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ARTICLE TITLE: Acetylcholinesterase Activity Staining in Freshwater Planarians

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ABSTRACT:

The serine hydrolase acetylcholinesterase (AChE) is an important neuronal enzyme which catalyzes the hydrolysis of the neurotransmitter acetylcholine and other choline esters. The breakdown of acetylcholine by AChE terminates synaptic transmission and regulates neuromuscular communication. AChE inhibition is a common mode of action of various insecticides, such as carbamates and organophosphorus pesticides. Freshwater planarians, especially the species *Dugesia japonica*, have been shown to possess AChE activity and to be a suitable alternative model for studying the effects of pesticides *in vivo*. AChE activity can be quantified in homogenates using the Ellman assay. However, this biochemical assay requires specialized equipment and large numbers of planarians. Here, we present a protocol for visualizing AChE activity in individual planarians. Activity staining can be completed in several hours and can be executed using standard laboratory equipment (a fume hood, nutator, and light microscope with imaging capability). We describe the steps for preparing the reagents, and the staining and imaging of the planarians. Planarians are treated with 10% acetic acid and fixed with 4% paraformaldehyde and then incubated in a staining solution containing the substrate acetylthiocholine. After incubation in the staining solution for 3.5 hours on a nutator at 4°C or stationary on ice, planarians are washed and mounted for imaging. Using exposure to an organophosphorus pesticide as an example, we show how AChE inhibition leads to a loss of staining. Thus, this simple method can be used to qualitatively evaluate AChE inhibition due to chemical exposure or RNA interference, providing a new tool for mechanistic studies of effects on the cholinergic system.

Basic Protocol 1: Preparing the staining solution

Basic Protocol 2: Fixing, staining, and imaging whole mount planarian specimens for visualization of acetylcholinesterase activity

KEYWORDS: flatworms, acetylthiocholine, enzyme activity staining, butyrylcholinesterase, AChE inhibition

INTRODUCTION:

Acetylcholinesterase (AChE) is an important enzymatic target for carbamate and organophosphorus insecticides. These compounds reversibly (carbamates) or irreversibly (organophosphorus pesticides) inhibit hydrolysis of acetylcholine by AChE in the central and peripheral nervous system (Russom et al., 2014). This inhibition leads to accumulation of acetylcholine and sustained synaptic transmission at cholinergic synapses, including neuromuscular junctions, causing overstimulation of the cholinergic system. Because these insecticides can inhibit AChE in various species, including humans, AChE activity has long been used as a validated biomarker for assessing poisoning in humans by these compounds (Lionetto et al., 2013). AChE activity is typically quantified in blood samples via an Ellman assay (Ellman et al., 1961).

Organophosphorus pesticides can also affect other targets besides AChE, including other hydrolases, acetylcholine receptors, and cytoskeletal proteins (reviewed in (Burke et al., 2017; Pope et al., 2005; Costa, 2018)). These additional targets are especially important in the context of low dose chronic exposure when AChE inhibition is low. Effects on alternative targets may also explain the distinct adverse outcomes that have been observed for low dose exposure to different organophosphorus compounds (Pope, 1999; Ireland et al., 2022b). Thus, to dissect how these insecticides damage the

brain, it is important to be able to differentiate which organismal effects are correlated with AChE inhibition and which are independent of AChE inhibition and may be due to action on other targets.

Freshwater planarians possess AChE activity (Bullock and Nachmansohn, 1942; De Simone et al., 1994; Zheng et al., 2011; Villar et al., 1994) and have been shown to be a well-suited organismal model for studying the neurotoxicity and developmental neurotoxicity (DNT) of organophosphorus compounds (Hagstrom et al., 2017, 2018a; Ireland et al., 2022a, 2022b; Zhang et al., 2019). Both Ellman assays and the activity staining described in this protocol have been used to study AChE inhibition in *D. japonica* planarians (Hagstrom et al., 2017, 2018a; Zhang et al., 2019; Ireland et al., 2022b, 2022a). These are complementary approaches, because the Ellman assay is an *in vitro* quantitative assay using clarified homogenates whereas the whole-mount histochemical staining allows for qualitative assessment of *in vivo* AChE activity in individual planarians. The histochemical staining method is advantageous when only small numbers of planarians are available, regional information is desired, or when reversible inhibitors such as physostigmine are evaluated, because the short-lived nature of this inhibition can be difficult to capture with *in vitro* Ellman assays (Hagstrom et al., 2018a).

The here presented protocol for histochemical staining of AChE in planarians stems from optimizing a method originally developed by Zheng *et al.* (Zheng et al., 2011) that was published in Chinese and translated by a native speaker. This optimized protocol includes an improved method of preparation of the staining solution, specimen fixation, activity staining, and slide preparation and imaging. The staining procedure is divided into two Basic Protocols. Basic Protocol 1 describes the preparation of the staining solution and Basic Protocol 2 describes the staining, mounting, and imaging. Staining can be completed in several hours and with standard laboratory equipment (a fume hood, nutator, and light microscope with imaging capability). The protocol here is written for use with *D. japonica* specimens but has been found to also work in the planarian *Schmidtea mediterranea*. Additionally, this method can be easily modified for detection of butyrylcholinesterase (BChE) activity in planarian samples by substitution of butyrylthiocholine for acetylthiocholine (Hagstrom et al., 2017).

