In vivo single-cell electroporation for transfer of DNA and macromolecules

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Single-cell electroporation allows transfection of plasmid DNA or macrocmolecules into individual living cells using modified patch electrodes and common electrophysiological equipment. This protocol is optimized for rapid *in vivo* electroporation of *Xenopus laevis* tadpole brains with DNA, dextrans, morpholinos and combinations thereof. Experienced users can electroporate roughly 40 tadpoles per hour. The technique can be adapted for use with other charged transfer materials and in other systems and tissues where cells can be targeted with a micropipette. Under visual guidance, an electrode filled with transfer material is placed in a cell body–rich area of the tadpole brain and a train of voltage pulses applied, which electroporates a nearby cell. We show examples of successfully electroporated single cells, instances of common problems and troubleshooting suggestions. Single-cell electroporation is an affordable method to fluorescently label and genetically manipulate individual cells. This powerful technique enables observation of single cells in an otherwise normal environment.

INTRODUCTION

Inferences about an individual cell's function, connectivity and development have historically been made from labeled cells in fixed tissues. The challenge today is to go one step further by making direct measurements of single labeled cells *in vivo* in order to understand the molecular and genetic mechanisms underlying their biological function.

Dye-labeling of living single cells is commonly achieved by single-cell iontophoresis[1]. However, this technique is limited to dye transfer. The need to transfect with genetically encoded markers, such as green fluorescent protein (GFP), has led to the development of several techniques to label living cells: microinjection[2], biolistic transfection[3], viral infection[4], lipofection[5] and transgenic technology[6,7]. While each of these techniques has its own advantages[8,9], single-cell electroporation (SCE) can be applied to a wider range of tissues and cell types with faster results and arguably less toxicity, expense and technical difficulty. Furthermore, SCE is an outstanding method for providing precise temporal and spatial control of gene or dye delivery.

SCE delivers DNA or fluorescent markers into individual cells in intact tissue or the whole animal. The underlying biophysical mechanism involves local electric currents that are thought to transiently open pores in the cell membrane, permitting the uptake of the transfer material carried in the electrode[10,11]. However, the precise mechanism of transfection by electroporation is still unclear[12]. Regardless, the technique is limited to the use of charged molecules, such as dextrans or DNA, whose direction of delivery is determined by the polarity of the electrodes.

Since this method can be conducted *in vivo*, it enables the repeated observation of cells over timescales of minutes to days. Furthermore, in combination with molecular biological reagents, it can be used to manipulate specific genes or biological functions of the single cell in its otherwise unaltered environment. This allows the dissection of cell-autonomous versus non-cell-autonomous effects that cannot be distinguished, for example, by systemic pharmacological manipulations. In addition, single-cell electroporation confers the ability to control temporally the molecular biology of the cell, which is a more challenging task in transgenic model systems, for instance.

Our protocol is optimized for the electroporation of neurons in the central nervous system of living albino *Xenopus laevis* tadpoles[13,14] (**Fig. 1**). Different transfer materials, such as plasmid DNA, morpholinos and fluorescently conjugated dextrans or a combination of them are described. This technique employs a pulled glass capillary pipette based on the electrode design used for typical patch-clamp electrophysiological recordings[15]; the technique is therefore limited to tissues that are accessible to the glass electrode. The electroporation stage is set up with long working distance optics to easily and rapidly position the tadpole. Given the microscope optics, we can identify the target region containing the cell bodies, but not the individual cell that will be electroporated. Such a strategy allows the electroporation of one animal in roughly 1 min, but with an overall lower efficiency of successfully labeled cells. With higher resolution optics (i.e., shorter working distance) and finer electrode manipulators, single cells could be identified and specifically targeted[16,17]. With this approach, the time to electroporate a single preparation will be longer, but with an expected higher efficiency of labeling. This latter strategy might be preferred if the model system is a limiting resource or if the experiments require the more precise identification of the cell.

