The Effect of Pipette Tip Roughness on Giga-seal Formation

M. Malboubi, H. Ostadi, S. Wang, Y. Gu and K. Jiang

***Abstract*— Reported is a study of applying nanofabrication technology to improve the surface roughness of micro glass pipettes to achieve giga ohm seal resistance in patch clamping processes. The surface roughness of pipette tips was first measured by 3D reconstruction of pipette tips using stereo imaging technique based on high resolution SEM images. Both the SEM images and the reconstructed images show that micro glass pipettes have rough and uneven tips which could be one of the causes of leakage in patch clamping. Then focused ion beam system was used to cut across the very end of the tip, producing a smooth and flat new tip. The average surface roughness Ra of a milled pipette tip was within a few nanometres. Patch clamping experiments were carried out using the polished pipettes on human umbilical vein endothelial cells (HUVEC), which were well known for their extremely flat shape making them very difficult to patch. The results show that above 3 Giga ohm seals were achieved in 60% of the experiments, as opposed to 1.5-2.0 GΩ in average with the conventional pipettes. The highest seal resistance achieved with a focused ion beam polished pipette was 9 GΩ, well above the 3 GΩ resistance, the usually best result achieved with a conventional pipette. The leakage current in single channel recording afterwards was found 0.3 pA, significantly smaller than 2-3 pA usually achieved using conventional pipettes. The research results demonstrate that the surface roughness of a pipette has a significant effect on the giga-seal formation of a patch clamping process.**

***Index Terms*— Focused ion beam, Giga-seal formation, Patch clamping, Pipette, Roughness.**

I. Introduction

Since the introduction by Neher and Sakmann in 1976 [1], patch clamp technique has been extensively used for cellular ion channel related studies. In patch clamping, a glass micropipette is in good contact with the surface of the cell in use and suction is applied to the other end of the pipette in order to form a high resistance seal which could reach giga-ohm in resistance. A giga-seal in patch clamping allows the recording of accurate currents through single ion channels of a cell with the minimum leakage current and high signal-to-noise ratio [2] – [4].

The physical and chemical mechanisms behind the giga-seal formation are not fully understood [5] – [7]. At present, the formation of a giga-seal seems to happen suddenly and in an all-or-nothing fashion [2]. Although other path clamping methods have higher throughputs, such as the automated conventional patch-clamping systems, planar patch clamping and lateral patch clamping [7] – [17], the pipette based conventional patch clamping method forms higher seals resulting in superior data quality statistically [2], [9]. The obtained seal resistances with pipettes are a factor 3 to 5 higher than those obtained with chips [7].

The important factors in giga-seal formation, based on the research literature, include cleanliness of both the pipette and plasma membrane [2], [4], [8], geometry of the tip, i.e. roundness [3], [18], [19], surface roughness of the site in contact with the cells [3], [7], [19], tip size [12], [20], hydrophilicity of the patch sites (since the hydrophilic cell membrane will not spontaneously interact with the hydrophobic surface in a way to form giga-seals) [12], [13].

This research work is designed to improve the sealing resistance in patch clamping and to acquire giga-seals more frequently. The roundness of the pipette tip was measured in the research for the first time by focused ion beam (FIB) nano-tomography and was reported in our pervious publication [21]. In this work, the effect of the surface roughness of glass micro pipettes on seal formation was examined. The tip of pipettes were imaged and reconstructed. The surface roughness was measured. The pipette tips were then milled using a focused ion beam (FIB) system resulting in a highly smooth surface. Extensive path clamp experiments were carried out to investigate the effect of the roughness on seal formation. We call this method "FIB polishing" in comparison with the "fire polishing method". Compared with fire polishing [22], [23], the pipettes polished using FIB have much smoother tip surfaces. The nanomachined pipettes were used in patch clamp recording experiments and much improved gigaseal formation has been achieved.

II. 3D reconstruction of a micropipette

The glass micro pipettes used in the experiments were made of borosilicate glass pipes with outer diameter of 1.5 mm and inner diameter of 0.86mm (BF150-86-10 Sutter Instrument). They were heated and pulled with flaming/brown micro pipette puller machine (Sutter Instrument Model P-97). The machine was set to produce pipettes with approximately 1.5μm in tip diameter. The 3D reconstruction of the pipette

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tip was based on high resolution scanning electron microscope (SEM) images [24], [25]. In this technique, 3D points are computed from 2D matched points in two SEM images taken from two angles between 5^o to 10^o away from the norm. Fig. 1 shows the configuration of SEM, tilting angle (α) and the projected coordination P1(X1, Y), P2(X2, Y).

