Answers to Prof. Tamás Vidóczy

I am grateful to Prof. Vidóczy for the positive assessment and the thoughtful reading of my work. I appreciate that he has given attention to several technical aspects of the experiments that I have not discussed in detail in the dissertation, but nevertheless I consider important to the validity of the results.

Remarks and questions:

1) *Fig. 2.2. shows the Perrin-Jablonski diagram of Chl. In the sister field of porphyrin research fluorescence from the upper excited state S_2 has been observed – has such an emission for Chls been detected?*

   In porphyrins S_2 corresponds to the Soret or B-band transitions. I am not aware of B-band fluorescence measurements in Chls. The internal conversion in Chls is at least an order of magnitude faster than in porphyrins. We measured the decay lifetime of the B-Q_y transition of Chl a around 140 fs (Bricker et al. 2015). With radiative lifetime in the ns range, the fluorescence quantum yield will be very low – in the order of 10^{-5}. In light-harvesting complexes, the internal conversion is thought to be further enhanced by carotenoids that mediate the energy transfer from B to Q band (Götse et al. 2014, ChemPhysChem 15:3392-401) making the probability for emission virtually zero.

2) *The orientation of samples in anisotropic CD measurements is crucial. Even though larger Even though larger structures like thylakoid membranes are more or less easy to align macroscopically, is it necessary to include some kind of averaging to account for the imperfect alignment?*

   Yes, indeed, perfect orientation is not physically achievable and the measurement averages over a distribution of angles. A formalism to calculate the distribution function has been elaborated (Ganago & Fock 1981, Spectrosc. Lett. 14:405-414). In the case of ACD measurements the effect of imperfect orientation can be approximated by treating the measured ACD as a linear combination of the ACD of an ideally oriented sample and the isotropic CD.

3) *On page 79 it is stated that ACD spectra were independent on the method of orientation –since neither of the two methods of orientation (gel compression and dehydrated film) is likely to result in perfect orientation, and the spectra have a lot of characteristic features (peaks and valleys), it would have been better to show, how much the spectra are similar for the two orientation methods.*

   I agree with the comment. We did show representative spectra obtained by both methods in the article on ACD (Akhtar et al. 2019). It is more correct to say that the ACD spectra of LHCII membranes oriented by dehydration and gel compression have qualitatively similar features. Minor differences can be observed comparing the isotropic CD spectra of the membranes in solution and in gel.

4) *Sample preparations is an important part of experimental techniques, due to the specificity of the nature of LHC II. Washing out the detergent without allowing protein aggregation has been checked with control experiments, where the washed gels were measured after incubation with detergent-*

containing buffer. I am not totally convinced, that such a control proves that the detergent has been washed out completely –please explain your line of reasoning.

Yes, this is an important issue. We adopted a thorough washing procedure to make sure that the detergent is completely removed. A 1.5-mm thick gel slab of was placed in 1 L washing buffer, diluting the medium 10,000 times, and incubated for 2 h on a shaker. Then the buffer was refreshed, and the sample was incubated overnight. We made sure that the detergent was completely washed out by checking the CD spectra and fluorescence lifetimes after repeated washing.

The reason to add detergent back to the washed gels was to check the effect of the detergent without any potential artefacts, such as partial pigment loss that may have occurred during the washing process.

5) *How much sample deterioration has been observed during the synchrotron radiation CD spectroscopy experiments at the Diamond beam line?*

We observed no sample deterioration under the beam. This is because UV light was blocked until the actual UV measurements and, more importantly, for long integration times the beam was not focused on the same spot, but the sample was translated using a motorized stage to scan a larger area. Lastly, the dehydrated LHCII films are extremely resistant to photodegradation – we are preparing a manuscript with this finding.

6) *The experimental technique used to measure fluorescence spectra at low temperature is intriguing. It is stated that the sample contains approximately 0,5 μgChl.cm^{-1}. Is this a typing mistake, should it read cm^{-2}?*

Yes, it should definitely be μg.cm^{-2} – apologies for the mistake.

7) *How was the formation of condensation on the cuvette faces avoided at liquid nitrogen temperature in the confined space of the fluorimeter sample chamber?*

In this experimental setup, condensation build-up could not be completely avoided and had to be monitored visually and removed as it appeared. Microcondensation lowers the signal and introduces noise but has otherwise no significant impact on the spectra. For more precise quantitative measurements we used an optical cryostat, which prevents condensation by design, but these data are not shown in the dissertation.

8) *Please explain the phrase "closing the RC by applying a blue light pulse" (top of page 52.). In the same experimental setup continuous actinic light is employed for the induction of NPQ -does this light not disturb the measurement of Chl fluorescence in the red region?*

The fluorescence induction spectrometer that we designed and developed to measure the NPQ spectra uses two LED light sources. One is used as actinic light to activate the NPQ processes in the leaves. This red light had moderate intensity that was not sufficient to keep the Photosystem II reaction centres in a fully closed state (reduced quinone acceptors). To estimate NPQ, we want to "close" the reaction centres so that the photochemical quenching is zero. Therefore, we apply short saturating pulses and measure the fluorescence intensity only at the end of each applied pulse.

9) *In time resolved fluorescence measurements the instrumental response function was measured using 0,2% milk as scattering medium. What is the advantage of using milk instead of silica? The natural ingredients of milk might give rise to autofluorescence –how can this be ruled out?*

We routinely use colloidal silica (Ludox) as scattering medium, with only one exception of experiments in Berlin, when Ludox was not immediately available. Although milk serves well as a scatterer in the visible region, Ludox is more convenient to store and use.

10) *In 2D spectroscopic measurements an optomechanical chopper was used to "detect and correct for" scattered light. Please explain how comes a mechanical device into play on the timescale used in 2D spectroscopy.*

Because the pump pulses are typically much stronger than the probe pulses, even a small fraction of the pump beam that is scattered by the sample onto the detector can interfere with the measurement. That is why we perform two consecutive measurements – a normal measurement synchronized with the probe and a "blank" measurement, when the probe beam is switched off. This is done by blocking every second probe pulse using a mechanical chopper wheel with a modulation frequency equal to one half of the pulse repetition rate. In this way, a background measurement is performed after each regular measurement. The background, which includes light scattered from the pump, is subtracted.

11) *Discussion of global lifetime analysis and the use of average lifetimes is described too late (on page 85 –Fig. 5.8. on page 63 shows average lifetimes much earlier).*

The global lifetime fitting and the term average fluorescence lifetime are not described specifically in the dissertation. They are widely used in the literature, but I admit it could have been useful to describe them for the non-specialist. I described the global lifetime analysis of 2DES data on page 85, because this approach was developed by me.

12) *I do not see any advantage in using average lifetimes when all the parameters of the multiexponential fitting are known.*

Indeed, the multiexponential fitting parameters convey more information about the kinetics than the average lifetime. The average lifetime is a useful measure of comparison, for example of the degree of quenching, in the context of non-photochemical quenching, or in the Stern-Volmer equation.

13) *It is stated that three lifetimes –0,54 ps, 4,7 ps and 3,2 ns –were necessary to obtain a good fit in the time window of 0,15 to 60 ps; parameter estimation so much outside of the experimental time window must contain a very high uncertainty.*

This is entirely correct – the final decay lifetime (3.2 ns) cannot be accurately determined from this measurement. I should have clarified that the decay lifetime was determined from fitting the kinetics in a longer time range up to 1 ns.

14) *How much does the excitation spectral width (~15 nm) affect the evaluation of downhill/uphill EET (page 88)?*

The excitation pulses used to record the 2DES spectra shown in Fig. 6.4 had spectral width ≥20 nm. This can be verified by observing the width of the 2D spectrum along the excitation wavelength dimension. The pulses span most of the Chl a Q_y band, but the extreme regions (>685 nm) are undetectable. This sets a limitation on the uphill/downhill EET analysis.

However, we have since performed similar measurements with significantly broader pulses, confirming our initial findings.

15) *Singlet-singlet exciton annihilation is mentioned several times (e.g. page 91) –what is known about this interaction? What are the products, where does the excitation energy dissipate?*

Singlet-singlet annihilation is explained in the framework of excitation collision in a molecular array (Suna 1970, Phys. Rev. B. 1:1716). There are different variants of exciton annihilation, but under the excitation conditions of our experiments, it is sufficient to consider S_1–S_1 annihilation (Valkunas et al. 1995, Biophys. J. 69:1117). It can occur when two mobile excitons (pigments in S_1 state) are generated in the connected pigment array. If the array is smaller than the exciton diffusion length, there is a high probability that the excitons collide during their lifetime, whereby the excitation energy of one molecule is transferred to the other. As a result, the first molecule relaxes to the ground state S_0 and the second molecule is in a higher state S_n, conserving the total energy. Because of a rapid $S_n \rightarrow S_1$ transition, after a short time (femtoseconds) the second molecule relaxes to S_1. In effect, one exciton vanishes and the exciton energy is dissipated as vibrational (thermal) energy.

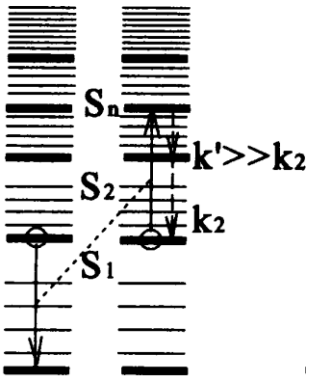


Diagram of electronic energy levels illustrating the S_1–S_1 annihilation pathway (Valkunas et al. 1995)

16) *The absorption features of Chl* a*and* b*are not widely different -are the changes in the Chl* a*and Chl* b*ratio in Bryopsis corticulans responsible for the increased blue-green absorption in this syphonous alga? Isn't the change in the carotenoid composition more important? How does this relate to the*

The absorption features of Chl *a* and *b* in LHCII are different – Chl *a* has absorption maximum between 660–680 nm, whereas Chl *b* absorbs at 640–660 nm. In addition, Chl *b* absorbs well in the blue-green region 480–490 nm. The increased Chl *b* content of Bryopsis enhances absorption of short-wavelength light compared to land plants. The specific carotenoids in Bryopsis further enhance the blue-green absorption around 500 nm.

17) *How does this relate to the mechanism of photoprotection, why is this alga a good model to study photoprotection?*

Bryopsis has a high capacity for photoprotection necessary to survive the fluctuating light conditions in the intertidal areas. One mechanism of photoprotection is the fast and efficient xanthophyll cycle that activates non-photochemical quenching in LHCII.

18) *The functional domain size determination using time resolved fluorescence spectroscopy is very interesting. Unfortunately, neither in the dissertation, nor in the original paper is mentioned, which of the three isomers of dinitrobenzene has been employed as quencher?*

This is an interesting question. I believe I used *m*-DNB but it is possible that *p*-DNB is a more effective quencher.

19) *I have to presume that within the photosynthetic community quenching of Chl fluorescence by PPQ or DNB are well know –as an outsider, I would be very much interested how these compounds quench fluorescence?*

Indeed, the quenching of Chl fluorescence by substituted quinone derivatives and similar compounds is well known and described in numerous reports. The mechanism of quenching is ascribed to electron transfer to the quinone and transient formation of a charge-separated state (Seely 1978, Photochem. Photobiol. 27:639-654), although I am not familiar with reports detecting the radical-ion pair.

20) *In the original publication it is well explained, why the measured quenching formally resembles dynamic quenching (this is not even mentioned in the dissertation) –however such a formal resemblance does not justify the publication of bimolecular quenching rate constants in the 10^{13} and $M^{-1}s^{-}11$range. Such high values are meaningless, impossible for bimolecular reactions, only their relative magnitude may bear importance.*

I agree with this. The absolute values of the quenching rate constants have little physical meaning other than to illustrate that quenching of the connected aggregates is significantly more efficient than pure bimolecular Chl-quinone quenching, which occurs with rate constants in the order of 10^{10} $M^{-1}s^{-1}$ (e.g. Gazdaru 2001, J. Optoelectr. Adv. Mat. 3:145).

21) *I would appreciate an explanation on why and how the same quencher is more effective, when quenching LHC II aggregates as compared to solubilized trimers (the enhanced activity is even more surprising, since the fluorescence lifetime of aggregates is considerably shorter, than that of the solubilized ones)?*

The apparent higher efficiency of the quencher when using LHCII aggregates as compared to solubilized trimers is the basis of our approach to estimate the exciton diffusion length, or functional domain size. We assume that quenching is more effective because of very fast exciton diffusion within the aggregate, which makes the quencher available to the entire aggregate at once and not just a single Chl. Because of the fast exciton diffusion, the apparent quenching rate constant increases with aggregate size, until it gets limited by the exciton diffusion length, determined by the exciton migration rate and lifetime.

22) *The data shown in Fig. 6. 25. are not understandable. It is claimed that panel A shows absorption spectra, while panel B shows the difference of this two spectra together with LHCII only membranes (illustrating, that the difference in panel A is due to LHC II). However the scale in panel A is between 0 and 1,5, it is surprising that the difference spectrum has the same range. While this might be the result of normalizing the spectra, there is a disturbing fact* i.e. *in panel A the difference between the spectra is larger just above 450 nm, as compared to that just below 450 nm, while the difference*

spectrum in panel B is more intense below 450 nm, which cannot be due to any normalization. Please explain.

