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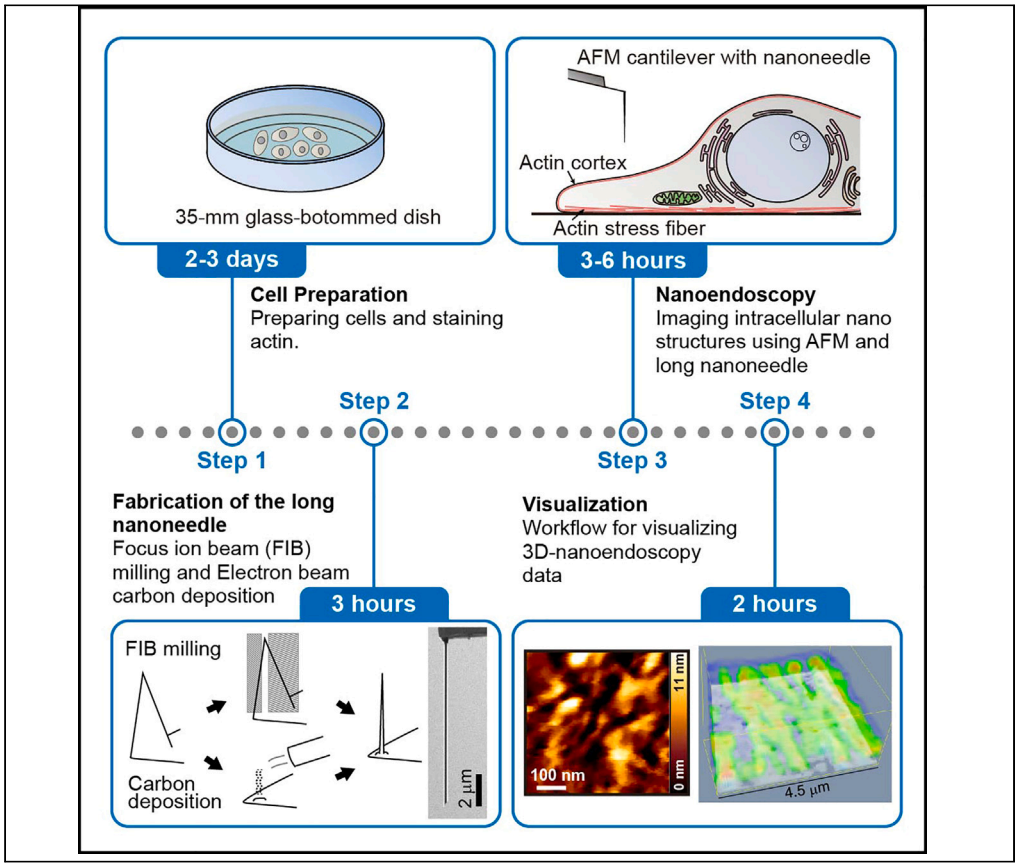
Protocol for live imaging of intracellular nanoscale structures using atomic force microscopy with nanoneedle probes



Atomic force microscopy (AFM) is capable of nanoscale imaging but has so far only been used on cell surfaces when applied to a living cell. Here, we describe a step-by-step protocol for nanoendoscopy-AFM, which enables the imaging of nanoscale structures inside living cells. The protocol consists of cell staining, fabrication of the nanoneedle probes, observation inside living cells using 2D and 3D nanoendoscopy-AFM, and visualization of the 3D data.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Fabrication of nanoneedle tip by EBD or FIB-milling

Step-by-step guide for 2D and 3D nanoendoscopy-AFM imaging

Visualization of 3D intracellular structures from 3D nanoendoscopy-AFM data

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Protocol for live imaging of intracellular nanoscale structures using atomic force microscopy with nanoneedle probes

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SUMMARY

Atomic force microscopy (AFM) is capable of nanoscale imaging but has so far only been used on cell surfaces when applied to a living cell. Here, we describe a step-by-step protocol for nanoendoscopy-AFM, which enables the imaging of nanoscale structures inside living cells. The protocol consists of cell staining, fabrication of the nanoneedle probes, observation inside living cells using 2D and 3D nanoendoscopy-AFM, and visualization of the 3D data.

For complete details on the use and execution of this protocol, please refer to Penedo et al. (2021)[1] and Penedo et al. (2021).[2]

BEFORE YOU BEGIN

In recent years, there has been an increasing demand to observe the dynamics of intracellular structures, such as organelles, protein complexes, and liquid-liquid phase-separated structures.[3,4] However, it has been difficult to observe intact intracellular nanoscale structures in living cells because electron microscopy has a high resolution but cannot be used for living cells. On the other side, fluorescence microscopy can observe living intracellular structures, but even super-resolution microscopy does not have enough resolution to observe a few nanoscale structures. Atomic force microscopy (AFM) can observe the nanoscale structures of living cells. Although several intracellular AFM imaging techniques have been proposed,[5–9] all of these techniques measure from outside the cell membrane; therefore, it is intrinsically difficult to visualize three-dimensional (3D) arrangements and could not achieve sub-10-nm spatial resolution. Recently, we developed a method for imaging the inside of living cells using AFM combined with a long nanoneedle tip.[1,2]

This protocol consists of four steps: cell staining, fabrication of the long nanoneedle probe, observation of the inside of the living cell using 2D- and 3D-AFM, and visualization of the 3D data.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Dulbecco's modified Eagle's medium (high glucose) with L-glutamine and phenol red	Fujifilm Wako	Cat#044-29765
Fetal bovine serum	Fujifilm Wako	Cat#553-36315
D-PBS(-)	Fujifilm Wako	Cat#045-29795
Penicillin-Streptomycin solution (×100)	Fujifilm Wako	Cat#168-23191
SiR-actin Kit	Cytoskeleton	CY-SC001
Dimethyl sulfoxide, dehydrated	Fujifilm Wako	Cat#040-18032
Leibovitz's L-15 Medium, no phenol red	Thermo Fisher	Cat#21083027
35-mm low-height glass-bottomed dish	ibidi	Cat#80137
15 mL plastic tube	Thermo Fisher	Cat#339650
ddH2O	N/A	N/A
Ethanol	Fujifilm Wako	Cat#057-00451
Cell-Tak	Corning	Cat#354240
Collagen Type I	Nippi	Cat# ASC-1-100-20
Poly-L-lysine Hydrobromide, M.W. >300,000	Fujifilm Wako	Cat#163-19091
Experimental models: Cell lines		
BALB/3T3 cell clone A31	JCRB Cell Bank	JCRB9005 RRID:CVCL_0184
Software and algorithms		
Voxler 4	Golden Software	https://www.goldensoftware.com/
Gwyddion Version 2.62	Czech Metrology Institute	http://gwyddion.net/ RRID:SCR_015583
JPK Data Processing Software Version 7.0.1	Bruker	https://japan.jpk.com/
LabView 2021	National Instruments	https://www.ni.com/ RRID:SCR_014325
Python 3.9	Python Software Foundation	https://www.python.oor RRID:SCR_008394
Custom-built analysis software	Takeshi Fukuma	https://github.com/fukuma-lab/3D-AFM_Analysis
Other		
Focused ion beam system	Thermo Fisher	Helios G4 CX Dual Beam system RRID:SCR_021773
AFM	Bruker	JPK Nanowizard 4
Inverted fluorescence microscope	Nikon	Eclipse Ti2 RRID:SCR_021068
EMCCD camera	Andor	iXon Ultra 888 RRID:SCR_023166
Fluorescence filter cube	Nikon	Cy5 HQ
High Accuracy Stage for Resolve BioAFM	Bruker	SPC-180713-04
Light source	Excelitas	X-Cite XYLIS
Sample heater	Bruker	RES-SAMPLE-HTR
Biohazard safety cabinet	ESCO	esc-ac2-4n7
CO_2 incubator	WakenBtech	Direct Heat CO_2 Incubator 320
Soft plasma etching system	Meiwafosis	SEDE-GE
AFM cantilever	Olympus	BL-AC40TS
AFM cantilever	OPUS	240AC-NG
Forceps	Dumont	#5

MATERIALS AND EQUIPMENT

Cell culture medium		
Reagent	Final concentration	Amount
Dulbecco's modified Eagle's medium (High Glucose) with L-Glutamine and Phenol Red	N/A	500 mL
Fetal bovine serum	10%	50 mL
Penicillin-Streptomycin Solution (×100)	1%	5 mL
Store at 4°C for up to 1 month.		

