Step-by-step tutorial for using EMC^2 tracking in Icy software

This supplementary material describes the step-by-step tracking of single neurons in calcium imaging of behaving animals with EMC^2 in the Icy software.

All .tif movies analyzed in the paper can be downloaded from the Biostudies website: https://www.ebi.ac.uk/biostudies/studies/S-BSST428

1. Downloading and installing Icy

Icy is an open-source software that can be downloaded here: http://icy.bioimageanalysis.org/download/

Many general Icy presentations and tutorials can be viewed on Youtube: https://www.youtube.com/results?search_query=icy+bioimage
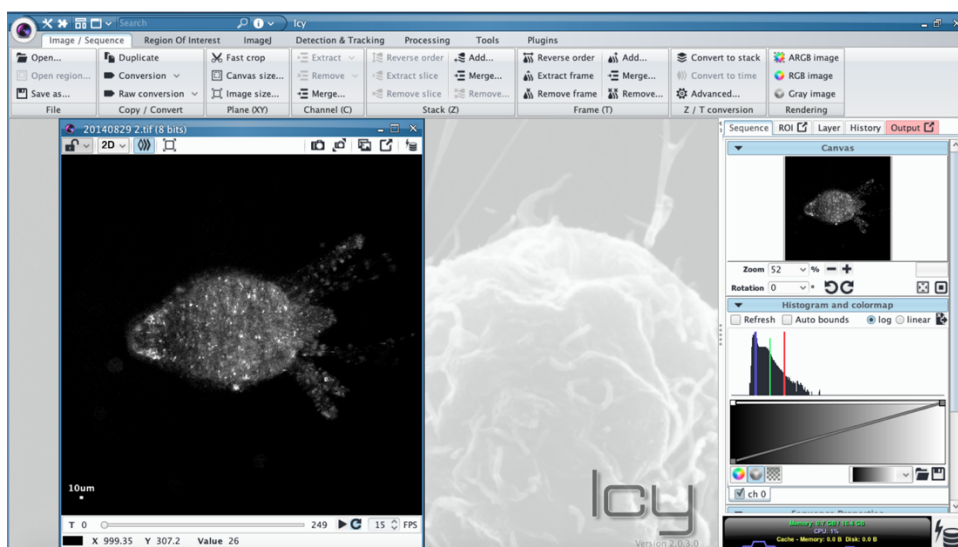
Also, a forum dedicated to Icy users can be found here: https://forum.image.sc/tag/icy

2. Opening a time-lapse sequence

Most image formats are supported by Icy. To open a time lapse sequence, you can either drag-and-drop the file into the software, or use the "Image/Sequence->Open" command in the upper-left tool bar of Icy
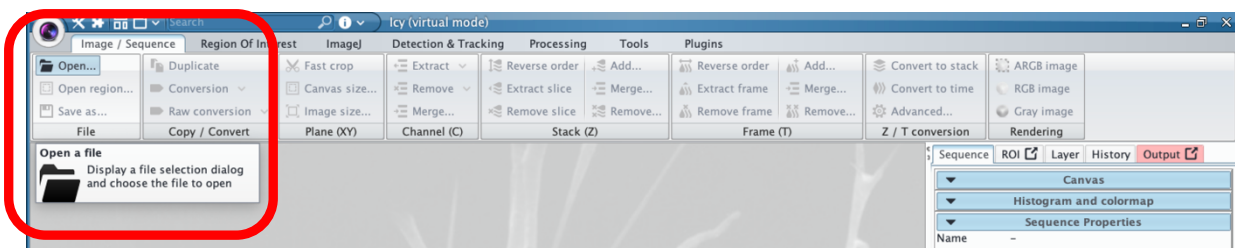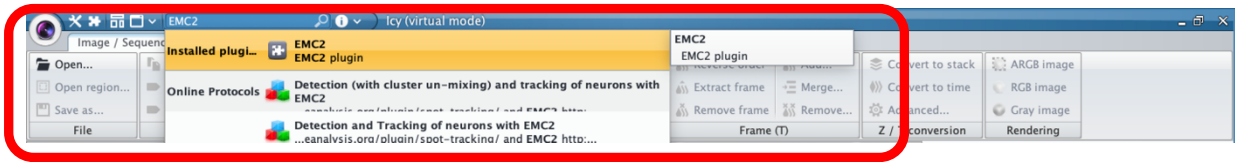

Example of an open sequence:

3. Using the Icy search bar for downloading plugins

Initially, Icy application contains a set of installed plugins but many of them, that are developed by many independent bio-image analysts worldwide, can be downloaded online using the search bar in the upper-right panel of the software. This includes the EMC^2 tracking suite:


4. Step-by-step tracking with EMC^2 using standalone plugins

a- Detect bright neuron spots using the Spot Detector plugin

The very first step of most tracking algorithms is the accurate and automatic detection of fluorescent neurons within each time frame of the sequence. This can be performed using the *Spot Detector* plugin in Icy.

