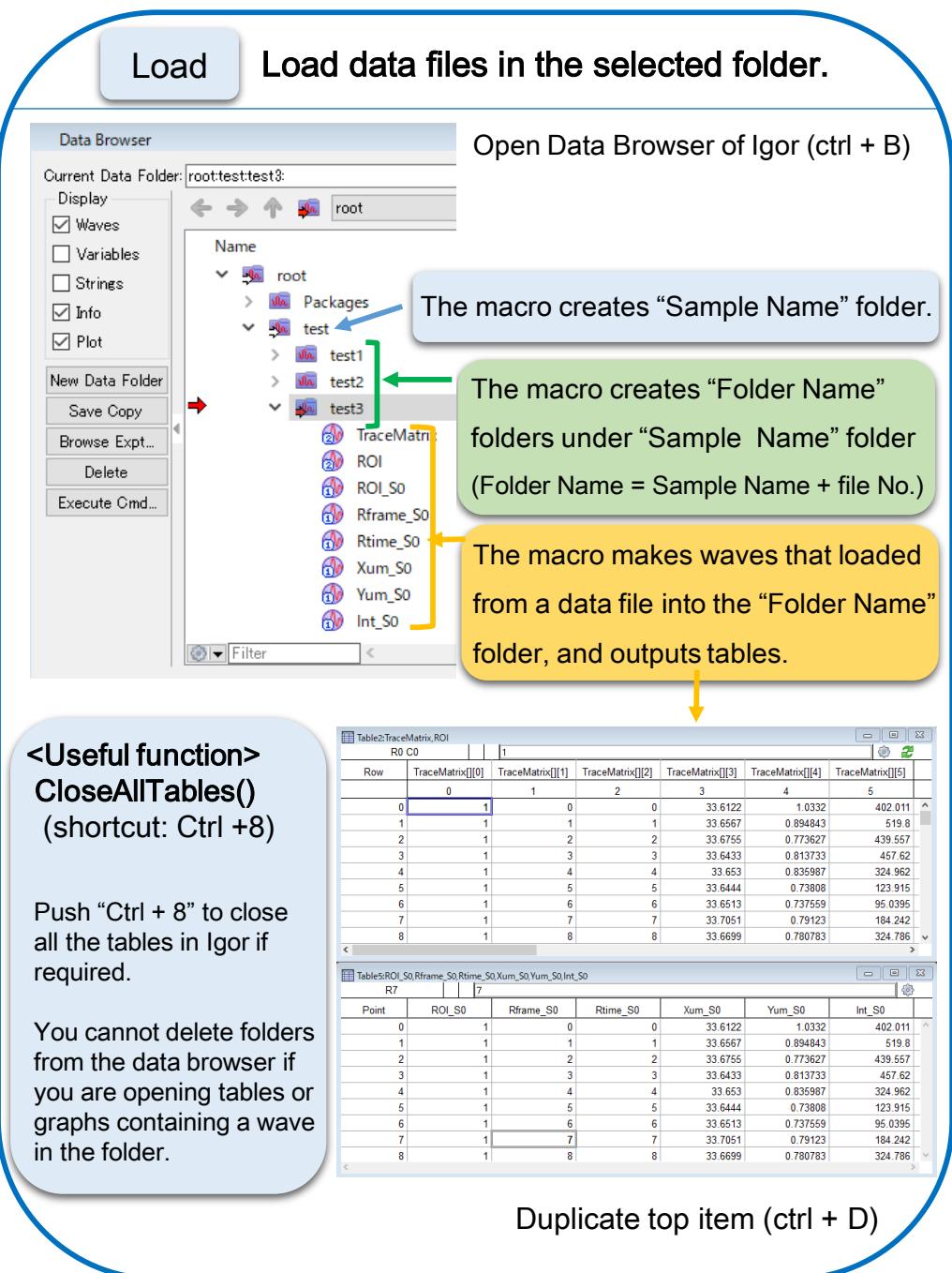
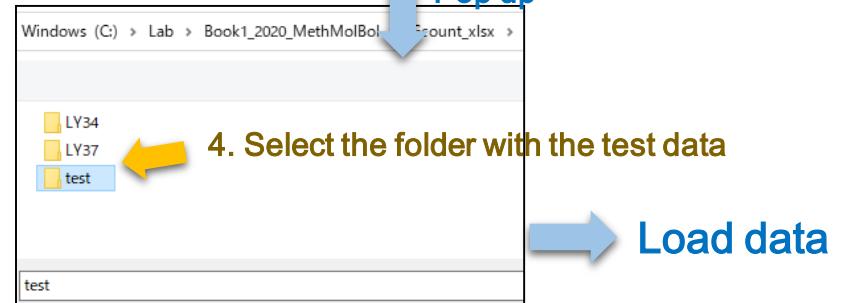
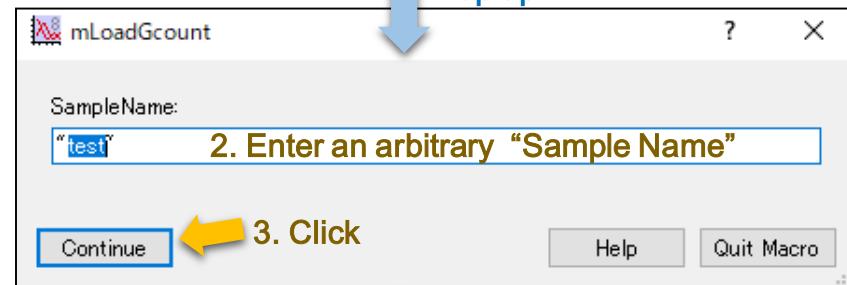
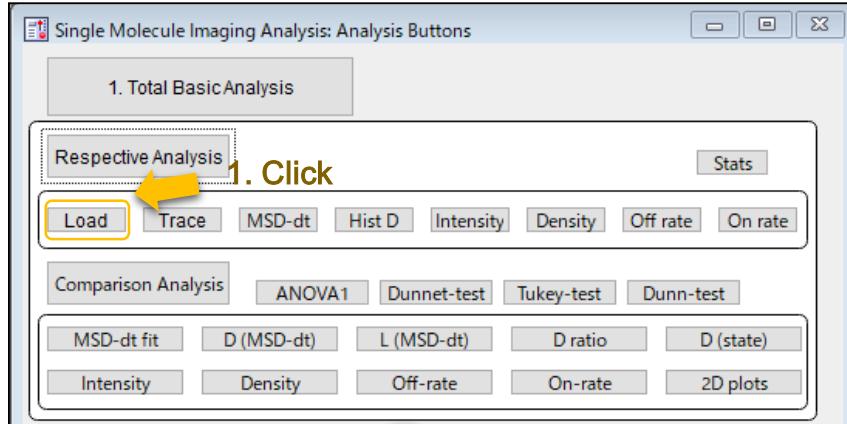
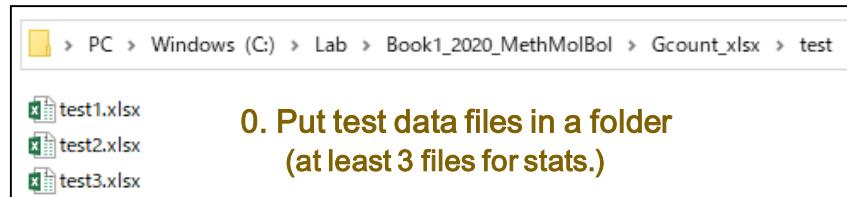


# Fig. S1 How to use the “Load” macro



# Fig. S2 How to use the “Trace” macro

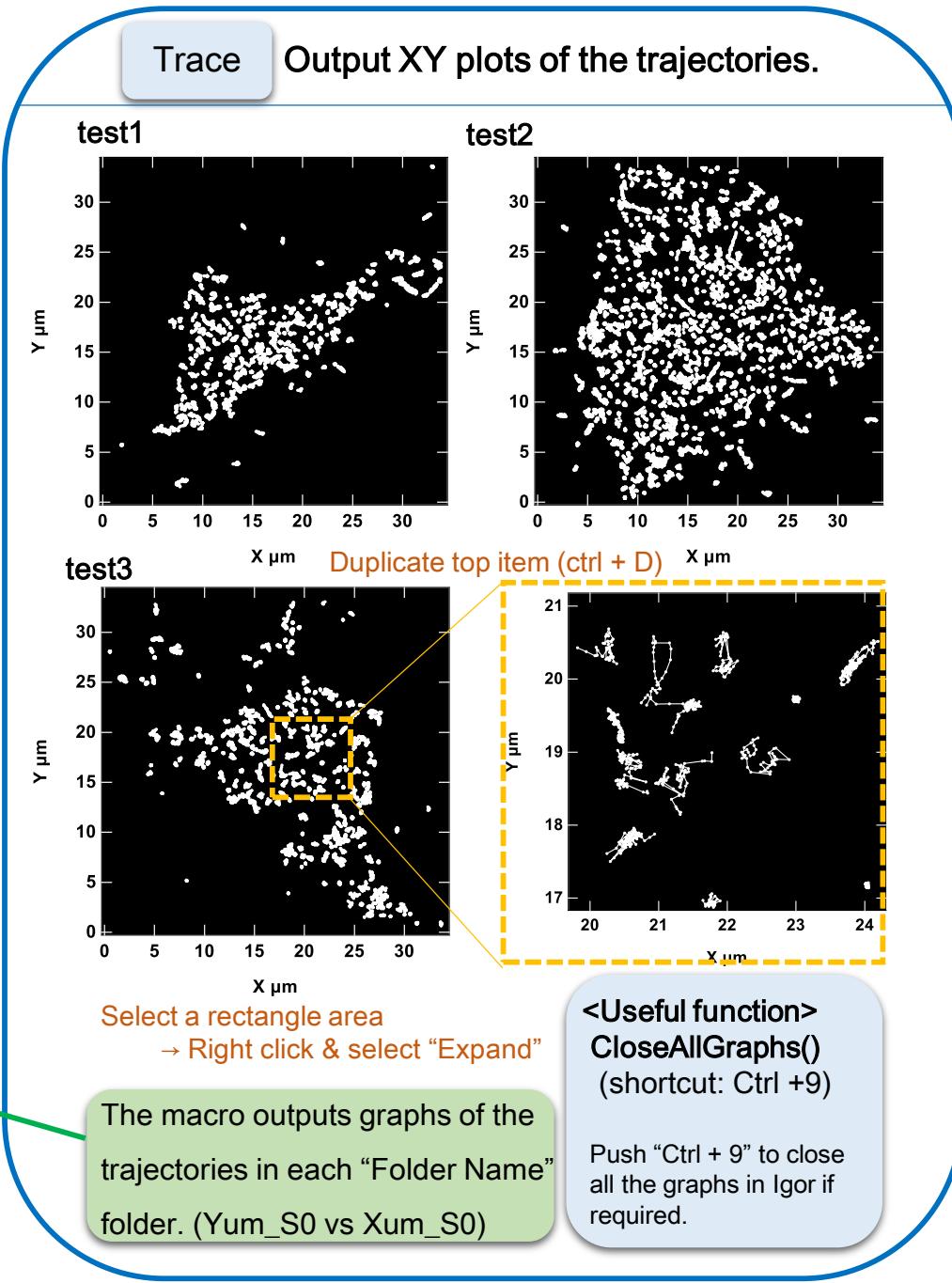
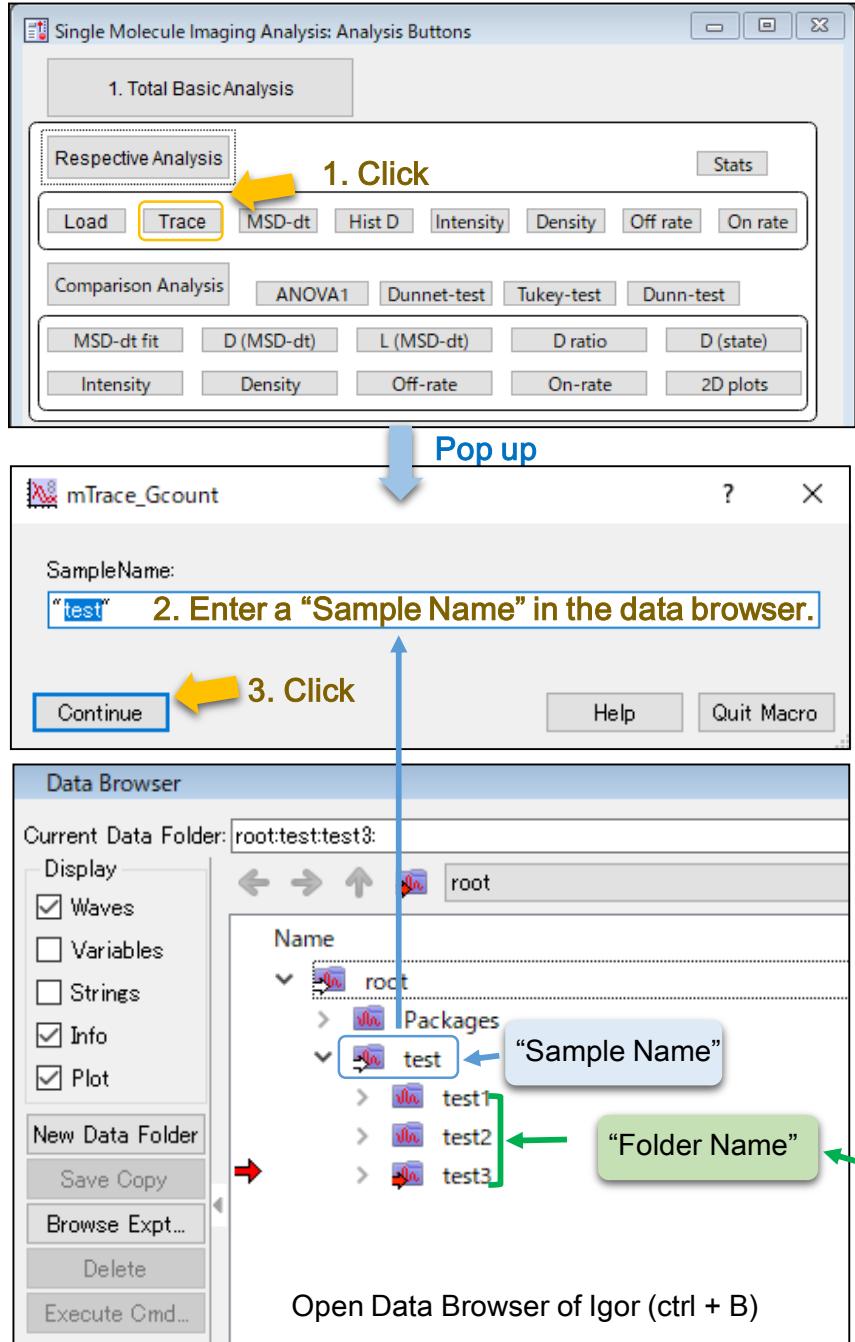
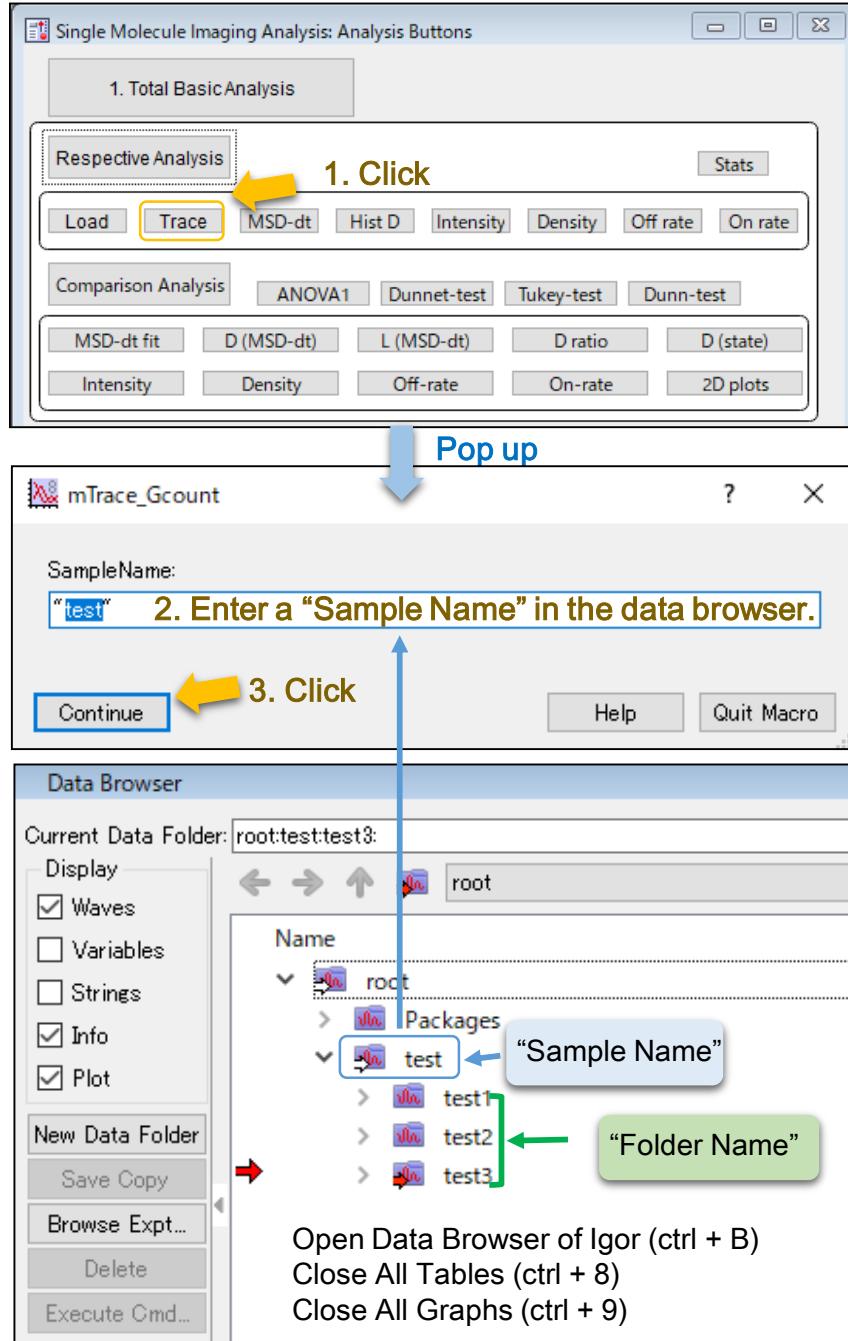


