Video Article

# Surgical Ablation Assay for Studying Eye Regeneration in Planarians

Jacob M. Morton*[1], Marwa A. Saad*[1], Wendy S. Beane[1]

[1]Department of Biological Sciences, Western Michigan University

*These authors contributed equally

Correspondence to: Wendy S. Beane at wendy.beane@wmich.edu

URL: https://www.jove.com/video/55594
DOI: doi:10.3791/55594

Keywords: Developmental Biology, Issue 122, Planaria, flatworm, eye ablation, eye resection, eye excision, ocelli, optic cup, stem cells, Schmidtea mediterranea, neuroscience, developmental biology, regeneration

Date Published: 4/14/2017

Citation: Morton, J.M., Saad, M.A., Beane, W.S. Surgical Ablation Assay for Studying Eye Regeneration in Planarians. *J. Vis. Exp.* (122), e55594, doi:10.3791/55594 (2017).

## Abstract

In the study of adult stem cells and regenerative mechanisms, planarian flatworms are a staple *in vivo* model system. This is due in large part to their abundant pluripotent stem cell population and ability to regenerate all cell and tissue types after injuries that would be catastrophic for most animals. Recently, planarians have gained popularity as a model for eye regeneration. Their ability to regenerate the entire eye (comprised of two tissue types: pigment cells and photoreceptors) allows for the dissection of the mechanisms regulating visual system regeneration. Eye ablation has several advantages over other techniques (such as decapitation or hole punch) for examining eye-specific pathways and mechanisms, the most important of which is that regeneration is largely restricted to eye tissues alone. The purpose of this video article is to demonstrate how to reliably remove the planarian optic cup without disturbing the brain or surrounding tissues. The handling of worms and maintenance of an established colony is also described. This technique uses a 31 G, 5/16-inch insulin needle to surgically scoop out the optic cup of planarians immobilized on a cold plate. This method encompasses both single and double eye ablation, with eyes regenerating within 1-2 weeks, allowing for a wide range of applications. In particular, this ablation technique can be easily combined with pharmacological and genetic (RNA interference) screens for a better understanding of regenerative mechanisms and their evolution, eye stem cells and their maintenance, and phototaxic behavioral responses and their neurological basis.

## Video Link

The video component of this article can be found at https://www.jove.com/video/55594/

## Introduction

Planarians are a powerful model organism for studying adult stem cell-mediated regeneration. These non-parasitic freshwater flatworms possess the ability to regenerate any and all missing tissues, including their central nervous system and brain[1]. Studied as far back as the 1700s[2], technological advances in the planarian field during the past 10-15 years (such as a sequenced genome, *in situ* hybridization, immunohistochemistry, RNA interference (RNAi), and transcriptomics) have updated this historical model organism. Specifically, planarians have recently gained popularity as an emerging model for eye research[3].

Planarians have prototypic eyes with only two tissue types, the photoreceptor neurons and pigment cells; this has enabled the characterization of its eye stem-cell population and demonstrated that many of the same genes regulating vertebrate eye development are conserved in planarians[4,5]. The optic cups are located dorsally and comprised of the white, unpigmented dendrites of the photoreceptor neurons and the semi-lunar black pigment cells, and the eyes innervate the brain via an optic chiasm. In addition to being a model for elucidating regenerative processes[6], the planarian eye is well suited to studying the evolution of visual mechanisms[7], behavioral responses to light (planarians display negative phototaxis)[8], and the neurological basis of behavior[9].

Eye regeneration in planarians has been largely studied in two main contexts: as part of head regeneration following decapitation[4,10], and following excision of just the eye tissues[11,12]. Most planarian studies on eye regeneration have used the decapitation method, as it is simple and straightforward. The most common planarian eye excision method to date has been via hole punch with a fine glass capillary tube[13,14], although some studies have also performed amputations just behind the eyes (partial decapitation)[15]. However, all of these methods involve the loss of many tissues other than just the eye (such as brain, intestines, and nephridia), potentially complicating interpretation of results. The eye ablation protocol presented here restricts excision to the eye tissues (specifically excluding brain), resulting in data that are more specific to the eye. Additionally, unlike decapitated worms that take 7-14 days to begin feeding, eye ablated worms will feed within 24 h of ablation[12], allowing RNAi experiments (where RNAi is delivered via food) to be performed concurrently.

Although eye ablation is technically harder to successfully perform than decapitation, current studies involving eye excision have not included detailed instructions on their procedures. The goal of this video article is to enable researchers to consistently remove the planarian optic cup without disturbing the underlying brain tissues and removing as few other tissues as possible. This method can be used for both single and double eye ablation and is applicable to a wide range of investigations. Like most regeneration assays, eye ablation is well suited for combination

 

with both pharmacological and genetic (RNAi) screens, as well as behavioral studies. Here we describe methods for the handling of worms, maintaining a planarian colony, and the eye ablation technique itself.

## Protocol

# 1. Animal Culture and Handling

NOTE: This protocol uses *Schmidtea mediterranea*, a diploid planarian species with a sequenced genome[16,17] that is commonly used for regeneration research. However, the assay is equally successful with other species, such as *Girardia tigrina* and *Girardia dorotocephala* (which are commercially available).

