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Short communication

Irradiance dependence of photobleaching of resorufin

Chia-Pin Chang*, David J. Nagel, Mona E. Zaghloul

The George Washington University, Department of Electrical and Computer Engineering, 2033 K St NW Suite 340E, Washington, DC 20052, USA

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ABSTRACT

Resorufin is a fluorescent reporter molecule, the end product in a variety of biochemical analyses. In such assays, excitation sources with high intensity are desirable for fast and precise results. However, high intensity sources bleach resorufin. We report quantitative bleaching rates for resorufin subjected to green excitation over an irradiance range of 5 to about 600 mW/cm^2.

1. Introduction

Microfluidic devices enable new, fast and highly capable chemical and biological analyzers. They have already proven to be commercially important [1,2]. Enzymatic recognition reactions permit the analysis of clinical and other samples without prior separations. Optical fluorescence is a major means of transducing the recognition events into measurable signals [3].

Resorufin ($C_{12}H_6NNaO_3$) is a fluorescent molecule that can be the endpoint of enzyme selectivity of different target molecules, including uric acid, glucose and cholesterol [4]. It is the reporter molecule for a commercial kit for analysis of uric acid [5]. We are developing a microfluidic point-of-care analyzer for uric acid that employs the following chemistry from the commercial kit:

$$\text{Uric acid} + O_2 \xrightarrow{\text{uricase}} \text{allantoin} + CO_2 + H_2O_2$$

$$H_2O_2 + \text{amplex red} \xrightarrow{\text{HRP}} \text{resorufin} + H_2O$$

HRP is horse radish peroxidase. In the course of the work, we observed photobleaching of resorufin, and quantified its behavior. This paper reports the methodology and results of our measurements. Such data are not available in the limited literature on the photosensitivity of resorufin [6–9].

2. Materials and methods

A compact test setup was made for the photobleaching measurements. A schematic of the apparatus is given in Fig. 1. The design of the setup provided relatively low fluorescent background signals, which were subtracted for all the readings in this work. The apparatus was machined from black Delrin plastic blocks.

A 90 lumens green LED (Luxeon Rebel 532 nm) was used for excitation. The LED emits the wavelength that is optimum for the excitation of the fluorescence of resorufin. It was powered with continuous, direct current from a commercial LED driver (Luxeon 3021-D-I-700). The LED was used without a collimator, in which case it had an angular emission width of ±60°. The LED power P_S in Watts was computed by using Eq. (1).

$$P_S \text{ (W)} = \frac{\text{lumens}}{683 \text{ lumens per Watt} \times \text{luminous efficacy}} \quad (1)$$

where the wavelength-dependent luminous efficacy is 0.86 for $\lambda = 532$ nm [10].

The diverging beam from the LED passed through a 1.5 cm focal length double convex lens placed at twice the focal length from the LED. The sample holder was placed at twice the focal length on the other side of the lens. The resulting 1:1 magnification allowed us to estimate the diameter of the irradiated area as 2.3 mm^2. From these values, estimates of the irradiance were obtained. These irradiance values may be about 30% high due to the LED and lens spectral distribution, but this correction is uncertain and was not included in the data presented below.

By using various combinations of neutral density filters (Lee filters 209), we adjusted the incident intensity of the excitation

* Corresponding author. Tel.: +1 5712399145.
E-mail address: chiapinc@gmail.com (C.-P. Chang).

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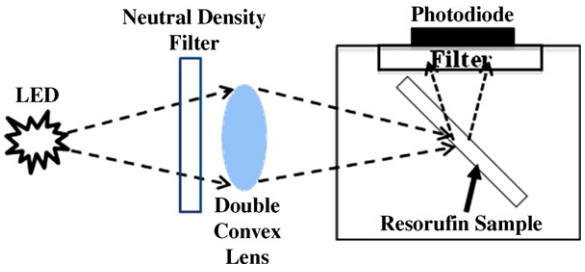

Fig. 1. The schematic of experimental apparatus. The LED and resorufin sample are placed on opposite sides of double convex lens at a distance of twice the lens focal length. Zero to several neutral density filters were employed between the LED and the lens to adjust the number of photons striking the resorufin. An amplified photodiode was used to detect the fluorescence in a range of 600 ± 20 nm as determined by an interference filter.

light over a range of two orders of magnitude, from 5 mW/cm^2 to 592 mW/cm^2.

An interference filter (Thorlabs FB600-40) was employed ahead of the detector to exclude scattered excitation radiation. It had a 40 nm FWHM centered at a wavelength of 600 nm. Its peak transmission was 80%. The fluorescence radiation from resorufin peaks at 585 nm. It was detected with an amplified photodiode (OptoDiode ODA-6WB-500M), which has remarkable output linearity from 1 mV to 5 V. The area of the detector element is 6 mm^2 (2 mm × 3 mm).

