Confocal laser-induced fluorescence detector for narrow capillary system with yoctomole limit of detection

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Abstract

Laser-induced fluorescence (LIF) detectors for low-micrometer and sub-micrometer capillary on-column detection are not commercially available. In this paper, we describe in details how to construct a confocal LIF detector to address this issue. We characterize the detector by determining its limit of detection (LOD), linear dynamic range (LDR) and background signal drift; a very low LOD (~ 70 fluorescein molecules or 12 yoctomole fluorescein), a wide LDR (greater than 3 orders of magnitude) and a small background signal drift (~ 1.2 -fold of the root mean square noise) are obtained. For detecting analytes inside a low-micrometer and sub-micrometer capillary, proper alignment is essential. We present a simple protocol to align the capillary with the optical system and use the position-lock capability of a translation stage to fix the capillary in position during the experiment. To demonstrate the feasibility of using this detector for narrow capillary systems, we build a 2- μm -i.d. capillary flow injection analysis (FIA) system using the newly developed LIF prototype as a detector and obtain an FIA LOD of 14 zeptomole fluorescein. We also separate a DNA ladder sample by bare narrow capillary – hydrodynamic chromatography and use the LIF prototype to monitor the resolved DNA fragments. We obtain not only well-resolved peaks but also the quantitative information of all DNA fragments.

Abbreviations:

LIF: laser-induced fluorescence
DNA: deoxyribonucleic acid
BaNC: bare narrow capillary
HDC: hydrodynamic chromatography
LOD: limit of detection
LDR: linear dynamic range
FIA: flow injection analysis
i.d.: inner diameter
o.d.: outer diameter
CE: capillary electrophoresis
bp: base pair
EDTA: ethylenediaminetetraacetic acid
tris: tris(hydroxymethyl)aminomethane
DDI: double deionized
TE: tris-EDTA
USB: universal serial bus
PMT: photomultiplier tube
Psi: pond per square inch

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1. Introduction

Laser-induced fluorescence (LIF) is one of the most sensitive techniques for molecular detection. It is widely used for monitoring compounds resolved by chromatography [1,2] and capillary electrophoresis (CE) [3,4] (including microfluidic chip-based analysis [5]). However, most of these instruments are either customer-made or specialized for particular utilizations. Only a few stand-alone LIF detectors are developed [6-10] and none is manufactured for low micrometer to sub-micrometer inner diameter (i.d.) capillary systems.

Genes are pieces of DNA that carry the genetic instructions used in developing all living organisms. Separating and analyzing DNA fragments is usually the first step toward understanding and manipulating how DNA works. DNA separations are commonly performed using slab-gel electrophoresis. To enhance the resolving power, reduce the separation time, and increase the throughput, researchers are now frequently using capillary gel electrophoresis or capillary array electrophoresis. All the above techniques utilize viscous gel sieving matrices (either cross-linked or linear polymers). As these matrices in capillaries need to be loaded and replenished after each run, loading/reloading viscous matrices into micrometer-bore capillaries is tedious and time-consuming.

We have recently developed an innovative separation technique for separating and sizing DNA without using sieving matrices [11-15]. Because the separation is preceded inside a Bare Narrow Capillary (BaNC) and the separation mechanism is based primarily on hydrodynamic chromatography (HDC) [16,17], we term this technique BaNC-HDC. DNA fragments from a few base pairs (bp) to more than one hundred kilobase pairs (kbp) have been nicely resolved [15]. Similar work has also been performed in other labs [18,19]. Owing to the small diameter (a

few hundreds of nanometers to a few micrometers) of the separation capillary, no detectors are commercially available for BaNC-HDC. Since narrow capillaries are commercially produced by Polymicro Technologies - a subsidiary of Molex, the lack of suitable LIF detectors has been a major barrier for other researchers to investigate and/or use BaNC-HDC. In order to address this issue, here we describe how to construct a confocal LIF detector and how to use it for BaNC-HDC. With this detector we also build the first 2- μ m-tubing flow injection analysis (FIA) system. To our knowledge, this is the smallest tubing FIA system reported so far. Reduced tubing size will lead to decreased reagent consumptions and sample quantity requirements. Characterizations of the instrument are also discussed.

