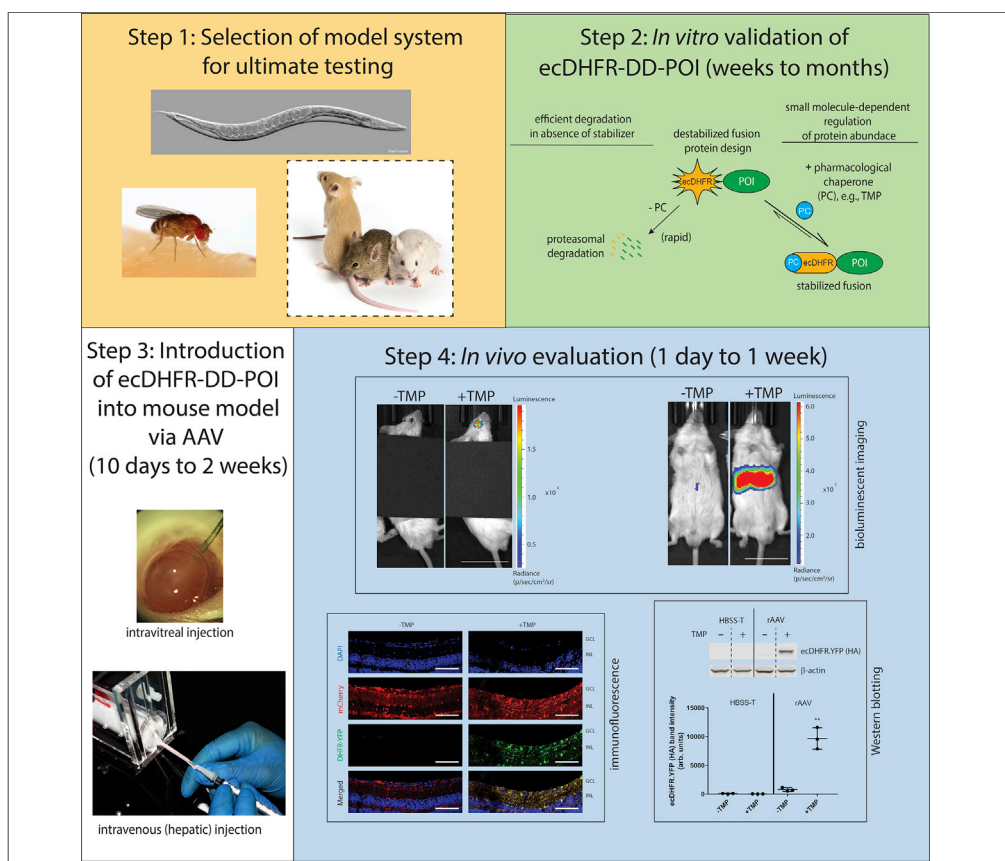


Protocol

Protocol for *In Vivo* Evaluation and Use of Destabilizing Domains in the Eye, Liver, and Beyond



Destabilizing domains (DDs) have been used successfully to conditionally control the abundance of proteins of interest (POIs) in a small-molecule-dependent manner in mice, worms (*Caenorhabditis elegans*), and *Drosophila*. However, development of such systems must account for delivery of the DD-POIs to the target tissue, accessibility of the target tissue to the small molecule, and quantification of stabilization. Here, we describe the considerations and steps to take in order to effectively implement a DD-POI in mouse ocular and hepatic tissue.

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HIGHLIGHTS
Destabilizing domains (DDs) rapidly regulate protein abundance in worms, flies, and mice

Use of the ecDHFR-DD has been thoroughly validated in the eye and liver of mice

Non-invasive bioluminescent imaging allows for assessment of longitudinal DD regulation

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Protocol

Protocol for *In Vivo* Evaluation and Use of Destabilizing Domains in the Eye, Liver, and BeyondPrerana Ramadurgum,¹ Steffi Daniel,¹ and John D. Hulleman^{1,2,3,4,*}¹Department of Ophthalmology, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75390, USA²Department of Pharmacology, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75390, USA³Technical Contact⁴Lead Contact*Correspondence: john.hulleman@utsouthwestern.edu
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SUMMARY

Destabilizing domains (DDs) have been used successfully to conditionally control the abundance of proteins of interest (POIs) in a small-molecule-dependent manner in mice, worms (*Caenorhabditis elegans*), and *Drosophila*. However, development of such systems must account for delivery of the DD-POIs to the target tissue, accessibility of the target tissue to the small molecule, and quantification of stabilization. Here, we describe the considerations and steps to take in order to effectively implement a DD-POI in mouse ocular and hepatic tissue. For complete details on the use and execution of this protocol, please refer to Datta et al. (2018), Ramadurgum and Hulleman (2020), and Ramadurgum et al. (2020).

BEFORE YOU BEGIN

Experimental Design Considerations

A destabilizing domain (DD) is an unstable protein sequence that is efficiently degraded by the proteasome under physiological conditions. When the DD is fused to a protein of interest (POI), the entire fusion product is rapidly degraded in untreated conditions. However, when desired, the DD-POI fusion protein can be stabilized upon the addition of a small molecule ligand (typically trimethoprim [TMP]), promoting protein abundance. Our primary goal of this STAR Protocols paper is to guide prospective users in utilizing and evaluating the DD system in the eye and liver of mouse models. While we focus on the eye and liver, similar techniques as described herein can be utilized in a variety of tissues, enabling the DD system to serve as a biologic tool or conditional gene therapy system.

Design and Evaluation of a DD Expression System for *In Vivo* Use

1. Prior to testing a DD-POI fusion for use in living organisms (i.e., *Caenorhabditis elegans*, *Drosophila*, or mice), we strongly recommend initially validating the system *in vitro*. Low basal levels of the DD-POI as well as effective stabilization *in vitro* usually correlates with an appropriately regulated system *in vivo*. In fact, anecdotally, we have noted that DD-POIs appear to be even better regulated *in vivo* than *in vitro*. Nonetheless, we encourage groups to confirm DD-POI functionality, subcellular localization, and dose-responsiveness *in vitro* prior to evaluating the DD-POI in more expensive, complex, and time-intensive systems such as animals. For the design and validation of the system *in vitro*, we recommend reading (Iwamoto et al., 2010; Ramadurgum et al., 2020; Sellmyer et al., 2012) and the corresponding *in vitro* STAR Protocols paper (Ramadurgum and Hulleman, 2020; <https://doi.org/10.1016/j.xpro.2020.100069>).



2. DDs conjugated to a POI have been shown to regulate protein abundance in multiple systems. Initially, a mutated FKBP12-DD was stabilized upon treatment with the small molecule, Shld-1, in NIH3T3 mouse fibroblasts and live mice (Banaszynski et al., 2006; Banaszynski et al., 2008). Later, an *Escherichia coli* dihydrofolate reductase (ecDHFR)-DD was developed, employing a commonly used antibiotic, trimethoprim (TMP), as a broadly penetrating and highly potent stabilizer in mammalian systems (Iwamoto et al., 2010). For the purposes of this protocol, we will focus here onward on *in vivo* application of the ecDHFR-DD system.
3. Due to its thermal instability only at elevated temperatures (37°C), modifications to the mammalian ecDHFR-DD system (i.e., either R12Y/G67S/Y100I [N-terminal ecDHFR] or R12H/N18T/V19A/G67S [C-terminal ecDHFR]) were used to allow for appropriate regulation in systems grown at lower temperatures (e.g., *C. elegans* and *Drosophila*) (Cho et al., 2013; Kogenaru and Isalan, 2018). The ecDHFR-DD mutations listed in these respective publications (e.g., R12H/N37D/N59D/G67S/D132G for *C. elegans*, or dual R12H/N23S/G67S/V78A/E120G/E134G/E153V/E157G ecDHFR domains in *Drosophila*) should be used as a starting point when developing an ecDHFR-DD-POI in these systems. We recommend the inclusion of a fluorescent or luminescent reporter gene wherever possible to allow for easy confirmation of POI regulatability. Previously we have used an enzymatic reporter such as firefly luciferase or Nano luciferase (Promega) due to the amplification of signal, allowing for easier detection and quantification *in vivo*.
4. *In vivo* validation of ecDHFR-DD-POI in mice has been accomplished in neuronal, ocular, and hepatic tissue. Whereas similar validation has been accomplished globally in *C. elegans* (Cho et al., 2013), and in *Drosophila* larval stages as well as adult eyes (Kogenaru and Isalan, 2018). While we see no reason why the system cannot be used in other tissues, this is an important aspect to test prior to beginning a full-scale project as it is likely that different tissues have varying levels of protein quality control, and thus degrade the ecDHFR-DD differently. Due to our experience with the mouse as a model system (Datta et al., 2019; Datta et al., 2018; Peng et al., 2019; Ramadurgum et al., 2020), and its wide use in biomedical research (Quintino et al., 2013; Sando et al., 2013; Tai et al., 2012), we have focused on this ecDHFR system in the protocol described herein.
5. There are multiple *in vivo* delivery approaches that an interested researcher can utilize for introduction of the ecDHFR-DD-POI, ranging from lentiviral or adeno-associated virus (AAV) infection to generating a knockin or transgenic mouse. Decisions to utilize one approach vs. the other will likely depend on the amount of time available for investment (viral approaches are quicker ways to introduce the ecDHFR-DD-POI), and the anticipated duration of use of the model system (knockin or transgenic mice allow for renewable, more consistent long-term experiments).
 - a. While producing a transgenic mouse line can be time- and labor-intensive, a ubiquitously expressed ecDHFR-DD-POI might prove more useful for the study of multiple, spatially distanced organs and cell types. In addition, this line could be bred with other mouse lines of interest for the study of specific processes. Furthermore, better control over levels of the ecDHFR-DD-POI may be achievable through knockin experiments than through the use of virus. However, if a fluorescence or bioluminescence reporter is used and broadly expressed across the mouse, it may be difficult to isolate signal originating from a single tissue in live animals (e.g., detect ocular-derived signal without detecting brain-derived signal). Dissection of individual organs would be one way to circumvent this shortcoming. Yet, such experiments are terminal and require the incorporation of much higher animal numbers.
 - b. Alternatively, viruses can serve as a powerful tool for the delivery of genetic material to specific target cells in an organism. The ever-increasing availability of new and unique serotypes through directed evolution allow for the production of virus that can target tissues of interest with increasing specificity (Bartel et al., 2012). Genome packaging size, genome integration vs. episomal expression, expression levels, and immunogenicity vary among conventionally used viruses and are just a few considerations when choosing the appropriate virus to accomplish a given task.

