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Laser fluorescence detector for capillary electrophoresis

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ABSTRACT

A simple, rugged, and relatively inexpensive laser-based fluorometer for detection in capillary electrophoresis is described. This is assembled from commercially available components and requires minimal experience for operation. Yet, the detection performance is comparable to those achieved in laser laboratories with sophisticated layouts. As an example, detection of a 3 p*M* solution of fluorescein is demonstrated. The design principles of laser fluorometric detection are critically examined, and the special adaptation for the present detection arrangement is discussed.

INTRODUCTION

Laser-induced fluorescence (LIF) detection has shown tremendous promise for applications in capillary electrophoresis (CE) [1–4]. Its acceptance naturally depends on having simple, inexpensive and reliable instrumentation for the routine user. Most laboratory systems are mounted on optical tables in partially darkened rooms for flexibility and ready access to the individual components. Certain optimization steps are also easier to perform in an open system. The need remains for a compact instrument that can be interfaced to any CE setup and which can provide close to state-of-the-art detection performance.

The requirements for good performance in fluorescence detection are (i) a stable light source, since it is the fluctuations in the residual background signal that determine the limit of detection (LOD); (ii) a rugged but adjustable mounting technique for the capillary column, since mechanical movements lead to misalignment and flicker noise; (iii) low stray light levels, which include room light plus scattering and fluorescence from the capillary walls.

In this paper we describe a compact laser fluorometer for CE that achieves high performance. It can be assembled from commercially available components with a minimum of additional engineering. The design addresses the main requirements outlined above. Operation and alignment are simplified to allow use by non-experts. The major part of the cost of the system is the laser source, which can range from several hundred dollars upwards.

EXPERIMENTAL

Chemicals and reagents

Trizma base [tris(hydroxymethyl)aminomethane, or Tris] was purchased from Sigma (St. Louis, MO, USA). Boric acid, HPLC-grade acetonitrile and 0.22-μm MSI Cameo filters were from Fisher Scien-

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tific (Bedford, MA, USA). Fluorescein, water soluble, dye content *ca.* 70%, was obtained from Aldrich (Milwaukee, WI, USA). Buffer (pH 8.7) was prepared by combining 0.8 ml of stock buffer (0.5 *M* Tris, 0.5 *M* boric acid, stored at room temperature), 35 ml of water and 4 ml of acetonitrile, giving pH 8.7. This buffer (10 m*M* Tris–borate, 10% acetonitrile) was filtered (0.22 μm) and degassed (bubbling with helium for 15 min) prior to use as a diluent to prepare solutions for injection using polypropylene tubes and pipetting tips.

Instrumentation

As depicted in Fig. 1, the device represents a simple and rugged design for LIF detection. It consists of 8 main components: a quartz 1-cm focal length lens (L), capillary holder (CH), 20× microscope objective (MO), microscope objective holder (MOH), mirror (M) and mirror mount (MM), photomultiplier tube (PMT) with filter (F), Plexiglass box (PB) and 2 light shields (LS). The laser beam enters the otherwise light-tight box PB through a 3-mm hole. L is rigidly mounted to PB so that the focal point of the excitation beam is uniquely defined and used as the reference point for all the other components. The capillary with a small section of its coating removed is mounted on a two-dimensional stage CH capable of 10 μm resolution. Two short pieces of quartz capillary 350 μm O.D. × 250 μm I.D. glued to CH serve to guide the separation capillary through the optical region. This can hold the common 150 μm O.D. capillary tubing. Alternatively, 350 μm O.D. separation capillaries can be inserted directly into CH. The mounted capillary is at about 20° with respect to the incident laser beam to minimize scattering off the capillary walls. We find that this configuration is rigid enough with regard to noise from mechanical vibrations. With this design, capillary change can be accomplished in less than 5 min.

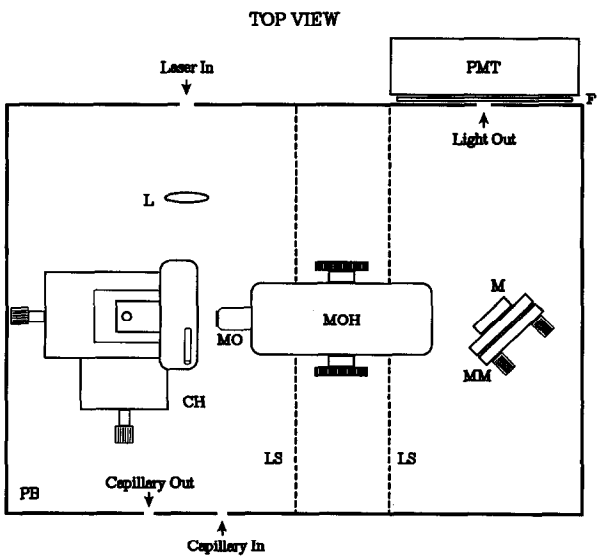

Fig. 1. Experimental arrangement for laser fluorometric detection. Abbreviations are explained in the text.

