Matlab tools for microscopy image processing and analysis

Hampus Du Rietz
Department of Oncology and Pathology, Lund University
hampus.du_rietz [at] med.lu.se

2019

Table of contents

Introduction

The microscopy image processing and analysis tools presented here were developed with the aim to streamline the analysis of intracellular vesicle events in live-cell widefield deconvolution microscopy data. Aspects of the data organization and operations are highly adapted to fit specific experimental and technological requirements. Nonetheless, parts of the framework can be used more or less free-standing, or can be modified to suit other specific needs.

Requirements that were central in the development of these tools include

- Automated image handling and analysis with batch processing options
- Possibility of processing large-size image datasets
- Efficient and adaptive automated or semi-automated tracking of intracellular structures
- Efficient options for data visualization
- Streamlining the analysis of many events of interest

To achieve this, three main strategies or components have been adopted

1. Pre-defined folder architectures and data organization
2. Standardized operations for data handling and processing
3. A MATLAB program with a graphical user interface

The image processing steps outlined below are only used for automated image handling and deconvolution, using Huygens deconvolution. For demo of the vesicle tracking and analysis functions, follow the instructions in blue in the corresponding sections.

Home folder and data repository organization

The MATLAB program works against a well-defined folder architecture on one or multiple data storage devices. Typically, our microscopy data is stored and accessed on a network associated storage (NAS). To make processing more efficient, some of the operations are performed on a local solid-state device (SSD) on the processing computer. The transfer of data from the primary storage device to the local SSD is performed automatically. Data can also be transferred and archived on external hard drives or data cartridges – all following a predetermined organization architecture.

On the NAS, the user has an individual or shared home folder. This directory contains the following subfolders

- Data repository | Containing all data in a hierarchical fashion, where acquisitions are organized in the folder of their corresponding experiment. The acquisition folder contains all the data associated with the specific dataset.
- Processing tools | Containing tools necessary for processing and analysis, including
    - Matlab | Containing all Matlab functions and code
    - Point spread functions | Containing all microscope PSFs that can be used for image deconvolution
    - Chromatic aberration correction model | Containing the model for using automatic correction of chromatic aberrations in the dataset
    - Tracking masks | Containing tracking masks used for object tracking and measurements
- Output data | The folder where collected data is exported

Download the zip-folder from the GitHub repository for either Mac or Windows. The file containes a Home folder `Home` with the subdirectories as outlined above. The MATLAB functions and the GUI is located in the folder `Home > Processing tools`. Run the GUI by clicking `widmip.mlapp`. The GUI opens in a separate window. In the main MATLAB window, set the current directory path to the Home folder

Matlab graphical user interface

The graphical user interface (GUI) enables a more intuitive and easier way to set up experiment parameters and use the processing tools. Experiment and metadata parameters are entered in the `Experiment tabs` section as described in the following sections. Some parameters are given as default values upon start of the application. These can be changed by configuring the application itself in the App Designer feature in Matlab.

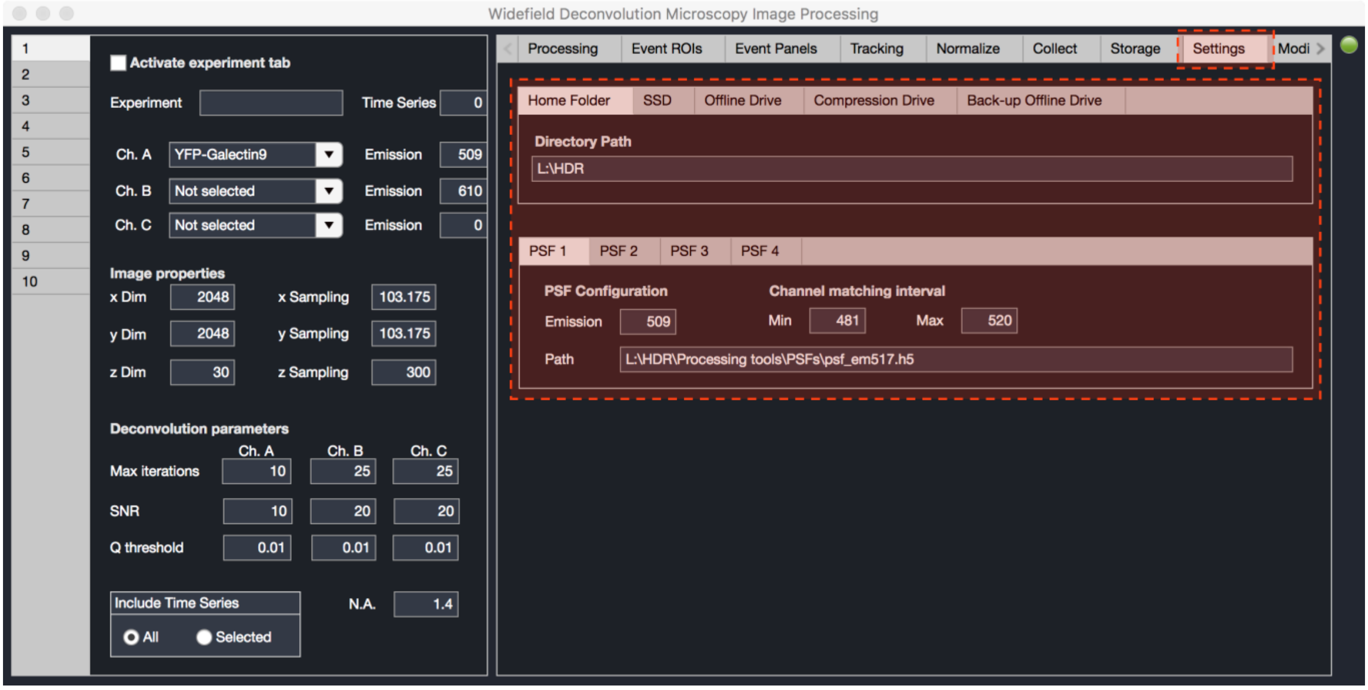
The directory paths of the cornerstone folders in the data organization should be entered in the `Settings` tab. Following the initial setup of the folder organization for a specific user, there is no need to change these paths. They include