- i. Adenovirus, with a genome capacity of up to ~35 kb, became the very first virus to be used for human gene therapy, and has up to 50 different serotypes (Crystal, 2014). Adenovirus DNA remains episomal, reducing the risk of genome disruption via integration (Benskey et al., 2019). However, adenovirus is highly immunogenic, resulting in an acute inflammatory response in most tissues (Thaci et al., 2011). This immune response is not only dangerous to the host, but also results in the eventual elimination of transduced cells as well as conferring a degree of resistance to secondary infection (Crystal, 2014). Thus, the safety profile of adenovirus does not make it ideal for gene therapy, especially with other available options.
- ii. Lentivirus is an integrating retrovirus which can produce stable expression with delivery of up to ~9 kb of genetic material (Benskey et al., 2019). It is easy to produce, has alterable pseudotype, and is unlikely to produce an immune response from the host (Benskey et al., 2019). However, lentivirus integrates randomly into the host genome, which increases the risk of insertional mutagenesis (Choudhury et al., 2017). Nonetheless, *ex vivo* lentiviral transduction of hematopoietic stem cells is currently being used in clinical trials for sickle cell disease, Fanconi anemia, among other diseases (Milone and O'Doherty, 2018). Furthermore, a study demonstrating robust transduction of hepatocytes in non-human primates with an accompanying conditional suicide gene herpes simplex virus-thymidine kinase (HSV-TK) established a method to ablate cells with insertional mutagenesis (Menzel et al., 2009). Yet, for the most part, lentivirus is not the first choice for *in vivo* experimentation due to its integration into the host genome and variability of integration locus from cell to cell.
- iii. AAV has become the safest and most prevalent virus used for gene therapy to date (Naso et al., 2017). AAV has the smallest carrying capacity of the three viruses listed herein, harboring only ~4.7 kb, but produces almost no human immune response within the host and can target specific cells through serotype altering (Benskey et al., 2019). However, AAV neutralizing antibodies to at least one of the almost 100 naturally occurring serotypes do exist in an estimated 70% of humans and non-human primates, which has proven to be a major limitation in human clinical trials (Lotfinia et al., 2019). AAV remains episomal and circularized in the nucleus following infection, providing persistent, long-term expression in nondividing cells (Choudhury et al., 2017). Due to its transduction efficiency, alterable serotype, and safety for use *in vivo*, we recommend the use of AAV for the introduction of the ecDHFR-DD-POI into living systems.

Note: The user will have to determine which AAV serotype is appropriate for their experiments depending on the target organ/tissue/cell type. For example, AAV2/2, AAV MAX, AAV8/8, AAV9/9 or AAV10/10 are highly neurotropic (Choudhury et al., 2017) and can target cells of the central nervous system. We previously used AAV MAX for intravitreal injections of the retina and AAV2/8 for intravenous injections targeting the liver (Ramadurgum et al., 2020). While generation and amplification of the AAV plasmid can occur in an individual laboratory, we recommend the use of a professional viral vector core to generate sufficiently high titers of pure AAV.

6. Once a route to introduce the ecDHFR-DD-POI into mice has been determined, the user should consider which genetic background of mouse to use. We recommend that the user select an inbred mouse strain such as Balb/c or C57BL/6J due to their wide availability and genetic homogeneity. While C57BL/6J mice are one of the most prevalent strains used in research, we note that the bioluminescent signal from C57BL/6J ocular tissue is about an order of magnitude lower than that of non-pigmented Balb/c mice. Thus, for bioluminescent purposes, Balb/c mice are recommended over pigmented strains (such as C57BL/6J), in a manner similar to the recommendation of using a white-walled multiwell plate for common luminescent reporter assays to bolster the signal-to-noise ratio.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
HA Tag Mouse Monoclonal Antibody (2-2.2.14)	Invitrogen	Cat# 26183
β-Actin Rabbit Monoclonal Antibody	LI-COR	Cat# 926-42210
IRDye® 680RD Goat anti-Mouse IgG (H + L)	LI-COR	Cat# 926-68070
IRDye® 800CW Goat anti-Rabbit IgG (H + L)	LI-COR	Cat# 926-32211
Invitrogen GFP Monoclonal Antibody (3E6)	Thermo Fisher Scientific	Cat# A-11120
Anti-mCherry antibody	Abcam	Cat# ab167453
Invitrogen Goat anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	Thermo Fisher Scientific	Cat# A-11029
Invitrogen Goat anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 633	Thermo Fisher Scientific	Cat# A-21070
Bacterial and Virus Strains		
Virus: AAV2/2 MAX Nano luciferase 2A ecDHFR firefly luciferase	Packaged by UNC Viral Vector Core	N/A
Virus: AAV2/8 Nano luciferase 2A ecDHFR firefly luciferase	Packaged by UNC Viral Vector Core	N/A
Chemicals, Peptides, and Recombinant Proteins		
Dimethyl Sulfoxide (DMSO)	Fisher Scientific	Cat# BP231-100
Carbowax™ PEG 400 (NF)	Fisher Scientific	Cat# P167-1
Tween™ 80	Fisher Scientific	Cat# BP338-500
Tween™ 20	Fisher Scientific	Cat# BP337-100
Cremophor EL	Millipore Sigma	Cat# 238470
Dextrose Anhydrous (Crystalline Granules/ Molecular Biology)	Fisher Scientific	Cat# BP350-500
Hanks buffered salt solution (HBSS)	Millipore Sigma	Cat# H6648
Trimethoprim (TMP)	Millipore Sigma	Cat# T7883
D-Luciferin Sodium Salt	Gold Biotechnology	Cat# LUCNA
0.9% Sodium Chloride Injection, USP	Hospira	N/A
Cyclopentolate hydrochloride 1% [w/v]	Alcon Laboratories	NA
Tropicamide 1% [w/v]	Alcon Laboratories	NA
GenTeal Severe Dry Eye Relief Lubricant Eye Gel	Alcon Laboratories	NA
AK-POLY-BAC antibiotic ointment	Alcon Laboratories	NA
Ketamine (30 mg/mL) Xylazine (4 mg/mL) Cocktail	UT Southwestern Animal Resource Center	NA
Critical Commercial Assays		
Maxi Prep Plus Kit	Qiagen	Cat# 12943
Experimental Models: Organisms/Strains		
Mouse: Balb/c Wild Type	Mouse Breeding Core, UT Southwestern	N/A
Mouse: C57BL6/J Wild Type	The Jackson Laboratory	Cat# 000664
Mouse: Agouti C3A.BLiA-Pde6b ⁺ /J Wild Type	The Jackson Laboratory	Cat# 001912

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Recombinant DNA		
pAAV2/2 MAX (7m8, QuadYF)	Reid et al., 2017	N/A
pAAV2/8	Zhu Laboratory, UT Southwestern	N/A
pTR smCBA Nano luciferase 2A ecDHFR firefly luciferase	Peng et al., 2019	N/A
Other		
BD Biocoat Disposable Syringe, Non-Sterile, Luer-Lok, 5 mL	Cole-Parmer	Cat# EW-07944-06
Hamilton 2.5 μ L Syringe	Hamilton Company	Cat# 7632-01
33 G $1/2$ inch 10° beveled needle	Hamilton Company	custom
25 G $5/8$ inch single use, sterile needle, regular bevel, regular wall, orange hub	BD Biosciences	Cat# 302886
30 G $1/2$ inch single use, sterile needle	BD Biosciences	Cat# 305107
1 mL Slip Tip Syringe with attached needle 27 G x $1/2$ inch, sterile, single use	BD Biosciences	Cat# 309623
Zeiss Stemi 305 Compact Greenough Stereo Microscope	Zeiss International	Cat# 435063-9010-100
Beaver Visitec International Ltd Cellulose Eye Spear	Fisher Scientific	Cat# NC0889513
Mouse Tail Illuminator Restrainer	BrainTree Scientific	Cat# MTI STD
JobSite Comfort Hand Warmers	Amazon	Cat# B005N8WEXU
IVIS Sepctrum <i>In Vivo</i> Imager	PerkinElmer	N/A
Odyssey CLx	LI-COR	N/A
Leica SP8 confocal microscope	Leica	N/A
Software and Algorithms		
Living Image Software	Perkin Elmer	Part # 128113

STEP-BY-STEP METHOD DETAILS

AAV Intravitreal Injections in a Mouse Model—ecDHFR-DD-POI Expression in the Retina

⌚ Timing: 10 days

Due to its serotype flexibility, low immunogenicity, and previously demonstrated efficacy for treating disease (Pierce and Bennett, 2015), we chose to use AAV as a means to introduce our DD-POIs into the eye as well as systemically in the liver.