Instrumental conditions

The photomultiplier was operated at 820 V and the laser at 5 mW. CE was performed in a fused-silica capillary (60 cm × 75 μm I.D. × 145 μm O.D.; PolyMicro Technologies, Phoenix, AZ, USA). The polyimide coating of the capillary was burned off to form a window 30 cm from the injection end (grounded anode end) of the capillary. The capillary was cleaned by vacuum siphoning the following sequence of solutions (5 drops each) over a 1.5 h period: (a) methanol–water (1:1, v/v); (b) 0.1 *M* NaOH; (c) methanol–water as before, and buffer. The data were collected at 20 Hz and processed by a Dynamax HPLC Manager utilizing software version 1.2 (Rainin, Woburn, MA, USA) interfaced to a Macintosh Classic computer.

RESULTS AND DISCUSSION

Equipment

The choice of the laser naturally depends on the application on hand. The 488-nm line of the Ar ion laser however does have an overall edge as a general purpose light source. Many fluorescence derivatization procedures have already been worked out for blue-green excitation for important biological molecules. Most notable are fluorescein and rhodamine derivatives of amino acids and nucleotides, including commercial chemistry kits for DNA sequencing. A continuous wave laser is a better choice than a pulsed laser, because the former usually have superior intensity stability, can be focused to smaller spots, and are less likely to damage the capillary column for a given number of excitation photons. Diode lasers are the most rugged devices, but pres-

ently are only available at 670 nm or higher, where few fluorescent tagging schemes have been developed [5]. They also produce output that can be collimated to around 100 μm, which is marginal for clean excitation in CE. The HeCd laser at either 442 nm or 325 nm is often used in the research laboratory because of the availability of good derivatization reagents. However, the HeCd laser tube has a significantly shorter lifetime and the output is sensitive to the temperature of the room, leading to larger intensity fluctuations. The UV lines of an argon ion laser and krypton ion laser lines are useful for exciting a broad range of chromophores, including protein native fluorescence [6] at 275 nm, but these systems are too large and too expensive for a routine instrument. Many commercial sources are available for small air-cooled Ar ion lasers with output of a few mW. We favor one with hard-sealed mirrors and a stable power supply, which is internally stabilized to ±0.1%. The lack of tunability among the various visible Ar ion laser lines is not a serious limitation regarding the selection of fluorophores. On the other hand, mechanical and optical ruggedness is significantly improved. There is a less expensive source of 543 nm light, *viz.* the HeNe "green" laser. The intensity is less stable and the derivatization chemistry is less well established. With time, this may become a good alternative to the Ar ion laser.

For efficient excitation and reduced stray light, the laser beam should be focused tightly to pass through the center of the capillary tube. A simple biconvex lens with around 1 cm focal length can produce a beam waist of 5 μm, which fits just about any capillary size commonly used. This lens can be mounted rigidly relative to the laser and never needs adjustment. To place the capillary column at exactly the laser beam waist, a holder with guides for the column is mounted on a microscope *x–y* stage. The latter is readily available and even the simplest models have sufficiently fine movement and minimal mechanical backlash. Translation is needed in line with the laser beam (focusing) and in a horizontal plane perpendicular to it (crossing). The capillary column with a liquid core is equivalent to a cylindrical lens. If the laser beam crosses that at exact center, its direction of propagation will not be altered. If the capillary column is placed symmetrically at the focal point of the lens, then the beam shape and size at far field should not deviate from those with the capillary removed. These criteria can be used to initially align the excitation beam. The holder for the capillary column consists of clearances drilled out to fit the 350-μm O.D. tubes, which are widely used in CE. With 1 cm or so between these clearances, the capillary column is held with almost no play. To replace columns, one simply threads the new one through the same clearances.

To collect fluorescence originating from the liquid core, a microscope objective is used. This need not be achromatic or of special flat-field design. For 50-μm columns, a 10× standard objective is adequate both in terms of magnification and numerical aperture. For columns 20 μm or narrower, a 20× objective can be used. This can be mounted on a commercial microscope focusing tube, without the eyepiece or its holder. That provides adequate movement for exact imaging of the fluorescence onto the spatial aperture. It is well known that specular reflections from the cylindrical tube can be a problem. This is confined to a plane perpendicular to the capillary tube. So, the capillary holder is designed to impose a 20° angle between this plane and the microscope objective. The region of the mount behind the capillary tube in direct view of the microscope objective is also cleared out to avoid reflections. The focusing tube can be set once and for all to coincide with the fixed beam waist of the laser beam to minimize alignment. For convenience, a mirror on a small integral mount is used to direct the collected fluorescence image onto the spatial aperture. Alignment is performed in a darkened room with a solution of 5 μ*M* fluorescein running through the capillary by pressure flow. It is essential to obtain a sharp image of the fluorescence, which appears as a line, so that reflections, scattering, and fluorescence from the capillary walls can be rejected by the aperture. With practice, one can look for the scattering centers corresponding to the entrance and exit of the laser beam at the column to define the fluorescence image, without the use of a dye stream.

