Chlorophyll Fluorescence

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Abstract

Here, we describe experiments and provide useful biological background knowledge pertinent to the scientific measurement of chlorophyll fluorescence in the time domain and its transient observed during active photosynthesis. This information will be used in Professor Clegg's Optical Spectroscopy—and perhaps Microscopy—classes, where more detail on the theory and other applications of fluorescence will be discussed. The instrumentation is versatile and easy to operate, and the method is applicable to a range of samples with lifetimes in the millisecond region or longer. The characteristics of fluorescence emissions in plants are revealing of the functionality and efficiency of their photochemistry [8]. These experiments and methods of observation convey the critical properties of photosynthesis and fluorescence, and the difficulties in studying them entails.

Introduction

Photosynthesis is the physical-chemical process of converting carbon dioxide, water, and light energy into stored chemical energy such as carbohydrates, ATP and NADPH while releasing oxygen as a byproduct. All photosynthesis takes place in

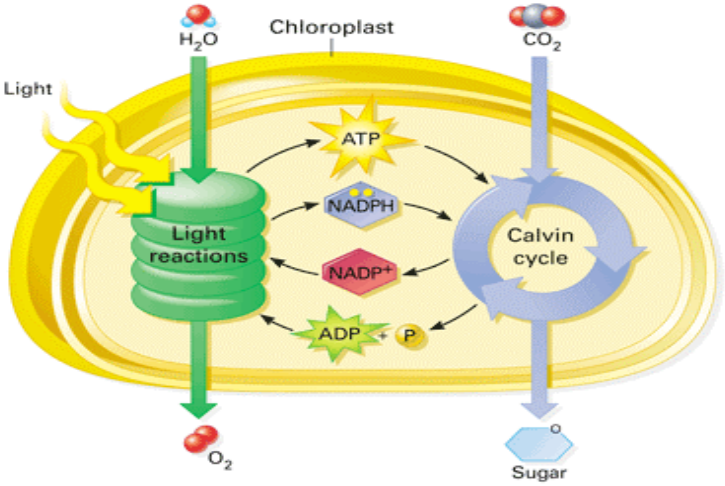

Fig. 1 A chloroplast. The light reactions take place mostly along the thylakoid membranes show here as the green disks. http://knight.noble-hs.sad60.k12.me.us/content/exploringLife/text/chapter8/08images/08-04.gif

chloroplasts—organelles typically found within mesophyll cells. Within the chloroplasts are stacks of thylakoids called granum surrounded by a dense fluid called stroma. Chlorophyll, along with the light reactions, resides on the thylakoid membranes. The process that produces carbohydrates that the plant uses as food is the Calvin cycle, which is supplied with energy from the light reactions (Fig. 1). [2] [5]

Incoming light is trapped by two separate protein complexes—Photosystem II (PSII) and Photosystem I (PSI), and its energy is carried along their respective antennae systems efficiently to the reaction centers by a non-thermal process known as resonance excitation energy transfer

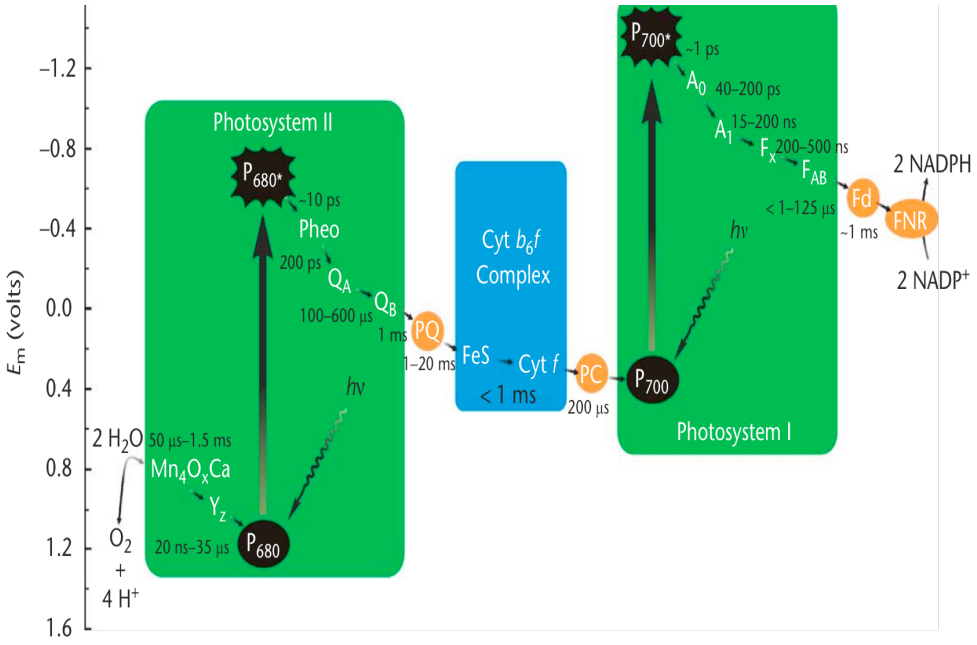

Fig. 2 The Z-scheme of photosynthesis including the two photosystems and the e- transport chain. Each major step in the process is labeled with the timescale in which it is completed [5].

(Fig. 3) [1] [5] [4]. The reaction center is a protein complex in which charge separation occurs, i.e., electrons are donated and accepted by a primary acceptor [2] [5] [7]. In Photosystem II, light energy is transferred to the reaction center where electrons are excited in the chlorophyll molecule P680, and then captured by pheophytin. The primary stable electron acceptor of PSII is Q_A (a plastoquinone), which transfers one electron at a time down the electron transport chain. In this chain, one of several functions is to utilize redox potential to convert ADP into ATP via photophosphorylation. [4] [5] [10]

The light reactions also use the light energy to split water so that hydrogen may reduce $NADP^+$; this is also the process that produces O_2.

At the end of the electron chain is PSI, in which chlorophyll P700 is excited in the reaction center, and A_0 (a specialized chlorophyll molecule) is the primary electron acceptor. From PSI, $NADP^+$ is reduced into NADPH (Fig. 2). [4] [5] [10]

The absorption spectra of chlorophyll *a* and chlorophyll *b* are slightly different due to the replacement of a methyl group in chlorophyll *a* by a formyl group in chlorophyll *b*, but both absorb best in the 400-490nm and the 640-700nm ranges. Their emission spectra are both in the red region: roughly around 660-690nm and 710-760nm (Fig. 4) [4]. Carotenoids are accessory pigments that can broaden the spectrum of light that can contribute to photosynthesis while also absorbing excess light if need be. Carotenoids are also responsible for the autumn coloration in leaves [1] [2] [5]. All of these pigments can be found in the light-harvesting antennae complexes of each photosystem in near-exact proportions.

