Construction of a multiwell light-induction platform for traceless control of gene expression in mammalian cells

Maysam Mansouri [1], Samson Lichtenstein [1], Tobias Strittmatter [1], Peter Buchmann [1] and Martin Fussenegger [1,2*]

[1] Department of Biosystems Science and Engineering, ETH Zurich, Basel, Switzerland.
[2] Faculty of Science, University of Basel, Mattenstrasse 26, CH-4058, Basel, Switzerland.

[*] Corresponding author:

Prof. Dr. Martin Fussenegger

ETH Zürich, Department of Biosystems Science and Engineering (D-BSSE)

Mattenstrasse 26,

CH-4058 Basel

Phone: 0041 61 387 31 60

FAX: 0041 61 387 39 88

E-mail: martin.fussenegger@bsse.ethz.ch

Abstract

Mammalian cells can be engineered to incorporate light-responsive elements that reliably sense stimulation by light and activate endogenous pathways, such as the cAMP or Ca^{2+} pathway, to control gene expression. Light-inducible gene expression systems offer high spatiotemporal resolution, and are also traceless, reversible, tunable, and inexpensive. Melanopsin, a well-known representative of the animal opsins, is a G-protein-coupled receptor that triggers a Gaq-dependent signaling cascade upon activation with blue light (≈470 nm). Here, we describe how to rewire melanopsin activation by blue light to transgene expression in mammalian cells, with detailed instructions for constructing a 96-LED array platform with multiple tunable parameters for illumination of the engineered cells in multiwell plates.

Key words: Optogenetics, Inducible gene expression, Cell engineering, Mammalian cells, Synthetic biology.

1. Introduction

The ability to remotely control the expression of genes of interest in mammalian cells is a central goal in both basic and clinical research. Cell-based treatment strategies in translational medicine usually employ engineered cells that sense input signals and respond appropriately [1]. Available input signals include a variety of endogenous or exogenous stimuli, such as biomolecules [2], chemicals [3], temperature [4], pH [5], light [6], gas[7] and many others. Among them, light offers a promising opportunity for traceless control of cellular behavior with rapid response, good reversibility and high spatiotemporal resolution [8]. The term optogenetics was coined to describe the use of light to achieve precise control of gene expression in engineered living cells [9,10]. Originally designed and most widely employed to manipulate neural activities, optogenetic tools are being increasingly used in biomedical research in fields outside neuroscience [11–15].

Photoactivatable proteins are at the core of the optogenetic tool kit. These proteins can be divided into two major groups, i.e., opsins and non-opsins (light switches). Non-opsin photoactivatable proteins are widely used in non-neural optogenetics (i.e. non-ion-flux-based optogenetics) [8,12]. Opsins are light-sensitive transmembrane proteins found in a variety of organisms ranging from microbes (Type I opsins) to primates (Type II opsins). Type II opsins are G-protein-coupled receptors (GPCR) that trigger a signaling cascade upon activation by light [16]. Melanopsin, a representative member of the animal opsins, is endogenously expressed in intrinsically photosensitive retinal ganglion cells (ipRGCs) of the inner retina [17]. It plays a crucial role in the adaptation of mammals to different light intensities, as well as in the circadian timing system [18]. Exposure to light induces isomerization of the retinal chromophore (11-cis-retinal to all-trans-retinal) of melanopsin, and this changes the conformation of the receptor, resulting in activation of the heterotrimeric G-protein and inducing dissociation of the Gαq subunit from the dimeric Gβγ complex. The Gαq subunit then activates phospholipase C, which initiates calcium-dependent signaling within mammalian cells. Ultimately, this leads to activation of the phosphatase calcineurin, which dephosphorylates the nuclear factor of activated T-cells

(NFAT). Dephosphorylated NFAT translocates to the nucleus and triggers expression of target genes [19] (Fig. 1).

Expression of melanopsin in mammalian cells provides functionality for blue-light dependent calcium influx into the cells. This feature allows us to rewire the signal transduction of light-activated melanopsin to a synthetic NFAT-responsive promoter, enabling the expression of gene(s) of interest to be triggered by blue light[20]. It is also possible to reprogram mammalian cells, including induced pluripotent stem cells (iPSCs), [21] for specific purposes by using blue light to alter endogenous pathways.

The following protocol describes in detail how to create a blue-light-inducible gene expression system for mammalian cells, including instructions for constructing a 96-LED array platform with multiple tunable parameters for stimulation of engineered cells in multiwell plates. Owing to the modularity of the LED array, the platform can easily be modified for use with other optogenetic receptors and light switches that operate at different wavelengths, simply by replacing the LEDs.

2. Materials

2.1. Construction of a programmable LED array

1. 60 Blue LEDs (475 nm, 20-30 cd, 50 mA, 5 mm, 15 o; Roithner Lasertechnik GmbH).
2. Electrical wire and tools (e.g., soldering iron, wire stripper, etc.).
3. Copper-terminal printed circuit board.
4. USB cable.
5. Photometer (Ophir, NOVA, P/N7Z01500).
6. Arduino™ microcontroller and software.
7. Arduoin drivers.
8. Step-up converter (SG3524) to achieve LED driving voltage.
9. Current mode switching circuit MLX10803 for LED current regulation.

2.2. Cell culture

1. Dulbecco's modified Eagle's medium (DMEM, Gibco™) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich) and 1% penicillin/streptomycin (Sigma-Aldrich).
2. Colourless DMEM (Gibco™) without phenol red, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin.
3. Medium (Gibco™) without FBS and penicillin /streptomycin antibiotics.
4. Trypsin-EDTA solution (0.05% (v/v), Gibco™).
5. Cell line (HEK-293T).
6. Black 96-well plates with transparent bottom (Greiner Bio-One).
7. Transparent 96-well plates (Nunc, Denmark).
8. 96 deep-well plate.
9. CO_2 tissue-culture incubator.
10. Sellotape
11. Polyethyleneimine (PEI) HCl MAX, Linear, Mw 40,000 solution (1 mg/mL, Polysciences Inc.)
12. All-trans retinal (ATR) (Sigma-Aldrich, 1 mM stock in ethanol)
13. Plasmids:
    Melanopsin expression vector (pHY42; P_{CMV}-OPN4-pA) [19].
    NFAT-driven secretory alkaline phosphatase (SEAP) expression vector (pHY30; P_{NFATx3}-SEAP-pA) [19].
    Filler DNA (plasmid without mammalian promoter (pDF145; PT7-SpAH-Env140ac) [22].
14. Plasmid miniprep kit (Zymo Research).
15. Cell counting device (CASY® cell counter).
16. Multichannel pipette and tray (reservoir)
17. Cell culture plasticware (100 mm petri dishes).
18. Centrifuge (e.g., Model 5810R, Eppendorf AG).

2.3. SEAP measurement

1. SEAP buffer (2x): 20 mM homoarginine (Acros Organics), 1 mM $MgCl_2$, 21% (v/v) diethanolamine, pH 9.8 (Acros Organics).
2. Substrate for SEAP: 120 mM para-nitrophenyl phosphate (pNPP) (Acros Organics) in 2x SEAP buffer.
3. 96-well plates (Nunc, Denmark).
4. Plate reader (TECAN).

3. Methods

3.1. Construction of a programmable LED array

The 96-LED array is designed to allow for programming of different light intensities as well as illumination patterns. For this, the following steps are needed:

1. Design and print a circuit board consisting of a 10x6 LED holder. Space the LEDs to the dimensions of a 96-well plate, so that one LED is located at the center of each well.
2. The LED's are organized in two rows of 5 LED's in series for each channel.
3. Based on the forward voltage of 4 V for the blue LED's, the output voltage of the Boostcircuit has to be set to 24 V to achieve accurate current regulation.
4. Each channel contains one switch-mode LED current-regulating circuit MLX10803. The MLX10803 offers the opportunity to add a PWM Signal (from Arduino) to achieve dimming of the LED in 0 - 255 steps.
5. To program the LED array, download the Arduino™ software from www.arduino.cc. Write software to set the desired illumination pattern (constant or pulsating), illumination time and light intensity. Upload the program to the Arduino™ Microcontroller by connecting the Arduino™ board to the computer via a USB cable and initiate the transfer from the Arduino programming GUI.
6. To correlate Arduino™ values to the light intensity, program Arduino™ with different values (0 to 255) and measure the emitted light from LEDs with a photometer following the manufacturer's instructions (Fig. 2 a-d).

3.2. Cell culture

The following protocols for cell culture experiments have been optimized for HEK-293T cells and would need to be modified for other cell lines.

1. Cultivate HEK-293T cells in 12 mL of complete DMEM (supplemented with 10% FBS and 1% penicillin/streptomycin) at 37 °C in an atmosphere of 5% CO_2.
2. After the cells reach 70–80% confluence, they can be used for light-induction experiments. Aspirate the medium, and expose the cells to 1 mL of 0.05% (v/v) trypsin/EDTA solution for 5 min at 37 °C in the incubator.
3. Remove the trypsinized cells from the incubator, and add 9 mL of complete DMEM to the cells. Detach the cells from the plate by gentle tapping.
4. Collect the cells from the plate, transfer them into a 15 mL conical tube, and centrifuge for 3 min at 450 ×*g.*
5. Discard the supernatant, and resuspend the cells in 10 mL of complete DMEM.
6. Count the cells using a CASY® cell counter according to the manufacturer's instructions.
7. Prepare 14 mL of seeding cell suspension in a 50-ml tube at the concentration of 1.5 x 10^6 cells/mL.
8. Close the 50 mL tube, invert several times to ensure proper mixing of the suspension, and pipette the cells into the multichannel tray reservoir.
9. Use a multichannel pipette to transfer 125 µL of the cell suspension into each well of a 96-well plate (mix the cell suspension by pipetting up and down).
10. Incubate the plate with the seeded HEK-293T cells at 37 °C under 5% CO_2 for 12–24 h prior to transfection.

3.3. Tranfection

We typically co-transfect HEK293T cells with 5 ng of pHY42 (P_{CMV}-OPN4-pA), 90 ng of pHY30 (P_{NFATx3}-SEAP-pA), and 30 ng of pDF145 ($_P$T7-SpAH-Env140ac), making a total amount of 125 ng DNA per well of a 96-well plate.

1. Prepare a master mix for 60 wells of a 96-well plate with pHY42, pHY30 and pDF145. Per well, add 50 µL of DMEM (without FBS and antibiotics) and 0.625 µL of PEI solution (1 mg/ml). This amounts to 60 x 50 µL = 3 mL of DMEM and 60 x

0.625 µL = 37.5 µL of PEI solution for the master mix. Vortex the PEI/DNA/DMEM mixture thoroughly for 20 seconds.

2. Incubate the PEI/DNA/DMEM mixture for 20 min at RT.
3. Transfer 300 µL of PEI/DNA/DMEM mixture to wells A2 to A11 of a deep well plate. Transfer 50 µL of the transfection cocktail with the multichannel pipette to each of the inner 60 wells of the 96-well plate (i.e. wells B2 to B11, C2 to C11, D2 to D11, E2 to E11, F2 to F11 and G2 to G11).
4. Row G will not be light-stimulated (dark) and serves as a control. It is protected from light by covering it with aluminium foil. Cut out 2 pieces of aluminium foil and glue them over rows F (which serves as a spacer between light-exposed and dark rows), G and H on the lid and the bottom of the 96-well plate.
5. Incubate transfected cells for 6–12 h at 37 °C under 5% CO_2. Then, pour the media out of the plate and gently tap the plate on a stack of paper towels to remove most of the remaining media. Replace with fresh complete DMEM and continue to incubate the cells for 24 h at 37 °C under 5% CO_2.

3.4 Light induction in transfected mammalian cells

Illumination should be started 24 hours after transfection to ensure good integration of cell membrane proteins. Program the LED array with Arduino software as follows:

1. Select the desired illumination pattern (i.e., constant or pulsating) and illumination time, as well as the light intensity (Fig.3). We employ a pulse pattern (5 sec ON

and 15 sec OFF) for successive periods of 12 h on and 12 h off (total: 48 hours) at 0.27 mW/cm^2 or 0.13 mW/cm^2.

2. Take 12 ml pre-warmed colourless medium (+ 10% FBS +1% Pen/Strep), add 5 µM ATR, and transfer into a pipetting reservoir.
3. Gently remove the medium from the transfected cell plate by tapping it upside-down onto a stack of paper towels.
4. Gently add 120 µL of the medium supplemented with ATR to each well of the 96-well plate using the multichannel pipette.
5. Put the LED array on top of the plate and connect it to the power supply. Illumination will start automatically as soon as the Arduino boots up.

3.4 Reporter expression analysis

We typically measure the reporter gene expression after 24 h and 48 h (Fig. 3), but SEAP analysis can be done at any desired time point. The fold induction is calculated as the ratio of SEAP level of stimulated cells to that of unstimulated cells. Measurement is done according to the following protocol.

1. Transfer 10 µL of the SEAP-containing cell supernatant to a fresh 96-well assay plate using a multichannel pipette.
2. Add 90 µL of ddH2O to the SEAP plate using a multichannel pipette.
3. Seal the SEAP plate with Sellotape and incubate the plate for 30 min at 65 °C and 350 rpm to inactivate endogenous phosphatases present in the cells.
4. Centrifuge the plate for 2 min at 14,000 g.

5. During the last 5 minutes of incubation, prepare the SEAP assay master mix for 60 wells as follows: For each well, use 80 µL of 2x SEAP buffer and 20 µL of pNPP solution (substrate).
6. Measure the absorbance at 405 nm every 30 s for 30 min with a TECAN plate reader.
7. Quantify SEAP levels according to Lambert-Beer's law:

$$E = \varepsilon \cdot c \cdot d \rightarrow c = \frac{E}{\varepsilon \cdot d}$$

E = increase in absorbance per minute (absorbance/min)
ε = molar extinction coefficient of the product, p-nitrophenolate (ε = 18,600 M−1 cm−1)
c = increase in concentration of p-nitrophenolate (M/min)
d = length of the light path in the sample (cm) (usually 0.5 cm for a 96-well plate)

8. Calculate the activity of SEAP (U/L) using the formula: SEAP activity = c × 10^6 × (200/10) (dilution factor).

Illumination with blue light activates the melanopsin receptor on the cell surface and triggers the Ca^{2+} signalling pathway, leading to transcription from the NFAT-responsive promoter and ultimately expression of the reporter gene (SEAP) (Fig. 1). The level of SEAP expression can be fine-tuned by adjusting the light intensity (Fig. 3c).

6. Notes

1. The ratio of receptor to reporter influences the fold induction. Thus, it may be necessary to optimize this ratio. The quality of the extracted DNA ($A_{260/280}$)

should always be checked before transfection to confirm that the plasmid is RNA-free.

2. Unwanted mutations can occur in the plasmid during bacterial propagation. Transfection of DNA from different bacterial clones is a useful way to find the best clone.
3. Transfection efficiency is highly dependent on the transfection reagent and cell type. A constitutively active reporter gene that can be multiplexed with the SEAP reporter (e.g. a fluorescent protein) can be co-expressed to monitor transfection efficiency.
4. Long-term blue-light illumination is toxic to mammalian cells. To monitor the cytotoxity, it may be necessary to include a suitable control. For example, pSEAP2-Control (P_{SV40}-SEAP-pA) from Clontech can be used to evaluate the cytotoxicity by comparing the expression levels in light-stimulated and unstimulated cells.
5. Illumination with blue light can up-regulate some endogenous transcription factors, leading to unwanted SEAP expression [23]. To monitor this type of artefact, it is necessary to have a control containing only the reporter (without receptor) to track non-specific induction.
6. The reporter gene utilized in the experiment (SEAP) can in principle be exchanged for any other desired reporter gene, such as genes encoding fluorescent proteins or luciferases.
7. Inserting a diffuser between the LEDs and cell plate generates a spatially more homogeneous illumination source.

8. Working with cells in ambient light may activate melanopsin. After transfection, cells should be handled under a hood with a safe red light, and all other lighting should be switched off.
9. Treatment of the 96-well plate with poly-L-lysine for 30 min at room temperature prior to cell seeding is recommended to avoid cell detachment during changes of media.
10. A high level of reporter gene expression in control cells increases the leakiness of the system. Reducing the reporter amount and also culturing light-protected control cells in a separate plate can ameliorate this kind of leakiness.

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Figure legends

Fig 1. Schematic of blue light-induced activation of endogenous signaling for gene expression in mammalian cells. Melanopsin signaling through the Gaq pathway leads to activation of the transcription factor NFAT (nuclear factor and activator of transcription). NFAT induces expression of the reporter gene, secreted placental alkaline

phosphatase (SEAP), from a synthetic promoter equipped with binding sites derived from the interleukin 4 promoter.

Fig 2. Experimental setup. a) Circuit diagram of the architecture of the LED array. b) Flow-Chart of the program running on the Arduino™. Pulses of 5 seconds ON and 15 seconds OFF were applied twice for 12h with a pause of 12h in between. c) View of disassembled parts. Left: bottom view showing LED holders; middle: fitting spacer to ensure correct orientation of LEDs; right: top view of the Arduino controller. c) 96-well plates with the bottom rows covered with aluminium foil to protect control cells from light (control). d) Operating the LED array on a 96-well plate inside the mammalian cell culture incubator.

Fig 3: Light intensity experiments. a) Calibration curve showing light intensity as a function of Arduino units. The curve needs to be established for every construction, because many parameters, including LED type, current and filters, affect the light intensity. b) Image of an operating LED array programmed with a light intensity gradient. c) Light irradiation pattern with 5 sec ON and 15 sec OFF at different light intensities. d) Light intensity-dependent SEAP expression levels.

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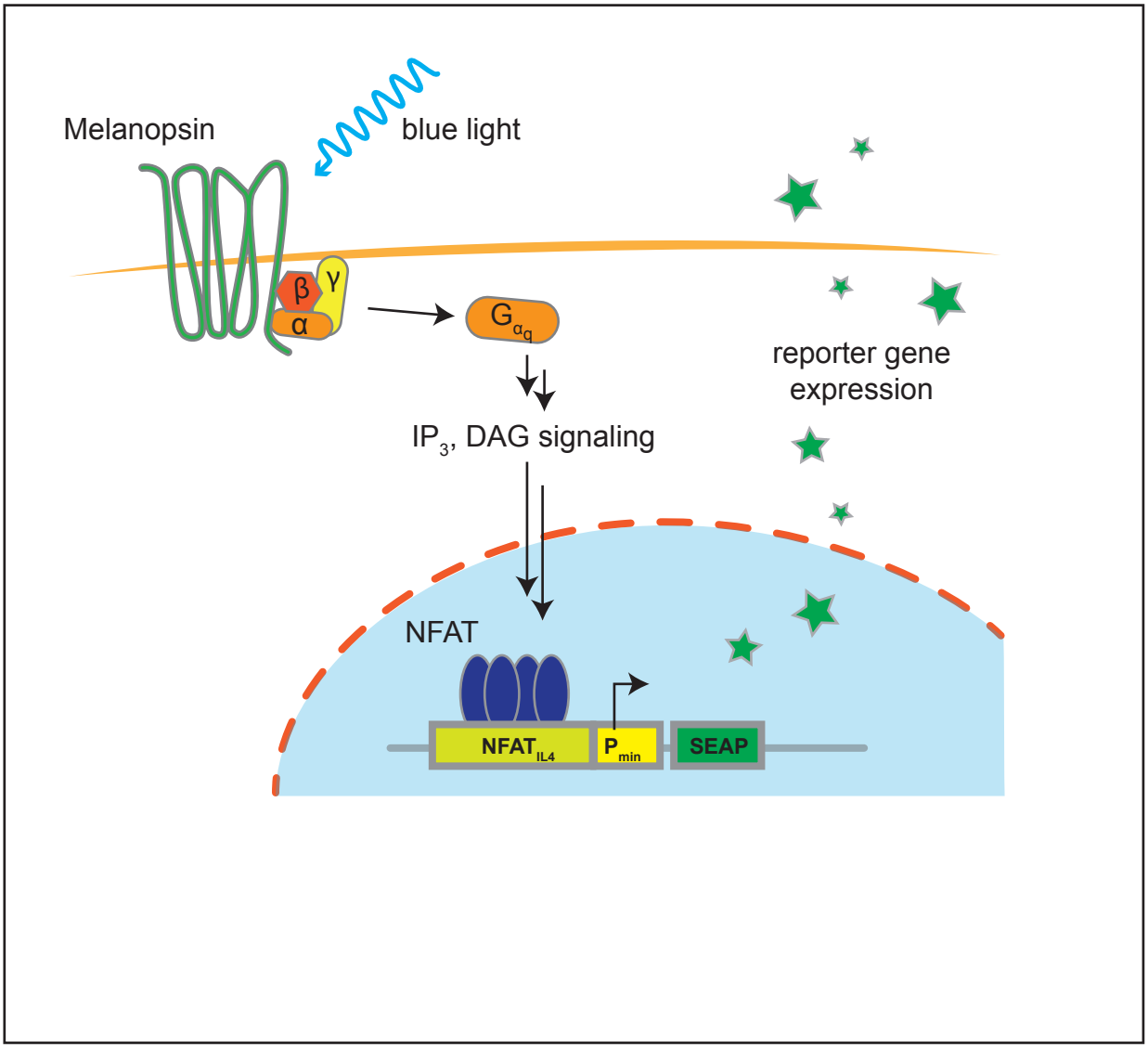
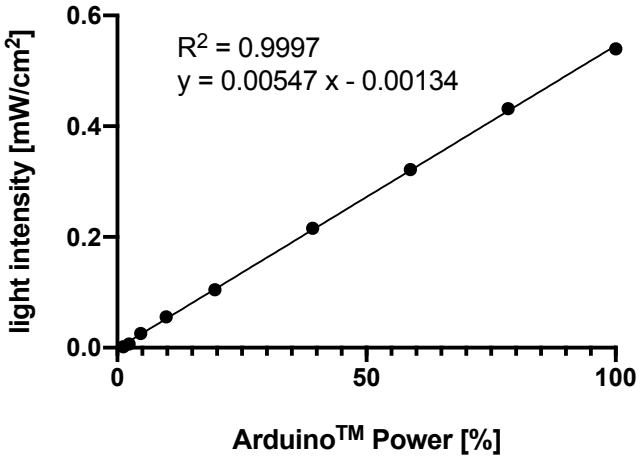

Melanopsin
blue light
β
γ
α
G_{α_q}
reporter gene expression
IP_3, DAG signaling
NFAT
$NFAT_{IL4}$
P_{min}
SEAP

a


R2 = 0.9997
y = 0.00547 x - 0.00134
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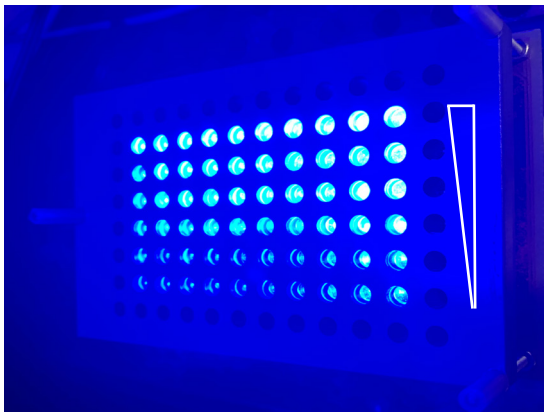
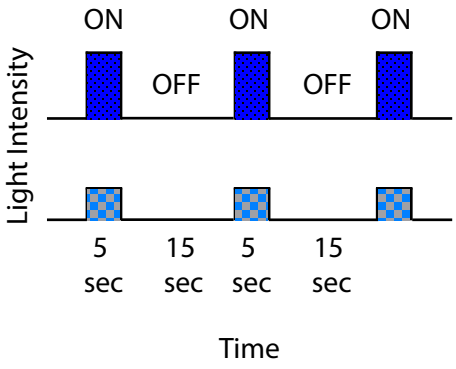
0.6
0.4
0.2
0.0
0
50
100
ArduinoTM Power [%]

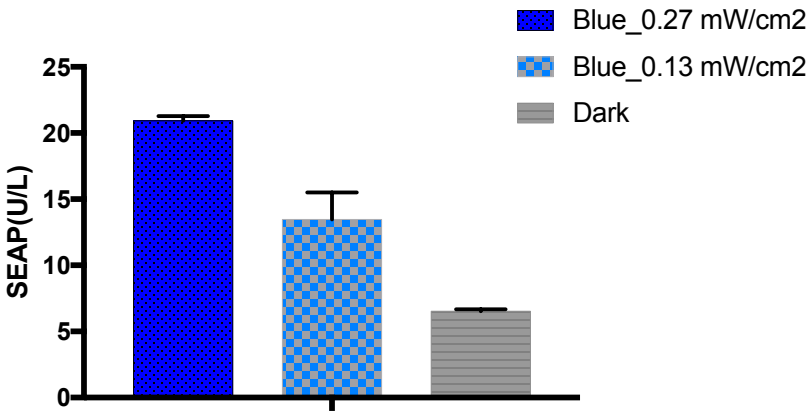
b



c


ON
ON
ON
OFF
OFF
Light Intensity
5 sec
15 sec
5 sec
15 sec
Time

d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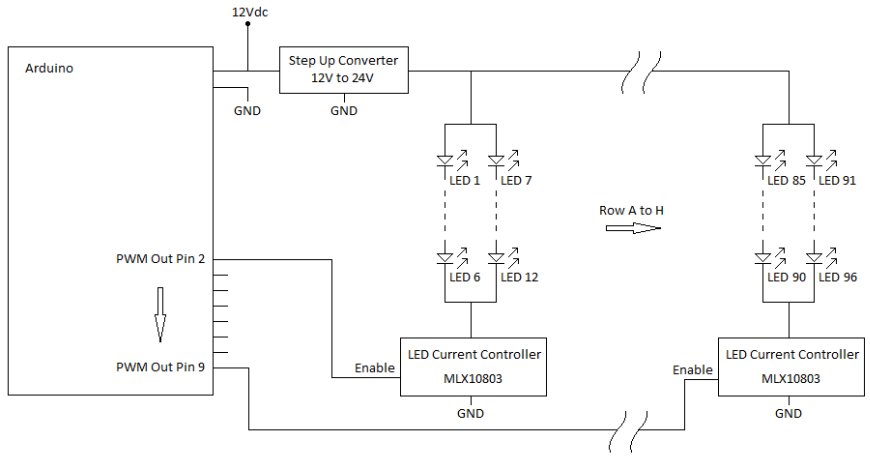
Blue_0.13 mW/cm2
Dark
SEAP(U/L)
25
20
15
10
5
0

a


12Vdc
Arduino
Step Up Converter
12V to 24V
GND
GND
LED 1
LED 7
LED 85
LED 91
Row A to H
PWM Out Pin 2
LED 6
LED 12
LED 90
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PWM Out Pin 9
Enable
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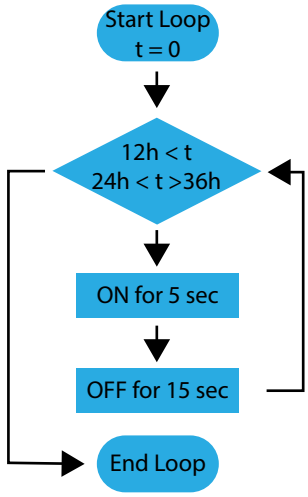
MLX10803
Enable
LED Current Controller
MLX10803
GND
GND

b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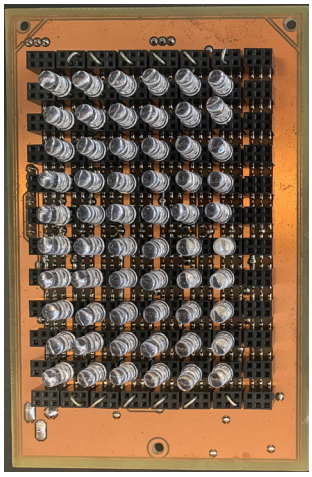
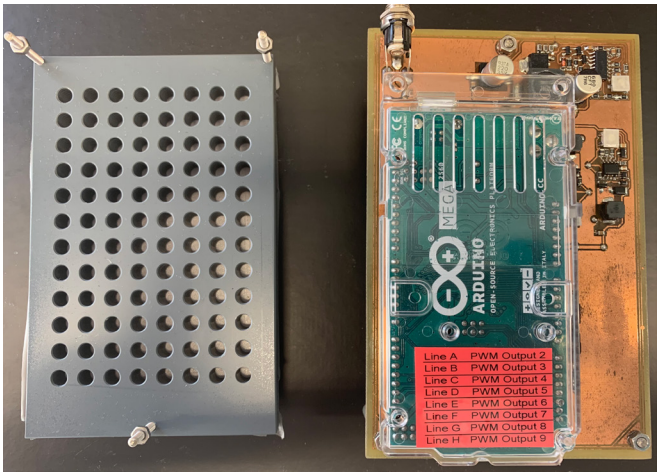
ON for 5 sec
OFF for 15 sec
End Loop

c

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Line E PWM Output 6
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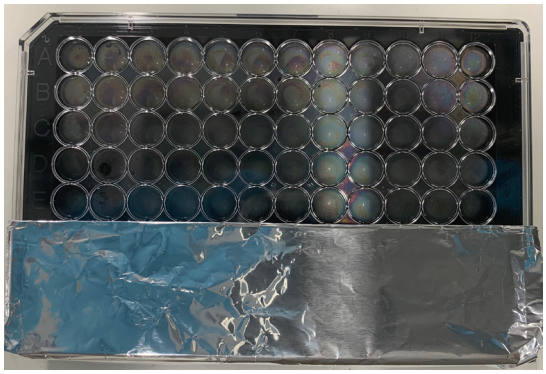
c



d