1. **Maintain worms in "worm water" made from 0.5 g/L sea salts in ultrapure (or filtered deionized) water. Use sterile polypropylene or glass containers to hold the worm water. See Supplementary File 1 for details on worm water preparation.**
   NOTE: Never use soap to clean containers (or other supplies) as soap is toxic to worms; instead wipe with 70% ethanol and allow to air dry.
   1. Wear gloves while working with planaria to protect them from contamination.
      NOTE: Worms are very sensitive to environmental toxins and chemicals, including soap, bleach, shampoo, conditioner, and hand lotion.

2. House colonies in polypropylene food containers, with the lid left partially open for air exchange at ~20 °C (room temperature). Store experimental worms in either petri dishes (20 worms per 100 mm dish) or non-treated tissue-culture plates (1 worm per well, for 12-well plates). To reduce stress, keep both colonies and experiments in the dark.
   NOTE: Colonies should have no more than 500-1,000 worms per liter of worm water. For experiments, leave lids fully in place, since airflow is designed into these dishes.
3. Feed non-experimental worms once a week with pureed organic beef or chicken liver by dropping puree from a transfer pipette, placing drops of food throughout the container. Allow worms 2 h to consume food in the dark before cleaning out container (see step 1.4). Prior to use, store puree at -20 °C for short term (weeks) or -80 °C for long term (months); do not refreeze puree for re-use (as bacteria may bloom and harm the worms).
   NOTE: Liver should contain no hormones or antibiotics and preferably not be previously frozen. Centrifuge puree prior to freezing (or prior to feeding) to remove air and prevent food from floating. Food should sink to the bottom of the container. 1 mL of puree is sufficient for 500-1,000 worms.
4. Exchange water once per week with fresh worm water for both colonies and experiments. Colony containers should also be wiped down with an unbleached paper towel (to remove mucus residues that trap wastes) once a week for starving worms, or twice a week for feeding worms (2 h after feeding and again 2 days later). In order to retain the biofilm, do not wipe more than 80% of the container.
5. **Move worms between containers (or surgery setup) using a transfer pipette. To free worms adhered to container surfaces, squirt water over/behind the worm to release it from the surface. Then suck up the worm into the pipette, while trying to keep the worm within the bottom inch (thinner) portion of the pipette.**
   1. Backload pipettes with a small amount of worm water before sucking them up.
   2. Try to move the water around the worm, rather than the worm itself, to help prevent tearing soft-bodied worms by touching them with the pipette.
   3. Keep experimental dishes covered unless transferring worms or replacing water.

# 2. Preparation

1. **Stop feeding worms at least one week prior to experiments.**
   1. Select worms that have been not been fed for ≥1 week and are at least 5-7 mm in length. Transfer the worms to a Petri dish 2/3 full of worm water, and confirm worm length by sliding a ruler underneath the dish. Measure worms while fully extended and moving.
      NOTE: While smaller (5-7 mm) worms work better when performing subsequent immunofluorescence and *in situ* hybridization analyses, larger worms (8-10 mm) are easier to work with – especially when learning to ablate.
   2. Ensure that worms are whole, undamaged, and not recently regenerating.
      NOTE: Regenerating worms will have much lighter (or no) pigment in the head and/or tail region as compared to normal worms.
   3. If performing RNAi or pharmacological treatments on worms, do so at this point. If worms were fed as part of the treatment (as for RNAi), wait at least 7 days after the last feeding before continuing to step 2.2.

2. **Clean the work space and dissecting microscope base with 70% ethanol and allow it to dry completely. Place the dish of selected worms to the left of the scope. To the right of the scope, place a pair of #5 forceps, a 31 G 5/16-inch insulin needle with a 1 mL syringe, and a clean transfer pipette.**
   1. Ensure that the instruments are kept from touching the bench (which could introduce contaminants). Use a rigid item (such as a chopstick rest) to keep the forceps, needle, and pipette clean. Alternatively, place instruments on top of a fresh brown (unbleached) paper towel.
      NOTE: These and subsequent instructions are written as they pertain to right-handed individuals. Left-handed individuals should reverse the right/left directions.

3. Next to the work space, place a box of lint-free tissue wipes, a source of fresh worm water, and some extra transfer pipettes (protected from the bench top). Place worm water in a plastic wash bottle for ease of dispensing. Also place a labeled 12-well plate (or 100 mm Petri dish) to the right of the scope for collecting ablated animals.
4. **If using a custom-made Peltier plate to immobilize worms, position the Peltier plate into the depression in the base of the dissecting microscope and adjust the output on the DC power source until the working surface of the Peltier plate is sufficiently**

**cool (typically ~5 V).Alternatively, make a cold plate by filling a 100 mm Petri dish ½ full with water and freeze for at least 24 h. Discard the lid and place the bottom of the 100 mm dish on the base of the dissecting scope, then place the lid of a 60 mm Petri dish upside down directly on the surface of the ice (Figure 1A).**

1. After the water has frozen in the 100 mm dish, a "volcano" of ice can appear in the center of the plate. If this happens, use a razor blade to scrape the ice flat, to remove any irregularities from the surface.
   NOTE: During surgeries the ice will melt, making ablations much more difficult. Prepare several ice dishes in advance, and replace frozen ones for melting ones during the assay as soon as the lid of the 60 mm dish starts floating.