However, not all light energy

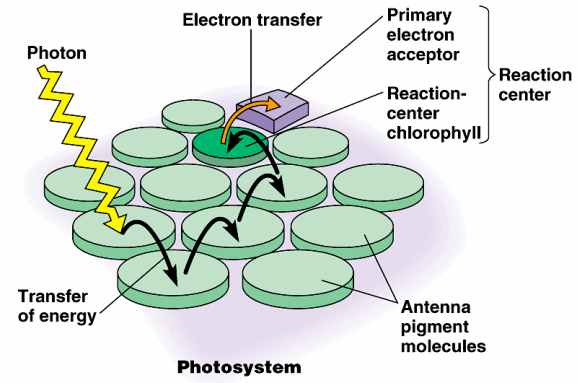

Fig. 3 Within each photosystem, light is absorbed and transmitted along the antenna complex to the reaction centers by a process named resonance excitation energy transfer. Antenna pigment molecules are chlorophylls and carotenoids.
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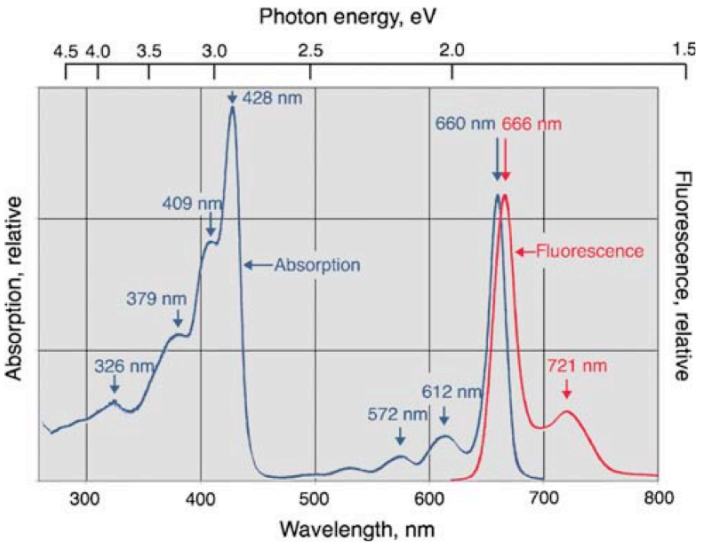


Fig. 4 Absorption and emission spectra of chlorophyll *a.* It is very similar to that of chlorophyll *b,* and the carotenoids' peak absorbance is nearer to the grean. [1]

absorbed in the photosynthetic membrane is transferred efficiently to perform useful chemical work; due to quantum physics, excitation is in direct competition with dissipation as heat and fluorescence [9] [7]. Fluorescence occurs when a photon (or in some cases, two) excite an electron to a higher energy level. Since the excited state is unstable, the electron will fall back down (after staying excited for a few tens of picoseconds to a few hundreds of nanoseconds), releasing radiation usually of a higher wavelength (lower energy) than that of absorption according to the Stokes Rule (Fig. 5)[1]. Lower energy emission is due to energy loss as vibrational relaxation in the excited state. However, excited molecules can undergo other de-excitation processes not shown in Figure 5, e.g., absorption by carotenoids and FRET. [7] [10] [11]

The characteristics of the emission are determined by the participating pigments, their orientation, how the energy is transferred along the antennae, and redox potential of the reaction centers (how full they are). [9]

When the reaction centers are empty (known as being dark-adapted), all quinones, particularly the primary acceptor, Q_A, are in the oxidized state (open). Under normal light conditions, if the reaction centers are open, then fluorescence is minimal (3-11% of energy dissipation in PSII) because electrons are in the excited state for a shorter period of time due to the efficient energy transfer leading into the reactions centers. Thus, fewer photons are emitted per second as either heat or fluorescence because there is a higher probability that energy will be captured in the reaction centers as long as they remain open (Fig. 6a). [4] [8] [10]

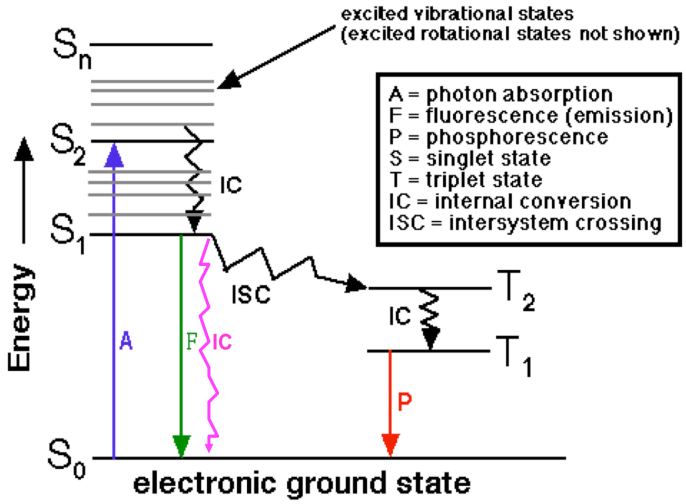


Fig. 5 A modified "Jablonski" diagram showing the different modes of molecular de-excitation in chlorophyll. We are mostly concerned with fluorescence, but for reference, crossing into the triplet state is especially damaging to cells because it can produce reactive oxygen species (oxygen naturally occurs in the triplet state) [5] [10]

[1] Electron spins are opposite in the singlet state, and they have parallel spins in the triplet states. Transitions from singlet to triplet states are possible if the spin states are not pure—perhaps due to spin-orbit coupling. A transition is usually slow, often releasing phosphorescence. [7]

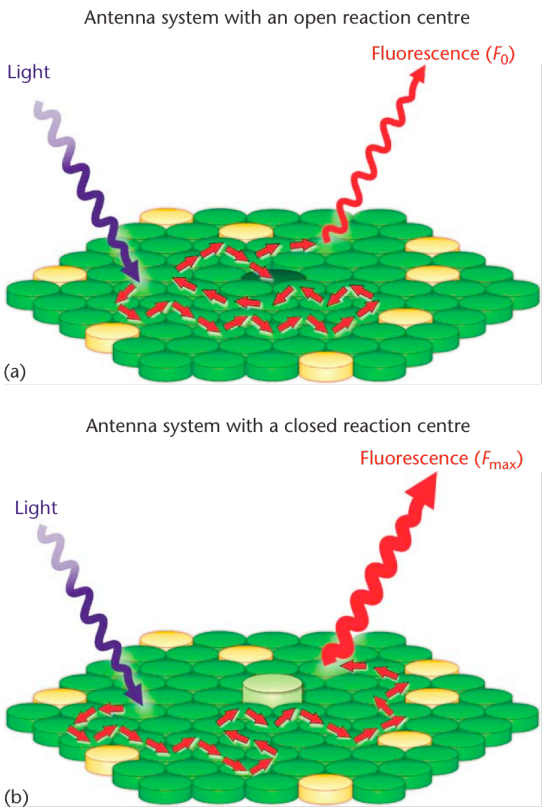


Fig. 6 When the reaction centers are oxidized, they absorb light energy and emit only minimal fluorescence. When they are reduced, nearly all energy is emitted as either fluorescence or heat [5].

