PatchTrackingTools Documentation

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1. Introduction

The PatchTrackingTools is a **Fiji** toolset to perform common tasks for quantitative fluorescence microscopy, such as movie reorganization and correction, fluorescent spot tracking (e.g. endocytic patches, nodes), molecule counting and position tracking.

Note that the new version of the PatchTrackingTools will **only work with Fiji** distributions of ImageJ.

2. Installation

1. Download the ActionBar plugin.
   http://imagejdocu.tudor.lu/doku.php?id=plugin:utilities:action_bar:start#installation
2. Copy the .jar file for the ActionBar plugin into your Fiji `plugins/` folder. Create the `ActionBar/` folder and the `icons/` subfolder (follow the example below)

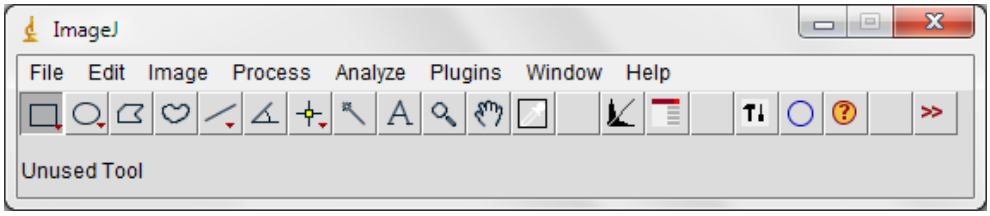
```
Fiji/plugins/action_bar202.jar          Action Bar plugin
Fiji/plugins/ActionBar/                 ActionBar folder for your bars
Fiji/plugins/ActionBar/icons/           icons folder
```

3. Copy the `PatchTrackingBarIcons/` folder into your Fiji `plugins/ActionBar/icons/` folder. You should end up with a folder `plugins/ActionBar/icons/PatchTrackingBarIcons/` with all images files with the icons in it.
4. Copy the file `PatchTrackingTools.ijm` into your Fiji `macros/toolsets/` folder
5. Copy the file `PatchTrackingBar.txt` into your Fiji `macros/` folder
6. Copy the folder "`macros for PatchTrackingTools/`" into your Fiji `macros/toolsets/` folder.
7. Copy the folder `patchTrackingTools/` into the Fiji `plugins/` folder.

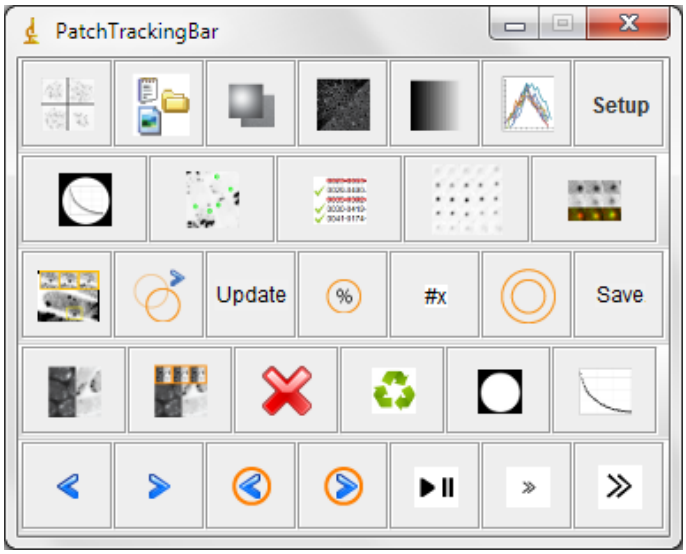
Note that you might be asked to install Jython the first time you run the patch finder tools. Just click OK and restart Fiji.

1. Getting started

1. Start (or Restart) Fiji.
2. Click on the last button of the toolbar  and select the toolset PatchTrackingTools



3. Click on the toolbox button  to show the extended toolbar.



The toolbar is organized into 5 rows:
1st row: tools for movie reorganization (from Andor iQ (legacy)) and corrections. It also contains the button to generate the final data files and the setup button.
2nd row: tools for finding patches.
3rd row: tools for tracking patches.
4th row: tools to modify the display of the images and manage the windows.
5th row: tools for navigating in the movies and the Rois.

Most of these tools have shortcuts. Fly over the icon with your mouse to find out a quick description of the function of the button and its shortcut (the letter in the brackets at the end of the description). Check also all the available macros and their shortcuts in Plugins>Macros>. Some of them do not have a button on the action bar.

Warning: some of the native shortcuts of Fiji and ImageJ will be replaced by shortcuts for PatchTrackingTools macros.

2. Raw data transformation and reorganization

a. *General movie reorganization*

Before starting!!

*Make sure you have a **backup copy of all your movies**. The macros might modify the original files and you might have troubles recovering the original data if there is a (very unlikely???) glitch in the macros.*

Important note for movies imaged on a single plane

The PatchTrackingTools have originally been developed to be used with spinning disc confocal data containing several z-slices. If your movie has only one z-plane, the PatchTrackingTools will still consider it as a hyperstack but with only one z-slice. Therefore the movie will be duplicated at some point to consider a hyperstack and a sum projection files (see below).

1. ***Always have a backup copy for all your movies!!***

2. Create one folder per movie. If you want to correct your movie for uneven illumination and camera noise using the automatic correction tools  , the folder name needs to be the same as the file name.

Example:

```
fim1mEGFP experiments/
    |_ 20110305 PM 051512 fim1mEGFP_GFP_alone_237_/
            |_ 20110305 PM 051512 fim1mEGFP_GFP_alone_237_.tif
```

If you do not want to correct the images for uneven illumination and camera noise:

If you do not plan to correct a movie and/or want to crop an area from a larger movie, you can use the rectangular selection tool (or CTRL+A to select the entire field) and run the macro 'Crop and save uncorrected HS and _SUM_Corrected file in new folder [R]' accessible in the Fiji menu *Plugins>Macros>'Crop and save uncorrected HS and _SUM_Corrected file in new folder [R]'* in the ***Other tools*** section or press SHIFT+R.

This macro will create a new folder with the same name as the original file, and two files within this folder, a hyperstack and a sum projection image, that have the right format for the other tracking tools.

Redo this step for all your movies and you're done (skip steps 3 and 4).

Example: if you use the macro *'Crop and save uncorrected HS and _SUM_Corrected file in new folder [R]' on the movie 20110305 PM 051512 fim1mEGFP_GFP_alone_237_.tif,* you will end up with something like that:

```
fim1mEGFP experiments/
    |_ 20110305 PM 051512 fim1mEGFP_GFP_alone_237_.tif
    |_ 20110305 PM 051512 fim1mEGFP_GFP_alone_237_/
            |_ 20110305 PM 051512 fim1mEGFP_GFP_alone_237__SUM_Corrected.tif
            |_ 20110305 PM 051512 fim1mEGFP_GFP_alone_237_HS.tif
```

3. Click on  to generate the files to correct for uneven illumination and the camera noise. You are asked to select the files that will be used for the corrections. The whole stack will be averaged to make a single frame images that will be used for the camera noise and the uneven illumination correction for all your movies. The correction images will be saved respectively as AverageCameraNoise.tif and AverageUnevenIllumination.tif in the parent folder of the selected files. If you do not want to correct your images, you can generate 'neutral' correction file (basically files that do not correct anything). To do so go to *Plugins>Macros>'Generate Neutral Correction Files'* in the ***Other tools*** section.

Example: At this stage, your movies should be organized like that:

```
fim1mEGFP experiments/
    |_ 20110305 PM 051512 fim1mEGFP_GFP_alone_237_/
            |_ 20110305 PM 051512 fim1mEGFP_GFP_alone_237_.tif
    |_ 20110305 PM 052345 fim1mEGFP_GFP_alone_237_1/
            |_ 20110305 PM 052345fim1mEGFP_GFP_alone_237_1.tif
    |_ 20110305 PM 053023UnevenIllumination _GFP_alone_237_/
            |_ 20110305 PM 053023UnevenIllumination _GFP_alone_237_.tif
    |_ 20110305 PM 053917 CameraNoise _GFP_alone_237_/
            |_ 20110305 PM 053917CameraNoise _GFP_alone_237_.tif
    |_ AverageCameraNoise.tif
    |_ AverageUnevenIllumination.tif
```

4. Click on  to correct all your movies. For each movie, it will generate 2 files starting with the name of the original file and ending with 'HS' and '_SUM_Corrected' for the corrected hyperstack and the sum projection of this hyperstack, respectively.
   Warning: these steps can take a bit of time since it will open and save a large number of files. Depending on the performances of your computer and hard drive and on the size of your movies, it might take several minutes or tens of minutes. Be also aware that your computer might have a hard time opening very large movies… Make sure that you allocated enough memory to Fiji (Edit>Options>Memory & Threads>Maximum memory). Typically, it should represent about 70% of your computer RAM.