To shield the optical system from room light, the entire set of components is enclosed in a black Plexiglass box. This also serves as protection against high voltage applied to the capillary tube. The cover of the box can be interlocked to the laser

power supply to allow operation as a Class I laser. The base of the Plexiglass box is extended for mounting the laser head and the phototube. The defocused laser beam after hitting the lens and the capillary column continues to the back wall of the box and is effectively stopped. Two cardboard light shield (LS in Fig. 1) basically divide the box into three compartments. This is extremely effective in eliminating stray light in the excitation region and the residual laser light not absorbed by the back wall. Two 1-mm holes are present in the plexiglass box to allow entrance and exit of the capillary column. These holes do not pass sufficient room light into the optical region to change the background level. The other two openings in the box are 3 mm and 5 mm for the laser input and the fluorescence output, respectively. At the inside of these openings, interference filters for the laser line and for the fluorescence wavelength are taped on to seal off almost all room light. The excitation filter is necessary also to eliminate plasma emission from the laser, which can be at the detection wavelength.

Almost any photomultiplier tube can be used to monitor the emission. A red-sensitive phototube is needed in this case. It is fairly straightforward to assemble this from an integral high-voltage power supply and a base with voltage divider. The output can be sent to a compact current-to-voltage converter (about 10^6 V/A) with switchable gain levels and time constants. The photomultiplier tube and all associated electronics can be placed inside a 10 × 15 × 15 cm metal box. It is important to also block room light from entering the phototube compartment. This can be accomplished by taping another emission filter onto the photomultiplier tube itself, exposing only a small region of the photocathode, combined with cardboard light shields inside the box. The opening to the photomultiplier tube can then be matched and sealed against the exit aperture of the optical isolation box. Typically, room light only results in a doubling of the dark-current output of the photomultiplier tube, so that maximum gain can be used in the detection electronics.

Fluorescein detection

Fluorescein standard solutions were prepared in buffer based on weight and assuming that the compound, as listed by the supplier, was 70% pure. Calibration curves were linear for both peak heights and areas (data not shown) from the lowest concentration injected ($3.2 \cdot 10^{-12}$ *M*) up to at least 10^{-8} *M*. For these curves, average values for duplicate injections were calculated, and the correlation coefficient was 0.999 in each case. This shows the high stability of the system. Shown in Fig. 2 is an electropherogram for the lowest concentration injected, in which the signal-to-noise ratio is about 3. The presence of acetonitrile in the running buffer contributed to band sharpness.

It can be difficult to compare different laser fluorescence detectors for capillary electrophoresis because several factors beyond the inherent performance of the detector can influence the detection limits that are reported: analyte tested, frontal (continuous) or plug injection of analyte, capillary dimensions, running buffer, type of injection technique (including any mechanism for analyte enrichment), condition of capillary tip, use of zone *vs.* a gel separation technique, how the signal-to-noise is calculated, whether the detection limit is observed or extrapolated, and whether a linear calibration curve is constructed. While there are some variables, it nevertheless appears that the sensitivity of our detector is comparable to what others have achieved for state-of-the-art laser fluorescence detection in a capillary electrophoresis system. For example, in terms of the concentration of the solution injected, Sweedler *et al.* [7] detected 6 p*M* fluorescein isothiocyanate (signal-to-noise ratio *ca.* 10), Zhang *et al.*

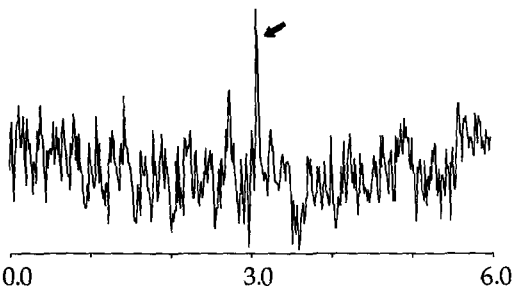

Fig. 2. Detection of $3.2 \cdot 10^{-12}$ *M* fluorescein (concentration of solution injected) by capillary electrophoresis with laser fluorescence detection. Injection and running buffer: 10 m*M* Tris–borate, pH 8.7, 10% acetonitrile. Injection technique: hydrodynamic, with anode end 5 cm higher for 20 s. Applied voltage: 30 kV.

[8] reported a detection limit of 20 p*M* for a fluorescein labeled deoxynucleotide triphosphate and 5 p*M* for a fluorescein-labeled 100-mer oligonucleotide [8], and Cheng and Dovichi [9] reported that the detection limit for a fluorescein-arginine conjugate was less than 5 p*M*. For tetramethylrhodamine isothiocyanate, a related analyte, Chen *et al.* [10] reported a linear calibration curve down to 64 p*M* ($r = 0.986$).

CONCLUSIONS

We have outlined here the design and performance of a home-built laser-based fluorometric detector for CE that has state-of-the-art performance. Commercial detectors of this type have just appeared on the market, but the present system is more flexible and much less expensive. In contrast to a commercial design using optical fibers to deliver laser light to the capillary column, the present systems offers better focusing properties and thus better rejection of stray light for working with small diameter capillaries. Light collection here is based on imaging and spatial filtering. This is similar to one commercial scheme which uses a fluorescence microscope, but is much easier to implement. The system here is also ready for UV excitation, *e.g.* at 275 nm [6], requiring only substitution of the optical filters.

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