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Letter to the Editor

STRAY LIGHT CORRECTION FOR MICROSPECTROPHOTOMETRIC DETERMINATION OF INTRACELLULAR ION CONCENTRATION

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ABSTRACT

A simple correction for the effect of stray light on absorbance measurements from cells filled with indicator dyes is described.

The intracellular injection of indicator dyes and the microspectrophotometric detection of changes in the concentration of particular cytoplasmic ions is an important and increasingly popular technique in cellular neuroscience. Three examples are the use of arsenazo III to measure changes in free calcium, Eriochrome Blue to measure changes in intracellular free magnesium, and phenol red to measure changes in internal pH (Brinley et al., 1977; Scarpa et al., 1978; Ahmed and Connor, 1980). The usual procedure is to measure the absorbance of a dye-filled cell at one or more wavelengths. Generally, the absorbance change at one wavelength especially sensitive to changes in a particular ion is compared to the absorbance at a second wavelength which is insensitive to variations in ionic concentration. The absolute magnitude of dye-related absorbance at the second wavelength, called the isosbestic point, is usually used to determine the amount of dye in the cell. This as well as changes in the absorbance at the first wavelength are used to quantify changes in the intracellular concentration of the ion of interest.

An often serious limitation in these techniques, that is frequently overlooked in their application, is that stray light, not passing through the dye-filled cell but sensed by the transmitted light detector, will confound both the measurement of the dye concentration estimated from the isosbestic absorbance and the measurement of changes in ionic concentration estimated from absorbance changes at the ion-sensitive wavelength. I offer here a method to correct for the effect of stray light on these absorbance measurements. For reasons of clarity and simplicity, the derivations and corrections are presented in a form immediately applicable to the detection of calcium with arsenazo III,

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rather than in a more abstract or generalized form. However, the correction is easily generalized to any indicator dye whose absorbance spectrum for varying ion concentration is known.

Dye Absorbance Measurement in the Presence of Scattered Light

To estimate the concentration of arsenazo III, X, or of some other dye in a cell, a beam of light is passed through the center of the cell and the increase in absorbance, A, of the cell is measured while injecting dye. The dye concentration, X, is calculated from $A = \varepsilon XD$, where ε is the molar extinction coefficient of the dye at its isosbestic point (577 nm for arsenazo III), and D is the cell diameter, which is the path length for the absorbance increase. The latter is defined as the reduction in the intensity of light transmitted through the cell according to

$$A_{577} = -\log_{10} \left.\frac{I_1}{I_0}\right|_{577} , \qquad [1]$$

where I_0 is the intensity of light transmitted through the cell before injecting dye and I_1 is the transmitted light intensity after dye injection, both at 577 nm.

Now suppose that an amount of stray light, S, passes around the cell and is picked up by the transmitted light detector. The total light sensed before filling the cell will then be $I_0 + S$, while the light sensed after filling the cell will be $I_1 + S$. We need now to know how to correct Eq. [1] for the effects of this stray light.

Let C be the fraction of total light sensed that passes through the cell,

$$C = \frac{I_0}{I_0 + S} \text{ at 577 nm}, \qquad [2]$$

and let F be the reduction in transmitted light intensity plus stray light on filling the cell with dye, expressed as a fraction of the transmitted plus stray light before dye injection,

$$F = \frac{(I_0 + S) - (I_1 + S)}{(I_0 + S)} . \qquad [3]$$

Then

$$1 - F = \frac{I_1 + S}{I_0 + S} . \qquad [4]$$

From Eqs. [2] and [3],

$$C - F = \frac{I_1}{I_0 + S} , \qquad [5]$$

and from Eqs. [2] and [5],

$$1 - F/C = I_1/I_0 , \qquad [6]$$

so from [1],

$$A_{577} = -\log_{10}(1 - F/C) = -\log_{10}[1 - F(1 - S/I_0)] \qquad [7]$$

gives the actual value of absorbance at any wavelength in the presence of stray light S.

Absorbance Changes in the Presence of Stray Light

When a change occurs in the concentration of an ion which forms a complex with the absorbant dye, the absorbance of the dye will normally change in a way that is proportional to the ionic concentration change, if the latter remains at a level below its binding constant with the dye (Brinley et al., 1978). Thus the change in absorbance, ΔA, of light passing through the cell can be used as an index of the ionic concentration change. In the case of arsenazo III, we typically measure the absorbance changes at 660 nm, ΔA_{660}, since this wavelength is most sensitive to changes in the calcium concentration. In the absence of stray light and for small changes in transmitted light, ΔI_1,

$$\Delta A_{660} = \log_{10}e \cdot \frac{\Delta I_1}{I_1}\Big|_{660} . \qquad [8]$$

We detect not only the light passing through the dye-filled cell, I_1, but also the stray light, S. So the problem is to estimate the fractional change in transmitted light intensity $\Delta I_1/I_1$, in the presence of stray light, all at 660 nm.