5. **Prepare the surgery surface. Cut a 5 cm x 10 cm piece of plastic paraffin film (4 $cm^2$ if using the petri dish cold plate) and place it on the center of the immobilization device. Fold a lint-free tissue wipe into a square roughly 2 $cm^2$ and place it on top of the film. Cut a piece of white filter paper 1.5-2 $cm^2$ and place it on top of the wipe. See Figure 1B-1E.**
   1. Hold the folded wipe in place with a gloved finger, and use worm water to lightly dampen the wipe to ensure that it remains folded. Use the side of a transfer pipette to roll the wipe flat.
      NOTE: Cold temperature helps to keep worms from moving. Working "dry" also helps in immobilization. The tissue wipe acts to wick water through the filter paper, keeping the worm moist during surgery without allowing worms to dry out completely (which is lethal).

## 3. Surgical Ablation

1. **Use a transfer pipette to place one worm dorsal side up onto the filter paper. Turn the light source on and direct the goosenecks so that the light is focused on the worm. Adjust the dissecting scope focus and magnification so that the eyes are clearly visible (the entire worm does not need to be in view); 5X magnification is a good starting point. Orient the worm by rotating the paraffin film so that the head is pointed towards the researcher (towards the front of the microscope), angled 30-40 degrees to the right.**
   1. Avoid use of bright light to prevent excess worm movement. Planarians display negative phototaxis and will attempt to evade bright light.
   2. If the worm is positioned ventral side facing up (with pharyngeal opening visible), use the dull side of the forceps to gently reposition the worm dorsal side up (with eyes visible). Avoid approaching the animal with the sharp tip of the forceps to minimize the chance of tearing through soft tissues when repositioning the animal.
   3. Adjust the microscope focus so that the eyes are clearly in focus. Also ensure that both the eyes and surrounding head tissues are in view.

2. **Hold a fresh needle/syringe in the right hand as though it were a pen (between thumb and index finger). Brace the left thumb against the Peltier plate (or Petri dish cold plate), and use the left thumb as a fulcrum on which the bottom portion of the syringe will rest (Figure 2A). Look through the microscope, and make sure the bevel of the needle is visible.**
   NOTE: Needles are very sharp. Be cautious when handling them. Wearing latex or nitrile gloves can offer some protection.
   1. Use the left thumb to help hold the needle/syringe steady throughout the procedure and avoid shaking hands.
   2. Make an angle of about 40° with the left thumb and left index finger (with the index finger on the surgery surface) to help hold the surgery surface stabile.

3. **With the bevel of the needle facing upward (like a spoon), position the needle at right angles to the eye (Figure 2B). Use the tip of the needle to gently penetrate the thin layer of tissue overlying the optic cup (white, unpigmented region) of the eye, scooping from right to left. Very gently remove any pigmented tissue located** within the optic cup, taking care not to puncture the bottom or destroy the borders of the optic cup.
   1. If the worm has been on the filter paper for >1-2 min at any point during the procedure, add one drop of worm water to rehydrate the worm.
      NOTE: Dehydration will increase the worm's susceptibility to ripping injuries.

4. Repeat step 3.3 until all of the black pigmented tissues (pigment cells), as well as all of the white tissues of the optic cup (dendrites of the photoreceptor cells), are removed. Remove excised tissues stuck to the end of the needle by carefully wiping with a tissue wipe. If double eye ablation is desired, ablate the second eye at this point.
   NOTE: To avoid injury, needles can be cleaned by grasping the needle with a clean tissue wipe held with the thumb and forefinger, then using the other hand to pull the needle through the wipe away from the thumb and forefinger. Alternatively, the needle can be wiped onto the surface of a wet tissue wipe and examined for cleanliness.
5. **When finished, use a transfer pipette to move the worm to a labeled dish containing fresh worm water. If analyzing group data, place up to 20 worms in a single 100 mm Petri dish. If tracking regeneration in the same individuals over time, place 1 worm into each well of a 12-well plate. Once all worms are transferred, rinse worms by removing all worm water from the dishes/wells and replacing with more fresh worm water.**
   1. To remove worms from the filter, backload the pipette with a small amount of worm water and release the water onto the worm. This should lift the worm off the filter paper, to be immediately sucked into the pipette for transfer.

6. Incubate experiments at ~20 °C (room temperature) protected from light and follow the regenerative process.
   NOTE: Worms can be stored in a temperature-controlled incubator to maintain a constant temperature, but this is not strictly necessary. Most room temperatures vary only by +5 °C, which will not affect the worm's ability to regenerate.
7. If desired, fix worms for immunohistochemistry (see **Figures 3-4**) and/or *in situ* hybridization analyses of gene expression. Different fixation methods exist for planarian immunohistochemistry[18,19,20] and *in situ* hybridization[21,22,23] protocols.
8. When experiments are concluded, sacrifice live worms by removing worm water from the dish and replacing with 70% ethanol. Incubate worms for 3-5 min, checking for worms to lyse and turn grayish.
9. Place non-treated worms in the normal waste stream once sacrificed. Avoid introducing live worms into the environment, particularly non-native species.

