**Atomic Force Microscopy (AFM) nanomechanical characterization of micro- and nanoplastics to support environmental investigations in groundwater**

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**Multifreq AFMSuite Guideline**

# Introduction

For a complete and advanced data analysis of multifrequency mechanical imaging, we developed Multifreq AFMSuite, which is a software similar to general mechanics software AFMech Suite1, but focused on multifrequency mapping. This suite is written in Matlab language, using event/object programming to provide an alternative tool for basic or advanced analysis of AFM nanomechanical maps. The analysis is real-time allowing the user to control each step of the analysis.

Multifreq AFMSuite is dedicated to beginners and experts in nanomechanics. The graphical user interface does not require knowledge of Matlab language. After the analysis, the user can export all graphical results using several formats. Moreover, the software can create custom metadata to save time and quickly reload previous analyses.

The purpose of this manual is to help unfamiliar users perform the analyses using all the software functions and illustrate the features operations. References will help interested users find additional materials and explanations about AFM nanomechanics. During the analysis, hints and warnings will support the users.

If problems or bugs regarding the software are experienced, please contact the authors and improve Multifreq AFMSuite. The software will be freely available on request.

List of commonly used abbreviations:

* AFM --- Atomic Force Microscopy
* E --- Elastic modulus/Young’s Modulus
*  --- Poisson`s ratio
* **[button]** --- Button name on the interface, between square brackets
* **<edit>** --- Edit or menu control, between angular brackets.

# Installation

If Matlab 2019Ra or Matlab Runtime Environment (MRE) version 9.6 is already installed in the operative system, the ‘Multifreq AFMSuite’ main file is ready to use. MRE can be downloaded also from: <https://www.mathworks.com/products/compiler/matlab-runtime.html>. When the Matlab runtime environment is installed ‘Multifreq AFMSuite’ can be launched as a standalone software. Technical details are collected in readme.txt in the downloadable archive.

# Data Import

The GUI MultifreqAFM suite Suite requires raw data in the most common format:

**Asylum Research**: raw data (‘.ibw’) from imaging in multifrequency AFM or bimodal mode containing at least frequency shift of second mode. Other formats from AFM companies will be available when this scan mode is implemented.

# Loading

The empty Multifreq AFMSuite interface is shown in Figure G1. In the panel ‘Loading’,the **[Load Map]** button allows loading .ibw raw file from AFM acquisition software. **[Load]** can use the complete identifier of the file (location directory + file name) contained in **<Path In>** edit. If **<Path In>** is empty or locating a directory, a menu choice appears to select the input file. **<Path Out>**, the edit string for the export/saving directory, is automatically updated accordingly with **<Path In>** selection, but can be user-overwritten to change the output directory. After loading, the morphology channel map will load automatically in Graph\_1: operations like masking and map visualization are active on this graph. Graph\_2 shows the amplitude vs. frequency of resonance curves for both the first and second modes. The quantitative histogram is shown in Graph\_3. Here, the vertical scale is user-controlled modifying ‘Low’ and ‘High’ values under **<Graphic limits>**.



Figure G1. Multifreq AFMSuite interface before loading data.

## Mechanical Analysis

After loading, the mechanical analysis can be performed automatically by pressing **[Execute]** after setting the parameters in ‘Probe-Sample Interaction’. Initially, all parameters are read from the .ibw as long they are set during AFM measurements. The overall parameters can be changed manually and the analysis refreshed by pressing **[Execute]**.

The software allows you to select up to 3 **<Tip Geometry>** relative to the contact mechanics model including Sphere, Punch, and Cone. The scale length can be set in nanometer as **<Radius Sphere>** **and <Radius Punch>** or in degree aperture in **<Tip Angle>**.

Depending on the selected model, different laws are employed. Here DK1 and DK2 are quantities summarizing the interaction tip-surface causing amplitude/phase shift of the first mode and frequency shift of the second mode, respectively.

