Fodis

A Software for Single Molecule Force Spectroscopy

User Guide

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Preface

Fodis (for "Force-distance software") is a program for the analysis of Single Molecule Force Spectroscopy data. It is mainly intended for automated analysis of force-distance curves of Protein Unfolding Events obtained during Atomic Force Spectroscopy experiments. However, it can be used for visualization and organization of any force-distance curve. Fodis provides a large number of force-curve visualization option, including function for basic statistical analysis. Since the developers are active AFM users, the program also contains some specific data processing methods intended for protein unfolding studies. Fodis is Free and Open Source software, covered by CC Public License. It was born in *Matlab* and we encourage third-parties to participate in developing functions and modules. Being a free software, it provides the source code to developers and users, which makes easier both the verification of its data processing algorithms and any further program improvements. Fodis was written in *Matlab 2016b*.

Installation

Fodis can be downloaded from `https://github.com/nicolagalvanetto/Fodis`. Users need to

- unzip Fodis folder and move it to the Matlab `Current Folder`
- right clik on the folder and select `Add to path folder and subfolders`
- type `Fodis` in the Command Window and `Enter`

Fodis requires *Matlab 2016b* of newer versions. It also requires:

- Signal Processing Toolbox
- Statistics and Machine Learning Toolbox
- Image Processing Toolbox

Non-Matlab users working on Windows, Mac or Linux need to launch the installer of their own version and follow the instructions. Please read the `README.txt` file before installation.

For any problem related to the software, please use GitHub issue section `https://github.com/nicolagalvanetto/Fodis/issues`.

Nomenclature

TSS	Tip Sample Separation: distance between the sample and the tip
Lc	Contour Length: length of the stretched polymer (segment)
Fc	Force: always referred in combination with *Lc*
F-d	Force-distance (curve): a SMFS trace
p	Persistence length
GHM	Global Histogram of the Maxima
CNG	Cyclic Nucleotide Gated channel

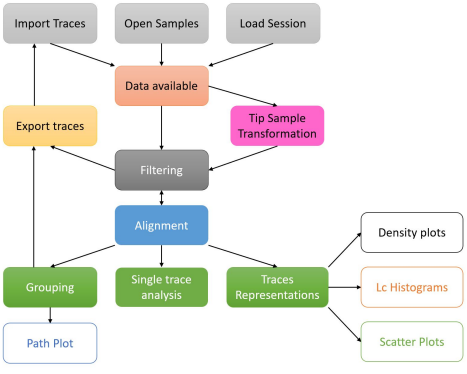
Chapter 1

Basics

In this chapter we present the basic working rules of the software and how to use it.

1.1 Software Framework

The general architecture of Fodis and the relationships among the various *blocks* is shown in this flow chart.



An ideal working session is here presented. After the collection of 1000 Force-distance curves the user imports these curves in Fodis.

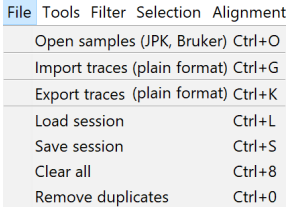
The gray boxes represent the **input options** (described in the section 1.2 and 1.3: the data are imported *as they are*.

If the imported data are in Volts (rather than in Newton) or in the absolute height (rather than in Tip Sample Separation(TSS)), it is possible to transform the force and position units with the **transformation module** (section 1.4). Then, the traces can be **aligned**, **filtered** and **analyzed** with all the tools available in

Fodis. Imagine a set 1000 consecutive Force-distance curves from a purple membrane patch (like in the famous experiment by Oesterhelt et al. [1]). The dataset contains many flat curves (without unfolding peaks), some traces with only non-specific adhesion, some traces with random peaks and some traces with real bacteriorhodopsin unfoldings. The basic filtering tool (section 1.9) and the selection through correlation (section 2.4) can be used to refine the selection of traces and go from 1000 to 10-20 traces. The remaining traces can be aligned with the manual alignment (section 1.11) or the automatic alignment (section 2.3). The user can then finalize his analysis with the remaining tools, focusing on single traces or to population statistics.

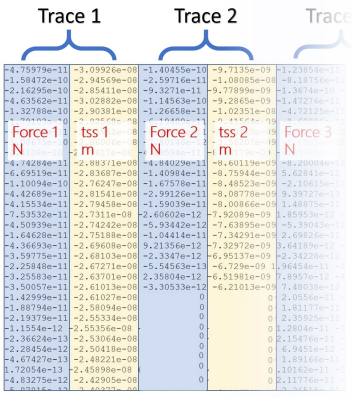
1.2 Open data

There are three modalities to import data and start working with Fodis.



- `Import traces` uploads matrices of numbers. This is the most universal import option in Fodis (see example below).
- `Open samples` uploads folders containing multiple files of single traces. The format allowed is listed in the GUI menu.
- `Load session` uploads .afm files previously generated by Fodis through `Save session`. These files contain not only the saved dataset, but also the specific configuration and parameters set in the previous working sessions.

1.2.1 Import traces (.txt files in Plain Format)

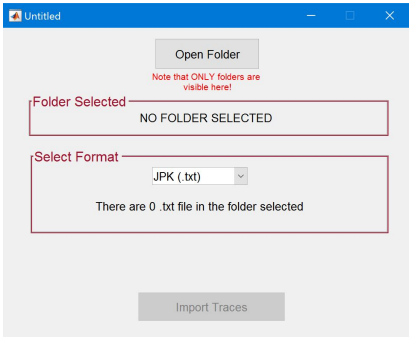


To import *.txt* files follow these rules:

- The .txt file must be organized in columns. The first column contains the force values of the first trace in Newton, the second column contains *tip-sample-separation* values (or polymer extension) in meters, and so on.
- Columns are space-separated.
- By convention, Fodis keep repulsive forces *negative*, attractive forces *positive*.
- If different traces have different number of points, the number of rows of the *.txt* will be equal to the number of point of the bigger trace. Zeros must be added at the end of short traces to fill the gap (see Figure).

N.B. If the user loads single traces (one couple of columns) with different number of rows, Fodis will manage the data anyway. The exported file will be created following the aforementioned rules.

1.2.2 Open samples



Open-sample user interface is designed to help the user uploading JPK or Bruker files. First select the desired format on the `Select format` menu and then `Open Folder`. An example of the various files can be found in *additional datasets*.

- **JPK .txt**: ASCII exported .txt files from JPK Data processing
- **JPK .jpk-force**
- **Bruker .txt**: with or without Header, ASCII exported in *Display* Units (Fodis reads the metric prefix of the units)
- **Bruker .spm (only for Windows in non-deployed versions)**: Bruker raw files cannot be decoded without their proprietary .dll libraries compiled in user's machine.
  Fodis can open .spm and .xyz (.084, .017, ...) files, but it requires some additional installation steps.

  1. Install Microsoft Windows SDK (Software Development Kit) `https://www.microsoft.com/en-us/download/details.aspx?id=8279`.
  2. Install the Matlab compiler MinGW-w64: from Matlab Home > Environment > Addons and search MinGW-w64 C/C++ Compiler or download it from `https://it.mathworks.com/matlabcentral/fileexchange/52848-matlab-support-for-mingw-w64-c-c++-compiler`
  3. Install Nanoscope
     64 bit: `ftp://anonymous@sboftp.bruker-nano.com/outgoing/GPTech/Software/NanoScope_Analysis_x86_v180r2sr3.exe`
     32 bit: `ftp://anonymous@sboftp.bruker-nano.com/outgoing/GPTech/Software/NanoScope_Analysis_x64_v180r2sr3.exe`

4. Install the AFM-Toolbox (chose custom and your Windows version) `ftp://anonymous@sboftp.bruker-nano.com/outgoing/GPTech/Software/AFM_MATLAB_Toolbox_Setup_for_NanoScope_Analysis_v180r2sr3.exe`
   and follow the instruction to add the folder to the path.

For more information please contact `fodis.help@gmail.com`, and for newer versions of Bruker's software contact `support.afm@bruker.com`.

