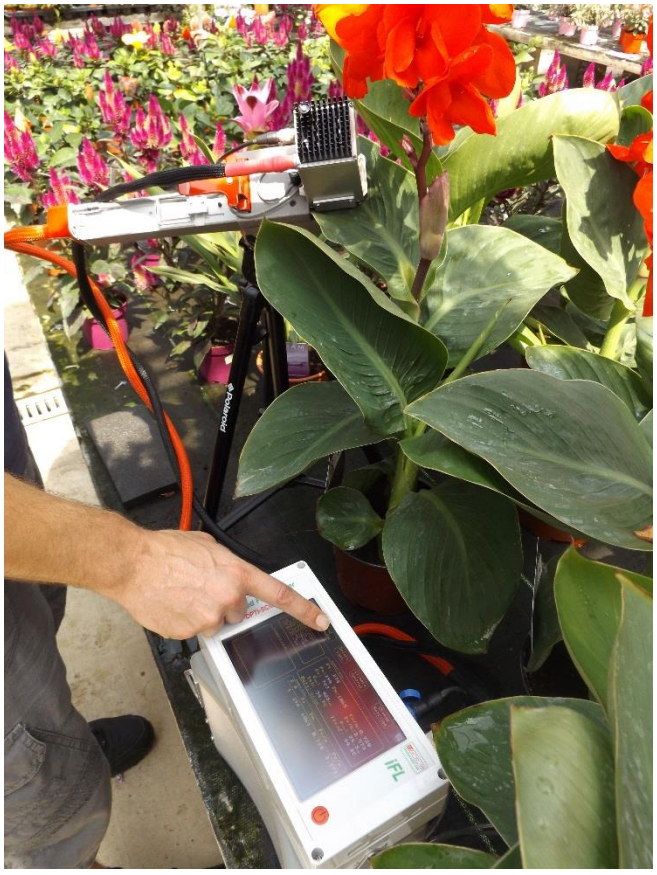
BioScientific Ltd.

Analysing the carbon cycle

***i*FL** *Integrated Fluorometer and Photosynthesis System*

OPERATING MANUAL



L.MAN-iFL
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Contents

Introduction to the i*FL* Integrated System:

The iFL is a joint venture between Opti-Sciences Inc. and ADC BioScientific. The directions for using the integrated system are contained in the first part of this manual. To operate the LCpro-SD separately from the iFL integrated chlorophyll fluorometer, refer to the second part of this manual, entitled “Using the LCpro-SD without the iFL”.

The i*FL* extraordinary capabilities include:

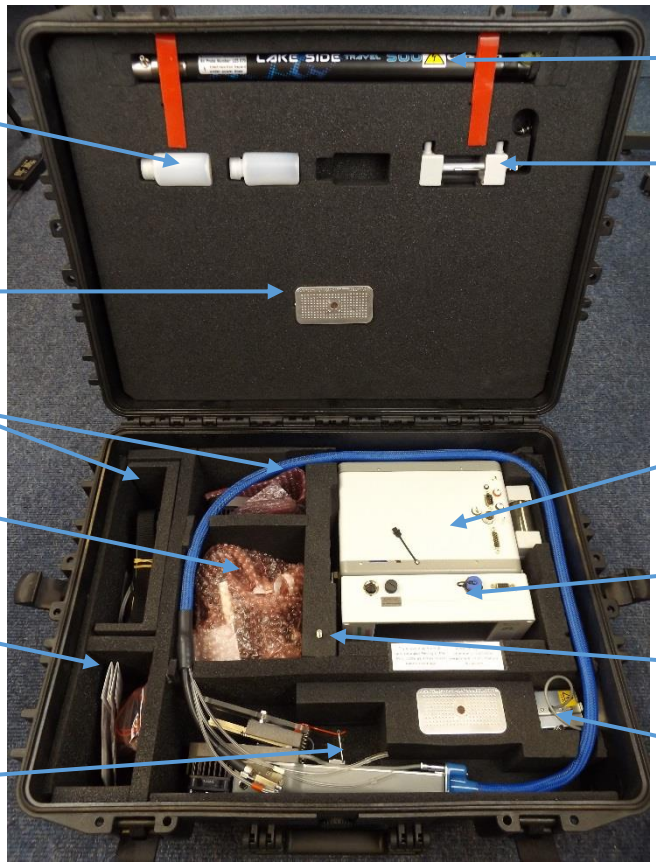
1. An intense white actinic light source with an intense blue spectrum. This light source allows chloroplast migration in a similar fashion to natural light conditions.
2. The iFL is the first integrated system to measure leaf absorptance. Leaf reflectance is measured using an RGB sensor. Leaf transmittance is measured using an RGB sensor.
3. An IR temperature sensor, covering approximately 80 of the leaf area. It was found that when chamber temperatures are substantially different from ambient conditions, point source measurement of leaf temperatures can lead to errors, whether a thermistor or a thermocouple is used. By measuring over a larger leaf area, results are more reliable (Pons 2009). Furthermore, these sensors are much more durable. The IR sensor is self-calibrating.
4. Walk away automation. Automatic IRGA zero, the walk away software platform, and long battery charge life (8 -16 hours) allow one to easily set up an experiment, run the experiment and come back hours later to see the results without intervention. Some of the longer protocols, like the Laisk protocol to determine R_d and Γ^*, may take more than 4 hours. These tests may be performed without intervention or battery change. The iFL always takes a "matched measurement", or single IRGA measurement every time a record is produced. Furthermore, the IRGA zero is automatically calibrated every time it takes a measurement, ensuring that every measurement is free of IRGA drift.
5. Protocols can be run one after another, using the automation platform. For example, after running the Laisk protocol, the R_d and Γ^* values may be automatically transferred to an A/Ci curve to provide an A/C_C curve and g_m calculations.
6. Automated built-in post-processing with curve fitting. Protocols and curve fitting allow graphing and automated parameter readout for the Laisk protocol, the Kok protocol, the Yin protocol and the Flexas chamber leakage protocol. The von Caemmerer Γ* Laisk correction is a selectable option. The instrument allows direct readout of R_d, Γ*, $\Gamma^*_{von\ C}$, g_m, and C_C. Furthermore, the R_d and Γ* values determined using the Laisk protocol and Flexas chamber leakage protocol can automatically be added to future measurements, for example in A/Ci curves to provide more reliable A/C_C curves.
7. F_M' correction according to Loriaux et al., 2013 ***with*** the option of standard square-topped flash for more reliable chlorophyll fluorescence measurements at high actinic light levels.
8. Lake Model quenching protocols with Kramer Lake Model, Hendrickson simplified Lake Model, and Puddle Model quenching parameters also provided.
9. A second PAR sensor, located on the top of the fluorometer, allows the environment inside the fluorometer chamber to approximate ambient conditions. The intensity may be set to maintain an ambient value, for faster steady state adjustment before measurement, especially on partly cloudy days. Alternatively, intensity may be set to track ambient conditions inside the chamber.
10. HDMI output allows the iFL measuring screen and all other *iFL* screens, including post processing graphs, to be displayed for teaching/presentation purposes.

Other, key capabilities:

1. Environmental control of actinic light intensity, temperature, humidity, and CO_2 concentration within the leaf chamber.
2. Programmable routines for A/Ci curves and A/Q curves.
3. Battery charge life of 8 to 16 hours.
4. AC battery charger, which may be used while running tests.
5. SD card for data storage and data transfer (via the internal memory).
6. USB port for data transfer to and from the internal memory.
7. Integrated chlorophyll fluorescence data and gas exchange data readout.
8. Measurement of chlorophyll fluorescence and gas exchange over the same leaf area. An important feature because under certain conditions, patchy fluorescence is known to occur. Under drought stress, under cold stress, and at low CO_2 levels found in A/Ci curves, using a system that averages chlorophyll fluorescence over the same measuring area will provide more reliable results.
9. Filter fluorometer design for more reliable chlorophyll fluorescence measurements.
10. Light weight. The entire iFL system weighs less than 4.48kg, or 16.5 lbs.
11. 1.9GB of data storage internally and additional storage available with additional data cards. The system will work with either 1 or 2 GB SD cards, but not larger than this.

Equipment list and case contents

The iFL is supplied in a convenient carrying case, containing:

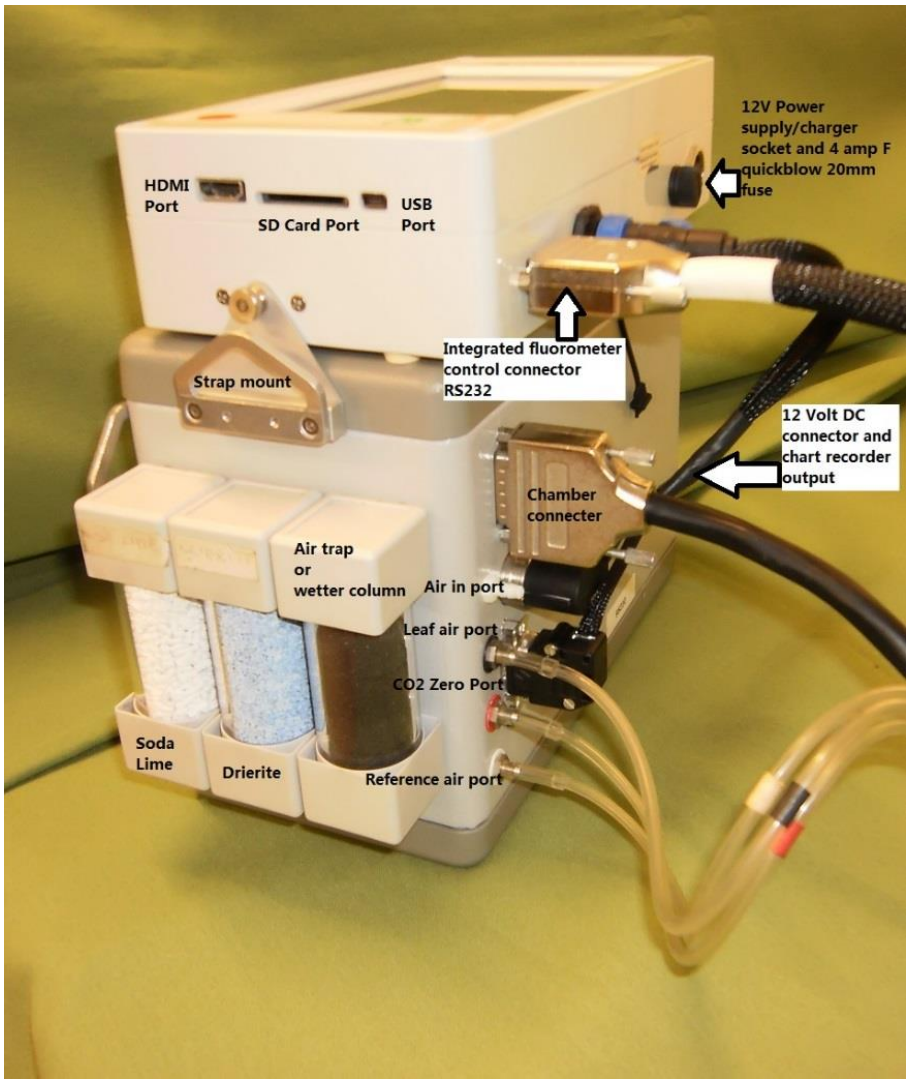


NOTE the cord must be stored in exactly this way.
Tuck the cord into the recess in the foam all the way around the edge, to prevent damage.

Please note – The supplied carry case is for 'By Hand' transportation only, if the instrument is being shipped by courier (for instance back to ADC for servicing) then it is highly recommended that suitable packaging – such as a cardboard box filled with polystyrene chips is used to protect both the case and instrument.

In addition to the above items there is a boxed "Spares kit" M.SKF-118 which contains some of the spares listed in Appendix 9.

Assembling the i*FL*



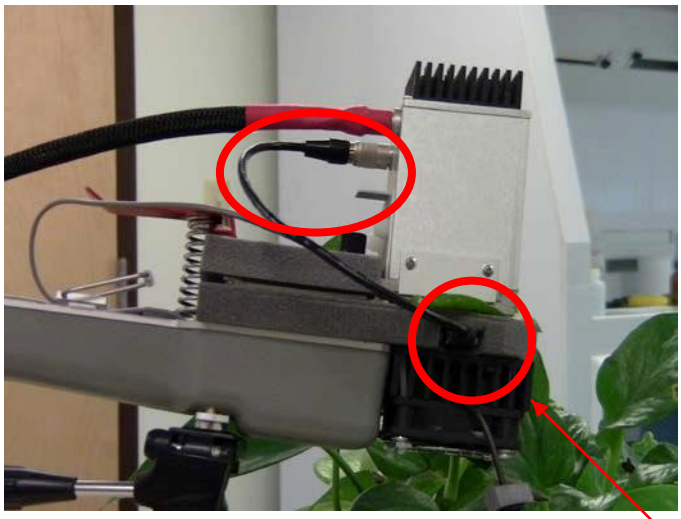
Assemble before turning the system on.

Console image showing HDMI port for high definition TV monitor and projector output of *iFL* viewing screen. The middle port shows the SD card inserted into the *iFL*. The USB port to the right is for downloading data files and images to computers. When using the *iFL*, these ports and connections should be used for data transfer and not the ports located on the LCpro-SD, shown on page 14.

Two Important notes on the SD card use.

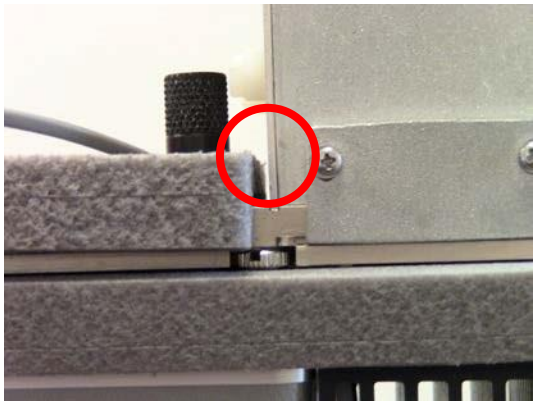
The SD card memory must not exceed 2GB.

The files on the SD card cannot be directly accessed via the USB cable, the USB cable will only access the IFL internal memory (a FLASH card on a PCB). Files may be transferred back and forth from the SD card to/from the internal IFL memory by touching the file on screen in the file memory then touching the copy option on screen.



Mount the *iFL* light unit onto the LCpro-SD leaf chamber as shown. The transparent radiation shield (not shown) must be removed before it can be attached, by loosening the three screws by hand. After positioning the light unit, lock it into place using the slot along the back of the measuring chamber. Plug the light unit cable into the LCpro-SD console.

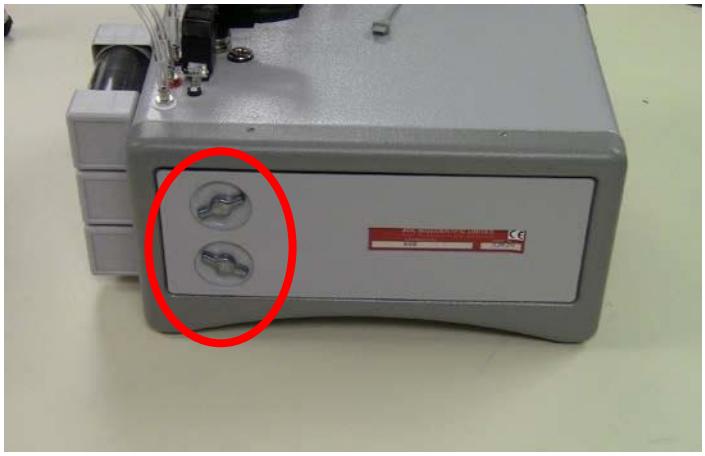
Connect the transmittance RGB sensor to the illuminator using the small cable with a mini USB connector on one side. The mini USB plugs into the side of the leaf chamber.

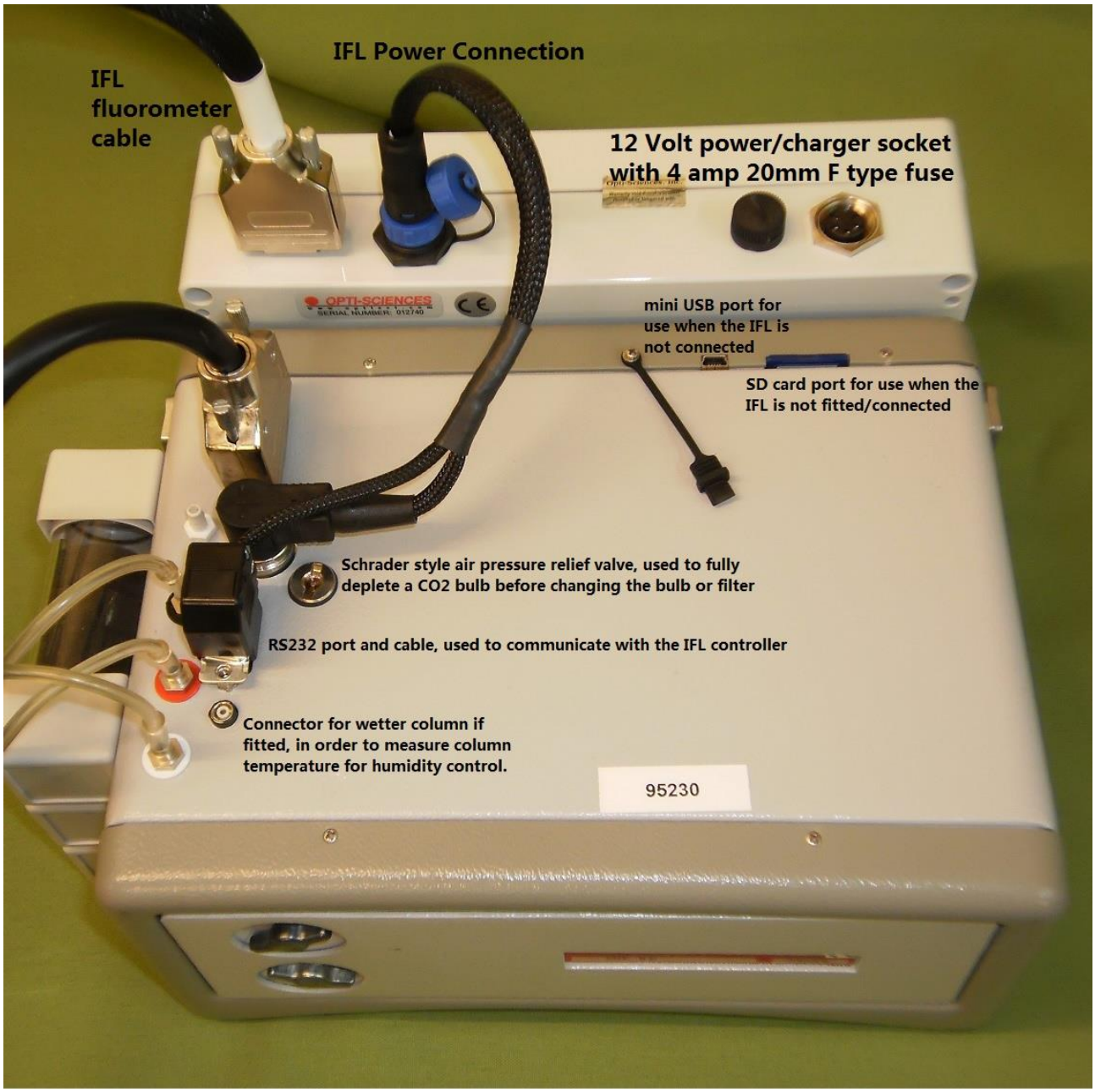


When mounting the iFL fluorometer head onto the leaf chamber, slide the back plate on the fluorometer head into the slot provided on the leaf chamber cuvette as shown on the left. Lock it in place.

The battery and CO_2 cartridges go into the base of the larger console. See the directions for installation elsewhere in this manual. *The Schrader valve should be depressed to eliminate cartridge pressure before removing existing CO_2 cartridges. The Schrader valve location is pictured on the next page.*

Before removal of the CO_2 cartridge, press the Schrader air pressure valve (shown in its location on the next page), until the CO_2 cartridge pressure has been released! ***This is not necessary when accessing the battery.*** Turn the two silver knobs on the bottom panel to release the bottom plate. This will expose the battery, and the CO_2 cartridge location. A coin can be used to remove the CO_2 cartridge cover that will appear after the bottom cover is removed.





Another assembled view and explanation of components.

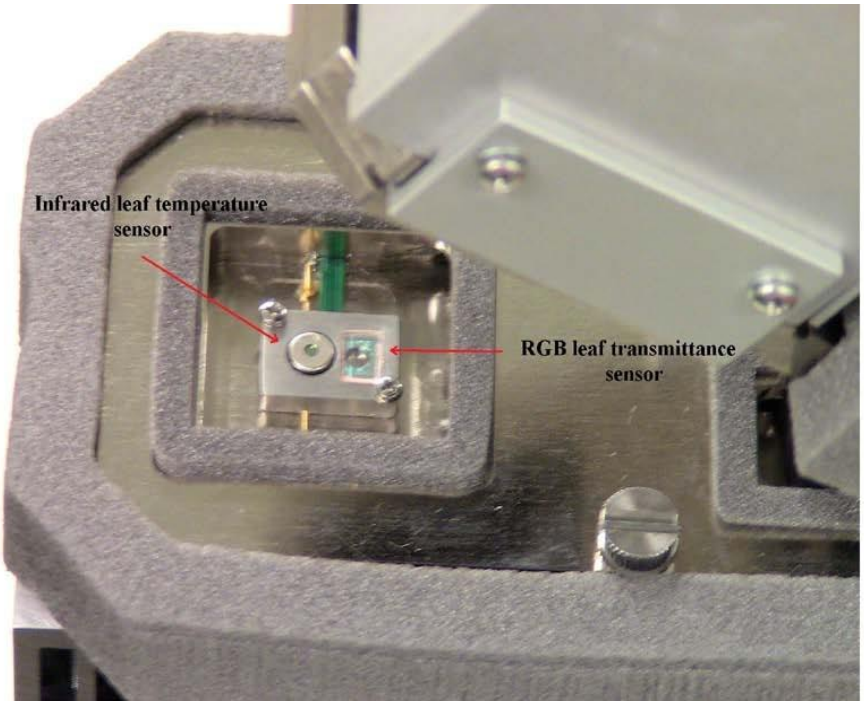
Switching on the i*FL*

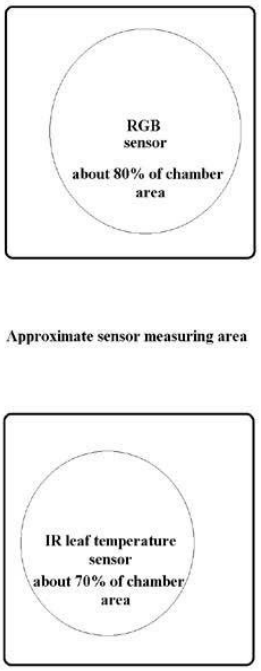
Turn on the system **only after everything has been connected properly.**

Use the orange button on the top of the iFL Console.

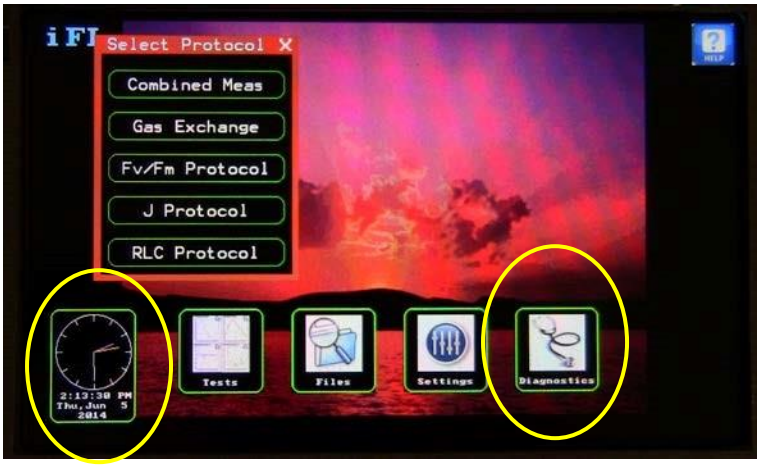
As the system boots up, a message may appear: 'analyser warming up'. It takes about 5 minutes for the IRGA to warm up before the system will allow further adjustment or measurement.

The picture below is of the Infrared leaf temperature sensor and RGB leaf transmittance sensor mounted in the *iFL* leaf chamber. The diagrams on the right display the approximate leaf area measurement for the two types of sensors.





Initial Adjustments



Clock and date

To adjust the date and time tap the clock on the bottom left of the opening screen. Changing the date and time will be self-explanatory.

Diagnostics - Calibration of leaf absorptance

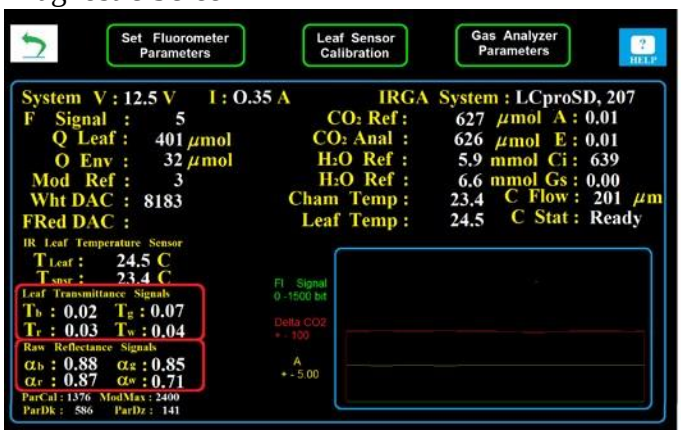
Every time the iFL illuminator head is reattached to the LCpro-SD gas exchange chamber, the leaf absorptance system must be re-calibrated. The system uses an RGB sensor below the leaf to measure leaf transmittance, and it has an RGB sensor above the leaf to measure leaf reflectance. The sensors break leaf reflectance and leaf transmission into Red, Green, Blue, and white spectrums. The white is not used in calculations. The iFL uses a special intense white light LED that radiates the same spectral ratio output throughout its intensity range. This means that the instrument radiates the same percentage of blue light and red light at 2,000 µmol as it does at 100 µmol.

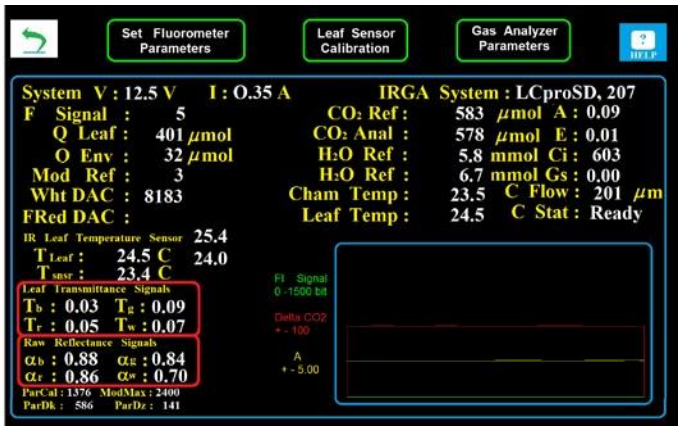
A PAR Sensor is located inside the light source to provide a stable white light output for extended periods of time to ensure steady state photosynthesis. The PAR sensor constantly monitors light source output and makes adjustments as necessary. Light source output is held stable to +- 3 µmol.

At the factory, we have compared measurements to using an integrating sphere with very favourable results. While it is not an integrating sphere, differences are very small. As a result, we are confident that the iFL will provide a strong approximation of measurements made with an integrating sphere. The alternative is either to create an integrating sphere using the same white light source and sensors, or to use averages that can produce J or ETR errors of more than 15.7% (Eichelman 2004).

To calibrate the iFL, assemble the instrument and make sure that the illuminator is securely fastened to the leaf cuvette. After turning on the instrument with the on off button located on the top of the console, let the instrument warm up. A sign will appear if the instrument has not yet warmed up. This can take a few minutes. Next select “Diagnostics” on the touch screen, and the screen shown below will appear.

Diagnostic Screen





The two screens above show how leaf transmittance changes due to chloroplast migration at high white actinic light levels over a 30 minute time scale. T_b is blue light transmission, T_g is green light transmission, T_r is red light transmission, and T_w is white light transmission. The α values are reflectance values for the same spectral regions.

To calibrate absorptance measurement:

1. Tap the Leaf Sensor Calibration button on top of the "Diagnostic Screen".
2. A box will appear. Select "Calibrate Leaf Absorptance".
3. A message will appear to place the absorptance standard in the leaf chamber covering the foam seal completely, with the black side facing up. When ready, tap "Continue".
4. Next a message will appear to place the measuring standard covering the foam seal completely with the white side facing up. When ready, tap "Continue".
5. Tap the white "x" on the orange box in the upper right hand corner. Then tap "Calibrate Transmittance". A message will appear that says to remove anything in the measuring path. When ready, tap "Continue".
6. Exit using the white "x" on the orange box when done.

Absorptance is now calibrated. Calibration should repeated every time the system is taken apart and reassembled. Absorptance measurement will occur in all light-adapted protocols using chlorophyll fluorescence. Measurements will be made before every saturation pulse if 'measure leaf absorptance' is selected in the protocol set-up window. Values may also be added in the set up window, or average values may be used. It is highly recommended that leaf absorptance be used for more reliable results.

$$J = \Phi PSII \times Q \times \alpha \times \beta$$

or Electron Transport Rate = Y(II) x PAR actinic light level x leaf absorptance x PSII to PSI reac. cent. ratio.

$$\alpha = ((\alpha_r)(B_r) + (\alpha_b)(B_b) + (\alpha_g)(B_g)) - (T_r/I_r + T_b/I_b + T_g/I_g)$$

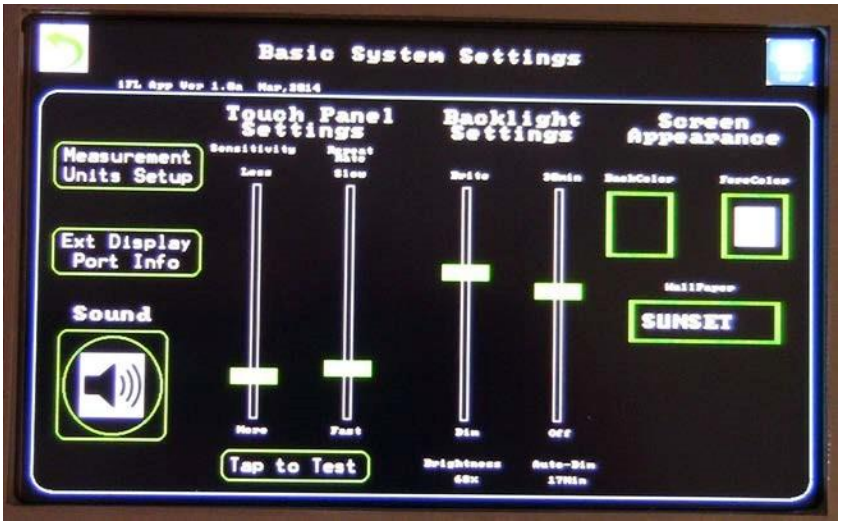
Here, absorptance = ((absorptance of red) (fraction of red radiation on the leaf) + (absorptance of blue) (fraction of blue radiation on the leaf) + (absorptance of green)

(fraction of green radiation on the leaf)) – (transmittance of red/raw red radiation incident on the leaf + transmittance of blue/raw blue radiation incident on the leaf + transmittance of green/raw green radiation incident on the leaf.)

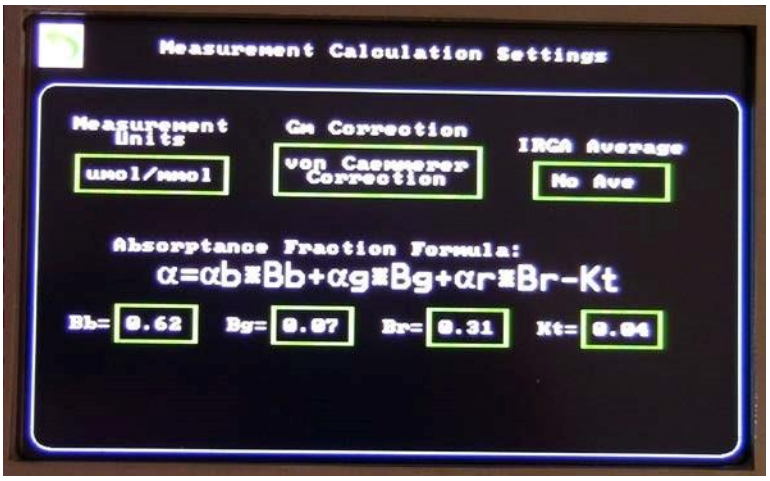
After calibrating the absorptance system, exit the "diagnostic screen" by tapping the green arrow in the upper left corner of the screen. Now go to "Settings".

Settings Window

On tapping the "Settings" window from the main screen, the screen below appears.



Here the type of measuring units can be selected (either µmol and mmol or ppm and mb). To set units, tap "Measuring Units Setup". When this is done the following screen appears:



Measuring Units

Measuring units may be reported in µmol and mmol or in ppm and mb. Tap the 'measuring units' window to select units.

g_m correction

The Laisk protocol is the most commonly used method to determine R_d and Γ* in C_3 plants which are in turn used to measure g_m and C_C. This function allows the Susan von Caemmerer correction to be included in the determination of Γ*. By tapping the "Gm Correction" window, this can be turned on or off.

IRGA Average

This window allows averaging of IRGA measurements. The default setting is "No Average". Other selections include 2, 4, and 8 measurement averaging. A single measurement is made in approximately 25 seconds, therefore it takes 50 seconds to produce a 2 measurement average.

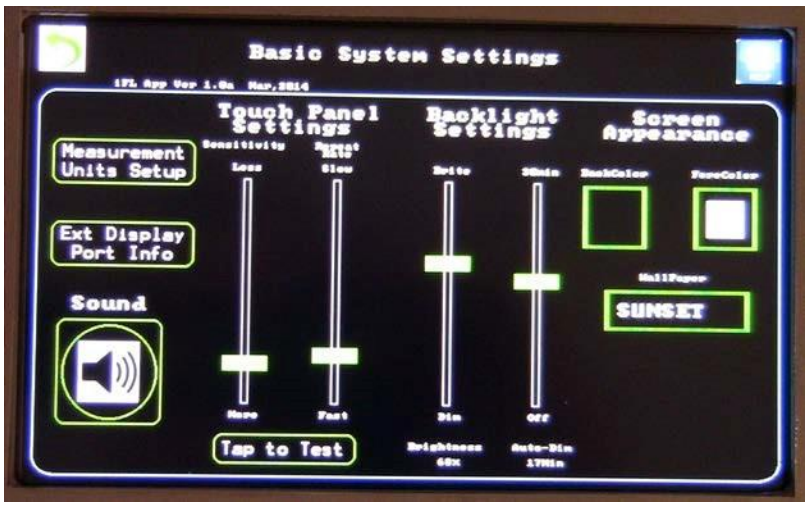
Absorptance Fraction Formula

This function serves two purposes. Below an actinic light level of 50 µmol, the leaf transmittance is no longer measured and the value that is put into the "Kt" window is used for the ratio of light transmitted through the leaf. The default value is 0.04, which may be changed by tapping the 'Kt' window.
The other values in the formula are determined at the factory. They represent the ratio of instrument LED light in that part of the spectrum that is incident on the leaf. B_b, for blue light, is 62%, Bg, for green light, is 7%, and Br, for red light is 31%. These ratios remain constant throughout the LED intensity range. The values are tested and are set at the factory. The factory set numbers are shown on the picture above.

When done, tap the green arrow in the upper left hand corner and the Basic System Settings screen appears. Information about the HDMI display port can be viewed by tapping the "Ext Display Port Info" button. Touch Panel Settings can be adjusted from very sensitive to less sensitive. Screen back lighting can be adjusted and the time for the screen to dim to save battery life can be adjusted from 0 to 30 minutes. If the instrument is set to 0 the instrument does not dim.

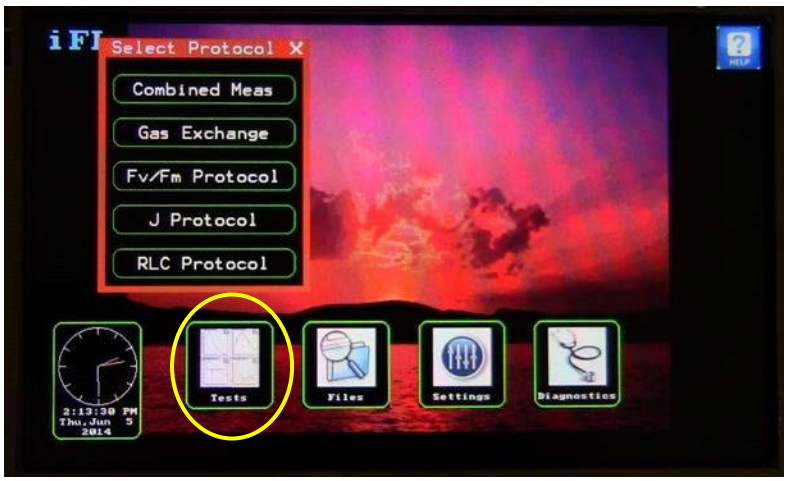
The background and lettering can be set by tapping the appropriate Screen Appearance button.

Wall paper may be added by transferring images using an SD data card and selecting the file under "Wallpaper" on this page, for the main screen. It may then be selected from this screen.



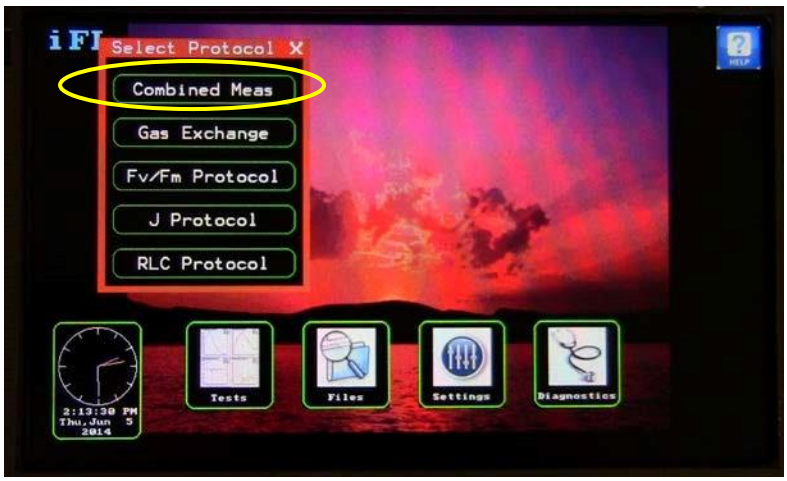
The next step is to set up the measuring method that will be used.

When finished with the setup, tap the green arrow in the upper left hand corner of the window.



Measuring Methods

1. Combined Gas Exchange and Chlorophyll Fluorescence
2. Gas Exchange Measurement Only
3. Y(II) light adapted chlorophyll fluorescence measurement method or $\Delta F'/F_M'$
4. F_V/F_M – a dark adapted chlorophyll fluorescence measurement method
5. RLC – Rapid Light Curves – a momentary dark adapted method that is designed to study the saturation characteristics of plants at different times of day. It is common to use this test on plants under canopy where the light is constantly changing, or to evaluate light adapted plants for saturation levels. I_K is the point where saturation starts to dominate, and I_M is the light intensity where maximum *J* or ETR occurs. I_M is where complete saturation of PSII occurs if the plant is steady-state light adapted to midday lighting and momentary dark adaptation is used.



Combined Gas Exchange and Chlorophyll Fluorescence

Operating this method

From the main screen, tap 'tests'. Select the "Combined Meas" method for integrated gas exchange and chlorophyll fluorescence. The main measuring screen for integrated use will appear.
Windows with green frames allow viewing more information.
The first page shows raw measuring data including "α" or leaf absorptance with leaf transmittance subtracted from the value. The measurements using red green and blue sensors are used here. The white values are not used.

Units shown in ppm and mb may be changed to μmol and mmol in the "Settings" section from the main screen.

Q_{Leaf} is actinic light level in the chamber
Q_{Env} is the ambient actinic light level outside of the chamber
C_{ref} is measured CO_2 before it enters the leaf chamber

ΔC is the difference between the CO_2 level of air leaving the chamber and the CO_2 level of air entering the chamber, corrected for IRGA (Infrared Gas Analyser) drift. Every measurement includes an automated correction made by scrubbing the air of all CO_2, measuring the scrubbed air, and correcting the IRGA zero.

e_{ref} is humidity in the air before it enters the chamber.
Δe is the difference between humidity of air leaving the measuring chamber minus the humidity of the air entering the chamber. ADC uses two Honeywell humidity sensors to measure H_2O to 1mol m^{-2} s^{-1}, the same resolution that is possible with IRGAs. However, new solid state humidity sensors provide more reliable measurements because they do not drift over time like IRGAs. Furthermore, they use much less battery power and require much less weight.

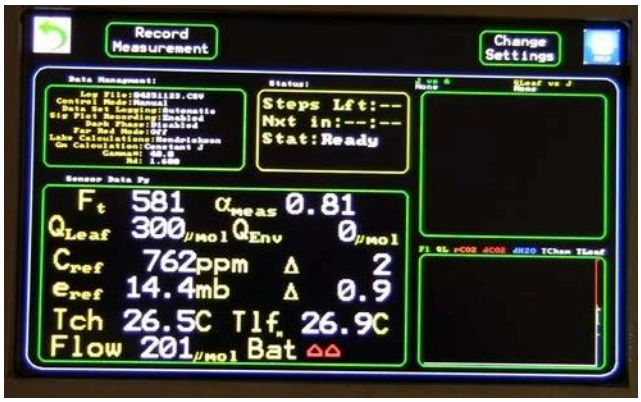
T_{ch} is leaf chamber temperature. It may be set to +-14^oC of ambient conditions.
T_{lf} is leaf temperature measured. The default method is with an IR sensor that provided an average leaf temperature over about 80% of the leaf chamber area. This method provides

a more reliable leaf temperature measuring method than others, especially when the chamber temperature is different than ambient (Ponds 2009).

Flow is the flow rate of air to the measuring chamber. The default setting 200 µmol but it may be set from 100 µmol to 500 µmol.

Bat is battery level charge. A full charge has 5 green pyramids. As the charge level goes down, the pyramid numbers are reduced, and they turn red when the number reaches 2. A full battery charge should last up to 16 hours if gas exchange alone is used, and up to 8 hours if integrated chlorophyll fluorescence and gas exchange are used.

F_t is the raw chlorophyll fluorescence signal as it is measured on a momentary basis.



If the green window on the bottom left is tapped, one can scroll through the other direct parameter readout values. The screen on the right above shows direct read out parameters derived from current and previous integrated measurements.

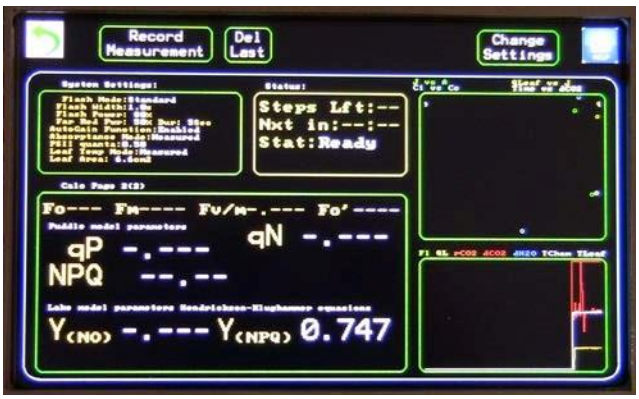
A is carbon assimilation.
E is transpiration
C_i is intracellular CO_2
Gs is stomatal conductance (g_s)

g_m is mesophyll conductance g_m– Before direct read out of g_m and C_C, R_d and Γ* must be measured using a protocol like the Laisk protocol, the Kok protocol, the Yin protocol, or the carbon isotope method. Measured values from internal protocols can be transferred directly to this measuring method or input in the "Data set up screen" when measured independently. The Laisk is the most used protocol for C3 plants, and the Kok protocol is commonly used for C4 plants, but it may also be used on C3 plants.

F' is the chlorophyll fluorescence level associated with the current actinic light level and plant conditions. **F_M'** is the chlorophyll fluorescence value associated with the last saturation flash at 7,000 µmols saturating light.

ΦPSII is the quantum yield of PSII or Y(II), or $\Delta F'/F_M'$ developed by Bernard Genty in 1989. ***J*** is electron transport rate also called ETR.

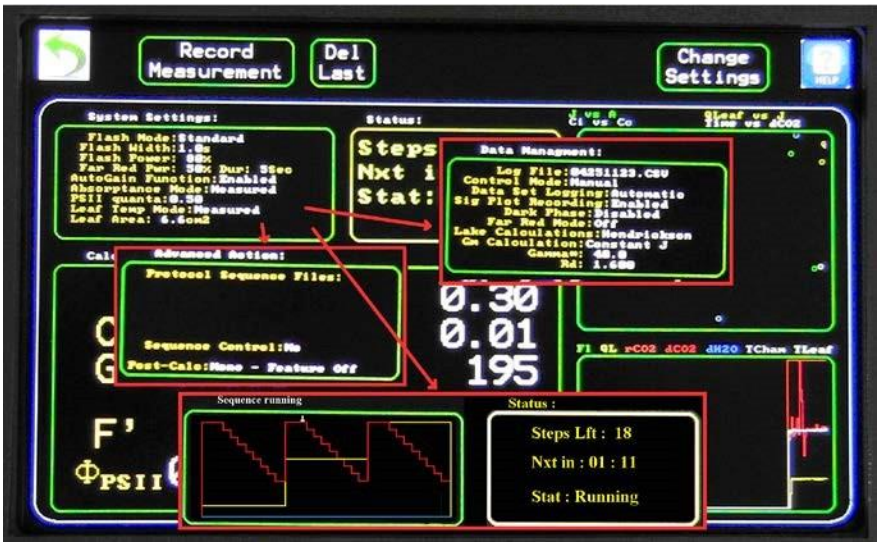
If the lower left hand window is tapped again, there is direct readout of quenching parameters as shown in the image below. One can choose either Kramer Lake Model parameters in conjunction with puddle model parameters, or the simplified Hendrickson Lake Model parameters with puddle model included.



See the section on quenching for definitions of quenching parameters.

By tapping the lower left hand window again, the data from an entire test series may be seen. Since the number of parameters that may be shown at one time is limited on the screen, one can select the reported parameters from the “Item List”. One can also scroll though data with the “Page Fwd” or “Page Back” buttons. The last measurement may also be deleted by tapping “Del Last”.
All measuring parameters are reported in the comma delineated data file that may be opened in any spread sheet without additional software.

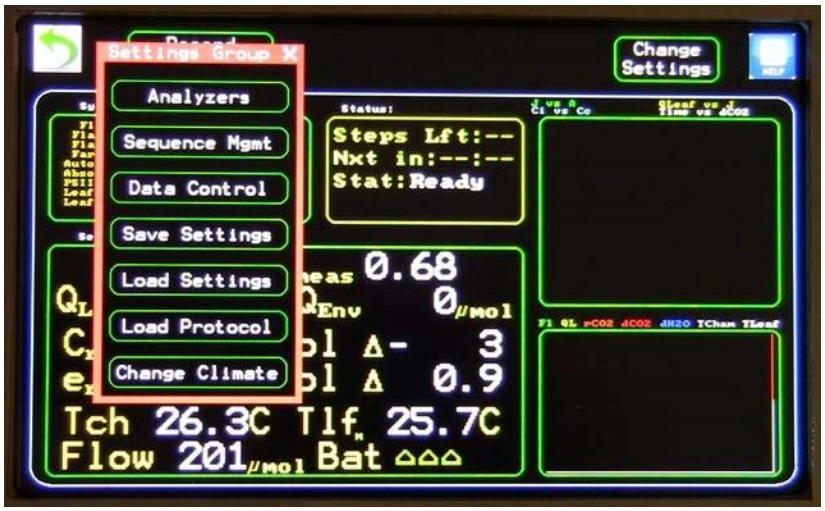
The upper left hand window allows viewing of the various setting that have been made for a test or sequence. By tapping this window, it will scroll through several windows that show many things.

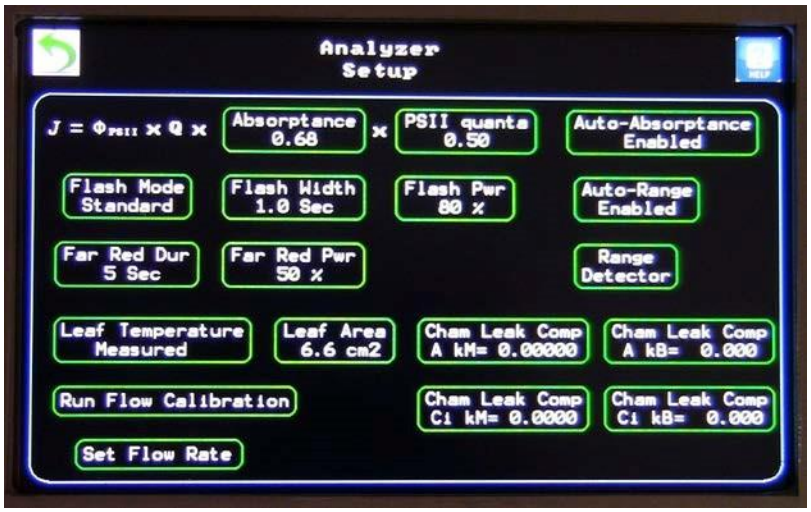


Many of the various settings programmed into a test, or sequence can be viewed here. In addition, as a sequence is running, a graphic representation may be viewed to show where in the sequence the test is now located. A white arrow points to the current test point and time.

To change the settings or to load or create a sequence, tap the “Change Settings” button on the upper right hand corner of the screen.

The following screen appears:





The $J = \Phi_{PSII}$ x Q x Absorptance x PSII quanta

Measuring Absorptance

The equation allows input into the *J* or electron transport rate equation. Using average values, the equation reads *J* = ΦPSII x Q x 0.84 x 0.50. However, Absorptance can vary from 0.7 to 0.9 in healthy plants (Eichelman 2004) and it changes with plant stress level, by species, leaf age, chlorophyll content, and light level (Cazzaniga *et al*., 2013). Cazzaniga *et al*., (2013) & Dall'Osta (2014) found that leaf absorptance changes with light level due to chloroplast migration. PSII ratio to PSI reaction centres can change with species, shade or sun growing conditions, and during extreme carbon deficiency. The range can be from 0.4 in some C_4 plants to 0.6 in some C_3 plants (Edwards 1993, Laisk 1996,). Measuring the ratio of PSII to PSI reaction centres is currently a destructive test at 77^{o}K (Zell 2010 & Anderson 1999). In many cases this ratio is published for specific sun grown plants (Edwards 1993, Laisk 1996).

If measured or estimated by other means, the Absorptance may be entered into the "Absorptance" window and the PSII ratio may be entered into the "PSII quanta" window. The window to the right of these settings is the "Auto-Absorptance" window. Here, auto absorptance can be enabled or disabled by tapping the window. If it is enabled, leaf absorptance will be measured automatically before every chlorophyll fluorescence measurement, and automatically added to the *J* or electron transport rate calculation. If it is disabled, then the value in the "Absorptance" window value will be used to calculate *J* or electron transport rate.

$$\alpha = ((\alpha_r)(B_r) + (\alpha_b)(B_b) + (\alpha_g)(B_g)) - (T_r/I_r + T_b/I_b + T_g/I_g)$$

or absorptance = ((absorptance of red) (fraction of red radiation on the leaf) + (absorptance of blue) (fraction of blue radiation on the leaf) + (absorptance of green) (fraction of green radiation on the leaf)) – (transmittance of red/raw red radiation incident on the leaf + transmittance of blue/raw blue radiation incident on the leaf + transmittance of green/raw green radiation incident on the leaf.)

Flash Mode

The choices here are "Standard" and Multi-Flash. Standard is the default setting; the standard square topped saturation flash used to determine F_M and F_M'. The other option is Multi-Flash, which allows F_M' correction using the Loriaux 2013 method. This group, which includes Bernard Genty, the researcher that developed the Y(II) light adapted test in 1989, say that at high actinic light levels, even the most intense saturation flash will not close all PSII reaction centres. They developed a method that correlates well with gas exchange measurements using a single multi-phases saturation flash at 7,000 µmol, a 20% down ramp and at a specific rate of change. By selecting Multi-Flash, the iFL uses the Loriaux 2013 method with all saturation flashes. See the section on F_M' correction for more details.

Flash Width

In most chlorophyll fluorometers, the width or time of the saturation flash is important, as F_M and F_M' values are usually averaged over the entire top surface of the saturation flash. If the flash is too narrow, PSII is not fully saturated and error can occur. If it is too wide, then there can be a downward rounding of the saturation flash, at the end of the flash, caused by saturation flash NPQ. This can also cause an error. The iFL, however, uses a special algorithm to prevent this problem. It uses a 25 ms, 8 point rolling average to measure the highest fluorescence value to prevent saturation flash NPQ from causing errors. As long as the saturation flash is wide enough, the measurement will be reliable. The default value is 0.8 seconds; however, higher plants' optimal times range from 0.5 seconds to 1.5 seconds (van Kooten and Snell 1990).

Flash Power

This represents a percentage of the total maximum saturation flash intensity of 7,000 µmol. Like the actinic light, the saturation flash uses white light. In the light, very intense saturation flashes do not harm the plant, even if they are two minutes apart. In the dark, frequent intense saturation flashes can damage the plant (Porcar-Castell 2008).

Auto-Range

Auto-Range provides the capability to set the iFL modulated light intensity and gain automatically and correctly. Auto-range must be enabled for the iFL to function. Modulated light should be set high enough for detection, but low enough to prevent any Q_A from being reduced, for reliable dark adapted F_V/F_M measurements. If it set too high, F_V/F_M will be reduced because F_O will be too high. The automated modulated light intensity adjustment may also be disabled. In this case, the intensity must be set manually.

For manual adjustment of light settings, dark adapt a leaf for at least 4 minutes on non-field plants, and at least 7 minutes on field plants. If the signal is too low, a message will appear stating that fact. It may be adjusted upward manually. Make sure that the "signal too low" message does not appear and that the Ft value on the measuring screen does not gradually rise over a 20 second time frame. If it does, the modulated light has become actinic, and it is reducing some Quinone A or Q_A. Auto-range prevents incorrect modulated light settings.

Range Detector

This allows a one-time auto adjustment of modulated light intensity and gain adjustment for modulated light only when this button is pushed. The "Auto-Range" button must be disabled for this function to work. This would likely be used once at the beginning of a test on a leaf.

Far Red Dur

When making quenching measurements or F_V/F_M measurements, the use of Far red light to stimulate PSI can be used to ensure that PSII is fully oxidized before measurement. This may be set to 0, 5, 10 or 15 seconds before the F_V/F_M measurement used in quenching protocols. At least one paper has found that 10 seconds works better than 5 seconds (Maxwell and Johnson 2000). However, this does not replace the dark adaptation relaxation times of q_E, q_M or q_I required for reliable quenching measurements. See the section on dark adaptation for more details.

Far Red Power

This button allows the setting to the Far Red light intensity. At 100%, the intensity is more than 90 μmol of light. Far red light does not stimulate PSII, only PSI. Far red light is filtered out of the Actinic white light. PSII is not stimulated by far red light; however, electrons transfer from PSII to PSI if PSI is excited by illumination.

Leaf Temperature

The options here are Measured or Calculated. If Measured is selected, the IR sensor is used to measure an integrated average leaf temperature over about 80% of the leaf chamber area to provide the most reliable leaf measurement. This becomes more important when the chamber temperature is different from ambient (Pons 2009). If the alternate accessory called a leaf thermistor is plugged into the side port near the chamber, the leaf thermistor becomes the leaf measuring method. If 'Calculated' is selected, it disables leaf measurement and calculates leaf temperature using chamber temperature in the standard way (see the 'formula' section for more details).

Leaf Area

The chamber leaf area is 6.6 cm^2. This is the default value. If a leaf that is smaller than the chamber is measured, or if the leaf does not completely cover the entire opening, then the leaf area should be measured and input here using this button. Leaf area is an important parameter.

Check Flow Calibration

This is a Flow check. It should be checked if you think there is a problem.

Note: The displayed values for "u" and "uset" are related to the Air Supply Unit (ASU) which provides flow to the leaf chamber. Although proportional to the ASU flow to some extent, the values displayed during a Flow Check calibration are the estimated flow through the analysis cell and the time allowed before the gas is stable and a reading taken. Typical values for broad, narrow and conifer chambers are shown in the table below. Analysis times for Soil chambers and Small chambers may be longer due to the larger chamber volume and lower advised ASU flow respectively.

It is strongly recommended to perform a flow check calibration if you change between chamber types or make a change to the chamber air supply flow larger than 30%. The flow check calibration checks that the cycle times are long enough for the gas in the analysis cell to become stable before the absorption is measured. The flow check adjusts the cycle times for both reference and analysis, therefore the chamber jaws must be fully closed before the check is started.

Changing the flow by greater than 30% without doing a flow check may result in insufficient settling time which may cause measurement errors because the gas concentration in the cell will not have had time to stabilize. It can cause an offset in the ΔCO_2 readings even with nothing in the chamber.

Typical flow values and normal variation from typical values that can be expected

ASU set flow ($\mu mol\ s^{-1}$)	Settling time (seconds)	Settling time (seconds)	Estimated flow ($\mu mol\ s^{-1}$)	Estimated flow ($\mu mol\ s^{-1}$)
-	Reference	Analysis	Reference	Analysis
200	4.45	3.86	83	97
300	4.32	3.36	85	110
Variation	5%	10% - 25%	5%	10% - 20%

Set Flow Rate

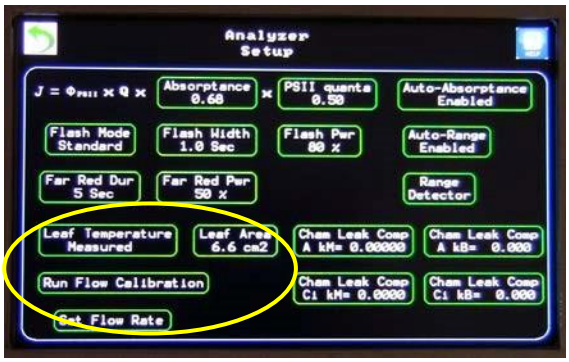
The flow rate may be set from 100ml/min to 500 ml/min. The default value is 200 ml/min for this measuring chamber. If different chambers are used, the flow rate should be reset to the recommended value for the chamber. Flow rates can also be adjusted for smaller leaves or leaves that exhibit low photosynthetic function.

Cham Leak Comp

This is the location where the results of running the Flexas leaf chamber leakage protocol are stored. The correction values are automatically transferred if transfer is selected after the protocol is run. The corrections will vary by type of leaf. Values can also be input manually. The default settings are zero for no leaf chamber leakage.

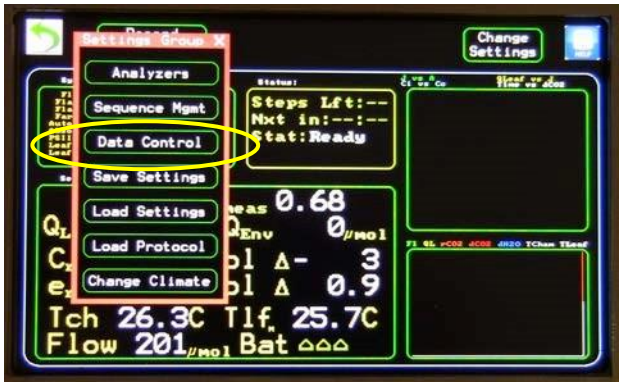
This becomes important when running A/C_i curves, or Laisk protocols as some CO_2 leakage occurs outward when chamber CO_2 values are higher than ambient, and some CO_2 leakage occurs inward if CO_2 levels are below ambient. See the section on the Flexas protocol for more information. It certainly makes sense to consider the method when working will g_m and C_C.

Tap the green arrow in the upper left hand corner to return to the combined measuring screen.



Data Control

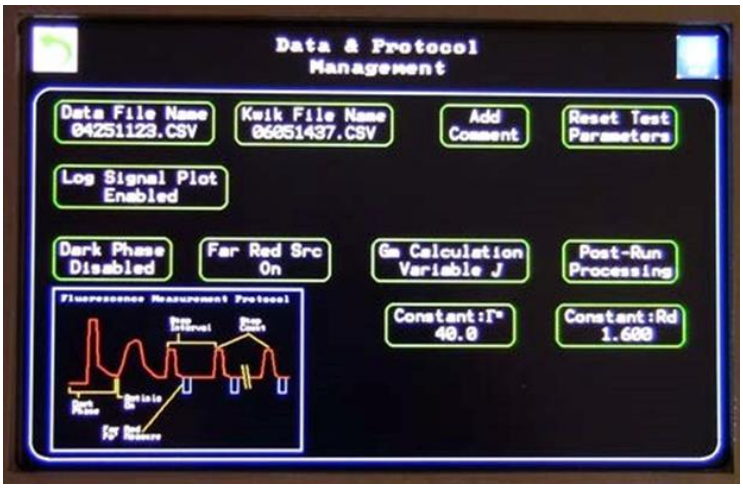
By tapping this button, the following screen appears:



After tapping “Data Control” the screen below appears:

1. Data File Name

Data File Name allows a custom alpha-numeric name of up to 8 characters. The suffix for such files is always .CSV.



2. Kwik File Name

This button allows quick naming of a measuring or protocol file. It uses the current date and time to provide the name.

3. Comment

This function allows up to a 32 character alpha-numeric comment to be added to the file for later review.

4. Reset Test Parameters

This function erases all existing test parameters.

Dark Phase

This function allows the measurement of F_V/F_M before a test begins. This also allows quenching measurements to be made. F_V/F_M is the measuring standard for all quenching parameters. By enabling the Dark phase, it is possible to monitor quenching parameters. If it is disabled, most quenching parameters will not report to the measuring screen or the data file. For more information about quenching, see the section on quenching measurement.

Far Red Src

This allows selection of the use of far red light. Far red light is not included in the white actinic illumination on purpose. The Actinic white light provides photosynthetically active radiation from 400nm to 700 nm. This range of light affects PSII but does not excite PSI. Far red light is used to excite PSI independently of PSII, or to completely oxidize PSII in F_V/F_M measurements, and to provide an F_O' value for Kramer lake model quenching parameters, or for some puddle model quenching parameters.
The selections here are "Pre" for oxidizing PSII before F_V/F_M measurements, "Post" for Kramer lake and puddle model quenching, "On" for illumination all the time, and "Off" for no far red light.

g_m Calculation

g_m, or mesophyll conductance, can be measured by using the constant *J* or variable *J* method. Here one can select either the variable *J* or the constant *J* calculation method of g_m calculation. This depends on whether CO_2 levels are above or below ambient.

Constant *J* equation:

$$g_m = \frac{A\,(4A + 4\,R_d - J)}{4AC_i + 4C_i\,R_d + 8A\,\Gamma^* - C_i\,J + 8\Gamma^*\,R_d + \Gamma^* J}$$

Constant *J* method:

1. When *J does not vary much* if CO_2 level is changed at higher CO_2 levels
2. Is normally used at CO_2 levels above ambient
3. *J* should be just below saturation for best results
4. "A" limited by RuBp
5. Sensitive to Γ* variation

Variable *J* equation

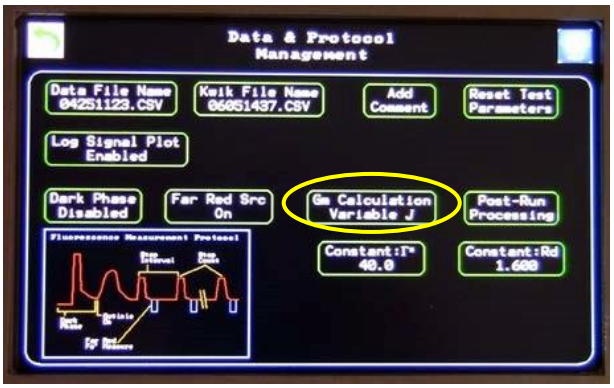
$$g_m = \frac{A}{C_i - \dfrac{\Gamma^* \cdot (J + 8 \cdot (A + R_d))}{J - 4 \cdot (A + R_d)}}$$

Variable J method:

1. When *J does vary* if CO_2 level is changed, usually used at lower CO_2 levels
2. It may be used above ambient; however; it is usually used where *J* is variable

3. Often used with 1% or 2% O_2 but not 0% O_2 in C_3 plants
4. More sensitive to Γ* and R_d than other methods
5. Is usually lower that the constant *J* method

"A" limited by Rubisco



Post-Run Processing

This button allows post processing of data measured for sequences and protocols. One can select post processing options for the following protocols:

Laisk protocol – considered authoritative for C_3 plants and it is the most used method for R_d and Γ* determination. Today it is common to use the von Caemmerer Γ* correction when using this protocol. This can be selected in the "set up screen" found on the main menu and then selecting "Measurement Units Setup".

