**Raman vibrational signatures of excited states of echinenone in the Orange Carotenoid Protein (OCP) and implications for its photoactivation mechanism**

**Supporting information**

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**Peak shapes in FSRS spectroscopy**

In classical Raman scattering, photons scattered with an energetic surplus must originate from an anti-Stokes process, characterized by extracting vibration energy from the molecule. Conversely, photons scattered with lower than input energy must originate from a Stokes process, where the molecule gains energy from the interaction. Additionally, there is only one possible resonance enhancement, and this is the resonance of the input wavelength with the molecular absorption1,2.

In contrast, in FSRS experiments, this problem is more complicated. In broadband stimulated Raman experiments, where the probe is white light, signals from both the Stokes and anti-Stokes regions result from the difference frequency beating originating from the superposition of pump and probe fields. Both can, in principle, lead to either loss or gain, posing no fundamental restriction to whether vibrational excitation or de-excitation is probed. In equilibrated thermodynamic conditions, the lower frequency modes are always more occupied, so the Stokes region is dominated by Raman gain, and the anti-Stokes region is dominated by Raman loss—both effectively Stokes scattering in the conventional Raman sense. However, in dynamic situations, both regions can be and often are a superposition of both gain and loss processes.

To complicate matters further, when Stokes and anti-Stokes probe regions of the white light are present simultaneously, they can operate in synergy. These poorly understood processes are probably responsible for generally stronger anti-Stokes loss signals compared to Stokes gain signals and a nonlinear response to probe light intensity. The anti-Stokes region differs from the Stokes region in that the probe itself is effectively the Raman pump, while the Raman pump operates as the probe. This means that anti-Stokes effectively sweeps its resonance across the detection range. There is well-established experimental evidence that strong resonant conditions can lead to peak distortion, both in the Stokes and anti-Stokes regions. The limited scope of theoretical works3,4 is inclined to ascribe it to the generation of multiple vibrational coherences simultaneously.

In the case of FSRS applied to the excited state dynamics of carotenoids, resonance with the S2 state absorption, for example, leads to the generation of vibrational coherence both at the S2 and higher excited states, possible even S0 via resonance with a stimulated emission, this is even more valid for the ICT state. These coherences might evolve differently and their interference produces distorted peak shapes. Another experimental observation we have made in our study is that peak distortion is significantly more pronounced in the case of anti-Stokes scattering, which is also more sensitive to vibrational cooling. Fortunately, none of these phenomena are related to the actual frequencies of the modes. So, while FSRS peaks, especially those in the anti-Stokes side of the Rp pulse, might appear dispersive or negative, especially when probing a hot state or a state heavily in resonance, the location of the peaks is always informative about the mode frequency.

In the particular case of the data presented in this work, we assume that especially the S2 state and ICT state are probed in the region where ESA and SE are superposed to various degrees and even evolve over time. This nature of probing two or more resonances simultaneously likely leads to peak shape distortion. This is much more pronounced for the S2 state and the ICT state, which are known to manifest stimulated emission together with ESA. In contrast to that, the S1 state is probed in the region where it has only a very mild S1-Sn resonance, and practically only vibrational coherence at the S1 state is generated and probed, so it does not show much peak distortion. Consistent with the established experimentally documented trend, only anti-Stokes peaks are distorted to a larger extent 3,4.

An additional effect evident in the presented data is referred to as "S2 state re-pumping." When the Rp pulse resonates with the excited state, there is a possibility, instead of forming vibrational coherence through co-interaction with Pr at the excited state, to establish a population at a higher excited state. This process effectively functions as a "re-pump" pulse, employing the terminology of "pump-dump-probe" spectroscopy 5. The re-excitation of the S1 and ICT states of the carotenoid to an even higher excited state most likely results in the formation of the S2 state or higher states, subsequently relaxing through the S2 state 6. The signature of this state then becomes apparent alongside the signature of the original state in resonance. Although the effect is relatively small, the resonance of S2 with the 790 Raman pump surpasses that of any other state, thereby even a minor quantity causing to induce noticeable features associated with the S2 state.

