

**Use and efficiency of morpholinos in Neotropical tadpole brains**

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**ABSTRACT****DOI:**

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in any medium, provided the original author and source are credited These parameters are a suggested starting point. Optimizing the protocol for species-specific applications to maximize transfection efficiency is recommended.

**Protocol status:** Working

We use this protocol and it's working

**GUIDELINES****MATERIALS****Injection, Electroporation, and *in vivo* Imaging Materials****Created:** Nov 16, 2022

Ethyl 3-Aminobenzoate Methanesulfonate (Millipore Sigma Catalog #10521)

**Last Modified:** Aug 20, 2023

**PROTOCOL integer ID:**  
72812

**Keywords:** amphibian, poison frog, vivo morpholinos, microinjection, electroporation, tyrosine hydroxylase

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 INSTRUCTIONS

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 5m
- 2 Store at 4C for up to one week

## 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

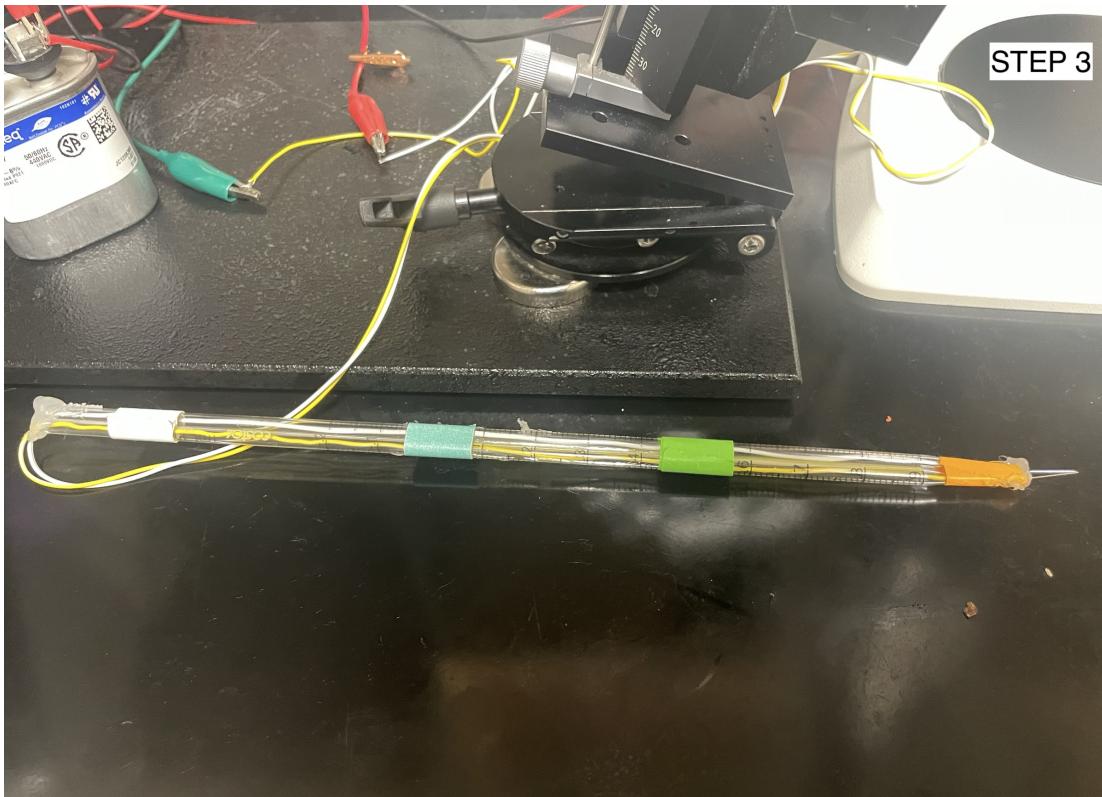


Figure 1. Two serological pipettes with electrode needles (see Step 4) attached together using electrical tape and hot glue to secure

- 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

## STEP 4

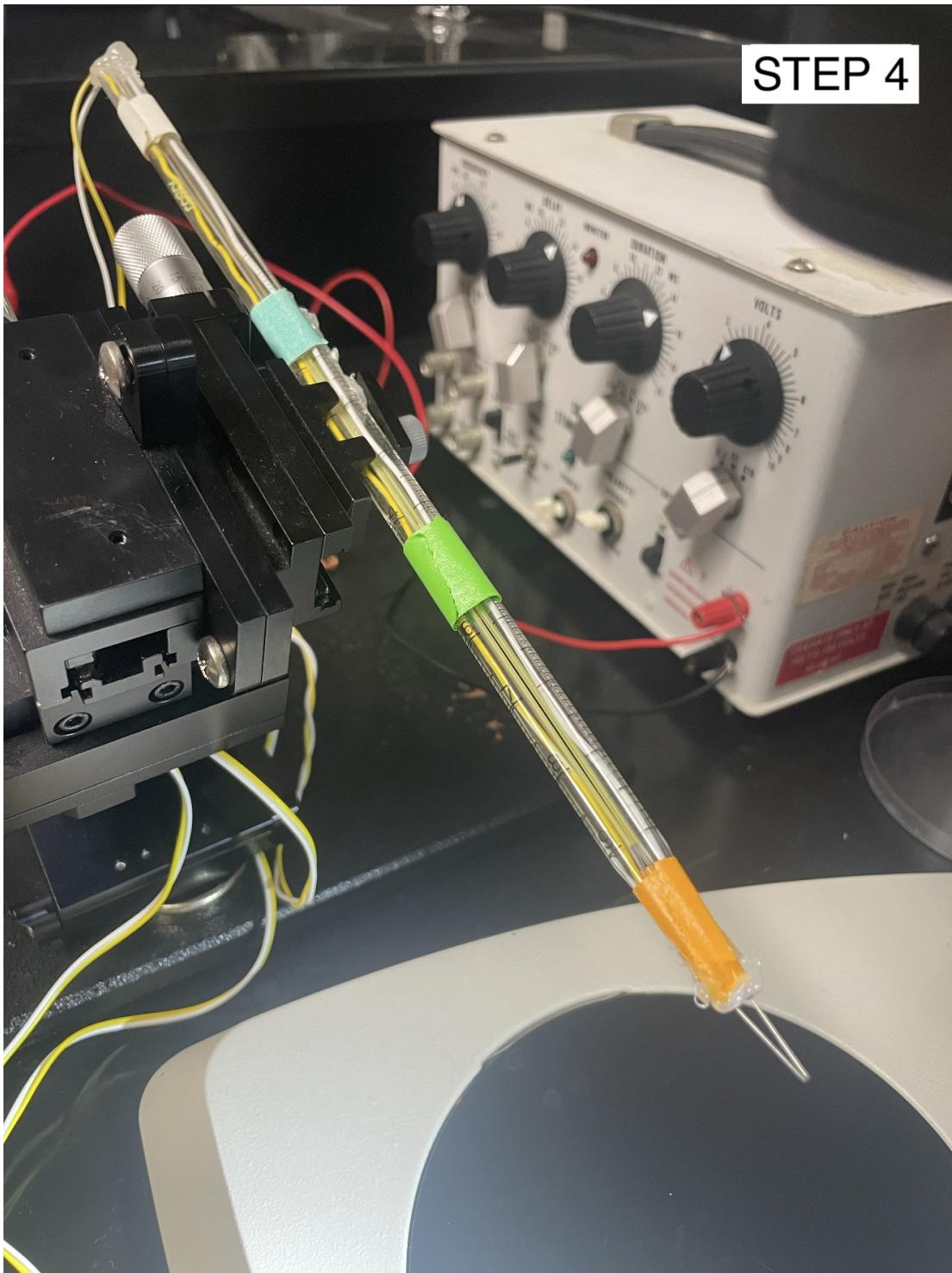


Figure 2. Two serological pipettes with electrode needles secured onto micromanipulator mount

- 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

## STEP 5

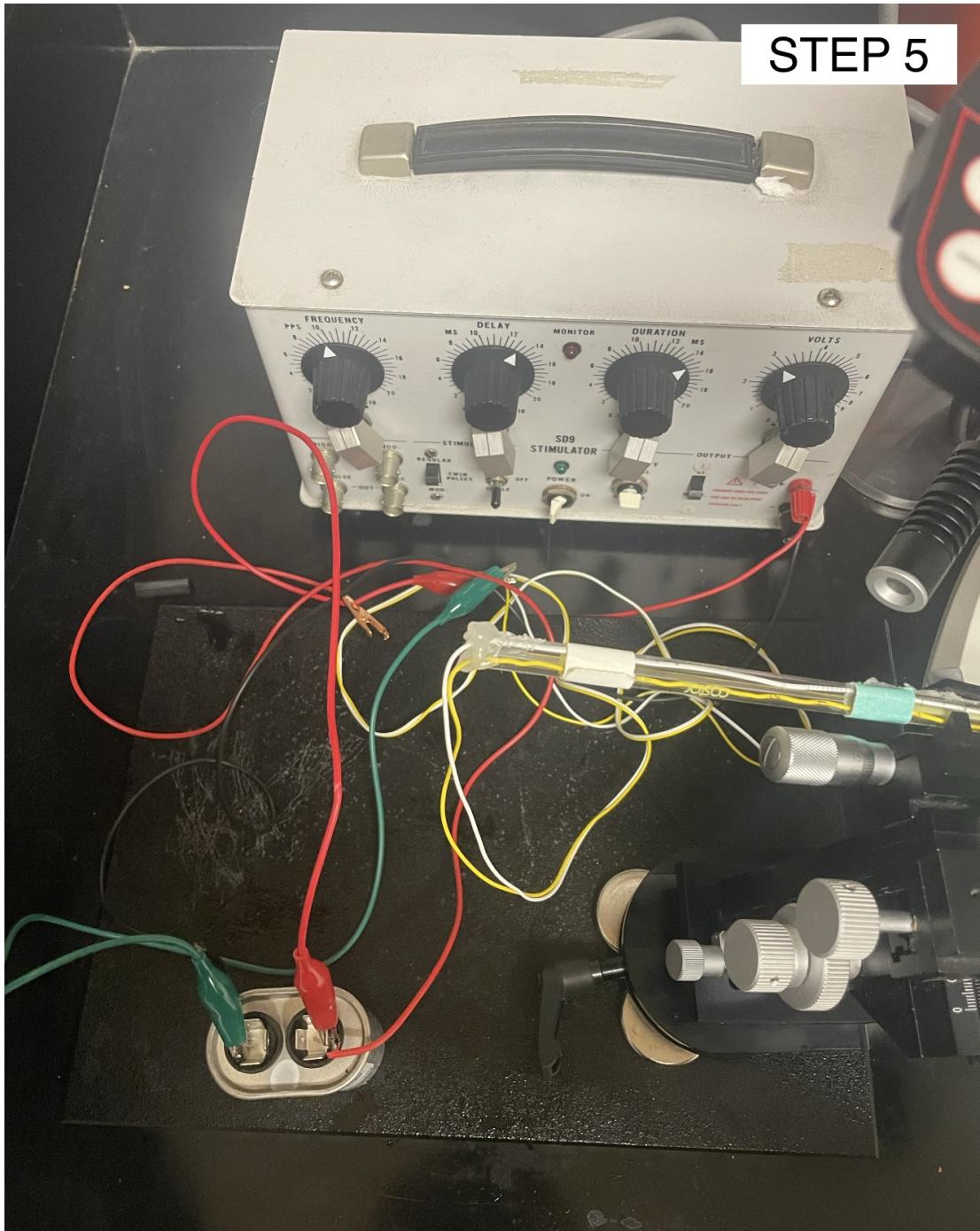
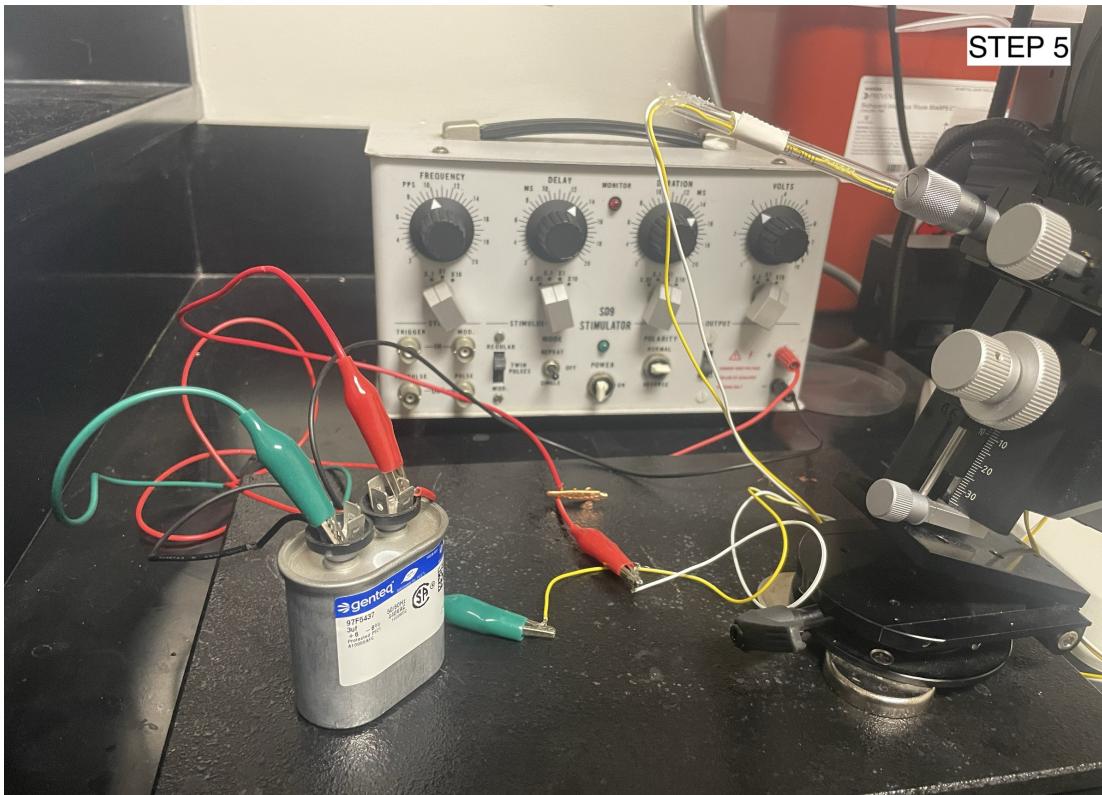


Figure 3. Top view of electrode needles, capacitor, and stimulator connected



STEP 5

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

# STEP 11

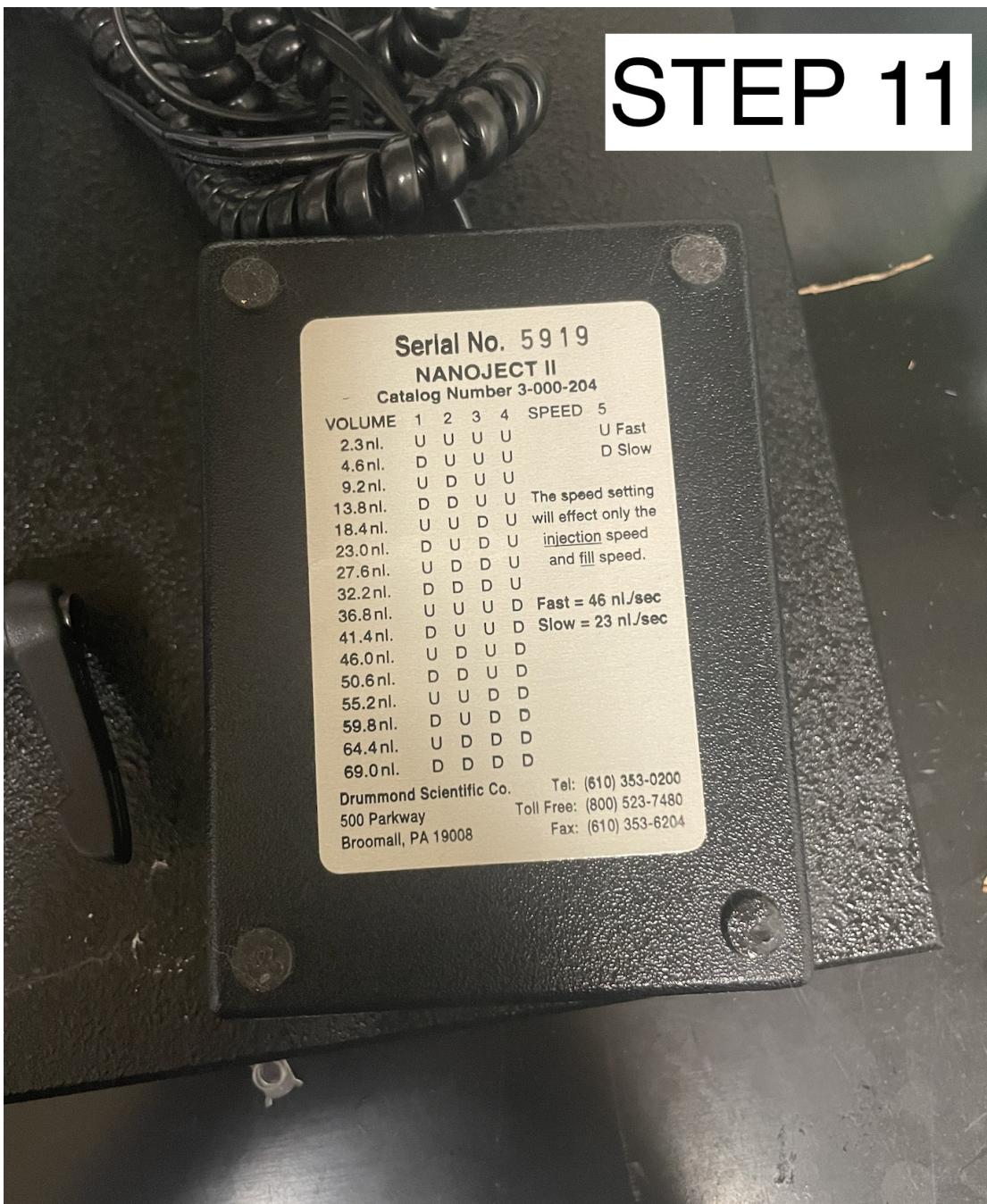


Figure 5. Nanoject II settings for injection volumes

STEP 11

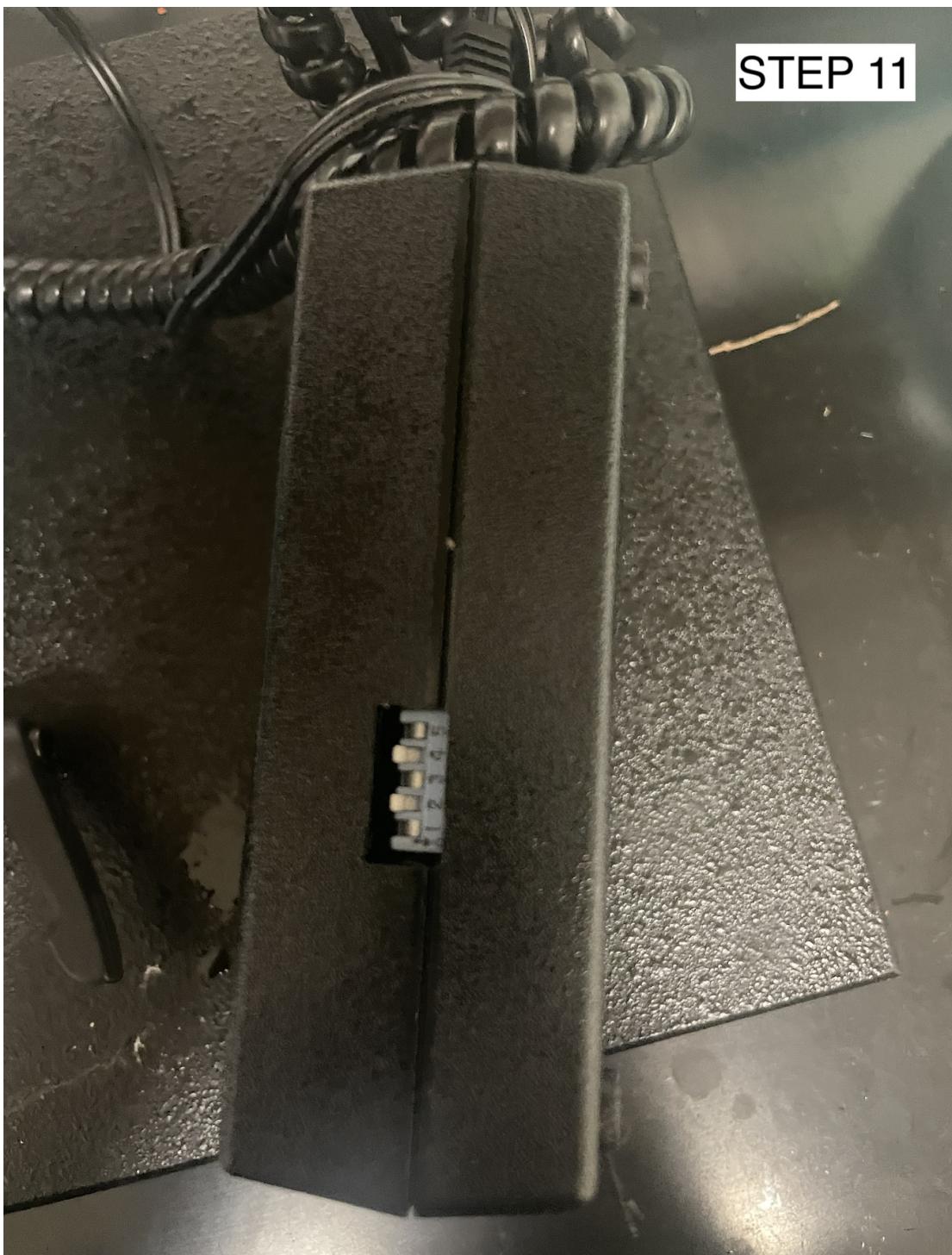


Figure 6. Example injection setting at 23.0 nl and slow

### 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

## STEP 15



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

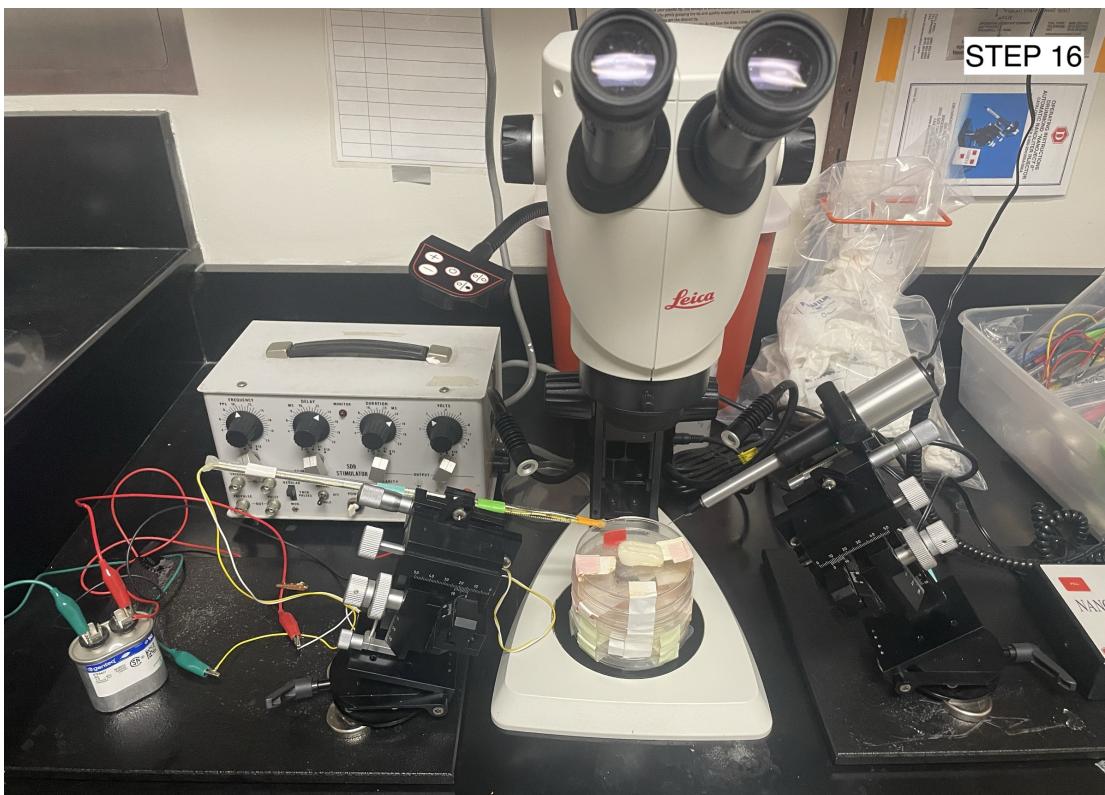


Figure 8. Full injection and electroporation set-up with clay tadpole platform positioned under dissection microscope

## 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 5m
- 18 Confirm the tadpole is completely sedated by checking for movement in response to stimuli 1m
- 19 Cover the clay platform with a Kimwipe damp with tadpole water 1m

20

Transfer the tadpole to the clay platform with a cut transfer pipette

1m

STEP 20

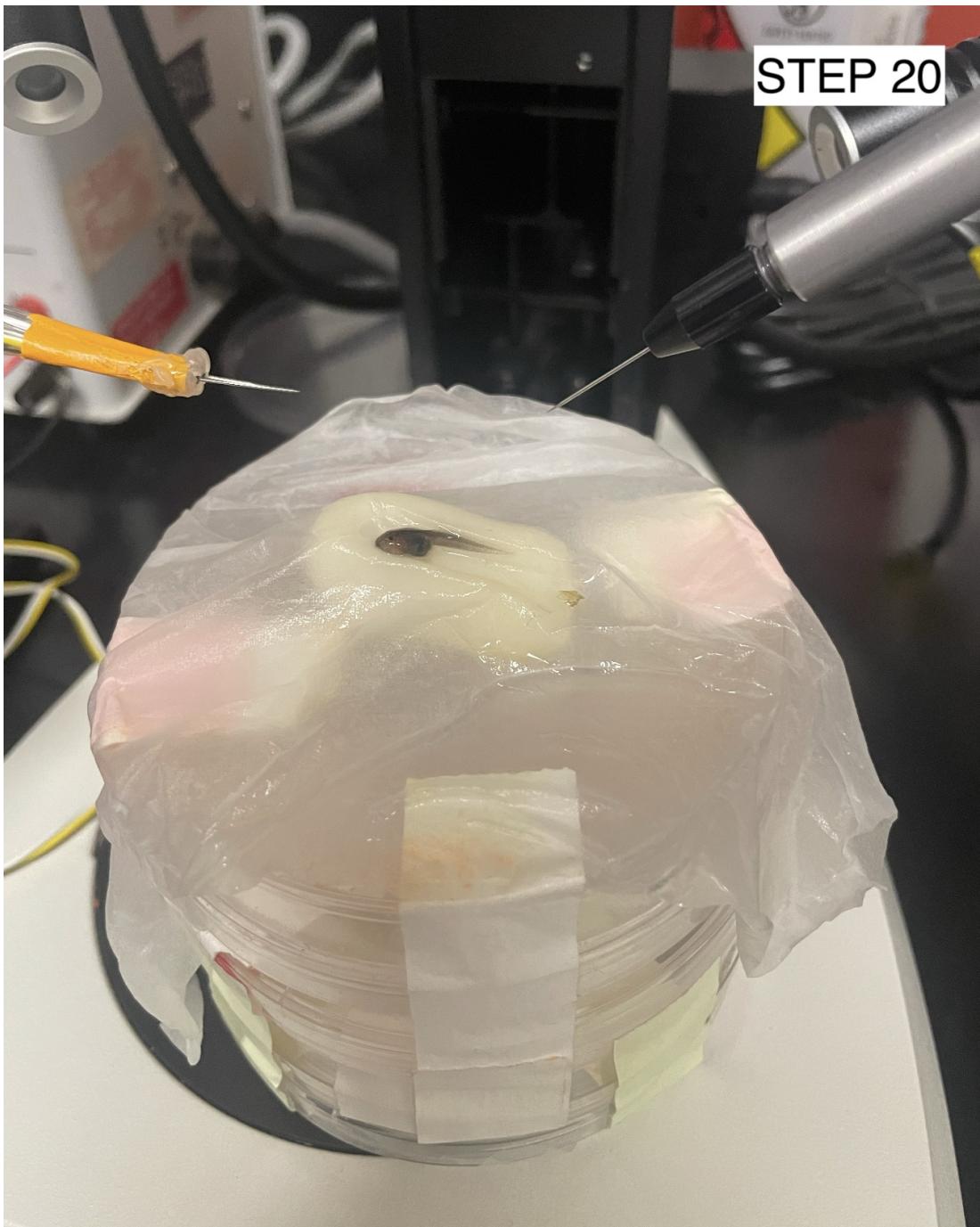


Figure 9. Anesthetized tadpole laying dorsal side up on top of Kimwipe moistened with frog water on the clay platform

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

## STEP 28

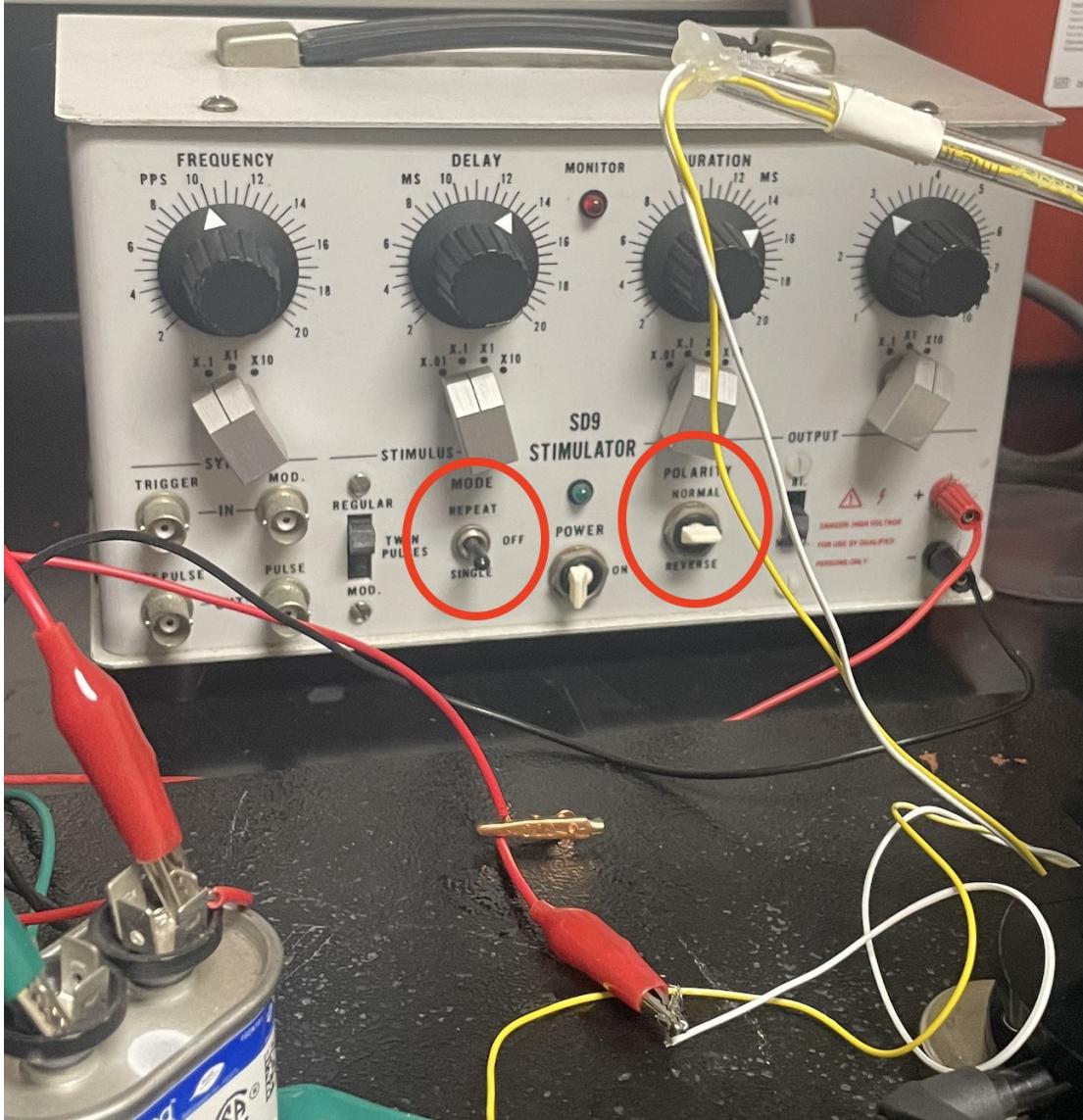


Figure 10. Close-up of stimulator settings with pulse switch and polarity switch circled in red

### 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 1m

- 33 Place the Petri dish underneath the fluorescent stereomicroscope 1m
- 34 Turn on the stereomicroscope and set the filter to GFP 1m
- 35 Open the imaging software 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