1.3 Data saving

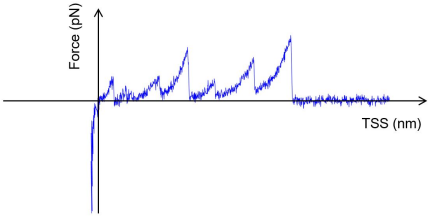
Any time, during a working session it is possible to save the data in two different modalities (`File > ...`):

- `Export traces` generates a .txt of the same format presented in the `Import traces` section. It is highly recommended to use this format for portability reasons.
- `Save session` generates a .afm file that can be uploaded only by Fodis through the `Load session`. These files contain not only the current dataset, but also the specific configuration and parameters set during the working session.

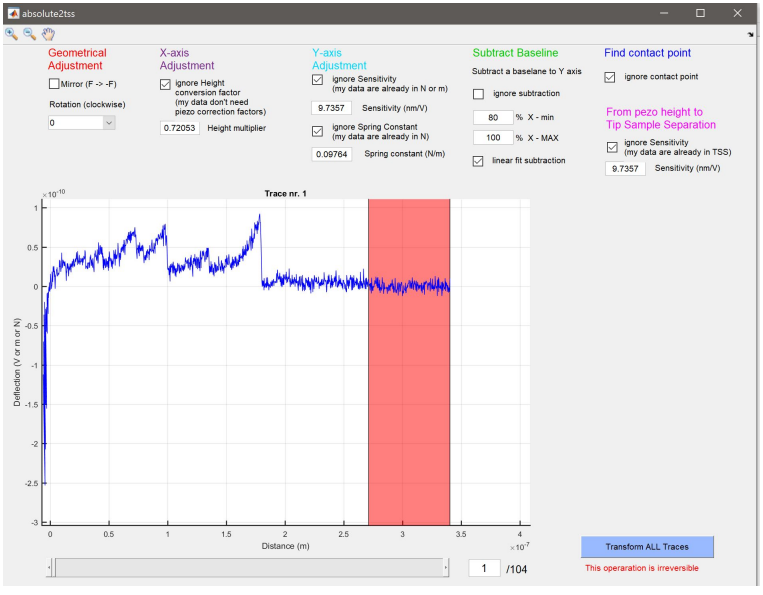
In case only partial information is needed, it is possible to Export plots or Excel tabels (`Tools > Export ...`):

- `Export plot` generates a .fig, or any other file format for figures of the current view.
- `Export Excel` generates a .xls file containing the list of selected traces and the relative Force and Positions of the peaks. (Due to Matlab-Excel poor compatibility this process is slow).

1.4 Data pre-processing



To correctly analyze a Force-distance curve in Fodis, it must have in the x-axis the Tip Sample Separation in meters (TSS) with the contact point on the left, and in the y-axis the force in Newton (baseline corrected).



The imported data may not be ready for the analysis (wrong units, TSS transformation needed, etc). To pre-process the curves please use this module that allows to perform 6 different type of transformations (go to `Tools > Pre-Processing (shift, TSS, Baseline)`). By default, no transformation is active, but a message pops up suggesting the necessary transformations that needs to be performed.

1. **Geometrical Adjustment**: the mirroring option inverts the values of the Force. The rotation option swaps the coordinates.
   N.B. the curves must have the contact point on the left, and the unfolding forces positive — tip-sample contact negative (as shown in the figure).

2. **X-axis Adjustment**: the x-axis values are multiplied by the constant Height multiplier (which is automatically read in the import files when present). This is necessary when the imported files contain only the non-corrected Height channel (e.g. in JPK files).

3. **Y-axis Adjustment**: if the vertical deflection of the cantilever is imported in Volts, the curves need to be rescaled with the Sensitivity constant. If the vertical deflection of the cantilever is imported in meters, the curves need to be rescaled with the Spring constant. (The constants are automatically read in the import files when present).

4. **Subtract Baseline**: if the Force values need to be vertically shifted, this option finds the baseline averaging the final part of the curve (red patch). The starting point and the ending point can be changed in the edit boxes. The **linear fit subtraction** interpolate the final part and allows to tilt the curve (Warning: curves with peaks within the selected region will be shifted in an inconsistent way).

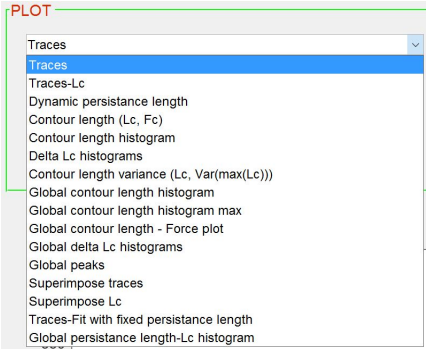
5. **Find contact point** finds the first positive value (starting from the left).

6. **From piezo height to TSS**: it transforms the x-coordinate in tip sample separation (TSS). (The constant is automatically read in the import files when present).

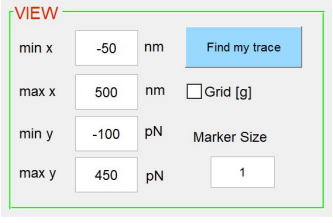
N.B. These transformations act on all `Valid` curves (see section 1.8). Mark the curves as `Not Valid` to skip the transformations.

1.5 Single trace representation - Plot data

In this section we present all possible trace representations of single events implemented in Fodis.

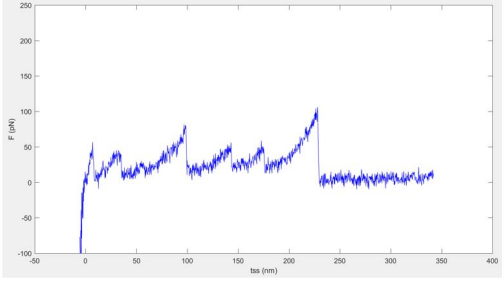


The plot-box at the top-left corner controls the graph displayed in the central panel. There are two major groups among these graphical representations: single trace representations and global representations of all selected traces.

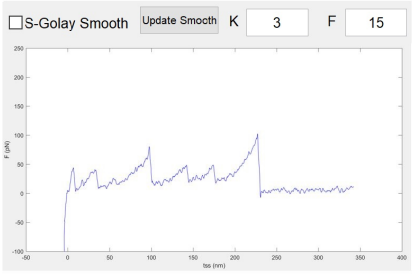


In the view-box, all the parameters necessary to control plotting range, marker size and grid are present. To perform `Export plot` click on `tool` in the menu bar: this creates a Matlab figure containing all the objects present in the Fodis graphic panel.
N.B. Use the button `Find my trace` in case no trace is visible in the screen.

1.5.1 Traces



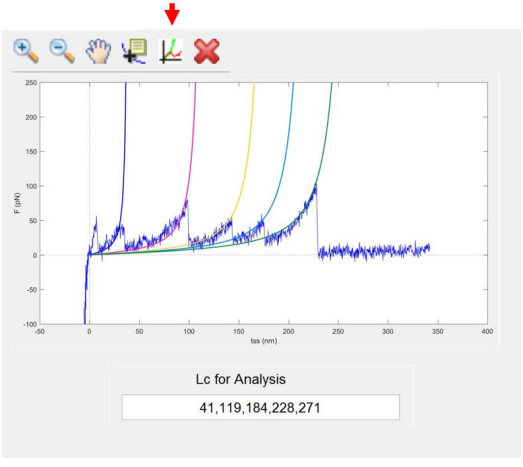
Typical *Force-tip sample separation* curve: exact representation of input numbers. The slide bar at the lower part of the panel allows to move across different loaded traces.



Trace smoothing performed through a discontinuity-preserving filter (Savitzky-Golay algorithm).
`K` is the polynomial order ot the interpolant curve while `F` is the number of points used for interpolation (`F` must be an odd number).

The smoothing acts on the trace shown in the screen when the check-box is checked, after clicking the button `Update Settings`. The button `Update Sel` performs the soothing onto all `Selected` curves (see section 1.8). To remove the smoothing, uncheck the check-box and `Update Settings`.

