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Stray Light Correction in the Optical Spectroscopy of Crystals

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Abstract

It has long been known in spectroscopy that light not passing through a sample, but reaching the detector (i.e., stray light), results in a distortion of the spectrum known as absorption flattening. In spectroscopy with crystals, one must either include such stray light or take steps to exclude it. In the former case, the derived spectra are not accurate. In the latter case, a significant amount of the crystal must be masked off and excluded. In this paper, we describe a method that allows use of the entire crystal by correcting the distorted spectrum.

Index Headings

Absorption flattening; Kinetic analysis; Membrane crystals; Stray light

INTRODUCTION

We are interested in studying the kinetics of the photocycle of bacteriorhodopsin (BR) in both its native membrane environment (purple membrane, or PM) and in crystals. It should be noted that according to convention, “bR” represents the protein and “BR” represents the ground state of the protein (before light activation). Bacteriorhodopsin crystals are hexagonal in shape (Fig. 1) and are most often obtained in sizes up to 100 μm . In our

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experience with the method of Landau and Rosenbusch,¹ crystals larger than 50 μm were rarely obtained. The number of photons passing through this small target is extremely low compared to the 1 cm diameter circle we have normally used for visible and IR spectroscopy with PM.² As a result, the signal-to-noise ratio (S/N) obtained in kinetic experiments is quite low. In a recent publication, Kubicek et al.³ described a modified in meso crystallization method yielding crystals up to a 200 μm size range. This increases the number of photons (compared to the 50 μm window) by 16-fold, which should raise the S/N by a factor of four.

However, as seen in Fig. 1, in order to use the entire crystal, light outside its dimensions must be included. Light that does not pass through the sample but that reaches the detector is called stray light. The spectrum obtained in the presence of stray light is reduced in amplitude and flattened compared to the true spectrum obtained in its absence. Under the constraint of a rectangular sample window, eliminating all of the stray light reduces the effective usable area of these hexagonal crystals to that of the inner rectangle in Fig. 1.

Many papers discuss the problems introduced by stray light, but we have not found any that offers a correction method to reverse the distortions of stray-light-induced absorption flattening.⁴⁻⁹ This paper describes a method for using the entire crystal and correcting for the spectrum-altering effects of stray light.

METHODS

Crystals

Crystals were prepared as described by Kubicek et al.³ For the purposes of this work, 200 μm crystals were not needed, as the hexagon shape is essentially the same for crystals of any size. However, the larger crystals are very important for the intended time-resolved X-ray diffraction studies and for increasing the S/N for fitting up to seven exponentials to time-resolved visible kinetic data.

Hardware

The system consists of the PhotonMax (Princeton Instruments), a 16-bit electron-multiplying charge-coupled device (EMCCD) with greater than 90% quantum efficiency and on-chip multiplication gain. The PhotonMax has a kinetics mode that allows microsecond acquisition of windows (one or more rows) until the full 1048 available rows (524 $\times$ 512 CCD, plus 512 $\times$ 512 transfer area plus reference rows) have been filled. The row pixel height is 16 μm . In our experiments, the camera is configured for kinetics mode using multiple triggers and a windows size of two rows (i.e., 32 μm high). The number of on-device averages can be set through a controlling Matlab program (The MathWorks). However, due to the large number of averages needed for an experiment, we perform additional averaging in software. By maximizing the number of averages done on-device, we keep our system repetition rate as high as possible. To lower noise levels, we set the camera's readout speed to 5 MHz, which limits us to an approximately 4 Hz repetition rate. The controlling Matlab program allows the user to specify the time collection schedule.

