1. Resource paper (RP)

This document explains the fundamental knowledge of our custom-built software, "Analysis Software," for analyzing data obtained by nanoendoscopy AFM. See the following paper for more details:

T. Ichikawa et al., "Protocol for live-imaging of intracellular nanoscale structures using AFM with nanoneedle probes", STAR Protocols, under review (2023).

2. How to install and run the "Analysis Software"

NOTE 1: Analysis Software is only worked on Windows OS. Mac OS and Linux OS are not supported.

NOTE 2: The following procedure assumes an environment in which Python and the required libraries are not installed. If all or some of these are installed, add or change the environment as necessary, depending on the situation.

I. Download the Python installer from the following URL: "https://www.python.org/downloads/windows/".
The recommended Python version is 3.7.8 with 64-bit for Analysis Software, and versions 3.6-3.9 with 64-bit Python may also be worked. Other versions are not supported.

II. Launch the Python installer and follow the on-screen instructions to proceed with the installation. On the screen immediately after launch, you should check the "Add Python 3.X to PATH" (3.X is your Python version) checkbox.

III. After installing Python, launch a command prompt, type each of the following strings, and press enter (Fig. S1):
- pip install numpy
- pip install matplotlib

This will install the "numpy" and "matplotlib" libraries for Python.

Fig. S1: Installation of "numpy" and "matplotlib" libraries.

IV. Download “AnalysisSoftwarePublicV1_0_0.zip” file from the Releases page on GitHub.

V. Extract the zip file and launch "setup.exe". Follow the on-screen instructions to proceed with the installation.

VI. After this installation, you can launch the Analysis Software.

3. How to use the "Analysis Software"

After launching Analysis Software, the main window appears (Fig. S2). Here we follow the procedure for intracellular measurement with nanoendoscopy AFM described in RP.

