



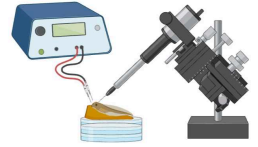
Aug 20, 2023

Version 1

🌐 Use and efficiency of morpholinos in Neotropical tadpole brains V.1

DOI

<https://dx.doi.org/10.17504/protocols.io.yxmvm23y6g3p/v1>



Sarah Ludington¹, Julie Butler¹, Chloe Golde¹, Lauren O'Connell¹

¹Stanford University

O'Connell Lab at Stanford



Lauren A O'Connell

Stanford University

Create & collaborate more with a free account

Edit and publish protocols, collaborate in communities, share insights through comments, and track progress with run records.

Create free account

OPEN  ACCESS



DOI: <https://dx.doi.org/10.17504/protocols.io.yxmvm23y6g3p/v1>

Protocol Citation: Sarah Ludington, Julie Butler, Chloe Golde, Lauren O'Connell 2023. Use and efficiency of morpholinos in Neotropical tadpole brains. **protocols.io** <https://dx.doi.org/10.17504/protocols.io.yxmvm23y6g3p/v1>

License: This is an open access protocol distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

Protocol status: Working

We use this protocol and it's working

Created: November 16, 2022

Last Modified: August 20, 2023

Protocol Integer ID: 72812

Keywords: amphibian, poison frog, vivo morpholinos, microinjection, electroporation, tyrosine hydroxylase, neotropical tadpole brains antisense morpholino, knockdown efficacy of each morpholino, tadpole physiology, study of tadpole physiology, knockdown evaluation in the neotropical tadpole, efficiency of morpholino, vivo morpholino delivery, morpholinos in the knockdown, mimetic poison frog, single tadpole brain, morpholino, morpholino knockdown, xenopus frog, morpholino delivery, tadpole behavior, extending morpholino delivery, knockdown protein abundance in target tissue, neotropical tadpole, protein knockdown assessment, enzyme in dopamine synthesis, tadpole swimming, knockdown protein abundance, dopamine synthesis, amphibian, protein level, common aquatic research organism, protein, limited to common aquatic research organism, amphibian species

Funders Acknowledgements:

NSF EDGE

Grant ID: NSF IOS 1827333

Rita Allen

Grant ID: Scholar's Award

Abstract

Antisense morpholinos are a common tool used to knockdown protein abundance in target tissues in fish and amphibians. However, protocols are largely limited to common aquatic research organisms, such as zebrafish or *Xenopus* frogs. The goal of this protocol is to enable in vivo morpholino delivery, visualization, and knockdown evaluation in the Neotropical tadpoles. We tested the efficiency of standard and Vivo-morpholinos in the knockdown of tyrosine hydroxylase protein, the rate limiting enzyme in dopamine synthesis, in the Mimetic poison frog (*Ranitomeya imitator*). We compared the knockdown efficacy of each morpholino at two different time points and the effect of knockdown on tadpole behavior. To quantify tyrosine hydroxylase knockdown efficiency, we developed a quick and inexpensive dot blotting method to evaluate protein levels within a single tadpole brain. We compared this method to chemiluminescent imaging and found both methods are sufficient to assess protein levels and discuss important differences in blot visualization techniques. Finally, we found morpholino knockdown reduced tadpole swimming, consistent with other studies implicating dopamine as important in motor behaviors. We complement this protocol with a discussion of common challenges, suggestions for troubleshooting, and ideas for future improvement. Extending morpholino delivery and protein knockdown assessment to diverse amphibian species and labs with low budgets will enable the field to move forward more rapidly in the study of tadpole physiology and behavior.

Guidelines

These parameters are a suggested starting point. Optimizing the protocol for species-specific applications to maximize transfection efficiency is recommended.



Materials

Injection, Electroporation, and *in vivo* Imaging Materials

Ethyl 3-Aminobenzoate Methanesulfonate (Millipore Sigma Catalog #10521)
Sodium Bicarbonate (Millipore Sigma Catalog #S6014)
Josh's Frogs R/O Rx
Parafilm M Sealing Film (Millipore Sigma Catalog #HS234526B)
Platinum Foil (Fisher Scientific Catalog #AA11509FF)
Lead Solder Wire (Amazon Catalog #B075WB98FJ)
Loctite Fun-Tak Mounting Putty Tabs (Amazon Catalog #1865809-12)
100 mm X 15 mm Petri Dishes (Fisher Scientific Catalog #FB0875713)
Serological Pipette (Fisher Scientific Catalog #12-567-600)
Electrical Tape (Fisher Scientific Catalog #19-047-280)
Micromanipulator (Sutter Catalog #MM-33)
Grass Instruments SD9 Square Pulse Stimulator
Disposable Paired 13 mm Subdermal Needle Electrodes (MFI Medical Catalog #RHL-RLSND121-1-0)
3.5" Replacement Glass Capillaries (Drummond Scientific Catalog #3-000-203-G/X)
Sutter Instrument Co P-97 (Sutter Catalog #P-P7)
Forceps (Fisher Scientific Catalog #12-000-157)
Mineral Oil (Millipore Sigma Catalog #M8410)
28 Gauge Metal Hub Blunt Point Needle (Fisher Scientific Catalog #14815616)
1 mL BD Disposable Syringe (Fisher Scientific Catalog #14-823-30)
Nanoject II Variable Volume Automatic Injector (Drummond Scientific Catalog #3-000-204)
GeneTools Standard and/or Vivo MOs designed against target of interest
Nuclease-Free Water (Millipore Sigma Catalog #W4502)
Kimwipes (Fisher Scientific Catalog #06-666)
Standard Disposable Transfer Pipettes (Fisher Scientific Catalog #13-711-7M)
Micro Detail Paint Brush
Stereoscope with a GFP filter

Dot Blot Materials

Tris-HCl (JT Baker Catalog #4103-01)
NaCl (Millipore Sigma Catalog #S9888)
10x SDS Stock (Invitrogen Catalog #24730020)
Tween-20 (Biotum Catalog #22002)
Forceps (Fisher Scientific Catalog #12-000-157)
Curved forceps (Fine Science Tools Catalog #91117-10)
Fine scissors (Fine Science Tools Catalog #91460-11)
Distilled water
cOmplete Mini EDTA-free table (Millipore Sigma Catalog #04693159001)



Motorized Tissue Grinder (Fisherbrand Pellet Pestle Cordless Motor Catalog #12-141-361)

Reusable or Disposable Pellet Pestles (Fisherbrand RNase-Free Disposable Pellet Pestles Catalog #12-1411364)

Invitrogen Qubit 4 Fluorometer Catalog #Q33238

Centrifuge

1.5 mL Eppendorf Tubes (Fisher Scientific Catalog #02682003)

Nitrocellulose Membrane (Thermo Scientific Catalog #88018)

5% Nonfat Dry Blotting Grade Dry Milk (Bio-Rad Catalog #1706404)

Bovine Serum Albumin (Millipore Sigma Catalog #A3059-100G)

Primary Antibody for target of interest (Millipore Sigma Anti-Tyrosine Hydroxylase MAB318)

Primary Antibody for housekeeping reference protein (Abcam Anti-GAPDH AB181602)

HRP-Conjugated Secondary Antibody (Abcam Goat Anti-Mouse Secondary with HRP AB6789)

ECL Visualization Kit (Bio-Rad Catalog #1705060)

Chemiluminescent Imaging System (Azure Biosystems Catalog #76501-636)

Opti-4CN Substrate Kit (Bio-Rad, Hercules, CA, ca# 1708235)

Safety warnings

- ❗ MS-222 is a respiratory irritant and the following personal protective equipment should be worn: labcoat, gloves and safety glasses.

Before start

Consult with your local animal ethics board prior to experimentation.

Anesthesia Preparation

5m

- 1 Mix 0.02g ethyl 3-aminobenzoate methanesulfonate (MS-222) and 0.08g sodium bicarbonate with 60 mL of tadpole water
- 2 Store at 4C for up to one week

5m

Electroporation Set-Up

8m

- 3 Remove the tips from two 1 mL serological pipettes using scissors and fasten them together with electrical tape and/or hot glue

5m

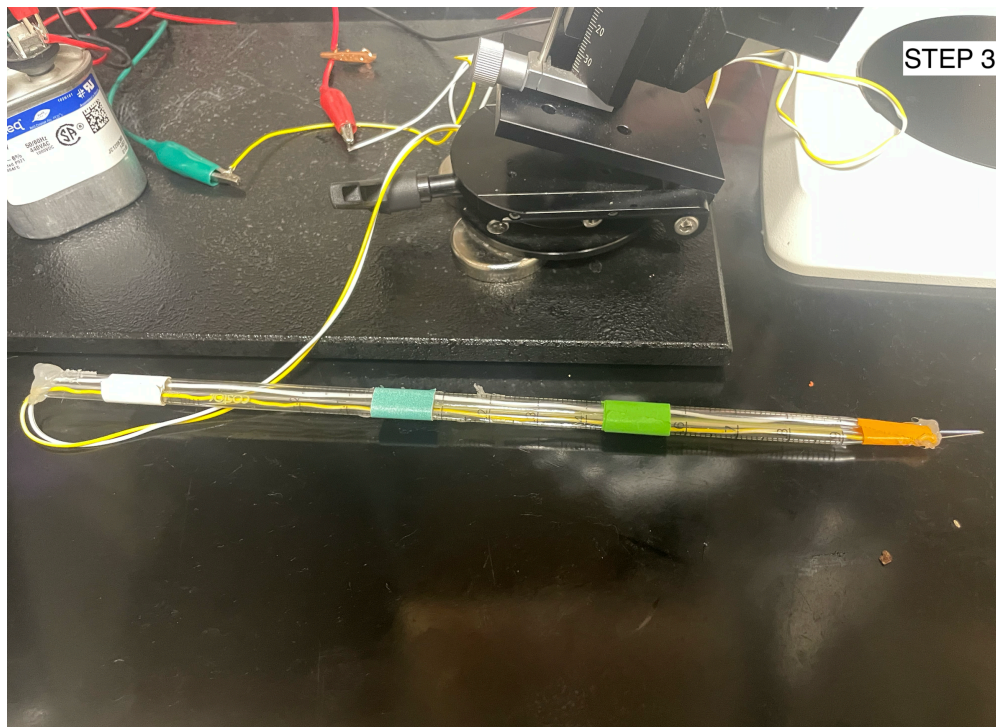


Figure 1. Two serological pipettes with electrode needles (see Step 4) attached together using electrical tape and hot glue to secure

- 4 Mount the pipettes onto the micromanipulator and run one needle electrode wire through each pipette. Ensure the electrode needles are 1 mm apart and parallel to one another

1m

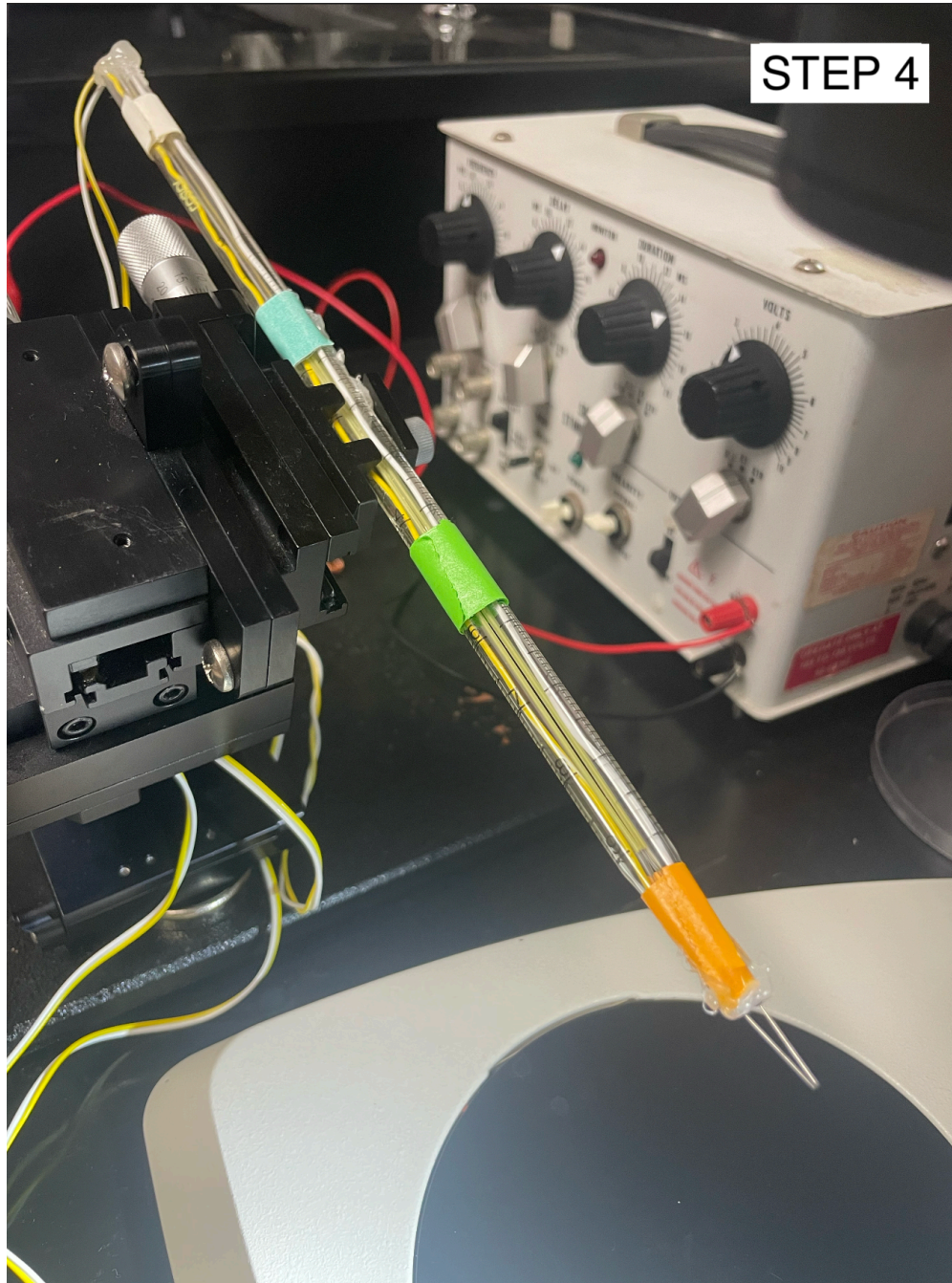


Figure 2. Two serological pipettes with electrode needles secured onto micromanipulator mount

- 5 Attach the wires from the electrode needles to the capacitor and then to the stimulator. Set the stimulator parameters to 1 pps, 15 ms duration, 1 ms delay, and 30-50 V

2m



Figure 3. Top view of electrode needles, capacitor, and stimulator connected

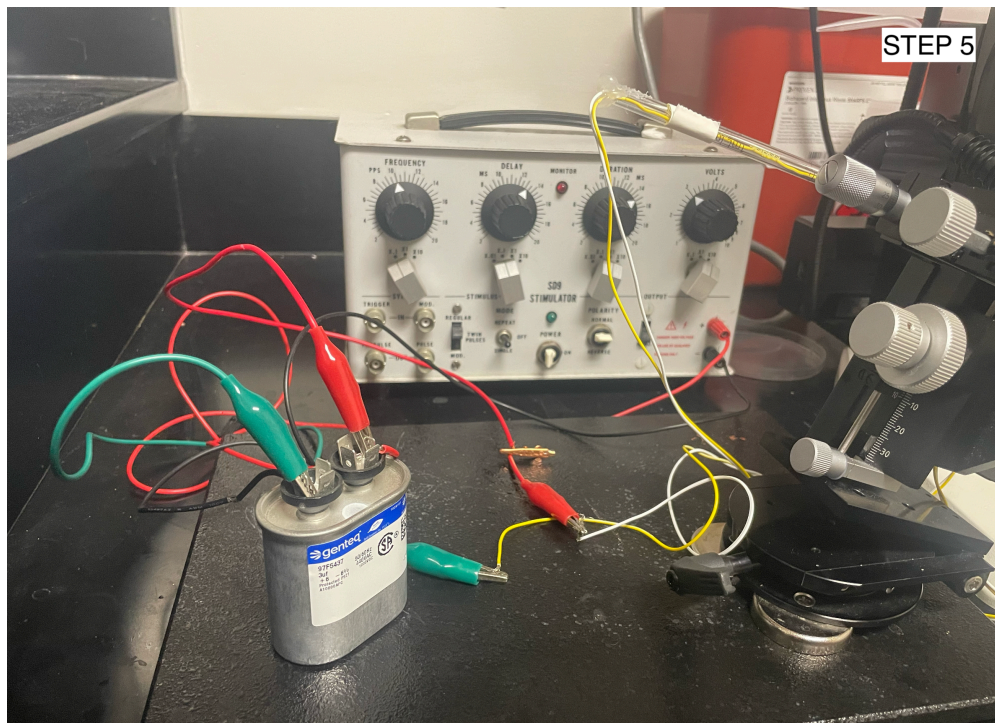


Figure 4. Side view of electrode needles, capacitor, and stimulator connected

Note

These parameters are a suggested starting point — optimizing the protocol for target molecules of interest and species-specific applications is recommended

Injection Set-Up

45m

- 6 Reconstitute morpholino solution (0.5 - 1.0 mM working concentration) in nuclease-free water 3m
- 7 Pull micropipettes from glass capillaries using a pipette puller 10m
- 8 Backfill the micropipette with mineral oil using a 28 gauge needle and 1 mL syringe 5m

- 9 Using forceps, break the micropipette at an angle to create a beveled tip. Performing this task using a dissection microscope is recommended 5m
- 10 Place the micropipette onto the injector plunger and tighten the collet 3m
- 11 Select an injection volume between 23-56 nL and set the injection rate to slow 1m



Figure 5. Nanoject II settings for injection volumes

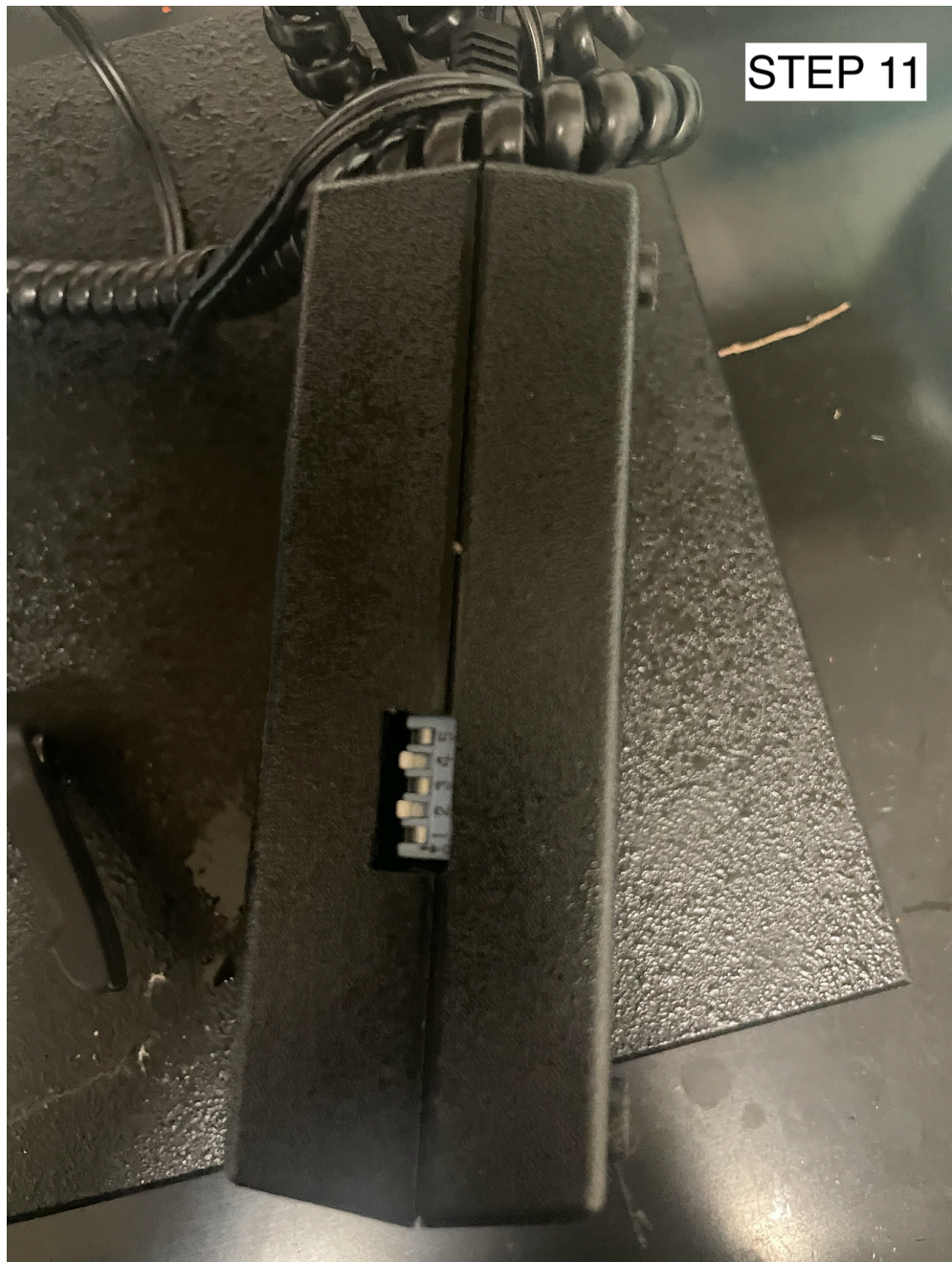


Figure 6. Example injection setting at 23.0 nl and slow

Note

This injection volume range is a suggested starting point. Morpholinos can be slightly toxic, so optimizing the appropriate injection volume for species-specific application is recommended.

- 12 Empty enough mineral oil to remove any bubbles and load 1-2 uL of morpholino solution 5m
- 13 Pipette 1-2 uL of morpholino solution onto a piece of Parafilm 1m
- 14 Gently dip the needle into the morpholino solution on the Parafilm and fill the micropipette without introducing air bubbles 2m
- 15 Construct a platform out of clay molded into the shape of a hill and fix on top of an empty Petri dish(es) 5m



Figure 7. Hill-shaped bed mounted on top of Petri dishes to hold tadpoles

- 16 Place the platform under a dissection microscope with the electrode on one side and the injector on the other

5m



Figure 8. Full injection and electroporation set-up with clay tadpole platform positioned under dissection microscope

Injection and Electroporation

22m

- 17 Anesthetize the tadpole by placing it in a Petri dish of room temperature 0.03% MS-222 for 3-5 minutes
- 18 Confirm the tadpole is completely sedated by checking for movement in response to stimuli
- 19 Cover the clay platform with a Kimwipe damp with tadpole water
- 20 Transfer the tadpole to the clay platform with a cut transfer pipette

5m

1m

1m

1m



Figure 9. Anesthetized tadpole laying dorsal side up on top of Kimwipe moistened with frog water on the clay platform

- 21 Adjust the position of the tadpole to be dorsal side up with its head in the clay depression 1m
- 22 Orient the tadpole so the head is facing away from the injector 1m
- 23 Lower the injector and insert the pipette needle into the brain, targeting the brain ventricle 5m



24 Inject the morpholino solution

2m

Note

We recommend injecting with 23-56 nL (detailed in Step 11) to start for species-specific optimization

25 Remove the pipette from the tadpole brain. Allow 10-20 sec before electroporation

1m

Note

If delivering standard morpholinos, continue with the protocol. If delivering Vivo MOs, skip to step 30

26 Orient the tadpole so the head is facing away from the electrode

1m

27 Lower the electrode needles until it is in full contact with the tadpole head on either side of the injection site

2m

28 Deliver the electrical pulses (4 total, half regular polarity and half reverse polarity)

1m

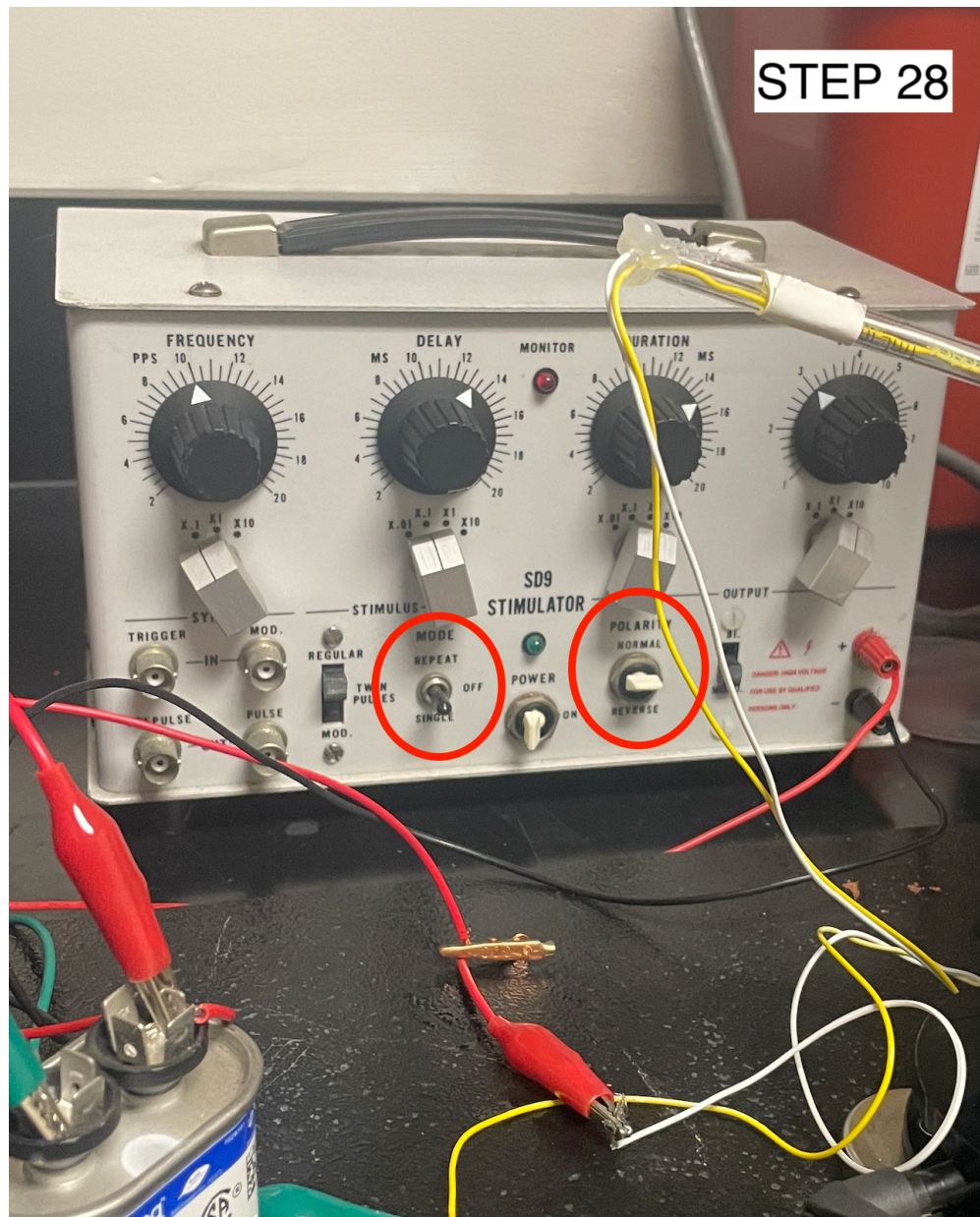


Figure 10. Close-up of stimulator settings with pulse switch and polarity switch circled in red

Note

The given pulse number is a suggested starting point. Optimizing the protocol for targets of interest and species-specific applications is recommended



- 28.1 Deliver 2 pulses on regular polarity with a 1s interval between each pulse
- 28.2 Switch the polarity to reverse
- 28.3 Deliver 2 pulses on reverse polarity with a 1s interval between each pulse
- 29 Remove the electrode from the tadpole skin
- 30 Transfer the tadpole to fresh tadpole water for several hours to recover

In vivo Visualization of Standard MO

1h 15m

- 31 At least 24 hrs after electroporation, anesthetize the tadpole by placing it in a Petri dish of room temperature 0.03% MS-222 for 3-5 minutes

5m

Note

Fluorescent signal can be imaged as soon as 24 hours post-electroporation. However, knockdown is most effective after previously translated protein of interest has been degraded. Optimizing timing based on target turnover rate and species-specific application is recommended

- 32 Move the tadpole to a new, small, and empty Petri dish using a cut transfer pipette
- 33 Place the Petri dish underneath the fluorescent stereomicroscope
- 34 Turn on the stereomicroscope and set the filter to GFP
- 35 Open the imaging software

1m

1m

1m

1m



36 Locate the tadpole under the stereomicroscope and zoom in, centered on the brain area

3m

37 Capture and save the fluorescent image

3m

38 Transfer the tadpole to fresh tadpole water for several hours to recover

1h

Preparing Protein Extraction and Dot Blot Solutions

30m

39 Prepare 1x Tris Buffered Saline (TBS)

15m

39.1 Mix 800mL distilled water with 6.05g Tris HCl and 8.76g NaCl

10m

39.2 Adjust pH to 7.6

4m

39.3 Add distilled water to bring to volume

1m

39.4 Store at 4C for up to 3 months

40 Prepare 2% SDS in 1x TBS

5m

40.1 Mix 800 uL of 1x TBS with 200 uL of 10x SDS

40.2 Store at 4C for up to 3 months

41 Prepare 7x protease inhibitor stock

5m

41.1 Add 1 cOmplete Mini EDTA-free tablet to 1.5 mL of distilled water



41.2 Store at 4C for up to one month

42 Prepare lysis buffer

5m

42.1 Mix 857 uL 2% SDS in 1x TBS with 143 uL 7x protease inhibitor stock

43 Prepare 1x TBST wash buffer

5m

43.1 Mix 500 mL of 1x TBS with 500 uL of Tween-20 (=0.01%)

Protein Extraction

2h

44 Anesthetize the tadpole by placing it in a Petri dish of room temperature 0.03% MS-222 for 5 minutes

5m

45 Sacrifice by rapid decapitation and dissect out tadpole brain

10m

46 Put the brain directly into 50-100uL of lysis buffer in a clean 1.5 mL microcentrifuge tube

1m

Note

For later lysis and processing, rapid freeze with dry ice and store at -80C

47 Homogenize the brain by hand with pestle or motorized tissue grinder

4m

48 Centrifuge tubes at 13000 rpm/18928 rcf for 90 min

1h 30m

49 Measure protein concentration on Qubit

30m

**Note**

Samples can be stored at -20 C before continuing the protocol. If proceeding from -20 C storage, allow samples to thaw on ice before proceeding with the dot blot

- 50 Calculate sample amounts for desired protein concentration (i.e. 15 ug protein) brought to volume in 1x TBS for dotting 6 ul of sample onto each of two nitrocellulose membranes 30m
- 51 Transfer the supernatant to a new 1.5 mL microcentrifuge tube 10m

Dot Blot**1d 1h 30m**

- 52 Using a narrow-mouth pipette tip, carefully dot 2-4 uL of samples onto two nitrocellulose membranes in the same pattern 30m
- 53 Allow the membranes to fully dry, then rewet in 1x TBST for 10 min 30m
- 54 Block non-specific binding in 5% dry milk in 1x TBST for 1 hour at room temperature with gentle agitation 1h
- 55 Incubate with primary antibody against target of interest on one membrane and primary antibody against reference protein on second membrane (1:100-1000) in 5% dry milk in 1x TBST for 1 hour at room temperature with gentle agitation 1h

Note

We recommend optimizing primary antibody concentrations using manufacturer's recommendation and pilot dot blots before data collection

- 56 Wash three times with 1x TBST for 10 min each at room temperature with gentle agitation 30m
- 57 Incubate with secondary antibody with HRP conjugate (1:2000-5000) with 2% BSA in 1x TBST for 1 hour at room temperature with gentle agitation 1h
- 58 Wash three times with 1x TBST for 5 min each at room temperature with gentle agitation 15m

**Note**

If chemiluminescent imaging only is desired, perform steps 59, 60 and 64. If brightfield imaging only is desired, skip to step 61. For both imaging techniques, perform the rest of the protocol as written.

59 Incubate with Clarity ECL substrate (equal volumes of each component, mixed) for 5 min in dark

5m

60 Image the membranes using a chemiluminescent imaging system (i.e. ChemiDoc)

10m

Note

We recommend trying different lengths of exposure to minimize background. If subsequent brightfield imaging is desired, we recommend waiting overnight to perform the colorimetric reaction.

61 Combine DI water, Opti-4CN dilutant, and Opti-4CN substrate according to Opti-4CN development kit and incubate membranes overnight

18h

62 Rinse membranes in DI water for up to 10 min. Let dry at RT for up to 30 min and seal with tape

45m

63 Image on brightfield next to normalized step ladder

15m

64 Measure optical density in ImageJ using mean gray values for each sample blotted across both membranes

1h

65 Calculate an optical density ratio of your protein of interest to that of your reference housekeeping protein

30m