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SHORT COMMUNICATION

# Filterless Fluorometry with Enhanced Sensitivity

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**Abstract** We describe a novel approach for fluorometry with light-emitting-diodes (LEDs) as excitation source that capitalizes on the principle of light shielding and total internal reflection to operate without optical filters. This scheme is practicable, demonstrated to significantly reduce the amount of excitation light overlapping the fluorescence signals at the photodetector, operated best with excitation light applied orthogonal to detection, and permitted higher measurement sensitivity of fluorophore emission.

**Keywords** Fluorometry · Light emitting diodes · Filterless · Spectrum

## Introduction

A fluorometer is an instrument for detecting and measuring fluorescence. Fluorescence provides rich information regarding biomolecules and their dynamics. It has been used in studies to monitor polymerization processes, detecting bases on DNA, measure diffusion coefficients, quantifying protein interactions and enzyme kinetics, investigate binding sites of antibodies and probe the internal polarity of proteins. Important breakthroughs in the life sciences today remain dependent on evidences derived using fluorometric measurements [1–3]. Much work in the improvement of the technique of fluorometry appears centered around the development of fluorophores [4]; albeit improvements in photonic instrumentation can also lead to significant advances. One important photonic instrumentation improvement was in the introduction of light emitting diodes (LEDs) which provided band limited wavelength excitation and extremely short duration pulses for lifetime measurements [5, 6].

Measurement sensitivity in fluorometry relies on (1) the ability to minimize the excitation whilst allowing maximal fluorophore emitted light to arrive at the photodetector, (2) eliminating cross-talk interference when the spectral bandwidth of the excitation light overlaps with that of the emission signal, and (3) maximizing emission photon collection at the photodetector. The use of monochromatic LEDs for illumination assists in factors (1) and (2) to a certain extent. Nevertheless, systems will commonly incorporate appropriate optical filter sets which unfortunately adds cost to the system and reduces the capability for compact design. In addition, depending on the transmission properties of the filter over the emission spectral bandwidth, signal attenuation can result in significant loss in measurement sensitivity. In the event that different fluorophores are used, a series of optical filters will be needed and increases instrumentation costs even further. Clearly a measurement scheme that eschews optical filters is desirable. Factor (3) has been addressed by the use of non-imaging optical designs [7, 8]. Such a specialty optical element will typically entail a high cost for its design and manufacture. As in the case of optical filters, obviating the requirement for specialty non-imaging optics will be a desirable development. In this work, we describe and demonstrate a simplified scheme to achieve high sensitivity fluorometric measurements by capitalizing on the principle of light shielding and total internal reflection.

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## Technique description

The basic setup for fluorometry is shown in Fig. 1a. Here, an optical fiber coupled to a photodetector (as an alternative to applying a photodetector directly) is used to harness the emission. Regardless of whether the excitation light from the LED is delivered from the top, side, or bottom; a measure of it will be sensed by the photodetector. In the case of excitation light delivered from the top, most of the excitation light will reach the photodetector through reflection off the liquid surface. In many fluorometry designs, illumination is applied from the side to minimize the excitation light sensed; albeit optical filters will still be used for improved measurement sensitivity. In the scheme proposed here, the optical fiber or photodetector is placed so that it hovers just above the liquid surface of the protein sample (Fig. 1b). This is not an impractical approach as (a) precise amounts of fluid (dispensed using micropipettes) are almost always used, (b) measurement is invariably done without any component movement, and (c) exact physical location of the optical fiber or photodetector is realizable. The premise with this arrangement is that excitation light rays from the LED above the liquid line will be shielded away while excitation light rays below the liquid line that arrive at the air–liquid interface at incident angles $\theta_i$ larger than the critical angle $\theta_c$ will be internally reflected back into the liquid. The critical angle can be found using the equation:

$$\theta_c = \sin^{-1}\left(\frac{n_2}{n_1}\right) \quad (1)$$

where $n_1$ and $n_2$ are the refractive indices of the liquid and air respectively. Clearly, this principle is similar to the case of light transmitted in optical fibers.

