Detection of immunoglobulins in a laser induced fluorescence system utilizing polydimethysiloxane microchips with advanced surface and optical properties

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We developed an automated laser induced fluorescence system utilizing microfluidic chips for detection and quantification of immunoglobulins. Microchips were fabricated from polydimethysiloxane (PDMS) using the so-called "prepolymerization technique." The microchip structure helped minimize the effects of PDMS autofluorescence and light scattering. Furthermore, a thin and uniform PDMS layer forming the top of the microchip enabled proper focusing and collection of the excitation beam and the emitted fluorescence, respectively. The developed system was tested for the detection of mouse immunoglobulins. The capturing antibodies were immobilized on internal microchannel walls in the form of a polyelectrolyte. We clearly show that this immobilization technique, if correctly realized, gives results with high reproducibility. After sample incubation and washing, secondary antibodies labeled by fluorescein isothiocyanate were introduced into microchannels to build a detectable complex. We show that mouse antibodies can be quantified in a wide concentration range, 0.01–100 μg ml^{-1}. The lower detection limit was below 0.001 μg ml^{-1} (6.7 pM). The developed laser induced fluorescence (LIF) apparatus is relatively cheap and easy to construct. The total cost of the developed LIF detector is lower than a typical price of plate readers. If compared to classical ELISA (enzyme linked immunosorbent assay) plate systems, the detection of immunoglobulins or other proteins in the developed PDMS microfluidic device brings other important benefits such as reduced time demands (10 min incubation) and low reagent consumption (less than 1 μl). The cost of the developed PDMS chips is comparable with the price of commercial ELISA plates. The main troubleshooting related to the apparatus development is also discussed in order to help potential constructors. © *2011 American Institute of Physics.*
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I. INTRODUCTION

Microfluidic devices become popular especially in medical diagnostics and other bioapplications. Microfluidic platforms enable an ultrasensitive fast low-cost automated detection of biological markers with a minimal consumption of samples and reagents (see, e.g., Refs. 1–7).

There are many ways how to detect specific biomolecules in microchips. The fluorescence detection is still the most popular optical method exploited in bioassays due to superior selectivity and sensitivity.[1] A variety of fluorescence excitation sources is available: (i) laser sources that produce coherent and low divergence beams, which are crucial in low volume detection [laser

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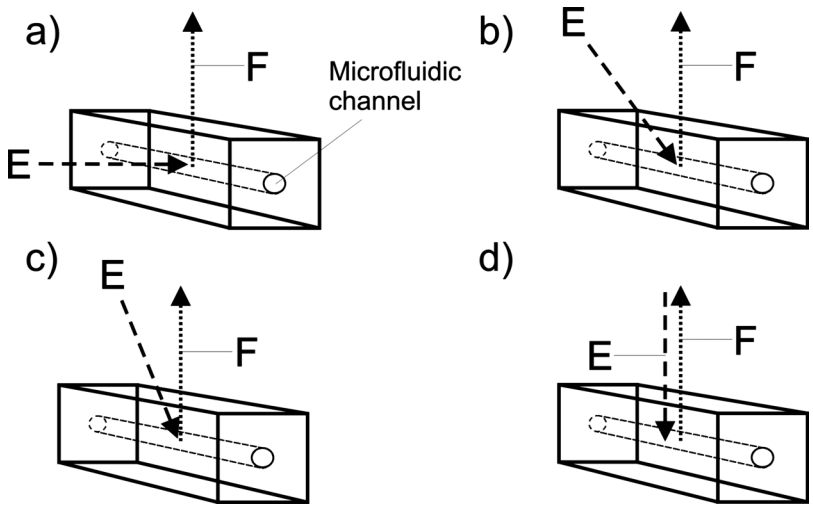

FIG. 1. Schematic picture of the typical arrangements of the LIF/microchip systems: E—excitation beam; F—fluorescence sensing. Panels (a)–(c) refer to the arrangement under different spatial angles of 90°, 45°, or 37° (Brewster's angle), respectively. Panel (d) refers to the reflection regime.

induced fluorescence (LIF) systems],[8,9] (ii) lamp-based excitation systems that are usually less expensive and less efficient than lasers,[10,11] and (iii) light-emitting diodes that are cheap and small; however, their beam spectra are wider than the laser spectra.[12]

Recent progress in the laser technology has produced stable laser sources that cover a wide range of wavelengths from ultraviolet to infrared region.[1] Modern lasers can be focused into very small detection volumes. This fact gives them a great advantage in the microscale detection. Excitation laser sources combined with photomultiplier tubes (PMTs), photon counting systems, or CCD devices attain the lowest detection limits.

