Optical parameters of the tunable Bragg reflectors in squid

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Cephalopods (e.g. octopus, squid and cuttlefish) dynamically tune the colour and brightness of their skin for camouflage and communication using specialized skin cells called iridocytes. We use high-resolution microspectrophotometry to investigate individual tunable Bragg structures (consisting of alternating reflectin protein-containing, high-refractive index lamellae and low-refractive index inter-lamellar spaces) in live and chemically fixed iridocytes of the California market squid, Doryteuthis opalescens. This subcellular, single-stack microspectrophotometry allows for spectral normalization, permitting use of a transfer-matrix model of Bragg reflectance to calculate all the parameters of the Bragg stack—the refractive indices, dimensions and numbers of the lamellae and inter-lamellar spaces. Results of the fitting analyses show that eight or nine pairs of low- and high-index layers typically contribute to the observed reflectivity in live cells, whereas six or seven pairs of low- and high-index layers typically contribute to the reflectivity in chemically fixed cells. The reflectin-containing, high-index lamellae of live cells have a refractive index proportional to the peak reflectivity, with an average of 1.405 + 0.012 and a maximum around 1.44, while the reflectin-containing lamellae in fixed tissue have a refractive index of 1.413 + 0.015 suggesting a slight increase of refractive index in the process of fixation. As expected, incremental changes in refractive index contribute to the greatest incremental changes in reflectivity for those Bragg stacks with the most layers. The excursions in dimensions required to tune the measured reflected wavelength from 675 (red) to 425 nm (blue) are a decrease from ca 150 to 80 nm for the high-index lamellae and from ca 120 to 50 nm for the low-index inter-lamellar spaces. Fixation-induced dimensional changes also are quantified, leading us to suggest that further microspectrophotometric analyses of this iridocyte system can be used as a model system to quantify the effects of various methods of tissue fixation. The microspectrophotometry technique described can be expected to provide deeper insights into the molecular and physical mechanisms governing other biophotonically active cells and structures.

1. Introduction

The structural iridescence displayed by cephalopods (e.g. octopus, squid and cuttlefish) is of interest because it is tunable [1–3] and proposed to be used for camouflage and communication [4,5]. These changes in colour and intensity of reflectance are accomplished by modulating multiple layers of alternating high- and low-refractive index material in subcellular structures that function as Bragg reflectors [6] in cells called iridocytes [7]. While earlier workers [1–4,8–17] 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 [18]. Multiple forays [3,7,17,19–23] 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 known as reflectins [24]. While the molecular
pathway and biophysical mechanism governing the neurotransmitter-activated [3], reflectin-mediated, tunable control of iridocyte reflectivity and colour have been elucidated recently [8,16,18], a comprehensive optical characterization of the Bragg reflecting structures in live tissue has remained elusive. Spectral measurements in previous studies [8,12,16,17,22] covered large areas and averaged signal 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. Likewise, analyses of physical cross sections of the Bragg stacks by electron microscopy [3,7,21] are compromised by the potential for artefacts arising from sample preparation, uncertainty of the section angle relative to orientation of the stack and difficulty in correlating the cross-sectional cell to the colour in the original sample. Here, we use microspectrophotometry to investigate the subcellular, tunable reflecting structures in live and fixed iridocytes of the California market squid, Doryteuthis opalescens [25], to determine the relevant physical parameters of the Bragg reflectors. Our high-magnification imaging spectrometer allows us to obtain normalized spectra of optically distinct sections of the individual, subcellular, multi-layer Bragg stacks in iridocytes. We use the well-established transfer-matrix model [26] to analyse the spectral data obtained from these cells, calculating the refractive indices and thicknesses of the high-index (reflectin protein-containing) lamellae and the inter-lamellar layers as well as the numbers of pairs of high- and low-index layers.

2. Material and methods

2.1. Spectroscopic sample preparation, live cells

Doryteuthis opalescens specimens were purchased live from Oterbanks Commercial Fisheries (Oxnard, CA, USA). They were transported under constant oxygen flow to the University of California, Santa Barbara where they were kept in tanks of fresh flowing sea water. If any squid died post-capture, they were placed on ice for a maximum of 24 h before dissection and analysis; the tissue was checked to make sure that it was live—i.e. checked for chromatophore activity—before use in optical analysis. 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 flat in a dissection tray. The dermis from the region under the fin was then carefully removed and pinned out to the native dimensions in a Sylgard 184 (Dow Corning, Midland, MI, USA) coated dish. We specifically used the iridocytes from the bright iridescent stripe directly under the fin; these particularly bright iridocytes, activated upon decapitation of the squid, yielded a better signal-to-noise ratio than other iridocytes in the dorsal part of the dermis (D. G. DeMartini 2012, unpublished data). We verified decapitation-induced activation by injecting acetylcholine post-decapitation into the dermis close to the iridocytes and observed no additional change in reflectivity of the iridocytes; consequently, all measurements were performed on decapitation-activated iridocytes. After pinning, a small section of the tissue was cut, wrapped around and pinned onto a cylindrical piece of Sylgard 184 so that the iridocyte layer was on the outside. A custom cover-glass-bottomed Petri dish was made with a layer of Sylgard 184 cured in the dish, and a central cylinder of the Sylgard 184 cut out to create a well above the cover glass, leaving a doughnut-shaped ring of Sylgard 184 in the dish. The tissue wrapped around the piece of Sylgard 184 was placed in the well and submerged in artificial sea water (ASW, 470 mM NaCl, 10 mM KCl, 27 mM MgCl₂, 29 mM MgSO₄, 11 mM CaCl₂, 10 mM 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid, pH 7.8). The central sample piece of Sylgard 184 was then pinned to the outside doughnut of Sylgard 184 to keep the sample from moving in the ASW during optical measurements. A cross-section schematic of the sample set up for measurement is shown in figure 1a. Note that the specific angle of the tissue and iridocytes relative to the sample stage is not an important factor because of the design of the measurement set-up as discussed in §2.4.

2.2. Spectroscopic sample preparation, chemically fixed cells

The chemically fixed tissue was prepared as above, except that after the initial pinning of the dermis, the sample was chemically treated to fix the tissue in ASW containing 2 per cent formaldehyde for 4 h at room temperature. Following the fixation, the tissue was washed extensively in ASW to remove excess fixative. The samples were then stored at 4°C and prepared for optical measurements as described earlier (figure 1a).
2.3. Refractive index of artificial sea water
Refractive index of ASW was measured using a Milton Roy LR45217 refractometer (Milton Roy Company, PA, USA).

2.4. Microspectrophotometry
Samples in the cover-glass-bottomed Petri dishes were placed on the stage of a Zeiss AxioObserver D1M inverted microscope (Carl Zeiss AG, Oberkochen, Germany), as shown in figure 1a. Increasing detail of the tissue, embedded iridocytes and the interaction with the collection set-up are shown in figure 1b,c and are discussed in detail later. Figure 2a shows a typical bright-field microscopic image of a few iridocytes taken with the microscope, illuminated with a broadband halogen lamp (with detectable light in the range of 200–800 nm) and imaged in reflection mode with a 50× objective (Zeiss EC EpiPlan-NEO-FLUAR, part no. 0000091156528000, 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 reflectivity. This observation is consistent with observations that each of these highly invaginated iridocytes [18] contains multiple stacks of curved lamellae that vary in the number of layers. Thus, each cell consists of several 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 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⁻¹, 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 this figure, the x-axis is wavelength; the y-axis corresponds to the vertical dimension of the rectangle in figure 2a, 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 2b shows spectra corresponding to the bright regions in the rectangle in figure 2a (and laid out along the y-axis), with one spectrum highlighted in red—the sample spectrum S_sample. Note that the spectra shown in figure 2b have the background spectrum subtracted. The sample spectrum was then normalized to the spectrum, S_reference, of a calibrated standard material (Ocean Optics STAN-SSH), with its dark spectrum subtracted, to obtain the normalized percentage reflectivity, R, of a single optical subcellular multi-layer structure using the formula $R = 100 \times S_{\text{sample}} / S_{\text{reference}}$. 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 is ±5 nm. The particular spectrum illustrated (as an example) shows a peak of absolute reflectivity of 6 per cent at approximately 510 nm, corresponding to the green colour seen in figure 2a. Such normalized reflection spectra were obtained from 40 live cells providing 185 spectra, and from 131 fixed cells, providing 521 spectra.

Care was taken to ensure that the spectra collected were from individual Bragg stacks. First, only isolated iridocytes (visually determined to be isolated by a minimum of five times the width of the collection slit in the x- and y-directions, and no other iridocyte in the z-direction) were used for spectral measurements to remove the likelihood of accidentally capturing light from closely neighbouring iridocytes. Second, a measurement set-up was chosen to have a depth of focus of 3.8 μm which is small enough to minimize the collection of light from other reflective parts of the same cell or other cells, yet large enough to cover a depth greater than the maximum number of Bragg lamellae found in the samples (11 × (160 + 140) nm = 3300 nm or 3.3 μm; cf. data from §3). Third, since the spectra are collected only through a small slit and not from the entire image, this isolated the collection area in the x, y and z dimensions; extraneous light was rejected in a fashion similar to that of confocal microscopy. A final test was simply to examine each spectrum to ensure that multiple reflection peaks were not observed. The few spectra (approx. 5% of cases) that displayed multiple peaks were discarded.

While the spectra collected were from within the cone of the numerical aperture of the objective (numerical aperture = 0.8, indicated by the orange triangle in figure 1a), the light collected was for essentially normal incidence and reflection for the Bragg stacks. This was verified by considering the following. For light incident and reflected at normal incidence from a Bragg stack, the spectrum is expected to be symmetrical around the centre wavelength. Since the illumination and collection geometry of bright-field imaging requires collection from a cone of angles, as long as the normal of the Bragg stack lies within the cone of illumination/collection, normal illumination and reflection is always collected. Owing to the geometry of the Bragg stack, any deviation from a normal angle of incidence and reflection
will result in an increase in the wavelength of the light reflected. Any deviation from normal incidence and collection would result in an asymmetric broadening of the measured spectra towards longer wavelengths. However, no such broadening was observed in the measurements, indicating that the light collected from the Bragg stacks is largely under the condition of normal incidence, for Bragg stacks oriented at any angle to the tissue as long as the normal to the Bragg stack lies within the cone of illumination/collection. Figure 1c shows the stack/cone geometry schematically. Only light incident on and reflected from Bragg stacks (dashed black rectangles) whose surface normal (black arrow) lies within the cone of the numerical aperture (orange triangle) of the objective lens is collected and results in productive interrogation; if the surface normal lies outside the cone of the numerical aperture of the objective, the reflection of light incident on the Bragg stack at oblique angles is not collected by the microscope objective and consequently no reflected light is collected from that particular Bragg stack. Thus, 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, and the collected light always includes the case for normal illumination. On the other hand, no light, normally or obliquely incident on the right-most Bragg stack, is collected as indicated by the red cross mark. The power of the method of optical characterization is that it automatically excludes data from Bragg stacks whose normal does not lie in the cone of illumination/collection of the microscope objective.

The measured spectra and reflectivities presented in this work were obtained from spectra normalized to a highly specular reflective standard (Ocean Optics STAN-SSH); this is in contrast to the previous common practice of using a diffuse reflection standard [16,17,27] used to normalize reflection spectra from large collections of cells or whole animals. This choice was governed by the fact that Bragg stacks, including the ones in the iridocytes, demonstrate directional reflectivity (iridescence); since we are able to study individual Bragg stacks (instead of collections of such Bragg stacks), it is appropriate to characterize their reflectance using a directionally reflective specularly reflective standard.

2.5. Modelling and fitting of measured spectroscopic data

To further understand the mechanism controlling the reflectivity and colour of the multi-layer stacks, we created a Matlab [28] routine based on the well-known transfer-matrix method [26] (discussed in detail in the electronic supplementary material) to model the reflectivity of the layers. This program calculates the reflectivity of a multi-layer dielectric stack consisting of a top spacer layer of refractive index \(n_T\), followed by \(N\) pairs of alternating layers of low- and high-refractive index \(n_L\) and \(n_H\), and a final bottom layer of refractive index \(n_B\). This is schematically shown in figure 3, illustrating a multi-layer stack of \(N = 7\). Because the low-index inter-lamellar regions are continuous with the extracellular fluid [18], we set the low-refractive index \(n_L = 1.34\) to match the measured refractive index of the ASW bath, which is in free exchange with the exposed iridocyes in the tissue. All refractive indices were assumed to be real (non-absorbing) and constant over the wavelength range considered in this work. A normal angle of incidence was assumed, based on the observation of symmetrical spectral peaks as discussed earlier.

Figure 4 shows the spectrum from figure 2c, with fits provided by the model described earlier. The grey trace is the normalized spectrum, and the dashed black trace is a spectrum calculated from the model, using parameters initially estimated from the peak reflectivity \(R_{\text{max}}\) and corresponding wavelength \(\lambda_{\text{max}}\). The parameters \(n_L, d_L, d_H\) and \(N\) were then varied using Matlab's least-squares fitting routine to minimize the residual, with the obtained fit shown by the solid black trace in figure 4. From these results, it is clear that the fitted reflection spectrum closely matches the reflection spectrum measured experimentally. The obtained fit parameters were \(N = 7\) pairs, \(n_L = 1.40\), \(d_L = 61\) nm and \(d_H = 127\) nm, as shown in the inset in figure 4. Fits for all spectra were obtained as described earlier, and the parameters extracted are discussed in §§3 and 4.

2.6. Transmission electron microscopy

For transmission electron microscopy (TEM) measurements, tissue samples of Doryteuthis opalescens were prepared as described earlier to pin out the dermis. The tissue was chemically fixed in ASW containing 2 per cent formaldehyde plus 2 per cent glutaraldehyde in ASW for 2 h at room temperature. Freshly degassed, deionized water was used for all of the following washing steps. Following chemical fixation, the tissue was washed with water at least three times and then post-fixed (2% OsO₄, 1 h at room temperature). The tissue was then dehydrated in a series of graded ethanol solutions: 25, 50, 75, 90, 100, 100 and 100% (ethanol); followed by solvent exchange into propylene oxide: ethanol solutions: 33, 66, 100, 100 and 100% (propylene oxide). The tissue was transferred to Spurr’s resin (Ted Pella Inc., Redding, CA, USA) through a gradient series of Spurr’s resin: propylene oxide : ethanol solutions: 33, 66, 100, 100 and 100% (ethanol). The tissue was then dehydrated in a series of graded ethanol solutions: 25, 50, 75, 90, 100, 100 and 100% (ethanol); followed by solvent exchange into propylene oxide: ethanol solutions: 33, 66, 100, 100 and 100% (propylene oxide). The tissue was transferred to Spurr’s resin (Ted Pella Inc., Redding, CA, USA) through a gradient series of Spurr’s resin: propylene oxide solutions: 33, 66, 100 and 100% (propylene oxide). The tissue was then dehydrated in a series of graded ethanol solutions: 25, 50, 75, 90, 100, 100 and 100% (ethanol); followed by solvent exchange into propylene oxide: ethanol solutions: 33, 66, 100, 100 and 100% (propylene oxide). The tissue was then dehydrated in a series of graded ethanol solutions: 25, 50, 75, 90, 100, 100 and 100% (ethanol); followed by solvent exchange into propylene oxide: ethanol solutions: 33, 66, 100, 100 and 100% (propylene oxide). The tissue was then dehydrated in a series of graded ethanol solutions: 25, 50, 75, 90, 100, 100 and 100% (ethanol); followed by solvent exchange into propylene oxide: ethanol solutions: 33, 66, 100, 100 and 100% (propylene oxide).

![Figure 3](http://rsif.royalsocietypublishing.org/Downloaded_from_http://rsif.royalsocietypublishing.org/) Schematic of a multi-layer stack of a Bragg reflector, with low- and high-refractive indices of \(n_L\) and \(n_H\), respectively, and corresponding thicknesses \(d_L\) and \(d_H\), respectively. (Online version in colour.)

![Figure 4](http://rsif.royalsocietypublishing.org/Downloaded_from_http://rsif.royalsocietypublishing.org/) Sample normalized reflectivity spectrum from a single multi-layer stack in an iridocyte (grey line). Data were fitted using the transfer-matrix method beginning with a starting fit (dashed black line) and ending with a best fit (solid black line) to the data, as described in §2. Inset text shows final parameters obtained from successive refinements.
Ultranthin sections (approx. 90 nm) were collected onto formvar-coated copper mesh grids and post-stained using 4 per cent uranyl acetate (10 min) followed by four water washes, and 2 per cent lead citrate (2 min) followed by four more washes. Samples were imaged on a JEOL 123 TEM (JEOL Ltd, Tokyo, Japan) operating at 80 kV.

3. Results

3.1. Microspectrophotometry

From each of the spectra obtained as described in §2, the maximum reflectivity $R_{\text{max}}$ and corresponding wavelength $\lambda_{\text{max}}$ are plotted in figure 5. The filled squares are data from spectra of live cells, and the open circles are data obtained from chemically fixed cells. These data show that the majority of the iridocytes analysed display a reflectivity in the range of 5–25%, with the rare brighter cells showing a reflectivity of up to 30 per cent. There is no observable correlation (Pearson’s $R = -0.13$ for live cells, and 0.09 for fixed cells) between the maximum reflectivity and the corresponding wavelength, indicating that the structural parameters that control these two aspects of the reflection are independent of each other. The spectra obtained from live cells all had maxima in the wavelength range 500–675 nm, whereas the spectra from fixed cells covered a much broader range from 425 to 675 nm—there were no blue live cells observed in the samples from which these specific data were taken, although we have observed live blue cells in other experiments, and many blue cells were observed in the chemically fixed tissue.

Figure 6 shows the fit-obtained values of $n_H$ of the reflectin-filled lamellae obtained from fits to individual multi-layer stacks, as a function of the peak wavelength, $\lambda_{\text{max}}$, of the corresponding reflection peaks. Filled squares are data from live cells, and open circles are data from chemically fixed cells. Values of $n_H$ typically lie between 1.38 and 1.44, with rare high values up to $n_H = 1.48$. Average refractive index of the reflectin-containing lamellae from the live cells was $n_{H,\text{live}} = 1.405 \pm 0.012$ and from the fixed cells was $n_{H,\text{fixed}} = 1.413 \pm 0.015$. There is no observed correlation between $n_H$ and $\lambda_{\text{max}}$ (Pearson’s $R = -0.06$).

whereas completely dehydrated (dry) reflectins have a refractive index in the range 1.56 [19] to 1.59 [20]. In addition, we see no observable correlation between $n_H$ and peak wavelength $\lambda_{\text{max}}$ (Pearson’s $R = -0.06$), indicating that the colour of the reflected light is not strongly dependent on the refractive index of the reflectin-containing layer.

Figure 7 shows a direct correlation between the fit-obtained $n_H$ and the peak reflectivity $R_{\text{max}}$, assessed with respect to each discrete number of pairs of layers $N$. While reflectivity and refractive index contrast are not generally linearly related, our data show that in the small reflectivity range (5–35%) of the iridocyte system, a linear approximation does describe the relationship fairly well; the Pearson’s $R$ values for each $N$ lie between 0.96 and 0.99 for live cells (figure 7a) and 0.90 and 0.99 (ignoring one outlying value for each set $N = 4$ and $N = 9$) for chemically fixed cells (figure 7b, fits not shown). This result demonstrates a clear positive correlation between $n_H$ and $R_{\text{max}}$, showing that brighter multi-layer Bragg reflectors typically have a higher $n_H$ value than dimmer ones. The grouping observed in figure 7 corresponding to the discrete values of $N$ is real; it is a result of the physical requirement that $N$ can have only integral values, and the finding (as expected, and confirmed by these data) that refractive index contrast makes the progressively greater contribution to peak reflectivity for those Bragg reflectors with the most layers. We note that the slight separation between the groups is not a physical requirement; it results from the formality of fitting the observed spectra to the Bragg model with an integral number of paired layers, all with the same refractive index, whereas in the tissue not all layers are necessarily well-formed.

Figure 8 shows the frequency of occurrence of the number of pairs of layers, $N$, obtained from fits of the model to the data. Again, the filled squares are for live cells, and the open circles are for chemically fixed cells. For the live cells, of the 185 spectra fitted, 60 per cent of the multi-layer Bragg stacks can be accounted for by $N = 8$ or 9, just under...
3.3. Transmission electron microscopy analysis

Figure 9c shows a sample TEM image of a cross section of portions of three iridocytes, fixed, stained and embedded in resin as described earlier. The dark areas correspond to the reflectin-filled platelets owing to the heavy metal staining of proteins, and the bright areas correspond to the inter-platelet spaces. Thicknesses of the high-index (dark) and low-index (bright) regions were obtained by taking 38 line profiles from separate areas of 18 TEM images corresponding to 38 separate Bragg stacks, and the thicknesses were averaged to obtain a mean thickness $d_{H,TEM}$ and $d_{L,TEM}$ for each cell. The range of thicknesses thus obtained were $d_{H,TEM} = 53–118$ nm and $d_{L,TEM} = 18–100$ nm, and the average thicknesses for the high- and low-index regions were $d_{H,TEM} = 89 \pm 16$ nm and $d_{L,TEM} = 53 \pm 20$ nm, respectively.

Attempts to obtain accurate values for the number of layers (N) from the TEM images pose a serious challenge as presented in §4. Randomly drawn transects, approximately three per cell (total of 95 counts), yield an average value of $N = 8.9 \pm 5$ pairs of layers, with values ranging from 2 to 30 layers. In contrast, transects drawn to choose the maximum number of layers possible give an average value of $N = 16.9 \pm 7$ pairs of layers.

4. Discussion

Analyses of the measured spectra show several important correlations. In figure 5, the observed lack of correlation between $R_{\text{max}}$ and $\lambda_{\text{max}}$ indicates that the colour observed in collections of iridocytes is due to a bias in that population of cells, rather than differences in the inherent brightness of particular colours of iridocytes. These results are in agreement with previous observations that colour and reflectivity variation is due to the physical differences from sample to sample over the wide range of colours observed in the cells.
Figure 9. (a) Reflectin-containing layer thickness, and (b) inter-lamellar space thickness as a function of peak wavelength, $\lambda_{\text{max}}$. For both plots, filled squares are data from live cells, and open circles are data from chemically fixed cells. The thickness of both is higher for redder colours. Note the weak positive correlation for both the reflectin-containing layers and the inter-platelet layers—Pearson’s $R \approx 0.59$ for both $d_h$ and $d_l$. (c) A sample TEM image used to obtain thickness measurements for the high- and low-index regions. The dark parallel stripes are the regions filled with reflectin; the intervening light regions are the inter-lamellar spaces.

Table 1. A summary of the fit-obtained thicknesses of the high- and low-index regions of live, fixed, and resin-embedded (for TEM analysis) cells.

<table>
<thead>
<tr>
<th>Thickness for Various Methods of Sample Preparation</th>
<th>Live Cells</th>
<th>Chemically Fixed Cells</th>
<th>Resin-Embedded Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low-index region, $d_l$</td>
<td>95 ± 14 nm</td>
<td>87 ± 15 nm</td>
<td>53 ± 20 nm</td>
</tr>
<tr>
<td>High-index region, $d_h$</td>
<td>120 ± 15 nm</td>
<td>115 ± 16 nm</td>
<td>89 ± 16 nm</td>
</tr>
</tbody>
</table>

The specific values of $n_H$ obtained from the calculations (1.38–1.44) bear discussion. The prevailing trend in the current literature has been to use values of 1.59 [4,14,15,27,29] or 1.56 [12,21] in calculations and models. Kramer et al. [20] used frustrated total internal reflection to measure the refractive index of recombinant reflectins dried to produce a dehydrated film, obtaining a value of 1.59. Similarly, Denton et al. [19] used interference microscopy in air and water to estimate a refractive index of 1.56 for dried samples of what they thought to be chitin, but later proved to be reflectin [16,30] from cells of the silvery tissue surrounding the squid eyes. In contrast to these studies of the dehydrated reflectins, our measurements show that the reflectin-containing lamellae in the activated (i.e. reflective) state in their live cellular environment have a refractive index in the range of 1.38–1.44, with an average refractive index $n_{H,\text{live}} = 1.405$ and a standard deviation of 0.012, while the fixed cells have an average refractive index $n_{H,\text{fixed}} = 1.413$ and a standard deviation of 0.015. We propose that the value $n_{H,\text{live}} = 1.405 \pm 0.012$ obtained from calculations based on measurements on live cells is more appropriate for use when analysing spectra taken from the intact iridocytes, and for theoretical analyses of their capabilities. We ascribe the difference in values relative to those reported previously to the higher level of hydration of the reflectin-containing lamellae in their native state [31], especially since our measurements show a slightly higher refractive index was obtained from the chemically fixed samples compared with the live tissue. We note that a similar value of 1.42 was obtained by Cloney & Brocco [7] when measuring the refractive index of isolated hydrated reflectin-containing lamellae in vitro [7].
water than the high-index regions—thereby providing a larger volume fraction for dehydration during the various steps of fixation. Brocco & Cloney [7,21] correctly anticipated and demonstrated some reduction in the thicknesses shown in the TEM images because of dehydration during preparation. Consistent with this suggestion, the thicknesses we determined from our spectroscopic analyses, when compared with our TEM measurements, indicate that a 25–45% decrease in thickness of the lamellae and inter-lamellar spacings resulted from the standardized procedure [3,8,10,11,21] of biological sample preparation for TEM, relative to the live tissue used for the optical measurements. While the changes in thickness measurements are dependent on the conditions used, and therefore not meant to be definitive, they indicate that shrinkage due to fixation should be taken into account when analysing TEM images of iridocytes (and other cells as well). The highly periodic nature of the Bragg stacks and the microspectrophotometric measurements presented here can be used as a model system for a detailed analysis of various methods of fixation and their effects on tissue—both protein rich (high-index lamellae) and protein poor (inter-lamellar spaces) tissue. As an example to illustrate the high sensitivity of the system to even mild shrinkage of 4–8% in the chemically fixed cells, microscope images of two cells before and after fixation are shown in figure 10a–d; the yellow cell in figure 10a changes to display green features upon fixation as seen in figure 10c, and the yellow cell in figure 10b shows a more pronounced change to green upon fixation as seen in figure 10f.

We also find from table 1 that the thicknesses of the reflectin-containing lamellae are higher than the inter-lamellar spaces in all cases. Brocco & Cloney [21] analysed TEM images of fixed and resin-embedded tissue to measure the thicknesses of the lamellae to be 90 ± 5.9 nm and the inter-lamellar spaces to be 60 ± 7.9 nm in the non-tunable iridocytes from the skin of the octopus Doryteuthis opalescens. Separately, this method of examining TEM images was used to measure the thicknesses of the reflectin-containing lamellae in the blue-green iris of the same octopus, yielding a value of 95 ± 14 nm [7]. Cooper et al. [3] measured TEMs for the thicknesses of a small number of lamellae from squid skin, finding values ranging from 80 to 180 nm, depending on the cell colour. Our measurements thus are consistent both with these prior studies and with our determinations from TEM images covering a similar range of thickness values for the high-index lamellae and low-index inter-lamellar spaces, as well as consistently showing the reflectin-containing lamellae to be thicker than the inter-lamellar spacing.

