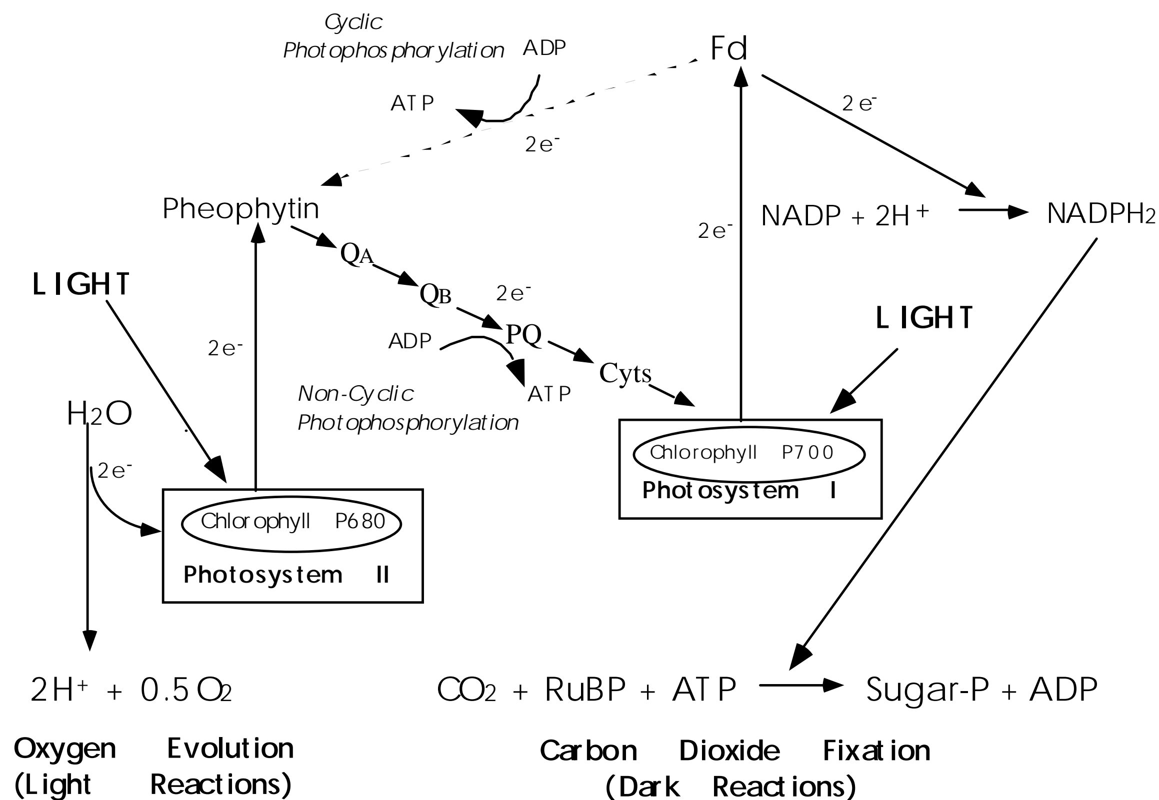
Chlorophyll Fluorescence and Photosynthetic Activity

The Light reactions of Photosynthesis

Photosynthesis involves the conversion of light energy into chemical energy mediated by light sensitive chlorophyll molecules in the leaf. Light-harvesting complexes involving numerous chlorophyll molecules intercept radiant energy and transfer this to a reaction center in Photosystem II (PSII). At the heart of the PSII reaction center a special chlorophyll molecule called P680 becomes excited and an electron from P680 is raised to a higher energy level. Under ideal conditions for photosynthetic activity this electron reduces an acceptor molecule called pheophytin (Fig.1).and is then transferred through a series of electron carriers including Q_A, Q_B Plastoquinone (PQ) and cytochromes (Cyts). This transfer of electrons is associated with the production of a transthylakoid proton gradient which may be coupled to the chemiosmotic production of ATP. This process is called non-cyclic photophosphorylation, and requires processing of 2 electrons from 2 P680 molecules for the production of ATP.

Fig. 1 Simplified Scheme for the Photosynthetic Light Reactions



Light harvesting complexes are also responsible for transferring energy to a second specialized chlorophyll molecule, called P700, at the heart of Photosystem I (PSI). When P700 becomes excited an electron is raised to a higher energy level and this may be involved in ATP production before it is returned to the oxidized P700. This process is called non-cyclic photophosphorylation and, like non-cyclic photophosphorylation, requires processing of 2 electrons from 2 P700 molecules. Alternatively, the electrons may reduce ferredoxin (Fd) which is then involved in the reduction of NADP to $NADPH_2$. The $NADPH_2$ may then be used in the reduction of CO_2 to sugar phosphates in the so-called “dark reactions” of photosynthesis as shown in Fig. 1.

If the electron released from P700 is used ultimately in the reduction of CO_2, the P700 molecule is left with a net positive charge. Electrical neutrality is restored by P700 accepting the electron released form P680 in PSII. Of course, the loss of an electron from P680 leaves this molecule with a positive charge and in this case the electron required to restore electrical neutrality is derived from the splitting of water. This reaction also generates the O2 gas that is released from the leaves of photosynthetic higher plants.

Alternative Pathways of Energy Dissipation

Like any other energy transduction system, conversion of light energy to chemical energy in photosynthesis is far from a perfect process. For various reasons, not all electrons raised to higher energy levels by irradiance are passed to electron acceptors and these electrons return to their parent chlorophyll molecules without the production of ATP and/or $NADPH_2$. The energy lost during the return to the ground state may be manifested as heat or light. If light is produced, it is emitted at a longer wavelength than the light that stimulated electron release from chlorophyll, and under these circumstances the chlorophyll molecule is said to **fluoresce**.

Chlorophyll fluorescence usually occurs maximally under conditions of high light when photosynthetic activity is inhibited. Consider, for example, a leaf that is exposed to high light after being maintained in the dark for several hours. During the dark period, several of the enzymes involved in the Calvin Cycle become deactivated and must be re-activated by light before they can operate. Also, the metabolites involved in the Calvin Cycle reactions must reach appropriate levels before CO_2 fixation can occur at optimal rate. Thus, there is a "Photosynthetic Induction Period" on the transfer of a leaf from dark to light during which CO_2 fixation is very slow.

During the photosynthetic induction period the electron acceptors in the leaf continue to accept electrons from excited chlorophyll molecules but have no way of dissipating the energy from these electrons because the "dark reactions" are not yet active or optimized. As a result, the number of these acceptors which can accept electrons quickly falls to zero since all reduction sites are occupied. Consequently, the electrons released from chlorophyll must dissipate their energy in another way. This is done partly by fluorescence, in which light is emitted as the electrons return to their initial energy level, and party by dissipation of energy as heat. Therefore, we would expect fluorescence to be high during the photosynthetic induction period, and then decline, as the CO_2 fixation reactions become more active.

Measurement of Fluorescence

Measurement of chlorophyll fluorescence using Qubit Systems' Chlorophyll Fluorescence Package requires a chlorophyll fluorometer with an LED light source to excite fluorescence and a detector to measure this fluorescence, a halogen light source for activating photosynthesis (actinic light), a leaf chamber or leaf clamp for positioning the leaf with respect to the light sources and detector, and a data acquisition system.

