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# Femtosecond anisotropy excitation spectroscopy to disentangle the $Q_x$ and $Q_y$ absorption in chlorophyll $a$ †

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Chlorophyll  $a$  (Chl  $a$ ) belongs to the most important and most investigated molecules in the field of photosynthesis. The Q-band absorption is central for energy transfer in photosystems and the relative orientation of the  $Q_y$  transitions of interacting chlorophylls governs the energy transfer. Chl  $a$  was well investigated, but a quantitative separation of  $Q_x$  and  $Q_y$  contributions to the Q-band of the Chl  $a$  absorption spectrum is still missing. We use femtosecond Vis-pump – IR-probe anisotropy excitation spectroscopy to disentangle the overlapping electronic  $Q_x$  and  $Q_y$  contributions quantitatively. In an anisotropy excitation spectrum we trace the dichroic ratio of a single vibration, *i.e.* the keto C=O stretching vibration at  $1690\text{ cm}^{-1}$ , as a function of excitation wavelength. The change in dichroic ratio reflects altering  $Q_y$  and  $Q_x$  contributions. We identified  $Q_{x00}$  (0–0 transition of  $Q_x$ ) and  $Q_{x01}$  transition at  $(636 \pm 1)\text{ nm}$  and  $(607 \pm 2)\text{ nm}$ , respectively, and the  $Q_{y01}$  and  $Q_{y02}$  at  $(650 \pm 6)\text{ nm}$ , and  $(619 \pm 3)\text{ nm}$ , respectively. We find that  $Q_x$  absorption, contributes to 50% to 72% at 636 nm and 49% to 71% at 606 nm to the Chl  $a$  absorption at room temperature. The Q band was well modelled by a single vibronic progression for the  $Q_x$  and  $Q_y$  transition of  $(700 \pm 100)\text{ cm}^{-1}$ , and the energy gap between  $Q_{x00}$  and  $Q_{y00}$  was found to be  $(820 \pm 60)\text{ cm}^{-1}$ . This precise description of the hexa-coordinated Chl  $a$  absorption spectrum will foster more accurate calculations on energy transfer processes in photosystems, and advance the detailed understanding of the intricate interaction of chlorophyll molecules with the solvent.

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## 1 Introduction

Absorption spectra of many important molecules such as photoreceptors, metal complexes and different kinds of tetrapyrroles, including chlorophylls, show spectrally overlapping contributions of different electronic transitions. A clear identification of the contributions at a given wavelength is typically connected with calculations in combination with measurements at different experimental conditions.<sup>1–4</sup> However, the interpretation of the experimental results depends strongly on the calculations, which become increasingly difficult for larger molecules. Here, we demonstrate a method based on polarization resolved femtosecond Vis-pump IR-probe spectroscopy to disentangle spectral contributions of different electronic transitions in the electronic ground state, without requiring

theoretical calculations. As an application example we have chosen the decomposition of the Q-band of chlorophyll  $a$  (Chl  $a$ ).

Chl  $a$  plays a fundamental role in photosynthesis, from the initial energy transfer in the light harvesting complex to the electron transfer in the reaction center.<sup>5</sup> In order to understand the different photosynthetic processes, an understanding of Chl  $a$  and its photophysical properties is crucial. Chl  $a$  shares many properties with other cyclic tetrapyrroles. As described by the Gouterman Model,<sup>6</sup> its chlorin macrocycle gives rise to two distinct broad absorption bands: the solet (B)-band at 350 nm to 450 nm and the Q-band at 550 nm to 680 nm. In addition, both the B- and the Q-band are further comprised of two distinguishable electronic states with different geometrical orientations:  $B_x$ ,  $B_y$  and  $Q_x$ ,  $Q_y$ .<sup>7</sup> Here,  $x$  and  $y$  indicate the expected directions of the transition dipole moments (tdm) for the transition between the ground-state and the respective excited-state within the chlorophylls macrocycle plane as depicted in Fig. 1 inset.

Particularly, the Q-band plays a key role in the efficient energy transport.<sup>5</sup> Thus, its physical properties are widely studied using different experimental techniques, including linear dichroism,<sup>8–10</sup> circular dichroism and magnetic circular dichroism (MCD),<sup>11–17</sup> spectral hole-burning<sup>18,19</sup> and

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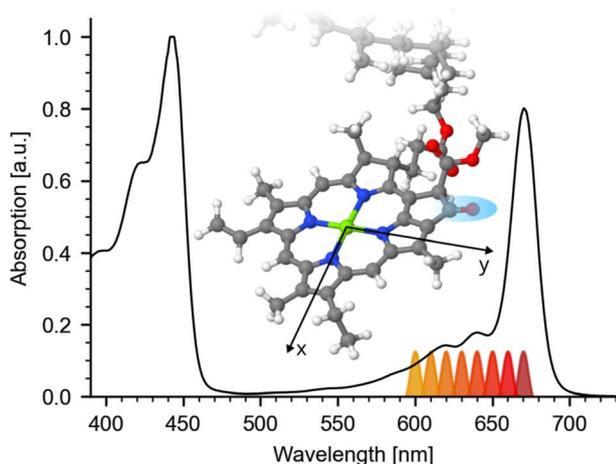


Fig. 1 Visible steady state absorption of hexa-coordinated Chl *a* in pyridine. The spectrum is normalized to its Soret-band at 443 nm. Q-band absorption is exhibiting a maximum at 670 nm followed by a shoulder around 644 nm and high energy side peaks at 619 nm and 585 nm. Different excitation wavelengths for the pump-pulse are indicated below the spectrum. Inset: Geometry optimized structure of Chl *a* macrocycle with *x* and *y* axis (taken from ref. 25). Atoms are color coded: carbon (gray), nitrogen (blue), magnesium (green), oxygen (red), and hydrogen (white). The investigated keto C=O group is marked by a blue region.

