

Probing Intra-Leaf Photo-Physics Via Differential Fluorescence Induction Kinetics

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INTRODUCTION

Chlorophyll *a* fluorescence induction (FI) has been the topic of research for several decades and can provide quantitative information concerning exquisite photo-physiology controlling the emission of chlorophyll fluorescence from photosystem (PS) II, the pigment-binding protein complex in which photosynthesis is initiated. The various transitions of the *fast phase* (*i.e.* within ~300-500 ms of turning on saturating light) of FI can provide detailed information concerning how the photo-physiology occurring within PSII can be impacted by various environmental stresses. Several types of fluorometers have been used during the decades of research aimed at studying FI. Here, we report the use of a newly designed fluorometer that takes advantage of both low intensity pulsed amplitude modulation (PAM) light and variably intense continuous light, the LEDs of which, while being separate, are spectrally identical (LEDs peak at 632 nm). Together they can be used to near simultaneously measure chlorophyll *a* FI using PAM and continuous light. Using the instrument's standard operating procedure, modest 2-3% differences were observed between the modulated and continuous FI signals measured using the two procedures in dark-adapted leaves. The amplitudes of the modulation pulses were varied between 10% and 100% of the peak modulation amplitude (~130 $\mu\text{mol m}^{-2} \text{s}^{-1}$) and the two types of FI kinetics were measured. Small differences (3-5%) were observed between the modulated and continuous FI signals, suggesting that the two light sources, while being significantly different in intensity, 'probed' similar phenomena throughout the depth of the leaves. By contrast, the standard fluorometer was altered such that the modulation LEDs were changed to blue LEDs (peak centered at 430 nm), while the saturating flashes continued to use the standard red LEDs. The resultant fractional differences between the modulated and continuous FI signals were: 1) about five times larger than when the LEDs were spectrally identical; and 2) characterized by a systematic bias toward negative values (*i.e.* the modulated FI signals were systematically higher than the continuous FI signal). Salient, oscillatory dynamics were also observed in the differences. These results suggest that being able to measure modulation and continuous FI simultaneously with red continuous and blue modulated light may provide an interesting new tool for studying intra-leaf, photo-physical phenomena.

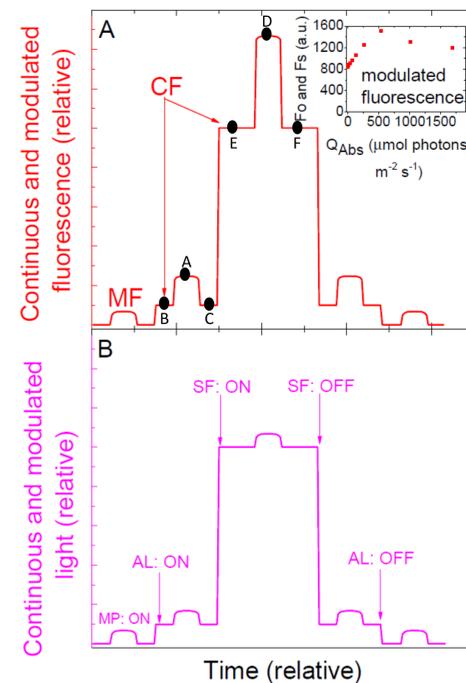


Figure 1. Schematic illustration of near simultaneous acquisition of continuous and modulated fluorescence Two "types" of fluorescence are described (A): continuous fluorescence (CF) and modulation fluorescence (MF). The former occurs in response to 'continuous' light in the form of actinic light (AL) and saturation flash (SF) light (B). MF occurs in response to low intensity modulation pulse (MP) light, the frequency of which is user-controlled. Take notice of the fact that the while the MP light source is depicted as being ON over the entire time period, at a constant peak amplitude and arbitrary frequency, the AL and SF light sources are depicted as being turned ON and OFF; the light intensities incident on the leaf are nonetheless depicted as being additive. Also, note that the figure is illustrative, such that the x-axis is not at all to scale. For example, the LI-6800 Chlorophyll Fluorometer takes advantage of modulation light in which the MP duration is ~2 μs , whereas a typical SF occurs on the 0.5 and 1 second time scale. The MF signal is electronically discerned from the CF signal by (A): $\text{MF} = \text{A (or D)} - [\text{B (or E)} + \text{C (or F)}] / 2$, whereas CF is determined from B, C, E, F and so on. Inset: minimum yields of chlorophyll fluorescence from a leaf (based on modulated light) in the dark (F_o) and during an ensuing light curve (F_s).