There are two main optical arrangements of LIF systems. The first one is based on focusing the laser beam into microfluidic channels under different spatial angles, typically 90°, 45°, or 37° (Brewster's angle) [see Figs. 1(a)–1(c)]. The emission light is then collected by an objective or lens perpendicular to the chip plane. These LIF optical arrangements enable highly sensitive detection; however, they can suffer from a high background noise generated by beam reflections and refractions in microchip structures. Yan *et al.*[11] developed a simple LIF detection system based on the above described optical arrangement. Solutions of sodium fluorescein and fluorescein isothiocyanate (FITC) labeled amino acids were used as model samples to demonstrate the LIF system performance. The detection limit of 1.1 pM fluorescein was obtained. Xu *et al.*[13] developed another LIF detection system for electrophoretic applications on a chip. As a key point of the system, a microgap with a dimension of 70 μm $\times$ 5 mm was inserted between the laser source and the microchip. The microgap substantially increased the separation efficiency of the proposed microsystem. A detection limit of 0.12 pM FITC was obtained. A LIF system was also used by Fister *et al.*,[14] who studied electrophoretic separation of dilute dye solutions. The obtained detection limits were 6.5 pM dichlorofluorescein and 21 pM fluorescein.

The second type of the LIF systems works in a so-called reflection regime when an excitation beam is imposed and the emission light is collected through the same pathway [Fig. 1(d)]. The same objective or lens is used for focusing the laser beam and collimation of the emission fluorescence light. A dichroic mirror or an optical filter is then used for wavelength separation. The reflection LIF systems are significantly simpler than other systems. Ros *et al.*[15] used a LIF system in the reflection regime for the detection of dyes and fluorescently labeled biomolecules in polydimethylsiloxane (PDMS) microdevices. Fluorescein samples gave linear concentration response in the range from 4 to 100 pM and the lower detection limit was equal to 0.1 pM. Similar results were obtained by Hellmich *et al.*[16] Shen *et al.*[17] combined the LIF detection with a contactless-

conductivity detector in polymethylmethacrylate chips. The detection limit of rhodamine B was less than 5 nM and less than 100 nM for the LIF system and the conductivity detector, respectively. Kong *et al.*[18] prepared an integrated microfluidic system for a clenbuterol immunoassay. The device consisted of an integrated microchip and a homemade LIF system. The homemade LIF scanner could be potentially exploited in high throughput assays.

In our work, we developed a relatively cheap and automated LIF system for the detection of antibodies in specially treated PDMS microfluidic chips. The total cost of the system including laser, reflection probe, photomultiplier, computer, multimeter, and other devices was about 10 000 EUR. The cost of our system is comparable to microscopy fluorescence detection.[19] However, our LIF device is fully automated and does not need highly specialized or skilled personnel to operate. The procedure of result evaluation is also automated. If we compare our LIF system to similar reported detectors,[11,13–15,18] we expect similar investment cost. On the other hand, we believe that the developed LIF detector in combination with the used PDMS microfluidic chip favors our system among others due to at least two reasons: (i) the developed PDMS microfluidic chip avoids the traditional use of expensive silicon stamps and oxygen plasma treatment and (ii) the exploited polyelectrolyte layer (PEL) technique of receptor immobilization significantly improves the intensity and homogeneity of the obtained fluorescence signals and can be, in principle, used in many heterogeneous sandwich immunoassays because immunoglobulins and other proteins can be successfully immobilized in this way.[20] The system was tested for the immunoassay of mouse antibodies (mIgG) in PDMS microchips. PDMS substrate was chosen due to good optical transparency, biocompatibility, nontoxicity, and undemanding microfabrication (see, e.g., Refs. 21–27). In principle, our LIF apparatus will work with microchips fabricated from other substrates with proper optical and chemical properties (glass, polystyrene, polymethylmethacrylate, etc.).[28]

In Sec. II, we shortly describe a "prepolymerization" method for the PDMS chip fabrication, the method used for PDMS substrate modification, the mIgG immunoassay procedure including the used immobilization technique, and the construction of the LIF detector. Immunoassay results, together with performance parameters of the LIF apparatus, are presented in Sec. III. The results are compared with those provided by a simple measurement on a lamp-based system. The effects of immobilization parameters are studied. We also discuss the main difficulties we met to help potential constructors in their own LIF system development.