An image intensifier VS4-1845HS (VideoScope International) is connected to the PhotonMax for additional gain and allows gating of the incoming light levels. As the BR

kinetics is captured in 524 time points, covering a range from microseconds to milliseconds, we use a quasi-logarithmic schedule. For the pre-laser flash ground state, the first 10 samples are collected at 80 μ s spacing. This is followed by 120 samples at 10 μ s spacing, 200 at 30 μ s spacing, and 194 at 50 μ s spacing. The image intensifier gating is critical for maintaining consistent exposure times as the sampling spacing changes. Timing for the system is handled through a custom device based on a MAX II complex programmable logic device (CPLD; Altera Corp., San Jose, CA). The CPLD generates all timing parameters from a master clock and controls the laser flash lamp, laser Q-switch, camera trigger, and image intensifier gating. The signal parameters (e.g., frequency, duty cycle, active high–low) are hardcoded into the device according to the respective manufacturer's specifications. The camera trigger can be disabled programmatically, and the sample-timing schedule is also programmable. The device is programmed using a commercial USB module (QuickUSB) and controlled through the main Matlab program.

In the studies reported here, it is important to emphasize that to focus attention on stray-light spectral aberrations and their correction, spectral changes due to photocycle turnover were eliminated by not firing the actinic laser.

Optical Connection of Monitoring Light from Its Source through the Microscope to the Entrance Slit of the Spectrometer

Light from two LED lamps (GE Vio/7.2W/841) that are positioned at right angles are combined via a 70/30 pellicle beam splitter to form a unified beam that is subsequently collimated by lenses. One of the LEDs has the phosphor dome removed to provide more intensity in the deep blue region of the visible spectrum. The collimated beam enters the external illumination port of the Bruker Hyperion 1000 IR microscope and is adjusted such that a uniform illumination is achieved through the condenser on to the sample plane. Neutral density attenuation can be inserted into the path of either LED or into the collimated beam to provide appropriate illumination levels for the detector system (described above). The interrogating beam, after illuminating the sample, is captured by the microscope objective and brought to a focus in the camera port plane, at which point a 10 $\times$ or 20 $\times$ Olympus objective focuses the image of the illuminated objective on to the polished end of a 600 μ m fused silica optical fiber. The diameter of the focused spot is closely matched to that of the fiber end. The distal end of the fiber is held in a three-axis positioner and adjusted to provide optimal illumination as determined by the measure of intensity of transmitted light recorded by a Newport model 1935-C power meter and a 818-ST2 wand-style calibrated photodetector. Finally, the light is passed through the entrance slit of an Acton 2500i spectrometer. At this step, the fiber height is adjusted so that the final image falling on the detector illuminates only the bottom two rows of the CCD array. The dispersed light from the spectrometer is brought to a focus on the photocathode of the image intensifier, and the intensified image is relayed to the image plane of a Princeton Instruments CCD camera.

Correction Procedure for Stray Light

In our analysis, the detected light intensity in digitized counts is used as a measure of transmittance (t). The 100% transmittance level obtained from a clear area of the dry cell is t_{100} . The dark background signal obtained with the light blocked is t_0 . The observed

transmittance (t_{obs}) when bR is present includes both light that passed through bR (t_{br}) and any stray light that is present. Stray light is expressed as a fraction of t_{100} as shown in Eq. 1:

$$t_{\text{obs}} = p \times t_{100} + t_{\text{br}} \quad (1)$$

where p is the fraction of stray light. When $p = 0$, t_{br} is equal to t_{obs} .

Transmittance curves are vector functions of t versus wavelength. Therefore, these curves are obtained by correcting t_{obs} to t_{br} at each recorded wavelength. The transmittance curve for t_{br} is used to obtain the true absorption curve called A . Transmittance curves obtained with stray light present produce distorted absorption spectra called A_x . The principle upon which the correction is based is to vary the value of p , as indicated in Eq. 2, using least squares regression analysis, to find a value of t_{brcor} that produces a value of A_x with the lowest residuals, R , shown in Eq. 3:

$$t_{\text{brcor}} = t_{\text{obs}} - p \times t_{100} \quad (2)$$

$$R = A - A_x \quad (3)$$

At $R = 0$, A_x is corrected to A .

