University of Szeged

Albert Szent-Györgyi Medical School

Doctoral School of Multidisciplinary Medical Science

Photonic application of proteins

PhD thesis booklet

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2023

List of publications

List of full papers directly related to the subject of the thesis

I. **Krekic, S.**; Nagy, D.; Taneva, S. G.; Fábián, L.; Zimányi, L.; Dér, A. Spectrokinetic Characterization of Photoactive Yellow Protein Films for Integrated Optical Applications. *Eur. Biophys. J.* **2019**, *48* (5), 465–473. https://doi.org/10.1007/s00249-019-01353-8. IF: 2.012

II. **Krekic, S.**; Zakar, T.; Gombos, Z.; Valkai, S.; Mero, M.; Zimányi, L.; Heiner, Z.; Dér, A. Nonlinear Optical Investigation of Microbial Chromoproteins. *Front. Plant Sci.* **2020**, *11*, 1567. https://doi.org/10.3389/fpls.2020.547818. IF: 5.753

III. Petrovszki, D.; **Krekic, S.**; Valkai, S.; Heiner, Z.; Dér, A. All-Optical Switching Demonstrated with Photoactive Yellow Protein Films. *Biosensors* **2021**, *11* (11), 432. https://doi.org/10.3390/bios11110432. IF: 5.743

IV. **Krekic, S.**; Mero, M.; Dér, A.; Heiner, Z. Ultrafast all-optical switching using doped chromoprotein films. *J. Phys. Chem. C* **2023**, *127* (3), 1499–1506. https://doi.org/10.1021/acs.jpcc.2c06232. IF: 4.177*

V. **Krekic, S.**; Mero, M.; Kuhl, M.; Balasubramanian, K.; Dér, A.; Heiner, Z. Photoactive yellow protein adsorption at hydrated polyethyleneimine and poly-L-glutamic acid interfaces. Submitted to *Molecules* on April 7, **2023**. https://doi.org/10.26434/chemrxiv-2023-zmtk4. IF: 4.927*

Cumulative impact factor of papers directly related to the subject of thesis: 22.612

List of full papers not related to the subject of the thesis

I. Szekeres, G. P.; **Krekic, S.**; Miller, R. L.; Mero, M.; Pagel, K.; Heiner, Z. The interaction of chondroitin sulfate with a lipid monolayer observed by using nonlinear vibrational spectroscopy. *Phys. Chem. Chem. Phys.*, **2021**, *23*, 13389-13395. https://doi.org/10.1039/D1CP01975A. IF: 3.945

Cumulative impact factor of other full papers: 3.945

*IF based on data for 2021

1. Introduction

The need for faster information transfer and data processing continues to grow every year, which pushes towards innovations that would go around the currently faced problems with miniaturization and heat effects. The implementation of photonic devices is one of the possible solutions to these emerging problems, because they are ideally all-optical, meaning their speed of operation is limited by the optical processes present in the circuit, omitting electronic components. All-optical integrated photonics (IO) is the equivalent of currently prevalent integrated electronics; however, every process is solely optical. The key building blocks of photonic devices are nonlinear optical (NLO) materials which possess properties that can modify light propagation or can store optical information. Photonic devices have started to take their hold in diverse fields such as optical communication, data processing and storage, holography, quantum computing, etc., and the possibilities to make them even more easily accessible and widespread are highly researched. One of the most challenging components of this research is finding or developing materials that possess optimal NLO properties for applicational purposes. Several materials are currently being tested for all-optical operations, however any of them are yet to be implemented in application. The application of biomaterials with π-conjugated electron systems has also been recently considered, since opposed to many other materials in development, they are readily available. Various organisms contain chromoproteins functioning as light detectors and energy converters, and have been perfected for these purposes through millions of years of evolution – making them in theory an ideal candidate for a similar filled purpose in IO. However, before photonic application, we need to consider the material's mechanical stability or the NLO properties which change corresponding with their environment. This makes a full NLO characterization and optimization unavoidable. A promising candidate is the photoactive yellow protein (PYP). PYP's IO characterization and possible application serves as the topic of this PhD thesis.

The photoactive yellow protein is a relatively small (molecular weight of 14 kDa; consists of 125 amino acids), water soluble protein found in purple sulfur bacteria. PYP belongs to the Per–Arnt–Sim (PAS-) domain superfamily, which is a signal transduction α/β pathway structure, commonly found in prokaryotic and eukaryotic organisms. During light-excitation, the PYP's chromophore goes into reversible trans-cis isomerization which prompts the PYP to go through several quasi-stable intermediates before de-isomerization, and return to the ground state. This reaction scheme is also called the protein's photocycle, and is the main factor when considering PYP for IO applications. Due to its size and water-solubility, PYP is a promising

biomaterial for IO applications, however, for application purposes, the refractive index changes accompanying the absorption changes of intermediates is the most important factor.

To extensively investigate the nonlinear properties of PYP in terms of its applicability, a variety of optical methods were applied throughout the thesis such as Z-scan, Broadband Vibrational Sum-Frequency Generation Spectroscopy.

The single-beam Z-scan technique was used to characterise the nonlinear optical properties of glycerol-doped (GL-PYP) films. During a Z-scan measurement, the investigated sample is moved along the optical axis through the focus of a Gaussian laser beam, while the transmitted power or energy in function of sample position (z) is measured through an aperture placed behind the focus in the far field. The Z-scan can either be done with open or closed aperture (OA and CA, respectively) – an OA measurement gives us information of the nonlinear absorption coefficients, while a closed, or small aperture trace yields the sign and magnitude of the nonlinear refractive index n_2. Due to the method's simplicity but high variability it was chosen as the method to monitor the nonlinear refractive index of the GL-PYP in terms of different applied pump intensities and laser repetition rates.

To address the problem of miniaturization in IO, using the PYP in a form that would not hinder the scalability of IO devices is also crucial. To investigate the PYP in quasi-monolayers, vibrational sum-frequency generation (VSFG) spectroscopy was used. VSFG is a second-order laser spectroscopic method, and it is a powerful surface-sensitive tool for the study of orientation, structure and dynamics of molecules located on interfaces between two centrosymmetric or random-oriented media.

To demonstrate switching capabilities, the techniques employing a Mach-Zehnder interferometer (MZI) as an IO-passive device, or by using the transient grating (TG) method have been used. Both techniques utilize the refractive index change and light interference on the sample they are used on, albeit in different ways. By using both techniques, a more thorough analysis could be done on the sample.

Objectives of the thesis

In this work, the structure and NLO properties of PYP films were investigated in consideration if the protein could be used as an IO active component in the future. Preliminary experiments were also done to demonstrate the switching capabilities of glycerol-doped PYP (GL-PYP) films under various circumstances. The thesis constitutes of four sections detailing the different properties, and preliminary switching application of PYP:

1. First, to investigate the possibility of utilizing protein films in IO, my aim was to study certain photocycle intermediates of PYP. Therefore, the photo-induced changes of PYP films were monitored at different relative humidity (RH) to find the optimal environment. To counteract the possible negative effects of humidity on the photocycle, the kinetics of doped PYP films were also investigated.

2. After characterizing the kinetics of the photocycle of PYP, the linear and non-linear refractive index of GL-PYP films had to be determined. The NLO refractive index of PYP films was investigated with the Z-scan technique as a function of the excitation laser pulse parameters, i.e., average and peak intensities, and repetition rate of the pulses.

3. To investigate the potential miniaturization of PYP films, along with their adsorption to solid substrates, the structure of PYP monolayers on top of a single and multilayers of polyelectrolytes was planned to be investigated by applying vibrational sum-frequency generation spectroscopy in different RH environments, in order to create protein monolayers for further IO applications.

4. After studying the structural, kinetic, and material properties of PYP films, the final goal was to demonstrate IO switching, utilizing the PYP's slow intermediate states by using a Mach- Zehnder interferometer with GL- PYP on the MZI's arms. Then, employing the fast, sub-10 ps, transitions at the beginning of the photocycle of PYP, the aim was to demonstrate ultrafast all-optical switching on GL-PYP samples with a transient grating setup.

2. Materials and methods

Film preparation

During the measurements, two different types of films were implemented. For the experiments involving the measurement of the proteins' photocycle kinetics and nonlinear refractive index, glycerol was added as a doping material, while for the vibrational spectroscopy measurements, the used multilayer structure was built by the layer-by-layer (LbL) deposition technique.