Fig. S3 How to use the “Trace” macro (HMM format)



Trace

Output XY plots of the trajectories.

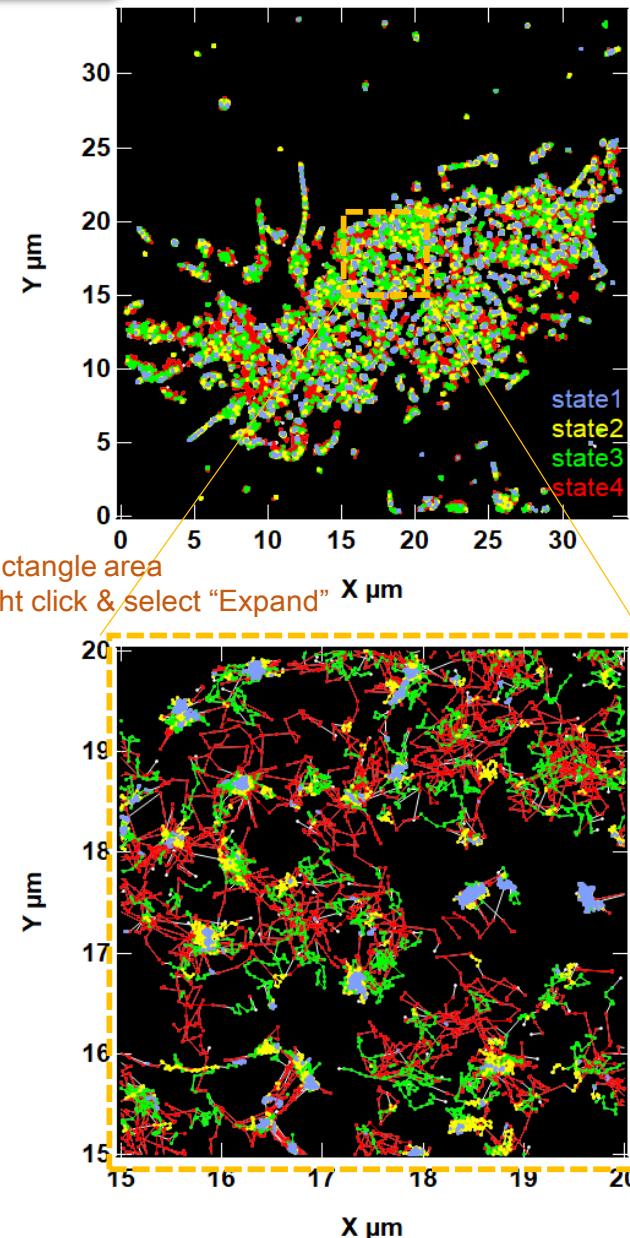
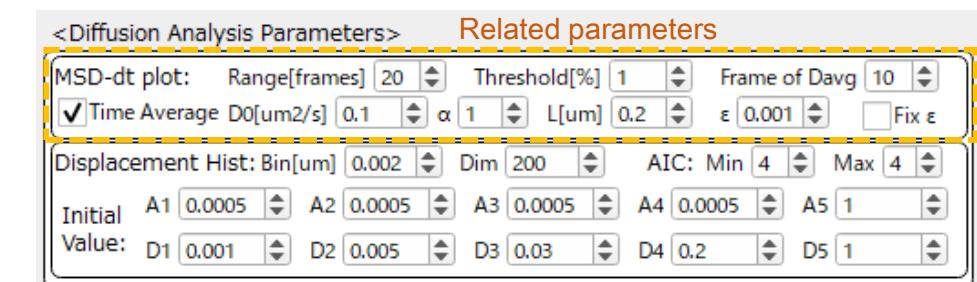
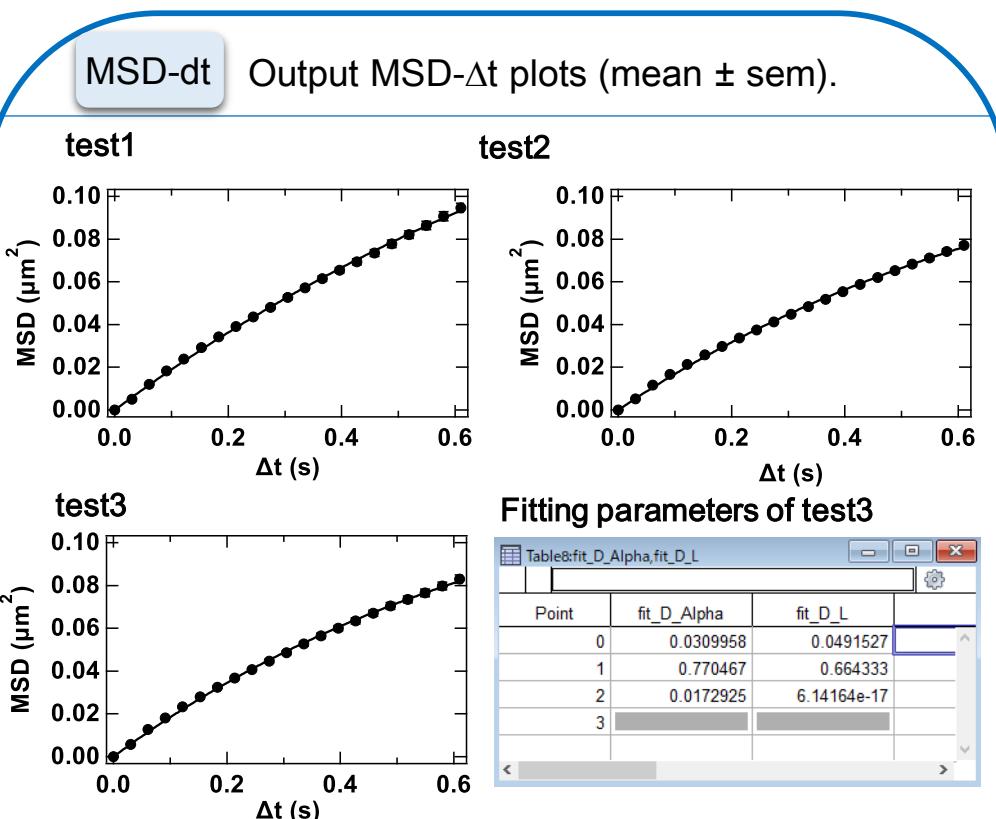
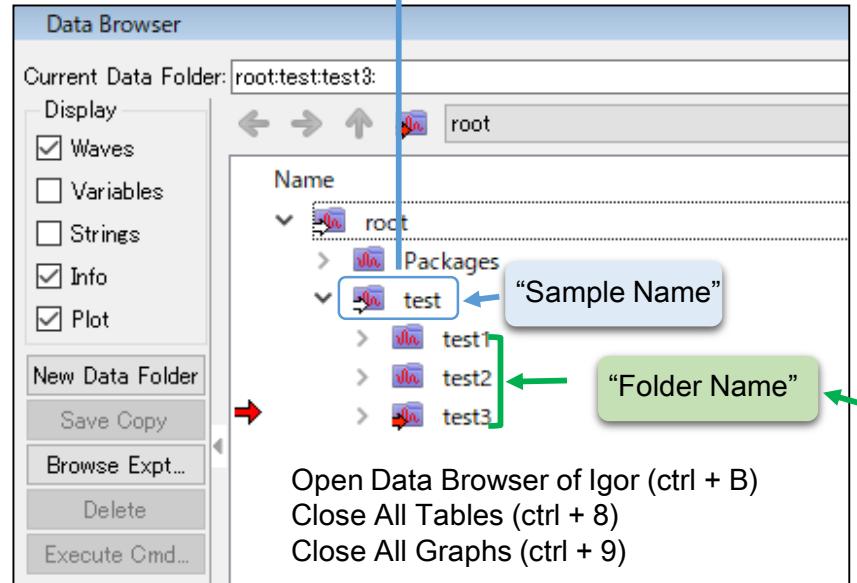
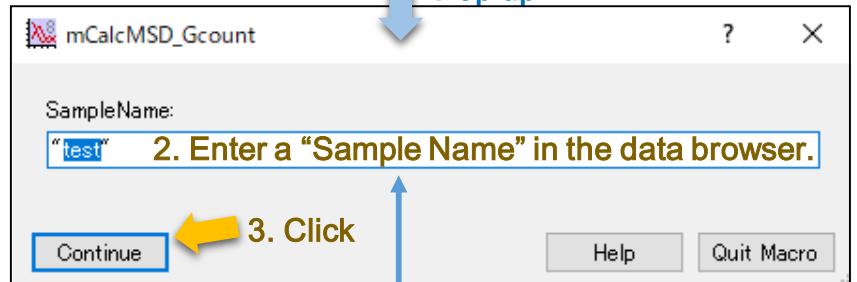
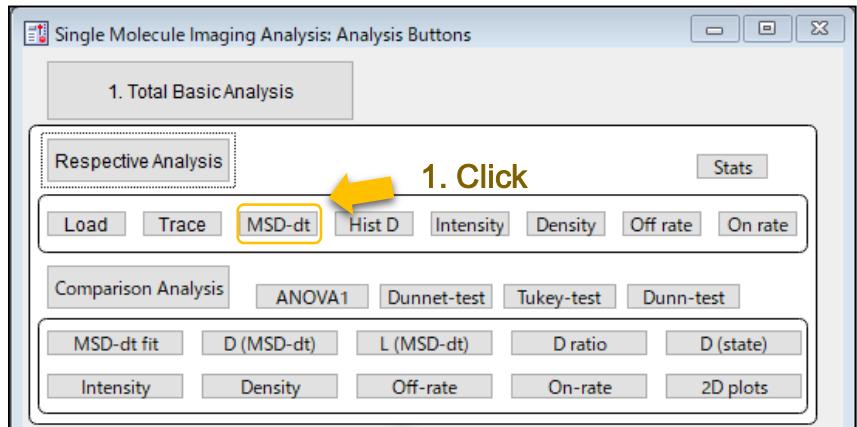
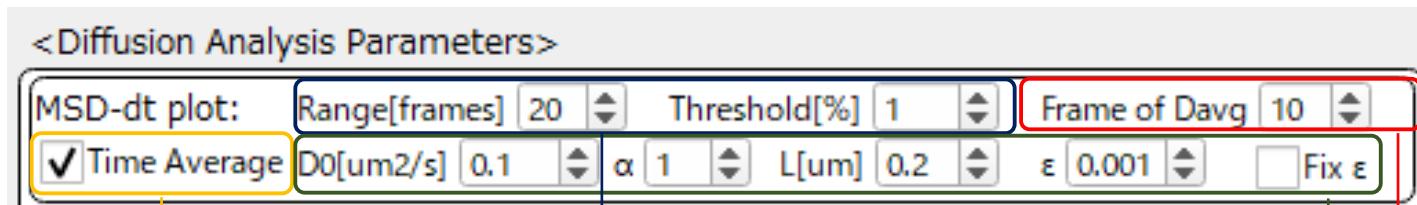


Fig. S4 How to use the “MSD-dt” macro

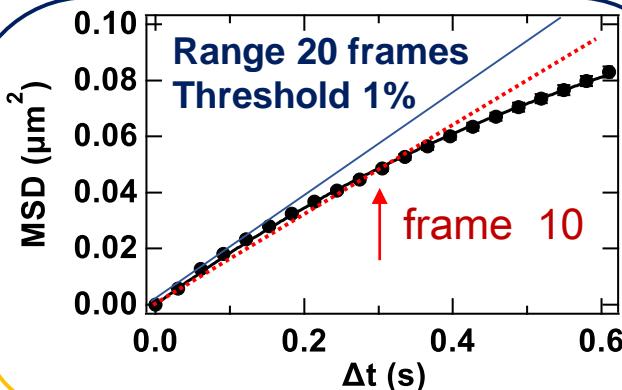


The macro outputs graphs of the MSD- $\Delta t$  plots and the related tables.

