Measuring and Quantifying Characteristics of the Post-Illumination Burst

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Abstract:

Leaf-level gas exchange enables accurate measurements of net CO_2 assimilation in the light, as well as CO_2 respiration in the dark. Net positive CO_2 assimilation in the light indicates that the gain of carbon by photosynthesis offsets the photorespiratory loss of CO_2 and respiration of CO_2 in the light (R_L), while the CO_2 respired in the dark is mainly attributed to respiration in the dark (R_D). Measuring the CO_2 release specifically from photorespiration in the light is challenging since net CO_2 assimilation is composed of three unique processes (the velocity of rubisco carboxylation; v_c, velocity of rubisco oxygenation; v_o, and R_L). However, by employing a rapid light-dark transient, it is possible to transiently measure some of the CO_2 release from photorespiration without the background of v_c-based assimilation in the dark. This method is commonly known as the post-illumination CO_2 burst (PIB) and results in a "burst" of CO_2 immediately after the transition to the dark. This burst can be quantitatively characterized using several approaches. Here, we describe how to set up a PIB measurement and provide some guidelines on how to analyze and interpret the data obtained using a PIB analysis application developed in R.

Key words:

C_3 photosynthesis, net CO_2 assimilation rate, Post-Illumination Burst, PIB, Photorespiration, Light Enhanced Dark Respiration.

1. Introduction:

Since leaf-level gas exchange only measures net CO_2 assimilation (A), specialized approaches are needed to estimate the component gross fluxes. The component gross fluxes associated with A in C_3 plants are CO_2 assimilation (velocity of rubisco carboxylation; v_c), the photorespiratory loss of CO_2 (usually depicted as $0.5v_o$, where v_o is equal to velocity of rubisco oxygenation) and respiration of CO_2 in the light (R_L) [1, 2]. Accurately measuring the gross CO_2 release from photorespiration in the background of CO_2 uptake can be challenging; however, it is possible to resolve values proportional to photorespiratory CO_2 release using a Post Illumination CO_2 Burst (PIB) [3, 4].

The PIB method uses leaf-level gas exchange to measure A across a light to dark transient. When a leaf is illuminated, net CO_2 assimilation reaches a steady state with $A = v_c + 0.5v_o + R_L$, while in the dark, CO_2 respiration reaches a steady state ($A = R_D$). Consequently, a "burst" of CO_2 commonly occurs during the first few minutes immediately following the light-dark transition presumably since the reactions of the C_3 cycle cease in the darkness much faster than the CO_2 release from the photorespiratory pathway ($0.5v_o$ in the previous equation) [5, 6].

Many photorespiratory-related metabolites have been hypothesized to contribute to the PIB. Glycine is suggested to be the primary contributor of CO_2 to the burst [3, 4, 7, 8]. Glycine, a photorespiratory intermediate that accumulates in the light, continues its metabolism in the dark longer than CO_2 is assimilated, so the CO_2 released is observed in the burst. In addition to glycine, there is evidence that CO_2 release from non-enzymatic decarboxylation between H_2O_2

and hydroxypyruvate and/or glycolate in the photorespiratory pathway are also captured in the burst [9].

Other non-photorespiratory metabolites that accumulate during photosynthesis, such as malate and pyruvate, have also been proposed to contribute to the burst. Since mitochondrial respiration is sensitive to light-dark transients, the decarboxylation of malate and/or pyruvate can stimulate initial rates of respiration in the dark after a period of illumination and has been denoted in the past as light enhanced dark respiration (LEDR) [4, 10, 11]. Substrate feeding experiments proved evidence that glycine (200mM) addition increased the burst size by 60% compared to water addition, while malate (200 mM), and pyruvate (200mM) were less effective at increasing the burst size [8]. While other metabolites have been suggested, it is likely that photorespiratory intermediates play a major role in contributing CO_2 to the PIB.

Although the PIB cannot directly measure the rate of photorespiration, since there are other non-photorespiratory contributors to the PIB and the release depends on pool sizes of photorespiratory intermediates that may or may not decarboxylate at the same rate in the dark as in the light, it is still an effective method for measuring an integrated signal of carbon emptying out of the residual carbon pools in the dark. Since there is a photorespiratory component contributing to the burst, changes in the total burst size are roughly proportional to the amount of photorespiration occurring.

Along with estimates of the absolute burst size, there are additional characteristics of leaf photosynthetic and photorespiratory performance that can be estimated from PIB measurements such as:

Steady-State Net CO_2 Assimilation Rate (A_s): A_s is the steady-state of CO_2 assimilation in the light before the leaf enters the dark and is given in units of μmol/m2/s.