One of the major requirements for the optimization of protein films for IO applications is that the films need to have a high optical quality. Due to crack formation when drying the PYP films, glycerol was added, which helped to maintain the homogeneity of the films while also stabilizing the relative humidity inside the samples. To create the samples, the PYP suspensions were mixed with an 87% glycerol solution so as the glycerol constituted 2% of the mixture. The mixtures were sonicated for 1-2 minutes, pipetted onto the surface of interest depending on the conducted measurement, then left to dry for at least 12 hours under an extractor fume hood. For the Z-scan and fast optical switching measurements, the films were sandwiched between two 200μm-thick microscope cover slips, with a 200μm-thick spacer used between the glass slabs, maintaining the film thickness constant. For optical waveguide lightmode spectroscopy (OWLS), absorption kinetics measurements, and the optical switching experiments demonstrated on PYP by CW lasers, the protein-glycerol mixtures were prepared the same way, however, sandwiching was not applied. The environment's relative humidity was kept at 33% during each experiment, while the temperature was kept at 23°C.

For the VSFG measurements, the PYP monolayer was adsorbed on top of a polyelectrolyte multilayer which was prepared by the spray-assisted LbL assembly technique. The chosen polyelectrolytes were the positively charged Polyethylenimine (PEI, branched type) and Poly-L-lysine (PLL); and the negatively charged Poly-L-glutamic acid (PGA), which were all purchased from Sigma-Aldrich and have a molecular weight of 600,000-1,000,000 (branched PEI solution, concentration of ~50% in H_2O), 50,000-100,000 (PGA sodium salt), and >30,000 (PLL hydrochlorid). Measurements were done on six different kinds of surfaces - PEI and PEI+PGA 'monolayer' surfaces, a multilayer finishing off with a PGA surface (PEI+(PGA+PLL)$_{6.5}$), and all these surfaces with PYP adsorbed on top. The prepared PEI stock solution had a concentration of 5 mg/ml, while the PGA and PLL solutions' concentration was 1 mg/ml. The stock solutions were sprayed on CaF_2 windows by first spraying PEI, then alternating between PGA and PLL up to 6.5 layers, ending in a PGA layer. Measurements on PYP were done at high (>85%) RH values, however, additional measurements were done at 3% and 100% in the amide region to monitor structural change on the surface due to shift in humidity. In case of sample reuse, the samples were put in a 100% relative humidity environment for an hour before measurements to ensure proper hydration.

Kinetic absorption spectroscopy

Time-resolved absorption spectra were measured with a home-built pump-probe apparatus on doped films to monitor how the photocycle changes in the presence of added glycerol. The applied pump source was Surelite II Nd:YAG laser with an OPO extension (Continuum, USA) with a wavelength of 445 nm, pulse energy was 2.80 mJ for the excitation of the initial state of PYP. The probe light was an unpolarized beam of a 35-W high-pressure Xenon lamp (Hamamatsu, Japan), and its wavelength was selected by using narrow-band interference filters. The pump and probe beams spatially overlapped on the sample, the repetition rate was 0.1 Hz. The output signals of the PMT were observed with a homemade current-voltage converter.

To measure the difference spectrum of the formed protein state after excitation, light-excitation experiments were carried out with illuminating the samples with a CW laser for an extended amount of time. The probe light's intensity was filtered by neutral density (ND) filters before reaching the sample. An iStar gated ICCD detector (Andor Technology, United Kingdom) was equipped to the measuring spectrograph. A 410-nm 40mW laser diode was used for the PYP film, which was illuminated for 10 seconds before the start of each measurement.

OWLS measurements

During OWLS, by measuring the probe light incidence angles' peak positions on the sample and by using the mode equations of the three-layer waveguide, the refractive index of the protein can be calculated. For the experiments, grating-coupled $Si(Ti)O_2$ slab waveguides created on a glass substrate (n_s=1.53) were used with a grating width of 1 mm, line density of 2400 mm^{-1}, and refractive index between 1.78-1.80 (MicroVacuum Ltd.). The waveguides were put on a rotational table (DPS, Ealing Electro Optics). The applied probe beam was from a 543 nm He-Ne laser (Melles Griot), and the angle of incidence of it on the sample was measured with high precision and with an accuracy of 10^{-4}. The intensity of the coupled light was measured by a photomultiplier tube (PMT, Hamamatsu, Japan) at the end of the waveguide, then amplified by a voltage converter, and recoded by a digital oscilloscope (LeCroy 9310-L).

Z-scan

The utilized laser source was a commercial Yb:KGd(WO$_4$)$_2$ laser oscillator-amplifier system operating at a central wavelength of 1028 nm with adjustable laser repetition rates up to 100 kHz. The sample was probed by linearly polarized 82-fs second harmonic pulses at 514-nm. The used Gaussian beam radius was measured to be 37 μm, while the measured M^2 values were

≤1.1 both in the horizontal and vertical planes. The pulsed Z-scan setup was additionally used for the CW measurements done at 405 nm, using single longitudinal mode, TEM_{00}-spatial-mode, temperature-stabilized diode laser. In this case, the Gaussian beam waist radius was measured to be 36.6 µm. All measurements were detected using a beam profiling camera, and the OA and CA Z-scan traces were extracted from the measured beam profiles using image processing. The OA traces were determined by adding the signal counts of all pixels of the camera in function of z, while the CA traces were obtained by adding the signal counts in a predefined 2D pixel area centered on the center of gravity of the beam profile.

Broadband Vibrational Sum-Frequency Generation Spectroscopy

The used pump laser source was a $Yb:KGd(WO_4)_2$ laser oscillator-amplifier system, which provided both the femtosecond mid-infrared (MIR) and the picosecond visible (VIS) beams. The tunable MIR pulses were used in either the 2725-3825 cm^{-1} or in the 1400-1700 cm^{-1} spectral range, while the second beam was used to generate the quasi-monochromatic VIS pulses with a center wavelength of 514 nm. A home-made purging system was used along the MIR beam's path to minimize the absorption by atmospheric water vapor and CO_2. The applied VIS laser pulses' energy was at 4 µJ, while for the MIR at 1267, 2980, and 3455 cm^{-1} had been 0.2, 0.7, and 0.7 µJ respectively, which was measured incident on the sample. The VSFG spectra were collected by an imaging spectrometer equipped with a Peltier-cooled CCD (Horiba, Ltd., Japan). The spectral resolution was ~3 cm^{-1}. VSFG spectra were collected in four different spectral ranges - the C-H, N-H and O-H stretching regions and the amide I region -, in two achiral (PPP and SSP) and three chiral (SPP, PSP, PPS) polarization combinations. The acquisition times ranged from 10 s to 120 s, depending on the spectral region and polarization combination. All measurements were done in a near total-reflection-geometry and were carried out using a home-built container. Measurements were done at high relative environmental humidity, and at 23°C room temperature. The measured spectra were finally fitted with Lorentzian peak profiles to accurately determine the position of the vibrational bands.

Optical switching

Switching with an IO Mach–Zehnder interferometer

A single-mode home-build MZI was used. A beam from a green laser diode (532 nm, 50 mW, Roithner, Austria) was coupled into the MZI by a single-mode optical fiber (S630-HP, Thorlabs

GmbH, Germany), which was positioned at the MZI's input by a micro-positioner (DC-3K, Märzhäuser Wetzlar GmbH & Co. KG, Germany), and was fixed with photopolymer glue (OP-66-LS, Dymax Europe GmbH, Germany). The GL-PYP stock solution was pipetted onto both arms of the interferometer in ~1 mm diameter patches and was left to dry before conducting the measurements. To excite different intermediates in the PYP's photocycle, two CW lasers were used at a wavelength of 445 nm and 405 nm, which had an intensity of 4.44 mW and 21.7 mW at the sample, respectively. The duration of the excitation was varied between 2 and 14 seconds. The bias point of the MZI was tuned by a heating wire, to which a voltage varied between 0–4.6 V was applied from a variable DC power supply (VLP 2403pro, Konrad Electronics, Germany). During the measurements, the environment's temperature was 23 °C, with a relative humidity of 33%.

Ultrafast switching demonstrated with transient grating technique

For the ultrafast TG experiments, the same 6 W Yb:KGd(WO$_4$)$_2$ laser system was used as for the VSFG spectroscopy and Z-scan measurements, to create two pump and a probe beams. During experiments, a simplified version of the folded BOXCARS geometry was used. The two pump beams were of either 450 nm or 480 nm, and were derived from the expanded input beam of 2 W, 2 mm diameter, and pulse energies between 1-12.5 nJ at a repetition rate of 1 Hz. The pump beams were focused on the sample by a 50 mm achromat lens to generate the grating and were spatially and temporally overlapping in the sample. The probe beamlet was derived from a beam with an average power of 4 W (i.e., 40 µJ). It had a pulse energy of 3 nJ on the sample, with a central wavelength of 514 nm, and was delayed with respect to the excitation pulses by using a translational stage with 20 fs step sizes. The pulse durations were 160 fs in both of the pump and probe pulses. The time-dependent diffracted probe signal was spatially separated by the incident beams by an output mask and was detected using a miniature fiber optic spectrometer (Ocean Optics, USB2000+, USA). A single-shot spectrum without averaging was recorded from -0.8 to 10 ps, which provided the time-dependent TG signals.