Apparent Young’s Modulus (E) is retrieved using the following relations:2

(punch) (sphere) (cone)

The final Young’s modulus for each measurement is obtained by approximating the elastic modulus and Poisson’s ratio of a probe:

Eprobe = 150 GPa probe = 0.17.3

And using

Maximum indentation is calculated as:

(punch) (sphere) (cone)

Average indentation is used automatically in the morphology channel to reproduce the zero-force morphology.

The stiffness of interaction is calculated as

(punch) (sphere) (cone)

Independently by probe geometry Loss-tangent:4

Finally, viscosity is calculated as

Where k1, Q1, A1free, A1, f1, k2, f2, f2, q, R are respectively the spring constant of the first mode, Q-factor of the first mode, the free amplitude of first mode, amplitude during interaction, elastic constant of second mode, frequency shift of second mode, frequency of second mode, cone angle and radius of the probe.

The results of the analysis can be selected in the list menu on top of the button **[Execute]**. An example of the results of the analysis is collected in the image Figure G2 containing:

1. Morphology (nm, Figure G2a)
2. Log10 Young’s Modulus (Log10(Pa), Figure G2b)
3. Young’s Modulus (Pa, Figure G2c)
4. Stiffness (N/m, Figure G2d)
5. Maximum Indentation (nm, Figure G2e)
6. Zero-Force Morphology (nm, Figure G2f)
7. Loss Tangent (a.u., Figure G2g)
8. Viscosity (Pas, Figure G2h)



Figure G2 | Output of data analysis in graphic form as maps. **a** Morphology; **b** Log10 Young’s Modulus; **c** Young’s Modulus; **d** Interaction Stiffness; **e** Maximum Indentation; **f** Zero-Force Morphology; **g** Loss Tangent; and, **h** Viscosity.

## Harmonic analysis

During automatic resonance peak analysis, Multifreq AFMSuite allows the loading of the resonance curves. The software can load the Amplitude(nm) vs. Frequency (Hz) data, as .ibw or .txt formats and visualize them in Graph2. The main advantage of harmonic analysis is to individuate the resonance peak with high precision in case of noise and spurious peaks.

In the panel ‘Resonance Frequency’ **[Load]** button allows the loading of the first and second harmonics from separate files. The software automatically makes a fitting using all the frequency intervals provided. If it is not a .ibw, the file must be a .txt containing 2 columns with Frequency (kHz) and amplitude (nm). The input file is based on the structure of the .txt exported from direct thermal noise Bruker/JPK acquisition software (🡪 export xz data). **[Load]** opens a menu choice appearing to select the input file. **<Update>** can be used to perform the analysis without reopening the external files from the beginning.

An example of results for the first and second resonance modes are collected in Figure G3a and Figure G3b. The software has also the ‘manual’ checkbox option to restrict the fitting interval on a selected location (see examples in Figure G3c and Figure G3d). After fitting, numerical values will appear focusing on Amplitude, Frequency, and Quality factor. Visualization of first and second harmonic, along with numerical values can be modified using **<Res Peak>** choice menu.

In air, the cantilever oscillates following the law of a simple harmonic oscillator. Parameters, such as zero frequency amplitude A, resonance frequency f0, and quality factor Q; can be retrieved by fitting the law:

Measurements in water result in more damped oscillation (lower Q), so the Lorentz model is more favorable for data analysis using respectively:



Figure G3| Harmonic graphs of amplitude vs. frequency for **a** first and **b** second resonance. Using the <manual> option it is possible to select the blue interval to perform the fit more accurately. Examples for **c** first and **d** second resonance curves.

## Statistical analysis

Statistical analysis can be visualized in Graph 3, represented by the histogram of selected results (elastic modulus, morphology, stiffness, etc). **<Binning>** allows changing the size of a single bin in a histogram. **[Execute]** will automatically perform the statistical analysis after showing Force Curves (FCs) with respective fittings. If the histogram is showing more than a single peak, the multi-Gaussian fit procedure will be the best choice to obtain quantitative information, selecting the number of peaks in **<Gaussians>**. Numerical results after data analysis can be selected by modifying the peak number in **<Peak Selection>**. Especially for multi-Gaussian procedures, when software is not recognizing automatically distribution peaks (see for example Figure G4a), manual mode can be activated using checkbox **<Manual>**.