2. Materials and methods

2.1 *Reagents and Materials*

Fused-silica capillaries were products of Polymicro Technologies (Phoenix, AZ). GeneRulerTM 1-kb plus DNA ladder (SM1331) was obtained from Fermentas Life Sciences Inc. (Glen Burnie, MD), and YOYO-1 was from Molecular Probes (Eugene, OR). Concentrated hydrochloric acid, ethylenediaminetetraacetic acid (EDTA), fluorescein, sodium hydroxide, and tris(hydroxymethyl)aminomethane (Tris) were purchased from Fisher Scientific (Fisher, PA).

2.2 *Solution Preparation*

Running buffer, 10 mM TE buffer, was composed of 10 mM Tris-HCl and 1.0 mM Na₂EDTA at pH 8.0. It was prepared using DDI water from a NANO pure infinity ultrapure

water system (Barnstead, Newton, WA). Before use, the running buffer was filtered through a 0.22- μ m filter (VWR, TX) and vacuum-degassed. 1 mM fluorescein stock solution was prepared by dissolving the desired amount in 10 mM TE buffer and, when in use, diluted to the required concentration with additional 10 mM TE buffer. The stock solution of 100 ng/ μ L 1-kb plus DNA ladder was prepared by mixing 39 μ L 10 mM TE buffer, 10 μ L 500 ng/ μ L DNA, and 1 μ L YOYO-1. Working standard DNA solutions were made by diluting the stock solution with 10 mM TE buffer at the ratio as needed. Running buffer and samples were stored at 4 $^{\circ}$ C.

2.3 LIF Detector Construction

Figure 1 illustrates how the LIF detector was constructed. Figure 1A presents a schematic diagram of a BaNC-HDC system in which major components of the LIF detector are shown. A 488 nm solid-state laser module (Melles Griot, Rochester, NY) was used as the excitation light source. After passing through a 488 nm laser clean-up filter (Melles Griot), the laser beam was reflected by a dichroic mirror (Semrock, Rochester, NY) with high reflectance below 491 nm and focused to the narrow capillary by an objective lens (0.32 NA, 16 $\times$ magnification, Melles Griot). The same objective lens collected and collimated the emitted fluorescent light. After the collimated fluorescence passed through the same dichroic mirror, it was reflected by a mirror (Melles Griot), filtered by a 510 nm long-pass filter (Semrock, Rochester, NY), and focused by a lens (Melles Griot) through a 800 μ m pinhole onto a photomultiplier tube (H5784-01, Hamamatsu, Shizuoka, Japan). The output signals was acquired by a USB data acquisition card (Measurement Computing, Norton, MA) and

displayed on a computer running an in-lab LabView program. Figure 1B presents a picture of the assembled LIF detector (note: the pressure chamber and the narrow capillary were not considered as part of the LIF detector), and Figures 1C–1F presents the details of how all components are arranged and packed together.

The backbone of the detector was a vertical support that was firmly attached to a base plate (see Figure 1C). A laser holder and a PMT holder were bolted onto the vertical support. There was a leveling plate on the laser holder and it was used to perform some fine adjustments of the laser height and direction. An optical tube was also bolted onto the vertical support and aligned with the laser and PMT (see Figure 1D). In order to show how optical components were arranged inside the optical tube, we made the tube wall transparent (see Figure 1E). The objective was fixed to the top of the tube via threads. The dichroic mirror was fixed to backside of the tube. The reflector was fixed at the bottom of the tube and it could be adjusted via an Allen wrench to direct the fluorescence light properly to the pinhole in front of the PMT. Figure 1F presents a view from the other side of the vertical support to which a waste reservoir holder was attached. A laser power supply was fixed to the base plate. An X-Z translation stage was bolted onto the back of the optical tube and a capillary holder was fixed to the translation stage.

Figure 1. BaNC-HDC system and LIF detector

(A) Schematic configuration of BaNC-HDC system. The narrow capillary had a total length of 47 cm (41 cm effective), an i.d. of 2 μm and o.d. of 150 μm . RC had a length of 6.5 cm an i.d. of 20 μm and an o.d. of 150 μm . (B) Picture of assembled LIF detector. (C) Detector backbone. (D) Arrangement of parts inside detector. (E) Optical component arrangement inside optical tube. (F) View from other side of vertical support.