CAUTION: This protocol involves the usage of hazardous chemicals. Some solutions must be prepared in a suitable fume hood with efficient ventilation (for details, see Reagents and Solutions). Lab coats, eye protection and reagent-impermeable protective gloves should be worn.

STRATEGIC PLANNING:

Users should prepare all reagents and materials before the start of this protocol. Basic Protocol 1 can be independently completed from and executed before Basic Protocol 2. We have successfully applied staining solution that was prepared up to 4 days in advance. To ensure consistency of timing in fixation, no more than 10 samples should be processed at a time. If more samples are to be tested, stagger the samples into different batches, such that fixation of the second set occurs after the first set has begun incubating in the staining solution. For chemical inhibition assays or RNA interference (RNAi) experiments, the timing of the treatment needs to be considered before staining can occur.

BASIC PROTOCOL 1: Preparing the staining solution

This protocol describes the steps to prepare a working staining solution. Visual quality checks should be performed at the end of this protocol to ensure that the staining solution will work prior to starting Basic Protocol 2.

Materials:

100 mM sodium acetate trihydrate (see recipe in Reagents and Solutions)
12% copper (II) sulfate pentahydrate (see recipe in Reagents and Solutions)
1.65% potassium ferricyanide (see recipe in Reagents and Solutions)
1% acetylthiocholine (ATCh; see recipe in Reagents and Solutions)
Optional: 250 mM butyrylthiocholine (BTCh; see recipe in Reagents and Solutions)
3% sodium citrate (see recipe in Reagents and Solutions)

15- and 50-mL polypropylene conical tubes
Pipettes (1000 μ L, 200 μ L and 20 μ L) and corresponding pipette tips

Protocol steps with *step annotations*:

Preparing staining solution

1. To a 15 mL polypropylene conical tube, add the following reagents in the order listed, vortexing each stock solution before it is added and the staining solution after addition of each component. Recipes and preparation guidelines for the stock solutions are provided at the end of this protocol in *Reagents and Solutions*.
 - 600 μ L 100 mM sodium acetate trihydrate
 - 10 μ L 12% copper (II) sulfate pentahydrate (10x stock)
 - 10 μ L 1.65% potassium ferricyanide (10x stock)
 - 180 μ L deionized H₂O (DI H₂O)
 - 100 μ L 1% ATCh
 - 100 μ L 3% sodium citrate

All reagents should be kept on ice.

Add reagents in the order listed. Watch for the correct color progression after addition of each component (Figure 1). If the final stain solution is not within shades of brown, the staining is unlikely to be successful and the staining solution should be remade.

The provided volumes are for 1 mL of stain which is sufficient for 1 tube if incubated on a nutator. 500 μ L of stain is sufficient for 1 tube if incubated stationary on ice. The volume of stain is independent of the number of worms per tube. For multiple samples, scale up to prepare a single volume of stain that will be sufficient for all tubes. It is important that all samples are

exposed to the same stain batch as there can be variation between batches. Since there may be some pipetting loss, it is helpful to prepare slightly more stain than needed (1 extra volume).

The protocol as written uses 10x stock solutions of copper (II) sulfate pentahydrate and potassium ferricyanide and adds 180 μ L DI H₂O. Alternatively, one can make and use 1x solutions of these compounds directly (i.e., 1.2% copper (II) sulfate pentahydrate and 0.165% potassium ferricyanide). Add 100 μ L of each 1x solution instead of the 10x solutions plus 180 μ L DI H₂O. We have found that using 10x stocks results in higher success rates because it is easier to accurately weigh out larger volumes of the chemicals. By making 10x stocks instead of larger volumes of 1x solutions, the chemical solutions are replaced more frequently and require less storage space. Note that expected colors of copper (II) sulfate pentahydrate and potassium ferricyanide stock solutions differ between the 1x stock and the 10x stock (Figure 2).

If staining for BChE activity instead of AChE activity, substitute 15 μ L 250 mM BTCh + 85 μ L DI H₂O for the 100 μ L 1% ATCh. Aliquots of BTCh should be thawed and kept at room temperature, as the solution will reprecipitate if put on ice.

2. Store the staining solution at 4°C in the dark (e.g., a foiled tube) for a minimum of one night and up to 4 days before use.

A red-brown precipitate will form overnight on the tube walls. If stain is used on the day of preparation, this precipitate may bind non-specifically to the exterior of the worms and make it difficult to see the actual desired stain. The pH of the stain, when prepared correctly, should be approximately pH 6.

BASIC PROTOCOL 2: Fixing, staining, and imaging whole mount planarian specimens for visualization of acetylcholinesterase activity

This protocol describes the steps for mucus removal, fixing the planarians using paraformaldehyde, staining for AChE activity using the staining solution prepared in Basic Protocol 1, and then imaging the stained samples. During fixation and staining, keep all solutions on ice unless otherwise noted.

