Analysing SPT data

Before describing the analysis of the experiments, two pieces of advice:

- ***Get off on the right foot***

  Good experiments (good labelling, good cells) = easy tracking and analysis.

  Bad experiments = headache!

- ***Be confident of your tracking***

  This is a mostly automatic process and you will always get numbers at the end. Be sure that they are meaninful! Control the tracking and learn how the analysis is done. This will help you not to waste your time.

  The controls of quality can be done at several steps:

  - Test of signals: Is the signal-to-noise ratio good enough? Are there too many particles that are not single? (use **Detection->Test of signals**)
  - Trajectories: Are the particles correctly followed? Are there particles outside cells? (use **Movies**)
  - Localization: Is there any shift between the images of different colours? (use **Movies**)

Running SPTrack_v6

You just need the movie file to perform the tracking. If you want to localize the trajectories, you need a localization image that was previously segmented (see the help file "Insights into SPT procedures").

Please note that your files must be recognized automatically by the programs. In consequence, their names must have the following formats:

- the movie file: *name*.tif or *name*.stk
- the localization image: *name*-**identifier**.tif

The analysis is divided in:

- **Detection:** will do the detection of objects (peaks). Creates *.pk files in \pk folder with the information about their position, intensity, etc.
- **Initial tracking**: Connects objects in .pk files between consecutive images. These initial trajectories are short on purpose for computing reasons. New files *.trc and *.traj are created \trc and \traj folders, respectively (see section Format of detection and tracking results below).
- **Reconnection**: Elongates the initial trajectories. Creates *.con.trc files and appends *.traj files with the new trajectories ('linked').
- **Localization**: Classify trajectories by their localization.
- **Diffusion analysis**: MSD and D calculation, analysis of confinement, dwell time, etc.

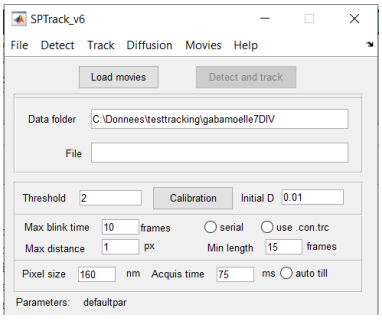
For each step of the analysis there is a progression bar. The program can be stopped at any moment closing the progression bar window. You can follow the progression also in the command window, where are shown the partial results (number of peaks, trajectories, etc).

Note: If the analysis is re-done, all the previous files are overwritten.

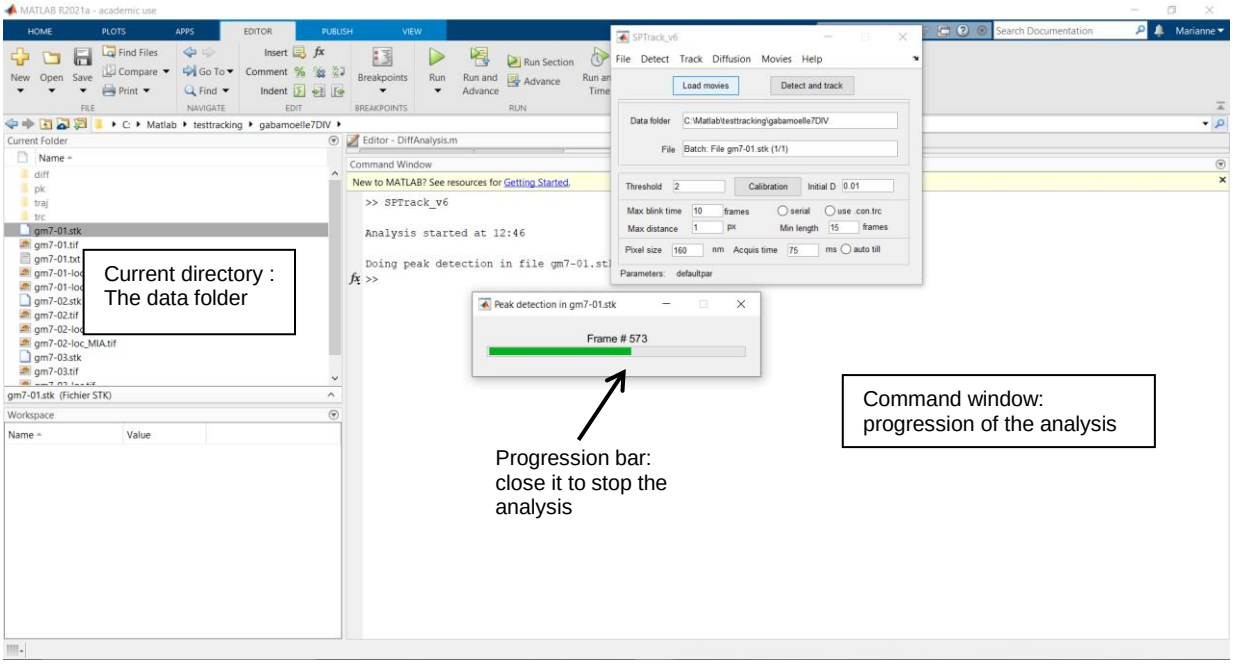
Quick list to perform SPT analysis:

See the sections below to have more information.

- Run SPTrack_v6 from the command window.
- The current directory must be that of the movies.
- Check the parameters on the window (see help file 'Choosing parameters').



- Load the movies with **File: Load movies** (Ctrl L) or the button **Load** to select the files to be analysed (one or more).
- If you want to use parameters other than the default, use **File: Load parameters** (Ctrl P) to choose your file, or type the name of the file and click 'enter'. You can also change the values by the window, and save the new set with **Save parameters**. For a detailed explanation about parameters, refer to the help file 'Choosing the parameters'.
- You can control the quality of detection and change its parameters using **Calibrate**.
- Click on **Detect and track** to perform peak detection, initial tracking and automatic reconnection of trajectories at once.



- You may want to select or clean trajectories before finalizing them: see Clean trajectories.
- Finalize tracking doing manual reconnection (and eventually localization).
- Run diffusion analysis.

Part by part

It is possible to run (and re-run) all the parts of the analysis independently.

- **Menu Detect -> Detection of particles**: will do the peak detection and save the information in pk*.pk files.
- **Menu Track -> Initial tracking**: will do the initial tracking using *.pk files. Creates trc*.trc and traj*.traj files not linked.
- **Menu Track -> Automatic** or **Manual reconnection**: To elongate the trajectories done by the initial tracking. Using *.trc files selected, they will create *.con.trc files and linked *.traj files. Localization can or not be added at this point.
- **Menu Track -> Localize over domains:** Adds the information of the localization to *.traj files.
- **Menu Diffusion -> Analysis on individual trajectories**: diffusion analysis for the selected *.traj or .trc files.

Note: if an initial step is re-done, do not forget to re-do all the following steps after.

Save report

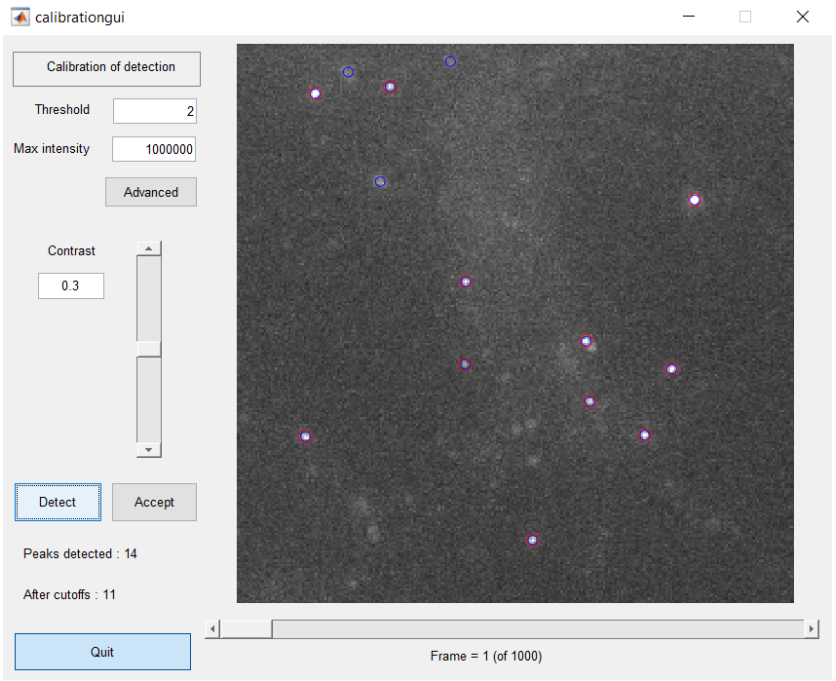
To maintain a record of the parameters and results obtained each time the analysis is run, click **File-> Save report**. This will create a *.txt file that has the information concerning the parameters, the number of detected peaks and trajectories obtained since the last time the button was click or since the program window was opened. The name of this file is the time at which it was created.

```
28-Apr-2022

Folder: C:\Matlab\testtracking\gabamoelle7DIV\

Parameters :
Threshold =2       Predicted D =0.01
Cutoffs : Intensity error = 0.33333        Max intensity =1000000
Sequential reconnection
Acquisition time =75        Size pixel =160
Min points =15      Calculation of D: fit MSD from point 2 to point 5.


Analysis started at 13:40

File: gm7-01.stk
Peak detection by Gaussian fitting
Image size (pixels): X= 250      Y= 251        1000 frames
There are in average 11.937 peaks per frame.
The initial tracking constructed 997 trajectories.

Sequential reconnection. Max blinking: 10 frames; Max dist: 2 pixels.
652 trajectories after reconnection and 460 trajectories after filtering the short ones (less than 3 points).

Sequential reconnection. Max blinking: 20 frames; Max dist: 3 pixels.
293 trajectories after reconnection and 123 trajectories after filtering the short ones (less than 15 points).

Analysis finished at 13:43
```

