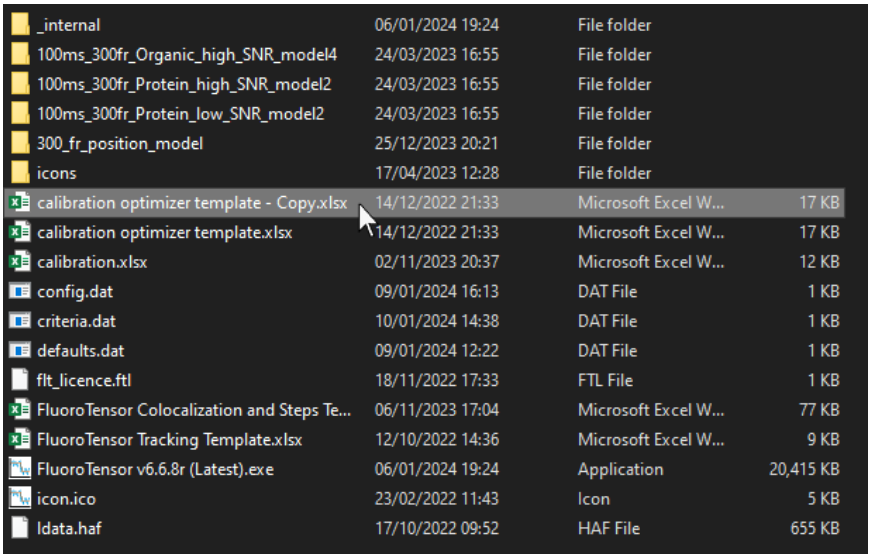
To begin analysing calibration movies, press ‘Start Calibration’. A message will pop up advising about laser powers, etc. This can be ignored as it pertains to a specific system we have. Users may have to optimise the conditions to find what works well for them.



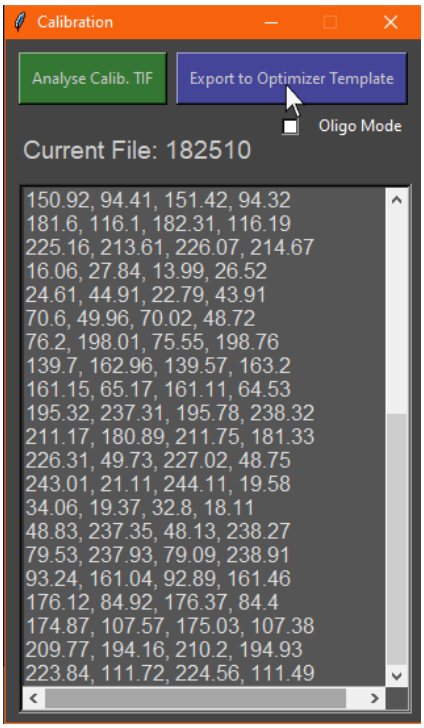
Press ok to dismiss the above message and the following window will appear. Click ‘Analyse Calib. TIF’ and select the first calibration movie after ensuring the frames are set correctly. Untick the ‘Analyse’ checkbox in the 56nm channel as it will not be required.

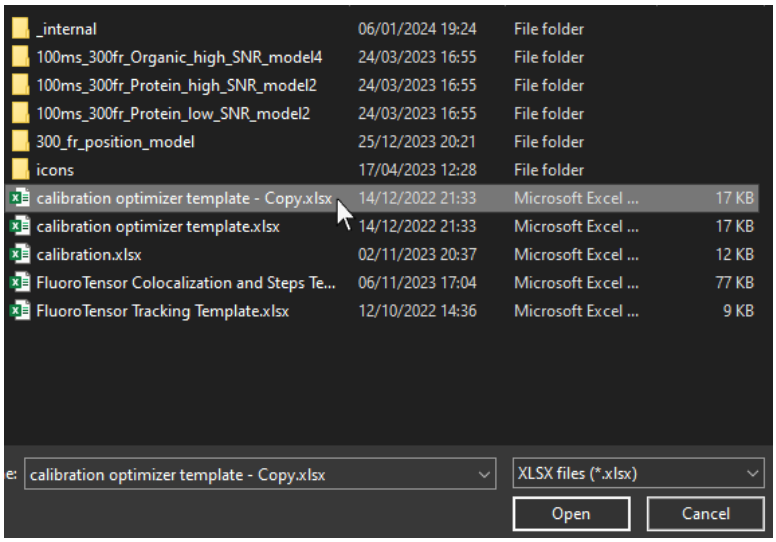


Users should ensure the distribution of beads is sparse as the program will automatically colocalize 488nm spots with 640nm spots within a larger threshold of 5 pixels. Spots will be detected automatically after the file is loaded and the 640nm-488nm coordinate pairs will appear in the window as shown above. Once the coordinates have appeared and the 'Analyse Calib. TIF' button has become green again, proceed to the next file and repeat. Once all the calibration files have been analysed. Find the 'calibration optimizer template.xlsx' in the FluoroTensor folder and make a copy of it. Name the copy appropriately and save it somewhere. Here we will simply keep the non-renamed copy for demonstration purposes:

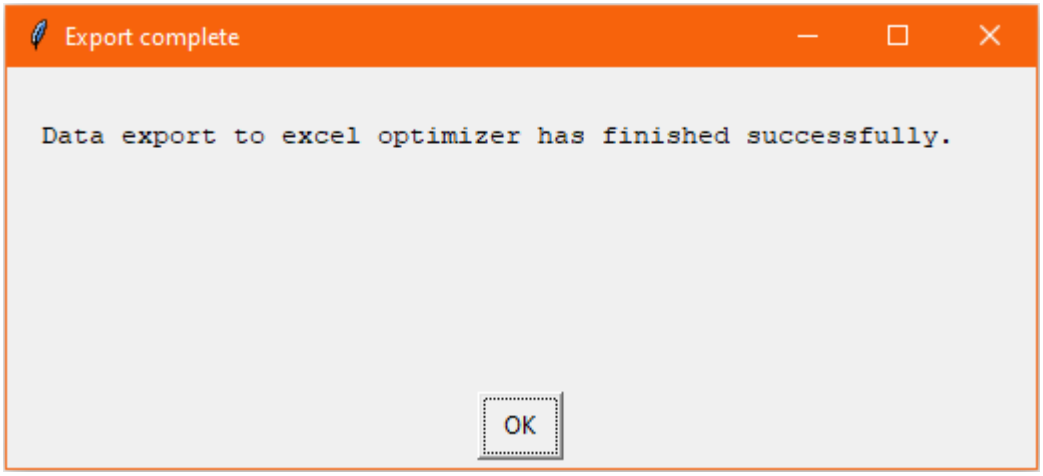


Next, go back to FluoroTensor and click ‘Export to Optimizer Template’ and then select the Excel file that was just copied and renamed. Make sure the file is not open in Excel at the time you export to it as the data will not be saved and FluoroTensor might hang in which case the raw analysis window will need to be closed and reopened from the main GUI window.





On success, a dialogue box will pop up to inform the user that the data was exported successfully:

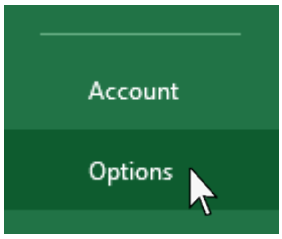


Next, open the Excel file the data was exported to. It will look something like this:

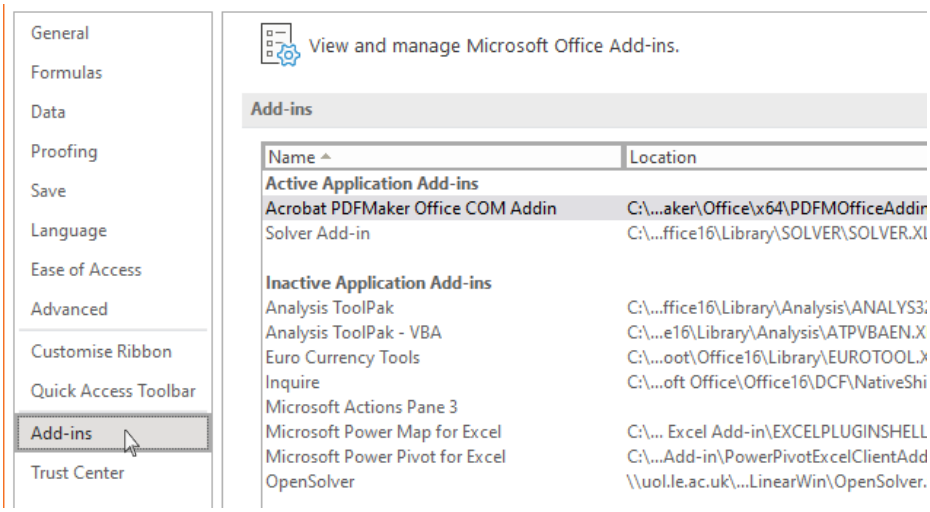
X	Y	Target X	Target Y	Trial X	Trial Y	DX	DY	SDX	SDY	TX - X	TY - Y
54.72	235.15	53.61	236.1	53.5892	236.385	0.02075	0.28468	0.00043	0.08104	1.11	0.95
75.99	129.08	74.68	129.03	75.077	129.099	0.39703	0.06897	0.15763	0.00476	1.31	0.05
97.63	48.42	96.42	47.39	96.9386	47.5145	0.5186	0.1245	0.26895	0.0155	1.21	1.03
146.92	204.8	146.29	205.4	146.733	205.687	0.44329	0.28683	0.1965	0.08227	0.63	0.6
176.42	23.32	176.12	21.82	176.535	22.1268	0.41534	0.30682	0.17251	0.09414	0.3	1.5
11.6	232.37	10.38	233	10.0277	233.573	0.35226	0.57282	0.12409	0.32812	1.22	0.63
49.73	32.52	48	31.16	48.5482	31.4323	0.54815	0.27226	0.30047	0.07413	1.73	1.36
102.9	41.38	101.89	40.31	102.263	40.3938	0.37256	0.08381	0.1388	0.00702	1.01	1.07
100.37	47.84	99.46	46.91	99.7067	46.9278	0.24666	0.01785	0.06084	0.00032	0.91	0.93
134.53	64.97	134.18	64.35	134.216	64.2542	0.03643	0.09582	0.00133	0.00918	0.35	0.62
134.42	210	134.03	210.68	134.105	210.946	0.0753	0.26643	0.00567	0.07098	0.39	0.68
46.24	229.12	46.16	230.19	45.0224	230.286	1.13758	0.09557	1.29409	0.00913	0.08	1.07
62.17	41.46	61.41	40.69	61.1155	40.4747	0.29447	0.21527	0.08671	0.04634	0.76	0.77
71.03	42.71	70.6	41.94	70.0662	41.7391	0.53375	0.20095	0.28489	0.04038	0.43	0.77
91.98	103.17	92.01	103.11	91.2308	102.892	0.77925	0.21799	0.60722	0.04752	0.03	0.06
124.26	232.32	124.63	233.47	123.841	233.522	0.78873	0.05225	0.62209	0.00273	0.37	1.15
155.5	17.04	155.91	15.81	155.401	15.7748	0.50886	0.03516	0.25894	0.00124	0.41	1.23
150.92	94.41	151.42	94.32	150.774	94.0316	0.64576	0.28839	0.417	0.08317	0.5	0.09
181.6	116.1	182.31	116.19	181.768	115.97	0.54162	0.2198	0.29335	0.04831	0.71	0.09
225.16	213.61	226.07	214.67	225.774	214.598	0.29561	0.0722	0.08738	0.00521	0.91	1.06
16.06	27.84	13.99	26.52	14.5334	26.6986	0.5434	0.17862	0.29529	0.03191	2.07	1.32
24.61	44.91	22.79	43.91	23.1709	43.9643	0.38095	0.05427	0.14512	0.00295	1.82	1
70.6	49.96	70.02	48.72	69.6318	49.0721	0.38816	0.35215	0.15067	0.12401	0.58	1.24
76.2	198.01	75.55	198.76	75.2892	198.819	0.26082	0.05901	0.06803	0.00348	0.65	0.75
139.7	162.96	139.57	163.2	139.439	163.367	0.13064	0.16728	0.01707	0.02798	0.13	0.24
161.15	65.17	161.11	64.53	161.109	64.4565	0.00101	0.07352	1E-06	0.00541	0.04	0.64
195.32	237.31	195.78	238.32	195.629	238.569	0.15114	0.24944	0.02284	0.06222	0.46	1.01
211.17	180.89	211.75	181.33	211.641	181.503	0.10885	0.17279	0.01185	0.02986	0.58	0.44
226.31	49.73	227.02	48.75	226.936	48.8395	0.08383	0.08951	0.00703	0.00801	0.71	0.98
243.01	21.11	244.11	19.58	243.807	19.8915	0.30284	0.31149	0.09171	0.09702	1.1	1.53
34.06	19.37	32.8	18.11	32.7177	18.1315	0.08229	0.02154	0.00677	0.00046	1.26	1.26
48.83	237.35	48.13	238.27	47.6389	238.61	0.49106	0.3399	0.24114	0.11553	0.7	0.92
79.53	237.93	79.09	238.91	78.6533	239.197	0.43672	0.28654	0.19073	0.08211	0.44	0.98
93.24	161.04	92.89	161.46	92.5037	161.425	0.38634	0.03472	0.14926	0.00121	0.35	0.42
176.12	84.92	176.37	84.4	176.232	84.4328	0.13773	0.03284	0.01897	0.00108	0.25	0.52
174.87	107.57	175.03	107.38	174.969	107.342	0.06053	0.03756	0.00366	0.00141	0.16	0.19
209.77	194.16	210.2	194.93	210.227	194.925	0.02681	0.00512	0.00072	2.6E-05	0.43	0.77
223.84	111.72	224.56	111.49	224.441	111.54	0.11912	0.05	0.01419	0.0025	0.72	0.23

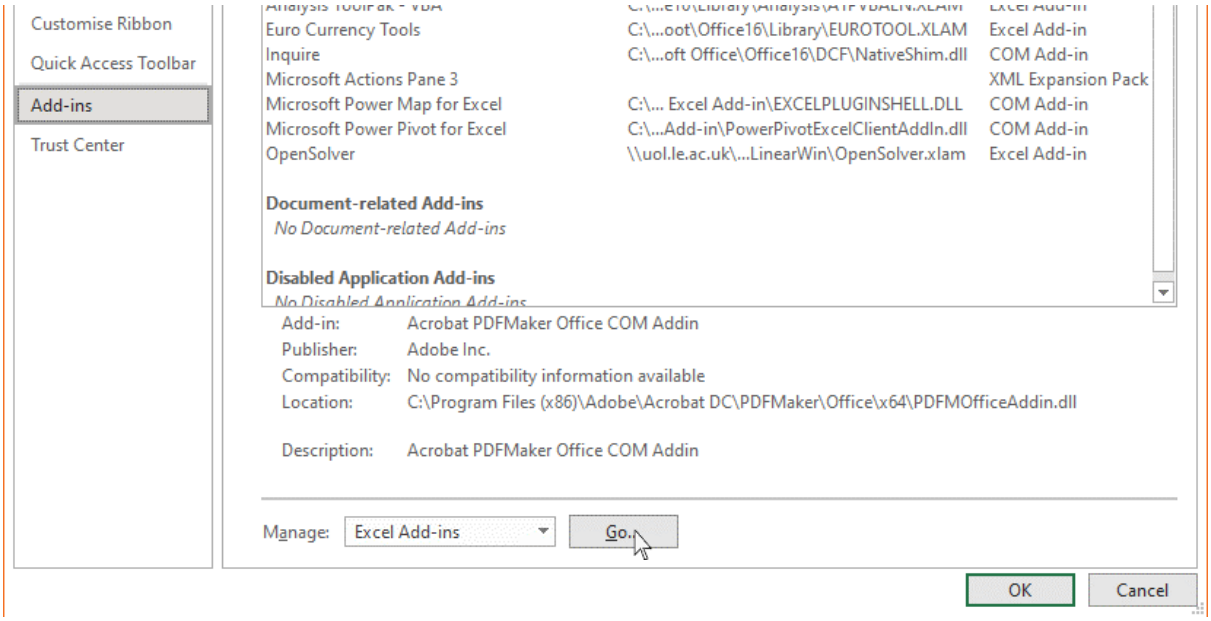
PARAMS		ERRORS	
Xc	165.1552558	SSDX	6.81397
Yc	127.4246436	SSDY	1.64866
SFx	97.66520135	SSD	8.46263
SFy	87.24950222		
ROUNDED		SUM DX	13.0443
Xc	165.155	SUM DY	6.29466
Yc	127.425	S(TX-X)	26.82
SFx	97.665	S(TY-Y)	30.15
SFy	87.25		
		MSEX	0.17932
		MSEY	0.04339
		STDEVX	0.25128
		STDEVY	0.12797

In Excel, if the solver add-in is not installed, go to File and then navigate to the options menu:

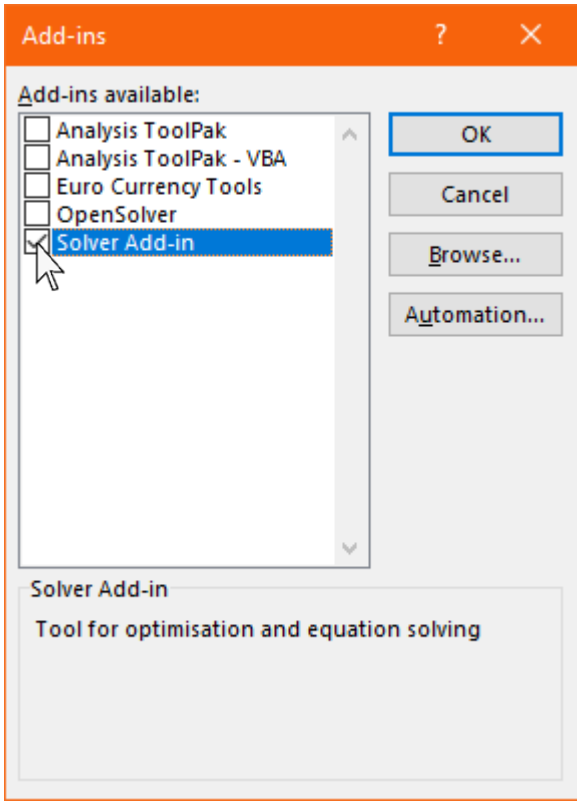


Then Click ‘Add-ins’ in the bottom right of the options window:



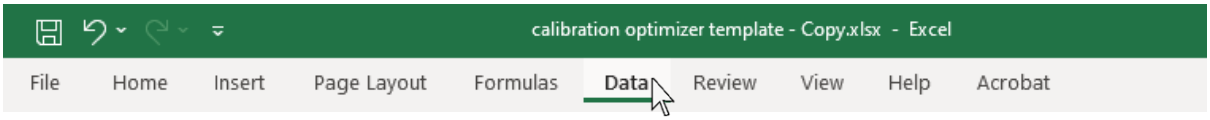


Select 'Excel Add-ins' in the dropdown menu at the bottom and then click 'Go…'

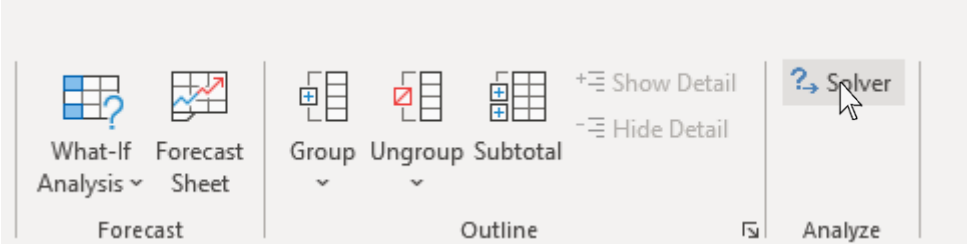


Make sure the 'Solver Add-in' box is checked and press 'OK'. The solver add-in should only take a moment to install.

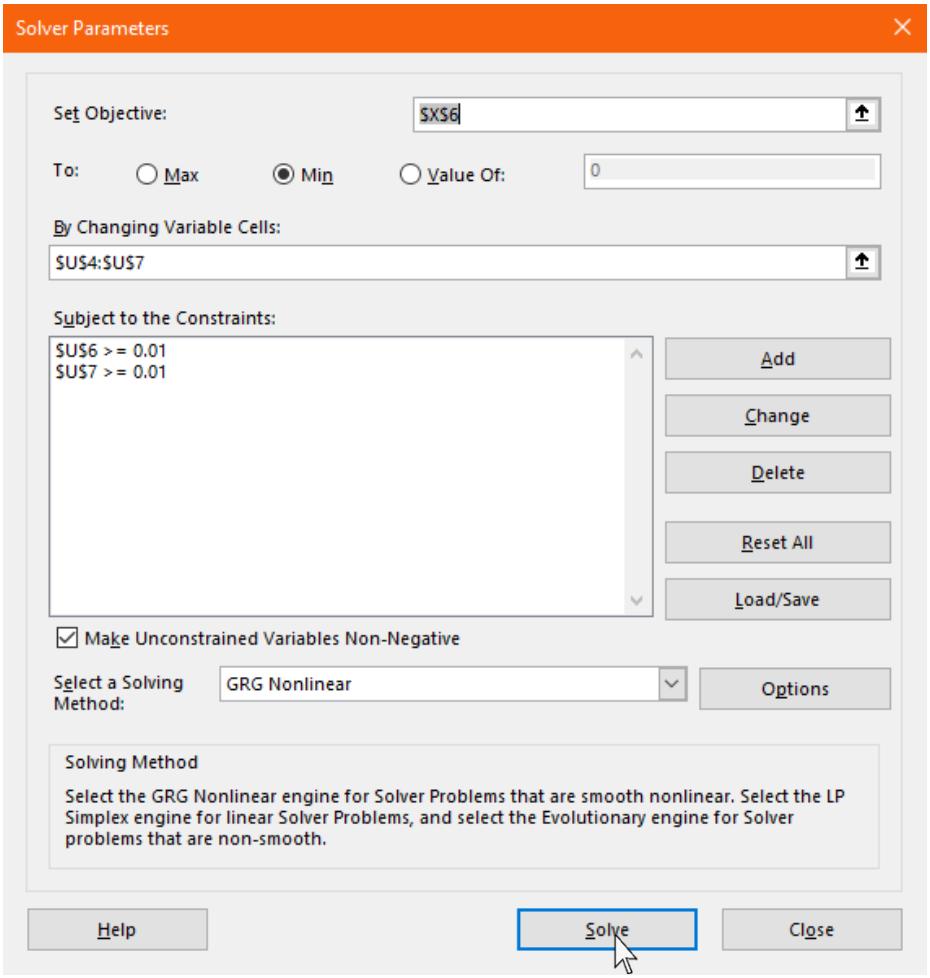
Once the solver add-in is installed, go to the 'Data' tab at the top of the toolbar in Excel:



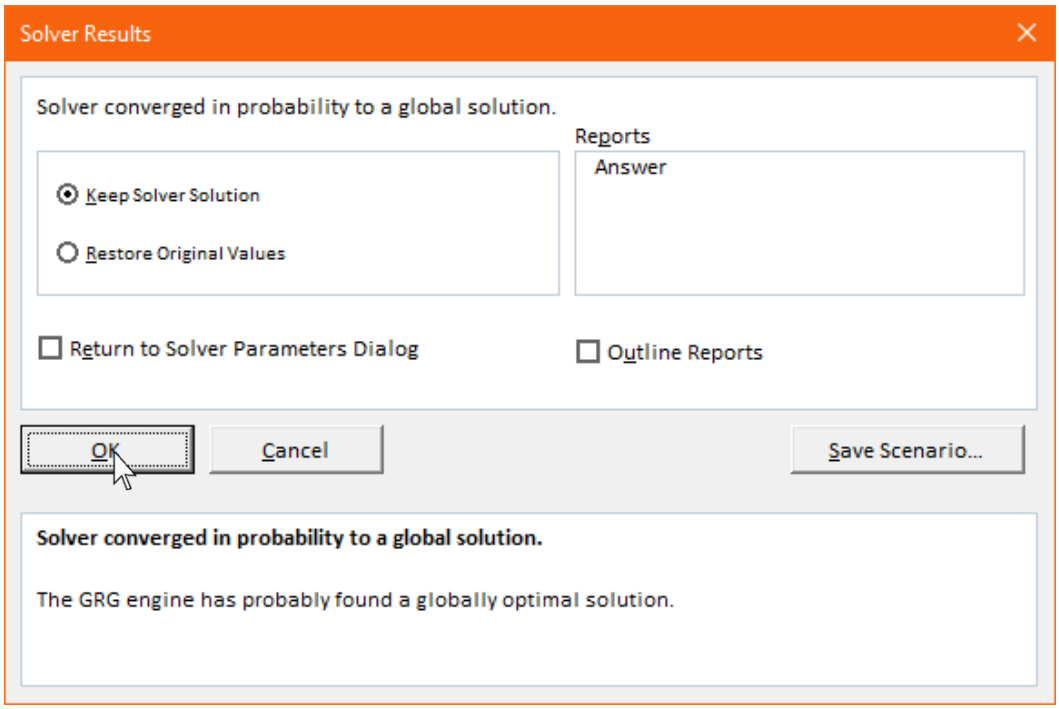
Then click on 'Solver' under the 'Analyse' section at the right-hand side of the toolbar ribbon:



Make sure the solver is minimising cell X6 by adjusting U4 through U7 as shown below. Ensure the solving method is set to 'GRG nonlinear' and constrained such that cells U6 and U7 are greater than 0.01. Then press solve:



On a successful solution, the following window will be displayed:



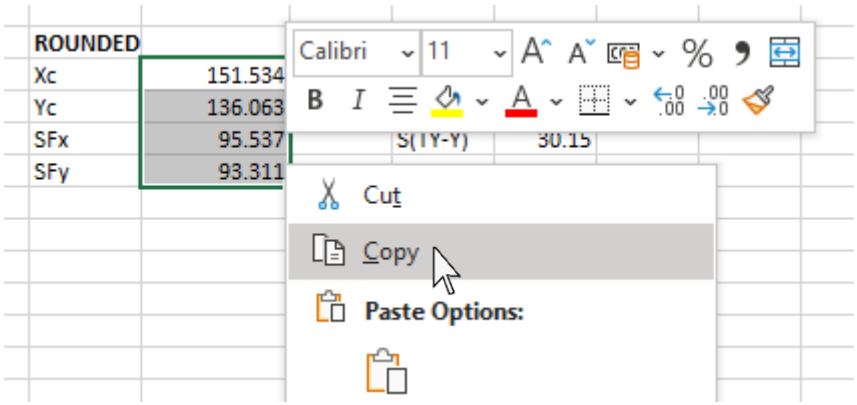
Ensure the 'Keep Solver Solution' radio button is selected, then press 'OK'. Now open the 'calibration.xlsx' file in the FluoroTensor folder. In a new column, add the current date in YYYYMMDD order in the first row:

L	M	N	O	P	Q
230217	20230301	20231102	20240108		
0	0	0			
0	0	0			
448.28	279.272	387.47			
151.358	108.487	191.799			
122.417	76.799	128.294			
102.274	69.06	104.801			
2	2	2			

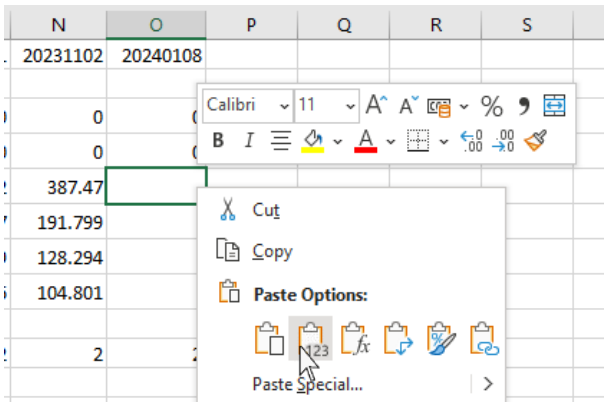
In rows 3 and four, enter 0, and in row 10 enter the colocalization threshold e.g., 2 pixels:

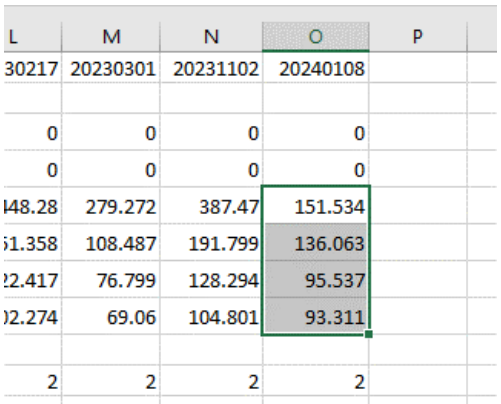
	M	N	O	P
7	20230301	20231102	20240108	
0	0	0	0	
0	0	0	0	
8	279.272	387.47		
8	108.487	191.799		
7	76.799	128.294		
4	69.06	104.801		
2	2	2	2	

Then go back to the calibration optimizer template that was just solved and select the rounded parameters in cells U10 through U13 and copy them:

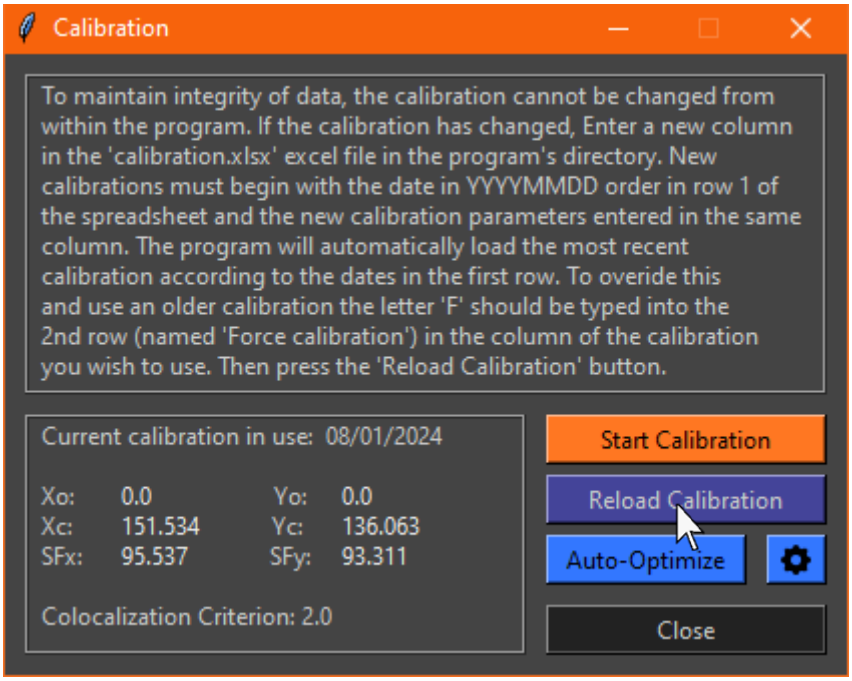


Go back to the 'calibration.xlsx' file and right click in row 5 of the calibration being added and select the 'Values' paste option, otherwise references will be copied and it won't work.





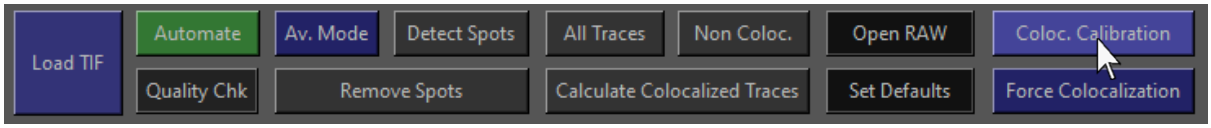
Once pasted, save the ‘calibration.xlsx’ file and go back to FluoroTensor. In the calibration window, click ‘Reload Calibration’, and the calibration that was just added will be loaded and selected in the future when the program is started.



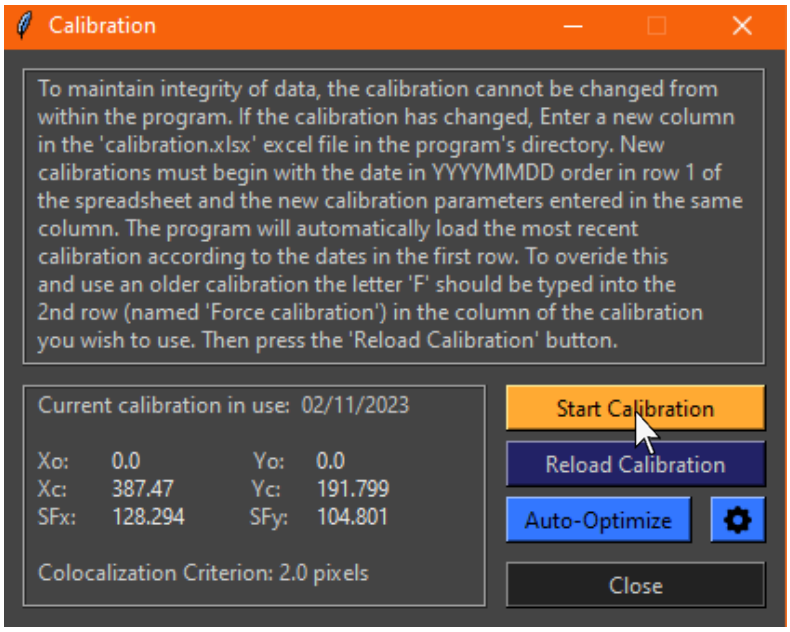
The new calibration will be applied immediately to spot detection and colocalization analysis.

3.3 Calibrating FluoroTensor with a Dual-Labelled Oligonucleotide

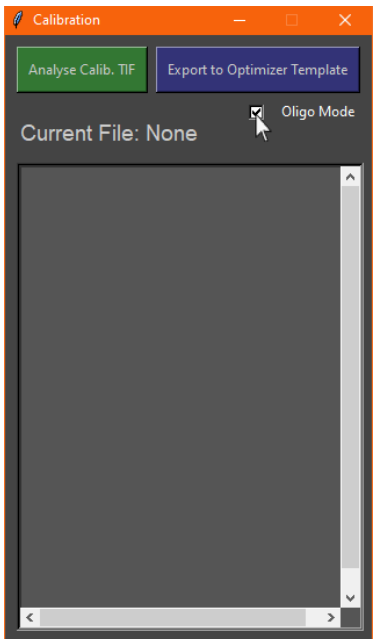
From the toolbar, open the calibration window:

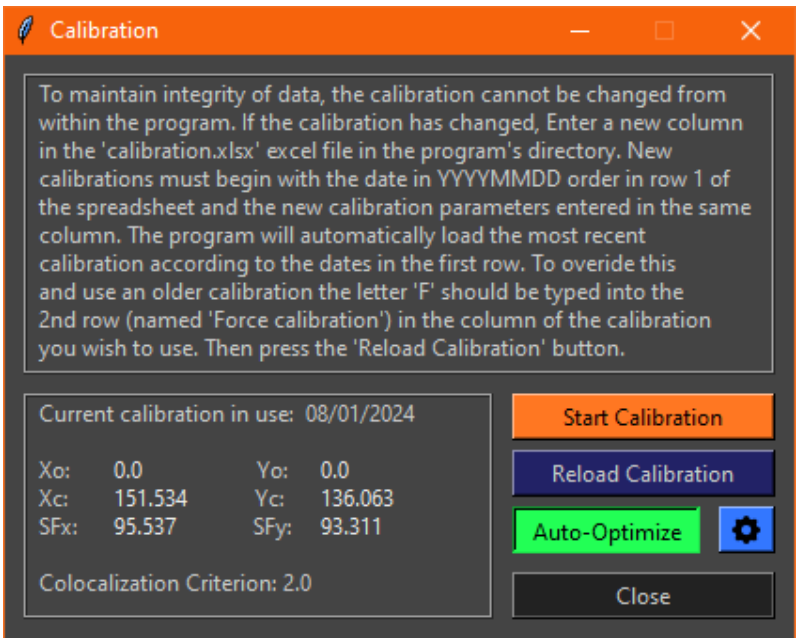


Press 'Start Calibration' just like when using fluorescent beads:



In the pop-up window, before beginning with analysis, click the 'Oligo Mode' checkbox which will automatically turn auto-optimise mode on and set the right enhancement:

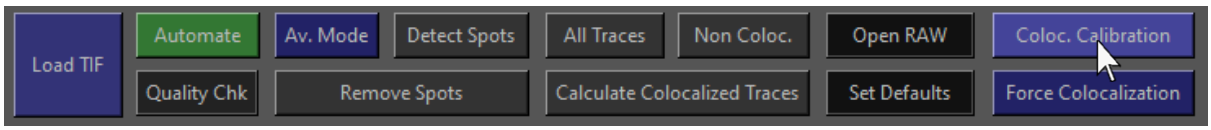




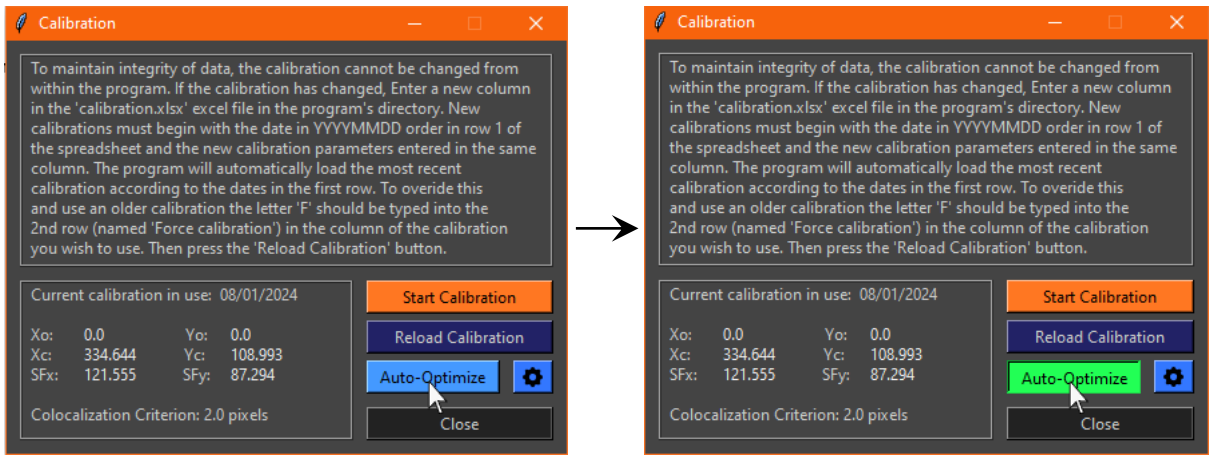
The rest of the process is identical to the fluorescent bead calibration method.

3.4 Automatic Optimization of Parameters

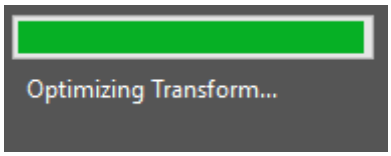
To automatically optimise the parameters while analysing data instead of using a fixed calibration, open the calibration window by clicking ‘Coloc. Calibration’ on the toolbar:



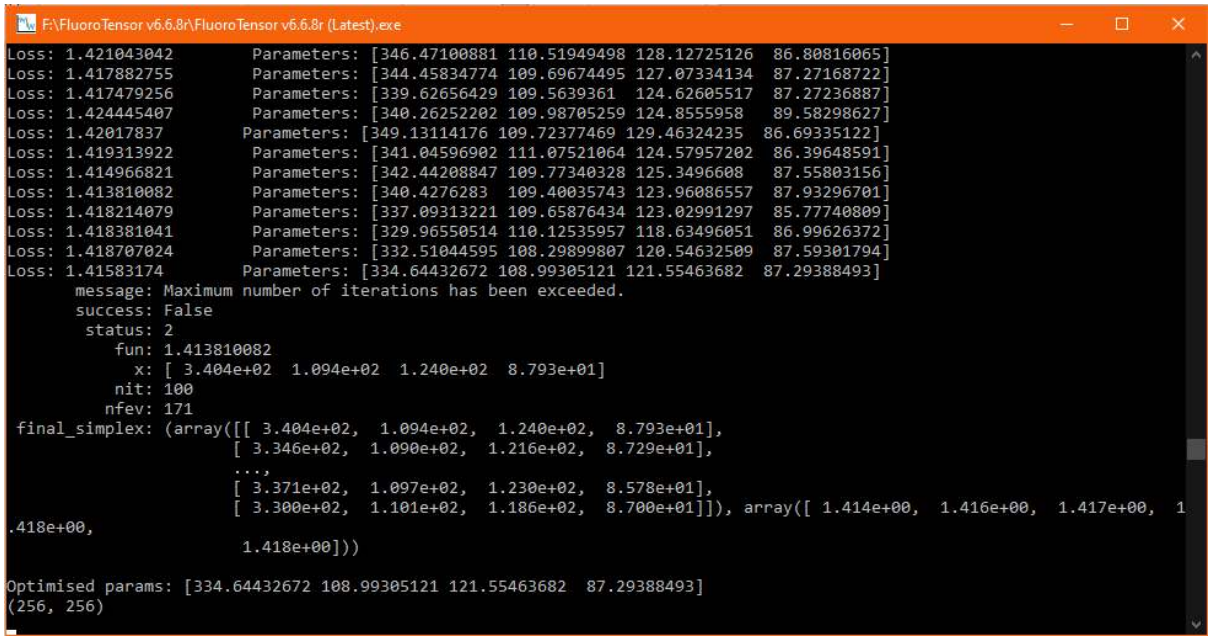
In the calibration window, toggle on automatic optimization by clicking ‘Auto-Optimize’:



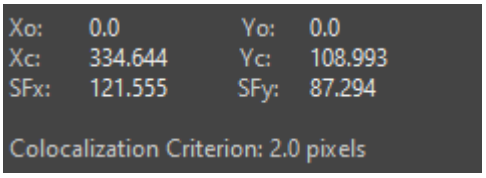
When Auto-Optimise is enabled, the transform will be optimised when the ‘Detect Spots’ button is pressed on the toolbar. The program will first detect spots in every channel. Then it obtains approximate parameters by grid search and optimizes them using a Simplex solver. Depending on the number of spots this may take a while, so don’t worry if the window becomes unresponsive. A progress bar will be shown in the toolbar while this process occurs:



In addition, the optimization steps are printed in the terminal that opens with the program:



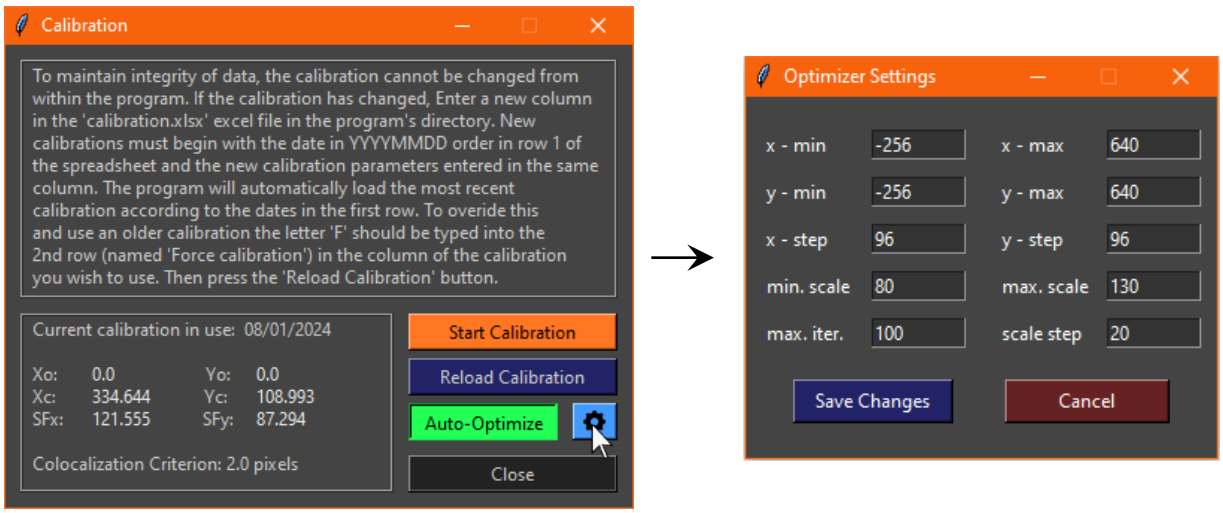
Once the solver has found an optimal solution, the parameters will be shown in the calibration window:



To return to the original calibration parameters, toggle off ‘Auto-Optimise’ in the calibration window and click ‘Reload Calibration’. If you wish to go back to the auto-optimised parameters without resolving them again, toggle on ‘Auto-Optimise’ again and press reload calibration.

3.5 Adjusting Auto-Optimizer Settings

To change the settings of the automatic optimizer, click the cog icon next to ‘Auto-Optimize’ and the settings window will appear:

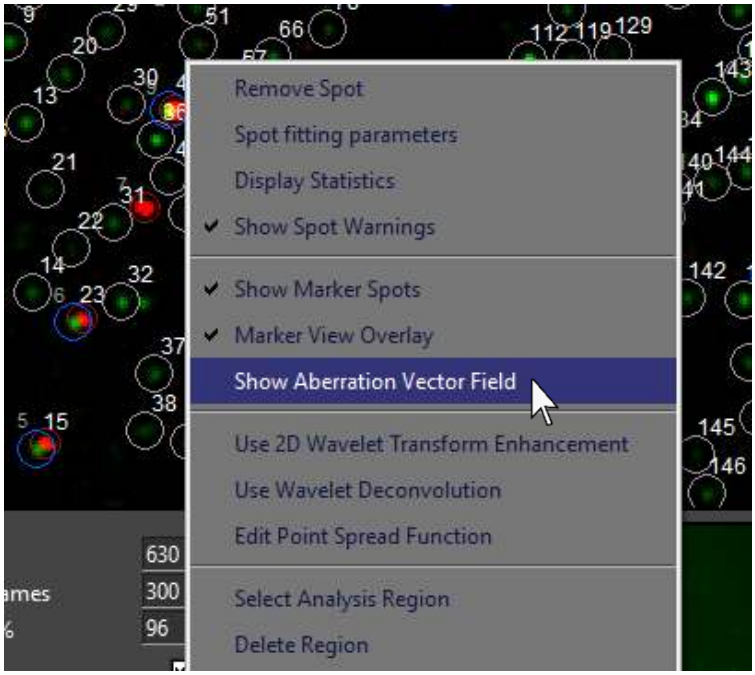


The top 4 inputs boxes control the range of coordinates the grid-search optimizer will cover. x-step and y-step are the steps it moves in; a smaller step size makes grid search more precise but slower, and we recommend no lower than 48 to achieve optimization in a reasonable time. Scale and scale step control the range and step of the scale factor, and max. iter. is the maximum number of iterations the Simplex solver will attempt. You can then save the changes or cancel.

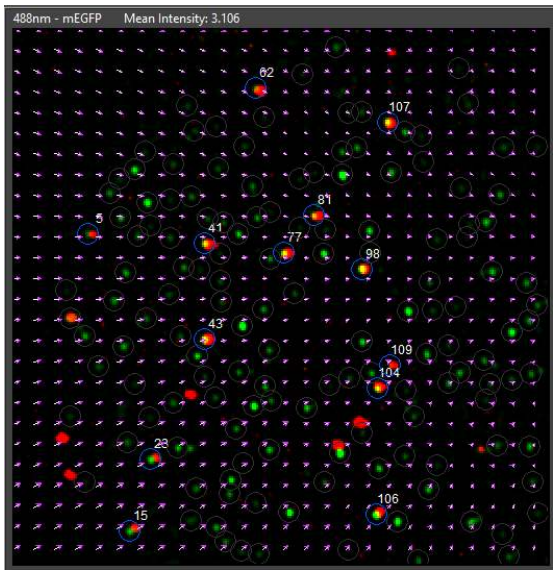
The optimizer isn’t perfect and may sometimes fail to find an optimal solution. The solver works better with more colocalized spots. The minimum number of spots to work reliably is 4.

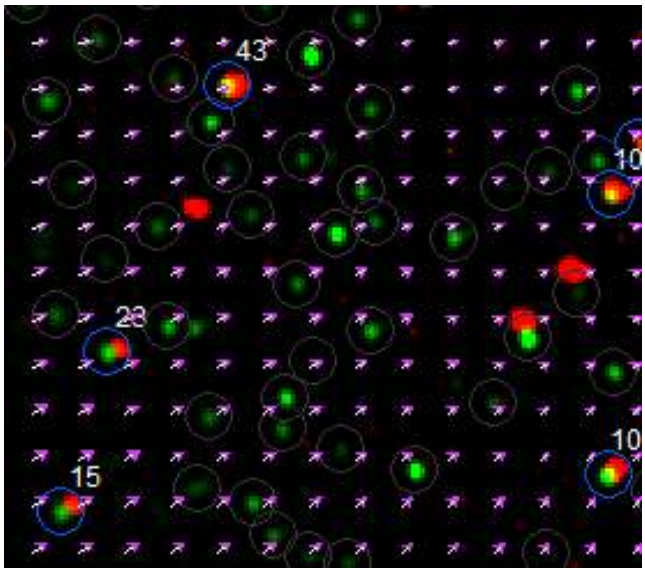
3.6 Plotting the Aberration Vector Field

It might sometimes be useful to visualize the transform that is being applied to correct chromatic aberration. This can be plotted as a vector field. To plot the transform vector field over the canvas, right click on the relevant canvas and select 'Show Aberration Vector Field' from the context menu. This toggles it on. To turn it off, right click and select it again:



The vector field will be displayed as white lines with magenta arrow heads pointing in the direction that maps secondary spots onto the markers. Unfortunately, antialiasing is not possible due to limitations of the canvas in the tkinter GUI library:





Chapter 4 – Automation

4.1 Prerequisites

Automation allows you to select a folder of movies, or load an index file which points to a curated list of quality-controlled movies, and the program will then run through them one by one, detecting foci and calculating traces.

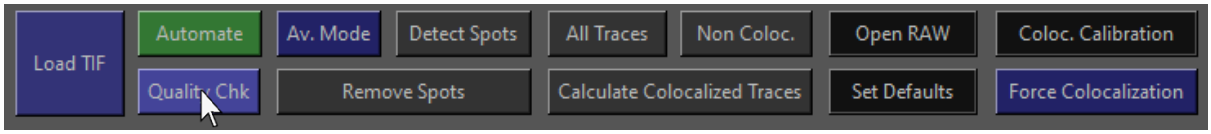
For automation, the frame boundaries of the channels must be identical from file to file. Thus, we recommend using this when data has been collected by automated acquisition on the microscopy system. In such a system the camera and laser shutters are precisely controlled such that each fluorophore type is illuminated for 300 frames. The first movie should then be loaded into the program to determine the frame boundaries that should be set at this time.

Before running automated analysis, ensure that the calibration is correct and properly maps coordinates to correct chromatic aberration. If using the automatic optimizer, ensure the colocalization rate in files is high enough. At least 4 colocalized spots are required per image and preferably more if the density of spots in secondary channels is high. Insufficient colocalized spots means the optimizer may solve false correlations and increase the rate of spots falsely identified as colocalized.

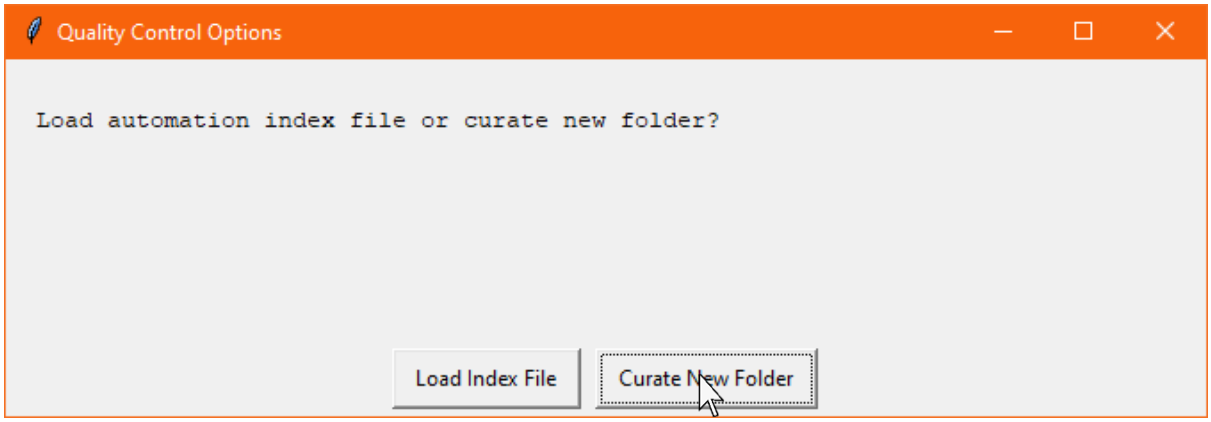
4.2 Quality Control

Before running automated analysis on a folder of .tif files, poor quality files should be removed. These include files with large aggregates or smears and where the images are out of focus. This can be done without using FluoroTensor, but it may be beneficial to use the inbuilt function which creates an index of good files rather than manually removing bad files from the folder.

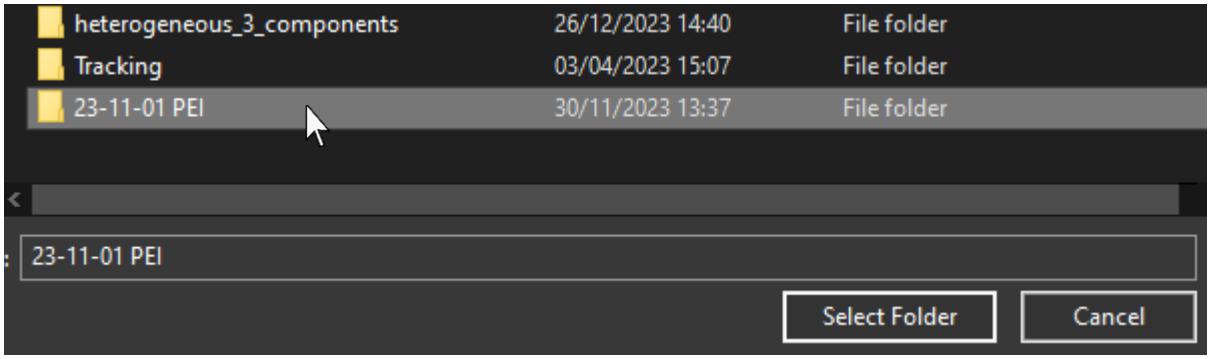
To start quality control, click 'Quality Chk' in the toolbar:



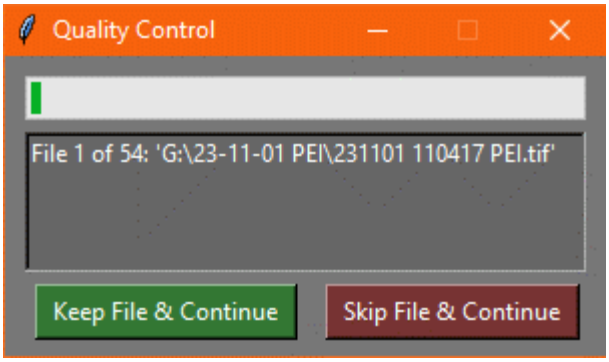
A dialogue box will appear prompting you to load an index file or curate a folder. In this case we want to start quality control. Press 'Curate New Folder':



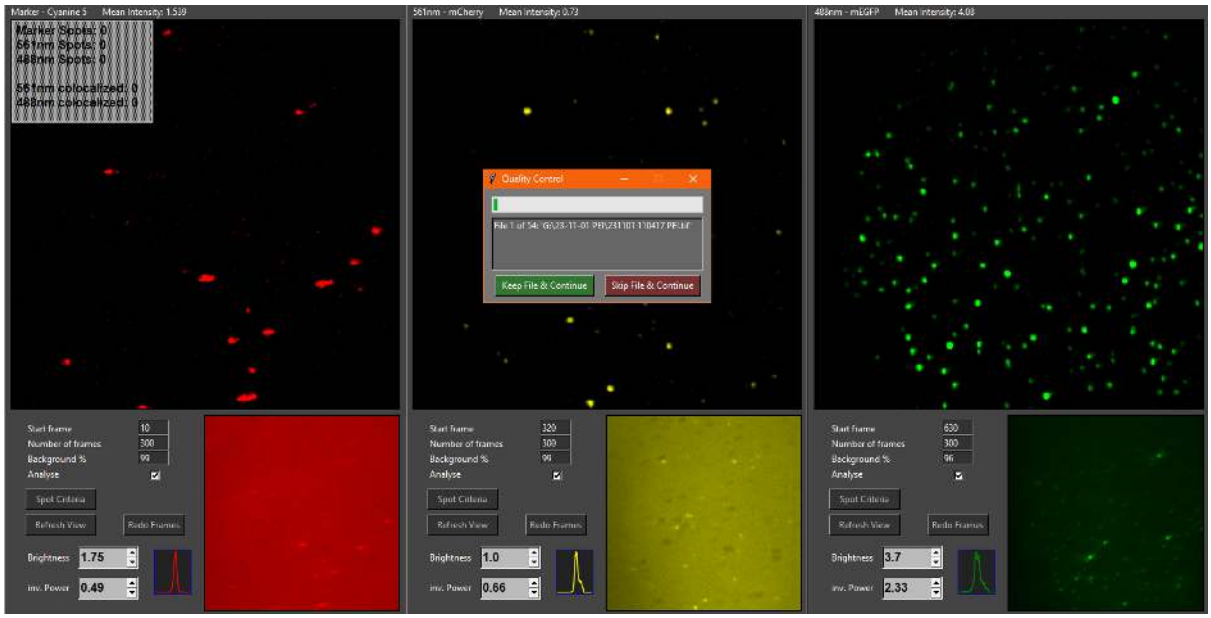
A file dialogue will appear from which you can select the folder you wish to curate:



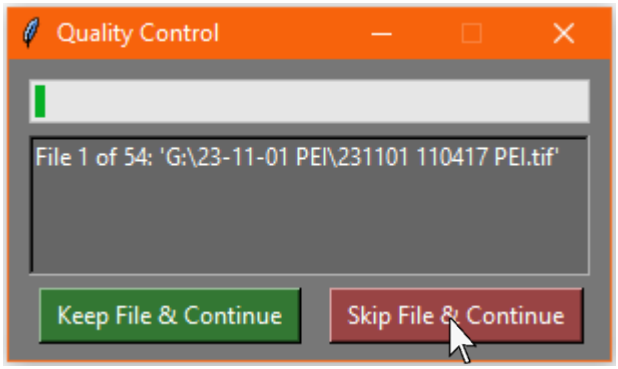
The program will then make a record of all .tif files in that folder and load the first one automatically. A window will be displayed where you can choose to add the file to the index if the quality is good, or skip it if the quality is bad:



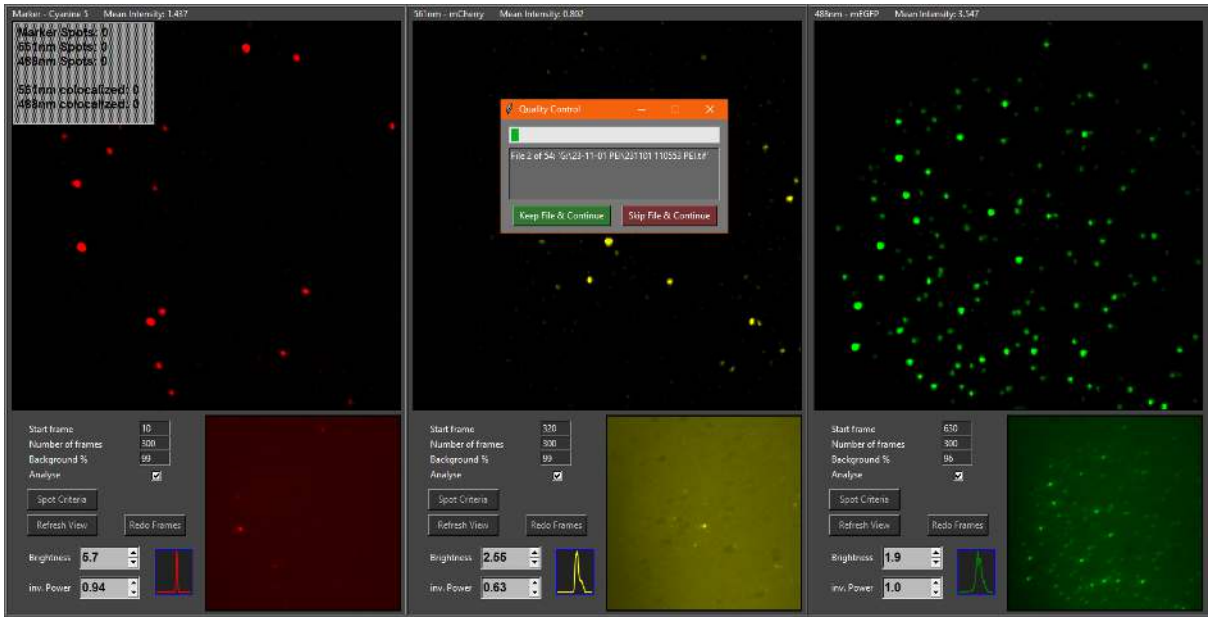
For example, the current file is out of focus in the marker channel:

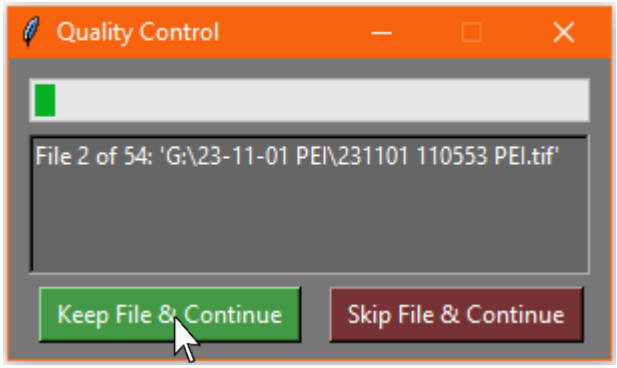


The file can then be skipped, and the next file will be loaded:

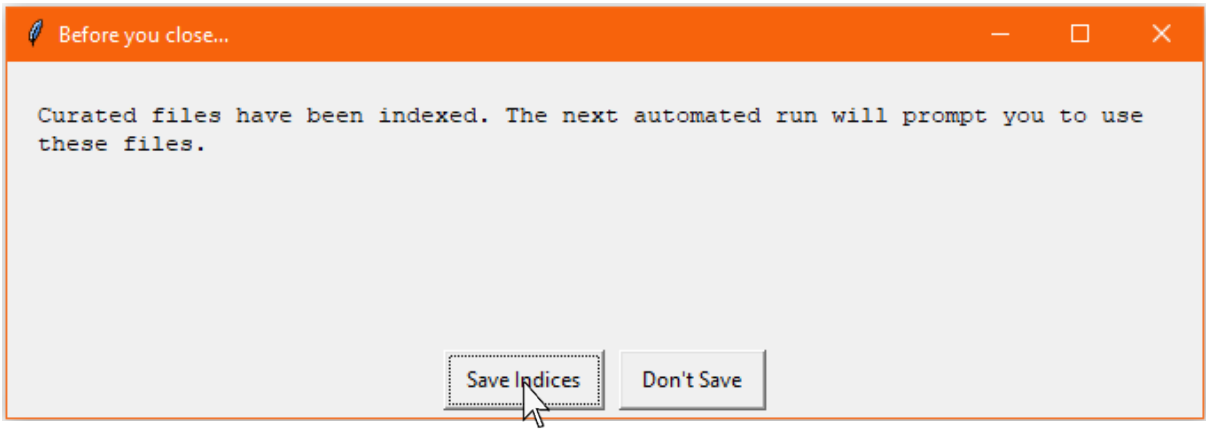


The next file is in focus and doesn't have large aggregates or smears and should be kept:





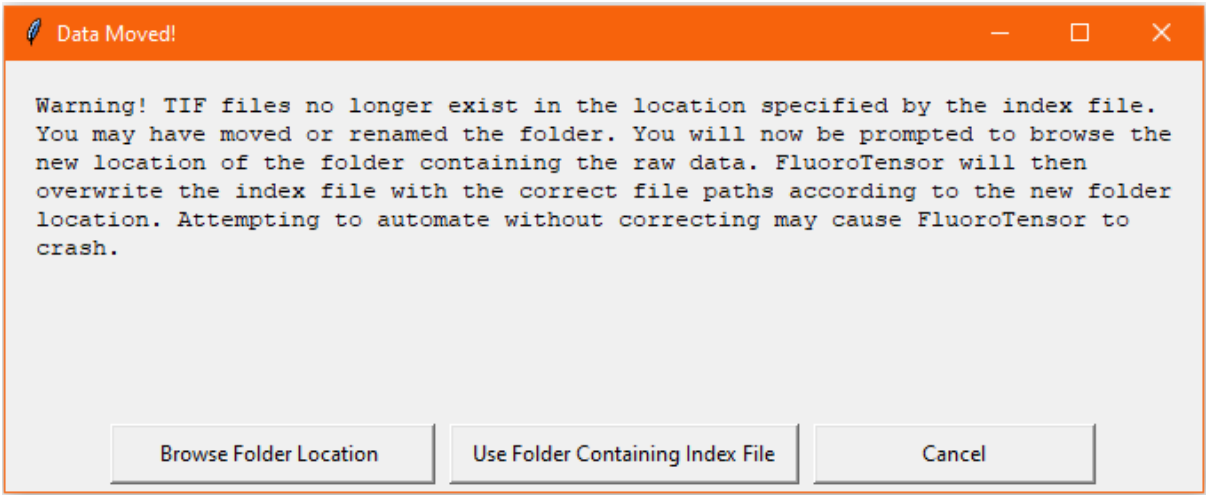
Once all files have been evaluated, you will be prompted to save the index:



If you do not save the indices, the curated list will only be available for automation during the current session and will be lost when the raw data analysis window is closed. If you choose to save them (recommended), a file dialogue will open where you can save the them as an index (.ind) file.

If you choose to close the quality control window before evaluating all files, you will also be prompted to save the index of the files kept so far.

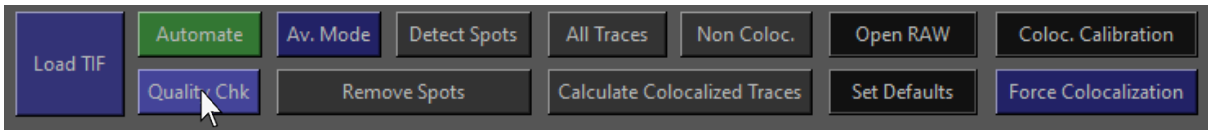
If you rename any of the .tif files that were indexed, the program will be unable to locate them if the index file is used and quality control will have to be done again. In the event that you move, copy and paste, or rename the folder that was indexed, the program will prompt you to browse the new location the next time the index file is loaded:



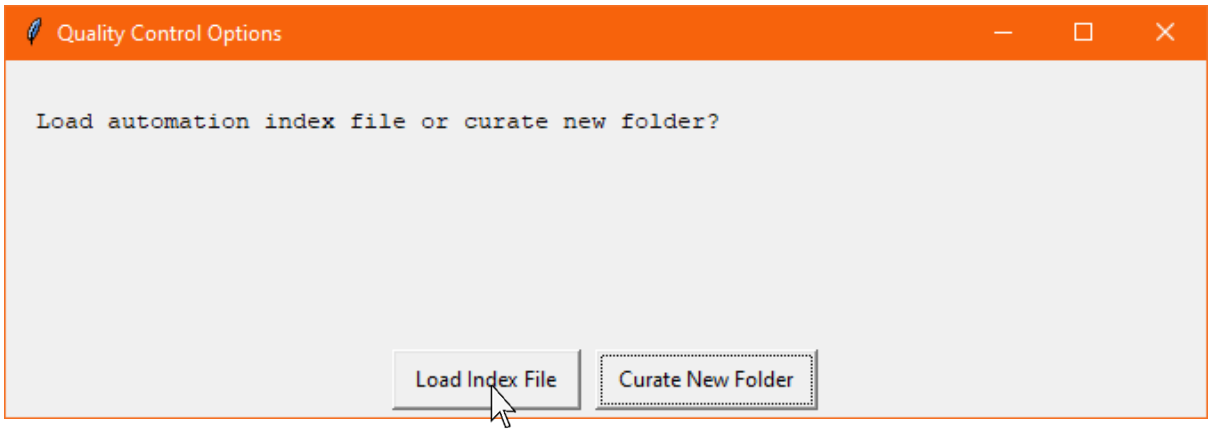
You can now browse for the folder where the data is located. Alternatively, the second option can be used if the index file is in the same folder as the data.

FluoroTensor will the rewrite the index file with the updated file locations.

To load the index file, click ‘Quality Chk’ in the toolbar:



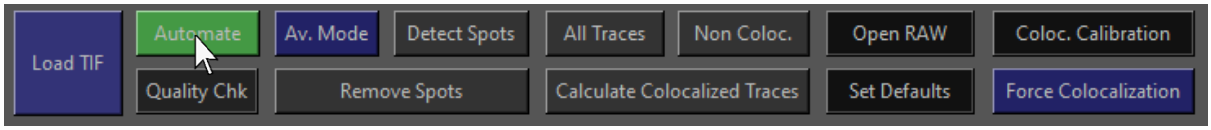
Then select ‘Load Index File’ in the dialogue box:



Unless there is an error with the index file or it has been moved it will be loaded, ready for the next automation run.