fluorescence spectroscopy,<sup>20–22</sup> as well as different time-resolved transient absorption<sup>23–25</sup> and 2D-absorption measurements.<sup>2,26,27</sup> Despite extensive research, the exact composition of the Q-band absorption is not yet fully understood. Even though, the low energetic Q<sub>y</sub> 0–0 transition (Q<sub>y00</sub>) could be assigned to the prominent low energy peak of the Q-band at 660 nm to 670 nm early on,<sup>8,10–13</sup> particularly the assignment of the Q<sub>x</sub> transition long remained controversial,<sup>1,8,10–17,19,20</sup> with different assignment options being considered. Moreover, changes in the macrocycle conformation, induced by penta- or hexa-coordination, induce a shift of the Q<sub>x</sub> band position of Chl *a*,<sup>14,17,20</sup> complicating conclusive assignment. Yet, only in the last decade, new qualitative information on the spectral position of Q<sub>x</sub> and Q<sub>y</sub> was obtained.<sup>17,20</sup> Furthermore, different polarisation resolved studies using femtosecond Vis-pump VIS-probe and 2DES spectroscopy investigated the spectral contributions of Q<sub>x</sub> and Q<sub>y</sub>.<sup>2,23,24</sup> They show strong polarization dependence as a function of probing and pumping wavelengths. Unfortunately, the broad excited state absorption (ESA) of Chl *a* with different anisotropic contributions,<sup>23</sup> together with the broad bleaching (BL) and stimulated emission contributions makes the interpretation very difficult without simplifications.<sup>2</sup> Yet, despite different approaches, up to now, to our knowledge no quantitative disentangling of Q<sub>x</sub> and Q<sub>y</sub> contributions was reported, so that the exact Q<sub>x</sub> and Q<sub>y</sub> absorption spectrum remains unknown.

Here, we apply anisotropy excitation spectroscopy tracing the impact of electronic excitation on the vibrational anisotropy at different excitation wavelengths. Compared to probing electronic transitions, the much narrower line-width and the multitude of vibrational bands enable a precise measurement

of anisotropy changes. However, even though in nature Chl *a* are largely penta-coordinated,<sup>28,29</sup> the two diastereotopic faces (*syn* and *anti*) of penta-coordinated Chl *a*,<sup>30,31</sup> make an unambiguous analysis of anisotropy changes difficult. Thus, using hexa-coordinated Chl *a* in pyridine allows for a clear analysis of its anisotropy excitation spectrum, providing a first clear separation of the Q<sub>x</sub> and Q<sub>y</sub> absorption spectra and a giving a more in-depth understanding of the electronic structure of Chl *a*.

## 2 Materials and methods

### 2.1 Sample preparation

Chl *a* from spinach was purchased from Sigma-Aldrich. All samples were dissolved in pyridine and prepared in sample cells with a thickness of 100 μm, with a sample concentration of 1.5 mM to 3 mM (corresponding to an absorption of ~2.5 OD at 671 nm). In pyridine, Chl *a* is hexa-coordinated and has a flat macrocycle.<sup>14,20,32,33</sup>

### 2.2 Transient absorption

Femtosecond laser pulses were generated starting from pulses delivered by a 1.088 kHz Ti:Sa laser system (Coherent Legend USP, 80 fs pulses at 808 nm). Spectrally tunable visible pump pulses were generated by cascaded OPAs generating fs output pulses of ~6 μJ and (130 ± 20) fs. In detail, a collinear BBO OPA pumped at 400 nm generated near-IR pulses at 1200 nm to 1350 nm, which were further amplified by two KTA amplification stages pumped with the fundamental at 800 nm. The near-IR pulses were then frequency doubled in a BBO-crystal, generating desired pump wavelengths between 600 and 670 nm with a spectral width of FWHM (10 ± 3) nm. A filter (Schott KG4) and a polarizer removed the remaining near-IR frequencies. To ensure photoselection conditions, the pump-pulses were further attenuated to 0.2 μJ to 0.5 μJ, exciting less than 20% of the sample. A λ/2-plate was used to set the polarization of the pump-beam, alternating between perpendicular and parallel pump – probe configuration. The diameter of the pump beam on the sample was (220 ± 40) μm (FWHM), the diameter of the two probe beams on the sample was (150 ± 30) μm (FWHM).

IR probe beams with energies 50 nJ were generated as reported elsewhere.<sup>34</sup> Two reflections of the fs mid-IR pulse were taken, one as probe beam and one as reference. Both beams pass through the same sample volume, with the reference pulse arriving 1.5 ns before the probe, further reducing the shot-to-shot noise.<sup>35</sup> The system response duration was about (300 ± 50) fs. Both IR pulses are detected by dispersing the beams with an imaging spectrograph and recording both beams simultaneously with a 2 × 32 element MCT-array from Infrared Associates. The spectral resolution was better than 3 cm<sup>-1</sup>. The sample was moved with a Lissajous-scanner to ensure a fresh sample volume between consecutive pump pulses. The probe pulses were delayed using a mechanical translation stage.

We excited the isotropic Chl *a* solution with linear polarized excitation pulses introducing a sub-set of oriented excited Chl *a* molecules (photoselection). This generates a transient anisotropy in the sub-set of excited molecules, which decays



through rotational diffusion with  $\tau \sim 90$  ps.<sup>25</sup> On a time-scale between 5 to 15 ps the change in anisotropy of the keto C=O stretching vibration is negligible within the S/N (see ESI Fig. SI 1†).

