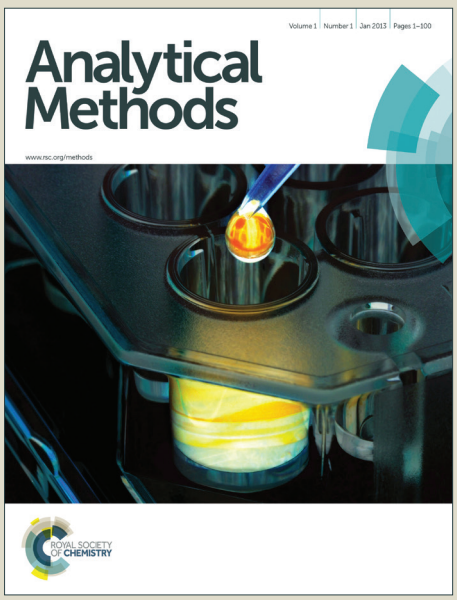
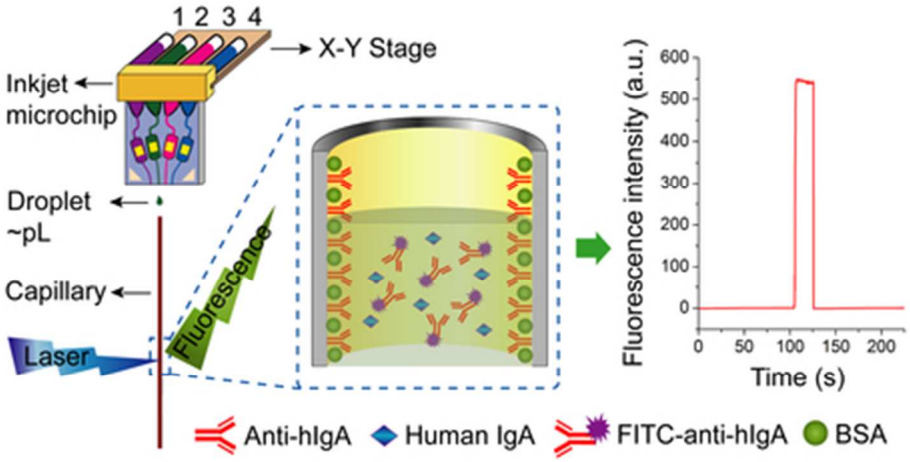
Analytical Methods

Accepted Manuscript



ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/methods



A quantitatively controlled immunoassay based on inkjet technology to perform nanoliter immunoassay in capillary microreactor was presented.
39x19mm (300 x 300 DPI)

Quantitative-nanoliter immunoassay in capillary immune microreactor adopted inkjet technology

Jianmin Yang, Hulie Zeng, Shuhua Xue, Fengming Chen, Hizuru Nakajima, Katsumi Uchiyama*

Department of Applied Chemistry, Graduate School of Urban Environmental Sciences, Tokyo Metropolitan University, Minamiohsawa, Hachioji, Tokyo 192-0397, Japan

* ***Corresponding author***. *E-mail: uchiyama-katsumi@tmu.ac.jp; Tel: +81-42-6772835, Fax: +81-42-6772821*

ABSTRACT

A quantitatively controlled immunoassay at the nanoliter level based on inkjet technology was developed. The volumes of solutions/samples introduced were accurately controlled at nanoliter level by a four-channel inkjet microchip. Antibody/antigen recognition was performed in an amino modified capillary with a short diffusion distance. As a proof-of-concept, a sandwich immunoassay of human IgA was conducted using the developed method. The results demonstrated a low detection limit (0.03 ng mL^{-1}) and a wide linear range (0.1-100 ng mL^{-1}, R^2 = 0.9959), comparable to currently used methods. For each capillary immunoassay, the volumes of the ejected solutions for human IgA, FITC conjugated anti-human IgA and the glycine-HCl dissociation solution were 52.15 ± 1.53 nL, 65.70 ± 2.06 nL and 37.51 ± 0.96 nL, respectively. The method, in which an inkjet functions as a novel "nanoliter pipette" in combination with a capillary for nanoliter immunoassays has promising applications in areas of clinical diagnosis and drug screening.

