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Application of a Submicroliter Spectrophotometer in Visual Pigment Studies

L. Marty Martinez,[1+] Rosalie K. Crouch,[1] and D. Wesley Corson[1,2*]

[1]Department of Ophthalmology, [2]Department of Pathology and Laboratory Medicine, Medical University of South Carolina Charleston, South Carolina

Absorbance measurements in the visible region of the spectrum are essential for determining pigment concentration in a number of biochemical assays involving recombinant visual pigments and in identifying pigments isolated from single retinas. As the amount of pigment available from a single retina (1) or from recombinant synthesis in COS cells (2) is limited, it is desirable to make these measurements in a small sample volume provided that this can be done with an acceptable level of pigment bleaching during the measurement. Although a variety of microcuvettes are available for conventional spectrophotometers, the practical minimum volume for these is on the order of 10 µl, and such cells are difficult to align and clean.

In addition to a small sample volume, it is desirable to use an instrument that minimizes scattering losses. Pigments used in these assays are often isolated in membrane fragments that may be partially solubilized with detergent. Scattering is problematic since the size of membrane fragments in a typical preparation are often on the order of the wavelengths of visible light. The SpectroPette (WPI, Sarasota, FL), a newly available diode-array spectrophotometer capable of measurements in the visible spectrum (380-760 nm) on submicroliter volumes, was tested for pigment bleaching and scattering losses with samples of bovine rhodopsin and pigment from single salamander retinas. For comparison, spectra also were obtained with a Hewlett-Packard (HP) 8452A diode-array spectrophotometer using an 80 µL microcell (Helma model 0100-1224) and a conventional Cary 2200 scanning spectrophotometer using the same cell.

Tiger salamanders, Ambystoma tigrinum, provide a widely used model for studies of visual transduction. As with other amphibians, a retina contains about a nanomole of a mixture of rhodopsin and porphyropsin pigments (3-5). Visual pigment was extracted from a single dark-adapted retina isolated under infrared illumination. The retina was suspended in 1 ml of 67 mM sodium phosphate buffer at pH 6.5 and pelleted for 10 minutes at 39,000g on a table top ultra centrifuge (Beckman Optimal TL Ultracentrifuge with a TLA 100.3 fixed angle rotor). The supernatant was decanted and the pellet was resuspended by trituration in 50 µl phosphate buffer. The suspension was sonicated for three 20 sec periods over a 5 minute interval in a bath sonicator (PUC-1-20s) supplied with the SpectroPette. A 50 µl aliquot of 2% dodecyl maltoside (Calbiochem #324355) in phosphate buffer was added and the sample was slowly rotated for 1 hour to extract the pigment. The sample was centrifuged for 10 min at 39,000 g to remove insoluble material, leaving the pigment solubilized in a 60 µl volume. A typical absorbance spectrum of visual pigment before and after bleach is shown in Figure 1 and illustrates the signal to noise ratio obtainable with small amounts of pigment; in this case, 5 pM out of 500 pM of extractable pigment from a single retina.

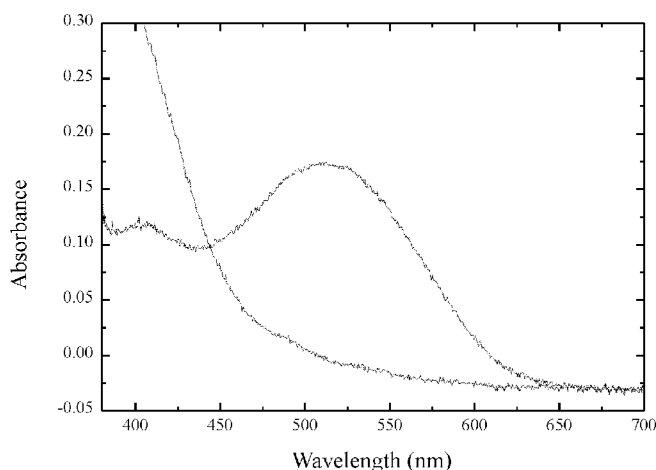

Figure 1. Single-scan pre-bleach (upper trace) & post-bleach (lower trace) spectra of pigment extracted from a single salamander retina. The spectrum was obtained with the SpectroPette using a 10 mm pathlength (600 nl volume) and a 132 ms integration time.