## Experimental and discussion

We used enhanced green fluorescent protein (EGFP) and *Discosoma* sp. red fluorescent protein (DsRed) isolated from genetically modified by *Escherichia coli* (*E. coli*) and purified by chromatography as samples to demonstrate the scheme. An experimental setup was arranged with a 1 mm diameter multimode optical fiber coupled to a spectrometer (Ocean Optics model USB4000). As the excitation spectra of EGFP and DsRed peak at 488 nm and 558 nm respectively, we used two separate LEDs, a blue (Avago Technologies HLMP-CB30-NRG00) and green (Avago Technologies HLMP-CM30-S0000), to excite the samples respectively. The typical emission peaks of EGFP and DsRed peak are at 509 nm and 583 nm [9–12].

A first experiment was conducted to determine the best illumination orientation. Protein solutions of volume of 100 μl of EGFP and DsRED at concentrations of 266 and 140 μg/ml respectively, were placed in separate wells of a 96-well clear flat-bottomed microplate. The optical fiber was placed so that it hovered just above the liquid line. Then, the respective LEDs were rotated around the sample to chart the spectral detection signal at different angular orientations. The results with the EGFP and DsRed samples are shown in Fig. 2a and b respectively. Clearly, the ratio of emission to excitation light detected was highest with side illumination; which allowed us to confirm that it was the best orientation to be used. It is noteworthy that the excitation light directed from below was so strong that it drowned out the fluorophore emission light. Excitation light directed from the top improved the situation, although a significant amount was still collected, particularly for the DsRED sample, as a consequence of reflection from the base.

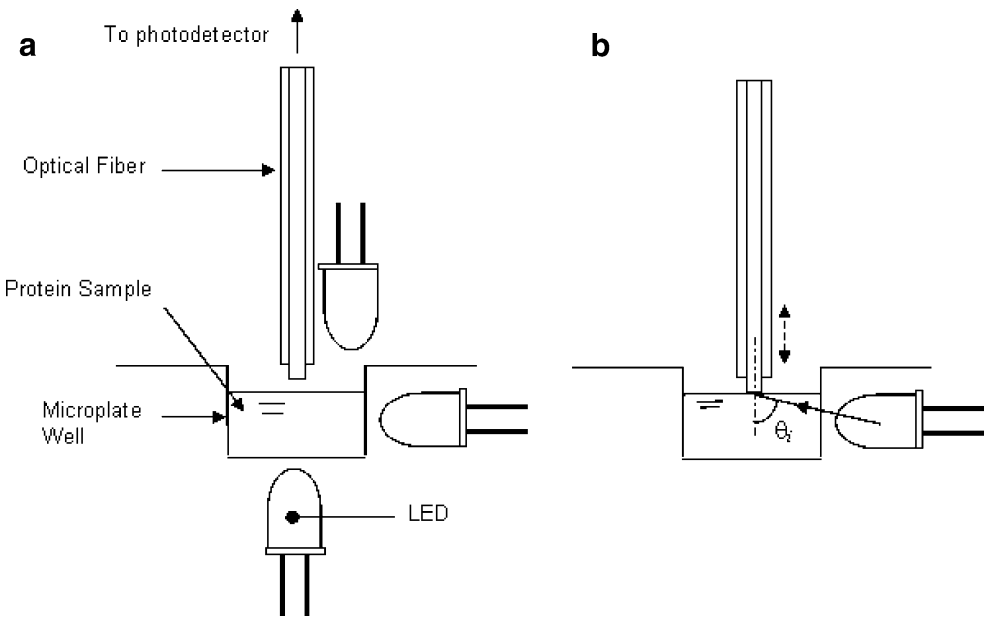


**Fig. 1** **a** Typical fluorometry measurement scheme, and **b** the proposed scheme with optical fiber just hovering about the liquid surface. In **b** excitation light arriving from the above and below the liquid surface are shielded and total internally reflected respectively from entering the fiber