**Detailed methods – FSRS experiment**

All experiments were conducted on a homebuilt set-up constructed around femtosecond Titanium Sapphire amplifier Femtopower (Spectra Physics), generating 4.2 mJ pulses of ~20 fs duration at a repetition rate of 1 kHz and Solstice amplifier (Spectra Physics) both sharing the fs oscillator. Amplifiers were synchronized both by means of electronic triggering and optical delay of the seed prior amplification. FSRS and TA experiment were integrated in one set-up as described below. The experiment is based on a controlled time overlap of three pulses in the sample, denoted as Probe (Pr -probing transient absorption and Raman transition), Raman pump (Rp -driving sample into vibration coherence with the Probe), and Actinic pump (Ap - triggering the desired photoreaction).

To generate the probe pulses, ~0.4 mJ of the laser output was split and used to pump a two-stage optical parametric amplifier to generate 1450 nm pulses of ~40 fs in duration. This output was used as a pump for a single filament supercontinuum generated in a 2-mm CaF2 plate, resulting in a white light with a spectrum covering the spectral region from 370 nm to the near infrared up to 1700 nm. The driving wavelength of 1450 nm was chosen to have the undesired spike in the probe intensity from the white light driving pump matching the first peak of water infrared absorption. A 1 cm cuvette with water was then used as a very efficient notch filter removing the 1450 nm spike resulting in a flat white light covering nearly the whole sensitivity range of the CCD detector. The probe was imaged onto the sample by a spherical mirror to a 50 µm diameter spot, and from the sample into the detection apparatus.

In the detection apparatus, the probe was split in two beams. One part was sent to a pair of a homebuilt grating based high resolution imaging spectrograph for Raman analysis in the Stokes region 750 nm to 950 nm and anti-Stokes region 650 nm to 850 nm. The other part was directed to a prism spectrograph to obtain transient absorption spectra in 370 nm to 1200 nm range. In all three spectrographs, a 58x1024 pixels CCD camera (Entwicklungsbuero Stresing) was used as a linear image sensor via operation in a full vertical binning mode. Cameras were triggered from the lasers at 1 kHz and provided full shot to shot detection with a dynamic range exceeding 30 000:1. Despite the low intensity of the single filament supercontinuum (~pJ/nm), it was possible to fully saturate the dynamic range of the sensors. At saturation level the readout noise of this detector is two orders of magnitude lower then optical shot noise at given intensity so all presented measurements can be considered only optical shot-noise limited.

The Raman pump was generated from ~1.5 mJ of the laser output transmitted via a 4f pulse shaper where a special disc with 96 shifted apertures was spinning at 10 Hz synchronized with the laser. Each aperture transmitted only one ~15 cm-1 interval of wavelengths at the time. This arrangement allowed to produce 96 Raman pulses for each 100 incoming pulses, where each was ~5 cm-1 shifted from each other. Shifted signals are then numerically recombined. Such approach helps to reduce fix-pattern noise and facilitate baseline correction. The Raman pulses were generated in the interval from 770 to 795 nm and resulting Raman spectra represents an average of signal from all Raman experiments conducted over this interval. Four pulses out of 100 were fully blocked to produce a pure transient absorption sequence along with the Raman experiment leading to the cyclic scheme where 96% of time the FSRS signal is measured and 4% of time pure TA signal is measured. The Raman pulses were guided via an optical delay line and then focused by a lens to ~100 µm diameter spot overlapped with the probe. Temporal overlap between Raman pump and Probe was adjusted to achieve maximal stimulated Raman gain while maintaining good spectral resolution. Average Raman pulse energy at the sample was ~3 µJ.

The actinic pulse was generated from 1.5 mJ of the laser output pumping two stage OPA combined with sum frequency generation (TOPAS, Light Conversion). The 480 nm output was guided via a motorized optical delay line and focused into the sample via a lens. The actinic pulse diameter at the sample was ~200 µm at 480 nm. The actinic pulse energy was adjusted to 200 nJ for all experiments, corresponding to photon fluxes of ~4 x 1015 photons per pulse per cm2 at 480 nm. Actinic pulse train was guided via an optical chopper reducing its repetition rate to 500 Hz. Resulting experimental sequence was 96 FSRS experiments and 4 pure transmission measurements recorded for each 100 pulses, in both cases half of them pumped and half of them un-pumped. As a result, for each 100 laser pulses, 48 transient Raman experiments and 2 transient absorption experiments were conducted. Such approach has benefit over the traditional method of using two ordinary choppers where FSRS and TA are measured at an equal fraction of time. This approach is more effective, since TA signals are typically orders of magnitude stronger and require much shorter acquisition times. The transient absorption signal was subtracted from the resulting Raman spectra and all spectrally shifted Raman signals were recombined based on the spectral calibration. Resulting spectra were baseline treated by fitting the baseline by the 6th order polynomial by the least absolute value residual method. We observed that such approach is less prone to artifacts than using the more common least square method. Region from 1450 cm-1 to 1900 cm-1 was excluded from the fit as it almost entirely consists of broad signals that would bias the baseline fit. Since the same probe pulse is used to record the FSRS and TA spectra, we unified the chirp correction for FSRS and TA signals, achieving nearly ideal temporal correspondence between TA and FSRS spectra. Samples were measured in a quartz flow cell with an internal thickness of 1 mm and outer cell wall thickness of 1.25 mm. The sample solution was flown by a peristaltic pump with a flow rate of about 6.0 mL/min.

**OCP** **Protein expression and purification**

To express the Orange Carotenoid Protein (OCP) from *Synechocystis* sp. PCC 6803, a targeted plasmid was engineered with OCP cDNA, optimized for E. coli expression using the GeneOptimizer algorithm. This was inserted into a modified pQE81L vector, introducing an N-terminal 6xHis tag. For incorporating carotenoid cofactors, E. coli was co-transformed with plasmids for β-carotene or zeaxanthin biosynthesis, leveraging *Erwinia uredovora* genes. Following transformation, cells were cultured on LB agar with antibiotics, then grown under specific conditions to ensure effective OCP expression and carotenoid integration, crucial for the protein's functionality.

For expressing the protein, cultures in LB-medium with chloramphenicol and ampicillin were initiated from a starter culture, reaching an optimal density before and after IPTG induction at controlled temperatures. Post-induction, cell harvesting revealed carotenoid synthesis. The cells were lysed using a phosphate buffer, lysozyme, and protease inhibitors, followed by freeze/thaw cycles. The lysate was then centrifuged to remove debris. The supernatant underwent affinity chromatography purification using specific columns, followed by concentration and desalting. This detailed procedure ensured the extraction of a pure protein solution, subsequently stored for future applications. Detailed description of the protein expression is available in the publication 7.

A graph showing a normalized pulse

Figure S1

Difference between OCPr and OCPo ground state FSRS Stokes spectra normalized by integral under the curve.

A graph showing a number of different types of signals

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Figure S2

Difference between OCPr and OCPo ground state FSRS anti-Stokes spectra normalized by integral under the curve.



Figure S3

Ground state FSRS spectra of echinenone in cyclohexane.



Figure S4

Full global analysis including the 70 fs and 140 fs components that are poorly resolved for echinenone in the solvents. In contrast to the EADS from OCPo, the 70 fs and 140 fs FSRS EADS tends to compensate each other in the solvents, cf. Fig.4.



Figure S5

Picosecond FSRS EADS of echinenone in OCP and in solvent with full TA spectra, cf. Fig.5.



Figure S6

Picosecond and longest components with exact peak positions marked, cf. Fig.5.



Figure S7

Longest fitted components. In case of OCPo the green component represents the S\* state, cf. Fig.5.

**Raw data overview**

A close-up of a measuring cup

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Figure S8

Stokes and anti-Stokes Raman raw spectra. Echinenone in cyclohexane. Spectra are not GVD corrected

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![A graph of a graph of a graph

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Figure S9

TA spectra. Echinenone in cyclohexane. Data are not GVD corrected.

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Figure S10

Stokes and anti-Stokes Raman raw spectra. Echinenone in methanol. Spectra are not GVD corrected. The Raman spectra amplitudes from a delay of 0.186 ps up to 20 ps were tripled for better visualization.

 A graph of a building

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Figure S11

TA spectra. Echinenone in methanol. Data are not GVD corrected.

A close-up of a graph

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Figure S12

Stokes and anti-Stokes Raman raw spectra, TA spectra. Echinenone in acetonitrile. Spectra are not GVD corrected.

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Figure S13

TA spectra. Echinenone in acetonitrile. Data are not GVD corrected.

A black and white image of a tower

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Figure S14

Stokes and anti-Stokes Raman raw spectra, TA spectra. Echinenone in acetonitrile. Spectra are not GVD corrected.

A graph on a white background

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Figure S15

TA spectra. OCPo WT. Data are not GVD corrected.



Figure S16

TA and FSRS spectra of OCPr on fs to ps time scales.

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