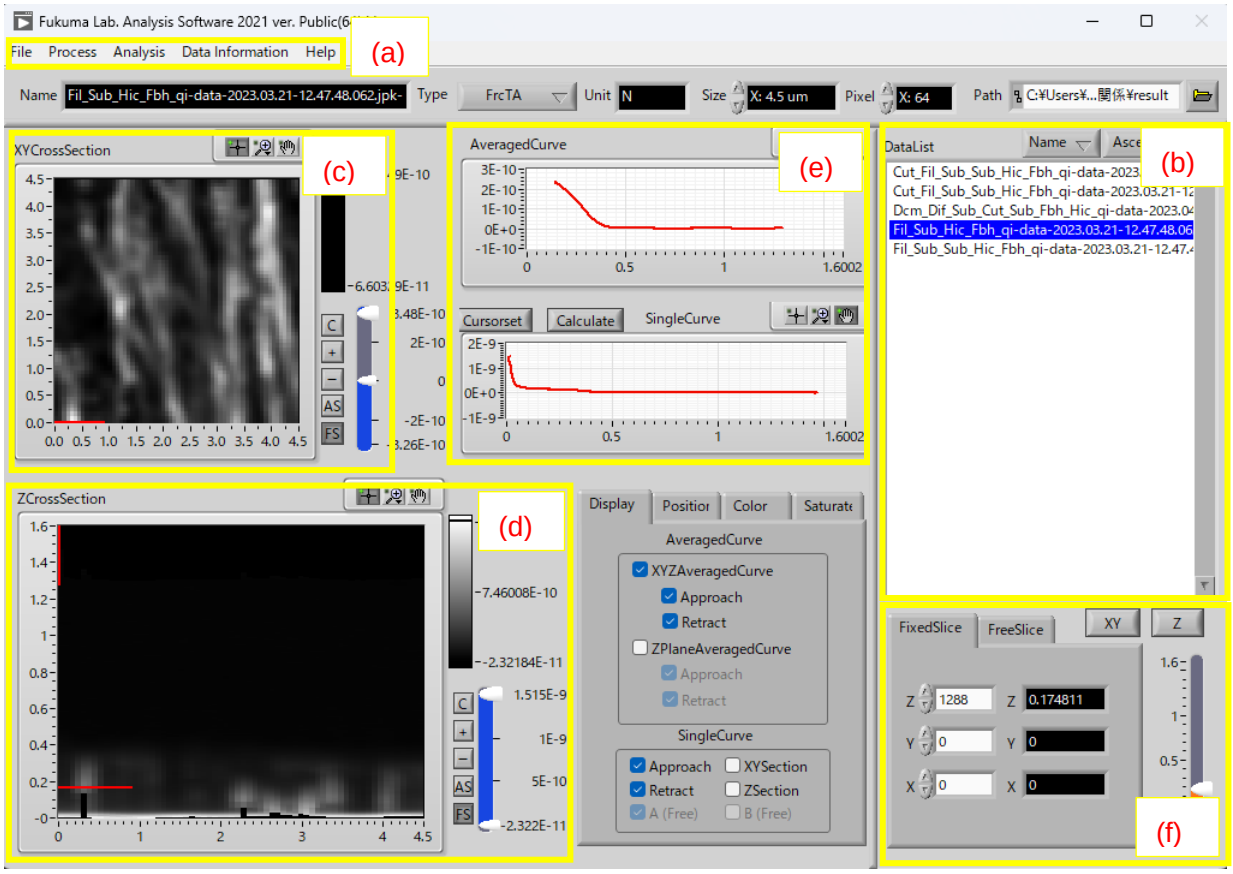

Fig. S2: Main window of Analysis Software. (a) Menu bar, (b) Data list, (c) XY cross-section, (d) XZ cross-section, (e) Force curve, (f) Slice selector for visualizing.

I. Load the QI Advanced Imaging data file obtained by the JPK-AFM system.

[RP Step 9a]

Go to the menu bar and select "File"> "Import 3D Data"> "JPK 3D Data(Fast Import Python)" (Fig. S3). At the upper left, specify the folder path, including the JPK-AFM data file, and then select its file at Data List. You should select others as follows:

- Read Data Type: "Approach".
- PythonVersion: Installed Python version.
- Height or time: "measuredHeight."
- Force: "vDeflection"

After that, you press OK to complete the file load.

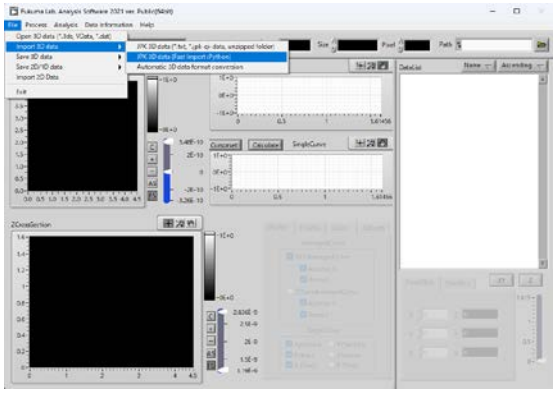

Fig. S3: Access to "JPK 3D Data(Fast Import Python)".

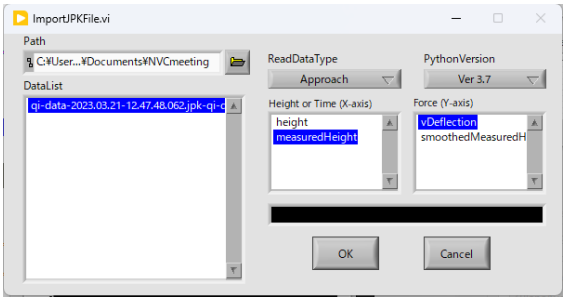

Fig. S4: "JPK 3D Data(Fast Import Python)" window.

After loading the file, we recommend that you save it in our original format (.3ds). To do it, go to the menu bar and select "File"> "Save 3D Data"> "3ds file (*.3ds)" (Fig. S5). This 3ds format is optimized for Analysis Software. When you select a folder path in the upper right corner of the main window, a list of 3ds files contained in that folder will appear in the DataList. By selecting one of the 3ds files in the DataList, you can immediately load it in Analysis Software. In addition, when the following processes are performed on 3ds file data, the data is automatically saved as a new file at the end of the process. This is useful for backing up data and reprocessing. (Note that this creates the file by appending a string derived from the process to the beginning of the file name so that the file will be overwritten if the same process is followed.) The process can proceed without saving to 3ds, but in this case, it will not be saved automatically.

II. Decimation [RP Step 9b]

Go to the menu bar and select "Process"> "Decimation"> "Z" (Fig. S5). Here, decimation is performed based on the following formula: (After decimated pixels) = (Current pixels)/Value. For example, setting Value to 2 reduces the number of pixels in Z by half. After setting Value, you press OK to complete the decimation.

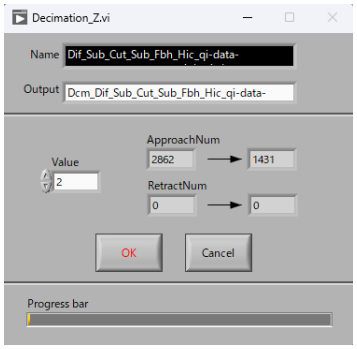

Fig. S5: "Decimation" window.

III. Flatten [RP Step 9c]

Go to the menu bar and select "Process"> "Flatten Bottom Height" (Fig. S6). You confirm ApproachList as "FrcTA" and press OK.

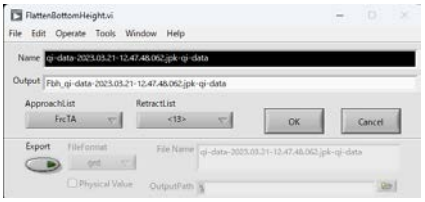

Fig. S6: "Flatten Bottom Height" window.

Then, go to the menu bar and select "Process"> "Height Correction" (Fig. S7). In this window, you can see the bottom 2D image of QI-mode 3D data. For flattening to eliminate the effect of sample slope, you select the parameter as follows:

- Method: "Line-by-Line (2)".
- DataType: "HeightTrace".

After that, you press OK to complete the flattening.

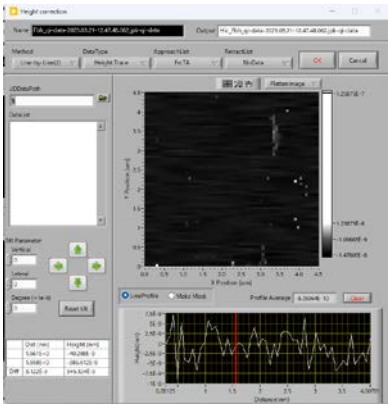

Fig. S7: "Height Correction" window.

IV. Linear background subtraction [RP Step 9d]

Go to the menu bar and select "Process"> "SubtractionLongRangeProfile" (Fig. S8). In this window, long-range force removal parameters can be determined by checking one force curve and applying them to all force curves. First, you select the base force curve by setting the lateral position from the lower left "X" and "Y". This base force curve is visualized on SingleProfle. Then, confirm the Function as "1 (Linear)" and check the RelativeRange checkbox. Adjust the two cursor positions on the SingleProfile to sandwich the areas where the effects of long-distance forces are more pronounced at relatively distant Z positions. It automatically performs a linear fitting and subtracts it from the original force curve, assuming that a linear long-distance force is applied within this region. This result appears on AfterSubtraction. When you press ApplyAll, these settings are used for long-range subtraction to all force curves.

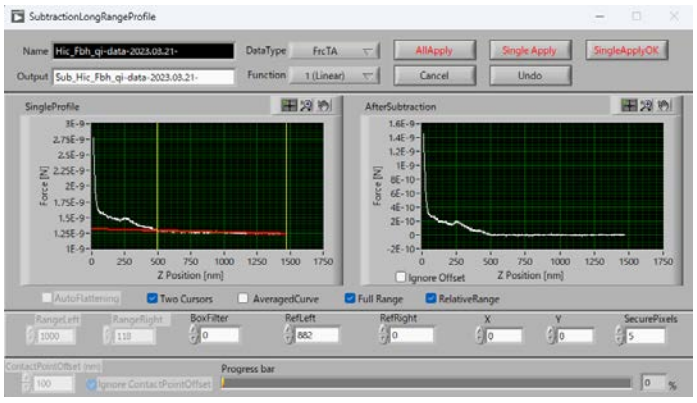

Fig. S8: "SubtractionLongRangeProfile" window

V. Averaging filter [RP Step 10a]

Go to the menu bar and select "Process"> "Filtering" (Fig. S9). You set the parameters as follows:

- Target: "X-Y".
- Method: "Average".

After that, you press OK to complete the filtering.

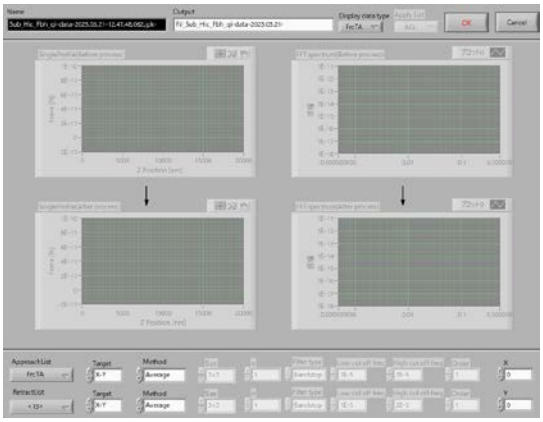

Fig. S9: "Filtering" window

VI. Export the text file for visualizing by Voxler [RP Step 10b]

Go to the menu bar and select "File"> "Save 3D data"> "text file (*.txt)" (Fig. S10). You set the parameters as follows:

- Column A: "X (Voxel)".
- Column B: "Y (Voxel)".
- Column C: “Z (Voxel)”.
- Column D: "Value".