When only weak light that does not fully reduce Q_A is shone on the leaf, it will yield minimal fluorescence (F_0). But with a bright saturating pulse, fluorescence emission rises quickly (after about 300ms) to a maximum value (F_m), passing through many distinct phases[2] along the way [9] [10]. The very immediate jump is due to the abundance of chlorophyll, which has a lifetime of about 18 nanoseconds, and after many milliseconds, the process described in the paragraph above is observed. The primary cause for the increase in fluorescent yield (known as he initial fluorescence transient rise or the Kautsky effect) is that the chlorophyll molecules will, at first, transfer excitation energy efficiently to the open reaction centers; but after milliseconds to hundreds of milliseconds, the reaction centers cannot transfer their energy to the electron chain (specifically Q_A) because the kinetics of the chain are slower [4] [10]. As energy enters the chain and reaction centers, they become reduced and unable to transfer energy. Thus, the chlorophylls not only have a greater probability of fluorescing, but also will fluoresce longer because electrons will remain in the excited state for a longer time (Fig. 7).

The fluorescence transient increase observed after the jump to $F_0$is due to the light being dissipated throughout the antenna complex because the reaction centers have closed and there is now one less pathway for de-excitation, at least until other processes become activated that will re-oxidize the electron chain (Fig. 6b). [8]

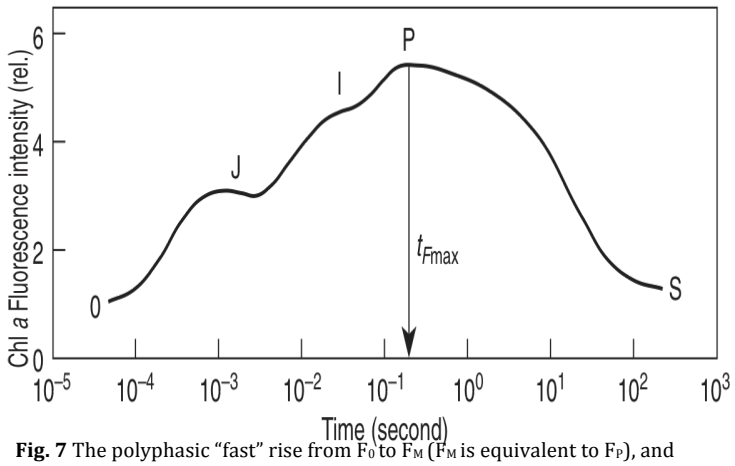


Fig. 7 The polyphasic "fast" rise from F_0 to F_M (F_M is equivalent to F_P), and the slower decline to the steady state F_S. The transient rise from F_0 to F_P is due to the accumulation of Q_A^- which clogs the e^- chain. From F_P to F_S, other processes activate that capture the excess energy—this phase is also the least understood [9].

[2] The complete list of phases is O,I,D,P,S,M, and T. They stand for, respectively, 'origin', 'intermediate', 'dip', 'peak', 'semi-steady state', 'a maximum', and a 'terminal steady state' also known as F_S [4].

Then, the emission signal will slowly decrease several minutes after reaching F_m to a steady state (F_T, F', or F_S). The reason for the existence of such a long delay before fluorescence yield decreases is due to the gap in the start of the carbon fixation process (associated with the Calvin cycle) [9] [10] and the xanthophyll cycle relative to the start of the light reactions [7]. The Calvin cycle uses the NADPH and ATP produced by the light reactions; and without sufficiently fast kinetics in either the electron chain or the Calvin cycle, the light reactions become clogged. Once the Calvin cycle has been activated, however, equilibrium in ATP/NADPH production and consumption is slowly reached, and a steady fluorescence emission is finally observed after several minutes. [10]

Also, there are methods of photoprotection that plants employ to defend against extreme light conditions often used in labs. One is a non-photochemical quenching (NPQ) of absorption (and thus quenching of fluorescence) by eliminating excess light via enhanced thermal dissipation. NPQ is characteristic of several processes, including the xanthophyll cycle, acidification of the thylakoid lumen, and other components of PSII [4] [10]. The xanthophyll cycle is exercised by the carotenoids to avert creation of reactive oxygen species (ROS) [5] [7]. It takes several minutes for this process to activate.

Materials and Methods

There are essentially two different experiments covered in this paper. One is performed in ESB with a fluorometer setup. There, students will spend time recording and analyzing emission data from samples like the leaf *Alnus incana* (Speckled Alder, Fig. 8). A substantial part of our experiment was designed around the methods used by Chandler *et al* [3]. Second, students will be using a fluorescence microscope—also in ESB—to visually acquire a sense for how biological samples fluoresce. Students may be able to use the confocal microscope at IGB to gather more precise data on fluorescence under a more controlled environment.

Fluorometer Experiment

Inside the fluorometer (Fig. 9), light from either a modulated Luxeon 3 star 538nm lamp; or a blue CL-2000 diode-pumped crystal laser (495 mW at 349-501nm) with a beam chopper (Laser Precision Corp. Model CTX534(HD)) and its corresponding controller (Laser Precision Corp. also

Fig. 8 Leaves from *Alnus incana* used in the experiments. On the left is the front, and on the right is the back.

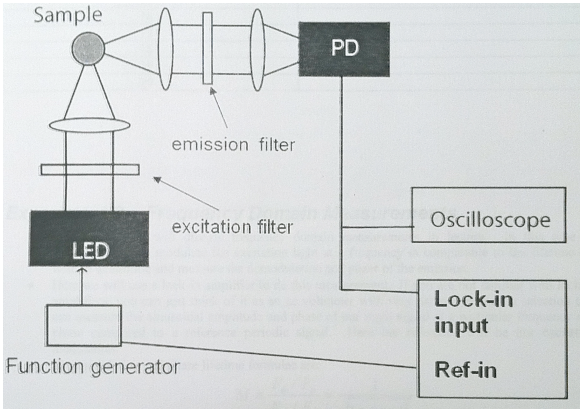


Fig. 9 The schematic of the optical setup used in ESB. The LED was modulated by a function generator. A lock-in was used to acquire the frequency data of the emission, and an oscilloscope was used to obtain the time series of the emission. A PMT was used instead of a photodiode. This design was based off the setup used by Chandler *et al* [3].

Model CTX534) is focused through optics into the housing (SLM Instruments Model OP450, Series 8403229). Although the lamp emits in the green, this is quite suitable for the plant because multiple layers of thylakoids allow for easy absorption of green light [1]. With the laser, a fiber optic cable may be required, and it is important to focus the laser to the smallest point to reduce noise in the signal [3]. For the lamp, light is modulated by a function generator (Agilent 20MHz Function/Arbitrary Waveform Generator). A pulse or square waveform works best. The generator's output must have a strength of at least 4 volts per pulse (VPP) to operate the lamp.

