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TITLE:

Reliably Engineering and Controlling Stable Optogenetic Gene Circuits in Mammalian Cells

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SUMMARY:

Reliably controlling light-responsive mammalian cells requires the standardization of optogenetic methods. Toward this goal, this study outlines a pipeline of gene circuit construction, cell engineering, optogenetic equipment operation, and verification assays to standardize the study of light-induced gene expression using a negative-feedback optogenetic gene circuit as a case study.

ABSTRACT:

Reliable gene expression control in mammalian cells requires tools with high fold change, low noise, and determined input-to-output transfer functions, regardless of the method used. Toward this goal, optogenetic gene expression systems have gained much attention over the past decade for spatiotemporal control of protein levels in mammalian cells. However, most existing circuits controlling light-induced gene expression vary in architecture, are expressed from plasmids, and utilize variable optogenetic equipment, creating a need to explore characterization and standardization of optogenetic components in stable cell lines. Here, the study provides an experimental pipeline of reliable gene circuit construction, integration, and characterization for controlling light-inducible gene expression in mammalian cells, using a negative feedback optogenetic circuit as a case example. The protocols also illustrate how standardizing optogenetic

equipment and light regimes can reliably reveal gene circuit features such as gene expression noise and protein expression magnitude. Lastly, this paper may be of use for laboratories unfamiliar with optogenetics who wish to adopt such technology. The pipeline described here should apply for other optogenetic circuits in mammalian cells, allowing for more reliable, detailed characterization and control of gene expression at the transcriptional, proteomic, and ultimately phenotypic level in mammalian cells.

INTRODUCTION:

Similar to other engineering disciplines, synthetic biology aims to standardize protocols, allowing tools with highly reproducible functions to be utilized for exploring questions relevant to biological systems[1,2]. One domain in synthetic biology where many control systems have been built is the area of gene expression regulation[3,4]. Gene expression control can target both protein levels and variability (noise or coefficient of variation, CV = σ/μ, measured as the standard deviation over the mean), which are crucial cellular characteristics due to their roles in physiological and pathological cellular states[5–8]. Many synthetic systems that can control protein levels and noise[4,9–12] have been engineered, creating opportunities to standardize protocols across tools.

One novel set of tools that can control gene networks that has recently emerged is optogenetics, enabling the use of light to control gene expression[13–17]. Similar to their chemical predecessors, optogenetic gene circuits can be introduced into any cell type, ranging from bacteria to mammals, allowing expression of any downstream gene of interest[18,19]. However, due to the rapid generation of novel optogenetic tools, many systems have emerged that vary in genetic circuit architecture, mechanism of expression (e.g., plasmid-based vs. viral integration), and light supplying control equipment[11,16,20–25]. Therefore, this leaves room for standardization of optogenetic features such as gene circuit construction and optimization, method of system utilization (e.g., integration vs. transient expression), experimental tools used for induction, and analysis of results.

To make progress on standardizing optogenetic protocols in mammalian cells, this protocol describes an experimental pipeline to engineer optogenetic systems in mammalian cells using a negative feedback (NF) gene circuit integrated into HEK293 cells (human embryonic kidney cell line) as an example. NF is an ideal system to demonstrate standardization since it is highly abundant[26–28] in nature, allowing protein levels to be tuned and noise minimization to occur. In brief, NF allows for precise gene expression control by a repressor reducing its own expression, thereby limiting any change away from a steady state. The steady state can be altered by an inducer that inactivates or eliminates the repressor to allow for more protein production until a new steady state is reached for each inducer concentration. Recently, an engineered NF optogenetic system was created that can produce a wide-dynamic response of gene expression, maintain low noise, and respond to light stimuli allowing the potential for spatial gene expression control[11]. These tools, known as light-inducible tuners (LITers), were inspired by earlier systems that allowed gene expression control in living cells[4,10,29,30] and were stably integrated into human cell lines to ensure long-term gene expression control.

Here, using the LITer as an example, a protocol is outlined for creating light-responsive gene circuits, inducing gene expression with a Light Plate Apparatus (LPA, an optogenetic induction hardware)[31], and analyze responses of the engineered, optogenetically-controllable cell lines to custom light stimuli. This protocol allows users to utilize the LITer tools for any functional gene they wish to explore. It can also be adapted for other optogenetic systems with diverse circuit architectures (e.g., positive feedback, negative regulation, etc.) via integrating the methods and optogenetic equipment outlined below. Similar to other synthetic biology protocols, the video recordings and optogenetic protocols outlined here can be applied in single-cell studies in diverse areas, including but not limited to cancer biology, embryonic development, and tissue differentiation.

PROTOCOL:

1. Gene circuit design

1.1. Select genetic components to combine into a single gene circuit/plasmid (e.g., mammalian DNA integration sites[32], light-responsive elements[33], or functional genes[34]).

1.2. Using any genetic engineering and/or molecular cloning software, store the DNA sequences for later use and reference, annotate each sequence, and examine all the necessary features (e.g., START codons, regulatory, or translated sequences)[35].

1.3. Develop or adopt parts according to the overall gene circuit design[36]. As an example, for optogenetic repressors as in the LITers[11], fuse a gene sequence coding for a domain capable of light-inducible degradation[37,38] or inactivation of a repressor's DNA binding ability[39], adjacent to and in the frame of the repressor gene (e.g., TetR)[4].

1.3.1. For optogenetic activators, ensure the presence of an activator domain[40] that promotes gene expression upon light-induced DNA binding[41]. For negative or positive autoregulation, ensure the presence of a regulatory binding site in the regulator gene's promoter (e.g., tetO sites in the TetR expressing promoter)[42].

1.4. Design the primers for DNA sequence amplification or sequencing of the plasmid using the molecular cloning software for each gene circuit.

1.5. Validate the primers computationally for plasmid construction through the built-in molecular cloning software features (e.g., sequence alignment).

1.6. Order oligonucleotide primers from the manufacturer. Plasmids constructed and used in this work can be found in the original supporting material[11] along with the primers designed and used.

1.7. Dilute the primers to 100 mM stock concentration in double-distilled water (ddH_2O).

1.8. Dilute the stock of 100 mM stock primers to 10 mM concentration for PCR.

1.9. Prepare the PCR mix with 1 µL of forward primer, 1 µL of reverse primer, 1 µL of template DNA to a total mass of 0.5–500 ng for genomic or 0.5 pg–5 ng for plasmid or viral DNA, 12.5 µL of DNA polymerase 2x master mix (or the volume that satisfies the manufacturer's dilution factor), and 9.5 µL of ddH_2O for a total reaction of 25 µL.

1.10. Incubate in a thermocycler at appropriate settings depending on the enzyme chosen[43]. Suggested reaction cycles include:
Step 1: One cycle of 30 s initial denaturation step at 95 °C.
Step 2: 40 cycles of 5 s of denaturation step at 98 °C.
Step 3: One cycle of 30 s of annealing step at 65 °C (determined by primers designed earlier).
Step 4: One cycle of 1 min of extension at 72 °C (~1 kilobase (kb) template fragment length).
Step 5: One cycle of a 2 min extension at 72 °C.
Hold the reaction at 4 °C until it can be tested via gel electrophoresis. This protocol will vary depending on the reagents used (e.g., polymerase and buffers).

1.11. Repeat step 1.9 to amplify all the fragments to be used in a DNA assembly reaction required for linking linear PCR products into a single circular DNA vector.

1.12. Run the PCR products on a 1% agarose gel followed by purifying the bands of the desired length.

1.13. Prepare the master mix for a DNA assembly reaction (**Table 1**).

NOTE: In this example, the mother-vector is split into two fragments to increase the efficiency of the PCR steps without causing significant changes to the overall assembly outcome.

1.14. Incubate the DNA assembly reaction master mix in a thermocycler at 50 °C for 1 h (unless otherwise specified in DNA assembly reagent protocol) and store the reaction at 4 °C to use the reaction product for bacterial transformation.

1.15. Set up bacterial transformation (chemical or electroporation) using competent *E. coli* (*Escherichia coli*) cells and the corresponding bacterial transformation protocol. After transformation, use the bacterial Luria-Bertani (LB) agar plates containing the chosen bacterial selection marker (e.g., Ampicillin) to plate the transformation mix. Incubate the plates at 37 °C overnight[44].

1.16. Check the plates the following day. To inoculate the colonies, pick individual colonies from the plates and resuspend them in a liquid LB broth with the corresponding bacterial selection marker in culture tubes. Incubate in a shaker incubator at 37 °C, 300 rpm overnight.

1.17. Perform plasmid preparation protocol to extract the plasmid DNA from the bacterial culture.

1.18. Validate the circuit in two steps. First, perform a test digestion using restriction enzymes as a crude verification to see whether the approximate plasmid product was obtained. Second, if the test digestion is passed/confirmed, submit the plasmid to a Sanger sequencing facility (or process using the available equipment) to obtain the precise DNA sequence to compare it to the expected sequence in the design software later.

1.18.1. To perform test digestion, select at least two restriction enzymes that produce at least two fragments, based on the molecular cloning software used. Once enzymes are selected, prepare the test samples by adding 1 µL of each enzyme, 5 µL of the generated DNA, and 13 µL of water with appropriate salts and buffers depending on the enzymes used. Incubate the reaction at 37 °C for 1 h, or as the enzyme manufacturer suggests. Run the test digestion products on a 1% agarose gel and determine whether the bands are correct.

NOTE: If the bands are correct, proceed to sequencing.

1.18.2. To perform sequencing, generate primers based on the DNA stored in the software so that the annealing regions of the primers are about 500 bp (base pairs) apart and cover the fragment of interest (gene circuit component) or the full plasmid. Dilute the primers with pure nuclease-free water (NF-H_2O) to 10 mM concentration. Prepare the sequencing samples by adding 1 µL of 10 ng/µL DNA, 1 µL of primer, and 8 µL of NF-H_2O to a 0.2 mL tube. Repeat this for each primer.

NOTE: When samples are prepared, drop them off at the sequencing facility and analyze the results with plasmid sequence using molecular cloning software.

1.19. At this step, generate approximately 100–1000 ng/µL of DNA sample for integrating the plasmids containing gene circuits into the appropriate cell line.

2. Stable cell line engineering

2.1. Order a mammalian cell line designed for rapid generation of stable sub-lines that ensure high-level expression of the protein of interest from a mammalian expression vector. The cell type can be variable, and the ease of cell line engineering can also be variable depending on what the users prefer.

2.1.1. For example, if users prefer cell line engineering with minimal intermediate steps, order the cells that contain a single stable integration site (e.g., FRT) at a transcriptionally active genomic locus. If more nuanced cell engineering is preferred, create integration sites at preferred locations using genetic engineering tools such as Cas9.

2.2. Grow cells in 5% CO_2 in humidified air at 37 °C. Adjust growth conditions as needed for the cell type.

2.3. Transfect the gene circuits designed above in the desired cells obtained from the previous steps to begin a stable cell-line generation process. To achieve this, use a liposome mixture[45] of the gene circuit DNA with appropriate recombinase (for example, Flp-recombinase for Flp-FRT recombination) or through other methods such as electroporation.

2.4. Two days after transfection, split the cells to 25% confluency.

2.5. Six hours after splitting the cells, begin antibiotic selection by exchanging the media to a fresh media containing 50 µg/mL of hygromycin antibiotic (or another antibiotic agent corresponding to the mammalian antibiotic resistance gene chosen during plasmid construction).

NOTE: There are a variety of mammalian antibiotic resistance genes utilized in gene circuit construction, each of which has a different kill curve. Therefore, whichever mammalian antibiotic resistance gene is chosen for circuit construction, a proper kill curve should be studied in the cells of interest. This step ensures that cells containing the gene circuit payload are enriched while those without the system are killed off.

2.6. Allow the cells to grow in the antibiotic selection media, change to fresh media every 2–3 days until the plate or flask has a few dozen foci. Passage adherent cultures when they are in the log phase before they reach confluence.

2.7. Once there are many foci, trypsinize the cells with 1 mL of 0.25% trypsin, 0.1% EDTA in Hank's balanced salt solution (HBSS) without Calcium, Magnesium, and Sodium Bicarbonate for several minutes. Neutralize the trypsin with fresh media and pass all the cells into a fresh container.

2.8. Once the cells in the fresh container are 80%–100% confluent, freeze them down in a mix of 45% old media, 45% fresh media, and 10% DMSO. Transfer the remaining cells to a sterile tube and perform single-cell sorting to isolate monoclonal cells into a 96-well plate.

2.9. Approximately 2–3 weeks post monoclonal sorting, wells within the 96-well plate should have foci. When approximately 50%–60% confluent, split the cells into a 12-well plate.

2.10. Once the 12-well plate is 80%–100% confluent, split into a tissue culture treated T-25 flask. Once the cells in the T-25 flask are 80%–100% confluent, freeze the cells and maintain a passage for characterization and testing of monoclonal cell lines.

