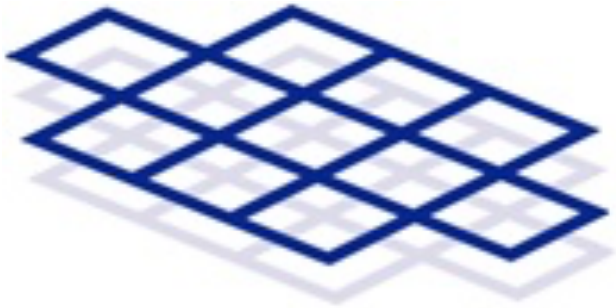
LumiCycle

User's Manual for USB versions

Luminometry for Circadian Biology



ActiMetrics

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About LumiCycle

LumiCycle performs high-throughput luminometry on self-luminous tissues, such as those from transgenic animals containing the luciferase gene. The system is equipped with 4 photon-counting photomultiplier tubes **(PMTs)**, each selected for low dark counts and high sensitivity in the green portion of the spectrum at which luciferase emits light. 32 tissue samples, each in a 35 mm Petri dish, can be counted at one time. 8 samples share each photodetector using an automated turntable that alternately brings each sample under the detector one at a time. The apparatus fits inside a standard incubator. An internal fan circulates the incubator air to maintain the proper temperature within the chamber. The turntable and photon counting are fully automated. System setup and operation is straightforward. In addition to its high-precision photon counting hardware, LumiCycle has the most flexible and easy-to-use software for the collection and analysis of circadian rhythms in luminometry data.

- Start and stop each counting channel asynchronously. You may, for example, start one experiment with 10 samples on one day, and start a second experiment with 22 samples the following day, stop the first experiment 5 days later and start yet a third set of samples in their place.
- With colored filters placed on 2 of the 4 photodetectors, 16 samples can be recorded in two separate colors simultaneously. Filters can be changed easily for different colors.
- Interrupt data collection briefly to perform experimental manipulations (such as adding a drug). After the manipulation, LumiCycle picks up where it left off. The small gap in the data will have little effect on subsequent analyses.
- Adjustable frequency at which each sample is counted, from once per hour, to once per minute.
- Automated, advanced signal processing algorithms compensate for baseline shifts and signal attenuation over the course of the trial. Extract dominant circadian period and phase and best-fit curves.
- Calculate rhythm parameters for the complete record or for multiple subsets of the record. For treatments given halfway through a record that are expected to create a period or phase change, the two halves of the record can be analyzed separately.
- View records during the data collection process. A single window shows data for all 32 channels at once. Or view the data for individual records in greater detail.
- During data collection, repeatedly copy the growing data files onto another computer (via a USB drive or the network) and analyze the data collected so far. Data collection continues uninterrupted until the end of the experiment.
- Extracted periods and phases can be exported to a text file for later manipulation in Excel or other analysis programs.
- Raw or baseline-subtracted records can be exported to ClockLab for further analysis using Periodograms, actograms, and activity profiles.

Before Getting Started

Before getting started, there are some important warnings that will help prevent damage to the unit. Please take a moment to look at them.

The incubator must NOT be humidified. Humidity will damage the PMTs. Instead of using humidity, seal your dishes and use a HEPES-based pH buffer.

The turntable can pinch fingers against the PMT plate while it is moving. Keep your hands away until the turntable has stopped moving.

Avoid exposing the PMTS to room light when they are on. LumiCycle automatically prevents light exposure: There is a switch on the inside of the door that turns off the PMTs when the light-tight cabinet is opened. Don't press the switch yourself while the cabinet is connected to the computer interface. If you disassemble the cabinet (doing so without consulting Actimetrics may void the warranty), make sure the light-tight cabinet is disconnected from the power supply.

The PMTs can store charge if they are exposed to strong light when they are off. This can cause a brief period of artificially high counts after the PMTs are turned on again. Because the light-sensitive windows of the PMTs are recessed between the PMT mounting plate and the turntable, the light reaching the PMTs with the door open is usually not strong enough to cause a problem. But if a period of high counts is consistently observed after the door has been opened, try dimming the room lights before opening the door.

Prepare the Computer

LumiCycle is designed to run 24 hours a day with little maintenance or oversight. You don't want the computer to crash on a weekend while no one is paying attention. To minimize the chances of a crash:

- Do not use the computer for surfing the net, playing video games, word processing, etc.

A number of Windows options can interfere with the long-term operation of LumiCycle:

- Disable any automated tasks such as virus scanners.
- Disable Windows Updates (in the Windows Updates Control Panel)
- Disable Sleep mode (in the Power Options Control Panel)
- Disable the "Automatically Adjust for Daylight Savings Time" option in the Date and Time Control Panel

For proper power failure recovery, put a short-cut to the LumiCycle Data Collection program in the Startup folder of your computer. For Windows 7, Click the Start button, click All Programs, right-click the Startup folder, and then click Open. Put a shortcut to LumiCycle into this folder. This will make the LumiCycle program start up automatically when the computer boots up. To access the startup folder in Windows 8/10, hold down the Windows key and press the "R" key. In the "Run" box type, shell:startup and then click on the OK button. Configure the computer to start up automatically after a power failure. The detailed steps necessary to activate this option vary from one computer to the next but it is usually an option in the BIOS.

Install the LumiCycle programs

Software is available on the Downloads page of the Actimetrics website.

User ID for download: behavioral
Password for download: Neuro11

Download, unzip and install the most recent version of the LumiCycle Data Collection and Data Analysis programs.

Download, unzip and install the CDM (FTDI) drivers for the USB interface ("Drivers for USB Interface" link on the Actimetrics' website).

Install the LumiCycle hardware

LumiCycles with separate interface box

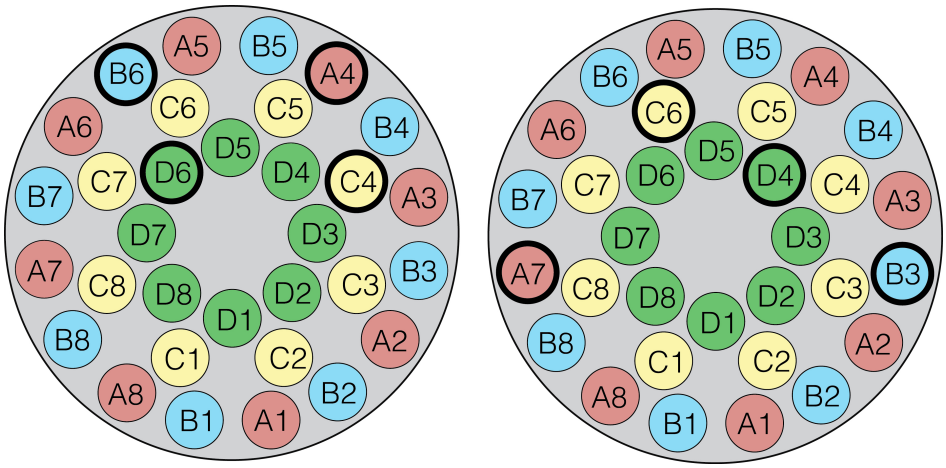
- Plug in the power supply to the USB interface via the 5 mm barrel connector, and plug the power supply into the wall. The green light on the interface should light up.
- Connect the interface to the computer using the USB cable provided.
- Connect the DB-9 (9-pin) connector on the left side of the light tight box to the interface box. If necessary, disconnect the round (blue) connector in the middle of the cable in order to fit the cable through the instrument port on the incubator. This cable carries power to the PMTs, control signals from the motor controller to the motor, and signals from the door and thermostat switches. All nine pins of the cable carry signals.
- Connect the 4 BNC (coaxial) cables to the 4 BNC connectors on the left side of the box. Feed these through the outlet of the incubator and plug them into the 4 BNC connectors on the interface box. Make sure you connect A to A, B to B, etc, or the data for the different samples will be swapped around into the wrong files. These cables carry the signals from the PMTs. All four BNC cables MUST be installed for proper operation.
- Do **NOT use BNC cables longer than the 6-ft cables that are provided. Longer cables may degrade the PMT signals and cause missed counts.**

All-in-One Units:

- User the included USB cable with threaded hood to connect to the light-tight cabinet. Plug the standard USB Type A connector on the other end of the cable into a free USB port on the computer. On desktop models, one of the USB ports in the rear is preferable.
- Connect the power supply to the barrel connector just below the USB connector on the light-tight cabinet, and plug the power supply into the wall with the provided cable.

Sampling

LumiCycle uses 4 PMTs (A-D) to count 32 samples. The 8 samples in each of 4 groups (A-D) share one PMT. If, for example, the counting interval (Channel Setup Window) is set to 10 minutes, then each sample gets counted for 1/8th of that time, or 75 seconds, minus the 5 seconds it takes to move the turntable from one position to the next.



Sample locations and PMT locations (thick border) for LumiCycle 32 (left) and LumiCycle 32 Color (right)

Note that the 4 samples being counted at any one time are not regularly spaced in the file list shown in the main window. For example, the left diagram above shows that in turntable position 4, samples A1, B1, C1 and D1 are located at the front of the cabinet and ready for loading (if the door were open). But samples A4, B6, C4 and D6 are in position to be counted (thick circles). Despite this somewhat confusing arrangement, at the end of each counting interval, all 32 samples will have been counted.

