Experimental determination of refractive index of condensed reflectin in squid iridocytes

Amitabh Ghoshal1,†, Daniel G. DeMartini1,2,†, Elizabeth Eck2 and Daniel E. Morse1,2

1Institute for Collaborative Biotechnologies, University of California, Santa Barbara, CA 93106-5100, USA
2Biomolecular Science and Engineering Program, University of California, Santa Barbara, CA 93106-9611, USA

Loliginid squid dynamically tune the structural iridescence of cells in their skin for active camouflage and communication. Bragg reflectors in these cells consist of membrane-bound lamellae periodically alternating with low refractive index extracellular spaces; neuronal signalling induces condensation of the reflectin proteins that fill the lamellae, consequently triggering the expulsion of water. This causes an increase in refractive index within the lamellae, activating reflectance, with the change in lamellar thickness and spacing progressively shifting the wavelength of reflected light. We used microspectrophotometry to measure the functionally relevant refractive index of the high-index lamellae of the Bragg reflectors containing the condensed reflectins in chemically fixed dermal iridocytes of the squid, Doryteuthis opalescens. Our high-magnification imaging spectrometer allowed us to obtain normalized spectra of optically distinct sections of the individual, subcellular, multi-layer Bragg stacks. Replacement of the extracellular fluid with liquids of increasing refractive index allowed us to measure the reflectivity of the Bragg stacks as it decreased progressively to 0 when the refractive index of the extracellular medium exactly matched that of the reflectin-filled lamellae, thus allowing us to directly measure the refractive index of the reflectin-filled lamellae as $n_{\text{condensed lamellae}} \approx 1.44$. The measured value of the physiologically relevant $n_{\text{condensed lamellae}}$ from these bright iridocytes falls within the range of values that we recently determined by an independent optical method and is significantly lower than values previously reported for dehydrated and air-dried reflectin films. We propose that this directly measured value for the refractive index of the squid’s Bragg lamellae containing the condensed reflectins is most appropriate for calculations of reflectivity in similar reflectin-based high-index layers in other molluscs.

1. Introduction

The structural iridescence displayed by cephalopods (e.g., octopus, squid and cuttlefish) is of interest because it is tunable [1–7] and consequently is used for active camouflage and communication [1,8]. These adaptations of colour and appearance are accomplished by modulating multi-layer Bragg reflectors [9] in cells called iridocytes [2]. While earlier workers also used this terminology or referred to these cells variously as iridophores or reflective cells, we use here the unambiguous convention of contemporary cell biology, referring to them as iridocytes (literally ‘iridescent cells’), with no specific photonic mechanism implied [10]. Multiple forays [2–5,11–14] have been made to optically characterize the iridocytes and their Bragg lamellae that are known to contain high concentrations of a unique family of proteins called reflectins [15]. The molecular pathway and biophysical mechanism governing the neurotransmitter-activated [2], reflectin-mediated, tunable control of iridocyte brightness and colour have been elucidated recently [10,16,17], confirming that the reflectance and colour are dependent on the condensation of the reflectin proteins and the resulting changes in thickness and spacing of the Bragg lamellae. While recent studies [18,19] have estimated the refractive index...
of reflectin assemblies based on optical measurements, a direct measurement of the functionally relevant refractive index of the reversibly condensed reflectins in the tunable Bragg lamellae of squid iridocytes has remained elusive. Spectral measurements in previous studies [13,14,16,17,20] covered large areas and averaged signals from many cells, yielding spectra from multiple Bragg stacks with unknown variation in spacings, refractive indices and orientations, thus making the data intractable for rigorous optical analysis, or for the refractive index measurements described herein. Our present study builds on our recent investigations using subcellular microspectrophotometry to characterize the tunable Bragg stacks and calculate the physiologically relevant optical parameters of these structures [18].

In the work reported here, we use micro-spectrophotometry to investigate the subcellular, tunable reflecting structures in chemically fixed (but only slightly altered [18]) iridocytes of the California Market squid, Doryteuthis opaline [21] and determine the refractive index of the high-index lamellae of the Bragg reflectors containing the condensed reflectins. Our high-magnification imaging spectrometer allows us to obtain normalized ‘pure’ spectra of optically distinct sections of the individual, subcellular, multi-layer Bragg stacks in iridocytes. We then replace the extracellular fluid with fluid of increasing refractive index, allowing us to measure the reflectivity of the Bragg stacks as it decreases with the expectation that the reflectivity will drop to 0 when the refractive index of the extracellular medium matches the refractive index of the reflectin-filled lamellae, thus allowing us to directly measure the functionally relevant refractive index of the condensed reflectin-filled lamellae, \( \eta_{\text{condensed lamellae}} \).

