Measurements of complex refractive index change of photoactive yellow protein over a wide wavelength range using hyperspectral quantitative phase imaging

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A novel optical holographic technique is presented to simultaneously measure both the real and imaginary components of the complex refractive index (CRI) of a protein solution over a wide visible wavelength range. Quantitative phase imaging was employed to precisely measure the optical field transmitted from a protein solution, from which the CRIs of the protein solution were retrieved using the Fourier light scattering technique. Using this method, we characterized the CRIs of the two dominant structural states of a photoactive yellow protein solution over a broad wavelength range (461–582 nm). The significant CRI deviation between the two structural states was quantified and analysed. The results of both states show the similar overall shape of the expected rRI obtained from the Kramers–Kronig relations.

Light-matter interaction is key to the characterization of a sample and is performed using a diverse range of existing methodologies, including visual inspection, scattering analysis, microscopy, and spectroscopy. The light-matter interaction represents the composite of the electric and magnetic responses of material. It can be quantified by a single parameter called the complex refractive index (CRI) described by the expression

\[ \text{CRI}(\lambda) = \sqrt{[1 + \chi_e(\lambda)][1 + \chi_m(\lambda)]} = n(\lambda) + i\kappa(\lambda). \]

Here, \( n \) and \( \kappa \) are the real and imaginary RIs, which describe reflection and transmission by bulk media, and \( \chi_e \) and \( \chi_m \) are the electric and magnetic susceptibilities, and \( \lambda \) is the wavelength of light, respectively. The real and the imaginary parts (\( n \) and \( \kappa \)) have extensively used separately as refractive index and extinction coefficient, respectively. As in Eq. (1), the CRI is a complex value that characterizes the electromagnetic response of the matter. The CRI has been importantly utilized in physical chemistry, analytical chemistry, and structural biology, where light is used as a probe1–7.

In particular, direct measurement of the CRIs of proteins is of great interest to biophysical chemists and because the CRI of a protein is related to its chromophore structure and chromophore environment. As an example, circular dichroism spectroscopy, which analyses the CRI difference between the two circular polarizations of light, has been utilized to determine the secondary structures of proteins8 and ultraviolet-visible spectrophotometry, which measures the imaginary part of the CRI (molar extinction coefficient) has been utilized to determine the protein concentration or property9.

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Unfortunately, despite their powerful molecular characterization capabilities, direct measurement of the CRIs of protein solutions over a wide range of wavelengths has been hindered by limitations in existing instruments. Generally, the real part (RI) and imaginary part (iRI) of the CRI can be separately measured using a reflectometer and an absorption spectrometer, respectively; however, the simultaneous measurement has been hardly realized. Simultaneous CRI measurement would be highly preferable for protein studies with the capability of performing spectroscopic measurements because the proteins are sensitive to surrounding environment such as temperature, pH, and ion concentrations, which may be perturbed during each individual measurement. Simultaneous CRI measurement is crucial especially for proteins that undergo conformational changes upon specific modulations because CRI can be used as a non-invasive and quantitative reporter for the alterations in proteins. Alternatively, Kramers–Kronig (K–K) relations can be utilized to estimate the RI from the measured iRI or vice versa. However, the K–K relations have a severe quantification problem unless the detection wavelength range is sufficiently broad. Spectroscopic ellipsometry can also be used to measure the CRI components simultaneously; however, the target sample needs to be a uniform thin film of known thickness. Thus, generally, the ellipsometry technique is primarily used to observe protein adsorption kinetics, rather than for the optical characterization of protein solutions.