Steady-State Dark Respiration Rate (R_D): R_D is the steady-state CO_2 dark respiration rate and is given in units of μmol/m2/s.

Area of the CO_2 Burst ($Burst_{Area}$): $Burst_{Area}$ is the entire amount of CO_2 released in the burst and is given in units of μmol/m2.

Time of the photorespiratory CO_2 burst ($Burst_{time}$)*:* $Burst_{time}$ is the length of time during the $Burst_{Area}$ and is given in minute units.

Maximum rate of CO_2 loss in the burst (Max_{lost}): Max_{lost} is the greatest rate of CO_2 lost during the $Burst_{Area}$ and is given in μmol/m2/s.

Along with these five unique parameters, it should also be noted that there are differences between species in the PIB response. Some species (i.e., *Arabidopsis thaliana*) give a single burst, where other species (i.e., *Nicotiana tabacum*) give two bursts (Figure 1). Sometimes portions of these bursts are referred to as LEDR. It is outside the scope of this method to explore the cause of these differences, but the protocol outlined below can capture and quantify these different burst types.

Here we describe how to set up a PIB measurement, what to pay attention to while making measurements, and provide some guidelines on how to analyze and interpret the results using some quantitative methods.

2. Materials:

1. Infrared gas exchange analyzer (IRGA) for leaves with power supply or batteries (see Note 1).
2. Light Source (see Note 2)
3. Tripod/Stand to mount the IRGA head.
4. CO_2 cartridges (see Note 3).
5. Soda lime.
6. Desiccant (such as Sorbead® Orange CHAMELEON silica gel beads or Dririte®).
7. Humidifier (If applicable for the IRGA; see Note 4).
8. Computer for data download and processing.
9. Software: ImageJ, Excel R, RStudio (Optional; see Note 5 and Note 6).

3. Methods:

3.1 Machine Setup and Calibration:

We use the LI-6800 portable photosynthetic system for gas-exchange measurements so there may be some differences in setup based on the specific manufacturer used.

1. Before turning the IRGA system on, be sure to refill/replace the CO_2 cartridge. CO_2 scrub, Desiccant, and Humidifier chemicals of needed according to the manufacturer's instructions.

2. If needed, switch the IRGA sensor head to the appropriate leaf chamber (e.g., for the LI-6800 there is the Multiphase Flash Fluorometer, or a Large/Small Light Source that can be used). Additionally, verify that the gaskets of the leaf chamber are fitted to the IRGA sensor head properly and are not damaged. Replace damaged or ill-fitting gaskets.
3. Place the IRGA system on a clear work surface, or in a climate-controlled cabinet set to the desired measuring temperature if measuring under conditions ~±6°C from ambient.
4. Power on the machine and check that the IRGA sensor head is communicating with the IRGA console. Check the temperature control on the IRGA system and set the IRGA system desired temperature. For optimal humidity control, ensure the IRGA temperature closely matches the ambient temperature of the instrument. The IRGA itself will have a limited range of temperatures that can be achieved relative to the ambient temperature. Let the machine equilibrate for at least 45-60 minutes with the chamber closed. This is especially important if you are measuring above or below room temperature.
5. Perform the warm-up test (and/or the start-up checks recommended by the manufacturer). The warm-up test should reveal any warnings or errors that need attention. If there are any warnings or error messages, resolve them before beginning the experiment for the day.
6. Set the desired environmental conditions for the leaf chamber (i.e., flow rate, CO_2 and O_2 concentration, vapor pressure deficit, light intensity, temperature).

3.2 Preparing the Leaf:

1. Place the leaf into the leaf chamber on the IRGA sensor head and close the chamber fully, taking care not to fold or damage the leaf. If the leaf does not cover the entire area

of the leaf gasket you will need to measure the area of the leaf after the measurement is taken (refer to *Collect Leaf Area section* below if applicable*).* Ensure that the thermocouple is making firm (but not too firm as to damage the leaf) contact with the bottom of the leaf. Leaf temperature is crucial to obtain accurate transpiration and stomatal conductance measurements. Ensure that the gaskets make a tight seal around the leaf and that there are minimal leaks.

2. Once you are confident with a secure fit onto the leaf, monitor the IRGA console. Verify that the environmental parameters are set correctly and watch the leaf parameters (i.e., A and g_{sw}) until steady state photosynthesis is reached. Visually, steady state photosynthesis will be reached once A and g_{sw} reach a plateau for more than 2-3 minutes.

3.3 Making a PIB Measurement:

PIB is measured during a light to dark transient that can be changed manually or written in an automated program. We recommend measuring A rate for 10 minutes in the light and 20 minutes in the dark to ensure good steady-states in the light and dark.

1. To establish a PIB program, create a sequence to control the light intensity of the leaf chamber. Begin the sequence by setting the desired light intensity (i.e., saturating, or sub-saturating) and end the sequence by setting the light intensity to zero (i.e., dark). Set the log interval to 1 second (or the fastest the IRGA system can log measurements). Set the entire program to run over 30 minutes (i.e., 10 minutes in the light, and 20 minutes in the dark) Ensure that there is no additional averaging of data above the log interval.
2. Open an empty log file on the IRGA system where the data can be stored during the measurement according to the manufacturer's directions.

3. Before starting the PIB measurement, it is important to switch the humidity and temperature controls to be constant during the transient, rather than depending on leaf performance (i.e., for the LI-6800 switch VPDleaf to H2O_r and Tleaf to Txchg). It is necessary to switch instrument controls to be constant because it will not result in artifactual changes in A or g_{sw} during the transient into the dark while the instrument works to compensate for the reduction in A and radiative load.
4. After setting the humidity and temperature controls to remain constant, wait for A and g_{sw} to restabilize before starting the measurement.
5. During the measurement one of two behaviors should be present:
    a. *Single Burst Observations*: In the light, the leaf will maintain A at a steady state. Immediately following the light-dark transition, a CO_2 burst will appear that will decelerate within minutes in the dark. After 10 minutes in the dark, the leaf will reach or approach an R_D rate at a steady state (Figure 1).
    b. *Double Burst Observations*: In the light, the leaf will A at a steady state. Once the lights turn off, an initial burst of CO_2 will occur within the first minute, followed by a much longer CO_2 burst. Depending on the species that is measured it is expected to take 15-20 minutes (perhaps longer, adjust program length accordingly) to reach a R_D rate at a steady state (Figure 1).

3.4 Collect Leaf Area (when necessary):

1. Once the PIB measurement is finished, mark the leaf with a marker where the gaskets are clamped on.

2. Release the leaf from the leaf chamber. Place a spare gasket overtop of the leaf and line it up with the marked spots. Place a known scale in the image field, such as a ruler, and take a picture of the leaf with the gasket placed overtop (ideally on a white surface).
3. Using a digital area analyzer, like ImageJ, determine the leaf area inside of the gasket [12].
4. Record the leaf area and correct the gas exchange data accordingly before analyzing the data (column 'S' on LICOR excel file).

3.5 Analyzing and Interpreting the PIB data:

The previous sections were to ensure that the highest quality of data is obtained for the PIB. The following section will provide insight into how to analyze and interpret the PIB data (Figure 2).

1. Plot A as a function of time and assess the quality of the data.
2. Perform initial quality control on the data. Visually, is there a lot of noise in the measurement? Does A rate reach a steady state in the light? Is there a distinct CO_2 burst immediately following the light-dark transition? Does R_D reach a steady state in the dark? If the data is satisfactory, then the PIB parameters can be estimated from the data.
3. To estimate A_s and R_D, average the last few points before the light transition, and the last few points in the dark before the measurement ends, respectively.
4. Add a linear regression overtop of the CO_2 burst, whereby the y-intercept is the solved $R_{D,}$ and the slope is set to zero.
5. To estimate $Burst_{Area}$, sum up the differences between the R_D linear regression and the measured assimilation values within the burst.

6. To estimate $Burst_{Time}$, find the difference between the first and last time point identified as the CO_2 burst.
7. To estimate Max_{Lost}, find the length from the R_D linear regression to the minimum assimilation in the CO_2 burst.

3.6 Using the ShinyApp to Automate Analysis:

The previous sections described how to check PIB data collected for quality, and how to analyze and interpret the PIB parameters manually. The following section introduces a custom-built program (i.e., ShinyApp) that can automate the analysis used to determine the PIB parameters (Figure 3).

1. Download the custom-built program from GitHub (https://github.com/L-Gregory/PIB_analyzer).
2. Open the script in RStudio and select Run App, this will open the application in another RStudio window.
3. Upload data by selecting the Browse button, or by "dragging and dropping" the data file into the box. Data must contain net assimilation rate and time columns.
4. Once data is uploaded successfully, select the columns containing time and assimilation. This will plot the data.
5. The data can be visually checked for quality now.
6. If the data are acceptable, click the "Fit" button. This will automatically estimate and display the five parameters of interest (A_S, R_D, $Burst_{Area}$, $Burst_{Time}$, Max_{Lost}) below the plot.

7. If you would like to change the R_D estimate for the linear regression – check the "Change Rd fit" box. Using the slider bar, select the number of points that you want to be included in the linear regression (default = 50).

Notes:

1. This method described here refers to gas exchange measurements made on the LI-6800 portable photosynthetic system (LICOR, Lincoln, NE, USA). The method could be adjusted for instruments from other manufacturers that measure CO_2 and H_2O gas exchange with high temporal resolution, as the general principle of operation, data collection, and analysis/interpretation still applies. However, we have not checked this method performance on other systems.
2. The simplest option is the LED light source made specifically for the leaf chamber in use, designed by the IRGA manufacturer. Depending on experimental requirements other light sources may be required (e.g., when a custom-built or clear top chamber is used, or when a certain light quality is required).
3. CO_2 cartridges should be purchased from the manufacturer if there is not a replaceable pre-filter. Use of generically branded CO_2 cartridges can introduce oil into the system and damage the instrument.
4. If you have a Nafion tube humidifier column, use deionized H_2O sources, instead of non-deionized H_2O as this can cause mineral buildup over time in the instrument.
5. Digital analysis for leaf area can be accomplished using ImageJ. However, this could be adapted to another application quite easily.
6. R code is provided in the supplemental section that automatically analyze and estimate the PIB parameters given PIB dataset.

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Table 1. List of parameters, descriptions, and their units

Parameter	Description	Unit
A	Net CO_2 assimilation rate	µmol/m2/s
A_s	Steady state net CO_2 assimilation rate	µmol/m2/s
g_{sw}	Stomatal conductance to water vapor	mol/m2/s [1]
C_a	The CO_2 partial pressure in the ambient air	Pa
C_i	The CO_2 partial pressure in the intercellular airspace of the leaf	Pa
R_L	Non-photorespiratory release of CO_2 in the light	µmol/m2/s
R_D	Non-photorespiratory release of CO_2 in the dark	µmol/m2/s
T_{leaf}	Leaf Temperature	°C
VPD	Vapor Pressure Deficit	kPa
$Burst_{Area}$	Total Area of the CO_2 burst	µmol m2
$Burst_{Time}$	Time of the CO_2 burst	minutes
Max_{Lost}	Maximum rate of CO_2 loss in the burst	µmol/m2/s
v_c	Velocity of rubisco carboxylation	µmol/m2/s
v_o	Velocity of rubisco oxygenation	µmol/m2/s

Figure 1. Example of a Single and Double Bursts in *Arabidopsis thaliana* and *Nicotiana tabacum*. There are two PIB behaviors that are seen across higher plant species. The first is a single burst (open points) whereby a CO_2 burst will appear immediately after the light-dark transition and will decelerate within minutes in the dark. The second PIB behavior is a double burst (closed points) whereby an initial burst of CO_2 will occur within the first minute, followed by a much longer CO_2 burst.

Figure 2. Estimated Parameters Overlayed onto Post CO_2 Illumination Burst. There are five unique parameters (A_s, R_D, $Burst_{Area}$, $Burst_{Time}$, Max_{lost}) that provide characteristics of leaf photosynthetic and photorespiratory performance that can be estimated from a PIB. The location of where these parameters are estimated are labeled onto of the PIB.

Figure 3. Custom-Written Application for Analyzing Post Illumination CO_2 Burst Data. Screen-shot from the custom-built program that automates estimation of the five unique parameters (A_s, R_D, $Burst_{Area}$, $Burst_{Time}$, Max_{lost}) from the uploaded PIB data. Upon executing the app, PIB data can be uploaded into the file selection box. After defining the axes, a plot of the data will appear in the main panel. Clicking on the “Fit” button will generate the five parameters of interest. If the linear regression is not fitting properly, the user can adjust the points that are included in it (default = 50).

Choose a CSV file

Browse | No file selected

Note: the CSV file being uploaded must contain a header row

Select column containing time in seconds

Time

Select column containing net assimilation rate

A

Data Quality

```
Check:
 1) Is the assimilation rate at a steady state in the light?
 2) Is the respiration rate at a steady state in the dark?
 3) Is the noise acceptable?
 4) Do you see one or two burst(s)?
```

Time	A
0.00	5.01
1.00	4.99
2.00	5.02
3.00	5.00
4.00	4.99