II. MATERIALS AND METHODS

A. Reagents

PDMS (Sylgard 184, Dow Corning), SU8-2025 (MicroChem), citrate buffer (Lach-Ner, 0.05*M*, *p*H=4), phosphate buffer (Sigma P-5368, 10 mM phosphate, 138 mM NaCl, 2.7 mM KCl, *p*H=7,4), Tris buffer (Sigma T-6664, 50 mM Tris, 138 mM NaCl, 2.7 mM KCl, *p*H=8), washing Tris-Tween buffer (Sigma T-9039, Tris buffer+0.05% Tween 20), sodium dextran sulfate (Sigma D4911-1G), glutardialdehyde (Sigma G400-4), capture antibodies hIgG (human IgG, Sigma I2511), mIgG (mouse antihuman antibody, Jackson Immuno LN85161), and secondary antibodies FITC-gIgG (FITC labeled goat antimouse IgG, Jackson Immuno LN 86837). The molar mass of IgG is approximately 1.5×10^5 g mol^{-1}.

B. Fabrication of PDMS chips

PDMS (Sylgard 184, Dow Corning) microchip (see Fig. 2) was prepared by means of a novel prepolymerization method described in Ref. 29. This method provides irreversible sealing of two PDMS slabs without any need of oxygen plasma or UV/ozone treatment.[25,30] Thus, the surface properties of the microchip should not be significantly altered.[31] Reversible microchip sealing mediated by van der Waals interactions was not exploited because the microchip has to withstand high pressure differences.[24]

The method[29] used was partially modified and can be shortly described as follows. A phosphore bronze plate 2.5 in. in diameter and 200 μm thick serves as the substrate for the master (mold). The substrate was etched in a diluted nitric acid (1 volume part of 68% acid in 2 volume

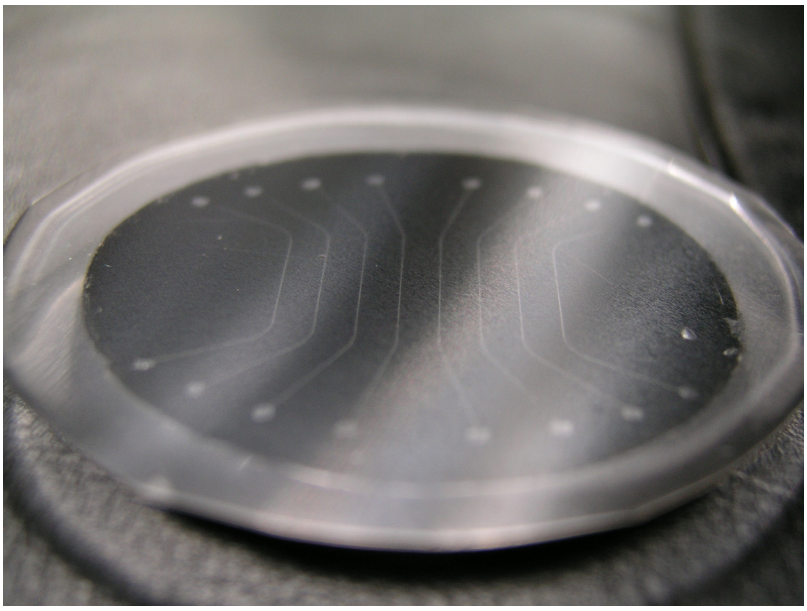

FIG. 2. The microfluidic device consists of eight parallel channels 75 μm deep and 100 μm wide.

parts of water), washed with de-ionized water and acetone, and finally dried in an oven for 30 min at 100 °C. This treatment increases the substrate roughness, which improves SU8 adhesion to the metal substrate and also enables the irreversible sealing of PDMS slabs. 75 μm high layer of SU8-2025 (MicroChem) was spin coated on the substrate. This layer was prebaked for 20 min at 100 °C on a hot plate, exposed through a mask with a desired microchannel pattern, postbaked for 4 min at 100 °C, and developed. Electron microscope images of the prepared structures are found in Ref. 29. PDMS was then prepared by thoroughly mixing prepolymer and curing agent in a weight ratio of 10:1 and adding 10 mg of a standard solid-type black printer ink (from laser printer toner) to 10 ml of this mixture. A similar approach was used by Roach *et al.*,[32] who suspended charcoal in PDMS. After degassing, PDMS was poured onto the phosphor bronze/SU8 master and cured in an oven for 60 min at 70 °C. PDMS cast was peeled off the master and cleaned with isopropyl alcohol and nitrogen. Another PDMS slab was prepared on a glass substrate and prepolymerized (approximately 10 min on a hot plate at 60 °C). Finally, these two PDMS slabs were brought in contact and placed in an oven at 95 °C for 30 min to complete the irreversible sealing. The fabricated PDMS michrochips contained eight microchannels with a cross section of 75 μm $\times$ 100 μm.

The black PDMS cast improves detection conditions. Transparent PDMS chips are inappropriate for immunoassay measurements because the transparent substrate supports light scattering and/or transmission among the microchannels. It definitely spoils fluorescence measurements.[32] Therefore, we fabricated black PDMS casts that suppress these negative phenomena. The PDMS autofluorescence is then also minimized. Uniform thickness of the PDMS layers was acquired with the use of the same amount of the PDMS mixture poured in the same molds under the same conditions.