This plugin is specifically designed to detect significant signal in noisy sequences. It is based on wavelet transformation and statistical thresholding of images (see Olivo-Marin J.-C*., Extraction of spots in biological images using multiscale products,* Pattern Recognition, 35(9) 2002).

Main parameters of the plugin are the used scale/threshold (small scale=small detected objects, and low threshold = low proportion of detected objects)
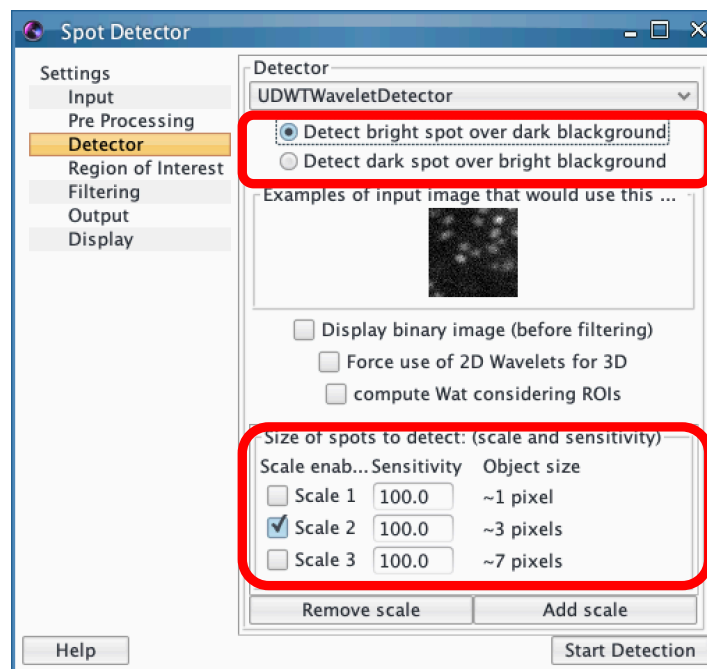
Also, it is important to check the "Import to swimming-pool" case in the "Output" menu of the plugin so that detections could be used within the tracking plugins
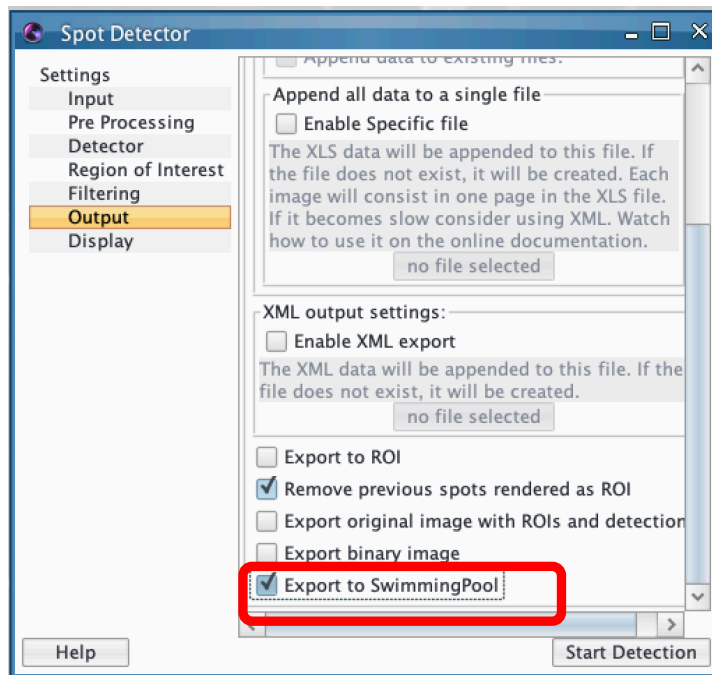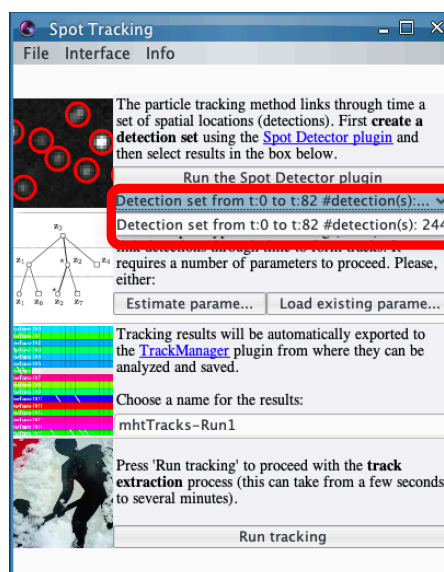
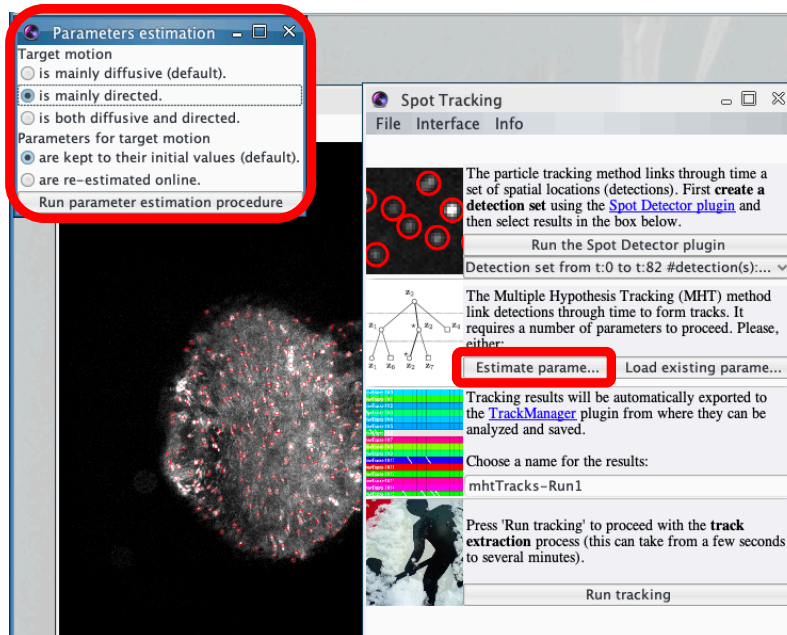### b- Bayesian tracking of detectable particles with Spot Tracking plugin