To measure fluorescence, the leaf is illuminated with a very weak LED light source (660 nm peak wavelength) that is pulsed 50 times per second (i.e. at a 50 Hz frequency). The duration of each flash is only 4µseconds. This light induces fluorescence to occur at the same frequency as the pulsed light, but the amount of light supplied to the leaf by the LED is, on its own, insufficient to drive photosynthesis. The chlorophyll fluorescence excited by the LED light is detected by a photodetector that has a long-pass filter so that it only detects wavelengths greater than 700 nm. Also, the signal from the detector is decoded by the fluorometer so that only fluorescence occurring at the same frequency as the pulsed LED light is measured. Thus, fluorescence caused by other light sources, including the actinic light source, is not measured by the system. The measured fluorescence is indicative of total leaf fluorescence, but because only the pulsed fluorescence signal is measured the signal from the photodetector does not saturate the detection system even under maximal fluorescence conditions. As a result, changes in

fluorescence can be measured under a very wide range of incident irradiances from darkness to conditions equivalent to many time full sunlight.

Fluorescence Yield and Photochemical Quantum Yield

When a photon of light excites a chlorophyll molecule a single electron is raised to a higher energy level as described above. This energy may be dissipated by use in photochemical reactions (P), by release of heat (D) or by fluorescence (F). Thus, the probability of each of these energy transduction processes occurring is given by the expression:

$$P + D + F = 1 \qquad \textbf{Eqn. 1}$$

where P is called the Quantum Yield of Photosynthesis and F is called the Fluorescence Yield. Of these parameters P is of greatest interest to plant scientists since this provides a measurement of the photochemical activity of the leaf. F may be measured quite easily by Qubit Systems' Fluorescence Package but P and D are very difficult to measure directly. Nonetheless, accurate measurements of P can be derived from measurements of F alone by the following method.

Again, consider a leaf that has been maintained in the dark for a time long enough to require a photosynthetic induction period on re-illumination. If, after this period, the leaf is exposed to an extremely bright flash of light, the chlorophyll molecules within the leaf are excited and pass their excited electrons to the electron acceptors of PSII and PSI. However, because the Calvin cycle reactions are not active, the $NADPH_2$ formed as a result of this photochemistry cannot be used and the pool of NADP available for reduction is very quickly exhausted. As a consequence, within a few microseconds of illumination, the electron carriers in PSI and PSII become unable to pass on their electrons and remain in the reduced condition. Therefore, electrons released from chlorophyll during continued illumination cannot be used for photochemical reactions.

As a result, during a saturating flash of light following dark-adaptation of a leaf quantum yield of photochemistry (P) declines to zero and Fluorescence yield (F) and Heat dissipation (D) reach maximal values (Fm and Dm)

Thus, during this flash of light:

$$Fm + Dm = 1 \qquad \textbf{Eqn. 2}$$

We can now express heat dissipation in terms of Fluorescence:

$$Dm = 1 - Fm \qquad \textbf{Eqn. 3}$$

It has been shown experimentally that the relative amounts of fluorescence and heat dissipation that occur during a brief (0.8 second) saturating flash of light are similar to those that occur under normal conditions of irradiance. Therefore:

$$Dm/Fm = D/F \qquad \textbf{Eqn. 4}$$

By substitution using Eqn. 4 we can derive the expression:

$$D/F = (1 - Fm)/Fm \qquad \textbf{Eqn. 5}$$

Therefore:

$$\mathbf{D = F(1 - Fm)/Fm} \qquad \textbf{Eqn. 6}$$

We can now substitute the D in Eqn. 1for the expression in Eqn. 7 thus:

$$\mathbf{P + [F(1 - Fm)/Fm] + F = 1} \qquad \textbf{Eqn. 7}$$

Therefore:

$$\mathbf{P = 1 - F - F(1 - Fm)} \qquad \textbf{Eqn. 8}$$

Simplifying:

$$\mathbf{P = 1 - F - F(1 - Fm) = (Fm - F)/Fm} \qquad \textbf{Eqn. 9}$$

The term (Fm-F)/Fm Eqn. 10 is usually expressed as Fv/Fm, where Fv is termed variable fluorescence since it represents the difference between maximum fluorescence (Fm) and background, or minimum fluorescence (see below).

Using the correct procedure of dark-adaptation of leaves prior to fluorescence measurements we can measure photochemical quantum yield purely in terms of chlorophyll fluorescence parameters thus:

$$\mathbf{P = Fv/Fm} \qquad \textbf{Eqn. 10}$$

It must be noted that chlorophyll fluorescence, and quantum yield, measured using Qubit Systems' Chlorophyll Fluorescence Package relates only to the activity of PSII, since it has been shown experimentally that only PSII (and not PSI) photochemistry is involved in inhibiting (quenching) the fluorescence measured by this system.

General Procedure for Measurement of Fluorescence Parameters

At the beginning of each experiment the background fluorescence from a dark-adapted leaf is measured when only the LED light is turned on. This value is termed Fo (see Figure 2 below). The output from the LED light is insufficient to drive photosynthesis and does not disturb the dark-adapted state.

Next maximum fluorescence yield of the leaf is measured i.e. the maximum fluorescence signal achieved when all chlorophyll molecules are saturated with light, and all electron acceptors are fully reduced. This is the Fm value. It is measured after applying a brief (0.8 second) saturating pulse of actinic light to the leaf after a period of dark equilibration. Quantum flux during the saturating pulse is in excess of 5000 µmol quanta/m^2/s.

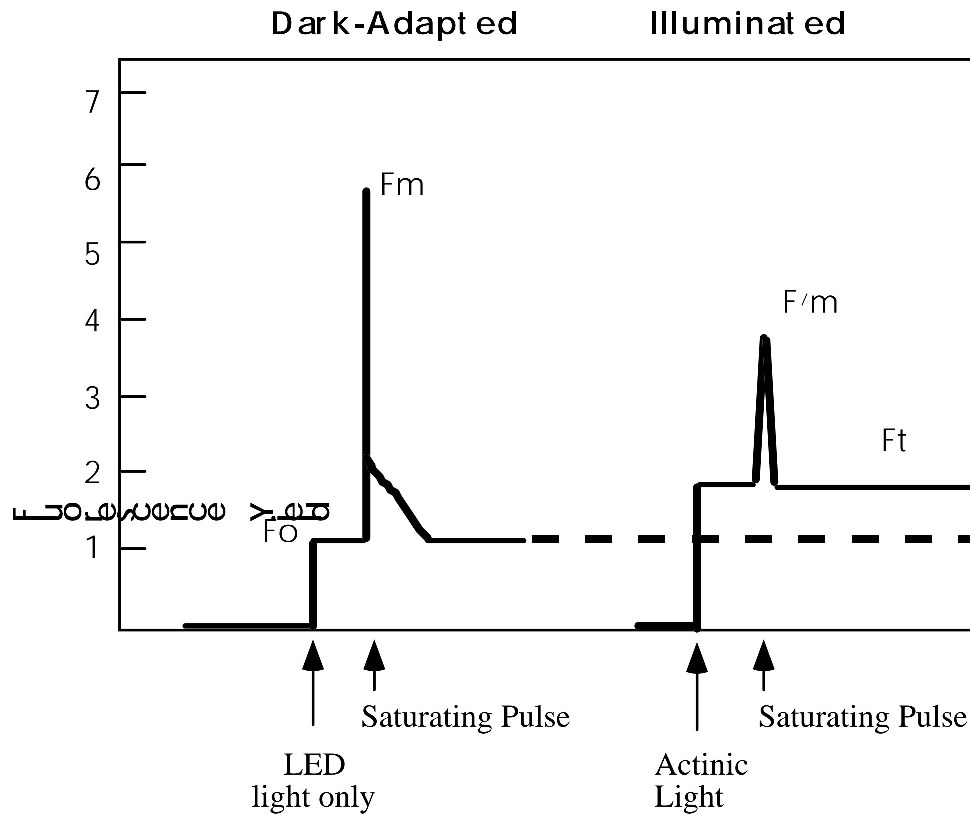
Quantum yield (P = Fv/Fm) of a dark-adapted leaf is calculated as (Fm – Fo)/Fm (see Eqn. 9 above). In healthy leaves this value is usually about 0.8.