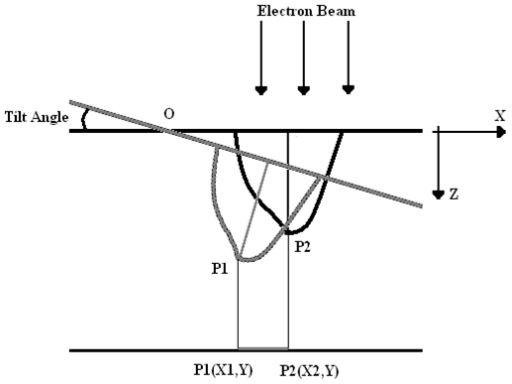


Fig.1. A pair of images of a single object for reconstruction of the object. P1 is the new position of P2 after a tilt of the stage about O.

The third dimension can be found from Equation (1) which can be derived based on the geometry of the projection.

$$Z = \frac{x_2 - x_1 + x_2(1-\cos\alpha)}{\sin\alpha} \qquad (1)$$

This process is used for every point of the object to find the shape of the structure [26], [27]. 3D surface profile of the pipette was obtained by analyzing three SEM images using a commercial software package Mex (Alicona) [28]. Fig. 2 shows the configuration of the FIB polishing and SEM imaging used in the experiments. Also, figures 3 (a) to (c) show the SEM images taken from the left, middle and right of the pipette. The tilting angle between (a-b) and (b-c) of the images is 9 degrees. The 3D reconstructed surface of the pipette tip is shown in Fig. 4 (a) and (b). It was found that the pipette tip was not only rough, but also wavy or inclined in its form. The surface parameters computed by considering both the roughness and shape of the tip are given in Table I.

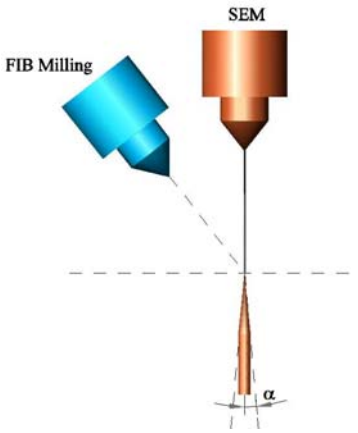
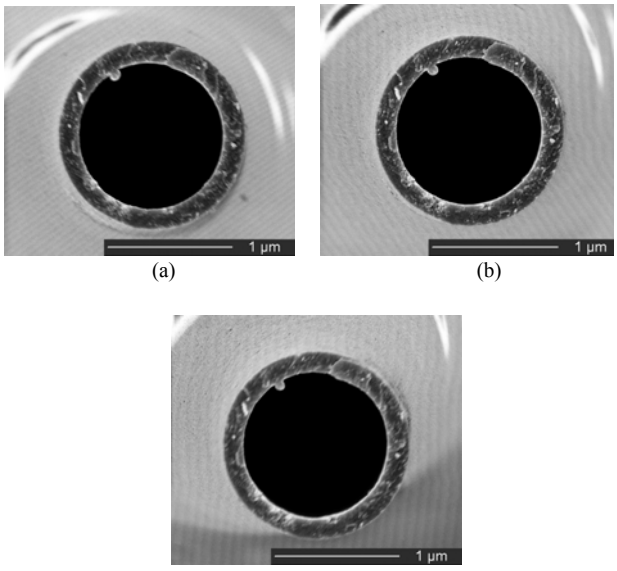


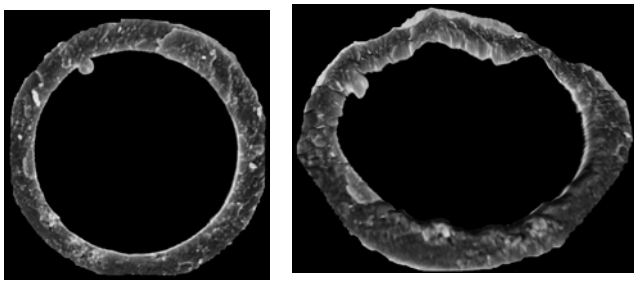
Fig.2. Schematic of the configuration of SEM and the tilting angle (α).



(a) (b)

(c)

Fig. 3. Stereo images of the pipette tip for 3D reconstruction; (a) left, (b) middle and (c) right.



(a) (b)

Fig.4. 3D reconstructed surface of the pipette tip shown in different viewing angles, (a) top view and (b) Angle view.

Table I. Surface parameters of the pipette tip.