Modifications to the basic SCE strategy that we present here have been made and successfully applied to other systems such as cultured chick dorsal thalamus explants, *Aplysia californica* ganglia, zebrafish larvae, mouse hippocampal slices and rat cerebella[18–22].

MATERIALS

REAGENTS

- Albino or wild-type *Xenopus laevis* tadpoles (obtained from lab colony or Nasco); we use stage 46–48 tadpoles[23] but other stages, earlier or later, can be used depending on the experimental question. For detailed instructions on how to obtain and maintain tadpoles, see ref. 22. No special procedures are necessary to rear tadpoles for electroporation.

Erratum: *In vivo* single-cell electroporation for transfer of DNA and macromolecules

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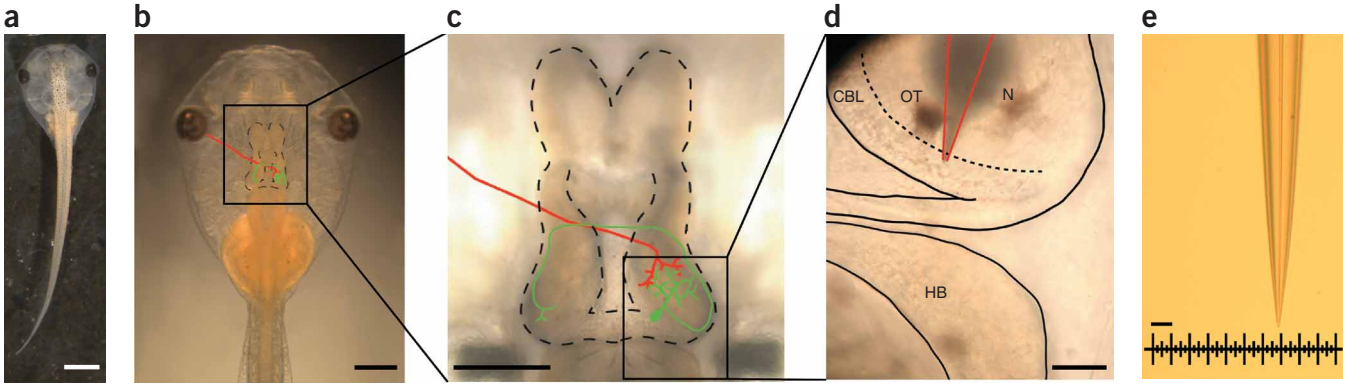

Figure 1 | Illustration of SCE in the optic tectum of the *Xenopus laevis* central nervous system. (**a**) Transparent albino *Xenopus laevis* tadpole. Scale bar = 2 mm (**b**,**c**). Magnification of forebrain and optic tectum with illustration of the contralateral projecting retinal ganglion cell axon (red) and optic tectal neuron (green). Scale bar = 1 mm. (**d**) Placement of single-cell electroporation electrode in the cell body layer of the optic tectum. The electrode is outlined in red. Scale bar = 200 μm. (**e**) Magnification of the electrode. Note the tip diameter is ~2 μm. Scale bar = 10 μm. N, neuropil; OT, optic tectum; CBL, cell body layer; HB, hindbrain.

- 100× and 1× Steinberg's Solution (see REAGENT SETUP).
- Anesthesia: 0.01% MS222 (3-Aminobenzoic acid ethyl ester or Tricane; Sigma) diluted in 1× Steinberg's Solution, adjusted to pH 7.4.
- Transfer material (diluted in H_2O or TE): Endo-free DNA at 1–5 μg/μl (Qiagen EndoFree Plasmid Maxi Kit), or Morpholino at 500 μM (Gene-Tools) or Fluorescent dextran (MW 10,000, 50 mg/ml stock solution; Invitrogen).