1. Prepare HBSS-T by adding Tween-80 to sterile HBSS until reaching a final concentration of 0.14%.
2. AAV is stable for multiple freeze-thaw cycles and can be thawed at room temperature (20°C–23°C). Do not leave at room temperature longer than 4 h.
3. Dilute the virus with HBSS-T. The user will need 1–2 μ L of virus per eye at a concentration of 3.5×10^{12} viral genomes/mL. We recommend preparing twice as much volume of virus as necessary when preparing for injections due to a high degree of error/dead volume associated with the injection syringe and the small volumes used.
4. Prepare a Hamilton 2.5 μ L syringe by removing the screw top and inserting a 33 $1/2$ G needle with a 10° – 12° bevel (Figure 1A). Carefully replace the screw top back onto the syringe so the needle is firmly fixed in position.

⚠ CRITICAL: The glass Hamilton syringe should be autoclaved before use. Do not autoclave the 33 $1/2$ G needles as this will scorch or damage the needle. Refer to step 6 in sterilizing the intravitreal 33 $1/2$ G needles.

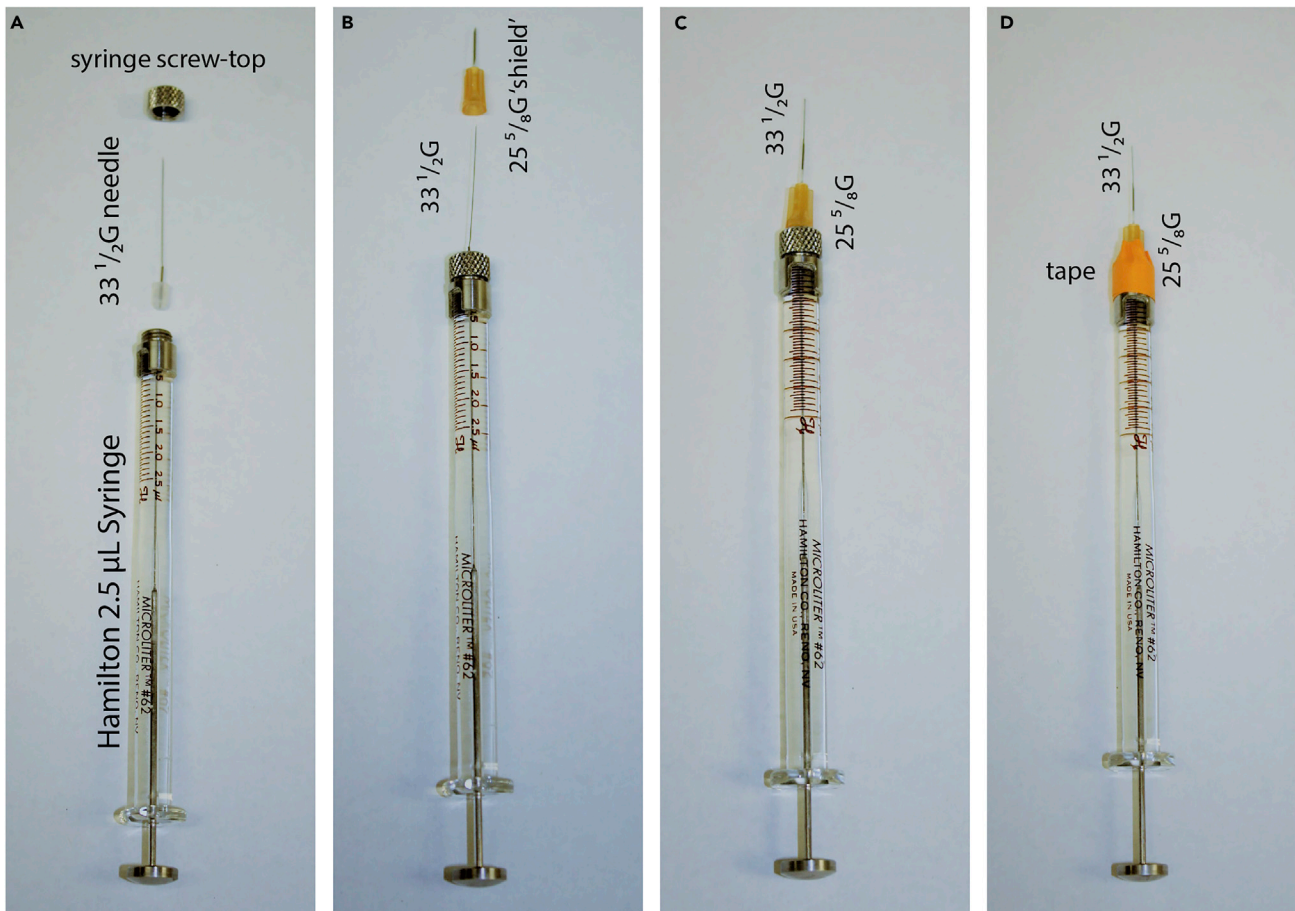


Figure 1. Demonstration of Syringe and Needle Setup for Intravitreal Injection

(A) To begin to assemble the needle and syringe for intravitreal injection, remove the syringe screw top and insert the 33 $\frac{1}{2}$ G needle, replace the screw top to secure the needle.

(B–D) Next, carefully fit the 25 $\frac{5}{8}$ G “shield” needle over the top of the 33 $\frac{1}{2}$ G needle (B and C) and fasten it securely to the syringe with tape (D).

- To prevent the 33 $\frac{1}{2}$ G needle from flexing or bending, thread it through a 25 $\frac{5}{8}$ G needle. This is referred to as the “shield needle”, (Figures 1B and 1C) Tape the shield needle into place carefully (Figure 1D).
- Rinse out the full syringe volume (almost 3 μ L) with HBSS-T 3–4 times. Then rinse out the 33 $\frac{1}{2}$ G needle with 5% bleach 3–4 times with only enough volume to rinse out the needle itself. The syringe has been sterilized and the user should avoid introducing bleach into the barrel of the syringe. Rinse the needle with only enough volume to rinse out the needle itself with HBSS-T another 8–10 times. It is now ready to use.
- If using multiple viruses or using HBSS-T vehicle control on one eye, label the needles accordingly. We recommend always using the virus syringe for virus only for all following injections and future experiments.
- Anesthetize the mouse with 96 mg/kg Ketamine and 12.8 mg/kg Xylazine of Ketamine (30 mg/mL) Xylazine (4 mg/mL) anesthetic cocktail and apply the initial dilator, cyclopentolate hydrochloride 1% [w/v], as soon as the mouse becomes immobile. If the dilator is applied too late, the ciliary muscles will no longer be able to react due to the anesthetic.
- After a minute, absorb excess dilator with an absorbent tissue or Kimwipe. Apply the secondary dilator, tropicamide 1% [w/v], and allow to sit for 1–2 min.
- Remove the excess dilator with a wipe and apply GenTeal eye gel to prevent corneal clouding.

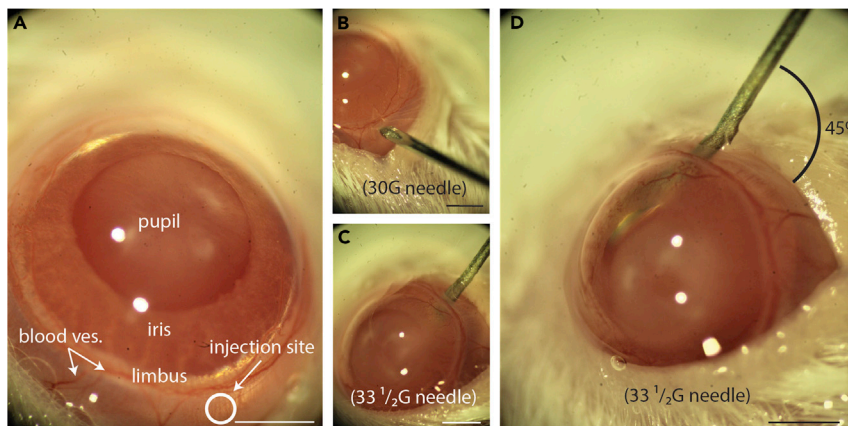


Figure 2. Demonstration of Intravitreal Injection Procedure in Balb/c Mice

(A) Ocular structures of a Balb/c eye under magnification from a Zeiss Stemi 305 Compact Greenough Stereo Microscope (Zeiss International, Oberkochen, Germany). The pupil, iris, limbus, and limbal blood vessels can be easily recognized. The injection site should be located below the limbus and avoiding any nearby blood vessels (white circle).