Materials:

Dugesia japonica planarians (4-5 mm in length)
4% paraformaldehyde (PFA; see recipe in Reagents and Solutions)
10% acetic acid (see recipe in Reagents and Solutions)
1x phosphate buffered saline (1x PBS; MP Biomedicals, cat. no. 092810306)
50% glycerol diluted in 1x PBS (Thermo Fisher Scientific, cat. no. 15514029)
Planarian water (1x Montjuic Salts (Cebrià and Newmark, 2005) or 1x Instant Ocean (0.5g/L; Spectrum Brands, Blacksburg, VA) or bottled spring water

1.7 mL polypropylene tubes

15- and 50-mL polypropylene conical tubes
Pipettes (1000 μ L, 200 μ L and 20 μ L) and corresponding pipette tips
Plastic transfer pipettes (Thermo Fisher Scientific, cat. no. 335-11)
Imaging slides (Fisher Scientific, cat. no. 12-550-A3; 25 x 75 x 1.0mm)
Coverslips (Corning, cat. no. 2975-225; 22 x 50 mm rectangular #1 or equivalent; 22 x 22 mm can also be used)
Double-sided tape (Duck tape DUC 1081698 or similar)
Razor blade or scalpel
Clear nail polish
Microscope (Leica 10446339 or equivalent) with camera setting (Leica 10446307 0.8x or equivalent)
Computer with imaging software installed (Point Grey FlyCap2 or equivalent)
60 mm petri dish
Black paper

Fixation

1. Select planarians to be stained.

At minimum, there should be 3 well stained and mounted worms per experimental condition. It is better to start with more worms than needed as they can easily get damaged during staining and mounting. Choose worms that are about 4-5 mm in length. Worms must be starved for at least five days before the experiment.

2. Using a transfer pipette, place one worm in a 1.7 mL tube.

For chemically exposed worms, first remove the chemical solution and wash 3x in planarian water before proceeding with fixation.

Worms may become sticky during fixation and staining. It is important to make sure that they are not stuck to the tube or to one another at any stage. Gently flicking the tube is the best way to dislodge stuck worms.

Multiple worms (we have tested up to 4) can be fixed and stained together in each tube. However, since planarians get sticky during the staining procedure, there is a risk that they will adhere to one another. If this occurs, they may not be adequately exposed to the stain and can also be damaged when separated for mounting.

Glass vials of equivalent size can be substituted for 1.7 mL polypropylene tubes. Additionally, 2 mL tubes or vials can be used, but the volume of stain in each tube should be proportionally increased.

3. Remove planarian water and immediately add 500 μ L cold 10% acetic acid. Keep the planarian in acetic acid for 10 seconds, continuously inverting the tube.

When removing solutions from tubes containing planarian samples, it is important to work with care and avoid touching the worms with the pipette to avoid damage. Solutions should be removed and added by pipetting at an angle. Allow planarians to settle to the bottom of the tube before removing liquid. It can also help to leave a small volume of liquid (~50 μ L) behind with each step.

Work with one tube at a time to keep timing consistent. Acetic acid kills the planarians and removes their mucus, making the worms more permeable to the stain.

4. Remove acetic acid and immediately replace with 500 μ L 4% PFA. Incubate on ice for 15 minutes.

Alternatively, planarians can be fixed on a nutator at 4°C for 15 minutes. Use 750-1000 μ L 4% PFA to ensure that worms remain in solution while moving as they can get stuck in the tube lid.

5. Fix (Steps 3-4) any additional tubes while the first one is incubating in PFA.

Repeat fixation steps for additional tubes one at a time while other tubes are in PFA. Work as quickly as possible to keep the timing consistent. For large numbers of tubes (>10), it may be helpful to divide the samples into multiple batches as discussed in Strategic Planning.

Staining

Steps 6 and 7 should be performed at cold temperatures (e.g., on ice, in a 4°C cold room, in a 4°C incubator or fridge).

6. Remove PFA, then wash quickly with 1 mL cold 1x PBS, inverting to mix.
7. Replace PBS with 500 μ L staining solution. Incubate on ice for 3.5 hours.

Alternatively, replace with 1 mL staining solution and incubate on a nutator at 4°C.

If staining on ice, periodically (every 30-60 minutes) invert the tubes to mix and ensure that the planarians are not sticking together. This is not necessary if staining is done on a nutator. Do not exceed the 3.5 hours incubation time if testing for loss of AChE activity. Longer incubation makes it challenging to detect differences in staining between wild type and treated worms.

All steps following staining are performed at room temperature.

8. Remove the staining solution. Quickly wash with 1 mL 1x PBS (room temperature) and invert the tube several times to remove most of the remaining staining solution.
9. Wash for 10 min in a second volume of 1x PBS, using a nutator to remove any residual and unbound stain.

Keep the wash within 8-10 minutes as longer wash steps can remove some of the AChE staining.

10. Remove 1x PBS and replace with 50% glycerol/1x PBS.

The worms are now ready for imaging but can be stored at 4°C in the dark for a few days before mounting and imaging. Specificity of the staining can be determined using concentrations of AChE inhibitors known to inhibit AChE in planarians, such as physostigmine or OPs like diazinon.

Slide preparation

11. Make a tunnel slide (Talbot et al., 2014) by attaching a piece of double-sided tape at one end of a clean microscope slide and another piece approximately 1 inch away (at a distance slightly less than the width of the coverslip to be used).

Leave enough space in the middle for the planarian(s) to be mounted.

Up to 3 layers of tape can be used depending on worm size and thickness. Use enough layers so that the planarians are not squashed by the coverslip.

Cut any excess tape off using a razor blade or scalpel.