Example: after this 4th step, your folder should look like that:

```
fim1mEGFP experiments/
    |_ 20110305 PM 051512 fim1mEGFP_GFP_alone_237_/
            |_ 20110305 PM 051512 fim1mEGFP_GFP_alone_237_.tif
            |_ 20110305 PM 051512 fim1mEGFP_GFP_alone_237__SUM_Corrected.tif
            |_ 20110305 PM 051512 fim1mEGFP_GFP_alone_237_HS.tif
    |_ 20110305 PM 052345 fim1mEGFP_GFP_alone_237_1/
            |_ 20110305 PM 052345fim1mEGFP_GFP_alone_237_1.tif
            |_ 20110305 PM 052345fim1mEGFP_GFP_alone_237_1_SUM_Corrected.tif
            |_ 20110305 PM 052345 fim1mEGFP_GFP_alone_237_1HS.tif
    |_ 20110305 PM 053023UnevenIllumination _GFP_alone_237_/
            |_ 20110305 PM 053023UnevenIllumination _GFP_alone_237_.tif
            |_ 20110305 PM 053023UnevenIllumination _GFP_alone_237__SUM_Corrected.tif
            |_ 20110305 PM 053023 UnevenIllumination _alone_237_HS.tif
    |_ 20110305 PM 053917 CameraNoise _GFP_alone_237_/
            |_ 20110305 PM 053917CameraNoise _GFP_alone_237_.tif
            |_ 20110305 PM 053917CameraNoise _GFP_alone_237__SUM_Corrected.tif
            |_ 20110305 PM 053917 CameraNoise _alone_237_HS.tif
    |_ AverageCameraNoise.tif
    |_ AverageUnevenIllumination.tif
```

b. THIS SECTION IS KEPT FOR LEGACY - THESE FUNCTIONNALITIES ARE NOT UPDATED ANYMORE

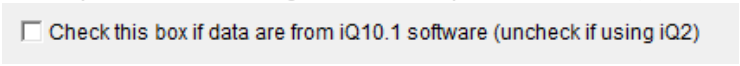
If you are using Andor iQ

Before starting!!

Make sure that you exported your movies from Andor iQ in the TIFF format as stacks (do not use any other format such as image sequences, the macros would not work). If you used the iQ10.1 software or the iQ2 software to acquire your data, for every .tif file, you should have one .txt file with the same name. If you used iQ2 and collected your data with a complex protocol with several channels, you will have an extra .txt file for every run of your protocol. It is important to keep all these files. Also make sure you have a ***backup copy of all your movies****. The macros might make some modifications on the files and you might have troubles to recover your original data if there is a glitch in the macros.*

1. ***Always have a backup copy for all your movies!!***
2. If you imaged several fields in each protocol, you have to split these files to have one tif file (with the movie) and one text file (with the protocol) per field. You can reorganize them using 
3. If you used iQ10.1, go to the setup window

(Extended toolbar) and check the box:



Warning: It is not sure the reorganization of movies from iQ10.1 still works fine since it has not been tested recently.

4. Click on  to reorganize the movies. You will have to choose the folder where your tif and txt files are. It renames each file by adding the date and time of creation of the movie at the beginning of the file name.
   Warning: it might not work well if you are using the iQ2 software and you took movies with protocols that are very different from each other (typically if you took 1-color movies and several-color movies the same day). In this case, we suggest you to move temporarily all the movies taken with the same protocol into a same temporary folder, reorganize these movies and then copy the newly generated folders back to your main folder.

5. If several channels have been imaged in the protocol (typically DIC and GFP), regroup (manually) the folders so that only the folder containing the channel that needs to be corrected is visible from the main folder (typically the GFP channel)

Example: if you took DIC and GFP images, at this stage you should have something like:

```
fim1mEGFP experiments/
    |_ 20110305 PM 051512 fim1mEGFP_GFP_alone_237_/
            |_ 20110305 PM 051512 fim1mEGFP_GFP_alone_237_.tif
            |_ 20110305 PM 051512 fim1mEGFP_GFP_alone_237_.txt
    |_ 20110305 PM 051512 fim1mEGFP_DIC_/
            |_ 20110305 PM 051512 fim1mEGFP_ DIC _.tif
            |_ 20110305 PM 051512 fim1mEGFP_ DIC _.txt
```

Which should be manually reorganized into:

```
fim1mEGFP experiments/
    |_ 20110305 PM 051512 fim1mEGFP_GFP_alone_237_/
            |_ 20110305 PM 051512 fim1mEGFP_GFP_alone_237_.tif
            |_ 20110305 PM 051512 fim1mEGFP_GFP_alone_237_.txt
            |_ 20110305 PM 051512 fim1mEGFP_DIC_/
                    |_ 20110305 PM 051512 fim1mEGFP_ DIC _.tif
                    |_ 20110305 PM 051512 fim1mEGFP_ DIC _.txt
```

So that only the folder containing the GFP movie that needs to be corrected (20110305 PM 051512 fim1mEGFP_GFP_alone_237_/) is visible from the main folder (fim1mEGFP experiments/)

6. If the multi-field protocols generated only one file, you should move the folders containing these original files into a separate folder, so that they won't be corrected. Each file, containing each field will be corrected independently.

Example: if you used a protocol with 3 fields, at this point you should have:

```
fim1mEGFP experiments/
    |_ 20110305 PM 051512 fim1mEGFP_GFP_alone_237_/
            |_ 20110305 PM 051512 fim1mEGFP_GFP_alone_237_.tif
            |_ 20110305 PM 051512 fim1mEGFP_GFP_alone_237_.txt
    |_ 20110305 PM 051512 fim1mEGFP_GFP_alone_237_-field0/
            |_ 20110305 PM 051512 fim1mEGFP_GFP_alone_237_-field0.tif
            |_ 20110305 PM 051512 fim1mEGFP_GFP_alone_237_-field0.txt
    |_ 20110305 PM 051512 fim1mEGFP_GFP_alone_237_-field1/
            |_ 20110305 PM 051512 fim1mEGFP_GFP_alone_237_-field1.tif
            |_ 20110305 PM 051512 fim1mEGFP_GFP_alone_237_-field1.txt
    |_ 20110305 PM 051512 fim1mEGFP_GFP_alone_237_-field2/
            |_ 20110305 PM 051512 fim1mEGFP_GFP_alone_237_-field2.tif
            |_ 20110305 PM 051512 fim1mEGFP_GFP_alone_237_-field2.txt
```

You should move the folder containing the original movie into:

```
fim1mEGFP experiments/
    |_ Original movies/
            |_ 20110305 PM 051512 fim1mEGFP_GFP_alone_237_/
                    |_ 20110305 PM 051512 fim1mEGFP_GFP_alone_237_.tif
                    |_ 20110305 PM 051512 fim1mEGFP_GFP_alone_237_.txt
    |_ 20110305 PM 051512 fim1mEGFP_GFP_alone_237_-field0/
            |_ 20110305 PM 051512 fim1mEGFP_GFP_alone_237_-field0.tif
            |_ 20110305 PM 051512 fim1mEGFP_GFP_alone_237_-field0.txt
    |_ 20110305 PM 051512 fim1mEGFP_GFP_alone_237_-field1/
            |_ 20110305 PM 051512 fim1mEGFP_GFP_alone_237_-field1.tif
            |_ 20110305 PM 051512 fim1mEGFP_GFP_alone_237_-field1.txt
    |_ 20110305 PM 051512 fim1mEGFP_GFP_alone_237_-field2/
            |_ 20110305 PM 051512 fim1mEGFP_GFP_alone_237_-field2.tif
            |_ 20110305 PM 051512 fim1mEGFP_GFP_alone_237_-field2.txt
```

Note: the name of the multi-field movies can also end with the pattern _t0000, _t0001, _t0002, ….