C. Protein immobilization

The receptor human antibodies hIgG were immobilized in the form of a PEL and then covalently interconnected (Fig. 3). The immobilization technique was adopted from Ref. 20. Such a technique is known to be capable of forming a thick multilayer of antibodies on a solid substrate.

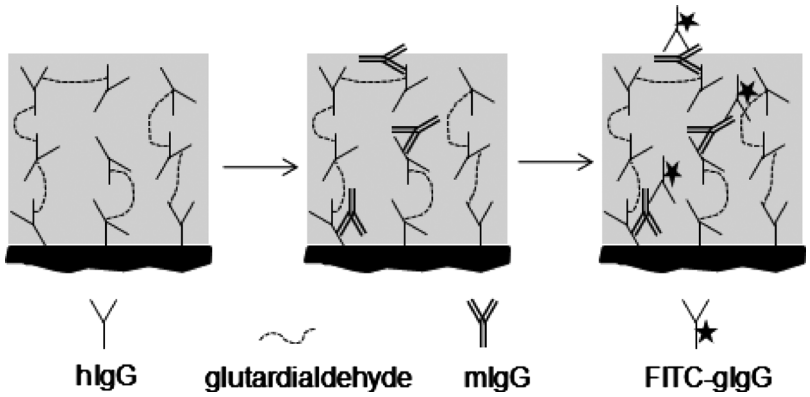

FIG. 3. Immunoassay procedure. The captured hIgGs are immobilized in the form of a multilayer.

A uniform PEL with high concentration of active antibody binding sites is obtained. More intensive and homogeneous fluorescence signal is expected when compared to a passive protein sorption.

PEL formation consists of several steps that result in a sandwich multilayer of positively charged molecules (antibodies) and negatively charged molecules (dextran) electrostatically attracted: (i) passive immobilization of hIgG (100 μg ml^{-1} in citrate buffer, 30 min), (ii) introduction of sodium dextran sulfate (1 mg ml^{-1} in citrate buffer, 10 min), (iii) introduction of hIgG (100 μg ml^{-1} in citrate buffer, 30 min), (iv) steps (ii) and (iii) were repeated several times to achieve a multilayer of capture antibodies. Citrate buffer was used as a washing solution between each two steps of the PEL formation. The procedure continues with steps: (v) antibody interconnection with glutardialdehyde (0,2% in citrate buffer, 30 min) and (vi) washing with phosphate buffer to electrostatically expel the auxiliary dextran molecules (antibody electric charge is inverted). The effect of the number of the antibody layers in the PEL, i.e., the number of repetitions of steps (ii) and (iii), on the obtained experimental results will be further discussed.

D. Immunoassay procedure

Two-step mIgG immunoassay followed (Fig. 3): (i) introduction of mIgG samples in Tris buffer and incubation (10 min) and (ii) introduction of the labeled FITC-gIgG (100 μg ml^{-1} in Tris buffer, 10 min). The fluorescence emission was then proportional to the mIgG concentration in the samples. The 10 min incubation highly exceeds diffusion time, which is necessary to transport antibodies to the immobilized receptors.

When the protein solutions were introduced, each channel was quickly filled with a syringe and then the flow was stopped for the chosen incubation period. Tris buffer was used as a washing solution between the two incubation steps. Tris-Tween buffer served as a final washing solution to suppress nonspecific bonds among proteins. All microchannels were continuously washed for about 2 min. 0.5 ml of a buffer was used in each microchannel.

E. Detection

1. Lamp-based system

We used a lamp-based system for reference detection and quantification of mIgGs on the same PDMS microchips. The obtained results are compared with the LIF data and both methods are discussed. The lamp-based system consists of a microscope (Olympus BX51WI) equipped with a CCD camera (Olympus Camedia C 5050) and a digital image processing. The whole system is described in detail in Ref. 33.

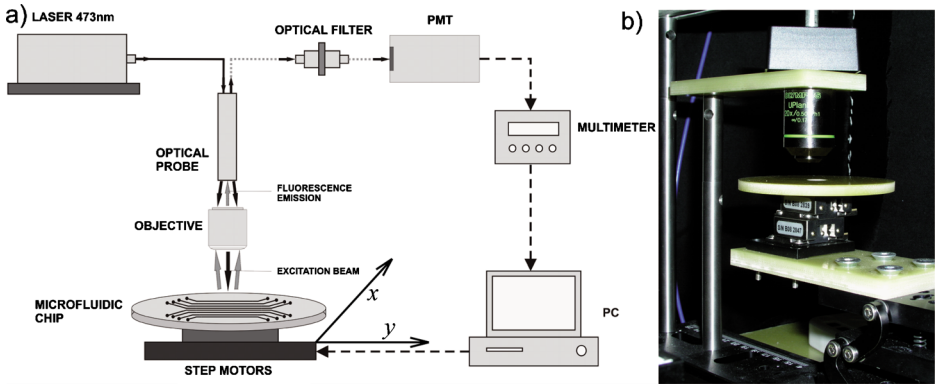

FIG. 4. Scheme of the LIF detection apparatus (a) and arrangement of the detection part (b). Symbols x and y represent the axis of the horizontal positioning.