4.3 Running Automated Analysis

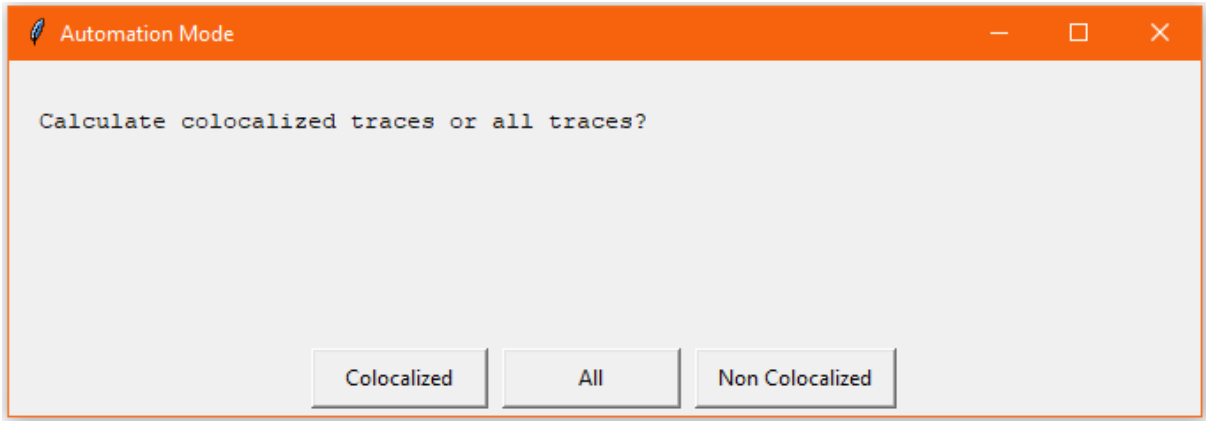
To run automated analysis, load the index file as mentioned in the previous section on quality control. Then click 'Automate' in the toolbar:



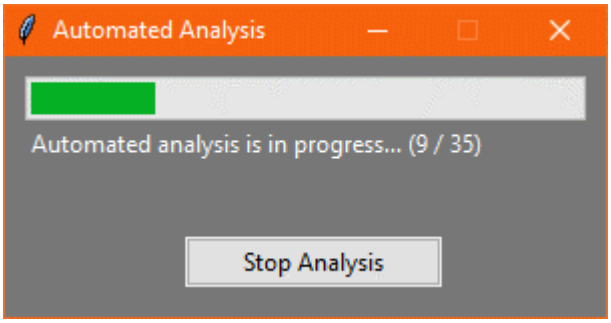
If you have loaded an index file prior to beginning automated analysis, a dialogue box will appear prompting you to choose whether to use the index file to load data, or to open data from a different folder:

If you haven't loaded an index file, or choose to open a different folder from the dialogue box, a file dialogue will be displayed where you can browse a folder location:

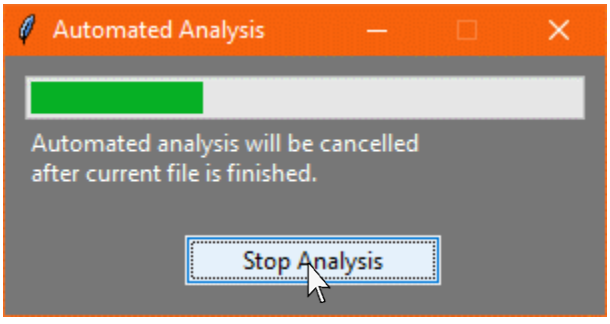
After this step, another dialogue box will appear prompting you to choose an experiment mode:



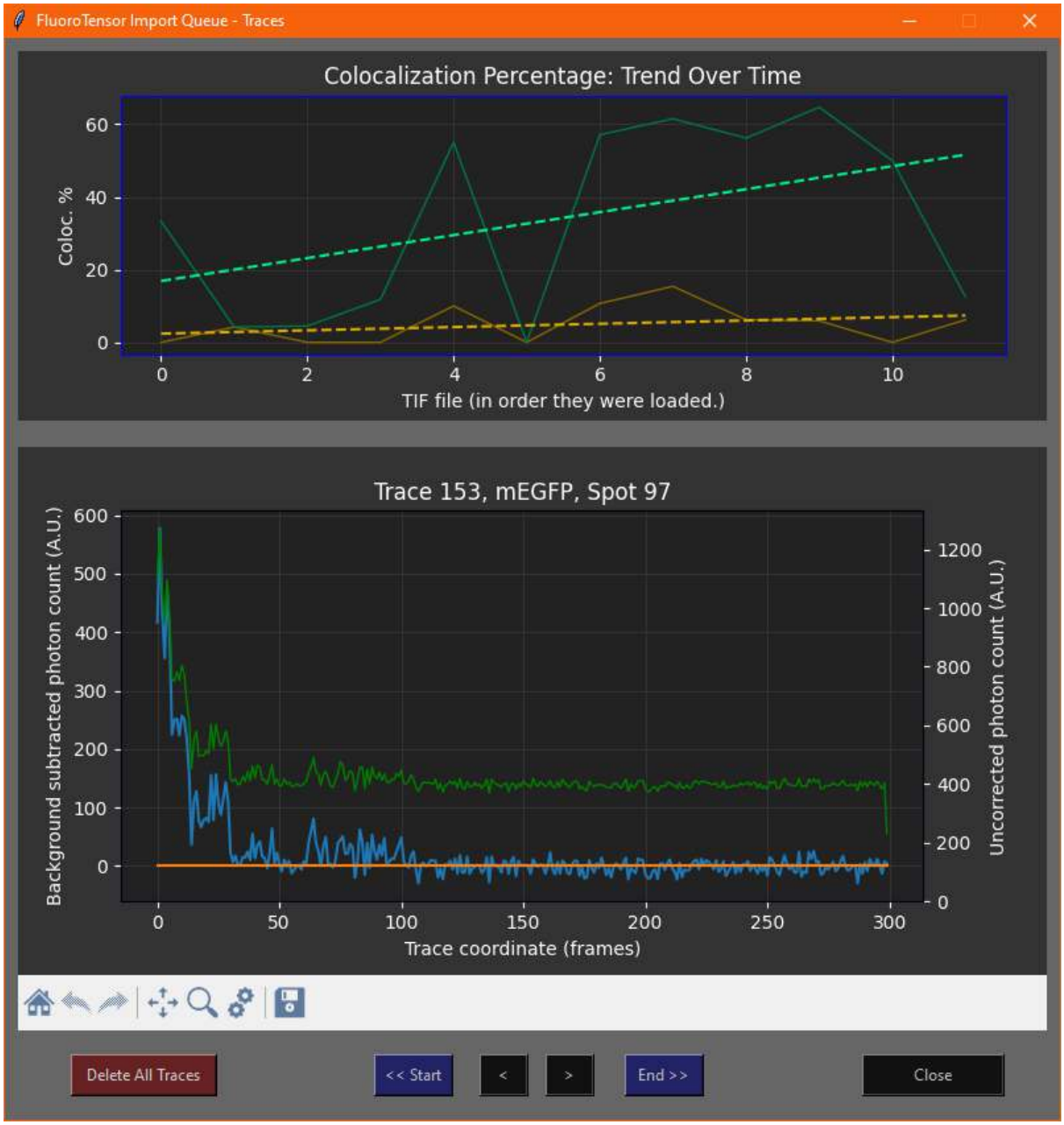
When a mode has been chosen, automated analysis will start immediately, sequentially loading the files in the index if applicable or in the selected folder if not. Each file will be split into channels based on the frames set at the beginning, enhanced and analysed. The relevant traces according to experiment mode will be calculated from the detected fluorescent signals and a progress bar will be displayed:



The run can be cancelled at any time by pressing 'Stop Analysis' in which case the program will finish analysing the current file and then stop:



Whether the automation run finishes naturally or is stopped early, the data will be kept and the trace window will pop up:



Chapter 5 – Step Detection and Traces

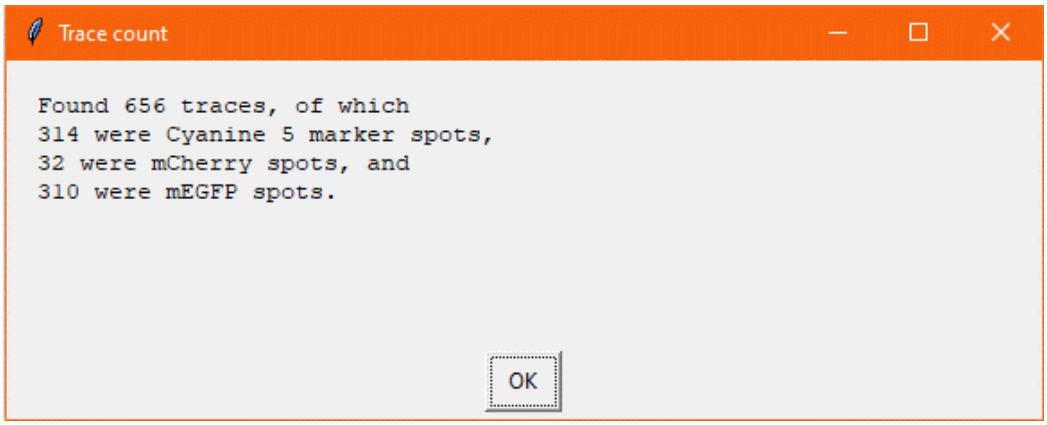
5.1 Importing Data from the Raw Analysis Window

Once the raw .tif files have been analysed, the traces are stored in an import queue ready to be transferred to the main interface. They can be viewed before importing, by clicking 'Open Traces' on the right-hand side of the toolbar:

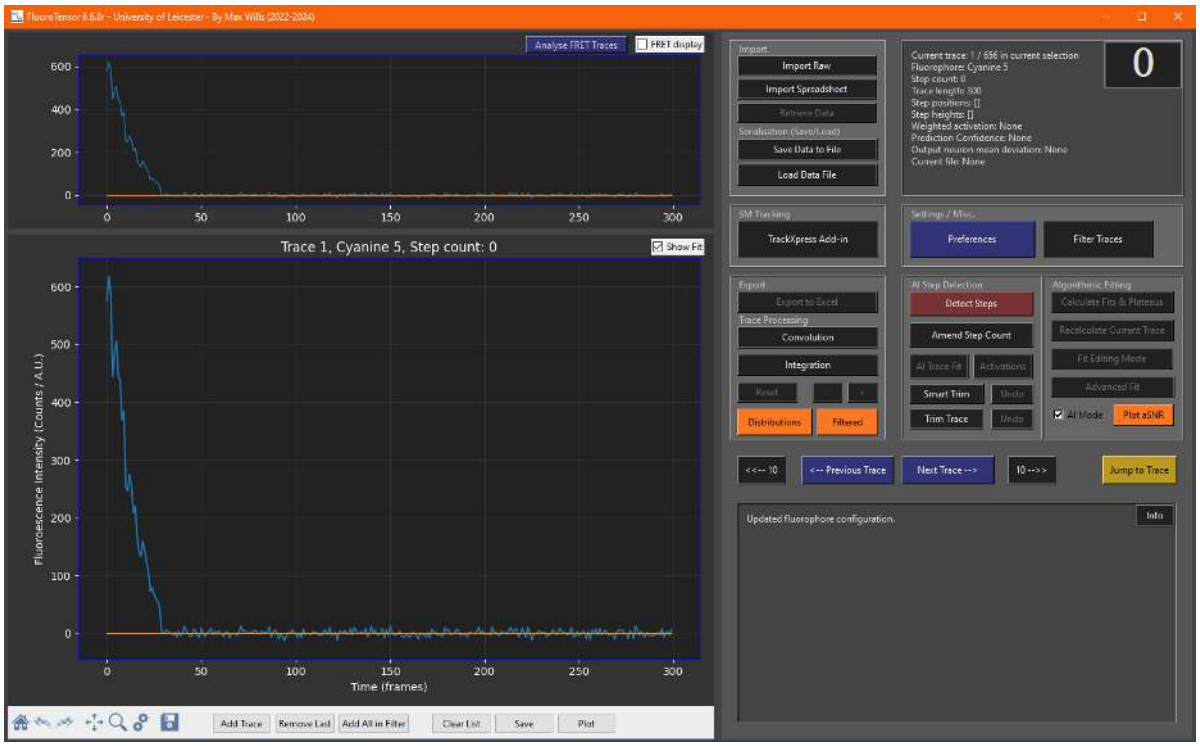
This will bring up the Import queue window that opens after traces are calculated or an automation run finishes. If a mistake was made, the traces can be deleted and the automation run restarted:

To import the traces into the main interface, click 'Import to FluoroTensor' at the bottom right of the toolbar:

The raw data analysis window and all its sub-windows will close automatically with this action and a dialogue box will appear notifying the user of the number of traces imported:



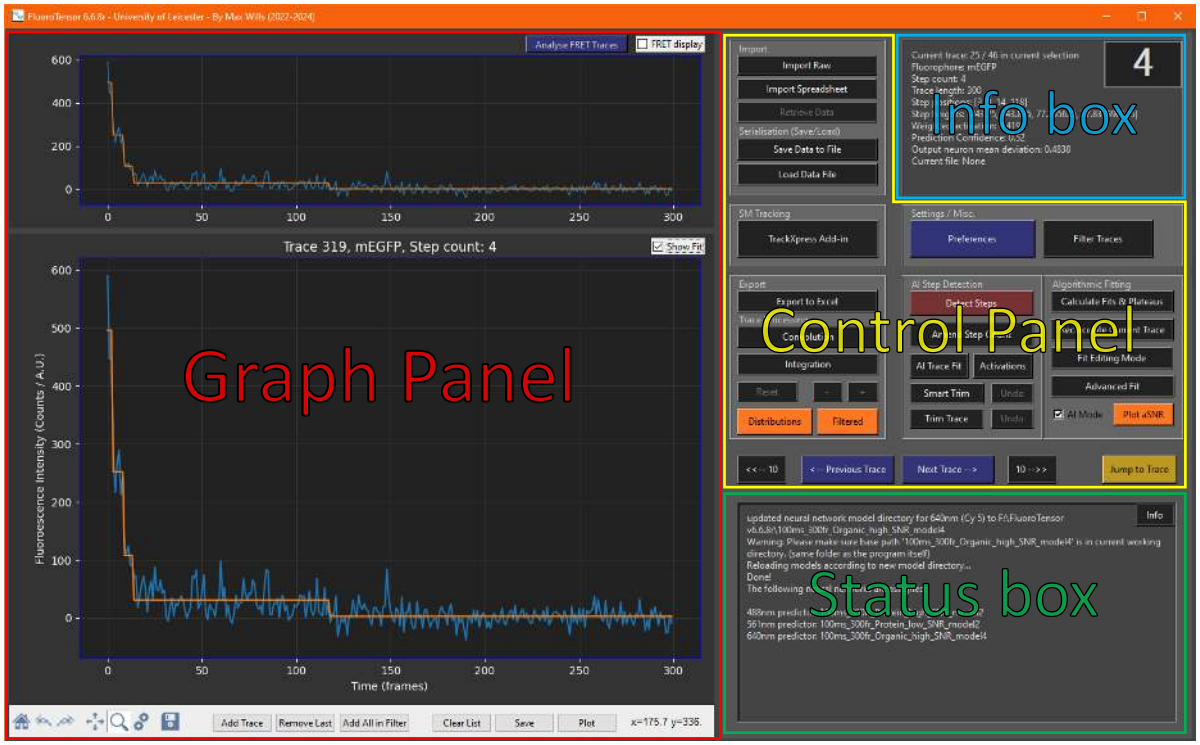
Click OK to continue to the main interface:



The next section will cover the layout of the interface.

5.2 Main Interface Layout

The GUI is split into four main areas. On the left is the graph panel where traces are plotted. On the right is the control panel where all the controls are for trace analysis. The info box shows information about the current trace plotted in the graph panel. The status box displays information about tasks being carried out in response to user input.

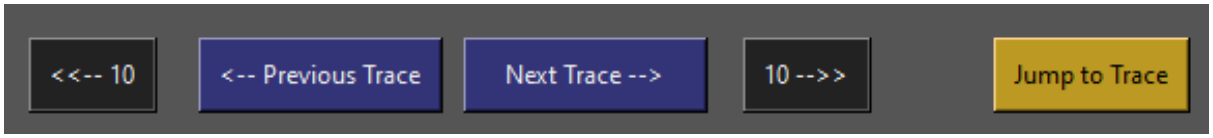


The control panel is split into sections with similar options clustered together. These sections include importing, exporting, AI step detection, trace fitting, statistics, trace processing, settings, filtering and navigation through the dataset.

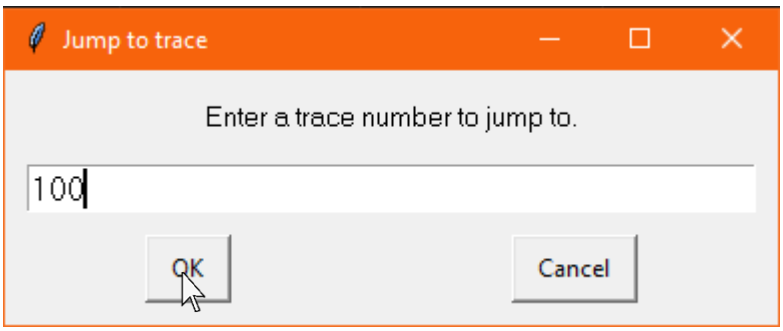
All of these functions will be covered in detail in the following sections and chapters of this user guide.

5.3 Navigating Through the Dataset

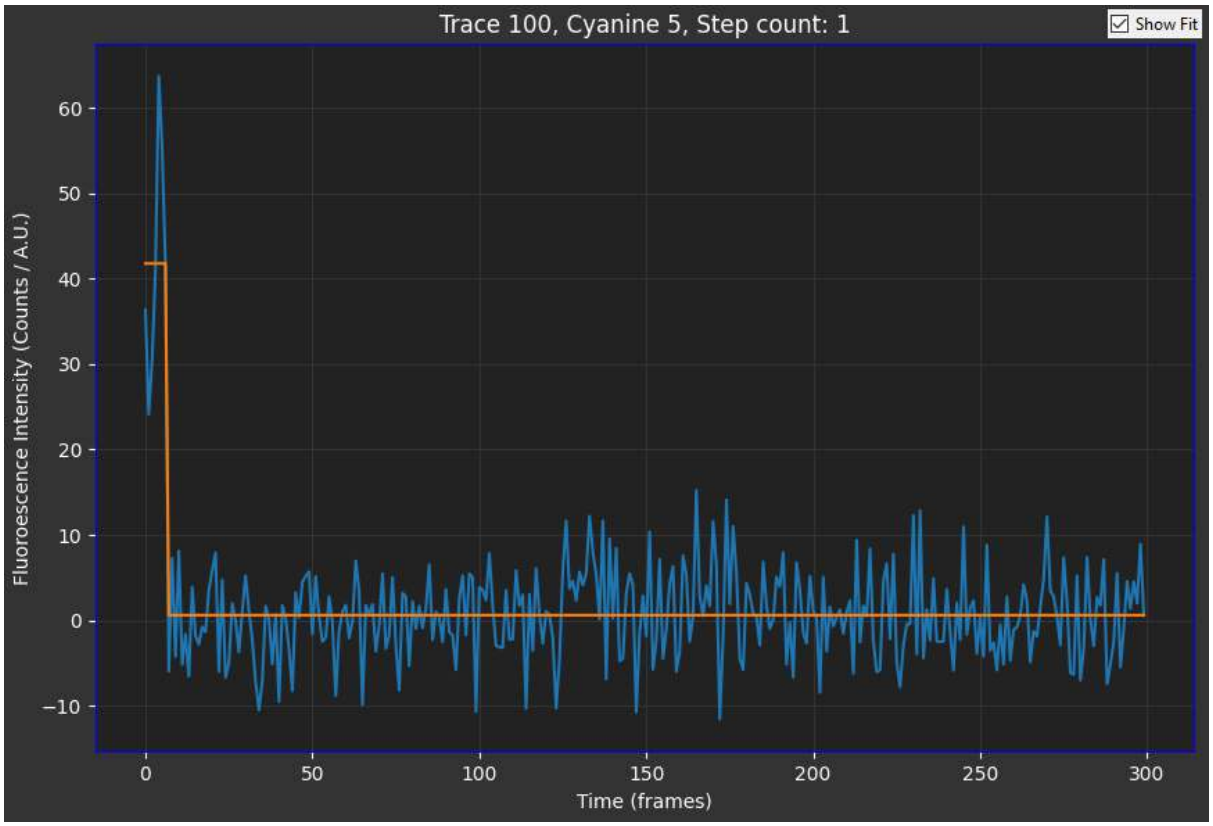
At the bottom of the control panel are buttons for navigating through the dataset:



To go to a specific trace in the dataset, press ‘Jump to Trace’ and enter the trace number into the text box that appears and press ‘OK’:

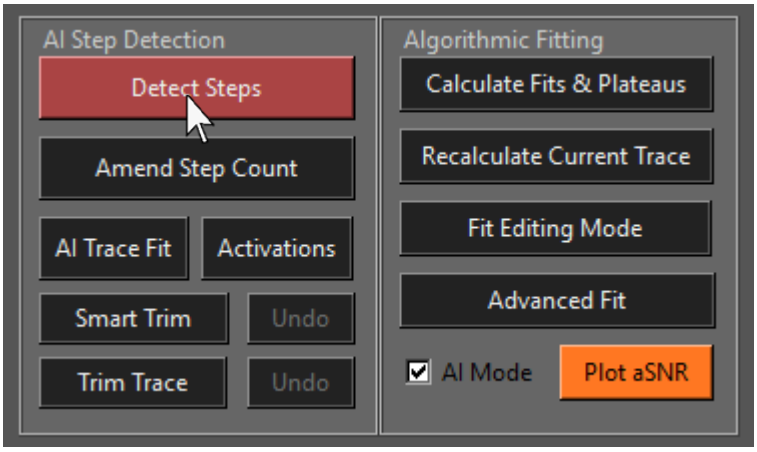


That trace will then be displayed in the graph panel, and the info box will be updated:

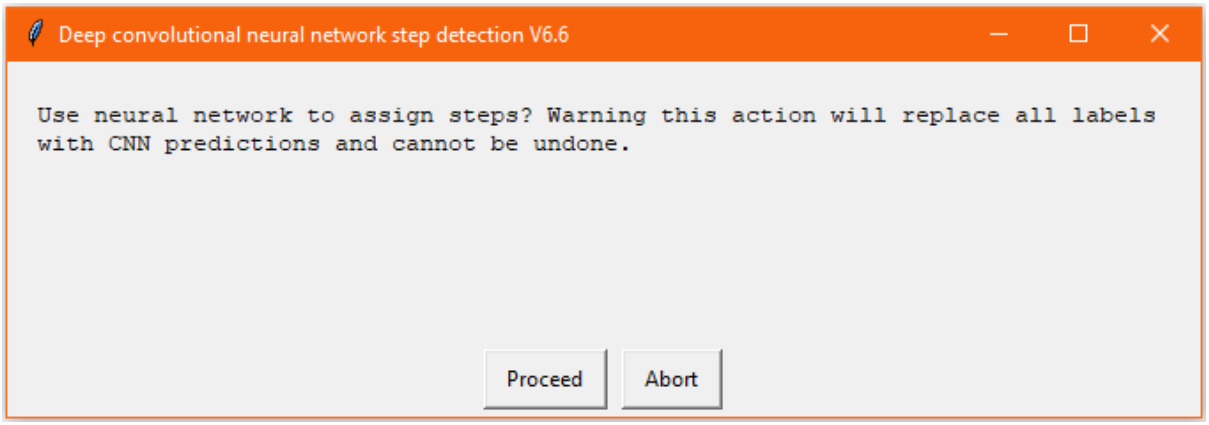


5.4 Step Detection

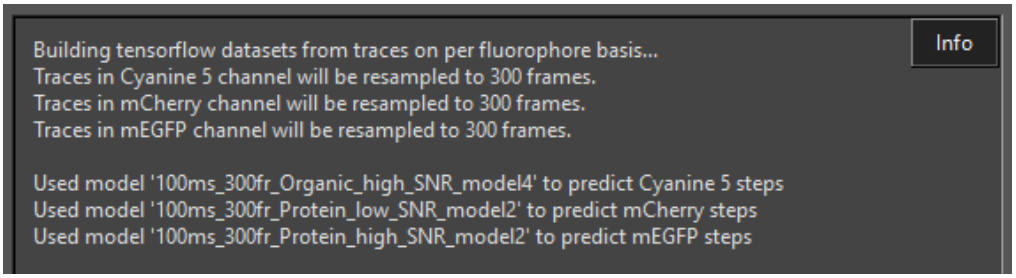
The core function of FluoroTensor is step detection in fluorescence intensity traces to determine the number of labelled molecules, and thus the stoichiometry of the complex. To detect steps, click 'Detect Steps' in the 'AI Step Detection' section of the control panel:



Next, a dialogue box will be displayed, asking the user to confirm:

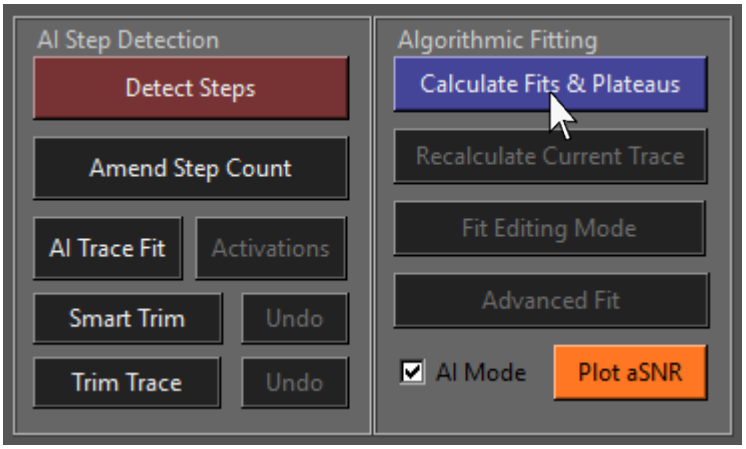


FluoroTensor will then use its neural network models to predict the number of steps in all traces in the dataset; this make take a little while, especially the first time this function is used in a session. The status box will display a message about which neural networks were used for each channel:

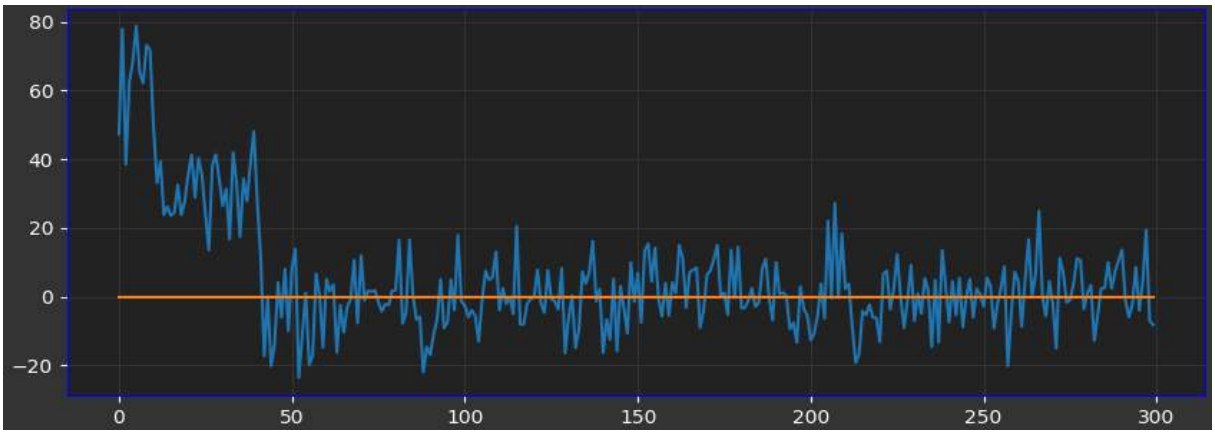


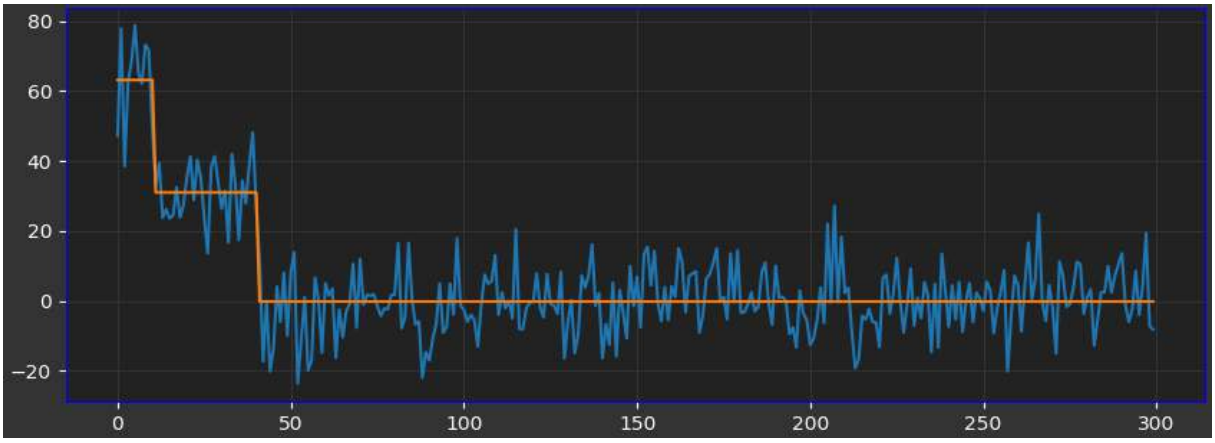
5.5 Plateau Fitting

Step detection only predicts the number of steps in a trace and works independently from the system that finds the positions in the trace to fit the plateaus. To fit the plateaus, click the 'Calculate Fits & Plateaus' button in the 'Algorithmic Fitting' section of the control panel:



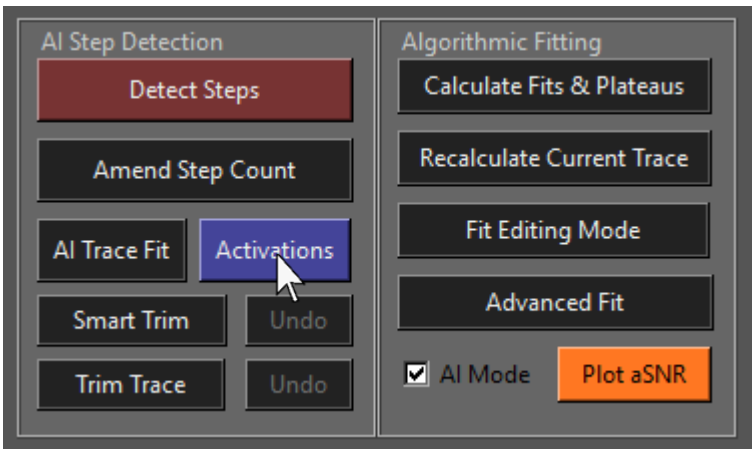
All plateaus will be fitted at once; this process may take a little while if the dataset is large (e.g., over 1000 traces). Once complete the trace plot will update:



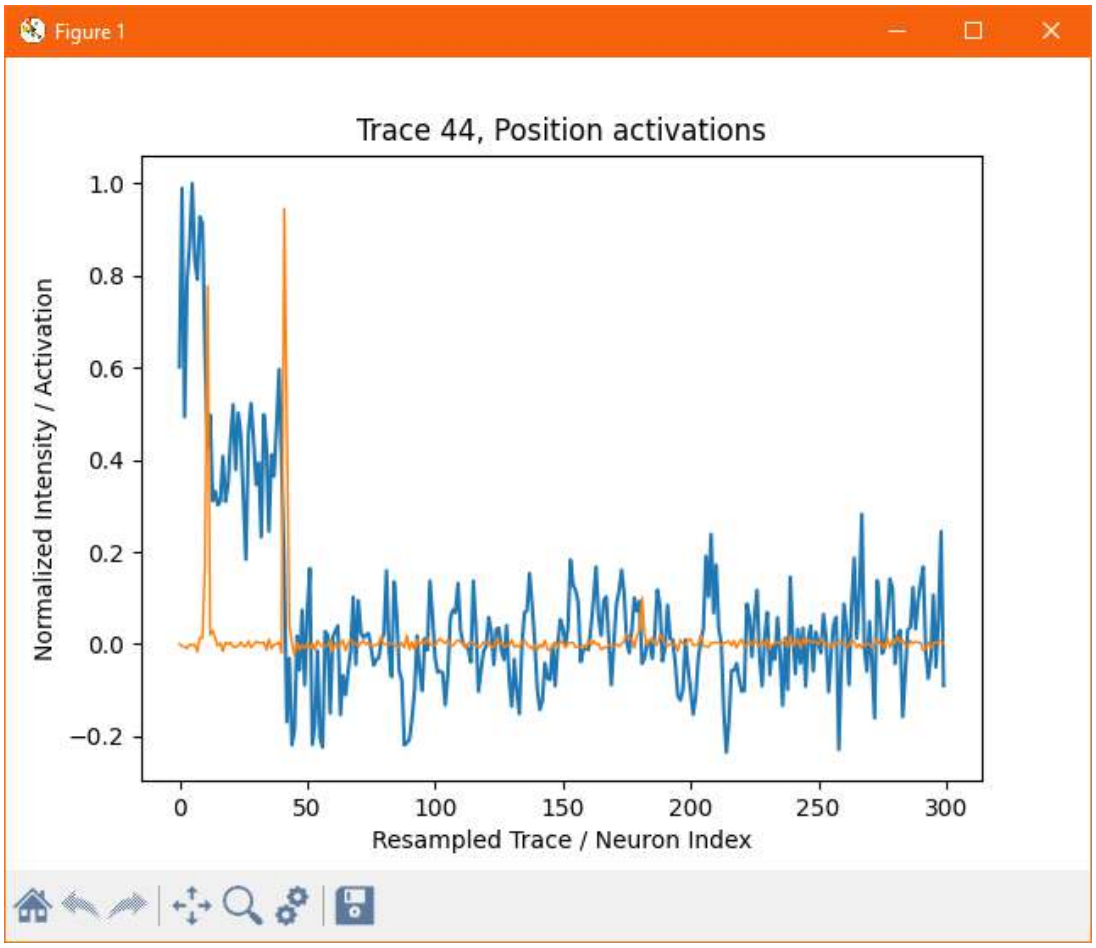


5.6 Viewing Positional Activations

The rest of this chapter is not essential but may be found useful. Sometimes it can be insightful to look at the activations of the neural network model that finds the positions of steps in the trace. To do this, click 'Activations' in the 'AI Step Detection' section of the control panel:



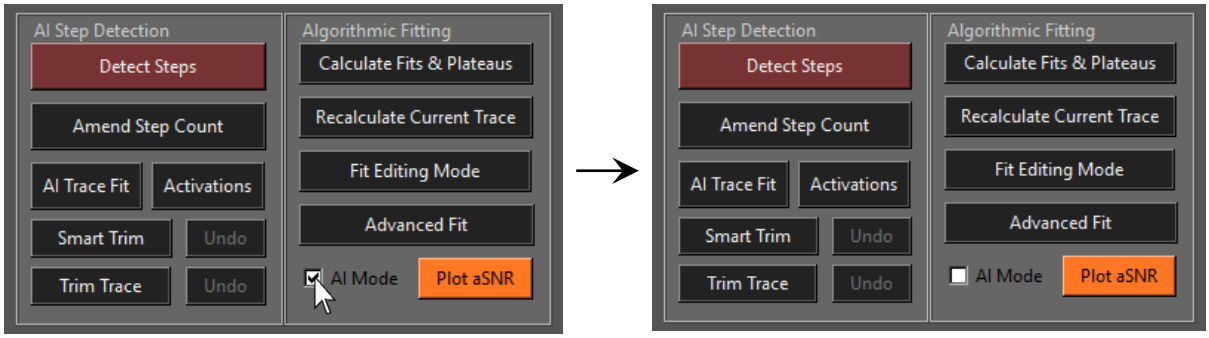
A pop-up window will appear with a graph of the normalized trace in blue and the output activations of the position detection neural network:



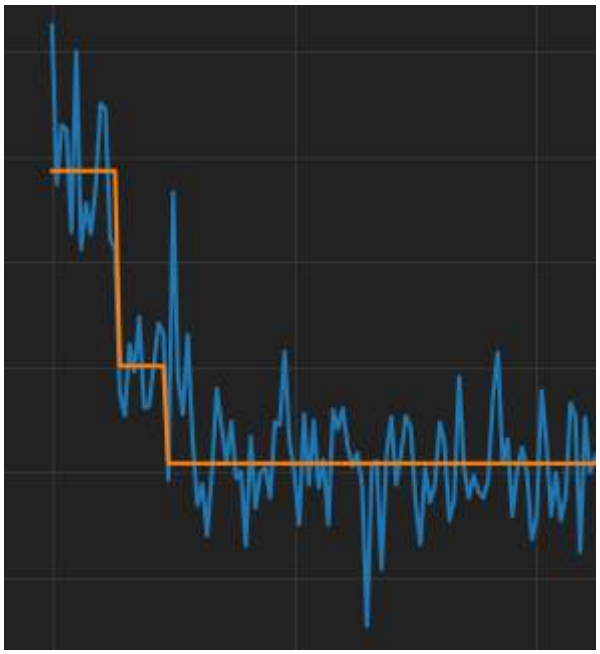
5.7 Switching to Algorithmic Fitting

The AI accelerated position detection system is the default method for fitting traces in this version of the program. Unpublished testing indicates it is more significantly more reliable than the moving-average-based approach to plateau fitting implemented previously.

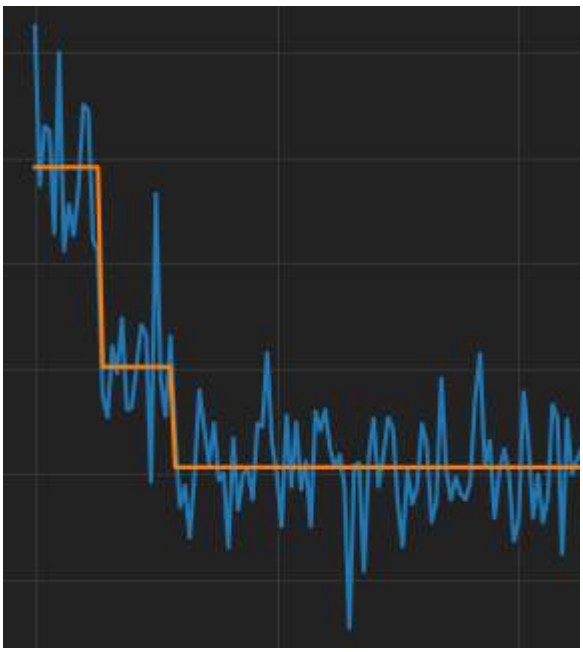
To switch to the older algorithm-based method, untick the 'AI Mode' checkbox in the 'Algorithmic Fitting' section of the user interface:



If any of the plateau fitting tools in the algorithmic fitting section are used, the old method will be used instead of the experimental AI tool. Bear in mind the old tool is generally less reliable at finding the correct fit and less robust for short plateaus:



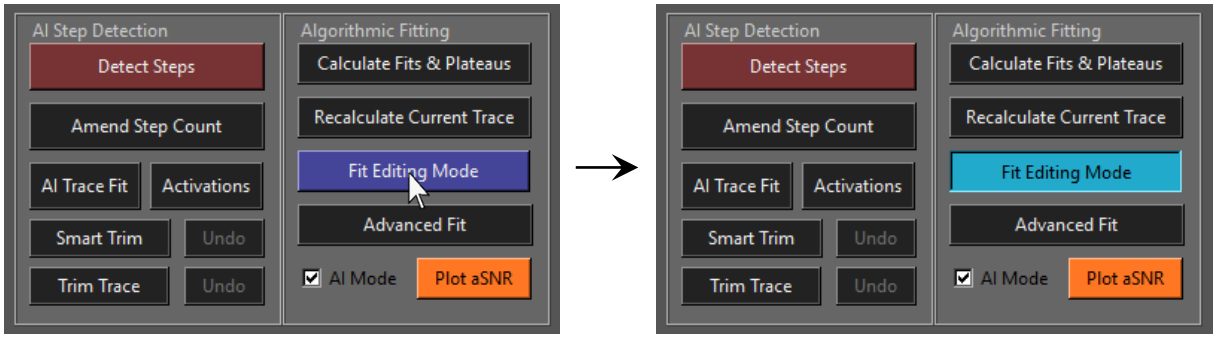
Moving average fitting tool



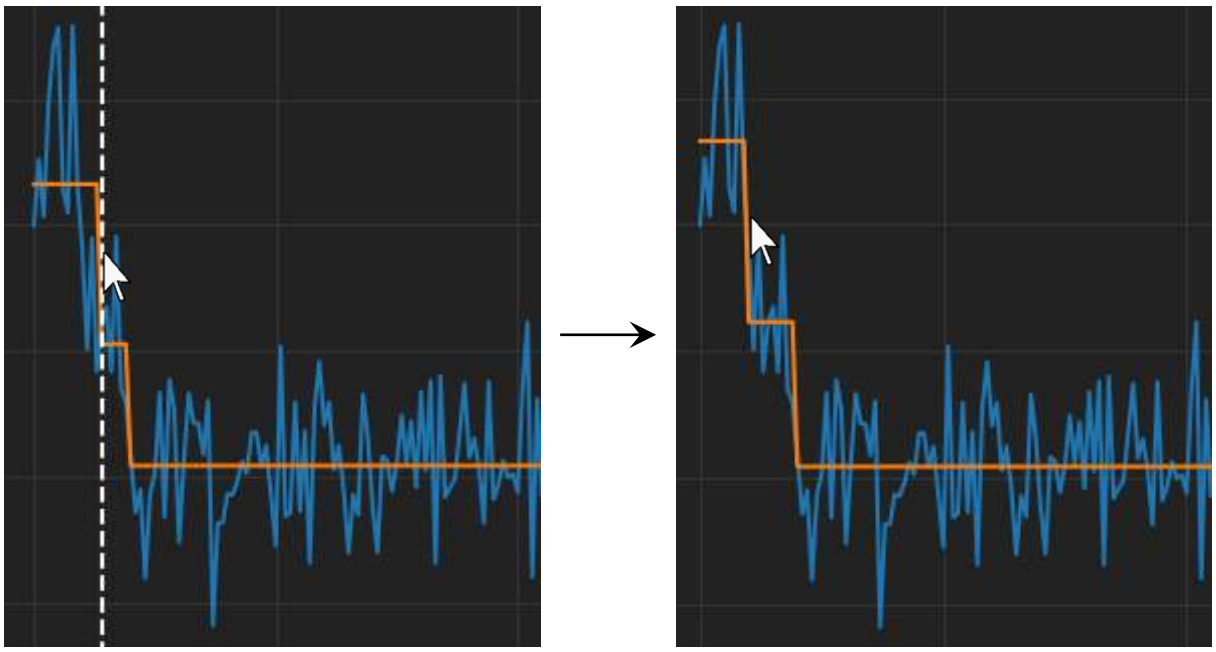
AI fitting tool

5.8 Manual Fit Correction

In the event that either the AI tool or moving average tool fit plateaus incorrectly, i.e., by placing the step in the wrong place, the mistake can be corrected manually. First, enable fit editing mode by clicking 'Fit Editing Mode' in the 'Algorithmic Fitting' section of the control panel. The button will remain light blue to signify the mode is enabled:

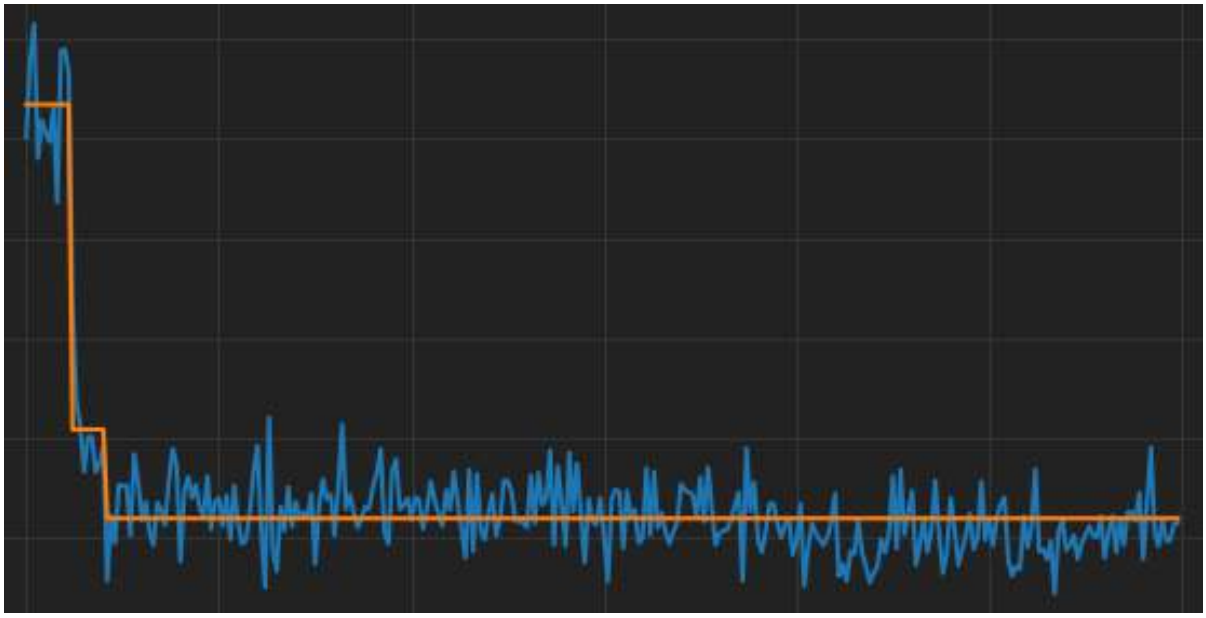


Next, click the step that you want to reposition, a white dotted line will appear and the program will wait for you to click the new position. When the new position is clicked, the dotted line will disappear and the step will be moved to the x – coordinate of where the mouse was clicked. The plateaus will be automatically recalculated based on the mean intensity between the step boundaries:

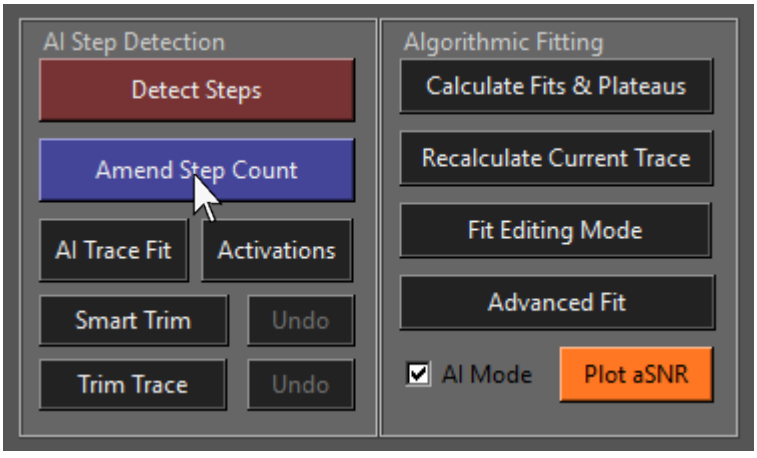


5.9 Manual Step Correction

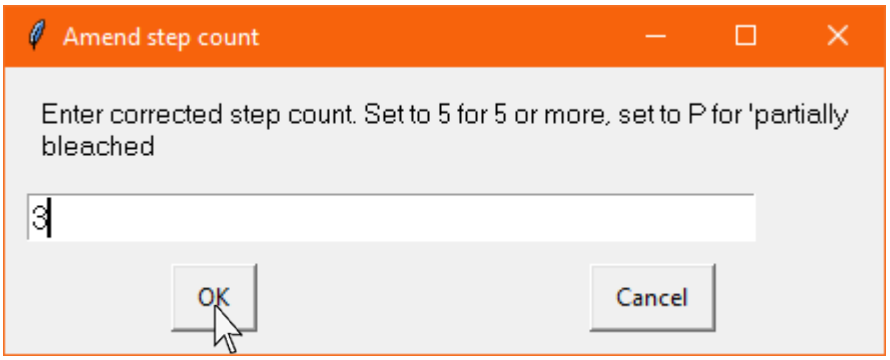
Sometimes, the neural network might incorrectly predict the step-count of a trace:



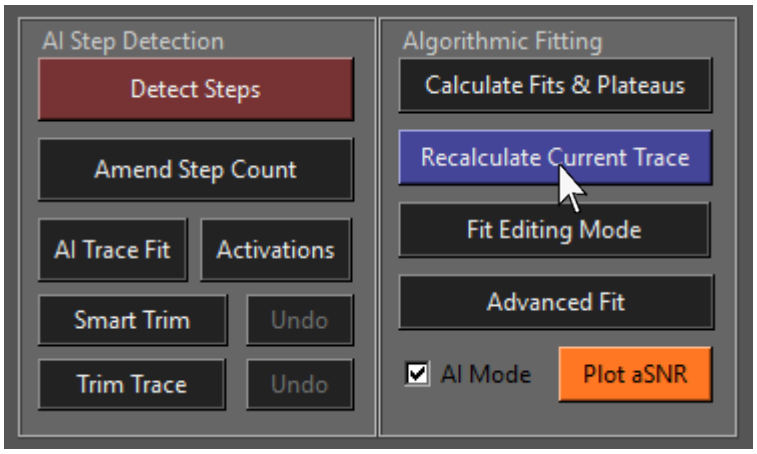
In the case where the neural network incorrectly predicts the step count of a trace or when manual assignment is preferred, click ‘Amend Step Count’ in the ‘AI Step Detection’ section of the control panel:



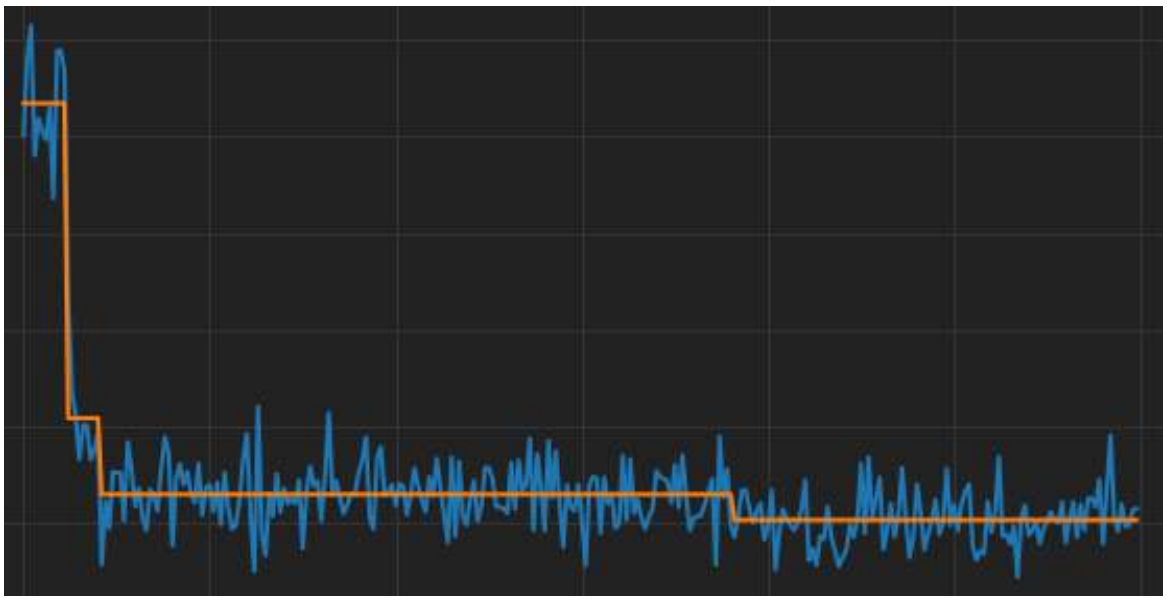
A textbox will appear where you can enter the corrected step count. Type 5 if it has 5 or more steps, or ‘P’ if it is partially bleached. In this case, the most likely correct step count is 3:



The program will then update the step count but the plateau fit will be incorrect. To re-fit the trace with the new step count, click ‘Recalculate Current Trace’ in the ‘Algorithmic Fitting’ section of the control panel:



The plateaus will then be refitted by the selected tool:

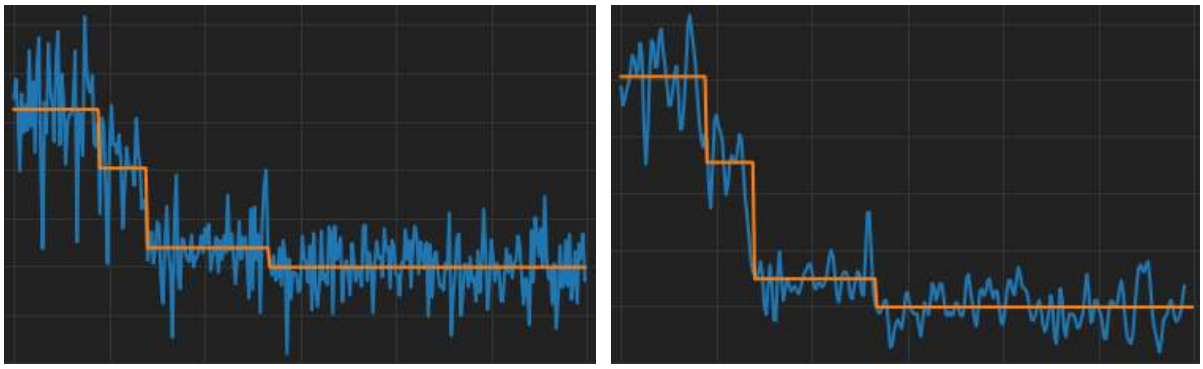


5.10 Convolution and Integration

FluoroTensor has two inbuilt tools to aid manual step assignment and fitting. They are two separate methods that help with the identification of very weak steps that can be easily missed by eye.

Convolution applies a kernel convolution to the trace which has a similar effect to Fourier filtering with a low-pass filter. This effect increases signal to noise ratio at the cost of temporal resolution. To display the convolved trace, click 'Convolution' under 'Trace Processing' in the control panel:

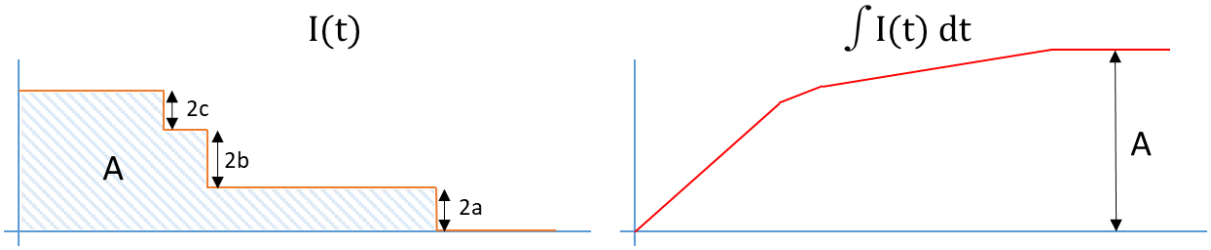
An example of convolution on a trace with very weak steps:



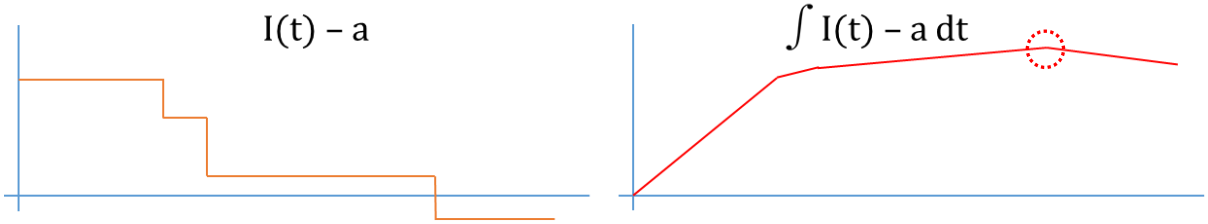
Original **Convolved**

An important thing to note is that the trace has not been altered, a copy of the trace is convolved and displayed but only the original will be analysed and exported. Convolution view can be turned off at any time by clicking 'Convolution' again to toggle it off.

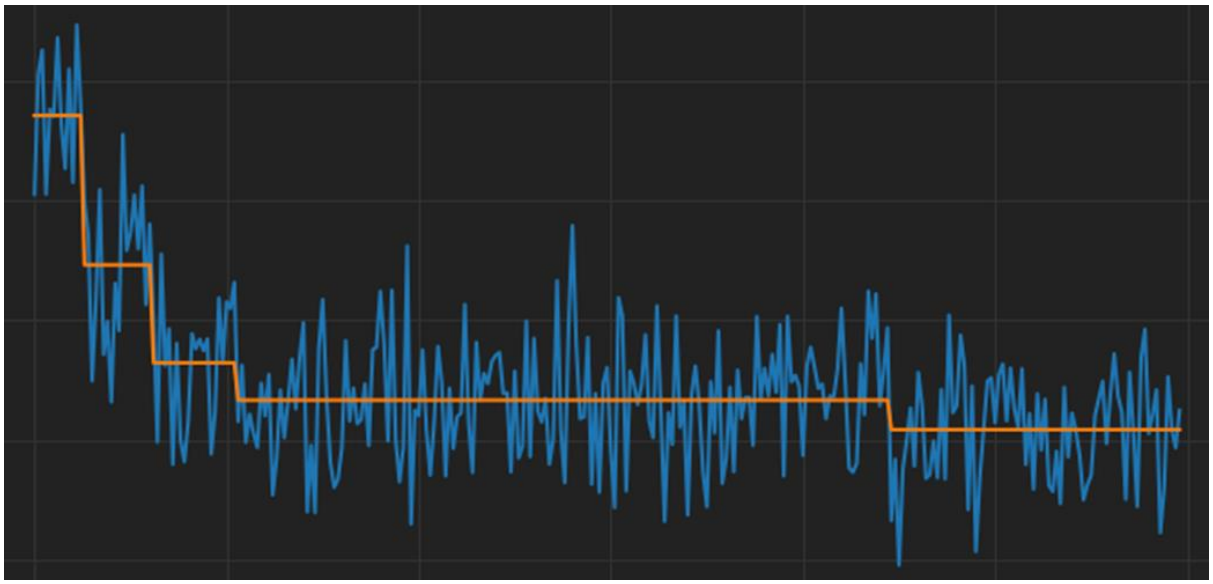
Integration is another method that can assist the user when trying to identify weak steps manually. Steps manifest as changes in gradient of the integration:



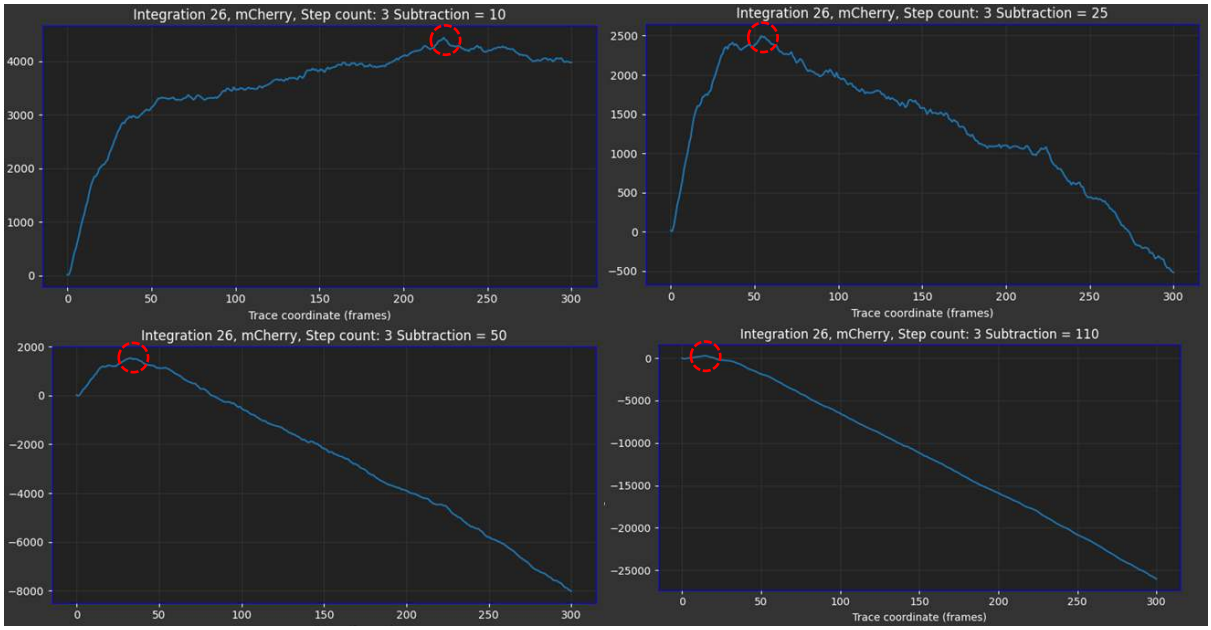
If the trace is subtracted by a constant at each frame, such that one plateau lies above zero and one lies below, the step between them will manifest as a maximum point:



The following are examples of the integration of a real trace and all its points transformed into maxima by subtracting each plateau below zero in turn:



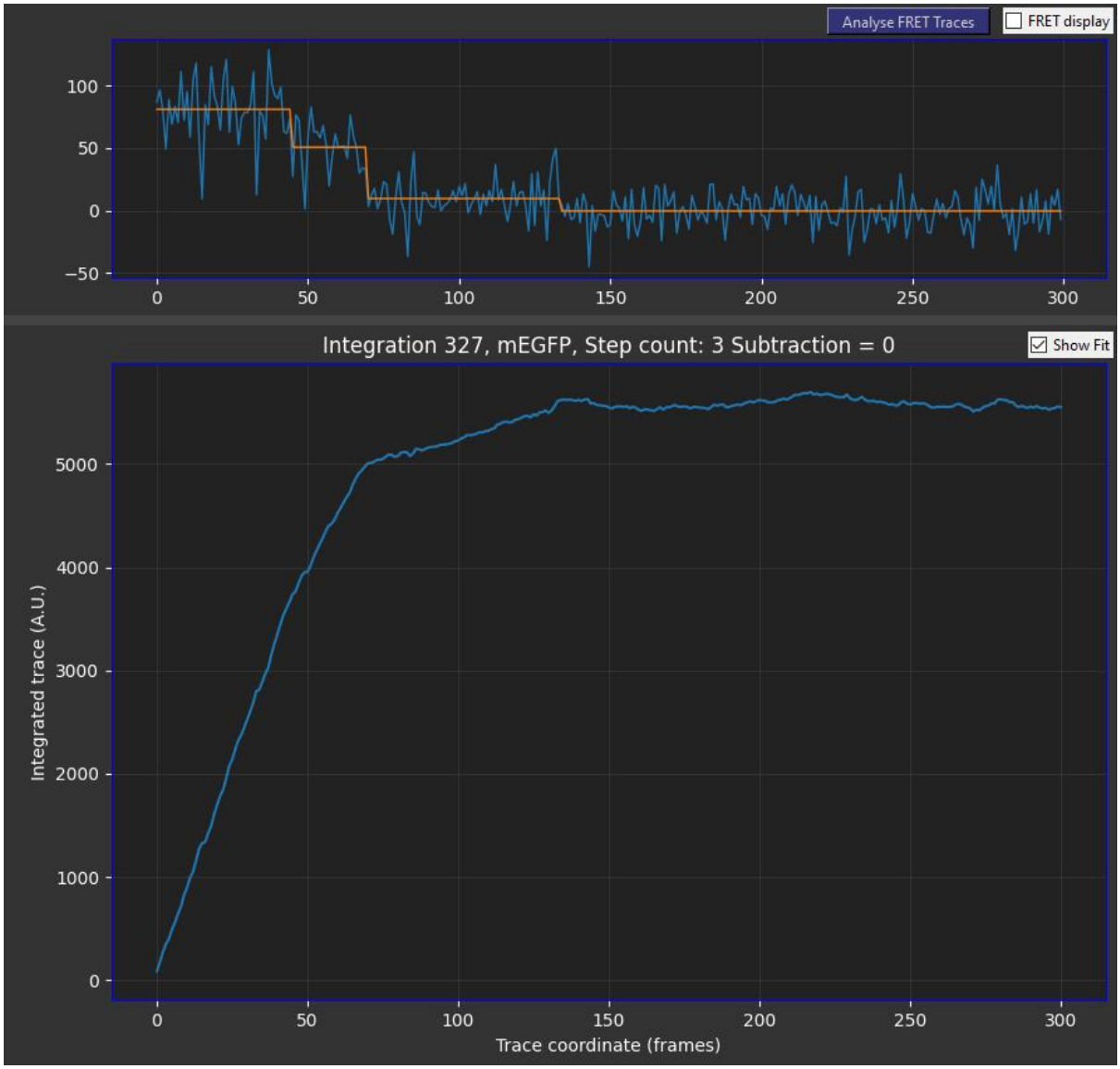
Original Trace



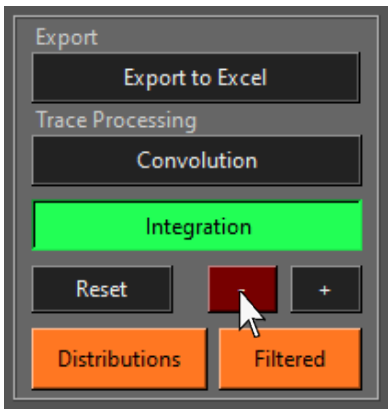
Integrated Representations

To turn on integrated view, click ‘Integration’ under ‘Trace Processing’:

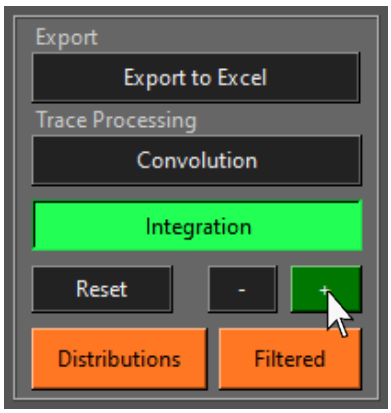
The integration will then be displayed on the main canvas while the original trace is displayed above it. It is then possible to compare the points in the integration where the gradient changes to the original trace.



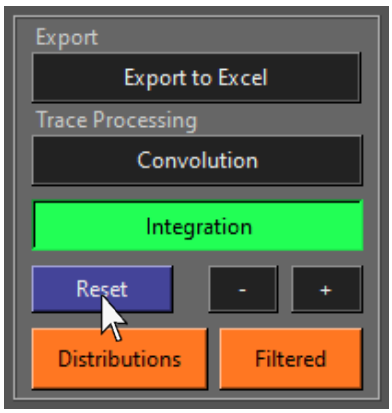
To increase or decrease the subtraction amount, click the + or – buttons under 'Trace Processing' in the control panel or press 'Reset' to reset the subtraction value to zero:



Reduce Subtraction

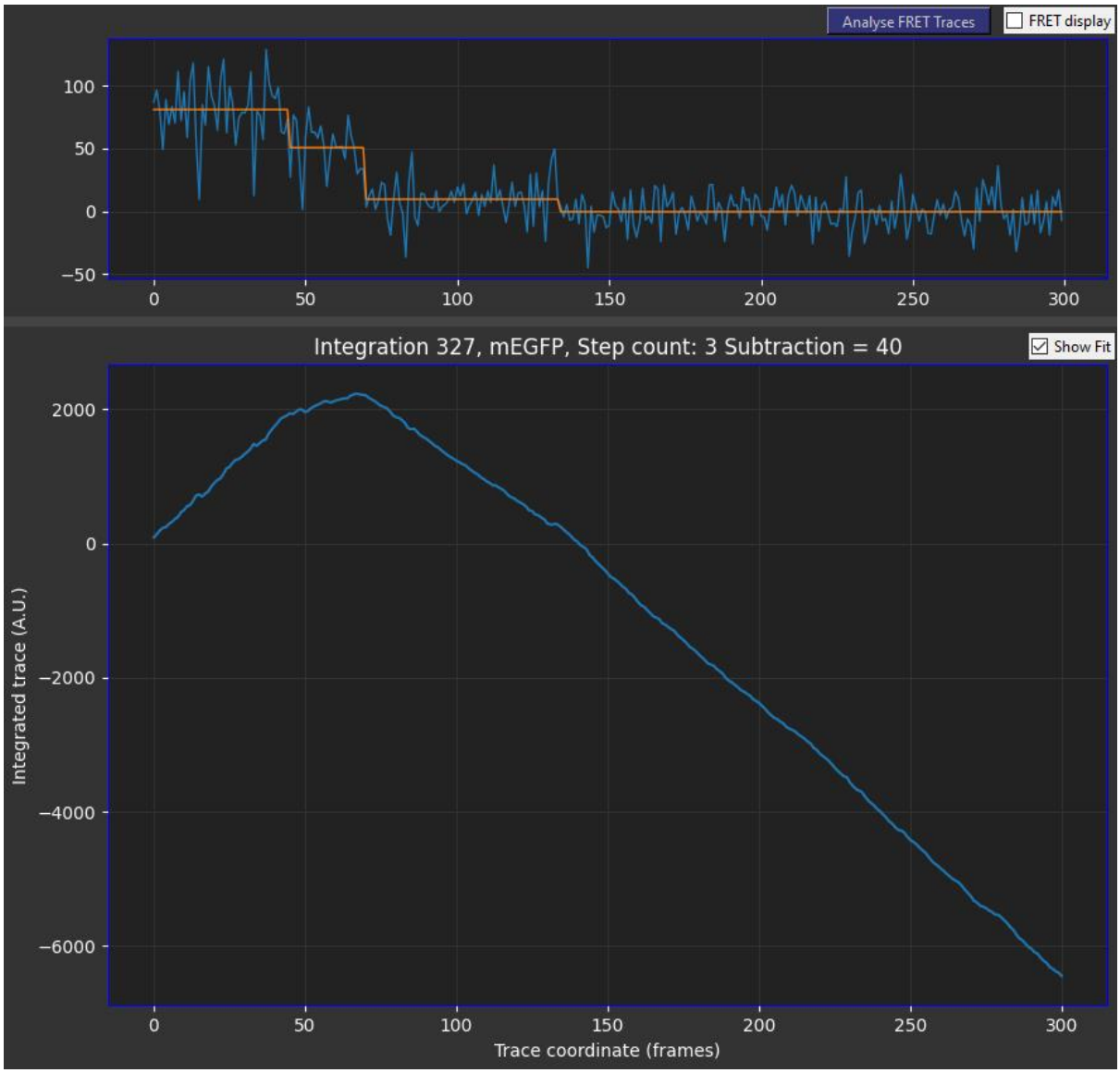


Increase Subtraction



Reset Subtraction

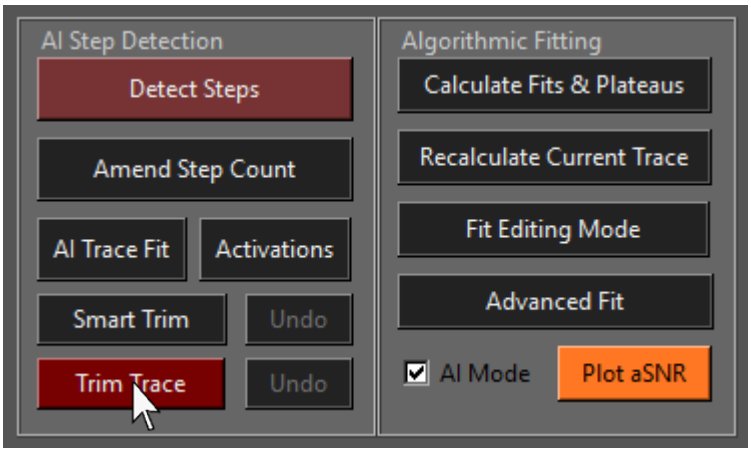
When the subtraction is increased, it is easier to see the changes in gradient of the integration for steps earlier in the trace:



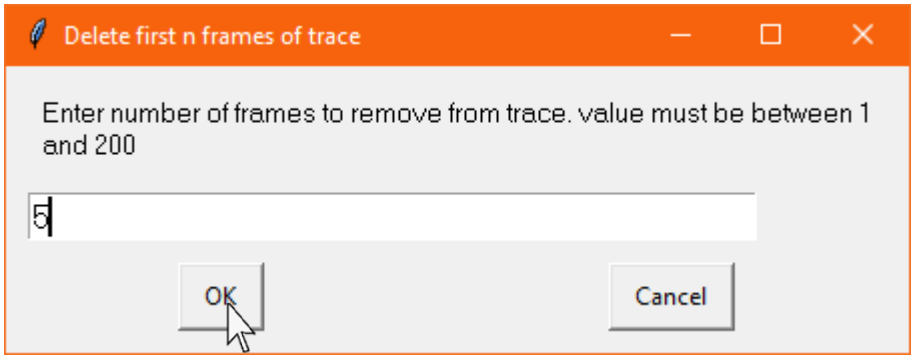
5.11 Trimming Traces

Sometimes if the autofocus takes a few frames to focus on the sample during data acquisition, the initial intensity of a trace may be lower for a few frames while the spot is out of focus. This shouldn't affect step detection but may affect plateau fitting. This can be done manually or automatically.

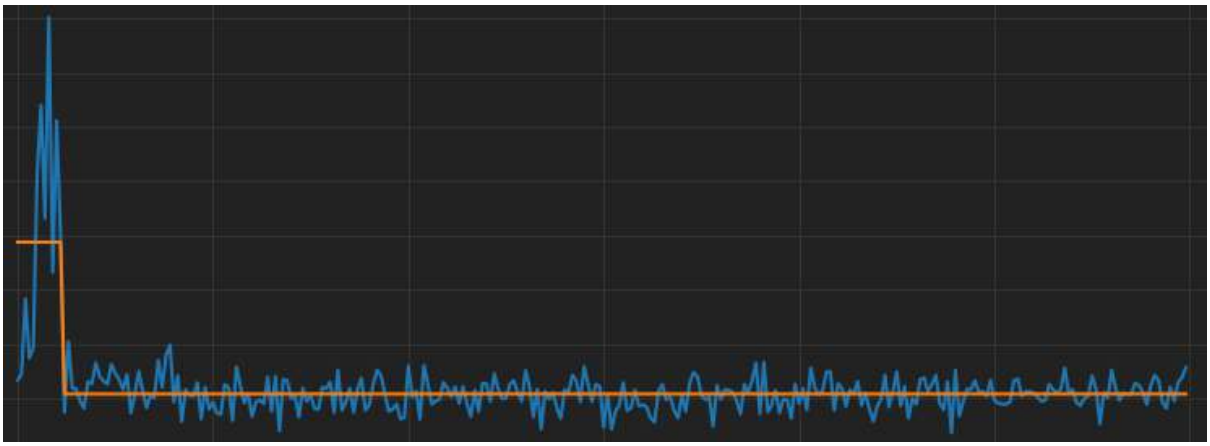
To manually trim frames from the beginning of a trace, click 'Trim Trace' in the 'AI Step Detection' section of the control panel:

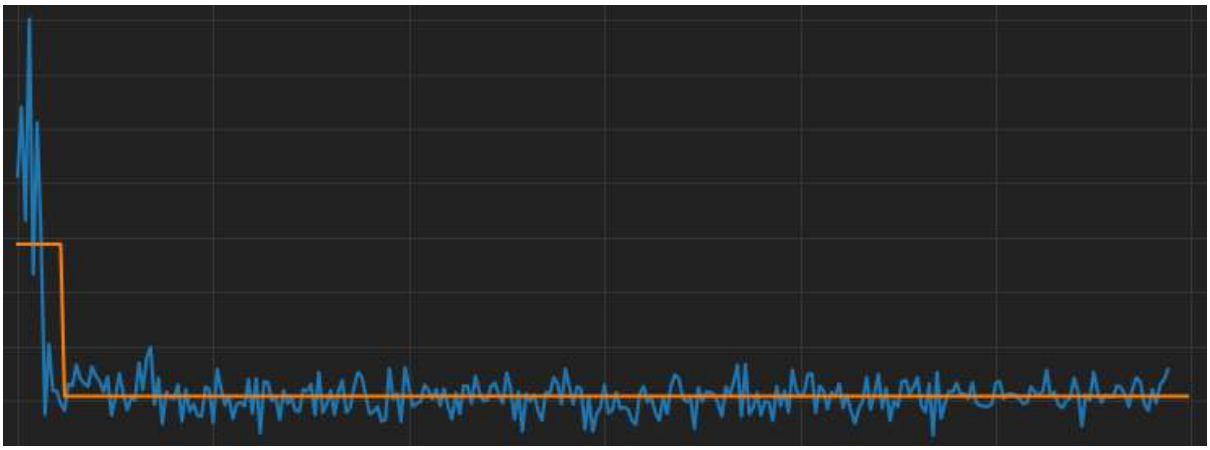


Then enter the number of frames to trim into the text-box that appears:

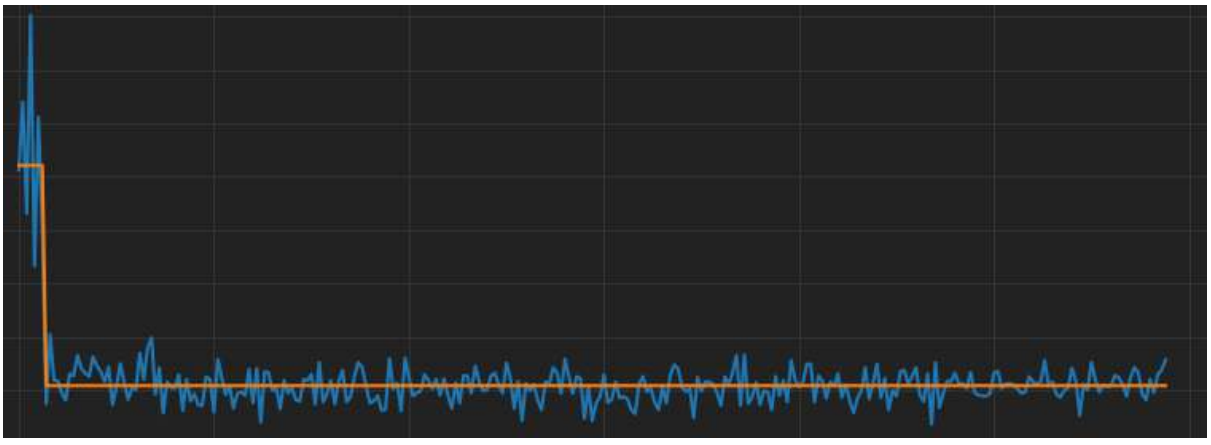


The trace will then be trimmed by deleting those frames from the start of the trace:

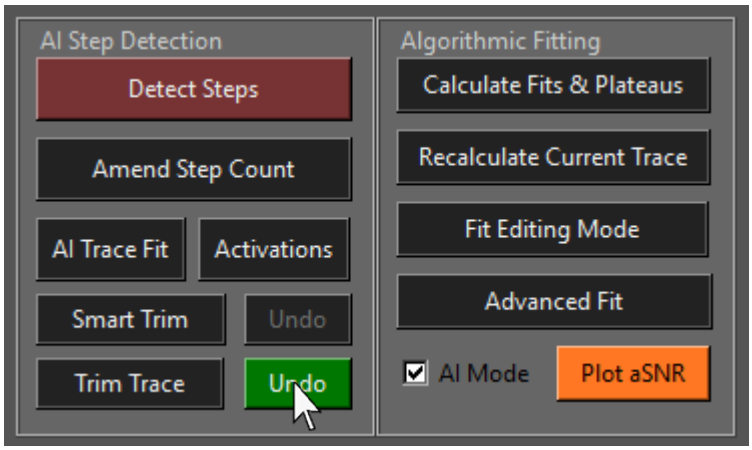




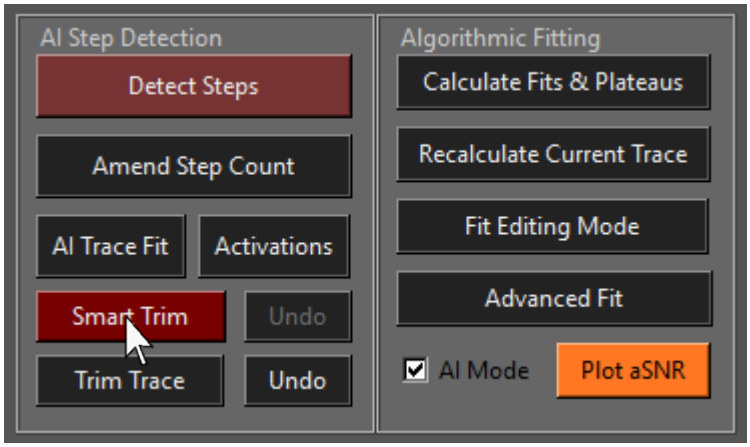
The trace will be trimmed but the fit is left alone and will now be offset from the data. To correct this, re-fit the trace by clicking ‘Recalculate Current Trace’ as described earlier.



