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CRISPR/Cas-Mediated Knock-in of Genetically Encoded Fluorescent Biosensors into the AAVS1 Locus of Human Induced Pluripotent Stem Cells

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Abstract:	Genetically encoded fluorescent biosensors (GEFBs) enable researchers to visualize and quantify cellular processes in live cells. Induced pluripotent stem cells (iPSCs) can be genetically engineered to express GEFBs via integration into the Adeno-Associated Virus Integration Site 1 (AAVS1) safe harbour locus. This can be achieved using CRISPR/Cas ribonucleoprotein targeting to cause a double-strand break at the AAVS1 locus, which subsequently undergoes homology-directed repair (HDR) in the presence of a donor plasmid containing the GEFB sequence. We describe an optimized protocol for CRISPR/Cas-mediated knock-in of GEFBs into the AAVS1 locus of human iPSCs that allows puromycin selection and which exhibits negligible off-target editing. The resulting iPSC lines can be differentiated into cells of different lineages while retaining expression of the GEFB, enabling live-cell interrogation of cell pathway activities across a diversity of disease models.

CRISPR/Cas-Mediated Knock-in of Genetically Encoded Fluorescent Biosensors into the AAVS1 Locus of Human Induced Pluripotent Stem Cells

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i. Running Head

Genetically-encoded fluorescent biosensors in iPSCs

ii. Summary/Abstract

Genetically encoded fluorescent biosensors (GEFBs) enable researchers to visualize and quantify cellular processes in live cells. Induced pluripotent stem cells (iPSCs) can be genetically engineered to express GEFBs via integration into the Adeno-Associated Virus Integration Site 1 (AAVS1) safe harbour locus. This can be achieved using CRISPR/Cas ribonucleoprotein targeting to cause a double-strand break at the AAVS1 locus, which subsequently undergoes homology-directed repair (HDR) in the presence of a donor plasmid containing the GEFB sequence. We describe an optimized protocol for CRISPR/Cas-mediated knock-in of GEFBs into the AAVS1 locus of human iPSCs that allows puromycin selection and which exhibits negligible off-target editing. The resulting iPSC lines can be differentiated into cells of different lineages while retaining expression of the GEFB, enabling live-cell interrogation of cell pathway activities across a diversity of disease models.

iii. Keywords:

Induced pluripotent stem cell, fluorescent biosensor, CRISPR/Cas, AAVS1, live cell imaging.

1. Introduction

Induced pluripotent stem cells (iPSCs) have enabled research into disease mechanisms in a manner minimally invasive for the donor, and particularly so for diseases that affect organs such as the brain that are otherwise inaccessible for cell and molecular studies [1–3]. Thus, differentiating iPSCs into neural cell types allows studies into how the functions of cells are affected by an individual's common genetic variants or disease-causing mutations [4–6]. For example, iPSC-derived cell types have given novel insight into disease mechanisms, examined biomarkers of disease, and identified targets for novel therapeutics [7–11].

The development of gene editing technologies has expanded the scope of iPSC-based disease modelling, enabling generation of isogenic iPSC lines in which disease-causing mutations have been introduced into control iPSC [12] or converted to the reference allele in disease-specific cells [13], or in which specific gene knock-out/insertions [14, 15] have been used to study gene function. Using CRISPR/Cas technology, it is also possible to target exogenous gene knock-ins to safe harbour sites (SHS), regions of the genome where genes can be inserted, function predictably, and not cause deleterious effects. One of the most well-characterized SHS is the adeno-associated virus integration site 1 (AAVS1), having previously shown robust transgene expression, without discernible phenotypic effects or deleterious damage to adjacent genes [16–18]. Targeted insertion into the AAVS1 locus using CRISPR/Cas technology requires a crRNA to recruit the Cas nuclease to DNA in a sequence-specific manner, and a homology-directed repair (HDR) template, typically a plasmid, with a cloning site positioned between two 'arms' homologous to the AAVS1 locus [18].

Genetically encoded fluorescent biosensors (GEFB) enable quantitation of how live cells respond to specific stimuli to determine if specific organelles or pathways are affected [19–21]. In this manner, they provide a 'readout' of alterations to cell states using assays that are non-destructive, in contrast to techniques such as immunofluorescence or cell-based assays

of metabolite levels which require cell fixation or lysis. In particular, GEFBs that enable multi-colour imaging are useful tools for quantitation of cell functions because the signal ratio can be calculated during time course experiments, and thereby indicate the extent of activity at specific time points with spatial precision. Because of these features, GEFBs have been developed to quantify changes in enzyme activities and metabolite fluxes, some of which are readily available from repositories such as *Addgene* (www.addgene.org).

Typical GEFBs utilise a fluorescent protein conjugated to a custom protein or peptide that renders the fluorescent protein sensitive to the presence of biomolecules (e.g., enzyme activity) or environmental changes such as pH, neurotransmitter levels, or ion flux [22-27]. Frequently, fluorescence resonance energy transfer (FRET) is used as a readout from a GEFB, for example, by engineering a biosensor composed of two different fluorescent proteins separated by a protease cleavage site [28]. Another creative method of reporting biological activity includes producing equimolar amounts of two different fluorescent protein conjugates, allowing one to serve as an internal control, whilst the other is degraded by a process of interest, such as autophagy [29]. Using this approach, the internal control protein will become predominant as the other fluorescent protein conjugate is degraded.

Here we detail protocols for design and generation of AAVS1-targeting HDR templates with GEFBs (*see* **Figure 1**) including protocols for Gibson assembly (*see* **Figure 2**), and using our previously described protocol for editing human iPSCs using CRISPR/Cas ribonucleoproteins [30]. The resulting iPSC lines serve as innovative approaches to understanding temporal relationships between diverse cell functions using high-throughput imaging-based experiments. We present an example of one such experiment from our laboratory including results from screening of predicted ‘off-target’ loci of the CRISPR/Cas crRNA (*see* **Figure 3**). We thereby present a well-characterised crRNA and HDR template backbone, alongside a protocol that is adaptable for other GEFBs or exogenous open reading frames.

2. Materials

2.1 Reagents and materials.

2.1.1 Molecular biology reagents

AAVS1-SA-puro-EF1-MCS plasmid (System Biosciences)

*Age*I-HF (NEB)

NEBuilder HiFi DNA Assembly Master Mix (NEB)

Competent *E. coli* (NEB)

LB agar

Ampicillin

DNA Gel Extraction Kit

Plasmid Extraction Kits

Genomic DNA Isolation Kit

BigDye Terminator v3.1 Cycle Sequencing Kit

CleanSEQ Dye-Terminator Removal Kit

Q5 High-Fidelity 2X Master Mix

Agarose, buffer and SYBR Safe for electrophoresis

PCR primers for CRISPR/Cas 'off-target' sites (*see* **Table 1**)

Primers to amplify GEFB (with *Age*I sticky ends)

2.1.2 CRISPR/Cas reagents.

Alt-R CRISPR-Cas9 tracrRNA ATTO 550 (IDT)

HiFi AltR-Cas9 Nuclease V3 (IDT)

Alt-R HDR Enhancer (IDT)

Alt-R cRNA to target AAVS1 locus (IDT):

/AltR1/rGrUrCrArCrCrArArUrCrCrUrGrUrCrCrCrUrArGrGrUrUrUrUrArGrArGrCrUrArUrGrCrU

/AltR2/

TE buffer

2.1.3. iPSC culture reagents

mTeSR Plus (Stem Cell Technologies)

Vitronectin XF (V-XF) (Stem Cell Technologies)

ReLeSR (Stem Cell Technologies)

ROCK Inhibitor (ROCKi/Y-27632)

P3 Nucleofection Kit, including P3 solution and supplement, pmaxGFP, and cuvettes (Amaxa)

Trypan Blue Stain

CloneR supplement (Stem Cell Technologies)

Dulbeccos's Phosphate-buffered saline (DPBS; Gibco)

Knockout Serum Replacement (Gibco, *see* **Note 1**)

DMSO for cell culture

2.2 Laboratory consumables.

Non-tissue culture treated plasticware

Molecular biology plasticware

Cell strainer (37 µm)

Syringe filter (0.22 µm)

2.3 Specific Equipment.

4D-Nucleofector Core unit, with X unit (Amaxa).