Introduction

Over the past few decades, immunoassays have been extensively utilized as a general approach to diagnosing various diseases. Traditional immunoassays on a 96-well microtiter plate typically involve the use of relatively large amounts of reagents, long assay times and tedious procedures. For these reasons, the development of new strategies for immunoassays with high-throughput and rapid analysis times, as well as lower reagent consumption and automatic operation would be highly desirable.[1-3] Among them, low volume immunoassays have attracted considerable interest, since they involve lower volumes of reagents and assay speeds are accelerated. The recently developed microfluidic chips,[4,5] digital microfluidic platform,[6,7] compact disk-like microfluidic platforms,[8,9] and SlipChips[10] have shown merits in the performance of immunoassays at the nanoliter level. However, complicated channel designs and fabrication procedures are needed, as well as a sophisticated control system.[11]

Although these microfluidic devices permit immunoassays to be carried out at the nanoliter level, sample/reagent introduction is still performed by a pipette at the microliter level.[4,8,10] A pump coupled with micro pipeline has also been used in microfluidic systems for delivering solutions.[12,13] However it is difficult to precisely control solution volumes at the nanoliter level. In addition, unavoidable reagent waste or dilution sometimes occurs. Reports regarding the direct manipulation of nanoliters of reagent for immunoassays are quite rare, because nanoliter volumes are very small and evaporate rapidly.[14,15] Actually, quantitative and reproducible liquid handling at the nanoliter level is very difficult or impossible when using conventional methods, such as pipettes/syringes on a microliter scale. The generations of droplets based on microfluidics have been widely reported, high-throughput nanoliter

droplets can be obtained when flexible chip designs and an immiscible oil phase are used.[16-18] However, it is difficult to apply such systems to complex immunoassays, due to the lack of controllability. Therefore, to accurately measure and manipulate nanoliters of solutions for nanoliter immunoassays, it is necessary to develop alternate approaches for solving this problem.

Recently, inkjet technology has attracted a great deal of interest because it permits nano-/pico-liter injections to be performed with spatial and temporal control. In addition, it has great advantages and potential for use in droplet manipulation and ejection for precise and ultra-small sample volumes and can be automated. Arrabito and coworkers [19] reported on a low cost and high performance drug screening methodology, based on an inkjet printing system for delivering molecules in a picoliter drop microarray format. Yasui et al [20] demonstrated that DNA droplets could be injected with an inkjet injector for microchannel array electrophoresis and was able to achieve the high throughput analysis of biomolecules. Inkjet technology has also been used as an injection tool for gas chromatography (GC),[21] capillary electrophoresis (CE),[22,23] chemiluminescence (CL),[24] and mass spectrometry (MS)[25]. However, there are few reports regarding the application of inkjet technology for immunoassays. In our previous study, a rapid ELISA performed using a nanoliter droplet on a PDMS microwell was reported,[26] in which inkjet technology was initially applied to an immunoassay. However, the use of a primary inkjet microchip had some drawbacks, such as, an additional reagent (glycerol) was needed to increase the ejection efficiency and decrease the evaporation. Herein, the improved hardware and software for an inkjet injector was

optimized to exclude additional reagent,[23] which will improve the performance of inkjet application on immunoassay.

In this work, we developed a quantitatively controlled immunoassay based on inkjet technology to perform nanoliter immunoassay in capillary microreactor. A noncontact injection system based on a four-channel inkjet microchip was exploited. Various solutions/samples could be injected and manipulated automatically at the nanoliter level by controlling the injection parameters. At the same time, a capillary, performing as a microreactor, can be used to effectively accumulate and accelerate an immunoassay by taking advantage of the enhanced surface area to volume ratio and the short diffusion distance. In addition, evaporation could be essentially eliminated with the reaction performed in the capillary. By integrating the inkjet injection technique with the capillary system, a novel strategy for conducting a nanoliter-level immunoassay was developed and successfully used in an assay for human IgA. It has great potential for use in immunoassays, as well as a promising tool for low-cost and rapid assays in disease diagnosis in the future.

