Video Article

Optogenetic Random Mutagenesis Using Histone-miniSOG in *C. elegans*

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Abstract

Forward genetic screening in model organisms is the workhorse to discover functionally important genes and pathways in many biological processes. In most mutagenesis-based screens, researchers have relied on the use of toxic chemicals, carcinogens, or irradiation, which requires designated equipment, safety setup, and/or disposal of hazardous materials. We have developed a simple approach to induce heritable mutations in *C. elegans* using germline-expressed histone-miniSOG, a light-inducible potent generator of reactive oxygen species. This mutagenesis method is free of toxic chemicals and requires minimal laboratory safety and waste management. The induced DNA modifications include single-nucleotide changes and small deletions, and complement those caused by classical chemical mutagenesis. This methodology can also be used to induce integration of extrachromosomal transgenes. Here, we provide the details of the LED setup and protocols for standard mutagenesis and transgene integration.

Video Link

The video component of this article can be found at <http://www.jove.com/video/54810/>

Introduction

Forward genetic screens have been widely used to generate genetic mutants disrupting various biological processes in model organisms such as *Caenorhabditis elegans* (*C. elegans*)¹. Analyses of such mutants lead to the discovery of functionally important genes and their signaling pathways^{1,2}. Traditionally, mutagenesis in *C. elegans* is achieved using mutagenic chemicals, radiation, or transposons². Chemicals such as ethyl methanesulfonate (EMS) and *N*-ethyl-*N*-nitrosourea (ENU) are toxic to humans; gamma-ray or ultraviolet (UV) radiation mutagenesis requires special equipment; and transposon-active strains, such as mutator strains³, can cause unnecessary mutations during maintenance. We have developed a simple approach to induce heritable mutations using a genetically-encoded photosensitizer⁴.

Reactive Oxygen Species (ROS) can damage DNA⁵. The mini Singlet Oxygen Generator (miniSOG) is a green fluorescent protein of 106 amino acids that was engineered from the LOV (Light, Oxygen, and Voltage) domain of *Arabidopsis* phototropin 2⁶. Upon exposure to blue light (~450 nm), miniSOG generates ROS including singlet oxygen with flavin mononucleotide as a cofactor⁶⁻⁸, which is present in all cells. We constructed a His-mSOG fusion protein by tagging miniSOG to the C-terminus of histone-72, a *C. elegans* variant of Histone 3. We generated a single-copy transgene⁹ to express His-mSOG in the germline of *C. elegans*. Under normal culture conditions in the dark, the His-mSOG transgenic worms have normal brood size and life span⁴. Upon exposure to blue LED light, the His-mSOG worms produce heritable mutations among their progeny⁴. The spectrum of induced mutations includes nucleotide changes, such as G:C to T:A and G:C to C:G, and small chromosome deletions⁴. This mutagenesis procedure is simple to perform, and requires minimal lab safety setup. Here, we describe the LED illumination setup and procedures for optogenetic mutagenesis.

Protocol

1. Construction of the LED Illuminator

NOTE: The required LED equipment is summarized in the List of Materials. The entire LED setup is small and can be placed anywhere in the lab, although we recommend it be placed in a dark room to control light exposure of worms⁴.

1. Connect the LED to a controller with cables (**Figure 1A, D**).
2. Connect the controller to a digital function generator/amplifier with a BNC cable (**Figure 1A, C, D**).
3. Fix the LED light 10 cm above the stage using a custom holder (**Figure 1A**).
NOTE: We made the holder with metal parts.
4. Cover the LED illumination setup partially, but avoid heat accumulation (**Figure 1B**), which makes worms sick.

NOTE: We use a custom-made hard plastic cover with open top and bottom (**Figure 1A**). The cover is not required for the mutagenesis itself, but is used to limit unnecessary exposure of the blue light to the surroundings. We advise to not cover the LED setup entirely (**Figure 1B**) to prevent overheating the worms.

5. Wear laser-protective glasses.

NOTE: We wear laser-protective glasses because the light is very bright. Sunglasses may work as well.

6. Switch the lever of the LED controller to 'Int.' for continuous illumination (**Figure 1D**) and set it at 65% of the maximum power (**Figure 1C**).
7. Use a photometer to measure the blue light intensity on the stage where the worms are placed using manufacturer's protocol. The setup gives 2.0 mW/mm² under continuous illumination.

2. Blue Light Treatment

NOTE: Use the strain CZ20310 *juSi164[Pmex-5-HIS-72::miniSOG-3'UTR(tbb-2) + Cb-unc-119(+)] unc-119(ed3)III^d*. The strain is available at the Caenorhabditis Genetics Center (CGC, <http://www.cgc.cbs.umn.edu/>).

1. Maintain the His-mSOG strain in the dark on standard nematode growth medium (NGM) plates with *E. coli* OP50 following a standard protocol^{10,11}.
NOTE: Under ambient light, *juSi164*-containing strains do not display a mutation rate above a spontaneous rate⁴. Routine strain passage using a dissecting scope with a halogen lamp generally does not cause mutations as well. Nonetheless, care should be taken to avoid unnecessary exposure to light, e.g., keeping the strains in a standard dark-inside incubator, or covering the strains with foil. It is advised to freeze aliquots of the strain after receiving it in case unnecessary mutations accumulate over time.
2. Pick gravid young adult (approximately 12 h post-L4) animals with a worm pick¹¹.
NOTE: Younger animals show less mutagenic effect⁴, while older animals may have lower brood size.
3. Cut a filter paper to fit a 60 mm plate with a 25 mm x 25 mm square hole and place onto an unseeded plate (**Figure 1E**).
NOTE: A square punch can be used but the size of the punch does not have to be precise.
4. Drop 100 μ L of 100 mM CuCl₂ on the filter paper evenly to restrict worms within the square hole on the plate.
5. Pick desired number of gravid young adult hermaphrodites (see Step 3 for an example) and transfer to the center of the CuCl₂ plate.
6. Wear laser protective glasses.
7. Switch ON the LED and the function generator.
8. Switch the lever of the LED controller to 'Ext.' to control it by the function generator (**Figure 1D**).
9. Set the controller at 65% of the maximum power and the function generator as sine wave, 4 Hz (**Figure 1C**).
10. Place the CuCl₂ plate from Step 2.5 without a lid on the stage under the LED (**Figure 1A**).
11. Illuminate the animals with blue light for 30 min.
12. Transfer healthy looking worms to a seeded plate as P₀.
NOTE: The desired number of P₀ depends on the type of screen. See Step 3 for an example. The P₀ worms may appear to be uncoordinated immediately after light treatment and will recover over time.
13. Keep worms covered using foil in an incubator or in the dark; and follow the standard procedure to begin screening desired phenotypes among their progeny^{2,10}.

3. Example of a Forward Genetic Screen

NOTE: This is an example of the clonal screen with 120 haploid genomes to check if the LED and strain are working. **Figure 2** shows a schematic of the procedure.

1. [Day1] After blue light treatment, pick five healthy looking worms onto a NGM plate with OP50 as P₀, keep them in a 20 °C incubator, and let them lay eggs for 1 d ('Day1' plate).
2. [Day2] Transfer P₀ onto a new seeded plate ('Day2' plate).
3. [Day3] Pick and kill P₀ on the 'Day2' plate by burning them.
4. [Day4] Pick 30 gravid young adult F₁ from the 'Day1' plate and transfer them onto individual plates (30 F₁ plates for 'Day1').
5. [Day5] Repeat Step 3.4 for the 'Day2' plate (30 F₁ plates for 'Day2').
6. [Day7-9] Check the abnormal morphologies and/or behavior of F₂ worms compared to the WT strain (**Figure 3A**) under a standard stereomicroscope when they become adults.
NOTE: A heritable mutation displays about a quarter of worms with the same phenotype among F₂ when it is recessive with high penetrance. However, some mutants may grow slowly and/or show partial penetrance. Therefore, it is possible that less than a quarter of the mutants can be found on a plate.
7. Pick worms with visible phenotypes individually and check the heritability of the phenotype in the next generation.

4. Example of an Extrachromosomal Transgene Integration

1. Inject the DNA of interest into CZ20310 *juSi164[Pmex-5-HIS-72::miniSOG-3'UTR(tbb-2) + Cb-unc-119(+)] unc-119(ed3)* and prepare transgenic worms with a low transmission rate (10-30%) following a standard injection protocol^{12,13}.
NOTE: Alternatively, established extrachromosomal arrays can be crossed into the *juSi164* strains. To avoid genotyping for *juSi164*, *juSi164* heterozygotes may be used as the P₀ although efficiency might be decreased. The genotyping method for *juSi164* is described in Section 5.
2. [Day1] Treat ~20 transgenic animals as P₀ with blue light as described in Step 2.
NOTE: More P₀ worms are necessary depending on the transmission rate. Transgenes can be identified by phenotypes or coinjection marker^{12,13}.
3. Pick 10 healthy looking P₀ onto individual plates, keep them in a 20 °C incubator, and let them lay eggs for a few days (P₀ plates)
4. [Day4] Single out 20 transgenic F₁ worms from each P₀ plate onto individual plates and let them lay eggs. (20 F₁ x 10 P₀ plates= 200 F₁ plates in total.)

5. [Day7] Find F_1 plates with >75% F_2 having transgenes.
6. Pick five transgenic animals from the >75% transmission rate F_1 plates (F_2 plates).
7. [Day10] Keep F_2 plates with 100% transmission rate as potential integrated lines.
8. Outcross the potential integrated lines using a standard procedure¹⁰ to check the Mendelian segregation and to remove potential background mutations.

5. Genotyping

1. Lyse 20 outcrossed F_2 worms in lysis buffer using a standard protocol¹⁴.
2. Perform PCR using the primers for MosSCI insertion⁹, *juSi164*, (**Table 1**) with an annealing temperature of 58 °C following a standard protocol¹⁴.
NOTE: It is unnecessary to genotype *unc-119(ed3)* because *unc-119(ed3)* can be detected by uncoordinated behavior after removing the rescuing transgene (*juSi164*) in the following Steps 5.4 and 5.5.
3. Run agarose gel electrophoresis¹⁵ and examine the band sizes (**Table 1**) to find the WT for *juSi164*.
4. Examine if the F_2 progeny has paralyzed worms due to the existence of *unc-119(ed3)*.
5. If uncoordinated worms are found on step 5.4, single several nonuncoordinated worms to obtain all nonuncoordinated worms in the next generation.

Representative Results

We performed a forward genetic screen with CZ20310 *juSi164 unc-119(ed3)* using 30 min light exposure following the standard protocol described in Sections 2 and 3. Among 60 F_1 plates corresponding to 120 mutagenized haploid genomes, we found 8 F_1 plates with F_2 worms displaying visible phenotypes such as body size defect (**Figure 3B**), uncoordinated movement (**Figure 3C**), and adult lethality (**Figure 3D**). We did not notice any difference in the number of mutants between 'Day1' and 'Day2' plates. All progeny of singled small F_2 worms and uncoordinated F_2 worms showed the corresponding phenotypes, suggesting that they are heritable mutants. The full observation is summarized in **Table 2**. On average, a clonal screen with 120 haploid genomes will result in about 10 F_1 plates containing worms with visibly detectable phenotypes such as body shape and locomotion that easily spotted under a stereomicroscope. More quantitative analysis is necessary to determine the mutation rate precisely⁴. The expected number of visible mutants from this screen suggests that the strain and setup are working properly for optogenetic mutagenesis.

Figure 1: The LED Setup. (A) The whole system. The LED was mounted on a custom-made holder. Details are found in the List of Materials. (B) The cover is made of plastic. It does not cover the system completely to avoid heat accumulation. (C) Controller and function generator. (D) The back side of the system. (E) An unseeded plate with CuCl_2 -soaked filter paper for blue light treatment. [Please click here to view a larger version of this figure.](#)

Figure 2: Experimental Procedure for a Clonal Screen. After blue light treatment, P_0 worms are transferred to a seeded plate and allowed to lay eggs. P_0 worms are transferred to a new seeded plate 1 d after light treatment and killed 2 d after light treatment. F_1 worms are singled from the P_0 plates. Phenotypes of F_2 worms are examined after they become adults. [Please click here to view a larger version of this figure.](#)

Figure 3: Example of Mutants. (A) Original strain for optogenetic mutagenesis: CZ20310 *juSi164 unc-119(ed3)*. This strain has normal body shape, behavior, and brood size. Scale bar: 0.5 mm. (B - D) F_2 progeny showing body size defect (B), uncoordinated (C), and lethal (D) phenotypes. [Please click here to view a larger version of this figure.](#)

Allele	Primers	Digestion	Fragment size (bp)
<i>juSi164</i>	oCF1513 (For.)	NA	wt: 371
	oCF1514 (Rev.)		<i>juSi164</i> : 519
	YJ10134 (Int. For.)		
<i>unc-119(ed3)</i>	YJ8515 (For.)	EcoRV	wt: 319 + 240
	YJ8516 (Rev.)		<i>ed3</i> : 559

Table 1: Genotyping of *juSi164* and *unc-119(ed3)*.

Plate #	Observation	Figure
Day1-10	About one quarter of F ₂ showed clear body color.	
Day1-22	About one quarter of F ₂ showed small body size.	
Day1-25	About one quarter of F ₂ showed uncoordinated movements.	Figure 3C
Day1-27	Small brood size. About one quarter of F ₂ showed protruded vulva and sterility.	
Day2-5	Small brood size. About one quarter of F ₂ showed body size defect.	
Day2-22	There were a dozen of dead adult F ₂ .	Figure 3D
Day2-24	About one quarter of F ₂ showed clear body color and small body size.	
Day2-26	Small brood size. About one quarter of F ₂ showed body size defect.	Figure 3B

Table 2: Example of Phenotypes from a Clonal Screen.

Discussion

Here, we describe a detailed procedure of optogenetic mutagenesis using His-mSOG expressed in the germline and provide an example of a small scale forward genetic screen. Compared to standard chemical mutagenesis, this optogenetic mutagenesis method has several advantages. Firstly, it is nontoxic, thereby keeping lab personnel away from toxic chemical mutagens such as EMS, ENU and trimethylpsoralen with ultraviolet light (TMP/UV). Secondly, the experimental procedure of mutagenesis can be used for a small number of the P₀ animals and can be finished within one hour. Thirdly, the optogenetic mutagenesis produces a broad spectrum of mutations including nucleotide substitutions and chromosome deletions. Finally, the optogenetic mutagenesis can be used for integration of extrachromosomal transgenes.

The critical step in this mutagenesis protocol is to ensure that the LED setup works on His-mSOG containing strains at the expected efficiency. We recommend performing a pilot clonal screen, as described in Protocol Section 3, with about 100 haploid genomes. The mutagenesis is easily scalable from the small scale pilot screens to nonclonal screens by increasing the number of P₀ for the light treatment. For large-scale nonclonal screens, we suggest synchronizing the worms to gravid young adults and transferring hundreds of them with M9 solution onto a CuCl₂ plate for light treatment. The standard condition described in Steps 2 and 3 is estimated to have approximately 4.4 times lower efficiency than the widely used 50 mM EMS mutagenesis method⁴. The mutation rate may be improved by increasing the light exposure time and/or increasing the intensity of the light. However, these modifications can cause phototoxicity and lead to small brood size and sickness of light-treated worms. To increase the efficiency, it may be possible to use miniSOG derivatives with higher ROS yield such as miniSOG(Q103L) although it may increase the background mutagenesis effect without light treatment^{16,17}. For the modification of the DNA construct, the *Pmex-5-HIS-72::miniSOG-3'UTR(tbb-2)* plasmid (pCZ886) is available commercially.

Under the conditions described here, integrated transgenes are generated at approximately one line per 200 F₁ on average⁴ when using an extrachromosomal transgenic strain with a transmission rate of 10 - 30%. This efficiency may be increased by using extrachromosomal transgenic strains with high transmission rates as previously reported for a UV radiation method¹⁸.

His-mSOG-mediated optogenetic mutagenesis induces mutations in a wide range of genes. Also, the frequency of loci recovered from the *rpm-1* suppressor screen using optogenetic mutagenesis is similar to those from the same screen using EMS⁴. However, it is possible that optogenetic mutagenesis using a histone gene may have a bias towards certain genes, due to unknown factors such as chromatin structure. To address this issue, it is necessary to analyze many optogenetically induced mutations by whole genome sequencing.

His-mSOG-mediated optogenetic mutagenesis causes a wide spectrum of mutations including single nucleotide substitutions and deletions ranging from 1 bp to a few hundred bp⁴. The spectrum of single nucleotide variants suggests that miniSOG-induced reactive oxygen species are the cause of the mutations⁴. It is also possible that miniSOG damages histone proteins and causes chromatin instability because miniSOG is used to inactivate proteins as well¹⁹. It may be possible to manipulate the type of mutations by using light of different intensities. Since optogenetic mutagenesis causes deletions, it is important to analyze deletions as well as single nucleotide changes in whole genome sequencing data for mapping²⁰.

It was previously reported that an epifluorescent microscope is used for miniSOG-mediated cell ablation^{21,22}. The light intensity of a standard epifluorescent compound microscope was measured to be ~0.5 mW/mm² without a lens, which is about 4x lower than that used in the standard optogenetic mutagenesis protocol. Thus, it is highly advisable to empirically determine the efficiency when using an epi-fluorescent microscope as a light source. One advantage of the LED is the stability over thousands of hours. Previous studies have shown that miniSOG is more potent in ROS-mediated cell ablation under pulsed light²², although the precise mechanism remains to be determined. We used a digital function generator/amplifier to make pulsed light. Another option is to connect the light source to a computer using a USB-TTL interface (**Figure 1A**) to regulate the LED light by a program. The LED system described here can also be used for other purposes, such as optogenetic control of excitable cells using channelrhodopsin²³, cell ablation using mitochondria- or cell-membrane-targeted miniSOG^{17,22}, and Chromophore-Assisted Light Inactivation (CALI) of proteins¹⁹. Furthermore, this optogenetic mutagenesis has the potential to be applied to other organisms, such as bacteria, yeast, fly, and zebrafish, as well as mammalian cell lines.

Disclosures

The authors have nothing to disclose.

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