From single neuron detections, short track (tracklets) can be reconstructed using classical single-particle-tracking algorithms, such as the probabilistic enhanced-Multiple-Hypothesis-Tracking (eMHT) method implemented in the *Spot Tracking* plugin of Icy (details about the tracking method can be found in Chenouard, N., Bloch, I., & Olivo-Marin, J. C. (2013). Multiple hypothesis tracking for cluttered biological image sequences. *IEEE transactions on pattern analysis and machine intelligence*, *35*(11), 2736-3750.)

First, detections that were previously exported to the *Swimming Pool* by the *Spot Detector* plugin have to be selected in the *Spot Tracking* plugin

Then, one has to click on the "estimate parameters" button to automatically estimate the best tracking parameters. A type of expected motion needs to be selected ("*directed motion*" here as neuron trajectories are not stochastic and follow directed paths locally)
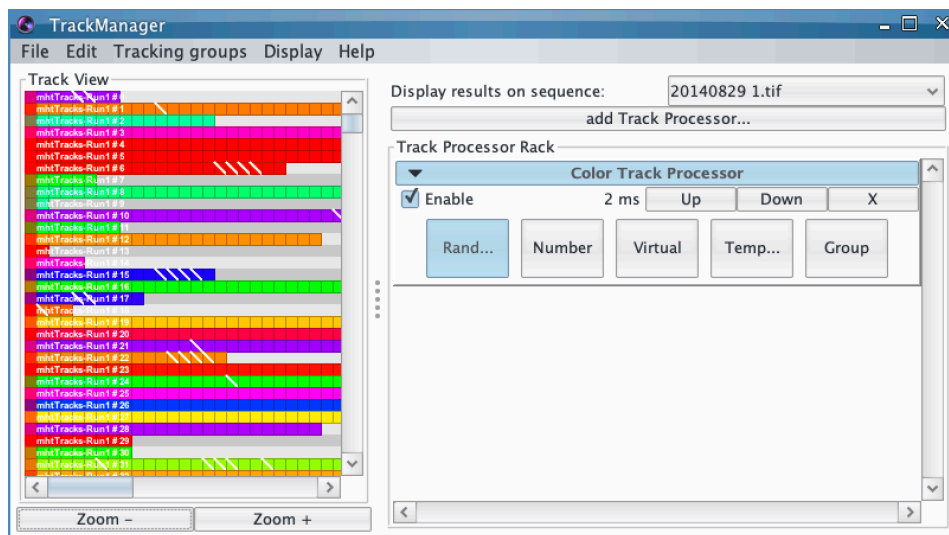

After the automatic estimation of tracking parameters, one can choose the name of the tracking result (*"mhtTracks-Run1"* by default) and click on the "*Run tracking*" button
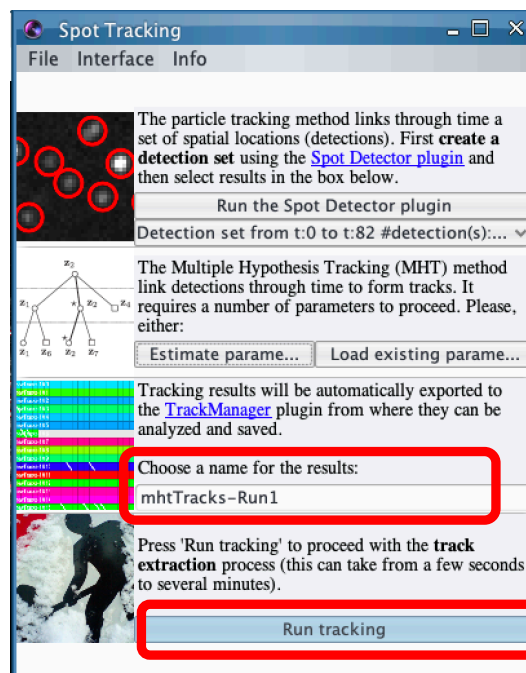
Once the tracking is terminated, *tracklets* of detectable particles are directly exported in the *Track Manager* plugin.

