生体物質を探索するための多機能サブマイクロピペットの開発

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Development of Multi-Functional Submicropipettes for Exploring Biomaterials

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抄録和訳

本総説は、工学院大学ナノ化学研究室で平成28年度から令和5年度までに行ってきた、直径1マイクロメートル以下のガラスピペットであるサブマイクロピペットに関する卒業論文と修士論文をまとめたものである。ガラス製サブマイクロピペットは細胞手術やナノ加工など様々な用途に使用されている。ここでは、その内径の空間分解能を利用した、イオン選択的検出、抽出、吸引などの多機能サブマイクロピペットについて解説する。さらに、ピペットの内部が複数に分かれていることで、抽出、吸引、測定などが同時に行うことが可能となるマルチバレルサブマイクロピペットを用いた研究についても解説する。

Abstract

This review paper summarizes the bachelor theses and the master thesis works between 2016 and 2023 fiscal years in Nanotechnology Chemistry

Laboratory, Kogakuin University, described on glass submicropipettes, the pipettes with their diameters less than one micrometer. Glass submicropipettes have been used for cell surgery, nanofabrication, and various applications. Here we show the capabilities of the multi-functional submicropipettes, such as ion-selective detection, extraction, aspiration, with the spatial resolution of their inner diameters. We also show the work on multi-barrel submicropipettes that can be used for simultaneous operation such as extraction, aspiration and measurements.

1. Preface

Glass submicropipettes have been used for various applications such as cell surgery, nanofabrication, and microscopy[1]. We have been exploring the capabilities of glass submicropipettes. In this review paper, our attempts to extend the function of glass submicropipettes by developing the component methods in order to realize beetle robot "*nano-mosquito*"[2], with showing the concept flag in Fig. 1. The design of the nano-mosquito is inspired from the beetle STM developed by Besocke. The quote "Learn from nature and create what is not in nature" is by Dr. Heinrich Rohrer who invented STM.

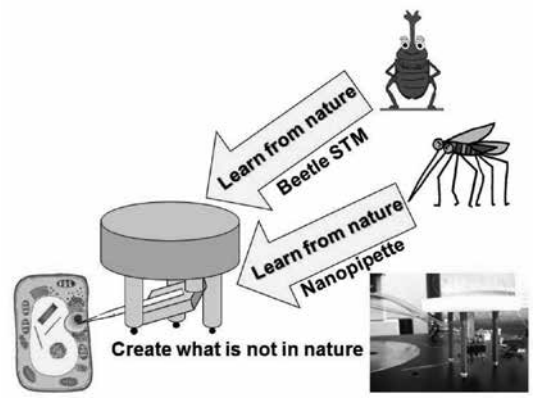

Fig. 1 Schematic concept of a "nano-mosquito"; three-legged beetle-type robot with a subkicropipette. See Ref. 2 for more details on the nano-mosquito. The illustrations were partly prepared by Miyuki Miyata.

2. Preparation of micro- and nano-pipettes

The glass pipettes were prepared with filament-based submicropipette puller (P-97/IVF, Sutter Instrument). A glass capillary loaded into the puller bar is softened by heating with a filament to a certain degree before the hard pull, and the capillary is then separated into two glass submicropipettes. This machine has four parameters to control the heat, pull, and time to adjust the shape of the as-prepared submicropipettes[3].

(1) HEAT: the output power of the filament heat, and consequently the amount of energy supplied to the glass capillary. It is noted that the HEAT value corresponds approximately to the puller filament temperature in ℃.

(2) VELOCITY: the velocity at which the puller bar must be moving before the hard pull is executed.
(3) DELAY: the timing of the start of the hard pull relative to the deactivation of the laser.
(4) PULL: the force of the hard pull.

Though there are slight differences between instruments, submicropipettes with diameters of 0.3–1 μm can be successfully prepared by synergistically adjusting these four parameters. Borosilicate glass is usually used to prepare these submicropipettes because borosilicate glass is easy to control the shape of the pipettes and cheap. As the heating temperature (HEAT value) was increased, the taper length of the resulting submicropipette tended to increase. Settings with a HEAT value exceeding 785 were not performed because of the risk of damage to the heating filament of the puller device. Also, when the HEAT value was lower than 740, the glass capillary was not heated sufficiently to soften the glass capillary, and thus could not be fabricated[4,5].

