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Femtoliter Volumetric Pipette and Flask Utilizing Nanofluidics

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Abstract

Microfluidics has achieved integration of analytical processes in microspaces and realized miniaturized analyses in the fields such as chemistry and biology. We have proposed a general concept of integration and extended this concept to the 10-1000 nm space exploring ultimate analytical performances (e.g. immunoassay of a single-protein molecule). However, a sampling method is still challenging for nanofluidics despite its importance in analytical chemistry. In this study, we developed a femtoliter (fL) sampling method for volume measurement and sample transport. Traditionally, sampling has been performed using a volumetric pipette and flask. In this research, a nanofluidic device consisting of a femtoliter volumetric pipette and flask was fabricated in glass substrates. Since gravity, which is exploited in bulk fluidic operations, becomes less dominant than surface effects on the nanometer scale, fluidic operation of the femtoliter sampling was designed based on utilizing surface tension and air pressure control. The working principle of an 11 fL volumetric pipette and a 50 fL flask, which were connected by a nanochannel, was verified. It was found that evaporation of the sample solution by air flow was a significant source of error because of the ultra-small volumes being processed. Thus, the evaporation issue was solved by suppressing air flow. As a result, the volumetric measurement error was decreased to ±0.06 fL (CV 0.6%), which is sufficiently low for using in nanofluidic analytical applications. This study will be a fundamental technology for the development of novel analytical methods for femtoliter volume samples such as single molecule analyses.

Introduction

Recently, microfluidics, lab on a chip or micro total analysis systems (μ-TAS)[1,2] have achieved the miniaturization/ integration of analytical processes into microspaces to realize improved analytical performance and are expanding in the fields of chemistry and biology. The benefits of integration and miniaturization include faster reactions due to a higher surface-to-volume ratio, smaller amounts of reagent and waste, and ease of automation, etc. Our group has proposed a general concept of integration, called micro-unit operation (MUO).[3] In MUO, the chemical process is first divided into individual operations such as mixing, reaction, extraction, and cell culture, and then each operation is performed at the micrometer scale. Finally, serial or parallel combinations of MUOs are used to integrate chemical processes in a microchip system. Nowadays, various kinds of complex chemical processes including blood tests,[4] drug synthesis[5,6] and environmental analysis[7] have been integrated into single microchip and microfluidic systems, and such analytical applications are now in a practical development phase.

The concept of MUO has been further extended to smaller dimensions, i.e., 10-1000 nm on glass substrates, which we have designated "extended nanospaces",[8] and thus pioneered the field of nanofluidics. Fundamental technologies for nanofluidics including top-down technology for nano-fabrication of 10-1000 nm channels on glass substrate[9] and highly sensitive methods to detect non-fluorescent molecules in nanochannels utilizing wave optics and the thermal lens effect were developed.[10] Using these fundamental technologies, we have been seeking the ultimate analytical

method for characterization of single molecules at the femtoliter (fL; 10^{-15} L) level. For example, a liquid chromatography (LC) system utilizing pressure-driven flow in a nanochannel was used to separate a femtoliter sample in a very short time (seconds) with very high efficiency (7,100,000 plates/m), contrasting strongly with that of a commercial high-pressure LC system.[11] Also, implementation of an immunoreaction in an extended nanospace (~fL) permits almost 100% capture of target proteins utilizing a smaller reaction field than in molecular diffusion (~10 μm on a seconds time scale).[12] This efficient reaction field permitted analysis of a single protein molecule by integration of an enzyme linked-immunosorbent assay (ELISA) into a nanochannel.[13] It is anticipated that such nanofluidics regimes will be eminently suited to novel analytical devices that process ultra-small sample volumes (e.g., single cell/single molecule analysis).

However, while the analytical process consists of three components, i.e., sampling, chemical processing and detection, the means for the initial sampling is still challenging in nanofluidic devices despite its importance. Several researchers have reported downsizing of the sampling method using microfluidics, but these methods are difficult to implement in nanofluidics. In one case, for example, aqueous picoliter droplets in an oil phase were generated and manipulated by surface acoustic waves [14,15] or by electrowetting.[16] However, use of an aqueous/oil interface can result in cross contamination whereas application of an electric current can bias sampling. In the work of Huang *et.al.*, a nanoliter liquid sampling method based on a microfluidic pneumatic valve was proposed,[17] which involved deformation of a soft material (e.g., polydimethylsiloxane) by pneumatic pressure; however,

fabrication of nanochannels in soft material is problematic using current technology.

