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Ratiometric Sensing Using Dual-Frequency Lifetime Discrimination

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Fluorescence sensing is a rapidly expanding area and is widely used in biotechnology. Most sensing applications rely on measuring changes in fluorescence emission intensity. However, measuring the absolute intensity of fluorescence has proven to be a difficult task—it depends on too many factors, like intensity of the excitation light, concentration of the fluorescent dye, length of the optical path, primary and secondary inner filter effects, and quenching (1). One possible solution is the use of lifetime measurements; however, most available dyes have lifetimes in the nanosecond to picosecond range. Measurements on this time scale require complex and sophisticated equipment (2). Furthermore, not every fluorescent dye that changes in intensity exhibits a significant lifetime change. Another possible approach is to use ratiometric probes; however there are few ratiometric probes, mostly for pH (3–6), calcium (7), and chlorides (8). Only recently was an O_2 ratiometric probe introduced (9, 10). An approximation of this method can be obtained by mixing two fluorophores with two different spectra (excitation or emission). They should respond in opposite directions to the analyte concentration (11) or one of them should serve as an analyte-insensitive reference (12). However, a specific problem is the requirement for a relatively narrow bandwidth of the excitation or the emission. If the demand is not met, the overlap of the spectra decreases the sensitivity of the measurement. A narrow bandwidth results in low light intensities, which imposes the use of highly sensitive photodetectors (photomultipliers or CCD-based detectors).

Another approach is to use modulation sensing (13, 14). The fluorophores used have significantly different lifetimes, so their spectra can overlap. Measurements of the modulation are generic, so they could be used in a variety of applications. However, this method requires exclusion of the ambient light or at least correcting for it. In real-life applications this is not always possible and may significantly complicate the measurement procedures. This restricts its application.

In this paper we are presenting a new approach for ratiometric sensing that is based on use of fluorophores with significantly different lifetimes. The method does not require narrow-bandpass devices (monochromators or filters); instead, it relies on simple long-pass filtering. Furthermore, the method is intrinsically insensitive to ambient light.

Theory. Let a fluorescent sample emit two emissions with steady-state intensities A_1 and A_2 and lifetimes τ_1 and τ_2 and $\tau_1 = 1000\ \tau_2$, respectively. The ratio A_1/A_2 is to be determined.

If the sample is excited by modulated light, the AC amplitude A of the emission will be dependent on the excitation frequency, the lifetimes, and the steady-state intensities by the equation

$$A = \sum_{i=1}^{2} A_i\,(1 + \omega^2\tau_i^2)^{-1/2}. \qquad [1]$$

Suppose that $\omega_1 = 0.1/\tau_1$. Then

$$A_{\omega_1} = 0.995 \cdot A_1 + A_2 \approx A_1 + A_2. \qquad [2]$$

Thus, at relatively low frequencies the amplitude of the total emission equals the sum of both partial emissions. Now, suppose that $\omega_2 = 100/\tau_1$. Then

$$A_{\omega_2} = 0.0099 \cdot A_1 + 0.995 \cdot A_2 \approx A_2. \qquad [3]$$

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In other words, there are frequencies at which the long-lifetime emission is almost completely demodulated, while the short-lifetime emission still retains significant modulation. When the sample is excited at these frequencies the AC amplitude of the emission is almost exclusively from the short-lived fluorophore.

Consequently, the intensity ratio of the two emissions (regardless of the degree of overlap between the spectra) can be determined by the equation

$$\frac{A_1}{A_2} = \frac{A_{\omega_1}}{A_{\omega_2}} - 1. \qquad [4]$$

Materials and methods. Three different fluorophores were used: fluorescein (Sigma, St. Louis, MO), Oregon green–BAPTA (Molecular Probes, Eugene, OR), and partially purified green fluorescent protein (GFP),[2] produced from pBAD–GFP-transformed *Escherichia coli* strain JM105 (15). The pH determinations were performed using phosphate buffers. The concentrations of the fluorophores were as follows: fluorescein—0.8 μM, calcium-sensitive dye—0.635 μM, and GFP—0.8 μM. Calcium determinations were performed using calcium calibration kit No. 2 (Molecular Probes).

The reference consisted of 1% (w/w) ruthenium tris-(bipyridyl) dichloride physically entrapped in cellulose acetate film by dissolving both in acetone and spread casting the mixture. As $Ru(bpy)_3Cl_2$ is water soluble, it was necessary to prevent its leaching. It was protected from contact with the water by a thin layer (0.2 mm) of RTV108 silicone rubber.

