ExoJ, a complementary tool for quantitative imaging of exocytosis

Exocytosis is a fundamental biological process where the intracellular vesicle eventually fuses to the plasma membrane, releasing its content into the extracellular space. To report exocytosis, we take advantage of pH-sensitive probes which are quenched in acidic environment and fluorescently bursts when exposed to a neutral pH environment (e.g. extracellular space).

We developed ExoJ, a computer-vision assisted tool implemented as a Fiji/ImageJ2 plugin to detect and record exocytosis features on single-cell basis. Built-in options allow end-users to immediately review each detected event and further the entire population within one cell.

We define a bona fide exocytosis event as a diffraction-limited object displaying a sudden burst of fluorescence followed by a decayed signal. Additional details are translated into a set of thresholding parameters to fully account of end-user experimental condition and type of vesicle.

Installation

The plugin ExoJ was successfully tested on computers (MAC OS Catalina and newer releases, Windows) with ImageJ2/Fiji 1.53s or newer versions and Java 8 installed.

It requires the prior installation of the plugin [Bio-Formats](#) (Linkert *et al.*, *J. Cell Biol.* 2010) which handles multiple image formats.

Download and copy the .jar file in Fiji/ImageJ2 plugin folder. Restart Fiji/ImageJ2, and the plugin ExoJ should appear in your plugin list under **Plugin/Project-ExoJ/ExoJ**.

Recommendation

The plugin was designed to automatically detect and record exocytosis from fluorescent time series. It can't handle 3D time series. We strongly advise to avoid time series displaying saturated pixels.

In case of lateral drift during live cell imaging, we recommend performing registration with available online plugins.

Procedure

The workflow comprises three main steps: **(1)** Detecting the bright spots seen as vesicles, **(2)** building 1D time-lapse trajectories and **(3)** identifying candidate exocytic events according to user definition.

At any points during the procedure, users can save and load detection settings (.dat file). A pop-up window lists opened files.

For newly-imported files, press Refresh to update the list.

Note that the preset parameters need to be tuned/adjusted.

STEP 1. Spot detection

METADATA
Upon file selection (Open), the plugin reads the metadata to fill the required information in the prompt-up window (Pixel size | Frame time interval | Frame rate).

DETECTION
For spot detection algorithm, we have implemented two options:

– **[default option]** a Wavelet-based approach *(Olivo-Marin 2002)*. Users are first asked to set a range of wavelet scales that roughly matches the size of the spots of interest (Min. and Max. fusion event apparent size). Pixel values are now accordingly converted to wavelet coefficient values. The detection threshold correspond to the Medial Absolute Deviation (MAD) calculated on wavelet coefficients ($\sigma_{wavelet}$). Upon activation, the resulting lowpass image is generated for further assessment (Show wavelet lowpass image).

– **[Untick Use wavelet filter]** The spot detection relies on the Maximum Finder tool implemented on Fiji/ImageJ2. The detection threshold correspond now to the MAD calculated on pixel intensity ($\sigma_{intensity}$)

The calculation is done on individual frames, and the result can be assessed by pressing **Preview**.

Both detection approaches can be carried out on first-order differential fluorescent time series (Transform to dF movie). This transformation can be reverted (*untick the option*). The subsequent steps are performed on raw fluorescent time series.

PHOTOBLEACHING CORRECTION

Live-cell imaging inherently faces photobleaching effect. To account for this, a correction can be performed upon clicking on the dedicated box (Correct Photobleaching). The correction is immediately applied and can be reversed (*untick the option*). Note that the presence of saturated pixels would most likely generate artifacts after correction.