The measurements described here were performed in-tissue, under conditions very close to the native hydration state of the reflectins; this is in marked contrast to previous works [11,12] in which refractive index measurements of reflectin proteins or peptides were performed on dehydrated and air-dried materials.

2. Material and methods

2.1. Iridocyte activation and tissue fixation

Doryteuthis opaline specimens were purchased live from Oterbanks Commercial Fisheries (Oxand, CA, USA) and were transported under constant oxygen flow to the University of California-Santa Barbara where they were kept in tanks of fresh flowing seawater. Fresh specimens were euthanized by quick decapitation. The squid mantles were cut along the ventral surface, the internal organs and gladius were removed, and the tissue was pinned out to the native dimensions in a Sylgard 184 (Dow Corning, Midland, MI, USA) coated dish. After the iridocytes were activated with acetylcholine [18], tissue samples were fixed in 4% formaldehyde in artificial seawater (ASW, 470 mM NaCl, 10 mM KCl, 27 mM MgCl₂, 29 mM MgSO₄, 11 mM CaCl₂, 10 mM HEPES, pH 7.8) for 4 h at room temperature. Following fixation, the samples were washed extensively in ASW to remove excess fixative.

Small samples (2 × 2 mm) were cut from the dorsal dermal tissue, placed in cover-glass-bottomed Petri dishes with the iridocyte layer down, and each was covered with a coverslip and a black painted aluminium weight to prevent movement and reduce background noise during the optical measurements.

2.2. Micro-spectrophotometry

Samples in the cover-glass-bottomed Petri dishes were placed on the stage of a Zeiss AxioObserver DIM inverted microscope (Carl Zeiss AG, Oberkochen, Germany), as shown in figure 1a. Details of the micro-spectrophotometer, the iridocyte-containing tissue and their interaction are shown in figure 1 and discussed later.

Figure 2a shows a typical bright-field microscopic image of a few iridocytes illuminated with a broadband halogen lamp (providing ample light in the measured range of 400–700 nm) and imaged in reflection mode with a 50× objective (Zeiss EC EpiPlan-NEOFLUAR, part no. 000001156328000, numerical aperture = 0.8, depth of field = 3.8 μm). A single iridocyte is marked by the dashed white oval. Note that rather than a single reflective area, the cell displays multiple iridescent regions of slightly varying colour and brightness. This observation is consistent with observations that each of these highly invaginated iridocytes [10] contain multiple stacks of curved lamellae forming optically distinct multi-layer structures of alternating low- and high-refractive index layers forming multiple Bragg stacks—each with its own characteristic material parameters that determine the colour and intensity of the reflected light.

An area of the image, marked by the narrow white rectangle in figure 2a, was imaged on the entrance slit (slit width 0.2 mm) of an imaging spectrometer (Horiba JobinYvon iHR320, Horiba Group, Kyoto, Japan). The light entering the spectrometer was dispersed horizontally using a grating (150 lines/mm, blazed for 500 nm), and the resulting image was captured with a
thermoelectrically cooled silicon charged-coupled device (CCD, Horiba JobinYvon Synapse detector) using an integration time of 0.05 s. A three-dimensional representation of the data collected by the CCD is shown in figure 2b. In figure 2, the x-axis is wavelength; the y-axis corresponds to the vertical dimension of the rectangle in panel (a), as indicated by the slice of the image next to the y-axis; the vertical z-axis is the measured brightness in CCD counts at a particular wavelength and position in space. Thus, figure 2 shows spectra corresponding to the bright regions in the rectangle in panel (a) (and laid out along the y-axis), with one of them highlighted in red—the sample spectrum \( S_{\text{sample}} \). Note that the spectra shown in panel (b) have the background spectrum subtracted. The sample spectrum was then normalized to the spectrum \( S_{\text{reference}} \) of a calibrated specular reflectivity standard (Ocean Optics STAN-SSH), to obtain the normalized per cent reflectivity, \( R \), of a single subcellular multi-layer reflective structure using the formula \( R = 100 \times \frac{S_{\text{sample}}}{S_{\text{reference}}} \). This normalized reflectivity, \( R \), is shown in figure 2c as a function of wavelength, smoothed using nearest neighbour averaging of 10 neighbouring data points. The spectral resolution of the spectrometer as set up for the measurements is ±5 nm. The particular spectrum illustrated (as an example) shows a peak of absolute reflectivity of approximately 6% at approximately 510 nm, corresponding to the green colour seen in the image in figure 2a. Such normalized reflection spectra were obtained for each visually distinguishable bright region from 11 cells from two different squids, with results closely consistent with our previously published measurements obtained from multiple other specimens by a completely independent method [18].