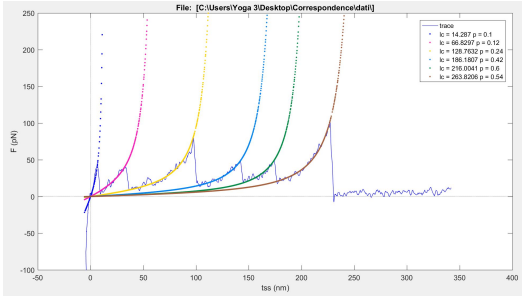
1.5.2 Traces-Lc (Peaks automatic detection)



`Traces-Lc` finds relevant peaks and fits them with the WLC equation. *Peak detection is enhanced on smoothed traces.* The numbers reported in `Lc for Analysis` are all detected contour lengths (in *nm*) of plotted rainbow curves. In case a peak is not detected, type the number in the edit box and then `Enter` or click the button (red arrow) then click the peak in the graph, and then `Enter`. That new Lc will be saved for all the following analyses.

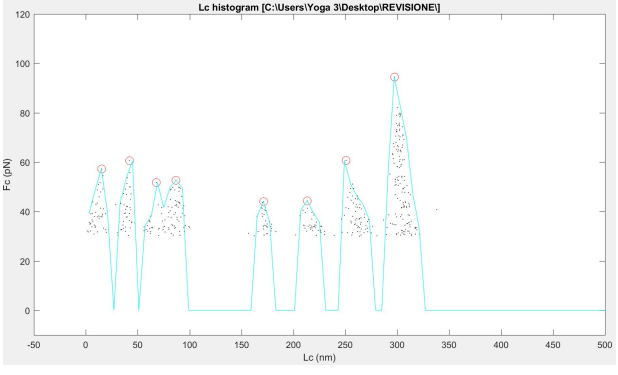
N.B. The detection of the peaks is based on the Force-Lc profile shown in section 1.5.4 (cyan line). The profile can be tuned acting on the parameters `Threshold N points` and `Min peak proximity` of the parameter box (section 1.7.2) to enhance peaks detection.

1.5.3 Dynamic persistence length



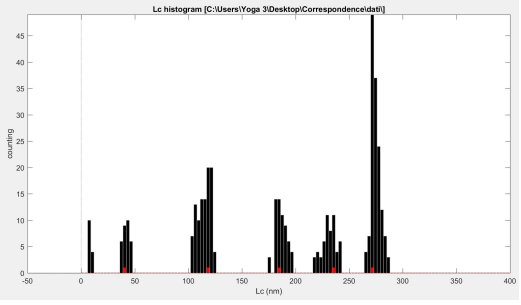
The persistence length, which is typically kept fixed (p=0.4nm for a.a. chains), in dynamic mode is relaxed to find the best p-Lc couple for each peak. The final parameters are plotted in the legend.

1.5.4 Contour length (Lc,Fc)



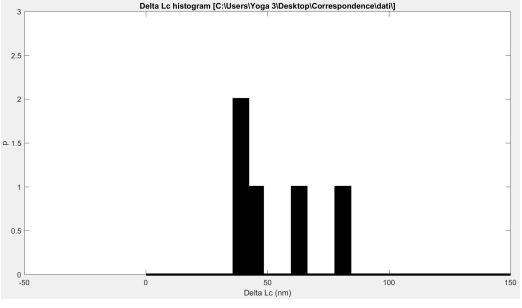
This representation transforms every point above a threshold force (in this case 30 pN, tunable in Parameters box `Min F`) from F-tss space to F-Lc space according to the transformation presented by Puncher and colleagues [2, 3].
N.B. The detection of the peaks is based on the Force-Lc profile shown (cyan line). The profile can be tuned acting on the parameters `Threshold N points` and `Min peak proximity` of the parameter box (section 1.7.2) to enhance peaks detection.

1.5.5 Contour length histogram



This is the histogram of `Contour length (Lc,Fc)` representation. It is also called Barrier position histogram [2]. The red bars represent the detected peaks. Bin width can be tuned in the Parameters box.

1.5.6 Delta Lc histograms

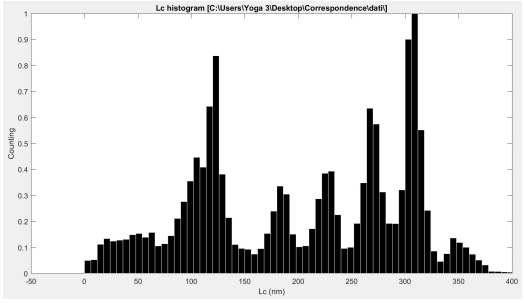


`Delta Lc histograms` shows the value of the difference in Lc between two consecutive peaks. The first peak's Delta-Lc, by definition, is equal to the first Lc.

1.6 Global trace representation

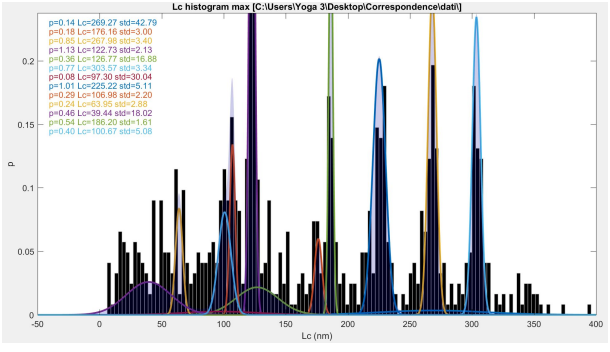
Single molecule force spectroscopy experiments generate a large amount of data. Single trace representations are useful to establish the quality and features of single events, but only a statistical evaluation of Lc distribution or other specific features allows to perform a quantitative analysis. For this reason, Fodis includes a variety of global representations for the population of traces.

1.6.1 Global contour length histogram



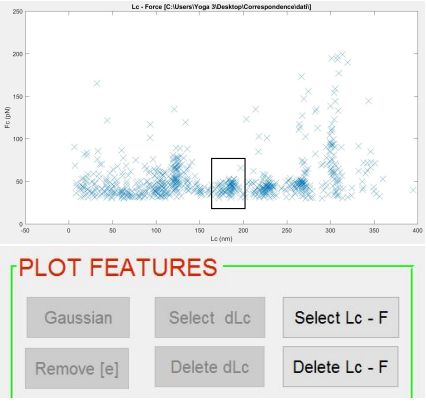
The `Global contour length histogram` is the sum of all `contour length histograms`, normalized on the highest bin. Bin size can be tuned in the Parameters box.

1.6.2 Global histogram max



The `Global histogram of Maxima` (also called *Contour length histogram* by Kawamura and colleagues [4]) counts the detected peaks of all selected traces (*i.e.* red bars shown in `Contour length histograms` section). The resulting distribution can be fitted with the Gaussian Mixture Model (GMM) checking `Tools > Automatic multi Gaussian` option on the menu bar. This representation is useful to determine the probability of a certain unfolding event happening along the Lc coordinate. (Bin size can be tuned in the Parameters box).

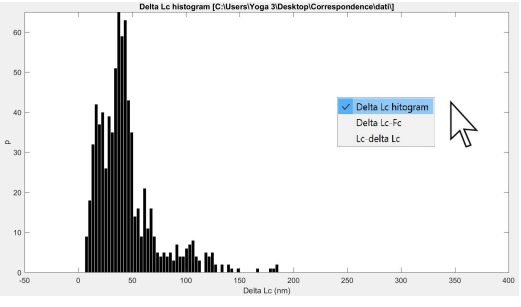
1.6.3 Global contour length - Force plot



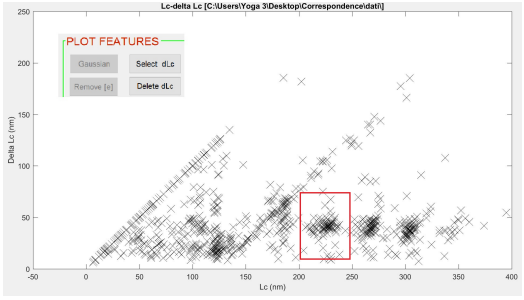
The *Lc - Force plot* shows the contour length *vs* Force of all detected peaks. This plot can be used also to detect the detachment force in binding experiments (e.g. with antibodies).

If the user is interested on the force distribution of a particular subgroup of peaks (i.e. black rectangle), using `Select Lc-F` button it is possible to select a region (draw a rectangle and double click inside de area). A force distribution of the selected points will be automatically generated.