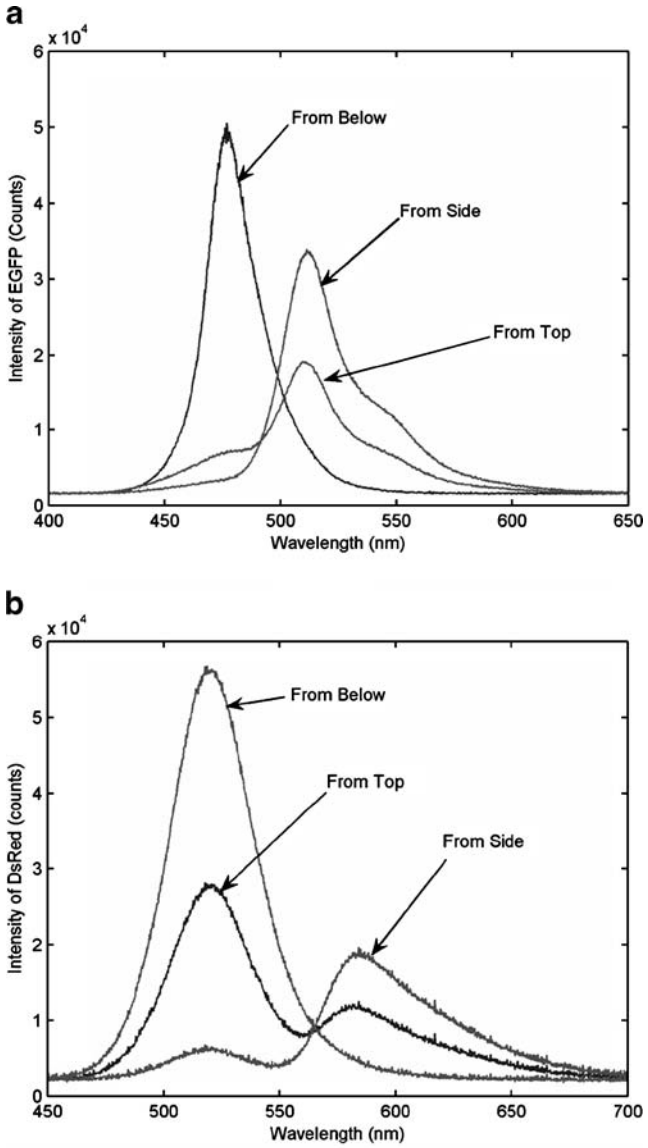


**Fig. 2** Spectrum measurements when LED was used to excite **a** EGFP and **b** DsRED protein sample from the top, below side of the respective well in a microplate

A second experiment was conducted to verify the ability of operating without optical filters. The same volume of 100 μl of EGFP and DsRED protein solution was used. With LED excitation light applied from the side, the distal end of the optical fiber was positioned a clear distance above the liquid surface and lowered in steps until it hovered just above the surface. Whilst it was possible to submerge the fiber's distal end into the liquid, this was eschewed because (a) in practice it would require cleaning at the end of each measurement, and (b) electrical problems would be an issue if a photodetector was used instead of an optical fiber. The spectra of light detected at each step are presented in Fig. 3a and b.

We first consider the part of the spectrum associated with excitation light. With the optical fiber above the liquid surface, excitation light collected was highest when furthest away from the liquid surface. This is obviously attributed to increased LED light rays from above the liquid surface arriving at the fiber end. As the distance of the fiber end from the surface was reduced, this amount reduced correspondingly until a minimal amount of light was collected with the fiber hovering just above the liquid surface. This confirmed the essential premise of the scheme.