In the present study, a sampling method for femtoliter volume measurement and sample transport was developed. In analytical chemistry, conventional sampling and transfer operations for liquids are performed at a microliter to liter scale, normally using volumetric pipettes and flasks. In this work, downscaling of the volumetric pipette and flask to the femtoliter was achieved. Given that gravitational effects, which are exploited in bulk fluidic operations, become much smaller than surface effects in a nanospace, we developed fluidic operations of the femtoliter volumetric pipette and flask which exploited the surface tension of the sample solution. To realize unbiased femtoliter sampling, the formation of a gas/liquid interface that was free from cross-contamination and controlled by surface tension and air pressure was required. We note that the concept of femtoliter sampling is incorporated in our most recent paper to achieve living single-cell protein analysis by nanofluidics.[18] Here, we report device design, principle verification, evaluation of performance, and development of fluidic operation with avoiding femtoliter sample evaporation. A nanofluidic device, which incorporated an 11 fL volumetric pipette and a 50 fL volumetric flask, was designed and fabricated, and the performance for volume measurement and transport was evaluated.

Working principle

Fig. 1 shows schematic diagrams of traditional and femtoliter volumetric pipettes and flasks. In a conventional volumetric pipette (Fig. 1(a)), a sample solution is sucked up by a mechanical pump until

it reaches the marked calibration line (volume measurement), and is then transported to the flask by gravity transport. Based on this approach, we propose a femtoliter volumetric pipette and flask that operates as shown in Fig. 1(b). The design challenges for a femtoliter volumetric pipette include the need for precise volume control and (2) a surface tension effect in the nanochannel as a replacement for gravity. With regard to the latter topic, Laplace nanovalves were selected to utilize the so-called Laplace pressure caused by the liquid surface tension.[19] As depicted in Fig. 1(b), the Laplace pressure exerts a force on the gas/liquid interface, and the Laplace pressure is governed by the surface wettability and the channel size as represented by the Young-Laplace equation:

$$\boldsymbol{P = -\frac{2\,\gamma\,cos\theta}{r}} \quad \#(\mathbf{1})$$

where γ is the surface tension, θ is the surface contact angle and r is the equivalent radius of the channel ($r = HW/(H+W)$ where H and W are height and width of nanochannel respectively). When the surface is hydrophobic ($\theta > 90°$), P takes a positive value and works as a pressure barrier against the introduction of liquid. For operation of the femtoliter volumetric pipette, two different Laplace valves were configured for use in the nanofluidic device. Laplace valve 1 utilized a difference in surface wettability and was used to halt the sample solution when no pressure was applied (initial state). Laplace valve 2 was situated in the main channel to define the sampling volume. To precisely control the sampling volume at the femtoliter level, the Laplace pressure was controlled via the channel size. According to Eq. (1), the Laplace pressure P is inversely proportional to the channel size r; consequently, a narrower channel has a higher Laplace pressure and can function as a valve. Using a

nanofabrication procedure it is possible to control the width, depth and length of the nanochannel with a resolution of 10 nm, which is appropriate for femtoliter volume control. A circular shape was adopted for the femtoliter flask, since this shape can accommodate and smoothly transport the sample via pneumatic pressure in the nanochannel where the surface tension dominates gravity.

The operation of the femtoliter volumetric pipette and flask is illustrated in Fig. 1(c). All operations are controlled by application of external pressure. Initially, without application of pressure, the sample solution is introduced to the main channel by capillary action but is stopped at the intersection point of the main channel and the air channel by Laplace valve 1. Then, when a pressure P_1 (greater than the Laplace pressure at the intersection point, P_{L1}, but smaller than the Laplace pressure in the narrow channel, P_{L2}) is applied, the solution moves up to Laplace valve 2. Next, the solution in the air channel is removed by applying a pressure P_2 from the air channel and a pressure P_3 from the right side of the main channel. Here, P_2 is greater than P_{L2} and less than the sum of P_{L2} and P_3. Consequently, only the solution from the intersection point of the main channel and the air channel to the Laplace valve 2 is left in place; this volume (*the volume measurement*) is at the femtoliter level and depends on the channel size and the distance to the Laplace valve 2. Finally, the solution is transported to the flask by removing pressure P_3 (*transport*).

3. Experimental

3.1 Design and fabrication of the femtoliter volumetric pipette and flask

As shown in Fig. 2(a), the volumes of the femtoliter volumetric pipette and flask were designed to be 11 fL and 50 fL, respectively. The femtoliter volumetric pipette had a width of 1600 nm for the wider channel and 900 nm for the narrower channel. The diameter of the femtoliter flask was 10 μm and the width of the downstream channel was 2100 nm. The depths of the femtoliter volumetric pipette and flask were 700 nm. In this design, P_{L1} and P_{L2} were estimated to be 100 and 130 kPa, respectively, assuming $\theta = 110°$ (contact angle measured in bulk octadecylsilane at surface) and $\gamma = 72.75$ mN/m in Eq. (1).