1.6.4 Global delta Lc histograms (and plots)

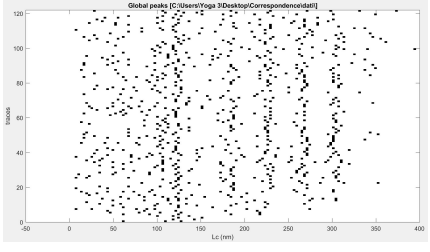


`Delta Lc` is defined as the Lc difference between two consecutive peaks. On the left, histogram of all Delta Lc values of the population.
N.B. Right clicking on the graphs allows the user to change the view to `Delta Lc-Fc` or `Lc-Delta Lc`.



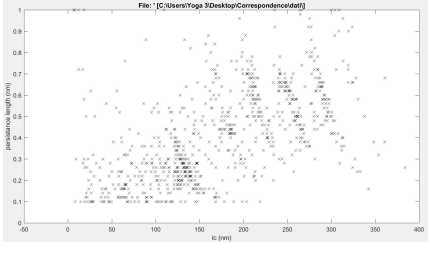
The `Lc-Delta Lc` plot shows the scatter plot of all detected peaks. If the user wants the `Delta Lc` distribution of a particular subgroup of peaks (i.e. red rectangle), using `Select dLc` button it is possible to select a region (draw a rectangle and double click inside de area). A `Delta Lc` distribution of the selected points will be automatically generated.

1.6.5 Global peaks (or Global Matrix)



Automatically detected peaks are used to form a string of ones and zeros for each trace (zeros where there are no peaks, ones where there are a peak). It is a sort of binarized coding for the traces. Each string is therefore plotted one above the other in `Global peaks` with white pixels for zeros and black pixels for ones.

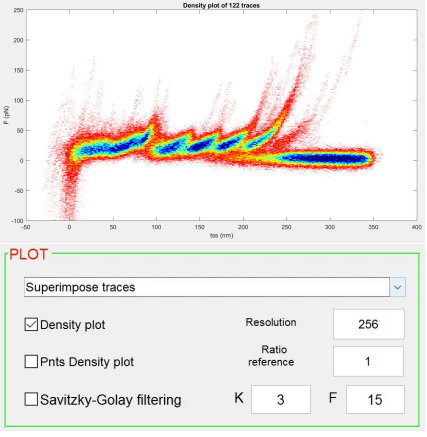
1.6.6 Global persistence length



All the peaks of the population are fitted with two free parameters - persistence length (p) and Lc as described in `Dynamic persistence length`- and plotted. This allows to find correlations between the peaks positions and their curvature(*i.e.* p).

1.7 Graphical features

1.7.1 Density plots



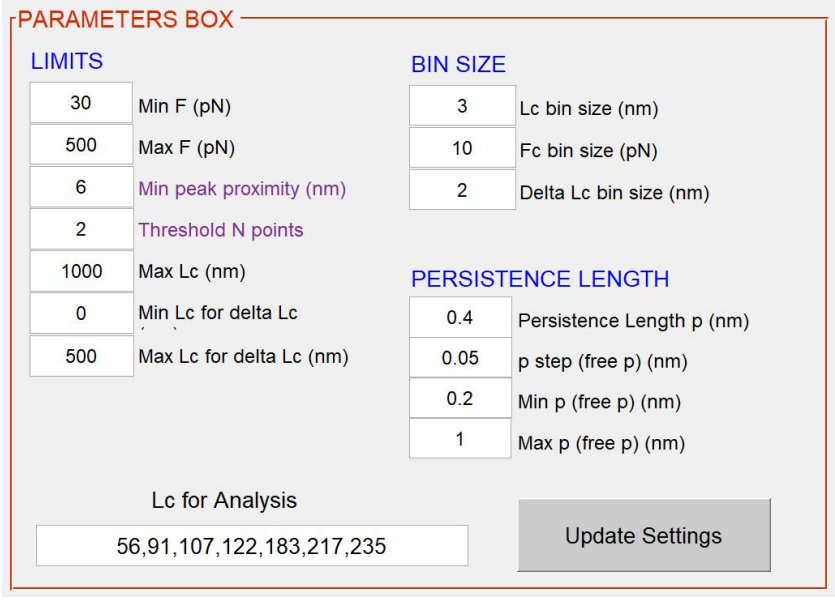
A heat map is plotted when the `Density plot` check box is selected. The image is constituted by pixels (`Resolution`) and the color of the pixel is proportional to the number of points contained in the pixel area. `Ratio reference` changes the heat map color limits. The `Points Density plot` highlights each point with the color of the corresponding pixel. Density plot is available for:

- `Superimpose Traces`
- `Superimpose Lc`
- `Global delta Lc histograms (Lc - Delta Lc)`

1.7.2 Parameters Box

Many parameters can be set to tune both graphical views and threshold limits (parameters not described here will be discussed later).

- `Min F`: minimum value of force valid for peaks detection or Lc evaluation;
- `Max F`: maximum value of force valid for peaks detection or Lc evaluation;
- `Min peak proximity`: minimum interval for automatic peaks detection (it tunes the sharpness of the cyan line in `Contour length (Lc,Fc) plot`, section 1.5.4);



- `Threshold N points`: parameter related to peaks detection. The automatic peaks detection operate on the `Contour length (Lc,Fc) plot` (section 1.5.4). N is the number of points that are removed from each bin (`Lc bin size`) after the transformation in Lc-Fc, to reduce the detection of spurious and isolated peaks caused by noise fluctuations.
  N.B. This number highly depends on the sampling rate of the curves. A heuristic rule could be to chose this value proportional to the total number of points of the curve:

$$Threshold\,N\,points \sim \frac{Total\,Points\,Number}{1000} \quad ;$$

- `Max Lc`: maximum value of Lc taken into account;
- `Min Lc for Delta Lc`: minimum value of Lc taken into account for Delta Lc plots;
- `Max Lc for Delta Lc`: maximum value of Lc taken into account for Delta Lc plots;

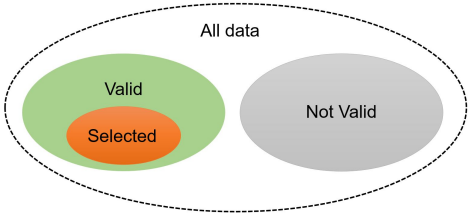
- `Lc bin size`: bin width of Lc histograms or Global Lc representations;
- `Fc bin size`: bin width of Force histograms or Global Force representations;
- `Delta Lc bin size`: bin width of `Delta Lc histograms` or `Global Delta Lc representations`;
- `Persistence Length p`: fixed value of persistence length for Lc evaluation;
- `p step (free p)`: minimum step when evaluating free persistence length (`dynamic persistence length`);
- `Min p (free p)`: minimum value for `dynamic persistence length` algorithm;
- `Max p (free p)`: maximum value for `dynamic persistence length` algorithm;
- `Lc for Analysis`: detected Lc values of a current trace (they can be edited);
- `Update Settings`: after parameters changes, click this button to update displayed results.

1.7.3 Export plot

Every plot can be extracted and saved individually in Matlab format .fig, or any other file format for figures, from the menu bar `Tools > Export plot`.

1.8 Selected & Valid Traces

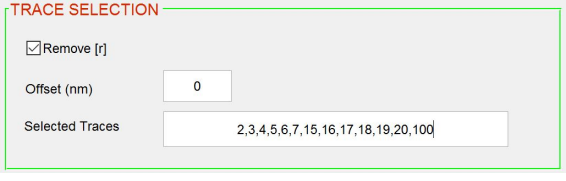
Loaded traces have a *visibility label*. There are only three possible labels:



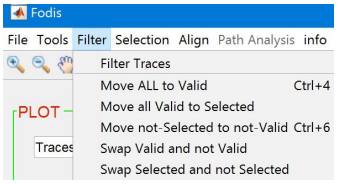
- `Selected`
- `Valid`
- `Not Valid`

By default, loaded traces are all `Selected`. Global representations act on `Selected` (that is a sub-group of `Valid`). All `Valid` traces are displayed moving the slide bar. `Not Valid` traces are not shown. Filtering procedures described in the next section and chapter, filter out traces labeling them as `Not Valid`.