7. Click on  to generate the files to correct for uneven illumination and the camera noise. You will be asked to select the files that will be used for the corrections. It will average the whole stack to make a single frame image that will be used for the camera noise and the uneven illumination correction for all your movies. The correction images will be saved respectively as AverageCameraNoise.tif and AverageUnevenIllumination.tif in the parent folder of the selected files.

   If you do not want to (or cannot) correct your images, you have to generate 'neutral' correction file. To do so go to Plugins>Macros>Generate Neutral Correction Files

8.

a. If you used **iQ2 with *ONLY 1 channel*** or **iQ10.1**, your movies should be named with the protocol name and you can skip this step .
b. If you used **iQ2 and a protocol with multiple channels** (typically a DIC image and a GFP movie), the software had renamed your file by adding the name of the channel at the end of the name of the file. You need to tell the plugin what is the pattern of the name of the channel you want to correct. Go to the setup window, and fill "Channel pattern" with the appropriate pattern for the channel. For example if you took your movies with the GFP channel, the name of the channel contains "_GFP_" and you can set this string of characters for the "Channel pattern".

9. Click on  to correct all your movies. For each movie, it will generate 2 files starting with the name of the original file and ending with 'HS' and '_SUM_Corrected' for the corrected hyperstack and the sum projection of this hyperstack respectively.
Warning: these steps can take a very long time since it will open and save a large number of files. Depending on the performances of your computer and hard drive and on the size of your movies, it might take several minutes or tens of minutes. Be also aware that your computer might have hard time to open very large movies… Make sure that you allocated enough memory to Fiji (Edit>Options>Memory & Threads>Maximum memory). Typically, it should represent about 70% of your computer RAM.

Example:

On March 5th 2011, starting at 5:15pm, You collected 2 one-color movies with IQ2. The name you gave for your protocol was 'fim1mEGFP' and you used the channel GFP_alone_237_.
You also collected one movie with the uneven illumination and one movie with the camera noise.

After exporting your files in the tif format you should end up with a folder you will name 'fim1mEGFP experiments' containing the following files:

```
fim1mEGFP experiments/
    |_ fim1mEGFP_GFP_alone_237_.tif
    |_ fim1mEGFP_GFP_alone_237_.txt
    |_ fim1mEGFP_GFP_alone_237_1.tif
    |_ fim1mEGFP_GFP_alone_237_1.txt
    |_ UnevenIllumination_GFP_alone_237_.tif
    |_ UnevenIllumination_GFP_alone_237_.txt
    |_ CameraNoise_GFP_alone_237_.tif
    |_ CameraNoise _GFP_alone_237_.txt
```

After the reorganisation of your movies (step 2), you will end up with:

```
fim1mEGFP experiments/
    |_  20110305 PM 051512 fim1mEGFP_GFP_alone_237_/
              |_ 20110305 PM 051512 fim1mEGFP_GFP_alone_237_.tif
              |_ 20110305 PM 051512 fim1mEGFP_GFP_alone_237_.txt
    |_  20110305 PM 052345 fim1mEGFP_GFP_alone_237_1/
              |_ 20110305 PM 052345fim1mEGFP_GFP_alone_237_1.tif
              |_ 20110305 PM 052345 fim1mEGFP_GFP_alone_237_1.txt
    |_  20110305 PM 053023UnevenIllumination _GFP_alone_237_/
              |_ 20110305 PM 053023UnevenIllumination _GFP_alone_237_.tif
```

```
        |_ 20110305 PM 053023 UnevenIllumination _alone_237_.txt
    |_ 20110305 PM 053917 CameraNoise _GFP_alone_237_/
        |_ 20110305 PM 053917CameraNoise _GFP_alone_237_.tif
        |_ 20110305 PM 053917 CameraNoise _alone_237_.txt
```

After generating the correction files (step 3), they will appear in the parent folder:

```
fim1mEGFP experiments/
    |_ 20110305 PM 051512 fim1mEGFP_GFP_alone_237_/
        |_ 20110305 PM 051512 fim1mEGFP_GFP_alone_237_.tif
        |_ 20110305 PM 051512 fim1mEGFP_GFP_alone_237_.txt
    |_ 20110305 PM 052345 fim1mEGFP_GFP_alone_237_1/
        |_ 20110305 PM 052345fim1mEGFP_GFP_alone_237_1.tif
        |_ 20110305 PM 052345 fim1mEGFP_GFP_alone_237_1.txt
    |_ 20110305 PM 053023UnevenIllumination _GFP_alone_237_/
        |_ 20110305 PM 053023UnevenIllumination _GFP_alone_237_.tif
        |_ 20110305 PM 053023 UnevenIllumination _alone_237_.txt
    |_ 20110305 PM 053917 CameraNoise _GFP_alone_237_/
        |_ 20110305 PM 053917CameraNoise _GFP_alone_237_.tif
        |_ 20110305 PM 053917 CameraNoise _alone_237_.txt
    |_ AverageCameraNoise.tif
    |_ AverageUnevenIllumination.tif
```

After correcting all the movies (step 4), you will have:

```
fim1mEGFP experiments/
    |_ 20110305 PM 051512 fim1mEGFP_GFP_alone_237_/
        |_ 20110305 PM 051512 fim1mEGFP_GFP_alone_237_.tif
        |_ 20110305 PM 051512 fim1mEGFP_GFP_alone_237_.txt
        |_ 20110305 PM 051512 fim1mEGFP_GFP_alone_237__SUM_Corrected.tif
        |_ 20110305 PM 051512 fim1mEGFP_GFP_alone_237_HS.tif
    |_ 20110305 PM 052345 fim1mEGFP_GFP_alone_237_1/
        |_ 20110305 PM 052345fim1mEGFP_GFP_alone_237_1.tif
        |_ 20110305 PM 052345 fim1mEGFP_GFP_alone_237_1.txt
        |_ 20110305 PM 052345fim1mEGFP_GFP_alone_237_1_SUM_Corrected.tif
        |_ 20110305 PM 052345 fim1mEGFP_GFP_alone_237_1HS.tif
    |_ 20110305 PM 053023UnevenIllumination _GFP_alone_237_/
        |_ 20110305 PM 053023UnevenIllumination _GFP_alone_237_.tif
        |_ 20110305 PM 053023 UnevenIllumination _alone_237_.txt
        |_ 20110305 PM 053023UnevenIllumination _GFP_alone_237__SUM_Corrected.tif
        |_ 20110305 PM 053023 UnevenIllumination _alone_237_HS.tif
    |_ 20110305 PM 053917 CameraNoise _GFP_alone_237_/
        |_ 20110305 PM 053917CameraNoise _GFP_alone_237_.tif
        |_ 20110305 PM 053917 CameraNoise _alone_237_.txt
        |_ 20110305 PM 053917CameraNoise _GFP_alone_237__SUM_Corrected.tif
        |_ 20110305 PM 053917 CameraNoise _alone_237_HS.tif
    |_ AverageCameraNoise.tif
    |_ AverageUnevenIllumination.tif
```

3. Patch finding

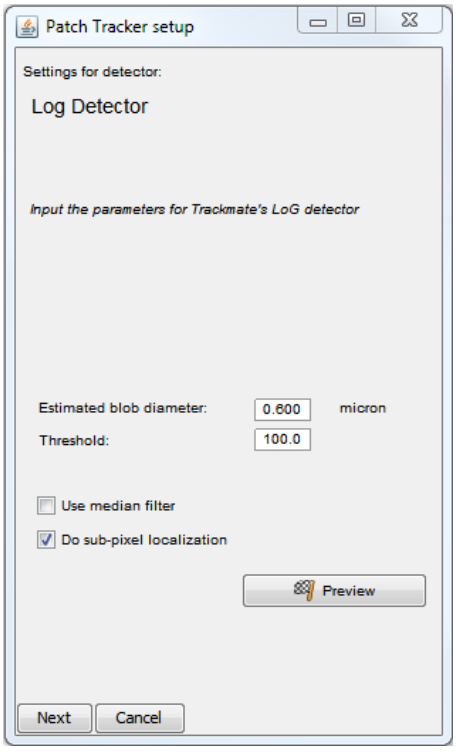
Important note: *if you are using a hyperstack, it is assumed that z-slices are separated by 0.5 microns, which is the optimum sampling for quantitative microscopy of diffraction limited spots. With these conditions, the majority of the fluorescence of a spot is contained on 3 consecutive slices.*

The automatic patch finder uses many functionalities of Trackmate, a single particle plugin included in the Fiji distribution of ImageJ. First, spots are found using Trackmate's "LoG detector" (Laplacian of Gaussian), then linked into tracks using Trackmate's "Simple LAP Tracker". A final step tags spots that will not be measured because a) they are not isolated (fluorescence intensity cannot be extracted from individual spots) or b) they are missing fluorescence (typically the maximum of their fluorescence is on the top or bottom slice of the stack, which means some fluorescence is missing). Tracks containing too many tagged spots will be rejected. Note that if you are using a movie containing a single plane, the tracker will not do part b) of spot tagging. Last, tracks are saved as Roisets in the folder of the image.