In Vis-pump IR-probe experiments, only signals from the excited sub-sets are detected. With polarization resolved mid-IR probing, the dichroic ratio  $D$  is given by  $D = \frac{A_{\parallel}}{A_{\perp}}$  for signal amplitudes of parallel  $A_{\parallel}$  and perpendicular  $A_{\perp}$  polarizations with respect to pump-pulse polarization. The ratio of the probed amplitudes reflect the projection of the vibrational tdm onto the excited electronic tdm. In particular, the relative angle  $\theta$  between the excited and probed tdm is given by  $\theta = \arccos(\sqrt{(2D-1)/(D+2)})$ .

### 3 Results and discussion

Steady state absorption of Chl *a* in pyridine is shown in Fig. 1, displaying Soret-band absorption at 390 nm to 460 nm and Q-band absorption at 570 nm to 670 nm. We performed photo-selection experiments creating an oriented sub-set of excited Chl *a* molecules and probed their dynamics by polarization resolved mid-IR spectroscopy. Photoexcitation of the Q-band initiates ultrafast 100 fs to 200 fs dynamics assigned to internal conversion from  $Q_x$  to  $Q_y$ ,<sup>2,23,24,26</sup> followed by population of the  $Q_y$  state, decaying on a nanosecond time-scale,<sup>5</sup> with negligible amplitude changes from 0.4 ps to 15 ps (see ESI Fig. SI 1†). Absorption spectra for different excitation wavelengths averaged over delay times from 5 ps to 15 ps are displayed in Fig. 2. Parallel and perpendicular polarizations with respect to the pump-polarization are shown in black and red, respectively. The spectral region of the keto C=O stretching vibration shows a prominent bleaching (BL) signal at  $1690\text{ cm}^{-1}$  and an associated excited state absorption (ESA) at  $1650\text{ cm}^{-1}$ .<sup>36</sup> Upon visible excitation at the low energy side of the Q-band at 670 nm, a pure  $Q_y$  contribution is observed.<sup>8,10-13</sup> In this case, the observed dichroic ratio  $D$  (670 nm) is around 2.59, with a  $1\sigma$  range from 2.47 to 2.73. This value corresponds to a relative angle  $\theta$  of  $(18 \pm 3)^\circ$  between the keto C=O vibrational tdm and the  $Q_y$  tdm in agreement with earlier findings.<sup>37</sup>

Upon excitation at shorter wavelengths the dichroic ratio decreases from 2.4 at 660 nm to 1.6 at 640 nm. Going to even shorter wavelengths, the ratio increases until 620 nm, then decreases again at wavelength of 600 nm. Since, the dichroic ratio is fixed for a given electronic transition,<sup>38</sup> this twofold decrease indicates two distinct contributions of another electronic transition, *i.e.* the  $Q_x$  transition. The dichroic ratio changes by about 1 as a function of excitation wavelengths, presenting a high anisotropy contrast for the keto C=O stretching vibration in connection to Q-band transitions. Thus, the keto C=O stretching vibration provides a well suited marker band for anisotropy excitation spectroscopy of Chl *a*.

We modelled the keto C=O ESA and BL signals for both polarizations simultaneously with a sum of Lorentzians, as depicted in Fig. 2. Since the dichroic ratio of the vibrational transition is expected to be identical for the BL and ESA band in

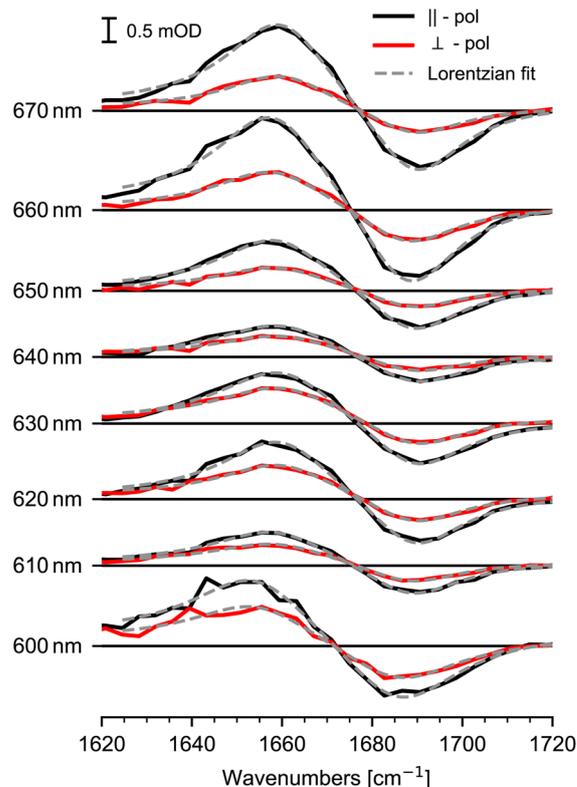


Fig. 2 Transient mid-IR absorption spectra of the keto C=O stretching vibration for different excitation wavelengths averaged over delay times from 5 ps to 15 ps for parallel (black) and perpendicular (red) polarization orientation. Bleaching (negative) signals at  $1690\text{ cm}^{-1}$  reflect the keto C=O stretching vibration in the electronic ground state and (positive) signals at  $1650\text{ cm}^{-1}$  display excited state signals in the  $Q_y$  state. The orientation of the detected sub-set is induced by the effective tdm given by the mixture of excited  $Q_x$  and  $Q_y$  tdm. Lorentzian fits are presented by gray dashed lines.

a rigid molecule,<sup>39</sup> we further constrained the fit by modeling both BL and ESA band with the same dichroic ratio. Errors are calculated using exhaustive search analysis.<sup>39,40</sup> The resulting changes in the dichroic ratio or anisotropy of the keto C=O vibration as a function of excitation wavelength are displayed in the excitation spectrum in Fig. 3a.

#### 3.1 $Q_x$ contribution in the Chl *a* spectrum

Changes in anisotropy directly mirror altering contributions of excited electronic transitions. The dichroic ratio  $D$  and anisotropy  $r$  can be used interchangeably ( $r = (D-1)/(D+2)$ ). Here, the dichroic ratio is directly obtained from modelling the data (see Fig. 3a). Thus, in order to unravel the  $Q_x$  and  $Q_y$  absorption, it is convenient to work with the dichroic ratio. For two contributing transitions  $Q_x$  and  $Q_y$ , the dichroic ratio for different excitation wavelengths  $D(\lambda)$  is a linear combination of the corresponding  $Q_x$  and  $Q_y$  dichroic ratios  $D_{Q_x}$  and  $D_{Q_y}$ :

$$D(\lambda) = (1 - c(\lambda))D_{Q_y} + c(\lambda)D_{Q_x} \quad (1)$$

with  $c(\lambda)$  being the relative contribution of the  $Q_x$  transition between zero and one, and  $1 - c(\lambda)$  being the relative



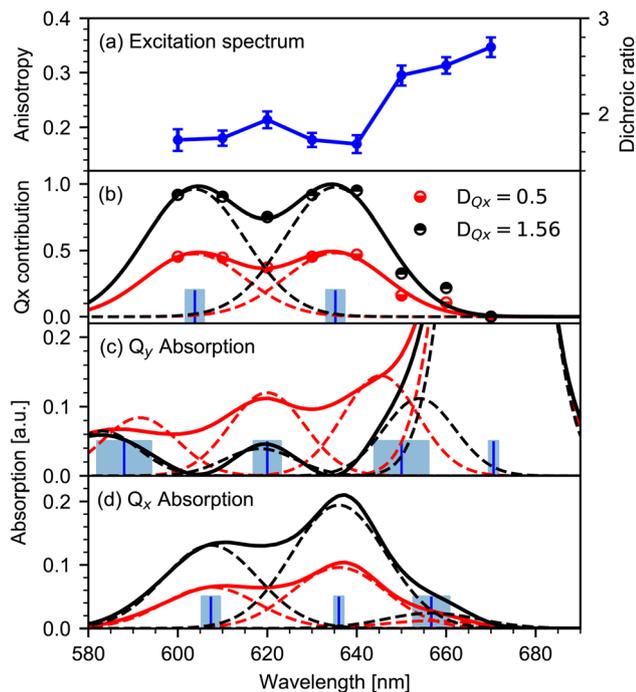


Fig. 3 Mapping the  $Q_x$  and  $Q_y$  absorption spectrum from the anisotropy excitation spectrum. (a) Excitation spectrum of the keto C=O stretching vibration (blue dots) as a function of excitation wavelength. Left scale anisotropy, right scale dichroic ratio. Errors ( $1\sigma$  ranges) are presented for the dichroic ratio. (b) Contribution  $c(\lambda)$  of the  $Q_x$ -transition to the Chl  $a$  absorption spectrum at different wavelengths (dots) calculated from (a). Modelling of  $c(\lambda)$  (solid lines) by two Gaussian peaks (dashed lines) for two limiting  $D_{Q_x}$  values of 1.56 (black) and 0.5 (red). Blue bars represent the center of the Gaussians. Error ranges for  $D_{Q_x}$  values of 1.56 and 0.5 are indicated in light blue. (c)  $Q_y$  absorption spectrum (product of Chl  $a$  absorption spectrum with  $(1 - c(\lambda))$ , solid lines) for the two limiting  $D_{Q_x}$  values (same color code as in b). Modelled spectrum by four Gaussians (dashed lines) with identical widths, and peaks  $Q_{y00}$  at  $(671 \pm 1)$  nm,  $Q_{y01}$  at  $(650 \pm 6)$  nm and  $Q_{y02}$  at  $(619 \pm 3)$  nm. Note, that the last peak at  $(588 \pm 6)$  nm is not covered by the anisotropy excitation experiment. (d)  $Q_x$  absorption spectrum (product of  $c(\lambda)$  with the Chl  $a$  absorption spectrum, solid lines) for the two limiting  $D_{Q_x}$  values (same color code as in b). Modelled spectrum with three Gaussians (dashed lines) with identical widths and peaks  $Q_{x00}$  at  $(636 \pm 1)$  nm,  $Q_{x01}$  at  $(607 \pm 2)$  nm, and  $Q_{x10}$  at  $(657 \pm 4)$  nm.

contribution of the  $Q_y$  transition. The relative contribution  $c(\lambda)$  of the  $Q_x$  transition to the absorption spectrum is given by:

$$c(\lambda) = \frac{D(\lambda) - D_{Q_y}}{D_{Q_x} - D_{Q_y}} \quad (2)$$

In general, the dichroic ratio of a band can vary between 0.5 and 3. However, since the  $Q_x$  contribution cannot exceed 100%, the largest possible value of  $D_{Q_x}$  is 1.56 (see Fig. 3a), therefore limiting the range of  $D_{Q_x}$  to 0.5 to  $-1.56$ .

For the limiting values of  $D_{Q_x}$ , the relative  $Q_x$  contribution  $c(\lambda)$  is presented in Fig. 3b. In the observed range, the relative contribution  $c(\lambda)$  exhibits two peaks at around 635 nm and 605 nm. We modelled the relative contribution  $c(\lambda)$  of  $Q_x$  with two Gaussians centered at  $(635 \pm 2)$  nm and  $(604 \pm 2)$  nm,

as depicted in Fig. 3b (solid lines). Contributions below 600 nm are needed to model the data. Note that for wavelengths below 600 nm the significance of the modelled contribution  $c$  decreases rapidly to shorter wavelengths.