The light sensed by the transmitted light detector, I_{tot}, includes both the light passing through the cell, I_1, and stray light, S,

$$I_{tot} = I_1 + S \quad \text{at 660 nm.} \qquad [9]$$

So the apparent absorbance change at 660 nm, $\Delta A_{660}'$, estimated from the fractional change in light sensed by the transmitted light detector, will be

$$\Delta A_{660}' = \log_{10}e \cdot \frac{\Delta I_1}{I_{tot}}\Big|_{660} = \log_{10}e \cdot \frac{\Delta I_1}{I_1 + S}\Big|_{660} . \qquad [10]$$

It is assumed that the stray light has a spectrum similar to that of the light coming through the unfilled cell. This will be true if the stray light passes through other cells near the cell to be filled. Then C of Eq. [2] will be independent of wavelength, and

$$S = (1/C - 1)I_0 \quad \text{at 660 nm.} \qquad [11]$$

To estimate the light transmitted through the cell at 660 nm, we note that the absorbance of arsenazo III, in excess KCl and magnesium and in low calcium, at 660 nm is about 1/3 its absorbance at 577 nm (Kendrick et al., 1977),

$$-\log_{10} \left.\frac{I_1}{I_0}\right|_{660} = A_{660} = 1/3\ A_{577} = -1/3\ \log_{10} \left.\frac{I_1}{I_0}\right|_{577} , \qquad [12]$$

or

$$\left.\frac{I_1}{I_0}\right|_{660} = \left(\left.\frac{I_1}{I_0}\right|_{577} \right)^{1/3} , \qquad [13]$$

and, from Eqs. [6] and [13],

$$I_1 = (1-C/F)^{1/3} I_0 \text{ at 660 nm.} \qquad [14]$$

Substituting Eqs. [11] and [14] into [9],

$$I_{tot} = \left[(1/C - 1) + (1 - F/C)^{1/3}\right] = KI_1 , \qquad [15]$$

where $K = \dfrac{(1/C - 1) + (1 - F/C)^{1/3}}{(1 - F/C)^{1/3}}$. Then

$$\Delta I_1/I_{tot} = \Delta I_1/KI_1 \quad \text{or} \quad \Delta I_1/I_1 = K\Delta I_1/I_{tot} \text{ at 660 nm,} \qquad [16]$$

and the actual change in the cell's absorbance at 660 nm, ΔA_{660}, will be related to the apparent absorbance change of Eq. [10] by

$$\Delta A_{660} = K\Delta A_{660}' . \qquad [17]$$

The factor K would be determined similarly for another wavelength or a different ion-sensitive dye. Only the exponent (1/3) would be different, reflecting the ratio of dye absorbance at the ion-sensitive and isosbestic wavelengths.

Eqs. [7] and [17], with the expressions [2] and [15] for C and K, may be applied to estimate the actual absorbance of a dye-filled cell at one wavelength and the real absorbance change at another wavelength, in the presence of a known amount of stray light. To be useful, the intensity of stray light, S, must be known. In practice, this may be estimated by filling a cell with excess dye until it appears dark; the remaining light sensed by the transmitted light detector provides an approximate measure of S, so long as the light transmitted through such an overfilled cell is truly negligible. That this is so can be confirmed by filling a cell with dye under favorable circumstances where stray light is likely to be minimal. When, for example, a large isolated neuron is filled with dye to darkness and the transmitted light is measured with a light guide of a diameter only half that of the cell and pressed against the cell's membrane, the transmitted light drops to about 1% on overfilling.

Once the correct values of A_{577} and ΔA_{660} are determined, the typical calibration factor relating $\Delta A_{660}/A_{577}$ to ΔCa can be used to estimate the change in internal calcium concentration. In the case of a first-order reaction, this factor is independent of dye concentration (Brinley et al., 1978), while for more complex dye reaction schemes, the factor may depend on the dye concentration (Thomas, 1979; Smith and Zucker, 1980). In that case, the dye

concentration is estimated from $A_{577} = \varepsilon XD$.

To illustrate the quantitative effect of stray light, I shall consider the application of these corrections to typical experimental data. In fact, this approach to correcting for the effects of stray light was used in previous work by this author (Smith and Zucker, 1980; Zucker, 1981a,b), although details of this corrction have never been published. To maximize absorbance signals, a light spot about the same diameter as the cell being studied is focused on the cell, and a light pipe connected to a photo-diode is brought up to the cell surface. To minimize the effect of a nonuniform light path through a rounded cell, the diameter of the light guide may be reduced to 80% that of the cell without too great a sacrifice in sensitivity. A common value for S in such a case is 20% of I_o, so from Eq. [2], C = 0.83. A 200 μm neuron was typically filled to an apparent absorbance at 577 nm, $-\log_{10}((I_1 + S)/(I_o + S))$, of 0.15. In the absence of stray light, this is the absorbance corresponding to 0.25 mM arsenazo III for a 200 μm path length and extinction coefficient of 2.98×10^4 $cm^{-1}M^{-1}$. However, with 20% stray light, from Eq. [3], F = 0.3. Thus, from Eq. [7], A_{577} was really 0.18, and the cell actually contained 0.30 mM arsenazo III.

Suppose a change in intracellular calcium occurs and an absorbance signal ΔA_{660} of 0.0015 is observed. If we ignored the effect of stray light and thought that A_{577} was 0.15, we would call this a relative absorbance change, $\Delta A_{660}/A_{577}$, of 0.01, which corresponds to a change in calcium concentration of about 1 μM (Smith and Zucker, 1980). But now, with 20% stray light, from Eq. [15], K = 1.23. Thus an apparent change in absorbance at 660 nm of 0.0015 represents a real absorbance change of 0.00185. With 0.30 mM arsenazo III, a change in calcium of 1 μM will generate a relative absorbance change, $\Delta A_{660}/A_{577}$, of 0.012, due to the second-order dependence of absorbance change on dye concentration (7). So now, in the presence of 20% stray light, our apparent relative absorbance change, $\Delta A_{660}'/A_{577}'$, of 0.0015/0.15 or 0.01 corresponds to a real absorbance change, $\Delta A_{600}/A_{577}$, of 0.00185/0.18 = 0.0103. This indicates a calcium concentration change of only 0.0103/0.012 or 0.86 μM calcium, rather than the 1 μM change it would indicate in the absence of stray light.

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