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The effect of photobleaching and velocity fluctuations on single-point LIF measurements

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Abstract This study investigates the effect of photobleaching on laser-induced fluorescence (LIF) measurements. Photobleaching causes the fluorescence to be velocity-dependent, which is undesirable if quantitative measurements are being made. To quantify this effect, simultaneous and coincident measurements of fluorescence and velocity were made within the measuring volume of a three-beam laser-Doppler anemometer (LDA), using both fluorescein and rhodamine 6G dyes in water. In addition, analytical expressions were developed for photobleaching in the LDA measuring volume, and a parameter was identified which predicts the degree of velocity sensitivity. Fluorescein was found to be far more susceptible to photobleaching than rhodamine 6G. Finally, the impact of photobleaching on statistical quantities (such as scalar fluxes) obtained from simultaneous LDA/LIF measurements is discussed.

1 Introduction

Laser-induced fluorescence (LIF) is a common technique for measuring the concentration of a scalar quantity in laboratory flows. In the LIF technique, the flow is selectively dosed with a fluorescent tracer, and then illuminated by a laser light source. For low dose strengths the resulting fluorescence at a point is proportional to the local dye concentration, and a light-sensitive device such as a photomultiplier or photodiode can be used to quantify the strength of the fluorescence. Depending on the optical configuration, the LIF technique can be used to measure concentrations in a plane (using a laser sheet; see Barrett 1989; Karasso 1994), along a line (using a laser beam; see Koochesfahani 1984), or at a single point (often at the intersection of two or more beams; see Durst and Schmitt 1984). The technique has the advantage of being non-intrusive, and it has an extremely fast response time. See Walker (1987) for a more complete description of the technique.

Previous studies have identified a variety of effects that can influence the magnitude of the laser-induced fluorescence. In general, the fluorescence of a fluid parcel moving through a laser-illuminated measuring volume depends on the concentration of the dye and the intensity of the illuminating laser (Guilbault 1973), as well as the temperature and pH of the fluid (Walker 1987). The intensity of the illumination source can be attenuated by the presence of dye in the fluid along the illumination path, resulting in reduced fluorescence at the measuring volume (Guilbault 1973). Koochesfahani (1984) also notes that the fluorescence can be attenuated by a separate process called photobleaching, where some dye molecules are rendered incapable of fluorescing after being subjected to laser illumination. Sugarman and Prud'homme (1987) applied a photobleaching analysis to Poiseuille flow through an on-column laser fluorescence detector. Saylor (1995) measured the fluorescence decay of fluorescein samples. Rička (1987) even took advantage of fluorescein's susceptibility to photobleaching to develop a full-field velocimetry technique.

The impact of photobleaching on LIF measurements has not been well documented. The physical and chemical processes responsible for photobleaching are not well understood and remain outside the scope of this paper. However, many studies (e.g. Imamura and Koizumi 1955) have provided empirical evidence that the photobleaching process bleaches organic dyes in accordance with a first-order rate equation.[1] The current study uses this empirical foundation to investigate the quantitative effect of photobleaching on LIF measurements with fluorescein and rhodamine 6G in water. In particular, this study details how photobleaching causes LIF measurements to be sensitive to the velocity of the flow being measured. As is discussed below in Sect. 4, knowledge of this sensitivity is especially important when correlations between velocity and concentration fluctuations (i.e. scalar fluxes) are being measured.

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[1] Some researchers (see, for example, Saylor 1995) have suggested that illumination may only *temporarily* render some of the dye incapable of re-fluorescing. The aspect of reversibility in photobleaching is not well understood and is beyond the scope of this paper. It is assumed (and the empirical evidence suggests) that the process still obeys a first-order rate equation even if some of the photobleaching is indeed reversible.