### 3.2 Extracting pure $Q_x$ and $Q_y$ absorption spectra

The individual  $Q_y$  and  $Q_x$  absorption spectra are calculated by the product of the Chl  $a$  absorption spectrum (normalized to the Q-band maximum at 670 nm) with the modelled contributions of  $Q_y$  ( $1 - c(\lambda)$ ) and  $Q_x$  as shown in Fig. 3b. The resulting absorption spectra of the pure  $Q_y$  and  $Q_x$  transitions for the limiting  $D_{Q_x}$  values of 0.5 and 1.56 are depicted in Fig. 3c and d, respectively.

### 3.3 Analysis of the $Q_y$ absorption spectrum

Modelling the  $Q_y$  absorption spectrum with Gaussians of the same spectral widths yields peaks at  $(670 \pm 2)$  nm,  $(648 \pm 5)$  nm and  $(617 \pm 2)$  nm (dashed lines in Fig. 3c). We assign the peak at 671 nm to the 0-0 transition  $Q_{y00}$  and the remaining two peaks to one vibronic progression with 0-1 transition  $Q_{y01}$  at 650 nm and 0-2 transition  $Q_{y02}$  at 619 nm. The frequency differences between adjacent  $Q_y$  peaks is  $(700 \pm 200)$   $\text{cm}^{-1}$ .

In order to model the data, an additional peak at shorter wavelength is needed, yielding a peak position at and  $(588 \pm 6)$  nm. As the peak is out of the spectral range of our measured anisotropy excitation spectrum, it is not possible to determine its exact contribution to the absorption spectrum. However, the spectral position matches the vibronic progression of  $(700 \pm 200)$   $\text{cm}^{-1}$ , suggesting possible 0-3 transition  $Q_{y03}$  at  $(588 \pm 6)$  nm.

Spectroscopic investigations<sup>24,26</sup> on penta-coordinated Chl  $a$  revealed coherent oscillations with a frequency of about 700  $\text{cm}^{-1}$ , showing a substantial coupling between vibrational modes and electronic excitations in this spectral range. Moreover, hole-burning fluorescence spectroscopy revealed vibrational contributions around 700  $\text{cm}^{-1}$  for penta- and hexa-coordinated Chl  $a$ .<sup>20</sup> The peak around  $(648 \pm 5)$  nm was observed before, but was associated either to the  $Q_x$  transition<sup>20</sup> or to the  $Q_y$  transition.<sup>10</sup> With our method we can assign this peak to the  $Q_y$  transition.

### 3.4 Analysis of the $Q_x$ absorption spectrum

The  $Q_x$  absorption spectrum is shown in Fig. 3d, displaying peaks at  $(657 \pm 4)$  nm,  $(636 \pm 1)$  nm and  $(607 \pm 2)$  nm (dashed lines). We assign the peaks  $(636 \pm 1)$  nm and  $(607 \pm 2)$  nm to 0-0 transition  $Q_{x00}$ , and 0-1 transition  $Q_{x01}$  of the  $Q_x$  absorption, respectively. The very small peak around 657 nm is tentatively assigned to the 1-0 transition  $Q_{x10}$ . This contribution should be strongly temperature dependent. Spectral position and assignment of both  $Q_x$  and  $Q_y$  bands is summarized in Table 1.

The frequency difference between  $Q_{x00}$  and  $Q_{x01}$  is  $(700 \pm 100)$   $\text{cm}^{-1}$ , and between  $Q_{x10}$  and  $Q_{x00}$  is  $(500 \pm 200)$   $\text{cm}^{-1}$ . Thus, a vibronic progression in the  $Q_x$  absorption spectrum with a frequency around 700  $\text{cm}^{-1}$  seems plausible.



Table 1 Spectral position of  $Q_x$  and  $Q_y$  absorption bands

Peak [nm]	Peak [ $\text{cm}^{-1}$ ]	Assignment
$671 \pm 1$	$14\,900 \pm 30$	$Q_{y00}$
$657 \pm 4$	$15\,220 \pm 100$	$Q_{x10}^a$
$650 \pm 6$	$15\,400 \pm 150$	$Q_{y01}$
$636 \pm 1$	$15\,720 \pm 30$	$Q_{x00}$
$619 \pm 3$	$16\,160 \pm 80$	$Q_{y02}$
$607 \pm 2$	$16\,480 \pm 60$	$Q_{x01}$

<sup>a</sup> Thermally excited ground state.

### 3.5 Vibronic progressions

The vibronic progression frequency of  $Q_x$  match the one from the  $Q_y$  transitions. Thus, we propose a single vibronic progression of about  $700 \text{ cm}^{-1}$  in the  $Q_x$  and  $Q_y$  absorption spectra. Other more complex interpretations are possible, but our simple model explains the spectroscopic findings in the absorption spectrum reasonably. From our analysis, the  $Q_y - Q_x$  energy gap is  $(820 \pm 60) \text{ cm}^{-1}$ , matching the reported value of about  $875 \text{ cm}^{-1}$ ,<sup>20</sup> and compatible with  $970 \text{ cm}^{-1}$ .<sup>1</sup> The energy gap is close to the frequency of the vibronic progression, making it difficult to reliably determine the contributions from the visible spectra alone. In this context we interpret the reported value of  $1294 \text{ cm}^{-1}$  for the vibronic progression.<sup>10</sup>