The nanofluidic device was fabricated using a process described elsewhere [8]. A Pyrex substrate (0.7 mm thick, 70 mm wide and 30 mm long) with microchannels (500 μm wide and 100 μm deep) fabricated by photolithography and wet etching was purchased from the Institute of Microchemical Technology Co. Ltd., Japan. Nanochannels were fabricated in fused silica (0.17 mm thick, 70 mm wide and 30 mm long) by electron beam lithography and reactive ion etching. Then, the two substrates were treated with Piranha solution and oxygen plasma and bonded before being heated at 110°C for 3 hours. The fabricated device is shown in Fig. 2(b). The width and depth of the nanochannels were in agreement with the target values.

The surfaces of the nanochannels were then modified with hydrophobic molecules (octadecylsilane). Octadecyldimethyl-N,N-diethylaminosilane (ODS-DEA), synthesized from diethylamine and octadecyldimethylchlorosilane (ODS-Cl), was used according to a procedure described elsewhere.[20] First, all of the nanochannels were flushed with toluene at 70°C for 30 min.

Then 20% (v/v) ODS-DEA/toluene was introduced from the bottom side of the vertical channel at 5 kPa and 70°C for 4 hours, flushing the solution once after two hours. After this modification, the channels were washed with toluene, hexane, acetone, ethanol and water (in that order) and dried by introducing air to all of the channels. Then, a part of nanochannel was silanolized by introducing 1M sodium hydroxide. The boundary between the sodium hydroxide solution and the air was controlled by adjusting the air pressure. Once the boundary reached the intersection point, the pressure was set at 5 kPa for 15 min. After silanolization, all nanochannels were washed with water and dried by passage of air.

3.2 Experimental setup

A schematic of the experimental setup for the operation of femtoliter volumetric pipette and flask is given in Fig. 2(a). An inverted microscope (IX71, Olympus, Japan) equipped with a CMOS camera (ORCA, Hamamatsu Photonics K.K, Japan) was used to observe the movement of solution in the nanochannels. An LED light source (Lumencor, USA) was used for excitation of sample fluorescence. The fabricated nanofluidic device was fixed on an aluminum chip holder and placed on the microscope stage. The inlets of the microchannels were connected with PEEK (polyether ether ketone) tubing via O-rings and Teflon screws (UF-C, Institute of Microchemical Technology Co. Ltd, Japan). The PEEK tubing was connected to vials containing reagents, which were linked to pressure controllers (MFCS^{TM}-EZ, Fluigent, France), and an air compressor. Water, air and surface-modification reagents were introduced into the microchannels and nanochannels through the PEEK tubing, according to a

programmed pressure-control sequence. The outlets of the microchannels were either connected with PEEK tubing for discharge to waste or sealed with Teflon screws for precise pressure control.

4. Results and Discussion

4.1 Confirmation of the stop/go function of the Laplace nanovalves

An illustration of the stop/go mode of operation is given in Fig. 3. To check the stop/go function of the Laplace valves, water was introduced into the device, and the movement of the water/air interface was observed as the air pressure was increased. When the applied pressure was 70 kPa, the air/water interface remained at Laplace valve 1, while the interface moved forward when the applied pressure was over 80 kPa. Thus, the stop/go function of Laplace valve 1 was confirmed. The stop/go function of the Laplace valve 2 was next investigated. When the applied pressure was adjusted to 140 kPa the water/air interface was at Laplace valve 2, while the interface moved beyond this point when the applied pressure attained 150 kPa. Thus, the stop/go function of Laplace valve 2 was confirmed. The estimated Laplace pressures on Laplace valves 1 and 2 were 70-80 kPa, and 140-150 kPa respectively, while the respective theoretical values were 100 kPa and 130 kPa. The estimated Laplace pressure on Laplace valve 1 was 20-30 kPa lower than the theoretical value. This difference in pressure may be attributed to the mitigation of surface hydrophobicity with the sodium hydroxide treatment. In the case of Laplace valve 2, the estimated Laplace pressure was 10-20 kPa higher than the theoretical value.

The surface roughness of the nanochannel (10 nm) may have caused an enhancement of the hydrophobicity.

