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Femtoliter-Scale Separation and Sensitive Detection of Nonfluorescent Samples in an Extended-Nano Fluidic Device

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ABSTRACT

The separation and sensitive detection of nonfluorescent molecules at the femtoliter (fL) scale has been achieved for the first time in a nanofluidic channel. Smaller sample volumes and higher separation efficiencies have been significant targets for liquid chromatography for many years. However, the use of packed columns hindered further miniaturization and improvement of separation efficiency. Our group recently developed a novel chromatographic method using an open nanofluidic channel to realize attoliter sample injection and a separation efficiency of several million plates/m. However, because of the extremely small optical path length, this detection method was limited to fluorescent molecules. Herein, we describe the combination of nanofluidic chromatography with differential interference contrast thermal lens microscopy (DIC-TLM), a sensitive detection method for nonfluorescent molecules developed by our group that has the ability to detect 0.61 zmol (370 molecules) with an optical path length of 350 nm. As a result, separation of a 21 fL sample containing 250 zmol was possible at the limit of detection (LOD).

1. Introduction

Smaller sample volumes and higher separation efficiencies have been major targets of separation science for many years. For instance, capillary electrophoresis greatly contributed to the decrease in the sample volume, and realized the integration of microfluidic chips with sensitive detection systems[1,2,3]. Pressure-driven liquid chromatography (LC) is also a powerful separation technique that can separate various types of molecules. Therefore, miniaturization of LC has been a significant focus of research, and a few studies on highly-efficient LC in microfluidic channels have been reported[4,5]. In addition, LC using nanocapillaries has recently been reported with a focus on the effects of electric double layer overlap[6] and much smaller sample volumes[7]. Thus, the field of separation analysis is moving from the micro to the nanoscale.

Separately, our group discovered that water possesses unique characteristics when confined in extended-nano (10^1–10^2 nm scale) fluidic channels[8], and has worked to understand the fundamentals of extended-nano fluidics in order to develop chemical devices that exploit these unique properties. For example, we have proposed an extended-nano chromatography method using an open nanochannel as a separation column[9]. Using a high speed, high pressure control system, we have developed a nanofluidic control technique that enables a minimum sample injection volume of 180 aL[10]. In addition, we verified that the surface dominant properties of the extended-nano space make it possible to achieve a high separation efficiency of 450,000 plates/m, and potentially 7,000,000

plates/m[11].

However, detection becomes quite difficult in such a nanospace because of small sample volume, small number of analyte molecules, and short optical path length. Laser induced fluorescence is the most frequently used technique for micro/nanofluidics[12,13], even though the number of molecules with high fluorescence quantum efficiency is limited and most molecules are nonfluorescent. Other detection methods such as electrochemical redox cycling[14] and plasmonic sensing[15,16] have been proposed for sensitive detection in nanochannels, but the available analytes for these methods are also very limited. Therefore, our group recently developed a novel detection technique called differential interference contrast thermal lens microscope (DIC-TLM) based on thermal lens spectroscopy (TLS)[17]. The novel detector can detect 390 molecules in a 500 nm deep nanochannel by utilizing wave optics that can be applied to nanospaces smaller than the wavelength of light[18], unlike conventional TLS[19,20], based on geometrical optics related phenomena such as refraction and deflection that are ineffective in nanospace. The greatest advantage of the thermal lens technique is that it can detect nonfluorescent molecules that have light absorption bands from the near infrared (IR) to the ultraviolet (UV) region. Thus, the combination of thermal lens detection with separation techniques is significantly important for analytical chemistry as a means for distinguishing different molecules that have absorption bands at the same wavelengths[21,22,23,24]. Previously, we considered the possibility of using DIC-TLM as a detector for extended-nano chromatography[25].

Herein, the separation and sensitive detection of nonfluorescent molecules were achieved using an extended-nano fluidic device for the first time. Both the detection conditions for DIC-TLM and separation conditions in the extended-nano fluidic device were optimized to confirm a separation efficiency of 150,000 plates/m (170 plates/1.1 mm). The injection volume and detection performance of DIC-TLM were then thoroughly evaluated. The results indicated that at the calculated limit of detection (LOD), a 21 femtoliter (fL) sample containing 250 zmol of analyte could be separated and detected.