EQUIPMENT

- Axoporator 800A (Axon Instruments).
- HS-2A-x10MGU headstage (Axon Instruments, **Fig. 2**).
- Grass stimulator SD9 (Grass Telefactor, **Fig. 2**).
- Micromanipulator with coarse and fine control (e.g., MM-3, Narishige).
- Oscilloscope (e.g., Hitachi V-252).
- Cables: a BNC cable, a BNC-banana plug, a ground wire (composed of a silver wire, diameter 0.25 mm, soldered to an insulated cable that can connect to the BNC banana plug) and at least four crocodile cables.
- Electrode holder with handle for Grass stimulator that allows wire to exit the back (e.g., MPH1, World Precision Instruments) or electrode holder for Axoporator headstage as part of the Axoporator equipment.
- Silver wire (0.25 mm; World Precision Instruments).
- Borosilicate capillary glass with filament: OD 1.5 mm, ID 0.86 mm, 75 mm (e.g. G150F-3; Warner Instruments).
- Electrode puller P-97 (Sutter Instrument) equipped with either box or trough filament (~3 mm).
- Upright light microscope with long working distance objective (e.g., Olympus BX50WI) with 20× water immersion lens (e.g., Olympus LMPlanFl).
- Paintbrush to position and handle tadpoles.
- 1-ml syringe (melted and pulled fine, see **Fig. 3**).
- Screening microscope with epifluorescence illumination (e.g., Nikon Optiphot-2) equipped with appropriate filter sets for viewing the fluorophore of choice (e.g., Chroma 41001 filter set for eGFP).

REAGENT SETUP

All chemicals are from Sigma unless otherwise noted.

100× Steinberg's stock: 0.5 g KCl, 0.8 g $Ca(NO_3)_2$• $4H_2O$, 2.1 g $MgSO_4$• $7H_2O$, 34 g NaCl, 119 g HEPES, to 1 l dH_2O, pH to 7.4, keep at 4 °C.

1× Steinberg's stock: 100 ml 100× Steinberg's to 10 l dH_2O. Add 500 μl penicillin/streptomycin (5,000 Uml^{-1}/5,000 $\mu g\,ml^{-1}$, Invitrogen), pH to 7.4. Keep and use at room temperature (18–22 °C).

EQUIPMENT SETUP

Optimization and preparation of electrodes

TIMING approximately 3–6 h: The optimization of the pipette is a time-consuming trial-and-error process. The objective is to make a pipette with a tip diameter smaller than the cell body size in order to target single cells and minimize damage. We have found that making the tip diameter smaller often results in a longer, more fragile tapered electrode. An optimal electrode shape is a balance between a small tip and a rigid pipette. Very small electrodes tend to clog; very long electrodes tend to break.

Using the instructions for "Patch Pipette Fabrication" in the *Micropipette Puller Operation Manual* (Sutter Instrument Company) as a guideline, make a patch pipette from a glass capillary that is pulled in four programming lines (tip diameter ~1–3 μm). Depending on the puller and the size of cells to be electroporated, it may be necessary to expand this to a five-line program in order to obtain an electrode with a shorter taper and smaller tip diameter than a typical patch pipette. For cells with a soma diameter of ~10 μm, a pipette with ~1–2 μm tip diameter is necessary to electroporate single cells. Tip diameters are measured with a micrometer ruler. On Sutter pullers, if the tip diameter is large (>2 μm), modification of the settings by increasing the velocity (by 3-unit increments) on the 4^{th} and 5^{th} lines and the heat (by 5-unit increments) on the $4^{th}/5^{th}$ line will result in patch pipettes with smaller tip diameters (~1–2 μm in diameter). Keep the taper as short as possible (**Fig. 1e**). It is necessary to test electrodes in the experimental system to determine if the electrode is suitable. Expect to modify these recommendations depending on the biological system and equipment used.

? TROUBLESHOOTING

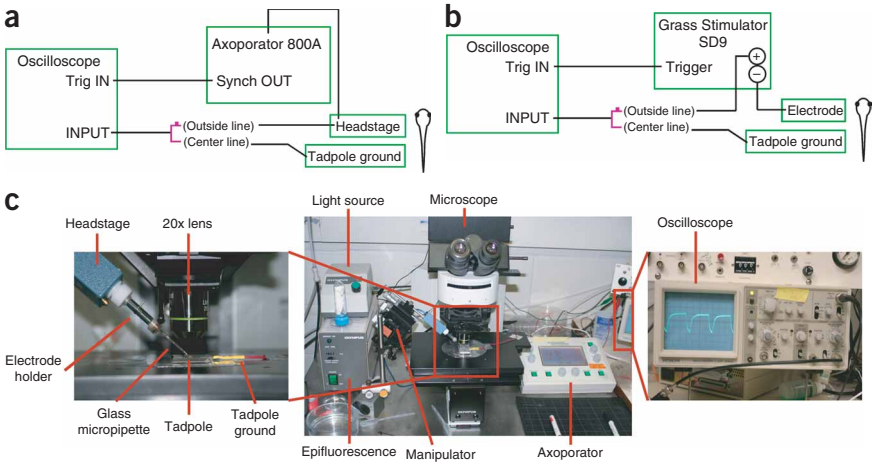

Figure 2 | The single-cell electroporation setup. Wiring of the single-cell electroporation setup for the Axoporator 800A (**a**) and the Grass Stimulator SD9 (**b**). Equipment is surrounded by green boxes, wiring is depicted by black lines, the BNC to banana plug adaptor is pink. (**c**) Pictures of the SCE setup. Note the angle of the electrode and the shape of the current pulse on the oscilloscope.

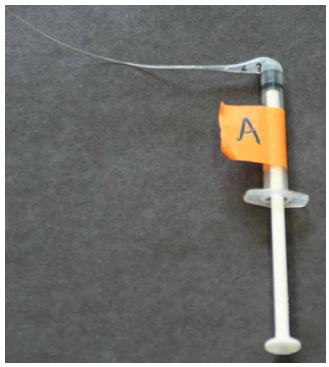

Figure 3 | Electrode loading syringe. A 1-ml syringe is melted over a flame. The melted tip drops and pulls into a fine wisp. After it cools, it is cut to size with a razor blade. Use caution, the melted plastic is hot.

Preparation of electrical equipment TIMING approximately 10 min: Connect the Grass stimulator or Axoporator, oscilloscope and electrode holder with the different cables according to the wiring diagram (**Fig. 2**). A minimum of five insulated cables is needed: (i) connect Trig IN port of the oscilloscope with the trigger or SynchOUT port of the stimulator using a BNC cable; (ii) link the oscilloscope INPUT to the +port or headstage of the stimulator using a BNC-banana plug; (iii) link the oscilloscope INPUT to the silver ground wire via soldered wires or crocodile cables that can connect to the BNC banana plug; (iv) connect the Grass stimulator (–port) to the silver wire in the electrode (see below) using crocodile cables; and (v) connect the Axoporator to the headstage with the cable that is supplied with the Axoporator. **! CAUTION** As with all electrical equipment, familiarize yourself with the safety precautions of the equipment. During assembly, the equipment should be turned off.
? TROUBLESHOOTING

Equipment assembly TIMING approximately 10 min: Tape a glass slide to the microscope stage over the light source. This is the stage upon which the tadpole will be placed and allows illumination of the preparation from below. Place the silver ground wire on the secured glass slide; tape it to the stage to hold it in place, if necessary. The ground must be in electrical contact with the electrode but the distance between them is not important because the circuit is closed through the tadpole. Place the electrode holder into the headstage (for Axoporator) or secure it directly to the manipulator (**Fig. 2c**). The headstage is the interface between the axoporator and the electrode. Make sure that a silver wire is secured in the electrode holder because it serves to connect the Axoporator/stimulator with the solution in the micropipette. The end of this silver wire has to be long enough to thread inside the electrode but short enough not to break the tip. In the Grass stimulator setup, the wire has to pass through the back of the holder where it is then connected via crocodile cables to the stimulator (see part (iii) above). Adjust the angle of the manipulator so that the electrode holder is at a 45–60 degree angle to the surface of the tadpole. This may need to be adjusted for different model systems and microscope configurations.

PROCEDURE

1| Fill the electrode with plasmid DNA, morpholino or dyes using a loading syringe (**Fig. 3**) or by back-filling the electrode. The solutions can be centrifuged before loading to minimize clogging of the electrode. Only a small volume is necessary (<1 µl). We recommend that new users start with fluorescently conjugated dextrans. Owing to their small size, they electroporate readily and can be seen under epifluorescence. This enables the tip position in the sample to be visualized and, upon electroporation, provides instant feedback on whether the equipment is functional and if the location of the tip is appropriate. Note, however, that prolonged exposure to epifluorescent light can induce photodamage.

2| Put the electrode into the electrode holder on the head stage or manipulator (see EQUIPMENT SETUP). The filament inside the glass capillary will wick the solution throughout the electrode, promoting the contact of the silver wire inside the electrode with the solution. This relieves the requirement of the silver wire to be visually in contact with the solution.

3| Anesthetize tadpoles in a Petri dish containing 0.01% MS222. Tens of tadpoles can be anesthetized simultaneously, but they should not be kept in the anesthesia for more than 1 h.

4| Cover the glass slide on the microscope stage (including the ground wire) with a wet Kimwipe. Use a plastic transfer pipette to put a tadpole onto the slide. Tadpoles do not have to touch the ground wire, but the Kimwipe and tadpole must be kept moist to complete the circuit and to keep the tadpoles from drying out.

5| Position the tadpole using a paintbrush so the head faces the electrode and the dorsal side is up so that the brain is accessible.

6| Adjust the light and contrast of the microscope to see the tissue structure and cell outlines. It may be useful to use off-center light and a closed diaphragm for better contrast.

7| Focus on the target region and poke the electrode through the skin of the tadpole to place the tip close to the cell bodies of interest. Use both the manipulator and the stage controls to position the tadpole, even after the electrode has penetrated the skin (**Fig. 1**).
? TROUBLESHOOTING

8| If using the Axoporator, monitor the electrode resistance in the tissue. Our pipettes typically range from 10–30 MOhm. The resistance of the electrode is an indicator of whether the tip diameter of the electrode is too small or clogged (high resistance) or if the tip diameter is too large or broken (low resistance). The absolute resistance cannot be measured on the Grass stimulator; however, the oscilloscope reading is an indirect indicator of the resistance of the electrode (also see Step 10).

9| Deliver the voltage pulse train. See **Table 1** for suggested electroporation settings for each transfer material.

TABLE 1 | Electroporation settings for different transfer materials.

	Electroporation settings					
Transfer material	**Polarity[b]**	**Pulse duration (ms)**	**Frequency (Hz)**	**Train duration (s)**	**Voltage (V)[c]**	**Offset (V)[d]**
Plasmid DNA	neg	1	200	1	~20–100	0
Morpholino	pos	1	200	0.5	~10–20	0
Dextran	pos	1	200	0.5	~5–10	0 or –1
Morpholino + dextran	bipolar	7	70	1	~20	0
DNA + dextran	bipolar	7	70	1	~20–100	0
siRNA[a]	*neg*	*1*	*200*	*0.5*	*~10*	*0*

[a]Entries in *italics* have not been tested, but suggested parameters are listed. [b]The Grass stimulator does not deliver bipolar pulses. Changing the polarity during train delivery may achieve the same result; this has not been tested, however. [c]Voltage settings need to be adjusted according to the resistance of the electrode. [d]Maintenance of a DC offset (possible with Axoporator) may prevent leakage of dye.