## Representative Results

For the first 1-2 h post surgery, animals may exhibit decreased movement compared to intact worms (however they will still move). If desired, worms will eat within 24 h of surgery (for instance, for RNAi feeding). When following eye regeneration in the same individuals over time, make sure to take a photograph of each worm both prior to surgery (intact) and at 1 h post ablation (hpa). By 4 days post ablation (dpa) regenerating pigment cells should be visible, and by 14 dpa the entire eye will have fully regenerated (**Figure 3A-B**). This includes the photoreceptor neurons and their innervation to the brain (**Figure 3C**), as well as functional recovery of the visual system (**Figure 4A-C**). Successful ablations will not disturb the underlying tissues of the brain (**Figure 4D-E**), nor introduce other injuries to the animal (**Figure 5A**). Unsuccessful ablations include animals with: an overly large excision that connects the two eyes (**Figure 5B**), tears to the lateral margin of the animal (**Figure 5C**), and/or wound sites that poke through the ventral epidermis (**Figure 5D**). Furthermore, unsuccessful ablations include incomplete removal of the entire optic cup, comprised of both the white unpigmented tissues and the black pigment cells (**Figure 5E-F**).

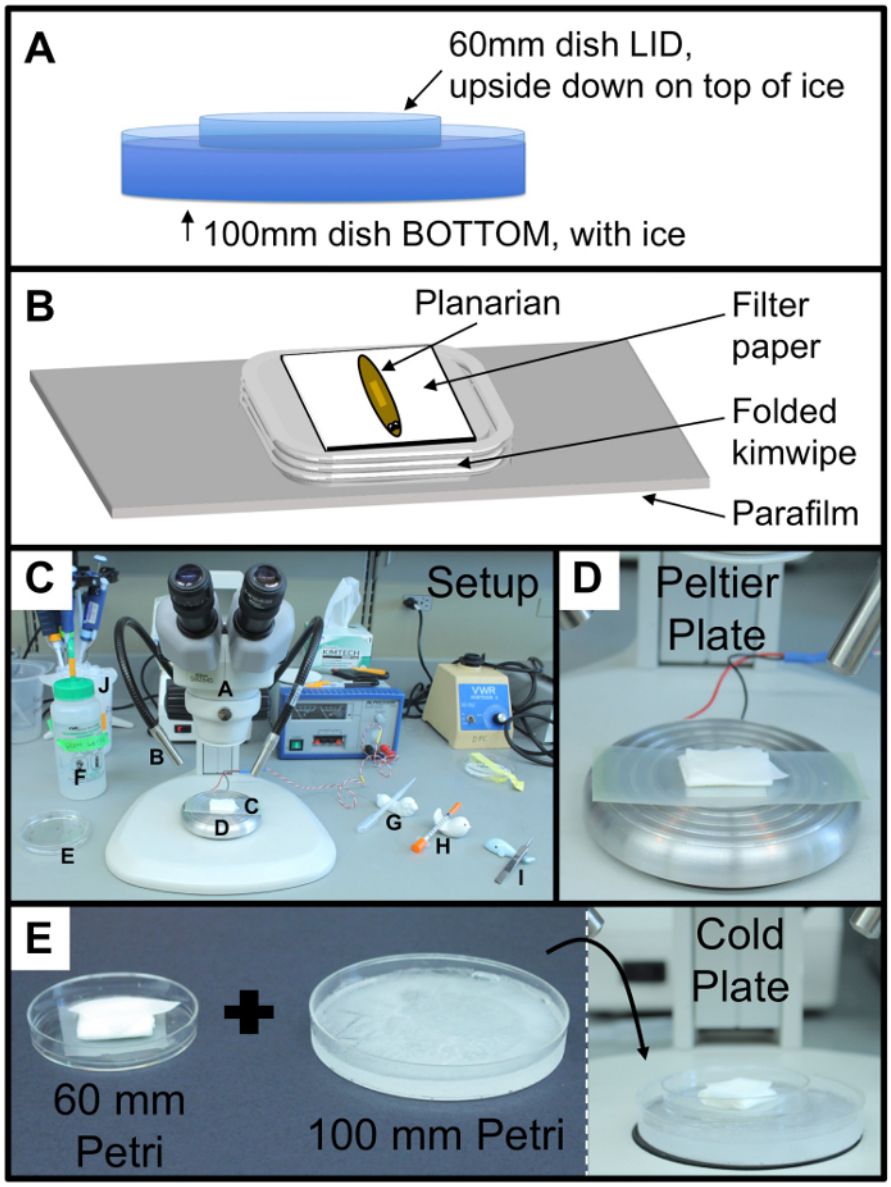


**Figure 1: Surgery preparation.** (**A**) Diagram of cold plate construction, using the bottom of a 100 mm Petri dish (filled with ice) and the lid of a 60 mm Petri dish (placed upside down on the ice surface). (**B**) Diagram of the surgery surface, made from a stack of (top to bottom) white filter paper, a damp folded tissue wipe, and a piece of paraffin film. (**C**) The surgery set up (for the right-handed). A: dissecting scope, B: gooseneck lighting, C: surgery surface, D: Peltier plate, E: dish of 5-7 mm worms ready for ablation, F: wash bottle of worm water, G: chopstick rest holding clean transfer pipette, H: chopstick rest holding needle/syringe, I: chopstick rest holding forceps, J: container of extra transfer pipettes. (**D**) Custom Peltier plate configuration. (**E**) Petri dish cold plate configuration. Please click here to view a larger version of this figure.