- Home folder | The path of the user specific home folder on the NAS
- SSD | The path of the user specific main folder on the local SSD of the processing computer
- Offline drive | The path of the external hard drive used for offline data storage and archiving
- Compression drive | The path of the local hard drive on the compression computer, used for performing the file compression before offline storage or archiving

For most of the operations to be executed, the Matlab path should be set to the folder containing the functions to be used, that is `Home folder > Processing tools > Matlab`. The Matlab path is often changed during the execution of the operations. For most instances, the 'Backwards' button can be used to quickly reach the function-containing folder.

Setting up experiment parameters

Experiment metadata and processing parameters should be specified in the experiment tab section in the GUI. To activate an experiment tab for processing, the top left box `Activate experiment tab` must be ticked. A maximum of ten experiments can be processed during the same run.



Widefield Deconvolution Microscopy Image Processing

☐ Activate experiment tab

Experiment | Time Series 0

Ch. A YFP-Galectin9 | Emission 509

Ch. B Not selected | Emission 610

Ch. C Not selected | Emission 0

Image properties

x Dim	2048	x Sampling	103.175
y Dim	2048	y Sampling	103.175
z Dim	30	z Sampling	300

Deconvolution parameters

	Ch. A	Ch. B	Ch. C
Max iterations	10	25	25
SNR	10	20	20
Q threshold	0.01	0.01	0.01

Include Time Series ◉ All ○ Selected

N.A. 1.4

Directory Path L:\HDR

PSF Configuration: Emission 509

Channel matching interval: Min 481 Max 520

Path L:\HDR\Processing tools\PSFs\psf_em517.h5

- Experiment | The name of the experiment with appropriate syntax

- Time series | This value specifies the number of acquisitions in the experiment. The value only needs to be specified to execute the 'Create Folders' operation.

- Ch. A, Ch. B, Ch. C | The name of the channels in the dataset. The order of the channels given in the GUI need to match the order of the channels in the dataset (c1, c2, c3). Enter any name or choose from the list.

- Emission | The emission wavelength for the corresponding channel (nm).

- x Dim, y Dim, z Dim | The spatial dimension of the datasets (pixels).

- x Sampling, y Sampling, z Sampling | The sampling intervals of the spatial dimensions (nm).

- Max iterations | The maximum number of iterations allowed during the deconvolution process.

- SNR | Signal-to-noise estimate of the raw data.

- Q-threshold | Quality threshold of the deconvolution process.

- N.A. | Numerical aperture of the microscopy objective.

- Include Time Series: All, Selected | Specify in all acquisitions of the experiments should be processed, or only hashtag-selected acquisitions.

If individual acquisitions of the same experiment have different z-dimensions or z-sampling intervals, the acquisitions should be processed separately (with matching datasets) using hashtag-selection.

In the first Experiment tab, check the `Activate experiment tab` box to activate the tab. As experiment name, enter `e000000_EXP0000` in the empty `Experiment` field. In the `Ch. B` list (Channel B), select `DY547-siRNA` and enter `547` in the `Emission` field to the right. Leave all other fields as pre-set.

For the deconvolution process, the full path of the appropriate point spread function (PSF) file should the entered in the PSF section in the Settings tab, along with the wavelength (nm) of the PSF and the minimum and maximum wavelengths for which the PSF should be used. Four PSFs can be used, that are matched to the wavelengths entered for each of the channels in the `Experiment settings tab`.

Adding experiment folders and data

To add new data into the organization, the GUI can be used to generate the appropriate folder structure of single or multiple experiments. In the `Experiment` tabs section, add the name/names of the experiments, the number of acquisitions (datasets) per experiment, and specify the channel names to be used. After this, simply press the `Create folders` button in the `Processing` tab to create all folders in the correct organization. Experiment names must start with a letter, and can only contain letters, numbers, underscores or dashes.

The raw data czi-files have to be manually transferred to `Data repository > Experiment > Acquisition > zenRaw`. Also, it is important that the raw data czi-file has a specific naming component indicating the number of stacks (time points) in the acquisition. The syntax preferably used is *e.g.* `filename_n100.czi` for a dataset with 100 time points. In this way, the naming of the files provides a simple piece of metadata used for the processing.

Add the available demo datasets in the following folders in the `Home > Data > Respository > e000000_EXP0000 > e000000_EXP0000_TS01`. Replace the existing `cellMetadata`, `eventMetadata` and `eventROIs` folder with the corresponding folders available at link, containing the demo dataset.

Hashtag selection of datasets for processing and analysis

The processing or analysis of specific datasets within experiments can be achieve by adding a hashtag [#] as the last character to the folder name of the datasets that should be included in the operation. Selecting individual acquisitions an experiment folder can be used for

- Image processing (export, deconvolution)
- Storage and archiving operations
- Creating event panels
- Creating event ROIs

For generating event panels and event ROIs, even more specific data selections can be used

- Selection of data associated with individual cells in an acquisition
- Selection of data associated with individual events

Make sure to deselect the folder after completing the operation.

No demo is available for this section.