2. Materials and methods

2.1. Fabrication of the micro/nanofluidic chip

Extended-nano channels were fabricated via electron beam (EB) lithography and plasma etching. First, an EB resist was spin coated on a 30 × 70 mm fused silica substrate, and a pattern of nanochannels was drawn and developed. Second, the nanochannels were fabricated by irradiating the patterned substrate with CHF_3/SF_6 plasma. The width and depth of the nanochannels was determined by the lithography and plasma irradiation times, respectively. In this study, the size of all of the nanochannels was 2.3 μm-wide by 350 nm-deep. Next, microfluidic channels were fabricated via UV photolithography and plasma etching on the same substrate. Cr (100 nm thick) was sputtered on the nanofabricated substrate, and the pattern of microchannels was transferred using a UV

photomask to fabricate microchannels that were 500 μm-wide by 5 μm-deep. Finally, the substrate with micro and nanochannels was bonded with another fused silica substrate with connection holes for liquid introduction using thermal fusion bonding in a vacuum furnace at 1080°C.

2.2. Experimental setup

Figure 1(a) illustrates the experimental setup for extended-nano chromatography, which is based on our previous report[9]. Two independent pressure controllers (PC-20, Nagano Keiki) were used to control the flow rates by pushing liquids in vials into the fused silica chip. Because of the slow time response of the pressure controllers, 3-way valves were used to switch the pressures on and off. The microchannels work as liquid reservoirs for the introduction of a solution of a sample and mobile phase into the nanochannels. In this study, the chip had orthogonally crossed nanochannels, including one loading nanochannel (longitudinal direction) and three separation nanochannels (lateral direction). Sample injection, separation, and detection were performed in three steps (Figure 1(b)). First, the sample was placed in the loading channel by applying the same pressure (0.1–0.5 MPa) from three sides. Second, the sample was injected into the separation channels by turning off the pressure on the upper and right sides and switching the flow direction. Finally, the mixed sample was separated via surface interactions with the nanochannel, and the analytes were detected using a DIC-TLM at a point 1.1 mm downstream from the nanochannel intersection.

The detection principles for DIC-TLM have been previously explained[26]. Briefly, in this study, an excitation laser was focused on the sample in order to produce heat and a local change in the refractive index (referred to as the photothermal or thermal lens effect) in the nanochannel via light absorption and nonradiative relaxation. The change in refractive index was then detected by an interferometer using a second probe laser. This technique is particularly effective for an optical path length shorter than the wavelength of the laser light because it is based on wave optics. In the DIC-TLM used in this study, an argon ion laser (488 nm) was used for excitation and a He–Ne laser (633 nm) was used as the probe. The power of the excitation beam was 30 mW under an objective lens with a numerical aperture of 0.75. The detection volume of the DIC-TLM was precisely determined to be 170 aL given the diameter of the focused excitation beam and the nanochannel depth. In addition, the time response of the DIC-TLM signal was determined by the time constant of the lock-in amplifier (t_c).

2.3. Reagents

The nonfluorescent dyes Sudan I and Sudan Orange G were used for normal phase mode separation[27]. The stationary phase was a bare fused silica surface with silanol and siloxane groups. Hexane with 0.3%–5% 2-propanol was used as the mobile phase. All reagents were purchased from Wako Pure Chemical Industries and used without further purification.

3. Results and discussion

3.1. Optimization of detection conditions

Figure 2 shows chromatograms for Sudan I and Sudan Orange G obtained at different t_c values when 1.0% 2-propanol was added to the mobile phase. The concentrations of the injected samples were 0.50 and 0.25 mM, respectively. The measurements were performed three times for each condition, and the reproducibility of the peak time and width was good (within 1 second variation). Because the raw chromatograms had small background signals (several microvolts) due to the minor light absorption of hexane at 488 nm, the background signal was subtracted. The chromatograms clearly show that the time response of the signal was slower for larger time constants, while the baseline noise was smaller. The resolution R was 1.71 $\pm$ 0.15 and 1.40 $\pm$ 0.03 for t_c values of 100 and 300 ms, respectively. Based on these results, a t_c of 300 ms was determined to be suitable for the subsequent experiments because the peak widths at 100 and 300 ms were nearly the same, and the value of R was approximately 1.5.