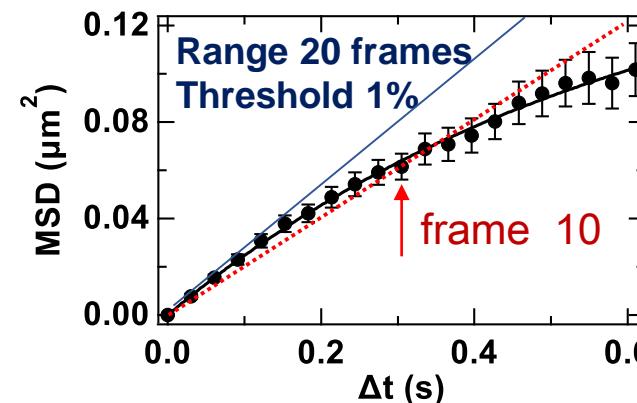
Fig. S5 Parameter settings of the “MSD-dt” macro



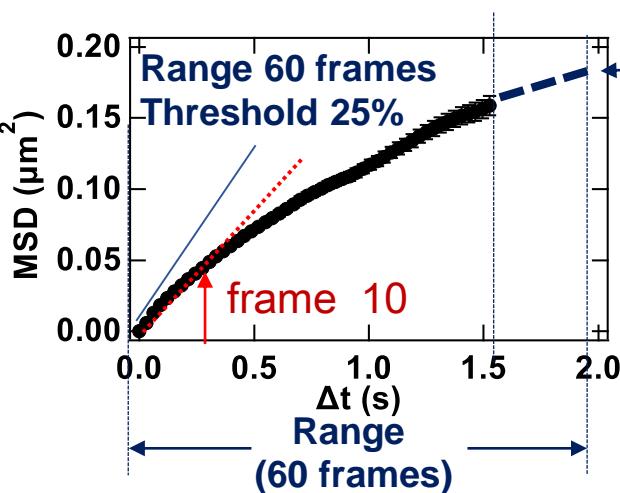
Time- & ensemble-averaged MSD



Ensemble-averaged MSD



$D_{\text{avg}}$  is related to the slope of the red dot line.



Uncalculated due to the number of data were below the threshold.  
(25% of total steps in this case)

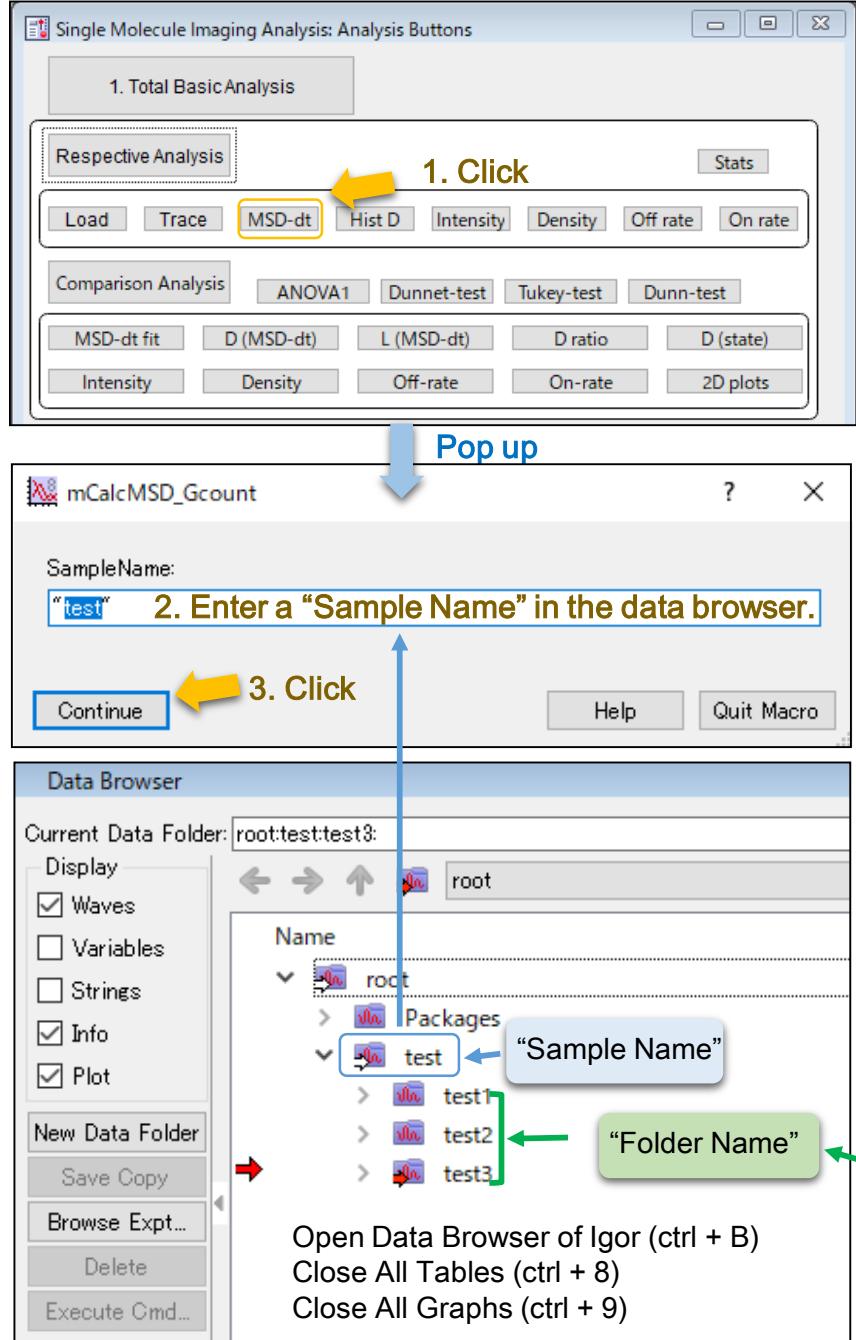
Fitting functions of the MSD- $\Delta t$  plots

$$1) \text{MSD}(\Delta t) = 4D_0\Delta t^\alpha - 4\epsilon^2$$

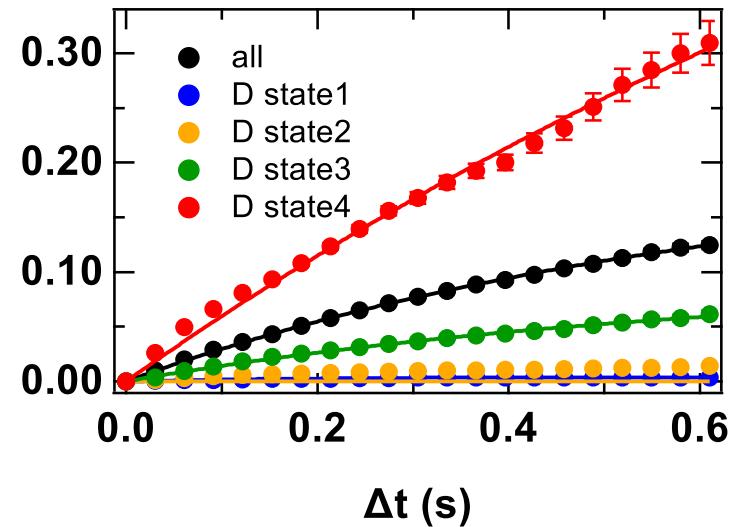
$$2) \text{MSD}(\Delta t) = \frac{L^2}{3} \left( 1 - \exp \left( \frac{-12D_0\Delta t}{L^2} \right) \right) - 4\epsilon^2$$

Macro performs fitting by the both functions, and  $D_0$  and  $L$  will be used in the comparison analysis.

Fig. S6 How to use the “MSD-dt” macro (HMM format)



MSD-dt Output MSD- $\Delta t$  plots (mean  $\pm$  sem).



<Diffusion Analysis Parameters>

Related parameters

MSD-dt plot: Range[frames]	20	Threshold[%]	1	Frame of Davg	10
<input checked="" type="checkbox"/> Time Average D0[ $\mu\text{m}^2/\text{s}$ ]	0.1	$\alpha$	1	L [ $\mu\text{m}$ ]	0.2
Displacement Hist: Bin[ $\mu\text{m}$ ]	0.002	Dim	200	AIC: Min	4
Initial Value:	A1: 0.0005	A2: 0.0005	A3: 0.0005	A4: 0.0005	A5: 1
	D1: 0.001	D2: 0.005	D3: 0.03	D4: 0.2	D5: 1

The macro outputs graphs of the MSD- $\Delta t$  plots of 4 diffusion state of HMM analysis

Fig. S7 How to use the “Hist D” macro

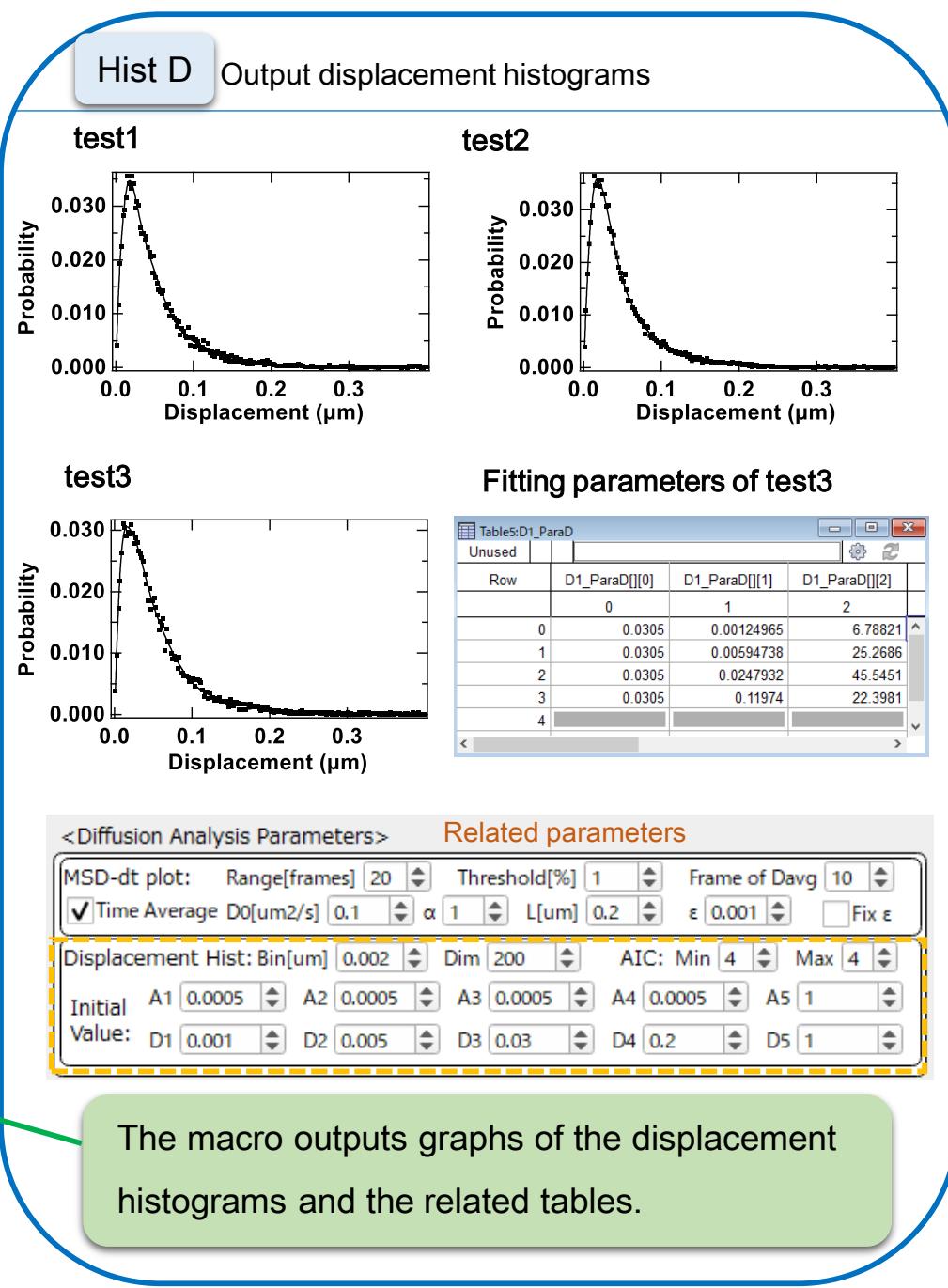
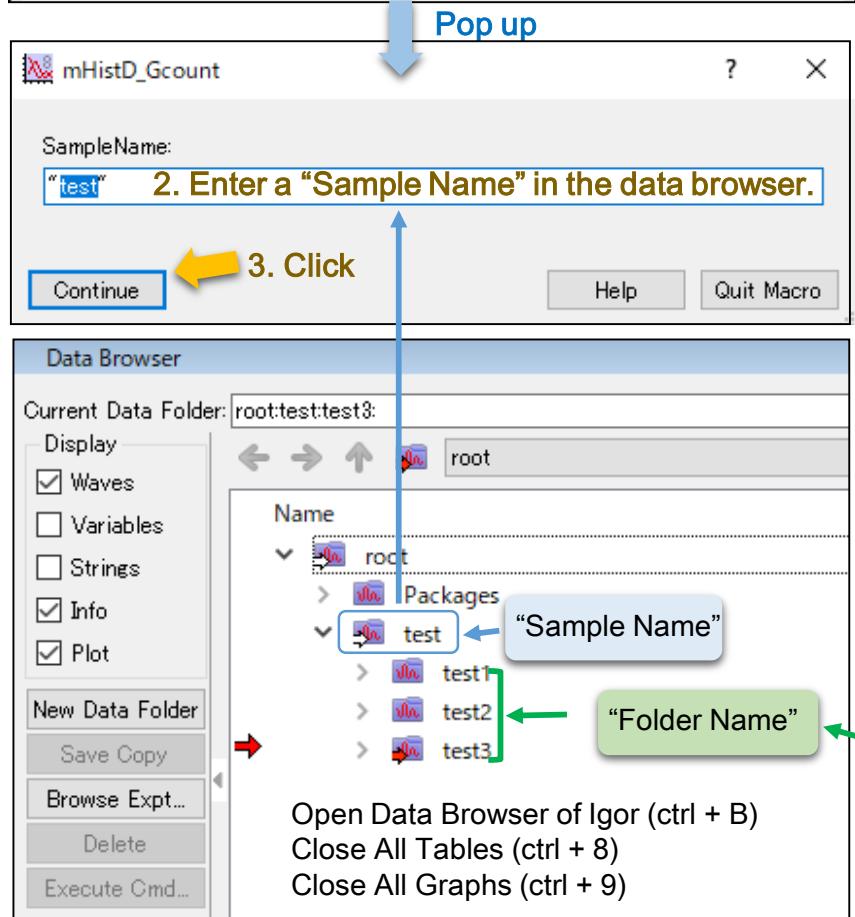
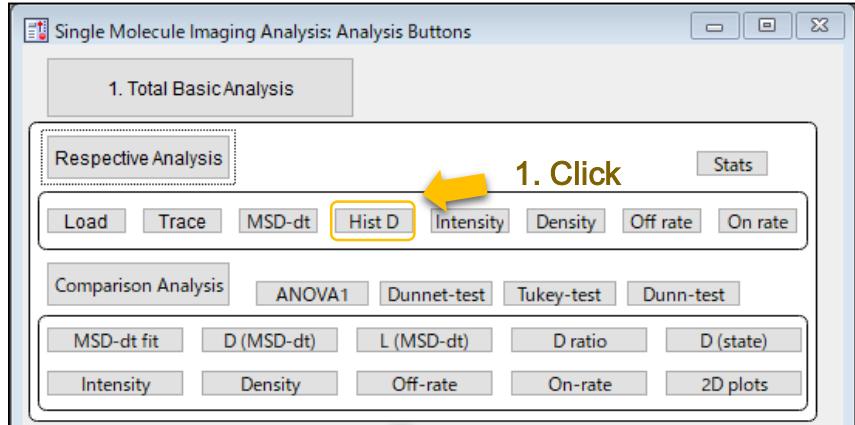
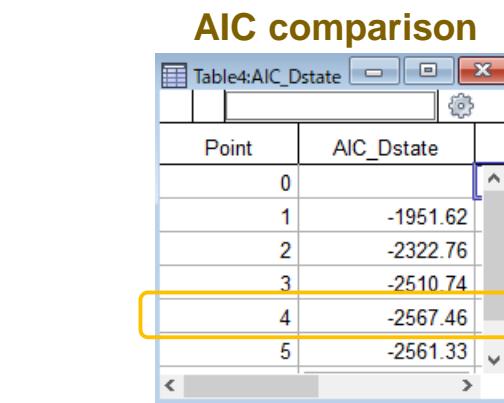
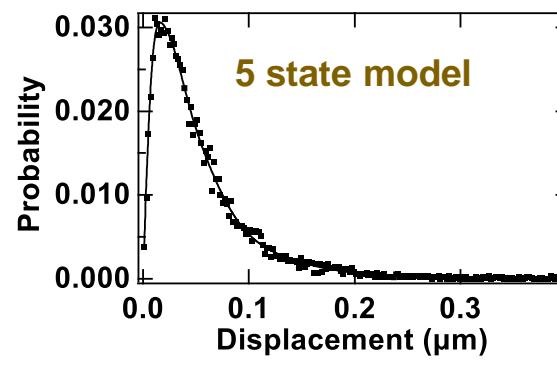
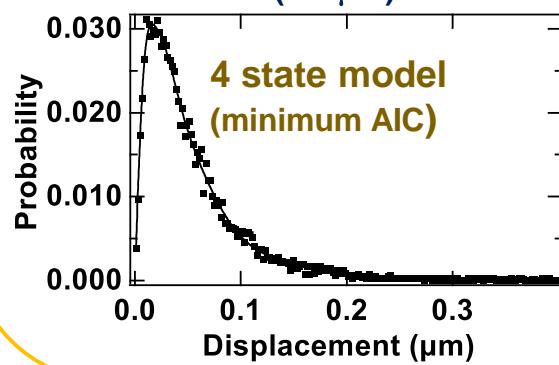
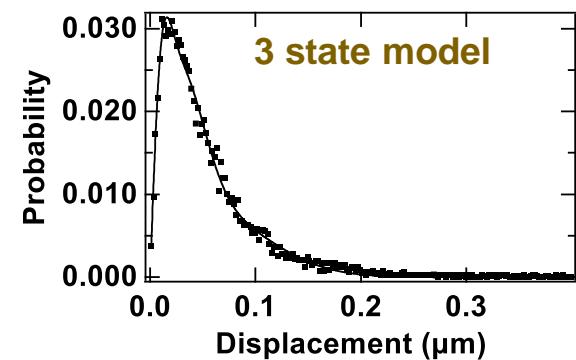
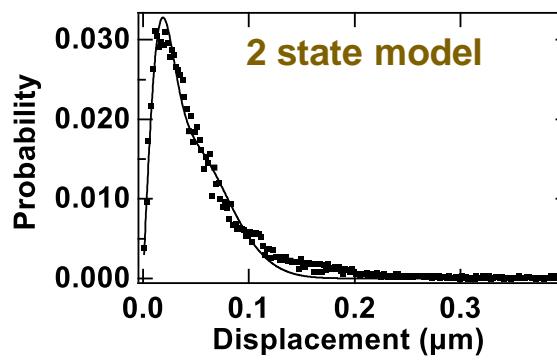
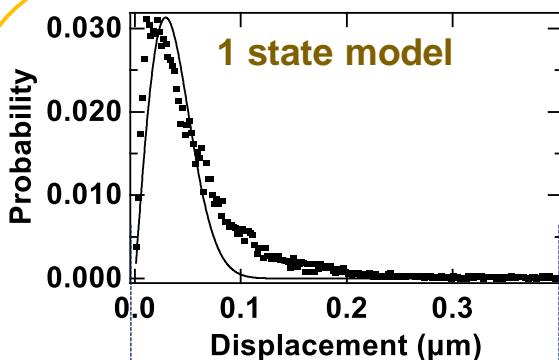