c- *Concatenate (stitch) short tracklets using the EMC² plugin*

The main problem with Bayesian tracking is that many tracklets might actually correspond to the same single particle (neuron) when it can be intermittently detected. Therefore, the last step of the tracking will specifically use the EMC² plugin to optimally concatenate (i.e. stitch) short tracklets that putatively correspond to the same particle (neuron).

The previous tracks computed by the Bayesian *Spot Tracking* plugin have to be selected in the *"Tracks"* menu of the plugin (last computed tracks = last item).

Parameters of the method are the maximum number of used fiducials (*"Max. number of fiducials"*) to estimate the elastic deformation of the time lapse sequence (for saving computer memory), the maximum distance for putatively concatenate tracklets after having corrected for the deformation of the field of view (*"Max. distance for track concatenation"*), the maximum time window (i.e. time-lapse) between the ending-point and starting-points of concatenated tracklets (*"Max. time window for track concatenation"*). This latter parameter prevents error propagations of the estimation of particle position during (too) long tracking gaps. Finally the "Alternative cost factor (for JV linear association)" is a technical parameter that modulates the precision (and the computational cost!) of the Jonker-Volgenant (JV) algorithm used to compute the (approximate) solution of the linear assignement problem for tracklet concatenation (increasing the cost parameter= increasing the precision and the computational load of the algorithm). This latter parameter needs to be tuned in very challenging tracking problems with an important density of tracklets. Finally, a *"no motion"* box can be checked if one wants to run the EMC² algorithm without correcting for the deformation of the field-of-view.
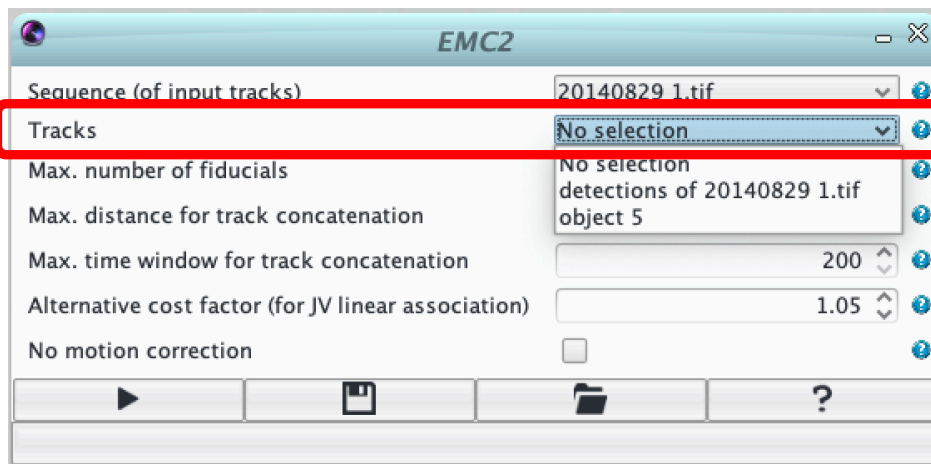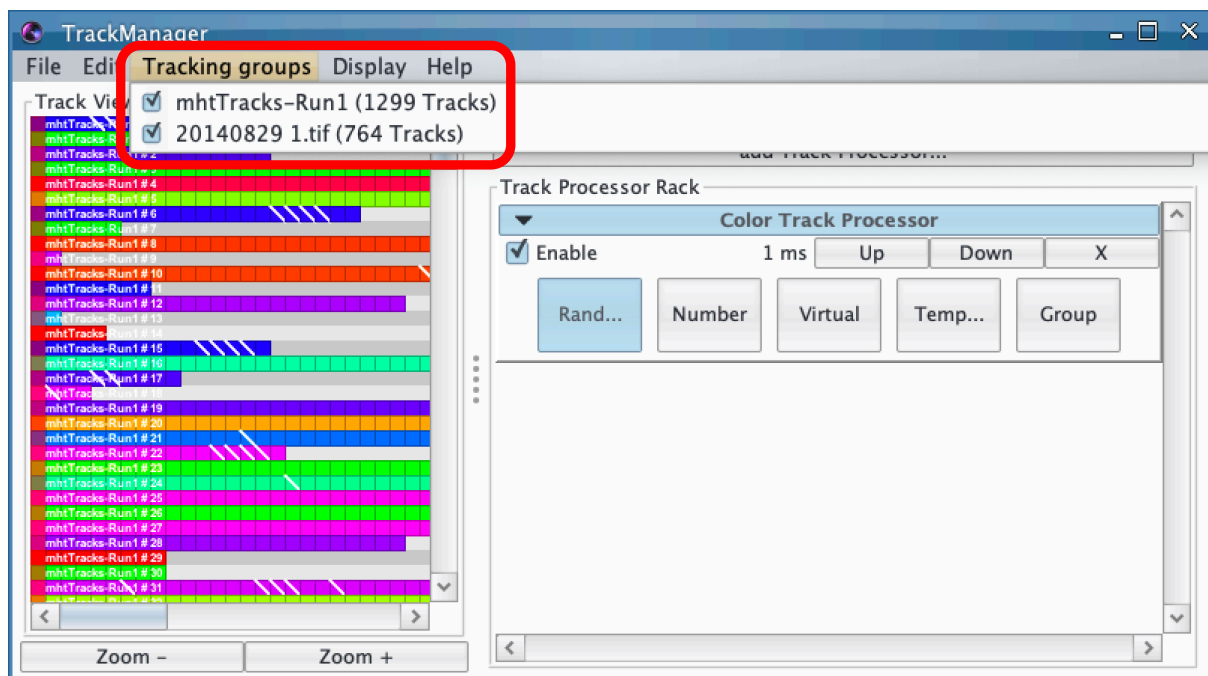
Finally, concatenated tracks ("*20140829.tif (764 Tracks)*" in this example) are exported to the *Track Manager*, together with previous short Bayesian tracklets ("*mhtTracks-Run1 (1299 Tracks)*" in this example).