3.2. Composition of the mobile phase

Figure 3 shows chromatograms obtained for 0.50 mM solutions of Sudan I and Sudan Orange G using different mobile phase compositions. Evaluation of these chromatograms revealed that the

separation mode was the normal phase mode because more hydrophilic Sudan Orange G was retained on the silica surface, while Sudan I was barely retained. Addition of 0.3%–1% 2-propanol in hexane was determined to be the most appropriate mobile phase in this study; however, the best composition varied day to day if the same chip was used. This variation suggests that the surface of the fused silica nanochannel was very delicate, and the conditions for both washing and storage of the nanochannel should be more carefully considered.

Next, although a van Deemter plot could not be obtained because of the low pressure range of the pressure controllers, the theoretical plate number was calculated to be approximately 150,000 plates/m (170 plates/1.1 mm) for the retained sample (Sudan Orange G) based on its bandwidth, which was of the same order as the previously reported value[11].

3.3. Evaluation of the injection volume and detection performance

Unlike analyses performed using a fluorescent microscope, evaluation of the injection volume when using the DIC-TLM is not simple. Thus, the injection volume was determined from the peak area and thermal lens signal intensity. Figure 4 shows chromatograms for samples containing different concentrations of Sudan I. Simultaneously, the thermal lens signal intensity for the corresponding Sudan I solutions (not injected, but flowing in the nanochannel) was measured at the same pressure and flow rate in order to eliminate the dependence of the signal on the flow rate. As can be seen in

the figure, the peak areas of the injected Sudan I samples were proportional to their concentrations, indicating that the injection volume was constant. Therefore, the peak area of a 1 mM sample [in μV·s] equaled the product of the thermal lens signal intensity of the same 1 mM solution (155 μV) and injection time, giving a calculated injection time of 0.19 ± 0.01 s. The injection volume was then calculated as the product of the injection time and flow rate, or 21 ± 1 fL. Here, the flow rate was calculated to be 120 fL/s using the peak time and nanochannel length, although the real flow rate in the injection channel may have been lower than that of the separation channel when the pressure was switched off. Thus, the injection volume was estimated to be less than 21 fL.

Finally, the LOD was calculated from Figure 4 using S = (the mean value of the baseline) + 2σ. The LOD of the concentration of the injected sample was determined to be 12 μM because the peak height was also proportional to the concentration, indicating that the separation and detection of 250 zmol was possible. Furthermore, the injected sample diffused in the nanochannel, and the concentration was determined from the peak height to be only 3.5 μM at the detection point. The number of molecules in the detection volume was calculated to be 0.61 zmol (370 molecules); thus, highly sensitive detection of absorbance was achieved in a nanochannel shorter than the wavelength of light.

4. Conclusions

The separation and sensitive detection of zmol-level nonfluorescent molecules in a femtoliter sample were integrated in a nanofluidic device for the first time. Two nonfluorescent dye molecules were chromatographically separated using an open nanofluidic channel, and then detected using a detection system based on wave optics that make sensitive detection possible in a nanospace smaller than the wavelength of the laser light. The separation efficiency of this extended-nano chromatography technique was 150,000 plates/m (170 plates/1.1 mm), and the LOD of the DIC-TLM was 370 molecules in a 2.3 μm-wide by 350 nm-deep nanochannel. Moreover, the injection volume and number of molecules were estimated to be 21 fL and 250 zmol at the LOD, indicating that the separation of a sample size 8 orders of magnitude smaller than that possible with high-performance liquid chromatography (HPLC) was achieved. Considering the 10^1–10^2 nm scale is the limit at which a liquid acts as fluid, the device investigated in this study is the extreme miniaturization of an LC system combined with an absorbance detector. Furthermore, because the injection volume was much smaller than a single cell (pL) and the number of molecules (zmol) was similar to the number of typical protein molecules in a single cell, this device is promising for single cell research in the future.

5. Acknowledgement

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References

[1] D. Chen and N. Dovichi, *Anal. Chem.,* 1996, **68**, 690-696.

[2] C. Effenhauser, G. Bruin, A. Paulus and M. Ehrat, *Anal. Chem.,* 1997, **69**, 3451-3457.

[3] J. Fister, S. Jacobson, L. Davis and J. Ramsey, *Anal. Chem.,* 1998, **70**, 431-437.

[4] J. P. Kutter, *J. Chromatogr. A,* 2012, **1221**, 72-82.

[5] W. De Malsche, J. O. De Beeck, S. De Bruyne, H. Gardeniers and G. Desmet, *Anal. Chem.,* 2012, **84**, 1214-1219.

