



Fast and direct carbon assimilation measurements utilizing a CO₂-sensitive colourimetric dye

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Fast and direct photosynthesis measurements utilizing a CO₂-sensitive colourimetric quantifier

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In the report's final version (after literature research and writing), the AI model ChatGPT-4 mini (accessible via: chatgpt.com) was used to improve grammar, spelling and fluency. The model made suggestions that were reviewed and used as a reference to improve the text. No AI output was directly copied to the final text but purely used as grammatical assistance. The author takes full responsibility for the content of the essay.

Abstract

Carbon assimilation is a fundamental process in plant growth and biomass accumulation, yet it remains largely unharnessed within plant breeding programs due to a lack of high-throughput measuring tools. To bridge this gap, this study explores the feasibility of utilizing a CO₂-sensitive dye (o-cresolphthalein) as a high throughput carbon assimilation quantification method. The dye's response to photosynthesis carbon assimilation and different environmental conditions was tested through experiments targeted to work with the natural physiological processes of a leaf. Simultaneous optical measurements (MultispeQ) and environmental control (LI-6800) were conducted, showing reversible and stable reaction patterns. Although carbon assimilation could not be measured with complete certainty using the current experimental setup, the developed open chamber approach, paired with optimized dye chemistry and an improved signal to noise ratio can be developed into a high throughput tool for plant phenotyping.

Keywords: Carbon assimilation, Plant breeding, Photosynthesis, CO₂-sensitive dye, Colorimetric sensing, High-throughput measurements, Optical measurements, Gas exchange

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1

Introduction

1.1 Background

Global food demand is outpacing agricultural production potential, creating a widening gap that threatens food security [1]. This widening gap between capacity and demand grows more severe as rapid population growth outpaces conventional plant breeding to hasten yield production methods for increasing crop yields. A complementary approach to conventional plant breeding is to target photosynthetic traits [2, 3, 4].

Photosynthesis is composed of both light and carbon assimilatory reactions. The light reactions convert sunlight energy into biochemical energy which is subsequently used in the carbon assimilatory reactions to assimilate CO₂ into sugars via Ribulose bisphosphate carboxylase/oxygenase (Rubisco) [5]. Both processes can be accurately quantified using optical and gas exchange methods [6, 7].

Instruments based on optical signals (e.g., MultispeQ, Walz) quantify the performance of the light reactions [8]. While they have the potential to be high-throughput (measurement time <1min), their information about the light reaction does not directly correlate with carbon assimilation and ultimately biomass accumulation. Alternatively, gas exchange measurements (LI-6800) quantify net carbon assimilation, however, they are time-consuming and very low-throughput (measurement time >2min). This delay arises from utilizing a non-dispersive infrared (NDIR) analyzer dependent system, to measure CO₂ concentration before and after passing through the chamber (containing a leaf) to determine net CO₂ assimilation [9]. The system's reliance on serial concentration measuring results in a time lag. The travel distance between the leaf chamber and the NDIR increases measurement time and limits the capturing of photosynthetic response to dynamic changes that research suggests have a significant impact on photosynthesis (Figure 1.1) [10, 11]. In the absence of high-throughput tools to reliably quantify carbon assimilation, selecting for the photosynthetic traits of carbon assimilation will remain unlikely to be included in plant breeding programs, resulting in

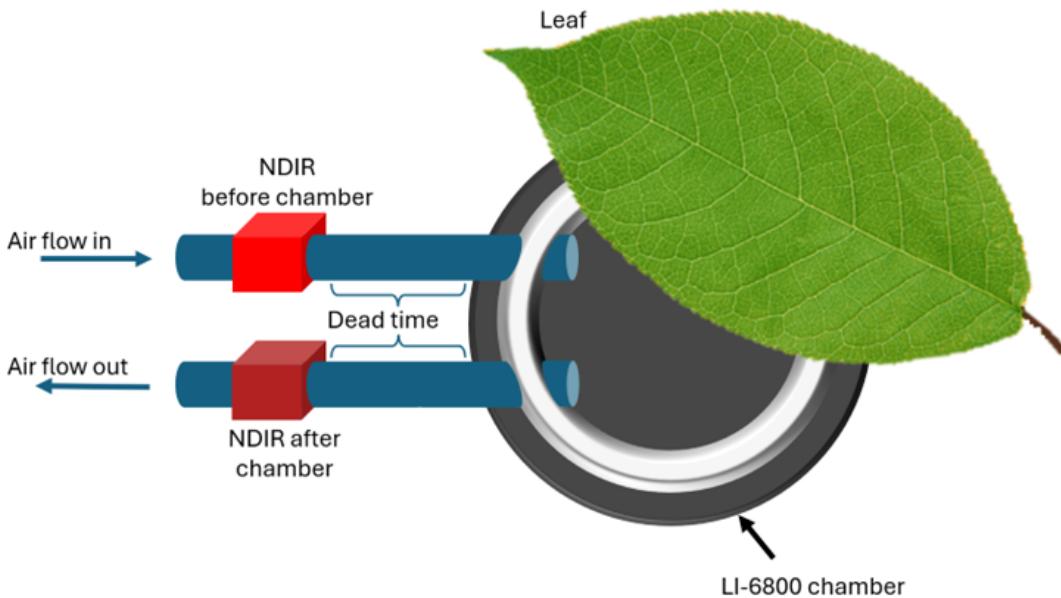


Figure 1.1: Top view of the LI-6800 chamber with a leaf inside, illustrating the dead time between NDIR sensor and leaf.

potential yield gains remaining unharnessed.

1.2 Bridging the gap

Within photosynthesis research carbon measurements are mainly based on infrared gas analysis. However, other fields have developed alternatives utilizing optical signals to quantify changes in CO₂ concentrations such as reversible colorimetric dyes sensitive to gas-phase CO₂ [12]. One such compound (Figure 1.2) has been proven effective across a multitude of applications: in aquariums, food packaging, medicine and as an indicator in air pressure measurements [13, 14]. The versatility of applications demonstrates the dye's potential as a fast, instantaneous and precise CO₂ sensor utilizing optical signals. This CO₂-sensing dye is based on o-cresolphthalein (Figure 1.2) which is a pH-dependent anionic chromophore. It shifts from purple (low CO₂) to colourless (high CO₂) when protonated (Figure 1.3). The protonation is driven by the acidic nature of CO₂ when dissolved in water, making the change both fast and optically measurable [14]. In this study, the o-cresolphthalein-based dye was tested for its ability to measure photosynthesis-driven changes in CO₂ concentration. Measuring photosynthesis with the CO₂-sensitive dye will allow the tracking of much faster change in direct proximity to the leaf which ultimately would translate in high-throughput measurements. This requires effective functioning of the dye within the context of photosynthesis. Effective functioning includes sufficient sensitivity to plant-induced CO₂ changes, reversibility under prolonged exposure, and adequate/stable response under dynamic environmental conditions (e.g., humidity, light, temperature).

To evaluate these properties, I designed a custom setup that integrated the CO₂-sensitive

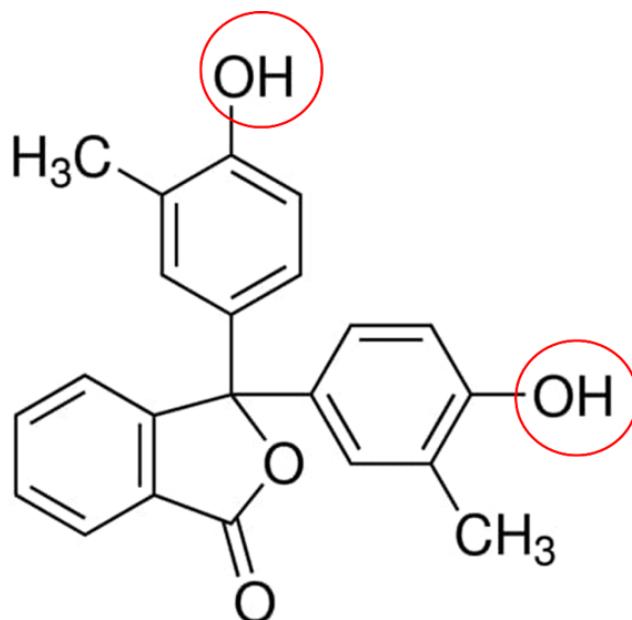


Figure 1.2: Chemical scheme of o-cresolphthalein, the chemical causing the purple-to-transparent colour shift of the dye when protonated at sites red highlighted [15].



Figure 1.3: Colourmetric colour shift from purple-to-transparent of o-cresolphthalein [14].

dye, immobilized on an Optical Carbon Tracking Operational Platform for Universal Support (OCTOPUS) (Figure 2.1). This platform was connected with a fiber optic to established measuring tools (LI-6800 and MultispeQ) for colour shift measuring and environmental control, optimizing dye performance and characterization. Dye performance and testability critically depend on the method of application, gas permeability and reflectivity of the materials, and thermal stability of the entire supporting structure. This setup allows for evaluation of the CO₂ sensitive dye under near in-situ photosynthetic conditions and as close to the leaf as possible.

1.3 Overall aims and research questions

The aim of this study is to validate the use of the o-cresolphthalein dye within the context of photosynthesis research. To achieve this the following research questions are outlined:

Main Research Question:

Can the CO₂-sensitive dye be effectively used to measure plant carbon assimilation, and how should it be applied?

Subquestions:

- *Response dynamics:* How does the dye respond to fluctuations in CO₂ concentration?
- *Sensitivity:* How do environmental conditions (humidity, temperature) affect the sensitivity of the dye under CO₂ fluctuations?
- *Material selection:* Which materials for dye application have the desired properties (white, gas-permeable, thermal stability), and which ones perform the best under photosynthetic conditions?
- *Application method:* What is the best method of dye application (e.g., vapor deposition, printing, pressure methods)?

Overall, this study lays the groundwork for developing a high-throughput quantification method to quantify net carbon assimilation for plant breeding selection.

1.3.1 Scope and limitations

Within the bounds of this study I focus on the feasibility of utilizing the CO₂-sensitive dye for carbon assimilation measurements and its response to environmental conditions. Ultimately with the goal to measure carbon assimilation of a leaf with the CO₂-sensitive dye providing proof-of-concept.

2

Experimental section

2.1 Measurement setup

To test the usage of the dye for carbon assimilation measurements this study utilizes a custom setup to validate the CO₂-sensitive dye as a measuring method. This measuring method allows simultaneous control of simulated environmental parameters as dye colour change data collection. The experimental setup (figure 2.1) integrates four core components: (1) an environmental control device (LI-6800) to regulate test conditions, (2) a device containing a spectrophotometer and light sources to measure changes in dye reflectance when exposed to different conditions (MultispeQ), (3) a fiber optic for signal transmission between the LI-6800 and the MultispeQ, and (4) a Universal Support structure, which secures the CO₂-sensitive dye to the fiber optic within the LI-6800 chamber (Optical Carbon Tracking Operational Platform for Universal Support; OC-TOPUS).

This framework enables study on how changes in environmental factors (light, CO₂, humidity, temperature) impact dye reflectance under CO₂ oscillations. By making a direct correlation of dye-induced optical response to regulated LI-6800 chamber conditions of CO₂ concentration, humidity, light and temperature. The framework also allows for the CO₂-sensitive dye to measure with close proximity to the leaf. Leaf proximity is important as it decreases the distance between phenomena and observation, a necessity when tracking the influence of dynamic parameters on carbon assimilation (Figure 1.1) [10]. The following sections detail each core component's implementation and procedures, beginning with a description of the signal path.

2.1.1 Signal path

To measure the CO₂-sensitive dye optically a light signal goes through the setup in figure 2.1. The LI-6800 (1) changes the environmental conditions that drive a change in dye reflectance. The optical signal path goes from emission, transmission, reflectance to detection and output:

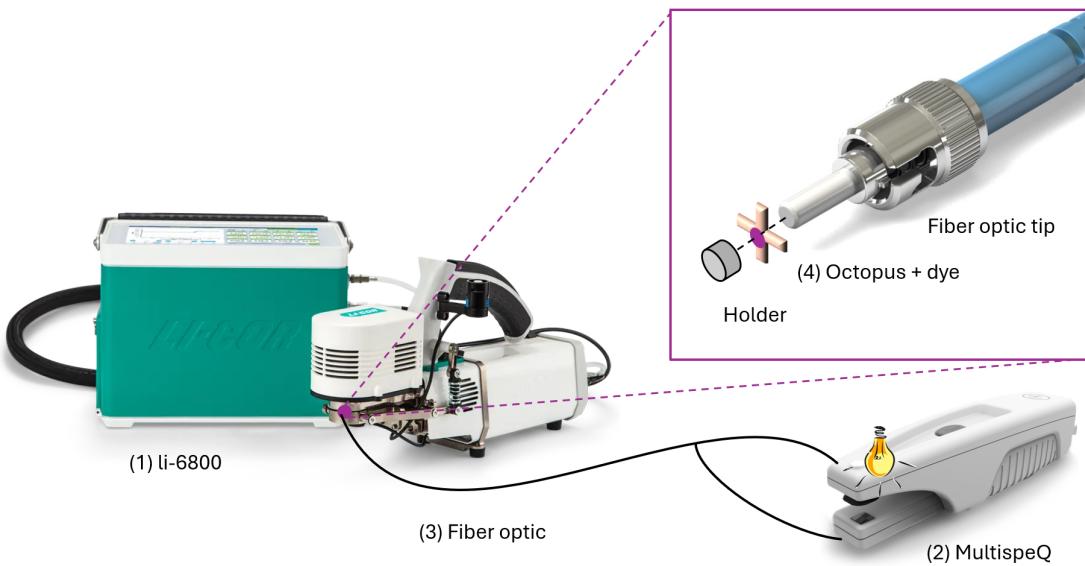


Figure 2.1: Cartoon of the core components: (1) LI-6800 device, (2) MultispeQ device, (3) fiber optic signal transmitter and (4) the CO₂-sensitive dye (purple dot) on a OCTOPUS, in the LI-6800 chamber

Emission: MultispeQ (2) emits 590 nm light from orange LED (led 3) [16]

Transmission: 3.9 mm diameter fiber optic (3) transmission to OCTOPUS containing dye (4) in LI-6800 (1) chamber

Reflection: Signal reflected back through fiber optic (3) (intensity varies with dye's purple-transparent color shift (Figure 1.3))

Detection: Reflected signal captures with MultispeQ (2) spectrophotometer (Detector 3) [16]

Output: Output as reflectance

2.1.2 Environmental control

Within the LI-6800 chamber, different environmental conditions were pre-programmed and simulated (see Appendix A.2). During each protocol, the dye is subjugated to the programmed conditions by adjusting the air before it enters the chamber [9].

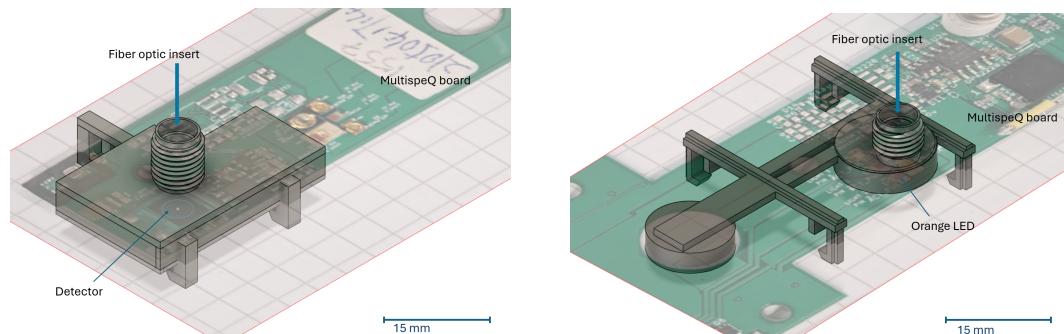
The parameters controlled during the protocols include environmental variables such as CO₂ concentration (CO_r), which were oscillated from 600-0-500 ppm to study dye response dynamics; relative humidity inside the chamber (RHcham); chamber air temperature (Tair); and light (Qin) during photosynthesis measurements. Operational parameters, such as airflow rate, were controlled to close the chamber when needed forming a closed chamber system (flow = 0) (Figure 3.6a). Additionally, the LI-6800 also allows changing the measurement frequency and the amount of measurements taken during a single run [9]. All programs can be found in '['github.com/monsterekie/Thesis-CO2-sensitive-dye-2025/Code/LI-6800/Programs'](https://github.com/monsterekie/Thesis-CO2-sensitive-dye-2025/Code/LI-6800/Programs)'.

2.1.3 Optical sensing

The MultispeQ emits 590 nm (amber) light (led 3) and captures reflected signal with a spectrophotometer absorption range 400nm - 700nm (detector 3) [16]. The detector is covered with a BG-18 bandpass filter with a center wavelength around 493 nm [17]. The absorption spectra of the CO₂-sensitive dye shows maximal absorption peak at $\lambda_{\max}(D^-) = 589$ nm between its purple and transparent states and a sensitivity of $\alpha = 200\%^{-1}$ at 20° [14]. The MultispeQs 590 nm amber LED was selected as it nears the max absorption peak and falls within the measurable range of the detector. The multispeQs detector synchronizes with LED pulse timing, this time resolved detection excludes continuous ambient light sources including those of the targeted wavelength [16]. The MultispeQ measurements were controlled by a custom python protocol script (see A.1.2).

Fiber optic-MultispeQ connector

To connect the MultispeQ to the fiber optic the original protective casing was removed and replaced with 3D-printed mounds that allow removable attachment of the fiber optic and MultispeQ board (containing the LED light) while maintaining 90° vertical alignment with the LED light (Figure 2.2). This standardized connection enables reproducibility of measurements.



(a) Connecting fiber optic to the detector on the MultispeQ circuit board
 (b) Connecting fiber optic to the orange LED light on the MultispeQ circuit board

Figure 2.2: Fusion designs of mounds connecting the fiber optic to the MultispeQ board (a) detector and (b) light source.

2.1.4 OCTOPUS Fabrication and Material Testing

To allow material testing and consistent measurements I designed an Optical Carbon sensitive dye Tracking Operational Platform for Universal Support (OCTOPUS; figure 2.4c). OCTOPUS immobilizes the CO₂-sensitive dye interchangeably to the fiber optic well limiting hindering of LI-6800 chamber closing mechanics. Based on photo-synthetic gas exchange dynamics and setup incorporation four critical design criteria were established:

- **Gas permeability:** to ensure unimpeded CO₂ diffusion to/from the dye

- **High reflectivity/low light absorption:** to enable optical measurements by reflectance
- **Thermal stability:** to maintain function under field conditions
- **Immobilization:** to securely but interchangeably attach to fiber optic within the LI-6800 chamber

OCTOPUS secures the dye on the fiber optic by acting as a dye application platform with a fiber optic attaching body. The OCTOPUS body has foldable tentacles to secure around the fiber optic tip, hold in place by the holder (figure 2.4b) without further adhesives (figure 2.4c). Dye is applied on a solid surface which is perforated into a circle and put into the center of the octopus during casting after a minimum drying period of 24 h. By casting gas permeable material for the body and the white reflective solid dye containing material in a flat 3D-printed mold (figure 2.4a) OCTOPUS is created and left to dry at room temperature to prevent dye degradation 2.4c.

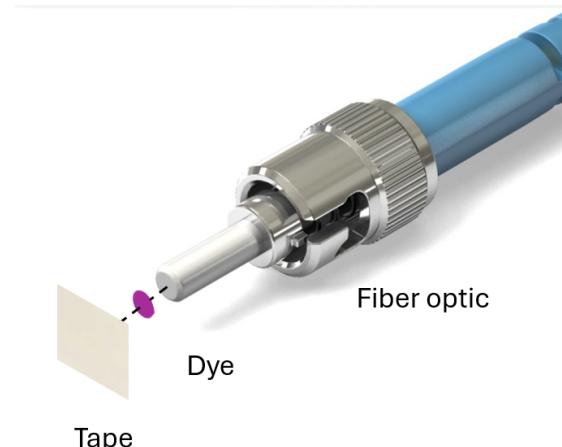
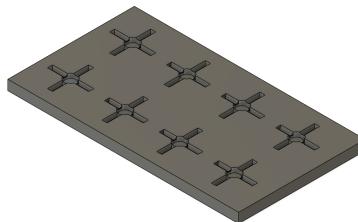


Figure 2.3: Cartoon of 'TapeOnly' measuring method.

All OCTOPUS's consists of a flexible body and a white reflective solid dye application material (Table 2.1). The only method that does not follow the standard OCTOPUS formula (figure 2.1 (4)) is the 'TapeOnly' method where dye is swiped directly on the fiber optic tip, and masking tape put on top is used as a reflective background (figure 2.3).

Method Name	Body Material	Platform Material	Notes
TapeOnly	None	Fiber optic + masking tape	Direct application, minimal setup
OCT-Eco	Ecoflex (1:1)	Ecoflex	non-reflective, non-functional
OCT-EcoPaper	Ecoflex (1:1)	White paper	Functional
OCT-PDMSPaper	PDMS (12:1)	White paper	Functional
OCT-PDMS.ABS	PDMS (12:1)	White ABS	Functional
OCT-PDMS.ABS.PDMS	PDMS (12:1)	White ABS	Non-functional, no oscillation

Table 2.1: Table containing the OCTOPUS body and application platform compositions of the different OCTOPUS's [18, 19].



(a) Mold for OCTOPUS production



(b) Holder folding OCTOPUS tentacles around fiber optic tip



(c) OCT-EcoPaper attached to the fiber optic tip

Figure 2.4: Fusion designs of OCTOPUS: (a) mold, (b) tentacle holder, and (c) OCTOPUS attached to the tip of the fiber optic.

2.2 Analytical programs

Data was collected from both the LI-6800 and MultispeQ separately. The LI-6800 is controlled via python programs (see A.1.1) and the MultispeQ is controlled via python script (see A.1.2). Both data sets were synchronized by timestamps and exported to R studio (v4.4.2) for further analyze using custom scripts. All data analyze scripts can be found in '['github.com/monsterkiek/Thesis-CO2-sensitive-dye-2025/Code/R studio'](https://github.com/monsterkiek/Thesis-CO2-sensitive-dye-2025/Code/R studio).

3

Results and Discussion

This chapter presents the experimental findings of applying the CO₂-sensitive dye to measure its response under different environmental parameters, OCTOPUS materials, application methods and eventually under plant photosynthesis to measure carbon assimilation. All experiments were conducted using the LI-6800 as an environmental controller, a MultispeQ for dye colour change monitoring and a fiber optic.

3.1 Sensitivity of Response Dynamics

Leaf photosynthesis operates under dynamic temperature, light and humidity conditions. Change in humidity conditions is brought both by the environment and by H₂O transpiration during photosynthesis [5]. Given the nature of the dye as a pH-dependent chromophore, humidity and temperature influence CO₂-sensitive dye response. The sensitivity of the dye to temperature and humidity was studied by subjecting the dye to consecutive CO₂ oscillation. CO₂ concentrations were repeatedly step-wise fluctuated from 600-0-500 under contrasting relative humidity conditions (70% (left) and 30% RH (right)) keeping all other environmental conditions constant (protocol A.2.6).

The reflectance revealed a consistent response that traced with the oscillations in CO₂ concentrations (figure 3.1). The pattern is repeating and stable over consecutive oscillations, with a response curve of recurring amplitude and period. However, these repeated oscillations show a smaller change in reflectance at elevated CO₂ concentrations (>500 ppm), meaning there is no change in reflectance as the dye is already transparent despite ongoing changes in CO₂ concentrations. The impeded response indicates a low sensitivity at high CO₂ concentrations. In other words, dye saturation decreases measurable sensitivity.

The response curve also revealed humidity-dependent characteristics (figure 3.1). A higher relative humidity (70%) consistently shows a higher baseline reflectance signal and smaller amplitude than at 30% RH. Initial trends also differ (downward at 70%

RH, upward at 30% RH) upon CO₂ exposure, needing several full oscillation cycles to stabilize.

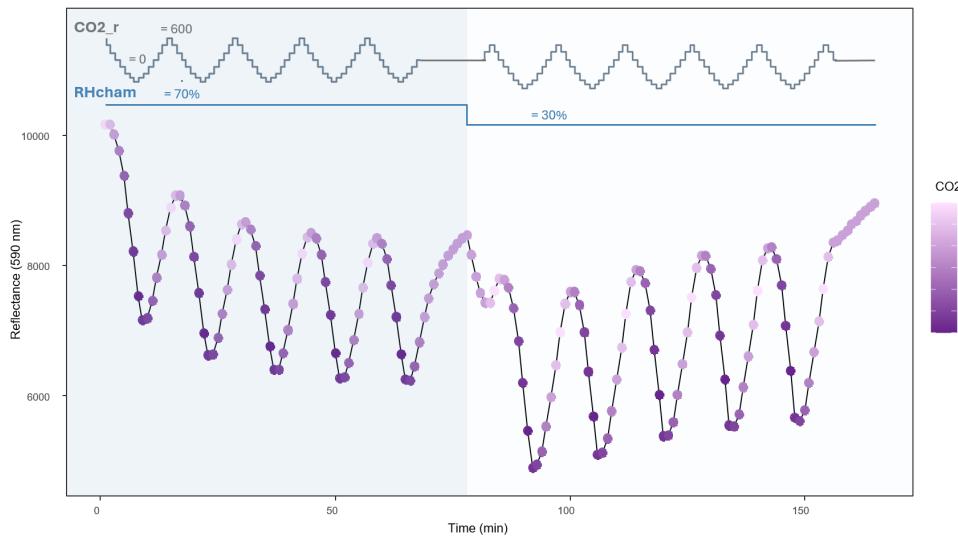


Figure 3.1: CO₂-sensitive dye response to oscillating CO₂ concentration and contrasting humidity conditions (70% and 30% RH) (protocol A.2.6).

Like humidity, temperature is a dynamic environmental parameter that has the potential to affect the reflectance reading of the dye. Higher temperatures (30° (right)) reveal larger measurable amplitude and lower reflectance measured compared to low temperatures (15° (left)) under CO₂ oscillation (figure 3.2).

Both humidity and temperature have measurable stable and reversible effects on dye response with individual differences in total signal and amplitude. A lower humidity 30% and higher temperature (30°) each increase amplitude, enhancing sensitivity to differentiate CO₂ concentrations. Overall the dye displays repeatability and remains stable across multiple oscillating CO₂ cycles.

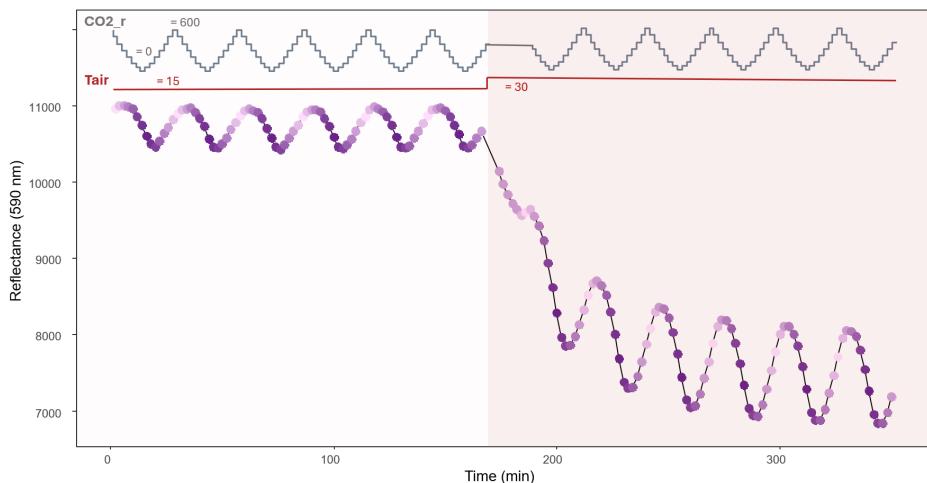


Figure 3.2: CO₂-sensitive dye response to oscillating CO₂ concentration and contrasting temperature conditions (15% and 30% RH) per protocol A.2.7.

3.2 Material selection

OCTOPUS is the supporting structure forming an attachment to the tip of the fiber optic and application platform for the dye (2.1.4). Several OCTOPUS material compositions, comprising of body and platform, were selected based on the critical design criteria (table 2.1) and tested under a standard response dynamic test (protocol A.2.6). While signal intensity and amplitude differed, the response pattern stayed consistent when overlaying reflectance peaks of measurements with different OCTOPUS/material methods (figure 3.3). This consistent response pattern indicate the material application method does not change the response pattern and thus dye functioning.

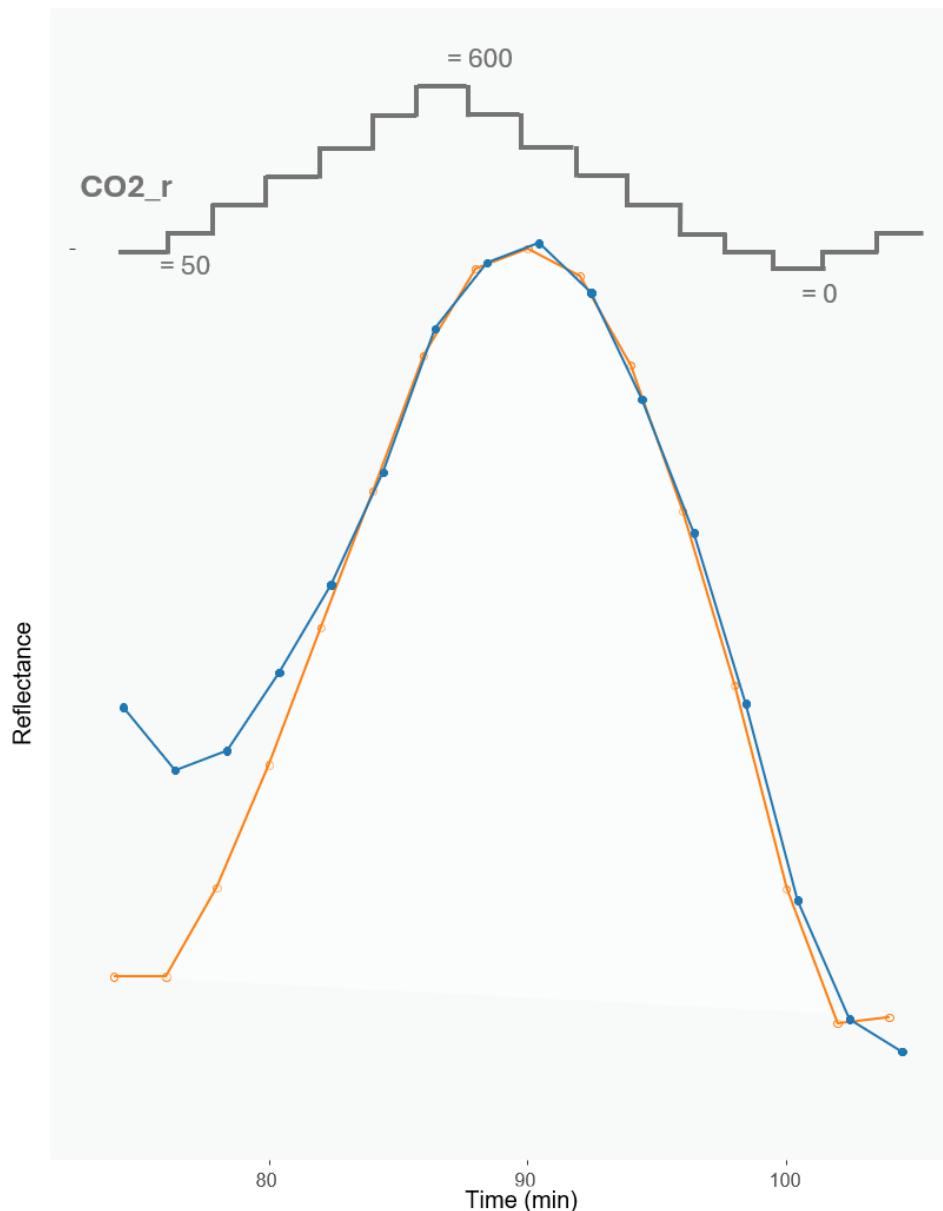


Figure 3.3: Overlay of two reflectance measurements during 0-600-0 CO₂ oscillation, using tape method (orange) and a OCTOPUS with ecoflex body and paper platform (blue), recorded 18 days apart (protocol A.2.6).

However, even the exact same measurement, with the same OCTOPUS, preformed at different dates showed different signal intensities. Signal intensity progressively lowered by up to 90% over the course of four months. In those four months the fiber optic has been carried around, moved and most importantly, slightly bended during use which could have potentially damaged the internal silica fibers, leading to decreased transmission efficiency.

3.3 Application method

Initial tests showed consistently that peak CO₂ concentration (600 ppm) and corresponding reflectance signals do not exactly align during CO₂ oscillations (figure 3.1) [13]. This suggests that the need for gases to diffuse through the dye results in a reaction delay. A long diffusion distance originated from a thick dye layer formed during manual application where the dye is swiped on the application platform with a nail polish brush (Figure 3.4). In such thicker layers, CO₂ can not penetrate completely within the 2 minutes of exposure to each CO₂ concentration, resulting in the colour shift of the deeper layers lagging behind the dye-air interface.

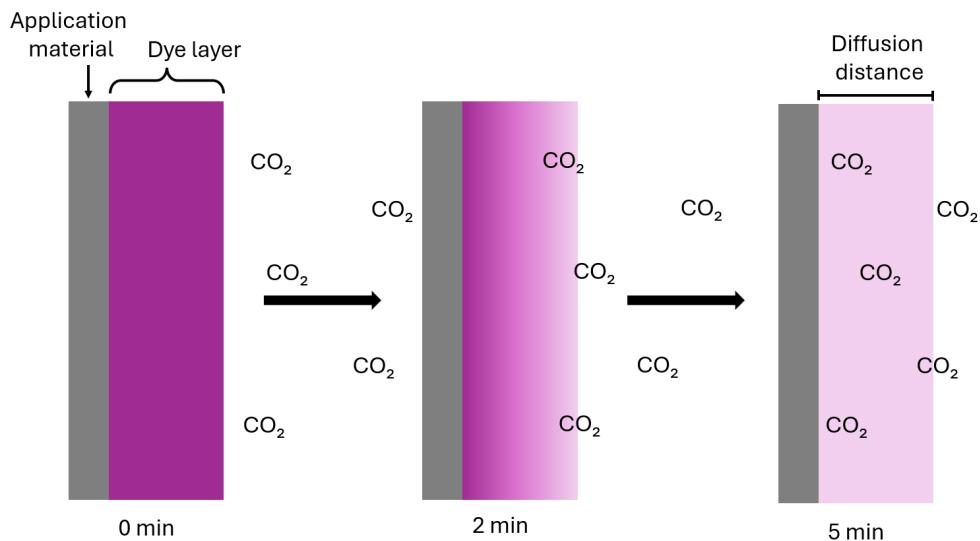


Figure 3.4: Cartoon of gas diffusion through the CO₂-sensitive dye spanning the diffusion distance over time.

When the dye was left to stabilize for an extended period of time (10 min), the reflectance signal aligns perfectly with peak CO₂ concentration (first data point figure 3.1), supporting that the lag originates from diffusion limitations into the dye. Decreasing diffusion distance by applying the dye as a thin layer with a k-bar coater should theoretically remove or minimize the lag. However, the implementation of this strategy did not produce the expected oscillations, instead forming a straight line (fig 3.5). This lack of response regardless of environmental changes suggest that though the dye may be reacting, the current setup is not sensitive enough to measure such small colour changes.

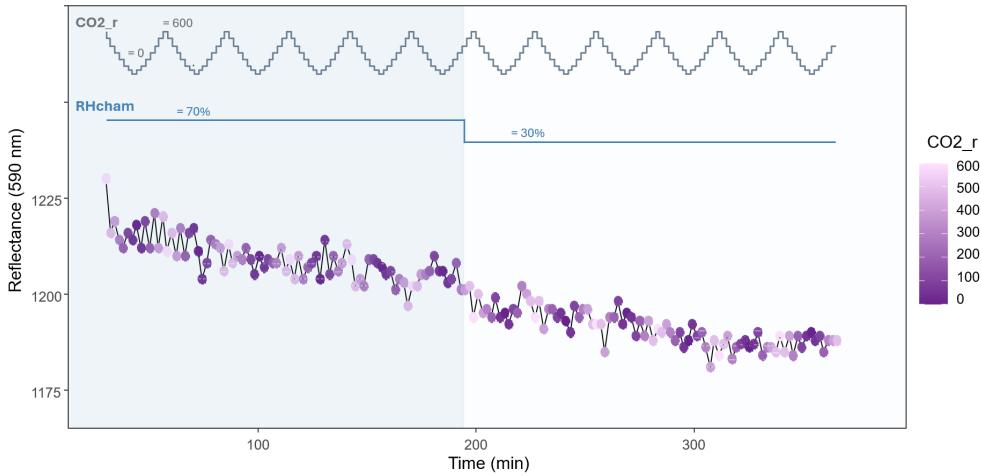
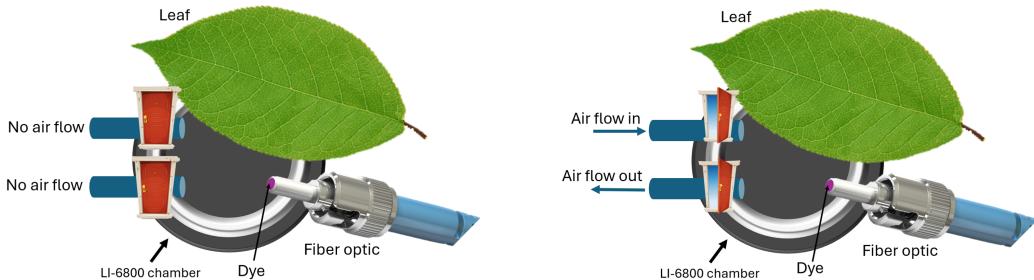


Figure 3.5: CO_2 -sensitive dye, applied with 24 μm wet film thickness, response to oscillating CO_2 concentration and contrasting temperature conditions (15% and 30% RH) (protocol A.2.6).

3.4 Measuring photosynthesis

The response of the CO_2 -sensitive dye to leaf photosynthesis was tested by measuring reflectance (590 nm) in closed and open gas exchange systems. Within a closed chamber there is no outside air flux into the testing chamber containing the dye and a leaf, leading to a progressive CO_2 draw-down as CO_2 is assimilated via photosynthesis (Figure 3.6a) [20]. In an open chamber, CO_2 and water vapor concentrations are kept constant and controlled by the LI-6800 (Figure 3.6b).



(a) Top view of closed chamber system setup containing a leaf (b) Top view of open chamber system setup containing a leaf

Figure 3.6: Top view cartoon of (a) closed and (b) open chamber system setup within the LI-6800 chamber during photosynthesis measurements.

CO_2 -sensitive dye response to leaf photosynthesis was first tested in a closed-chamber system, where CO_2 concentration decreases with photosynthesis. In the LI-6800 chamber the CO_2 -sensitive dye applied on an OCTOPUS and a barley leaf were clamped under continuous light exposure and actively stabilized conditions of CO_2 (200 ppm) concentration, temperature (25°) and humidity (30%) (protocol A.2.1) until a steady state was reached. Upon reaching steady state the air flow in to the chamber was repeatedly stopped creating subsequent closed-chamber environments. Within this transient closed-chamber, deviations in observed reflectance signal of the CO_2 -sensitive dye

measured with the MultispeQ were assumed to be driven by leaf gas exchange. After stopping the air flow into the chamber, reflectance immediately increases until a steady state (plateau) is reached (figure 3.7). This observation is contradictory to expectations. I expected that as CO₂ is assimilated by the leaf, and environmental CO₂ concentration drops, the dye would shift towards a darker purple, resulting in less measured reflectance (Figure 1.3). Instead the rise in reflectance suggests confounding effects.

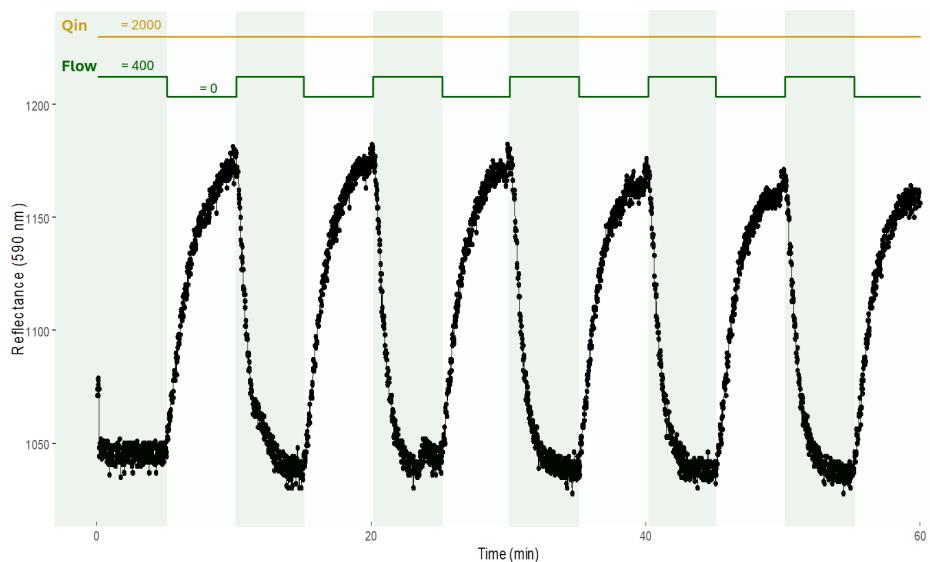


Figure 3.7: Dye reflectance response during consecutive closed-chamber photosynthesis measurements (protocol A.2.1)

This apparent confounding factor can be understood when considering the complete photosynthesis gas exchange process. During photosynthesis, the molar exchange ratio of H₂O transpiration and CO₂ assimilation is around 1000:1 [3]. Water is a key component in dye function as the colour change is driven by the pH changes brought from dissolved CO₂ [14]. The dye colour shift caused by an increase in relative humidity outpaces the CO₂ concentration dependent decrease in reflectance. Relative humidity induced dye colour change was confirmed by oscillating relative humidity under constant CO₂ concentration of 200 ppm (figure 3.8).

To isolate the CO₂ dependent optical change from transpiration noise, relative humidity needs to be stable within the chamber during photosynthesis [20]. To achieve this stability a bean leaf inside a closed-chamber was first allowed to reach a steady state (plateau) under photosynthesis conditions. Upon reaching steady state in order to induce changes in CO₂, that are driven by photosynthetic carbon assimilation, and minimize changes in other external factors, lights inside the chamber were turned on (light column) and off (dark column) every 2 min (figure 3.9) [10]. Light is a necessity for carbon assimilation so with humidity at a plateau the induced dye response should be carbon assimilation [5].

During periods of light an expected decrease in reflectance is observed albeit with low

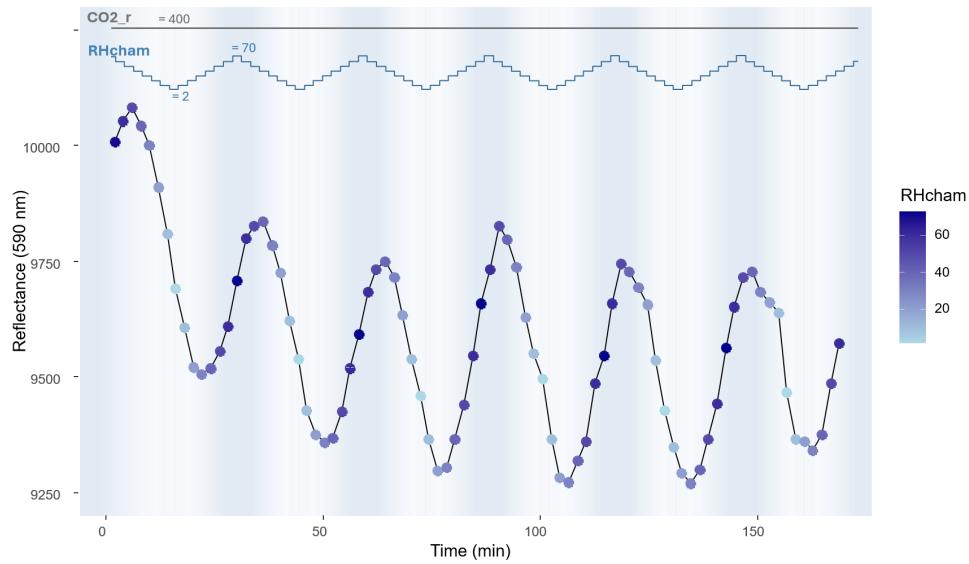


Figure 3.8: Dye reflectance response during oscillating RH of 70-60 under 200 ppm (protocol A.2.4)

amplitude (figure 3.9). While this pattern is consistent with CO₂ uptake the light oscillations required to induce photosynthesis introduce thermal fluctuations (>1°C). By oscillating temperature 1°C the individual contribution of light source radiated heat was isolated (figure 3.10). Though an increase in temperature decreases measured optical signal, on the scale of >1°C this effect is not distinguishable thus negligible making the observed decrease measured carbon assimilation.

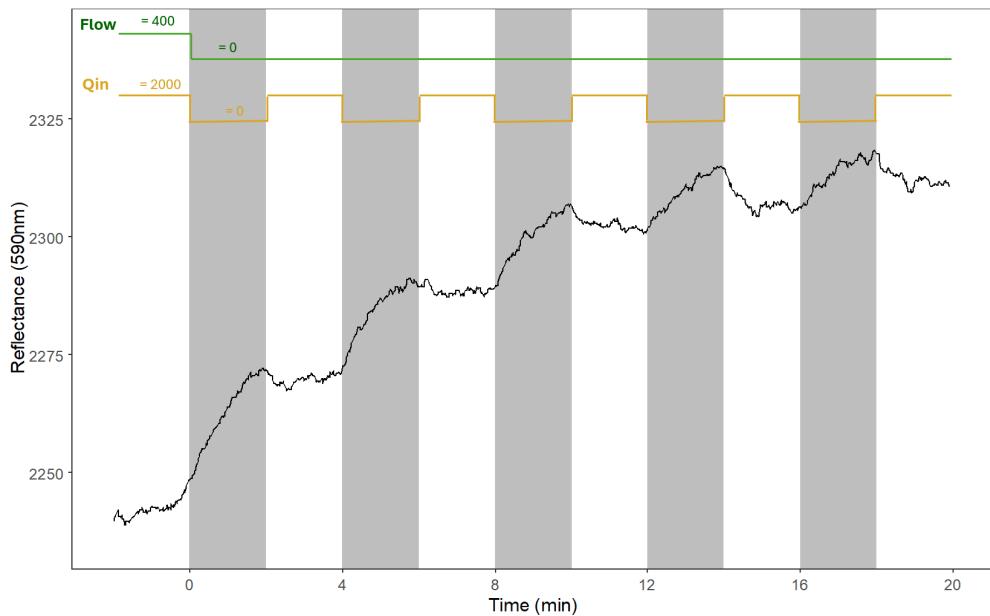


Figure 3.9: CO₂-sensitive dye response during light oscillations isolated from transpiration effects by humidity stabilization (protocol A.2.2). Gray columns indicate light off and white columns light on.

With the plateau reaching closed-chamber approach suspected carbon assimilation was isolated and measured, but using this approach it's not possible to simultaneously measure the other parameters in the chamber leaving a blind spot of potential error

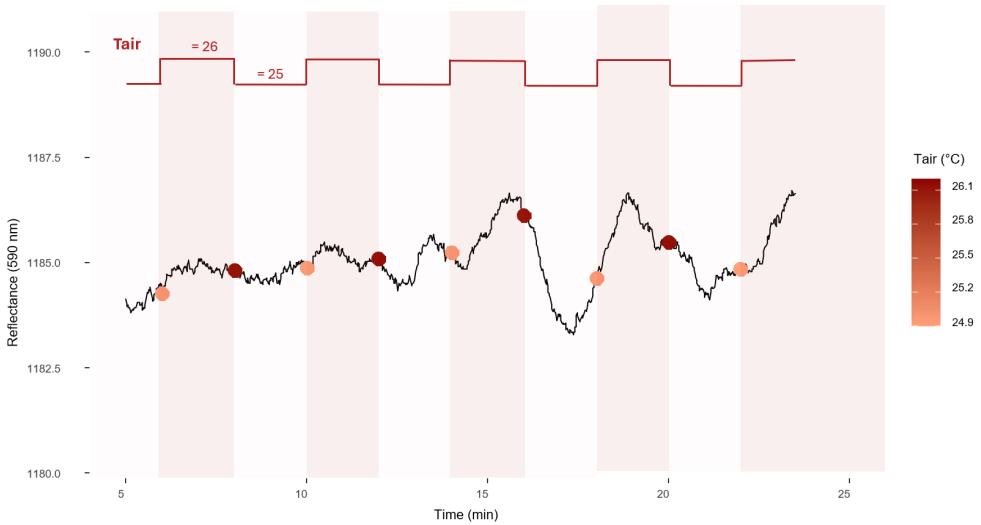


Figure 3.10: Thermal fluctuations induced by 25°-26° temperature oscillations (protocol A.2.5).

and unknown confounding factors. To remove this blind spot there needs to be air flow controlled by the LI-6800 into the chamber. With air flow, parameters are adjusted and recorded continuously, then the LI-6800 data can be cross referenced to dye data forming an alternative open-chamber photosynthesis measuring approach [9]. Within this open-chamber the carbon assimilation effect can be isolated by taking advantage of plant physiology. When a leaf is illuminated, meeting photosynthesis conditions, carbon uptake is rapidly initiated upon light exposure. This rapid initiation contrasts with transpiration that depends on stomatal regulation. Stomatal regulation is much slower to react than carbon assimilation [21]. This suggests that when a leaf is exposed to light oscillations with dark periods (2 min) short enough to allow intermittent carbon uptake but continuous H₂O transpiration, the initial change in reflectance should correlate with the photosynthetic rate of assimilation in the light. This would stabilize the humidity effect and thus uncouple assimilation from transpiration. During periods of illumination (light column) there is no repeated visible change (figure 3.11 a)). However, I know there is a 10 ppm CO₂ concentration change from the LI-6800 data well during short periods of darkness (gray column), photosynthesis lacks energy to assimilate CO₂, and the concentration inside the chamber goes back to 400 ppm (figure 3.11 b)). Notably, even with 2 minutes light oscillations the leaf is able to increase the humidity. The increase in humidity coincides with an increase in CO₂ concentration change from 10 ppm to 15 ppm, suggesting an increase in stomatal conductance over time. The increase in humidity after 15 minutes is not reflected into reflectance data. Neither is the temperature change, brought by light, of 0.5°C as it is within the 1° negligible range (figure 3.10 d)). This suggests the open-chamber approach managed to stabilize humidity and temperature induced colour shifts for 8 minutes, isolating carbon assimilation.

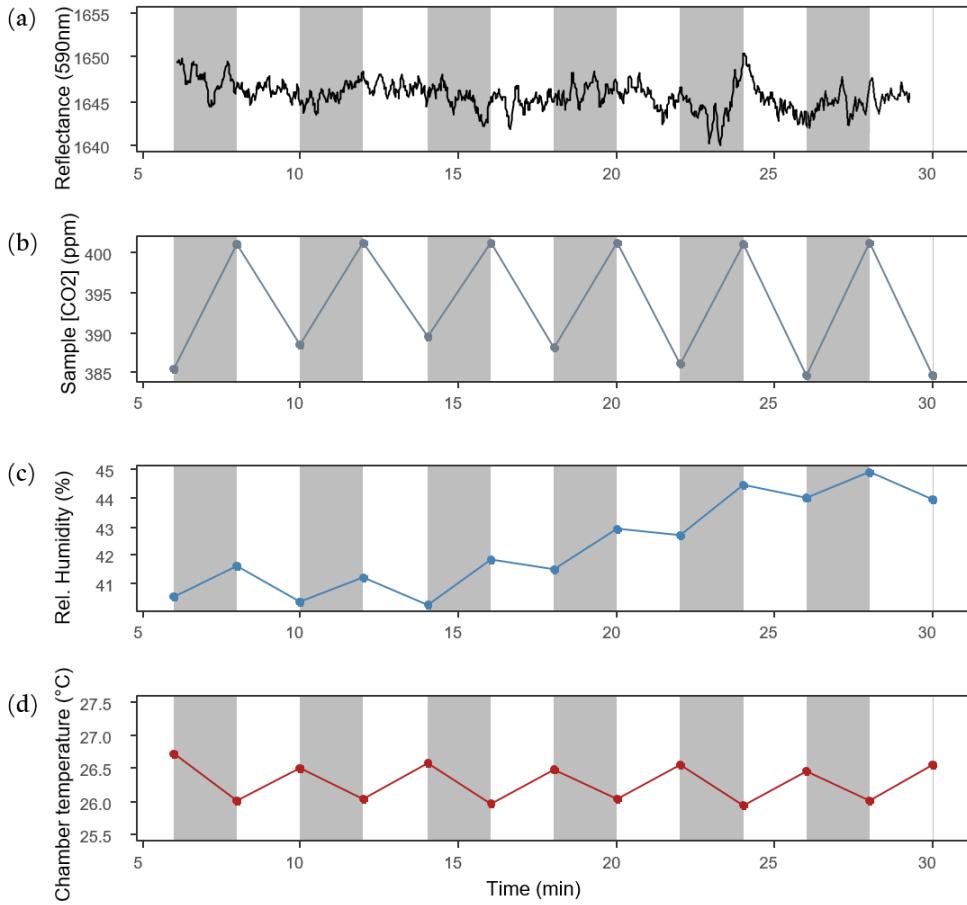


Figure 3.11: (a) CO_2 sensitive dye response, (b) CO_2 concentration, (c) relative humidity and (d) temperature during open-chamber approach to isolating carbon assimilation response (protocol A.2.3).

Despite the different approaches tested both plateau reaching closed-chamber and open-chamber approaches reveal limitations that hinder quantification of dye response into carbon assimilation values with the current setups signal amplitude. Within the plateau reaching closed-chamber approach although a suspected carbon assimilation driven reflectance decrease was measured, the lack of environmental monitoring introduces a blind spot. This brings doubt of the observed decrease being actually carbon assimilation or an unaccounted variable. In contrast, the open-chamber approach does simultaneously control as measure environmental parameters and measure reflectance, but no variable pattern of decrease attributed to carbon assimilation is observed, despite CO_2 concentrations changing. This suggests that the amplitude of carbon assimilation signal is too low, with the current setup, to distinguish from background noise. A consequence of both the increases signal to noise ratio of the open-chamber approach [20], near ambient CO_2 concentration and significant signal loss in the current setup.

4

Conclusion

4.1 Key Findings

This study aimed to validate if the CO₂-sensitive dye can be effectively used to measure photosynthetic carbon assimilation, through experiments targeted to work with the natural physiological processes of a leaf.

The results showed that the CO₂-sensitive dye's response profile to CO₂ fluctuations remained stable and reversible despite varying environmental conditions. Nonetheless, confounding environmental effects need to be addressed to avoid inaccurate quantification. Conditions as lower humidity and higher temperatures showed an increase in dye's signal amplitude, enhancing sensitivity to changes in CO₂ concentration. However, the dye approaches saturation at ambient CO₂ concentration, limiting its ability to distinguish small fluctuations at higher concentrations (>500 ppm).

Though direct comparison is limited by fiber optic degradation, response patterns stayed consistent across OCTOPUSs when different dye application materials were tested. Making the OCTOPUS a key development enabling standardized and repeatable dye evaluation. Still, the fiber optic and thus OCTOPUS approach suffered significant signal loss, suggesting that they may need to be scraped in future setups.

The lag between peak CO₂ concentration and peak response curve, originating from diffusion limitations highlight the importance of dye layer thickness. This is a crucial factor effecting the dyes temporal accuracy, and was found to be removed by increasing the dyes stabilization time. Even with optimized timing the current setup is not sensitive enough to with certainty measure carbon assimilation. While carbon assimilation can not be quantified with this setup, the experiments suggest the open-chamber system approach would work. The open-chamber system, when paired with an improved dye and reduced optical loss, is a promising option for quantification of dye response into carbon assimilation values. While quantification is within reach, widespread adoption into the broader photosynthetic research community would require the dye to be developed into an inexpensive and rapid CO₂ sensor.

5

Recommendations

5.1 Future Direction

The findings of this study highlight key opportunities and challenges within the utilization of CO₂-sensitive dye for carbon assimilation. As the dye successfully traced potential carbon assimilation brought during periods of light with the plateau reaching closed-chamber approach, the dye is also saturated around ambient CO₂ concentration (400 ppm) and measurements experience significant signal loss due to the fiber optic. For both of these challenges, there is a direct path for further potential using available tools.

Replacing o-cresolphthalein, a new thymol blue based CO₂ sensitive dye with an approximately 12 times lower sensitivity has recently become available. With this lower sensitivity the dye will not reach saturation at ambient air CO₂ concentrations (400 ppm).

Signal loss on the other hand requires setup adaptations. I recommend removing the fiber optic from the setup entirely and measuring transmittance instead of reflectance decreasing signal loss. This can be achieved by placing the detector and light source, adjacent to each other inside of the LI-6800 chamber. The LI-6800 chamber is too small to contain the MultispeQ but recent work at Jan Ingenhouze Institute suggest the potential development of a sensor containing both a light source and spectrophotometer small enough to be placed inside a leaf chamber. This sensor is originally designed to remotely measure photosynthesis over extended periods of time. Considering their small size, two of them can be placed inside the LI-6800 chamber flanking the dye from either side while environmental parameters are controlled, reusing the already established LI-6800 programs (Figure 5.1).

Within this potential setup the LED light can be changed to an optimal wavelength further reducing signal loss. The solution of thin layer application to decrease diffusion distance should be retested using the small sensor setup and the alternative dye applied on transparent material. Lastly, considering environmental conditions as hu-

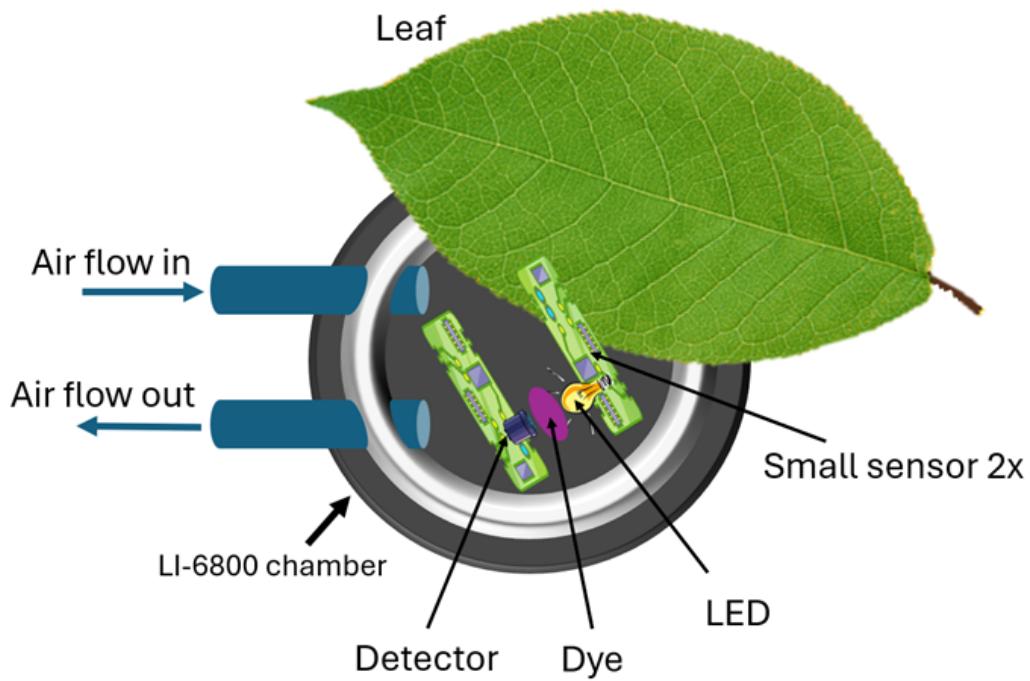


Figure 5.1: Cartoon of recommended setup within LI-6800 chamber for transmittance measurements using two small sensors with a detector and a light source flanking the CO₂-sensitive dye.

midity and temperature, consistently showed repeatable and reversible response to CO₂ concentration they can be corrected for using calculations based on known environmental effects.

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Appendices

A

Showcasing the First Appendix

All mentioned files are available in the GitHub Repository: github.com/monsterkiek/Thesis-CO2-sensitive-dye-2025

A.1 Measurement Startup

A.1.1 Measurement Startup li-6800

Stepwise walkthrough of li-6800 startup. This procedure was followed for all measurements:

- Turn on li-6800
- Change CO₂ cartridge and refill water, silica gel and soda lime if necessary.
- Close chamber and start warm up test (takes about ~30 min)
- Open new log file and give custom name
- Load configuration 'Configuration'
- Start background program 'BackgroundProgram.py' (only for overnight runs)
- Load the appropriate program in repeated_responses, depending on the intended environmental control and measurement protocol.
- Check for fails in warm up test, continue with measurement only if no fails detected
- Insert fiber optic tip with OCTOPUS/dye in the li-6800 chamber
- Check if chamber leak is <25% before proceeding
- Click auto match
- Start the selected program

File Locations in Repository:
Code/li-6800/Configuration - Sets all li-6800 settings and parameter setting to a default, changes to the default are explicitly controlled with the program Code/li-6800/BackgroundProgram.py - Ensures automatic log closing and device shutdown after a set duration if measurement needs to run overnight.
Code/li-6800/Programs - Load based on experimental design

A.1.2 Measurement Startup MultispeQ

Stepwise walkthrough of MultispeQ startup. This procedure was followed for all measurements:

- Open 'protocol_590nm-Kiek.py' (I used visual studio code)
- Fill in how many data points are needed "measurements" (same as in li-6800 to allow data matching)
- Fill in "pulse_distance" and "measurements_delay" for time between measurements (Note: the licors 2 minute measurement is actually 120500 ms so fill in 120500 ms for synchronization)
- Save changes
- Open 'AM - CO2 dye 5.ipynb' in a second tab
- Plug in MultispeQ, with connected fiber optic, in a USB port and ensure the corresponding port is recognized by filling in '_connection = _device.connect('COM11')'
- Create a file path where you collected data will be posted
- Fill in the same custom file name as given to the corresponding li-6800 file: "YOUR_FILENAME.csv"
- Save changes
- Click restart
- Click Run all 1 min 45 sec after li-6800 started

File Locations in Repository:

Code/MultispeQ/protocol_590nm-Kiek.py – Main MultispeQ measurement protocol
 Code/MultispeQ/AM - CO2 dye 5.ipynb – Measurement execution and file saving

A.2 Experimental Protocols

The following subsections contain the complete information about the experiments. Each experiment covers all relevant information about dye application and material selection as well as the li-6800 program followed (Github 'Code/li-6800/Programs') and the ensuing data analysis in the repository. Except for changing programs the start up process described in A.1 was followed.

All gathered raw data can be found in Github 'RawData' and the processed files used for Data analysis in Github 'DataAnalysis'. The R Studio code used to analyze the processed data files is in Github 'Code/R studio'. The file 'Code/R studio/All li6800-MultispeQ analysed data(R studio).R' contains the initial analyzation of all collected data including what did not make it into the report. Further analysis of selected graphs is located in individual files that will be referenced to in the subsection covering that experiment.

A.2.1 Closed chamber leaf test

During the closed chamber photosynthesis experiment conducted with a barley plant (*Hondeum Vulgare*) on 2025-05-22, a PDMS octopus with paper as background (OCT-PDMSPaper; 2.1) was used and the 'CCS flow' li-6800 program was loaded. Data analyses of the experiment can be found in 'CCS photosynthsis (R studio)'.

Full file name: 2025-05-22-1130_ICOS_flowAndFan+leafTest_purpleDye_licor+PDMS+paper+octopus+multispec

File Locations in Repository:

Code/R studio/CCS photosynthsis (R studio).R - Data analysis

Code/li-6800/Programs/CCS flow.xlsx - li-6800 program

A.2.2 Plateau reaching closed chamber leaf test

During the plateau reaching closed chamber photosynthesis experiment conducted with a bean plant (*Phaseolus Vulgaris*) on 2025-05-29 a PDMS octopus with ABS as background (OCT-PDMS.ABS; 2.1) was used and the 'PCCS phothosynthsis' li-6800 program was loaded. Data analyses of the experiment can be found in 'PCCS+OCS photosynthsis (R studio)'.

Full file name: 2025-05-29-1333_ICOS_leafTest+plateau+lightOscillations_purpleDye_licor+PDMS+paper+octopus+multispec

File Locations in Repository:

Code/R studio/PCCS+OCS photosynthsis (R studio).R - Data analysis

Code/li-6800/Programs/PCCS phothosynthsis.xlsx - li-6800 program

A.2.3 Open chamber leaf test

During the open chamber photosynthesis experiment conducted with a bean plant (*Phaseolus Vulgaris*) on 2025-06-02 a PDMS octopus with ABS as background (OCT-PDMS.ABS; 2.1) was used and the 'OCS photosynthesis' li-6800 program was loaded. Data analyses of the experiment can be found in 'PCCS+OCS photosynthsis (R studio)'.

Full file name: 2025-06-02-1447_ICOS_leafTest+lightOscillations2min+outputRHline_purpleDye_licor+PDMS+paper+octopus+multispec

File Locations in Repository:

Code/R studio/PCCS+OCS photosynthsis (R studio).R - Data analysis

Code/li-6800/Programs/OCS photosynthesis.xlsx - li-6800 program

A.2.4 Relative humidity oscillations test

During the relative humidity oscillations experiment conducted on 2025-05-08 the dye was applied directly on the fiber optic and covered with markers tape as reflective background (TapeOnly; 2.1). The 'RH oscillations' li-6800 program was loaded and data analyses of the experiment can be found in 'RH oscillations (R studio)'.

Full file name: 2025-05-08-1707_ICOS_RHoscillations_licor+tape+directApply+multispec

File Locations in Repository:

Code/R studio/RH oscillations (R studio).R - Data analysis

Code/li-6800/Programs/RH oscillations.xlsx - li-6800 program

A.2.5 1 degree temperature oscillations test

During the 1 degree difference temperature oscillation experiment conducted on 2025-06-06 a PDMS octopus with ABS as background (OCT-PDMS.ABS; 2.1) was used and the '25-26 temp oscillations' li-6800 program was loaded. Data analyses of the experiment can be found in '25-26 oscillations (R studio)'.

Full file name: 2025-06-06-1305_ICOS_tempOscillations25-26_purpleDye_licor+PDMS+paper+octopus+multispec

File Locations in Repository:

Code/R studio/25-26 oscillations (R studio).R - Data analysis

Code/li-6800/Programs/25-26 temp oscillations.xlsx - li-6800 program

A.2.6 Relative humidity sensitivity

During the relative humidity sensitivity experiment conducted on 2025-04-24 the dye was applied directly on the fiber optic and covered with markers tape as reflective background (TapeOnly; 2.1). The 'SS_600-0-500_Cycle_30-70RH' li-6800 program was loaded and data analyses of the experiment can be found in 'SS_600-0-500_Cycle_70-30RH+15-30Temp (R studio)'.

Full file name: 2025-04-24-1437_ICOS_CO2cycle_basetest_licor+tape+directApply+multispec

File Locations in Repository:

Code/R studio/SS_600-0-500_Cycle_70-30RH+15-30Temp (R studio).R - Data analysis

Code/li-6800/Programs/SS_600-0-500_Cycle_30-70RH.xlsx - li-6800 program

The same program was followed for different materials and OCTOPUS's functioning as a comparative standard protocol. Two of these were compared further by overlapping their response curve. Data analysis of the comparison can be found in the repository; Code/R studio/Overlaying graphs (R studio).R

A.2.7 Temperature sensitivity

During the temperature sensitivity experiment conducted on 2025-05-06 the dye was applied directly on the fiber optic and covered with markers tape as reflective background (TapeOnly; 2.1). The 'SS_600-0-500_Cycle_30-70RH' li-6800 program was loaded and data analyses of the experiment can be found in 'SS_600-0-500_Cycle_70-30RH+15-30Temp (R studio)'.

Full file name: 2025-05-06-1646_ICOS_tempChange+Oscillations_licor+tape+directApply+multispec

File Locations in Repository:

Code/R studio/SS_600-0-500_Cycle_70-30RH+15-30Temp (R studio).R - Data analysis
Code/li-6800/Programs/SS_600-0-500_Cycle_30-70RH.xlsx - li-6800 program