Details Box shows the numbers:
85 `Selected`,
101 `Valid`,
122 `Valid` plus `Not Valid`, i.e. all loaded data.



Trace Selection Box allows to edit the list of `Selected` traces. The `Remove[r]` check box removes the current trace from the selection (to `Valid` but `not Selected`). `Offset` add a shift to all selected traces. `Selected Traces` edit box lists the indexes of `Selected` curves. It allows also, by changing the list, to add or to remove traces from `Selected` group.



In the Filter menu bar the user can manage the *visibility labels* of loaded data:

- `Move ALL to valid`: all `Not Valid` are labeled `Valid`;
- `Move all Valid to Selected`: all `Valid` are labeled `Selected`;
- `Move not-Selected to not-Valid`: all `Valid` but not `Selected` are set `Not Valid`;
- `Swap Valid and not Valid`: all `Valid`, therefore also `Selected`, are labeled `Not Valid`, and vice versa;
- `Swap Selected and not Selected`: all `Selected` are labeled `Valid`, and vice versa; `Not Valid` remains unchanged.

1.9 Trace filtering: basic tool

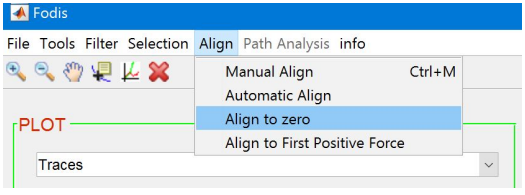
SMFS experiments typically generate a large amount of data. In this section we present a basic filtering tool that allows the user to select traces upon two characteristics: points position in F-tss space and peaks position. Traces that do not satisfy desired requirements will be labeled as `Not Valid`.

The top panel displays single traces. On this panel, the user can draw two kinds of rectangular ROIs: mandatory regions (green) and forbidden regions (red). Green ROIs define Force and tss intervals in which at least one point of the trace must fall into, otherwise the trace is filtered out. Red ROIs define Force and tss intervals in which no point of the trace can fall into, otherwise the trace is filtered out. The ROIs are equal for all the traces. To draw a ROI, the user needs to click on `Draw Rectangle ROI` and then move on the panel and click&drag; to erase a ROI, just click `Erase ROI`. Multiple ROIs can be drawn.
The bottom panel displays the single contour length histograms in red and the detected peaks in black. Color limits are set by the four edit boxes on the right. The Blue section defines the area in which at least one peak of the trace must fall, otherwise the trace is filtered out. The Pink section defines the area in which the last peak of the trace must fall, i.e. no peaks on higher Lc values, otherwise the trace is filtered out.
N.B. We suggest to perform this filtering procedure with traces smoothed with Savitzky-Golay algorithm in order to enhance peaks detection.
Once all the desired limits have been set, `Compute Filter` finds the traces

to be removed and `Update Valid Traces` updates the `Not Valid` group.

1.10 Align to `zero`

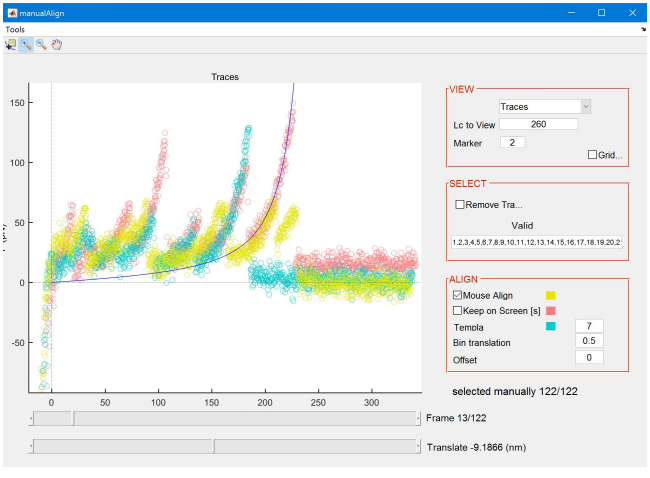
Loaded traces can be all aligned to a zero position. There are two `zero` definitions:



- `Align to zero`
- `Align to first positive force`

`Align to zero` finds the average value of tss corresponding to negative forces between -200pN and -400pN, and translates each trace accordingly. Sometimes this procedure may not work because the contact phases are not homogeneous: in this case `Align to first positive force` sets to zero the tss corresponding to the first positive value of Force of each trace.

1.11 Manual Alignment



Manual Alignment tool (accessible from the menu bar `Align > Manual alignment`) allows the user to slightly refine the relative shift among valid traces, typically caused by misalignment of the base line. The graphical panel shows three traces of different colors:

- Yellow - chosen trace, its number is visible in the `Frame` text box;
- Green - template trace, editable in the `Template` edit box;
- Pink - trace(s) saved on screen, `Keep on screen` check box.

Traces can be moved only horizontally, both with the Translate slide bar and clicking&dragging on the graphic panel. Only chosen trace (yellow trace) can be translated. To move across valid traces, use Frame slide bar. `Select box` contains all the information related to Selected traces, the chosen trace can be removed editing the selected edit box or by clicking on `Remove trace` check box. `View box` allows to decide the trace representation and to set an Lc of reference.

Chapter 2

Advanced Tools

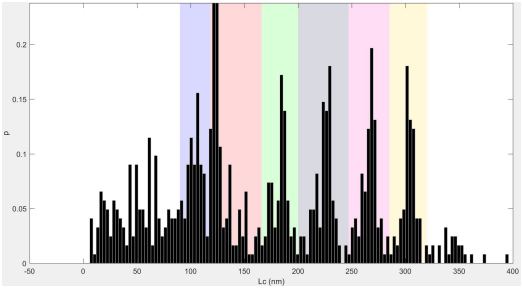
In this chapter we describe the tools implemented in Fodis. These tools were developed in order to solve some common issues in SMFS experiments, like clustering and alignment.

2.1 Unfolding Pathways Analysis

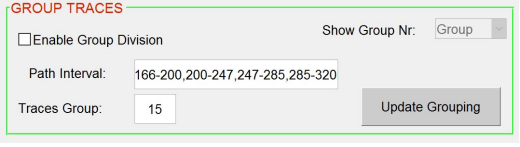
Given a population of traces that represent the unfolding of a certain protein, an important issue in SMFS is to determine *how* this unfolding happens. Global representations presented in Chapter 1, for instance, are very useful to generate statistics in an *aggregated* fashion. Here, with Unfolding Pathways Analysis we intend to present three representations that, in spite of being *global*, show unfolding pathways in a *disaggregated* fashion.

2.1.1 Group division

The first step in an Unfolding Pathways Analysis is to identify different groups of traces depending on the position of their force peaks.



The idea is to generate a graphical representation of the different unfolding pathways of a set of traces, and cluster them. Starting from Global Histogram of Maxima (GHM), we can divide the Lc coordinates in intervals (different colors).



The extremities of each interval can be set in the `Path Interval` edit box. Intervals cannot overlap.

There are two different ways to construct intervals:

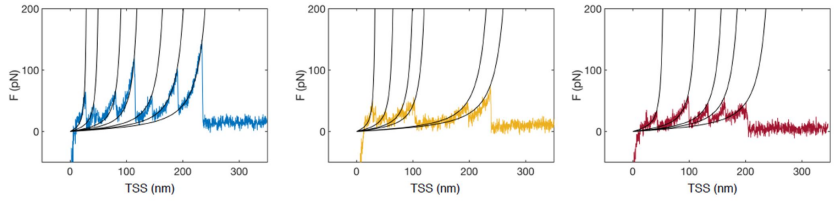
1. `min1-max1,min2-max2,...` in nm;

2. `N` where N is an integer: divide Lc coordinates in N equal intervals.

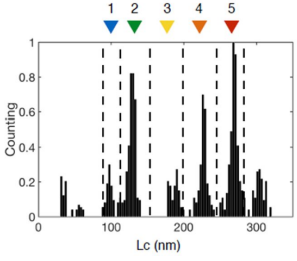
Once the intervals have been chosen, the user can update the group division by clicking on `Update Grouping`. Then checking `Enable Group Division` it is possible to move across different resulting groups with the menu `Show Group Nr.`
N.B. A good rule to prepare the intervals is to set the borders in the minima of the GHM.