(B) To make the initial puncture, approximately half of the tip of a 30G needle is inserted below the limbus and then removed.

(C and D) The intravitreal 33 1/2 G needle is inserted into the hole made in (B) until the bevel can be visualized through the pupil (shown in D). At this point, the intravitreal needle is at an ~45 degree angle from the horizontal (D). Scale bars, ~1 mm.

11. Once the mouse is fully anesthetized, use a triangle spear to comb back the whiskers and eyelashes using the GenTeal gel as the “glue.” Only do this for the eye you intend to inject at this moment.
12. Place the animal under the Zeiss stereo microscope with the target eye facing up. Focus the microscope and ensure no whiskers or eyelashes are in the way. In addition, confirm that the pupil is fully dilated.
13. Gently proptose the eye with two fingers of the user’s nondominant hand, avoiding putting too much pressure as this might suffocate the mouse.
14. In the microscope, look for the limbus of the eye. Once located, focus the microscope to fully visualize the surrounding blood vessels. Look for areas directly underneath the limbus without many blood vessels (Figure 2A).
15. Once the user finds an area with little to no blood vessels, use a 30G needle and gently puncture the area, making sure to only insert the tip of the bevel (Figure 2B). If the puncture hole is too large, there will be backflow of the injected virus or leaking of the aqueous humor.

△ CRITICAL: Replace the 30G puncture needle after 2–3 eyes. It dulls easily and will make the puncture much more traumatic to the surrounding tissue if blunt.

Note: Use your nondominant hand during this entire process to keep the eye gently proptosed.

Note: If the user accidentally punctures a blood vessel, soak up the blood with a triangle spear and continue as planned. There will be less visibility and greater chance of the virus entering the bloodstream, but this should not hinder the rest of the injection process.

16. Pick up the Hamilton syringe and insert the needle at a 45° angle from the horizontal until the user is able to see the needle through the pupil (Figures 2C and 2D). Be careful not to insert the needle too far, just until the user is able to see it through the pupil is enough.

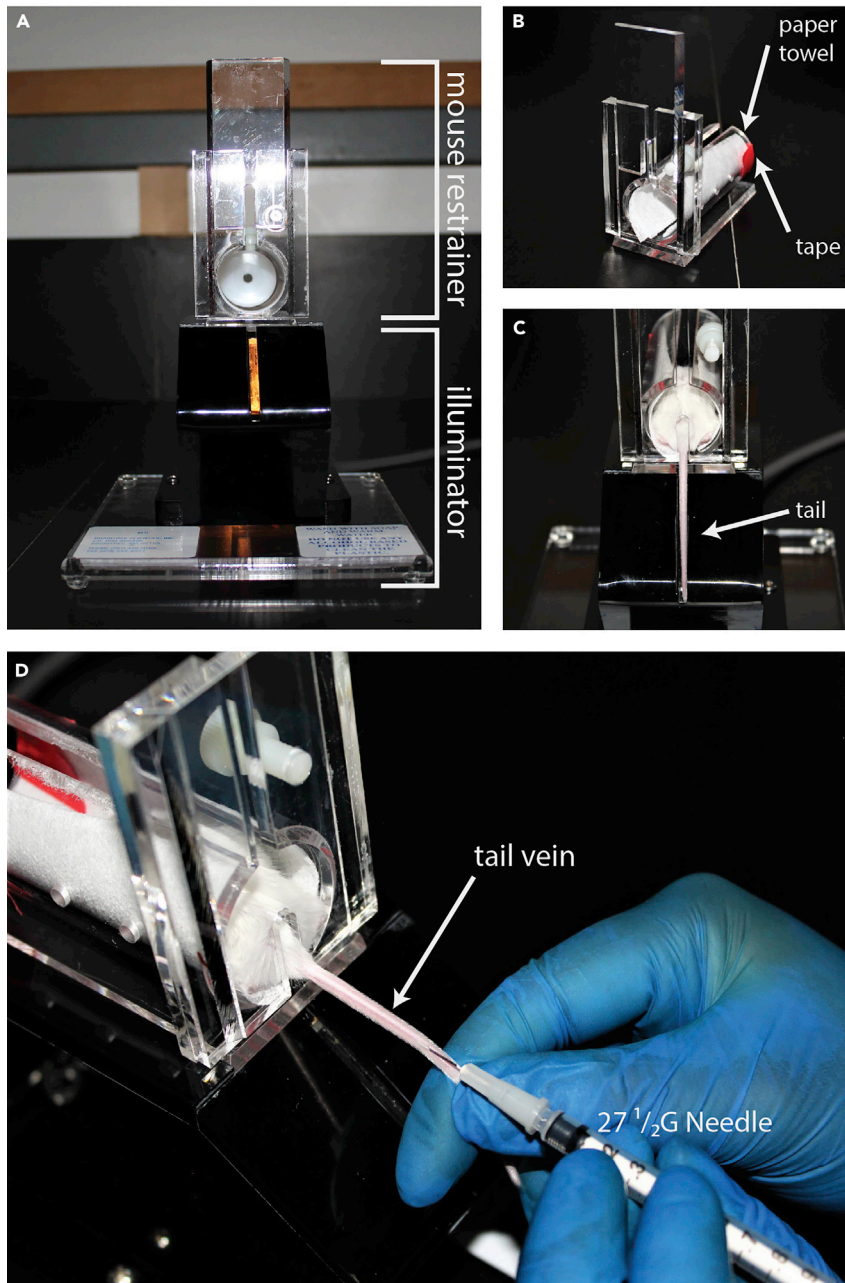


Figure 3. Setup and Procedure for Intravenous Injection of AAV in Balb/c Mice

(A) Braintree Tail Vein Illuminator (Braintree Scientific, Braintree, MA, USA) has two distinct components, the restrainer and tail vein illuminator.

(B) To prevent the mouse from turning, we recommend lining the restrainer tube with paper towel and taping it into place.

(C) The mouse can be allowed to crawl into the restrainer and the gate at the front of the restrainer can be closed, allowing only the tail through the slot and onto the illuminator deck.

(D) Once the tail has been warmed and the vein easily visualized, the vein can be injected with a 27 $\frac{1}{2}$ G needle.

17. The user should remove their nondominant hand from the mouse and gently push down the plunger of the Hamilton syringe over the course of 60 s.

△ CRITICAL: releasing the virus solution too quickly may disturb other ocular structures or cause backflow out of the eye.

Note: If the user injected a small air bubble into the eye, this should not be an issue. It will dissipate over time.

18. Once the plunger is fully released, hold the Hamilton syringe in place for another 60 s so that all the virus may sink down undisturbed.
19. Carefully remove the needle.
20. Apply AK-POLY-BAC antibiotic ointment to the eye. On top of this, add GenTeal gel.
21. Proceed to inject the other eye using the same protocol.
22. Put the mouse on a warm surface and note that the GenTeal will melt over time. Replace the gel every 15 min until the mouse wakes up to avoid corneal clouding.
23. Full transduction and expression of the viral construct will take at least 10 days. Some groups recommend 21 days, but we have found that the signal of reporter proteins is stable after 10 days. If a self-complementary AAV (scAAV) is used, expression will plateau more quickly than conventional single-stranded DNA-based AAV. Nonetheless, in the absence of retinal degeneration, we have noticed sustained ocular expression for 6 months or longer.

AAV Tail Vein Injections in a Mouse Model—Hepatic Expression of ecDHFR-DD-POIs

⌚ Timing: 2 weeks

24. The mouse liver is a large and easily accessible organ through a tail vein injection. Hepatocytes are robustly transduced by a number of serotypes including AAV2/2, AAV5/5, AAV8/8 and AAV10/10 (Pipe et al., 2019). We have specifically utilized the AAV2/8 serotype developed for even and strong transduction at a lower titer than the conventionally used AAV8/8 serotype, making it much more translatable to human disease in which viral dose must be kept as low as possible (Cingolani et al., 2012). In (Peng et al., 2019; Ramadurgum et al., 2020), we have utilized the liver as a control organ to mirror results observed in ocular tissue due to its accessibility through the tail vein. However, because hepatocytes are continually dividing, we recommend using the mice in 1–3 days after the 2 week incubation period. We have anecdotally noted a decrease in luminescent signal following imaging 3 weeks post-injection. Before starting, place the mouse cages on a warmer. This will increase the mouse's blood flow and will make it easier to visualize the tail vein.
25. AAV is stable for multiple freeze-thaw cycles and can be thawed at room temperature (20°C–23°C). Do not keep virus thawed longer than 4 h at room temperature (20°C–23°C).
26. Dilute the virus with HBSS. The user will need 200 μ L of virus per mouse at a concentration of 2.5×10^{10} viral genomes/mL. We recommend preparing 300–400 μ L extra, as any excess virus can be frozen again at -80°C .
27. The user has the option to restrain the mouse using equipment like a tail vein illuminator (Figure 3A) or by anesthetizing with isoflurane. We have utilized a tail vein illuminator and restrainer in this protocol.
28. If using a restrainer, line the inside partly with paper towel (Figures 3B and 3C). The mouse will not be able to move around as much.
29. Load a 27 $\frac{1}{2}$ G syringe with 200 μ L of diluted virus. Adjust the needle so the bevel faces up.

⚠ CRITICAL: Throughout these injections, use the same syringe. Otherwise, the user may risk losing virus when loading a new syringe with each injection. We do, however, recommend using a new needle with every mouse. Injection through the tail flesh very easily dulls the needle.

Note: Many animal training facilities recommend the use of a 30G insulin needle for tail vein injections. While this needle is smaller and is able to enter the tail vein much more easily, it is

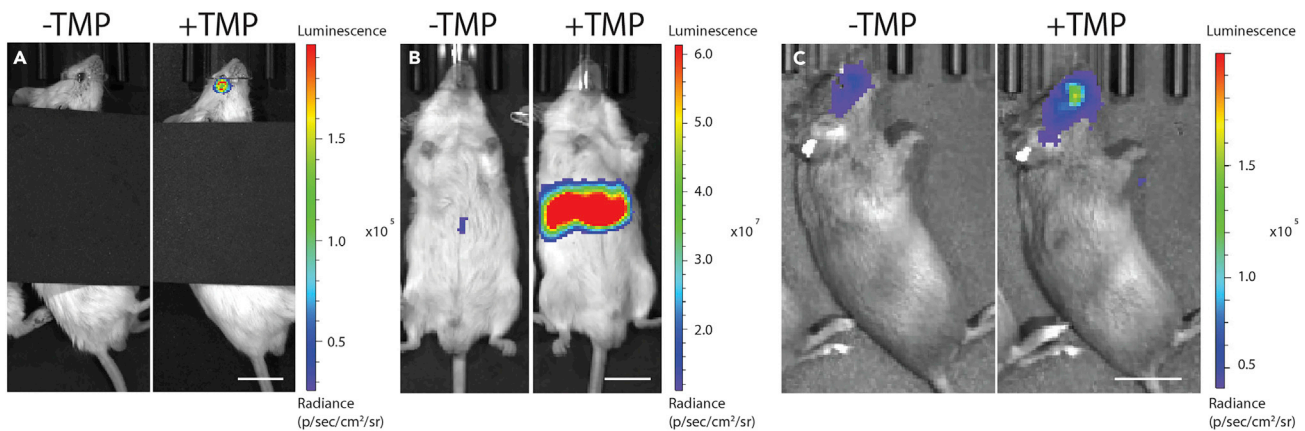


Figure 4. Example Ocular and Hepatic Imaging of Wild-Type Balb/c Mice Demonstrating an Increase in Signal Post-treatment with TMP

(A) Balb/c intravitreally injected with AAV2/2 NLuc 2A ecDHFR-Fluc show bioluminescent signal in ocular tissue after stabilization with TMP (6 h, 40 mg/kg, gavage).
 (B) Balb/c intravenously injected through the tail vein with AAV2/8 Nano luciferase 2A ecDHFR-firefly luciferase show efficient transduction of hepatocytes, which a high level of signal post TMP addition (6 h, 40 mg/kg, gavage).
 (C) A wild-type agouti mouse subretinally injected with AAV2/2 NLuc 2A ecDHFR-firefly luciferase displays more scattered signal due to injection method. All images and measurements were acquired on the IVIS Spectrum (PerkinElmer Inc., Waltham, MA). Scale bars, ~15.7 mm.

also very flexible. This increased flexibility can make it difficult to properly insert the needle into the tail vein.

30. Hold a hand warmer or reusable heat pack against the mouse's tail for 30–60 s. Wrap the warmer with a paper towel as mice have sensitive skin.
31. Once the tail vein can be clearly visualized, insert the needle and perform the injection. Inject slowly and steadily while holding the tail taut (Figure 3D).
32. The virus will take 14 days to fully transduce the liver and express. We recommend utilizing the mice within 1–3 days of this 2 week mark as hepatocytes are turned over continuously and cells expressing the AAV will divide and eventually dilute AAV-based expression.

In Vivo Bioluminescence Imaging

⌚ Timing: 2 days

The two primary ways to determine *in vivo* stabilization in real time in live mice are via fluorescence or luminescence. We have successfully used each of these techniques in the mouse eye. While initially we chose fluorescence (via an ecDHFR-DD-YFP 2A mCherry AAV), we have more recently transitioned to bioluminescence (via an ecDHFR-DD-firefly luciferase 2A Nano luciferase AAV) because it avoids the use of extremely bright, potentially phototoxic light administered directly to the eye. Here we describe the visualization of bioluminescence signal from firefly luciferase in mouse ocular or hepatic tissue using the IVIS Spectrum (Perkin Elmer, Waltham, MA, USA). We recommend receiving training on using the IVIS Spectrum as well as its accompanying isoflurane anesthesia machine.

Prior to using a small molecule stabilizer, basal levels of the ecDHFR-DD-POI should be evaluated by *in vivo* imaging, if possible, but also through alternative or orthogonal means such as western blotting or immunofluorescence. Examples of data obtained by these means can be found later in this protocol.

33. At least 10 days post intravitreal injection or 2 weeks post tail vein injection, the mice exhibit stable signal from the AAV encoding for the ecDHFR-DD firefly luciferase fusion protein. Because

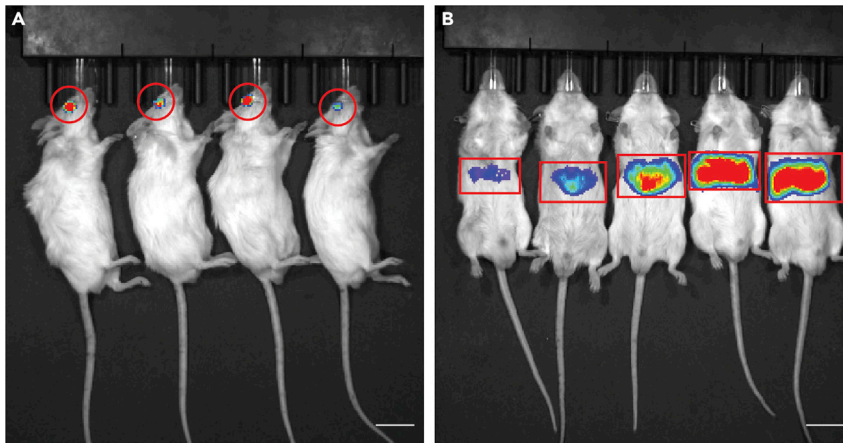


Figure 5. Region of Interest (ROI) Is Specified in the IVIS Spectrum Imaging Software to Measure Total Bioluminescent Signal, or Flux, from a Particular Area

(A) Ocularly transduced Balb/c mice have equally sized ROI rings drawn over their eye. Eye size is consistent from mouse to mouse, regardless of age and size, and so ROI rings must remain the same for ocular imaging. (B) Hepatically transduced Balb/c mice have rectangular ROI's drawn over their upper abdominal area where the liver is located. The size and shape of this area can vary greatly from mouse to mouse. ROI size must be adjusted for each mouse when imaging larger organs such as the liver. Scale bars, ~15.7 mm.

retinal cells are nondividing, the signal should theoretically never decrease over the lifetime of an intravitreally injected mouse. Transduced hepatocytes will, however, continue to divide and the titer will decrease in daughter cells over time. Therefore, in the 1–3 days post 2 weeks after the tail injection, the mice must be imaged.

34. Dissolve 40 mg of D-luciferin in 1.0 mL of 0.9% saline buffered solution. A 25 g mouse will receive an 80 μ L dose (3.2 mg per 25 g mouse or 128 mg/kg).

△ CRITICAL: Keep the D-luciferin solution protected from light and store in 4°C. If the color changes, discard and make fresh luciferin solution.

△ CRITICAL: Do not store dissolved luciferin longer than 1 week.

35. Load 80 μ L into insulin needles for i.p. injection. If the user has a different syringe/needle combination they feel more comfortable using, that is fine. Load one syringe per mouse.

Note: We recommend pre-loading one syringe per mouse due to timing issues. All mice must be injected with luciferin within a few seconds of each other. This also reduces differences in dosage from inaccurate dispensing.

