# 7

# AFM Study of Surface Structure Changes in Mouse Spermatozoa Associated With Maturation

**Hiroko Takano and Kazuhiro Abe**

## 1. Introduction

If a sample has a comparatively even surface and is fixed on a sample stage, atomic force microscopy (AFM) will give a clear image of the surface structure at subnanometer level ***(1,2)***. Because a sperm head is flat and can be attached on the slide glass firmly after it is fixed, we consider that AFM is the competent tool for the study of the sperm surface structure.

Spermatozoa are produced in the testis and transferred into the epididymis. In the epididymis, they acquire a fertilization ability and mobility, which is called sperm maturation ***(3)***. It has been proved biochemically and immunocytochemically that glycoproteins on or in the sperm plasma membrane are altered, masked, or replaced by new glycoproteins of epididymal origin in the epididymal duct ***(4–6)***. These changes are considered to be necessary for fertilization ***(3,7)***. Thus, it is probable that the sperm surface structure changes progressively in the epididymal duct ***(8)***. We reported the changes in the surface structure of the spermatozoa in the hamster epididymis by AFM ***(9)***. Subsequently, we have studied the surface structure of the spermatozoa from mouse epididymis by AFM. In these studies we developed several tricks for improving AFM images. In this chapter, we will show our recent results and the materials and methods used , including the tricks we learned in these studies.

Histologically, the mouse epididymis is divided into five regions (segments I–V) in adult male mice **(Fig. 1;** refs. ***10,11***). These segments perform different roles in the process of sperm maturation ***(12)***. Spermatozoa are immature in segment I but mature in segment V ***(13)***. The epithelial cells in segment II appear to secrete glycoproteins for sperm maturation ***(10,14,15)***, so we examined the segments I, II, and V of the mouse spermatozoa by AFM.

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

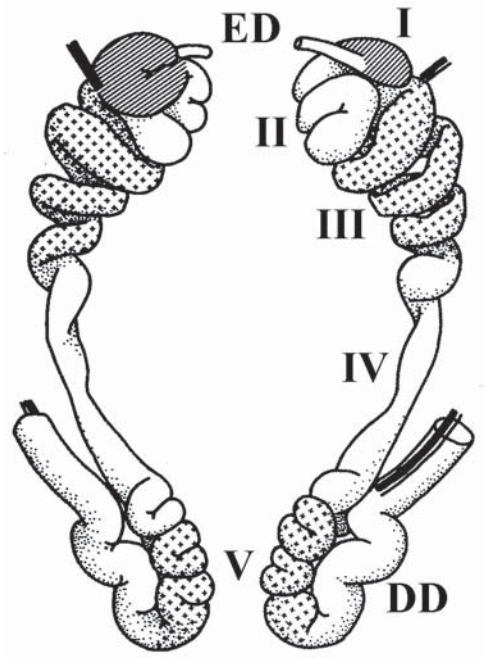


Fig. 1. Schematic diagram of the histological segmentation of the mouse epididymis. ED, efferent duct; I–V, epididymal segments; DD, deferent duct.

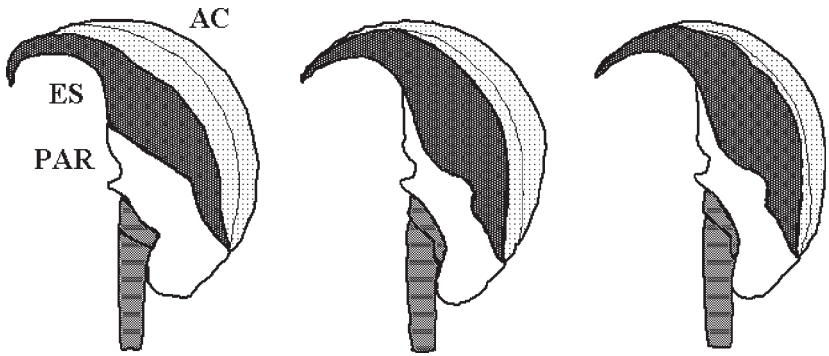


Fig. 2. Diagrams traced from the AFM images of the mouse spermatozoa from segments I *(left),* II *(middle),* and V *(right).* AC, acrosomal cap; ES, equatorial segment; PAR, post-acrosomal region.

