Journal of Undergraduate Chemistry Research, **2023**, 22(4), 92

UTILIZING REPEATED LED EXPOSURE TO MEASURE PHOTOBLEACHING KINETIC DECAY RATE OF ACRIDINE ORANGE

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Abstract

Fluorescence photobleaching is the permanent loss of a fluorophore's ability to excite and then emit photons. In this paper we show the development of a technique utilizing a mounted LED light source to induce photobleaching and study the resulting loss in fluorescence intensity. We were able to successfully induce photobleaching in acridine orange and were able to extract decay rate constants from the resulting intensity loss.

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Keywords: fluorescence, photobleaching, decay rates, acridine orange, 3D printing

Received: August 31, 2023 Accepted: September 14, 2023 Published: September 21, 2023

Introduction

Fluorescence is an important tool in biological imaging and is used to capture highly detailed images, which would otherwise be difficult to discern due to the noise or resolution limit of most imaging techniques.[1] These techniques range from instances used to highlight areas of interest, such as labeling cells and staining gels, to techniques designed to take advantage of the photophysical properties of the fluorophores, such as anisotropy or Förster resonance energy transfer.[2-4] Most fluorescent probes are small molecules, which can be attached to a target of interest to highlight it from its surroundings.[5-6] When choosing which probe to use, there are many considerations including excitation and emission wavelengths, suitability for attachment, and photophysical stability.[7] Photobleaching can be of particular concern as it is a limiting factor in how long a particular sample is able to be studied, which is a matter of importance in single molecule studies for which long term dynamics are important.[8-9]

Fluorescence photobleaching is the process in which fluorescence molecules decay over time due to prolonged exposure to excitation light.[10-11] There are many causes of photobleaching but the primary cause is oxidative damage due to the presence of singlet oxygen reacting with the excited molecule and removing the fluorescence electron.[12-13] This eliminates the fluorophore's ability to fluoresce and can cause the molecule to permanently go dark unless it is able to recover. There have been efforts to reduce the impact that photobleaching has through environmental additives.[14-15] Additionally, a number of fluorescent probes, such as the Alexa Fluor series, have been developed to be photobleaching resistant and new probes are being developed to also help mitigate the effects that photobleaching has on the probe's performance.[16-17]

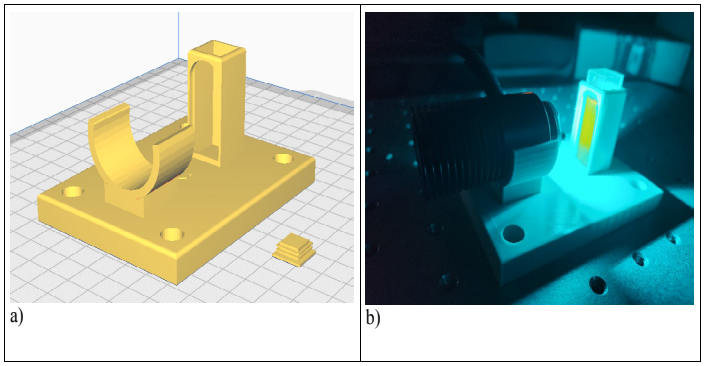
Traditional methods of measuring photobleaching kinetics rely on imaging a sample repeatedly, utilizing the spectrometer's excitation source to cause photobleaching, and then inferring the photobleaching decay rate based on image analysis of the decreasing intensity of the sample's fluorescence.[14, 18-19] There is a need for creating an assay that can determine the photobleaching rate at an early stage in probe development in an efficient manner. However, standard fluorescence spectrometers are ill-equipped to measure photobleaching kinetics due to the long timescales involved in photobleaching and issues such as diffusion in the cuvette.