36. Ensure the oxygen tank connected to the IVIS contains enough oxygen to image. In addition, check the level of isoflurane.
37. Initialize the IVIS Spectrum, this will take around 30 s.
38. Place a clean, non-reflective black mat on the base of the IVIS imaging station.

△ CRITICAL: While the imaging stage is already matte black, we recommend the use of an additional mat due to the warmth generated by the stage. The mice will overheat or incur burns if left on the bare stage.

39. Anesthetize the mice with isoflurane at a flow rate of 2.0%–2.5%. Make sure the oxygen flowrate is 1.0–1.8 L/min and the vacuum pump is turned off. This will allow isoflurane to properly

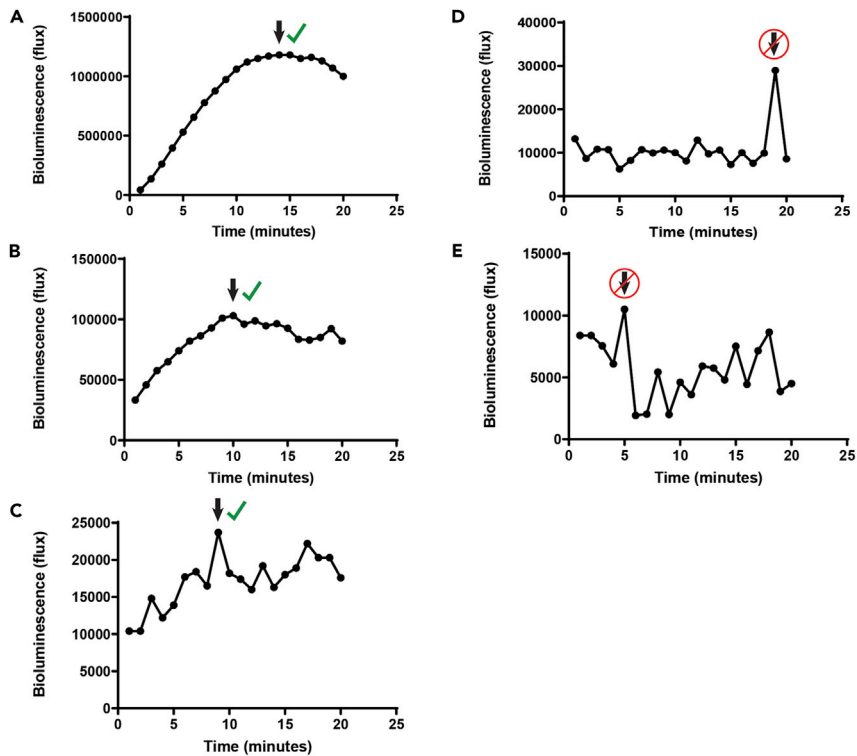


Figure 6. Evaluation of Acceptable and Unacceptable Flux Readouts

(A–C) Examples of acceptable flux curves. Post luciferin injection, the resulting flux values were graphed over the 20 min imaging period. This kinetics curve is important in determining the strength and consistency of signal. (A) An example of an ideal curve that follows a parabolic path and peaks somewhere between 10–15 min. There are no outliers or inconsistencies. This curve is indicative of strong signal and a successful i.p. injection of luciferin. (B) Example of an acceptable, but non-ideal curve. The values follow a general parabolic trend and the peak is still somewhat midrange. Such a peak can be a result of poor signal or a low luciferin dose due to poor injection technique. (C) An example of another acceptable, but again, non-ideal curve. Certain injections, such as subretinal, can result in low signal and consistently produce such curves. However, if a mouse which normally produces an ideal curve displays these inconsistent increases and decreases in flux, assume a failed i.p. injection of luciferin. (D and E) Unacceptable signal curves that have an inconsistent shape and signal variation. In (D and E) we can note a “peak” but this signal is not in line with the rest of the curve and therefore cannot be used. This is most likely due to low overall signal from the mouse, indicating poor transduction or a failed i.p. injection of luciferin. Images and measurements were acquired on the IVIS Spectrum (PerkinElmer Inc., Waltham, MA).

accumulate in the anesthesia chamber and prevent the mouse from waking up while being placed in the imager.

40. After a few minutes of exposure to isoflurane, turn on the flow to the IVIS stage under the same settings. Insert the nosecones into the IVIS nosecone ports and wait a minute for flow to regulate.
41. Place mice in numerical order on their stomach on the IVIS stages. Apply GenTeal gel to their eyes to prevent drying out.
42. Remove the first mouse and inject i.p. with the premeasured luciferin solution. Place the mouse back in its respective nosecone on either its side (if looking for ocular signal) or on its back (if looking for hepatic signal). If measuring ocular signal, the eye containing the reporter should face up. If both eyes contain the reporter, the user has the option of placing the mouse on its stomach or imaging one eye at a time.
43. Once all mice are injected with luciferin, place a non-reflective black strip over the ear tags. If the mice do not have ear tags, this may be skipped.

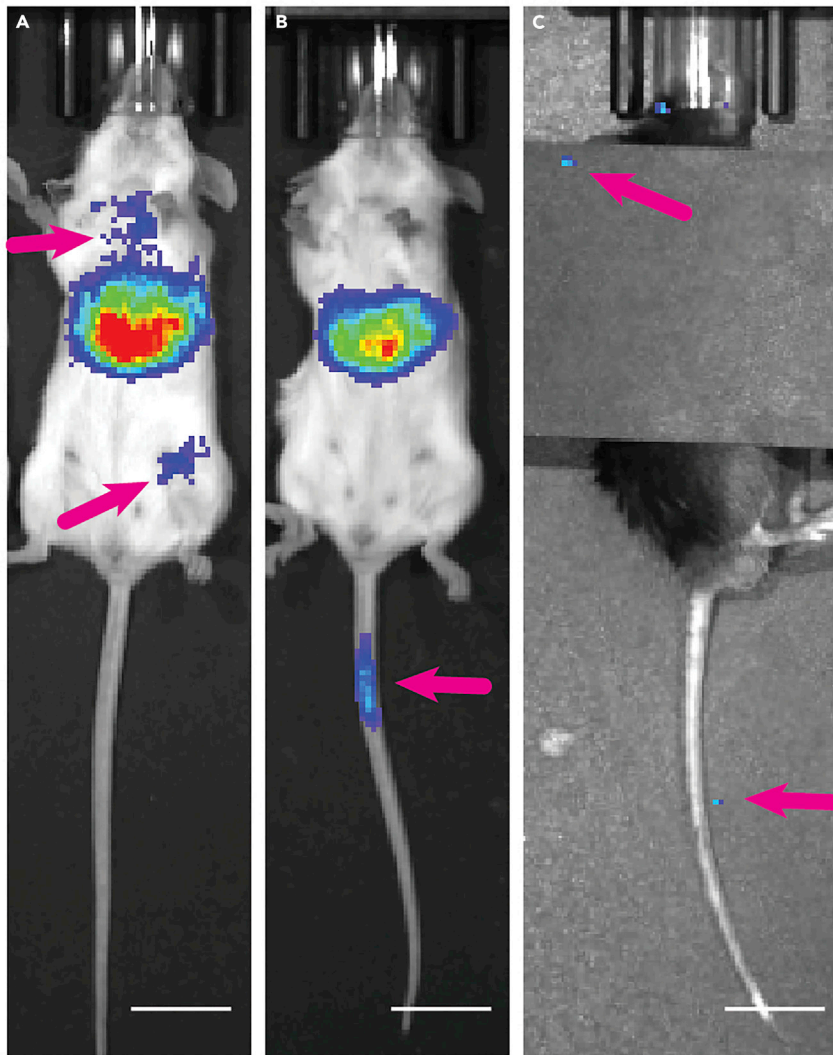


Figure 7. Presence of Background Flux Can Either Be a Result of Viral Transduction of Non-target Cells or Low Overall Signal

(A) Background signal around the Balb/c mouse's chest and lower abdominal area, in addition to around the hepatic region. In this case it can be safely assumed there was transduction of non-target organs. We do not recommend using a mouse with transduction in the gastrointestinal area as this can be indicative of poor intravenous injection technique.

(B) This mouse displays some signal in the tail, at the site of injection, but nowhere else besides the liver. Transduction of muscle tissue at the site of injection should not interfere with other organs or bioluminescent readings, and this mouse can be used for experiments.

(C) Low signal after intravitreal injection of a C57BL6/J mouse with some background scatter. The presence of background signal (arrows) indicates a very low signal originating from the eye. This can be a sign of poor transduction or a failed luciferin dose. If this is the level of ocular signal post stabilizer, then we recommend to not include this mouse due to low signal (or it is possible that the stabilizer does not work *in vivo* in the indicated tissue (see [Troubleshooting](#) for further recommendations). All images and measurements were acquired on the IVIS Spectrum (PerkinElmer Inc., Waltham, MA). Scale bars, ~15.7 mm.

△ CRITICAL: Ear tags are reflective and therefore need to be covered. Any reflectivity in the chamber will affect the resulting measured signals.