2.4 Alignment of Capillary with LIF detector

A detection window was opened at ~ 41 cm from the sampling end of a narrow capillary (47-cm-long $\times$ 2- μm -i.d. $\times$ 150- μm -o.d.) by removing the polyimide coating with flame. The detection window was then fixed onto the capillary holder coarsely aligned with the objective lens using the X-Z translation stage. Then, a fluorescein solution (1 μM fluorescein in 10 mM TE buffer at pH ~ 8.0) was flow through the capillary at a constant flow rate and the fluorescence signal was monitored. The position of the detection window was adjusted via the X-Z translation stage until the maximum signal output was reached. At this time, the detection window was

considered to be aligned with the LIF detector and the X and Z positions of the translation stage were locked.

2.5 DNA Sample Preparation

The stock solution of 100 ng/ μ L 1-kb plus DNA ladder was prepared by mixing 39 μ L of 10 mM TE buffer, 10 μ L 500 ng/ μ L DNA, and 1 μ L YOYO-1. Working standard DNA solutions were made by diluting the stock solution with DDI water at the ratio as needed. Eluent and DNA samples were stored at 4 °C.

2.6 Flow injection analysis of fluorescein

To prepare for flow injection analysis right after capillary alignment, the capillary was thoroughly washed with a carrier solution (10 mM TE buffer) until the fluorescence signal reached the background level. Referring to Figure 1A, as the carrier solution was driven through the 60-nL injection valve to the microfabricated Chip-T, it was split into two streams; one stream went to the narrow capillary while the other went through a restriction capillary (RC). When a sample was injected by the injection valve, only a small portion of the sample was injected into the narrow capillary. Under the indicated conditions and a chamber pressure of ~500 psi, the splitting ratio was about 70,000:1; leading to an injection volume of ~0.85 pL. Five fluorescein solutions were injected from low concentration to high concentrations and each solution was injected five times at one minute interval.

2.7 BaNC-HDC Separation of DNA ladder

Referring to Figure 1A, 10 mM TE buffer at pH 8.0 was loaded in the reservoir inside the pressure chamber as eluent. During the separation, the eluent was constantly driven through the 60-nL injection valve to the microfabricated Chip-T; one small portion of eluent went to the narrow capillary. When a sample was injected by the 60-nL injection valve, ~0.85 pL sample was injected into the narrow capillary. Fluorescence signal was continuously monitored by the LIF detector. After all the DNA fragments were eluted out, the next sample was injected for analysis. No column reconditioning was needed!

3. Results and Discussion

3.1 Linear Dynamic range and Limit of Detection

Figure 2A presents the fluorescence signal obtained when fluorescein solutions (0 nM, 5.3 nM, 10 nM, 53 nM, 0.1 μ M, 0.53 μ M, 1.0 μ M and 5.3 μ M in 10 mM TE buffer) were flushed through the narrow capillary. The inset presents a Y-axis-expanded version of the same results from 0 to 53 nM. Excluding the data point for 5.3 μ M, good linear relationship existed between fluorescence intensity and fluorescein concentration (see Figure 2B, $R^2=0.9993$). Using the criterion for signal-to-noise ratio of 3, we determined the limit of detection (LOD) to be 0.8 nM. Therefore, the linear dynamic range of the detector was greater than 3 orders of magnitude. If we assume the laser spot on the narrow capillary had a diameter of ~50 μ m, the volume of the solution that was illuminated was $\pi \times (1 \mu\text{m})^2$

$\times 50 \mu\text{m} = 1.6 \times 10^{-22}$ L. That is to say, the LOD of the LIF detector was ~ 70 fluorescein molecules or 12 yoctomoles.

Figure 2. Fluorescence intensity as function of fluorescein concentration (A) Fluorescence signals obtained as different fluorescein solutions were flushed through the narrow capillary (total length of 47 cm (41 cm effective), i.d. of $2 \mu\text{m}$ and o.d. of $150 \mu\text{m}$) under a pressure of 500 psi. (B) Calibration curve of fluorescein from 5.3 nM to $1.0 \mu\text{M}$.