12. Use a transfer pipette (or other wide-diameter pipette, such as a cut or wide-bore 1000 μ L pipette tip) to slowly move the planarian onto the slide between the two sets of tape. Avoid air bubbles. Keep the planarian in a small volume (100-200 μ L) of 50% glycerol/1x PBS. Once the worm is lying flat, cover the planarian with a transparent coverslip (see Figure 3).

The staining is most clearly observed on the ventral side of the planarian, as the planarian central nervous system is ventrally located. Additionally, darker pigmentation on the dorsal side can make it difficult to see the staining. It is thus best to mount with the ventral side facing up. If mounted with the dorsal side up (the eyespots will appear darker), turn the slide over when imaging to view the ventral side. See Figure 4C and D for a comparison of ventral vs. dorsal imaging.

The planarian can be positioned with fine micropipette tips; these are also helpful to straighten out curled worms.

To avoid the introduction of air bubbles and avoid damaging the samples, carefully place one edge of the coverslip on the slide first and slowly lower it from an angle.

Mount multiple planarians of the same treatment group on one slide to save time and supplies.

13. Seal the edges of the coverslip with clear nail polish. Allow the nail polish to dry for a few minutes before imaging or storing the slides for future imaging.

Sealing the slides reduces evaporation of mounting solution if slides are stored for later imaging. Additionally, nail polish adheres the coverslip to the slide so that the slide can be turned over for imaging if a worm was mounted dorsal side up.

If the slide will be imaged at a later time, store prepared slides at 4°C in a slide holder box (or equivalent).

Imaging

Samples are best imaged within a week of staining, but the staining can persist for up to a year if stored properly (4°C in a slide holder box). Image all samples within an experiment using the same lighting, exposure, and magnification settings. Use a dark background against which the stain will appear white.

14. Turn on the computer and open the imaging software. Connect the microscope camera to the computer.
15. Place a piece of black paper on the microscope stage to provide a dark background. Put a 60 mm petri dish (without a lid) on the paper and place the slide on top of the open dish.

The petri dish serves to separate the slide from the background and thus gives an out-of-focus background for better imaging.

If using a microscope with a mirror transillumination base, the angle of the mirror can be adjusted to provide a dark background in lieu of using black paper.

16. Turn on overhead lighting and place the slide so that the planarian is centered in the imaging frame. Set magnification such that the worm nearly fills the field of view. Adjust focus and lighting so that the stain is clearly visible.

It is best to image wild type/control specimens first and then use the same settings for treatment samples.

Lighting should be uniform and from above, with minimal shadows from the specimens. This can be accomplished either with the microscope's overhead lighting, or from external sources such as gooseneck lamps.

If the planarian appears too bright and detail of the stain cannot be seen, reduce the exposure or dim the lighting.

17. Capture and save the image. Repeat for all remaining samples.

*See Figures 4, 5 and 6A-B for example staining in *D. japonica* planarians.*

REAGENTS AND SOLUTIONS:

NOTE: Volumes can be adjusted as needed.

Acetic acid, 10% (v/v)

- 9 mL DI H₂O
- 1 mL 100% acetic acid (Sigma-Aldrich, cat. no. 695092; CAS no. 64-19-7)
- Can be stored at 4°C for up to 1 month.
- *Always add acid to water.*

Acetylthiocholine, 1% (w/v)

- 0.1 g acetylthiocholine iodide (ATCh; Sigma-Aldrich, cat. no. 01480; CAS no. 1866-15-5)
- 10 mL DI H₂O
- Prepare a large volume and make aliquots of 500 µL in 1.7 mL tubes, which can be stored for at least several months at -20°C. Thaw necessary aliquots and place on ice before preparing staining solution.
- *Acetylthiocholine iodide is toxic if swallowed or in contact with skin; prepare solution in a fume hood.*

Butyrylthiocholine, 250 mM (optional)

- 0.79 g butyrylthiocholine iodide (Sigma-Aldrich, cat. No. 20820; CAS no. 1866-16-6)
- 10 mL DI H₂O
- Prepare a large volume and make aliquots of 500 µL in 1.7 mL tubes, which can be stored for at least several months at -20°C. Thaw necessary aliquots at room temperature before preparing staining solution. Vortex thoroughly to ensure a homogenous mixture. BTCh should not be placed on ice before staining as the solution will reprecipitate.
- *Butyrylthiocholine iodide causes skin and eye irritation; prepare solution in a fume hood.*

Copper (II) sulfate pentahydrate, 12% (w/v)

- 0.12 g copper (II) sulfate pentahydrate (Acros Organics, cat. No. 197722500; CAS no. 7758-99-8)
- 1 mL DI H₂O
- Vortex to make sure all solid is dissolved. Solution should look blue (see Figure 2A).
- *Can be stored at 4°C in the dark for up to 1 month.*
- *Copper (II) sulfate pentahydrate is harmful if swallowed, causes serious eye damage and is highly toxic to aquatic life; prepare solution in a fume hood.*

Paraformaldehyde, 4% (v/v) (PFA)

- 10 mL 16% paraformaldehyde (Ted Pella, cat. no. 18505; CAS no. 50-00-0)
- 30 mL diluent: either planarian water or 1x PBS
- *Can be stored at 4°C in the dark for up to 1 month.*
- *Paraformaldehyde is combustible and harmful if in contact with skin or eyes, or if inhaled. Prepare solution in a fume hood and keep away from ignition sources.*