The method requires two sets of experimental data. One set is data with no stray light to obtain A , and the other includes stray light to obtain A_x .

Simulation of Data to Test the Method

Data for a true BR spectrum were taken from a film of purple membrane (PM) where all stray light was blocked. The isolation of PM, composition of the stock suspension, and formation of the film are as described in Hendler et al.² Stray-light-distorted spectra were obtained by computing t_{obs} using Eq. 1 with p varied from 0 to 0.25, in steps of 0.05. These simulated data spectra are shown in Fig. 2.

Using Experimental Data Obtained with a Crystal

In the case of simulated data, only one t_{100} was needed to convert t_{obs} to t_{brcor} (Eq. 2). In the case of experimental data using a crystal, the true spectrum, A , was obtained by using knife edges to block all stray light around the crystal as indicated by the inner rectangle in Fig. 1, which is $\sim 80 \times 50 \mu\text{m}$. The stray-light-distorted spectrum A_x for the full crystal was obtained by opening the knife edges to $\sim 80 \times 80 \mu\text{m}$. Converting the transmittances with and without stray light to absorbances requires two t_{100} , one for each case. Because of the two different window sizes, it was necessary to add a second fittable parameter (p_2) to allow for any adjustment of amplitude, if needed (Eq. 4). Parameter p_1 is the fraction of stray light. In practice, the fits were improved by using the second parameter. The experimental data are an average of 15 000 repeats (compare to Eq. 2):

$$t_{\text{brcor}} = p_2 \times t_{\text{obs}} - p_1 \times t_{100} \quad (4)$$

In turnover studies the photocycle is initiated with a 3 mJ pulse of 532 nm light in a ~5 ns pulse, produced by a frequency-doubled, Surelite 1–10 Continuum Nd : YAG laser. This actinic pulse is fired 1 ms after the start of data collection (sample no. 30). Time samples numbered 3 to 28 then are averaged into a 512×1 vector for the ground state spectrum prior to turnover. The columns from numbers 30 to 524 form a 512×496 matrix where the time-resolved columns contain the kinetic information of turnover. However, in these studies, the laser was not fired in order to avoid complications of spectral changes due to changing amounts of intermediates.

RESULTS

With Simulated Data

Spectra of the simulated data are shown in Fig. 2. The true curve with 0% stray light is the blue one at the top. Difference spectra with reference to the blue curve in Fig. 2 as the value of p was raised are shown in Fig. 3 where absorption flattening distortions are quite apparent. The curves show the amount of spectrum lost compared to the true spectrum at each level of stray-light contamination. Stray-light levels >15% (the lowest curves in Fig. 2) show (impossible) negative absorbances. Because the purple membrane was stray light free, corrections of >15% led to overcorrections where t_{brcor} dipped below the t_0 (dark background) level. When this happens, the log of the ratio of $(t_{100} - t_0)/(t_{\text{obs}} - t_0)$ becomes negative, and the absorbance is returned as an imaginary number, where only the real part is used. Figures 4 to 8 show that the correction procedure for retrieving the undistorted spectra worked at all levels of added stray light, even those >15% (Figs. 7 and 8).

Corrections for the simulated absorption curves with 5%, 10%, 15%, 20%, and 25% stray light are shown in Figs. 4 to 8, where absorption (A) is plotted against wavelength. In all cases, the top (a) panels show three curves: (1) true absorption with no stray light (blue), (2) absorption with a known amount of added stray light (green), and (3) residuals (red). The bottom panels (b) show both the true curve and the light-contaminated curve after the correction described above. These curves are superimposed. The residuals are shown in red. Statistics for the quality-of-fit of the parameter p in Eq. 2 are shown in Table I. Designations “Pin” and “Pout” are for the input and output values of the fitted parameters, respectively. In all cases, the input value of 0 converged to a fitted value with an extremely low standard error. Upon convergence, the sum of squares was dramatically decreased from that of the initial input value of 0. Dependency values are important when more than a single parameter are used as in cases where crystal rather than simulated data are used. In such cases the dependency values can vary from one to infinity. These values indicate the independence of individual parameters. Higher values indicate over-parameterization. Values <10 show that the tested parameters are independent and required.^{2,10}

Experimental Data

Crystal data using the vector and matrix are shown, respectively, in Figs. 9 and 10.