The surface of the sperm head is classically divided into two domains, the acrosomal region and postacrosomal region (**Fig. 2**). The acrosomal region is subdivided into the acrosomal cap and equatorial segment (**Fig. 2**). These domains play the different roles at fertilization ***(16,17)***. The plasma membrane of the equatorial segment is maintained after acrosome reaction and fuses with

the plasma membrane of an oocyte at fertilization ***(3)***. Because of this, our study focused on the changes in the surface structure of the equatorial segment.

Dynamic force mode AFM, as used in our laboratory, provides images in constant height mode, amplitude mode, and phase mode. Images in constant height mode offer the topographical information of the sample height (**Fig. 3**). Those in amplitude mode and phase mode offer well-defined contours of the projections and hollows based on the changes in oscillation amplitude and phase, respectively (**Figs. 4–6**). Using a combination of these modes, the sperm head was shown to be covered with particles whose diameter was < 100 nm. The size of the surface particles differed between the acrosomal region and postacrosomal region in the same spermatozoon. Particularly, the particles covering the equatorial segment changed in size dramatically during the passage through the epididymal duct **(Figs. 4–6**). The surface of the equatorial segment was covered with the particles of 20–50 nm in diameter in segment I, 50–80 nm in segment II, and 20 nm in segment V. These size differences generated the different morphological surface features among the spermatozoa from segments I, II, and V (**Figs. 4–6**). Because the epithelial cells in segment II appear to secrete acid glycoproteins, which play a role as a sperm maturing factor ***(10,14,15)***, large particles covering the segment II sperm surface may be glycoproteins secreted from the epithelial cells in segment II.

AFM images also demonstrated the changes in shape of the acrosomal cap. The acrosome cap is flat and wider in the immature spermatozoa from segments I and II than in mature spermatozoa from segment V (**Figs. 2** and **3A** through **6A**). Thus, an application of AFM for the study of the surface structure of the mouse spermatozoa brought us noteworthy new findings.

## 2. Materials

1. A mature male dd-mouse at 90 days of age.
2. Modified tyrode solution (this medium is used for making sperm suspension and for washing spermatozoa by centrifugation): 500 mL distilled water, 2.05 g NaCl, 0.1 g KCl, 0.1 g $CaCl_2$(anhyd.), 0.05 g $MgCl_2/H_2O$, 0.025 g $NaH_2PO_4/H_2O$, 1.5 g $NaHCO_3$, approx pH 8.0. This stock solution can be used for one month if stored at 4°C.
3. Fixative: 2% glutaraldehyde in 0.1 *M* cacodylate buffer.
4. Ethyl alcohol (anhyd.).
5. 3-methylbutyl acetate.
6. Nitrogen gas.
7. A double-edged razor.
8. Small vials.
9. Test tubes.
10. Test-tube stand.
11. Disposable pipets.
12. Plastic dishes for ethyl alcohol.

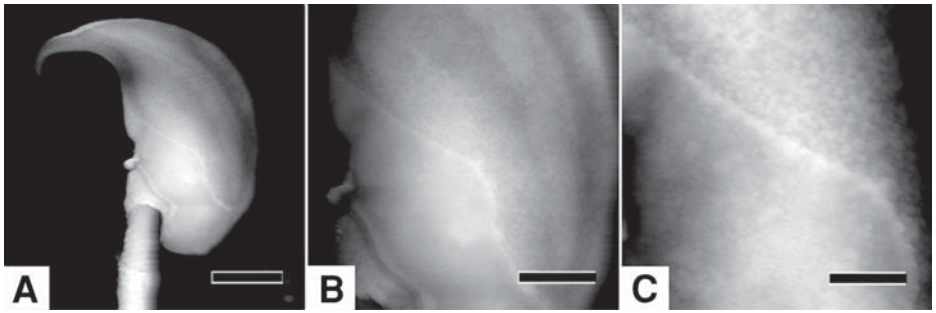


Fig. 3. AFM image in constant force mode of epididymal segment I.