Potassium ferricyanide, 1.65% (w/v)

- 0.0165g potassium ferricyanide (Acros Organics, cat. no. 424120050; CAS no. 13746-66-2)
- 1 mL DI H₂O
- Solution should look bright yellow (see Figure 2B). *Can be stored at 4°C in the dark for up to 1 month.*
- *Potassium ferricyanide causes serious eye irritation and is toxic to aquatic life with long lasting effects; prepare solution in a fume hood.*

Sodium acetate trihydrate, 100 mM

- 0.272g sodium acetate trihydrate (Sigma-Aldrich, cat. no. 236500; CAS no. 6131-90-4)
- 20 mL DI H₂O
- *Can be stored at 4°C in the dark for up to 1 month.*

Sodium citrate, 3% (w/v)

- 0.30g sodium citrate (LabChem, cat. no. LC236501; CAS no. 6132-04-3)
- 10 mL DI H₂O
- *Can be stored at 4°C in the dark for up to 1 month.*

COMMENTARY:

Background Information: The cholinergic system is a common target of insecticides and other chemical agents. Cholinergic neurotransmission, controlled by the neurotransmitter acetylcholine, controls a variety of cognitive, peripheral autonomic, and somatic motor functions. AChE acts as a key regulator of cholinergic neurotransmission by hydrolyzing acetylcholine to end synaptic transmission.

Inhibition of AChE causes cholinergic overstimulation and can have drastic physiological consequences, including paralysis and death at high enough levels in many organisms, including humans (Russom et al., 2014). Because of its importance and ease of measurement from blood samples, AChE activity is a commonly used biomarker for the detection of pesticide poisoning (Lionetto et al., 2013).

Several research groups have shown that AChE activity can be detected in various planarian species (Bullock and Nachmansohn, 1942; De Simone et al., 1994; Zheng et al., 2011; Villar et al., 1994). We have shown that AChE activity can be detected during all stages of neurodevelopment in *D. japonica* planarians (Ireland et al., 2022a). This species is also sensitive to and can metabolize organophosphorus pesticides (Ireland et al., 2022a; Hagstrom et al., 2017). Together, this makes *D. japonica* planarians an especially suitable invertebrate model for studying developmental neurotoxicity of these compounds.

D. japonica planarians have two putative cholinesterase genes, *Djche-1* and *Djche-2*, which have been shown using RNAi to be the primary enzymes responsible for ATCh catalysis in this species (Hagstrom et al., 2018a). Results from Ellman assays using either ATCh and butyrylthiocholine (BTCh) as substrates in the presence of various classical inhibitors demonstrated that *D. japonica* cholinesterases are hybrid esterases that display both AChE-like and butyrylcholinesterase (BChE)-like activity (Hagstrom et al., 2017). Thus, while we refer only to AChE activity in planarians for simplicity, this hybrid activity needs to be kept in mind.

A method for staining AChE activity in planarians has been previously described (Zheng et al., 2011) based on similar methodologies used in mammalian brain sections (Hedreen et al., 1985). Here, we present an optimized and accessible version of this staining, as the previous publication was only available in Chinese. In our protocol, AChE activity stains white and can be visualized against a dark background. Hydrolysis of ATCh by AChE produces free thiocholine which reduces Cu^{++} to Cu^+ and forms cuprous thiocholine iodide in the presence of iodide. This results in the white Koelle precipitate (Koelle and Friedenwald, 1949), which is likely the white staining observed in our protocol. The Koelle reaction competes with the reduction of ferricyanide to ferrocyanide by thiocholine, which combines with Cu^{++} in the staining solution to produce cupric ferrocyanide (Hatchett's Brown or Karnovsky precipitate; reviewed in (Tsuji and Larabi, 1983). Both precipitates indicate sites of AChE activity, but which reaction dominates depends on the specific ion concentrations (Tsuji and Larabi, 1983). Given the dark pigmentation of planarians, the white Koelle precipitate is desirable as it makes visualization easier.

With a single alteration (substitution of BTCh for ATCh as the substrate), this protocol can be used to stain for BChE activity instead of AChE activity. The BChE activity stain is otherwise conducted in the same manner. Because of the hybrid nature of the *D. japonica* cholinesterases, staining with either ATCh or BTCh produces the same activity pattern in this species (Figure 6C).

This protocol provides a means to qualitatively characterize AChE activity in freshwater planarians, allowing one to visualize its localization as well as relative activity levels. This staining method may be more applicable in certain cases over the *in vitro* Ellman assay, such as when only a limited number of specimens per treatment condition are available or when assaying short-lived reversible inhibition. We previously found that, using the same exposure conditions, inhibition by the reversible inhibitor physostigmine could not be quantified by an Ellman assay, but was obviously decreased using the AChE activity staining method presented here (Hagstrom et al., 2018a). However, this staining method does not quantify AChE activity and thus must be complemented by an Ellman assay or other approaches if such quantitative measurements are needed. Additionally, it is important to consider that this protocol detects catalysis of ATCh (or BTCh), which may be achieved by other non-AChE enzymes as well. The

contribution of other esterases can be minimized by using a short incubation time, as proposed here, as hydrolysis by non-specific esterases occurs at a slower rate (Koelle and Friedenwald, 1949).