Preparing Java Robot class operations

In order to achieve image export and deconvolution, other commercially available software programs are used during the raw data processing operations. The programs are handled via Java Robot class commands in Windows itself, virtually running the computer according to the code in the Matlab functions. When the Java Robot is running, any additional mouse or keyboard operations (movements, clicks) will disrupt the Java operations. Before initiating any Java Robot class operation, the mouse pointer will run diagonally from the top-left to the bottom-right corner of across the screen, as a sign to refrain from any manual work at the computer during the process.

The raw data files that are handled are czi-format files compatible with the ZEN Blue software. The Java Windows controller tools are configured to work with version X. Modifications to the `runZEN.m` function can adapt the execution according to the specific needs.

The image deconvolution is performed using the *Batch processing* interface of the Huygens Professional Deconvolution software. The available code will work for version X, using empirical PSF files compatible with the Huygens software. Modifications to the `runHuygens.m` function can adapt the execution according to the specific needs.

Before initiating the raw data processing (export and deconvolution), some preparations are required to successfully execute the Java Robot class operations:

1. Start the ZEN Blue software. Select the *Batch* mode and *Image export*.

2. Start Huygens Professional software main window.

3. Maximize the program windows, and in the windows bar, put the ZEN icon to the far left of all icons, with the Huygens icon following second.

Raw data processing

No demo is available for this section.

After preparing the processing in the GUI and initiating the programs as described above, the data processing can be initiated. In the GUI, it is possible to delay the start of the processing with a timer (slider). This can be of use if the raw data czi-files are being transferred from the acquisition computer to the NAS, in which case the processing can be delayed until the transfer of the files is completed. Remember that the raw czi-files must contain the appropriate *n*-string for processing before initiating the transfer if the processing is set to start immediately after completing the transfer.

The data processing procedure contains the following steps, which are executed in a serial mode for all of the acquisitions selected for the processing:

1. The raw data czi-file is copied to the SSD device.

2. Raw image export is performed using the ZEN Blue software. Images are exported as single plane, single channel 16-bit tiff-files.

3. The raw tiff-images are resampled using Matlab, so that all z-planes of individual stacks are contained in a single tiff-file. This is a requirement for appropriate deconvolution of the stack in Huygens.

4. Image deconvolution is performed using the ‘Batch processing’ interface in Huygens. The deconvolution is performed using the experiment parameters specified in the GUI and the experimental PSFs provided in the settings section of the GUI.

5. Deconvolved images and deconvolution metadata are transferred to the NAS.

6. Maximum intensity projections of the deconvolved data is created using Matlab.

7. The remaining files on the SSD is deleted.

Confocal or other microscopy system data

The general data organization and analysis tools available with the Matlab GUI can be used for other microscopy data than widefield data or Zeiss data files.

For microscopy data acquired on a Zeiss microscopy system (*e.g.* confocal microscope) and saved as czi-files, the GUI can be used for image export and organization. This requires that the `Skip deconvolution` option in the `Processing` tab is enabled. All other experiment parameters should be specified in the `Experiment tabs` section as usual (except deconvolution parameters). The image processing procedure will then include image export using ZEN, resampling and sorting of the image files, and creating maximum intensity projections of *z*-stacks. The raw data images will be located in the `Experiment > Acquisition > decon` folder, even though the deconvolution was not performed. This is to enable subsequent analysis of the data without using special options or routines.

For microscopy data acquired on other microscopy systems, the image files should be exported as raw data tiff-files and organized in separate folders in `Experiment > Acquisition > decon`. The GUI can be used to generate the appropriate folder organization. No standard processing (deconvolution or generating maximum intensity projections) is available using the GUI, but the processing procedure could be tailored to fit other microscopy system software. In that case, replacing or modifying the `runZEN.m` and `imProcess.m` functions would be a good place to start. `imProcess.m` contains the full routine of the operations included in the processing, and `runZEN.m` is responsible for actuating the control of the ZEN software using Java Robot class operations.

Visualizing images in ImageJ/Fiji

No demo is available for this section.

Visualizing the complete dataset becomes ever more challenging as the size of the data increase. Images can be opened in ImageJ/Fiji as virtual stacks, where the data is only contained in the RAM when the specific image is being view when toggling the dataset. Although minimizing the RAM usage, the virtual stack mode is often unsatisfactory because of significant lag as the data is constantly being transferred to the RAM during toggling of the dataset, and also because of difficulties maintaining the appropriate window settings (brightness/contrast) when toggling. For most applications, the most efficient way to visualize the data is read all of the data in the RAM, in which case the maximum intensity projections often have to be used to fit all data in the limited RAM. For many applications MIPs are also more appropriate for quickly visualizing and inspecting the 3D volume.

The data is imported via the Bioformats plugin. As the data is organized in separated folders for each channel, the channels are opened separately and then merged in the software using *Image* > *Channels* > *Merge channels*.

No demo is available for this section.

Event calling in ImageJ/Fiji

For the purpose of vesicle or structure analysis, individual events of interest can be identified manually in the ImageJ/Fiji software, followed by further analysis. Open the dataset of interest, and open the ROI manager located in *Analyze* > *Tools*. The typical procedure is performed on a per cell level:

1. Pan the data and identify events of interest

2. Pin-point the event of interest using the *Point tool* (hair cross). In the case of *e.g.* marker recruitment or change of state identification, pin-point the structure at the time point close to the change of the state.

3. Click the *Add* button in the ROI manager, or press *T* to add the point to the ROI list

4. Continue and add the remaining events of interest to the ROI list one by one.

5. After adding all points of interest, change to the *Freehand selections* tool.

6. At the time point of the first event of interest identified in the cell, draw an outline of the cell and add to the ROI list. Repeat this process at the time point for the last event. If only one event of interest has been identified, simply add two identical copies of the ROI to the list. It is necessary that the two cell outline ROIs are listed last in the ROI list and in the correct order.