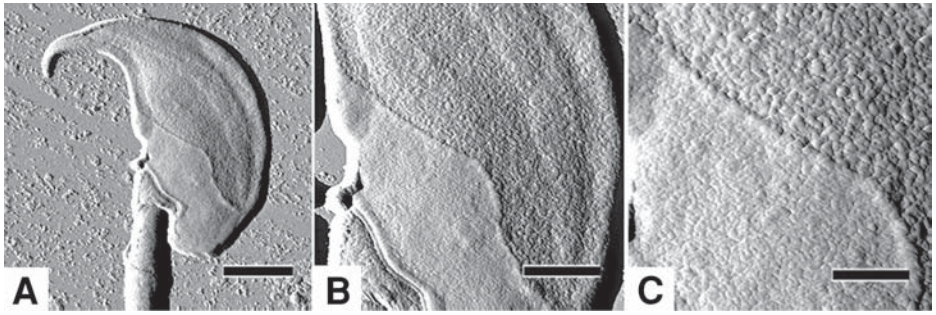


Fig. 4. AFM image in amplitude mode of the same spermatozoon as in **Fig. 3.**

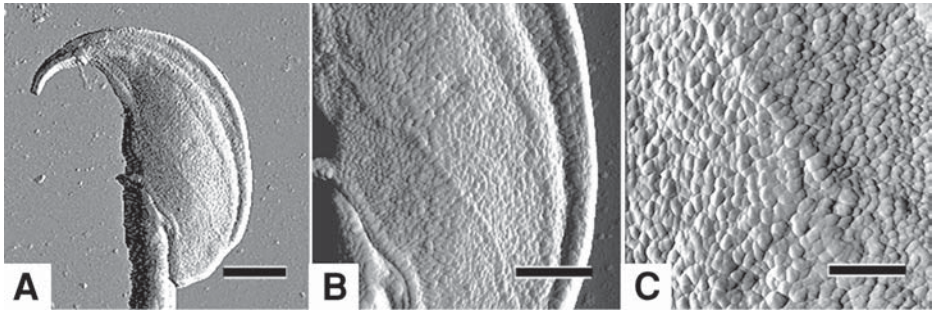


Fig. 5. AFM image in amplitude mode of the spermatozoon from segment II.

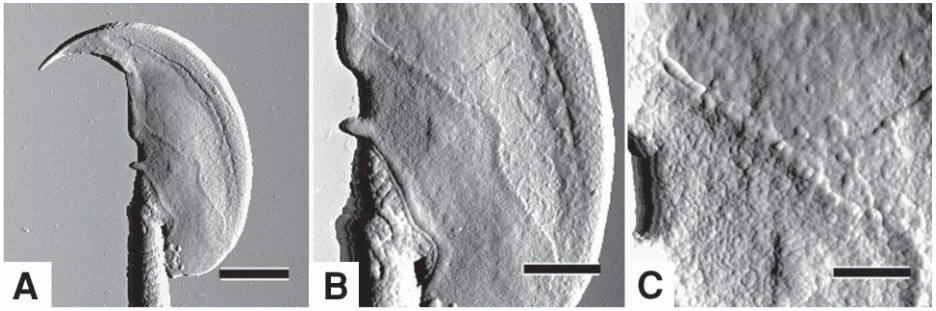


Fig. 6. AFM image in amplitude mode of the spermatozoon from segment V.

13 A glass dish for 3-butyl-ethyl acetate.
14. Micro slide glass coated with adhesive material (Superfrost, Matunami Glass IND., LTD, Japan).
    a. Cut the micro slide glass into 7-mm squares.
    b. Mark the surface of one side of of the slide glass squares with the glass cutter to distinguish the side of the sperm attachment.
15. Centrifugal separator.
16. Critical point drier.
17. AFM.
    a. Probe station/Unit SPI3800 /SPA300 (Seiko Instruments, Japan).
    b. Scanner table (a maximum scan range is 20 μm × 20 μm × 3 μm, (*x*, *y*, *z* direction).
    c. Lever table (20 N/m).
    d. Cantilever tips SI-DF20 (Seiko Instruments, Japan).
    e. Optical microscope.
    f. Antivibration platform and nitrogen gas bomb.