The goals of this study are to:

- Develop an analytical relationship that describes the photobleaching process for a combined LDA/LIF probe
- Perform experiments to measure photobleaching with both fluorescein and rhodamine 6G dyes in water, comparing the experimental results with the analysis
- Determine the net effect of photobleaching on combined LDA/LIF measurements, particularly on fluxes

2 Photobleaching theory

2.1 Basic equations

Continuous excitation of organic dyes leads to a reduction in the ability of the dye to absorb and re-emit (fluoresce) light. This reduction results when some dye molecules are either temporarily or permanently bleached by the illumination source. If the concentration of a given dye is defined as the number of *non-bleached* dye molecules per unit volume, then photobleaching results in a net reduction in concentration with time. Imamura and Koizumi (1955) performed experiments to show that the photobleaching process could be represented by a simple first-order rate process. This assumption allows the concentration of unbleached dye molecules C remaining after continuous illumination from time t_0 to time t to be expressed as (Koochesfahani 1984)

$$C(t) = C_0 \exp\left[-Q_b \sigma \Phi (t - t_0)\right] \tag{1}$$

where C_0 is the concentration of the fluid parcel at time t_0, Q_b is the quantum bleaching efficiency, σ is the absorption cross section, and Φ is the photon flux of the excitation source through the fluid parcel. Q_b is defined as the number of dye molecules that are bleached per absorbed photon, and can vary with the type of dye solvent, the dye concentration, the excitation wavelength, and the photon flux (Karasso 1994). The photon flux is given by $\Phi = I/h\nu$, where I and ν are the intensity and the frequency of the monochromatic excitation source, respectively, and h is Planck's constant. The absorption cross section is defined as $\sigma = \varepsilon / N_{AV}$, where N_{AV} is Avogadro's number, and ε is the extinction coefficient based on the natural logarithm as defined by Walker (1987). If a fluid parcel is exposed to a time-varying excitation intensity, then the concentration of unbleached dye molecules remaining at time t is given by

$$C(t) = C_0 \exp\left[\frac{-Q_b \sigma}{h\nu} \int_{t_0}^{t} I(\tilde{t})\, \mathrm{d}\tilde{t}\right] \tag{2}$$

where $\tilde{t}$ is a variable of integration. Alternatively, if an infinitesimal fluid parcel moves in the x-direction from x_0 to x with velocity U through a spatially varying excitation field, the concentration at position x is

$$C(x) = C_0 \exp\left[\frac{-Q_b \sigma}{h\nu U} \int_{x_0}^{x} I(\tilde{x})\, \mathrm{d}\tilde{x}\right] \tag{3}$$

For most optical configurations, the laser used for illuminating the measuring volume must first pass through background fluid which itself has dye in it. This causes the illumination source to be attenuated along its path due to absorption. This results in a non-linear fluorescence vs. concentration relationship (see Walker 1987). However, for sufficiently low dye concentrations, the laser attenuation due to absorption can be neglected, allowing the fluorescence at a point to be described linearly as

$$F = \alpha I C \tag{4}$$

where α is a constant of proportionality. Equation (4) forms the basis for the theory developed in the following section.

2.2 Application of photobleaching theory to an LDA measuring volume

In this section, the basic photobleaching theory developed above is applied to the specific optical geometry of the experiments performed in this study. The details of the experimental setup are described below in Sect. 3.1. The measuring volume for the combined LDA/LIF apparatus is formed by the oblique intersection of three laser beams, each having a two-dimensional Gaussian cross section. The measuring volume that results at the intersection of the beams is shown in Fig. 1. The laser intensity distribution of the measuring volume is Gaussian along all three axes, and the surface on which the laser intensity is reduced to e^{-2} of the maximum value is an ellipsoid with major and minor axes of dimension $2a \times 2b \times 2a$. In this study, the measuring volume is placed in a flow whose mean component is aligned with the x-axis.