7. Save the list in the data organization in `Experiment > Acquisition > celldata` by creating a new folder named `cell_`*n*, where *n* is the number of the cell processed in the dataset written as `01, 02, 03,`.... To save the list, click *More* > *List*, right-click in the window and select *Save as.* Save the list as `data.txt` in the folder. Be sure to add the `.txt` extension to the name of the file.

8. Save the cell outline ROIs by selecting the individual ROIs in the list (marked blue), go to *Analyze* > *Tools* > *Save XY Coordinates*. Save the file as `startFrame` or `endFrame`, respectively. No file name extension is required. The files have to be saved separately.

9. Clear the ROI manager list and repeat the process for all cells to be analyzed.

10. After identifying the events of interest in all cells, select a region of the background in the image at the first time point in the dataset and add this to the ROI list. Save

this ROI in `Experiment > Acquisition > background` in the same way as described in above, with the filename `background` without extension.

Creating ROIs of events

No demo is available for this section. The dataset used for demo contains event ROIs.

After object or event identification, the relevant data for further analysis of individual objects can be extracted as event ROIs of the full dimensional dataset, with some associated whole-cell data and metadata. In the GUI, options for creating the ROIs should be specified accordingly:

- Extend ROI | Specifies the lateral *xy*-dimension of the ROI, indicated as the pixel distance from the center pixel given by the event location. For example, the value 75 will create a ROI with 2 × 75 + 1 = 151 pixels in both *x*- and *y*-dimension.

- Times Pre | Specifies the number of time points before the indicated event to include in the ROI. To include all time points, set the value to the number of time points in the complete dataset.

- Times Post | Specifies the number of time points after the indicated event to include in the ROI. To include all time points, set the value to the number of time points in the complete dataset.

Note that all *z*-planes and all channels will be included in the ROI. A specific processing option, `File Access Mode`, makes it possible to read the data from the NAS or using predefined criteria to determine if transfer to the SSD will yield the shortest processing time. This depends on the number of events to be processed, the i/o speed of the various storage devices, the number of i/o operations per event and the transfer speed between the storage units. These parameters can be modified to meet the hardware and processing criteria in `benefitSSD.m`.

If the experiment and ROI parameters have been adequately provided in the GUI, the processing will begin by creating a new directory for all events identified in each cell in the Experiment > Acquisition > eventdata directory. Each event will have its own folder, containing all the metadata and other data used for further analysis. The completed event ROIs will be saved as mat-files in Acquisition > eventROIs > cell_*m* > event_*n*. Specific measurements of the cell in which the event occurred, as well as image background information, will be saved in the event folder as well.

If the generation of an event ROI is reiterated, *e.g.* if the time or spatial dimension have to be modified, the Remake option in the Select Task section in the GUI should be selected to correctly execute the operation.

Chromatic aberration correction

Some specific processing tools and options are used to minimize effects of chromatic aberration in the microscopy system. A linear correction model for each spatial dimension and for each channel can be generated using analysis of images of fluorescent beads (Tetraspeck). The correction model can then be applied to the event ROIs to shift the frames of the channels to minimize the aberration. The correction procedure can be automatically applied every time an already generated event ROI is processed by Matlab, *e.g.* when creating event ROI panels or performing tracking analysis, although the original event ROI data is always kept uncorrected.

If a correction model is not currently available or should not be used, the Skip chromatic aberration correction option can be enabled under the Event panel or Tracking tab in the GUI.

The chromatic aberration correction is enabled by default and will correct the demo dataset according the the correction models present in the `Processing tools` directory in the Home folder. To turn off the correction, check the box next to `Skip chromatic aberration correction` in the `Event panels` or `Tracking` tabs as appropriate.

Create visualization panels of event ROIs

To make the inspection and visualization of event ROIs easier and less time consuming, ROIs can be put together in new datasets called event panels using the GUI. Event panels can be put together with only the event ROI data itself, or with the addition of tracking data in a separate channel. All event ROIs are required to have the same number of channels, but can have spatial and temporal dimensions of different sizes. Different options are available as how to display the *z*-dimension and any available tracking data:

- MIP Untracked | This option will create a maximum intensity projection of the event ROIs and will not include any available tracking data in the event panel. This option is suitable for obtaining a quick view of the event ROI data, *e.g.* verifying the appropriate cropping of the ROIs. The *t*=0 time point will be the frame in which the original coordinates of the event were located during event identification.

- MIP Tracked | This option will create a maximum intensity projection of the event ROIs and will also include available tracking traces in a separate channel. This option is suitable for inspecting the accuracy of the tracking procedure. The *t*=0 time point will be the frame in which the event is detected in the tracking analysis

- Z-Cropped Tracked | This option will create a dataset containing a number of individual *z*-planes as specified by the Extend Planes option, and will also include available tracking traces in a separate channel. The *t*=0 time point will be the frame in which the event is detected in the tracking analysis, and the tracked plane will always be kept at the center *z*-plane in the event panel data stack.

- Z-Complete Untracked | This option will create a dataset containing all individual z-planes of the event ROIs, and will not include any available tracking data in the event panel. The size of the *z*-dimension is likely to generate event panels of a significant size using this option, making visualization in ImageJ/Fiji cumbersome.

There are some specific variables determining the layout and the dimensionality of the event panels

- Extend Planes | Specifies the number of planes to include in the maximum intensity projections in MIP Tracked and MIP Untracked, or the number of planes to include in the Z-Cropped Tracked visualization options. Typically, this value should correspond to the number of z-planes in the dataset.