## 3. Methods

### *3.1. Preparation of Samples*

1. Remove both sides of the epididymis from a mature dd-mouse. Separate the tissue of segments I–V of the epididymis (**Fig. 1**; **ref. *10***).Cut tissue blocks of segments I, II, and V into small pieces with razors and place in the bottom of small vials separately.
2. Pour 3 mL of medium into each small vial and make the small pieces of tissue separate in the medium with tweezers. Wait a few minutes for spermatozoa to submerge from the stumps of the epididymal duct.
3. Transfer 2 mL of sperm suspension to a centrifugal tube and centrifuge at 240*g* for 8 min.
4. Pour 3 mL of medium into each centrifugal tube to wash the spermatozoa, and centrifuge at 240*g* for 8 min twice more.
5. Fix the spermatozoa in 2% glutaraldehyde in 0.1 *M* cacodylate buffer solution for 1 h.
6. Adjust the total amount of the sperm suspension to be 0.3 mL with distilled water after centrifugation.
7. Place one drop of sperm suspension on the slide glass square. As the glass is coated with adhesive material, spermatozoa adhere to the surface of the glass in 5–10 min. Make 3 or 4 samples.
8. Move the slide glass in distilled water to allow the release of loosely-attached spermatozoa from the slide glass.
9. Check the density of the spermatozoa on the slide glass under a light microscope.
10. Transfer the slide glass with spermatozoa in turn to 80 (5 min), 90 (5 min), 95 (5 min), and 100% (10 min, twice) ethyl alcohol solutions for dehydration.

---

Figs. 3–6. *(facing page)* AFM images of mouse epididymal spermatozoa. **(B)** and **(C)** are enlarged images of **(A)**. Bar is 2 μm in **(A)**, 1 μm in **(B)**, and 0.5 μm in **(C)**.

11. Immerse the samples in 3-butyl-ethyl acetate solution for 15 min.
12. Put a sample in a cage and perform critical point drying.

### 3.2. Scanning Operation Method of Dynamic Force Mode (DFM)

1. Allow the nitrogen gas to flow into the antivibration platform.
2. Turn on the antivibration platform and CCD camera controller.
3. Turn on the light of the optical microscope.
4. Set the scanner table.
5. Turn on the power at the center of the front panel of SPI3800N probe station controller.
6. Turn on the computer and the display. Microsoft Windows startup menu will appear.
7. Double click the icon of *Spise132* on the desktop to open the application for AFM.
8. Select "DFM" in "SPA300/400" frame and click "OK"; the main program, *SPIWin,* will start.
9. Set the sample stage on the mount on top of the scanner.
10. Set the sample on the sample stage.
11. Select a spermatozoon and move it to the center of the eye field of the optical microscope by hand.
12. Install the DFM cantilever holder with a DFM cantilever into the unit.
13. Select "CCD Monitor" in the setup menu to display "CCD". Check the position of the cantilever and the specimen by CCD image. Use the Move-in command (Low or High) and set the distance between the sample and the cantilever at approx 0.1 mm (which corresponds to a 180°- rotation of the fine-focus dial of the optical microscope).
14. Set the laser unit on SPA 300.
15. Adjust the laser light spot to be in right position.
    a. Rotate adjustment knobs "Laser X" and "Laser Y" to put the laser light spot on the top of the cantilever under the optical microscope.
    b. Rotate adjustment knobs "Laser X" and "Laser Y" to maximize ADD output. The maximum value of ADD output is around 5 or 13 depending on the cantilever.
    c. Rotate adjustment knob "DIF" so that the output may be in the range from –1 to 1 V
    d. Rotate adjustment knobs "DIF" and "FFM" so that the output may be in the range from 0 to 1 V.
16. Measure the Q-curve.
    a. Select "Q-curve" in the Scan menu to display "Q-curve Console."
    b. Put the initial value of the parameter of the Q-curve Console. One example follows:

| | |
|---|---|
| Freq. High | 400 kHz |
| Low | 1 kHz |
| Gain | 1 |
| Vib. Voltage | 1 V |
| LPF | 1 kHz |

| | |
|---|---|
| HPF | 1 kHz |
| Time | 5 s |

c. Select "Left" in the Vib. Freq. frame.
d. Check "Phase," "Calibration," and "Auto Set."
e. Click "Configuration" button to display configuration dialog.
f. Set the value of the auto set. "Amplitude" is 1.000 V and "Frequency" is 3.000 kHz.
g. Click "Start" to measure the Q-curve and the phase curve. Computer calculates the optimal value for the vibration frequency (operation point) immediately and displays the values with the Q-curve. An example follows:

| | |
|---|---|
| Freq. High | 128.410 kHz |
| Low | 125.410 kHz |
| Gain | 1.000 |
| Vib. Voltage | 1.511 V |
| LPF | 1.0 kHz |
| HPF | 1.0 kHz |
| Time | 5 s |
| Vib. Freq. | 126.800 |
| Amplitude | 1.068 V |
| Peak Freq. | 126.918 kHz |
| ΔF | 0.335 |
| Q | 379.273 |
| Phase | –56.520° |

h. Click "Close" to close, on the Q-curve Console

18. Approach the force area.
    a. Select "Image" in the Scan menu to display "Approach" and "Scan Console."
    b. Preset the parameters in the Scan Console follows:
       Amplitude Reference -0.101
       I-gain 0.177, P-gain 0.0488, A-gain 0, S-gain 0
       when the maximum ADD value is around 5.
       I-gain 0.0592, P-gain 0.0122, A-gain 0, S-gain 0
       when the maximum ADD value is around 13.
    c. Open "Sub Console" to confirm the filter values for topography.
       Data Type Topo (servo)
       LPF 1.000 kHz, HPF 0.000 Hz, Range 1583.20 nm, Offset 0.715V
    d. Approach the sample to the cantilever by using the Approach button.
    e. Lower the value of the Amplitude Reference until the scanning voltage becomes stable.
    f. Make the Amplitude Reference 0.3–0.5 down from the value of getting stability of the scanning voltage.
19. Separate the sample from the cantilever until the scanning voltage displays –20.
20. Turn off the laser light.
21. Insert the head of the spermatozoa under the tip of the cantilever under the optic microscope.
22. Perform the test scan at 512 × 128 points in the area of 15,000 $nm^2$ at the scan speed of 1 Hz.

23. Approach the force area by using the "Approach" button and click "Start."
24. Execute **steps 21–23** again when the object is not displayed in the canvas.
25. Change the rotation angle, center of the image, and scan area to get the proper compositon. Use "Zoom" to change the center of the image.
26. Check the composition of the image by test scan.
27. Write the sample information in the column for comments.
28. Change the number of the scan points to 512 × 512.
29. The relation between "Scan Area" and "Scan Speed follows."

| *Scan area (nm)* | *Scan speed (Hz )* |
|---|---|
| 9,000 | 0.28 |
| 8,000 | 0.28 |
| 6,000 | 0.37 |
| 4,000 | 0.41 |
| 3,000 | 0.56 |
| 2,000 | 0.85 |
| 1,000 | 1.23 |

    It takes 40 min to get an image of 9,000 nm square.
30. Start to scan.
31. Set the S-gain value between 3 and 8 if the image is improved by this.
32. Separate the sample from the cantilever after completing the scan.
33. Save the measured data into HDD or MO as soon as possible.
34. When all measurements are finished, close the *SPIWin* software and *CCD* monitor.
35. Shut down the system.
36. Turn off the light of the optical microscope.
37. Turn off the power of CCD camera controller and antivibration platform.
38. Stop nitrogen gas to flow to the antivibration platform.
39. Turn off the power at the center of the front panel of SPI3800N Probe station controller.

## 4. Notes

1. When the ADD output does not move freely, readjust the position of the laser light on the cantilever or the distance between the specimen and the cantilever to be adequate (approx 0/1 mm).
2. The adequate value of the Amplitude Reference often moves in the minus direction during scanning. In this case the scanned image will become blurred and disappear at last. To avoid this, the value of the Amplitude Reference is better set 0.3–0.5 lower than the value necessary to stabilize the scanning voltage.
3. Caution the following items to prevent the cantilever tip from damages.
    a. Scanning should not be stopped on the way of image delineation, even if the image is not good.
    b. The rotation angle and the scan area of the image should not be changed when the sample is in the force area.
    c. When the computer freezes, the sample should be separated from the cantilever by using up-down lever equipped in SPA300 before application is stopped.
    d. FFM knob should not be rotated during scanning.