We next consider the part of the spectrum associated with emitted light. Quite clearly, the intensity was highest when the fiber was located just above the liquid surface and

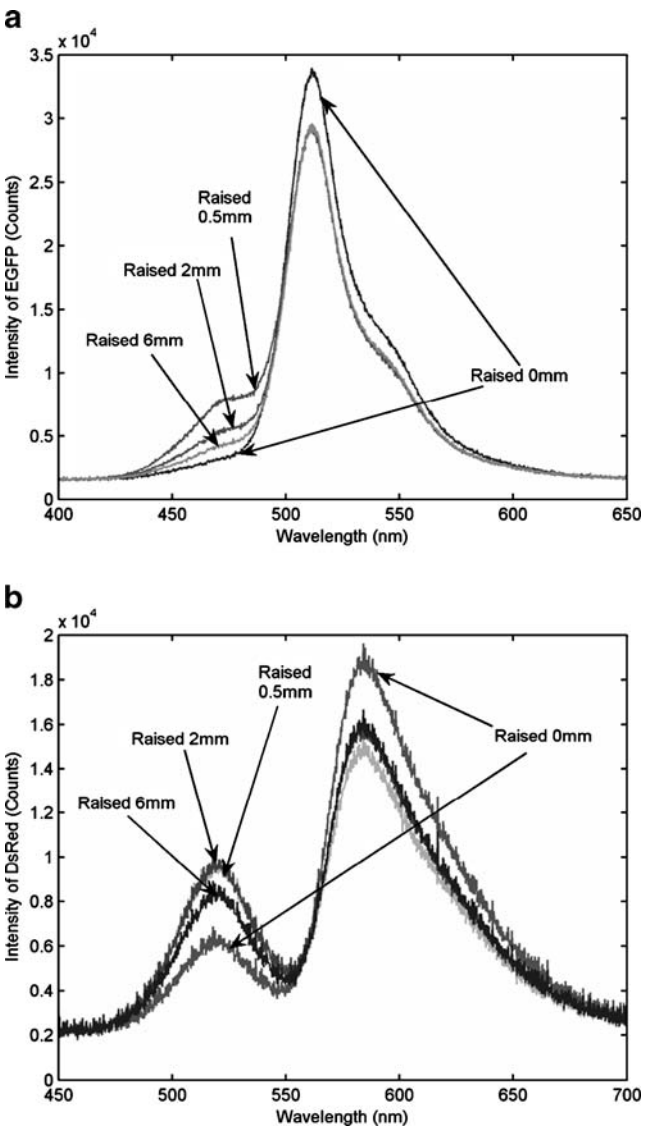


**Fig. 3** Intensity of EGFP and DsRed protein emission detected by spectrometer at different optical fiber position from the surface of the protein solution. Placing the fiber just above the liquid surface reduces the excitation light intensity whilst increasing the emission light intensity

reduced with increasing distance from the surface. This is attributed to the well known fact that fluorophore emission is isotropic and a higher degree of light should be gathered when a collector (fiber end) is located closer to the source. The higher amount of emission light collected infers that recording with the optical fiber just above the liquid surface offers a relatively higher degree of sensitivity from any measurement made.

A last experiment was conducted for measurements at different protein concentrations with the fiber located just above the liquid surface. Varying protein concentrations of EFPG and DsRed were prepared and the emission measured using the recording scheme. The spectral distributions are shown in Fig. 4a and b. Table 1 presents the result in terms of the spectral value at peak emission (509 nm for EGFP and 583 nm for DsRed) as well as summation from 400 nm to 750 nm. The latter corresponds to sensing using a typical visible range photodetector with flat spectral response. These results are presented in the graphs of Fig. 5a and b. There is clearly good linear response ($r^2$>0.98) between fluorescence intensity and protein concentration with EGFP and DsRed using both sets of metrics. The result in Fig. 5b indicates that the measurement method with a photodetector is able to distinguish concentrations between 2.5 μg/ml to 250 μg/ml. The detection limit of the fluorescent proteins at 2.5 μg/ml compares well with that of micro protocols of conventional dye-binding or BCA-based protein assays that typically detects down to 1 μg/ml and 0.5 μg/ml respectively. We believe that such levels of operation may be possible when our system is optimized. Looking more closely at Fig. 3a and b we find that a certain degree of excitation light remains detectable which is likely due to the irradiation characteristics of the LEDs used. The LED that we used had a beam spread of 30° and it is plausible that some light rays did not meet the total internal reflection condition of Eq. (1). We believe that this can be overcome with careful illumination tailoring of the LED light source [13]. In addition, the blue LED used had a spectral peak of around 470 nm which is closer to the excitation maxima of EGFP at 509 nm; as opposed to the green LED with spectral peak at around 520 nm relative to the excitation maxima of DsRED at 583 nm. The proportion of photons not used for excitation will constitute stray light that is detectable when the total internal reflection condition is not met. This very likely accounted for the higher residual excitation component present for DsRed in Fig. 3b. Another direction towards optimization will hence be in seeking light sources with wavelengths as close as possible to excitation.