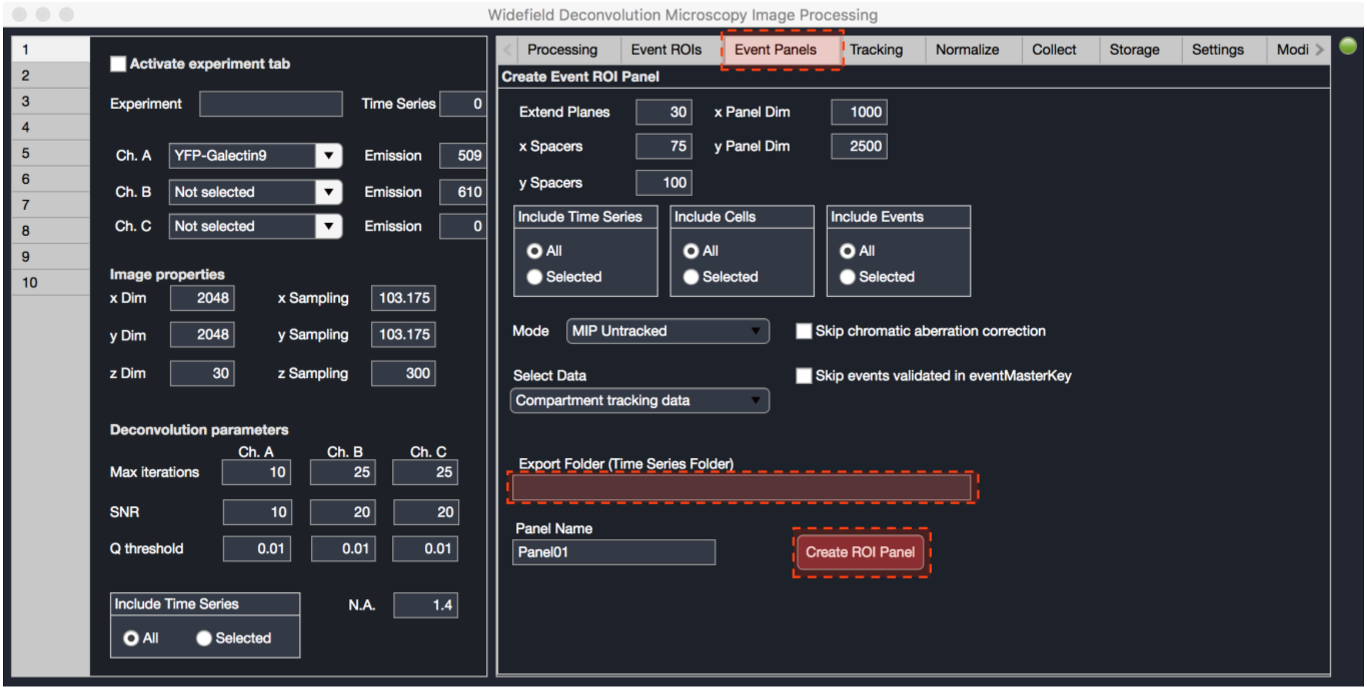
- X Spacers, Y Spacers | Specifies the horizontal and vertical distances, respectively, separating individual event ROIs in the panel.

- X Panel Dim, Y Panel Dim | Specifies the horizontal and vertical dimension of the event panel, respectively.

After running `Create ROI Panel`, the `eventPanel` variable in the Matlab workspace have to be exported to an appropriate location, specified in ________. Provide a name of the panel and click the `Export event panel` button. `eventPanelMetadata.m` will be saved to the same folder, for later use during manual event tracking. The event panel can be imported and visualized in ImageJ/Fiji in the same way as described previously for the complete MIP data.

To create a visualization panel using the demo data, go to the `Event Panels` tab. Use all pre-set parameters. In `Export folder` field, enter the path corresponding to `Home > Data repository > e000000_EXP0000 > e000000_EXP0000_TS01`. Click on the red `Create ROI Panel` button. The top left lamp on the GUI will shift from green to orange as MATLAB is running, and will return to green when the operation has finished. The output files are located in the selected Export folder, in a subdirectory called `eventPanels > Panel01` **`date`** `> panelFiles`. Open the exported panel and all channels in Fiji using

Compartment tracking – Automated object tracking



□ Activate experiment tab

Experiment
Time Series 0

Ch. A YFP-Galectin9 Emission 509
Ch. B Not selected Emission 610
Ch. C Not selected Emission 0

Image properties
x Dim 2048 x Sampling 103.175
y Dim 2048 y Sampling 103.175
z Dim 30 z Sampling 300

Deconvolution parameters

	Ch. A	Ch. B	Ch. C
Max iterations	10	25	25
SNR	10	20	20
Q threshold	0.01	0.01	0.01

Include Time Series ○ All ● Selected
N.A. 1.4

Create Event ROI Panel

Extend Planes 30 x Panel Dim 1000
x Spacers 75 y Panel Dim 2500
y Spacers 100

Include Time Series ○ All ● Selected
Include Cells ○ All ● Selected
Include Events ○ All ● Selected

Mode MIP Untracked
□ Skip chromatic aberration correction
Select Data
Compartment tracking data
□ Skip events validated in eventMasterKey

Export Folder (Time Series Folder)

Panel Name
Panel01

Objects or structures in the event ROIs can be analyzed using automated tracking. In principal, the default tracking procedure consists of:

1. Identification of the time point and location at which the event of interest occurs'

2. Forward tracking of the object of interest

3. Pre-event stationary measurements performed at the location where the object or event is first identified

This specific procedure is referred to as compartment tracking. A set of tracking parameters are needed to properly perform the analysis, and some adjustments might be needed to successfully track a set of events with a certain variability:

- Track Times Pre-Event **|** Specifies the number of frames to include in the analysis prior to the event (the number of static measurements)

- Track Times Post-Event | Specifies the number of frames to include in the analysis after identification of the event (the number of tracking measurements)

- Min Times Pre | Specifies the minimum number of time points required.