In the SpectroPette, the probe beam passes through the entire volume of the small sample and bleaches a fraction of a given photosensitive sample depending on its absorbance and the spectral distribution and duration of the probe pulse that is gated by a shutter. For bleaching determinations, membrane fragments from bovine rod outer segments were prepared from frozen retinas (Lawson, Lincoln, NB) using the method of Papermaster and Dreyer (6). The fragments were pelleted in a Beckman Ultracentrifuge (Model L5-75, SW-28 rotor) at

[+]Current Address: L. Marty Martinez, Spartanburg Regional Medical Center, 101 E. Wood Street, Spartanburg, South Carolina 29303, phone (803) 853-0864

[*]To whom correspondence should be addressed: D. Wesley Corson, Department of Pathology and Laboratory Medicine, Medical University of South Carolina, Charleston, South Carolina, Phone (803) 792-7673, Fax: (803) 792-1723 email: corsondw@musc.edu

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40,000 RPM at 4˚C for 15 min. The supenatant was removed and 0.2 M sodium phosphate buffer solution containing 2% CHAPS (Calbiochem #220201, La Jolla, CA) at pH 6.7 was added. The pellet was dissolved by rocking for 1.5 hours. Rhodopsin absorbance measurements were obtained following a second centrifugation to remove undissolved material (7). All procedures were carried out under dim red light.

The WPI spectrophotometer was set to an integration time of 132ms. The initial absorption spectrum was recorded with the first scan for each new sample. The spectrum was then recorded at intervals following repeated scans. Measurements were taken every 20 min to allow for the decay of pigment photoproducts between measurements (8). As shown in Figure 2, some bleaching was produced by the measuring beam as

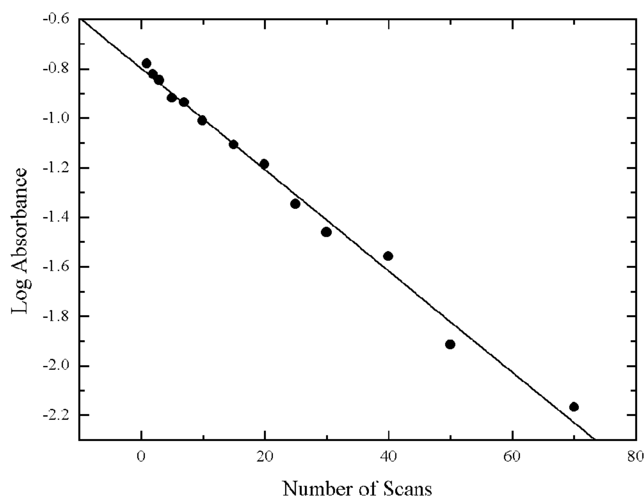

Figure 2. Depletion of solubilized bovine visual pigment with repeated scanning in the SpectroPette spectrophotometer. 4.0 µM rhodopsin was prepared as described in the Methods and scanned with 132 msec integration time and a 10 mm pathlength.

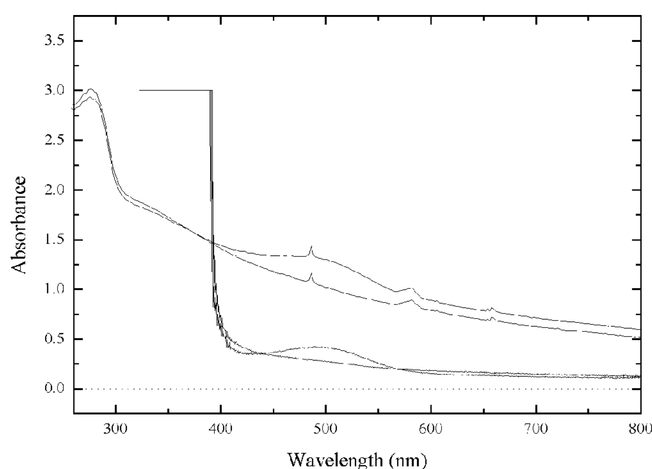

Figure 3. Differences in scattering losses arising from different optical configurations when using partially solubilized bovine rhodopsin. The lower spectra were obtained with a SpectroPette and show less apparent density attributable to scattering throughout the spectrum than the upper spectra, which were obtained with a HP 8452 spectrophotometer.

the pigment decayed exponentially with the number of scans. The amount of bleaching caused by each scan in 3 independent measurements was calculated to be 3.5% (1.6% (mean ± s.d.) and was acceptably small, making this a useful tool in our studies of visual pigments.

Figure 3 shows the initial and bleached spectra of partially solubilized pigment on the SpectroPette (lower traces) and HP instruments (upper traces). This figure illustrates the lower scattering losses with the WPI instrument relative to the Hewlett-Packard instrument. Spectra from the Cary spectrophotometer, a scanning instrument, gave results similar to those of the Hewlett-Packard instrument.

On the basis of our evaluation, the WPI spectrophotometer should prove to be a very useful tool in the study of visual pigments. It should be cost effective in cases where the small sample chamber and adjustable path length permit meaningful measurements to be obtained without consuming large amounts of expensive materials. Additionally we have demonstrated that interference from scattering is minimal and that the extent of bleaching caused by the measuring beam is acceptably small permitting quantitative work.

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