3. Methods

All steps involving medium exchange or passaging of cells should be performed using sterile technique in a biological safety cabinet. Approval from relevant institutional committees is required for research involving human iPSC. The work described herein was performed with approval from HREC-Tasmania (H0014124) and from the University of Tasmania Institutional Biosafety Committee.

3.1 Design and Generation of AAVS1-Fluorescent Biosensor Homology-directed Repair Template

Benchling's molecular biology suite (http://www.benchling.com) provides comprehensive tools for planning of cloning steps, crRNA/primer design and performing diagnostic restriction digests. The AAVS1-donor vector (AAVS1-SA-puro-EF1-MCS) is supplied purified by the manufacturer but should be re-transformed into competent cells for use in experiments to avoid original stock depletion. Linearization of the AAVS1-donor vector using *Age*I must be completed for subsequent Gibson assembly with the fluorescent biosensor fragment.

3.1.1 Gibson assembly of GEFB with AAVS1-donor vector

1. Import the GEFB of interest and the AAVS1-donor vector plasmid sequence files into Benchling.
2. Using the Assembly Wizard, create a Gibson assembly of the AAVS1-donor vector (backbone) with desired GEFB (insert) ligated via *Age*I sticky ends (*see* **Note 2** & **Figure 2**).
3. Create an appropriate number of LB agar plates, with antibiotic selection, (*see* **Note 3**).
4. Obtain the bacterial stab(s) from Addgene (stored at 4^{o}C).
5. Create a streak plate of the bacterial stab and incubate at required temperature overnight (*see* **Note 4**).
6. Create liquid broth cultures (with selection antibiotic) by picking single colonies from the streak plate.
7. Incubate overnight at respective temperature on an orbital shaker (*see* **Note 5**).
8. Remove from the orbital shaker and prepare a glycerol stock by adding 500 µL of sterile 50% glycerol to 500 µL of culture in a screw-top tube. Transfer glycerol stocks to -80^{o}C as soon as possible.

9. Perform a miniprep using Promega Wizard Plus DNA Purification System kit, or similar, following manufacturer's protocol.
10. Measure the concentration of the DNA using a Nanodrop spectrophotometer (*see* **Note 6**).
11. Store purified DNA product at -20°C.
12. Linearize the AAVS1-SA-puro-EF1-MCS plasmid with *Age*I (*see* **Table 2**).
13. Incubate at 37°C for 15 minutes (overnight digestion is also safe).
14. Perform PCR for fluorescent biosensor using Q5 High Fidelity 2 × Master Mix.
15. Electrophorese both the fluorescent biosensor PCR reaction and the *Age*I-linearised AAVS1-SA-puro-EF1-MCS vector through an agarose gel.
16. Gel extract and purify each DNA fragment using a DNA Gel Extraction Kit following manufacturer's instructions.
17. Measure DNA concentration using a Nanodrop spectrophotometer.
18. To perform the Gibson assembly with two fragments, use 0.02-0.2 pmol of DNA fragments in a 1:2 ratio of vector:insert, and make up to 10 µL with ultrapure nuclease free water (*see* **Note 7**).
19. Add 10 µL of Gibson Assembly NEBuilder Hifi DNA Assembly Master Mix to the PCR tube.
20. Incubate at 50°C for 1 hour.
21. Place the tube on ice and store at -20°C until needed.
22. To amplify the assembled vector, prepare LB agar plates (with ampicillin selection).
23. Thaw competent *E. coli* on ice.
24. Add 1-5 µL (containing 1pg-100ng) of plasmid DNA to the *E. coli* (once thawed), carefully tap the tube to mix, and then place back on ice for 30 minutes (avoid vortexing).
25. Heat-shock the cells in a dry block at 42°C for 30 seconds**.**
26. Place the cells immediately back on ice for 2 minutes.

27. Add 500 µL of SOC medium to the cells, secure the tube horizontally on an orbital shaker (at 250 rpm), and incubate at 30°C for 1 hour.
28. During this time prepare a sterilised work area and warm selection plates to 30°C.
29. Centrifuge the tube at 5,000 rpm for 5 minutes, aspirate 400 µL of supernatant (without disrupting cell pellet), and resuspend pelleted cells in the remaining 150 µL of solution.
30. Pipette 50 µL of the cell solution onto an agar plate with ampicillin and evenly spread over the surface using a microbial cell spreader.
31. Incubate plates at 30°C overnight.
32. Create LB broth culture via single colony pick and incubate overnight at 30°C.
33. Generate glycerol stocks and perform maxiprep using an endotoxin-free purification kit following the manufacturer's instructions.
34. Measure the concentration of the DNA using a Nanodrop spectrophotometer.
35. Store purified plasmid DNA at -20°C.

3.1.2. Quality Control for AAVS1-Fluorescent Biosensor Plasmid

The first step to ascertaining whether the Gibson assembly was successful is to perform a diagnostic restriction digest. Expected results can be visualised using Benchling, before confirmation with purified plasmid. Following this, Sanger sequencing is performed to check for any erroneous ligation or mutation events.

1. Open the desired AAVS1-FB file in Benchling (or similar).
2. Select 'Digests' (depicted by a scissor icon), and search through the different enzymes for one that generates 2-6 cuts.
3. Confirm that there is at least one cut through each fragment being ligated.
4. Select 'Run Digest' and then 'Virtual Digest' to visualise a virtual gel with expected fragments that can confirm correctly assembled plasmid (*see* **Figure 4**).

5. Using the restriction enzyme selected in step 37 and purified plasmid from step 33-35, perform a restriction digest to validate successful assembly of the AAVS1 donor vector with GEFB.
6. For verification by Sanger sequencing, generate a reaction master mix of BigDye Buffer, BigDye Terminator and nuclease-free H_2O (*see* **Table 3**).
7. Distribute 8 µL to the appropriate number of PCR tubes (2 per plasmid to be sequenced), followed by a forward or reverse primer (not both) and plasmid.
8. Using a thermocycler, denature DNA at 96^{o}C for 1 minute and run 25 cycles consisting of denaturation at 96^{o}C for 10 seconds, annealing at 50^{o}C for 5 seconds and extension at 60^{o}C for 4 minutes.
9. Prepare a wash buffer by diluting 100% molecular grade ethanol to 85% using nuclease-free water; 300 µL is needed for each reaction.
10. Once the PCR has finished, centrifuge the PCR tubes briefly.
11. Vortex CleanSEQ magnetic beads and add 10 µL of CleanSeq beads to each PCR tube.
12. Add 42 µL of 85% ethanol to each PCR tube.
13. Invert to mix and then briefly centrifuge the PCR tubes to pellet the beads.
14. Place the PCR tubes on a magnetic separation rack.
15. Ensuring the PCR tube remains on the magnetic rack, aspirate and discard cleared solution.
16. Add 100 µL of 85% ethanol to each tube.
17. Incubate for 30 seconds to wash.
18. Adjust pipette to 130 µL to ensure full removal and aspirate.
19. Add another 100 µL of 85% ethanol.
20. Remove the clear solution using 130 µL pipette volume.
21. Using a P20 pipette, remove any residual fluid.
22. Allow the tubes to dry for three minutes.
23. Add 30 µL of ultrapure nuclease free water.

24. Flick the PCR tubes gently so the beads release the product (keep flicking until the magnetic beads are homogenous with the solution, *see* **Note 8**).
25. Centrifuge the tubes briefly (*see* **Note 9**).
26. Carefully pipette 15 µL of cleared supernatant containing the sequencing reaction products onto MicroAmp optical 96-well reaction plate with septa (*see* **Note 10**).
27. Using a thermocycler, denature the DNA at 95ºC for 5 minutes to remove secondary structures (*see* **Note 11**).
28. Run the plate on the ABI Sanger sequencer.
29. Align the resulting sequence against the expected sequence using Benchling (or similar), to exclude any nucleotide changes or the presence of indels before continuing to further steps.

3.2 iPSC editing using CRISPR-Cas and Homology-directed Repair

3.2.1 Assembly of CRISPR/Cas Ribonucleoprotein

1. To prepare crRNA solutions, spin the tubes down to collect all the powder at the bottom of the tube and resuspend the 2 nmol of each oligo in 20 µL of nuclease free IDTE buffer or nuclease free duplex buffer resulting in 100 µM solution.
2. In a PCR tube, create a final crRNA:tracrRNA duplex concentration of 50 µM by adding 2 µL of AAVS1 crRNA (100 µM) to 2 µL of tracrRNA-ATTO-550 (100 µM) (*see* **Note 12**).
3. Briefly centrifuge, then heat at 95ºC for 5 min in a PCR thermal cycler, protected from light.
4. Briefly centrifuge, spray with 70% ethanol and place back in the biosafety cabinet. Allow to cool to room temperature (20–25ºC).
5. Mix 3 µL of the assembled crRNA:tracrRNA duplex with 2.1 µL Cas9 purified, recombinant protein, gently swirling the pipet tip while pipetting.
6. Incubate at room temperature for 10–20 min to assemble the RNP complex.

3.2.2 Nucleofection of iPSC and puromycin selection

Cells are ready for nucleofection when at ~80% confluency, and free of differentiation and microbial contamination. The AAVS1-donor vector contains a puromycin resistance gene that enables selection of cells in which the plasmid HDR template has been incorporated into the AAVS1 locus. If not already known for the iPSC line being used, a kill curve will need to be established to determine the minimum concentration that kills 100% of parental iPSCs in 7-10 days before proceeding with CRISPR/Cas-mediated knock-in (*see* **Note 13**). Similarly, if nucleofection conditions are not optimised, consider testing several programs and assessing the percentage of cells nucleofected and their viability.

1. Coat a non-tissue culture-treated 6-well plate with 10 µg/mL V-XF in CellAdhere Dilution buffer, and leave the plate at room temperature for 1 hour.
2. Aspirate the V-XF solution and wash with CellAdhere Dilution buffer.
3. Immediately add 1.7 mL of prewarmed (37ºC) mTeSR Plus medium with ROCKi and HDR Enhancer (24 µM) added per well.
4. Aliquot mTeSR Plus and add 10 µM ROCKi (see **Note 14**).
5. Prepare complete Nucleofector Solution (100 µL per reaction; 82 µL P3 Nucleofector Solution with 18 µL Nucleofector Supplement).
6. Aspirate spent medium from the culture vessel (*see* **Note 15**).
7. Rinse wells once with 2 mL DPBS.
8. Add 1 mL TrypLE-select to cells, and incubate the vessel at 37°C, 5% CO_2 for 5 minutes.
9. Gently tap the plate to detach cells.
10. Gently pipette the cells up and down 5−10 times with a 1000 µL pipette to generate a single cell suspension (*see* **Note 16**).
11. Transfer the cell suspension to a conical tube containing 3 mL of mTeSR Plus medium (+ ROCKi) to dilute the dissociation reagent.
12. Centrifuge the iPSCs at 200 × *g* for 3 minutes, aspirate and discard the supernatant.

13. Flick the tube 3−5 times to loosen the pellet, then resuspend the cells by pipetting them up and down 5−10 times in 2 mL of mTeSR Plus medium (+ ROCKi).
14. Count viable cells using Trypan blue and your preferred method of counting cells.
15. For each sample, centrifuge the required number of cells ($0.8 – 1 \times 10^6$ cells per sample) at 200 × *g* for 3 min at room temperature.
16. Remove supernatant (*see* **Note 17**), and resuspend the cell pellet carefully in 100 µL room temperature complete Nucleofector Solution per sample.
17. Add 5 µL RNP and 1 µL Alt-R Cas9 Electroporation Enhancer to each tube, as well as 0.5 µg of AAVS1-GEFB plasmid to each sample tube. Ensure that DNA is sufficiently concentrated to avoid adding a volume more than 10% of the reaction volume.
18. Add 100 µL cells in complete Nucleofector Solution, gently mix and immediately transfer cell/DNA suspension into a nucleofection cuvette (*see* **Note 18**) and close with cap.
19. Insert the cuvette with cell/DNA suspension into the Nucleofector Cuvette Holder, close lid and apply the nucleofection program optimized for the iPSC line being used. We have found that program CB150 provides high viability and sufficient efficiency.
20. Once the program has finished, remove the cuvette from the holder, sterilize it and place it in the biosafety cabinet.
21. Add 200 µL of mTeSR Plus medium (+ ROCKi) to each cuvette. Using the sterile transfer pipette supplied with the nucleofection reagents, seed the entire contents of the cuvette into one well from the prepared 6-well plate. Use one well per nucleofection reaction.
22. Incubate the cells in a humidified 37°C, 5% CO_2 incubator.
23. Change medium after ~24 hours using mTeSR Plus (without ROCKi). Use 1 mL tips to change media so as to avoid contamination.
24. Continue replacing medium daily until cells reach 80% confluence, then passage each culture into multiple wells of a 6-well plate and continue to refresh medium daily.

25. Once cells reach 80% confluence, perform puromycin selection for 7-10 days replenishing medium (with puromycin) daily. Use appropriate iPSC as a negative control; we typically use cells nucleofected with the CRISPR/Cas RNP but without the donor HDR plasmid template.
26. Once only puromycin resistant colonies are left, expand the cultures for cryopreservation.

3.2.3 Cryopreservation of Cell Cultures

Cryopreserve extra cell cultures for future experiments once they reach approximately 80% confluency, as a reserve of polyclonal cell cultures if subsequent generation of monoclonal cell lines is unsuccessful.

1. Prepare freezing solution: 10% DMSO in KnockOut Serum Replacement.
2. Transfer the freezing solution to 4ºC until needed.
3. Aspirate spent medium and wash cells with 1 mL/well DPBS.
4. Add 1 mL/well of ReLeSR and aspirate ReLeSR within 1 minute.
5. Incubate at 37ºC for 5 min.
6. Add 1 mL of the pre-chilled freezing solution to each well using a serological pipette.
7. Gently tap the plate on the sides for 30-60 seconds to dislodge cells.
8. Using a 5 mL serological pipette, transfer 1 mL of cell suspension (*see* **Note 19**) to each cryotube, transfer tubes to a freezing container (e.g. CoolCell or Mr. Frosty), store at -80ºC for 24 hours and then transfer cells to vapour phase of liquid nitrogen for long term storage.

3.2.4. Isolation and Expansion of AAVS1-GEFB Monoclonal iPSC Line

1. Prepare CloneR medium; add 2.5 mL of CloneR to 22.5 mL of complete mTeSR Plus and mix thoroughly (*see* **Note 20**).
2. Culture puromycin-resistant, polyclonal iPSC to ~80% confluency, aspirate spent medium and wash the cells twice with DPBS.