4.2 Operating conditions for the femtoliter volumetric pipette

The operating conditions for the femtoliter volumetric pipette were next investigated. After water was introduced to the femtoliter volumetric pipette, a pressure of 400 kPa was applied to the air channel to cut and isolate the sample. As shown in Fig. 4(a) top, although the sample was successfully cut, it disappeared within 4 s of cutting. There are two possible explanations for this phenomenon: leakage of the water (loss of both solute and solvent) or evaporation (loss of solvent). In an attempt to clarify the reason for the sample loss, the same operation was conducted but with the use of a fluorescent solution as the sample. As shown in Fig. 4(a) bottom and 5, the fluorescence intensity of the sample remained constant, but the total volume of the solution decreased dramatically. Based on the consideration that the fluorescence intensity corresponded to the number of fluorescence molecules contained within the solution, it was concluded that evaporation was responsible for the decrease in solvent while the amount of solute within the nanochannel was retained. Noted that decrease of the fluorescence intensity after 3 s as shown in Fig. 5 is considered to be because of concentration quenching by high concentration of fluorescence molecules with the loss of solvent. This evaporation was attributed to airflow caused by the applied pressure. When 400 kPa was applied to the air channel, all of the air (nanoliter volume) passed over the sample solution (femtoliter volume) in seconds,

causing evaporation to occur. This suggests that when the sample volume is at the femtoliter level, as is often the case in nanofluidic devices, evaporation by airflow would be a significant issue.

To overcome the evaporation issue, static pressure was adopted (Fig. 4(b)) rather than dynamic pressure (Fig. 4(a)). As shown in Fig. 4(b), applying air pressure from both sides of the air channel minimized the overall air flow (static pressure). The time-course for the sample volume in this approach is shown in Fig. 6. Compared to the operation using dynamic pressure, the use of a static pressure effectively reduced the rate of evaporation by *ca.* 10 times.

Consequently, static pressure was selected as the operating mode for the femtoliter volumetric pipette. Also, the operating time was set as 0.7 s so that the loss of sample solution by evaporation was kept to a minimum (less than 1%) as shown in Fig. 6.

4.3 Demonstration of femtoliter volumetric pipette and flask

To demonstrate the working principle of the femtoliter volumetric pipette and flask, the operation of sampling defined in the section 4.2 was conducted using pure water as a model sample. Fig. 7(a) shows the time-course microscopic images for femtoliter-scale sampling and transport. First, water was introduced to the femtoliter volumetric pipette by application of pressure P_1 = 100 kPa (Fig. 7(a) top). Then, air was introduced from both the upper and lower sides of the air channel by application of a pressure of P_2 = 200 kPa, while applying a back pressure of P_3 = 140 kPa from the right side of the main channel (Fig. 7(a) middle). After 0.1s, pressure P_3 was turned off and the sample solution was transported to the femtoliter flask (Fig. 7(a) bottom). As shown in Fig. 7(a), the liquid sample was

successfully introduced, cut, and transported to the flask. Thus, the working principle of the femtoliter volumetric pipette and flask was verified. Since the effect of airflow on the evaporation of liquid sample was minimized, the sampling of 11 fL, which is 85 times smaller than our most recent report,[18] was realized.

Performance of the femtoliter volumetric pipette was quantitatively evaluated. Here, the variation of fabrication, surface modification, and liquid operation were considered as error factors. The volume of sampling chamber of the femtoliter volumetric pipette was 11.74 ± 0.73 fL (CV 6.2%) with repeated fabrication, whereas the designed volume was 11.20 fL (width × depth × length = 1600 × 700 × 10000 nm). The variation of hydrophobic-surface modification by octadecylsilane (ODS) was 103.3 ± 1.0° (CV 1.0%) as the value of the static contact angle. As shown in Fig 7(b), the variation of the measured sampling volume was 10.83 ± 0.06 fL (CV 0.6 %) based on pixel counting for 10 repeated liquid operations when the volume of fL sampling chamber was 11.06 fL (width × depth × length = 1580 × 700 × 10000 nm). Effect of above factors on the accuracy and precision of femtoliter volumetric pipette were discussed. The size variation of fabrication affects the accuracy but not the precision of the sampling volume because the femtoliter volumetric pipette is integrated in a nanofluidic device and repeatedly used. The variation of surface modification can affect the Laplace pressure up to 9.8 kPa, but as the pressure tolerance of Laplace valve 1 and 2 are designed 25.1 kPa, it does not affect the operational pressure. Thus, in principle, the variation of surface modification does not affect the error of sampling. In contrast, the variation of liquid operation can affect both accuracy and precision of

sampling volume owing to the different meniscus volume of the air/liquid interface at each time of operation. Comprehensively, the accuracy of sampling volume was affected by the difference of fabricated volume (+4.6%) and sampled liquid volume by operation (-2.1%) from the designed volume while the precision was affected by the variation of sampled liquid volume by operation (0.6%). Although the precision was three times larger than that for pipetting in a traditional volumetric analysis (about 0.2%), we consider this performance is acceptable for quantitative applications at femtoliter level because the error is less than 1% of sample volume.