Kok protocol – commonly used for C_4 plants. It may also be used for C_3 plants but values vary from the Lasik method for R_d measurement. Γ* is calculated.

Yin protocol – this a newer protocol for R_d determination. It uses chlorophyll fluorescence and higher actinic light levels, making the error in R_d determination smaller relative to the measurement; Γ* is calculated.

Flexas CO_2 chamber leakage protocol – This protocol is designed to measure the CO_2 leakage into the measuring chamber and out of the measuring chamber at CO_2 levels above and below ambient for specific types of plant leaves. See the section on the Flexas protocol for more information.

When these post processing protocols are complete, and show the results, the various measured parameters such as R_d, Γ*, and chamber leakage correction can to forwarded to other work and allow direct readout of g_m, C_C, and values corrected for chamber leakage.

For more information on these methods see the section devoted to each.

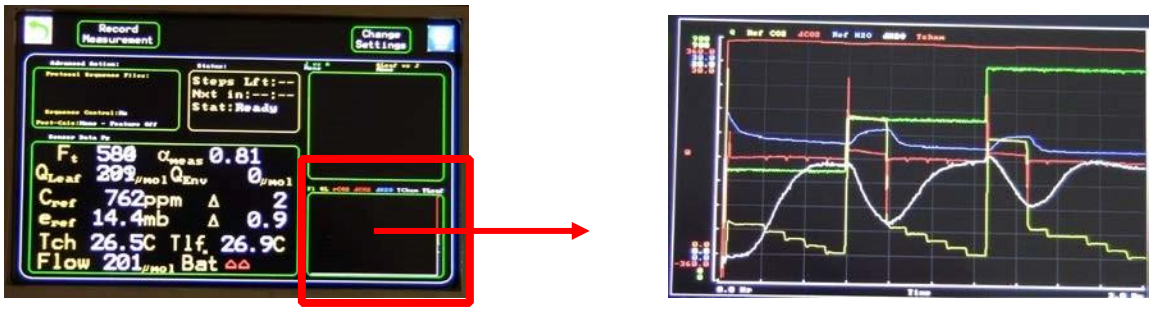
Constant Γ*

This is either the value forwarded from one of the protocols listed above, or it may be entered at this location if the testing is done independently, via the carbon isotope method, or some other way. Along with R_d, these values allow direct readout of g_m and C_C.

Constant Rd

This is either the value forwarded from one of the protocols listed above, or it may be entered at this location if the testing is done independently, via the carbon isotope method, or some other way. Along with Γ*, these values allow direct readout of g_m and C_C.

When done tap the green arrow in the upper left hand corner to return to the combined measuring screen.



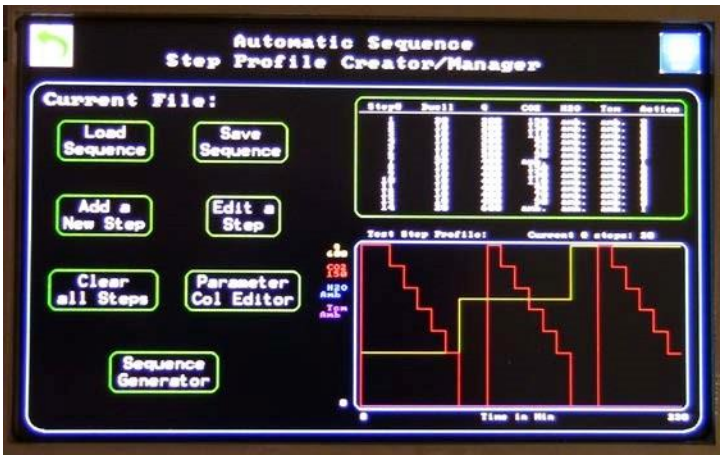
Log Signal Plot

If this function is enabled, the lower right hand screen on the combined measuring screen will log all of the raw data for viewing during the test and after the test is over. See the picture on the right above as an example. Actinic light level, CO_2 ref, ΔCO_2, leaf temperature, H_2O ref, ΔH_2O, and chlorophyll fluorescence signal. If the graphing is not wanted, disable this function.

Sequence Mgmt

This is the preferred location to create custom measuring sequences, load existing measuring sequences, or to load protocols as templates to make custom sequences. All plants are different and require some fine tuning of protocols. Plants grown in the shade respond differently than those grown in the sun. In addition other growing conditions and plant stress may require sequence adjustment.

Tap “Sequence Mgmt” and the following screen appears:



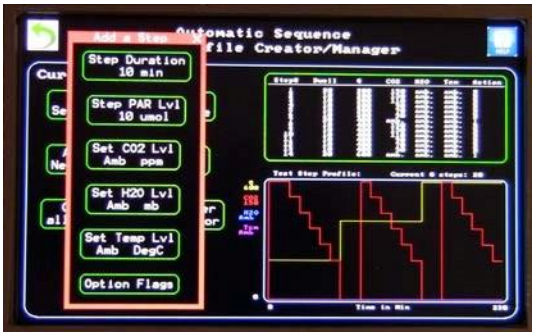
The screen above is used for custom sequence generation. The sequence shown above is an example of a Laisk protocol to determine R_d, and Γ*. It includes three consecutive A/Ci curves at low CO_2 levels at three different actinic light levels, all *below saturation* level. Red lines represent CO_2 level, yellow lines represent actinic light level or Q, purple represents temperature and blue represents humidity.

To load an existing protocol or sequence, tap “Load Sequence” and the following window appears:

Tap the sequence file (all end in .SQS.) of interest. Next, tap the white “x” in the orange window to go to back to the “Sequence Mgmt.” window. When loaded, a graphic representation of the steps are shown in the lower right hand window. The values selected for the steps are shown in the upper right hand window. One can scroll through long lists of steps by touching the bottom of the window.

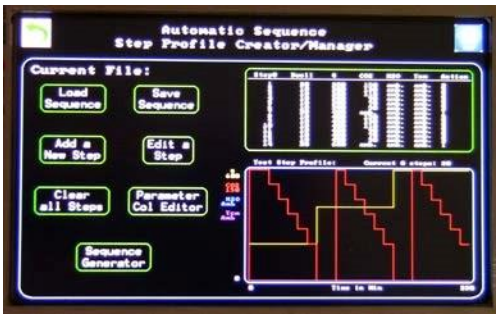
To edit a step, tap the “Edit a Step” button and the following window appears:

Tap the step that is to be changed and the next screen appears:

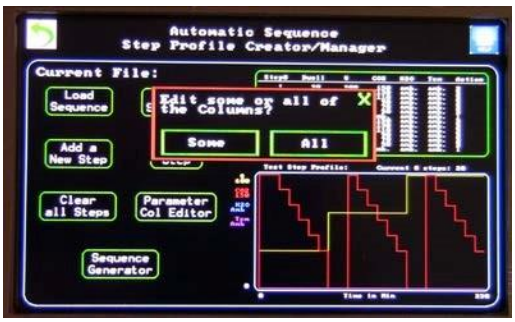


Here, one can choose to edit time, light or PAR level, CO_2 level, H_2O level, Temperature or change 'Option flags' that indicate when a sequence ends. The window looks the same for "add a new step". Tap the white "x" in the orange window when ready.

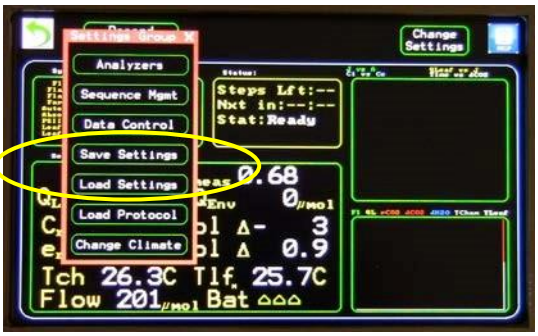
"Parameter Col Editor" enables parameters to be changed by column. A column may be changed partially or fully. Tap the "Parameter Col Editor" and a window appears:



If one chooses to edit some of the column, another window will appear asking for the step where the change starts. Another question will follow asking the step where the change ends. The return key in the window is used to make the change. When ready, exit using the white "x" in the orange window.



"Sequence Generator" should be used when custom sequences or protocol are wanted. It will: walk the user through the steps, allow almost an unlimited number of steps, show a graphic representation of parameter values in the sequence, and will show the actual settings that are input.



Save Settings

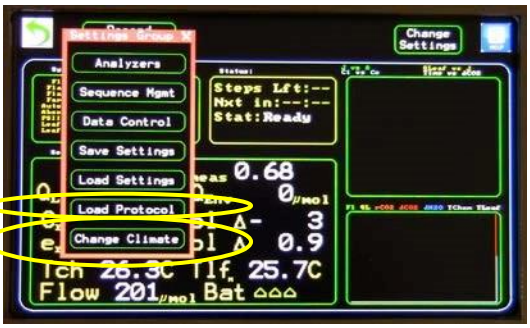
New settings can be given a new sequence name or values can be appended to an existing sequence.

Load Settings

To load an existing sequence file.

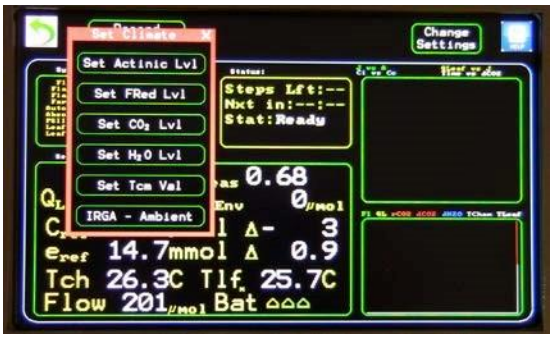
Load Protocol

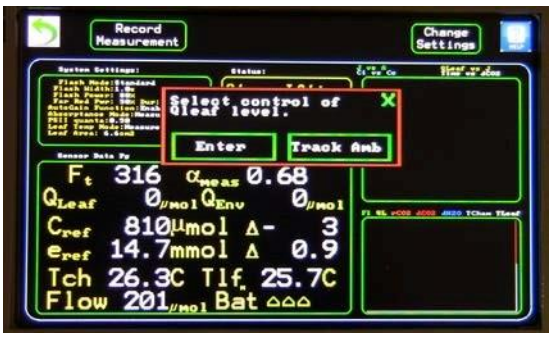
At this time there are protocols that can be loaded and modified as a new sequence; however these protocols remain in memory unchanged. This ensures that they cannot be erased.
The options here include: Laisk protocol, Kok protocol, Yin protocol and the Flexas chamber leakage protocol.



Change Climate

This function allows changing the various chamber climate values without creating a sequence. When "Change Climate" is tapped the screen below appears:





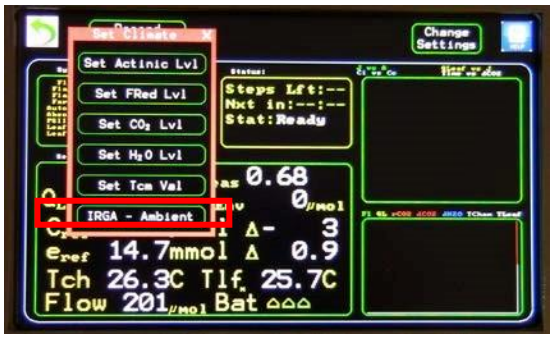
"Set Actinic Level"
Allows setting of the white actinic LED intensity from 0 to 2,000 µmol at the leaf plane. The light intensity at any given wavelength is always proportional to its intensity at 2,000 µmol. Furthermore, the light intensity is held constant over time using a feedback loop with the internal PAR sensor. The PAR sensor measures the actinic light irradiated onto it and it corrects for any intensity changes, automatically. Since most chlorophyll fluorescence and gas exchange measuring parameters are designed to be used at steady state photosynthesis, it is very important that the light intensity does not change inside the chamber, or steady state is not achieved. The actinic light level can also be set to "Track ambo" (ambient) lighting conditions, or measure and lock an ambient measuring value by tapping "Enter". This feature is valuable when making measurements in the field in partially cloudy conditions. The PAR sensor on the top of the iFL illuminator can be used to measure ambient conditions and match the intensity inside the chamber. If a cloud is about to block the sun and ruin a measurement, the ambient value can be measured and locked, to maintain a constant light level.

"Set Fred Level" - Far red light is not part of the iFL white light actinic spectrum. It is supplied by a far red diode in the illuminator. The white light actinic diode drives photosystem II and variable chlorophyll fluorescence. Far red light drives photosystem I, which does not exhibit variable chlorophyll fluorescence. The iFL may be used while far red light is turned on or off. When turned on, the intensity at 100% is more than 90 µmol. Far red light is also used in the Kramer Lake Model quenching protocol and in some Puddle Model quenching parameters to measure F_O'. It can also be used in the F_V/F_M protocol to pre-illuminate a dark-adapted sample. This ensures complete oxidation of PSII before measurement. For more on this topic, see 'Variable Chlorophyll Fluorescence'.

"Set CO_2 Level"
This allows the chamber CO_2 level, for air entering the chamber, to be set in a range from 0 to 2,000 ppm.

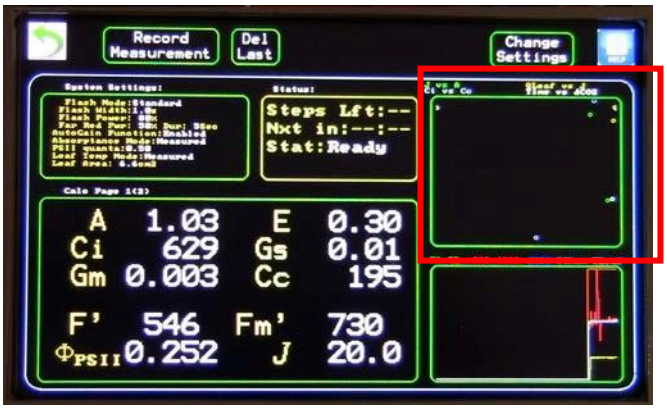
"Set H_2O level"
This function allows H_2O level to be set from above ambient down to zero when the wetter column is used with iron oxide. It allows adjustment from ambient down to zero when using the black foam H_2O traps.

"Set Tch Val"
This allows the chamber temperature to be set +- 14°C above or below ambient temperatures using the broad leaf chamber and +-10°C above or below ambient with other measuring chambers. It uses a Peltier module for heating and cooling.



"IRGA Ambient" This sets the CO_2 level to ambient conditions.

Plot window - in combined measurement. If one taps the plot window below, highlighted in red, values measured can be plotted against other measurements of interest.



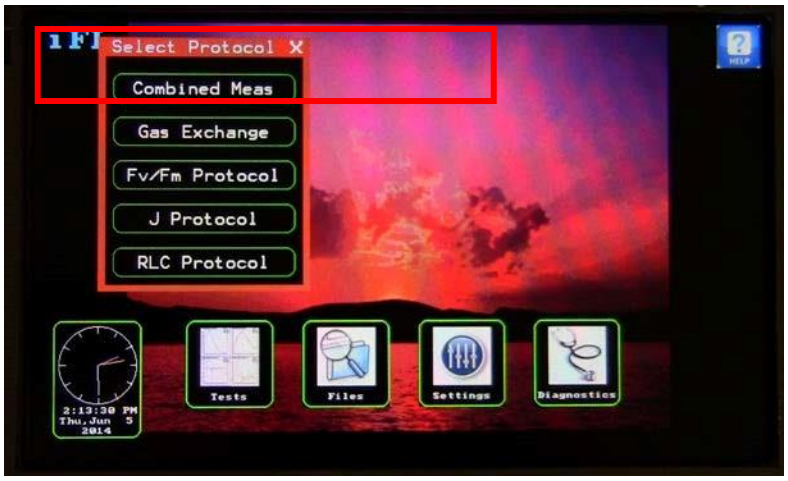
The plot above shows J vs. A, Ci vs. Cc, Q vs. J, and time vs. ΔCO_2

Tap the “Choose XY- Plots” and the following window appears. Graphs can be produced using straight lines that connect measured points or using circles. Tap the “Style” button to choose (in general, circles tend to be preferred).

When one of the four possible plot windows is tapped above, the “x” and then the “y” axis choices for graphing appear. Tap the parameter of interest and move on. Tap the white “x” to exit the screen.

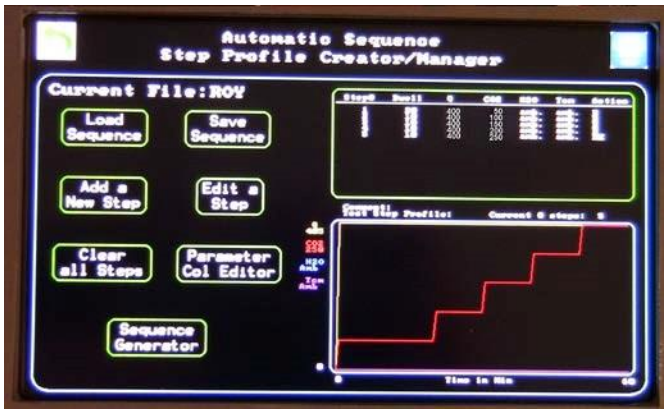
When ready, tap the green arrow in the upper left hand corner to return to the combined measuring screen.

Gas Exchange – only

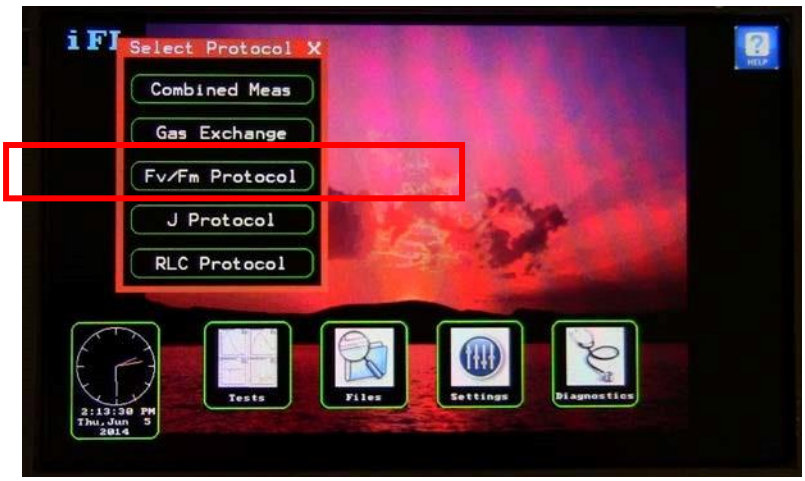


The gas exchange only protocol works like the combined Measure protocol without chlorophyll fluorescence. Tap the "Gas Exchange button and the Gas Exchange only measuring screen appears. The lighting is the same with the Combined and the Gas Exchange only, as are all of the adjustment screens, sequence set up, and other parameters, but without measuring chlorophyll fluorescence. *See the "Combined Meas" section of this manual for operating instructions. Disregard the parts involved with chlorophyll fluorescence.*

The operator can choose ppm and mb, or µmol and moles in the "Settings window".

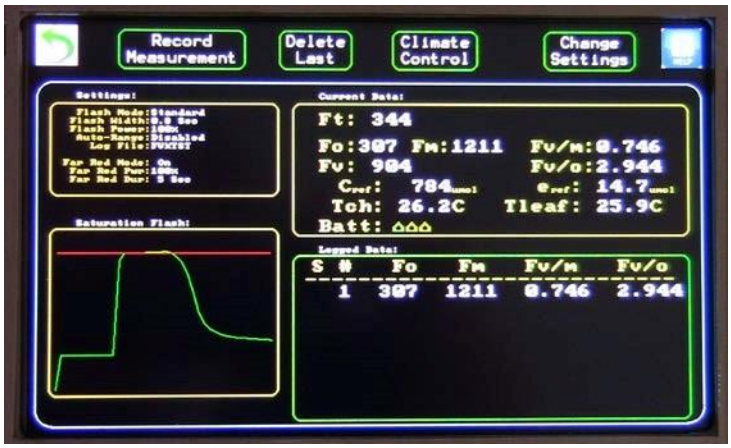


This is an A/Ci curve sequence created with the Sequence Generator. See the "Combined Meas" instructions for details.



F_V/F_M Protocol

This protocol is for dark adapted chlorophyll fluorescence measurements with measuring chamber control. It allows measurements at various chamber temperatures, CO_2 levels, and H_2O levels. F_V/F_M can also be measured in the "Combined Meas" protocol.



This protocol allows Auto-adjustment of the modulated light intensity with "Auto-Range". If tapped, auto-ranging will occur with every measurement. A single automated adjustment of the modulated light intensity can be performed with "Range".

"Saturation Flash Power" intensity from 0 to 100% with 100% at about 7,000 µmol. Dark adapted sun grown field plants can be saturated with the use of about 1,000 µmols of light (Ralph 2005). Very intense saturation flashes will not damage plants unless they occur too frequently (Porcar-Castell 2008).

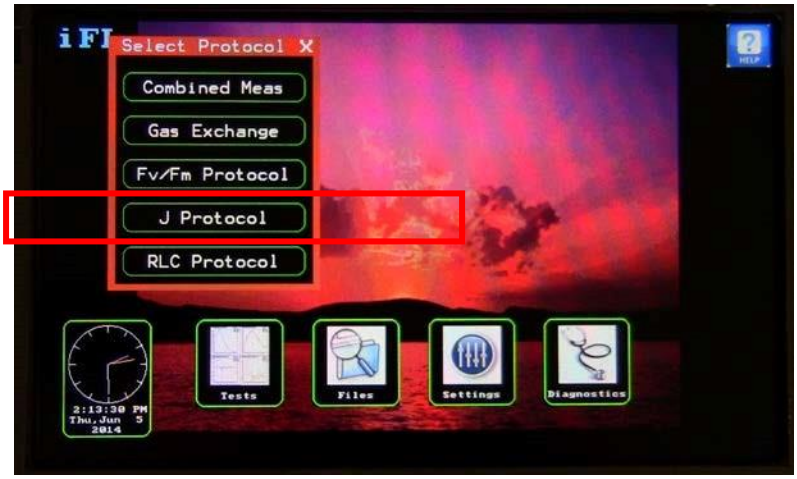
"Saturation Flash Width" from 0.1 to 1.5 seconds. The default is set at 0.8 seconds. The instrument uses a special 8 point 25 ms rolling average algorithm to determine maximum F_M. This means that as long as the saturation flash is wide enough, the correct F_M value will be measured even if some saturation flash NPQ exists.

"Far Red Mode" The options here are Pre, Off, or On. Far red light stimulates photosystem I. In the Pre mode, the Far red light before measurement ensures that photosystem II is fully oxidized for a better and higher F_V/F_M measurement. It is common to use 5 seconds or 10 seconds of Pre far red light. Options include: 0, 5, 10, and 15

seconds. Research shows that 10 seconds works better than 5 second. The most important thing is consistency. Using far red light does not replace dark adaption (see the section on dark adaption for more details).

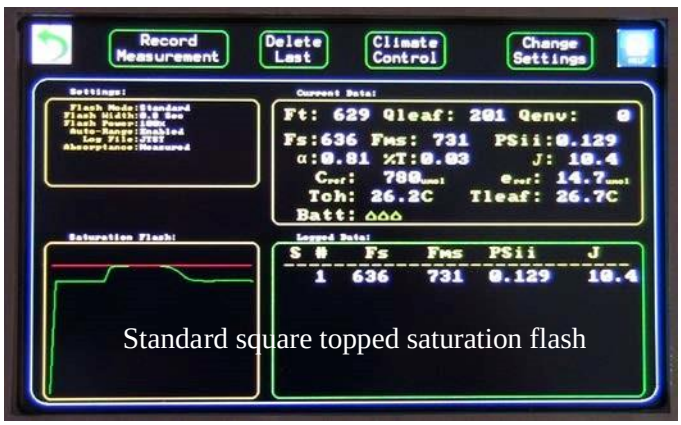
The graph on the lower left hand side shows the saturation flash. The red line represents F_M.
Data from multiple measurements can be viewed in the lower right hand window. One can scroll through hundreds of measurements by touching the bottom or the top of the data window.

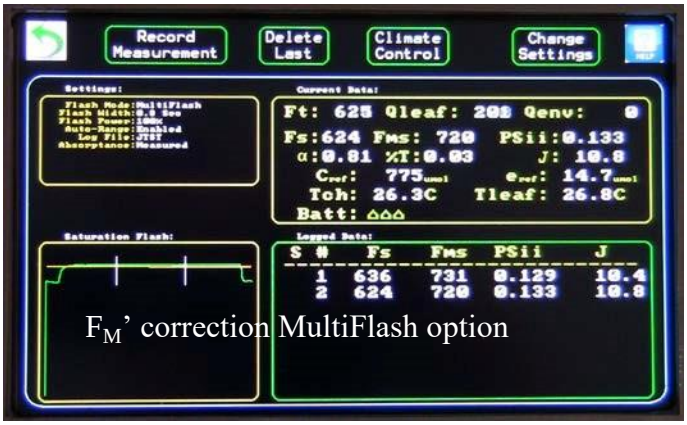
J Protocol



The *J* Protocol is the chlorophyll fluorescence light adapted test for measuring Y(II) or $\Delta F'/F_M'$ and Electron Transport Rate, or *J*. $\Delta F'/F_M'$ or Y(II) is the quantum yield of PSII developed by Bernard Genty in 1989. *J*, or electron transport rate, can also be measured in the "Combined Meas" protocol. This Protocol is designed to focus on just light adapted chlorophyll fluorescence measurement, its use with an adjustable environmental chamber, and leaf absorptance measurement. An in-depth discussion is available under the variable chlorophyll fluorescence section on light adapted measurements, see Y(II).

Tap the *J* Protocol button and the *J* Protocol measuring screen appears:





Buttons exist for: "Record Measurement"
"Delete Last" deletes the last measurement made
"Climate Control" allows adjustment of actinic light intensity, CO_2 level, humidity, and chamber temperature.
"Change Settings" allows additional parameter changes.
Tap "Change Settings" and the screen below appears:

The buttons on the top allow retrieval of saved / saving of new measuring configurations.

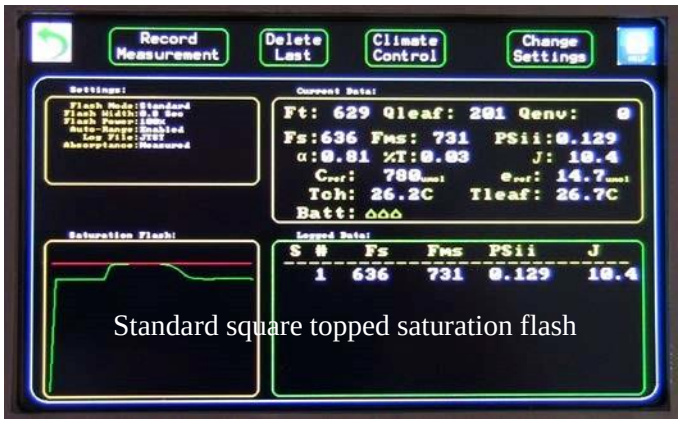
The first line inside the white window is the formula for *J* or ETR (electron transport rate). The terms used by people trained in chlorophyll fluorescence are different that those trained in gas exchange. The following list compares the two sets of terms.

"J" ETR (electron transport rate).
"Φ_{PSII}" Y(II) or $\Delta F'/F_M'$ or quantum yield of PSII.
"Q" PAR or "photosynthetically active radiation" between 400nm and 700nm.
"Absorptance" The amount of light incident on the leaf that is absorbed by the leaf. Here it measures the light incident on the leaf, the light reflected from the leaf and the light transmitted by the leaf. A calibration standard is used when the instrument is assembled.
"PSII quanta" The ratio of PSII reaction centres to PSI reaction centres.

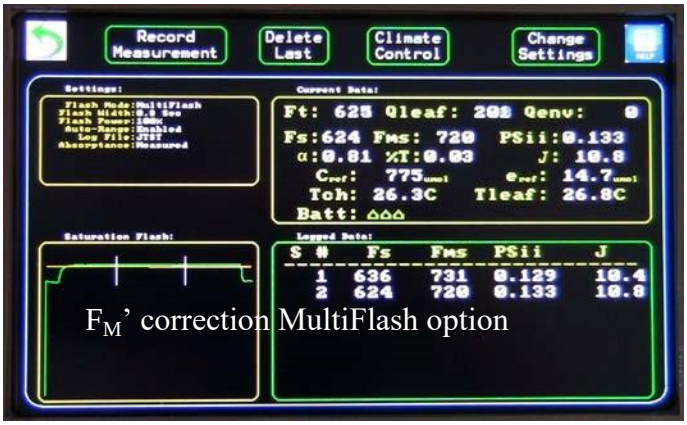
Values may be input here for "Absorptance" and for PSII reaction centre ratio if measured by some other means. Those numbers will then be used to calculate *J* if "Auto-Absorptance" is disabled. If "Auto Absorptance" is enabled, then the value measured by the i*FL* overrides the value inserted into the Absorptance window.

"Flash Mode" Allows switching from the "Standard" square topped saturation flash to the Loriaux 2013 F_M' correction method of F_M' measurement. The Loriaux 2013 method is called "MultiFlash". "MultiFlash" should be considered if the sample is under high actinic light conditions. Under lower light conditions, "MultiFlash" gives approximately

the same values as the "Standard" square topped saturation flash. See the section under MultiFlash for more information.



Standard square topped saturation flash

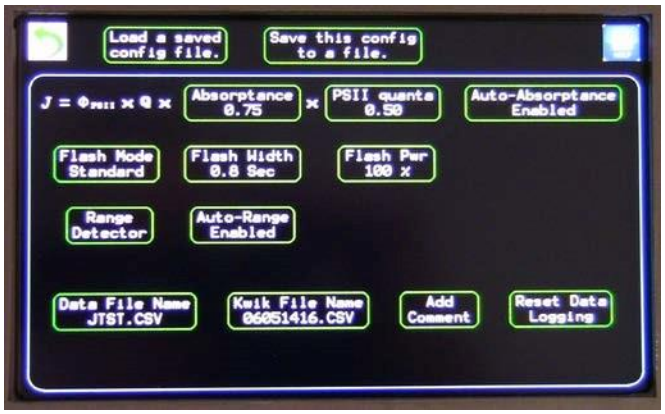


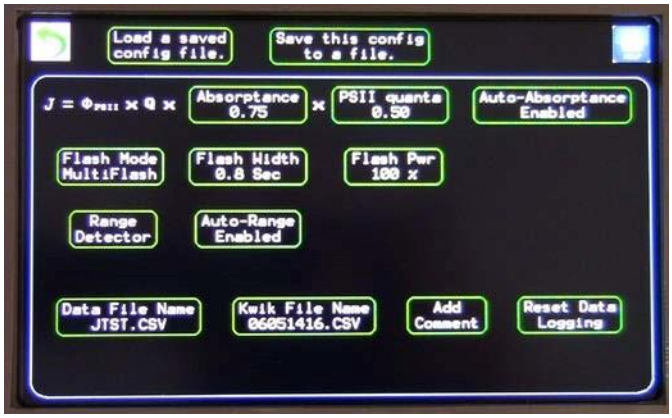
F_M' correction MultiFlash option

"Flash Width" is the duration of the saturation flash. It can be set between 0.1 seconds to 1.5 seconds, and the default value is 0.8 seconds. The iFL uses a special 8 point 25 ms rolling average algorithm to determine the highest 25 ms F_M' value. As a result, the instrument will make reliable measurements even if the saturation flash is set too long, as saturation flash NPQ does not affect measurements.

"Range detector" automatically sets the modulated light intensity once.

"Auto-Range" automatically sets the modulated light intensity every time the "Record Measurement button is tapped.





"Data File Name" Allows an 8 digit alpha-numeric name for new measuring data files.

"Kwik File Name" Uses the current date and time to create a new data file name.

"Add a Comment" Allows a 32 character alpha-numeric comment for each measurement. It appears in the data file.

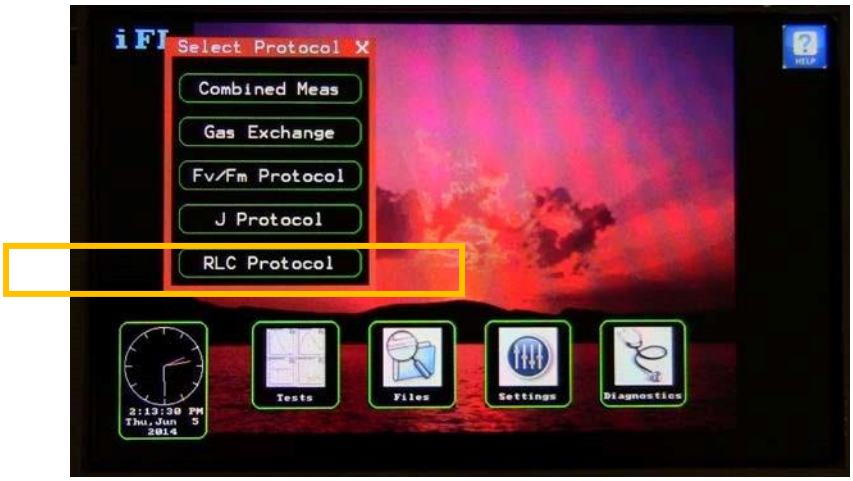
"Reset Data Logging" Erases all data in the measuring file and starts the file over again.

"Climate Control" Allows pre-illumination of samples to set actinic light intensities before Y(II) measurement. It also allows chamber temperature to be set to +-14ºC above or below ambient, Changing CO_2 levels from 0 -3000ppm, and to adjust humidity from 0 to above ambient with the "wetter column" supplied. The screen below asks if one wants to track ambient light levels to cut down on times to reach steady state, or to enter a fixed value that is either at ambient, or at some level between 0 - 2,000 µmol.

"Logged Data" is available in the lower right hand corner. One can scroll through the data by touching the bottom of the window or the top of the window.

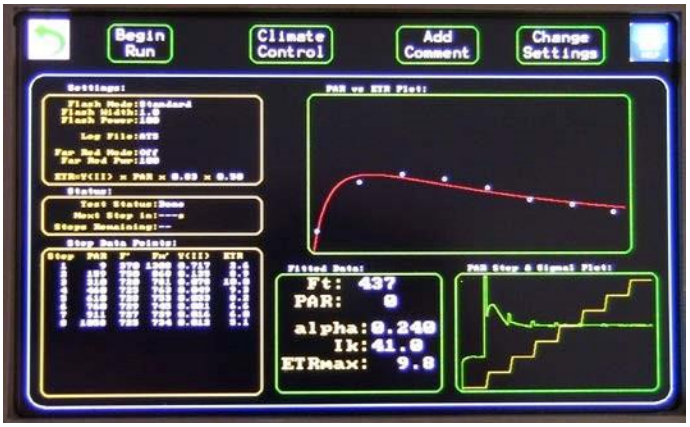
Under climate control of Q level, the option exists to allow the chamber to track ambient lighting conditions inside the chamber. This allows plants that are already at steady state photosynthesis to remain at steady state, allowing for faster ambient measurements. By tapping "Enter", one can either choose a new actinic light level or set the current ambient light level inside the chamber, to ensure a continuous light level and so steady state measurements even on partially cloudy days.

RLC Protocol



This is the Rapid Light Curve Protocol. Most measuring protocols are designed to work at steady state photosynthesis. However, for plants that are under canopy, the light can be rapidly changing. Rapid light curves were developed for such measuring conditions. Under changing light conditions, Y(II) and *J* can overstate RuBisCO conditions, while the parameters $\mathbf{ETR_{MAX}}$, α, $\mathbf{I_k}$, and $\mathbf{I_m}$ strongly correlate to RuBisCO activity. The method is good for investigation into the saturation characteristics of plants at different times of day. One method (Ralph 2005), uses 10 second dark adaptation times to determine saturation characteristics under current conditions of plant adjustment and lighting conditions. Curves taken on plants at steady state photosynthesis near mid-day can show the light saturation level for a particular plant.

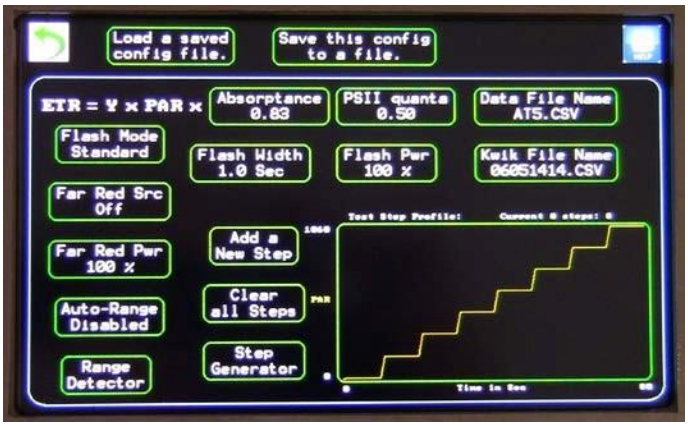
The i*FL* uses Eilers and Peeters curve fitting algorithms resident in the iFL software. The measuring screen below is the result of a RLC measurement.



Settings are listed in the upper left hand corner. Measuring status is next, and measurement values are listed on the bottom left side. The calculated parameters are from curve fitted calculations, and the fluorescence curve is overlaid against the RLC light steps used. The fitted curve is shown above on the right. Each step was programmed to be 10 seconds.

ETR_{MAX} Maximum ETR level at current light adapted conditions calculated by curve fitting.
α Slope of the initial fluorescence rise.
I_k Point at which light saturation starts to dominate, according to curve fitting.
I_m Calculated actinic light intensity where ETR_{MAX} occurs according to curve fitting. **I_m** is also the saturating light level as calculated by the software.

Tap the "Change Settings" button to reach the screen below:



The ETR formula is the same as the *J* (*J* = ETR) formula in other light adapted protocols

"Flash Mode" this function allows switching from the "Standard" square topped saturation flash to the Loriaux 2013 F_M' correction method of F_M' measurement. The Loriaux 2013 method is called "MultiFlash" and should be considered if the sample is under high actinic light conditions. Under lower light conditions, "MultiFlash" gives approximately the same values as the "Standard" square topped saturation flash. See the section under MultiFlash for more information.

"Flash Width" is the duration of the saturation flash. It can be set between 0.1 seconds to 1.5 seconds, and the default value is 0.8 seconds. The iFL uses a special 8 point 25 ms rolling average algorithm to determine the highest 25 ms F_M' value. As a result, the instrument will make reliable measurements even if the saturation flash is set too long, as saturation flash NPQ does not affect measurements.

"Range detector" automatically sets the modulated light intensity once.

"Auto-Range" automatically sets the modulated light intensity every time the "Record Measurement" button is tapped.

"Data File Name" allows an 8 digit alpha-numeric name for new measuring data files.

"Kwik File Name" uses the current date and time to create a new data file name.

"Add a Comment" allows a 32 character alpha-numeric comment for each measurement. It appears in the data file.

"Reset Data Logging" erases all data in the measuring file and starts the file over again.

"Climate Control" This allows pre-illumination of samples to set actinic light intensities before Y(II) measurement. It also allows chamber temperature to be set to +-14ºC above or below ambient, Changing CO_2 levels from 0 -3000ppm, and to adjust humidity from 0 to above ambient with the "wetter column supplied.

Choose to track ambient light levels to cut down on time taken to reach steady state, or, to enter a fixed value that is either at ambient, or at any level between 0-3,000μmol.

"Step generator" This can be used to create a rapid light curve using a variable number of steps with variable actinic dwell times and variable step durations. The Ralph (2005) approach is to use 8 steps with at least 2 steps above leaf saturation levels for 10 seconds at each step. This offers a few advantages. Ralph recommends RLCs at different times of day, a 10 second dark adaptation period, and a duration that does not allow plant adjustment. He uses 10 second durations. However, saturation pulse NPQ will build up with each step saturation flash, as it takes 60 to 120 seconds for it to dissipate (van Kooten & Snell 1990). For that reason, fewer steps may provide a more reliable result. Other approaches have also been used with different times for dark adaption and step duration (see Rascher 2000).

The "Add a new Step" and "Clear All Steps" are self-explanatory.

Plant stress measurement

With integrated chlorophyll fluorescence and gas exchange equipment

The relationship of the light reaction to the dark reaction of photosynthesis is not always straightforward especially in C_3 plants (Rosenqvist & van Kooten, 2006). Under non-photorespiratory conditions, in C_3 plants, there is a curvilinear correlation between the chlorophyll fluorescence light adapted parameter, Y(II), and the gas exchange dark reaction measurement of "A" or carbon assimilation. Furthermore, in C_4 plants, there is a linear correlation between light adapted Y(II), and the dark reaction, "A", carbon assimilation (Schreiber 2004).

Chlorophyll fluorescence, by itself, has been very successful in measuring many types of plant stress (Desk Top Plant Stress Guide). Under photorespiratory conditions in C_3 plants, *J* (or ETR) from the light reaction continues to produce high energy molecules even after CO_2 assimilation in the dark reaction of photosynthesis starts to fall. This has been shown to be true during drought stress in C_3 plants and to a lesser degree, heat stress. There is no significant photorespiration in C_4 plants (Flexas 1999, 2000). In C_3 plants, photorespiratory conditions allow energy molecules ATP and NADPH, created by the light reaction, to combine with various forms of oxygen instead of CO_2. In this case, photorespiration provides other electron sinks that maintain higher chlorophyll fluorescence values until severe drought stress sets in. While there are now special chlorophyll fluorescence assays to measure early drought stress (Burke 2007, Burke 2010), the use of an integrated chlorophyll fluorometer and gas exchange system provide

an excellent tool for the drought stress studies. It also provides advantages when investigating cold stress in C_3 plants where the chlorophyll fluorescence parameter Y(II) has been reported to be three times higher than expected compared to gas-exchange measurements. The chlorophyll fluorescence parameter Y(II) has also been shown to detect heat stress at 35^{o}C, while gas-exchange can detect heat stress at 30^{o}C (Haldiman P, & Feller U. 2004). In CO_2 stress, Y(II) is not a sensitive detection tool while gas exchange works well (Siffel & Braunova 1999). At least in these areas, the integrated combination of gas exchange and chlorophyll fluorescence offers advantages over separate systems.

Measurement of the same leaf area by chlorophyll fluorescence and gas exchange

It is also important to measure the chlorophyll fluorescence *over the same measuring area* as gas exchange. It has been found the chlorophyll fluorescence has been patchy under some plant stress conditions, and so averaging the fluorescence value over the same measuring area has become important. Patchy fluorescence can occur under drought stress, cold stress, and at low CO_2 levels found in A/C_i curves and A/C_C curves (Baker 2008).

Integrated chlorophyll fluorescence and gas exchange measurement provides more reliable basic research

In the past, some integrated photosynthesis parameters were estimated or calculated by estimating leaf absorptance of PAR radiation. The range in healthy plants can be significant. A value range of 0.7 to 0.9 was reported by Eichelman (2004). Now that chloroplast migration has also been shown to change leaf absorptance at different light levels, measurement has becomes even more important. *J* or electron transport rate is affected dramatically by leaf absorptance and it is a key component in other combined integrated measuring parameters, including g_m and Cc. Furthermore, an increase of leaf transmittance at high actinic leaves due to chloroplast migration, using white light, will even affect $\Phi PSII$ and even carbon assimilation values. Chloroplast migration as it happens in nature requires an intense white light or an intense blue light, there is no significant chloroplast migration using an intense red light.

Assuming that the difference between *J*, electron transport rate values and A, carbon assimilation values, is caused by photorespiration is suspect for C_3 plants. While linear correlation with C_4 plants and curvilinear correlation with C_3 plants is possible, exact correlation is not possible. Most chlorophyll fluorescence occurs near the top surface of the leaf and not from deeper layers as is true in gas exchange (Schreiber 2004). Furthermore, even if a red spectrum actinic light is used to more fully penetrate the leaf, re-fluorescence caused by the re-absorption of original fluorescence from lower layers, causes a significant error (Gitelson 1999).

With the i*FL* integrated system, *new options are possible.* The i*FL* uses a filter fluorometer to minimize the re-fluorescence error (Schreiber 2004). Chlorophyll fluorescence is measured above 700 nm. It uses white actinic light with an intense blue spectrum to ensure chloroplast migration occurs as it does in nature, since chloroplast migration can affect non-photochemical quenching by up to 30%. Chloroplast migration can affect all $\Phi PSII$ measurements and most gas exchange measurements as well because

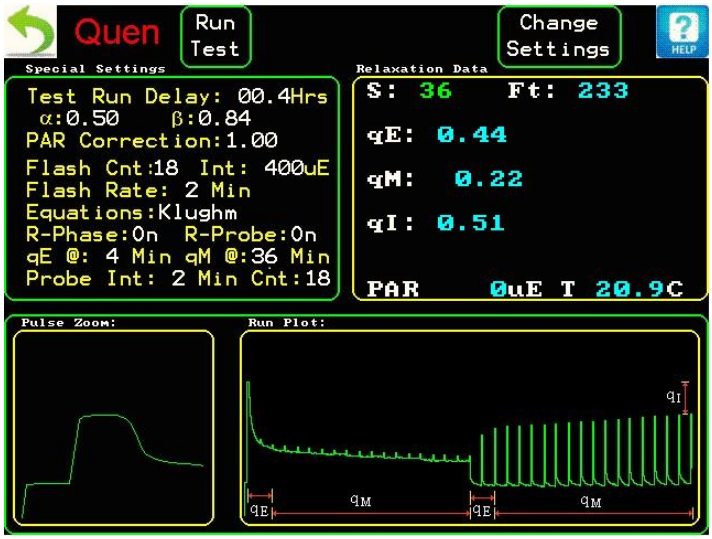
it changes leaf light transmission at high light levels. The i*FL* also measures leaf absorptance, to provide a more reliable *J*, g_m and C_C.

Chloroplast migration or q_M

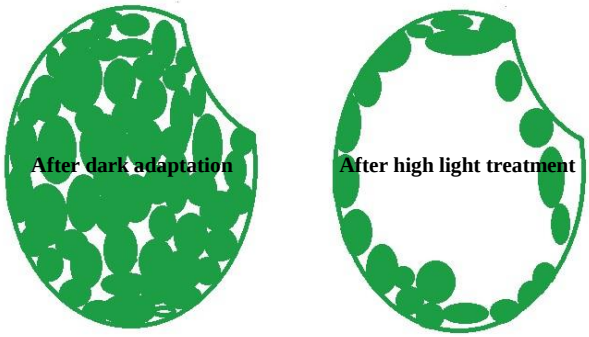
Chloroplast migration replaces *state transitions* and *acute photoinhibition* as the intermediate time-frame chlorophyll fluorescence change in C_3 plants (Cazzaniga *et al*., 2013) (Dall'Osta 2014). Chloroplast migration also occurs in C_4 plants and it is modulated by blue light intensity (Maai 2011).

It is now known that about 30% of fluorescence NPQ (non-photochemical quenching), at high actinic light levels, is due to q_M (chloroplast migration). Chloroplast migration, as it occurs in nature, only occurs under intense white light or intense blue light, not intense red light (Cazzaniga *et al*., 2013).

This recent research shows that substantial measuring artefacts are possible when using a low intensity blue light source for chlorophyll fluorescence measurement. ETR or *J*, Y(II) or Φ_{PSII}, NPQ, g_M, C_C and q_I may all include measuring errors without a reliable light source.



Opti-Sciences OS5p+ Quenching relaxation test representation at high actinic light levels. See the graphic



Representation of Arabidopsis cell chloroplasts in a cell, viewed from the top with a microscope after dark adaption on the left, and viewed from the top after high light treatment on the right.

Furthermore, chloroplast migration, as it occurs in nature, *changes leaf absorptance* at higher light levels. If intense blue light is not part of the actinic light used, chloroplast migration does not occur significantly and it can affect most gas exchange measurements for a given high light level, as well as A/Q and A/Ci curves at higher light levels.

Question: What causes the non-photochemical quenching (NPQ) fluorescence relaxation change that is greater than a few minutes and less than thirty five minutes?

Discussion: *Under higher actinic light levels and near saturating light conditions,* the latest data supports chloroplast migration as the source of fluorescence change and quenching relaxation in C_3 plants (Cazzaniga *et al*., 2013) (Dall'Osto 2014). Chloroplast migration modulated by blue light intensity has also been shown to exist in C_4 plants (Maai 2011). Since q_M is responsible for about 30% of NPQ in samples tested, it becomes a game changer for most chlorophyll fluorescence measuring parameters and protocols. *This research affects the type of actinic light sources that should be used to measure most light adapted parameters under high light conditions, the times recommended for dark adaptation, and the time required to reach steady state photosynthesis under high light conditions.*

Until recently, it was believed that as a plant goes from a dark adapted state to a high light level or from a high light adapted state to a dark adapted state, there were three basic mechanisms involved in chlorophyll fluorescence measurement of non-photochemical quenching (NPQ) ; q_E, q_T, and q_I.
The parameter q_E can be described as a rapid photo-protective adjustment of photosystem II caused by ΔpH of the thylakoid lumen and the xanthophyll cycle. It can take q_E several seconds to several minutes to adjust, and it tends to be longer in field plants (Baker 2008, Murchie 2011, Nilkens 2010).
For most greenhouse or indoor plants, q_E is complete after about 4 minutes; however, it can take up to 7 minutes in some field grown plants (Baker 2008).

Traditionally, q_T was thought to be caused by state transitions, that q_T could take up to fifteen or twenty minutes and it had been described as a fluorescence change that overlapped somewhat with q_E. Changes that took longer were related to q_I or photoinhibition (Ruban 2009). There is now significant evidence to show that the fluorescence change measured as q_T in quenching relaxation measurements is likely due to other chloroplast migration, at least *under high actinic light conditions in many land plants.*

State transitions – the older classical view:

According to classical state transition theory, state transitions are thought to be a low light level survival mechanism that allows balancing of light between photosystem II (PSII) and photosystem I (PSI). It was believed that LHCII antenna trimers, or peripheral phosphorylated light harvesting complex II antenna, migrated from PSII complexes to PSI complexes. The movement would occur from one thylakoid membrane to another when they were very close or adjacent to one another. The movement and reaction took place on the stroma side of the thylakoid membrane allowing the LHCII antenna to serve as a PSI antenna. When dephosphorylated, the LHCII antenna favoured movement back to PSII. The result was that at low light levels, movement was favoured to PSI. LHCII phosphorylation was a prerequisite for dynamic regulation of relative balance of PSI/PSII excitation under artificially induced state transitions with different qualities of light. However, this process has not been viewed, without reservation, in PSII rich thylakoid membranes.

State transitions – a more recent view:

A more recent view has emerged from there being no clear evidence to support the actual movement of LHCII (light harvesting complex II antenna) in stroma-exposed PSII rich thylakoid membranes, to PSI reaction centres. LHCII phosphorylation does not collect light energy for PSI in these cases (Tikkanen 2012). Only in the margins of the grana thylakoid membrane, do LHCII antenna behave according to the traditional view of phosphorylation-induced state transition (Tikkanen 2008). Tikkanen also states that there is substantial evidence to show that the classical mechanism of state transitions is not the sole method for energy balance between the two different photosystem types. There is also evidence to show that LHCII phosphorylation probably connects the regulation of light balance between PSII and PSI through unknown non-photochemical quenching mechanisms (Tikkanen 2012), not only with PSII but also PSI (Tikkanen 2010). It was also noticed that when light intensity is increased, the PsbS protein is protonated, turning the LHCII antenna into a dissipative state for PSII (Li 2004, Tikkannen 2012). At lower light levels, LHCII activity is restored, and PSII activity is increased. Furthermore, phosphorylation is controlled by the enzymes STN7 and STN8 kinases and their opposing phosphatases; that are in turn, closely controlled by light intensity. These kinase functions are completely synchronized with PsbS and the xanthophyll cycle (Tikkanen 2012).

Previous contender - q_Z - due to an unknown longer term xanthophyll cycle mechanism

In 2010, Nilkens and others used NPQ Arabidopsis mutants to determine that *under saturating light conditions*, q_T or state transitions did not significantly contribute to fluorescence change and resulting quenching relaxation in the dark. Furthermore, samples were tested at moderate illumination to rule out q_I or photoinhibition as the contributor to this fluorescence change. The changes related to q_Z were complete by 30 minutes. It was proposed that under steady state, *saturating light conditions*, NPQ should be divided into q_E, q_Z, and q_I.

As described by others, q_E is a process that is created and relaxes in the ten second to two hundred second time frame, and is dependent on Δph of the thylakoid lumen, the PsbS protein and zeaxanthin formation. The longer portion of q_E from 100 seconds to about 200 seconds is limited by zeaxanthin synthesis. Relaxation of q_E regulates the Δph of the thylakoid lumen.

According to the Nilkens group, the proposed q_Z is created in the ten to thirty minute time frame. PsbS is not involved in q_Z, but is wholly dependent on zeaxanthin formation. Relaxation depends on the reconversion of zeaxanthin to violaxanthin. It should be stated that the group found a sample that seemed to contradict the other q_Z results; however, they thought this was likely due to a retarded relaxation of a large fraction of q_E and not normal middle range fluorescence relaxation characteristics. The test plant was a zeaxanthin devoid mutant npq1.

Photoinhibition, q_I, was shown to form after 30 minutes and was dependant on illumination time, intensity and genotype. It was also found that the state transitions, q_T, were not a significant contributor to NPQ at saturating light intensity.

However, since the chloroplast migration mechanism has been found it both C_3 plants and C_4 plants, strong evidence not points to chloroplast migration as the mechanism responsible for intermediate time frame chlorophyll fluorescence change (Cazzaniga *et al.*, 2013),(Dall'Osto 2014),(Maai 2011).

q_M – due to chloroplast migration

Cazzaniga S, Osto L.D., Kong S-G., Wada M., Bassi R., (2013) used multiple methods, Arabidopsis mutants and wild type Arabidopsis plants to determine that the fluorescence change was caused by *chloroplast migration.* They found, as others have, that under high light levels, chloroplasts move from the top of cells to the sides of cells, partially shading other chloroplasts. This was verified with light microscopy using samples treated to prevent migration after high light migration. They also found that leaf transmittance increased and therefore leaf absorptance was lowered with chloroplast migration. The research concludes that the cause of q_M is a decrease in light photon absorption which creates lower fluorescence yield, rather than a true quenching process. This is thought to be another avoidance process to protect leaves from high light levels. They found that the time scales for adjustment and fluorescence intensity-change mirrored the previously used q_T and acute photoinhibition, with time ranges of 20 to 30 minutes and up to 35 minutes with some mutant plants. Chloroplast migration has been known and studied for a while; it was stated by Brugnoli in 1992 that chloroplast migration affected chlorophyll fluorescence. The Cazzaniga *et al*., (2013) paper is the first to name chloroplast migration as the source of the q_T and q_Z fluorescence change.

Researchers found that high white actinic light was more effective than high red actinic light at inducing the photoprotective functions of q_M. Chloroplast migration was shown to be controlled by high blue light intensity (Kagawa T. 2001). Arabidopsis mutants that were devoid of q_E (npq4) were tested and Arabidopsis mutants devoid of q_E and chloroplast migration (npq4 photo2) were also tested along with other mutants. In addition, targeted reverse genetic analysis was used to eliminate other contending possibilities by creating a series of double and triple mutants that covered the remaining spectrum of mechanisms that affected chlorophyll fluorescence, including; the composition of various parts the photosynthetic apparatus, carotenoid biosynthesis, and state transitions as sources of q_M.

In regard to q_T being replaced by q_Z, it was found that by using mutants devoid of q_E and zeaxanthin, the magnitude of q_M did not change but the recovery in the dark took longer. Plants were grown at 150 μmol photons m^{-2} s^{-1}, and tested at 400, 800 and 1,200 μmol photons m^{-2} s^{-1}. The adjustment time for q_M ranged up to 35 minutes for some mutants.

The Cazzaniga *et al*., (2013) observation that the stn7 mutant, devoid of state transitions, has NPQ measurements very similar to the wild type in Arabidopsis, casts strong reservations in regard to the relevance of q_T in Arabidopsis.

A second paper was also published on the subject:

Dall'Osto L., Cazzaniga S., Wada M. and Bassi R. (2014) On the origin of a slowly reversible fluorescence decay component in the Arabidopsis npq_4 mutant, Phil. Trans. R. Soc. B 2014 369, 20130221, published 3 March 2014.

In 2011 a paper was published by Maai that confirmed chloroplast migration as a mechanism in C_4 plants driven by intense blue light. While it was written before the Cazzaniga *et al* paper (2013), it confirms the mechanisms presence in C_4 plants as well.

Chloroplast migration - conclusions, and the ramifications for chlorophyll fluorescence and gas exchange:

The evidence shows that changes previously reported as q_T in quenching relaxation tests are not due to state transitions *at higher light levels or saturating actinic light intensities*. The latest evidence points to chloroplast migration and the resulting reduced photon absorption as the source of fluorescence change during light adaptation and during quenching relaxation, in dicot land plants at the very least.

Some prominent researchers see evidence that intermediate component mechanisms that are part of an NPQ measurement, may not be the same in all photosynthetic organisms. There could be a relationship between the phosphorylation found in state transitions and NPQ regulation found in some monocots (corn, barley and rice). Maai found in 2011 that intense blue light was required for chloroplast migration in C_4 plants as well. While this was before Cazzaniga *et al*., (2013) made the connection between the intermediate change in fluorescence and chloroplast migration, it shows that chloroplast migration is a mechanism in C_4 monocots as well, and intense blue light is required in C_4 plants. There is also some strong evidence that q_T fluorescence, from state transitions, exists in the green algae: Chlamydomonas reinhardtii (Depège N., Bellafior e S., Rochaix J-D., 2003).

However, The fact that *higher intensity white or high intensity blue actinic light* is required to properly activate q_M and the fact that q_M represents about 30% of NPQ under these condition, indicates the need for either a white light source or a high intensity blue light and red light instead of using a high intensity red and lower intensity blue actinic light. This new research shows that substantial measuring artefacts are possible when using a low intensity blue light source for chlorophyll fluorescence measurement. ETR or *J*, Y(II) or $\Phi PSII$, NPQ, g_M, C_C and q_I may all include measuring errors without a reliable light source.

Furthermore, chloroplast migration at higher light levels increases leaf transmittance. As a result, many gas exchange parameters would be affected for a given light intensity. A/Q curves and A/Ci curves would certainly be affected at higher light levels.

Times for steady state photosynthesis

Chloroplast migration also changes the time required to reach steady state photosynthesis, and recommendations for dark adaptation. Until now, Maxwell & Johnson (2000) has been the most sighted paper for reliable steady state photosynthesis conditions at any given light level. It lists 15 to 20 minutes as the time required for 20 wild land plants to reach steady state photosynthesis. Prominent researchers, Lichtenthaler (1999) and Ruban (2009), list the dark adaptation time required for quenching relaxation of q_T at the same 15 to 20 minutes.

With this new chloroplast migration evidence from Cazzaniga *et al*., (2013), and Dall'Osto (2014), dark adaption times and the time to reach steady state lighting conditions should be extended, at least at higher light levels. They showed that it takes from *20 minutes to 30 minutes* for chloroplast migration to adapt to higher light levels and for relaxation in the dark. As a result, the times for each step of an A/Q curve should be extended to 35 minutes if the plant's absorptance characteristics have not been measured, and the initial step of an A/Ci curve should be extended to 30 minutes if the plants absorptance characteristics have not been measured. In addition, the initial light changing steps of a Laisk protocol should be extended to 30 minutes for the same reason (some mutant plants require up to 35 minutes for complete chloroplast migration). Research shows that it takes about 10 minutes for most plants to adjust to different CO_2 levels and H_2O levels at one specific light level.

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Parameters Measured and Protocols included:

Selectable readout in either µmol, mmol and moles, or in ppm and mbar.

Φ_{PSII}: Quantum Yield of PSII (or) ΔF/Fm' or Y(II))

***J*:** Electron transport rate (ETR)

PAR: Photosynthetically Active Region value (with optional PAR clip)

α: Leaf absorptance of PAR spectrum using RGB sensors above the leaf and below the leaf. Corrected for Leaf transmittance.

Values for reflectance and transmittance may be viewed on the diagnostic screen.

On the diagnostic screen additional leaf absorptance values may be measured including:

α_b blue leaf absorptance without leaf transmittance included

αg green leaf absorptance without leaf transmittance included,

αr red light leaf absorptance without leaf transmittance included,

αw white light absorptance without leaf transmittance included,

T_b blue light leaf transmittance,

T_g green light leaf transmittance,

T_r red light leaf transmittance,

T_w white light leaf transmittance (note: white light values are not used in instrument calculations).

Chamber temp: thermistor

Leaf T: Leaf temperature -infrared sensor measures approximately 80% of leaf area and provides an average value for more reliable measurement especially when chamber temperatures are different than ambient.

Laisk protocol for R_d and Γ* measurement - von Caemmerer correction option included

Yin protocol for R_d. Γ* is calculated

Kok protocol for R_d. Γ* is calculated

g_m Mesophyll conductance

C_C CO_2 at the site of carboxylation

Γ* Compensation point absent of day respiration

R_d Respiration in the light

(R_d , Γ* and other parameters or constants may also be measured independently and input manually, as well as being measured by the iFL).

Light curves & A/Q light response curves

A/Ci curves, A/C_C curves

F_V/F_M: Maximum Photochemical efficiency of PSII

F_V/F_O: A more sensitive detector of stress than Fv/Fm, but it does not measure plant efficiency.

F_O: Minimum fluorescence – used in F_V/F_M. F_V/F_O and Hendrickson Lake Model quenching.

F_O': Quenched minimum fluorescence used in Kramer Lake Model and Puddle Model quenching.

F_M: Maximal fluorescence

Fv: Variable fluorescence

F_M': Maximal fluorescence with actinic illumination

F_S (or F): Fluorescence under steady state conditions (prior to saturation pulse)

Y(II): (ΦPSII or $\Delta F/F_M'$) Quantum Yield of PSII - Formula = (F_M'-F_S) / F_M'

RLC: Rapid light curves using Eilers and Peeters curve fitting formula.

$rETR_{MAX}$: a measure of a leaf's photosynthetic capacity or maximum electron transport rate

α: The initial slope of line at low PAR values created by relating ETR to PAR. A measure of quantum efficiency

I_k: A measurement of the light intensity where light saturation dominates, or the minimum saturation level

I_m: The optimum actinic light intensity for optimal production or calculated intensity for rETR.

Hendrickson Quenching with NPQ: Y(NPQ), Y(NO), Y(II), NPQ, F_V/F_M

Kramer Quenching: q_L, Y(NPQ), Y(NO), Y(II), F_V/F_M

Puddle Model parameters

NPQ, qN, qP, Y(II), F_V/F_M

Walk away automation sequence builder.

Build a sequence, build a protocol, or stitch multiple protocols together.

Definitions:

Chlorophyll fluorescence

F_V/F_M and Y(II) Definitions:

Actinic light source – Any light source that drives photosynthesis (sunlight or artificial light). Some higher end fluorometers contain one or more built-in artificial actinic light sources for experimentation with specific repeatable radiation (or light) levels. The OS5p+ uses a stable high intensity white light LED that is designed for pre-illumination of samples and quenching measurements at stable intensities when used with the PAR Clip. The LED provides an intense blue spectrum that ensures proper chloroplast migration as found in nature. This light source also allows the measurement of qM or chloroplast migration (Cazzaniga *et al*., 2013).

