High power light emitting diode based setup for photobleaching fluorescent impurities

Tobias K. Herman,[a] Stephan A. Mackowiak, and Laura J. Kaufman[b]
Department of Chemistry, Columbia University, New York, New York 10027, USA

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Single molecule fluorescence experiments, with their associated low signals, require very low background fluorescence in the sample. Even high purity liquids will often possess large numbers of fluorescent impurities that are difficult to completely remove through standard purification techniques such as distillation and recrystallization. We have constructed a simple setup in which such impurities can be photobleached before final sample preparation. The instrument consists of high power light emitting diodes, and it delivers almost 10 W of light to the sample without the heating associated with more conventional light sources or the cost and safety concerns associated with a high power laser. © *2009 American Institute of Physics.* [DOI: 10.1063/1.3073736]

From the first appearance of single molecule spectroscopy less than 20 years ago, improvements in optical techniques and detector sensitivity have transformed the technique from a specialized procedure usable at cryogenic temperatures to one that can be used to acquire high speed video images at room temperature.[1] In fact, much of the single molecule microscopy work being performed today is in molecular biology.[2]

The fluorescent probes used in single molecule applications have unusually high absorption cross sections and fluorescence quantum yields. However, even the most stable single molecule fluorophore has a limited number of excitation-emission cycles before "photobleaching," irrevocably transforming into a nonfluorescent form through a variety of chemical pathways.[3] For fluorophores such as rhodamine, the total number of emitted photons can approach 10^6 and for some large aromatic fluorophores that number may exceed 10^7. However, with typical collection efficiencies of about 5%, a very limited number of signal photons reach the detector over the duration of the experiment. With such low signal levels, minimizing background photons becomes paramount.

While background minimization is important in all single molecule experiments, it is even more crucial in some. Much single molecule microscopy is currently performed using total internal reflection fluorescence (TIRF) microscopy, a technique in which an evanescent wave that penetrates ~150 nm into the sample is used to excite fluorescence from probe molecules.[1] The limited sample volume excited in this configuration results in a relatively high signal to background ratio. While TIRF microscopy is ideal for many *in vitro* biological studies, it cannot be used in all single molecule applications. For example, to interrogate the potentially heterogeneous dynamics in a supercooled liquid near its glass transition temperature, the sample is often maintained in a cryostat. The additional glass through which the excitation light must travel makes the implementation of TIRF difficult and encourages the use of standard epifluorescence microscopy. Here, with significantly greater amounts of sample illuminated and with greater excitation intensities required, reduction of fluorescent impurities in the sample becomes even more critical.

The fluorescent impurities can manifest in microscopy images and interfere with data interpretation in two ways. A strongly fluorescent impurity may be bright enough to be mistaken for a fluorescent probe molecule or many weakly fluorescent impurities may appear as a low level background "haze." Since the concentration of fluorescent probe molecules used in many single molecule experiments is on the order of $10^{-9}M$, even exceedingly small quantities of impurities can obscure the faint fluorescence signal. Traditionally, to remove impurities from a substance, one distills or recrystallizes the substance iteratively until the level of impurity is below the minimal acceptable level. However, we found that when working with a variety of organic liquid samples, distillation was not sufficient to remove the fluorescent background. Recrystallization, when possible, was somewhat more effective at removing the background haze but still left many bright impurities that appeared similar to our fluorescent probes.

As an alternative or supplement to separating impurities from the sample, we explored the possibility of photobleaching them before adding the fluorescent probes to the sample, an idea that had been considered previously.[4] To avoid the high cost of operating a laser for simple photobleaching purposes, we designed a simple but efficient lighting system using light emitting diodes (LEDs). By using the newest generation of high power LEDs, it was possible to generate high optical power in a relatively small volume without generating excess heat. The entire system is energy efficient and compact, fitting inside a discarded computer case that can be tucked underneath a laboratory bench. Total optical power rivals that of standard laboratory pump lasers, although here the light is diffuse, spread out over an area of a few square

[a]Present address: Department of Physics, Engineering and Geosciences, Montgomery College, Rockville, MD 20850.
[b]Author to whom correspondence should be addressed. Electronic mail: kaufman@chem.columbia.edu.

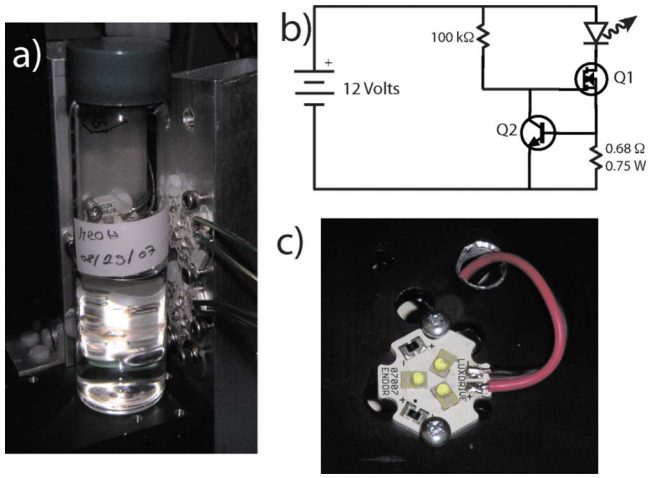

FIG. 1. (Color online) LED setup. (a) A vial in place for bleaching. Four sets of LEDs are shown, and the eventual setup used nine sets. (b) Circuit schematic: Q1 is an *N*-channel MOSFET (STF20NF06) and Q2 is an NPN general purpose amplifier (2N5088). (c) Closeup of a single set of LEDs.

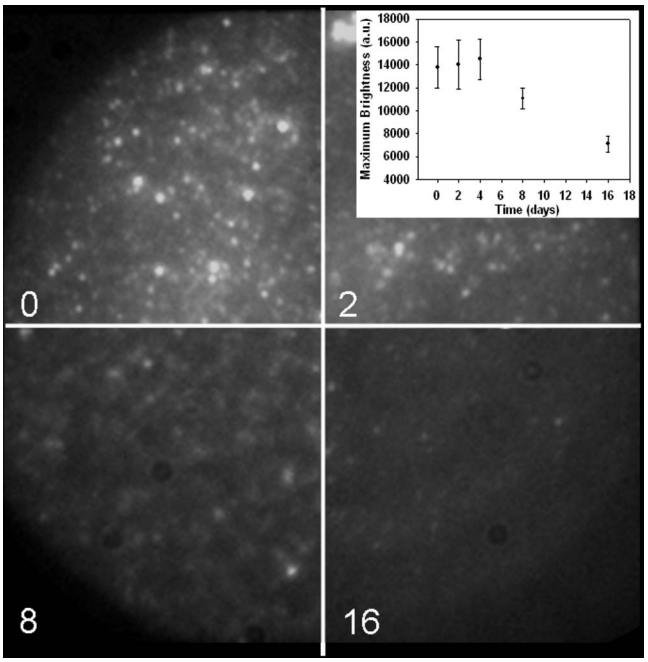

FIG. 2. One quadrant of images obtained from individual thin film samples prepared from the same propylene carbonate solution. 100 frames of 0.2 s exposure are summed in each case. Images are presented on the same scale. Number of days after the beginning of the bleaching experiment is shown in the lower left hand corner. Inset shows maximum pixel intensity averaged over three 100 frame movies vs time. The error bars represent one standard deviation.

centimeters rather than a collimated beam of several square millimeters.

The core of the system uses high current, high efficiency LEDs. In our case, we used Phillips LumiLEDs Luxeon Rebel LEDs with emission wavelengths in the blue region (center wavelength 455 nm) since we typically use fluorescent probes excited in this region. Longer wavelength LEDs could be substituted by groups working with dyes exciting in the red region of the spectrum. The LEDs have a rated radiometric output of 275 mW per LED for a driving current of 350 mA. Since the LED driving current can be raised as high as 1 A, the radiometric output can reach 700 mW per LED.