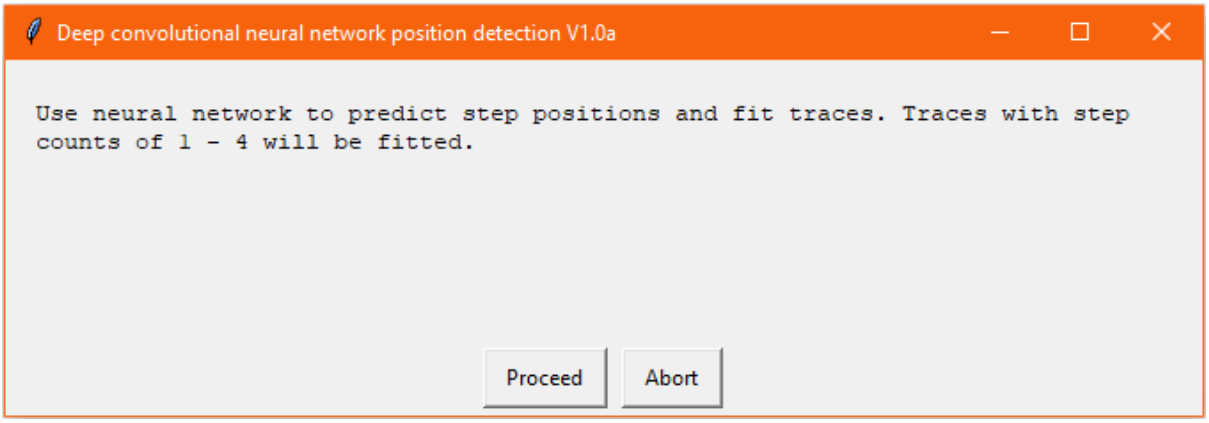
If the wrong number of frames were removed, the trim action can be undone by pressing undo:



Alternatively, the auto-trimming function will automatically trim low intensity frames from the beginning of all traces. This function can be unreliable in very noisy conditions if the signal is weak and fail to trim traces at all. Click 'Smart Trim' to auto-trim all traces:

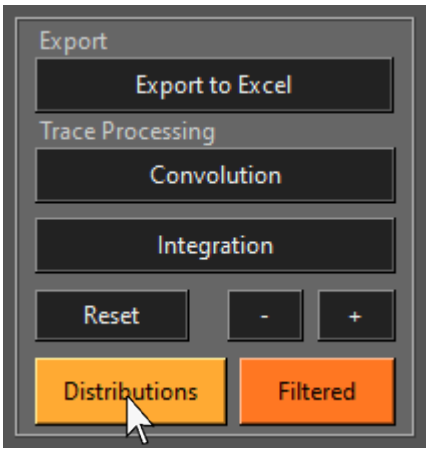


A dialogue box will appear after the smart trim function has finished asking if traces should be refitted automatically:

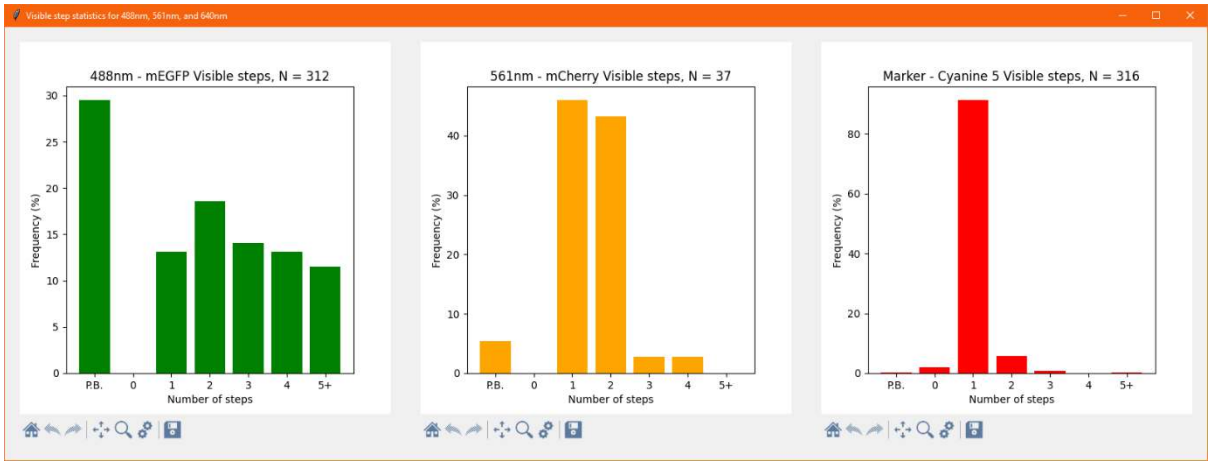


5.12 Plotting Step Distributions

To plot the step distributions of each channel for any preliminary observations before exporting, Click ‘Distributions’ under ‘Trace Processing’ section of the control panel:

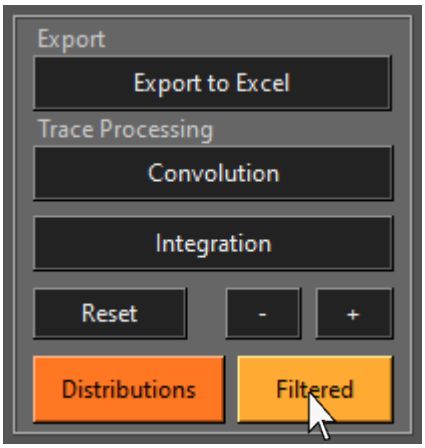


A window will then appear with step distribution histograms for each channel:

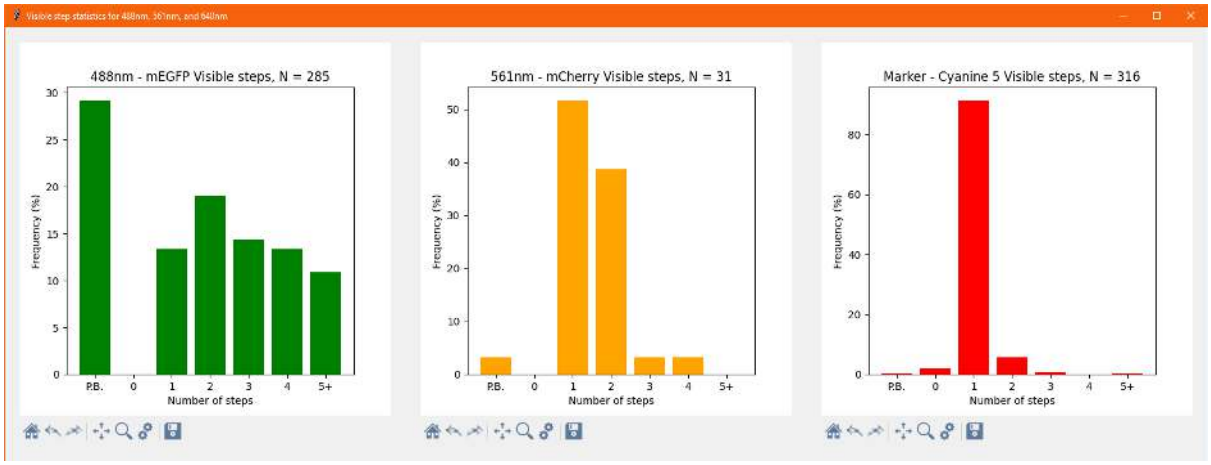


In some experiments, for example when looking at splicing factors assembling on pre-mRNA, we wish to observe the distribution of steps of labelled proteins that colocalize with only a single RNA molecule. In cases such as this, the non-marker channels should exclude steps from traces in the distribution that colocalize with a marker with more than one bleaching step.

To display the distributions of secondary channel steps filtered by colocalization to a marker trace with a single bleaching step, click ‘Filtered’:



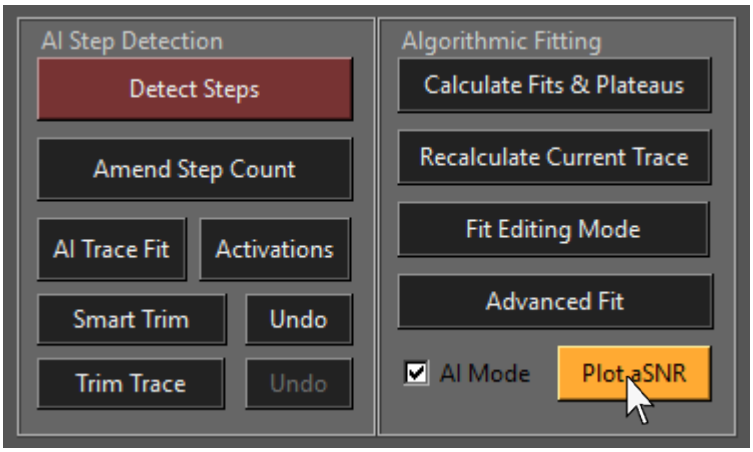
The same window will then appear but the distributions in the 488nm channel and 561nm channel are filtered as described:



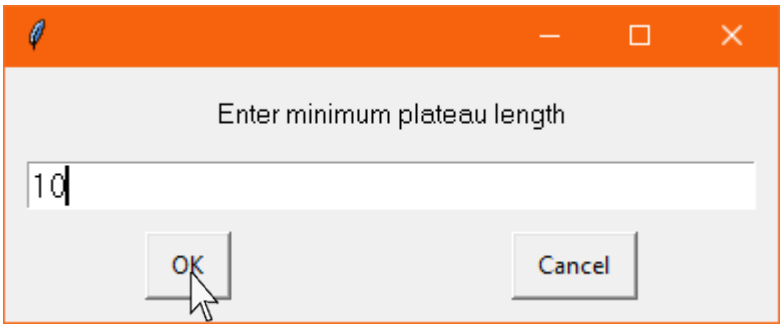
If the marker is supposed to be a multimer, the program is unable to show preliminary filtered distributions of secondary channel steps colocalized with multiple markers at this time, however this kind of filtering can be applied when the data is exported which will be described in the next chapter.

5.13 Plotting the Signal-to-Noise Ratio of the Dataset

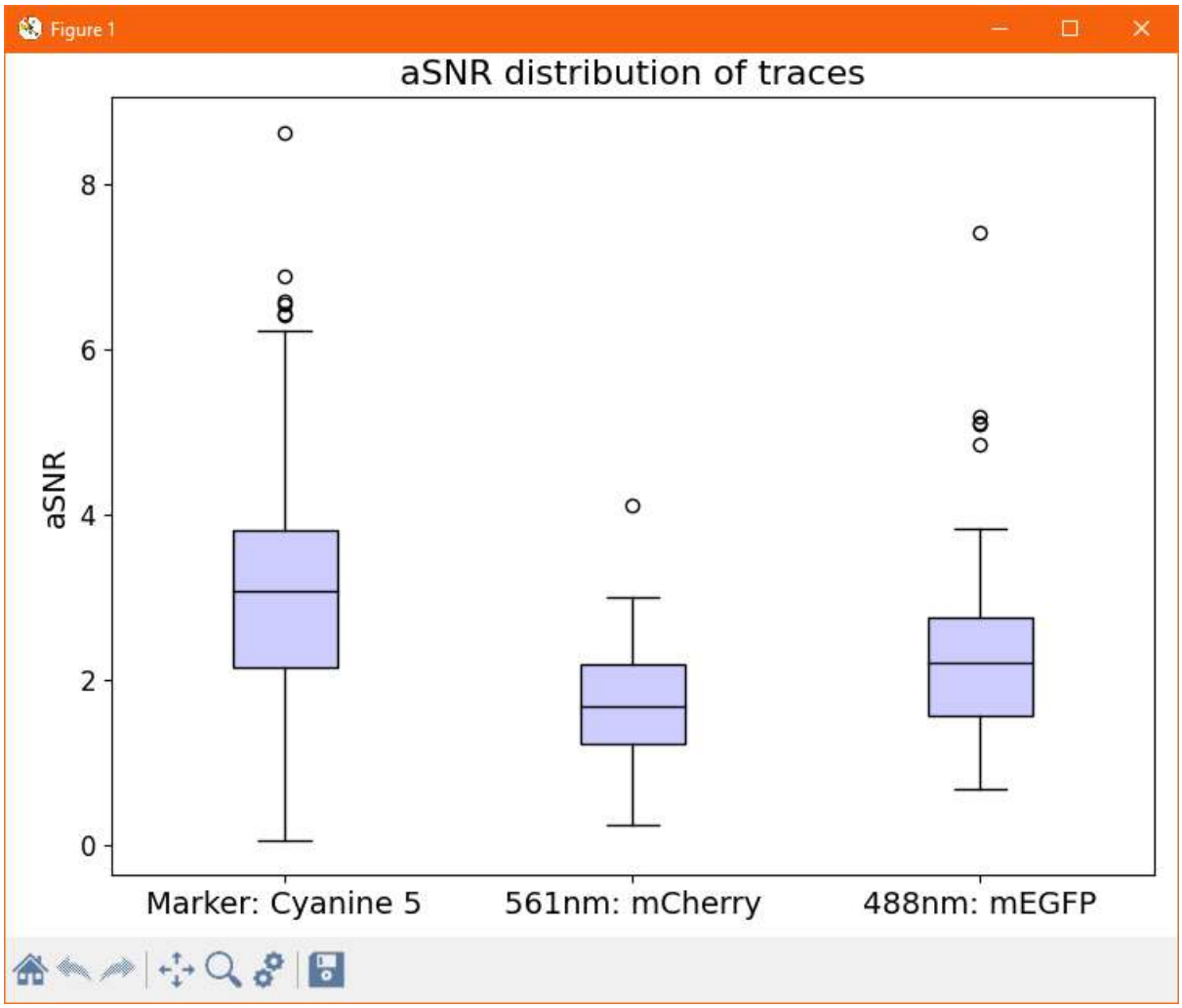
After step counts and plateau fits have been calculated, the distribution of average signal-to-noise ratios of traces can be plotted for each channel. To do this, click 'Plot aSNR' in the 'Algorithmic Fitting' section of the control panel:



A text-box will then appear prompting the user to enter the minimum plateau length to be used to calculate signal-to-noise ratios of traces. The lower this number, the more data can be used to calculate the distribution, but the less precise the calculated standard deviation of intensities of the plateaus will be. Typically, 10 frames will give a good estimate. We don't recommend using less than 5.



A window will then appear, displaying the signal-to-noise ratio distributions for each channel in the form of box-and-whisker plots:

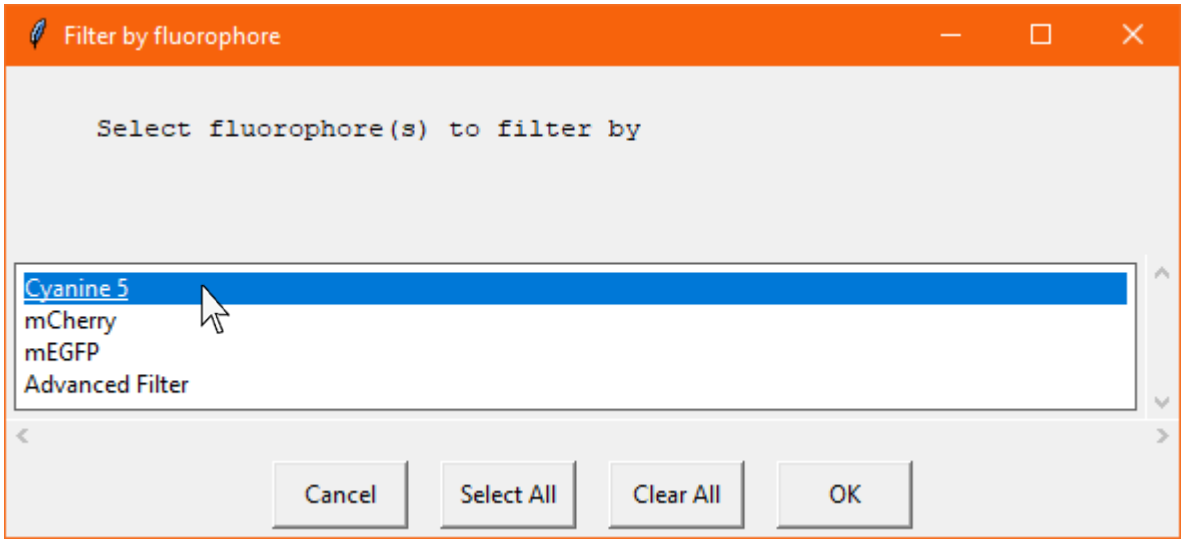


5.14 Filtering Displayed Traces

The user may wish to only look through traces that have a particular property. For example, filtering by fluorophore type to only look at Cy5 traces with 1 bleaching step. Currently, the program supports filtering by fluorophore type and / or the number of steps.

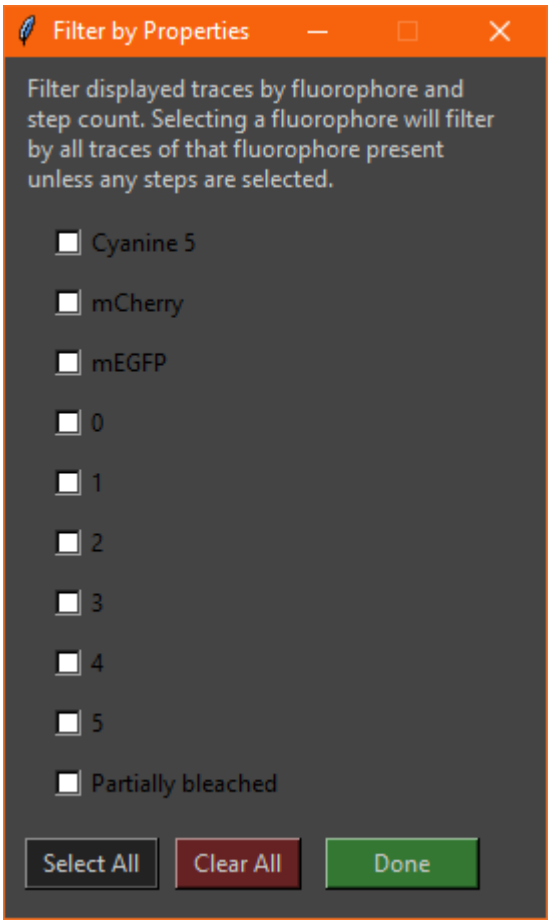
To filter the displayed traces, click on 'Filter Traces' in the miscellaneous section of the control panel:

If filtering only by fluorophore type, choose a fluorophore in the selection box and press OK:

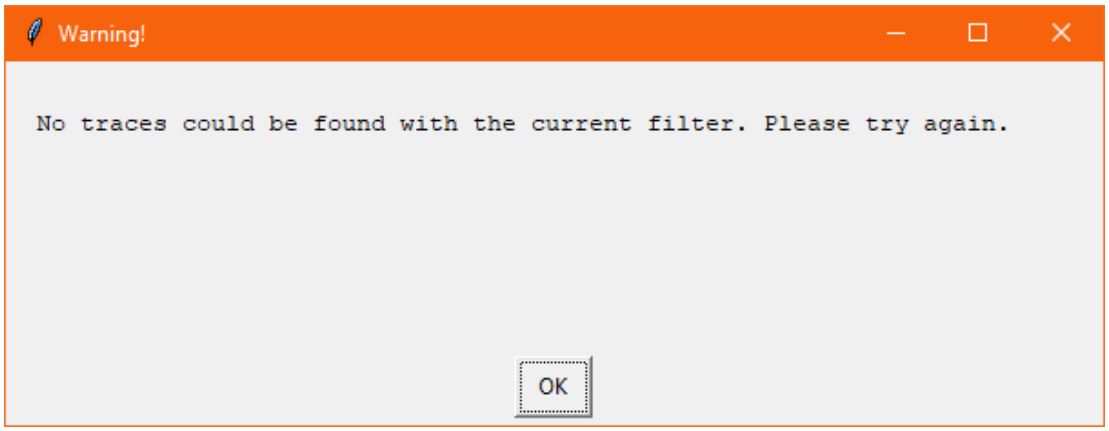


For example, in this case, only Cy5 traces will be shown to the user as they click through. To filter by step count as well, select 'Advanced Filter' from the list and click OK.

The advanced filter window will then appear:



Tick the checkboxes for options you wish to include in the filter. For example, picking mCherry, 2, and 3, will only show the user mCherry traces with step counts of 2 and 3. Multiple options can be selected in each case. If no traces with these properties exist in the dataset, the relevant checkboxes will be greyed out. This isn't foolproof since the step count 3 checkbox cannot be disabled if mCherry has no traces with step count 4 but mEGFP does. If no traces can be found the following warning will be displayed:

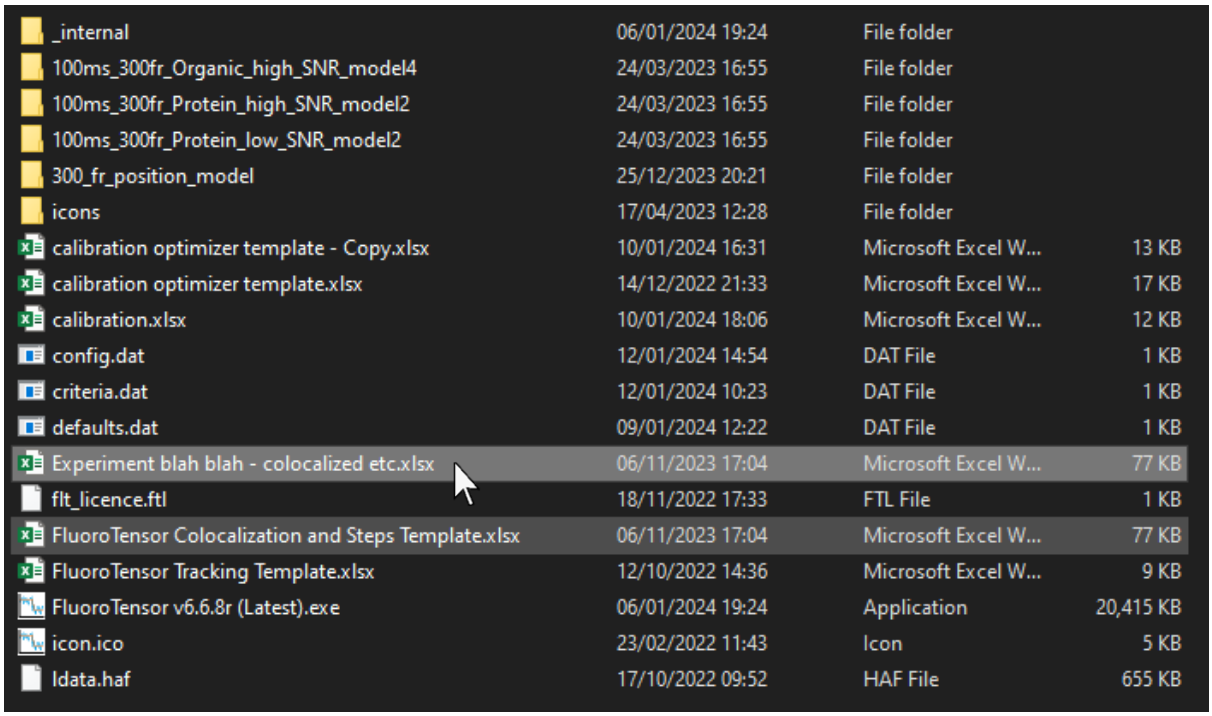


Chapter 6 – Exporting Results

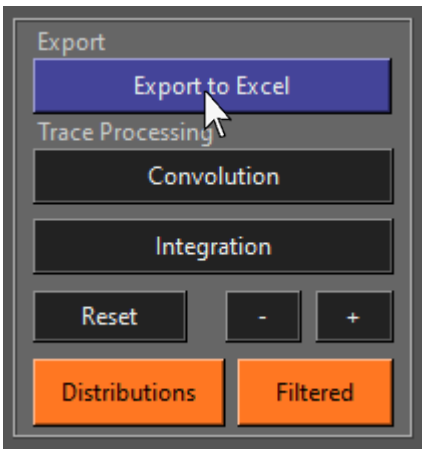
6.1 Exporting Data to Excel

Once traces have been analysed, steps detected and plateaus fitted, the data can be exported to a preformatted Excel spreadsheet.

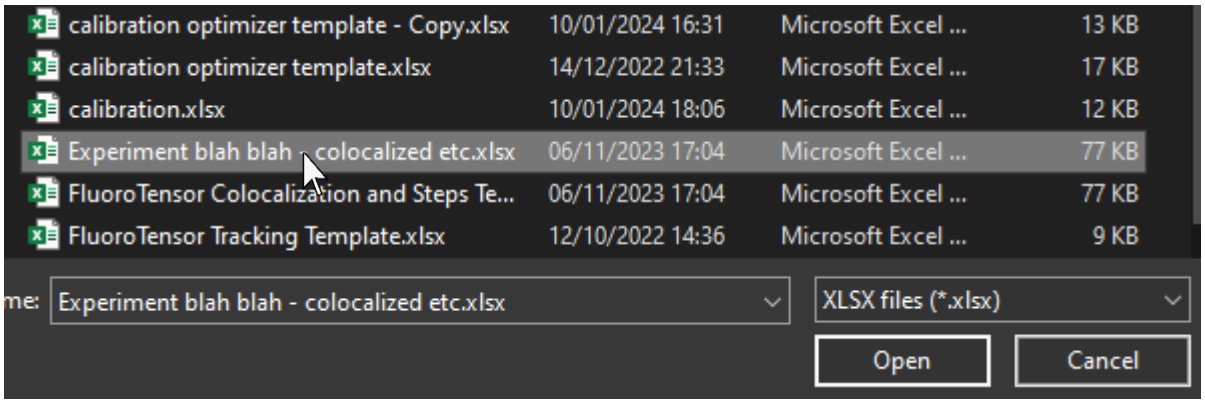
In the FluoroTensor folder, find the file named 'FluoroTensor Colocalization and Steps Template.xlsx' and make a copy of it. Rename the copy appropriately based on the experiment and analysis mode:



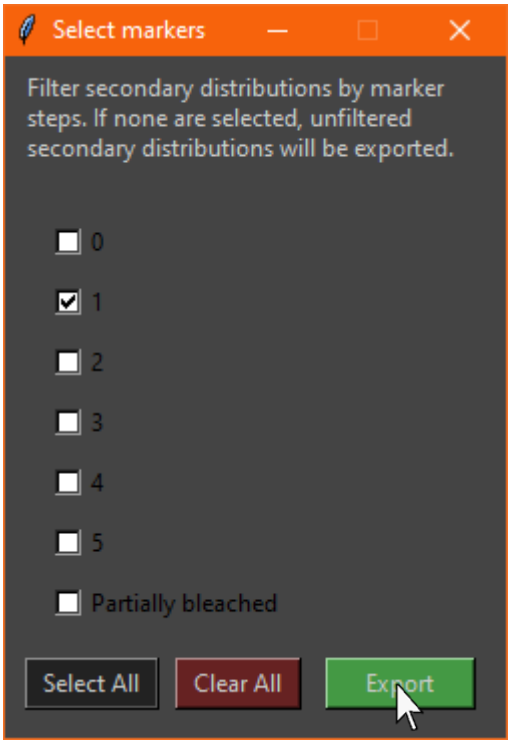
In the program, click 'Export to Excel' under 'Export' in the control panel:



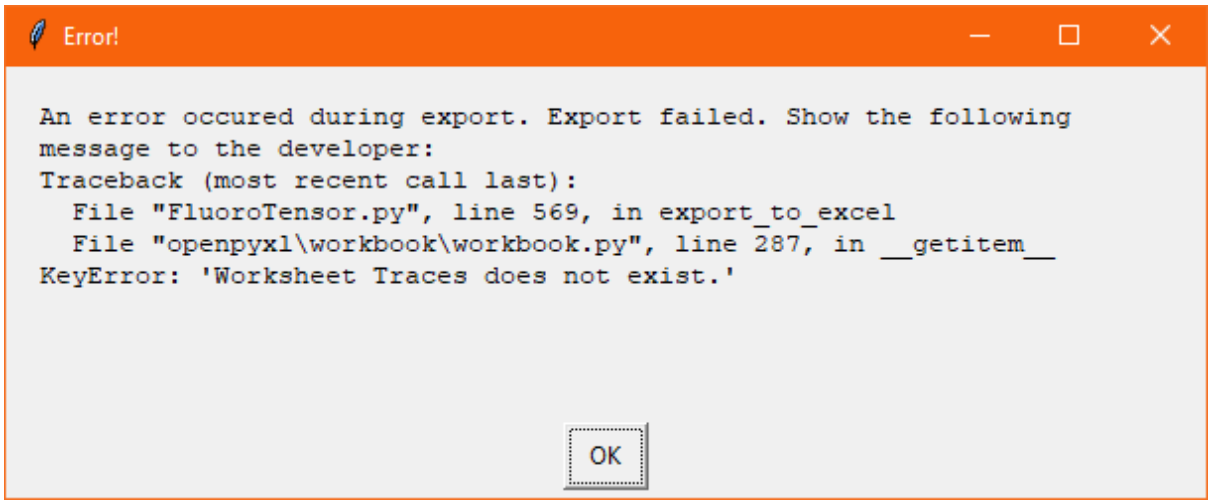
A file dialogue will then be displayed, prompting the user to select a valid file for the data to be exported to:



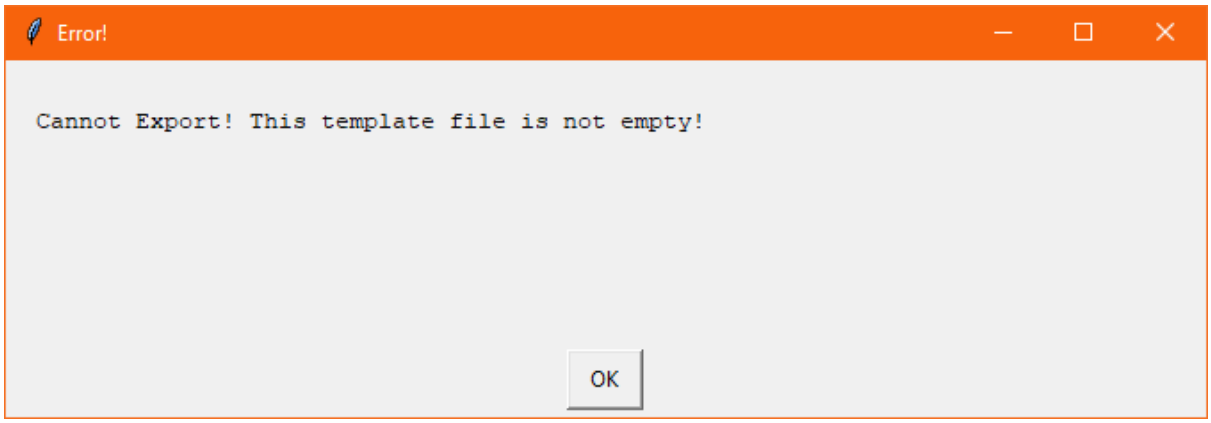
Next, the user will be prompted to select the export filter. This determines which steps will be exported to the secondary step distributions based on the number of steps in the colocalized marker. This will be explained in more detail in the next chapter with examples. In short, selecting all will export raw colocalized distributions with no filtering. Selecting specific steps will generate distributions based off colocalized markers with that step count and selecting none will export pure step distributions of all traces disregarding colocalization. This is necessary when analysing non-colocalized molecules. Once the desired filter is chosen, click 'Export':



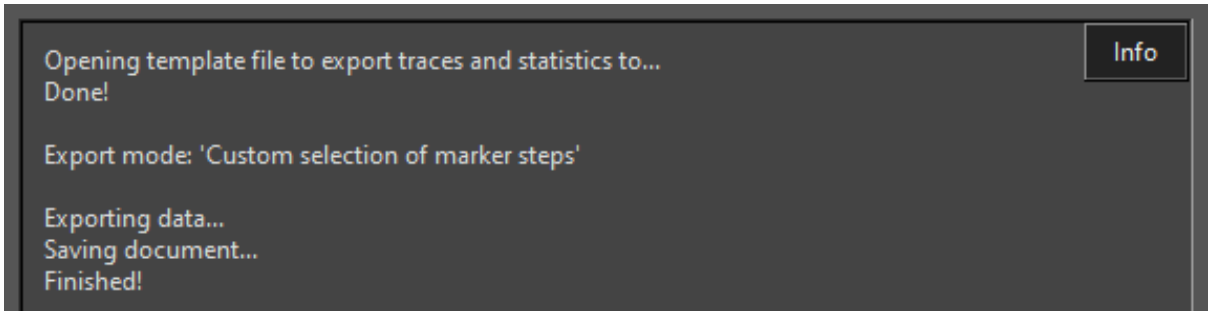
If an invalid file was selected for export, an error message will appear:



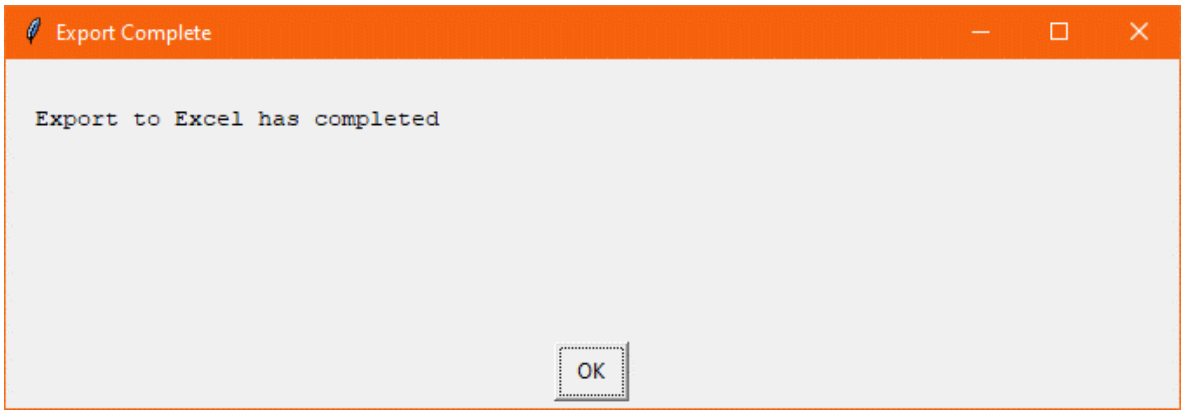
Likewise, if a non-blank template with data already exported to it is selected, an error message will appear at this stage:



Once the export process begins, progress will be reported in the status box:

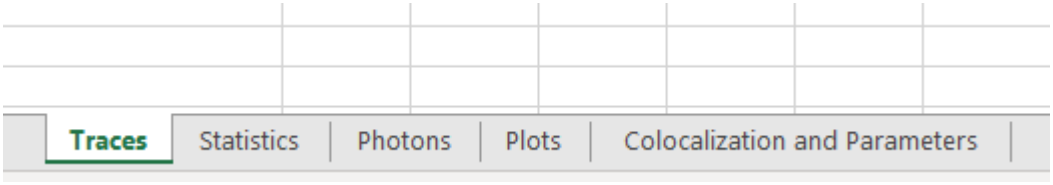


When the export has successfully finished, a dialogue box will open to notify the user:

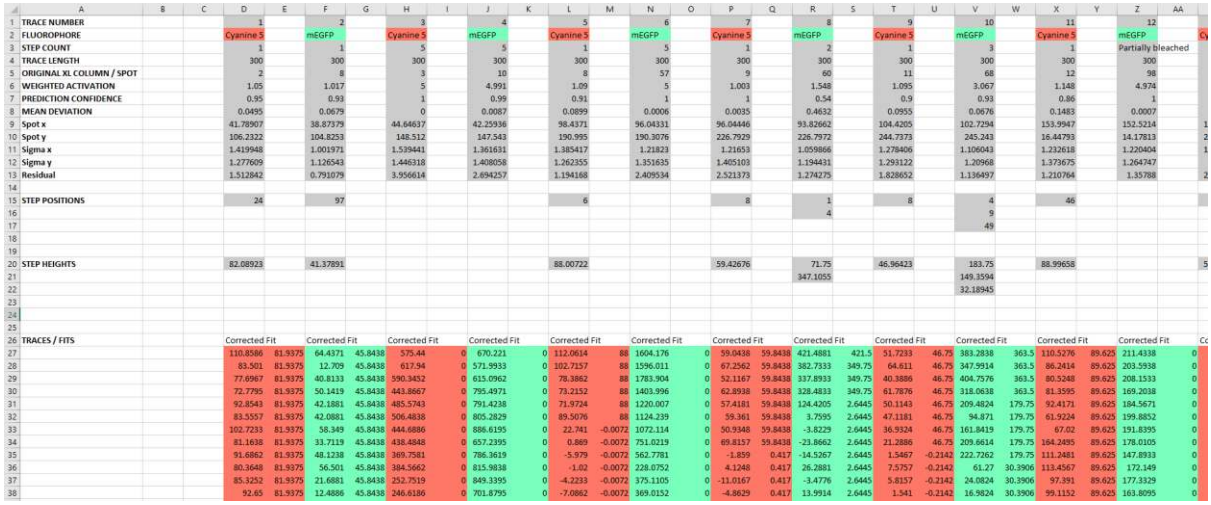


6.2 The Colocalization Template – Traces

The colocalization template is a Microsoft Excel Workbook with several sheets. Different information can be found among them:



The first sheet is called 'Traces' and keeps a record of the traces and spot fitting parameters:



	A	D	E	F	G	H	J	L	N	P	R	T	V	X	Z
1	TRACE NUMBER	1		2		3	4	5	6	7	8	9	10	11	12
2	FLUOROPHORE	Cyanine 5		mEGFP		Cyanine 5	mEGFP	Cyanine 5	mEGFP	Cyanine 5	mEGFP	Cyanine 5	mEGFP	Cyanine 5	mEGFP
3	STEP COUNT	1		1		5	5	1	5	1	2	1	3	1	Partially bleached
4	TRACE LENGTH	300		300		300	300	300	300	300	300	300	300	300	300
5	ORIGINAL XL COLUMN / SPOT	2		8		3	10	8	57	9	60	11	68	12	98
6	WEIGHTED ACTIVATION	1.05		1.017		5	4.991	1.09	5	1.003	1.548	1.095	3.067	1.148	4.974
7	PREDICTION CONFIDENCE	0.95		0.93		1	0.99	0.91	1	1	0.54	0.9	0.93	0.86	1
8	MEAN DEVIATION	0.0495		0.0679		0	0.0087	0.0899	0.0006	0.0035	0.4632	0.0955	0.0676	0.1483	0.0007
9	Spot x	41.78907		38.87379		44.64637	42.25936	98.4371	96.04331	96.04446	93.82662	104.4205	102.7294	153.9947	152.5214
10	Spot y	106.2322		104.8253		148.512	147.543	190.995	190.3076	226.7929	226.7972	244.7373	245.243	16.44793	14.17813
11	Sigma x	1.419948		1.001971		1.539441	1.361631	1.385417	1.21823	1.21653	1.059866	1.278406	1.106043	1.232618	1.220404
12	Sigma y	1.277609		1.126543		1.446318	1.408058	1.262355	1.351635	1.405103	1.194431	1.293122	1.20968	1.373675	1.264747
13	Residual	1.512842		0.791079		3.956614	2.694257	1.194168	2.409534	2.521373	1.274275	1.828652	1.136497	1.210764	1.35788
14															
15	STEP POSITIONS	24		97				6		8	1	8	4	46	
16											4		9		
17													49		
18															
19															
20	STEP HEIGHTS	82.08923		41.37891				88.00722		59.42676	71.75	46.96423	183.75	88.99658	
21											347.1055		149.3594		
22													32.18945		
23															
24															
25															
26	TRACES / FITS	Corrected Fit		Corrected Fit		Corrected Fit	Corrected Fit	Corrected Fit	Corrected Fit	Corrected Fit	Corrected Fit	Corrected Fit	Corrected Fit	Corrected Fit	Corrected Fit
27		110.8586	81.9375	64.4371	45.8438	575.44	0 670.221	0 112.0614	88 1604.176	0 59.0438	59.8438	421.4881	421.5	51.7233	46.75 383.2838 363.5 110.5276 89.625 211.4338 0
28		83.501	81.9375	12.709	45.8438	617.94	0 571.9933	0 102.7157	88 1596.011	0 67.2562	59.8438	382.7333	349.75	64.611	46.75 347.9914 363.5 86.2414 89.625 203.5938 0
29		77.6967	81.9375	40.8133	45.8438	590.3452	0 615.0962	0 78.3862	88 1783.904	0 52.1167	59.8438	337.8933	349.75	40.3886	46.75 404.7576 363.5 80.5248 89.625 208.1533 0
30		72.7795	81.9375	50.1419	45.8438	443.8667	0 795.4971	0 73.2152	88 1403.996	0 62.8938	59.8438	328.4833	349.75	61.7876	46.75 318.0638 363.5 81.3595 89.625 169.2038 0
31		92.8543	81.9375	42.1881	45.8438	485.5743	0 791.4238	0 71.9724	88 1220.007	0 57.4181	59.8438	124.4205	2.6445	50.1143	46.75 209.4824 179.75 92.4171 89.625 184.5671 0
32		83.5557	81.9375	42.0881	45.8438	506.4838	0 805.2829	0 89.5076	88 1124.239	0 59.361	59.8438	3.7595	2.6445	47.1181	46.75 94.871 179.75 61.9224 89.625 199.8852 0
33		102.7233	81.9375	58.349	45.8438	444.6886	0 886.6195	0 22.741	-0.0072 1072.114	0 50.9348	59.8438	-3.8229	2.6445	36.9324	46.75 161.8419 179.75 67.02 89.625 191.8395 0
34		81.1638	81.9375	33.7119	45.8438	438.4848	0 657.2395	0 0.869	-0.0072 751.0219	0 69.8157	59.8438	-23.8662	2.6445	21.2886	46.75 209.6614 179.75 164.2495 89.625 178.0105 0
35		91.6862	81.9375	48.1238	45.8438	369.7581	0 786.3619	0 -5.979	-0.0072 562.7781	0 -1.859	0.417	-14.5267	2.6445	1.5467	-0.2142 222.7262 179.75 111.2481 89.625 147.8933 0
36		80.3648	81.9375	56.501	45.8438	384.5662	0 815.9838	0 -1.02	-0.0072 228.0752	0 4.1248	0.417	26.2881	2.6445	7.5757	-0.2142 61.27 30.3906 113.4567 89.625 172.149 0
37		85.3252	81.9375	21.6881	45.8438	252.7519	0 849.3395	0 -4.2233	-0.0072 375.1105	0 -11.0167	0.417	-3.4776	2.6445	5.8157	-0.2142 24.0824 30.3906 97.391 89.625 177.3329 0
38		92.65	81.9375	12.4886	45.8438	246.6186	0 701.8795	0 -7.0862	-0.0072 369.0152	0 -4.8629	0.417	13.9914	2.6445	1.541	-0.2142 16.9824 30.3906 99.1152 89.625 163.8095 0

At the top of each trace is information regarding spot fitting, steps, neural network info and the step positions and heights:

TRACE NUMBER	7	8	9	10
FLUOROPHORE	Cyanine 5	mEGFP	Cyanine 5	mEGFP
STEP COUNT	1	2	1	3
TRACE LENGTH	300	300	300	300
ORIGINAL XL COLUMN / SPOT	9	60	11	68
WEIGHTED ACTIVATION	1.003	1.548	1.095	3.067
PREDICTION CONFIDENCE	1	0.54	0.9	0.93
MEAN DEVIATION	0.0035	0.4632	0.0955	0.0676
Spot x	96.04446	93.82662	104.4205	102.7294
Spot y	226.7929	226.7972	244.7373	245.243
Sigma x	1.21653	1.059866	1.278406	1.106043
Sigma y	1.405103	1.194431	1.293122	1.20968
Residual	2.521373	1.274275	1.828652	1.136497
STEP POSITIONS	8	1	8	4
		4		9
				49
STEP HEIGHTS	59.42676	71.75	46.96423	183.75
		347.1055		149.3594
				32.18945

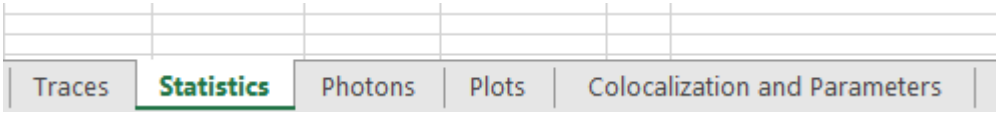
Under the information is the full trace and the fit line, these can be easily plotted in origin or GraphPad prism for publication quality figures.

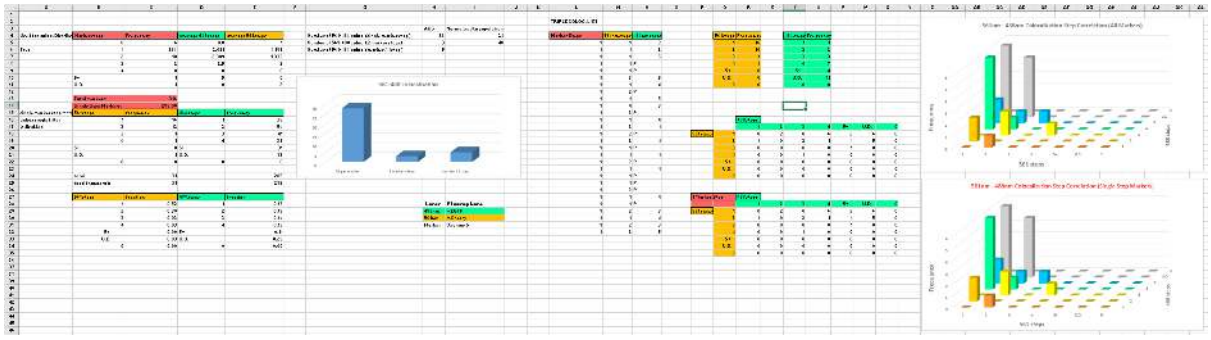
Corrected	Fit	Corrected	Fit	Corrected	Fit	Corrected	Fit
59.0438	59.8438	421.4881	421.5	51.7233	46.75	383.2838	363.5
67.2562	59.8438	382.7333	349.75	64.611	46.75	347.9914	363.5
52.1167	59.8438	337.8933	349.75	40.3886	46.75	404.7576	363.5
62.8938	59.8438	328.4833	349.75	61.7876	46.75	318.0638	363.5
57.4181	59.8438	124.4205	2.6445	50.1143	46.75	209.4824	179.75
59.361	59.8438	3.7595	2.6445	47.1181	46.75	94.871	179.75
50.9348	59.8438	-3.8229	2.6445	36.9324	46.75	161.8419	179.75
69.8157	59.8438	-23.8662	2.6445	21.2886	46.75	209.6614	179.75
-1.859	0.417	-14.5267	2.6445	1.5467	-0.2142	222.7262	179.75
4.1248	0.417	26.2881	2.6445	7.5757	-0.2142	61.27	30.3906
-11.0167	0.417	-3.4776	2.6445	5.8157	-0.2142	24.0824	30.3906
-4.8629	0.417	13.9914	2.6445	1.541	-0.2142	16.9824	30.3906
1.609	0.417	2.6086	2.6445	-14.1871	-0.2142	17.1143	30.3906
4.2567	0.417	30.8657	2.6445	-2.7081	-0.2142	28.6019	30.3906
-1.711	0.417	-4.329	2.6445	2.3329	-0.2142	71.789	30.3906
-2.0533	0.417	2.231	2.6445	-2.129	-0.2142	136.2852	30.3906
5.9395	0.417	-22.5567	2.6445	5.2829	-0.2142	59.2424	30.3906
3.2919	0.417	12.5524	2.6445	5.2376	-0.2142	58.6186	30.3906
2.3367	0.417	16.4919	2.6445	4.2529	-0.2142	32.8376	30.3906
-1.2343	0.417	15.2171	2.6445	-2.1695	-0.2142	24.8886	30.3906
7.0576	0.417	18.0286	2.6445	4.5081	-0.2142	0.08	30.3906
-1.5176	0.417	6.8281	2.6445	-8.3967	-0.2142	34.2014	30.3906
-4.471	0.417	18.0029	2.6445	-5.0976	-0.2142	0.6338	30.3906
2.6971	0.417	40.2114	2.6445	4.7795	-0.2142	9.5448	30.3906
-2.6052	0.417	4.329	2.6445	-3.3976	-0.2142	11.9862	30.3906
1.7571	0.417	-6.0838	2.6445	-6.9357	-0.2142	66.7048	30.3906
-4.2471	0.417	-5.9719	2.6445	11.0781	-0.2142	-1.7286	30.3906
-0.5181	0.417	6.4919	2.6445	7.3276	-0.2142	50.469	30.3906
-4.9871	0.417	-11.9833	2.6445	0.9581	-0.2142	13.4281	30.3906
4.1629	0.417	-4.4805	2.6445	1.78	-0.2142	4.5238	30.3906

Due to limitations of Excel, if there are more than 4000 traces in the dataset, only the first 4000 will be exported to this sheet, otherwise the program risks addressing a cell that doesn't exist and will fail to save the document and crash. Another way of exporting all traces will be covered at the end of this chapter.

6.3 Step Distribution Statistics

Step distribution statistics can be found in the 'Statistics' sheet in the export template:





On the left are the step distributions for colocalized markers and secondary colocalized step distributions:

4	Used for coloc. Distribs	Marker steps	Frequency	average 488 steps	average 561 steps
5		0	6	0.8	1
6	True	1	288	2.021	1.516
7		2	18	2.389	1.333
8		3	2	2.5	2
9		4	0	0	0
10		5+	1	5	0
11		U.D.	1	0	2
12					
13		Total markers:	316		
14		Single Step Markers:	288.00		
15	single marker step ---->	561 steps	frequency	488 steps	frequency
16	unless single filter	1	16	1	38
17	is disabled	2	12	2	54
18		3	1	3	41
19		4	1	4	38
20		5+	0	5+	31
21		U.D.	1	U.D.	83
22		0	0	0	0
23					
24		total:	31		285
25		total (nonzero):	31		285
26					
27		561 steps	fraction	488 steps	fraction
28		1	0.52	1	0.13
29		2	0.39	2	0.19
30		3	0.03	3	0.14
31		4	0.03	4	0.13
32		5+	0.00	5+	0.11
33		U.D.	0.03	U.D.	0.29
34		0	0.00	0	0.00

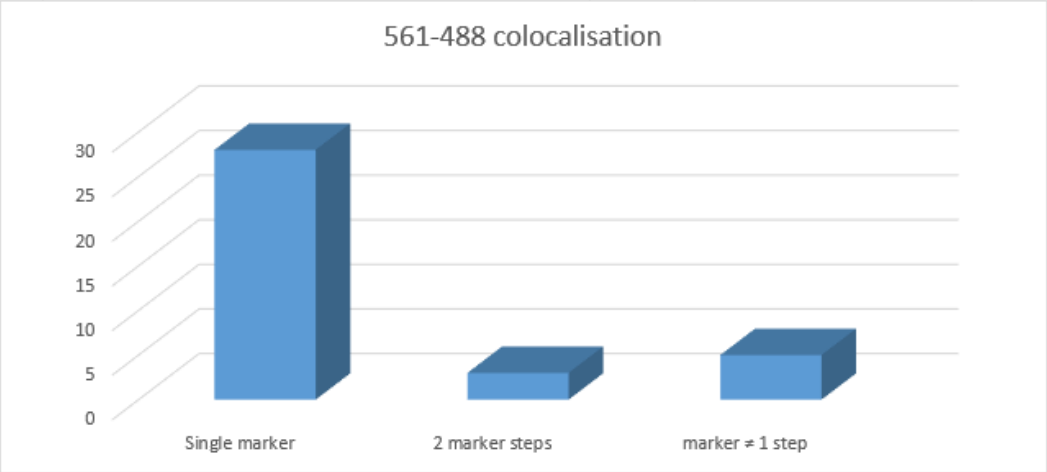
MARKERS

561nm Coloc.

488nm Coloc.

On the right of these distributions is information about triple colocalized spots, that is markers that are colocalized with both secondary channels. The first Is simply a count of the number of triple-colocalized events:

	ABS	Normalised to population
Number of 561-488 coloc. (single marker step)	28	28
Number of 561-488 coloc. (2 marker steps)	3	48
Number of 561-488 coloc. (marker ≠ 1 step)	5	



Laser	Fluorophore
488nm	mEGFP
561nm	mCherry
Marker	Cyanine 5

ABS is the absolute number of events, normalized to population extrapolates the number of events to the ratio of marker steps. Underneath this is a record of which fluorophores correspond to which channel.

To the right of this is a list of all triple colocalized events and distributions:

TRIPLE COLOC. LIST

Marker Steps	561nm steps	488nm steps
1	1	2
1	1	2
1	1	5
1	1	P
1	1	P
1	3	5
1	1	4
1	2	P
1	1	5
1	4	3
1	2	P
1	1	4
1	2	1
1	2	P
1	2	4
1	1	P
1	1	4
1	2	P
1	1	4
1	1	P
1	1	P
1	2	P
1	1	4
1	1	P
1	2	3
1	1	4
1	2	3
1	2	5

561 steps	Frequency
1	16
2	10
3	1
4	1
5+	0
U.D.	0
0	0

488 steps	Frequency
1	1
2	2
3	3
4	7
5+	4
U.D.	11
0	0

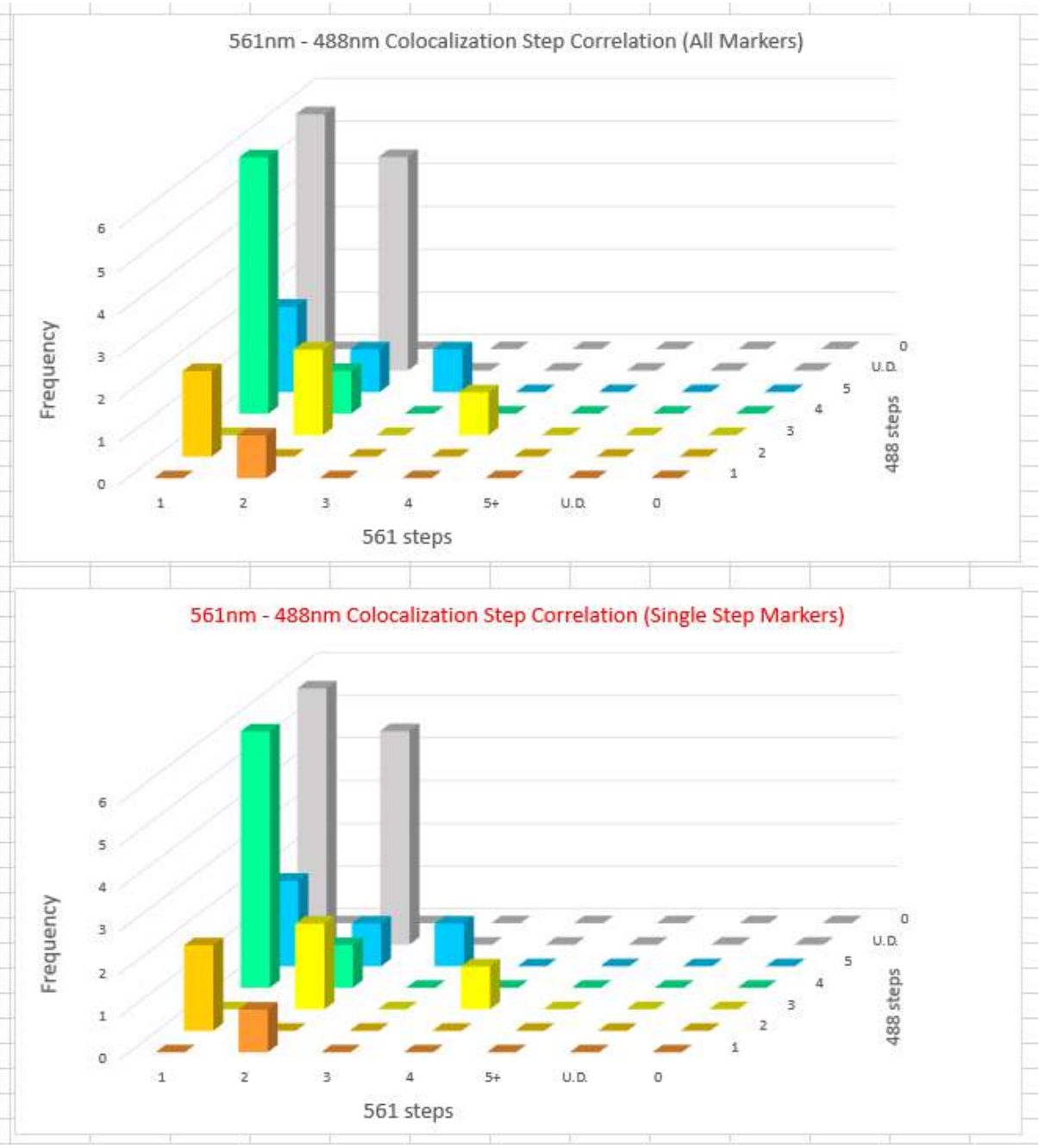
	488 steps							
561 steps	1	2	3	4	5+	U.D.	0
1	0	2	0	6	2	6	0
2	1	0	2	1	1	5	0
3	0	0	0	0	1	0	0
4	0	0	1	0	0	0	0
5+	0	0	0	0	0	0	0
U.D.	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0

1 Marker Step	488 steps						
561 steps	1	2	3	4	5+	U.D.	0
1	0	2	0	6	2	6	0
2	1	0	2	1	1	5	0
3	0	0	0	0	1	0	0
4	0	0	1	0	0	0	0
5+	0	0	0	0	0	0	0
U.D.	0	0	0	0	0	0	0
0	0	0	0	0	0	0	0

The top two tables are the step distributions of 561nm and 488nm channels that are triple colocalized with a marker. Only the triple colocalizations for marker steps selected in the export filter are shown in the list.

The tables in the bottom right correlate the number of steps of each channel in a histogram format, showing the frequency of a certain number of steps in one channel correlating to the number of steps in the other channel. The upper of these two tables does this for all triple colocalizations in the exported list whereas the lower of the two filters this list where the colocalized marker has a single bleaching step.

To the right of this information are the correlation tables plotted as histograms:

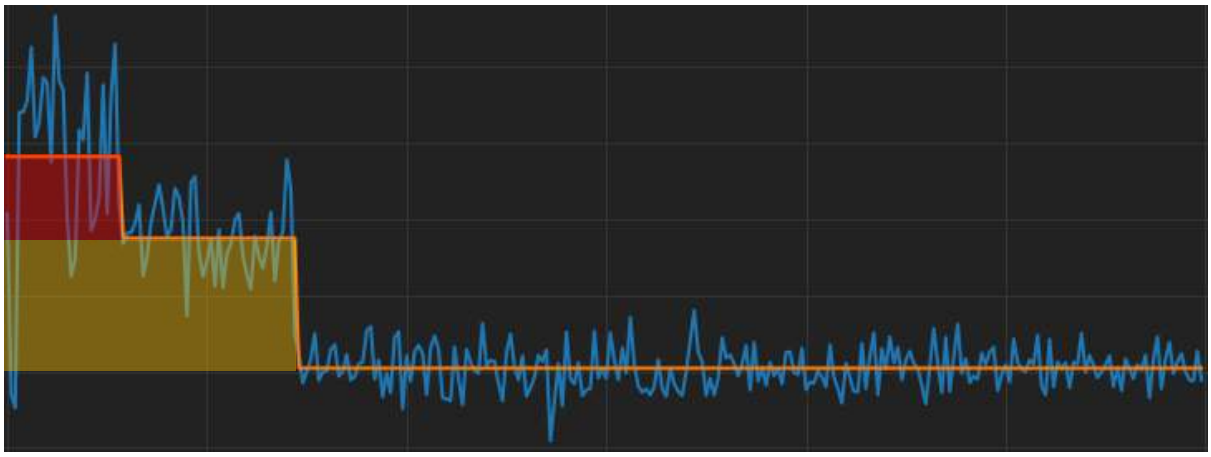


This information can help identify whether proteins in the complex associate cooperatively or competitively which is especially useful when investigating regulatory complexes with activator or repressor factors that may bind concurrently.

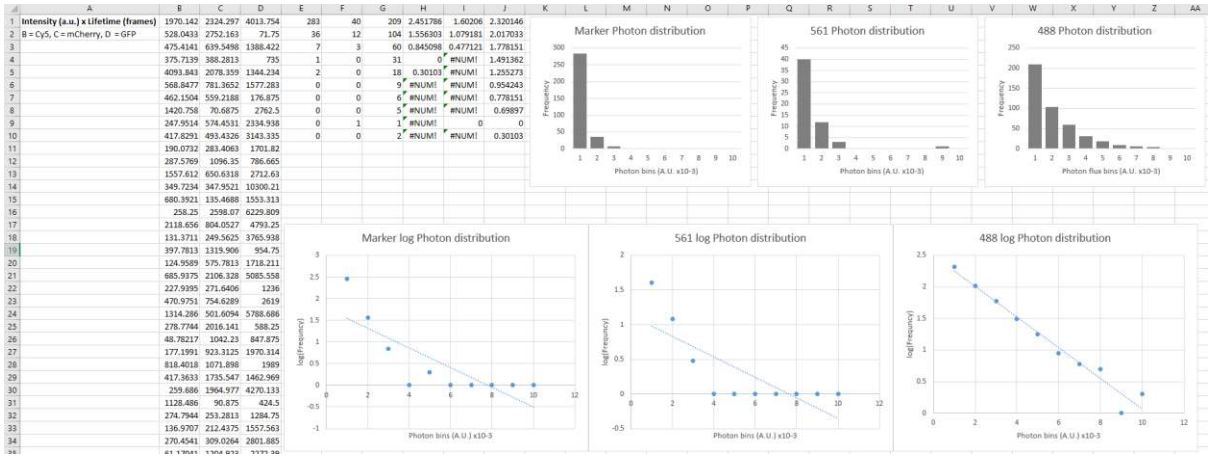
6.4 Total Photons Emitted

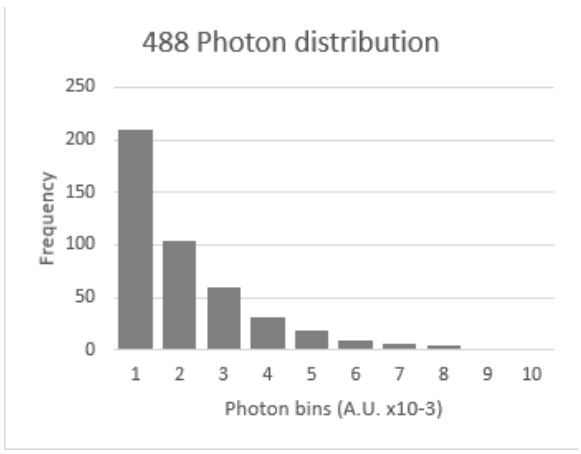
Each time a fluorophore is excited there is a chance it will remain in a reactive triplet excited state and photobleach via a reaction of the triplet with dissolved oxygen species in solution. The rate of photobleaching depends on the number of photons absorbed per unit time, thus weak photobleaching steps from fluorophores in orientations that result in non-optimally aligned transition dipole moments have the close to the same integrations as optimally aligned fluorophores which have high intensity steps and bleach rapidly.

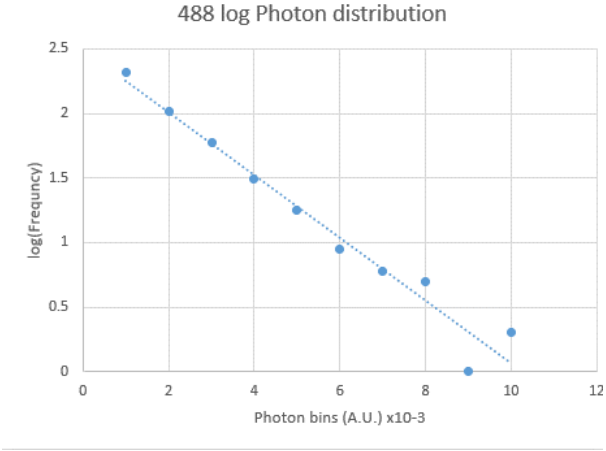
The program integrates plateaus from the beginning of the trace to the fitted step boundaries to calculate the total photons emitted by each fluorophore in arbitrary units. The distribution should be close to exponential and thus the log plot can be fitted with a straight line.



The 'Photons' sheet has a list of the raw integrations as well as pre-binned lists with graphs plotted from them. Currently the auto-binning system is set up *ad hoc*. We encourage users to use plot their own histograms from the raw integrations rather than using the inbuilt binning system.



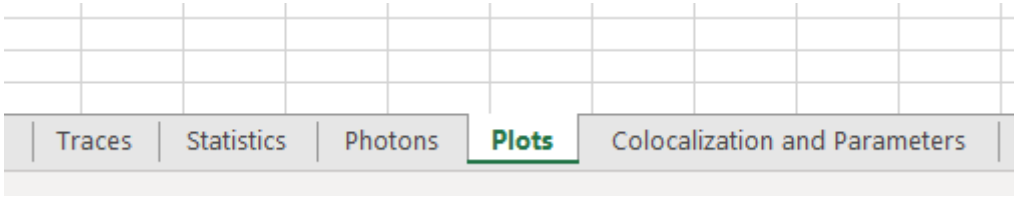


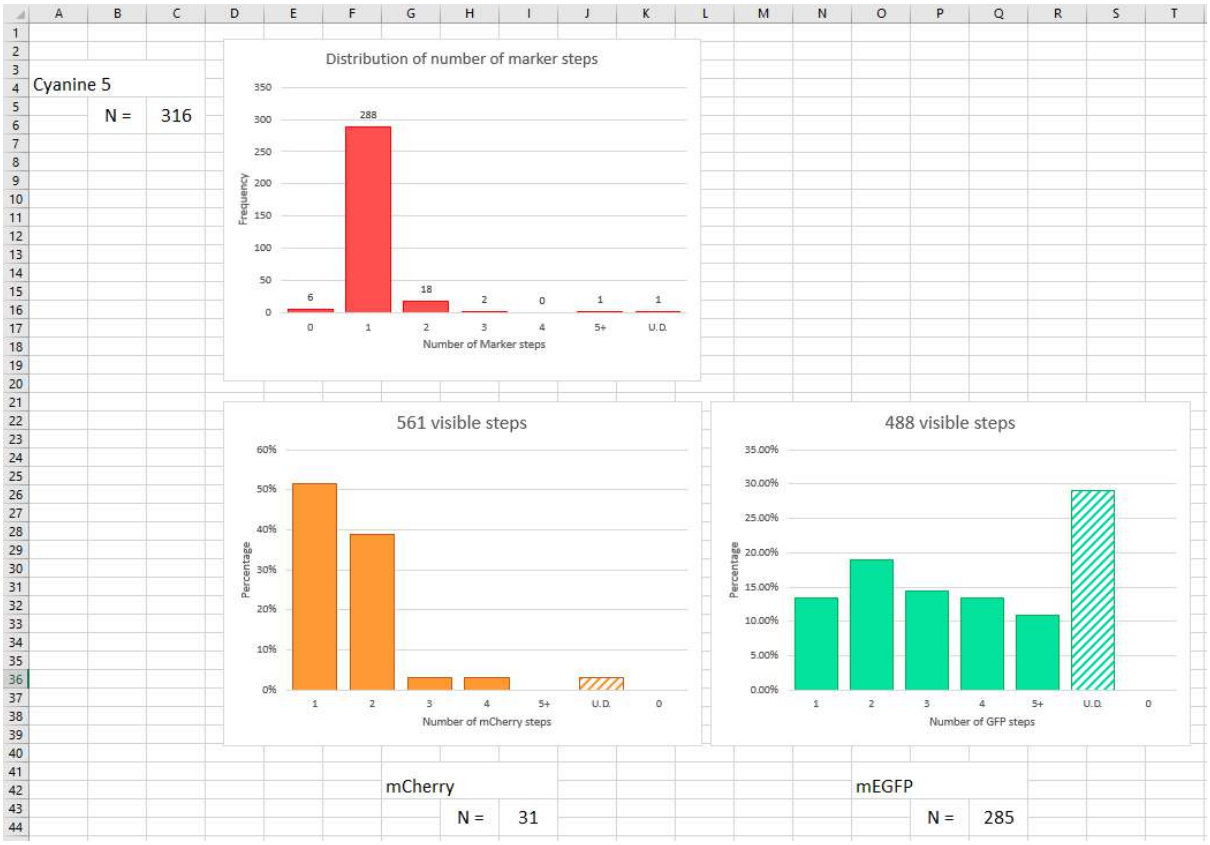


When plotted on a log axis, the exponential distribution can be fitted with a straight line.

6.5 Step Distribution Plots

Step distribution plots can be found in the ‘Plots’ sheet in the export template:

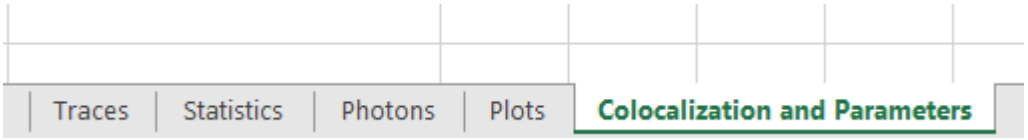


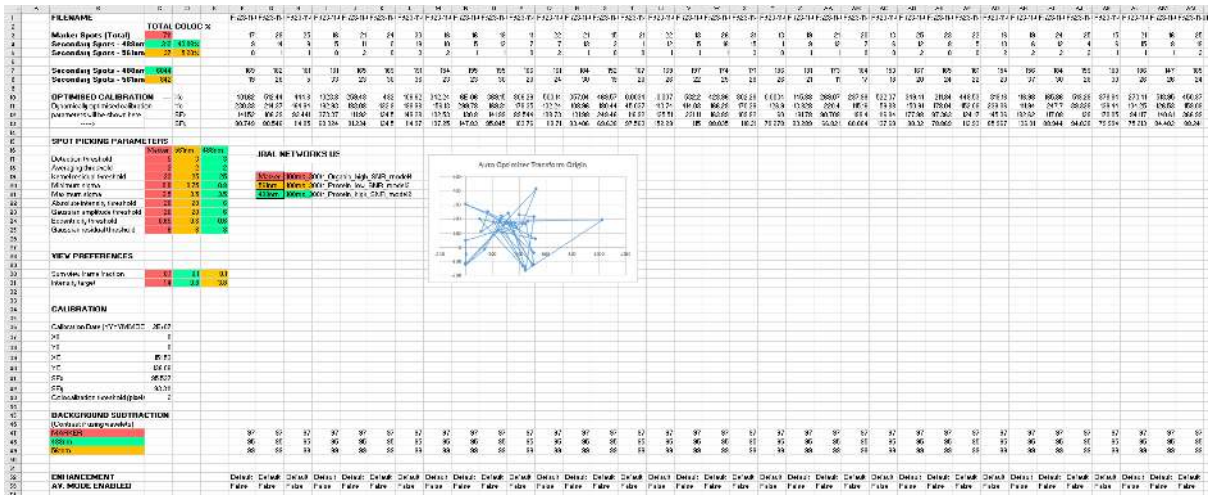


0-step traces are not included in the percentage shown in the graphs as they are spots that were aberrantly detected and had no discernible fluorescence signal or step count according to the neural network prediction. U.D. represents undefined spots, those which are labelled as ‘partially bleached’ in the program.

6.6 Colocalization and Settings Information

The final sheet ‘Colocalization and Parameters’ has a record of the number of spots detected and spots colocalized for each .tif file analysed as well as the settings and calibration used:





In the top left are the colocalization percentages, disregarding the marker step filter. Thus, it simply shows the number of spots in each secondary channel, colocalized out of all spots in the marker channel. Colocalization is calculated as the number of marker spots with a colocalized secondary spot out of the total number of marker spots expressed as a percentage:

FILENAME				F:\23-11-0	F:\23-11-0	F:\23-11-0	F:\23-11-0
	TOTAL	COLOC %					
Marker Spots (Total)	711			17	28	25	16
Secondary Spots - 488nm (Coloc.)	312	43.88%		9	14	9	5
Secondary Spots - 561nm (Coloc.)	37	5.20%		0	1	1	0
Secondary Spots - 488nm (Total)	6044			169	182	181	181
Secondary Spots - 561nm (Total)	842			19	26	5	33

The full file path of the tif file is also recorded in the top row.