In addition to pure water as a model sample used in the present study, the concept of the femtoliter volumetric pipette and flask was applied to real samples of aqueous solution in our recent report, in which sampling of cell culture medium and protein standard solutions were demonstrated.[18] In contrast, currently, sampling of organic solvents (e.g. methanol, acetonitrile, etc), is difficult because the surface of the nanochannel is hydrophobic but oleophilic. In the future, sampling of organic solvents can be achieved by applying oleophobic surface[21] to a surface of femtoliter volumetric pipette. On the other hand, in many cases of analytical chemistry, it is necessary to measure different calibrated volumes. Regarding a usage of the femtoliter volumetric pipette as a graduated pipette, a multiple of 11 fL (the sampling volume of current femtoliter volumetric pipette) is feasible by repeating the sampling operation with desired times.

Utilizing the femtoliter sampling method, high-performance nanofluidic analytical devices exploiting single-molecule ELISA[13] and attoliter-to-femtoliter chromatography with one-million

theoretical plates[11] can be realized. For example, we have realized the quantification of countable-number proteins secreted from single cell, which is an important achievement for single cell life sciences.[18] Accordingly, the present study developed the femtoliter sampling method in nanochannel, which has been missing part of three components of analysis (sampling, chemical processing and detection) in nanofluidics, and will lead to novel analytical applications such as single-molecule or single-cell analyses.

5. Conclusions

In the context of nanofluidics, we developed a femtoliter volumetric pipette and flask which utilize air pressure and surface tension for operation. The nanofluidic device incorporating an 11 fL volumetric pipette and a 50 fL flask was fabricated in glass substrates and reliable operation conditions required for manipulation of the gas/liquid interface in the nanochannels using Laplace valves was established. Initially, evaporation of the liquid sample owing to the airflow generated during cutting of the liquid sample was significant, but we suppressed the evaporation by minimizing the airflow and shortening the operational time to 0.7 s, less than the time for evaporation. Finally, the working principle of the femtoliter volumetric pipette and flask with 11 fL sample volume was verified. The sampling volume was measured as 10.83 ± 0.06 fL (CV 0.6 %) with repeated operations. Generally, the sampling process is fundamental in analytical chemistry. The present study realized the sampling at femtoliter volume in nanofluidic device, which is a key technology for analyses of single cell or

analyses at single-molecule level, and hence advance knowledge in various fields including biology and medicine.

Conflicts of interest

There are no conflicts to declare

Acknowledgements

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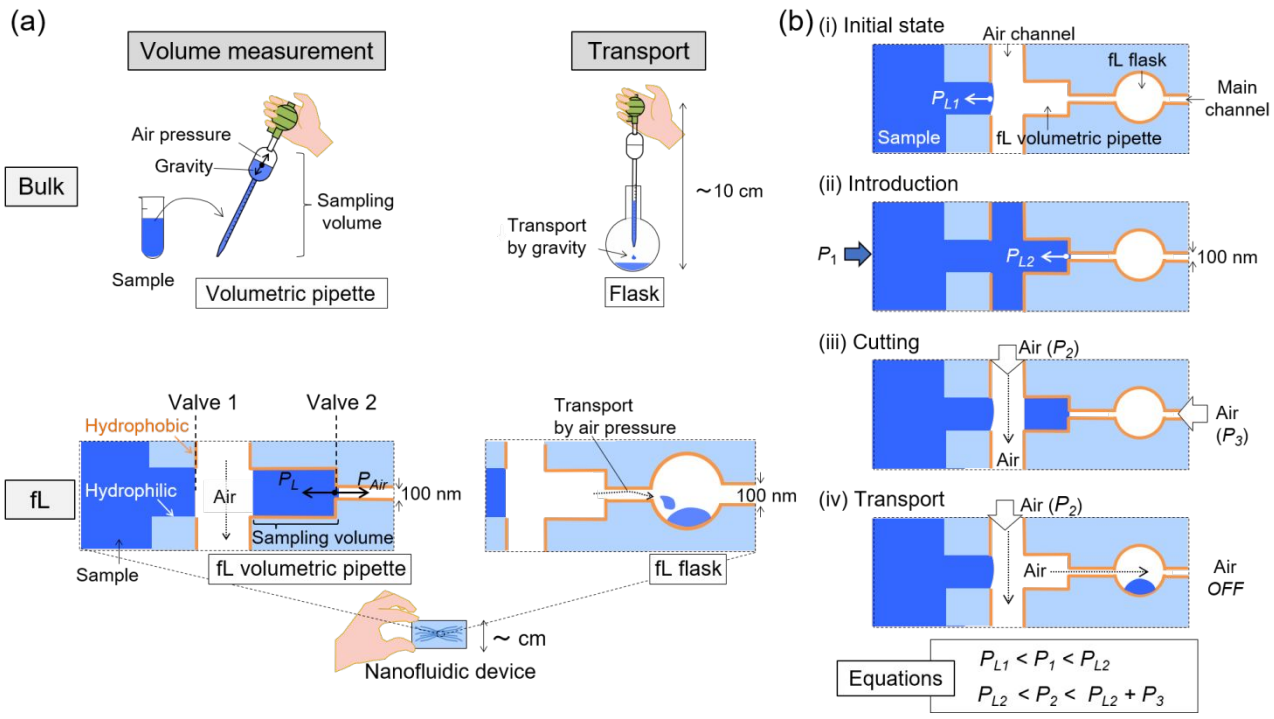