Color recording. For units capable of color recording, the locations of the PMTs are different (right diagram above), but the principle is the same. PMTs A and B are also fitted with removable slides that can hold 25-mm colored filters.

When the program is recording in color mode (Channel Setup Window), the turntable takes up 16 different positions during a sample interval, instead of the usual 8. In this way, PMTs A and B can record in turn from each of the 16 samples in the outer ring (groups A and B). For each sample, the counts recorded on PMT's A and B are written into one data file. Note that in color mode, the total count time for each sample in the outer ring is the same as it is for broadband recording, but the counting time is now split evenly between the A and B PMTs.

In Color mode, the 16 samples in the inner two rings (Groups C and D) are recorded in broadband by their corresponding PMTs. These samples only align with their PMTs for 8 out of the 16 turntable positions (1, 3, 5, etc.). While the turntable is in the alternate positions (2, 4, 6, etc.) no samples from the C and D groups are recorded. As a result, the total counting time for these samples is half what it is during broadband recording.

Main Window (Data Collection)

Context Help. Most controls and indicators in the program contain information about their contents and use. To display this information, select the Help->Show Context Help menu item and then hover the cursor over a control or indicator.

File Controls and Indicators

The current counting interval and how many intervals there are per hour are shown in the **Interval** indicators at the upper right of the main window. Which set of samples is currently being counted is indicated by the **Position** indicator. At each position, as each set of samples is being counted, the blue sliding **Time** indicator keeps track of the time within the current counting period. The slide is dark blue while the turntable is moving and lighter blue while the samples are being counted.

Save. Turns on or off data storage for the corresponding channel. When you turn on the save button for a channel, you will be prompted to specify a file in which data will be saved. Navigate to the directory in which you would like to place the file and either select an existing file for data to be appended or type in the name of a new file. Different files can be in different directories. For example, if there are two sets of samples from two different experiments running at once, you may want to save the two sets of files in different directories.

The array of 32 controls and indicators to the left of the main window are used to keep track of data files. There is one file for each sample being counted.

#. The channel number. Corresponds to one sample in the LumiCycle cabinet.

File names. Displays the name of the file in which data are being saved for each channel. The display cannot be changed directly. To change the filename for a particular channel, click the Save button off and then on again. You will be prompted for a new file name in a standard open-file dialog. The display is blank when the corresponding Save button is off.

Days. Displays the number of days and hours of data contained in the current file. Updated once per hour.

Error. Turns red if data are not being written to the file correctly. An error might occur if you delete the directory in which the file sits, for example, or if a file is open from another program.

Count. Displays the accumulated (total) counts for the current counting cycle. This indicator is cleared to 0 at the beginning of each counting interval and continues to read 0 until it becomes time for the corresponding sample to get counted.

Color recording. With color recording selected, the Count display shows 48 values, 2 for each of the 16 channels that are recorded in color (groups A and B), and 1 for each of the channels that are recorded in broadband. The background colors of the currently counting channels indicate the colors being recorded, as set in the Channel Setup window.

Time. Displays the current Standard time. To avoid problems with the shift into and out of Daylight Savings Time, the computer should be set NOT to adjust automatically for DST. You will find this setting in the Date and Time control panel by clicking on the Change Time Zone button. With this setting, the Time indicator in the program window will NOT reflect Daylight Savings Time during the summer months, and so will be 1 hour behind the indicated System Time. LumiCycle will then store all data

according to Local Standard Time in order to avoid confounding 1-hour shifts at the beginning and end of each summer.

Open Counter Test Panel. This button only appears at startup while the motor is being initialized. Clicking the control once will cause the counter test panel to be open when the motor initialization is completed. The test panel shows a graph of the count rate, which is updated once per second. To measure the dark counts of the PMTs, open this panel when there are no samples in place.

Error and Warning Indicators

All warning and error indicators are normally not visible unless the specified condition occurs.

Initializing Turntable Warning. Appears only at the start of program operation while the turntable is brought into its start position. This time is variable depending on the position that the turntable was in when the program last ran. It should take no more than 70 seconds.

Counter Error. Appears if an error occurs in the operation of the high-speed counters.

Low Count Warning. Appears if counts on any one of the PMTs falls to 0 for an entire counting interval. Indicates a faulty PMT, faulty connection from the PMT to the Interface box, or a problem in the USB connection between the interface box and computer.

Serial Error. Appears if an error occurs in the serial communication with the motor controller.

Motor Timeout. As the turntable rotates into position 1, a "home" or "limit" switch is tripped by a small lever on the turntable (if the lab is not too noisy, you may hear an audible click when the switch is tripped). If the program does not detect the closing of this switch within a specified time after initiating the rotation, a timeout error indicator will appear.

Position Error. If it takes too many or too few steps to complete a revolution of the turntable, this indicator will appear. This error can occasionally appear and then clear itself on the full next rotation of the turntable. If it is displayed for several minutes or appears repeatedly, contact Actimetrics.

Overheat Error. Appears if the temperature in the box rises too high, for example, if the fan fails or the air intakes on the front of the light-tight box are blocked. If overheating occurs, a thermostat will turn off the PMTs to protect them from damage. The program will also stop the counting sequence and shut down the motor. Check that the incubator is at the correct temperature. Also check that the LumiCycle's fan is working. If you listen carefully at the lower opening on the front of the unit, you should be able to hear the fan. Contact Actimetrics if the overheat error condition persists.

Menus

File Menu

Quit. Stops the program.

Settings

Channel Setup. Opens the Channel Setup window for setting the counting interval, number of samples being recorded, and – if available – color recording.

Channels

Sequence Names. Often, files for the different channels will be named sequentially, such as Sample_01, Sample_02, etc. Instead of laboriously clicking on each ON button individually and entering the name for that channel, you can instead enter the base name for the files and a starting value. All available channels will be activated and named sequentially starting with the Starting Value. For channels that are already active, the filenames will be replaced by the sequence.

Stop All. Turns all active channels off.

Comment. Allows you to enter comments that will be saved in the data files. Each comment is saved (and then cleared) the next time the corresponding sample is counted. Comments can be viewed in the LumiCycle Analysis program.

View

All... Opens the Thumbnail Window, which displays 32 graphs showing the data collected so far for each channel. Use the Y Scale Listbox to the left of the window to adjust the vertical scale on each graph. Double click a graph to view its data in more detail.

Selected... Opens the Data Window, for viewing the data from one channel in some detail.

Help

Context Help. Most controls and indicators in the program contain information about their contents and use. To display this information, select the Help->Show Context Help menu item and then hover the cursor over a control or indicator.

Selected... Opens the Data Window, which allows you to view the data from one channel in some detail.

LumiCycle Help. Opens this manual.

About. Displays the program version number.

Channel Setup Window

Number of samples. LumiCycle normally records from each of the 32 sample locations in one sample interval. If fewer samples are being monitored, however, it is more efficient to skip those locations where no samples are present. The program therefore gives the option to record from 16 or 8 locations, instead of 32. The diagram to the right of the control shows which locations will be recorded. In addition, the Loading Window will indicate which locations are active.

Sample Interval. Determines how often each sample will be recorded (once every minute, once every 2 minutes, etc.). The duration of the counting period for individual samples is also indicated. The counting duration is a function of the interval, the number of locations being recorded, and how long it takes for the turntable to rotate between each position. The times shown are approximate.

Note that as the sample interval decreases, LumiCycle will spend a larger proportion of its time turning the turntable and a smaller proportion counting. It takes about 5 seconds for the turntable to move 1 position. As a result, with a sample interval of 2 minutes and 32 locations active, the program will spend 30% of the time turning and 70% counting. With a 10-minute interval, the program spends less than 10% of its time turning. The longer sample interval gives data with lower temporal resolution, but the longer counting times give a higher signal-to-noise ratio for each data point.

Color Recording. On LumiCycles equipped with filter holders, setting the **Color** control to ON sets the program to record in two colors from the outer ring of 16 locations. The colors are determined by the colors of the filters placed in the filter holders. The color locations – 1 through 8 A, and 1 through 8 B – are indicated by the gold coloring in the turntable diagram. The remaining samples – those in groups C and D – are each recorded by only a single PMT with no filter (broadband), and are indicated in white.

The filters' colors can be stored in the data files and displayed in the program's display graphs by setting the two **Colorbox** controls just underneath the **Color** switch. The left colorbox should be set to the color of the filter on the left front PMT in the LumiCycle cabinet. The right colorbox to the color of the right PMT.

With Color recording selected, and fewer than 32 locations selected, the pattern of active locations on the turntable is asymmetrical. The active locations are indicated in the Loading window to facilitate proper placement of samples.

Photomultiplier Tubes

The photomultiplier tubes (PMTs) are highly sensitive, delicate and expensive instruments, designed to count single photons. LumiCycle uses PMTs hand selected to have low dark counts. The dark counts – defined as the number of counts registered by the PMT in complete darkness – are a function of thermal noise. The dark counts cannot be reduced to zero without compromising the sensitivity of the detectors, but the dark counts should be less than 10/sec at room temperature, and 30-60/sec at 35 degrees C. Higher values might mean that the detectors have been compromised, or that the box is no longer light tight.

The PMTs are temperature sensitive. Temperature fluctuations inside the light-tight box of less than 1 degree can visibly change the dark counts. The more tightly controlled the temperature, the more stable the system will be. Poorly regulated incubators have significant temperature fluctuations with a period of an hour or so. These fluctuations will be clearly visible in your records.