Comparing the number of pairs of layers, N, obtained from the fitting of spectra of live and chemically fixed cells shows that 60 per cent of N-values for live cells is accounted for by N = 8 or 9, whereas about 60 per cent of N-values for chemically fixed cells is accounted for by N = 6 or 7. We tentatively attribute this difference to misalignment of some layers during the process of fixation, thus excluding such layers from the optical measurements, and consequently from the spectra-derived results. In addition, we find that the range of N shown in figure 8 is higher than the two to seven pairs of layers typically observed for the dermal iridocytes of D. opalescens [7] which are less bright than the iridocytes we have analysed from under the fin (D. G. DeMartini 2012, unpublished data). Efforts to obtain accurate estimates of the number of layers (N) from TEM images pose a significant challenge (cf. §2 and figure 9c) leaving it up to the experimenter to guess which cross section is optically relevant for the calculation of N. Such estimates are thus extremely prone to the possibility of error and subjective bias. To further complicate matters, we were not able to determine exactly what changes occur in a particular cell during fixation, embedding and dehydration prior to electron microscopy. It is possible that there were more voids (or thick regions of low refractive index) that were collapsed during these processes, but there is no clear way to track such a phenomenon. We are thus unable to directly compare the values of N obtained from the optical measurements and TEM images. However, the TEM images do serve to confirm that the data obtained from the spectra are in the same range as the number of layers seen in TEM images. We expect that the observed voids serve to break up a complete Bragg stack into multiple Bragg stacks with fewer layers, and that these are the determinants of the observed optical behaviour in the live cells. Further analysis of these voids awaits future study.

5. Conclusions

We report the accurate measurement, in live, dynamically tunable iridocytes from Doryteuthis opalescens, of the reflectance spectra of the subcellular Bragg stacks consisting of reflectin-filled lamellae and their inter-lamellar spaces; and using these measurements, we calculate the optical and physical properties of the Bragg stacks. We measured spectra from individual multi-layer Bragg stacks instead of from collections of cells as reported previously [8,9,12,16,17]. This microspectrophotometry allowed us to accurately normalize the spectra using a known specular reflectivity standard, and consequently use the standard model of Bragg reflectance to extract all the parameters of the multi-layer dielectric stack, including the refractive index of the reflectin-containing lamellae. The results of the model show that the reflectin-containing lamellae of the activated iridocyte have an index of refraction ranging from 1.38 to 1.44 with an average of $n_{	ext{av}} = 1.405 \pm 0.012$. We also find that peak reflectivity is proportional to the refractive index $n_1$. The number of layers that contribute to the observed reflectivity is typically eight or nine pairs of low- and high-index
layers. The changes in dimensions required to tune the reflected wavelength from 675 (red) to 425 nm (blue) are a decrease in thickness from ca 150 to 80 nm for the high-index lamellae and a decrease in thickness from ca 120 to 50 nm for the low-index inter-lamellar spaces. Comparison of the results deduced from these optical analyses of live tissue with measurements from TEM indicate that shrinkage of the lamellar thickness of ca 25–45% results from processes used to fix and embed the iridocytes in resin for TEM analysis, but only 4–8% shrinkage results from fixation alone. In this work, we have presented the first analyses of iridocytes with a microspectrophotometric tool; we expect that this tool will aid in gaining deep insight into the molecular and physical mechanisms important in the functioning of similar biophotonically active cells and structures. Additionally, we have used the tool to measure dimensional changes of tens of nanometres owing to the process of fixation; we propose that the iridocyte system studied in the microspectrophotometry set-up can be used as a model system to quantify the effects of various methods of tissue fixation.

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