Figure 1. Concept of femtoliter-scale sampling method. Conventional volume measurement and transport in bulk: volumetric pipette and flask (top). femtoliter volume measurement and transport: femtoliter volumetric pipette and flask (bottom). (b) Operation of femtoliter volumetric pipette and flask.

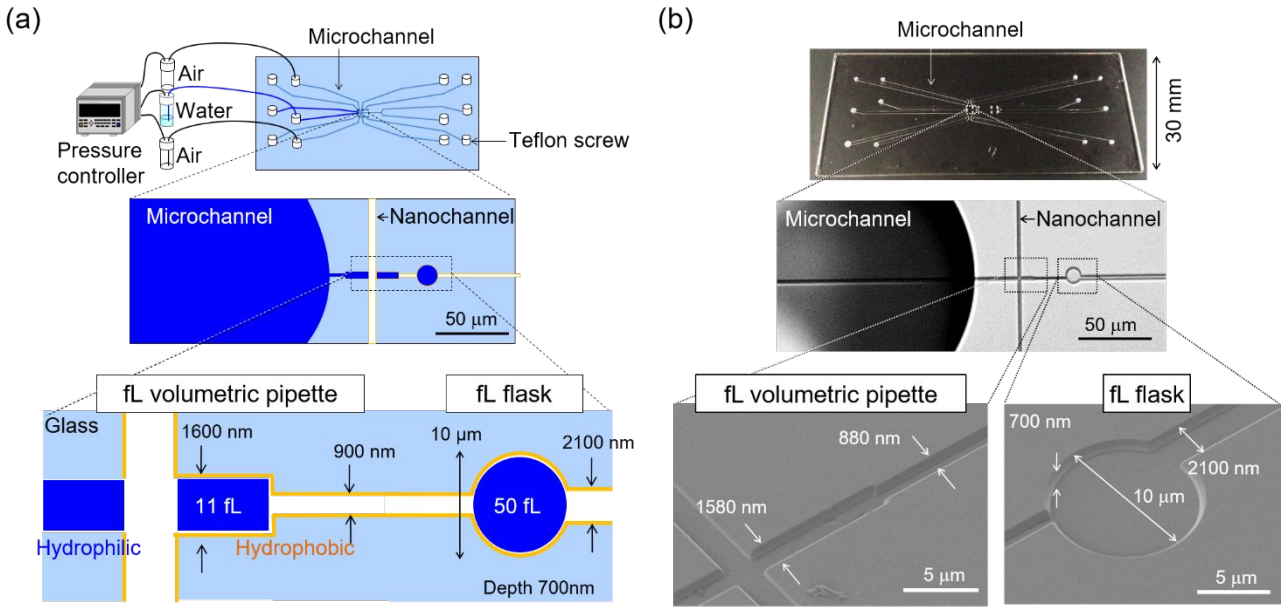


Figure 2. (a) Design of femtoliter volumetric pipette and flask. (b) Microscopic and scanning electron microscopic images of the fabricated femtoliter volumetric pipette and flask.