MISCELLANEOUS

MAD: Median Absolute Deviation is a measure of dispersion based on the calculation of the median of the absolute deviation from the data's median.
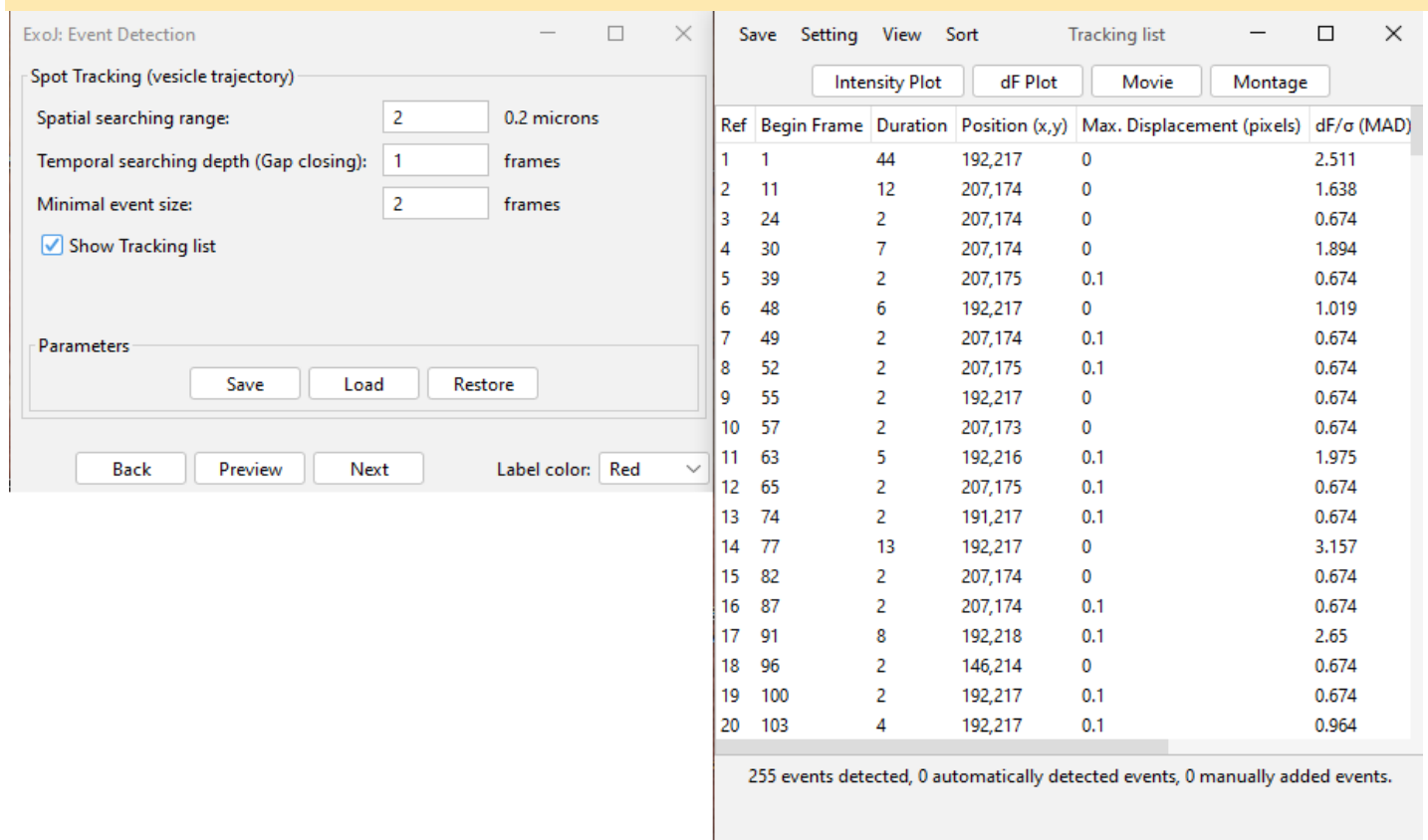
The color of the detected spots can be changed using the drop down list (bottom right-hand side, **Label color**).
Press **Run** to start the Spot detection.
You can **Save** your detection parameters or **Load** previous ones.

STEP 2. Spot tracking

The next step consists in building 1D time lapse trajectories using previously detected spots.

ExoJ: Event Detection			
Spot Tracking (vesicle trajectory)			
Spatial searching range:	2	0.2 microns	
Temporal searching depth (Gap closing):	1	frames	
Minimal event size:	2	frames	
☑ Show Tracking list			

Parameters: Save | Load | Restore

Back | Preview | Next | Label color: Red

Save   Setting   View   Sort   Tracking list

Intensity Plot | dF Plot | Movie | Montage

Ref	Begin Frame	Duration	Position (x,y)	Max. Displacement (pixels)	dF/σ (MAD)
1	1	44	192,217	0	2.511
2	11	12	207,174	0	1.638
3	24	2	207,174	0	0.674
4	30	7	207,174	0	1.894
5	39	2	207,175	0.1	0.674
6	48	6	192,217	0	1.019
7	49	2	207,174	0.1	0.674
8	52	2	207,175	0.1	0.674
9	55	2	192,217	0	0.674
10	57	2	207,173	0	0.674
11	63	5	192,216	0.1	1.975
12	65	2	207,175	0.1	0.674
13	74	2	191,217	0.1	0.674
14	77	13	192,217	0	3.157
15	82	2	207,174	0	0.674
16	87	2	207,174	0.1	0.674
17	91	8	192,218	0.1	2.65
18	96	2	146,214	0	0.674
19	100	2	192,217	0.1	0.674
20	103	4	192,217	0.1	0.964

255 events detected, 0 automatically detected events, 0 manually added events.

PARAMETERS
Three user-defined parameters are required to define spot trajectories

– Spatial searching range: spots located within a circular region centered on the detected spot, with a user-specified radius will be linked. If two or more spots fall within the region, the "closest" will be chosen.