STEP-BY-STEP METHOD DETAILS

Cell preparation and actin staining

Timing: 2–3 days for cell culture, staining 1 h (for steps 1 to 3)

We refer to the fluorescence image to choose the scanning area for the Nanoendoscopy-AFM. In this protocol, the target structure is an actin filament. Therefore, we stain the actin filament using a chemical dye. This step varies depending on the experiment, and different fluorescent dyes or fluorescent protein-expressing cells can also be used. The following steps describe the procedure for chemical staining using the acting-binding fluorescent dye (SiR-Actin kit, CY-SC001, Cytoskeleton). The protocols follow the datasheet of the products (https://www.cytoskeleton.com/pdf-storage/datasheets/cy-sc001.pdf).

⚠ CRITICAL: The SiR-Actin is based on jasplakinolide, which inhibits actin depolymerization.[10] Therefore, treatment with excess SiR-Actin should be avoided because it decreases actin dynamics.

1. Preparation of cells.
   a. Seed 0.1–5 × 10^4 BALB/3T3 cells on a 35 mm low-height glass-bottomed dish in culture medium.

   Note: This step should be performed in a biohazard safety cabinet.

   b. Culture cells in a 5% CO_2 incubator for 2 days.

   Note: Nanoendoscopy-AFM imaging is easier when the cell height in the target area is low. Therefore, cells such as fibroblasts are relatively easy to measure because they are usually spread out and thin.

   Note: Cultivate the appropriate number of cells so that they do not touch each other, but multiple cells are visible in the field of view (cell density range: 0.4–2 × 10^4 cells/cm^2).

   Note: We use a low dish (μ-Dish 35 mm low Glass Bottom, ibidi) because a tall dish has a risk of contact with the AFM head when the cantilever reaches the bottom.

2. Staining cells.
   a. Prepare 1 mM SiR-Actin stock solution by dissolving the reagent in anhydrous Dimethyl sulfoxide.
   b. Prepare a staining solution by adding a 1:1000 volume of 1 mM SiR-actin stock solution to the culture medium (DMEM with 10% FBS and 1% penicillin-streptomycin).
   c. Change the cell culture medium to the staining solution in the safety cabinet and incubate in a CO_2 incubator for 0.5–1 h.

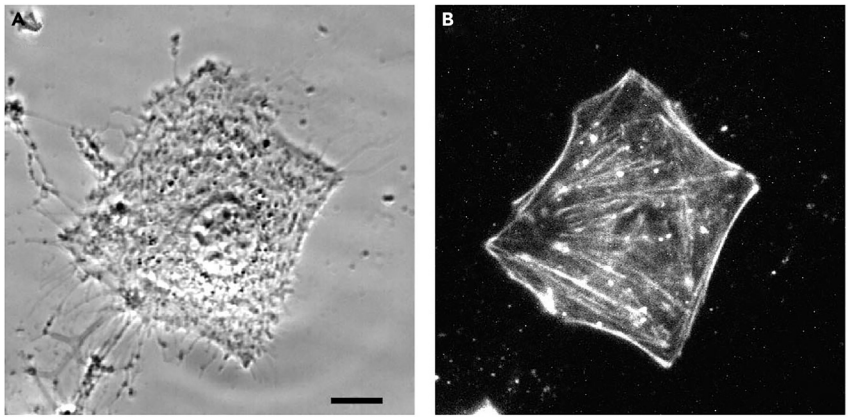


Figure 1. Bright-field and fluorescence images of a BASL 3T3 cell
(A) Bright-field image.
(B) Actin-stained fluorescence image of the same cell as A. Scale bar indicates 10 μm.

d. Change the medium to Leibovitz L-15 supplemented with 1% penicillin/streptomycin.

3. Checking the fluorescence.
   a. Turn on the inverted fluorescence microscope (Eclipse Ti2, Nikon) equipped with the EMCCD camera (iXon Ultra 888, Andor).
   b. Select the Cy5 filter (excitation 620/60, dichroic 660, barrier 700/75).
   c. Adjust the excitation intensities and exposure times to avoid photobleach and phototoxicity (laser power: 1%–10%, exposure time: < 100 ms).
   d. Take pictures. Compare with the bright image (result example: Figure 1).

Note: If the staining efficiency is low, add 10 μM verapamil, which is included in the SiR-Actin kit, to inhibit efflux pumps.

Note: Use Phenol Red-free L-15 medium for fluorescence measurement because Phenol Red increases the background when the orange or red fluorescent dye is used.

Note: Using a medium without FBS for measurement may be preferable because it reduces the frequency of cell division during nanoendoscopy.

Fabrication of the nanoneedle probes

Timing: 3 h

We describe two methods for fabricating nanoneedle probes. One method is to mill a commercial probe using a focused ion beam (FIB).[2] Another method is electron beam deposition (EBD) which deposits carbon at the tip of the cantilever.[11,12] The FIB-milled tip is longer and harder, but it requires skills and takes time to fabricate. EBD can be fabricated relatively easily in a short time, but it is shorter. The FIB milling method requires 3 h, and the EBD method requires 1 h.

4. Fabrication of FIB-milled tip.
   a. Setting cantilevers in FIB-SEM.
      i. A cantilever (240AC-NG, OPUS, spring constant 2 N/m) is placed on the custom-built cantilever holder for FIB milling (Figure 2A).
      ii. The cantilever folder is placed on the 45° inclined surface of the stage in a Helios G4 CX Dual Beam system (FEI, Thermo Fisher Scientific) and fixed it with a screw (Figure 2C).

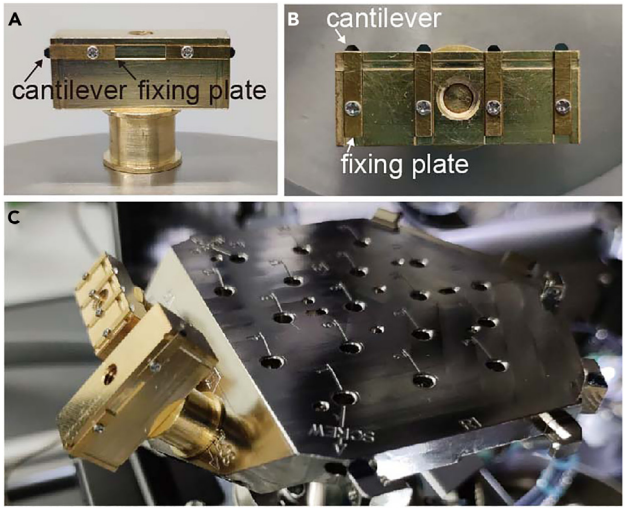


Figure 2. Cantilever holder for fabrication of the long nanoneedle tip

(A) Cantilever for focused ion beam (FIB)-milling tip.