See also Points 5-d below

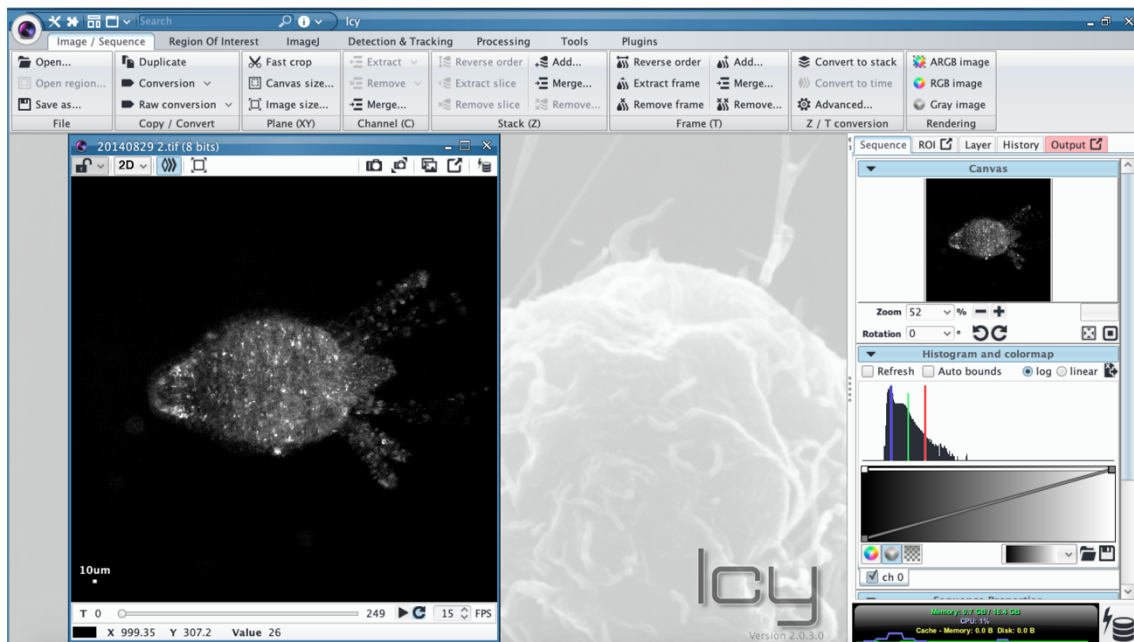# 5. Step-by-step tracking with EMC² using protocols

We highlight that there are two protocols: one (*Detection (with cluster un-mixing) and tracking of neurons with EMC2*), described in the main manuscript, that detect bright spots and de-cluster big spots with Laplacian of Gaussian (LoG) convolution and local maxima, before tracking individual neurons with EMC^2 algorithm, and another (*Detection (with cluster un-mixing) and tracking of neurons with EMC2*) that does not de-cluster big spots. The latter protocol, while being less accurate, is faster especially for long (>500 frames) sequences.