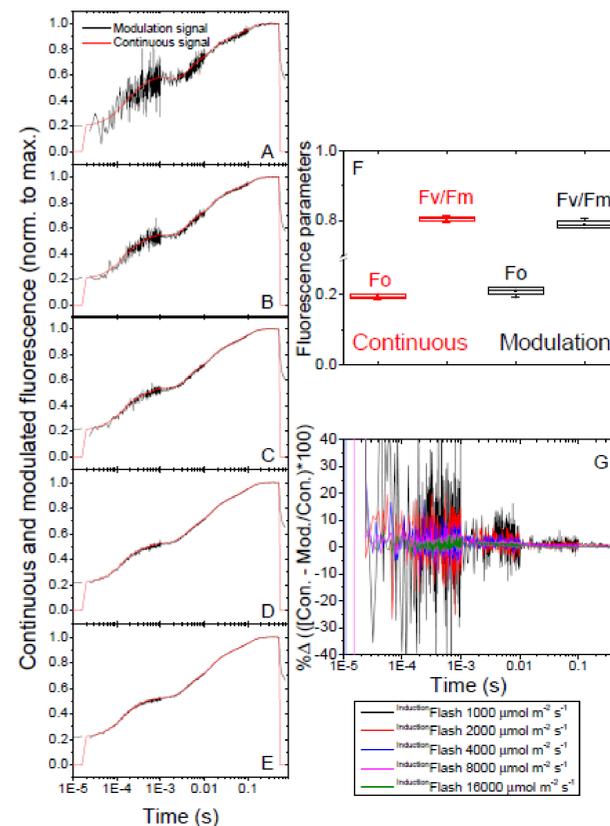


Figure 2. Induction dynamics of continuous and modulation fluorescence Continuous and modulation fluorescence induction dynamics (A) (normalized to maximum) were measured using a range of induction flash intensities (shown in Panel A), whereas the peak modulation pulse amplitude was constant at ~130 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ at a frequency of 250 KHz. Continuous and modulation fluorescence signals are represented by smooth and somewhat more 'noisy' signals, respectively. Inset: Box plots of the respective values of maximum fluorescence (F_m), the minimum fluorescence (F_o), and the maximum quantum yield of photosystem II electron transfer (F_v/F_m); data are averaged over the five respective continuous and modulation traces. In order to quantify the differences between the two types of fluorescence signals during the saturation flashes, the percent differences (B) were quantified as: continuous ("Con.") signal minus the modulation ("Mod.") signal, divided by the continuous signal, followed by multiplication by 100.

Figure 4. Differential fluorescence induction kinetics versus peak modulation pulse amplitude Saturation flashes (16,000 $\mu\text{mol m}^{-2} \text{s}^{-1}$) were applied while measuring continuous and modulated fluorescence as a function of variable peak modulation amplitude. The peak modulation amplitudes were randomly changed using 10% (A), 20% (B), 40% (C), 80% (D), and 100% (E) of the peak modulation amplitude (100% peak modulation amplitude was 130 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$). The continuous and modulation fluorescence signals, which were normalized to their maximum values, are represented by the red and black traces, respectively. Box plots of the minimum yields of fluorescence (F_o) are shown along with the maximum quantum yields of PSII electron transfer based on both continuous and modulation fluorescence (F). Note the break in the y-axis. The percent differences (G) between the continuous and modulation fluorescence signals were quantified as described in legend of Figure 2.

CONCLUSIONS

There are three LI-6800 design features that are important to emphasize in concluding remarks: 1) The standard peak modulation pulse intensity is ~130 $\mu\text{mol m}^{-2} \text{s}^{-1}$; 2) The modulation and continuous light sources are separate, yet spectrally identical; and 3) The instrument was designed to measure both modulated and continuous fluorescence signals.

- 1) The series of modulation and continuous fluorescence induction traces shown in Figure 2 exhibit very small differences, even though the pairs of traces at the given flash intensities represent $n = 1!$ This suggests that the signal-to-noise ratios of the modulated signals are quite good, owing to the high intensities of the modulated pulses. In addition, these data suggest that the two types of signals report on similar photo-physical phenomena throughout the depth of the leaf, a result that is a function of the spectral overlap of the continuous and modulation light sources. For example, when the spectral composition of the modulated LEDs was changed to a blue color, while the continuous LEDs remained red, obvious differences were observed between the modulation and continuous fluorescence induction signals (Figure 3). Red and blue light are well known to penetrate differentially into the depth of the leaf; thus when operated in this non-standard mode, the two signals report different photo-physical phenomena within the depth of the leaf.
- 2) A longstanding assumption, often mentioned in the literature, is that modulation light is of such a low intensity that it only probes the upper layers of leaf cells, possibly precluding explicit comparison of modulation fluorescence parameters with those of, for example, gas exchange. While the peak modulation amplitudes were changed between 10% and 100% of the peak modulation amplitude of 130 $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ (Figure 4), the continuous and modulation fluorescence signals reported very similar photo-physical and redox dynamics using induction flashes of 16,000 $\mu\text{mol m}^{-2} \text{s}^{-1}$, calling into question the above mentioned assumption.
- 3) The above conclusions were quantitatively supported by comparisons of the modulated and fluorescence signals.