2. LIF detection system

The LIF detection system [Fig. 4(a)] is composed of a laser excitation source (blue laser 473 nm, Photop Suwtech Inc., DPBL-9020F), optical fibers (Ocean Optics, P-200-UV), an objective (Olympus 20×), a reflective probe (Ocean Optics, R-200-7-UV-VIS), a narrow-band optical filter (Newport, DFS-001887, 520 ± 10 nm), a Hamamatsu 5784-04 (No. 4490010) PMT module together with a ±15 V plus ground power supply (STATRON 2229.81) and a homemade potentiometer for setting the gain, two step motors (Agilis, AG-LS25), multimeter (Agilent, 34970A), and a computer. The apparatus works in the reflection regime. The laser beam is guided from the laser source by an optical fiber to the reflective probe and then to the focusing objective that is located above the microfluidic device. The probe and the objective are perpendicularly oriented to the microchip surface. The fluorescence emission, together with other light, is collected by the objective, guided back through the reflective probe and through the band optical filter to PMT. Signals from PMT are transferred to the multimeter and then processed in the computer.

The probe and the objective are held at a fixed position above the microchip that is laid on a plastic plate [Fig. 4(b)]. Horizontal positioning of the microchip is controlled by two step motors connected to a computer. Fluorescence profiles of the microchip are scanned perpendicularly to the longitudinal dimension of microchannels (x-horizontal direction). The microchip is moved by the step motors in small discrete steps ($\sim 6 \times 10^{-2}$mm). The period between two steps is 2.5 s. Within this period, a small microchip area ($\sim 4 \times 10^{-3}$mm^2) is illuminated and the emitted fluorescence is detected. This is repeated until the entire microchip profile, which includes all microchannels, is scanned. The scanning procedure can be repeated at any other part of the microchip (at any different y-position). The whole detection procedure is fully automated and controlled by the computer.

III. RESULTS AND DISCUSSION

A. LIF detection of mIgG

The results of three independent immunoassay experiments on three different microchips are shown in Fig. 5. Each immunoassay is represented by a single fluorescence profile of the microchip. The three profiles are slightly shifted in space to clearly see differences in the three independent measurements. Five groups of similar peaks are plotted in Fig. 5. For example, all the left peaks correspond to the first experiment. The x-axis (position) is only useful to see distances on a particular fluorescence profile. The peak groups correspond to the antibody concentrations of 100, 10, 1, 0.1, and 0.01 μg ml^{-1} in five adjacent microchannels. Blank experiments (0 μg ml^{-1}) were carried out in two outside microchannels. The upper detection limit is above 100 μg of protein per 1 ml (67 nM). In this arrangement, the detector is sensitive to the mIgG concentration at least in the interval of 0.01–100 μg ml^{-1}. The detector sensitivity decreases with decreasing antibody

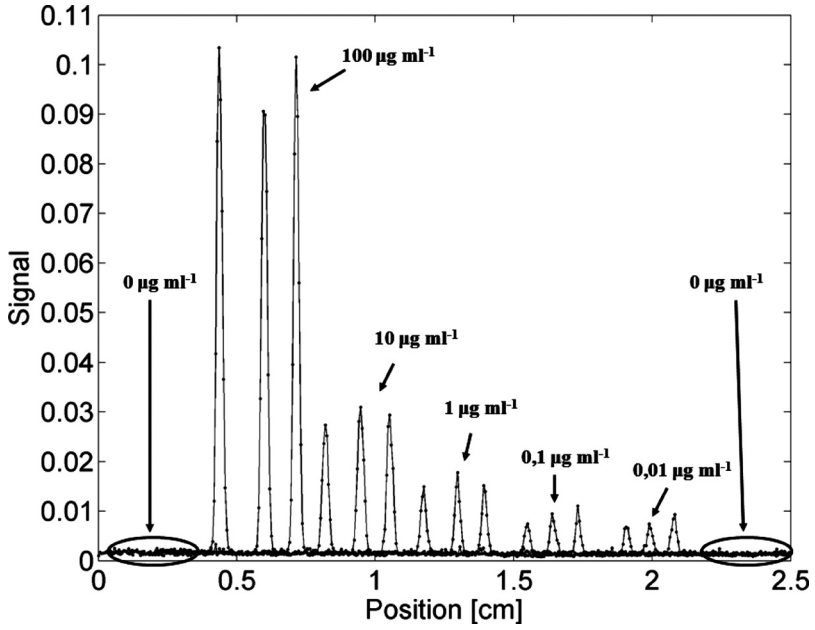

FIG. 5. Profiles of the fluorescence signal intensity given by the LIF system. Concentrations of mIgG in a sample are marked at each peak. Four layer PEL.