To measure quantum yield under illuminated conditions the actinic light source is turned on and steady state fluorescence (the Ft value) is measured. A saturating pulse of light is then applied (of the same intensity and duration of that applied to the dark-adapted leaf) to obtain maximum fluorescence yield under illuminated conditions (the $F'm$ value).

$F'm$ has a lower value than Fm, and the $F'm$ value tends to decrease with increasing irradiance. This is because under high light conditions the proportion of photosynthetic electron acceptors that are oxidized

at any one time decreases, and heat dissipation with respect to fluorescence increases. The latter is due, in part, to the development of the transthylakoid proton gradient. As a result, the quantum yield of photochemistry, calculated as $(F'm - Ft)/F'm$ (see Eqn. 9) is reduced under high light conditions.

Fig. 2 Measurement of Chlorophyll Fluorescence Parameters



Measurement of Electron Transport Rate

The relative quantum yield of photochemistry (P) can be used to estimate the rate of photosynthetic electron transport in the leaf provided that the photon flux incident on the leaf is known. Since 1 µmol of photons causes the excitation of 1 µmol of electrons from chlorophyll, and P represents the proportion of these electrons that are used in photochemistry, the electron transport rate (ETR) is related to product of P and the photon flux density of photsynthetically active radiation at the leaf surface (PAR). However, not all the light incident on a leaf is absorbed by chlorophyll molecules, since some is transmitted through the leaf and some is reflected. About 84% of incident light is absorbed by chlorophyll molecules in the average leaf, with 50% of the photons activating chlorophyll molecules associated with PSII and 50% activating PSI. Therefore, an estimate of electron transport rate can be derived from the following:

$$\mathbf{ETR = P \times PAR \times 0.42} \qquad \textbf{Eqn. 11}$$

PAR is measured by a light sensor that is supplied with the fluorescence package.

Components of the Chlorophyll Fluorescence Package

The Chlorophyll Fluorescence Package consists of the following components:

(1) A chlorophyll fluorometer with a gain potentiometer to control the level of the LED light, a DIN cable to take the fluorescence signal to the computer interface and a ground cable.

(2) An actinic light control box with a potentiometer for controlling the level of the actinic light, a timer to control the frequency of saturating light pulses from the actinic light, a manual push switch to provide saturating light pulses as required, and a cable to take the irradiance signal from the light source to the computer interface.

(3) A pulse-modulated LED chlorophyll excitation light on a cable for attachment to the fluorometer

(4) A chlorophyll fluorescence detector on a cable for attachment to the fluorometer

(5) A laboratory stand

(6) A filtered 50W actinic halogen light source mounted in a lamp housing and attached to a metal bracket for positioning on the laboratory stand

(7) An aluminum bracket attached to the actinic lamp holder. This incorporates a leaf clamp for holding the leaf in a stable position with respect to the LED light and fluorescence detector, and fittings for mounting the LED light and fluorescence detector above the leaf clamp It also holds a light sensor that is calibrated to measure the PAR incident on the leaf.

(8) A DC power supply specific for your local power grid.

(9) A 4 channel Universal Lab Interface and Logger Pro software or 2 channel Serial Box Interface and Data Logger software.

Note that you may also use the chlorophyll fluorescence package in conjunction with the CO_2 analysis package manufactured by Qubit Systems to allow simultaneous measurements of chlorophyll fluorescence and photosynthetic CO_2 exchange. In this case the leaf is housed in a leaf chamber rather than being held on the leaf clamp and fluorescence measurements are made through the upper surface of the chamber.

Installation and Use of Logger Pro Software

Logger Pro software and customized set-up software for the fluorescence package are provided on 3.5" diskettes or a single CD. A full description of Logger Pro software may be found in the manual prepared by Vernier Software Inc. Installation procedures for Macintosh and PC computers are summarized below.

The students will be using Logger Pro data acquisition software to display data outputs from the analytical instruments in this package. Logger Pro is a Windows-and MacOS-based program that displays data graphically and in table-form as they are collected. The program has numerous data manipulation functions some of which the students will use for data analysis.

Macintosh installation instructions for Logger Pro and Qubit Systems' set-up files

- If using CD installation place CD in drive and double click on Install to load Logger Pro
- If installing from disk, place the first Logger Pro floppy disk into Drive A. Double click on Drive A icon.
- Install the MAC Logger Pro application program from the three installation disks. (Follow the on-screen instructions.) Choose the full installation (default) option, then choose the default destination which is in the root directory of your hard drive. Re-boot your computer.
- Open the Logger Pro folder on your hard drive. Drag and drop the 'Calibrations' and 'Experiments' folders into the trash and empty the trash.
- Place the Qubit Systems' C404-MAC disk into Drive A. Double click on the Drive A icon.
- Copy (drag and drop) the 'calibrations' folder from the diskette in drive A to the Logger Pro folder on your hard drive. Copy (drag and drop) the 'Fluores' folder from the diskette in drive A to the Logger Pro folder on your hard drive.
- The calibration files and fluorescence set-up files are now installed on your hard drive.
- To start Logger Pro using a particular set-up file, open the 'Fluores' folder and double click on the desired file (Fluores or FluorCO_2).
- To facilitate quick access to your set-up files, make alias files for the fluores set up files that are in your 'Fluores' folder. (Select each set-up file, then choose 'make alias' from the 'file' menu.)
- Place (drag and drop) the set-up aliases onto your Desktop.
- To lock the set-up files, select each file then choose 'get info' from the file menu. Click on 'lock'. This will prevent users from writing over the set-up files when saving their data.
- You should also create a User (student) folder on your hard drive. Create a new folder on your Desktop and call it 'Student Data Files'. Folders for individual students can then be created within this folder.
- To start Logger Pro with a particular set-up file in place, simply double click on the appropriate set-up file alias on the desktop.

Windows 3.1 installation instructions for Logger Pro and Qubit Systems' set-up files

- If using CD installation place CD in drive and the installation program will start automatically.
- If installing from disk, place the first Logger Pro installation disk into Drive A.
- In Program Manager go to 'File' on the menu bar and select 'Run'. In the Command Line type 'a:\setup' and click 'OK'.
- A series of dialog boxes will step you through the installation procedure. Proceed as instructed.

- When you reach the 'Set Up Type' screen, select 'Custom'. From the list of file options select only 'Program Files', 'Calibration Files' and 'On-Line Help Files'.
- The destination path for Logger Pro should read C:\Vernier\LoggrPro. If it does not, the Qubit Systems' calibration and set-up files will not be installed into the correct directory structure. Use the 'Browse' function to select the correct destination on your hard drive.
- If your computer lacks Microsoft WIN32s set-up files you will be prompted to load these before the Logger Pro installation is complete. These files can be downloaded from the Vernier web site at www.vernier.com "Free Stuff" page or requested from Vernier Software.
- Insert the Qubit Systems' diskette C404-PC and in Program Manager go to 'File'.
- Select 'Run' on the Menu bar.
- In the Command Line type 'a:\install3'. This will install the Qubit Systems' calibration and set-up files.
- To start Logger Pro, select 'Programs' under the 'Start' icon, and then 'Vernier Software' and Logger Pro.
- Open the calibration files in the following directory:
- C:\Vernier\LoggrPro\Calfiles.
- Open the set up files in the following directory:
- C:\Vernier\LoggrPro\fluores

Windows 95 and 98 installation instructions for Logger Pro and Qubit Systems' set-up files

- If using CD installation place CD in drive and the installation program will start automatically.
- If installing from disk, place the Logger Pro installation disk into Drive A.
- Open the 'My Computer' folder on desktop.
- Double click the Drive A icon.
- Double click the 'Install' Icon for Logger Pro.
- A series of dialog boxes will step you through the installation procedure. Proceed as instructed.
- When you reach the 'Set-Up Type' screen, select 'Custom'. From the list of file options select only 'Program Files', 'Calibration Files' and 'On-Line Help Files'.
- The destination path for Logger Pro should read C:\..\Vernier Software\Logger Pro. If it does not, Logger Pro will not be installed into the correct directory structure. Use the 'Browse' function to select the correct destination on your hard drive. This should be C:\Program Files\Vernier Software\Logger Pro
- Insert the Qubit Systems' diskette C404-PC (calibration files and fluorescence set-up files) into the A Drive.
- Double click on the Drive A icon in the 'My Computer' folder.
- Double click on the 'Install.bat' icon. This will install the calibration files and the fluorescence set-up files.
- Start Logger Pro. (Double click on the Logger Pro icon.)
- When Logger Pro loads, it will call up a default set up which will not be the set up that you will require for the experiments in this package. To change the set up, click on 'File' and then 'Preferences.' A dialog box will appear which will show the directories that contain the files you may select.
- First, check the path shown in the 'Experiment' folder. If it reads 'Program files\Vernier Software\Fluor,' click on 'O.K.' and leave the 'Preferences' dialog box. Then click on 'File' and 'Open'. The set-up files should be listed on the left side of the new dialog box. Double click on your choice of set-up file. This will open the file. You are now ready to collect data. If the path

shown in the 'Experiment' folder is not 'Program Files\Vernier Software\Fluores,' you must use the following procedure to specify the correct path:

- Select 'Modify' next to the Experiment folder box. Then scroll down the directory options until you reach 'Program Files'. Double click on 'Program Files.' Scroll down to find 'Vernier Software.' Double click on 'Vernier Software.' Scroll down to find 'Fluores.' Double click on 'Fluores.' Click on 'O.K'. and leave the 'Preferences' dialog box. Then click on 'File' and 'Open'. The set-up files should be listed on the left side of the new dialog box. Double click on your choice of set-up file. This will open the file. You are now ready to collect data.
- To make your set-up files read-only, select each set-up file in turn, choose 'properties' from the 'file' menu, click on 'read only', then 'apply' and then 'O.K.' This will prevent users from writing over the set-up files when saving their data.

Running Logger Pro

When the program starts, you should see a graph or series of graphs appear on your computer screen. If the ULI is not connected to the default port (COM 2) on your computer, the message 'ULI cannot be found on COM 2' will appear on your screen. If this happens, click on COM 1 (if this is where the ULI is connected) or click on 'Scan port'. If you receive a message stating 'ULI2 Rev 1.00', click on 'OK'.

If you still have a problem, re-check the connection to the port on the computer and to the back of the ULI. Also, check that the 9V jack is plugged into the ULI and that the adapter is plugged into the power source. If there is a problem with your power source, the green light on the back of the ULI will be off.

Creating your own custom set-up files

After your set-up file loads, a graph or combination of graphs will appear on the screen. Each custom set-up file will show the output from one or more of the instruments supplied with the package. You may create your own custom set-up file by changing the sensor inputs, calibration files, graph ranges, running time and data collection rate as described below. For additional details, please refer to the "How to" section of your Logger Pro manual.

Selecting Sensor Inputs and Calibration Files

Ensure that each sensor you wish to use is connected to an appropriate DIN port on the ULI. Click on the blue ULI icon to the left of the 'Collect' icon above the displayed graphs. When you click on a DIN icon you will see the sensor to which that port has been assigned. You may change the sensor assignment by scrolling through the sensor options in the dialogue box, and highlighting the sensor that you have attached to the DIN port. You should also ensure that the correct calibration file is loaded for the sensor you select. Do this by scrolling through the calibration file options in the calibration dialog box.

Graph Ranges

To modify the ranges of the X- and Y- axes click on the maximum and/or minimum values in turn, type in the value you desire and then click on the Return Key. Note that the maximum time value that you select for the X-axis limits the time over which you can collect data for a particular run. Data collection will stop automatically at the end of the time period specified. If your experiment is still running when the data collection period expires select 'Data' from the main menu and then 'Store Latest Run'. You may then restart data collection. (Click on 'Collect'). Data from the first part of your experiment will

remain on the screen as a faint trace and new data will be plotted in bold. You may collect numerous runs in this way. Each run will be collected to a separate data table. When you save your data, all the runs will be saved under the same file name. You can customize most of the options of any graph by double clicking on appropriate portion of the graph (such as the graph title, data line, etc.) and selecting options from the dialogue box that is displayed.

Running Time and Data Collection Rate

To set the running time for your data collection, select ‘Set Up’ from the main menu and then ‘Data Collection’. Select ‘Sampling’ from the dialogue box, and type in the collection time that you require in the box labeled ‘Experiment Length’. You may also specify the length of the data collection period by altering the maximum value on the X-axis, as described previously.

The default data collection rate is five points per second for Fluorescence set up files, and one point per second for all other set-up files. For long experiments, you may wish to reduce the sampling rate to avoid the creation of very large data files. For experiments where important transient conditions are being investigated, you may wish to sample at a faster rate. Logger Pro can collect a maximum of 30,000 data points per input channel, per session, so you should set your sampling rate and collection time accordingly.

Collecting Data and Saving your Data Collection File

To start data collection, click on the ‘Collect’ icon above the displayed graphs. Once data collection has started, this icon changes to read ‘Stop’. You may click on this icon to stop data collection at any time.

If you wish to save your data, stop data collection, select ‘File’ from the main menu, then select ‘Save As’. Type in an appropriate file name. If you do not type in a new name and your set-up file is not locked, your data will be written over your set-up file. You should save your data files under a new name and store them in an appropriate location on your hard drive or on a 3.5” disk in drive ‘A’. To recall your stored file for viewing or analysis, select ‘File’ then ‘Open’ from the main menu. You may then navigate to your stored file via the ‘Look In’ dialog box.

Configuration of the Chlorophyll Fluorescence System

Refer to Figure 3 and configure the chlorophyll fluorescence system as follows:

(1) Attach the lab stand to the lab bench by using the clamp and thumb screws

(2) If you are measuring CO_2 exchange at the same time as chlorophyll fluorescence attach the leaf chamber to the lab stand at a height suitable for the plant you will be using. Remove the leaf clamp from the aluminum bracket attached to the actinic light support, and then attach this to the lab stand sliding it down the stand until base of the aluminum bracket touches the upper surface of the leaf chamber.

(3) If you are not measuring CO_2 exchange leave the leaf clamp attached to the aluminum bracket and slide the actinic light fitting down the lab stand until the leaf clamp is at an appropriate height for the plant you are using.

(4) Place the brass fitting holding the LED chlorophyll excitation light into the smaller of the two holes in the aluminum bracket and secure it in place with the nylon screw. Plug the cable from the LED light into the socket labeled "LED" on the front of the chlorophyll fluorometer.

(5) Place the brass fitting holding the chlorophyll fluorescence detector in to the larger of the two holes in the aluminum bracket, and plug the cable from the detector into the socket labeled "Light Sensor" on the chlorophyll fluorometer.

(6) Attach the ground plug on the green cable to the green socket on the back of the fluorometer and attach the alligator clip to any point on the aluminum bracket.

(7) Attach the plug on the cable from the actinic light and light sensor to the socket labelled "Bulb" on the rear of the actinic light control box.

(8) Attach the plug on the FL1 DC power supply to socket labelled 12 V Power on the rear of the fluorometer.

(9) Attach the red and black banana plugs on the power supply cable to the positive and negative sockets of the large DC power supply and attach the plug on the other end of the cable to the socket labeled "Actinic Power" on the rear of the actinic light control box. Plug the power supply into the mains socket and turn it on.

(10) Attach the 5 pin DIN plug on the cable from the rear of the fluorometer to the DIN 3 socket on the Universal Lab Interface.

(11) Attach the 5 pin DIN plug on the cable from the rear of the actinic light control box to the DIN 4 socket on the Universal Lab Interface.

(12) Before placing a leaf in the leaf chamber or leaf clamp ensure that the potentiometers labeled "Actinic" and "Auto Flash" on the light control box are turned fully counter-clockwise and that the power switch is set to the 'Off' position. It is also advisable to turn the Gain dial on the fluorometer fully counterclockwise before powering the unit. This minimizes irradiance of the leaf from the LED measurement light.

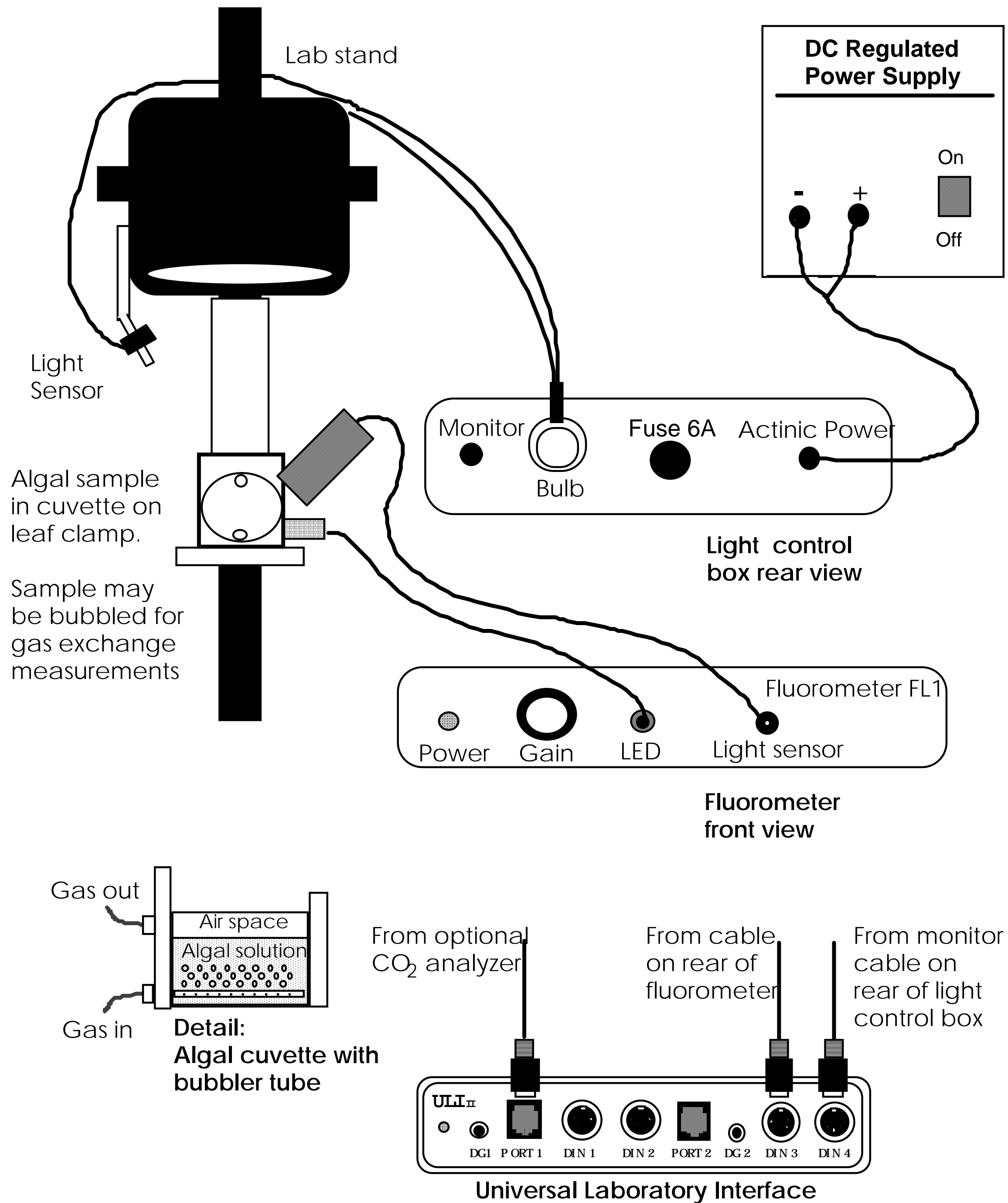
Note that the LED excitation light fitting and the fluorescence detector fitting may be moved back and forth within the aluminum bracket by adjusting the tension of the nylon screws that hold them in position. Also, the aluminum blocks that house the fittings may be tilted to different angles by loosening the screws that attach them to the bracket. These features allow the user to adjust the position of the LED and the detector to optimize the fluorescence signal.

It is also important to ensure that the actinic light source is centered above the leaf clamp or the leaf chamber. The values of actinic light incident on the leaf measured by the photodetector are only accurate if the lamp is placed vertically above the leaf sample. The angle of the lamp housing may be corrected, if necessary, by adjusting the black screws that hold the housing in place.

The best signals are usually obtained when the LED and fluorescence detector are placed as close as possible to the leaf sample, without shading it from the actinic light, and angled at approximately 45• to the leaf surface. When measuring chlorophyll fluorescence of a leaf in a leaf chamber, the LED and detector fittings should not protrude beyond the position of the neoprene gasket within the chamber or they will obscure part of the leaf from the actinic light. If positioning of the fittings is difficult, the lateral position of the leaf chamber itself may be altered slightly by loosening the wing nuts that attach the chamber to its bracket and moving the chamber sideways.

Figure 3

Configuration of Chlorophyll Fluorescence System



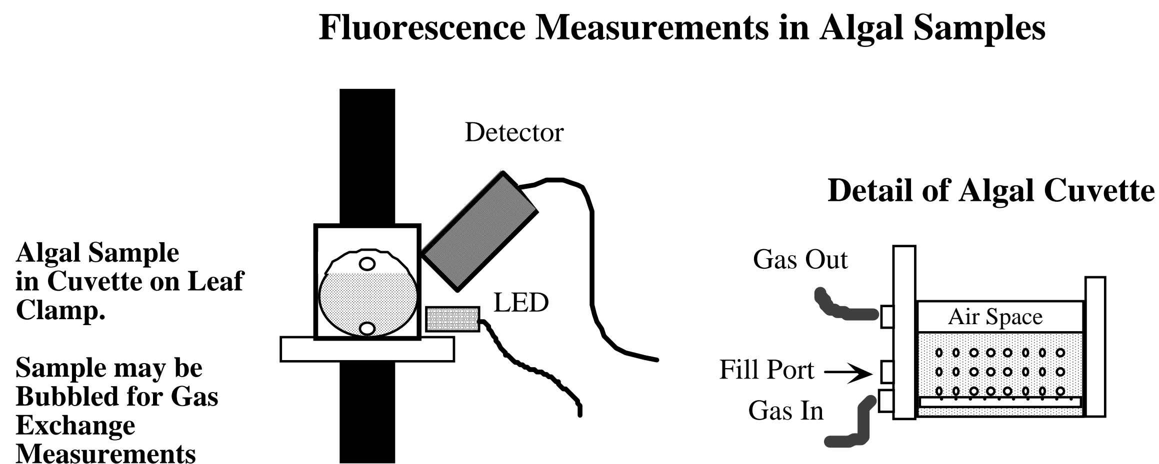
Measurement of Algal Fluorescence with Qubit System's Fluorescence Package

As well as providing measurements of chlorophyll fluorescence parameters in leaves of higher plants, Qubit Systems' Fluorescence Package may also be used to measure these parameters in algal samples in the aqueous phase. Set up of the fluorescence package hardware is exactly the same as that for use with leaf samples as described in the previous section of this manual.

The algae are placed in a transparent cuvette that is supported on the base of the leaf clamp. The LED chlorophyll excitation light is placed below the photodiode that measures fluorescence (see Figure 4), and both are positioned close to the wall of the sample vial. Actinic illumination is provided from above by a 50 W halogen lamp that provides from 0 – 2000 µmoles quanta PAR m^2/s at the surface of the vial. The same lamp also provides a saturating flash of >5000µmoles quanta PAR for measurements of Fm and $F'm$. It is not possible to calibrate the light sensor for measurements of PAR within the algal sample since this will depend on the optical density of the sample.

Figure 4

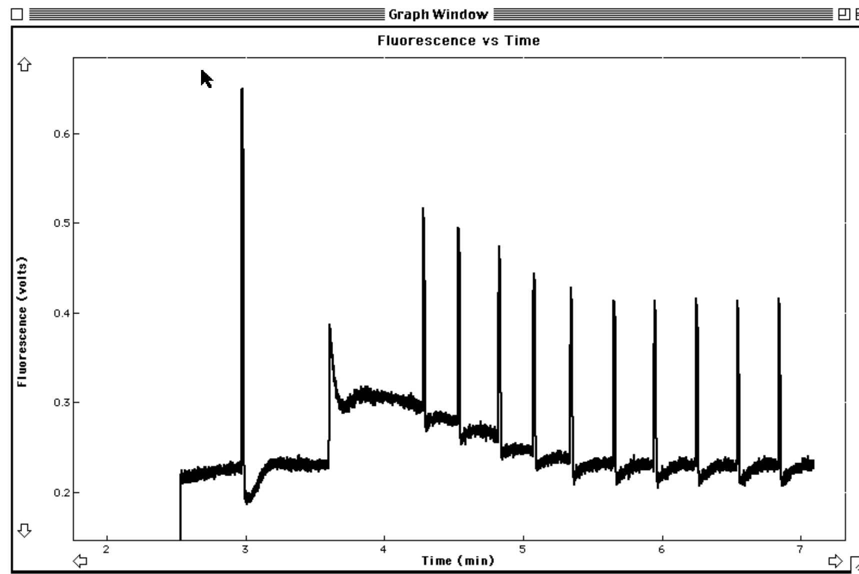
Fluorescence Measurements in Algal Samples



The data shown in Figure 5 were collected using a sample of Chlamydomonas (0.6 mg/L total chlorophyll) maintained in the algal cuvette. These data allow calculation of quantum yield in the dark-adapted and illuminated condition, as well as estimation of PS II electron transport rate if the PAR in the sample is measured.

Figure 5

Chlorophyll Fluorescence Characteristics of *Chlamydomonas*



The sample may be bubbled during measurements of fluorescence with only a slight increase in the noise of the fluorescence signal. Measurement of CO_2 concentration in the effluent gas stream using Qubit Systems' S151 CO_2 analyzer allows simultaneous measurement of CO_2 exchange if the sample has sufficient activity. Also, the effects of changes in environmental parameters, and the effects of adding metabolites or inhibitors, on both the light and dark reactions of photosynthesis may be evaluated simultaneously with chlorophyll fluorescence parameters..

Filling and Emptying the Algal Cuvette

The algal cuvette has a Luer port for inlet gas at its base and a Luer port for outlet gas at the top of the sample chamber. Each port is coupled to a length of Excelon tubing by a Luer connector. The tubing attached to the inlet port is supported by a clamp at the top of the cuvette to keep it in a vertical orientation. This, and the very small holes in the bubbling tube, ensures that the sample does not leak from the chamber if gas flow is interrupted.

A fill port is located at the base of the cuvette slightly offset from the gas inlet port. This is plugged during use, and is used only when filling, draining and cleaning the cuvette.

To fill the algal cuvette:

- Detach the plug from the fill port of the algal cuvette.

- Take a measured sample of your algal suspension in a syringe and attach the male Luer of the syringe to the female Luer fitting on the fill port of the cuvette.

- Tilt the cuvette on its side and inject the sample slowly. Remove the syringe and replace the plug in the fill port

- Tilt the cuvette back to vertical ensuring that the surface of the suspension remains below the level of the gas outlet port.

To empty the algal cuvette:

(6) Remove the plug from the fill port and the Excelon tubing from the gas outlet port.

(7) Tilt the cuvette to drain the algal suspension.

(8) If required, flush the cuvette with water or a sterilizing solution.

(9) You may dry the cuvette by blowing dry air at a high flow rate into the gas OUTLET port while keeping the fill port unplugged. Do not blow air at high flow rate through the gas inlet port as this may cause detachment of the bubbling tube.

Qubit Systems manufactures two algal cuvettes; one suitable for samples of 10 mL and below, and one for samples of 30 mL and below

A Gas Exchange System for Measuring Algal CO_2 Exchange

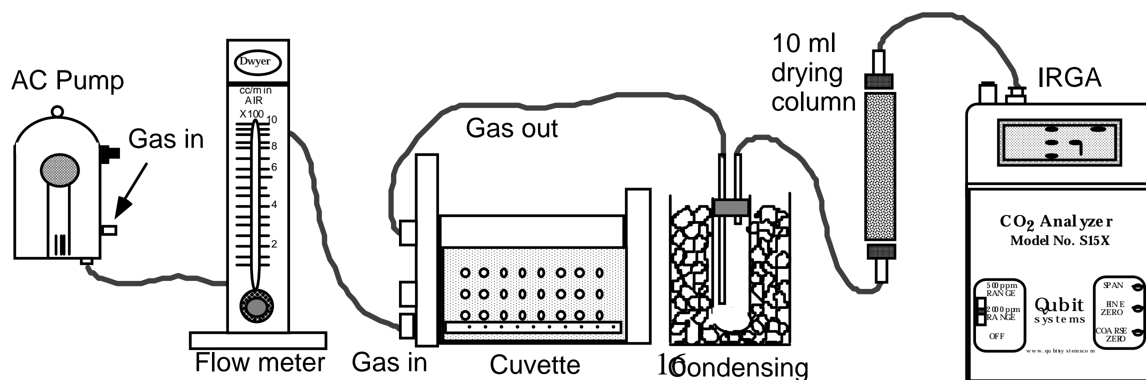
The algal cuvette is designed for use in a gas exchange system as shown in Figure 5. Gas with a stable CO_2 concentration is attached to the inlet of the pump. This may be provided from a pressurized supply or from a gas bag that is filled with ambient air. The CO_2 concentration of the gas supply must be measured before the start of an experiment.

The gas is pumped through a flow meter, and flow is regulated by a needle valve to a rate that produces a measurable difference in CO_2 concentration between the gas entering the algal cuvette (reference gas) and leaving the cuvette (sample gas). The chosen flow rate will depend on the density of the algal sample its biological activity.

The sample gas leaving the cuvette must be dried before it enters the CO_2 analyzer. A column containing a magnesium perchlorate drying agent is provided with the CO_2 analyzer, **but it is vital that the majority of water in the sample gas is condensed out of the line before it enters the magnesium perchlorate column.** This is achieved by passing the gas through a test tube (or other container) kept in an ice bath. The gas enters through a long tube that reaches the bottom of the container, and exits through a short tube (see Figure 5). This arrangement must not be reversed since it may result in condensate entering the exit gas line and saturating the magnesium perchlorate.

Figure 6

A Gas Exchange System for Measuring Algal CO_2 Exchange



Magnesium perchlorate liquefies when saturated and if this liquid enters the IRGA serious damage will result. **Qubit Systems is not responsible for damage caused to the IRGA in this way.** The condition of the magnesium perchlorate must be checked each time the IRGA is used, and it must be replaced whenever signs of exhaustion (clumping or shrinking at the column inlet) are observed.

Drierite may be used as a non-deliquesent alternative to magnesium perchlorate. This is not as effective a drying agent, but it presents no melt-down hazard. Silica gel should not be used as a drying agent since this may absorb and desorb CO_2.

Calculation of CO_2 Exchange Rate

Measurements of photosynthetic rate in photosynthetic organisms are usually expressed as rates of CO_2 exchange per unit time per unit tissue parameter. The units most commonly used are µmoles of CO_2 per second per m^2 (for leaves), or per unit weight of tissue or chlorophyll. To express your data in these units use the following calculations:

(1) Calculate the difference between the CO_2 concentration in the reference and sample gases. For example, if an experiment was conducted in air of 350 ppm CO_2, at a flow rate of 50 mL/min, the depletion of CO_2 due to uptake in photosynthesis may result in a sample gas CO_2 concentration of 310 ppm. The difference between the reference and sample gas streams (δCO_2) in this example would be 40 ppm.

(2) Convert the δCO_2 value from ppm into umoles per litre thus:

$\delta CO_2 / 22.413\ ([T+C]/T)$

where C is the temperature in • C and T is the absolute temperature (273K).

At a temperature of 20• C, and a δCO_2 of 40 ppm, the δCO_2 would be equivalent to 1.66 µmol CO_2 per liter.

(3) Multiply the δCO_2 value by the flow rate (in liters per second) used in your experiment to obtain a CO_2 exchange rate per second. A flow rate of 50 mL/min is equivalent to 0.00083 L/s. So the CO_2 exchange rate in our example would be 0.0014 µmol/s.

(4) Express your CO_2 exchange rate on a tissue parameter basis by dividing the CO_2 exchange rate per second by that parameter. For example, if your algal sample had a total chlorophyll content of 0.6 mg/L and you used a 10 mL sample in your experiment, the total amount of chlorophyll in your sample would be 0.006 mg. The CO_2 uptake rate in your sample would therefore be 0.23 µmol CO_2 per mg chlorophyll per second.

Carbon Dioxide and Bicarbonate

Note that the CO_2 exchange rate measured using the algal cuvette will provide an accurate measurement of carbon uptake by the algae only if the CO_2 supplied in the gas stream is the only source of carbon available to the algae. If the algae are maintained in a nutrient medium that is rich in bicarbonate, there

may be little difference observed between the CO_2 concentrations in the reference and sample gas streams, since the dissolved bicarbonate will provide the major source of inorganic CO_2 for photosynthesis.

To maximize CO_2 uptake from the gas stream the algal samples should have been been cultured in a bicarbonate-free medium equilibrated with air. Alternatively, if the algae were cultured in the presence of high bicarbonate concentrations, a sample may be separated from the culture medium by centrifugation and then re-suspended in air-equilibrated bicarbonate-free medium before injection into the algal cuvette. Note that when working with blue-green algae (cyanobacteria) it may be necessary to add carbonic anhydrase to the medium for rapid attainment of the CO_2-bicarbonate equilibrium when bubbling with air.

Addition of Metabolites and Inhibitors to the Algal Cuvette

Many interesting photosynthetic phenomena may be observed by the addition of inhibitors of the light and/or dark reactions of photosynthesis to an algal suspension during measurements of CO_2 exchange and chlorophyll fluorescence. Effects of metabolites may also be investigated in the same way.

The most convenient way to add these compounds to the suspension is to unscrew the Luer fitting from the gas outlet port of the cuvette and to inject a solution containing a the compound using a syringe fitted with a needle. Turbulence created by the bubbling tube should be sufficient to distribute the compound throughout the algal suspension within a period shorter than the response time of the gas exchange system.

Operation of the Chlorophyll Fluorescence System

Software and Set Up Files

Install Logger Pro software on your computer according to the instructions given in the "Installation and Use of Logger Pro Software" section of this manual. The set up files you will be using with the fluorescence package are provided on a floppy disk and are called "Fluores.MBL" and "FluorCO_2.mbl".

If you are measuring CO_2 exchange as well as fluorescence, you will use the FluorCO_2 set-up file. Attach the analog output from the CO_2 analyzer to DIN1 of the Universal Lab Interface, the output of the fluorescence sensor to DIN 3 and the output of the actinic light source sensor to DIN4. You may attach the output of a humidity sensor, or any other sensor, to DIN2, but ensure that you specify the chosen sensor under the "Sensor Set-Up" option of the Logger Pro menu. The humidity sensor has been selected as the default sensor for DIN 2 in the FluorCO_2 set-up file. After you have loaded FluorCO_2, four graphical displays will appear on your computer screen showing the outputs from the chosen sensors. You may start collecting data at any time by clicking on the "Collect" button at the top of the screen.

If you are measuring only fluorescence and actinic light level, you will use the Fluores set-up file. Attach the output of the fluorescence sensor to DIN 3 and the output of the actinic light source sensor to DIN 4. After you have loaded Fluor, two graphical displays will appear on your computer screen showing the outputs from the fluorescence sensor and the level of actinic light incident on the leaf. You may start collecting data at any time by clicking on the "Collect" button at the top of the screen.

The Chlorophyll Fluorometer

The fluorometer has a potentiometer for adjusting the gain of the fluorescence signal. With the system activated, but with zero actinic light and a dark-adapted leaf in the leaf chamber, you should adjust the gain potentiometer to increase or decrease the fluorescence signal so that an optimum signal to noise ratio is obtained. The maximum gain setting is appropriate for many circumstances. With very large signals, or if noise levels are high, the gain setting may be reduced.

The Actinic Light Control Box

The potentiometer on the front left of the actinic light control box is used to activate the actinic light and to control irradiance level. The irradiance level is monitored by a light sensor exterior to the lamp housing that sends a signal to the control box where it is conditioned and amplified. The signal is sent to the Universal Lab Interface via a cable from the back of the control box, and is monitored in Logger Pro software. Continuous Actinic irradiance can be adjusted between 0 and approximately 2500 $\mu E/m^2/s$.

At any given background irradiance, the actinic light can provide a 0.8 second saturating pulse of light (>5000 $\mu E/m^2/s$) to obtain values of Fm or $F'm$. The pulse can be activated by pressing the "Flash" button on the front of the actinic light control box, or a series of pulses can be programmed at regular intervals using the potentiometer on the front right of the control box. The maximum frequency of pulses is 1 pulse per 8 seconds and this is attained by turning the potentiometer fully clockwise.

Typical Experimental Procedure

A typical experiment would involve the following:

(1) Attach the DIN connectors from the fluorescence monitor, irradiance monitor, CO_2 analyzer and other sensor (if required) to the appropriate DIN sockets on the Universal Lab Interface, and load the FLUORES set-up file in Logger Pro.

(2) Click on the Collect icon to start collecting data. Obtain a zero fluorescence reading without any leaf in the leaf chamber. If the numerical value for fluorescence on screen reads 0.00, use a small screw-driver to adjust the zero control on the rear of the fluorometer until the value increases to just above zero (e.g.0.05). This is necessary because Logger Pro cannot read negative voltages and the system may have a significant zero offset unless a true zero is measured.

(3) If measuring CO_2 exchange as well as fluorescence, place a dark-adapted leaf into the leaf chamber and place an opaque card (such as a business card) over the window of the chamber to maintain the dark condition. If not measuring CO_2 exchange keep the sample leaf in a dark condition while you configure the fluorescence apparatus as described above.

(4) Turn on the light control box by turning the switch to the 'On' position and turn the "Intensity" potentiometer clockwise until it clicks. This will maintain the leaf in the dark until the potentiometer is turned clockwise. You may also set the potentiometer to a desired light setting before selecting the 'On' position with the switch. This will allow you to expose the leaf immediately to the desired actinic irradiance without moving through a range of lower irradiances.

(5) Place the dark adapted leaf on the leaf clamp or remove the business card from the leaf chamber window and observe the voltage signal from the fluorescence detector both on graphically and numerically in the data box at the bottom of the computer screen.

(6) Adjust the gain control on the fluorometer, if necessary, to set Fo at an appropriate value with a low signal noise. An Fo value between 0.2 and 0.5 is usually optimum.

(7) Press the saturation pulse button on the light control box and observe the transient increase in the chlorophyll fluorescence signal. The peak value represents Fm. If this value is too high, and saturates the fluorescence detector, you may adjust the gain control on the fluorometer to bring it within a measurable range. However, you will need to dark adapt your leaf again to obtain an Fm value.

(8) Having measured Fm, turn on the actinic light source and adjust it to the desired irradiance. This will cause fluorescence to increase transiently to a level close to that of Fm, and then decline to a new steady state value (Ft). This transient fluorescence response on a dark-light transition is called the Kautsky effect, and you may investigate it by applying saturating pulses of light, either manually or automatically, with the "Rate" potentiometer, during the non-steady state condition.

(9) You may now proceed with your experiment and measure maximum fluorescence ($F'm$) periodically by either pressing the saturation pulse button when desired, or adjusting the flash frequency dial on the actinic light control box to select an appropriate flash frequency.

Data Analysis

Having completed your experiment you may analyze your data and obtain values of Fo, Fm, Ft, $F'm$ and $F'o$. Select "Analyze" from the main menu, and then "Examine.". A vertical line will appear on each of your graphs which can be moved to any point in the data sets. Boxes will also appear that show the data value at any time point selected. Therefore, to measure Fm, for example, position the line at the maximum fluorescence value after the saturating pulse was applied to the dark-adapted leaf. Record this value and subtract the value of Fo to obtain the Fm value. Repeat this procedure for the measurement of $F'm$, and subtract the Ft value to obtain ΔF. To obtain accurate measurements you may need to use the Zoom features as described below.

Zooming In

If you run experiments over longer periods you may find it difficult to identify the fluorescence peaks accurately when examining your data. To simplify this you may Zoom In on any part of your data. Position the cursor at the start of the data you wish to examine and click and hold on the mouse. A black box will appear around your selected data as you move the cursor across the screen. When you have selected the data set, unclick on the mouse and select “View” and then “Zoom In”. Your selected data set will fill the entire screen and you may use the Examine function from the Analyse menu to obtain specific values.

Using the Statistics Function to Obtain Values

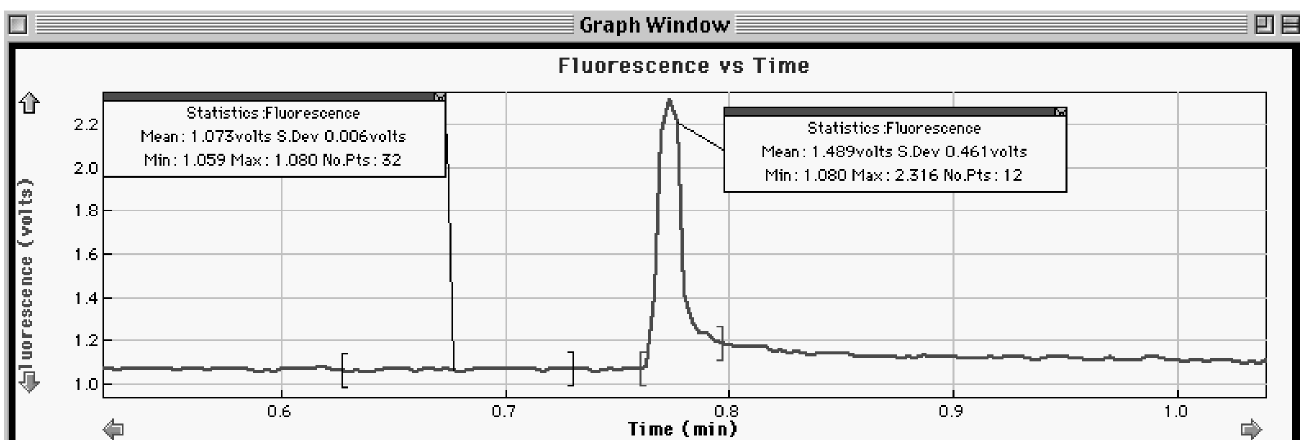
If there is noise on the fluorescence signal, or the $F^{/}m$ value is only slightly greater than the F value, you may use the Statistics function of Logger Pro to obtain the best values for your fluorescence parameters. Zoom In on your data as described above and then highlight the data you wish to examine by clicking and dragging over the data with the mouse. A black box will appear around the selected data, which will remain when you unclick on the mouse. Select “Analyse” from the main menu and then “Statistics”. A box will appear on the screen showing the mean, maximum and minimum values of all the data in the box, as well as other statistical parameters. From this data you can obtain, for example, the mean value of F or Fo, before a saturating light pulse was applied. If you use the statistics function to analyze the data following a saturating pulse, the maximum value shown in the statistics box identifies the maximum fluorescence value (Fm or $F^{/}m$). You may delete the statistics boxes from the screen by clicking on the icon in the top right hand corner of the box.

Annotating Your Data

If you wish to make notes on your data, you may select “Make Annotation” from the “Analyse” menu. A box will appear on the screen in which you may type your comments. The box may be dragged with the mouse to an appropriate location on the screen. The box may be deleted by clicking on the icon in the top right hand corner.

Example of Experimental Data

In the following display the Zoom In function has been used to produce a full screen display of fluorescence data just before, during, and just after a saturating pulse of light is applied to the leaf. The statistics function has been used to show the mean value of F before the flash, and to identify the maximum fluorescence value ($F^{/}m$) during the flash. The range of data included in the statistical analyses are marked by the parentheses visible on the display.



Experiments

Note that the following four experiments are given exactly as they appear in the Student's Manual, except that additional notes are provided to the Instructor. The reference to instructions for set up of the chlorophyll fluorescence system and running Logger Pro, refer to the instructions in the Student's Manual and not to the more detailed instructions provided in this manual.

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