Online documentation for using Protocols in Icy can be found on the Icy webpage of the plugin: http://icy.bioimageanalysis.org/plugin/protocols/

a- *Search and download the desired protocol from the search bar of the software (see previous point 3)*
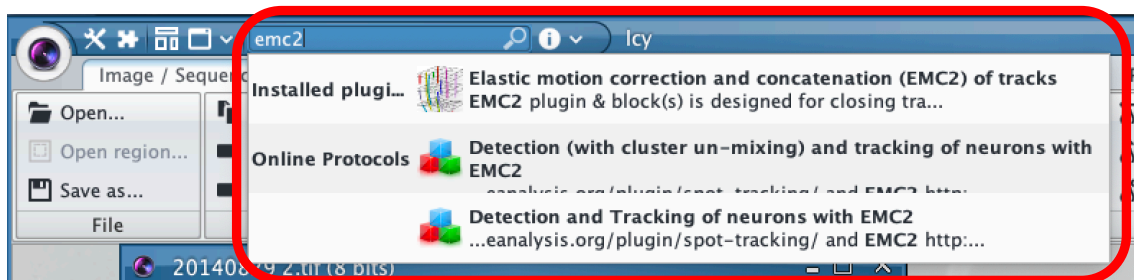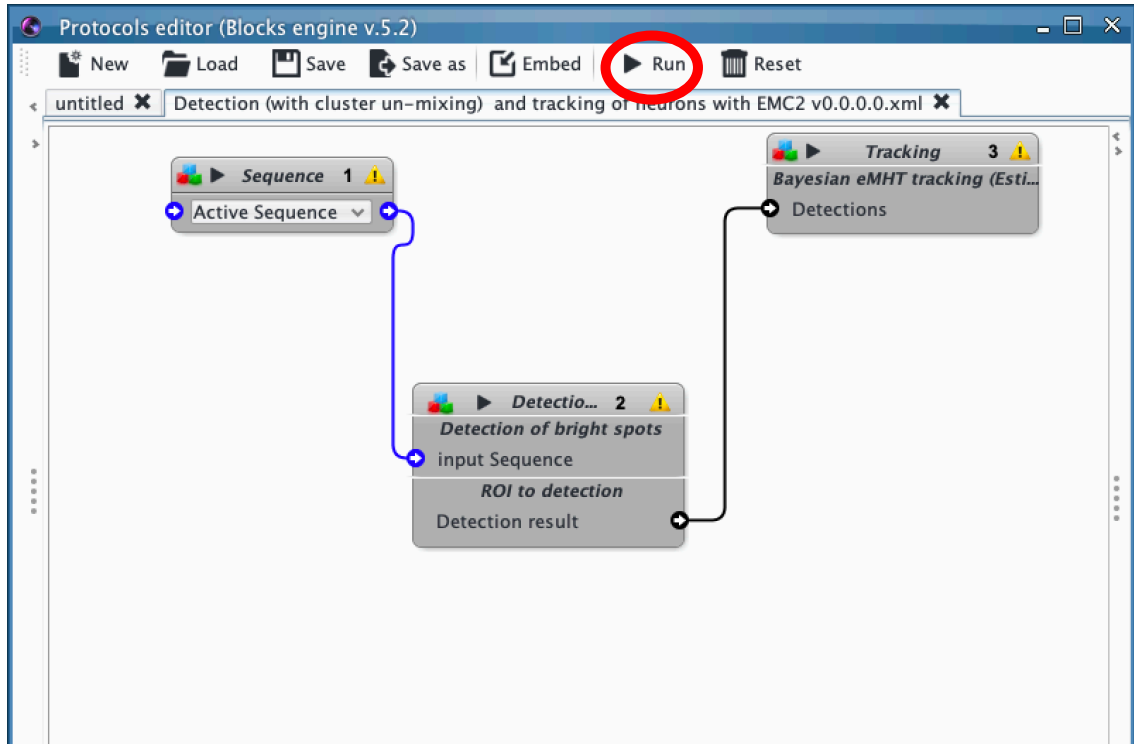