Name	Value	description
S_a	27.24nm	Average height of selected area
S_q	34.85nm	Root-Mean-Square height of selected area
S_p	104.5nm	Maximum peak of selected area
S_v	150.54nm	Maximum valley depth of selected area
S_{sk}	-0.225	Skewness of selected area
S_{ku}	3.26	Kurtosis of selected area
S_{dq}	0.877	Root mean square gradient
Sdr	34.98%	Developed interfacial area ratio

III. Focused Ion Beam Polishing

The uneven surface of the pipette tip was corrected by cutting the top of the pipette across using FEI dual beam focused ion beam system. Because of the conic shape of the pipette, cutting the tip changes the tip size which is an important factor in patch clamping as it determines the pipette resistance. It is also well known that a giga-seal is not likely to be achieved with big tip sizes. So care was taken not to cut more than 1µm from the top. Since the roughness of the tip of the pipette was in nanometres cutting 1µm from the top should be sufficient to remove all rough edges without increasing the tip size significantly. In the FIB milling process, the pipettes tips were cut using Ga^+ ions with 50 pA current for 100 seconds and dwell time of 1µs (Fig. 5). The pipette before and after milling is shown in figures 6 (a) and

(b). The image shown in Fig. 6 (b) has a resolution of 4.5 nm. No feature could be identified on the milled surface for producing roughness parameters at this magnification. Therefore, the average surface roughness (Sa) of the milled pipette tip should be less than 4.5nm.

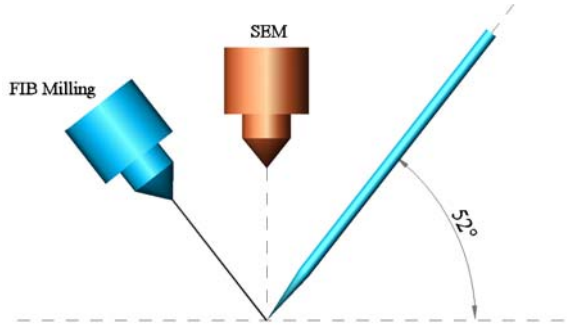


Fig. 5. The configuration of glass micro pipette milling in the SEM/FIB chamber. The stage was tilted by 52° so that the ion beam was perpendicular to the pipettes.

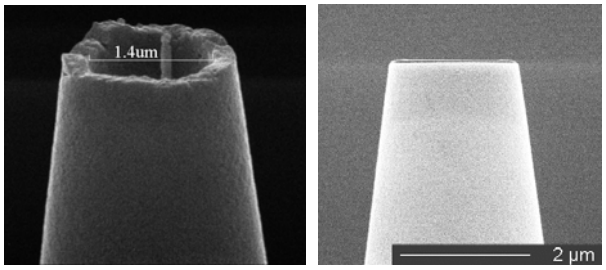


Fig. 6. (a) A micro glass pipette before milling, (b) the same pipette after the milling. No surface roughness could be identified after milling, so the surface roughness should be smaller than the resolution of the SEM image, which is 4.5nm.

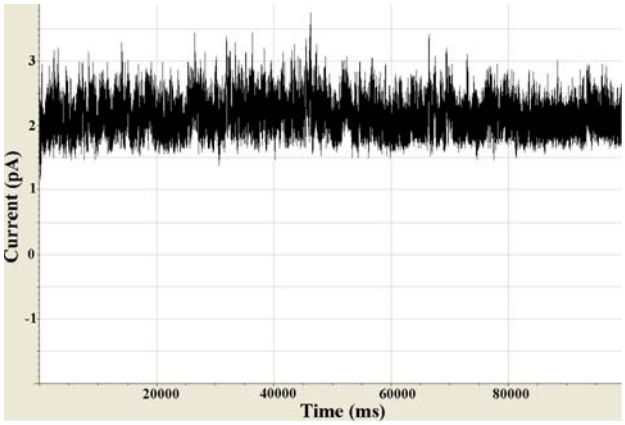
IV. Patch Clamp Experiment

Human umbilical vein endothelial cells (HUVECs) were utilized to investigate the performance of the FIB polished micro pipettes in achieving giga-ohm seals. HUVECs were cultured in EBM medium (Lonza Co., CC-3121) on cover slips two to three days before the experiment and incubation was done at 37ºC. At the time of experiments, the confluence of the cells is over 80% and all the cells were firmly attached to the bottom of the cover slips. HUVECs are well known for their extremely flat shape and their thicknesses are usually 1-2 micrometers. Thus, it is one of the most difficult cell types for patch clamping, especially when they are grouped and fully stretched, which was the case in the experiments. During experiments, individual cover slip was directly taken out from incubator and sited in the recording chamber.