### 3.6 Refined absorption spectra

Up to this point, no additional information on the relative orientations of the electronic tdm were taken into account. Following the generally accepted Gouterman Model,<sup>6</sup> the relative angle between the  $Q_x$  and  $Q_y$  tdm is expected to be close to  $90^\circ$ . This is confirmed by experimental studies on hexa- and penta-coordinated Chl *a* reporting angles between  $Q_y$  and  $Q_x$  tdm of about  $70^\circ$  to  $74^\circ$ .<sup>2,10</sup> If we assume the angle between  $Q_y$  and  $Q_x$  tdm to be in the range of  $70^\circ$  to  $90^\circ$ , and combine this information with a maximal angle of  $20^\circ$  between the keto C=O tdm and  $Q_y$  tdm ( $D_{+1,0}(670 \text{ nm}) = 2.47 \hat{=} 20^\circ$ ), we get a maximal relative angle range of  $50^\circ$  to  $90^\circ$  between keto C=O tdm and  $Q_x$  tdm. This correspond to a value range of  $D_{Q_x} = 0.5$ – $1.15$ . Taking this into account, the disentangled  $Q_x$  and  $Q_y$  absorption spectra of Chl *a* in pyridine and their possible variation due to different  $D_{Q_x}$  values are presented in Fig. 4. The sum of  $Q_x$  and  $Q_y$  spectra of corresponding  $D_{Q_x}$  values give the measured Chl *a* absorption spectrum. We find that the  $Q_x$  contribution to the Chl *a* Q-band absorption spectrum between  $590 \text{ nm}$  and  $700 \text{ nm}$  is always smaller than  $(72 \pm 5)\%$ , and is in the range of  $50\%$  to  $72\%$  at  $636 \text{ nm}$  and of  $49\%$  to  $71\%$  at  $606 \text{ nm}$ .

Using the disentangled absorption spectrum, we can determine the oscillator strength ratio between  $f(Q_x)$  and  $f(Q_y)$ . With normalized  $Q_{y00}$  absorption at  $671 \text{ nm}$  and  $Q_{x00}$  absorption strength at  $f(636 \text{ nm}) = 0.14 \pm 0.04$ , the ratio  $f(Q_x)/f(Q_y)$  is between  $5.6$ – $10$ . This is in agreement with a value of  $5.9$  reported for Chl *a* in pyridine<sup>1</sup> and with a value of  $7.5$  for penta-coordinated Chl *a* in ethanol.<sup>24</sup> In addition, from the relative intensities of the  $Q_x$  and  $Q_y$  absorption bands, we calculated the relative Franck–Condon factors (see Table 2) in agreement with earlier findings.<sup>10</sup>

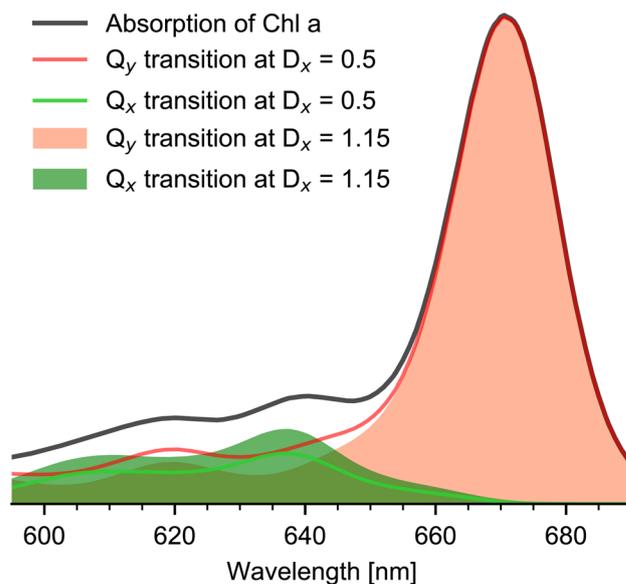


Fig. 4 Chl *a* absorption spectrum and  $Q_x$  and  $Q_y$  contributions. Hexa-coordinated Chl *a* absorption spectrum (black line) in pyridine solution and  $Q_y$  (orange) and  $Q_x$  (green) absorption spectra for the two limiting  $D_{Q_x}$  values  $0.5$  (minimal, solid lines) and  $1.15$  (maximal, filled area). The sum of  $Q_x$  and  $Q_y$  spectra at the same  $D_{Q_x}$  value gives the experimental Chl *a* absorption spectrum.

### 3.7 Discussion

The optical properties of Chl *a* are strongly affected by temperature, macrocycle coordination, and interaction with solvent molecules.<sup>14,20,32,41,42</sup> In the gas phase recent calculations report a  $Q_x - Q_y$  energy gap of  $2400 \text{ cm}^{-1}$ .<sup>43</sup> In condensed phase the energy gap was reported to be around  $900 \text{ cm}^{-1}$ ,<sup>1,20</sup> matching our findings of  $(820 \pm 60) \text{ cm}^{-1}$ . The fraction of  $Q_x$  contribution to the Chl *a* absorption spectrum was estimated between  $10\%$  to  $25\%$  (ref. 1) in agreement with our findings of  $14\%$  to  $27\%$  in the spectral region from  $590 \text{ nm}$  to  $720 \text{ nm}$ .

Moreover, a strong vibronic coupling was predicted that inseparably mixes  $Q_x$  and  $Q_y$  transitions.<sup>1</sup> From our measurements we can state that such a mixing has no or negligible effect on the orientation of the electronic tdm, since the tdm are well distinguishable and are the cause for our observed anisotropy contrast. Hence, the orientation of the excited electronic  $Q_x$  tdm is preserved on a hundred picosecond time-scale. Upon  $Q_x$  excitation, ultrafast energy relaxation to  $Q_y$  is reported, with a time constant of  $150 \text{ fs}$  to  $200 \text{ fs}$ .<sup>2,23,24,26</sup> We found that excitation of Chl *a* in the spectral range between  $606 \text{ nm}$  and  $636 \text{ nm}$  leads to an approximate  $1 : 1$  excitation of  $Q_x$  and  $Q_y$  transitions. Thus, excitation with linear polarized light generates populated

Table 2 Relative Franck–Condon factors of the  $Q_x$  and  $Q_y$  transition calculated from the calculated  $Q_y$  and  $Q_x$  absorption spectra, see Fig. 3c and d (dashed lines), respectively