In order to select the frequencies for measurements, the lifetime of the reference was determined on ISS Koala frequency domain lifetime fluorometer (ISS, Champaign-Urbana, IL) using a blue LED as the excitation source. Literature values were used for the lifetimes of the dissolved sensing fluorophores (1). Measurements of the modulation of the combined sensing dye and the reference were also performed on ISS Koala.

Right-angle format was used for the dual-frequency measurements. In-house low-cost excitation and detection systems were used. The reference film was placed inside the test cuvette. The excitation source was a blue LED LNG992CFBW (Panasonic, Secaucus, NJ), directly driven from the TTL output of a 200-MHz lock-in amplifier SR844 (Stanford Research Systems, Sunnyvale, CA); it was filtered using a 460 ± 30-nm interference filter (Intor, Soccorro, NM). Fluorescence emission was measured through long-pass filters 500LH or 550LH (Andover, Salem, NH) using a C5460 APD module (Hamamatsu, Bridgewater, NJ) with a cutoff frequency of 10 MHz. Additionally, short-pass filters (600SP or 650SP) were used to adjust the desired ratio. The signal from the APD module was fed into the lock-in amplifier for detection. The amplifier was controlled through the serial port by a computer running in-house developed software using Labview 5.0 (National Instruments, Austin, TX). The algorithm of the operation was as follows: (a) set the frequency to 25 kHz; (b) read the output 10 times and average; (c) set the frequency to 1 MHz; (d) read the output 10 times and average; (e) calculate the ratio of the first and second average. All the measurements were performed without any protection from the ambient light.

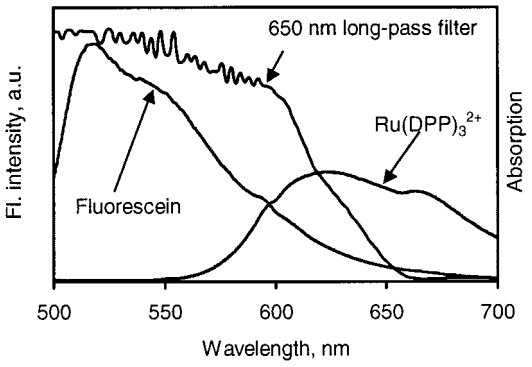

FIG. 1. Emission spectra of the sensing and reference dyes and filter absorption. Emission intensities of the fluorophores not shown to scale.

Results and discussion. Ratiometric approach in fluorescence intensity measurements helps to eliminate the errors caused by variations in excitation intensity, as well as possible changes in the length and absorptivity of the light path.

The emission spectra of the sensing and reference dyes as well as the absorption of one of the long-pass filters are presented on Fig. 1. Only one of the three sensing dyes used (fluorescein) is presented, as their spectra are actually quite similar. It is readily seen that using filters with different cutoff wavelengths allows the contribution from the different emissions to be varied and consequently the actual value of the ratio can be adjusted.

The proposed method uses low-cost semiconductor light sources for excitation. LEDs are easily modulated up to hundreds of MHz. They have been used in sensing—oxygen (16) and pH (17), for microscopy illumination (18), and in some areas of bioengineering (19). Their ability to be quickly switched between the modulation frequencies is crucial for the method. However, the actual frequencies to be used depend on the lifetimes of the dyes used. The frequencies selected—25 kHz and 1 MHz—are determined by the lifetime of the $Ru(bpy)_3^{2+}$ reference, which was 927 ns. This choice allows for practically 100% modulation of both emis-

[2] Abbreviation used: GFP, green fluorescent protein.

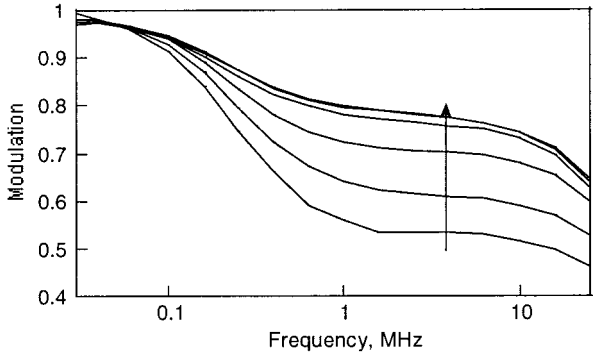

FIG. 2. Frequency-domain modulation measurements of the fluorescein-Ru(DPP)Cl_2 system. Curves are obtained at pH 4.5, 5, 6, 7, and 8.

