APPLICATION NOTE

# Modification of LI-6400/LI-6400XT to Control at Low [$CO_2$]

The LI-6400/LI-6400XT can be modified to control [$CO_2$] down to 0 $\mu mol_{CO_2}$ $mol^{-1}_{air}$. However, with the low-$CO_2$ modification, the LI-6400/LI-6400XT cannot simultaneously control [$CO_2$] and automatically adjust the flow rate to maintain [$H_2O$]. As always, LI-COR Biosciences' staff is available to assist with any technical questions about the instrument.

## Introduction

As plant scientists work to improve crop productivity to meet global demands for food and fuel, greater focus is being placed on improving or transferring the $CO_2$-concentrating mechanism in $C_4$ plants. The $CO_2$-concentrating mechanism in $C_4$ plants increases efficiency at low $CO_2$ concentration ([$CO_2$]) by increasing [$CO_2$] in the bundle sheath cells, which competitively blocks the oxygenation reactions. The greater efficiency of the $C_4$ pathway is evident in the lower intercellular $CO_2$ ($C_i$) that corresponds to a net positive carbon assimilation rate or $CO_2$-compensation point ($\Gamma$). Typically in $C_4$ plants, $\Gamma$ is $\leq$ 10 $\mu mol_{CO_2}$ $mol_{air}^{-1}$, whereas $\Gamma$ in $C_3$ plants ranges between 40 and 50 $\mu mol_{CO_2}$ $mol_{air}^{-1}$ (Lambers et al., 2006, von Caemmerer, 2000).

Empirical gas-exchange measurements of the assimilation rate at very low $C_i$ can be accomplished either by decreasing the [$CO_2$] outside the leaf ($C_a$) to low levels or by inducing low stomatal conductance, thereby decreasing $CO_2$ diffusion into the leaf. The first method is preferable since it will achieve low $C_i$ while leaf chamber conditions can be optimized for high stomatal conductance to decrease the variance in measured gas exchange. In differential gas-exchange systems such as the LI-6400/LI-6400XT, the difference between the air entering and exiting the chamber is used to calculate the net change in the gas concentration, which is then used to calculate the net flux (see LI-COR, 2008). Leaf stomatal aperture is regulated to control the water loss from the leaf and consequently restricts $CO_2$ diffusion into the leaf. The stomatal conductance to water ($g_s$) is used to calculate $C_i$ (see LI-COR, 2008). As $g_s$ approaches 0, the uncertainty in the calculated $C_i$ increases; conversely, higher $g_s$ decreases the uncertainty in the calculated $C_i$. Therefore, high $g_s$ and low $C_a$ are important to be able to make gas exchange measurements at low $C_i$ values.

While there is some variation between systems, a typical LI-6400/LI-6400XT equipped with the 6400-01 $CO_2$ mixer assembly can control [$CO_2$] down to between 30 and 50 $\mu mol_{CO_2}$ $mol_{air}^{-1}$. With the $CO_2$ mixer turned off, a [$CO_2$] of 0 $\mu mol_{CO_2}$ $mol_{air}^{-1}$ can be achieved. If [$CO_2$] between 0 and 50 $\mu mol_{CO_2}$ $mol_{air}^{-1}$ are needed, the LI-6400/LI-6400XT console flow path can be modified by the user to accomplish this and easily returned to normal factory flow path later if desired (Figure 1a). The altered flow path places the $CO_2$ mixer on the positive pressure side of the pump (Figure 1b), forcing the injector pressure to work against the system pressure, effectively decreasing the flow of pure $CO_2$ from the mixer and thereby allowing lower [$CO_2$] control points.

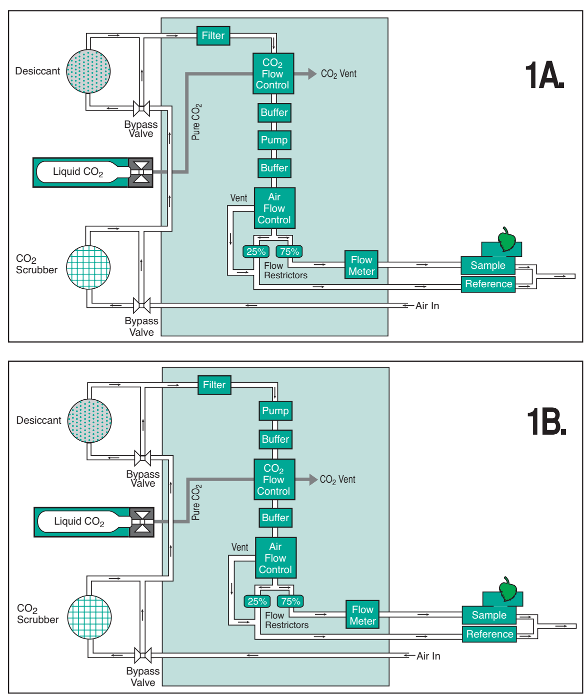


***Figure 1.*** *Simplified flow schematic of LI-6400/LI-6400XT in either the a)standard factory configuration, or b) modified low-[$CO_2$] control configuration.*

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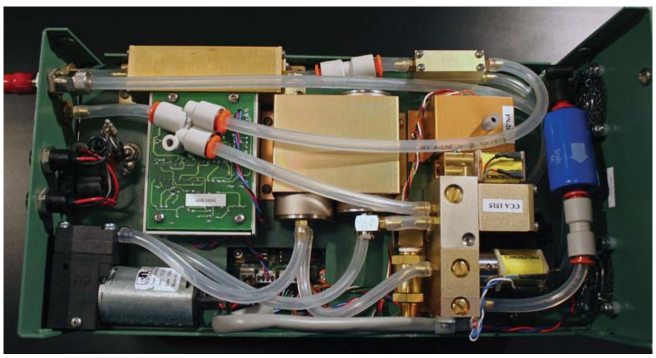
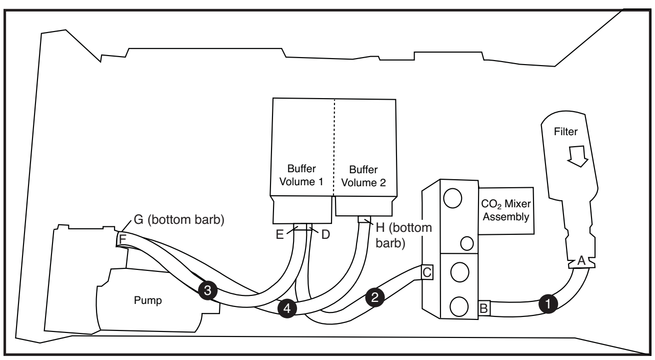
## Flow modification to LI-6400/LI-6400XT

Modification of a LI-6400/LI-6400XT equipped with a $CO_2$ mixer to control at low [$CO_2$] is easily done with minimal tools and equipment. The modifications should take two hours or less to complete, and can easily be reversed to return an instrument back to the original configuration. The modification involves removing the existing Bev-a-line® tubing from the air filter, $CO_2$ mixer, first buffer volume and pump; the rest of the flow pathway remains unaltered. LI-COR staff will be able to assist with any questions regarding the modifications.

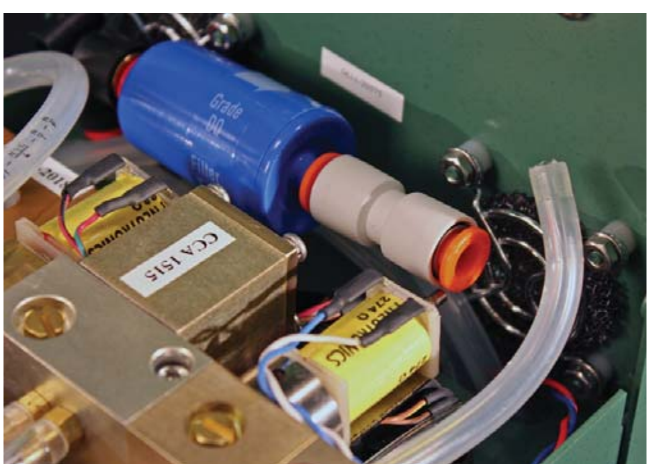
1. With the unit powered off and disconnected from mains and/or battery power, remove the console shell and turn the console upside down. Instrument is shown in standard factory configuration and the color of components may vary.



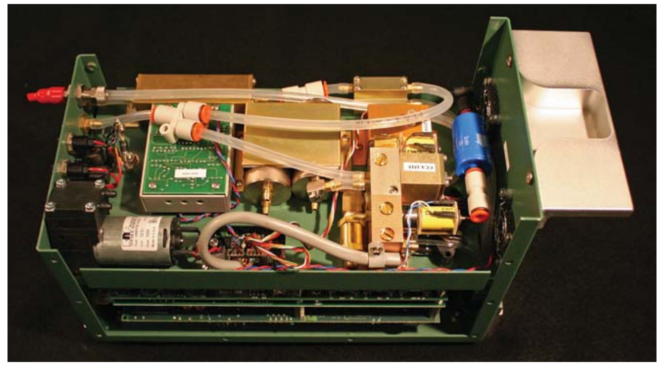



*LI-6400/LI-6400XT component identification.*

2. Remove tubing ❶ from the quick connect fitting (A) on the outlet end of the filter.



3. Remove ❶, ❷, ❸, ❹ tubing from the inlet (B) and outlet (C) of the $CO_2$ mixer, the inlet (D) and outlet (E) of buffer volume 1, and inlet (H) of buffer volume 2.



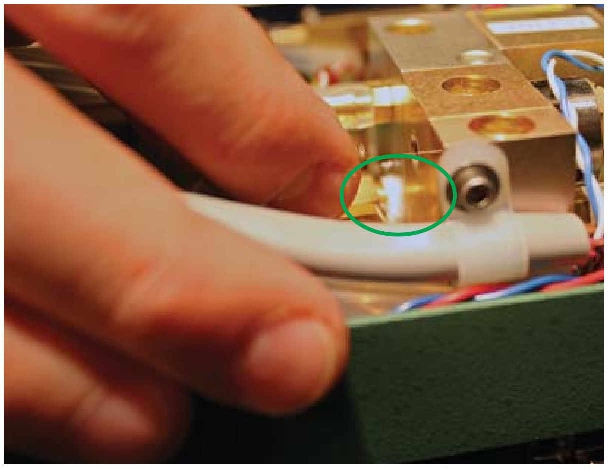
   a. The hose barbs are plastic (on the pump) or brass (on the rest of the components) and can be easily damaged by any hand tool or cutting device. If a hose barb is damaged during tube removal, it must be replaced. Even very small scores in the fitting surface are channels that will allow bulk air leaks, which will impact the stability of the $CO_2$ and $H_2O$ control. Additionally, bulk leaks will cause a significant offset in the IRGA zeros, impacting all subsequent measurements of $CO_2$ and/or $H_2O$.

   b. The tubing can be removed by grasping it on top of the barb with a pair of side-cut pliers and stretching the tube.

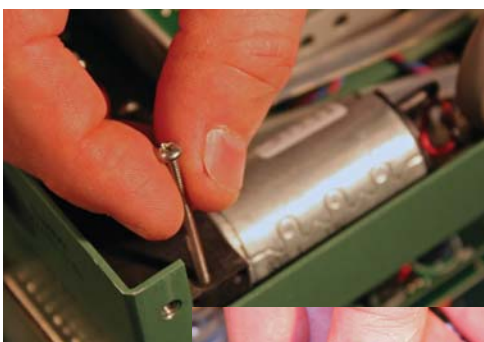
   The tubing can also be cut at an oblique angle to the tube, which will make the tube wall thinner and therefore more easily removed without damaging the barb.

   c. If damaged, the hose barbs can be removed easily with a 1/4-inch or adjustable wrench by turning the barb counter-clockwise. New hose barbs can be installed by turning clockwise to tighten. Be sure that there is a black rubber fitting on the hose barb and that the fitting is free of cracks and debris.

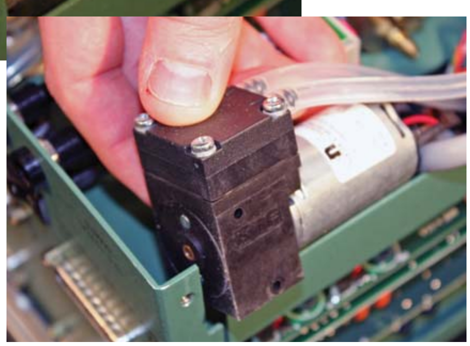
d. ***If you are removing the hose barb on the outlet of the $CO_2$ mixer assembly, be very careful of the very small GLASS tube (below) that runs inside this hose barb.*** This glass tube can be sheared off if the hose barb is taken off or installed at an angle. Broken glass tubes will require a return for factory repair.



4. Remove the screw to make accessing the tubing easier by repositioning the pump (see below). Remove tubing from the inlet (F) and outlet (G) of the pump, being careful of the plastic hose barbs.



*Remove the screw securing the pump...*



*and lift the pump out of the shell to access the hose barbs.*

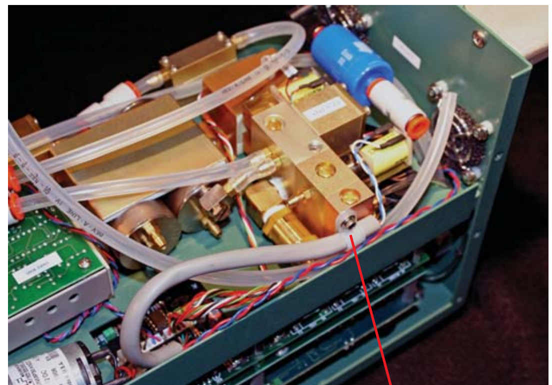
5. From a new supply of Bev-a-line tubing (LI-COR P/N 8150-250), cut four lengths of tubing: ① = 30 cm; ②= 13 cm; ③ = 34.5 cm; and ④ = 11.5 cm. It is important to use Bev-a-Line or similarly diffusion-resistant tubing to limit diffusion leaks.

   a. When cutting the tube, ensure the cut is at right angles and the tubing is not crushed or deformed (see examples of damaged tubes below).

*Make sure tubing is not crushed...*

*or deformed in any way.*

6. Route ① under the wire clamp on the side of the $CO_2$ mixer and connect to the quick connect coupler (A).

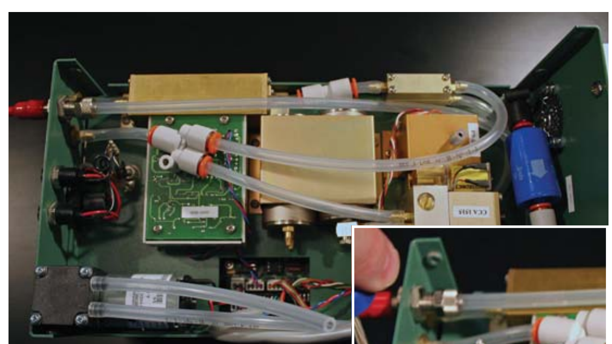


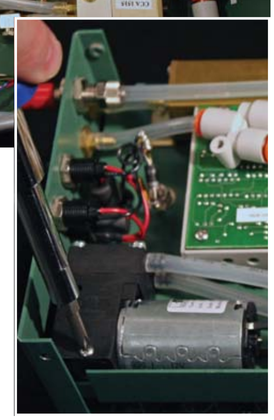
*Route hose ① under this wire clamp.*

   a. Lightly lubricate with silicone grease (p/n 210-1958-1) or teflon grease (p/n 210-05774) the outside of the tubing to ensure the o-ring inside the quick connector seats and seals around the tubing.

7. Connect other end of ① to the inlet side (F) of the pump. The pump body has arrows that indicate the direction of the flow.

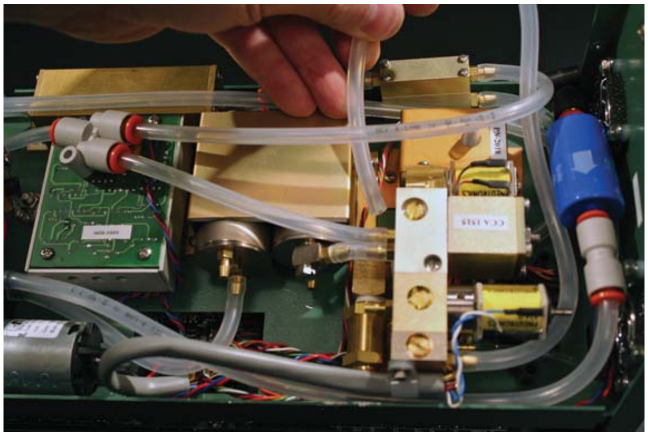
8. Connect ② to the outlet (G) of the pump and then reinstall the pump with the screw.





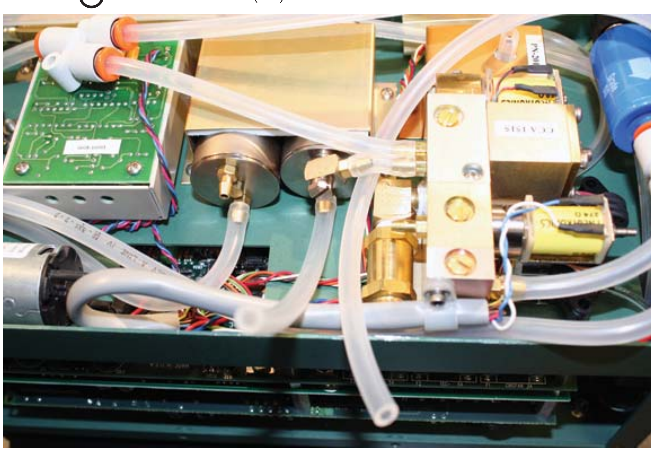
9. Connect the other end of ② to the inlet hose barb (D) on buffer volume 1.

10. Connect ③ to inlet (B) of the $CO_2$ mixer. Gently form tubing ③ into a loop to route the tubing under the hose from outlet of buffer volume 2.

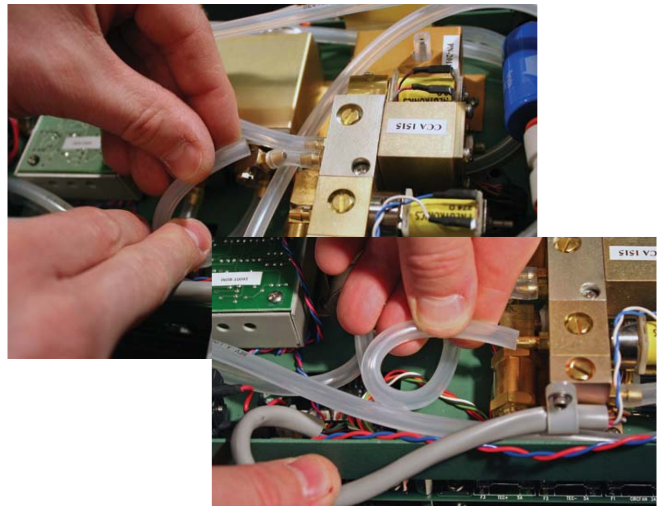


   a. The tubing should only be bent into curves such that the internal volume is not decreased. If the tubing is bent too severely (below), the tubing wall will crease, causing a diffusion leak through the damaged area.

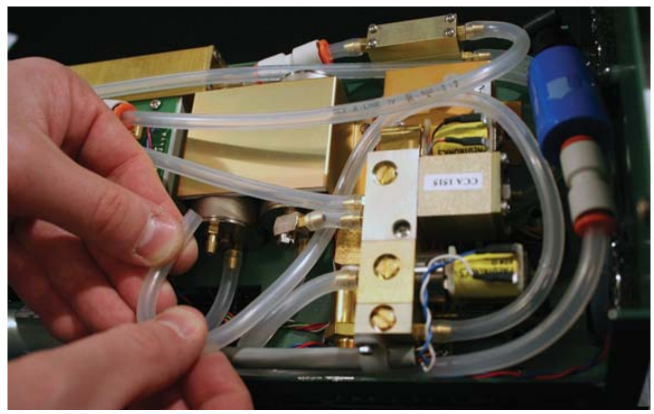
11. Install ④ on the inlet (H) of buffer volume 2.



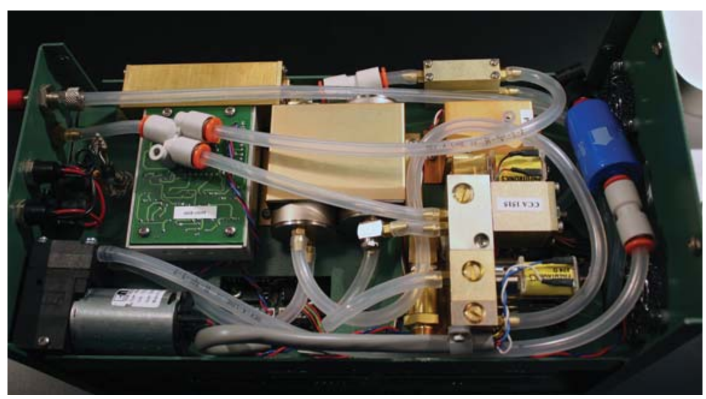
12. Gently form a loop in tubing ④ (clockwise corkscrew) and connect to the $CO_2$ mixer outlet (C).

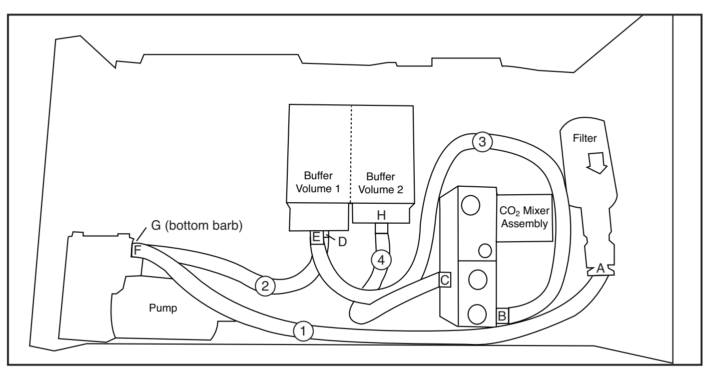


13. Gently form tubing ③ into an "S" to route the tubing to outlet hose barb (E) on buffer volume 1.



When routing the tubing for the connections, position it to avoid crushing by the console shell. Summary of Connections (barb-tubing-barb): A-①-F; G-②-D; E-③-B; and C-④-H.





Once the flow modification is complete, the system should be tested for leaks. Right the console and connect the power. **Care should be taken not to touch any of the electrical components to prevent injury to the user or any damage to the instrument from a short.** Fill the $CO_2$-scrub tube with fresh soda lime and place on full scrub. Bypass the desiccant. Connect the sensor head cables and tubing and power-on the instrument. In New Measurements (**F4**), monitor the [$CO_2$] in the sample and reference cells. Once the [$CO_2$] is stable, blow under the console to increase the ambient [$CO_2$] around the flow-path components. If the [$CO_2$] increases in both the reference and sample, the flow path has a leak. Blow through a tube/straw around the different components to isolate the leak. Any leaks should be repaired before using the instrument for measurements.

## Reverting back to standard $CO_2$ control

To return the instrument to the standard, factory flow-path, remove tubing from connections A - H. From a new supply of Bev-a-line tubing (LI-COR PN 8150-250), cut four lengths of tubing: ❶ = 7.4 cm; ❷ = 11.5 cm; ❸ = 12.7 cm; and ❹ =16.5 cm. Connect them in the following sequence (barb-tubing-barb): A-❶-B; C-❷-D; E-❸-F; and G-❹-H. Undo the edits to the $CO_2$ Mixer Calibrate program (see next section).

## Configuration and calibration for modified $CO_2$ control

With the modification of the flow path, the response of the 6400-01 $CO_2$ mixer assembly to the control voltage signal will change. The LI-6400/LI-6400XT relies upon a calibration curve for determining the control voltage to achieve an initial set-point for a given requested [$CO_2$] for either $CO_2R$ or $CO_2S$ control. Within the Calibration Menu (**F3**), $CO_2$ Mixer Calibrate generates a calibration for the $CO_2$ mixer. For instruments running OPEN v6.1.4 and below, the system code for the automated process needs to be modified to include additional set points at low [$CO_2$]. The file can be modified either via "Access the Filer" in the Utilities Menu (**F5**) or via a PC connection using LI6400Finder (networked instruments only).

1. Access the Filer
2. Press **D** to change the directory to /sys/open

```
=====Select a Directory=====
 ▶ ├Closed
 ▶ ├Factory
 ▶ ├Lib
 ▶ ├Open
 ▶ └Utility
+ ▼/▶ ◆ Add ◆ Purge ◆ CANCEL◆ SELECT
```

3. Using the arrow keys, highlight the file Open.mxr.

```
/Sys/Open          Open.lp
Filter=            Open.mch
Total: 49          Open.msr
Bytes: 347053      Open.mxr
Tagged: 0          Open.soil
Bytes: 0           Open.spc
                              ← →
1 Dir ◆ filter◆ Sort ◆ Tag ◆ purGe
```

Press **E** for edit.

```
=====Open.mxr=====
/*
 OPEN.MXR

 CO2 mixer support

 950410 - version 2
 960812 - added power on to 'C'
```

4. Press **end** and then **pgup** 23 times to locate the section of the file to be edited.

5. Change:

   a. PUB mixerSignalOrig[8] to PUB mixerSignalOrig[10]

```
=====Open.mxr=====          5233=
/* ---------- External ------------- */
:STATIC 0
:DOUBLE
 PUB mixerSignalOrig[10] { 5000 3000 150
    PUB mixerSignal[10] {  }
    PUB mixerPPM[10]          { 2400 1100
```

   b. PUB mixerPPM[8] to PUB mixerPPM[10]
   c. Add 50 and 0 to the end of PUB mixerSignalOrig to get PUBmixerSignalOrig[10]{ 5000 3000 1500 1000 500 300 200 100 50 0}
   d. Add 15 and 0 to the end of PUB mixerPPM[10] to get PUB mixerPPM[10] { 2400 1100 450 270 120 70 50 30 15 0}

```
=====Open.mxr=====          5334=
------ */


0 3000 1500 1000 500 300 200 100 50 0}

400 1100  450  270 120  70  50  30 15 0}
```

6. Press **Escape,** then **U** for update, and **Q** for quit.

```
=====Exit Menu=====          5334=
----| <esc> - Resume editing
    | U - Update file Open.mxr
    | S - Store as new file
0 30| Q - Quit editor          50 0}
    | X - eXecute
400 |                       30 15 0}
```

7. Restart instrument by powering off and then on.

The file will appear as in Appendix 1; much of the file has been omitted to save space.

## Nuances and limitations of the modification

### $CO_2$ mixer setpoints altered

The flow path modification imposes some limitations to the LI-6400/LI-6400XT systems that must be considered when making measurements. Placing the $CO_2$ mixer on the positive pressure side of the pump forces injection of $CO_2$ against the pump pressure decreasing [$CO_2$] at all settings by about 10% (Figure 2). For most LI-6400/LI-6400XT instruments, the maximum attainable [$CO_2$] typically will be > 2000 $\mu mol_{CO_2}\ mol_{air}^{-1}$.

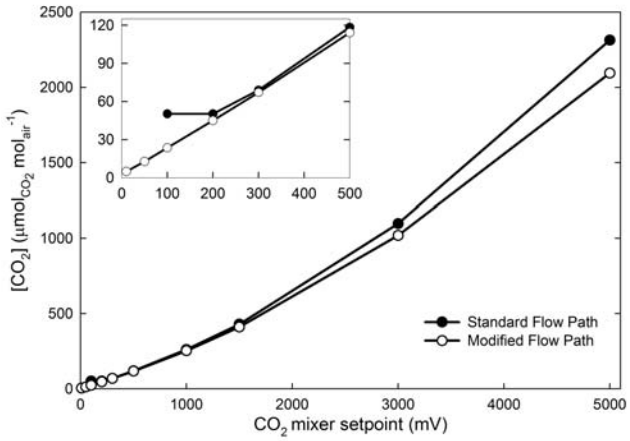



*Figure 2. Calibration curves for the 6400-01 $CO_2$ mixer configured in different system flow paths. The attained $CO_2$ concentrations plotted against the control voltage. The inset examines voltages < 500 mV; note the increased low-end range of the modifications.*

The response time of the $CO_2$ mixer increases slightly (Table 1), but is still rapid enough for survey-style measurements to be taken in approximately 90 - 120 seconds. The adjustment times from a higher concentration to a lower concentration are the same (data not shown) because they are controlled by system flow rate and not the $CO_2$ mixer. Even at the lower $CO_2$-mixer set points, the moment-to-moment noise is similar to that in the standard flow path. Across the range of $CO_2$ set points, the [$CO_2$] variance resulting from $CO_2$ mixer is comparable for the standard and modified flow paths (Table 1).

*Table 1: Comparison of performance parameters for $CO_2$ mixer configured with different system flow paths as illustrated in Figure 1. Values are for a single representative LI-6400XT.*

| Response metric for $CO_2$ mixer | Standard flow path | Modified flow path |
|---|---|---|
| Minimum $CO_2$ set point (ppm) | 50.6 | 1-2 |
| Maximum $CO_2$ achieved (ppm) | 2328.3 | 2090.8 |
| Noise (60s Std.Dev.) at: Minimum (ppm) | 0.044 | 0.017 |
| 100 (ppm) | 0.053 | 0.080 |
| 400 (ppm) | 0.11 | 0.11 |
| Maximum (ppm) | 0.11 | 0.12 |
| Time to maximum from turned off (not initial pressurizing) (seconds) | 26 | 46 |
| Step time: | | |
| Minimum to Maximum (seconds) | 103 | 132 |
| Minimum to 100 ppm (seconds) | 17 | 52 |
| 100 ppm to 400 ppm (seconds) | 30 | 35 |

**Simultaneous flow and [$CO_2$] control**

The modified system flow does have one major limitation; it hampers the system's ability to simultaneously control [$CO_2$] and to continuously vary the flow rate. In the LI-6400/LI-6400XT, there are two separate controls of the sample water concentration ([$H_2O$]), the flow and the desiccant scrub position (see Chapter 3, Tour #3 LI-COR, 2008). When configured in the standard flow path, we recommend using the desiccant to coarse adjust the [$H_2O$] entering the sample chamber. The entering $H_2O$ plus the transpired $H_2O$ will determine the chamber [$H_2O$]. As air flows through the sample chamber, longer retention in the chamber allows greater amounts of transpired $H_2O$ to be added. The flow rate controls the retention time of the chamber air; greater flow rates flush the chamber faster resulting in the air passing through the chamber picking up less $H_2O$. Conversely, the longer the air remains in the chamber, the greater quantity of $H_2O$ that is taken up by each air unit which increases the chamber [$H_2O$]. The flow rate has a greater control range on the sample chamber [$H_2O$] than does the entering [$H_2O$] (do Tour #3, Experiment #1 to prove this, LI-COR, 2008). With the standard flow path, the $CO_2$ mixer will operate independently of the flow to control chamber (or reference) [$CO_2$] at the desired set-point.

When the LI-6400/LI-6400XT flow path is modified for low [$CO_2$] control, we recommend only operating on constant flow control because changes to the flow rate result in a momentary change in the pressure at the pump. This transient pressure change momentarily relieves the backpressure that is working against the pure $CO_2$ injection from the $CO_2$ mixer causing a brief spike in [$CO_2$]. If the system is operating at a constant flow rate, there will not be any [$CO_2$] perturbations. However, flow rate changes cause [$CO_2$] to noticeably change. In the automated [$H_2O$] control, the LI-6400/LI-6400XT uses continuously variable flow to maintain a constant [$H_2O$] in the sample chamber and subsequently the sample relative humidity (RH%) and vapor pressure deficit of the leaf (VPDl).

In a modified instrument, every adjustment of the flow rate causes a change in the [$CO_2$], which causes the photosynthetic rate to change, resulting in altered stomatal aperature controlling the $g_s$. The altered $g_s$ changes the transpiration rate, which affects the [$H_2O$] in the chamber resulting in flow rate changes to maintain the requested chamber [$H_2O$]. This becomes an endless loop of change and subsequent adjustment making measurements impossible as seen in the measured $CO_2$ response curve for *Zea mays* (Figure 3). A leaf was measured with the standard flow path and using the automated flow control to maintain H2OS constant. The system flow path was modified and the same leaf was measured again using the automated flow control. As the [$CO_2$] in the chamber changed, the resulting increased $C_i$ caused the stomata to begin responding. This began the feedback loop described and resulted in the unstable calculated rates of photosynthesis.

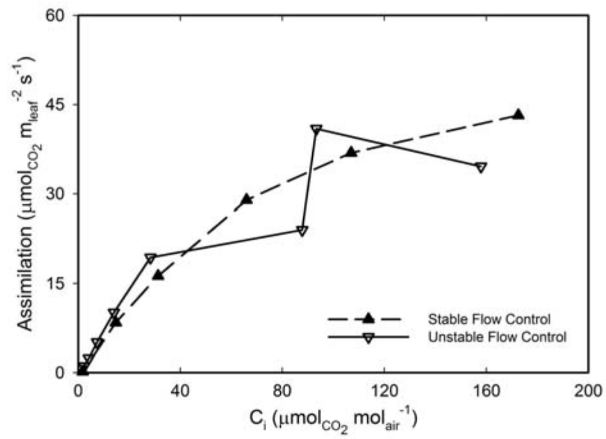



*Figure 3. Photosynthetic response to increasing [$CO_2$] for* Zea mays. *Stable $CO_2$ and automated flow control result in smooth transitions in photosynthesis with changes in [$CO_2$]. However, unstable $CO_2$ rates results in large variance in photosynthesis.*

**Diffusion at low [$CO_2$]**

The most common use of low [$CO_2$] control will be to measure the photosynthetic response to [$CO_2$] in $C_4$ plants. Typically these measurements will be at [$CO_2$] that are much different than ambient. The large concentration differences drive diffusional fluxes that can be a significant error in the measured photosynthesis rate. When the chamber [$CO_2$] is controlled at a low set point, the diffusion flux is into the chamber countering the net removal of $CO_2$ by leaf photosynthesis. This effectively decreases the calculated photosynthesis and can be a large error in the measurement (Flexas et al., 2007, Rodeghiero et al., 2007, Long and Bernacchi, 2003, LI-COR, 2008). At the low [$CO_2$] that result in a large diffusional flux, the photosynthetic rates are often very low compounding the error effect (LI-COR, 2008). Fortunately, there are methods for correcting the calculated rate of photosynthesis including a method for calculating a diffusion correction (see LI-COR, 2008). When measuring at low [$CO_2$], the effects of diffusion become proportionally larger errors in the photosynthetic measurements. Fortunately, the errors can be accounted for and corrected. Each user will have to determine the impact that the diffusion may have on their experiments.

The photosynthetic response to [$CO_2$] was measured on three *Zea mays* leaves for chamber [$CO_2$] < 400 µmol$_{CO_2}$ mol$_{air}^{-1}$ (Figure 4). Glasshouse grown plants in 13 L pots of MiracleGro® potting mix were measured at approximately growth stage V5. Leaves were acclimated to 1900 µmol$_{CO_2}$ m$_{leaf}^{-2}$ s$^{-1}$ light, approximately 380 µmol$_{CO_2}$ mol$_{air}^{-1}$ in the sample chamber, 29 °C, and relative humidity ranging between 60 - 75 %. An autoprogram was used to change the reference $CO_2$ stepwise from 400 to 0 µmol$_{CO2}$ mol$_{air}^{-1}$. With the low $CO_2$ modification, the LI-6400/LI-6400XT measured was able to measure numerous points < 20 µmol$_{CO_2}$ mol$_{air}^{-1}$ $C_i$.

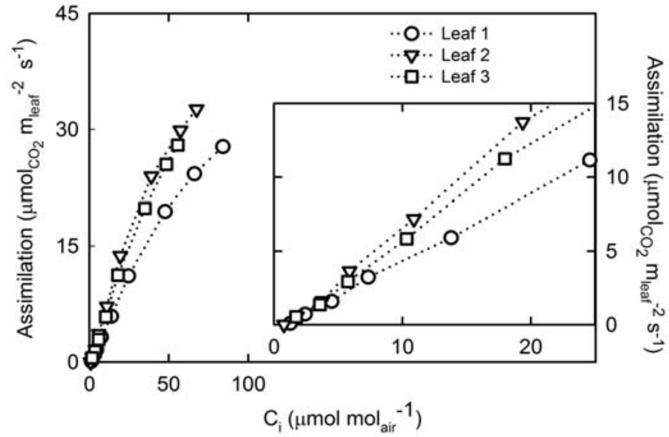



*Figure 4. Photosynthetic response curves to [$CO_2$]. a) photosynthetic response to $C_a$ < 400 µmol$_{CO_2}$ mol$^{-1}_{air}$ for three leaves of* Zea mays. *Photosynthetic rates are as reported by the LI-6400/LI-6400XT. Inset is photosynthesis at very low [$CO_2$] showing the increased number of points obtained with the low $CO_2$ modification.*

## Bibliography

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Lambers, H., Chapin III, F. S. & Pons, T. L. (2006) Plant Physiological Ecology. Springer Science+Business Media, LLC, New York.

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Long, S. P. & Bernacchi, C. J. (2003) Gas exchange measurements, what can they tell us about the underlying limitations to photosynthesis? Procedures and sources of error. Journal of Experimental Botany, 54 (392): 2393-2401.

Rodeghiero, M., Niinemets, Ü. & Cescatti, A. (2007) Major diffusion leaks of clamp-on leaf cuvettes still unaccounted: How erroneous are the estimates of Farquhar et al. model parameters? Plant, Cell and Environment, 30, 1006-1022.

von Caemmerer, S. (2000) Biochemical Models of Leaf Photosynthesis. Techniques in Plant Science. CSIRO Publishing, Collingwood, VIC, Australia.

## Appendix I

Note underlined changes in the code.

```
/*
       OPEN.MXR

       CO2 mixer support

       950410 - version 2
       960812 - added power on to 'C'
       960822 - changed # cal pts, ignore
       status < 300 mv
       970425 - mixer cal lookup change
*/
:STATIC 1
:CHAR initMess[] "Soda Lime should be on FULL
SCRUB\n  when using the CO2 mixer..."
:INT
       mixerSlopesComputed 0
       messageThreshold    5
:LONG
       lastChange                 0
       effSampVol                 5000
:DOUBLE
       mixerSlope[4]      {  } /* coeffs for
(y=ppm/mv, x=mv) relation */
:FLOAT mixerPrevTarget 0  /* last REF ppm target
*/
```

**...(about 5200 bytes omitted)...**

```
/* ----- External ------ */
:STATIC 0
:DOUBLE
       PUB mixerSignalOrig[10] {5000 3000 1500
1000 500 300 200 100 50 0}
       PUB mixerSignal[10] {  }
       PUB mixerPPM[10] {2400 1100 450 270 120
70 50 30 15 0}

:INT
       mixerTargetIndex    1
    PUB mixerAvail         0
    PUB mixerRefId         2
    PUB mixerSmpId         3
       mixerIsStable       0
       mixerIgnoreBelow   300
       allowMixerControl   1

:FLOAT
       PUB   mixerFudge 1
:PTR mixerPtrs[] {Noop Noop Noop Noop}

PUB CO2ControlList[]
```

**...(omitted)**

## Supplies Required

| | Part No. |
|---|---|
| Bev-a-Line tubing | 8150-250 |
| Hose barbs (optional) | 300-02547 |
| Silicone grease | 210-1958-1, or |
| Teflon grease | 210-03774 |
| Side cut pliers | |
| Utility knife | |
| 1/4" adjustable wrench | |
| #2 Philips head screwdriver | |
| #1 Philips head screwdriver | |

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