The light passes through a 530nm excitation filter for the lamp, and a 440nm filter for the laser, when it is focused onto the sample. Since plants fluoresce in the red, a 630nm emission filter is used. The emission is captured by a PMT (PDA 100A Si Detector sensitive within 400-1100nm with a gain of 0-70dB). The detector's output can be conveyed visually by either a lock-in amplifier for frequency domain measurements (Stanford Research Systems Model SR830 DSP) or an oscilloscope for time domain measurements (Tektronix TDS3012B Two Channel Color Digital Phosphor Oscilloscope), from which it can be analyzed by any scientific program. If the lock-in is not being used, the "sync" on the function generator must connect to the "external trigger" port on the oscilloscope. Samples should not be exposed to light for an extended period of time as, especially so with the laser, photobleaching may diminish signal strength. Samples were exposed to light only long enough for the oscilloscope to adequately average the signal.

Sample Preparation

In keeping with the right angle design of a fluorometer, the flat sample must be at a 45-degree angle relative to both the LED and the PMT. Given the flimsy nature of the thin leaf, it was found that a support was needed onto which the sample could remain completely flat. Any curvature or wrinkle in the sample will cause scattering and interfere with the signal. The support we found to work best was a rectangular cutout of thin Teflon fitted perfectly to

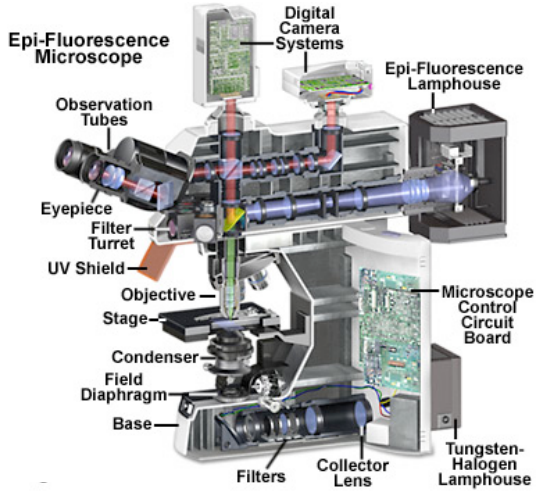


Fig. 10 Schematic of a fluorescence microscope. More information can be found at http://store.amscope.com/fm320-8m.html or http://microscopyu.com/articles/fluorescence/fluorescenceintro.html

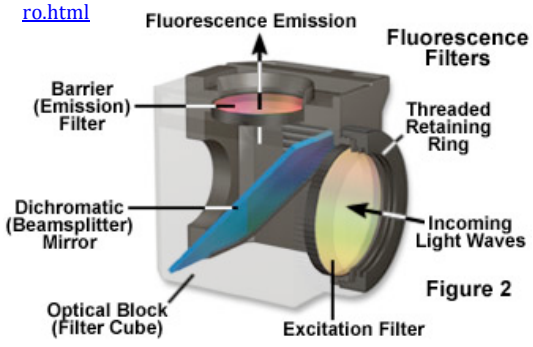


Fig. 11 The dichroic mirror is the distinguishing characteristic of fluorescence microscopes. http://microscopyu.com/articles/fluorescence/fluorescenceintro.html

the diagonal of the cuvette. Teflon has minimal fluorescence, so it is unlikely that it will interfere with the data. If there is trouble in keeping the sample attached smoothly to the support, a drop of water or double-sided tape should adhere the sample to the support.

Students will want to observe both the front and back of the leaf in their experiments. Students are also encouraged to use an abrasive technique—as we did—on the epidermis to try to increase fluorescence emission from the front or back of the leaf. The epidermis is the thick, protective coating on the outside of leaves and stems. In addition to the cuticle (a waxy coating in the epidermis designed to prevent water loss), this thick coating may reduce fluorescence. Removal of this coating can be attempted by sandpapering, but it is unclear whether it increases light penetration to the mesophyll as intended, or only damages the sample. No conclusive evidence was gained, however, to prove that it increased fluorescence, but it is interesting to try.

It is also ideal to place the sample in a medium such as water for an optimal signal.

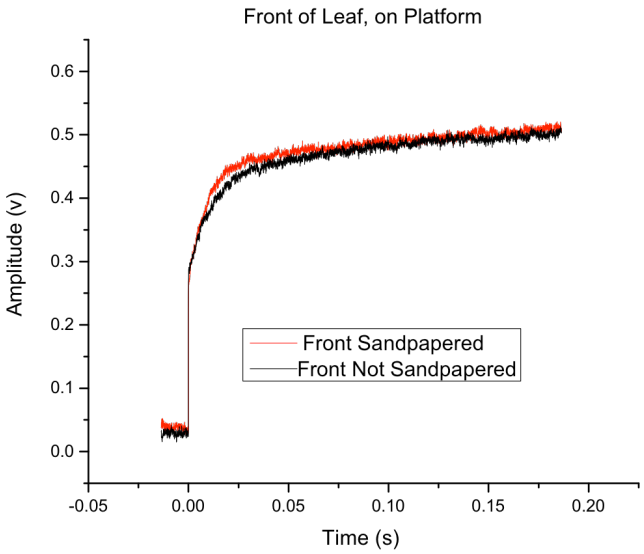
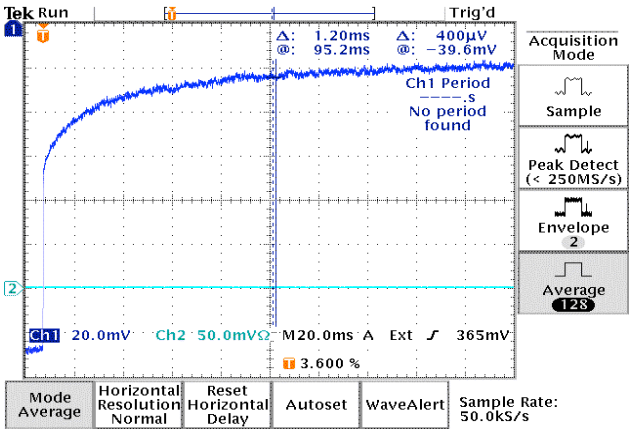


Fig. 13 A sample was excited repeatedly by a 1s pulse with a 200ms width from a 538nm lamp. Take note of the similarity of the two plots.

Fig. 12 The fluorescent transient of the front of an *Alnus incana* (Speckled Alder) leaf. The sample was immersed in water for its ideal optical properties. Notice the immediate jump followed by a slow relaxation.