3. Results and Discussion

Photocycle of the glycerol-doped Photoactive Yellow Protein film

Based on the glycerol concentration present in the sample, it is estimated that the water activity inside the sample to be equivalent of a glycerol-devoid PYP film placed in a 80% environment, however, when comparing the PYP films' photocycle in high RH environment, the GL-PYP film has a significantly slower photocycle. This can be attributed to viscosity effects, opposed to water-structure mediated kosmotropic effects, which would be expected to accelerate the photocycle due to destabilization of open conformations - such as the pB intermediate. According to the accumulation measurements, after 10 s illumination with 410-nm, 40mW CW diode laser, most of the protein is in the pB state, hence we can assume that PYP is driven to a steady state of pB and the initial states. The ratio of the intermediates in this steady-state is dependent on and adjusted by the average exciting light intensity applied during Z-scan and switching experiments, and specific simplified photocycle schemes will need to be considered relative to these parameters.

Determining the linear and nonlinear refractive index of PYP films

To successfully access the Δn and n_2 values of the PYP sample from the Z-scan experiments, the linear refractive index values of GL-PYP film need to be determined. By applying the OWLS method, high-resolution scans were done by measuring the angle of incidence in the range where light could be coupled inside the waveguide. The measurements were first conducted with a bare waveguide, then followed by coating the same waveguide with the PYP film. With the applied probe wavelength of 543 nm, the PYP's linear refractive index was measured to be 1.460, which value was then used as basis for the Z-scan experiments.

Z-scan experiments were carried out at multiple different average and peak intensities and laser repetition rates, and with CW excitation as well, to determine their effect on the nonlinear refractive index of the PYP. During Z-scan experiments, it was found that nonlinear response is driven by the average intensity rather than the peak intensity. The absolute value of the nonlinear refractive index is inversely proportional to the average intensity. In case of average intensity being in the range of 0.1 to 1 W/cm^2, n_2 values fall between the range of 10^{-3} to 10^{-4} cm^2/W. At low average intensities, i.e., <0.1 W/cm2, our obtained Δn values of $\sim 10^{-4}$ for PYP is comparable to those of the best solid-state materials.

Investigation of oriented PYP films

Chiral and achiral VSFG spectroscopy was performed to study the adsorption properties of PYP on polyelectrolyte films and layer stacks. Chiral VSFG spectra of PYP on polyelectrolyte multilayers were also measured to monitor the effect of the humidity on the adsorbed PYP layer. It was found that employing multilayer stack leads to a much more homogenous top layer, since structural homogeneity is heavily tied to increasing vibrational band amplitudes and decreasing bandwidths. Acquiring chiral spectra revealed details about the β-scaffold of PYP, while the achiral signal was not specific to the protein.

In chiral VSFG spectra of adsorbed PYP, the homodyne N-H stretch signal shows a redshift for negatively charged PGA+PYP compared to PEI+PYP. The relative red shift in the PGA+PYP case suggests that PYP keeps its tertiary structure to a higher degree when interacting with PGA than with PEI. It was also found that at low humidity, in spite of a stable beta-sheet secondary structural motif, the PYP loses its external hydrogen shell, which can be observed by the changes in the amide I and II bands in the 1500-1700 cm^{-1} range. At the same time, some minor denaturation, i.e., partial loss of tertiary structure, is also detectable.

Optical switching

IO switching on the GL-PYP was demonstrated with two techniques, utilizing different parts of the protein's photocycle. First, switching using a MZI was demonstrated by continuously illuminating the sample on the interferometer's arms, influencing the intensity at the output of the interferometer. By using two different excitation wavelengths simultaneously, the PYP molecules were first pushed to be in the pB intermediate by CW-excitation at 445 nm, followed by shortcutting the whole photocycle by illuminating the sample by a 405-nm CW laser, which targeted the accumulated pB intermediate. The second excitation not only returns the protein to the ground state faster than the regular decay of the pB state, but enables an extra control of light-switching via the PYP photocycle.