LEDs were mounted on metal core printed circuit boards (Endor 07007 Star, LEDdynamics, Inc.) by soldering on a hotplate, with three LEDs per 1 in. hexagonal board [Fig. 1(c)]. Each set of three LEDs was driven, in series, from a 12 V source using the simple constant current driver shown in Fig. 1(b). The *N*-channel metal-oxide-semiconductor field effect transistor (MOSFET) and *NPN* transistor act as a negative feedback loop, controlled by the voltage drop across the 0.68 Ω current sense resistor.

Eight LED boards were mounted on four pieces of aluminum square tube stock. These were then assembled with an aluminum base holding a ninth LED board to form a square tubular cavity about 1 in. across that accommodates 8 and 25 ml glass sample vials. Figure 1(a) shows this setup with some of the LEDs removed for clarity. A few watts of heat is generated at the LED, requiring heat sinking and forced air cooling of the apparatus. Several small heat sinks and two small fans ensured that the LEDs' operating temperatures remained within tolerance, while at the same time minimizing unnecessary heating of the sample. The sample temperature remained under 40 °C, allowing us to use the setup with fairly volatile liquids. The rated long lifetime of the LEDs (brightness >70% after 50 000 h) ensures that they never need to be replaced.

The 12 V power source was a repurposed computer power supply. In order to maintain good voltage regulation of this switching power supply, a 10 W load (2.5 Ω resistor) was placed across the 5 V output. In total, there were nine sets of LEDs operating at 700 mA, placing a mild 70 W load on the power supply. The use of a recycled computer power supply suggests the use of a recycled computer case to contain the apparatus. Indeed, the entire setup fit within the case and was made relatively light proof with the addition of a few baffles. The setup has been running for more than a year in our microscope room without any problems, and it has become a necessary step in the preparation of all our samples.

To characterize the amount of optical power reaching our liquid samples, we made a simple calorimetric measurement. By placing a vial of absorbing (black) solution in the setup and measuring its warming, we calculated optical power levels of 8.2 and 4.5 W reaching the samples in our 25 and 8 ml vials, respectively. The illumination area is measured directly, yielding average intensities of ~0.3 W/cm^2.

Samples of propylene carbonate (Sigma-Aldrich, HPLC grade, 99.9%) were prepared before bleaching and following 2, 4, 8, and 16 days of bleaching. Aside from bleaching time, sample preparation was identical. Samples were spun coat at 4000 rpm for 20 s onto a clean silicon wafer. They were then placed in a cryostat (Janus, ST-500-LN), and the temperature was lowered to 160 K. Images were acquired in epifluorescence mode on a homebuilt microscope fit with an electron multiplying charge coupled device camera (Andor iXon DV887). Excitation and detection were identical for all samples. Specifically, 7.5 mW of 532 nm light (Coherent Verdi) was used for excitation, and dichroic and bandpass filters (Semrock and Chroma) were used to separate excitation light from fluorescence. Camera exposure time was 0.2 s and 100 frames were collected consecutively and analyzed as described below.

Figure 2 shows quadrants of unscaled images of particular samples following 0, 2, 8, and 16 days of bleaching. Reductions in both bright, resolvable single fluorophore impurities and the diffuse hazy background fluorescence are evident over time. Averaged over three excitation spots on a single film prepared from these samples, from day 2 to day 16 the mean pixel intensity of the images decreases linearly at a rate of 2% per day. Similarly, we find that the maximum brightness decreases substantially with time, as shown in the inset of Fig. 2. This quantity is closely related to the number of fluorescent impurities that may be confused with fluorescent probes in a single molecule experiment. Indeed, chosen manually, the five brightest individual 5×5 pixel spots in the day 0 images have an average intensity 1.5 times that in the day 16 images. In addition to the results presented above, we note that the same trends were observed for other liquid samples including glycerol and toluene as well as for excitation of the propylene carbonate at 488 rather than 532 nm.

In sum, we present a small, inexpensive unit suitable for bleaching fluorescent impurities in solvents. While some fluorophores may persist under the low intensity conditions described here, the fact that mean pixel intensity was found to decrease linearly with time to at least 16 days suggests most impurities present in such samples can be bleached under these conditions.

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