DISCUSSION

The purpose of this study was to test a new method for correcting the spectral distortions due to stray light. To our knowledge, no other procedure has been shown to accomplish this. The results show that the correction procedure based on optimization using least squares regression analysis can correct distorted spectra to their stray-light-free shapes.

Simulated data with stray light at 5%, 10%, 15%, 20%, and 25% levels produced spectra that were progressively more distorted in amplitude and shape, panels (a) of Figs. 4–8. The fitting procedure converged on the known amount of added stray light in each case (statistics of the fits are shown in Table II) and produced spectra that were identical to the spectra with no stray light, panels (b) of Figs. 4–8.

Crystal-derived data are shown in Figs. 9 and 10. As with the simulated data, Fig. 9 shows that the correction procedure reduced the residuals obtained with the vector shown in panel (a) to near zero shown in panel (b) with a close overlap of the stray-light-distorted and stray-light-free spectra, especially in the range that covers all of the known intermediates (from 380 to 650 nm).

The data matrix depicted in Fig. 10 shows a distributed spread of spectra. The absorption flattening for these spectra is seen in panel (a). The corrected spectra and residuals in panel (b) show that the correction procedure restored the correct spectral shapes. The reason for the greater divergence of spectra shown in Fig. 10 than in Fig. 9 is that all of the individual columns (i.e., time points) are shown, whereas in Fig. 9 only the averages of all columns are shown. In a kinetic experiment where the actinic laser is fired, there are large changes in absorbance as BR goes through its photocycle. When the absorbance scale is increased to accommodate these changes, the relative amplitudes of the background scatter seen in Fig. 10 are greatly diminished.

It should be recognized that the methods described in this paper will not correct some other forms of absorbance flattening arising from crystals not having perfectly parallel front and back surfaces, slanted edges with respect to the parallel planes, or grossly inhomogeneous sample thicknesses. There are additional potential sources of distortions, such as (1) the lack of perfect collimation of the measuring beam, which results in a distribution of effective path lengths, that is, a short path length for the rays most perpendicular to the front and back surfaces, and longer path lengths for rays traveling more obliquely; (2) the breakdown of the ray picture of light, that is, the involvement of diffraction, when the gap around the small crystal starts to resemble a narrow slit; and (3) nonuniformity of sample concentrations throughout the sample crystal, as when a spatially nonuniform photolysis flash creates a higher degree of photoreaction at some areas within the crystal than at others.

Because our procedures were able to correct the distorted spectra back to the shapes seen with non-crystalline bacteriorhodopsin, it is obvious that these other forms of absorbance flattening were absent or too small to be significant. Therefore, the first test with any crystal is to determine if the procedures described here can reproduce spectra seen with the native protein.

If any other protein crystal intended for kinetic studies is to be used and it does not produce the same spectra as seen in the non-crystalline state, it may still be usable for kinetic studies. Most or all of the complications listed above such as nonparallel planes, slanted sides, and inhomogeneities are also constants. These distorted spectral shapes should remain constant. The kinetics of changes due to transitions of sequential intermediates should not be affected. Thermodynamics and concentrations of the actual chemical substances will still produce the true kinetic constants and associated spectral changes. If there is any reason to suspect altered kinetics due to changes in the already distorted spectral shapes, singular value decomposition (SVD) should be used.¹¹ Concentration-dependent kinetic changes are fit by exponentials. There is no reason to believe that changes in spectral shape, if they occur, are also concentration dependent. Kinetic eigenvectors produced by SVD for such spectral changes would not be fit by exponentials. Should any such non-exponential eigenvectors be present, they would be separated from the kinetic ones that would be fit by exponentials.