2.1.2 Path Plot

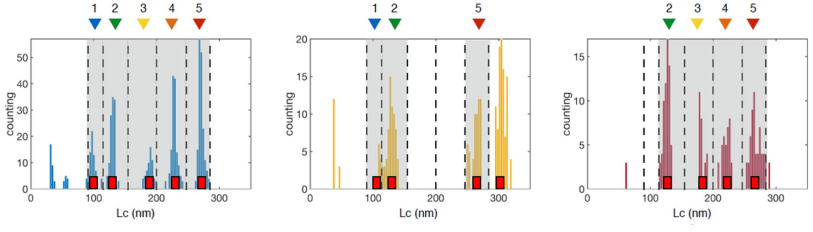
The *Path Plot* is a representation that clusters a population of traces depending on the position and on the number of Force peaks. Group division, and resulting *Path Plot* generation (`Path Analysis > Path Plot`), is based on the following 5 steps.



Traces are fitted with the WLC model, transformed into Lc histograms and grouped in the Global Histogram (or GHM).



The Global Histogram is divided into intervals according to the main ensembles of occurring events, to partition the Lc coordinates in regions with distinct maxima.



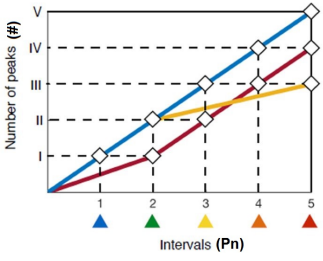
Following the aforementioned rule, in this explanatory panel, we selected 5 intervals 90-118,118-155,155-200,200-247,247-285nm.

	1	1	1	1	1
Pn =	1	2	3	4	5
# =	1	2	3	4	5

	1	1	0	0	1
Pn =	1	2			5
# =	1	2			3

	0	1	1	1	1
Pn =		2	3	4	5
# =		1	2	3	4

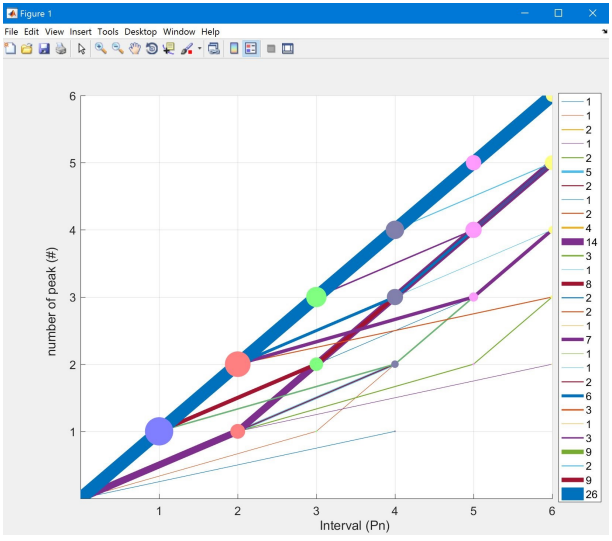
On the base of this division, each trace is coded in a binary string of 5 digits: 0 is assigned if no force peak is detected within the interval, 1 is assigned if at least one event is detected. From each string, we created two additional sequences: # and Pn. # is the sequence referred to the order of appearance of the force peak along the trace (in a trace with 2 peaks, the 1st peak has #=1 and the 2nd peak has #=2). Pn refers to the interval position occupied by a peak (a peak that falls within the 3rd interval has Pn=3).



Traces so coded are finally plotted as broken lines into an orthogonal #-Pn space, the line-width is proportional to the number of traces that follow the same path. This method provides a representation to distinguish different unfolding behaviors/clusters of a given set of traces, based on the number and position of occurrence of unfolding events.

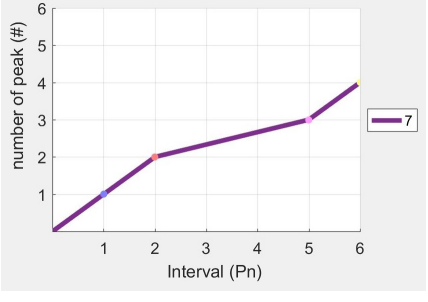
Path Plot of a larger population

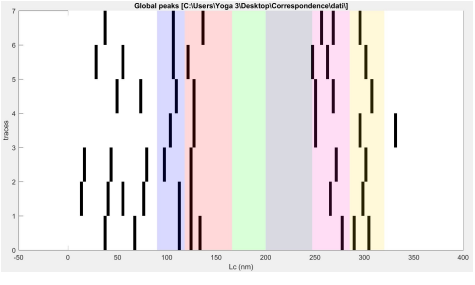
The previous example shows how the Path Plot is constructed, now we present how it looks when generated on a larger dataset (CNG dataset).

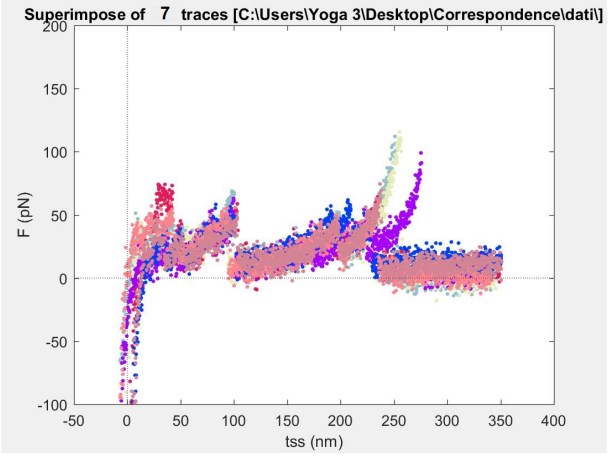


Each line width is proportional to the number of traces that cover that specific path. The size of the point at a node is proportional to the number of traces that pass through that node.

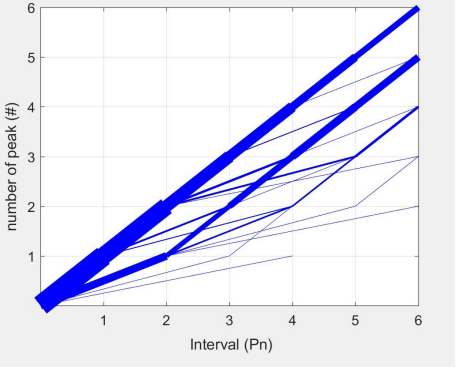
By `Enabling Group Division` we can select only one group, the 18th in this case. We can see the peaks distribution and relative Path Plot of 7 traces that has no peak in intervals 3 and 4, indeed, in the *Path Plot* representation, it does not have any node in the 3rd and 4th intervals.







2.1.3 Combined Path Plot

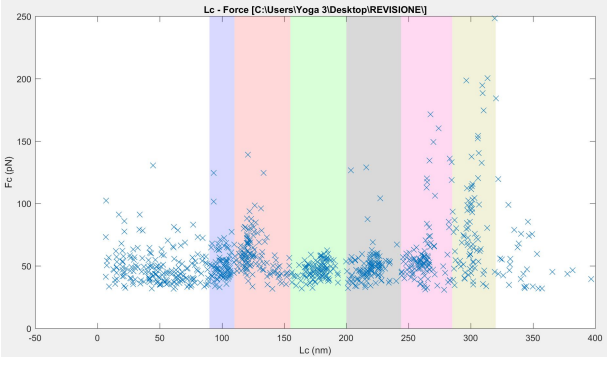


Similar to the Path plot, the *Combined Path Plot* displays the #-Pn space but with no color distinction. Each segment-width is proportional to the absolute number of traces that travel over that particular segment path.

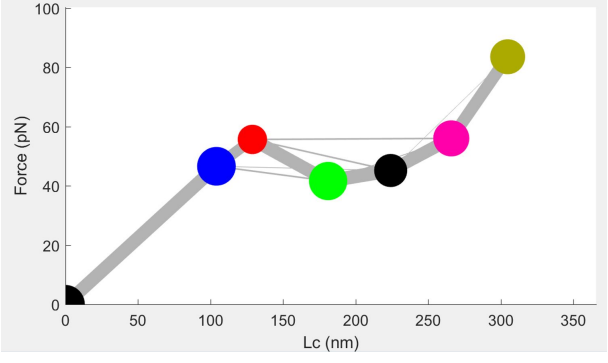
2.1.4 Force-Lc segmentation plot (Thoma et al.)