In this paper we show the development of a technique to extract the photobleaching kinetics of the acridine orange fluorophore using a conventional fluorescence spectrometer by introducing the sample to time-controlled exposure to a wavelength specific LED. We selected acridine orange as it is a readily available fluorophore while also not being particularly photobleaching resistant.[20] In our results we were able to show that this technique allows for repeatable photobleaching decay rates to be determined. By moving the bleaching process to an external light source, we can confidently and more accurately measure the exposure time without needing to interpret instrument parameters, which might give an inaccurate assessment of the actual exposure time.

Experimental Methods

3D printing

The bleaching stage and cuvette lids were custom designed and 3D printed for this project (Figure 1a). The bleaching stage consisted of a mountable base with screw holes designed to fit a standard mounting breadboard, a LED mount, and a cuvette holder with a single window facing the LED mount. The mount was positioned to securely hold a LED horizontally 2 cm from the sample and vertically aligned with the center of the sample,



a) b)

Figure 1. a) 3D model for the bleaching stage and the cuvette cap. b) Sample cuvette in the bleaching stage.

and a cuvette holder with a single window facing the LED mount. All designs were created in FreeCAD (v0.18 and v0.19). Once the design was modeled a 3D mesh of the model was transferred to Ultimaker Cura (AV_Mk5_2) to be processed and sliced for the gcode instructions. The bleaching stage was printed on the Anycubic Vyper 3D printer using 1.75mm white PLA (polylactic acid). White was chosen for the stage to maximize reflective exposure. Cuvette caps were printed on a Flashforge Finder 2.0 3D Printer using 1.75mm PLA of various colors. Cuvette caps were designed to be snug fit and air tight. All 3D printing models are available on request.

Photobleaching procedure

A 100 µM NHS (N-hydroxysuccinimde ester) acridine orange (Chemsavers) solution in 1x PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4 [pH 7.4]) buffer (VWR) was prepared. The solution was dispensed into two standard fluorescence cuvettes and then fitted with a custom designed 3D printed cuvette lid to seal the cuvette. One sample was placed in the bleaching stage while the other was kept in the dark. For acridine orange, a 490 nm LED (ThorLabs) powered by a T-Cube LED Driver (ThorLabs) set to the maximum intensity of 1200 mA was used to bleach the sample (Figure 1b). The LED was hooked up to an outlet timer set for 30 minutes. The whole stage was covered to prevent excess light leakage. After the 30-minute exposure period ended the sample cuvette was transferred to a FluoroMax-4 (Horiba) spectrophotometer and a fluorescence curve was taken. Excitation wavelength was set to 490 nm with a slit size of 5 nm. Emission spectra were collected from 470 nm to 570 nm at an increment of 1 nm with a slit size of 5 nm. After the measurement the sample was returned to the bleaching stage to repeat the 30 min exposure over a period of around 6 hours. Immediately following the scan of the bleaching sample, the control sample was scanned using the same parameters but kept in the dark between measurements.

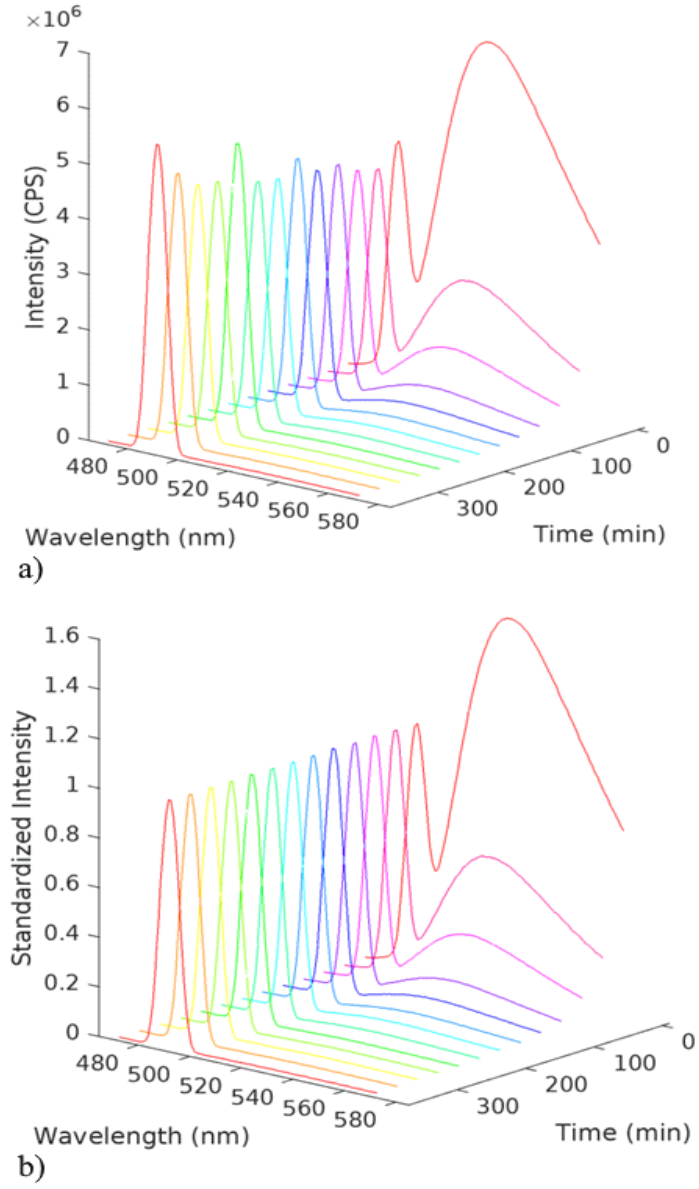


Figure 2. Fluorescence curves of a single sample acridine orange after repeated 30 minute exposures to 490 nm LED. a) The raw intensity counts per second (CPS) plotted vs wavelength and exposure time. b) To compare between scans each curve was internally normalized based off of the excitation peak at 490 nm.

Results and Discussion

The fluorescence emission of acridine orange was observed over a period of six hours of exposure to a 490 nm LED. Successive curves of a bleaching trial of acridine orange taken after 30 minutes of exposure across the whole of the 6-hour exposure process is shown in Figure 2a. Each curve was normalized based on their intensity at the excitation peak of 490 nm so that the relative heights of the fluorescence emission peak could be compared (Figure 2b). Peak fluorescence emission was observed to be at 525 nm and decayed over time due to photobleaching. Figure 3 shows the 525 nm emission wavelength plotted against exposure time for the LED exposed sample. A dual exponential decay was fitted to the photobleaching decay after determining that a single exponential decay rate did not fit the data. This indicates that the photobleaching process for acridine orange is biphasic. The rate

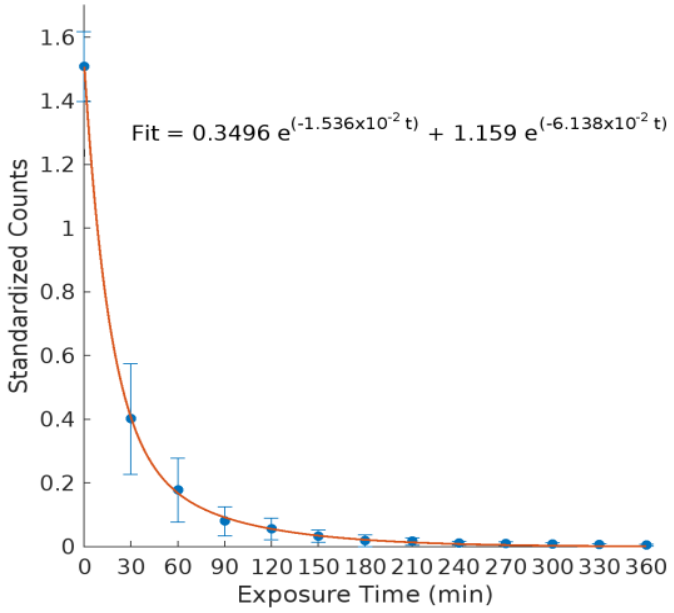


Figure 3. Acridine orange photobleaching decay curves over 6 hour exposure time period. Curves were fit with a biphasic exponential decay curve. Samples were measured in triplicate with error-bars to represent the spread in measurements. Data was standardized based on counts per second at the excitation peak at 490 nm.

constants for the dual exponential are 1.5×10^{-2} min^{-1} for the fast rate and 6.1×10^{-2} min^{-1} for the slow rate.

