FINAL SCRIPT: APPROVED FOR FILMING

Submission ID #: 68779
Scriptwriter Name: Sulakshana Karkala
Project Page Link: https://review.jove.com/account/file-uploader?src=20977643

Title: Rapid Optimization of a Light-Inducible System to Control Mammalian Gene Expression

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Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes, all done**

3. **Filming location:** Will the filming need to take place in multiple locations? **Yes**

   The distance between locations is about 1 mile and is normally walked to preserve sample integrity. It's about a 3-5 min drive between locations.

4. **Testimonials (optional):** Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **No**

Current Protocol Length
Number of Steps: 26
Number of Shots: 52

Introduction

Videographer: Obtain headshots for all authors available at the filming location.

INTRODUCTION:

~~What is the scope of your research? What questions are you trying to answer?~~

1.1. **Shruthi Garimella:** This project aims to develop a high-throughput method of optimizing and characterizing an multicomponent optogenetic tool called LACE, for mammalian gene expression.

1.1.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

CONCLUSION:

~~What advantage does your protocol offer compared to other techniques?~~

1.2. **Shruthi Garimella:** This protocol enables high-throughput evaluation of samples in technical replicate using the OptoPlate to program varied light conditions and pulse frequencies.

1.2.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera.

Videographer: Obtain headshots for all authors available at the filming location.

Protocol

2. Plating HEK293T Cells in a 96-Well Glass Bottom Format for Transfection Assay

Demonstrator: Shruthi Garimella

2.1. To begin, place a cell culture flask containing HEK293T *(H-E-K-2-9-3-T)* cells inside a biosafety cabinet **[1].** Use a serological pipette to aspirate the spent media **[2]**.

2.1.1. WIDE: Talent placing a flask with HEK293T cells inside a biosafety cabinet.

2.1.2. Talent aspirating spent media from a cell culture flask using a serological pipette.

2.2. Pipette 10 milliliters of DPBS *(D-P-B-S)* to one corner of the flask and gently swirl to wash the cells **[1]**. Then aspirate the wash solution **[2]** and dispose both the DPBS and the aspirator into the waste container **[3]**.

2.2.1. Talent adding Dulbecco's phosphate-buffered saline to the flask corner and gently swirling it.

2.2.2. Talent aspirating the Dulbecco's phosphate-buffered saline.

2.2.3. Talent discarding solution and aspirator in the waste container.

2.3. Now add 1.5 milliliters of 0.05% Trypsin-EDTA to the flask to cover the surface and incubate **[1-TXT]**. Gently tap the flask to loosen the cells from the surface **[2]**.

2.3.1. Talent adding Trypsin-ethylenediaminetetraacetic acid to cover the surface of the flask. **TXT: Incubation: RT, 1 min**

2.3.2. Talent gently tapping the flask to dislodge cells.

2.4. Next, pipette 8.5 milliliters of fresh DMEM into the flask **[1]**. Aspirate the solution up and down until no aggregates are visible to resuspend the cells **[2]**. Then transfer 9 milliliters of the cell suspension into a 15-milliliter conical tube **[3]**.

2.4.1. Talent adding Dulbecco's Modified Eagle Medium into the flask.

2.4.2. Talent pipetting up and down to resuspend the cells.

2.4.3. Talent transferring cell suspension into a 15 milliliter conical tube.

2.5. Add 9 milliliters of fresh DMEM to the flask **[1]**. Incubate the suspension until confluency under 5% carbon dioxide at 37 degrees Celsius **[2].**

2.5.1. Talent adding medium to the flask.

2.5.2. Shot of the flask being placed in an incubator.

2.6. Now, using a hemocytometer, mix 10 microliters of the suspended cells with 10 microliters of Trypan blue dye at a 1 to 1 ratio to calculate cell concentration **[1]**.

2.6.1. Talent mixing suspended cells and Trypan blue on a hemocytometer.

2.7. Seed approximately 35,000 cells in 100 microliters into each well of a high-performance number 1.5 black 96-well glass bottom plate **[1]**. Place the plate into an incubator set to 37 degrees Celsius and 5 percent carbon dioxide for 24 hours **[2]**.