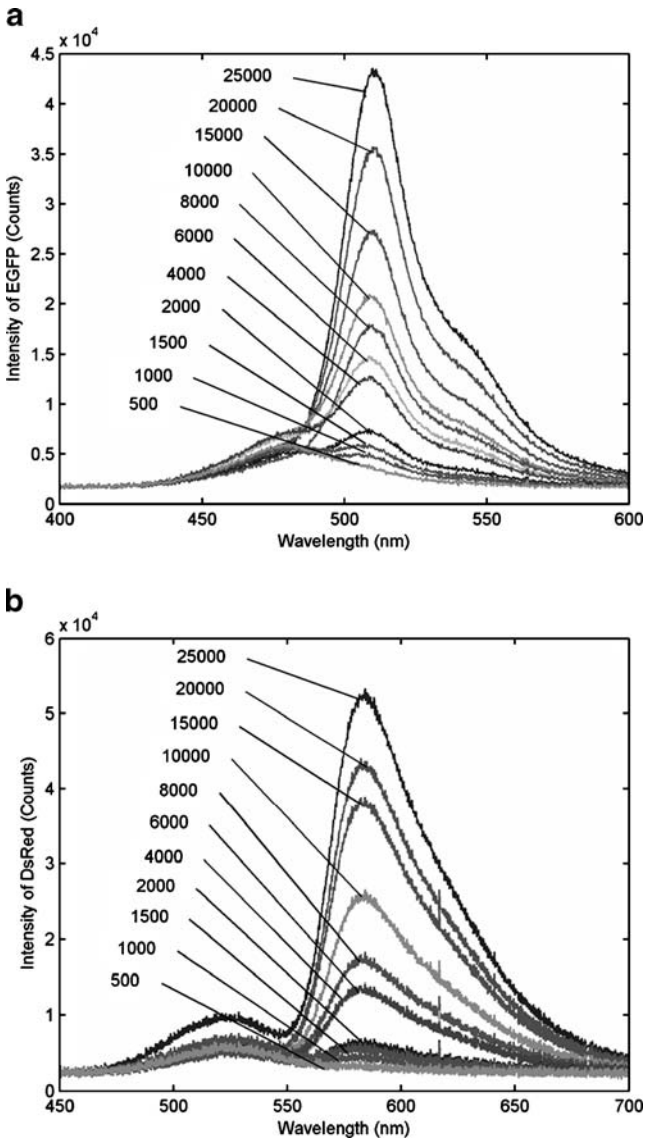


**Fig. 4** Spectrum measurements at various concentrations (μg/ml) of **a** EGFP and **b** DsRED protein by placing the optical fiber just above the liquid surface

## Conclusions

In summary, the fluorometry recording scheme described here is verified to be able to make use of the principle of light shielding and total internal reflection to eliminate the need for optical filters. It operates best with the LED excitation applied orthogonal to light collection. In addition, it offers maximal collection of emitted light from the fluorophore source to improve measurement sensitivity. Experiments with different protein concentrations conducted demonstrate the ability to distinguish between 2.5 and 250 μg/ml for EGFP and DsRed. The technique can be optimized by using light sources with wavelengths as close as possible to excitation and through careful optical design. We believe that this seemingly simple and novel scheme will positively impact fluorometry instrumentation design.