b- *Open the time-lapse sequence with fluorescence activity of individual neurons (see previous point 2)*
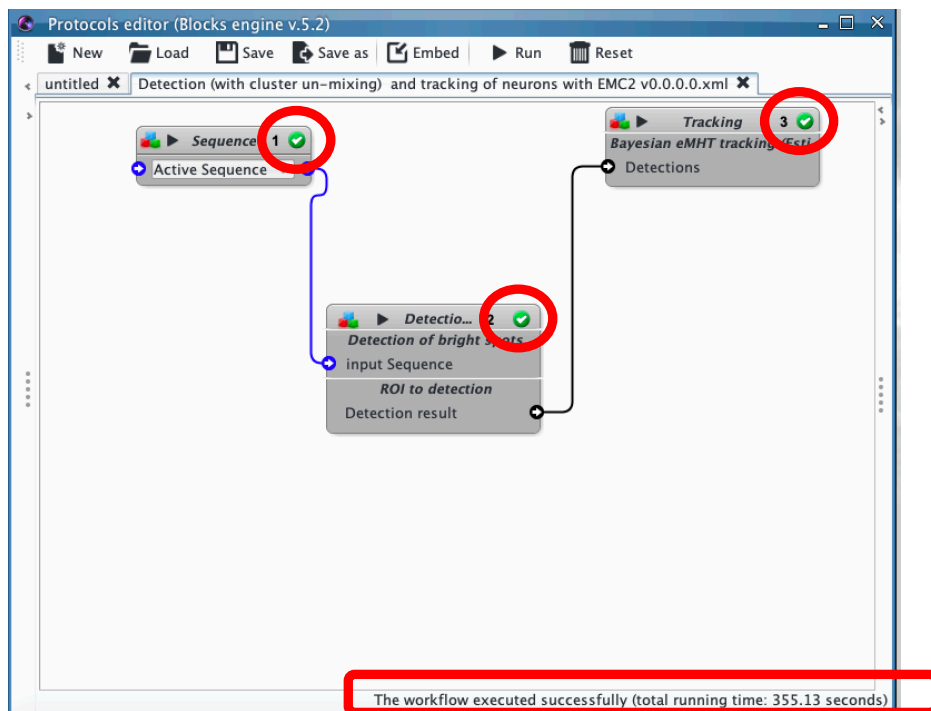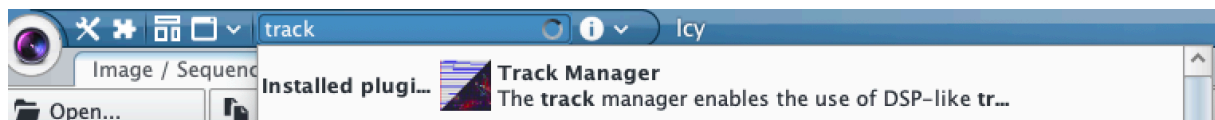

c- *Open and Run the protocol*

Detailed documentation of the protocol can be found here : http://icy.bioimageanalysis.org/protocol/detection-with-cluster-un-mixing-and-tracking-of-neurons-with-emc2/

Once the protocol is correctly executed…


Search and launch the Track Manager plugin

Track Manager plugin, through different *processors*, will help at visualizing and exporting track attributes (such as intensity along time)

Online documentation for Track Manager can be found on the Icy webpage of the plugin: http://icy.bioimageanalysis.org/plugin/track-manager/

d- *Visualize tracks with "Track Clipper" and "Display Track Number Track Processor" processors*

You can select the sequence on which you want to display single tracks, and export intensities for further analysis (highlighted below with a red rectangle)
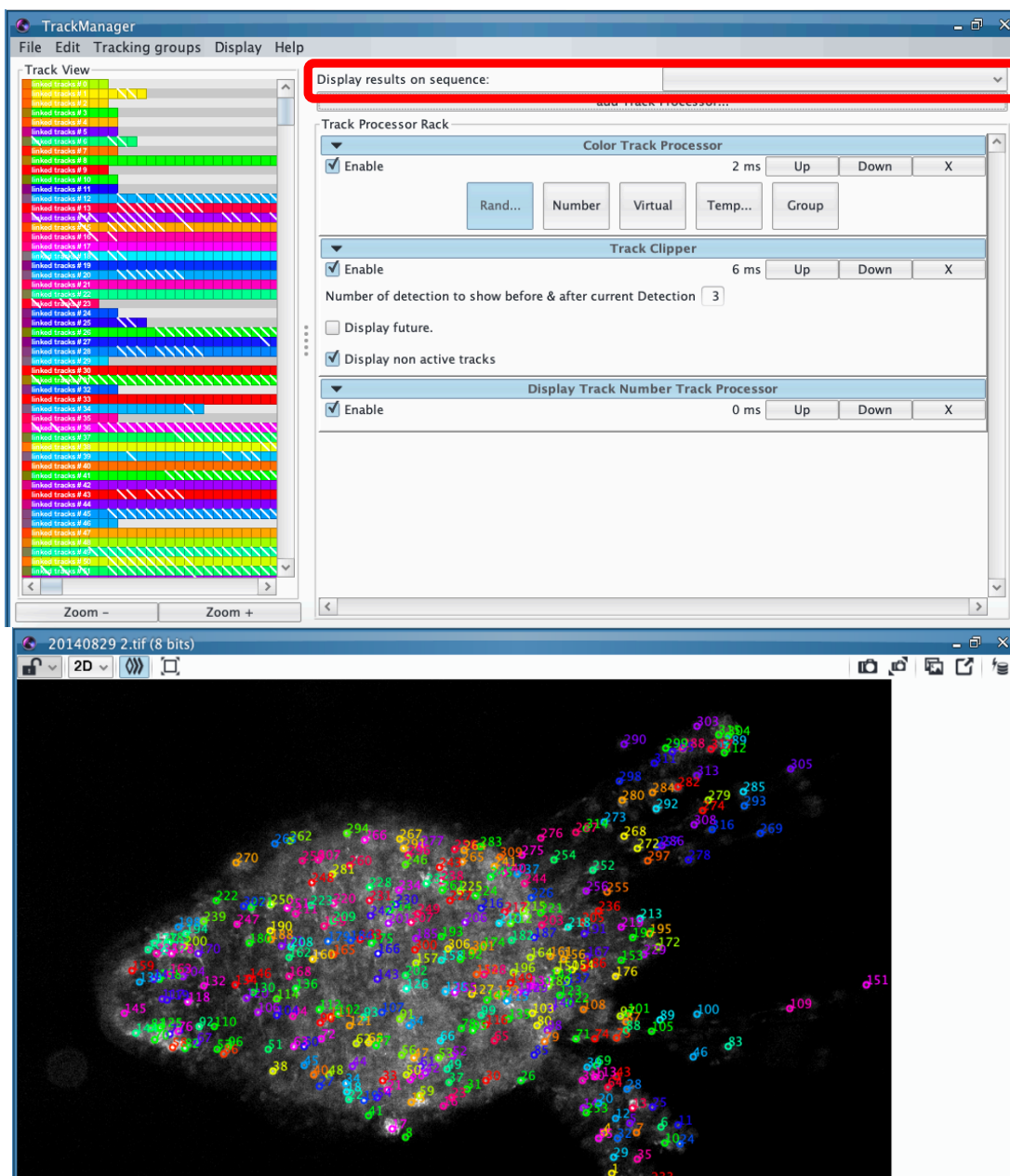
e- *Export track intensity for further analysis (spike extraction…) with "Intensity Profile" processor*