Figure 3 presents the results of a drift test as 10 mM TE buffer was flushed through the capillary for an hour. As can be seen, the background signal was pretty stable; signal drift was 1.2 standard deviation of the background signal over an hour. To check whether the LIF detector worked properly, we injected a fluorescein sample (10 nM in 10 mM TE buffer) to see how it responded to it. The signal between ~100-150 s was obtained when 10 nM fluorescein was flushed through the capillary. The signal was comparable to what we got in Figure 2.

Figure 3. Drift and Noise characterization over 1 hour

The experiment was performed by flushing 10 mM TE buffer (pH=8.0) through the narrow capillary under 500 psi. The peak signal was obtained by flushing 10 nM fluorescein in 10 mM TE buffer through the narrow capillary under the same conditions. Dynamic drift of the background signal (relative fluorescence intensity) was calculated to be 4.0×10^{-4} per hour, while noise was calculated to be 3.4×10^{-4} .

3.2 Flow injection analysis of fluorescein

To demonstrate the performance of the newly developed LIF prototype, we performed a flow injection analysis of fluorescein using the system as presented in Figure 1A. This is probably the smallest tubing flow injection system ever reported. Samples were injected via the injection valve at a rate of one injection per minute. Figure 4 presents the results; there were five fluorescein solutions, each was analyzed five times. A linear regression equation was established in the concentration range of 50 nM – 1 μ M. The linear regression coefficient (R^2) was 0.996. When we used the criteria of $S/N = 3$, an LOD of ~ 17 nM fluorescein or 14 zeptomole ($17 \text{ nM} \times 0.85 \text{ pL}$) of fluorescein was obtained. This also revealed the dispersion factor of this flow injection system was ~ 21 .

Figure 4. Flow injection analysis of fluorescein. The capillary had a total length of 47 cm (41 cm effective), an i.d. of 2 μm , and an o.d. of 150 μm . The carrier solution was 10 mM TE buffer at pH $\sim$ 8.0, and the fluorescein solutions were prepared with the carrier solution. The injection volume was estimated to be 0.85 pL. (A) Diagrams. (B) Linear regression results.

3.3 Analysis of DNA Ladder

We coupled the developed LIF prototype to BaNC-HDC. Figure 5A presents the chromatograms of separating a standard sample of 1 kb plus DNA ladder at five different concentrations, and Figure 5B presents the calibration curves of all fifteen DNA fragments in the range of 0.075 – 20 kbp. The linear regression coefficients were in the range of 0.985 – 0.991. For large DNA fragments we could detect them at the zeptomole level. It should be noted that each DNA molecule was intercalated with many YOYO dye molecules.

Figure 5. Using developed LIF detector for monitoring BaNC-HDC separation BaNC-HDC conditions: the capillary had a total length of 47 cm (41 cm effective), an i.d. of 2 μ m, and an o.d. of 150 μ m, the injection volume was estimated to be 0.85 μ L, the eluent was 10 mM TE buffer at pH 8.0, and the elution pressure was 500 psi. (A) Chromatograms. The total concentrations of 1-kbp plus DNA marker were indicated in the figure. (B) The calibration curves for different DNA fragments.

Relative peak areas at different concentrations were obtained from Figure 5A. The trend-lines had linear regression coefficients of 0.985 – 0.991.

4. Conclusion

We have built a prototype LIF detector for monitoring fluorescence signal within a narrow capillary. The component arrangement has been described in detail, which should be helpful if one wants to construct similar detector on his/her own. We have also characterized the detector. Using a 2- μm -i.d. capillary as a separation column, we have determined the limit of detection of the detector to be 0.8 nM fluorescein, which is corresponding to ~ 70 fluorescein molecules in the detection volume. We have also determined the linear dynamic range of the detector to be more than 3 orders of magnitude. To demonstrate the feasibility of using the prototype for monitoring resolved analytes in a narrow capillary, we used a 2- μm -i.d. capillary to separate a 1 kb plus DNA ladder by BaNC-HDC. The detector not only can monitor all the fifteen resolved DNA fragments but also can quantitate each fragment with good precision and accuracy.

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LIF Detector for Submicron Capillary System