Experimental

Instrumental setup for immunoassay

The immunoassay system with accurate nano-injection was constructed as illustrated in Fig. 1, and consists of an inkjet injector, a capillary immune microreactor, and a laser-induced fluorescence (LIF) detection system. As described in our previous reports,[22-24] sample/solution introduction was carried out by a four-channel inkjet microchip (Fuji Electric, Tokyo, Japan), which was positioned by means of an electromotive X-Y stage. A piece of piezoelectric ceramic was tightly attached to each loading chamber of the inkjet microchip.

The solution loaded in chamber was pressed by the bended piezoelectric ceramic when a pulse voltage was applied, at which time, the droplets were ejected from the nozzle. The two-dimensional X-Y stage was used to hold the inkjet microchip and control its exact position to match the inlet of the capillary tip.

The capillary immune microreactor was fabricated from a fused silica capillary with a UV transparent coating (i.d. 100 μm, L 10.0 cm and L_{eff} 5.0 cm, GL Science, Tokyo, Japan) and a syringe pump (NE-300, New Era Pump Systems, Inc, New York, USA). A 55037-U PEEK screw bolt and a silicon tube (3.0 mm i.d.) were used to connect the capillary and syringe pump. An orthogonal excitation configuration LIF detection system was assembled to detect the fluorescence signal (see ESI Part B for detailed information).

Modification of the internal surface of the capillary

To improve the sensitivity of the immunoassays, amine functionalization of inner surface of the fused silicon capillary was performed with 2% (v/v) 3-aminopropyltriethoxysilane (APTES) at 75 ℃ for 1 h.[27-29] Detailed information can be found in ESI (Part C and Fig. S1a-c).

Immunoassay procedure

For the antibody immobilization, 100 μL of 20.0 μg mL^{-1} anti-human IgA containing 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and n-hydroxysulfosuccinimide (SNHS)[29] was introduced into the modified capillary by the syringe pump (Fig. S1d). After a 10 min incubation at room temperature (23 ± 1 ℃), the unbounded anti-human IgA was washed out with 100 μL of Tween 20-PBS. The capillary, containing the covalently immobilized anti-human IgA, was then blocked with 1% (w/v)

BSA (Fig. S1e) for 10 min at room temperature and subsequently washed out with 100 μL of Tween 20-PBS. The capillary immune microreactor was stored in a freezer (-10 ℃) until used.[30]

During the assay, the human IgA solution (diluted with PBS), FITC labeled anti-human IgA (FITC-anti-hIgA, 5.0 μg mL^{-1}), 0.05% (v/v) Tween 20-PBS, and 0.1 M glycine-HCl (pH 2.2) were sequentially loaded into the inkjet microchip. The capillary immune microreactor was cut into a 10.0 cm section in advance. Initially, 300 droplets (52.15 ± 1.53 nL) of the human IgA solution was introduced onto the tip of the capillary, then it was sucked into the capillary at 1.0 cm min^{-1} by the syringe pump (Fig. S1f). The capillary was then washed three times with 100 droplets (82.33 ± 2.26 nL) of Tween 20-PBS at the same velocity by the syringe pump. Then, 300 droplets (65.70 ± 1.53 nL) of 5.0 μg mL^{-1} FITC-anti-hIgA were introduced into the capillary by the same procedure as described above for human IgA introducing (Fig. S1g). After washing with Tween 20-PBS, 200 droplets (37.51 ± 0.96 nL) of 0.1 M glycine-HCl (pH 2.2) solution were introduced to remove the complex of human IgA and FITC-anti-hIgA, as shown in Fig. S1h. In this case, the glycine-HCl acted as an extractant to dissociate the FITC-anti-hIgA, which was then eluted from the inlet to the detection point (Fig. S1i). The fluorescence intensity of the eluted FITC-anti-hIgA was measured at least three times and the averages of the fluorescence intensities are shown.

Results and discussion

Optimization of the inkjet ejection conditions and evaluation of the LIF

As described previous report,[24] the size and volume of the droplets were easily manipulated via controlling the driving voltage and pulse duration. To obtain stable and uniform picoliter

droplets, the influence of pulse duration on the volume of the ejected droplet was investigated, as listed in Table S1. The optimal ejection conditions for each solution were nearly the same except for the glycine-HCl solution as shown in Table 1. To determine the volume of each introduced solutions/samples droplet, 1000 droplets of solutions/samples were weighed and the weight was divided by its density using the burst gravimetric method.[31] As shown in Table 1, the droplet volumes of each solution were at the picoliter with high reproducibility. Totally consumption of each immunological solution was kept at tens of nanoliters, and the droplet numbers were in the range from 100 to 300.