To show that this decay was brought about by the direct exposure to the sample from the LED a second sample was prepared at the same time and was monitored immediately following the photobleaching sample. The photobleaching decay curve at 525 nm for the dark control sample is shown in Figure 4. Both the single exponential decay and a double exponential decay was fit to the dark control. The double exponential decay fitting model returned only a poorly fit single exponential. The exponential decay rate for the dark control was 2.1×10^{-4} min^{-1}. Due to the spread in error there is very little confidence in the value of this decay rate, but because it is two orders of magnitude slower than either of the photobleaching rates of the exposed sample there is high confidence that the LED setup does induce a measurable photobleaching effect. Any bleaching that is occurring is likely the result of the repeated exposure of the fluorescence scanning.

These results show that we were able to observe photobleaching induced by LED exposure with our setup. As this technique is easily adaptable by changing the LED source to the wavelength of excitation of the target fluorophore, it can be modified for future studies on alternative fluorophores.

Supplementary Information

Supplementary information, including all 3D printer files and designs, is available upon request from the corresponding author <david.cooper@nevadastate.edu>

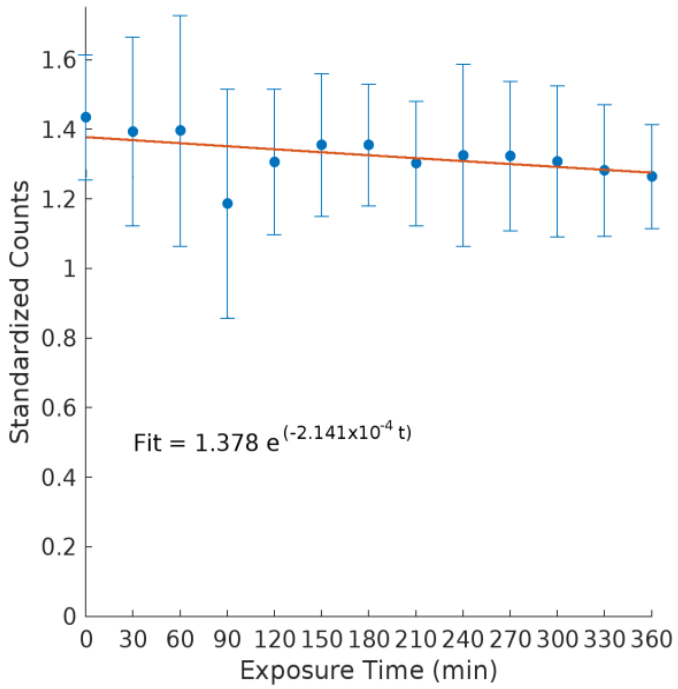


Figure 4. Acridine orange dark control. Each data point was measured after the counterpart sample was exposed for 30 min to the LED. Data was standardized based on counts per second at the excitation peak at 490 nm. The data was fit with the same model as the photobleached samples.

Acknowledgements

The authors would like to thank Nevada State University for space and funding of this project. This material is based upon work supported in part by the National Science Foundation under Grant No. OIA- 2148788 as well as NASA grant #80NSSC20M0043 to the Nevada Space Grant Consortium. This publication was made possible by a grant from the National Institute of General Medical Sciences (GM103440) from the National Institutes of Health. This publication's contents are solely the responsibility of the authors and do not necessarily represent the official views of NIH.

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