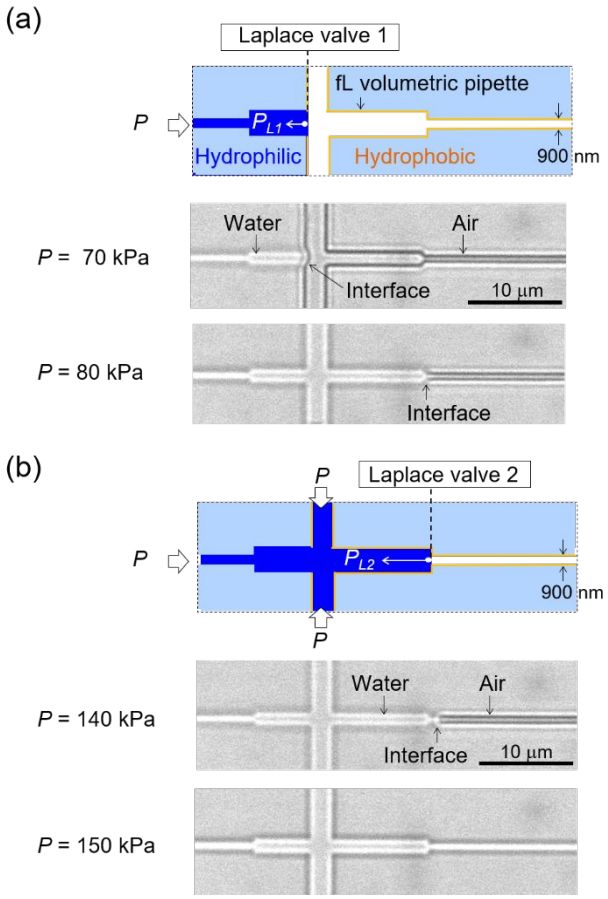


Figure 3. Schematic diagram (top) and microscopic photos (middle and bottom) for investigation of stop/go function of Laplace valve 1 (a) and 2 (b).

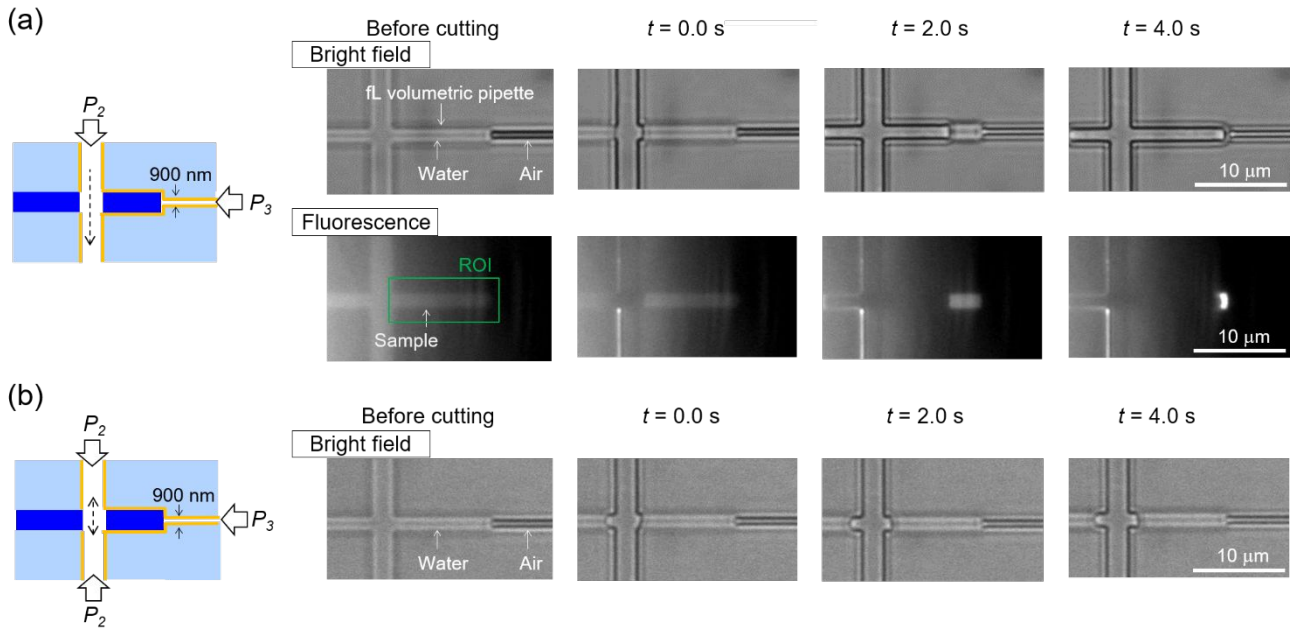


Figure 4. Schematic and time-course microscopic images for the operation of femtoliter-scale sampling with (a) dynamic pressure and (b) static pressure. Rectangle frame (green) represents the region of interest (ROI) for measurement of fluorescence intensity