concentration. The lower detection limit is below 0.001 μg ml^{-1} (6.7 pM); however, the obtained signal is almost the same as that given by the protein concentration 0.01 μg ml^{-1}. Hence, the protein quantification cannot be carried out for such low concentrations due to a limited sensitivity of the detector.

We also carried out blank experiments, i.e., only Tris buffer instead of a mIgG sample was introduced in microchannels in the first incubation step. The second incubation and the washing steps were realized in a manner similar to that in other experiments. Intensities of the fluorescence emission from the "blank" microchannels were undistinguishable from the background signal (Fig. 5).

B. Lamp-based mIgG detection

In order to evaluate the performance of the LIF system, we compared the obtained results with those given by the simple lamp-based system. According to the image analysis method described elsewhere,[33] the mIgG concentrations of 100, 10, 1, and 0.1 μg ml^{-1} correspond to normalized fluorescence signal intensities of 150, 100, 50, and 48, respectively. It is obvious that the mIgG concentrations of 1 μg ml^{-1} and lower are hardly distinguishable with the lamp-based system. The lower detection limit for the lamp-based system is shifted by several orders of the concentration magnitude. With the lamp-based system, it is not easy to scan the fluorescence emission in a larger number of microchannels on a single microchip due to limited area below the microscope objectives. Moreover, the data processing relying on the digital image analysis is not automated.

Figure 6 gives us also information about the quality of the immobilized layer of the capture antibodies. The fluorescence signal from a particular microchannel is relatively homogeneous except the vertical sidewalls. This effect takes place due to intensive fluorescence emission from the vertical walls because the detectable protein complex is formed on the entire internal surface.

C. mIgG quantification

In experiments on three PDMS microchips, we obtained 8–11 independent peaks for each mIgG concentration (c_A). The arithmetic means and 95% confidence intervals of the fluorescence signal are plotted in Fig. 7. The signal is represented either with the peak maximum (Signal_M) or

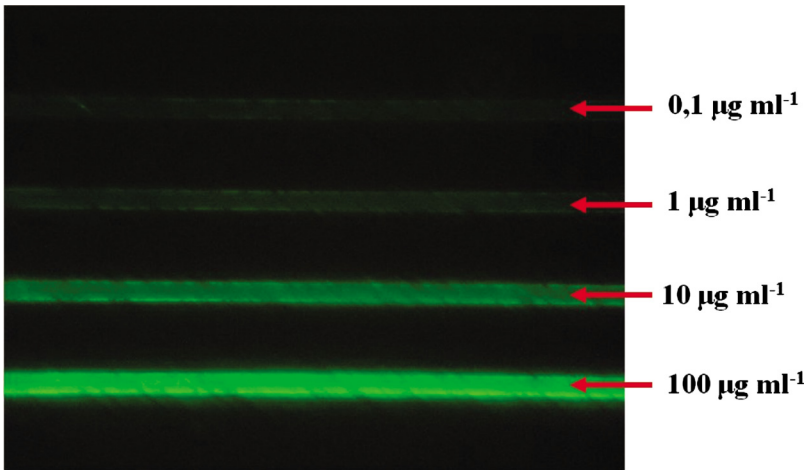

FIG. 6. Lamp-based fluorescence detection of mIgG. Concentrations of mIgG in a sample are marked at each channel. Four layer PEL.

with the peak area ($Signal_S$). We expected that the peak area to be less sensitive to random signal fluctuations. However, we found that there is no significant difference in the shapes of these dependences and the data variations. The dependences can be well fitted by means of quadratic functions that can serve as calibration curves (Fig. 7). It can be seen that the dynamical range of the LIF detection is four orders of the concentration magnitude. The sensitivity markedly increases with increasing concentration. The reproducibility of the immunoassay and detection procedure is