The DC output of the photodiode was digitized using the ELVIS module and LabVIEW from National Instruments. Measurements were taken at 5 s intervals for 5 min. We measured the absolute absorption spectra with an Ocean Optics spectrometer USB2000 over the wavelength range of 450–650 nm.

Resorufin (AnaSpec #80003) was dissolved in deionized water to form a 0.85 mM solution with a measured pH of 8.5. The absorption and emission spectra of resorufin are available on the web [11]. The resorufin solution to be measured was held between a precleaned conventional glass microscope slide and a 22 mm × 22 mm square glass cover slip. They were held parallel and together by the use of one or two layers of 90 μm thick double-sided adhesive tape (Scotch Tape Cat 665) to determine the thickness of the sample volume. The double-sided tape was placed under two of the edges of the cover slip. The resourifin sample thickness was determined by micrometer measurements. Quantitative pipetting of the solution was also employed to verify the sample thickness. The overall dimensions were measured as 22 mm × 15 mm × 0.09 mm or 0.18 mm. Hence, 60 μL of resorufin was employed for each measurement.

3. Results

Fig. 2 shows the absolute absorption spectra of resorufin measured in two holders with the noted sample thicknesses. The molar extinction coefficient of 53,450 M^{-1} cm^{-1} at the excitation wavelength of 532 nm was computed from the two absorption spectra. Resorufin has the highest extinction coefficient of 91,600 M^{-1} cm^{-1} at wavelength of 572 nm, according to our experiments, compared to 54,000 M^{-1} cm^{-1} from the literature [12].

The bleaching histories for 0.85 mM solutions from the same stock solution and different values of irradiance are shown in Fig. 3. The samples receiving the highest irradiances clearly bleach relatively rapidly. The curves show an initial, relatively fast, exponential-like decay followed by a much slower decay rate. The slower decay is probably due to diffusion of bleached resorufin molecules out of the irradiated region and their replacement by unbleached molecules from the un-irradiated region.

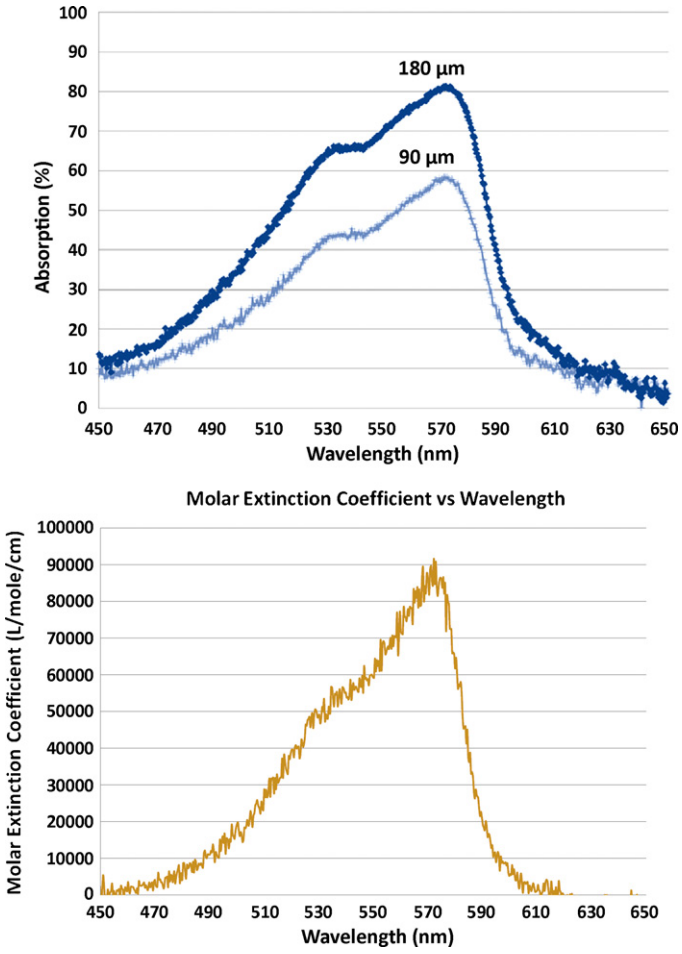

Fig. 2. Top: absorption spectra of resorufin. Samples of 0.85 mM resorufin (pH 8.5) were placed in two different thickness holders (90 μm and 180 μm). Bottom: by using the data in the top figure, we computed the molar extinction coefficient of resorufin.

That is, the data at longer times are due to a combination of photochemistry and diffusion. Separation of these two effects at longer times would require detailed knowledge of the irradiance distribution and the diffusion coefficient, and their use in a computational model. Such analysis is not needed to give us the practical short-time information we need for development of our analyzer. We used an estimated diffusion coefficient to determine that the short-time data is free of molecular migration effects [13].

The first 20 s of the bleaching curves were fitted to the single-exponential first order process given in Eq. (2) by using Excel to obtain values for the photobleaching lifetime τ.

$$I(t) = I(0) \times e^{-t/\tau} \quad (2)$$

$I(0)$ is the initial fluorescence intensity, and the $I(t)$ is the fluorescence intensity at time t.

Fig. 4 shows how the photobleaching lifetime depends on the irradiance of the green light. It is seen from this figure that when excitation irradiances are more than 100 mW/cm^2, the fluorescence emitted from the resorufin is substantially photobleached in a few minutes.

4. Discussion

We determined quantitative photobleaching lifetimes as a function of the absolute irradiance at one wavelength. The data are

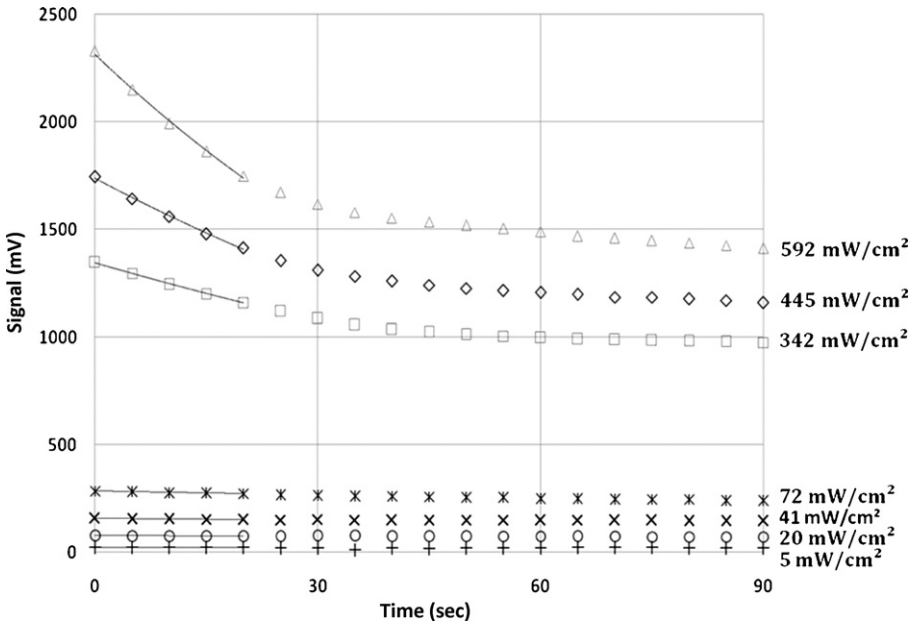

Fig. 3. Photobleaching of resorufin under different irradiances. We recorded the data every 5 s for 300 s. The first 90 s are displayed here. The lines are exponential fits to the first 20 s to determine the bleaching rate at each irradiance.

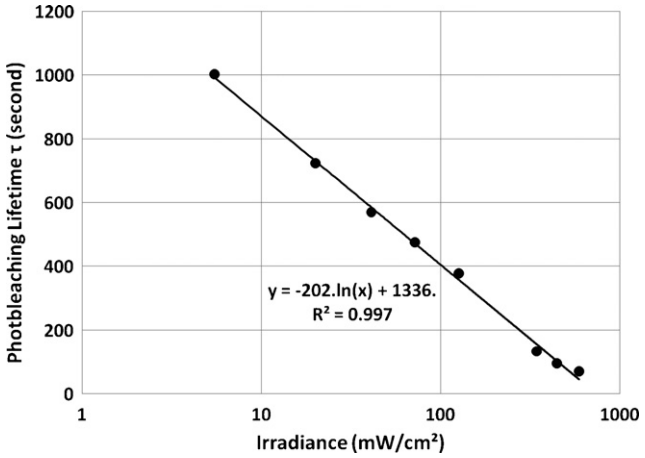

Fig. 4. Resorufin photobleaching lifetime τ as a function of irradiance. This figure was made from the data collected in Fig. 3. The one point at irradiance of 126 mW/cm^2 in this figure was collected by using different setup from that described previously. The unit of irradiance power can be easily converted from mW/cm^2 to photons/s/mm^2 by multiplying 2.67×10^{13}.

adequate for the development of a uric acid analyzer. A more detailed study of the photo response of resorufin would be desirable. Dependence on the incident wavelength and on the solution pH would be interesting. Determination of kinetic factors would be worthwhile. Our results give a starting point for further studies of the photo degradation of resorufin.

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