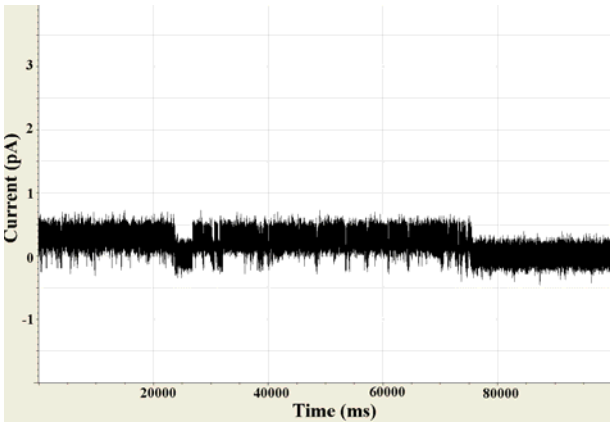
Experimental equipment setup was the same as the normal patch clamping setup widely adopted in other laboratories, consisting of Axon 1D amplifier, Flaming/Brown micro pipette puller (Sutter Instrument Model P-97) and glass micro pipettes (BF150-86-10 Sutter Instrument). The opening of the pipette tip is about 1.4µm in diameter. The backfill solution contained (in mM): kcl 40, K-gluconate 96, K_2ATP 4, GTP 2, HEPE 10, pH 7.2, and the bath solution contained (in mM): NaCl 110, KCl 5, $MgCl_2$ 1, $CaCl_2$ 1, HEPEs 5, HEPE-Na 5 (mM), pH 7.2.

A 10mV pulse was constantly applied on the recording electrode from the time that pipette tip was just immersed in the bath solution till it touched the cell membrane. A negative pressure was immediately applied to the pipette and then the voltage pulse was raised to 60mV to monitor the seal resistance precisely.

To investigate the effect of the roughness of pipette tips, experiments were carried out with polished and unpolished pipettes under the same conditions and the results were compared. When there was no contact between recording pipette and cell membrane, the total resistance ranged from 6.0 to 6.5 MΩ. With the FIB polished pipettes, above 3 Giga ohm seals were achieved in 60% of the experiments (n=20) and the highest seal resistance reached 9 GΩ. In comparison, the seal resistance achieved using the conventional polished or non-polished pipettes are 1.5-2.0 GΩ in average and the seal resistance could reach 3 GΩ in some excellent cases. The leakage current in single channel recording afterwards was found 0.3 pA, significantly smaller than 2-3 pA usually achieved using conventionally treated pipettes. Fig. 7 shows the filtered leakage currents of unpolished pipettes and polished pipettes.



(a)



(b)

Fig. 7. Single channel recording form HUVECs. (a) Unpolished pipettes: leakage current is about 2.1 pA, b) FIB polished pipettes: leakage current is about 0.3 pA.

The improved patch clamping performance with polished pipettes can be understood as that the smoother surface of the pipette tip leaves little concave area to hold water, opposed to the unpolished pipettes, as illustrated in Fig. 8. This greatly reduces the chance of current leakage. Also a flat and smooth tip enables more cell membrane to be sucked into the pipette, increasing the contact area between the pipette and the cell membrane and resulting in better sealing effects. Since in patch clamping the membrane can be destroyed at the tip of the pipette and the seal is still retained [5], [29], the second

factor is the dominant, i.e. the membrane has moved inside the pipette more.

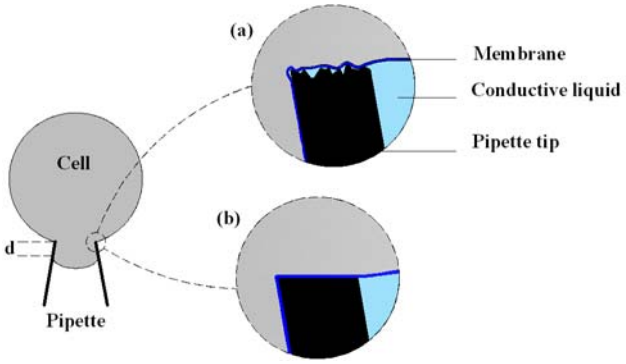


Fig. 8. Schematic of pipette-membrane interaction.

V. Conclusion

A giga-seal in patch clamping will produce improved signal-to-noise ratio and enables ion channel signal measurement to be more accurate. Currently, the formation of a giga-seal in patch clamping occurs in a sudden and all-or-nothing way. A large number of parameters affect the seal formation, making it hard to understand the physical and chemical mechanisms behind it. In this research, the SEM stereo imaging techniques were used to inspect the surface roughness of micropipettes. The high magnification images revealed the surface nature of the tips to be in contact with cells. Then the contact tips of pipettes were cut across, leaving a very smooth surface at the top of the pipettes. A large number of patch clamping experiments were conducted on HUVECs using the polished pipettes and 60% of the experiments achieved above 3 Giga ohm seals and the highest seal resistance reached 9 GΩ. The leakage current in single channel recording afterwards was found 0.3 pA, significantly smaller than 2-3 pA usually achieved using conventionally treated pipettes. The results show that nanomachined micro glass pipettes have improved the giga-seal formation in patch clamping.

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