After that, select the output destination and press OK to generate the file.

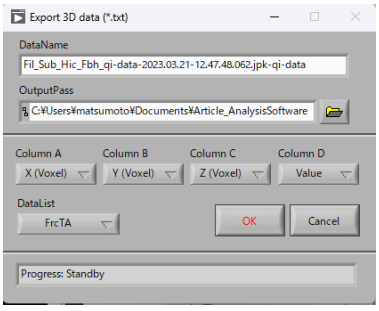

Fig. S10: “Export 3D data (*.txt)” window

VII. Fitted curve subtraction [RP Step 11a]

Load the data after the IV process is completed, and then go to the menu bar and select "Process"> "SubtractionLongRangeProfile" again (Fig. S11). Check the RelativeRange checkbox, and select Function as "3 Weighting". Then, adjust the two cursor positions in SingleProfile to sandwich an area of almost zero force far from the surface. After that, press ApplyAll to complete the subtraction.

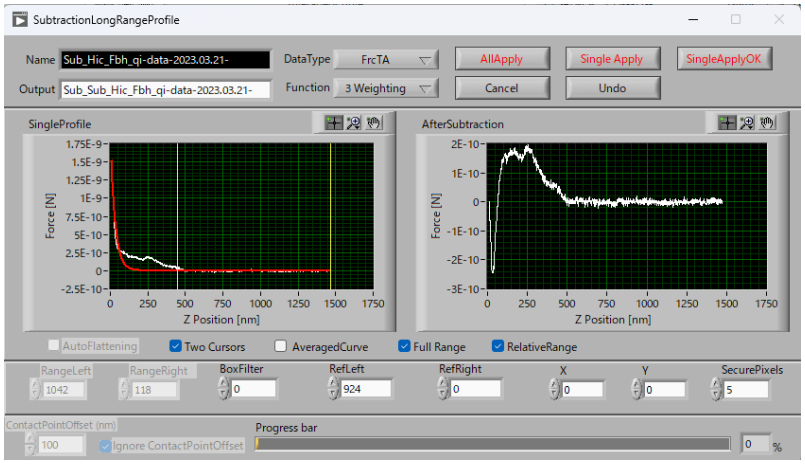

Fig. S11: "SubtractionLongRangeProfile" window (2).

VIII. Averaging filter [RP Step 11b]

This process is the same as V.

IX. Export the text file for visualizing by Voxler [RP Step 11c]

This process is the same as VI.

X. Differentiation and Inversion of data sign [RP Step 12a and b]

Load the data after the VIII process is completed, and then go to the menu bar and select > "Process"> "Differential" (Fig. S12). Select the Differential Method as "2nd Order Central" and check the Inverse checkbox. After that, press OK to complete the differentiation and inversion of the data sign.

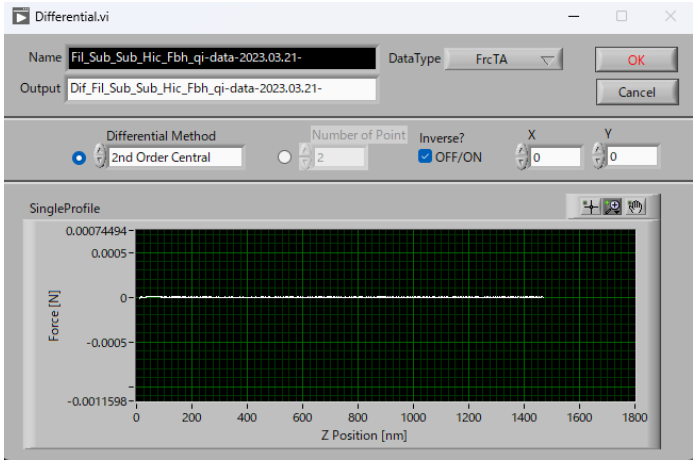

Fig. S12: "Differential" window.

XI. Export the text file for visualizing by Voxler [RP Step 12c, d, and e]

This process is the same as V.

4. Terms of Use / Copyright

✓ **Using Analysis Software**

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[1]. No redistribution, public transmission, modification, and translation.

[2]. You retain proprietary rights to the results obtained from the analysis of your data by this software. However, in publishing such results, it is necessary to cite the following article in the section describing the experimental method:

- T. Ichikawa et al., "Protocol for live imaging of intracellular nanoscale structures using atomic force microscopy with nanoneedle probes", STAR Protocols 4(3), 102468 (2023).

[3]. The developers of this software bear absolutely no responsibility for any action taken by the user involving the use of this software.

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