In order to simultaneously measure the CRI components of a protein solution, we employed quantitative phase imaging (QPI) for measurement, and Fourier transform light scattering (FTLS) techniques for analysis of the QPI data. QPI uses the principle of holography to quantitatively and precisely measure both the optical attenuation and phase delay images when light passes through the sample. In other words, QPI uses the optical field image of a sample, which is directly related to the sample’s CRI. The optical field images of both non-biological and biological samples, including colloidal particles, red blood cells, neurons, and tissues, have been successfully measured using QPI techniques. FTLS converts the measured optical field images into light scattering information using numerical far-field propagation. Unlike conventional scattering analysis of bulk samples, FTLS specializes in light scattering analysis of individual microscopic samples, such as microspheres, colloidal particles, red blood cells, bacteria, and tissues. In this work, we induce light scattering inside the protein solution by Immersing a known microsphere in the target solution. Because the light scattering information is highly sensitive to the CRI of the surrounding medium, the CRI of the protein solution can be calculated precisely from the light scattering information using Mie theory.

Using this method, we measured the CRI as a function of wavelengths of both a photoactive yellow protein (PYP) and its excited state by illumination with a blue light. PYP is a protein related to phototaxis signal transduction in *Halorhodopsin halophila*. PYP has been extensively investigated using various spectroscopic techniques because it serves as an important model system for the study of the relation between structural change and signal transduction process. PYP is also of interest as a photosensory module with potential as an optogenetic tool or artificial protein machine because of its small molecular size (125 amino acids, 14 kDa), high solubility, and photoactivity coupled with large structural change. The chromophore of PYP, deprotonated p-coumaric acid, absorbs blue light and demonstrates the conformational change cycle. When irradiated with blue light, the ground state of PYP (pG) decays into two intermediates, pB1 and pB2, at λmax = 465 nm. The pB1 and pB2 decay with timescales on the order of microseconds and hundreds of microseconds, respectively. The other intermediate states between pR1 and pR2 decay with timescales on the order of milliseconds to seconds. Therefore, for the rest of this paper, we simply denote the ground and excited states of PYP as the pG and pB states, respectively.

The absorption spectrum or iRI has been one of the most popular spectroscopic probes, the rRI of PYP has not been extensively studied and only estimated qualitatively. In this work, we precisely quantify the rRI of PYP simultaneously for both the pG and pB states of PYP solution, using the proposed holographic techniques.

**Results**

**Experimental procedure.** In order to obtain the CRI of PYP, we first measured the multi-wavelength light field images of a 100-μm-diameter polymethyl methacrylate (PMMA) microsphere immersed in PYP solution (Fig. 1). The illumination wavelength for the optical field measurement (probe beam) is systematically scanned over a broad visible spectral range. The centre wavelength and bandwidth of the probe beams are defined by the prism, lens, and pinhole used in the illumination. For optical field measurements, a quantitative phase imaging unit (QPIU) was implemented. The QPIU is a common-path full-field interferometer that uses the principle of lateral shearing interferometry (see Supplementary Fig. S1 online for the detailed optical setup). The measurements were performed with and without the pump beam (445 nm peak; M455L3, Thorlabs Inc.) to measure the CRI of both the pG and pB states. To ensure state equilibrium of the PYP solution, we included sufficient idle time (>10 seconds) after switching the LED on or off before taking holograms. For each state, we obtained holograms at eleven different wavelengths ranging from 461 to 582 nm. The wavelengths were carefully selected to fully characterize the CRI of the PYP solution (see Supplementary Table S1 and Fig. S2 for detailed probe beam specifications). The nonlinear effects from the probe beam were negligible, and the intensities of probe beams were normalized in the data analysing process.

From each measured raw holographic image (Fig. 1b), the amplitude and phase images of the immersed microsphere were obtained with a conventional field retrieval algorithm (Fig. 1c,d). The retrieved light field images were converted into angle-resolved light scattering plots using FTLS, as shown in Fig. 1e. In FTLS, the measured optical field of a sample is numerically propagated to the far-field, which creates a direct 2D Fourier
transformation of the optical field information of the sample. The angle-resolved light scattering plots are achieved by azimuthally averaging the 2D light scattering patterns, which greatly increase the signal-to-noise ratio (SNR) of the measurements. As the spatial analogous to Fourier-transform infrared spectroscopy, FTLS provides an unprecedented SNR in measuring scattered light signals owing to Fellgett’s advantage\(^51\). The azimuthal averaging is possible because the imaging target (a microsphere) is azimuthally symmetric. Then, the CRI was extracted by fitting the obtained FTLS results to the Mie scattering theory, which is the exact solution to Maxwell’s equations for light scattering from homogeneous spheres\(^52,53\).