Be careful to remove/disable the "Track Clipper" processor before exporting track intensity, otherwise only the few frames of the time clip will be processed and saved
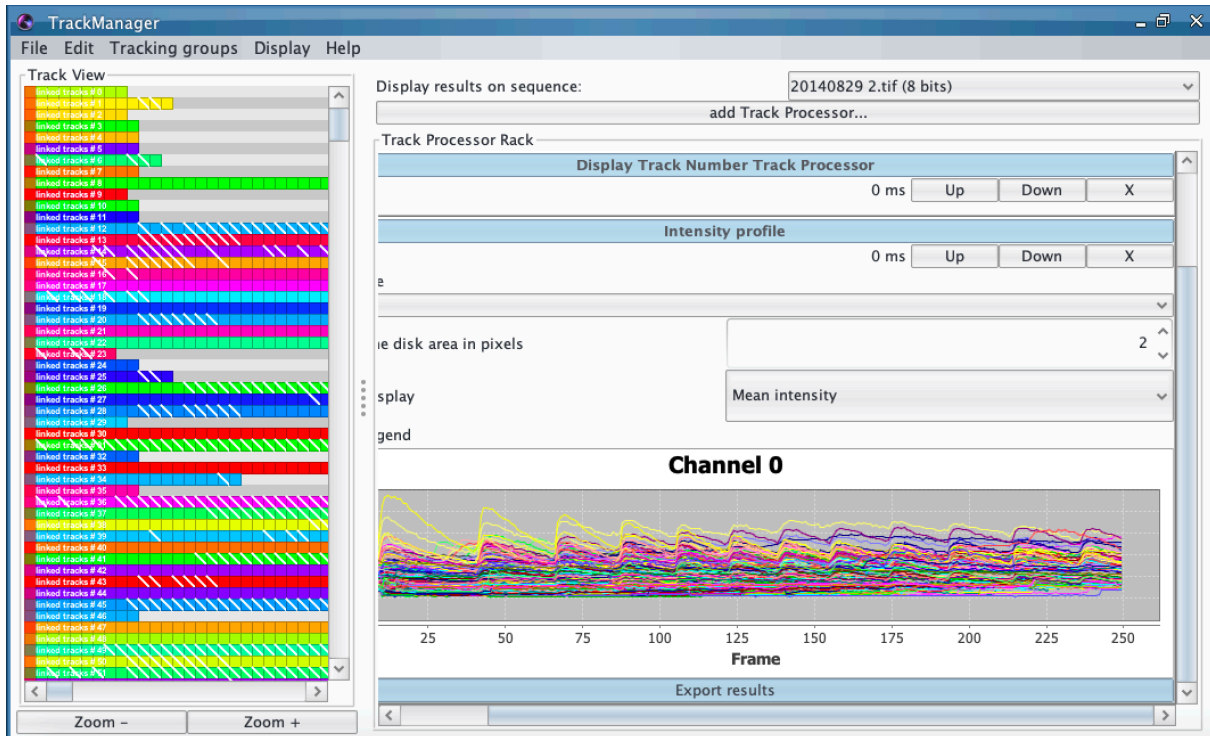
The fluorescence intensity of each individual track is then saved in an excel file that can be further used with any mathematical/statistical software (like R and Matlab) to analyze data (denoising, spike extraction …).