Fig. S8

## Parameter settings of the “Hist D” macro

Displacement Hist: Bin[um]	0.002	Dim	200	AIC: Min 1	Max 5
Initial Value:	A1 0.0005	A2 0.0005	A3 0.0005	A4 0.0005	A5 0.0005
	D1 0.001	D2 0.005	D3 0.03	D4 0.2	D5 1



Fitting function of the displacement histogram

$$P(r) = \sum_{i=1}^n A_i \frac{r}{2D_i \Delta t} \exp\left(\frac{-r^2}{4D_i \Delta t}\right)$$

You can compare Min (1) to Max (5) state fitting models by AIC, but the fitting result is sensitive to the initial values. Check the fitting result of each model if the error message outputs as below .

error: singular matrix or other numeric error

Fig. S9 How to use the “Hist D” macro (HMM format)

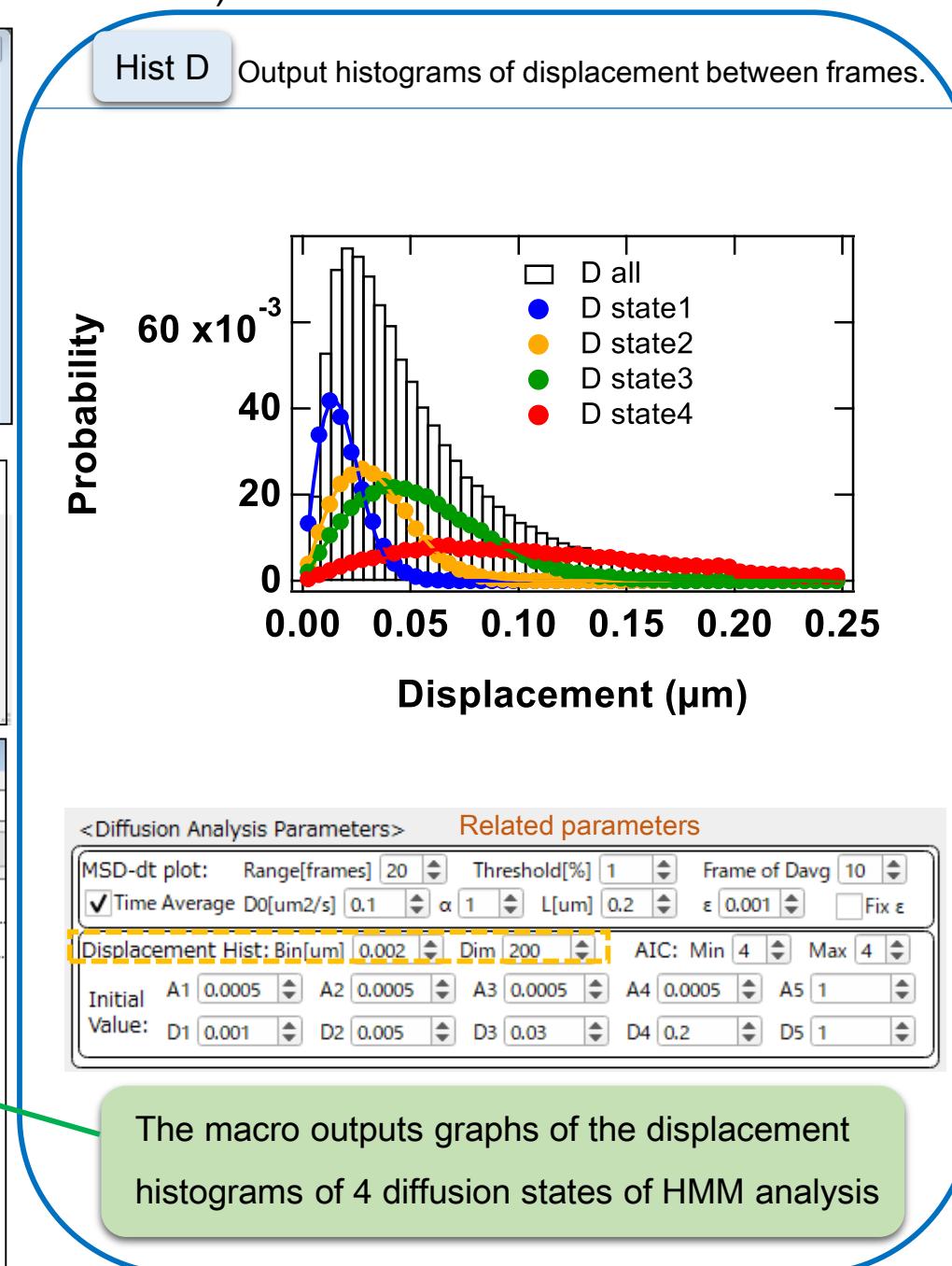
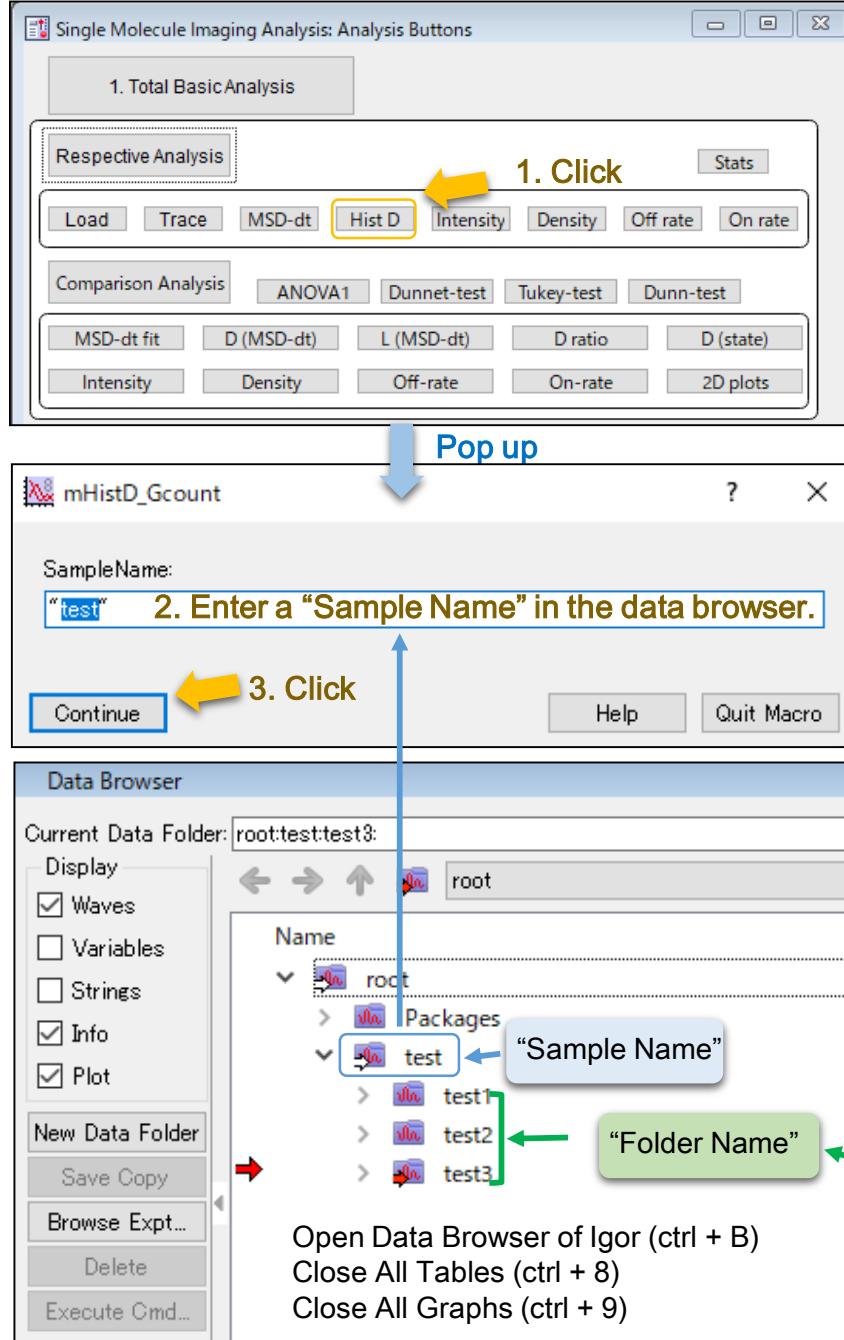


