A new and compact fluorescence biosensor is constructed. The distinguished feature of this setup is the use of an ellipsoidal reflector as an optical collector of the fluorescence light. A traditional collecting lens with numerical aperture of 0.5 can only collect a few percent of the available signal, while the incorporation of the ellipsoidal reflector enables the apparatus to provide a signal increase of at least 66 times. A very good linear relationship between the concentration of fluorescent dye and the fluorescence intensity is achieved with correlation coefficient \( R = 0.99979 \). The 3\( \sigma \) detection limit is 5 nM for microliter volume sample. Meanwhile, it is found that laser light scattering by dirt and scratches on the sample cell's windows and by turbidity in the sample solutions is the dominant limitation to increase the detection sensitivity. Approach to solve this problem is suggested.

**Keyword:** Optical biosensor; Laser-induced fluorescence; Ellipsoidal reflector

1. Introduction

Laser-induced fluorescence (LIF) technique has been widely used for a variety of applications, from clinical diagnosis to food safety and environmental monitoring [1–3]. It is also getting increasingly important in biodefense field [4,5]. The LIF is a process where molecules are excited to higher electronic energy states via laser absorption and subsequently fluoresce. The intensity of the emitted fluorescence is, in general, a function of the species concentration. Fluorescence-based biosensors utilize fluorescent probe (fluorophore) as a marker. Fluorophores are usually chemically or physically tuned to bind to antibodies, DNA molecules, viruses and other target entities. For example, a sandwich assay is reported [4,5], in which target pathogens are first captured by antibodies specific to the pathogens. Then a second antibody tagged by a fluorescent dye is bound onto the captured pathogen, forming a sandwich structure with pathogen in between.

While stimulated by a laser beam, the fluorescent dye glows, signaling the presence of the harmful elements as well as quantifying its concentration according to the intensity of the fluorescence. Another example, in which DNA molecule is involved [4,5], recognizes the target analyte by comparing its DNA with a single-stranded DNA probe labeled by fluorescent dye. When the target DNA matches the probe DNA to form stable complexes, based on the complementary nature of DNA molecules, the emitted fluorescence allows researcher to identify the target.

Standard instrumental requirements of LIF detection consist of a laser, sample cell, collection optics, wavelength filter to separate the fluorescence signal from laser-based scattering, and photodetector. Nevertheless, conventional LIF detectors always use regular lenses as collection optics and fluorescence light is always collected at 90° with respect to the incident excitation beam, despite the fact that fluorescence is emanated isotropically in 4\( \pi \) steradians. This paper describes a new set-up of laser-induced fluorometer biosensor. In this setup, the collection of the fluorescence light is accomplished by utilizing an ellipsoidal reflector. Sample is placed onto the primary focal point of the ellipsoidal reflector. The fluorescence signal is reflected by the inner reflecting wall of the reflector and focused.
toward the secondary focal point. By this means, larger solid angle of light collection is achieved, thereby significantly enhance the efficiency of fluorescence collection. The incorporation of ellipsoidal reflector enables the apparatus to provide a signal increase of at least 66 times. As biological analysis systems shrink to smaller dimensions, the detection of small volume samples on microliter level is demanded. Most commercial units maximize sensitivity by using a sample cell that is much longer than the absorption length of the laser light, because the longer the pathlength of the cell, the lower the concentration that can be detected. This is not an acceptable approach to a biochip fluorescent detection. The thickness of our sample cell is only 0.76 mm and our experiment is conducted on the sample volume of 2.4 μl. Cy5-AP3-DUTP manufactured by Amersham Pharmacia Biotech Inc. is used as fluorescent dye for concentration calibration. The absorption peak and the emission peak of Cy5 are 649 and 670 nm, respectively (Amersham Pharmacia Biotech Inc.).

2. Design and structure of fluorescence biosensor

A new fluorescence biosensor based on the above idea to improve the collection efficiency of the fluorescence light has been designed and constructed. The objective of the work is to design a fluorescence reader that includes a pocket to house a disposable biochip. The biochip is to fulfill biorecognition reaction of biowarfare agents and other pathogens. Fig. 1 is the schematic diagram of the sensor.

An 8 mW semiconductor diode laser (56DOL687, Melles Griot, Colorado, not shown in the figure) is used to excite the fluorescent dye. The emission line of the laser light is at 642.0 nm. The exciting laser beam passing through a small hole (2 mm in diameter) directly impinges onto the sample chip, which is inserted into the sample chip slot. A beam blocker just in front of the sample (the black rod on the optical axis) is employed to prevent incident laser light from striking on the detector directly.

An ellipsoidal reflection mirror (02REM005, Melles Griot, Colorado) is used to collect the isotropically emitted fluorescent light from the sample. The ellipsoidal reflector was cut off about 1 inch long along the focal axis by diamond saw and is placed firmly in the inner part of the housing space. In order to keep the inner part of the sample-mirror complex hermetically sealed, both ends of the reflector are sealed with O-rings, so that the inner space can be filled with oil, which is able to reduce the total reflection at the interface of the sample cell window, hence, to further enhance the transmission of the fluorescence light. A chip slot for housing the sample is formed by using electrical discharge machining. The chip slot is located at the position that the sample can be situated right across the primary focal point of the ellipsoidal reflector, so that the isotropically emitted fluorescent light can be collected and reflected effectively by the ellipsoidal mirror and focused onto the secondary focal point of the ellipsoidal reflector. Light signal over the solid angle corresponding to the scope of 42–65° and 295–318° on the cross-section plane is able to be collected. Such a design with the ellipsoidal reflection mirror dramatically enhanced the collection efficiency of...
the fluorescent light in more than an order of magnitude, hence, increased the sensitivity of this new sensor much significantly.

An adjustable collimating lens is placed beyond the secondary focal point along the optical axis to receive all the reflected fluorescent light convergent at the secondary focal point and turn it into collimated light beam, so that the fluorescent light beam can strike the interference filters at normal incidence. Otherwise there will be a band-pass shift to shorter wavelength from normal incidence to oblique incidence, causing undesired wavelengths or false signals passing through. The collimating lens can be moved, via thread, back and forth with respect the fixed sleeve. Two high-resolution band-pass interference filters (670FS10-50, Andover Corporation, New Hampshire), which are centered at 670 nm with 10 nm full-width at half-maximum (FWHM) and with 65% peak transmission, are placed along the optical axis. The function of the band-pass filter is to discriminate the fluorescence signal from reflected and scattered laser light, if there is still any, only allowing the light in the range of fluorescence emission spectrum to pass through. According to the characteristic spectrum of the filter, the attenuation of the incident laser light by this doublet filter assembly can be as high as 110 db, while the attenuation of the fluorescent light is only about 7.5 db. A plano-convex objective lens is placed further along the optical axis to focus the parallel fluorescent light beam onto the detector. The objective lens is mounted in a threaded sleeve that allows adjusting back and forth.

A Hamamatsu photomultiplier tube (PMT) module is used as a detector to convert the optical signal into electrical signal. A voltmeter with 100-nV accuracy manufactured by Keithley (Model 2010) is used to read out fluorescence intensity. The surface of the inner housing space was anodized to black to absorb the scattered incident light and prevent it from reaching the detector keeping the background noise in an extremely low level.

Not shown in the figure is a beam limiting aperture that prevents laser light from scattering off of the sides of the sample cell. This aperture is placed far enough out of the focal plane, which coincides with the sample cell, that light scattered from the aperture itself is not collected.

With the above design, very high signal to noise ratio has been achieved by maximizing the collection efficiency of the fluorescent light and minimizing the scattered incident light. As a result, collecting efficiency is dramatically enhanced by the using of the ellipsoidal reflection mirror. The enhancement factor $k$ can be calculated with simple geometry of the optic system as follows:

$$k = 1 + 2(\cos \theta_1 - \cos \theta_2)/(r^2/l^2),$$

where $r$ is the radius of the collimating lens, $l$ is the distance from the sample cell surface to the collimating lens surface, $\theta_1$ and $\theta_2$ are the angles at the cross section plane cutting by the corresponding solid angle, defined by the ellipsoidal reflection surface. In our case, about 32% of the available fluorescence signal can be collected and a theoretical enhancement factor $k$ of 66 has been achieved.

3. Experimental

The calibration experiment was carried out with the Cy5-AP3-DUTP manufactured by Amersham Pharmacia Biotech Inc. This dye has an absorption maximum at 649 nm and a fluorescence maximum at 670 nm. The corresponding Stokes shift is about 21 nm. Extinction coefficient is 250,000 M$^{-1}$cm$^{-1}$ and quantum yield is about 28%. Fig. 2 shows the spectra of Cy5 fluorescent dye [6].

In order to perform the calibration test, we design and fabricate our own sample cell to insert into the chip-slot described in Fig. 1. As shown in Fig. 3, the effective testing dimension of the sample is 2 mm in diameter, 0.76 mm in thickness and hence 2.4 µl in volume. This is a significantly small volume comparing to almost all commercial units, in which they try to maximize sensitivity by using a cell with a
much longer path length for excitation light to pass through. However, small volume of sample is becoming increasingly important in realizing integration and scaling down of the biosensor.

Phosphate buffered saline (PBS) was used to dilute the concentrated dye and serial molar concentrations of 2.44, 4.88, 9.76, 19.53, 39.06, 78.12 and 156.25 nM were prepared for testing. First, background response was measured when the sample cell was filled with pure PBS. The background response was defined as the difference in PMT output voltage with the laser on and off. The measurements were repeated ten times and the buffer solution was changed for each measurement. The background signal was $26.7 \pm 3.5 \text{ mV}$ ($1\sigma$). Since, the noise of the PMT is only 0.01 mV, this signal must be due to either background fluorescence of the PBS or the scattered laser light. We then measured the response of the dye samples of above concentrations, subtracting the background signal from the total response in each case. Fig. 4 shows the result of fluorescence signal versus dye concentration. A least-squares fit through the data shows very good linear relationship between concentration and fluorescence signal with correlation coefficient $R = 0.99979$. The standard deviation is 20 mV and the $3\sigma$ detection limit is 5 nM. The fluorescence signal at low concentration region is attached on the left-upper part of Fig. 4. It can be seen from the graph, the linear relationship is well extended down to very low nanomole concentration region. Detection of concentration down to 2 nM and lower with this new setup is possible.

We found that scattered light beyond what can be removed by the interference filters caused by dusts and scratches on the window surfaces as well as bubbles and turbidity of the sample solution will be collected by the fluorescence detector. Thus, cleanliness must be carefully maintained during the measurements to avoid false signal readings, which will be discussed in detail in the next part.

4. Discussion

The detection limit is clearly a major feature of any biosensing method. The background signal of the setup is a key factor to improve the detection limit. Many studies have been conducted to examine the origin of the background signal as well as how to reduce it and keep it stable.

The background signal as stated in the previous section is defined as the difference of PMT output voltage with the laser on and laser off. The laser off-state voltage of our setup was $-4.60 \pm 0.10 \text{ mV}$, which was very low and stable throughout the entire work. While the laser on-state voltage was strongly depending on the configuration and condition of the setup. A systematic study has been devoted to explore the origin of the on-state voltage and to reduce and stabilize it. The results of the study are summarized in Table 1. The on-state voltage with no sample cell inserted into the setup was $+6.1 \text{ mV}$, resulting in a background voltage of 10.70 mV. An aperture was incorporated in between the ellipsoidal reflector and the collimating lens. Adjusting the position and the diameter of the aperture thus that all the reflection light from the ellipsoidal reflector could be collected, while the scattered light from the rest part of the setup would be blocked, the on-state voltage much reduced down to $-3.9 \text{ mV}$, resulting in a background voltage down to 0.70 mV. This is a strong indication that the on-state voltage was mainly due to the scattering light of the laser beam. Keeping the aperture in the same position and inserting the sample cell with only one
window glass plate facing the aperture, the background voltage increased up to 10.1 mV. Put the second window plate onto the sample cell at the farer side of the aperture, the background voltage increased to 19.3 mV. We believe that the increase of the background voltage is mainly due to the reflection and scattering light caused by the window surfaces of the sample cell. Using dry nitrogen gas to blow the possible dusts on the window surface, the background voltage could be effectively reduced as stated in Table 1. Injection of the PBS buffer fluid into the sample cell with a syringe through the injection hole of the cell, the background voltage dramatically increased and gradually decayed and stabilized at about 62.0 mV. The time-dependent change of the background voltage was due to the bubble formation and turbidity of the fluid from the injection.

The cleanness of the sample cell and the syringe used for injection of the buffer fluid strongly affected the background voltage. For a stained sample cell, the background voltage could be easily hiked to 100–200 mV range. For example, a sample cell filled with PBS buffer fluid the background voltage measured as 135 mV, if we drain the PBS fluid out and refill it with the same fluid without any cleaning, the background voltage increased up to 190–200 mV. If the sample cell was cleaned with flushing alcohol and then refilled with PBS buffer fluid, the background voltage would be reduced to its original level of 130–140 mV. Repeatedly flushing the sample cell with alcohol and refilling it with PBS, after nine tries, the background voltage could be further reduced down to 80–135 mV.

The quality of the PBS buffer fluid is also important in reducing the background voltage. The buffer fluid used in the above study was provided by our collaboration partner, which was prepared long time ago. Using a freshly prepared PBS buffer fluid and cleaning the sample cell thoroughly by dismounting it and ultrasonic cleaned in alcohol, and using a pump to drain the PBS fluid out. The background voltage can be reduced down and stabilized in the range of 26.1–28.5 mV. The measurements were repeated for ten times, a background voltage of 26.7 ± 3.5 mV (1σ) was achieved as stated in the previous section. The experimental results presented in the above section were based on the extensive and systematic studies.

Discussing on the reduction of background voltage, we would also further stress the importance of the geometrical alignment of the setup, specifically the parallelness of the window plates of the sample cell. Deviation from the parallel alignment will result in multireflection within the sample cell between the window surfaces and cause the increase of scattering of the laser beam, hence dramatically increase the background voltage.

The above discussion has revealed that the background voltage is mainly depending on the scattering of the laser beam from various objects. Although our study on the reduction of the background voltage to improve the detection limit is quite successful, a further improvement of the detection limit should be relying on the exploration of better fluorophores. The effect of scattering light of the laser beam is ultimately caused by the narrow window or Stokes shift between the excitation and emission wavelength of today’s fluorophores, which is usually only 20–50 nm (The Stokes shift of Cy5 dye is 21 nm). Even the best band-pass filter cannot screen out all scattered excitation light with the wavelength so close to the fluorescence emission wavelength. For example, a little tilting of the interference filter from normal incidence to oblique incidence will cause the shift of the band pass of the filter to shorter wavelength, which increases the transmittance of the scattered exciting light, since wavelength of the excitation light is always shorter than that of emitted fluorescence.

Therefore, explore a new fluorohore with wider Stokes window is worth for consideration, while at the moment, too much concerns are focused on developing the instrumental component. With wide wavelength separations between absorption and emission of fluorescent dye, a simple and cheap colored glass filter, instead of bulky and expensive interference filter, is good enough to absorb every scattered exciting photon and completely eliminate the scattered light from real fluorescence signal. Meanwhile, the use of glass filter is very helpful for biosensor miniaturization.

5. Conclusion

We have demonstrated a new laser-induced fluorescence biosensor with a 3σ detection limit of 5 nM concentration for a 2.4 µl sample volume, as well as an excellent linearity between concentration and fluorescence intensity. The distinguished feature of this set up is the use of ellipsoidal reflector as the collection optics, by which about 32% of available fluorescence signal can be collected, which is at least 66 times more than a regular collecting lens. The design of the new fluorescence biosensor is successful. We believe that the dominant limitation to increase the

<table>
<thead>
<tr>
<th>Configuration of the fluorescence setup</th>
<th>Background voltage (mV)</th>
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</thead>
<tbody>
<tr>
<td>1  Sample cell not inserted</td>
<td>10.7</td>
</tr>
<tr>
<td>2  An aperture added</td>
<td>0.7</td>
</tr>
<tr>
<td>3  Sample cell inserted with one window glass plate</td>
<td>10.1</td>
</tr>
<tr>
<td>4  Blowing with dry N₂ gas once</td>
<td>9.45</td>
</tr>
<tr>
<td>5  Blowing with dry N₂ gas again</td>
<td>9.01</td>
</tr>
<tr>
<td>6  Sample cell inserted with two window glass plates</td>
<td>19.3</td>
</tr>
<tr>
<td>7  Sample cell filled with PBS buffer fluid (stock fluid)</td>
<td>62.0</td>
</tr>
<tr>
<td>8  Sample cell filled with freshly prepared PBS buffer fluid</td>
<td>26.7</td>
</tr>
<tr>
<td>9  Sample cell stained</td>
<td>100–200</td>
</tr>
<tr>
<td>10 Stained sample cell flushed with alcohol</td>
<td>80–135</td>
</tr>
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sensitivity is the scattering of laser light by dirt and scratches on the sample cell’s windows and by turbidity in the sample solution. Sensitivity can be improved by more than 1000 times before detector noise becomes a limitation.

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References