2.7.1. Talent seeding 100 µL of cell suspension into each well of a 96-well plate.

2.7.2. Talent placing the 96-well plate into the incubator.

3. 2pLACE Transfection and Optogenetic Activation in HEK293T Cells

3.1. For transfection, first aliquot 11 microliters of warm serum-free DMEM into a 1.5 milliliter microcentrifuge tube for one well and 10 percent excess **[1]**.

3.1.1. Talent aliquoting 11 µL warm serum-free medium into a microcentrifuge tube.

3.2. Prepare various plasmid mass ratios of CRY2 *(Cry-Two)* -enhanced green fluorescent protein to CIBN *(C-I-B-N)*-guide RNA **[1-TXT].** Aliquot 110 nanograms per well of the prepared solutions into each tube containing the serum-free medium aliquots **[2]**.

3.2.1. Shot of the labeled prepared plasmid mixtures. **TXT: CRY2-eGFP: CIBN-gRNA plasmid mass ratios: 1:9, 2:8, 3:7, 4:6, 5:5, 6:4, 7: 3, 8:2, and 9:1**

3.2.2. Talent adding 110 ng/well DNA plasmid mixtures into microcentrifuge tubes with serum-free medium.

3.3. Then pipette an additional 11 microliters of serum-free DMEM for each well, into separate tubes for transfection reagent dilution **[1]**.

3.3.1. Talent aliquoting 11 µL serum-free medium into new microcentrifuge tubes for transfection reagent dilution.

3.4. Add the diluted transfection reagent to the DNA-medium mixture and incubate to form the transfection complexes **[1-TXT]**. Then pipette 20 microliters of the prepared transfection complex to each well of the 96-well plate containing the HEK293T cells **[2]**.

3.4.1. Talent combining transfection reagent with DNA. **TXT: Incubate at RT, 12 min**

3.4.2. Talent adding 20 µL transfection complexes into individual wells.

3.5. Wrap the 96-well plate in aluminum foil **[1]** and place it into an incubator set at 37 degrees Celsius under 5 percent carbon dioxide for 24 hours **[2]**.

3.5.1. Talent wrapping the plate in aluminum foil.

3.5.2. Talent placing it into the CO_2 incubator.

3.6. To begin the activation process, modify the microcontroller input code to illuminate the

desired wells using the specified script lines **[1]**. Set the light-emitting diode intensity to 9.27 milliwatts per square centimeter using the code lines indicated **[2]**.

3.6.1. SCREEN: 68779_screenshot_4 0:00:11-0:00:37

3.6.2. SCREEN: 68779_screenshot_5 00:21-0:00:31

3.7. Adjust the pulse length to 1 second **[1]**, then set the pulse frequency to 0.067 hertz, equivalent to every 15 seconds **[2]**.

3.7.1. SCREEN: 68779_screenshot_6 00:08-0:00:17

3.7.2. SCREEN: 68779_screenshot_7 0:00:17-0:00:24

3.8. Spray the 3D printed lid of the OptoPlate *(opto-plate)* with 70 percent ethanol **[1]** then allow it to dry in a biosafety cabinet **[2]**.

3.8.1. Talent spraying the 3D printed lid with ethanol.

3.8.2. Talent placing the sprayed lid inside a biosafety cabinet to dry.

3.9. Now, turn on a red light lamp in the darkroom **[1].** Place the 96-well plate into the biosafety cabinet **[2]**. Replace the plate lid with the dried 3D printed lid **[3]**.

3.9.1. Talent turning on a red lamp in a darkroom.

3.9.2. Talent moving the 96-well plate into the biosafety cabinet and removing the aluminum foil.

3.9.3. Talent replacing the original plate lid with the dried 3D printed lid.

3.10. Place the 96-well plate onto the LED array to assemble the activation apparatus **[1]**. Connect the microcontroller, light-emitting diode, and fan ports to a power source **[2-TXT]**.

3.10.1. Talent positioning the plate onto the LED array structure.
AUTHOR'S NOTE: Please move 3.11 after 3.10.1

3.10.2. Talent plugging in microcontroller and peripheral connections to the power source. **TXT: Incubate for 24 h**

3.11. Place the fully assembled LED array apparatus into an incubator set at 37 degrees Celsius and 5 percent carbon dioxide for 24 hours **[1]**.

3.11.1. Talent carrying and placing the LED apparatus into the incubator.

4. Flow Cytometry Gating, Data Acquisition, and Instrument Cleaning

4.1. To perform flow cytometry, run the system startup program on the CytExpert *(Site-Expert)* software **[1].** Load 2 milliliters of deionized water into the sample loader **[2]**.

Run quality control using the provided quality control beads **[3]**.

4.1.1. SCREEN: 68779_screenshot_8 00:45-0:00:52, 03:36-03:45

4.1.2. Talent loading 2 milliliters of deionized water into the sample loader.

4.1.3. SCREEN: 68779_screenshot_9. 0:01:25-0:01:36

4.2. Set up and specify the sample wells for acquisition **[1]**.

4.2.1. SCREEN: 68779_screenshot_10 00:02-00:03, 00:08-00:12, 00:20-00:27,00:33

4.3. Create the plots and tables for Side Scatter versus Forward Scatter, Side Scatter Height versus Side Scatter Area, Side Scatter Area versus FITC-A *(Fit-C-A)*, and the FITC-A statistics table **[1]**.

4.3.1. SCREEN: 68779_screenshot_11 0:01:20 – 0:01:57

4.4. Now, snap the V-bottom 96-well plate into the plate loader of the cytometer **[1]**. Select an untransfected well and click on **Initialize**, followed by **Run [2]**.

4.4.1. Talent placing the plate into the plate loader.

4.4.2. SCREEN: 68779_screenshot_12 well 0:06:20 – 0:06:25

4.5. Adjust the side scatter and FITC voltages to center the population of interest on the SSC-A vs FSC-A plot **[1]**. Create a polygon to gate the healthy cell population **[2]**, a second polygon to gate for doublet discrimination on the SSC-H vs SSC-A plot **[3]**. ~~and shift the FITC voltage to place untransfected cells to the left of the SSC-A vs FITC-A plot **[4].**~~

4.5.1. SCREEN: 68779_screenshot_12 06:50 – 07:15

4.5.2. SCREEN: 68779_screenshot_14 0:00:12 – 0:00:26
AUTHOR'S NOTE: Please move 4.5.2-4.5.3 after 4.7.1

4.5.3. SCREEN: 68779_screenshot_14 0:00:28 – 0:00:44

~~4.5.4. SCREEN: Modifying FITC voltage to align untransfected cells on the left side of the SSC-A vs FITC-A plot.~~

4.6. Click on **Run** then select a CMV *(C-M-V)* -enhanced green fluorescent protein transfected well **[1]**.

4.6.1. SCREEN: 68779_screenshot_13 00:13-00:24

4.7. Adjust the FITC voltage to include both autofluorescent and fluorescing cells in the SSC-A vs FITC-A plot **[1]**. Create a polygon gate to distinguish fluorescing cells from non-fluorescing ones using the gate from the untransfected cells as a reference **[2]**.

4.7.1. SCREEN: 68779_screenshot_13 0:01:15 - 0:01:30

Adjusting FITC voltage slider.

4.7.2. SCREEN: 68779_screenshot_14 01:00 - 0:01:15.

4.8. Auto-record samples at 60 microliters per minute until either 200 seconds have elapsed or 10,000 events have been reached **[1]**. Then export the Mean FITC values as a CSV file and analyze the data **[2]**. Clean the flow cytometer by selecting the **Daily Clean** option **[3]**.

4.8.1. SCREEN: 68779_screenshot_15 02:05 – 02:45

4.8.2. SCREEN: 68779_screenshot_16 0:02:11 – 0:02:30

4.8.3. SCREEN: 68779_screenshot_17 0:00:02-00:14, 00:35 – 00:40

Results

5. Results

5.1. Fluorescence imaging showed that at a mass ratio of 1 to 9 blue light activation induced lower maximal eGFP *(E-G-F-P)* expression **[1]** compared to the 5 to 5 ratio which also exhibited increased leakiness in the dark condition **[2]**.

5.1.1. LAB MEDIA: Figure 2. *Video editor: Highlight the green fluorescence in the upper-left panel under "1:9" and "Blue Light (ON)"*

5.1.2. LAB MEDIA: Figure 2. *Video editor: Highlight the green fluorescence in the lower-right panel under "5:5" and "Dark (OFF)"*

5.2. Flow cytometry showed that over 95% of gated events in P1 were singlets across all conditions **[1]**, and untransfected cells had less than 0.1% eGFP-positive population **[2]**.

5.2.1. LAB MEDIA: Figure 3A–D. *Video editor: Highlight the middle plots labeled P1 with ">95%" values*

5.2.2. LAB MEDIA: Figure 3A. *Video editor: Highlight the green rectangle in the far-right P2 plot*

5.3. The CMV-eGFP control had approximately 99% eGFP-positive cells **[1]**, while 2pLACE *(Two-place)* -transfected cells had approximately 57% **[2]**.

5.3.1. LAB MEDIA: Figure 3B. *Video editor: Highlight the green rectangle in the far-right CMV-eGFP panel*

5.3.2. LAB MEDIA: Figure 3D. *Video editor: Highlight the green rectangle in the far-right CMV-eGFP panel*

5.4. The CMV-eGFP transfected cells showed strong constitutive eGFP expression under both light and dark conditions **[1]**, while untransfected cells showed no visible fluorescence **[2]**. It also confirmed successful transfection of 2pLACE and activation of eGFP expression under blue light, with lower expression than CMV-eGFP **[3]**.

5.4.1. LAB MEDIA: Figure 4A. *Video editor: Highlight the greenfield panels for "CMV-eGFP" under both "Blue light (ON)" and "Dark (OFF)" columns*

5.4.2. LAB MEDIA: Figure 4A. *Video editor: Highlight the black greenfield panel under "Untransfected (UT)" and "Blue light (ON)"*

5.4.3. LAB MEDIA: Figure 4A. *Video editor: Highlight the greenfield panel under "2pLACE" and "Blue light (ON)"*

5.5. A 3-fold increase in eGFP expression was observed in light-activated 2pLACE samples

compared to dark conditions **[1]**. The percentage of eGFP-positive cells in 2pLACE-transfected samples was approximately 60% **[2]**.

5.5.1. LAB MEDIA: Figure 4B. *Video editor: Highlight the light blue bar for "2pLACE"*

5.5.2. LAB MEDIA: Figure 4C. *Video editor: Highlight the bar labeled "2pLACE"*

5.6. 3 to 7 mass ratio produced the highest mean fluorescence intensity and dynamic range **[1]**.

5.6.1. LAB MEDIA: Figure 5A and B. *Video editor: Highlight the tallest light-blue bar under the 3:7 ratio*

5.6.2. LAB MEDIA: Figure 5A and B. Video editor: Highlight the cluster of darker blue bars for 6:4 and 9:1 ratios to show reduced expression

Pronunciation Guide:

- **optogenetic**

Pronunciation link: https://www.merriam-webster.com/medical/optogenetics Merriam-Webster+2Cambridge Dictionary+2
IPA: /ˌɑːpˈtoʊ-dʒəˈnɛtɪk/
Phonetic spelling: ahp-toh-juh-NET-ik

- **hemocytometer**

Pronunciation link: https://www.merriam-webster.com/dictionary/hemocytometer Merriam-Webster+1
IPA: /ˌhiː.məˌsaɪˈtɑːm.ə.t̬ɚ/
Phonetic spelling: hee-muh-sigh-TAH-muh-ter

- **transfection**

Pronunciation link: No confirmed link found
IPA (approx): /trænsˈfɛkʃən/
Phonetic spelling: trans-FEK-shun

- **microcentrifuge**

Pronunciation link: No confirmed link found
IPA (approx): /ˌmaɪkroʊˈsɛn.trɪˌfjuːdʒ/
Phonetic spelling: my-kroh-SEN-tri-fyoog

- **plasmid**

Pronunciation link: No confirmed link found
IPA (approx): /ˈplæz.mɪd/
Phonetic spelling: PLAZ-mid

- **cytometry**

Pronunciation link: No confirmed link found
IPA (approx): /saɪˈtɑː.mə.tri/
Phonetic spelling: sigh-TAH-muh-tree

- **incubator**

Pronunciation link: No confirmed link found
IPA (approx): /ˈɪŋ.kjəˌbeɪ.tər/
Phonetic spelling: ING-kyuh-BAY-ter

- **ethylenediaminetetraacetic** *(from "Trypsin-EDTA")*

Pronunciation link: No confirmed link found
IPA (approx): /ˌɛθ.əˈliː.nˌdaɪ.əˌmeɪn ˌtɛtrəəˈsɛtɪk/
Phonetic spelling: eth-uh-LEEN-die-uh-MAYN tet-ruh-uh-SET-ik