Dark-adapted or Dark Adaptation – This is a term that means that an area of a plant, or the entire plant to be measured, has been in the dark for an extended period of time before measurement. Dark adaption requirements may vary for dark-adapted tests. Dark adaption times of twenty minutes to sixty minutes are common, and some researchers use only pre-dawn values. Dark-adapted measurements include F_V/F_M, and non-photochemical quenching parameters. Longer dark adaption times are common for quenching measurements. In this case, it is common to use times of twelve hours, or overnight. For a detailed discussion of dark adaptation, refer to the application note on dark adaptation.

Far red light source– Provides wavelengths above 700 nm to drive PSI, drain PSII of electrons, and to allow the rapid re-oxidation of PSII. It is used extensively for the determination of quenching parameters in Quenching protocols, and for pre-illumination and rapid re-oxidation of PSII in F_V/F_M measurements. F_o' is used in determining Kramer's quenching parameters, as well as Puddle model, q_N. Both require the use of far red light to determine quenched F_o.

F_M: Maximal fluorescence measured during the first saturation pulse after dark adaption. Fm represents multiple turnovers of Q_A with all available reaction centres closed. All available energy is channelled to fluorescence.

F_S: Also known as F' at steady state, Fs is the fluorescence level created by the actinic light. Initially, the value is high and then decreases over time to steady state values due to the initiation of electron transport, carboxylation and non-photochemical quenching. F_s has also been used to designate steady state F' conditions.

F_M': The saturation flash value that is not dark-adapted. At a lowered value due to NPQ or non-photochemical quenching. When this parameter has reached steady state, it is used to calculate Yield of PSII or Y(II) or ΔF/Fm' along with F_s.
$F_{M'}$ at steady state is also used to calculate q_N, NPQ, q_P, qL, Y(NPQ), Y(NO), q_E, q_T, and q_I.

F_O: Minimal fluorescence after dark adaptation. Measured with a modulated light intensity too dim to drive chemical reduction of Q_A and yet bright enough to detect "pre-photosynthetic" antennae fluorescence.

F_t: The current instantaneous fluorescent signal shown on the fluorometer measuring screen. It is used to set the modulated light source intensity [see 'setting the modulated light source intensity'].

F_V/F_M = $(F_M - F_O) / F_{M..}$ Maximum quantum yield. This is a dark adapted test. This ratio is an estimate of the maximum portion of absorbed quanta used in PSII reaction centres (Kitajima and Butler, 1975). Another way to look at F_V/F_M is a measurement ratio that represents the maximum potential quantum efficiency of Photosystem II if all capable reaction centres were open or oxidized. 0.79 to 0.83 is the approximate optimal value range for most land plant species with lowered values indicating plant stress. It is important to properly dark adapt samples for this test. F_O will be raised and F_M will be lowered if dark adaption is inadequate. Since dark adaption requirement can vary with species and light history, and type of dark adapted test, testing should be done to ensure proper dark adaption, [see the section on dark adaptation]. This test is a normalized ratio.

F_V/F_O = $(F_M - F_O) / F_{O.}$ A more sensitive plant stress detector than F_V/F_M. It is important to properly dark adapt samples for this test. F_O will be raised and F_M will be lowered if dark adaption is inadequate. Since dark adaption requirement can vary with species and light history, and type of dark adapted test, testing should be done to ensure proper dark adaption, [see the section on dark adaptation]. This test is a normalized ratio. Measurements commonly range from 0 to 4.

Modulated light source - The light source that makes light adapted quantum photosynthetic yield measurements possible along with direct measurements of F_o and F_o'. The modulated light source is used at an intensity range that is too low to drive photo-chemical reduction of Q_A and yet allows fluorescence measurement of pre-photosynthetic F_o, and photosynthetic F_o'. This light source is turned off and on at a particular frequency. The frequency is adjusted automatically for optimal application usage. Intensities are adjusted between 0 to 0.4 µmol. The intensity and frequency must be set differently for light and dark adapted methods, Vredenburg OJIP quenching and quenching relaxation tests. It is critical to adjust the intensity of this lamp correctly in dark adapted protocols to prevent driving photo-chemical reduction of Q_A in F_V/F_M, and quenching measurements. For more details see the application note on dark adaptation. The OS5p+ has an automated routine that can be used to automatically set the modulated light intensity correctly. It can also be set manually if desired. The frequency is set automatically for the test that is being used.

PAR – Photosynthetically Active Radiation between 400nm and 700nm. Measured in either µmol or µE. PAR can be measured in different dimensions such as Watts per meter or in micro-moles (µmol) or micro-Einsteins (μ_E). When using a PAR Clip, dimensions will always be in µmol.

PAR Clip – This is a fluorometer accessory that allows the measurement of PAR or PPFD and Leaf Temperature along with Yield measurements. Since Y(II) change with

PAR radiation (or light) levels and temperature levels, the ability to record Yield values with these parameters provides control over important variables. Since changes in Y(II) can be due to changes in plant stress or light level, it is important to control and measure light level when comparing values. A PAR Clip allows the calculation of relative ETR. When used with internal fluorometer actinic illuminators, it will measure reproducible and repeatable controlled values. PAR clips are recommended for field use with quantum photosynthetic yield measurements. See the section on quantum photosynthetic yield for an in depth discussion.

PPFD - Photosynthetic Photon Flux Density of PAR. Measured in either µmol or μ_E, PPFD is the number of PAR photons incident on a surface in time and area dimensions (per meter squared per second, m^{-2} s^{-1}). These terms are equivalent for PAR Clip leaf radiation measurements. Furthermore, both can be presented in either of the equivalent dimensions, micro-moles (µmol) or micro-Einsteins (μ_E).

Quantum Yield of PSII = Y(II) or (F_M' – F_S) / F_M' - This test is also known as $\Delta F/F_M$'. Yield of PSII is a fast light-adapted test taken at steady state photosynthesis levels. This ratio is an estimate of the effective portion of absorbed quanta used in PSII reaction centres (Genty, 1989). It provides a measure of actual or effective quantum yield. Y(II) is affected by closure of reaction centres and heat dissipation caused by non-photochemical quenching (NPQ). It allows investigation of the photosynthetic process whilst it is happening. No dark adaption is required. When a sample is exposed to light, it normally takes several minutes for photosynthesis to reach steady state. According to Maxwell and Johnson (2000), it takes between fifteen to twenty minutes for a plant to reach steady state photosynthesis. To obtain a reliable yield measurement, photosynthesis must reach steady state. Recent research has shown that under high actinic light conditions it can take 20 minutes to 35 minutes to reach steady state photosynthesis due to chloroplast migrations (Cazzaniga *et al*., 2013).

This is usually not a concern when using ambient sunlight or artificial greenhouse light, however, clouds and light flecks below a canopy level can cause problems. If one uses a built-in fluorometer actinic illuminator to measure yield, make sure that steady state photosynthesis has been reached [see the discussion on Quantum Yield of PSII for more information]. Remember that ambient sun light contains far red illumination for activation of PSI. It is something to consider when using an internal illuminator for Y(II) measurements. Far red illumination is an option when using internal actinic illumination for yield measurements (see section on quantum yield of PSII).

Y(II) has been found to be more sensitive to more types of plant stress than F_V/F_M, however one must only compare measurements at the same light level, as the value changes at different light levels. A PAR clip should be used with the fluorometer to measure Y(II) in all field applications. This allows for proper comparisons of values and the determination of ETR or electron transport rate, a parameter that includes both yield and actinic light level [see the Stress Guide for more details].

Light adapted measurements include Y(II) or $\Delta F/F_M$', ETR, PAR (or PPFD), and Leaf Temp, quenching measurements, Rapid Light Curves, and Light Curves.

Saturation pulse - A short pulse of intense light designed to fully reduce all available PSII reaction centres. For higher plants, the optimal duration of the saturation pulse is between 0.5 seconds and 1.5 seconds (Rosenqvist and van Kooten 2006). It is typically a white light that has to be high enough to close all available PSII reaction centres. With leaves that have a very intense actinic light history, this may not be possible using a standard square flash. For these samples, Multi-Flash is recommended (see the section on Multi-Flash for a detailed explanation). On the OS5p+ an LED light source is used. Opti-Sciences uses 0.8 seconds as the default saturation pulse duration for higher plants. And 1.1 seconds in the Multi-Flash F_M' correction protocol after Loriaux 2013. The duration is adjustable from 0.1 to 2.0 seconds; however, the OS5p+ uses an eight point 25 ms rolling average to determine F_M and F_M'. This ensures that the optimal measurement F_M, or F_M' values even if the duration is set too long as long as the duration is long enough.

µE – is a **micro Einstein**, a dimension that involves both time and area. It is equivalent to the

µmol. Both terms have been used extensively in biology and radiation measurements.

µmol - is a **micro mol** (also abbreviated µml, or µmol $m^{-2}s^{-1}$). This is a dimension that involves both time and area (per meter squared per second). It is equivalent to the micro Einstein. Both terms have been used extensively in biology and radiation measurements.

1µE

$= \quad 1\ \mu mol\ m^{-2}\ s^{-1} \ = \quad 6.022 \times 10^{17}\ photons\ m^{-2}\ s^{-1}$

For Quenching definitions and equations go to the
Quenching section.

Definitions - Lake Model Parameters

Y(NPQ) is a lake model quenching parameter that represents heat dissipation related to all photo-protective mechanisms also called regulated heat dissipation. (David M. Kramer, Giles Johnson, Olavi Kiirats & Gerald E. Edwards 2004) (Klughammer and Schreiber 2008).
A low Y(NPQ) at high light levels is an indication of sub-optimal photo-protective mechanisms. (Klughammer and Schreiber 2008).

Y(NO) is a lake model quenching parameter that represents all other components of non-photochemical quenching that are not photo-protective. They include non-radiative decay, and fluorescence. Part of Y(NO) includes photoinhibition (David M. Kramer, Giles Johnson, Olavi Kiirats & Gerald E. Edwards 2004). Klughammer and Schrieber define Y(NO) as the "fraction of energy that is passively dissipated in the form of heat and fluorescence mainly due to closed PSII reaction centers". Hendrickson calls Y(NO) constitutive heat dissipation. A high Y(NO) value after dark adaptation is an indication of photo-damage. (Klughammer and Schreiber 2008). According to Klughammer (2008), Y(NO) is the only quenching parameter that does not need to be taken as steady state photosynthesis.

q_L is the lake model quenching parameter that represents photochemical quenching. It is a measure of the fraction of still open PSII reaction centres. (David M. Kramer, Giles Johnson, Olavi Kiirats & Gerald E. Edwards 2004). It is available in the Kramer equations but not the Hendrickson – Klughammer equations.

Y(II) = quantum yield of photosynthetic energy. The equation is the same as for $\Delta F/F_M'$. $(F_M' - F_S) / F_M'$

Puddle model parameter reconciled with the lake model
(Klughammer and Schreiber 2008)

NPQ = Y(NPQ)/Y(NO) or NPQ= $(F_M-F_M')/F_M'$ Klughammer and Schreiber reconcile NPQ with the lake model using simplified parameters. NPQ, [resurrected puddle model parameter valid in Klughammer simplified Lake model equations, see above] is non-photochemical quenching and is a measure of heat dissipation. NPQ is an alternate expression of non-photochemical quenching. NPQ provides an estimate of quenching without knowledge of F_O'. The advantage of NPQ over q_N depends on the specific application. NPQ is more heavily affected by non-photochemical quenching that reflects heat-dissipation of excitation energy in the antenna system. So, it may be thought of as an indicator of 'excess excitation energy'. Alternatively, NPQ is relatively insensitive to the part of non-photochemical quenching associated with q_N values lower than 0.6. The range of NPQ is affected by ΔpH of the thylakoid lumen, which is an important aspect of photosynthetic regulation, state transitions and photoinhibition. Numbers range from zero to infinity with typical samples measuring in the 0.5 to 3.5 range (Maxwell and Johnson 2000), (Bilger & Björkman, 1990), (Muller P., Xiao-Ping L., Niyogi K. 2001).

Definitions - Puddle Model Parameters

NPQ is non-photochemical quenching and is a measure of heat dissipation. NPQ is an alternate expression of non-photochemical quenching. It provides an estimate of quenching without knowledge of F_O'. The advantage of NPQ over q_N depends on the specific application. NPQ is more heavily affected by non-photochemical quenching that reflects heat-dissipation of excitation energy in the antenna system. So, it may be thought of as an indicator of 'excess excitation energy'. Alternatively, NPQ is relatively insensitive to the part of non-photochemical quenching associated with qN values lower than 0.6. Much of the range of NPQ is affected by ΔpH of the thylakoid lumen which is an important aspect of photosynthetic regulation, it is also affected by state transitions and photoinhibition. Numbers range from zero to infinity with typical samples measuring in the 0.5 to 3.5 range (Maxwell and Johnson 2000). (Bilger & Björkman, 1990), (Muller P., Xiao-Ping L., Niyogi K. 2001).

q_N is similar to NPQ but requires F_O' in the calculation. q_N is defined as the coefficient of non-photochemical fluorescence quenching. The original definition of this term implied that fluorescence quenching affects primarily the 'variable fluorescence' (F_V) and not the minimal fluorescence (F_O). In cases where q_N is greater than 0.4 this may not be a good assumption. When q_N is above 0.4, F_O' should replace F_O in q_P equations. q_N is less

sensitive than NPQ at higher values (Maxwell and Johnson 2000). By using the Far-Red source after actinic illumination is turned off, the PSII acceptors are re-oxidized and PSI is reduced. An $F_{o'}$ value is measured and used for corrections to the quenching coefficients. Numbers range from zero to one [puddle model], (Van Kooten & Snel, 1990).

q_P is the quenching parameter that represents photochemical quenching. It is a measure of the fraction of still open PSII reaction centres. q_P is defined as the coefficients of photochemical fluorescence quenching. The original definition of this term implied that fluorescence quenching affects primarily the 'variable fluorescence' (F_V) and not the minimal fluorescence (F_O). In cases where q_N is greater than 0.4 this may not be a good assumption. When q_N is above 0.4, F_O' should replace F_O in q_P equations. By using the Far-Red source for post illumination, the PSII acceptors may be re-oxidized through the illumination effect on PSI. A new F_O' value may be measured and used for corrections to the quenching coefficients. This assumes the PSI acceptors are properly activated, which may not be the case in a dark adapted sample. Therefore, the F_O' determination should be done after induction of photosynthesis has reached steady state. Numbers range from zero to one [Puddle model], (Van Kooten & Snel, 1990).

Quenching Relaxation Definitions for Lake and Puddle Model

q_E, q_M, q_Z, q_T, q_I

q_E -(puddle model and lake model parameter) is the quenching parameter that represents the photo-protective mechanisms in the leaf that allow rapid compensation for changes in light levels due to cloud cover and increased light intensity. It is directly related to ΔpH of the thylakoid lumen and the xanthophyll cycle. (Muller P., Xiao-Ping L., Niyogi K. 2001) This process is completed in two to four minutes after an actinic light is turned on but may be as long as seven minutes in field grown leaves (Baker 2008), (Lichtenthaler 1999). It is delineated from NPQ by using a quenching relaxation method. Some researchers in the past have also divided q_N into q_E, q_T, and q_I instead of NPQ (Lichtenthaler 1999) The relaxation characteristics of field plants can vary with changing environmental conditions, for example q_E may take as long as seven minutes (Baker 2008).

q_T -(puddle model and lake model parameter) is not true quenching. Instead, the parameter represents state 1 and state 2 transitions. This value is negligible in higher plants at high light levels but may be substantial at low light levels (Lichtenthaler 1999) (Baker 2008). According to Ruban (2008) state transitions require between fifteen and twenty minutes to complete. It can be delineated from NPQ by using a quenching relaxation method (Muller P., Xiao-Ping L., Niyogi K. 2001). For more information on state transitions, and how they affect fluorescence measurement contact Opti-Sciences for the application note on state transitions. The relaxation characteristics of field plants can vary with changing environmental conditions (Baker 2008). At near higher light levels and near saturation light conditions fluorescence changes starting before q_E is ended and lasting for between 20 minutes and 35 minutes has been shown to be the result of chloroplast migration or q_M in dicots (Cazzaniga *et al*. 2013). It is also likely in moncots (Maai 2011).

q_I - (puddle model and lake model parameter) is the quenching parameter that represents photo-inhibition and photo-damage. (Puddle model) (Muller P., Xiao-Ping L., Niyogi K. 2001) According to Lichtenthaler (1999, 2004) chronic photo-inhibition starts to relax after forty minutes in the dark and may take up to sixty hours. It can be delineated from NPQ by using a quenching relaxation method. The relaxation characteristics of field plants can vary with changing environmental conditions (Baker 2008). There is some residual photoinhibition built into fluorescence measurements on sun leaves after a sunny day in the summer. Light history should be evaluated before comparisons between measurements.

q_Z - due to an unknown longer term xanthophyll cycle mechanism. In 2010, Nilkens and others used NPQ Arabidopsis mutants to determine that under saturation light conditions, q_T or state transitions did not significantly contribute to fluorescence change and resulting quenching relaxation in the dark. Furthermore, samples were tested at moderate illumination to rule out q_I or photoinhibition as the contributor to this fluorescence change. The changes related to q_Z were complete by 30 minutes. It was proposed that under steady state, saturating light conditions, NPQ should be divided into q_E, q_Z, and q_I.

As described by others, q_E is a process that is created and relaxes in the ten second to two hundred second time frame, and is depends on Δph of the thylakoid lumen, the PsbS protein and zeaxanthin formation. The longer portion of q_E from one hundred seconds to about 200 seconds is limited by zeaxanthin synthesis. Relaxation of q_E is regulated the Δph of the thylakoid lumen.

According to the Nilkens group, the proposed q_Z is created in the ten to thirty minute time frame. PsbS is not involved in q_Z, but is wholly dependent on zeaxanthin formation. Relaxation depends on the reconversion of zeaxanthin to violaxanthin. It should be stated that the group found a sample that seemed to contradict the other q_Z results; however, they said that is was likely due to a retarded relaxation of a large fraction of q_E and not normal middle range fluorescence relaxation characteristics. The test plant was a zeaxanthin devoid mutant npq1. Photoinhibition, q_I, was shown to form after 30 minutes and was dependant on illumination time, intensity and genotype. It was also found the state transitions, q_T, were not a significant contributor to NPQ at saturating light intensity.

q_M – chloroplast migration

See detailed discussion in ‘Chloroplast Migration’ Section on previous pages.

Chlorophyll fluorescence quenching equations:

Kramer and Hendrickson / Klughammer and Schreiber's Lake Model parameters account for all light that is absorbed by PSII. All parties agree with the following equation:

Kramer's equation: $1 = Y(II)+Y(NPQ)+Y(NO)$
$Y(II)$ is quantum yield of photochemical energy also known as $)F/F_M'$ or $(F_M'- F_S')/ F_M'$

For comparison purposes, the differences in the equations are listed below.

Kramer's equations:
$Y(II) = (F_{M'} -$
$F_S)/F_{M'}$ or $\Delta F'/$
F_M' $q_L = q_P (F_o'/F_s)$
$Y(NO) = 1/(NPQ+1+q_L(F_M/F_O -1))$
$Y(NPQ) = 1- Y(II)-Y(NO)$

Hendrickson's equations and NPQ resurrected to the lake model from the puddle model by Klughammer and Schreiber:
$Y(II) = (F_{M'} -F_S)/F_{M'}$ or
$\Delta F_M'/ F_M'$ $Y(NO) =$
F_S/F_M or F'/F_M
$Y(NPQ) =(F_S/F_{M'})-Y(NO)$ or $(F'/F_M') - Y(NO)$
$NPQ= Y(NPQ)/Y(NO)$ or $NPQ = (F_M-F_M')/F_M'$

Puddle Model Parameters:
$q_P = (F_{M'} - F_S)/(F_{M'} -F_O)$ Above 0.4, F_o' should replace F_o
$q_N = 1- ((F_{M'}-F_O) /(F_M-F_O))$ or $q_N = 1- ((F_M'-F_O) / (F_M-F_O))$ Above 0.4, F_o' should replace F_o $NPQ = (F_M-F_{M'})/F_{M'}$

Quenching relaxation valid with Hendrickson Lake Model and Puddle Model equations:
$NPQ = q_E+q_T+q_I$ or $NPQ = q_E+q_Z+q_I$ or $NPQ =q_E+q_M+q_I$ $q_E = ((F_{ME}-F_{M'})/(F_M-F_{M'}))$ is the relaxation saturation value at 4 to 10 minutes in the dark (time is adjustable).

$q_M = ((F_{MM}-F_{ME})/ (F_M-F_{M'}))$ is the relaxation saturation value at 20 to 35 minutes in the dark. Time is adjustable; 35 minutes being the default value.
$q_Z = ((F_{MZ}-F_{ME})/ (F_M-F_{M'}))$ is the relaxation saturation value at 20 to 30 minutes in the dark. Time is adjustable; 30 minutes being the default value.
$q_T = ((F_{MT}-F_{ME})/ (F_M-F_{M'}))$ is the relaxation saturation value at 15 to 20 minutes in the dark. Time is adjustable; 20 minutes being the default value.

$q_I = ((F_M-F_{MM}) / F_M-F_{M'}))$ Relaxation of qI starts at about 40 minutes and can take up to 60 hours.
qI can be determined from the dark adapted F_M measurement and the saturation pulse at 35 minutes used for q_M .

Variable Chlorophyll Fluorescence – Overview (2014)

Chlorophyll "a" absorbs light most effectively in the red and blue parts of the visible spectrum. Chlorophyll fluorescence is light that is re-emitted at a longer wavelength after being absorbed by chlorophyll molecules at shorter wavelengths. Variable chlorophyll fluorescence is only observed in chlorophyll "a" in photosystem II. By measuring the intensity and nature of variable chlorophyll fluorescence, using developed protocols, plant physiology can be investigated (Baker 2004). The variable nature of chlorophyll fluorescence allows research into the light reaction of plants, plant photo-protection mechanisms, heat dissipation, correlation with photosynthesis carbon assimilation, and measurement of most types of plant stress at usable levels (Baker 2004). As stated earlier, the sole origin of variable chlorophyll fluorescence is chlorophyll "a" in photosystem II (Zhu 2005). Light energy entering photosystem II can be converted to chemical energy by photochemistry. It can also be re-emitted as chlorophyll fluorescence or it can be re-emitted as heat. These three processes are in competition, so that when photochemistry output is high, chlorophyll fluorescence and heat are lower. Conversely, if fluorescence is maximized, then the other two paths are minimized.

While photosystem I does also emit chlorophyll fluorescence, it is at a much lower level and it is not variable. For that reason, chlorophyll fluorescence of photosystem II is of much greater interest
(Schreiber 2004).

Photosynthesis is comprised of a light reaction and a dark reaction. The light reaction converts light energy into chemical energy that can be used in the dark reaction. The dark reaction uses the energy molecules NADPH and ATP, created by the light reaction, to produce simple sugars in conjunction with the assimilation of CO_2 from the air. The relationship between the two may be correlated, but it is not always straightforward (Rosenqvist and van Kooten). Correlation of variable chlorophyll fluorescence in the light reaction, and carbon assimilation in the dark reaction has a linear relationship in C_4 plants, and a curve-linear relationship in C_3 plants (Schrieber 2004). This correlation in C_3 plants can break down under special conditions that create photorespiration such as drought and heat stress. Under photo-respiratory conditions, oxygen and possibly other electron sinks, alternatively use the energy molecules generated in the light reaction, even after leaf carbon assimilation has been reduced. Under these conditions, chlorophyll fluorescence levels may be unchanged until severe plant stress occurs (Baker 2008), (Flexas 1999). In C_4 plants, there is no significant photorespiration. Fortunately, special chlorophyll fluorescence assays or methods have been developed to overcome the most interesting problem areas, including: drought stress in C_3 plants, nitrogen stress in C_3 plants and C_4 plants, and heat stress in C_3 and C_4 plants (Desk Top Plant Stress Guide).

How does the variable chlorophyll fluorescence work? (Dark adapted tests)

Upon illumination of a leaf that is dark-adapted (either overnight or by artificial means), there is a rapid rise in fluorescence from Photosystem II (PSII), followed by a slow decline. This displays the variable nature of chlorophyll fluorescence in photosystem II. It was first detailed by Kautsky in 1931, and this is called the Kautsky Effect (Govindgee 2004), (Kautsky 1931).

In Photosystem II, light is absorbed by accessory pigment-protein complex molecules called antenna (Zhu 2005), and transferred to PSII reaction centres. Zhu divides antenna into peripheral antenna and core antenna. Core antenna are tightly bound chemically and adjacent to the reaction centre. Peripheral antenna are near the core and chemically bound either strongly, at a medium level or loosely. They are, however, in close proximity to either photosystem II or photosystem I reaction centres (Schneider 2013).
In the photosystem II reaction centre are D_1 and D_2 pigment-protein complexes that coordinate the specialized chlorophyll "a" photoactive reaction centre structure, P_{680} (Papageorgiou 2004). There are different models that show P_{680} acting as various types of dimer (Papageorgiou 2004), (van Gronelle 2004), (Razewski 2008), or a monomer (Takehashi 2009); however, the Zhu (2005, 2012) papers, written by some of the most prominent chlorophyll fluorescence researchers, focus on fluorescence and provide a conservative approach that is currently the most accepted.

The core antenna complexes are known as CP43 and CP47. They are chlorophyll-protein complexes that are adjacent and associated with the D_1D_2 P_{680} PSII reaction centre.

[For more details concerning these structures see Diagrams C or refer to the application note on Variable chlorophyll fluorescence at www.optisci.com].

There are several different pigment types associated with peripheral antenna, including: Chlorophyll a, Chlorophyll b, lutein, xanthophylls, beta carotene, and lycopene. The antenna absorb light in different wavelength ranges, and transfer the energy to nearby photosystem I and II reaction centres. As the energy transfer occurs to the reaction centre, a small amount of energy is lost as passive heat. The antennas transfer energy to photosystem II reaction centres and to photosystem I reaction centres. Both photosystems are located in thylakoid membranes. Thylakoids are lumened structures stacked inside plant chloroplasts. They are pictured in the diagrams below (Diagram B). There are two basic types of photosystems called either photosystem II, or photosystem I (Diagram C). While it has been shown that chlorophyll "b" can show a slight fluorescence when energy cannot be transferred to chlorophyll "a", the emission spectrum in the 660 nm to 665nm range is normally filtered out by chlorophyll fluorometers and it does not directly affect F_O, or F_V/F_M. No fluorescence has been observed in chlorophyll "b" when energy transfer to chlorophyll "a" is normal (Govingee, 1978).

There are also two varieties of photosystem II reaction centres that affect variable chlorophyll fluorescence. Energy is transferred to either Q_B^- reducing reaction centres, capable of being used in photochemistry, or to Q_B^- non-reducing reaction centres, that are not capable of photochemistry. Q_B^- non-reducing reaction centres do not transfer their energy to other reaction centres and so the absorbed energy is re-emitted as either heat or non-variable chlorophyll fluorescence at a low level (Diagram C).

Q_B^- non-reducing reaction centres have smaller core antenna, an oxygen evolving complex, and no peripheral antenna. There is also no electron transfer beyond Quinone A or Q_A (Quinone B is designated Q_B). A higher number of Q_B^- non-reducing reaction centres in the leaf increase the minimum fluorescence, F_O, which is measured in a dark adapted state, and decrease the F_V/F_M measurement parameter to be discussed in detail later (Zhu 2005).

Q_B^- reducing reaction centres that can be used in photochemistry, can be either opened or closed. They are open if they are chemically oxidized and are closed if they have been chemically reduced. Closed Q_B^- reducing reaction centres can transfer additional energy

to other, open Q_B^- reducing reaction centres. In a properly dark adapted state, most or all Q_{B-} reducing reaction centres will be open (Zhu 2005). When Photosystem II Q_B^- reducing reaction centres receive an adequate threshold of light energy, charge separation is driven in photosystem II (Zhu 2005). This, and the electron transfer to the A_O molecule in photosystem I, are the only steps where light energy is converted into chemical energy (Zhu 2005).

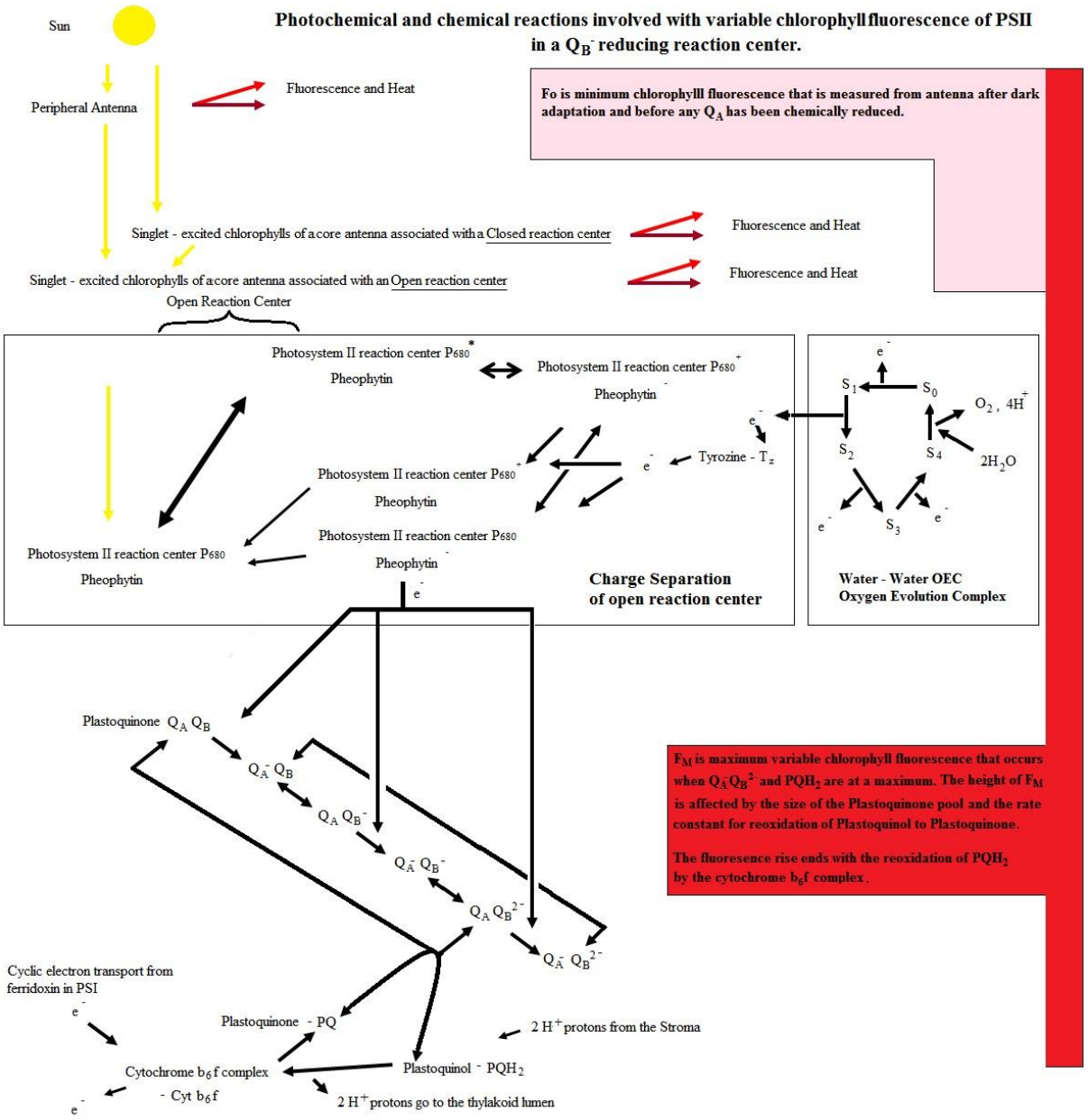
At charge separation in photosystem II, an electron is transferred from P_{680}, the primary electron donor, to the primary electron acceptor, pheophytin. The chemical process for charge separation is shown in diagram A. During this process, an electron is added from Tyrozine, Y_Z, generated from the Oxygen Evolving Complex, through the Mehler reaction. The oxygen evolving complex involved in this process is also shown in the diagram C below (Zhu 2005). The energy levels of the remaining steps in the light reaction of photosystem II are all downhill in oxidation-reduction reactions (Zhu 2005).

Measuring fluorescence in a dark adapted state begins with measuring minimum fluorescence generated by peripheral and core antenna before any Q_A has been chemically reduced (Zhu 2005). This is commonly done using a modulated chlorophyll fluorometer that excites minimum fluorescence. The modulated light source is adjusted high enough to allow minimum antenna fluorescence measurement, but it is set low enough to prevent the reduction of any Q_A. Additional sources of minimum fluorescence also include Photosystem I, and PSII Q_B^- non-reducing reaction centres (Zhu 2005), (Opti-Sciences F_V/F_M checklist application note).

The rise of variable chlorophyll fluorescence starts at charge separation and continues through the reduction of Q_A, Q_B, and the reduction of the Plastoquinone Pool. Evidence shows that the rise ends with the re-oxidation of PQH_2 (Plastoquinol) to PQ (plastoquinone) by the Cytochrome b_6f complex. Maximum variable fluorescence or F_M, occurs when $Q_AQ_B^{2-}$ and PQH_2 are at a maximum (Zhu 2005). The height of F_M is affected by the size of the Plastoquinone pool and the rate constant for reoxidation of PQH_2 to PQ. A higher k_{ox}, reoxidation constant, or a larger plastoquinone pool reduces the F_M value (Zhu 2005).

Different types of plant stress affect PSII differently, therefore one should consult the "Desk Top Plant Stress Guide" (provided), to determine the best measuring protocol or special assay before working. Research referenced in the Plant Stress guide shows that while some types of plant stress affect chlorophyll fluorescence of a plant in a dark adapted state (F_V/F_M), measuring some types of plant stress at a sensitive level requires the light adapted Y(II) or $\Delta F/F_M$' or special assays.

(Diagram A): Adapted from Zhu (2005), page 116, with more in-depth descriptions for purposes of understanding. It represents one reducing Q_B^- PSII unit.



(Diagram B):

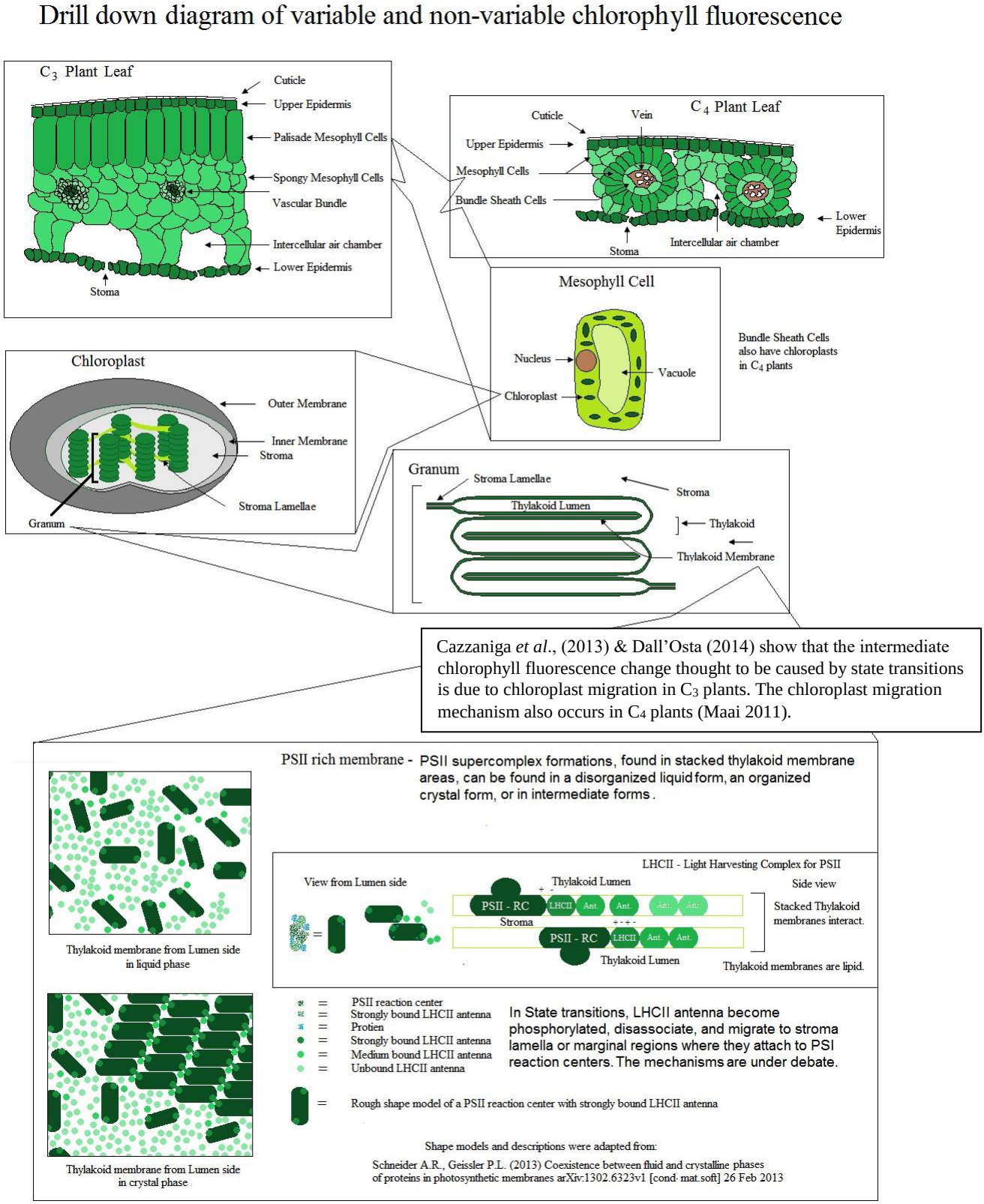
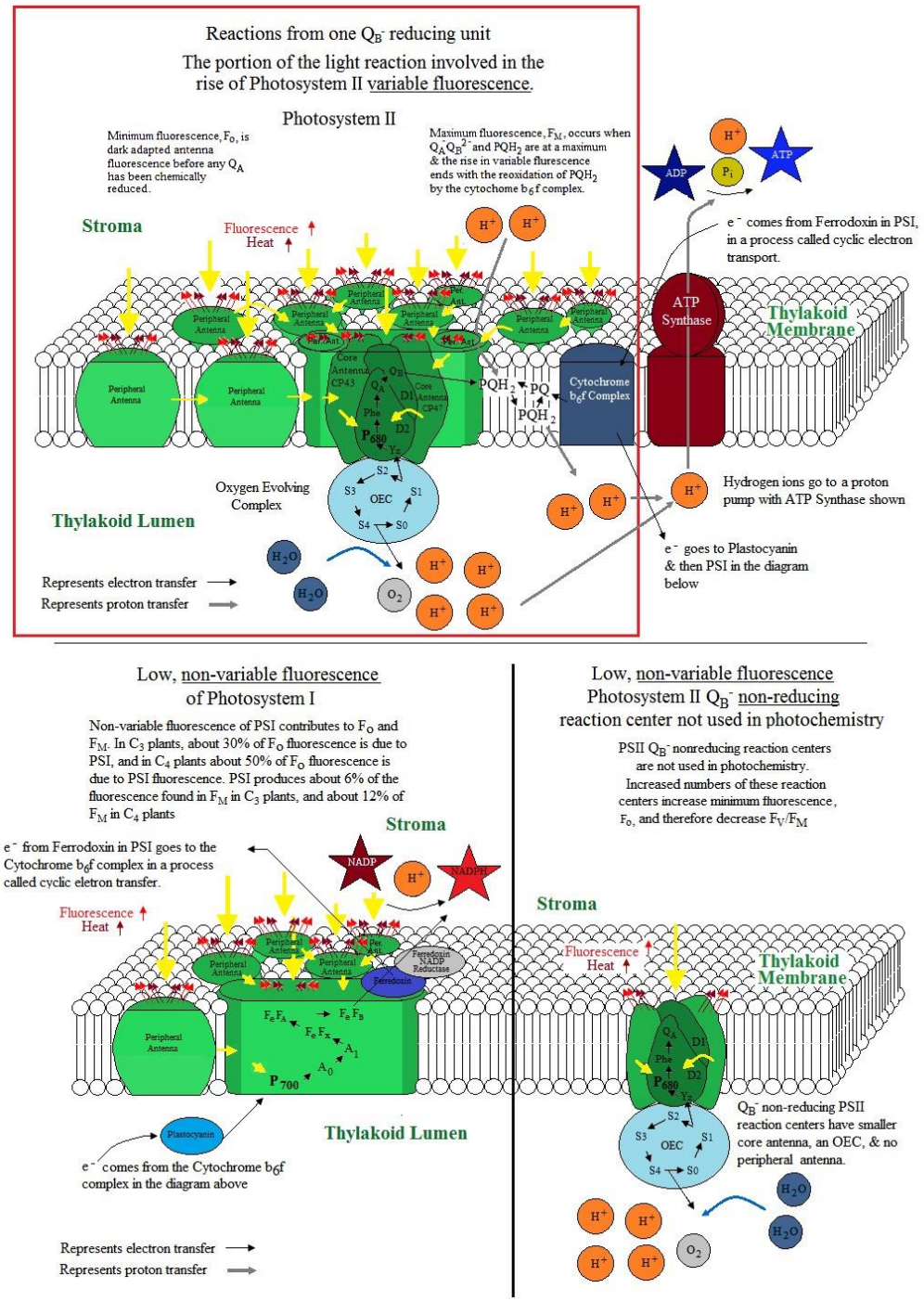

Drill down diagram of variable and non-variable chlorophyll fluorescence
C3 Plant Leaf
Cuticle
Upper Epidermis
Palisade Mesophyll Cells
Spongy Mesophyll Cells
Vascular Bundle
Intercellular air chamber
Lower Epidermis
Stoma
C4 Plant Leaf
Cuticle
Vein
Upper Epidermis
Mesophyll Cells
Bundle Sheath Cells
Lower Epidermis
Stoma
Intercellular air chamber
Mesophyll Cell
Nucleus
Vacuole
Chloroplast
Bundle Sheath Cells also have chloroplasts in C4 plants
Chloroplast
Outer Membrane
Inner Membrane
Stroma
Stroma Lamellae
Granum
Granum
Stroma Lamellae
Stroma
Thylakoid Lumen
Thylakoid
Thylakoid Membrane
Cazzaniga et al., (2013) & Dall'Osta (2014) show that the intermediate chlorophyll fluorescence change thought to be caused by state transitions is due to chloroplast migration in C3 plants. The chloroplast migration mechanism also occurs in C4 plants (Maai 2011).
PSII rich membrane - PSII supercomplex formations, found in stacked thylakoid membrane areas, can be found in a disorganized liquid form, an organized crystal form, or in intermediate forms.
Thylakoid membrane from Lumen side in liquid phase
LHCII - Light Harvesting Complex for PSII
View from Lumen side
Thylakoid Lumen
Side view
PSII - RC
LHCII
Ant.
Stroma
Stacked Thylakoid membranes interact.
Thylakoid Lumen
Thylakoid membranes are lipid.
PSII reaction center
Strongly bound LHCII antenna
Protien
Strongly bound LHCII antenna
Medium bound LHCII antenna
Unbound LHCII antenna
In State transitions, LHCII antenna become phosphorylated, disassociate, and migrate to stroma lamella or marginal regions where they attach to PSI reaction centers. The mechanisms are under debate.
Rough shape model of a PSII reaction center with strongly bound LHCII antenna
Thylakoid membrane from Lumen side in crystal phase
Shape models and descriptions were adapted from:
Schneider A.R., Geissler P.L. (2013) Coexistence between fluid and crystalline phases of proteins in photosynthetic membranes arXiv:1302.6323v1 [cond-mat.soft] 26 Feb 2013

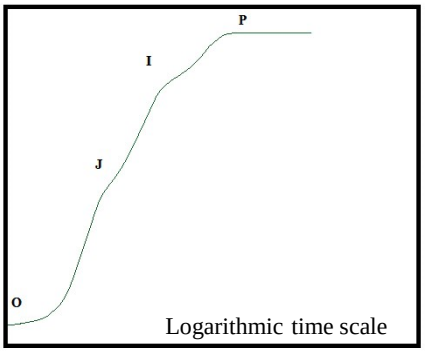
Diagram C

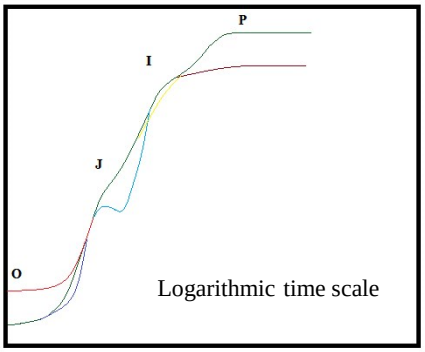
A drill down diagram of the mechanisms that affect variable chlorophyll fluorescence. It designed as a more visual representation of the most accepted understanding of the sources of chlorophyll fluorescence and variable chlorophyll fluorescence. Chlorophyll fluorescence generating sources for Photosystem II were taken from Zhu 2005, & 2012. Photosystem I information was taken from Schreiber (2004).



OJIP and F_V/F_M - Understanding the fluorescence rise and steps

If the rise in chlorophyll fluorescence is examined with high speed time resolution in the range of microseconds and milliseconds, specific steps appear during the rise. The following descriptions represent a synopsis of information available from a paper written by Xin-Guang Zhu, Govindjee, Neil R. Baker, Eric deSturler, Donald R. Ort, and Stephen P. Long in 2005. The information was reaffirmed in a second paper in 2012 by Xin-Guang Zhu,Yu Wang, Donald R. Ort, and Stephen P. Long. These are some of most respected names in chlorophyll fluorescence and photosynthesis. While there is still some debate regarding some of the details of the OJIP fluorescence rise, this approach is the one that is most accepted.

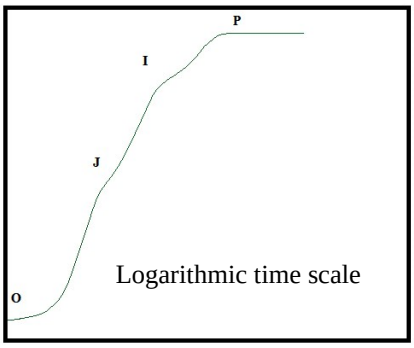


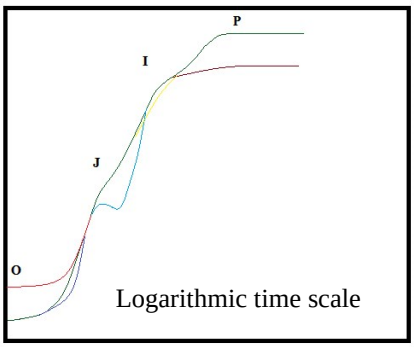


'O' is commonly measured at 20 µsecs after the start of actinic illumination in continuous fluorometers and is not equal to F_O measured by modulated fluorometers. Continuous fluorometers use linear regression analysis to estimate F_O, or minimum fluorescence in a dark adapted state, before any Q_A has been chemically reduced.
The height of both O and F_O are affected by the ratio of the size of peripheral antenna to core antenna. A larger ratio of peripheral antenna causes O and F_O to be lower. Both O and F_O are affected by the ratio of Q_B^- non-reducing reaction centres to Q_B^- reducing reaction centres. As the ratio of Q_B^- non-reducing reaction centres increases, both O and F_O values increase. This is shown in the lower diagram. The green line represents a sample with significantly fewer Q_B^- non-reducing reaction centres, and the red line represents a much higher percentage of Q_B^- non-reducing reaction centres.

O to J – The slope of the rise in fluorescence is affected by the probability that excitation energy will migrate from a closed Q_B^- reducing reaction centre to an open Q_B^- reducing reaction centre. Higher probability delays the rise as shown in the lower diagram in dark blue. Q_B^- non-reducing reaction centres do not transfer energy to open Q_B^- reducing reaction centres. Energy absorbed by these reaction centres is converted to heat and fluorescence only. They are not involved in photochemistry. The O-J rise represents the photochemical reduction of pheophytin and Q_A. J represents maximum values for $Q_AQ_B^-$ and $Q_A^-Q_B^-$. J becomes more defined and lower if the dark adapted Oxygen Evolving Complex ratio of the specific states of S_1 to S_0 move from 1:0 to 0:1. The dip after J becomes more defined with a higher S_0 value. It provides a greater P_{680}^+ concentration that is a strong fluorescence quencher. This dip is shown in the lower diagram in light blue. A separate new step called the K step can appear at 300 µsecs. It only appears at

high light levels (Vredenberg 2004), when there is severe nitrogen, iron, or sulfur deficiency (Strasser 2004). The Zhu 2005 paper shows that the timing of J is somewhat variable. However, in the Strasser JIP protocol, used for plant stress measurement, it is fixed at 2 msec.





J to I - This rise represents the photochemical reduction of Q_B . "I" represents the first shoulder in the $Q_AQ_B^{2-}$ chemical equation that ends at P with a maximum for $Q_A^- Q_B^{2-}$. If properly dark adapted, the J to I rise starts with the ratio of Q_B: Q_B^- = 1:0 and ends with the ratio at 0:1.The dark adapted ratio prior to light exposure of Q_B: Q_B^- affects the slope and height of "I" as shown in the lower diagram by the yellow line.

Again, the Zhu group shows that the time to reach I is also somewhat variable. However, in the Strasser JIP protocol, used for plant stress measurement, it is fixed at 30 msec.

P = F_M or Maximum variable chlorophyll fluorescence. This value represents a maximum for chemical values of $Q_A^-Q_B^{2-}$, & PQH_2. The rise in fluorescence ends with the cytochrome b_6f complex re-oxidizing PQH_2 to PQ. The height and slope of the rise to P or F_M are affected by the reoxidation rate constant of PQ, k_{ox} and by the size of the plastoquinone pool.

A higher rate constant and a larger PQ pool reduce the value for P. The time to reach P is variable in the Zhu paper and in the Strasser JIP protocol. A high rate constant reduces the time to reach P, and a larger PQ pool extends the time to reach P. The time to reach P is reported in the Strasser protocol. A larger PQ pool is shown in the lower diagram in dark red crimson. In 2004, Wim Vredenberg discovered that the OJIP graph changes dramatically at different actinic light levels. In fact, the K step only appears under very high light levels and under specific severe plant stress conditions. For this reason and to ensure comparable results, it is common to calibrate the light source of OJIP instruments. The OS30p+ provides automatic actinic light calibration when the instrument is turned on (Vredenberg 2004).

The quality of light can also be a factor. It has been found that red actinic light penetrates the entire leaf, while blue light does not. For this reason, it has been common to compare work done using the same type of saturating actinic light sources. The OS30p+ offers a red calibrated light source with intensities that may be set at 3,500 µmol for the Strasser protocol and up to 6,000 µmol for other work. Various light sources are used for measurement of F_V/F_M. Industry options include: red, red and blue, white light halogen, white LED, and Xenon light sources. A paper comparing xenon and red saturating light sources provided results that correlated well with slightly lower values using a red light source (Cessna 2010). The paper found poorer correlation with blue saturating light. For longer measuring protocols used in quenching measurements, light curves, or for

extended pre-illumination of shorter light adapted tests, white actinic light sources have advantages. The apertures of plant stoma are mediated by blue actinic light (Kinoshita 2001). In addition, chloroplast migration can be responsible for up to 30% of non-photochemical quenching at high actinic light levels at steady state photosynthesis. Chloroplast migration only occurs when white actinic light is used or actinic light sources with an intense blue spectrum; there is no significant chloroplast migration with intense red light. Chloroplast migration also changes leaf absorptance (Cazzaniga *et al*., 2013).

Photochemical and Non-photochemical quenching

After proper dark adaptation to a known state, a leaf is exposed to a photosystem saturating light. Initially, a maximum amount of the saturating light absorbed by the leaf and used in F_V/F_M goes to variable chlorophyll fluorescence. Smaller amounts go to unregulated heat dissipation and photochemistry. The same is true when using a saturating actinic light in OJIP protocols for the initial rise of fluorescence.

There are other mechanisms, slower reacting, that affect variable chlorophyll fluorescence. After dark adaptation and the initial rise in chlorophyll fluorescence, these mechanisms begin to respond. Depending on the type of plant, peak fluorescence is maintained from 0.5 seconds to 1.5 seconds in land plants (Schreiber 1995) and from 25 milliseconds to 50 milliseconds in algae (Schreiber 1995). The fluorescence output then begins to drop due to the initiation of photosynthesis (whereby more light is used in photochemistry), in a process called photochemical quenching. This, together with photo-protective mechanisms, begin to adapt to existing, actinic light levels.

The xanthophyll cycle and the ΔpH of the thylakoid lumen convert absorbed light into regulated heat dissipation, in a form of non-photochemical quenching considered as a photoprotective mechanism. At higher light levels, there is more NPQ. This process takes from several seconds to minutes in greenhouse plants (Lichtenthaler 2004), but can take up to 7 minutes in field plants (Baker 2008). q_E is a parameter that is used to measure the NPQ photoprotective mechanisms and is used in conjunction with quenching relaxation protocols (Muller 2001). q_L from the Lake Model and q_P from the Puddle Model are parameters designed to measure photochemical quenching or open PSII reaction centres.

There are still other, slower acting mechanisms that continue to lower fluorescence output after the initial fluorescence rise. Some prominent researchers see evidence that these intermediate but slower mechanisms that are part of an NPQ measurement, may not be the same in all photosynthetic organisms. There could be a relationship between the phosphorylation found in state transitions and NPQ regulation found in some monocots (corn, barley and rice). There is also some strong evidence that q_T fluorescence, from state transitions, exists in the green algae *Chlamydomonas reinhardtii* (Depège N., Bellafiore S., Rochaix J-D., 2003). The relevance of state transitions measured as q_T fluorescence is highly questionable for a number of land plants including Arabidopsis. The evidence shows that changes previously reported as q_T in quenching relaxation tests are not due to state transitions at higher light levels or saturating actinic light intensities. The latest evidence points to chloroplast migration and the resulting reduced leaf photon absorption as the source of fluorescence change during light adaptation and during quenching relaxation, in dicot land plants at the very least (Cazzaniga *et al*., 2013).

Question: What causes the non-photochemical quenching (NPQ) fluorescence relaxation change that is greater than a few minutes and less than thirty five minutes?

Discussion: In 2010 (Nikens) it was found that q_T did not exist at near saturation light conditions. It was though that it must be an unknown slower acting mechanism related to zeaxanthin, and it was named q_Z (Nilkens 2010). Since that time, there has been new evidence. Under higher light levels and near saturating light conditions, the latest data supports chloroplast migration as the source of fluorescence change and quenching relaxation. This research also affects the type of actinic light sources that should be used to measure most light adapted parameters, quenching measurements, and under high light conditions, the times recommended for dark adaptation, and the time required to reach steady state photosynthesis under high light conditions (Cazzaniga *et al*., 2013).

Until recently, it was believed that as a plant went from a dark adapted state to a high light level or from a high light adapted state to a dark adapted state, there were three basic mechanisms involved in chlorophyll fluorescence measurement of non-photochemical quenching (NPQ) ; q_E, q_T, and q_I. q_E can be described as a rapid photo-protective adjustment of photosystem II caused by ΔpH of the thylakoid lumen and the xanthophyll cycle.

It can take q_E several seconds to minutes to adjust, and it tends to be longer in field plants (Baker 2008, Murchie 2011, Nilkens 2010). Traditional q_T was thought to be caused by state transitions. q_T could take up to fifteen or twenty minutes has been described as a fluorescence change that overlapped somewhat with q_E. Changes that took longer were related to q_I or photoinhibition (Ruban 2009). There is now significant evidence to show that the fluorescence change measured as q_T in quenching relaxation measurements is likely due to other chloroplast migration at least under high actinic light conditions in many land plants (Cazzaniga *et al*., 2013).

State transitions – a classical view:

According to classical state transition theory, state transitions are thought to be a low light level survival mechanism that allows balancing of light between photosystem II (PSII) and photosystem I (PSI). It was believed that LHCII antenna trimers, or peripheral phosphorylated light harvesting complex II antenna, migrated from PSII complexes to PSI complexes. The movement would occur from one thylakoid membrane to another when they were very close or adjacent to one another. The movement and reaction took place on the stroma side of the thylakoid membrane allowing the LHCII antenna to serve as a PSI antenna. When dephosphorylated, the LHCII antenna favoured movement back to PSII. The result was that at low light levels, movement was favoured to PSI. LHCII phosphorylation was a prerequisite for dynamic regulation of relative balance of PSI/PSII excitation under artificially induced state transitions with different qualities of light. However, this process has not been viewed, without reservation, in PSII rich thylakoid membranes.

State transitions – a more recent view:

A more recent view of state transitions is as follows: There is no clear evidence to support the actual movement of LHCII (light harvesting complex II antenna) in stroma-exposed PSII rich thylakoid membranes, to PSI reaction centres. LHCII phosphorylation does not collect light energy for PSI in these cases (Tikkanen 2012). Only in the margins of the grana thylakoid membrane do LHCII antenna behave according to the traditional view of phosphorylation-induced state transition (Tikkanen 2008). Tikkanen also states that there is substantial evidence to show that the classical mechanism of state transitions is not the

sole method for energy balance between the two different photosystem types. There is also evidence to show that LHCII phosphorylation probably connects the regulation of light balance between PSII (photosystem II) and PSI (photosystem I) through unknown non-photochemical quenching mechanisms (Tikkanen 2012), which work not only with PSII but also PSI (Tikkanen 2010). It was also noticed that when light intensity is increased, the PsbS protein is protonated, turning the LHCII antenna into a dissipative state for PSII. (Li 2004, Tikkannen 2012). At lower light levels, LHCII activity is restored, and PSII activity is increased. Furthermore, phosphorylation is controlled by the enzymes STN7 and STN8 kinases and their opposing phosphatase; that are in turn closely controlled by light intensity. These kinase functions are completely synchronized with PsbS and the xanthophyll cycle (Tikkanen 2012).

A previous contender - q_Z - due to an unknown longer term xanthophyll cycle mechanism

In 2010, Nilkens and others used NPQ Arabidopsis mutants to determine that under saturation light conditions, q_T or state transitions did not significantly contribute to fluorescence change and resulting quenching relaxation in the dark. Furthermore, samples were tested at moderate illumination to rule out q_I or photoinhibition as the contributor to this fluorescence change. The changes related to q_Z were completed in under 30 minutes. It was proposed that under steady state, saturating light conditions, NPQ should be divided into q_E, q_Z, and q_I.

As described by others, q_E is a process that is created and relaxes in the 10 to 200 second time frame and is dependent on Δph of the thylakoid lumen, the PsbS protein and zeaxanthin formation. The longer portion of q_E from 100 seconds to about 200 seconds is limited by zeaxanthin synthesis. Relaxation of q_E is regulate the Δph of the thylakoid lumen.

According to the Nilkens group, the proposed q_Z is created in the 10 to 30 minute time frame. PsbS is not involved in q_Z, but is wholly dependent on zeaxanthin formation. Relaxation depends on the reconversion of zeaxanthin to violaxanthin. It should be stated that the group found a sample that seemed to contradict the other q_Z results; however, they thought this was likely due to a retarded relaxation of a large fraction of q_E and not normal middle range fluorescence relaxation characteristics. The test plant was zeaxanthin devoid mutant npq1. Photoinhibition, q_I, was shown to form after 30 minutes and was dependant on illumination time, intensity and genotype. It was also found the state transitions, q_T, were not a significant contributor to NPQ at saturating light intensity.

q_M – due to chloroplast migration

Cazzaniga S, Osto L.D., Kong S-G., Wada M., Bassi R., (2013) used multiple methods, Arabidopsis mutants and wild type Arabidopsis plants to determine that the fluorescence change (previously thought to be the result of state transitions, or more recently, a longer lasting xanthophyll cycle process) was caused by chloroplast migration. They found, as others have, that under high light levels, chloroplasts move from the top of cells to the

sides of cells, partially shading other chloroplasts. This was verified with light microscopy using samples treated to prevent migration after high light migration. They also found that leaf transmittance increased and therefore leaf absorptance was lowered with chloroplast migration. The research concludes that the cause of q_M is a decrease in light photon absorption which creates lower fluorescence yield, rather than a true quenching process. This is thought to be another avoidance process to protect leaves from high light levels. They found that the time scales for adjustment and fluorescence intensity change mirrored the previously used q_T and acute photoinhibition, extending up to 30 minutes with some plants and up to 35 minutes in some mutants. Chloroplast migration has been known and studied for a while, and it was stated by Brugnoli in 1992 that chloroplast migration affected chlorophyll fluorescence. The Cazzaniga *et al*., paper is the first to name chloroplast migration as the source of the q_T and q_Z fluorescence change along with the change thought to be acute photoinhibition as defined by Theile (1998) & Lichtenthaler (2004).

Researchers found that high white actinic light was much more effective than high red actinic light at inducing the photo-protective functions of q_M. Chloroplast migration was shown to be controlled by high blue light intensity (Kagawa, T. 2001). Several Arabidopsis mutants; some devoid of q_E (npq4), some devoid of q_E and chloroplast migration (npq4 photo2) were tested. In addition, targeted reverse genetic analysis was used to eliminate other contending possibilities by creating a series of double and triple mutants that covered the remaining spectrum of mechanisms that affected chlorophyll fluorescence, including; the composition of various parts the photosynthetic apparatus, carotenoid biosynthesis, and state transitions as sources of q_M and acute photoinhibition. In regard to q_T being replaced by q_Z, it was found that by using mutants devoid of q_E and zeaxanthin, the magnitude of q_M did not change but the recovery time in the dark was lengthened. Plants were grown at 150 μmol photons m^{-2} s^{-1}, and tested at each of 400, 800 and 1,200 μmol photons m^{-2} s^{-1}. The adjustment time for q_M ranged up to 35 minutes for some mutants.

The Cazzaniga S, Osto L.D., Kong S-G., Wada M., Bassi R., (2013) observation that the stn7 mutant , devoid of state transitions, has NPQ measurements very similar to the wild type in Arabidopsis, casts strong reservations in regard to the relevance of q_T in Arabidopsis.

The fact that higher intensity white or high intensity blue actinic light is required to activate q_M, indicates the need for either a white light source or a high intensity blue light and red light, rather than using high intensity red and lower intensity blue actinic light (Cazzaniga *et al*., 2013). This new research shows that significant measuring artefacts are possible when using a low intensity blue light source for chlorophyll fluorescence measurement. ETR or J, Y(II) or F_{PSII}, NPQ, g_M, C_C and q_I may all include measuring errors without a reliable light source. Furthermore, all gas exchange measurements contain an error if blue light intensity is not high enough to allow q_M to occur for a specific high light level.

This may also change the times required for proper dark adaptation measurements and the time to reach steady state photosynthesis under light adapted conditions. Until now, Maxwell & Johnson (2000) has been the most sighted paper for reliable steady state photosynthesis conditions at any given light level. It lists 15 to 20 minutes as the time

required for 20 wild land plants to reach steady state photosynthesis. Prominent researchers, Lichtenthaler (1999) and Ruban (2009), list the dark adaptation time required for quenching relaxation of q_T at the same 15 to 20 minutes. With this new evidence from Cazzaniga *et al.* (2013), it is apparent that dark adaption times and time taken to reach steady state lighting conditions should be extended (certainly at higher light levels). Cazzaniga *et al*. (2013) show that it takes from 20 to 30 minutes for chloroplast migration to adapt to higher light levels and for relaxation in the dark. Current research will likely provide some additional surprises in this area, moving forward.

Photoinhibition

Photoinhibition is a process that can occur at high light levels for extended periods of time. It was thought that the effects of high light levels for shorter periods of time, an hour or two, was a form of acute photoinhibition could be reversed with 20 to 30 minutes of dark adaption (Theile, Krause & Winter, 1998). *Recent research indicates that this is more likely due to chloroplast migration due to the relaxation times tested* (Cazzaniga *et al*., 2013). Chronic photo-inhibition, caused by several hours of high light exposure, starts to relax or repair at about 40 minutes and may take 30 to 60 hours to fully relax or repair under dark adaptation (Lichtenthaler & Babani, 2004), (Theile, Krause & Winter 1998)).