(B) Cantilever for electron beam deposition (EBD) tip.

(C) Fixed state to the 45° inclined plane of the stage of FIB-SEM.

Note: The cantilever holders are made of brass to avoid magnetization.

b. Finding the tip position.
   i. Close the door and start evacuation of the vacuum chamber.
   ii. After confirming that the pressure in the chamber is less than 5×1^{-4} Pa, press "Beam On" to start the electron and ion beams.
   iii. Set the electrical voltage to 15 kV, and the current to 43 pA in SEM mode.
   iv. Adjust the focus and link the Z position of the stage and the distance between the sample and the lens.
   v. Bring an AFM tip on the cantilever into the field of view.
   vi. Bring up the stage to 4 mm and tilt 17° with respect to the horizontal plane. Then, adjust the focus and link it again.
   vii. Switch to the FIB mode and set the acceleration voltage to 16 kV and the current to 15 pA (observation mode).

⚠ CRITICAL: When using the FIB mode, set the lowest electrical current possible and avoid unnecessary imaging to reduce unwanted milling.

Note: Because the ion beam line is tilted 52° from the vertical axis, the angle between the cantilever on the 45° inclined surface and the ion beam line becomes 10° by tilting the stage by 17°.

   viii. Increase the magnification step by step up to 15,000× and focus on the AFM tip.
   ix. Record the position as "side_(cantilever number)".

c. Milling the tip.
   i. Place the rectangle area for milling inside of the probe (Figure 3A).

Note: Initially, place the rectangle relatively away (~2 μm) from the center line of the probe and then gradually close it to the center by decreasing the electrical current.

   ii. Set the electrical current to 0.4 nA.

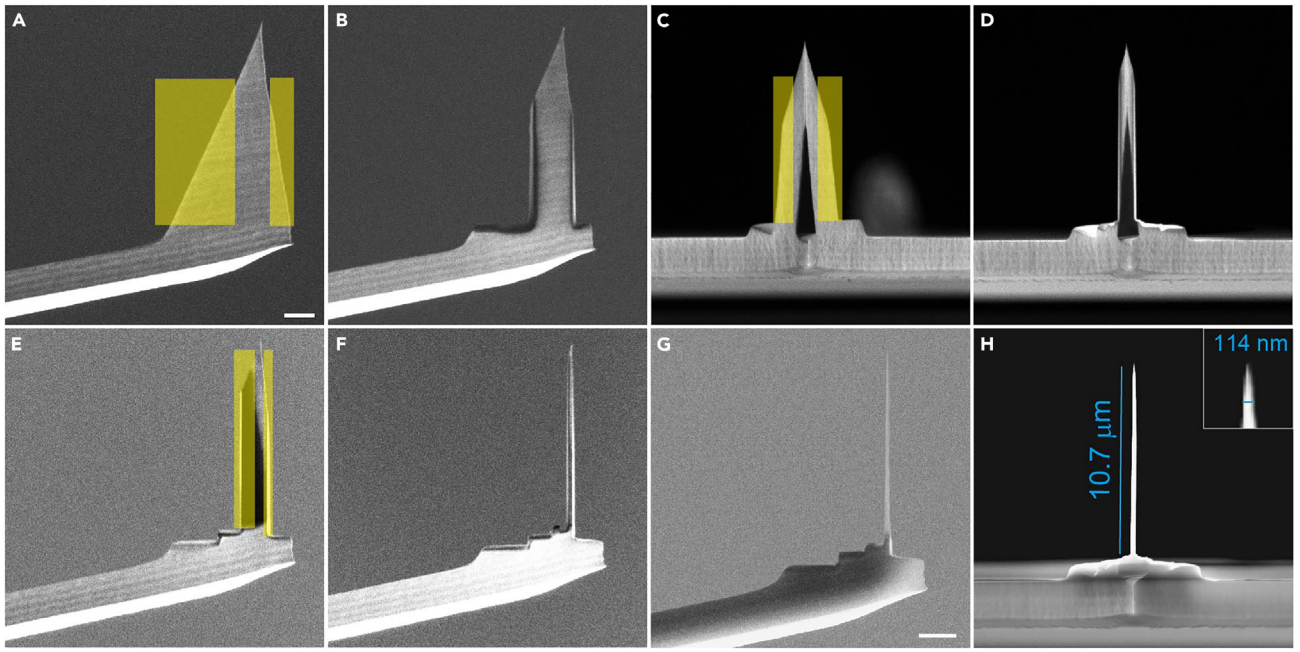


Figure 3. Fabrication process of the FIB-milled tip

(A) Side view of the intact 240AC tip overlaid with the milling rectangle (yellow area). Scale bar indicates 2 µm. FIB image.
(B) Image after milling in A.
(C) Front view after A overlaid with the milling rectangle. FIB image.
(D) Image after milling in C.
(E) Side view of D overlaid with the milling rectangle. FIB image.
(F) Side view image after milling in E.
(G) Side view image after milling. SEM image. Scale bar indicates 2 µm.
(H) Front view image after milling. Inset shows the magnified tip image.

iii. Set the Properties in the Pattern Control panel to Application Si, Time 5–10 min, and Scan Direction right to left.

Note: It is not necessary to adjust other parameters such as Z size and Dwell Time.

Note: The Scan Direction should be set in the manner that the scans start away from the center of the tip and go toward the center.

iv. Scan once just before milling and confirm that the tip is not drifting. If significant drift was observed, wait 5–10 min and readjust the milling position.
v. Start the milling.
vi. When the size of the object becomes small, stop the milling and reduce the size of the milling rectangle. And then mill it again.

Note: The smaller the milling rectangle area, the faster the milling speed.

vii. Replace the milling rectangle on the other side of the probe.
viii. Perform the milling (after milling: Figure 3B).
ix. After milling from the side of the probe, reduce the electrical current to 15 pA.
x. Return the Tilt to 0° and lower the Z to 10–20 mm, and then rotate the stage by 180°.

Note: Make sure to lower the stage not to hit the lens or the nozzle of the Gas Injection System (GIS).

xi. Move the cantilever into the field of view. Set the Z position to 4 mm and Tilt to 7°.
xii. Focus and record the position as "front_(cantilever number)".
xiii. Set the Scan Rotation to −90° so the probe's tip faces up.
xiv. Place the milling rectangle on one side of the probe. Set the electrical current to 45 pA (Figure 3C).

Note: Put the milling rectangle upstream of the drift first.

xv. Scan once and start milling.
xvi. Move the rectangle to another side of the probe and mill it again (after milling: Figure 3D).
xvii. Decrease the electrical current to 15 pA and return to the "side" position.
xviii. Place the rectangle inside the probe (Figure 3E). Set the electrical current to 0.13 nA.
xix. Scan once and start milling.
xx. As mentioned above, repeat the process 2–3 times while reducing the electrical current until the width of the probe is less than 200 nm.

Note: Be sure to scan once before every milling to avoid cutting unwanted areas.

Note: Be sure to set the electrical current to 15 pA (for observation) after milling.

Note: When the width of the probe is close to 200 nm, we suggest placing a reference rectangle with a width of 180 nm at the center of the probe and using it as a shaving guide.

d. Finishing fabrication of the tip.
i. When the diameter of the probe is less than 200 nm, tilt the milling rectangle by 5° and taper off the tip in the front view.
ii. Do it again by tilting the milling rectangle by −5°(after milling: Figures 3G and 3H).