Sutter's manual[3] mentions that the tip inner diameter becomes smaller as the HEAT value is increased. The desired result is obtained when the temperature is increased too much because the tip inner diameter exceeds the softening point of borosilicate glass by much more than 730℃. The stability of the tip inner diameter obtained depends on the heating temperature. However, too high a temperature will not give the desired results because it is well above the softening point of borosilicate glass, 730℃. On the other hand, too low a temperature below the softening point will result in a very unstable tip. On the other hand, setting too low a temperature below the softening point results in unstable conditions for making the tip bore, resulting in failure to burn out the tip. Therefore, the optimum heating temperature for 3-step pulling is considered to be 742, which is close to the softening point as the HEAT value[4,5].

Next, we will discuss the 4-step pulling. In making submicropipettes with pullers, four-step pulls are not mentioned in the Sutter manual[3], because in general, more than four-step pulls are difficult and unstable results have been obtained. In this study, we also tried various 4-step pulling conditions, but the tubing burned out before the 4th step, making it impossible to fabricate a stable pipette or even a pipette. In this situation, we succeeded in pulling the pipette in four steps and obtained a stable parameter of 845 ± 5 nm. The reason why this 4-step parameter was obtained is thought to be that the problem of the inability to burn out the tubules at low HEAT was solved by lowering the VELOCITY and DELAY values, and the tubules were burnt out firmly even at low temperatures[4,5].

On the other hand, it is possible that not only heating temperature but also dust, oil, and humidity adhering to the fine tube may affect the variation of the tip inner diameter.

However, since a large amount of experimental data would be required to demonstrate the effects of humidity and other factors, there is room for further investigation of these factors[4,5].

Although the taper length tends to increase as the HEAT value is increased, the difference is not as great as expected and does not change at temperatures above 770℃. Therefore, it is considered difficult to further increase the taper length while maintaining the tip inner diameter in the 3-step pulling process. Therefore, it is expected that the 4-step pulling method, which requires a greater number of pulls, will result in a longer taper length than the 3-step pulling method, though there is room for further investigation of this issue. In anyway, we found stable parameters to produce submicropipettes as shown in Tables 1 and 2[4,5].

Table 1 Stable parameter to achieve505 ± 20 nm pipettes using 3-stage process[4, 5].

	HEAT	PULL	VELOCITY	DELAY
1st	742	0	30	250
2nd	732	0	40	250
3rd	722	30	55	250

Table 2 Stable parameter to achieve845 ± 5 nm pipettes using 4-stage process[4, 5].

	HEAT	PULL	VELOCITY	DELAY
1st	727	0	10	230
2nd	717	0	20	230
3rd	717	0	30	230
4th	707	30	40	230

We considered that the application of this 4-step pull parameter provides the key to searching for parameters to stably fabricate “multi-channel pipettes,” such as the two-part “theta pipette”, which is difficult to fabricate in the past and is not shown in the Sutter manual[3].

The suitable parameters to fabricate dual-barrel submicropipettes were not clear and no reports on these parameters to fabricate double-barrel pipettes with high accuracy and homogeneity. Kaneko found that these parameters determine the stability and accuracy of the pipette fabrication; unsuitable parameters sometimes resulted in the half-open of the double-barrel or the standard deviation of the inner diameter is more than 10%[6]. We tried 32 kinds of the combination of the parameters, and more than three times fabrications were conducted for each parameter. In this way, we have obtained a parameter to fabricate double-barrel borosilicate submicropipette at 1 μm, as shown in Table 3[6]. The average total inner diameter of the prepared micropipettes was 513 ± 25 nm. With our

efforts, the limit of the small diameters and the short taper length of the double-barrel glass pipettes were 0.5 μm and 5.2 mm, respectively, using our filament-type puller. Laser-puller could fabricate small-diameters and shorter-taper double-barrel submicropipettes[6].