**Figure 2: Position of hands and needle/syringe during ablation.** (**A**) Placement of hands (for the right-handed). Note that the left thumb is used to support the syringe (held in the right hand) to minimize movement. (**B**) Placement of the needle in relation to the worm's eye. Note that the beveled surface of the needle faces upwards. Please click here to view a larger version of this figure.

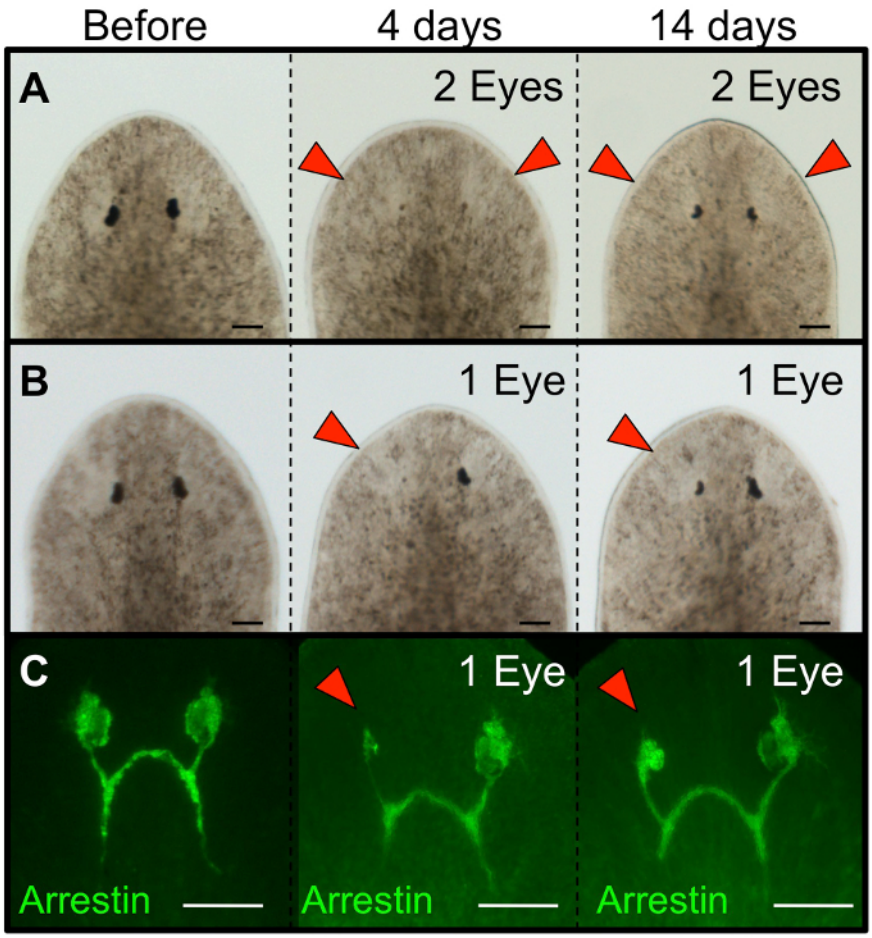


**Figure 3: Ablated planarian eyes regenerate.** Morphology of *Schmidtea mediterranea* planarians regrowing eyes following (**A**) double eye ablation and (**B**) single eye ablation. (**C**) Immunohistochemistry showing regeneration of the photoreceptor neurons (anti-arrestin) following single eye ablation. Intact worms are shown before surgery, and regenerates are shown at days 4 and 14 after ablation. For single eye ablations, the left eye was ablated and the right eye serves as an internal (uninjured) control. Red arrowheads: ablated eyes. Scale bars = 100 µm. Please click here to view a larger version of this figure.