1. Calibrate:

Clicking on this button opens a new window to visualize the detections and to verify detection parameters. The movie analyzed is the one entered with **Load movies** or, if a list of movies was selected, the first of the list.

Detect re-does detection with the new parameters. If you agree with the result, click **Accept**: the selected values will be transferred to the SPTrack_v6 window.

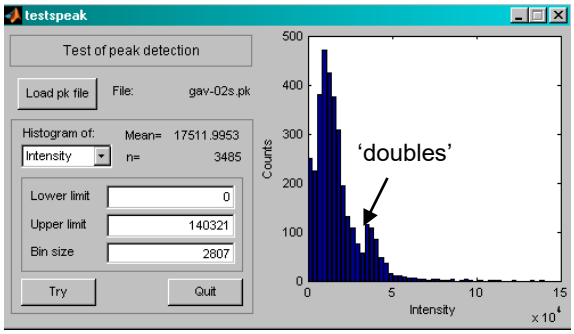


All the detected peaks appear circled in blue, and the ones retained for tracking are circled in red. Using the arrows or sliding bar at the bottom it is possible to visualize different frames. The contrast of the image can be changed by the vertical sliding bar. An additional factor (on the left of the bar) can be entered to change the intensity of the effect. Click on the bar after changing it to refresh the image.

The Advanced button allows changing some others parameters concerning detection, but these values will be used only in the present analysis session and will be lost once the program window is closed.

2. Test of signals:

This program plots histograms of the intensity of the peaks, their width and the offset (noise). These are important features to know if the labelling and detection were satisfactory.



- **Load** the .pk file
- Choose the data (**Histogram of** intensity, offset or width)
- **Try** a first histogram
- Set other limits and bin size if necessary.

Intensity histogram:

Ideally, it has to display a normal distribution with only one local maximum that corresponds to the intensity of single particles. The presence of another peak at intensities that double the first peak is an indication of the presence of double particles. These can be just particles that by chance came together by diffusion (and this is normal) or particles that are bound to the same molecule (not good). This second peak has to be as small as possible.

Width histogram:

The detection procedure is optimized for objects that are 2-4 pixels wide. If you don't get similar values, you may have detected objects that are not single particles, or your single particles do not have the proper size (check the pixel size).