Table 3　The best parameters for the fabrication of 1 μm double-barrel pipette[6].

	HEAT	PULL	VELOCITY	DELAY
1st	761	0	30	200
2nd	751	0	40	200
3rd	751	50	50	200

We also investigated the influence of the direction of the glass tube set in the puller. We tried (1) perpendicular, (2) horizontal, and (3) 45° tube setting by using the parameter shown in Table 1, and the standard deviations were (1) 5.07%, (2) 7.09%, and (3) 18.10%, respectively. Therefore, we concluded that perpendicular setting of the glass tube is suitable for the stable fabrication of the double-barrel submicropipettes[6].

3. Observation of the tip top of submicropipettes by scanning electron microscopy

The observation of the edge of insulators such as glass usually suffered from charge up during the observation with electron beam by scanning electron microscopy (SEM). Here we demonstrate the method to prevent tip top glass pipettes from charge up.

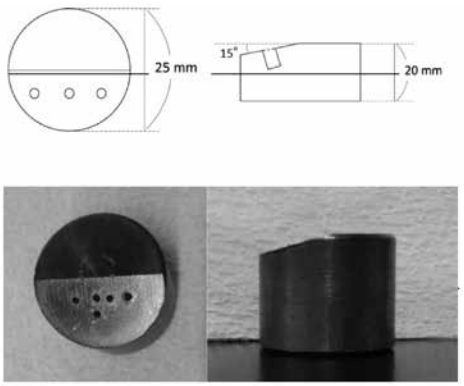

Fig. 2　Top view (left) and side view (right) of the copper sample stage for the observation of the tip top of glass pipettes; the schematic drawings (upper) and the photos (lower)[7].

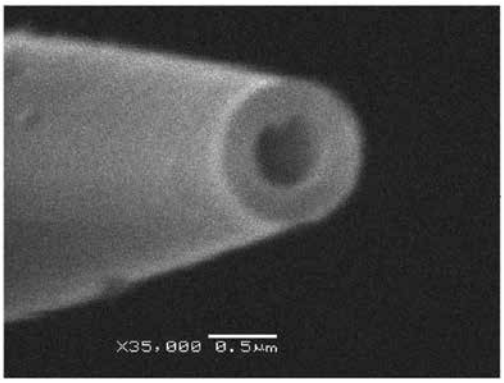

Fig. 3　Scanning electron microscope image of the tip top of the glass submicropipette, observed at the tilted angle of 15°[7].

Ono used conventional antistatic spray (Elegard® manufactured by Lion Inc.)[7]. Among the components of Elegard®, those involved in antistatic properties are polyethylene glycol and alkyl trimethyl ammonium salt, and they pretend like ionic liquids. Thus, strong Coulomb forces act between the nitrogen ions of the alkyl trimethylammonium salt and the oxygen ions of the polyethylene glycol, and they exhibit the same properties as an ionic liquid.

Moreover, Ono prepared a copper sample stage to observe the tip top of glass submicropipettes at the suitable angle 15°[7]. Figure 2 shows the photo of the prepared sample stage. Figure 3 shows a typical SEM image of the glass submicropipettes. The sample stage allows the glass submicropipettes to be tilted 15° from the vertical when viewed from the side, as shown in Fig. 3, making it easier to observe the tip with SEM[7].

4. Evaluation of inside of submicropipettes with gas flow method, using Gomperz function for simulating the shape of submicropipettes

The outer shape of the fabricated submicropipettes can be examined with SEM observation. However, the inner shape and cleanliness cannot be observed because electron is not transparent to glass. Therefore, we have developed the gas flow method to investigate the inner shape and cleanliness of sub-micropipettes[8-12].