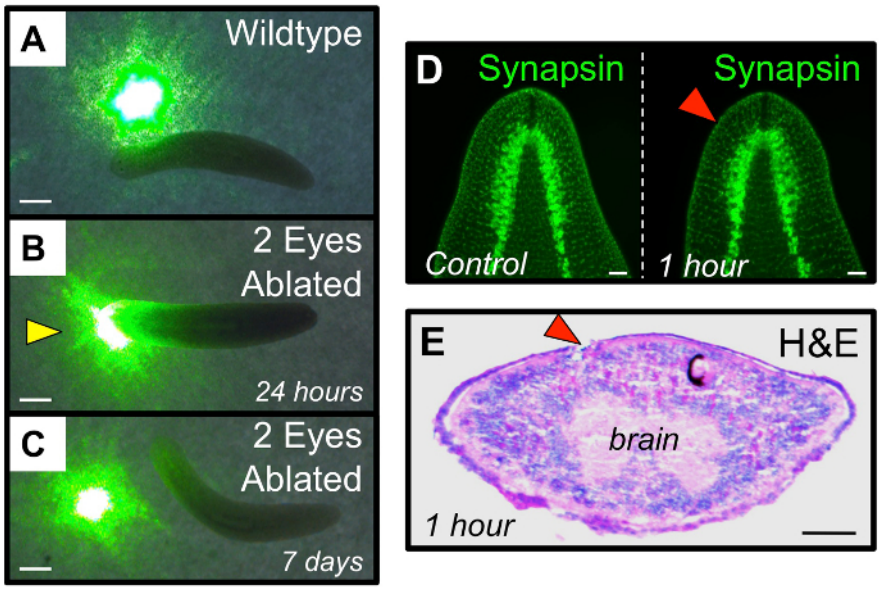


**Figure 4: Functional recovery of the visual system after ablation.** (**A**-**C**) Functional assay to test planarian photophobia. (**A**) Intact worms avoid traveling through areas of light, such as a spot from a green laser pointer. (**B**) At 24 h post ablation, "blind" double eye ablated worms travel through light spots. (**C**) At 7 days post ablation, double eye ablated worms have regenerated their visual system sufficiently to recover light avoidance. Yellow arrowhead: aberrant behavioral response. (**D**-**E**) Eye ablation is restricted to the tissues of the optic cup, and does not disturb the underlying brain tissues. (**D**) Immunohistochemistry showing brain architecture (anti-synapsin) is unchanged from before ablation to 1 h post ablation. (**E**) Hematoxylin and eosin (H&E) staining at 1 h post ablation in a transverse section showing damage is largely restricted to the site of the ablated optic cup. Right eye (with black pigment cells) serves as internal control. Red arrowheads: ablated eyes. Scale bars = 1 mm in (A-C) 1 mm, 100 µm in (D-E). Please click here to view a larger version of this figure.