Meanwhile, the stability and reliability of the LIF system was tested by the fluorescence detection of various concentrations of fluorescein sodium salt. A linear relationship from 10 to 10000 ng mL^{-1} was obtained with an R^2 value of 0.9991, and the RSDs were below 1.2 % (Fig. S2). The detection limit for Fluorescein was 0.5 ng mL^{-1}.The results confirmed that the stability and reliability of the current system were satisfactory for LIF measurements at the nanoliter level.

Optimization of the immunoassay procedure

The density of the antibody on the internal surface of capillary played an important role in the immunoassay characteristics. It was mainly dependent on both the reaction time and temperature at the immobilization step. To determine the optimum reaction time between the antibody and the internal surface of the amine functionalized capillary, FITC-anti-hIgA was used as a model to optimize the influence of reaction time on the immobilization of the antibody. In an initial run, 1.0 μg mL^{-1} of EDC/SNHS activated FITC-anti-hIgA was placed in the amine functionalized capillary and incubated at room temperature. The unbound

FITC-anti-hIgA was removed by washing with Tween 20-PBS. The fluorescence intensity from the internal surface of the capillary (only the area of detected point) was measured. As shown in Fig. S3, the fluorescence intensity was increased with the increasing incubation time, and reached a plateau after 7 min. The results indicated that the density of the antibody on the capillary internal surface would be saturated at 7 min. In view of the assay efficiency, 10 min was selected as the optimal reaction time for the immobilization of anti-human IgA in subsequent experiments.

The droplets ejected from the inkjet microchip were introduced into the capillary via the negative pressure created by the syringe pump (Fig. 2A). Therefore, the times for each step in the immunoassay such as binding, washing and dissociation were controlled by the line velocity. As shown in Fig. 2B, a lower line velocity resulted in better sensitivity but sample throughput was reduced. High sensitivity was obtained when the line velocity was below 2 cm min^{-1}. Considering the assay performance and the complete assay time, 1 cm min^{-1} was selected as the optimal velocity at which the time for each step was only about 5 min.

Analytical performance

Under optimal conditions, a human IgA measurement was carried out and the performance of the immunoassay system was evaluated. Fig. 3 shows the relationship between fluorescence intensity and the concentration of standard human IgA (from 0.001 ng mL^{-1} to 100 μg mL^{-1}). A detection limit of 0.03 ng mL^{-1} ($Mean_{blank}$ + 3 SD_{blank}) was achieved by the method, which was comparable to that for the conventional method using a micro-titer plate. RSD values for the measurements for the concentration range tested were below 6.5%. Sigmoidal calibration curve with a logarithmic scale was obtained in the range 0.1 ~ 100 ng mL^{-1} for human IgA with an R^2 of 0.9959.

Analysis of an actual sample

To confirm the practical application of the proposed quantitative introduction of solutions/sample for a nano immunoassay system, human IgA in an actual saliva sample was assayed utilizing the method. The approach to saliva collection and pretreatment is described in ESI Part D. Meanwhile, the assay results were compared with a conventional ELISA on 96-well plates in Table S3. The results showed that the concentrations of human IgA in the five saliva samples were between 201.8 to 239.2 μg mL^{-1} with RSDs of less than 5%, which were consistent with the results from a traditional ELISA on 96-well plates. Additionally, the performance of the present method was completed within 1 h, which was significantly faster than 96-well microplate format (normally need about 5 h).

Conclusions

In summary, we report on the successful design and fabrication of a new immunoassay system in which nanoliter level volumes are introduced at the nanoliter level. The method is faster and the sensitivity is comparable to classical methods that area currently in use. The highly repeatable and accurate introductions of solutions/samples at the nanoliter level were achieved by using an inkjet microchip. The immunoassays were carried out in a capillary microreactor, in which solution evaporation is negligible and provides for an adjustable reaction field. Utilizing the present system, a low detection limit and wide linear range was obtained for human IgA, and the results of an assay of a saliva sample indicated its potential application for clinical diagnosis. The present method is expected to be a potential “nanoliter pipette” that can permit the accurate introduction of nanoliter volumes in immunoassays.

Acknowledgements

This work was financially supported by the High Technology Research Project by Tokyo Metropolitan Government.

Supplementary information available

Additional information as noted in text.

References

1 H. Yang and M. A. Gijs, *Anal. Chem.*, 2013, **85**, 2064-2071.

2 L. Gervais, M. Hitzbleck and E. Delamarche, *Biosens. Bioelectron.*, 2011, **27**, 64-70.

3 M. Herrmann, T. Veres and M. Tabrizian, *Anal. Chem.*, 2008, **80**, 5160-5167.

4 F. Lacharme, C. Vandevyver and M. A. Gijs, *Anal. Chem.*, 2008, **80**, 2905-2910.

5 J. Zhang, S. Liu, P. Yang and G. Sui, *Lab Chip*, 2011, **11**, 3516-3522.

6 R. Sista, Z. Hua, P. Thwar, A. Sudarsan, V. Srinivasan, A. Eckhardt, M. Pollack and V. Pamula, *Lab Chip*, 2008, **8**, 2091-2104.

7 R. S. Sista, A. E. Eckhardt, V. Srinivasan, M. G. Pollack, S. Palanki and V. K. Pamula, *Lab Chip*, 2008, **8**, 2188-2196.

8 S. Lai, S. Wang, J. Luo, L. J. Lee, S. T. Yang and M. J. Madou, *Anal. Chem.*, 2004, **76**, 1832-1837.

9 S. Morais, L. A. Tortajada-Genaro, T. Arnandis-Chover, R. Puchades and A. Maquieira, *Anal. Chem.*, 2009, **81**, 5646-5654.

10 W. Liu, D. Chen, W. Du, K. P. Nichols and R. F. Ismagilov, *Anal. Chem.*, 2010, **82**, 3276-3282.

11 J. Atencia and D. J. Beebe, *Nature*, 2005, **437**, 648-655.

12 Z. Fu, G. Shao, J. Wang, D. Lu, W. Wang and Y. Lin, *Anal. Chem.*, 2011, **83**, 2685-2690.

13 S. Ota, H. Kitagawa and S. Takeuchi, *Anal. Chem.*, 2012, **84**, 6346-6350.

14 N. S. Lynn, C. S. Henry and D. S. Dandy, *Lab Chip*, 2009, **9**, 1780-1788.

15 D. E. Cohen, T. Schneider, M. Wang and D. T. Chiu, *Anal. Chem.*, 2010, **82**, 5707-5717.

16 F. Guo, M. I. Lapsley, A. A. Nawaz, Y. Zhao, S.-C. S. Lin, Y. Chen, S. Yang, X.-Z. Zhao and T. J. Huang, *Anal. Chem.*, 2012, **84**, 10745-10749.

17 K. S. Deal and C. J. Easley, *Anal. Chem.*, 2012, **84**, 1510-1516.

18 L. Frenz, A. E. Harrak, M. Pauly, S. Bégin-Colin, A. D. Griffiths and J.-C. Baret, *Angew. Chem. Int. Ed.*, 2008, **47**, 6817-6820.

19 G. Arrabito and B. Pignataro, *Anal. Chem.*, 2010, **82**, 3104-3107.

20 T. Yasui, Y. Inoue, T. Naito, Y. Okamoto, N. Kaji, M. Tokeshi and Y. Baba, *Anal. Chem.*, 2012, **84**, 9282-9286.

21 H. L. Zeng, N. Seino, T. Nakagama, Y. Kikuchi, H. Nagano and K. Uchiyama, *J. Chromatogr. A*, 2009, **1216**, 3337-3342.

22 H. Zeng, Y. Weng, S. Ikeda, Y. Nakagawa, H. Nakajima and K. Uchiyama, *Anal. Chem.*, 2012, **84**, 10537-10542.

23 F. Chen, Y. Zhang, Y. Nakagawa, H. Zeng, C. Luo, H. Nakajima, K. Uchiyama and J.-M. Lin, *Talanta*, 2012, **107**, 111-117.