[6] X. Wang, J. Kang, S. Wang, J. J. Lu and S. Liu, *J. Chromatogr. A,* 2008, **1200**, 108-113.

[7] X. Wang, C. Cheng, S. Wang, M. Zhao, P. K. Dasgupta and S. Liu, *Anal. Chem.,* 2009, **81**, 7428-7435.

[8] T. Tsukahara, K. Mawatari and T. Kitamori, *Chem. Soc. Rev.,* 2010, **39**, 1000-1013.

[9] M. Kato, M. Inaba, T. Tsukahara, K. Mawatari, A. Hibara and T. Kitamori, *Anal. Chem.,* 2010, **82**, 543-547.

[10] R. Ishibashi, K. Mawatari, K. Takahashi and T. Kitamori, *J. Chromatogr. A,* 2012, **1228**, 51-56.

[11] R. Ishibashi, K. Mawatari, and T. Kitamori, *Small,* 2012, **8**, 1237-1242.

[12] P. Dittrich and A. Manz, *Anal. Bioanal. Chem.,* 2005, **382**, 1771-1782.

[13] J. T. Mannion and H. G. Craighead, *Biopolymers,* 2007, **85**, 131-143.

[14] B. Wolfrum, M. Zevenbergen and S. Lemay, *Anal. Chem.,* 2008, **80**, 972-977.

[15] N. Ly, K. Foley and N. Tao, *Anal. Chem.,* 2007, **79**, 2546-2551.

[16] Y. Oh, S. Park, M. Kang, J. Choi, Y. Nam and K. Jeong, *Small,* 2011, **7**, 184-188.

[17] J. Harris and N. Dovichi, *Anal. Chem.,* 1980, **52**, 695A-706A.

[18] H. Shimizu, K. Mawatari and T. Kitamori, *Anal. Chem.,* 2010, **82**, 7479-7484.

[19] R. Snook and R. Lowe, *Analyst,* 1995, **120**, 2051-2068.

[20] T. Kitamori, M. Tokeshi, A. Hibara and K. Sato, *Anal. Chem.,* 2004, **76**, 52A-60A.

[21] T. Nolan, D. Bornhop and N. Dovichi, *J. Chromatogr.,* 1987, **384**, 189-195.

[22] F. Li, A. A. Kachanov and R. N. Zare, *Anal. Chem.,* 2007, **79**, 5264-5271.

[23] F. Kitagawa, Y. Akimoto and K. Otsuka, *J. Chromatogr. A,* 2009, **1216**, 2943-2946.

[24] S. Hiki, M. Tokeshi, M. Kakuta, K. Mawatari, Y. Kikutani, K. Sato, A. Hibara, K. Shimura, N. Uchida and T. Kitamori, *Bunseki Kagaku,* 2007, **56**, 1-7.

[25] H. Shimizu, K. Mawatari and T. Kitamori, *J. Sep. Sci.,* 2011, **34**, 2920-2924.

[26] H. Shimizu, K. Mawatari and T. Kitamori, *Anal. Chem.,* 2009, **81**, 9802-9806.

[27] P. Botek, J. Poustka and J. Hajslova, *Czech. J. Food Sci.,* 2007, **25**, 17-24.

Figure captions

Fig.1 Illustration of the extended-nano chromatography method (a) Setup of the fluidic control system and (b) the sample loading, injection, and detection procedures. (c) SEM image of injection point and (d) detection point (end of nanochannel).

Fig. 2 Chromatograms of Sudan I and Sudan Orange G recorded using different lock-in amplifier time constants.

Fig. 3 Chromatograms of Sudan I and Sudan Orange G in normal phase mode.

Fig. 4 Quantitative performance of the differential interference contrast thermal lens microscopy (DIC-TLM). Chromatograms of Sudan I for three different concentrations.