Note: Adjust the angle to achieve the desired tip aspect ratio.

iii. Tilt 38° from the "side" position (total Tilt 45°). Change to SEM mode and take a photo of the probe.

5. Fabrication of the electron beam deposition (EBD) tip (optional).
a. Setting the cantilever in the chamber of the FIB-SEM.
i. Place a cantilever (BL-AC40TS-C2, Olympus) on the cantilever holder for EBD fabrication (Figure 2B).
ii. Fix the cantilever holder on the 45° inclined surface of the stage (Figure 2C).
b. Preparation and finding the tip.
i. Close the door and start evacuation of the vacuum chamber.
ii. After confirming the pressure in the chamber is less than 5×10^{-4} Pa, press "Beam On" to start the electron and ion beams.
iii. Set the electrical voltage to 15 kV and the current to 43 pA.
iv. Adjust the focus on the surface and link the stage's Z value to the distance between the sample and the lens.
c. Flattening the tip.
i. Rotate 180°.
ii. Bring up the stage to 4 mm.
iii. Bring the cantilever into the field of view.
iv. Tilt the stage by 7°.
v. Select the FIB mode and set the electrical voltage to 16 kV and the current to 15 pA.

Note: Set the lowest electrical current and reduce the imaging time as possible to avoid unwanted milling.

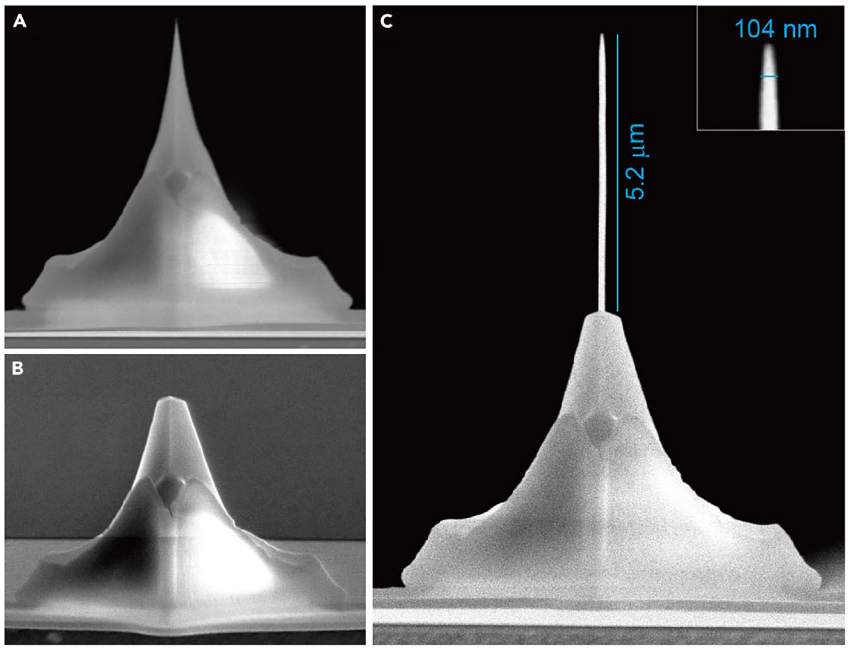


Figure 4. Fabrication process of the EBD tip

(A) Intact BL-AC40TS-C2 tip. SEM image.

(B) After cutting the tip.

(C) After the fabrication of EBD. Inset shows the magnified tip image.

vi. Focus the AFM tip in FIB mode.

vii. If the head of the probe is not flat, flatten the surface using FIB. Change the electrical current to 45 pA and place the milling rectangle at the tip of the probe (Figures 4A and 4B).

viii. Take one image and perform milling for 2 min.

d. Deposition of carbon on the tip.

i. Change to SEM mode.

ii. Set the Tilt to 35°.

iii. Find the place for the deposition.

iv. Magnify to 150,000× and adjust the focus.

v. Set the electrical current to 0.17 nA. Place the deposition circle and set the properties with the following values: Outer Diameter 22 nm, Dwell Time 20 min, Scan Direction Inner To Outer.

vi. Insert the carbon GIS nozzle. Repeat opening and closing a few times to release air from the nozzle.

vii. Take one image. If significant drift is observed, wait for about 5–10 min and readjust the position of the deposition circle.

viii. Start deposition.

Note: Ensure that Z is approximately 4 mm. A longer Z distance decreases the deposition efficiency.

ix. After deposition, retract the nozzle and delete the deposition circle. Change the electrical current to 45 pA.

x. Return to Translation 0° and Rotation 0°.

xi. Tilt 45°.

xii. Find the tip position and take a photo (Figure 4C).

Note: Other types of cantilevers can be used in the same manner.

Note: The nanoneedle probe can be stored in the desiccator for about several months.

Nanoendoscopy

Timing: 3–6 h (for step 6)

Timing: 2–4 h (for steps 7 to 8)

There are two types of scanning methods; 2D scanning and 3D scanning. 2D scanning is the so-called "Tapping", "Dynamic", or "AC" mode. The cantilever oscillates at the resonance frequency and detects the surface of the structures. In this mode, the cantilever moves along the surface. In 3D scanning mode, the cantilever is forcibly retracted by a set length (~ 0.1–10 μm) at each pixel, and the resistance strength is detected until it reaches the set point during the approach. Intracellular structures can be extracted from the 3D force data.

6. 3D Nanoendoscopy.
   a. Setting up AFM.
      i. Turn on the AFM (JPK Nanowizard 4, Bruker), stage controller (SPC-180713-04, Bruker), dish heater (RES-SAMPLE-HTR, Bruker), AFM-equipped fluorescence microscope (Eclipse Ti2, Nikon), light source (X-Cite XYLIS, Excelitas), and EMCCD camera (iXon Ultra 888, Andor).
      ii. Set the temperature of the dish heater to 37°C.

Note: If freshly fabricated probes are not used, plasma cleaning for 10 min at 2 mA (Meiwafosis) is recommended.

      iii. Place the dish on the stage. Open the lid and fix the dish with fixing plates.
      iv. Place the fabricated nanoneedle probe into the cantilever holder and fix it.
      v. Place the cantilever holder on the AFM head.
      vi. Take 50 μL of the medium from the dish with a micropipette and fill the space between the cantilever and cantilever holder with the medium.

Note: If this step is skipped, bubbles will appear on the cantilever and obstruct the optical path of the detection laser.

      v. Start the AFM software.
      vi. Place the AFM head on the stage.

Note: Ensure space between the cantilever and the bottom of the dish. If there is insufficient space, raise the Z-position of the AFM head.

      vii. Choose Force Spectroscopy mode.
      viii. Lower the AFM head and dip the cantilever in the medium.

Note: Be careful not to touch the cell or the bottom of the dish with the tip.

      ix. Bring into the focus of the cantilever using a low-magnification lens (10 or 20×) while observing the bright-field image.
      x. Align the AFM detection laser to the cantilever probe by maximizing the SUM value and centering the laser spot on the photodiode.

xi. Calibrate the cantilever using the calibration manager of the AFM software.

Note: If it is difficult to find the detection laser spot in the bright-field image, first check whether the laser cutting and fluorescence filters have been removed. Then, turn off the light of the bright field, increase the camera contrast, and find the tail of the laser spot.

xii. Choose the data saving folder in Saving Settings and turn on Autosave.