Fig. S10 How to use the “Intensity” macro

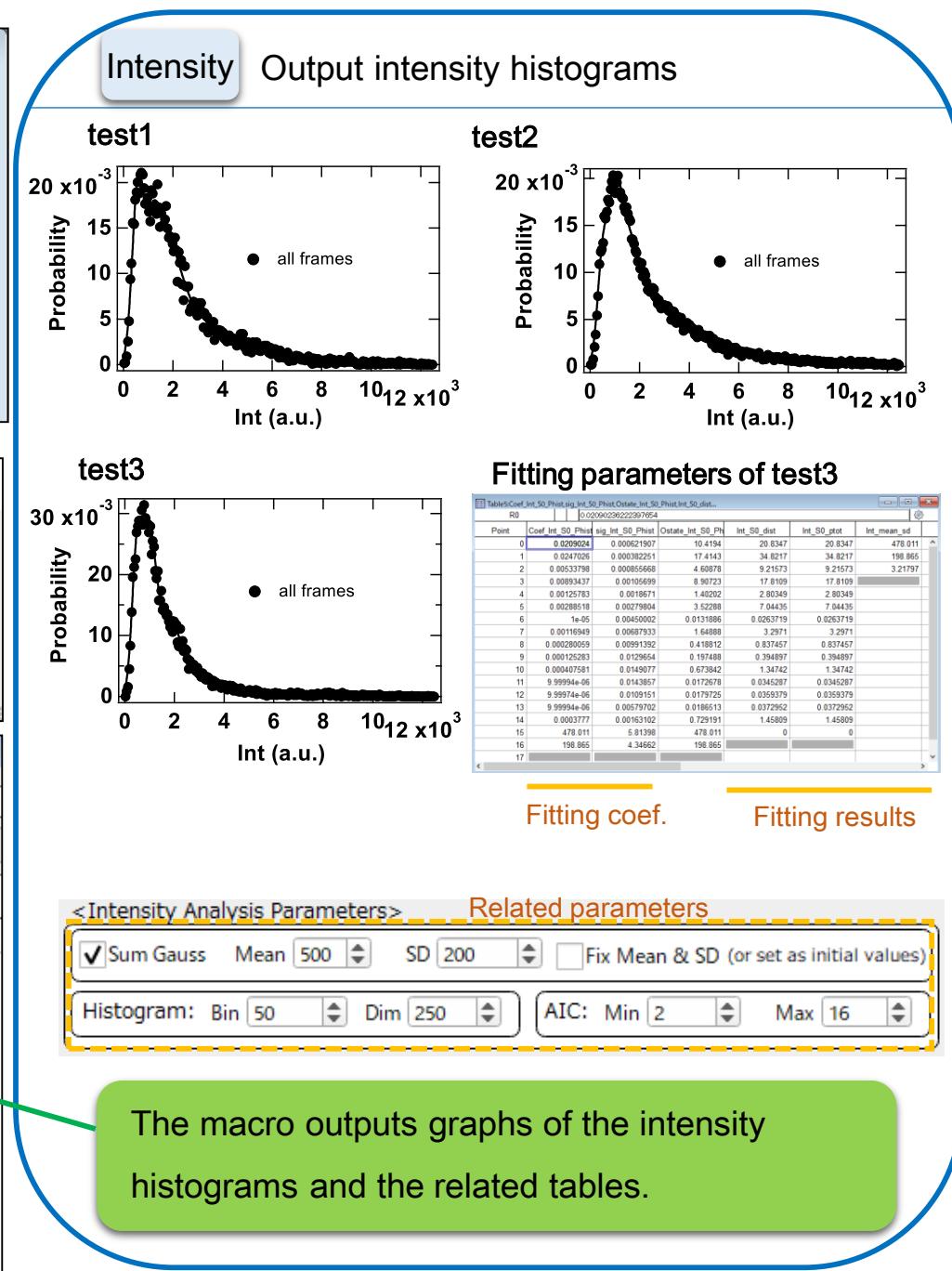
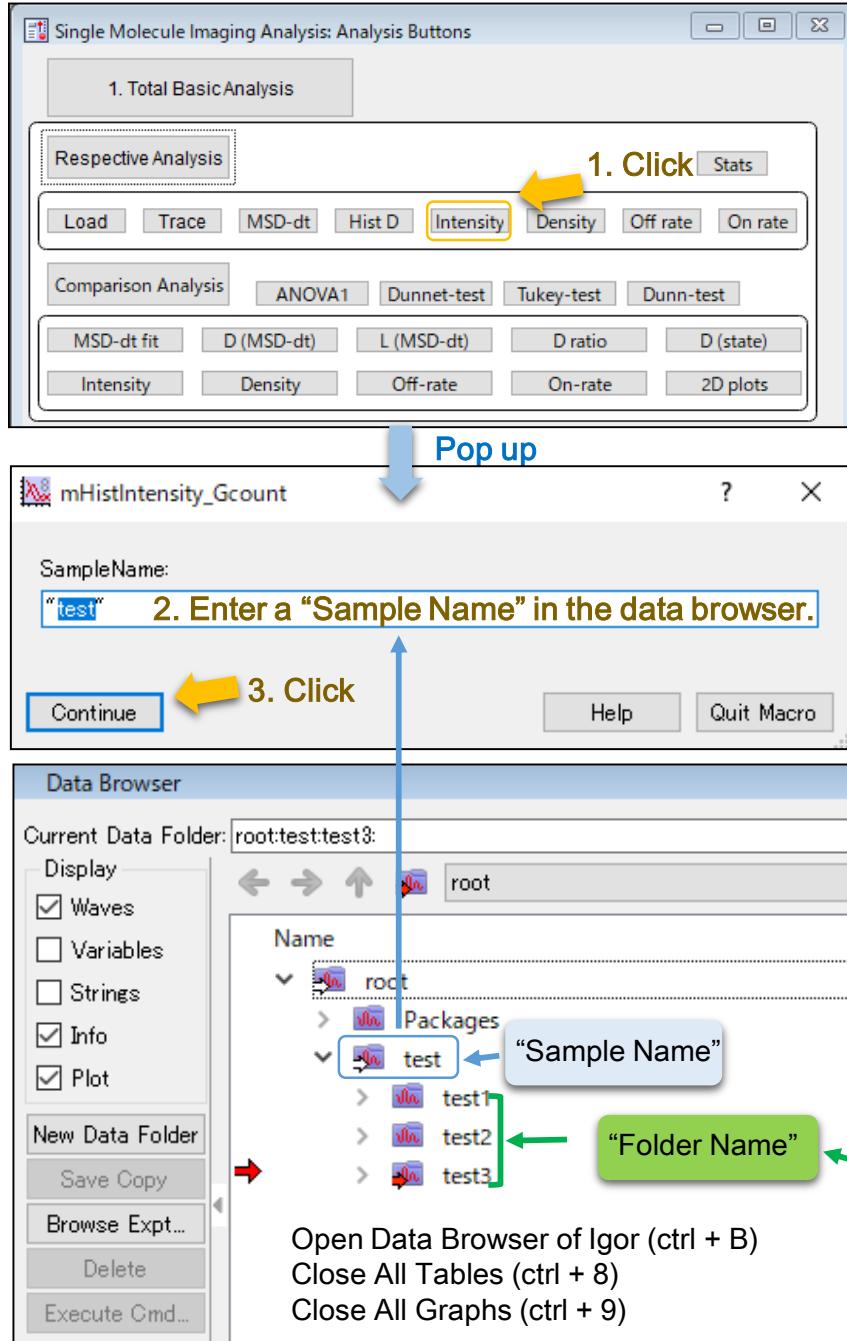
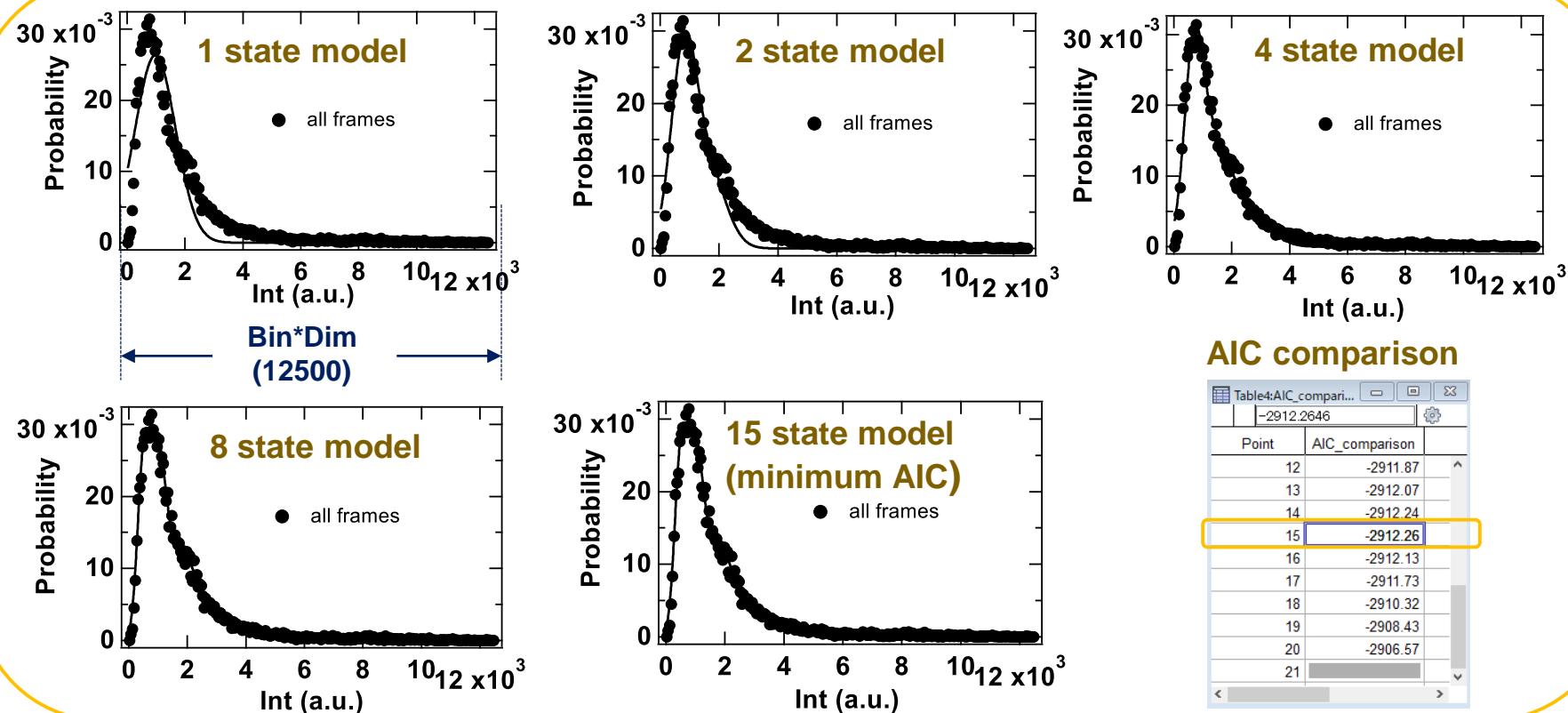


Fig. S11 Parameter settings of the “Intensity” macro

<Intensity Analysis Parameters>

<input checked="" type="checkbox"/> Sum Gauss	Mean 500	SD 200	<input type="checkbox"/> Fix Mean & SD (or set as initial values)
Histogram: Bin 50	Dim 250	AIC: Min 2	Max 16



Fitting function of the intensity histogram

$$P(x) = \sum_{n=1}^N A_n \exp\left(-\frac{(x - nI)^2}{2n\sigma^2}\right)$$

You can compare Min (1) to Max (20) state fitting models by AIC. Check the fitting results to confirm the Mean and SD values are within the appropriate range as a single-molecule intensity.

The Mean and SD values are fixed to the input values when the checkbox is checked.

Fig. S12 How to use the “Intensity” macro (HMM format)

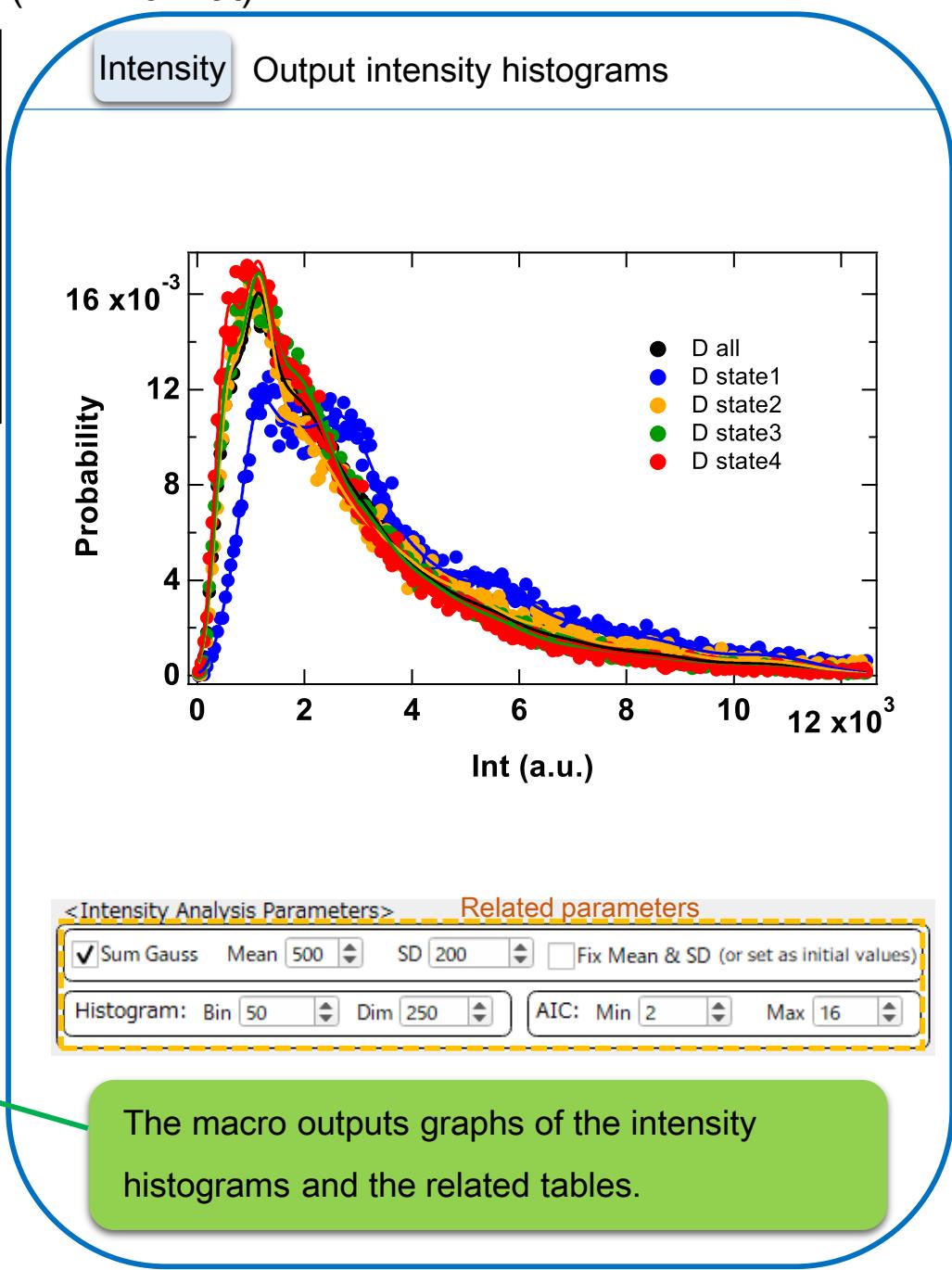
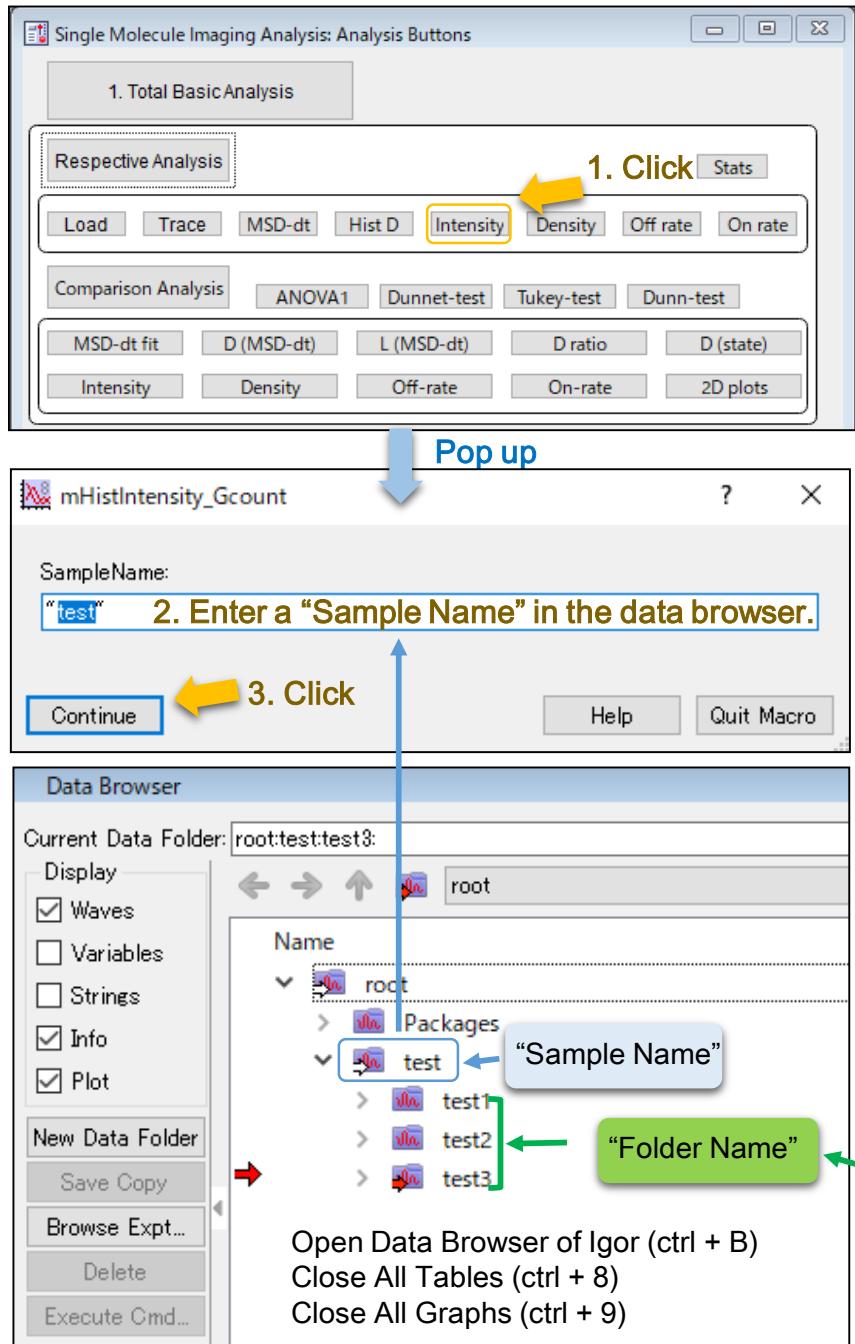