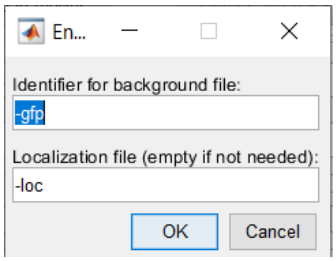
Offset histogram:

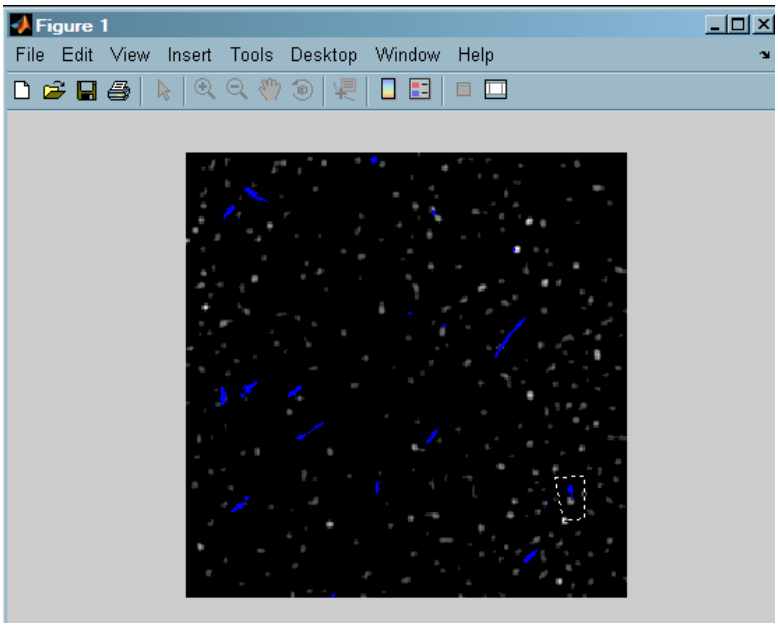
It shows the distribution of intensities of the background: the infamous noise. This value is the average intensity immediately around the signal of the particle (the square area used to do the gaussian fit). The size of this area can be seen and modified using the Calibration window (**Advanced** parameters)

Note: not all the peaks that appear here will be used for tracking. The trajectories will be constructed with those who pass the quality control, set to keep only the detections that fulfil the characteristics of a single particle (intensity peak close to the PSF of the set up).

3. Clean trajectories:

This tool deletes trajectories that are visualized and chosen by hand. First, you are asked to choose a trajectory file and a background file to display the trajectories. If you want to include a localization step at the end, indicate the identifier of the localization file.





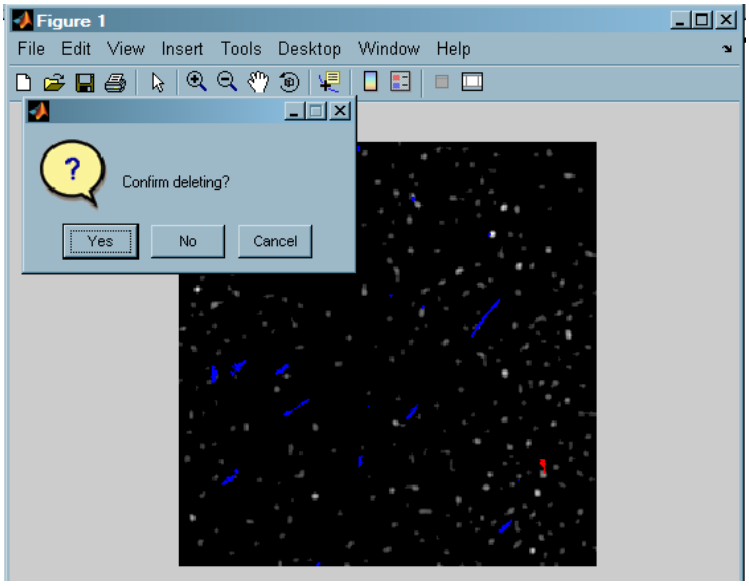
In the figure that appears, select with the mouse the trajectories that you want to delete.

If you select only a part of a given trajectory, the program will delete the entire trajectory anyway.

Before deleting, you can observe the selected trajectories and confirm or not the deletion. You can choose several areas (trajectories) in a given image.

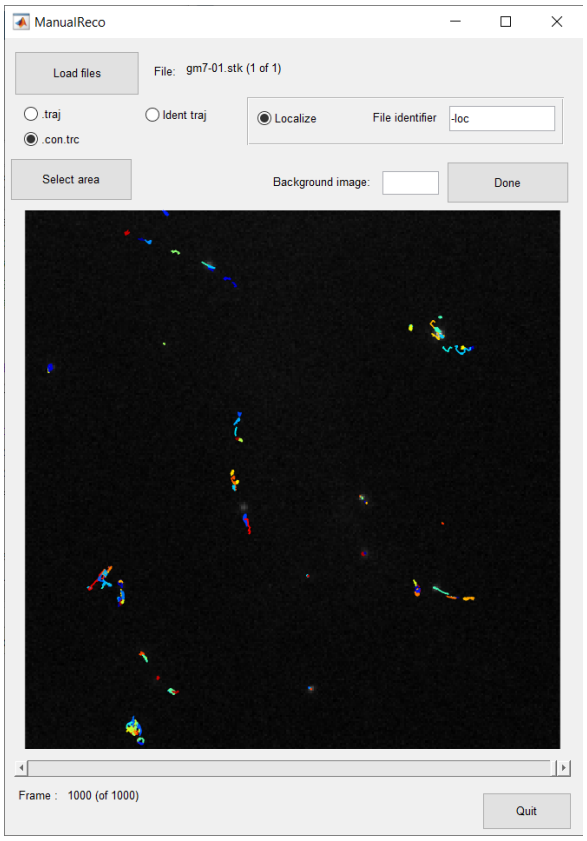
Please note: results (.trc and .traj files) are saved separately in the **\clean** folder created by the program (not to overwrite the original results).

Thus, if you want to analyse diffusion on these cleaned files, do not forget to move these new files to the \trc and \traj folders.



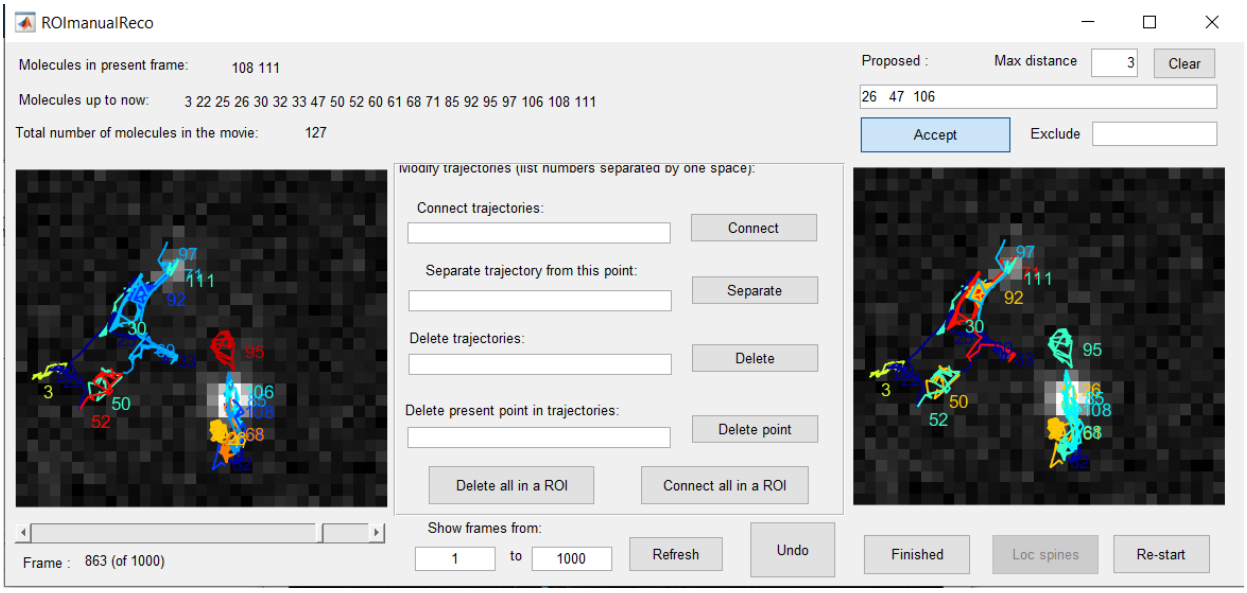
4. Manual reconnection:

With this program it is possible to reconnect trajectories manually or to modify them to correct wrong connections. The window *ManualReco* shows the overlay of trajectories (each one of them in a different color) and the movie. The trajectory file can be .trc or .traj.