Thoma and colleagues, in 2017, presented a different Unfolding Pathways Analysis based on the **Contour length position** and **average Force** of Force peaks [6]. In this representation, the points coordinates

are the average Force and Contour length of each different class (intervals of different colors in the figure below); the segments connect the points showing the distribution of the unfolding pathways. The group-division implemented in Fodis is based on the partition shown in section 2.1.1. This representation can be generated clicking (`Path Analysis > Force-Lc segmentation plot`). The radius of the points is proportional to the number of Force peaks that are present in the relative intervals; the width of the segments is proportional to the number of Force-distance curves that show that specific point-to-point pathway.
N.B. This analysis is enabled only after setting the intervals (section 2.1.1).

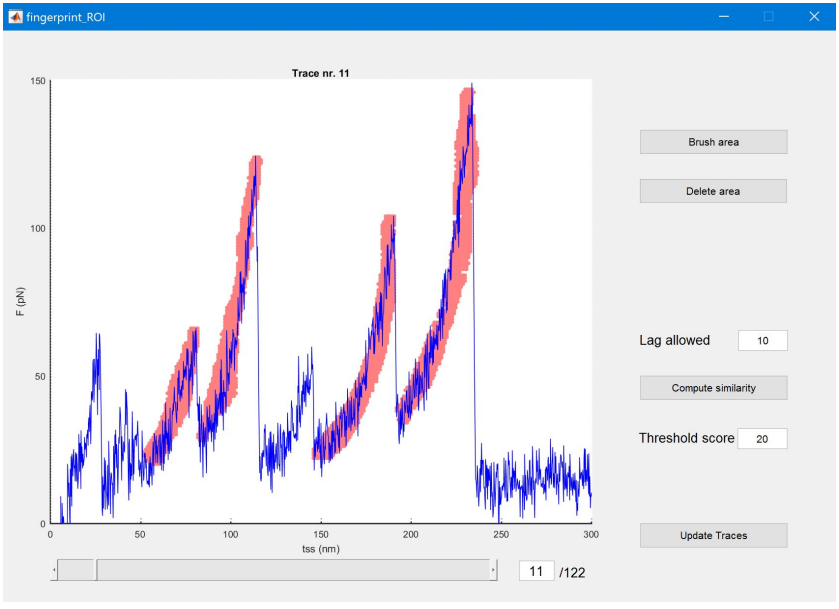


Global force plot and group division



Thoma plot relative to the above population of peaks

2.2 Fingerprint ROI



Fingerprint ROI is a tool intended to find specific pattern over the population of traces. It is accessible from the menu bar `Selection > Fingerprint ROI`.
This graphic interface shows the plot panel and the slide bar. The general idea behind this tool is to evaluate how many points of a trace fall into the drawn area (pink area). By clicking the `Brush area` button, move the mouse cursor on the graph and finally click&drag to draw a continuous ROI. The user can draw as many ROIs as needed just by clicking the button `Brush area` for any new ROI. The `Delete area` button deletes all ROIs previously drawn. Once all desired ROIs have been drawn, the evaluation can start. `Lag allowed` edit box sets how many nm each trace is shifted from its starting position. The maximum value of intersection between ROIs and a trace is saved and displayed with `Compute similarity`. The `Threshold score` edit box sets the threshold value of the score: the `Update Traces` button filters out all the traces that score lower than the threshold value and then they are available in the main Fodis interface.

2.3 Automatic Alignment

The Automatic Alignment (menu bar `Align > Automatic Alignment`) is a tool intended to solve the critical and highly operator-dependent issue of *traces alignment.*

Align to a reference trace

The `Align to this trace` option takes the contour length histogram of the current trace (the trace in the view box) and it calculates the cross correlation of all contour length histograms against the current trace. After this process, all the traces will be horizontally shifted to maximize the cross correlation against the current trace.

Reference-free Alignment

This tool is based on the work of Bosshart and colleagues [3], with some specific improvements developed to extend its applicability to more general SMFS datasets.
In particular, they proposed an algorithm consisting of 4 steps. Starting from the `contour length histogram` of every trace, they:

- subdivided the traces into groups of homogeneous traces (i.e. traces with the same number of peaks);
- recursively aligned traces into the same group with the maximum-correlation principle, building an average contour-length reference for each group;
- formed a *global reference*;
- aligned all the traces of the dataset to the *global reference.*

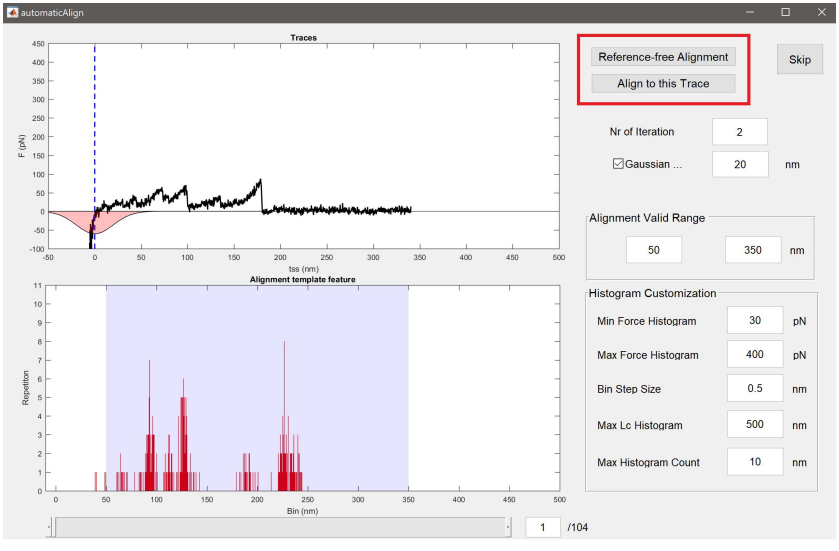
To this remarkable computational procedure, we added two features that help to manage a larger variety of traces that do not display regular occurrence of peaks.

1. In addition to the contour-length histogram, we assign to every trace a zero-point, that is the point of tip-sample contact. Given the correlation curve of two traces, we then multiply the correlation curve with

a Gaussian curve centered at the point in which the zero-points of the two traces match with each other (red Gaussian curve). The idea is to apply a "potential well" to reduce the maximum displacement of the two zeros.

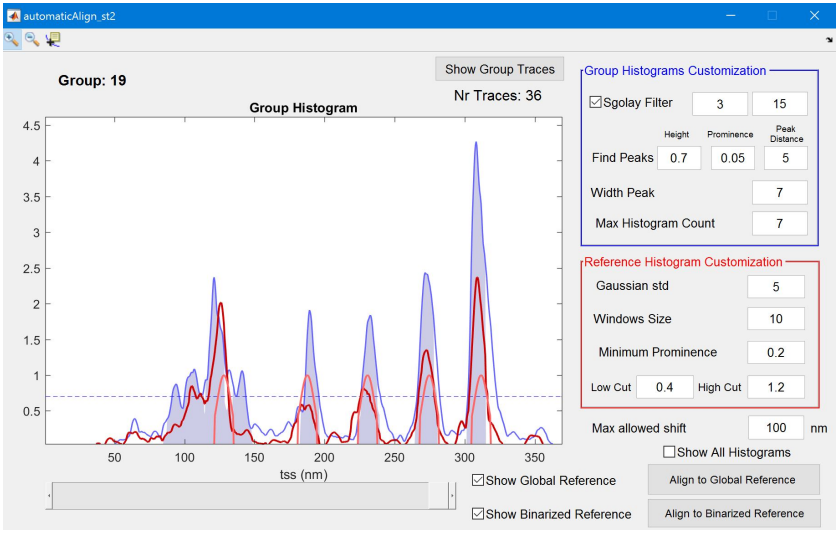
2. Group division proposed by Bosshart and colleagues works only if all the traces with the same number of peaks have the peaks in the same position, but this is not generally true for F-D curves of the same protein. Therefore, we used a group division following the method described in the *Path Plot* section. In this way, we imposed two constrains for a given trace to be part of a given group: to have a specific number of peaks and to have those peaks in a specific position.
   N.B. Group division is done as shown in *Path Plot* section, with custom or equally-spaced intervals.