24 F. Chen, Z. Lin, Y. Zheng, H. Zeng, H. Nakajima, K. Uchiyama and J.-M. Lin, *Anal. Chim. Acta*, 2012, **739**, 77-82.

25 C. Luo, Y. Ma, H. Li, F. Chen, K. Uchiyama and J.-M. Lin, *J Mass Spectrom.*, 2013, **48**,

321-328.

26 Y. Kudo, M. Nishiwaki, Y. Inoue, N. Seino, T. Nakagama and K. Uchiyama, *Chem. Lett.*, 2008, **37**, 536-537.

27 N. Aissaoui, L. Bergaoui, J. Landoulsi, J. F. Lambert and S. Boujday, *Langmuir*, 2012, **28**, 656-665.

28 M. Zhu, M. Z. Lerum and W. Chen, *Langmuir*, 2012, **28**, 416-423.

29 C. K. Dixit, S. K. Vashist, B. D. MacCraith and R. O'Kennedy, *Nat. Protoc.*, 2011, **6**, 439-445.

30 T. G. Henares, E. Tsutsumi, H. Yoshimura, K. Kawamura, T. Yao and H. Hisamoto, *Sensor. Actuat. B*, 2010, **149**, 319-324.

31 R. M. Verkouteren and J. R. Verkouteren, *Anal. Chem.*, 2009, **81**, 8577-8584.

Figure captions

Fig. 1 Schematic illustration of the setup for immunoassay.

Fig. 2 (A) Images of the droplet injection process from the inkjet to the capillary. (a) Solutions being loaded into the inkjet microchip for injection, (b) droplet ejected from the inkjet microchip to the capillary tip, (c) and (d) the injection solution is allowed to flow into the capillary by the pull force created by the syringe pump. (B) Influence of the line velocity in the immunoassay procedure. Human IgA concentration was 100 ng mL^{-1}. Line velocities were set 0.5, 1, 2, 5, 10, 20, and 60 cm min^{-1}.

Fig. 3 Calibration curve for human IgA with the detected concentrations from 0.001 ng mL^{-1} to 100 μg mL^{-1}. The inset shows the linear relationship between the fluorescence intensity and the logarithm of the IgA concentration (0.1-100 ng mL^{-1}).