**CRI of PYP solutions in \(pG\) and \(pB\) states.** The CRI values of both the \(pG\) and \(pB\) states of the PYP solution are shown in Fig. 2. The precision or standard deviation of the proposed method at each wavelength is depicted by the error bars. The mean precisions of the CRI are 8.2 \(\times\) 10\(^{-5}\), and 4.2 \(\times\) 10\(^{-5}\), respectively. The decrease in accuracy with increasing wavelength is caused by the bandwidth widening of the probe beam, which reduces the interference efficiency.

The CRI of the PYP decreases monotonically as the wavelength increases (Fig. 2a) and converges to zero for wavelengths longer than 500 nm. The CRI values in the presence of the pump beam (pump—on case) are approximately five times smaller than those of the PYP in the absence of the pump beam (pump—off case). The significant decrease in the CRI in the pump—on case indicates a PYP population transition from the \(pG\) to the \(pB\) state. Since the absorbance of \(pB\) is negligible for the current wavelength range (see Supplementary Fig. S3), we deduce that the non-zero CRI in the pump—on case is caused by the presence of a \(pG\) population. Therefore, the molecular density or concentration of \(pG\) \((\rho_{pG})\) can be determined simply by

\[
\text{CRI}(\rho_{pG}, \lambda_b) = \frac{\ln(10)}{4\pi} \rho_{pG} \lambda_b e(\lambda_b),
\]

where \(\lambda_b\) is the wavelength of the probe beam and \(e\) is the known molecular extinction coefficient of \(pG\). The \(\rho_{pG}\) were measured as 3.44 \(+\) 0.1 mM and 0.56 \(\pm\) 0.1 mM for the pump—off and pump—on cases, respectively (Fig. 2a). Thus, the concentration of the \(pB\) state is found to be 2.88 \(\pm\) 0.1 mM, and the \(pB\) population ratio \((R_{pB} = \text{amount of } pB/\text{amount of } pG)\) is 0.837 \(\pm\) 0.035. Because a continuous light source was used as the pumping source, \(R_{pB} = 1\) is not achievable in this two-state equilibrium system\(^54\). A previous report that used LEDs for continuous illumination showed a similar \(R_{pB}\)\(^55\).

The CRI values of the PYP decrease monotonically as the wavelength increases (Fig. 2b), which is a general phenomenon occurring in protein solutions due to the CRI of water. For a wavelength ranging from 461–582 nm, the CRI in the pump—off case decreased from 1.3518 to 1.3452, and the CRI in the pump—on case decreased from 1.3510 to 1.3450. The pump—on case shows lower CRI values than the pump—off case for the entire wavelength range. Unlike the CRI values, the quantitative CRI values have not been reported previously. Therefore, we verified the measured CRI values using a conventional refractometer (R-5000, ATAGO Co., Ltd). The verification was conducted with an identically prepared PYP solution in the pump—off case. The CRI values cannot be measured...
for short wavelength probes, owing to their lower intensities. The verification results are shown by the dotted grey lines in Fig. 2b, where the error bars indicate the minimum scale of the refractometer.

Similar to the iRI in Eq. (2), the rRI is also a function of molecular density. In order to characterize the PYP independent of the molecular density, we calculated the refractive index increment; that is, the density derivative of the real RI ($\alpha$ = $\partial \mathrm{RI} / \partial \rho$). The refractive index increment of the PYP in the $pG$ and $pB$ states can be calculated individually using the linear equation:

$$
\begin{bmatrix}
\alpha_{pG,\text{pump-off}}(\lambda) \\
\alpha_{pG,\text{pump-on}}(\lambda)
\end{bmatrix}
= \begin{bmatrix}
0 & \rho_{pG,\text{pump-off}} \\
\rho_{pB,\text{pump-on}} & \rho_{pG,\text{pump-on}}
\end{bmatrix}
\begin{bmatrix}
\alpha_{pG}(\lambda) \\
\alpha_{pB}(\lambda)
\end{bmatrix}
+ \alpha_{H_2O}(\lambda),
$$

where $\rho_{pG}$ and $\rho_{pB}$ correspond to the molecular densities of the $pG$ and $pB$ states in the pump-on or pump-off cases, as denoted by the subscript; $\alpha_{pG}$ and $\alpha_{pB}$ are the refractive index increments ($\partial \mathrm{RI} / \partial \rho$) of the PYP in the $pG$ and $pB$ states, respectively; $\alpha_{pG,\text{pump-off}}$ and $\alpha_{pG,\text{pump-on}}$ are the rRI values of the PYP solution in the pump-off and pump-on cases, respectively; and $\alpha_{H_2O}$ is the known rRI of distilled water. The results are shown in Fig. 2c and tabulated in Supplementary Table S2. The difference between $\alpha_{pG}$ and $\alpha_{pB}$ was maximized (approx. 0.3 M$^{-1}$) at 470 nm, and decreased as the wavelength increased.

The $pB$ population ratio ($R_{pB}$) of a state is related to the kinetics of the transition between the $pG$ and $pB$ states of the PYP. The relaxation time $\tau$ of the $pB$ state to $pG$ state can be obtained from the measured $R_{pB}$ with the following equation (see Supplementary Information for details)\cite{57,58}.

Figure 2. Measured CRI and refractive index increment of PYP solution. (a) The red line represents the iRI value of the PYP solution in the absence of pump beam illumination (pump-off). The green line represents the iRI value of the PYP solution in the presence of pump beam illumination (pump-on). The grey dotted line represents the fitted graphs of both of these values, based on Eq. (2). Corresponding concentrations of the ground state PYP are indicated in brackets on each line. (b) The rRI values of the PYP solution in the pump-off (red) and pump-on (green) environments. The grey dotted line represents the rRI result for a pump-off PYP solution obtained from a conventional refractometer for comparison. The inset shows the result after subtracting the rRI of distilled water from the PYP solution. (c) Refractive index increment ($\alpha$) of $pG$ (black) and $pB$ (blue) states. The error bars indicate the standard deviation from five measurements with different microspheres immersed in identical PYP solutions.
\[ \tau = \frac{1}{\phi} \ln\left(10 \cdot \frac{1 - R_{pB}}{R_{pB}} \right) \int \varepsilon(\lambda_p) \frac{\partial I_p}{\partial \lambda_p} d\lambda_p, \]

where \( \hbar \) is Planck’s constant, \( N_A \) is Avogadro’s constant, \( \lambda_p \) is the wavelength of the pump light, \( \phi \) is the photo-cycle quantum yield of PYP, and \( \partial I_p/\partial \lambda_p \) is the spectral density of the pump beam. Inserting \( \phi = 0.35 \) from the literature and \( \partial I_p/\partial \lambda_p \) (see Supplementary Fig. S2) and \( R_{pB} = 0.837 \pm 0.035 \) from the measurement, \( \tau \) was calculated to be \( 77 \pm 27 \) ms. We note that the relaxation time determined here is smaller than those reported by typical time-resolved pump-probe experiments (0.15–2 s)\(^{44,61,62}\). The discrepancy may be related to the different modes of data collection (continuous illumination vs. pump-probe), but the exact origin is not clear at this stage. The accuracy of this calculated \( \tau \) is mainly determined from the uncertainty of \( R_{pB} \) due to the high \( R_{pB} \) sensitivity of \( \tau \) in Eq. (4).

**Discussion**

The deviation in the \( iRI \) between the two structural states of the PYP is to be expected caused by the well-known different extinction coefficients between two states. The \( rRI \), however, is a quantity that relates to the mass or density of the material\(^{63,64}\). Because only conformational change, not mass variation, occurred when the pump beam was turned on or off, the deviation in the \( rRI \) can be considered to arise from density variations resulting from the conformational change.

To explain the results of these measurements, we employ the K–K relations. The K–K relations connect the \( rRI \) and \( iRI \) based on the causality of the response functions. The K–K relations allow for calculation of the \( rRI \) from the \( iRI \) by

\[ rRI(\omega) = 1 + P \int_{-\infty}^{+\infty} \frac{d\omega'}{\pi} \frac{iRI(\omega')}{\omega' - \omega}, \]

or vice versa. Here, \( \omega \) is the angular frequency of light. Although the K–K relations do not provide an exact quantitative solution due to their inherent infinite-integral form, we are still able to obtain qualitative trends for the \( rRI \) from the well-known values of the \( iRI \). The calculated results are shown in Fig. 3. The overall shape of the expected \( rRI \) obtained from the K–K relations matches well with the experimental results of both the pump-on and pump-off cases and shows the largest deviation at a wavelength of 470 nm, in agreement with our result. In order to retrieve the reliable \( rRI \) results from the K–K relations, we find that the nearest absorption peaks should be considered at least. In this works, we expect \( iRI \) measurements over 250 nm – 2,000 nm is required for reliable \( rRI \) results, regarding the second absorption peak of PYP (280 nm)\(^{65}\) and water absorption peaks in infrared regime\(^{66}\).

To provide a more intuitive explanation for the \( rRI \) deviation resulting from the molecular structure change, we introduce the concept of atomic refraction (AR). Historically, AR studies have tabulated the contribution of individual atoms and atomic bonding to the \( rRI \) of a molecule, and in this way have been able to closely predict the \( rRI \) of unknown chemicals\(^{67-69}\). By considering the AR as a microscopic version of our preconception about the relationship between the \( rRI \) and density/mass, the deviation in the \( rRI \) resulting from molecular structure changes can be explained. Further, the AR implies that the \( rRI \) results may help to reveal atomic bonding changes that occur during the protein conformational change.

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**Figure 3.** Comparison between calculated \( \Delta rRI \) trends of the PYP based on the K–K relations (dotted lines) and measurements (solid lines). The solid lines are identical to the inset in Fig. 2b. The red and green curves indicate the case in the absence (pump-off) and the presence (pump-on) of the pump beam, respectively. The \( y \)-axis offset of the dotted lines is set discretionarily because of the ambiguity arising from the integral forms of the K–K relations.
The relation between the CRI and electromagnetic susceptibilities [Eq. (1)]70 should be emphasized once again. Because the susceptibilities of molecules strongly relate to their electric and magnetic dipole moments, measurements of the CRI can provide clues to protein structure. For example, Tamasaku et al. visualized the electron cloud distributions of diamonds with a resolution of 0.54 Å by using extreme-ultraviolet (103 Å–206 Å) light, and the non-linear susceptibility relationship between X-rays and the chosen frequencies71. However, in the current study, the CRI measurements of PYP proteins were performed in solution which makes it challenging to directly translate our CRI measurements into structural changes in the PYP. This is because proteins in solution have arbitrary orientations, which result in the smoothing of directional information. When the directional- and/or polarization-dependent CRIs or the electromagnetic susceptibilities of proteins are measured systemically, they have the potential to provide more useful information on the structure of proteins.

Conclusions
In this work, we presented a method to precisely and quantitatively measure the CRIs of photoactive proteins and their excited states over a wide range of wavelengths. Using a QPI equipped with a wavelength-sweeping source and FTLS, the CRIs of the PYP solution were measured for wavelengths ranging from 461–582 nm.

We found a significant difference in the CRI values of PYP for the absence of a pump beam (pump-off case) and the presence of a pump beam (pump-on case) as a function of wavelength; not only for the rRI, but also for the rRI. We retrieved the refractive index increment values of PYP for both the pG and pB states. The maximum difference between αpG and αpB is approx. 0.3 M⁻¹ at 470 nm. We also explained the reason for the unexpected deviation in rRI by employing the K–K relation and atomic refraction. We expect the extension of measurable wavelength in the UV regime will help the direct examination of the CRI changes in pB states.

The present method measures both the rRI and rI values of photoactive proteins simultaneously over a wide wavelength range, and it will be useful for real-time measurements as well as adding to the body of comprehensive, precise, and quantifiable information on light–matter interactions and directional information. Furthermore, precise measurement of the CRI can provide insight into the application of photoactive proteins in the field of material science and optics.

Methods

Sample preparation. The purified PYP solution (see Supplementary Information) was mixed with PMMA microspheres having a diameter of 100 μm (74214 FLUKA, Sigma-Aldrich, Inc.) that had been washed three times with distilled water. Ten microliters of the mixture were sandwiched between the coverslips, and the edges were sealed with epoxy adhesive.

CRI extraction by Mie theory fitting. To determine the CRI of the PYP solution, we performed nonlinear fitting of the measured FTLS signals using the Mie theory (Fig. 1e). The solution describes the scattering of an electromagnetic plane wave by a homogeneous sphere. We used three fitting parameters: the rRI (n) and the rRI (c) of the PYP solution, and the diameter of the PMMA microspheres. For a robust and automated fitting analysis, the angle-resolved light scattering signals were prepared as a function of \( \sin \theta \) instead of \( \theta \) in order to ensure the same scale for the horizontal axes while adjusting the rRI (n) value because light scattering is dependent on refractive index. During this experiment, we took a photographic image of the microsphere and its surroundings using a CCD camera. We discovered that moving further away from the microsphere’s centre caused the thickness of the PYP solution to increase, causing the CRI to change in response.

Due to the highly oscillatory features of the angle-resolved light scattering curves (Fig. 1e), the direct application of conventional fitting algorithms either shows non-convergent results or is dependent on the initial fitting parameters. Instead, we used a process prior to the fitting processes to find appropriate initial fitting parameters by minimizing the total variance of the extreme positions between the measurement and the Mie theory. These initial parameters yield highly reproducible fitting results. Thereafter, we used a nonlinear fitting algorithm (nlinfit, a built-in function of MatLab76) with these predetermined initial fitting parameters. We expect the fitting method can be further simplified by employing global optimization algorithms such as genetic algorithms72.

PMMA microspheres CRI calibration. Mie scattering is highly dependent on the CRI of both the surrounding medium and the homogeneous sphere. In order to measure the CRI of the PYP solution, the CRI of the PMMA microspheres used should be well-known. However, we found that the CRI of polymers such as PMMA can slightly vary product-by-product, depending on the manufacturing procedure. Therefore, the CRI of the PMMA microspheres should be calibrated beforehand.

For the calibration, we used an identical experimental procedure with PMMA microspheres immersed in distilled water, whose CRI value is known66. The fitting parameters were the rRI, the rRI, and the diameter of the PMMA microsphere. The calibrated CRI of the PMMA microsphere was used for the known parameters in the PYP solution CRI measurements.

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**Author Contributions**

Y.-K.P and H.I conceived and supervised the study. Y.K.P prepared the samples. K.R.L. and Y.K. performed the optical measurements and analysed the data. J.H.H. built the optical setup and provided the analysis tool. All authors discussed the experimental results and wrote the manuscript.

**Additional Information**

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