When making longer quenching and quenching relaxation measurements related to photo-inhibition and photo-damage mechanisms (common in chronic high light stress, high heat stress, cold stress and over-wintering stress), one should understand that it can take days for full relaxation or repair of the non-photochemical quenching parameters, $\mathbf{q_I}$, to pre-stress conditions. To get an accurate control value for Fm and Fo under chronic photoinhibition conditions (components of non-photochemical quenching parameters) it is common to dark-adapt for a full night, or 24 hours (Maxwell & Johnson 2000).
To study photoinhibition, one could partially shade samples for a few days before testing, to ensure that all residual photoinhibition has relaxed or repaired. It is expected and accepted that there is some residual unrelaxed photoinhibition or NPQ in field plants using dark-adapted samples, after bright days during summer. For this reason, it is important to compare samples with similar light history. Samples that do not have the same F_V/F_M must never be compared, because F_V/F_M is the benchmark reference in determine these values. For more information on quenching measurements and light history, refer to the corresponding OSI application notes.

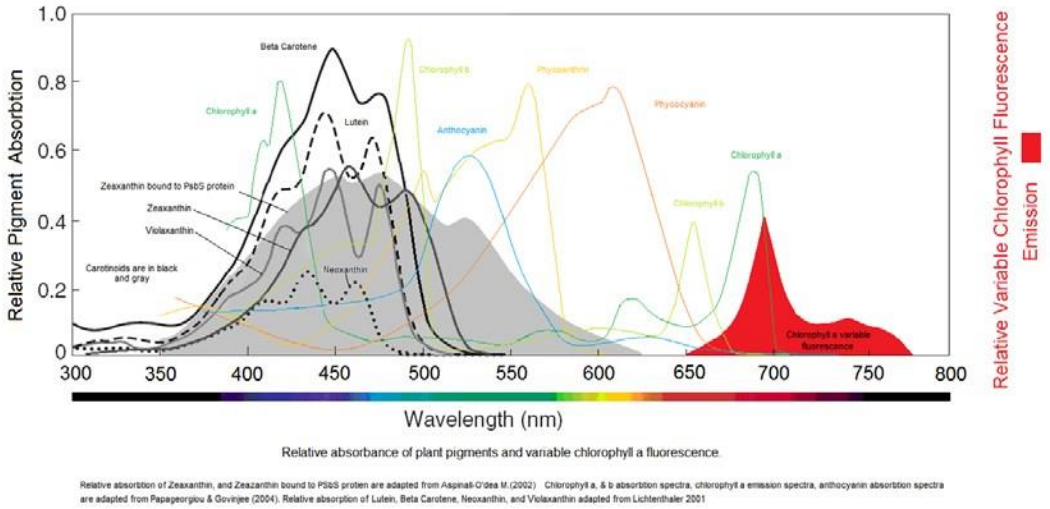
Other plant pigments

Carotenoids are present in the antenna systems and reaction cores. They include alpha and beta carotene, and xanthophylls: lutien, zeaxanthin, violaxanthin, antheraxanthin and neoxanthin. Carotenoids are involved in a number of processes including: acting as antenna in the transfer of energy to PS II and PSI reaction centres; the xanthophyll cycle is used in plant photoprotection to dissipate excess light energy, the blocking of free oxygen and organic radical, and the quenching of chlorophyll excited states as well as singlet oxygen (Gitelson, 2002).

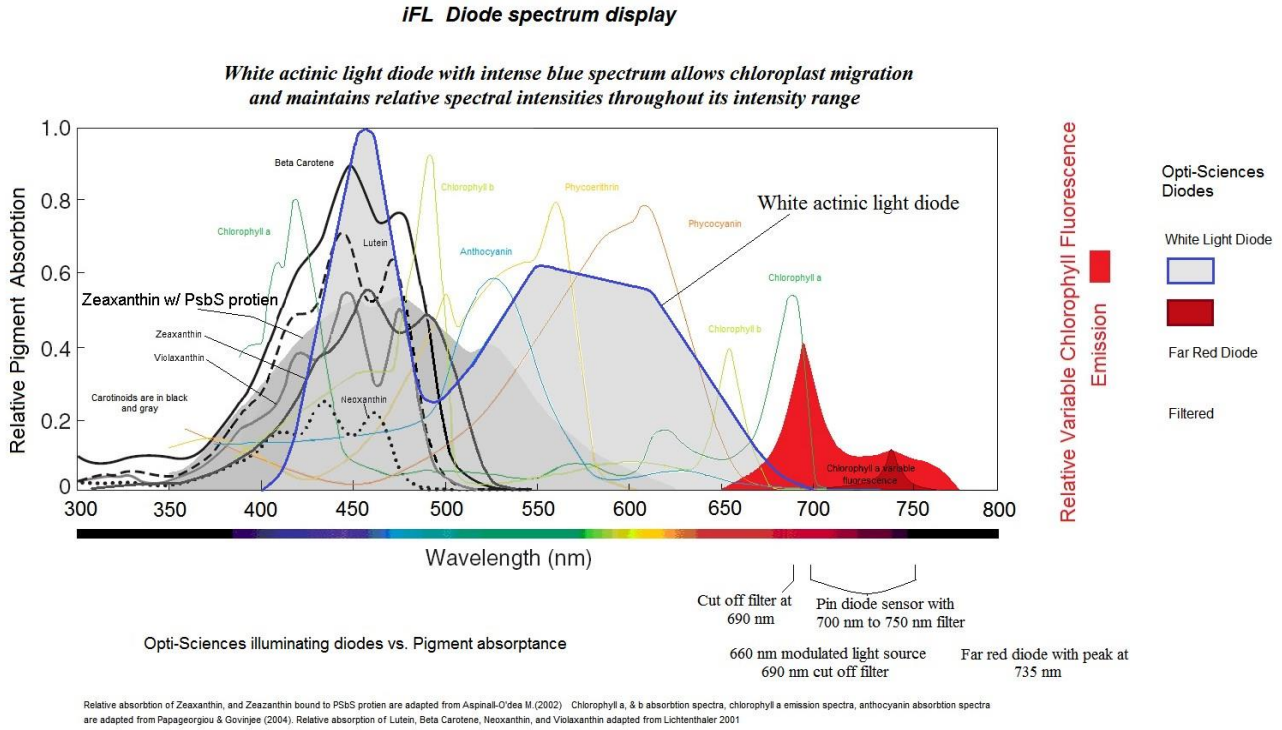
The ratio of red light leaf absorption to blue light leaf absorption changes with actinic light intensity level (Bernacchi, 2002), (Cazzaniga *et al.*, 2013). There is significant

evidence to show that violaxanthin converts to zeaxanthin as light levels increase, and that zeaxanthin works in conjunction with the protein PsbS in a photoprotective role that shifts the absorption spectrum (Aspinall-O'Dea, 2002). In addition, zeaxanthin, in stoma guard cells, has been linked to stoma aperture size and appears to be mediated by blue light (Kinoshita, 2001), (Ziegler, 1998).

The function of anthocyanins in leaves, has been the subject of debate. They have been found most commonly in cell vacuoles but may be found in all plant tissues. There is evidence to show that they function as longer term photo-protective mechanisms, help serve in the protection of shade leaves from high intensity sunflecks, help provide protection against UV-B, function in antioxidant activity, act as attractors to animals for pollen and seed distribution, or act indirectly in signalling mechanisms involved in plant growth, and development, plant stress response, and gene expression. The affects can vary from one species to the next (Gould, 2009).



Graph of plant pigment absorption, & chlorophyll fluorescence



The graphs above show the relative intensity of white light LED actinic light source at various wavelengths vs. the absorption spectrum of zeaxanthin bound to a PSII reaction centre with PsbS protein, and other plant pigments. Chloroplast migration, a mechanism responsible for up to 30% of NPQ at high actinic light levels, is modulated only by intense blue or white spectrums, not red spectrums. The blue & red diode spectrums above represent the approximate relative maximum intensities of another system on the market.

F_V/F_M: definition and background (or $F_{V/M}$ on the screen)

It is a dark adapted test - a measurement ratio that represents the maximum potential quantum efficiency of Photosystem II if all capable reaction centres were open. 0.79 to 0.83 is the approximate optimal value for most plant species with lowered values indicating plant stress (Maxwell & Johnson, 2000). Developed by Kitajima and Butler in 1975, F_V/F_M has a photochemical component and a non-photochemical component (Baker 2004). F_V/F_M is a fast test that usually takes less than two seconds. However, when using pre-illumination with far red light, the test can take several seconds [see the section on dark adaptation and our desktop plant stress guide for detailed review].

The assumptions are that before the test, PSII is fully oxidized and quenching mechanisms have relaxed to a known dark-adapted level. With the F_V/F_M test, it is important to dark adapt the sample being tested to get reliable measurements. If dark adaption is not complete, F_M will be lower than it should be, F_o will be higher than it should be and F_V/F_M will also be lowered. F_V/F_M dark adaption times can vary due to light history and the goal of the measurement. With recent research regarding q_M, relaxation of chloroplast migration can take between 20 and 35 minutes (Cazzaniga *et al*., 2013). Some scientists only work with pre-dawn dark adapted samples [for more information, see the section on dark adaptation].

Non-Photochemical Quenching normally lowers F_V/F_M when a plant is exposed to illumination. By dark adapting, one is allowing the re-oxidation of PSII and the relaxation of non-photochemical quenching. Experiments should be done on plants to be tested to find the appropriate dark adaption times for best results. If that is not possible, then 35 minutes is a safe time for samples with similar light histories, and where the measurement of photoinhibition is not the goal.

A Checklist before making reliable F_V/F_M and OJIP measurements

Accuracy is the ability to hit the bull's eye. In many types of measurements, accuracy is determined by calibrating to a measuring a standard that is traceable to the National Agency. With such measurements, tolerances are always involved.

Repeatability is the ability to achieve the same measurement again and again to a certain tolerance level.

A Reliable measurement is one that is accurate and repeatable.

With Chlorophyll fluorometers, accuracy is determined by following recommended methods and understanding the limitation of the measurement.

F_V/F_M & OJIP

The biggest advantage of F_V/F_M is that it is a measure of PSII performance that puts all samples in the same known dark adapted state before measurement. F_V/F_M is a normalized ratio that does not use a traceable standard. Instead, its accuracy is determined by properly using the instrument and following the lessons learned by several great plant physiologists. For most species, the optimal F_V/F_M reading for stress free plants is in the range of 0.79 to 0.83 (Maxwell & Johnson, 2004). It is important to know that the checklist for OJIP is the same as for F_V/F_M. The only difference is light intensity. As long a saturation light source is high enough to saturate samples it will work just fine for F_V/F_M. *However, with OJIP, the intermediate fluorescence values for K, J, and I, along with parameters that use these values in their calculation, can change with light intensity (Vredenberg 2011). For that reason, it is important to use the same intensity every time. In the past, 3,000 µmol was used in the Strasser OJIP protocol, more recently 3,500 µmol is used. The OS5p+ comes with the actinic light set at 3,500 µmol; however, it may be set up to 5,800 µmol.*

To get a reliable measurement, one has to follow tested guidelines:

1. Dark-adapt properly, knowing the plant's light history. It takes only a few minutes for the xanthophyll cycle and the ∆ph of the thylakoid lumen to return to a dark-adapted state. State transitions however, take between 15 to 20 minutes (Ruban 2009), (Lichtenthaler 1999). These times can vary in field plants and can take slightly longer. At high light levels found in the field, chloroplast migration takes between 20 and 35 minutes to fully relax (Cazzaniga *et al.*, 2013). Deactivation of Rubisco in the dark, takes between 12 and 18 minutes in vascular plants and 9 to 28 minutes in some phytoplankton (MacIntyre 1997). In addition, field plants and plants that have been exposed to photoinhibition conditions for a number of hours, will retain a certain amount of NPQ for up to 60 hours (Lichtenthaler 2004). This means that even if dark adaptation is overnight, there will almost always be some residual NPQ built into most summer field measurements of Fv/Fm. This is alright as long as samples with similar light history are compared. Light history should always be taken into account when comparing samples. It is common for researchers to choose dark adaptation times

anywhere from 20 minutes to overnight (using pre-dawn values). Shorter times may be used to study the effects of plant mechanisms. For more information contact OSI for the 'dark adaptation application note' (these guidelines are different for quenching measurements and for Rapid Light Curves).

2. Modulation light intensity setting
   $F_V/F_M = (F_M-F_O)/F_M$.
   Minimum fluorescence or F_O, is a dark adapted value measured by exposing the leaf antennae to a very low intensity modulated light before any Q_A has been reduced by an actinic light source. The intensity must be set properly to allow detection, but not high enough to drive any photochemical reduction of Q_A. If it is set too high, it will drive photochemical reduction of Q_A and provide an F_O value that is too high. When setting the modulating light intensity, the Ft value or fluorescence signal should not rise over a 15 to 20 second period when a leaf is used. If it does, the intensity must be lowered. *OSI now offers an automated modulated light set up routine for their new OS5p+.*

3. Shade leaves vs. Sun leaves. The F_V/F_M ratio will be slightly higher on sun leaves than on shade leaves (Lichtenthaler 2004).

4. F_V/F_M will be higher with a white saturation pulse than a red saturation pulse. Some fluorometers use a red saturation pulse (Cessna, 2010). This is not an issue for comparative measurements of plant stress with similar instruments, but values measured on a fluorometer with a white saturation pulse should not be directly compared to measurements of a fluorometer with a red saturation pulse. There is evidence to show that systems with a red saturation pulse correlate but measure consistently lower than systems with white light saturation lights (Cessna, 2010).

5. Maximum F_V/F_M values vary with species. The average maximum F_V/F_M value is between
   0.79 - 0.83 (Maxwell & Johnson, 2000).

6. Compare samples with a similar light history. Field plants should only be compared to field plants with a similar light history and green house plants should be compared to green houseplants with a similar light history. Due to the fact that it can take up to 60 hours for chronic photoinhibition to relax, photoinhibition can be involved in some measurements more than others (Lichtenthaler 2004).

7. It is common to use the youngest fully mature leaf blade for diagnosis of deficiencies in plants (Reuter & Robinson 1997).

8. To reduce error in F_V/F_M measurements, the duration of the saturation pulse should be between 0.5 and 1.5 seconds for higher plants, and 25 to 50 milliseconds for Phytoplankton and cyanobacteria (Schreiber, 1995). Shorter durations prevent complete saturation of PSII regardless of the light intensity. Longer durations create a form of saturation pulse NPQ that rounds the tail end of the pulse maximum value, and reduces the average maximum saturation pulse value. Opti-Sciences provides a moving 25 ms average to determine the highest F_M. This ensures that a reliable value will be measured even if the saturation pulse width or duration is too long.

9. Saturation pulse intensity. Dark adapted leaves saturate easily with lower saturation pulse intensities. It may take a few hundred Δmol to saturate shade leaves and sun leaves will saturate below 1,500 Δmol. Lower values may not fully saturate PSII, and provide an error. Higher values always work with dark adapted samples (Ralph 2005). Requirements are different for Y(II).

10. Some F_V/F_M fluorometers have the ability to pre-illuminate dark adapted leaves with far-red light such as the i*FL*. When this feature is used for five to ten seconds before an F_V/F_M measurement takes place, it activates PSI, and ensures that all electrons have been drained from PSII before the measurement of F_O. While this feature ensures that PSII is completely re-oxidized, it does not relax the xanthophyll cycle, chloroplast migrations, state transitions, or photoinhibition. Time is still required in a darkened environment to relax all forms of NPQ and to obtain a reliable F_V/F_M measurement (Maxwell & Johnson, 2000), (Cazzaniga *et al*., 2013).

11. Fluorescence heterogeneity, or patchy leaf fluorescence, presents itself as different F_V/F_M measurements on different parts of the leaf. It has been found to occur under cold stress conditions, with biotic stress, and under drought stress conditions (Baker 2008). The iFL averaged chlorophyll fluorescence measurements over the same large area as the gas exchange measurement preventing heterogeneous fluorescence from being a problem.

12. Part of the minimum fluorescence, the F_O parameter, in F_V/F_M $(F_M – F_O)/F_M$), contains PSI fluorescence as well as PSII fluorescence. With F_V/F_M, one is trying to measure the maximum variable fluorescence of PSII in a dark-adapted state. PSI fluorescence is not variable, but the low fluorescent signal from PSI does overlap with PSII. This produces an error. In C_3 plants, about 30% of F_O fluorescence is due to PSI, and in C_4 plants about 50% of F_O fluorescence is due to PSI fluorescence. PSI produces about 6% of the fluorescence found in Fm in C_3 plants, and about 12% of Fm in C_4 plants (Pfundle, 1998). This not a problem when comparing F_V/F_M measurements for plant stress because PSI fluorescence does not change. It remains constant and F_V/F_M is a normalized ratio.

There are fluorescence solutions and assays available that are sensitive to most types of plant stress. F_V/F_M is not as sensitive as Y(II) for many types of plant stress. F_V/ F_M is not a sensitive test for drought stress, heat stress, nitrogen stress, nickel stress, sulfur stress, zinc stress, some herbicides and salt stress in some types of plants (Opti-Sciences Plant Stress Guide 2010). It can be used effectively in most other types of plant stress. For specific research results on specific types of plant stress, see the Plant Stress Guide offered by Opti-Sciences Inc.

Y(II) -Quantum yield of PSII

An in depth discussion of the value and limitations. Y(II) or $_{\Delta}F/F_M$' or F_M' – Fs / F_M') is a time tested light adapted, normalized ratio parameter that is more sensitive to more types of plant stress than F_V/F_M according to a survey of existing research (see the OSI Desk Top Plant Stress Guide). While F_V/F_M is an excellent way to test for stress and the health of Photosystem II, Quantum Photochemical Yield is a test that allows the measurement of the efficiency of the overall process under actual environmental and physiological conditions.

Quantum Yield of PSII is a measurement ratio that represents achieved efficiency of photosystem II under current steady-state photosynthetic lighting conditions (Genty 1989), (Maxwell & Johnson, 2000), (Rascher 2000). It is affected by closure of reaction centres and heat dissipation caused by nonphotochemical quenching (Schreiber 2004). It is also affected by chloroplast migration (Cazzaniga *et al.*, 2013), (Dall'Osta 2014). Y(II) was developed by Bernard Genty in 1989.

As ambient light irradiates a leaf, an average of about 84% of the light is absorbed by the leaf and an average of 50% of that light is absorbed by the antennae associated with PSII and transferred to PSII (Photosystem II) reaction centres. Leaf Absorptance can range from 70% to 90% (Eichelman H., 2004) and PSII absorption can range from 40% to 64% (Edwards, 1993), (Laisk, 1996). Leaf absorptance changes with *plant stress, leaf age, chlorophyll content, species and light level* (Cazzaniga *et al*., 2013). The i*FL* is designed to take a leaf absorptance measurement with every Y(II) measurement. It measures leaf reflectance in the visible spectrum and leaf transmittance as well in the process. At higher light levels, chloroplast migration changes leaf light transmission in C_3 plants (Cazzaniga *et al*., 2013), (Dall'Osta, 2014) and C_4 plants (Maai, 2011). As a result, estimating leaf absorptance, instead of measuring leaf absorptance, can add a significant error to all integrated measuring parameters including *J*, g_m, and C_C. Under normal non-stressed conditions, most light energy is channelled into photochemistry with smaller amounts of energy channelled into heat and fluorescence. In photosystem II, this process is competitive so that as plant stress occurs, mechanisms that dissipate heat, photo-protect the leaf, and balance light between photosystem II and photosystem I, change the output of fluorescence and heat. In other words, conditions that maximize photochemistry minimize fluorescence and heat dissipation and conditions that maximize fluorescence minimize photochemistry and heat dissipation.

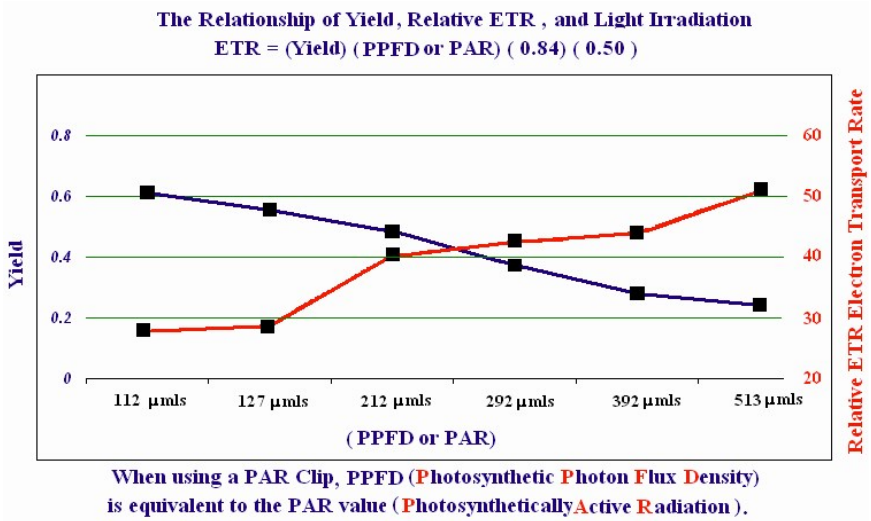
Once these mechanisms have achieved an equilibrium at a specific light level and temperature, steady state photosynthesis has been achieved, a process that was until recently, was thought to take fifteen to twenty minutes (Maxwell & Johnson, 2000). Recently, it was found that it can take 20 to 35 minutes at high light levels found under field conditions to reach steady state photosynthesis. This extended time is due to the time required by different plants to complete chloroplast migration (Cazzaniga *et al*., 2013), (Dall'Ost, 2014). At this point, a modulated fluorometer can be used to expose a plant to a very intense short light pulse called a saturation pulse. It is designed to momentarily close or chemically reduce all remaining capable PSII reaction centres, while at steady state photosynthesis. Apart from the known exceptions listed under "Correlation to Carbon Assimilation" later in this discussion, quantum photochemical yield will reflect changes in the function levels of PSII antennae, PSII reaction centres, electron transport, and regulatory feedback mechanisms.

Quantum yield of PSII is measured only at steady state photosynthesis.
F_s is the fluorescence level at steady state photosynthesis, and F_M' maximum fluorescence value measured during a saturation pulse, and is taken to mean that all PSII reaction centres are closed or photo-chemically reduced. In a high light environment, this may not be true and the Multi-flash F_M' correction method may be required. See Multi-flash for more details on pages 71-74.

Y(II) will change at different actinic light levels and temperatures so it is necessary to use a PAR sensor that measures yield relative to visible light intensity or irradiation level and temperature. PAR sensors measure Photosynthetically Active Radiation between the wavelengths of 400 nm and 700nm. When the dimensions per square meter per second in micro-mols or micro-Einstein's are added, this parameter becomes Photosynthetic Photon Flux Density or PPFD (µmol and μ_E are equivalent, and when using a PAR sensor, PAR and PPFD are equivalent).

PAR sensors also allow measurement of *J*, or relative ETR or relative Electron Transport Rate. *J* is a parameter designed to measure the electron transport rate of PSII. It has also been found to correlate well with CO_2 assimilation. Since most chlorophyll fluorescence comes from the upper leaf layers, J is a relative value that correlates with carbon assimilation and it is not absolute. Carbon assimilation also occurs at deeper layers (Schreiber, 2004).

For reliable Y(II) and *J* measurements, photosynthesis must be at steady state and with illumination on the same side of the leaf that is being measured (see number eight under correlation to carbon assimilation). Steady state photosynthesis is an equilibrium condition reached after a several minutes of exposure to existing light radiation conditions. Recent work done by Cazzaniga *et al*., (2013) indicates that under high light conditions, it can take between twenty minutes to thirty five minutes to reach steady state photosynthesis due to chloroplast migration [see the application note on q_M and its ramifications for chlorophyll fluorescence at www.optisci.com]. Measurements taken under variable lighting conditions may not provide reliable Y(II) results (Rascher, 2000). No dark adaptation is required for Y(II) measurements.



Correlation to Carbon assimilation:

In 1989, Genty developed the yield measurement and provided strong evidence of a linear correlation between Yield measurements, Electron Transport Rate, and CO_2 assimilation for C_4 plants (Baker & Oxborough, 2004) and many others have confirmed the relationship (Edwards & Baker 1993), (Krall & Edwards 1990, 1991), (Siebke, 1997). It was found that a curve-linear correlation between Yield and CO_2 assimilation exists for C_3 species where photorespiration can also use significant products of electron transport (Genty, 1990), (Harbinson, 1990), (Baker & Oxborough 2004). Psydo-cyclic electron transport and other electron sinks may also be involved.

Relative Electron Transport Rate

Relative Electron Transport Rate – *J* or ETR Δmol = (YII) (PAR) (PSII ratio to PSI) (Leaf absorptance)

J or Relative Electron Transport Rate is a parameter that is measured using the equation above. *J* is a relative measurement that provides comparative electron transport rates for PSII at different light or radiation levels and different leaf absorptance levels. It is considered relative because chlorophyll fluorescence does not correlate exactly with absolute gas exchange measurements. While most fluorescence occurs in the upper most layers of the leaf, gas exchange measures the effects of photosynthesis in deeper layers as well (Schreiber, 2004).

Even with this in mind, *J* can be extremely valuable. While exact correlation to gas exchange carbon assimilation is not possible, linear correlation is possible with C_4 plants (Genty 1989) and a curvilinear correlation is possible with C_3 plants (Genty 1990). While four electrons must be transported for every CO_2 molecule assimilated, or O_2 molecule evolved, differences from gas exchange measurements can occur under conditions that promote of photorespiration, cyclic electron transport, and nitrate reduction (Schreiber 2004), (Baker & Oxborough 2004).

The equation for Relative *J* is *J*= (Y(II) or ΔF/Fm') (leaf absorptance) (PSII / PSI) (PPFD or PAR). In this equation, Y(II) represents overall PSII yield. While other instruments use the average value 0.84 for leaf absorptance, the iFL measures the value with each fluorescence measurement. In healthy plants, leaf absorptance varies from 0.7 to 0.9 (Eichelman 2004) and it can vary with plant stress, leaf age, species or actinic light intensity (Cazzaniga *et al*., 2013). Leaf transmission increases at higher light intensities as chloroplast migration occurs, and it decreases at lower light intensities as chloroplasts move back. This process takes between 20 minutes and 25 minutes (Cazzaniga *et al.* 2013). The portion of light provided to PSII reaction centres is estimated to be 0.5 or 50%. However, if it is measured by other means, it may be input into the equation. PSII ratio varies from 0.4 in some C_4 plants to 0.60 in some C_3 plants (Edwards 1993), (Laisk 1996). PSII ratio varies by plant type (C_3 or C_4), species, sun or shade growth, and carbon deficit.

The most common way to measure *J* is destructive involving temperatures at 77^oK (Anderson 1999) (Zell 2010). The end result is a much closer approximation of *J*, g_m, and C_C can be measured and used for relative evaluation of different samples. Actinic light

irradiation is measured with a PAR sensor PPFD is PAR irradiation measured very near the leaf in μmol or μ_E (equivalent units). The iFL uses a silicon diode PAR sensor from 400 nm to 700 nm, which provides a desired relatively flat response.

The PAR Correction factor allows for PAR measurement correction of different types of light sources other than sun light, and the internal actinic light source. Under most conditions, it should be set at 1.00. It also allows for distance and location error correction capability. When using an artificial light source, the location of the PAR sensor may cause an error relative to the location of the leaf. This correction factor allows following the correction procedure in a paper by Rascher (2000).

As mentioned earlier, the relative electron transport rate provides an estimate of CO_2 assimilation. C_4 plants have been found to correlate in a linear manner with CO_2 assimilation (Genty 1989, 1990). In C_3 plants, Correlation with CO_2 assimilation is curvilinear due to photorespiration, pseudocyclic electron transport or other electron sinks (Fryer 1998),(Genty 1990). Under some forms of plant stress, such as heat stress, and water stress, this relationship and correlation can be delayed due to photorespiration and other electron sinks in C_3 plants. There is no significant photorespiration in C_4 plants.

Since J is provided by measuring absorptance and using average values for PSII reaction centre ratio the iFL provides a more accurate J, gm, and CC value than other systems. Note: Four electrons must be transported for every CO_2 molecule assimilated or O_2 molecule evolved.

Y(II) can vary significantly with light level and with temperature. Without controlling irradiation and temperature it is possible to misinterpret results. In fieldwork where both light and temperature can vary, a lower Yield measurement on one plant as compared to another could be misdiagnosed as stress, when it may only be an increase in irradiation or a change in temperature on the leaf. When a PAR Clip is used to take Yield measurements, the combination can be formidable. Only samples at similar light levels should be compared for plant stress using Y(II).

A PAR Clip is a leaf cuvette that allows the holding of the sample leaf at a repeatable angle and distance from the measuring probe while measuring Yield of PSII, ETR, PAR, and leaf temperature. These values are contained in same data file with a time and date stamp. PAR (Photosynthetically Active Radiation) is irradiated light between the wavelengths of 400 nm and 700nm and it is measured very near the sample measuring area and at the same angle as the leaf. When PAR is measured using a PAR Clip and the dimensions per square meter per second are used, the value becomes PPFD (Photosynthetic Photon Flux Density). When using a PAR Clip the terms PAR and PPFD are interchangeable.

The iFL offers the ability to pre-illuminate samples at programmable light intensity. When changing light levels and the chloroplast migration characteristics are unknown it is safe to use 35 minutes to reach steady state photosynthesis.

When in the field, the time to reach steady state photosynthesis can be substantially reduced if ambient light is desired. The iFL can be set to track ambient conditions and

closely duplicate them in the leaf chamber, or ambient values can be measured and set inside the chamber for partly cloudy conditions.

The programmed PAR value is measured by the PAR sensor every second, and the intensity is held constant though out the desired protocol, step or measurement. The light irradiation is therefore held constant using a feedback loop involving the PAR sensor.

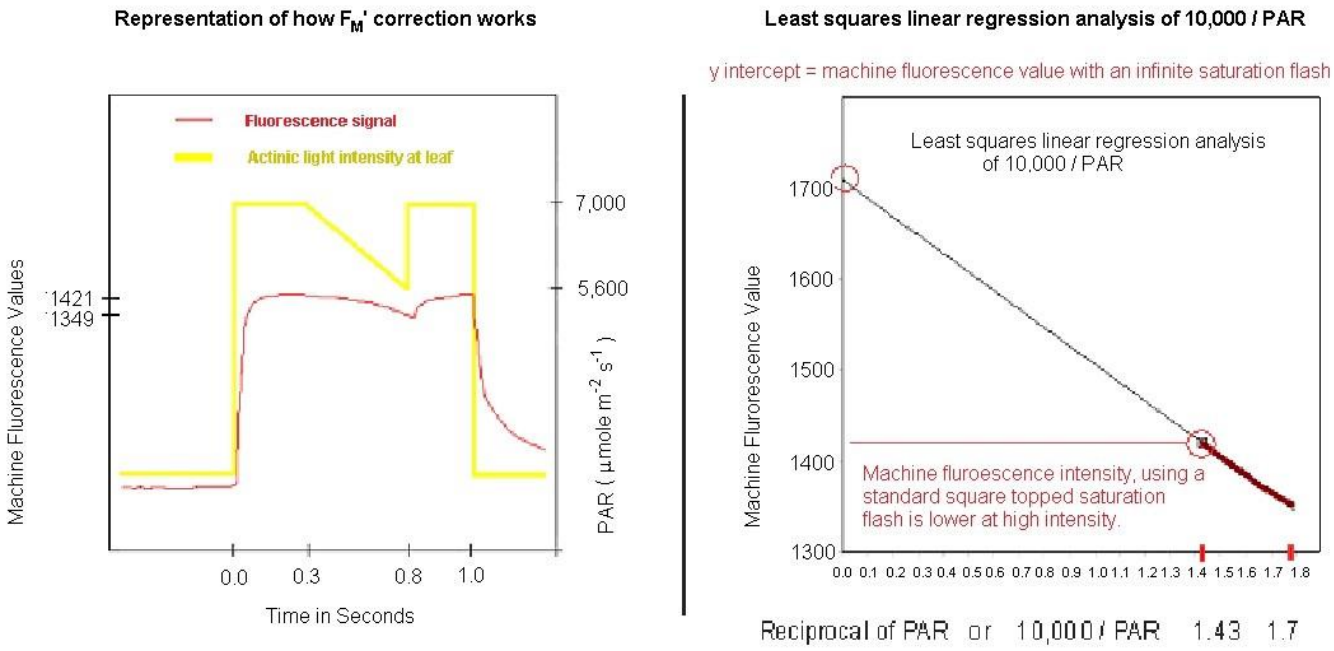
Multi-flash F_M' correction - based on Loriaux (2006) & Loriaux (2013)

Saturation pulses used with modulated fluorometers are designed to close all PSII reaction centres. The maximum fluorescence intensity value, of the saturation flash, FM', is used in most measurements including, quantum yield of PSII or Φ_{PSII} (also called Y(II) or $\Delta F'/F_M'$), *J* (or ETR), and in all quenching protocol parameters.

While it is possible to reduce or close all reaction centres in a properly dark adapted sample, with a relatively low amount of light, it has been found that in light adapted samples, with a high actinic light history, complete closure of all PSII reaction centres becomes problematic with even the highest amounts of saturation light. It is thought that complete reduction of Q_A is prevented by fast turnover of the plastoquinone pools (Margraph 1990, Loriaux 2013). With this in mind, Y(II) and ETR measurements taken under these conditions, can be underestimated. In a poster, researchers that included Bernard Genty, the developer of quantum yield of PSII, verified the issue, and developed a method for F_M' correction. It involved a multiple phases single saturation pulse with multiple light intensities, and the use of least squares linear regression analysis of the reciprocal of PAR (Photosynthetically Active Radiation), to determine the FM' fluorescence level using an infinitely intense saturation pulse, without causing damage to the plant and without closing all of the reaction centres.

Studies by Earl (2004) and Loriaux (2006), have compared chlorophyll fluorescence measurement results with gas exchange measurements and found that by using multiple saturation flashes, and regression analysis, an infinite fluorescent saturation light flash intensity can be determined and used to correct Φ_{PSII} or (Y(II)) and *J* (ETR) measurements.

This standard option is provided on the OS5p+, the i*FL*, and OS1p instruments. It is available for all Light adapted and quenching protocols, and it can be turned off or on. The method described by Loriaux, Avenson, Welles, McDermitt, Eckles, Riensche, & Genty (2013). Research has shown that Y(II) measurements, taken under high actinic light conditions, can be underestimated with up to a 22% error, and there can be up to a 41% error in ETR values if this method is not used. Loriaux, Burns, Welles, McDermitt, & Genty (2006) and expanded by provides the most accepted method currently available. According to Loriaux 2013, the iFL provides the optimal saturation intensity of 7000 µmol, optimal light ramping of 20%, and a ramping rate less than 0.01 mol $m^{-2}s^{-1}$. While some adjustment is possible, the default protocol has been optimized for most applications.



The first saturation flash step, shown on the left, is at 7,000 µmol for 0.30 seconds to saturate PSII. The saturation flash intensity is then ramped downward by 20%, making a large number of fluorescence measurements along the way, to 5,600 µmol. The ramping rate is less than 0.01mol photons $m^{-2}s^{-2}$. The final phase is at 7,000 µmol to check for saturation pulse NPQ. Recent studies have shown that those setting provided optimal results for plants that have been tested (Loriaux 2013). A rolling 25ms eight point average is used to determine maximum F_M'.

The graph on the right represents the Loriaux (2006) & Loriaux (2013) method for estimating F_M' with an infinitely intense saturation flash. Least squares linear regression analysis of the reciprocal of PAR (or 10,000 / PAR) allows determination of the y intercept, which represents the machine fluorescence value with an infinite saturation flash.

The final period at 7,000 µmol is used to determine if saturation flash NPQ occurs with the sample. Bernard Genty recommends not using the method if F_M' is lower for the second measurement than the first, as this indicates that saturation duration is too long for the species being measured.

A checklist for making reliable Y(II) measurements

The Limitations of Y(II)

The strong relationship between Y(II) and CO_2 assimilation correlation has been reaffirmed repeatedly by many researchers with the following caveats:

1. At high actinic light levels, the correlation between ETR and CO_2 assimilation breaks down. Y(II) can show an error of up to 22%, and ETR can show an error of up to 41% (Loriaux 2006). It is thought by some to be caused by the inability of the most intense saturation light sources to completely close all PSII reaction centres under high light stress conditions. To compensate for this issue, Earl (2004) uses saturation pulses at various levels and extrapolates the results of a saturation pulse at infinity using linear regression analysis. Loriaux (2006, & 2013) develops a single multiple phased saturation flash with least squares linear regression analysis to correct for this error. In the latest refinement of the process, Loriaux (2013) found that using a saturation intensity of 7,000 µmol to 13,000 µmol, a 20% intensity down ramp, and a ramp rate less than 0.01mole $m^{-2}s^{-1}$ provided the most accurate results. This method restores the correlation of ETR and CO_2 assimilation and it is an option called Multiflash that is offered in the i*FL*, Opti-Sciences OS5p+ and the OS1p.

2. There is small percentage of chlorophyll fluorescence that comes from photosystem I that does not change with light intensity (PPFD) or plant stress. Therefore, the error is greatest at very high light levels when yield is minimized and PSI fluorescence remaining constant. This error is not large (Baker Oxborough 2004).

3. "Super-saturating flash" error is produced by using a very intense saturation light source that is longer that 2 ms causing multiple turnovers of primary PSII receptor Q_A and the reduction of plasotoquinone to plastoquinol. This raises F_M' and can cause an overestimate of Yield by less than 10% (Baker and Oxborough 2004), (Schreiber 2004). Use of a super-saturation flash is by far the most common method of measuring Y(II) in higher plants.

4. Cold stress can produce a non-linear correlation with CO_2 assimilation. Electron transport of PSII in cold stressed corn far exceeds the requirements for CO_2 assimilation by more than three to one, indicating that under these conditions other electron sinks are at work. The ratio of ETR (a product of Y(II), PAR, leaf absorption ratio, and PSII absorption ratio) to CO_2 assimilation under cold stress can be diagnostic for cold stress. (Fryer M. J., Andrews J.R., Oxborough K., Blowers D. A., Baker N.E. 1998).

5. The ratio of *J* to CO_2 assimilation can be diagnostic for water stress in C_3 plants. C_3 plants exhibit strong electron transport rates for early and moderate levels of water stress even when CO_2 assimilation has decreased due to water or drought stress. This indicates that there are other electron sinks for electron transport. (Ohashi 2005). This problem of early water stress measurement and detection may be overcome by using the Burke assay (Burke 2010). Y(II) can be used to measure very early water stress (Burke 2007 and Burke 2010).

6. Mangrove leaves growing in the tropics. Here again electron transport rate is more than three times that of CO_2 assimilation. It is believed that this is mostly due to reactive oxygen species as an electron sink (Baker Oxborough 2004), (Cheeseman 1997).

7. Measurements not taken at steady state photosynthesis can lead to non-linearity caused by chloroplast migration. The error can be up to 30% (Cazzaniga *et al*., 2013) (Dall'Osta 2014), but can be avoided by allowing plant samples to reach steady state. Photosynthesis, a process that takes between 20 and 35 minutes (Cazzaniga *et al*., 2013).

8. While linear correlation and curvilinear correlation are possible (Genty 1989), (Genty 1990), (Baker & Oxborough 2004), exact correlation between fluorescence ETR and gas exchange ETR is not possible due to the fact that fluorescence comes from only the upper most layers of the leaf while gas exchange measurements also measure lower layers (Schreiber, 2004).

9. In CAM plants, gas exchange measurements are not possible during daylight hours so Y(II) measurements can provide insights into daytime light reactions (Rosenqvist and van Kooten 2006). As illustrated by the exceptions listed above, in some cases …"the relationship between light reactions and dark reactions is not straightforward"… The energy molecules ATP and NADPH can be used for carbon fixation and for photorespiration (Rosenqvist & van Kooten 2006), or light reaction electrons may flow to other electron sinks (Ohashi 2005), (Baker & Oxborough 2004), (Fryer M. J., Andrews J.R., Oxborough K., Blowers D. A., Baker N.E. 1998). For this reason, it is not uncommon for authors to differentiate between work done under non-photorespiratory conditions and under photorespiratory conditions (e.g. Earl 2004), (e.g. Genty B, Harbinson J., Baker N.R., 1990).

10. Samples should be compared that have similar light histories. It takes between 40 minutes and 60 hours for chronic photoinhibition to relax or repair. As a result, there is always some residual
photoinhibition built into chlorophyll fluorescence measurements after a sunny day in the field. Plants that have a few overcast days may not have this residual photoinhibition built into measurements (Lichtenthaler, 2004).

11. Chlorophyll fluorescence heterogeneity or patchy fluorescence occurs under some conditions. This is not an issue with the iFL because it averages chlorophyll fluorescence over the large area of the leaf chamber. Furthermore, it measures over the same leaf area as gas exchange, preventing heterogeneity from being an issue. Heterogeneity happens during drought stress, cold stress, and under low CO_2 levels (Baker, 2008).

Y(II) is the more versatile fluorescence measuring parameter than F_V/F_M, but it is best to use a system that offers multiple test parameters for diverse stress applications. While systems that provide true Y(II) measurements tend to cost more than ones that provide just F_V/F_M measurements, they offer greater capability for plant stress measurement. See the Opti-Sciences "Desk Top Plant Stress Guide" for more information www.optisci.com.

Quenching measurements and understanding the quenching fluorescence curve

This article is an overview of the value and limitations of quenching kinetic traces and provides a basic understanding of photochemical and non-photochemical quenching measurements. The puddle model, the Kramer lake model, and the Hendrickson lake model parameters that include NPQ resurrected to the lake model by Klughammer & Schrieber will be reviewed. Practical considerations are added to the discussion.

Quenching traces are used in measuring photo-protective mechanisms, chloroplast migration (there is now substantial evidence that change in fluorescence thought to be due to state transitions may be due to chloroplast migration in the cell), photoinhibition, and passive energy dissipation state transitions (where they exist). Most of the quenching measuring parameters require steady state photosynthetic conditions for reliable results; however, Klughammer states that Y(NO) is not limited to steady state measurement. Without a good understanding of the mechanisms that affect the light trace, machine artefacts and user errors can be included in measurements.

Chlorophyll fluorescence signal

Variable chlorophyll fluorescence has been found to provide significant information regarding the light reaction of photosynthetic processes, plant health, and plant stress measurement. Variable chlorophyll fluorescence from light that is absorbed by PSII has been shown to vary with these conditions and allow measurement, whereas PSI fluorescence is low, and does not vary. The fluorescence signal that comes from PSII is the result of a competitive process with photochemistry and heat dissipation (heat dissipation can further be divided into non-radiation decay and photo-protective regulated heat dissipation). For example, when most of the light is used by photochemistry, less is given off as fluorescence and heat. PSII has also been found to be sensitive to many types of plant stress (Baker 2008).

Actinic Light Source

The iFL uses a white actinic light source with an intense blue spectrum to drive photosynthesis. The programmable irradiation intensity is held constant using a feedback loop involving the internal leaf chamber PAR sensor, to ensure a stable actinic light intensity is maintained over the course of an experiment, a protocol or an experimental step. The actinic light intensity is measured and adjusted at least ten times per second and sometimes more often, depending on the measuring protocol.

Why do it this way?

Most built-in fluorometer light sources used as actinic sources for quenching measurements, and light curves, decline in intensity during these measurements. This is due to the fact that heat from the internal light sources reduces light output. It can happen to halogen light sources and to LED light sources. When this happens, the photosynthetic sample may never really reach steady state photosynthesis, a process that takes between 20 and 35 minutes at a specific light level (Cazzaniga *et al*., 2013), (Dall'Osta 2014). Furthermore, the intense blue spectrum allows chloroplast migration very much like it does in nature. Chloroplast migration only happens with an intense blue spectrum, there is not significant chloroplast migration with intense red light. Chloroplast migration can be

responsible for about 30% of NPQ at higher actinic light intensities. In addition, the relative spectral output of the light source does not change with intensity.

Lake Model and Puddle Model Quenching Parameters

Understanding of the organization of antennae and reaction centres has changed over the years. It is now understood that a single antennae does not link only to a single reaction centre as was previously described in the Puddle Model. Current evidence indicates that reaction centres are connected with shared antennae in terrestrial plants. q_P, the parameter that has been used in the past to represent the fraction of PSII reaction centres that are open, is a puddle model parameter. Dave Kramer (2004) has come up with a set of fluorescence parameters that represent the newer shared antennae paradigm called the lake model.

Others have also come up with more simplified equations that eliminate the need for the measurement of F_o and F_o' and approximate the measurements made by Kramer. Hendrickson's (2004) work offered such a solution with Y(NPQ) measurements that are consistently and only marginally lower values than Kramer's work, and Y(NO) measurements that are consistently and marginally lower except at high light levels and low temperatures than Kramer's work. He speculates that the differences in values between Kramer and his own were possibly due to the difficulties in making F_o' measurements. Furthermore, Hendrickson does not provide a parameter like q_L to estimate the fraction of open PSII centres.

From Hendrickson's work, and earlier works by Cailly (1996) and Genty (1989, 1996), Klughammer and Schreiber derive simplified equations that allow for Hendrickson's parameters, and also allow users to reconcile NPQ puddle model measurements with the lake model.

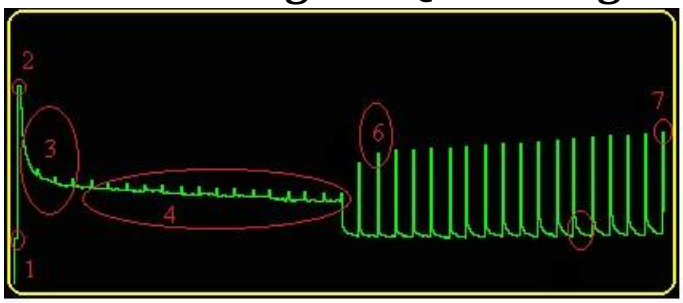
One will be able to choose Kramer parameters, Hendrickson with NPQ parameters, or Puddle Model parameters. The use of Puddle Model parameters have been retained because they allow for the separation of photo-protective mechanisms, state transition measurements, and photo-inhibition in quenching relaxation protocols. There is also a significant volume of work done using the older puddle model parameters that may be valuable for comparison. In addition, the reconciliations of NPQ with Lake Model by Klughammer and Schreiber allow separation of q_E, state transitions q_T, and photoinhibition q_I with the Hendrickson lake model. In 2013, Cazzaniga replaced q_T with q_M for chloroplast migration.

The loss of light energy in the plant due to fluorescence, comes primarily from the variable PSII reaction. When leaves have been dark-adapted, the pools of oxidation-reduction intermediates in the electron transport pathway return to a oxidized state and quenching mechanisms relax. After dark adaptation, a low intensity modulated light turns on and off and the minimal fluorescence signal, F_o, is measured. The modulated light source is at an intensity too low to drive chemical reduction of Q_A, but high enough allow measurement. Upon saturation illumination of a dark-adapted leaf, there is a rapid rise in variable chlorophyll fluorescent light emission from PSII as all available reaction centres are closed, and the maximum amount of light is channelled to variable chlorophyll

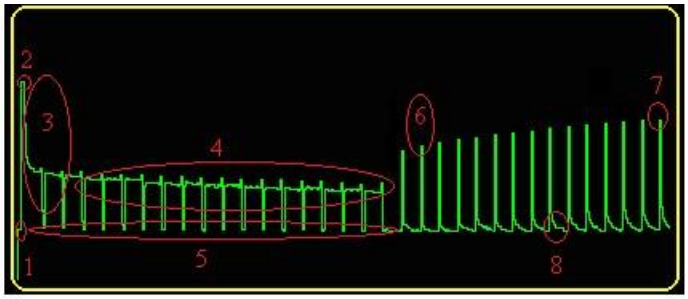
fluorescence. Multiple turn-overs of the Q_A molecule occur before maximal fluorescence, F_M.

As the actinic light is turned on, things start to happen.

Understanding the Quenching Mode Trace



Hendrickson lake model quenching & quenching relaxation



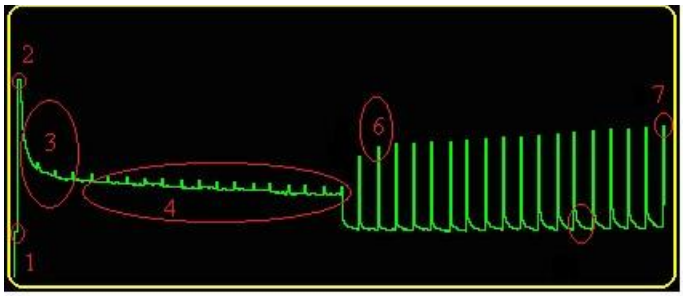
Kramer lake model quenching & quenching relaxation

#1
Represents a condition that is normally dark adapted (proper dark adaption is required when making non-photochemical quenching measurements) with the sample shielded from any actinic light that would drive photosynthesis. The only light on at this time is the modulated measuring light at about 0.1 or 0.2 µmol intensity. This is not enough to drive photochemical reduction of Q_A but it is enough to detect and measure minimum fluorescence from the leaf antennae. In most commercial fluorometers, 30% of the intensity of F_O in C_3 plants is the result of fluorescence from PSI. In C_4 plants, 50% of the intensity of F_O is the result of fluorescence from PSI. This contributes to a small error in F_V/F_M measurements and creates an underestimation of maximum quantum efficiency. Fluorescence from PSI is not variable, it is constant.

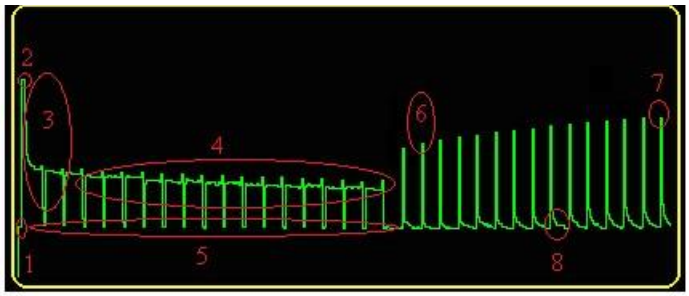
#2
Shows the first saturation pulse flash. This is a very intense short lasting flash of light that is designed to saturate PSII and close all available reaction centres. For higher plants, the optimal time duration for a saturation pulse has been found to be between 0.5 seconds to 1.5 seconds (Rosenqvist & van Kooten 2005). For Algae and cyanobacteria the optimal duration of the saturation pulse is shorter, 25 to 50ms (Schreiber 1995). Opti-Sciences uses an eight point rolling average over a 25 ms period to determine maximum F_M and F_M'. This prevents saturation pulse NPQ from causing an error if the saturation pulse duration is set to be too long, and ensures reliable results on land plants or with algae.

Furthermore, either square topped saturation flashes or Multiflash (F_M' correction for light adapted samples) may be used during quenching measurements if desired (see the Multi-Flash section for more details). With dark adapted samples, complete closure of all PSII reaction centres can be accomplished with minimal saturation intensity. PAR Light values of 1,500 µmol or higher are commonly used. Certainly, values of 1500 µmol or higher will fully saturate any properly dark adapted sample (Ralph 2005). However, due to the need to close reaction centres in light adapted samples, it is common to use 7,000 µmol for saturation intensity.

The rise from F_O to F_M represents multiple turn overs of Q_A, the primary quinone receptor. PSII is fully reduced at F_M with all available PSII reaction centres closed or reduced. At this point, a maximum amount of fluorescence is measured. Most light energy goes to fluorescence at F_M. For more details about F_M, see the review of variable chlorophyll fluorescence in this document.



Hendrickson lake model quenching & quenching relaxation



Kramer lake model quenching & quenching relaxation

#3

After F_M, the white light LED actinic light source is turned on to the pre-programmed light level. The fluorescence signal competes for energy with photochemistry and heat dissipation. Since photochemistry and heat dissipation mechanisms have just been initiated, most of the energy goes to fluorescence. The fluorescence signal starts to fall as electron transport and carboxylation begins (Schreiber 2004). The photo-protective mechanisms of the xanthophyll cycle and Δ pH of the thylakoid lumen are also beginning to affect the signal to drive down the intensity values of both the saturation flashes and the fluorescence signal itself at higher light intensities. These are heat dissipation mechanisms. The fluorescence signal continues to drop as full activation of Rubisco continues. Full activation of Rubisco in both algae and higher plants takes between 3 to 4 minutes (Baker 2008).

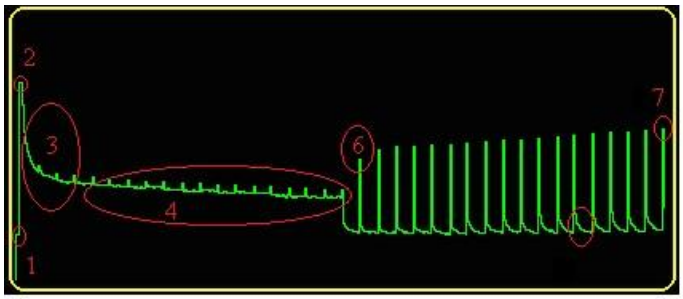
In addition, non-photochemical quenching, q_E, or a measure of the photo-protective xanthophyll cycle and ΔpH of the thylakoid lumen take between about two minutes to seven minutes to adjust to a new light level (Baker 2008). Some have claimed it can take up to ten minutes in field plants, however, since no references have been found, seven minute may be the limitation. Field plants take longer that other plants to adjust. The saturation pulse at about four minutes is a likely place for the end of q_E adjustment (the third saturation pulse). It is believed that there is an overlap of the initial q_E mechanisms and intermediate plant mechanisms.

#4

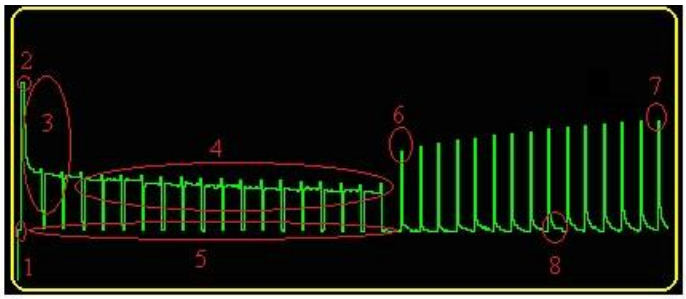
At low light levels it is still possible that q_T or state transitions overlap with q_E, in plants where they exist. At higher light levels in higher plants (C_3 and C_4 plants), q_M or chloroplast migration is also believed to overlap with q_E (Cazzaniga *et al*., 2013). At higher light levels that exist in the field, Chloroplast migration is responsible for the fluorescence adjustment that is seen from about 4 minutes to about 20 to 35 minutes (Cazzaniga *et al*., 2013). This change, as it occurs in nature, can represent up to 30 % of non-photochemical quenching when an intense white or an intense blue actinic light are used. There is no significant chloroplast migration under a high red actinic light. This has been shown in dicots, and it is likely also the case in monocots because chloroplast

migration also occurs in monocots (Maai 2011). While the mechanisms of chloroplast migration and state transitions are not truly non-photochemical quenching, they are grouped in NPQ just the same. NPQ includes mechanisms involved in photo-protection, chloroplast migration, state transitions, and photoinhibition. State transitions, where they exist, take between 15 to 20 minutes to adjust. (Baker 2008) Saturation flash intensity values are driven down by non-photochemical quenching. Previously it was thought that steady state photosynthesis took between fifteen and twenty minutes (Maxwell & Johnson 2000). However, it has been found that under high light levels chloroplast migration takes between 20 to 35 minutes to complete (Cazzaniga *et al*., 2013). Under photo inhibitory conditions D1 protein degradation found in PSII reaction centres close some PSII reaction centres. Other mechanisms have also been suggested for being involved in photoinhibition. It is common to take quenching measurements at steady state photosynthesis after the leaf has fully adapted to a specific light level. Most light adapted parameters are defined as measurements at steady state. However, Klughammer claims that Y(NO) can be measured at other times. Graphing quenching values at non steady state conditions through arrival at steady state helps to understand the process (MacIntyre, Sharkey, Guider 1997). State transitions take between fifteen and twenty minutes to occur (Ruban A.V., Johnson M.P., 2009), (Allen J. F., Mullineaux C.W., 2004), (Lichtenthaler H. K., Burkart S., 1999). The fluorescence signal and saturation flash intensity are affected by light level, heat and cold, as well as many other forms of plant stress, and the plants ability to deal with plant stress [see the Opti-Sciences Desk Top Plant Stress Guide www.optisci.com].

The saturation qI measurements may take several hours under photoinhibitory light conditions (Ruban & Johnson, 2009), (Allen & Mullineaux, 2004) (Lichtenthaler & Burkart, 1999). The picture below shows 18 saturation flashes 2 minutes apart. The final flash in the light adapted portion of the test is at steady state photosynthesis and should be the one used for most quenching parameters except Y(NO) that may be used at any time.



Hendrickson lake model quenching & quenching relaxation



Kramer lake model quenching & quenching relaxation

At steady state photosynthesis, after the last light adapted saturation flash, the actinic light source is turned off, and a far red light is turned on for several seconds to activate PSI and drain all remaining electrons from PSII. This results in a quenched measurement of F_O called F_O', the minimum quenched fluorescence value measured. F_O' is used in the quenching parameters q_P, q_N, and Kramer's Y(NPQ), q_L, and Y(NO). I has also been used in q_E , q_T , and q_I when q_N is used in place of NPQ. After five to ten seconds, the far red light is turned off. The Far red light at 735 nm is at too long a wavelength to drive PSII, but it will drive PSI.

#5

This shows the fluorescence trace with F_O' displayed. After the saturation flash, Far red light with a peak at 735 nm is used, usually for 5 to 10 seconds, to illuminate the leaf sample. Far red light stimulates Photosystem I and not Photosystem II. It allows the available electrons in PSII to be drained for use in PSI and allows the measurement of F_O' or quenched F_O. F_O' is used in most of the Kramer quenching parameters and puddle model parameters q_N and q_P, however, it is not used in NPQ. Hendrickson on the other hand, uses simplified Kramer equations without F_O' use. See the discussion about how these values relate to one another under Kramer and Hendrickson definitions.

#6

This section of the graph has the actinic light turned off and it is used to study the relaxation characteristics of NPQ. #6 is the second saturation flash after 4 minutes in the dark. It is the likely relaxation of NPQ due to q_E or the photo-protection segment of the trace. It takes the same time to adjust in the light as it does in the dark to relax. The saturation flashes beyond #6 to #7 are the result of chloroplast migration adjustment in the dark. During this phase of the graph, the actinic light is automatically turned off and the sample is in the dark. Only the modulated light that does not drive photosynthesis, and saturation pulses are used here. The increase in the peak height is the result of the relaxation mechanisms grouped into non-photochemical quenching including; photo-protective mechanisms, state transitions (where they exist), q_M chloroplast migration, and eventually photoinhibition. Lichtenthaler found that the relaxation of photo protective mechanisms that involve ΔpH of the thylakoid lumen and the xanthophyll cycle takes between 2 and 4 minutes. A saturation pulse at the end of this period can be used to measure q_E photo-protective mechanisms (Lichtenthaler 1999). The relaxation characteristics of field plants can vary with changing environmental conditions. Baker (2008) states that q_E may take up to 7 minutes in field plants.

The relaxation of state transitions (where they exist) takes between 15 and 20 minutes, so a saturation pulse after twenty minutes in the dark can provide a measurement of q_T. A saturation pulse after 20 to 35 minutes in the dark can be used to measure q_M or chloroplast migration. q_I or photoinhibition can also be determined with this q_M peak, because F_M is known from the dark adapted first pulse and the difference is considered to be photo inhibition. Chronic photoinhibition starts to relax at about 40 minutes in the dark and Lichtenthaler (1999, 2004) found that it could take up to 60 hours for complete relaxation of photoinhibition. It is common for researchers to dark adapt overnight (Maxwell and Johnson 2000) when making quenching measurements.

Note: q_Z is term developed by Nilkens (2011) to name some unknown xanthophyll cycle mechanism. It appears that q_M is likely the source of q_Z.

q_M replaces q_T and acute photoinhibition as the intermediate source of chlorophyll fluorescence change. Mutant Arabidopsis plants devoid of competing mechanisms were evaluated to come to this conclusion and it was discovered that chloroplast migration takes between 20 minutes and 35 minutes to complete.

It should be noted that NPQ should only be used to compare plants of the same species and with the same F_V/F_M values (Baker 2008), (Maxwell and Johnson 2000).

In addition, with the reconciliation of NPQ to Lake Model parameters, it becomes possible to use quenching relaxation with the Hendrickson lake model. It is also possible that at high light levels that there is some overlap between q_E and q_M (Cazzaniga *et al*., 2013).

It is recommended to use a tripod to measure all quenching parameters. This ensures that all measurements are made at steady state photosynthesis at a specific light level. An internal PAR sensor measures the illumination output at the leaf, and maintains the programmable light setting desired by measuring light irradiation at the leaf every 0.1 seconds, and adjusts the intensity as necessary to maintain the stable light level for any length of time. The relaxation characteristics of field plants can vary with changing environmental conditions (Baker 2008).

8
After a saturation flash, the tailing off of the signal is the result of NPQ caused by the saturation flash. According to Rosenqvist and van Kooten (2006) it takes between one and two minutes for complete dissipation of saturation pulse NPQ in the light. With this in mind, saturation pulses should be spaced to avoid a build-up of NPQ. It is also mentioned that photo-damage can occur to samples when saturation flash intensities are too high in the dark, or too frequent, when there is no actinic light. There is no evidence that damage occurs on samples in the light (Rosenqvist 2006).

In some cases, Opti-Sciences have found that saturation flash NPQ takes longer than 2 minutes in the dark, especially during long quenching *relaxation* protocols with many saturation flashes. If one is concerned, space the saturation flashes to 3 or 4 minutes apart for relaxation work. Problems show themselves as the fluorescence bottom signal rising over time. This represents either a build-up of saturation flash NPQ or an actinic modulated light setting that is too high and driving reduction of Q_A. Relaxation saturation flashes may be made at a specific minute time after the actinic light intensity has been turned off. Make sure that the spacing between the programmed saturation flashes (for example: every four minutes) does not allow saturation pulse NPQ to affect the measurements of q_E, q_M, or q_I if specific time selections are made. For example: if the specific time for q_M is set at 35 minutes, make sure that the measurement occurs at least 2 minutes after the last regular sequenced programmed saturation flash occurs. One can run regular sequenced saturation flashes up to 32 minutes, and then have the final flash at 35 minutes.

Checklist before making NPQ and other quenching measurements

Quenching measurement parameters, such as NPQ, are the least understood, and most often misused parameters that are available with advanced chlorophyll fluorometers. This application note is designed to improve the understanding of proper quenching protocol usage.

While the Puddle Model, (based on a one antennae per reaction centre model) is no longer credible, Puddle Model-based parameters continue to be used. The newer, Lake Model, is based on shared antennae. The Kramer Lake model (Kramer 2004) uses F_O' or quenched F_O in most of its measuring parameters. In addition, it provided q_L or a photochemical parameter to replace q_P, a Puddle Model parameter. The Hendrickson Lake Model offers simplified Kramer equations without F_O' (Hendrickson 2004). The Hendrickson Lake Model equations do not have an equivalent to qL or photochemical quenching, however, Klughammer shows that NPQ can be resurrected from the Puddle Model to the lake model using Hendrickson's equations, NPQ= Y(NPQ)/Y(NO). Furthermore, both sets of equations provide Y(II), Y(NPQ), and Y(NO). For an in-depth discussion of the differences of each set of equations see Quenching application note available for free at www.optisci.com. To get reliable measurements, one should follow tested guidelines.

1. Dark-adapt properly knowing the plant's light history. It takes only a few minutes for the xanthophyll cycle and the ∆pH of the thylakoid lumen to return to a dark-adapted state. State transitions (where they exist), however, take between fifteen to twenty minutes. These times can vary somewhat in field plants and can take slightly longer (Baker 2004). Recently, it was found that the fluorescence change that was thought to be due to state transitions and acute photoinhibition was really caused by chloroplast migrations (Cazzaniga *et al*., 2013), (Dall'Osta 2014). q_M, the fluorescence intensity value change caused primarily by chloroplast migration, can represent about 30% of NPQ at high actinic light levels. Furthermore, it takes from 20 to 35 minutes for chloroplast migration to completely adjust in the light, and relax in the dark.