Infinitesimal fluid parcels passing through various parts of the finite measuring volume each fluoresce with an intensity proportional to the parcel's instantaneous dye concentration and the local excitation intensity, as described by Eq. (4). This fluoresced light is imaged onto a photomultiplier tube (PMT). At each instant in time, the PMT receives fluoresced light from all of the fluid parcels that currently reside in the measuring volume. If we define normalized spatial variables $x^* = x/a$, $y^* = y/a$, and $z^* = z/b$, then the total normalized fluorescence received by the PMT can be defined as

$$F^* = \iiint I^*(x^*, y^*, z^*)\, C^*(x^*, y^*, z^*)\, \mathrm{d}x^*\, \mathrm{d}y^*\, \mathrm{d}z^* \tag{5}$$

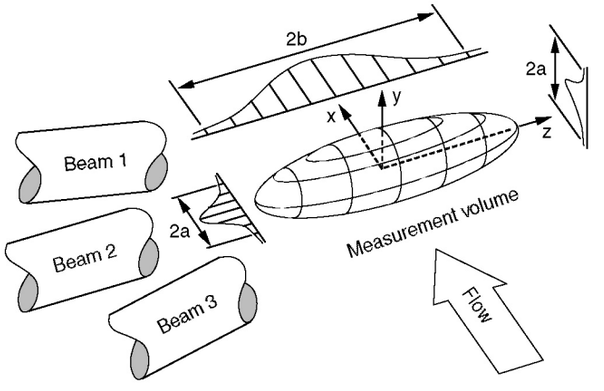

Fig. 1. Measuring volume geometry

where C^* and I^* are the normalized concentration and excitation intensity distributions, respectively. C^* and I^* are normalized (see Eq. (8) and (10)) such that F^* is unity when there is no photobleaching, and zero when the photobleaching completely obliterates the original dye. By definition, the total power P in the three laser beams which fluxes across the x–y plane is

$$P = \iint I(x^*, y^*, z^* = 0)\, \mathrm{d}x\, \mathrm{d}y \tag{6}$$

Assuming Gaussian beam cross sections, the excitation intensity can therefore be expressed as

$$I(x^*, y^*, z^*) = \frac{2P}{\pi a^2} \exp\left[-2(x^{*2} + y^{*2} + z^{*2})\right] \tag{7}$$

If the excitation intensity distribution is normalized such that

$$\iiint I^*(x^*, y^*, z^*)\, \mathrm{d}x^*\, \mathrm{d}y^*\, \mathrm{d}z^* = 1 \tag{8}$$

the resulting expression for I^* is

$$I^*(x^*, y^*, z^*) = \frac{2\sqrt{2}}{\pi^{3/2}} \exp\left[-2(x^{*2} + y^{*2} + z^{*2})\right] \tag{9}$$

If the concentration distribution is normalized such that

$$\lim_{U \to \infty} C^* = 1 \tag{10}$$

the resulting expression for C^* is

$$C^* = \exp\left\{-B\left[1 + \mathrm{erf}(\sqrt{2}x^*)\right] \exp\left[-2(y^{*2} + z^{*2})\right]\right\} \tag{11}$$

where B is the photobleaching parameter, defined as

$$B = \frac{PQ_b\sigma}{\sqrt{2\pi}\, ahvU} \tag{12}$$

Substitution of Eqs. (9) and (11) into Eq. (5) results in an equation that can be numerically integrated to obtain the normalized fluorescence as a function of the photobleaching parameter, B. It can be easily shown that

$$\lim_{U \to 0} F^* = 0 \quad \text{and} \quad \lim_{U \to \infty} F^* = 1 \tag{13}$$

as expected.

The results of the numerical integration of Eq. (5) are shown as the solid line in Fig. 2 (the normalized experimental data shown in the figure are discussed later in Sect. 3.2). For small values of B (large U), the effect of photobleaching is small. For large values of B (small U), significant amounts of the dye are bleached out of the fluid during its transit across the measuring volume. To ensure that F^* is accurate to within one percent of the unbleached value, it is necessary that $B < 0.020$, or $U > 20\, PQ_b\sigma/ahv$.