- Event Detection Search Radius | Specifies the maximum xy-distance from the given coordinates of the object within which to initially detect the event. If there are many objects present in the near vicinity of the object to be detected, the parameter should be set lower.

- Event Tracking Search Radius | Specifies the maximum *xy*-distance to search for the objects between frames. If there are many objects present in the near vicinity of the object to be detected, other objects with higher intensity will be erroneously tracked is they are located within the event tracking search radius. If the objects which is tracked is highly mobile, the parameter should be adjusted to correspond to the maximum *xy*-distance travelled by the object between two frames.

- z-Search Distance | Specifies the maximum *z*-distance to search for the objects between frames. If the objects which is tracked is highly mobile, the parameter should be adjusted to correspond to the maximum *xy*-distance travelled by the object between two frames.

- Start Event Detection | Specifies?

- Confirm Event Detection | Specifies the number of time points used in the confirmation of the event identification. It represents the number of time points following the initial identification of an object with an intensity higher than the event detection threshold at which the intensity must also exceed this limit.

- Event Detection Threshold | Specifies the sensitivity of the event detection. The parameter represents the number of standard deviations from the mean signal in the object mask that corresponds to a significant recruitment or accumulation of the marker that defines the event.

- Object Mask Threshold | Specifies the sensitivity of the algorithm determining the size of the object.

The channel order of the dataset should be specific in the GUI the properly execute the tracking of objects using either the `Compartment Tracking` or `Cargo Tracking` method:

- Primary channel | The channel used for event (recruitment) detection and for post-recruitment tracking.

- Secondary channel | The channel containing compartment markers (compartment tracking), or cargo (cargo tracking)

- Tertiary channel | The channel containing the second compartment marker (if dual compartment tracking), or the single compartment marker (if cargo tracking with cargo as secondary channel)

After parameter setup, the tracking analysis is performed for all events selected. During the analysis, only the raw image data in the event ROIs and the ROI metadata are required. This makes the analysis fast and efficient, and the work required for re-analysis or parameter adjustment is minimized. The tracking data is automatically saved in `Acquisition > trackingdata > cell_m > event_n`.

> Select the `Tracking` tab and use all pre-specified parameter settings. The tracking parameters have already been individualy adjusted for some of the events, using the `Modify Tracking Parameters` section, to achieve adequeate tracking for the majority of events. Press the red `Track Compartments` button to start the object tracking. The top-rigtht lamp will turn green when the tracking is completed. To visualize the trajectories, continue to 'Visualize and validate object tracking' below

Cargo tracking – Semi-automated object tracking in ImageJ/Fiji

A specific strategy can be used to track small and highly mobile structures in a crowded environment, where a successful automated event tracking is hard to achieve. The original idea of this tool was to use automated tracking of the structure of interest, but extend the tracking of the object before recruitment of a certain marker. This alternative approach relies on manual identification of the specific structure of interest until significant recruitment or accumulation of the marker has occurred. As an example, to measuring the release if endosomal cargo from intracellular vesicles in the presence of a membrane damage sensor like a galectin, the releasing vesicle is manually traces up until a clear galectin recruitment has occurred. In the following quantification process, the structure of interest will be automatically tracked in using the galectin signal after significant recruitment, and the manual tracking data will be used for the analysis at the time points prior to the galectin accumulation.

To perform the analysis, start by creating a `MIP Untracked` event panel with the events to be analyzed (events that will not be tracked can still be included in the panel), and export the panel and the panel metadata. After this:

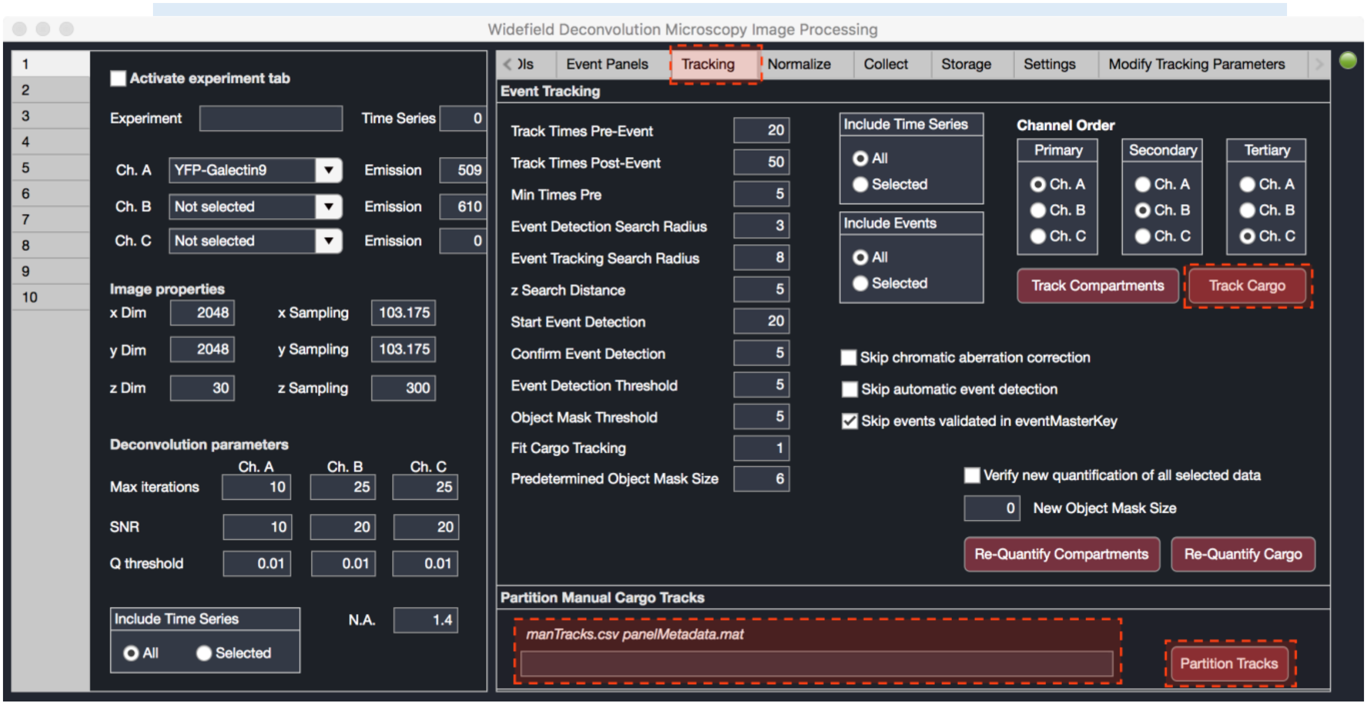
1. Open the event panel in ImageJ/Fiji
2. Go to *Plugins* > *Tracking* > *Manual Tracking*
3. Before starting the tracking, make sure that the first channel in the stack is selected with the channel slider. This affects the indexing of the tracking coordinates.
4. Click *Add track*.
5. Locate the structure of interest in the ROI, and track it from a time point of choice prior to the recruitment of the marker.
6. After clear recruitment of the marker, stop the tracking by pressing *End track*.
7. Repeat the process for all events to be analyzed. Specific tracking coordinates of whole tracks can be deleted using the plugin options.
8. When the tracking of the events is completed, save the coordinates by right clicking on the window of the table and save the data as `manTracks.csv` in the same folder as the event panel and event metadata.
9. In the Matlab GUI, enter the path of the event metadata and the tracking coordinates.
10. Press `Partition Tracks` to partition all tracking coordinates. The tracking coordinates of individual events will be saved in `Acquisition > eventMetadata > cell_`*`m`*` > event_`*`n`*.

After generation and partitioning the tracking coordinates, the data extraction and additional tracking can be performed using the GUI:

1. Specify the tracking parameters as described in the Automated event tracking section.

2. Fit Cargo Tracking | Specifies the number of pixels with which to fit the object mask to the object, in order to reduce inaccuracy in the manual tracking.

3. Run the tracking by pressing `Track Cargo`



☐ Activate experiment tab

Experiment | Time Series 0

Ch. A YFP-Galectin9 | Emission 509

Ch. B Not selected | Emission 610

Ch. C Not selected | Emission 0

Image properties

x Dim	2048	x Sampling	103.175
y Dim	2048	y Sampling	103.175
z Dim	30	z Sampling	300

Deconvolution parameters

	Ch. A	Ch. B	Ch. C
Max iterations	10	25	25
SNR	10	20	20
Q threshold	0.01	0.01	0.01

Include Time Series: ◉ All ○ Selected

N.A. 1.4

Event Tracking

Track Times Pre-Event	20
Track Times Post-Event	50
Min Times Pre	5
Event Detection Search Radius	3
Event Tracking Search Radius	8
z Search Distance	5
Start Event Detection	20
Confirm Event Detection	5
Event Detection Threshold	5
Object Mask Threshold	5
Fit Cargo Tracking	1
Predetermined Object Mask Size	6

Include Time Series: ◉ All ○ Selected

Include Events: ◉ All ○ Selected

Channel Order

Primary	Secondary	Tertiary
◉ Ch. A	○ Ch. A	○ Ch. A
○ Ch. B	◉ Ch. B	○ Ch. B
○ Ch. C	○ Ch. C	◉ Ch. C

☐ Skip chromatic aberration correction

☐ Skip automatic event detection

☑ Skip events validated in eventMasterKey

☐ Verify new quantification of all selected data

0 New Object Mask Size

Partition Manual Cargo Tracks

manTracks.csv panelMetadata.mat

Visualize and validate object tracking

To inspect the tracking, create a new event panel with the vesicle tracks as a separate channel. In the Event Panels tab, change Mode from MIP Untracked to MIP Tracked. In the Select data list, select Compartment tracking data or Cargo tracking data as appropriate to include the tracking data. Use the same Export folder as before, a new subdirectory will be created. The following aspects of the object tracking typically requires validation

- Event detection (*t*=0) is set to the first frame with detectable marker recruitment
- Fitting and adequacy of the cargo tracking
- Pre-recruitment stationary tracking is performed at the position of event detection
- The tracking mask follows the center of the object throughout the tracking. If the object makes a leap exceeding the maximum tracking distance of the algorithm, either the object center will be missed, generating a deviation in the data, or the track of the object will be fully lost.
- The object tracking mask adequately fit the apparent size of the object

If the track of the object is lost or disrupted, but an acceptable part of the event has been correctly tracked, the erroneously tracked last part can be manually excluded from the dataset later, without the need of further optimizing the tracking procedure.

Modify tracking parameters and reiterate tracking

The tracking parameters of individual events can be modified in the `Modify Tracking Parameters` tab in the GUI. The specified parameters will be saved in the `roiMetadata` file of the event ROI, and will overrun the general tracking parameters in the GUI during analysis. Specify the following parameters in the GUI tab:

- Experiment | Full experiment name, *e.g.* EXP001
- Acquisition | Full name of the acquisition, *e.g.* EXP001_TS01

- Cell | Cell ID, *e.g.* cell_01

- Event | Event ID, *e.g.* event_01

To specify individual tracking parameters, enter a value in the parameter boxes. If parameters are not specified, the general parameter settings in the GUI will be used for the tracking. The parameters are further described in the Compartment Tracking section.