Care was taken to ensure that the spectra collected were from individual Bragg stacks. In addition, while the spectra collected were from within the conical angle of the numerical aperture of the objective (N.A. = 0.8, indicated by the orange triangles in figure 1a,c) the light collected was for essentially normal incidence and reflection for the Bragg stacks. Thus, in figure 1c, the reflected light from the four Bragg stacks on the left is collected by the measurement set-up as indicated by the green check marks, while no light, normally or obliquely incident on the right-most Bragg stack is collected as indicated by the red cross mark. As we were able to isolate spectra from individual Bragg stacks, the spectra were normalized appropriately to a highly specular reflective standard (Ocean Optics STAN-SSH); this is in contrast to the previously common practice of using a diffuse reflection standard [14,16,22] used to normalize reflection spectra from large collections of cells or whole animals. A detailed discussion of the experimental set-up and its advantages and limitations can be found in the work by Ghoshal et al. [18].

2.3. External refractive index fluid

As it is known that the low-refractive index intra-lamellar layers of the iridocyte Bragg stacks are continuous with the extracellular medium [10], the refractive index of the low-refractive index (non-reflectin-containing) region was modified by replacing the extracellular medium with fluids of differing refractive index (but the same osmotic pressure as ASW). We prepared multiple homo-osmolar solutions of different concentrations of Optiprep (a liquid iodixanol density gradient medium of refractive index 1.43—Sigma-Aldrich, MO, USA) buffered with 5 mM tris(hydroxymethyl) aminomethane (pH = 7.4). Optiprep was used to control the refractive indices of the solutions while the buffered sodium chloride solution was used to control osmolarity and prevent cell dessication. The concentration of the saline solution used in each of the different solutions was determined using a Vapro, vapour pressure osmometer 5520 (Wescor Inc., UT, USA). Osmolarities of all of the solutions were adjusted to 170 ± 5 mOsm kg\(^{-1}\), and the concentration of Optiprep was varied—from 0 to 100%, in steps of 10% to obtain corresponding refractive indices, \( n \), of 1.33–1.43 in steps of approximately 0.01 (measured using a Milton Roy LR45217 Refractometer, Milton Roy Company, PA, USA).

Each sample was stored for at least 8 h at 4°C in a particular solution to allow for complete diffusion of the solution into the intra-lamellar spaces of the iridocyte tissue [10], and spectra were taken the following day in the manner described above.

3. Results

As described above, iridocytes were activated with acetylcholine; because this study requires measurements of reductions in reflectivity, only notably bright iridocytes were selected for analysis. For a series of spectra taken from the same Bragg stack immersed in solutions of progressively higher \( n \) ranging from 1.33 to 1.43, the maximum reflectivity, \( R_{\text{max}} \), was identified as indicated in figure 2c and the variation of \( R_{\text{max}} \) as a function of refractive index of the immersion solution is plotted in figure 3a. This process was repeated for a total of 14 spectra from 11 cells (with some spectra taken from different regions of the same cell), as shown in figure 3b.

To verify our results, as a final step each sample was immersed in ASW, equilibrated as described above, and a spectrum was taken; in each case, the final reflectivity was seen to match the previously measured initial values for immersion in ASW (not shown).
Figure 3. (a) Peak reflectivity, \( R_{\text{max}} \), as a function of the refractive index of extracellular fluid, \( n \), for a sample cell. \( R_{\text{max}} \) decreases as \( n \) is increased, pointing to a reflectivity at \( n \approx 1.44 \)—the condition where \( n \) matches the refractive index of the reflectin-containing lamellae \( n_{\text{condensed lamellae}} \). (b) The same data as panel (a), but for all cells studied (11 cells, 14 spectra). Note that in all cases \( R_{\text{max}} \) goes to zero at \( n \approx 1.44 \). For both panels (a,b), the increase in \( R_{\text{max}} \) at intermediate values of \( n \) was calculated to be due to the layers in the Bragg stack meeting the perfect Bragg condition.