2.10.1. Characterize the monoclonal cell lines by microscopy and flow cytometry assays to report gene expression profiles based on the induced fluorescent reporter production. Verify functional protein production via fluorescent antibody labeling and immunofluorescence assay. Test the transcriptional-level gene expression induction via quantitative real-time PCR (qRT-PCR). Validate the genetic sequence and integration accuracy via local and whole-genome sequencing of the clones.

3. Light plate apparatus induction assays

3.1. Construct an LPA device[31,46] to be used for light induction of engineered cells. Briefly, there are several broad steps crucial for creating LPA to use for controlling gene expression. These include 3D printing LPA frame components, circuit board construction, programming the circuit via microcontroller programmer, assembling the components into a final LPA, programming the memory card via IRIS software, and calibrating the finished device. For a more thorough explanation, refer to the above references.

3.1.1. Print the 3D parts as outlined in Gerhardt et al. (2016 and 2019)[31,46].

3.1.2. Assemble the circuit board as outlined in Gerhardt et al. (2016 and 2019)[31,46].

3.1.3. Add the firmware to the assembled LPA circuit using microcontroller programmer as outlined in Gerhardt et al. (2016 and 2019)[31,46].

3.1.4. Combine 3D printed parts and the assembled circuit board as outlined in Gerhardt et al. (2016 and 2019)[31,46]. This includes taking the mounting plate, the circuit board, the LED (light-emitting diodes) spacer, the plate adaptor, a 24-well plate, a plate lid, mounting bolts, and wing nuts and stacking components as shown in the filmed video and **Figure 2**.

3.1.5. For memory card programming and calibration of the device, follow the steps described below.

3.2. Use the IRIS software available on the Tabor Lab website[47] to program an SD card for the Light Plate Apparatus (LPA)[31] and explore appropriate light conditions needed to begin an optogenetic experiment.

NOTE: The IRIS software is a web-based application for programming the optogenetic electronic hardware, known as the LPA, developed by the Tabor Lab. The software allows programming of relative IRIS values that control the individual light-emitting diodes (LEDs) in each well of the LPA hardware.

3.3. Choose the LPA (4 x 6) drop-down option, followed by clicking the appropriate illumination approach (Steady-state, Dynamic, Advanced).

NOTE: For this manuscript, all the assays will focus on the steady-state examples. However, the advanced setting examples can be used for pulse durations and duty cycle regimes.

3.4. Choose whether the top or the bottom LEDs will be illuminated by entering values in the cells corresponding to the wells of the plate. For the LPAs used in this work, blue LEDs placed in the top position were used in all experiments. Each LED, once programmed, can provide continuous light exposure with a constant light intensity. The IRIS software allows for 4096 intensity levels which can be programmed.

3.5. Once the LED locations are chosen, enter intensity values for the desired experimental outline. For example, enter 8 different light intensities (or pulse durations or duty cycles) with three technical replicates per plate (**Table 2**). G.s. refers to grayscale units—the light intensity level measurement values used for LPA programming in the IRIS software.

3.5.1. When designing IRIS experimental files, keep in mind edge effects from adjacent wells on the LPA device, light toxicity from increasing intensities of blue light exposure, and determining the type of dose-response desired (e.g., monotonic vs. non-monotonic).

3.5.2. If users program cells in the LPA in ascending/descending order of a particular light parameter (e.g., intensity), wells may produce edge effects of light crosstalk or even heat which can influence adjacent wells. This can inadvertently influence the outcome of measured outputs when experiments are complete. To alleviate this, users can implement a randomization matrix on the IRIS software to scramble well locations, minimizing edge effects. An example is described in the represented results below (**Figure 4A–B**).

3.5.3. In addition, higher intensities of blue light have been found to interfere with cellular growth and viability[48]. Therefore, to mitigate light toxicity, it is important to produce a light-intensity, pulse duration, or duty cycle response curve depending on the modality being investigated.

3.5.3.1. For example, program 8 light intensity values with three replicates per 24-well plate, with a range from no light to a max intensity of the LPA. Then, run these samples on a flow cytometer with an SSC-FSC gate or a live stain such as propidium iodide to quantify the population cell survival (living cells compared to total events including dead cells/cellular debris) at each light value.

3.5.3.2. Then, determine the ideal amount of population survival for the experimental setup, since any stimulus may adversely affect proliferation, gene expression, or survival (e.g., setting 80% cell survival as an appropriate tradeoff). For example, in this work, once calibrated, the intensity did not exceed 3000 g.s. units (~3/4 the maximum intensity of the LPA device). An example of this is described in the represented results below.

3.5.4. In addition to restricting light values because of toxicity, users may wish to restrict light values to characterize a particular part of the dose-response, such as the range of the monotone response.

3.5.4.1. To achieve this, initially scan a wide range of light intensities, pulse durations or duty cycles depending on the modality being analyzed when determining the desired dose-response (**Table 2**) to narrow in on the light regime of interest, e.g., where gene expression correlates positively with increasing light values for a monotonic dose-response.

3.5.4.2. To determine the light range of interest, program a single LPA with up to 24 wells of different intensity/pulse duration/duty cycle/etc. or more wells (e.g., 48, 72, 96, etc.) depending on whether multiple LPAs are calibrated to deliver equivalent light amounts and proceed with the cell culture work or assays outlined below. Therefore, start characterization of an optogenetic system with a wide dose range of light stimuli to determine the range interval that gives the desired gene expression and subsequently perform experiments in that refined dose range.

3.5.4.3. For example, in this work, once 3000 g.s. units was determined as the threshold for toxic light intensity; this threshold was used as the upper bound of light for assays outlined below (e.g., immunofluorescence).

NOTE: The steps above are independent of the optical calibration of the LPA and refer to a molecular-level calibration for each optogenetic system.

3.6. Once the appropriate light intensity values are programmed in IRIS, insert a memory card with the USB 3.0 outlet into the LPA to download and transfer the files.

3.7. If calibration of the LPA is complete[31], proceed to cell culture work for initiation of light-induction assay. If the calibration has not been done, calibrate the LPA using an Image analysis method (steps 3.7.1–3.7.3) once the LPA is programmed with the microcontroller programmer.

3.7.1. Briefly program the LPA to have the same IRIS level as described by Gerhardt et al. (2016)[31].

NOTE: For calibration, the LPA was programmed to have a value of 2000 g.s.

3.7.2. Once the device is programmed with the memory card and the reset button (physical button on the LPA) is pressed, acquire an image of the entire device (e.g., gel station imager, scanner device, etc.). For the calibration, acquire two images with the device rotated by 180°.

3.7.3. Then, use open-source software designed by Gerhardt et al. (2016)[31] to show the variability in LED intensity on the LPA plate and calculate the LED compensation values to make the intensity equal across wells. An example of this is described in the represented results below (**Figure 3**). Once this calibration is complete, proceed to cell culture work for initiation of light-induction assay.

3.8. Obtain flasks of engineered monoclonal cells from the previous section to investigate light-induction effects on gene expression.

3.9. Remove the old media from the flask.

3.10. Add 1 mL of trypsin to the cells and incubate for 5 min.

3.11. After 5 min, neutralize trypsin by adding 4 mL of Dulbecco-modified Eagle's medium (DMEM or other desired media) supplemented with the necessary chemicals (this work used 50 µg/mL of hygromycin, 1% penicillin/streptomycin solution, 10% fetal bovine serum, FBS).

3.12. Add ~75,000 cells per well in a 24-well black plate in a total volume of 500 µL. The number of cells seeded can vary depending on the duration of the experiment desired and the cell type used.

NOTE: Additionally, note that cell seeding density affects the culture maintenance and potentially the duration of the experiment. Starting at a lower number of cells per well ensures longer time durations before the culture reaches confluency. Furthermore, the type of optogenetic components integrated into the gene circuits of interest will influence when gene expression reaches a steady state and therefore affect the duration of the experiments. Other factors that may be considered are cell line-specific growth rates, media composition, and conditional growth effects (i.e., light).

3.13. After plating, place the cells in a humidified incubator with 5% CO_2 to allow them to settle for 2–6 h.

3.14. After the incubation, transfer the cells to a tissue culture hood, remove the plastic lid, and add an adhesive foil strip to the top of the plate (**Figure 2D**). This step allows minimal light transfer between wells, as the top and the sides of the wells are coated, and the light can now only enter from the bottom of the well where the LED is placed.

3.15. Place the plate in the fitted 3D parts of the LPA. Then, cover the plate with the 3D-printed LPA lid, which fits over the device screws on each corner.

3.16. Plug the device into the power source and push the reset button on the LPA device to ensure that the updated LPA experiment settings are applied.

3.17. Incubate the cells within the experimental system for an appropriate amount of time, depending on the original seeding density, cell line-specific growth speed, and growth conditions. In this example, the cell lines were induced for 3 days continuously for gene expression to reach a steady state. However, it should be noted that many optogenetic systems (e.g., VVD) may reach steady-state gene expression much sooner (e.g., 24 h), and therefore experimental induction times can be reduced or prolonged as needed.

3.18. At the end of the light induction experiment, utilize the samples for any of the following four assays to characterize the engineered cell lines (sections 4–7).

4. Fluorescence microscopy of light-induced engineered cells

4.1. 24–72 h post-induction, remove the cells in the LPA from the incubator and place them in a tissue culture hood.

4.2. Remove the foil strip and let the plate sit for 1–2 min. This prevents condensation from forming on the plastic lid, if placed immediately on the plate.

4.3. After 1–2 min of sitting, put the original plastic lid back on the plate.

4.4. Image the cells with the appropriate phase contrast or fluorescence microscope.

4.5. Depending on the instrument, adjust the exposure time, light source intensity, and gain for displaying engineered cells. In this experiment, the following parameters were applied: 50 ms for FITC/GFP (green fluorescent protein) light source exposure time and 1–5 ms for phase-contrast exposure time at 100% intensity for each.

NOTE: It should be noted that optimal exposure times, gain levels, and light source intensities are often derived empirically from the experience of this work to minimize oversaturation in the fluorescence reporter, minimize cellular damage, and capture adequate images for qualitative and quantitative analysis. When determining the levels of each of these parameters, the aspects to keep in mind include maximizing signal-to-noise ratios, minimizing phototoxicity, minimizing oversaturation of fluorescence signals, and increasing the ability to amplify weak fluorescence signals.

4.5.1. Optimize these parameters grossly ad-hoc; however, previous experimental values (e.g., light source intensity causing oversaturation of fluorescence reporter) can guide future experimental settings when applicable. For example, the gain settings, exposure times, and light intensity initial values from one circuit (LITer1.0) or experimental setup (e.g., light intensity) can be used as a starting point when moving to a similar but different gene circuit (LITer2.0)[11] or a different light modality (e.g., light duty cycle).

4.6. Streamline plate imaging by a coordinate template programmed into the microscopy software allowing the entire plate size (e.g., 24 wells) to have automatized image acquisition from multiple image locations per well.

5. Flow cytometry of light-induced engineered cells

5.1. 24–72 h post-induction, remove the cells from the LPA in the incubator or from the microscope after imaging and place them in the tissue culture hood.

5.2. If removed directly from the LPA, remove the foil strip, and let the plate to sit for a minute or two.

5.3. Aspirate the media from each well (of the entire 24-well plate if all the wells are used).

5.4. Add 100 µL of 0.25% trypsin to each well, cover the plate with a plastic lid, and place it back in the incubator.

5.5. Leave the cells undisturbed in the incubator for 5 min.

5.6. After 5 min, remove the plate and return it to the tissue culture hood.

5.7. Neutralize each trypsinized well with 400 µL of DMEM.

5.8. Label a 5 mL polystyrene round-bottom tube with cell strainer (or appropriate flow cytometry tube that can be filtered to remove large clumps of cells not fully trypsinized) with a name corresponding to each well.

5.9. Use a P1000 pipette to pipette the contents of each well up and down 6–8 times to break cell clumps and create single-celled samples for flow cytometry[49].

5.10. Transfer the entire contents of each well (~500 µL) to the labeled tubes with the strainer.

5.11. Bring cells to the appropriate flow cytometry instrument with lasers of the correct wavelengths (can bring tubes with cells on or off ice).

NOTE: The flow cytometer used in this work was a part of a core facility located at the university hospital.

5.12. Create a forward- and side-scatter gate (FSC and SSC, respectively) to capture the single cells of the appropriate size and granularity to exclude debris and cellular clumps on the flow cytometry software.

5.13. Once the gate is set, capture approximately 10,000 cells with the appropriate gate. Adjust this number depending on the amount of cellular data the users are seeking. Repeat for each tube containing the cells from the experiment.