   In addition, field plants and other plants that have been exposed to high light photoinhibition conditions for a number of hours, will retain a certain amount of NPQ or chronic photoinhibition for up to 30 to 60 hours (Lichtenthaler 2004). This means that even if dark adaptation is overnight, there will almost always be some residual NPQ built into summer field measurements of F_V/F_M, and other displayed quenching parameters. For this reason, it is important to only compare samples with a similar light history. When performing quenching measurements on field plants, it is common for researchers to choose pre-dawn dark adaptation values. It is not common to use shorter times to study quenching (see dark adaptation application note from Opti-Sciences.) If studying photoinhibition, it is a good idea to partially shade samples for a few days, if possible, to ensure that all photoinhibition has relaxed or repaired to give a baseline.

2. **Compared samples must have the same F_V/F_M values.** Quenching measurements of different samples with different F_V/F_M values should not be compared (Baker 2008). F_V/F_M is used as the measuring standard for non-photochemical quenching measurements, and if the measuring standard is different, the quenching values are

meaningless. Comparing values from samples with different F_V/F_M values is like measuring items with a ruler that has dimensions that change. Since it can take up to 60 hours for photoinhibition to relax, light history is important. If q_I is being measured, it may be best to partially shade samples for at least 60 hours to eliminate residual photoinhibition before testing.

3. **Modulation light intensity setting** (F_V/F_M is $(F_M-F_O)/F_M$).
   F_O, or minimum fluorescence is a dark adapted value made by exposing the leaf antennae to a very low intensity modulated measuring light, that allows measurement, but not high enough to photochemically reduce Q_A in the sample. This phenomenon appears as a rise in the base line chlorophyll fluorescence signal, F_t, over a 10 or 15 second time frame when the sample is exposed only to the modulated light. The modulated light may be set manually or it may be set using the "Auto Setup" button under "Change Settings" in the quenching protocol. The automated modulated light intensity setting allows easy adjustment.

4. **Leaves must be at steady state photosynthesis for reliable measurement.** It was previously thought that for most quenching measurements, steady state was only reached after 15 to 20 minutes at a new light level (Maxwell and Johnson 2000). However, recent research shows that chloroplast migration takes between 20 minutes and 35 minutes to fully adjust, and replaces not only state transitions, but also acute photoinhibition as the source of chlorophyll fluorescence change. There is strong evidence that chloroplast migration can be responsible for up to 30% of NPQ at high actinic light levels in dicots (Cazzaniga *et al*., 2013) and probably monocots as well (Maai 2011). The fluorescence change thought to be due to state transitions and acute photoinhibition is most likely due to chloroplast migration. *For this reason, longer pre-illumination times are likely to be required for reliable measurement.* For example, if there are 18 saturation pulses spaced 120 seconds apart, the leaf will be exposed to the actinic light for 36 minutes after dark adaptation. Since an internal fluorometer artificial light source is normally used, the test allows one to compare leaves as long as the F_V/F_M values are the same. According to Klughammer (2008), the only non-photochemical parameter that does not have to be taken at steady state photosynthesis is Y(NO) from Hendrickson.

5. **Use a fluorometer with a stable actinic light output**. Depending on the brand and type of fluorometer, the intensity output of the actinic light can change over time. When an actinic light is on, it can heat the fluorometer and cause a lowering of the light output. The intensity of the actinic LED light source output changes as the heat from the lamp changes the lamp temperature. The iFL, the OS5p+, the OS5p, and the OS1p uses the PAR Clip to measure actinic light output, and adjust the intensity of the light source as necessary to maintain a constant light level to +- 3 μmol over long periods of time. (Use the PAR clip on a tripod. A dark shroud, pre-dawn measurement, or a darkened room are suggested be used for measurement). Changes in actinic light intensity during measurement can be the source of significant error.

6. **Shade leaves vs. sun leaves.** The Y(II) ratio will be higher on Sun leaves than on shade leaves (Lichtenthaler 2004).

7. **Leaf orientation** is not important because an artificial actinic light source is used.

8. **Leaf maturity.** Most commonly, the youngest fully mature leaf blade is used for diagnosis of deficiencies in plants (Reuter and Robinson 1997).

9. **The duration of the saturation pulse** should be between 0.5 seconds and 1.5 seconds for higher plants (Rosenqvist and van Kooten 2006), and 25 to 50 milliseconds for Phytoplankton and cyanobacteria (Schreiber 2005). Times outside these ranges increase the error in Y(II) and quenching measurements. Shorter durations prevent complete saturation of PSII regardless of the light intensity. Longer durations can create a form of saturation pulse NPQ that rounds the tail end of the saturation pulse maximum value, and can reduce the average maximum saturation pulse value. (Rosenqvist and van Kooten 2006). The iFl, OS1p, OS5p+ and the OS5p use a special algorithm to prevent errors caused by saturation pulse NPQ. A rolling 25 ms., 8 point average of the highest F_M and F_M' values is used to prevent this error. This ensures that the correct value is reported as long as the saturation pulse width is wide enough.

10. **Saturation pulse intensity** is more of an issue with the light adapted Y(II) than with dark adapted F_V/F_M . While shade leaves will saturate at a few hundred μmol, sun leaves will usually saturate below 1,500 μmol (Ralph 2005). A very intense saturation flash intensity does not damage light adapted samples, but may damage dark-adapted samples under cold stress conditions, if the saturation flash happens too frequently in the dark (Porcar-Castell 2008). Multiflash (F_M' correction after Loriaux 2013) or square saturation flashes may be selected and used. See Multiflash for more details. For a single test, it is unlikely that intense flashes in the dark are a problem; however, for extended periods, the frequency of saturation flashes should be considered. It is recommended that maximum intensity should be used for all quenching measurements. Saturation pulses used at night for extended tests, should be at least ten minutes apart to one hour apart to prevent photo-damage from saturation pulses (PorcarCastell A. 2008). The iFL, and OS5p+ offers single relaxation saturation flash measurements at specifically selected times for q_E and other parameters, or a series of general relaxation saturation flashes spaced at a programmable distance apart. Both schemes can be used together, but saturation flashes should be at least two minute apart to prevent a build-up of saturation pulse NPQ.

11. **The time between saturation pulses** is important. Rosenqvist and van Kooten (2006) state that between one to two minutes is required for complete relaxation of saturation pulse NPQ. If saturation pulses are not separated by this distance range, then an error caused by this type of saturation pulse NPQ will result. It will accumulate with each saturation pulse. When in doubt, space saturation pulses 120 seconds apart or more. Opti-Sciences have found that relaxation saturation pulses in the dark, may require longer times for relaxation. In tests where there are many saturation pulses in the dark we have found that it is better to space relaxation saturation flashes 3 to 4 minutes apart to prevent a build-up of saturation flash NPQ. 12. Overlap of PSI fluorescence -Part of the minimum fluorescence, the F_O parameter, in F_V/F_M ($(F_M - F_O)/F_M$), contains PSI fluorescence as well as PSII fluorescence. With F_V/F_M, one is trying to measure the maximum variable fluorescence of PSII in a dark-adapted state. PSI fluorescence is not variable, but the low fluorescent signal from PSI does overlap with PSII. This produces a small non-variable error. In C_3 plants, about 30% of F_O fluorescence is due to PSI, and in C_4 plants about 50% of F_O fluorescence is due to PSI fluorescence. PSI produces

about 6% of the fluorescence found in F_M in C_3 plants, and about 12% in C_4 plants. (Pfundle 1998). This not a problem when comparing quenching measurements for plant stress because, PSI fluorescence does not change with light level or plant stress and comparisons are usually done on the same species for comparison.

13. **Leaf fluorescence heterogeneity** presents itself as patchy fluorescence on measurements of different parts of the leaf. It has been found to occur under cold stress conditions, with biotic stress, with low CO_2 conditions, and under drought stress conditions. This is not a problem with the iFL. The iFL averages chlorophyll fluorescence measurements over the same large area as gas exchange measurements or over the entire leaf chamber area.

14. **The q_E and q_M (or q_T & q_Z) values can be set to any minute value**. However, there could then be additional saturation flashes between the general saturation flashes and therefore saturation flash NPQ could create an error if they are less than 120 seconds apart. For example: If general quenching relaxation saturation flashes are 2 minutes apart, q_E might be set at 4 minutes and q_M at 36 minutes. q_E and the intermediate value parameter may also be set without measuring general relaxation saturation flashes, at any minute time setting. It is also possible to provide relaxation saturation flashes four minute apart ending at 32 minutes, and the last saturation flash at 35 minutes to prevent saturation flash NPQ from being a problem yet demonstrating relaxation of q_M over time. It is believed that there is some overlap between q_E and intermediate relaxation parameters. q_E can be set from 1 minute to 10 minutes. Baker (2008) states that it is shorter for non-field plants and longer for field plants. He states that times up to 7 minutes are possible in field plants. q_M or chloroplast migration takes between 20 to 35 minutes (Cazzaniga *et al.*, 2013), q_T or state transitions range from 15 minutes to 20 minutes (Ruban 2009). q_Z or an unknown longer NPQ mechanism ranges from 20 minutes to 30 minutes (Nilkens 2010).

15. **Far red light**. The built in actinic light has been filtered to eliminate far red light that excites PSI. This allows more control. Far red light can be selected during all phases of the quenching protocol, as desired. Of course, sun light contains far red light. Far red light may also be turned on/off for various parts of quenching tests, or for the entire quenching protocol. Far-red light has been used to pre-illuminate dark-adapted leaves. When this feature is used for five to ten seconds before an $F_{V/M}$ measurement takes place, it activates PSI, and ensures that all electrons have been drained from PSII before the measurement of F_O. While this feature ensures that PSII is completely re-oxidized, it does not relax the xanthophyll cycle, state transitions, chloroplast migrations, or photoinhibition. Time is still required in a darkened environment to relax all forms of NPQ and to obtain reliable quenching values. Far red light is used after saturation flashes to measure F_O', or quenched F_O (a parameter used in many Kramer Lake Model and Puddle Model protocols). PSI fluorescence is involved in all measurements and does not vary with light level or plant stress (Schreiber 2004). With this in mind, comparing samples with similar light histories allows comparison of many types of plant stress. The Plant Stress Guide provided by Opti-Sciences www.optisci.com, references papers that deal with specific types of plant stress and limitations of different chlorophyll fluorescence parameters for measuring plant stress.

Running the iFL measuring protocols

Protocols

The iFL supplies measuring protocol templates that cannot be erased. They may, however, be modified and stored as a custom test name based on the protocol template. This allows fast and easy test set up, and it helps ensure that the custom test takes

important variables into account. R_d and Γ* are both needed to determine g_m and C_C.

iFL templates exist for the following protocols:

1. Laisk protocol for R_d, and Γ* measurement – considered authoritative for C_3 plants. The von Caemmerer correction option is included.
2. Kok protocol for R_d measurement in C_4 plants, Γ* is calculated.
3. Yin protocol for R_d measurement in C_3 plants and C_4 plants, Γ* is calculated.
4. gm & CC protocol - Variable J and Constant J methods
5. Flexas Chamber leakage protocol
6. A/Q - light response curves
7. A/C_i curves & A/C_c curves

R_d, Γ*, and other parameters may also be entered into formulas when measured by other methods.

It is also possible to piggy back multiple protocols together to further improve automation.
For example, a Laisk test may take about 240 minutes if done properly, and no battery change is required.

With the iFL, it now becomes possible to automatically take the R_d and Γ* parameters determined in a Laisk protocol, and input them into A/C_i curves to make A/C_C curves. It is possible to do a Flexas chamber leakage protocol and automatically transfer that data to correct the Laisk protocol that follows.
It is possible to do an A/Q curve and an A/C_C or an A/C_i curve back to back.

Sequences

In the combined chlorophyll fluorescence – gas exchange protocol, the Automatic Sequence generator allows the creation of new custom sequences and protocols, as well as the retrieval of existing standard or custom protocols. While protocols can provide a template for development of tests, it is the sequence that becomes the custom test that will be used for actual work.

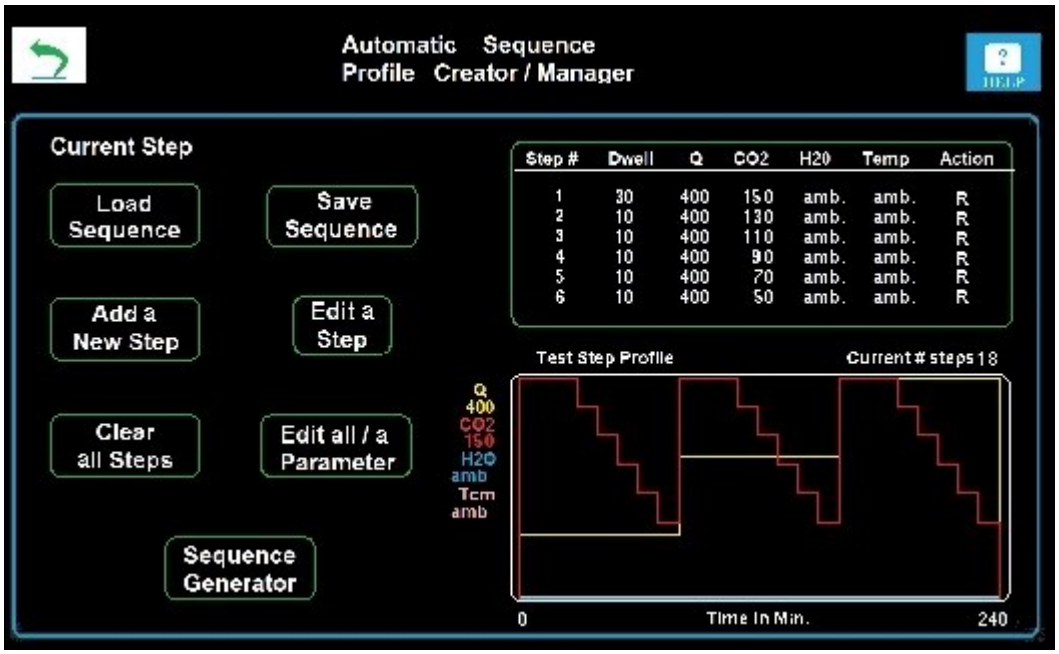
The page shown below allows loading of an existing protocol by tapping the Load Sequence protocol. From here a list of protocols and tests will appear with other files.

The “Save Sequence” allows saving new sequences or protocols that may be recalled and used later.

“Add a Step” allows adding additional steps to an existing or new protocol or sequence.

“Edit a Step” lets one change existing steps light intensity, dwell time, CO_2 level and dwell time, or H_2O level and dwell time.

The graph on the bottom right illustrates the test steps that have been created. It shows a Laisk protocol test that allows chloroplast migration to happen after 30 minutes at a set light level, and then the CO_2 level is dropped to lower levels for measurement. The process is repeated at a different light level and then a third light level, all below light saturation. It is this screen that allows an almost unlimited sequence of events and times.



Sequences may be created from this screen or sequences that have been created before, may be loaded into the active memory of the instrument from this screen. Sequences can be customized by loading a protocol and changing parameters or steps to customize the protocol into a new sequence. The sequence may then be saved under a new name that may be recalled later under “Load a Sequence”.

Sequences may also be created from scratch. They may be made, one step at a time, or one column at a time. Parts of columns may also be changed. If “Edit all / a Parameter” is tapped, a small screen appears and asks if “Some” or “All” values should be changed in a column. If “Some” is selected than a second screen appears that asks at which step the change should start, and next, a screen appears asking when the change should end. Tap the register return to go to the next step. When complete, tap the white “x” in the upper right hand corner of orange window to return to the measuring screen.

The window on the upper right side displays step numbers and the values for the various parameters in that step. Step numbers may be selected for change.

The window on the bottom right provides a graphical display of the sequence created. Different colours represent different parameters. Q is actinic light intensity, CO2 is chamber CO2 level, H2O is humidity level, and Tcm is chamber temperature. Check this graph for an understanding of the sequence.

Due to Chloroplast migration, changes in actinic light intensity require times of between 20 to 30 minutes for steady state lighting conditions to occur (Cazzaniga *et al*., 2013). It is common for CO_2 level changes to take between 10 and 12 minutes to reach steady state conditions.

Combined Chlorophyll Fluorescence/Gas Exchange Parameters g_m, C_C

1. g_m should be a constant that is measured in post processing using Rd and Γ* determined in the Laisk method or other methods. The constant can be reapplied after post processing to the various lines or A/Ci steps related to individual steps of the Laisk method as a constant, and not a variable. When using the Laisk method, both Laisk $Γ^*_{Laisk}$ and $Γ^*_{von\ C}$ can be reported. In addition, we provide:
   $g_{m\ Laisk}$ $g_{m\ vonC}$, and $C_{c\ Laisk}$, and $C_{c\ vonC}$
2. The introduction of the constant g_m allows determination of the variable C_c for each previous step, and for future work. It may be applied automatically to A/Ci curves to provide direct readout and graphing of A/C_C curves.
3. g_m, Γ*, and R_d <u>are only constants at a specific temperature.</u> All change with temperature.
   Furthermore, g_m changes under high light conditions. As a result, g_m vs temperature curves require determination of Γ*, and Rd at all temperatures measured. Converting to a constant temperature is not as reliable as testing at actual temperatures, however it is done.
4. A source of measuring error with these smaller values is chamber leakage. The Flexas protocol for chamber leakage is recommended before starting to work with Rd, Γ*, and g_m. Since the values are small, using this method will provide a more reliable result.
5. Another source of error is respiration in the light under the instrument gasket. The Pons protocol for under gasket respiration correction can be used to obtain a more accurate g_m and Cc in smaller chambers used. It involves using a chamber large enough to enclose an entire leaf for R_d determination and then correcting the value in smaller chambers. This requires an additional chamber. For this reason, it is used less often.

Formulas for g_m using the constant *J* method, and the variable *J* method.

g_m is measured using two methods. The variable *J* method, and the constant *J* method. To determine which is best, a curve of *J* vs. Ci is created when the CO_2 level is varied. If *J* stays relatively constant, the constant *J* method is used. If *J* varies with CO_2 level, then the variable *J* method is used to determine g_m (Bernacchi 2002).

This will be a constant for every temperature

Constant J - J stays the same with CO_2 level

$$J = ((A + Rd)) \left(\frac{4((Ci - A/g_m) + 2\,\Gamma^*)}{(Ci - A\,g_m) - \Gamma^*} \right)$$

Solve for g_m - Constant J

$$g_m = \frac{A(4A + 4Rd - J)}{4(A)(Ci) + 4(Ci)(Rd) + 2(A)(\Gamma^*) - (Ci)(J) + 2(\Gamma^*)(Rd) + (\Gamma^*)(J)}$$

Variable J - J changes with CO_2 level

$$g_m = \left(\left(Ci - \frac{A}{\frac{\Gamma^*(J + 8\cdot(A + Rd))}{J - (4(+Rd))}} \right) \right)$$

For von Caemmerer correction

$$\Gamma^*_{von\,C} = \Gamma^*_{Laisk} + R_{d\,Laisk}/gm_{Laisk}$$

Solve for g_m - Constant J

$$g_{m_{von\,C}} = \frac{A(4A + 4Rd - J)}{4(A)(Ci) + 4(Ci)(Rd) + 2(A)(\Gamma^*_{von\,C}) - (Ci)(J) + 2(\Gamma^*_{von\,C})(Rd) + (\Gamma^*_{von\,C})(J)}$$

Variable J - J changes with CO_2 level

$$g_{m_{von\,C}} = \left(\left(Ci - \frac{A}{\frac{\Gamma^*_{von\,C}(J + 8\cdot(A + Rd))}{J - (4(+Rd))}} \right) \right)$$

$$Cc_{von\,C} = C_i - A / gm_{von\,C}$$

$\Gamma^*_{von\,C}$	This will be a constant for every temperature
$gm_{von\,C}$	This will be a constant for every temperature
$Cc_{von\,C}$	This will be a variable depending on Ci & A

$C_C = C_i - A/g_m$ This is the standard formula for C_C or CO_2 at the site of carboxylation in chloroplasts.

Source of von Caemmerer Γ* correction:

Pons T.L, Flexas J., von Caemmerer S., Evans J.R., Genty B, Ribas-Carbo M., and Brugnoli E., (2009) Estimating mesophyll conductance to CO2: methodology, potential errors, and recommendations,
Journal of Experimental Botany, Vol. 60, No. 8, pp. 2217–2234, 2009,
doi:10.1093/jxb/erp081 Advance Access publication 8 April, 2009

g_m, Γ*, and R_d are only constants at a specific temperature. All change with temperature. Furthermore, g_m changes under high light conditions. As a result, g_m vs. temperature curves require Laisk method determination of Γ*, and R_d at all temperatures measured. Converting to a constant temperature is not as reliable as testing at actual temperatures.

While the parameters A, and C_i can be determined with standard gas-exchange measurements, Rd, and Γ* require additional tests to obtain the values. Some methods involve the use of gas exchange instrumentation only and one method uses the combination of chlorophyll fluorescence – gas exchange instrumentation.

R_d is the rate of mitochondrial day respiration, separate from photorespiration.

Γ* is the CO_2 compensation point, or the point where there is the absence of day respiration, at R_d.
It can be measured or estimated from the methods shown below for the most reliable results.
Some researchers have entered values into the calculation from Bernacchi *et al*. (2001)**.**
Γ* is the point where $C_c = C_i$. The compensation point of CO_2, Γ*, is also defined as the CO_2 concentration at which net CO_2 fixation is zero at a given O_2 level and temperature. O_2 is usually listed at 0.21 or 21% in most calculations.

*Methods for determining R_d and Γ**

Both R_d and Γ* are measured by the Laisk protocol. R_d is measured, and Γ* is estimated, in the Kok and Yin protocols:

The Kok method (from 1948) uses a light curve at very low light levels along with "A", carbon assimilation values, to determine R_d in C_4 plants. It may also be used for C_3 plants but the Laisk method is considered the authoritative method for C_3 plants (Ribas-Carbo 2010).

Plant respiration in the light is a difficult to measure, with lower values come a relatively high error rate. To get the measurement, very low light levels must be used, along with some higher light levels. The higher light levels produce "A" values that decrease with

light intensity in a linear fashion. When the "A" values drop off and become non -linear to the first trend linear line, a second linear trend line is drawn that represents the "A" values after the drop off. The intersection of the two lines is Γ*, the compensation point.

Next, using linear regression from Excel, the first trend line using the higher light values is extended to the Y axis, or the zero light level setting. The value on the Y axis becomes Rd. Rd is always zero or a negative value. However, when it is written, it is written as a positive value. The values generally range from 0.00 µmol to 2.00 µmol. Two decimal places are used. It is common for values to be in the range of 0.50 µmol to 1.5 µmol. Sometimes 1.00 µmol is used as a rough estimate for Rd.
Γ* is in µbars.

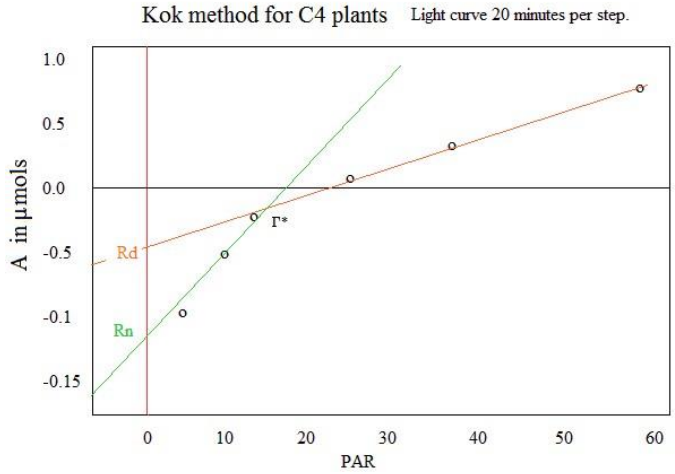
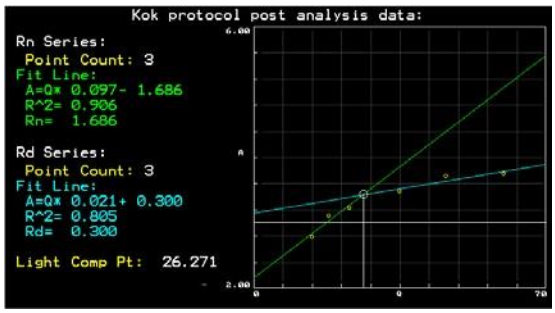


Image of Kok protocol post processing graph for Rd determination and Γ* estimation



The Laisk method 1977 is the more authoritative method for C_3 plants, but it only works for C_3 plants (Ribas-Carbo 2010). Here, three A/C_i curves are created at three different PAR levels. 600 µmol, 400 µmol, and 200 µmol are recommended and commonly used. However, the intensities should be at lower than saturation light levels.

The common point where the lines intersect is the CO_2 compensation point, Γ* read from the "X" or C_i axis, and R_d is the linear regression y axis value at 200µmol and Ci value of 0. The intensities can vary depending on the growing conditions of the plant. *The highest intensity should be below saturation light intensities.* While the graph below is an ideal representation showing a single point intersection, it is more common that all three A/C_i curves rarely intersect at the same point. As a result, the iFL uses a special algorithm to

determine the nearest point of coincidence, and it displays a red curve that represents every point on the graph and how close points on the three A/C_i curves come to a single coincidence point. The closer the line is to the Y axis zero line, the closer all three lines come to a single point of coincidence. This is the most commonly used method for C_3 plants.

The Kok , Laisk, and the Loreto stable isotope method are reviewed by Ribas-Carbo (2010). Since both the Kok method and the Laisk method are performed with either low light levels or small carbon assimilation rates, the variability is larger with these types of protocols. The Ribas-Carbo group also states that it is still possible that day respiration varies with light level even though researchers assume that it does not.
"To summarize, measuring respiration is a very challenging task, especially in the light, when substrates and products are interchanged by different and opposing reactions that operate simultaneously." Ribas-Carbo.

All light intensities used in the Laisk protocol should be less than saturation light intensities. While I have not been able to find a reference for this concept, it is a notion supported by prominent researchers. By using the RLC (Rapid Light Curve) protocol, sample saturation light intensity characteristics can be estimated. ETR_{Max} and the light intensity associated with it can serve as a starting point to develop a Laisk protocol test where all intensities are lower than saturation levels. These values change in the field during the day. ETR_{MAX} and the light level associated with it, I_m, are somewhat higher than values at steady state photosynthesis (Scheiber 2004), as a result the maximum actinic light intensity should be somewhat lower than the I_m or optimal light intensity value that provides ETR_{MAX}. Dark adapting for 5 or ten seconds and then running a RLC will provide saturation characteristics of a light adjusted plant at that time of day. For dark adaptation see (Ralph 2005). For effects of RLC at different times of day see Rascher.

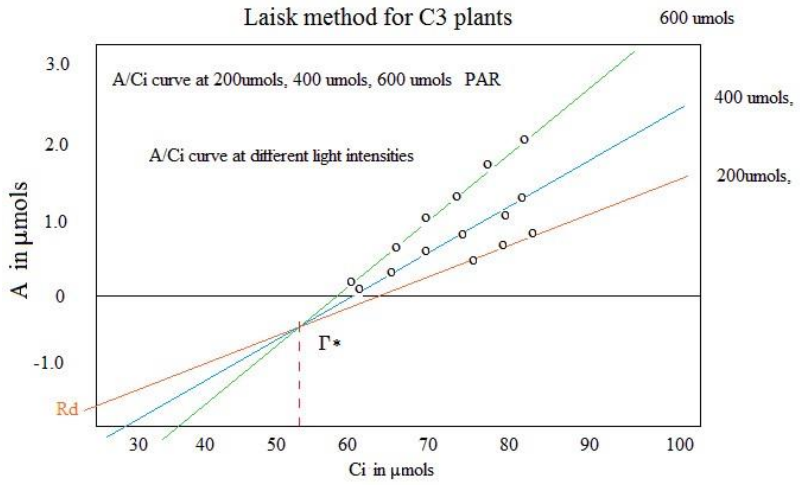
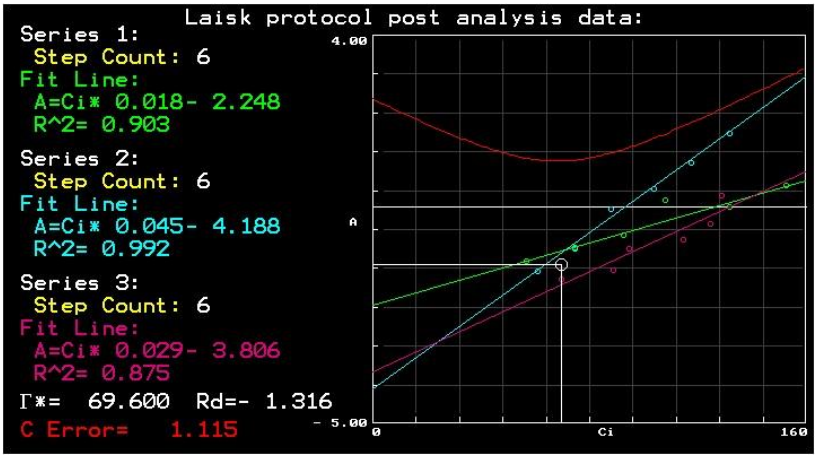


Image of Laisk protocol post-processing showing Rd, and Γ* without the von Caemmerer correction, but with nearest point of coincidence, and the red coincidence graph line.



The Yin chlorophyll fluorescence – gas exchange method (Yin 2009, 2011).

This newer method uses combined gas exchange and chlorophyll fluorescence to determine R_d, and other parameters. It can be used on C_3 or C_4 plants. Values agree with the Laisk method for C_3 plants. The advantage of the method is that it can be used at any CO_2 level, not just lower CO_2 levels, where errors are higher relative to the amount of CO_2. Values are consistently somewhat higher than the Kok method for C_3 plants due to the gas exchange – chlorophyll fluorescence correction of the non-linear relationship between "A" and irradiance. The method works better under non-photorespiratory conditions or lowered O_2 levels in C_3 plants. Linear regression is used on the data set when comparing A on the y axis and I_{ing} Φ_2/ 4 on the X axis. This is "A" values vs. (Photon flux density incident to leaves) x Y(II) /4 , or PAR x Y(II) /4 is calculated and = 0.5 x the oxygen partial pressure / relative CO_2/O_2 specificity factor for Rubisco (Yin 2011).

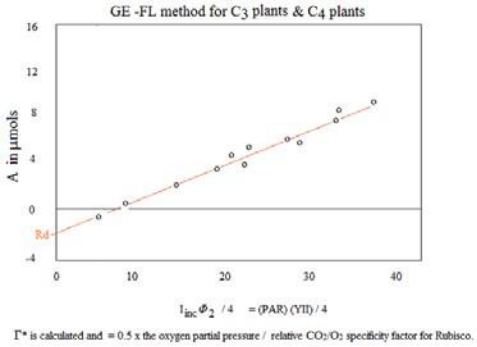
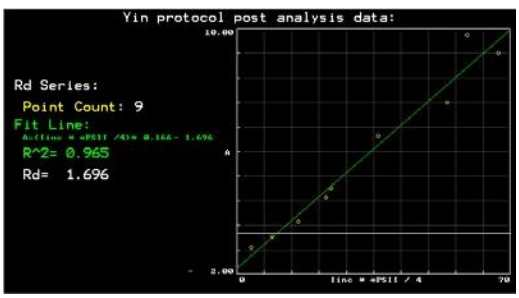


Image of i*FL* Yin protocol post processing graph for R_d determination and Γ* estimation.



Other methods for determining R_d

The Cornic method (1973) involves putting an illuminated leaf in a CO_2 free environment, noting the CO_2 production rate in 21% oxygen or in nitrogen, then darkening the leaf. When the leaf is darkened, the re-fixation of photo-respired CO_2 disappears and the peak of CO_2 production is measured.
In this method, *R_d = CO_2 production in the light in 21% oxygen - CO_2 production in the light in nitrogen - peak of CO_2 production in the dark + R_n.* In the Cornic method, values for R_d may be entered into the instrument equation manually (Ribas-Carbo 2010).

The Loreto (2001) stable carbon isotope method is also used. Here, day respiratory substrates are consumed more slowly than photo-respiratory substrates. Plants grown in a $^{12}CO_2$ environment are put into a $^{13}CO_2$ environment. $^{12}CO_2$ production measured in a $^{13}CO_2$ environment is R_d. Corrections for re-fixation of respired CO_2 are also made. *Here, the value for R_d may be input into the instrument equation manually, or the gas exchange system environment may be used with a $^{13}CO_2$* supply cylinder (Ribas-Carbo 2010).

Parameter definitions (From Bernacchi, 2002):

C_c: CO_2 at site of Carboxilation - Determined from g_m calculation
$C_c = C_i - A/g_m$
C_c: Reported in µbar Range 0 to 1000 µbar
J : Photosynthesis term for ETR or electron transport rate derived from chlorophyll fluorescence.

J = Y(II) (measured by iFL) * PAR at leaf (measured by iFL)* leaf absorbance (measured by iFL)* ratio of PSII reaction centres to PSI reaction centres. Average values are the default value of 0.5, literature values may be input, or values can be measured by the Zell method at 77°K separately. (Zell 2010)

The ratio of leaf reaction centers varies for C_3 plants and C_4 plants. In C_4 plants the ratio is lower. For Example, in corn, the ratio is 0.4 while in C_3 plants it can be as high as 0.60. The ratio for many types of common crops is available in the literature. However, the ratio value can vary by sun or shade growing conditions or under severe carbon deficit. (Baker 2008)

Γ*- CO_2 compensation point when $C_C = C_I$. R_d – Day mitochondrial respiration. g_m – "The conductance of CO_2 transfer from the intercellular airspaces to the site of carboxylation"

Definitions from Yin (2009, 2011):

J_{MAX} – The maximum value of *J* under saturated light (Yin 2009)
$S_{C/O}$– The relative CO_2 / O_2 specificity factor for Rubsico (Peterson 1989) (Yin 2009)
VC_{MAX} - Maximum rate of Rubisco activity-limited carboxylation
(Yin2009)
T_P - Rate of triose phosphate export from the chloroplast (Yin 2009)

Other useful graphs:

***J* / C_i graph or ETR vs. C_i.** This is used to determine whether to use the variable or constant *J* method for determining g_m.
A /C_C graph. This is a more reliable form of the A/ C_i curve that works better at high temperatures.
g_m / A, g_m / C_i, g_m / g_s curves are also commonly used.
g_m / Temperature graph -10ºC to 45ºC , with the graph showing 0 to 45ºC
This is useful for determining the limiting effect of g_m.

Leaf Absorptance Measurement

Measuring leaf absorptance is now possible over the entire PAR range. Leaf transmittance is also measured for the most reliable absorptance measurement.

J, or electron transport rate, should never be used for comparing different leaves without measuring leaf absorptance (Baker N. 2008) for the following reasons: absorptance can vary with plant stress level, it can vary by species, leaf age, chlorophyll content (Eichelman H. 2004), (Evans 2001) and light intensity (Cazzaniga *et al*., 2013). In addition, electrons can flow to other electron sinks other that photosynthesis like photorespiration. This has been found in plants under chilled conditions (Fryer 1998) and in C_3 plants under drought conditions (Flexas 1999, 2000). Eichelman (2004) reports leaf absorptance variations from 0.7 to 0.9 on samples tested. Recently, Cazzaniga *et al.* (2013) found the chloroplast migration changed leaf transmittance at high actinic light levels.

Using papers written by J.R. Evans & H.Poorter (2001) and C. J. Bernaccchi C. Pimentel, & S. P. Long (2003) as a guide for measuring absorptance, we used an integrated sphere, the white actinic light source used in the i*FL*, and a scanning spectrophotometer to measure leaf reflectance, leaf transmittance and leaf absorptance. This was then compared to measurements made in the i*FL* leaf chamber. After calibration, differences between the two methods were in the range of electronic noise. Since the i*FL* uses a white actinic light source, RGB sensors are located above the leaf, and below the leaf to measure leaf transmission.

Red, green, and blue sensors, located above the leaf, are used to measure reflectance. Red, green and blue sensors are also located under the leaf to measure leaf transmission for the most reliable leaf absorptance measurement. The change in leaf transmittance after chloroplast migration can be measured.

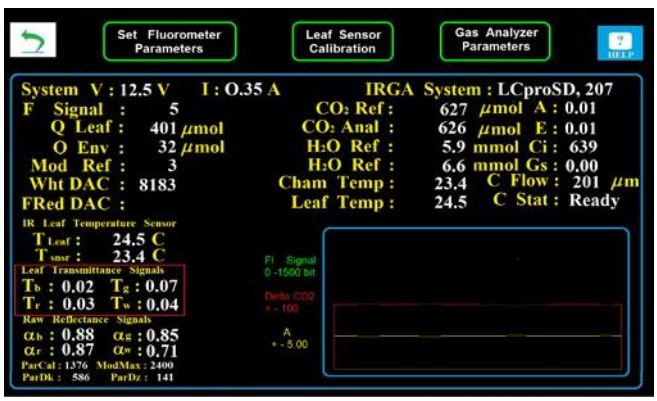
$$J = \Phi PSII \cdot Q \cdot \alpha \cdot \beta$$

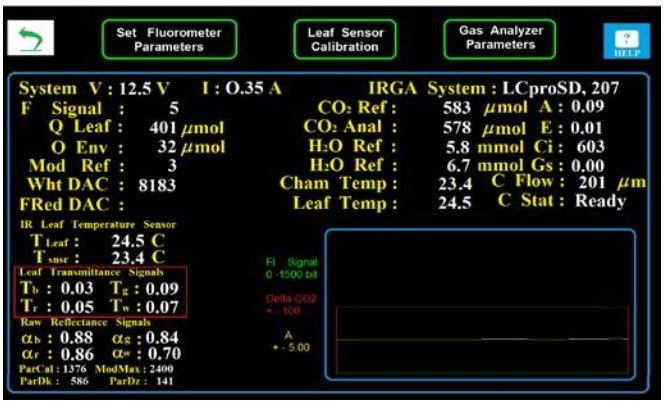
$$\alpha = ((\alpha_r)(B_r)+(\alpha_b)(B_b)+(\alpha_g)(B_g)) - (T_r/I_r + T_b/I_b + T_g/I_g)$$

J = electron transport, Φ_{PSII} = Y(II) or yield of PSII (F_M' - F_S / F_M'), Q = PAR or photosynthetically active radiation at the leaf, a is leaf absorptance using the equation shown above. β is the ratio of PSII reaction centres to PSI reaction centres in the leaf. The spectral characteristics of the White light LED are determined at the factory. They do not change significantly with light intensity or with age. Calibration is done with a white and black sample supplied with the i*FL*. While calibration may be checked from time to time it should hold for at least several weeks. α is derived in the following way: The amount of red, blue and green-yellow radiation incident on the leaf is measured, B_r, B_b & B_g are the fractions of each spectral range incident on the leaf, determined at the factory; α_r , α_b , α_g , or absorptance in each spectral range, are determined by measuring the amount of light

reflected from the leaf in each spectral range, and subtracting that amount from the light incident in each spectral range on the leaf; then, the amount of light transmitted through the leaf, in each spectral range, is measured (represented by T_r ,T_b,& T_g). The values I_r, I_b ,I_g are the raw red, blue and green-yellow radiation incident on the leaf. The light transmitted through the leaf is then subtracted from the equation. The result is a reliable leaf absorptance measurement, and much more reliable *J*, g_m and C_C measurements.

The ratio of PSII reaction centres to PSI reaction centres varies from 0.4 in some C_4 plants to 0.6 in some C_3 plants (Edwards 1993, Laisk 1996,). While 0.5 is sometimes used for an average value, the most used method for measuring the ratio of PSII to PSI, involves the use of spectral analysis of samples at 77°K (Anderson 1999), (Zell 2010); This ratio varies by type of plant, C_3 or C_4, by plant species, by sun grown leaves vs. shade leaves, and in carbon deficient leaves.





The change in leaf absorptance can be demonstrated in the diagnostics part of the program shown above. By subjecting a leaf in the chamber to near saturation light intensity, watch the change in leaf transmission as a plant goes from a dark adapted state to full chloroplast migration.

The screen above on the left shows leaf transmittance values in the blue, green and red parts of the spectrum on an indoor leaf, at near saturation light levels of 400 µmol, after 4 minutes. The screen on the right shows transmittance values after full chloroplast migration, or after 35 minutes. Notice the change; leaf absorptance changes with light intensity (Cazzaniga *et al*., 2013), (Dall'Osto 2014).

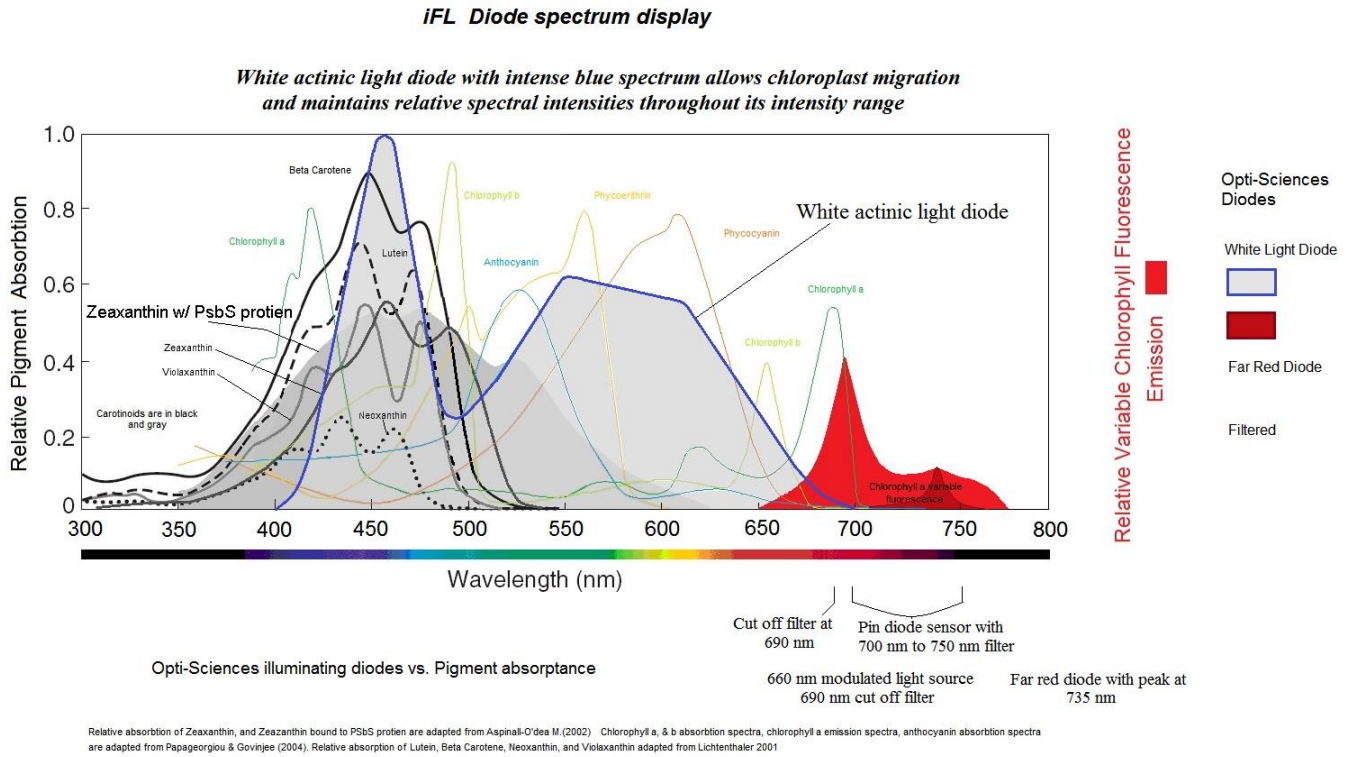
Here, T_b is the portion of leaf transmittance in the blue spectrum, T_g is the portion of leaf transmittance in the green spectrum, and T_r is the portion of leaf transmittance in the red spectrum. α_b, α_g , α_r, values represent leaf absorptance using reflectance only in their respective colour ranges. Absorptance displayed in all protocols use both reflectance and transmittance values for calculation. The white light values T_w, and α_w are provided by the sensors but not used in any calculations. They are only displayed here. Touch the screen on the lower left hand side and more screens will appear:

Absorptance as shown from the main measuring screen.

While in either the combined fluorescence mode may be viewed by touching the green window on the lower left hand side of the main measuring screen. Tap the window with a finger and the four screens shown below will scroll. The first window is calculated data, the second window represents raw measured data, the third window, quenching data, and the fourth window will be data from all measurements in this file. Up to 8 parameters may be selected for display at the same time. In the Y(II) protocol, absorptance may be viewed on the main measuring screen as α_{meas} .

Notice α in the upper right hand window.

iFL Spectral chart



The Graph above shows relative plant pigment light absorption, chlorophyll fluorescence spectrum, the iFL white light LED spectrum and the Far red light LED spectrum.

Plants grow better under white light than under just red and blue light. In addition, some plants developed foliar edema when grown under just red and blue illumination (Massa 2008). However, the most important reason for an intense blue spectrum-white light illumination is to allow chloroplast migration to occur as it does in nature. Chloroplast migration is responsible for about 30% of NPQ at higher light levels, and it does not occur significantly with intense red light (Cazzaniga *et al*., 2013), (Dall'Osta 2014).

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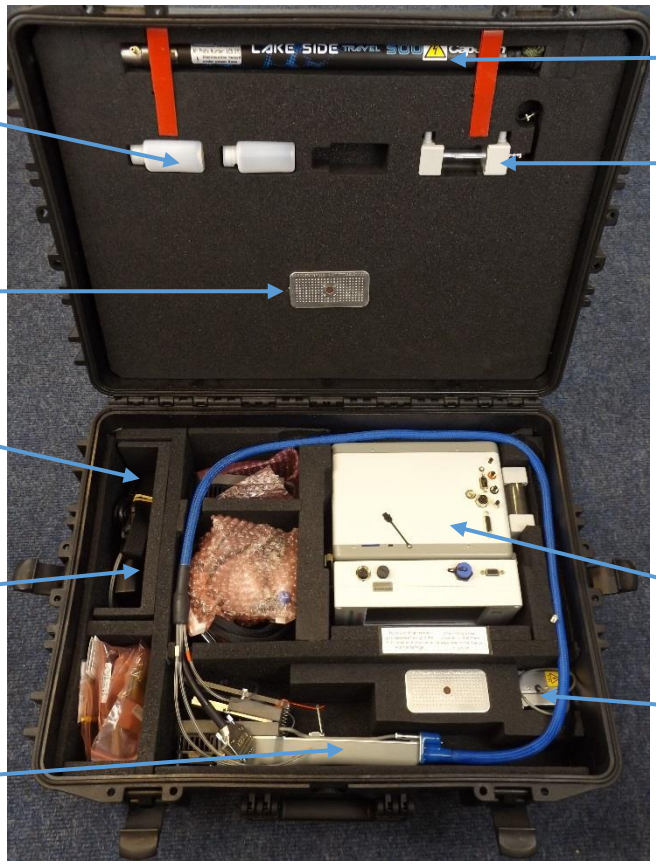
Using the LCpro-SD without the i*FL*

Section 1. Introduction

This manual covers the operation and maintenance of the LCpro-SD Portable Photosynthesis System. This part of the manual is to be used when the iFL chlorophyll fluorometer is not being used. It contains gas exchange equations, constants, appendices on relevant gas exchange topics, definitions, and recommendations for proper use. It provides information about the battery, CO_2 cartridges, chemicals, thermistor use, using calculated temperature, and other topics. Review the table of contents to find the section of interest.

Equipment list (without the iFL parts)

The LCpro-SD Analyser is supplied in a convenient carrying case containing the following items.
In addition to the above items there is a boxed "Spares kit" M.SKF-118 which contains some of the spares listed in Appendix 9.



NOTE the cord must be stored in exactly this way.
Tuck the cord into the recess in the foam all the way around the edge, to prevent damage.

Please note – The supplied carry case is for 'By Hand' transportation only, if the instrument is being shipped by courier it is highly recommended that suitable packaging, such as a cardboard box filled with padding material, is used to protect both the case and instrument.

Description

The LCpro-SD is specifically designed for portability and field use, and provides internal battery power suitable for between 4 to 16 hours of continuous operation, depending on the functions used. Its purpose is to measure and control the environment of a leaf contained in the chamber, and to calculate the photosynthetic activity of the leaf or, when used with a soil pot, to measure the gas exchange associated with soil biomass respiration.

The instrument comprises a main console with signal conditioning, air supply, microprocessor control, SD card data storage, a 5-button keypad and a leaf chamber connected by an umbilical cord. Where applicable the chamber is equipped with temperature control and a removable light unit. The main console supplies air with controllable CO_2 and H_2O concentrations to the chamber at a measured rate. The CO_2 and H_2O concentrations are measured, and the air is directed over both surfaces of the leaf. The discharged air leaving the chamber is analysed, and its (generally decreased) CO_2 content and (increased) H_2O content determined. In the case of the soil pot the CO_2 concentration of the air entering the hood is measured and the discharged air that has passed over the soil is analysed and its (generally increased) CO_2 content is analysed. An excess of air is provided to the hood over that extracted, and a pressure relief vent ensures that the hood is not pressurised as this would interfere with the gas exchange at the soil/air interface.

From the differences in gas concentration and the airflow rate, the assimilation and transpiration rates are continuously calculated with a complete analysis cycle taking approximately 16-20 seconds. A small fan in the chamber ensures thorough mixing of the air around the leaf. Measurement of CO_2 is by an infrared gas analyser (IRGA). H_2O measurement is by two high quality humidity sensors. Similarly with the soil pot these measurements are used to calculate soil respiration. The system also measures leaf (or soil) temperature, chamber air temperature, PAR (Photosynthetically Active Radiation), and atmospheric pressure. The PAR level at the leaf and the radiant energy balance of the leaf are calculated, see appendix 3.

Measured and calculated data is displayed on the Liquid Crystal Display (LCD) on the front panel of the console. The first three screens of data, (as listed in the Log? column of Appendix 1), can be logged on to a SD memory card. The card, which is located in a socket at the front of the unit, can be removed by pressing the card. The stored log (file) can be viewed on the display, sent via the serial link to a PC or a printer, or loaded directly into a spreadsheet on a PC equipped with a SD card socket.

The measurements are carried out in an ‘Open System’ configuration in which fresh gas (air) is passed through the PLC (Plant Leaf Chamber) or soil pot on a continuous basis. Measurements are carried out on the state of the incoming gas (the ‘reference’ levels) and after passing the leaf/soil specimen (the ‘analysis’ levels); the gas is then vented away. This arrangement tolerates some outward gas leakage and ad/absorption by the materials used in the gas path.

By comparison, in a ‘Closed System’, a gas sample is continuously circulated and measured over a period of time to establish rates of change in the parameters measured. This is therefore less tolerant to leakage and material ad/absorption.
Further information on photosynthesis and the measurement of it can be found in

"Photosynthesis" by Hall and Rao, Pub. Cambridge University Press
"Plant Physiological Ecology field methods and instrumentation" by Pearcy, Ehleringer, Mooney and Rundel, Pub. Chapman and Hall
"Techniques in Bioproductivity and Photosynthesis" by Hall, Long and Scurlock, Pub. Pergamon Press.

Further information on soil respiration and the measurement of it can be found in

"Quantitative Comparison of In Situ Soil CO2 Flux Measurement Methods" by Knoepp and Vose. Research Paper.

The Internal Calculations

A complete list of Units and Symbols used, either for display, or for the purpose of calculations, are given in Appendix 1.

A number of internal calculations are performed repetitively using the measured parameters and various correction factors. These produce intermediate results and values for various photosynthetic parameters derived from established formulae. Derivations for these and the soil respiration calculations are given in Appendix 3.

The calculated values are displayed on the screen to serve their main purpose of providing a check on the validity of the measured data. This is useful for reference just before a record is taken, and as a means of checking that the leaf is photosynthetically stable or equilibrium is reached in the soil pot.

For a typical leaf, CO_2 flux will be between -10 to +100 µmol m^{-2} s^{-1} and H_2O flux will be between 0 to 15mol m^{-2} s^{-1}. The analyser performs some checks on magnitudes of readings, particularly of certain settings, which have pre-set limits (for example minimum airflow rate). There is, however, a wide tolerance on 'allowable' settings for which the user is responsible (for example leaf area), and which can significantly affect the validity of the photosynthesis measurements.

Section 2. Getting Started

Note this section assumes that the Broad Leaf Chamber, supplied with iFL, is being used.

2.1 Checking the Chemical Columns

The removable column nearest to the connectors optionally is either empty (for ambient H_2O use) or, using the column with the built-in temperature probe, holds iron sulphate (7 hydrate), which is used to increase the water concentration of air supplied to the leaf chamber.

The middle column is used to decrease the water concentration and should contain a drying chemical such as 'Drierite'. (Drierite is recommended as it contains an indicator).

The furthest column from the connectors contains soda lime, which is used to strip carbon dioxide. This is used both as a zero gas, and as a diluent when controlling the CO_2 concentration in climate control mode. On delivery the soda lime column is filled with an indicating Soda Lime, which is recommended.
To maintain the performance of the LCpro-SD, always replenish the Soda Lime when the colour first changes from white to violet. This colour change indicates that the soda lime is becoming exhausted for practical purposes. **See also section 6.1.**

IMPORTANT NOTE: The soda lime colour change is temporarily reversible until it is fully depleted.

To remove a column pull it outwards at the top and bottom, lift off the top cap, and fill it to just below the column top with the chemical. Compact the chemical by tapping the column on a solid surface several times. Make sure that there are no chemical granules remaining on the top edge of the column. Replace the top cap and refit the assembly. Ensure that all 'O' rings are lightly greased with the silicone grease supplied and that both ends are located tightly to prevent gas leaks.

2.2 Initial Preparation and air supply

If the chamber has been left with the jaws closed for a few hours or more, the gaskets will need to reform. This is achieved by leaving the jaws open for at least half an hour before use (longer for badly flattened gaskets). In severe cases, it might be necessary to replace the gaskets, which are self-adhesive, but it is recommended first to leave the jaws open overnight, ideally somewhere warm to make certain the gaskets will not recover before replacing. (See Appendix 9 - Spares & Accessories) for part numbers)

With the chemicals installed, the leaf chamber is connected to the analyser with the 26-pin plug, and the three pieces of tubing (matching the three coloured sleeves on the pipes to the entries). Be careful not to put the instrument on a soft surface with these entries downwards, as they may become blocked, e.g. with soil.

The LCpro-SD system requires a fresh air supply and preferably one that will not be affected by operator breathing or local crop conditions as far as CO_2 &/or H_2O levels are concerned. The air supply should be taken from a region where the levels are reasonably stable, preferably 3-4 meters above ground level.

The ADC air probe attached to the ground spike inserted into the ground provides such an arrangement, drawing air in 3 metres above ground level. The Air probe can also be fixed to a tripod. In use, the probe should be extended to its full length. Note that the air probe type LC4-070 shipped up to the end of 2015 is glass fibre, but its replacement type, LC5-070 is carbon fibre, which is conductive and should not be extended under overhead high voltage power lines due to the danger of electrocution. For laboratory use, a length of tube to the outside of the building away from traffic and chimneys will normally suffice. Good buffering against ambient changes can be obtained with a plastic 25-litre container by making two gas chance of water reaching the LCpro-SD. A much smaller volume, typically 4.5 litres may be used to buffer an external air supply used while working inside, see appendix 7.

The instrument contains connections in the lid, and arranging for the inlet pipe to reach to the bottom. This ensures maximum mixing and minimum an internal hydrophobic filter, which will protect against both dust and water, but this will quickly become blocked if the air supply is dusty and an external filter is not fitted. This is especially true in CO_2 climate control mode, when the instrument continuously draws in high flows (about 720 ml/min) of fresh air. To minimise this problem, a plastic bodied filter is supplied in the spares kit and should be fitted using 6mm bore PVC pipe as an adapter, in the air supply pipe, either at the instrument end or the far end, as convenient. Alternatively, there is a metal-bodied filter which is smaller but not so effective for dust. If used, fit it with the largest part of the body on the inlet side.

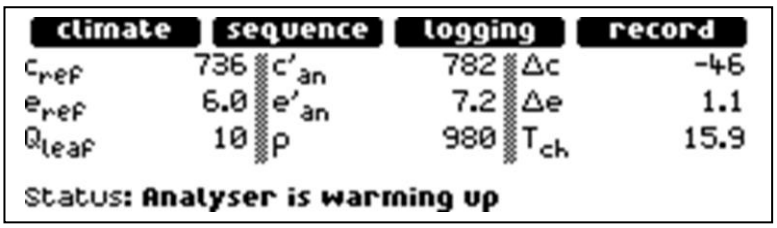
If elevated CO_2 is needed, a cylinder of gas should be fitted in the holder under the battery cover, see section 6.6.

2.3 Switching On

The LCpro-SD is delivered ready for use with the internal battery fully charged and connected and the Soda-lime and Drier columns filled. The LCpro-SD defaults to the factory-installed settings, some of which may need to be changed for immediate use (e.g. time & date, serial link). It can be switched ON by pressing the front panel switch key at the top right-hand side of the display (shown in the text as |).

Note that after first switching on, the LCpro-SD requires about five minutes for CO_2 measurements to stabilise. It will display an 'analyser is warming up' message during this time, and will beep when it is ready. If you wish to bypass the warm up timer, press the left button just after the LCpro-SD has been switched on and is displaying the software version and serial number. Note that if warm-up is bypassed the LCpro-SD will need to complete at least one full analysis cycle before normal readings are displayed.
A few seconds after switch-on, the screen displays a set of parameters and values. Pressing the 'page' or 'on' key will cycle between the three main pages. Appendix 7 – Menu Structure shows all the pages and how they are related. The **'**function**'** headings along the top correspond to keys on the keypad above. The power off key is the only means of turning off (apart from disconnecting the battery). If the screen is very light or dark the contrast may need adjusting (see section 2.5).

Screensho



Close the leaf chamber head, check that the chamber fan is rotating (usually it can be heard) and check that the chamber gaskets are sealing. (It may take a few minutes before new gaskets are compressed sufficiently by the spring. In the case of Conifer chambers, ensure that the clip is latched).

2.4 Electrical Connections

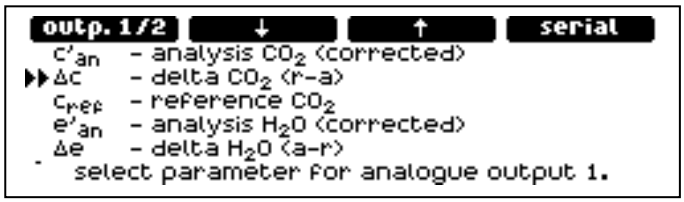
(See picture on page 13 for locations)

Power Socket

This is provided for an external 12-volt supply or the battery charger connection, and is current limited. Reverse current flow is not prevented, which allows you to power external equipment from the LCpro-SD battery provided that the power requirements are modest.

This socket also provides two analogue output channels of 0-5V, being voltage sources intended for connection to a high input impedance (1MΩ) recorder channel. They are protected against an accidental short circuit to ground.
The connections are: Channel one = pin 4 Channel two = pin 1 0V ground = pin 5

On entry to the output menu page output 1 is shown.
The analogue output parameters are selected from the output menu page. (See screenshot). The outp1/2 key toggles between output 1 & 2 and the ↑ & ↓ keys are used to select the parameter required.
(See "An o/p column in appendix 1 for available parameters).



The power socket mates with a standard 5 pin 240° DIN audio plug connector. This is provided in the spares kit, pre-wired as follows:

braid	= signal ground (0V)
blue lead	= analogue 1 signal
red lead	= analogue 2 signal
red 4mm shielded plug	= +12V.DC
black 4mm shielded plug	= 0V

USB Connector

The USB Mini B connector mates with a standard USB A to USB Mini B connector often used with digital cameras. When connected to a PC the LCpro-SD looks like a drive and data files may be dragged and dropped.

RS232C Connector

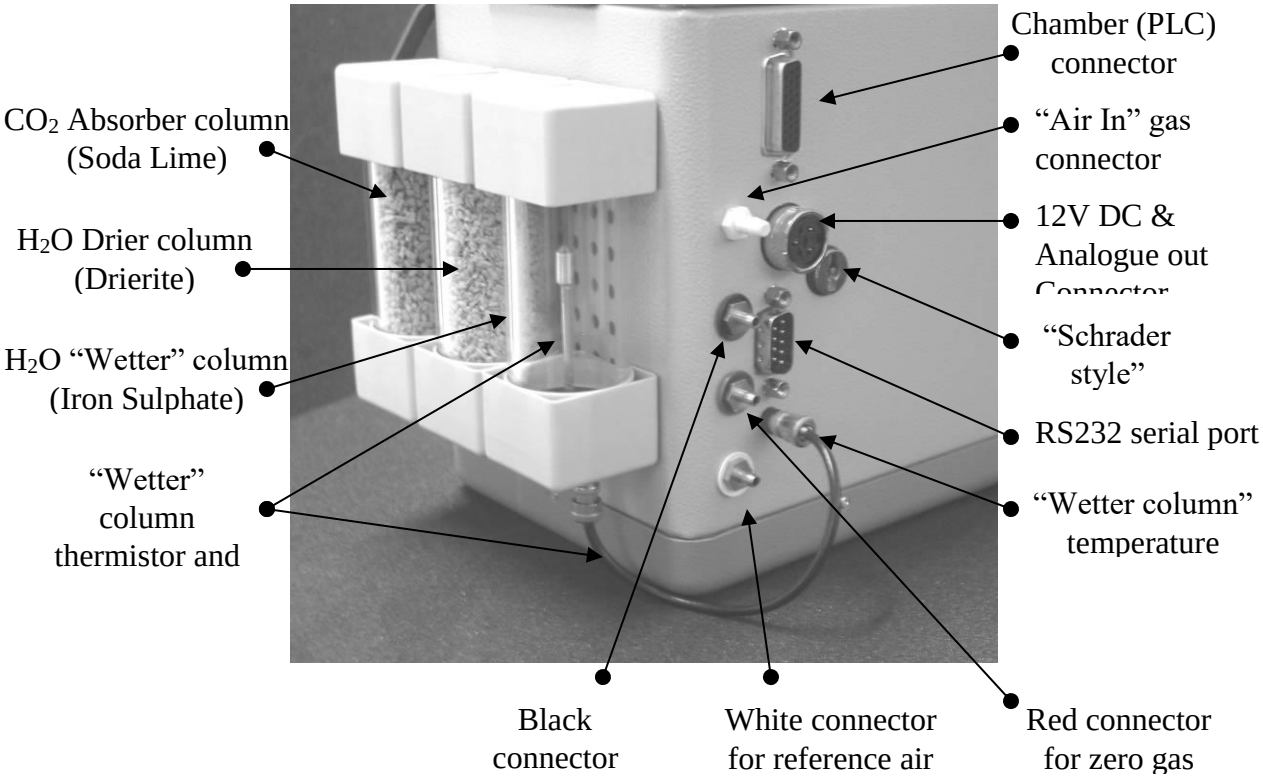
The RS232C connector mates with a standard 9 pin 'D' type null-modem serial link cable socket (female). A suitable cable is included in the spares kit. It provides RS232C signals and handshake lines to suit standard printers, VDU's, PC's etc. The user can set the baud rate and handshake protocols. The socket connectivity is PC standard.

Column Temperature connector

It is intended that the column assembly fitted with the temperature probe is used with the humidifier chemical (iron sulphate), and connected to this socket with the lead assembly supplied. This allows the instrument to calculate the maximum humidity level available, and set the mix ratio accordingly for any lower level which may be requested.

2.5 Display

The display unit is a LCD type with an adjustable contrast control. If the user prefers a different contrast level, adjustment is available on the PCA-288 'digital' board. using potentiometer RV102 (Located in the right hand corner of the board).
(See section 16.2 for how to gain access to the potentiometer).