Note: Make sure to include Vertical Deflection data for saving.

b. Setting up fluorescence imaging.
   i. Start the fluorescence image acquisition software (NIS-Elements, Nikon).
   ii. Change to a high-magnification lens. To use the oil-immersion lens, place a drop of oil on the lens.
   iii. Bring the cells into focus.
   iv. Change to epifluorescence mode. Select the Cy5 filter (excitation, 620/60; dichroic, 660; barrier, 700/75).
   v. Take an image. Find the cell you want to observe.
c. Approaching the bottom of the cell.
   i. Approach the bottom surface of the dish. Set the Z position as 0.
   ii. Retract the cantilever to enough height so that the nanoneedle probe does not touch the sample (10–15 μm).
   iii. Move the cantilever to the desired position on the cell.
d. Approaching the cell surface and taking one force curve.
   i. Approach to the cell surface. Calculate the height of the cell to be measured.

Note: For approaching the cell surface, the set point should be less than 0.5 nN.

ii. Set the setpoint to 1–2 nN in the case of a 0.1 N/m spring constant cantilever, Z length 2 μm, Z speed 15 μm/s, 2 px/nm (sample rate 3000 Hz), approach speed 3 μm/s.

Note: The parameters should be adjusted depending on the cell type or target structure.

iii. Take one force curve.

Note: If the force curve shows a small peak around the cell height, it indicates that the probe successfully penetrated the cell membrane (indicated by the arrow in Figure 5A). If the force curve shows a smooth exponential increase without peaks, it indicates that the probe did not penetrate the cell membrane (Figure 5B). In this case, increase the set point, change the position, or change the nanoneedle.

Note: The 2D nanoendoscopy uses the same procedures until this step.

Note: Both types of probes (FIB and EBD nanoneedles) can be used.

e. 3D nanoendoscopy measurement.
   i. Change the mode to QI Advanced Imaging.
   ii. Set the setpoint to 1–2 nN, Z length 2 μm, Z speed 15 μm/s, 2 px/nm (Sample rate 3000 Hz), approach speed 3 μm/s.

Note: We usually use 64 × 64 pixel and scan size 0.5–20 μm. It takes about 30 min.

iii. Start scanning.

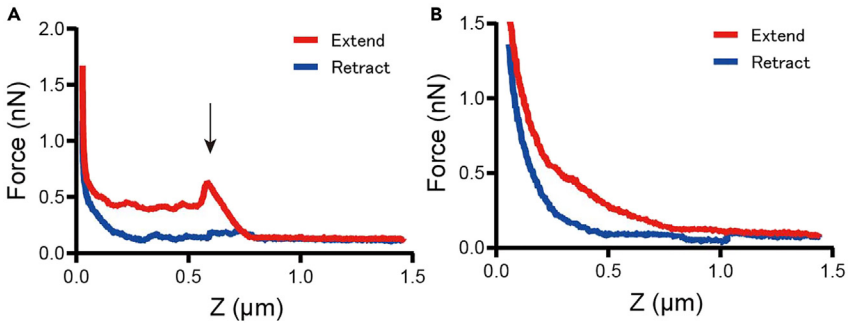


Figure 5. Comparison of the penetration for successful and not successful force curves

(A) Successful examples of the force curve. The small peak indicated by the arrow indicates the penetration of the cell membrane.

(B) Non-successful example of the force curve. The extend curve increases exponentially without any peaks.

7. 2D Nanoendoscopy (optional).
   a. The AFM setup is the same until Step 5d (iii) of 3D Nanoendoscopy.

   Note: Both types of probes (FIB and EBD nanoneedles) can be used.

   b. Set up for AC mode.
      i. Change to AC imaging mode.
      ii. Start the AC Feedback Mode Wizard and press the Run or Infinite icon to run a frequency sweep.
      iii. Zoom into the resonance peak and set the Drive Frequency and Setpoint.

   Note: Choose the setpoint at 50%–70% of the left side of the Lock-in amplitude peak.

   Note: We usually set 25 kHz as Drive Frequency and 5–10 nm as Setpoint (Peak to peak amplitude: ~12 nm) when we use BL-AC40TS-C2 (spring constant: 0.1 N/m).

      iv. Set the IGain to 50–150.
   c. 2D nanoendoscopy measurement.
      i. Start approach.

   Note: If the approach stopped at around the cell membrane, increase the setpoint to around 90%, change the place to be observed, or change the nanoneedle probe (See trouble-shooting problem 3).

      ii. Wait for 2 min to stabilize the nanoprobe inside the cell.
      iii. Start imaging. Compare the trace and retrace images. If these are the same, imaging is successful. Otherwise, readjust the setpoint or IGain.

   Note: We usually use 128 × 128 pixels and scan size 0.2–2 μm. It takes about 2 min. It is possible to increase pixel size as necessary.

   d. Post-scanning.
      i. After taking an image, change to Force Spectroscopy.
      ii. Take a force curve and check if the height of the cell is changed by comparing the force curve before and after the 2D imaging.
      iii. Approach the bottom of the dish and take an image of the substrate.

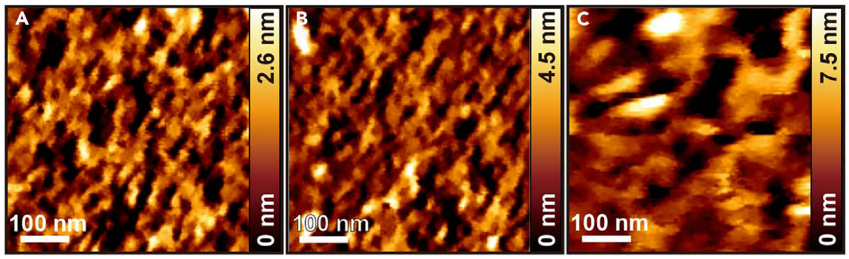


Figure 6. Bottom effect in 2D nanoendoscopy

(A and B) (A) Image of the dish bottom (B) Image of the intracellular bottom membrane. The structures appear to be affected by the bottom surface. Setpoint: 3 nm.

(C) Image of the bottom of the inside of the cell, which is not affected much by the bottom surface structures. Setpoint: 10 nm.

iv. Compare the substrate image with the 2D nanoendoscopy image because 2D nanoendoscopy at the bottom of the cell is likely to be affected by substrate corrugation (Figures 6A and 6B).

8. Cleaning.
   a. After the measurement is completed, take the AFM head off the stage and detach the cantilever holder. Remove the cantilever.
   b. Clean the cantilever holder in ddH_2O by sonication for 1 min.
   c. Discard the dish in the waste bag for disposal of biohazard materials.

Note: Do not throw dispose of the cell culture medium in the sink.

Note: Do not place the cell dish back in the CO_2 incubator.

   d. Close the AFM and fluorescence software. Turn off all the equipment that has been started up.
   e. The locking part of the cantilever holder in the AFM head should be cleaned using ddH_2O and ethanol.
   f. Clean the lens if you use the oil immersion lens using lens paper and a cleaner.
   g. Transfer data.

Visualization

Timing: 2 h

For 3D visualization of 3D nanoendoscopy data, the data is first pre-processed using custom-built Python and LabVIEW (National Instruments) programs. Next, the data are imported into Voxler 4 (Golden Software) and visualized. For 2D nanoendoscopy, JPK Data Processing Software (Bruker) or Gwyddion (open-source Software)[13] is used with normal processing (Plane Fit, Line Leveling, and Median Filter). In this section, we describe the protocol for the visualization of 3D nanoendoscopy-AFM.

Note: We uploaded the custom-built programs (https://github.com/fukuma-lab/3D-AFM_Analysis).

The process flow is illustrated in Figure 7. This process varies depending on what is to be visualized and the type of data. The common process is described in Step 7. We perform the following processing to visualize the upper membrane (Step 8), actin fibers (Step 9), and the lower membrane (Step 10).

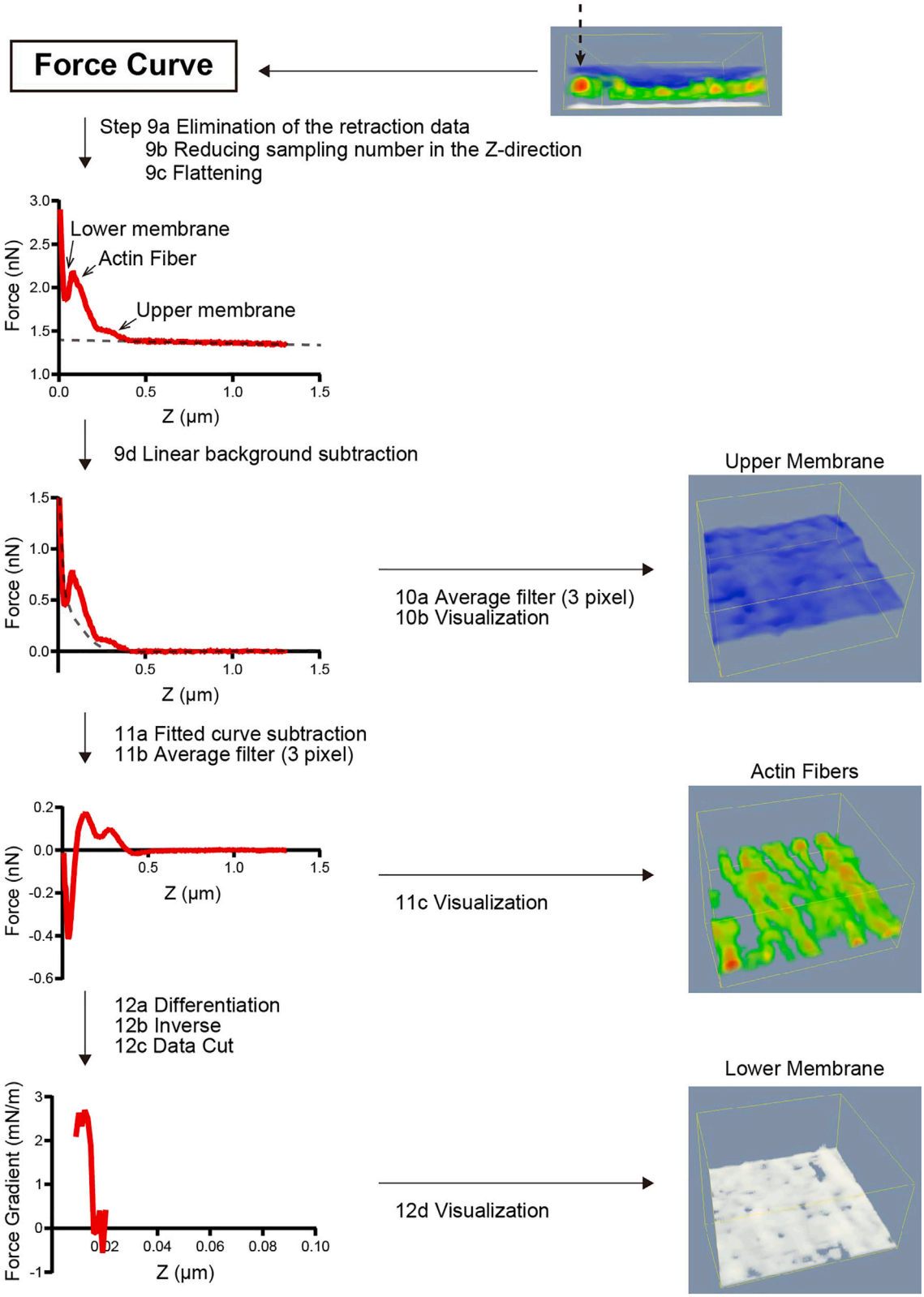
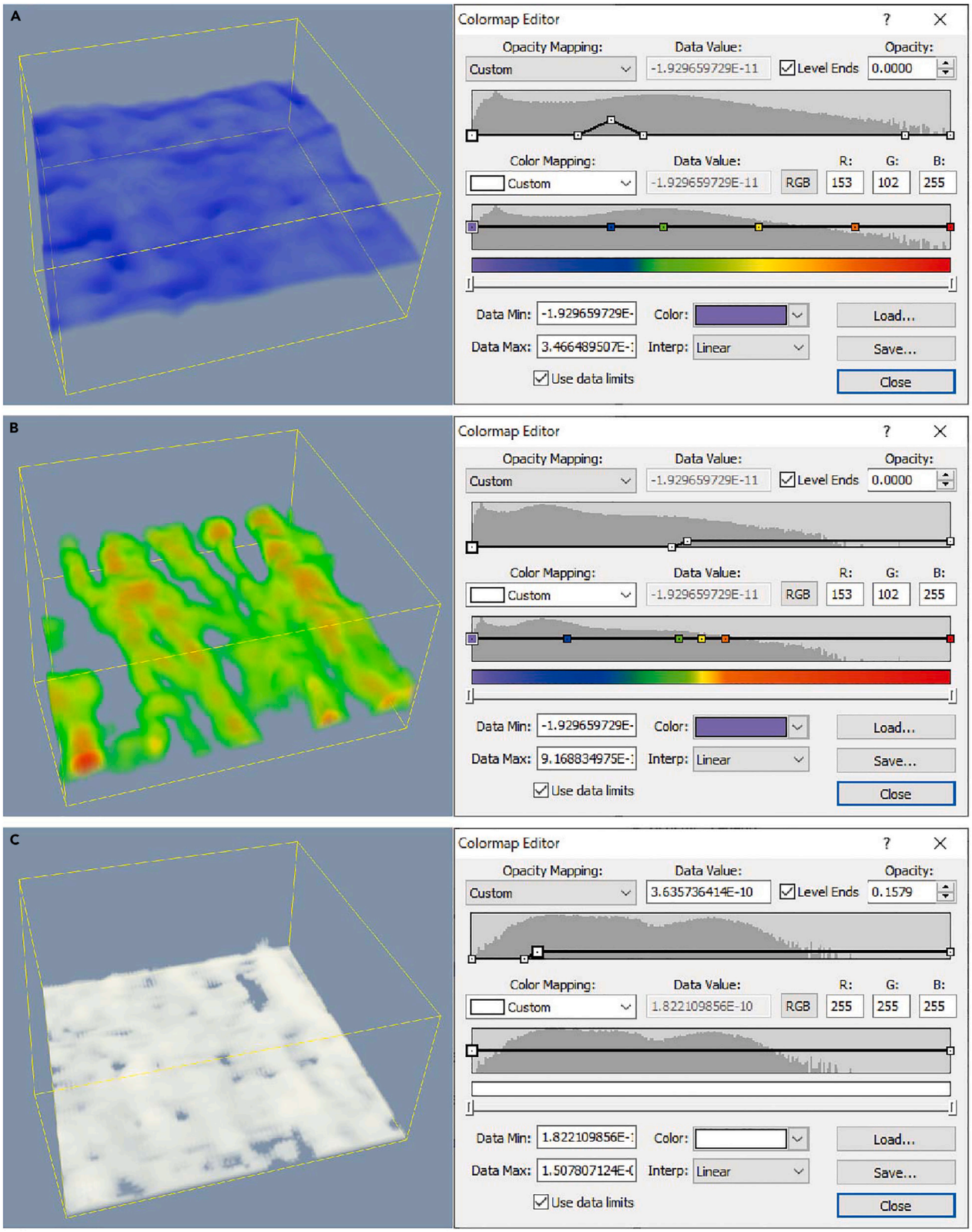


Figure 7. F-z curves processing diagram flow
Each step corresponds to the step in the text.


A
Colormap Editor
Opacity Mapping:
Data Value:
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-1.929659729E-11
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Data Min:
-1.929659729E-
Color:
Load...
Data Max:
3.466489507E-
Interp:
Linear
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Colormap Editor
Opacity Mapping:
Data Value:
Opacity:
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Opacity Mapping:
Data Value:
Opacity:
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3.635736414E-10
Level Ends
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Color Mapping:
Data Value:
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1.822109856E-10
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1.507807124E-
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Figure 8. Colormap and transparency settings for visualization of 3D nanoendoscopy
(A) The setting of Colormap and Opacity Map for the upper membrane.
(B) The setting of Colormap and Opacity Map for actin fibers.
(C) The setting of Colormap and Opacity Map for lower membrane.

9. Common processing.
   a. Eliminate the retraction data from 3D nanoendoscopy data.
   b. Reduce the sampling number in the Z-direction.

   Note: Because the resolution in the Z direction is usually higher than that in the XY direction, the resolution in the Z direction can be reduced by at least 1/2 to reduce the file size.

   c. Flatten the image.
   d. Subtract the background with liner fitting.

   Note: All visualizations use the same processing up to this point.

10. Visualization of the upper membrane.
    a. Process with a 3-pixel averaging filter.
    b. Import the data into Voxler 4. Visualize the upper membrane using VolRender. Adjust the Colormap and Opacity Map as shown in Figure 8A.
11. Visualization of the actin stress fiber.
    a. Subtract the background with the following fitted curve from the force curve to the data after Step 7d.

$$F(z) = A\exp\left(\frac{-(z - z_0)}{\lambda}\right)$$

    A and Z_0 are fixed so that the curve passes through the point closest to the substrate on the force curve. In addition, the force values are used as the standard error of fitting for force-dependent weighting.
    b. Perform a 3-pixel averaging filter.
    c. Import the data to Voxler 4 and visualize the actin filament in VolRender by setting the Colormap and Opacity Map as shown in Figure 8B.
12. Visualization of the lower membrane.
    a. Calculate the differentiation of each force curve using the data after Step 9b.
    b. Reverse the positive and negative numbers.
    c. Import the data to Voxler 4.
    d. Eliminate the data except where the lower membrane exists using Subset command.
    e. Visualize the lower membrane in VolRender by setting the Colormap and Opacity Map as shown in Figure 8C.

EXPECTED OUTCOMES

Successful application of this protocol allows users to fabricate long, narrow needle-like tips with a diameter of less than 200 nm (Figures 3 and 4), find the target position based on the fluorescence image (Figure 1), and observe the overall 3D live cell image using 3D nanoendoscopy (Figure 9) and the nanoscale intracellular structure image of a living cell in 3D and 2D nanoendoscopy (Figures 10 and 11). We successfully observed intact actin stress fibers and the actin cortex with unprecedented resolution. This technique can be used to observe other organelles inside living cells, such as microtubules, intermediate filaments, nuclei, mitochondria, focal adhesions, endoplasmic reticulum, lysosomes, Golgi apparatus, organelle connections, and liquid-liquid phase-separated structures. This protocol can be expected to become a standard tool for studying nanoscale structures in living cells.

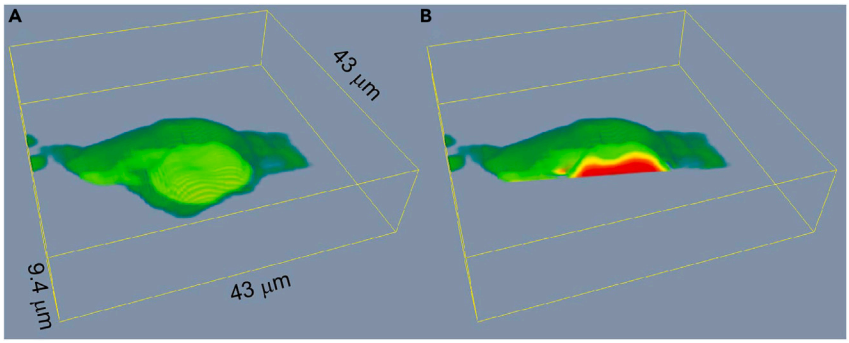

Figure 9. Overall cell image using 3D nanoendoscopy
(A) Overall BALB 3T3 cell image using 3D nanoendoscopy.
(B) Cross-section image of A.

LIMITATIONS

As cells move and intracellular structures change dynamically, images can be distorted during observation. 3D nanoendoscopy takes more than 30 min to obtain one 64 × 64-pixel image with a 2 µm Z height. Occasionally, the image is affected by XY drift, but it is possible to reduce the scan time by reducing the Z height or by increasing the scan speed within a range that does not affect the image quality. Since 2D nanoendoscopy takes approximately 2 min per image (Line Rate 2 Hz, 128 × 128 pixels), the effect of XY drift is small, but it may be harder to detect the surface of intracellular structures than with 3D nanoendoscopy.

TROUBLESHOOTING

Problem 1

The tip bent during FIB fabrication in Step 3.

Potential solution

Cutting a large area using a high electrical current may cause bending. Therefore, the cantilever tip should be gradually sharpened using relatively low currents, particularly when the needle width is less than 500 nm.

Problem 2

The EBD tip length cannot be increased in Step 4.

Potential solution

Make sure the distance between the cantilever and the lens is approximately 4 mm. A long-distance decreases the deposition efficiency. Alternatively, drift results in thicker and shorter EBD tips. Waiting 10–30 min before starting carbon deposition may improve this situation.

Problem 3

It does not appear to penetrate the cell membrane in Step 5.

Potential solution

If a small increase followed by a plateau or a sharp drop is observed around the height of the cell membrane in the force curve, the cell membrane can be considered to have been penetrated; however, if the force continuously rises during the approach, it means that the cell membrane has not been penetrated (Figure 5). In this case, the following three methods can be attempted: increasing the setpoint, changing the positions, or changing the nanoneedle tip.

Problem 4

Cells detach from the dish during or after nanoendoscopy in Step 5 or 6.

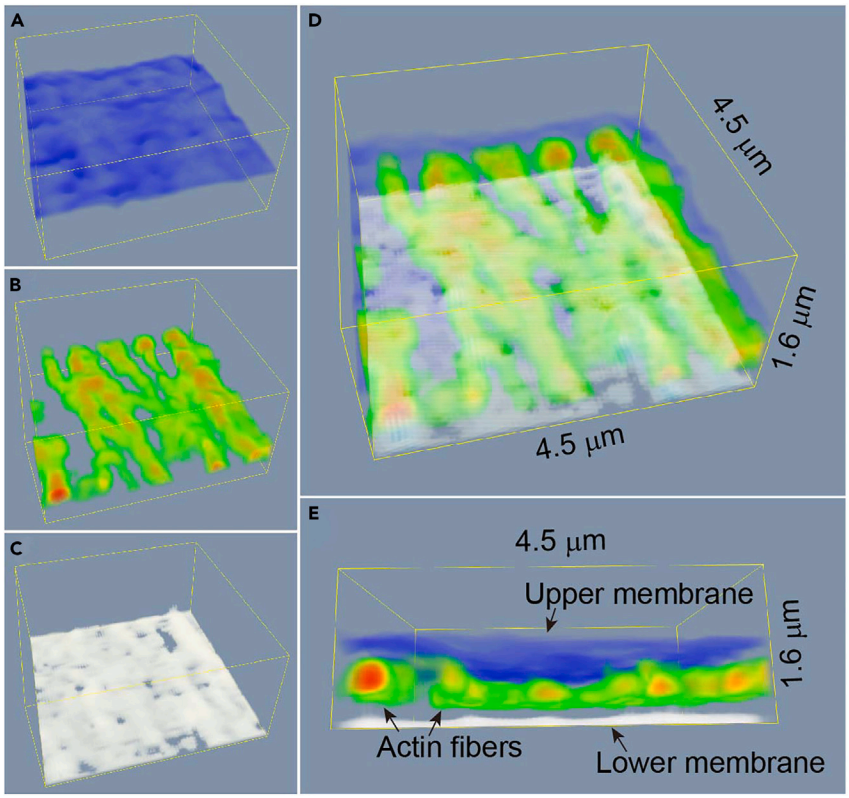

Figure 10. Expected result of intracellular high-resolution 3D nanoendoscopy

(A) Upper membrane.

(B) Actin fibers.

(C) Lower membrane.

(D) Overlaid image of three components.

(E) Side view of D.

Potential solution

If the cells are not in good condition, they tend to detach from the dish. Therefore, we examined cell culture conditions under which this problem occurred. Thinner probes are less likely to cause cell detachment. Coating the dish with collagen, poly-L-lysine, or Cell-Tak™ may prevent cell detachment.

Problem 5

The image is very noisy or distorted in Steps 5 or 6.

Potential solution

Optimize the parameters so that the image quality is improved. The setpoint is the first parameter to be adjusted. Cell migration can also lead to image deterioration. Cell-Tak™ can be used to inhibit cell migration.

Problem 6

The 2D nanoendoscopy image appears to be similar to the bottom of the dish in Step 6.

Potential solution

Increase the setpoint (80%–90% of the resonance amplitude). Alternatively, an approach in Force Spectroscopy mode with a low setpoint and switch to AC mode can be used.

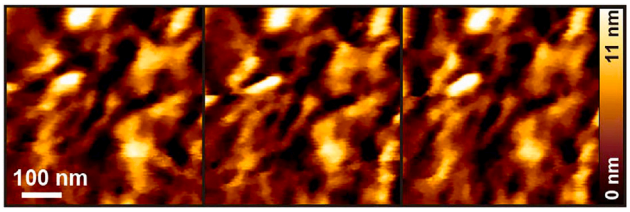

Figure 11. Expected results of intracellular high-resolution 2D nanoendoscopy, showing the continuous three images acquired every 2 min

Structures that can be corresponded in each frame indicate that these structures are not artifacts but real structures.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact Takeshi Fukuma (fukuma@staff.kanazawa-u.ac.jp).

Materials availability

This study did not generate new unique reagents.

Data and code availability

The custom-built analysis software is available on the GitHub site (https://github.com/fukuma-lab/3D-AFM_Analysis).

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AUTHOR CONTRIBUTIONS

M.P., K.Miyazawa, H.F., and C.N. established the method for the fabrication of AFM nanoneedles. M.P. and T.F. established the nanoendoscopy methods. K.Miyazawa and Miyata maintained the AFM system. T.I. established a method to evaluate cell variability. K.M. and S.F. developed the custom-built analysis software. T.I. and M.S.A. performed the experimental work. T.I. wrote the manuscript. All of the authors reviewed the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Penedo, M., Miyazawa, K., Okano, N., Furusho, H., Ichikawa, T., Alam, M.S., Miyata, K., Nakamura, C., and Fukuma, T. (2021). Visualizing intracellular nanostructures of living cells by nanoendoscopy-AFM. Sci. Adv. *7*, eabj4990. https://doi.org/10.1126/sciadv.abj4990.

2. Penedo, M., Shirokawa, T., Alam, M.S., Miyazawa, K., Ichikawa, T., Okano, N., Furusho, H., Nakamura, C., and Fukuma, T. (2021). Cell penetration efficiency analysis of different atomic force microscopy nanoneedles into living cells. Sci. Rep. *11*, 7756. https://doi.org/10.1038/s41598-021-87319-3.

3. Decelle, J., Veronesi, G., Gallet, B., Stryhanyuk, H., Benettoni, P., Schmidt, M., Tucoulou, R., Passarelli, M., Bohic, S., Clode, P., and Musat, N. (2020). Subcellular Chemical Imaging: New Avenues in Cell Biology. Trends Cell Biol. *30*, 173–188. https://doi.org/10.1016/j.tcb.2019.12.007.

4. Leis, A., Rockel, B., Andrees, L., and Baumeister, W. (2009). Visualizing cells at the nanoscale. Trends Biochem. Sci. *34*, 60–70. https://doi.org/10.1016/j.tibs.2008.10.011.

5. Garcia, R. (2020). Nanomechanical mapping of soft materials with the atomic force microscope: methods, theory and applications. Chem. Soc. Rev. *49*, 5850–5884. https://doi.org/10.1039/d0cs00318b.

6. Stühn, L., Fritschen, A., Choy, J., Dehnert, M., and Dietz, C. (2019). Nanomechanical subsurface mapping of living biological cells by force microscopy. Nanoscale *11*, 13089–13097. https://doi.org/10.1039/c9nr03497h.

7. Guerrero, C.R., Garcia, P.D., and Garcia, R. (2019). Subsurface Imaging of Cell Organelles by Force Microscopy. ACS Nano *13*, 9629–9637. https://doi.org/10.1021/acsnano.9b04808.

8. Shekhawat, G.S., and Dravid, V.P. (2005). Nanoscale imaging of buried structures via scanning near-field ultrasound holography. Science *310*, 89–92. https://doi.org/10.1126/science.1117694.

9. Tetard, L., Passian, A., Venmar, K.T., Lynch, R.M., Voy, B.H., Shekhawat, G., Dravid, V.P., and Thundat, T. (2008). Imaging nanoparticles in cells by nanomechanical holography. Nat. Nanotechnol. *3*, 501–505. https://doi.org/10.1038/nnano.2008.162.

10. Lukinavičius, G., Reymond, L., D'Este, E., Masharina, A., Göttfert, F., Ta, H., Güther, A., Fournier, M., Rizzo, S., Waldmann, H., et al. (2014). Fluorogenic probes for live-cell imaging of the cytoskeleton. Nat. Methods *11*, 731–733. https://doi.org/10.1038/nmeth.2972.

11. Miyazawa, K., Izumi, H., Watanabe-Nakayama, T., Asakawa, H., and Fukuma, T. (2015). Fabrication of electron beam deposited tip for atomic-scale atomic force microscopy in liquid. Nanotechnology *26*, 105707. https://doi.org/10.1088/0957-4484/26/10/105707.

12. Uchihashi, T., Kodera, N., and Ando, T. (2012). Guide to video recording of structure dynamics and dynamic processes of proteins by high-speed atomic force microscopy. Nat. Protoc. *7*, 1193–1206. https://doi.org/10.1038/nprot.2012.047.

13. Necas, D., and Klapetek, P. (2012). Gwyddion: an open-source software for SPM data analysis. Cent. Eur. J. Phys. *10*, 181–188. https://doi.org/10.2478/s11534-011-0096-2.