5.14. Once the experiment is complete, import the data to the available flow cytometry data software for analysis.

5.15. Create an FSC-SSC gate (as before during acquisition) and apply it to each batch of experimental data. A reference well of the un-induced cell population is used for creating this gate in this manuscript, but other metrics for gate creation exist, such as density-based gates.

5.16. With the experimental conditions gated with an FSC-SSC gate, plot the flow cytometry data as histograms or represent in other ways to illustrate the obtained expression patterns. In this experiment, the fluorescence was captured by the GFP/FITC or PE/TexasRed channels.

6. RNA extraction and quantitative PCR of gene circuit components

6.1. 24–72 h post-induction, remove the cells from the LPA in the incubator or from the microscope after imaging and place them in the tissue culture hood.

6.2. If removed directly from the LPA, remove the foil strip, and let the plate sit for a minute or two.

6.3. Aspirate the media from each well (of the entire 24-well plate if all the wells are used).

6.4. Proceed to extract RNA from the cells using the appropriate RNA extraction kit.

6.5. Once the RNA extraction is complete, perform a reverse transcription reaction of each sample (**Table 3**).

6.6. Further, perform quantitative PCR of each sample (**Table 4**). Utilize a DNA polymerase and associated protocol to set up PCR reactions. For this step, set up a multiplexed reaction with a housekeeping gene and a gene of interest, or create separate reactions. In this example, GFP, KRAS, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels were probed. After completing the qRT-PCR experiment, proceed with the analysis via available software to illustrate gene circuit fold change at the RNA level.

7. Immunofluorescence of gene circuit components

7.1. Use an ice bath or refrigerator/freezer to cool down methanol.

7.2. 24–72 h post-induction, remove the cells from the LPA in the incubator or from the microscope after imaging and place them in the tissue culture hood.

7.3. If removed directly from the LPA, remove the foil strip, and let the plate sit for 1–2 min.

7.4. Aspirate the media from each well (of the entire 24-well plate if all the wells are used).

7.5. Add 100 μL of 0.25% trypsin to each well, cover the plate with a plastic lid, and place it back in the incubator.

7.6. Leave the cells undisturbed in the incubator for 5 min.

7.7. After 5 min, remove the plate and return it to the tissue culture hood.

7.8. Neutralize each trypsinized well with 400 μL of DMEM.

7.9. Label mini-centrifuge tubes with names corresponding to each well.

7.10. Use a P1000 pipette and pipette the contents of each well up and down 6–8 times to break the cell clumps.

7.11. Transfer the entire contents of each well (~500 µL) to the labeled tubes.

7.12. Centrifuge the cells for 5 min at 400 x *g*.

7.13. When complete, discard the supernatant and move the samples to a chemical fume hood.

7.14. Resuspend the cells (use a P1000 pipette and pipette up and down 6–8 times in each tube to break cell clumps) in 750–1000 µL of 4% paraformaldehyde (diluted in phosphate-buffered saline, PBS).

7.15. Allow the cells to sit for 15 min at room temperature.

7.16. After the incubation, add 750–1000 µL of PBS. Pipette up and down several times.

7.17. Centrifuge the cells for 5 min at 400 x *g*.

7.18. When complete, discard the supernatant and move the samples to a chemical fume hood.

7.19. Resuspend the cells (use a P1000 pipette and pipette up and down 6–8 times in each tube to break cell clumps) in 750–1000 µL of ice-cold methanol.

7.20. Allow the cells to sit for 30 min on ice or in a -20 °C freezer.

7.21. After the incubation, add 750–1000 µL of PBS. Pipette up and down several times.

7.22. Centrifuge the cells for 5 min at 400 x *g*.

7.23. When complete, discard the supernatant and move the samples to a chemical fume hood.

7.24. Depending on the type of antibodies used, modify the protocol from this point forward. Follow either steps 7.24.1-7.24.14 or 7.24.15-7.24.22.

7.24.1. If using a primary and secondary antibody, resuspend the cells using a P1000/P200 pipette and pipette up and down 6–8 times in each tube to break cell clumps in 100 µL of primary antibody. In this case, a dilution of 1:800 for stock antibodies was made, including KRAS antibody or ERK antibody, and was allowed to sit for 1 h at room temperature. Antibodies were diluted in an incubation buffer made from 1x PBS with 0.5 g of BSA.

NOTE: Determine the exact dilution of the antibody empirically by creating a standard curve of antibody dilutions vs. the antigen of interest.

7.24.2. After adding antibodies, cover the tubes with a foil to prevent light effects on labeled antibodies.

7.24.3. After incubation, add 750–1000 µL of the incubation buffer. Pipette up and down several times.

7.24.4. Centrifuge the cells for 5 min at 400 x *g*.

7.24.5. When complete, discard the supernatant and move the samples to a chemical fume hood.

7.24.6. Resuspend the cells using a P1000/P200 pipette and pipette up and down 6–8 times in each tube to break cell clumps in 100 µL of secondary antibody. In this case, cells were resuspended in 100 µL secondary antibody at a dilution of 1:800 for KRAS antibody or 1:2000 for ERK antibody and allowed to sit for 30 min at room temperature. Similar to primary antibodies, dilute the secondary antibodies in the incubation buffer as described above. Determine the dilutions of the secondary antibodies based on the empirical findings of a standard curve.

7.24.7. After adding antibodies, cover the tubes with a foil to prevent light effects on the labeled antibodies.

7.24.8. After the incubation, add 750–1000 µL of the incubation buffer. Pipette up and down several times.

7.24.9. Centrifuge the cells for 5 min at 400 x *g*.

7.24.10. When complete, discard the supernatant and move the samples to a chemical fume hood.

7.24.11. Resuspend the cells using a P1000 pipette and pipette up and down 6–8 times in each tube to break cell clumps in 500 µL of PBS.

7.24.12. Transfer the entire contents of each tube (~500 µL) to the labeled tubes with strainers.

7.24.13. Bring the cells to appropriate flow cytometry instruments with lasers of the correct wavelengths (can bring tubes with cells on or off ice).

NOTE: It should be noted that having several controls are important for progressing with flow cytometry measurement and analysis of engineered cell gene expression. For example, having completely unstained cells, cells stained with primary antibody alone, and cells stained with secondary antibody alone can be useful for comparing results with background signals from antibodies.

7.24.14. From this point, proceed with the analysis as described in section 5.

7.24.15. If using only a primary antibody, resuspend the cells using a P1000/P200 pipette and pipette up and down 6–8 times in each tube to break cell clumps in 100 µL of labeled primary antibody. Determine appropriate antibody dilution and incubate for 1 h at room temperature. Determine the antibody dilution from the standard curve empirically.

7.24.16. After adding antibodies, cover the tubes with a foil to prevent light effects on labeled antibodies.

7.24.17. After incubation, add 750–1000 µL of the incubation buffer. Pipette up and down several times.

7.24.18. Centrifuge the cells for 5 min at 400 x *g*.

7.24.19. Resuspend the cells using a P1000 pipette and pipette up and down 6–8 times in each tube to break cell clumps in 500 µL of PBS.

7.24.20. Transfer the entire contents of each tube (~500 µL) to labeled tubes with strainers.

7.24.21. Bring the cells to appropriate flow cytometry instruments with lasers of the correct wavelengths (can bring tubes with cells on or off ice).

NOTE: It should be noted that having several controls are important for progressing with flow cytometry measurement and analysis of engineered cell gene expression. Having completely unstained cells and cells stained with primary antibody alone are useful for comparing results with background signals from antibodies.

7.24.22. From this point, proceed with the analysis as described in section 5.

REPRESENTATIVE RESULTS:

Gene circuit assembly and stable cell line generation within this article were based on commercial, modified HEK-293 cells containing a transcriptionally active, single stable FRT site (**Figure 1**). The gene circuits were constructed into vectors that had FRT sites within the plasmid, allowing for the Flp-FRT integration into the HEK-293 cell genome. This approach is not limited to Flp-In cells, as FRT sites can be added to any cell line of interest anywhere in the genome using DNA editing technology such as CRISPR/Cas9[50].

Once appropriate cell lines were constructed and validated for the correct insertion, the LPA and IRIS software were chosen as a standardized protocol for light induction of gene expression[31,51]. The LPA system allows 24-wells to be programmed for induction of multiple conditions of mammalian cells depending on the light intensity, pulse duration, and duty cycle, to name a few types of experimental conditions (**Figure 2**). Within this article, spatially uniform light intensity, pulse duration, and duty cycle were prioritized. However, researchers can use this software and the LPA for more advanced programming of temporal light wave patterns.

A crucial aspect about the LPA to be addressed before starting experiments is the optical calibration which can be performed for single or multiple devices. The previously described image analysis method[31] used for calibration requires an image of the entire LPA with all LEDs of interest turned on, which can be acquired from a variety of sources[52], including a gel imager. Here, a computer scanner was used for image acquisition (**Figure 3A**). Once the image was acquired, the open-source software created by Gerhardt et al. (2016)[31] was used to calculate compensation values for each LED to ensure each LPA emits the same intensity at a given grayscale value. The software subtracts the background signal, thresholds the image into a binary category, and calculates the pixel intensity (**Figure 3B**). From the calibration, a minimal variation among the LEDs was found, with a CV of 0.04 between 96 wells (or 4 plates calibrated, **Figure 3C**). Lastly, using the calibration software demonstrated the LED variation by location (**Figure 3D**) and created a grayscale adjustment (**Figure 3E**) so that each LED is normalized to the same intensity.

In addition to calibration of the LPA, other important aspects of using the LPA include integrating several controls in the experimental pipeline that can help users minimize systemic errors, confounding variables such as light-toxicity effects and design limitations based on experimental materials. For example, **Figure 4** illustrates two different configurations for programming different light intensities in the LPA wells. The first subpanel of **Figure 4A** shows an ascending organization of light intensity. This can make for ease of programming and data analysis but can produce edge effects where the bottom row of the LPA has the highest light intensity and can potentially cause unwanted heat and light induction of nearby wells. Similarly, in the second subpanel of **Figure 4A**, a randomization matrix has been applied to the IRIS software, creating various light intensities in random positions. This can minimize systemic influences of edge effects. A descrambled matrix (**Figure 4B**) is created with the IRIS software, which can then be utilized to determine the experimental conditions after the completion of the experiment and during analysis. Similar to the randomization of wells, users should also be aware of light toxicity effects that may occur (blue light within this work). In **Figure 4C**, two different light intensities used for gene circuit induction are shown with the same FSC-SSC gate based on the un-induced population of engineered cells. Increasing blue light induction drastically increased the number of events categorized as cellular debris, clumps, or dead cells. As such, users should perform a dose-response on the light modality of interest (e.g., pulse, duty cycle, etc.) to determine light toxicity effects that can occur at the high end of exposure (**Figure 4D**). Here, increasing blue light levels caused dose-dependent cellular debris, dead cells, and clumps compared to non-stimulated cells. As such, the continuous light intensity was restricted to around ¾ of the maximum LPA intensity (~3000 g.s. units).

Once proper equipment was set up, gene expression was then induced in engineered LITer gene circuits via fluorescence microscopy (**Figure 5**). Cells were induced at different light pulse durations on the LPA, which gave a light dose-response of gene expression at the population level. Cells within this work were imaged for GFP expression using 50 ms for FITC/GFP light source exposure time and for bright field imaging using 1–5 ms for phase-contrast exposure time. Given the robustness of this cell line-engineering protocol, any fluorescence marker could be used instead of GFP. The cells can be induced with multiple light regimes and produce a large range of

responses (**Figure 5**). The latter is beneficial in further controlling the expression of functional genes (e.g., oncogene in the original work[53]).

Immediately after fluorescence microscopy, cells could be analyzed in a variety of experimental protocols, including flow cytometry, qRT-PCR, and immunofluorescence. In this work, flow cytometry was performed as a first validation procedure and carried out following the methods outlined above (**Figure 6**). Similar to microscopy, cells were induced at different pulse length values, and showed a dose-response of gene expression quantified at the population level of over four-fold change from un-induced states (**Figure 6A–B**). Similarly, gene expression noise reduction can be shown by comparing various light intensity values (**Figure 6C–D**) for the LITer system versus a positive regulation system such as the VVD/LightOn[54]. When comparing the LITer with the VVD system, negative feedback achieves over five-fold gene expression noise reduction. The same pre-induced cells imaged on microscopy could also be analyzed via qRT-PCR (**Figure 7**). Similar to flow cytometry, the engineered LITer cells could express RNA levels in a dose-responsive manner (over 10-fold induction from un-induced states), matching the dose-response of protein levels quantified by GFP expression on flow cytometry. The fluorescent microscopy and flow cytometry data mentioned can be calibrated using non-fluorescence cells, constitutively expressing fluorescence cells, and fluorescent 6-8-peak validation beads (e.g., FL1-channel green, fluorescent beads). Each of these components can allow the normalization of gene expression in a single experiment. However, these factors can also allow normalization of circuits and engineered cells across different experimental plates, experimental conditions, and days to allow comparison and standardization of gene expression data.