Using the Fluorescence Microscope

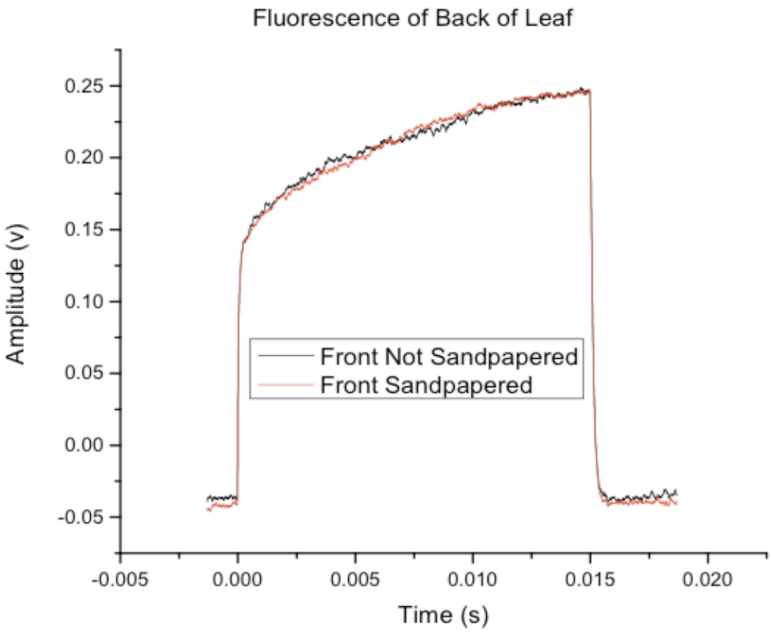
The Fluorescence microscope (AmScope EPI Fluorescence Trinocular Microscope with 8M Digital Camera (Fig. 10) can excite a specimen using light from an arc-discharge lamp (in our case, a mercury lamp that emits 366, 405, and 437nm light) through a vertical illuminator within the microscope. An excitation filter can be fitted upon the microscope. Fluorescence objectives were used—total magnification was either 10x or 40x.

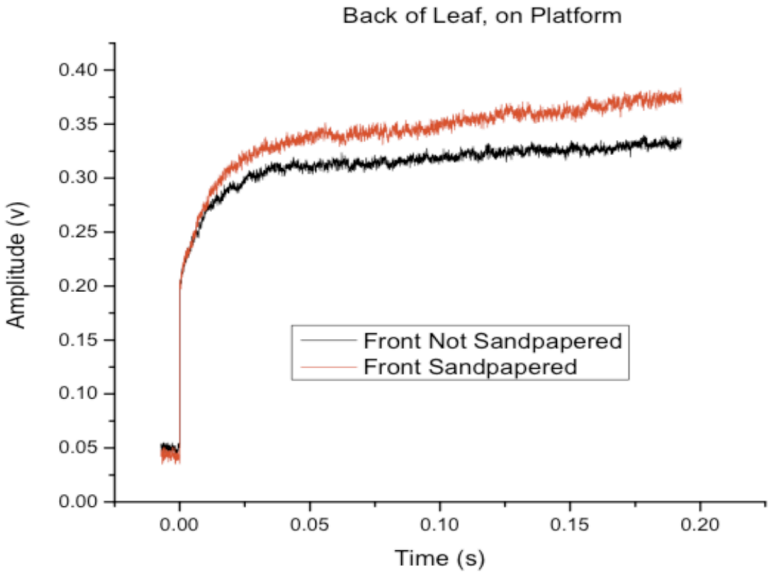
Excitation photons reflect off the dichroic mirror (Fig. 11), through the objective, and onto the sample. The emission light passes through the dichroic mirror to be filtered (filters are already installed within the device). Students can capture the images they see through the eyepiece with a CCD

camera (Amscope MD1800) that can be mounted atop the microscope.

Results

Most data was collected from, and analyzed by, our instrumentation in ESB, as will likely be the case for students. Measurements in the time domain were obtained via the oscilloscope described above, and analyzed in Origin. Images of cells were captured using the fluorescent microscope and its accessory camera described above.





Fluorometer Experiment

A rectangular cutout of *Alnus incana* (Speckled Alder) leaf was placed onto the Teflon support platform in the cuvette with the front facing at a 45° toward the excitation source. The sample was excited with a square wave repeating at 2 Hz. Water was used as a medium because of its ideal optical properties; it filled the cuvette. Figure 12 shows the time domain measurement of this setup.

There was also effort to remove the cuticle and perhaps part of the epidermis in the hope of increasing light penetration to the spongy mesophyll, and therefore, increase fluorescence. Figures 13-15 show the final results of this experiment.

Fluorescence Microscope Images

To accompany the graphical representation of the observed properties of chlorophyll fluorescence, images from a fluorescence microscope with a CCD camera are given.

For reference, an image of the sample under 10x magnification under only white light is given (Fig. 17). The bodies of what look to be cells are actually large cell groups. Individual cells can be distinguished by careful observation. The white, curved lines are veins that supply water and nutrients to these groups. Under white light, there is no visible difference

On previous page: Fig 14 (top) The back of the sample was excited by a 530nm lamp modulated at a 200ms pulse with a 15ms width. The red line represents the signal after the epidermis was scratched with sandpaper, while the black line represents the absence of abrasion. For simplicity, the sample was in air. **Fig 15 (bottom)** A separate sample was used here. The back was excited by 530nm light modulated to a repetitive 1s pulse with a 200ms width. Notice the similar energies and relaxation patterns of the two plots in Figure 15. The sample was in air. Figure 14 is a magnification of the experimental time window during the initial transient rise (as the vertical jump begins to relax) shown in Figure 15, but the two figures show different samples being excited.

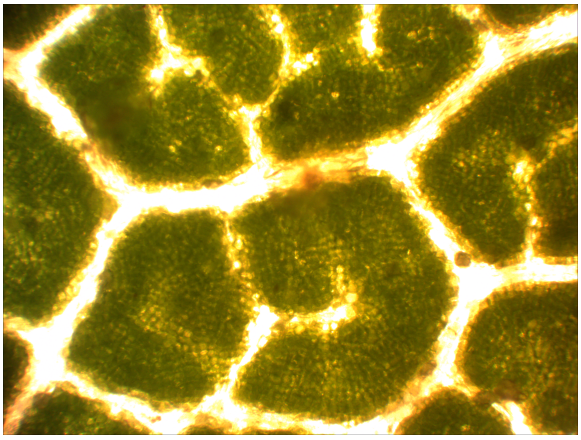


Fig. 17 (above) Cell groups under white light. A 10x achromatic DIN objective was used: total magnification is 10x. Individual cells can be seen.

between a sandpapered leaf and one that has not been sandpapered.

Under blue excitation light from the arc-discharge lamp, images were taken of the same samples (Fig. 18). Impurities and other fluorescent objects are clearly visible because no emission filter was used so that maximum image depth and clarity could be achieved.

Also, unlike under white light, the effects of abrasion on the epidermis are visible because they interfere with the fluorescence emission (Fig. 19). When a sample was sandpapered in one direction only, parallel scratch marks are apparent and probably scatter emission light. It is clear however, that the scratches do not penetrate into the

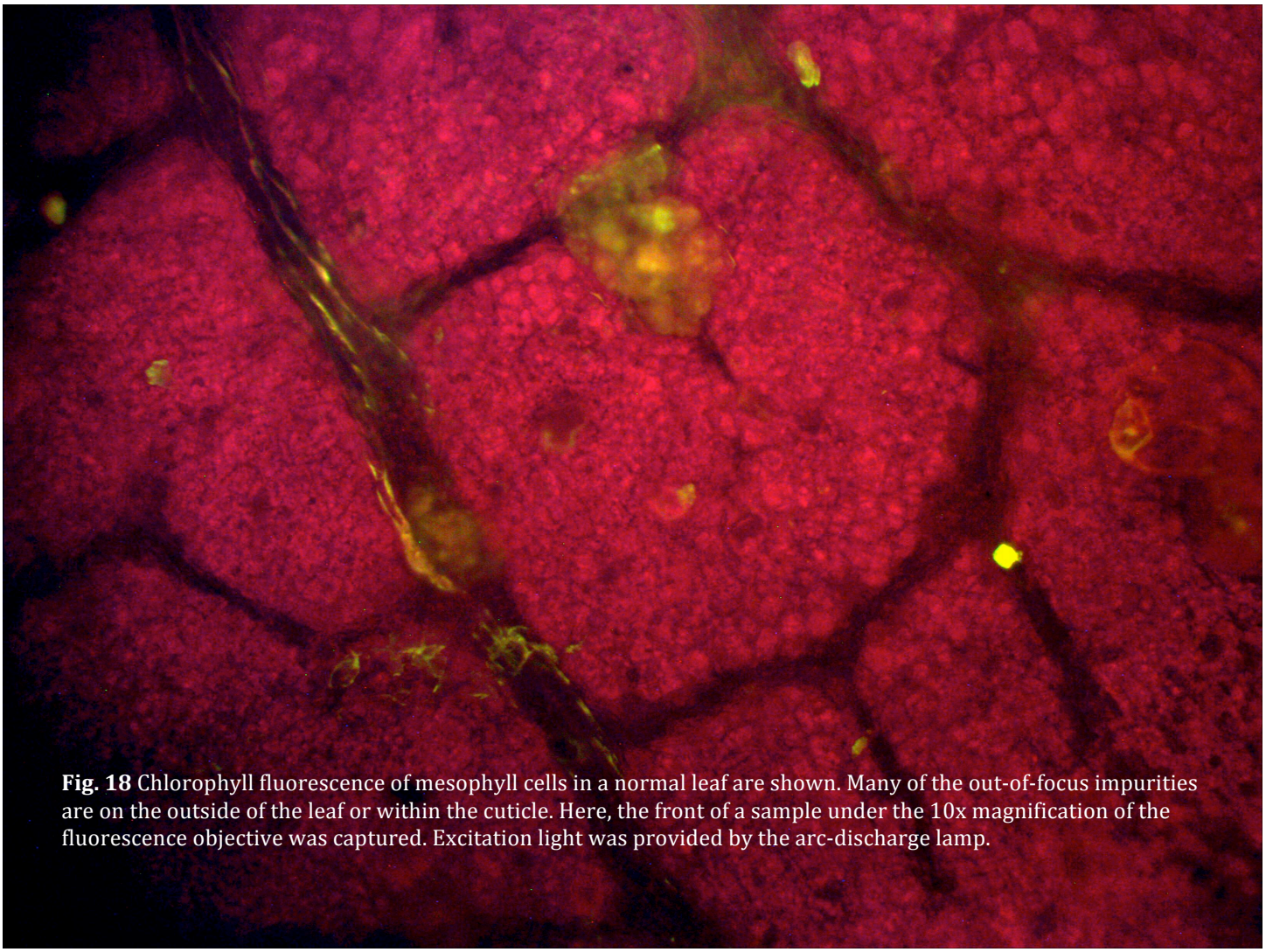


Fig. 18 Chlorophyll fluorescence of mesophyll cells in a normal leaf are shown. Many of the out-of-focus impurities are on the outside of the leaf or within the cuticle. Here, the front of a sample under the 10x magnification of the fluorescence objective was captured. Excitation light was provided by the arc-discharge lamp.

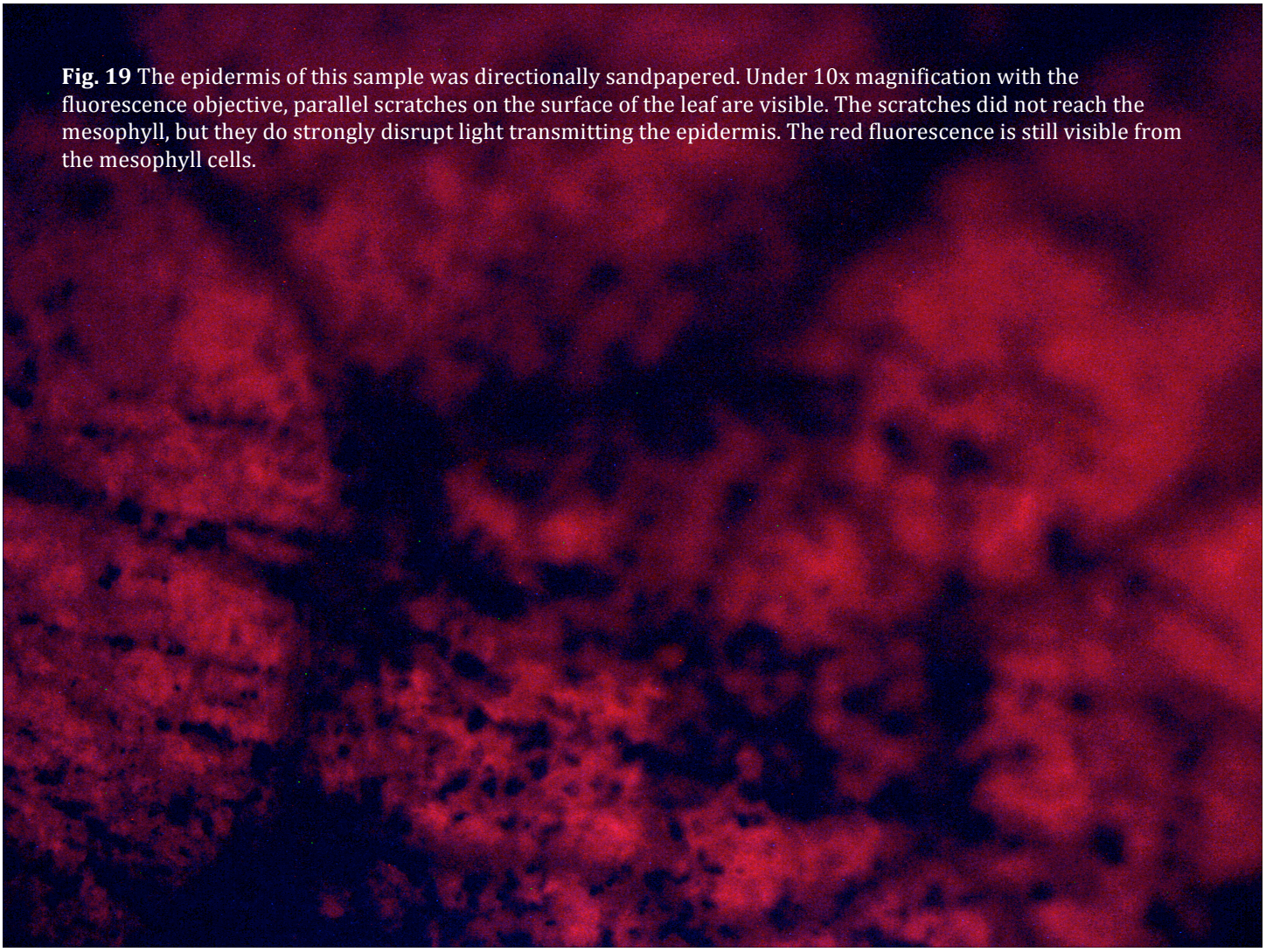
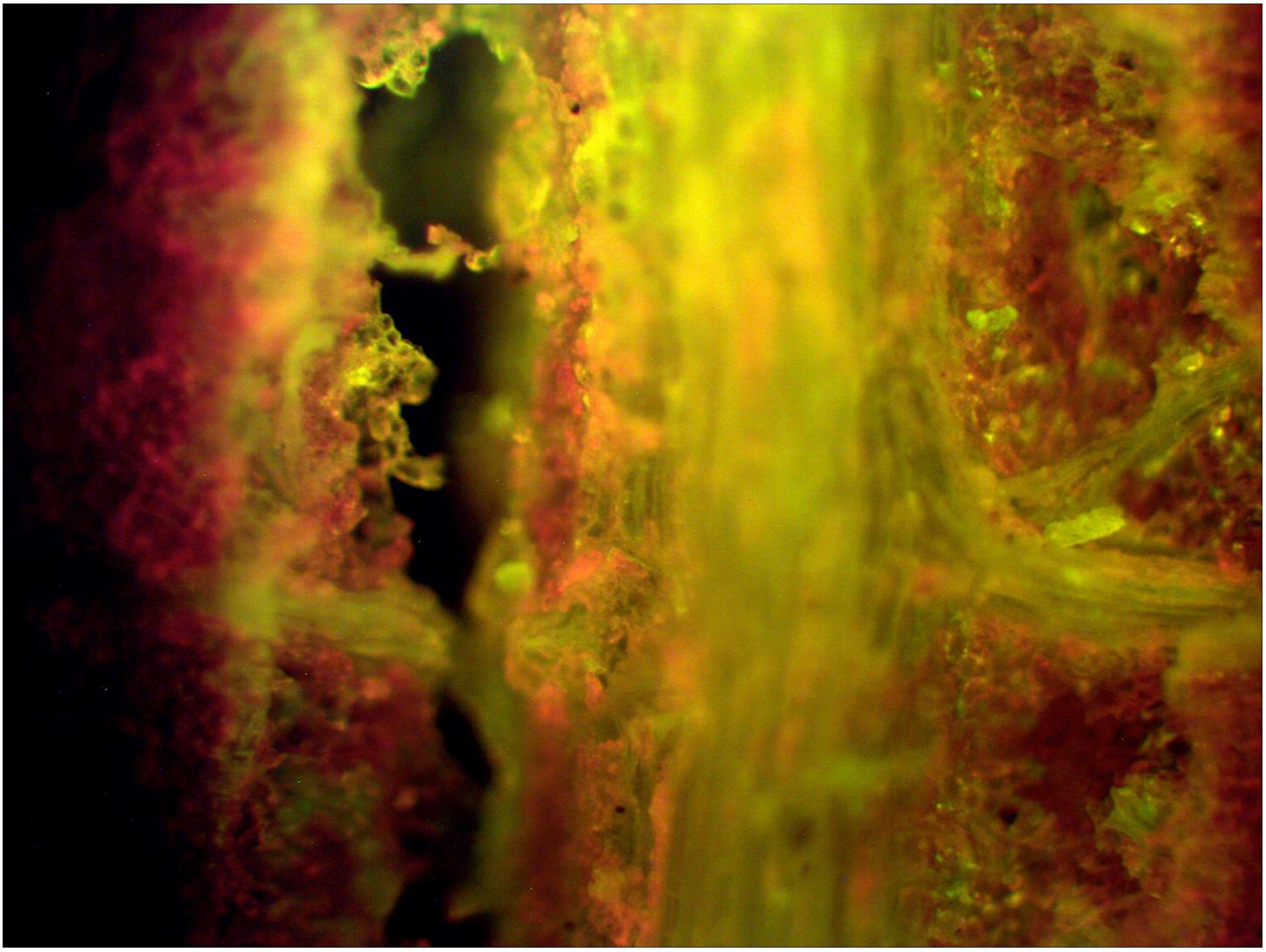


Fig. 19 The epidermis of this sample was directionally sandpapered. Under 10x magnification with the fluorescence objective, parallel scratches on the surface of the leaf are visible. The scratches did not reach the mesophyll, but they do strongly disrupt light transmitting the epidermis. The red fluorescence is still visible from the mesophyll cells.

mesophyll, and thus, may not increase fluorescence.

Additionally, a very intense abrasion was applied to the leaf in attempt to break through the epidermis (Fig. 20). It was successful, but also so destructive that it ripped through the leaf. The mesophyll was exposed in the process, and fluorescence intensity spiked in this region. In fact, the intensity was 80% higher where the mesophyll was penetrated. However, the lesions seemed to be concentrated near the veins where the most friction occurred during sandpapering. Outside a few tenths of a millimeter away from the lesion, fluorescence is characteristic of a normal leaf (Fig. 21). This agrees with our fluorometer experiment because extreme sandpapering was never attempted on those samples, at least not to the point where visible lesions were made, because the warped nature of the sample would interfere with the signal.



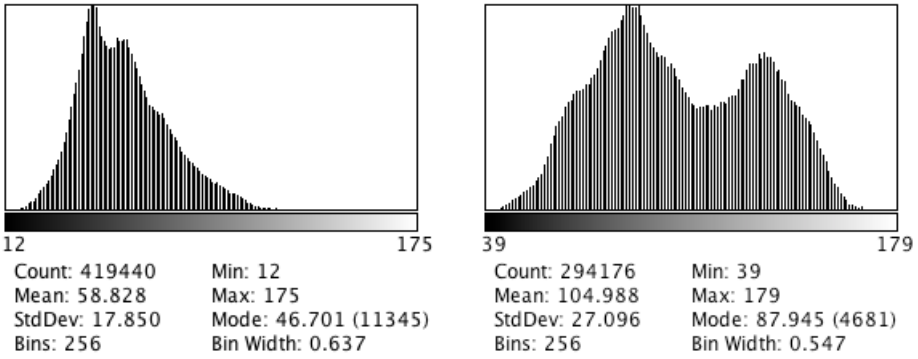


Fig. 20 (above) A leaf under 40x magnification was heavily sandpapered to the point where lacerations penetrated the entire leaf—shown here as a black canyon next to the vein. The mesophyll was exposed (especially to the right of the vein). Extensive damage was done to the tissue immediately surrounding the veins. Because of their relative height above the rest of leaf, this region received the most friction from sandpapering.

Fig. 21 Above, two histograms compare intensity in two different regions of Figure 20. For convenience, the figure was first transformed into 32-bit grayscale image for analysis. The "Count" number merely indicates the quantity of pixels selected for analysis. **(left)** The intensity of the upper right quadrant was analyzed, where the mean intensity (of the color weight applied to each pixel) is 58.8. The light green vein was not included in the sample, as it contains no chlorophyll. **(right)** The intensity of a thin, rectangular area adjacent to both the vein and lesion penetrating though to the bottom was analyzed. The mean intensity was 104.9, or 80% higher than sections without deep lacerations.

Discussion

The primary motivations behind our experiments were to increase fluorescence intensity, and also determine how the photochemical functions in plants affected that fluorescence transient. Our methods were rather crude but they yielded consistent results, and they provided insight into a still mysterious science. There are other recently discovered methods that hold the potential to better achieve these goals, as the study of chlorophyll lifetimes is a rather new and less developed field than the strict chemistry of photosynthesis.

One such method that we would have liked to pursue was applying a perfluorodecalin (PFD) solution to the leaf because it would penetrate into the mesophyll and remove both O_2 and CO_2 pockets. We suspect that this would have been a substitution for the harsh abrasion method that we employed because, without as many air pockets to cause diffraction or reflection, both excitation and emission photons would have a higher probability of transmittance across tissue layers, perhaps also increasing fluorescence. [6]

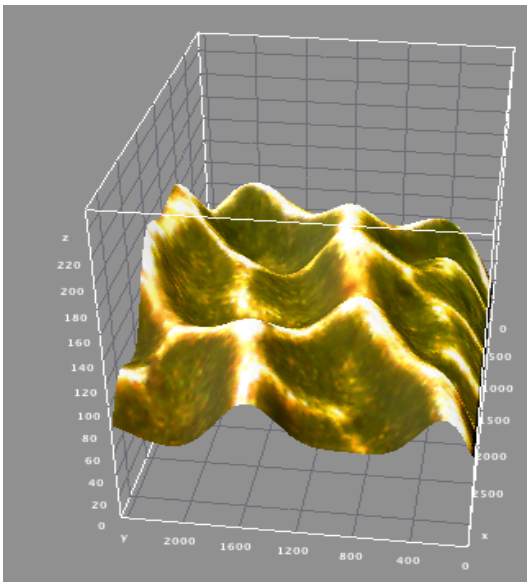


Fig. 22 This is a smoothed 3D surface plot of Fig. 17. By the translucent properties of water, the veins have a more intense signal than their chlorophyll neighbors when excited though the bottom by white light. Thus, a fairly accurate surface plot could be constructed—based solely on intensity—to demonstrate the relative height difference between cell groups and veins.

Another interesting experiment is measuring the Kautsky effect after the application of an inhibitor like DCMU (a herbicide) that blocks the re-oxidation of the plastoquinone pool—specifically targeting the electron acceptor, Q_A. This essentially kills the electron chain after all the quinones have been reduced [10]. Curiously, an inhibition in the electron flow beyond Q_A causes a more rapid fluorescence rise to F_P, and that without an inhibitor (only a light pulse), Chlorophyll fluorescence was about 10-20% more intense when the whole plastoquinone pool was fully reduced. Other experiments also yielded interesting results when electron inhibition was imposed on the donor side of PSII (between water splitting and P680). [4]

Fluorometer Experiment

The commonality of all data plots gathered from the oscilloscope in the fluorometer experiment is the fluorescence transient, which is in agreement with the well-established OJIP transient. The total relaxation time after the initial jump is approximately 200ms—our experimental time window. With the information currently known in this area, there two implications of our data: 1) that the signal we are seeing is

between F_I and F_P because the timescale in which we are operating is appropriate for such an observation (refer to Fig. 7), and 2) that the bulk of the emission is coming from the light-harvesting antennae complexes of PSII.

As explained in the introduction, the transient rise is due to the reduction of the reaction centers and the quinones in the electron chain. Without an oxidized molecule to absorb the electron, the probability drastically increases that light absorbed in the antenna complex will cause fluorescence—which is one of several competing modes of chlorophyll de-excitation. It is through the results of this competition that the most valuable information is gained concerning the photochemical health and functionality at the molecular level in plants [7]. The transient, after rising, then approaches a highly energetic steady state, at least for a few hundreds of milliseconds to seconds as F_P or F_M, because the plastoquinone pool in the electron chain has been reduced but not yet re-oxidized [4].

We do not believe that there is enough time for the xanthophyll cycle to activate and start absorbing excess energy from the antenna complex because no decrease from the steady state was observed. We also believe that the majority of our signal is coming from PSII and not PSI because about 90% of chlorophyll *a* is found in PSII, and PSI fluoresces more weakly for many highly sophisticated, yet interesting reasons. Specifically, we believe that the majority of the fluorescence from PSII is from the minor antenna complexes CP-43 and CP-47. However, some studies point out that a good part of the emission may be from the recombination of excited P680 with reduced Pheophytin [4]. We were not observing a decrease in fluorescence (from F_P to F_S) because such a decrease would take longer than one second, which is beyond our experimental window.

Fluorescence Microscope

From the images gathered using the Fluorescence microscope, a few conclusions can be made.

Firstly, sandpapering does not penetrate the epidermis into the mesophyll except for extreme cases in which the leaf was practically destroyed. With normal sandpapering, part of the epidermis may be removed, but there is no marked increase in fluorescence. In fact, there is little evidence to suggest that sandpapering the top of the leaf affects signal emission when either the front or the back is excited.

Secondly, in the extreme cases of abrasion, the mesophyll was penetrated and a very large increase in intensity (of about 80%) was measured (refer to Figs. 20 and 21). However, this phenomenon was only observed within the immediate vicinity of the large lacerations that resulted from the extreme abrasion. In fact, tissue within tenths of millimeters away from these lacerations was—in terms of their emission intensity—quite normal for a sandpapered sample. Lacerations were localized on or near veins, depending upon the direction of sandpapering, because these features protrude from surface and thus receive the greatest friction (Fig. 22).

Thus, other methods of removing the light-disrupting features—either air pockets or the thick epidermis, or others—should be contemplated and pursued for more accurate measurement of the chlorophyll transient, and subsequently, more accurate data on the chemical and physical health of a plant's most important operational components.

Importance of Chlorophyll Lifetimes

Fluorescence emission from photosynthesizing organisms is a highly valuable technique from which to gain insight to the immensely complex photochemical processes that no microscope can see and no chemistry lab can easily isolate. The fluorescent lifetimes and transient emissions are sensitive to even minute disruptions or abnormalities in photosynthesis, making it a cornerstone of biology. Using chlorophyll lifetimes is a useful tool to detect, monitor, and diagnose plants in adverse conditions [7]. There are many applications in a wide variety of fields for such diagnostics, including agriculture, quality control for commercial produce, plant pathogen detection and prevention, toxin detection—specifically acid rain, and even skin cancer treatments. [8] [9]

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