Please refer to Trackmate's documentation and Jaqaman et al (2008) for more details about the LoG detector and the Simple LAP Tracker.

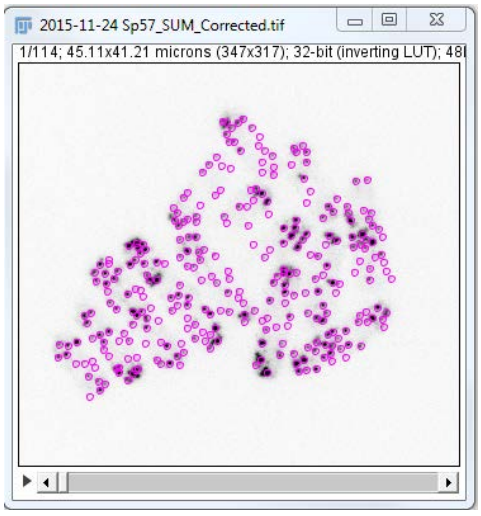
- http://fiji.sc/TrackMate
- Jaqaman et al. *Robust single-particle tracking in live-cell time-lapse sequences*. **Nat Methods** (2008) vol. 5 (8) pp. 695-702

1. Open the sum projection file (ending with '_SUM_Corrected.tif') for the movie you want to analyze.
2. Click on  to start the automatic patch finder. This opens Trackmate's LoG Detector setup window:

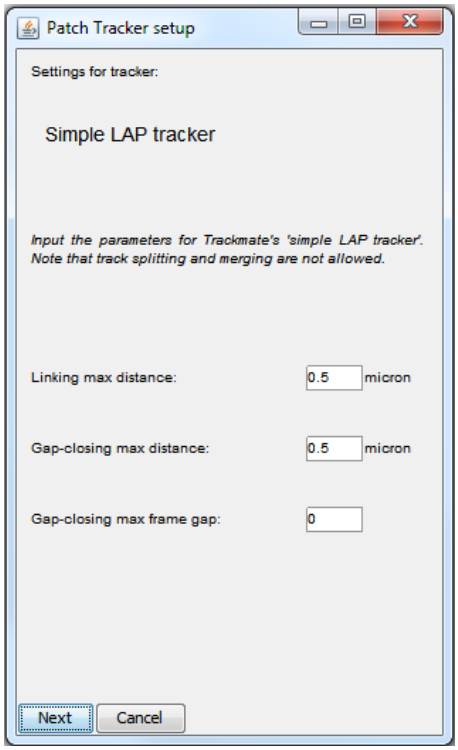


Estimated blob diameter: diameter of the spot to be tracked
Threshold: background threshold under which spots are not detected

You can preview the effect of these parameters on the current slice of the movie by clicking the *Preview* button

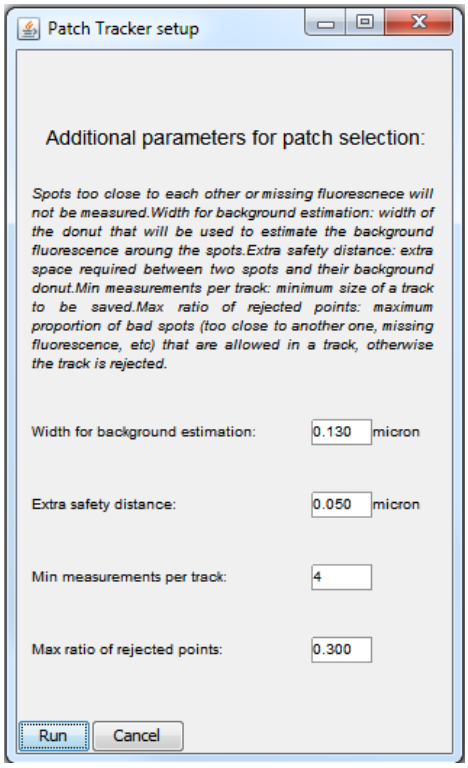


After clicking on the Next button, Trackmate's Simple LAP Tracker setup opens:



Linking max distance: maximum distance between spots to link them into the same track
Gap-closing max distance: if tracks are allowed to skip time frames (the "Gap-closing max frame gap" value is larger than 0), spots should still be within the "Gap-closing max distance"

Last, extra parameters to reject bad quality spots need to be set up:



Spots too close to each other will not be measured. The distance between spots is estimated as twice the sum of the spot radius, the width for cytoplasmic background estimation and the extra safety distance.

Width for background estimation: thickness of the donut used around the spot to estimate cytoplasmic background.
Extra safety distance: extra empty space between two measured spots
Min measurements per track: tracks containing fewer untagged (aka rejected, aka not measured) spots than this number will be rejected
Max ratio of rejected spots: tracks containing more than this ratio of tagged spots will be rejected

After clicking the *Run* button, the macros will find tracks that fit all the criteria set up. The tracks found will be saved as RoiSets (zip files) in the same folder as the image with a name starting with "RoiSetAuto-".

<u>Be patient!</u> This step can take a bit of time and freezes ImageJ (we are working on a fix)**.**

3. Saved tracks should be individually checked since it may happen that two tracks are mistakenly linked to each other, some tracks are clearly not individual spots, etc.

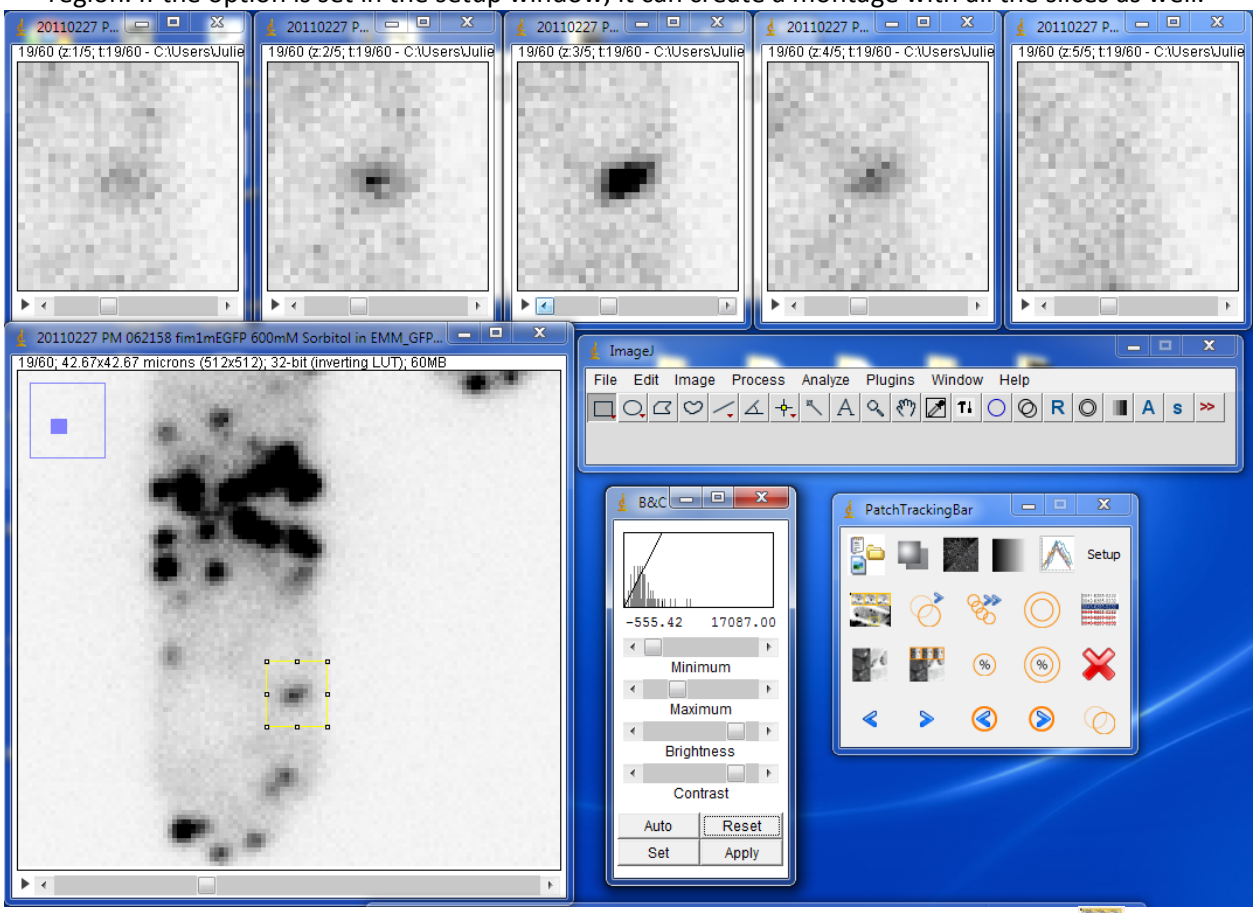
   Click on the button  to automatically make montages of all Roisets.

   - If the montage looks OK, just close it.
   - If it seems that the patch finder picked up something clearly wrong, click on the montage and press SHIFT+B. This will add an underscore "_" to the Roiset filename, which will prevent it to be used when performing measurements (ex: _RoiSetAuto-12.zip). You can then close the montage.
   - If it seems that two tracks were linked to each other, you can tag the Roiset by pressing SHIFT+H. "M_" will be added to its name (ex: M_RoiSetAuto-12.zip). You can then go back, reopen the RoiSet, edit it, split it in half, etc and save it with a different name. You can then close the montage.

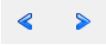
   Note that in some tracks, spots saved in the RoiSet may overlap with another spot in the image in some frames. These spots are tagged in the Roiset file and will not be measured. You can check it by opening the Roiset zip file in Fiji.

4. Patch tracking

1. Open the sum projection file (ending with '_SUM_Corrected.tif') for the movie you want to analyze.
2. You might find if easier to work on the negative image (dark dots on white background) or with the fire LUT (Lookup table, i.e. color coded intensities). Click on  or press the shortcut 'o' to switch between these 3 LUTs.
3. Find a patch you want to track. Draw a square region around it.
   Alternatively, if you have found potential patch candidates with the patch finder tools, load the zip file corresponding to the patch you want to track.
4. You can check quickly if the patch is a good one by making a montage with all the slices by clicking on the second row button  . The length of the montage can be changed in the setup. The montage is centered on the current time point.
5. Click on  or press the shortcut 'd'. That will open the different slices of the movie for this region. If the option is set in the setup window, it can create a montage with all the slices as well.



Example of patch tracking. *Draw a square box around the patch in the projection image (bottom left). Click on*  *or press the shortcut 'd'. The projection image will be move to the bottom left corner and the 5 z-slices in the selection box will appear over the projection image.*

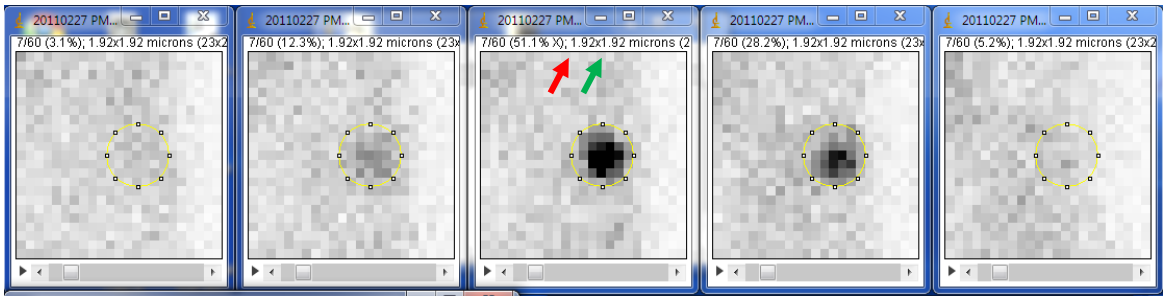
You can navigate in time with the arrows  or with the shortcuts 'k' and 'l'. It will update all the slices at the same time. This allows you to figure out if the patch you chose is actually a single patch or multiple patches, if another patch interferes with it at some point and if it has been imaged fully and you are not missing any slice.

You can play all the slices automatically (like a movie) by clicking on  or typing 'y'. You can increase the playing speed by clicking on  or typing 'F' (shift+F) or slow it down with  or 'f'.
You can select a new region on the projection image at any time and see the different slices by repeating step 3.
At any time, you can close all the slices, except the current image, by typing 'x' or clicking on  .
Typing 'X' (shift+X) will close all the images.

6. If you found a patch you want to track, go to the time point when it appears for the first time, select the circle tool  on the **ImageJ toolbar**. Then click on the center of the patch. An ROI will be created in the ROI Manager. (Make sure that it was empty before selecting your patch).
Note: you can change the default diameter of the patch in the setup window. However, be aware that all your ROIs for all your movies have to be the same size to be comparable quantitatively.

7. Track the patch on the next time point by clicking  or typing 'n'.
At any time you can refine the position of the ROI by moving it (you first need to make sure that one of the geometry tools from the native ImageJ toolbar  is selected. Do not forget to update the position, clicking on  or typing 'w'.

8. When an ROI is selected, you can check which slice contains the maximum intensity (an 'X' appears just above the image, green arrow on next figure) and what percentage of the total intensity each slice contains (red arrow) by pressing 'g' or clicking on  . The intensity is corrected for cytoplasmic background intensity, evaluated as a 1 pixel torus ('donut') around the ROI.
A 'good' endocytic patch usually spreads on 3 slices and contains about 40-50% of intensity on its middle slice.

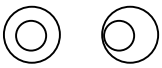


9. If, at a given time point, you think that the patch you are tracking doesn't meet your quality standards (e.g. it overlaps with another one), you can tag this time point so that the intensity won't be measured and taken into account in the intensity data by pressing 'b' or clicking on  . The name of the current roi will then be tagged with '#x' at the end. You can untag it by clicking again on  or pressing 'b'. Warning: If this particular roi is tagged, its intensity won't be measured but be aware that its position will still be measured.

0030-0305-0106
0031-0304-0103
0032-0303-0104#x
0033-0304-0104
0034-0303-0104
0035-0304-0105

10. Once you are done tracking the patch, you can generate automatically all the ROIs that will be used for the cytoplasmic background correction  . Those are concentric circles around the ROI.

    You can navigate between the background correction ROIs ( or 'K' and 'L') and move them if they overlap with another patch (Do not forget to update them!). However, you cannot move them too much since they have to include the pixels circled by the corresponding patch ROI. In other words, with the default values, you cannot move the background ROIs more than 1 pixel in each direction.

    OK:  Not OK: 

    Note: you can change the default diameter of the background ROIs in the setup window. However, be aware that all your ROIs for all your movies have to be the same size to be comparable quantitatively.

10 VS. You can also do the cytoplasmic background correction by choosing a zone in the cytoplasm that does not contain any patch (a.k.a 'Volodia's method'). After tracking the patch, add one Roi in the cytoplasm on the 1st frame when the patch appears. Run this macro to spread (copy) the cytoplasmic Roi on the next frames. Save this RoiSet with a name that ends with "_VS.zip". This name pattern is crucial during the data analysis and the generation of the data files.

11. Finally, save the ROISet by clicking 'Deselect' in the ROI Manager, then 'More>>' > 'Save'. Alternatively you can press 'S' (shift+S) or click on  . The name should start with the pattern that can be specified in the setup window (Default is 'RoiSet'), in order to be taken into account during the measurements.
    Note: Make sure you deselected all the ROIs before saving. The default name that is proposed should end with '.zip'

12. *Photobleaching correction ROIs:*
    Select ROIs inside the cells that are not in the nucleus, not in the vacuoles and do not overlap with any patch during the entire movie. These ROIs are used to estimate the decrease in fluorescence due to photobleaching. You can select several regions by keeping CTRL pressed. Once you have selected regions that are big enough, click on  . It will save this ROI (RoiForPhotoBleaching.zip) and show the bleaching curve and the fit to an exponential. If it really doesn't look like an exponential, refine your ROI and make sure there is no patch crossing it anytime during the movie.
    You can choose in the setup which formula to use for the photobleaching fit. Usually it gives more robust (but a bit less accurate) results if you use the exponential with no offset.
    You can also set yourself the photobleaching coefficient in the setup.
    Note: you should combine several ROIs in different cells.
    Tip: It is easier if you generate a projection file with all the frames of the movie to select the photobleaching ROI.

5. Measurements

Click on  or type 'A' to automatically collect all the intensity and position data for all the patches you have tracked and whose name starts with the pattern set in the setup window. It will generate several files with the intensities, the positions and other statistics about the patches

Note:

- The position information of an Roi is measured as the center of mass of the intensity of the pixel of this Roi, on the slice that contains the largest intensity (if the box is checked on the setup window, otherwise, it is measured on the sum projection image).
- The rois that are tagged as bad (#x) don't have their intensity measured, but the position information is still measured.

Files generated

The files that are generated can be chosen in the setup window.

- *CorrectedAlignedIntensities.xls*
  This file contains the intensities for each Roi that are corrected for cytoplasmic background and for photobleaching. The intensity is the sum of the intensities of consecutives slices centered on the slice that contains the maximum intensity. Quality control can be performed by checking the extra columns in the file. Each RoiSet has two extra columns, one containing the percentages of fluorescence on each slice, the other one containing the index of the slice with the maximum intensity.

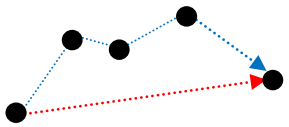
RoiSet-3.zip Quality	RoiSet-3.zip IndexMax
'-1.4 / 6.6 / 45.2 / 36.9 / 12.8 '	3
'-1.2 / 9.4 / 38.3 / 39.6 / 14.0 '	4
'2.0 / 10.0 / 39.6 / 37.6 / 10.8 '	3
'1.3 / 12.7 / 44.2 / 32.1 / 9.7 '	3
'1.2 / 14.2 / 43.0 / 32.9 / 8.7 '	3
'0.3 / 13.3 / 45.4 / 33.9 / 7.1 '	3
'1.5 / 12.1 / 43.8 / 33.2 / 9.3 '	3
'1.1 / 11.1 / 38.4 / 38.2 / 11.2 '	3
'0.5 / 12.4 / 44.2 / 34.3 / 8.7 '	3
'1.1 / 14.0 / 46.6 / 32.8 / 5.4 '	3
'1.4 / 18.8 / 44.3 / 28.5 / 7.0 '	3
'2.5 / 21.4 / 44.6 / 27.0 / 4.4 '	3
'2.6 / 21.8 / 46.5 / 23.0 / 6.1 '	3
'2.6 / 21.6 / 48.9 / 22.1 / 4.8 '	3
'2.4 / 27.1 / 44.7 / 21.9 / 3.8 '	3
'7.6 / 28.0 / 45.4 / 14.7 / 4.4 '	3
'3.6 / 33.7 / 46.4 / 15.3 / 0.9 '	3
'3.9 / 20.2 / 42.9 / 25.0 / 7.9 '	3
'2.2 / 11.2 / 33.7 / 35.7 / 17.2 '	4
'1.4 / 4.7 / 39.0 / 40.4 / 14.5 '	4
'1.5 / 13.6 / 36.6 / 33.9 / 14.3 '	3
'0.5 / 9.5 / 60.7 / 22.6 / 6.7 '	3

Example of quality control data for a 5 slice-movie.

- *ConcentrationVsDistance.xls*

TimePointNb	time	RoiSet-1.zip Distance px	RoiSet-1.zip Path Length	MSD	Speed	Concentration		RoiSet-2.zip Distance px	RoiSet-2.zip Path Length	MSD	Speed	Concentration
0	0											
1	1											
2	2											16324.8531
3	3							0.0304	0.0304	0.0067	0.0304	33209.6877
4	4					14253.814		0.0251	0.0443	0.0163	0.0139	59889.7716
5	5	0.0237	0.0237	0.0092	0.0237	32757.2176		0.0167	0.0657	0.0211	0.0214	80126.2402
6	6	0.0062	0.0414	0.0119	0.0177	63312.9355		0.063	0.114	0.0198	0.0483	106901.207
7	7	0.0471	0.0828	0.0128	0.0413	79825.8425		0.0638	0.1159	0.0221	0.0018	141976.1313
8	8	0.0711	0.1076	0.0184	0.0248	108303.6261		0.0891	0.1445	0.028	0.0286	167129.2013
9	9	0.0777	0.1325	0.0203	0.025	124846.6665		0.0985	0.1706	0.0387	0.0261	177306.6761
10	10	0.0819	0.1368	0.0268	0.0043	147986.9913		0.0984	0.1999	0.0509	0.0293	214841.4743
11	11	0.0893	0.1602	0.0311	0.0234	186334.1277		0.1004	0.22	0.0629	0.0201	212888.5017
12	12	0.1305	0.2571	0.0374	0.0969	209386.7069		0.1442	0.2639	0.0657	0.0439	242309.5395
13	13	0.1156	0.2967	0.0446	0.0396	223271.6282		0.1984	0.3182	0.0671	0.0543	241192.2307
14	14	0.148	0.3516	0.0532	0.0549	222457.4435		0.2294	0.3778	0.0734	0.0596	264489.8655
15	15	0.1617	0.3981	0.0607	0.0464	244373.7594		0.2414	0.4001	0.083	0.0223	259158.3845
16	16	0.1974	0.4368	0.0716	0.0388	220733.1786		0.1935	0.5055	0.0861	0.1054	228852.6567
17	17	0.2032	0.4482	0.0841	0.0114	217036.4275		0.1644	0.5414	0.0882	0.0359	232592.6948
18	18	0.2066	0.4554	0.099	0.0072	184768.7047		0.241	0.6234	0.0874	0.082	229538.3011
19	19	0.2574	0.5094	0.1147	0.054	152773.3603		0.277	0.6779	0.0862	0.0545	175346.1545
20	20	0.4461	0.6993	0.1257	0.1898	120081.0041		0.2212	0.8209	0.0855	0.1431	169416.0643
21	21	0.301	0.8459	0.1163	0.1467	98033.5482		0.1787	0.8788	0.0896	0.0579	136239.0247
22	22	0.3112	0.9221	0.1226	0.0761	80789.2665		0.306	1.0446	0.0943	0.1658	88234.4103
23	23	0.4246	1.0414	0.1304	0.1193	65913.797		0.2776	1.0801	0.0928	0.0355	
24	24	0.3163	1.2495	0.1142	0.2081	44034.0767		0.207	1.1824	0.0933	0.1022	43923.3545

For each patch, a block of column is generated. It contains the distance from the first position, the cumulative path length of the patch (ie the sum of the displacement of the patch at each time point, from the beginning), the MSD (Mean Squared Displacement), its speed (distance travelled between two time points divided by the time delay between two images (see setup), the corrected intensity of the patch.
The rois that are tagged as bad (#x) (red arrow) don't have their intensity measured, but the position information is still measured.



Distance from starting point
Cumulative path length (displacement)

- *AlignedDisplacements.xls*
  Contains the cumulative path length for each patch (ie the sum of the displacement of the patch at each time point, from the beginning).