Fig. S13 How to use the “Density” macro

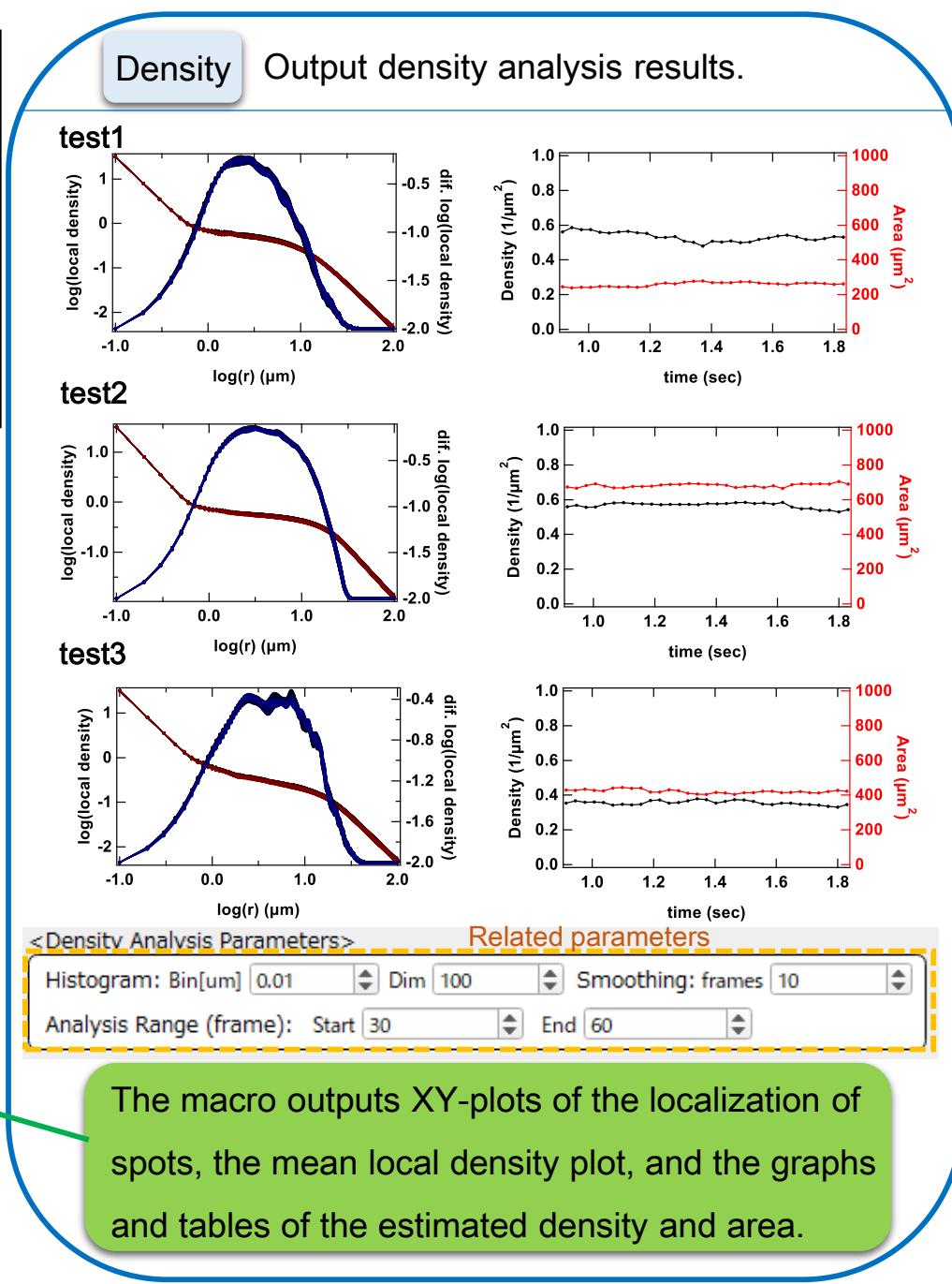
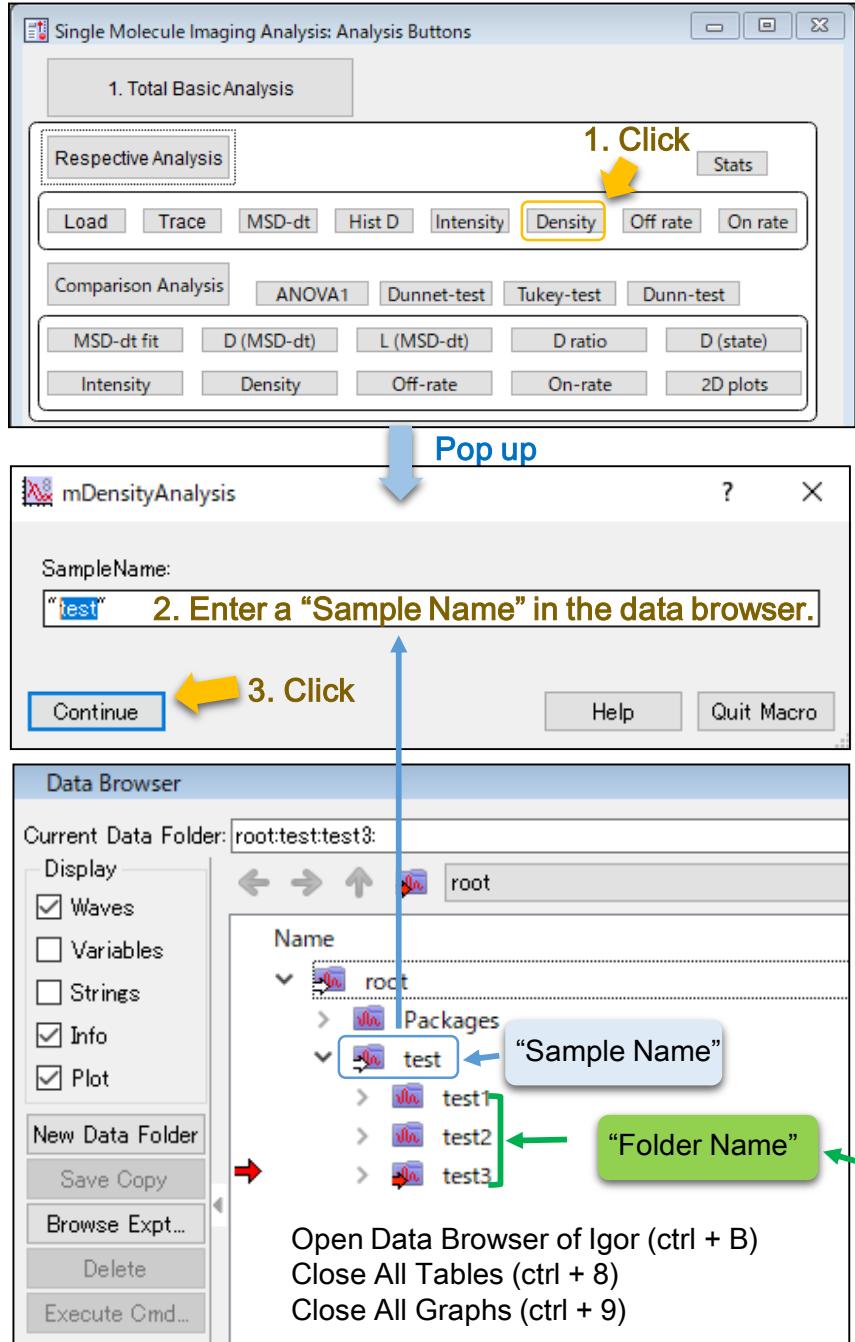
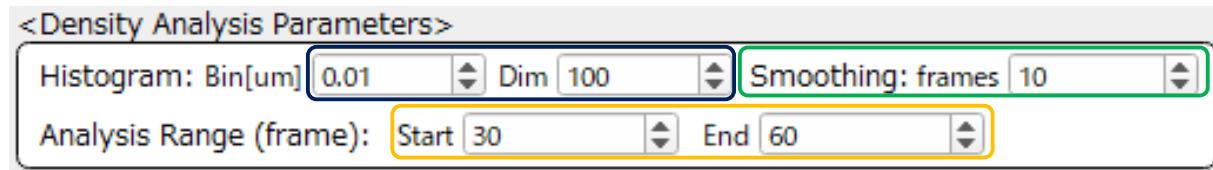
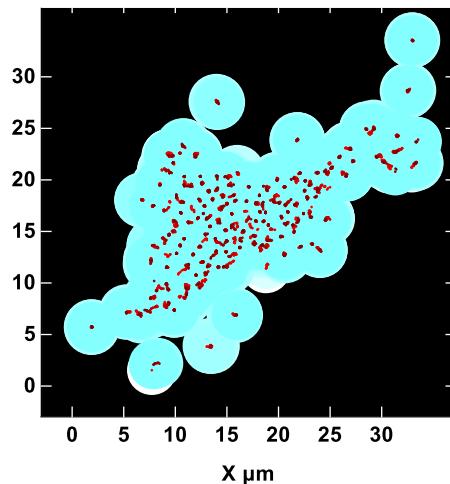


Fig. S14 Parameter settings of the “Density” macro



Single particle localization



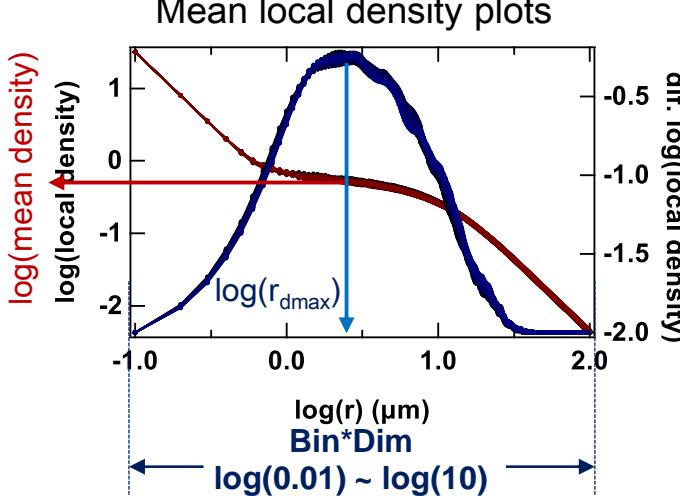
<Red dots>

The particle localization within the selected frames (Start: 30 ~ End: 60) are plotted.

<Blue circles around the red dots>

Circles with radius ( $r_{\text{dmax}}$ ) that gives the plateau of the mean local density curve (middle panel) are plotted around each localization.

Mean local density plots



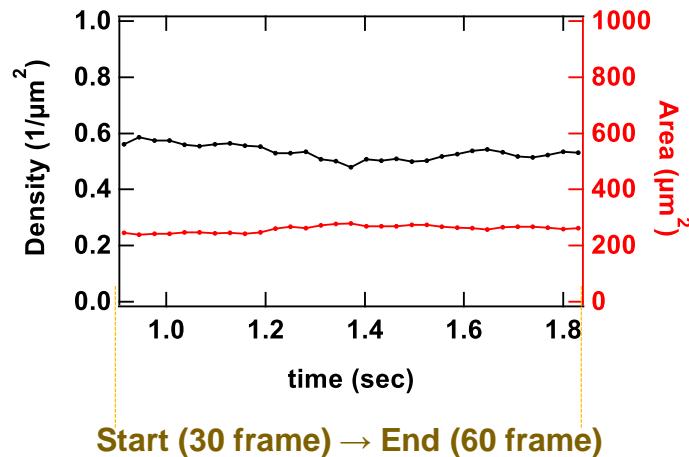
<Red curves>

The mean local density around in the vicinity of  $r$  from each localization within a frame are plotted against  $r$ .

<Blue curves>

First-order derivative of the mean local density curves. The local density in the vicinity of  $r_{\text{dmax}}$ , which gives the peak, is adopted as the mean density of particles at each frame.

Estimated density and area



<Black plots>

The mean density at each frame are plotted against time.

<Red plots>

The area of cell region that was estimated from the mean density and particle number are plotted against time.

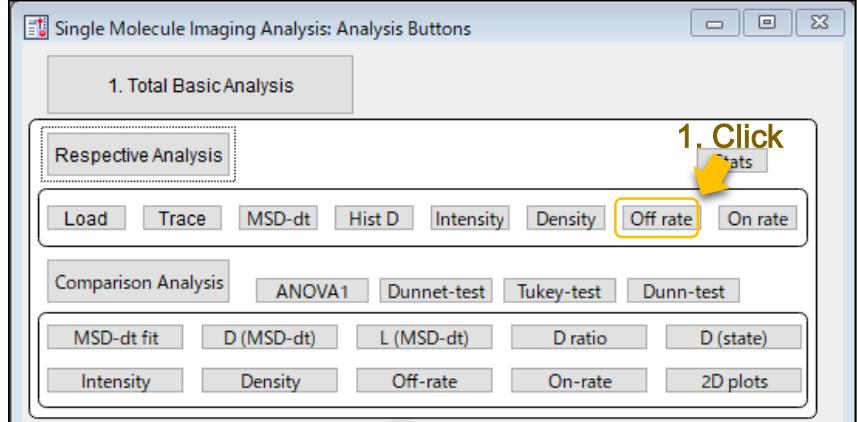
### The mean local density function

$$d_{avg}(r) = \frac{1}{n} \sum_{i=1}^n \frac{N_{Pi}(r)}{\pi r^2} \quad (N_{Pi}(r): \text{number of points } N \text{ within a distance } r \text{ of point } i)$$

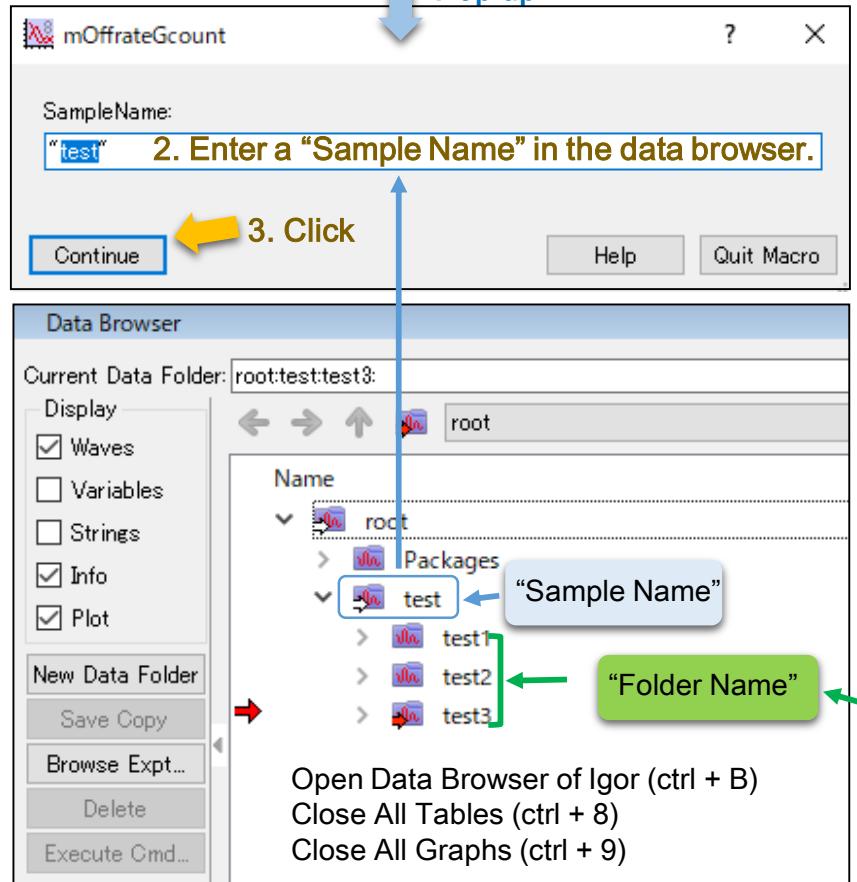
A parameter to find peak ( $r_{\text{dmax}}$ ) in the middle panel.