2.6 Error, Warning & Status Messages

If after switch on, the screen remains blank – check the battery is fitted &/or charged.

A number of parameters are displayed on the screen, including values for CO_2 & H_2O. With the jaws shut and no leaf in the chamber, CO_2anl should equal ambient CO_2ref and H_2Oanl should equal ambient H_2Oref.
PAR should also reflect ambient conditions while T_{ch} (chamber temperature) will initially equal ambient temperature but will rise 3-4 degrees above it after a while due to the local heating effect of the infrared source and electronics.

If the instrument is switched on but does not respond to key presses, pressing simultaneously the page key (top right) and the two leftmost keys will invoke a hardware reset.

Status messages are those that indicate the functional state of the LCpro-SD, and are generally associated with time-consuming tasks that are occupying the processor, and during which time other normal functions are suspended. Since these messages usually relate to the function or facility involved, they should not be disturbed in the meantime. For example, do not disable the printer whilst the 'printing record' status message is on the screen.

A warning message indicates that it is not possible to comply with a user request. The text of the message always describes why compliance is not possible, offering the user the opportunity to correct the situation.

Warning messages usually appear with an OK function label, which, if operated, will allow the user to continue anyway.

2.7 Low Battery Voltage

The internal battery voltage is monitored to detect if the battery is close to being fully discharged. This occurs at 10.8 volts, whereupon a **'Battery Low'** warning message is overwritten on the screen.
At this point, there is typically about 5 minutes life left in the battery. This should be sufficiently long for the user to either connect a charger or complete his current record. If the warning message is ignored the LCpro-SD will switch itself off when the battery voltage has fallen to 10.5 volts!
The battery power is shown as a bar graph at the bottom of screen page 3, and numerically on the diagnostics page. The battery should be recharged (see section 6.3) after any significant period of use or if it is less than 12V

2.8 Fitting/changing a chamber

This information applies to all chamber types and to the Soil pot.
Note that only the "conventional" Broad, Narrow and Conifer leaf chambers have separate upper and lower jaws whilst the Arabidopsis and Small leaf chambers and the Soil pot feature an "interface block". All references to "jaw" in this section also apply to the "interface block" where applicable.

Description

The jaw is fitted to the handle using three captive screws. The three screws carry the analysis stirrer fan signal, the built-in leaf thermistor (where applicable – see next paragraph) and the ground return.

Note that only the Broad and Narrow leaf chamber jaws feature the built-in leaf thermistor. The Conifer leaf chamber jaw, Arabidopsis and Small leaf interface blocks and the Soil pot do not feature a built-in thermistor and the screw is grounded.
In all cases care should be taken as described below.

Fitting a chamber (see photographs on the following two pages)

Before fitting the chamber, check to see that the five "O" rings are all in place (two gas stems and three sensor housings) and that the three captive spacers are free to move and not engaged on the screw threads. (There should be about a millimetre free play up and down the shaft of the screw). There is a spare set of 'O' rings in the spares kit.
When fitting a chamber, press down on the jaw itself then turn the three captive screws evenly USING THE FINGERS OR AN ADC JAW SCREW ADJUSTMENT TOOL ONLY.

DO NOT USE A SCREWDRIVER OR COIN as overtightening the screws may destroy the electrical connections through to the circuit board inside.

DO NOT PUSH DOWN ON THE SCREWS as this may dislodge the mounting bushes from the handle baseplate, causing loss of the electrical connections. To ensure that the jaw forms a good gas-tight seal it is only necessary to push down on the jaw itself.

Removing a chamber (see photographs on the following two pages)

When removing a chamber it is permissable to use a small coin to unscrew the three captive screws but again it should be stressed that no downwards pressure should be applied to the screwheads.

Check to ensure that the small "O" rings on the two gas stems in the handle baseplate remain in place and are not carried away in the jaw.

Removing and fitting jaws on the LCPro-SD.

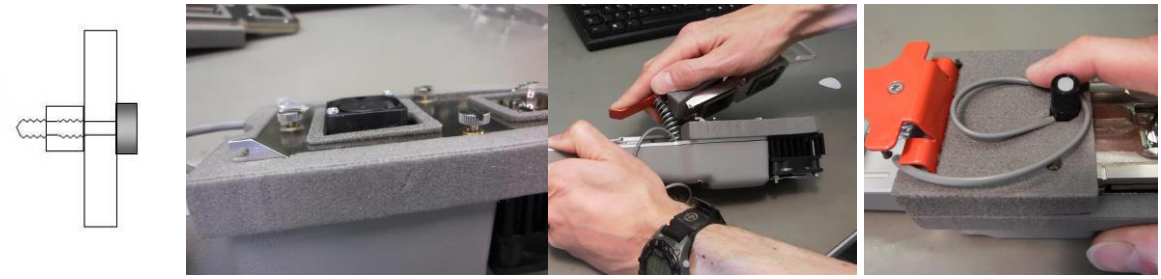
Broad jaws, upper and lower, collectively known as the chamber (first photograph above left).
Remove from the handle as follows…
Remove the radiation shield, pull out the PAR sensor then remove the upper jaw as shown.

Use the correct tool (LCi-220) to undo the screws, remove the cable from under the cable clip (LCM-166) and pull the peltier plug out of its socket. If this is difficult then push the plug against the socket, keep it held tightly together (it consists of two parts) and pull it out. DO NOT PULL THE CABLE!

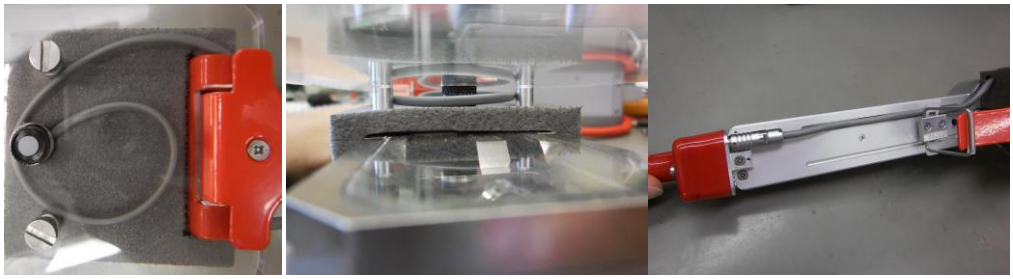
Remove the lower jaw and retrieve any o rings that have come off with the jaw (a cocktail stick or blunt tweezers is ideal), take care not to damage the o rings.
Ensure the spacers/o rings are present and correct on the handle as shown above in the second photograph.

A jaw may now be fitted to the handle…

Make sure the jaw screws each have a threaded nylon spacer, and that each spacer is fully threaded onto the screws as far as they will go, the jaw screw spacers are half threaded and must go onto the screws as shown in the first photograph below:



Push the lower jaw onto the handle, tighten each screw in turn by hand then with the jaw screw tool (LCi-220) until the tool just starts to bend. **Do not over tighten with a coin/screwdriver.**
Attach the jaw spring to the upper jaw and fit as shown above (third photograph).
Mount the PAR sensor as shown in the final photograph above, with the cable laid as shown.



Fit the radiation shield as shown above, taking care not to trap the PAR cable.
Finally plug in the peltier cable, tuck it under the cable clip (LCM-166), and latch the jaw lever open if the jaws are not to be used right away.

Always inspect the gaskets on a closed jaw before use to make sure that there are no gaps, gaps may be reduced by temporary use of an elastic band around the jaw or by squeezing and releasing the jaw by hand to compress the foam gaskets.

The Jaw Screw Adjustment Tool (LCi-220).

Tighten the screw as shown opposite , until the tool just starts to bend and then stop.

Careful use of the jaw screw adjustment tool, will ensure the screws are tight enough for the O rings to make a good seal, but not tight enough to break a delicate wire just under the screw head.

Section 3. The Leaf Chamber

3.1 General Description

The PLC consists of a handle and an interchangeable leaf chamber or soil pot (See section 5 for Soil pot description). There are five styles of leaf chamber available. These are Arabidopsis, Small, Broad, Narrow and Conifer leaves. Refer to section 4 for a description of Arabidopsis and Small leaf chambers, which are very different in design to the other chambers. The Broad, Narrow and Conifer leaf chambers consist of a top jaw, which may be fitted with a radiation shield or light unit, and a bottom jaw containing a Peltier cooler (see paragraph 3.6). Broad and Narrow leaf chambers also feature a built-in leaf thermistor commonly known as a "Spider" and "Snail" respectively because of their appearance (see paragraph 3.5).

The handle contains a jack socket for use with a detachable leaf temperature sensor, a 'record' switch, and an electronics board providing sensor amplifiers for signals to the LCpro-SD console.
A cable is attached to the handle linking the electrical signals and gas lines to the LCpro-SD console. Repeated flexing can break the cable. When storing the chamber try to avoid tight bends especially where the cable joins the handle and the "D"-type plug and do not store the cable by wrapping it around the handle.

To fit the light unit instead of the radiation shield (which is fitted as standard to Broad, Narrow and Conifer leaf chambers), remove the two slotted and knurled screws. The spacers underneath are held captive with threads. The method of mounting the light unit depends on the type of leaf chamber. (See also section 3.7).

If the chamber is used without the radiation shield, the transmission loss (T_{rw}) will be for the window only and therefore lower than the default values, which are for window and shield combined. This also applies if the light unit is fitted. (See section 8 for alternative values).

In order to access or change any parts of the chamber, loosen the radiation shield mounting screws a few turns (it is not necessary to remove them completely). Lift off the radiation shield and pull the PAR sensor off its mounting plate. While holding the jaw fully open against the spring, twist it slightly so that the hinge pin slides out on the slotted side. To change a chamber or access the temperature and humidity sensors, the three fixing screws

and lower jaw can now be unscrewed about 6 turns. It is not necessary to completely remove them, as they remain captive in the jaw. When replacing the jaws, note that the coin slots in the knurled screws are intended to assist with removal rather than tightening: Finger tight is generally sufficient and over-tightening should be avoided as damage may be caused.

If the chamber is changed, it is necessary to inform the analyser using configure setup and change + or change – until the chamber type displayed matches the chamber type fitted. In so doing, the appropriate factory default values of r_b, H_{fac}, and T_{rw} (see below and section 3.3) are automatically chosen, and they may then be individually adjusted if required. Any changes made to the values are saved at power off.

The Broad chamber has a square (6.25cm^2) aperture sealed around the edge, and can be used for any flat leaf, whether the leaf fills the aperture or not.

The Narrow chamber has a rectangular (5.8cm^2) aperture sealed around the edge, and can be used for long flat leaves, i.e. grasses etc.

The Conifer chamber is cylindrical in design with sealed edges and can be used for non-flat plant material e.g. conifer needles, small fruits etc.

3.2 Operation

To minimise noise on the measurements, the chamber should be held as steadily as possible during the measurement. To assist the user in this, the underside of the chamber has a thread for a ¼" Whitworth tripod screw.

Prior to taking measurements on a leaf, the chamber sensors can be checked as follows. With the chamber closed, after a few seconds the CO_2ref & CO_2anl readings on the LCpro-SD display should stabilise to give similar CO_2 levels. The H_2O levels should also be checked for similarity and that the PAR and chamber temperatures (Tch) readings are in accordance with ambient conditions.

If these checks are satisfactory, leaf measurements can be made.

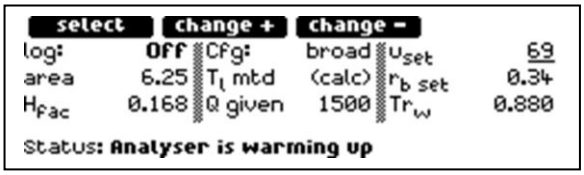
Once the leaf is enclosed in the chamber, it may take up to 2 minutes to re-adjust to its new microclimate. During this period CO_2 & H_2O values will gradually stabilise. Generally a good indication of stability is when the value for Ci (substomatal CO_2) has stabilised.

After readings are stable, a 'record' may be taken (see section 12.2).

3.3 Leaf Chamber Constants

The design of the leaf chamber affects various parameters, which are constants for a particular design or type. These include 'r_b' (boundary layer resistance), 'H_{factor}' (the level of radiation energy affecting the leaf (referred to as 'Trans' on LCA2 & 3)), and 'Tr_w' (the transmission factor of the chamber windows to PAR). These constants may be changed with configure set up select . When the appropriate parameter is underlined, it can be modified with the change + and change – keys.

Screenshot:



'r_b' The value for 'rb' is influenced by the efficiency of gas mixing within the chamber, ab/ad-sorption of CO_2/H_2O of the materials used, and 'dead' volume. [see Section 8.1 for typical values].

'H_{factor}' Previously defined as 'Trans' in LCA2 & 3 references, Hfactor is affected by the material used for the shield (if fitted) and the chamber window. This is due to the different transmission factors at the wavelengths in the visible and infrared regions, the position of the PAR sensor (inside or outside the chamber), and the type of light source. [see Appendix 4].

'Tr_w' On the chambers, the measurement of PAR is via a sensor mounted on the shield above the window. The value for PAR at the leaf (Q_{leaf}) is therefore less than that measured (Q) by factor 'Tr_w' – the transmission factor of PAR introduced by the arrangement of the chamber shield &/or window.

See Section 8 for typical values.

3.4 Leaf thermistor

The leaf temperature may be measured as an alternative to calculating it. The parameter used to switch between the two options is *Tl mtd* in the configure setup menu. In general, if the Broad chamber has a large broad leaf with a known area, or is so big that it completely fills the chamber, so that its area is 6.25cm^2, then calculated leaf temperature is best. If the area is uncertain, e.g. conifers, the temperature will need to be measured. This measurement is made with a microchip thermistor attached to a jack plug with thin wires (supplied). The plug connects to a socket on the chamber handle and the thermistor is rested on the leaf with the wires trapped between the jaws together with the leaf. To hold the thermistor in position it is sometimes easier to insert it in a small cut made in the leaf with a scalpel, or by taping the wires to the edge of the chamber. Broad and Narrow style chambers offer an additional integral microchip thermistor mounted in the leaf chamber. (see paragraph 3.5). This integral thermistor is disabled if the external thermistor is plugged in.

3.5 Leaf Spider

(Broad and Narrow style heads only)

The integral thermistor is a similar microchip on a springy ‘spider’ mounting that is fitted in the Broad and Narrow jaws and touches the underside of the leaf. It is held in place in the lower jaw with two pins. To fit it, remove the top jaw, connect the horizontal pin of the spider by pushing on the back of the connector socket. When it is fully connected, align the vertical connector with its mating pin, and push it together by pressing on the back of the connector socket. To remove the spider, use a pair of thin nose pliers with serrated jaws, or a strong pair of tweezers with serrated jaws. Hold the end of the vertical socket and pull it off gently, ensuring that there is no sudden snatch when it finally disengages. Then hold the horizontal socket between the two plates and pull the connector off about 1mm. With a cocktail stick or similar, hold the free side of the spider above the edge of the jaw, whilst pushing apart the connector with another stick.

3.6 Chamber Cooler

Broad, Narrow and Conifer chambers are fitted as standard with a Peltier heat exchange module. The module is fixed directly under the chamber and controls the air within the chamber to a user-selected temperature. In practice this allows temperatures that deviate by a minimum of 10°C from ambient to be achieved. Heat from the module is dissipated at the back with a heatsink and fan. The temperature of the module is directly monitored with a thermistor, independent of the chamber thermistor.

3.7 Light Units

Light units are available suitable for Broad, Narrow and Conifer jaws. They slide or clip onto the top jaw, (after removing the shield), with the lead towards the cable end of the chamber. The cable rests in the narrower of the two grooves in the handle, and it plugs into the adjacent socket. Align the red dots before plugging in. To remove the plug, push in lightly on the end of the connector to depress the spring latch then pull back on the section of the body that has the 4 grooves and a red dot whilst still pushing lightly on the end.

WARNING. The plug will not disengage if you pull on the cable or the rear section of the plug that has the two flats. Pulling on the cable may cause damage.

The Broad and Narrow light unit is removed by first disconnecting the plug, then lifting the spring loaded metal tab on top, above the cable. With the tab lifted, the light unit can be pushed off the jaw. Once the light unit has moved about 2mm, it is not necessary to continue to hold the tab up. The Conifer light unit includes a hood to prevent outside light from entering the chamber through the lower jaw and uses plastic clips to keep it in position. It can be fitted/removed with a moderate pressure.

When the light unit is attached to the top jaw, the join is not totally light proof. For the most accurate results, shield the light unit from direct sunlight when in use, especially when the light unit is running at low light levels.

With a light unit fitted, the transmission loss (T_{rw}) will be for the window only. (See the table below "T_{rw}" in section 8).

The diode array contains blue LED's at 470nm wavelength and red ones at 660nm. Between 5-10% of the photons are blue. The output of the array is monitored with a light sensor, which adjusts the power, so that the light output is constant at the value set by the user.

The Broad and Narrow light units can produce up to 2000µmol of light, and the Conifer can produce up to 1500µmol. The software will set these limits as appropriate provided that the correct chamber type has been selected in the config menu.

If the chamber type has not been set to Conifer, but a Conifer lamp is fitted; the software will allow the user to enter levels as high as 2000µmols without apparent error. In this instance levels greater than 1500 may be set but not achieved due to the higher power demand of the Conifer light unit's larger LED array, and there is the risk that the light power supply may be damaged by the excessive loading.

Conversely, if the chamber type has been set to Conifer but a Broad or Narrow type lamp is fitted, the software will not allow levels above 1500µmols to be set.

WARNING

The console supplies all the light units with around 110V, current limited. Be sure that the light unit is not operated in conditions where moisture could condense or drop on it. Do not poke conductive material inside it during operation as this could result in an electrical shock to the operator.

3.8 Options and Q options

The Q menu page has two buttons, (hold Q and release Q) that hold or release the PAR (Q_{leaf}) reading. This feature is not needed with the light unit supplied with the system, as it has its own integral light sensor. It is useful for users who wish to use their own light source, whose dimensions are such that a PAR sensor and a leaf cannot both be simultaneously illuminated. In that case, this feature can be used as follows: The PAR sensor is removed from its usual position and placed in the chamber with the sensor facing the light source. Pressing the hold Q button holds the resultant PAR reading (Qleaf). This value does not have the window transmission factor applied to it because it is assumed that it was made with the sensor under the window. The PAR sensor can then be removed from the chamber, and replaced by the leaf. All subsequent calculations are based on the frozen value, which can be used for many leaves. Normal operation is restored with the release Q button.

3.9 Enter given Q value

In addition to the two buttons referred to in paragraph 3.8, the first main menu page also has a button (given Q) that allows a given value of PAR (Q) outside the chamber to be used. The "given" value is entered from the configure set up menu by selecting */Q set/* then using the change + and change – buttons to enter the required value. The default value is 1500µmols m^{-2} s^{-1}. Any value between 0 and 3000 in increments of 5 may be entered in each of the seven configuration set-ups. Once given Q is pressed the given value will conform to whatever configuration is being used at the time. Note that the corresponding window transmission factor is applied to it, so it will generally be less than the value entered. Press release Q to return to measured values. If hold Q is pressed whilst "*given Q*" is being used, the current value for Q will be held even when changing to an alternative configuration set-up. Note however that when release Q is pressed, the value for Q will return to *measured* not *given*.

3.10 Climate Q

This button displays the value of Qref corresponding to what the climate control system is doing at the time. If it is off, ie at ambient, the climate Q and release Q buttons will both display the Q_{leaf} value as measured by the PAR sensor. If the light unit is switched on, climate Q will display the Q_{leaf} value as measured by the light sensor in the light unit, and release Q will display the value measured by the PAR sensor. Once a non-ambient light level has been set in the climate control menu the Q_c reading will be displayed even if ambient light has been reselected. The release Q button will need to be pressed to return to normal Q readings. Entering a non-ambient value for Q in a sequence programme will also have the effect of placing the Q measurement method to Q_c. The method will remain Q_c even after the sequence has ended.

Section 6. Routine Maintenance

6.1 Chemicals

CO_2 Stripper

The performance of the LCpro-SD is dependent on the satisfactory condition of the soda lime stripper, which is in the column furthest from the connectors. The life expectancy of the soda lime before it becomes exhausted depends on use and ambient conditions; but is approximately 200 hours at normal CO_2 (air) levels, if CO_2 control is not being used. Exhausted soda lime may lead to negative ΔCO_2 readings even with nothing in the chamber. This is especially true when the soda lime needs to work harder to control CO_2 levels in climate control mode. If the soda lime has changed colour by half its length or more it should be replaced for CO_2 climate control work. Otherwise, it will work until 90% has changed colour. The soda lime supplied is an indicating type, which turns from white when fresh, to violet when exhausted. Some water content is necessary to assist the chemical reaction, which is to convert CO_2 to calcium carbonate + H_2O. Re-conversion back to soda lime is not practicable.

Soda lime is commonly available, but is usually a non-indicating type – this will lead to erroneous CO_2 measurements if the soda lime is used (unknowingly) in an exhausted state. It is acceptable to mix indicating and non-indicating types together.

Note: A warning message will appear on the status line if the reference CO_2 reading falls below 100 ppm. The message prompts the user to check the Soda lime, which may be exhausted.

Drier

This is “Drierite”, anhydrous calcium sulphate, with an indicator, which changes from blue when fresh to a pale pink when exhausted. This can be regenerated as follows: Spread the granules in layers one granule deep and heat for 1 hour at 210° C or 425° F. The regenerated material should be placed in the original glass or metal container and sealed while hot. The colour of the indicating Drierite may become less distinct on successive regenerations due to the migration of the indicator into the interior of the granule and sublimation of the indicator.

Humidifier

This is iron (ferrous) sulphate, 7 hydrate. It has a colour change from a pale lime green when fresh to white when exhausted. It is not practical to rejuvenate it.

6.2 Dust Filters

The gas connections on the front may become blocked if the instrument is placed with this surface downwards. Note that the bottom three, with metal tubes, are outlets. If they are blocked, it may be possible to poke at the blockage with the instrument running, so that the debris is expelled. As a general precaution, fit two loops of pipe to the four entries when the chamber is not connected. If the instrument is placed with the entry side downwards, the two pipes will protect the ends of the entries against damage and prevent debris entering.

Although 'clean' chemicals are supplied, in practice fine dust particles can be given off, which eventually may cause a malfunction of the mass flow sensors and/or the optical bench. This will also be the case if dust or pollen is drawn in from the air supply. The filters used are designed to prevent this, but will gradually restrict the airflow in the process. If difficulty is experienced in obtaining the maximum (PLC) flow of 341 μmol $sec^{-1.}$ (i.e. the indicated flow 'u' is very much less than 341 μmol sec^{-1} and pump "racing"), this can be taken as a sign that filters should be changed (or cleaned if they can be dismantled).

The most likely filter to become blocked is the external plastic bodied one, if fitted. Otherwise, check the 3cm diameter disc filter with a Luer connector, located under the top bezel. In dusty atmospheres, with continuous operation, and no other external filtering, this can become blocked in less than a week. If in doubt, compare its colour with the one in the spares kit. The other filters are not transparent so cannot be checked visually.

The next filter to check is the external metal bodied one, if fitted. It contains a 25μm gauze filter element that can be cleaned with a small brush. Replace it with the largest part of the body on the inlet side as this will ensure that trapped dirt is on the outside of the mesh and can be easily removed.

With the exception of the "Wetter" column all filters are made from Vyon which is impervious to the acidic effects of Soda Lime. The "Wetter" column filter is made from foam and should be inspected and changed if signs of degradation are apparent.
The columns should be removed and washed in soapy water from time to time.

6.3 Battery Charging

The LCpro-SD has an internal sealed re-chargeable lead-acid battery, which, when fully charged, operates the system for about 10 hours. Battery power is shown on a bar graph, and also as a numerical voltage 'V_{batt} in the configure diagnose page.

The battery can be re-charged in situ via the five-pin power socket on the side, using the charger lead supplied. The LCpro-SD can also operate from an external 12-volt supply of at least 2A capability (using the charger lead), without the internal battery fitted. Be aware that there is no diode to prevent power flow back out of the battery (although there is a thermal self-resetting 5A fuse). If the charger is disconnected from the mains it should also be disconnected from the LCpro-SD to prevent draining the battery.

The main battery will give several years of service, **providing** the following precautions are taken:

Never over-charge the battery as this can damage it. An indication that a battery is being overcharged is a noticeable rise in its temperature. When the instrument is being used in the field on a daily basis, an overnight charge will be sufficient; do not leave the battery on continuous charge for more than a day.

Never store the battery in a discharged condition – this will shorten its life.

Never charge the battery using a constant current supply, commonly used to charge Ni-cad batteries – this can over-charge it. An ADC battery charger or a constant voltage supply only should be used, i.e. one in which the charge current (which must be monitored) is set by adjusting the supply voltage.

In the field, the battery can be charged to some extent by connecting it to a vehicle battery with the power cable supplied. It will be more fully charged if the vehicle engine is running. For field operations, spare, fully charged batteries will extend operating time.

When the battery in the LCpro-SD is near to a discharged state, a **warning** – **low battery voltage** message is flashed on the display. In this event, terminate the work as soon as possible, switch the LCpro-SD off and, either recharge, or replace the battery. If a suitable external DC power source is on hand, connect it to the LCpro-SD as soon as the message appears. In this case, work can continue undisturbed.

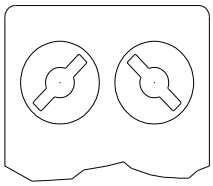
If you plan to store the instrument, fully charge the battery first. Giving it an 8-hour top up charge at least once every 6 months will maximise its life. It is not necessary to remove the battery, but if it is removed for more than a few weeks, the rechargeable clock battery may become discharged. In this case the clock will require resetting.

6.4 Battery Replacement

Battery replacement can be done at any time but, before doing so, switch the LCpro-SD OFF via the power off function. The Configuration used at the time will then be preserved. Batteries cannot be expected to last beyond 5 years. Symptoms of a faulty battery are a short running time even after it has been left on charge for 8 hours, or a very small charging current, even if it appears to be discharged.

In order to swap to a fully charged battery without switching off, it is necessary to first connect an external supply to the 5 pin power socket. The supply could be from a battery charger (supplied), or from another 12V battery using the crocodile clips.

The battery and the supply fuse are located in the base of the console, and are accessed by rotating the two spring fasteners on the bottom panel and opening it. The battery can then b ter disconnecting the two spade terminals.



With the instrument upside down, undo the two fasteners on the base plate of the LCpro-SD by rotating them a quarter turn; the base plate can then be removed to expose the battery. Disconnect the battery by holding the spade terminals and not the wires. Turn the instrument the right way up to withdraw the battery. Reconnect a replacement, ensuring that the LCpro-SD RED lead terminal is connected to + and the black terminal to –. When removing or refitting the battery, ensure that the metal tabs on the battery do not touch the chassis. Fit the battery into the LCpro-SD and refit the base plate by locating its 'tongue' into the chassis then rotating the two fasteners to the positions shown in the figure above and pressing firmly until they are heard to click.

6.5 Battery Fuse

The battery fuse is a 20mm glass type located in a bayonet type holder next to the two fasteners under the base plate. This 3.15 Amp time delay glass fuse is connected in series with the battery 'positive'.
Under normal conditions, the fuse should not fail. If it does, it could be due to an internal fault, by a high voltage applied externally, by an external supply reversal or by the battery over-charging which can cause its terminal voltage to increase. Providing the cause of fuse failure/s is removed, and the fuse is replaced (a spare is provided), the LCpro-SD will have been protected from permanent damage.

6.6 CO_2 cartridge

The CO_2 cartridge is located under the battery cover, next to the battery fuse.. The cartridge holder can be loosened with the special "key" provided in the spares kit (M.LCM-146) or with a well-fitting coin, then unscrewed by hand. The instrument is supplied with an empty test cartridge fitted. This is to help exclude atmospheric water vapour, which will freeze with the sudden expansion of the gas from a new cartridge. The resulting ice crystals will cause unstable CO_2 control for the first few hours. It is recommended that you leave an empty cartridge in place to keep moisture excluded, until you need CO_2 control again.

When replacing with a full cartridge, the cover can be screwed most of the way by hand, until resistance is felt. This is caused by a sharp pin inside the regulator about to pierce the seal on the cartridge. Continue to tighten with the special "key" or well fitting coin. After about ¼ turn, the housing will become much stiffer to turn as the cartridge is pierced and high-pressure gas (about 8Mpa) is released into the regulator. The gas is prevented from escaping by the 'O' ring seal but the pressure on the end of the cartridge causes friction on the housing threads. To be sure the 'O' ring is completely sealed and the cartridge is completely pierced, continue to tighten for about two turns. Using the special "key" is recommended as it provides sufficient leverage to ensure that the cartridge housing is tightened sufficiently. The "ground spike" can also be inserted through the hole in the key to give additional leverage if required.

If the cartridge holder is removed before the cartridge is empty gas will escape rapidly through the side of the housing, and the 'O' ring may be damaged. To prevent this happening use the special "key" or a coin to depress the "Schrader style" valve located at the front of the console (see photo on page 13) to bleed off the remaining gas. Alternatively use the knurled brass plug LCM-039 supplied in the spares kit; screw it in finger tight then back out one t. This valve should be kept open for 30 minutes, even if the gas can no longer heard as it hisses out. If the valve is released sooner then the O ring which seals the gas bottle may balloon to several times its size and require replacement (ADC part number 653-126).

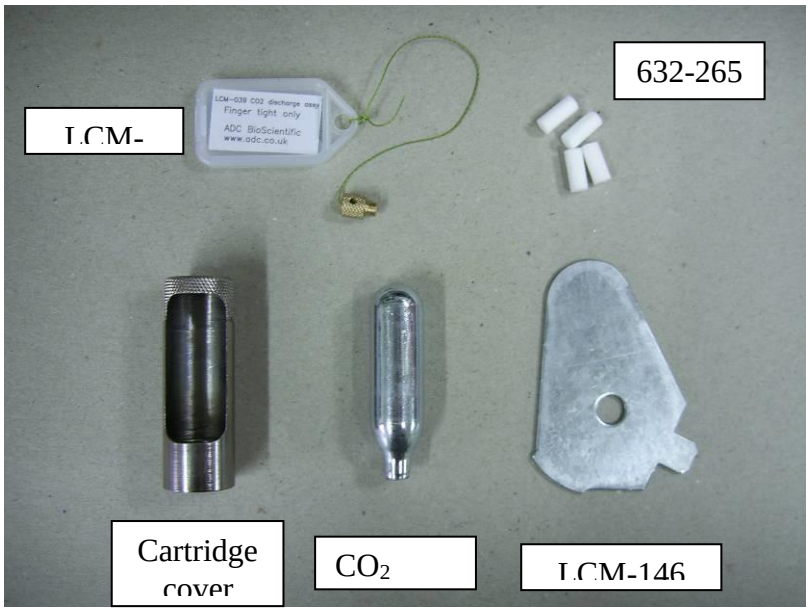
The "Schrader style" valve should be also used if you need to remove the cartridge when it might have a significant quantity of gas remaining in it. After the gas has been released there may still be CO_2 molecules under pressure contained within the rubber "O" ring.
To prevent the "O" ring seal from being damaged by sudden decompression, allow 30 minutes before finally unscrewing the cartridge holder.

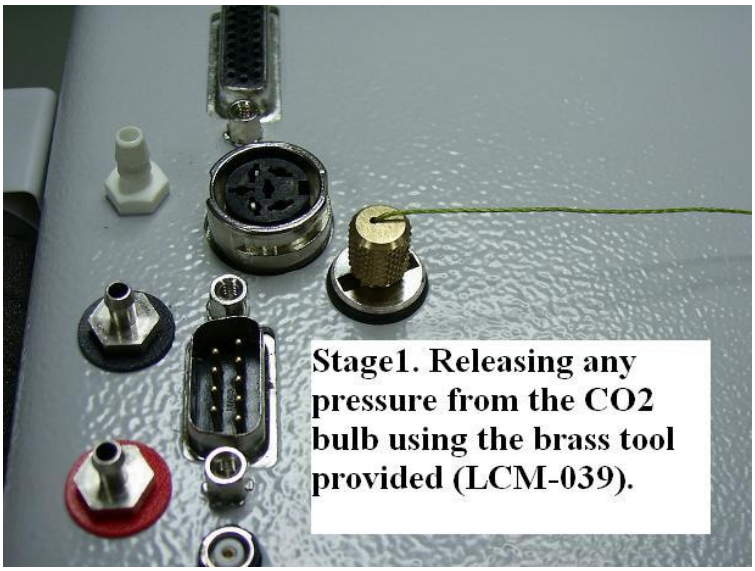
After every ten cartridges the filter (632-265) in the "Schrader style" valve housing should be changed. Using the special "key" provided or a coin, depress the "Schrader style" valve and unscrew the housing in the front of the console.

WARNING!

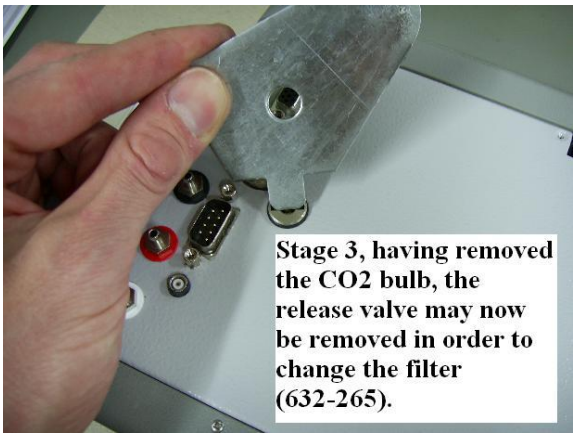
DO NOT ATTEMPT TO REMOVE THE FILTER HOUSING WITHOUT RELEASING THE PRESSURE VIA THE VALVE. FAILURE TO DO SO MAY RESULT IN THE FILTER HOUSING BEING BLOWN OUT AND CAUSING INJURY.

Once the housing is removed the filter can be hooked out with a small screwdriver, tweezers or a paper clip. Fit the new filter by simply pushing into the housing as far as it will go then replace the Schrader valve housing into the regulator (see photographs on following page).


LCM-
632-265
Cartridge cover
CO_2
LCM-146


Stage1. Releasing any pressure from the CO2 bulb using the brass tool provided (LCM-039).


Stage 2. Removing the CO2 holder and the CO2 Bulb, using the tool provided. (LCM-146).


Stage 3, having removed the CO2 bulb, the release valve may now be removed in order to change the filter (632-265).


Stage 4. Remove and replace the old filter, a sharp pair of tweezers is ideal, as shown below.

6.7 Suggested Maintenance Schedule

1. O-rings, check the handle o-rings for wear or damage each time a jaw set is changed or removed. Check the o-rings on the chemical columns each time they are removed. A tiny amount of silicone grease on all o-rings helps to make a good seal, damaged o-rings should be replaced using the spare o-rings provided in the spares kit. Additional o-rings may be ordered from ADC or an approved agent.

2. Chemicals, should be replaced when the colour changing indication shows that approximately 80% of the chemical has been used, take care not to damage the large o-rings on the columns when re assembling the columns, keep these o-rings free from chemical granules and lightly greased with silicone grease (supplied in the spares kit).

3. External filters should be used with the console and inspected every time the system is setup for use, two types are provided in the spares kit, x2 plastic ones which are disposable and very effective at stopping fine dust (630-980), and a metal one (631-180) which can be dismantled and cleaned out (both supplied in the spares kit). Great care must be taken to make sure these filters are always connected in the same direction of air flow, otherwise dust will get into the console which may clog the internal filter. These filters should be regularly inspected to ensure they are not clogged. A badly clogged filter may damage the console as the console has to work harder to overcome the impedance caused by the restricted airflow.

4. The internal filter (630-693), this should be checked if it is suspected that an external filter has failed. See section 16.2 and 16.5.

5. The CO_2 regulator is expensive, so it's filter should be replaced after ten CO2 bulbs, because they contain traces of oil which will damage the mechanism (see section 6.6).

6. Carry case drier (black plastic flight cases), this changes colour from blue to red when saturated, and can be baked in an oven to dry it out, see the instruction card in the flight case for details (normally located under the drier canister). Inspect every time the instrument is put away after use in the field, and keep the case closed at all times when access is not required.

7. Solenoid valve in the handle, when the system is switched on a clicking noise should be heard on start-up, if not then a valve exercise function should be used to get the valve working (it may be stuck after a period of inactivity), this is done in the console screen using *config>diagnose>sys.info>SV check*. If the valve still does not click then it may be faulty.

8. Leak tests, once a year or if a user suspects the instrument's results may not be correct. See section 16.4.

9. Recalibration, every 4 years is the recommended interval for recalibration, best done by ADC Bioscientific Ltd. Calibration dates are to be found in the calibration screen. See section 7 for more details.

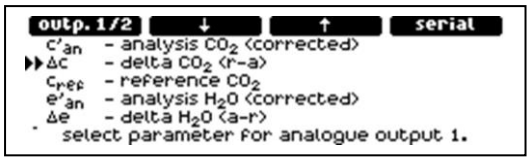
Section 7. Set-Up and Calibration

All the current attributes and settings are retained indefinitely by a non-volatile electrically alterable ROM (EEPROM). These settings are read into Random Access Memory (RAM) when the LCpro-SD is switched on and resaved from RAM to EEPROM when the LCpro-SD is switched off. This ensures that any changes to the configurations made by the user are retained. A small rechargeable battery supplies the clock. If the LCpro-SD supply is removed (e.g. its battery is discharged), this battery will become discharged, typically after 7 weeks. It will automatically be re-charged when LCpro-SD power is re-applied, but it will be necessary for the user to re-set the date and time.

7.1 Analogue Output Port Set-up

Pressing output displays the parameters list, from which two can be selected for the two chart recorder outputs. Pressing outp 1/2 toggles between analogue output channels 1 and 2

Screensho



The An o/p column in Appendix 1 lists the parameters available for the analogue outputs. The following three columns list the unit of measurement, the range and derivation. An explanation of the derivation codes follows the table in Appendix 1.
The port output is scaled at 0.0V = zero or offset and +5.0V = full scale readings. Further information such as Units per Volt is shown in Appendix 2.

7.2 Serial Link Port Set-up

Pressing output then serial displays the menu from which the the baud rate and handshake protocol can be selected.
Set the required baud rate by highlighting it with select then use change + and change - to cycle through the options of 300, 1200, 2400, 4800, 9600, 19200 and 38400 baud. Highlight the handshake to cycle through the options of "CTS", "use CTS for record", "xon-xoff", and "none".
Note that if "CTS for record" is selected it is not possible to send recorded data over the serial port. Any attempt to do so will result in the message 'Serial port set for record trigger'.

7.3 Time & Date Set-up

Pressing configure then time/date displays the Time and Date menu. Press select to step through hours; minutes; seconds; day; month; year Pressing change + or change - increments or decrements the chosen parameter (except for seconds which resets to zero).
The clock is in a 24-hour format.

7.4 CO_2 signal phase correction (stable air supply required)

Pressing configure diagnose sys.info. displays the system information page and the auto-phase function soft key. It is not normally necessary to re-set the CO_2 signal phase correction unless a new infrared source or detector has been fitted or a large adjustment has been made to the CO_2 zero.
The set phase operation is fully automatic but can be escaped from without effecting a change by pressing any button. During the set phase operation, the instrument performs a series of checks in one-degree steps between 65 degrees and 100 degrees to find the phase correction that gives the best CO_2 signal energy. Typically, the phase correction angle is between 70 and 80 degrees. When the scan is completed, it will ask whether you wish to save the new setting.

7.5 Calibration Menu

Pressing calibrate accesses the calibration menu. From here the various options can be chosen using the select key and the + and – keys to set the value where applicable. Press do calib. to start the calibration.

7.6 Flow check (stable air supply required)

Note: The displayed values for u and u_{set} are related to the Air Supply Unit (ASU) which provides flow to the leaf chamber. Although proportional to the ASU flow to some extent, the values displayed during a Flow Check calibration are the estimated flow through the analysis cell and the time allowed before the gas is stable and a reading taken. Typical values for broad, narrow and conifer chambers are shown in the table below.
Analysis times for Soil chambers and Arabidopsis style chambers may be longer due to the larger chamber volume and lower advised ASU flow respectively.

It is strongly recommended to perform a flow check calibration if you change between chamber types or make a change to the chamber air supply flow larger than 30%. The flow check calibration checks that the cycle times are long enough for the gas in the analysis cell to become stable before the absorption is measured. The flow check adjusts the cycle times for both reference and analysis, therefore the chamber jaws must be fully closed before the check is started.

Changing the flow by greater than 30% without doing a flow check may result in insufficient settling time which may cause measurement errors because the gas concentration in the cell will not have had time to stabilise. It can cause an offset in the ΔCO_2 readings even with nothing in the chamber.

Typical flow values and normal variation from typical values that can be expected

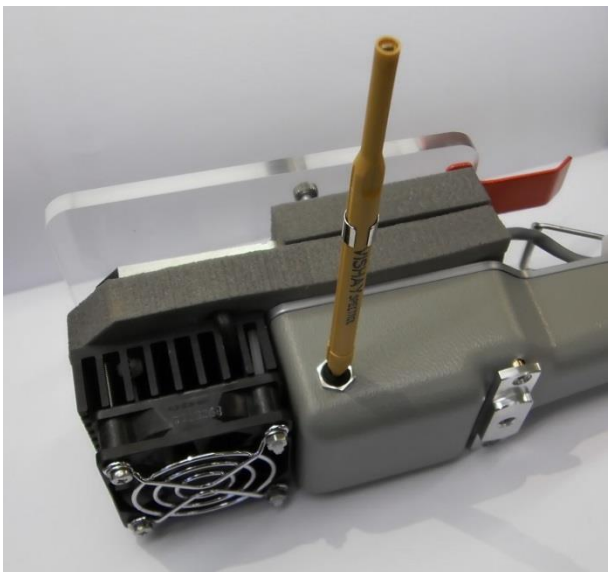
ASU set flow (µmols s^{-1})	Settling time (seconds)		Estimated flow (µmols s^{-1})	
	Reference	Analysis	Reference	Analysis
200	4.45	3.86	83	97
300	4.32	3.36	85	110
Variation	5%	10% - 25%	5%	10% - 20%

7.7 CO_2 Zero

The CO_2 zero setting is automatically maintained by a software adjustment during each zero cycle. The adjustment effectively changes the gain in order that the signal level, when zero gas is flowing, is constant. For this to be performed correctly, the soda lime column must be kept in a non-exhausted state. If this is not the case, there will be an apparent reduction in measured (span) values and a warning message 'cref low, check absorber' when the soda lime is completely exhausted. This effect may therefore appear to indicate that a 'span' calibration is necessary, when in fact it will not be. Prior to reaching this conclusion, ensure that the chemical has been checked.

The degree of software zero adjustment being applied can be checked with configure diagnose The C(z) reading should lie between 45,000 and 60,000 counts (with the optimum being 52500). If this is not the case, the warning message "cref low, check absorber" will already be displayed and a hardware adjustment can be made.

The CO_2 Zero adjustment potentiometer is located inside the jack socket where the leaf thermistor can be connected (See figure). The potentiometer is adjusted using the thin end of the long (13 cm) trim tool supplied in the spares kit. This tool is the correct length and diameter to fit in the jack socket, and correctly engage in the slot of the pot without misalignment and damage. Insert the tool in the jack socket and gently press, (you will feel resistance as it passes the rear contact set). Turn the tool whilst gently pressing until it engages the slotted adjustment screw of the pot. Zero adjustment can now be performed.



In the calibration menu, select *CO_2 zero*, then press do calib. Adjust the pot to reduce the displayed count to within 200 counts of zero, turning it clockwise if the displayed value shows a down arrow. A value within 10% of the range will be functional, and will cause 'OK' to be displayed. If the adjustment is very wrong, or if there is another fault, other messages will be displayed; "CO_2 low energy", or "CO_2 signal over-range".

The chemical in the column MUST be in a good condition at all times for correct zero operation. **If the check indicates maladjustment, check the state of the chemical before any potentiometer adjustment.**

7.8 CO_2 span

The CO_2 span calibration setting may be recalibrated by one of two methods, the first method is somewhat crude and not recommended unless an instrument has lost its stored calibration setting, the second method is to be preferred.

Note before using either method

Using a stable air supply either from a small volume or from the extended air probe (or the bottled gas if using method 2)
Set the flowrate to 341µmol/sec, perform a phasecheck *config>diagnose>sysinfo>auto phase*, followed by a CO_2 zero calibration (in the calibration menu – you will need a potentiometer adjustment tool to insert into the handle jack socket and rotate until the screen displays "ok" when in CO_2 zero calibration mode).
Lastly perform a CO_2 flowcheck, from the calibration menu (the jaws must be correctly fitted with all 5 o rings making a good seal from the jaws to the handle. Make sure the jaw gaskets have no gap around them, a small gap can be fixed with an elastic band around the jaws).

Method 1. The ambient method.

This can only be done in an open area, several Km away from and ideally not downwind of any major sources of CO_2 such as cars, buildings etc. It should be done in an open area not for example in a forest environment. A good location would be on a coast with light wind or an onshore breeze. Connect the console air in port to a buffer volume or the air probe (extended and held upright by use of the groundspike) using 3 or 4mm bore PVC tubing.
Next enter the calibration menu and perform a CO_2 span calibration, having first adjusted the span gas ppm level in the calibration menu to 400 ppm (in 2017) this being a typical global average. After calibration exit the calibration menu and monitor Cref for a couple of minutes, redo the calibration if Cref deviates more than 10ppm away from the span gas value.

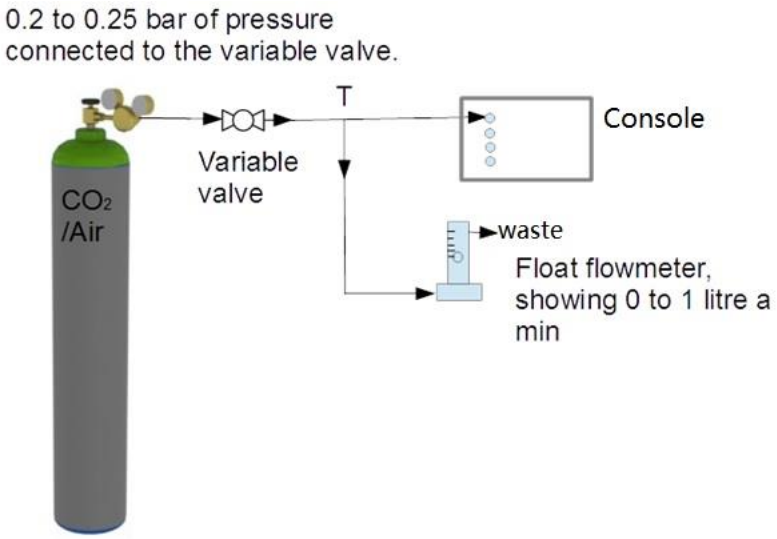
Method 2. The bottled gas method.

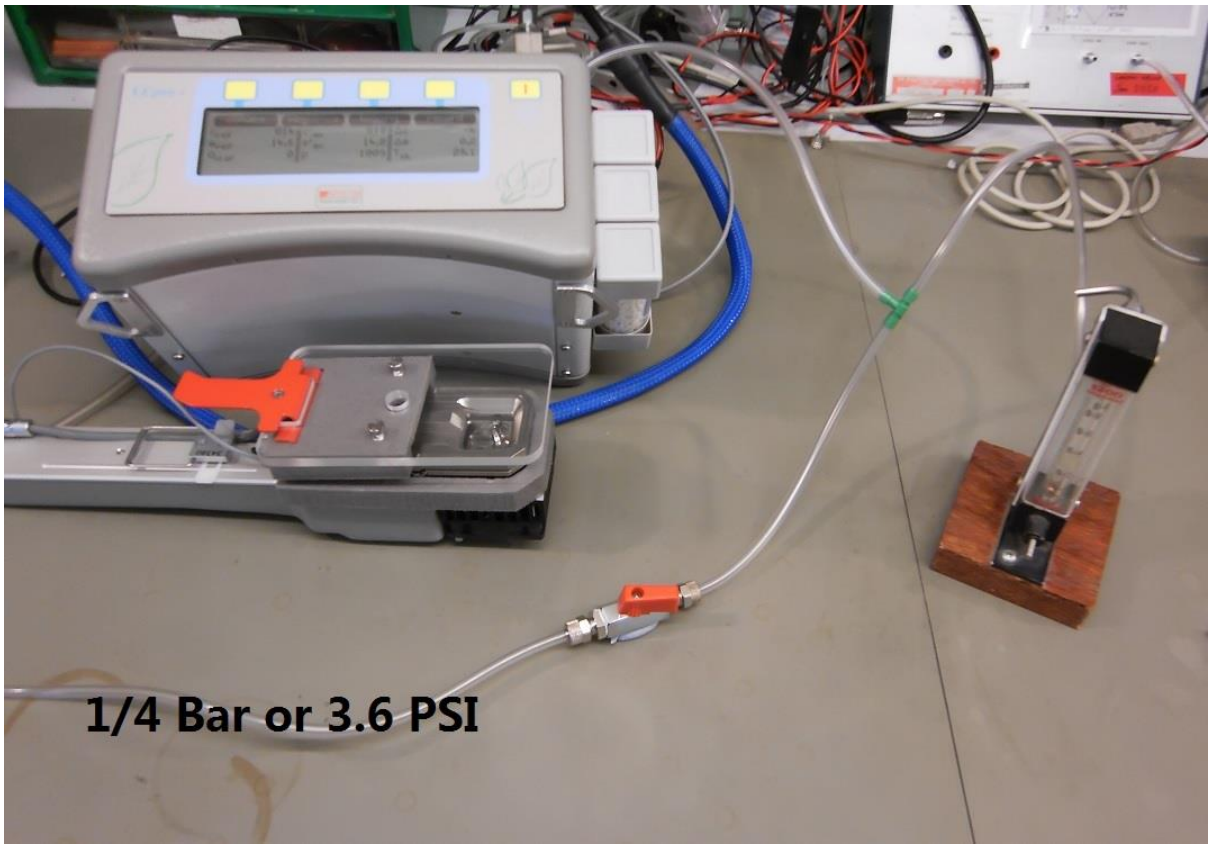
To do this you will need a pressurized bottle/cylinder of gas made from a mixture of CO_2 and Nitrogen. The PPM value of the CO_2 must be known to a good level of certainty e.g 1% accuracy. The span gas should be 40-99% of the maximum range of the instrument.

Equipment:
You will also need a two stage pressure regulator fitted to the gas bottle/cylinder to reduce the pressure to approximately ¼ bar or 3.6 PSI, a ball or needle valve, some 3 or 4mm bore PVC tubing, a T piece and a float flowmeter with a range of or close to 100ml to 1000 ml or more per minute. The flowmeter must allow enough reverse flow for the console to run at maximum flow rate – most float flowmeters do but some ball type flowmeters do not, this should be tested as follows:
Set the console flowrate to 341µmol/sec, use a piece of PVC tubing, 3 or 4 mm bore to connect the inlet of the flowmeter to the air in port of the console. If the console pump becomes very noisy then disconnect the flowmeter quickly – it will not be suitable. A small increase in pump noise is acceptable.

Setting up the Equipment:

Connect the output of the two stage regulator to the ball/needle valve, then connect the remaining entry of the valve to one air entry of the T piece. Connect one of the remaining air entries of the T piece to the air in port of the console. Connect the last air entry of the T piece to the flowmeter air in entry. The diagram and photograph below show this setup:





Method:

Turn on the LCP/LCi and set the flow to 341µmol/sec. Turn off the variable valve (ball or needle).
Next use the two stage pressure regulator to set the pressure to or slightly under 0.25BAR or 3.6 PSI. Very slowly open the variable valve until the flow meter reads about 200 ml/min – this reading will fluctuate over the instruments CO_2 cycle.
Adjust the control valve so that the flowmeter never drops below 100 ml/min, watch this for a minute to make sure and continue to monitor this while performing the calibration. The excess flow ensures that the system always has a surplus of span gas and never dilutes this by sucking in non span gas.
After the system has finished its warm up cycle – this will clear from the status line of the display and the console will make a beep noise to indicate this, perform the steps laid out in "notes before using either method".
After 5 minutes enter the calibration menu and adjust the CO_2 Span value to the value of the gas being used, then perform the CO_2 span.
After calibration exit the calibration menu and watch the Cref reading for a couple of minutes, repeat the span calibration if Cref deviates more than 0.5%+4ppm away from the span gas value.

7.9 H2O Calibration

Recalibration and Small ∆ e offsets.

RH recalibration should not normally be required, unless a sensor has become faulty and been replaced. As long as a system is returned every 4-5 years for service by ADC (which includes RH calibration and a linearity test), then recalibration should not be necessary.
A small **∆e** error due to component drift does not have a significant effect on the final calculated value of transpiration rate (**E**).
A small **∆e** error may however be eliminated by first making sure the air supply is stable i.e. via a volume; second, by making sure the jaws are correctly fitted with no gaps in the gaskets, no loose screws, no missing 'o' rings, and finally if a delta e is still present, then a H2O span may be done in the calibration menu using the measured value of **e'ref** as the span level. This should only be done after both **e'ref** and **e'an** have been stable for 20 minutes or more at a flowrate of 200 µmol s^{-1} or higher.

Special note on Replacement Sensors.

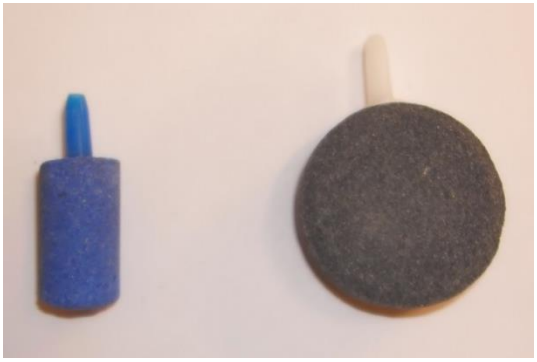
If replacing an RH sensor before recalibration, please be aware there are two types.
The original sensors will only work on the LCP,LCP+, LCP-SD up to and including software version 3.01.
The new type sensors will work in the LCP-SD with at least 3.03 software. The software version may be easily upgraded by parties other than ADC, but a hardware modification may be required.
Contact ADC Bioscientific for more information on software upgrading if required. The software version is displayed on the startup screen.

In the photo opposite, the original sensor is shown on the right, it has a rectangular shaped sensor face. The new type is on the left, it has a small circular hole for the sensor face.
The ADC part numbers are for LCP old type – LCM-032 and new type LCM-037.

Full H_2O Calibration

This may be done outside of ADC Bioscientific, but the following equipment will be necessary. (If a dew point generator is being used then not all the following may be needed depending on the additional features of the dewpoint generator, e.g whether it has a variable air pump/flowmeter).

1. A **source of dry air or granular drying agent**.
2. A **float flowmeter** (air) of, or close to, the range 0-500ml/min (1000 ml/min if using method 2 of the H_2O zero calibration). The company, Cole Parmer, supply suitable flowmeters.
3. A **low voltage DC air pump** that can go up to 500 ml/min (1000 ml/min if using method 2 of the H_2O zero calibration) or more with a **variable power supply**. ADC Bioscientific can supply a suitable pump if required, but inexpensive alternatives are easily found online.
4. **Two water bubblers** (see appendix 11 for construction details), one of which must have the means of allowing the water temperature to be accurately measured. Water bubblers can be made using glass jars with air entries mounted through the lids, one entry (air in) will need a length of tube below the water line as shown in the photographs. The ends of these tubes should be attached to "air stones" which are made for aquariums, to ensure small bubbles, which are important. Ideally two types of air stone should be used and the one that makes the smallest bubbles used on the second water bubbler which also contains a thermocouple to measure the water temperature. If making water bubblers from glass jars they must be airtight. ADC Bioscientific can supply plastic air entries to mount through the lid. The lids should be sealed with PTFE tape around the glass threads. Alternatively, a dew point generator can be used.
5. An **empty water bubbler jar** (see appendix 11) to act as a water trap between the flowmeter/pump and the water bubblers – when the pump is powered down, a small amount of water is sucked out of the first bubbler and may reach the flowmeter without this water trap.
6. Some means of measuring the water temperature in the final water bubbler, e.g. a submerged thermocouple/**digital thermometer** probe through a hole in the lid, then sealing the lid hole with glue (hot or resin glue) works well. Digital thermometers must be calibrated and regularly tested to a high standard.
7. Some short lengths of PVC tubing bore diameter 3 to 4mm, one length of 4mm x 60cm.
8. A **room thermometer** of good accuracy **or a second thermocouple** if using a digital thermometer.
9. If a linearity test (optional) is to be done, a good quality RH meter will also be required. Preferably one that has been tested using saturated salts as a calibration medium, an internet search will explain this.



Two types of "air stones" commonly used in aquariums. The larger more expensive one on the right produced smaller bubbles. It is preferred if the second water bubbler makes smaller bubbles than the first one.

Method:

The calibration menu shown below will need to be used for the H_2O zero and Span calibration.

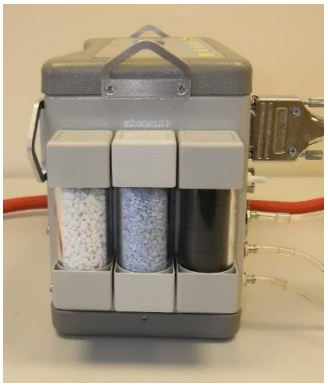
H_2O zero calibration

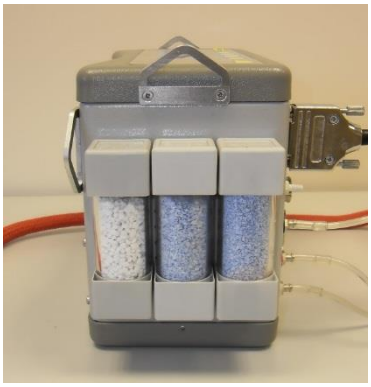
First make sure the console has the correct time and date. This can be seen and edited by going to ***config>time/date***.

Method 1.

This method requires a granular drying agent such as Drierite, it is easier to do than method 2 but slightly less accurate. The normally inert chemical column of the LCP column (normally contains four pieces of removable foam) should be removed (switch the console off first), and the pieces of foam removed and replaced with the granular drying agent.
Refit the chemical column, turn on the instrument and run the machine at a flowrate of 341 µmol s^{-1} for 20 minutes, reduce the flowrate to 200 µmol s^{-1} for 20 minutes then perform a H_2O zero from the calibration menu. Afterwards monitor the **e'ref** and **e'an** values, they should be within 0.1mb of 0.0 mb over a period of 5 minutes, if not then repeat the zero calibration.

The photos below show the console chemical columns fitted as normal (left) and fitted for a H_2O zero calibration (right) with a drying agent, in this case blue Drierite.





Method 2 (recommended).

With the instrument switched on and warmed up, supply dry air into the black ringed handle tube at a rate of 800-1000ml/min, this normally connects to the console as one of the three colour coded tubes. Pure nitrogen may be used for this purpose. The air must be completely dry. The black ringed handle tube goes directly to the closed jaws which is where the two RH sensors are located.
Allow 20 minutes at this flowrate of 800-1000ml/min, then reduce the flowrate to about 300 ml/min, allow this to run for 20 minutes, then enter the calibration menu and calibrate by selecting the H_2O zero option.
Afterwards, monitor the **e'ref** and **e'an** values, they should be within 0.1mb of 0.0 mb over a period of 5 minutes, if not then repeat the zero calibration.

H_2O Span Calibration

Warning One:
The temperature of the water in the second bubbler should be <u>equal to or less than</u> ambient temperature, otherwise water will drop out of the air in the tubing en route to the black ringed handle pipe. If water does get into the black ringed handle pipe then disconnect the pipe from the water bubblers, remove the jaws and blow compressed air down the small brass entry sticking out of the handle (make sure it is the smaller one of the two), water will be expelled (out) at the console end of the black ringed handle pipe.
This risk is eliminated if you perform the calibration in a room which is slowly increasing in temperature during the day, provided the water has been exposed to a lower temperature overnight in the same room. This way the water temperature will slightly lag behind the temperature of the room.

Warning Two:
When using the water bubblers, the Tch value on the console screen should be ≥ 1 °C above ambient temperature before connecting the black ringed handle pipe to the two water bubblers. If this is not the case then running the system for 20 minutes is normally enough for Tch to become ≥1 °C above ambient temperature. Make sure the black ringed handle pipe is connected to the console during the warm up period.

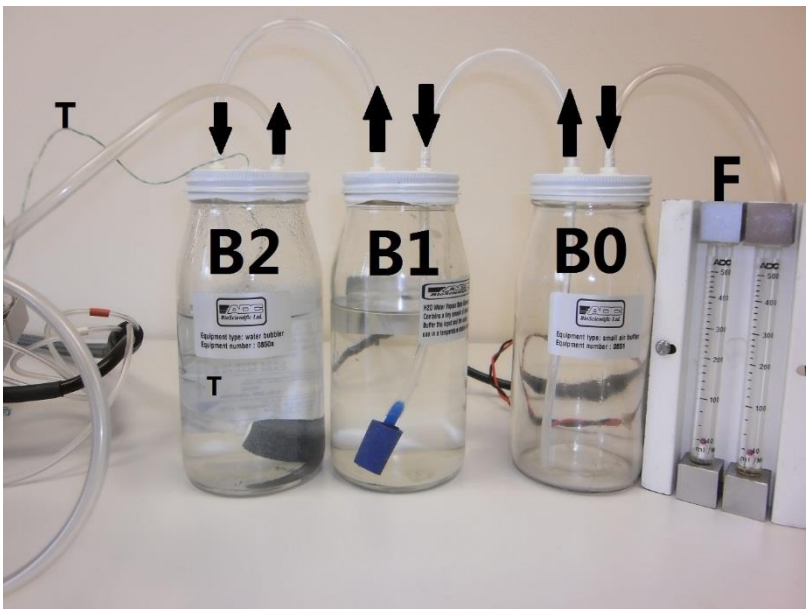
Dew Point Generators

If using a dew point generator instead of the water bubbler method described, set the dew point temperature 5 °C below the ambient temperature in which the calibration is being done. Refer to the table in appendix 5 to get a mb value for the H_2O span air.
Setting a dew point temperature below that of ambient reduces the risk of condensation.

IMPORTANT NOTE:

Before connecting everything together it is a good idea to raise the temperature of the first water bubbler slightly above the second or final water bubbler. This ensures the air is over saturated when leaving the first bubbler but reduced to the calculated saturation value, accurately determined by water temperature, when leaving the second bubbler. This increase in water temperature is easily realised by adding approximately a tablespoon (25ml added to about 250ml of room temperature water) of boiling hot water to the first bubbler and mixing it up before replacing the lid. The water in the first bubbler should be 5-10 °C warmer than in the second bubbler.

Connect the “air out” entry of the pump to the “air in” port of the flowmeter, leaving the “air in” entry of the pump free to suck in room air.
Connect the “air out” port of the flowmeter (F) to the buffer jar (B0) “short” port (the one without the internal tube fitted), connect the other port of the buffer jar to the first bubbler (B1) “air in” port (the one with the tube that connects to the air stone), connect the “air out” of the first bubbler to the “air in” port (the one with the tube that connects to the “air stone”) of the second bubbler (B2), this second bubbler should have some means of measuring its water temperature e.g a submerged thermocouple probe.
Finally connect the “air out” port of the second bubbler to the black ringed handle pipe (P), use a piece of 4mm bore PVC tubing about 60cm long. Form a loop in the 4mm section secured with a cable tie as shown in the second photograph below, this way if water vapour condensates in this piece of connecting pipe it can be seen and quickly disconnected.
The first photo below shows the buffer jar and the two water bubblers connected in series, the arrows indicate the direction of air flow. The second photo below shows complete setup including the loop in the section of pipe which connects to the black ringed handle pipe.

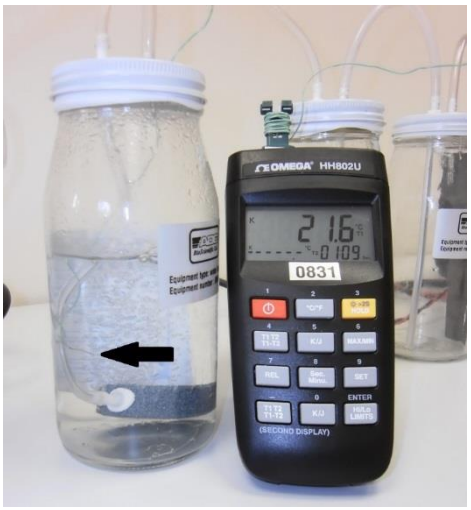


T = Thermocouple probe for measuring water temperature.
B0 = Buffer jar.
B1 = Water bubbler 1.
B2 = Water bubbler 2.
F = Float flowmeter.

The photo below shows the complete setup for H_2O span calibration. Note that the loop formed in the connecting pipe is attached to the black ringed handle pipe with the connection made above the lowest part of the loop. Watch this loop during calibration and disconnect the handle pipe quickly if water forms in the loop. If this occurs then blow out the water with compressed air and try again having first reduced the temperature (by a few °C) in the second water bubbler.

Before powering up the pump (shown above connected to a variable power supply), check that the water temperature is slightly cooler than ambient conditions, power up the pump at a flowrate of 500ml/min. Carefully watch the pipework coming out of the bubbler system and make sure no condensation is visible. Stop at once if it is present! **As a precaution you can disconnect the black ringed handle pipe and start the pump and see if condensation occurs before reconnecting the black ringed handle pipe.**

The photo below shows the water temperature being measured using a submerged thermocouple (indicated by the black arrow and not touching the glass!) in the second bubbler.

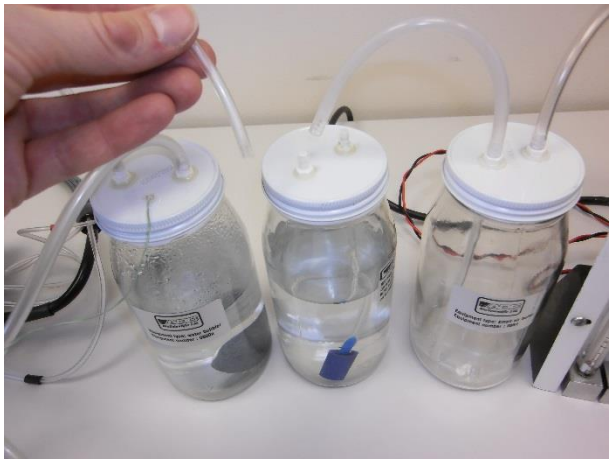


IMPORTANT NOTE:
The thermocouple shown above in the second bubbler should not be in contact with the side of the jar.

IMPORTANT NOTE!
Make sure the thermocouple lead is sealed around the hole in the lid where it enters the second water bubbler as shown opposite. Glue from a hot glue gun is good for a few weeks or months but may need to be replaced over time. Two part slow setting resin based glues are best for long term use e.g araldite 2011.
Make sure the thermocouple is not touching the side of the jar.

Run the pump at a flowrate of 500ml/min for 10-15 minutes, reduce the flowrate to 250-300ml/min and continue to run the pump for another 10-15 minutes.
With the pump still running you are now ready to perform a H_2O span calibration from the calibration menu. First you must calculate the H_2O saturated vapour pressure (SVP) in mb value which is determined by the water temperature and the fact that the water bubblers give an output of 100% RH.
Using the same formula as the LCP/I which is based on the Arden Buck 1981 formula for saturated vapour pressure, the table in **Appendix 8** can be used to quickly give this value in mb.

Read the table down first then across, for example 20.5 °C would be the row labelled 20 and the column labelled 0.5 which gives a SVP value of 24.2mb.
Enter the value obtained from the table as the span level in the calibration menu, perform the span calibration. Afterwards, monitor the **e'ref** and **e'an** values. They should be within 0.1mb of the span value over a period of 1-2 minutes. If not, leave the system alone for 10 minutes with the pump still running at 250-300ml/min then repeat the span calibration using a new SVP value from the table, if the water temperature has changed.



Switching off
When the pump is switched off the glass jars will be lightly pressurised which will normally result in water flowing backwards up the pipes. To prevent this, simply **remove both pipes from the first water bubbler as soon as the pump is switched off**, as shown opposite.

Empty the water away and allow the jars to dry out before placing them into storage.

Linearity Test (optional)

This can be done as a basic check but is not necessary. You will need a good RH sensor and a thermometer next to the console and close to the "air in" entry on the console.
Connect the black ringed handle pipe to the black coloured air port on the console.
Position the LCP system in a quiet corner and leave it to run on full flowrate (341µmol sec^{-1}) for 30 min. Note the e'ref value, when it is stable over a period of 10 minutes then the linearity check may be done.
Obtain a value of SVP using the table, based on the air temperature. Multiply this by the RH value/100, this calculated value is the water vapour **partial** pressure (PVP) of the air being sucked into the LCi.
Compare this to the **e'ref/e'an** values on display, typically they will be within 2 mb of each other.
The test is limited by the accuracy of the thermometer and the RH sensor next to the LCP/I console, commercial RH meters are very rarely accurate and fast responding.

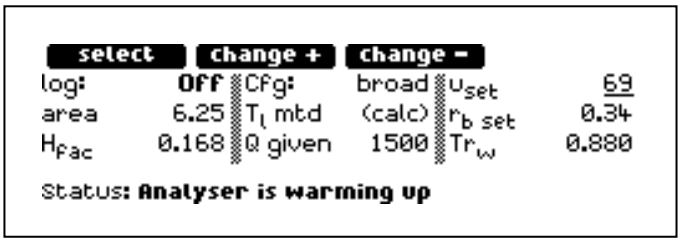
Section 8. Measurement Configuration

8.1 The 'config' Function Menu

Refer to Appendix 7 for the menu tree

Use configure to get to menu time/date set up diagnose SD card The various options can be chosen with set up select. Use change + and change - to alter the parameter selected.
The type of chamber */Cfg/* you are using can be selected from: Broad, Narrow, Conifer, Soil pot, Small, Arabdidopsis, user1, user2, user3. The chamber parameters, uset, area, T_l method, rb, Hfac, Qgiven and Trw are stored separately for each type, and the LCpro-SD is factory set with suitable default values. You can change the parameters to suit your chamber and the leaf being measured. Note that when the soil pot is selected some of these parameters are not used and therefore not displayed.

Screensho



When the LCpro-SD is switched on, it will select whatever configuration was last in use. The table below defines the default values:

	U set	Area	Tl mtd	rb	Hfac	Trw	
Units	µmols s^{-1}	cm^2	n/a	m^2 s mol^{-1}	n/a	n/a	
Range	68 to 341	0 to 100 *2	n/a	0.1 to 1.00	0.1 to 1.000	0.25 to 1.000	
Steps *1	1	0.01 *2	n/a	0.01	0.001	0.01	
Chamber						with shield	without shield
Broad	200	6.25	Calc.	0.17	0.168	0.870	0.920
Narrow	200	5.80	Calc.	0.30	0.168	0.870	0.920
Conifer	200	100.00	Calc.	0.35	0.177	0.860	0.910
Soil pot	200	97.5 *2	Meas. *3	n/a	n/a	n/a	n/a
Small	68	2.16	Calc.	0.25	0.168	n/a	0.920
Arab.	68	0.95	Calc.	0.25	0.168	n/a	0.920
User 1	68	6.25	Meas.	0.17	0.168	0.870	
User 2	341	5.80	Meas.	0.30	0.168	0.870	
User 3	200	100.00	Meas.	0.35	0.177	1.000	

*1 The steps are greater when the /+/ or /-/ keys are held down.
*2 The soil-hood range is 0 to 400cm^2 in 0.5cm^2 steps, the value given is for a version 2 soilhood and assumes use with a soil collar.
*3The Tl method cannot be changed from measured.

Refer to the table on the previous page for the default values.

/Uset/ is used to set flow rates through the Leaf Chamber/Soil pot.

/area/ is used to input the effective leaf area exposed to PAR or the area enclosed in the soil pot.
The area exposed depends upon the type of Leaf Chamber in use, and how much of the leaf is within the window area. When using Conifer Chambers, the 'area' may have to be established by experiment.
See section 5.7 for the Soil pot
Note; within some experiments, some 'constants' may vary from one specimen to another (e.g. area), and must be re-entered.

/T_l mtd/ is used to determine how the leaf temperature is obtained and toggles between */calc* / and */meas/*. The soil pot is set to */meas./* and cannot be changed.

/calc/; selects the value as calculated by the LCpro-SD from the energy balance equation.
/meas/; uses the temperature measured by whichever leaf temperature thermistor is connected.

/rb/ is used to input the value of 'boundary layer resistance to water vapour', which is a function of the leaf chamber type.
For Conifer type chambers, rb will be about 0.35, but is dependent on plant morphology and should be determined by experiment.

For all other chambers refer to the table on the previous page for default values.

r_b is not applicable to the Soil pot.

/H_{fac}/ is used to enter the absorption factor of the broad band radiant energy onto the leaf chamber by the exposed leaf surface. This factor is dependent upon the materials used in the construction of the shield and/or window of the Leaf Chamber. (For LCA2 & 3 types of analysers, this factor is referred to as '*Trans*'). Appendix 4 gives the derivation of H_{fac}.
H_{fac} is not applicable to the Soil pot

/Qgiven/ is a value of Q entered by the user, to be (optionally) used in the calculations. See section 3.9. The default value is 1500 µmols m^2 s^{-1} for all leaf chambers and is not applicable to the Soil pot.

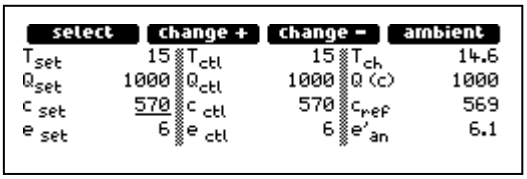
/T_{rw}/ is the transmission factor of PAR into the leaf chamber at the exposed leaf surface. ie it is the factor which *Q* is multiplied by to obtain Q_{leaf}. It is dependent upon the materials used in the construction of the Leaf Chamber window and, where applicable, the radiation shield. A radiation shield is supplied with Broad, Narrow and Conifer leaf chambers but may, at the users' discretion, be removed. In this case T_{rw} should be increased by 0.05 to compensate.
Note that Arabidopsis and Small leaf chambers do not have a radiation shield and therefore the default value should not be changed.
Note that holding down the change + or change – key causes the parameter's steps to be increased tenfold.

Section 9. Microclimate Control

9.1 The climate menu (Single Point Environmental Control)

Pressing climate displays the microclimate control menu. With the select change+ change- and ambient keys it is possible to separately set the chamber temperature (T_{set}), light level (Q_{set}), CO_2 concentration (c_{set}) and humidity (e_{set}). Press the select key to scroll through the functions and the change+ or change- keys to set the value. Holding down the change+ or change- keys will increase the size of the steps that the value is incremented or decremented by. The selected value is held continuously, and is shown as both the set and ctl value. Temperature and light levels are not relevant when used with the Soil pot and also with the Arabidopsis and Small leaf chambers as they do not have this facility.
(*Note: A Broad leaf Light may be used with Arabidopsis and Small leaf chambers:- Application notes can be requested and are automatically supplied with both chambers when purchased*).
Pressing the ambient key disables the climate control for whatever function is selected at the time.

Screenshot:



9.2 Temperature

The temperature can be set in steps of 1°C over the range 1°C to 40°C or it can be set to track the temperature as measured at the Taux input. The starting point is 20°C. To set the tracking to the Taux input simply press the change+ or change- keys until 'track' is displayed.

The cooling or heating is done with a solid state Peltier module. This is an array of semiconductor blocks sandwiched between two thin ceramic plates. Heat is pumped from one plate to the other, with a magnitude and direction dependent on the current flow.

To achieve the fastest stable control, the temperature of the metalwork is monitored next to the Peltier element. A feedback loop will make this point the same as the set temperature. The air temperature inside the chamber will differ by a few degrees due to the gradient across the metal-air interface. The air is monitored by a separate precision thermistor (Tch), and this value is used for logging and the calculations. If necessary, the set-point can be increased or decreased by a few degrees to offset the difference.

9.3 Light

The light level in the illuminator is measured with a sensor adjacent to the LED array. This is used to maintain a constant level, independent of age and temperature. The light level is calibrated so that the value achieved in the chamber is the level set on the screen, reduced by the window and radiation shield transmission factor. This is so that the illuminator calibration is the same as using the PAR sensor with sunlight. Since the LED array is not used with the radiation shield, the transmission factor should be changed to 0.92 or 0.91 as

shown in the table. Do not forget to change the value back again when the LED array is not used. The light can be set in steps of 10 between 0 and 1500µmols m^{-2} s^{-1} for Conifer heads and 0 and 2000µmols $m^{-2}s^{-1}$ for all other chamber and user configurations.

9.4 Carbon Dioxide

The CO_2 concentration is controlled using a solenoid valve (SV10) driven by a variable duty cycle. (see gas circuit, section 15). The duty cycle of the valve is varied by the instrument to maintain the setpoint requested by the user. The CO_2 level can be set between 0 and 2000 ppm in steps of 10ppm. To set above ambient levels a CO_2 cartridge will need to be installed. The maximum achievable concentration is reported in the CO_2 options page. Once the cartridge's pressure drops below a useable level, and the set concentration can no longer be achieved, a warning message will appear. The control system will set the level as requested until the cartridge pressure drops, when it will eventually fail to control, and the controlled level will fall. A full cartridge with a good seal on the 'O' ring will last at least a whole day. The cartridge is used at a constant rate even if the CO_2 control is turned off.

The CO2 control function works best at a flowrate of u=200 or u=68. For other user determined flowrates, it is suggested that a leafless log be performed and the average offset of the photosynthetic rate determined from the log file, by plotting "A" as a simple line graph and noting the middle position of the line. This can then be added or subtracted to the results obtained from the leaf present logfile later on.
A flowrate of u=200 is maximum that should be used in elevated CO2 mode.

In elevated CO2 mode it is highly recommended that log files be used with the logging rate set to the highest possible frequency for reasons given below, see "timing mode" in section 12.2 of this manual.
Cref and Can will fluctuate up and down slightly due to the mixing action of the solenoid valves and some small variation in the CO2 regulator flowrate (the calculated value for photosynthetic rate will also fluctuate as Cref and Can are factors of the photosynthetic rate), so an instantaneous value of Cref/Can/A may not be the most representative value of what is going on.

The instrument can be used to control at moderate CO_2 levels, all the way down to zero, using ambient air, i.e. with an empty cartridge.

At high, elevated CO_2 levels, there is a tendency for diffusion out of the chamber through the gaskets. This is made worse by draughts and by small leaks caused, for example, by very uneven leaves. A small positive ΔC will be caused, and can be checked for by closing the jaw without any leaf in it, or with an inactive (dead) leaf.

If there is a problem, it can be eliminated or minimised by holding the jaws closed more than the spring would normally achieve. Using a rubber band wrapped round the upper and lower jaws for example. In addition, enclosing the jaws, as much as possible, in a plastic bag, can eliminate draughts.

"Bottle Average" is shown in the options CO2 mode page. When the LCpro-SD is first switched on this will show a default value of 3600 (ppm). When a cartridge is installed and

CO_2 climate control started this value will start to change, eventually stabilising at a nominal value of 3000. This value is the maximum level of CO_2 concentration generated by the instrument before reaching the solenoid valve mixer control. When the cartridge approaches exhaustion this value will start to fall, eventually dropping below 2000 ppm (which is the maximum available climate setting) then falling below whatever level has been set by the user. At this point a status message will appear warning the user that the required concentration can no longer be held.

CO_2 Control Tip:

Every time the system is turned off and back on again the bottle average is reset to the default value of 3000. The performance of the CO_2 control will be improved if the system is using a bottle average value more accurately known.

To do this simply close the jaws, supply a stable air supply (via a volume or airprobe) to the console, run a flow check, and make sure there is a CO_2 bulb in the console which has been there for less than 24 hours, then set the CO_2 level in the climate menu to 2000 (normal mode not fast mode). After about 20 minutes the bottle average value should be accurately determined by the software and cref should be 2000ppm ±10ppm. This will make it easier for the system to change from one level to another at the start of an experiment as it will already know the bottle average as long as the system is not switched off. The determined bottle average value will be automatically updated by the software during the experiment.

Refer to the maintenance section 6.6 for details on replacing the CO_2 cartridge and filter. We advise that the filter should be replaced every tenth cartridge.

9.5 Carbon Dioxide Control Options

The analyser will control the CO_2, and H_2O levels of either reference or analysis. It is usual to control reference, i.e. the air supplied to the chamber. However, it may be useful to control analysis, i.e. the stirred air in which the leaf is working. The disadvantage of this arrangement is that changes in photosynthesis need to be matched by changes in the control of the analyser. This method therefore has longer settling times.

To change between the two (See Appendix 7 - Menu Structure) select options then either CO_2 mode or H_2O mode as required. In both cases, the option of reference or analysis is presented. In the CO_2 mode page the “bottle average” is also shown. (See section 9.1.3 for further details).

Further options have been added to the options CO2 mode page with version 3.00 software. These are norm/fast which determines the mode of operation and source which is set dependent upon the gas input.

norm/fast toggles the mode between and fast. Normal mode controls the CO2 to that requested by making an initial duty cycle estimate and then homing in on the value after which constant adjustments are made to maintain it. In fast mode the controller makes an estimate of the duty cycle to achieve the requested CO2 concentration but makes no attempt to adjust it subsequently until a new CO2 value is entered, in which case it makes a new estimate based upon the data obtained from the previous request. Fast mode is intended under circumstances where a rapid change is required with a relative short time spent at each CO2 concentration such as A/ci curves. Since the estimate is made from the

previous setting, to get the best results the instrument should be first used in normal mode set at a high level between 1500 and 2000ppm. This will provide live data upon which to make the estimation, otherwise a default value is used which can give poor estimates. Once a setting has been reached the data for the estimate is retained until the instrument is switched off.

source toggles the gas input setting between ambient air and zero and should be set according to the gas input used by the user. It is used only at initialization to give a better estimate of the PWM setting for the requested CO_2 level.

9.6 Humidity

As with the CO_2 controller, the H_2O level is controlled using a solenoid valves (SV12) with a variable duty cycle. (See gas circuit, section 15).

The Instrument is provided with two columns, one being the "wetter" column fitted with a thermistor and the other being a normal "empty" column. The Instrument is despatched with the "empty" column installed and the "wetter" option set to empty. In this configuration only below ambient levels can be set.

Note: care should be taken to always ensure that the instrument configuration matches the column installed. If for example, the wetter column is installed but the configuration is set to empty. The air will be saturated as all the flow will pass through the wetter column.

Crystal Temperature (°C)	Vapour Pressure (mb)
20	13.1
22	14.2
23	15.3
24	17.7
25	18.9
26	20.4
27	21.8
28	23.5
29	25.2
30	27.0
31	28.9
32	31.0
33	33.1
34	35.3
35	38.0
36	41.8
37	43.4
38	46.4
39	49.4
40	52.3

The humidity level can be set in 1 mb steps. It is controlled between zero and the maximum level achievable at the temperature of the humidifier (wetter) column. This temperature is monitored by a thermistor in the column, connected to the console by a double-ended coaxial cable. The instrument will only permit the user to set a humidity level up to the maximum achievable, as shown in the left-hand table:

If desired, it is possible to increase the maximum available by warming the column. However, care must be taken to ensure that the maximum humidity available from the column does not exceed the dew point of the instrument, as shown in the right-hand table.

Ambient Temperature (°C)	Vapour Pressure (mb)
15	17.0
16	18.2
17	19.4
18	20.6
19	22.0
20	23.4
21	24.9
22	26.4
23	28.1
24	29.8
25	31.7
26	33.6
27	35.7
28	37.8
29	40.1
30	42.4
31	44.9
32	47.6
33	50.3
34	53.2
35	56.2

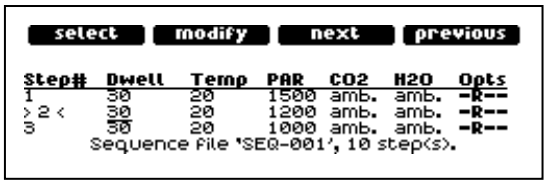
If a sequence is running, the sequence value will take preference, and the ctl value will be that at which the sequence is controlling. At the end of the sequence, the control value will go back to the set value.

Section 10. The “Sequence” Function

Description

Pressing sequence enters the sequence menu page. From here the user may write a new sequence file, using edit, load or delete an existing file from the SD card, using file load seq. then load seq. or delete seq. or save a sequence by using save seq.. The recv.seq. and trans.seq. keys are used to receive or transmit a sequence file to or from a computer over the serial link. The format for sequences produced on a computer is shown in appendix 5.

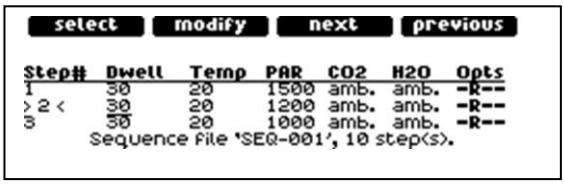
Screenshot:



If the file is new or to be edited, press edit, the select modify next previous menu keys are then displayed. The next and previous buttons move up and down the list of steps, as indicated by the < > symbols.
Note that temperature and light are available even when a Soil pot configuration is selected even though the Soil pot does not have these facilities. This maintains compatibility of sequence files. Setting temperature or light when using a Soil pot will cause no harm except a small power penalty.

The select button moves the cursor to select the dwell time, the four climate variables or the options (Opts). If dwell is selected, the modify key changes the menu keys to ambient change+ change- as prev. The change keys increment or decrement the time in 1 minute steps up to 100, whilst as prev is used to set the dwell time to that in the previous step.

Screenshot:



When the desired time has been chosen, pressing the page key will cause the menu to change back to the select modify next previous menu so that the next item for change can be selected. The default setting for the four variables is ambient, which means that the control is turned off.

If opts (options) is selected for modification, the possibilities are:

Ignore where the step does nothing. This is a way of turning off a step, (since there is no delete function) and being able to easily turn it back on.

Record which causes a record to be made at the end of the dwell time, prior to the next step starting.

End seq which stops the climate sequence running, even if it is not the last entry. (This is a way of changing the length of a sequence).

Power dn which turns off the analyser completely at the end of the step, and is useful for lengthy unattended experiments.

Each of these four buttons toggles on/off. When the climate sequence is complete, it can be saved using file, using either the default name which has been assigned (seq-nnn), or changing it with the + and – keys.

The sequence can be initiated with start, which changes the menu to run next previous
The last two keys allow the sequence to start from a point that is not the start of the file.
run actually starts the sequence, which can be left running in the background by pressing the page key . When the sequence has finished, a message “Control sequence stopped. Encountered stop flag in step NN” appears.
If more than one record per step is required then the timed log function can be run simultaneously (see section 12.2).

The SD card is supplied with an example sequence file named demo, which exercises each of the four chamber variables that the analyser can control i.e. light, temperature, CO_2, and H_2O. The files consist of lines of information. Each line has a sequential step number, a dwell time for which it is active, the values of the four variables, and an option to ignore, log, power off or finish.

Section 11. Graphical Display

11.1 Introduction

The LCpro-SD has the facility to display parameters in graph form.

The graphing function is particularly useful to see if an experiment has settled and/or proceeding as expected.

Two types of graph are available that plot either single parameter (bar chart) or dual parameter (X-Y plot). Dual parameter graphs have each data set shown as a cross, the most recent of which is shown “flashing”.

For all modes, the graphs are scaled automatically to make best use of the display resolution.

The parameter(s) to be plotted are selected from the analogue output settings (See section 7.1)

Either type of graph is capable of displaying up to 200 data sets. After this, the earliest sets will be replaced by the most recent set. In the case of the single parameter Bar chart type, this has the effect of appearing to scroll the graph from right to left as each new data set is added.

The graph options are:

Single parameter bar chart.

1. O/P 1 parameter against time
2. O/P 1 parameter against record number

   Dual parameter X,Y plot chart.
3. O/P 1 parameter against O/P 2 parameter (triggered by time)
4. O/P 1 parameter against O/P 2 parameter (triggered by record number)

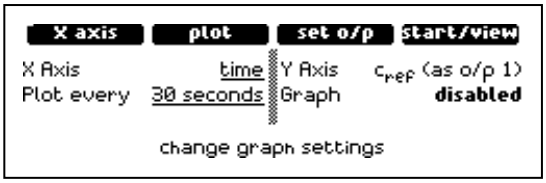
   Time may be set to 15 or 30 Seconds.

11.2 Operation

Before entering the graphs menu it is advisable to set up the analogue outputs to the parameters required. This is detailed in section 7.1. Although the analogue outputs page can be accessed from the graph set up, once exited the LCpro-SD returns to the top level menu necessitating re-entering the graph set up menu once again to complete the setting-up procedure.

Graphs are set up by pressing graph from the output, calibrate, graph, record menu followed by set up.

Screenshot:



The X axis button toggles the *X axis* setting between '(as o/p2)' and 'time' or 'log record' the latter being determined by the plot setting.

The plot button determines when a plot is taken. It cycles the *plot every* value between '15 seconds', '30 seconds' and 'log record'.

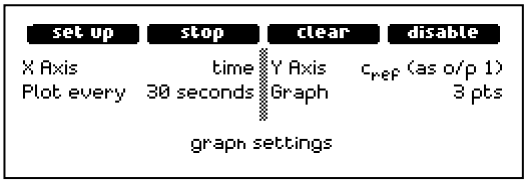
The set o/p button accesses the analogue output page where the graph parameters may be set.

The start/view button starts the plotting. It opens up the graphical display screen as a fourth top level screen.

To set O/P 1 against time for example first use X axis and select either 15 or 30 seconds. Use X axis to toggle to 'time' and press start/view when ready.

To stop the graph press graph in the output, calibrate, graph, record screen.

Screenshot:



The stop button stops the graph being updated but does not erase it in case it is required for later reference.

The clear button erases the graph data but allows it to be restarted with the current parameters.

The disable button erases the graph and removes it from the top level screens.

Finally the set up button may be used to start a graph with new parameters as previously explained.

11.3 Pause Function

The graph may be paused. This allows an operation to be carried out which would otherwise alter the scaling of the graph. Changing the jaws for example. Data is ignored during pause but the time function is maintained so no data is shown during pause.

To activate the pause function press any soft key on the graph page which will pop up the pause button. To restart the graph again press any soft key and press the resume button.

11.4 Copy Function

A bit map copy of the graph screen can be made to the SD card.

To create the copy press any soft key on the graph page which cause the copy button to pop up. A pop up message giving the filename will be displayed once the copy has been made.

Section 12. Recording a Log

12.1 The nature of a record

The data record is associated with a log file, in which a single record is stored for every 'record' action. A single SD card can store a number of log files. The maximum number of log files that can be accessed is 60. Additional records will be saved to the card, but the oldest will not be available to the filing system. The 'record' is a single recording of all the parameters listed in the Log (column 1 of Appendix 1). The number of records that can be accumulated depends on the size of the SD card and the amount of data already on it. A warning message is displayed when the SD card is full.

12.2 Taking a record

A record can be taken by any one of four methods.

1/ By pressing any of the record buttons displayed on the three top-level menus.
2/ By pressing the 'record' pushbutton switch on the handle of the PLC.
3/ By sending "r" or "R" over the serial port from a dumb terminal.
4/ By closing a remote switch connected between pins 7 (12V) and 8 (CTS) on the 9 pin RS232 connector. To enable the last option, it is necessary to set up the serial port (see Section 7.2)

After a record is successfully taken, the LCpro-SD will beep. If a log file has not been set, a message appears "log file not set, set now?". If you do not wish to take a record, the message can be cleared by a second 'record' action or by pressing the No button. When a record is 'taken', it is appended sequentially in the current log 'filename' on the SD card. The 'record number', which starts at '1', is automatically incremented. Parameter values are stored as signed integers, or in exponential form; the associated units of measurement are NOT stored.

If records are to be taken at regular intervals, the timed log function can be used. It allows log intervals of 1 minute to 100 minutes in minute increments to be chosen or the intervals can be synchronised with the gas cycle in which case the increment will vary dependant on the ASU flow. The time can be scrolled from 1 minute to 100 minutes using change - allowing longer times to be selected quickly.
Select logging timed log change+ or change– The analyser will default to 1 minute increments and automatically take a record and store the results after the selected number of minutes has passed. In order to select intervals synchronised with the gas cycle press timing which toggles the mode.

Screenshot:

Selection of intervals synchronised to the gas cycles allows the maximum meaningful update rate and optimises noise performance.

The timed log will continue until it is switched off by selecting logging timed log manual or the SD card becomes full. This function can be used simultaneously with the sequence function to give more records for each step of the sequence.

12.3 Deleting a record

If you have taken an unwanted record, it can be marked as 'deleted' on the log file, but it still has a unique record number attached to it. To do this, hold the record button down continuously until the message "hold record key to delete last record" appears. Continue to hold the button pressed until "last record deleted" appears. If you release the button before the second message, no action is taken, and the message "record not stored" appears.

In regard to taking or deleting a record, the record pushbutton on the chamber handle works in the same way as the one on the front panel.

12.4 Sending a serial record

Rather than storing a record, it can be sent directly to the serial port using logging to serial. In this case, the record is not appended to a log file. Sending a record successfully requires the serial link [Section 7.2], to be set-up so that it matches the protocol settings of the receiving device. (See section 12.6). If a record is sent when the serial port has its CTS line enabled to initiate a record, an error message will be displayed.

Screenshot:

A record can also be requested via the serial port: a "P" or "p" sent to the port will cause the LCpro-SD to transmit a single record.

The serial data is sent in csv (comma-separated-value) format, without labels or headers, in the following sequence:
record number, date, time, e ref, delta e, c ref, delta c, Q leaf, chamber temp (t_{ch}) ,leaf temperature (t_l) , ASU flow (u), ambient pressure (p), ci, E, gs, A, area, rb, tl_{mthd}, Q_{mode}.
Appendix 1 gives further details of the parameters recorded and their units of measurement

12.5 Deleting a serial record

A serial record can also be marked as deleted. If you are recording to the serial port, and follow the method for deleting in section 12.3 above, the 'record number' for the deleted record is transmitted a second time, with the message, 'record deleted'. As for recording to a file, the 'record number' will continue to be incremented as if you had not deleted the record.

12.6 Receiving a serial record

This applies to Windows 95, 98, ME, NT & XP. For non-Windows systems, you will need to use a terminal emulator.

1. Select Hyperterminal from the start menu: “START”, “PROGRAMS”, “ACCESSORIES”, and “HYPERTERMINAL”

2. Select (double click on) the Hypertm.exe icon.

3. Name your new connection e.g. LCpro, and choose an icon if desired. Click on “OK”. This will save all your settings so that it is easy to repeat the transfer.

4. Ignore the telephone number and click on the “connect using” option window. Select the COM port number that you intend using on your PC. The other options on this window will then be automatically deselected. Click on “OK”. A window will then appear asking you to set the COM port settings.

Select :	bits per second	9600 (or as set on the LCpro-SD)
	data bits	8
	parity	none
	stop bits	1
	flow control	xon-xoff

5. Click on “OK”
   Ensure that the settings match those on your LCpro-SD before transmitting data (see section 7.2)

6. As a check, each time you press a “p” on the PC you will receive one data record.

7. Click on the “transfer” button and select “capture text”.

8. Enter a filename and click on “start”

9. The PC should then be ready to receive data from the LCpro-SD, which can be sent by pressing logging to serial on the LCpro-SD.

10. To stop data transfer, click on “call”, “disconnect”.

Tip: if you give your file a csv extension you will be able to import it directly into most spreadsheet programs

Section 13. Data Files & Using the SD Card

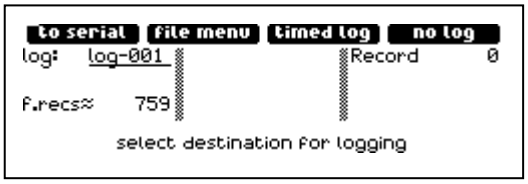
Never remove a card while you are recording or transferring files.

The SD card supplied has a minimum capacity of 1GB. All files are allocated in 512B blocks. Log files vary in length depending on the recorded data. An empty 1GB card will hold a single log file of around 8000 to 16000 records.

13.1 Selecting a File

When you first switch on, no file is selected. To set a file, install the SD card, press logging file menu then either use the arrow buttons to select an existing file or leave the arrow cursor pointing to *new file*. Press the set log button. If you select an existing file, records will be added to it otherwise if *new file* is chosen the LCpro-SD will choose a default file name with a value one higher that that currently on the card. You can change the name if you wish by using the > + - and del buttons. If your file names are numeric, the numeric part should not have leading zeroes suppressed if you wish the filing system to display them in correct order, for example log-100 will be displayed before log-2, but after log-002.

Screenshot:



13.2 Reviewing Log Files

Press the logging file menu buttons then select a file as described above. Press options review The data may be reviewed sequentially using next and previous or switched between first and last record using 1st/last. Holding down next or previous for one second will increase the steps to ten at a time (or return to single steps if pressed again for one second).
Note: Reviewing a log file in reverse (previous) takes longer to step through. Depending on the length of the file and the position of the record to be reviewed it may be preferable to select the first record then step through using next.

13.3 SD Card Data Format

SD cards are preformatted in a DOS format and the LCpro-SD stores data on the SD card using this format so files may be read with a PC which has a suitable card reader. Most current PC's and laptops have a card reader and if not external card readers are readily available at a modest cost. Since SD cards are suitably preformatted a format function is not required on the LCpro-SD. In the unlikely event that the format of a card has been corrupted then it can be formatted on a PC.

13.4 Delete (Erase) Existing Files

Press file menu, select a file as described above then press options delete. You will then be asked to confirm Yes or No. If you wish to abort the deletion press No otherwise press Yes. 'File erased' will then be displayed. Press yes to acknowledge the confirmation message.
If you currently have the selected file in use as a logging destination, you will not be allowed to delete it. If you still wish to delete the file deselect it by pressing page then no log then starting the process again.

13.5 Copying Files using the USB

Files may be moved or copied using the USB connection. When a PC is connected over the USB the SD card in the LCpro-SD will looks like a mass storage device and will appear as another drive on the PC.

Note: When the LCpro-SD is connected using the USB no file operations can be carried out from the LCpro-SD front panel. To do so may corrupt the file system.

13.6 Storing Cards

In common with all computer storage media, they must not be exposed to extremes of temperature, dampness or dirty environments.

The construction of the cards protects them from normal environments and handling but are best kept in their plastic case or a suitable anti-static container when not in use.

13.7 Using Alternative Card Types

SD Cards are available from different manufacturers and with various capacities and all those compatible with the SD card format should work in the LCpro-SD. However only those supplied by ADC BioScientific have been tested and guaranteed to work. If using SD cards supplied from elsewhere it is suggested that cards from recognised manufacturers such as **Sandisk, Kingston** or **Transend**, are used.

Section 14. How The Analyser Works

14.1 Infrared Gas Analysis

The LCpro-SD uses the principal of Non Dispersive Infrared (NDIR) for the CO_2 measurement. This relies on the fact that CO_2 absorbs energy in the infrared region in a proportion related to the concentration of the gas. The gas sample to be measured is passed through a tube (or cell). A source of infrared is directed down the cell, which is gold plated to maximise the intensity of the source. A solid state detector at the receiving end of the cell measures the amplitude of the infrared signal, which will be reduced if CO_2 is present in the gas sample. A thin film filter (TFF), with a pass band of 4.24μm, is fitted in front of the detector to narrow the bandwidth being measured to one which includes a strong absorption band for CO_2.

The reference (TO the chamber) and analysis (FROM the chamber) gases are alternated with 'zero' gas during a measurement cycle which typically lasts 16-20 seconds. The 'zero' gas is generated by passing the air through soda lime, which removes all of the CO_2. The cycle time allows for the cell to re-fill, and is automatically adjusted to suit the current flow rate, if requested by the user. This arrangement provides measurement of the CO_2 content in both the reference and the analysis gases, while eliminating much of the drift due to temperature change etc.

The infrared source is pulsed at 8Hz to give an alternating waveform. The waveform varies in amplitude with the energy absorbed by the gas, being a minimum when full-scale concentration is present and a maximum when 'zero' or non-absorbing gas is present. The waveform is rectified, with the resultant DC voltage at the zero condition providing a reference for the subsequent measurement cycle. Any change in the zero reference condition is applied ratiometrically to the measurement. This system provides very stable gain settings, which are independent of the IR source condition (unless this has deteriorated appreciably) and are only slightly affected by deterioration of the optical elements.

14.2 Gas Correction

Measurement of a gas concentration using its IR absorption properties provides a comparative measurement against a standard gas of known concentration. However, once the system is calibrated, secondary effects relating to the state of that gas being measured can subsequently affect the accuracy of measurement. This is also true of the stability of the optical system.

The absorption properties are affected by changes in temperature and atmospheric pressure. Variations due to changes in temperature are minimised with a thermal jacket around the cell assembly. Ambient pressure is monitored by a sensor in the main unit and used to compute a correction to the measured values.

The presence of water vapour in the CO_2 measurement introduces ‘interferent’, ‘density’, and ‘pressure broadening’ effects which are dealt with as follows.

As an interferent, H_2O partly shares the CO_2 IR absorption band. Its presence, therefore, appears as a proportionate level of CO_2. The effect, however, is relatively small and is eliminated by computing a reduction of the signal as a function of H_2O.

H_2O in the gas displaces CO_2 and therefore reduces the density of CO_2. At known temperature and pressure, the effect is predictable from physical laws, and its computed corrections are applied.

H_2O also has the more significant effect of broadening the CO_2 IR response band and therefore of increasing the signal for a given concentration of CO_2. As part of the design, in which the optical filter can also influence the results, the appropriate compensation has been established experimentally, and a computed correction is applied based on this.

All the values used or displayed for CO_2 and H_2O are after full correction i.e. there are no ‘raw’ values used.

14.3 Other measurements

PAR is measured with a silicon-based sensor.

Chamber temperature is measured with an accurate thermistor sensor mounted in the leaf chamber.

Leaf temperature is measured by a miniature thermistor mounted on a spring, which presses against the leaf (Broad and Narrow chamber types only). An optional miniature loose thermistor sensor can be positioned in the chamber at the user’s discretion (used for the Conifer, Arabidopsis and Small leaf chambers). This thermistor plugs into the jack socket on the handle lid and disables the spring-mounted thermistor. The user can also select between the value measured with the thermistor or an internally calculated value derived from the energy balance equation (See Appendix 3 – Leaf surface temperature).

Gas flow rate to the chamber is measured by an accurate air mass flow sensor and controlled to either a default or user-selected level.

Section 15. Gas Circuit Description

Fresh air is drawn in by the internal air supply pump P70 via the external particulate filter, and then the hydrophobic filter. The hydrophobic filter removes dust particles and helps to prevent water being sucked in. It contains a porous PTFE membrane, which prevents the flow of water by using the effect of surface tension. If the water contains impurities, which substantially reduce the surface tension, e.g. detergent, the water may be sucked in. Just before the pump is a solenoid valve SV62, to switch in the CO_2 enhancement system, and the internal (large) volume, the purpose of which is to average out fluctuations of CO_2 and H_2O concentrations that occur naturally in the background. This greatly reduces noise on differential measurements. The air probe, if connected, will also help to reduce noise, having a nominal volume of 460cc when extended.

The air then passes either directly through SV10 or via the soda lime column. In the latter case the air passes through the soda lime column to remove CO_2 and then through a dust filter to remove any soda lime dust. Soda lime generates water vapour as a by-product of the conversion process. This causes the air leaving the column to be very damp and, if the analyser is taken from a hot place to a cold place, condensation will form inside the 'zero' tube to the chamber. To minimise this effect, the air passes through an equilibriator pipe that matches the water vapour concentration inside the tube to that of the outside.

The stripped air is then passed directly to the analysis cell as 'zero' air when SV1 is on and SV2 is off. In addition, the stripped air can be mixed with non-stripped air in a variable ratio dependant on the duty cycle of the solenoid valve SV10. This air, containing a controllable concentration of CO_2 passes, via a buffer volume, through a second solenoid valve SV12, which also has a variable duty cycle, allowing the air to be either wetted or dried. Small dust filters after the wetter and drier columns remove any dust.
The air then passes either directly to the analysis cell as 'reference' air when SV1 and SV2 are both off, and continuously through the mass flow meter as air supply to the leaf in the chamber. The mass flow meter F10 acts with the pump P70 in a closed loop feedback system to keep the air supply constant despite changes in pump loading due to the various states of SV1 and SV2.

The air supply to the chamber first passes through a temperature equilibration loop that brings it to the chamber temperature. The air is stirred around the chamber with a fan, which also blows air through the analysis cell when SV2 is on. Excess air is allowed to escape via a waste valve in the top half of the chamber. This air would otherwise pressurise the leaf if the jaws were tightly shut.

When CO_2 climate control is selected and a non-ambient concentration set, pump P140 and solenoid valves SV62 and SV140 will all be turned on. Normal air is then drawn through CV1, and mixed with pure CO_2 from the cylinder. The ratio of the flow through CV1 and the flow through SV140 define the available concentration of CO_2. The two gasses pass through a volume to allow them to mix thoroughly before passing through a mass flow meter F130. The flow meter is in a closed feedback loop with P140 to maintain a constant flow and hence a constant mix. The air leaving F130 through SV62 is used by P70 in the usual way, with excess flow escaping to waste via a tee. When ambient climate is set, P140, SV62 and SV140 are all off. SV140 then wastes the CO_2 (which continues to be released from the flow controller) to atmosphere (to avoid over pressurising). SV62 then only allows air to flow directly from the filter.

Section 16. Maintenance

16.1 Tools

Note: When the LCpro-SD is first switched on, the screen shows the 'Software version', and 'Instrument Serial Number'. Always quote these in correspondence relating to the instrument.
There are no special tools needed to dismantle the LCpro-SD and replace parts. The use of a small sized thermostatically controlled s**oldering iron** is recommended to replace electronic components, as is an **anti-static wrist strap**, especially when working on the digital board. All screws are metric except the hexagonal pillars on the 'D' type connectors. All screw heads are **'Pozidrive' (crosshead)** types. A **sphygmomanometer** without the cuff is useful for testing for leaks or, alternatively, a **water manometer** connected with pipe and a tee to a 100ml disposable syringe can be used. A **small paintbrush** is good for general cleaning, and **cotton wool buds** and acetone or **alcohol** are good for cleaning the cell.

16.2 Accessing the Inside of the Main Instrument

With the LCpro-SD switched off, unscrew the 4 M3 screws securing the two upper strap clips, and then the 5 M2 securing screws around the top bezel. The display panel can then be lifted up and to one side.
All pipes are push-on although some have been fitted using 'Hellerman'™ oil, which allows pipes to push on easily, but sticks them in place when dry. If a pipe will not pull off easily, do not continue to tug as the pipe tends to become thinner and grip even tighter, instead use a pair of **thin nosed pliers** with one jaw either side of the connector to push on the end of the pipe. This particularly applies to barbed plastic fitting, which might otherwise be damaged. Note that if you remove a pipe from a barbed plastic fitting by cutting along the length of the pipe with a sharp knife, you will probably damage the barb and could introduce a small leak.

CAUTION:
Care should be taken to protect the display membrane as it can be easily damaged.
The digital board (PCA-288p) is attached to the display panel and, unless you are taking static precautions, you should avoid touching the electronics. Do not pull on the electrical cables.

To gain further access to the analogue board (PCA-281) and the piping. Unclip and remove the battery cover plate then remove the six M2 screws on the outside of the lower bezel, and the two M3 screws fixing the lower bezel under the battery cover then lift off the lower bezel.
(*Note the position of the long M2 screw at the opposite end to the chemical columns*). Now remove the four M3 screws at either side of the curved plate and the two nearer to the middle. Extract the spacer (if fitted) from between the volume housing and the central chassis bracket. The curved plate can now be depressed slightly and withdrawn upwards, taking care to prevent fouling the hank bushes on the volume and gas piping.

CAUTION:
Do not try to spring the right hand edge of the case, as this may cause the 12-way connector between the analogue and interface boards to unplug. The angle at which it rests does not allow it to be easily reconnected.

There is usually no advantage in dismantling the LCpro-SD further.

When replacing a set of screws it is best to have all of them inserted a few turns before tightening any of them fully.

16.3 Air Flow (Mass Flowmeter)

The mass flow meters are in closed feedback loops with their pumps, and will drive them faster or slower until the set flow is achieved. If a pump has stopped or is going as fast as possible, the mass flow meter may be faulty. If there is a leak inside the console, in the piping between a flow meter and its pump, the pump will run faster than normal, but insufficient flow will emerge from the flow meter. See leak testing, below.

The air mass flow meter is very stable. If its calibration changes, the cause is almost certainly contamination inside it. If this happens, a subsequent re-calibration cannot be considered reliable and a replacement of the flow meter and its interface board (PCA-272) is recommended. It might be possible to blow out the contamination. The board is supplied pre-calibrated and, as such, replacement is a simple matter of removing the pipes [See Section 16.1 if you experience difficulty], and pulling the flow meter and its board off the mounting pillars. Fitting the new board is a reversal of the removal procedure, but ensure that the 5-way electrical connector is properly engaged into the socket on the analogue board (PCA-281) before pushing the board onto its pillars. Support the flow meter with one hand while pushing the pipes back on with the other. In the case of F130, which is soldered to the board, a replacement flow meter could be fitted. Calibration consists of setting zero volts at TP130 (using RV130), with the pump disconnected electrically, and a flow of 715 to 730ml/min (using RV134) when it is switched on. i.e. CO_2 control is requested.

16.4 Leak Testing

There is an easy method for testing for a leak anywhere in the console that requires the use of a water filled manometer. First wait until the CO_2 cylinder, if fitted, is empty. Ensure the wetter column is empty (the vapour pressure from a full column will confuse results). Link or block the three connections to the chamber, red, white, and black, and apply about 10cm water gauge of pressure to the manometer and inlet. Wait a few seconds for the manometer reading to settle then note the reading. Wait a further 10 seconds then, if you cannot detect a fall in the reading the system is sufficiently leak tight.
Repeat the test but pressurise to between 25cm and 30cm water gauge. The pressure will drop fairly rapidly to between 15cm to 25cm, then stop suddenly. This tests the operation of the 3“ water one way valve CV1 and waste pipe.

16.5 Filters

Filters must be replaced if there is evidence that the pump is being overloaded, as indicated by an inability to achieve maximum airflow for example. Otherwise replacement should be based on an assessment of previous use in dust-laden conditions, or on visual inspection.

There is a main filter inside the console and filters at each end of the columns (see section 16.7). The filters in the console unit can be accessed by removal of the top (and side for maximum convenience) described under section 16.2 above. Individual filters can be disconnected from their piping in each part of the gas circuit, and a new one inserted. If during these operations piping is damaged, or a good seal cannot be achieved with existing piping, then the section of pipe should be replaced with a new piece. (See Appendix 9 for details). If a section of pipe is seen to contain any debris, it is easier and safer to replace the pipe. The spares and accessories list (Appendix 9) gives details of the necessary piping, excluding the pipe in the umbilical cord connecting the chamber, repair of which is beyond normal maintenance.

In the chamber, there is a permanent mesh filter under the stirrer, which is best cleaned of large debris with a small paintbrush. The fan cannot be removed to assist cleaning and so the paintbrush must be small enough to pass between the blades of the fan. If there is fine dust on this filter, there is a risk that it will be pushed through the filter with the brush. It is best to remove lower jaw from the handle and blow backwards through the mesh filter while you disturb the dust with the brush.

There is another permanently fitted mesh filter in the corner of the stirrer cavity in the upper jaw, leading to the waste valve. The same general comment applies with regard to dust, but you should not blow backwards through the valve, which is delicate.

16.6 Pumps

The pumps are held in position on the analogue board by a “tie wrap” which enables easy access for servicing. The pumps are supported by a foam pad which acts as an anti-vibration mount and reduces pump noise. To remove a pump simply unplug its electrical lead, ease the tie-wrap towards the back of the pump (away from the pipes) then gently ease the front end of the pump upwards and forwards from under the tie-wrap. Once free of the tie-warp the pipes can be disconnected if required but note should be taken which pipe attaches to which entry on the pump. Refitting is the reverse of removal but care should be taken to ensure that the pipes do not become kinked over and collapse as this will restrict the flow.
The most common pump problem is insufficient flow and is caused by dirt contamination under the flap valves. These can be accessed by removing the four self-tapping screws that retain the head. Note the orientation of the parts. Some pumps have parts that will fit two ways round but only one way will work! Wipe the flaps, even if they look clean, with a smooth cloth. Reassemble, but only tighten the screws enough to make the pump leak tight. After much use (a few years), the motor bearings will become slack and noisy, and the pump will need replacing. The two pumps are identical, and since P140 is only used when the CO_2 supply system is running, it may be substituted for P70 as an emergency repair or for fault finding.

16.7 Chemical Column filters

Maintenance on the chemical columns is limited to checking the general condition of the ‘O’ rings and the filters in the column caps. Occasionally the columns should be cleaned in soapy water and left to dry before replacement.
Air seals should be maintained around all of the ‘O’ rings. The use of silicon grease provided will greatly assist this and help to keep the ‘O’ rings in good condition.

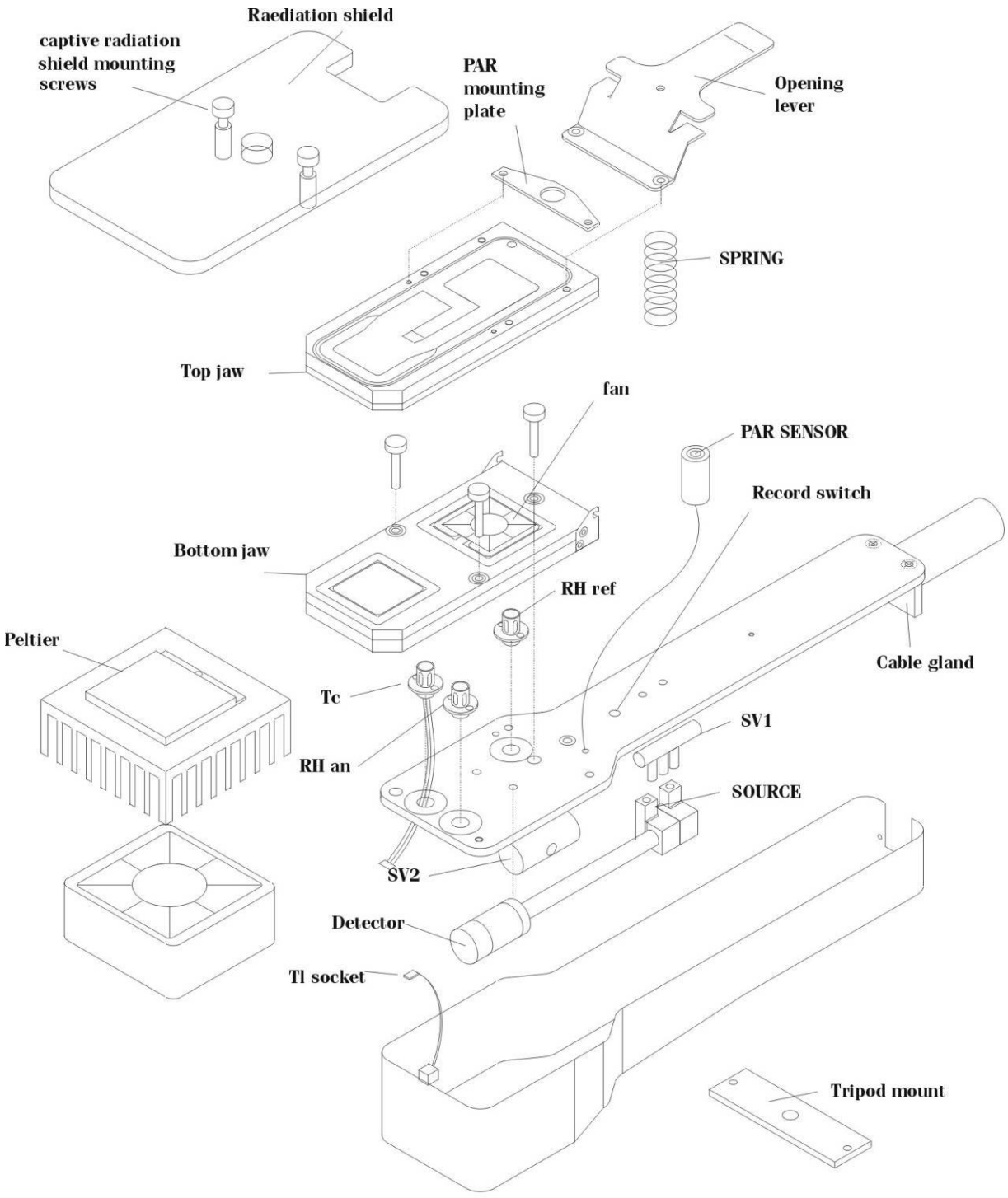
16.8 Display Contrast Setting

The normal contrast setting for the display changes little with variations in ambient temperature. Manual re-adjustment to suit operator preferences is via the **'contrast'** potentiometer on circuit board PCA-288p indicated in section 2.5. This can be accessed by removal of the top display section of the LCpro-SD as previously described.

16.9 Software and Serial Number

The Software Part number and version and the instrument Serial number for the LCpro-SD are shown on the display when the LCpro-SD is first switched on. If, for some reason, the LCpro-SD cannot be switched on the software part number and version are shown on the label attached to the digital board (PCA-288p) and the Serial number label is attached to the battery cover. **These details should be quoted in any correspondence.** The current ADC part number for the software is PRD1063.

16.10 Dismantling the Chamber



CHAMBER EXPLODED DIAGRAM

The radiation shield can be easily removed without affecting the functionality of the chamber. You may wish to do this where solar radiation is not a problem, and the shield is too bulky. Loosen, but do not fully remove, the two knurled screws, and slide it away from the opening lever. From serial number 31453 the shield is hard coated on both sides to minimise scratching.

The par sensor is permanently connected electrically but the sensor head can now be removed by pulling it out of its mounting plate. This is preferable if you wish to remove the upper jaw.

The top jaw can be removed by pressing against the spring so that one hinge pin is no longer hooked in place. The jaw can then be twisted to disengage the pin then pulled away from the other hinge. If the leaf spider thermistor is present, either it should be removed, see section 3.5, or care should be taken not to damage it while the top jaw is being removed. The top jaw (Narrow and Broad only) contains the waste valve, which is only likely to cause problems if dirt gets on the seat. The valve spring is in a hole that can be seen through the window. The bottom of the spring pushes on the diaphragm, which is transparent, and the valve seat can be seen through it. The top jaw also contains a magnet to operate the jaw-open detector switch.

The Broad and Narrow windows are made from polycarbonate. If one is badly scratched, it should be polished or replaced. For chambers manufactured from 2003 onwards, the polycarbonate windows are hard coated, which greatly reduces the risk of scratching. Scratches cannot be polished out of these coated windows. Conifer windows are made from PTEG, and cannot be hard coated.

The bottom jaw can be removed by unscrewing the three knurled screws. Take care not to lose the three captive nylon spacers on the knurled screws. If the screws are too stiff to loosen by hand, you can use a coin, but the slot should not be used for tightening. It is not necessary to unscrew the screws so far that they become detached, they just need to be loose. While the bottom jaw is removed, check that the fine mesh filter under the fan does not have dirt in it. If it does, use a small paintbrush between the blades of the fan, or blow clean air into the pipe. The bottom jaw is constructed of two pieces of aluminium, screwed together with air tight gasket compound. It is important for electrical continuity that the screws between the jaw pairs are tight, especially the hinge screws. There are wires between the plates, connecting from the fixing screws to the fan and jaw-open switch sensor. It is not practical to dismantle it to this extent. If you have problems with the fan or sensor, return the jaw to ADC BioScientific or your local Service Centre.

Once the lower jaw has been removed the reference and analysis humidity sensors and the chamber temperature sensor (which looks like a black bead inside its metal housing) are exposed. Take care not to lose the three spacers and ‘O’ rings on the sensors or the two ‘O’ rings on the gas stems. (The two gas stem ‘O’ rings can sometimes be caught up in the foam insulation in the lower jaw assembly. If you spot an ‘O’ ring missing that is the first place to look).
The humidity sensors can be withdrawn by unplugging them after the two M1.6 slot-headed countersunk screws have been removed. Note that the software stores separate span and zero constants for each of the sensors, so be sure to put them back in their original locations if you wish to avoid re-calibration. If it is possible that the sensors have become swapped when you reassemble it, and you have no calibration facility, choose the locations that make the sensors most closely agree when there is no leaf in the chamber. The sensors are interchangeable to within 5%RH without re-calibration. Lightly grease the five ‘O’ rings with the silicone grease supplied before re-assembly, and be sure that the Nylon spacers and

'O' rings around the two humidity sensors and the temperature sensor are pushed completely down to the flange before re-assembly.

The temperature sensor has wires and a socket on the back and it can only be removed by taking off the handle lid (see below) and disconnecting it. The socket will pass through the sensor hole with care. The plug is not polarised and so a note should be taken of its orientation. If you are unsure, no damage will result from an incorrect orientation, but the temperature reading will be obviously in error. The sensors are interchangeable to better than 0.1°C without re-calibration.

The handle lid is removed by extracting the two upper M2 screws either side of the cable gland, and the two M3 screws that fix the tripod mount. Remove it carefully as the leaf temperature jack socket assembly has two twisted wire cables and two sockets, which should be unplugged. The detector socket (white twisted pair) is polarised but the leaf thermistor socket has no polarising key, so make a note of the orientation before removal. The position and function of the connectors and the potentiometers is shown on a label inside the lid.

Before proceeding further, note that the metal plate is at ground potential. If you remove the circuit board or do anything which will short circuit the back of the board to the plate while the instrument is switched on and connected to the chamber, you are likely to blow the fuse and/or damage the electrical components.

The status of the solenoid valves is indicated by the light emitting diodes LED10 and LED90 (see gas circuit). The solenoid valves are both replaceable items and cannot be dismantled. If leak testing them, note that SV2 is of a latching type; that is, it stays in its last position without power, while SV1 has a spring return. If SV2 leaks, it is probably dirt on the seal, which might be possible to dislodge as follows. Ensure that the valve is switched so that the leaky direction is open. Strip back 4mm of the insulation from some 7/0.1 tinned copper wire and, while turning it, push it into the valve entry that leaks. The wires will spread sideways and dislodge the dirt from under the seat. Remove the wire, and blow clean air into the leaky port, to blow the dirt out. When replacing the valves, refer to the piping diagram.

A low detector signal can be attributed to a faulty source, faulty detector or a contaminated cell

The infrared source is the first item to check, as it is the easiest. A source with a working filament has enough light escaping through the exhaust of the tubeset for it to be visibly flashing under normal indoor illumination. Alternatively the filament can be tested for continuity with a multi-meter, it should read about 125 ohms. To change the source it is necessary to, at least, partially remove the cell. Unscrew the single M3 screw under the lower jaw, then the two M3 screws that retain the jaw open clip. The cell can then be raised high enough to access the two M2 screws that hold the source housing to the cell. Remove the housing complete with the source and insulation. The source is a small light bulb, which has been pre-aged to minimise drift. It has a thin envelope to minimise infrared loss, and a low-mass, fast response filament. The envelope on a good source should be clear. If it looks black or silvery, it should be replaced.

The detector can be removed with the cell in place. It is a static sensitive device and so static precautions should be observed. Unplug its connector, and remove the large piece of insulation around the detector housing. Unscrew the knurled nut by turning it counter-clockwise, but try to avoid turning the circuit board. The detector complete with socket and lead, may now be withdrawn. Do not touch the window on

the detector. Any fingerprints need to be removed with alcohol and cotton wool. If the detector is removed from its socket, note its orientation with respect to the circuit board. (The "tab" on the detector case should line up with the semi-circular cut-out on the socket. Also note that there is a thin film filter (TFF) assembly remaining in the end of the tubeset. It is a loose fit, and may fall out. Replacement is a reversal of the removal procedure. Tighten the knurled nut with your fingers only, do not use pliers, and do not turn the circuit board.

If the detector signal falls so far that CO_2 zero cannot be manually corrected with the potentiometer or dirty water has entered the analyser, it is possible that the analysis cell will need cleaning. It is best to first remove the cell as described above then pull the cell off the pipe that connects it to SV2. ***Note that the detector is static sensitive and suitable precautions should be taken.*** Unplug the detector and source leads from the board. Remove the large insulation around the detector. Pull back the insulation around the cell sufficiently to remove the two M2 screws that retain the detector housing. Remove the source and its two M2 screws, and the flow sensor housing and its two screws. You can now look through the cell, which should appear uniformly shiny. If it appears dull or patchy, it may be possible to clean it.

The cell is gold plated internally and can be cleaned with care with cotton buds or cotton wool wrapped around a thin stick. For persistent dirt, alcohol or acetone can be used. If the cell has had aqueous liquid in it for a few days, it is possible that there is corrosion under the plating, in which case, it will need to be re-plated or replaced by ADC BioScientific.

16.11 Removing the Handle Cover

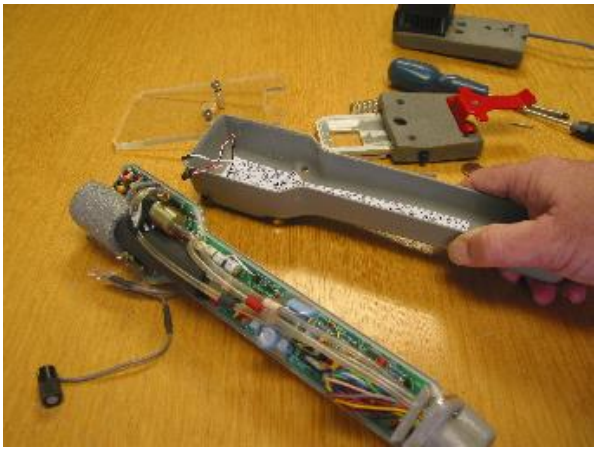
Unscrew the two retaining screws from the tripod boss. Note the orientation of the boss – On some instruments the fixing screws are not in the middle.

Remove the two screws furthest from the baseplate, which pass through the cable gland assembly.

Sometimes these screws are cross head rather than slotted.

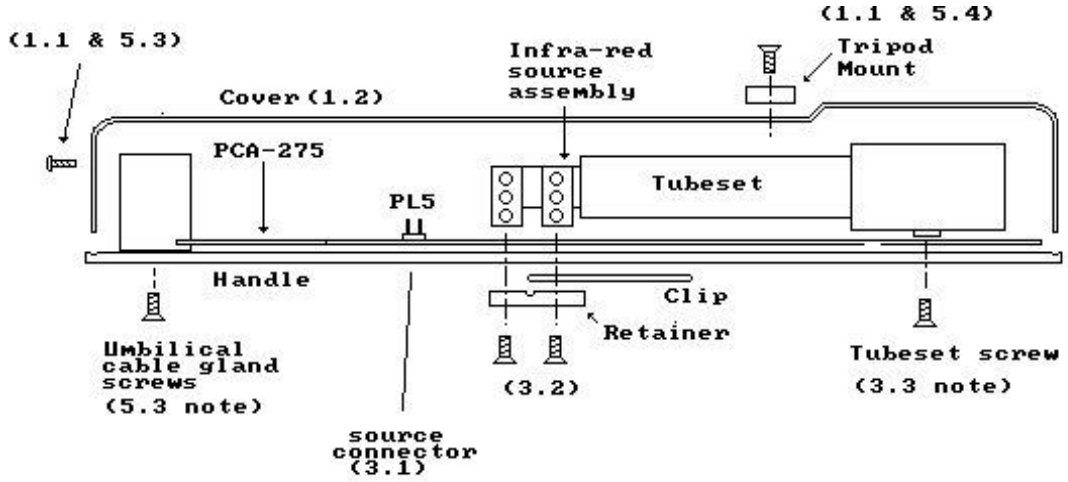
If you wish to completely remove the handle cover, take a note of the orientation of the connectors (there is a diagram inside the handle cover) and unplug the two sockets of the Tleaf thermistor and the CO2 zero potentiometer wires from the PCB.

On early models that have not been upgraded there is only a single twisted pair of wires from the leaf thermistor jack socket to PL20 on the board.



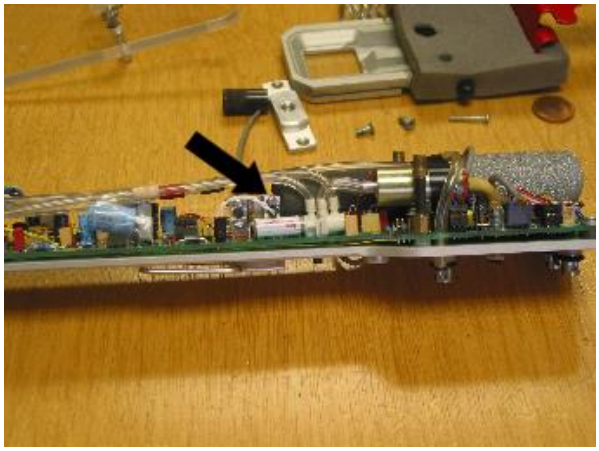
Later models have the CO2 zero potentiometer mounted in the handle cover instead of on the board so that an additional twisted pair of wires runs from the jack-socket assembly to RV9 on the board

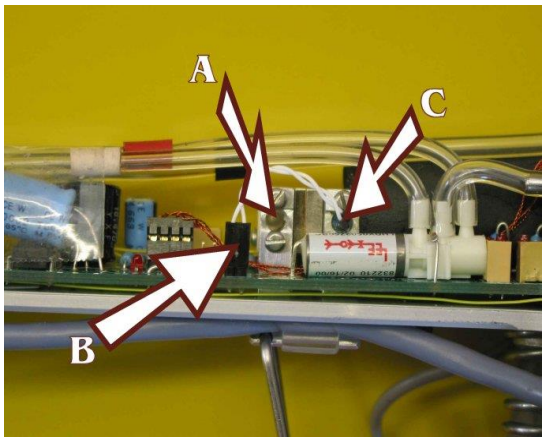
On the diagram/label in the handle:
R indicates red
B indicates black
W indicates white
X indicates no connection (polarising pin)



16.12 Checking the Source

First remove the jaws and then the handle cover, (see previously). It is not necessary to completely remove the cover.
The source is indicated by the black arrow in the above picture. It is a small light bulb, which has been pre-aged to minimise drift. It has a thin envelope to minimise infrared loss, and a low-mass, fast response filament.

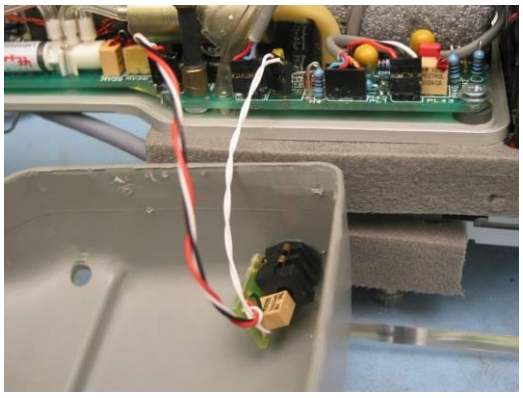




Look for flashing coming from the small window (A). This is best done in a dark area.
If the source has a white body then light can be seen through the body.
If there is no sign of flashing then unplug the source connector (B) and check resistance to see if the source bulb is open circuit.
If there is no flashing and the source is open circuit then the source (C) will need to be replaced. It will also need to be replaced if it is blackened or in any way dark.

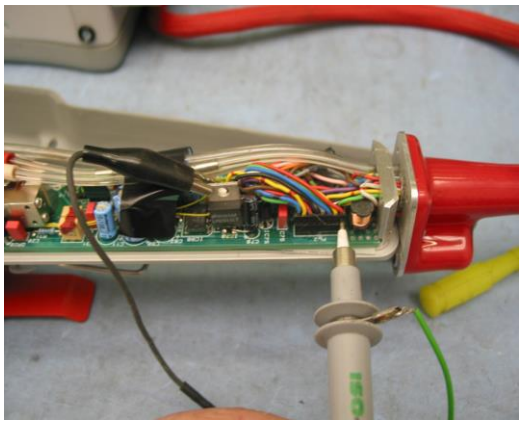
The resistance of a good source is about 125 ohms. If there is flashing then carry out the next test.

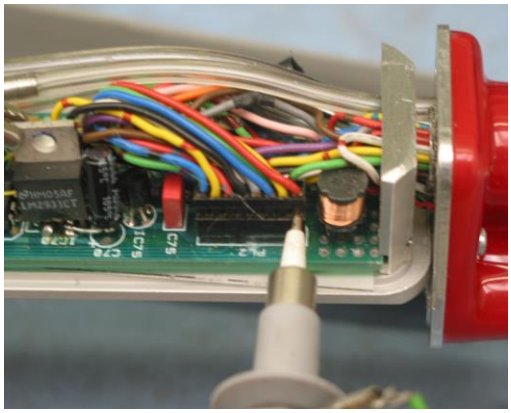
16.13 Checking the Detector



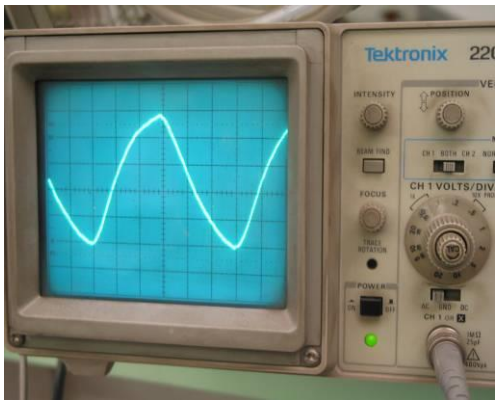
Before checking the detector, check the source is OK. Remove the jaws and handle cover, without disconnecting it electrically.
Check that the wires are not broken and that the connector is securely fitted to the PCB pins.

Place the earth clip of an oscilloscope probe on the metal body of the regulator as shown.





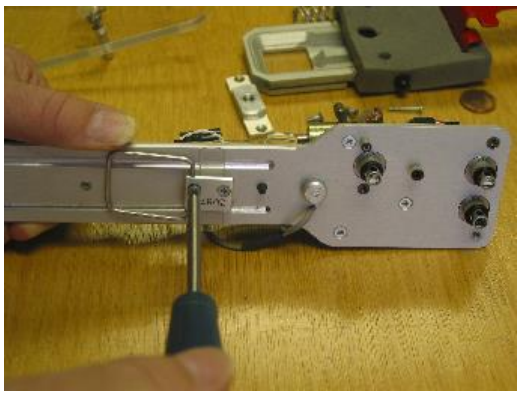
With a narrow oscilloscope probe measure the signal on the red wire of the connector. This can be done through the small hole in the side of the connector.



The detector signal should be an approximate sine wave between 3.5 and 2 volts peak to peak, depending on (amongst other things), the setting of the CO2 zero pot. If you do not have an oscilloscope measure the voltage with an AC Voltmeter. If there is no detector signal, then the detector is probably faulty and will need to be replaced.

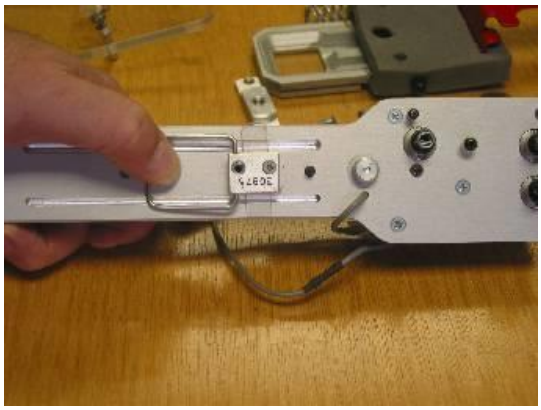
The detector can be removed with the cell in place. It is a static sensitive device and so static precautions should be observed as for changing the EPROM. Unplug its connector, and remove the large piece of insulation around the detector housing. Unscrew the knurled nut by turning it counter-clockwise, but do not turn the circuit board. The circuit board, the socket, and the detector may now be withdrawn. Do not touch the window on the detector. Any fingerprints need to be removed with alcohol and cotton wool. If the detector is unplugged, note its orientation with respect to the circuit board. Also note that there is a thin film filter (TFF) assembly remaining in the end of the tubeset. It is a loose fit, and may fall out. Replacement is a reversal of the removal procedure. Tighten the knurled nut with your fingers only, do not use pliers, and do not turn the circuit board.
If you do have a detector signal then reassemble the handle cover, replace the cable clamp securing screws and fit the camera tripod boss and its securing screws.
Leave the instrument to warm up for 10 minutes and reset the CO2 zero as indicated in the manual.

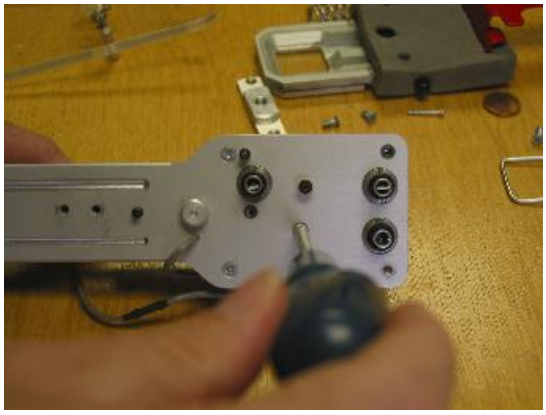
16.14 Replacing the Source



Unscrew and remove the two screws from the lever catch body.

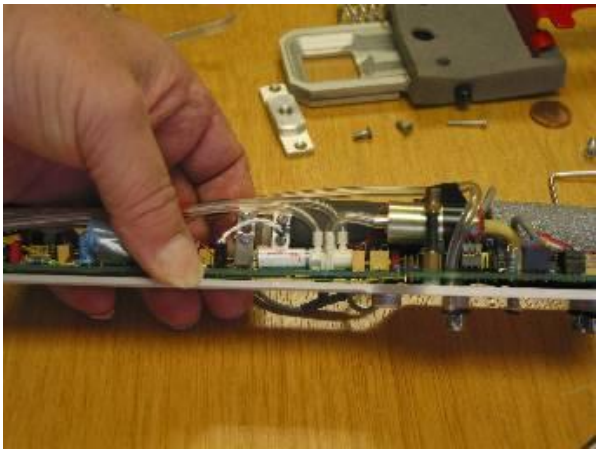
Note that the retaining screws are off-set and not central. Remember this when reassembling the handle.

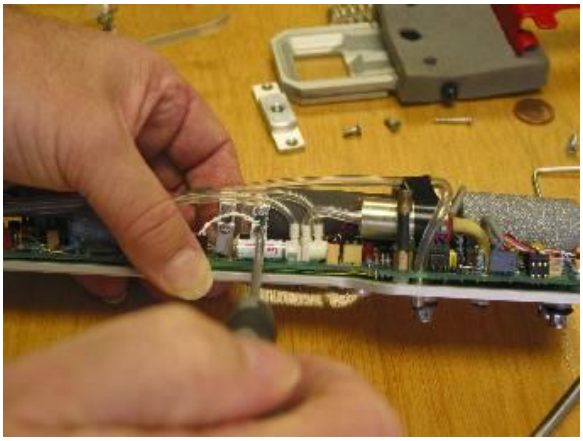




Unscrew the analyzer retaining screw TWO TURNS. Do not remove this screw completely because it locates the analyser cell at the detector end and prevents its pipe connection and detector wires being strained.

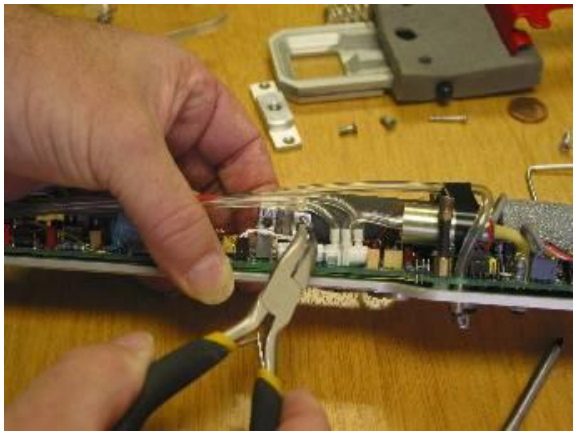
This will give you just enough slack to lift the source end of the analysis cell, giving you access to the lower source retaining screw. If there is not enough movement to get a screwdriver on to the sources lower screw then go back to the previous instruction and unscrew the analyzer retaining screw another half a turn and try again.

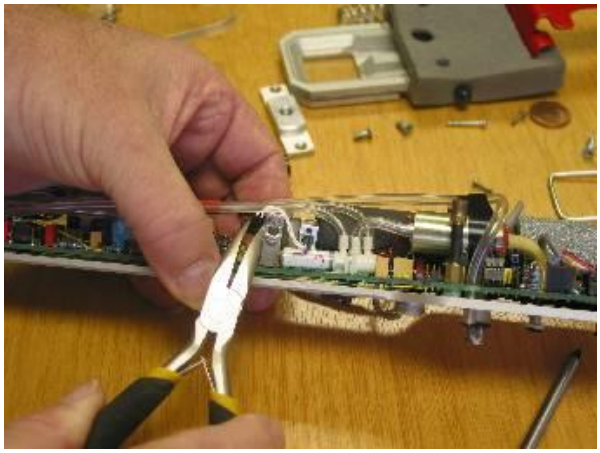




Using a small flat bladed screwdriver, unscrew and remove the upper and lower source retaining screws. These screws are stainless steel – Do not replace them with mild steel types.

Using tweezers or long nose pliers gently pull out the old source from the analyzer cell. Take care not to allow any debris to get inside the analysis cell.



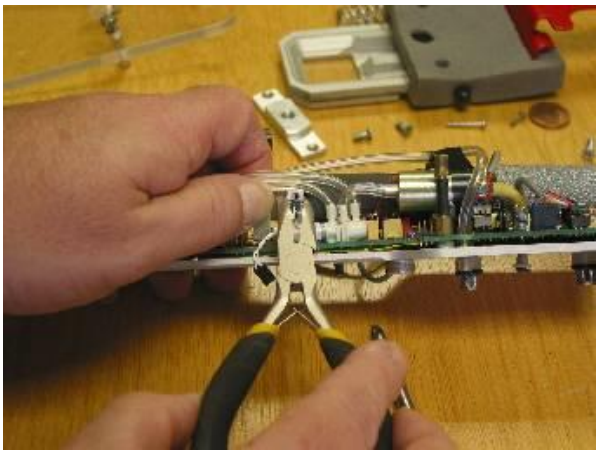


Disconnect the sources electrical connection from the circuit board.

Then remove the old source.

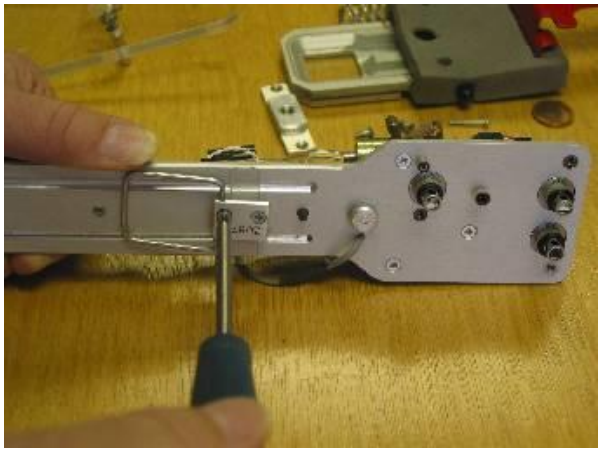
A thermal isolation gasket should have come off with the old source.

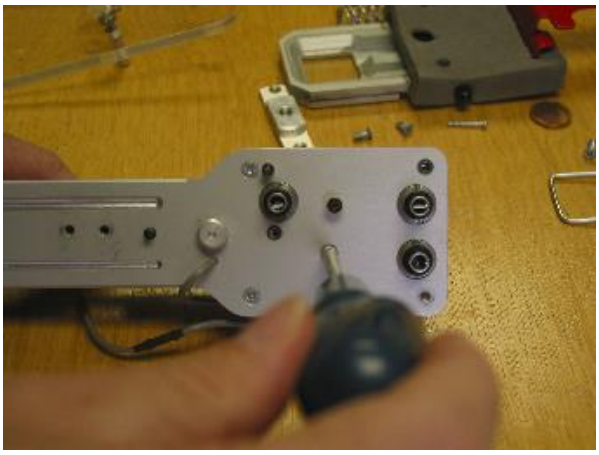
Remove the thermal isolation gasket from the old source and fit it onto the new replacement source.



Fit the new source in to the analyzer cell and replace the two retaining screws. Connect the electrical connections to the circuit board on connector PL5. The source is not polarized.

Refit the lever catch body, ensuring the correct orientation so that the body sits centrally on the handle.





Tighten the analyzer retaining screws but DO NOT OVERTIGHTEN, the threads are into plastic spacers.

Refit the Handle cover and Jaws.

IMPORTANT! TWO THINGS TO RECALIBRATE AFTER FITTING A NEW IR SOURCE.

- Allow the machine to warm up and perform a CO2 zero calibration, see section 7.5.2 of this manual.
- Run a phase check, see section 7.4 of this manual

16.15 Peltier Cooler and Light Unit.

The peltier cooler, mounted on the lower jaw, is not serviceable by the user, and should be returned to ADC BioScientific or your local Service Centre if there is a problem with it. It is made from semiconductor blocks sandwiched between thin ceramic plates, and is brittle, so it can easily be broken if the jaw is dropped.

Servicing the light unit is limited to loosening any dirt with a soft brush, and blowing it out. Contact ADC BioScientific or your local Service Centre for any other problem with the light unit. (See inside front page for contact details)

Section 17. Error messages and Fault finding

Fault (warnings in quotes)	Possible cause	Remedy
Analyser will not switch on	Battery discharged Fuse blown	Recharge battery Replace fuse
Analyser will not respond to key presses	Software bug causing it to ignore keypad	Invoke hardware reset by pressing, simultaneously, the left 2 keys and the right key
Pump running fast but not enough flow	Any pipe (red, white or black) is disconnected. The air inlet (top connector) is partially blocked or supply pipe is too thin or long The black pipe is squashed flat inside the chamber handle.	Reconnect Remove blockage or use bigger supply pipe Reposition the pipe.
Pump supplies enough air but is noisy	Pump bearings worn	Replace pump
Difficulty removing or dis-mantling chemical column	'O' ring seals are dry	Apply a thin wipe of silicone grease to the 'O' rings.
"CO_2 signal failure"	The detector signal is out of range of the A-D converter. Due to: either CO_2 zero cal. or Source failure or Detector failure	Recalibrate CO_2 zero. Look for source flashing- light escapes through the base. Measure resistance (125Ω) Using oscilloscope look for about 15mVpk-pk triangle wave on pin 2 (red) of PL1, on PCA-275A
"CO_2 low energy"	Gain set too high Soda lime exhausted Dirt in the cell	Recalibrate CO_2 zero. Check soda lime Remove and clean cell
"C_{ref} low, check absorber" Can and Cref readings very low or zero	Soda lime exhausted or red (zero) pipe blocked, or valve SV1 stuck in Normally Open (NO) position	Check soda lime, if OK check pipe, if OK check SV1
Low or negative CO_2 values (reference and analysis)	Soda lime exhausted SV2 is stuck	Check soda lime Use the exercise SV2 option in config>diagnose>sys info>sv check , the valve should click loud enough to be heard within 1 metre of the instrument. Contact ADC for help if the valve does not work.
Only C_{an} low or zero	Fan stopped (this happens if jaws are open) Or if a jaw screw has been over tightened, which can break a wire attached to the jaw. No jaw pressure, caused by a big leak. Fan outlet blocked	Check fan, shut jaws. Check jaw gasket and O rings. Check outlet filter
"CO_2 signal over range"	Signal at A-D converter is out of range. Gain is set too high	Recalibrate CO_2 zero.
"span gas reading is too low"	The analyser cannot reduce its span coefficient low enough for the value you have set.	Check that the span gas is not being diluted. Check that the value you have entered matches the cylinder
"span gas reading is too high"	The analyser cannot increase its span coefficient high enough for the value you have set.	Check that the analyser is not being pressurised Check that the value you have entered matches the cylinder
"Current log file cannot be deleted (or renamed)"	You cannot delete a file if it is enabled to receive records	Switch logging off before deleting.
"Chamber flow not as set"	Pipe not connected or kinked in the handle Air supply to analyser partially blocked	Check Check
"T_{leaf} probe error"	The Tleaf reading is outside the A-D converter range.	Check probe is connected, and is not broken (should be 2kΩ at 25°C)
Both the "C" readings are the same or nearly the same and do not change.	Confirm this by briefly opening the jaw and blowing into it then closing, C_{an} should go very high or o/r (over range), try this twice. SV2 may be stuck	Use the exercise SV2 option in config>diagnose>sys info>sv check , the valve should click loud enough to be heard within 1 metre of the instrument. Contact ADC for help if the valve does not work.

Appendix 1. LCpro-SD Parameter Information

Symbol	Description	Screen	Log?	An o/p	Displayed Units	Range	Type
A	Photosynthetic rate	2	16	-	µmol m^{-2} s^{-1}	0-100	Ca
Area	projected leaf surface area	3, cfg	17	-	cm^2	0.1-100	G
c_{ref}	CO_2 reference	1	6	y	vpm	0-2000	M,Co
c'_{an}	CO_2 analysis (corrected for dilution)	1	-	y	vpm	0-2000	M,Co
Δc	Delta CO_2 (Cref - C'an)	1	7	y	vpm	+/-2000	Ca
c_I	Sub-stomatal CO_2	2	13	y	vpm	0-2000	Ca
[cab]a	Infra-red absorption due to analysis CO_2	diag	-	-	%	0-40	-
[cab]r	Infra-red absorption due to ref CO_2	diag	-	-	%	0-40	-
[c]z	Raw CO_2 zero reading	diag	-	y	adc counts	-	-
C_e	Soil Respiration	2	-	y	µmol s^{-1}		Ca
Cfg:	Chamber type / configuration set	3, cfg	-	-	-	-	-
Dt	Date (text)	diag	2	-	-	-	-
E	Transpiration rate	2	14	-	mmol m^{-2} s^{-1}		Ca
e_{ref}	H_2O reference, as partial pressure	1	4	y	mBar	0-75	Ca,Co
e'ad	H_2Oanalysis, dilution corrected	1	-	y	mBar	0-75	Ca,Co
Δe	Delta H_2O (e'_{an}-e_{ref}), partial p.	1	5	y	mBar	+/-75	Ca
gs	Stomatal conductance of H_2O	2	15	y	mmol m^{-2} s^{-1}	0-1	Ca
Hfac	H factor - energy conversion factor	3, cfg	-	-	-	0.1-1	F,G
Log:	Name of log file	3, cfg, log	-	-	-	--	G
Mem.	Free space on memory card	log	-	-	Kilo Bytes	-	-
NCER	Net CO_2 Exchange Rate	2	-	y	µmol m^{-2} s^{-1}	0-100	Ca
P	atmospheric pressure	1, hid	12	y	mBar	600-1100.	M
Phase	CO_2 rectifier phase shift	hid	-	-	°	-	-
Power	Bar graph showing battery state	2	-	-	-	10.5-14.3	M
Q	P.A.R. at window	hid	-	-	µmol m^{-2} s^{-1}	0-3000	M
Qleaf	P.A.R. incident on leaf surface	1	8	y	µmol m^{-2} s^{-1}	0-3000	Ca
Q_{mode}	Light measurement method	-	20	-	-	-	-
r_b	Boundary resistance to H_2O	3	18	-	m^2 s mol^{-1}	0.1-9	G
r_b set	Boundary resistance at full flow	cfg	-	-	m^2 s mol^{-1}	0.1-9	G
Record	Current record number	2, log	1	-	-	-	-
rs	Stomatal resistance to H_2O	-	-	-	m^2 s mol^{-1}	0.1-2000	Ca
Tch	leaf chamber temperature	1	9	y	°C	-5 to +50	M
Tl mtd	Leaf temperature determination method	3, cfg	19	-	-	-	-
Tleaf	Leaf surface temperature	2	10	y	°C	-5 to +50	M,G
Tm	Time of day	diag	3	-	-	-	-
Trw	Chamber window transmission factor	3, cfg	-	-	-	0.25-1	F,G
u	ASU mass flow (measured)	2	11	y	µmol s^{-1}	68-341	M
U_a	Last value of an flo, from flowcheck	cfg	-	-	µmol s^{-1}	-	Ca
u_s	Flow per unit leaf area	-	-	-	mol m^{-2} s^{-1}	-	Ca
u_{set}	Desired molar air flowrate	2, 3, cfg	-	-	µmol s^{-1}	68-341	G
Va(±20%)	Measured analyser flow	-	-	-	µmol s^{-1}	-	-
Vaux	Aux input, scaled as volts	-	-	-	Volts	-	-
Vbatt	Battery voltage	diag	-	-	Volts	10.5 -14.3	-
W_{ref}	H_2O reference, as %RH	diag	-	y	%RH	0-100	M,Co
w'_{an}	H_2O analysis, corrected, as %RH	cfg	-	-	%RH	0-100	M,Co
w'_{ad}	H_2O analysis, dilution corrected	diag	-	y	%RH	0-100	Ca,Co
Δw	Delta H_2O (w'an-Wref), as %RH	-	-	y	%RH	+/-100	Ca
[w]a	Raw H_2O analysis reading	hid	-	-	adc counts	-	-

[w]r	Raw H_2O reference reading	hid	-	-	adc counts	-	-
W_{flux}	Net H_2O Exchange Rate	2	-	y	mmol m^{-2} s^{-1}	-	Ca

Column explanations and type derivation codes:

"Screen" column.
This gives the location (where applicable) of displayed parameters.
There are three main screens (1, 2, & 3) and four sub screens (described below).
The sub screens are diag = diagnostics screen
cfg = configuration set up screen
log = log set up screen
hid = hidden screen for ADC use only

"Log?" column
This column shows the position of a parameter in the log record (where applicable). If no number is shown the parameter is not logged.

"An o/p" column
This column indicates whether the parameter can be monitored from the analogue output port.
See Appendix 2 for scaling details.

"Type" column
This column indicates the method of derivation, according to the following code:
Ca = calculated (generally by a formula given in the appendices)
Co = corrected (by terms defined in the appendices)
F = Factors (established by experiment or other means)
G = Given (i.e. entered by the user)
K = constants (physical or scientific)
M = measured raw values (by transducers in the LCpro-SD)
(H_{fac} was Trans on LCA-3)

Appendix 2. Analogue Output Scaling

Parameter & Symbol		Units	Reading @ 0V	Reading @ 5V	Units/V
Atmospheric pressure	(p)	mBar	600	1100	100
Analysis CO_2	(c'an)	vpm	0	2000	400
Delta CO_2	(Δc)	vpm	-200	+200	80
Reference CO_2	(cref)	vpm	0	2000	400
Analysis H_2O	(e'an)	mBar	0	100	20
Delta H_2O	(Δe)	mBar	-5	+5	2
Reference H_2O	(eref)	mbar	0	100	20
Analysis humidity	(w'an)	%RH	0	100	20
Delta humidity	(Δw)	%RH	-5	+5	2
Reference humidity	(wref)	%RH	0	100	20
Leaf chamber temperature	(Tch)	°C	-5	+50	11
Flow	(u)	μmol s^{-1}	0	342	68.4
Leaf temperature (meas/calc'd)	(Tl)	°C	-5	+50	11
Qleaf (PAR at leaf surface)	(qleaf)	μmol m^{-2} s^{-1}	0	3000	600
Sub-stomatal CO_2	(C_i)	vpm	0	20	4
Photosynthetic Rate	(A)	μmol m^{-2} s^{-1}	-2.5	25	5.5
Transpiration Rate	(E)	mmol m^{-2} s^{-1}	0	50	10
Net CO_2 Exchange Rate [*1]	(NCER)	μmol m^{-2} s^{-1}	0	100	20
Net H_2O Exchange Rate [*1]	(W_{flux})	mmol m^{-2} s^{-1}	0	50	10
Soil Respiration [*1]	(C_e)	vpm	0	20	4
Raw CO_2 zero at TP20 [*2]	([c]z)	Volts	4.05	5	0.19
Raw CO_2 zero Diagnostic [*3]	([c]z)	A-D count	45000	60000	3000
Stomatal Conductance	(G_s)	mol m^{-2} s^{-1}	0	1	20
Column Temperature	(Taux)	°C	-5	50	11
CO_2 Bottle Mix. Conc.	(CO_2 bot)	vpm	300	10000	1940

[*1] Applies to Soil pot configuration only.
[*2] Volts as measured at TP20 during zero parts of gas cycle or CO2 zero calibration
[*3] A-D count optimally 52500 for "perfect" CO2 zero calibration.

Appendix 3. LCpro-SD Calculated Parameters & Constants

CO_2 Concentration

The IRGA cell measures the absorption of infra-red due to the presence of CO_2. This value must be scaled and linearised to get the actual concentration. The processing is done in several steps as shown below for the analysis channel, the reference channel is treated the same, substituting subscript 'r'.

$$[c_{ab}]_a = \frac{z_a - r_a}{z_a}$$

Where $[c_{ab}]_a$ absorption due (mainly) to CO_2
z_a detector signal at zero
r_a detector signal at current reading

The reading is now linearised and scaled according to the calibration set during span adjustment:

$$c_{an} = L_c([c_{ab}]_a s)$$

Where L_c linearisation function for CO_2
$[c_{ab}]_a$ absorption of infra-red due to CO_2
s span factor; determined during calibration (span adjustment)

The reading is now compensated for changes in atmospheric pressure. The LCpro-SD leaf chamber and IRGA cell are very close to ambient pressure.

$$c_{an}' = c_{an}\ (1 + \frac{(p_{ref} - p)\ a}{p_{ref}})$$

Where c_{an}' pressure corrected CO_2 value
p_{ref} ambient pressure at last span adjustment, mbar
p ambient pressure, mbar
a pressure compensation factor (1.4)

The IRGA CO_2 reading is slightly influenced by the presence of water vapour (pressure broadening). The water vapour readings are used to compensate the measured readings:

$$C_{an}'' = C_{an}' + (C_{an}' wm_{an})$$

Where c_{an}'' reading compensated for the presence of H_2O
wm_{an} H_2O concentration, as a molar fraction

Finally, leaf transpiration causes the net volume of air leaving the leaf chamber to be higher than that entering. This volume increase tends to dilute the CO_2 concentration, causing c_{an} and w_{an} to be lower. Dilution compensation removes this effect, so that the Δc Δe value reflects the differences due to absorption by the leaf, not transpiration. This compensation is only applied to the analysis reading.

$$c_{and} = c_{an}''(\frac{1-wm_{ref}}{1-wm_{an}})$$

Where c_{and} Final, compensated reading, as displayed
wm_{ref} Reference water vapour concentration, as molar fraction
wm_{an} Analysis water vapour concentration, as molar fraction

APPENDIX 3 (Continued)

Molar flow of air per m² of leaf surface

symbol: u_s (mol m^{-2} s^{-1})

$$u_s = \frac{u}{100 * area}$$

Where u molar air flow in µmol s^{-1}
$area$ projected leaf area in cm^2

Difference in CO2 concentration

symbol: ΔC, vpm(≡µmol mol^{-1})

$$\Delta c = c_{ref} - c'_{an}$$

where C_{ref} CO_2 flowing into leaf chamber, µmol mol^{-1}
C'_{an} CO_2 flowing out from leaf chamber, µmol mol^{-1}, dilution corrected

Photosynthetic Rate (Rate of CO_2 exchange in the leaf chamber)

symbol: A (µmol m^{-2} s^{-1})

$$A = u_s \Delta c$$

where u_s mass flow of air per m^2 of leaf area, mol m^{-2} s^{-1}
Δc difference in CO_2 concentration through chamber, dilution corrected, µmol mol^{-1}.

Water vapour pressure in and out of leaf chamber

The calculation for reference is show for illustration. Substitute e'_{an} and rh_{an} for the analysis calculation.

symbol	e_{ref}	into leaf chamber (mbar)
	e_{an}	out of leaf chamber

$$e_{ref} = \frac{rh_{ref}}{100} e_s$$

where	rh_{ref}	water vapour concentration as %rh (as measured)
	e_s	saturated vapour pressure, mbar (see later)

APPENDIX 3 (Continued)

Calculation of molar concentration of water vapour in and out of leaf chamber

The calculation for reference water vapour is shown for illustration.

symbol	wm_{ref}	into leaf chamber (ratio)
	wm_{an}	out of leaf chamber

$$wm_{ref} = \frac{e_{ref}}{p_{amb}}$$

where	e_{ref}	water vapour pressure into chamber, mbar
	p_{amb}	ambient pressure, mbar

Difference in water vapour pressure

Note that Δw and ΔRH are calculated in exactly the same way. The dilution corrected analysis value is used.

symbol **Δe** (mbar)

$$\Delta e = e'_{an} - e_{ref}$$

where e_{ref} water vapour pressure into leaf chamber, mbar
e'_{an} water vapour pressure out of leaf chamber, mbar, dilution corrected

Transpiration rate

symbol: E (mol m^{-2} s^{-1})

$$E = \frac{\Delta e u_s}{p}$$

where Δe differential water vapour concentration, mbar, dilution corrected
u_s mass flow of air into leaf chamber per square metre of leaf area, mol s^{-1} m^{-2}
p atmospheric pressure, mBar

APPENDIX 3 (Continued)

Leaf surface temperature

Where calculated. This value may also be measured or given.

symbol: T_{leaf} (^{O}C)

$$T_{leaf} = T_{ch} + \left(\frac{(Q \, x \, H_{factor}) - \lambda E}{\left(\frac{0.93 \, M_a \, C_p}{r_b} \right) + 4\sigma (T_{ch} + 273.16)^3} \right) \quad *1$$

*1 Energy Balance Equation for calculating leaf temperature: PARKINSON, K.J. (1983)
Porometry in S.E.B. Symposium of Instrumentation for Environmental Physiology, Cambridge University Press

where

T_{ch}	leaf chamber temperature, °C
Q	photon flux density incident on leaf chamber window, μmol m^{-2} s^{-1}
H_{factor}	energy conversion factor (was TRANS on LCA-3) J/μmol
λ	latent heat of vaporisation of water, J mol^{-1}
E	Transpiration rate, mol m^{-2} s^{-1}
M_a	molecular weight of air
C_p	specific heat at constant pressure, J g^{-1} K
r_b	boundary layer resistance to vapour transfer, m^2 s^{-1} mol^{-1} (0.93 is conversion factor for above to give boundary layer resistance to heat)
σ	is Boltzmann's constant, $Wm^{-2}K^{-4}$

Stomatal resistance to water vapour

symbol: r_s (m^2 s mol^{-1})

$$r_s = \frac{(w_{leaf} - wm_{an})}{\left(\frac{\Delta eu_s}{p}\right)} - r_b$$

where w_{leaf} saturated water vapour concentration at leaf temperature, mol mol^{-1}, thus:-

$$w_{leaf} = \frac{e_s}{p}$$

e_s	saturated vapour pressure at leaf surface temp, mBar
p	atmospheric pressure, mBar
Δe	differential water vapour concentration, mbar, dilution corrected
wm_{an}	water vapour concentration out of leaf chamber, mol mol^{-1}
r_b	boundary layer resistance to water vapour, m^2 s mol^{-1}
u_s	mass flow of air per m^2 of leaf area, mol m^{-2} s^{-1}

APPENDIX 3 (Continued)

Sub-stomatal cavity CO_2 concentration

symbol: c_i (μmol mol^{-1})

$$C_i = \frac{((g_c - \frac{E}{2})c'_{an}) - A}{g_c + \frac{E}{2}} \quad *1$$

where

$$g_c = \frac{1}{1.6\,r_s + 1.37\,r_b}$$

c'_{an}	CO_2 flowing out from leaf chamber, μmol mol^{-1}, dilution corrected.
E	Transpiration rate, mol m^{-2} s^{-1}
A	photosynthetic rate of CO_2 exchange in the leaf chamber, μmol m^{-2} s^{-1}
r_b	boundary layer resistance to water vapour, m^2 s^{-1} mol^{-1}
r_s	stomatal resistance to water vapour, m^2 s^{-1} mol^{-1}

Saturated vapour pressure of water at leaf surface temperature

symbol: e_s (bar)

For $T_{leaf} >= 0$

$$e_s = 6.13753\,10^{-3}\,e^{\left(\frac{T_{leaf}\left(18.564 - \frac{T_{leaf}}{254.4}\right)}{T_{leaf}+255.57}\right)}$$

For $T_{leaf} < 0$*, above water*

$$e_s = 6.13753x10^{-3}\,e^{\left(\frac{17.966T_{leaf}}{T_{leaf}+247.15}\right)}$$

(Arden L Buck, Journal Appl. Meterology vol 20 1981 pp1527-1532)

where T_{leaf} leaf surface temperature, °C

*1 Calculation for Ci, Substomatal CO2 von CAEMMERER, S. and FARQUHAR, G.H. (1981)
Some relationships between the biochemistry of photosynthesis and the gas exchange of leaves. Planta 153:376-387

APPENDIX 3 (Continued)

Stomatal conductance of water vapour

symbol: g_s units: mol m^{-2} s^{-1}

$$g_s = \frac{1}{r_s}$$

where r_s stomatal resistance to water vapour, m^2 s^{-1} mol^{-1}

P.A.R. incident on leaf surface

symbol: Q_{leaf} units: μmol s^{-1} m^{-2}

$$Q_{leaf} = Q \; x \; Tr_w$$

where Q Photon flux density incident on leaf chamber window, μmol m^{-2} s^{-1}
Tr_w Leaf chamber window transmission factor to P.A.R. (given)

Soil Respiration (Net Molar Flow of CO_2 in/out of the Soil)

symbol: C_e (μ mol s^{-1})

$$C_e = u \; (-\Delta c)$$

where u molar air flow in mol s^{-1}
Δc difference in CO_2 concentration through soil pot, dilution corrected, μmol mol^{-1}.

Net CO_2 Exchange Rate (C_e per unit area)

symbol: ***NCER*** (μmol s^{-1} m^{-2})

$$NCER = u_s(-\Delta c)$$

where ***u_s*** molar flow of air per square meter of soil, mol m^{-2} s^{-1}
Δc difference in CO_2 concentration through soil pot, dilution corrected, μmol mol^{-1}.

Note: *This is equivalent to -A*

APPENDIX 3 (Continued)

Net H_2O Exchange Rate (Soil Flux)

symbol: ***W_{flux}*** (m mol s^{-1} m^{-2})

$$W_{flux} = \frac{\Delta e u_s}{p}$$

where ***u_s*** molar flow of air per square meter of soil, mol m^{-2} s^{-1}
Δe differential water vapour concentration, mbar, dilution corrected
p atmospheric pressure, mBar

Note: *This is equivalent to E*

Appendix 4. Constants

Volume of 1 micro-mole of air at 20˚C and 1 Bar (Vm_{20C})
Value used is 2.4387x10^{-2} m^3.

Latent heat of vaporisation of water (λ)
Value used is 45064.3 - (t_{ch} x 42.9) Joule mol^{-1}

Boltzmann's constant (σ)
Value used is 5.7 x 10^{-8} W m^{-2} K^{-4}.

Molecular weight of air (M_a)
Value used is 28.97

Specific heat at constant pressure (C_p)
Value used is 1.012 J g^{-1} K^{-1}

Appendix 5. Derivation and Measured Values For Hfactor

The leaf temperature can be calculated from the energy balance, as shown in Appendix 3. This requires knowledge of the total incident radiation H absorbed by the leaf, which in sunlight lies between 0.4 and 3.0 microns. This therefore includes the PAR radiation (0.4 – 0.7μ) and near infrared radiation (0.7 – 3.0μ).

The Hfactor is used to convert the measured PAR value into a figure for the total energy absorbed, by the leaf, which will depend upon the visible/infrared ratio of the incident radiation. This in turn is determined by the nature of the energy source and conditions, and also by the absorption properties of the leaf and the leaf chamber windows.

$$H = Q \times Hfactor$$

A value for Hfactor is given by the following equation:

$$Hfactor = a.e.f. + a^1.c.d.$$

Where;

H = energy absorbed by the leaf in W/m^2

Q = PAR in mol m^{-2} s^{-1}

a = conversion from incident photon flux density between 0.4 & 0.7μ to radiant energy

a^1 = conversion from incident photon flux density between 0.7 & 3.0μ to radiant energy

[a & a^1 vary with light source and type of light sensor – a silicon type is used with the LCpro-SD]

c = the fraction of infrared transmitted by the chamber windows and shield if fitted

d = the fraction of infrared absorbed by the leaf

e = the fraction of visible transmitted by the chamber windows and shield if fitted

f = the fraction of visible absorbed by the leaf

Typical values for the above factors are;

a = 0.2188 for sunlight(K.J.McCree,1972,Agricultural Meteorology,10, p443-453 etc.)

a^1 = 0.1205 (based on 361.5wm^{-2}/3000μmol m^{-2} s^{-1} at λ ave = 0.992μ)

c = 0.6

d = 0.2 (for typical leaves)

e = 0.88 (Broad & Narrow chambers), 0.93 (Conifer chamber)

f = 0.8 (for typical leaves)

These values give Hfactor = 0.168 (Broad & Narrow chambers) – for sunlight

= 0.177 (Conifer chamber)

Other values have been obtained for sunlight and various light sources, based on the Broad and Narrow chambers and using a silicon PAR sensor. These are given in the following table, and generally are to be recommended.

Appendix 6. Measured Hfactor values/conditions

	PLC with Windows and Perspex Shield	
LIGHT SOURCES	**PAR sensor outside chamber**	**PAR sensor inside chamber**
Sun & Sky	0.168	0.214
Tungsten 3000°C	0.340	0.429
Warm white fluorescent	0.109	0.139
Cold white fluorescent	0.113	0.144
Grolux fluorescent	0.118	0.150

Appendix 7. Specification for Sequence Files

Basic Specification

The climate sequence file is a comma-separated text file, which can be easily read, created and generated on a PC.

The file consists of lines of text, which contain either the detail for one step in the sequence, or comment (descriptive notes or other text which is ignored when the sequence runs.)

Blank lines are not acceptable (even at the end of the file).

Mandatory Sections

The first *two* lines in a climate sequence file *must* be comment lines. Any additional comment lines are optional.

The first comment line normally contains details about the creator of the file, but the content is unimportant. Only the initial '#' character is mandatory.

The second comment line holds the column headings. The presence of the heading is used to validate the file, so at a minimum, the second line in a sequence file must start with:
'#Dwell'. We recommend that the standard second line is used as this identifies the columns when the sequence file is viewed on a spreadsheet. See the example file on page 94.

Comment Lines

If a line starts with a '#' character at the START of the line, it is treated as comment, and will not be displayed or executed. Any text up to the end of the line is ignored.

Comment lines (after the first two lines in the file) are optional. Be aware that comment lines use space, and excessive comment may make the sequence file too big.

The LCpro-SD on loading a sequence file will strip off any optional comment lines, so these will be lost if the LCpro-SD subsequently saves the file onto a memory card. Optional comment is not removed when the file is *first* transferred to the LCpro-SD.

Data Lines

Each data line in the climate sequence file consists of one step, and these are numbered and executed in sequence from the start to the end of the file.

The line consists of 6 'fields', separated with commas. Except for the last, each field must be present, with a number or indicator as allowed. The LCpro-SD will attempt to interpret invalid fields, but the result may be unexpected.
NOTE: The step number (as shown on the LCpro-SD screen) is *not* present - the analyser adds it when the file is loaded.

Indicators

Generally numbers are required for the fields, but the following 'indicators' can be used in place of the number for special values:

PREVIOUS - use the value from this field in the previous step.

Notes

This indicator is not allowed in the first step.

This indicator can be abbreviated to 'p', 'prev.' or indeed anything beginning with upper or lower case P.

AMBIENT - this parameter is no longer controlled, and where possible, will revert to ambient.

This indicator can be abbreviated to 'a', 'amb.' or indeed anything beginning with upper or lower case A.

Only ONE indicator is allowed per field.

Field Descriptions

The fields are as follows (numbered left to right):

1. Dwell Time

This field sets the time for which this step executes in minutes. The 'p' indicator is allowed. Integer values between 1 and 100 are accepted. Invalid values are changed to 5 minutes.

2. Temperature

This field sets the temperature control demand value in Celsius. The acceptable temperature range is currently 1 to 40°C. Integer values are required. Temperature control is turned off if the 'a' indicator is used, and the 'p' indicator is also allowed.

3. PAR

Sets the light level, where a suitable illuminator is fitted. Valid PAR values range from 20 to 2000 (subject to illuminator), and the value of zero is accepted as dark. If the ambient indicator is used, this also turns off the lamp. The 'p' indicator is allowed.

4. CO_2 Concentration

This field sets the reference or analysis concentration in vpm. The channel controlled will depend on the CO_2 option setting. The range of concentrations will depend on whether or not a CO_2 cylinder is fitted. The 'a' and 'p' indicators are allowed, but note that truly ambient CO_2 is not possible with the cylinder fitted, as all incoming air is enriched by the cylinder.

5. H_2O Concentration

This field sets the reference or analysis water concentration. The channel controlled will depend on the H_2O option setting. The range of concentrations will depend on whether or not a 'wetter' column is fitted. The 'a' and 'p' indicators are allowed, but note that truly ambient H_2O is not possible with the wetter column fitted, as all incoming air is wetted.

6. Options

This field is unique in that it can be blank, and can only hold 'flag' letters. Each flag enables a particular option for the step, and flags can be present in any combination. Any flag can appear once only.

The flags are single letters, (either case) as follows

I Ignore - this step is skipped when the sequence runs
R Record - take a log record at the end of this step
E End - stop the sequence running at the end of this step (after any log record taken)
P Power down - as 'E', but turn off the power as well

Creating A Climate Sequence With Excel™

A spreadsheet program is the best tool to create and edit climate sequence files. Providing simple rules are followed, a file can be created very quickly.

Starting with a blank sheet, insert the mandatory comment lines at the top:

1. The top left cell should hold '#' followed by any note you may want to make about the file - keep this text in the first cell.
2. On the second row, enter the following column headings into the left six cells:
   #Dwell | Temp | PAR | CO2 | H20 | Flags
   (take care with this - it must be on the second row in the sheet, and the first cell must read exactly as shown, or the file will be rejected.)

Now, simply enter the climate steps in subsequent rows. Do not use commas - the spreadsheet software will insert these automatically when you export the data. Do not add spaces. Use only integer numbers (no decimals).

Comments

If you want to add a comment line, put a '#' in the leftmost cell, with the comment following it in the same cell.

Saving the File

From the file menu select 'Save As'. From the 'Save as Type' drop down list, select 'CSV (Comma Delimited)'. Select a file name as usual. Excel will use the extension '.CSV' at the end of the file name.

If you are saving onto a memory card directly, note that the file must be renamed with a '.SQS' extension for the LCpro-SD to recognise it. If you are using transfer to send the file to the LCpro-SD, the extension will be changed automatically.

Editing an Existing File With Excel™

If necessary, select 'All files (*.*)' from the list - this is necessary if the file has been loaded from a memory card directly, as the file will have a '.SQS' extension that Excel does not normally recognise.

The file will be loaded ready for editing. Follow the rules given in the previous section.

When saving the file, ensure that the 'save as' option is 'CSV' and, if the file extension is changed to '.CSV', remember to change it.

Technical Notes

File Size Limits

Sequence files are currently limited to 8000 characters maximum, including comments and carriage return/linefeeds. There is also a limit of 100 data lines.

Attempting to load or transmit an oversize file to the LCpro-SD will result in the file being truncated.

End-Of-Line

The LCpro-SD recognises the end of line conventions used by both DOS/Windows (carriage return, linefeed pairs) and UNIX type systems (carriage return only). Files created on the LCpro-SD, or saved after editing on the analyser will use DOS/Windows conventions.

Be cautious when creating files on a Mac, as the Apple convention for end of line is linefeed only - this is not acceptable to the LCpro-SD.

Spaces

The LCpro-SD is fairly tolerant of trailing spaces in the file, but these waste space. Avoid leading spaces in fields.

Example File

```
# ADC BioScientific  LCpro-SD  System Climate Sequence
#Dwell,Temp,PAR,CO2,H2O,Options
1,amb.,1,amb.,amb.,
2,amb.,200,amb.,amb.,R
2,amb.,300,amb.,amb.,IR
2,amb.,450,amb.,amb.,R
1,prv.,prv.,prv.,prv.,R
prv.,prv.,prv.,prv.,prv.,
1,35,prv.,prv.,prv.,RE
```

Appendix 8. Saturated Vapour Pressure

SATURATION VAPOUR PRESSURE OVER WATER (SVP)

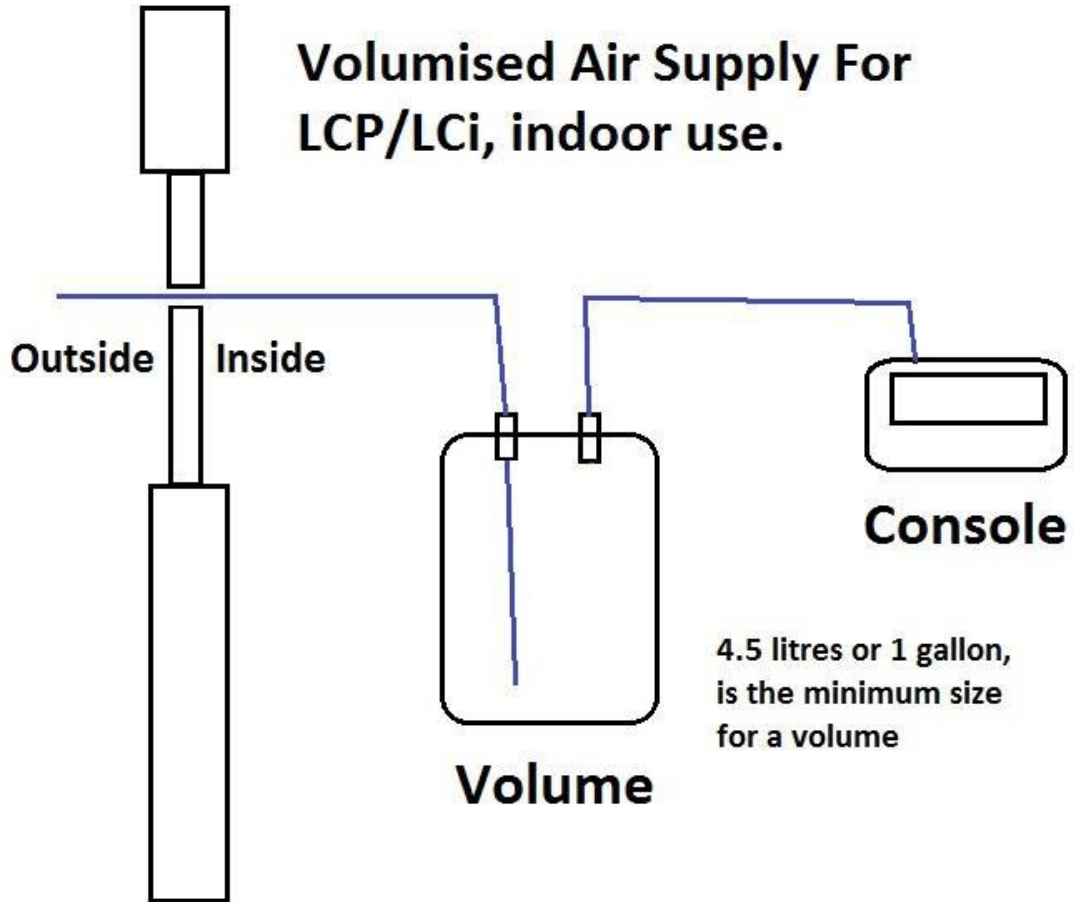
Values obtained using the LCP_SD software based on A Buck formula 1981

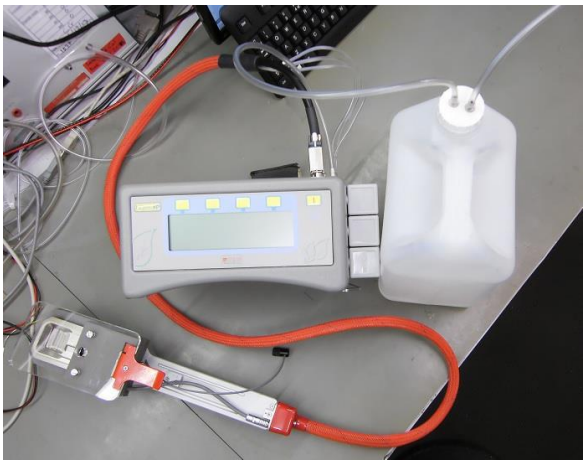
Metric Units (millibars)

°C	0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
15	17.1	17.2	17.3	17.5	17.6	17.7	17.8	17.9	18.0	18.1
16	18.3	18.4	18.5	18.6	18.7	18.8	19.0	19.1	19.2	19.3
17	19.5	19.6	19.7	19.8	20.0	20.1	20.2	20.3	20.5	20.6
18	20.7	20.9	21.0	21.1	21.2	21.4	21.5	21.7	21.8	21.9
19	22.1	22.2	22.3	22.5	22.6	22.8	22.9	23.0	23.2	23.3
20	23.5	23.6	23.8	23.9	24.1	24.2	24.4	24.5	24.7	24.8
21	25.0	25.1	25.3	25.4	25.6	25.7	25.9	26.1	26.2	26.4
22	26.5	26.7	26.9	27.0	27.2	27.4	27.5	27.7	27.9	28.0
23	28.2	28.4	28.6	28.7	28.9	29.1	29.3	29.4	29.6	29.8
24	30.0	30.1	30.3	30.5	30.7	30.9	31.1	31.2	31.4	31.6
25	31.8	32.0	32.2	32.4	32.6	32.8	33.0	33.2	33.4	33.6
26	33.8	34.0	34.2	34.4	34.6	34.8	35.0	35.2	35.4	35.6
27	35.8	36.0	36.2	36.4	36.7	36.9	37.1	37.3	37.5	37.7
28	38.0	38.2	38.4	38.6	38.9	39.1	39.3	39.5	39.8	40.0
29	40.2	40.5	40.7	40.9	41.2	41.4	41.6	41.9	42.1	42.4
30	42.6	42.9	43.1	43.4	43.6	43.9	44.1	44.4	44.6	44.9
31	45.1	45.4	45.6	45.9	46.2	46.4	46.7	47.0	47.2	47.5
32	47.8	48.0	48.3	48.6	48.9	49.1	49.4	49.7	50.0	50.2
33	50.5	50.8	51.1	51.4	51.7	52.0	52.3	52.5	52.8	53.1
34	53.4	53.7	54.0	54.3	54.6	54.9	55.2	55.6	55.9	56.2
35	56.5	56.8	57.1	57.4	57.7	58.1	58.4	58.7	59.0	59.4
36	59.7	60.0	60.3	60.7	61.0	61.3	61.7	62.0	62.4	62.7
37	63.0	63.4	63.7	64.1	64.4	64.8	65.1	65.5	65.8	66.2
38	66.6	66.9	67.3	67.6	68.0	68.4	68.7	69.1	69.5	69.9
39	70.2	70.6	71.0	71.4	71.8	72.1	72.5	72.9	73.3	73.7

Values obtained using the LCP software based on the Arden Buck 1981 formula..

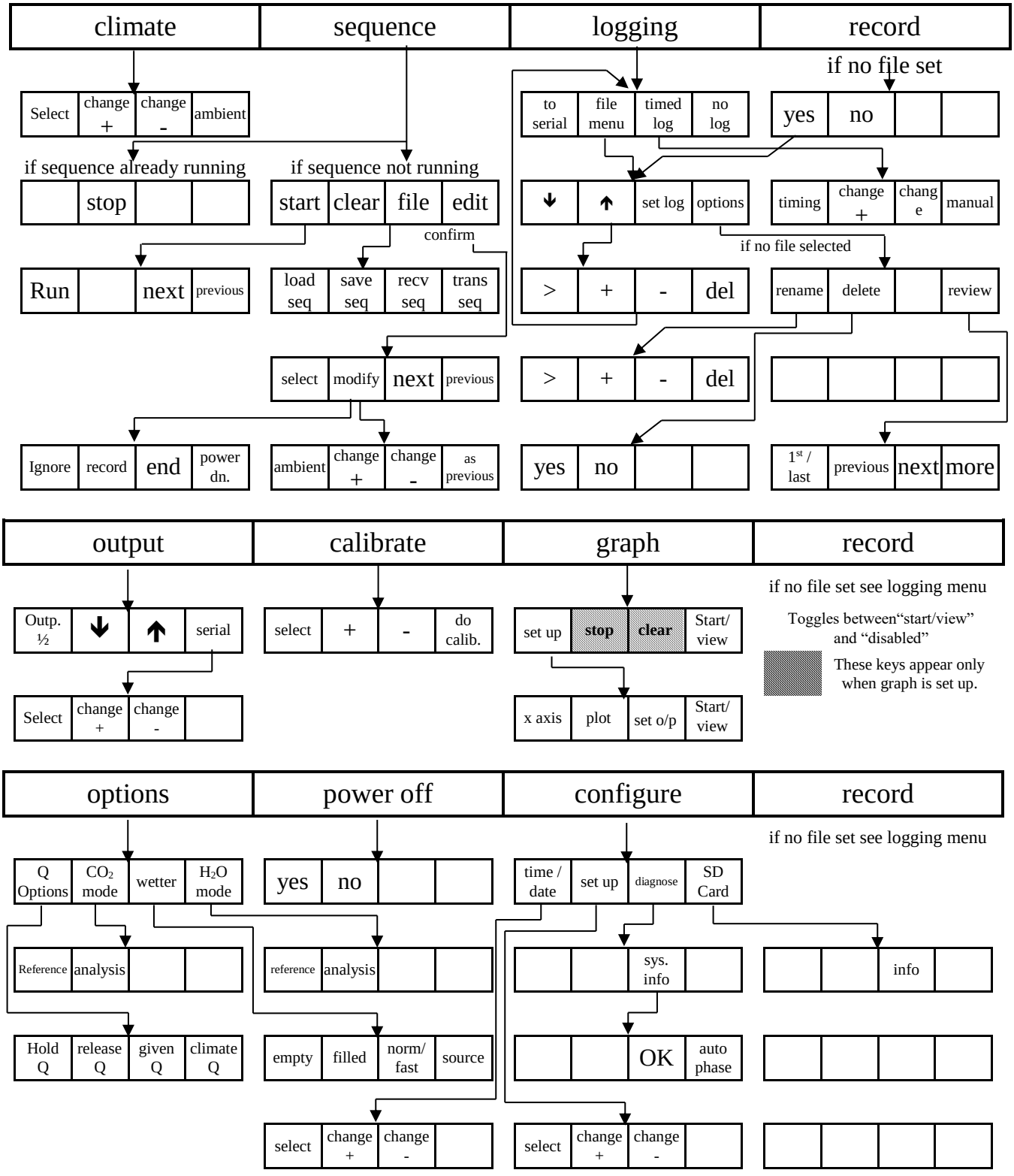
Appendix 9. Volumised Air Supply for Indoor Use.





Appendix 10. LCpro-SD Menu Structure

Pressing the "Page" key in "sub-level" menus returns to previous level except where shown. Pressing the "Page" key in the "top-level" menu steps through the three main pages.



Appendix 11. iFL System Technical Specifications

Fluorometer Module Specifications:

Excitation sources:
Saturation pulse: White LED with 690nm short pass filter
0-7,500 µmol m^{-2} s^{-1}
Modulating light: 660nm LED with 690nm short pass filter
Actinic light: White LED 0-2,000µmol m^{-2} s^{-1}
Far red: Above 740nm LED

Blue/red/green absorptance sensors

PAR Sensors - Two silicon diodes. One is inside the fluorometer chamber to measure PAR at the leaf level. It maintains a constant actinic light irradiation level throughout measuring routines, to ensure constant and proper intensity for steady state measurements. It is also used to measure absorptance diode intensity.

The second PAR sensor is on top of the fluorometer. It can be used to measure and track or set ambient light irradiation at the angle of the leaf. This sensor can be used to match ambient PAR intensity in the leaf chamber in an automatic fashion. When it is desired, this feature can be used set light level on partly cloudy days to either sunny conditions, or cloudy conditions to minimized the time required to reach steady state conditions for ambient light measurements.

Detection method: Pulse modulation

Automated setting of modulated light intensity: Adjustable On/Off

Automated Multi-Flash Fm' correction: Adjustable On/Off

Detector: PIN photodiode with 700 - 750nm filter

Sampling rate: 10 to 10,000 points per second, dependent on phase of test

Test duration: Adjustable 20 seconds to 4,000 hours

Detection method: Pulse modulation method.

Detector & Filters: A PIN photodiode with a 700 ~ 750 nm band-pass filter.

Sampling Rate: Auto-switching from 10 to 10,000 points per second, depending on phase of test. Automated routine to optimally set the modulated light intensity. The modulated light may also be set manually.

Multi-Flash F_M' correction for all light adapted protocols. (According to Loriaux 2013) It may be turned on or off.

Test Duration: Adjustable from 2 seconds to 4000 hours.

Storage Capacity: 2 Gigabyte of non- volatile internal flash memory, supporting unlimited data sets and traces

Digital Output: USB, SD 2 GB data cards.
Video output: HDMI

User Interface:
Display: Graphic colour touch screen
Menu driven touch screen.

Battery Life: 8 hours of continuous combined operation.

LCpro-SD Specifications:

Measurement range and technique:	CO_2:	0-3000 ppm, 1ppm resolution Infra red gas analysis, differential open system, auto zero, automatic atmospheric pressure and temperature compensation.
H_2O:		0-75mbar, 0.1 mbar resolution Two laser trimmed, fast response RH sensors.
PAR:		0-3000 μmol m^{-2} sec^{-1} Silicon photocell
Chamber temperature:		-5°C to 50°C. Precision thermistor. ±0.2°C accuracy
Leaf temperature:		Energy balance or microchip thermistor or self positioning thermistor
Gas Exchange Repeatability:		CO_2: 0.1% of reading @ 370ppm H_2O: 0.5% R.H.
Linearity:		CO_2: 0.5% of reading H_2O: 0.5% RH
Temperature effect on span		CO_2: <0.05% of f.s.d. per °C
Flow rate in PLC:		100ml to 500ml min^{-1}
Flow rate accuracy:		± 2% of f.s.d.
Automatic environmental control: CO_2:		Up to 2000 ppm with integral controller
H_2O:		Above ambient, down to zero

Temperature		±10°C or better from ambient. (Software limited between 1 to 40)
PAR		Up to 2000 μmol m^{-2} sec^{-1} by red/blue LED array
Display:		240 x 64 dot matrix super twist LCD
Warm up time:		5 minutes at 20°C
Recorded data:		Secure Digital.
Battery:		7 AH lead acid 12V to give 16 hours max
Battery charger:		90 to 260V, 50/60 Hz
Analogue output:		0 to 5V on user selected parameter
RS232 output: USB Slave Peripheral		User selected rates up to 19200 baud
Electrical connections	Power: Analogue out: RS232: Chamber: USB:	5 pin DIN 5 pin DIN 9 pin D type. “AT” pin configuration. 15 pin high density D type USB Mini B
Gas connections:		3mm barbed
Power requirements:		2A @ 12V DC
Operating temperature:		5°C to 45°C
Dimensions H x W x D overall	Console: Chamber:	280 x 135 x 190 mm 300 x 80 x 75 mm
Weight (typical)	Console: Chamber:	4.4 kg 0.8 kg

Integrated iFL System Specifications:

Data storage: 2GB internal memory for thousands of data sets and traces. Removable SD cards

Digital output: SD cards, USB and HDMI

User interface: Large, colour, menu driven, graphic touch screen display (14.5cm x 8.5cm)

Battery: 7.0Ah 12 V lead acid battery.
Up to 8 hours of battery life as iFL system

Weight and Dimensions:
Console total: 31cm x 11cm x 24cm, 5.2kg (incl. battery)

Leaf Chamber: 30cm x 8cm x 16cm

USER NOTES: