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Building a simple and versatile illumination system for optogenetic experiments

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Corresponding Author:	Phillip Kyriakakis, Ph.D. University of California San Diego La Jolla, CA UNITED STATES
Corresponding Author's Institution:	University of California San Diego
Corresponding Author E-Mail:	pkyriaka@ucsd.edu
Order of Authors:	Phillip Kyriakakis, Ph.D.
	Lourdes Fernandez de Cossio
	Patrick Howard
	Sivleng Kouv
	Marianne Catanho
	Vincent Hu
	Robert Kyriakakis
	Molly Allen
	Yunhan Ma
	Marcelo Aguilar-Rivera
	Todd Coleman
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TITLE:

Building a Simple and Versatile Illumination System for Optogenetic Experiments

AUTHORS AND AFFILIATIONS:

Phillip Kyriakakis[1], Lourdes Fernandez de Cossio[2], Patrick Wade Howard[1], Sivleng Kouv[1], Marianne Catanho[1], Vincent J. Hu[3], Robert Kyriakakis[1], Molly E. Allen[1], Yunhan Ma[4], Marcelo Aguilar-Rivera[1], Todd P Coleman[1]

[1]Department of Bioengineering, University of California, San Diego, 9500 Gilman Drive, La Jolla, California, United States

[2]University of California, San Diego, 9500 Gilman Drive, La Jolla, California 92093-0412, United States

[3]Department of Mathematical Computational, and Systems biology, University of California, Irvine 402 E Peltason Dr, Irvine, CA, United States

[4]Department of Biomedical Engineering, Duke University 4101 Science Drive, Durham, NC, United States

Corresponding Author:

Phillip Kyriakakis (pkyriaka@ucsd.edu)

Email Addresses of Co-Authors:

Lourdes Fernandez de Cossio (l4fernandezdecossiog@health.ucsd.edu)
Patrick Howard (phoward@ucsd.edu)
Sivleng Kouv (sikouv@ucsd.edu)
Marianne Catanho (mcatanho@eng.ucsd.edu)
Vincent Hu (huvj@hs.uci.edu)
Robert Kyriakakis (rkyriakakis@gmail.com)
Molly Allen (meallen@eng.ucsd.edu)
Yunhan Ma (yunhan.ma@duke.edu)
Marcelo Aguilar-Rivera (mia003@eng.ucsd.edu)
Todd Coleman (tpcoleman@ucsd.edu)
Phillip Kyriakakis (pkyriaka@ucsd.edu)

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SUMMARY:

This protocol describes how to perform optogenetic experiments for controlling gene expression with red and far-red light using PhyB and PIF3. Included are step-by-step instructions for building a simple and flexible illumination system, which enables the control of gene expression or other optogenetics with a computer.

ABSTRACT:

Controlling biological processes using light has increased the accuracy and speed with which researchers can manipulate many biological processes. Optical control allows for an unprecedented ability to dissect function and holds the potential for enabling novel genetic therapies. However, optogenetic experiments require adequate light sources with spatial, temporal, or intensity control, often a bottleneck for researchers. Here we detail how to build a low-cost and versatile LED illumination system that is easily customizable for different available optogenetic tools. This system is configurable for manual or computer control with adjustable LED intensity. We provide an illustrated step-by-step guide for building the circuit, making it computer-controlled, and constructing the LEDs. To facilitate the assembly of this device, we also discuss some basic soldering techniques and explain the circuitry used to control the LEDs. Using our open-source user interface, users can automate precise timing and pulsing of light on a personal computer (PC) or an inexpensive tablet. This automation makes the system useful for experiments that use LEDs to control genes, signaling pathways, and other cellular activities that span large time scales. For this protocol, no prior expertise in electronics is required to build all the parts needed or to use the illumination system to perform optogenetic experiments.

INTRODUCTION:

Optogenetic tools are becoming ubiquitous and new technology is constantly being developed to optically control biological processes such as gene expression, cell signaling, and many more[1–3]. The ability to control cellular processes with light allows for fast kinetics, tight spatial control, and dose-dependent regulation that can be controlled by light intensity and exposure time. To use these tools, a device to control these parameters is necessary. We have recently developed a genetically encoded PhyB-PIF3 mammalian gene switch that reversibly activates and deactivates genes using red/far-red light, respectively[4]. This system was tested in several mammalian cell lines and enabled the unparalleled induction of gene expression even with very small amounts of light, including pulses of light. Researchers wishing to use the PhyB switch and similar tools[5,6] frequently request information on methods to control illumination intensity and duration. Therefore, we developed this protocol with step-by-step instructions to enable broader adoption of these tools for optogenetics.

Before the widespread use of LEDs, broadband light sources with filters were used to study light-responsive proteins such as phytochromes[7]. Recently, some LED illumination systems have been published along with optogenetic tools[8–12], but those protocols can require significant expertise in electronics/software, require specialized equipment (e.g., 3D printers, laser cutting machines, or photomasks), or do not provide the step-by-step instructions some researchers would need to deploy for their research needs. While independent control of individual wells in a multiwell plate can be useful, it is often unnecessary when researchers only need to compare several different samples in light and dark or red light versus far-red light. Also, many existing commercial systems are expensive, with limited customization capability. However, the LEDs described in this protocol are cost-effective, bright, and can be mounted in many ways; therefore, they can be used to illuminate several different types of samples. With the protocol and the software provided, LEDs ranging from ultraviolet (UV) to NIR can be used and controlled with software to perform optogenetic experiments using UVR8[13,14], Dronpa[15,16], LOV domains[17,18], Step Function Opsins[19,20], CRY2[21,22], PhyB[4,23–25], bacterial phytochromes[26–29] and other light-responsive systems[30–32].

This protocol constitutes a tutorial for the assembly of the circuits and other hardware needed to control different parameters for light stimulation as well as the molecular/cellular tools to run an optogenetic experiment. In addition, we report plasmids optimized from Kyriakakis et al.[4] that are smaller and more stable for cloning. Through this protocol, biologists without expertise in electronics and optics can build illumination systems that are flexible and robust. In a step-by-step fashion, we show how to build LED systems, removing the technical bottleneck for the broader adoption of optogenetic tools. This system can easily be used in most cell culture incubators, even if they do not contain wire ports. For example, we have kept the LED system in a humidified CO_2 incubator continuously for more than 6 months with no decrease in performance. We also explain how to connect the LED system to a computer and interface it with open-source software we provide on GitHub (https://github.com/BreakLiquid/LED-Control-User-Interfaces). Building a system using this protocol provides researchers the basic knowledge to debug potential issues, replace parts, and improve/extend functionalities.

System overview

Building the illumination system involves (1) building the electronic circuit, (2) building the peripherals (power supply cord, power switch, etc.), (3) building the LEDs, (4) assembling all of these components, and (5) installing the software to control the LEDs with a user interface (**Figure 1A**). Once completed, the illumination system can control up to four LEDs independently with a user interface (**Figure 1B**). The user interface enables each LED to pulse at specified time intervals and shut off after a specified time. There is also a start-delay to begin illumination programs at a specified time. Potentiometers (POTs) regulate each LED's intensity independently or can be used for manual LED control without a computer. The wires to the LEDs can be any custom length, allowing them to be easily placed in an incubator or lab space. Due to these LEDs' high power, they can be used to illuminate a large area with a single LED from a distance.

LED driver description

To power and control the LEDs' intensity, this protocol will go through steps to build an "LED driver". Each LED has a range of voltages in which it operates (**Figure 1C**). During operation, the regulator's output voltage, which controls the light intensity, can be tuned by a potentiometer. The POT varies the resistance, adjusting the output voltage/brightness. Tuning with a 1kΩ (1 kilo-ohm) POT gives what we call the "high-voltage circuit" and has a range of 1.35 V to 2.9 V. Since 2.9 V is too high for operating the lower voltage LEDs (**Figure 1C**), we show a single modification (Resistor 3 or "R3" **Supplementary Figure 1A**) that limits the range to match the low voltage LEDs. R3 serves to decrease the maximum voltage applied to the LEDs to 1.85 V (assembly detailed in **Supplementary Figure 8**) when in parallel with the potentiometer. By using voltage to control brightness instead of current, the system is more flexible for LEDs with different operating voltages. **Figure 1C** contains a list of the high and low voltage LEDs to guide the optimal circuit selection. This design keeps the minimum voltage low enough so that the LED is completely off when the potentiometer is off and does not allow the voltage to go above the typical operating voltage of the LED. For PhyB optogenetics, we use deep red and far-red LEDs, which use the low-voltage circuit.

LED computer control system description

The LED illumination system can be used for constant illumination without a computer or microcontroller. However, for pulsing programs and for controlling individual LED timing, a microcontroller must be installed. To use a microcontroller to control the LEDs, a transistor is required to connect the microcontroller to the circuit. This transistor senses voltage from the microcontroller and switches from being conductive or insulating. To control the "on" and "off", we use what is called an "NPN switching type transistor" (2N2222) as a controllable shunt across R2 (**Supplementary Figure 1A**). When the voltage from the microcontroller is applied to the transistor base, the transistor becomes conductive and makes the LED voltage low, turning the LED off. Thus, the LED and transistor on and off states are directly controlled by the microcontroller, which is controlled by the software installed on the PC.

For making the illumination system, the following steps are required: Build the electrical circuit; build the power supply, manual power switch, POTs, and microcontroller connection; build the LEDs; accommodate a black box to fit the illumination system; connect all of the wiring and devices; install the LED control software, stimulate the cells with light; measure gene expression using a dual luciferase assay.

PROTOCOL:

1. Build the electrical circuit

NOTE: The protocol for building a single circuit for an available LED is described here. Instructions to expand this up to four LEDs are included in the supplementary info.

1.1. Turn on the smoke absorber and the soldering iron. Add water to the wiping sponge, have the solder at hand.

CAUTION: Make sure to take safety precautions to remove smoke and prevent burns.

1.2. Begin soldering circuit components to the printed circuit board (PCB board) in the order shown in the supplementary panels.

NOTE: Use a small amount of solder on the soldering iron tip to heat the metal of the component and the PCB board first and melt additional solder directly onto the components; flux can help a lot.

1.3. Solder jumper wires and components (**Supplementary Figure 2** and **Supplementary Figure 3**).

1.3.1. For the jumper wires (the insulated wiring that connects two points on the circuit board), use two pieces of orange [7.6 mm (0.3 inch)] and yellow [12 mm (0.4 inch)] wires from the jumper kit.

1.3.2. Clip the PCB board onto the "helping hands" and insert the jumper wires in the following pinholes, bend the terminals 45 degrees and add flux (**Figures 2, Supplementary Figure 2** and **Supplementary Figure 3**): a1 and a3 → ground (-) (orange), a7 → power supply (+) #7 (yellow), d2 → d6 (yellow).

1.3.3. Solder and then trim the back of the wires.

1.3.4. Insert LM317T voltage regulator in the following pinholes, bend the pins, and add flux (**Figures 2** and **Supplementary Figure 4**): Adj → e5, V_{out} → e6, V_{in} → e7.

1.3.5. Solder the left and right terminals first, trim them, then solder and trim the middle terminal.

1.3.6. To set the low voltage range of the circuit, insert an 820 Ω resistor all the way down into pinholes, solder and trim c2 → c5 (**Figures 2** and **Supplementary Figure 5**).

1.3.7. To enable LED control by the microcontroller, insert the transistor into b3–b5 (**Figures 2** and **Supplementary Figure 6**): Collector → b3, Base → b4, Emitter → b5.

NOTE: Be aware of the orientation of the transistor to insert correctly; check the specs to find the Collector, Base, and Emitter designation.

1.4. Solder the wire-to-wire connectors for the POT, LED, microcontroller, and power source.

NOTE: Pay attention to the color of the wires of the wire-to-wire connectors and whether using a female or male wire-to-wire connector.

1.4.1. Determine whether a "low voltage" circuit or "high voltage" circuit is required for the desired LED (**Figure 1C**).

NOTE: If the LED is on the "low voltage" list, a resistor in parallel with the POT is required.

1.4.2. For the "low voltage" or "high voltage" circuit, place the wire from a female wire-to-wire connector through hole a5 (**Supplementary Figure 7**). Do not solder into place yet if making the low voltage circuit.

NOTE: Twist the bare wire ends so that the small wire hairs don't flake out. If the wire seems too thick to push through the pinhole without fraying, cut 2–6 strands and then twist them back together (**Supplementary Figure 7B–D**).

1.4.3. If making the "high voltage" circuit, skip to step 1.4.5. If making the "low voltage" circuit, push a 560Ω resistor through the same hole (a5) and solder with the wire-to-wire connector lead.

1.4.4. Connect the other end of the resistor to the ground (**Supplementary Figure 7G**).

1.4.5. Insert the other end of the female wire-to-wire connector soldered into a5 hole connecting it to the ground and solder it (**Supplementary Figure 8A,B**).

1.4.6. For the microcontroller connection, insert one end of a male wire-to-wire connector into hole a4 and the other into a hole connected to the ground (**Supplementary Figure 9A–C**).

1.4.7. For the LED connection, insert one end of a female wire-to-wire connector into hole a2 and the other end into a hole connected to the ground (**Supplementary Figure 9D,E**).

2. Build power supply, manual power switch, POTs, and microcontroller connection

2.1. Build the power supply.

2.1.1. Solder an orange [7.6 mm (0.3 inch)] jumper from a29 to the ground (**Supplementary Figure 10**).

2.1.2. Solder a female wire-to-wire connector from a30 to the power supply (+) (**Supplementary Figure 11A–C**).

2.1.3. Solder a male wire-to-wire connector from c29 to c30 (**Supplementary Figure 11D–F**).

2.1.4. Cut the connector off a power supply cord, expose the wires, and strip them (**Supplementary Figure 12A–C**).

2.1.5. Add flux to the wires prior to soldering using a flux pen (**Supplementary Figure 3G**).

2.1.6. Place a 3.18 mm (1/8 inch) shrink tube around a male wire-to-wire connector and a thicker piece 4.76 mm (3/16 inch) over the power supply wire (**Supplementary Figure 12D**).

2.1.7. Twist the wires from the power supply and the male wire-to-wire connector together and solder (**Supplementary Figure 12E, 13A,B**).

2.1.8. Place the smaller diameter shrink tube 3.18 mm (1/8 inch) over the connections and shrink them with a heat gun (**Supplementary Figure 13C,D**).

2.1.9. Place a larger diameter shrink tube 4.76 mm (3/16 inch) over the smaller shrink tube 3.18 mm (1/8 inch) and heat again (**Supplementary Figure 13E,F**).

2.2. Build the manual power switch.

2.2.1. Place the shrink tube 3.18 mm (1/8 inch) over the wires for the switch (**Supplementary Figure 14A**).

2.2.2. Twist and solder the wires of a male wire-to-wire connector (**Supplementary Figure 14B,C**).

2.2.3. Place the shrink tube 3.18 mm (1/8 inch) over soldered sections and shrink with a heat gun (**Supplementary Figure 14D,E**).

2.3. Connect the male wire-to-wire connector to the POT.

2.3.1. Loop the wire-to-wire connector's black wire around the middle terminal of the POT (**Supplementary Figure 15B**).

2.3.2. Twist the wire that is looped tightly around the terminal and solder it (**Supplementary Figure 15C**).

NOTE: Small precision pliers can assist in making a tight twist.

2.3.3. Repeat with the red wire connection to the terminal, as in **Supplementary Figure 15D**.

2.3.4. Use pliers to break the metal tab near the red arrow (**Supplementary Figure 15E,F**).

2.4. Build the microcontroller connection (only necessary for computer-controlled LEDs).

2.4.1. If making a LED driver for more than one LED, cut off the black wires from all but one female wire-to-wire connector (**Supplementary Figure 16A**).

2.4.2. Crimp the ends of the wire-to-wire connectors, as shown (**Supplementary Figure 16B–D**).

2.4.3. Push the crimped ends through the rectangular connector (**Supplementary Figure 16E**).

3. Build the LEDs

3.1. Strip the wire ends (~5 mm) and apply flux using a flux pen as in **Supplementary Figure 3G**.

NOTE: To efficiently solder the wires onto the LED base, flux must be added to the contacts on the LED base and the wires.

3.2. Tin the wire by heating the wire from below and adding solder from the top (**Supplementary Figure 17B**).

3.3. Use the flux pen to place flux onto the surface contact of the LED base (**Supplementary Figure 17C**).

3.4. Place a generous amount of solder onto a large soldering tip (~4–5 mm) (**Supplementary Figure 17D**), use it to heat the LED base at the contact (**Supplementary Figure 17E**). After a few seconds, drag the solder across the contact (**Supplementary Figure 17F**). Repeat steps 3.3–3.4 on the other contact (**Supplementary Figure 17G**).

CAUTION: The LED base can get very hot during soldering. Place the LED base on a surface that will not melt or burn.

3.5. Clip the black wire onto the contact "C+" (the cathode) using the hair clips (**Supplementary Figure 18A**).

3.6. Place a generous amount of solder onto the large soldering tip (**Supplementary Figure 18B**) and press it down on the wire until the solder on the LED base melts (**Supplementary Figure 18C**). Hold down the wire (**Supplementary Figure 18D**) and remove the soldering iron while holding the wire in place (**Supplementary Figure 18E**).

3.7. Place a small amount of solder paste onto the pads for the LED connections (**Supplementary Figure 19A,B**) and place the LED over the pads using forceps (**Supplementary Figure 19C**).

NOTE: If the placement is a little off, it is okay; it will go into place once the solder paste melts.

3.8. Hold the red wire on the "A+" (anode) and clip it with a hair clip (**Supplementary Figure 20A–C**).

3.9. Place a generous amount of solder onto the large soldering tip (**Supplementary Figure 20D**) and press it down onto the wire until the solder on the LED base and the solder paste under the LED melts (**Supplementary Figure 20E**).

NOTE: After the solder paste melts, the color becomes silver (**Supplementary Figure 20H,I**).

3.10. Choose the length of the wire needed for the desired set up. Strip the LED wires, and a male wire-to-wire connector (**Supplementary Figure 21A**) then add flux as in **Supplementary Figure 3G**.

3.11. Place the shrink tube over the wires. Use a 3.18 mm (1/8 inch) shrink tube over the wire-to-wire connectors and a 4.76 mm (3/16 inch) shrink tube over the wire (**Supplementary Figure 21B**).

3.12. Clip the wire-to-wire connector with a "helping hands" and twist the connector end with the wire (**Supplementary Figure 21C**) and solder them. Repeat with the other wire (**Supplementary Figure 21D,E**).

3.13. Place the 3.18 mm (1/8 inch) shrink tubes over the solder and shrink (**Supplementary Figure 21F–G**).

3.14. Place the 4.76 mm (3/16 inch) shrink tube over the 3.18 mm (1/8 inch) shrink tube and shrink (**Supplementary Figure 21H–I**).

3.15. Clip the LED wires the "helping hands" with tape under it (**Supplementary Figure 22A**).

3.16. Mix epoxy according to the manufacturer's instructions and spread over the top of the soldered LED (**Supplementary Figure 22B**). Leave overnight to cure.

3.17. If mounting using a touch fastener, cut a small piece of the touch fastener (**Supplementary Figure 23A**) and press it against the back of the LED for 30 s.

3.18. Use a high-speed rotary tool to make a notch on the lid of a black box (**Supplementary Figure 23C–E**).

3.19. Build a mounting for a single LED through a privacy film.

3.19.1. Using the spade drill bit, drill a 1.75 cm (11/16 inch) hole through the top of a black box where the LED will be placed (**Supplementary Figure 24A**).

3.19.2. Using a high-speed rotary tool, make a notch on one side of the hole to make room for the LED wire, as shown in **Supplementary Figure 24A**.

3.19.3. Cut a piece of privacy film (25–30 mm) and tape onto the inside of the black box covering the hole that the LED will illuminate through (**Supplementary Figure 24A**).

3.19.4. Place the LED outside the black box on top of the hole with privacy film and tape in place with electrical tape (**Supplementary Figure 24B–E**).

4. Accommodate a black box to fit the illumination system

4.1. For a four LED system, drill four 0.83 cm (21/64 inch) holes on the lid 3.81 cm (1.5 inch) apart where the potentiometers will be attached (**Supplementary Figure 25**).

4.2. Using a high-speed rotary tool, cut a 1.19 cm x 1.90 cm (0.47 inch x 0.75 inch) rectangular hole in the top-left corner (**Supplementary Figure 25**).

4.3. Using the spade drill bit, drill a 1.75 cm (11/16 inch) hole on the black box (**Supplementary Figure 26**).

4.4. File the holes and insert the grommet into the hole drilled in (**Supplementary Figure 26**).

4.5. For the computer-controlled LEDs, sandpaper the area where the microcontroller will be glued in a black box, as well as the bottom side of the microcontroller holder.

4.6. Snap the microcontroller onto the holder before securing the holder in the black box and then epoxy them in place (**Supplementary Figure 27A**).

4.7. Use sandpaper to sand the bottom of two clips and the area in a black box where the circuit will be placed and secure the clips inside the black box with the epoxy (**Supplementary Figure 27A**).

4.8. Secure the PCB board into clips (**Supplementary Figure 27B**).

4.9. Push the power switch through the square hole in the lid made in **Supplementary Figure 25** and snap it into place (**Supplementary Figure 28A**).

4.10. Push the POTs through the holes on the lid, screw into place (**Supplementary Figure 28A**), and put the knob onto the POT (**Supplementary Figure 28B**).

5. Connect all of the wiring and devices

5.1. Label the wire-to-wire connectors (e.g., LED, POT, COM) (**Supplementary Figure 29A**).

5.2. Attach the crimped connectors made in step 2.4 (**Supplementary Figure 16**) to the male wire-to-wire connector between the two female connectors (POT and LED) (**Supplementary Figures 7A and S37**).

5.3. Connect the crimped ends into the microcontroller (**Supplementary Figure 30**).

5.4. Pull the USB cable through the grommet and plug it into the microcontroller.

5.5. Pull the wires for the LEDs through the grommet and connect to the female wire-to-wire connector on the left of the microcontroller connection (**Supplementary Figures 9D and 38**).

5.6. Pull the wire for the power supply through the grommet and connect it to the male wire-to-wire connector on the right side of the PCB board (**Supplementary Figure 11D**).

5.7. Connect the male wire-to-wire connector from the power switch to the female wire-to-wire connector on the right of the PCB board (**Supplementary Figure 11A**).

5.8. Connect the male wire-to-wire connectors from the POTs on the lid to the female wire-to-wire connectors on the PCB board (**Supplementary Figures 8 and 36**).

NOTE: Do not turn on the circuit without the potentiometers connected.

6. Install the LED control software

NOTE: See the detailed Software Installation Instructions in the supplementary file on Github. https://github.com/BreakLiquid/LED-Control-User-Interfaces

6.1. Download and install the software for programming the microcontroller

6.2. Download and install the package manager.

6.3. Program the microcontroller.

6.4. Download and install the runtime engine.

6.5. Download the user interface.

7. Stimulate the cells with light

7.1. Transfect HEK293 cells.

7.1.1. Plate HEK293 cells at 100k cells per well in a 24-well plate.

7.1.2. Use the example table to calculate serum-free media, Polyethylenimine (PEI), and DNA volumes (**Supplementary Figure 39**) and transfect using the manufacturer's protocol.

7.2. Stimulating cells with light.

NOTE: Cells must be kept in the dark after transfection or handled using a light source that does not excite the optogenetic system.

7.2.1. Decide what type of stimulation will be used on the cells (continuous light, pulsing intensity, etc.).

7.2.2. With the POTs turned off (counterclockwise), turn on the LED power supply.

7.2.3. Place a light meter inside the black box where the cells will be placed and place the lid with the LED over the meter. Adjust the light intensity as needed.

7.2.4. If using the computer to control the LEDs, open the user interface software.

7.2.5. Program the user interface (**Figures 5A**,**B**).

7.2.5.1. On the top left panel, select the COM port for the microcontroller and click on **Connect**.

7.2.5.2. Use the panels on the right to program each LED. For continuous light, select any time except for zero in the "Time On" and set the "Time Off" to zero.

7.2.5.3. On the bottom right panel, program the main timing control.

7.2.5.3.1. To delay illumination, select a start delay (HH:MM).

7.2.5.3.2. To shut off all LEDs after a designated time, select a run time (HH:MM).

7.2.5.3.3. Start the illumination program by clicking on the **Run** button (**Figure 5A**).

8. Measure gene expression using a dual luciferase assay

8.1. Prepare luciferase reagent by mixing 10 mL of luciferase buffer with luciferase reagent and aliquot in 1 mL tubes to be stored at -80 °C for up to 1 year.

8.2. Prepare lysis buffer 5x into 1x for 100 µL for N + 2 wells. e.g., for 30 samples, 30 x 20 µL of 5X lysis buffer, and 30 x 80 µL of MQ H_2O.

8.3. Prepare Renilla substrate solution: 20 µL of Renilla substrate for 1 mL of Renilla buffer (this amount is suitable for 10 assays).

8.4. Remove cells from the incubator, aspirate the media, add 100 µL of 1x lysis buffer per well and place it on a shaker at 100 RPM for 15 min.

8.5. Place in -20 °C for at least 1 h.

8.6. Add 100 µL of luciferase reagent per sample into a well of a white 96-well plate.

8.7. Set up the plate reader for luminescence. Using the luminometer module of the plate reader, set the integration for 1 s.

8.8. Add thawed lysates in wells below the luciferase reagent. Using a multichannel pipette, mix 20 µL of sample into the luciferase reagent and measure luminescence immediately.

8.9. After the readings plateau, add 100 µL of Renilla substrate solution and scan again.

8.10. Divide the Luciferase signal by the Renilla signal to account for transfection efficiency.

8.11. Compare luciferase signals normalized for transfection efficiency (e.g., compare the signal from red light illuminated and far-red light illuminated samples).

REPRESENTATIVE RESULTS:

Once the power circuit, power supply, power switch, the POTs, and an LED is assembled (up to **Supplementary Figure 21**), the circuit can be tested. With all the POTs in place, the POT will control the LED intensity. Once assembly is completed up to **Supplementary Figure 29**, the system can be used manually for optogenetics or other applications. The entire system power can be manually controlled with the power switch. The intensity of each LED can be controlled independently using the POT connected to each circuit.

After installing the software and programming the microcontroller, the user interface can communicate with the microcontroller. With the user interface, the LEDs can be controlled temporally in several ways: (1) each LED can be programmed to stay on for a specified time, (2) each LED can be programmed to pulse, (3) a global start delay (e.g., when transfecting and shining light 24 h later) can be programmed (**Figure 6B**), (4) the total time for the program to run after the delay. There are two User Interfaces, one with larger buttons that can control two LEDs at a time and another that can control four LEDs (**Figure 5A,B**). The two LED User Interface is optimized for tablets and is sufficient to control red and far-red LEDs for many experiments.

For larger experiments, the second user interface can be used to control up to four LEDs. When inducing gene expression, the anticipated result depends on several parameters. These include induction time, induction levels (e.g., amount of light or drug), and copy number of the inducible construct in the cell. To show this, we transfected the PhyB gene switch along with different amounts of reporter DNA (pPK-202) (0.5%, 1%, 2%, 4%, and 8% of the transfected DNA) (**Figure 6A**) and illuminated as shown in **Figure 6B**. In samples containing PhyB, but no plasmid to produce phycocyanobilin (PCB-chromophore) (i.e., unresponsive to light), luciferase gene expression/leakiness increases with the amount of reporter DNA (**Figure 6C**) (Far-red $P < 0.0001$, Linear regression followed by a Wald test), (Red $P < 0.0001$, Linear regression followed by a Wald test). In addition, when the entire PhyB gene switch, including the PCB-chromophore producing plasmid (light-sensitive cells), are illuminated for Far-red light, Luciferase expression also increases with the increasing reporter construct amounts in the transfection mix (**Figure 6C,D**) (Far-red light $P < 0.0001$, Linear regression followed by a Wald test). Similarly, when the light-sensitive cells are illuminated with red light, luciferase expression also increases with increased reporter amount ($P < 0.0001$, Linear regression followed by a Wald test). When comparing induction levels of the red light treated cells to the far-red light treated cells, we found a small decrease in the fold activation with increasing reporter amount (**Figure 6E**) ($P = 0.0141$, Linear regression followed by a Wald test).

FIGURE AND TABLE LEGENDS:

Figure 1: A basic circuit for a single LED. (**A**) A flow chart showing an overview of the steps needed to build the LED illumination system. (**B**) The LED illumination control system. (left) Control box for regulating LED intensity and timing. (middle) A PC tablet running user interface for controlling LEDs. (right) A black box for mounting LEDs and placing cells for optical stimulation. (**C**) Table for determining whether the LED requires a high or low voltage circuit.

Figure 2: Instructions for soldering the components into place. (**A**) An example of the step-by-step cartoon instructions for building the circuit. (**B,C**) Example instructions with pictures of the device being assembled. (**D**) Example instructions for assembling multiple circuits simultaneously.

Figure 3: Views of an assembled LED control system. (**A**) A top outside view of the assembled system. (**B**) An inside view of an assembled four LED illumination system.

Figure 4: Instructions for reflow soldering the LED onto the heat sink. (**A**) The LED base and a close up of a deep red LED. (**B**) Placement of solder paste onto the LED base. (**C**) Picture of soldered LED. Red arrows point to soldering pads. Compared to gray before soldering (**A**), after soldering, the solder appears metallic/shiny.

Figure 5: Software for controlling optogenetic experiments. (**A**) A two LED user interface with large buttons for easy use with an inexpensive tablet. (**B**) A four LED User Interface. Both interfaces allow independent LED control. For pulsing, LEDs can be programmed to turn on and off for specific pulse widths and specified durations. The pulsing can also have a start delay and a predetermined total run time. (**C**) The LED control tablet mounted onto a cell culture incubator. (**D**) Illustration of the PhyB gene system when illuminated with far-red light. Far-red light keeps the gene in the “off” or “dark” state. (**E**) Illustration of the PhyB gene system when illuminated with red light. Red light induces gene expression by promoting the interaction between PhyB and PIF3. This interaction localizes the gene activation domain (AD) fused to PIF3 to the UAS promoter, activating the reporter gene.