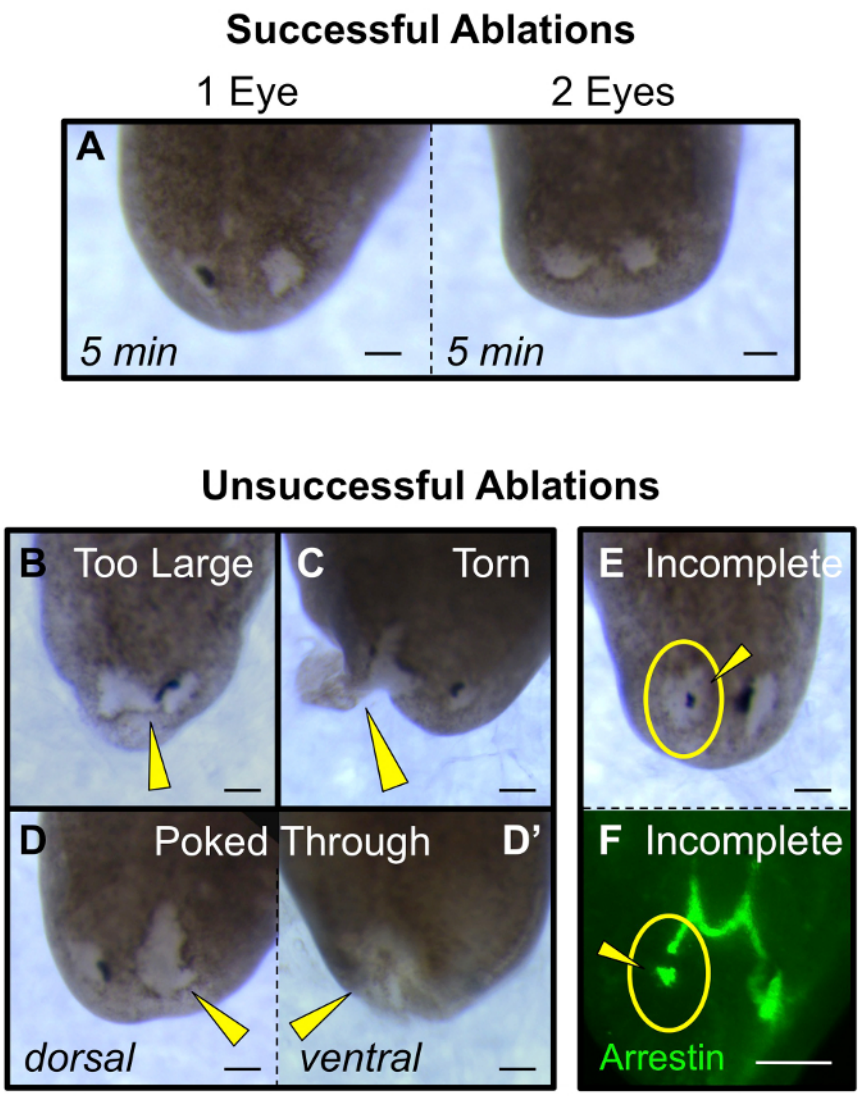


**Figure 5: Hallmarks of successful and unsuccessful ablations.** (**A**) Successful single and double eye ablations (at 5 min post ablation) have wounds that are roughly similar in size to the original optic cup. (**B**-**E**) Unsuccessful ablations: (**B**) too much tissue is removed or the wound connects the two eyes, (**C**) the wound site tears and extends to the margin, (**D**) the wound is too deep and pokes through from the dorsal (**D**) to the ventral (**D'**) side, and (**E**-**F**) not all of the optic cup tissues are removed, visualized both morphologically (**E**) and by anti-arrestin staining of the photoreceptor neurons (**F**). Yellow arrowheads: aberrant ablations. Yellow circles: ablation site. Scale bars = 100 µm. Please click here to view a larger version of this figure.

## Discussion

This eye ablation technique improves on the current methods (such as hole punch) by excluding brain tissues and restricting excision mainly to the eye tissues. With practice, this technique can be performed by most individuals, from technicians experienced in microsurgeries to inexperienced but conscientious undergraduate students. It is recommended that this technique be practiced many times prior to using ablations in experiments, including (when possible) confirmation of complete removal of all eye tissues by immunohistochemistry or *in situ* hybridization for eye marker(s)[4,5,13]. The most critical step in this protocol is the scooping out of tissues. It is important to not remove either too much or too little tissue. This can be avoided by not scooping too deeply with the needle, to avoid penetrating through to the ventral side of the worm. The tip of the needle should only be inserted about 0.4 mm deep, and the beveled part of the needle should never go all the way through the eye. Special care should be taken to not damage the fragile tissue between the two eyes (the medial edge of the black pigmented region of each eye represents the borders of each eye). Damage between the eye and the distal margin of the worm should also be avoided. Smaller worms are more likely to rip or be unintentionally injured, so the use of larger worms (particularly for practice) is recommended. The main limitations of this technique are that it is relatively time intensive (and not appropriate for high-throughput screens), and it requires an initial investment in practicing to ensure the accuracy of the results. However, with practice 10 eyes can be ablated in ~15 min.

A major area for troubleshooting is in keeping planarians still during the procedure. The two main reasons worms are not immobilized sufficiently to perform ablations are (a) too much water and (b) loss of cold temperature. (A) Excess planarian water that pools on the surgery surface while transferring worms will allow worms to move and complicate the surgery. The surgery surface (lint-free tissue wipe and filter paper) should

remain moist only. If at any point during the procedure the wipe becomes saturated with worm water, it should either be replaced or a transfer pipette can be used to gently remove excess fluid from the tissue wipe (always draw from the wipe and not the filter paper). Tissue wipes can also be used to remove excess water pooling on the surgery surface. This process may need to be repeated many times, depending on the number of worms being ablated. (B) If using the petri dish cold plate set up, remember to replace the cold plate as soon as the ice starts melting and the 60 mm Petri dish lid begins to float. This will ensure both a stable surgery surface and an appropriately cold surface. Alternatively, if using the Peltier plate set up, be aware that after 45 min to 1 h of use, the heat in the outer rings will overcome the cold plate in the center and all cooling will be lost. If this happens, turn off the Peltier plate for 5-10 min to allow it to come to room temperature before resuming ablations.

Another area of troubleshooting is during worm transfer between the dish and the surgery surface and back again. If ablated worms are hard to remove from the filter paper, place a drop of worm water on the worm until the liquid pools on top of the filter paper. Worms can then be sucked into the transfer pipette as usual. If problems persist, try flipping the worm onto its ventral side first (using the dull side of the forceps). If a worm gets stuck inside the transfer pipette, this is usually a sign that too much water has been taken up (worms should be drawn into the transfer pipette no farther than about an inch). To remove a worm stuck in a transfer pipette, draw up 1-2 mL of worm water into the pipette, so that the worm is covered with water. Firmly flick the side of the pipette until the worm detaches from the wall of the pipette and is floating.

If the needle becomes dull, replace with a new needle/syringe. Consider replacing the needle after using it to ablate ≥30 eyes. Excessive removal of resected tissues by wiping with a tissue wipe can also dull the needle, making ablations more difficult. It is okay if resected tissues stay within the beveled part of the needle throughout the procedure (even across multiple animals). Always use a fresh needle/syringe when changing between worms of differing conditions (*e.g.* wildtype *vs.* RNAi worms), as well as when beginning new ablation sessions (*i.e.* on different days).

This surgical eye ablation technique is a powerful means to investigate light-induced behaviors (and their genetic and neurological basis) in planarians, particularly when combined with pharmacological treatments or RNA interference. Eye ablation is also a great means by which to elucidate the mechanisms that regulate endogenous planarian eye regeneration (and eye stem cell maintenance and differentiation) *in vivo*. As most vertebrates have very limited abilities to regenerate eye tissues, understanding how planarians are able to regenerate their eyes will be important in identifying possible mechanisms for translation into future therapies.

## Disclosures

The authors have nothing to disclose.