Critical Parameters:

The most critical component of this protocol is the preparation of the staining solution in Basic Protocol 1. Figure 1 shows the progression of the color of the solution after the addition of each component (Figure 1A), as well as the expected final color of the solution, which should be brown (Figure 1B). If stain reagents are not made correctly or have been stored for too long, the final stain color will be incorrect, often green instead of brown (Figure 1C). When the staining solution is prepared, its color should be observed after each component is added to ensure that it is being made correctly.

Our previous experiments have shown that brown precipitates can form in the staining solution. When the staining solution is made fresh, the precipitates tend to bind to the planarian bodies, making it harder to see the AChE activity staining. When the solution is prepared at least a day before, the brown precipitate binds to the sides of the solution storage tube rather than the specimens, resulting in clearer staining and image quality.

The duration of staining is important when looking for qualitative differences in AChE activity levels between samples. If staining proceeds for longer than 3.5 hours, it may be difficult to observe the reduction of AChE activity. However, longer incubations (up to 6 hours) may be appropriate if greater resolution of activity is desired, such as to observe AChE activity over the course of regeneration (Ireland et al., 2022a). For any sample comparisons, the same staining solution must be used since staining outcomes can be variable depending on the quality of the staining solution.

Lastly, it is important to handle the samples with care as they are delicate and easily damaged. The planarians become sticky during the procedure and can get stuck to each other (if multiple worms are stained in a single tube), or to pipette tips and tubes. It helps to make sure that the planarians are floating freely in solution throughout the protocol, by gently tapping on the tube periodically during the staining incubation or by using a nutator.

Troubleshooting:

Table 1. Troubleshooting Guide for Acetylcholinesterase Staining

Problem	Possible Cause	Solution
Stain is not brown.	Component reagents not prepared correctly or have degraded.	Prepare fresh component solutions and remake staining solution.
Red-brown stain on surface of worms after staining.	Staining solution not prepared in advance.	Make staining solution at least 1 day prior to use.

Worms are curled.	Most likely a problem with fixation.	If possible, fix new samples, being careful to only incubate in acetic acid for 10 seconds and to use fresh solutions. If new samples cannot be made easily, fine micropipette tips can be used to straighten out the worm. Be gentle to avoid damage.
Worms are stuck together in tube.	Not enough inversion/movement of the tube during the protocol.	Stain worms on a nutator, or periodically invert the tube throughout staining. Alternatively, only use one worm per tube.
No difference in AChE activity between treatment groups.	If inhibition was expected, incubation time may be too long.	Shorten the incubation time to allow for the detection of smaller differences in AChE activity.
Planarians look damaged on slide.	Planarians get squashed when mounting for imaging.	Use more layers of double-stick tape when mounting. Try mounting fewer planarians on the same slide.

Understanding Results:

This protocol will result in images of whole-mount planarians stained for AChE activity. The staining will appear white against the planarian body when imaged as described in Basic Protocol 2. Figure 4 shows various possible staining patterns that may be seen after AChE staining of wild type *D. japonica* worms. All show AChE activity in the nervous system, but different parts of the nervous system may stain more strongly than others at times. Variation in staining is dependent on the batch of stain as well as on the exact fixation conditions. Thus, it is important to always include control specimens stained at the same time as experimental ones, as well as to stain multiple worms per condition (minimum n=3). Imaging conditions must be kept identical when imaging different experimental groups. Due to differences in the coloration of individual planarians as well as a red-brown precipitate which can sometimes occur during the staining (Figure 4E), AChE activity staining is best presented as grayscale images (seen in Figures 5 and 6). Figure 4 contains color images to illustrate the red background that can occur.

The results from this staining protocol are qualitative, showing localization of AChE activity as well as relative levels of activity. This method can thus be used to demonstrate changes in AChE activity levels as the result of a gene knockdown or chemical exposure. Figure 5 shows an example of reduction of AChE activity in *D. japonica* as a result of a 12-day exposure to 1 μ M diazinon, an organophosphorus insecticide. The control specimen (Figure 5A) shows AChE staining throughout the nervous system, including the brain, ventral nerve cords and commissures. In contrast, the diazinon-exposed specimen (Figure 5B) only shows faint staining in the head. Importantly, only strong reductions in activity can be

detected with this method. If no obvious differences are found between control and experimental groups, the staining time can be reduced as this could potentially increase any existing differences between groups. However, we found that below 2 h of staining, even control planarians show little activity signal. In this case, an Ellman assay can be performed to detect any potential small differences in AChE activity which would be missed with the staining. Exposure to AChE inhibitors under conditions (exposure and timing) that have been shown by an Ellman assay to significantly inhibit AChE activity in *D. japonica*, such as in (Hagstrom et al., 2018b; Ireland et al., 2022a, 2022b), can be used as a negative control for the specificity of the stain, as staining should be absent or decreased under these conditions, like in Figure 5B.

While prepared slides are ideally imaged fresh, we have imaged slides that were stored for nearly 12 months without significant signal loss (Figure 6A-B). We have used this protocol with minor modifications with BTCh as the substrate (Figure 6C) and with asexual *S. mediterranea* planarians (Figure 6D). Further optimization of the procedure may be required for AChE activity staining in other planarian species.