3. Add 1 mL TrypLE-select, incubate the vessel at 37°C, 5% CO_2 for 5 min.
4. Gently aspirate and expel the TrypLE-select with a 1000 µl pipette to generate a cell suspension.
5. Dilute dissociating reagent by transferring the cell suspension to a conical tube containing 3 ml CloneR medium.
6. Centrifuge the iPSC suspension at 200g for 3 minutes and then discard the supernatant.
7. Resuspend the cell pellet in 1 ml of CloneR medium.
8. Perform a cell count, and seed 2×10^3 cells per well in a V-XF coated 6 well plate in CloneR medium, and culture for 5-7 days. Change the medium to fresh mTeSR Plus every second day.
9. Monitor cell growth using an inverted microscope; when colonies derived from single cells are 200-250 µm in diameter, they are ready to be manually picked.
10. Coat a 12-well plate with V-XF at room temperature for 1 hour.
11. Aspirate V-XF from the 12-well plate and add 1 mL CloneR medium to each well.
12. Pre-equilibrate the 12-well plate to receive the picked colonies in a 37ºC and 5% CO_2 incubator until needed.
13. Wash the well with single cell-derived colonies for picking with 2 mL DPBS.
14. Add 1 mL ReLeSR and aspirate within one minute.
15. Gently add 2 mL CloneR medium immediately.
16. Using a stereomicroscope fitted with a heated stage in a Class II biosafety cabinet, use a 200 µL micropipette to collect a single colony in 50 µL medium and transfer it to a single well of a U-bottom 96 well plate.
17. Gently pipette up and down to break up the colony and transfer the entire 50 µl to a single well of the pre-equilibrated 12-well plate with CloneR medium.
18. Repeat steps 16. and 17. until a single colony has been transferred to each well of the 12-well plate.
19. Return the 12-well plate to the incubator and do not disturb for 48 hours. Thereafter, perform a full- medium change with CloneR medium and incubate for 24 hours at 37ºC.

20. At day 4+ perform medium changes with mTeSR Plus until colonies are 60-80% confluent.
21. Using a microscope or other instrument capable of fluorescent imaging of live cells, view each well of the 12 well plate using the appropriate excitation/emission wavelengths for the selected GEFB.
22. Identify wells with colonies that have the most uniform expression of the GEFB across all cells. These should be harvested, expanded and cryopreserved, and subjected to subsequent quality control assays. Any remaining monoclonal iPSC cultures can also be cryopreserved if desired.

3.3 Quality Control of Monoclonal iPSC Lines

Ascertaining whether the newly generated GEFB iPSC line is of high-quality and has not been significantly perturbed through off-target CRISPR/Cas editing is vital before proceeding to experiments investigating factors that modulate activity of the GEFB (*see* **Note 21 & Figure 5**). Using PCR and Sanger sequencing, regions predicted to be susceptible to off-target editing of CRISPR/Cas can be analysed for any insertion/deletion events.

1. Extract iPSC gDNA from a 80% confluent well using a QIAmp DNA Micro Kit following manufacturer's instructions.
2. Perform PCR for each off-target site (*see* **Table 2**) using Q5 MasterMix and confirm single amplicons of expected size are produced using agarose gel electrophoresis.
3. Gel extract the amplicons and purify using a DNA Gel Extraction Kit.
4. Perform Sanger sequencing of the purified PCR products, and compare to appropriate control sequences to identify any indel mutations (*see* **Note 22**). Other standard quality control assays for iPSC should also be conducted (*see* **Note 23**).

3.4 Testing Fluorescent Biosensor Model Functionality

Where appropriate, functionality of the generated GEFB model can be tested using relevant drugs to elicit a response prior to differentiation. In this example from our own lab, we used

the proteasome inhibitor MG132 to induce autophagy in an iPSC line in which we have used the protocol described here to knock-in the GFP-LC3-RFP-LC3ΔG autophagy biosensor [29] [31], and used live-cell imaging to record levels of GFP and RFP fluorescence at 20 minutes intervals over the course of five hours (*see* **Figure 6**). Consistent with the use of this biosensor in other models, in response to exposure to MG-132 GFP fluorescence decreased over time, indicating induction of autophagy and degradation of GFP-LC3, whereas the autophagy resistant RFP-LC3ΔG remained relatively constant.

4. Notes

1. Thaw KnockOut Serum Replacement overnight at 4°C. It is stable at 2°C to 8°C for up to 4 weeks, protected from light. Working volumes can be aliquoted and stored at −20°C. Thaw aliquots as needed, avoiding additional freeze-thaw cycles.
2. Once complete, under the 'Assembly' tab, the primers can be checked for efficiency before being ordered. This includes checking the FWD and REV primers for mispriming, appropriate GC clamp, difference in primer melting temperature, etc.
3. LB agar plates can be made beforehand and stored at 4ºC for several days or weeks depending on the stability of the required antibiotic. The antibiotic required can be found on the Addgene plasmid product page under 'Growth in Bacteria.'
4. The required temperature for the bacteria to grow will vary from plasmid to plasmid and can be found on the Addgene plasmid product page under 'Growth in Bacteria.'
5. Tubes are often placed at an angle on the swirling rack with a loose cap to better ventilate the liquid culture.
6. Ensure the 260/280 value is close to 1.8.
7. Typically, our lab has used 0.02 pmol of vector and 0.04 pmol of insert.
8. Do not vortex the PCR tubes during this step as it shears the long DNA fragments.
9. There may be magnetic beads that do not dissolve; flick the tube and repeat centrifugation.

10. If there are empty wells in any of the used columns, fill them with 15 µl of nuclease free water.
11. Do not heat the lid of the thermocycler.
12. Work without light in the biosafety cabinet to protect fluorescent tracrRNA. The duplex amount provided is for 1 reaction. As a minimum you would require two reactions – one for the AAVS1-FB plasmid and the other for a no plasmid control.
13. Generally, iPSC lines are sensitive to approximately 1 µg/ml of puromycin. The puromycin kill curve will need to be applied to iPSC cultures that are at similar confluence (usually 80%) to be an accurate and effective measurement. Perform the puromycin kill curve (recommended 0.5-2 µg/ml) in a 6-well plate as this will better reflect the future CRISPR/Cas experiment also performed in a 6-well plate.
14. During this experiment, all steps using mTeSR Plus should also contain 10 µM ROCKi or CloneR to avoid significant cell death.
15. Using a stereomicroscope, iPSC cultures can be manually cleaned of differentiated cells if necessary.
16. To check that a single cell suspension has been generated, view the cells under an inverted microscope. If there are cell clumps, continue to resuspend up and down in the biosafety cabinet and check again on the microscope. Repeat until cells are singular.
17. Use 1 mL pipette tip to discard supernatant followed by a 200 µL pipette tip to get rid of most of the medium closer to the cell pellet.
18. The cell suspension must cover the bottom of the cuvette without air bubbles.
19. To ensure optimal survival of iPSCs, keep cell clumps large and avoid breaking up aggregates too small.
20. If not used immediately, store at 2 - 8^{o}C for up to one week.
21. Embryoid body formation should be performed to identify whether the GEFB line can differentiate into all three germ layers (ectoderm, endoderm and mesoderm), and karyotyping performed to ascertain whether there are any chromosomal abnormalities.

22. There may be single nucleotide polymorphisms within the sequencing data, however this must be compared between the unedited ‘wildtype’ iPSCs and the edited HDR knock-in iPSCs.
23. A T7 Endonuclease I mismatch cleavage assay may be performed to detect on/off-target genome editing [30].

Acknowledgements

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of cyan fluorescent proteins towards a quantum yield of 93%. Nat Commun 3:751

Figure Captions

Figure 1. Overview of the concept for generating individual-derived GEFB iPSC models for high-throughput imaging analysis. A) Identify population of interest. B) Obtain iPSC-derived from healthy controls or patients. C) Perform CRISPR/Cas9 gene editing of individual's iPSCs to generate GEFB model suited to visualising hypothesis. D) Ensure newly established GEFB iPSC models are pluripotent and not genetically perturbed. E) Perform custom experiment to test hypothesis. F) Visualise effect using automated fluorescent live-cell imaging system.