## Acknowledgements

The authors would like to thank Michelle Deochand for perfecting this eye ablation technique, Taylor Birkholz for assistance with the functional assay, Michael Levin for the anti-arrestin antibody, and Junji Morokuma for information on the Peltier plates. This work was supported by an SFSA grant from Western Michigan University to WSB.

## References

1. Gentile, L., Cebria, F., & Bartscherer, K. The planarian flatworm: an in vivo model for stem cell biology and nervous system regeneration. *Dis Model Mech.* **4** (1), 12-19 (2011).
2. Elliott, S. A., & Sanchez Alvarado, A. The history and enduring contributions of planarians to the study of animal regeneration. *Wiley Interdiscip Rev Dev Biol.* **2** (3), 301-326 (2013).
3. Emili Saló, R. B. in *Animal Models in Eye Research.* (ed Panagiotis A. Tsonis) Ch. 3, 15-26 Academic Press. (2008).
4. Lapan, S. W., & Reddien, P. W. dlx and sp6-9 Control optic cup regeneration in a prototypic eye. *PLoS Genet.* **7** (8), e1002226 (2011).
5. Lapan, S. W., & Reddien, P. W. Transcriptome analysis of the planarian eye identifies ovo as a specific regulator of eye regeneration. *Cell Rep.* **2** (2), 294-307 (2012).
6. Inoue, T. *et al.* Morphological and functional recovery of the planarian photosensing system during head regeneration. *Zoolog Sci.* **21** (3), 275-283 (2004).
7. Pineda, D. *et al.* The genetic network of prototypic planarian eye regeneration is Pax6 independent. *Development.* **129** (6), 1423-1434 (2002).
8. Paskin, T. R., Jellies, J., Bacher, J., & Beane, W. S. Planarian Phototactic Assay Reveals Differential Behavioral Responses Based on Wavelength. *PLoS One.* **9** (12), e114708 (2014).
9. Raffa, R. B., & Martley, A. F. Amphetamine-induced increase in planarian locomotor activity and block by UV light. *Brain Res.* **1031** (1), 138-140 (2005).
10. Sandmann, T., Vogg, M. C., Owlarn, S., Boutros, M., & Bartscherer, K. The head-regeneration transcriptome of the planarian Schmidtea mediterranea. *Genome Biol.* **12** (8), R76 (2011).
11. Vasquez-Doorman, C., & Petersen, C. P. The NuRD complex component p66 suppresses photoreceptor neuron regeneration in planarians. *Regeneration (Oxf).* **3** (3), 168-178 (2016).
12. Deochand, M. E., Birkholz, T. R., & Beane, W. S. Temporal regulation of planarian eye regeneration. *Regeneration.* **3** (4), 209-221 (2016).
13. Sakai, F., Agata, K., Orii, H., & Watanabe, K. Organization and regeneration ability of spontaneous supernumerary eyes in planarians -eye regeneration field and pathway selection by optic nerves. *Zoolog Sci.* **17** (3), 375-381 (2000).
14. Asano, Y., Nakamura, S., Ishida, S., Azuma, K., & Shinozawa, T. Rhodopsin-like proteins in planarian eye and auricle: detection and functional analysis. *J Exp Biol.* **201 (Pt 9)** 1263-1271 (1998).
15. Cross, S. D. *et al.* Control of Maintenance and Regeneration of Planarian Eyes by ovo. *Invest Ophthalmol Vis Sci.* **56** (12), 7604-7610 (2015).
16. Robb, S. M., Gotting, K., Ross, E., & Sanchez Alvarado, A. SmedGD 2.0: The Schmidtea mediterranea genome database. *Genesis.* **53** (8), 535-546 (2015).

 

17. Robb, S. M., Ross, E., & Sanchez Alvarado, A. SmedGD: the Schmidtea mediterranea genome database. *Nucleic Acids Res.* **36** (Database issue), D599-606 (2008).
18. Beane, W. S., Tseng, A. S., Morokuma, J., Lemire, J. M., & Levin, M. Inhibition of planar cell polarity extends neural growth during regeneration, homeostasis, and development. *Stem Cells Dev.* **21** (12), 2085-2094 (2012).
19. Forsthoefel, D. J., Waters, F. A., & Newmark, P. A. Generation of cell type-specific monoclonal antibodies for the planarian and optimization of sample processing for immunolabeling. *BMC Dev Biol.* **14** 45 (2014).
20. Ross, K. G. *et al.* Novel monoclonal antibodies to study tissue regeneration in planarians. *BMC Dev Biol.* **15** 2 (2015).
21. Cardona, A., Fernandez, J., Solana, J., & Romero, R. An in situ hybridization protocol for planarian embryos: monitoring myosin heavy chain gene expression. *Dev Genes Evol.* **215** (9), 482-488 (2005).
22. King, R. S., & Newmark, P. A. In situ hybridization protocol for enhanced detection of gene expression in the planarian Schmidtea mediterranea. *BMC Dev Biol.* **13** 8 (2013).
23. Pearson, B. J. *et al.* Formaldehyde-based whole-mount in situ hybridization method for planarians. *Dev Dyn.* **238** (2), 443-450 (2009).

 