2.3 Velocity sensitivity

It is of interest to determine how the magnitude of B affects the sensitivity of the fluorescence to velocity fluctuations. To simplify the analysis, consider the fluorescence at a single point

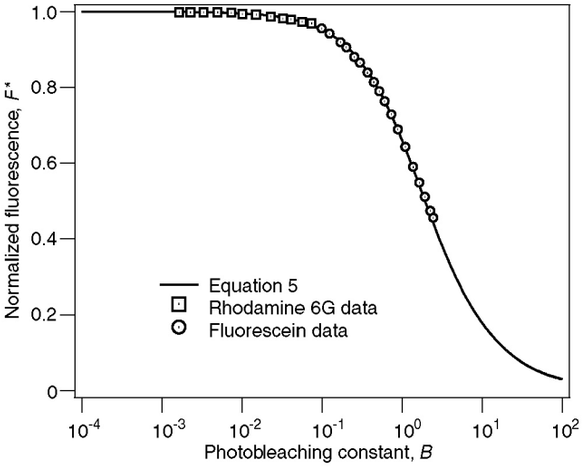

Fig. 2. Normalized fluorescence vs. the photobleaching parameter, B

in the measurement volume (say, the origin), rather than the spatially-integrated fluorescence. At the origin, we have

$$F^*(0,0,0) = I^*(0,0,0)\, C^*(0,0,0) = \frac{2\sqrt{2}}{\pi^{3/2}} \exp(-B) \tag{14}$$

Then, the change in the fluorescence given a change in the velocity is

$$\frac{\partial F^*(0,0,0)}{\partial U} = \frac{2\sqrt{2}}{\pi^{3/2} U} B \exp(-B) \tag{15}$$

This function has an absolute maximum when $B = 1$ (the point where the magnitude of the slope in Fig. 2 is greatest). Thus, while the magnitude of the fluorescence in the measuring volume decreases monotonically as U decreases (that is, as B increases), the sensitivity of the fluorescence to *changes* in velocity is highest when $B = 1$. As will be discussed in Sect. 5, the sensitivity of the photobleaching phenomenon to velocity changes is often more important to the experimentalist then the actual magnitude of the photobleaching.

3 Experiments with fluorescein and rhodamine 6 G

3.1 Experimental setup and procedure

The experiments were conducted in the Stanford Benthic Boundary Layer Facility (BBLF). The BBLF is a recirculating-flow open-channel flume with glass sidewalls in the test section, and is described in detail by O'Riordan et. al (1993). Dye concentrations and flow velocities were measured in the potential core of a dyed, coflowing jet as shown in Fig. 3. Measurements were made for 18 discrete jet velocities ranging from 1 to 45 cm/s, and these measurements were performed twice using two different dyes. The jet was positioned in the freestream of the flume flow, away from the bottom and sidewall boundary layers. The flume was operated at a constant

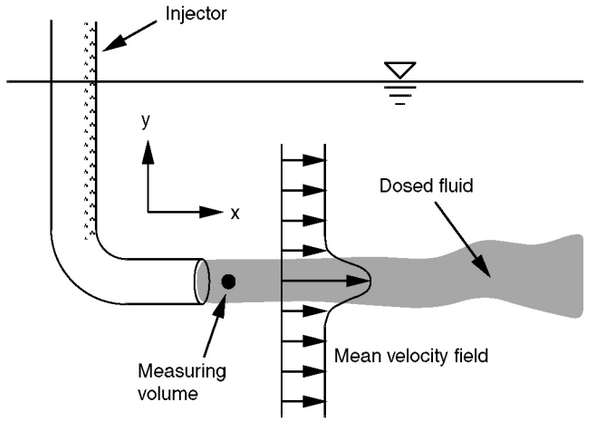

Fig. 3. Dye jet configuration

speed of 4 cm/s in order to advect the dyed jet plume downstream from the measurement volume. The jet was dosed with 4 ppb of dye for fluorescein, and 2 ppb for rhodamine 6G. The jet diameter was 0.75 cm at the jet origin, and dye accumulation in the 8000 l flume during the experiments was negligible.