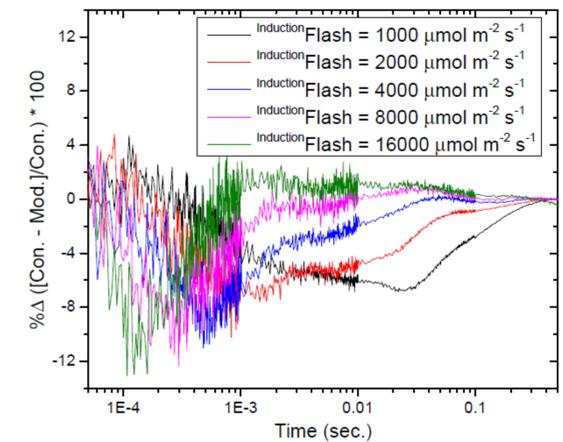
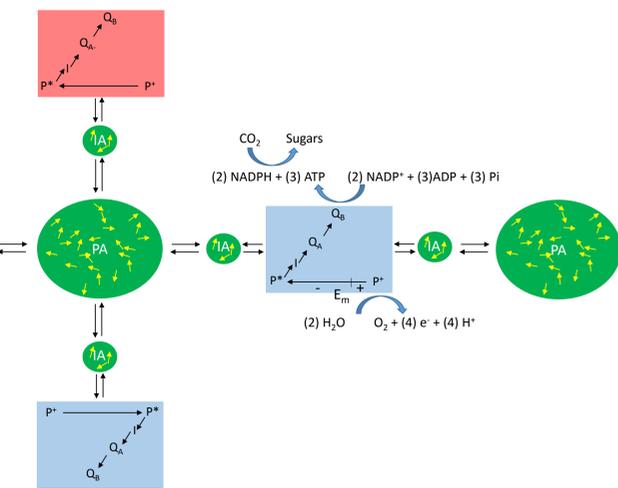


Figure 3. Differential induction kinetics based on spectrally different continuous and modulated light sources The exact same experiment was performed as described in Figure 2, except the optics of the fluorometer were altered by changing the modulating LEDs to a blue color with a central peak at 430 nm. Take note of the fact that the LEDs used for the saturating flashes were not altered and they have a peak at 632 nm. While the continuous and modulation fluorescence induction traces are not shown, the percent differences between the traces are shown. The inset shows the intensities of the saturation flashes used for performing fluorescence induction.

The LI-6800 Photosynthesis System The LI-6800 is virtually a complete renovation of its predecessor, the LI-6400. Just to name a few differences: 1) the reference and sample gases are split within the head; 2) automated control of all environmental variables, including water; 3) larger leaf area aperture; 4) combined measurements of gas exchange and chlorophyll *a* fluorescence; 5) the weight of the system is 30% lighter; 6) and a beautiful touch screen and user friendly interface.



LI-COR



ANTENNAE-REACTION-CENTER 'CENTRIC' PHOTOSYNTHESIS MODEL

Absorption of photons occurs in peripheral antenna (PA) and inner antennae (IA). The resultant excitons (yellow arrows) can very rapidly and reversibly transfer within and between the respective antennae, as well as the reaction centers, which are comprised of special pairs of chlorophyll (P^+ and P^*), corresponding to the oxidized and excited states of the special pair, respectively), pheophytin (I), the primary electron acceptor (Q_A), and the secondary electron acceptor (Q_B). The midpoint potential (E_m) of P^+ is capable of participating in water oxidation; the liberated electrons participate in a series of proton-coupled electron transfer reactions, the details of which are not all shown, that ultimately generate NADPH and ATP. These electron transfer reactions are thermodynamically favorable (*i.e.* $\Delta G = -n^*F^*\Delta E_m$) and the resultant energetic intermediates are used by the enzymology of carbon metabolism to assimilate CO_2 from the atmosphere into sugar phosphates. Chlorophyll fluorescence induction (FI) can provide information as to the intricacies of the initial photo-physical and redox reactions of the antennae and reaction centers.

METHODS

All experiments were performed with the recently introduced photosynthesis system, the LI-6800, which is capable of measuring combined chlorophyll *a* fluorescence and gas exchange. Tobacco plants were dark-adapted over night and then clamped into the chlorophyll fluorometer chamber of the LI-6800. The chamber conditions were controlled using: a flow rate of 700 $\mu\text{mol air s}^{-1}$; a fan speed of 10,000 rpm; a sample chamber $[\text{CO}_2]$ of 400 $\mu\text{mol CO}_2 \text{ mol air}^{-1}$; a VPD_{Leaf} of 2 KPa; and a T_{Leaf} of 25°C. Saturation flashes for measuring fluorescence induction were 500 ms in duration and variable intensities (see Figure legends); flashes were randomized and applied every 15 minutes. The frequency of the modulation light during induction was 250 KHz and the peak modulation amplitude varied and is noted in figure legends.