In the next four rows are the optimized transform parameters to correct chromatic aberration for each tif file if the auto-optimizer was used:

9											
10	**OPTIMISED CALIBRATION ----->**		Xc	401.6234	512.4362	444.8032	1023.796	259.431	431.9999	106.6161	
11	Dynamically optimised calibration		Yc	230.3335	214.3709	-164.939	192.9344	183.0846	-132.8	198.9791	
12	parameters will be shown here		SFx	141.5165	106.2876	93.44124	373.3655	111.9239	124.5	149.2845	
13	----->		SFy	90.74912	80.54593	114.8484	68.32413	91.2337	124.5	114.9676	
14											

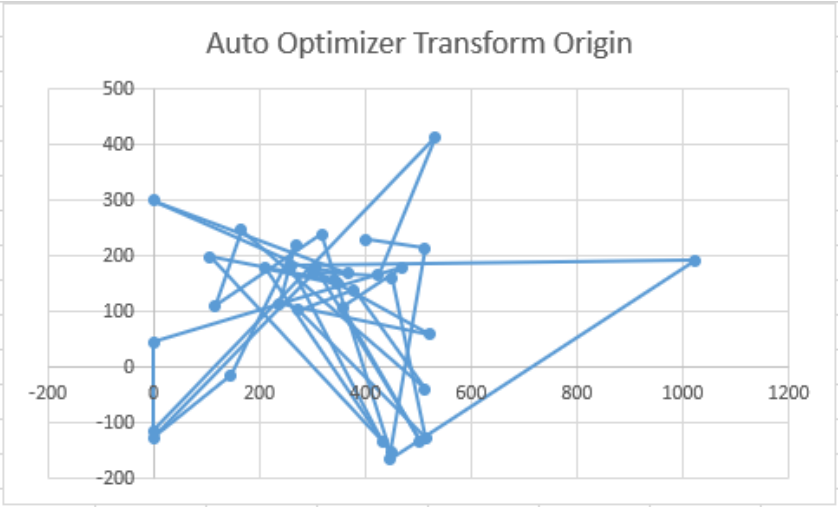
Below this are a record of the exact spot detection thresholds used when analysing the raw .tif files and the proportion of frames at the beginning of the files used to create the enhanced views which were analysed. On the right are the names of the neural networks used to detect steps for each channel:

SPOT PICKING PARAMETERS			
	Marker	561nm	488nm
Detection threshold	5	3	3
Averaging threshold	2	2	2
Kernel residual threshold	22	25	25
Minimum sigma	0.8	0.75	0.8
Maximum sigma	3.5	3.5	3.5
Absolute intensity threshold	20	20	6
Gaussian amplitude threshold	20	20	6
Eccentricity threshold	0.65	0.6	0.6
Gaussian residual threshold	6	6	8
VIEW PREFERENCES			
Sum view frame fraction	0.1	0.1	0.1
Intensity target	1.4	0.8	3.8

NEURAL NETWORKS USED

Marker	100ms_300fr_Organic_high_SNR_model4
561nm	100ms_300fr_Protein_low_SNR_model2
488nm	100ms_300fr_Protein_high_SNR_model2

On the right of these is a plot which shows the origin of the chromatic aberration transform for each .tif file in pixel coordinates:



At the bottom of the sheet is the calibration that was loaded and used for analysis if not using the auto-optimizer. Under this is a record of the background subtraction for each .tif file and the enhancement type used:

CALIBRATION								
Calibration Date (YYYYMMDD)	20240108							
X0	0							
Y0	0							
XC	151.534							
YC	136.063							
SFx	95.537							
SFy	93.311							
Colocalization threshold (pixels)	2							
BACKGROUND SUBTRACTION								
(Contrast if using wavelets)								
MARKER				97	97	97	97	97
488nm				95	95	95	95	95
561nm				99	99	99	99	99
ENHANCEMENT				Default	Default	Default	Default	Default
AV. MODE ENABLED				False	False	False	False	False

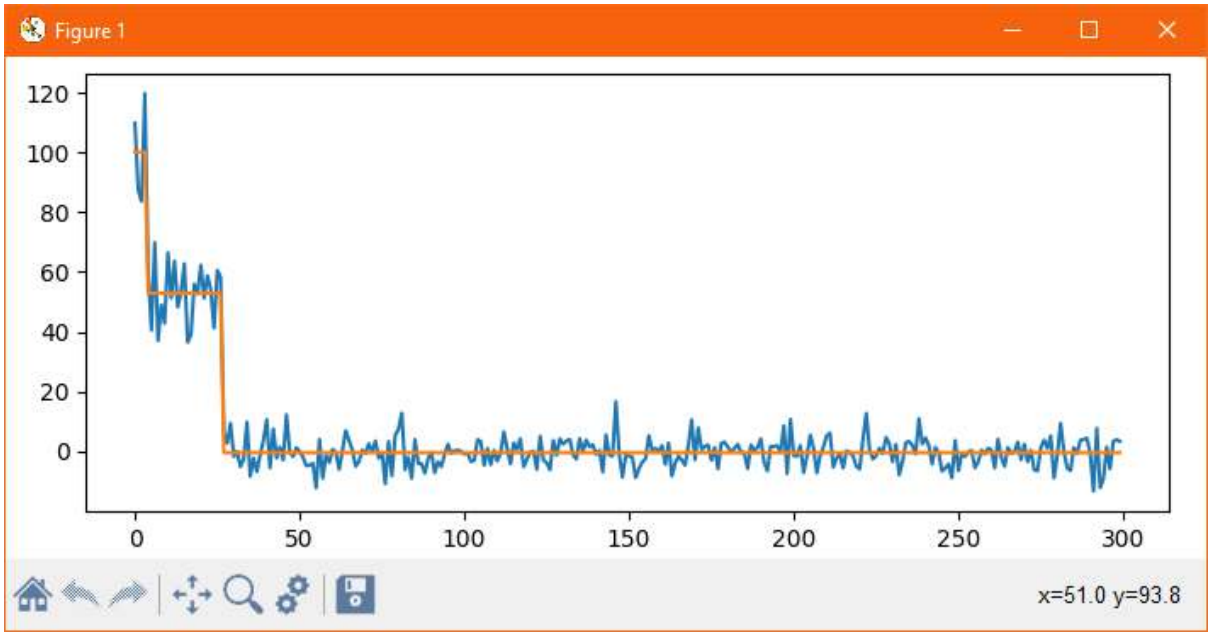
6.7 Saving and Loading Traces

FluoroTensor has an inbuilt file format (.trcs) for saving and loading traces. The file format is binarized data using the pickle library which converts python objects to bytes. Three objects are saved and loaded. These are trace_info which is a list containing information about traces and is structured as follows: [[fluorophore: string, step count: int, frame count: int, spot number: int, step positions: list, weighted activation: float, confidence: float, mean deviation: float, step heights: list, [x, y, [sigma x, sigma y, amplitude, residual]]],]. The second object is all_traces or a subset of this list depending on the filter which is the set of traces stored in a list in the from [[int 0, int 1, int2, …, int299],]. The third object is all_fits or a subset of this list in the same format as all_traces. This data can easily be extracted using a short python script:

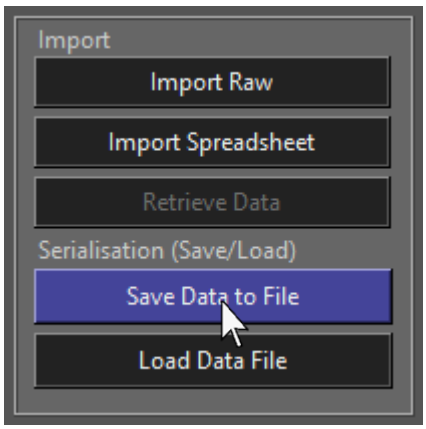
```
import matplotlib.pyplot as plt
import pickle

with open("F:/FluoroTensor v6.6.8r/demo traces.trcs", "rb") as data:
    trace_info = pickle.load(data)
    all_traces = pickle.load(data)
    all_fits = pickle.load(data)

print(trace_info[0])
plt.plot(all_traces[0])
plt.plot(all_fits[0])
plt.show()
```

This will print the information of the first trace in the dataset and plot it along with its fit line:

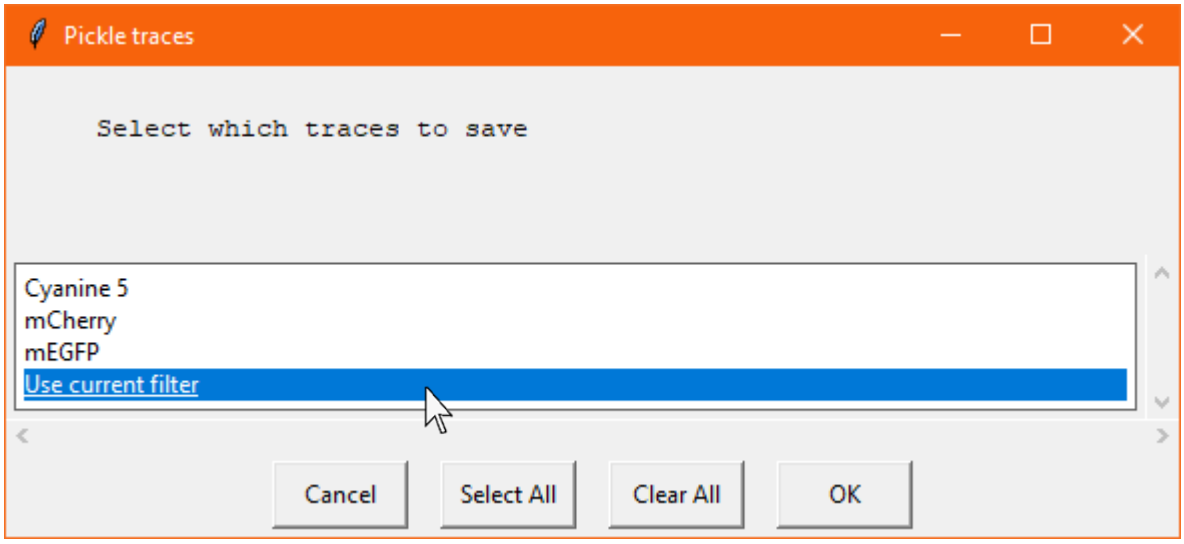
```
['Cyanine 5', 2, 300, 6, [4, 27], 2.075, 0.92, 0.0758, [47.25, 53.33935546875], [71.9074982306415,
222.15402575524712, [1.6926157950200122, 1.5813987007469976, 324.0181037772983, 5.262283819039296]]]
```



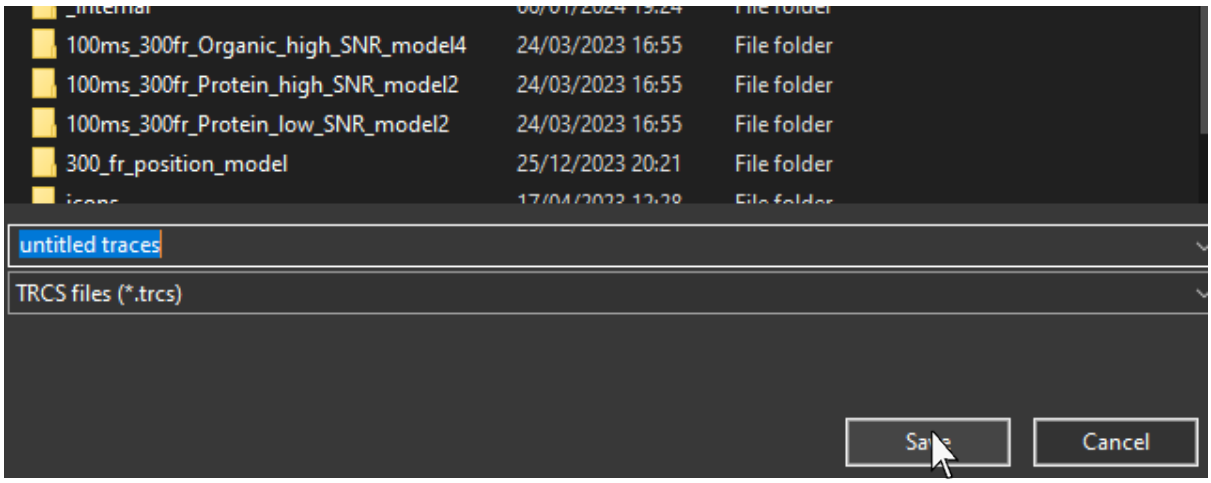
To save traces in FluoroTensor, click ‘Save Data to File’ under ‘Serialisation (save/load) in the control panel:



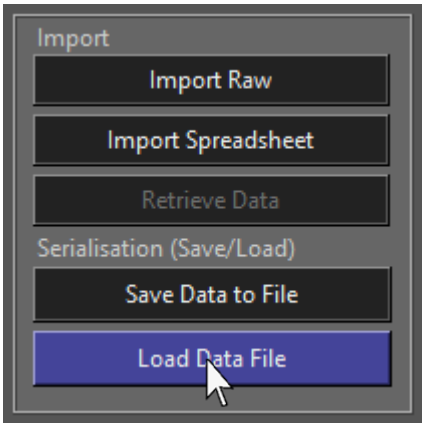
You can then select which traces to save from the list box. Using the current filter will save all of the traces displayed by the filter which is described at the end of chapter 5:



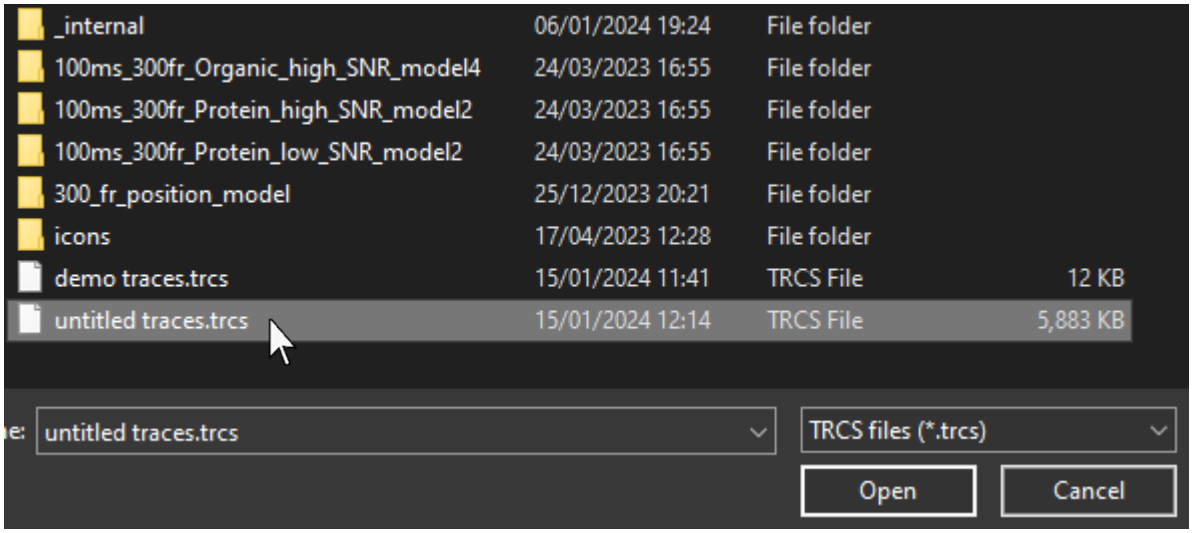
A file dialogue will then be displayed where you can save the traces as a .trcs file:



The file can be reloaded by clicking ‘Load Data File’:



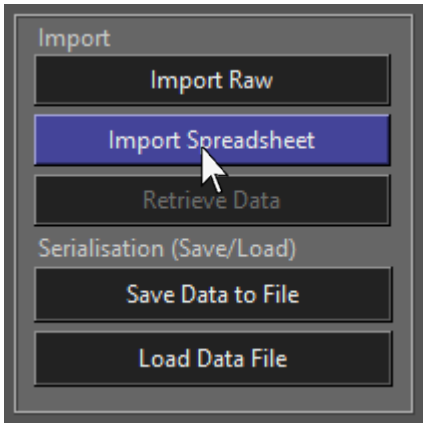
You can then select the file you want to load from the file dialogue:



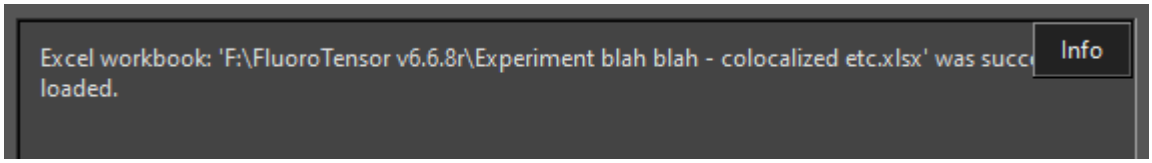
Note that this method only saves traces and their specific information and is not the same as a full export. Information about colocalization will be lost.

6.8 Loading Traces from Excel Template

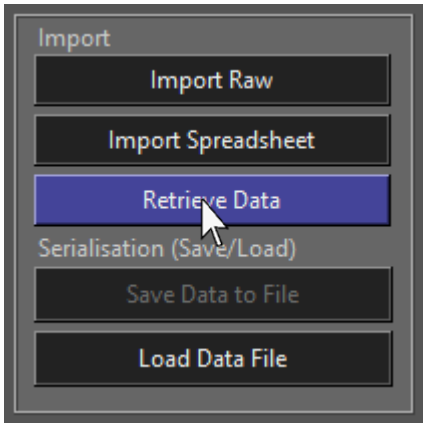
Traces exported to Excel can be reloaded along with the spot criteria and calibration used for the analysis if the data is to be reanalysed from the raw .tif stack in the same session. To load traces / settings from an output template, click 'Import Spreadsheet' in the 'Import' section of the control panel:



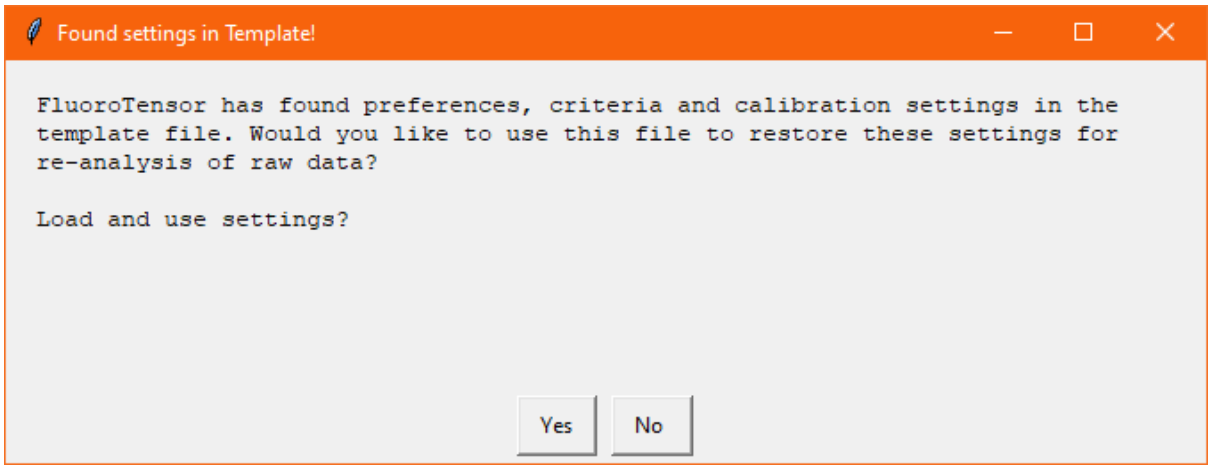
This may take some time especially for large datasets. Progress will be shown in the status bar:



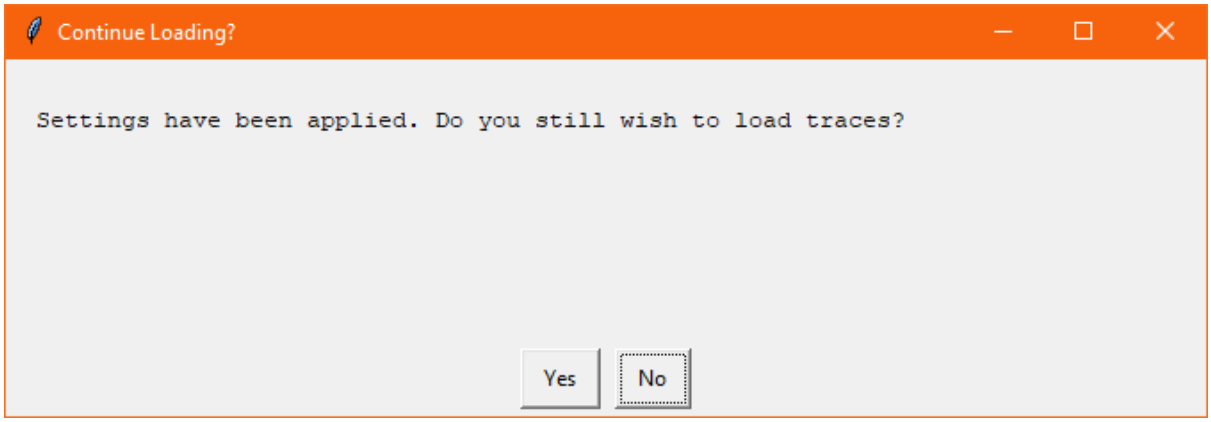
Once the workbook has been loaded, click 'Retrieve Data' under 'Import':



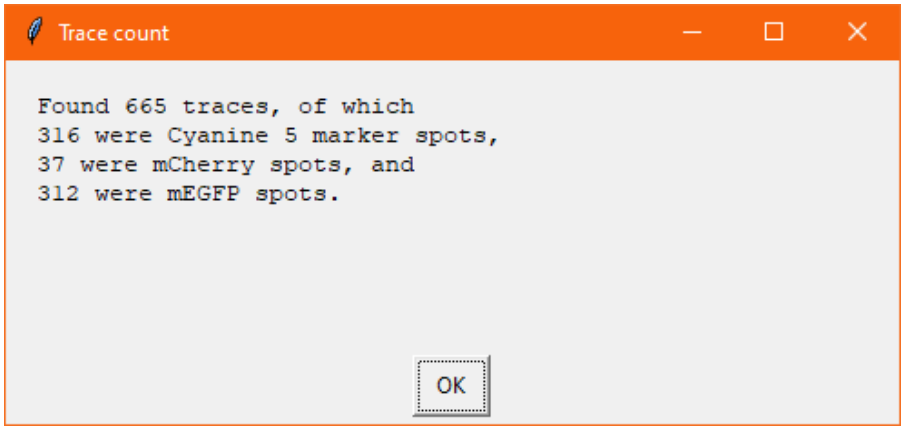
A dialogue box will then appear, prompting the user to load the analysis settings if applicable:



If not, traces will be loaded. If the settings are loaded another dialogue box will appear asking if it is still necessary to load the traces, since you may only be using the Excel template to load the settings:



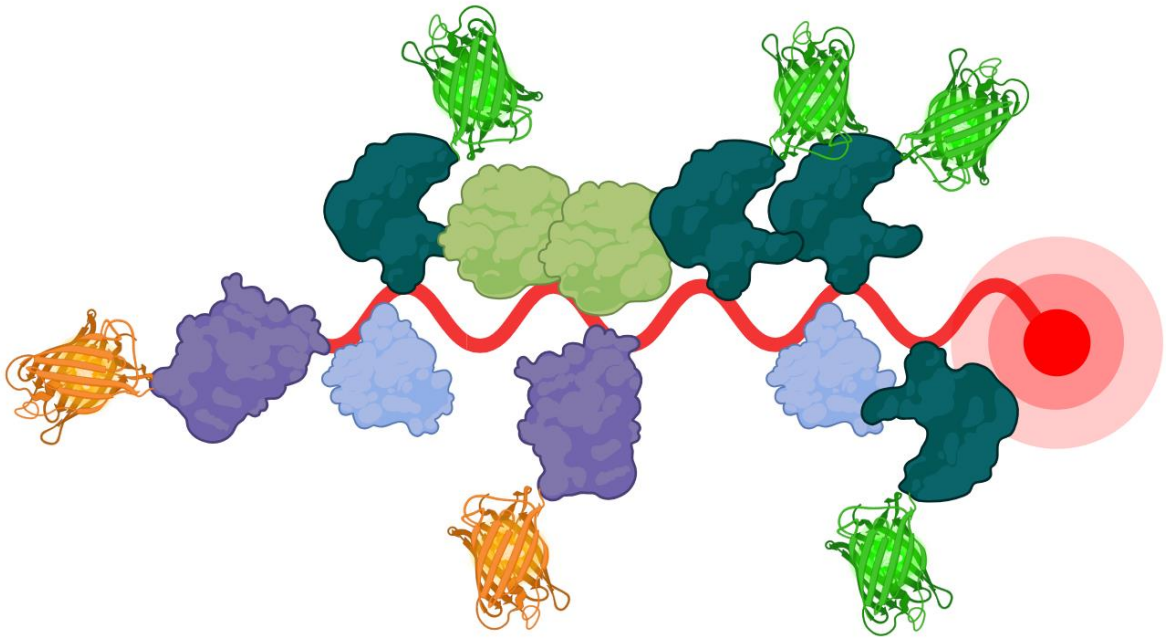
If / when traces are loaded, a pop-up will notify the user of which traces were found in the file:



Chapter 7 – Examples of Automated Experiments

7.1 Colocalization of Splicing Factors with pre-mRNA

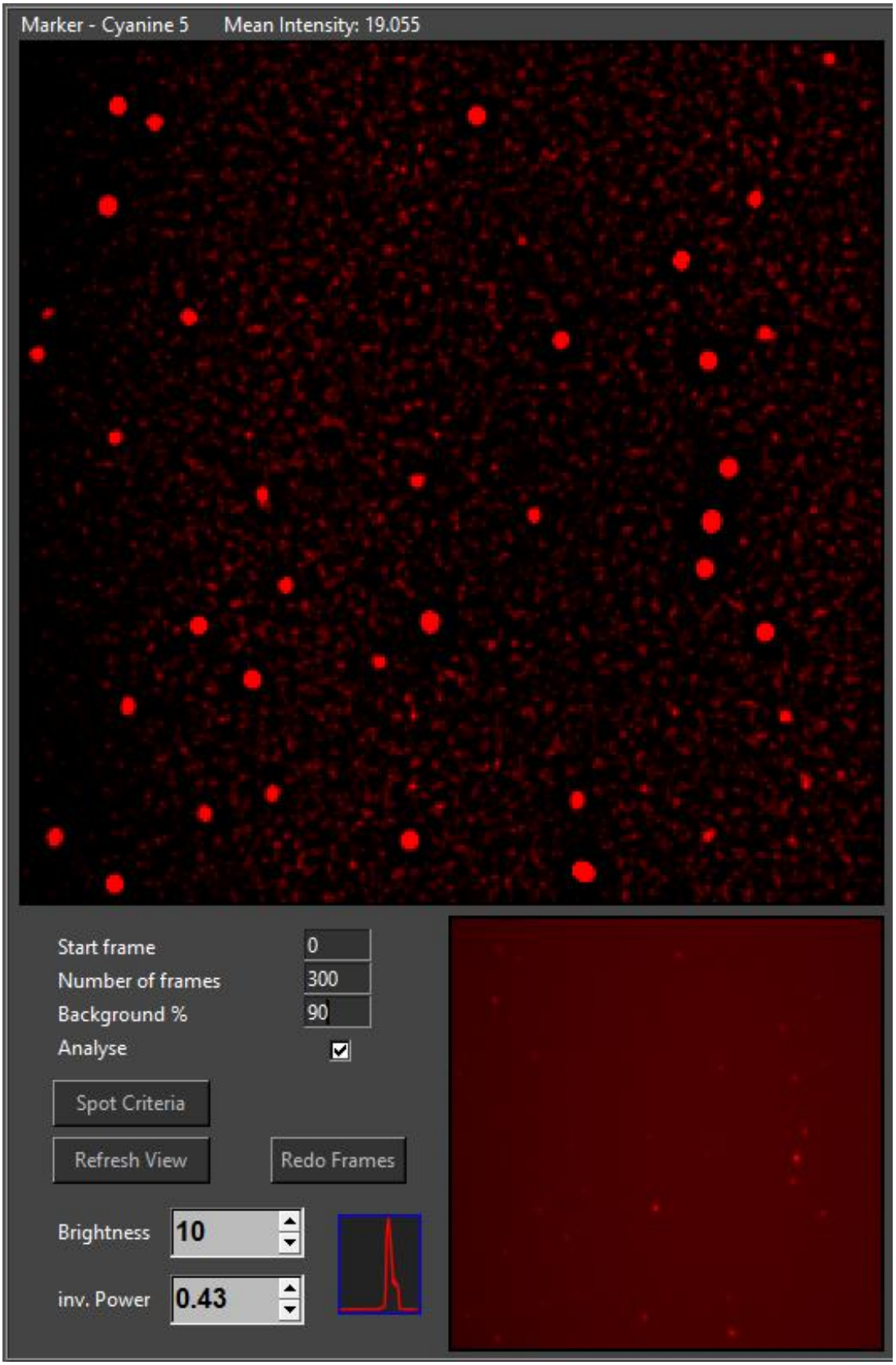
Consider a complex formed of regulatory splicing factors assembled on a Cy5-labelled pre-mRNA strand. Two different regulatory proteins of interest are expressed as fusion proteins conjugated to fluorescent proteins, in this case mCherry and mEGFP respectively:



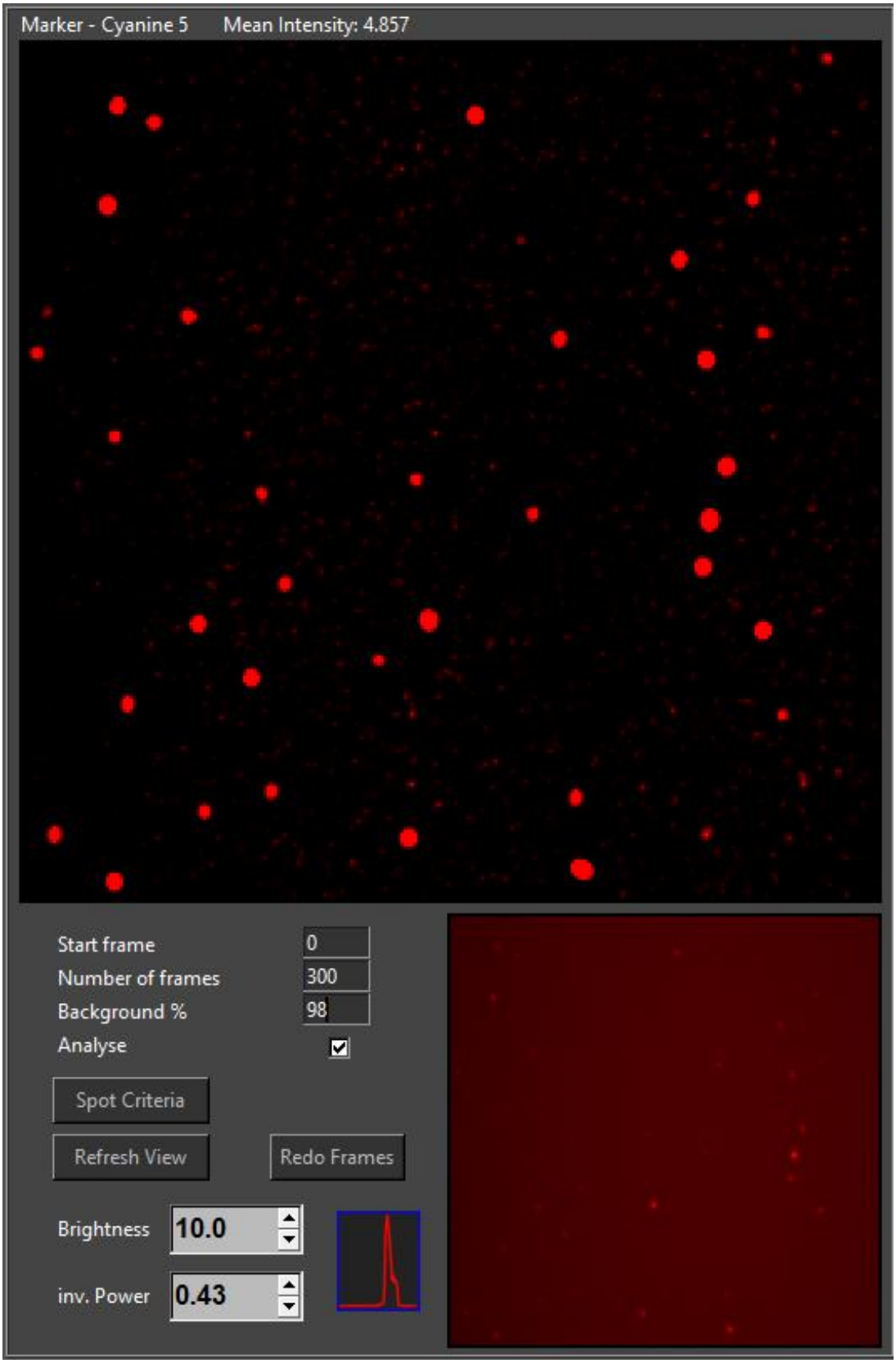
This diagram shows an ideal state where all the proteins of interest are labelled. Usually they are between 50-80% labelled according to western blot assays of exogenous fusion vs total expression. Thus, in practice, the output distributions from FluoroTensor should be run through some kind of binomial solver which can work backwards from the proportion labelled and the distribution to find the true stoichiometry.

This example will be demonstrated with simulated data.

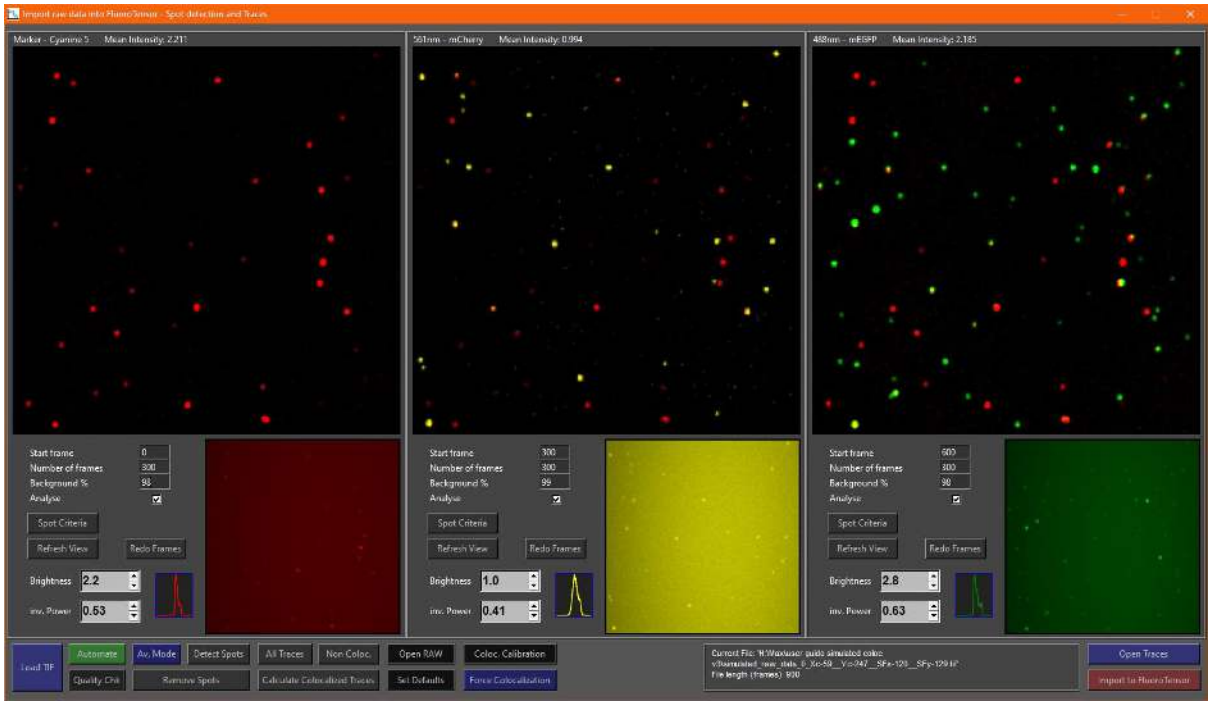
First, a file was loaded to check the frames, number of spots and thus the percentile of the background that should be removed in each channel. To do this, the brightness was increased until the spots were blown out and the background was creeping into the image:



Next, the background subtraction percentage was adjusted until the spots remained but the background was almost fully truncated from the enhanced image. This was then repeated for the other channels as well.



Once the background subtraction was optimal for each channel, the intensity targets were set which helps maintain a sensible brightness, purely for the user to be able to observe spots by eye.

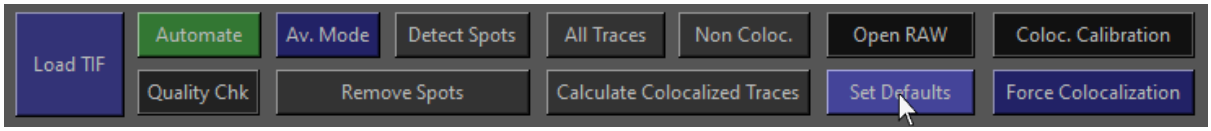


At the top of each channel is the channel name, fluorophore name and the mean intensity of the enhanced view taking the brightness into account:

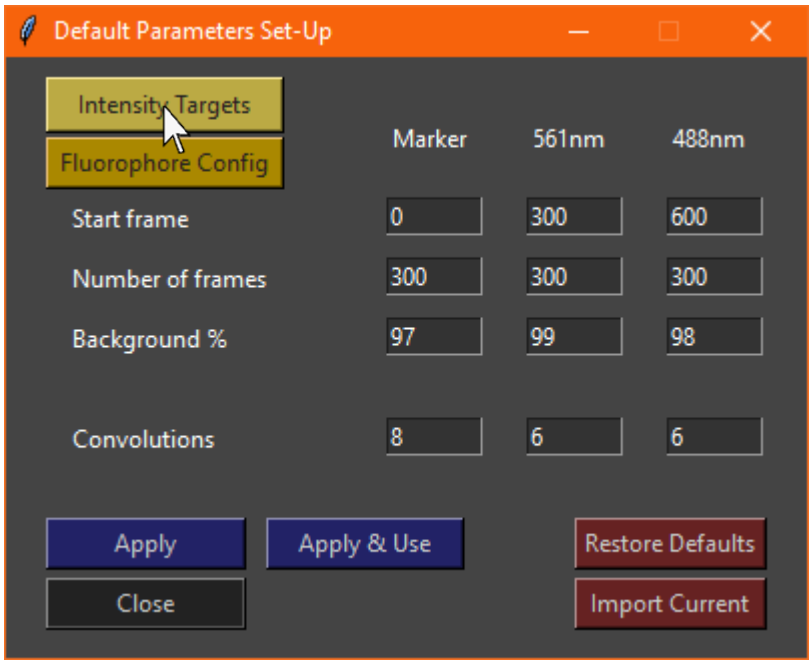
The brightness was adjusted until the user was satisfied:

The mean intensity of 2.76 is now the target the program should aim for when automatically adjusting the brightness of the enhanced image when the file is loaded or ‘Redo Frames’ is pressed.

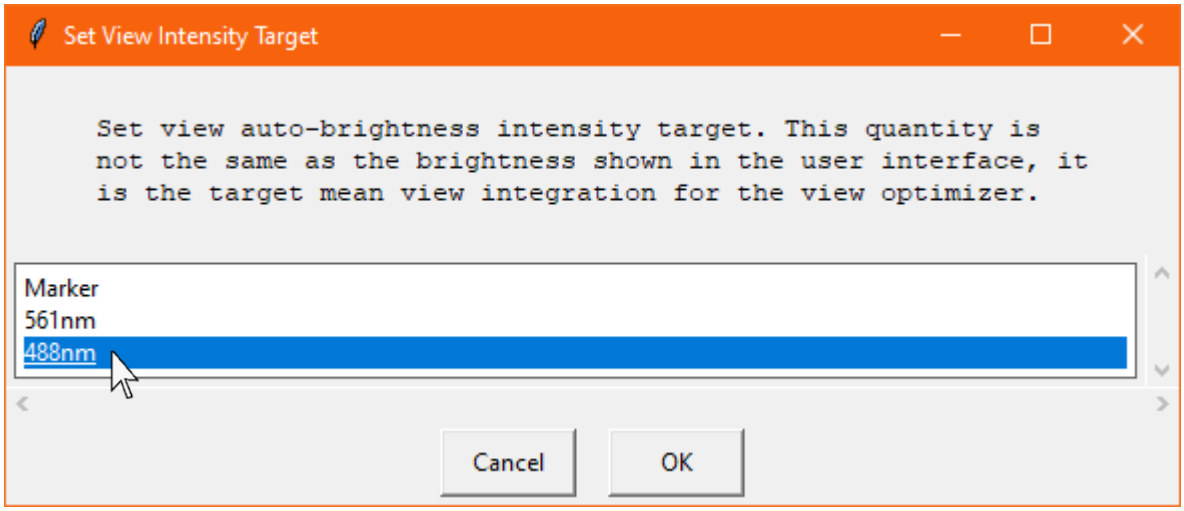
The intensity targets can be changed by clicking ‘Set Defaults’ in the toolbar:



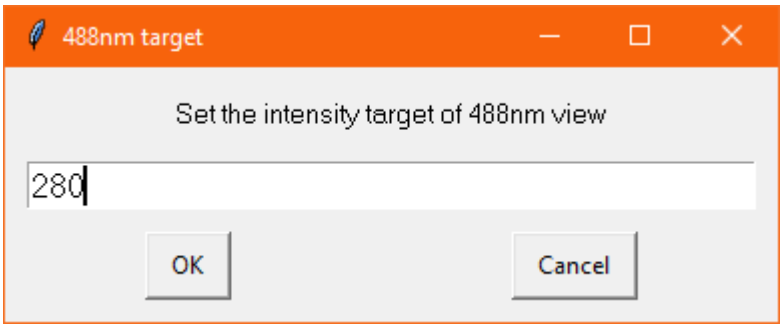
Then, in the default settings window click ‘Intensity Targets’:



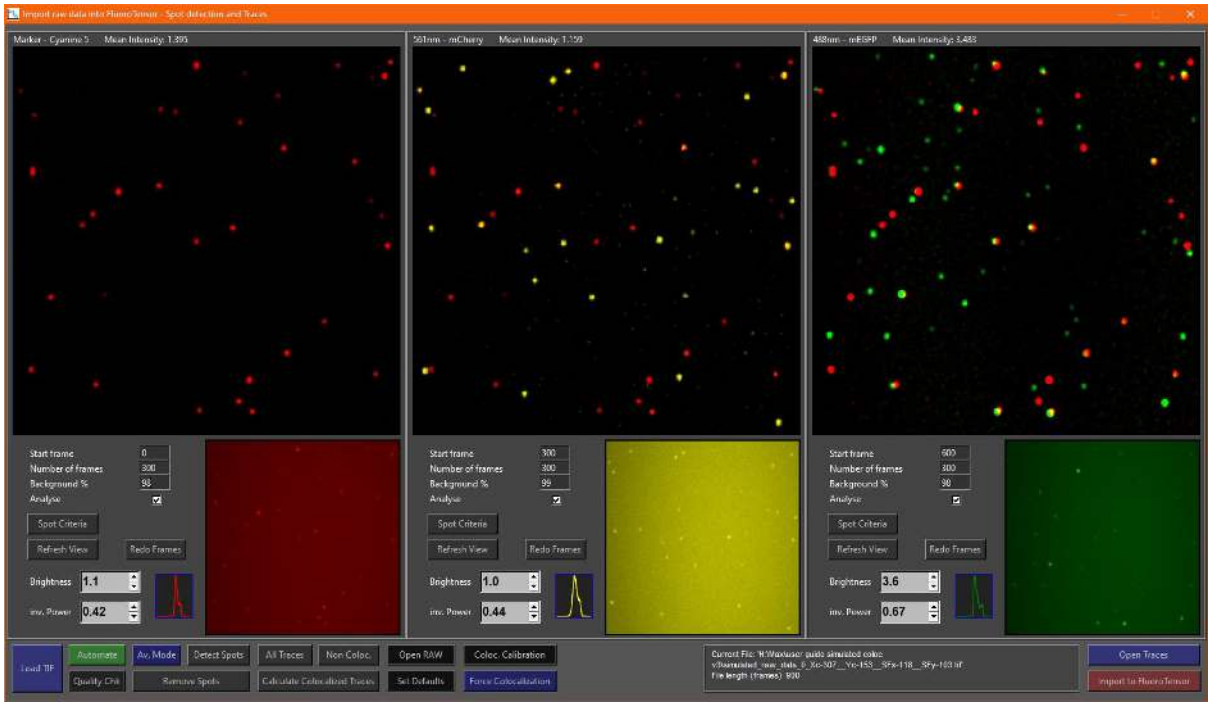
Then the channel can be selected from the listbox:



The intensity target should be set as a multiple of 100 of the mean intensity of the view. An approximate value is fine:

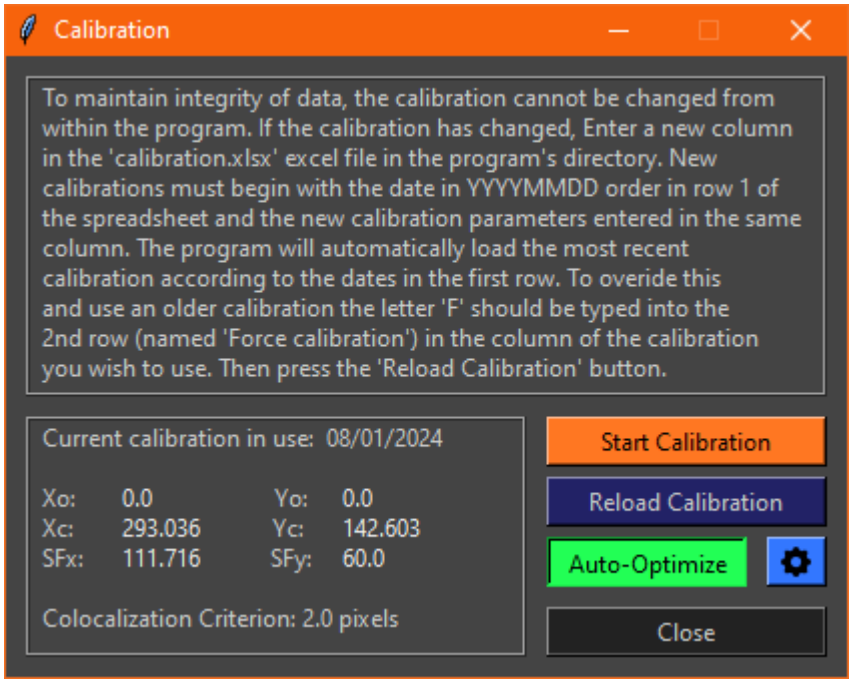


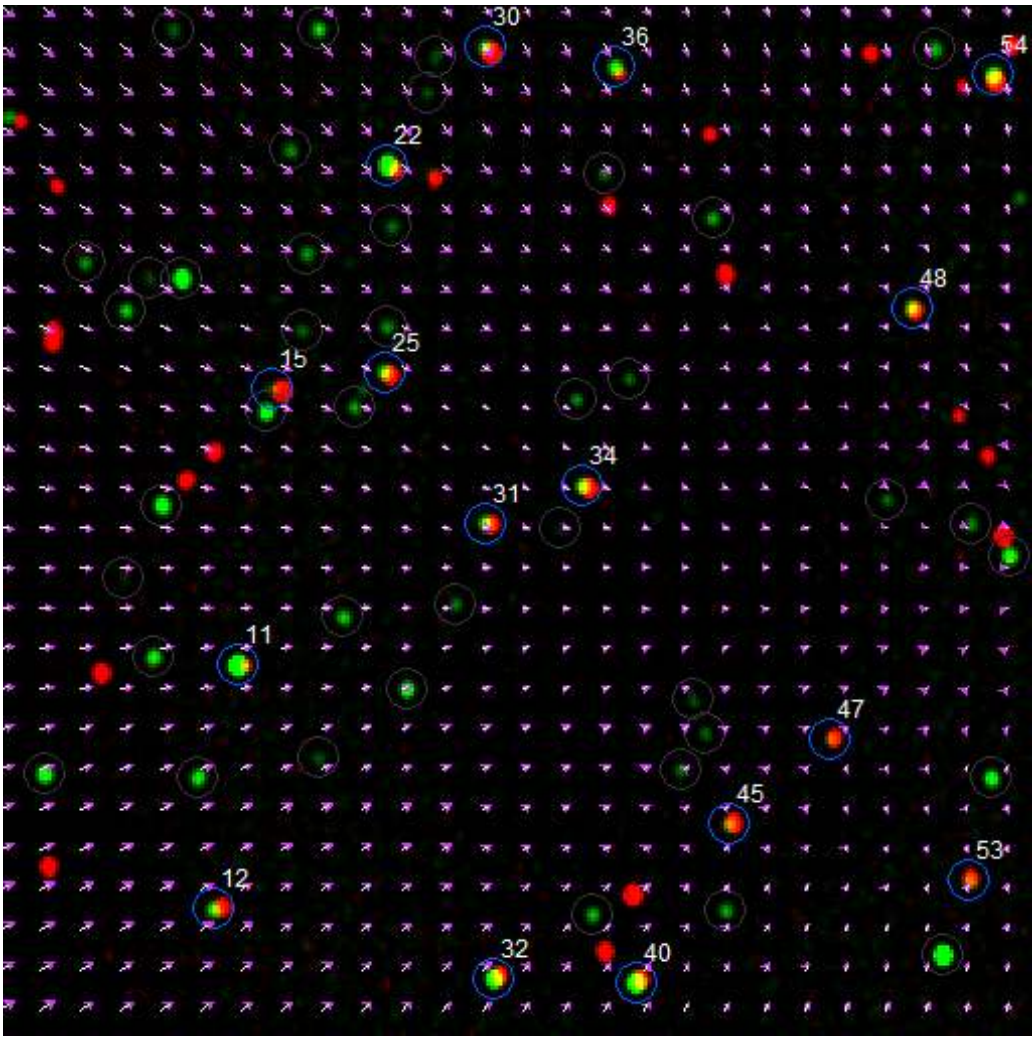
Next, another file should be loaded to check that the intensity targets and background subtraction are roughly consistent from one file to the next in the dataset:



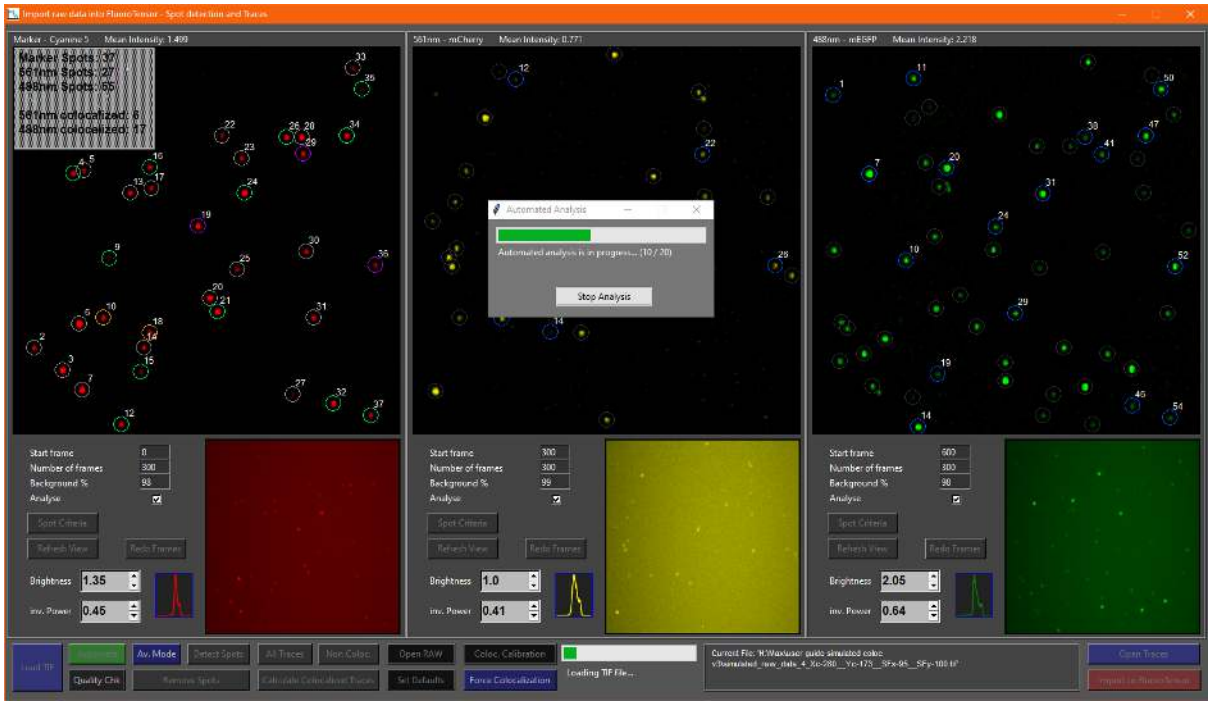
Normally it would be useful now to quality check the files before an automation run (This demonstration uses synthetic data and thus this step isn't helpful here).

Finally, since colocalization levels are high, the auto-optimizer was enabled and checked on the current file:



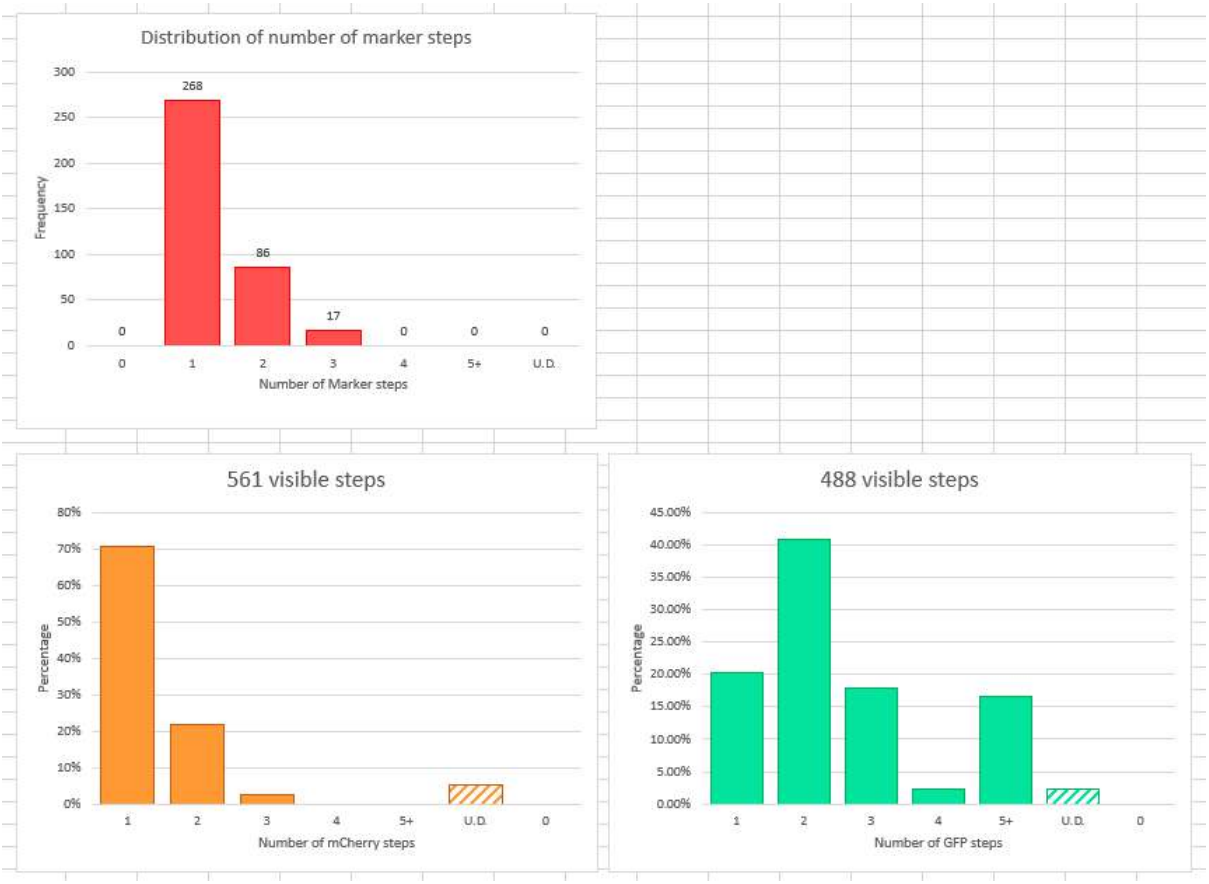


The automation run was then started in colocalization mode:

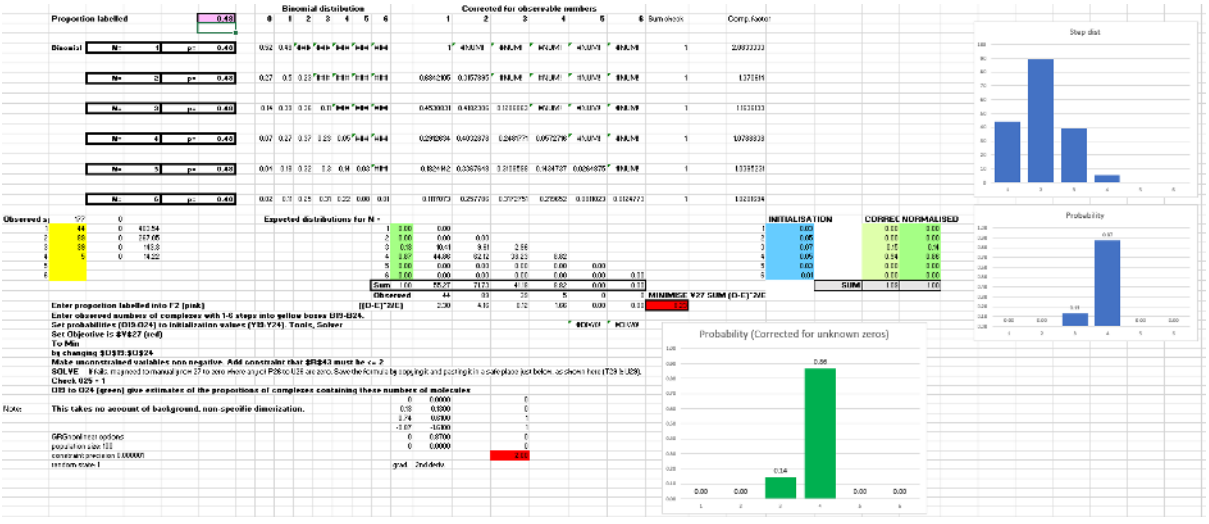


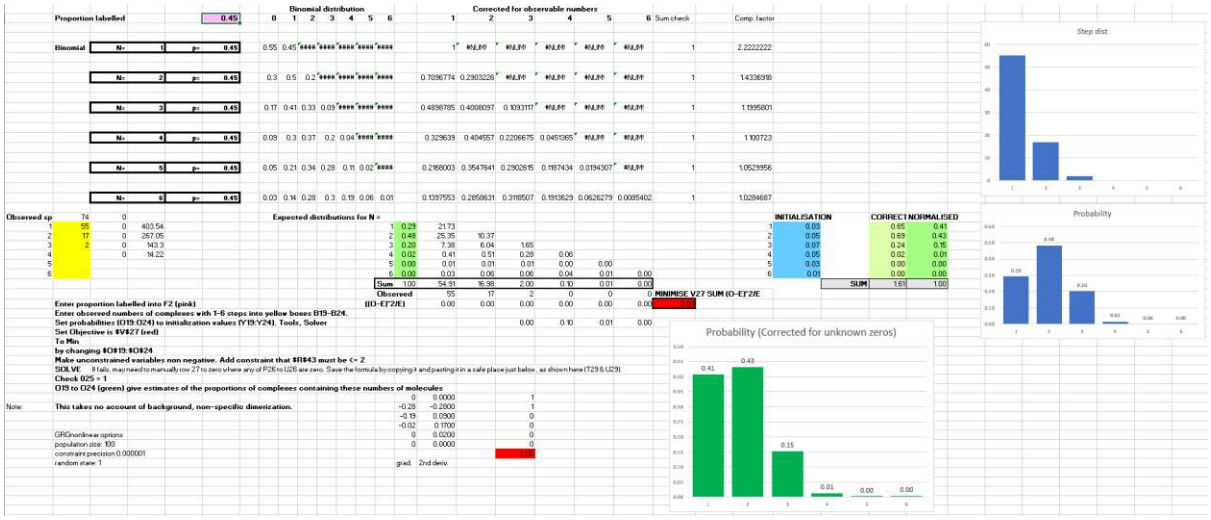
The traces were then imported into the main window and steps were detected / traces fitted:

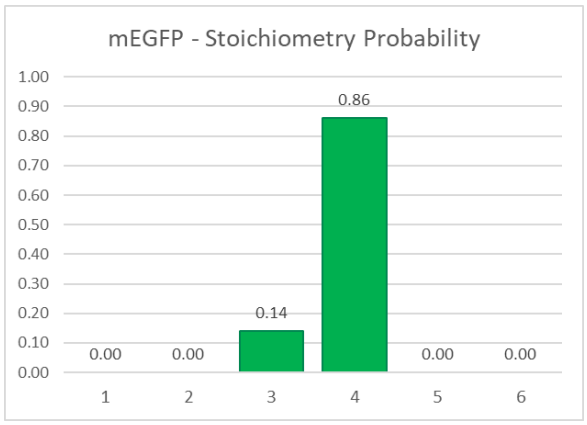
The data was then exported to the colocalization template using a single Cy5 step as the filter since we're only interested in complexes that form on a single RNA molecule:

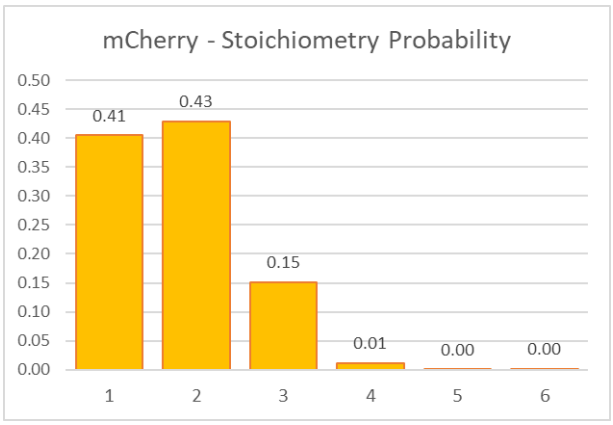


The distributions were then solved using the labelling efficiency and by fitting with a binomial optimizer the expected stoichiometry (4 mEGFP labelled units and 2 mCherry labelled units) is yielded:

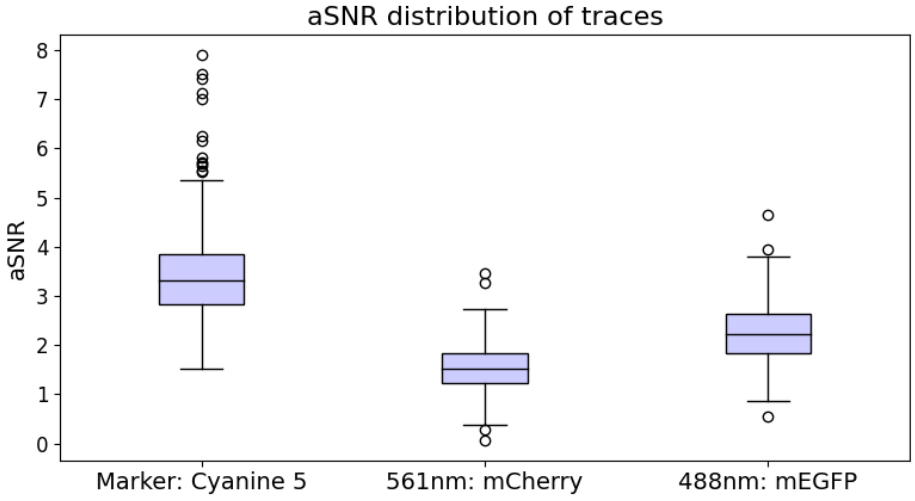






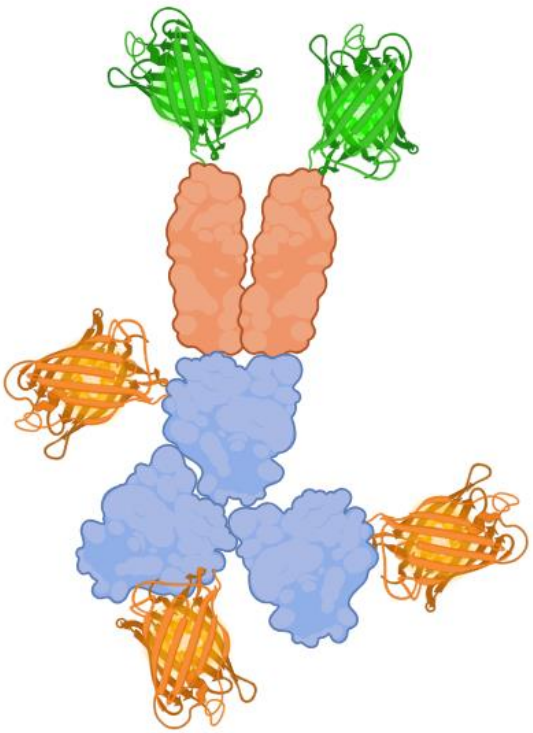


The wider distribution of probable stoichiometries was expected for mCherry since the foci were simulated with very weak intensities and thus fewer 2-step traces were detected than were expected. The signal-to-noise ratios were plotted and mCherry was found to be on the edge of the detection limit for FluoroTensor at a median aSNR of 1.5:



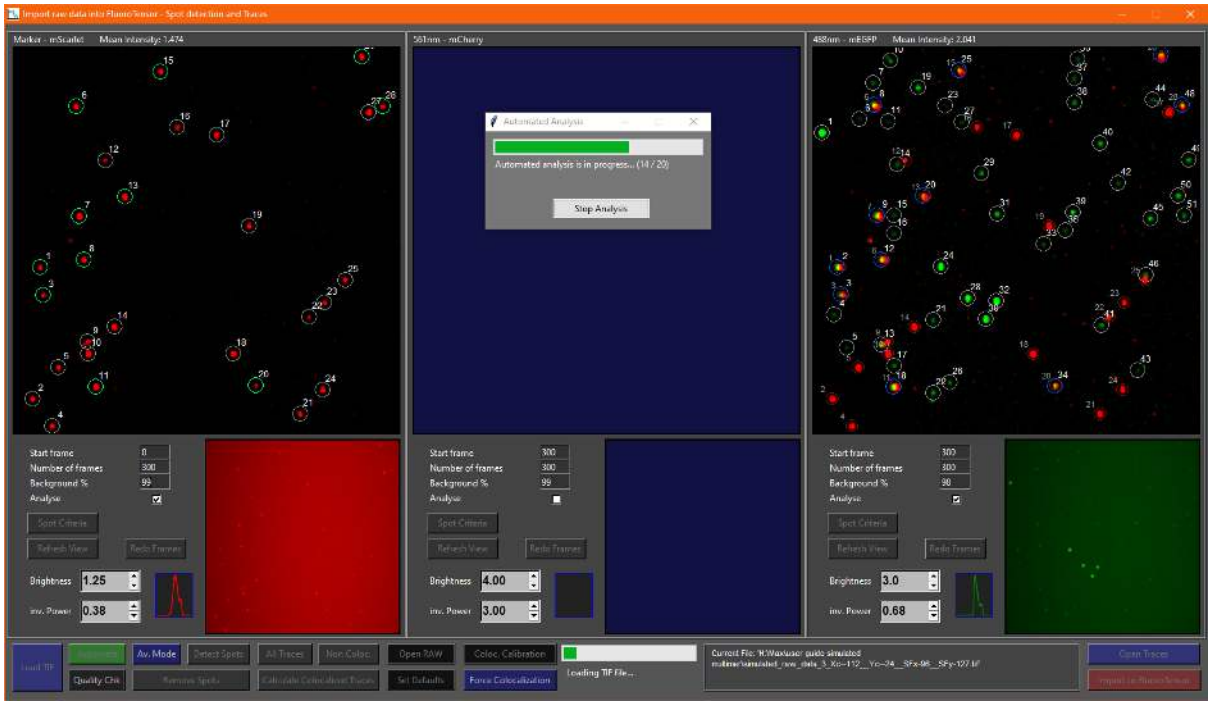
7.2 Stoichiometry of a Hetero-Pentamer

Consider a multimer of two different kinds of proteins, each expressed as fusion proteins with a fluorescent protein attached. Perhaps, the literature says they interact but the stoichiometry is uncertain. In this case FluoroTensor can be used to analyse the SM data to determine the stoichiometry of the components of the multimer by photobleaching analysis.



In this simulation, one component is labelled with mEGFP and the other with mScarlet. For simplicity, mScarlet will be chosen as the marker. In reality they should both be used as markers in different analyses to compare colocalization.

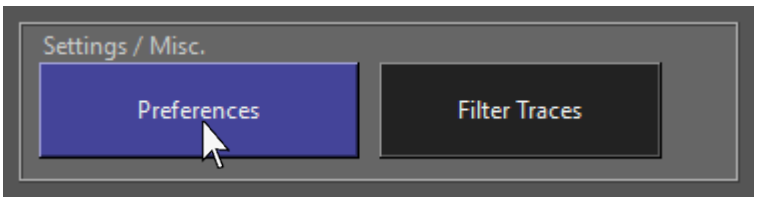
After the marker fluorophore name is loaded and the frames are set. The process is the same as the previous example until the right background subtractions are found. First, an automated run for colocalization was started using only the marker and 488nm channel, with auto-optimization enabled:

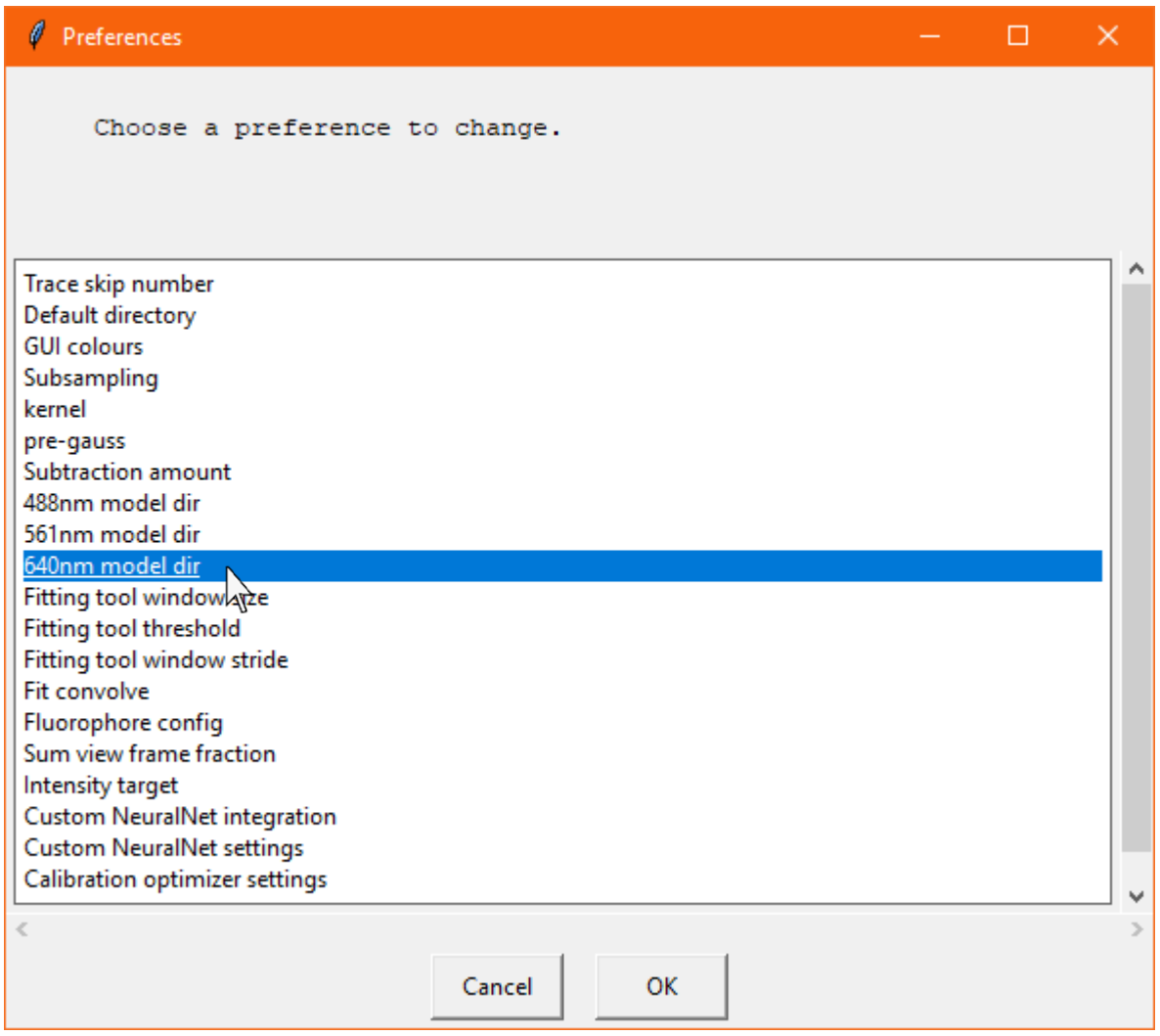


The data was then imported to the main interface:

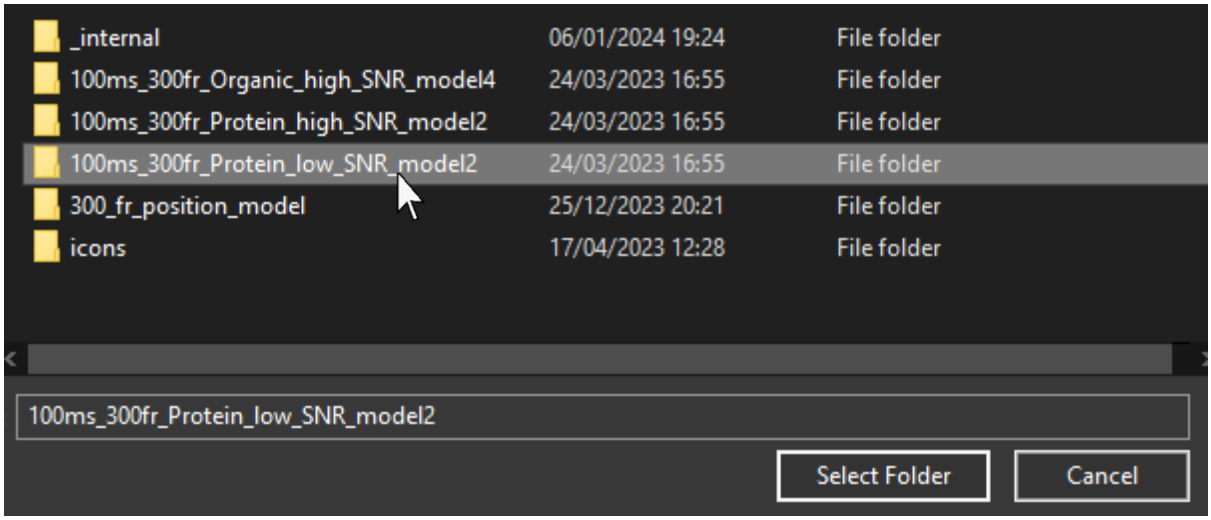
Since the marker traces are now mScarlet and not Cy5, the neural network model used to predict steps must be changed from the organic dye model (default) to the low signal-to-noise model for fluorescent proteins:

This can be done by clicking ‘Preferences’ in the control panel:

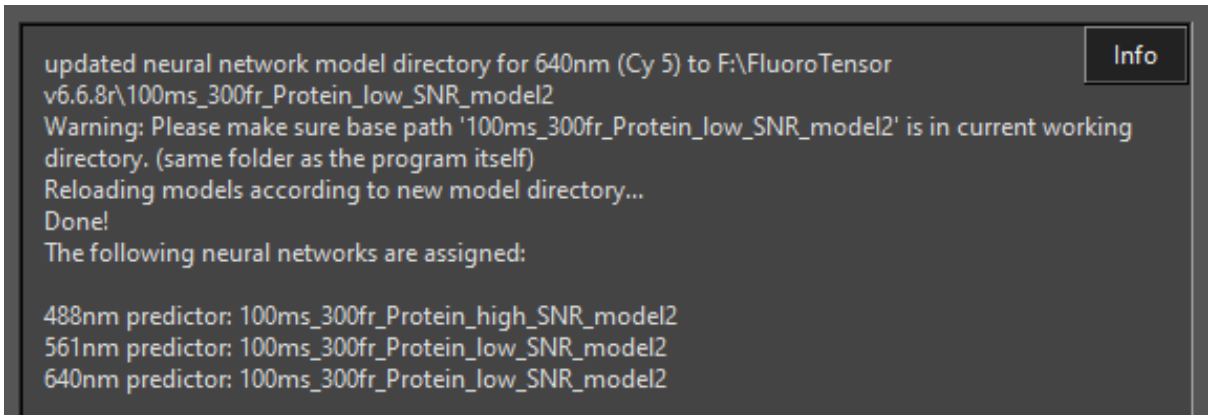




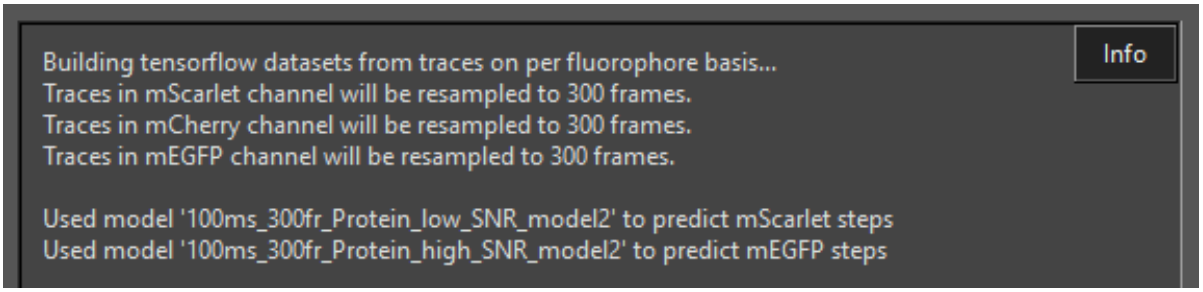
Select ‘640nm model dir’ from the settings and a file dialogue will be displayed:



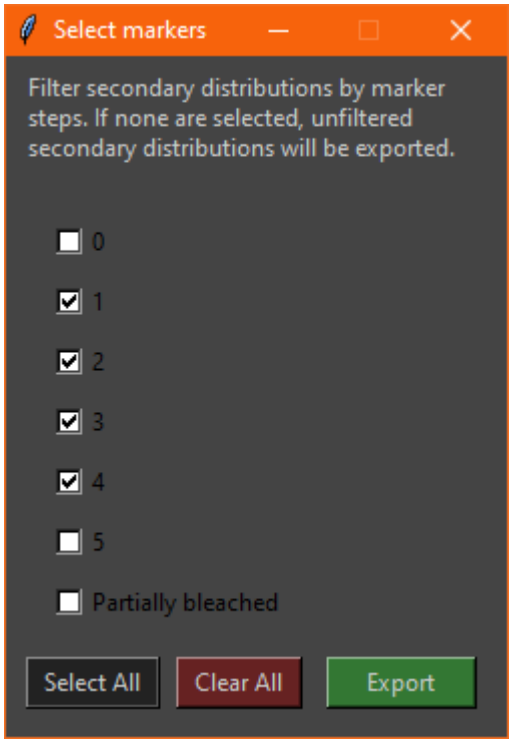
Select the ‘100ms_300fr_Protein_low_SNR_model2’ folder inside the FluoroTensor folder. Models will then be reloaded according to the new configuration and that new configuration will be shown in the status box:



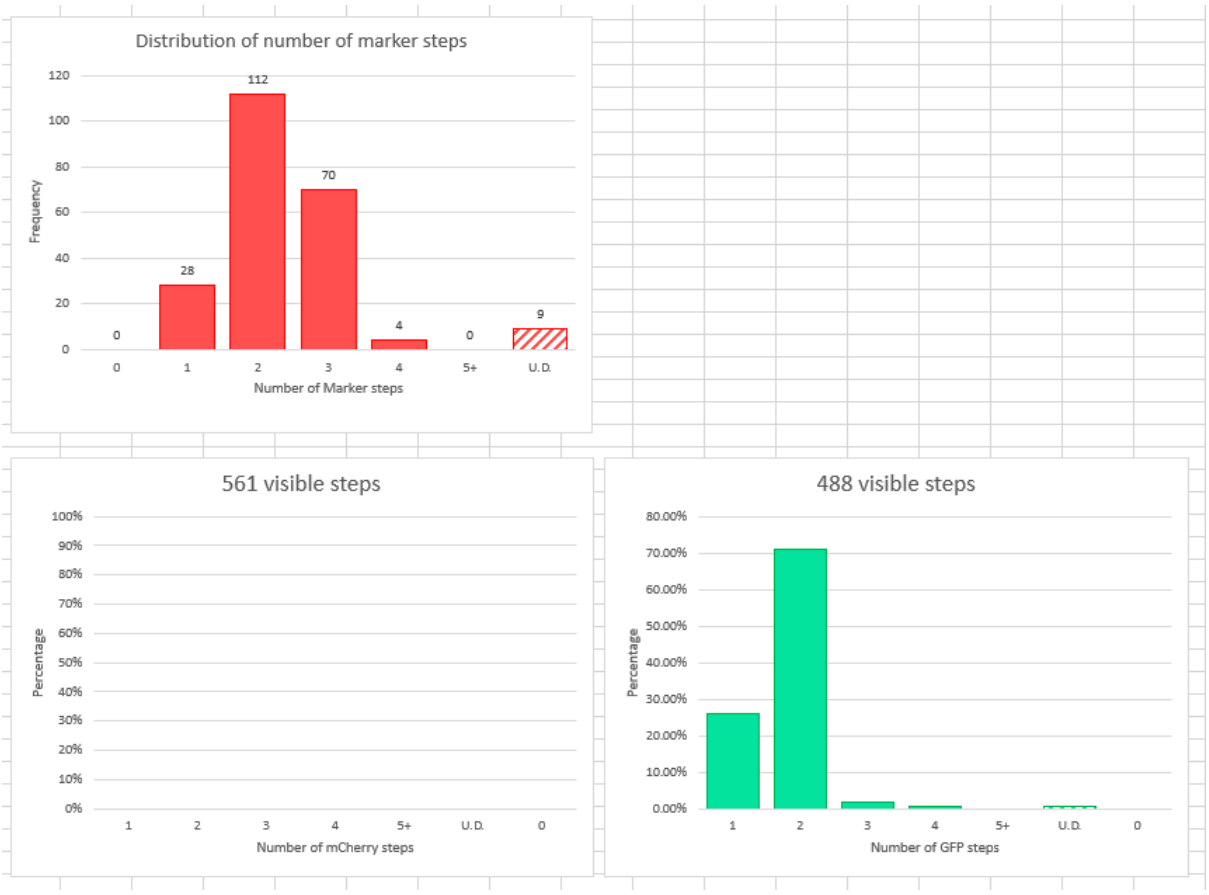
Steps for mScarlet and mEGFP were then detected as normal:

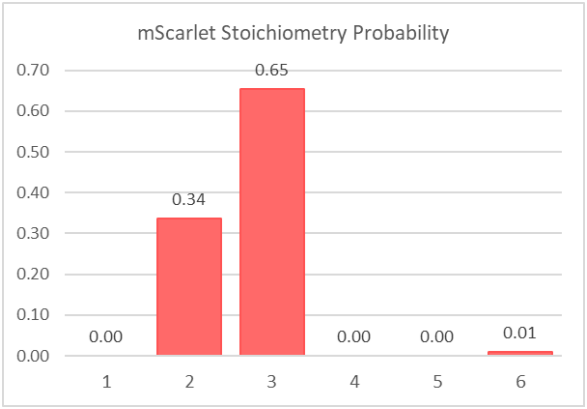


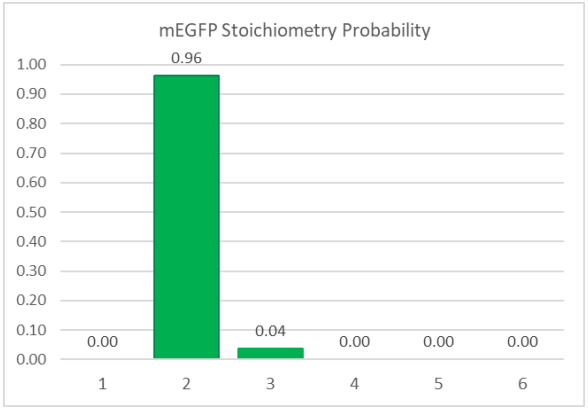
Since the marker is not necessarily expected to be a single unit like in the case of the RNA binding proteins, the export filter for the marker is selected to be valid steps from 1-4:



Based on labelling efficiencies and expected binomial distributions, the hetero-pentamer is composed of 3 mScarlet labelled components and 2 mEGFP labelled components:

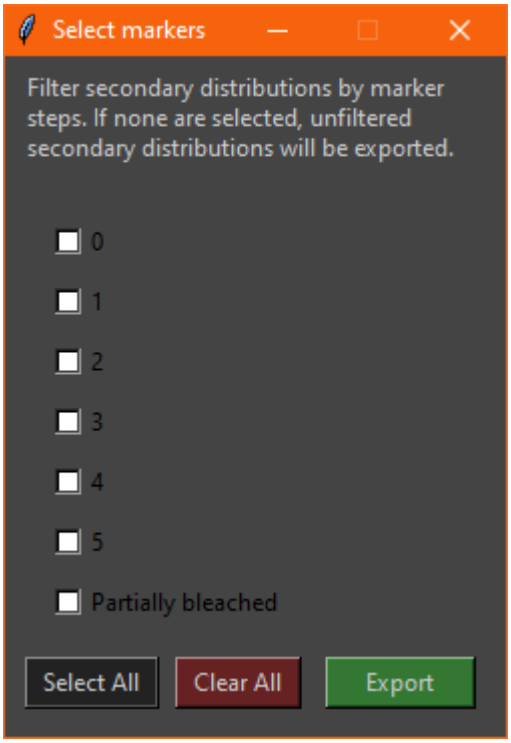




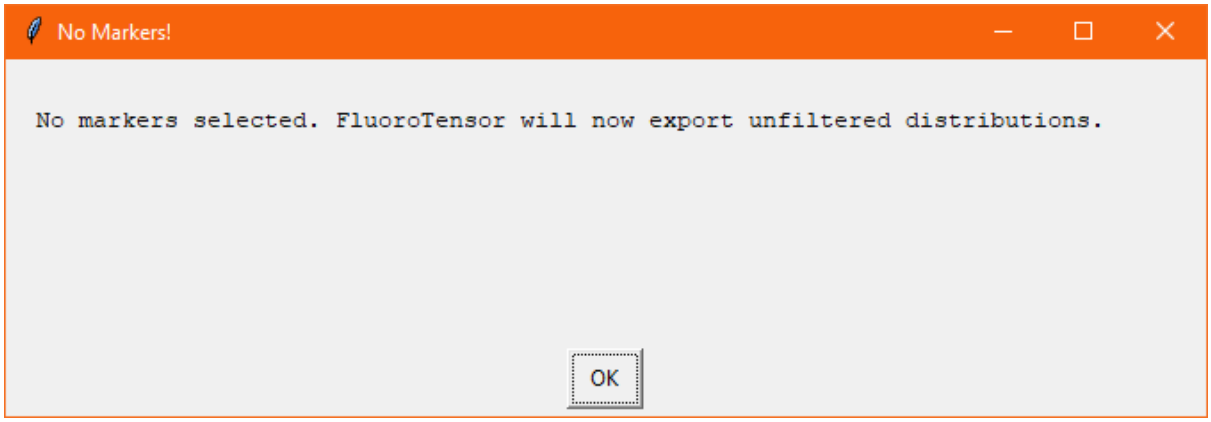


The data was then reanalysed in non-colocalized mode to observe the distribution and multimerization state of non-colocalized spots. Some of these will be multimers with only one of the components labelled where the other components are endogenous protein, invisible to the fluorescence microscope. Others may be incompletely assembled or dissociated multimers, single labelled proteins, or multimers of a single one of the two component proteins.

