**Protocols** Steady-state light and CO2 response curves for LI-6800

**Version** CIW - DGE - Fall 2020 adapted for UQ custom LI-6800 SIF system

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| **Step** | **Measurement/activity** | **Time** |
| 1 | Instrument set up/warm up | 45-60 mins |
| 2 | Assessment of dark acclimated Fv/Fm | 30 mins (if overnight dark acclimated) |
| 3 | Light response curve | 1.5 hours + 30 min acclimation period |
| 4 | CO2 response curve | 1.5 hours + 30 min acclimation period |
| 5 | Data collection/instrument shut down | 15-30 mins |
| **Total** |  | **About 6 hours** |

1. **Set up procedure**
   1. Replace reagents and gaskets as necessary; warm up instruments (LI-6800, spectrometers, and HAL light source); load configuration file (***UQ\_lab***); complete warm-up tests and fluorometer tests; check fluorometer calibration; only proceed once all tests are passed.
   2. Configure the cuvette environment for the first measurement (PPFD = 0, Tleaf = 25°C, CO2\_S = 400 ppm, RH\_S = 70%, O2 = 21%, Flow = 500 umol s-1, Fan = 10,000 rpm).
   3. Configure the fluorometer (Output rate = 1000 hz, Before/after pulse = 10 points; Dark, light, and flash modulation rates 1, 20, 250 kHz; Averaging for Fo and Fs = 15s; RF = 0.9s duration, **5000 umol PPFD m-2 s-1 maximum intensity**; MPF = 0.9s duration, 5000 umol PPFD m-2 s-1 maximum intensity, Phases 1-3 with 0.3/0.3/0.3s each and a ramp of -25% in second phase for determination of Fm’, follow with 5s dark pulse for determination of Fo’).
   4. Configure QEPs to 10ms integration time, 1 scan to average, non-linearity correction ticked, and electric dark current ticked (note the latter corrections will not matter when recording DNs(?) – these can be applied in a post-processing stage). Double check that the thermo electric set point is 10 degrees C (to minimise condensation forming) – Right click on the Device in OceanView > New Acquisition > Thermo-Electric.
   5. Check that the QEP hal ref is saturated at around 580nm and that the HAL PAR is < 1 umol m-2 s-1 using the white reference panel. ***[work out the Absolute Irradiance Intensity values for RGB and also the equivalent DNs]***
   6. Measure dark current: ensure IO-2 and IO-3 are in position ‘Low’, meaning there is no light passing through the beam splitter. Collect 100 spectra labelled ‘DC’. Ensure Fluorometer MB is OFF.
   7. Measure white reference (WR\_100\_str): Insert the white reference panel in the chamber (correct orientation with dimples on the bottom) and collect 100 spectra (no averaging): ensure fluorometer measuring beam ‘MB ON’ and that the halogen is ON. Ensure the file names are correctly labelled before the measurement is collected. Ensure that the AUX on the 6800 is set to IO-1-3 are all ‘High’.
   8. Then change IO-3 to low and collect a direct reference measurement of the HAL light source (‘HAL\_100\_str’ denoting 100 HAL spectra at the start of the experiment). Swap IO-3 back to high (default position).
   9. An additional optional measurement is to measure the dark panel (DP\_100\_str) with the QEP as a reference. Ensure that the top QEP is recording the cuvette and not the reference light source.
   10. Insert the diffuser and measure with MB ON. Only the transmitted data is needed. Remove the diffuser and close the cuvette. The diffuser reference measurement is required to calculate transmittance (instead of using the 100% transmitted light from the top fiber).
   11. Note: perhaps have the HAL source blocked out here so that the effect of adding the HAL to the dark state leaf can be quantified with the Fs trace. *Alternatively, pick a second leaf and instert it in the chamber, wait for Fs to stabilise, then look at the effect of turning the HAL on on the Fs trace.*
   12. Match the IRGAs and then confirm there are no appreciable leaks.
   13. Open a log file. Go into the “Log files” tab and press “Open a log file”. Create a new log file. You will now be able to press the Log(0) tab to record manual measurements. In “Log settings” make sure that “also log to an Excel file” is selected and select the option to always include a dark pulse for assessment of Fo'. In “Match options”, select “match always.”
   14. Start recording the continuous fluorescence trace, then turn on the measuring light in the fluorometer. Make sure the display graphs are configured so you can easily monitor Fs throughout the experiment.
   15. Insert the leaf into the chamber, taking care to avoid/minimize exposure to stray light, ensure that thermocouple is in good contact with leaf surface, and that the clamp is adjusted so as to securely hold leaf without being so tight as to cause damage. Match the IRGAs again.
   16. Turn on the HAL light (if not already done so) and look at the effect on Fs of adding the new light.
   17. Start recording with the QEPs.
   18. Record in lab notebook the software version, the name of the configuration file used, the name of the log file, and the configuration details used for the fluorometer.
2. **Measure dark-adapted Fv/Fm and Rd**
   1. After the dark-adapted leaf has been introduced to the cuvette, monitor the respiration rate and steady-state fluorescence level to ensure a steady-state is achieved. If the cuvette conditions are similar to the ambient conditions, this will be relatively rapid.
   2. Wait a minimum of 15 minutes. Use the “Log” function to sample gas-exchange and fluorescence with MPF pulse. Then wait another at least 15 minutes. Watch Fs and wait until it returns to the pre-pulse state.
   3. Check the RF geometry to verify steady-state achieved within pulse and MPF geometry to verify there are no errors in extrapolating the ramped phase at very low slopes. Also check to ensure that the recorded Rd value is comparable to values observed before log and ensure there are no errors with matching routine.
   4. If errors apparent in PAM or gas-exchange measurements, troubleshoot using the leaf in the cuvette. Recognize that in the dark, long intervals between saturating pulses are required to achieve strictly comparable results. Resolve the instrument issues. Then discard the leaf from the cuvette, replace with a fresh leaf that has been overnight dark-adapted and begin again.
   5. Once clean Fv/Fm and Rd measurements have been acquired, proceed to measure the light response.
3. **Measure light response**
   1. Continue in the same log file and continuous fluorescence recording file.
   2. While leaf is still darkened, check that actinic light specification is: 90R10B.
   3. Load the “LightResponse\_WW\_IO3\_v2.py” autoprogram. ***Ensure that IO 1 and IO 2 are ‘High’*** – i.e. Hal light ports are open. The autoprogram cycles through the light levels and automatically includes a sequence for toggling the upper fiber (IO 3) so that the reference can be periodically collected. 5 min intervals will suffice for practical purposes.
   4. Check autoprogram for light response, with intensities of 10-20-40-80-150-300-450-600-750-900-1200-1500-1800-2100-2400-1500 spaced at 5 minute intervals.
   5. Let 5 minutes elapse after the dark MPF measurement, then begin the light response autoprogram. This will require about 1 hour and 25 minutes. Monitor the progression of the measurements so that any issues are detected (and corrected) promptly.
   6. The last light intensity is set to the value used for the CO2 response measurements, which are next. It will take some time for the leaf to relax back from the highest light intensities. Give at least 30 minutes to permit re-equilibration before proceeding.
   7. Configuration notes: ***Make sure that each step is long enough to achieve a steady-state at least for fluorescence and gas-exchange***. Remember that the stomata will be the slowest part of the system and both fluorescence and gas-exchange will be ‘captive’ to stomatal movements***. The range and speed of the above curve is appropriate only for high light grown plants with rapid stomatal movements***. For low light grown plants and/or those with slow stomatal movements, consider scaling the upper end of the light response to align with the highest light intensities the plants experienced during growth and extending the duration of each step.

Post- light- or CO2- response steps:

1. Re-measure reference measurements (white reference, dark reference, and dark current).

1. **Measure CO2 response:**
   1. Load “*CO2Response\_IO3\_single\_v1.py*” autoprogram (see ‘*double’* version for 10 mins per level with 5 min intervals).
   2. Continue in the same log file and continuous fluorescence recording file.
   3. Configure autoprogram for CO2 response if the defaults levels are to be changed, with CO2\_S of: 400-0-50-100-150-200-250-300-350-400-600-800-1000-1200-1400-1600-1800-2000-400 spaced at 5 minute intervals.
   4. ***Ideally, wait until the leaf has relaxed back to the physiological state observed during the 1500 measurement in the middle of the light response. Then begin the CO2 response autoprogram.*** This will require about 1 hour and 35 minutes. As before, monitor the progression of the measurements so that any issues are detected (and corrected) promptly.
   5. The last CO2 level is set to the initial value. It will take some time for the leaf to relax back from the highest values. *Give at least 30 minutes to permit re-equilibration* and verify that the original state can be recovered. Manually log this final point.
   6. Configuration notes: The same general principles about achieving the steady-state apply to these measurements. The light intensity that is chosen for this curve will largely determine whether the leaf is driven into an electron transport limited or export limited state at the highest CO2 levels. While the electron transport limited state should be highly reproducible, the export limited state is likely to be sensitive to the leaf’s photosynthetic and nutritional history. Finally, the range of CO2 levels above is designed for a C3 leaf, and can be adjusted for a C4 with more dense sampling at low CO2 and more sparse sampling at high CO2.

**5. Daily shut down procedures, including COVID-related precautions:**

1. When any autoprograms finish and the experiment is done, stop the continuous fluorescence recording. Put the instrument into standby to minimize use of reagents/power/etc.
2. Remove leaf from cuvette. Take photograph for determination of leaf area within cuvette. Characterize R/T/A in integrating sphere if desired. Harvest leaf discs for short-term (24-48 hr) storage at 4°C (i.e., determination of abaxial/adaxial anatomical maximum stomatal conductance via light microscopy), drying at 55°C for 72 hours (i.e., determination of water content and specific leaf area), and other discs for freezing at -80°C (i.e., save for future determination of pigments, proteins, or other major constituents).
3. Take reference measurements for RT:
   1. Measure dark current: ensure IO-2 and IO-3 are in position ‘Low’, meaning there is no light passing through the beam splitter. Collect 100 spectra labelled ‘DC’. Ensure Fluorometer MB is OFF.
   2. Measure white reference (*WR\_100\_end*): Insert the white reference panel in the chamber (correct orientation with dimples on the bottom) and collect 100 spectra (no averaging): ensure fluorometer measuring beam ‘MB ON’ and that the halogen is ON. Ensure the file names are correctly labelled before the measurement is collected. Ensure that the AUX on the 6800 is set to IO-1-3 are all ‘High’.
   3. Then change IO-3 to low and collect a direct reference measurement of the HAL light source (‘*HAL\_100\_end*’ denoting 100 HAL spectra at the start of the experiment). Swap IO-3 back to high (default position).
   4. An additional optional measurement is to measure the dark panel (*DP\_100\_end*) with the QEP as a reference. Ensure that the top QEP is recording the cuvette and not the reference light source.
   5. Insert the diffuser and measure with MB ON (*DIFF\_100\_end*). Only the transmitted data is needed. Remove the diffuser and close the cuvette.
4. Collect the day’s data, including (a) the log file, (b) the fast kinetics files from each PAM pulse, and (c) the continuous fluorescence recording. If you made any updates to the config file, save a new copy of that, too. Upload these files to somewhere in the shared drive. Perform any area corrections, plot corrected data, and identify any outliers.
5. Disinfect the equipment, per manufacturer: <https://www.licor.com/env/support/LI-6400/home.html>

* *Wipe all touchable surfaces with a soft, clean cotton cloth that has been dampened with lab grade ethanol (70% or more), isopropyl, or hydrogen peroxide (3% or less) cleaning solution.*
* *Dampen the cloth with a spray bottle of cleaning solution and wipe buttons, handles, touchscreens, and cable connectors.*
* *Do not apply liquid cleaning solutions directly onto an instrument. Avoid wetting electrical components such as circuit boards. Do not use bleach, acetone, or other germicidal cleaning solutions on the instrument, especially on touchscreens. Damage caused by cleaning agents is not covered under the warranty.*