
DISCUSSION

Chairman: We begin with a question from an anonymous referee: The statement in your Results (page 349) that there is no difference between 685 and 735 nm fluorescence as a function of pulse intensity seems to contradict the earlier statement that 735 nm fluorescence is quenched more easily than the 685 nm fluorescence.
GEACINTOV: There is no difference in the fluorescence efficiencies at 685 and 735 nm when single picosecond laser pulses are used for excitation. When trains of picosecond pulses are used, or when microsecond excitation pulses are utilized, the quenching at 735 nm is more pronounced than at 685 nm due to triplet excitons, which appear to be more effective as quenchers in PS I than in the light-harvesting pigment system (refs. 4, 5, 12, and 13). Quenching of singlets by triplet excitons is not important when single picosecond pulses are used because of the short time scales involved.

BERGER: In Fig. 1 C you show two curves: one, \(K[Q]\), is the quenching of PS I fluorescence determined experimentally; the other \(\gamma_{TS}[T]\), is the theoretical curve calculated from Eqs. 2, 3, and 4. Can you explain the significance of these curves, and in particular, why their shapes are different?

GEACINTOV: First of all there is the experimental \(K[Q]\) curve inferred by using dynamic quenching changes in the singlet’s lifetime as given by the Stern-Volmer equation. The other curve is a one-compartmental model calculation of \(\gamma_{TS}[T]\), using Eqs. 2 and 3 given in the text. We did not consider a two-compartmental model in which we have rates for singlet and triplet excitons migrating from the light-harvesting system (LH) to PS I. If singlet excitons had migrated from LH to PS I and then formed triplet excitons by intersystem crossing, we would not observe the large delay in quenching, since the rise time of the 735-nm emission is approximately 140 ps. The calculated curve is only semi-quantitative and demonstrates that the lag time is a real effect. The absolute scale on the \(\gamma_{TS}[T]\) curve is unimportant. The density of triplet excitons is not known but from unpublished microsecond and pulse train studies we can infer a value of the singlet-triplet rate constant, \(\gamma_{TS}\), of approximately \(7 \times 10^{-9} \text{ cm}^3 \text{s}^{-1}\).

KAUFMANN: Have you noticed on the triplet quenching PS I any effect of magnesium iron concentration?

SWENBERG: We have not done that experiment yet.

NASH: The data of decay lifetime vs. pulse number (Fig. 1 B) show that no quenching occurs before pulse 4, which indicates that the triplet formation occurs entirely in the light-harvesting pigments. However, the data of Fig. 1 B pulses 1, 2, and 4 have a large error and thus could be fit in several ways. A more pessimistic fit would indicate quenching as early as the second pulse. Two questions: (a) Does restricting triplet formation to the light-harvesting pigments seem reasonable, and do you have other convincing data indicating the delay of quenching to pulse 4? (b) Could the onset of quenching be pulse number-dependent rather than time-dependent, and have you varied pulse frequency to test this?

SWENBERG: Yes, you could take a pessimistic view. There are published experiments by Breton and Geacintov (ref. 5) where the wavelength dependence of the fluorescence after 1, 4, and 20 pulses has been measured. The data show that there is very little change during the first 4 pulses but very pronounced quenching at the end of 20 pulses. I think that strengthens our interpretation that there is a time lag.

GEACINTOV: For your second question, do you mean the spacing between the pulses?

NASH: Yes.

GEACINTOV: No. This spacing is fixed by the round-trip time of the pulses in the laser cavity.
NASH: It is reasonable to consider then that the pulse quenching is pulse number-dependent rather than time-dependent? For example, the number of quenchers could be incremented by a fixed amount in each pulse, and it would not necessarily be a time process.

GEACINTOV: Well, the data speak for themselves. There is a buildup of the quenchers with time, but there is also a lag in the buildup of quenchers in PS I. Thus there is a time dependence.

MAUZERALL: To amplify that suggestion given by Dr. Nash, we have models of these photosynthetic units where there are multiple traps, and in fact if there are three or four traps per unit and for some reasons you fill only one per pulse, then you would get in fact this delay of four and then fill up with the quenchers after the traps are filled. That's the model that amplifies.

SWENBERG: I will comment on that. I agree with Dr. Mauzerall that there are multiple-trap problems, even with regard to the singlet fluorescence quantum yield. I currently don't know how to include these effects in terms of a simple kinetic model, but I do agree with you on that particular point.

BADEA: I would like to ask three questions. (a) The fluorescence decay time of spinach chloroplasts has been determined to be 1.5 ± 0.4 ns. This ±24% interval of confidence is typical for the measurement of nanosecond relaxation times. What is it in picosecond technology? (b) Regarding the monoexponentiality of fluorescence decay, how accurately can it be ascertained at the present time with single picosecond pulses? How distinct should the two exponentials be to make possible their separation in the picosecond domain? (c) What is the minimum spacing of two pulses on the nanosecond time scale beyond which the fluorescence decay initiated by the first pulse is significantly perturbed by the second? Would the authors give a rough estimate for both mono- and biexponential decay in terms of the ratio: average lifetime/spacing (or the inverse ratio)?

SWENBERG: With regard to the accuracy of ± 25%, I think that this is a problem of reproducibility. The errors in the paper are standard deviations from six independent measurements. It probably can't be done any better than that right now.

Concerning your second question: It is easy to add exponentials in fitting curves; however, considering the accuracy of the experiment, I call this an example of over-interpretation. Whenever you have any type of bimolecular processes that occur in a finite domain, you do not necessarily expect standard decays. Instead the decay curves are infinite-series sums of exponentials: the smaller the domain size, the more terms in the series.

With regard to the third question, do you want to place the second pulse at varying time delays after the first pulse and see when the effect of the first pulse occurs on the second pulse emission? Is that the question?

BADEA: No, what I was asking is how widely spaced do you have to have the pulses one after the other to measure, for example, nanosecond fluorescence with any degree of accuracy.

SWENBERG: Well, first of all there are instrumental problems. You are not automatically able to position the second pulse after the first one. Probably Dr. Rentzepis can talk about that. The other important point is that the quenching states produced by either autoionization after bimolecular singlet fusion or intersystem crossing into triplet manifolds need time to occur. Singlet intersystem crossing rates are the order of 10^-9/s; thus you need at least that much time before a sufficient number of triplet excitons accumulate. Furthermore, you don't get any
of these nonlinear effects unless you have sufficient intensity. This is a crucial point and considerable disagreement exists in the literature because many investigators are not being careful about the number of pulses. Many of their experiments are in the intensity regime, where all sorts of nonlinear processes occur. I don’t know if I have answered your question.

RENTZEPI: The pulse separation is determined by twice the cavity length $l$, $2l/c$. Of course there are two ways to vary the pulse separation. One can enlarge or decrease the cavity length by moving the mirrors or one can place an optical modulator in it that transmits one every third pulse, etc. I think that Dr. Kaufmann is very anxious to make a comment.

KAUFMANN: I would just like to remind Dr. Rentzepis that a few years ago some elegant papers by Huppert and Rentzepis showed that you could easily use two pulses to do such experiments. They did things such as preparing solvated electrons in sodium methylamine solutions and showed a very nice way of varying the pulse rate. If I remember correctly, they varied the difference between pulses in the order of 300 ps or so but that technique obviously can be extended to 30 or 40 ns without too much trouble. This is especially apt for this kind of work, when you need pulses of very low intensity.


KAUFMANN: Another point on the technology is that in our laboratory in one system we have measured the singlet lifetime with the streak camera to be about 110 ps, and when we measured the singlet lifetime by absorption techniques, measuring ground state repopulation, it was on the order of $90 \pm 20$ ps. I think that there is reasonable agreement for this kind of time measurement.

RUBIN: What is the quantum yield conversion to triplet state?

SWENBERG: In our calculations we have taken the quantum yield for chlorophyll-a fluorescence and its lifetime in ethanol and have inferred an intersystem crossing rate of approximately $1.3 \times 10^8/s$. We assumed that this value is unchanged in vivo. No firm knowledge concerning this approximation is known. At least we don’t know of any.

RUBIN: But is it a certain concentration of triplet state that contribute to fluorescence? I am talking about the quantum yield of the triplet state appearance in the light-harvesting system.

SWENBERG: Are you asking how many of the triplets formed in the light-harvesting unit actually make their way to PS I?

RUBIN: Yes.

SWENBERG: Right now I don’t know the answer. However, since PS I emits at longer wavelengths then LH and PS II and has basically only chlorophyll-a molecules, I would assume that most triplets formed probably make their way there. That’s why there is more quenching occurring in PS I.

RUBIN: Are you monitoring the triplet state concentration and its decay or chlorophyll ions?

SWENBERG: The triplet excitons are actually monitored by triplet-triplet absorption. Probably
Dr. Geacintov would wish to comment on this. Using microsecond pulse excitation and observing the actual fluorescence decay at various wavelengths, one can get some estimate of the number of triplet excitons present. This can then be compared directly with triplet-triplet absorption spectroscopy.

GEACINTOV: Breton and myself in France have done experiments of this type where we monitored both the ion information and the triplet formation during and after laser pulses of various lengths (not picosecond lasers). Within experimental error, which was rather large, we did not detect any chlorophyll ions. Our limit of detection was about 1 chlorophyll ion per 100 molecules. On the other hand we could easily detect and observe between 1 and 3 carotenoid triplets per 100 chlorophyll molecules.

RUBIN: Now I want to make some comments about the experiments in which we use the same high-intensity picosecond pulses as Geacintov. One problem arising is the nonlinear dependence of the lifetime of the chlorophyll in vivo on the intensity of light. This is known and everyone is careful now to use nonintense pulses. But there are other problems, such as the nonstability of the photosynthetic system under high intensity light. If you compare with the structure of the chloroplast before the laser pulse, you will see physical damage after a $10^{15}$ quanta pulse and then finally severe damage with the $10^{17}$-$10^{18}$ quanta pulse. If you look at the “softest” of the chloroplast membranes, you can see the structure well, with a lot of particles correlated with the photosynthetic activity of the chloroplast. You can see the surface of the chloroplast membrane and the drastic damage by the high intensity, $10^{18}$ quanta/cm$^2$ pulse. The damage threshold is $\sim 10^{15}$ quanta/cm$^2$. So you must be careful. Have you any evidence that you do not destroy your chlorophyll sample?

GEACINTOV: We have also been very successful in destroying chloroplasts at various times. This type of irreversible effect is easily observed. Your findings are very interesting. I would just like to say that we performed controls in all of our experiments; these controls consisted of monitoring the fluorescence yield under low excitation intensity conditions both before and after our high-intensity laser experiments. As far as the fluorescence yields are concerned, we did not observe any changes. Therefore if you do observe changes in the chloroplast structure in the low-intensity limits, these do not seem to have any influence on the fluorescence yield. But this needs to be investigated further.

SWENBERG: I do not expect this effect to be important over small domains but I do believe there is quite a bit of structural change on the stacking of the grana.

MAUZERALL: Yes, in our experiments on the algae we also noted that there is a great variability among the different plant material. Some are particularly resistant to our nanosecond pulses, but some blue-green algae are very rapidly damaged by such a pulse system and one must of course check the reversibility of these systems.

RUBIN: I can tell you that chloroplasts are not very resistant to laser radiation and the threshold of killing them is about $10^{15}$ quanta/cm$^2$.

MAUZERALL: That’s just about what we had measured, too. That’s why our data quit at that point.