Note: If a whole-body image of the mouse is desired, the user may leave off the black strip for the first minute of imaging and pause imaging. Once paused, the black strip may be replaced over the ear tags and imaging can be resumed.

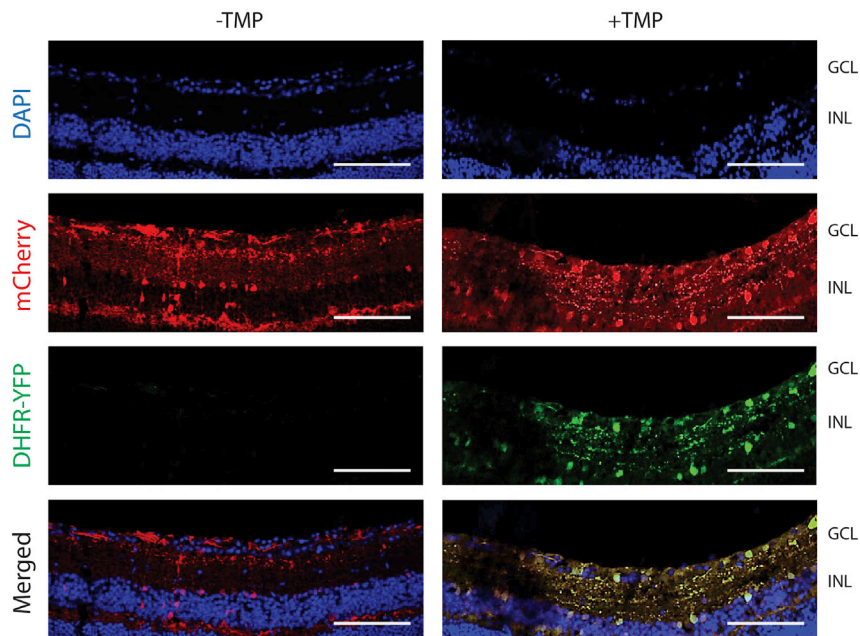


Figure 8. Example Immunofluorescence Images of the Inner Retina after Injection with AAV Expressing smCBA-Driven ecDHFR-YFP 2A mCherry in Untreated Mice (Left Hand Column) and Mice Treated with TMP (6 h, 40 mg/kg, Gavage, Right Hand Column)

Representative images of retinal cross-sections post immunostaining with YFP (green) and mCherry (red) antibodies, as well as DAPI (blue) counterstaining. Magnification, 25 \times ; scale bar, 100 μ m; GCL, ganglion cell layer; INL, inner nuclear layer. Images obtained using a Leica SP8 confocal microscope (Leica, Wetzlar, Germany).

44. In the IVIS menu, set calibration to bioluminescence for mice with measurements taken every minute over the course of 20 min.
45. After imaging, the mice can be placed back into their respective cages. They should awaken within 30 second to 1 min.
46. In the LivingImage software, set the units to radiance. Example images obtained before and after TMP administration are shown in [Figures 4A–4C](#).
47. Opening up the minute 1 image, there may be signal around the eyes. The software has automatically determined the minimum and maximum signal based on the background signal and has assigned colors to specific radiance or flux values. These may be adjusted if “Individual” is selected. Adjusting the minimum or maximum will not change the absolute bioluminescent signal value, but rather alters the presentation of the signal.
48. In the region of interest (ROI) Menu, select “Apply to All” and create a circular or rectangular ROI. Drag and reshape circular ROIs over the first mouse’s eye ([Figure 5A](#)). Make sure the ROI is slightly larger than the eye and surrounding bioluminescent signal. Select “Measure” in the ROI menu. The software will pull up all bioluminescent flux values over the course of 20 min (listed as total flux). Copy-paste this into a designated Excel sheet.
49. If performing ocular imaging, drag the exact same ROI over each mouse and retrieve each one’s total flux values over the 20 min. If imaging the liver, draw a new rectangular ROI for each mouse since the sizes of each mouse may vary ([Figure 5B](#)).

Note: Be careful that no ROI boxes overlap as signal may be double-counted in this situation.

50. Once all values are in the designated Excel sheet, graph the “total flux” column over 20 min. The resulting scatterplot should be a curve ([Figures 6A–6C](#)). If it is not a curve, this could be indicative

that the i.p. injection of luciferin failed or the detected signal was too low to form a curve (Figures 6D and 6E). Refer to [Troubleshooting](#) if curves seen in Figures 6D and 6E are observed.

51. Select the peak of the curve, the highest flux value will be used to represent the bioluminescent signal from this mouse.
52. If the mouse is untreated, this bioluminescent value is indicative of its baseline abundance of ecDHFR-DD-firefly luciferase fusion protein.

Note: There should be a small, albeit detectable amount of basal bioluminescent signal, even in the absence of stabilizer.

53. On the following day, treat each mouse with 40 mg/kg of trimethoprim (TMP, see below for recommended administration regimen). 6 h post treatment, image the bioluminescent signal once again and calculate the peak flux signal from each mouse.
54. The fold-change difference between these two days for each mouse should be calculated. This value should be fairly consistent (although the raw flux values will not be).

△ **CRITICAL:** The frequency of animal imaging in the IVIS Spectrum is limited by the time period for full excretion of the small molecule stabilizer and luciferin. We have previously noted that signal from TMP administered through oral gavage (3 mg) does not return to baseline until 9 days after ([Datta et al., 2018](#)). By using a smaller dose (1 mg) or alternative administration regimen, the TMP washout time period post gavage can be reduced to a couple of days or even overnight. While other stabilizers may not have the same tissue penetrance and retention as TMP, we recommend further research into the specific pharmacokinetics new compounds. Regarding luciferin, we have noted that this substrate appears to be metabolized or excreted 24 h post administration. Therefore, mice cannot be re-administered with luciferin multiple times within a day to gauge real-time kinetics. Bearing all of these factors in mind, in addition to the welfare of the mouse, we do not recommend imaging mice more than 4 times a week, as repeated isoflurane doses in a short frame of time can be toxic.

Note: If the user wishes to test multiple different stabilizers in a set of mice, we recommend using fully grown adult mice whose size and weight will not change significantly during this experimental period and who can endure multiple rounds of anesthesia. Changes in weight may affect distribution and absorption of small molecules as lipophilic compounds may be retained within fat pockets. In addition, metabolic changes associated with aging may affect the readings. Typically we wait at least 1 week between administrations of different stabilizers to ensure their metabolism and excretion. Before addition of the next stabilizer, we also confirm that baseline signal in the mouse has returned back to normal levels observed prior to stabilizer.

Oral Gavage Preparation and Administration of Small Molecules for *In Vivo* ecDHFR-DD-POI Stabilization

⌚ **Timing:** 1 day

Administration of small molecule stabilizers can be achieved through multiple different regimens. Previously, we demonstrated that oral gavage (1 – 3 mg, 40–120 mg/kg assuming a 25 g mouse), drinking water (0.4 mg/mL [a saturated amount], equivalent to ~100 mg/kg based on imbibing ~6.25 mL/night and a 25 g mouse), or eye drops (~120 μ L of a 1 mg/mL solution) could be used to successfully control ecDHFR-DD-POI abundance in the eye ([Datta et al., 2018](#); [Peng et al., 2019](#)). The different routes of administration resulted in different “On/Off” kinetics of controlling the ecDHFR-DD-POI. See ([Datta et al., 2018](#)) for more information on regimen-dependent kinetics. Gavage allows the user to better

define the timing of administration of the stabilizer within a short period of time. Drinking water is a convenient medium for stabilizer administration, but requires that the compound be water soluble and requires large amounts of compounds. Additionally, since mice drink throughout the night, the temporal onset of stabilization via drinking water is likely broad and not well-defined like gavage administration is. Polytrim (polymyxin/TMP sulfate) eye drops are also a convenient way to stabilize a ecDHFR-DD-POI in the eye and possibly topically, but is an approach that does not allow for accurate control of the amount of stabilizer that can reach a tissue (due to run-off, unknown routes of absorption, etc). The final route by which a researcher chooses to administer the stabilizer will have to take these differences into account when deciding appropriate regimens.

While TMP is a cheap and effective stabilizer that has good tissue penetration across a range of tissues (Iwamoto et al., 2010; Quintino et al., 2013; Quintino et al., 2018; Sellmyer et al., 2017a; Sellmyer et al., 2017b; Tai et al., 2012), other stabilizers such as pralatrexate are rather expensive, and/or have limited solubility. For these reasons, we prefer to administer stabilizers other than TMP via gavage. As mentioned above, this also allows for better control of timing of administration.

Below, we describe the preparation of the oral gavage solution containing small molecule compound for administration in mice. We recommend the use of a bath sonicator to assist with drug solubilization.

55. Dissolve 1 mg of small molecule compound in 20 μ L of DMSO. Vortex until dissolved. Warm in a 37°C water bath, if necessary.

Note: Allowing the 1 mg of compound to sit at room temperature in DMSO (20°C–23°C) for 5–10 min can improve solubility.