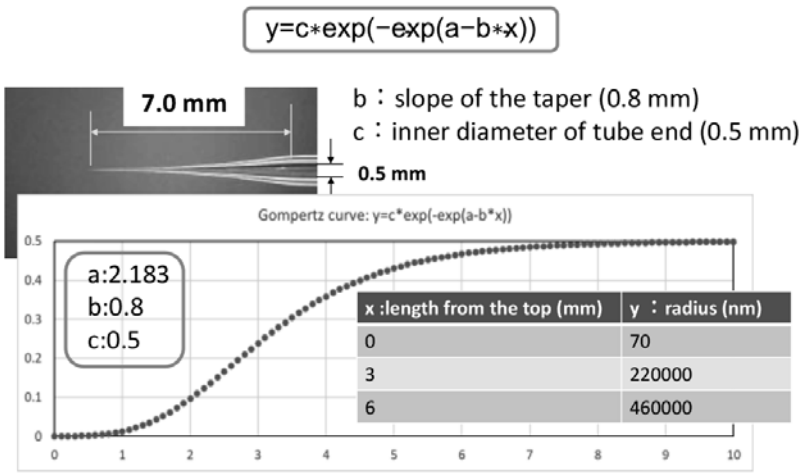

Fig. 4 How to fit the shape of submicropipette with Gompertz curve[11].

For the simulation of the gas flow through glass submicropipettes in order to investigate the inner shape and cleanliness, we need a function to fit the shape of

submicropipettes. We found a suitable function of Gompertz function,

$$G(x) = c \exp[-\exp(a - bx)],$$

where *a*, *b*, and *c* are the constant parameters. The Gompertz function is commonly used for the growth curve with its applications such as economical management and population growth model[11,12]. Figure 4 shows how we fit the Gompertz function to the shape of submicropipette[11]. In this way, we have established a method to evaluate the fabricated submicropipettes with non-distractive measurement combined with the shape simulation[11,12].

5. Various ion-selective electrodes prepared in glass pipettes

Son reported separate detection of sodium and potassium ions by preparing ion-selective membrane (ISM) in submicropipettes[13], and Deng reported calcium-ion ISM in glass submicropipettes[14]. We have ever prepared cesium[15,16] and chlorine[17] ISMs. We have explored the signal detection system using rock-in[18] or common patch cramp amplifier[19,20].

Nishiyama developed a method to prevent ISM from mixing with water during the preparation process[15,16]. Figure 4 shows the photos showing the process to fabricate the ISM in submicropipette. It is noted that the photos show upside down. First, pure water was filled in the submicropipette to the shank end using a micro-syringe (A-1). Then, we poured *ca.* 0.01 μL cyclohexane (Sigma-Aldrich, purity >99.0%, CAS 110-82-7) on the water (A-2). After making the cyclohexane solvent layer on the water, 10 μL of (i) 32 mg of poly (vinyl chloride) (PVC) (Sigma-Aldrich, high molecular weight, CAS 9002-86-2) for the main resource of the membrane, (ii) 62 mg of 2-Nitrophenyloctylether (NPOE, Dojindo, purity >99.0%, CAS 37682-29-4) for the plasticizer, (iii) 5 mg of 1,3alternate thiacalix [4] biscrown-6,6 for the cesium ion-selective ionophore, and (iv) 1 mg of tetrakis [3,5-bis (trifluoromethyl) phenyl] borate, sodium salt (TFPB, Dojindo, purity >99.0%, CAS 79060-88-1) for the anion repeller, which were dissolved in 1 mL tetrahydrofuran (THF, Sigma-Aldrich, purity >99.9%, with 250ppm dibutyl-hydroxytoluene (BHT) for the stabilizer, CAS 109-99-9), which we call the mix solution "PVC solution" in the caption of Fig. 5 (B-1), and were poured in the submicropipette. The solution put in submicropipette was dried to cure the PVC membrane (B-2). It is noted that the cyclohexane solvent was finally transpired with THF on the drying process. In this way, using cyclohexane before pouring the solution is the improved point in this study[15,16].