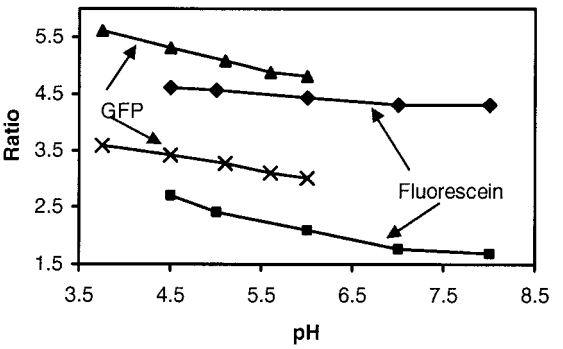

FIG. 3. Calibration curves for pH determination using fluorescein, GFP, and different combinations of emission filters. ■, 550LP and 600SP; ◆, 500LP; ▲, 550LP; ×, 500LP and 600SP.

sions at the lower frequency, while at 1 MHz only the emission from the sensing dye is modulated. This is also confirmed by the modulation measurements on the frequency-domain fluorometer (Fig. 2). The complete demodulation of the long lifetime results in a plateau in the modulation graphs for frequencies between 1 and 10 MHz. This fact significantly decreases the requirements for the stability of the modulation frequency. Measurements performed on any frequency in this region will yield the same result, allowing for the use of very inexpensive devices.

The fact that the discrimination between the emissions is performed through the use of different modulation frequencies rather than through measuring on different wavelengths allows the use of fluorophores with significant overlap in their emission spectra. The excitation unit is simplest if the excitation wavelengths are the same, but when that is not possible, two different sets of excitation LEDs could be used. Given the low cost of the light sources and the ease of their control, this does not overly complicate the measuring system.

As the discrimination between the sensing and the reference dyes is lifetime-based rather than wavelength-based, it is possible to use a simple long-pass filter for the emission collection. This strongly enhances the signal level and allows the use of less sensitive photodetectors. As a result, the whole system (both excitation and emission units) was entirely semiconductor-based, which allows operation in even strong ambient light without damaging the detector.

Actual results from pH calibration using fluorescein and GFP are shown on Fig. 3. The same filter set was used for both dyes. The range of the ratios obtained using GFP as a pH sensor is smaller than results from fluorescein. This is due to the fact that the GFP used in this study (20) is significantly less sensitive to the pH of the environment. Still, the properly selected reference intensity works as a scaling factor, resulting in a high absolute change of the ratio. The results clearly indicate that the method could be very useful for measurements of near-neutral pH in environments where it is difficult to exclude ambient light. When better sensitivity is needed, there are several GFP mutants with an enhanced response to pH changes (21). Since GFP is already used as a marker in many applications, this method could enable localized pH sensing.

Calcium concentration measurements are extremely important in biological systems. Measurements of calcium concentrations rely on a variety of dyes; however, the need for a reference had created the use of mixed indicators (22). The proposed method offers an alternative to this approach. The results of Ca^{2+} calibration are shown on Fig. 4. As can be seen, the method allows for easy and reliable measurements within physiological concentrations. The desired range of the ratio values is easily adjusted using different combinations of filters in the excitation and/or emission path. The dye used (Oregon green–BAPTA) also exhibits a change in the lifetime as the calcium concentration changes; however, determination of such short lifetimes (0.74 ns in the absence of Ca^{2+} and 3.8 ns at high Ca^{2+}) requires highly sophisticated lifetime fluorometers.

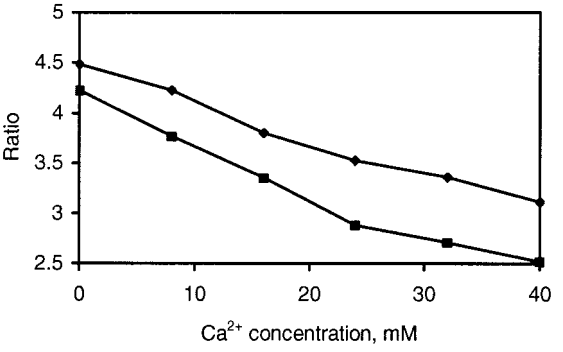

FIG. 4. Calibration curves for Ca^{2+} determination using Oregon green–BAPTA and different combinations of emission filters. ■, 550LP and 600SP; ◆, 500LP and 650SP.

The proposed method is generic and could be used with a great variety of dyes and references. Given the fact that even dyes with overlapping spectra could be used, as well as the enhanced levels of the output signal and the low cost of the system, it could find application in a variety of fields.

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