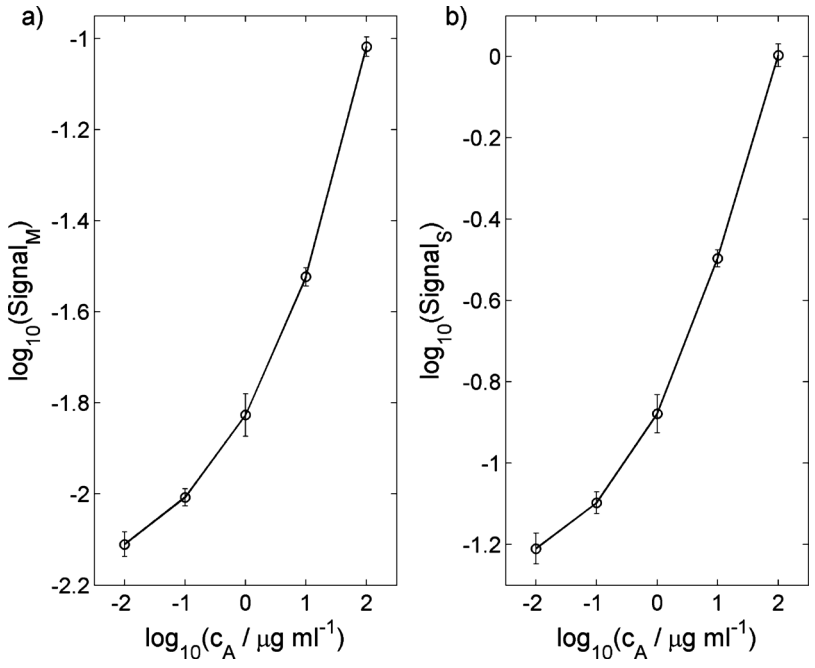

FIG. 7. Dependences of the obtained fluorescence signal on the mIgG concentration, the LIF detection, four layer PEL. The fluorescence signal is represented (a) with the peak maximum and (b) with the peak area. The experimental data in the logarithmic representation can be fitted with quadratic functions: (a) $Y=0.0667X^2+0.2666X-1.8303$ and (b) $Y=0.0668X^2+0.3028X-0.8696$.

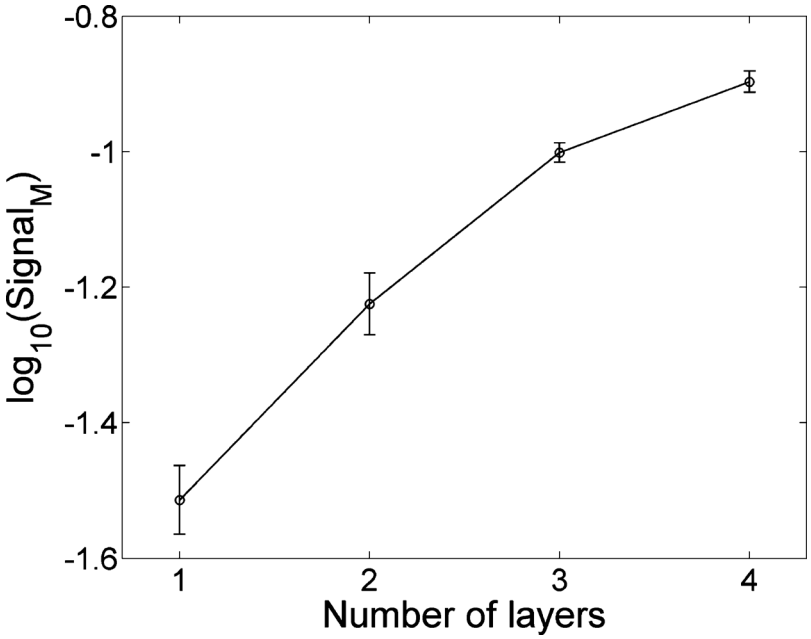

FIG. 8. Fluorescence signal obtained for different PELs, $c_A = 100\ \mu g\ ml^{-1}$.

very good, which is demonstrated by low variance of the measured data. High reproducibility of the experiments indicates that the LIF system is capable of protein quantification at a large concentration interval.

D. Effect of PEL thickness

In all previously described experiments, the PEL was formed by four layers of antibodies. Because this way of protein immobilization is quite time demanding, we decided to test the effects of the number of the antibody layers on the signal intensity and reproducibility. Each point in Fig. 8 represents the arithmetic mean of four independent immunoassay experiments in four different PDMS microchips. 95% confidence intervals are also plotted.

The obtained intensity of the fluorescence signal monotonously increases with the number of the antibody layers. Growing amount of the capture antibodies results in an increasing number of active binding sites available for ligands. Another observation is that the experimental data obtained on the microchips with one or two antibody layers suffer from a substantially higher variance. One layer immobilization, in fact, corresponds to the immobilization based on passive sorption. Protein layers passively adsorbed on polymer substrates are often inhomogeneous,[34] which results in a decrease of the immunoassay reproducibility. The microchips with three and four antibody layers give significantly lower variance of the data.

