

TITLE:

High-throughput optogenetics experiments in yeast using the automated platform Lustro

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SUMMARY:

This protocol describes how to use the automated platform Lustro for the high-throughput characterization of optogenetic systems in yeast.

ABSTRACT:

Optogenetics provides precise control of cellular behavior through genetically encoded light-sensitive proteins. However, optimizing these systems to achieve the desired range of functionality often requires many design-build-test cycles, which is time-consuming and labor-intensive. To address this, we designed Lustro, a platform which integrates light stimulation with laboratory automation to enable high-throughput screening and characterization of optogenetic systems. Lustro utilizes an automation workstation equipped with an illumination device, a shaking device, and a plate reader. A robotic arm is programmed to move a microwell plate between the devices to stimulate optogenetic strains and measure their response. Here we present a protocol for using Lustro to characterize optogenetic systems for gene expression control in the budding yeast *Saccharomyces cerevisiae*. The protocol describes how to set up the components of Lustro, integrating an illumination device with an automation workstation, and provides instruction for programming the illumination device, plate reader, and robot.

INTRODUCTION:

Optogenetics is a powerful technique that uses light-sensitive proteins to control the behavior of cells with high precision¹⁻³. However, prototyping optogenetic constructs and identifying optimal illumination conditions can be time consuming, which makes it difficult to optimize optogenetic systems^{4, 5}. High-throughput methods to rapidly screen and characterize the activity of optogenetic systems can accelerate the design-build-test cycle for prototyping constructs and

exploring their function.

We therefore developed Lustro, a laboratory automation technique designed for high-throughput screening and characterization of optogenetic systems that integrates a microplate reader, illumination device, and shaking device with an automation workstation⁶. Lustro combines automated culturing and light stimulation of cells in microwell plates (**Figure 1**), allowing for the rapid screening and comparison of different optogenetic systems. The Lustro platform is highly adaptable and can be generalized to work with other laboratory automation robots, illumination devices, plate readers, cell types, and optogenetic systems, including those responsive to different wavelengths of light.

In this protocol, we demonstrate how to set up Lustro and use it to characterize an optogenetic system. We use optogenetic control of split transcription factors in yeast as an example system to illustrate the function and utility of the platform by probing the relationship between light inputs and the expression of a fluorescent reporter gene, mScarlet-1⁷. By following this protocol, researchers can streamline the optimization of optogenetic systems and accelerate the discovery of new strategies for the dynamic control of biological systems.

PROTOCOL:

1. Set up the automation workstation.

- 1.1. Equip the automated workstation with a Robotic Gripper Arm (RGA) capable of moving microwell plates (see **Figure 1**).
- 1.2. Install a microplate heater shaker into the automated workstation (**Figure 1.1**) with an automatic plate locking mechanism that allows access to the RGA.
- 1.3. Secure a microplate reader adjacent to the automated workstation (**Figure 1.2**) that allows access to the RGA.
- 1.4. Install a microplate illumination device (**Figure 1.3**) that allows easy access to the RGA, such as the optoPlate⁸ (as used here) or LITOS⁹.

2. Prepare the illumination device.

- 2.1. Construct and calibrate the optoPlate (or other illumination device) according to established methods^{8, 10, 11}.
- 2.2. Use an adaptor on the illumination device that allows access to the RGA.
- 2.3. Program the optoPlate from a spreadsheet input¹⁰ (or via a graphical interface¹²). Considerations for programming light stimulation programs are detailed below.

3. Design a light stimulation program. Determine the light conditions that will be used for the sample plate and flash (load) them onto the illumination device.

- 3.1. Enter desired light conditions (including light intensity, light start time, pulse length, pulse number, and interpulse duration) into a spreadsheet and flash onto

the optoPlate (as described in the following github repository: github.com/mccleanlab/Optoplate-96). Note when programming the light conditions into the illumination device that the sample plate will not receive illumination while in the microplate reader or on the heater shaker. The duration and frequency of these events may need to be optimized for specific experimental needs.

- 3.2. Include dark conditions for each strain for proper background measurements to be taken.
 - 3.3. Use high light intensity for initial characterization experiments to determine functionality of transformants⁶. Note that light intensity should be optimized for more sensitive experiments as excessive light is phototoxic to yeast¹³.
4. **Prepare the microplate reader.** Configure the microplate reader to measure the quantity of interest prior to performing experiments. In the example presented here, we configure the microplate reader to measure the amount of fluorescence from a reporter expressed by the strain of interest. Outputs such as luminescence or optical density can also be used, depending on experimental needs.
- 4.1. Grow the strain of interest (and a nonfluorescent control) in synthetic complete (SC) media¹⁴ (or another low fluorescence media) to the highest cell density that will be measured, pipette into a glass-bottom black-walled microwell plate, and measure to determine optimal microplate reader settings. Verify the plate dimensions are correctly entered to ensure accurate readings are taken. Measure the plate from below to ensure accuracy.
 - 4.2. Consult fpbase.org to determine approximate absorption and emission spectra of the target fluorescent protein¹⁵.
 - 4.3. Determine the z-value (the distance between the plate and reader) for the plate by performing a z-scan on wells with the fluorescent strain and well with the nonfluorescent strain. Select the z-values that yield the highest signal-to-noise ratio.
 - 4.4. Optimize absorption and emission spectra by using the absorption scan and emission scan on the fluorescent and nonfluorescent strains to determine the optimal signal-to-noise ratio.
 - 4.5. Measure the fluorescent strain with the gain set to optimal to determine the highest optical gain that can be used without returning an overflow measurement error. This optical gain should be manually set across experiments with a given strain to ensure consistency in results.
 - 4.6. Prepare a measurement script (see **Figure 2**) in the microplate reader software. This measurement script configures the instrument to measure the optical density of the cultures and fluorescence spectra of any fluorescent proteins to be measured. Measure optical density of strains at 600 nm unless they produce red fluorescent proteins. If the strains express a red fluorescent protein, measure optical density at 700 nm to avoid bias¹⁶.
 - 4.7. Set the script to maintain an internal incubation temperature during

measurements of 30 °C.

- 4.8. Set the measurement script to export the data into a spreadsheet (or ASCII files, if desired).

5. Program the robot. Set up the worktable definition in the automated workstation software according to the physical layout of the carriers (e.g., heater shakers, nest platforms, illumination devices, etc.) and the labware (i.e., the 96-well plate). Prepare a script on the automation workstation software to perform light induction and measurement (see **Figure 3**) as follows.

- 5.1. Turn off any internal sources of illumination to avoid background activation of optogenetic systems.
- 5.2. Set the heater shaker to 30 °C. The plate will be at ambient temperature (22 °C) while not on the heater shaker.
- 5.3. Use loops, a timer, and a loop counting variable to repeat the steps of inducing and measuring the cells over regular intervals.
- 5.4. Prior to recording any measurements, shake the sample plate to ensure all cells are suspended (60 s at 1,000 rpm with a 2 mm orbital is sufficient to resuspend S288C *S. cerevisiae* cells) to avoid bias in measurement.
- 5.5. The robot arm then moves the sample plate to the microplate reader and removes the lid (to avoid bias in optical density measurement). Control software will automatically remove (and replace) the lid to the designated position if the microplate reader carrier is defined as not allowing lids.
- 5.6. Run the microplate reader measurement script (described in step 4).
- 5.7. The robot arm then replaces the lid to the sample plate and moves the plate to the illumination device.
- 5.8. Set the script to wait until the timer reaches 30 min (multiplied by the loop counting variable), and then repeat the whole loop 48 times (for a 24-hour experiment).
- 5.9. Run an empty plate through the steps of the script loop described above several times to troubleshoot potential errors and ensure that the carrier and labware definitions are set properly and that the RGA can correctly and precisely pick up and place the plate at each carrier site.
- 5.10. Set up user alerts to notify the user in the case of any instrument state changes (such as errors).

6. Set up the sample plate. Grow up the strains of interest and load them into a glass-bottom black-walled 96-well plate.

- 6.1. Grow yeast strains on rich media plates, such as YPD¹⁷ agar. Include a nonfluorescent (negative) control. Yeast strains used here (see **Table of Materials**) grow well between 22 °C - 30 °C and in a range of standard yeast media.
- 6.2. Pick colonies from these plates and grow them overnight in 3 mL SC¹⁴ (or another low fluorescence media, such as LFM¹⁸) at 30 °C on a roller drum. Keep cultures in

- the dark (or under a non-responsive wavelength of light, such as red light for blue light-responsive systems) during incubation and for downstream processing steps.
- 6.3. Measure the optical density of the overnight cultures by diluting 200 μL of each into 1 mL (final volume) SC and measuring the optical density at 600 nm (OD_{600}) with a spectrophotometer or microplate reader.
 - 6.4. Dilute each overnight culture to $\text{OD}_{600}=0.1$ in glass culture tubes. Where higher strain-testing throughput is desired, automated dilutions can be performed in microwell plates.
 - 6.5. Pipette the diluted cultures into the 96-well plate. Perform each condition in triplicate (i.e., three identical wells with the same strain and light condition) to determine technical variation. Include blank media and nonfluorescent cells as negative controls for determining background fluorescence and optical density.
 - 6.6. Incubate the plate at 30 $^{\circ}\text{C}$, shaking for 5 hours before beginning the light induction experiment. Dilution amounts and incubation times may need to be optimized for specific strains and experimental conditions.

7. Perform the experiment.

- 7.1. Load the sample plate onto the heater shaker and start the automation script (described in step 5).
- 7.2. Start the light stimulation program (described in step 3) once the first measurement on the plate reader has been taken.
- 7.3. If the automation workstation isn't located in a dark room, cover it in a blackout curtain to prevent background illumination.

8. Analyze the data. Use Python or another programming language to process the data exported by the microplate reader during the experiment.

- 8.1. Prepare a spreadsheet map for the experiment. This map should correspond to the 8x12 layout of the 96-well plate and include the names of the strains being measured in one grid and descriptions of the light conditions used in another grid.
- 8.2. Use a Python script (or other preferred coding language) to analyze the data. Read the map spreadsheet into an array with the strain names and condition names for each well of the plate. Find sample code here: <https://github.com/mccleanlab/Lustro>. Alternatively, use an app that can parse data from a variety of plate readers¹⁹.
- 8.3. Read the data from the experiment spreadsheet export into another array.
- 8.4. Plot the optical density values and fluorescence values of each strain and condition versus time (as shown in **Figure 4**).
- 8.5. Use the optical density plots to determine when cultures are in the exponential growth phase or reach saturation and can thus be used to select appropriate timepoints for comparing fluorescence measurements between strains or conditions (see **Figure 5**).

REPRESENTATIVE RESULTS:

Figure 4A shows the fluorescence values recorded over time from an optogenetic strain (with the expression of a fluorescent reporter driven by a light-inducible split transcription factor) induced by different light conditions. The duty cycle (percentage of the time the light is on) of the light stimulation is proportional to the overall level of fluorescence measured. **Figure 4B** shows the corresponding OD₇₀₀ values for the same experiment. The consistency of the optical density readings between different light conditions indicates that the experimental technique does not result in significant differences in growth rate between different light conditions. Measuring fluorescence and optical density over time can lend more insight into how optogenetic systems respond to different light stimulation programs compared to techniques that only record the output at a single time point. This time-course data can also be used to inform which time point measurements should be used for comparing different strains and conditions. **Figure 5** shows a single time point (measured at 10 hours into light induction) comparing two different optogenetic strains induced by different light stimulation programs. These strains use a light-inducible split transcription factor to drive expression of a fluorescent reporter. Varying light pulse intensity, period, and duty cycle elicit different responses in the strains.

FIGURE AND TABLE LEGENDS:

Figure 1. Worktable layout and experimental workflow. Screenshot of a sample worktable layout, denoting the movement of the sample plate in LustrO. The plate is moved by the robotic arm from a heater shaker (1) to the microplate reader (2), and then to the illumination device (3). Photographs are included in Supplementary Figure 1.

Figure 2. Plate reader measurement script. Sample screenshot of a plate reader script setting the microplate reader to incubate at 30 °C and record fluorescence and optical density measurements.

Figure 3. Automated workstation script. Sample screenshot of an automated workstation script for LustrO. The script starts a timer, ensures the interior light is turned off, sets a loop counting variable to an initial value of 0, and sets the heater shaker to incubate at 30 °C. Within each loop, the plate is locked, shaken for 1 minute, moved to the plate reader and measured, then moved to the illumination device and the robot is set to wait for the remainder of the 30-minute loop interval. At the end of this time, the loop counter variable is increased by one and the loop is repeated.

Figure 4. Induction time course. Sample light induction time course data from a Gal4BD-eMagA/eMagB-Gal4AD split transcription factor strain with a pGAL1-mScarlet-I reporter (yMM1734⁶). Fluorescence of mScarlet-I⁷ is measured at 563 nm excitation and 606 nm emission with an optical gain of 130. Light intensity is 125 $\mu\text{W}/\text{cm}^2$ and error bars represent standard error

of triplicate samples. Vertical red dotted line shows when cultures reach saturation. (A) Fluorescence values from the strain over time. Light patterns (as indicated) were repeated for the full duration of the experiment shown. Inset shows that light pulse times are interspersed with dark interpulse times, repeated over the course of the experiment. (B) Optical density (measured at 700 nm) values for the experiment shown in (A).

Figure 5. Comparison of different optogenetic systems. Comparison of different light induction programs between CRY2(535)/CIB1 and eMagA/eMagBM split transcription factor strains with pGAL1-mScarlet-I reporters (yMM1763 and yMM1765⁶, respectively). Fluorescence of mScarlet-I⁷ is measured at 563 nm excitation and 606 nm emission with an optical gain of 130. Light intensity used is 125 $\mu\text{W}/\text{cm}^2$, except where otherwise noted. Error bars represent standard error of triplicate samples (indicated as dots). Fluorescence values shown were recorded 10 hours into induction.

Supplementary Figure 1. Representative images of the devices used in LustrO. Picture of the LustrO setup and zoomed-in images of the devices used. The robotic arm moves the sample plate from the heater shaker to the plate reader, and then to the illumination device in a cycle over the course of the experiment. Components are numbered with a legend on the side.

DISCUSSION:

The LustrO protocol provided here automates culturing, illumination, and measurement to allow for high-throughput screening and characterization of optogenetic systems⁶. This is accomplished by integrating an illumination device, microplate reader, and shaking device in an automation workstation. In this protocol we specifically demonstrate the utility of LustrO for screening different optogenetic constructs integrated into the yeast *S. cerevisiae* and comparing light induction programs.

This protocol emphasizes several crucial steps that are integral to the effective utilization of LustrO. It is essential to carefully design customized light programs that align with the specific kinetics of the optogenetic construct under investigation. Additionally, precise calibration of the plate reader to achieve the desired output is necessary to obtain reliable measurements. Thorough dry runs of the experiments on the robot, including adjusting the timings as necessary to ensure proper synchronization with the light programs, are critical to ensure the script runs properly.

The sample protocol detailed here describes comparing a light-inducible split transcription factor driving expression of a fluorescent reporter to a nonfluorescent control over a range of light stimulation conditions. We measure fluorescence from each well in the plate at 30-minute intervals with one minute on the heater shaker prior to measurement. As described and implemented in this protocol, LustrO is suitable for use directly with blue light-responsive optogenetic systems integrated into many non-adherent cell types, including bacteria and other yeasts. However, the protocol is easily extended to different cell types, optogenetic systems, and experimental designs with small modifications. Minor changes to the plate reader settings would

allow measurement of outputs other than fluorescence, such as bioluminescence. Measurements could be taken more frequently where finer temporal resolution is needed. Incubation on the heater shaker could be repeated more frequently as needed for specific cell types where shaking and temperature control are critical. Gas and environmental control, for example through an incubated hotel, would allow incorporation of mammalian cell lines. The iteration of Lusto described here uses specific instrumentation; however, the Lusto platform can be easily adapted to work with other laboratory automation robots or microplate readers. Illumination devices, such as the LPA²⁶ or LITOS⁹, could be substituted for the optoPlate to stimulate different optogenetic systems. A particularly exciting future modification of the Lusto platform would be to incorporate liquid handling to facilitate automated dilutions for continuous culture applications. This would also allow Lusto to be adapted for cybergenetic feedback control, where real-time measurements inform changes in light or culture conditions to reach or maintain a desired response^{5, 21, 22}.

High-throughput techniques are important for optimizing and taking advantage of the dynamic nature of optogenetic systems. Lusto overcomes many of the limitations of existing protocols. For example, while bioreactor-based optogenetics techniques allow for constant readout and culturing conditions, they are limited by low throughput^{23–26}. The optoPlateReader²⁷ device shows great promise for performing real-time optogenetics experiments in microwell plates, but currently suffers from low throughput due to the high number of replicates needed to obtain reliable results and doesn't provide access to continuous culturing. Lusto is able to perform high-throughput screening of optogenetic systems to characterize their dynamic activity. Nevertheless, some limitations of the Lusto protocol remain. Intermittent shaking in Lusto does cause a small lag in growth for yeast cells⁶, but that could be remedied by adapting an illumination device to shake. An additional limitation of the Lusto system is that the sample plate is not incubated while on the illumination device and is maintained at ambient temperature (22 °C). While the small volume of each sample allows for high-throughput screens to be performed, it is possible that additional optimization of the illumination steps would need to be undertaken when scaling to larger reaction volumes for bioproduction or other applications^{28, 29}.

Overall, Lusto enables the rapid development and testing of optogenetic systems through high-throughput screening and precise light control. This automated approach allows for efficient characterization and comparison of different optogenetic constructs under various induction conditions, leading to faster iteration and refinement of these systems. With its adaptability to different cell types, optogenetic tools, and automation setups, Lusto paves the way for advancements in the field of optogenetics, facilitating the exploration of dynamic gene expression control and expanding possibilities for studying biological networks and engineering cellular behavior.

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DISCLOSURES:

The authors have nothing to disclose.

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