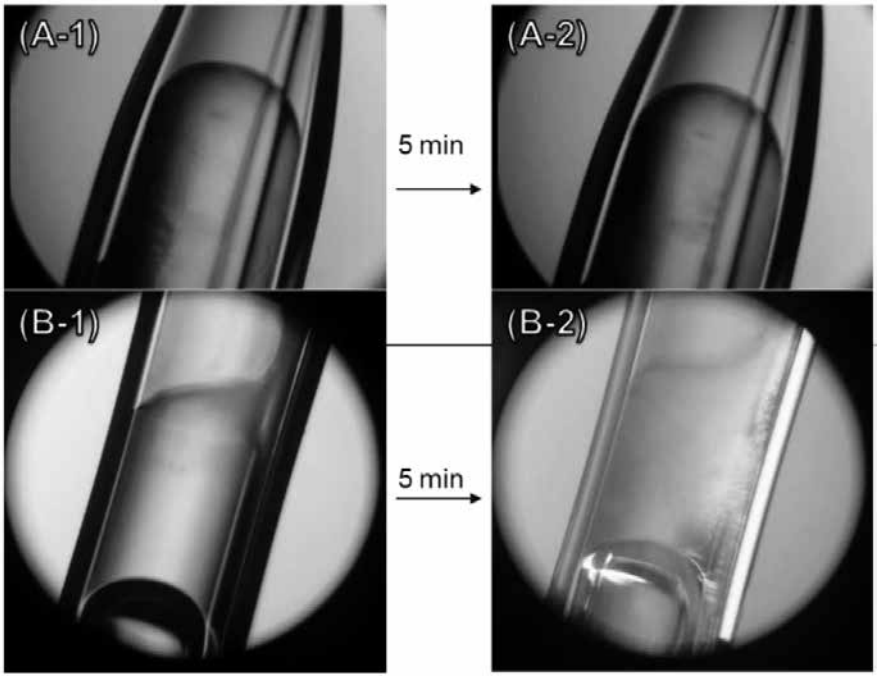

Fig. 5 Photos of submicropiette tube during the curing process of PVC membrane inside the submicropipette. (A-1): After pouring the PVC solution on the distilled water covered with cyclohexane. (A-2): Five minutes later after taking the photo (A-1). (B-1): After pouring the PVC solution on the distilled water, without using cyclohexane. (B-2): Five minutes later after taking the photo (B-1)[15, 16].

6. Double-barrel ion-selective electrodes

Kaneko found the best parameter to fabricate double-barrel submicropipettes[6]. Akutsu succeeded in preparing different ISEs in each barrel of double-barrel submicropipettes[21], which we reported the results in eJSSNT[22].

Akutsu discovered a way to prepare double-barrel submicropipettes to avoid the formation of bubbles in the ISM caused the disturbance of the ion-selective signal[21]. Even after confirming the absence of bubbles with an optical microscope, bubbles sometimes appear when the dried ISM is checked again with an optical microscope, which resulted in no signal detected as well. For solving the bubble formation problem, the tip of a double-barrel pipette was immersed in pure hot water (90 ℃) during the dry process to form ISM in the double-barrel pipette. Even if there are no bubbles after drying, the ISM might peel off from the inner glass wall when the electrolyte was filled in the pipette, which used

to occur in the case that the adhesive area between the ISM and the inner wall of the glass pipette was small[21]. This problem could be solved by fabricating the ISM closer to the tip, as reported previously[23], and by reducing the filling volume of the ISM solution to 1 μL[23].

7. Double-barrel micropipette used for tuning folk non-contact microscope

Yoneda developed frequency-modulation atomic force microscope (FM-AFM) with a double-barrel submicropipette, by modifying an existing SPM apparatus (JEOL JSPM-5200)[24]. A theta-tube submicropipette was glued to a quartz tuning folk (QTF) oscillator, and the Q value was estimated. The operation of the external device was confirmed by determining the resonance frequency of the QTF with pipette. An image based on frequency modulation was obtained as FM-AFM using a double-barrel submicropipette. Figure 6 shows the block diagram of the FM-AFM with a double-barrel submicropipette. The developed apparatus would be a future study of simultaneous action of measurement and fabrication.