Time Considerations:

The entire protocol takes about 2 days to complete. The preparation of the staining solution in Basic Protocol 1 takes 20 minutes if all stock solutions have been prepared in advance. The preparation of the stock solutions takes about 30 minutes per stock solution for a novice user and 15 minutes for an experienced user. Prepare the staining solution at least a day before the experiment for better results (see Troubleshooting section). Staining solution can be stored at 4°C in the dark for up to four days. Basic Protocol 2 takes about 6-8 hours to complete, depending on user experience and number of samples. The fixation and wash steps take 45 minutes for a minimal number of samples (2-4 tubes). For experiments where qualitative differences between samples are expected (e.g., if the goal was to test inhibition of AChE activity), the staining step should be kept to 3.5 hours. Otherwise, staining time can be extended up to 6 hours. The post-staining washes take 30 minutes, followed by slide preparation and mounting, which can take between 20 and 60 minutes, depending on user experience. We recommend novice users practice slide setup without specimen first. Imaging takes 15-30 minutes per slide if there are multiple worms on the slide. Prepared slides can be saved and stored at 4°C for several months without significant loss of staining (Figure 6A-B).

AUTHOR CONTRIBUTIONS:

Christina Rabeler: Investigation, Methodology, Original draft writing, Review and editing; TaiXi Gong: Methodology, Visualization, Original draft writing, Review and editing; Danielle Ireland: Conceptualization, Methodology, Original draft writing, Review and editing; Olivier Cochet-Escartin: Conceptualization, Methodology; Eva-Maria S. Collins: Funding acquisition, Supervision, Conceptualization, Methodology, Original draft writing, Review and editing.

CONFLICT OF INTEREST STATEMENT:

EMC is the founder of Inveritek, LLC, which offers planarian screening commercially.

DATA AVAILABILITY STATEMENT:

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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FIGURE LEGENDS:

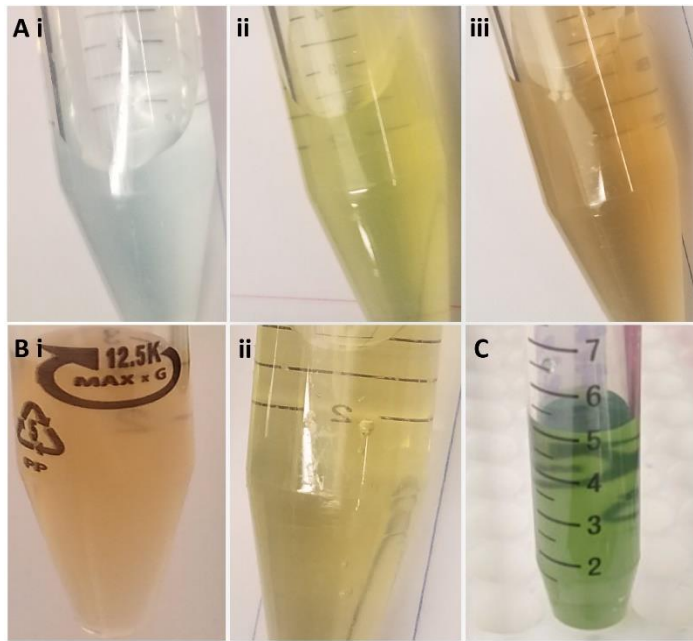


Figure 1 Color progression of stain after adding each component. **(A)** Examples of correct color progression after addition of **(i)** copper sulfate, **(ii)** potassium ferricyanide, and **(iii)** ATCh. **(B)** Examples **(i, ii)** of shades of correct color of the staining solution. **(C)** Example of an incorrect coloration of the final staining solution.

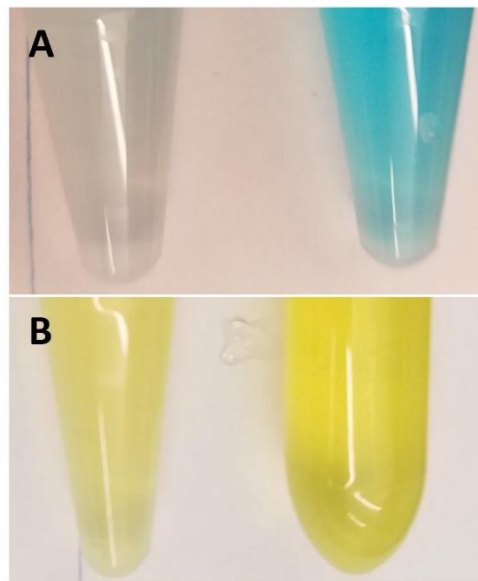


Figure 2 Coloration of 1x (left) and 10x (right) reagents. **(A)** copper sulfate **(B)** potassium ferricyanide.