10| Monitor the amplitude of pulses on the oscilloscope (**Fig. 2c**). Alter the voltage to maintain a 1–1.5 μA amplitude. If the pulse amplitude decreases, it is indicative of a clogged electrode. If the pulse amplitude increases, it is likely due to a broken tip.

? TROUBLESHOOTING

11| Retract electrode.

12| The electrode can be reused until it clogs. Repeat the procedure either at different sites in the target area or in a new tadpole. When using multiple transfer materials, rinse the silver wire with dH_2O after use and before inserting the new electrode to avoid contamination of the solution in the electrode. We have found this to be sufficient for maintenance of the silver wire. We typically electroporate 2–3 times per hemisphere in the tectum, spaced apart so that cells do not overlap. This is to increase the chances of obtaining 4–6 single cells in one animal.

? TROUBLESHOOTING

13| Place the tadpole in a Petri dish containing Steinberg's rearing solution to recover.

14| Screen for successful electroporation of single cells with an epifluorescence microscope, using settings and filters appropriate for the label used. Beware of photodamage by prolonged exposure. Cells electroporated with dextrans and morpholinos can be detected as soon as 30 min after electroporation and as soon as 12 h later for plasmid DNA.

● **TIMING**

Steps 1–2: approximately 1 min
Step 3: approximately 5 min
Steps 4–13: approximately 1 min with experience
Step 14: 30 min–12 h wait time, approximately 30 s for screening

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 2**.

TABLE 2 | Troubleshooting table.

PROBLEM	POSSIBLE REASON	SOLUTION
Electrode[a] (see **Fig. 1** and **Table 1**)		
Low current reading on oscilloscope that does not respond to increased voltage	Tip size too small	Modify electrode puller program. For example, decrease velocity or heat parameters.
	Clogged electrode	Discard and replace electrode. Impurities in transfer material can clog electrodes, therefore spin down transfer material before filling electrode.
High current reading with low voltages	Tip size too big	Adjust puller program by increasing heat and velocity parameters.
	Broken electrode	Adjust puller program if puller creates a broken electrode. Check puller filament.
Equipment (see **Fig. 2**)		
No signal on oscilloscope	Improper wiring/open circuit	Consult **Figure 2**; make sure ground wire and electrode form a circuit through the preparation.
	Headstage malfunction	Consult Axoporator manual.

TABLE 2 | Troubleshooting table (continued).

PROBLEM	POSSIBLE REASON	SOLUTION
Results (see **Fig. 5** and **Table 1**)		
No labeled cells or evidence of unhealthy or dead cells	Failure to penetrate tissue	Adjust angle of manipulator. Adjust light/contrast so that electrode tip can be visualized in the tissue. Make sure electrode is located in the target region. If electrode is blunt or breaks, adjust puller program.
	Tip size too big	Decrease voltage and/or modify puller program.
	Toxic concentration of transfer material	Adjust concentration.
	Inappropriate voltage setting	Adjust voltage. Excessive voltage can kill cells; too low of voltage will not label cells.
Dim cells	Transfer material concentration too low or contaminated	Double check solutions.
	Tip size too small	Adjust puller program.
	Voltage settings too low	Increase voltage. If problem persists, increase train duration.
Multiple cells	Tip size too large	Adjust puller program.
	Voltage settings too high	Decrease voltage. If problem persists, decrease train duration.

[a]Consult the operation manual for the electrode puller for electrode design.