Figure 6: Anticipated results using the LED system to control PhyB. (**A**) A plasmid encoding PhyB+PIF3 two-hybrid partners (pPK-351), a plasmid encoding phycocyanobilin (PCB-chromophore) synthesis enzymes (pPK-352), and a Luciferase reporter plasmid (pPK-202). (**B**) Timeline of light induction experiments for C–E. (**C**) Basal transcription levels (AKA leakiness) with increasing amounts of reporter DNA. “Leak” samples are not transfected with pPK-352 (i.e., unresponsive to light), but are illuminated with red or far-red light. Light Switch (LS) samples include all light-gene switch plasmids and are illuminated with red or far-red light. (**D**) Light induction levels in response to red and far-red light. (LS-Far-red light is the same data in C and D.) (**E**) Fold induction of luciferase in cells illuminated with red light/far-red light.

Supplementary Figure 1: Electronic Driver Circuit for multiple LEDs. (**A**) The circuit diagram for a single LED system. (**B**) The circuit diagram for a four LED system.

Supplementary Figure 2: Placing the circuit Interconnections. (**A**) Clip your PCB board onto your helping hands. (**B**) Position of main circuit jumpers into the through holes in the picture. (**C**) Diagram of wire connectors mapping the coordinates. For the four LED systems, draw lines dividing each circuit as shown (black vertical lines). **Supplementary Figure 31–38** describe the assembly of four circuits simultaneously.

Supplementary Figure 3: Soldering the wires onto the PCB. (**A**) Bend jumpers so that they make direct contact with the PCB and stay in place while soldering. (**B**) Another view of the bent wires. (**C**) Wires after soldering. (**D**) Trimmed wires on the PCB. (**E**) Shrunken insulation after heating with solder. (**F**) Moving the insulation into position to cover the ground through-hole (blue arrow) (**G**) Adding flux to a wire end or terminal.

Supplementary Figure 4: Soldering the voltage regulator into place. (**A**) Map of the voltage regulator coordinates. (**B**) Placement of the voltage regulator. (**C**) Bent voltage regulator leads. (D) Voltage regulator terminals after soldering.

Supplementary Figure 5: Soldering the R1 resistor into place. (**A**) Map of the R1 resistor (820Ω) coordinates. (**B**) Pulling the resistor through by the lead using pliers (**C**) The pulled resistor close to PCB. (**D**) The soldered resistor close to PCB.

Supplementary Figure 6: Soldering the transistor into place. (**A**) Map of the transistor coordinates and orientation. (**B**) Note the orientation of the transistor; the label in this model is facing the voltage regulator (LM317T). Double-check the specification of the transistor to make sure the “Emitter”, “Base”, and “Collector” are in the correct holes. (**C**) The transistor with the terminals bent before soldering.

Supplementary Figure 7: Soldering the wire-to-wire connector for the potentiometer into place (plus a 560Ω resistor for the low voltage circuit). (**A**) Map of the coordinates of the wire-to-wire connector (plus the R3-560Ω if building the low voltage circuit, the wire-to-wire connector is placed in the hole before the resistor). (**B**) A female wire-to-wire connector. (**C**) To facilitate fitting the resistor and the wire-to-wire connector into the through-hole, 3–5 strands of the braided wire are bent. (**D**) The strands are cut off with wire cutters as close to the insulation as possible. (**E**) Inserted red wire of a female wire-to-wire connector through the a5 through hole (for the low voltage circuit insert R3 through the same through hole). (**F**) Underside view of the resistor and wire-to-wire connector before soldering. (**G**) Image of the soldered R3 resistor connected to the ground (F = Female).

Supplementary Figure 8: Soldering the wire-to-wire connector for the potentiometer to the ground. (**A**) Map of the coordinates of the ground connection for the potentiometer wire-to-wire connector. (**B**) Top view of the potentiometer wire-to-wire connector in parallel with R3 (F = Female).

Supplementary Figure 9: Soldering the microcontroller and LED wire-to-wire connectors. (**A**) Map of the coordinates of the wire-to-wire connector for connecting the 2N222A and the ground to the microcontroller. (**B**) Soldered male wire-to-wire connector. (**C**) Top view of (**B**). (**D**) Map of the female wire-to-wire connector coordinates for connecting the input of the circuit and ground to the LED. (**E**) Soldered female wire-to-wire connector (F = Female, M = Male).

Supplementary Figure 10: Soldering the jumper for the power supply circuit. (**A**) Map of the coordinates of the orange jumper for connecting the power supply to the ground. (**B**) The orange jumper soldered in place. (**C**) The underside view of the jumper soldered in place.

Supplementary Figure 11: Soldering the power switch and power source wire-to-wire connectors. (**A**) Map of the coordinates of the female wire-to-wire connector for connecting the power switch. (**B**) The female wire-to-wire connector soldered in place. (**C**) Another view of (**B**). (**D**) Map of the coordinates of the male wire-to-wire connector for connecting the power source. (**E**) Soldered male wire-to-wire connector. (**F**) Another view of (**E**) (F = Female, M = Male).

Supplementary Figure 12: Connecting the power supply to a male wire-to-wire connector. (**A**) The unmodified power supply. (**B**) Cutting off the power supply wires. (**C**) The power supply wires stripped and with excess insulation cut away. (**D**) Placement of shrink tube around power supply wires. Tubing separating the two connections (red arrows) and tubing to hold the separated wires (yellow arrow). (**E**) Twisted wires connecting the power supply to the female wire-to-wire connector.

Supplementary Figure 13: Soldering and insulating the power supply connection to a male wire-to-wire connector. (**A**) The soldered connection between the power supply ground and a female wire-to-wire connector. (**B**) The soldered connection between the positive terminal of the power supply and a female wire-to-wire connector. (**C**) Shrink tube pulled over the soldered individual connections (red arrow). (**D**) Both power supply connections soldered and with heat-treated shrink tube. (**E**) Placement of shrink tube over individual connections (yellow arrow). (**F**) Completed power supply.

Supplementary Figure 14: Soldering the power switch to a male wire-to-wire connector. (**A**) Power switch with stripped wires and shrink tube placed over the wires (red arrows). (**B**) Wires connecting the switch and male wire-to-wire connector twisted together before soldering. (**C**) Placing the shrink tube over the soldered connections. (**D**) Connections covered with the heat-treated shrink tube. (**E**) A power switch assembled with a male wire-to-wire connector.

Supplementary Figure 15: Wiring a potentiometer to a male wire-to-wire connector. (**A**) The potentiometer parts. (**B**) A male wire-to-wire connector twisted and bent to hook around the middle terminal of the potentiometer. (**C**) A male wire-to-wire connector twisted around the middle terminal of the potentiometer. (**D**) Soldered wire-to-wire connections. (**E**) Red arrow pointing to the metal tab before removal. (**F**) The potentiometer after metal tab removal.

Supplementary Figure 16: Wiring the microcontroller connection. (**A**) Wires for female wire-to-wire connectors stripped and cut in preparation for crimping. (**B**) Placement of the crimp on the wire-to-wire connector. (**C**) Crimping of the wire-to-wire connector. (**D**) Crimped wire-to-wire connector. (**E**) Fully assembled microcontroller connection.

Supplementary Figure 17: Soldering wires and LED onto the LED base Part 1. (**A**) Materials needed to solder the LED to the LED base. (**B**) Tinning the tip of the stripped wire. (**C**) Applying flux onto the contact of the LED base. (**D**) Adding solder to the large soldering tip to tinning the LED base. (**E**) Placement of solder onto the contact to heat the LED base. (**F**) The LED base after dragging the soldering tip across the contact. (**G**) The same procedure on the other contact.

Supplementary Figure 18: Soldering wires and LED onto the LED base Part 2. (**A**) A tinned wire clipped to the contact using a hair clip. Note that the black wire is soldered to the cathode "C-". (**B**) Addition of a generous amount of solder to the soldering tip. (**C**) The soldering tip pressing down on the wire, melting the solder on the LED base and the wire. (**D**) Holding down the wire so that it stays put when the soldering iron is removed. (**E**) Holding the wire in place until the solder hardens.

Supplementary Figure 19: Soldering wires and LED onto the LED base Part 3. (**A**) Using a sharp tip to place solder paste onto the LED base for mounting the LED. (**B**) The LED base with the soldering paste in place. (**C**) Placement of the LED onto the LED base such that the contacts of the LED and LED base match.

Supplementary Figure 20: Soldering wires and LED onto the LED base Part 4. (**A**) The black wire still clipped to the contact by the hair clip. (**B,C**) Using a second hair clip, the red wire is held into place. Note that the red wire is soldered to the anode "A+". (**D**) Addition of a generous amount of solder to the soldering tip. (**E**) The soldering tip pressing down on the wire, melting the solder on the LED base and the wire as well as the solder paste under the LED. (**F**) The hot LED base cooling after soldering. (**G**) The LED base with the wires and LED soldered on. (**H,I**) Red arrows point to soldering pads. After soldering, the solder appears metallic/shiny (compared to gray before soldering (**Supplementary Figure 16D**)).

Supplementary Figure 21: Connecting the LED wire to a male wire-to-wire connector. (**A**) Stripped wires and male wire-to-wire connector next to the shrink tube cut in half (1/8 inch and 3/16 inch). (**B**) Shrink tube placement over the wires before soldering. (**C**) Wires twisted together before soldering. (**D**) The soldered connection from the wire to the wire-to-wire connector. (**E**) Both the red and black wires soldered together. (**F**) Placement of the 1/8 inch shrink tube over the soldered connection. (**G**) The shrink tube after shrinking with the heat gun. (**H**) Placement of the 3/16 inch shrink tube over the smaller shrink tube. (**I**) The connection soldered and sealed with the shrink tube.

Supplementary Figure 22: Securing the wires and LEDs to the LED base using epoxy. (**A**) Using a wooden applicator to place epoxy into the LED base. A tape is placed below to catch any dripping epoxy. (**B**) Epoxy is spread evenly over the entire surface. (**C**) The LED is left overnight to cure.

Supplementary Figure 23: Mounting LEDs inside a box lid. (**A**) An LED with a touch fastener piece attached for easy mounting. (**B**) Different color LEDs mounted on the inside of a black box using a touch fastener. (**C**) A notch on the black box's lid made by a high-speed rotary tool to make room for the LED wire. (**D**) A black box for stimulating the cells with touch fasteners for mounting the LED. (**E**) Placement of a multiwell dish inside of the touch fastener version of the LED box.

Supplementary Figure 24: Mounting LEDs outside a box lid. (**A**) Hole drilled into the black box's lid with a notch from the high-speed rotary tool to make room for the wire (red arrow). (**B**) LED placed into the hole with the wire in the notch, held in place with electrical tape. (**C**) Two more pieces of tape are used to secure the LED. The backside of the heat sink is exposed to maximize heat exchange. (**D**) Privacy film taped over the hole where the LED will be placed. The red arrow points to the privacy film. (**E**) A black box for stimulating the cells with an LED mounted outside the box and with privacy film for diffusing the illumination. (**F**) Placement of a multiwell dish inside of the external LED + privacy film version of the LED box.

Supplementary Figure 25: Drilling holes on the box lid for the power switch and potentiometers. (**A**) A CAD drawing with annotated dimensions of the box lid. (**B**) The box lid with the potentiometer and power switch holes.

Supplementary Figure 26: Preparing the wire outlet hole. (**A**) A CAD drawing with annotated dimensions. (**B**) Image of the drilled hole with the drill bit. (**C**) Smoothing of outlet hole with high-speed rotary tool or filing tool. (**D**) Placing grommet in outlet hole.

Supplementary Figure 27: Placement of the microcontroller and the PCB in the box. (**A**) The microcontroller holder (orange) and PCB holders inside the box. (**B**) The microcontroller and PCB secured into the box.

Supplementary Figure 28: Placement of the potentiometers and the power switch. (**A**) A front view of a box lid with a power switch and four POTs. (**B**) A front view of the box lid with potentiometer knobs added. (**C**) A rear view of the box lid with the attached components.

Supplementary Figure 29: The assembled LED control system. (**A**) An open control box with the wires labeled with a label printer and zip tied for organization. (**B**) The box once it is fully assembled with each POT labeled along with the PIN.

Supplementary Figure 30: Placement of the crimped wire-to-wire connector. (**A**) Picture of the crimped wire-to-wire connectors for a four LED-microcontroller system. (**B**) Placement of the crimped connector into the microcontroller ports.

Supplementary Figure 31: Placing the jumper wires. (**A**) A circuit board with the coordinates of the red jumper wires labeled. (**B**) A circuit board with the coordinates of the yellow jumper wires labeled.

Supplementary Figure 32: Placing the jumper wires. A circuit board displaying coordinates of the yellow jumper wires.

Supplementary Figure 33: Adding the voltage regulators. The LM317T voltage regulators are added to the circuit with their coordinates labeled in the diagrams.

Supplementary Figure 34: Inserting the 820Ω resistors. The R1 resistors are added to the circuit with their coordinates labeled in the diagrams.

Supplementary Figure 35: Inserting the transistors. The 2N2222A transistors are added to the circuit with their coordinates labeled in the diagrams

Supplementary Figure 36: Inserting the Female wire-to-wire connectors and Resistors (optional) for the POT connection. The wires and resistors are added to the circuit with their coordinates labeled in the diagrams. (**A**) Insert the red wire, followed by the R2 resistor (560Ω) (for the low voltage circuit only). (**B**) Insert the other end of the resistor into the indicated ground hole. (**C**) Insert the black wires into the marked holes to connect to ground. Note: R2 (560Ω) is parallel to the potentiometer.

Supplementary Figure 37: Inserting male wire-to-wire connectors for the microcontroller connection and power supply. The wires are added to the circuit with their coordinates labeled in the diagrams. (**A**) Insert the red wires into the indicated holes. (**B**) Insert the black wires into the marked holes.

Supplementary Figure 38: Adding LED wire-to-wire connectors. (**A**) Female wire-to-wire connectors with the red lead coordinates highlighted. (**B**) Female wire-to-wire connector with the black lead coordinates highlighted.

Supplementary Figure 39: Setting up a PhyB-PIF3 gene switch experiment. (**A**) An example table of a master mix containing Renilla for the internal control. (**B**) An example table for setting up the DNA mixture for a Dual-Luciferase Reporter Assay of a PhyB-PIF3 optogenetic experiment. (**C**) An example table for setting up PEI transfection reagent and aliquoting the mixture onto cells (dropwise). (**D**) Placement of the light meter for setting the LED brightness.

DISCUSSION:

The LED system described here has been used in our lab to optimize, characterize, and work with several optogenetic tools. In Kyriakakis et al.[4], we tested many combinations of PhyB-PIF gene switches in parallel. We then used this system to test pulses of light at different frequencies to measure the gene switch kinetics and effective light intensity. This system was also used to optimize and characterize two optogenetic systems that use blue light for stimulation[5,6]. Since only one LED needed to be bright enough to activate most optogenetic tools, buying a system with large numbers of LEDs over each well is not always necessary. This setup is inexpensive, reliable, easy to reconfigure, and requires no prior electrical expertise to follow the assembly protocol.

In the supplementary **Supplementary Figures 31–38**, we describe how to incorporate up to four LEDs into the system. While this may limit some experiments requiring a large number of parallel conditions, more LEDs can be added by replacing the 9 Volt power supply used in this protocol with a higher wattage one. Similarly, several lower power LEDs can be connected in parallel to each circuit. In this latter arrangement, some LEDs will not be controlled individually, but this can be useful when many LEDs are required to cover a larger area. Once familiar with the electronics of this system, there are many ways to customize it. Additional strategies for customizing the system include placing the LED farther or closer to the sample and illuminating through filters/diffusers for homogenous illumination conditions or to prevent heating as in (**Supplementary Figure 23**) and Allen et al.[5]. Another notable feature of our LED design is that it is encapsulated in epoxy and has a touch fastener on the back; this allows the LED to be securely placed with ease virtually anywhere: in incubators, fish tanks, animal cages, walls, etc.

Many experiments that use optogenetics to control genes, signaling pathways, and other cellular activity often require pulsing, span large time scales, or need to be performed in an incubator, therefore requiring automatization or remote manipulation without a microscope. This LED system has been tested continuously for several months inside a humidified CO_2 incubator without any issues. Additionally, with reversible systems such as PhyB optogenetic systems, the experimenter may need to program-specific pulsing illumination schedules. In our previous work[4], we used pulsing programs to test the reversibility dynamics of a PhyB-PIF3 switch in mammalian cells through the user interface. Using the methodology described in this manuscript, programming a pulsing protocol is easy, providing the flexibility and autonomy needed for many types of optogenetic experiments in a user-friendly manner.

The most critical steps in building this system include putting together the electrical circuit on the PCB board and connecting the components, which are detailed in section 1 and section 2. It is essential to carefully follow each step in these sections and double-check the pinhole numbers line-by-line before soldering each component. Section 2 explains how to set up the components that will be connected to the circuit. So that the components connect in the correct orientation, it is particularly important to ensure that the colors of the black and red wires on the wire-to-wire connectors match. Small oversights in these two sections will very likely affect the system's functionality. Indeed, the first step in troubleshooting this method will be to check that the circuit was built correctly and that all the connections are in place. Secondly, checking the soldering quality for loose connections and the wires for flaring wirehairs that may be shorting the circuit is of particular importance. A third step would be to ensure that the LEDs are working correctly, which can be done using a power supply or a 1.5 V battery by clipping the LED's two terminals with alligator clips. Another potentially critical consideration is to prevent heating (when using the LEDs at high power) or diffusing the light for wider spread illumination. To address these considerations, the LEDs can be mounted outside of a black box with "privacy film" on the inside, as described in **Supplementary Figure 23** and Allen et al.[5]. Because of the simplicity of this system, taking it apart to verify, modify, upgrade, or repair modular components is not difficult.

Another critical factor for inducible gene systems is to consider how much activation is required or how much leakiness is acceptable for the biological system being controlled. As shown in **Figure 6**, these can vary with the amount of reporter DNA. In addition, the transfection efficiency and, therefore, the copy number of reporter constructs in each cell will vary. It may be advantageous for some experiments to make a cell line with a fixed amount of reporter or PhyB gene-switch components and screen for clones with the desired range of induced expression, as is commonly done with drug inducible systems. Due to the size and instability of lentiviral plasmid pPK-230[4], we also made non-lentiviral plasmid versions of the PhyB switch in the pcDNA backbone pPK-351 (Addgene #157921) and pPK-352 (Addgene #157922).

By building this LED illumination system following this protocol, users have all the components necessary to perform a wide array of optogenetics experiments in vitro and in vivo. Combined with the instructions for using PhyB-PIF3 in mammalian cells, this protocol will allow non-engineers and biologists to, flexibly and effectively, use PhyB-based optogenetics in a variety of contexts.

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DISCLOSURES:

The authors have no conflicts of interest to disclose.

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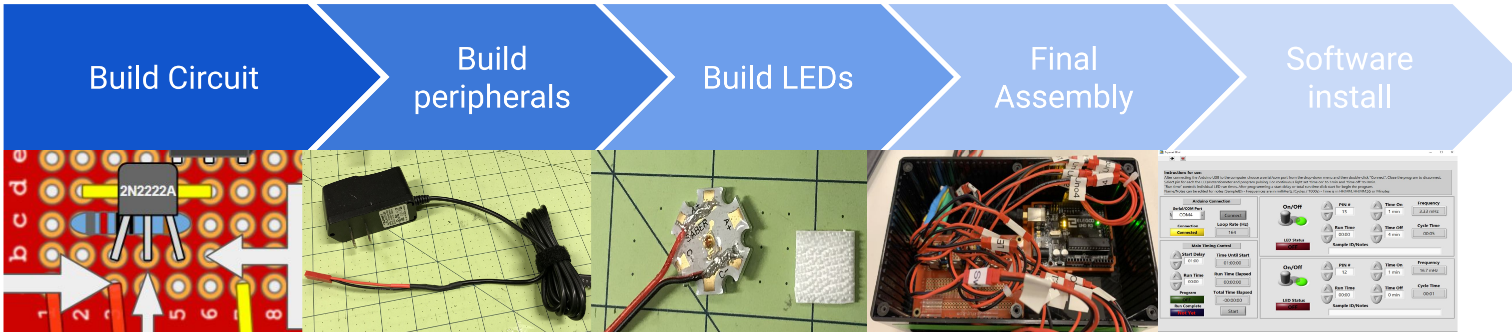

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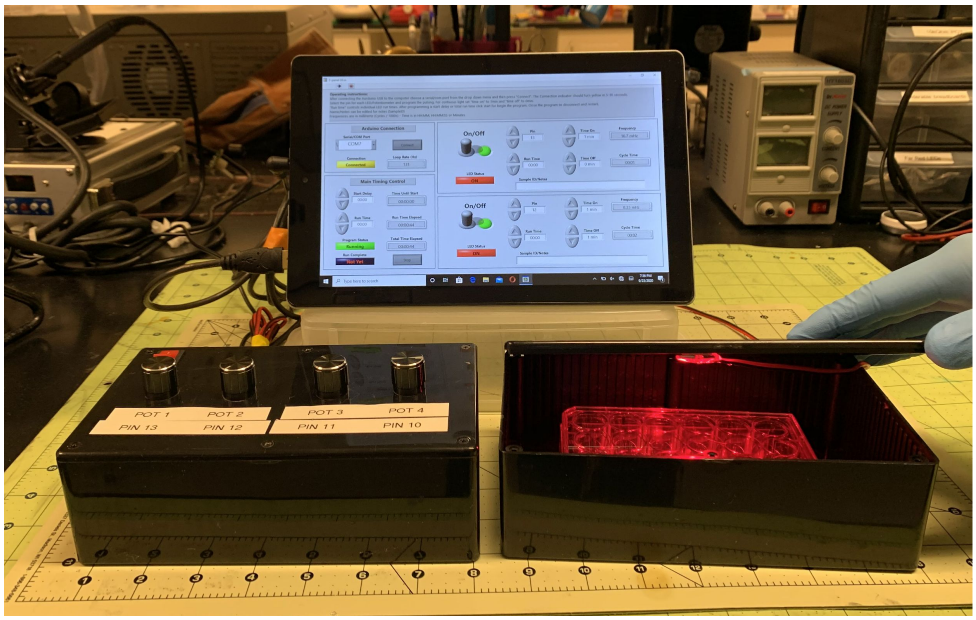

C

Table 1: List of compatible LEDs

High Voltage				Low Voltage			
Color	Forward Voltage (Vf)			Color	Forward Voltage (Vf)		
	Min	Typical	Max		Min	Typical	Max
Red	2.31	2.90	3.51	Far Red 850nm	-	1.50	1.90
Red-Orange	2.31	2.90	3.51	Far Red 810nm	-	1.90	2.50
PC Amber	2.55	3.05	3.51	Far Red 780nm	-	2.10	2.60
Amber	2.31	2.90	3.51	Far Red 740nm	-	2.00	2.40
Lime	2.60	2.75	3.00	Far Red 720nm	1.60	1.80	2.40
Green	2.55	3.21	3.51	Deep Red	1.80	2.10	2.80
Royal Blue	2.55	2.95	3.51	Red	1.80	2.10	2.80
Blue	2.55	2.95	3.51	Red-Orange	1.80	2.10	2.80
Cyan	2.55	3.17	3.51	Amber	1.80	2.10	2.60

Figure 2

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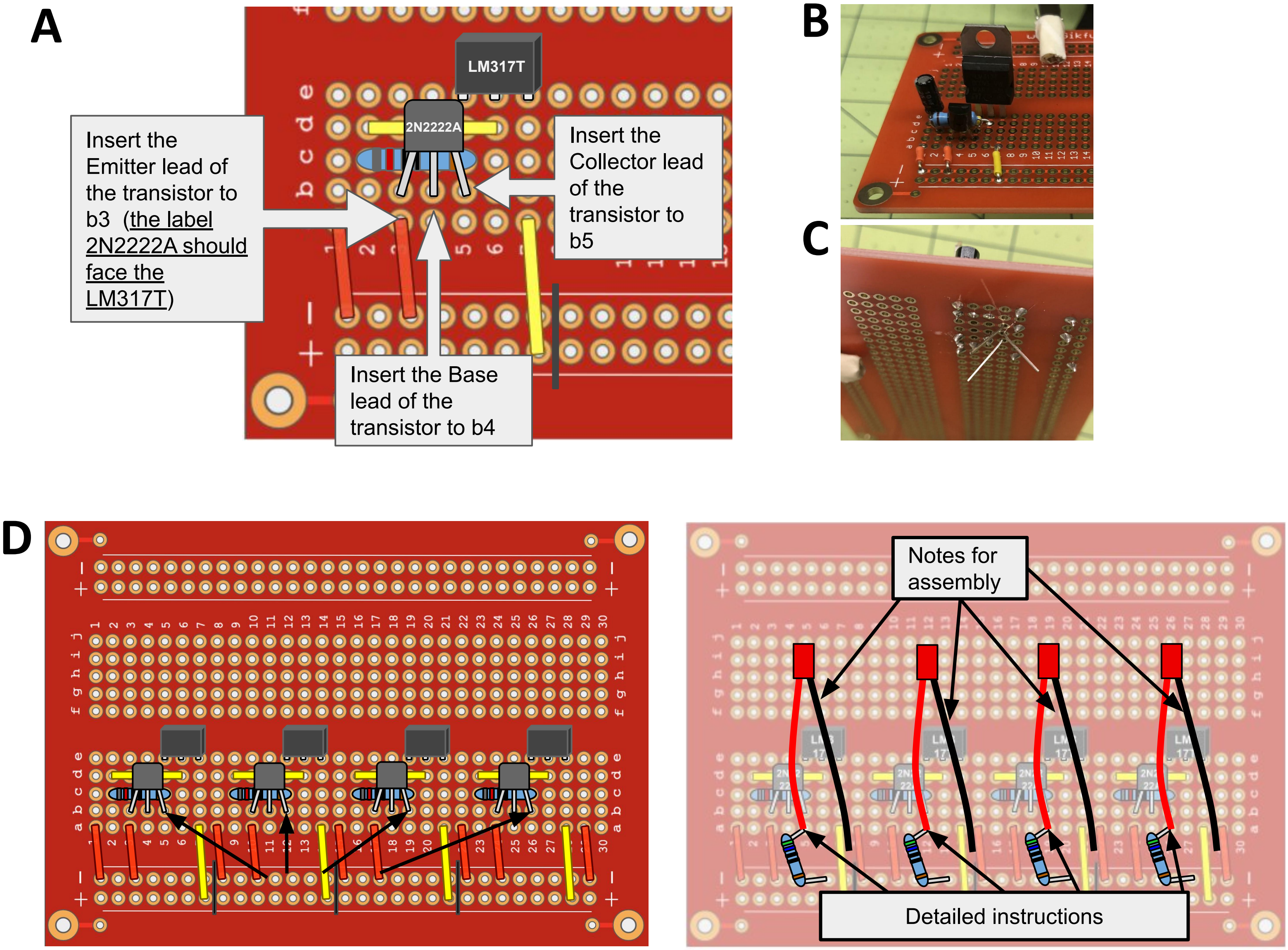

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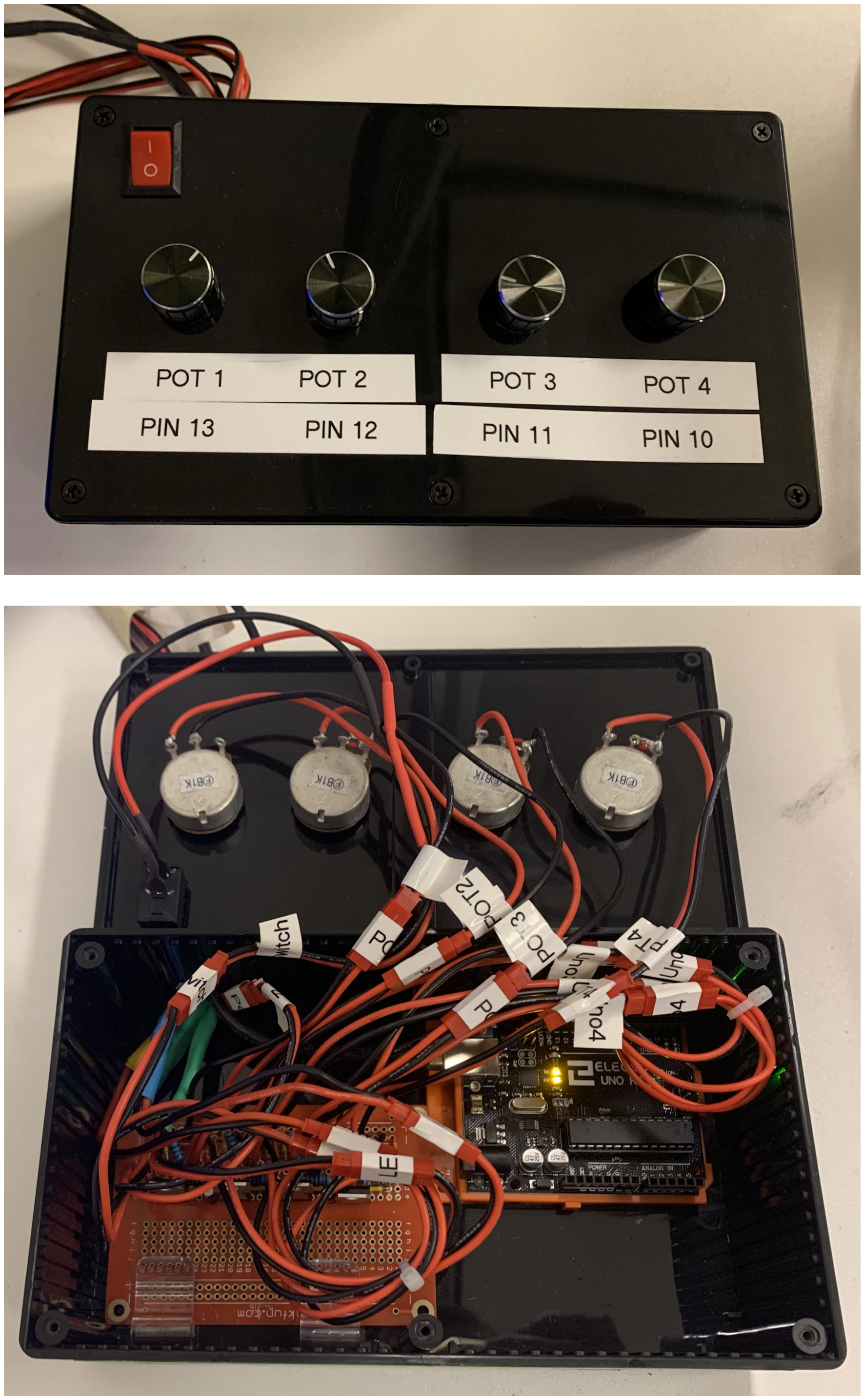


Figure 4

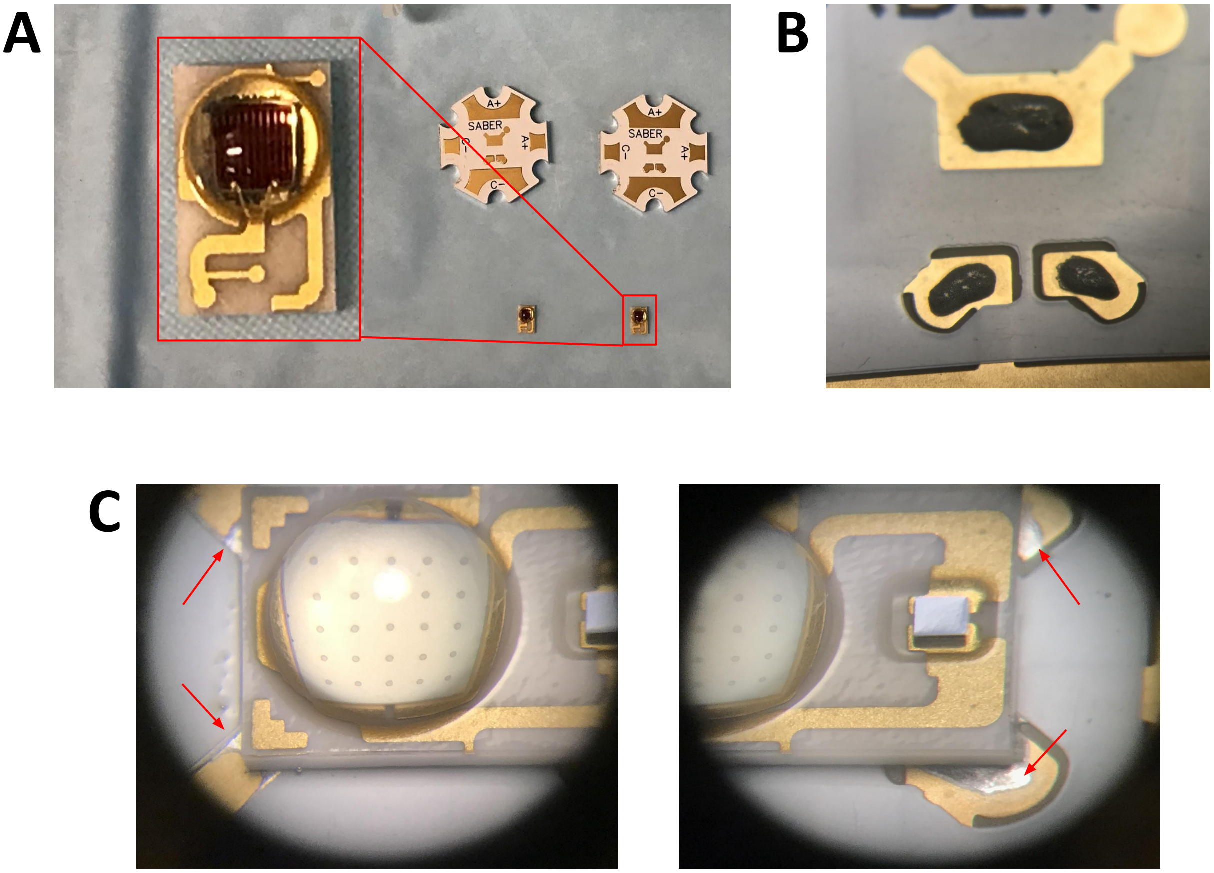


Figure 5

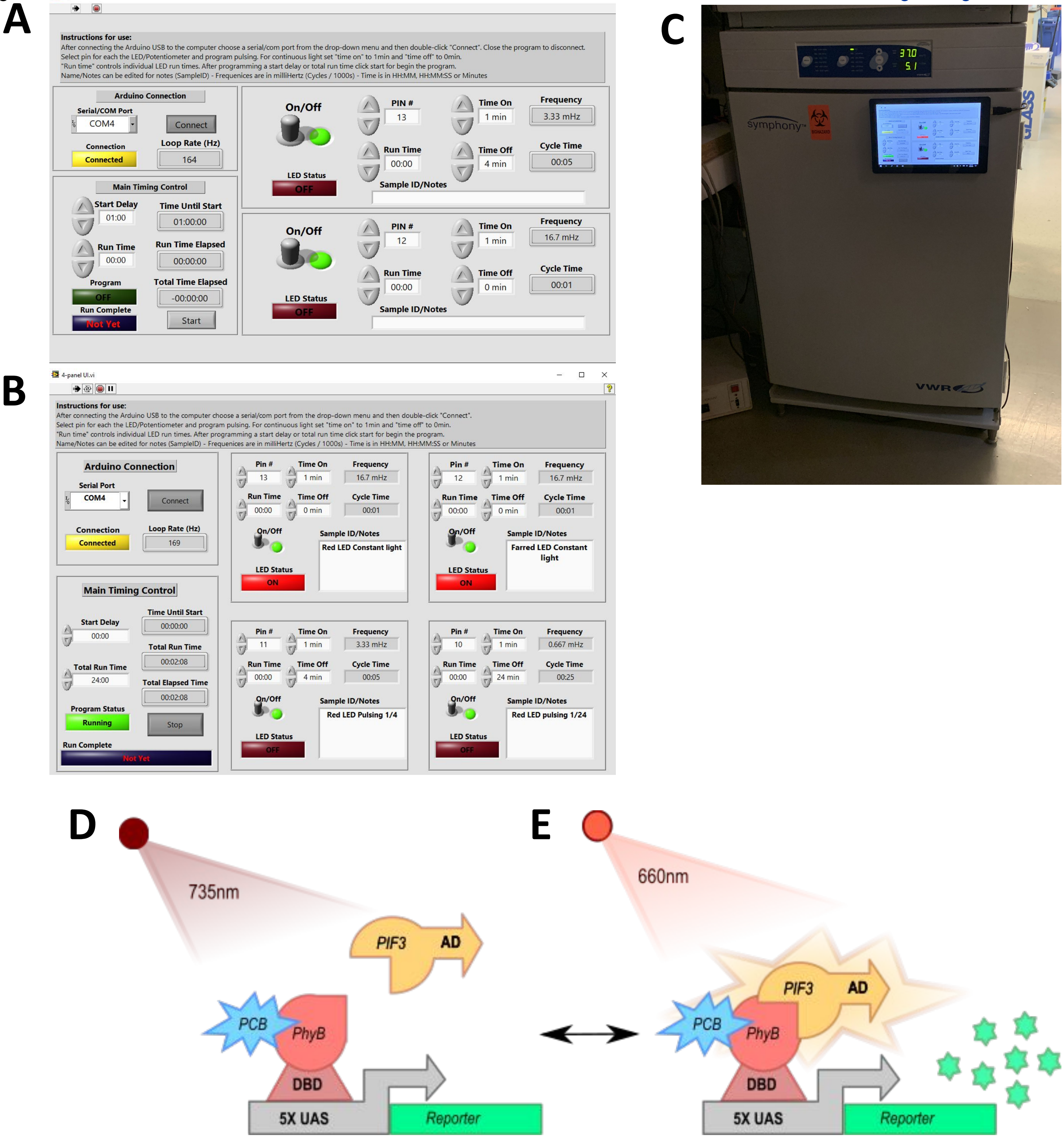
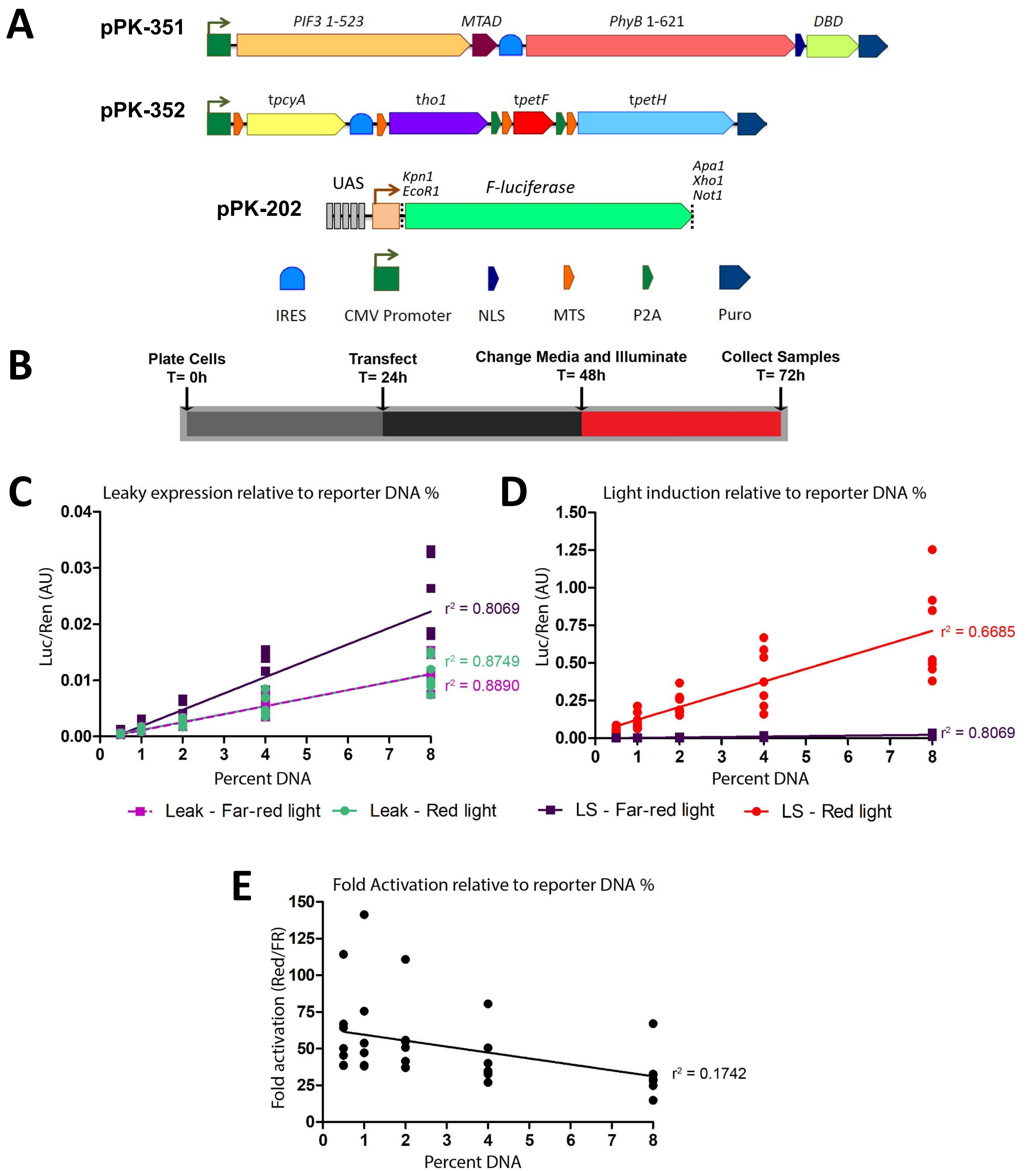

A
2-panel UI.vi
Instructions for use:
After connecting the Arduino USB to the computer choose a serial/com port from the drop-down menu and then double-click "Connect". Close the program to disconnect.
Select pin for each the LED/Potentiometer and program pulsing. For continuous light set "time on" to 1min and "time off" to 0min.
"Run time" controls individual LED run times. After programming a start delay or total run time click start for begin the program.
Name/Notes can be edited for notes (SampleID) - Frequencies are in milliHertz (Cycles / 1000s) - Time is in HH:MM, HH:MM:SS or Minutes
Arduino Connection
Serial/COM Port
COM4
Connect
Connection
Connected
Loop Rate (Hz)
164
Main Timing Control
Start Delay
01:00
Time Until Start
01:00:00
Run Time
00:00
Run Time Elapsed
00:00:00
Program
OFF
Total Time Elapsed
-00:00:00
Run Complete
Not Yet
Start
On/Off
LED Status
OFF
PIN #
13
Time On
1 min
Frequency
3.33 mHz
Run Time
00:00
Time Off
4 min
Cycle Time
00:05
Sample ID/Notes
PIN #
12
Time On
1 min
Frequency
16.7 mHz
Run Time
00:00
Time Off
0 min
Cycle Time
00:01
B
4-panel UI.vi
Arduino Connection
Serial Port
COM4
Connect
Connection
Connected
Loop Rate (Hz)
169
Main Timing Control
Start Delay
00:00
Time Until Start
00:00:00
Total Run Time
00:02:08
Total Run Time
24:00
Total Elapsed Time
00:02:08
Program Status
Running
Stop
Run Complete
Not Yet
Pin #
Time On
Frequency
Run Time
Time Off
Cycle Time
On/Off
LED Status
ON
OFF
Sample ID/Notes
Red LED Constant light
Farred LED Constant light
Red LED Pulsing 1/4
Red LED pulsing 1/24
13
12
11
10
16.7 mHz
3.33 mHz
0.667 mHz
24 min
00:25
C
symphony
VWR
D
735nm
PIF3
AD
PCB
PhyB
DBD
5X UAS
Reporter
E
660nm
PIF3
AD
PCB
PhyB
DBD
5X UAS
Reporter

Figure 6



A
pPK-351
PIF3 1-523
MTAD
PhyB 1-621
DBD
pPK-352
tpcyA
tho1
tpetF
tpetH
pPK-202
UAS
Kpn1
EcoR1
F-luciferase
Apa1
Xho1
Not1
IRES
CMV Promoter
NLS
MTS
P2A
Puro
B
Plate Cells
T= 0h
Transfect
T= 24h
Change Media and Illuminate
T= 48h
Collect Samples
T= 72h
C
Leaky expression relative to reporter DNA %
Luc/Ren (AU)
r² = 0.8069
r² = 0.8749
r² = 0.8890
Percent DNA
D
Light induction relative to reporter DNA %
Luc/Ren (AU)
r² = 0.6685
r² = 0.8069
Percent DNA
Leak - Far-red light
Leak - Red light
LS - Far-red light
LS - Red light
E
Fold Activation relative to reporter DNA %
Fold activation (Red/FR)
r² = 0.1742
Percent DNA

Click here to access/download;Table of Materials;Copy of JoVE_Table_of_Materials and Equipment.xlsx

Name of Material	Company	Catalog Number
18AWG 2pin RED Black wire	Amazon	15M-28AWG-2468
1K Ohm potentiometer	Amazon	52161500
20 Gauge Silicone JST Connector	Amazon	SIM&NAT 5.9 inch 2 I
22 AWG solid jumper wires	Amazon	WJW-60B-R
560 ohm 1/2watt 1% tolerance	Amazon	a14051600ux0301
820 ohm 1/2watt 1% tolerance	Amazon	TTL-A-8035-50Ea
A Male to B Male Cable (10 Feet)	Amazon	Part# 30-001-10B
Ardiuino UNO equivilent	Amazon	Elegoo EL-CB-001
Arduino holder	Digikey	X000018
Black boxes for circuits and light chambers	Amazon	1591ESBK
Blue LED	Digikey	LXML-PB01-0040
Cable ties	Amazon	sd027
Command Fridge Clips	Amazon	17210CLR
Cyan LED	Digikey	LXML-PE01-0070
Electrical tape - 3M Scotch #35 Electrical Tape Value Pa	Amazon	03429NA
Farred LED 720nm	Luxeon Star LEDs	LXML-PF01
Farred LED 740nm	Ushio	EDC740D-1100-S5
Farred LED 780nm	Ushio	EDC780D-1100
Farred LED 810nm	Ushio	EDC810D-1100
Farred LED 850nm	Ushio	EDC850D-1100
Grommets	Amazon	Pico 6120D
Hair/Alligator Clips	Amazon	
LED base	Luxeon Star LEDs	LXB-RS20A
LED PCB fopr Ushio LEDs	Adura LED solutions	STAR XP 3535 Packag
Loctite Epoxy Clear Multi-Purpose, 0.85-Fluid Ounce Sy	Amazon	1943587
NTE Heat Shrink 2:1 Assorted Colors and Sizes 160 PCS	Amazon	B000FIDTYG
Picture Hanging Velcro Strips	Amazon	PH204-16NA
Power supply	Amazon	tb013
Power switch Rocker Switch	Amazon	SIXQJZML
Rectangular Connectors - for crimped wires	Digikey	2183-1905-ND
Red LED	Digikey	LXM3-PD01

Sandpaper	Amazon	B002NEV6GS
Solder for soldering wires and circuit components	Amazon	Mudder Lead Free Sc
Solder-able Breadboard for building the circuit	Amazon	GK1007
Spade drill bit	Amazon	Irwin 88811
Transistor	Newark	2N2222A
Voltage regulator	Newark	LM317T
Windows 10 tablet	Amazon	B08BYTT79Y
Cell Culture Reagents		
Human Embryonic Kidney 293 cells HEK293	ATCC	ATCC CRL-1573
Fetal Bovine Serum	ThermoFisher	26140079
Dulbecco’s Modified Eagle Medium High Glucose	ThermoFisher	11965–092
10,000 units/mL of penicillin and 10,000 µg/mL of	ThermoFisher	15140122
White Corning 96-Well Solid Black or White Polyst	ThermoFisher	07-200-589
PEI MAX - Transfection Grade Linear Polyethylenir	PolySciences	24765-1
Name of Equipment		
Diagonal Cutting Plier (110mm)	Amazon	Proskit 1PK-037S
Dremil 3000 with cutting tool and grinder	Amazon	Dremel 3000
Dremil cutting and grinding tool	Amazon	Dremel 200-1/15
Dremil grinding tip	Amazon	Dremel 84922
EDSYN The Original Deluxe SOLDAPULLT	Amazon	DS017
Helping Hand with Magnifying Glass	Amazon	SE MZ101B
Pointed Nose Micro Pliers	Amazon	Hakko CHP PN-20-M
Small screw drivers	Amazon	Wiha 26197
Soldering iron	Amazon	Yihua 939D+ Digital S
TraceTech No-Clean Flux Pen	Amazon	2507-N
Weller WSA350 120v Bench Top Smoke Absorber	Amazon	WSA350
Wire strippers	Amazon	CSP-30-7
IWISS IWS-3220M Micro Connector Pin Crimping Tool (	Amazon	IWS-3220M

Comments/Description	Link
Inexpensive wire to connect LEDs to the power circuit.	https://www.amazon.com/gp/product/B072KGYH1M/ref=oh_aui_detailpage
2 x 1K Ohm potentiometer potential + 2 x black control Knob.	https://www.amazon.com/gp/product/B00XIWA2GO/ref=oh_aui_detailpage
These are very common and there are many equivalents.	https://www.amazon.com/gp/product/B071XN7C43/ref=oh_aui_detailpage_
Jameco Valuepro WJW-60B-R Wire Jumper Kit 350 each 22 AWG, 14	https://www.amazon.com/Jameco-Valuepro-WJW-60B-R-Jumper-Lengths/d
Uxcell a14051600ux0301 60 Piece Axial Lead 1% Tolerance Colored	https://www.amazon.com/a14051600ux0301-Tolerance-Colored-Resistor-Re
Set of 50Ea Metal Film Resistor 820 Ohm 1% 1/2W (0.5W).	https://www.amazon.com/50Ea-Metal-Film-Resistor-0-5W/dp/B00VGU2SS0
The cable that comes with the Arduino doesn't fit well in the box.	https://www.amazon.com/gp/product/B001MSU1HG/ref=oh_aui_detailpage
UNO R3 Board ATmega328P ATMEGA16U2 with USB Cable for Ardui	https://www.amazon.com/gp/product/B01EWOE0UU/ref=oh_aui_detailpag
Fits very snug.	https://www.digikey.com/product-detail/en/arduino/X000018/1050-1150-N
Hammond 1591ESBK ABS Project Box Black.	https://www.amazon.com/gp/product/B0002BSRIO/ref=oh_aui_detailpage_
LED LUXEON REBEL BLUE SMD. Uses "Saber 20 mm Star base"	https://www.digikey.com/product-detail/en/lumileds/LXML-PB01-0040/141
Tarvol Nylon Zip Ties (Pack of 100) 8 Inch with Self Locking Cable Tie	https://www.amazon.com/Tarvol-Nylon-Locking-Cable-White/dp/B01MRD0
Clips for holding circuit board inside of the black box. Command stri	https://www.amazon.com/gp/product/B0084M69YM/ref=oh_aui_detailpage
LED LUXEON REBEL CYAN SMD. Uses "Saber 20 mm Star base"	https://www.digikey.com/products/en?keywords=1416-1031-1-nd
Scotch 700 Electrical Tape, 03429NA, 3/4 in x 66 ft.	https://www.amazon.com/Scotch-Electrical-Tape-4-Inch-66-Foot/dp/B001U
Far Red (720nm) LUXEON Rebel LED. Uses "Saber 20 mm Star base"	https://www.luxeonstar.com/lxml-pf01-far-red-luxeon-rebel-led-260mW
Uses "STAR XP 3535" base	https://www.ushio-optosemi.com/jp/products/led/power/pdfs/edc/EDC740
Uses "STAR XP 3535" base	http://www.ushio-optosemi.com/jp/products/led/power/pdfs/edc/EDC780
Uses "STAR XP 3535" base	http://www.ushio-optosemi.com/jp/products/led/power/pdfs/edc/EDC810
Uses "STAR XP 3535" base	http://www.ushio-optosemi.com/jp/products/led/power/pdfs/edc/EDC850
These are very common and there are many equivalents.	https://www.amazon.com/Pico-6120D-Vinyl-Grommets-Package/dp/B0002
1-3/4 Inch (45 Mm)- Hair Clips Single Prong Metal Alligator Clips Hai	https://www.amazon.com/gp/product/B00K09T3L8/ref=oh_aui_detailpage_
Saber 20 mm Star Blank Aluminum MCPCB Base For Rebel LEDs	https://www.luxeonstar.com/saber-20mm-star-blank-mcpcb-base-for-a-rebe
Fits many other LEDs by Ushio	http://aduraled.com/product/pcb/1901-star-xp-3535-package-led
Loctite Epoxy Clear Multi-Purpose, 0.85-Fluid Ounce Syringe.	https://www.amazon.com/Loctite-Multi-Purpose-0-85-Fluid-Syringe-194358
These are very common and there are many equivalents.	https://www.amazon.com/NTE-Heat-Shrink-Assorted-Colors/dp/B000FIDTY
With these you can hang the LEDs in many places.	https://www.amazon.com/Command-Picture-Hanging-16-Pairs-PH204-16ES/
Any other 9V 1.5Z AC/DC converter will do becuase we cut the end	https://www.amazon.com/gp/product/B06Y1LF8T5/ref=oh_aui_detailpage_
These are very common and there are many equivalents.	https://www.amazon.ca/COOLOOdirect-Solder-Rocker-Switch-Toggle/dp/B0
6 Rectangular Connectors - Housings Black 0.100" (2.54mm)	https://www.digikey.com/product-detail/en/pololu-corporation/1905/2183-
LED LUXEON REBEL DEEP RED SMD. Uses "Saber 20 mm Star base"	https://www.digikey.com/products/en?keywords=1416-1701-1-nd

3M Wetordry Sandpaper, 03022, 800 Grit, 3 2/3 inch x 9 inch.	https://www.amazon.com/3M-03022-Imperial-Wetordry-Sandpaper/dp/B00
These are very common and there are many equivalents.	https://www.amazon.com/Mudder-Solder-Electrical-Soldering-0-22lbs/dp/B0
Gikfun Solder-able Breadboard Gold Plated Finish Proto Board PCB D	https://www.amazon.com/gp/product/B071R3BFNL/ref=oh_aui_detailpage_
These are very common and there are many equivalents.	https://www.amazon.com/Speedbor%C2%AE-Blue-GrooveTM-Standard-Leng
Can buy from many places.	http://www.newark.com/nte-electronics/2n2222a/bipolar-transistor-npn-40
Equivilent to NTE956.	https://www.newark.com/stmicroelectronics/lm317t/adjustable-linear-regu
Any Windows 10 PC will do.	https://www.amazon.com/gp/product/B08BYTT79Y/ref=ppx_yo_dt_b_asin_

Common Cell line.	https://www.atcc.org/products/all/CRL-1573.aspx
These are very common and there are many equivalents.	https://www.thermofisher.com/order/catalog/product/26140079#/2614007
These are very common and there are many equivalents.	https://www.thermofisher.com/order/catalog/product/11965118?SID=srch-
These are very common and there are many equivalents.	https://www.thermofisher.com/order/catalog/product/15140122?SID=srch-
White plates are preferred. Do not use clear plates.	https://www.fishersci.com/shop/products/costar-96-well-black-white-solid-p
Can be replaced with another transfection reagent.	https://www.polysciences.com/default/catalog-products/life-sciences/

These are very common and there are many equivalents.	https://www.amazon.com/iExcell-Diagonal-Cutting-Nippers-Chrome-Vanadiu
Dremel 3000-2/28 Variable Speed Rotary Tool Kit- 1 Attachments &	https://www.amazon.com/Dremel-3000-2-28-Attachments-Accessories/dp/I
Any similar Dremil will work.	https://www.amazon.com/Dremel-200-1-Two-Speed-Rotary-Tool/dp/B002B
Silicon Carbide Grinding Stone.	https://www.amazon.com/Dremel-84922-Silicon-Carbide-Grinding/dp/B000
For removing solder/mistakes.	https://www.amazon.com/EDSYN-The-Original-Deluxe-SOLDAPULLT/dp/B00
These are very common and there are many equivalents.	https://www.amazon.com/SE-MZ101B-Helping-Magnifying-Glass/dp/B000RE
Steel Super Specialty Pointed Nose Micro Pliers with Smooth Jaws, 1	https://www.amazon.com/Hakko-PN-20-M-Specialty-Pointed-Pliers/dp/B00I
These are very common and there are many equivalents.	https://www.amazon.com/26197-Precision-Slotted-Phillips-Screwdrivers/dp/
These are very common and there are many equivalents.	https://www.amazon.com/Professional-Digital-Soldering-Station-Switch/dp/
Tech Spray 2507-N No-Clean Flux Dispensing Pen, 11.5 mL.	https://www.amazon.com/Tech-Spray-2507-N-No-Clean-Dispensing/dp/B00I
For soldering safety.	https://www.amazon.com/Weller-WSA350-Bench-Smoke-Absorber/dp/B000
These are very common and there are many equivalents.	https://www.amazon.com/Hakko-CSP-30-7-Stripper-Maximum-Capacity/dp/
These are very common and there are many equivalents.	https://www.amazon.com/gp/product/B078WPT5M1/ref=ppx_yo_dt_b_sea

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FZPGUBI/ref=sr_1_1?s=hi&ie=UTF8&qid=1519268153&sr=1-1&keywords=Hakko+CHP+PN-20-M+Steel+Super+Specialty+Pointed+Nose+Micro+Pliers+w
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219869297712!p!!g!!lm317t&CMP=KNC-GUSA-SKU-MDC

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points The authors have made these changes.

3. Please provide an email address for each author. The authors have added the email address of each author in the manuscript.

4. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Arduino, Labview, LUXEON Rebel Color Line, Windows PC, LXB-RS20A, TraceTech No-Clean Flux Pen, LARII, etc. The authors have made these changes.

5. Please remove commercial terms from the keywords as well. Please do not italicize the keywords. The authors have made these changes.

6. Please define all abbreviations during the first-time use. The authors have made these changes.

7. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s) without any brackets. Please use Calibri 12 as font and size for the citation numbers as well. The authors have made these changes.

8. Each step and substep in the protocol should be numbered and numbering should follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, alphabets, or dashes. The authors have made these changes.

9. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” The authors have made these changes.

10. The Protocol should contain only action items that direct the reader to do something. The authors have made these changes.

11. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step. The authors have made these changes.

12. Please use complete sentences to describe the action being performed. The authors have made these changes.

13. Only one note and one caution can follow one step. In the JoVE Protocol format, “Notes” should be concise and used sparingly. They should only be used to provide extraneous details, optional steps, or recommendations that are not critical to a step. Any text that provides details about how to perform a particular step should either be included in the step itself or added as a sub-step. The authors have removed many notes and have edited remaining ones to be more concise.

14. Please revise the protocol text to avoid the use of any personal pronouns in the protocol (e.g., "we", "you", "our" etc.). The authors have made these changes.

15. Please ensure you answer the “how” question, i.e., how is the step performed? The authors have edited many steps to make how the steps are performed more clear.

16. There is a 10-page limit for the Protocol, but there is a 3-page limit for filmable content. Please highlight 3 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. The authors have highlighted the areas for filming and are within the page limits.

17. Please remove the figure legends from the figure. All figure legends need to be placed after the representative results section. The authors have made these changes.

18. Please upload each supplementary figure individually (with all the panels combined) to your editorial manager account. Are supplementary figures for review purpose only? Do you want these to be published along with the manuscript? Is it possible to reduce the number of figures in the second case? The authors have separated the supp figures from the legends and uploaded them separately. Feedback from non-engineers was that these figures printed alongside the legends were immensely helpful in building the device. One issue with the previously published protocol is access to the supplementary info and less granular details. We think that having all the steps will enable its wider adoption. Since we selected it to be open access, anyone interested in using this protocol will have access to all parts needed.
The authors have kept the software installation as a separate document as it has redundancies for different types of installations. It is possible to host this file on Github along with the software, however the reviewers suggested we actually expand on details related to the software. The

authors have removed DNA sequences and plasmid maps since the information is available on Addgene.

19. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].” All of the figures in this manuscript are original. The authors have not reused any figures from a previous publication.

20. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol The authors discuss several critical steps related to soldering/assembly, testing the system,deciding what LEDs and how to mount them. The authors also discuss the concept of leakiness and activation variable and how they depend on the copy number of the reporter DNA and possibly transfection efficiency.
b) Any modifications and troubleshooting of the technique The authors discuss modifying the system to have more LEDs or power and how to arrange the LEDs increase light diffusion of illuminate incubators, fish tanks, animal cages etc..
c) Any limitations of the technique The authors write about the limitations in terms of number of LEDs, power, as well as data and figures showing the activation levels and leakiness of the system.
d) The significance with respect to existing methods The authors explain that our system does not use many LEDs compared to other systems, but its simplicity to build and lower cost as well as its flexibility make it stand out.
e) Any future applications of the technique The authors indicate that this system has been used for three different optogenetic tools and even used in vivo in mice, which opens up many possible applications.

Reviewers' comments:

The authors thank all the reviewers for the thoughtful feedback and suggestions. Below, we address each concern individually.

Reviewer #1:
Manuscript Summary:
The manuscript describes the assembly and interfacing of an LED controller based on commercially available components. The assembly is detailed down to the most simplest aspects.

Major Concerns:

In most cases, scale bars are missing in the figures. The authors thank the reviewer for pointing this out. Most of the images are of standard parts (where dimensions are defined) and were taken with a camera from different angles and distances to help show the details of each step. We will note that many images are taken on top of a mat with a measuring grid.

It would have been helpful to see a completed system in operation together with the petri dishes comprising the cell cultures. The authors thank the reviewer for this helpful suggestion. We have added an image of the completed system in Figure 1B, described in lines 112-117, and noted additional images published in Allen *et al.* 2019. Lines (770-771 and 782-785)

Figure captions are missing (e.g. pages 70/71). The authors thank the reviewer for pointing this out. We have removed the figures with plasmid details for the sake of brevity and because all of this information is available on Addgene.

I recommend to limit the manuscript to the description of the technical tool(s) and leave out the transfection aspects. The authors thank the reviewer for this suggestion. However, since we include data from these transfections in the manuscript, we want to ensure the methods used here are sufficiently detailed. As another reviewer suggested, we have moved Supplementary Figure 30 (Now Figure S39) and removed the text in the protocol for the routine part of mammalian cell transfection.

Minor Concerns:
Figure 4 - Panel (E) is not given; I assume that in the Figure caption, (E) should read(C). Author have to cross check this issue for all other figure captions. The authors thank the reviewer for pointing this out. This omission has been fixed.

Sections are highlighted in yellow on pages 6-10 - please remove that. This highlighting is necessary to mark the sections for filming.

The web links on pages 26 and following are not given in a way such that they can be used. This needs to be solved by the authors. We think this is just due to the PDF format for the reviewed document. The links seem to work and the entire link should be visible once the Excel file is published, but we will be sure to check again before final publication.

Reviewer #2:
Manuscript Summary:
The manuscript presents a step by step protocol to setup an inexpensive light box suitable for optogenetic applications. This light box is easy to build and provides control over important parameters for optogenetic applications such as light intensity, light wavelength and pulsed light treatments.

Major Concerns:

Enough detail is provided to build the circuit however, more information about the software used to control the light box needs to be included in the main text. The authors have included additional instructions in section 6 (lines 405-415) and section 7 (lines 438-454) of the main text for installing and running the software.

Minor Concerns:

Below I provide some comments and suggestions:

Lane 30. Change Cry2 for CRY2 The authors thank the reviewer for pointing this out. This error has been fixed.

Lane 63. Include a reference of a review The authors thank the reviewer for this recommendation. We have added references of three reviews.

Lane 63. Consider changing the sentence: "to control genes expression and other cellular processes " The authors thank the reviewer for this recommendation. We have rewritten this sentence to be more concise and clear.(lines 72-74)

Briefly mention that optogenetic tools are dose dependent, and the dose can be regulated by tuning the light intensity and the exposure time. Having a tunable light source, as the one presented here, makes possible to adjust the light dose. The authors thank the reviewer for this recommendation. Authors have included this suggestion in the manuscript in lines (lines 74-75).

Lane74. Include reference to "Hernandez-Candia, C. N., Wysoczynski, C.L., and Tucker, C.L., Advances in optogenetic regulation of mammalian cells using cryptochrome 2 (CRY2). (2019) Methods." Here a step by step protocol to build a similar light box is presented. The authors have included this reference.

Lane 85. Explain more about the plasmids. What are they coding for? The authors thank the reviewer for pointing this out. We have removed the figures with plasmid details for the sake of brevity and because all of this information and more is available on Addgene.

Lane 94. Before explaining the details of each section, provide a general overview of the system. You could show a flowchart of the steps needed to build the light box. The authors thank the reviewer for this recommendation. We have included a new Figure 1A with a flow chart and a section for a system overview. (lines 112-123)

Also explain the difference between using a high or low voltage LED and mention what needs to be considered by the user to select a proper LED. The authors have rewritten the section on the LED driver in the introduction to explain the reason for the two circuits and how to select a proper LED. (lines 124-138) and lines (205-211)

Lane 124-132. Include a step to install the light box software and to setup the light treatment parameters. Authors have included additional instructions in section 6 and section 7 (lines 405-415 and 438-454) of the main text for installing and running the software.

Lane 256. First time the computer control is presented as an option. Mention since the beginning that the light box can be used without a computer, but that limits the system to constant illumination. The authors thank the reviewer for this recommendation. We have included this in the introduction in lines (292-293)

Lane 365. Instructions for the software installation and how to use it must be included here. You can refer to Supplementary file 2, but at least a general overview must be included in the main text. The authors thank the reviewer for this recommendation. The authors have included a section here for installing the software. We have also included further instructions for using the software in the next section "7. Stimulating the cells with light". (lines 405-415 and 438-454)

Lane 394. Mention that samples must be maintained in the dark and to change media a proper light source that do not excite the optogenetic system must be used. The authors thank the reviewer for pointing this out. We have added a note at the beginning of that section explaining this. (lines 427-428)

Figure 4. Legend refers to panel E) instead of panel C) The authors thank the reviewer for pointing this out. This error has been fixed.

Supplementary figure S30 can be moved after Supplementary figure S39 The authors thank the reviewer for this recommendation. The authors have switched the order of the figures accordingly.

Reviewer #3:

Manuscript Summary:

The authors present a detailed protocol on the construction of an illumination device for optogenetic experiments. The device controls 4 high powered LEDs that are each tunable through potentiometers. The device is controlled by an Arduino, which can be programmed by a nice-looking custom GUI. The authors have taken great care to detail every step of assembly, with clear diagrams and descriptions. I applaud the authors for the effort in assembling this comprehensive protocol. My main concern is that in recent years, a number of more sophisticated devices have been described (PMID 32521262, 31235951), which also have step-by-step protocols, and appear to be faster and simpler to assemble. Thus I'm not sure the extent to which a scientist would choose this technique over others. Although this device is "simpler" as the authors state, it doesn't necessarily seem simpler to assemble in terms of number of parts or time of assembly. The authors should explicitly compare and contrast this device vs the others in more detail, and indicate the use cases where this device would be more suited for a particular application.

Major Concerns:

-the introduction should be more specific about what will actually be constructed, as this is not clear. An annotated image or diagram of the final working device should be presented and described upfront. As I was reading the protocol, I wasn't sure if the LEDs were pointing into the box, or out of the box, or if that was something you could choose, or whether the cells were in the box, or outside of the box. I'm still not entirely sure. The authors thank the reviewer for suggesting this. We have included a diagram and image of the entire system in Figure 1A and 1B and reference them in a new section describing the system as a whole. (lines 112-123)

-the introduction should also discuss the other available alternatives (especially PMID 32521262, 31235951 as mentioned above), compare and contrast the authors' technology with these alternatives, and mention specific use cases where the authors' technology would be preferable. The argument on line 75 that other devices don't have step by step protocols and require more expertise than this protocol are not true, eg for the two publications cited above.

The authors thank the reviewer for pointing out these differences. Having reviewed and compared the protocols PMID 32521262, 31235951 (and Hernandez-Candia, C. N., Wysoczynski, C.L., and Tucker, C.L., Advances in optogenetic regulation of mammalian cells using cryptochrome 2 (CRY2). (2019) Methods.") with ours it seems that the three references are more sophisticated and more complex to assemble. In addition, to complete some of the steps with the instructions in those publications would require a high level of expertise and they call for much more costly mechanical and electronic parts. The authors have rewritten the paragraph referenced by the reviewer to better compare each device . In addition, the authors have added details explaining when and why this LED system would be preferable to the others (lines (85-94).

-the choice to tune LED intensity with variable voltage should be discussed. The voltage-brightness relationship is nonlinear, and for this reason LEDs are more commonly tuned by changing current, which has a linear relationship with brightness. At a minimum this should be acknowledged and discussed.

The authors thank the reviewer for pointing this out. The linear relationship between current and brightness is particularly useful when calibrating for a specific current to run at a specific intensity, as for a system that would automate the brightness. In addition, by having a power supply that controls LED voltage instead of current, it can power LEDs that use different voltages without modifying the design. The authors have included this explanation in Lines 133-134. However, this system has a manual light intensity adjustment (using the potentiometer) that is important to manually calibrate with a light meter before each experiment anyway. We have added instructions on how to measure the light intensity routinely using a lightmeter prior to each experiment (lines 433-446). We have now included a figure showing how to place the light meter in Figure S39D.

There is very little characterization of important illumination parameters that have been discussed in reports of previous devices. For example, there is no characterization of light intensities, heterogeneity of intensities, or how intensity scales with tuning the potentiometer Is this device best used for single large plates? Multiwell plates? Do you have spatial heterogeneity within a plate depending on where you place it relative to LEDs?
Since the system can be set up in many ways, the spatial heterogeneity depends on how they are used. By using the LEDs on the top of the boxes (Figure 1B, S22 and S23) we are illuminating all the cells in box with excess light (for PhyB optogenetics), so the amount of heterogeneity is not an issue for using the LEDs to control genes. When higher power is needed heterogeneity could be an issue. Allen et al. characterized this for the nMag-pMag-based optogenetic system in Figure S2. The authors have updated the text adding this reference in the discussion. (Lines 770-771 and 782-785)

Does this limit how much analog control you can have over your optogenetic construct (in that it works great for fully ON or OFF stimulation, but would have too much variability at intermediate intensities?) How far away from the samples should the LED box be placed? This device uses high-powered LEDs, which draw lots of current and get very hot. Is there heating at the sample? Does this constrain how far the sample has to be from the LEDs? These are important factors that, if not characterized, should at a minimum be discussed.
Intermediate intensities should not be an issue to achieve because the intensity is adjusted before each experiment using a light meter. Using the voltage in the circuit we designed, we don't feel any noticeable heating to the touch, even at the highest intensity for the low voltage LEDs and very little heat for the high voltage LEDs. When higher powers are used, LEDs can be set up farther from the cells, physically separated, as shown in Figure S2 and in Allen *et al.* 2019 to both minimize heat near the samples and decrease heterogeneity of the light intensity. These points are discussed in Lines 770-771.

Minor Concerns:
-some figures are introduced out of order (the first figure cited is figure S7)
-line 129 - it is unclear what box is being referred to. This would be fixed by a more detailed discussion of the device in the intro The authors thank the reviewer for this suggestion. We have included a section called "system overview" as well as a picture of the entire system (Figure 1B) referenced in this section. (lines 112-123)

-line 147. To solder a joint, you want to make sure both the metal on the board and component are heated before adding solder. This protocol suggests only heating the component, which will lead to a poor joint. The authors thank the reviewer for pointing this out. This note has been rewritten to clarify best soldering techniques. (lines 172-174)

-it's unclear why large portions of the text are highlighted in yellow This highlighting is necessary to mark the sections for filming.

-line 177 it is unclear why flux helps solder "with minimal heating".

Should be more specific about what user has to do with the flux, and why this helps solder at lower temperatures

The authors thank the reviewer for pointing this out. We have changed this section, removing the Note and added steps for adding flux. (Lines 182-183, 187-188, 243, 287-289, 293, 318-319).

-line 348 what does it mean to "sand clips to the base"? Perhaps this is a type-o The authors thank the reviewer for pointing unclear instruction out.The authors have rewritten this section to make the meaning clear. Lines (366-367 and 372-373)

Supplemental File 1: Assembly and transfection instructions

Table of contents:

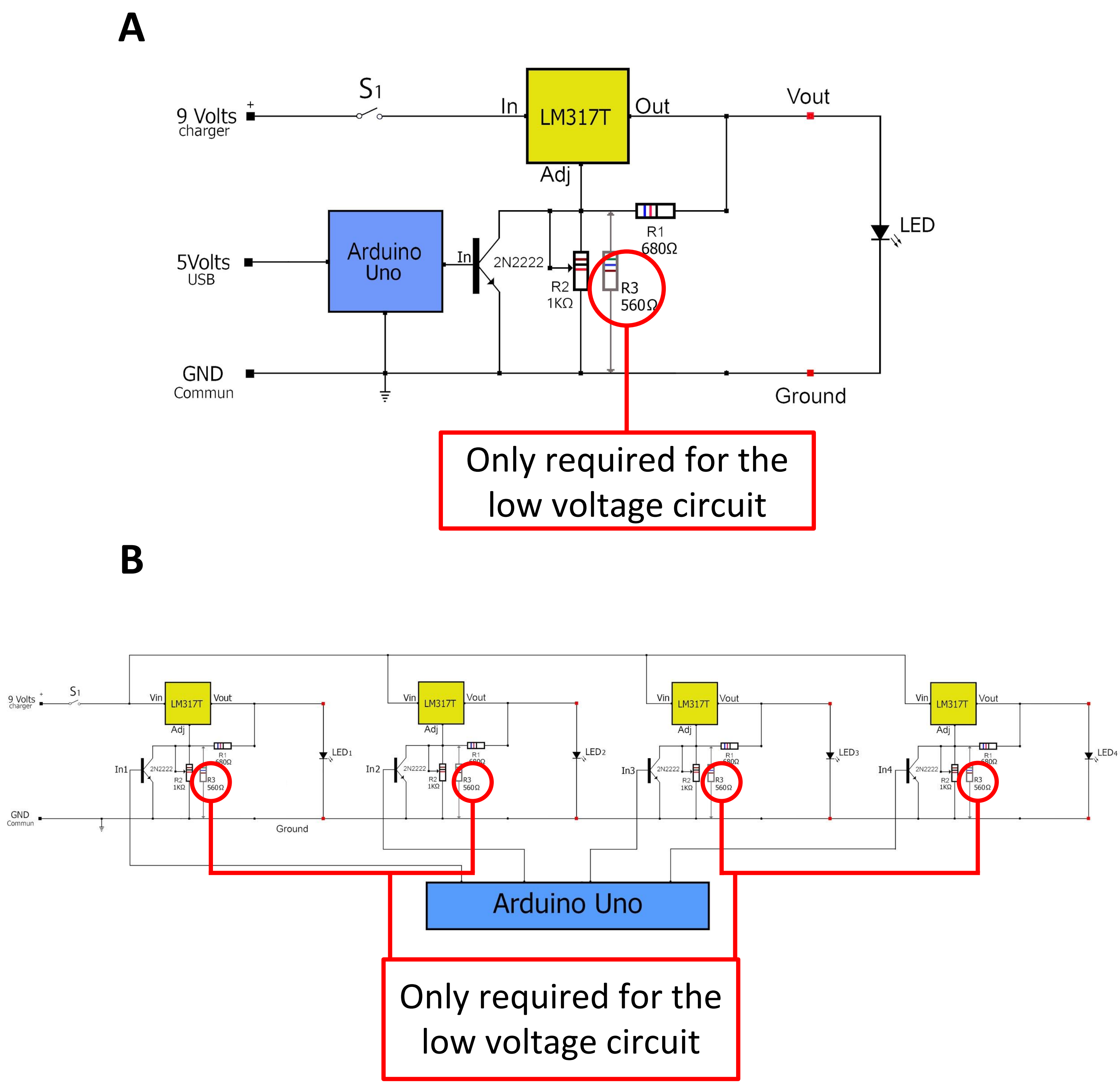


Figure S1: Electronic Driver Circuit for multiple LEDs. (A) The circuit diagram for a single LED system. (B) The circuit diagram for a four LED system.

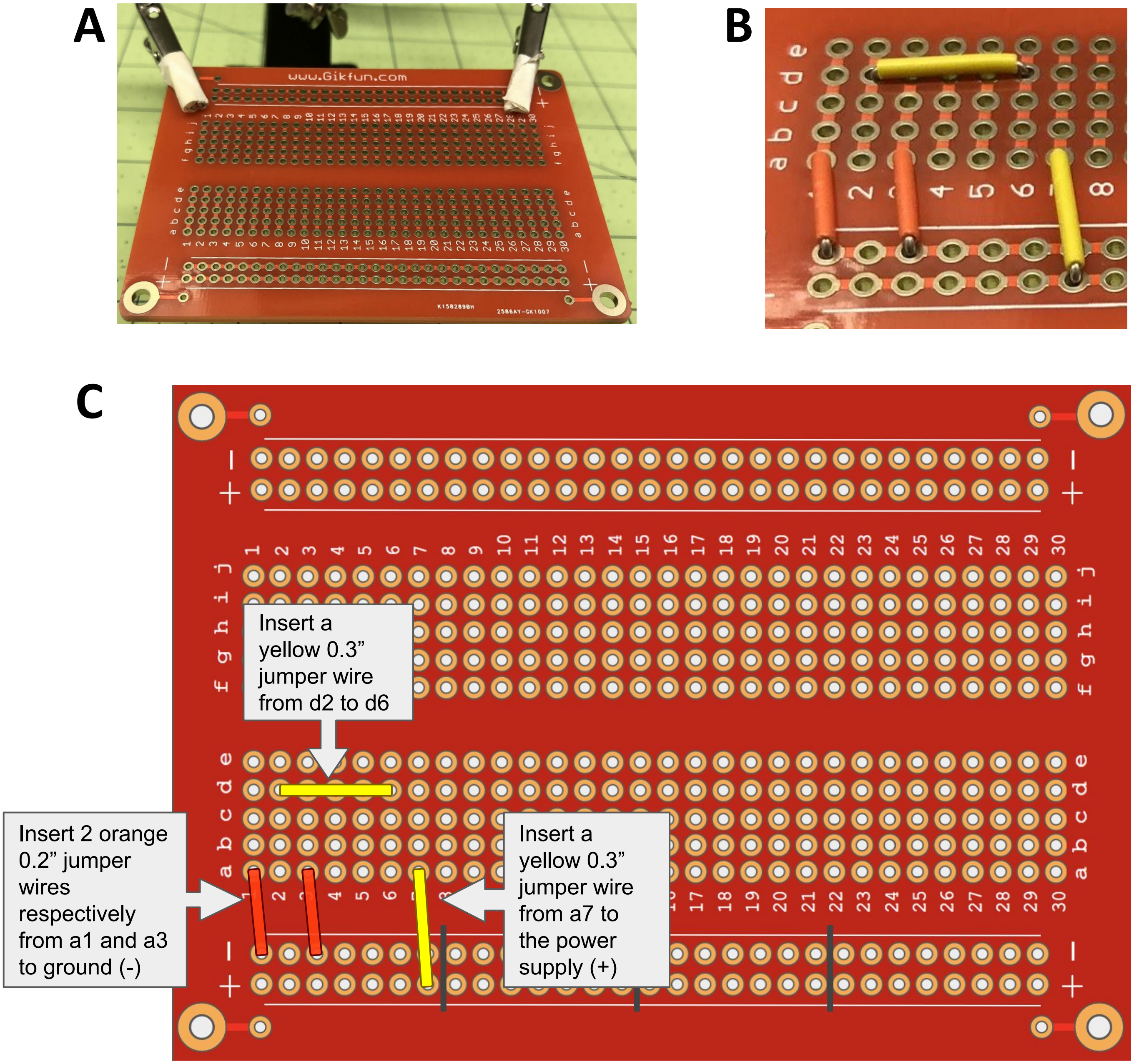


Figure S2: Placing the circuit Interconnections. (A) Clip your PCB board onto your helping hands. (B) Position of main circuit jumpers into the through holes in the picture. (C) Diagram of wire connectors mapping the coordinates. For the four LED system, draw lines dividing each circuit as shown (black vertical lines). Figures S31-S38 describe the assembly of four circuits simultaneously.

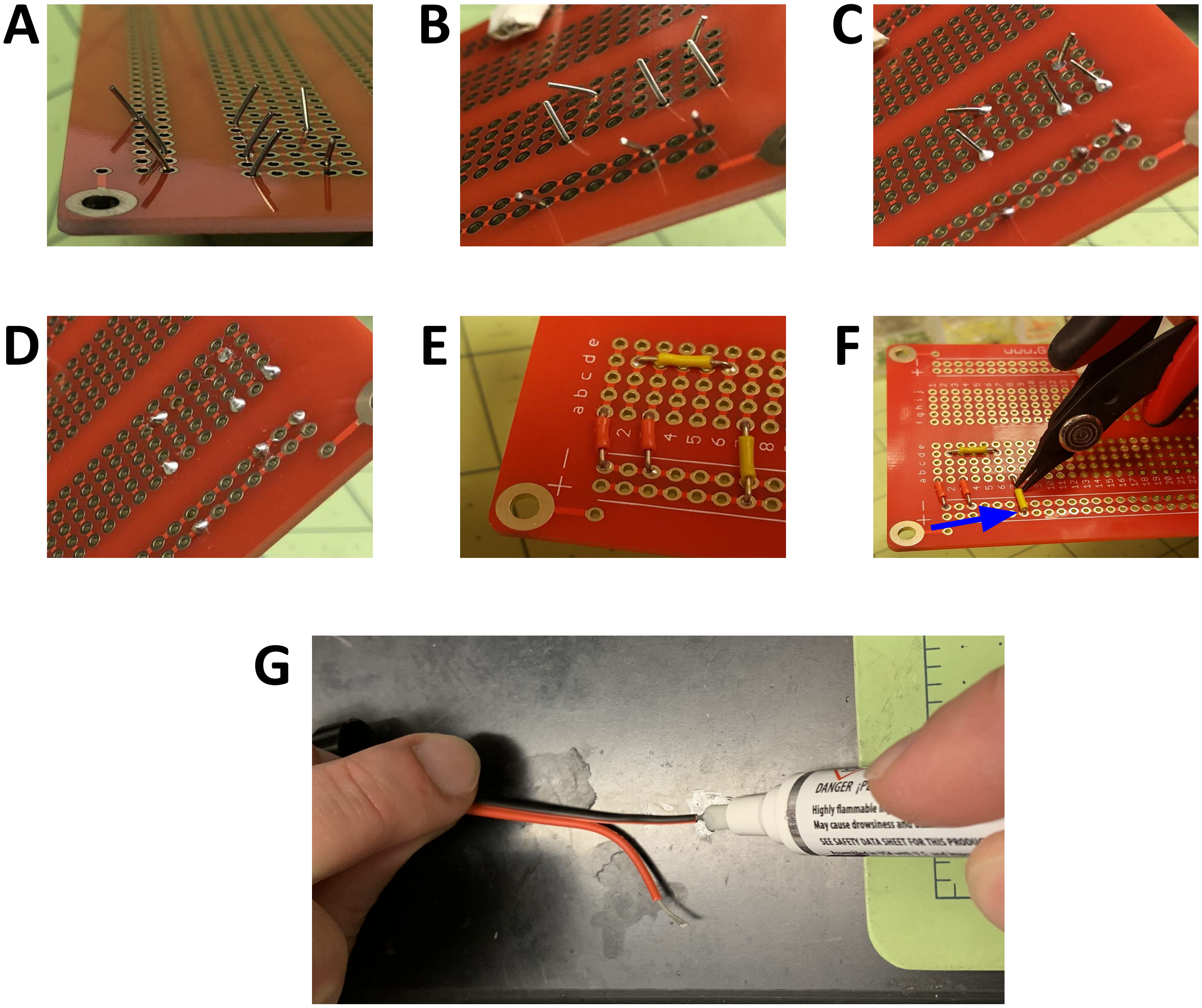


Figure S3: Soldering the wires onto the PCB. (A) Bend jumpers so that they make direct contact with the PCB and stay in place while soldering. (B) Another view of the bent wires. (C) Wires after soldering. (D) Trimmed wires on the PCB. (E) Shrunken insulation after heating with solder. (F) Moving the insulation into position to cover the ground through hole (blue arrow) (G) Adding flux to a wire end or terminal.

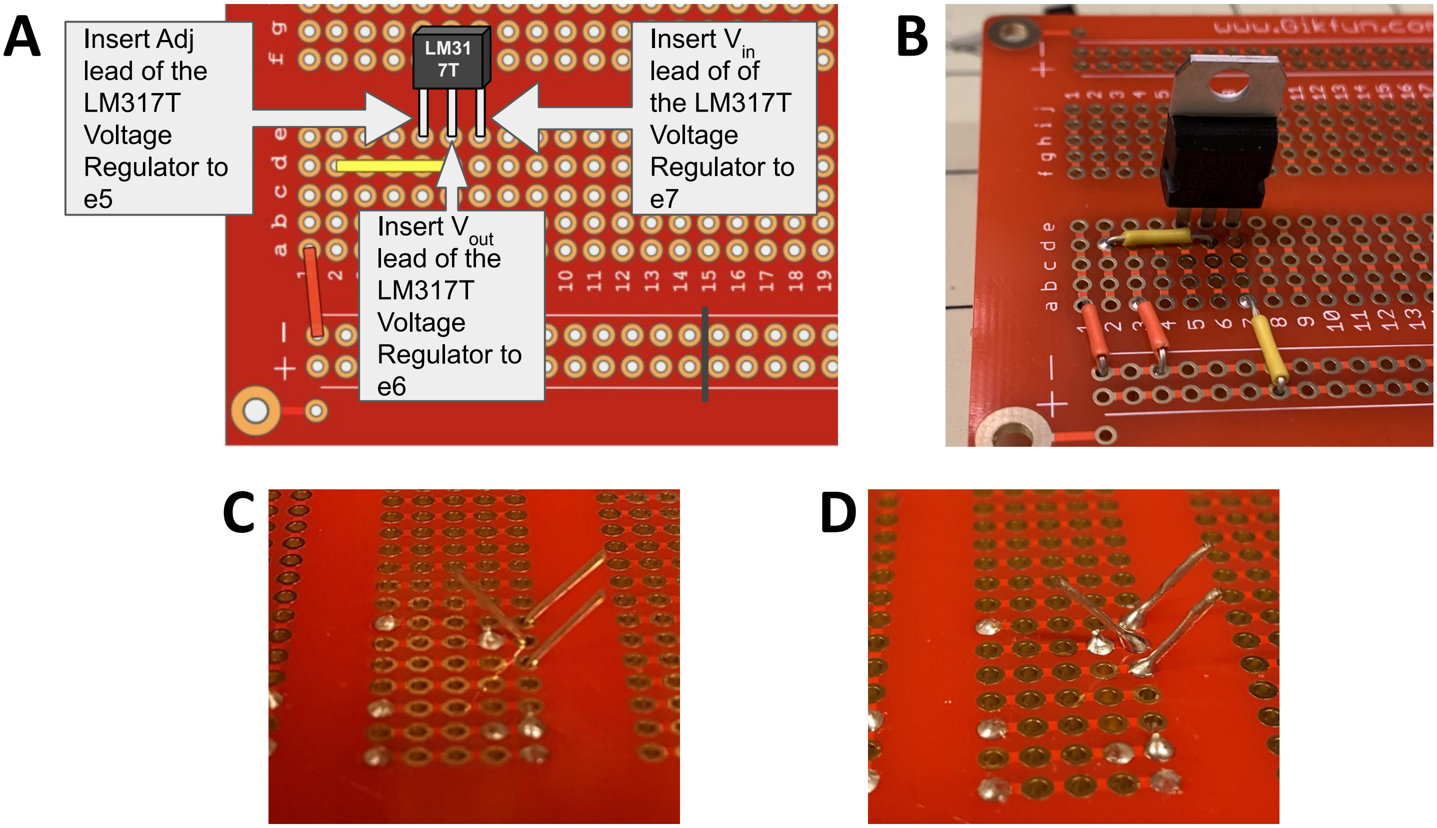


Figure S4: Soldering the voltage regulator into place.(A) Map of the voltage regulator coordinates. (B) Placement of the voltage regulator. (C) Bent voltage regulator leads. (D) Voltage regulator terminals after soldering.

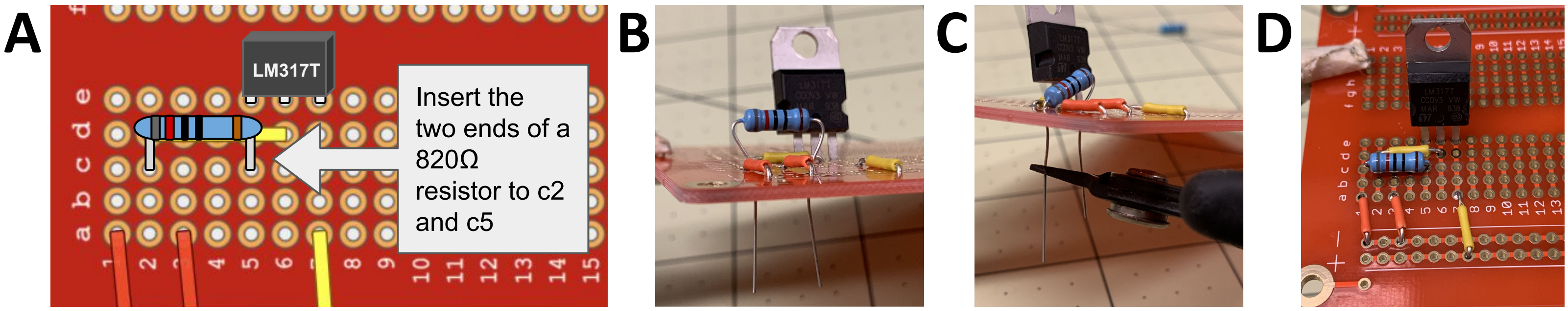


Figure S5: Soldering the R1 resistor into place. (A) Map of the R1 resistor (820Ω) coordinates. (B) Pulling the resistor through by the lead using pliers (C) The pulled resistor close to PCB. (D) The soldered resistor close to PCB.

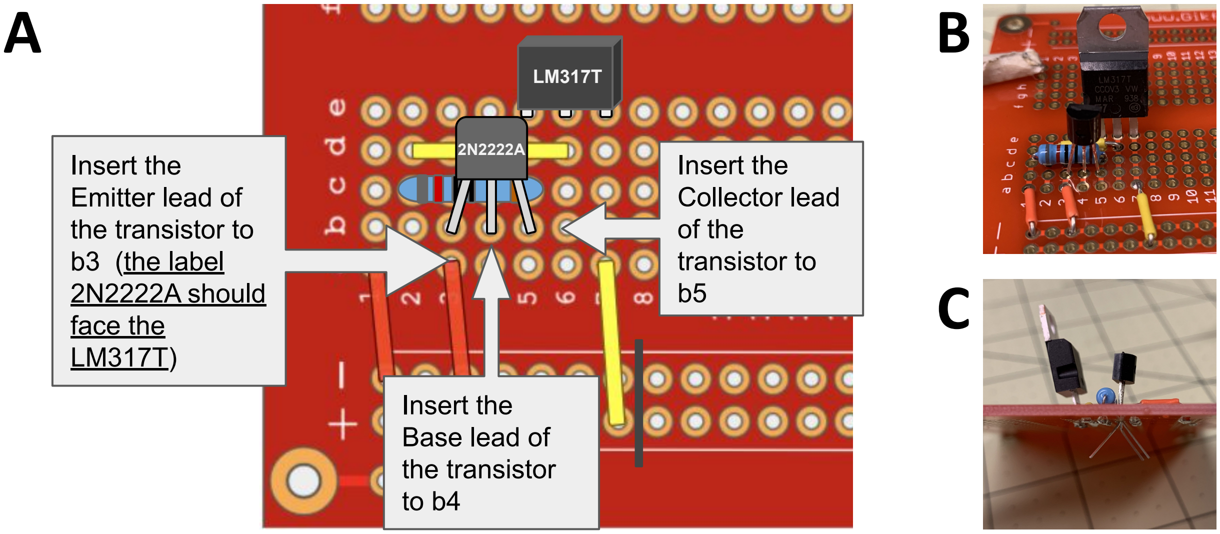


Figure S6: Soldering the transistor into place. (A) Map of the transistor coordinates and orientation. (B) Note the orientation of the transistor, the label in this model is facing the voltage regulator (LM317T). Double check the specification of the transistor to make sure the “Emitter”, “Base”, and “Collector” are in the correct holes. (C) The transistor with the terminals bent prior to soldering.

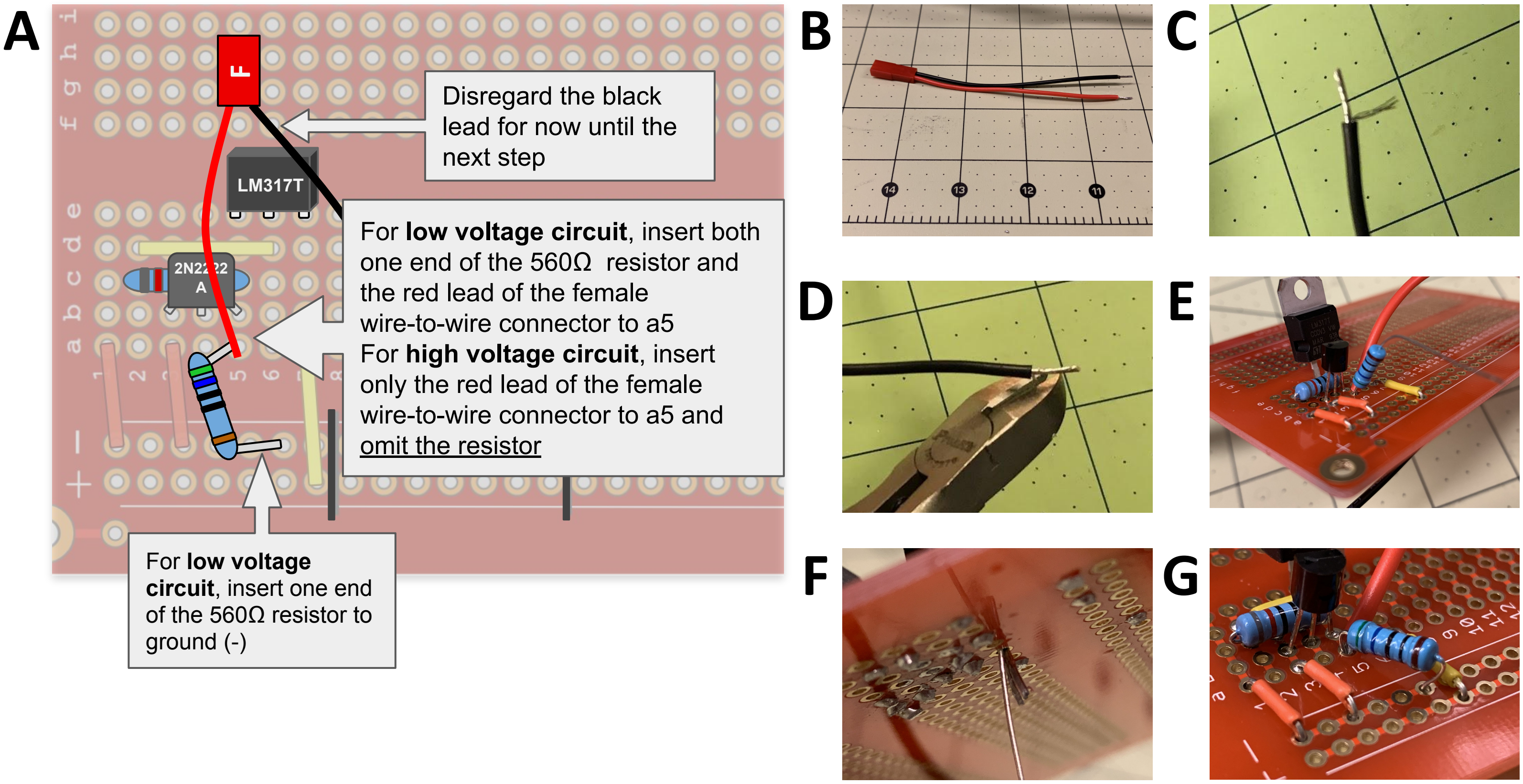


Figure S7: Soldering the wire-to-wire connector for the potentiometer into place (plus a 560Ω resistor for the low voltage circuit). (A) Map of the coordinates of the wire-to-wire connector (plus the R3-560Ω if building the low voltage circuit, the wire-to-wire connector is placed in the hole before the resistor.) (B) A female wire-to-wire connector. (C) To facilitate fitting the resistor along with the wire-to-wire connector, 3-5 strands of the braided wire are bent. (D) The strands are cut off with wire cutters as close to the insulation as possible. (E) Inserted red wire of a female wire-to-wire connector through the a5 through hole (For the low voltage circuit insert R3 through the same through hole.) (F) Underside view of the resistor and wire-to-wire connector before soldering. (G) Image of the soldered R3 resistor connected to ground. (F = Female)

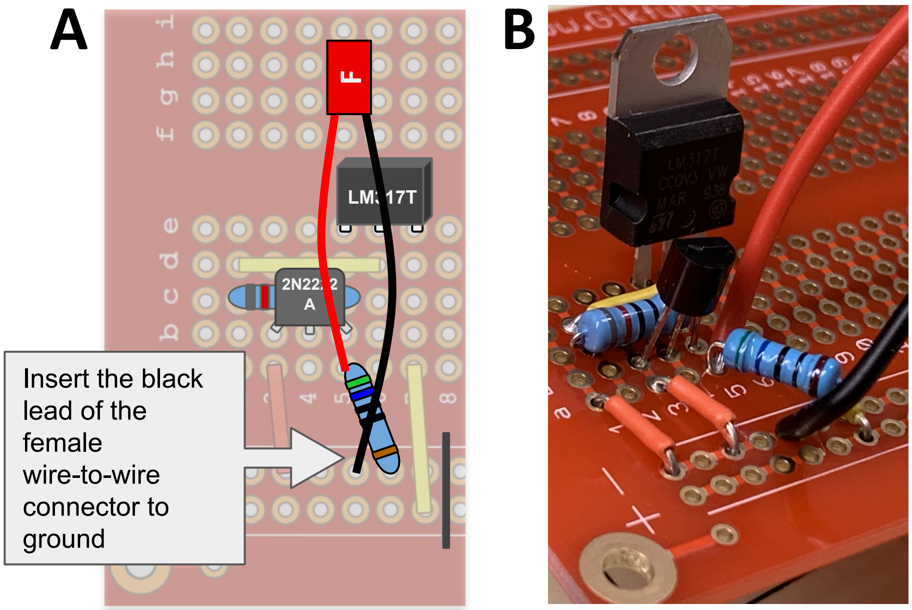


Figure S8: Soldering the wire-to-wire connector for the potentiometer to the ground. (A) Map of the coordinates of the ground connection for the potentiometer wire-to-wire connector. (B) Top view of the potentiometer wire-to-wire connector in parallel with R3. (F = Female)

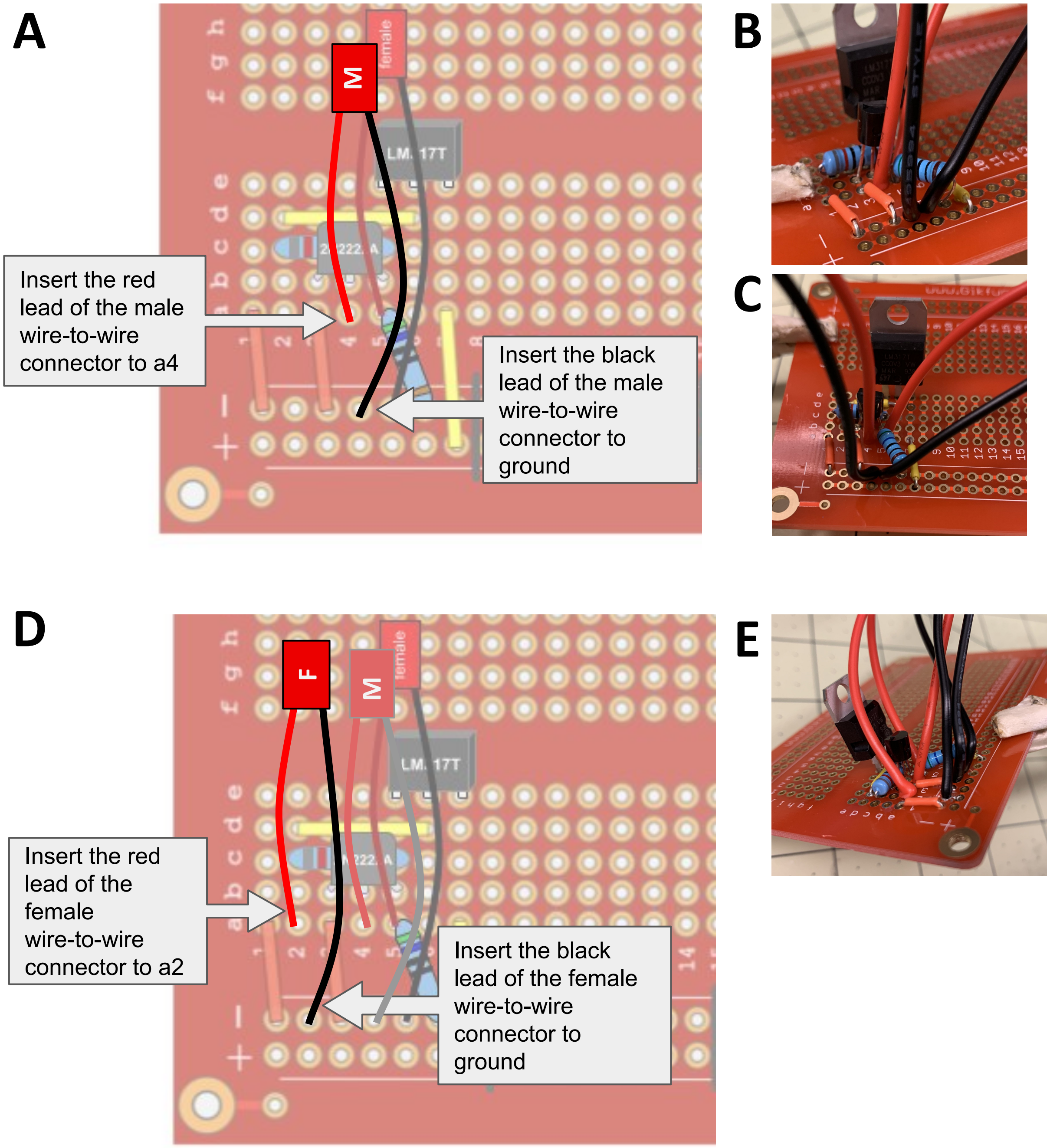


Figure S9: Soldering the microcontroller and LED wire-to-wire connectors. (A) Map of the coordinates of the wire-to-wire connector for connecting the 2N222A and the ground to the microcontroller. (B) Soldered male wire-to-wire connector. (C) Top view of (B). (D) Map of the coordinates of the female wire-to-wire connector for connecting the input of the circuit and ground to the LED. (E) Soldered female wire-to-wire connector. (F = Female, M = Male)

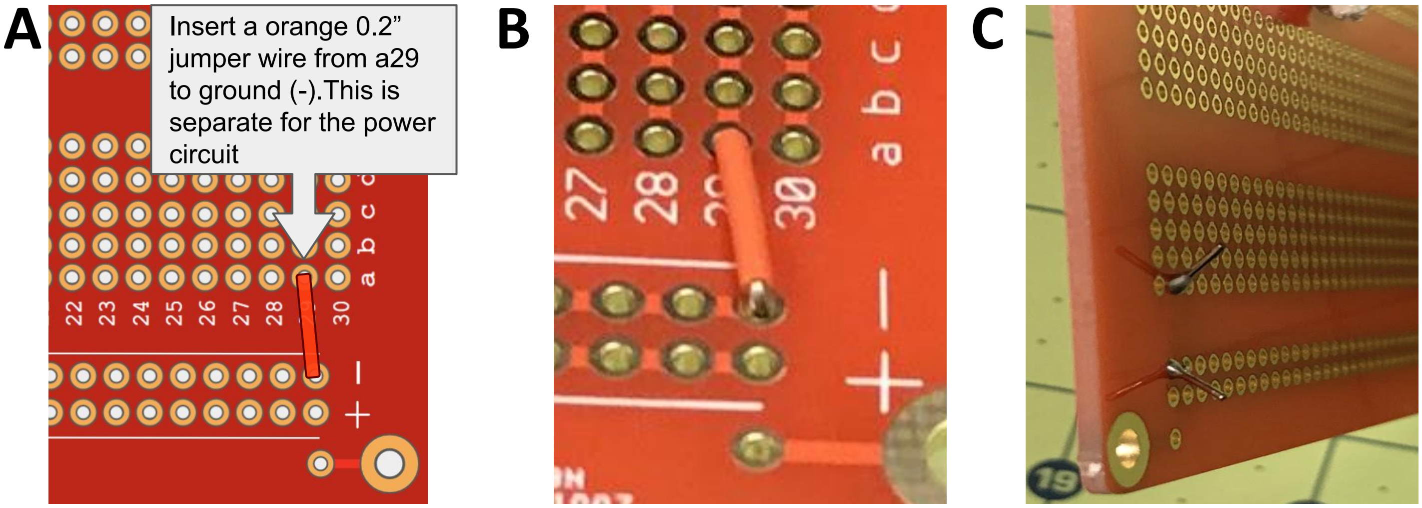


Figure S10: Soldering the jumper for the power supply circuit. (A) Map of the coordinates of the orange jumper for connecting the power supply to ground. (B) The orange jumper soldered in place. (C) The underside view of the jumper soldered in place.

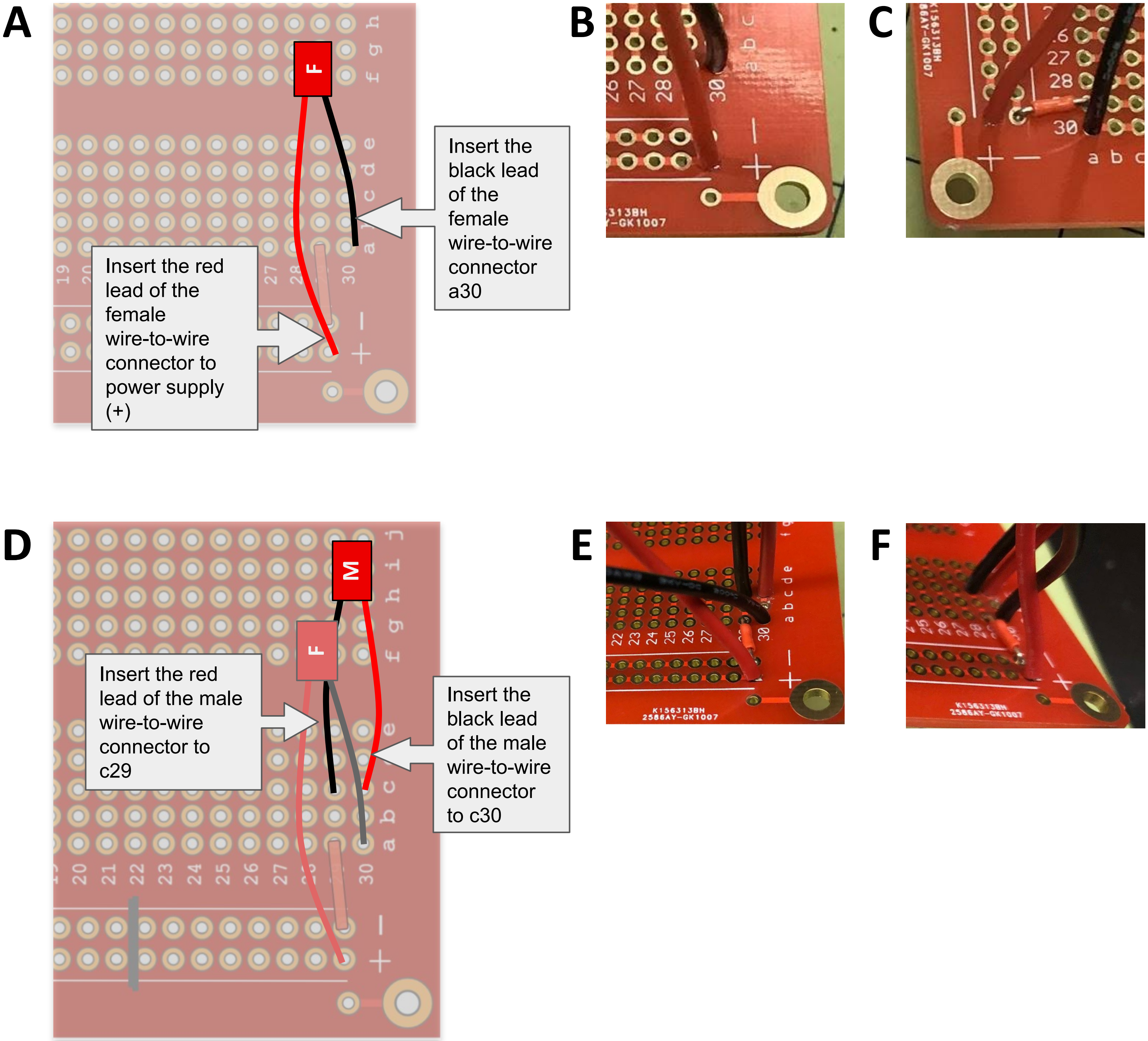


Figure S11: Soldering the power switch and power source wire-to-wire connectors. (A) Map of the coordinates of the female wire-to-wire connector for connecting the power switch. (B) The female wire-to-wire connector soldered in place. (C) Another view of (B). (D) Map of the coordinates of the male wire-to-wire connector for connecting the power source. (E) Soldered male wire-to-wire connector. (F) Another view of (E). (F = Female, M = Male)

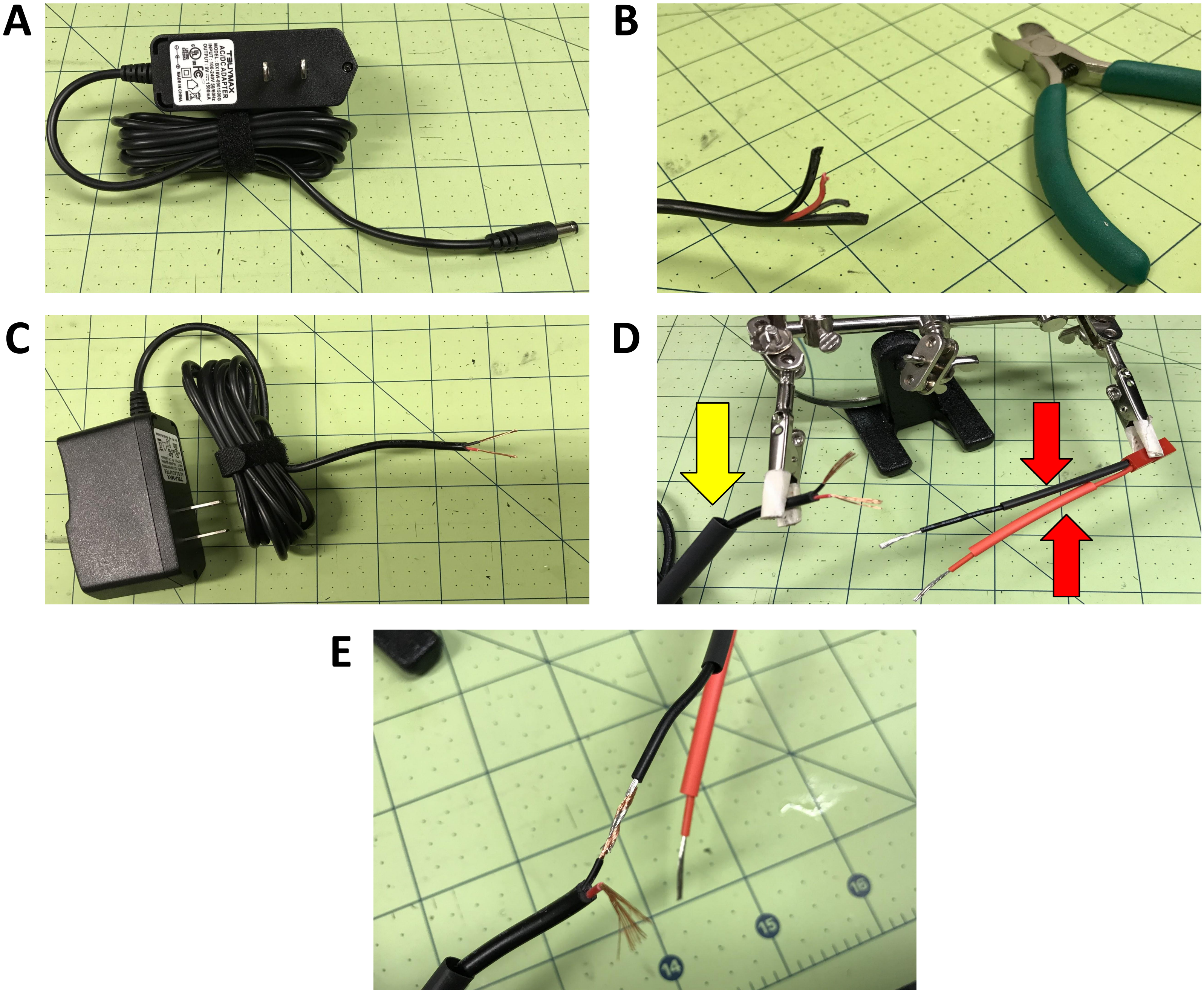


Figure S12: Connecting the power supply to a male wire-to-wire connector. (A) The unmodified power supply. (B) Cutting of the power supply wires. (C) The power supply wires stripped and with excess insulation cut away. (D) Placement of shrink tube around power supply wires. Tubing separating the two connections (red arrows) and tubing to hold the separated wires (yellow arrow). (E) Twisted wires connecting the power supply to the female wire-to-wire connector.

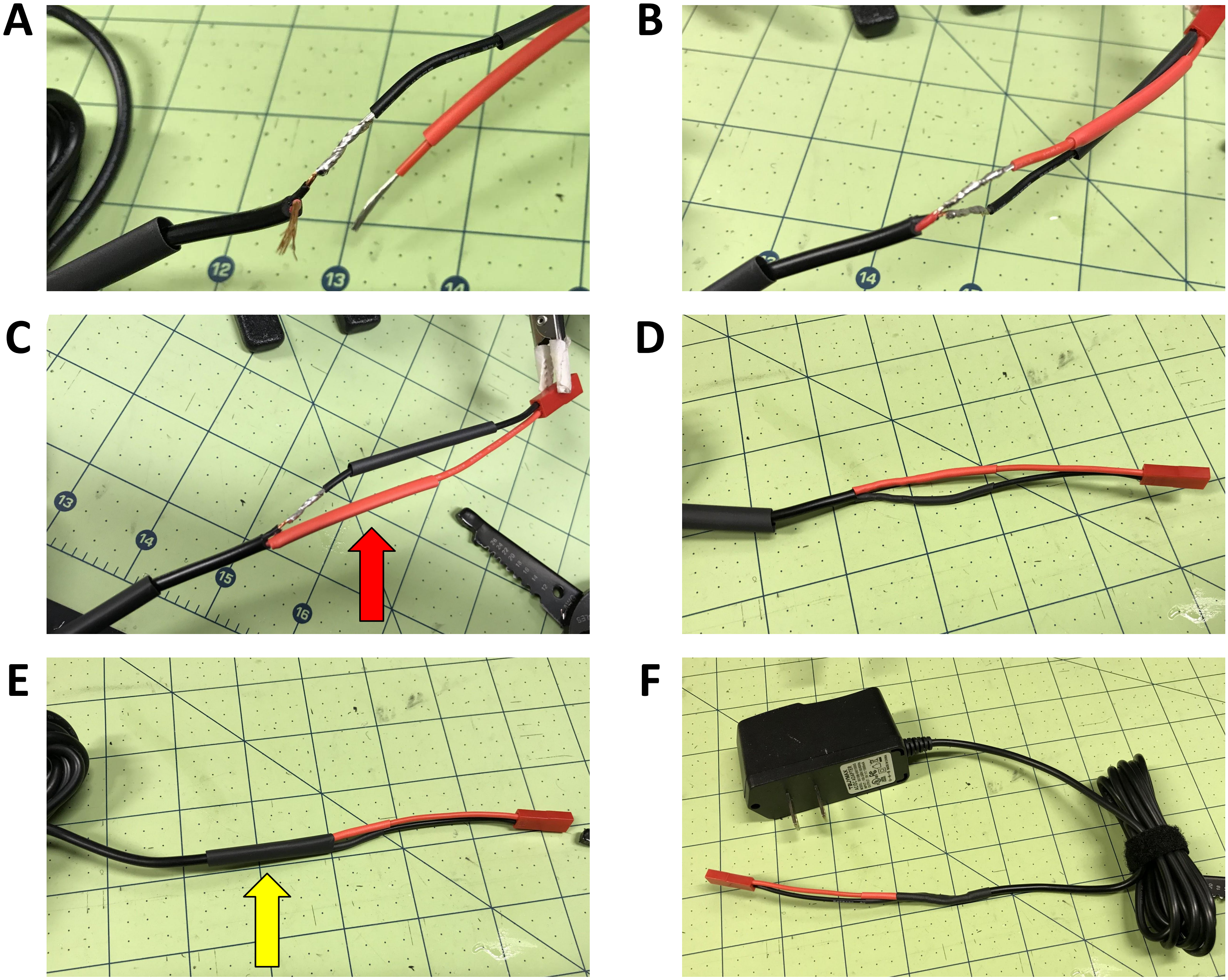


Figure S13: Soldering and insulating the power supply connection to a male wire-to-wire connector. (A) Soldered connection between the power supply ground and a female wire-to-wire connector. (B) Soldered connection between the positive terminal of the power supply and a female wire-to-wire connector. (C) Shrink tube pulled over the soldered individual connections (red arrow). (D) Both power supply connections soldered and with heat treated shrink tube. (E) Placement of shrink tube over individual connections (yellow arrow). (F) Completed power supply.

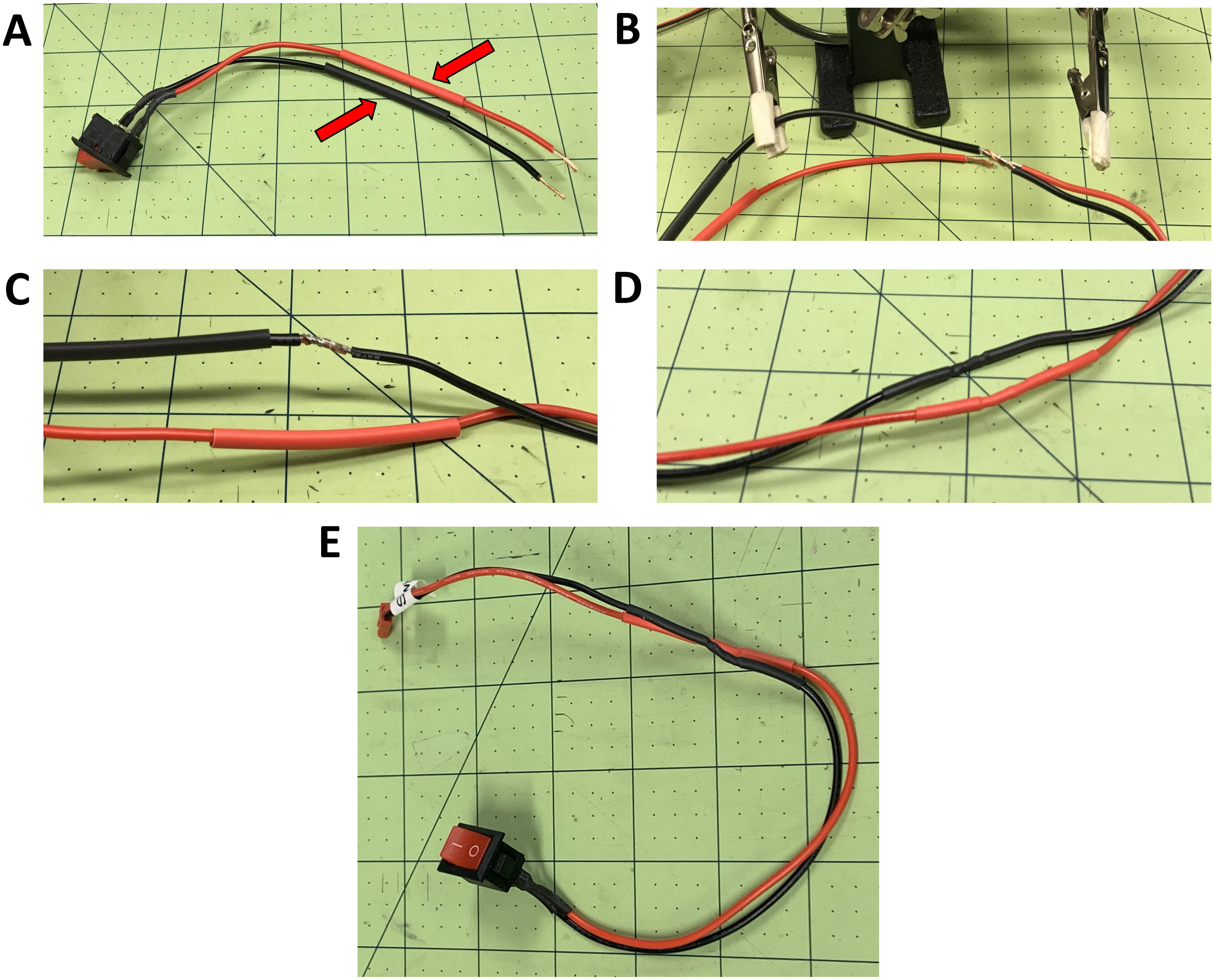


Figure S14: Soldering the power switch to a male wire-to-wire connector. (A) Power switch with stripped wires and shrink tube placed over the wires (red arrows). (B) Wires connecting the switch and male wire-to-wire connector twisted together before soldering. (C) Placing the shrink tube over the soldered connections. (D) Connections covered with the heat treated shrink tube. (E) A powerswitch assembled with a male wire-to-wire connector.

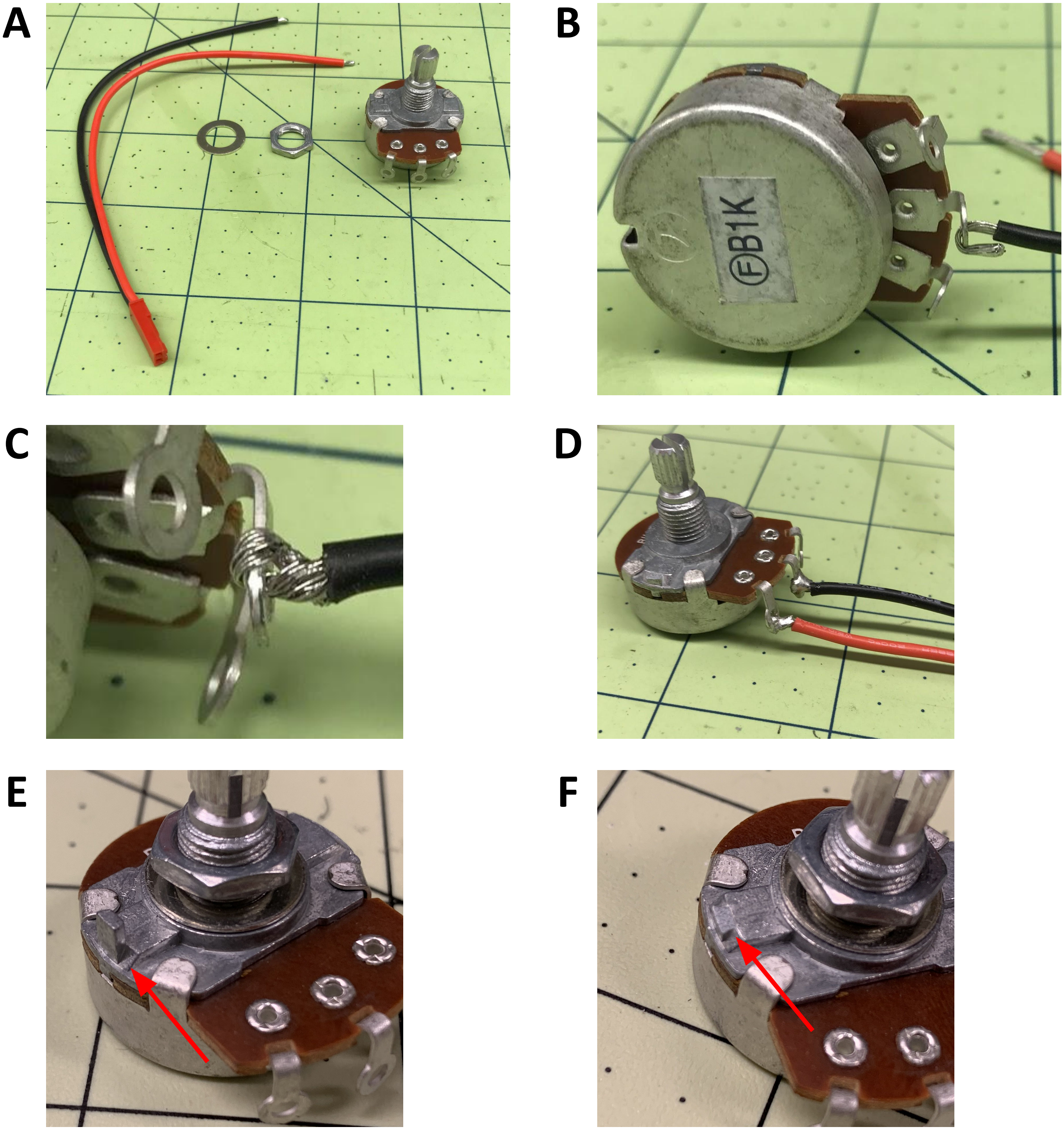


Figure S15: Wiring a potentiometer to a male wire-to-wire connector. (A) The potentiometer parts. (B) A male wire-to-wire connector twisted and bent to hook around the middle terminal of the potentiometer. (C) Male wire-to-wire connector twisted around the middle terminal of the potentiometer. (D) Soldered wire-to-wire connections. (E) Red arrow pointing to the metal tab prior to removal. (F) The potentiometer after metal tab removal.

A B C D E

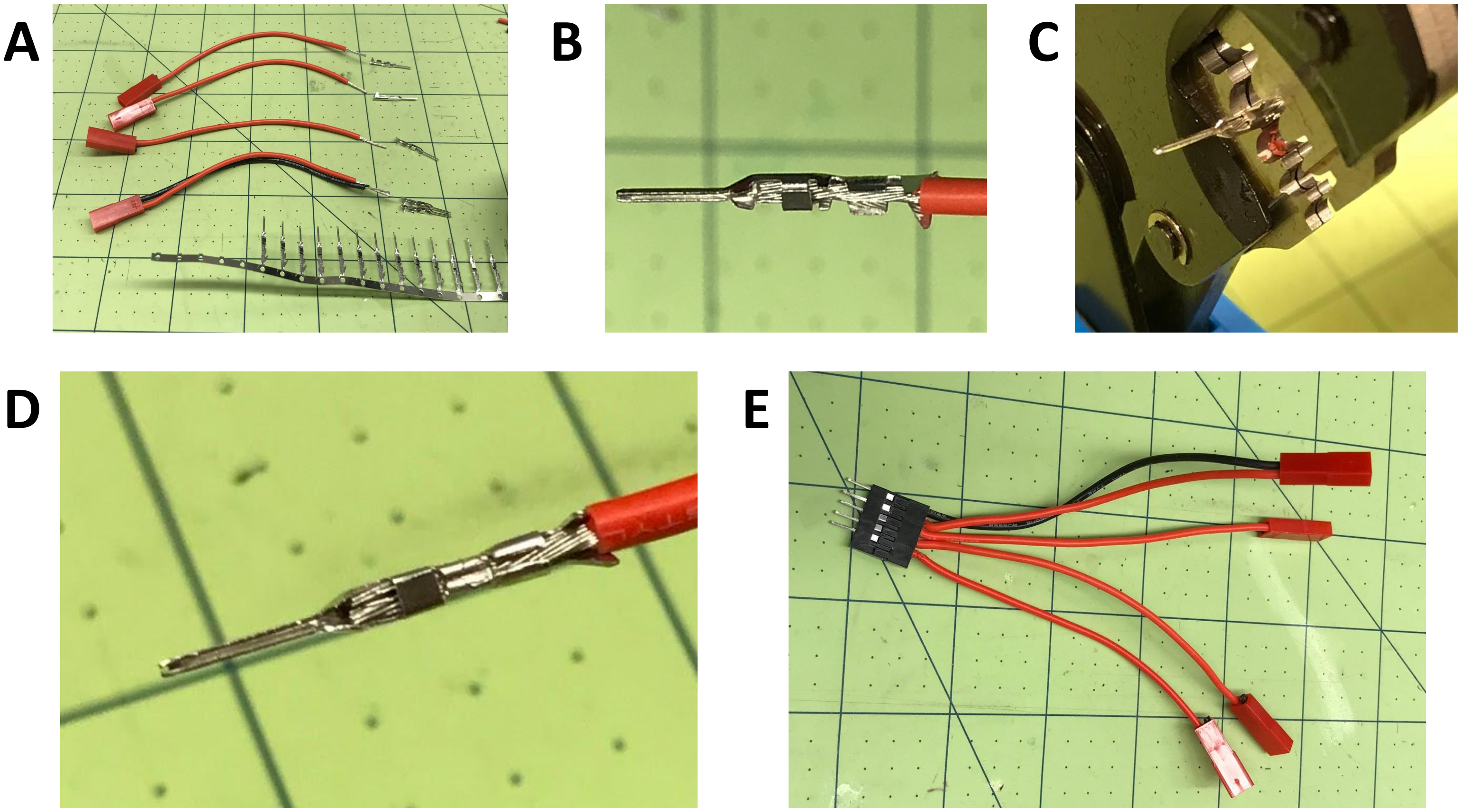


Figure S16: Wiring the microcontroller connection. (A) Wires for female wire-to-wire connectors stripped and cut in preparation for crimpring. (B) Placement of the crimp on the wire-to-wire connector. (C) Crimping of the wire-to-wire connector. (D) Crimped wire-to-wire connector. (E) Fully assembled microcontroller connection.

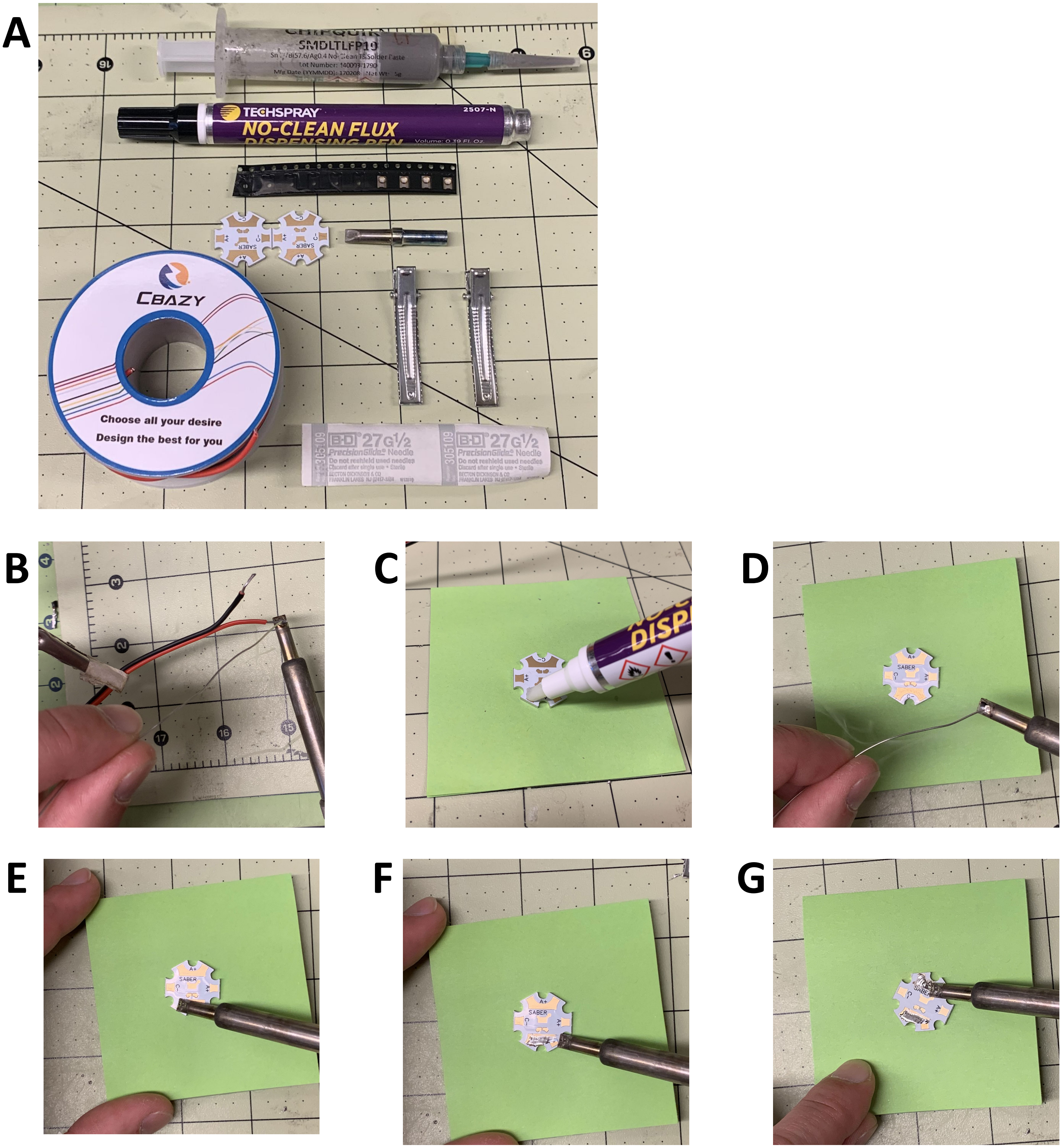


Figure S17: Soldering wires and LED onto the LED base Part 1. (A) Materials needed to solder the LED to the LED base. (B) Tinning the tip of the stripped wire. (C) Applying flux onto the contact of the LED base. (D) Adding solder to the large soldering tip to tinning the LED base. (E) Placement of solder onto the contact to heat the LED base. (F) The LED base after dragging the soldering tip across the contact. (G) The same procedure on the other contact.

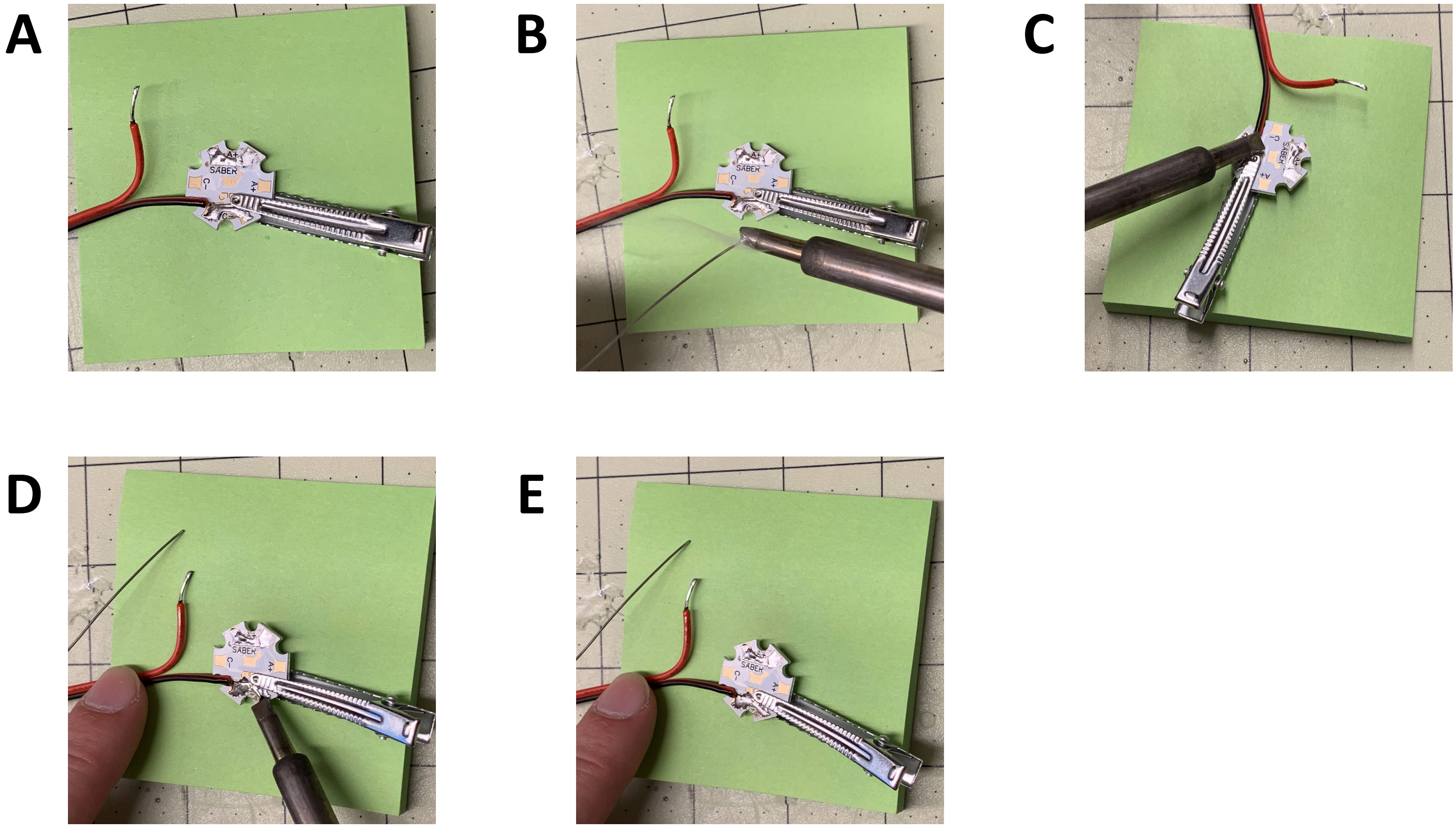


Figure S18: Soldering wires and LED onto the LED base Part 2. (A) A tinned wire clipped to the contact using a hair clip. Note that the black wire is soldered to the cathode “C-” (B) Addition of a generous amount of solder to the the soldering tip. (C) The soldering tip pressing down on the wire, melting the solder on the LED base and the wire. (D) Holding down the wire so that it stays put when the soldering iron is removed. (E) Holding the wire in pace until the solder hardens.

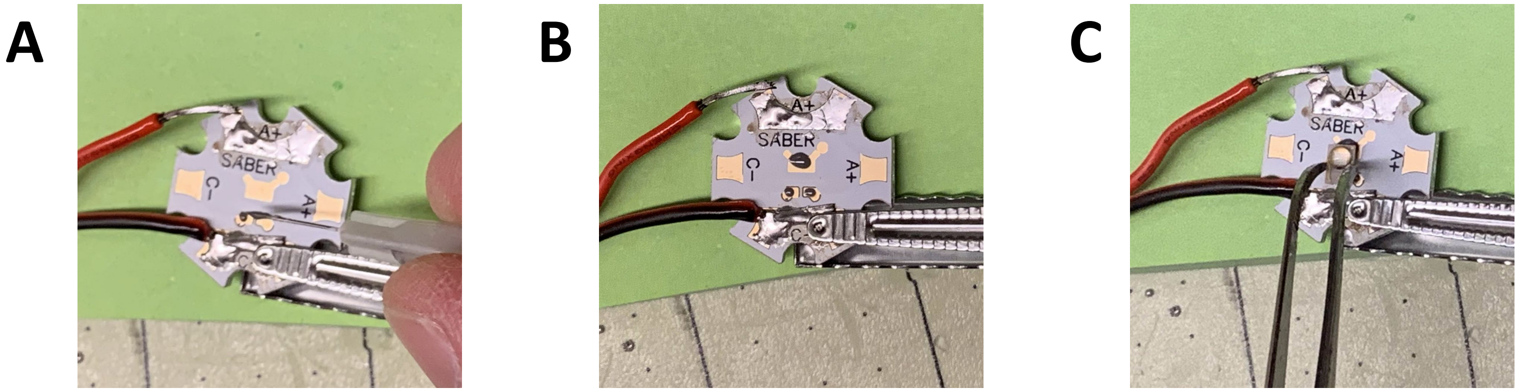


Figure S19: Soldering wires and LED onto the LED base Part 3. (A) Using a sharp tip to place solder paste onto the LED base for mounting the LED. (B) The LED base with the soldering paste in place. (C) Placement of the LED onto the LED base such that the contacts of the LED and LED base match.

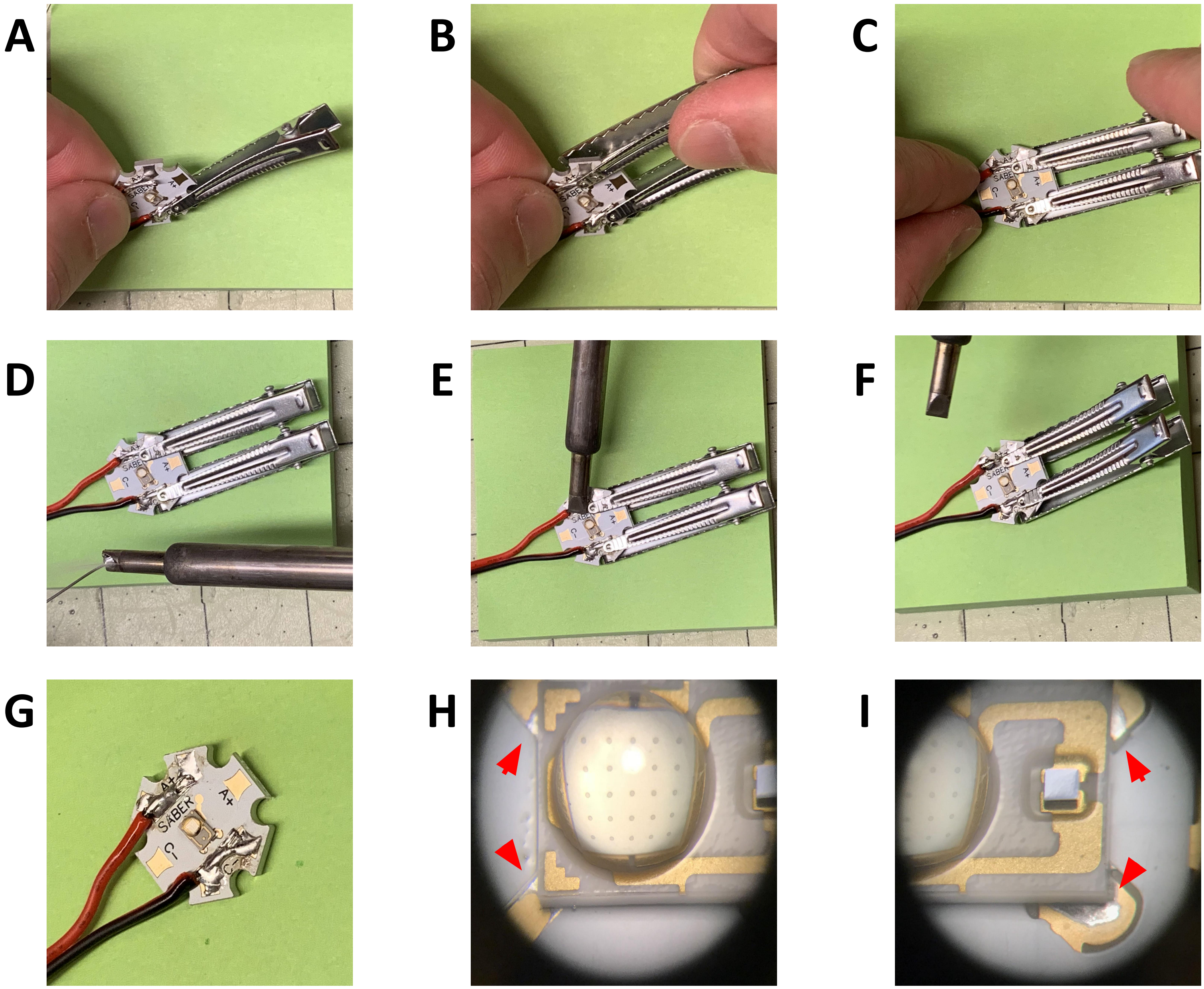


Figure S20: Soldering wires and LED onto the LED base Part 4. (A) The black wire still clipped to the contact by the hair clip. (B,C) Using a second hair clip, the red wire is held into place. Note that the red wire is soldered to the anode “A+”. (D) Addition of a generous amount of solder to the the soldering tip. (E) The soldering tip pressing down on the wire, melting the solder on the LED base and the wire as well as the solder paste under the LED. (F) The hot LED base cooling after soldering. (G) The LED base with the wires and LED soldered on. (H,I) Red arrows point to soldering pads. After soldering, the solder appears metallic/shiny (Compared to grey before soldering (Figure S16D)).

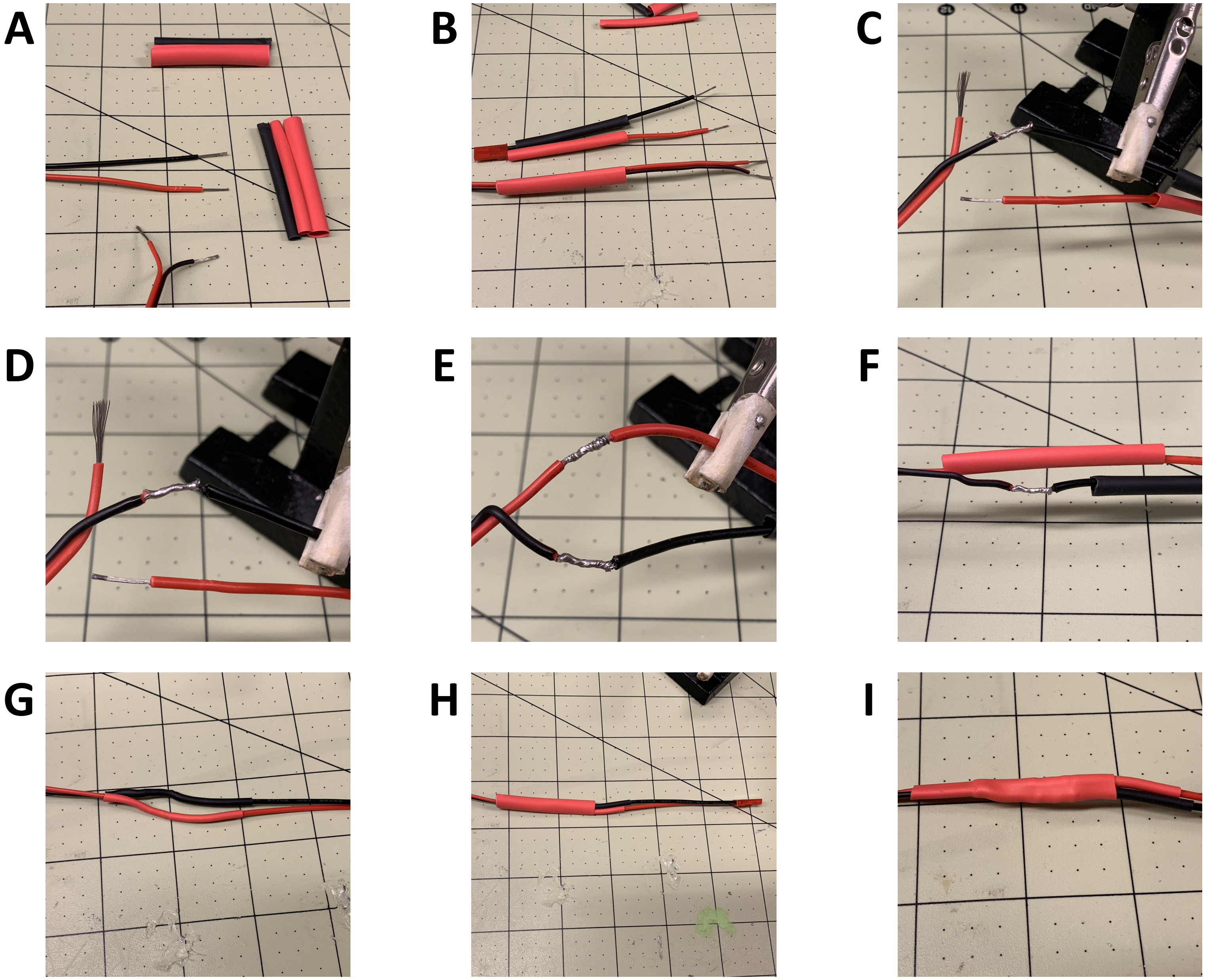


Figure S21: Connecting the LED wire to a male wire-to-wire connector. (A) Stripped wires and male wire-to-wire connector next to the shrink tube cut in half (1/8'' and 3/16''). (B) Shrink tube placement over the wires prior to soldering. (C) Wires twisted together before soldering. (D) The soldered connection from the wire to wire-to-wire connector. (E) Both the red and black wires soldered together. (F) Placement of the 1/8 ''shrink tube over the soldered connection. (G) The shrink tube after shrinking with the heat gun. (H) Placement of the 3/16'' shrink tube over the smaller shrink tube. (I) The connection soldered and sealed with shrink tube.

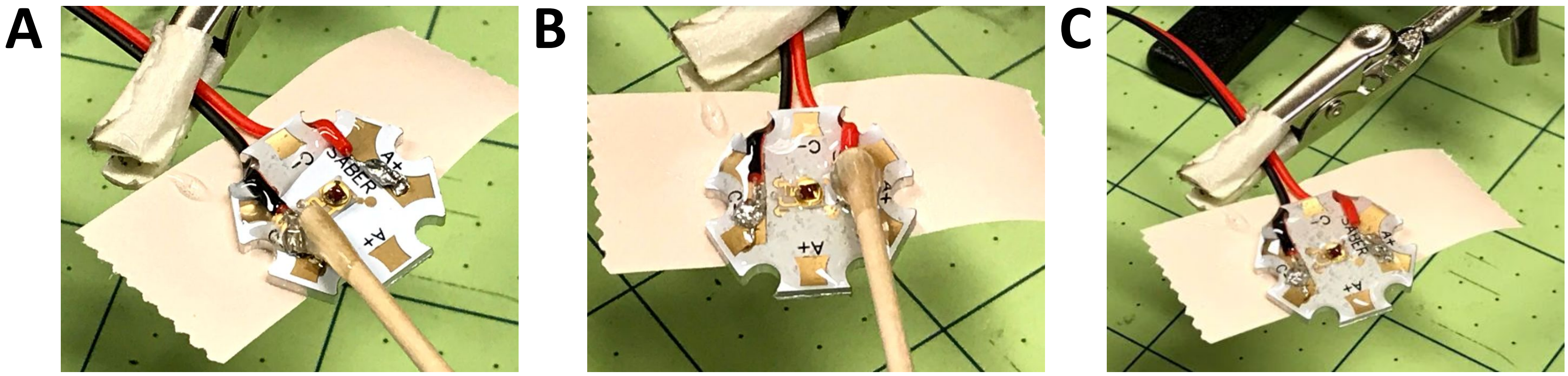


Figure S22: Securing the wires and LEDs to the LED base using epoxy. (A) Using wooden applicator to place epoxy into the LED base. Tape is placed below to catch any dripping epoxy. (B) Epoxy is spread evenly over the entire surface. (C) The LED is left overnight to cure.

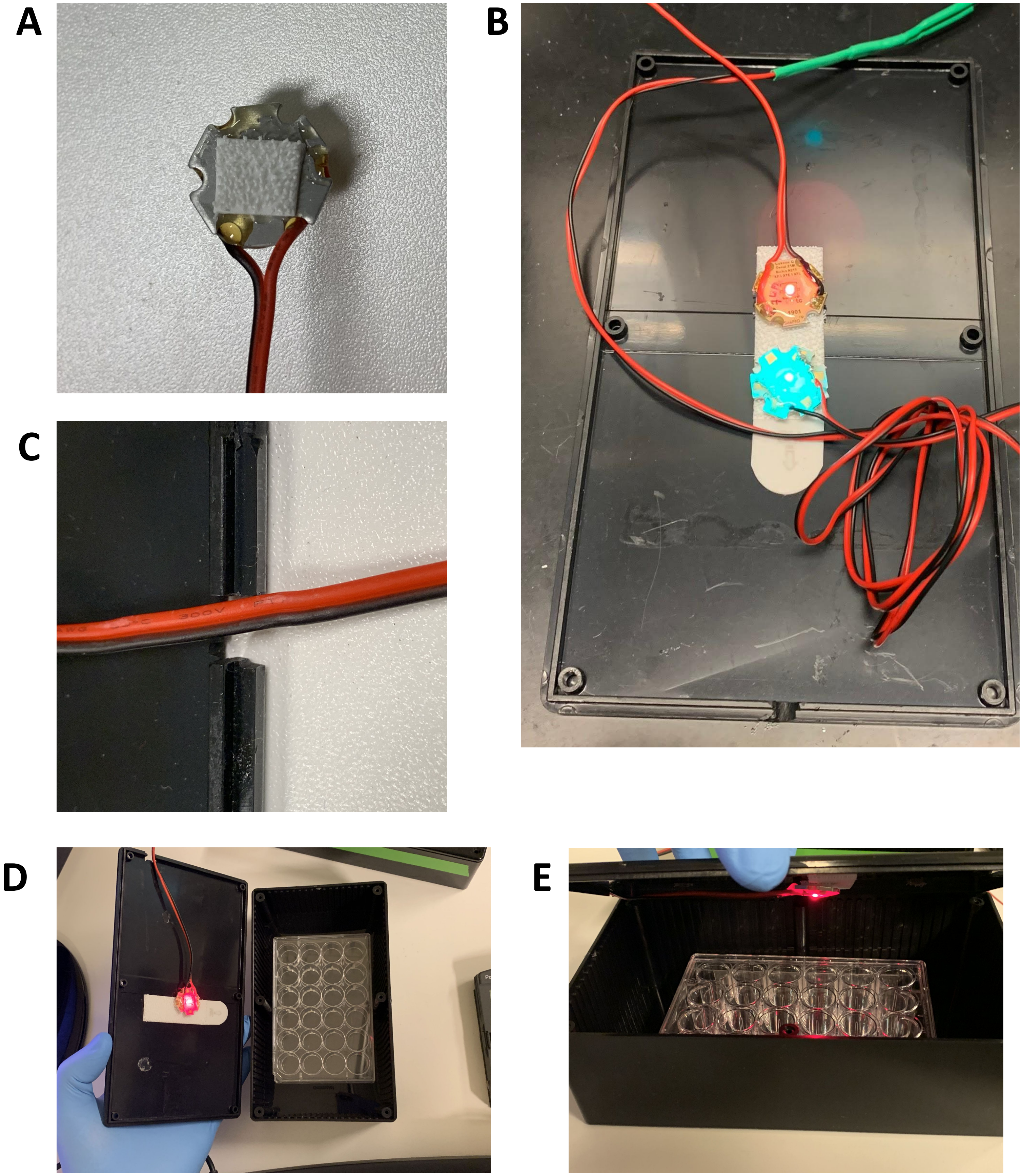


Figure S23: Mounting LEDs inside a box lid. (A) An LED with a command strip piece attached for easy mounting. (B) Different color LEDs mounted on the inside of a black box using a command strip. (C) A notch on the lid of the black box made by a Dremel to make room for the LED wire. (D) A black box for stimulating the cells with touch fasteners for mounting the LED. (E) Placement of a multiwell dish inside of the touch fastener version of the LED box.

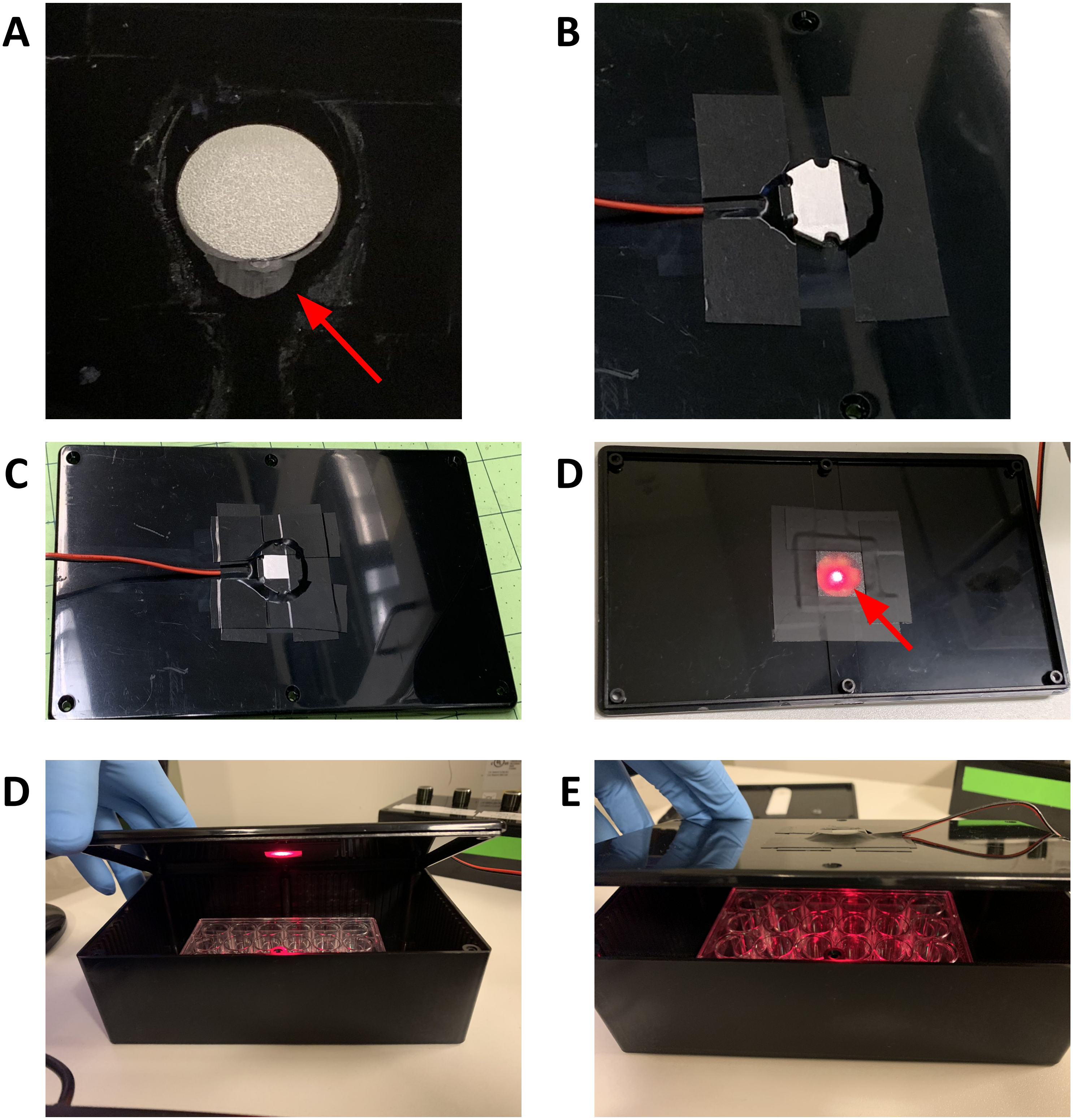
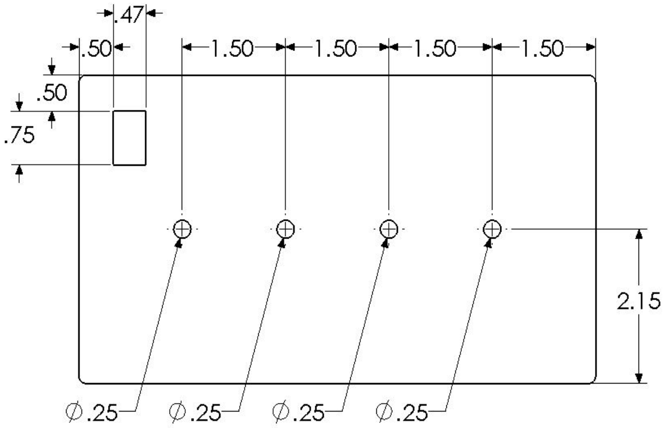


Figure S24: Mounting LEDs outside a box lid. (A) Hole drilled into the lid of the black box with a notch from the Dremel to make room for the wire (red arrow). (B) LED placed into the hole with the wire in the notch, held in place with electrical tape. (C) Two more pieces of tape are used to secure the LED. The backside of the heat sink is exposed to maximize heat exchange. (D) Privacy film taped over the hole where the LED will be placed. The red arrow points to the privacy film. (E) A black box for stimulating the cells with an LED mounted outside the box and with privacy film for diffusing the illumination. (F) Placement of a multiwell dish inside of the external LED+privacy film version of the LED box.

A



B

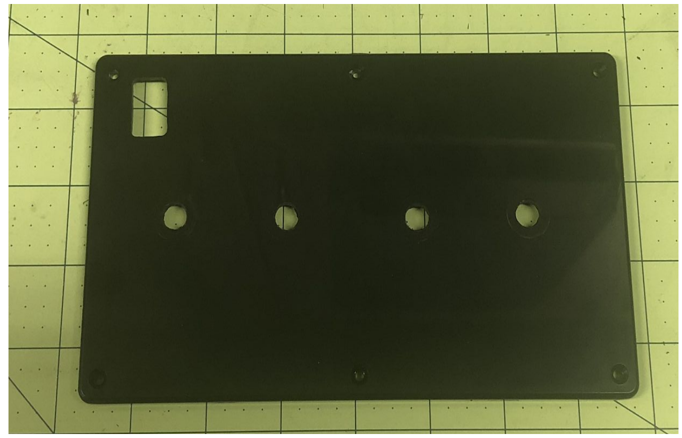


Figure S25: Drilling holes on the box lid for the power switch and potentiometers. (A) A CAD drawing with annotated dimensions of the box lid. (B) The box lid with the potentiometer and power switch holes.

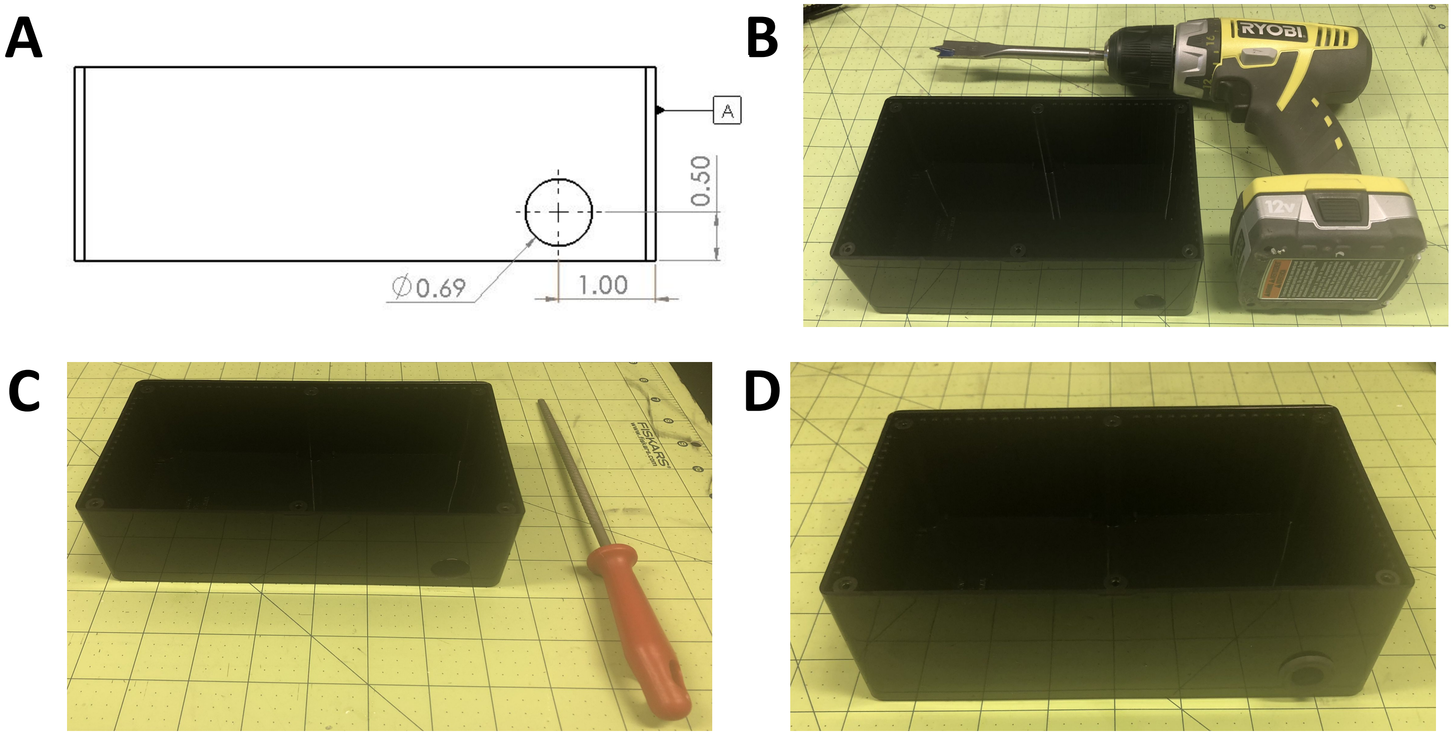


Figure S26: Preparing the wire outlet hole. (A) A CAD drawing with annotated dimensions. (B) Image of the drilled hole with the drill bit. (C) Smoothing of outlet hole with Dremel or filing tool. (D) Placing grommet in outlet hole.

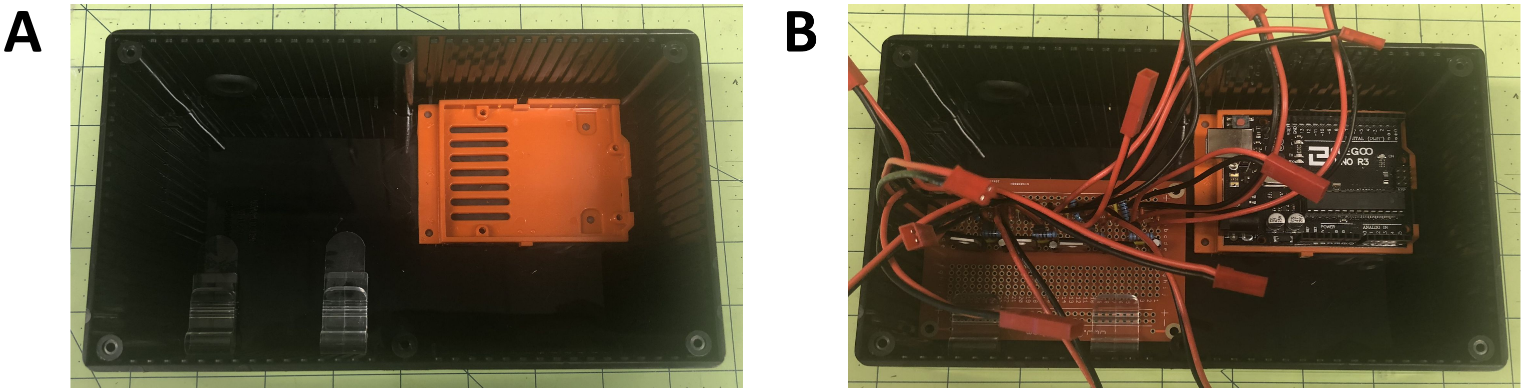


Figure S27: Placement of the microcontroller and the PCB in the box. (A) The microcontroller holder (orange) and PCB holders inside the box. (B) The microcontroller and PCB secured into the box.

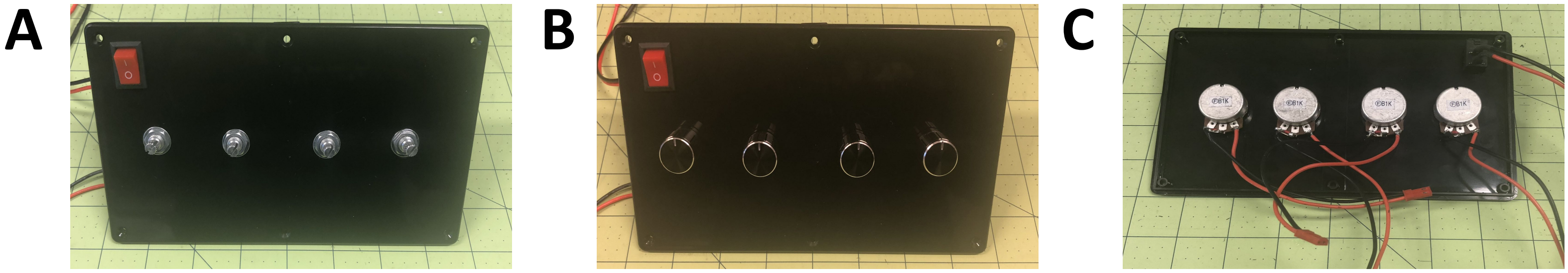


Figure S28: Placement of the potentiometers and the power switch. (A) A front view of a box lid with a power switch and four POTs. (B) A front view of box lid with potentiometer knobs added. (C) A rear view of box lid with attached components.

A

B

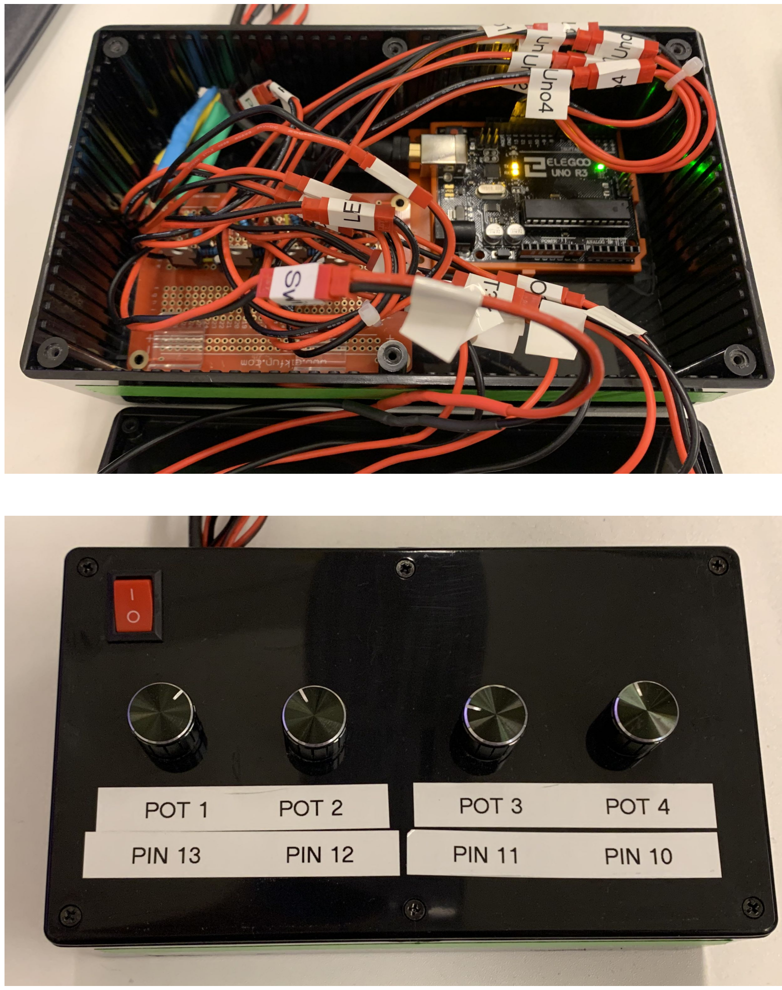


Figure S29: The assembled LED control system. (A) An open control box with the wires labeled with a label printer and zip tied for organization. (B) The box once fully assembled with each POT labeled along with the PIN it is connected to.

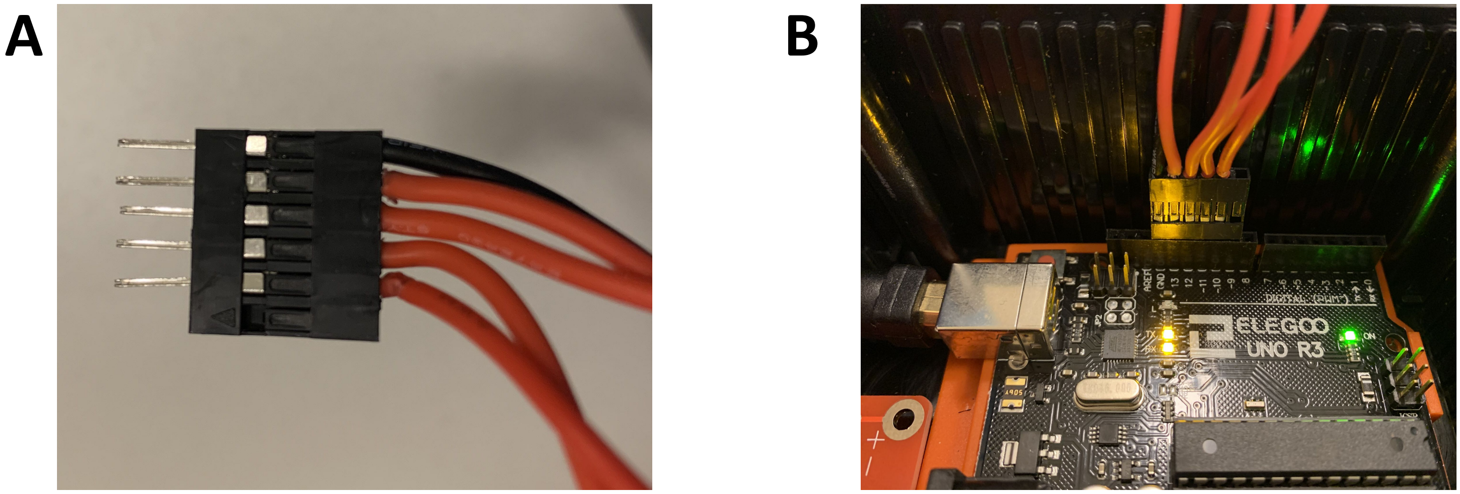


Figure S30: Placement of the crimped wire-to-wire connector. (A) Picture of the crimped wire-to-wire connectors for a four LED-microcontroller system. (B) Placement of the crimped connector into the microcontroller ports.

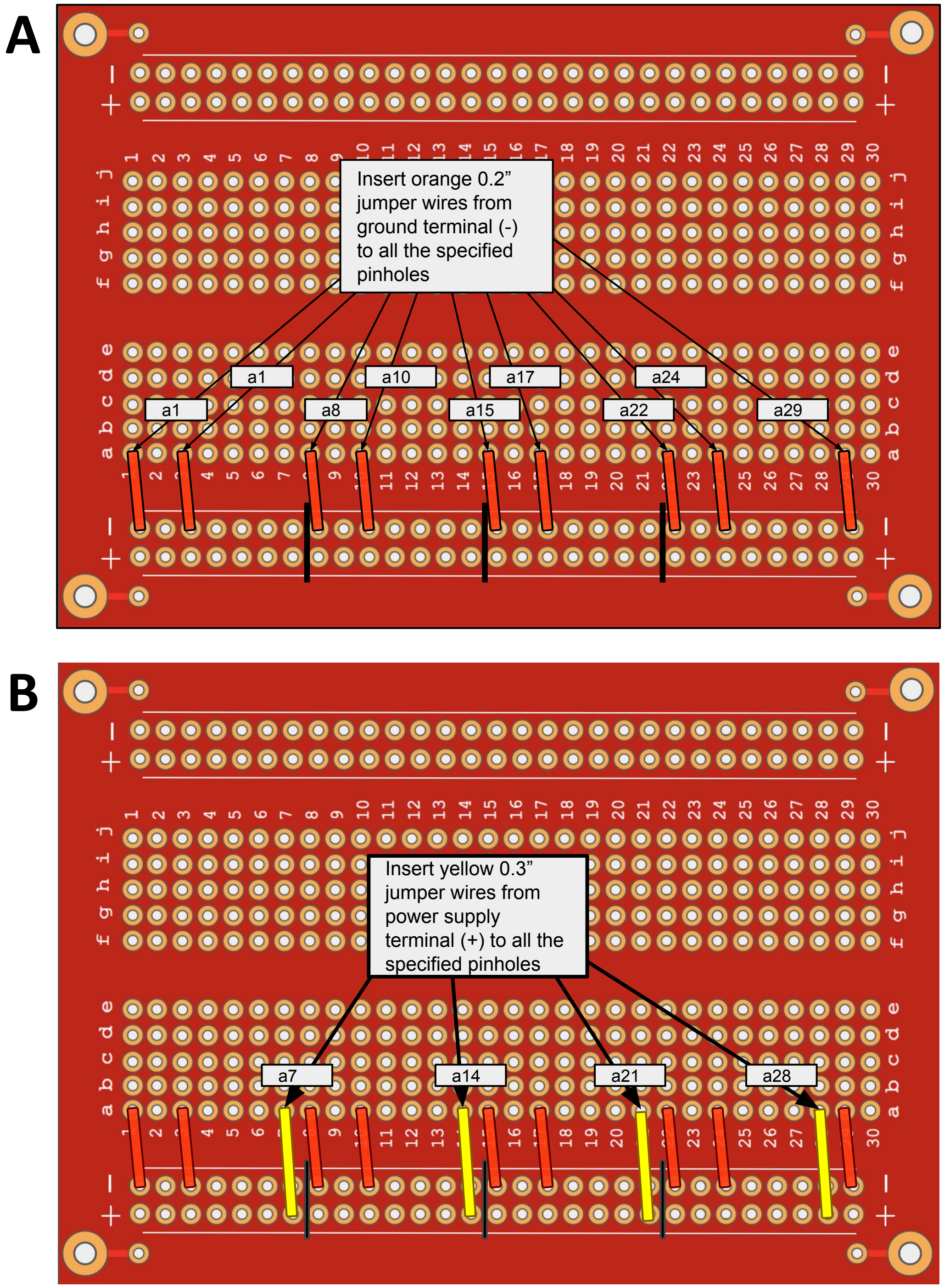


Figure S31: Placing the jumper wires. (A) A circuit board with the coordinates of the red jumper wires labeled. (B) A circuit board with the coordinates of the yellow jumper wires labeled.

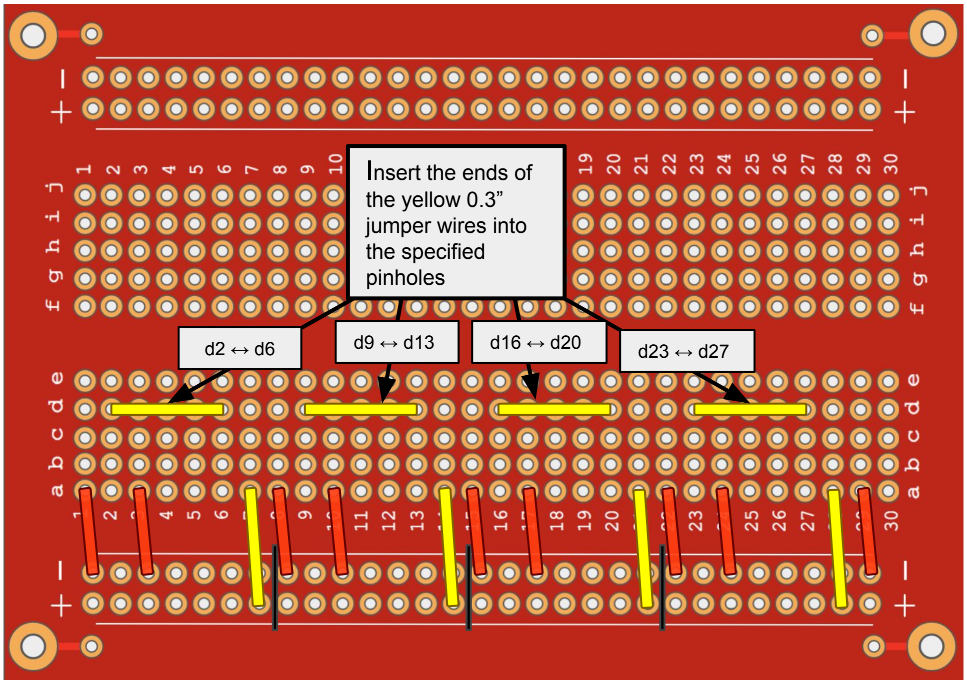


Figure S32: Placing the jumper wires. A circuit board displaying coordinates of the yellow jumper wires.

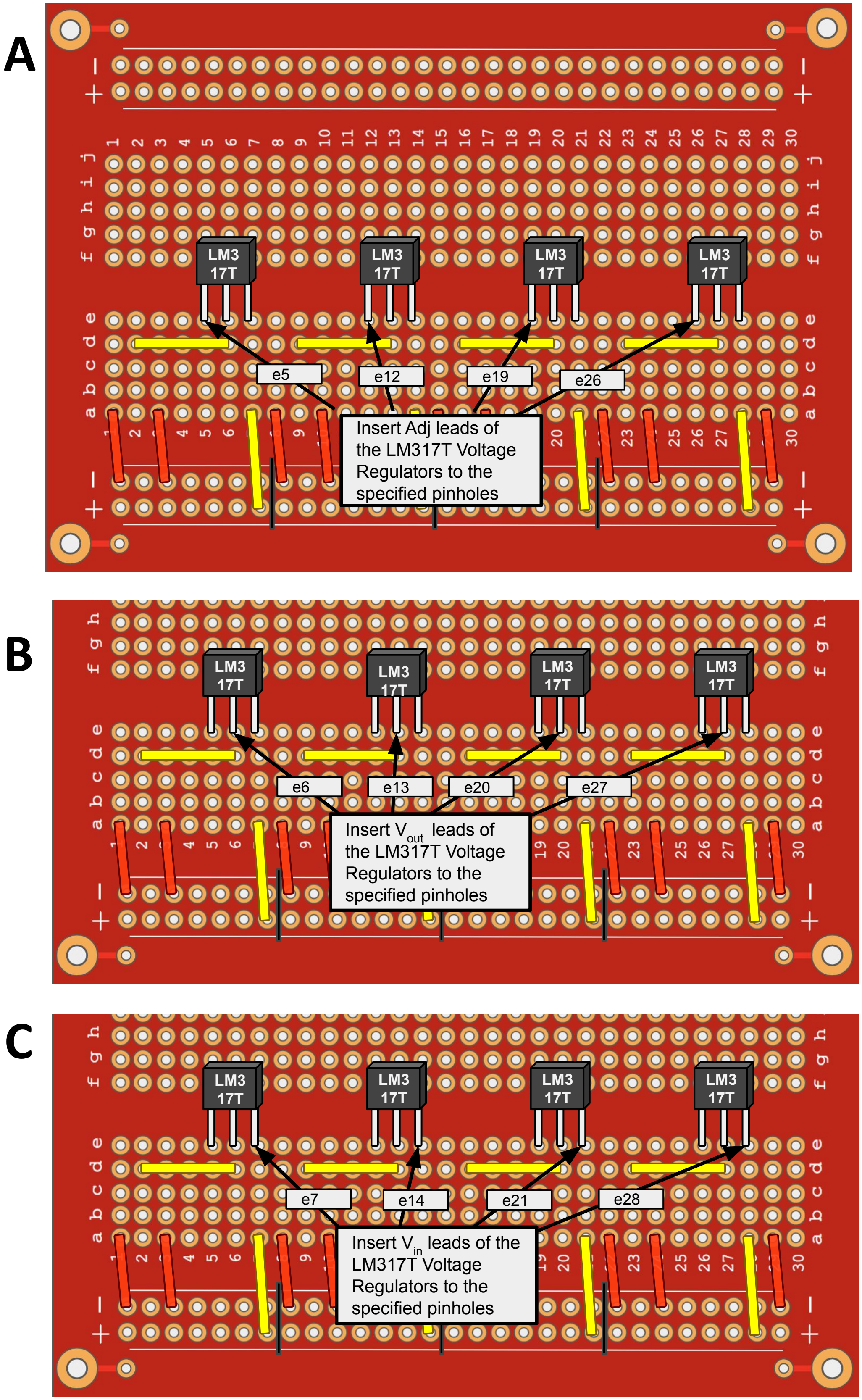


Figure S33: Adding the voltage regulators. The LM317T voltage regulators are added to the circuit with their coordinates labeled in the diagrams.

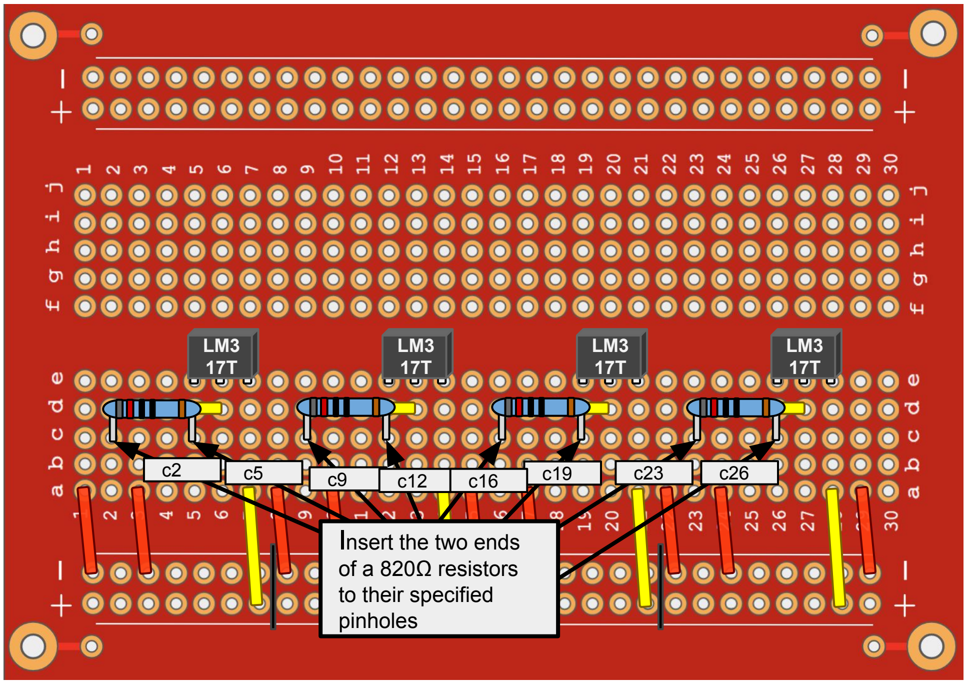


Figure S34: Inserting the 820Ω resistors. The R1 resistors are added to the circuit with their coordinates labeled in the diagrams

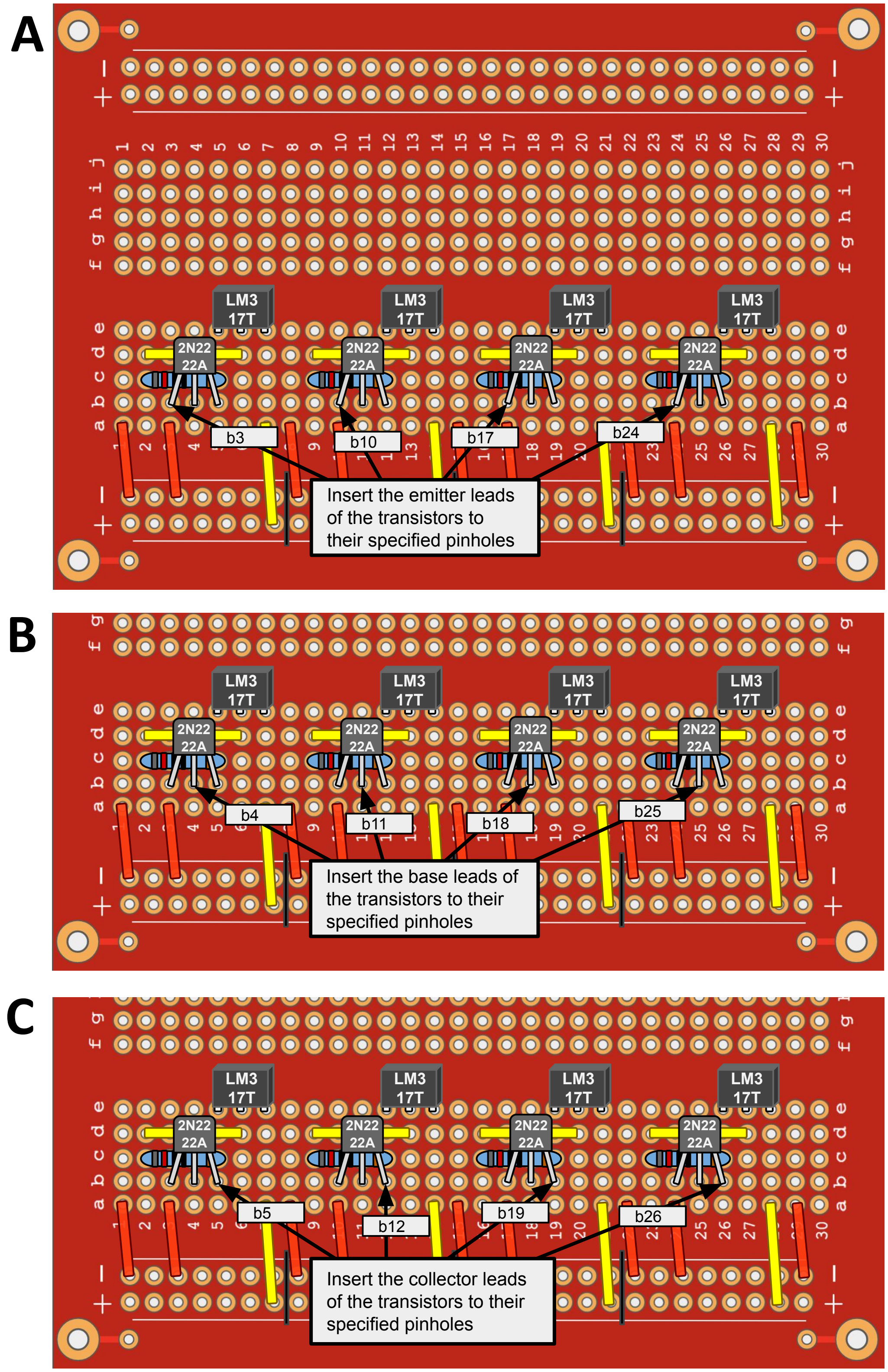


Figure S35: Inserting the transistors. The 2N2222A transistors are added to the circuit with their coordinates labeled in the diagrams

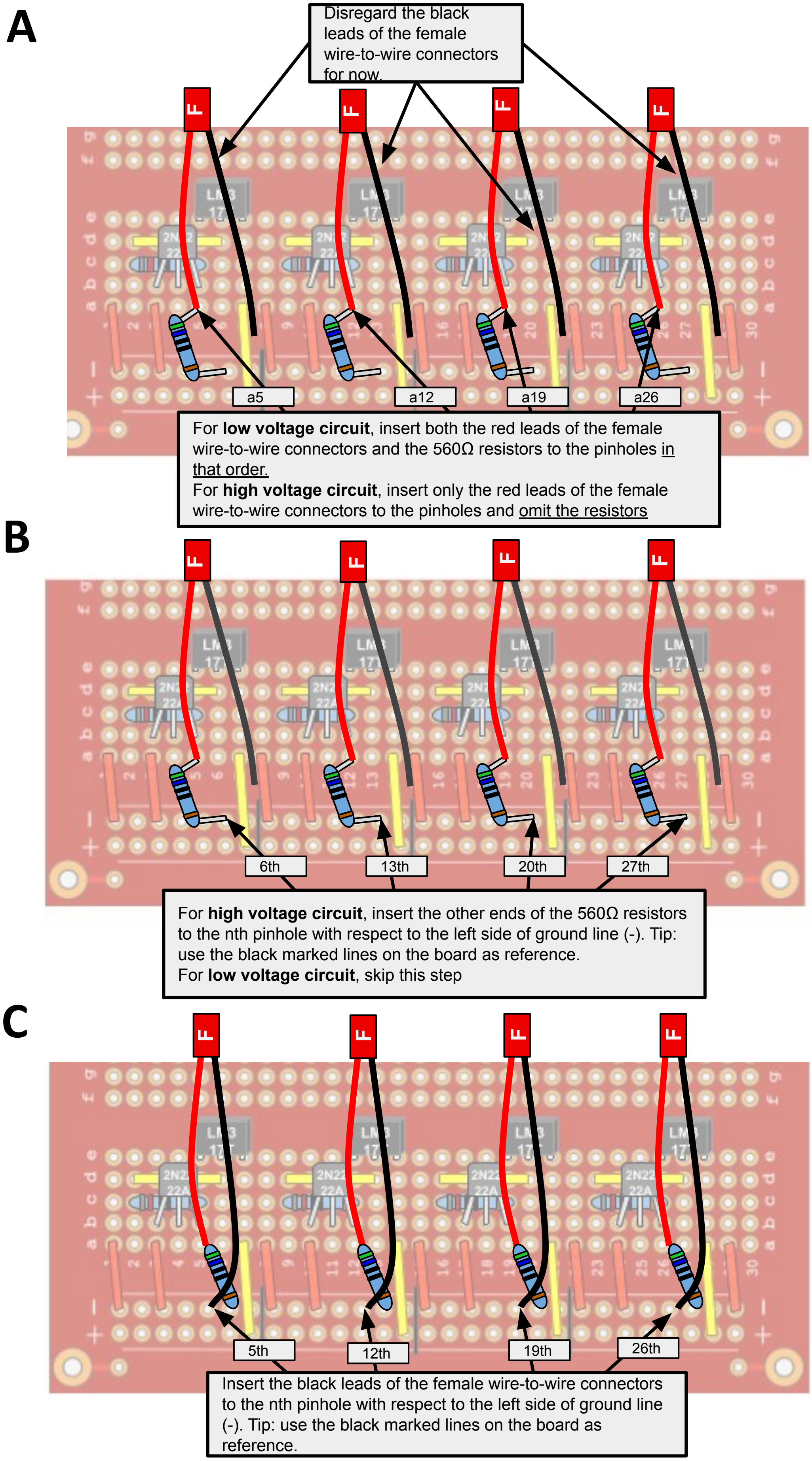
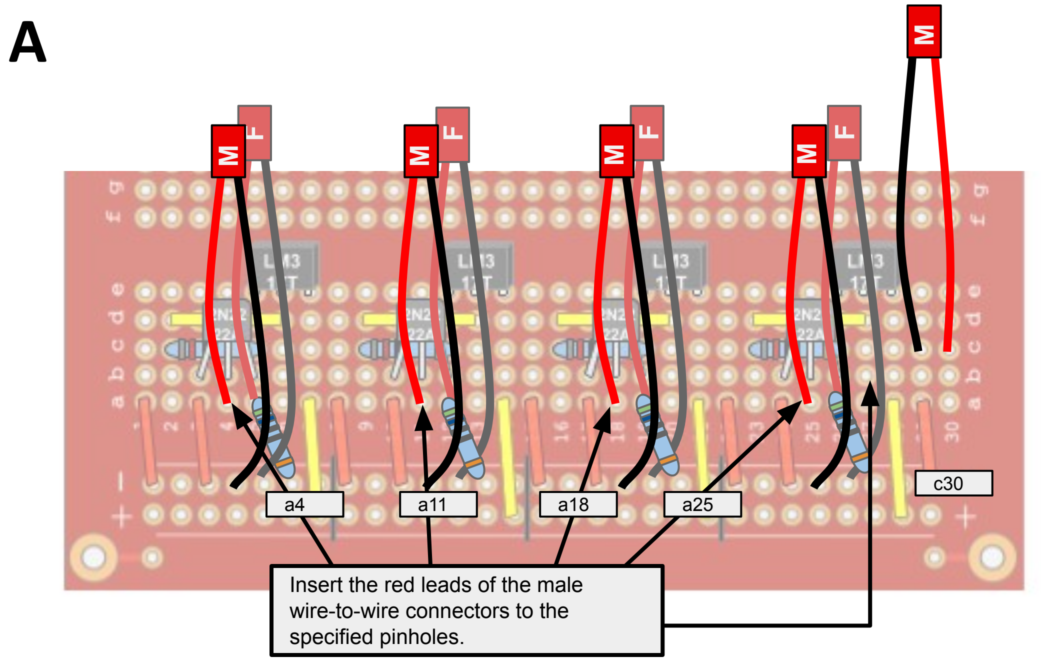


Figure S36: Inserting the Female wire-to-wire connectors and Resistors (optional) for the POT connection. The wires and resistor are added to the circuit with their coordinates labeled in the diagrams. (A) Insert the red wire, followed by the R2 resistor (560Ω) (for the low voltage circuit only). (B) Insert the other end of the resistor into the indicated ground hole. (C) Insert the black wires into the indicated holes to connect to ground. Note: R2 (560Ω) is in parallel with the potentiometer



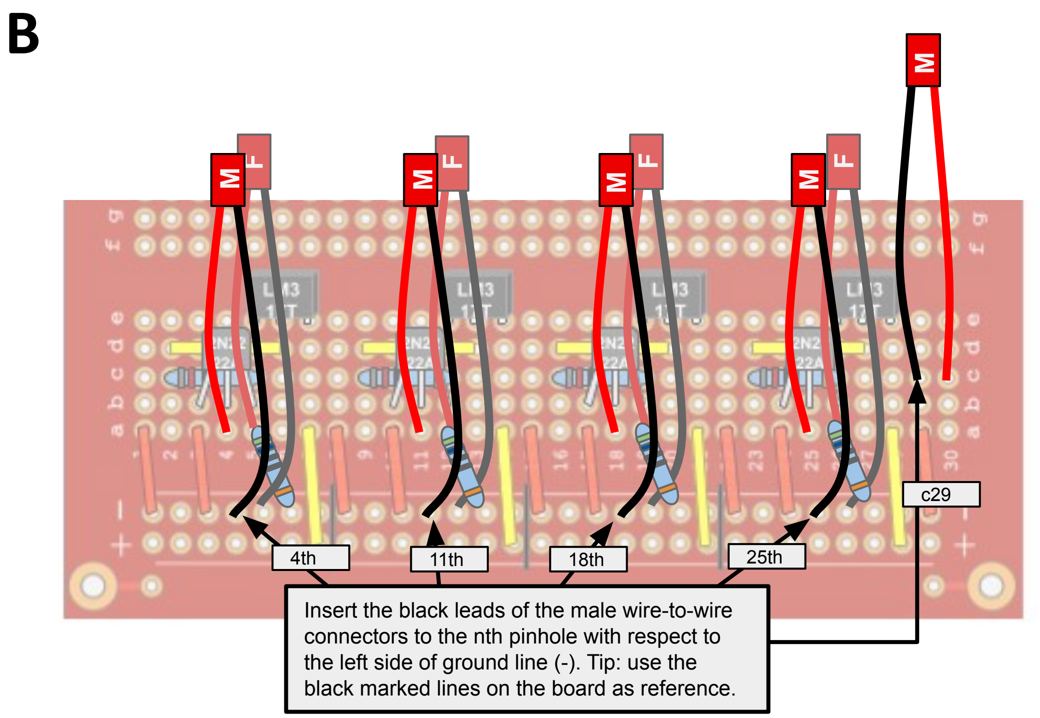


Figure S37: Inserting male wire-to-wire connectors for the microcontroller connection and power supply. The wires are added to the circuit with their coordinates labeled in the diagrams. (A) Insert the red wires into the indicated holes. (B) Insert the black wires into the indicated holes.

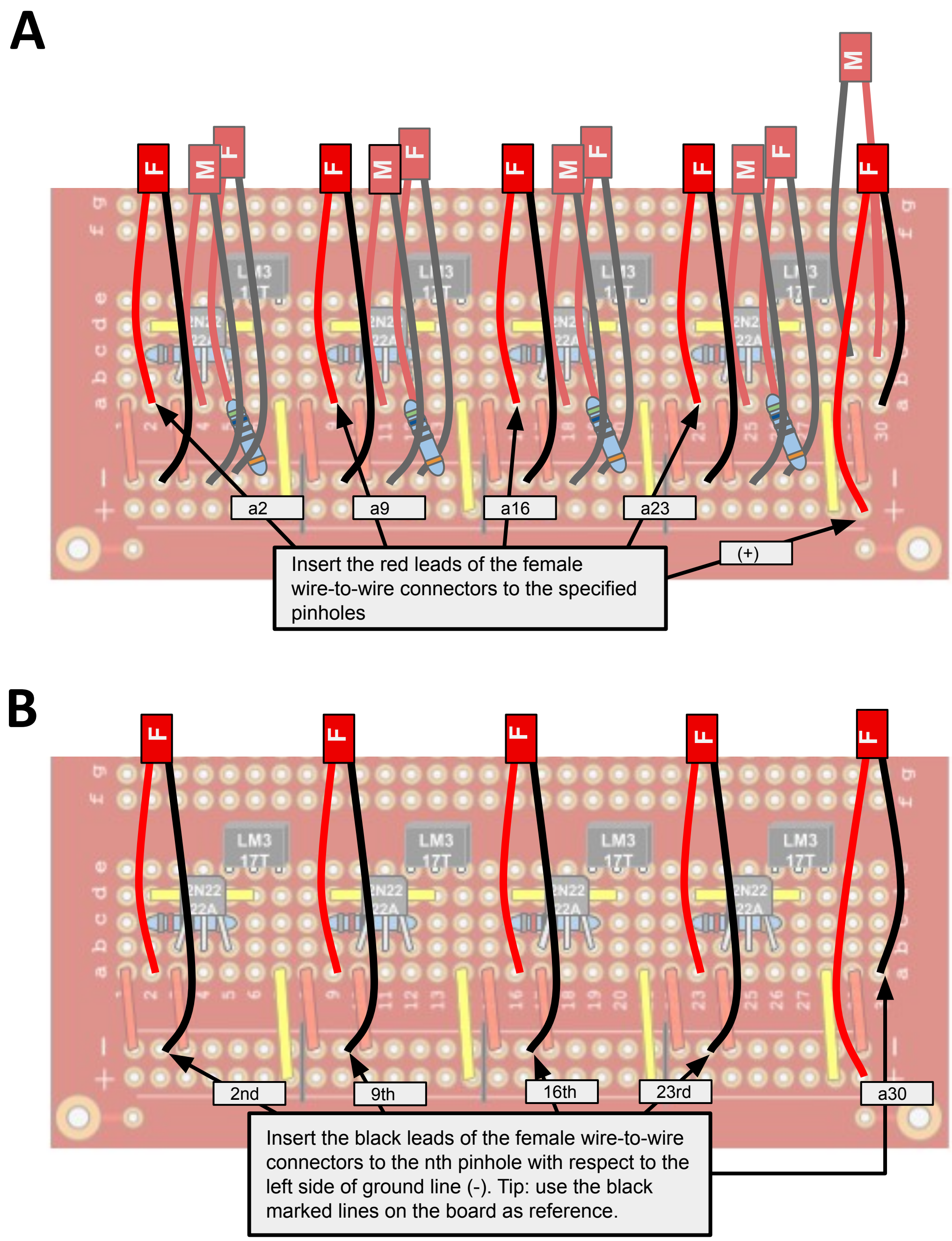


Figure S38: Adding LED wire-to-wire connectors. (A) Female wire-to-wire connectors with the red lead coordinates highlighted. (B) Female wire-to-wire connector with the black lead coordinates highlighted.

A

Do this first:						
Wells using Master mix (MM)		20				
Master Mix DNA	Conc.	% of MM	DNA Mass	DNA Vol/well	DNA Vol all wells	10% extra
pPKm-121	723	4.0000	20	0.028	0.553	0.609
	Total			0.028	0.553	0.609
			MM Optimem	Optimem/well	Optimem all wells	
			MM Optimem	19.972	199.723	219.696
			Vol/ condition			20
Aliquot the mastermix:		Volume per condition (ul)	20			

B

							# of wells	2.2
A	Condition	Plasmid	Construct	DNA ratio	DNA mass	Conc. ng/ul	Vol/well (ul)	FV
	3:1 Phy:PCB	Master Mix DNA		4	20.0			20.00
		pPK-351	A-CMV-PIF3-MTAD-IRES-PhyB-G4	66	330	422.4	0.78	1.72
		pPK-352	DNA-CMV-PCYA-IRES-HO1-FD-FN	22	110.0	234.4	0.47	1.03
		pPKm-202	UAS-Luciferase	8	40.0	92.69	0.43	0.95
			Total	100			Optimem	3.80
							# of wells	2.2
	Condition	Plasmid	Construct	DNA ratio	DNA mass	Conc. ng/ul	Vol/well (ul)	FV
B	Leaky	Master Mix DNA		4	20.0			20.00
		pPK-351	A-CMV-PIF3-MTAD-IRES-PhyB-G4	66	330.0	422.4	0.78	1.72
		pPK-178	ABE-EGFP-P2A-mito-his-sfGFP-Pu	22	110.0	106.1	1.04	2.28
		pPKm-202	UAS-Luciferase	8	40.0	92.69	0.43	0.95
			Total	100			Optimem	2.55
							# of wells	2.2
	Condition	Plasmid	Construct	DNA ratio	DNA mass	Conc. ng/ul	Vol/well (ul)	FV
C	3:1 Phy:PCB	Master Mix DNA		4	20.0			20.00
		pPK-351	A-CMV-PIF3-MTAD-IRES-PhyB-G4	66	330.0	422.4	0.78	1.72
		pPK-352	DNA-CMV-PCYA-IRES-HO1-FD-FN	22	110.0	234.4	0.47	1.03
		pPK-178	ABE-EGFP-P2A-mito-his-sfGFP-Pu	4	20.0	106.1	0.19	0.41
		pPKm-202	UAS-Luciferase	4	20.0	92.69	0.22	0.47
			Total	100			Optimem	3.86
							# of wells	2.2
	Condition	Plasmid	Construct	DNA ratio	DNA mass	Conc. ng/ul	Vol/well (ul)	FV
D	Leaky	Master Mix DNA		4	20.0			20.00
		pPK-351	A-CMV-PIF3-MTAD-IRES-PhyB-G4	66	330.0	422.4	0.78	1.72
		pPK-178	ABE-EGFP-P2A-mito-his-sfGFP-Pu	26	130.0	106.1	1.23	2.70
		pPKm-202	UAS-Luciferase	4	20.0	92.69	0.22	0.47
			Total	100			Optimem	2.61

C

Calculations	Volume *per* well	Volume for *all* wells
PEI	1.5	30
Optimem	11	220
Number of wells	20	
Make this	20% extra	Volume to add to each master mix
PEI	36	27.5
Optimem	264	
Volume (ul) of master mix per w	25	<---- Add this to each cell culture well

D

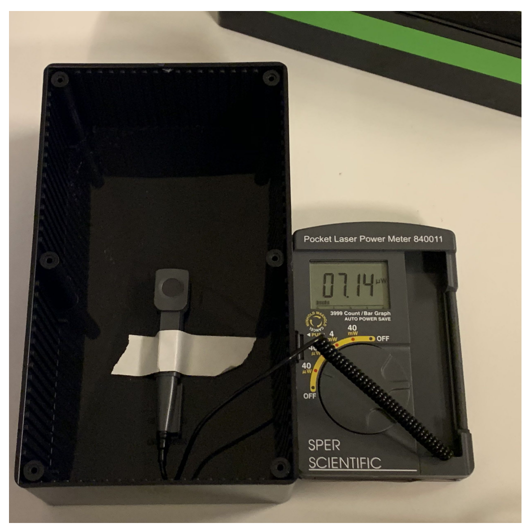


Figure S39: Setting up a PhyB-PIF3 gene switch experiment. (A) An example table of a mastermix containing Renilla for the internal control. (B) An example table for setting up the DNA mixture for a Dual Luciferase Reporter Assay of a PhyB-PIF3 optogenetic experiment. (C) An example table for setting up PEI transfection reagent and aliquoting the mixture onto cells (dropwise). (D) Placement of the light meter for setting the LED brightness.