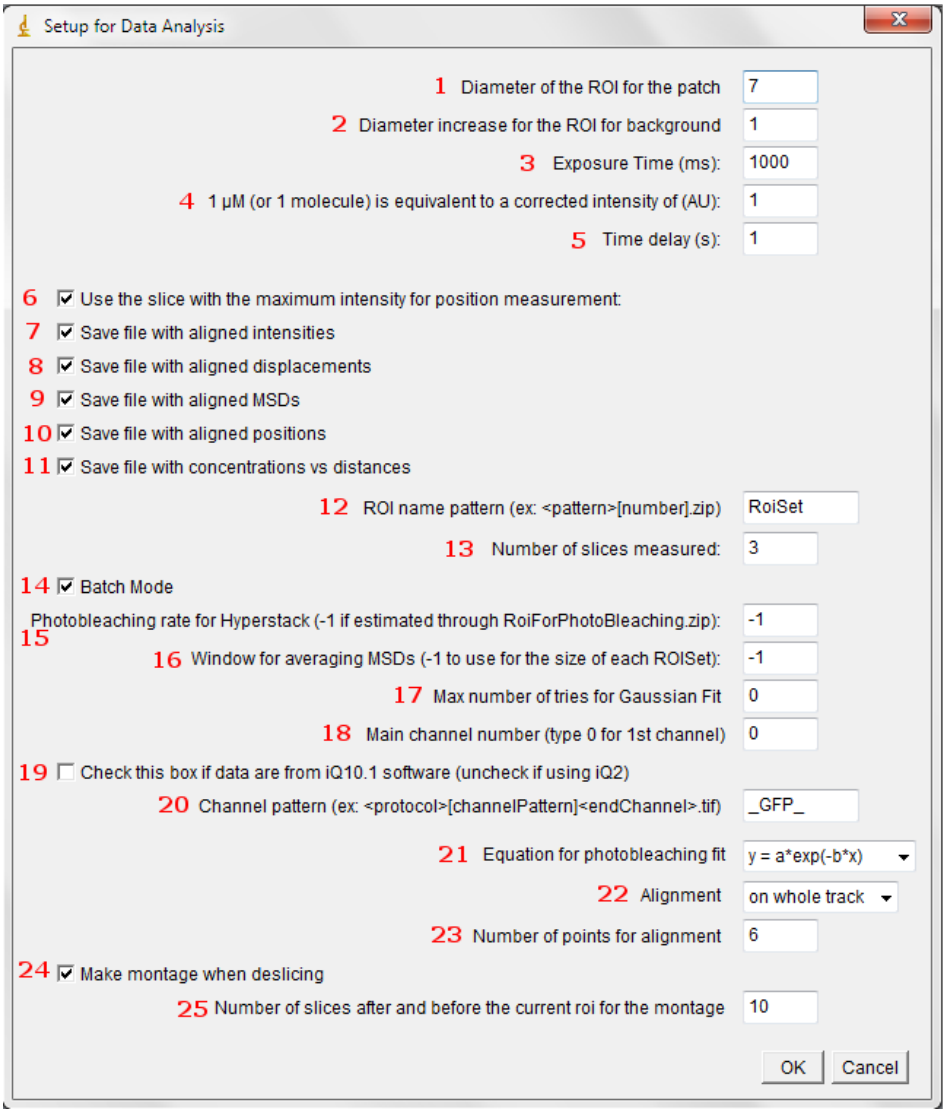
- *AlignedMSD.xls*
  Contains the mean square displacement for each patch

- *AlignedPositions.xls*
  Contains the position information for each patch. The columns whose name end with 'px' are calculated as the center of mass of fluorescence, the columns without 'px' are estimated from the Gaussian fitting of the fluorescence (doesn't work well and the Gaussian fit for each coordinate is done independently). Quality control information for the Gaussian fit are available in the extra columns.

- *Log.txt*
  Contains what has been written in the log window during the analysis (for example the photobleaching coefficient, …).

6. Setup Window

This window contains most of the parameters that can be changed. However, be aware that everytime the PatchTrackingTools are reloaded or Fiji is restarted the parameters will be back to the default options (see window below). If you want to change the defaults you should edit the toolset file /macros/toolset/PatchTrackingTools.txt.



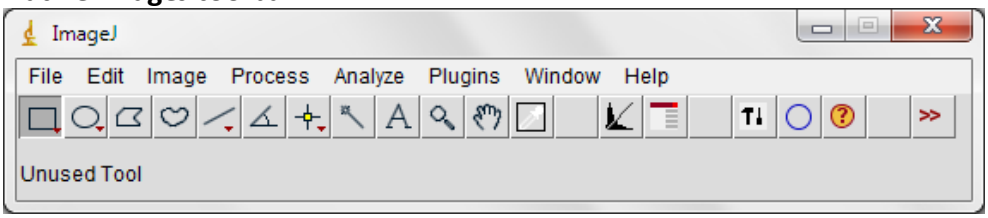
1. Diameter of the circle tool  in pixel
2. Thickness of the torus ('donut') used for the cytoplasmic background correction 
3. Exposure time of the movie in milliseconds
4. Conversion factor for the intensity values, estimated from the calibration curve. The number represents the intensity that would be measured for 1 micromolar (or 1 molecule) of fluorescent protein in 1 second.
5. Time step between 2 consecutive frames of the movie.

6. Check this box if you want the position of the patch to be measured from the slice with the maximum intensity only. If the box is unchecked, the position will be measured from the projection image. Note that the measured coordinates are the coordinates of the center of the mass of intensity of the ROI.
7. Check if you want the file with aligned intensities to be saved
8. Check if you want the file with aligned displacements (cumulative path length) to be saved
9. Check if you want the file with aligned MSDs to be saved
10. Check if you want the file with aligned positions to be saved
11. Check if you want the file with all the data to be saved
12. Pattern for the names of the Roisets that are measured during the analysis. Only the files starting with this pattern will be taken into account
13. Number n of slices measured. The measurement of intensity adds the intensity of n consecutive slices, centered on the slice with the maximum intensity.
14. Check if the macros should run in batch mode. Highly recommended!
15. Photobleaching rate. Set it to '-1' to estimate the photobleaching rate from the roi in the file RoiForPhotoBleaching.zip, which is generated through the photobleaching macro  . Otherwise, the number that is set is the photobleaching rate, representing the loss of fluorescence every time one image is taken (thus based on the photobleaching of the hyperstack and not the sum projection image).
16. Maximum window to calculate the MSDs
17. Number of tries for the Gaussian fit. 0 if no Gaussian fit should be done. Warning: The Gaussian fit is done on each coordinate independently. Use at your own risks!
18. For multi-channel movies, sets the channel that has to be taken into account for all the measurements (0 for the 1st channel).
19. Check this box if the data are from Andor iQ 10.1 version.
20. Pattern for the channel name. Used during the correction of movies containing several channels(?).
21. Equation used for the estimation of the photobleaching coefficient. The single exponential usually gives a more robust result, even if the fit is less good. In general, if the movie is not long enough, the offset of autofluorescence is hard to estimate and the parameter fit is not always consistent. Setting it to zero makes the estimation of the photobleaching coefficient more consistent, even if it might a bit less accurate. However, it usually gives pretty good results.
22. Mode of alignment of the data. The options are:
    a. *On the whole track*: the algorithm tries to align the intensity data so that all the tracks are superimposed. Technically, it uses the longest track as a reference and it aligns the other tracks to minimize the square difference between the reference intensities and each track, at each time point.
    b. *On beginning*: same idea as above but the algorithm uses only the ***N first*** data points to align the data. The number ***N*** can be set in the next box of the setup. The data are thus aligned so that the assembly phases overlap.
    c. *On end*: same as *'on beginning'* but uses the ***N last*** data points instead. The data are thus aligned so that the disassembly phases overlap.

23. Number of points for the data alignment if the alignment mode is *'on beginning'* or *'on end'*
24. Check this box if you want to generate a multi-slice montage when deslicing ([d] or )
25. Number of time points (frames) used before and after the current time point (frame) to make the multi-slice montage ([j] or ).

8. Toolbar, Macros and Shortcuts

Native ImageJ toolbar



 : Contrast and Brightness balance

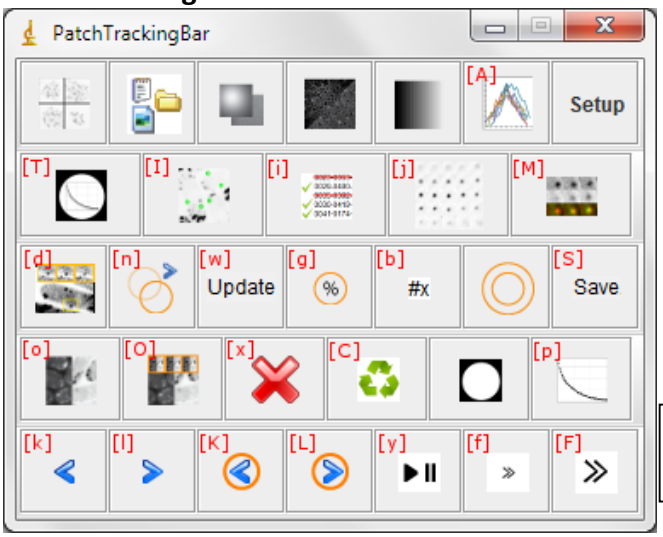
 : RoiManager

 : PatchTrackingToolbar

 : Circular Roi tool

 : About the PatchTrackingTools

Patch Tracking Toolbar



Patch tracking toolbar. In red are the shrotcuts to the different tools

More macros are available under Plugins>Macros. The letter in brackets at the end of a macro name is its shortcut.

