Multiplex CRISPR genome regulation in mouse retina with hyper-efficient Cas12a

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Method Article

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Abstract

CRISPR-Cas nucleases and their nuclease-deactivated dCas variants have revolutionized the field of genome editing and gene regulation. Cas12a possesses intrinsic RNAse activity and can process multiple functional crRNAs from a single long transcript, making it a powerful tool for multiplex gene targeting. We engineered a dCas12a variant termed hyperCas12a with superior efficacy in gene editing and multiplex gene regulation, especially at restrictive crRNA concentrations. Here, we describe a step-by-step protocol for constructing and validating a crRNA array, and using it with the hyperdCas12a system for multiplex gene regulation *in vivo* by subretinal delivery in mice.

Introduction

Reagents

Molecular biology and cell culture:

- Plasmid DNA: pSLQ10704, pSLQ10844, others (on Addgene)
- Enzymes: Esp3I (NEB), XhoI-HF (NEB), NEBuilder HiFi DNA Assembly (NEB), T4 DNA Ligase (NEB)
- P19 cells (ATCC, CRL-1825)
- Alpha-MEM with nucleosides (Thermo Fisher, 12571063)
- Penicillin-streptomycin (Thermo Fisher Scientific, 10378016)
- FBS (MilliporeSigma, F0926)
- Trypsin-EDTA 0.05% (Thermo Fisher, 25300120)
- Triton X-100 (MilliporeSigma, T9284)
- Mirus X2 (Mirus, MIR 6003)
- Puromycin (MilliporeSigma, P8833)
- Hygromycin B (MilliporeSigma, 31282-04-9)

QPCR

- RNeasy Plus Mini Kit (QIAGEN, 74136)
- iScript cDNA synthesis kit (Biorad, 1708891)
- iTaq™ Universal SYBR® Green Supermix (Biorad, 1725121)

Example antibodies for immunostaining:

- Pax6 (Thermo Fisher, Rabbit polyclonal, 42-6600) 1:500 (in vivo)
- RBPMS (PhosphoSolutions, Guinea Pig polyclonal, 1832-RBPMS) 1:4000 (in vivo)
- HA (Roche, Rat HA clone 3F10, 11867423001) 1:100 (in vivo) 1:200 (in vitro)
- Oct4 (BD bioscience, mouse clone 40/Oct-3, 611203) 1:100 (in vivo), 1:200 (in vitro)
- Sox2 (Cell signaling, rabbit polyclonal, 14962) 1:100 (in vivo), 1:200 (in vitro)
- Klf4 (R&D, goat polyclonal, AF3158) 1:100 (in vivo), 1:200 (in vitro)
- Donkey anti-rat Cy3 (Jackson ImmunoResearch, 711-166-152) 1:1000 (in vivo)
- Donkey anti-goat Cy5 (Jackson ImmunoResearch, 705-175-147) 1:1000 (in vivo)
- Donkey anti-rabbit Cy5 (Fisher Scientific, NC0254454) 1:1000 (in vivo)
- Donkey anti-mouse Cy5 (Jackson ImmunoResearch, 715-605-151) 1:1000 (in vivo),
- Donkey anti-mouse Alexa Fluor 488 (Thermo Fisher, R37114) 1:500 (in vitro)
- Donkey anti-rabbit Alexa Fluor 647 (Thermo Fisher, A-31573) 1:500 (in vitro)
- Donkey anti-goat Alexa Fluor 647 (Thermo Fisher, A32849) 1:500 (in vitro)

For immunohistochemistry:

- 10 mm diameter tweezer electrode (Model #522; BTX Instruments)
- Normal donkey serum (Jackson ImmunoResearch Laboratories, 017-000-121)
- Sucrose (Fisher scientific, S5)
- PFA (Electron microscopy sciences, 15710)
- PBS (Fisher scientific, BP2944100)
- Fluoromount-G (Southernbiotech, 0100-01)

Equipment

Equipment:

- Biorad real-time system (CFX384, Biorad)

· Leica cryostat (Leica Microsystems, CM3050S)

· Zeiss Confocal inverted laser scanning microscope with airyscan (Zeiss, LSM 880)

· Leica inverted microscope with a Leica DFC9000 CT camera (Leica, DMi8)

Software:

· SnapGene 4.3.11 (GSL Biotech LLC, San Diego, CA 92108)

· Fiji image J (open source)

Code:

· Matlab script for imaging quantitation: https://github.com/QilabGitHub/dCas12a-microscopy

Procedure

Screening of crRNAs in mouse P19 cells

1. For each target gene of interest, its promoter region is identified on ENCODE, and all canonical Cas12a PAMs (TTTA, TTTC, or TTTG) are identified. Please note that hyperCas12a can also target non-canonical PAMs (i.e., TTTT, TTCA, TTCC, CTTA) albeit at lower efficiency. CrRNA between 20-23bp can be used.

2. The crRNA can be purchased as a paired oligos and annealed with standard protocol (95°C for 5min, then stepped down 5°C per minute until room temperature), then ligated into pSLQ8453 (available on Addgene) that has been digested with Esp3 (NEB). Ligation reaction consists of 0.5uM of each oligo duplex, and 1ul of 10x T4 DNA ligase buffer (NEB) and 0.5ul of T4 DNA ligase in a total 10ul reaction incubated at 24C for 10 minutes. Please note that pSLQ8453 expresses a fluorophore (BFP) and the antibiotic resistance marker to hygromycin. Then, transform ligation reactions into Stellar competent cells, select colonies and verify by Sanger sequencing.

3. For CRISPR-activation, the crRNA plasmid should be co-transfected with hyperdCas12a-miniVPR (or a similar transcriptional activator), such as pSLQ10844. For CRISPR-repression, the crRNA plasmid should be co-transfected with hyperdCas12a-KRAB (or a similar transcriptional repressor), such as pSLQ10875. If enrichment of dual-transfected cells is desired by dual antibiotic selection, please ensure that both plasmids have distinct antibiotic resistance markers (e.g., pSLQ8453 which has PuroR, pSLQ10844 which has HygroR).

4. Culture mouse P19 cells in alpha-MEM with nucleosides (Invitrogen) with 10% FBS.

5. For transient transfection, seed P19 cells (in 6-well, 12-well, or 24-well plates) the day before transfection at $2x10^5$ cells/mL to aim for ~60-80% cell confluency at the time of transfection.

6. Perform transient transfections with 3 µl of Mirus X2 transfection reagent (Mirus) per µg of total plasmid according to manufacturer protocols.

7. For enrichment of transfected cells using double-selection, the cells can be treated the day after transfection with 500 ug/ml of hygromycin and 2 µg/ml of puromycin.

8. At 48 hours after selection (3 days post-transfection), collect the cells for analysis. The level of gene activation or repression and be assayed by qPCR or immunostaining, as described further in next sections.

Constructing crRNA arrays for multiplex gene targeting

1. CRISPR arrays for multiplex gene targeting can be first designed with the molecular cloning software SnapGene 4.3.11, with each crRNA separated by the direct repeat for LbCas12: AATTTCTACTAAGTGTAGAT, and the whole array driven by the U6 promoter. For an example, please refer to pSLQ10704.

2. The crRNA array can divided into ≤60 nt sequences with unique 4-nt 5' overhangs and ordered as oligos with standard desalting purification. Each pair of oligos can be annealed and then ligated as described in section above. Transformation can be carried out as described above. Verify the correct clone by Sanger sequencing. An initial screen by colony PCR can be used to reduce the number of candidate clones for sequencing.

Confirming changes in target gene expression levels

1. To quantify changes in mRNA levels of target genes by qPCR, isolate RNA from transfected cells with Qiagen RNeasy plus kit (Qiagen), followed by reverse transcription of 100ng RNA per condition into cDNA using iScript kit (Biorad). Perform quantitative real-time PCR (qRT-PCR) with SYBR master mix (Biorad) per manufacturer's protocol. Primers used for our gene targets of interest are included in supplementary information of our main paper. Quantify RNA expression by ΔΔCt, with normalization based on expression of glyceraldehyde 3-phosphate dehydrogenase.

2. To detect changes in protein expression of targeted genes, immunohistochemistry can be performed if there exist high-quality antibodies to the proteins of interest. Seed transfected P19 cells into black flat-bottom 96-well plates 2 days after transfection (thus 1 day after the start of dual-antibiotic selection), keeping them in dual-antibiotic selection media. The next day, aspirate out the media and fix cells with 4% formaldehyde in 1x PBS for 15 minutes. Then, aspirate out the formaldehyde solution and permeabilize cells with 0.25% Triton X-100 in 1x DPBS for 5 minutes, and apply blocking solution (5% donkey serum in DPBS) for 0.5-1hr. Then apply primary antibodies diluted in blocking solution, and move the plate to 4C

overnight after wrapping in aluminum foil. Next day, wash each well 3x with 1x DBPS and apply secondary antibody Alexa Fluor-conjugated 488nm or 647nm secondary antibodies diluted in blocking solution. Please refer to our antibody list for catalog numbers and dilutions that we used for our proteins of interest. Please note that since our dCas12a plasmid (pSLQ10844) contains mCherry, we did not use any 594nm secondary antibodies. Lastly, each cell was washed 3x with 1x PBS and kept in 1x PBS for imaging. Since our crRNA plasmid (pSLQ8453) contains BFP, we did not use a nuclear dye. Imaging was done with Leica DMi8 inverted microscope with 20x objective and a Leica DFC9000 CT camera.

3. For quantitation of cell fluorescence intensities, it is possible to use a semi-automatic image analysis pipeline based on Matlab (version R2019a), available at https://github.com/QilabGitHub/dCas12a-microscopy. Since our target genes were all nuclear-localized transcription factors, and since dCas12a-NLS is directly fused to mCherry (i.e., without a 2A self-cleaving peptide), we were able to use threshold-based segmentation based on the mCherry channel (which is predominantly nuclear). Morphological operations can be applied to remove noise and thus yields masks for single cells. Based on the masks, mean fluorescent intensities of all corresponding channels for every cell can be collected for further statistical analyses.

Multiplex gene regulation in the mouse retina by *in vivo* electroporation

1. For *in vivo* targeting, the U6-crRNA array and CAG-dCas12a-miniVPR (without mCherry) were combined into a single plasmid using NEBuilder HiFi DNA Assembly (NEB). Please refer to pSLQ10873 as an example.

2. For in vivo electroporation experiments, pregnant CD1 mice were purchased (Charles River Laboratories), and maintained under standard conditions until they gave birth. The neonatal mice can be used for electroporation at post-natal day 0-3 (with electroporation efficiency diminishing around day 4).

3. Make a mix of crRNA-array-dCas12a plasmid (e.g., pSLQ10873) and a CAG-GFP plasmid (pSLQ10301, used as marker of electroporation boundary) at ~5:1 ratio while maintaining the total concentration of 2μg/μl. Please only use high-quality DNA preps for in vivo delivery (Qiagen endotoxin-free midiprep or maxiprep kit).

4. For details on subretinal injection and in vivo electroporation, please refer to published methods paper[1]. Briefly, anesthetize newborn mice on ice and carefully incise the lids open with a sharp 30-gauge needle. Then, inject 0.3 - 0.5 μl of DNA solution (with 0.1% FastGreen dye as an injection tracer) into the subretinal space. Place the head of the injected pup between a 10mm diameter tweezer electrode and apply 5 pulses of 80V, 50ms each, at an interval of 950ms.

5. Sac the animals at the desired timepoint. It is known that GFP expression is detectable after 3 days[1] and up to several months[2] post-electroporation. Dissect out the electroporated eye and fix in 4%

paraformaldehyde (PFA) in 1x PBS (pH 7.4) for 2 hours at room temperature. Then, dissect out the retina and equilibrate into a series of sucrose solutions at room temperature (5% sucrose in 1X PBS, 5 min; 15% sucrose in 1X PBS, 15 min; 30% sucrose in 1X PBS, 1 hr; 1:1 mixed solution of OCT and 30% sucrose in PBS, 4°C, overnight), then freeze and store at -80°C.

6. Using a Leica CM3050S cryostat (Leica Microsystems) or equivalent, prepare 20 µm cryosections and place onto Superfrost microscope slides (Fisherbrand), which can be stored at -80°C.

7. For immunostaining of cryosections, wash the slides briefly in 1X PBS, then incubate in 0.5% Triton in 1X PBS for 20 min, then block for 30 min in blocking solution of 0.1% Triton, 1% bovine serum albumin and 10% donkey serum (Jackson ImmunoResearch Laboratories) in 1X PBS.

8. Incubate slides overnight in primary antibodies diluted in block solution, in a humidified chamber at 4°C. Then, after washing in 0.1% Triton in 1X PBS three times, incubate slides in secondary antibodies and DAPI (Sigma-Aldrich; D9542) for 1-2 hr, then wash three times with 0.1% Triton in 1X PBS and mount the slides with Fluoromount-G (Southern Biotechnology Associates). For the antibodies we used for our proteins of interest, please refer to “Antibodies” section above.

9. Image slides with LSM 710 or LSM 880 Confocal inverted laser scanning microscope, with Plan Apochromat objective with 405, 488, 561 and 633 lasers.

Troubleshooting

Time Taken

Anticipated Results

References

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