There is a small amount (< 0.5%) of crosstalk between the samples. That is, a strong source in one sample will create extra counts in some of the adjacent samples. This is caused by some of the photons bouncing through the small gap between the top of the turntable and the PMT mounting plate. Take this into consideration when placing samples that you anticipate having very strong signals next to others that have much weaker signals.

Precautions

- Don't drop or jar the light-tight box. The PMTs are not cushioned inside the box.
- Don't block the air intake or outlet on the front surface of the box. Otherwise the box could overheat and damage the PMTs before the thermostat has a chance to turn them off.
- **Do not turn on the PMTs while the door of the light-tight box is open!** The door latch turns on the power to the PMTs when the door is closed by activating a small switch mounted on the inner wall of the box, just inside the door. If this switch is pressed while the door is open, power will go to the PMTs and the light entering the box will overwhelm them. The PMTs can handle strong light while they are off (otherwise you could never open the door at all), but exposure to more than a small amount of light while they are on can damage them. **So don't poke, explore, mess with or otherwise get near the switch**.

Loading Samples

- **Pinch hazard.** Don't go near the turntable while it is moving. There is a lot of force behind the movement of the turntable, which could pinch your fingers and damage the mechanism.
- Dishes MUST be sealed and the incubator must not be humidified. Humidity will damage the PMTs. Because the incubator is kept dry, the dishes must be sealed. We recommend using silicon grease and 40 mm cover glasses from Fisher Scientific (40 Circle #1)

LumiCycle is designed to fit Falcon 3001 35-mm dishes.

To load samples into the Light-tight box, simply open the door while the program is running. **If the turntable is moving, wait until it has stopped**. Remove the turntable cover from the opening in the cover plate. LumiCycle detects that the door is open and will display the Loading Window. The window displays which samples are currently front-most in the box and in position to be loaded. If you have chosen to record from fewer than 32 samples in the Channel Setup Window, then only some locations will be counted during a sample interval. The text labels of those locations that are NOT being counted will be dimmed in the Loading window.

> **Color recording.** If the program is in color mode, then the sample locations that are being recorded in color will appear a light orange/gold color and locations being recorded in broadband will be white.

You may use the **space bar** to advance the turntable by one position, or hit the **keys 1 - 8** on the computer keyboard to advance to the corresponding position.

Note that 5 sample locations are exposed in a pentagonal pattern, but only 4 locations—those corresponding to the labeled positions in the diagram—should be loaded at a time. Once these 4 samples are loaded, advance to the next position using the keyboard commands.

The program will return to its counting sequence when the door is closed and the handle turned all the way clockwise. **Don't forget to replace the turntable cover before closing the door.**

If the program does not close the Loading Window and resume counting once the door is closed, check that the handle is turned firmly clockwise, and check all connector cables. The window can be closed in an emergency by hitting the Q key, but this will also stop the program.

You may wish to stop the counting sequence, open the door and add pharmacological agents or perform other manipulations to the samples in the middle of an experiment. As with the loading procedure, simply open the door of the Light-tight box and remove the turntable cover. The program will stop and display the Loading Window. Use the keyboard commands to access all the samples and perform whatever manipulations are necessary. When the door is closed again, the program will pick up where it left off, using the same files as before. There will, of course, be a small gap in each of the data files, but the analysis program is designed to accommodate such gaps.

Managing Time with LumiCycle

LumiCycle uses the Windows System Clock to tell time, and Windows system clocks can drift over time. To keep your data as accurate as possible, check and set your clock regularly. If your computer has an Ethernet connection, set Windows to synchronize with the Internet Time Server in the Date and Time Properties control panel. (This is usually the default setting.)

Set your clock NOT to "Adjust automatically for Daylight Savings Time". To avoid problems with the shift into and out of DayLight Savings Time, the computer should be set NOT to adjust automatically for DST. You will find this setting in the Date and Time control panel by clicking on the Change Time Zone button. With this setting, the Time indicator in the program window will NOT reflect Daylight Savings Time during the summer months, and so will be 1 hour behind. LumiCycle will then store all data according to Local Standard Time in order to avoid confounding 1-hour shifts at the beginning and end of each summer.

Thumbnail Window

Selecting the View All... menu item in the main program window opens the Thumbnail window, which displays a graph of the data being collected on each of the 32 channels.

Use the **Y Scale Listbox** to adjust the vertical scale on all the graphs.

Double click a graph to see its data in more detail.

Data Window

Select a channel to display using the File popup menu at the top of the graph. Or move from file to file using the >> and << buttons.

Set the units on the vertical scale to Counts per second or Counts per minute using the Y Scale toggle switch.

Set the units on the horizontal scale to Hours or Days using the X Scale toggle switch.

The data can be smoothed using the Smoothing button. Smoothing is done with a median filter. When the smoothing value is set to 10, for example, each point in the graph is replaced by the median of the surrounding 10 points.

The appearance of the graph can be changed using the Graph Legend to the upper right of the graph. Point style, line style, point and line colors, graph style, interpolation, and more can all be changed.

To zoom in on a part of the data, click the Zoom icon (the magnifying glass below the graph) and then click-and-drag on the desired part of the graph. Zoom out using the X, Y or All buttons.

Close the window using the Close menu item.

Maintenance

Computer Maintenance

Because of the complexity of modern operating systems, they will not always run without problems for long periods of time. To reduce the likelihood that your computer will crash and lose data, we recommend a few quick maintenance procedures.

- Reboot your computer and restart LumiCycle once a month.
- If your computer is not on the network, check your system clock regularly.
- Do not run any programs other than LumiCycle. Running programs other than those required will make it more likely that your computer might crash while unattended. Do not try to do data analysis or word processing on the data collection computer.

Device Maintenance

You can clean the various surfaces of the unit (top and side, turntable, etc) with a damp cloth or paper towel. Alcohol is also permissible. Try not to drip water or alcohol into the box below the turntable.

No lubrication is necessary.

Running Two LumiCycles on One Computer

Once you have installed the LumiCycle program, find the folder C:\Program Files (x86)\LumiCycle.

Make a copy of the whole folder. Rename the original “LumiCycle 1” and the copy “LumiCycle 2”.

In the 1st folder, rename LumiCycle.exe to be “LumiCycle Unit 1.exe”.

In the 2nd folder, rename LumiCycle.exe to be “LumiCycle Unit 2.exe”.

The names must be exactly as shown.

Make shortcuts to both of these .exe files and put them on your desktop for starting up the programs.

Plug in just one interface box. Start up the LumiCycle Unit 1.exe while holding down any two keys on the keyboard. You should see a window that lets you select which interface box for the program to use. Select the interface being shown.

Unplug the one interface, plug in the other, and repeat the procedure for LumiCycle Unit 2.exe.

You should now be able to run both units simultaneously. The color of the two windows for the two versions of the program should be slightly different, and the title bars will show the names Unit 1 and Unit 2.

Troubleshooting LumiCycle

The Hardware Error dialog appears when the program starts up. The LumiCycle interface has not been properly recognized by the computer.

Check that the USB cable between the interface and the computer is securely plugged in.

Check that the interface box is receiving power. The power supply should be correctly plugged in and the green LED on the front of the interface should be on.

Check that the drivers for the interface have been installed. The drivers are available on the downloads page of the Actimetrics web site.

Check that the interface is being recognized by the computer. Open the Device Manager. In the Universal Serial Bus Controllers section, the LumiCycle interface should appear as 2 lines at the bottom of the list: “USB Serial Converter A” and “USB Serial Converter B”.

The Low Count indicator appears in the main program window.

Check that BNC cables between the interface box and the cabinet are properly connected and locked onto each connector.

Switch the cable connection to the interface box between the bad PMT and one of the remaining good ones. Run the unit for a while and see if the problem stays on the same PMT or switched to the formerly good one. This will determine whether the problem is with the PMT itself, or with the counting input on the interface.

The Overheat indicator has appeared in the main window.

Check to see that the air inlets on the front surface of the light-tight box are not blocked.

Check the incubator temperature. It should not be higher than 37 degrees C.

Check that the fan is operating. Place your ear next to the lower opening on the face of the cabinet while you plug and unplug the 9-pin connector on the side of the cabinet. You should hear the fan start and stop.

The cabinet door is closed, but the program claims that it is not.

Close the door and turn the handle to the open and closed position. There should be an audible click as the tab on the handle hits the On/Off switch for the PMTs. This switch sits just inside the door. **Don’t press the switch with the door open unless the 9-pin cable to the cabinet is unplugged!**

If the click is not audible, with the light-tight box disconnected as above, reach inside the door and check the switch. Depress the metal tab by hand and make sure that it clicks audibly. Contact Actimetrics if the switch appears to be broken or not engaging properly.

Replacing a PMT in LumiCycle

UNPLUG ALL CONNECTORS TO THE LIGHT-TIGHT BOX.

Place the unit on a counter with the rear panel facing you.

Remove the cover of the light-tight box by removing the top screws.

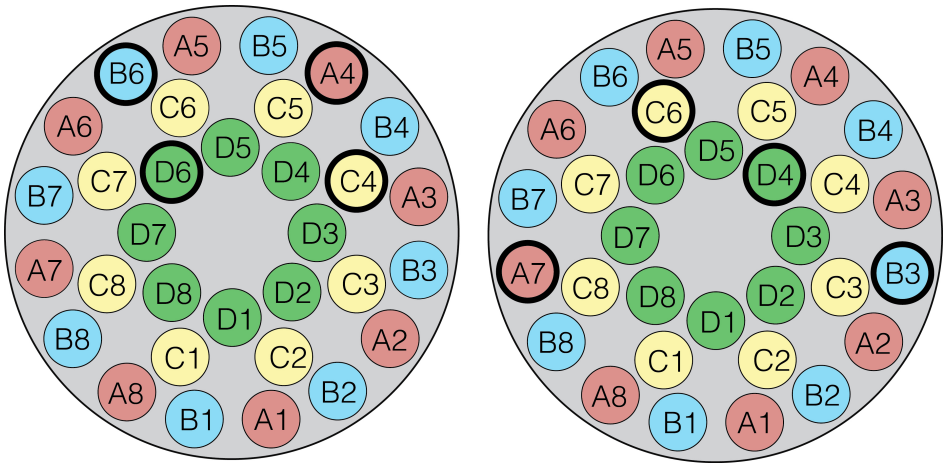
If the rear panel is secured by screws, remove it by removing the three screws located along each side. Leave the top center screw in place in place.

If there are no screws on the rear panel, simply lift the panel out vertically. Find and save the rubber gasket material inserted into the vertical corner channels. Remove the vertical corner channel to your left. It is held by a single flat-head screw at the corner of the bottom plate.

Unplug the white plastic power connectors to the PMTs. There are 4 of them that are hanging in a bundle just below the PMT plate at the back. These are the white connectors with one red and one black wire coming from each PMT. **DO NOT PULL ON THE WIRES.** Grab the smaller white plastic part (toward the bottom) with pliers and pull off the larger upper part with your fingers.

Unplug the 4 BNC (coaxial) connectors on the inside wall of the box. Note they are marked with different numbers of cable ties: 1 = A, 2 = B, etc.

The diagram that shows the PMT positions (thick circles) for LumiCycle (left) and LumiCycle Color (right). Location A1 in the diagram is at the front of the cabinet.



Cut the black and white cable-ties that hold the wires going to and from the broken PMT and unravel them. **Be careful not to cut the wires as you cut the cable ties.**

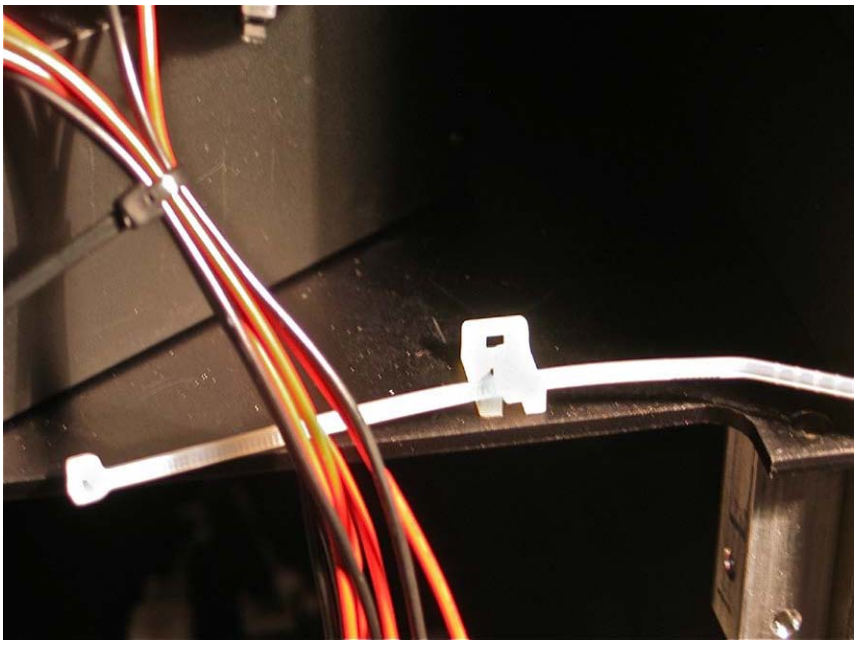
Remove the socket-head screws that hold the PMT plate at its corners. Use the large yellow-handled hex wrench.

Slide out the PMT plate by tilting and pulling it toward you. Handle it gently. Protect the glass PMT windows.

Remove the bad PMT by taking out the 4 flat-head screws that hold it in place (red-handled 2-mm hex wrench).

Remove the tape covering the face of the new PMT and screw it in place. Don't over tighten the screws. Firm but not too tight. Make sure the window of the PMT is over the hole in the mounting plate.

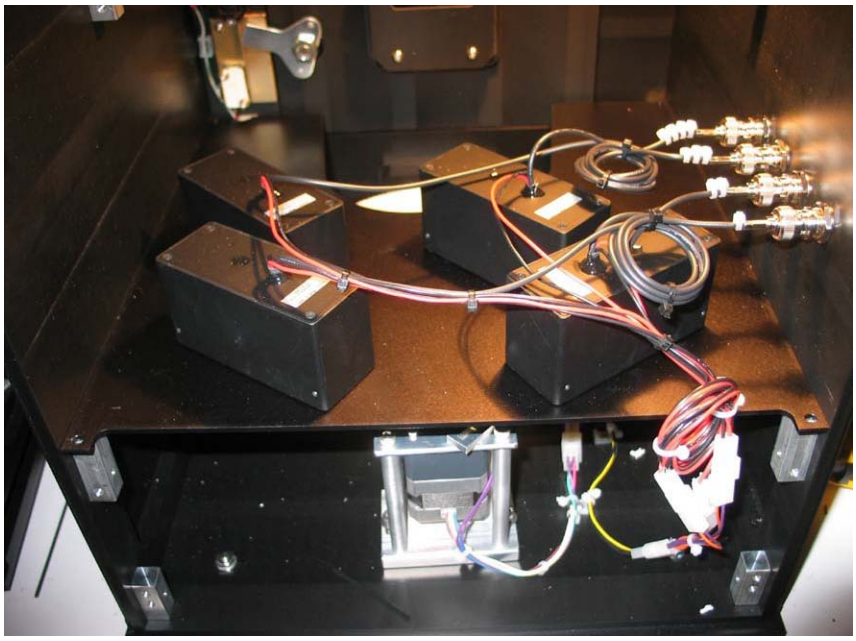
Reinstall the PMT plate and replace the 4 corner screws that hold it in place. There is a small semicircular cut at the front edge of the PMT mounting plate. Make sure that the wires running up the inside of the front panel are located in the cut, so that the edge of the plate does not pinch the wires. Push the wires to the right if necessary so that they lie inside the cut.



Connect the front two PMT's signal (BNC cables). First coil up the excess from each into a 5 cm coil (see photos), place a couple of cable ties on the coil, and then plug the connectors in to the front two BNC connectors (A to 1, B to 2). The cable ties should be snug, but not tight.

Connect the rear two PMT signal cables. Again, coil the excess wire and secure the coils with cable ties.

Route the red and black power connections from the PMTs as shown in the photos. Use cable ties to secure them. For the cable tie down at the rear of the PMT plate, use two cable ties here. One goes in the loop of the tie-down. The second threads through the first.



Plug the 4 power connectors into their mating connectors underneath the PMT plate. It does not matter which one goes to which connector. They are all equivalent. Make sure the wires hang so that they are not near the turntable.

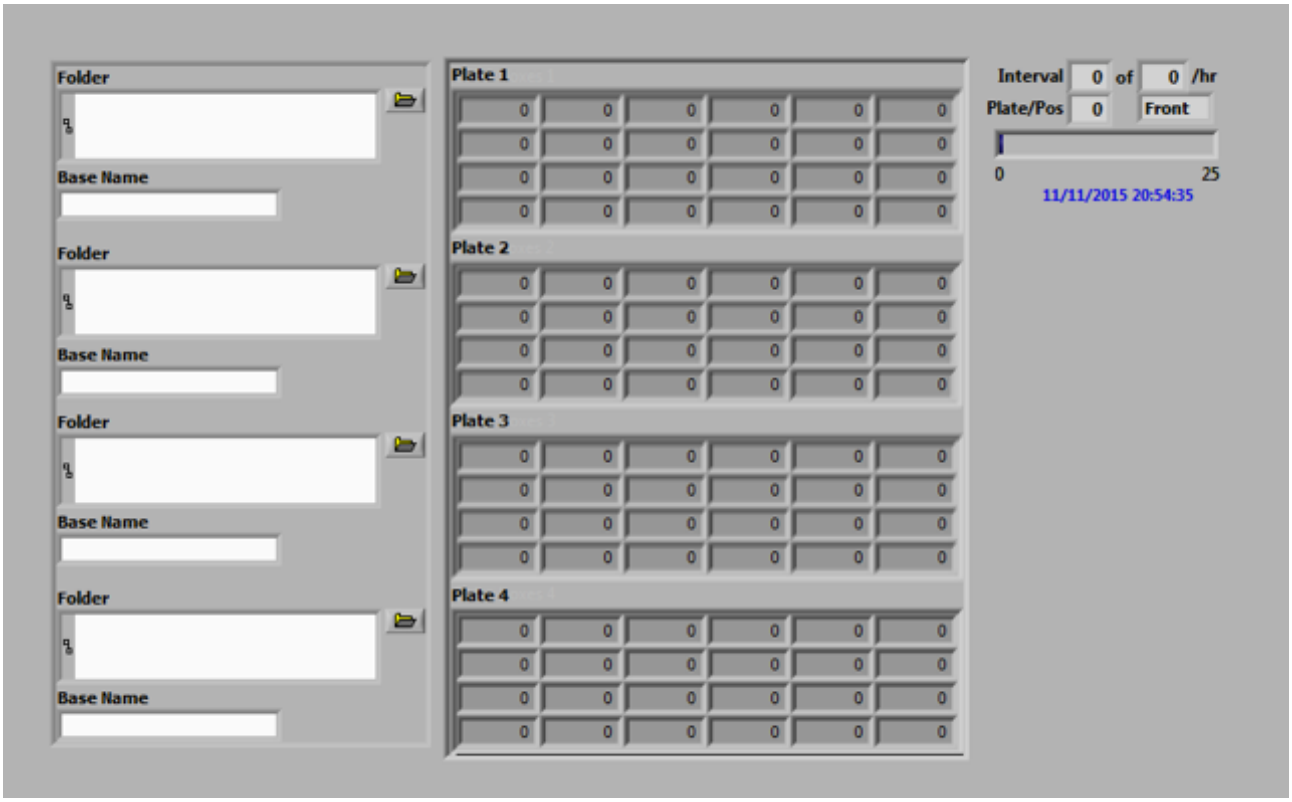
Replace the rear panel and top cover. Make sure every screw is in place, or the screw holes will leak light.

Use the COUNTER TEST PANEL when you start up the program to check that everything is working. The new PMT might start out having high counts, but it should settle after several hours of running.

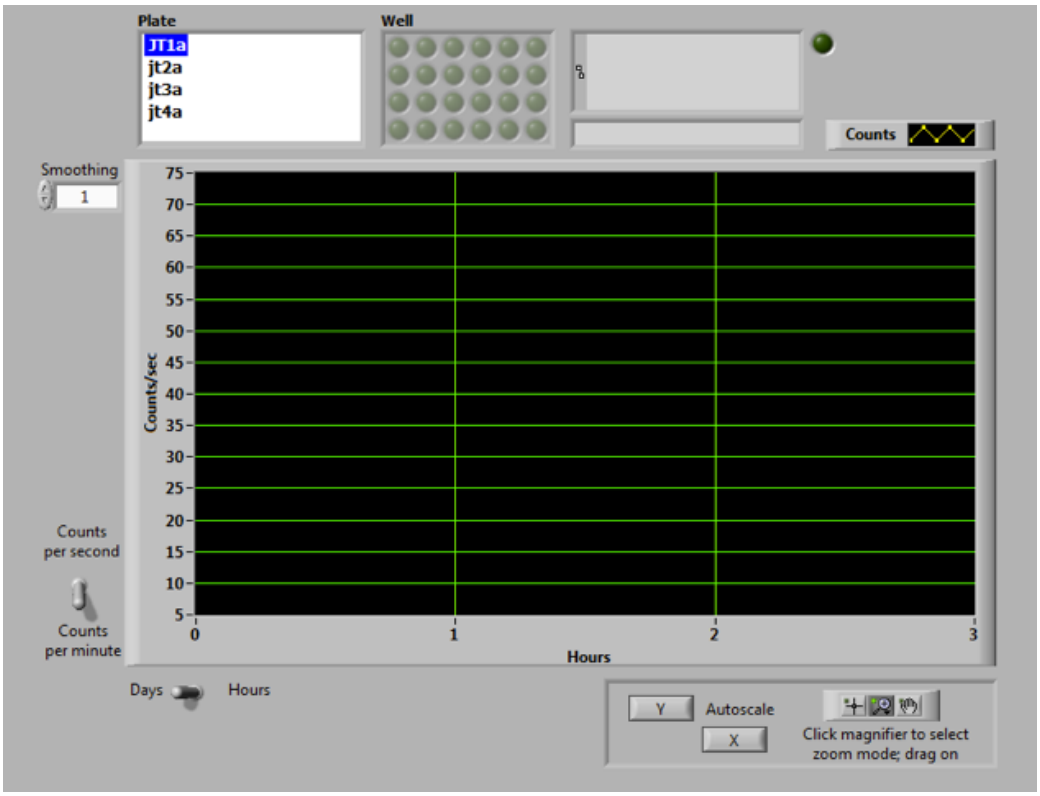
LumiCycle 96

There are very few differences between the LumiCycle 32 (LC32) and the LumiCycle 96 (LC96). In LC96, instead of setting the file names for each sample, you set the file name for each of the 4 plates. There is a browse button on a file control that you use to the set the folder where the files will go, one for each plate. There is also is a string control where you enter the base name of the files. Once you do that, the 24 files are named with the base name plus a number for each well, (A1, etc, through to D6).

The counts for the current counting period show up in the arrays labeled Plate 1 through Plate 4. The eight currently being counted at any moment will be highlighted in yellow.

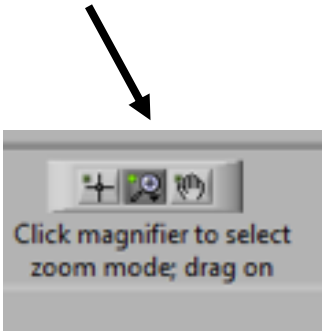


Double-clicking a number in the table opens up the window below, which shows all 24 traces for the selected plate (top left). Clicking on one of the 24 buttons in the “Well” section will highlight the trace for the selected well in yellow. Arrow keys navigate through the well, moving the selected well left/right or up/down. The Y and X buttons force autoscaling for the corresponding axis. The little graph palette next to the Y and X buttons allows you to zoom in on the graph in different ways (vertically, horizontally, stretch or condense).



If you click on the hand and place your cursor on the motion index graph at the top of the analysis window, you can drag the graph, left, right, up, or down.

Clicking on the magnifying glass will display a set of six icons.



The function of each icon, going left to right starting on the top row:

- Zoom horizontally and vertically over selected area of graph
- Zoom horizontally over selected area of graph
- Zoom vertically over selected area of graph

Bottom row of icons going left to right

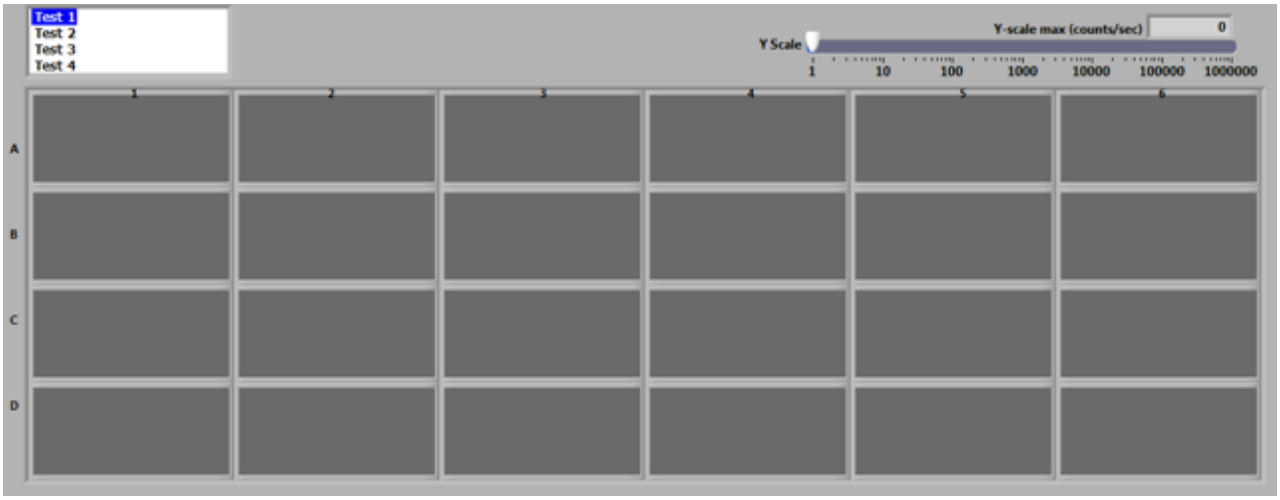
- Return graph to auto-scaled display
- Zoom graph vertically and horizontally
- Retract graph vertically and horizontally

After clicking on any of the top three icons, place your cursor on the Motion Index graph at the top of the analysis window, click and hold the left mouse button and then drag to the left or right (horizontal zoon), up or down (vertical zoom) or right/left and up/down (horizontal and vertical zoom).

After clicking on the second or third icon on the bottom row, just click on the graph. Each click will expand the graph (second icon on bottom) or retract the graph (third icon on bottom).

The effect, zooming, expanding, and retracting will take effect on the motion index graph, the bout indicator just below the motion index graph and the stimulus display below the bout indicator. The display will remain as you set it until you click on the first icon on the bottom, change the threshold or bout duration values or click on the motion index threshold graph on the bottom of the screen.

Another way to view all the traces (see picture below) as an array of graphs. The slider at the top changes the Y scale. It is accessed from the View All menu item. Channel setup allows you to select the count time for each set of samples. I STRONGLY recommend the longest sample times. 60 or 90 seconds. It saves wear and tear on the motors and connecting wires.



LumiCycle *In Vivo*

Operation of LumiCycle *in Vivo* is similar in principle to that of LumiCycle 32 and LumiCycle 96. Each unit contains 2 PMTs that record from one animal. The counts from the two PMTs are stored together in one data file, which can be opened and analyzed in the LumiCycle Analysis program. There, the two count streams can be analyzed separately, or as a sum.

Install the LumiCycle *in Vivo* program and CDM drivers from the Actimetrics web site.

Connect the power supply to the barrel connector on the side of the light-tight cabinet and plug the power supply into the wall power socket using the cable provided.

Connect the USB cable with the threaded hood to the corresponding connector on the side of the light-tight cabinet, and plug standard USB-A connector on the other end of the cable into a USB port, preferably on the back of the computer. You may connect as many as 4 LumiCycle *in Vivo* units to one computer, but each must be set internally to be a different unit ID.

Run the LumiCycle program. Depending on how many units you have connected to the computer, up to 4 lines of data will appear in the program.

Data display:

- Save: whether the data are being stored or not for this channel. The LumiCycle program runs continuously. Use the Save buttons to start and stop recording from different units. Normally, when you start a trial in one of the light-tight cabinets, you will start a recording with the Save

button and select a unique file name based on the animal's ID or on the experimental details. Stop the recording when the trial is finished. At any time during a trial, you can copy the data file to another computer and view its contents in the analysis program.

- Channel number.
- File name where the data are being stored.
- File length in days and hours.
- Counts for the two PMTs (third indicator is currently unused).
- Wait: After the internal light has been on, the program will wait a specified time before resuming recording. This indicator shows how much of the wait period remains.
- Light. Whether the internal LED light is on.
- Shutter: If the light is on, or the door is open, or the program is recording dark counts, the rotating shutter will close over the PMT openings. When you open or close the door, you will hear the shutter motor briefly as it opens or closes the shutter.
- Gate: An electronic (silent) gate signal that turns off the high-voltage power supply of the PMTs. The gate will "close" when the lights are on or the door is open.
- Temperature/Humidity: The current temperature and humidity in the cabinet.
- Lux: The current light level recorded in the cabinet in lux. Note that the detector is located on the wall of the cabinet and will not necessarily report the same level at the level of the animal.
- Light: The current brightness setting of the internal light.

Scrolling Count Display: The graph in the lower half of the main window shows a scrolling display of the counts one unit. Select which unit to view with the Channel control at the upper left of the graph. The two lines of the graph show the counts from the two PMTs.

Interval: The counts are collected for 1 minute and stored in the data file. The blue slider shows progress during each counting period, and the numerical indicators show which period of the current hour is being recorded.

Dark count correction

The PMTs' dark counts – thermally triggered counts in the absence of light – are enormously sensitive to temperature. Standard LumiCycles are usually kept in a temperature-controlled incubator, so the dark counts never vary. LumiCycle in Vivo is often kept in a the lab or animal quarters where the temperature can vary several degrees, often on a daily cycle. The resulting changes in dark counts can obscure or distort the rhythms in the animal's luminescence.

To control for temperature-induced variations in dark counts, LumiCycle can regularly measure the dark counts by recording for 1 minute with the internal shutter closed. These dark-count measurements are stored in the data file and later subtracted from the records to obtain a corrected measurements of luminescence. If dark counts are recorded, the subtraction is done automatically in the ClockLab Analysis program.

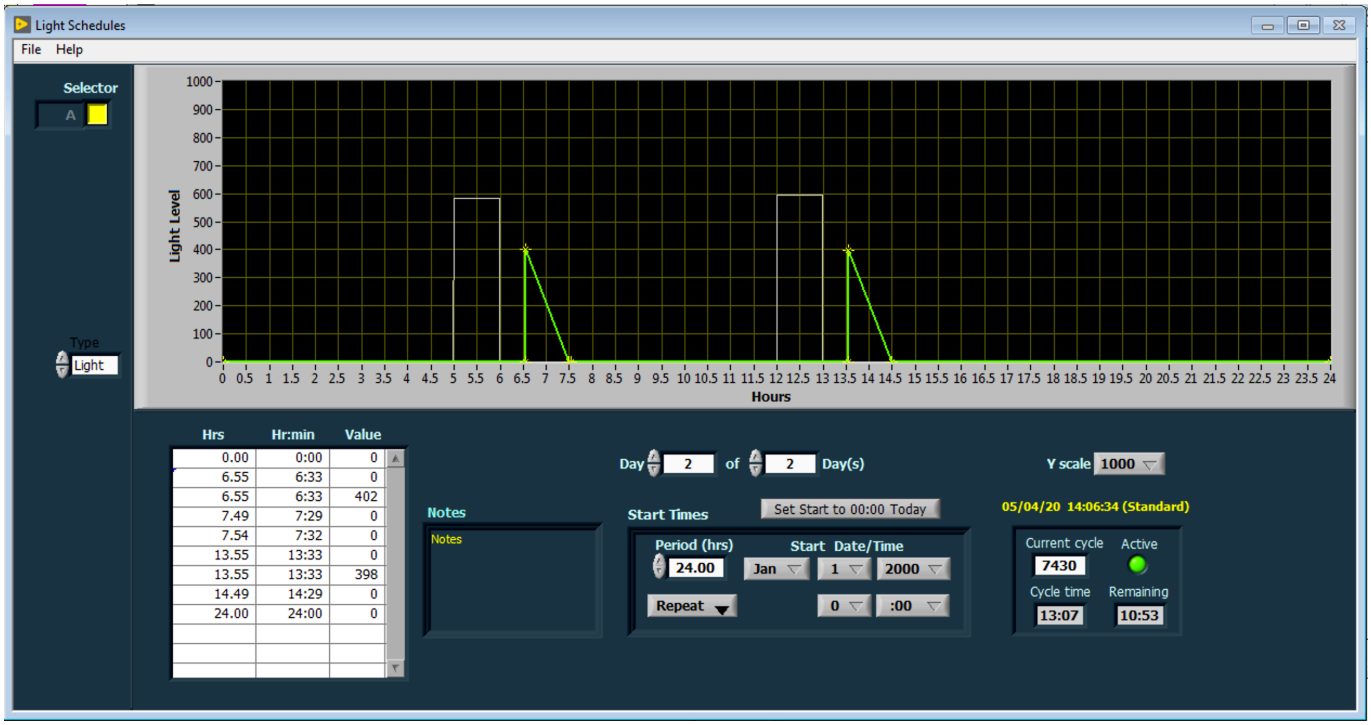
Settings Window

Select Settings->Recording from the main window's menus to set the two parameters that control recording.

- **PMT timeout**: While the lights are on, the PMTs, even though they are powered down, can sometimes collect charge and give artefactual counts for some time afterwards. To prevent these counts from distorting the recordings, the program can be set to wait for some minutes to start recording after the lights are turned off. How long to wait can depend on how bright the lights were. You can explore the proper setting for the timeout by recording from an empty cabinet with Timeout set to 0. See if there are extra counts after the lights are turned off.
- The **Dark Count recording Interval** settings determines how often the dark counts are recorded. The trade-off is higher resolution and accuracy in the dark count time measurements against a reduction in the proportion of time spent recording. 10 to 15 minutes is usually fast enough to provide a good dark-count measurement.

Light Schedules

The light-tight cabinet has a built in bright LED for creating daily light cycles for the animal. The light is located near the top of the cabinet on the left wall, just in front of the air vent. When the light is on, the high-voltage power to the PMTs is turned off, and the rotating shutter closes over the PMT opening. The shutter is not completely light-tight and so some counts will still appear on the PMTs. See PMT Timeout above.



To set a schedule for the lights, select the Settings->Light Schedules… menu item. The Light Schedule window is shown below.

Context Help. To display information about each control or indicator, select the Help->Show Context Help menu item and then hover the cursor over a control or indicator.

Selector. Selects the Channel (Chamber) to be configured. The selector array will have two columns if each chamber has two different light sources to be configured (Sensor board and Control board). Click on one of the buttons to select a Channel.

Set Start to 00:00 Today. Click this button to set the start of the schedule to midnight of the current data.

Start Times. This cluster of controls is used to set the Start Time of the schedule, the Period of the cycles in the schedule, and whether the schedule repeats when completed or is only performed once.

Current Status. This cluster of controls shows the current status of the Schedule: Which cycle of the schedule (if it is repeating), time into the cycle, time remaining in the cycle, and whether it is active. A cycle will be inactive if it set to repeat once and has yet to begin or is already finished.

Day/Day(s). **Day(s)** sets the number of days in the complete schedule. **Day** selects one of those days for editing in the Schedule Graph.

Schedule graph. This graph is an active control, used to configure the light output waveform over time. The Light or Temperature graphs for all Days of the cycle are superimposed. The active graph (the one that is currently being edited) is shown in green; the rest are shown in gray.

Note that the units on the Y axis of the Light Schedule graph are arbitrary (and therefore not Lux), where1000 represents the maximum current that can be supplied to the LED lights. The Y-axis scales can be altered using the Y-scale control.

- To select one of the plots (i.e. one of the circadian days of the schedule) for editing, *double-click* it. It becomes green. Alternatively, use the Day control.
- To modify the waveform, *drag* the yellow cursors or the green line segments. To constrain the motion of the cursor or segment to be horizontal or vertical, hold the *shift key* down BEFORE clicking.
- To add or delete a cursor (and therefore a line segment), *right-click* and select the appropriate item from the short-cut menu that appears.
- To force a line segment to be perfectly vertical or horizontal, *right-click* and select the appropriate item from the short-cut menu that appears.
- To copy the plot for one day, *right-click* and select Copy Day, change to a different day using the Day control, or by double-clicking on the corresponding plot, and then *Right-click* again and select Paste Day.

- To copy a complete schedule from one chamber to another, right-click and select Copy Schedule, use the Selector buttons to move to another chamber, then right-click and select Paste Schedule.

In the example shown above, a 2-day schedule has been set, with two square waves on day one and two triangle waves on day two. Note that 8 cursors (inflection points) are required to make the wave-form on day 1 and 6 cursors for day two.

Schedule Table. The table at the lower-left of the window shows the locations of all the cursors in the currently selected day. The values in the first and last columns of the table can be edited. Double click a cell, type in the desired value and hit the Enter key. The Schedule graph should change accordingly.

Notes. Any notes entered here will be stored and displayed again when the Chamber is selected.

View Selected

Use this window to view the graphs of current trials. The counts from both PMTs are shown.

LumiCycle Analysis Program

The LumiCycle Analysis program is available on the Software download page of the Actimetrics web site. To download, you will need a user ID and password.

User ID: behavioral

Password: Neuro11

You may install the analysis program on as many computers as you like. It's best not to use the data collection computer for analysis, however.

Open a data file using the FILE->OPEN menu (or double-click on the file indicator at the top of the window), which will present a standard Open File dialog. Use the FILE->NEXT (or FILE->PREVIOUS) menu item to open the next file (or previous) in the current folder (in alphabetical order). You may also open one of the sample files provided with the program (FILE->OPEN SAMPLE FILE).

Note that the OPEN and NEXT commands pay attention to the PREFERENCES->USE FILE EXTENSION setting. If this option is checked, the open-file dialog will show and open only those files with the extension 'lcfd'. If it is left unchecked, then all files will be shown. Similarly, the NEXT command will look for lcdf files or all files depending on the setting of this option.

Baseline fit: When a file is opened, the data are displayed in the upper graph in the window. The program then attempts to remove any baseline drift from the data. It does so in one of three ways, selected by the tabs at the top left of the main window.

Polynomial Fit: Fits a polynomial curve to the data. The polynomial is shown as the smooth red trace and is subtracted from the raw data (white) to give the curve displayed in the lower Baseline Subtracted graph in the window. The polynomial is of the order specified in the **Poly Order** control. This number roughly corresponds to the number of inflections or bends that are allowed in the fitted curve. Choose the Polynomial Order carefully. Too large a number will allow the fitted curve to follow (and thereby remove) the daily fluctuations that are the significant parts of the data. Too small a number will prevent the program from fitting the baseline drift properly. Start with a number that is approximately the number of days included in the data. The **Smooth** control is used to smooth the small-amplitude, rapid fluctuations in the data. When set to 5, for example, each point in the trace is replaced by an average of itself and the 4 surrounding points (2 on each side).

Running Average: The baseline curve (red) is calculated as a running average of the data. As for Polynomial Fit, the baseline curve is subtracted from the raw data to derive the Baseline Subtracted curve. When 12 **Hours** is selected, for example, each point in the baseline curve is an average of the corresponding point in the raw data and the surround 12 hours of data. The Smooth control operates the same as for Polynomial Fit (above).

Butterworth: The raw data are passed through a Butterworth digital band-pass filter to arrive at the Baseline Subtracted graph. The filter **Order** (number of poles), **high cutoff period** and **low cutoff period** are set in the corresponding controls.

Sometimes a record will have a large, seemingly spurious decay at the start of a trace, as the tissue adjusts to being in culture. Better baseline fits are obtained when this part of a record is not included in the fit calculation. To limit the portion of the data to which the baseline fit is applied, use the light blue vertical **cursors** in the baseline fit graph. Move the cursors by dragging them. Or you can CTRL-click in the

graph to move the nearest cursor to the point you click on (also helpful if the cursors are off the edge of the graph and not visible). The baseline subtraction is updated whenever the cursors are moved, with the baseline subtracted data appearing in the lower graph.

Smooth. Smooths the trace by averaging N adjacent points together, where N is the setting in the Smooth control.

Period calculation. Adjust the cursors in the lower (baseline-subtracted) graph to set the portion of the data on which the period calculation is performed. There are 6 methods available for finding the dominant period in the data. Select the method from the pull down menu, and start the calculation by hitting the **Fit** button (or hit the <Enter> key). You may also have the program automatically calculate the fit when each file is opened by checking the **Auto** button near the Fit button.

You may select a portion of the Baseline-Subtracted data for fitting using the vertical blue cursors. As for the Raw data graph, CTRL-clicking in the graph brings the nearest cursor to the clicked point. For example, if you have applied a drug in the middle of a trial that you expect to change the period or phase, you can estimate these two parameters for the two parts of the data independently by setting the cursors and clicking the **Fit**.

For most of the period calculations, a best-fit curve is displayed in red (derived from the most likely period and damping parameters) and the **Goodness of Fit** is displayed. The goodness of fit (%variance accounted for) is calculated from the least-squares (point-by-point) distance between the data and the fitted curve, divided by the total variance of the data (relative to the mean). The number shown is 100 times 1 minus this ratio.

Sin fit. With this method, the program looks for the largest sinusoidal component in the data. Note that this calculation is related to a Fourier transform but differs in significant ways. The program calculates the dot product between the data, $Y(t)$, and sine and cosine curves of a range of closely spaced periods. The corresponding amplitude, A, of the sinusoidal component for each period is the square root of the sum of the squares of the two dot products:

$$A(f) = \sqrt{\left(\sum Y(t) \times \sin(2\pi f t)\right)^2 + \left(\sum Y(t) \times \cos(2\pi f t)\right)^2}$$

A is plotted as a function of Period (1/frequency) in the Amplitude spectrum graph at the lower left. The best fit sine wave (the one that yields the largest A) is shown in the lower data graph in red. The period of the peak of the graph, and the phase of the best fit sine wave are shown in the slider controls at the lower right.

Sin fit (damped). Fit to a damped sine wave. Once the best frequency is found from the equation above, the damping of the oscillations in the data are fit to a negative exponential. The exponential is fit to the values of Y occurring at the times of the peaks and troughs of the best fit sine. The fitted curve (red) is now the best-fit sine multiplied by the best-fit exponential. The damping constant (tau) is reported in the slider controls to the lower right.

Sin fit (undamp). Amplitude, frequency, phase and damping are calculated and reported as in the **Sin fit (damped)** case, above. In this case, however, the data values, Y(t), are divided by the best-fit exponential in order to reconstruct the underlying, undamped sine wave. Note that the reconstruction gets noisier as the amplitude of the oscillations in the original data wane.

LM Fit (Damped Sine). In the above fits, the frequency and damping are determined independently (in sequence). That is, the frequency is found first from the sine-wave fitting, and the damping is found afterward on the basis of this assumed frequency. In the LM calculation, 5 parameters – Amplitude, frequency, phase, damping tau, and offset (*C*) – are allowed to vary independently in a gradient-descent fitting procedure. The Levenberg-Marquardt algorithm is used to find the set of parameters that gives the least-squared distance between the data and the equation:

$$Y(t) = Asin(2\pi ft + \phi)e^{\frac{t}{\tau}}+\mathrm{C}$$

The starting values given to the LM algorithm for A, f, t, τ, and C are taken from the damped-sine fit. This method is slower than the purely spectral methods, often gives better overall fits to the data, but usually gives similar results for the derived period.

LM Fit (Sin). Similar to LM Damped Sine fit, but here tau of the exponential decay is assumed to be infinite (no damping).

Periodogram. Uses the Bushell-Sokolov algorithm (*J Theor Biol.* 1978, **72**:131-60) for the chi-square Periodogram to find the best period. No damping is calculated.

FFT. Performs an FFT on the subtracted data and displays it in the Spectrum graph. No fit is performed. Instead, the Amplitude indicator shows the fraction of signal power (or amplitude) of the signal contained in the range of periods delineated by the yellow cursors in the spectrum graph. Power is shown when the **Power Spectrum** checkbox is checked. FFT Amplitude is shown when the checkbox is not checked. The data can be windowed by a **Blackman-Harris window** before the FFT is performed by selecting the checkbox. Windowing smoothes the FFT and reduces edge effects that occur at the abrupt ends of the data.

By definition, the frequencies (periods) of the FFT are spaced at fixed interval determined by the length of the record, *T*. The shorter *T* is, the wider the spacing between sampled frequencies (periods). For a 10-day record, the FFT will give amplitude values for points that are spaced over 2 hours apart in the region around 24 hours. Since the dominant period of a record rarely falls on the one of the sampled frequencies, the power of the oscillations end up being split between the two adjacent frequencies. Or, if the period changes somewhat during the record, the power will be distributed among several of adjacent frequencies.

Exporting Fit Parameters. You may export the fitted parameters to an Excel-compatible text file by selecting the EXPORT->FIT PARAMETERS (CURRENT) menu item. This adds a list of the fitted parameters to the currently specified file. The default file is "Cycle Parameters.csv", located in the Analysis Folder, which is created in the folder containing the currently open data file. Along with the fit parameters and goodness of fit, also exported are the locations of the blue cursors and the time of occurrence of the maximum in the fitted curve (t-max). By selecting EXPORT->FIT PARAMETERS (ADVANCE TO NEXT FILE), the parameters are exported and the next data file in the current folder is opened (as if CTRL-N had been pressed).

Use the EXPORT->FIT PARAMATERS (BATCH) to select multiple files for analysis. Each file will be opened in turn, and the fit parameters will be added to Cycle Parameters.csv.

Exporting Data as Text. The data making up each trace can be exported using the EXPORT->AS TEXT menu items. Either raw data, or baseline-subtracted data can be exported. Note that only the portion of the

data within the cursors in the appropriate graph are exported. The exported text files can be opened in ClockLab or any text editor.

Maxima and Minima. The program automatically detects peaks and troughs in the circadian data. To do so, it fits each 8-hour portion of the trace to a 2nd-order polynomial and uses the coefficients from the fit to determine whether a peak or valley is present. The peaks and troughs are visible in the subtracted trace as green points. These can be made hidden or shown using the PREFERENCES->SHOW/HIDE MAXIMA/MINIMA menu item.

Export the locations and amplitudes of the Maxima and Minima using the EXPORT->MAXIMA/MINIMA menu item. The values are written to a csv file in the Analysis Folder.

Composite View. It is sometimes useful to compare data from a number of different trials or to superimpose different traces for making slides and figures. It is possible to do so using the Composite feature of the program. Send a trace to the Composite window using the EXPORT->TO COMPOSITE menu items. Open the Composite window with the VIEW->COMPOSITE menu item. The Composite window used to be modal (it was always on top and had to be closed to return to the main window). As of version 1.30, it can be sent to the background or minimized without closing.

Actogram. The Actogram window will display the data in a form similar to an actogram used for circadian activity records. It is useful finding phase shifts and period shifts caused by manipulations of a sample in the course of a trial.

Periodograms. You can display a Chi-square Periodogram of the baseline-subtracted data by selecting the VIEW->PERIODOGRAM menu item. The calculation is derived from Sokalove, G. and Bushell, W. (1978) The Chi Square Periodogram: its utility for analysis of circadian rhythms. Journal of Theoretical Biology 72:131-160.

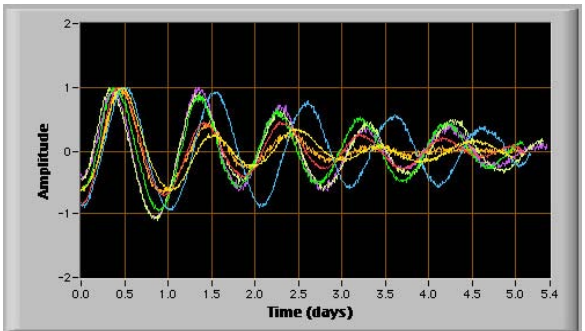
The VIEW->PERIODOGRAM menu item displays an animated version of the Periodogram calculation. For each value of Tau, the count record is broken up into pieces, each of length Tau. These are superimposed (the light-blue traces) and averaged (the yellow trace). The value of the growing Periodogram is calculated as the standard deviation of the yellow trace for each value of tau. You'll see that when the tau is such that the blue traces are more or less in synch with one another, the yellow trace grows in amplitude, along with its standard deviation. You can slow down or speed up the animation with the Display Speed control. The significance line (red line in lower graph) is controlled by the Significance pop-up menu (see Sokalove and Bushell).

Composite Window

The Composite Window, and its Display sub window, are used to superimposing and comparing multiple traces. Most features of the graphs can be altered to taste.

Once a graph appearance is set to your liking (below), right click on it and select **Copy Data**; then Paste the graph into Photoshop or similar programs. Or use the ALT-PRNT SCRN key to copy a picture of the screen into the clipboard, then paste into Photoshop. The Large Format window (below) may also be printed using its **Print** button.

Add traces (Raw of Baseline-Subtraced) to the Composite view using the EXPORT->TO COMPOSITE menu items in the main window. Only the portion of each trace between the cursors is sent to the composite. The maximum number of traces that can be added to the composite is 30.



Once all the traces have been added to the graph, it can be saved to a file (extension "lccd") using the FILE->SAVE menu item. This file can be read in (FILE->OPEN menu) at a later time. New traces can then be added to the graph.

To view the Composite window without sending a new trace to it, select the VIEW>COMPOSITE menu item in the Main window. The Composite window can remain open even after you return to the Main window. The Composite window can also be minimized.

The **Files** listbox shows the list of traces that have been added to the composite graph. To figure out which trace is which, click the **Highlight Selected** button on (pointing to the right) and click on a line in the listbox. The corresponding trace will be displayed one point-size larger than the rest of the traces.

Delete a trace by selecting it in the Files listbox and selecting the EDIT->DELETED SELECTED menu item. Delete all traces with EDIT->DELETE ALL menu item.

The **Linewidth** control changes the width of the displayed lines.

The **Background** and **Grid** controls change the background and grid colors of the graph.

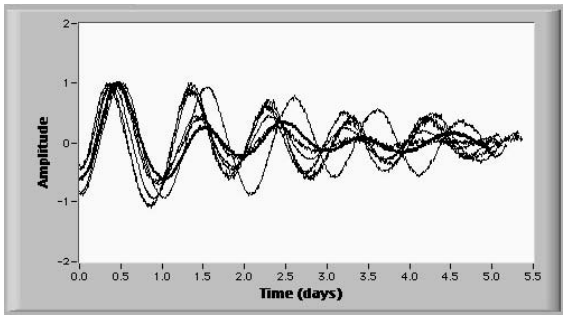
For example, you can select a white background and grid with black traces to make a figure for publication.

The **Trace** control allows you to select the color of the traces. The **Defaults** button returns the background and grid colors to their defaults.

The **Smooth** control sets the smoothing of the traces. The smoothing is done using a median filter, in which each point of the trace is replaced by the median of the N+1 surrounding points, where N is the control setting.

The **Normalize** button scales each trace to a maximum height of one.

The **Align** button aligns the displayed traces to start at time zero (instead of the real time of day of each trace).



Once the various features of the graph are chosen and the desired traces have been imported, graph can be opened in larger, high-resolution format by selecting the VIEW->LARGE FORMAT menu item. A new window is opened with the same graph attributes but with additional features:

- In the large format graph, the traces are anti-aliased so that they will look better in print.
- Adjust the size of the graph using the **Width** and **Height** controls.
  Adjust the attributes of each trace by clicking on corresponding line in the **Graph Legend** to the right of the graph. Line width, plot style, point style and so on are endlessly variable.
- Change the X or Y scale of the graph by double-clicking the top or bottom tick labels on the Y scale, or the left or right tick labels on the X scale and typing in the desired value.

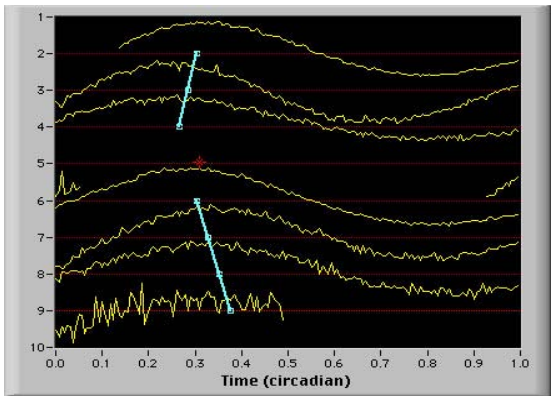
The display of the traces in the Large Format graph can be slow because of the anti-aliasing calculation done on each trace.

Actogram Window

The Actogram Window presents the data from a single trial in a form similar to an actogram used to display circadian activity data.

Selecting the EXPORT->TO ACTOGRAM (SET 1) menu item in the main window sends the data between the cursors in the lower baseline-subtracted graph of the main window to the Actogram window. The data are displayed in multiple lines, each line representing one circadian day of the record. The length of the day is specified by the **Tau** control. The **24** button and the **Period** button set **TAU** to 24 hours, or to the period determined by the Fourier Fit performed on the data in the main window (displayed in the **Period** indicator in the Actogram window).

The **Normalize** switch determines whether or not each line is scaled to the same amplitude in the display or whether they are displayed according to their relative height in the original record. The **Scale** slider control scales each line relative to their vertical spacing.



The Offset control shifts the data left or right be the specified number of hours in the display.

A **Cursor** (red asterisk) is provided in the graph. Drag the cursor to any point of the graph to display clock time and circadian time of the point in the cursor indicators to the right of the graph.

In addition to the baseline-subtracted data, the time of the peak from each cycle of the record is shown (open blue symbols). These peak times are calculated from the Fourier transform of the entire imported part of the record, not just from the local maximum in the data.

Phase Shift Calculations

It is possible to calculate phase shifts between two portions of record.

- Select the first portion of the data to be measured using the cursors in the lower (baseline-subtracted) graph of the main window.
- Click the FIT or UNDAMP button to calculate the Fourier transform.
- Select the EXPORT->TO ACTOGRAM (SET 1) menu item.
- Close the Actogram.
- Select the second portion of the data, again using the cursors in the lower graph of the main window.
- Click the FIT or UNDAMP button to calculate the Fourier transform.
- Select EXPORT->TO ACTOGRAM (SET 2) menu item.
  The Actogram will now show both sets of data, with separate linear fits to the peaks of the two portions.
  Adjust the **TAU** and **OFFSET** so that the two fitted lines are as closely vertical as possible.
- Place the red cursor over the line representing the day on which phase shift occurred.

The Phase Shift indicators will show the horizontal distance between the two fitted lines at the point at which they cross the selected day, both in circadian and clock time.