Ultrafast all-optical switching was demonstrated on GL-PYP films by using a TG setup in a BOXCARS geometry. The temporal evolution of the laser-induced concentration grating in the films was monitored for up to 10 ps, while employing either 450 nm or 480 nm wavelength pump laser pulses. The excitation wavelength has a drastic effect on the switching time, due to the varying contribution of stimulated emission from the Franck-Condon to the ground state. The time-dependent diffracted probe intensity was modelled with coupled rate equations. From this, it was determined that the early stage of the PYP's photocycle model was

needed to be extended to obtain a better fit between experiment and theory. Based on this result, creating all-optical logic components is possible by PYP films on a sub-ps timescale, which would enable THz switching speed. Different CW laser intensities at 405 nm were used to bleach the sample, and investigate its effect on the photocycle's dynamics. The diffracted probe intensity decreased in proportion to the bleaching intensity but did not completely vanish. It is expected, that shifting the wavelength towards the red edge of the PYP's ground state absorption spectra, the diffraction can be fully eliminated.

4. Summary

The goal of this thesis was the investigation of photoactive yellow films in the aspect of future integrated optical application. To investigate the topic thoroughly, in the first part of the thesis, doped PYP films were investigated using a variety of spectroscopical methods, to monitor the changes during the photocycle. It was previously demonstrated that at high relative humidities, undoped PYP films have a similar photocycle to that of the protein in solution, however, for the PYP to be applicable in IO further down the line, it needs to be doped for it to remain optically homogenous, at the same time allow high protein concentration. Glycerol was chosen as the doping agent when creating PYP films, since optical homogeneity could be maintained.

In currently used integrated electrical devices, the topic of miniaturization is a pressing issue, and IO faces the same upcoming problems, as well. While applying proteins provides a good alternative, the passive structures need to also be scalable, which poses the question if PYP films could get to as close to monolayer thickness as possible. I investigated this possibility in my thesis by adsorbing PYP onto polyelectrolyte multilayers created by PEI, PGA and PLL with the LbL technique, and then characterized these layers by chiral and achiral VSFG spectroscopy. While the initial reason to investigate PYP on top of polyelectrolyte layers was more in terms of IO applicability, valuable fundamental research results were also obtained.

In the third part of the thesis, the PYP was investigated in terms of applicability, be performing optical switching with a MZI interferometer; and ultrafast optical switching with a transient grating setup. These techniques utilized different parts of the protein's photocycle, and were used to demonstrate the PYP's switching capabilities at different timescales. It was shown that the film-dopant material had minor influence on the switching speed of PYP films in either timescale. In contrast, the excitation wavelength had a drastic effect on the switching. During MZI experiments, it was shown that by applying different excitation wavelengths, the photocycle can be short-circuited, giving opportunity for faster switching than when only applying one-wavelength excitation. During the transient grating experiments, the excitation wavelength had a drastic effect

on the switching time, possibly due to the varying contribution of stimulated emission from the Franck-Condon to the ground state. The results show that an all-optical logic component based on PYP can be envisioned on the sub-ps timescale, enabling THz switching speed.

Based on the findings summarized in this thesis, GL-PYP films are valid options as active materials in IO applications. Producing PYP is cheap, and films made of it possess a high enough refractive index change; meanwhile being small enough and water-soluble to be easily combined with nanostructured materials. Doping the PYP film with glycerol also maintains high optical quality and protein concentration in the films, preventing crack formation, and enabling the photocycle to take place even in low humidity environments, due to the glycerol's water retention. The comprehensive investigation of the GL-PYP's NLO properties was performed by characterizing absorption kinetics, linear and nonlinear refractive indices, and it's IO-switching capability. Investigation of the PYP's monolayer structure on polyelectrolytes was also performed.

For further practical applications, high-repetition-rate switching experiments should be performed. It can be done, e.g., by utilizing the photosensitivity of the primary intermediates of the photocycle, and driving the protein back to the ground-state conformation, similarly as demonstrated in some switching experiments done with MZI. Another way to accomplish high-repetition-rate switching by PYP could utilize the chemical modification of the chromophore by removing the p-coumaric acid via hydroxylamine treatment and subsequently reconstitute the protein-pigment complex by non-isomerizable analogues.