- **Load** the file (.stk). The trajectories appear in different colors. Move the slider to the last frame to see them all. **Ident traj** adds the numbers of the trajectories on the image. If you click on it, move the slider again to refresh the visualization. If an identifier for a **background image** is provided, the trajectories will be plot over the background image, not the movie file.
- Click on **localize** if you want to do the localization over the files that contain the **File identifier** in their names.
- To do the reconnection, select the area of interest pressing **Select area** and using the mouse. This will open a new window (reconnection window, see below)
- When you have finished the area selected previously will be marked with a red rectangle.
- **Done** will save the modifications on the present movie and load the next one (in case of selecting several movies).

Reconnection window:



On the reconnection window, you will find the numbers of molecules present at each frame as well as all the previous ones ("Molecules up to now"). It also gives the total number of trajectories of the movie. Use the sliding bar to visualize tracers and trajectories along the movie. You can select a given period with **Show frames from** and **Refresh**.

You can modify the trajectories entering their numbers in the window:

- **Connect trajectories**:

  Indicate the number of the trajectories to be connected (they must not happen at the same time). Write the numbers separated by one space.

- **Separate trajectory from this point**:

  It cuts the trajectory indicated into two parts, the first one will keep the same number, the new trajectory will have the number that you enter (it has to be higher than the maximum number of trajectories).

- **Delete trajectory**:

  It deletes the indicated trajectories.

- **Delete present point in trajectories**:

  It deletes only the present point from the indicated trajectory.

  You can manually select a region to **Delete all in a ROI** or **Connect all in a ROI**.

  Use **Undo** to undo the last modification. **Re-start** comes back to the initial state.

Note: only closing the window with **Finished** will save the new trajectories (that will overwrite the previous .traj and .trc files).

In addition, the program proposes connexions (up to ten trajectories each time). The resulting trajectories are shown on the right image. The numbers of the trajectories that are proposed appear on the window.

The possibilities are:

- **Accept**:

  Accept the reconnection proposed or try another one. The proposed trajectories are not saved if they are not accepted. The trajectories that will be saved are always those one the left image.

- **Max distance** is the threshold in distance to look for proposed connexions. To try new connexions, change the value of this threshold, click **Clear** to reinitialize the list of trajectories proposed and click **Try** to look for new connexions.

- **Clear**

  It clears the list of proposed connexions.

- **Exclude**

  The trajectories indicated here will be excluded from the proposed ones.

Note: the propositions will be accurate only if the trajectories are well done (i.e. they correspond to only one particle)…

Format of detection and tracking results

The analysis will create four folders in the data directory to save the results in text files or MatLab format files (.mat):

1) **pk folder**: text files with the information about the peaks that were detected (all of them, independently of cut-offs).

The format of this files (*name*.pk) is:

1.0000000e+000	1.8499393e+002	7.5884616e+001	2.4469606e+000	1.6107000e+005	5.0813681e+003	4.3407125e-002	4.
1.0000000e+000	1.5381968e+002	1.5974734e+002	2.3305483e+000	4.9349747e+004	4.5186872e+003	5.8951635e-002	5.
1.0000000e+000	1.1011960e+002	9.1221703e+001	2.4784089e+000	5.1832693e+004	5.2278425e+003	7.2535086e-002	7.
1.0000000e+000	1.3310520e+002	6.1945936e+001	2.5208663e+000	5.7287578e+004	4.9888592e+003	6.5745706e-002	6.
1.0000000e+000	9.9087740e+001	9.7623393e+001	2.5028885e+000	6.4257246e+004	5.1201365e+003	7.0970094e-002	7.
1.0000000e+000	4.6111851e+001	1.4902105e+002	2.3501667e+000	5.0157373e+004	5.6509336e+003	8.7209555e-002	8.
1.0000000e+000	1.2904919e+002	1.0662387e+002	1.9950019e+000	5.2288149e+004	5.5859894e+003	6.2674369e-002	6.
1.00(							
1.00(							
1.0000000e+000	1.3452157e+002	2.8116747e+001	2.6033936e+000	9.7303521e+004	5.5317687e+003	6.4074832e-002	6.
1.0000000e+000	1.4582354e+002	1.0520250e+002	2.7021176e+000	1.0655700e+005	5.2480803e+003	5.0535650e-002	5.

Image # x position y position size (pixels) intensity offset …..