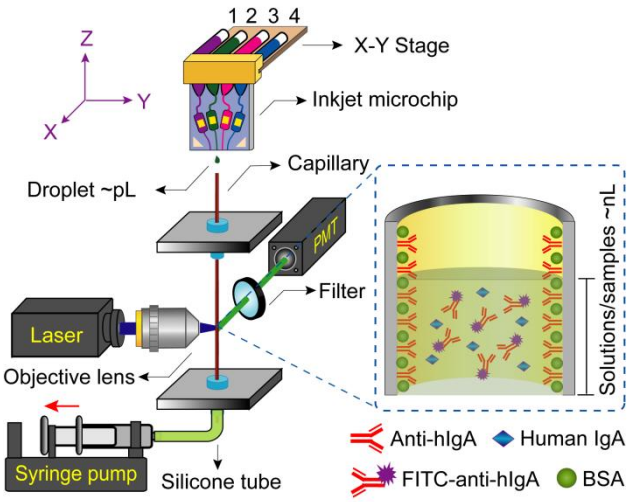


Fig. 1

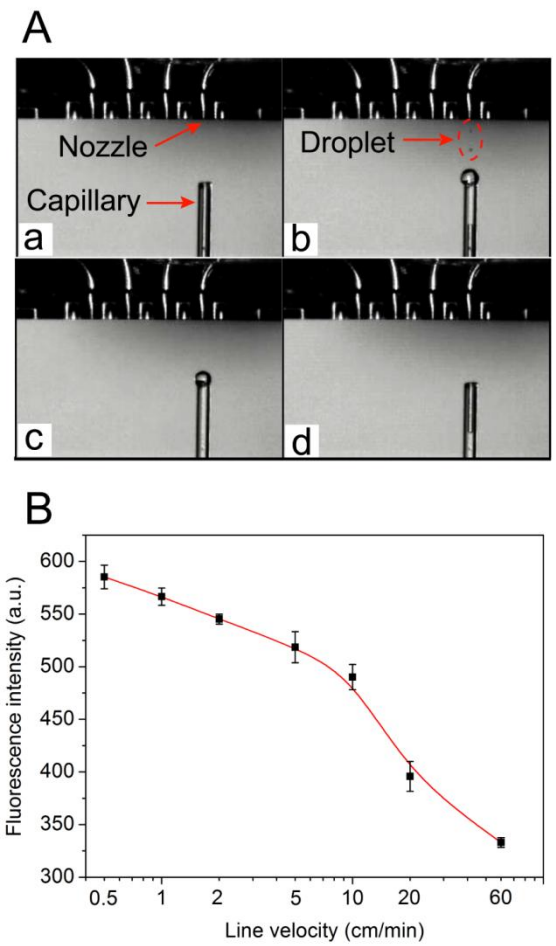


Fig.2

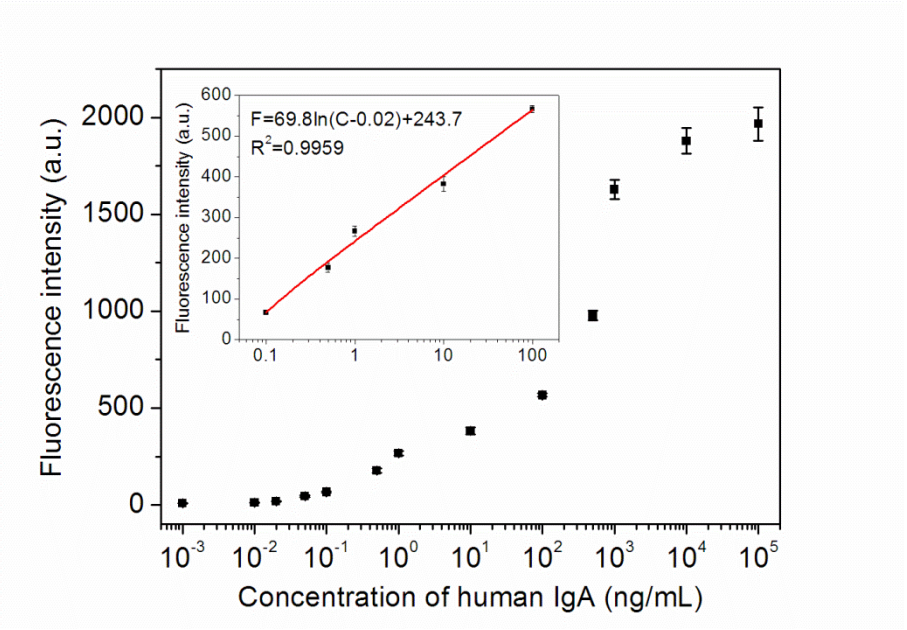
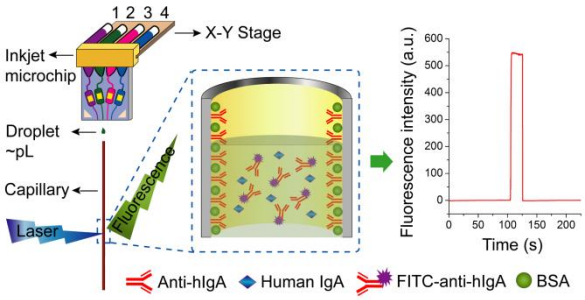


Fig. 3

Table 1 Ejection conditions and droplet volumes for each solution.

Channel	Solution	Ejection condition		Density (g mL^{-1})	Each droplet volume (pL)	Droplet number	Volume (nL)
		Driving voltage (V)	Duration time (μs)				
1	FITC-anti-hIgA (5 μg mL^{-1})	40	40	0.9985	219	300	65.70 ± 2.06
2	Tween 20-PBS (0.05% v/v)	40	40	1.0067	823	100	82.33 ± 2.26
3	Human IgA (0.1 μg mL^{-1})	40	40	0.9936	174	300	52.15 ± 1.53
4	Glycine-HCl (0.1 mol L^{-1})	40	25	1.0016	188	200	37.51 ± 0.96

Table of contents



A quantitatively controlled immunoassay based on inkjet technology to perform nanoliter immunoassay in capillary microreactor was presented.