**** Andor iQ2 movies reorganization ****

Split multi-field movies: use this macro to split the tif files of a folder in as many fields as it contains. Each file generated will have the same name as the original tif file, with the field number added at the end. It also creates a text file with the protocol.

Reorganize movies from microscope: reorganize the files from a given folder to create one folder per movie. It will also contain the protocol text file and all the files for multi-channel movies (Not sure it works well. Need to test again??).

Combine Channels: makes one file from multi-channel movies that have been saved as several files (Not sure it works well. Need to test again??).

**** Movie Corrections ****

Generate Correction Files: Generates the uneven illumination and the camera noise files from movies.

Generate Neutral Correction Files: Generates 'fake' uneven illumination and camera noise files that actually do not contain any correction. Useful if you want to use the patch tracking toolbox on raw data (without correcting your files).

Correct all movies: correct all the movies in all the subfolders of the selected folder.

Spread ROI for Photobleaching Analysis: use this macro after selecting Rois in the cytoplasm of the cells to generate the file RoiForPhotoBleaching.zip that will be used for the photobleaching corrections.

**** Patch Finder ****

Start tracking [T]: Reduces the field of the image to the areas that have been illuminated at least 50% of the maximum illumination and does a rough correction for photobleaching, based on the variations of the total intensity of the sum projection image at each time point.

Find patch candidates [I]: automatically finds spots that look like patches.

Curate patch candidates [i]: goes through all the Rois in the RoiManager, makes a multi-slice montage and allows you to save the Roi in a single zip file in the folder of the image.

Save Current Roi [J]: saves the selected Roi from the RoiManager into a zip file.

**** Patch tracking ****

Circle Tool [c]: adds to the RoiManager a circle Roi which is centered on the pixel where the user has clicked. The diameter of the circle tool can be changed in the setup.

Make montage one row per slice [j]: makes a montage with one row per slice (in z) and centered on the current selection on the image (current Roi from the RoiManager or rectangular selection). The length of the montage can be changed in the setup.

Deslice Around ROI [d]: opens one window per slice (in z). Each window is cropped around the current selection.

Recenter next slice [n]: tracks the patch on the next frame and adds the Roi to the RoiManager.

Recenter current slice [r]: recenters the current Roi around the patch

Update [u]: updates the current Roi and goes to the next Roi in the list

Regular Update ROIManager [w]: updates the current Roi

Get Intensities [g]: measures the corrected intensities for each slice (in z) and prints the percentage of total fluorescence above the image. Also adds an 'X' above the image that contains the Roi with the maximum intensity.

Mark ROI as bad/good [b]: tags the current Roi as bad and adds '#x' at the end of its name. If it is already tagged as bad it turns it into a good patch and removes the tag. The intensity of rois tagged as bad will not be measured. However their position information will be taken into account.

Background ROI Action Tool: adds at the end of the Roi list of the RoiManager the rois for the cytoplasmic background correction. These rois are centered on the Rois of the list but with a larger diameter that can be changed in the setup.

Spread Cytoplasmic Background Roi (VS Style): After tracking Roi, add one Roi in the cytoplasm on the frame with the first time point for the patch. Run this macro to spread (copy) the cytoplasmic Roi on the next frames. Save this RoiSet with a name that ends with "_VS.zip".

Save ROISet [S]: save the current list of Rois of the RoiManager into an RoiSet

Plot intensities for ROI [P]: plots the raw and corrected intensities of the patch along with the cytoplasmic background. Requires a patch to have been tracked and its Rois for the cytoplasmic background correction to have been added.

AutoRecenter: recenter the current roi to encircle the patch. Does not work very well…

Follow the Point Source: keeps tracking the patch on next frames. Press escape to stop it!

**** Data Analysis ****

New Data Analysis [A]: Collects the data for the current sum projection image and generates the files set in the setup

Setup: opens the setup window.

**** Display ****

OptimizeContrast [o]: changes the LUT used and optimize the contrast. Every time this macro is called it circles between the regular black and white, the inverse black and white and the fire LUT (color coded intensities).

Same Contrast For All [O]: Sets the same LUT and the same contrast to the images for the different slices

Reduce field to 50% of illumination: reduces the field to the areas that have been illuminated by at least 50% of the maximum illumination

Close all except current [x]: close all images except the current one.

Close all [X]: close all the open images

Zoom to selection [0]: zoom to the current selection

Set photobleaching Correction [p]: does a rough correction for photobleaching, based on the variation along time of the whole field of the sum projection movie.

Cancel photobleaching Correction: cancels the rough photobleaching correction

**** Player ****

Play [y]: plays all the windows at the same time

Slower [f]: plays slower

Faster [F]: plays faster

1st Frame [1]: moves the sum projection movie and the movies for each slice to the first frame

Previous Frame [k]: moves the sum projection movie and the movies for each slice to the previous frame

Next Frame [l]: moves the sum projection movie and the movies for each slice to the next frame

Previous ROI [K]: shows the previous ROI in the sum projection movie and the movies for each slice

Next ROI [L]: shows the next ROI in the sum projection movie and the movies for each slice

**** Other Tools ****

Garbage collector [C]: closes all the invisible or unused windows and recycles the memory ('garbage collector').

Make Montage [M]: make a montage for a 2-channel movies: 1 row per channel and 1 row combining both colors

Circle cells: automatic cell circling. Use with black cell on a bright background.

Correct for Photobleaching: Corrects the current window for photobleaching. Requires another movie to have already been corrected for photobleaching (ie after using the macro *Set photobleaching Correction [p]*). The same correction factors will be used.

Change RoiNames: Renames all the RoiSets in a folder by adding at the end of their name the position information of the first Roi.

Synthesis Patch: Adds to the RoiManager one Roi for each of the Roisets in the folder of the current sum projection image

printExposureTime: prints the exposure time for the movies in all the subfolders.

7. Known problems and solutions

- Sometimes, when trying to show the different slices (pressing 'd') or doing something else, you might get an error message.
    a. First try to close all the images except the projection image (you can select this one and press 'x'). Navigate between the frames (typing 'k' or 'l'). Then retry want you wanted to do.
    b. If that didn't work, reload the macros toolset (Click on the last button of the toolbar  and select SemiManualTracking.iQ2Data.dev). Navigate in the image and retry. If it still doesn't work, close all images ('X') and reopen the projection image and retry.
- If you get an 'Out of memory ' message while correcting movies, you should try to increase the memory of ImageJ (Edit>Options>Memory & Threads…). The optimum maximum memory should be about 70% of the total RAM of your computer.
  If that doesn't work, you should try to make smaller movies…
- If you get 'unreproducible' errors, that could be due to a problem with the synchronization of the macros. Open the file PatchTrackingTools.txt in the macro/toolsets/ folder and increase the lag time: var IJSynchronizationLag=200;
- Note that some of the native shortcuts of ImageJ will be replaced by shortcuts for the PatchTrackingTools.
- If you reorganized your movies manually, many problems can happen. The most common is that the dimensions of the hyperstacks and the sum projection movies are wrong. Make sure the number of "slices" is the number of "z" slices and the number of "frames" corresponds to the time.
- If you have 'weird' tracks in the excel files, make sure that the Rois don't contain extra time points and that there is exactly one background Roi for each Roi in the RoiSets. Make sure the background Rois are at the end of the file and that their shape 'contains' totally the shape of the patch Roi.
- Alignment of the data with the PatchTrackingTools may not be very accurate.

9. How to report a glitch?

If you think you found a glitch or the patch tracking tools have a 'weird' behavior, ideally you should:

1. Make sure you are using the tools the way they should be used to do what you mean to do
2. Make sure you screened through the documentation for an answer
3. Make sure the problem is reproducible:
    a. on the same dataset
    b. on another dataset
4. Try to identify the minimum requirements on your dataset to reproduce the problem, ie try to identify when it works and when it doesn't work.
5. Send an email describing precisely the problem (also describing when it works and when it doesn't work) to julien.berro@yale.edu

If you have hard time to narrow down the reasons of the glitch, or if you have any question, any suggestion to improve the PatchTrackingTools and its documentation, send an email to julien.berro@yale.edu.