4. Pay attention to the appearance of double images or two or more triangle-shaped particles arranged in the same direction, which reflect damage to the cantilever tip. If these signs appear, replace the cantilever immediately.
5. When an image does not appear in the NC-force canvas, try again and you will get the image.
6. A test scan is recommended after the scan area is changed because the scan position often shifts after that.
7. When the scanning voltage shows 200, the following three reasons should be taken into consideration.
   a. The laser beam is now off.
   b. The proper value of the amplitude reference is changed spontaneously to minus direction. In this case remeasurement of Q-curve is effective.
   c. FFM value is out of ±1.0 V.
8. Check the FFM value just before scanning, because FFM value is easy to change.

## References

1. Ushiki, T., Hitomi, J., Ogura S., Umemoto, T., and Shigeno M. (1996) Atomic force microscopy in histology and cytology. *Arch. Histol. Cytol.* **59,** 421–431.
2. Tojima, T., Hatakeyama, D., Kawabata, K., Abe, K., and Ito, E. (1999) Reexamination of fine surface topography of nerve cells revealed by atomic force microscopy. *Bioimages* **7,** 89–94.
3. Yanagimachi, R. (1994) Mammalian fertilization, in *The Physiology of Reproduction,* Vol. 1, 2nd ed . (Knobil, E. and Neill, J. D., eds.), Raven Press, New York, pp. 189–317.
4. Brooks, D. E. and Higgins, S. J. (1980) Characterization and androgen-dependence of proteins associated with luminal fluid and spermatozoa in the rat epididymis. *J. Reprod. Fertil.* **59,** 363–375.
5. Jones, R., Pholpramool, C., Setchell, B. P., and Brown, C. R. (1981) Labelling of membrane glycoproteins on rat spermatozoa collected from different regions of the epididymis. *Biochem J.* **200,** 457–460.
6. Echieverria, F. M. G., Cuasnicu, P. S., and Blaquier, J. A. (1982) Identification of androgen-dependent glycoproteins in the hamster epididymis and their association with spermatozoa. *J. Reprod. Fertil.* **64,** 1–7.
7. Moore, H. D. M. (1981) Glycoprotein secretions of the epididymis in the rabbit and hamster. Localization on epididymal spermatozoa and the effect of specific antibodies on fertilization in vivo. *J. Exp. Zool.* **215,** 77–85.
8. Bearer, E. L. and Friend, D. S. (1990) Morphology of mammalian sperm membranes during differentiation, maturation, and capacitation. *J. Electron Microsc. Tech.* **16,** 281–297.
9. Takano, H. and Abe, K. (2000) Changes in the surface structure of the hamster sperm head associated with maturation, in vitro capacitation and acrosome reaction: an atomic force microscopic study. *J. Electron Microsc.* **49,** 437–443.
10. Takano, H. (1980) Qualitative and quantitative histology and histogenesis of the mouse epididymis, with special emphasis on the regional difference. *Acta Anat. Nippon.* **55,** 573–587 (in Japanese).

11. Abe, K., Takano, H., and Ito, T. (1983) Ultrastructure of the mouse epididymal duct with special reference to the regional differences of the principal cells. *Arch. Histol. Jpn.* **46,** 51–68.
12. Abe, K., Takano, H., and Ito, T. (1982) Response of the epididymal duct in the corpus epididymidis to efferent or epididymal duct ligation in the mouse. *J. Reprod. Fertil.* **64,** 69–72.
13. Pavlok, A. (1974) Development of the penetration activity of mouse epididymal spermatozoa in vivo and in vitro. *J. Reprod. Fertil.* **36,** 203–205.
14. Lea, O. A., Petruz, P., and French, F. S. (1978) Purification and localization of acidic epididymal glycoprotein (AEG): a sperm coating protein secreted by the rat epididymis. *Int. J. Androl. Suppl.* **2,** 592–607.
15. Flickinger, C. J. (1983) Synthesis and secretion of glycoprotein by the epididymal epithelium. *J. Androl.* **4,** 157–161.
16. Koehler, J. K. (1982) The mammalian sperm surface: an overview of structure with particular reference to mouse spermatozoa, in *Prospects for Sexing Mammalian Sperm* (Amann, R. P. and Seidel, G. E Jr., eds.), Colorado Associated University Press, Boulder, pp. 23–42.
17. Peterson, R. N. and Russell, L. D. (1985) The mammalian spermatozoon: a model for the study of regional specificity in plasma membrane organization and function. *Tissue Cell* **17,** 769–791.