4. Discussion

Figure 3b shows a progressive decrease in the observed reflectivity \( R_{\text{max}} \) from 6 to 0.2% with an increase in the refractive index of the immersion solution from 1.33 to 1.43. Thus, as expected, reducing the refractive index contrast between the refractive index of the external solution \( n \) and that of the condensed reflectins in the iridocyte Bragg lamellae \( n_{\text{condensed lamellae}} \) reduces the reflectivity. Using the line in figure 3b as a guide to the eye allows us to estimate that the refractive index range at which \( R_{\text{max}} \) would go to 0, and thus satisfy the zero refractive index contrast condition, is between 1.43 and 1.45; that is, \( 1.43 < n < n_{\text{condensed lamellae}} < 1.45 \). In agreement with this estimate, linear regression analyses indicate a value for \( n_{\text{condensed lamellae}} \approx 1.437 \pm 0.002 \), but we note that the observed variability in refractive index from condensed layer to layer and from sample to sample do not warrant reliance on any apparent statistical significance.

We further note that this directly measured refractive index of the condensed lamellae \( n_{\text{condensed lamellae}} \approx 1.44 \) is consistent with the refractive index range (1.38–1.45) that we determined by analyses of the Bragg lamellae from other specimens using an independent method [18]. That the present measured values of \( n_{\text{condensed lamellae}} \) fell towards the high end of the previously obtained range is easily understood by considering that in this study we only chose the brightest of the iridocytes for the measurements; thus it should be expected that the condensed reflectin-containing lamellae in these cells would have a higher refractive index than in the average iridocyte. (Additionally, as we showed previously, brightness is also a function of the number of lamellae in the Bragg stack [18].) Also as shown previously, the measured values of \( n_{\text{condensed lamellae}} \) are from tissue that is only slightly altered by chemical fixation [18].

It is interesting to note the increased reflectivity (\( R_{\text{max}} \)) in the range \( n = 1.35–1.40 \) (figure 3a,b). This increase in reflectivity was confirmed to be due to the special condition in which both the reflectin-filled lamellae and the interlamellar spaces satisfy the Bragg requirement of periodically repeated structures of optical path length equal to one-fourth of the wavelength of the peak reflectivity (calculations not shown).

We know that the reflectins comprise approximately 18.5% by mass of the total dry weight of the dorsal iridocyte tissue analysed [23], and that all of the reflectins are contained within the Bragg lamellae [15,17]. Our quantitative microscopic analyses of the volumetric density of the Bragg lamellae in the iridocyte tissue thus allow us to consider the value of \( n_{\text{condensed lamellae}} \) to be a close approximation of the refractive index of the condensed reflectins themselves. Our directly measured value of \( n_{\text{condensed lamellae}} \) is lower than values of 1.56 [11] and 1.59 [12] previously reported for reflectin, but those values may be high because they had been obtained for dehydrated films of the reflectin proteins, or owing to inclusion of other components used for tissue preparation for the \( \text{in vivo} \) measurements. The present measurements are consistent both with our previous calculations for the reflectins in their condensed but hydrated state within the Bragg lamellae of live cells [18] and with Cloney and Brocco’s measurements of the refractive index of hydrated reflectins measured \( \text{in vitro} \) [5].

5. Conclusion

We have directly measured the functional and relevant refractive index of the tunable iridocyte Bragg lamellae containing reversibly condensed reflectin proteins in chemically fixed squid dermal iridocytes [18]. The measured value of \( n_{\text{condensed lamellae}} \) from these bright iridocytes falls within the range of values recently determined by an independent optical method [18] is estimated to closely approximate the refractive index of the condensed but hydrated reflectins \( \text{in vivo} \) and is lower than values previously reported for dehydrated and air-dried reflectin films [11,12].

Acknowledgements. We thank Tim Athens at Outerbanks Commercial Fisheries for providing healthy live squid.

Funding statement. This research was supported by the Army Research Office (through grant no. W911NF-10-1-0139 and through contract no. W911NF-09-D-0001 to the Institute for Collaborative Biotechnologies) and by the Office of Naval Research (through MURI award N00014-09-1-0133 to Duke University). E.E. was supported by a Fellowship from University of California Santa Barbara’s Materials Research Laboratory, a Materials Research Science and Engineering Center supported by the National Science Foundation (through grant no. DMR-0080034).
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