Once the automation run is complete, the steps are detected as before but when exported, no steps are selected and the filter is disabled since the spots are non-colocalized:



A message will appear, confirming this choice:

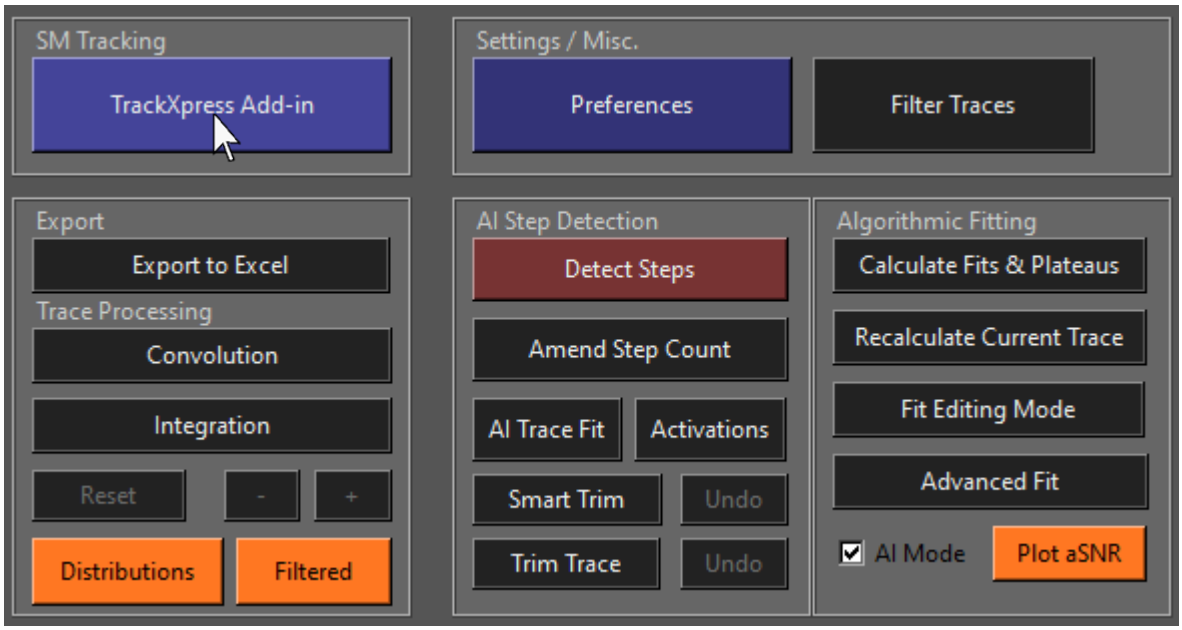


From the non-colocalized distributions and binomial analysis it appears as if the mScarlet labelled component exists in primarily dimeric or trimeric form with a small proportion of monomer, whereas the mEGFP labelled component exists in almost equal parts monomeric and dimeric forms, slightly in favour of monomeric.

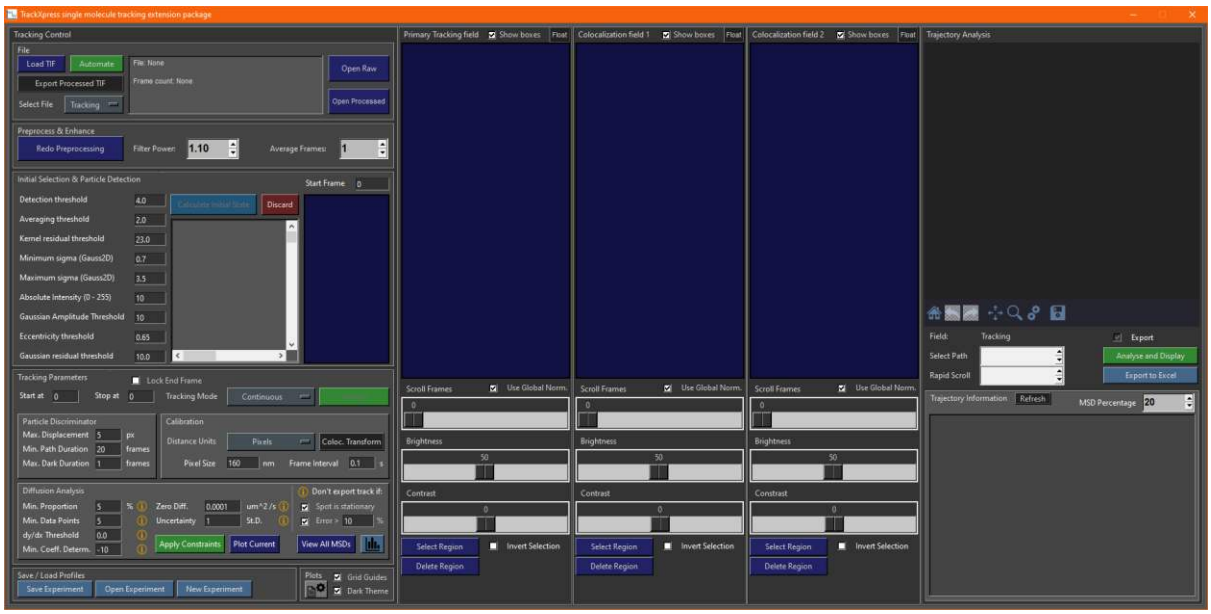
Chapter 8 – Tracking

8.1 Launching the TrackXpress Add-in

To launch the TrackXpress single molecule tracking add-in within FluoroTensor, click ‘TrackXpress Add-in’ under ‘SM Tracking’ in the main interface:

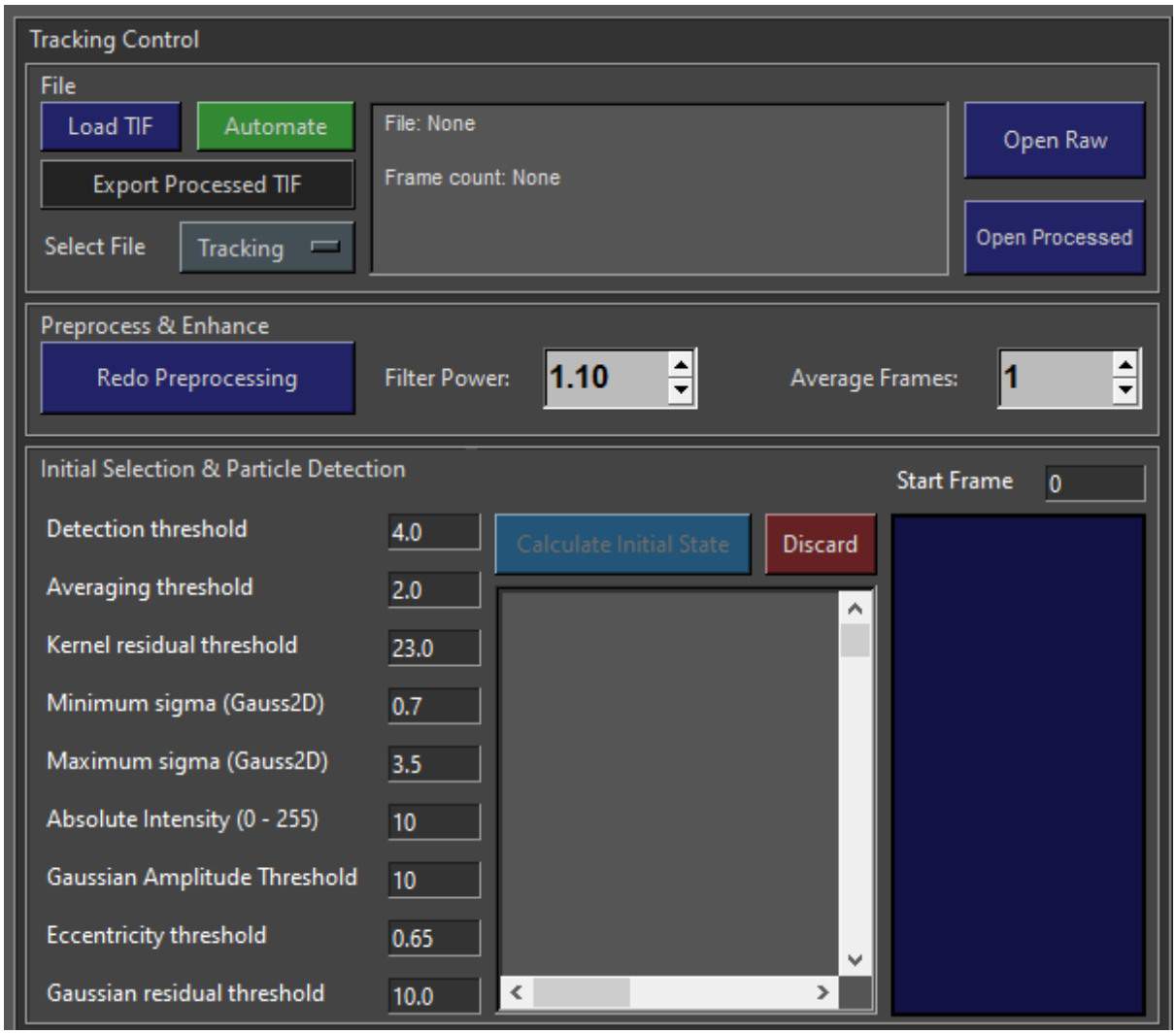


The tracking interface should then appear shortly:



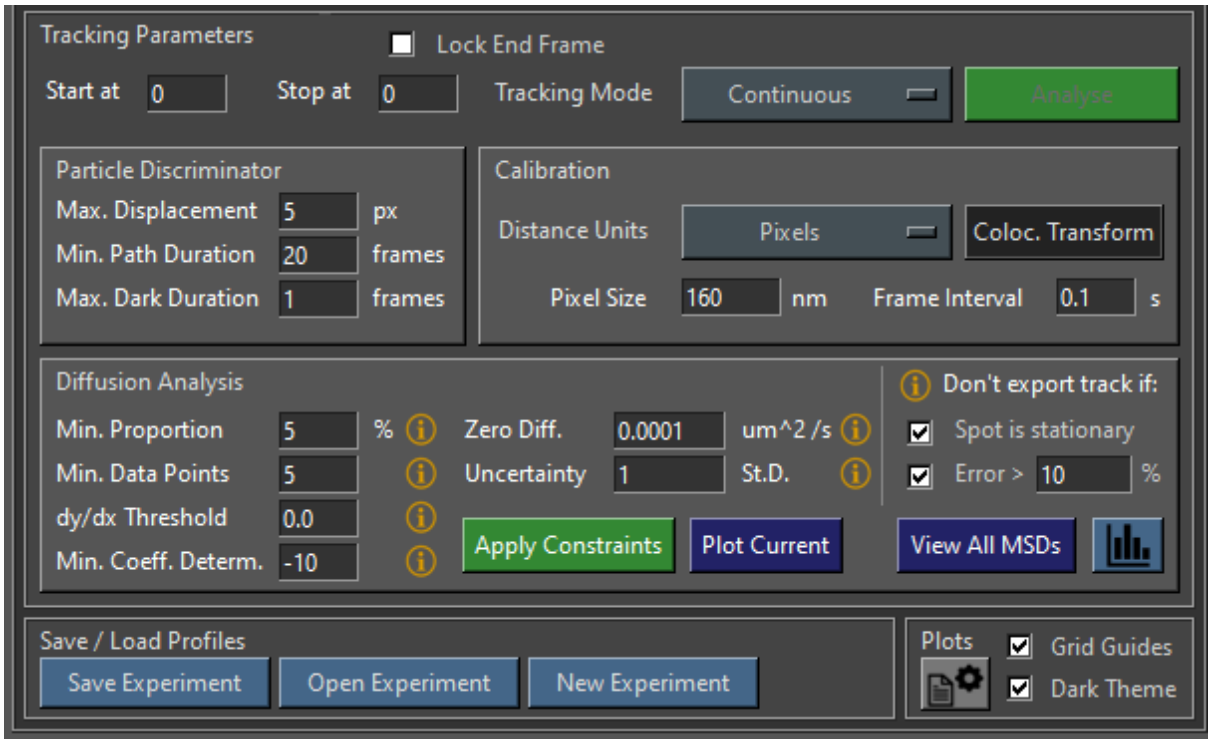
8.2 An Overview of the Tracking Interface

The left side of the interface is the control panel. At the top is the 'File' section where data can be loaded or saved, viewed, or automated. Below this is the 'Preprocess & Enhance' section for enhancing data before tracking. Next is 'Initial Selection & Particle Detection' which contains thresholds for particle detection:

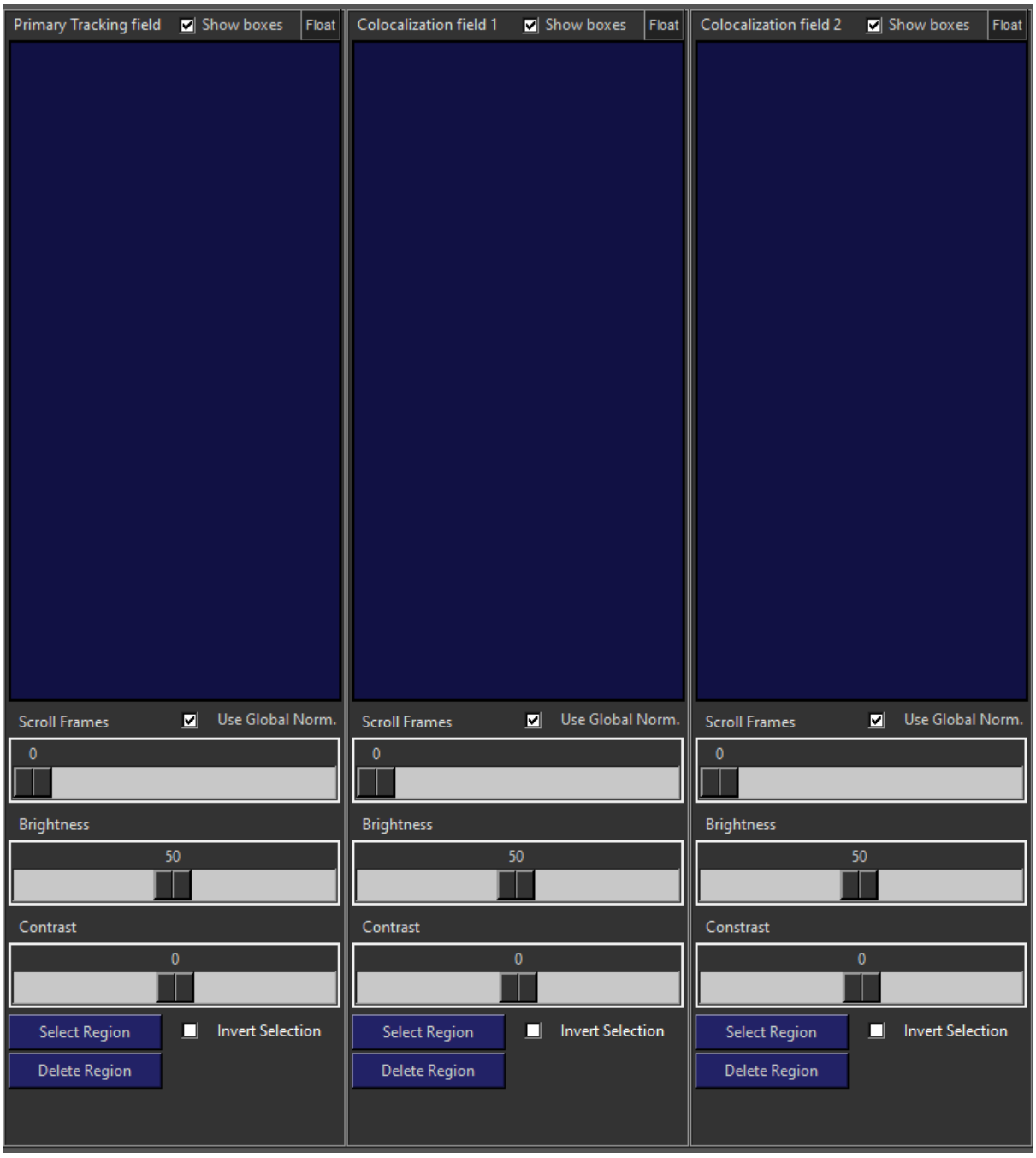


Underneath this is the ‘Tracking Parameters’ section where the settings for tracking are located. This include the particle discriminator which thresholds for minimum track length, maximum leap distance and maximum time in a dark state before discarding or terminating the track. ‘Calibration’ contains settings to do with setting the pixel size and length of a frame so distance and time measurements are correct for diffusion analysis. ‘Diffusion Analysis’ contains all of the settings and thresholds for fitting mean-square displacement (MSD) plots and filtering the tracks based on quality.

Underneath this are sections for saving and loading the tracking data and analyses as ‘Tracking Experiment’ (.txp) files and adjusting the font size and appearances of graphs in the interface.

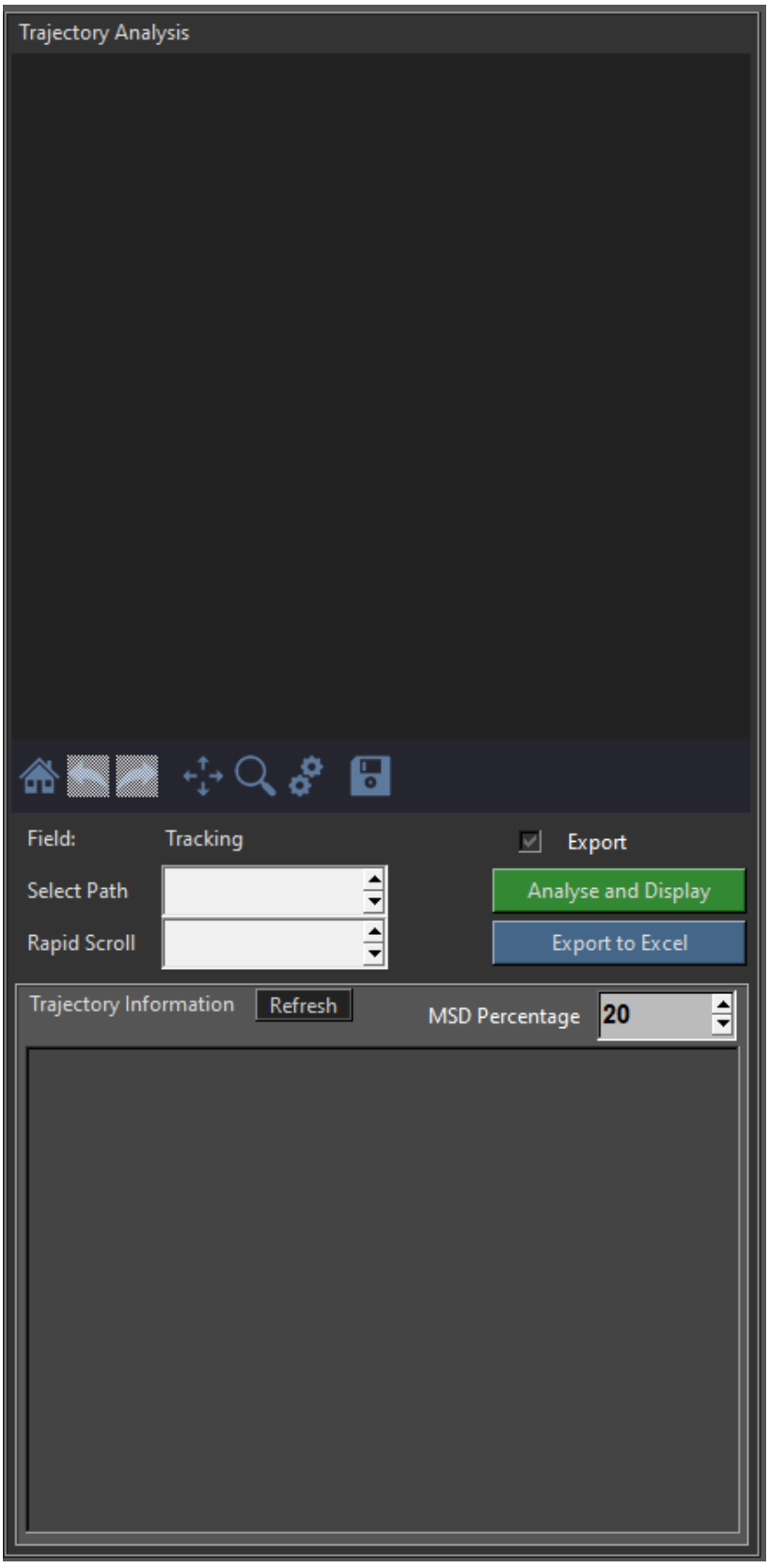


In the middle of the interface are the channels, each with a canvas where the data is displayed and settings such as brightness and contrast can be adjusted:



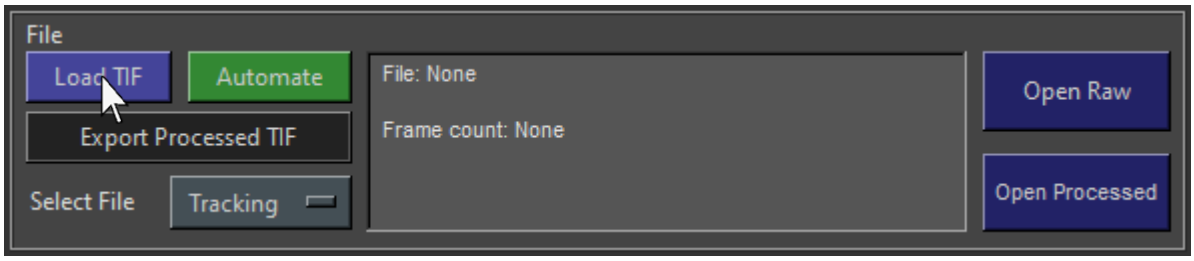
The first channel is the primary tracking field, the other two would be colocalization fields, however colocalized tracking has not yet been implemented in this version. Currently all three channels can be used for independent files at the same time.

On the right is a display area where tracks are plotted and information displayed. Time series plots can be viewed from here as well as the data exported to an Excel template.

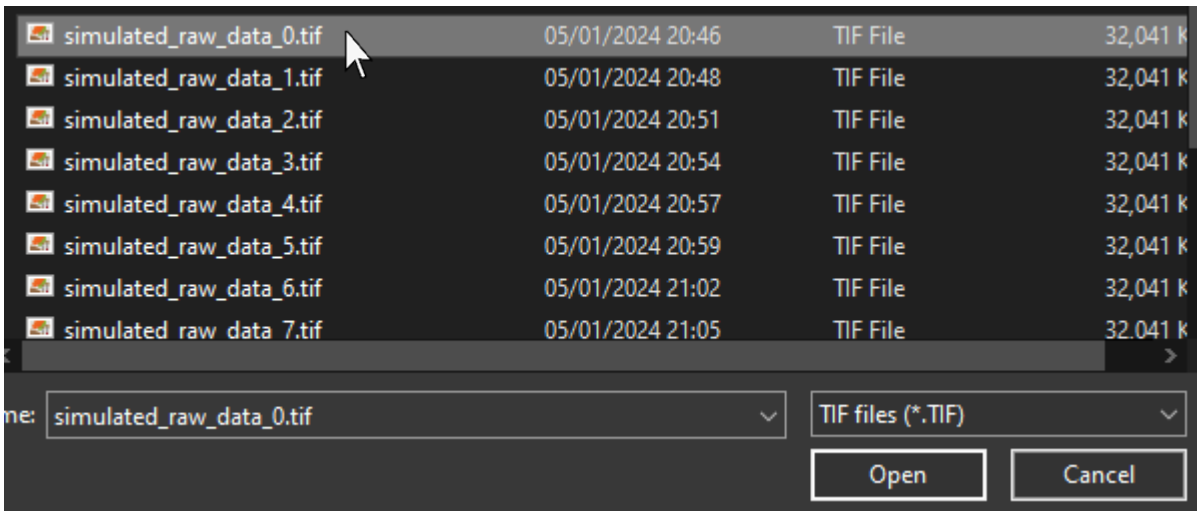


8.3 Loading a TIF File for Tracking

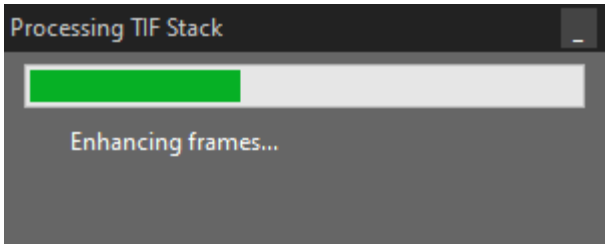
To load a .tif file, click ‘Load TIF’ in the ‘File’ section of the control panel:



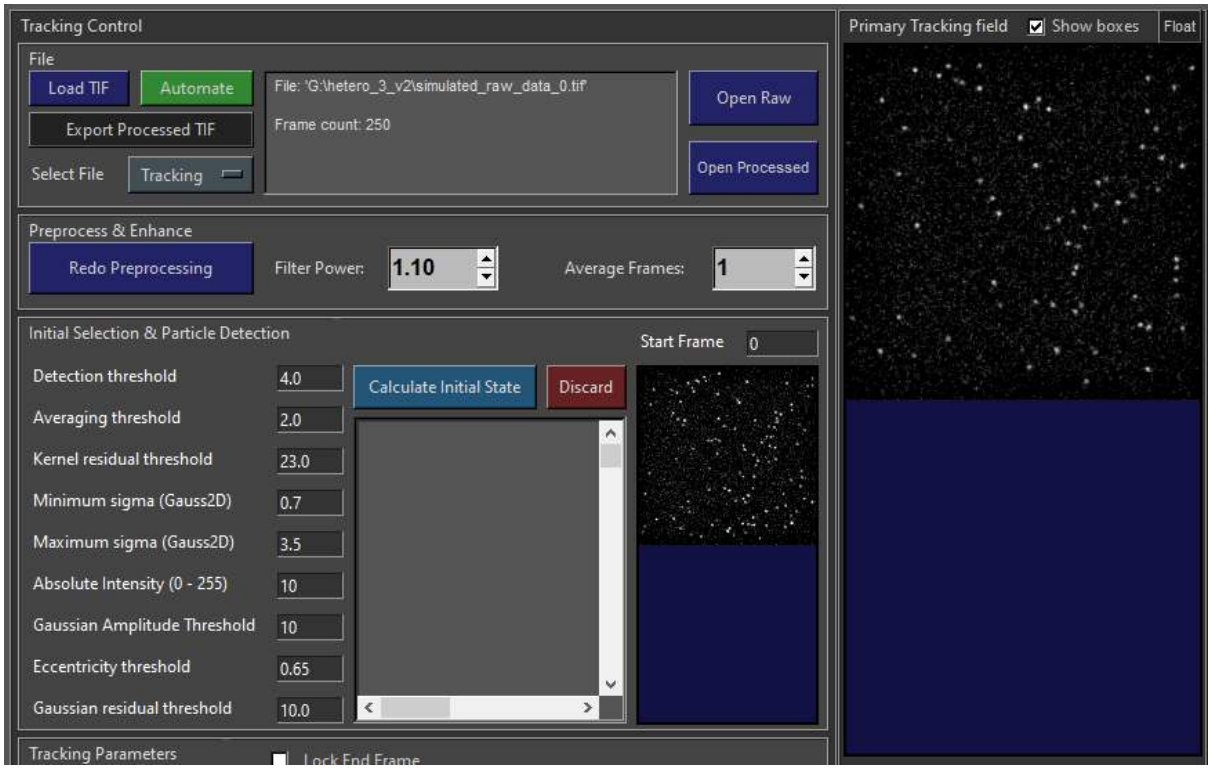
A file dialogue will then appear where you can choose a .tif file:



The file will then load and after a short time, a progress bar window will appear as the frames are enhanced:



Once the movie is loaded and enhanced, it will appear in the leftmost ('primary tracking field') channel:



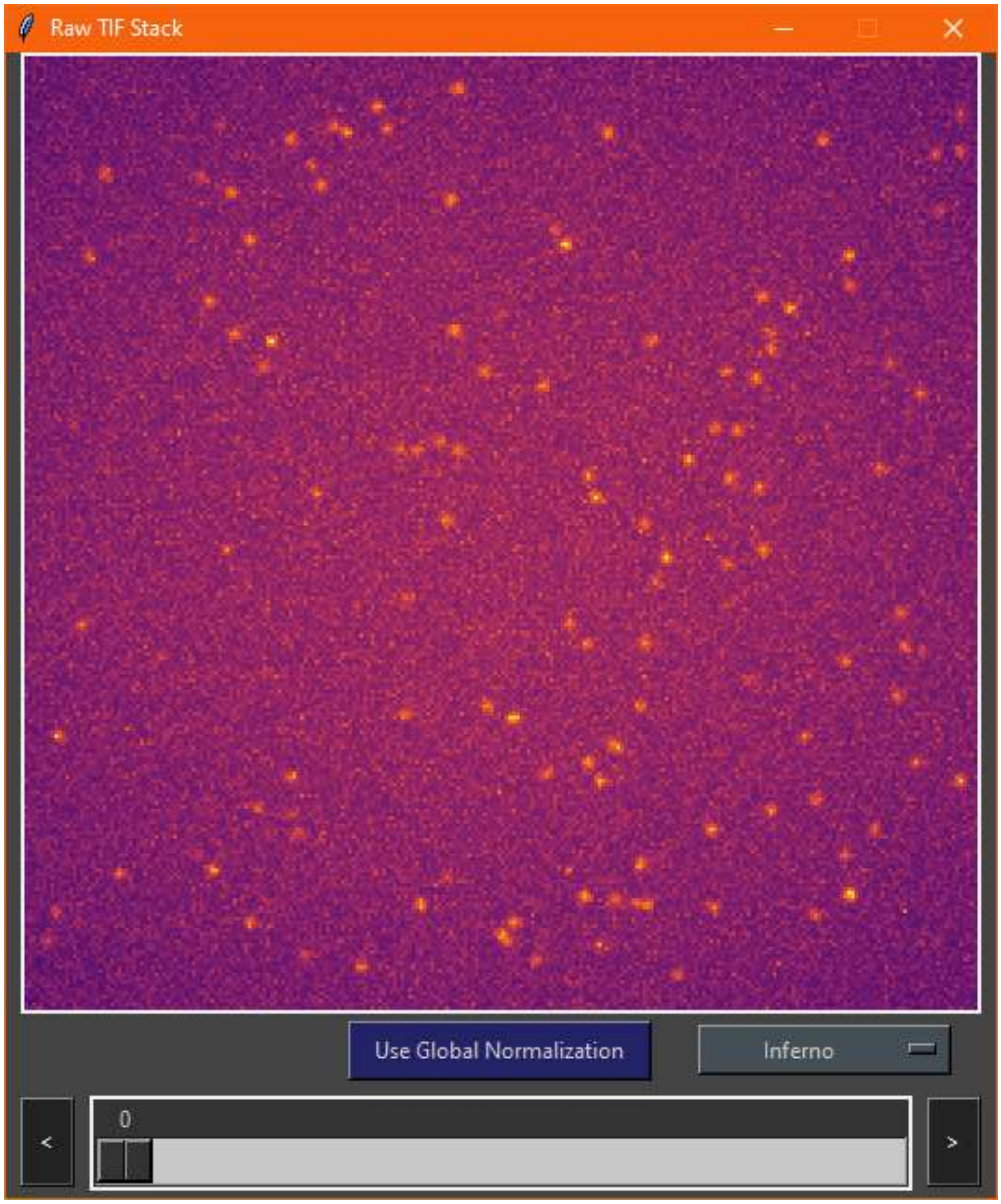
Unlike the colocalization program, any resolution is technically possible for the tracking program, but we don't recommend anything over 512x512 by 500 frames or 256x256 by 2000 frames since the analysis may take a very long time or even crash the program in the extreme. The canvas will only display up to 256x512 but can be lifted into a new window which will be explained later.

On the left, a frame of the movie is also displayed in the particle detection section. This can be used to aid in setting the spot detection thresholds correctly for tracking. The frame count and file path will be displayed in the info box in the file section at the top left of the control panel:

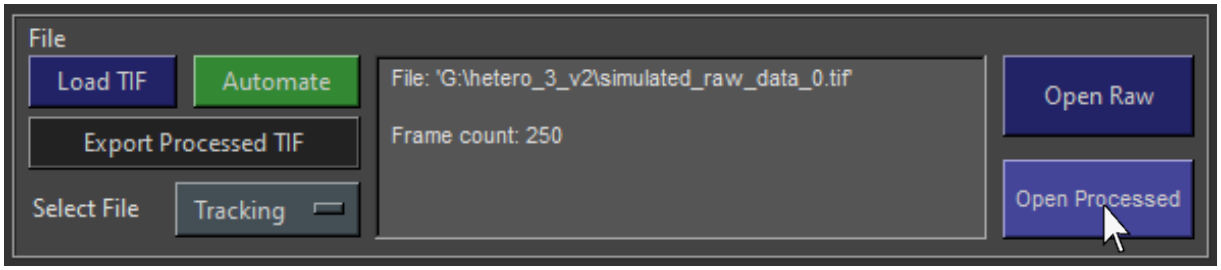
8.4 Viewing Raw and Processed Data

To view the raw unprocessed .tif stack, click ‘Open Raw’ in the file section of the control panel:

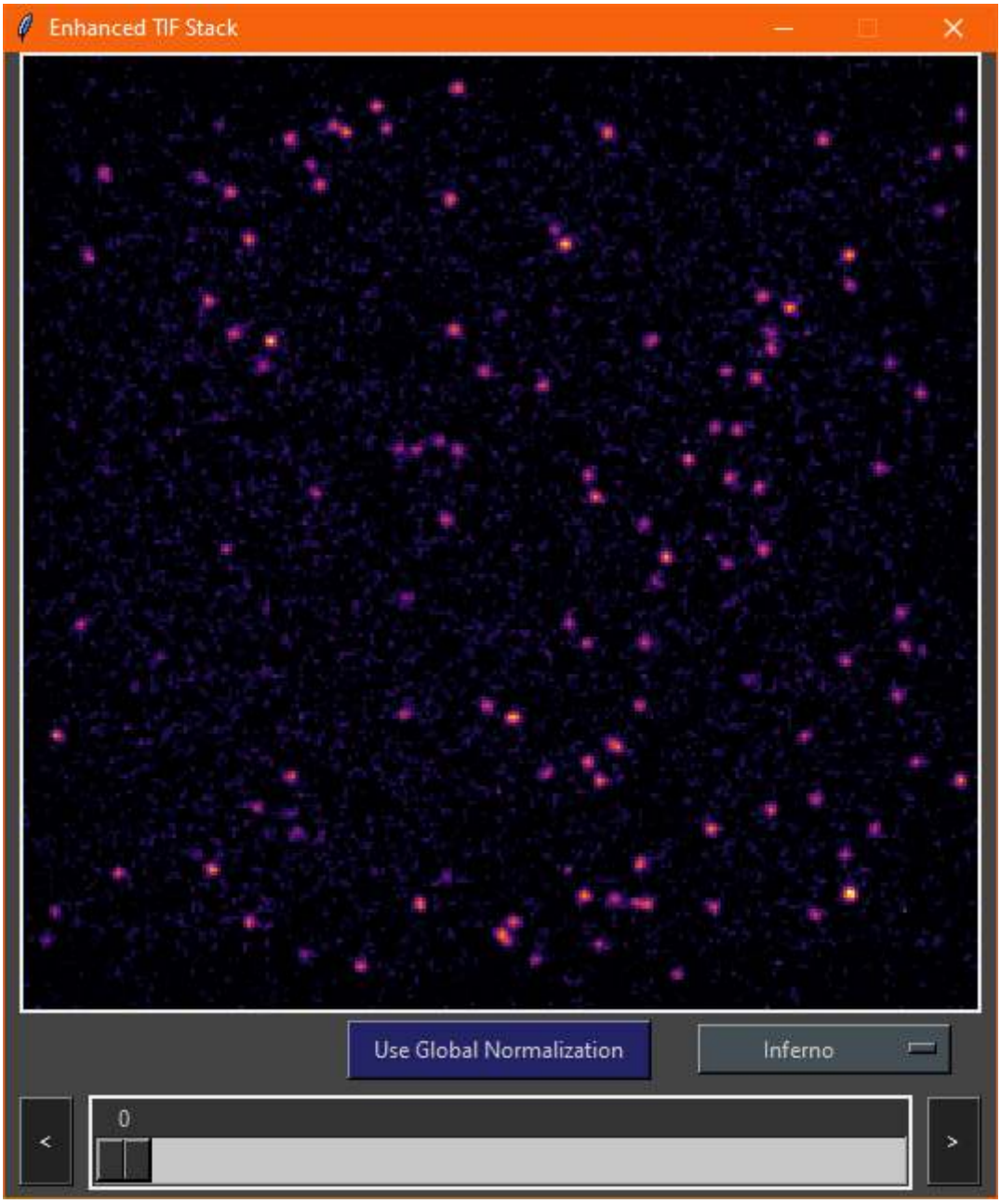
In the same way as in the colocalization a window will appear where you can scroll through each frame of the data one by one or move to a specific frame:



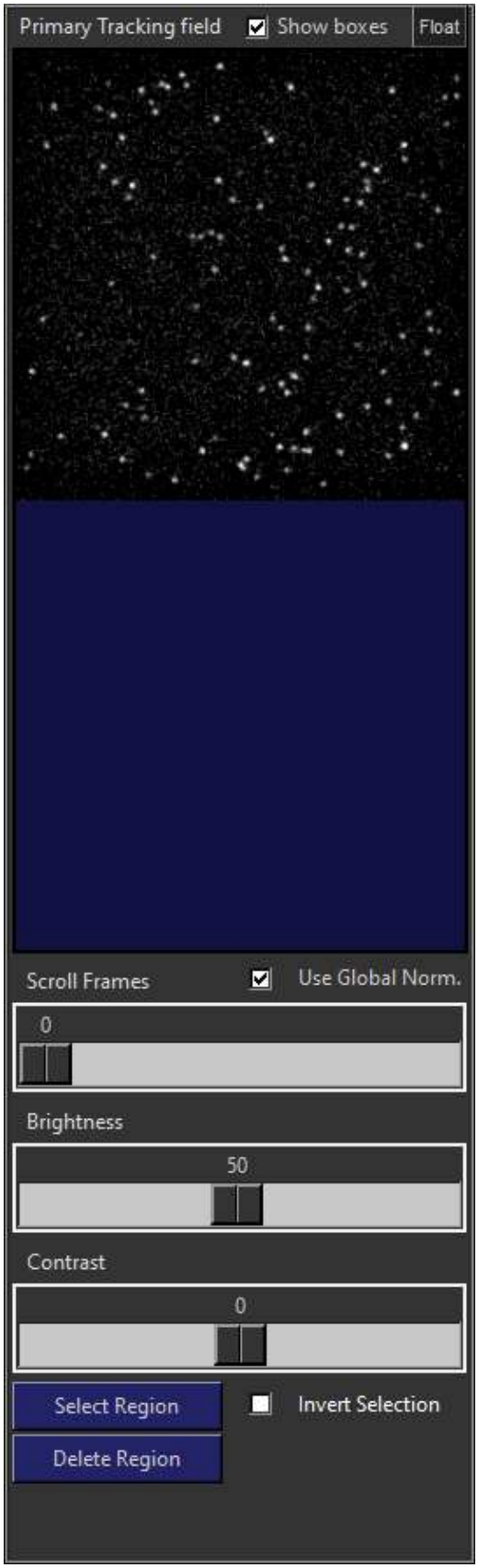
The same can be done for the enhanced stack. Click ‘Open Processed’ in the file section:



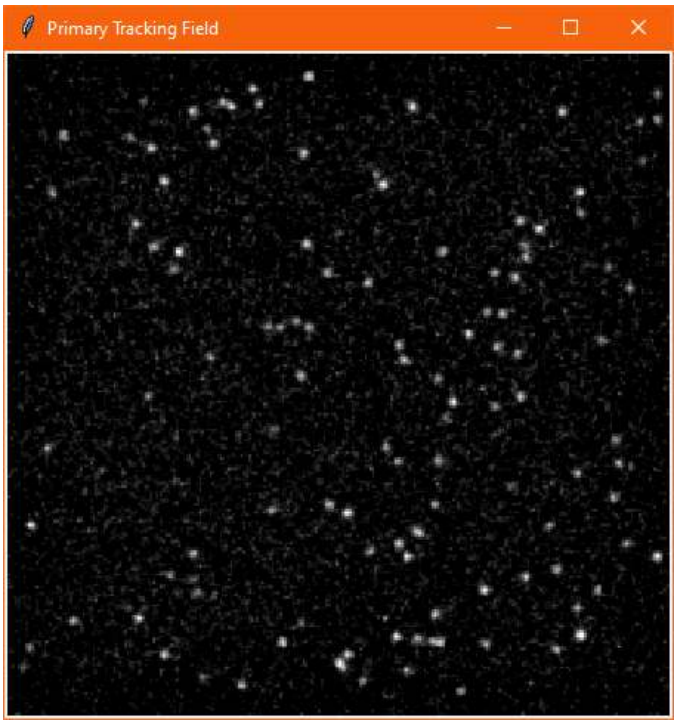
Both the raw and enhanced stacks can be displayed at the same time for comparison.



8.5 An Overview of a Channel

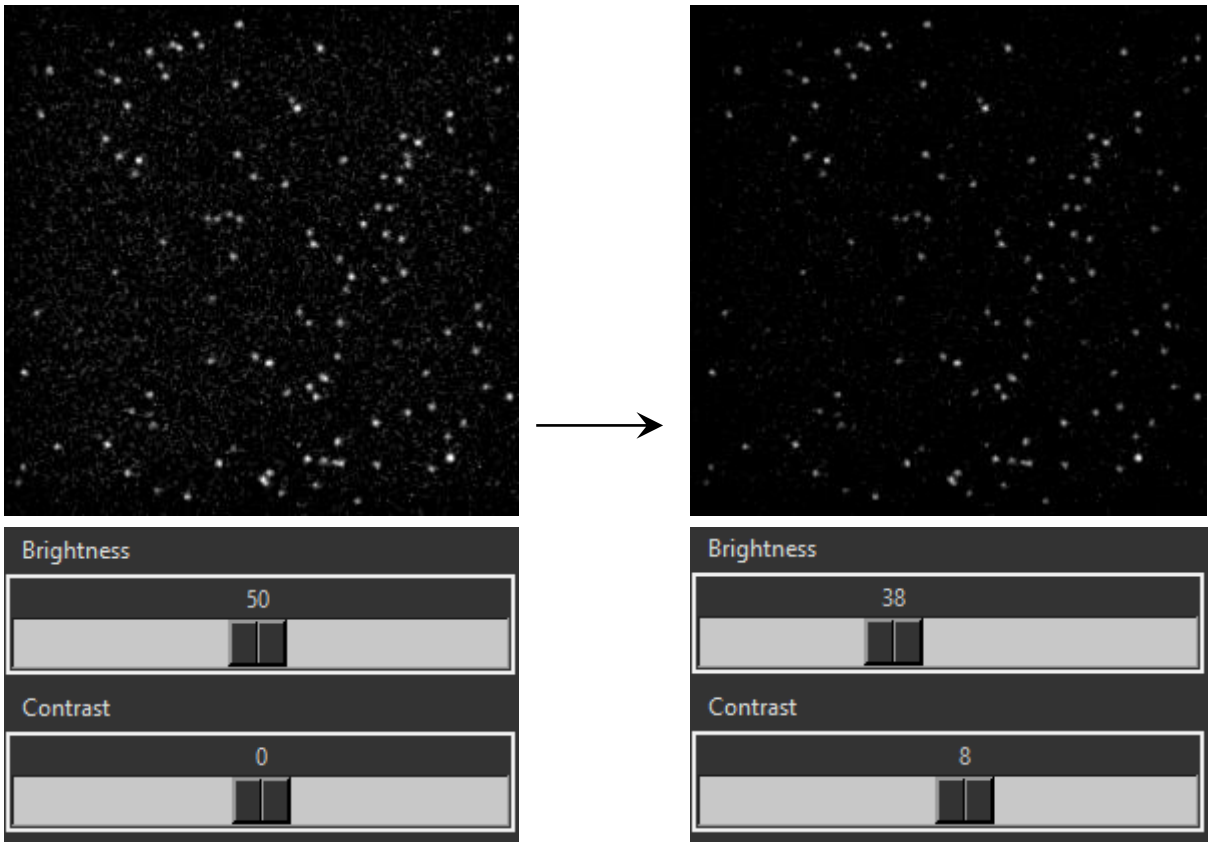


At the top is the channel label, in this case the 'Primary Tracking Field'. 'Show boxes' toggles whether to display an overlay of boxes around the tracked spots. 'Float' opens a new window and displays the movie in an enlarged canvas at 1.75x magnification in that window:



Global normalization normalizes the entire movie with respect to the brightest frame, instead of normalizing the current frame with respect to the brightest pixel within it. Global normalization should always be enabled for tracking.

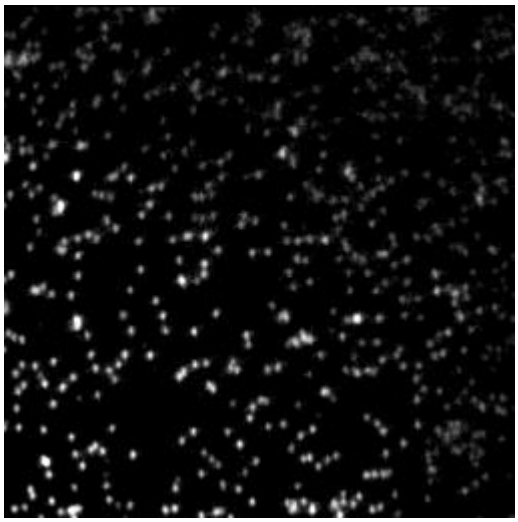
Global normalization, brightness, and contrast are all parameters which affect tracking. The brightness and contrast controls can be used as additional enhancement to try and remove spurious noise left over after high-pass filtering. Care should be taken not to over-adjust the contrast as weak signals may be lost and spots will begin to flicker. Increased variance in fitting parameters due to flickering may result in poor results when tracked.



Shown above is an example of a good enhancement using brightness and contrast for this file. Note that this will vary depending on your setup. Signal intensity and noise strength within the image will affect this. Generally, the better the enhancement and the cleaner the image, the more computationally efficient tracking analysis will become since many fewer false positives will be attempted to be fitted.

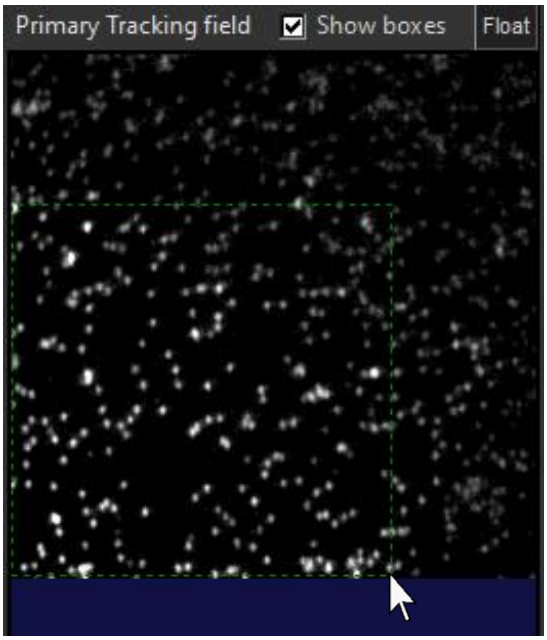
8.6 Selecting a Custom Analysis Region

Sometimes you may wish not to track the entire field of view but only part of it. For example, optical aberrations or poor focus at the edge of the field of view will be tracked, to the detriment of the analysis:



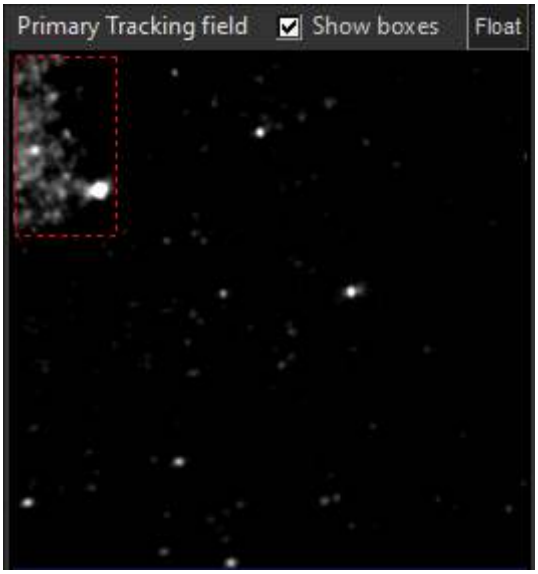
To only analyse the high-quality portion of the field of view, click 'Select Region' at the bottom of the channel to toggle on selection mode:

Then, click and drag the cursor over the area you wish to select:



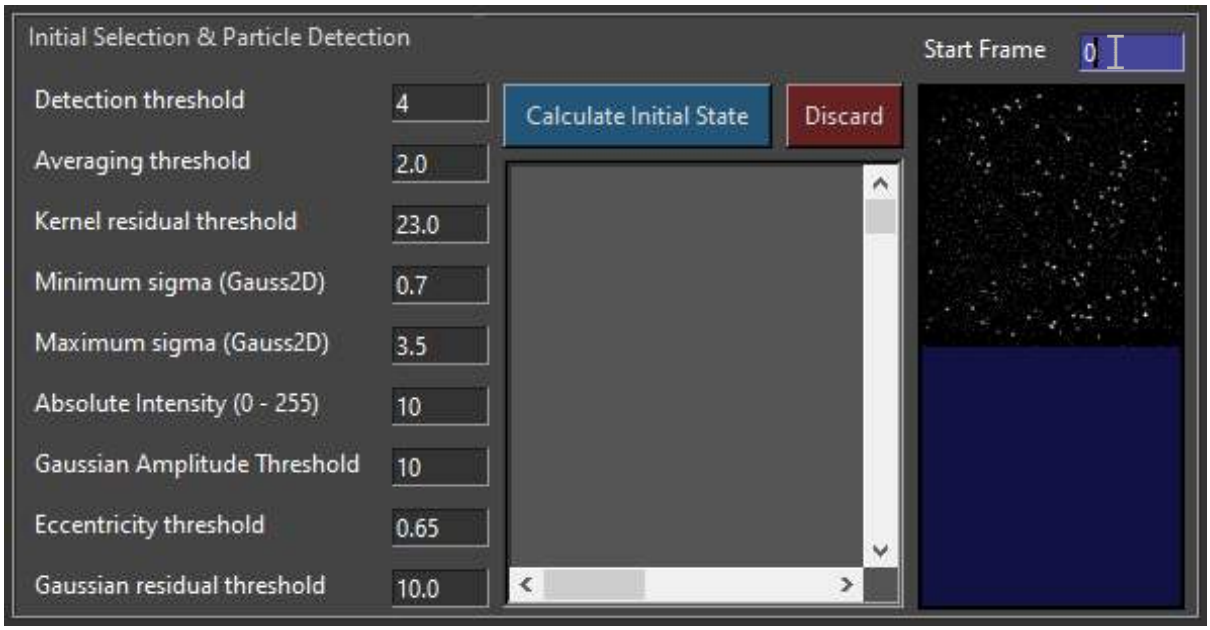
By default, the selection box will be shown in green. This means that the region within the selection will be analysed. If instead a specific region should be excluded, tick the 'Invert selection checkbox and the region within the selection will be ignored and the box will appear red:

For example, in this file there is a patch of aggregated fluorescent material which can be excluded like so:

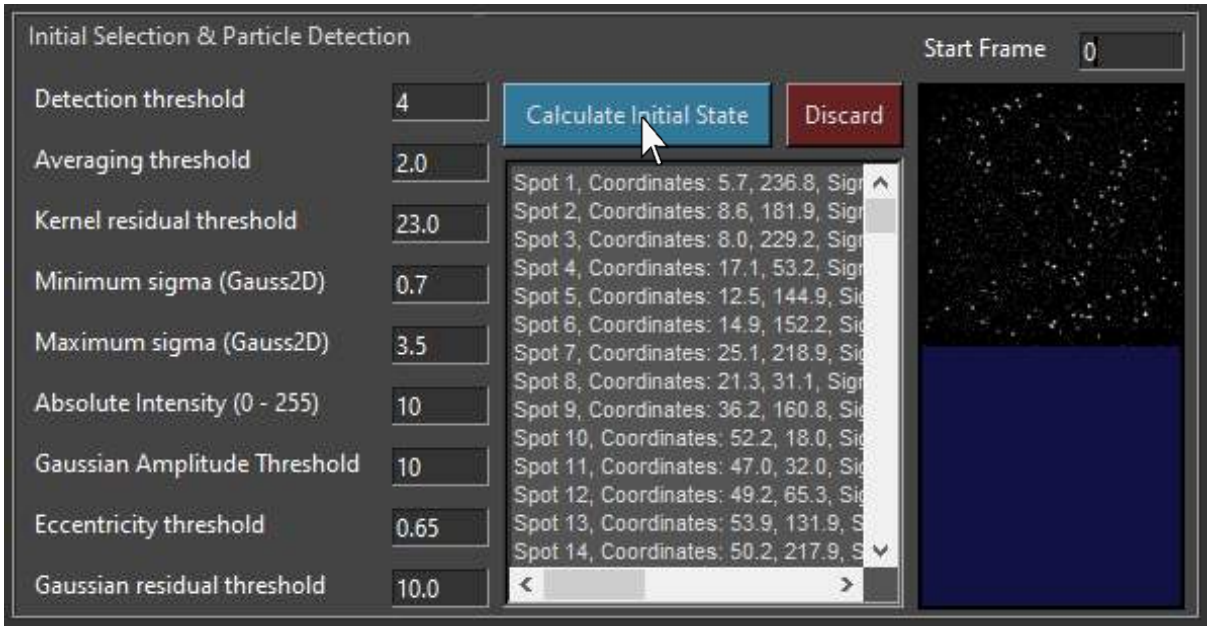


8.7 Setting Up Particle Detection Parameters

First, set the start frame in the top right of the 'Initial selection & Particle Detection' section to the first frame of your data:

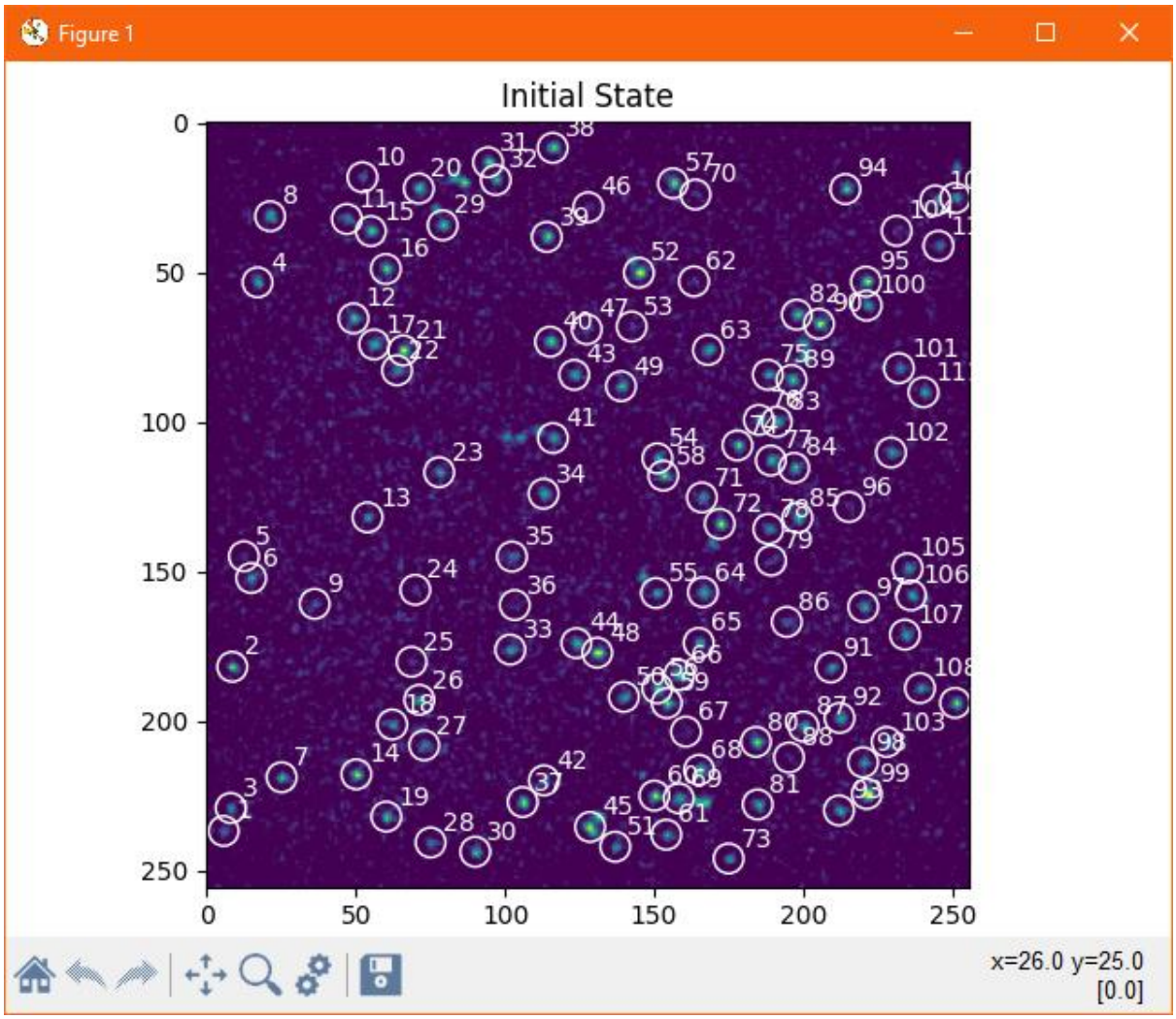


Then, click 'Calculate Initial State':

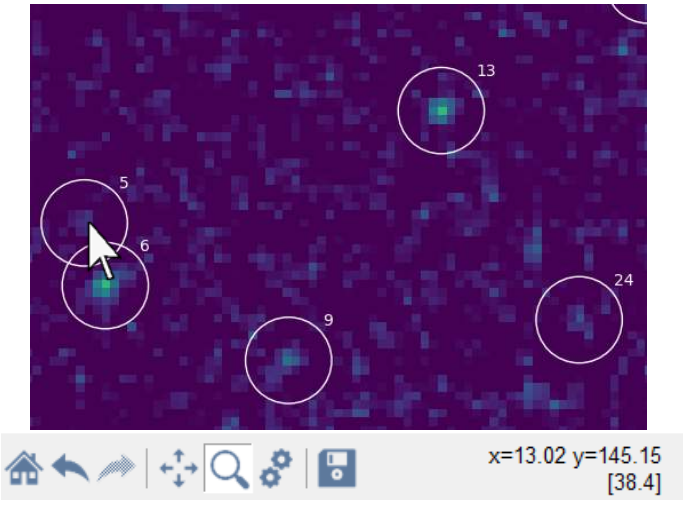


The coordinates and fitting parameters of the spots found in that frame will be displayed in the scroll box beneath, this can be highlighted and copied to the clipboard via CTRL – C.

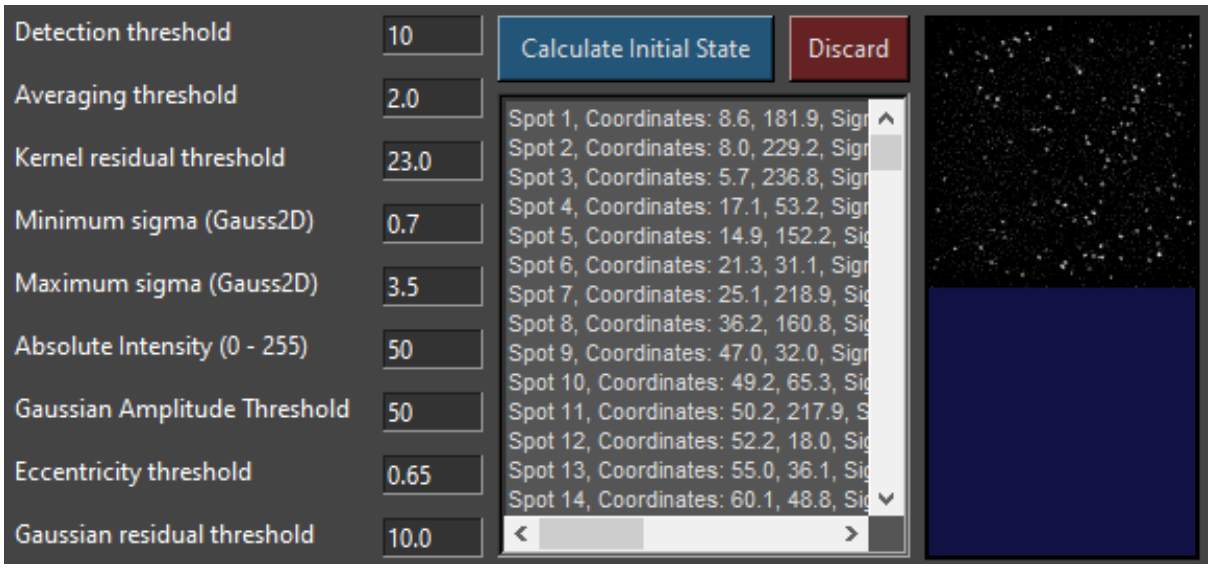
In addition, a window will pop up showing the fluorescent signals that were detected in that frame:

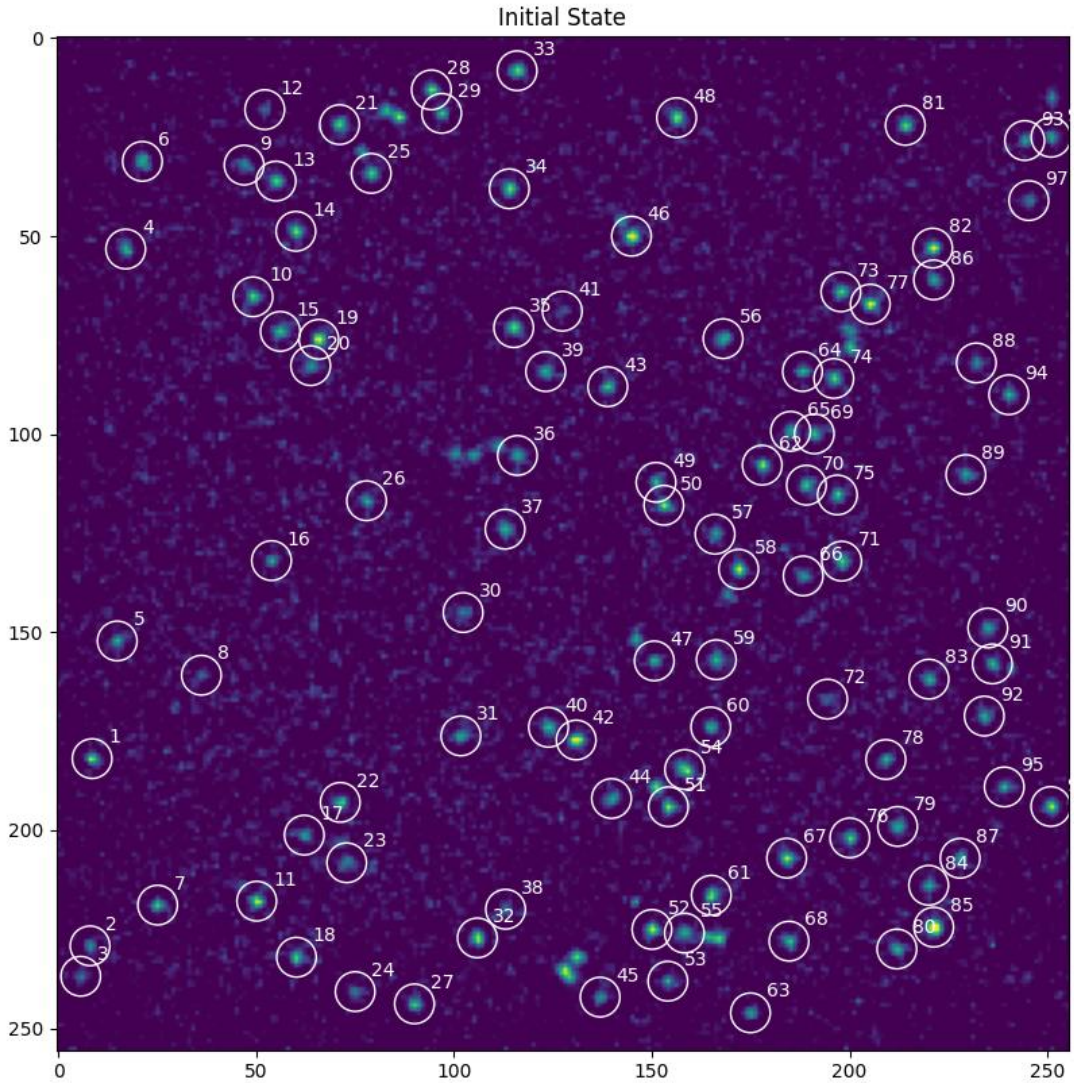


Zooming in and hovering the cursor over a pixel will give its coordinates and intensity in the bottom right corner of the plot toolbar:



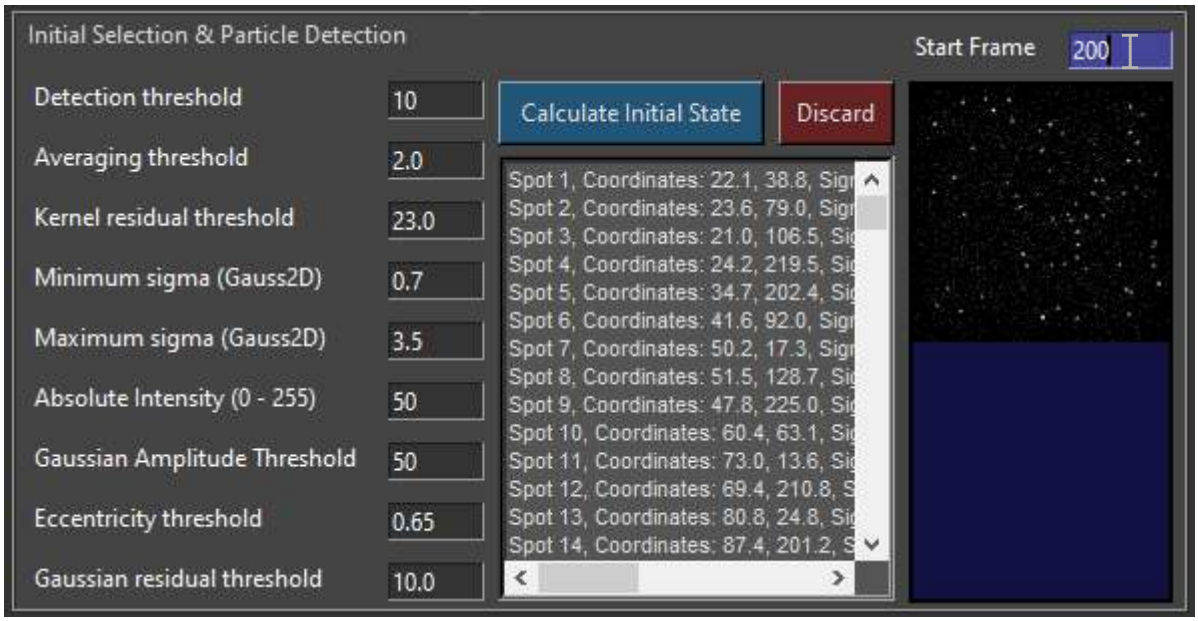
Close the window and adjust the thresholds until spurious noise is no longer detected as particles without losing actual fluorescent signals when the initial state is recalculated:



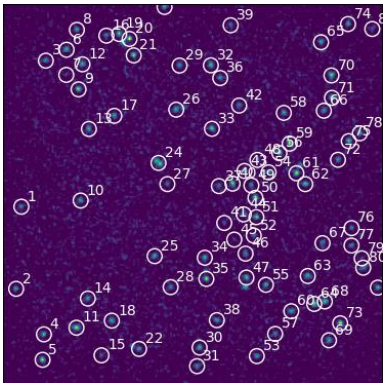


This will vary between experimental setups, so copying the numbers in the example is unlikely to work. The detection threshold, absolute intensity, and Gaussian amplitude are the main settings that should be changed for this. The other settings work well to ensure that very close or coalescing spots will be ignored.

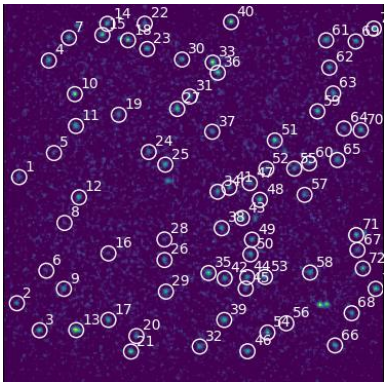
Next, it is important to check that these parameters work consistently throughout the movie. Set the frame to some point in the middle or towards the end of the movie:



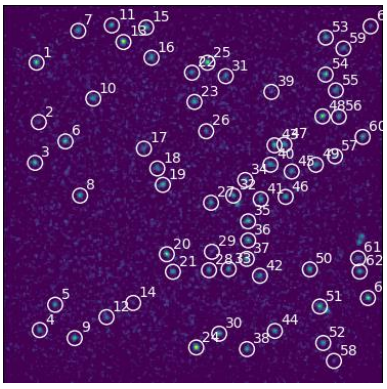
Once it has been verified that the parameters select spots consistently throughout the file, tracking settings can be adjusted; this will be explained in the next section.



Frame 100



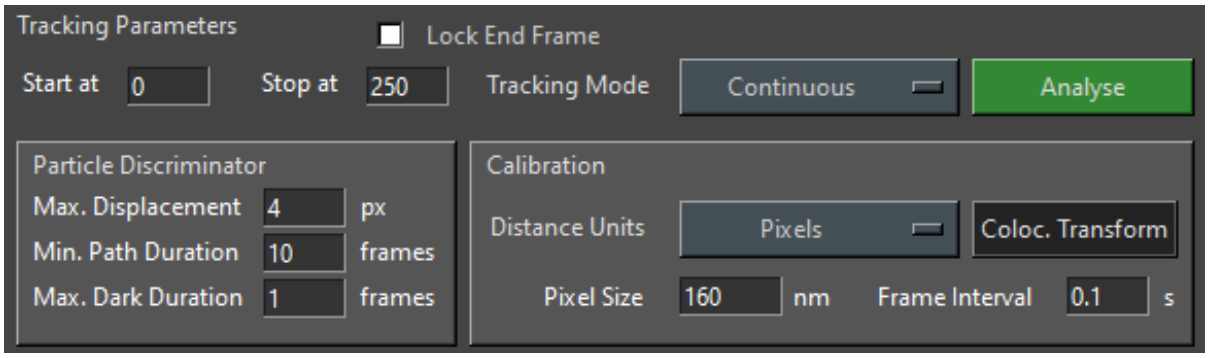
Frame 150



Frame 200

8.8 Setting Up Tracking Parameters

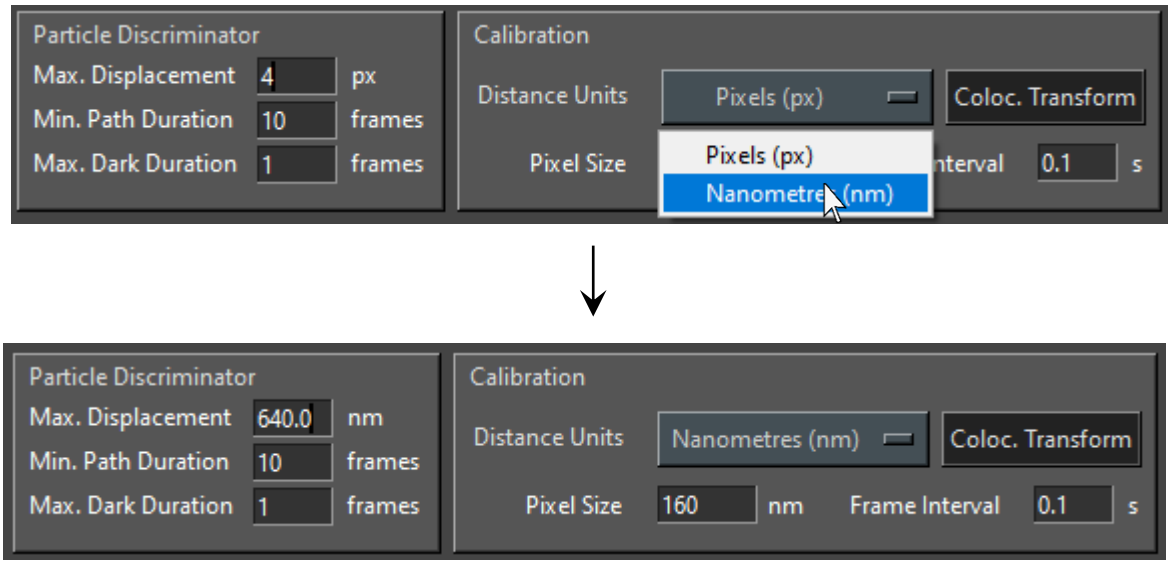
Once the parameters that govern spot detection have been adjusted, the same needs to be done for the parameters that govern how motion is tracked from frame to frame.



At the top, enter the frame range you wish to track. By default, this will be the first frame all the way to the end of the movie.

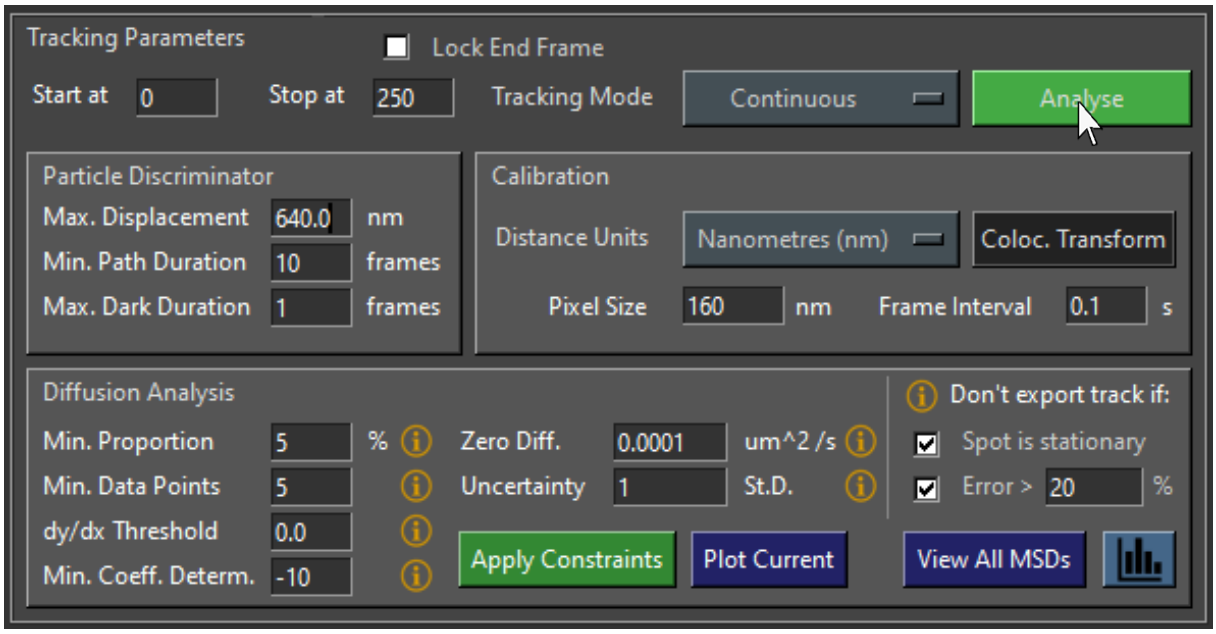
'Max. Displacement' is the maximum distance a particle can 'hop' in a single frame to be considered the same object in a different location in the next frame. 'Min. Path Duration' is the shortest track length in frames. 'Max. Dark Duration' is the maximum amount of time in frames for which a fluorescence signal can disappear for it to be considered the same object when it reappears. To the right, the pixel size and frame interval must be set according to the experimental setup and acquisition.

Using the drop-down menu, it is possible to change the distance units from pixels to nanometres for the 'Max Displacement' property:

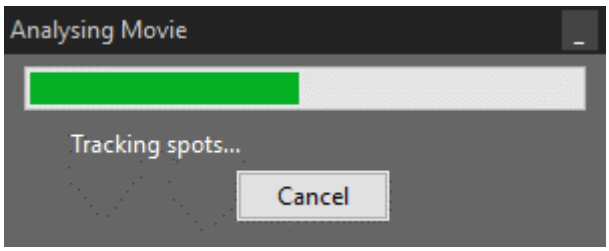


8.9 Tracking

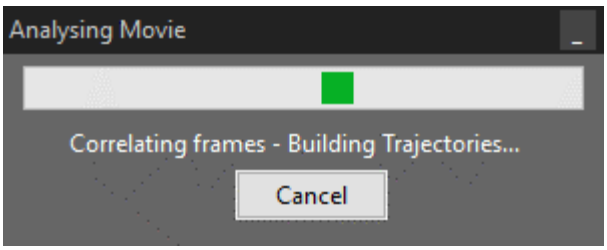
Once all the necessary parameters have been set appropriately, the data can be tracked. To begin tracking single molecule motion, click 'Analyse':



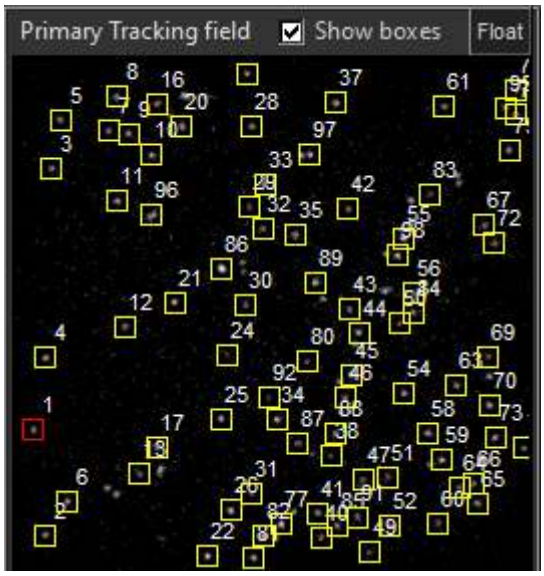
The program will then begin tracking and a progress bar will be displayed. This process may take some time depending on the size of the file and the number of spots. First the program will try to detect molecules in every frame:



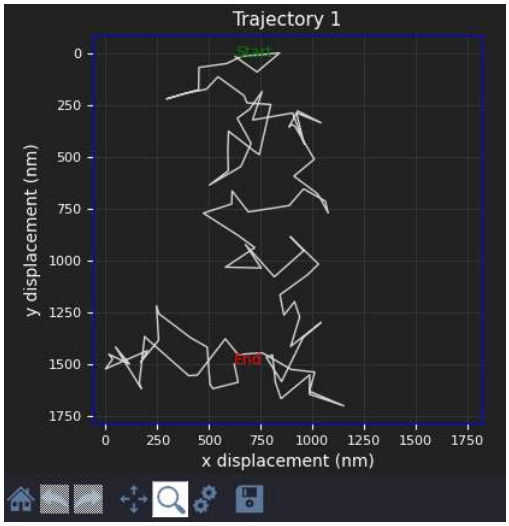
Then it will connect paths between the detected spots:

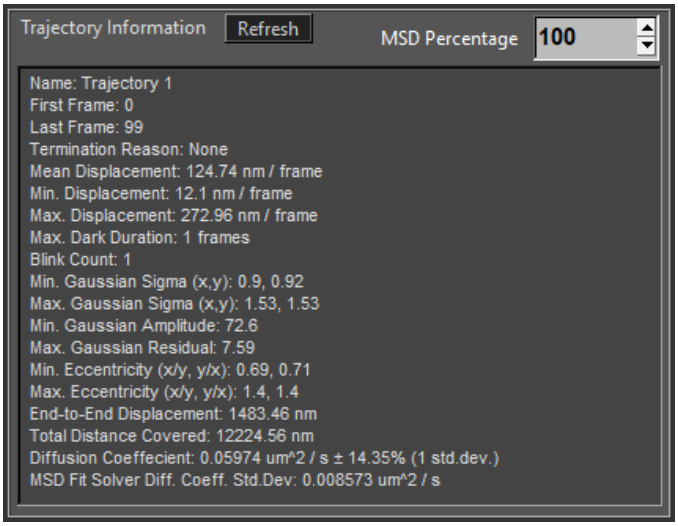


After this process has completed, spots that have been tracked will be outlined by yellow boxes. The currently selected track will be outlined by a red box:



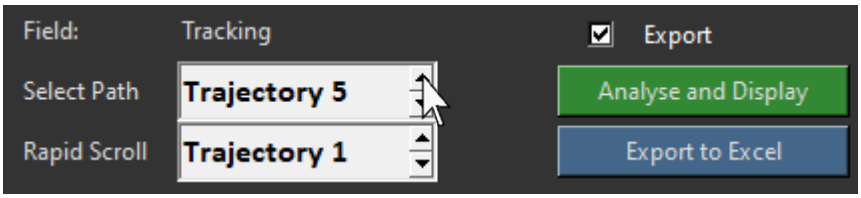
On the right of the interface, the currently selected track will be plotted and the info box below will contain information about the track:



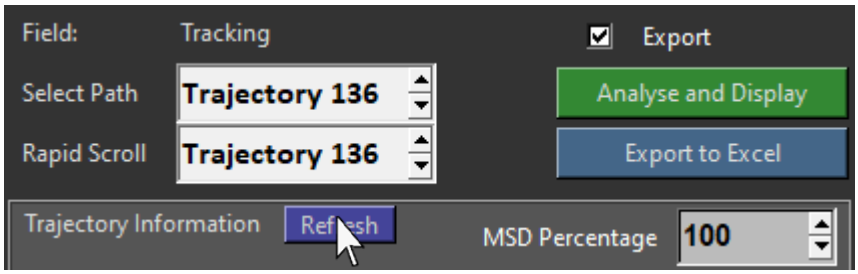


8.10 Navigating Through Tracks and Visualizing Time Series

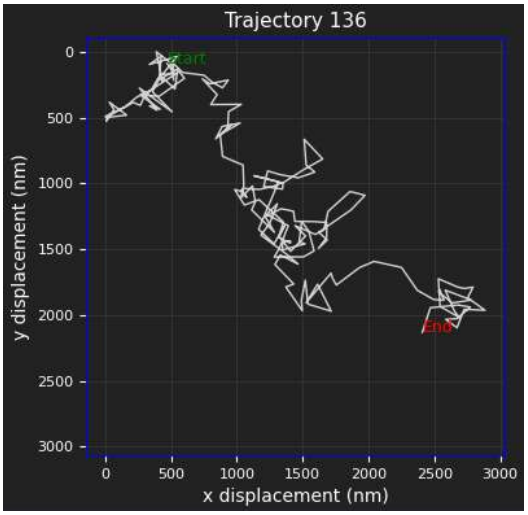
There are a couple of ways to select a track and display its trajectory and information. The first is to scroll through tracks in order using the 'Select Path' spin box:

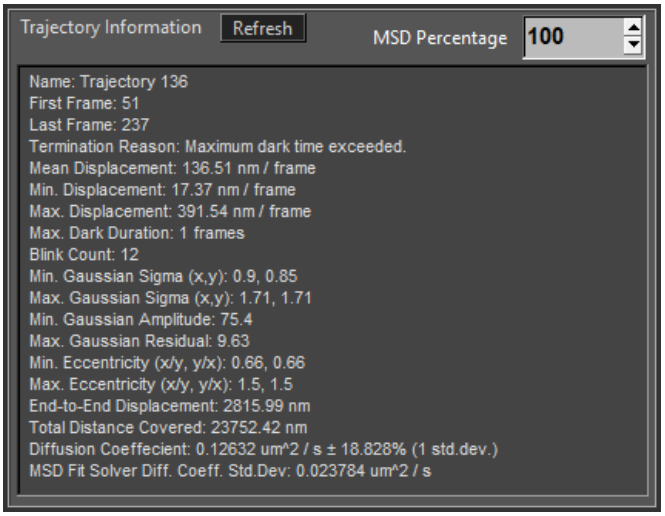


The 'Select Path' spin box changes the selected path and updates the plotted trajectory. Since this is a slow process, it may be more convenient to rapidly scroll to a specific track number. Use rapid scroll to quickly reach the track to plot, this will not update the information immediately when the value in the spin box changes, instead click 'Refresh' to update the trajectory plot and information:

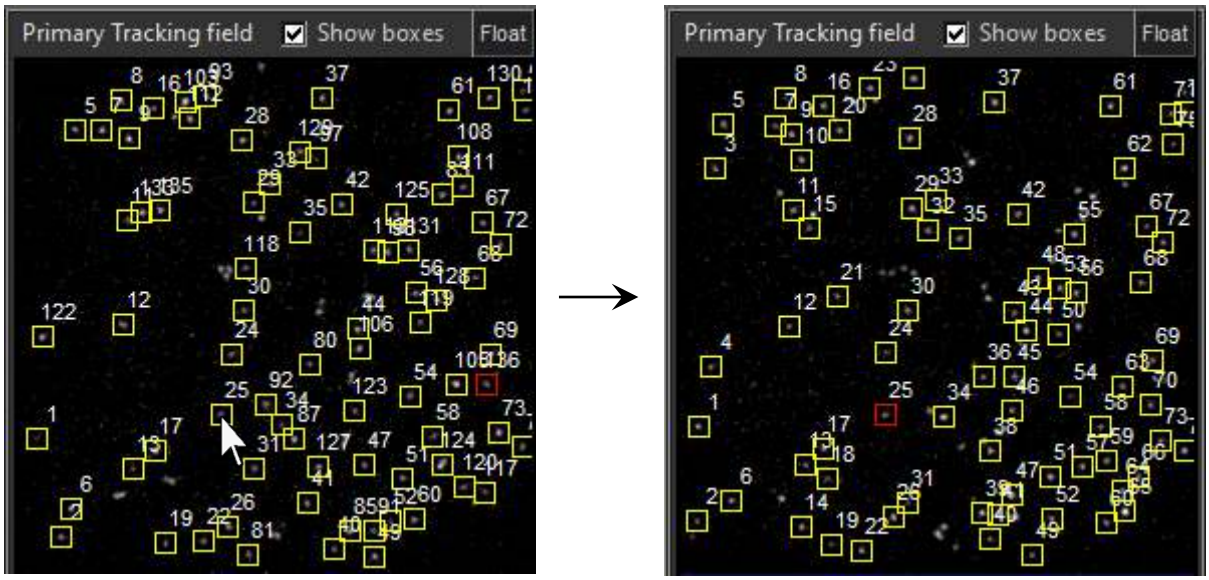


The selected track will then be displayed:

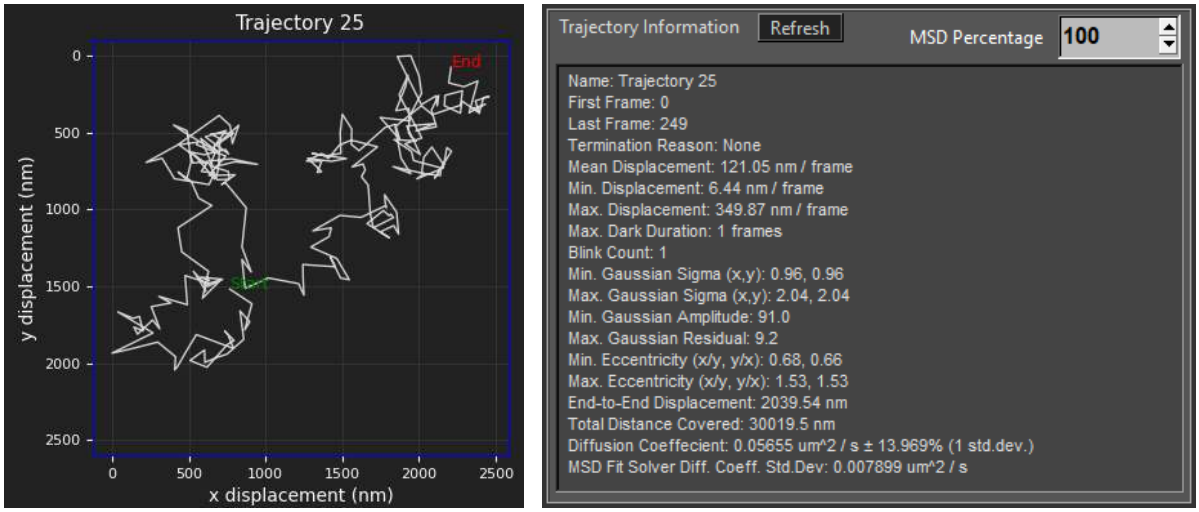




Alternatively, a spot in the canvas can be selected. Click a spot in the canvas to display its track and information:

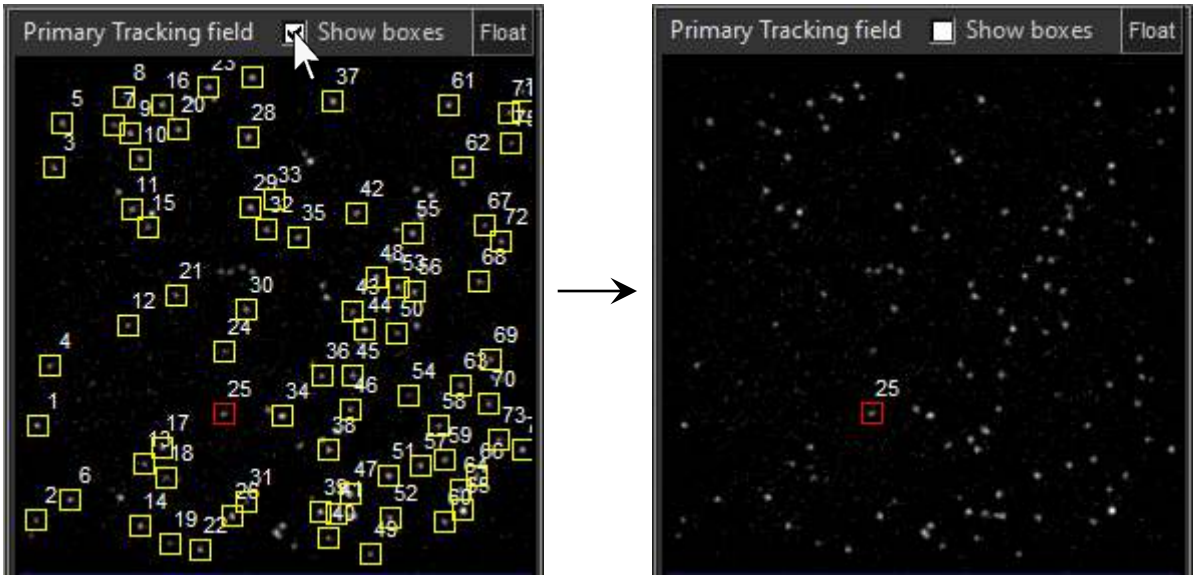


The frame slider will be automatically set to the first frame of the selected track and its information will be displayed on the right:

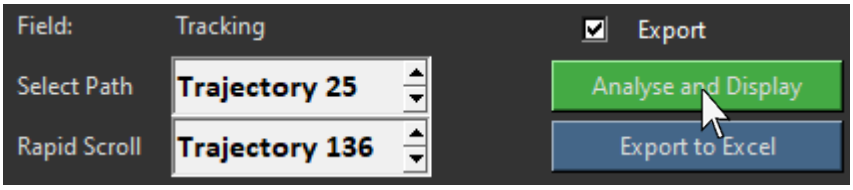


Track information is calculated ‘just in time’ from the coordinates each time it is displayed and when it is exported rather than during tracking. This means that the pixel size and time step can be updated without needing to re-track the movie and MSDs / diffusion coefficients can be dynamically updated in real time.

Sometimes, with all of the boxes and numbers, the canvas can become very cluttered, especially when the spot density is high. To declutter the canvas, untick the 'Show boxes' checkbox at the top of the canvas, and only the currently selected track will be outlined:



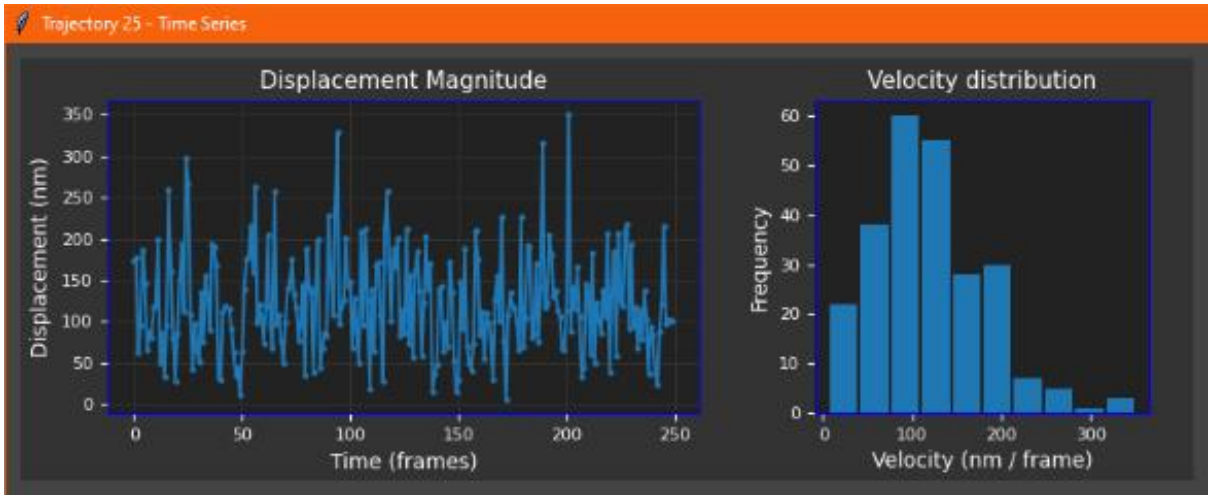
To display time series information including fitting parameters in each frame, velocity distributions and the fluorescence intensity trace, click 'Analyse & Display':



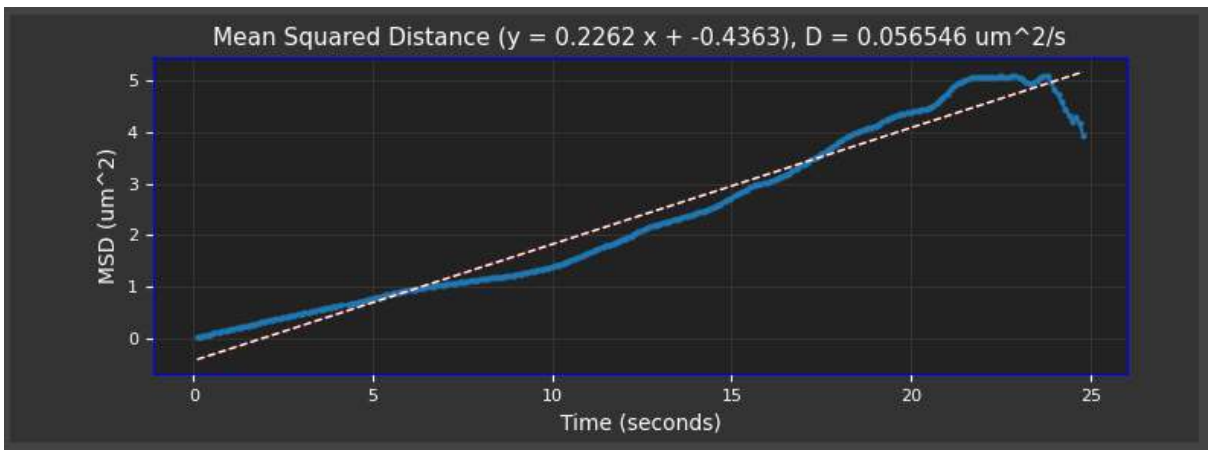
A window will then appear displaying time series, these are also calculated 'just in time' and can therefore be dynamically updated if the calibration is corrected:

8.11 Time Series

In the time series window are a number of plots relating to properties of the current track. In the top left the displacement, or 'hop' distance for each frame is plotted. To the right of this is a histogram showing the velocity distribution per frame, which is in essence a histogram of the displacement magnitude series:

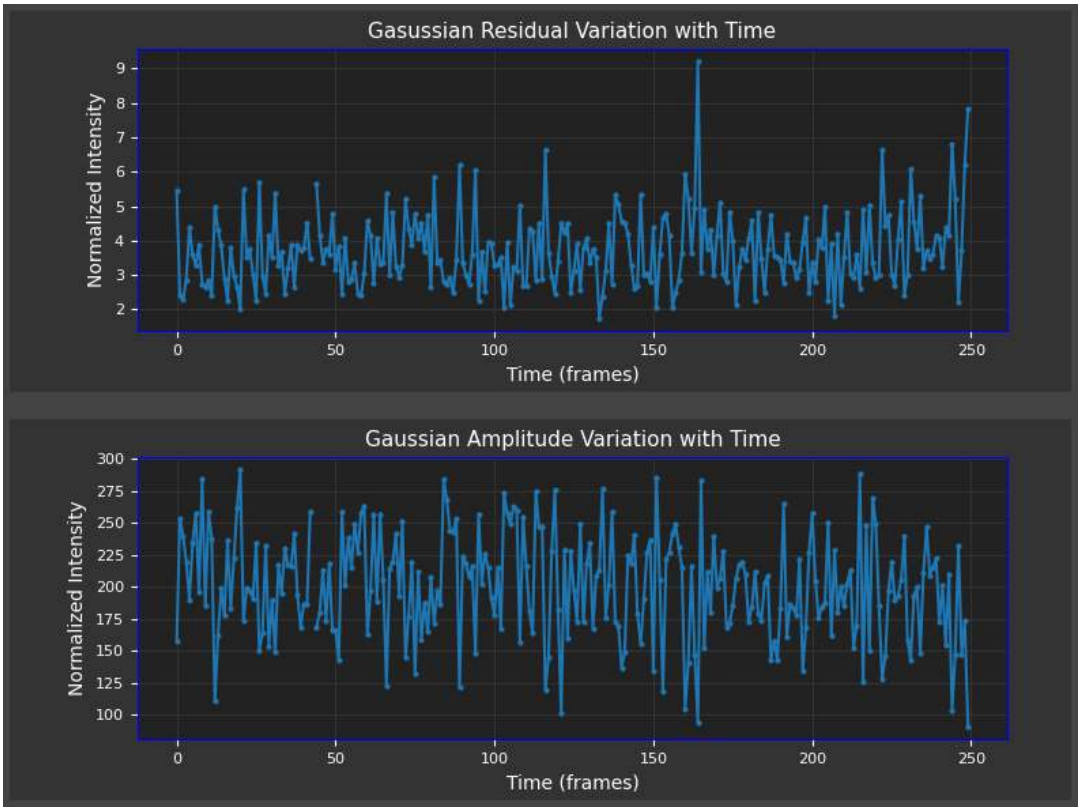


In the top right of the window is a plot of the mean-square displacement, fitted via linear regression to calculate the diffusion coefficient. By default, all MSDs are fitted without optimization, using 100% of the plot:

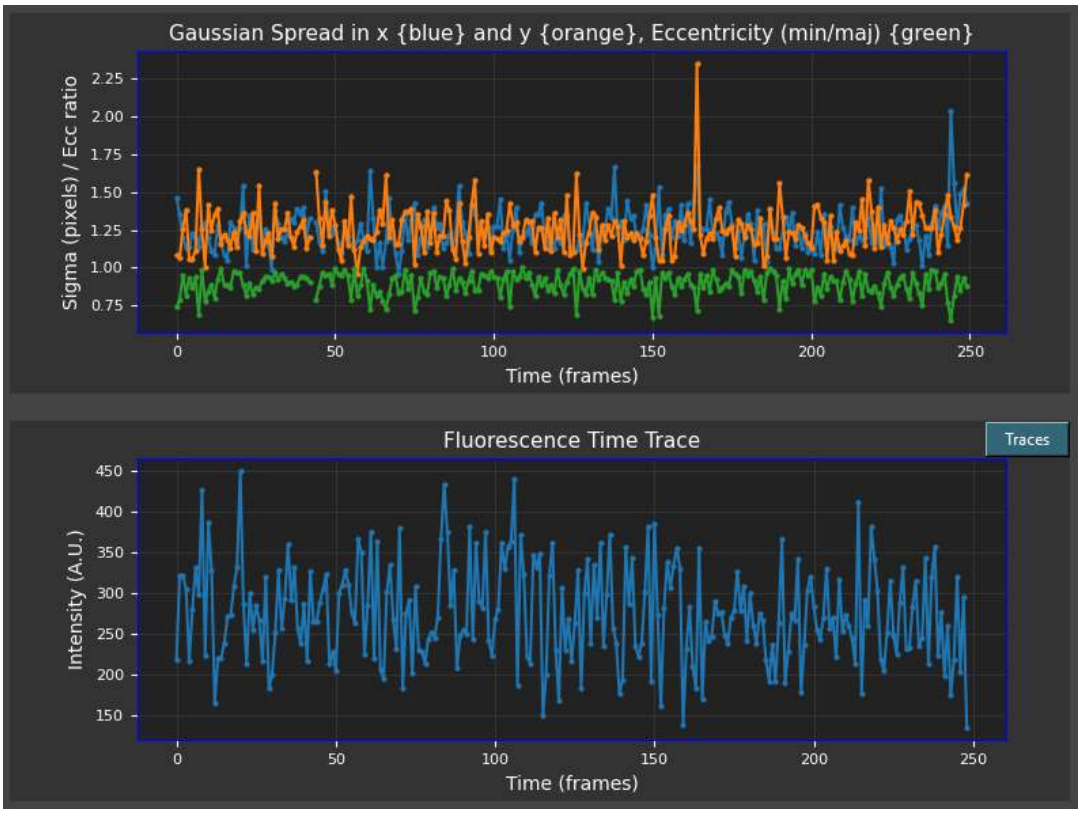


The next chapter will cover how to properly fit and filter MSD plots for much more accurate diffusion coefficient distributions.

In the bottom left of the window are the Gaussian fitting parameters of the spot for each frame it was detected:

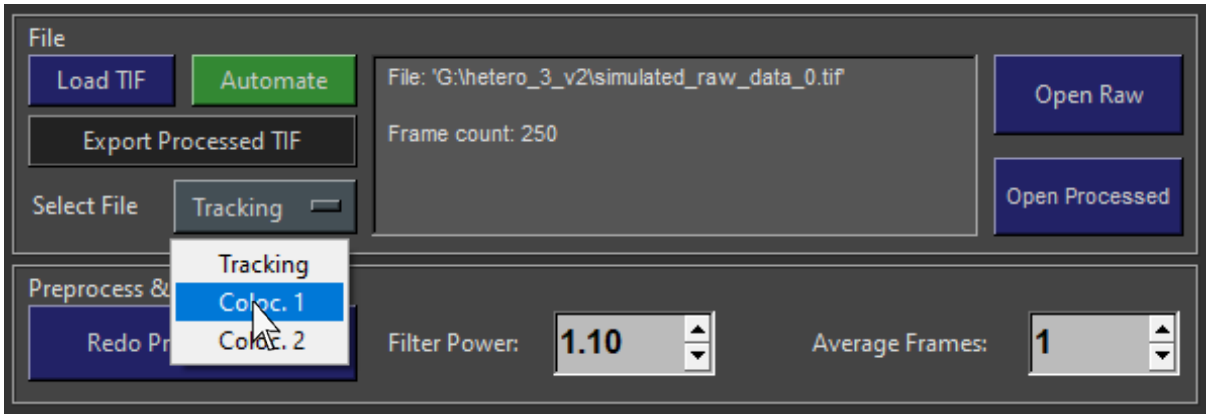


On the bottom right are the spot width, eccentricity, and fluorescence intensity trace:

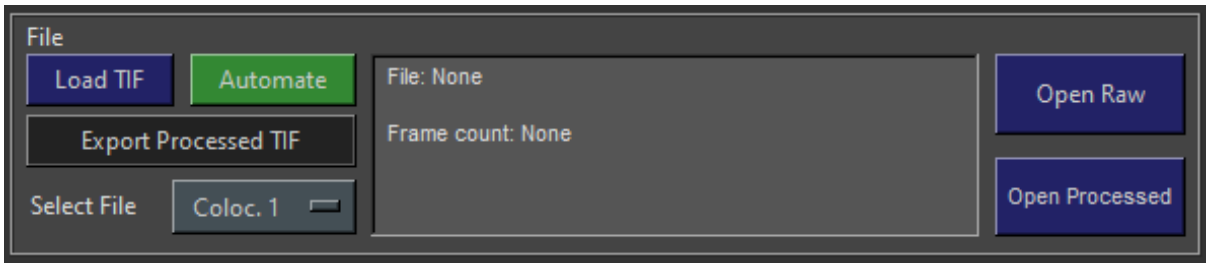


8.12 Switching to Another Channel

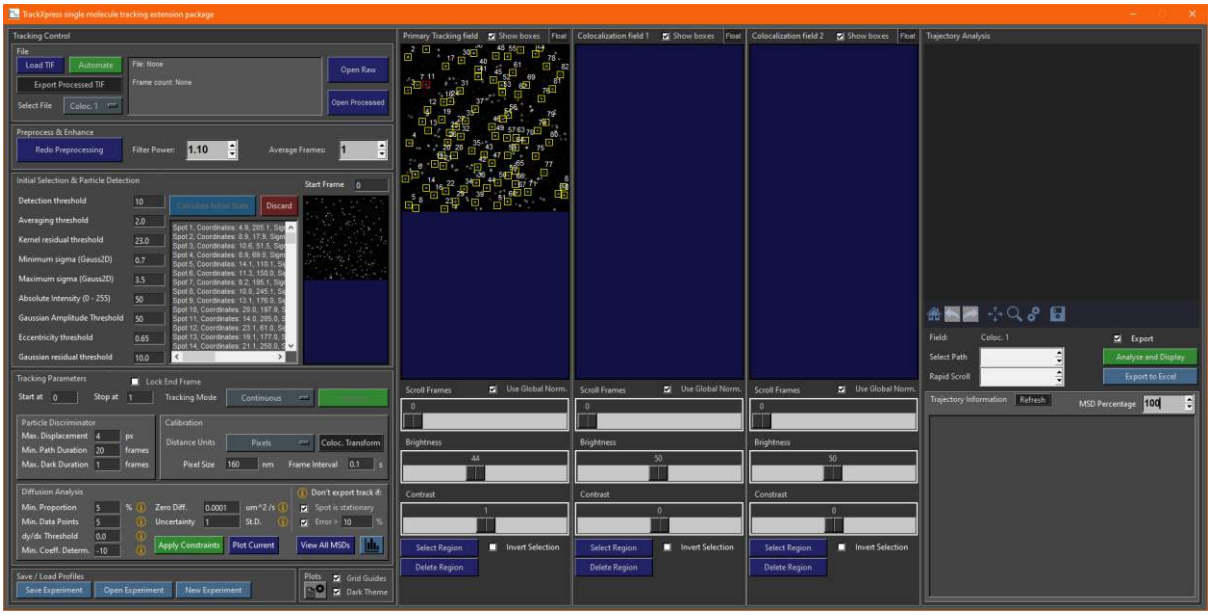
By default, data will be loaded into the ‘Primary Tracking Field’ and analysed. To switch to another channel, select it in the file section of the control panel:



Notice that the information box is now empty. The data and analysis for the ‘Primary Tracking Field’ is still in memory, but the program is only showing information about ‘Colocalization Field 1’:

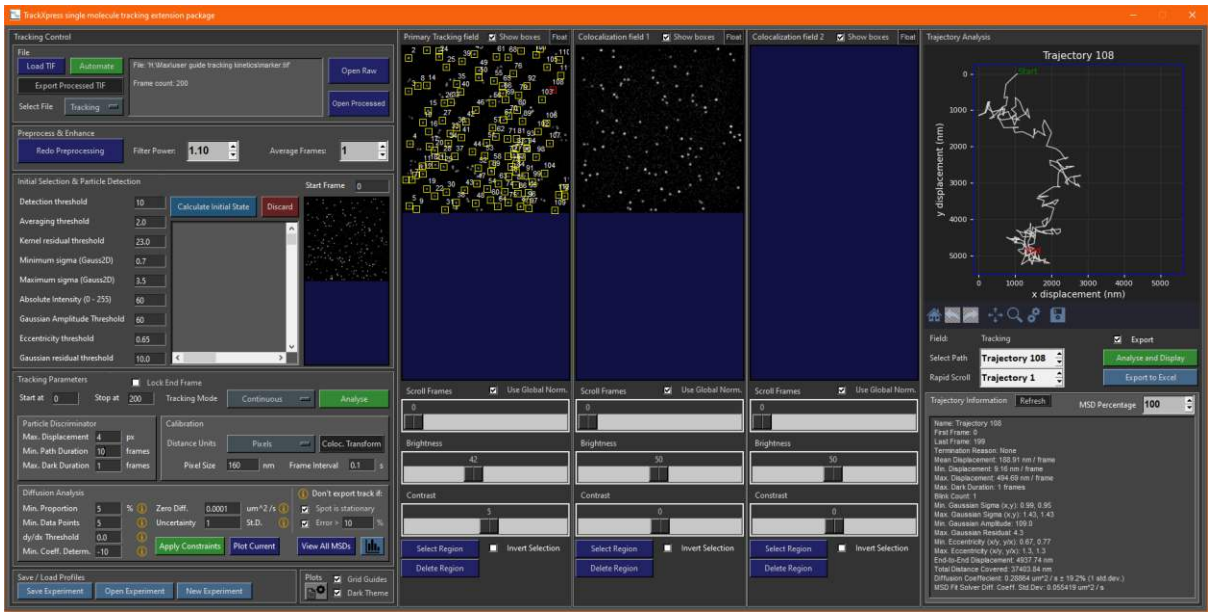


Similarly, the program will no longer show the results from the first channel:



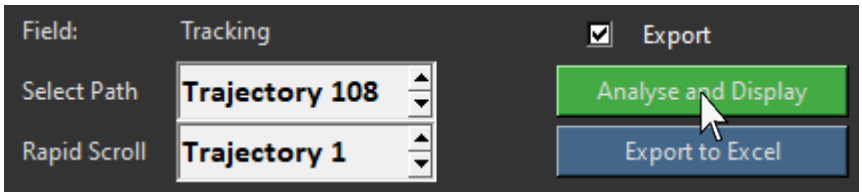
All information is stored and you can switch back and forth between channels freely from the dropdown menu. Only change the channel when FluoroTensor is not running a task such aa tracking, enhancing, MSD fitting etc, otherwise this will result in a crash or data corruption.

It is possible to use multi-channel tracking to observe binding kinetics between fluorescently labelled diffusing membrane proteins and a ligand with another dye. Load the data from the diffusing membrane protein into the 'Primary Tracking Field' and the data from the ligand into 'Colocalization Field 1' and then track the primary field, using it as a marker similarly to the colocalization analysis part of the program described in previous chapters:

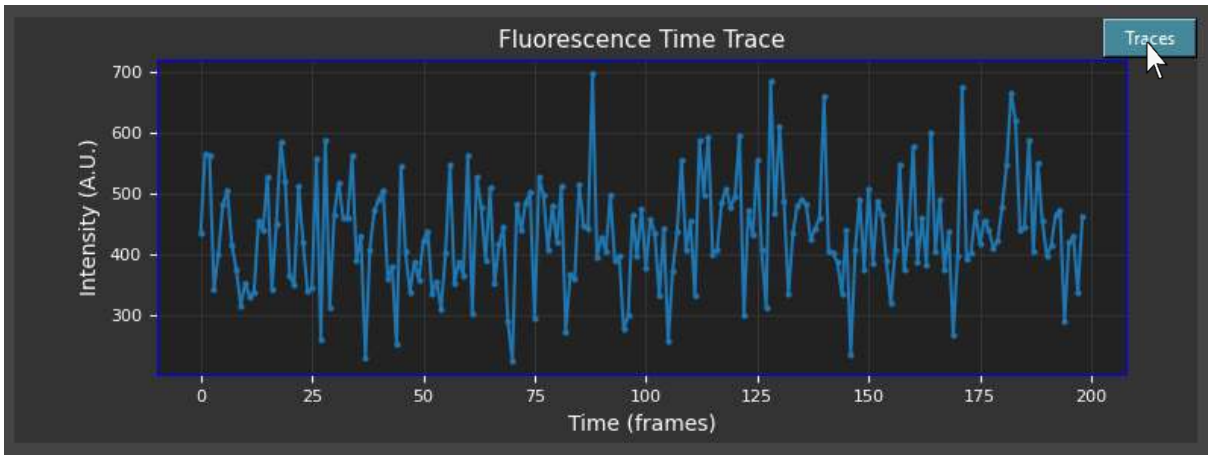


This is currently an experimental feature and currently does not support chromatic aberration correction in its current state. The fluorescence signal is calculated both for the tracked marker and its position in the colocalization channel. Thus, fluorescent molecule binding and unbinding will show up in the colocalized trace.

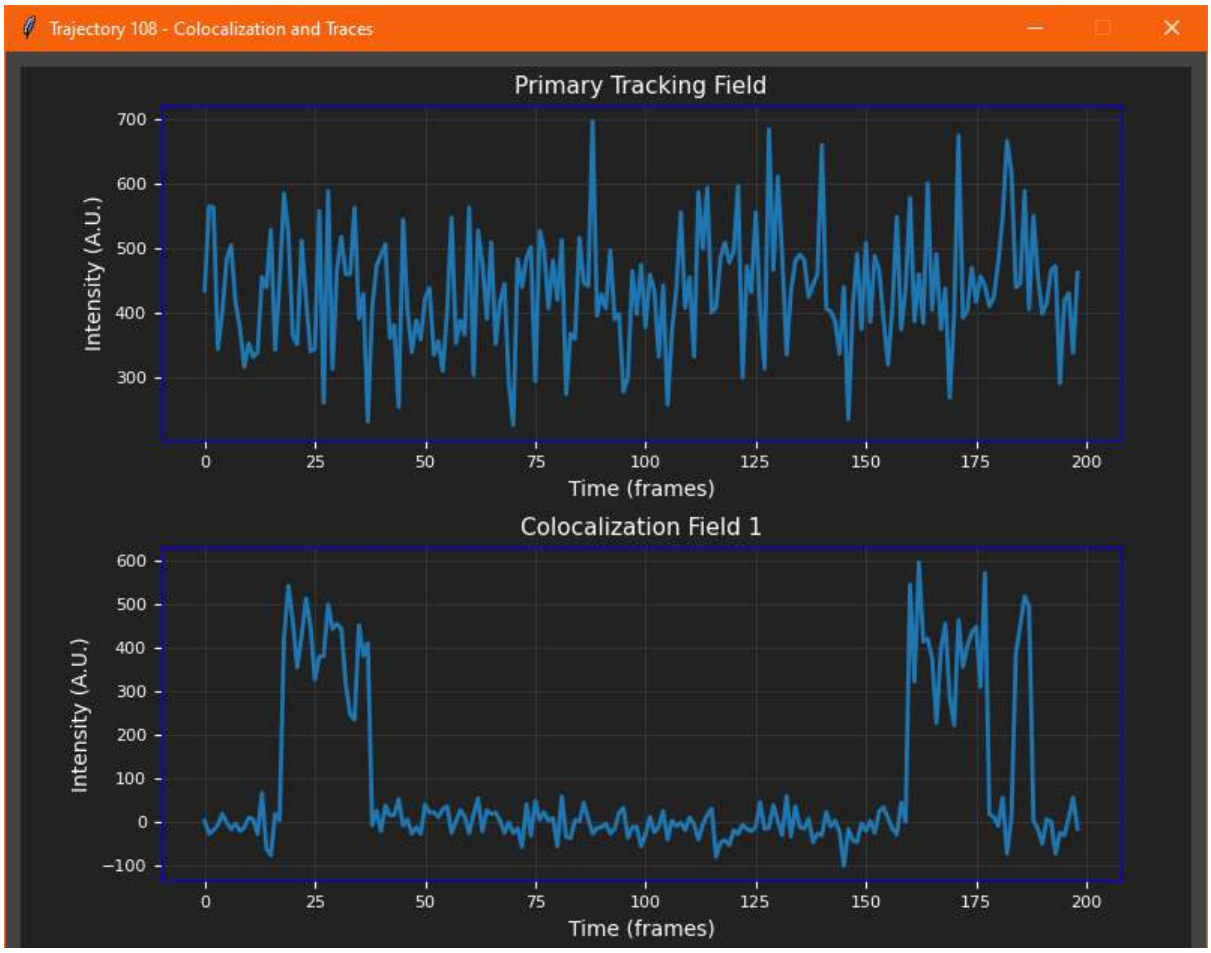
To access the colocalized trace, open the time series window first:



Then in the time series window, find the fluorescence trace in the bottom right corner and click ‘Traces’ in the top right of the plot:



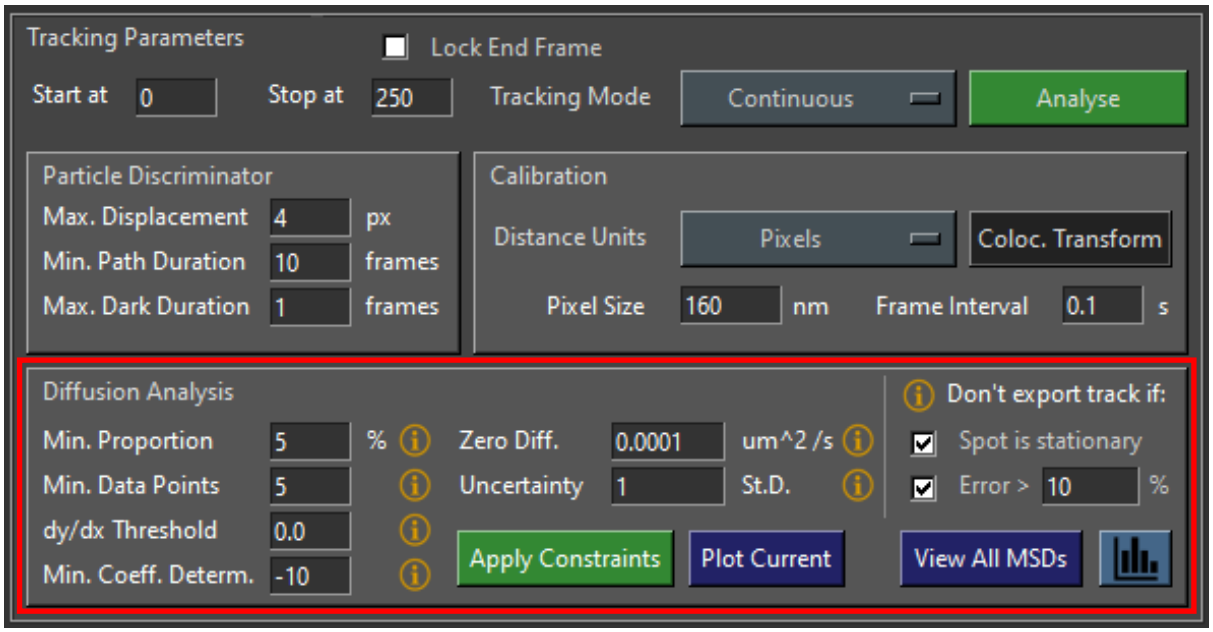
A new window will pop up with the fluorescence traces of all three channels calculated from the region following the x, y position of the track in the marker channel. A binding and unbinding fluorescent ligand will show up as fluctuating intensity in the colocalization channel:



Chapter 9 – Fitting MSDs and Plotting Diffusivities

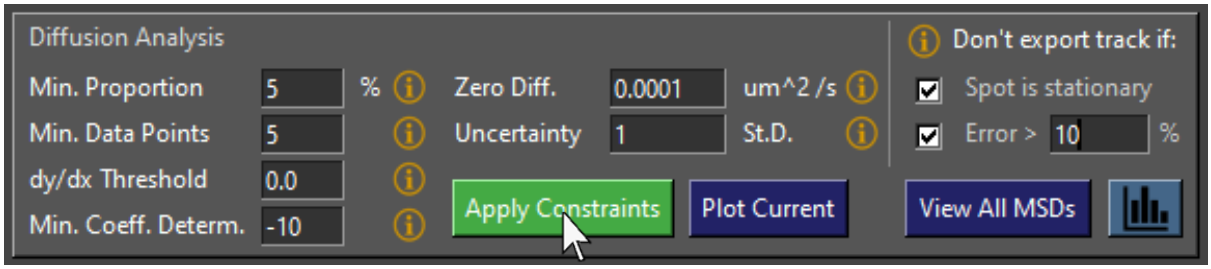
9.1 MSD Autofit

Under the tracking parameters is the control panel for diffusion analysis:

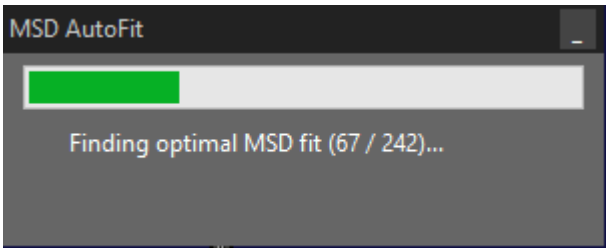


The parameters are explained in the accompanying paper. In brief, the autofit function fits a number of proportions of the MSD with the aim of maximising R^2 to find the point of optimal fit towards the beginning of the MSD plot before it deviates from linearity. If R^2 is less than minus 10 by default and the calculated diffusion coefficient is less than the 'Zero Diff' threshold, the spot will be marked as stationary. If the checkboxes are selected, tracks that are marked as stationary or have a fitting error greater than a certain threshold will be removed from the export list (unless manually re-added) and will not be included in the diffusivity histogram.

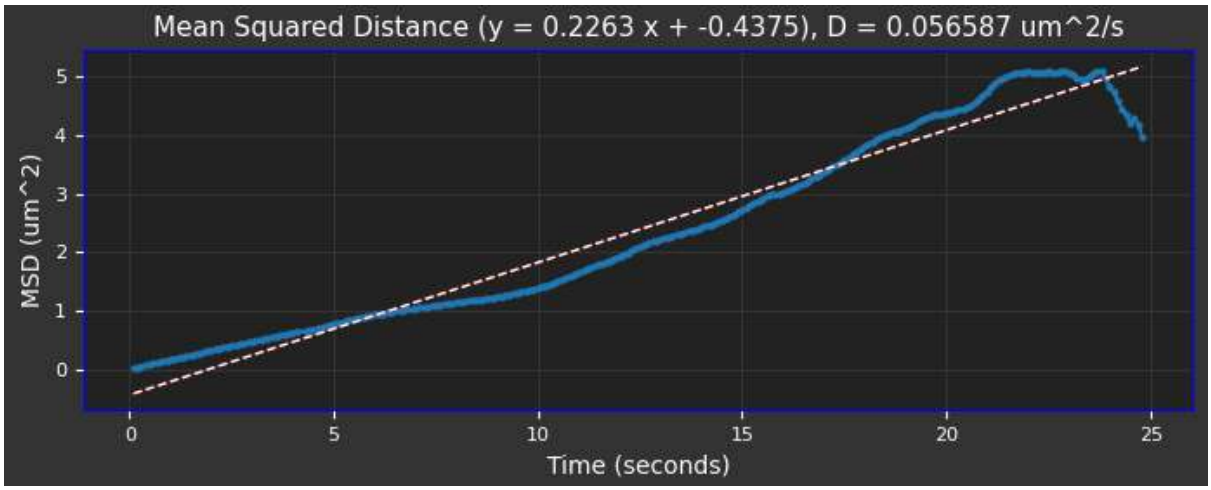
To use MSD autofit, click 'Apply Constraints' after the data has been tracked:

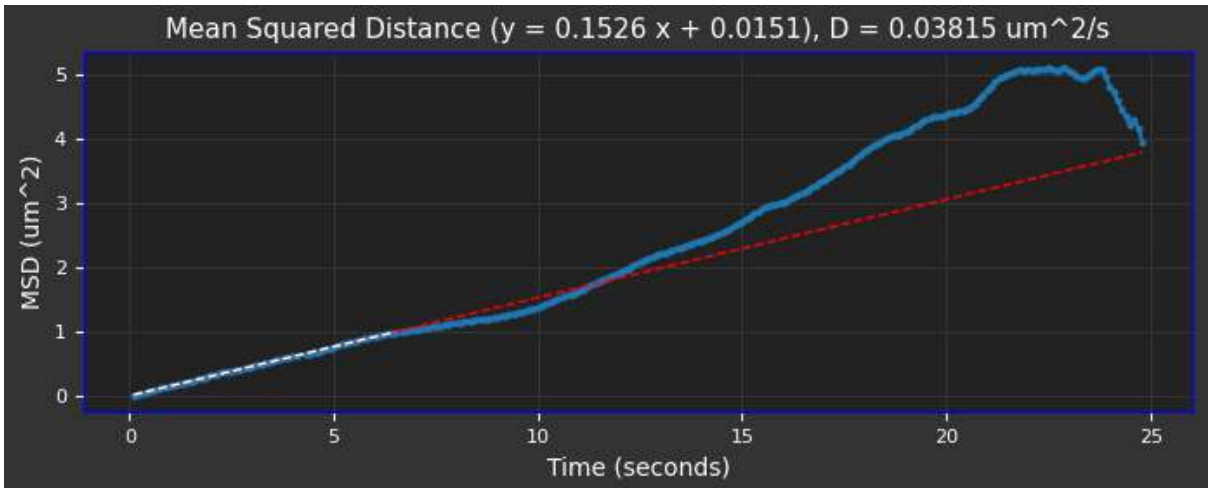


The program will then calculate the optimal MSD fit based on the parameters set in the 'Diffusion Analysis' section of the control panel:



Comparing the linear regression using the full MSD function compared to the optimized fit:

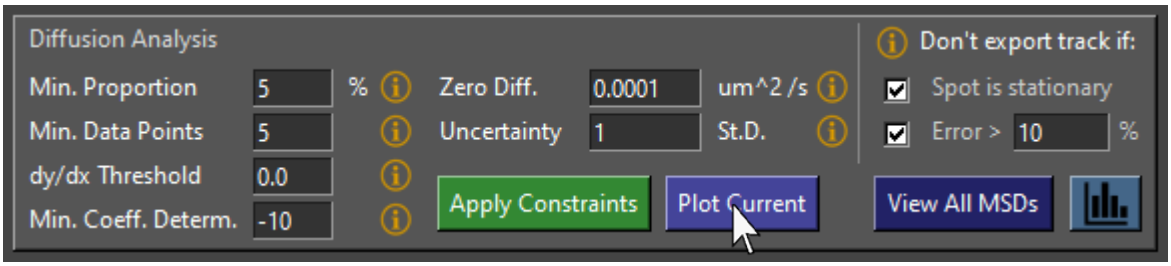




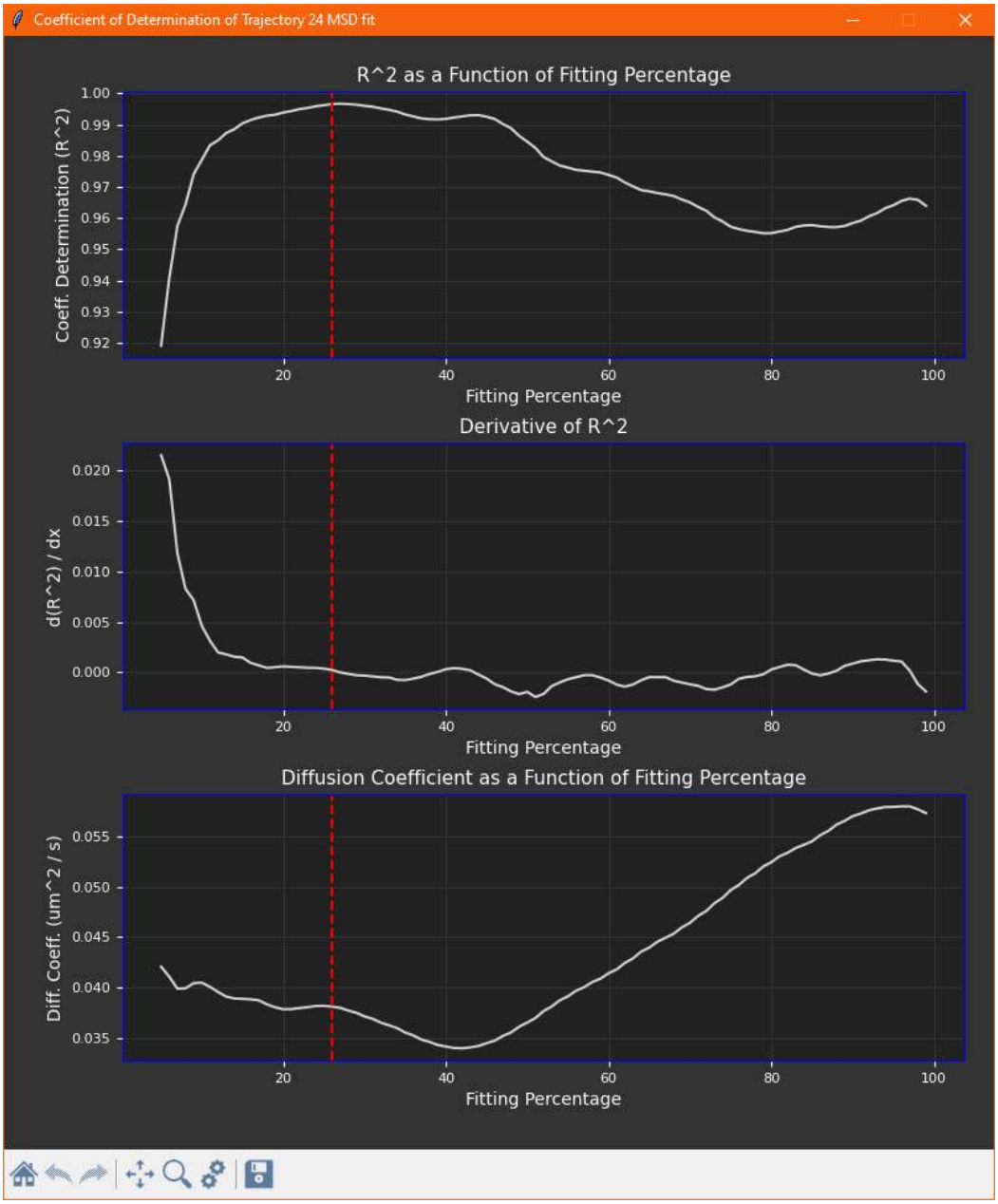
The fitted proportion is shown in white and extrapolated over the unfitted region in red. The ground truth diffusivity of this track is 0.040 μm^2 / s.

9.2 Plotting the Autofit Function of the Current Track

To plot the functions used to find the MSD fit of the current track, click 'Plot Current' in the control panel:

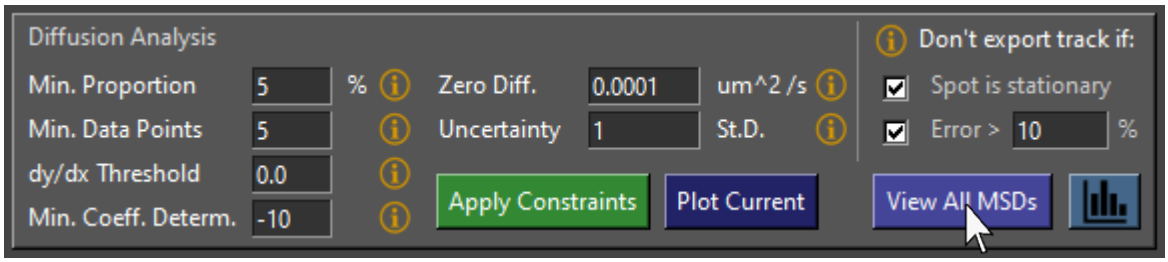


A window will then appear with a plot of R^2 as a function of the proportion of the MSD plot fitted, its gradient, and the diffusivity at each proportion:

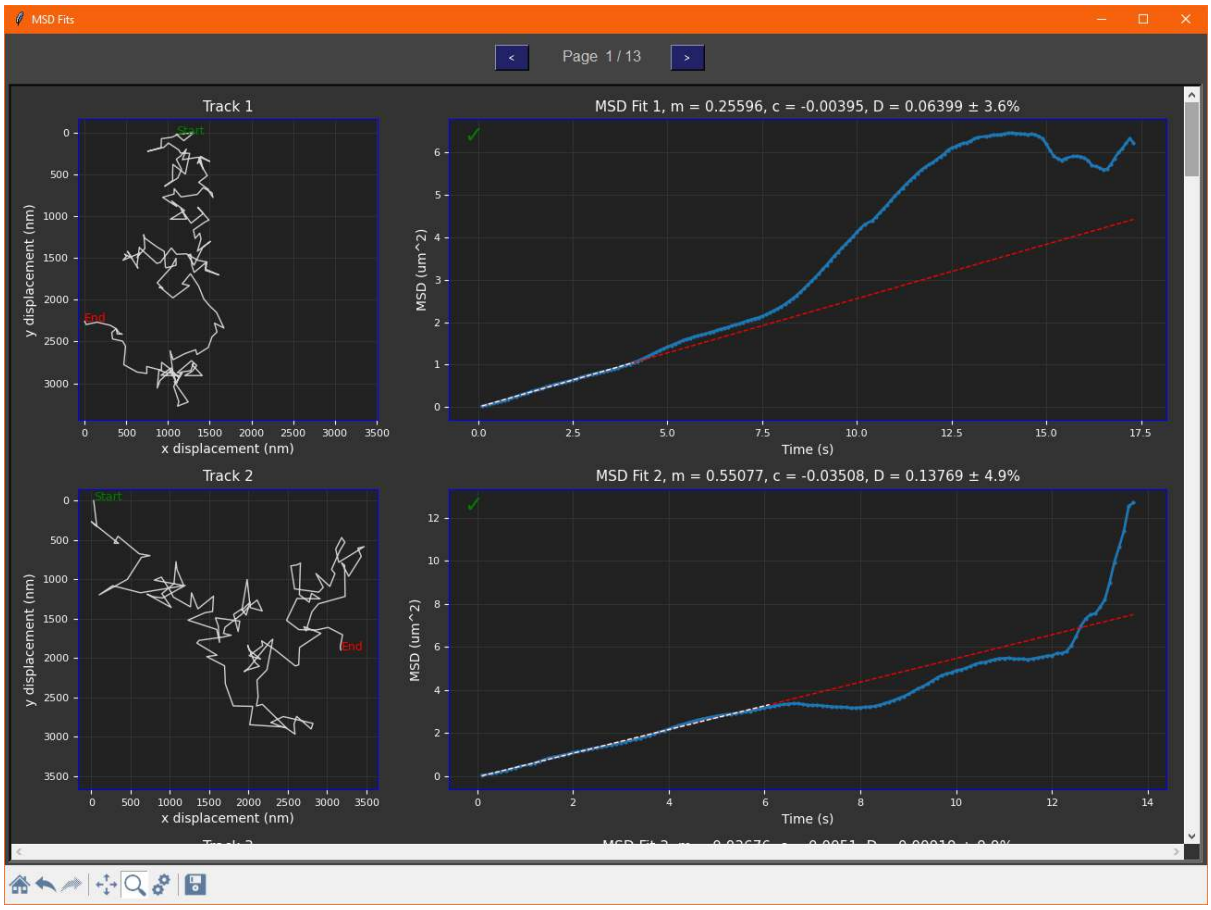


9.3 Viewing All MSD Plots

To view the MSD plots of each track without needing to open the time series window every time, click ‘View All MSDs’ in the control panel:

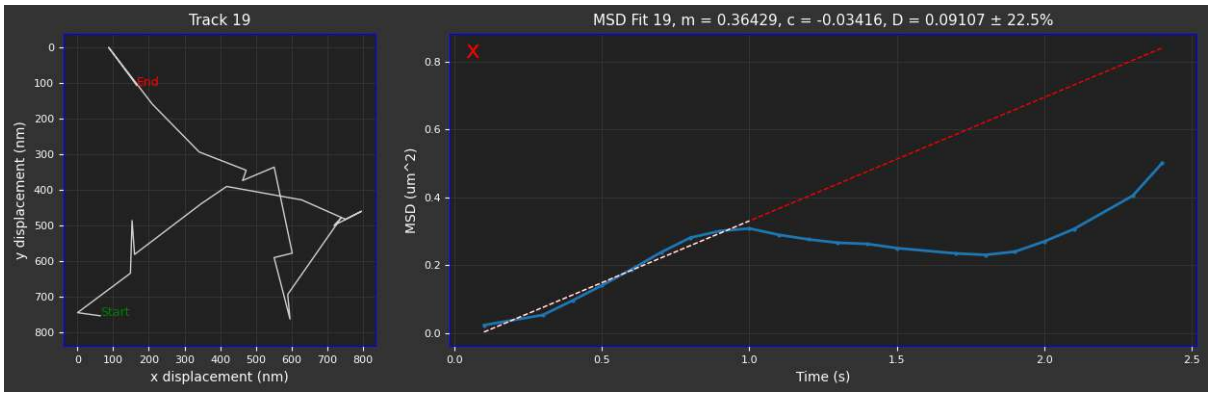


A window will then appear with pages of tracks that can be scrolled through:



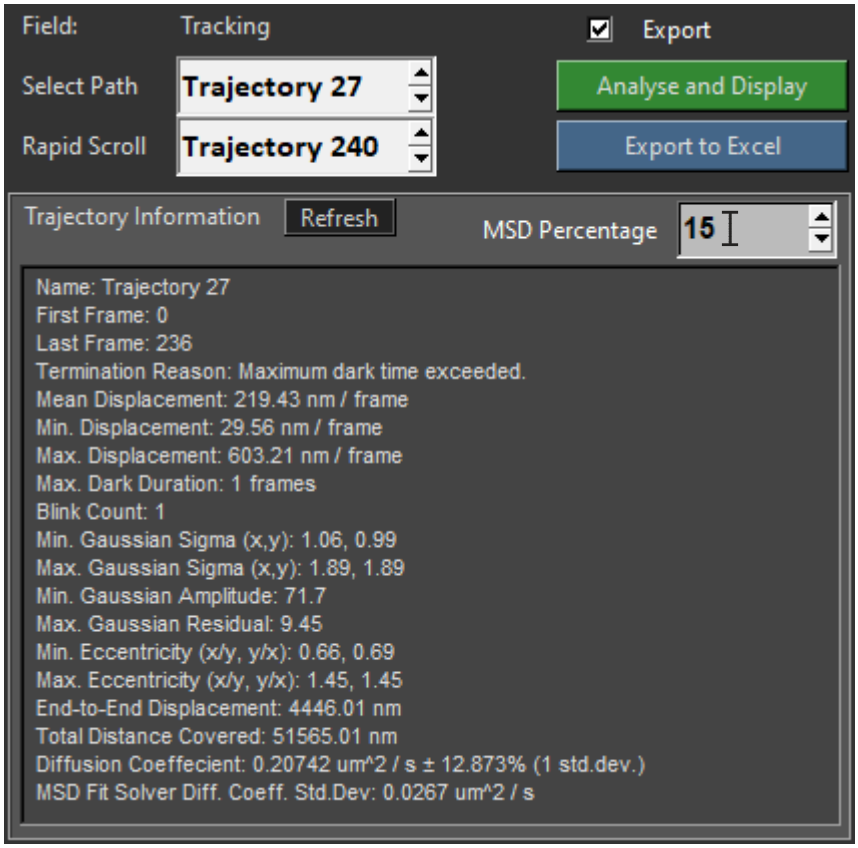
Note that the percentage error is not the uncertainty of the diffusion coefficient of the molecule (this will be calculated from the diffusivity distribution later) but rather, the standard deviation of all the diffusion coefficients of each fitting proportion of the fitted portion of the

MSD which is used as a measure of fitting quality. The green tick means the track has been kept for export. A red cross would mean it was discarded from the dataset:

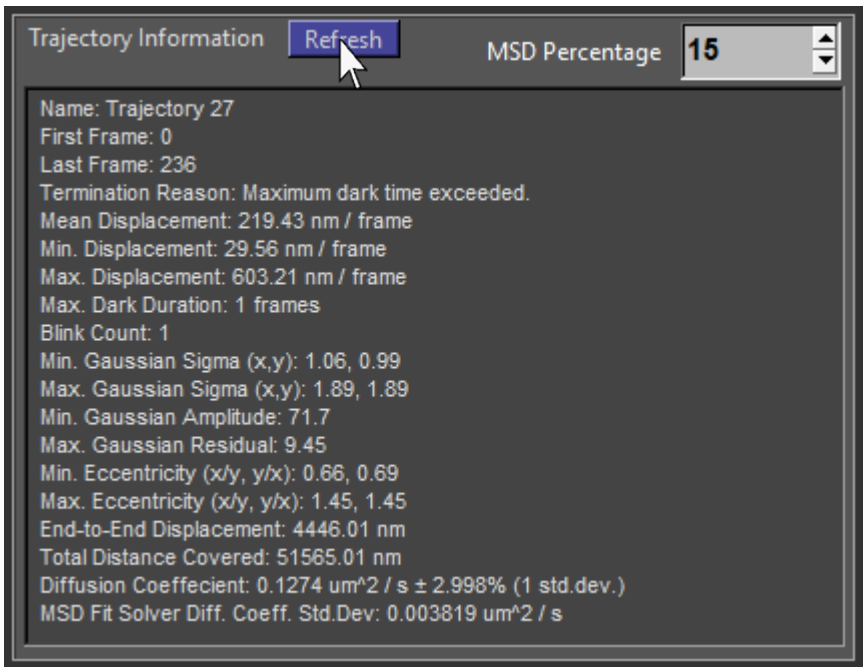


9.4 Manually Setting MSD Fit Proportion

To set the proportion of the MSD plot to fit manually, enter the percentage into the spin box or scroll through using the arrows:

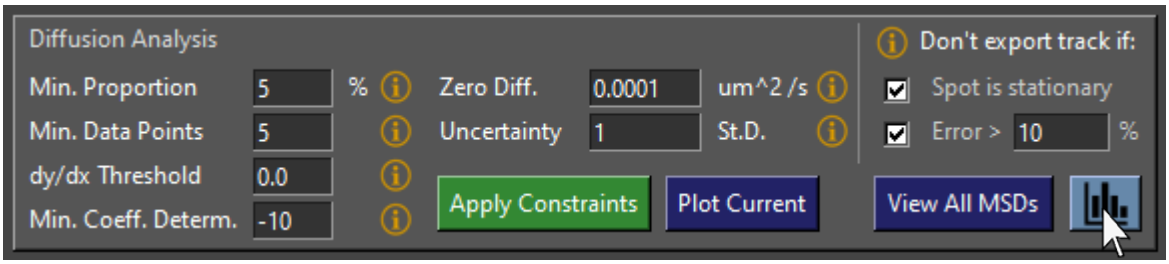


Then click 'Refresh' and the information in the box below will update:

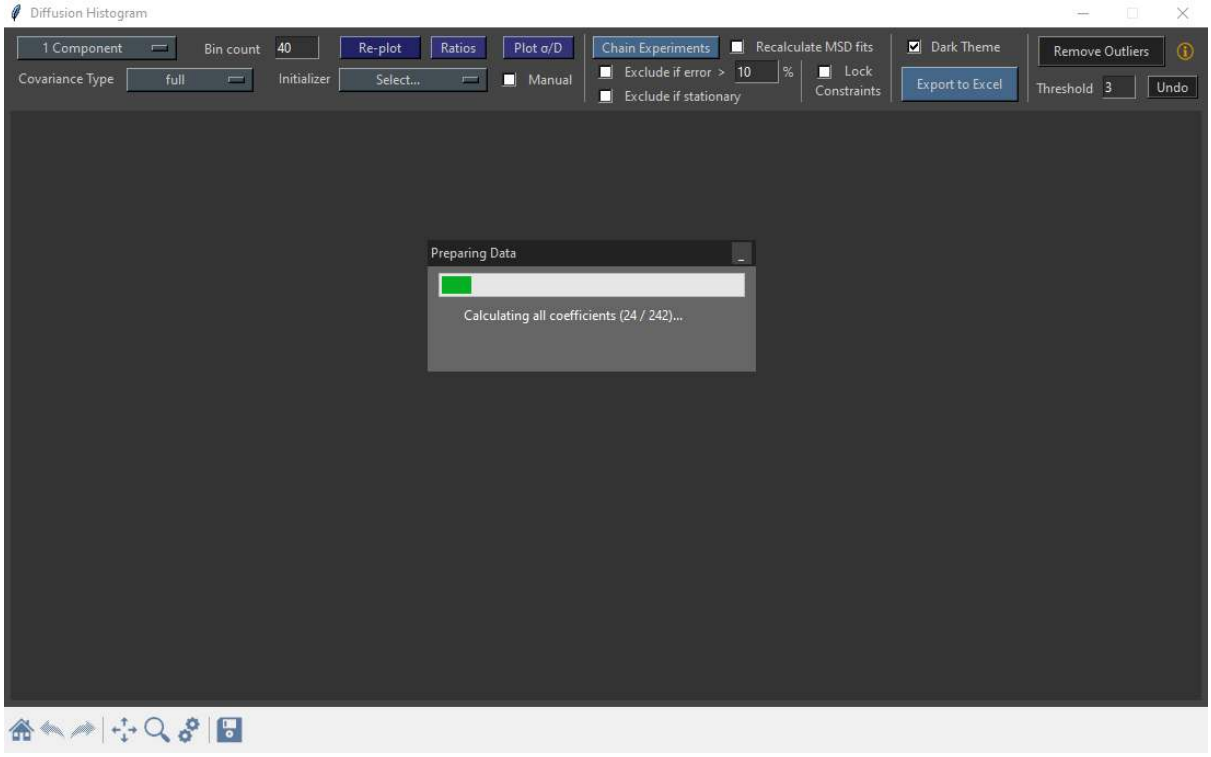


9.5 Plotting Diffusivity Distributions

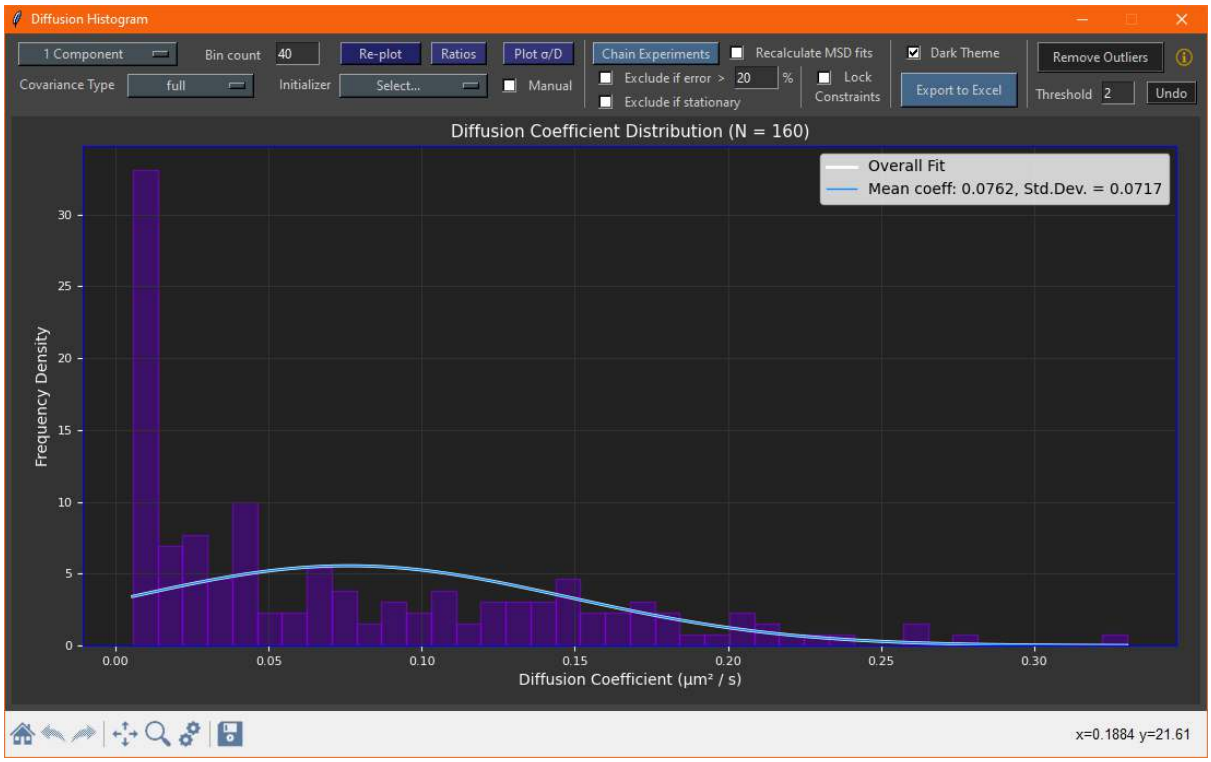
To plot the diffusion coefficient distribution of all tracks in the dataset, click the histogram icon in the 'Diffusion Analysis' section of the control panel:



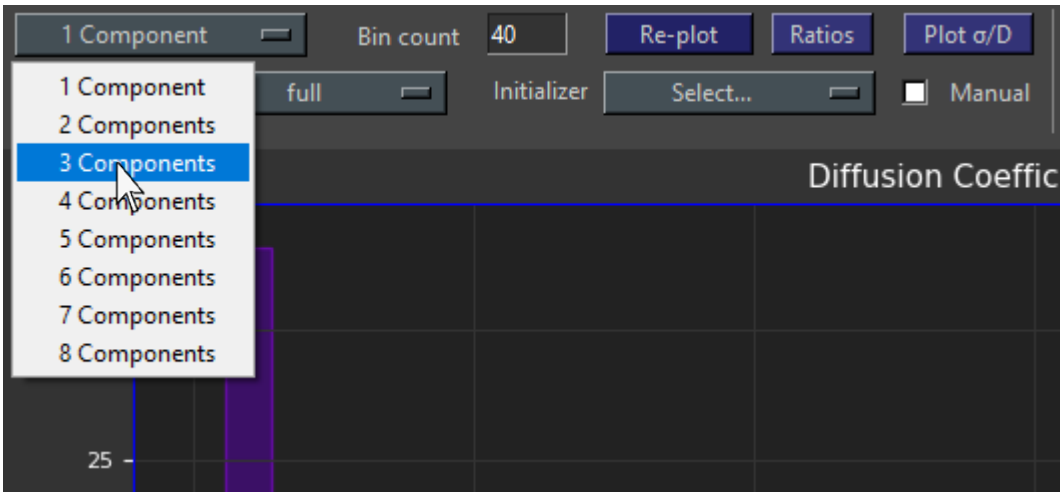
The diffusivity histogram window will appear, and the program calculates all diffusion coefficients based on the MSD fit of each track:



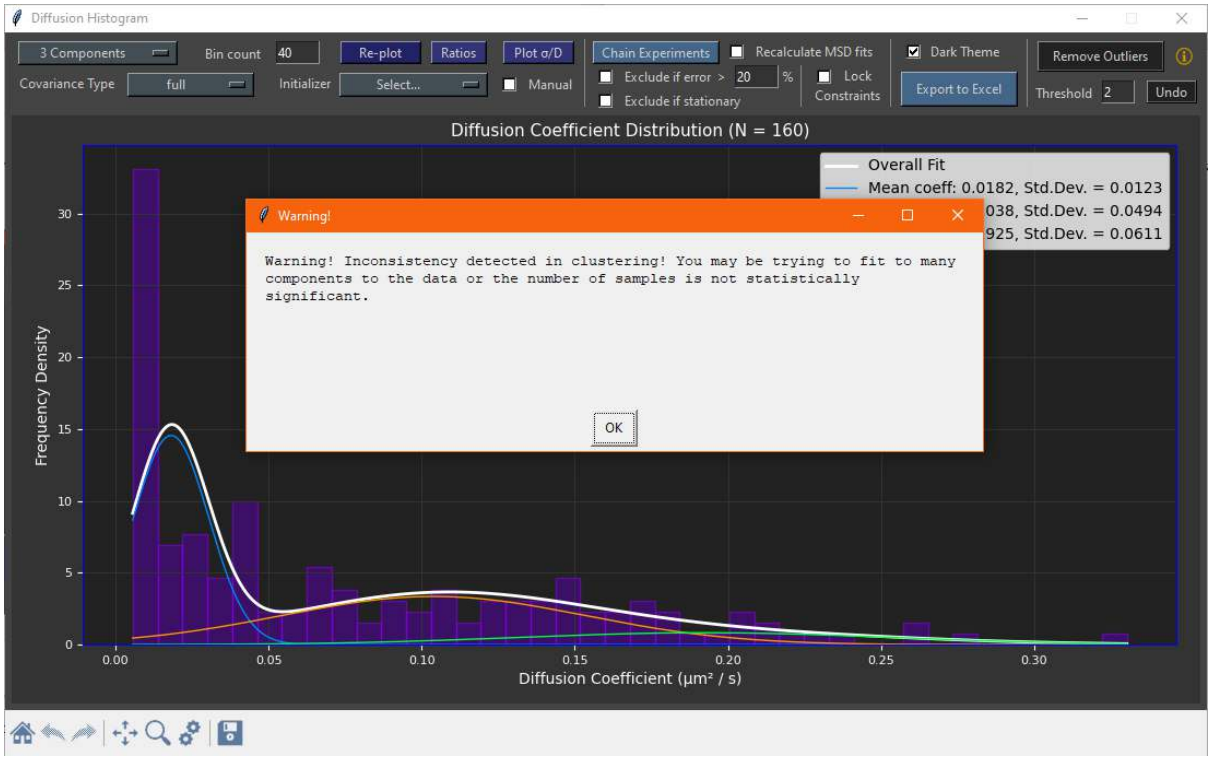
Once this process has completed, the histogram is plotted and fitted with a Gaussian mixture model:



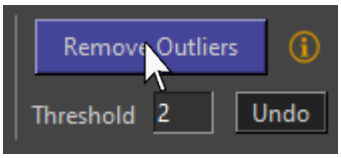
By default, the number of components is set to 1. This can be changed by selecting the number of components from the drop-down menu in the toolbar:

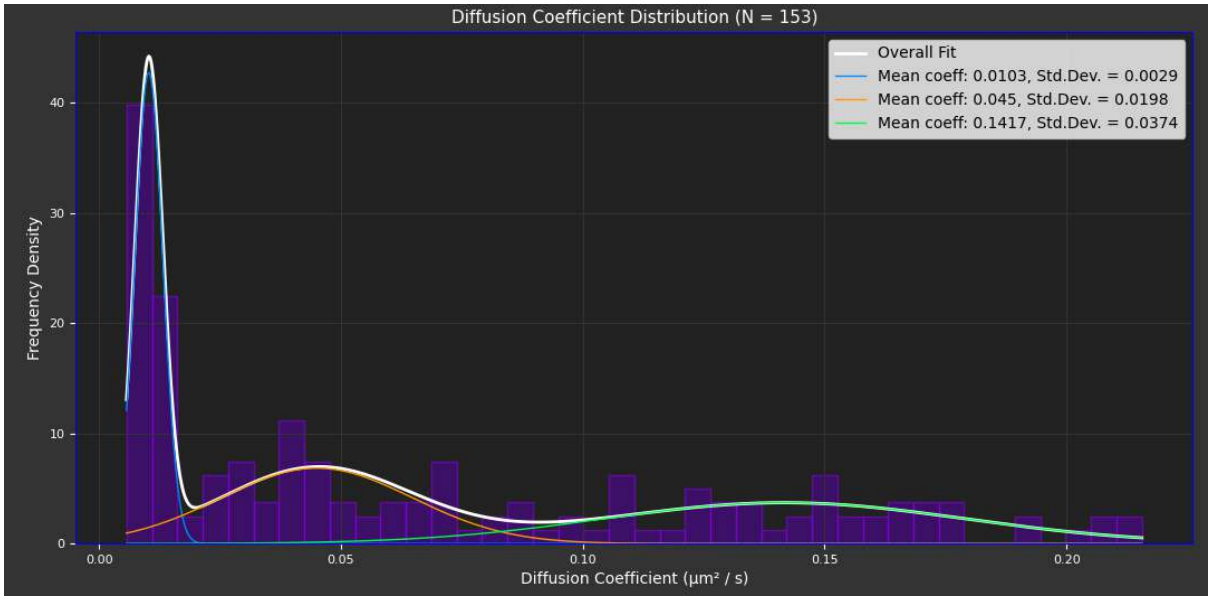


Gaussian mixture models are sensitive to outliers and may have inconsistent clustering results:

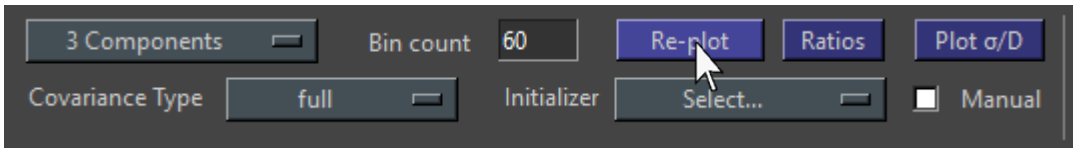


Click ‘Remove Outliers’ to remove points that are n standard deviations greater than the mean of the distribution where n is the threshold in the box below:

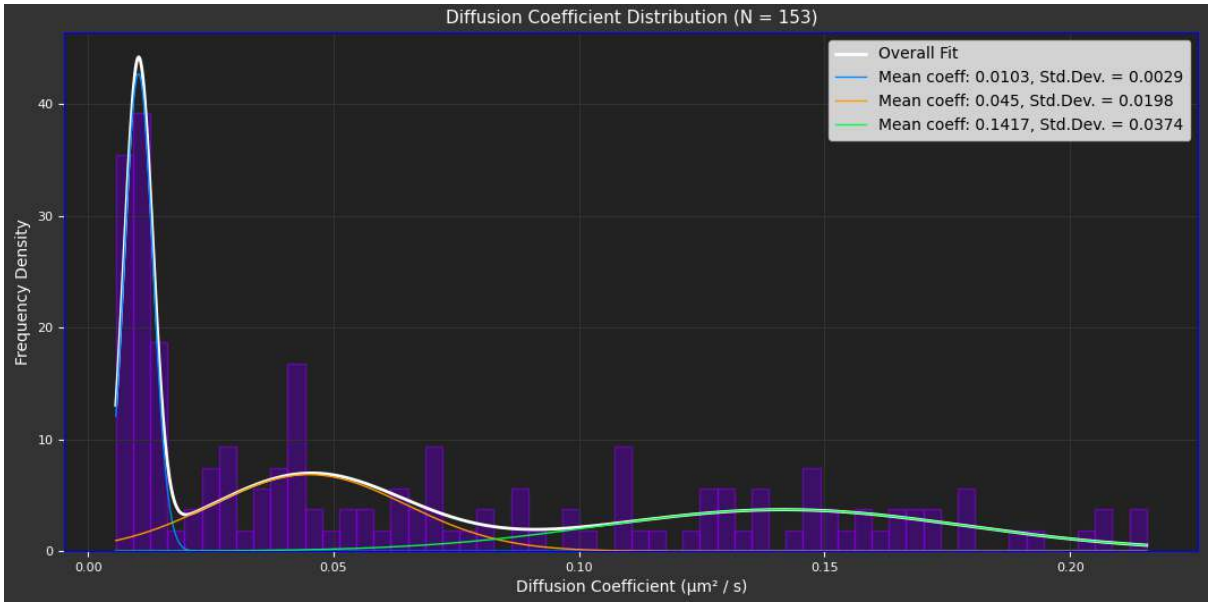




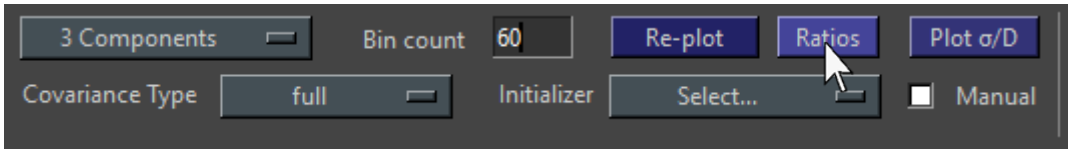
To re-bin the histogram, set the value in the ‘Bin count’ box and click ‘Re-plot’:



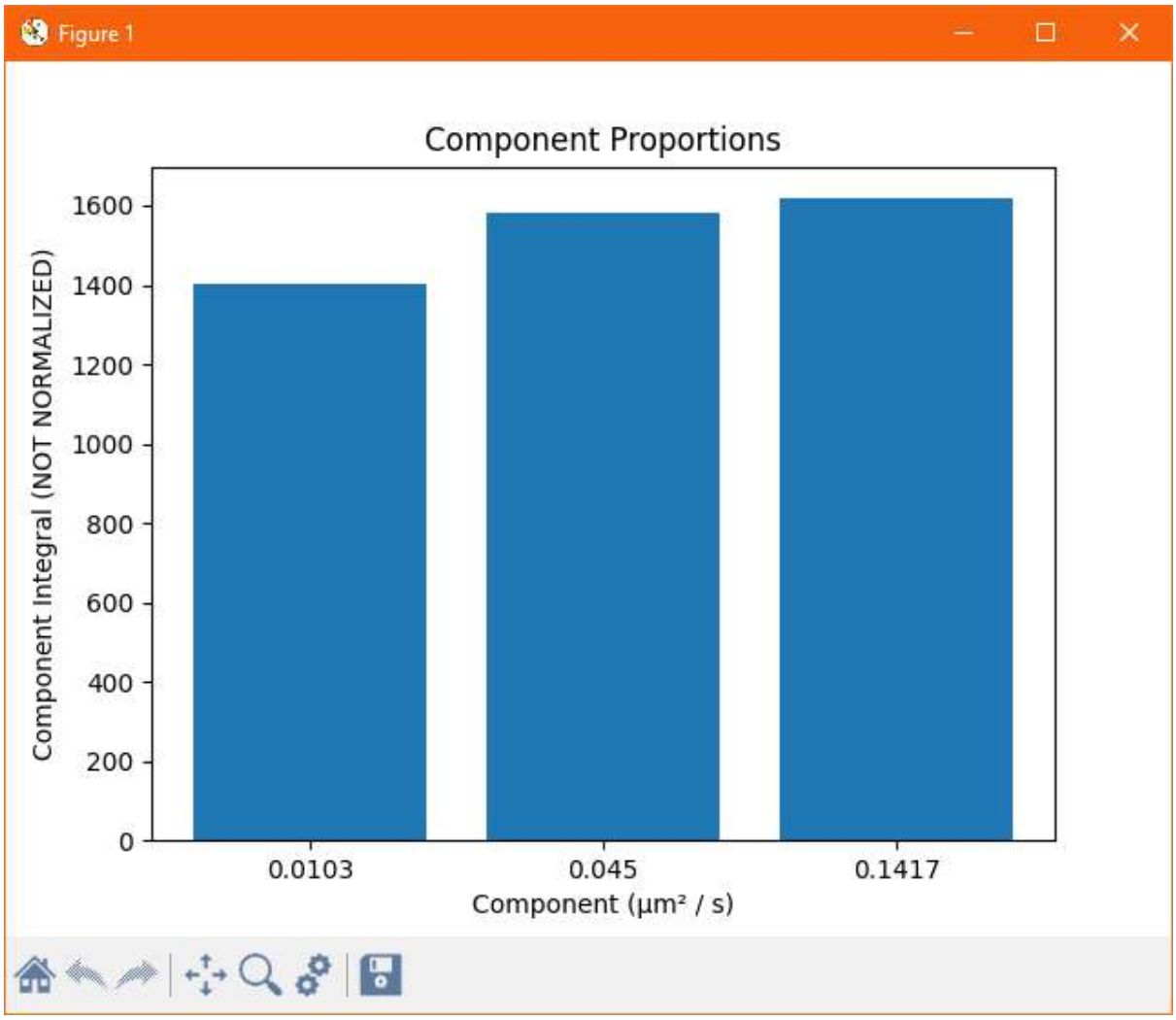
The distribution will be re-plotted with the new number of bins:



To plot the proportion of each component (the relative integrals of the Gaussians), click ‘Ratios’ in the toolbar:

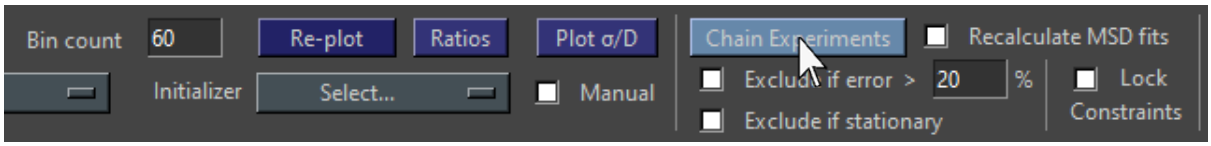


A graph window will then appear with a bar chart showing the relative proportion of each component:

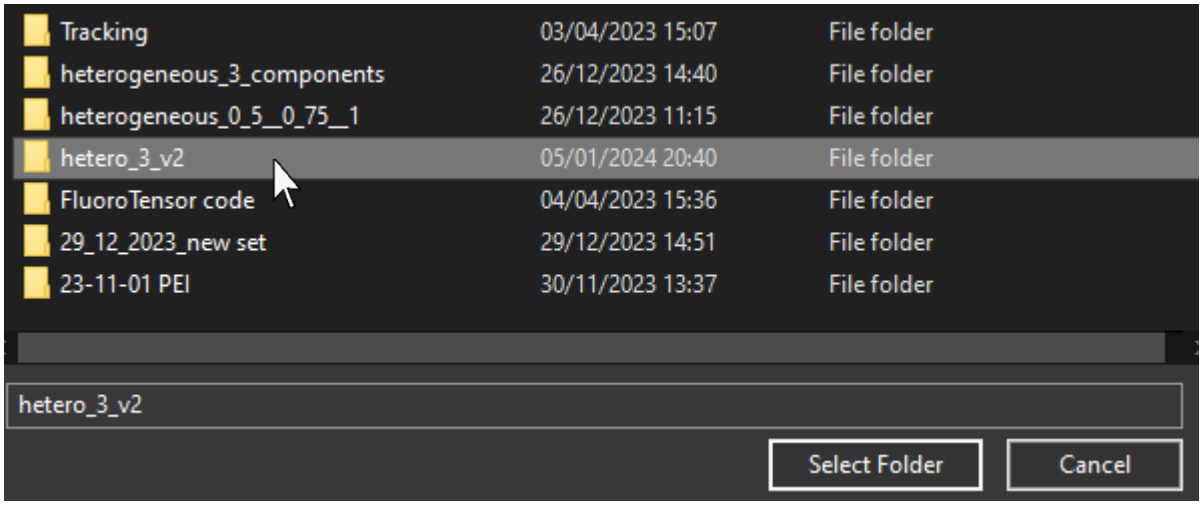


9.6 Plotting Diffusivities from Multiple TXP Files

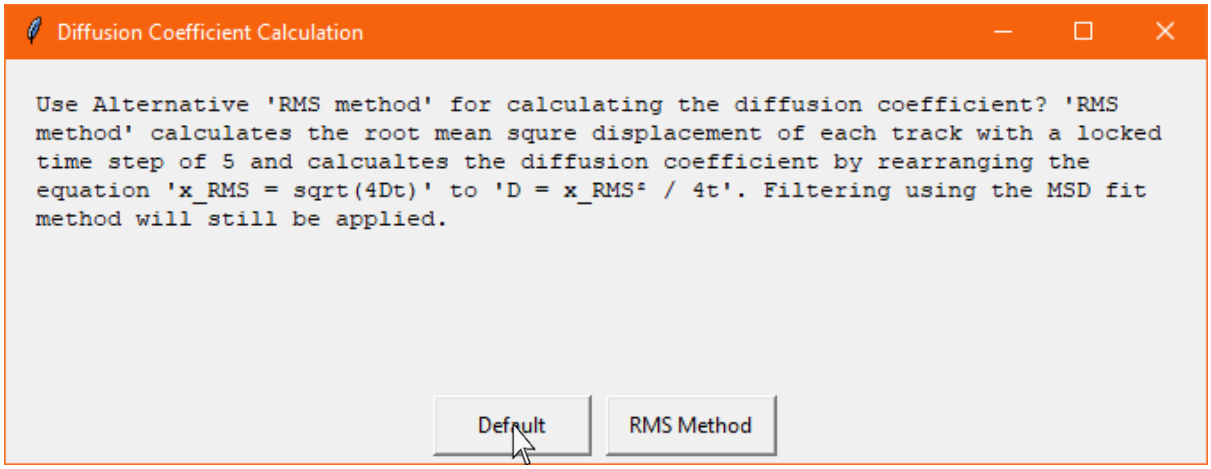
If several acquisitions were made from the same sample and analysed as separate files, the Tracking eXPeriment (.txp) files can be chained together to combine all the results into a single histogram which will likely lead to a better fit. To do this, click 'Chain Experiments':



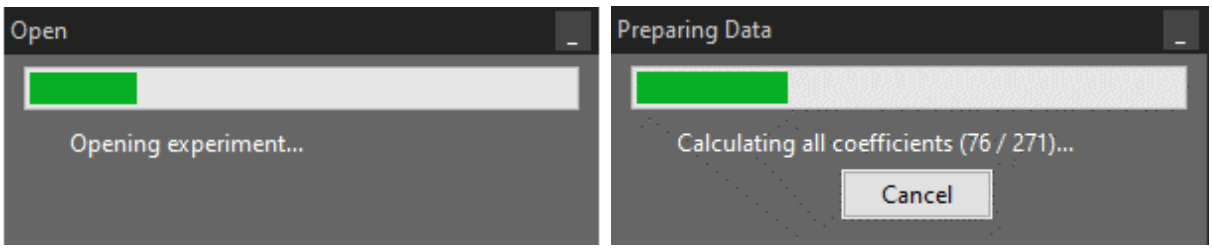
A file dialogue will appear; select the folder where the experiment files are located:



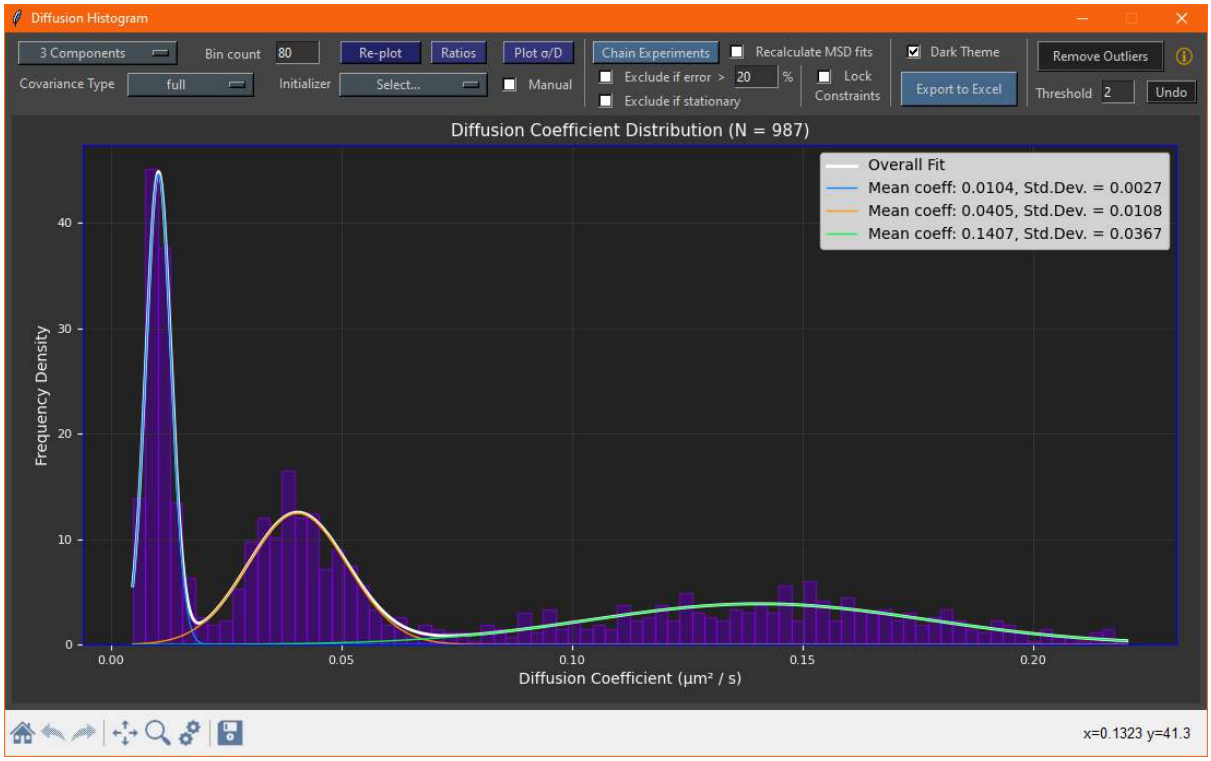
Then click 'Default' in the dialogue box that appears:



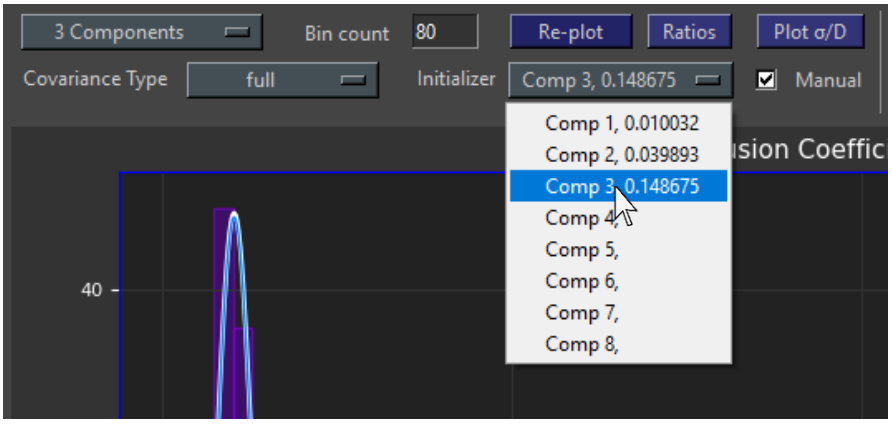
The program will then sequentially load each .txp file in the folder and calculate the diffusion coefficients of all the tracks, adding them to the distribution:



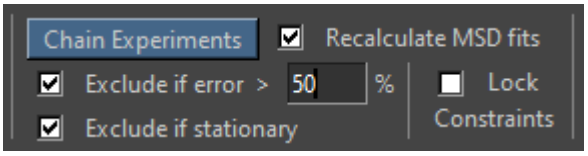
Once this process is completed, the full dataset of the combined files will be plotted and fitted:



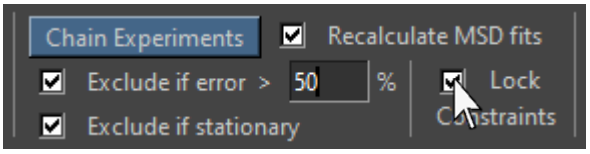
Sometimes, especially with outliers, fitting can be temperamental. Clustering can be initialised manually by selecting components from the dropdown menu and clicking on the histogram to select the centre of a perceived distribution:



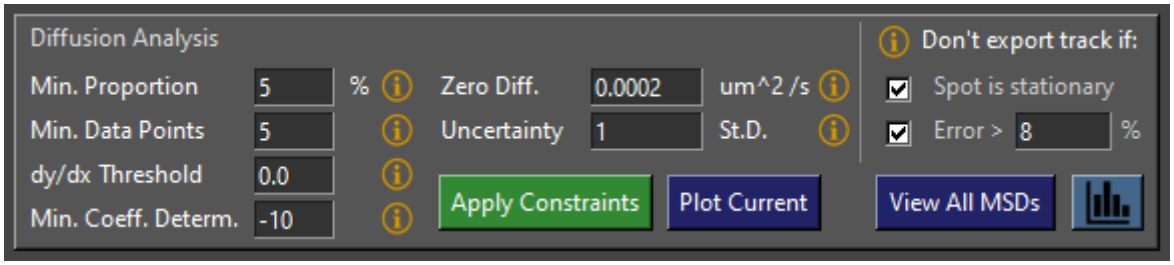
By default, all of the settings used for analysis and MSD fitting are loaded from the .txp file and diffusion coefficients are calculated from these automatically. The MSDs can be recalculated and the tracks used filtered when these files are loaded from the histogram window:



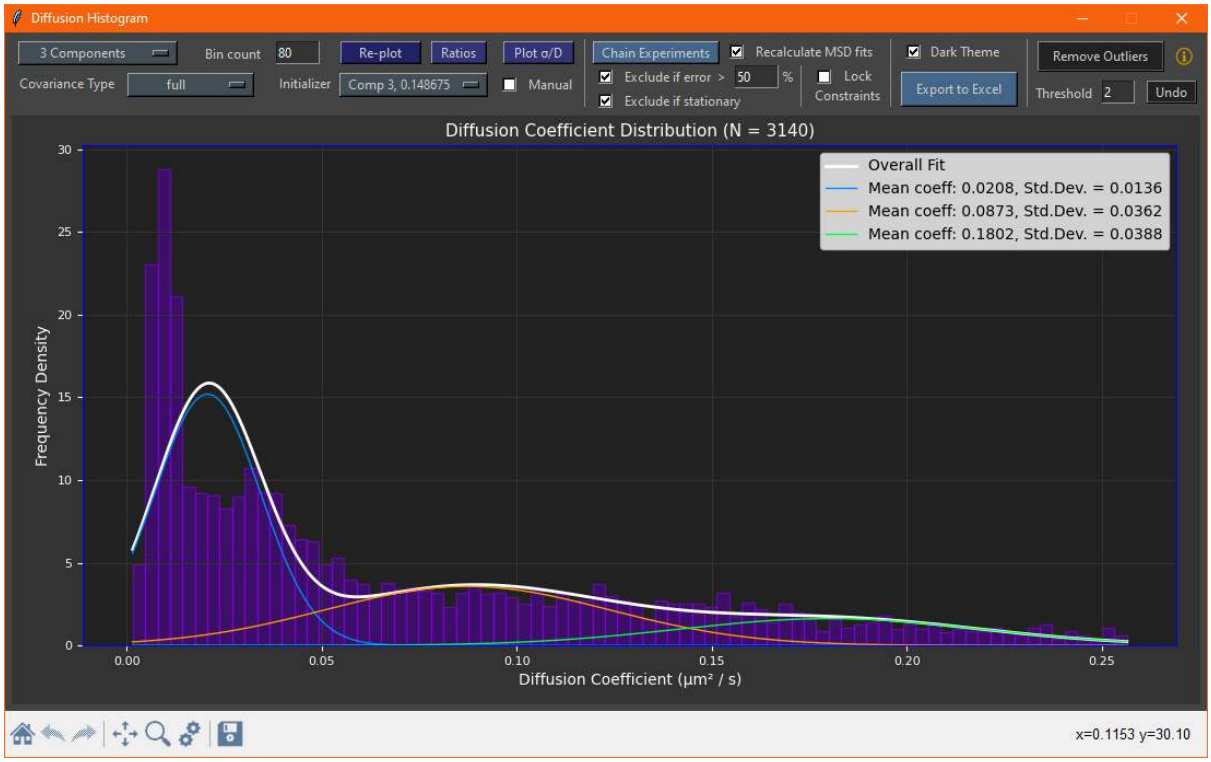
If you want to use custom settings for calculating the MSD fit instead of those loaded from the file, tick the 'Lock Constraints' checkbox:



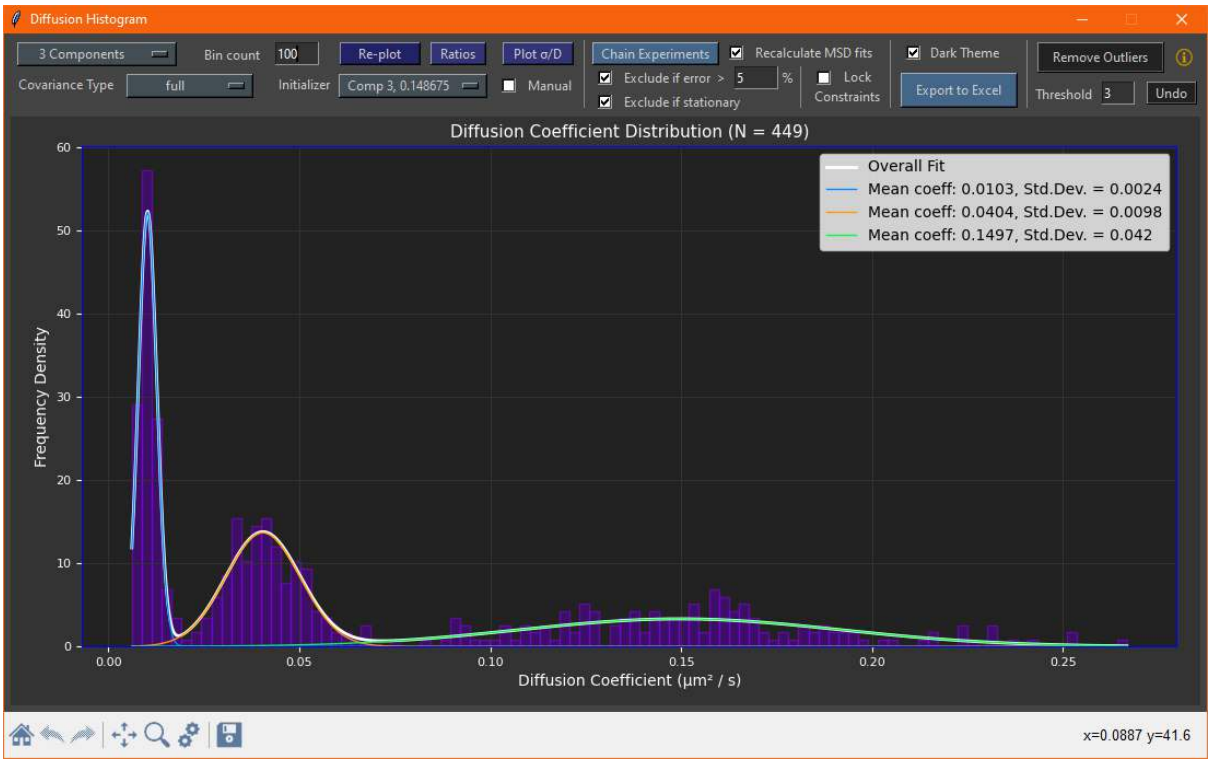
If 'Lock Constraints' is enabled, the settings in the main control panel used to fit the MSDs will not be loaded from the file. Instead, the settings currently in the control panel will be used instead. These can still be changed manually:



In this model three component system (simulated tif stacks), including all tracks up to an estimated MSD fitting error of 50% results in the following distribution of 3140 tracks:

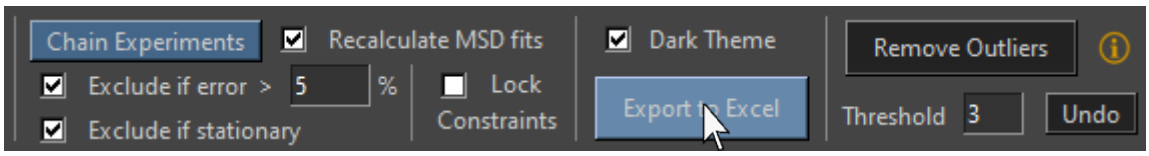


The distribution is convolved due to outliers and short tracks with variable MSDs. The Gaussian mixture model has difficulties correctly fitting the distribution. Compare this to the distribution from exactly the same data and system but filtering to only include tracks with an estimated fitting error of 5% (ground truth = 0.010, 0.040, 0.153 $\mu m^2/s$):



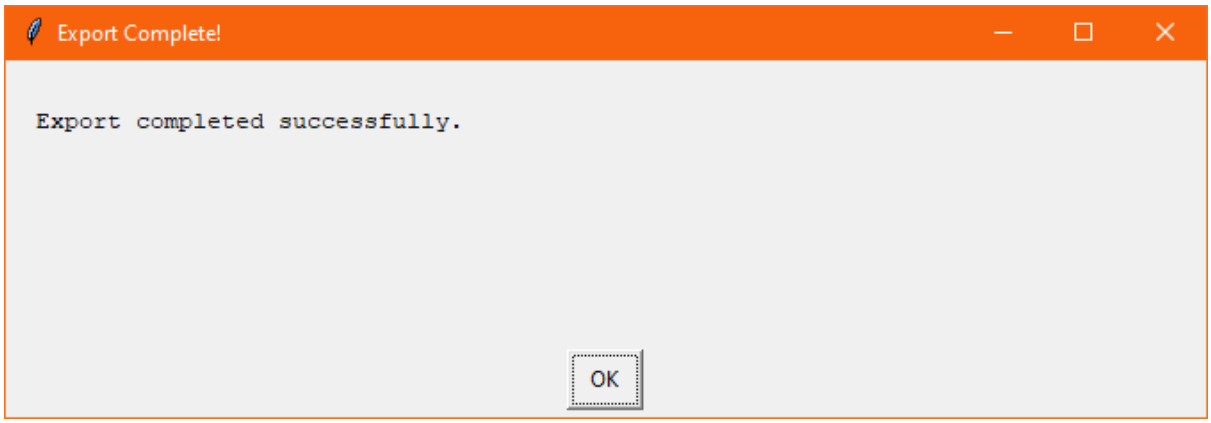
9.7 Exporting the Fitted Distribution to Excel

To export the fitted distribution to an Excel spreadsheet, click ‘Export to Excel’ in the toolbar:

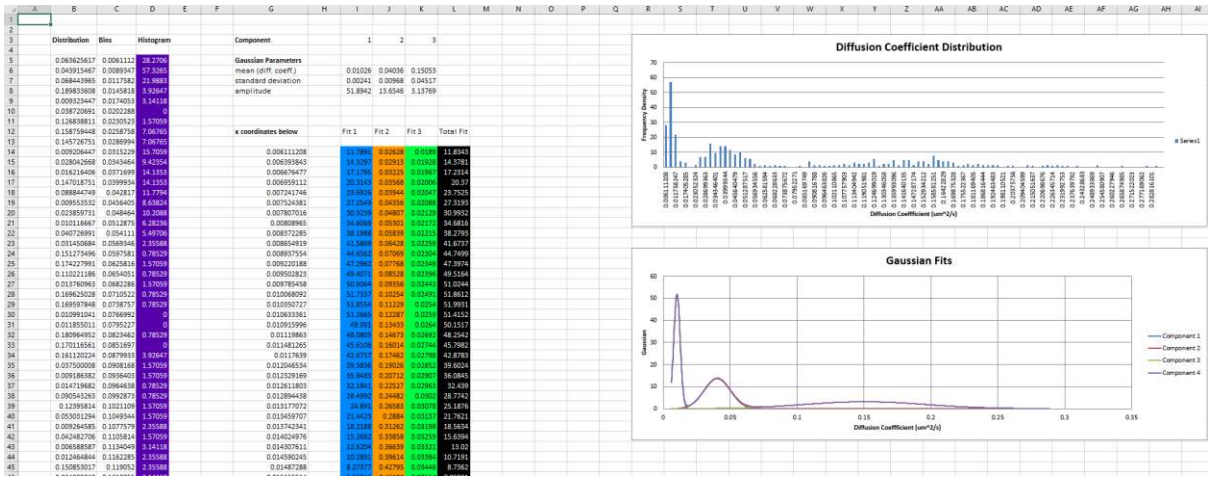


A file dialogue will then appear where you can save the data as an Excel ‘.xlsx’ file:

The data will then be exported and a message will appear when it has completed successfully:



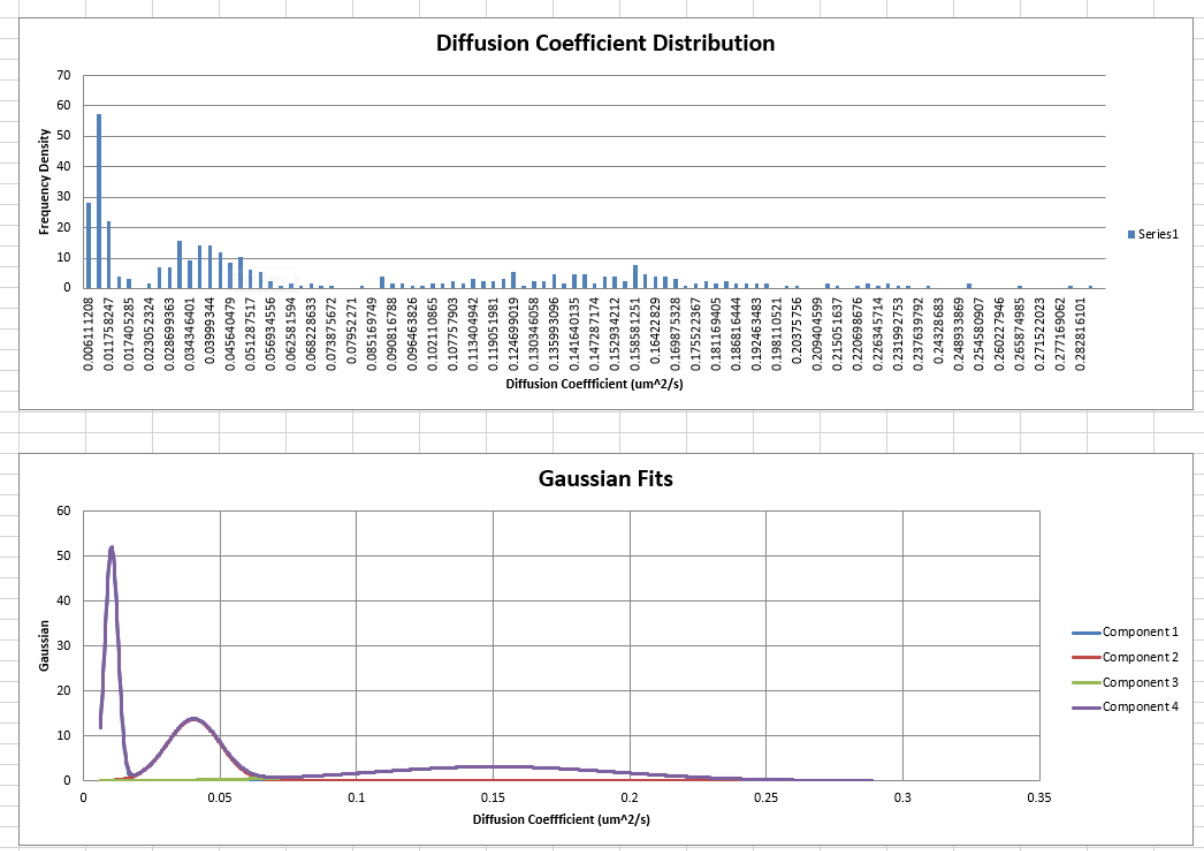
The Excel file is shown here:



On the left side is the distribution as a sequence of all diffusion constants of all tracks. Then the bins and histogram (purple) are listed exactly as what was plotted in the program. On the right of this is a list of the component fitting parameters, underneath which are the Gaussian functions in list form following the same colour layout as in the program:

Distribution	Bins	Histogram		Component	1	2	3	
0.063625617	0.00611121	28.27061		Gaussian Parameters				
0.043915467	0.00893473	57.32652		mean (diff. coeff.)	0.010258	0.040359	0.150533	
0.068443965	0.01175825	21.98825		standard deviation	0.002408	0.009684	0.045166	
0.189833608	0.01458177	3.926474		amplitude	51.89416	13.65462	3.137686	
0.009323447	0.01740529	3.141179						
0.038720691	0.0202288	0						
0.126838811	0.02305232	1.57059						
0.158759448	0.02587584	7.067653		x coordinates below	Fit 1	Fit 2	Fit 3	Total Fit
0.145726751	0.02869936	7.067653						
0.009206447	0.03152288	15.7059		0.006111208	11.78911	0.02628	0.018897	11.83429
0.028042668	0.0343464	9.423537		0.006393843	14.32965	0.029125	0.019278	14.37806
0.016216406	0.03716992	14.13531		0.006676477	17.17945	0.03225	0.019667	17.23137
0.147018751	0.03999344	14.13531		0.006959112	20.3143	0.035681	0.020062	20.37004
0.088844749	0.04281696	11.77942		0.007241746	23.69264	0.039442	0.020465	23.75255
0.009553532	0.04564048	8.638242		0.007524381	27.25487	0.043563	0.020875	27.3193
0.023859731	0.048464	10.20883		0.007807016	30.92385	0.048074	0.021292	30.99322
0.010116667	0.05128752	6.282358		0.00808965	34.60686	0.053006	0.021717	34.68158
0.040726991	0.05411104	5.497063		0.008372285	38.1988	0.058395	0.02215	38.27934
0.031450684	0.05693456	2.355884		0.008654919	41.58687	0.064277	0.02259	41.67373
0.151273496	0.05975808	0.785295		0.008937554	44.65619	0.070691	0.023038	44.74992
0.174227991	0.06258159	1.57059		0.009220188	47.29619	0.077679	0.023494	47.39737
0.110221186	0.06540511	0.785295		0.009502823	49.40713	0.085284	0.023958	49.51638
0.013760963	0.06822863	1.57059		0.009785458	50.90637	0.093555	0.02443	51.02436
0.169625028	0.07105215	0.785295		0.010068092	51.73371	0.102541	0.024911	51.86116
0.169597848	0.07387567	0.785295		0.010350727	51.85541	0.112294	0.0254	51.9931
0.010991041	0.07669919	0		0.010633361	51.26648	0.12287	0.025897	51.41524
0.011855011	0.07952271	0		0.010915996	49.99101	0.134328	0.026404	50.15174
0.180964952	0.08234623	0.785295		0.01119863	48.08053	0.146729	0.026919	48.25418
0.170116561	0.08516975	0		0.011481265	45.61059	0.160138	0.027443	45.79817

On the right of the document are plots of the histogram and fit as they were plotted in the program, but on separate graphs due to limitations with openpyxl:

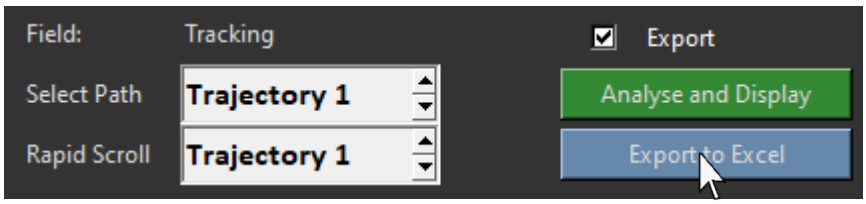


This spreadsheet is not recommended for final plotting; instead, the distribution can be exported to origin or GraphPad prism and refitted or plotted using the existing fit from the list of diffusion coefficients and Gaussian parameters.

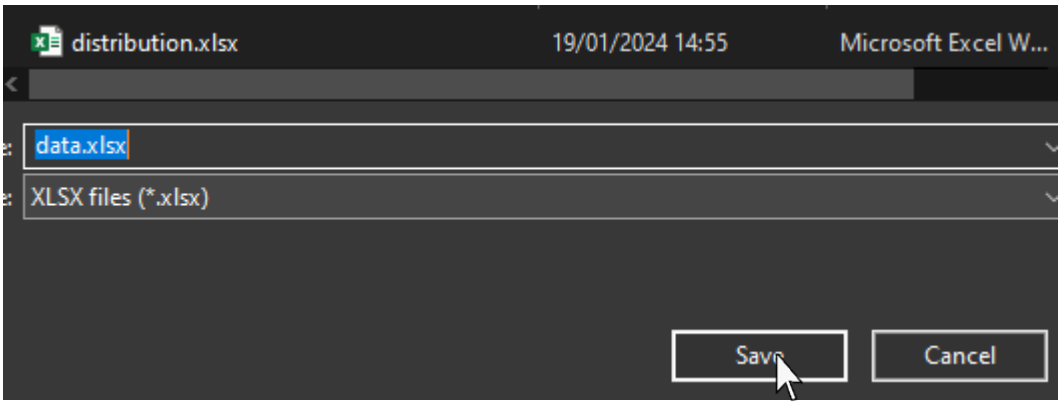
Chapter 10 – Exporting and Saving Data and Results

10.1 Exporting Tracking Data to Excel

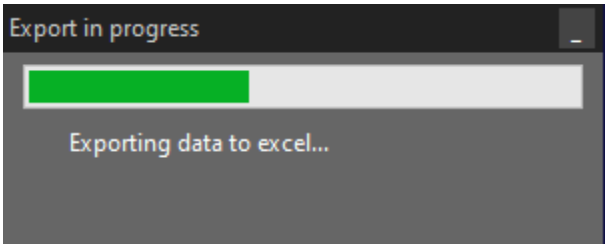
To export the data from the current active channel e.g., 'Primary Tracking Field', click 'Export to Excel' under the track plot:



A file dialogue will then appear where you can save the Excel '.xlsx' file:



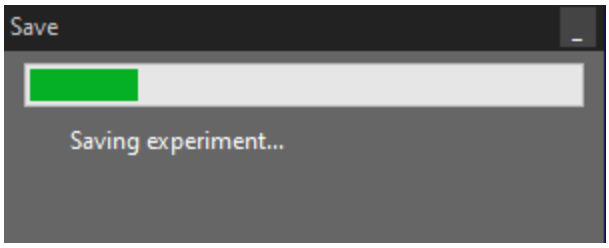
The program will then calculate the properties of each track based on the coordinates on demand as it exports them to ensure changes in the calibration are applied. A progress bar will be displayed as this happens:



Once the export has completed, another file dialogue will be displayed, prompting you to save the traces in .trcs format:

Finally, another dialogue will be displayed prompting you to save the experiment as a TXP document which can be reopened in the program in the exact current state including MSD fits, settings etc:

A progress bar will appear while the document is saved:



When opened the Excel spreadsheet will have each track and its information / time series listed in the first sheet 'Raw Data':

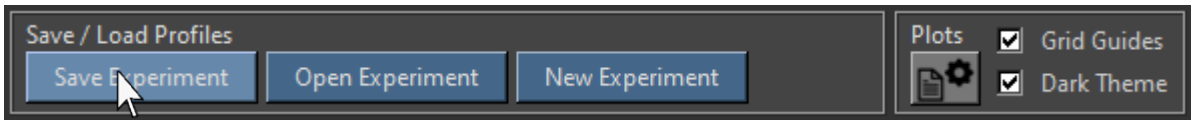
	A	B	C	D	E	F	G	H	I	J	K	L
1	Name:	Path 1									Name:	Path 2
2	First frame:	0									First frame	0
3	Last frame:	174									Last frame	138
4	Mean displacement (um):	0.126686									Mean displ	0.2002
5	Min. displacement (um):	0.012128									Min. displ	0.018853
6	Max. displacement (um):	0.29955									Max. displ	0.573742
7	Max. dark time (frames):	1									Max. dark	1
8	Blink count:	1									Blink coun	1
9	Min. Gauss. sigma [x, y] (pixels):	0.756	0.81								Min. Gaus	0.916
10	Max. Gauss. sigma [x, y] (pixels):	1.787	1.88								Max. Gaus	1.633
11	Min. Gauss. amplitude:	86.02485									Min. Gaus	99.01808
12	Max. Gauss. residual:	8.585711									Max. Gaus	6.011203
13	Min. Eccentricity (x/y, y/x):	0.662	0.624								Min. Eccer	0.657
14	Max. Eccentricity (x/y, y/x):	1.601	1.511								Max. Ecce	1.356
15	End-to-end displacement (um):	2.493343									End-to-en	3.675232
16	Total 'taught' path length (um):	21.91671									Total 'taug	27.42733
17	MSD fitting percentage:	25									MSD fittin	39
18	Diffusion Coefficient (um^2/s):	0.063626									Diffusion	0.142661
19	Std. Dev. (um^2/s):	0.001836									Std. Dev. (	0.012617
20												
21	Frame	Time (s)	x coord. (px)	y coord. (px)	displacement (um)	msd (um)	trace (A.U.)				Frame	Time (s)
22	0	0	8.612452634	181.9002753			242.3887603				0	0
23	1	0.1	9.205405423	182.3647561	0.12051	0.01958617	266.757686				1	0.1
24	2	0.2	9.922153399	181.7821813	0.14778	0.03826007	260.8310744				2	0.3
25	3	0.3	8.748727292	181.8960931	0.18863	0.060106697	237.5676033				4	0.4
26	4	0.4	8.306167359	182.1077327	0.07849	0.085109773	278.7980165				5	0.5
27	5	0.5	7.490472961	182.2198043	0.13174	0.111487913	319.957686				6	0.6
28	6	0.6	7.475127547	182.8891397	0.10712	0.135152245	130.9282645				7	0.7
29	7	0.7	6.484837841	183.1682817	0.16462	0.158570021	207.254876				8	0.8
30	8	0.8	7.025914069	183.0282587	0.08942	0.183607149	248.2532231				9	0.9
31	9	0.9	7.702480122	182.871802	0.11111	0.21247863	269.962314				10	1
32	10	1	8.042788882	182.5389066	0.07617	0.241136536	353.208595				11	1.1
33	11	1.1	8.827519344	183.0706889	0.15167	0.268902912	212.6109091				12	1.2
34	12	1.2	8.931859244	183.2895035	0.03879	0.297368664	291.4042975				13	1.3
35	13	1.3	9.636915973	183.3285273	0.11298	0.3292966	225.1332231				14	1.4
36	14	1.4	9.302614934	184.8596956	0.25076	0.356540419	208.5295868				15	1.5
37	15	1.5	9.094469939	184.7062699	0.04137	0.382024508	302.6512397				16	1.6
38	16	1.6	8.369101612	184.1424906	0.14699	0.407422874	277.6813223				17	1.7

The second sheet, 'MSD' contains a list of diffusion coefficients and MSDs for each track only along with the time taken until the particle stopped being tracked, most likely when it bleaches:

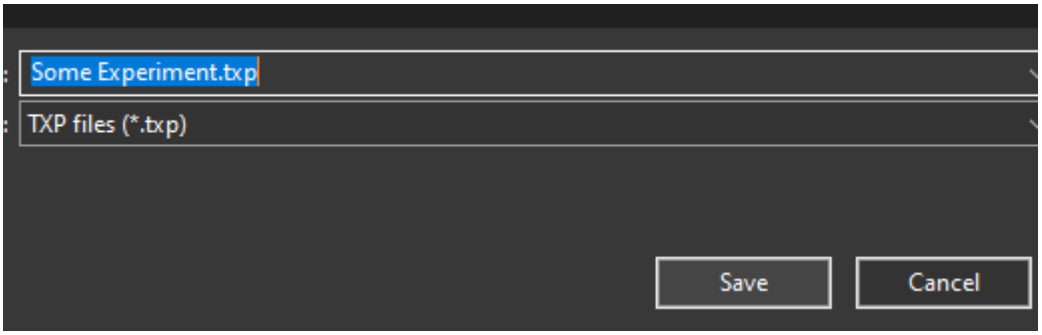
	A	B	C	D	E	F	G	H	I	J	K
1	name	Path 1	Path 2	Path 3	Path 4	Path 5	Path 6	Path 7	Path 8	Path 9	Path 10
2	diffusion coeff	0.063626	0.142661	0.00942	0.204042	0.043915	0.007287	0.068444	0.007331	0.005653	0.189834
3	bleaching time (s)	17.4	13.8	2.1	12.8	24.9	24.9	15.2	24.9	11.2	4.3
4		msd cumulative									
5		0.019586	0.052764	0.008801	0.069574	0.020336	0.005679	0.019488	0.00664	0.006712	0.074979
6		0.03826	0.109232	0.011948	0.135243	0.035497	0.009117	0.040312	0.01071	0.01024	0.147005
7		0.060107	0.162499	0.016083	0.198934	0.053521	0.013112	0.062793	0.015525	0.013674	0.220567
8		0.08511	0.211135	0.019976	0.281892	0.069988	0.016657	0.08773	0.020177	0.01882	0.300385
9		0.111488	0.265979	0.026123	0.356088	0.089039	0.020332	0.113293	0.024568	0.021864	0.389958
10		0.135152	0.333894	0.025893	0.43166	0.106265	0.02379	0.140437	0.028531	0.025167	0.462622
11		0.15857	0.397103	0.027107	0.492803	0.122058	0.027414	0.168748	0.033041	0.02668	0.535443
12		0.183607	0.462408	0.023865	0.551522	0.138576	0.030764	0.198636	0.035753	0.029132	0.603033
13		0.212479	0.535629	0.020836	0.626556	0.154918	0.033621	0.227387	0.039553	0.031326	0.696904
14		0.241137	0.609667	0.017597	0.703054	0.169266	0.036867	0.25726	0.042727	0.033566	0.779427

10.2 Saving Tracking Experiments as TXP Files

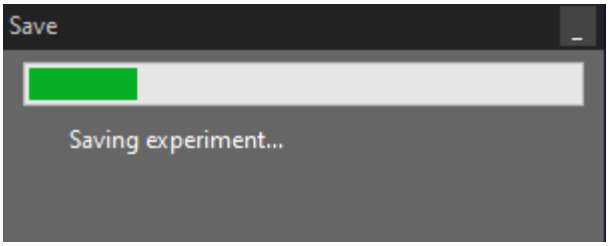
To save the whole state of TrackXpress including the raw data, enhanced data, analysis, MSD fits and settings into a single document that can be reopened, click ‘Save Experiment’ in the ‘Save / Load Profiles’ section in the bottom left of the control panel:



A file dialogue will appear where you can choose where to save the file:



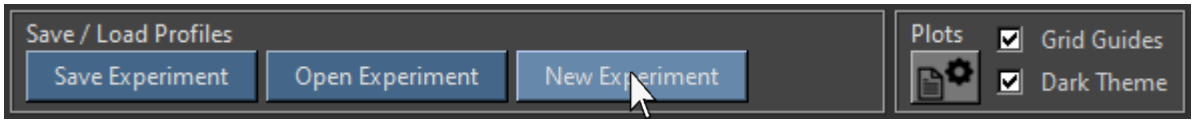
A progress bar will appear as the file is saved:



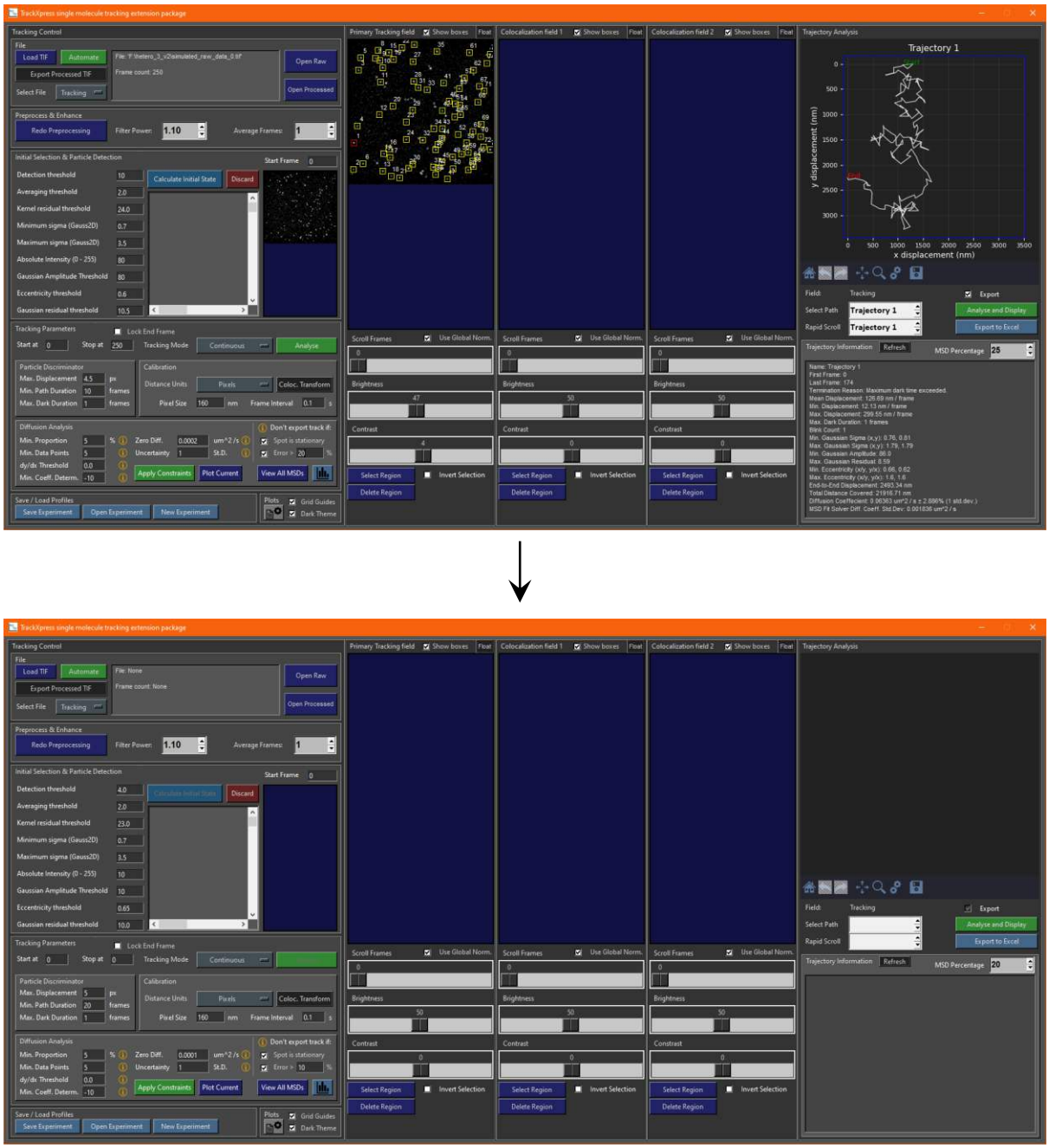
Once this is complete, the program can be closed, and next time the experiment document is opened it will load up exactly as it was when it was saved.