**Table 1** Spectrum peak values (a) and spectrum summation values (400–750 nm) (b) plotted against protein concentrations of EGFP and DsRed

| EGFP | | | DsRed | | |
|---|---|---|---|---|---|
| Protein concentration (μg/ml) | Spectrum value at 509 nm | Spectrum summation (400–750 nm) | Protein concentration (μg/ml) | Spectrum value at 583 nm | Spectrum summation (400–750 nm) |
| 2.5 | 3,237.84 | 3,512,841.93 | 2.5 | 2,895.83 | 4,157,902.91 |
| 5 | 3,686.34 | 3,570,649.83 | 5 | 3,205.07 | 4,531,442.04 |
| 10 | 4,792.25 | 3,719,420.44 | 10 | 4,599.74 | 4,708,546.39 |
| 15 | 5,631.91 | 3,945,700.35 | 15 | 5,633.96 | 5,502,056.67 |
| 20 | 7,161.75 | 4,149,752.14 | 20 | 6,104.99 | 5,458,350.63 |
| 40 | 12,597.06 | 5,556,425.95 | 40 | 13,379.38 | 7,690,411.34 |
| 60 | 14,409.51 | 5,890,754.82 | 60 | 13,274.93 | 7,426,407.77 |
| 80 | 17,956.59 | 6,154,736.13 | 80 | 17,293.05 | 8,780,399.79 |
| 100 | 20,727.49 | 7,162,768.54 | 100 | 25,548.41 | 11,397,572.69 |
| 150 | 27,354.72 | 8,262,835.21 | 150 | 38,008.25 | 15,354,147.89 |
| 200 | 35,751.39 | 9,920,004.48 | 200 | 43,150.70 | 16,854,375.20 |
| 250 | 43,566.44 | 11,409,180.54 | 250 | 52,497.63 | 20,504,701.38 |

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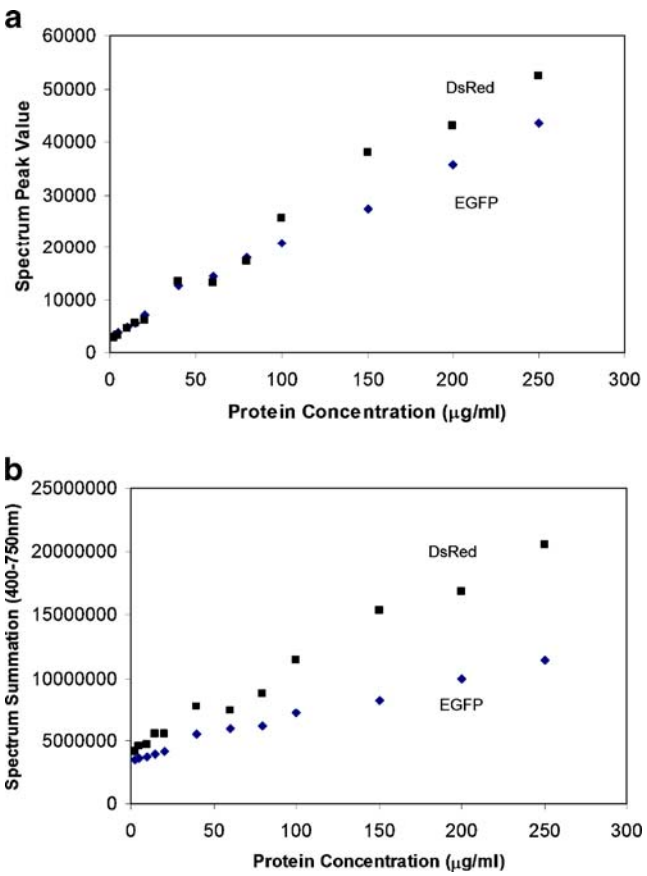


**Fig. 5** Spectrum peak values (**a**) and spectrum summation values (400–750 nm) (**b**) plotted against protein concentrations for EGFP and DsRed

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