The measurements in this study were made using a combined LDA/LIF system that permits simultaneous and coincident measurements of velocity and concentration. The LDA system is based on a Dantec three-beam, two-component LDA. Two of the three beams are electronically shifted (by 40 and 70 MHz), allowing all three of the beams to be operated from a single line of an argon-ion laser. An appropriate laser line was chosen depending on the excitation frequency, λ_{ex}, of the fluorescent dye that was being used. The dimensions of the LDA measuring volume (to the e^{-2} intensity contour) are $2a = 0.07$ mm, and $2b = 0.9$ mm (see Fig. 1).

The measuring volume is imaged by two separate PMTs – one for the LDA, and one for the LIF system. Both PMTs have pinhole masks that reduce stray light. The PMT for the LDA was placed in the forward-scatter location to take advantage of the high signal-to-noise ratio associated with the strong forward-scatter lobes. Since fluorescence is an omnidirectional phenomenon, the PMT for LIF could be placed in the backscatter location (within the LDA optics) without loss of signal. The advantage to operating the LIF system in backscatter is that it is easier to ensure that the PMT optics are consistently imaging the same portion of the Gaussian measuring volume, thus eliminating the need to recalibrate the LIF system every time the LDA is moved. The two PMTs were each optically shielded by narrow bandpass filters (20 nm bandwidth) that selectively passed the appropriate LDA or LIF signal. The LDA signal has the same wavelength as the laser wavelength, λ_{laser}, and the LIF signal is centered at λ_{em} (the fluorescence emission peak of the particular dye used). Table 1 summarizes the peak excitation and emission wavelengths for the two dyes used in this study (data from Penzkofer and Leupacher 1987; Smart and Laidlaw 1977), along with the laser wavelengths, λ_{laser}, and incident laser power, P, used for each of the two dye experiments.

Table 1. Parameters for fluorescein and rhodamine 6G

Dye	λ_{ex} (nm)	λ_{em} (nm)	λ_{laser} (nm)	P (W)	$Q_b\sigma$ (cm^2)
Fluorescein	490	520	488	0.56	2.8E-20
Rhodamine 6G	528	555	514.5	0.58	4.5E-22

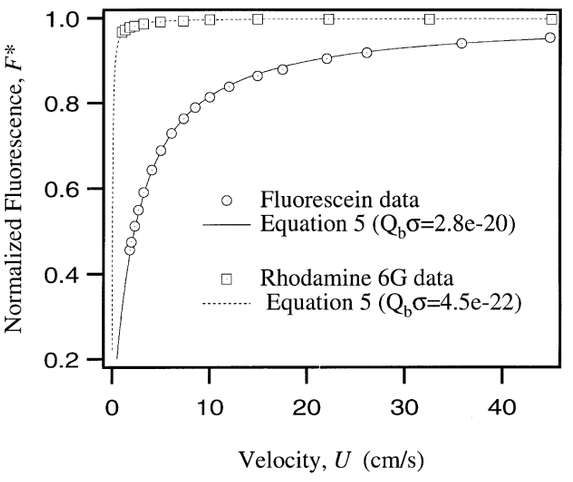

Fig. 4. Normalized fluorescence vs. velocity, U

3.2 Experimental results

As the flow velocity through the measuring volume increases towards infinity (and the transit time thus decreases towards zero), the effect of the photobleaching is eliminated (see Eq. (13)). Therefore, the fluorescence data were normalized so that they asymptote to unity as U gets large. To determine the asymptote of the data, a least-squares fit of the equation $C_1 + C_2 \exp(-C_3/U)$ was performed on the data values for which $U > 10$ cm/s. The asymptotic value $C_1 + C_2$ predicted from this fit was then used to normalize the data.