We conclude that our experimental arrangement requires at least three immobilized layers in order to obtain reproducible immunoassay results with a satisfactory intensity of the fluorescence signal. Antibody immobilization in the form of the four layer PEL is quite time consuming. However, the microchips with immobilized capture proteins can be prepared independently before an immunoassay in a similar manner as functionalized ELISA (enzyme linked immunosorbent assay) plates. The prepared chips stored under proper conditions (4 °C and filled with Tris buffer) can be used at least after a week of storage. We note that the intensity of the fluorescence signal plotted in Fig. 8 does not correspond to that in Fig. 7 because of slightly different setups of the laser source in these two experimental series.

E. Discussion on LIF apparatus troubleshooting

Our experiences with the LIF apparatus development will be discussed in this paragraph to help a future constructor. The general rule is that the apparatus should be as simple as possible because more parts means larger losses of excitation and/or emission signal. For the same reason, the optical waveguides must not be significantly deformed.

The light emitted from a laser has to be accurately focused by a lens into an optical waveguide to maximize the excitation light intensity. The emission light (fluorescence light) has to be led through an optical filter to remove all wavelengths that do not correspond to the fluorescence emission. In this study, we used an interference filter with ± 10 nm band. A ± 2 nm narrower filter, which we also tested, gave significantly lower signal intensity. On the other hand, filters with larger bands can fail because the excitation and the emission peaks of FITC and some other labels are quite close. The used optical filter has to be fixed perpendicular (with high precision) to optical waveguides. We recommend using a commercial filter holder with connectors to optical waveguides and possibly with focusing lenses.

Another problem is focusing the excitation light in the microchannels. When we used the reflective probe without the objective, the obtained signal/noise ratio was often insufficient. The objective enables focusing the excitation light into a small spot and the same objective collects the emission light back to the reflective probe. The optimal height of the objective above the microchip surface has to be found experimentally. In order to obtain reproducible results, the microchips' thickness has to be as uniform as possible to keep the constant distance between the microchip and the objective.

In our arrangement, complexes of biological molecules are not detected on the microchip surface. The fluorescence signal is generated on the internal walls of the microchannels. We found that the thickness of the microchannels wall, i.e., the distance between the internal microchannel surface and the external microchip surface, should be as thin as possible. Small thickness of the wall facilitates focusing the excitation light and minimizes absorption and scattering of the emitted fluorescence.

The entire apparatus was covered by black velvet. It helped decrease light noise produced by auxiliary devices and a computer. Microchips have to be always clean because dust or fingerprints on microchips surface can destroy the entire experiment.

Scanning the fluorescence profile of the microchip was controlled by linear stages. One has to properly set space steps and speed of the movement. Very slow movement can result in unacceptable duration of experiments and in bleaching of the fluorophore. On the other hand, too fast movement can result in an insufficient data acquisition from a particular microchannel.

The crucial step of the LIF apparatus development was the selection of a proper immobilization procedure. As discussed above, simple passive sorption of the receptors on microchannel walls is for the given purpose unsuitable. We found that an immobilization procedure based on the PEL formation is one of plausible techniques.

IV. CONCLUSIONS

We developed a LIF detection system for immunoassays. The total cost of the developed LIF detector is lower than a typical price of plate readers. The apparatus is simple and, in principle, can be constructed in a short time. Typical problems, which we met already, and their solutions are discussed in this paper.

Immunoassay experiments were realized in the PDMS microfluidic chips. The microchips were constructed by means of the prepolymerization technique, which should guarantee relatively stable surface properties of the PDMS chips. The surface properties may be important when proteins are immobilized in microchannels or electrokinetic transport is used for sample dosing (it was not subject of this study). The prepolymerization technique was modified to obtain microchips with reduced autofluorescence and light scattering effects. If compared to classical ELISA plate systems, the detection of immunoglobulins or other proteins in the developed PDMS microfluidic

devices brings other important benefits such as reduced time demands (10 min incubation) and low reagent consumption (less than 1 μl). The cost of the developed PDMS chips is comparable to the price of commercial ELISA plates.

The developed system was tested for immunoassay detection of mouse antibodies. We chose a heterogeneous two-step arrangement of the immunoassay. Receptors (human antibodies) were immobilized on microchannel walls with the use of a technique relying on the polyelectrolyte layer formation.[20] We determined that at least three layers of the captured antibodies have to be immobilized on the walls. We showed that mouse antibodies can be quantified in a wide concentration range, 0.01–100 μg ml^{-1}. The lower detection limit was below 0.001 μg ml^{-1} (6.7 pM), which is comparable to other published results (see, e.g., Refs. 13–15 and 35). High reproducibility of the experimental results was attained.

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