Increase it if the peak detection was improper due to the noise.

Fig. S15 How to use the “Off rate” macro



1. Click



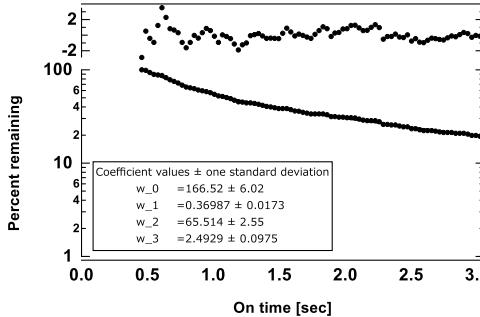
Pop up

3. Click

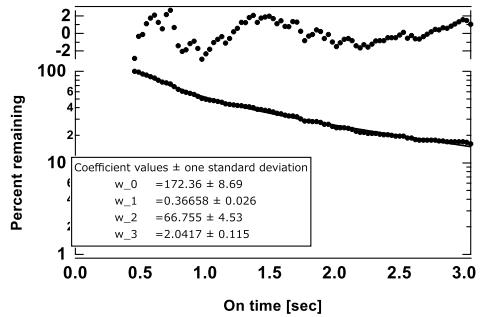
Off rate

Output decay curves of the duration of trajectories.

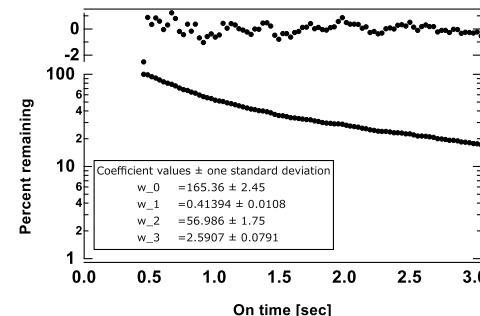
test1



test2



test3



Fitting parameters

Point	ParaDuration
0	71.0029
1	0.369874
2	27.9342
3	2.49286
4	1.06292
5	2.49286
6	

<Off-rate Analysis Parameters>

Exponential fitting: Min 1 Max 3 Frame Edge correction

A1	100	A2	50	A3	5	A4	1	A5	0.5
Tau1	0.3	Tau2	1	Tau3	3	Tau4	5	Tau5	10

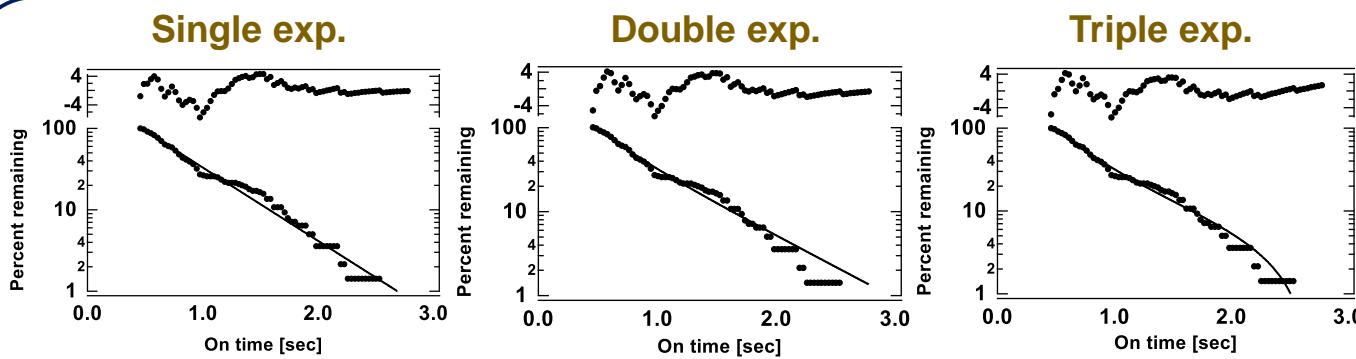
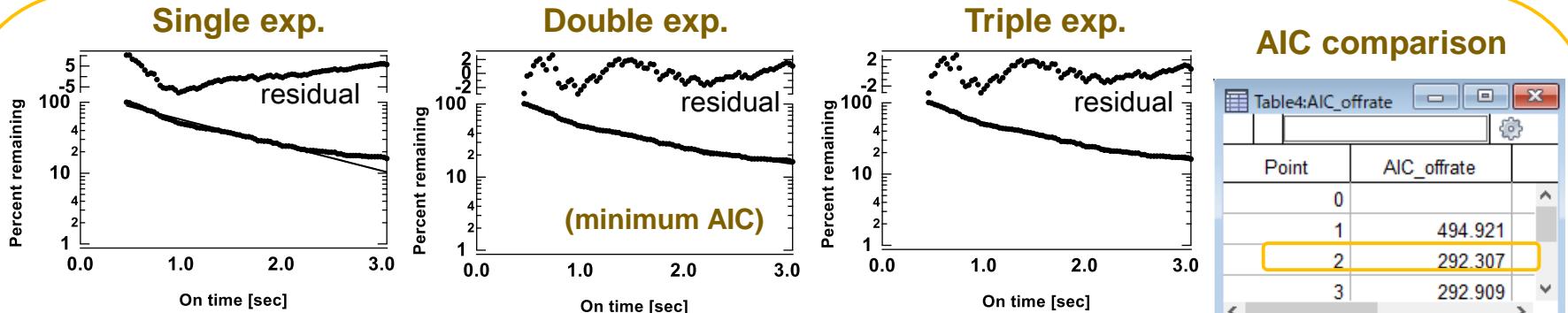
Related parameters

The macro outputs decay curves of the duration of trajectories, and the related tables.

Fig. S16 Parameter settings of the “Off rate” macro

<Off-rate Analysis Parameters>

Exponential fitting	Min 1	Max 3	<input type="checkbox"/> Frame Edge correction						
A1	100	A2	50	A3	5	A4	1	A5	0.5
Tau1	0.3	Tau2	1	Tau3	3	Tau4	5	Tau5	10



If the check box is checked, the plot is created except for the trajectories that start in the first frame or ends in the last frame.

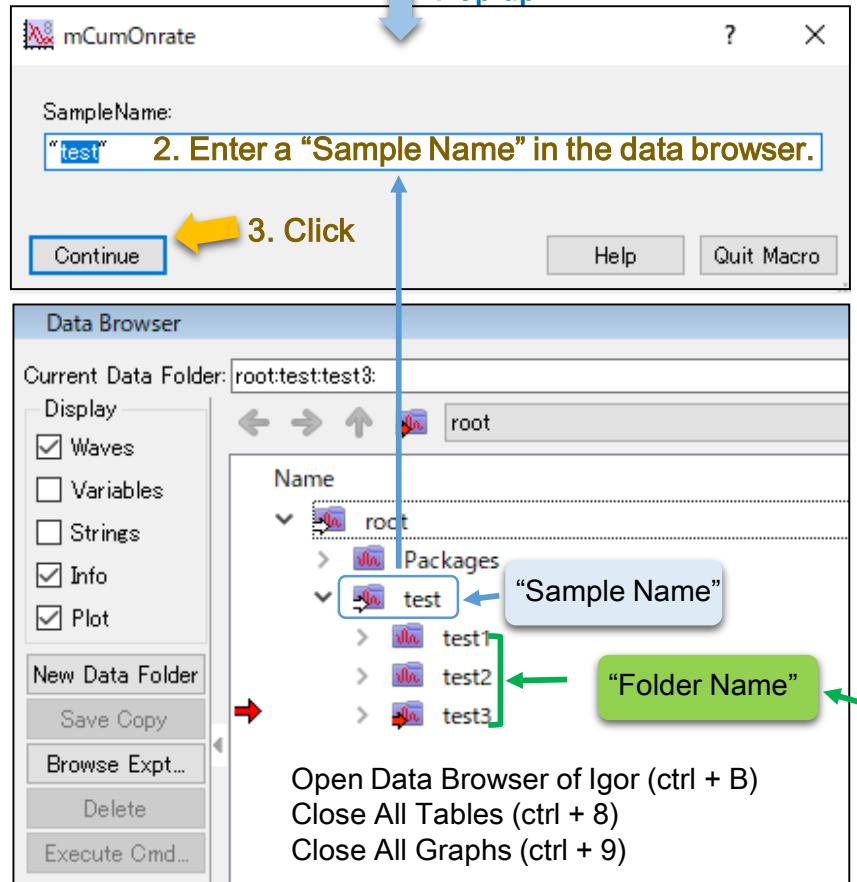
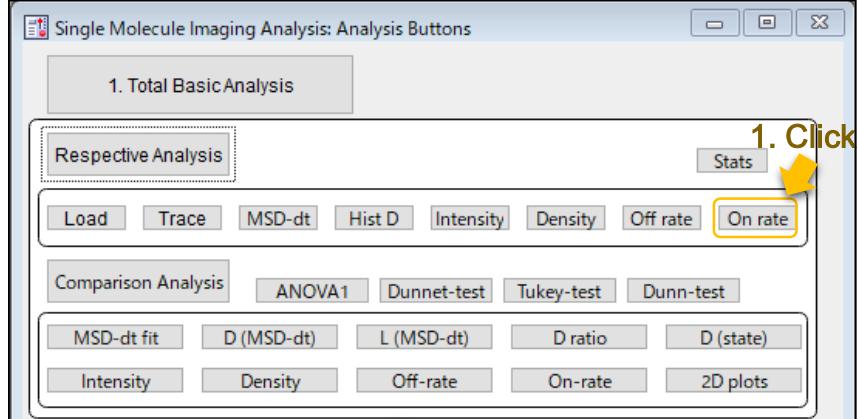
Fitting function of the decay curve

$$P(t) = \sum_{i=1}^n A_i \exp\left(\frac{-t}{\tau_i}\right)$$

You can compare Min (1) to Max (5) state fitting models by AIC, but the fitting result is sensitive to the initial values. Check the fitting result of each model if the error message outputs as below .

error: singular matrix or other numeric error

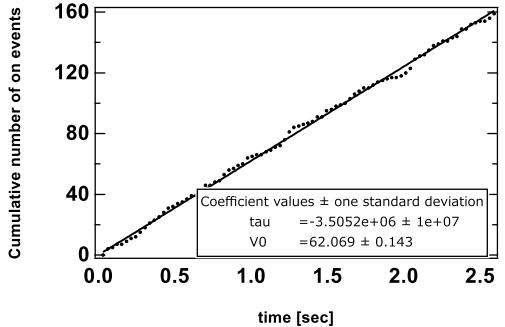
Fig. S17 How to use the “On rate” macro



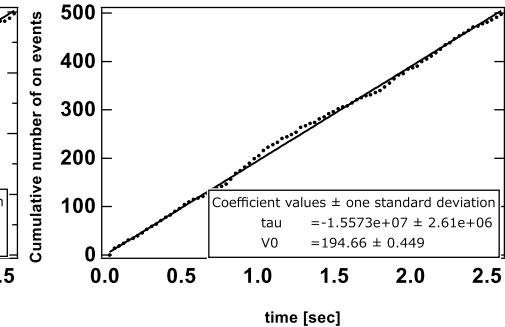
On rate

Output cumulative event number plots of the starting time of trajectories.

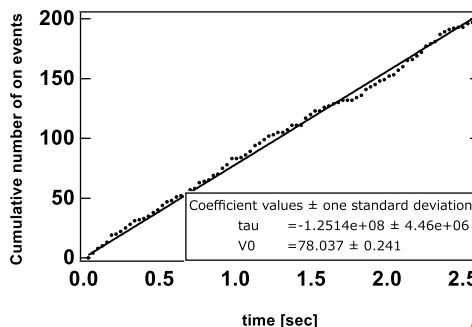
test1



test2



test3



Fitting parameters

Table2:ParaOnrate

Point	ParaOnrate
0	-1.25141e+08
1	0.185969
2	0.186602
3	

Related parameters

<On-rate Analysis Parameters>

Initial value:  $V_0$  1 Tau (sec) 1  Fix Area [ $\mu\text{m}^2$ ] 1177

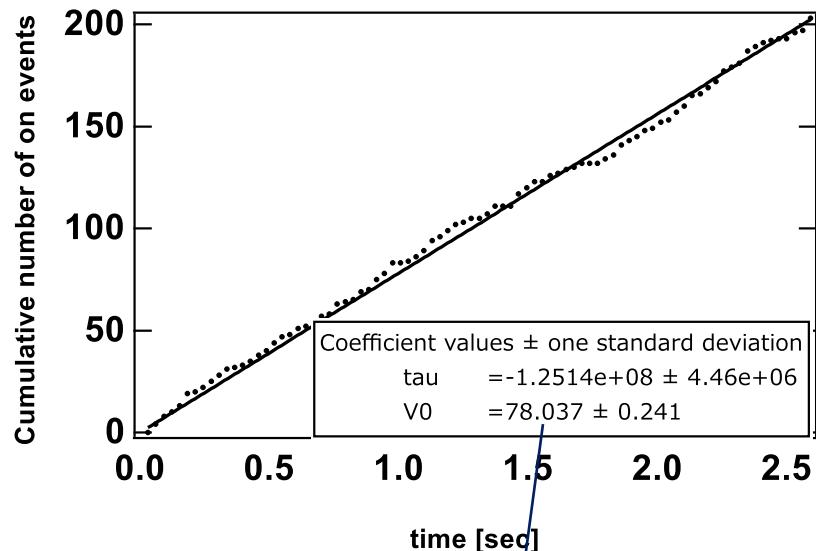
The macro outputs cumulative event number plot of the starting time of trajectories, and the related tables.