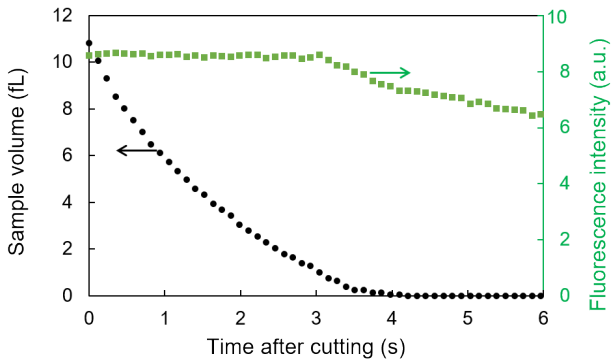


Figure 5. Time-course measurement of sample volume and fluorescence intensity. Black circular dots represent the sample volume in femtoliter volumetric pipette during cutting operation. Green square dots represent fluorescence intensity from the region of interest (ROI) shown in Fig. 4.

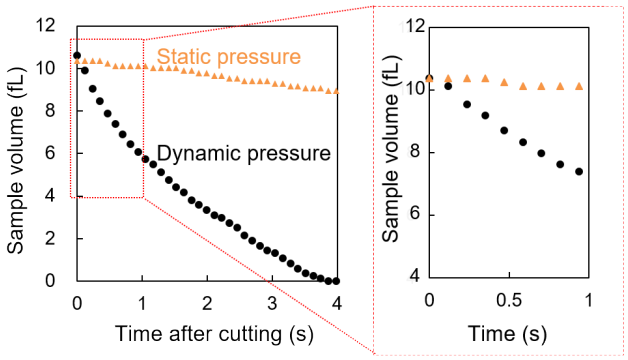


Figure 6. Time-course measurement of sample volume with the operation using dynamic pressure and static pressure.

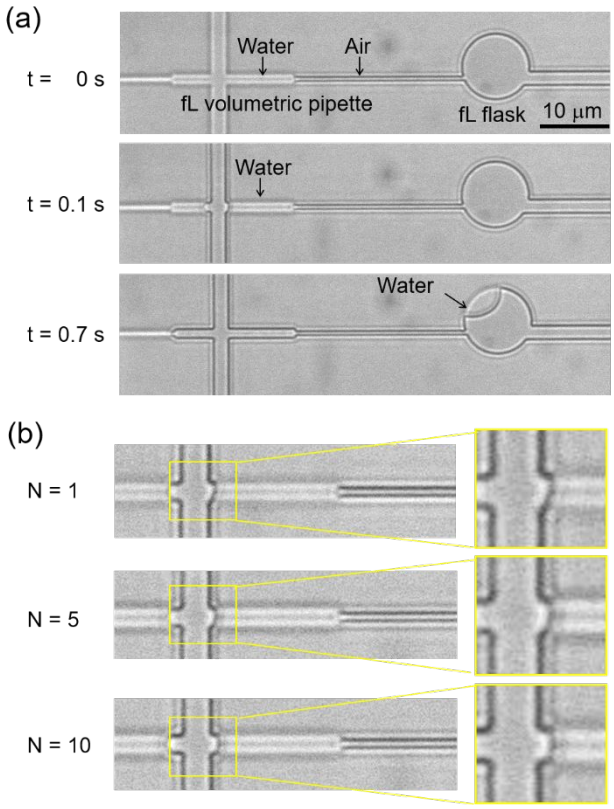
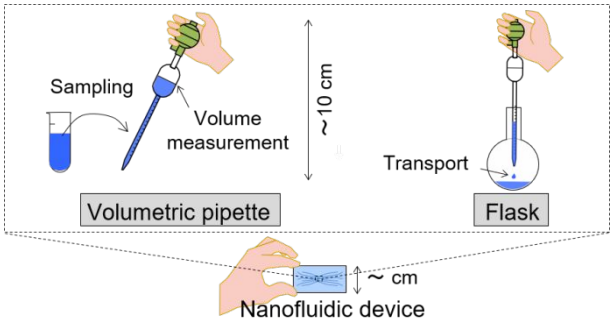


Figure 7. (a) Demonstration of working principle for femtoliter volumetric pipette and flask. (b) Three examples of femtoliter liquid sampling (11 fL). The meniscus of the femtoliter liquid sample is the dominant source of sampling error.



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A femtoliter sampling method, which includes volume measurement and sample transport, was realized by femtoliter volumetric pipette and flask fabricated on a glass-made nanofluidic device