Figure 2. Overview of achieving Gibson assembly of AAVS1-donor vector and FB. A) General plasmid map of AAVS1-donor vector. B) Linearization of AAVS1-donor vector using AgeI restriction digest. C) Purification of gel extracted FB amplicon and mixing with AAVS1-donor vector in addition of Gibson assembly components. D) Incubation period for ligation. E) Transformation of Gibson assembled plasmid in competent cells.

Figure 3. Sanger sequencing of A) TTN-AS1, B) Chromosome 10, C) TRPM7 and D) THSD4, confirming absence of off-target editing at sites predicted using Synthego program.

Figure 4. Example of diagnostic restriction digest following Gibson assembly of AAVS1 donor vector and pMitoTimer [32]. A) Virtual restriction digest generated using Benchling. B) Gel electrophoresis of AhdI digested AAVS1-pMitoTimer, matching virtual digest and therefore confirming successful ligation. C) Benchling generated overview of restriction digest, including corresponding length of amplicons (dashed box).

Figure 5. Example of karyotyping for AAVS1-Tubulin, generated from AAVS1 donor vector and pmTurquoise2-Tubulin [33]. A) Fluorescent imaging of AAVS1-Tubulin iPSC colony. B) Whole genome virtual karyotyping plot of AAVS1-Tubulin obtained using HumanCytoSNP-12.

Figure 6. Example biosensor experiment using AAVS1-GFP-LC3-RFP-LC3ΔG iPSC line. After application of MG132, iPSCs were imaged every 20 minutes for 5 hours in an automated fluorescent imaging system. Key timepoints are shown and exhibit the increasing level of autophagy with time. The gradual degradation of GFP-LC3 can be seen as time progresses in the GFP channel, resulting in RFP-LC3ΔG becoming the predominant signal.

Table 1. PCR Primers used to screen for off-target editing.

Gene/Locus	Primer Sequence	Annealing Temp (∘C)	PCR Product size (bp)
THSD4	FWD – tgccttcttgccttgcatcctagc REV – ttaaaactgcaggcgggagagc	72	446
TRPM7	FWD – ttcacacagactcgtggcatcc REV – agcactatcctcaccctgctttgc	72	519
RP11-212121.2	FWD – tggtccatgtcagtggttctca REV – ggtaaatgaggcactgagggtg	72	258
Chromosome 10	FWD – aaggtcacttctgggcttaggc REV – tgcacacatacatgcctgcacc	72	797
ARHGAP31	FWD – aacttcaaactccacggctccagc REV – tgaacaccagcattgcagaggc	72	1083
TTN-AS1	FWD – ctgggaatagaaagactacttcc REV – tcaggcatagtctgtgaggtcaaa	67	627
JAKMIP1	FWD – tgtgctttgatggttcccagcg REV – agaagatgcagtcagggcaaagc	72	817
APPL2	FWD – aaaatgagctgggtgtggtggtgc REV – atccatgctcagcaccgttgatgg	72	911

Table 2. Reaction mixture for *Age*I linearization of AAVS1-donor vector.

Component	50 µL Reaction
AAVS1-SA-puro-EF1-MCS plasmid DNA	1.0 µg
10X CutSmart Buffer	5.0 µL
*Age*I-HF	1.0 µL
Nuclease-free Water	to 50 µL

Table 3. Reaction mixture for Sanger sequencing.

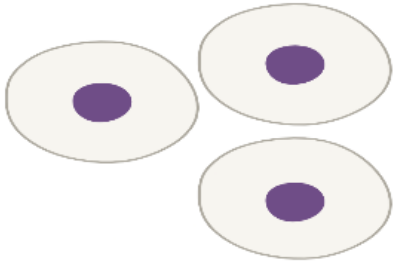
Reagent	Vol per Reaction (µL)	
BigDye Buffer	1.75	1.75
BigDye Terminator	0.25	0.25
Plasmid (100 ng/µL) - [add this after distributing master mix]	1	1
Forward Primer (10 µM) - [add this after distributing master mix]	1	0
Reverse Primer (10 µM) - [add this after distributing master mix]	0	1
Nuclease free H_2O	6	6

Table 4. Predicted off-target sites related to crRNA for AAVS1 gene knock-in.

Sequence	Mismatches	Chromosome	Cut site	Locus/Gene
GUCACCAAUCCUGUCCCUAG	0	19	55,115,786	*AAVS1*
GCCACCACTCCTGTCCCTGG	3	15	71,357,395	*THSD4*
GCCACCACTCCTGGCCCTAG	3	15	50,552,136	*TRPM7*
GCCACAAATCCTGTCCCTGG	3	16	55,430,781	*RP11-212121.2*
GTCACCAATCCTAGCACTAG	3	10	36,766,500	-
GCCACCAAGCCTGGCCCTAA	4	3	119,376,206	*ARHGAP31*
GACACTGATCCTGTCCCTAA	4	2	178,603,028	*TTN-AS1*
AGCACCAATCTTGTCCCTGG	4	4	6,074,123	*JAKMIP1*
GTAACCAAGACTGTCACTAG	4	12	105,200,591	*APPL2*

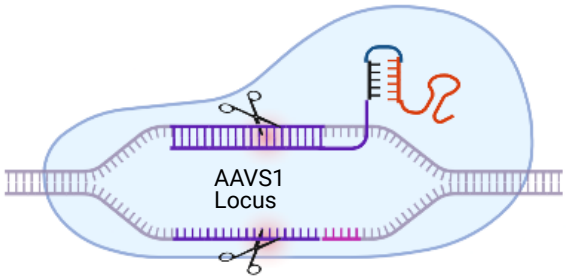
Figure1

A Individual donor

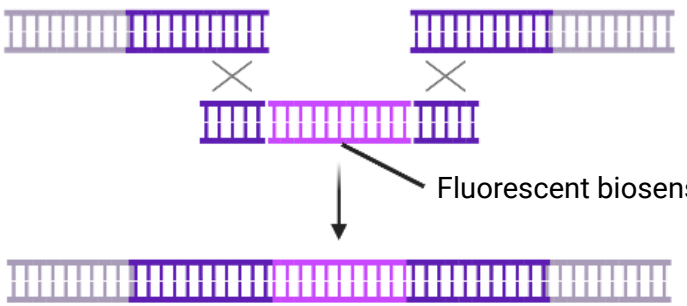


B Donor's iPSC

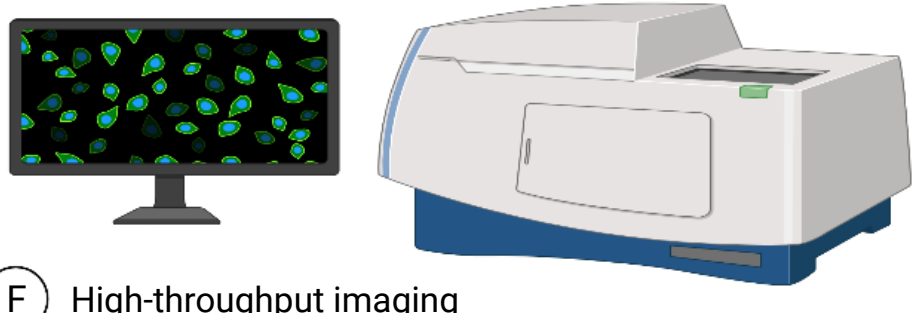
C CRISPR/Cas HDR



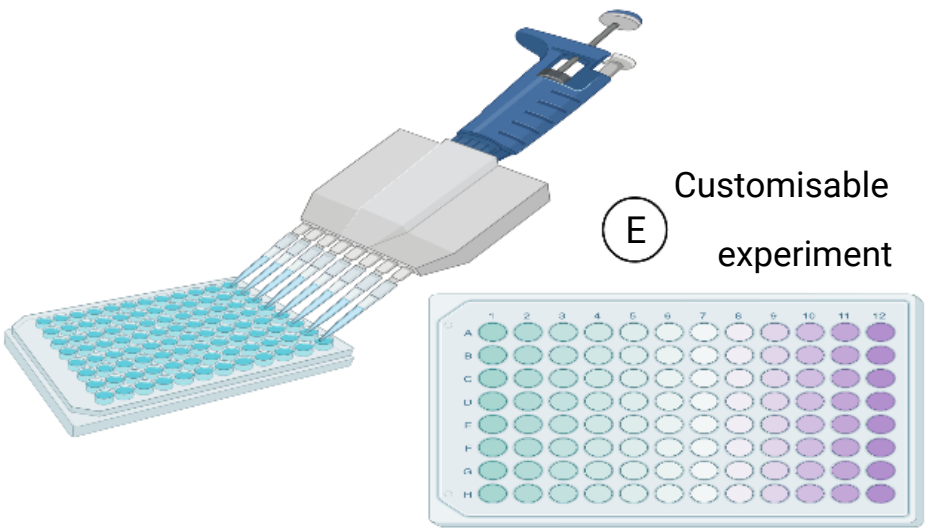
Homologous regions



Fluorescent biosensor integration



F High-throughput imaging



E Customisable experiment

D Quality control

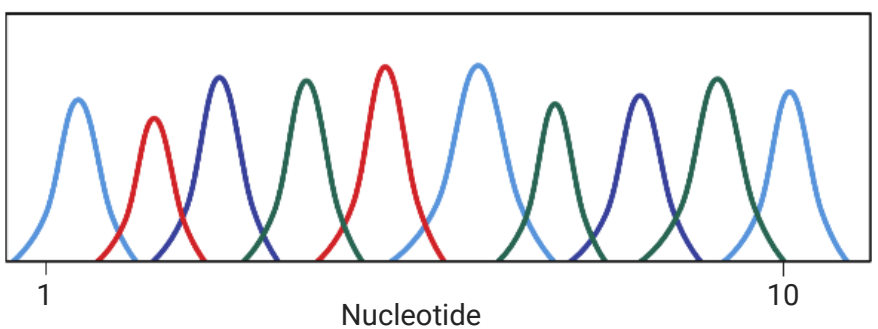


Figure2

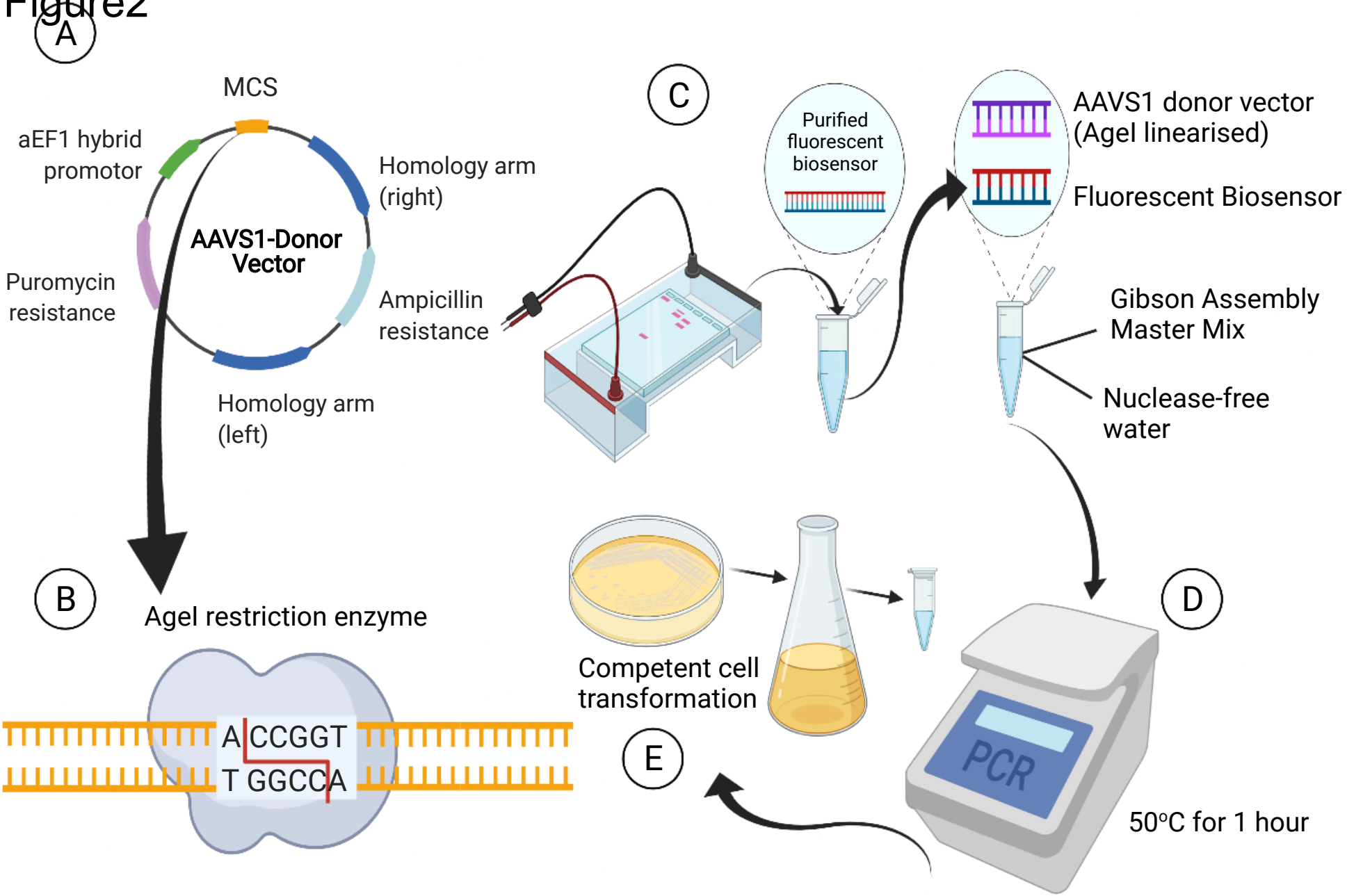

A
MCS
aEF1 hybrid promotor
Homology arm (right)
AAVS1-Donor Vector
Puromycin resistance
Ampicillin resistance
Homology arm (left)
B
AgeI restriction enzyme
A CCGGT
T GGCCA
C
Purified fluorescent biosensor
AAVS1 donor vector (AgeI linearised)
Fluorescent Biosensor
Gibson Assembly Master Mix
Nuclease-free water
D
PCR
50°C for 1 hour
E
Competent cell transformation

Figure3

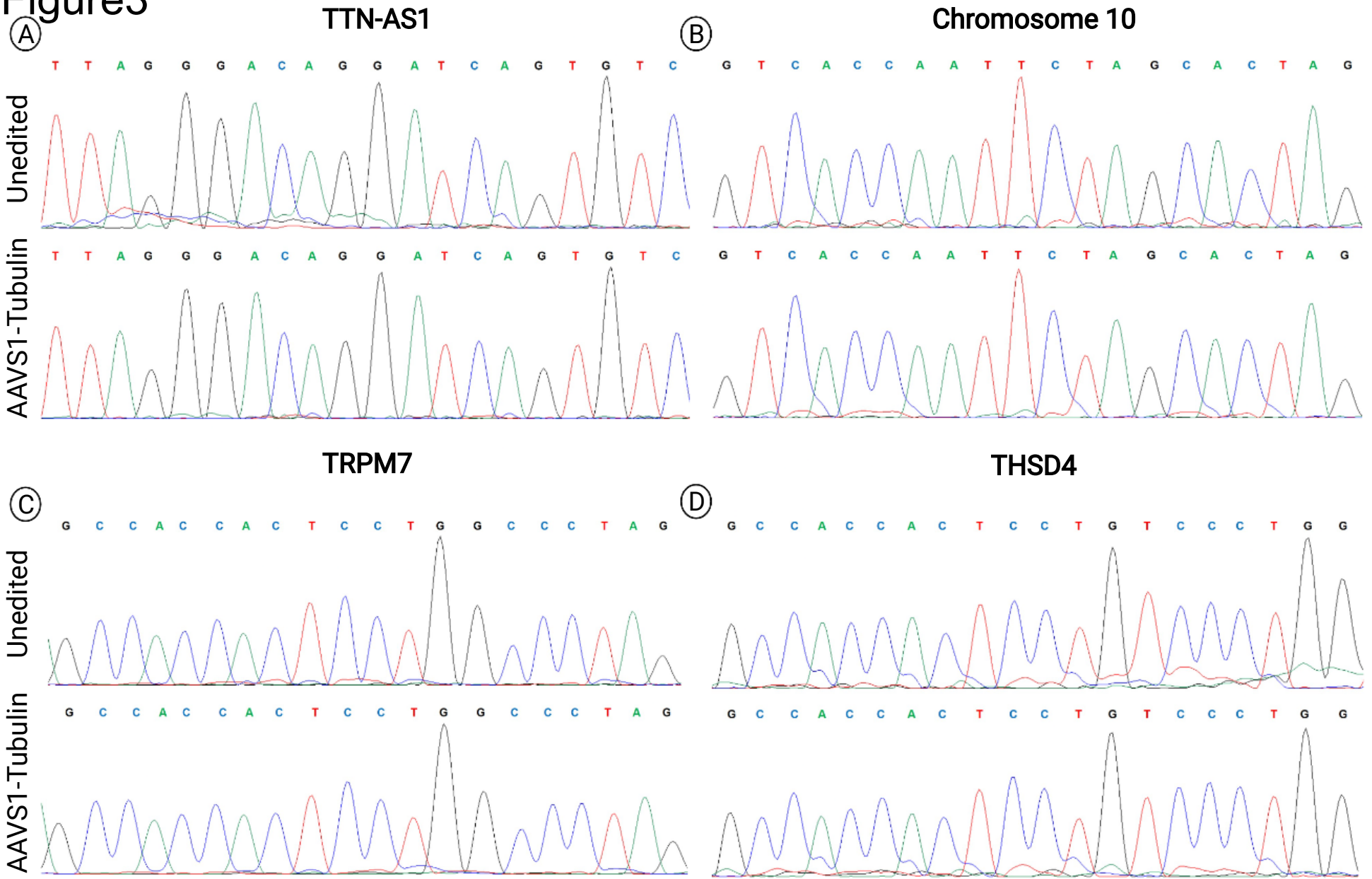
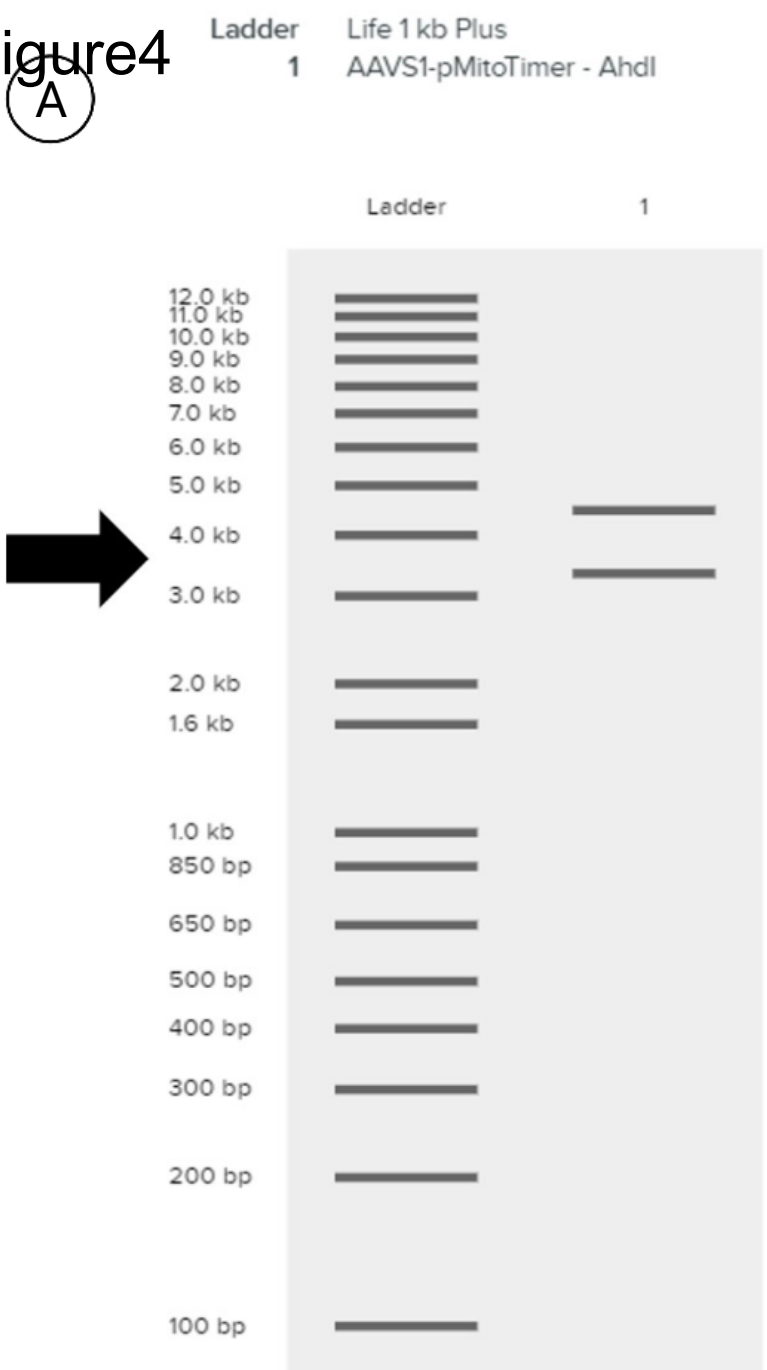

A
TTN-AS1
T T A G G G A C A G G A T C A G T G T C
Unedited
T T A G G G A C A G G A T C A G T G T C
AAVS1-Tubulin
B
Chromosome 10
G T C A C C A A T T C T A G C A C T A G
G T C A C C A A T T C T A G C A C T A G
C
TRPM7
G C C A C C A C T C C T G G C C C T A G
Unedited
G C C A C C A C T C C T G G C C C T A G
AAVS1-Tubulin
D
THSD4
G C C A C C A C T C C T G T C C C T G G
G C C A C C A C T C C T G T C C C T G G

Figure4

A



B

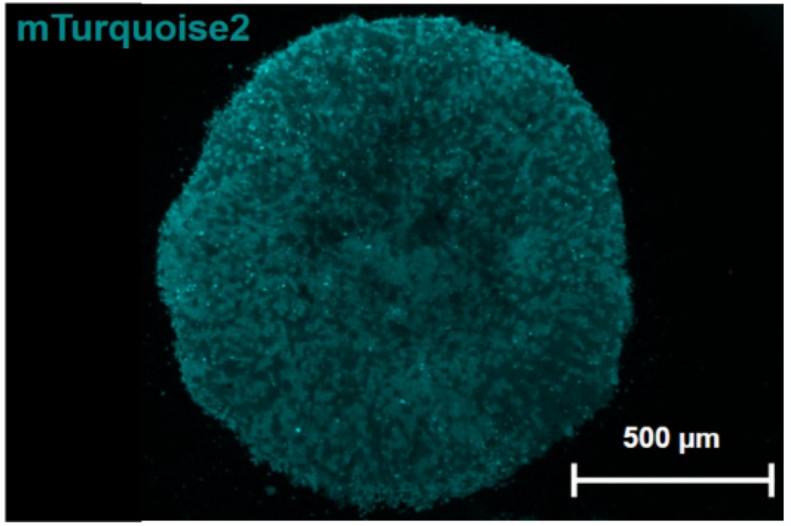
C

Enzymes	Cuts	Temp.	1.1	2.1	3.1	4/CS
AhdI	2	37°C	25	25	10	100

Start	End	Length	Left Cutter	Left Overhang	Right Cutter	Right Overhang
2842	7299	4458	AhdI	3'	AhdI	3'
7300	2841	3341	AhdI	3'	AhdI	3'

Figure5

A AAVS1-Tubulin IPSC Colony



B AAVS1-Tubulin Karyotyping

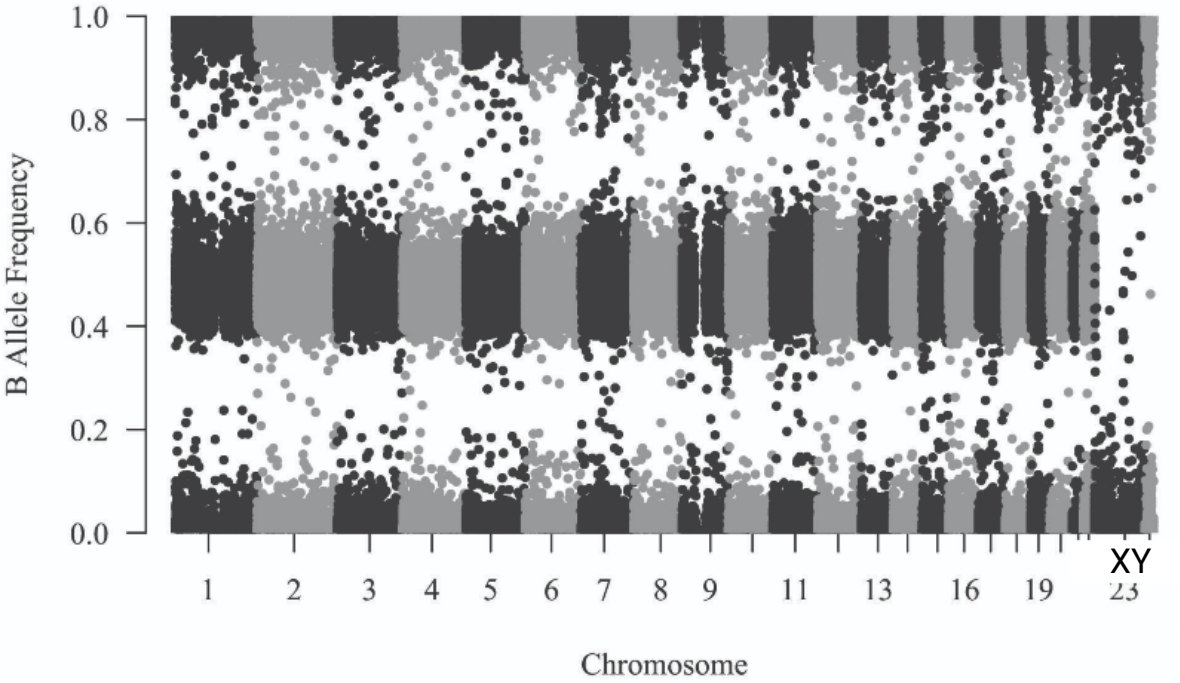
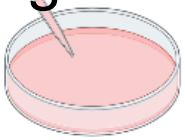


Figure6



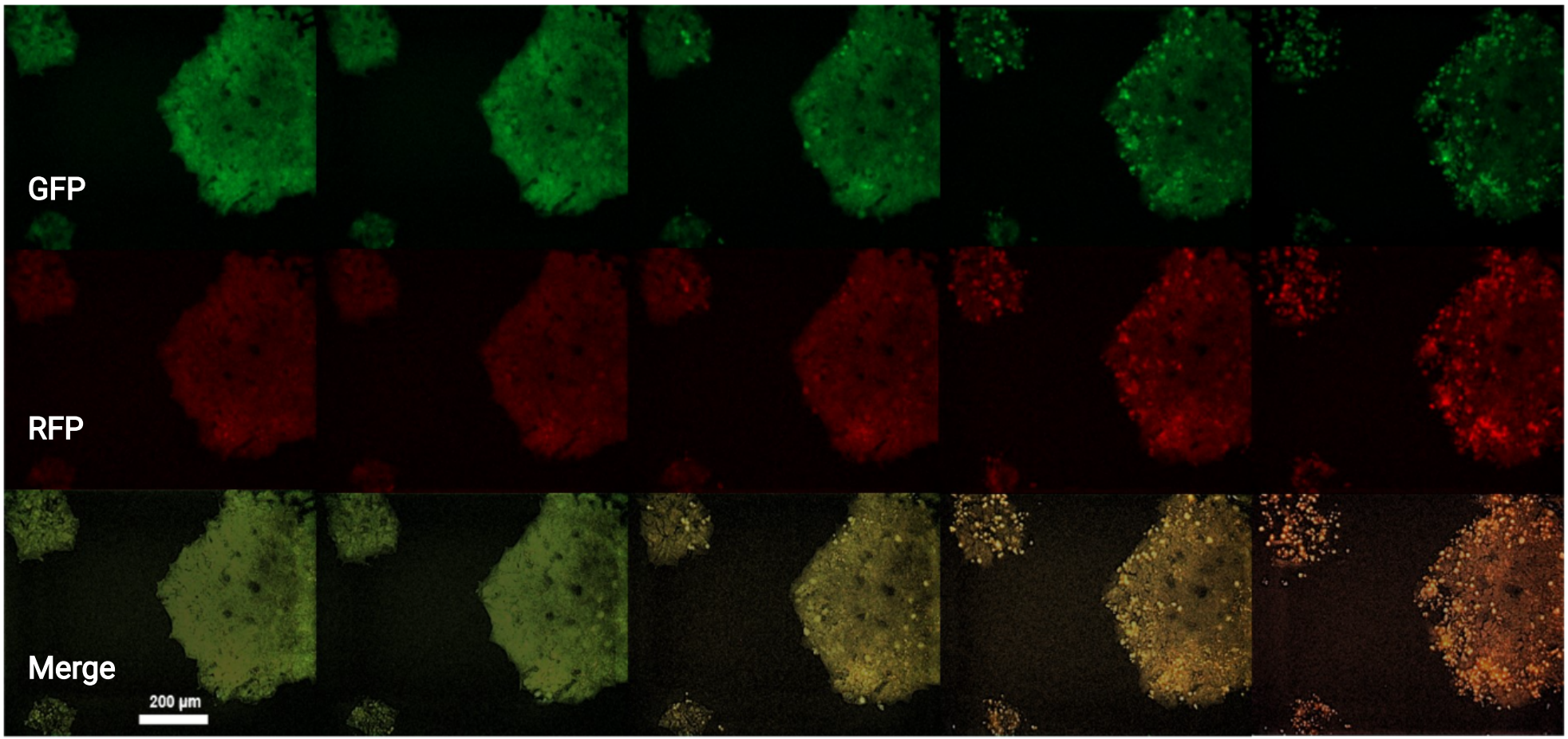
MG132 (5 μM) application

Increased degradation of GFP-LC3

Internal control - RFP-LC3ΔG

Autolysosome

0 — 20 — 100 — 200 — 300 Minutes



LOW — Autophagy — HIGH