Fig. 1

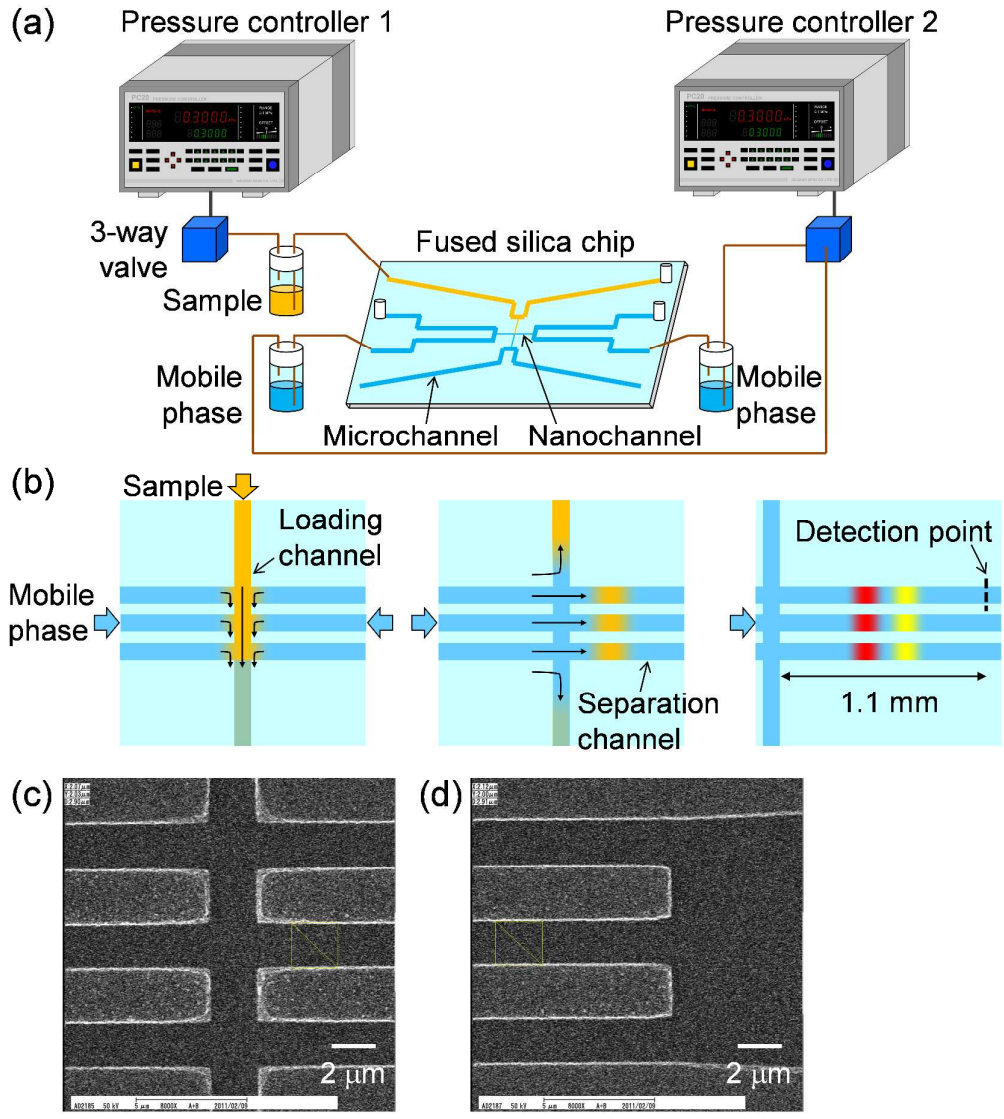


Fig. 2

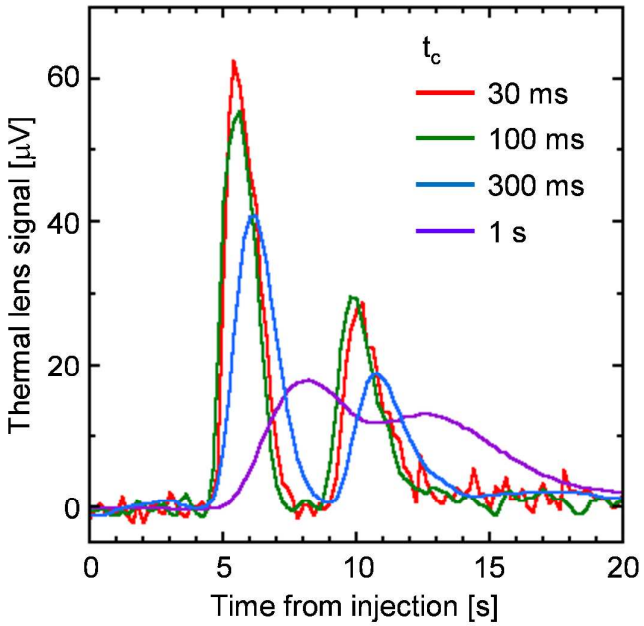


Fig. 3

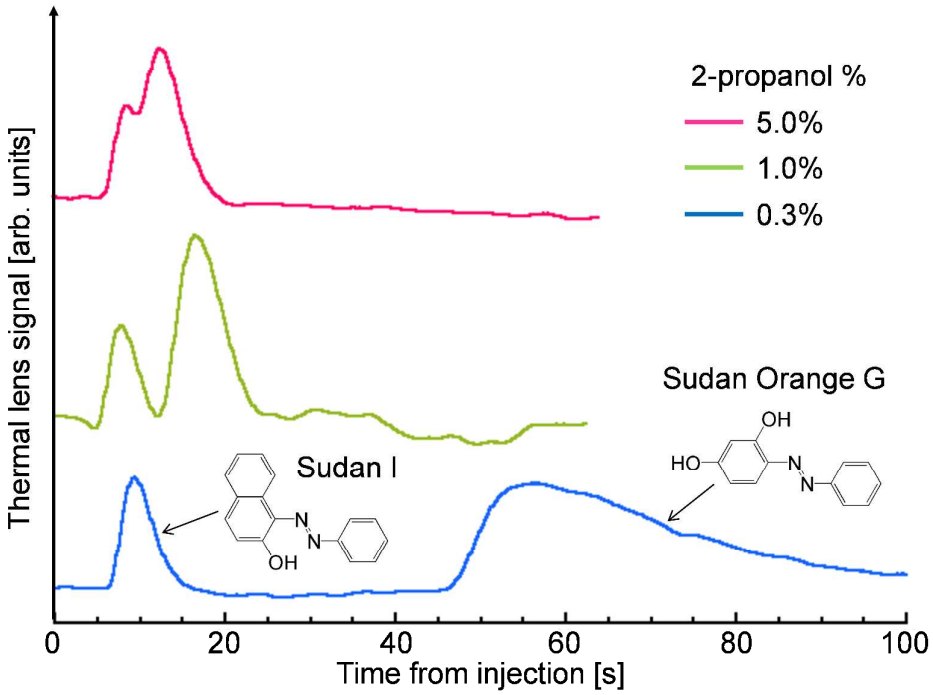
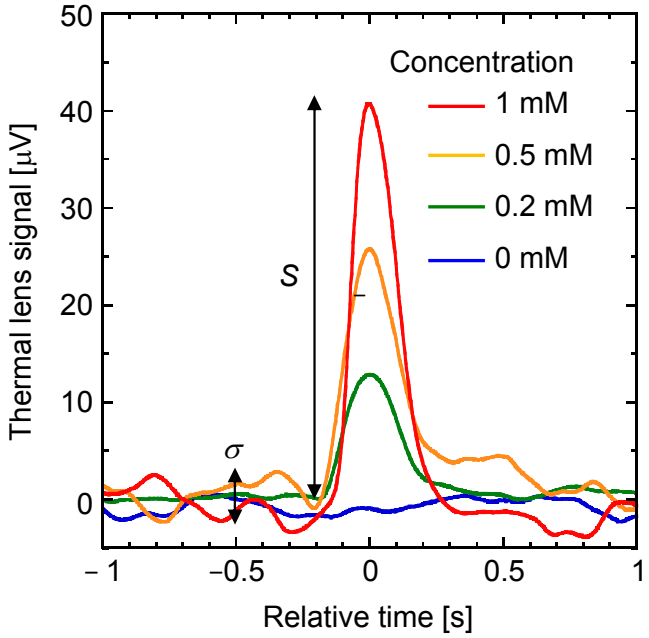


Fig. 4



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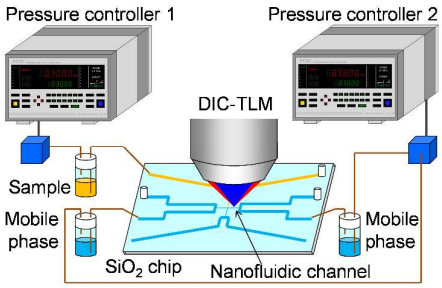

Liquid chromatography using nanofluidic chip and DIC-TLM realized separation and detection of 21 fL, 0.61 zmol nonfluorescent sample.