$Q_x$	$Q_y$
$(Q_{x00} : Q_{x01}) \text{ nm} = 1.5 \pm 0.2$	$(Q_{y00} : Q_{y01}) \text{ nm} = 7 \pm 2$
—	$(Q_{y00} : Q_{y02}) \text{ nm} = 9 \pm 3$



$Q_y$  states along the  $x$ - and  $y$ -axis direction. This is essential for simulating energy transfer processes at various excitation wavelengths in photosystems, e.g. in LHC II.<sup>37,44,45</sup>

We localized the  $Q_{x00}$  transition at 636 nm. For wavelength longer than 650 nm only the  $Q_y$  transition contributes to the Chl  $a$  spectrum, revealing a shoulder at  $(650 \pm 6)$  nm. Up to now, this shoulder remained largely unnoticed, leading prior studies to locate the  $Q_{y01}$  at 613 nm to 619 nm.<sup>1,10,20</sup> We assign the  $Q_{y01}$  to the peak at  $(650 \pm 6)$  nm.

Consequently, in contrast to earlier findings, we explain the substructure of the absorption spectrum of the hexa-coordinated Chl  $a$  between 600 nm to 720 nm by a single vibronic progression with a frequency of about  $700 \text{ cm}^{-1}$ .

## 4 Conclusion

Femtosecond Vis-pump – IR-probe anisotropy excitation spectroscopy is an efficient tool to identify spectral positions and contributions of different electronic transitions within the electronic absorption spectrum. Tracing the pump wavelength dependence of vibrational anisotropy allows for separation of electronic transitions with distinct tdm orientations.

Applying this method on hexa-coordinated Chl  $a$ , we separated  $Q_x$  and  $Q_y$  contributions to the Q-band absorption quantitatively, with 0–0 transition of  $Q_y$  ( $Q_{y00}$ ) at  $(671 \pm 1)$  nm, and  $Q_{x00}$  at  $(636 \pm 1)$  nm. The investigated spectral Q-band range is well modelled by a single vibronic progression with a single frequency of  $700 \text{ cm}^{-1}$  for both the  $Q_x$  and the  $Q_y$  transition. We clearly identified the  $Q_{y01}$  and  $Q_{y02}$  vibronic progression peaks at  $(650 \pm 6)$  nm, and  $(619 \pm 3)$  nm, respectively, and the  $Q_{x01}$  peak at  $(607 \pm 2)$  nm. The  $Q_x$  and  $Q_y$  transition tdm's are well separated and not mixed. The energy gap between  $Q_{x00}$  and  $Q_{y00}$  was specified to  $(820 \pm 60) \text{ cm}^{-1}$ . We identified distinct relative Franck–Condon factors for the  $Q_y$  and  $Q_x$  transitions. The ratio of  $Q_{y00}$  to  $Q_{y01}$ , and  $Q_{y02}$  is  $7 \pm 2$  and  $9 \pm 3$ , respectively. In contrast the ratio of  $Q_{x00}$  to  $Q_{x01}$  is  $1.5 \pm 0.2$ , reflecting a substantially stronger vibronic coupling in the  $Q_x$  transition. The foundation for this contrast may be attributed to the difference in symmetry, but further studies need to explore the details of this contrast. We expect that extending the studies to multiple vibrational transitions may increase accuracy and provide more detailed information.

Our results provide an essential framework for further calculations of energy transfer processes on photosystems and a starting point for comparing Chl  $a$  properties with penta-coordinated macrocycle. Anisotropy excitation spectroscopy is a powerful and straight-forward tool to unravel different electronic ground-state transitions and their vibronic sub-structure in a variety of important photoactive molecules such as porphyrins, corroles, linear tetrapyrroles, metal complexes and photoreceptors.

## Data availability

Data for this paper, including transient polarization resolved data are available at <https://box.fu-berlin.de/5YbB4AamN4jZiYLB>.

## Author contributions

K. H. designed the experiment; C. Z. performed the Vis-IR spectroscopy and analyzed the data, T. S. and K. H. contributed to analyzing the data, C. Z. and K. H. wrote the manuscript with input from all authors.

## Conflicts of interest

There are no conflicts to declare.

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## References

- 1 J. Reimers, Z.-L. Cai, R. Kobayashi, M. Rätsep, A. Freiberg and E. Krausz, *Sci. Rep.*, 2013, **3**, 2761.
- 2 Y. Song, A. Schubert, E. Maret, R. K. Burdick, B. D. Dunietz, E. Geva and J. P. Ogilvie, *Chem. Sci.*, 2019, **10**, 8143–8153.
- 3 F. Santoro, A. Lami, R. Improta, J. Bloino and V. Barone, *J. Chem. Phys.*, 2008, **128**, 224311.
- 4 M. S. Katelyn, J. Dasgupta, J. C. Lagarias and R. A. Mathies, *J. Am. Chem. Soc.*, 2009, **131**, 13946.
- 5 R. E. Blankenship, *Molecular mechanisms of photosynthesis*, Wiley-Blackwell, London, 2nd edn, 2014.
- 6 M. Gouterman, *J. Mol. Spectrosc.*, 1961, **6**, 138–163.
- 7 M. Gouterman and L. Stryer, *J. Chem. Phys.*, 1962, **37**, 2260–2266.
- 8 L. L. Shipman, T. M. Cotton, J. R. Norris and J. J. Katz, *J. Am. Chem. Soc.*, 1976, **98**, 8222–8230.
- 9 D. Bauman and D. Wrobel, *Biophys. Chem.*, 1980, **12**, 83–91.
- 10 M. Fragata, B. Norden and T. Kurucsev, *Photochem. Photobiol.*, 1988, **47**, 133–143.
- 11 C. Houssier and K. Sauer, *J. Am. Chem. Soc.*, 1970, **92**, 779–791.
- 12 C. Weiss, *J. Mol. Spectrosc.*, 1972, **44**, 37–80.
- 13 M. V. Belkov and A. P. Losev, *Spectrosc. Lett.*, 1978, **11**, 653–669.
- 14 M. Umetsu, Z.-Y. Wang, M. Kobayashi and T. Nozawa, *Biochim. Biophys. Acta, Bioenerg.*, 1999, **1410**, 19–31.
- 15 M. Umetsu, Z.-Y. Wang, K. Yoza, M. Kobayashi and T. Nozawa, *Biochim. Biophys. Acta, Bioenerg.*, 2000, **1457**, 106–117.
- 16 J. L. Hughes, R. Razeghifard, M. Logue, A. Oakley, T. Wydrzynski and E. Krausz, *J. Am. Chem. Soc.*, 2006, **128**, 3649–3658.
- 17 J. R. Reimers and E. Krausz, *Phys. Chem. Chem. Phys.*, 2014, **16**, 2315–2322.
- 18 R. Avarmaa and K. Rebane, *Spectrochim. Acta, Part A*, 1985, **41**, 1365–1380.
- 19 J. L. Hughes, B. Conlon, T. Wydrzynski and E. Krausz, *Phys. Procedia*, 2010, **3**, 1591–1599.
- 20 M. Rätsep, J. Linnanto and A. Freiberg, *J. Chem. Phys.*, 2009, **130**, 194501.