Manual mode asks the user’s input to recognize multi-peak positions. First, as shown in Figure G4a, the interval used for multi-Gaussian fitting must be selected (selection will be highlighted in blue) (e.g., Figure G4b). Then, starting positions must be individuated using a left-click mouse: first, the center and height of distribution, then, width at semi-height of distribution (stars in Figure G4c). The operation must be repeated for every distribution selected in **<Gaussians>**. Results could appear graphically as in Figure G4d, while numerical values are shown under **<Results>** and controlled by **<Peak Selection>**. Value and error of quantity in logarithmic scaleare just the center and semi-width of the single-mode Gaussian distributions converted from lognormal to linear distribution using:

Where exp10 is exponential base 10, ELIN, LIN, and ELOG LOG are respectively linearized mean value (Pa), linearized standard deviation (Pa); center and semi-width of the lognormal distribution.



Figure G4 | **a** Selection of interval before fitting in manual mode **b** peaks finding procedure for each peak: first center and height (triangles) and width at semi-height (squares) **c** Final results after multi-Gaussian fitting for center and height of the distribution and **d** the entire distribution.

## Using the Mask

As pointed out in the previous section, the creation of a mask on a map is crucial for data selectivity. Data selectivity is mostly used to balance the content of heterogeneous surfaces or erase bad points from a map. An example can be seen in Figure G5. The mechanical map shows 2 phases (Figure G5a) but one phase has many points in comparison with its counterpart. The histogram shows only 1 peak like in Figure G5b. Mask can be created to remove excessive points from 1 phase.

**[Mask]** allows the selection of a rectangle on Graph\_1 to create (if ‘stop’ is selected) or remove (if ‘pass’ is selected) a manual mask. Playing with ‘stop’ and ‘pass’ options, masks with complex shapes can be created. As an example, the map with a mask is shown in Figure G5c, finally showing a histogram with easy determination of different contributions (Figure G5d).

Finally, the mask can be removed by pressing the **[Reset Mask]** button.



Figure G5 | Example of a map with **a** strong contribution from substrate and **b** correspondent histogram. **c** the mask can be created to remove large contribution of substrate and **d** finally show a histogram evidencing different contributions.

## Export & Save



Figure G6 | Example of Multifreq AFMSuite interface after data analysis and ready to export data or images.

All the graphics produced in AFM Suite like in Figure G4 can be exported during any stage of analysis using buttons **[Export]** found under every graph. Exported files are automatically saved in the directory designated by respective **<Path Out>** edit.

Using **[Export]**, images will be exported in the following formats:

1. .tif *Tagged Image File Format* no compression, 300 dpi
2. .fig *Matlab Figure*
3. .mat *Matlab Data*, the structure containing Matlab variables
4. .tif(3D) *Tagged Image File Format*, 300 dpi, 3D representation of map
5. .fig(3D) *Matlab Figure*, 3D representation of map

For the resonance curve of Graph2 and Gaussian analysis of Graph 3, using **[Export],** formats available are listed below:

1. .tif *Tagged Image File Format* no compression, 300 dpi
2. .fig *Matlab Figure*
3. .mat *Matlab Data*, the structure containing Matlab variables
4. .txt *Plain text with no special formatting,* data, and/or fitting in columns
5. .xls *Excel* table, data, and/or fitting in columns
6. .opj *Origin* table and graphs

If Matlab software is not available .fig and .mat files cannot be opened. Origin export in .opj is not creating or opening if Origin is not installed on the machine.

Saving data is possible through the button **[Save].** A snapshot of the analysis, variables, and structures is saved and named *#filename#*\_metadata inside the **<Path Out>** directory. All data will be saved in one click: saving will also work with partial analysis allowing the user to pause analysis and restart later opening the metadata file.

Metadata can be loaded only with **[Load Metadata]** in the ‘Loading Files’ panel.

# References

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