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Fig. 1. Photograph of a typical hexagonal BR crystal in its crystallization well with an edge-to-edge diameter of 80 μm . Superimposed are two rectangular frames. The outer frame captures the entire crystal, but also includes stray light. For a regular hexagon, this would be 25% of the enclosed area. The inner frame, which excludes all stray light, contains approximately two-thirds of the crystal.

Fig. 2.

The ordinate shows the measured absorption for absolute BR spectra obtained at different levels of stray light. Curves from top to bottom contain 0%, 5%, 10%, 15%, 20%, and 25% stray light, respectively. Vertical markers at 412, 532, and 568 nm show no steady state photocycle intermediate M or laser interference and the A_{max} for BR, respectively.

Fig. 3. Difference spectra at different levels of stray light. Curves from bottom to top are difference spectra at increasing levels of p for each of the curves shown in Fig. 1 obtained by subtracting each stray-light-contaminated curve from the blue stray-light-free curve. The color identities correspond to those in Fig. 2.

Fig. 4.

Panel (a) shows the no-stray-light spectrum (blue); the 5% stray-light spectrum (green), and residuals (red). Panel (b) shows the corrected 5% stray-light absolute spectra superimposed on the stray-light-free curve and the residuals curve (red).

Fig. 5.

Panel (a) shows the no-stray-light curve (blue); the 10% stray-light curve (green), and residuals (red). Panel (b) shows the corrected 10% stray-light curve superimposed on the stray-light-free curve and the residuals curve (red).

Fig. 6. Panel (a) shows the no-stray-light curve (blue); the 15% stray-light curve (green), and residuals (red). Panel (b) shows the corrected 15% stray-light curve superimposed on the stray-light-free curve and the residuals curve (red).

Fig. 7.

Panel (a) shows the no-stray-light curve (blue); the 20% stray-light curve (green), and residuals (red). Panel (b) shows the corrected 20% stray-light curve superimposed on the stray-light-free curve and the residuals curve (red).

Fig. 8.

Panel (a) shows the no-stray-light curve (blue); the 25% stray-light curve (green), and residuals (red). Panel (b) shows the corrected 25% stray-light curve superimposed on the stray-light-free curve and the residuals curve (red).

Fig. 9.

Panel (a) shows the no-stray-light curve (blue); the raw vector data from the crystal with stray light as shown in Fig. 1 (green), and the residuals (red). Panel (b) shows the corrected vector data, which are superimposed on the stray-light-free curve. The residuals curve is in red.

Fig. 10.

Panel (a) shows the no-stray-light curve (blue); the raw matrix data from the crystal with stray light as shown in Fig. 1 (green), and the residuals (red). Panel (b) shows the corrected matrix data, which are superimposed on the stray-light-free curve. The residuals curve is in red.

TABLE I

Statistics for quality-of-fits to Eq. 2.

Added stray light (%)	Pin	Pout	Sum of squares			Standard error	Dependency
			Start	Converged			
5	0	0.0500	0.46817	4.5288e-7		3.7272e-5	1.0000
10	0	0.1000	1.7191	4.5288e-7		3.7272e-5	1.0000
15	0	0.1500	3.5736	4.5288e-7		3.7272e-5	1.0000
20	0	0.2000	5.8993	4.5288e-7		3.7272e-5	1.0000
25	0	0.2500	8.5964	4.5288e-7		3.7272e-5	1.0000

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TABLE II

Statistics for quality-of-fits to Eq. 4.

Pin	Pout	Sum of squares		Standard error	Dependency
		Start	Converged		
512 × 1 vector data from crystal					
0	0.06276	11.92	0.062174	0.19686	4.5079
1	1.2411			0.19686	4.5079
328 × 496 matrix data from crystal					
0	0.08521	16988	490.55	0.24142	4.3964
1	1.0811			0.24142	4.3964