56. Add 40 μ L of PEG-400 and vortex. It is not uncommon for the compound to precipitate, still proceed if this occurs.
57. Add 4 μ L of Tween-80 and vortex.
58. Add 20 μ L of cremophor EL and vortex.
59. Add 116 μ L of dextrose 5% in water (D5W) and vortex. This can be made by dissolving dextrose to achieve a 5% concentration in water (5 g in 100 mL of water).
60. Incubate the solution in a bath sonicator for 10–15 min at 37°C. The compound should be fully dissolved at this point.

Note: If the compound does not dissolve when following these steps, warm the solution between every step in a 37°C bath. If necessary, the user may bath sonicate the solution for 2 min between each step as well.

Pause Point: The gavage solution can be used immediately or stored at –20°C for up to 1 week. If frozen, thaw for 10 min in a 37°C water bath and vortex before use.

61. When ready to treat the specified mice, preload the gavage syringe with attached gavage needle.
62. Scruff the specified mouse around the ears (instead of around the shoulder area) so that the head and neck is immobile. Because the grip must be fairly strong, we recommend the use of latex gloves as this will give the user better hold of the mouse.
63. Holding the mouse sideways, insert the ball-tip of the gavage needle into the mouth. Gently push the mouse's head back (so it is facing towards the ceiling) and angling the syringe so it is parallel to the mouse (pointing straight down into the esophagus).
64. At this point, the user should loosen their grip on the gavage needle and allow it to gently fall down the esophagus. The mouse may gag or bite on the needle, but do not force the needle down in any way. Gavage needles have a ball-point at the end to prevent entrance into the lungs, but are still able to do so with a little force.

Note: If the needle encounters resistance or otherwise stops, it is most likely it has hit a ridge. Lift the needle 0.5–1 cm out of the mouse, angle it towards the front of the mouse and let it drop once again. This may need to be done several times before successfully inserting the gavage needle.

65. Once almost the entire needle is inside the mouse, slowly dispense the 200 μ L.
66. Slowly remove the needle from the mouse to avoid damaging of any internal structures. We recommend observing the mouse for a minute and listening to its breath. If correctly gavaged, breathing should be silent as usual. If solution has entered the lungs, the breath will sound “wet.”
67. Image mice as described in “In Vivo Bioluminescence Imaging” section.

Note: We have imaged as early as 6 h post gavage, but it is possible that an increase in signal could be observed even earlier. This will have to be determined empirically by the user.

EXPECTED OUTCOMES

After performing the appropriate virus injections (Figures 2 and 3), the resulting increase in signal from TMP treatment can be measured by various means (Figures 4, 8, and 9). Upon observing the resulting luminescent flux values, poorly transduced mice or failed luciferin administration can be identified (Figure 7). We also suggest the user examine the images taken by the IVIS Spectrum to ensure they resemble those shown in Figure 5 and do not show aberrant signal such as in Figure 7. *In vivo* imaging should always be verified by orthogonal approaches such as immunofluorescence (Figure 8) and/or western blot (Figure 9).

LIMITATIONS

The ecDHFR-DD system of regulation is dependent on efficient ubiquitination and degradation by the proteasome. In our research (Datta et al., 2019; Datta et al., 2018; Peng et al., 2019; Ramadurgum et al., 2020), we have demonstrated the efficacy of this system in healthy mice. However, dysfunction of the proteasome is associated with a number of diseases, including neurodegeneration, as well as in the aging process (Rousseau and Bertolotti, 2018). Thus, it is possible that in certain instances, like aging or degeneration, the ecDHFR-DD system may not faithfully regulate protein abundance. Potential limitations such as these will need to be explored in future experiments and might be organ/tissue dependent.

TROUBLESHOOTING

In the described experiments, issues may arise due to mishandling of materials or contamination of reagents. Please ensure that all reagents and materials are stored according to instructions, are handled properly, and are freshly aliquoted to minimize contamination. Use of calibrated pipettes is strongly recommended as is the use of aerosol barrier tips wherever possible.

Problem 1

Low stabilized signal from the ecDHFR-DD fusion protein.

If bioluminescent signal resembles Figure 7C ($< 10^3$ p/s/cm²/sr), with background spots, this can be indicative of too low overall signal or a failed luciferin dose. Additionally, a low signal will be immediately apparent when the resulting flux values are graphed and compared to those provided in Figure 6.

Potential Solution

In our experience, with a smCBA-driven ecDHFR-DD firefly luciferase AAV construct, we typically observe 10^4 – 10^5 p/s/cm²/sr at baseline for intravitreal injections and 10^5 – 10^7 p/s/cm²/sr for baseline hepatic transductions. If an animal demonstrates bioluminescence values well below these standard numbers, we recommend to re-perform the luciferin i.p. injection to see if the value changes. If the

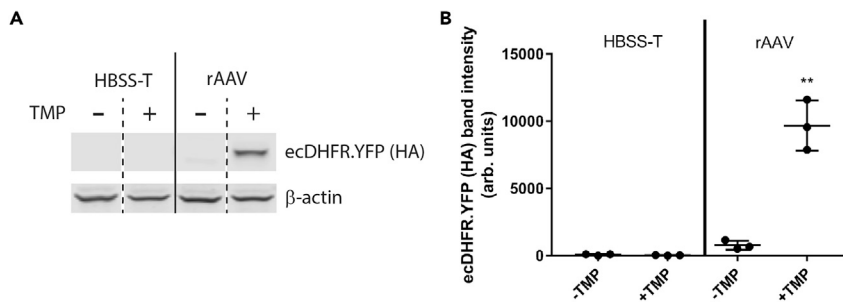


Figure 9. Example Western Blotting Data Obtained from Mice Transduced Intravitreally with ecDHFR-YFP(HA)-Encoding AAV

(A) C57BL/6J mice were intravitreally injected with either HBSS-T or AAV encoding for ecDHFR-YFP with an HA tag, followed by treatment with TMP (6 h, ~112 mg/kg, gavage). Neural retina were isolated and subjected to western blotting. Images were obtained and quantified using an Odyssey CLx (LI-COR, Lincoln, NE) and its associated software. Original images were published previously in (Datta et al., 2018). ** $p < 0.01$, unpaired, 2-tailed t-test assuming equal variance compared to “- TMP (naïve).”

resulting flux curve resembles those in Figure 6A, 6B, or 6C, it is likely the low signal was a result of a failed i.p. injection or weak stabilization by the gavaged compound. If the curve resembles that of 6D or 6E, it may be the case that the signal is too low to be properly detected by the IVIS. At this point, we then recommend to evaluate that mouse’s response to a conventional stabilizer like TMP. If the TMP-treated animal shows a flux curve similar to 6D or 6E at this point, it is safe to assume that the target tissue has not been properly transduced.

Problem 2

Low (or no) fold change post small molecule stabilizer addition.

Potential Solution 1

It is possible that particular mice may vomit post gavage administration, and therefore did not receive the compound. To prevent this, the user can observe the mice for 1–2 min after gavage to ensure they do not vomit.

Potential Solution 2

If treatment of the small molecule was through drinking water, we suggest considering administering the compound through oral gavage instead to be more certain of administration. It is possible, although improbable, that the mouse did not drink overnight.

Potential Solution 3

Another possibility if using an unvalidated small molecule stabilizer is that the compound is quickly metabolized, excreted or does not penetrate your organ/tissue of interest. If this is suspected to be the case, try TMP as a positive control as this molecule has excellent penetrance in most tissue, including the central nervous system. Additionally, presence of the stabilizing compound can be verified and quantified by mass spectrometry as described in (Datta et al., 2018).

Problem 3

Bioluminescence signal from wrong organ.

If the user notes signal from non-target organs upon imaging, such as what is observed in Figure 7A (liver transduction), this may be indicative of incorrect injection technique, or a failed injection.

Potential Solution

We recommend the reevaluation of this particular mouse to ensure the signal is true. Next, the user should observe this mouse when anesthetized by isoflurane to observe any movement. Some mice

will twitch and move when under isoflurane, this movement can be enough to register signal coming from the wrong location. After the initial photograph taken of the mouse, the IVIS does not take additional pictures as it monitors for luminescence. It just assumes that the mouse is immobile, it just measures luminescence. If this particular mouse does happen to move under isoflurane, we recommend imaging this mouse separately with a higher flow rate of isoflurane (around 2.5%, at maximum). If these suggestions do not resolve the issue, it is likely there was an issue during injection. This particular mouse should not be used in this case.

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, John D. Hulleman, Ph.D., john.hulleman@utsouthwestern.edu.

Materials Availability

No new mouse lines were generated in this study. C57BL6/J (cat# 000664) and agouti mice (cat# 001912) were obtained from The Jackson Laboratory (Bar Harbor, ME). Balb/c mice were obtained from the UT Southwestern Mouse Breeding Core.

Data and Code Availability

No new data or code were generated for this study.

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AUTHOR CONTRIBUTIONS

Conceptualization, J.D.H.; Writing – Original Draft, P.R. and J.D.H.; Writing – Review & Editing, P.R., S.D., and J.D.H.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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