– Temporal searching depth (Gap closing): this parameter defines the maximum number of consecutive frames that can separate two linked spots. By default, a gap tolerance of 1 frame enforces strict temporal continuity, requiring spots to be detected in every frame. For a gap of 2 and more, the missing frames are filled with a virtual spot corresponding to the timepoint preceding the gap.

– Minimal event size: a threshold is applied to the minimum number of spots required to be considered as a valid trajectory.

PRACTICAL CONSIDERATION

These three parameters are highly dependent on imaging modality and settings, such as pixel size and frame rate.

We assumed that fusion events are recorded across at least two consecutive images (i.e. two detected spots). Otherwise, detecting a single spot might indicate a vesicle moving in and out of focus, not necessarily a genuine fusion event. If you proceed with single-frame detections, ensure the Minimal event size parameter is set to 1 to prevent self-imposed limitations on ExoJ detection performance (Lachuer et al., 2024).

MISCELLANEOUS

Tracking results can previewed (**Preview** button). The list of reconstructed trajectories can be displayed if Show Tracking List is activated prior to pressing **Preview** or **Run** .

You can use the **Back** button to go back to the previous step (**Spot detection**).

STEP 3. Event Identification

To identify bona fide exocytosis events, the last step is made fully user-configurable.

PARAMETERS

Fitting procedure (mean lifetime and estimated apparent radius)

– Min. points for fitting procedure: minimal number of data points required to start fitting procedure. We recommend to leave it to the default value 5.

– Expanding frames (pre/post peak): number of additional frames on top of reconstructed trajectories (from step 2).
Increasing the number of pre-peak frames would refine the local background calculation (and hence the Detection threshold).
Increasing the number of post peak frames will affect the Gaussian fitting procedure for estimating the **mean lifetime**.
The Fixed position option enables additional frames to be either tracking maximum intensity (*unticked*) or stationary at their previous spatial position (ticked).

– Min. R^2: If ticked, minimal Goodness-of-fit is used as a hard threshold to select bona fide events.

Exocytosis event

– Detection threshold: Similarly to step 1, events are selected according to the MAD calculated on each trajectory (+/- pre/post peak frames). Only candidate events above the detection threshold value will be considered.

– Upper/Lower Decay limit: Setting the allowed range for how long events can last (**mean lifetime** is derived from a **2D Gaussian function**)
If no Lower Decay limit is set, the fitting process can yield negative mean lifetime values. For users who prefer alternative fitting functions, the raw data for each identified event is available in the latter step for further analysis.

– Upper/Lower apparent size limit: The apparent size is derived from the intensity profile (at the onset of the event) by fitting a **2D Gaussian function**. The Full Width Half Maximum is used as a proxy for the **event apparent size**. The raw data for each each identified event is also available in the latter step.
If none of these two parameters are activated, **the apparent size won't be derived from the 2D Gaussian fitting procedure**.

– Max. displacement: Maximal displacement allowed throughout the exocytosis event.

PRACTICAL CONSIDERATION

Increasing the number of pre/post peak frames could hinder the detection of two closely-timed events at the same location.
User-input values are in pixel unit for Upper/Lower Decay limit, Upper/Lower apparent size limit and Max. displacement.

MISCELLANEOUS

Results for identified events can previewed by pressing the **Preview** button).
A new window (Result table named *ExoJ: Detected exocytosis list*) will pop up, listing all events and the associated features and parameters.
You can use the **Back** button to go back to the previous step (**Spot tracking**).

Results

A pop-up table now provides a detailed list of all identified exocytosis events, including their normalized intensity (density), mean lifetime (duration) and estimated size.

| Save | Setting | View | Sort | | | ExoJ: Detected exocytosis list | | | | | | — | □ | × |

| Peak Intensity (F) Plot | (dF) Plot | Size | Spatial dynamics | Movie | Montage | Distribution Map | Event Counts | Size Stat. | Tau Stat. |

Ref	Property	Begin Frame	Duration	Peak Frame	Position (x,y)	Max. Displacement (pixels)	τ (sec)	R^2 for Decay Est.	dF/σ (MAD)	F0	ΔF	R^2 for Gaussian fit	Size (FWHM, microns)
53	Automatic	519	15	522	196,259	1	0.549	0.947	4.952	427	495	0.917	0.339
54	Automatic	529	18	532	203,298	1	1.256	0.956	13.69	395	522	0.922	0.343
55	Automatic	544	358	548	378,321	2	18.655	0.813	4.167	813	144	0.655	0.413
56	Automatic	562	16	565	317,272	2.236	0.772	0.924	7.42	479	318	0.912	0.28
57	Automatic	565	29	570	293,338	0	-3.785	0.849	5.662	414	435	0.953	0.246
58	Automatic	572	20	575	268,341	0	0.93	0.983	18.526	390	1,211	0.99	0.296
59	Automatic	576	15	579	307,351	1	0.317	0.95	8.964	371	452	0.977	0.279
60	Automatic	589	19	592	203,204	0	1.153	0.924	25.766	424	1,311	0.982	0.273
61	Automatic	600	19	603	193,323	1	0.548	0.993	24.64	386	1,129	0.962	0.288
62	Automatic	607	16	610	212,380	0	0.446	0.983	9.378	518	404	0.942	0.288
63	Automatic	615	15	618	246,150	1	0.239	0.983	22.048	331	510	0.965	0.305
64	Automatic	629	26	632	257,339	1	0.903	0.979	16.881	512	849	0.983	0.272
65	Automatic	647	16	650	245,380	1	0.43	0.976	14.095	460	587	0.904	0.314
66	Automatic	656	19	664	254,414	0	0.575	0.959	6.352	383	5	0.957	0.291
67	Automatic	678	30	683	269,413	3.162	-2.299	0.926	8.484	204	295	0.933	0.288
68	Automatic	680	23	684	276,320	0	1.198	0.977	9.464	287	1,535	0.989	0.338
69	Automatic	687	25	690	214,222	2.236	-52.145	0.881	11.954	349	321	0.854	0.474
70	Automatic	691	15	694	390,313	1	0.295	0.951	5.444	336	152	0.758	0.342
71	Automatic	692	15	695	65,393	0	0.482	0.807	5.786	485	106	0.843	0.369
72	Automatic	699	22	702	329,241	0	0.015	0.992	22.146	400	1,734	0.991	0.268

83 events detected, 83 automatically detected events, 0 manually added events.

Set a ROI over the missing event in the result window to add it | Add | Remove | Export current table | Export all intensity plots | Export all table

Top buttons are shortcuts to display:

– **Peak Intensity (F) Plot**: Fluorescence peak intensity F over time
– **(dF) Plot**: First-order differential Fluorescence peak intensity dF over time
– **Size**: 2D intensity profile and its associated Gaussian fitting
– **Spatial dynamics**: Recording the radial peak intensity profile over time. Event XY position is used as a central point.
– **Movie**: Generating a 9-by-9-pixel timeseries tracking the selected event
– **Montage**: Generating a custom montage using the **Movie**
– **Distribution Map**: Spatial distribution of identified events
– Histograms (**Event counts**, **Size Stat.**, **Tau Stat.**): Generating the corresponding histogram on the number of identified events, their apparent size and mean lifetime.

Selected Secretion Event   — □ ✕

[+] [-] ☑ Fixed position

Ref	Property	Frame	Intensity	x	y	R² for Gau...	Size (FWH...
0	Expanded	562	479.0	317	272	0	0
1	Expanded	563	508.0	317	272	0	0
2	Expanded	564	511.0	317	272	0	0
3	Automatic	565	797.0	317	272	0.28	0.912
4	Automatic	566	643.0	315	273	0.395	0.82
5	Automatic	567	658.0	315	273	0.396	0.809
6	Expanded	568	618.0	315	273	0	0
7	Expanded	569	605.0	315	273	0	0
8	Expanded	570	568.0	315	273	0	0
9	Expanded	571	581.0	315	273	0	0
10	Expanded	572	547.0	315	273	0	0
11	Expanded	573	512.0	315	273	0	0
12	Expanded	574	531.0	315	273	0	0
13	Expanded	575	505.0	315	273	0	0
14	Expanded	576	498.0	315	273	0	0
15	Expanded	577	509.0	315	273	0	0

[+] [-]

[View size curve] [View selected vesicle radius] [View movie] [View image]

TIPs

Raw data can be accessed by double-clicking on the corresponding identified event. A pop-up table (Selected Secretion Event) provides the recording of quantitative features for the selected event.

+/-: add frames pre and post peak *a posteriori*. Table will be updated to include additional frames (added frames will be **Ref**erenced with **0** and **Property** named as **Custom**).
View size curve: Displaying the event FWHM over time.
View selected vesicle radius: First, select a frame (i.e. line in the table). Display the XY plot used to derive the FWHM

View movie: Generating a 9-by-9 pixels timeseries centered on the selected event.
View image: Generating a still image corresponding to the selected timepoint in the table.

Mean lifetime and **Size (FWHM, microns)** estimation could be flagged in red for the following reasons:
– for **mean lifetime** ≤ 0 (unit), the estimated value will be colored in red. The button Lower

apparent size limit was not activated (unticked). This is not a perfect solution, but this allowed the detection of exocytosis events with "positive slope"

– for the **estimated apparent size (FWHM)** the estimated value is compared to the theoretical resolution (STEP 1) if filled by users. *Note that the **estimated apparent size** won't be displayed if one of both buttons related to the apparent size limit is/are not activated (unticked).*

References

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