Some general strategies to optimize the tracking can be used, either by changing the general GUI tracking parameters or event specific tracking parameters:

- Event is not detected

- Adjacent object is erroneously detected as the event

- The event is correctly detected but the tracking jumps to an adjacent object

- The object moves longer distances than the tracking algorithm can keep up with

After modifying tracking parameters, re-tracking of the events can be performed. If the general GUI tracking parameters have not been changed, the outcome of the tracking will not be changed unless tracking parameters of the individual events have been modified. This means that either all events can be included in the re-analysis, or individual events can be selected using the hashtag selection option to speed up the process.

Normalize and collect tracking data

Since the data associated with individual events are located in the individual folder of the single event, certain tools are required in order to preform data normalization and to collect tracking data from a larger number of acquisitions or experiments, which for some purposes could comprise hundreds of individual events, to a single sheet.

There are several options for data normalization in the GUI:

- Cell objects | The local background intensity, calculated as a rolling average over the number of time points specified as the `nRoll` parameter, is subtracted from the object intensity. The object values are then divided by the median value of all objects in the individual cell. This number is calculated as the median of all pixel values in the cell higher than the mean + n × standard deviation of common logarithm (base 10) transformed cell pixel values, where n is specified in the `Normalize data` tab.

- Cell background | The local background intensity, calculated as a rolling average over the number of time points specified as the `nRoll` parameter, is subtracted from the object intensity. The object values are then divided by the background intensity of the cell, calculated as the median pixel value of the cell.

- Max of series | The local background intensity, calculated as a rolling average over the number of time points specified as the `nRoll` parameter, is subtracted from the object intensity. The object values are then divided by the maximum object value in the individual object trace.

- Image background | No subtraction of the local background intensity from the object intensity is performed. Only the image background (detector) intensity is subtracted from the object values.

- Local background | No subtraction of the local background intensity from the object intensity is performed. The object intensity is divided with the local background intensity calculated as a rolling average over the number of time points specified as the `nRoll` parameter. The image background (detector) intensity is subtracted from both the object and local background values.

For the `Cell objects` and `Cell background` normalization options, a linear regression model is fitted to the two calculated numbers (corresponding to values calculated using the first frame in the ROI of the first event and the last frame in the ROI of the last event in the cell, for which cell data is extracted using the cell outlines created in ImageJ/Fiji during the generation of the event ROIs.) The models are then used to normalize the object values to the calculated cell object or cell background values at the corresponding time.

The collecting of normalized tracking data can be performed after specifying the kind of data to be collected. Collected events are organized in column and arranged so that *t*=0, *i.e.* the first time point at which the event was detected, is located at row 100 for all events. The collected data is exported to a new folder in the `Output data` directory in the `Home folder`, where it can be further explored and exported using manual operations.

Offline data storage and archiving

Raw data czi-files can be transferred to offline external storage hard drives after event ROIs have been properly made, to save online storage capacity. For organizational reasons, all acquisitions in an experiment are preferably transferred to the external hard drive at the same occasion or in successive order, to avoid splitting of the data on multiple drives. The available space on the hard drive will be compared with the total size of all acquisitions of the experiment, and the transfer operation will only be performed if there is enough space available to transfer all acquisitions. This is to avoid splitting of the data of single experiments across multiple offline storage drives.

Bring Offline Raw Data | This option in the GUI will copy the czi-files of the acquisitions listed in the GUI to an experiment specific folder on the external hard drive. The czi-files on the online storage will then be deleted.

Metadata | If enabled, Java Robot class operations will export a metadata-containing xml-file using the ZEN software to the online and offline storage directories.

Compression | If enabled, the raw data file will be compressed before being copied to the external HDD. The first step of this procedure is that the file is copied to a specified local directory on the computer. This file will then be compressed and transferred to the offline storage hard drive. Since the external hard drive has a lower I/O speed, the compression is performed locally to speed up the process. Also, note that the file that is compressed will not be automatically copied by the compression operation. The file is copied initially rather than performing the compression on the original file. If the system or process fails, the original file will not be corrupted.

Bring Offline All Data | This option can be used for archiving all data associated with an experiment (e.g. processing data, tracking data, ROIs, metadata etc.). Two alternatives of data inclusion or selection is available for this procedure in the 'Include'-panel:

- From list | If the czi-files have not already been transferred to an external drive, the option will move all data associated with one experiment and the acquisitions associated with it, as is specified in the `Experiment` tabs section in the GUI, will be transferred to the external hard drive. If the czi-files have already been transferred to the external hard drive, only the remaining associated data will be copied. If associated experiment data have already been copied to the external hard drive, but the acquisition is still included in the operation, the associated data on the external hard drive will be replaced by the data present on the online storage unit.

- From drive | If czi-files of experiments have already been transferred to the external drive, and the remaining data is to be archived, this operation will evaluate the experiments present on the drive, and archive all remaining online data of these experiments. No experiments need to be listed in the GUI. Note that if some experiments on the drive have already been fully archived, the data will be replaced with the current online data associated with the acquisition.

Hashtag selection of acquisitions can be used to transfer or archive single acquisitions in experiments, using either the `Bring Offline Raw Data` or `Bring Offline All Data` options. Note that if the `Bring Offline All Data` operation is selected, the `From list` option must be selected in the `Include` section. To enable the hashtag selection, the `Include - Selected` option should be selected in the `Experiment` tab of the specific experiment.