2) **trc folder**: text files with the information about the trajectories.

There are several .trc files, depending on the different steps of the analysis that were performed.

Initial tracking : *name*.trc

1.0000000e+000	1.0000000e+000	1.5381968e+002	1.5974734e+002	1.9923065e-001
1.0000000e+000	2.0000000e+000	1.5390920e+002	1.5970688e+002	1.9902105e-001
1.0000000e+000	3.0000000e+000	1.5404362e+002	1.5970862e+002	1.9931722e-001
1.0000000e+000	4.0000000e+000	1.5398947e+002	1.5976557e+002	1.9925981e-001
1.0000000e+000	5.0000000e+000	1.5407786e+002	1.5973974e+002	1.9938376e-001
1.0000000e+000	6.0000000e+000	1.5401896e+002	1.5974573e+002	1.9800292e-001
1.0000000e+000	7.0000000e+000	1.5425990e+002	1.5977814e+002	1.9771094e-001
1.0000000e+000	8.0000000e+000	1.5450888e+002	1.5968371e+002	1.9281971e-001
1.0				
1.0				
2.0000000e+000	1.0000000e+000	1.3310520e+002	6.1945936e+001	1.9910794e-001
2.0000000e+000	2.0000000e+000	1.3321505e+002	6.1895810e+001	1.9945456e-001
2.0000000e+000	3.0000000e+000	1.3320545e+002	6.1871880e+001	1.9905264e-001
2.0000000e+000	4.0000000e+000	1.3326024e+002	6.1989354e+001	1.9899402e-001

molecule # image # x position y position ….

Reconnection : *name*.con.trc

1.00	1.00	153.81968000	159.74734000	0.19923065
1.00	2.00	153.90920000	159.70688000	0.19902105
1.00	3.00	154.04362000	159.70862000	0.19931722
1.00	4.00	153.98947000	159.76557000	0.19925981
1.00	5.00	154.07786000	159.73974000	0.19938376
1.00	6.00	154.01896000	159.74573000	0.19800292
1.00	7.00	154.25990000	159.77814000	0.19771094
1.00	8.00	154.50888000	159.68371000	0.19281971
1.00	9.00	154.00512000	159.81614000	0.19728599
1.00	10.00	154.28252000	159.71045000	-1.00000000
2.				
2.				
2.00	4.00	133.26024000	61.98935400	0.19899402
2.00	5.00	133.15708000	61.89707000	0.19823971

Mol # image # x pos y pos ….

3) **traj folder**:

The files with the extension .traj are databases in MatLab format. They contain several variables of ‘structure’ type. That is, they contain several ‘fields’. These are some of them:

- Variable ‘source’: contains the fields (within others)
  - chemin: data directory
  - fichier: movie file

- Variable ‘recadrage’:
  - Nz: number of frames

- Variable ‘information’:
  - Te: integration time (acquisition time) in ms

- Variable ‘parametres.physiques’:
  - parametres.physiques.NA = numerical aperture of the objective
  - parametres.physiques.lambda = wavelength
  - parametres.physiques.a = pixel size (nm)
  - parametres.tracking.Dini = Initial D for tracking

- Variable ‘fit’: all the information about trajectories
  - spot: trajectories from the initial tracking. Contains the fields:
    - 'nb_points'
    - 'nb_segments'
    - 'segment':
      - ‘coordinates’: x, y, frames
      - ‘length’: number of points

The code corner:

-for each *numero* of particle (trajectory) we have:
`fit.spot(numero).nb_points`: number of points of the trajectory
`fit.spot(numero).nb_segments`: number of non-blinking periods

-for each *num_seg* non-blinking period:
`fit.spot(numero).segment(num_seg).coordinates`=[x y frame];
`fit.spot(numero).segment(num_seg).length`=number of points

  - nb_spots: number of trajectories
  - new_spot: linked trajectories.
    - 'spot': with the information of linked trajectories
      - 'nb_points'
      - 'nb_segments'
      - 'segment'
      - 'localisation': with fields
        - ‘coord’ (x, y, frame, localization),
          The localization is
          ‘0’: extra-domain or
          even number: inside the domain
          odd number: inside the peri-domain
        - ‘perisyn’: size of peri-domain ring
        - ‘nb_points’: number of points
    - 'nb_spots'
    - 'methode'