The normalized fluorescence data, F^*, are plotted in Fig. 4 as a function of the dimensional velocity, U. There is one set of data values for each of the two dyes tested. From an experimental standpoint, the data in Fig. 4 can be discussed in terms of the dye choice and the velocity dependence.

Dye choice The effect of photobleaching at a given flow velocity is much more pronounced for the experiments with fluorescein dye than for the experiments with rhodamine 6G. The deviation of F^* from unity $(1 - F^*)$ is as much as 62 times greater for fluorescein than for rhodamine 6G at a given velocity.

Velocity dependence The effect of photobleaching is clearly seen to be most prominent at lower velocities. Normalized fluorescence values as low as 0.46 were measured when using

fluorescein at low velocities (1.8 cm/s). At higher velocities, F^* asymptotes towards unity. Normalized fluorescence values of 0.998 were measured when using rhodamine 6G at high velocities (45 cm/s).

Also shown in Fig. 4 are theoretical curves generated by numerical integration of Eq. (5), where the product $Q_b\sigma$ was varied to give the best fit to the data for each of the two dyes (the resulting $Q_b\sigma$ values are summarized in Table 1). Note that the product $Q_b\sigma$ (which appears in the photobleaching parameter, B) is a parameter which completely characterizes the photobleaching characteristics of a given dye. The $Q_b\sigma$ values obtained by fitting the data in Fig. 4 were then used to plot the normalized fluorescence data versus the photobleaching parameter, B, as shown in Fig. 2. Note that the data collapses onto the single curve generated by numerical integration of Eq. (5). The photobleaching parameter, B, is a single parameter which incorporates the effect of velocity (U) and dye choice ($Q_b\sigma$). The experimental data deviates from the theoretical curve by less than one percent over the range of velocities tested.

4 Effect of photobleaching on flow statistics

Photobleaching results in an explicit correlation between the measured fluorescence and the velocity of the flow. Although the measured velocity is unbiased, the measured concentration (and statistical quantities that contain concentration) can be biased by photobleaching. The combined LDA/LIF system provides simultaneous records of velocity, $U(t)$, and fluorescence, $F(t)$. Equation (4) transforms $F(t)$ into $C(t)$ (where $\propto I$ is usually determined with an in-place calibration at some velocity U_{cal}). Any biases in $F(t)$ from photobleaching are then contained in $C(t)$ as well.

To analyze these effects we first decompose the velocity and concentration records into mean and fluctuating quantities:

$$U(t) = \bar{U} + u(t), \qquad C(t) = \bar{C} + c(t) \tag{16}$$

Here, $\bar{U}$ and $\bar{C}$ are the time-averaged velocity and concentration, respectively, and $u(t)$ and $c(t)$ are their fluctuating components. Correlations such as $\overline{uu}$, $\overline{cc}$, and $\overline{uc}$ can be calculated by time-averaging the products of the fluctuation records. All of the concentration quantities ($\bar{C}$, $c(t)$, $\overline{cc}$, and $\overline{uc}$) can potentially be biased by the presence of mean velocities $\bar{U}$ and fluctuating velocities $u(t)$ because of photobleaching. The effect on each of these quantities is addressed individually below, where, for simplicity, it is assumed that all velocity values are always positive.

- $\bar{C}$: The mean concentration value obtained from the fluorescence data increases from the calibrated value when $\bar{U} > U_{cal}$, and it decreases when $\bar{U} < U_{cal}$. However, $\bar{C}$ is *not* affected by velocity perturbations $u(t)$ about $\bar{U}$ so long as these perturbations are small enough that the resulting fluorescence (and concentration) perturbations are relatively linear. Note, again, that this also assumes that none of the perturbations are large enough to cause $U(t)$ to go negative.
- $c(t)$: The concentration fluctuation values will be artificially low during periods where $U(t) > U_{cal}$ and artificially high when $U(t) < U_{cal}$. Note that $c(t)$ is sensitive to both $\bar{U}$ and $u(t)$.
- $\overline{cc}$: The concentration variance is weakly affected by $\bar{U}$, in the sense that concentration fluctuations are scaled up or down as $\bar{C}$ is increased or reduced by photobleaching. More significantly, the true variance is increased directly by $u(t)$ due to the resulting artificial fluorescence fluctuations that influence the concentration measurements.
- $\overline{uc}$: Since photobleaching causes the concentration measurement and the velocity to be positively correlated (for positive velocities), the calculated scalar flux $\overline{uc}$ will be artificially high.