- 21 M. Rätsep, Z.-L. Cai, J. R. Reimers and A. Freiberg, *J. Chem. Phys.*, 2011, **134**, 024506.
- 22 M. Rätsep, J. M. Linnanto and A. Freiberg, *J. Phys. Chem. B*, 2019, **123**, 7149–7156.
- 23 P. Martinsson, J. A. Oksanen, M. Hilgendorff, P. H. Hynninen, V. Sundström and E. Åkesson, *Chem. Phys. Lett.*, 1999, **309**, 386–394.
- 24 Y. Shiu, Y. Shi, M. Hayashi, C. Su, K. Han and S. Lin, *Chem. Phys. Lett.*, 2003, **378**, 202–210.
- 25 M. Linke, A. Lauer, T. von Haimberger, A. Zacarias and K. Heyne, *J. Am. Chem. Soc.*, 2008, **130**, 14904–14905.
- 26 E. Meneghin, C. Leonardo, A. Volpato, L. Bolzonello and E. Collini, *Sci. Rep.*, 2017, **7**, 11389.
- 27 N. H. C. Lewis and G. R. Fleming, *J. Phys. Chem. Lett.*, 2016, **7**, 831–837.
- 28 P. Jordan, P. Fromme, H. Witt, O. Klukas, W. Saenger and N. Krauß, *Nature*, 2001, **411**, 909–917.
- 29 A. Ben-Shem, F. Frolow and N. Nelson, *Nature*, 2004, **426**, 630–635.
- 30 T. S. Balaban, P. Fromme, A. R. Holzwarth, N. Krauß and V. I. Prokhorenko, *Biochim. Biophys. Acta, Bioenerg.*, 2002, **1556**, 197–207.
- 31 M. O. Senge, A. A. Ryan, K. A. Letchford, S. A. MacGowan and T. Mielke, *Symmetry*, 2014, **6**, 781–843.
- 32 S. Krawczyk, *Biochim. Biophys. Acta, Bioenerg.*, 1989, **976**, 140–149.
- 33 K. Saito, T. Suzuki and H. Ishikita, *J. Photochem. Photobiol., A*, 2018, **358**, 422–431.
- 34 R. A. Kaindl, M. Wurm, K. Reimann, P. Hamm, A. M. Weiner and M. Woerner, *J. Opt. Soc. Am. A*, 2000, **17**, 2086–2094.
- 35 T. Stensitzki, Y. Yang, V. Kozich, A. A. Ahmed, F. Kössl, O. Kühn and K. Heyne, *Nat. Chem.*, 2018, **10**, 126–131.
- 36 A. S. Holt and E. E. Jacobs, *Plant Physiol.*, 1955, **30**, 553–559.
- 37 M. Kaucikas, K. Maghlaoui, J. Barber, T. Renger and J. Thor, *Nat. Commun.*, 2016, **7**, 13977.
- 38 Y. Yang, D. Jones, T. von Haimberger, M. Linke, L. Wagnert, A. Berg, H. Levanon, A. Zacarias, A. Mahammed, Z. Gross and K. Heyne, *J. Phys. Chem. A*, 2012, **116**, 1023–1029.
- 39 M. Theisen, M. Linke, M. Kerbs, H. Fidder, M. E.-A. Madjet, A. Zacarias and K. Heyne, *J. Chem. Phys.*, 2009, **131**, 124511.
- 40 M. Newville, T. Stensitzki, D. B. Allen and A. Ingargiola, *LMFIT: Non-Linear Least-Square Minimization and Curve-Fitting for Python*, 2014.
- 41 S. B. Broyde and S. S. Brody, *J. Chem. Phys.*, 1967, **46**, 3334–3340.
- 42 I. Renge and R. Avarmaa, *Photochem. Photobiol.*, 1985, **42**, 253–260.
- 43 A. Sirohiwal, R. Berraud-Pache, F. Neese, R. Izsák and D. A. Pantazis, *J. Phys. Chem. B*, 2020, **124**, 8761–8771.
- 44 L. O. Pålsson, M. D. Spangfort, V. Gulbinas and T. Gillbro, *FEBS Lett.*, 1994, **339**, 134–138.
- 45 Y. Shibata, S. Nishi, K. Kawakami, J.-R. Shen and T. Renger, *J. Am. Chem. Soc.*, 2013, **135**, 6903–6914.