Fig. S18 Parameter settings of the “On rate” macro

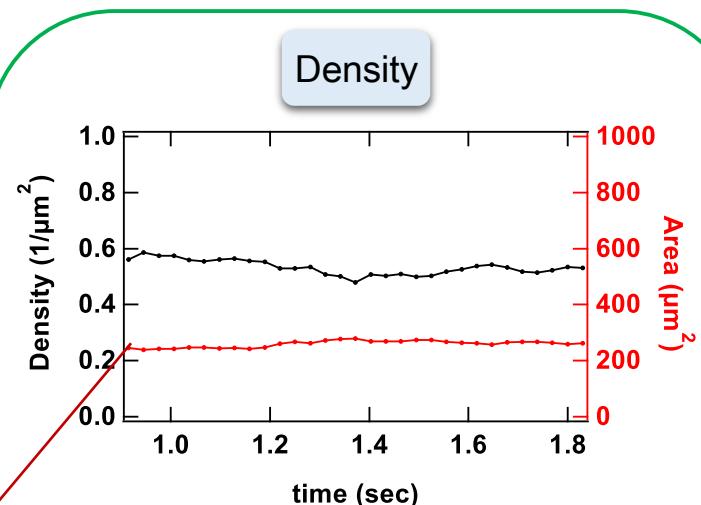
<On-rate Analysis Parameters>

Initial value:	V <sub>0</sub> 1	Tau (sec) 1	<input type="checkbox"/> Fix Area [μm <sup>2</sup> ] 1177
----------------	------------------	-------------	---

On-rate analysis should be done after the density analysis



$$\text{on-rate} = V_0 / \text{Area}_{\text{cell}}$$



If the check box is unchecked, the  $\text{Area}_{\text{cell}}$  estimated from the density analysis is used for the calculation of the on-rate.

If checked, the input value is used for the calculation of the on-rate.

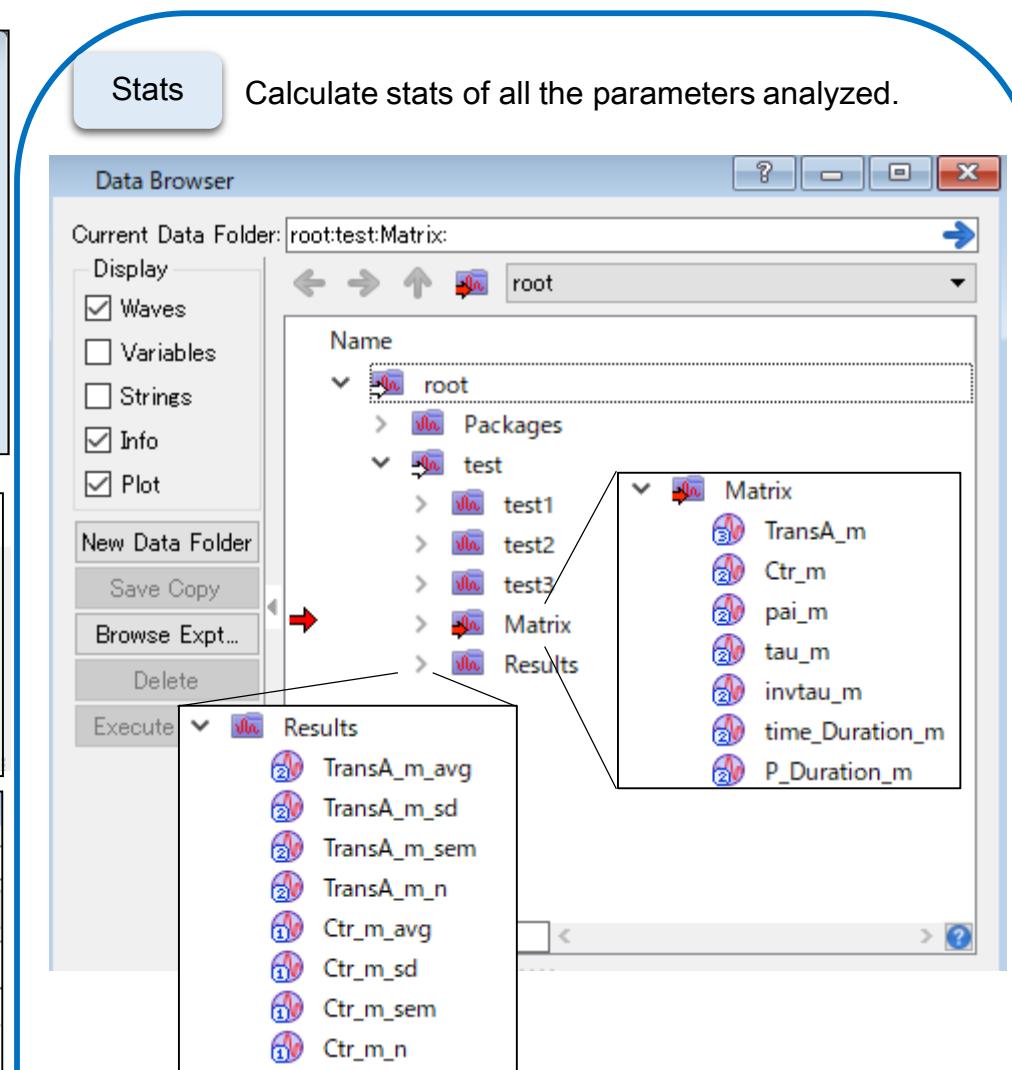
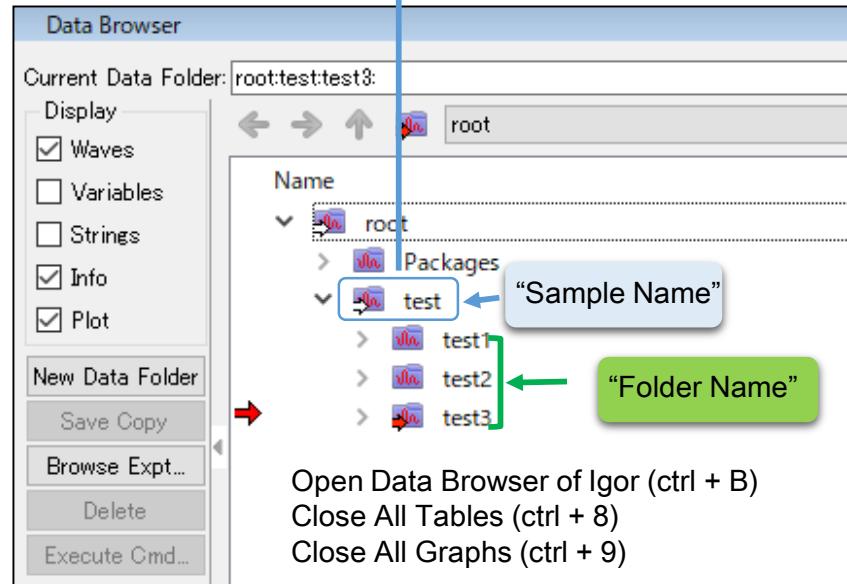
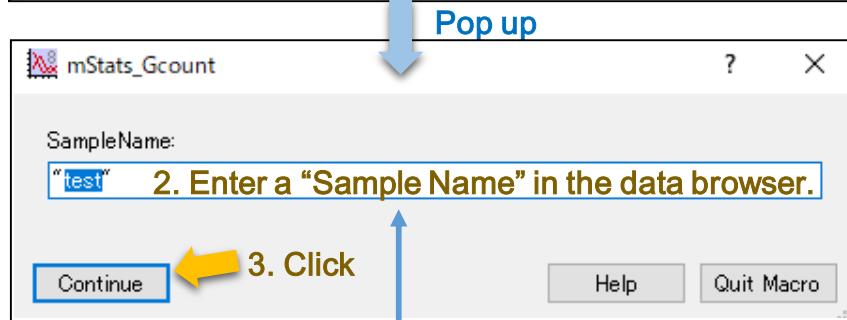
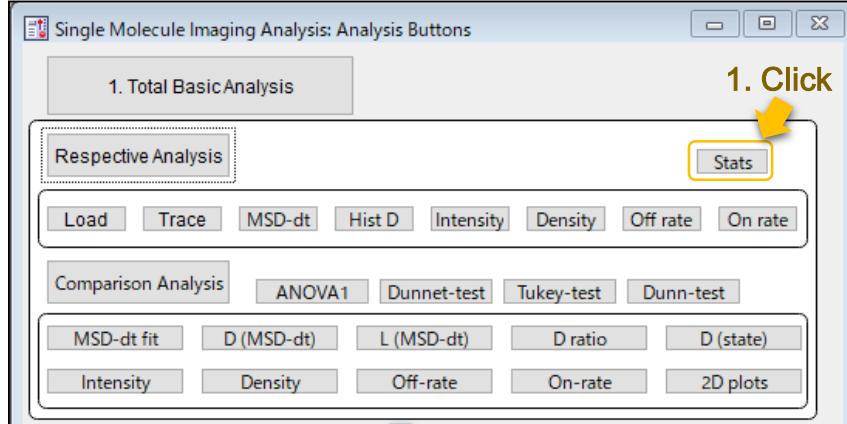
Fitting function of the plot

$$f(t) = V_0 \left( 1 - \exp \left( \frac{-t}{\text{tau}} \right) \right)$$

V<sub>0</sub> is the initial slope of the cumulative event number plot.

Tau is required when the cumulative event number plot is not linear due to the photo bleaching.

Fig. S19 How to use the “Stats” macro



The macro creates Matrix and Results folders.

Matrix folder contains a side-by-side matrix of each analysis results. Results folder contains the waves of stats (mean, sd, sem, and n).

# Fig. S20 Example waves in the Matrix and Results folders

## Matrix folder

### Parameter matrix wave of MSD

Row	MSD_avg_S0_m[]	MSD_avg_S0_m[]	MSD_avg_S0_m[]
0	0	1	2
1	0.00500314	0.00526875	0.00576375
2	0.0120773	0.0115625	0.0127474
3	0.0182476	0.0166603	0.0181497
4	0.0238967	0.0213793	0.0232293
5	0.0292303	0.0257554	0.0279257
6	0.0342909	0.029819	0.0324053
7	0.0391057	0.0337428	0.036684
8	0.0436207	0.0374983	0.0406618
9	0.0480769	0.041147	0.0446136
10	0.052624	0.0447818	0.0486081
11	0.0570795	0.0483549	0.0526086
12	0.0613937	0.0518142	0.0564456
13	0.0654452	0.0552962	0.0600148
14	0.0693923	0.0587669	0.0634982
15	0.0734701	0.0620452	0.0669969
16	0.0777791	0.0652375	0.0703611
17	0.0821415	0.0683141	0.0734828
18	0.086438	0.0712027	0.0765974
19	0.0906515	0.0742011	0.0796915
20	0.0945471	0.0770692	0.0829351
21			

test1    test2    test3

Waves with suffix “\_m” are created.

## Results folder

### mean

Point	MSD_avg_S0_m_avg
0	0
1	0.00534521
2	0.0121291
3	0.0176859
4	0.0228351
5	0.0276371
6	0.0321717
7	0.0365108
8	0.0405936
9	0.0446125
10	0.0486713
11	0.052681
12	0.0565512
13	0.0602521
14	0.0638858
15	0.0675041
16	0.0711259
17	0.0746461
18	0.0780793
19	0.0815147
20	0.0848505
21	

### sd

Point	MSD_avg_S0_m_sd
0	0
1	0.000386025
2	0.000594152
3	0.000889502
4	0.00130422
5	0.00175532
6	0.00224508
7	0.00268563
8	0.00306178
9	0.00346495
10	0.00392148
11	0.00436276
12	0.00479061
13	0.00507863
14	0.00532327
15	0.00572928
16	0.0063057
17	0.00698673
18	0.007725
19	0.00837537
20	0.00889501
21	

### sem

Point	MSD_avg_S0_m_sem
0	0
1	0.000222872
2	0.000343034
3	0.000513554
4	0.00075299
5	0.00101343
6	0.0012962
7	0.00155055
8	0.00176772
9	0.00200049
10	0.00226407
11	0.00251884
12	0.00276586
13	0.00293215
14	0.00307339
15	0.0033078
16	0.0036406
17	0.00403379
18	0.00446003
19	0.00483552
20	0.00513554
21	

### n

Point	MSD_avg_S0_m_n
0	3
1	3
2	3
3	3
4	3
5	3
6	3
7	3
8	3
9	3
10	3
11	3
12	3
13	3
14	3
15	3
16	3
17	3
18	3
19	3
20	3
21	

Waves with suffix “\_m\_avg”, “\_m\_sd”, “\_m\_sem”, “\_m\_n” are created.

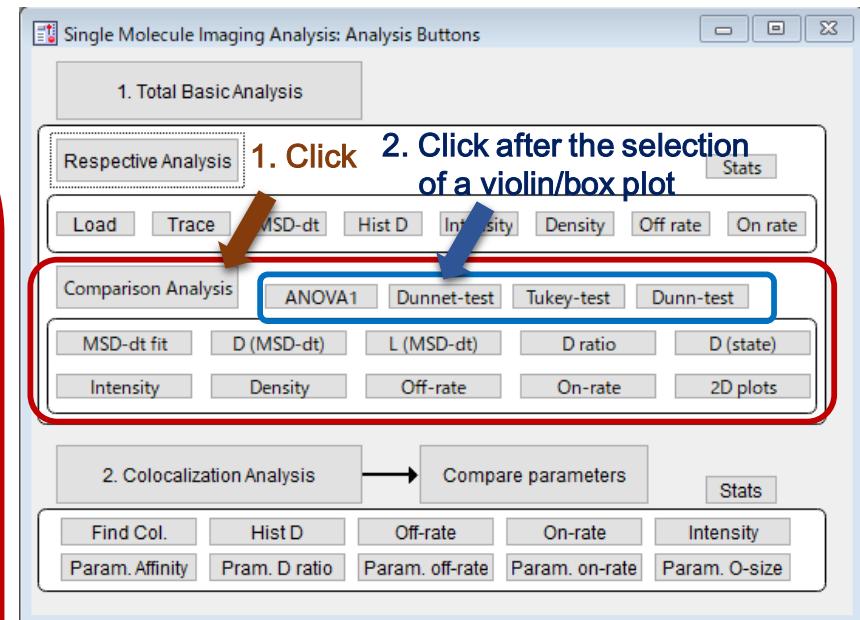
Calculation of stats

Note: At least 3 data are required to use the stats macro.

Fig. S21 Work flow of the “Comparison Analysis” and “Multiple comparison test” macros

### Comparison Analysis

- MSD-dt  
Output the MSD- $\Delta t$  plot (mean  $\pm$  sem of the cells).
- D<sub>MSD-dt</sub>  
Output violin/box plot of the diffusion coefficient (D) estimated from the MSD- $\Delta t$  plots.
- L<sub>MSD-dt</sub>  
Output violin/box plot of the confinement length (L) estimated from the MSD- $\Delta t$  plots.
- D<sub>ratio</sub>  
Output violin/box plot of the diffusion state ratio estimated from the displacement histogram analysis.
- D<sub>state</sub>  
Output violin/box plot of the D of each state estimated from the displacement histogram analysis.
- Intensity  
Output the intensity analysis results.
- Density  
Output violin/box plot of density analysis results.
- Off rate  
Output decay curves of the duration of trajectories and violin/box plots of the off rate parameters.
- On rate  
Output violin/box plot of the on rate parameters.
- 2D plots  
Output 2D plots of the diffusion, intensity, and density parameters of the single-cell.



### Multiple comparison test

- ANOVA  
Output result table of one-way ANOVA among groups in a selected violin plot.
- Dunnett  
Output result table of Dunnett's test among groups in a selected violin plot.
- Tukey  
Output result table of Tukey's test among groups in a selected violin plot.
- Dunn  
Output result table of Dunn-Holland-Wolfe's test among groups in a selected violin plot. (Non-parametric)

Fig. S22 Example of the violin/box plots (MSD- $\Delta t$  plot analysis) and the multiple comparison test