5 Recommendations and conclusions

The analytical and experimental work presented above make it clear that caution must be exercised when designing an LIF experiment. In particular, the choice of flow velocities, laser power, and fluorescence dye must be made in light of the resulting range of photobleaching parameter values, B. If the only information required from the LIF probe is mean concentration values, then the problem is likely moot, as discussed in Sect. 4. In this case, it is usually possible to obtain very accurate results simply by calibrating the LIF probe in place at the mean velocity values where the experiments will be performed. However, if quantities like $C(t)$, $\overline{cc}$, or $\overline{uc}$ are of interest, photobleaching can lead to unacceptable errors.

This study identified the photobleaching parameter, B, from an analysis performed for photobleaching within an LDA measuring volume. This parameter places any given LDA/LIF experiment on the analytical curve (Eq. (5)) presented in Fig. 2. The location of an experiment along this curve tells the experimentalist two things. First, the magnitude of F^* at B determines the amount of photobleaching that will occur due to the mean velocity. The amount of photobleaching increases monotonically as B increases. Secondly, the slope of the curve at the B-location determines the sensitivity of the photobleaching to fluctuations in the flow velocity about the mean. This sensitivity has a maximum at $B = 1$, and decreases monotonically on both sides of unity.

Rhodamine 6G proved to be far superior to fluorescein with regard to photobleaching. In this study, the product $Q_b\sigma$ was found to be 62 times lower for rhodamine 6G than for fluorescein. The actual values of $Q_b\sigma$ found in this study are summarized in Table 1. It should be noted that the actual $Q_b\sigma$ value for a particular dye might depend on a variety of factors that are beyond the scope of this study. It should also be noted that there are very few published values for Q_b and σ for common fluorescent dyes, and almost none of the published values are for dyes in water (Ippen et al. 1971 do, however, report values for fluorescein and rhodamine 6G in ethanol). Future research in this area would be extremely useful.

The analysis and experiments presented in this study suggest several guidelines for the design of LIF experiments:

– Ideally, the experiment should be designed so that B is as far below unity as possible (ideally, B should be well below 0.1). This will minimize both the magnitude of the photobleaching due to the mean flow velocity as well as the sensitivity of the photobleaching to velocity fluctuations. The value of B can be lowered by choosing a dye with a lower $Q_b\sigma$ value, by increasing the flow velocity, or by decreasing the laser

power (which, unfortunately, decreases the signal-to-noise level of the PMT output).

– Calibrate the LIF probe at the same mean flow velocity at which the experiment will be run. This completely eliminates the problem of photobleaching due to the mean flow velocity. The analysis presented in this study allows the calibration performed at a single flow velocity to be extended to correct for a wide range of flow velocities. However, velocity fluctuations about the mean can still corrupt many of the concentration statistics.
– If concentration statistics are the most important desired results, then the most important feature of the experimental design is to move the value of B as far away from unity as possible (since the sensitivity of photobleaching to velocity fluctuations is maximized at $B=1$). This means that if it is impossible to decrease B well below unity (and ideally well below 0.1), it is actually advantageous to *increase* B well beyond unity. This will decrease the magnitude of the fluorescence (which can be corrected for) and decrease the signal-to-noise level from the PMT, but it will minimize the corruption of the concentration statistics by photobleaching.

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