10.3 Resetting the TrackXpress GUI

To reset the interface to its default state ready for a new experiment, click ‘New Experiment’ in the control panel:

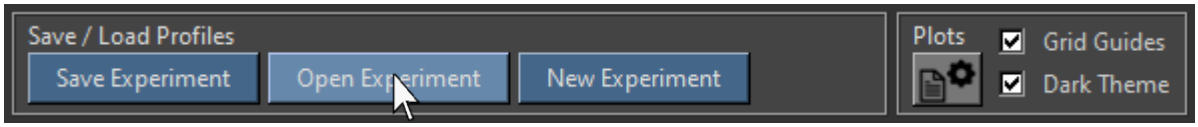


The current TrackXpress window will then close and reopen in the default state:

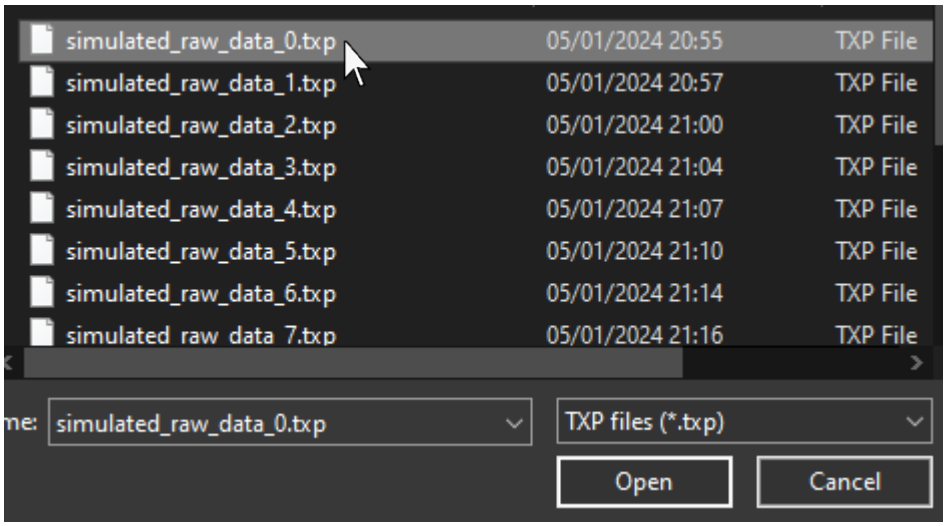


10.4 Loading a TXP File

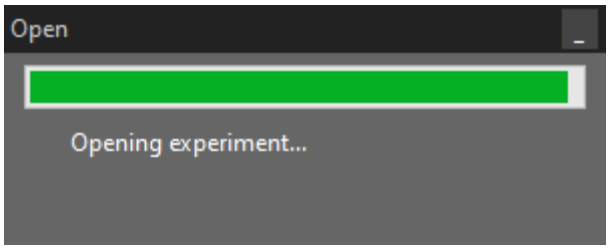
To load a .txp file, click ‘Open Experiment’ in the control panel:



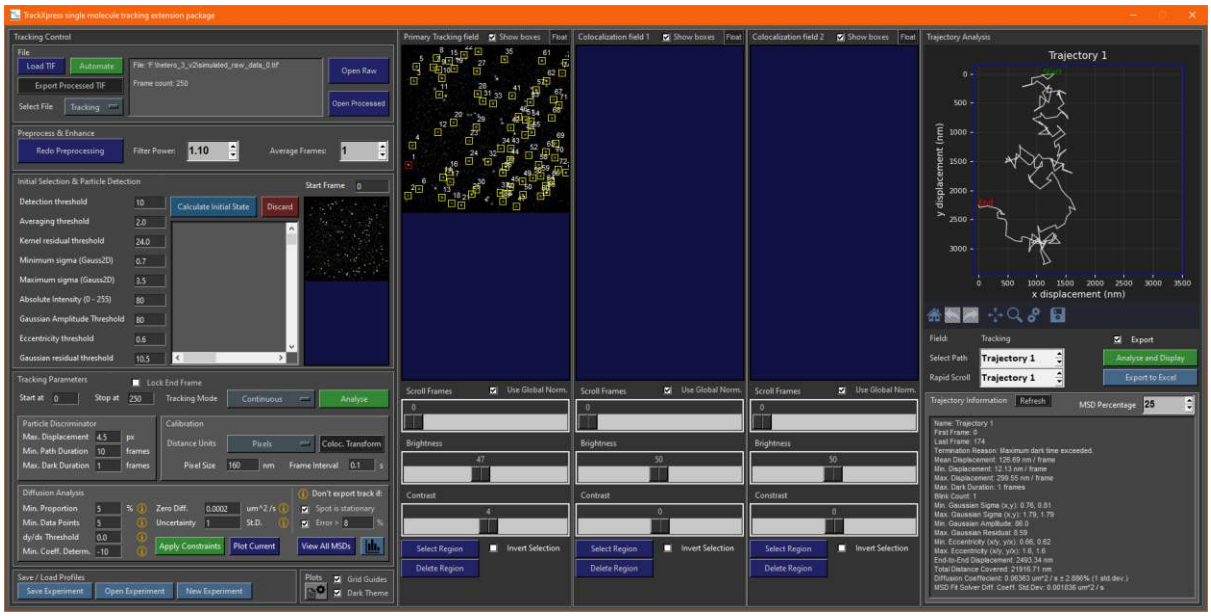
Then select the correct .txp file in the file dialogue:



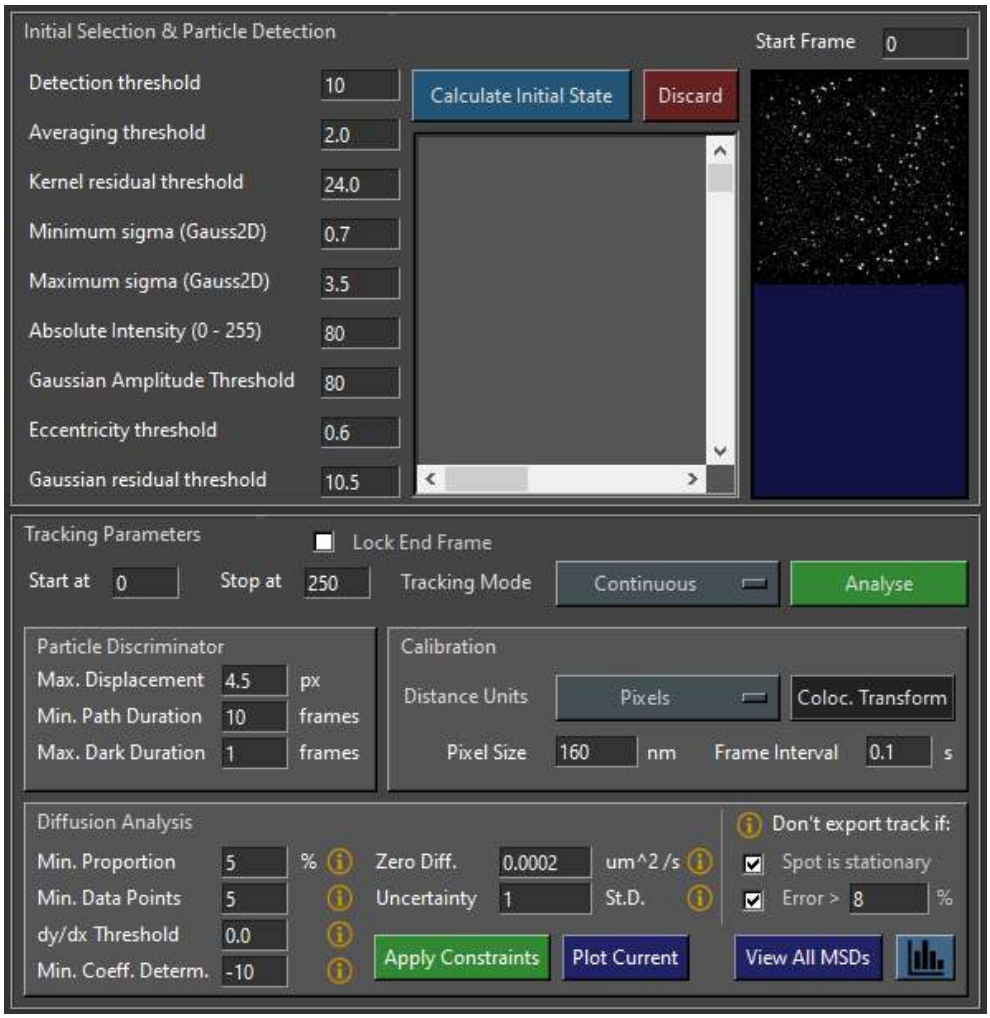
A progress bar will appear as the file is opened:



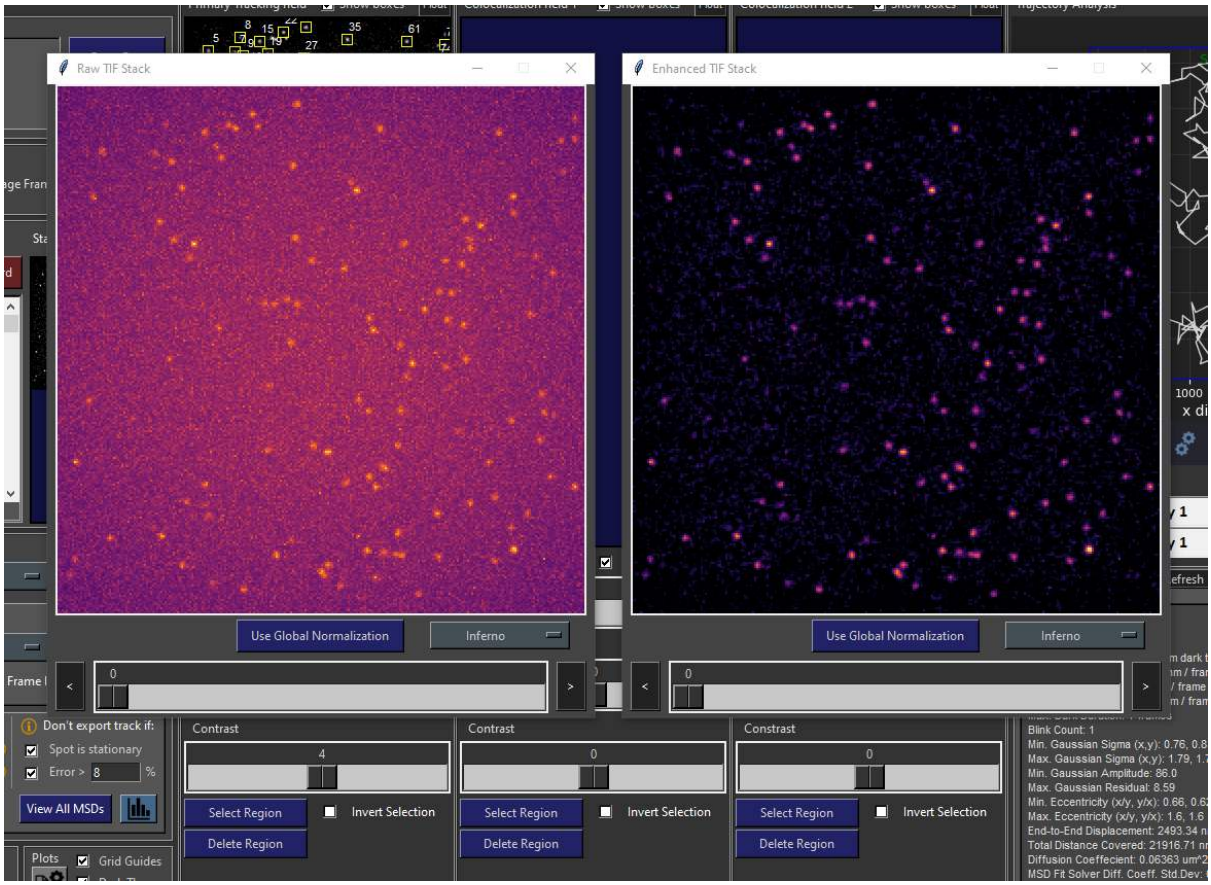
Once the .txp file has been opened the data and analysis that was saved will be opened in the exact state it was saved in:



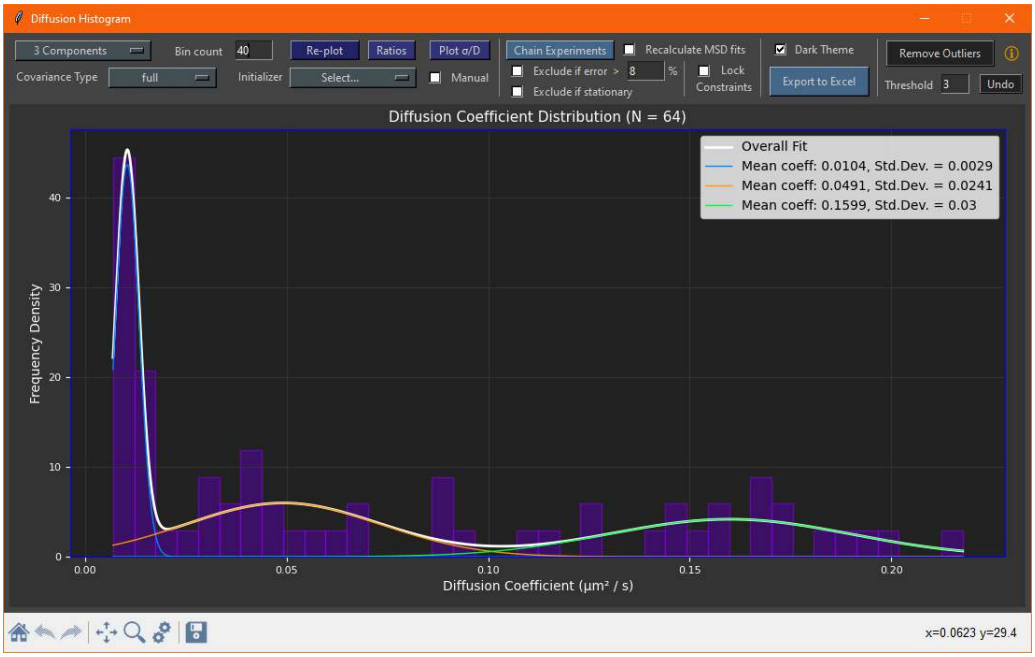
This includes all of the properties in the control panel:



And the raw / processed data:



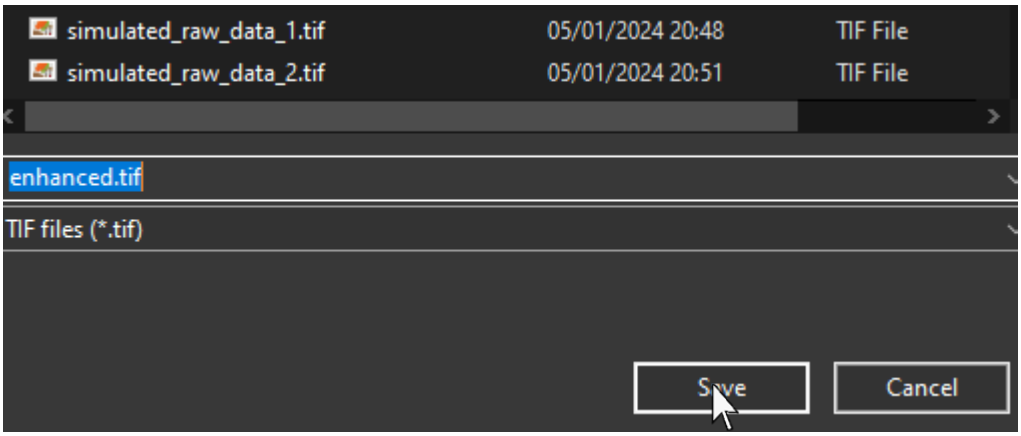
This means, the data can be re-tracked, or the settings recovered to track a different file with the same analysis settings. All tracking data is present so histograms can be replotted and / or exported:



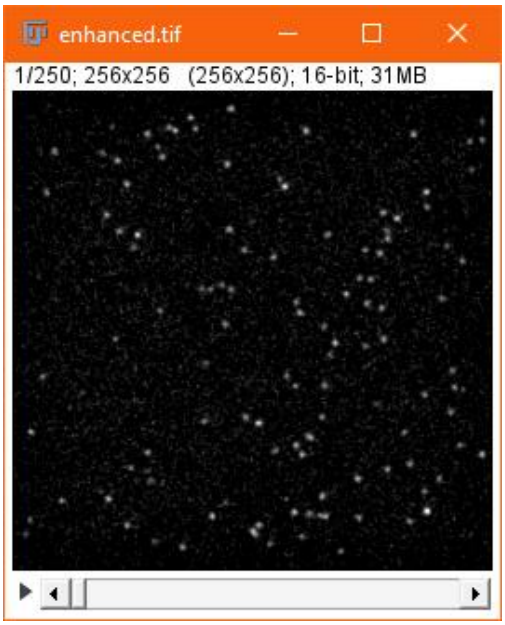
10.5 Exporting the Enhanced Tiff File

To export the enhanced frames of the movie as a .tif stack, click ‘Export Processed TIF’ in the file section of the control panel:

Then select a location to export the .tif file using the file dialogue:

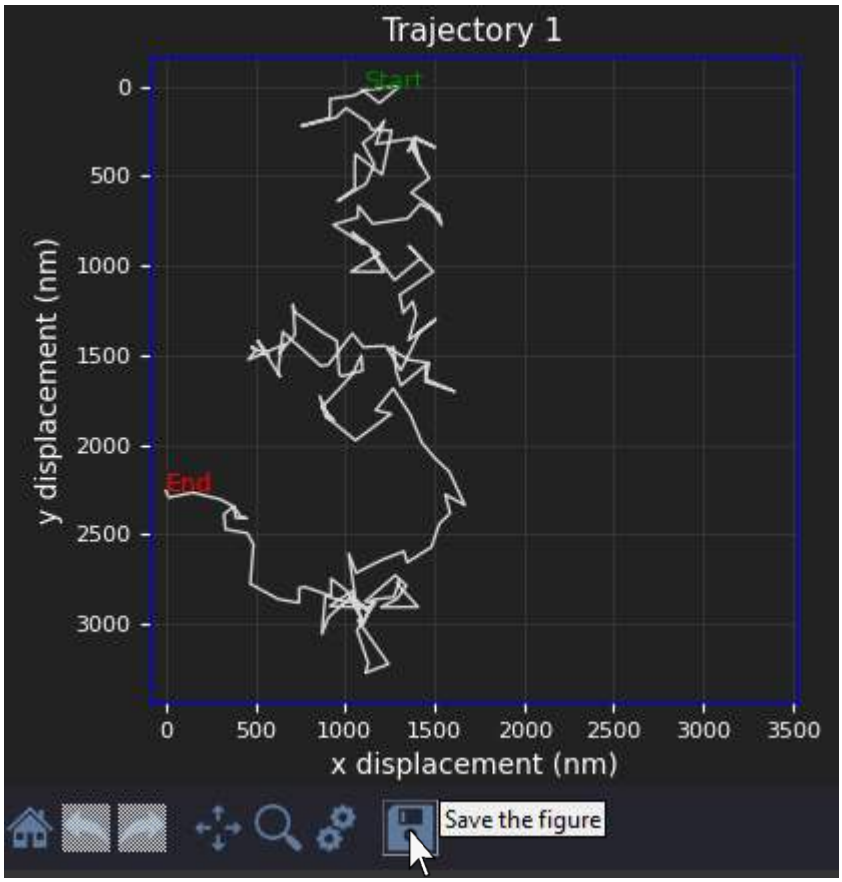


The enhanced data will be converted into a .tif file and saved in the specified location. It can then be loaded in an external software package such as ImageJ:

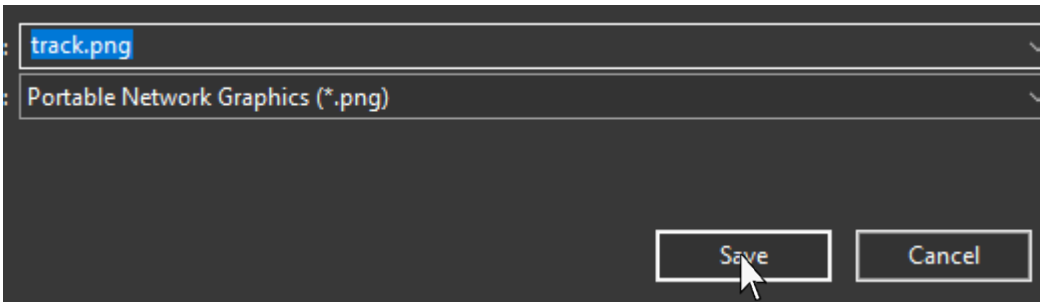


10.6 Saving Figures Directly from TrackXpress

Any graph in TrackXpress with a toolbar can be saved directly as a .jpeg / .png directly from the program. For example, in the track display canvas, click the save icon:



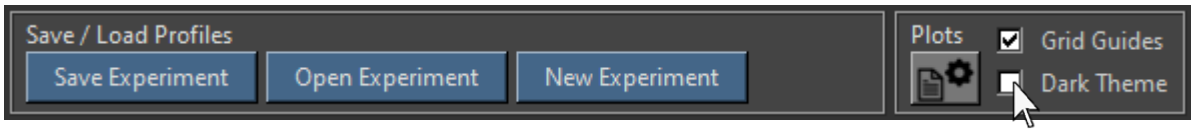
And then select a location to save it:



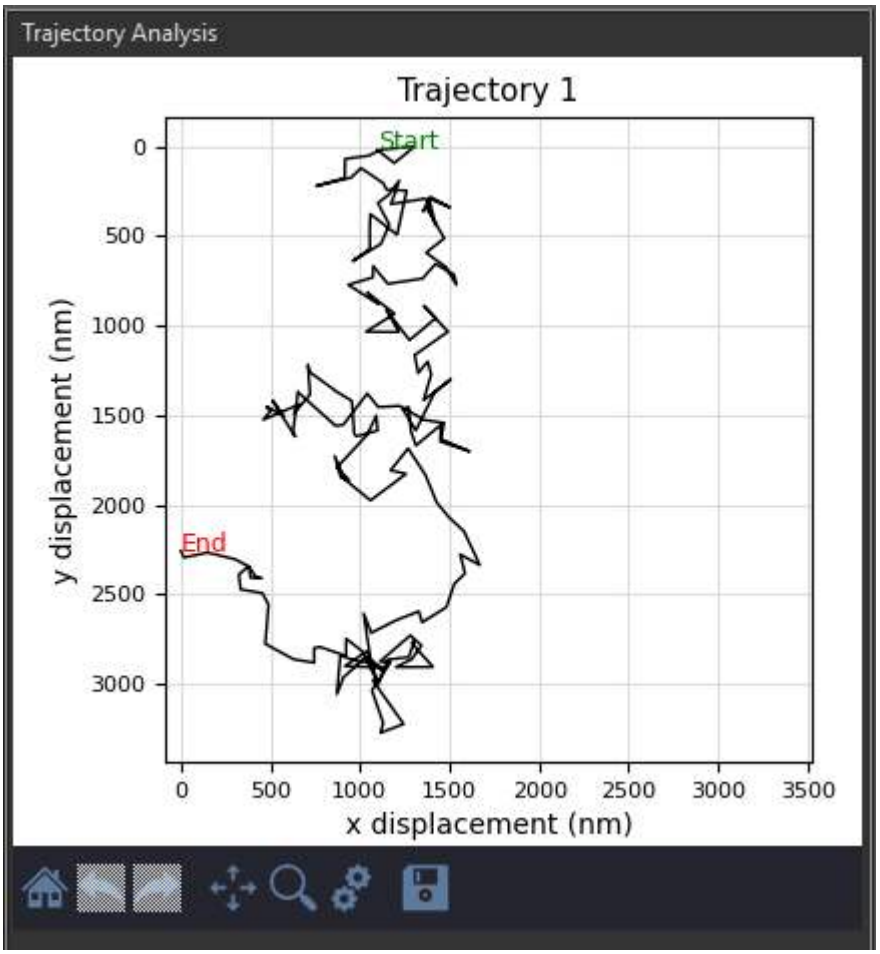
The magnifying glass icon and the directional arrows allow you to zoom with a zoom rectangle at specific parts of the plot and move it around respectively. Home resets the graph to the default view.

10.7 Plot Settings

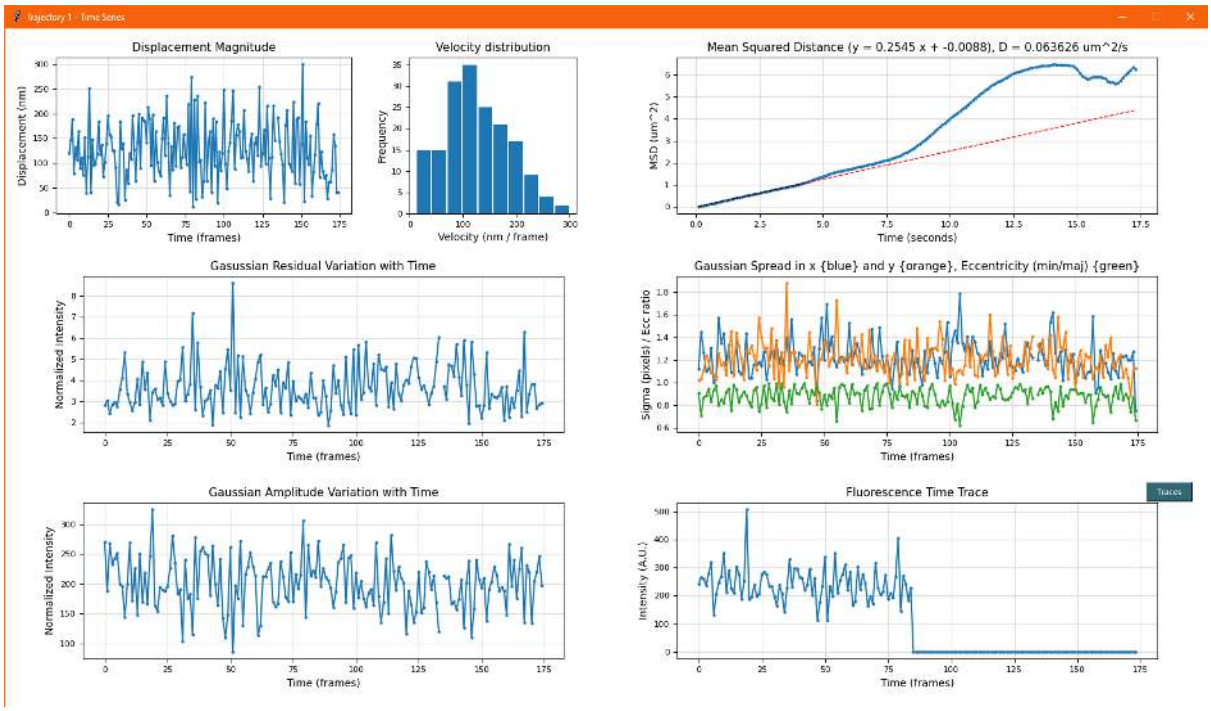
For presentations it is often inconvenient to display plots in ‘dark mode’ i.e., a light-coloured line on a black / grey background, since many presentations use a white background. To do this, untick the ‘Dark Theme’ checkbox in the bottom right of the control panel:

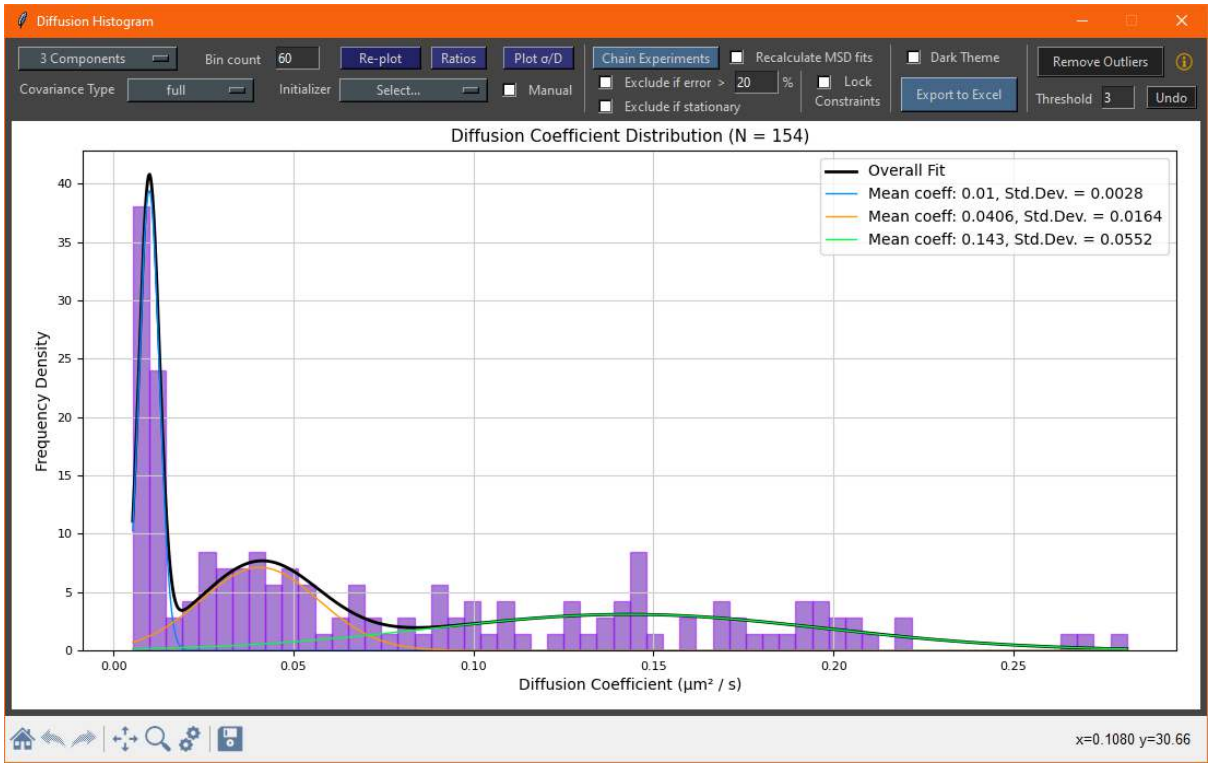


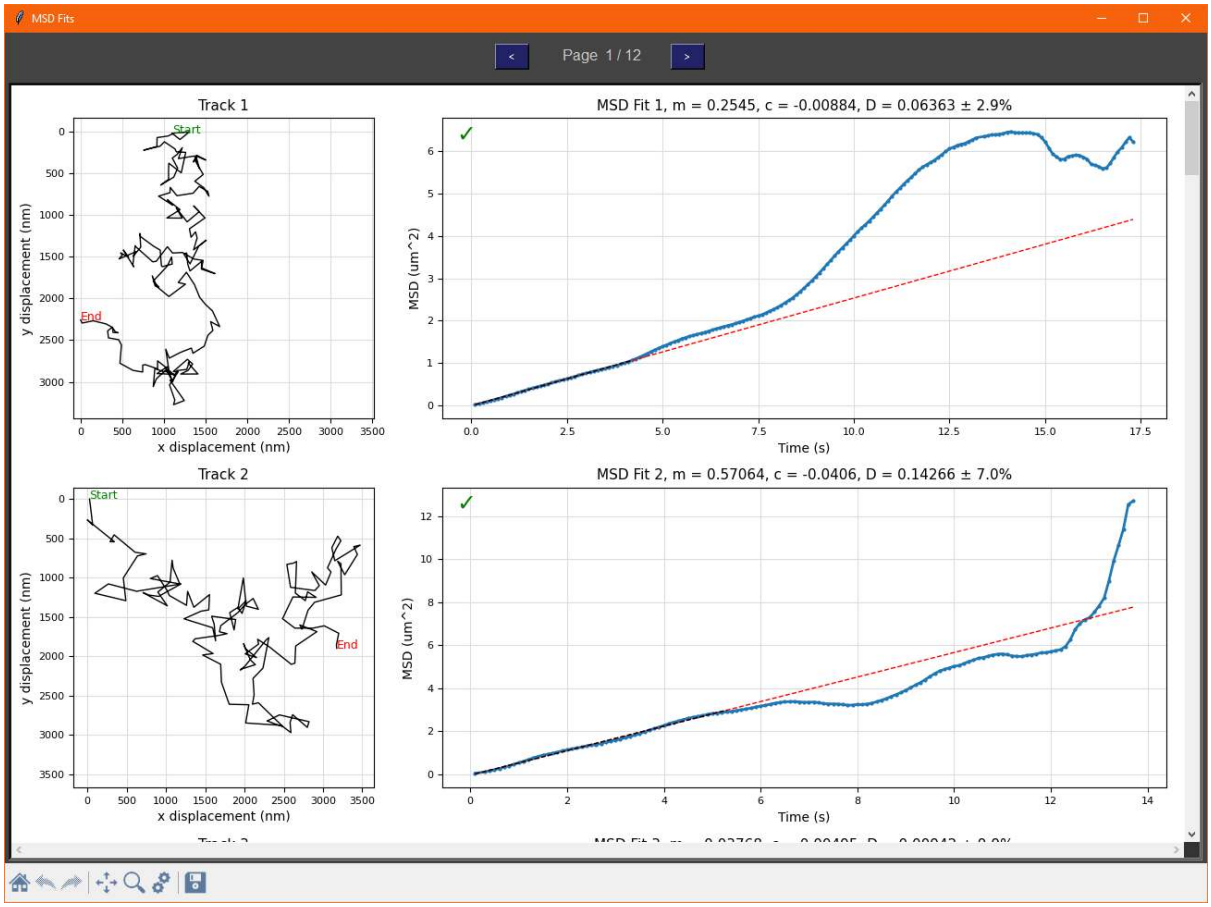
The display of the track will then change to light mode:



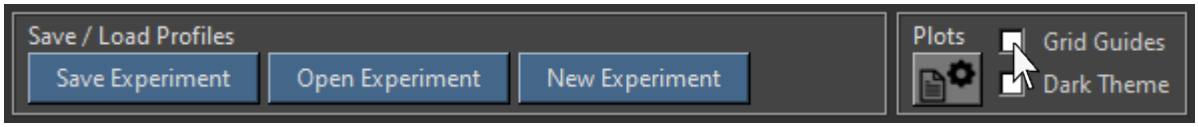
Any windows with plots in them will also be plotted in light mode the next time they are opened / reopened:



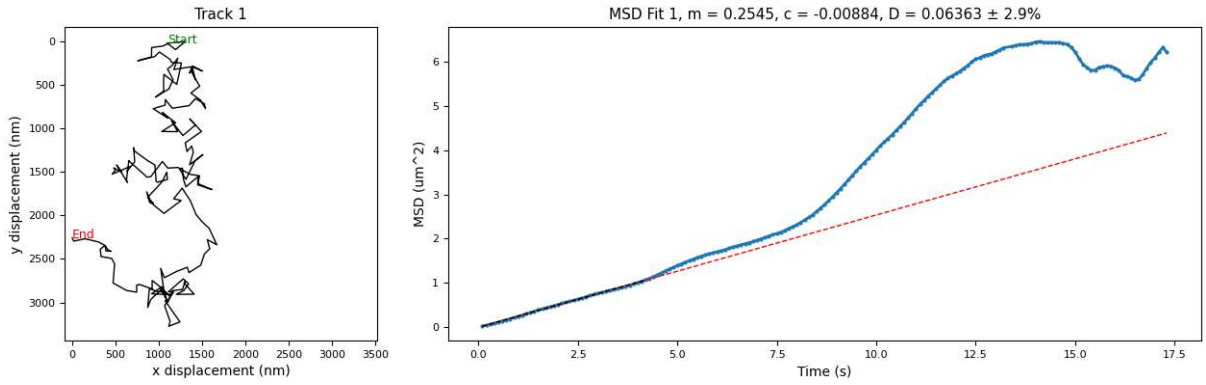




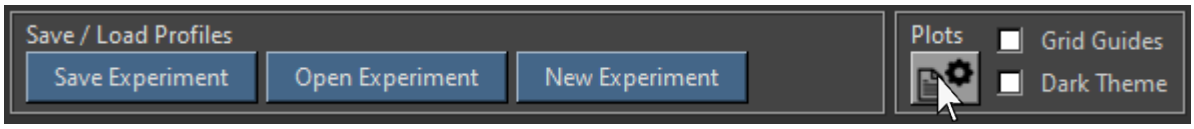
To disable gridlines on plots, untick the ‘Grid Guides’ checkbox in the control panel:



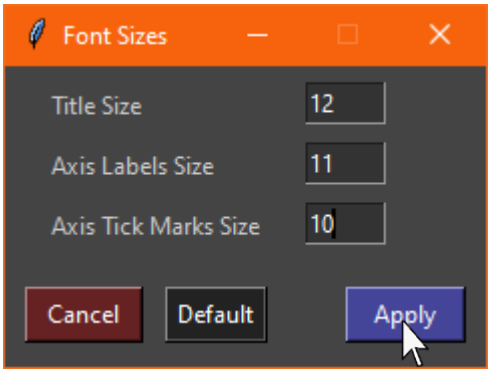
Plots will then be displayed without gridlines:

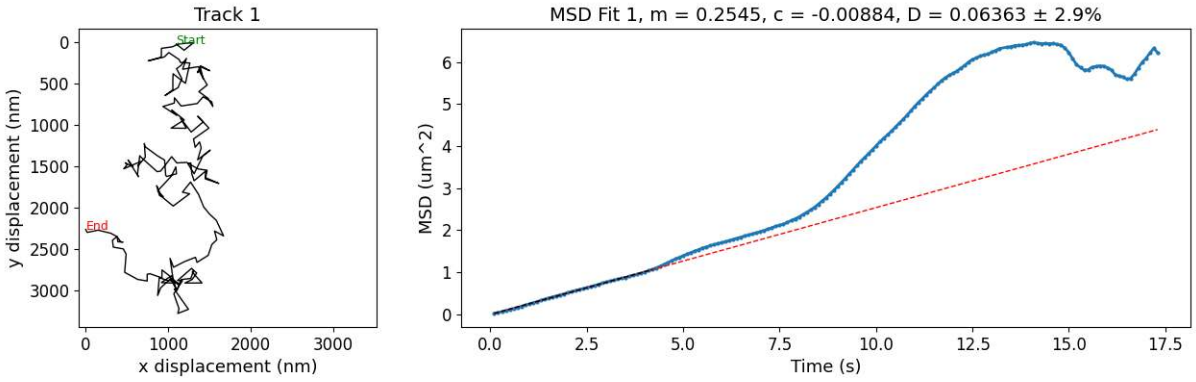


Font sizes can also be changed if text is too small. To do this, click on the text options icon in the control panel:



Then set the desired font sizes in the pop-up window and click 'Apply':





This will affect all plots next time they are refreshed and will be saved even if the program is closed and reopened. Defaults can be restored by clicking 'Defaults' then 'Apply'

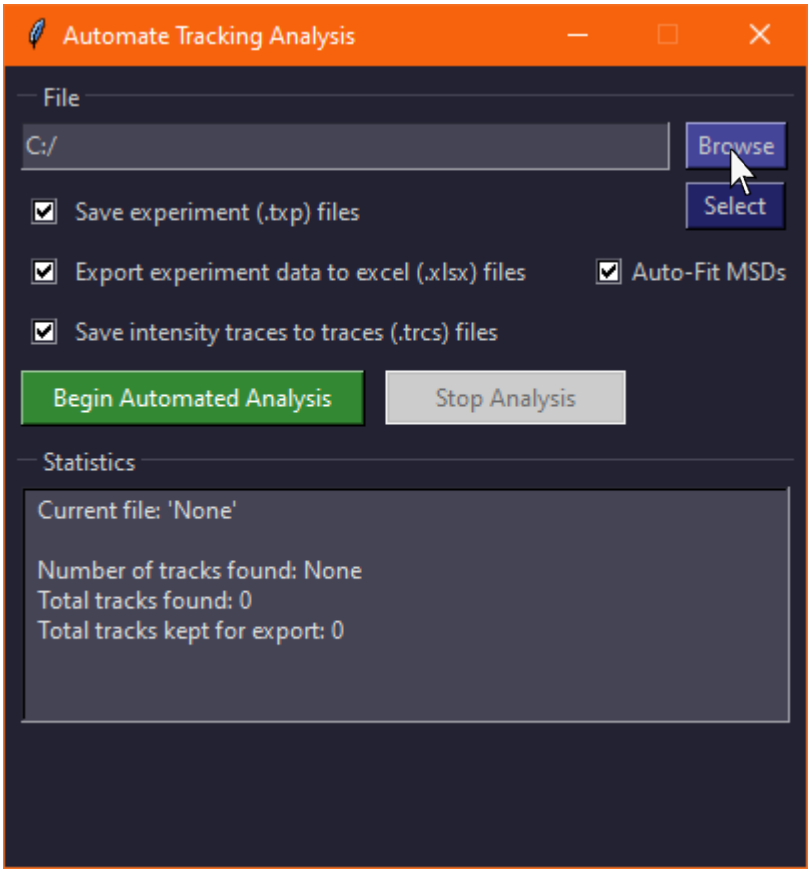
Chapter 11 – Tracking Automation

11.1 Setting Up Automated Tracking

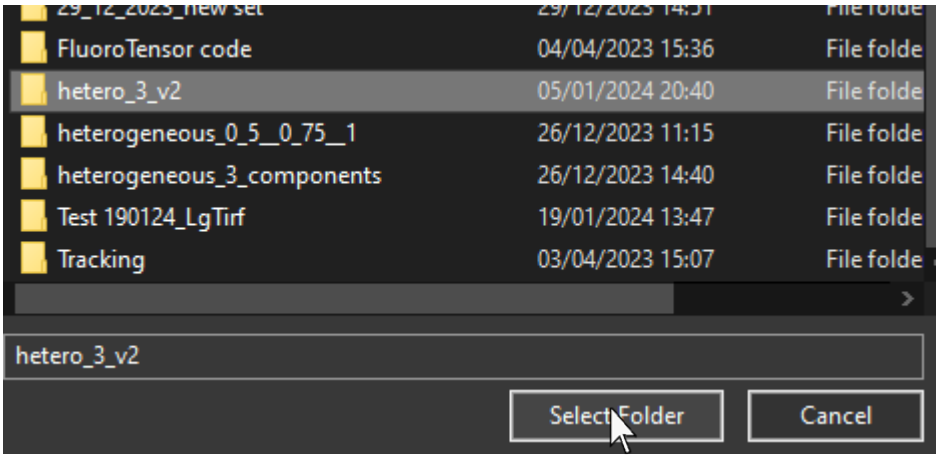
The first step of automated tracking is the same as manual tracking. All files should first be trimmed, and checked to make sure they are good using an external program such as ImageJ. Then they should be loaded into the TrackXpress Add-in and the tracking parameters set accordingly.

Then, to set up automated tracking, click 'Automate' in the file section of the control panel:

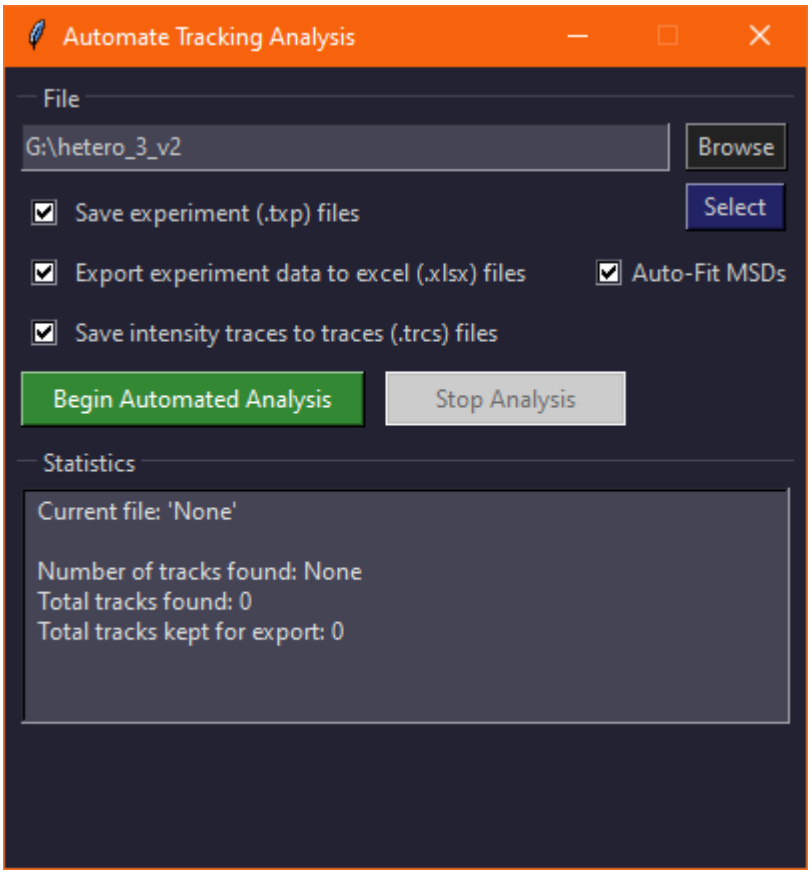
The automation window will then appear. Click 'Browse' to locate the folder containing the movies that should be tracked:



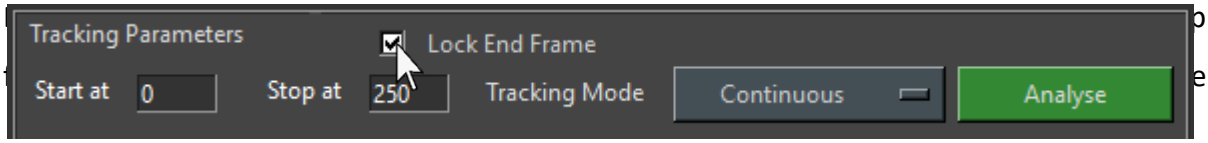
Select the folder from the file dialogue:



The folder name will then be displayed in the automation window and all .tif files in that folder will be automatically indexed and selected for automation:

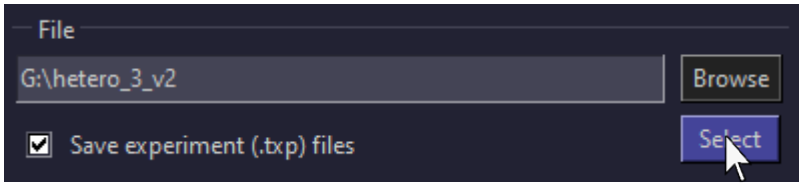


When a .tif file is loaded the end frame is set automatically in the tracking range. The start and end frame can be set manually. To ensure it doesn't change, tick 'Lock End Frame':

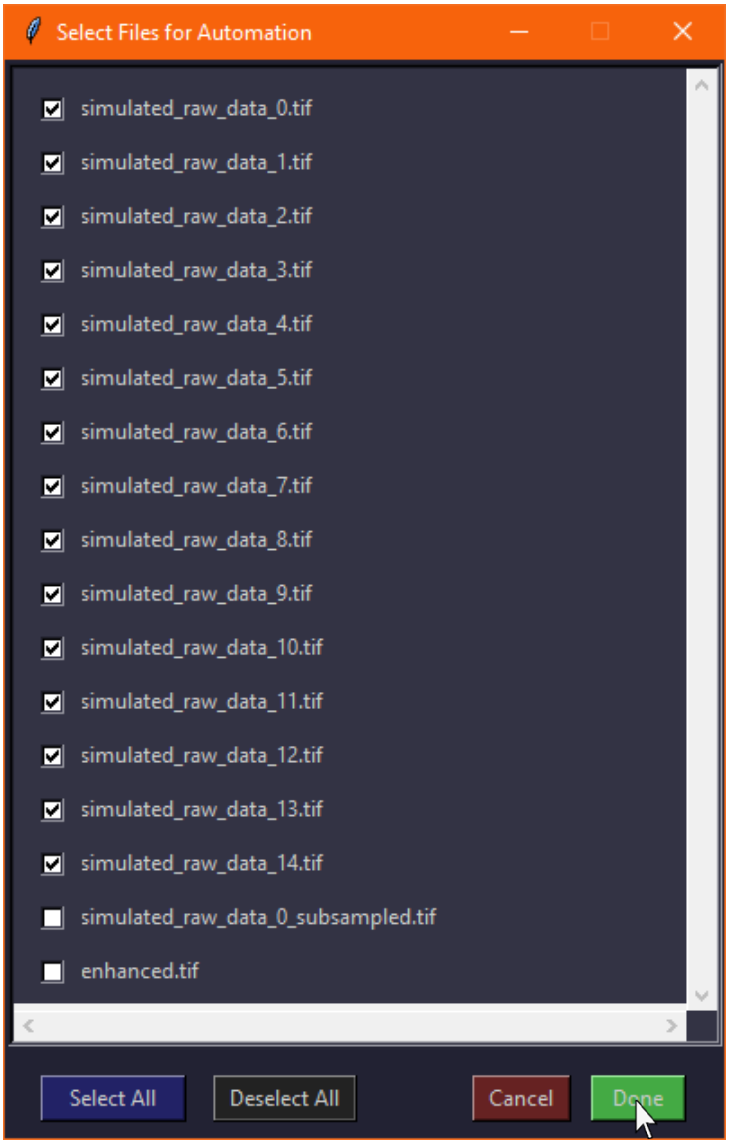


options can mostly be unchecked individually, however, traces cannot be calculated if Excel export is unchecked at this time.

By default, all files in the folder will be analysed. To select a custom list, click ‘Select’:

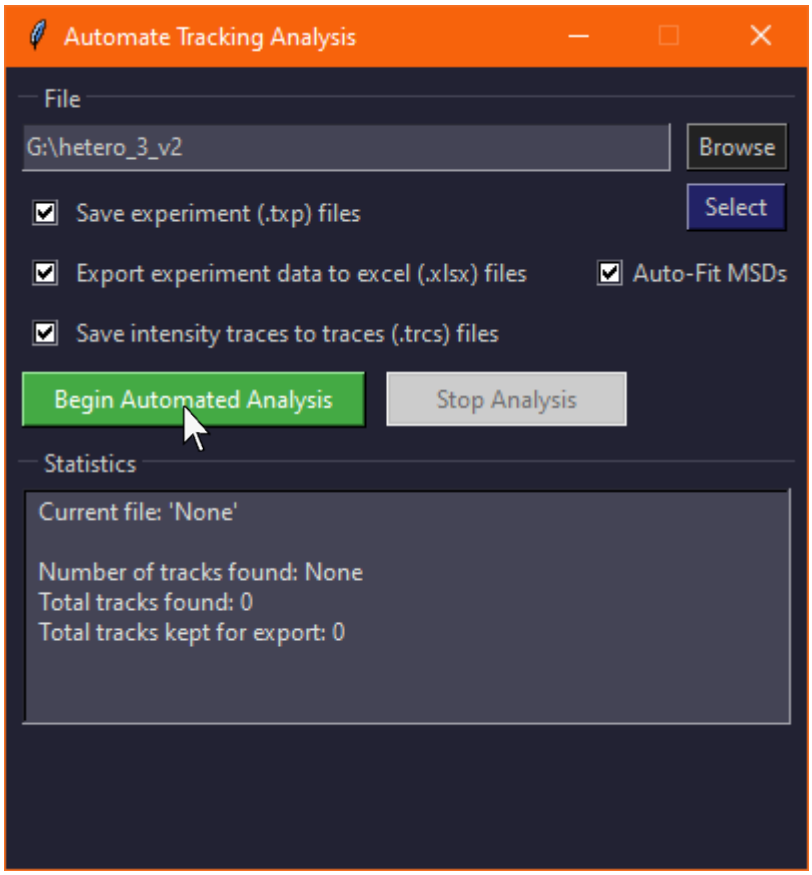


A selection window will then appear where files can be checked or unchecked to include / exclude them from the automation list, then click ‘Done’:

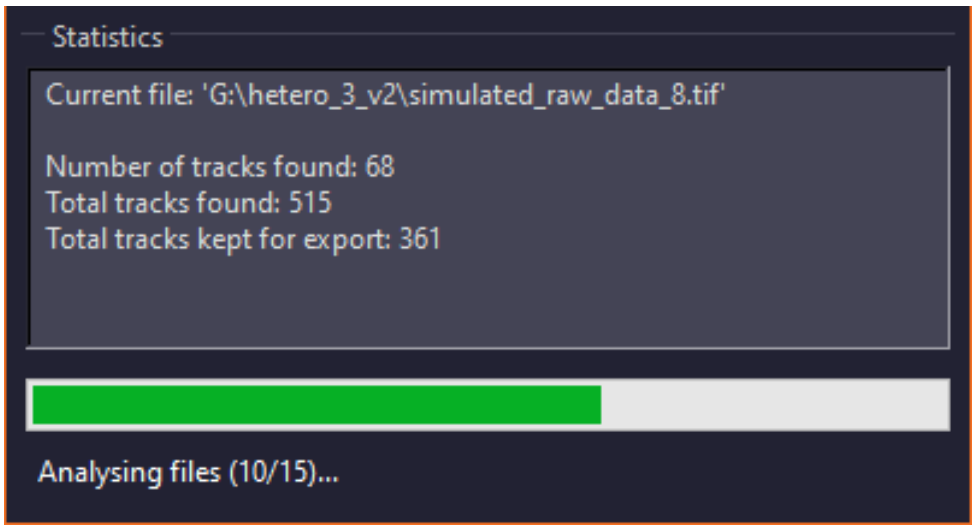


11.2 Automated Tracking

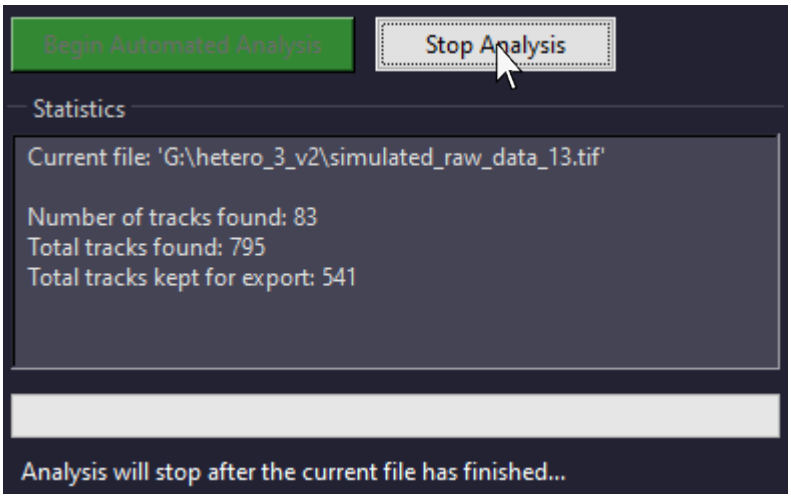
Once everything is set up, click 'Begin Automated Analysis' in the automation window:



As the files are analysed, the automation window keeps track of the last file that was completed and shows the number of tracks found in that file as well as the total number of tracks found so far in all analysed files and the number of those which were kept after filtering tracks based on estimated MSD fitting error:



To stop automated analysis, click ‘Stop Analysis’ and tracking will stop once the current file has completed:

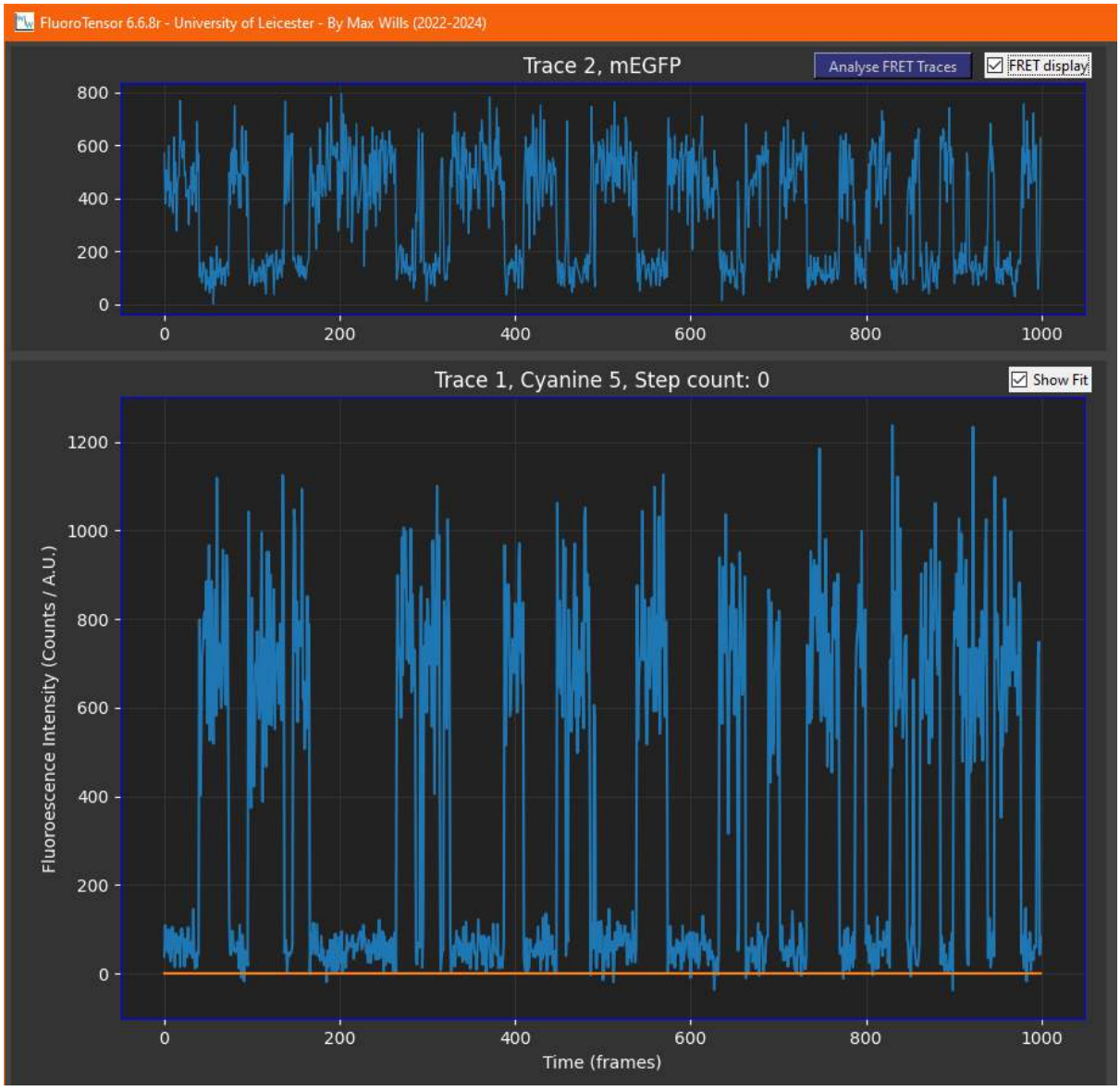


Appendix – Settings / Misc. Functions

FRET Analysis

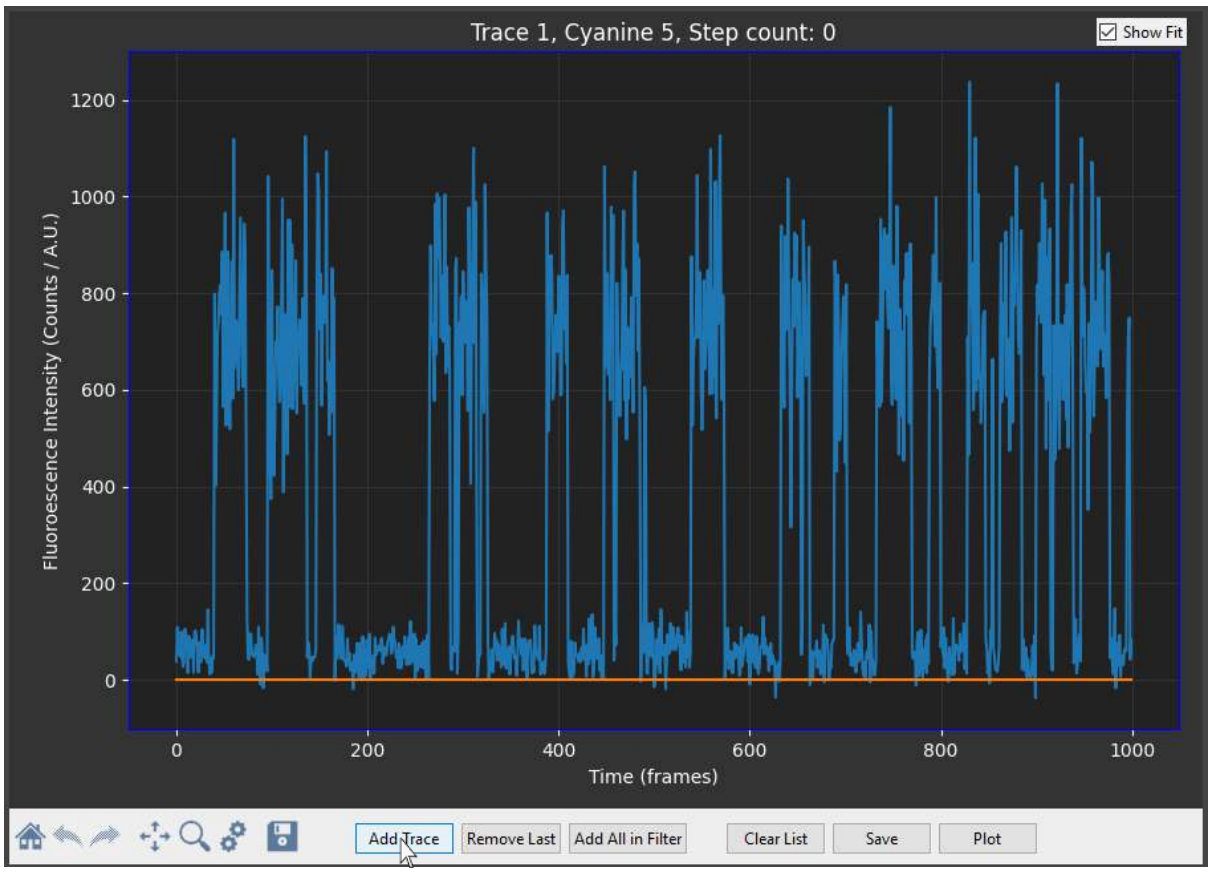
Currently, FRET analysis is not fully supported in FluoroTensor, traces can be fitted but the process is largely manual and there are no options to export the analysis at this time.

After analysing colocalization and calculating traces from a FRET experiment, enable 'FRET display' in the main interface by ticking the checkbox in the top right of the graph panel:



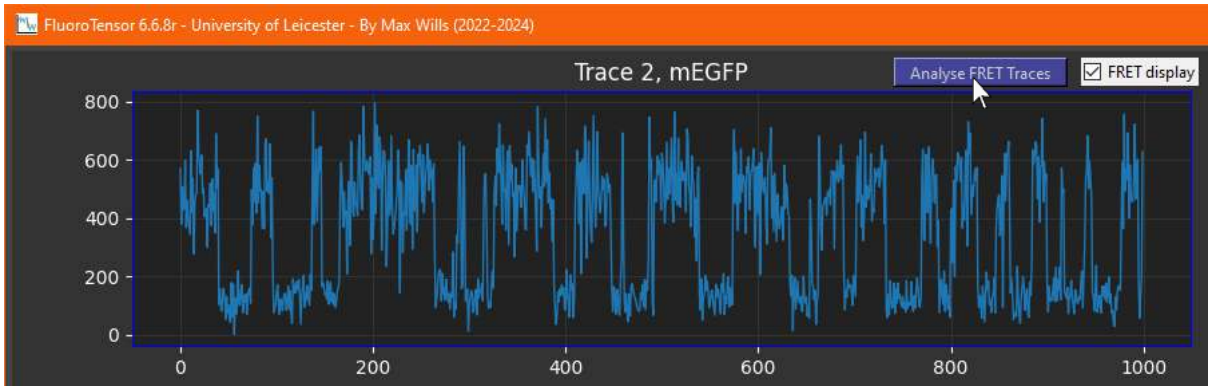
This places the marker trace in the lower (main) canvas and the colocalized anti-correlated trace in the upper (secondary) canvas. At this time, traces will have to be checked visually to see if they're anti-correlated.

If you are satisfied the two traces are an anti-correlated FRET pair, click ‘Add Trace’ in the toolbar below the main graph canvas:



If ‘FRET display’ is enabled, this will add the pair of traces currently being displayed will be added to a separate dataset which persists even if new traces are loaded into the main interface. These traces can be saved as .trcs files.

Once the dataset has been curated and any number of FRET pairs added, click ‘Analyse FRET Traces’ at the top of the graph panel:

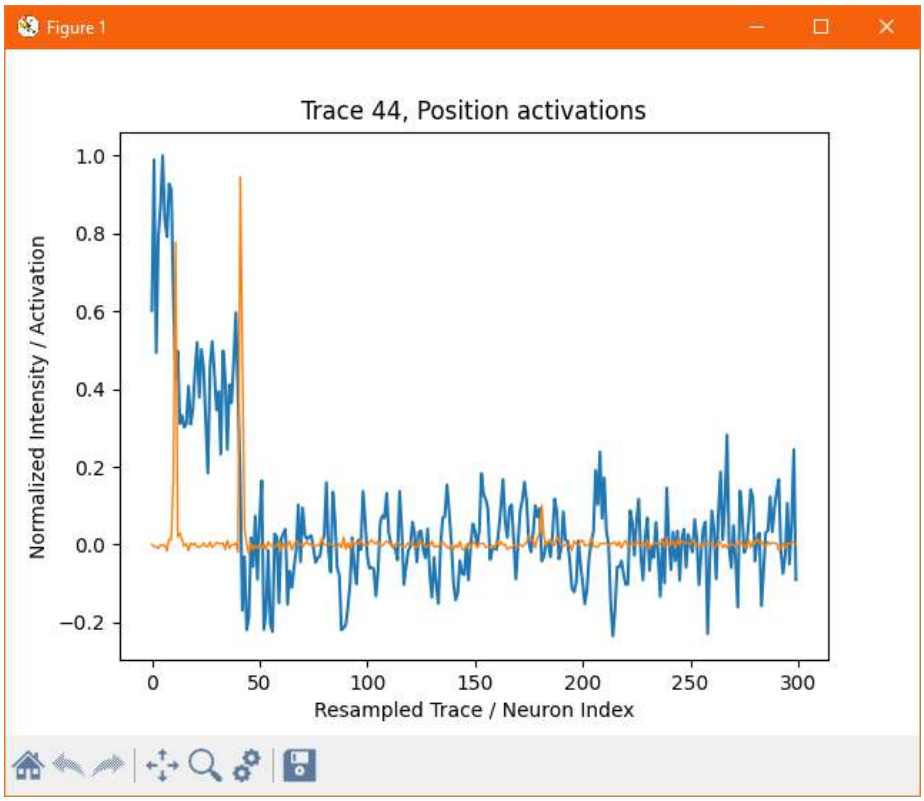


The FRET Analysis window will then appear and use a Gaussian mixture model to threshold on and off states to fit the trace automatically:

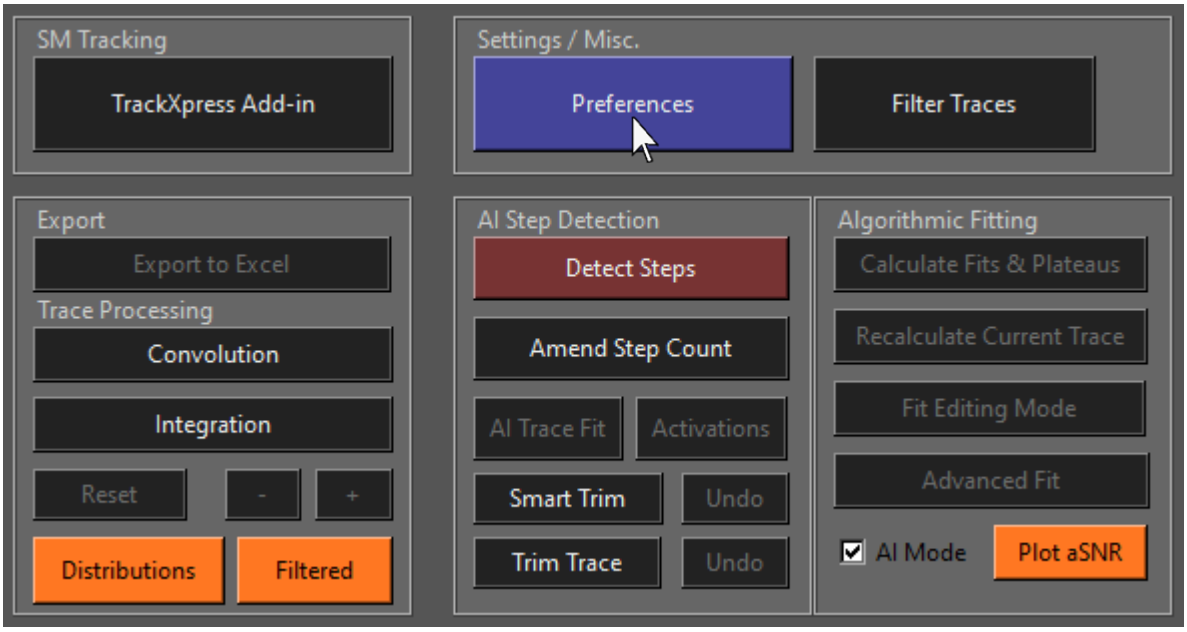
Since this is only a demonstration of a prototype feature, only first order kinetic information is calculated and the data cannot be exported at this time.

Integrating Custom Neural Network Models

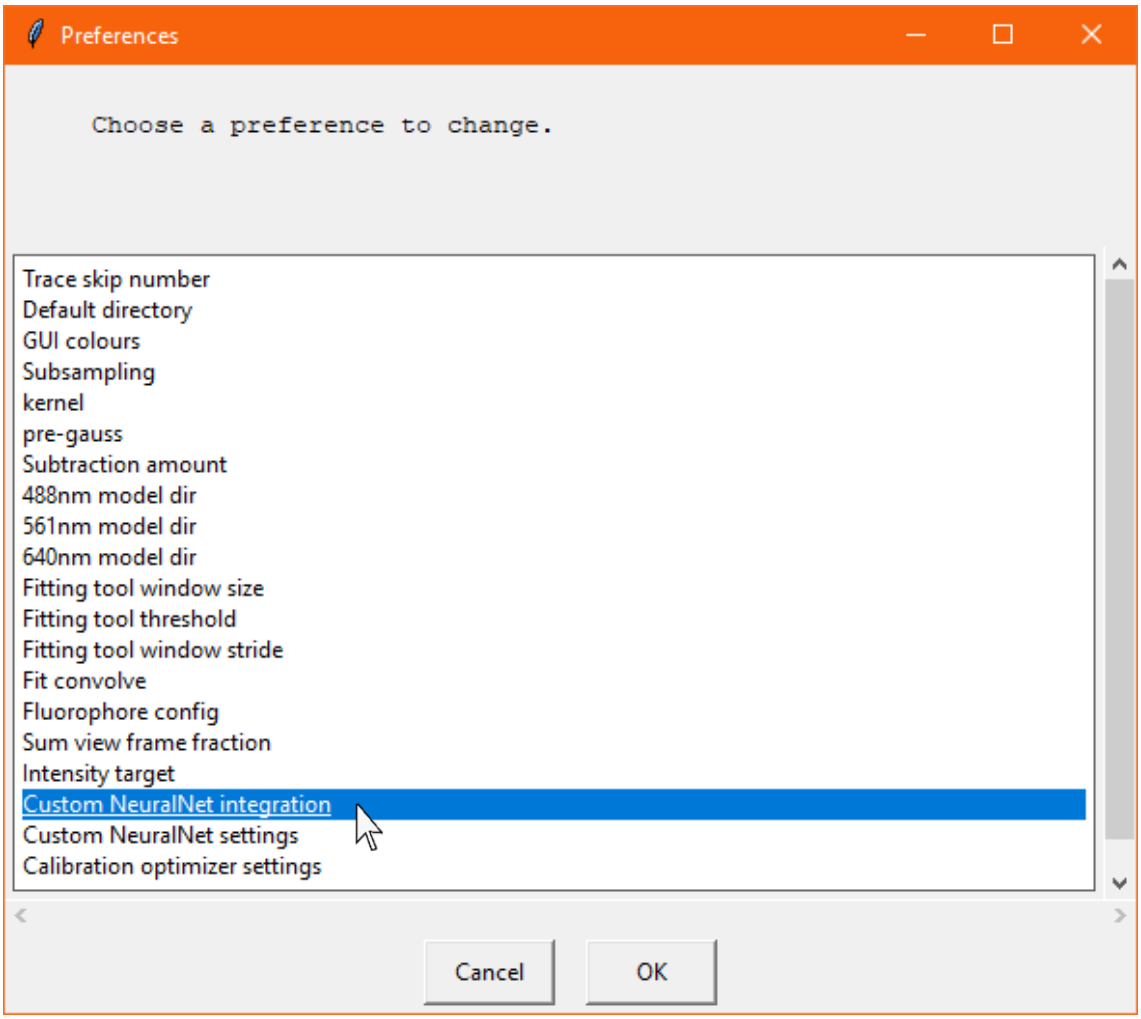
FluoroTensor, as a platform, was designed such that it can be used immediately with pre-supplied general prediction models for step detection and trace fitting. These may not always perform optimally and are limited by the number of frames traces must have as an input for reliable results. Thus, we have designed the software to allow the integration of neural networks trained by end users. In this example we will demonstrate how to integrate the 2019 Xu et al CLDNN model into FluoroTensor. CLDNN models have their own normalization requirements that were implemented; however, other networks are required to use standard normalization of data in the training set such that the mean of a fully bleached trace is centred on 0 (noise can dip into the negative) with a maximum value of 1 for the highest intensity in the trace as in this example:



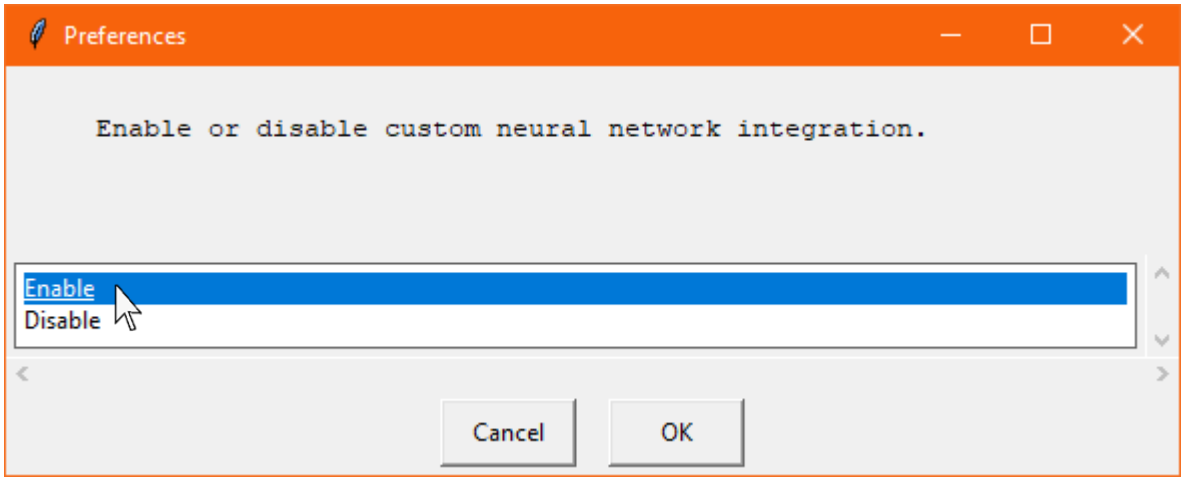
To integrate a custom neural network into FluoroTensor, click ‘Preferences’ in the main control panel of the main window:



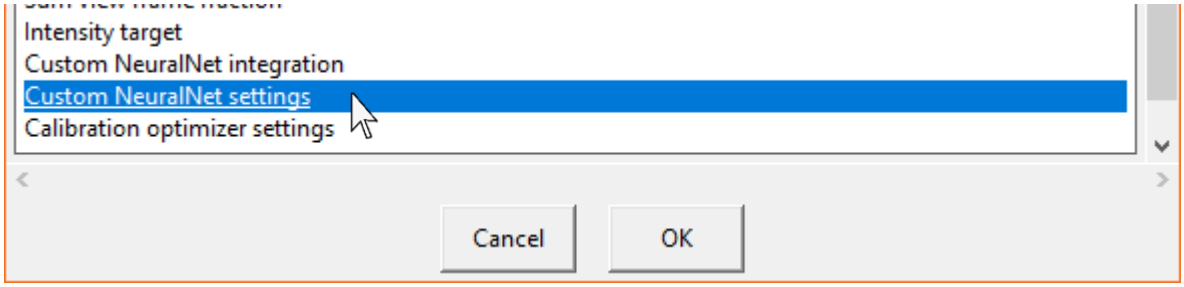
Then select ‘Custom NeuralNet integration’ from the available settings:



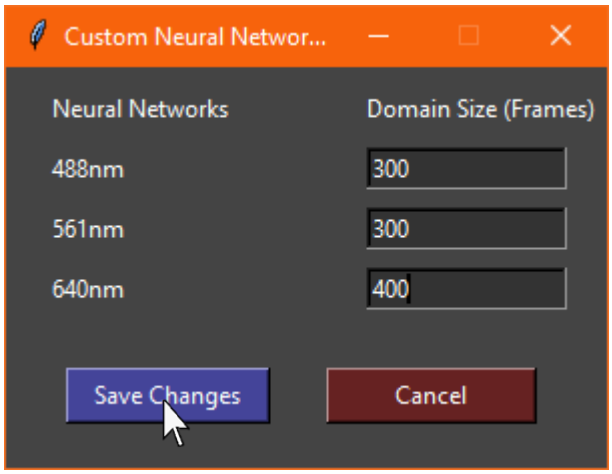
Then select ‘Enable’ in the list box and press OK:



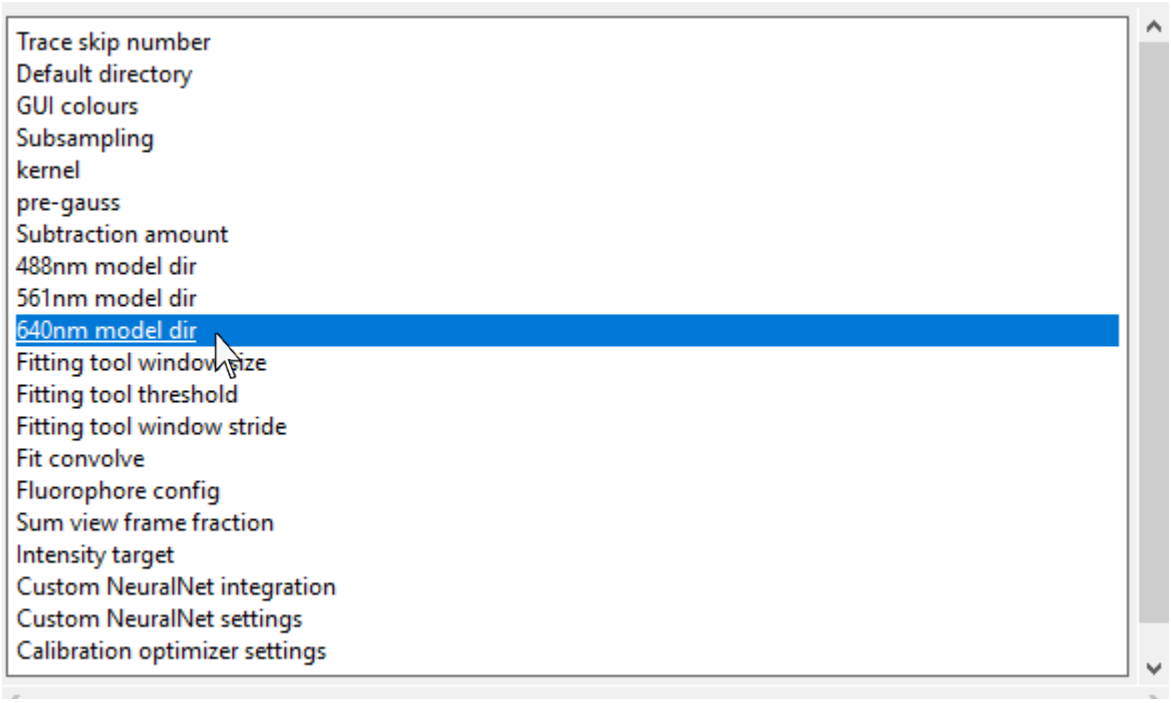
This means FluoroTensor will no longer attempt to get the required trace resampling length by parsing the name of the neural network. Instead, it will use the custom neural network resampling lengths from the preferences. To set these, click ‘Custom NeuralNet settings’:



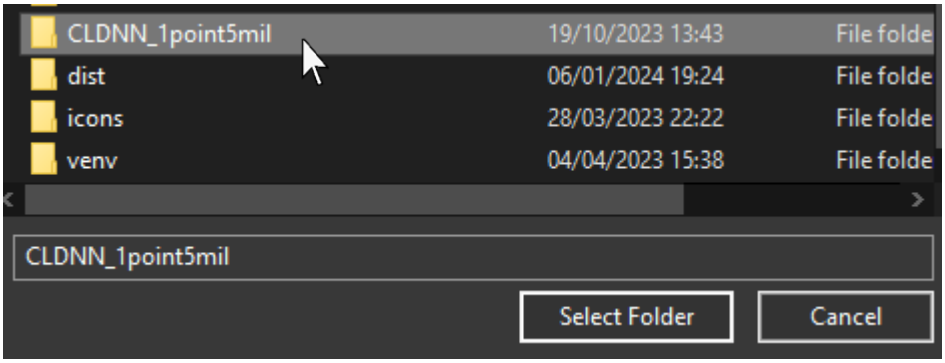
Ensure that the resampling length matches the number of input neurons in the custom neural network for the correct channel in the settings window that appears and save changes:



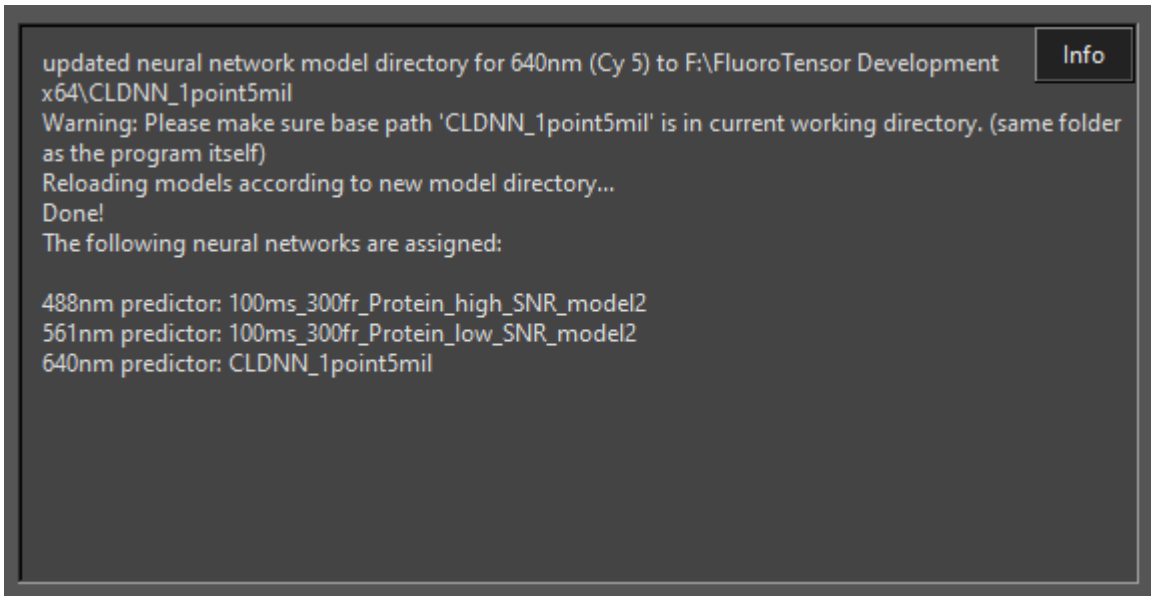
Finally, make sure the neural network is saved in the current working directory of the program like the other models and select the relevant model directory setting to load the neural network for the relevant channel(s):



Then select the model folder in the file dialogue:



The model will then be loaded for the selected channels and information displayed in the status box:



Next time steps from that channel are detected, the custom model will be used: