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Measurement of Mechanical Properties of Intact Endothelial Cells in Fresh Arteries

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1. Introduction

Atomic force microscopy (AFM) has been successfully applied not only to the topography of biological specimens but also to the measurement of their local mechanical properties. This technique is very useful for imaging such biological specimens as cells, proteins, and DNA, because no special treatments of samples are required ***(1,2)***. The measurement of local mechanical properties in a living cell is achieved with a nanoindentation technique ***(3)***. The method has been applied to several kinds of cultured cells, including Madin-Darby canine kidney (MDCK) cells ***(4)***, myocytes ***(5)***, fibroblasts ***(6,7)***, and endothelial cells ***(8,9)***. However, morphology and properties are different between cells cultured in vitro on substrate and intact cells in vivo ***(10)***.

Vascular endothelial cells form a lining of the inner surface of blood vessel and are always exposed to blood flow. Because their morphology, internal structure, functions, and mechanical properties are closely related to each other and are strongly affected by hemodynamic factors, the measurement of the mechanical properties of the cells *in situ* is very important. Unlike the other cell types, endothelial cells are not embedded in tissues. Therefore, if a blood vessel is cut opened, we can observe intact endothelial cells and measure their mechanical properties with AFM ***(11)***.

This chapter explains an AFM method for the measurement of the local mechanical properties of intact endothelial cells in fresh arteries. The artery in living animals is exposed under anesthesia, and marked with stain dots on the outer surface along the axial direction. The distances between the dots and the external diameter of the artery are measured to give the in vivo axial and circumferential lengths, respectively. Immediately after sacrifice, the artery is excised, and temporarily stored in Hanks' balanced salt solution (HBSS) at 4°C.

From: *Methods in Molecular Biology, vol. 242: Atomic Force Microscopy: Biomedical Methods and Applications*
Edited by: P. C. Braga and D. Ricci © Humana Press Inc., Totowa, NJ

First, the sensitivity of the AFM system is determined using a cantilever for each measurement and a glass cover slip. Immediately before AFM observation, strip specimens are cut out from the artery. Each specimen is fixed on silicone rubber with the endothelial side up using pins at its in vivo axial and circumferential lengths. The specimen attached to the rubber is covered with HBSS of room temperature and is mounted onto the sample stage of the AFM. After the surface topograph of the endothelium is determined, force curves are obtained at various locations in each endothelial cell. Force–indentation relations are determined from the force curves. Stiffness is calculated from the force–indentation relations.

2. Materials

1. Living animal.
2. Pentobarbital sodium solution.
3. Gentian violet solution: dissolve gentian violet pellets in distilled water to make saturated solution; store the solution in a refrigerator at 4°C (*see* **Note 1**).
4. HBSS: mix 136.8 m*M* NaCl, 5.3 m*M* KCl, 0.3 m*M* $Na_2HPO_4 \cdot 12\ H_2O$, 0.4 m*M* KH_2PO_4, 0.5 m*M* $MgCl_2 \cdot 6H_2O$, 0.4 m*M* $MgSO_4 \cdot 7H_2O$, 1.3 m*M* $CaCl_2$, 4.2 m*M* $NaHCO_3$, and 5.6 m*M* dextrose in distilled water, filtrate with a membrane filter having the pore size of 0.2 μm, adjust the pH to 7.4, and store in a refrigerator at 4°C (*see* **Note 2**).
5. Silicone rubber: cut a 3-mm thick silicone rubber sheet into appropriate size (e.g., 5 × 5 mm). Ultrasonically wash it with acetone, ethanol, and then distilled water for 10 min for each, and dry it.
6. Stainless steel pin: cut an approximately 0.3-mm diameter stainless-steel wire into short pieces each having the length of about 4 mm and bend one end of each to an angle of about 90° to make an L-shape. Ultrasonically wash them in the same way as that for the silicone rubber, and dry (*see* **Note 3**).

3. Methods

3.1. Resection of Arterial Segment

3.1.1. Exposure of Artery Under Anesthesia

1. Induce general anesthesia to an animal by the injection of pentobarbital sodium into the vein or the abdominal cavity (*see* **Note 4**).
2. Shave and incise the skin.
3. Carefully expose an artery, and dissect it from the surrounding tissues using forceps (*see* **Note 5**).

3.1.2. Measurement of Arterial Dimensions

1. Measure the external diameter of the artery with a caliper (*see* **Note 6**).
2. Dot with gentian violet on the outer surface along the axial direction at 3- to 5-mm intervals.
3. Measure the distances between the dots with a caliper.

3.1.3. Resection of Arterial Segment and Storage

1. Inject an excess of pentobarbital sodium solution into the vein, and wait until cardiac arrest (*see* **Note 7**).
2. Immediately after sacrifice, cannulate the artery with a syringe needle, and gently flush it with HBSS of room temperature to wash out blood (*see* **Note 8**).
3. Ligate the artery at proximal and then at distal position with threads.
4. Resect an arterial segment with a surgical scissors between the ligations.
5. Immediately immerse the resected segment in HBSS of room temperature in a Petri dish and gently wash the segment (*see* **Note 9**).
6. Put the segment in a bottle with fresh HBSS room temperature and store it at 4°C.

3.2 Preparation of Arterial Wall Specimen

3.2.1. Cutting Out Specimen Strips

1. Transfer the arterial segment from the bottle to a Petri dish.
2. Measure the external diameter and the distance between the gentian violet dots on the outer surface. Calculate the in vivo circumferential and axial extension ratios (ratio of in vivo dimension to in vitro one).
3. Cut out rectangular specimen strips from the segment using a microscissors and a surgical blade (*see* **Note 10**).

3.2.2. Attachment of Specimen to Silicone Rubber

1. Place each specimen strip on a silicone rubber with the endothelial side up and cover the endothelium with a droplet of HBSS to keep wet.
2. Fix the specimen to the rubber with L-shaped stainless-steel pins, stretching to the in vivo axial and circumferential length.
3. Soak the specimen in HBSS of room temperature.

3.3. AFM

3.3.1. Mounting of Specimen on AFM Sample Stage

1. Mount a clean glass cover slip on the sample stage of AFM. Attach a cantilever to the cantilever holder of AFM and place it over the cover slip. After putting a drop of HBSS at room temperature on the cover slip and soaking the cantilever in the drop, adjust a laser beam from AFM head so as to strike the backside of the end part of the cantilever. Then, scan the cantilever or the cover slip, and obtain an image of the cover slip surface. Subsequently, determine the sensitivity of the system using the function of sensitivity measurement of AFM (*see* **Note 11**).
2. Input the values of the cantilever's spring constant and the above-determined sensitivity (**item 1**; *see* **Note 12**).
3. Obtain a force curve from the glass cover slip in the force curve mode of AFM, and confirm the suitability of the cantilever (*see* **Note 13**). Remove the cover slip from the sample stage.
4. Mount a specimen attached to the silicone rubber onto the sample stage of the AFM, and cover it with HBSS at room temperature (*see* **Note 14**).

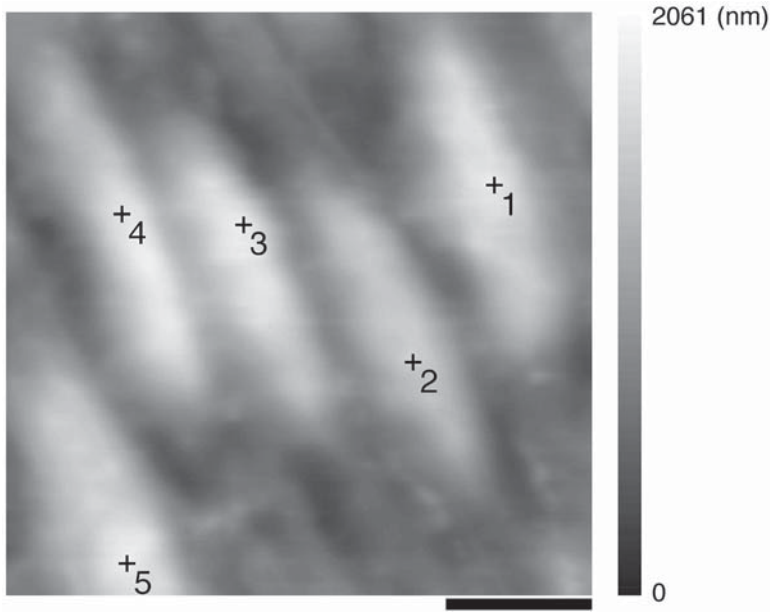

Fig. 1. AFM image of living endothelium in a rabbit abdominal aorta. Plus symbols indicate the highest points in individual endothelial cells. Black bar is 10 µm. Grey scale shows relative height.

3.3.2. Topography of Endothelial Surface

1. Set the *x-y* scanning range as large as possible.
2. Take a topograph of the endothelial surface in the contact mode at a low scanning rate (less than 1 Hz). Keep imaging force as low as possible to avoid the damage of endothelial cells. Change HBSS every 30 min.
3. Using the zoom function of AFM and monitoring the image, reduce the scanning size to the area of interest, and scan again to obtain a magnified image (*see* **Fig. 1** and **Note 15**).

3.3.3. Measurement of Force Curve

1. Obtain force curves from endothelial cells (*see* **Note 16**).
2. Force–indentation relation is determined from each force curve, where indentation is obtained from the difference between the vertical displacement of the piezo and the cantilever deflection (*see* **Fig. 2**). From the force–indentation relation, stiffness is determined (*see* **Note 17**).

4. Notes

1. The addition of very small amount of formaldehyde may help the stain attach to the adventitial surface of the artery.

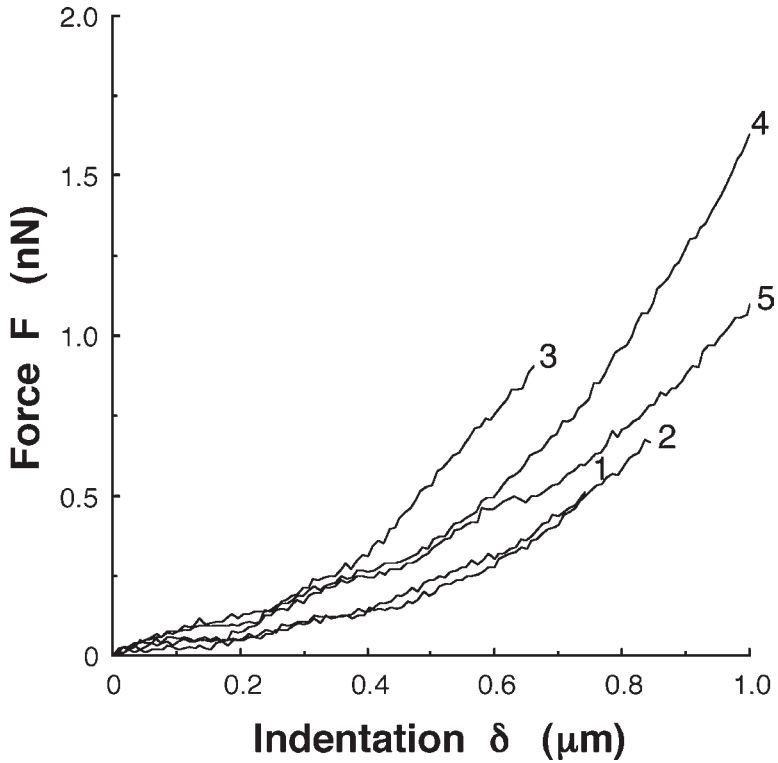

Fig. 2. Force–indentation curves obtained from the highest points in the endothelial cells shown in **Fig. 1.** The numbers attached to the curves correspond to the locations indicated in the AFM image.

2. HBSS is commercially available.
3. One of the tips of the wire should be made sharp so as to be easily pierced into the silicone rubber through the arterial wall.
4. Do not apply too much pentobarbital sodium to avoid respiratory failure. The dosage depends on animal species and weight. Inhalation anesthesia can also be used.
5. When exposing and resecting arterial segments, avoid bleeding as far as possible. Arteries contract when contacting with blood, which makes difficult to precisely measure the in vivo external diameter. Never grip arterial wall itself with forceps to avoid wall damage and detachment of endothelial cells. Rubbing and stretching of arterial wall also should be avoided as far as possible. Always keep the artery wet with HBSS at room temperature during the procedure.
6. If a noncontact measurement method is available, it is recommended.
7. If arteries for study are located in the legs or neck, they may be resected before sacrifice.

8. Do not flush the artery at high flow rate to avoid the detachment of endothelial cells.
9. When handling the arterial segment, hold loose fibers on the outer surface. Do not touch arterial wall itself. If blood remains inside the resected artery, gently wash it out.
10. Do not scrape the inner surface of the segment to prevent the detachment of endothelial cells. Always keep the specimen wet with HBSS.
11. Sensitivity defines a relation between the displacement of the cantilever tip (or the deflection of the cantilever) and the voltage applied to the piezo of the AFM, which is determined by pressing the tip against the cover slip using the piezo. The laser beam is reflected from the backside of the end part of the cantilever toward a segmented photodiode in the AFM head. The photodiode senses the shift of the reflected laser beam, which is induced by the displacement of the cantilever tip. The sensitivity is expressed as a relation between the output voltage from the photodiode and the voltage applied to the piezo. Thus, the displacement of the cantilever tip (or the deflection of the cantilever) can be obtained from the voltage output of the photodiode and the sensitivity. Because the sensitivity is changeable depending on the striking position of the laser beam on the cantilever, do not change the alignment of the beam until all force curve measurements are completed. The method for the determination of sensitivity is specified for each AFM apparatus and software.
12. A spring constant is given for each cantilever. Because the actual value may be slightly different from the nominal value, it is advisable to measure or calculate it in advance. There are several methods for the determination of the spring constant of a cantilever, including a thermal vibration method.
13. A force curve shows a relation between the force applied to a specimen and the displacement of the piezo. Force is calculated by multiplying the spring constant by the deflection of the cantilever (output voltage from the photodiode). The deflection of cantilever is obtained from the sensitivity and the voltage applied to the piezo as mentioned in **Note 11**. The force curve of a glass cover slip is obtained from pushing the cantilever tip against the cover slip by the drive of the piezo only in z direction at a constant rate. In case the initial linear portion of the curve is not clearly observed, discard the cantilever and use a new one. The method for the determination of force curve is different in each AFM apparatus and software. A large-area piezo scanner having the maximum x–y scanning range of about 100×100 μm and the z range of more than 10 μm should be used, partly because the length of endothelial cells is 20–50 μm and partly because the arterial wall is not flat even if it is pinned under tension. Select a soft cantilever having a spring constant of, for example, less than 0.1 N/m and a pyramidal or a conical tip.
14. The specimen should be firmly fixed to the sample stage to obtain a good image. The silicone rubber easily adheres to the surface of the sample stage without glue.
15. A clear image is necessary to obtain a good force curve. The cantilever should be withdrawn from the specimen surface before setting the new (smaller) scanning area, because the thickness of arterial wall is not uniform and the endothelial surface is not flat. If the cantilever tip remains in contact with the specimen sur-

face, it may scratch and destroy the endothelium, and debris from cells and/or tissue may stick to the tip. This should be avoided because good images and force curves cannot be obtained with such a contaminated tip.

16. All the measurements should be completed within 12 h after the sacrifice of animals to avoid the deformation and structural change of endothelial cells.
17. There are various methods for the analysis of force–indentation relations.

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