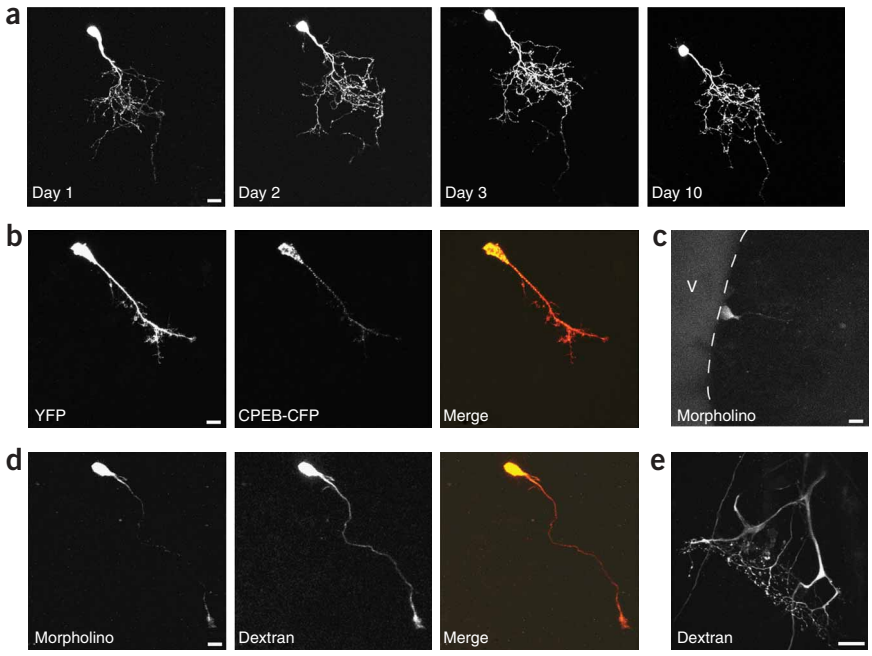

Figure 4 | Anticipated results of single-cell electroporation. Examples of labeled neurons and glia from *Xenopus laevis* tadpoles imaged with two-photon microscopy. (**a**) An optic tectal neuron labeled with green fluorescent protein (GFP), imaged over 10 d. (**b**) A glia transfected with two plasmids encoding yellow fluorescent protein (YFP) and cytoplasmic polyadenylation element binding protein (CPEB) tagged with cyan fluorescent protein (CFP), imaged 2 d after electroporation. (**c**) A tectal neuron transfected with fluorescein-tagged morpholino, imaged 1 d after electroporation. (**d**) A tectal neuron labeled with fluorescein-tagged morpholino and Alexa Fluor 568–conjugated dextran, imaged 1 d after co-electroporation. (**e**) An example of a motor endplate in the tadpole tail transfected with Alexa Fluor 568–conjugated dextran, imaged ~30 min after labeling. Scale bar, 15 μm. V, ventricle.

ANTICIPATED RESULTS

Expect to observe a range of results. We regard SCE as successful when it produces an individual, brightly labeled cell that can be imaged and whose complete dendritic arbor could be digitally reconstructed. Even the most distal and fine processes of neurons, for example, are clearly labeled (**Fig. 4**). With these criteria, our yield of successfully electroporated cells in the *Xenopus laevis* optic tectum is a single cell in ~25% of electroporated tadpoles. In **Figure 4** we show examples of cells that were successfully electroporated with DNA, morpholinos and dextrans. **Figure 5** illustrates examples of what we consider to be unsuccessful electroporation attempts, such as faintly labeled and multiple labeled cells. Note, however, that depending on the experimental question, these results could still be useful.

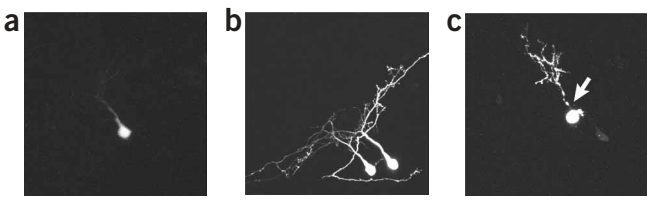

Figure 5 | Examples of electroporation results requiring troubleshooting. Examples of GFP-labeled neurons from *Xenopus laevis* tadpoles imaged with two-photon microscopy. (**a**) A faintly labeled cell. (**b**) A pair of neurons. (**c**) A typical dying cell with fragmented processes and a pinched-off soma (arrow).

COMPETING INTERESTS STATEMENT
The authors declare that they have no competing financial interests.

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