Lastly, the LITer gene circuits can be engineered to express functional genes, such as the KRAS (G12V, **Figure 8**). The versatility of this pipeline allows users to utilize the LITer architecture with cell engineering for any functional gene of interest, possibly incorporating additional architectures such as positive feedback. The LITer showed increasing amounts of blue light that resulted in increased levels of the gene circuit output (KRAS (G12V) protein levels) and, consequently, phosphorylated-ERK levels, both of which can be quantified via immunofluorescence assays.

FIGURES AND TABLE LEGENDS:

Table 1: DNA assembly reaction master mix calculation (step 1.13). The dark, gray-shaded cells are custom filled based on the size of the DNA fragments assembled and the concentration of these fragments after PCR amplification and agarose gel extraction (step 1.11). The light-gray shaded cell (total reaction volume) can be modified; however, the stated volume is recommended. Unshaded cells are generated automatically.

Table 2: IRIS programming of light intensities (g.s.) for light induction experiment in a 24-well plate with three replicates (step 3.4). Color gradient from white to dark green indicates the increase in light intensity from 0 to 3000 g.s. G.s.—grayscale units. This can be randomized by the IRIS software as needed.

Table 3: Reverse transcription master mix calculation (step 6.5). The reaction recommended total volume is 20 µL, and its components are the reverse transcriptase enzyme master mix (here: 5x), RNA template, and nuclease-free water. In this example, the RNA concentrations of samples 1, 2, and 3 are 500, 333, and 250 ng/µL, respectively.

Table 4: Quantitative PCR master mix calculation (step 6.6). The reaction recommended total volume is 20 µL and its components are the DNA polymerase enzyme master mix (here: 2x), GFP and/or GAPDH probes, cDNA (100 ng total), and nuclease free water (NF-H_20).

Figure 1: Circuit design and cell engineering. (**A**) Cell engineering tools, including LITer synthetic gene circuit with FRT integration sites, recombinase enzyme (Flp recombinase) for circuit integration, drug used for selection of appropriate genetic clones, and cell line of interest used for creating desired optogenetic mammalian cell lines. (**B**) Systems can be integrated at a specific FRT-site containing locus within the human genome. These genomic loci can then express specific antibiotic resistance or fluorescent reporter genes for selection of properly integrated genetic cassettes. Gene circuit integration can be initiated with the use of recombinases that recognize specific sites within the original genetic cassette. This introduces a novel drug resistance selection gene which can ensure generation of correctly engineered cells with the desired gene circuit. At the bottom of panel B is the gene circuit architecture for the optogenetic negative feedback system, LITer2.0. This circuit is composed of a self-repressing TetR protein fused with a Light-oxygen-voltage-sensing domain (LOV2), a linker sequence P2A which enables a multicistronic transcript, and GFP. When blue light is applied, the TIP sequence opens from the LOV2 domain, inhibiting the TetR protein, and allowing increased, dose-responsive transcription of both TetR and the GFP reporter. The inhibited TetR protein is unable to bind and repress at the DNA operator site, thus increasing transcription. This negative feedback keeps the gene expression noise low and allows a high-fold change of gene expression. FRT - flip recognition target, HygR - hygromycin resistance, LacZ - β-galactosidase, ZeoR - zeocin resistance, T - TetR, tetracycline repressor protein, D2ir is a CMV-based promoter, LOV2 is Light-oxygen-voltage-sensing domain, TIP is tet-inhibiting peptide which inhibits the TetR protein. This figure has been modified from Guinn and Balázsi (2020)[55].

Figure 2: LPA experimental programming. (**A**) Experiments can be programmed using the tools listed on Tabor lab website[47] with flexibility for steady-state, dynamic, or advanced LPA programming[31]. Furthermore, electronic files can be saved for future use for technical replicates or experimental induction of alternative cell lines. (**B**) LPA device with main components viewed from the top and side (**C**). (**D**) Images of foil-sealed plates to reside on LPA. LPA - Light Plate Apparatus, LED - light-emitting diode. Elements of this figure have been modified from Guinn (2019)[11].

Figure 3: Calibration of the light plate apparatus. (**A**) Representative image of LPA for LEDs set to 2000 g.s. based on IRIS software. (**B**) Image from panel (A) with background subtracted and threshold used to binarize the image to calculate pixel intensity of each LED. (**C**) Histogram of pixel intensities (determined by MATLAB image analysis) of 96 LEDs (4 LPA plates) set to the same IRIS software value (e.g., 2000 g.s.). The red line represents mean LED pixel intensity, and CV in

the panel represents the coefficient of variation for the 96 wells. (**D**) Heatmap showing the LED intensities of the LPA image in panel (A) normalized to the maximum intensity LED. (**E**) Calibration value heatmap determined for the LPA image in panel (A) to create equal intensity production for each LED when programmed for the LPA. LPA - Light Plate Apparatus, LED - light-emitting diode, CV - coefficient of variation.

Figure 4: Randomization of LPA and light toxicity effects. (**A**) Representative image of two programmable LPA LED configurations set from 25 to 4000 g.s, based on IRIS software. The first configuration is more likely to allow systematic errors such as edge-effects of heat and light to cross over (e.g., the lower row of the LPA affecting the 2nd lowest row). The second image randomizes the location of intensities, therefore minimizing systematic error (bias) caused by edge effects. (**B**) Matrix showing randomization location for light intensities in the LPA shown in panel (A), which can be used to determine the intensity-dependent results of a given experiment. (**C**) Flow cytometry data for two different light intensities (1250 and 4000 g.s.) for 3 days of induction at continuous illumination. The black gate is based on uninduced cells. (**D**) Bar graph showing flow cytometry data such as panel (C) but for a wide range of light intensities. LPA - Light Plate Apparatus, FSC - forward scatter, SSC - side scatter, g.s. - light intensity value measured in grayscale. LITer cells[11] had a dose-responsive decrease in cell survival that was statistically significant using ANOVA 1-tailed test with a p-value of 0.0022.

Figure 5: Representative microscopy images of engineered optogenetic cell lines. Cells were imaged on an inverted microscope with a camera (14-bit) for acquiring phase contrast and fluorescence imaging. Cells were exposed for 5 ms for phase contrast (**Panel A**, representative bright-field image) and 50 ms of light source for GFP/FITC acquisition (**Panel B**) at 100% light source intensity. Cells can be imaged at various time points depending on desired steady-state acquisition or dynamic response, leading to a steady state. Cells here represent a pulse intensity titration at fixed intensity (1000 g.s.) ranging from no light exposure to 3 days of light exposure. The unit g.s represents a light intensity value measured in grayscale. This figure has been modified from Guinn (2019)[11].

Figure 6: Flow cytometry of engineered optogenetic cell lines. Cells were analyzed on a BD LSRFortessa flow cytometer, with approximately 10,000 cells collected within an SSC-FSC (side scatter-forward scatter) gate. The cells were then analyzed with the FCS Express software. Histogram or scatter plots could be generated to quantify the expression of the gene of interest (e.g., GFP). (**A**) Engineered cells plotted on SSC-FSC axes for gating within the black line. (**B**) Representative LITer cells induced with varying duration of light pulses at 1000 g.s. up to 72 h of induction, illustrating dose-response of gene expression. (**C**) Representative dose-response data for light intensity titration comparing the LITer gene circuit (TIP-based system) versus VVD or LightOn system[40], which is a positive regulation system with no feedback. (**D**) Histogram corresponding to the VVD system, illustrating wider distribution (and therefore higher gene expression noise) compared to the LITer system. CV is the coefficient of variation, a metric for gene expression noise. FSC - forward scatter, SSC - side scatter, FITC - fluorescein isothiocyanate, g.s. - light intensity value measured in grayscale. This figure has been modified from Guinn (2019)[11].

Figure 7: Quantitative real-time PCR of engineered optogenetic cell lines. Representative data showing that multiple gene expression levels can be induced and quantified using light as a stimulus. The unit Rq represents relative quantification of expression fold-change compared to control. LITer cells[11] had a dose-responsive increase in RNA expression that was statistically significant using ANOVA 1-tailed test with a p-value of 0.0352 and 0.0477 for GFP and KRAS levels, respectively. This figure has been modified from Guinn (2019)[11].

Figure 8: Immunofluorescence of engineered optogenetic cell lines. Representative data showing protein-level estimates based on immunofluorescence. (**A**) Protein levels of KRAS(G12V) and (**B**) protein levels of phosphorylated-ERK, which the LITer gene circuit induces according to increasing light amounts directly and indirectly, respectively. Data shown in bars are mean values, error bars are standard deviation (n = 3). A.u. - arbitrary units, g.s. - light intensity value measured in grayscale. LITer cells[11] had a dose-responsive increase in protein levels that was statistically significant using ANOVA 1-tailed test with a p-value of 2.79E-04 and 0.016 for KRAS and phosphorylated-ERK levels, respectively. This figure has been modified from Guinn (2019)[11].

DISCUSSION:

Readers of this article can gain insight into the steps vital for characterizing optogenetic gene circuits (as well as other gene expression systems), including 1) gene circuit design, construction, and validation; 2) cell engineering for introducing gene circuits into stable cell lines (e.g., Flp-FRT recombination); 3) induction of the engineered cells with a light-based platform such as the Light Plate Apparatus (LPA); 4) initial characterization of light induction assays via fluorescence microscopy; and 5) final gene expression characterization with a variety of assays, including flow cytometry, quantitative real-time PCR (qRT-PCR), or immunofluorescence assays.

Additionally, the methods outlined above are highly modular for essentially any gene circuit architecture expressing genes-of-interest, with the main potential modifications to the protocols at the circuit construction step and the assays conducted. In the case of gene circuit construction, the flexibility of molecular cloning allows any gene of interest to be exchanged or co-expressed with a fluorescence marker with minor modifications to primer design or assembly protocols. Additionally, while the procedures outlined here focus mostly on a NF gene circuit design for precise (low-noise) gene expression control, other architectures such as positive regulation or positive feedback (PF) can be implemented to achieve different features such as high-fold change or high gene expression noise, respectively[56,57]. Using a variety of gene circuit architectures (e.g., PF, NF, etc.) can allow researchers to explore diverse biological questions such as the roles protein magnitudes and noise play in drug resistance or metastasis[58]. The protocols listed here also focus on various ways of gene expression quantification, but any number of functional assays (e.g., cell motility, wound-healing, proliferation, etc.) can be added after microscopy acquisition with little to no effect on the preceding methods. This is especially relevant to single-cell studies where optogenetics can use spatiotemporal induction to study behaviors such as pulsatile expression dynamics[59].

Complementing method modularity, troubleshooting is another important feature of each main protocol. For circuit construction, the main area of variability resides in the appropriate circuit-specific primer design, while later methods such as assembly and plasmid amplification via bacterial growth are more standardized and typically include their own extensive troubleshooting procedures. Cell engineering can be modified with drug titration curves depending on the cell line used. Additionally, if a commercial cell line is used, troubleshooting guides can be obtained directly from the cell line manufacturer. Also, unintended light effects are important to quantify on the engineered cell lines. For example, due to the phototoxic effects of 470 nm light, a light intensity curve should be created to investigate the induction of death or damage in a population. Once a variety of light values are tested, users can create an FSC-SSC gate or utilize a method such as propidium iodide staining to quantify dead/damaged cells, and therefore, serve as a tool to determine appropriate light ranges to use experimentally.

For LPA troubleshooting, LEDs can produce edge effects of light and heat crosstalk with adjacent wells. For example, high-intensity wells may cause some induction in adjacent wells if there is an improper seal or a large heat/light gradient between wells. This can be maximized if the LPA is programmed in a particular order (e.g., ascending/descending) of a light parameter. To combat this, the IRIS software allows users to employ a randomization matrix to randomize the well intensities and therefore reduce systematic error. For troubleshooting aspects of fluorescence microscopy, exposure time, gain settings (controlling camera sensitivity), and light source intensity are the main parameters that can be adjusted. Tuning these parameters can allow for optimal image production and ideal signal-to-noise ratios as well as avoiding oversaturation and phototoxicity.

For flow cytometry troubleshooting, photomultiplier tube voltages and cellular gating are the main aspects that can be adjusted. Tuning these parameters can allow ideal comparisons between experimental conditions and control samples, proper signal acquisition for both weak or strong fluorescence signals, and exclusion of cellular debris or unwanted cell populations. It should be noted that flow cytometry voltages are ideally kept constant across experiments to allow proper comparisons. Additionally, for optogenetic systems requiring multiple fluorescence outputs (e.g., FITC and PE), users will need to be aware of fluorescence compensation given crosstalk between fluorophores. In such scenarios, controls are crucial for determining proper fluorescence signals. Controls that will be necessary for users to perform include fluorescent beads, stained cells with the marker of interest, or cells constitutively expressing the fluorescence protein that can be used for creating a compensation matrix in the flow cytometry software of interest. Additionally, whether using one fluorescence marker or more, all users should routinely measure fluorescence signals of each marker for non-fluorescent cells, which yield autofluorescence/background signals. qRT-PCR troubleshooting includes varying cDNA amounts and probe design, which are often project-specific. Lastly, for immunofluorescence troubleshooting, antibody concentrations are the biggest variable for assay quantification, necessitating optimal concentration determination.

One troubleshooting aspect that is relevant to most of the above methods is the isolation effect of using a sealed plate with cells covered with foil. This method may impede the carbon dioxide

gas exchange needed for the proper growth of various cell lines. From previous experimental experience, this was not a significant impediment to cellular growth for a 3–5 day experiment using the engineered cell lines within this manuscript but may become important for other cell lines or experimental durations. To address this concern, one adaptation implemented with the sealed plates includes using carbon dioxide independent media, which has not affected the growth of cells used in this study. It should be noted for other assays or cell lines where metabolism, pH levels, and cell densities are important, other interventions may be needed such as changing media or nutrients more frequently.

The limits of the methods outlined here reside in the gene circuit used, the optogenetic technology precision, and LPA interface with other equipment such as fluorescence microscopes. The technology described here is affordable, quick to build, and easy to validate[46] for populations of optogenetically engineered cells. However, there are certain spatial limitations, such as the inability to control single cells without modifications to the light induction setup, such as utilizing digital mirror devices (DMDs), which has recently demonstrated beneficial applications in living cells[60,61]. Furthermore, the methods outlined here are limited in live-cell tracking during optogenetic stimulation, allowing only for the end-point data acquisition of the entire experimental time course.

Despite these limits, the optogenetic methods outlined here are complementary to the existing technology such as the DMDs, which have the flexibility to control single cells in real-time but are limited by the number of samples one can stimulate. Furthermore, the stable cell line engineering methods discussed here contrast existing synthetic biology methods which often characterize systems via transient transfections. Transient transfection approaches are inherently noisier due to the greater gene circuit copy number variation among cells. In contrast, the methods presented here involve monoclonal cell variants, each containing one gene circuit copy. Stable cell lines allow exploring cellular aspects such as gene expression variation, protein level effects on phenotypic landscapes, and single-cell methods with finer precision since there is higher confidence that each cell is identical to its neighbors.

The work here demonstrates a platform for designing any optogenetic gene circuit of interest, inducing such systems with reliable experimental tools, and characterizing them with a variety of gene expression and functional/phenotypic assays in a standardized manner. Future improvements of these protocols may include the integration of these methods with live-cell tracking using other optogenetic technologies such as the DMDs, which will allow spatiotemporal control of gene expression and functional applications at the single-cell level. Such advances will allow light utilization to produce the gene expression pattern of interest in single cells, establish transcription/translation dynamics, quantify protein levels, and study their role in diverse biological processes, including cell migration, proliferation, metastasis, and differentiation.

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DISCLOSURES:

The authors declare no competing financial interests.

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Figure 1

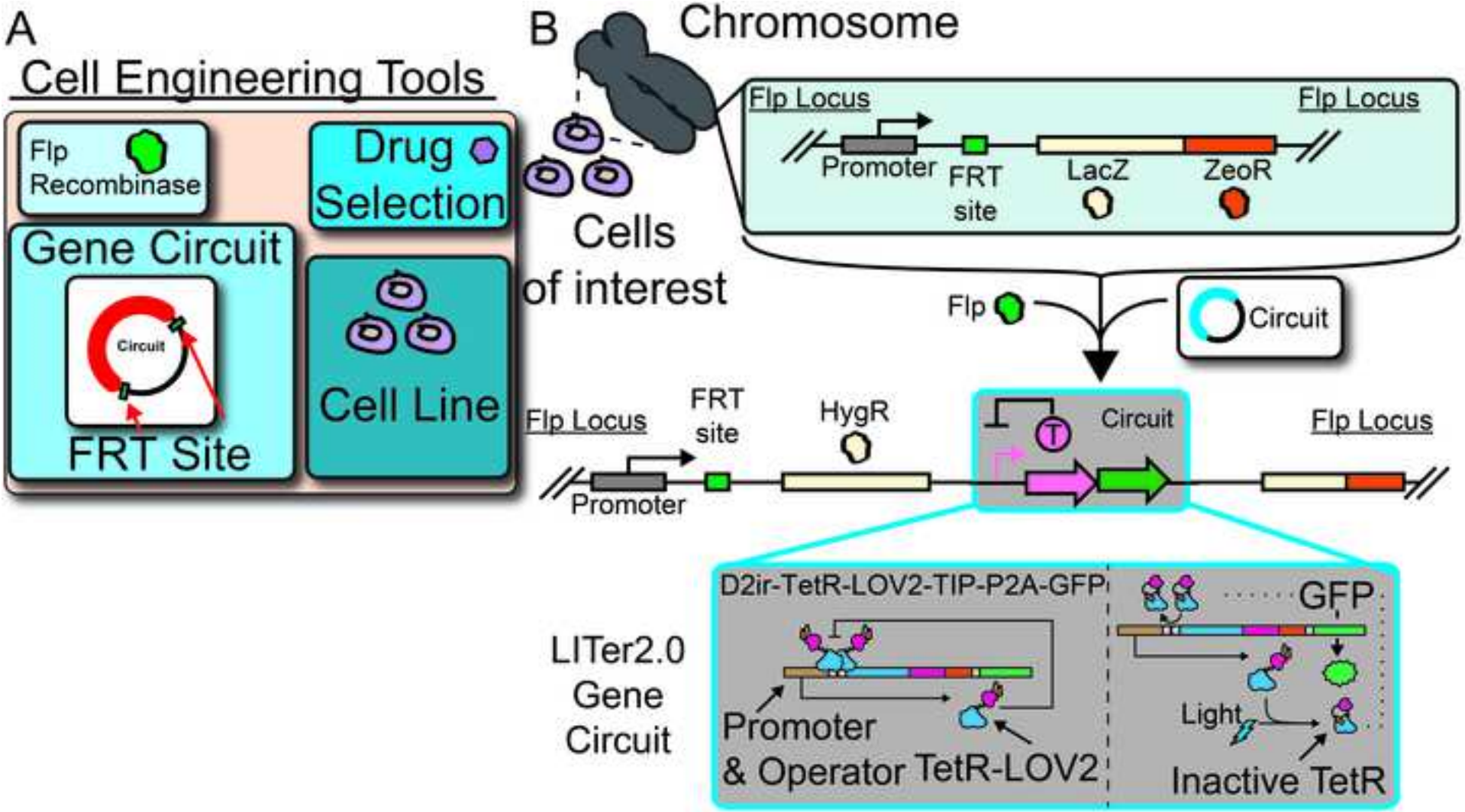


Figure 2

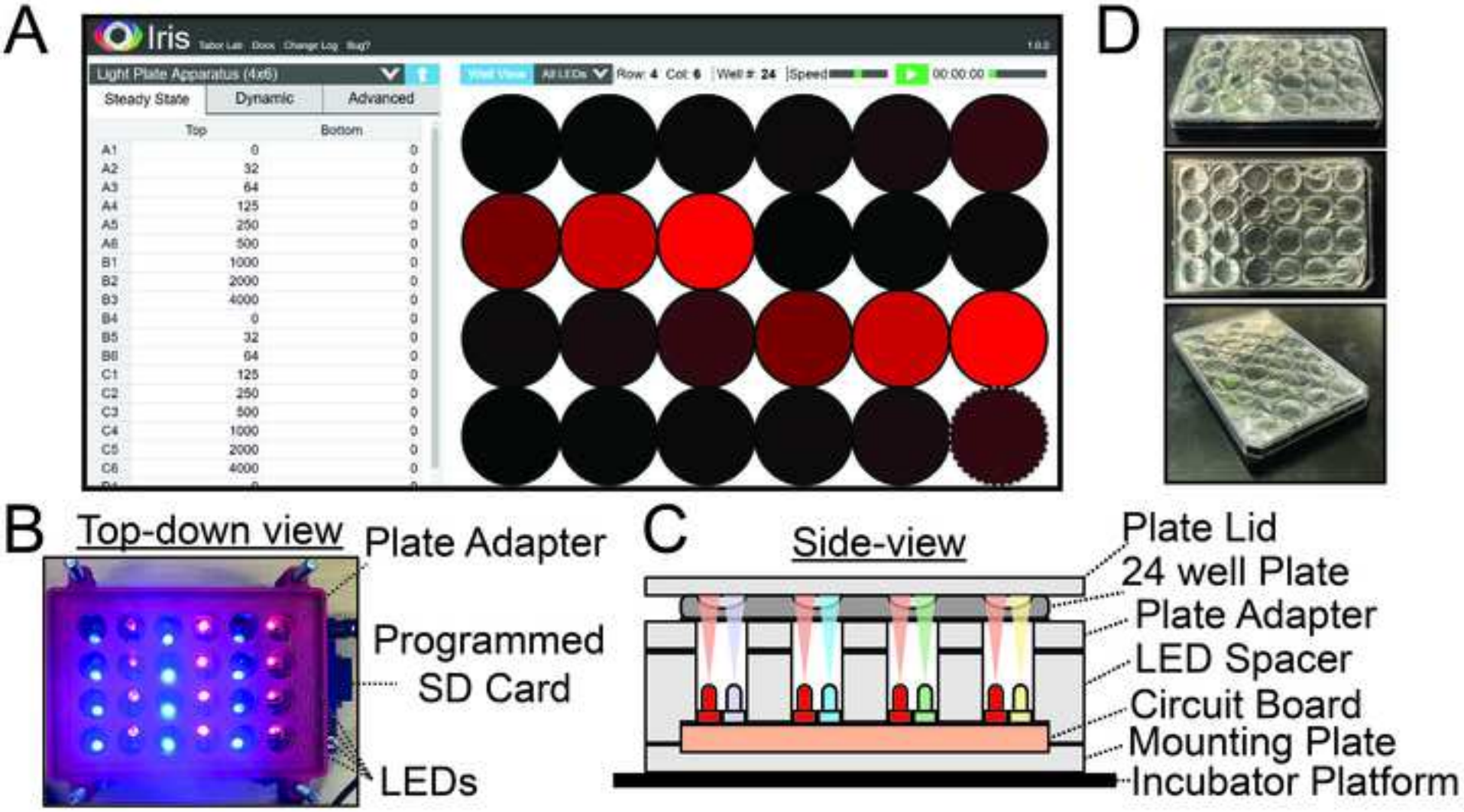


Figure 3

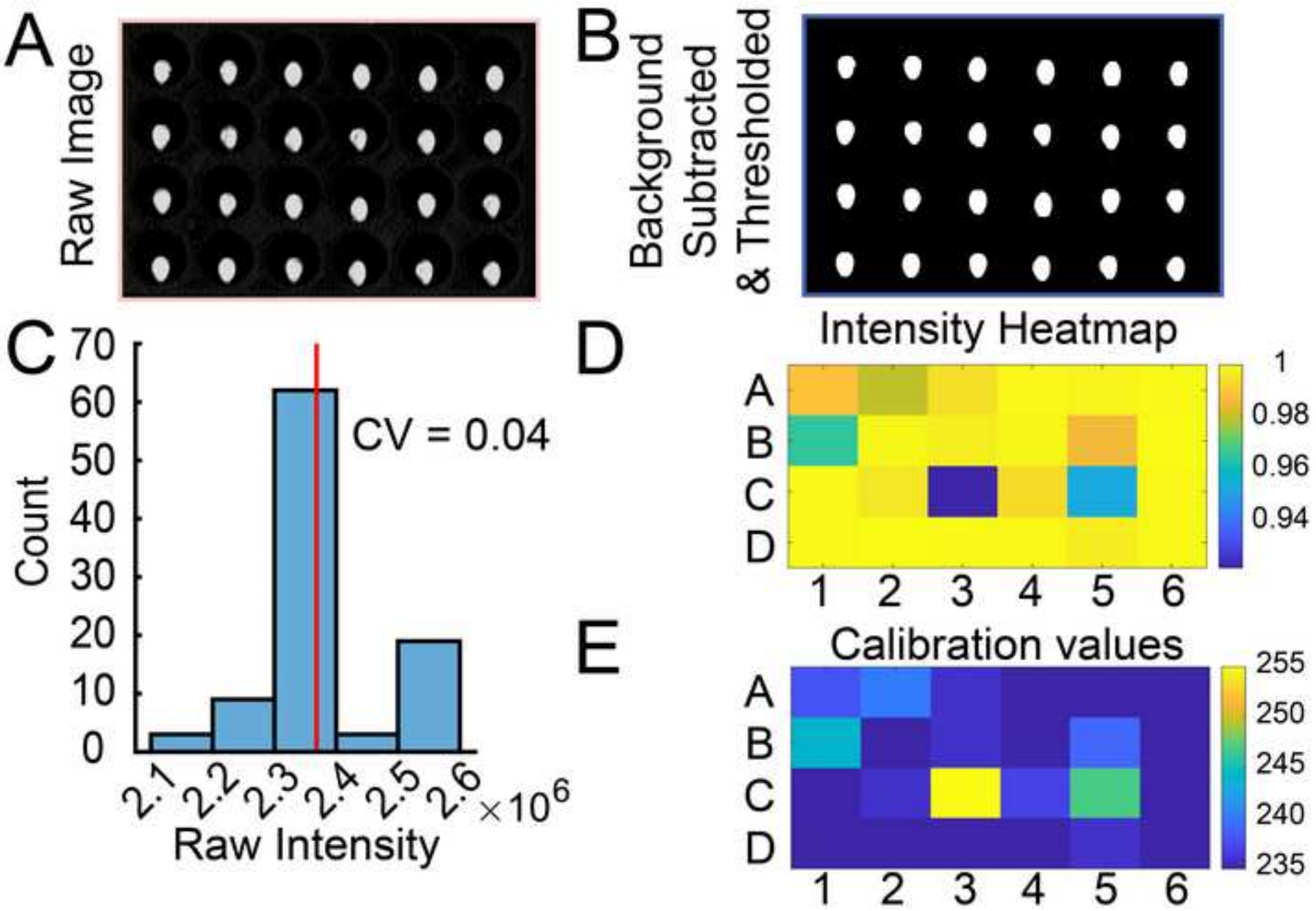
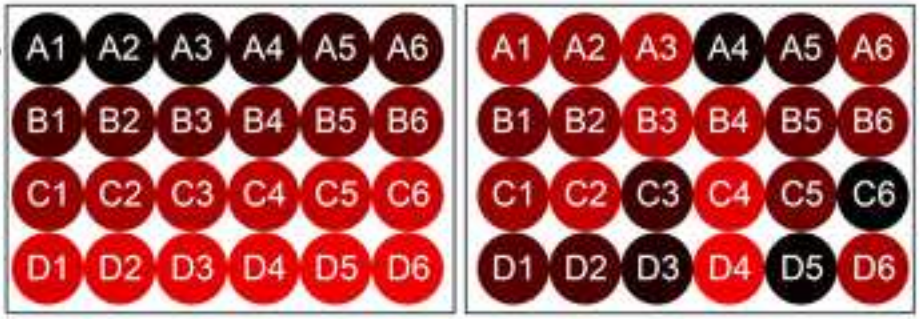


Figure 4

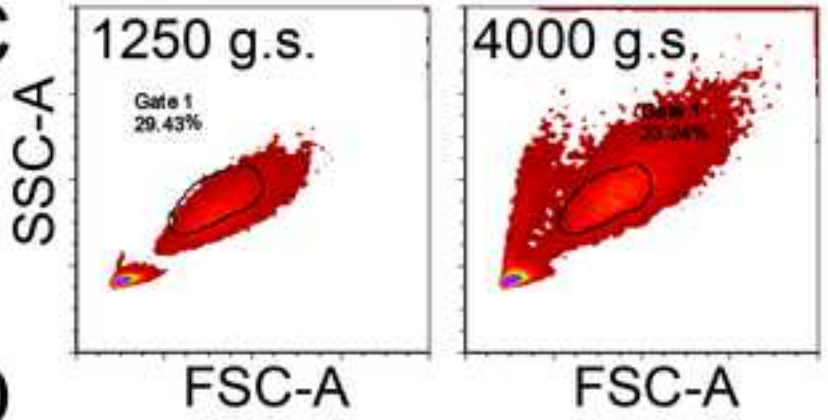
A



B

Plate Well Index	Plate Location	Descrambled Well Index (Randomization Matrix)	Light Intensity
1	A1	16	2000
2	A2	14	1500
3	A3	20	3000
4	A4	3	100
5	A5	5	300
6	A6	13	1250
7	B1	18	2500
8	B2	12	100
9	B3	21	3250
10	B4	19	2750
11	B5	9	700
12	B6	11	900
13	C1	15	1750
14	C2	22	3500
15	C3	6	400
16	C4	23	3750
17	C5	10	800
18	C6	1	25
19	D1	8	600
20	D2	7	500
21	D3	4	200
22	D4	24	4000
23	D5	2	50
24	D6	17	2250

C



D

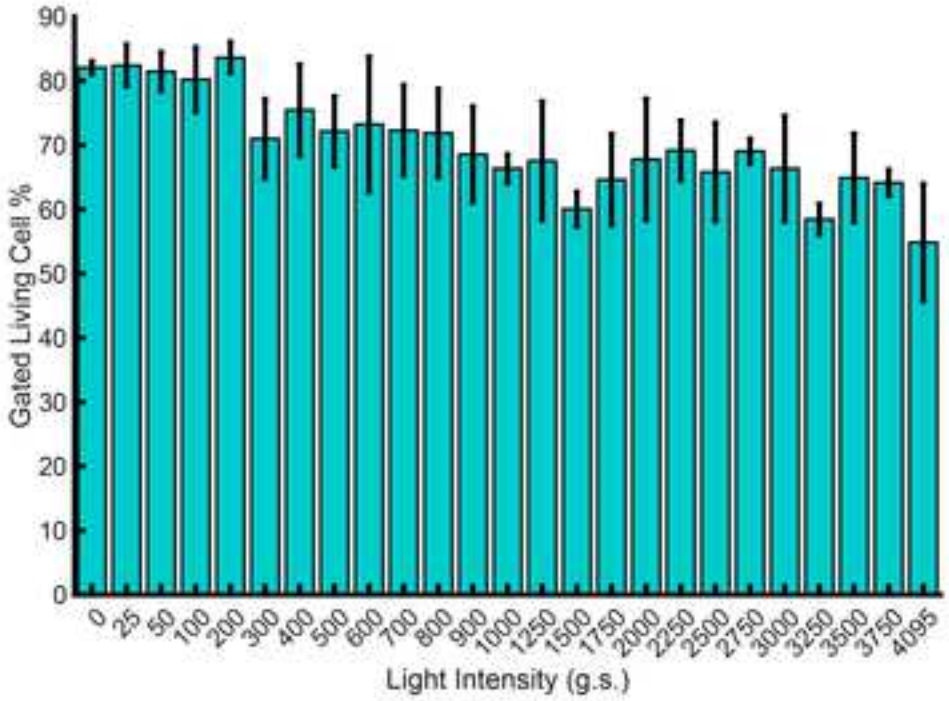


Figure 5

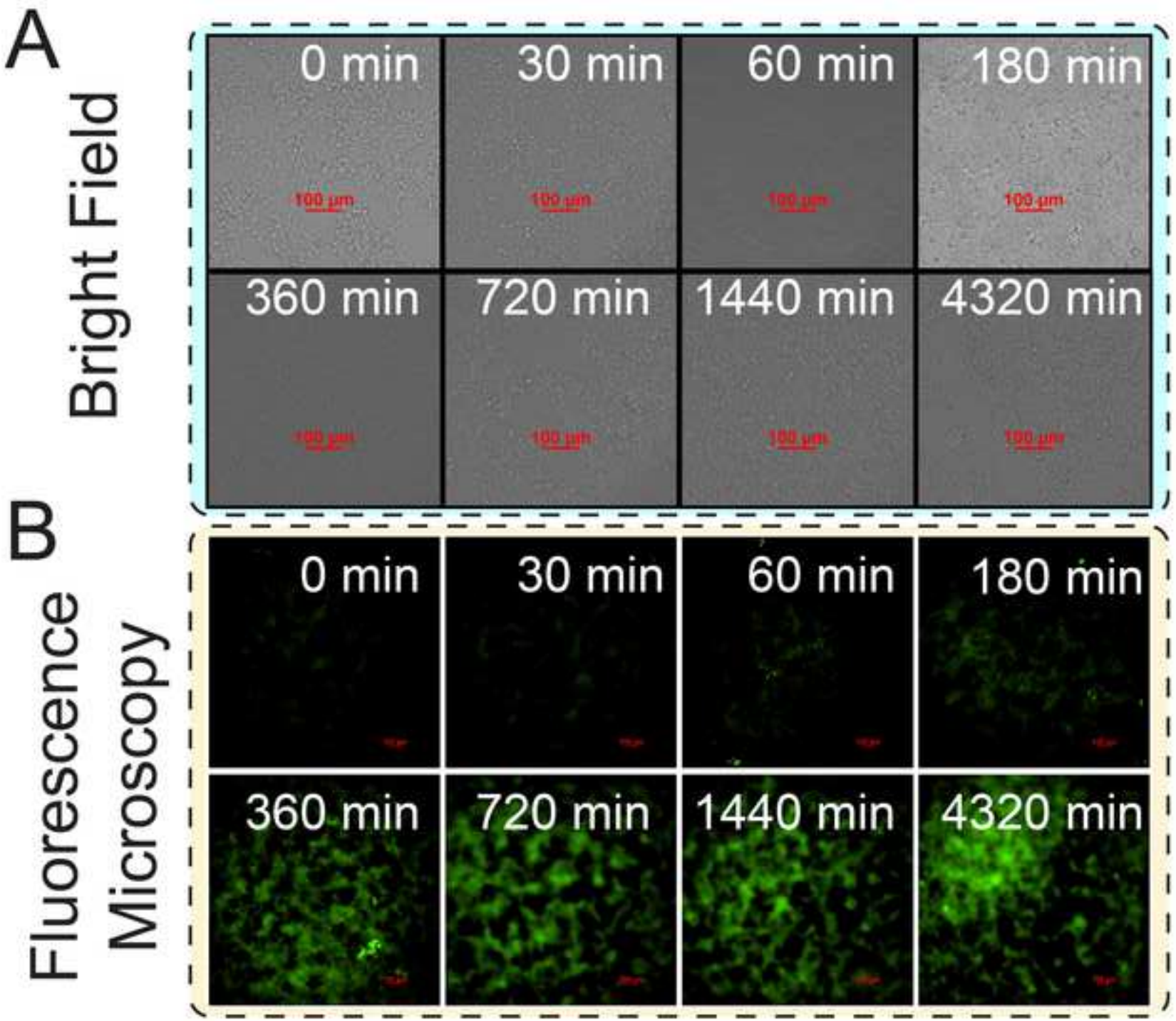


Figure 6

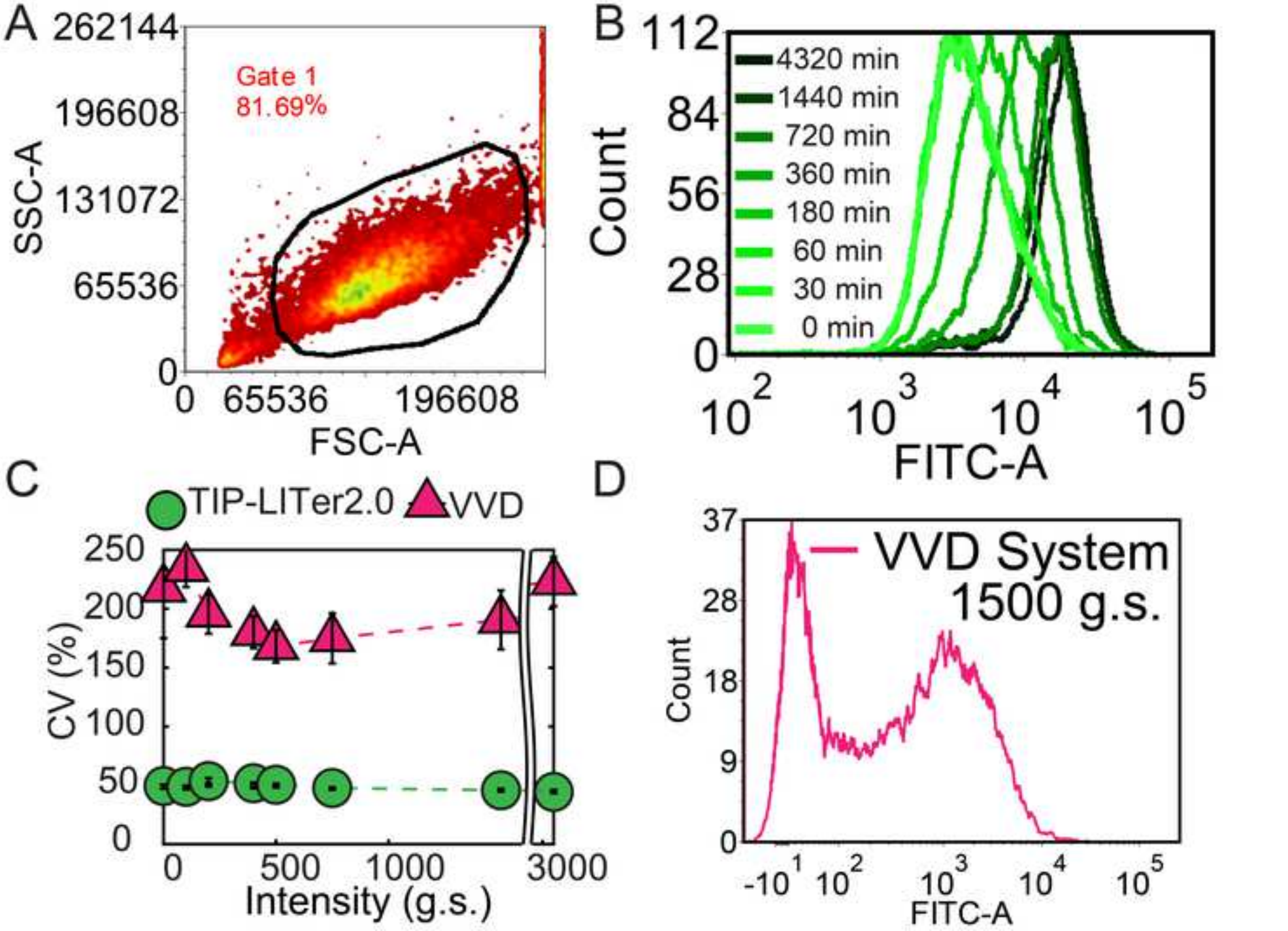


Figure 7

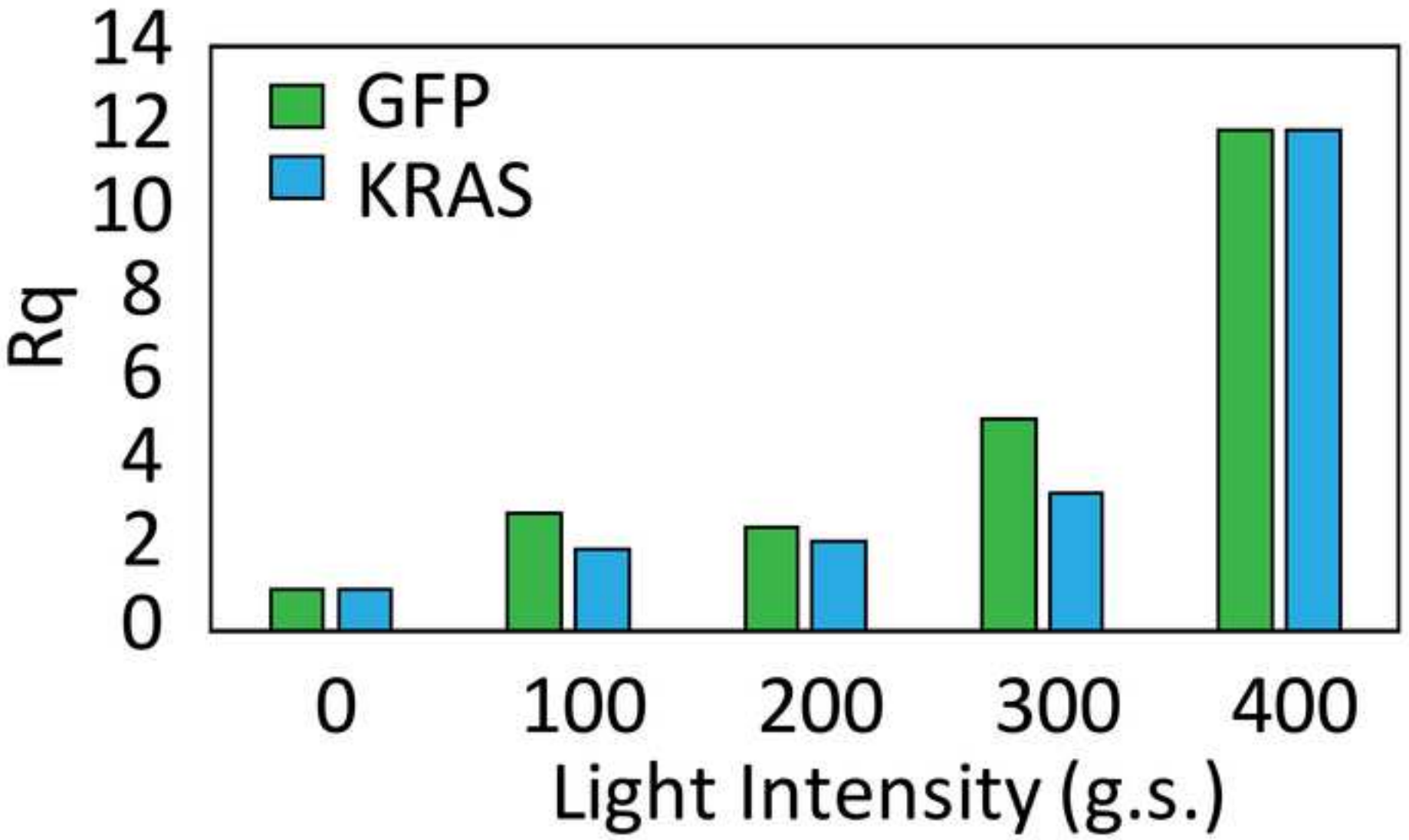


Figure 8

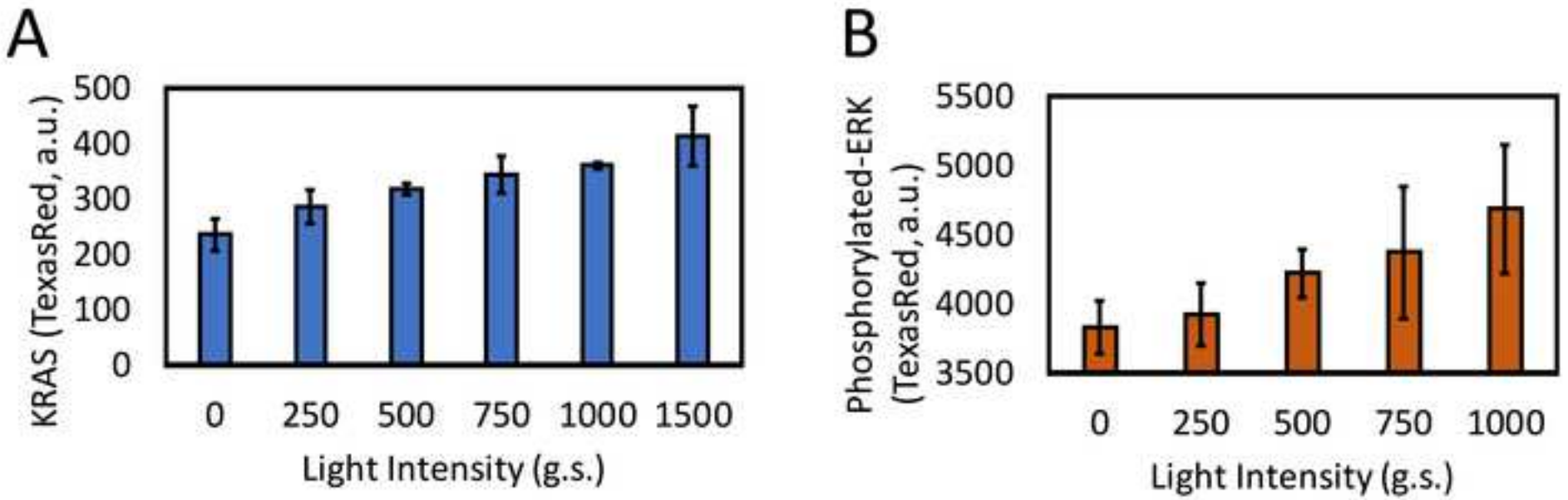


Table 1.

Fragment	Ratio	Size (bp)	DNA fragment weight concentration (ng/µL)	DNA fragment molar concentration (fmol/µL)
Mother Vector fragment 1	1	3414	18.2	8.626608476
Mother Vector fragment 2	1	4642	18.1	6.309684121
Gene of Interest	1	549	37.9	111.7018505
Total				

Volume (µL)	Resulting DNA molar mass (fmol)
4.09093293	35.29087669
5.593128913	35.29087669
0.315938156	35.29087669
10	105.87

Table 2.

0	100	200	400	500	750
1500	3000	0	100	200	400
500	750	1500	3000	0	100
200	400	500	750	1500	3000

Table 3.

	Sample 1	Sample 2	Sample 3
Reverse Transcriptase Master Mix Volume (µL)	4	4	4
RNA Template (1000 ng) Volume (µL)	2	3.003003	4
NF-H_20 Volume (µL)	14	12.996997	12
Total Volume (µL)	20	20	20

Table 4.

	Sample 1	Sample 2	Sample 1 & 2 Multiplexed
GFP Probe Volume (µL)	1	-----	1
GAPDH Probe Volume (µL)	------	1	1
DNA polymerase Master Mix Volume (µL)	10	10	10
cDNA (100 ng) Volume (µL)	2	2	2
NF-H_20 Volume (µL)	7	7	7
Total Volume (µL)	20	20	20

Reviewers' comments:

Reviewer #2:

Manuscript Summary:

The protocol "Reliably Engineering and Controlling Stable Negative Feedback Optogenetic GeneCircuits in Mammalian Cells" describes the construction of optogenetic circuits, their integration into stable cell lines and discusses the technical setup and protocols for light-induction. It also gives guidelines for the characterization of light-inducible cells. The article is well written and will be a very useful contribution and guideline for research groups that have no or little experience with optogenetics. The abstract and introduction now clearly state the content as well as relevant information, and our previous major and minor concerns have been fully addressed. We recommend the article for publication.

We thank Reviewer 2 for the opportunity to improve this work and share the findings with the other research groups.

Reviewer #3:

Manuscript Summary:

In this manuscript the authors provide a detailed end-to-end protocol that will aid others who wish to apply optogenetics in their synthetic biotechnological research. They cover the important steps in this process, from design to biological implementation to experimentation. Particularly commendable is the attention they pay to calibration and measurement, as these are often difficult to standardize in optogenetic systems and between laboratories and many studies priv. The authors also discuss the potential benefits of negative feedback for optogenetic systems, and present some data to support this. The manuscript has previously been through review, and the authors have effectively addressed most of the reviewers' critiques. My comments below are therefore mostly minor, addressing a few outstanding concerns.

We thank Reviewer 3 for the comments and opportunity to improve this work with the listed suggestions. We have attempted to address both major & minor concerns below.

Major Concerns:

1. My only major concern is one that echoes points previously raised by both reviewers. Namely, the manuscript's primary goal seems to be an outline of experimental processes for application of optogenetics in Synthetic Biology. However, the authors then go on to spend a significant amount of space in introductory sections expounding on the potential benefits of negative feedback circuits (largely re-stating conclusions from their previously published work). Overall I do not believe this split message _significalty_ detracts from the manuscripts goals, but it was potentially confusing for a first-time reader of their work. For example, as I read I was waiting for a section on experimental design/tuning/realisation of negative feedback systems which never came (aside from a brief mention of promoter design on line 131).

   Perhaps to address this point the authors might reconsider how they present the key motivations of their work in the Introduction. Specifically, rather than focusing on negative feedback as an important regulatory feature in and of itself (which they do not subsequently develop further), it could be mentioned more as a "means-to-an-end", i.e. as a method via which they will demonstrate their optogenetic protocol's ability to measure cell-to-cell variability. In essence, the underlying message of the introductory sections could be changed from (excuse my paraphrasing) "Optogenetics is useful. Also, negative feedback is useful." to something more like "Optogenetics is useful. Here we demonstrate this with a negative feedback system."

   a. Thank you for this comment. Indeed, the focus is on the optogenetic methods with the NF system serving as an example for potential applications and the work would benefit from rephrasing this. We have attempted to rephrase the Introduction to give more focus to the optogenetic method, showing that the NF system is one particular example to demonstrate this, but that the methods can be used for other optogenetic gene circuits.

Minor Concerns:

1. If the authors elect to leave significant discussion of the properties/benefits of negative feedback in their manuscript, some of this should be tightened up. For example, negative feedback alone does not counteract "any" (line 67) change from setpoint and take the expression level "back to the setpoint" (line 69) unless the control system is perfectly adapting (i.e. integral control), which is typically not achieved by simple negative autoregulation architectures. That is, negative autoregulation does _reduce_ variability,

but it does not eliminate it entirely. As another example, the paragraph beginning Line 66 might highlight some of the tradeoffs inherent in negative feedback (i.e. it can also reduce absolute expression levels, fold-change of output, change response/rise time, etc).

   a. Thank you, we adjusted the Introduction to deemphasize NF. Additionally, we changed the paragraph mentioned in this comment, referring deviations from steady-states.

2. There have been some recent studies on optogenetic control of noise which the authors would do well to cite (for example: http://dx.doi.org/10.1038/s41467-018-05882-2) given this closely parallels their work and may indeed represent an interesting future application for their light plate.
   a. Thank you for your suggestion. This and other references to recent works in noise controlling optogenetic gene circuits were added to the Introduction and Discussion.
3. Initial steps in the protocol (i.e. 1.1, 1.2) regarding design of gene circuits might benefit to other protocol or review papers that provide greater detail. Given that genetic design is not the core message of this protocol it seems reasonable to leave these sections quite tearse, but it would be beneficial to include references that would provide the reader further background (if they are interested).
   a. This is a good point and we have adjusted steps 1.1 & 1.2 to be briefer with appropriate references for more background readers may wish to explore.
4. Following from the above, step 1.3 might include additional references. The instruction "fuse a domain to the repressor" could be interpreted in many ways (and if done incorrectly would lead to a non-functional system!). Are there any citations (even to the author's past work) that could be included here for the interested (or uninformed) reader?
   a. Thank you for pointing out this ambiguous point. We have attempted to clarify this step and also added additional references that may be of interest to readers wishing to learn more on the topic.
5. Step 3.5.2 - it might be useful to tell the reader exactly what "changes [that] occur with variation in light parameters" (line 343) they should be looking for, and how these observed changes can thus be used to "determine the ideal range for the experimental setup" (Line 344). Admittedly some of this is subsequently discussed around Line 723, but perhaps some of these features would better be mentioned earlier.
   a. Thank you for pointing out this protocol point that can be better specified. We have attempted to clarify the main changes to look for, including cell survival, and have listed other features that may be of interest to readers.
6. Following the above, it would be worthwhile to carefully read the manuscript and add detail/citations to the (many) steps where reference is made to other/standard laboratory techniques. As another specific example, Line 133 starts with "Proceed to primer design (minimum 2)." Minimum 2 what? Primers?
   a. We have attempted to address this comment by adding citations for the principles of gene circuit design, molecular cloning techniques, and cell culture. Additionally, we clarified line 133 by changing it to: “Design primers for DNA sequence amplification or sequencing of the plasmid using the molecular cloning software for each gene circuit.”

Reviewer #4:

Manuscript Summary:

The methodology of constructing a robust light driven synthetic negative feedback loop gene circuit in mammalian cells is introduced. Step by step introduction to gene circuit design, cell line engineering, light induction and fluorescence detection are given. Examples of the protocol use are presented on several figures.

The original MS was carefully evaluated by two referees. Their major concerns all have been addressed by the authors in this resubmitted version. These changes greatly improved the MS, especially in introducing the whole concept and background to optogenetics.
I do not have further concerns and suggest the acceptance of this revised version.

We thank Reviewer 4 for the comments and opportunity to share the findings with the other research groups.

Reviewer #5:

Manuscript Summary:

This methods article provides steps for creating and characterizing optogenetic gene circuits for controlling downstream functional genes of interest. The paper outlines detailed steps for generating mammalian cell lines with stable integration of the gene circuit, constructing a high-through light induction device and characterizing the constructed cell line using different measurement techniques. The entire pipeline is demonstrated with a previously published gene circuit by the authors. The protocol is well written and will be a useful tool for a broad range of users. Particularly, researchers looking to adopt optogenetics for noise control will find this paper helpful for quickly getting up to speed. However, the following points should be addressed.

Major Concerns:

1. Users might not a priori know for what range of the induction light intensity the light-sensing domain exhibits a monotonic response. If the users desire a monotonic response, as is the case most of time, it will be instructive if the authors include brief steps for identifying this range. I assume this range of intensity values will be a factor affecting the LPA calibration step contingent upon the experimental requirements and the properties of the light-sensing domain. The authors previously observed a non-monotonic response for the VVD system in their original LITer publication (reference 18). Maybe they can briefly reproduce their strategy in that paper here.
    a. Thank you, this is a great point that is not discussed much in literature. To address this point, we have added the following section in the protocol section to help readers approach this issue in their own optogenetic system:
    b. “3.5.4 In addition to restricting light values because of toxicity, users may wish to restrict light values to characterize a particular part of the dose-response, such as the range of monotone response. To achieve this, users should initially scan a wide range of light intensities, pulse durations or duty cycles depending on the modality being analyzed when determining the desired dose response (Table 2) to narrow in on the light regime of interest, e.g., where gene expression correlates positively with increasing light values for a monotonic dose-response. To determine this light range of interest, users should program an LPA with 24 light conditions for a single plate or more conditions (e.g. 48, 72, 96, etc.) depending on if multiple LPAs can be calibrated to deliver equivalent light amounts and proceed with the cell culture work or assays outlined below. Therefore, users should always start characterization of an optogenetic system with a wide dose-range of light stimuli to determine the range interval that gives the desired gene expression, and subsequently perform experiments in that refined dose range. For example, in this work once 3000 g.s. units was determined as the threshold for toxic light intensity, this threshold was used as the upper bound of light for assays outlined below (e.g. immunofluorescence).
    c. NOTE: These steps above are independent of the optical calibration of the LPA and refer to a molecular-level calibration for each optogenetic system.”
    d. Regarding calibration, in our experience the optical and molecular LPA calibration are two independent, important and necessary calibration steps. More specifically, the optical calibration of the LPA is performed independently of the biological system used, where calibration results in the intensity of light from one LED (well) to match the intensity of light from another LED (well) as long as both LEDs are programmed to output the same intensity. Optical calibration is required initially, and later occasionally if changing the actual LEDs. On the other hand, the dose-

response calibration in our experience has to be done for each new optogenetic system tested.

2. Negative feedback based genetic circuits, LITer in particular, are presented as systems for precise control of mean expression with low gene expression noise. The representative results and the original LITer publication (reference 18) demonstrate this in comparison to a positive regulation system like VVD. However, another aspect to gene expression control could be increasing expression heterogeneity rather than decreasing noise. Increased expression noise is relevant for many biological processes such as fate decisions in viruses, development in stem cells or drug resistance in cancer. The LITer system or other feedback topologies can potentially be combined with bursty promoters to precisely control (increase or decrease) expression noise, in addition to the mean, by using the protocol presented in this paper. I suggest that the authors expound on this in the discussion section to broaden the scope for application of the presented method.
    a. Thank you for this valuable suggestion. We have adjusted the discussion to have a more developed section on this topic: “Additionally, the methods outlined above are highly modular for essentially any gene circuit architecture expressing genes-of-interest, with the main potential modifications to the protocols at the circuit construction step and the assays conducted. In the case of gene circuit construction, the flexibility of molecular cloning allows any gene of interest to be exchanged or co-expressed with a fluorescence marker with minor modifications to primer design or assembly protocols. Additionally, while the procedures outlined here focus mostly on a NF gene circuit design for precise (low-noise) gene expression control, other architectures such as positive regulation or positive feedback (PF) can be implemented to achieve different features like high-fold change or high gene expression noise respectively. Using a variety of gene circuit architectures (e.g. PF, NF, etc.) can allow researchers to explore diverse biological questions such as the roles protein magnitudes and noise play in phenomenon such as drug-resistance or metastasis. The protocols listed here also focus on various ways of gene expression quantification, but any number of functional assays (e.g. cell motility, wound-healing, proliferation, etc.) can be added after microscopy acquisition with little to no effect on the preceding methods. This is especially relevant to single-cell studies where optogenetics can use spatiotemporal induction to study behaviors such as pulsatile expression dynamics.”

Minor Concerns:

1. Coefficient of variation (CV) is mentioned as a 'measure of noise'. However, readers unfamiliar with noise biology research might be confused by this. Consider giving the exact definition of CV (= σ/μ). Standard deviation and mean should be known to the community at large.
    a. Thank you, we added the definition of CV in the Introduction.
2. Was a live stain, such as propidium iodide (PI), used for the flow-cytometry experiments or only forward and side scatter were used to gate for live cells?
    a. In this study, no live stain was used given the region of dead cells/debris is well known in HEK293 and its derivative FLP293, however we adjusted the protocol to state using propidium iodide as a suitable alternative for calculating the cell survival percentage in a population.
3. On line 355, what image analysis method is used to perform the calibration step? Are steps 3.7.1 to 3.7.3 the aforementioned analysis method? If yes, please make this explicit

in step 3.7. Maybe something like, '...(here an image analysis method (3.7.1-3.7.3) is used)...'.

   a. Thank you for pointing out this ambiguity. We have adjusted the protocol, so it now reads as follows: “calibrate the LPA using an Image analysis method (steps 3.7.1-3.7.3).”

4. On lines 361 and 362, it should be '...and the reset button (physical button on LPA) pressed...'.
   a. We have corrected this statement so it is clear that the reset button must be pressed in order to start the LPA.
5. On line 398, should be '...(Figure 2D)'.
   a. We have changed the protocol as suggested.
6. On line 418, should be '...growth...' and not '...grow...'.
   a. Thank you, we have changed the manuscript as suggested.
7. On lines 447 to 451, the authors state that 'NOTE: Optimal exposure times, gain levels, and light source intensities are often derived empirically from the experience of this work...'. Further, it is stated that these parameters should be selected to maximize signal-to-noise ratios, minimize phototoxicity, minimize oversaturation of fluorescence signals, and increase the ability to amplify weak fluorescence signals. Was this multi-objective optimization performed ad-hoc or an optimization heuristic based on experimental data was used for this purpose? If the latter was used, it will be helpful to briefly mention the outline of such a heuristic.
   a. Thank you for this question. The optimization was grossly performed ad-hoc, however previous experimental values (e.g. oversaturation of fluorescence) guided future experimental settings when applicable. To clarify this, we added the following to the text: “Users can optimize these parameters grossly ad-hoc, however previous experimental values (e.g. light source intensity causing oversaturation of fluorescence reporter) can guide future experimental settings when applicable. For example, the gain settings, exposure times, and light intensity initial values from one circuit (LITer1.0) or experimental setup (e.g. light intensity) can be used a starting point when moving to a similar but different gene circuit (LITer2.0) or a different light modality (e.g. light duty cycle).”
   b. “It should be noted optimal exposure times, gain levels, and light source intensities are often derived empirically from the experience of this work to minimize oversaturation in the fluorescence reporter, minimize cellular damage, and capture adequate images for qualitative & quantitative analysis.”
8. On lines 502 and 503, it should be '...for creating this gate...' and not '...for creating this well...'.
   a. Thank you, this was changed.
9. On lines 787 and 788, Guinn and Balázsi (2020) should be reference 27 and not 8.
   a. Thank you, we corrected the citation.
10. On lines 815, 842, 852, 866 and 871, Guinn (2019) should be reference 18 and not 8.
   a. Thank you, we have corrected the citations mentioned.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
   a. Thank you, this was completed.
2. Line 27-37: Please rephrase the Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words. Consider moving lines 32-37 to the discussion.

    a. The summary was shortened and rephrased, with previous information moved to the discussion.
3. Line 230-233: Please elaborate on the step by adding more details. A citation would suffice.
    a. Thank you, a citation was added.
4. Line 258-259: Please specify the seeding density.
    a. This was clarified to indicate 1 cell per well.
5. Line 262/264: Please mention the percentage of confluency.
    a. This was clarified to indicate the percentage of confluence.
6. Line 277-278: Please ensure both the citations are relevant. Ref 23 is Nevozhay, D. and Zal, T., Balazsi, G (2013) and ref 25 is Gerhardt et al. (2016). There cannot be two reference numbers superscripted to one reference. Please revise the same in steps 3.1.1-3.1.5.
    a. Thank you, the references were changed to the correct references.
7. Line 358-359/366: Please check and insert the correct reference number.
    a. This has been corrected.
8. Line 416-425: The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.
    a. This section of the protocol was removed from the methods section and added to the discussion.
9. Line 787-788: Please include the correct reference number. The authors, year, and the reference number do not match (Figures 1,2,5, 6, 7, 8).
    a. This has been corrected.
10. Line 836-837/ 865-868/ 870-875: Please include the details of the statistical analysis performed.
    a. This has been added to the figures.
11. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in the imperative tense.
    a. Thank you, this was completed.

Noise-reducing optogenetic negative-feedback gene circuits in human cells

Author: Guinn, Michael Tyler; Balázsi, Gábor
Publication: Nucleic Acids Research
Publisher: Oxford University Press
Date: 2019-07-03

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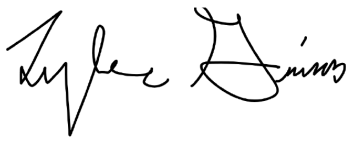

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