The difference spectrum is calculated in the following way. First, the absorption spectrum of PSI:LHCII membranes is decomposed by linear least-squares fit into the absorption spectra of PSI and LHCII, obtaining the linear coefficients *a* and *b*, such that:

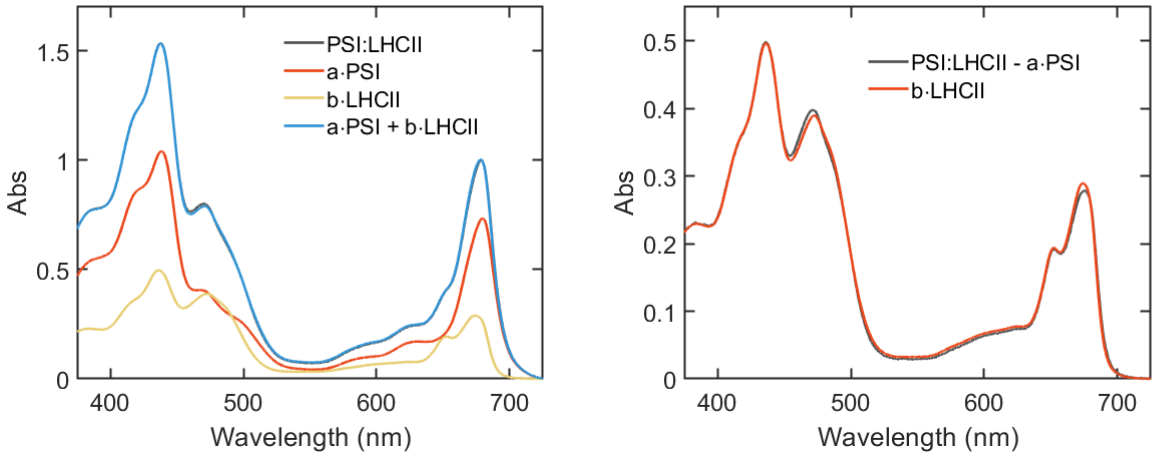
[PSI:LHCII] = *a*·PSI + *b*·LHCII

where PSI, LHCII, and [PSI:LHCII] stand for the respective spectra in Fig. 6.25. Rearranging the formula, we get

LHCII = ([PSI:LHCII] – *a*· PSI) / *b*

The right part of the equation is precisely how the difference spectrum in Fig. 6.25 was calculated. It is, as expected, nearly identical to the measured spectrum of LHCII.

The next figure shows the same spectra where PSI and LHCII are scaled with the coefficients *a* and *b*, respectively. The sum *a*·PSI + *b*·LHCII is identical to the measured PSI:LHCII spectrum. The purpose of this comparison is to prove that 1) the PSI:LHCII stoichiometry of the reconstituted membranes is as expected; 2) the PSI and LHCII complexes undergo no structural changes that could affect their excitation properties and the energy transfer model.



23) In Fig. 6.28. panel B three spectra are shown. Isn't it possible that the three spectra are almost identical (a normalization would easily answer this)?

This is correct – the DAES of LHCII have a similar shape. However, the figure shows that the long-lived components have the highest amplitudes. In contrast, in PSI-LHCII membranes, these long-lived components have the lowest amplitudes. We take this as evidence for energy transfer to PSI.

24) The review would not be complete without stating the opinion of the reviewer on the thesis (in the dissertation called "Summary) of the work. The first one states that the structure and excitation properties of LHCII are sensitive to the molecular environment of the complex. While I fully agree with this statement, this is a triviality –the absorption and excited state characteristics of almost all naturally occuring complex systems are indeed very sensitive to the molecular environment. Nevertheless, points a. and b. nicely summarize the findings of the work. I accept all 7 points as new scientific results, noting that ACD spectroscopy seems to me a more-or-less qualitative tool (as

compared to absorption spectroscopy), which is natural for an emerging new technique. In my opinion items 2, 4 and 6 are the most valuable contribution.

I partly agree with Prof. Vidóczy's assessment. It is true that some degree of sensitivity to the environment can be expected for any complex system; however, we have shown significant sensitivity in LHCII, contrary to the behaviour of other similar complexes, such as the PSII and PSI core complexes or the bacterial LH antenna. Therefore, the sensitivity of LHCII is far from trivial. It is now established but only after considerable opposition from our peers when publishing our results. I believe that it is a unique attribute of LHCII, related to its function as a self-regulating antenna that senses the external conditions.

Szeged, 28 February 2023

(Petar Lambrev)