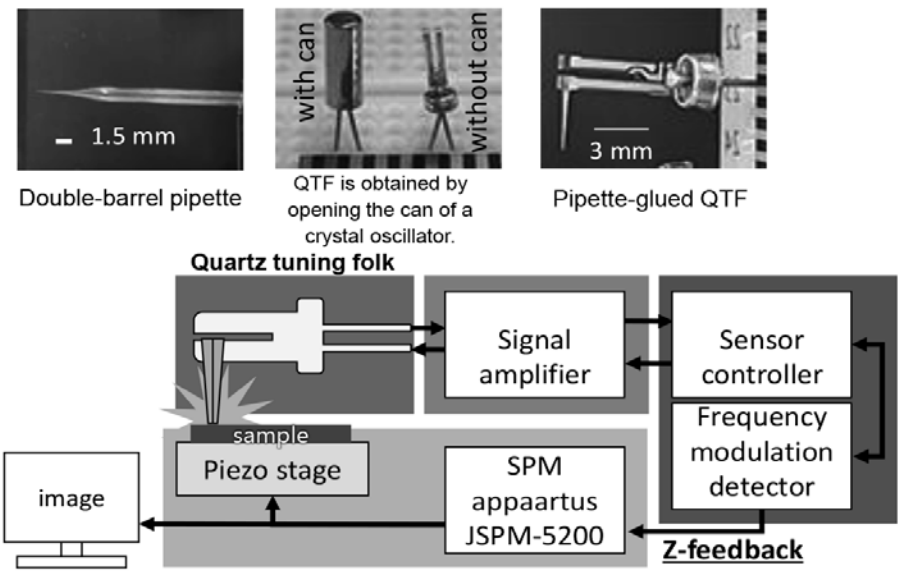

Fig. 6　Schematics of FM-AFM with double-barrel submicropipette developed in our laboratory[24].

8. Automatic injection to living cells

Yokogawa Electric Corporation developed Single Cellome™ Unit, SU10, which would be a promising tool to explore molecular dynamics in living cells[25]. We rent the apparatus from Yokogawa, and tested the injection of fluorescent molecule into a HeLa cell, one of the famous cancer cell. Figure 7 shows typical results of automatic injection, conducted by a master course student, after learned how to use from Yokogawa Electric Corporation for several hours[24].

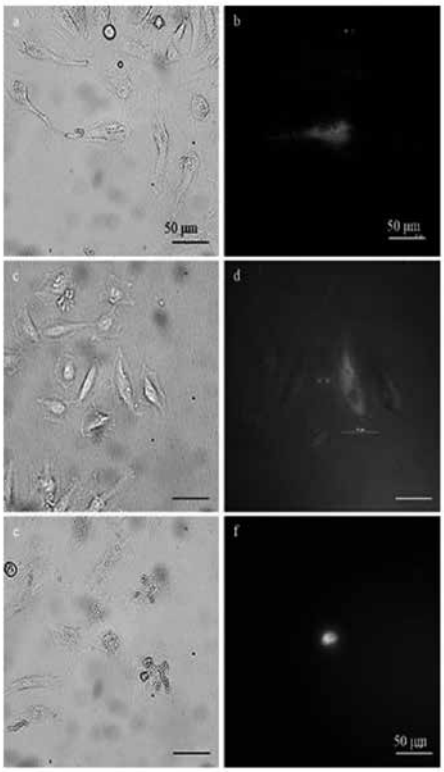

Fig. 7 Demonstration of SU-10[25]; injection of FITC, fluorescent molecule, to HeLa cell[24].

9. Remarks

We have developed component methods to realize automatic injection robot, "*nano-mosquito*"[2], by integrating the developed methods of pipette fabrication[4-6], evaluations by SEM[7] and gas flow method[8-12], ISEs for various ions[15-20], double-barrel ISE micropipettes[21,22], combination of FM-AFM with QTF[24], and automatic injection system developed by Yokogawa Electric Corporation[24,25]. We promise that the developing tool will contribute to the development of various fields such as cell engineering and nano-fabrication.

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