The user performing Automatic Alignment (menu bar `Align > Automatic Alignment`) in *Fodis* is guided across the aforementioned steps with the help of two Graphic Interfaces.



Graphic interface 1 for *global reference* construction

The first interface helps the user to set all relevant parameters for the formation of the *global reference*. The `Histogram Customization` box is crucial to determine the shape of the histograms to be aligned. Default parameters are generally adequate, but in case of some specific requirements, the user is allowed to change them. The `Alignment Valid range` edit boxes set the limits over which histograms are set to zero. `Gaussian std.` sets the standard deviation of the Gaussian "potential" and `Nr. of Iterations` sets how many iterations the algorithm executes: iterations are time consuming, but only one iteration generally gives unstable results. Use the button `Align!` to execute the algorithm, `Skip` if first alignment has already been performed thus to go directly to the second Alignment interface.



Graphic interface 2 for *global reference* tuning

The scope of the second alignment step is to prepare the desired *global reference* (i.e. the reference histogram onto which every single histogram is finally aligned). Default parameters should be adequate, but for specific requirements, it is possible to change some settings.
The tuning process consists of:

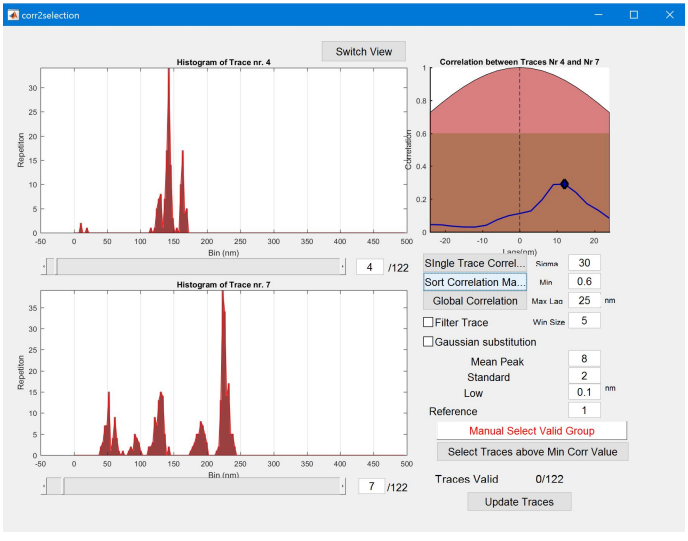
- tuning shape of the blue profiles (i.e. *group references*, resulting his-

tograms from alignment *intra*-group). This can be done in the `Group Histograms Customization` box moving through different groups with the slide bar.
N.B. Group division is done as shown in the *Path Plot* section, with custom or equally-spaced intervals;

- tuning shape of the red profile (i.e. *global reference*) and pink profile (i.e. *binarized reference*). This can be done in `Reference Histogram Customization` box.
  The *global reference* is the weighted average of *group references*. The *binarized reference* is based on the *global reference*. It contains peaks found in the *global reference*, but they are leveled and normalized.
  `Align to Global-Custom Reference` to finally re-align all the traces to the reference.

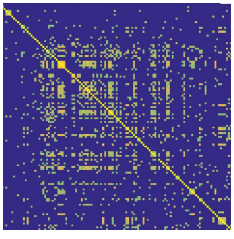
2.4 Selection through correlation

A common problem in SMFS is finding and isolating *relevant* traces among all collected data. The *Basic Filtering Tool* described in Chapter 1 allows the operator to filter out traces longer or shorter than the expected ones; the *Fingerprint ROI* tool, described in this Chapter, looks at a specific pattern drawn by the user and can list the traces accordingly. All these tools need the intervention of the user to choose some specific interval or shape, in contrast with the purpose of an unsupervised filtering procedure.

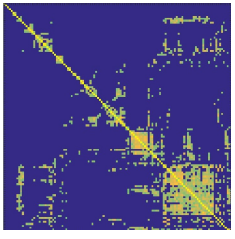


The *Selection through correlation* (`Selection > *`) calculates the cross correlation between every couple of loaded (`Valid`) traces forming a symmetric *similarity matrix*. This matrix can then be sorted (ordered) with the MATLAB function `symamd` (symmetric approximate minimum permutation algorithm).
The slide bars change the visualized contour length histograms. The blue curve in the top-right panel is the calculated cross correlation of the two visualized histograms of *trace 1* and *trace 2*. The diamond points the maximum value, the red area is the Gaussian curve that weights the correlation (as described in *Automaic Alignment*).

- `Single trace correlation` button displays the values of correlation relative to *trace 1* (the one shown in the upper panel);
- `Sort Correlation Matrix` applies the symmetric approximate minimum permutation algorithm to the *similarity matrix* (i.e. it changes the order of the traces to find clusters of similar traces);
- `Global Correlation` shows the *similarity matrix*;
- `Sigma` determines the width of the Gaussian curve that weights the correlation curve;
- `Min` is the threshold value for the *similarity matrix*;
- `Max Lag` is the maximum lag allowed when calculating the correlation between two traces.



Similarity matrix



Sorted *similarity matrix*

At this point the user has two options: he can decide to select a specific cluster in the sorted *similarity matrix*, or he can select traces similar to the reference *trace 1* (the trace visualized in the upper panel).

With the first button, the sorted *similarity matrix* pops up and, with the cursor, the user can select a cluster of traces (click-drag&drop plus double-click on the segment). The selected group (identified by a **X**) of traces will

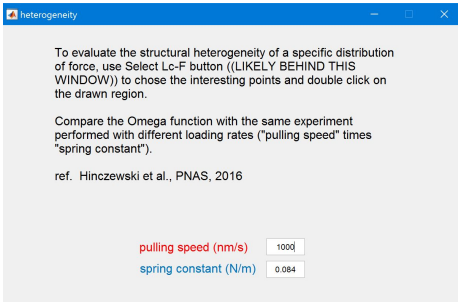
be visible in the main interface only after clicking `Update traces`. If the selection does not satisfy the user, he can `Filter > Move ALL to Valid` and redo all the process.
The second button allows to select the traces above the `Min` threshold, that are similar to the reference trace in the upper panel. The selected traces are previously visible clicking `Single trace correlation` button.

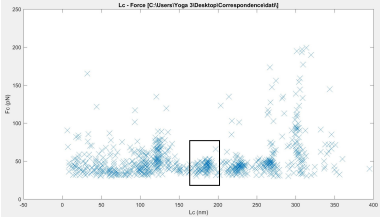
A further refinement of the calculation of the correlation is possible tuning the contour length histograms with the options:

- `Filter trace` applies a smoothing filter to the contour length histogram of every trace;
- `Gaussian substitution` substitutes a sum of Gaussian curves to each contour length histograms. It detects the peaks with the parameters on the right.

2.5 Structural heterogeneity determination

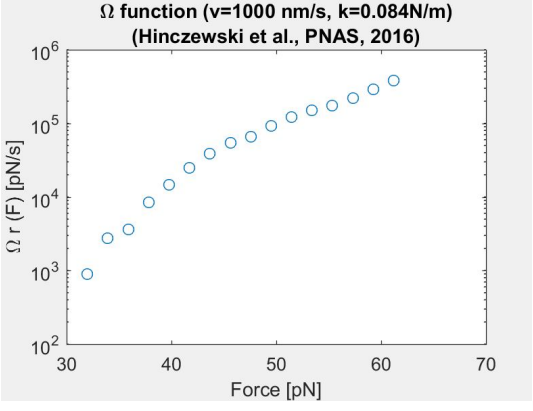
Hinkzewski and colleagues in 2016 found a function that give information about the structural heterogeneity of a protein [5]. This function Ω can be determined performing SMFS at different pulling speed. To perform this analysis in Fodis go to `Tools > Structural heterogeneity`.





Global Contour length-Force plot (*section 1.6.3*).

The user have to select the region of interest containing the Force peaks, and double click inside the region. Fodis will compute Ω. Then the user have to repeat the procedure to the same Force peaks obtained at a different pulling speed, saving and comparing the Ω graphs.



Ω function of the force

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