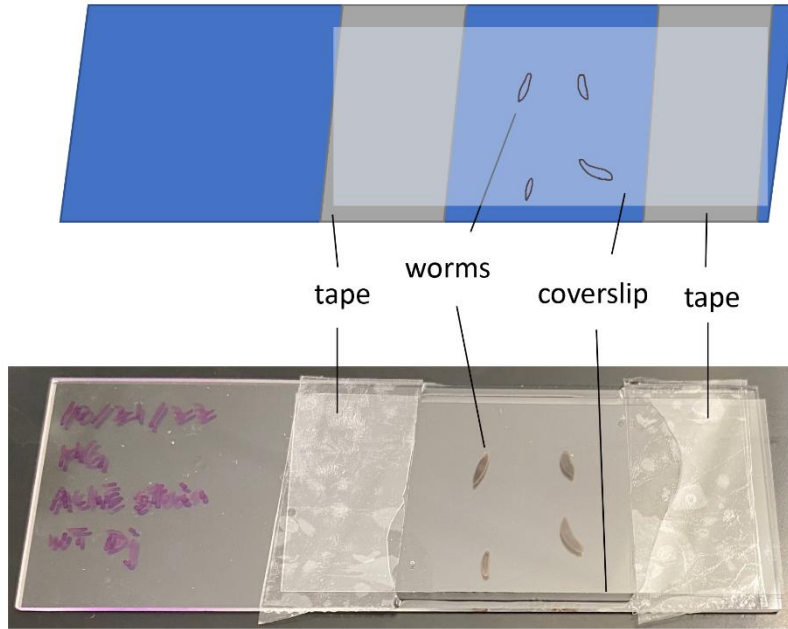


Figure 3 A schematic drawing (top) and an example (bottom) of slide preparation. Nail polish is applied around the rim of the coverslip.



Figure 4 Example images of AChE staining of *Dugesia japonica*. (**A, B**) Examples of correct staining of the nervous system. Different components can show variability in the strength of staining. In (**A**) the brain, ventral nerve cords (VNCs), and commissures are strongly stained while in (**B**) the brain staining is weaker but more peripheral nervous system is stained. (**C-D**) show the same planarian from the (**C**) ventral and (**D**) dorsal side, respectively. The staining is more clearly seen from the ventral side. (**E**) If stain is improperly prepared, red-brown precipitate can adhere to the sample. Scale bar is 0.1 mm.



Figure 5 AChE staining can be used to show reduced AChE activity following chemical exposure. (A) The control group was exposed to 0.5% DMSO for 12 days. (B) Worms treated with 1 μ M diazinon for 12 days had a reduction of staining. *D. japonica* planarians were used. Scale bar is 0.1 mm.

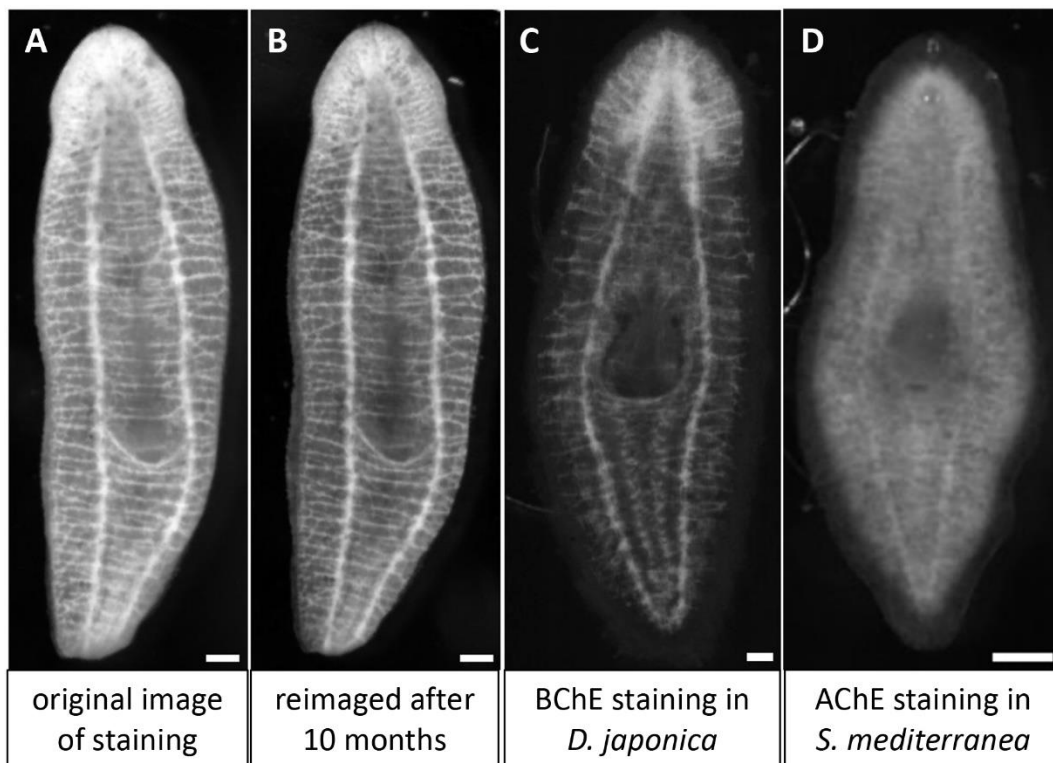


Figure 6 Staining outcomes for specific circumstances. (A) AChE staining on a wildtype *D. japonica* imaged shortly after the protocol was completed, and (B) the same sample imaged 10 months later. (C) BChE staining in *Dugesia japonica* using BTCh as the substrate and (D) AChE staining in *Schmidtea mediterranea* using the described protocol. Scale bar is 0.1 mm.