**TITLE:**

**Electroporation method for *in vivo* delivery of plasmid DNA in the adult zebrafish telencephalon**

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**KEYWORDS:**

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**SUMMARY:**

Here, we are reporting an electroporation method for plasmid DNA delivery and ependymoglial cell labeling in the adult zebrafish telencephalon. This protocol is a quick and efficient method to visualize and trace individual ependymoglial cells and it opens new possibilities to apply electroporation for a broad range of genetic manipulations.

**ABSTRACT:**

Electroporation is a transfection method in which an electrical field is applied to cells in order to create temporary pores in the cell membrane and increase its permeability, thereby allowing different molecules to be introduced to the cell. In this paper, electroporation is used to introduce plasmids to ependymoglial cells, which are lining the ventricular zone of the adult zebrafish telencephalon. Fraction of these cells has stem cell properties and generates new neurons in the zebrafish brain; therefore, studying their behaviour is essential in order to study their role in both neurogenesis and in regeneration. The introduction of plasmids via electroporation enables long-term labelling and tracking of a single ependymoglial cell. Moreover, electroporation allows reliable identification of cellular morphology and behaviour, setting it up as necessary tool for chronic live *in-vivo* imaging. Furthermore, plasmids such as Cre recombinase or Cas9 can be delivered to single ependymoglial cells, which enable gene recombination or gene editing, providing a unique opportunity to assess the cell autonomous gene function in otherwise control, natural environment. Ultimately, we are demonstrating a detailed step-by-step electroporation protocol used to obtain successful introduction of plasmids into a large number of single ependymoglial cells.

**INTRODUCTION:**

Zebrafish are an excellent animal model to address the question of brain regeneration after stab wound injury. In comparison to mammals, on the evolutionary ladder less evolved species such as zebrafish, generally show higher rates of constitutive neurogenesis and broader areas of adult neural stem cell residence, leading to constant generation of new neurons throughout most brain areas in the adult life. This feature appears to correlate with significantly higher regenerative capacity of zebrafish in comparison to mammals1, as zebrafish have remarkable potential to efficiently generate new neurons in most brain injury models studied until now2-8. More specifically, we are looking at the zebrafish telencephalon, since it is a brain area with prominent neurogenesis and contains regions homologous to subventricular and subgranular zone in mammals as well as a neocortex like region, whereas in rodents neural progenitors are silent9-11.

Ependymoglial cells act as resident adult neural stem cells and are responsible for generation of new neurons both in the intact and in the regenerating brain3,5. Lineage tracing experiments have shown that ventricular ependymoglia react to injury, proliferate and generate new neuroblasts that migrate to the lesion site5. Due to everted nature of zebrafish telencephalon, ependymoglial cells are lining ventricular surface and building the ventral ventricular wall. The dorsal ventricular wall is formed by dorsal ependymal cell layer that has all characteristics of classical ependymal cells in the mammalian brain (see Fig. 1A).

In order to be able to follow *in vivo* behavior of single ependymoglial cells during regeneration, they need to be reliably labeled. Various methods of *in vivo* cell labeling for fluorescent microscopy have been previously described, such as endogenous reporters or lipophilic dyes12. These methods, in contrast to electroporation, might need longer time and often do not offer the possibility of single cell labeling or permanent long-term tracing. Electroporation, however, besides single cell labeling, offers the possibility of introducing new DNA into the host cell. Moreover, compared to other methods of DNA transfer into the cells,electroporation has been demonstrated to be one of the most efficient methods 13-16 .

Here, we are reporting electroporation protocol which was refined for the purpose of labeling single ependymoglial cells in the adult zebrafish telencephalon. With this protocol, we are able to label single ependymoglial cells in order to either follow them long-term17 or manipulate specific pathways in cell autonomous manner18,19.

**PROTOCOL:**

# 1. Preparation of Plasmid Mixture for Electroporation

## Dilute the plasmid of interest in sterile water and add fast green stain stock solution [1mg/mL]. The final concentration of the plasmid should be ̴1 mg/µL. Fast green stain should be added at a concentration of no more than 3%, as its purpose is only to color the solution and visualize ventricular injection.

* 1. Once prepared, mix the plasmid solution by pipetting up and down several times or by finger tapping. Store at room temperature until usage.

NOTE: To simultaneously co-electroporate two plasmids into the same cell, the concentration of each individual plasmid used in the mixture should be at least 0.8 mg/µL with molar ratio 1:1. This should yield 80 - 90% co-electroporation.

# 2. Preparation of Injection Capillaries

## Prepare the glass capillaries necessary for injection in the needle pulling apparatus. In order to inject the correct amount of plasmid (see above), we pull the capillary at a temperature of 68.5°C with two light and two heavy weights (see Table of materials for puller specifications). This ensures the correct pulling force for the necessary length and width of the capillary for injection. In case that the different puller is used the capillary should be calibrated to deliver the appropriate volume of electroporation mix.

#  Zebrafish Anesthesia

* 1. Prior to anesthetization, prepare a stock solution of anesthesia with 0.2% MS222. Dilute this stock 1:10 (i.e. to 0.02% MS222) using fish water. The fish are anesthetized by keeping them in this working solution until the movement of the body and gills subsides (typically for a couple of minutes).

**NOTE: All experiments have to be approved by the local authorities.**

# Preparation of Injection and Electroporation Devices

* 1. Set the Injection device to an injection pressure of 200hPa and a constant pressure of 0hPa. Set injection time to manual mode and control with foot pedal.
	2. Set electroporation device to “LV mode” with 5 pulses at 54-57V, 25ms each with 1sec interval. Connect the electrodes to the device.

# Preparation of Clean Water Tank

* 1. Prepare one fish tank with clean fish water where the fish will be awakened from the anesthesia after the electroporation procedure. Aerate the water by keeping the air stone attached to the air pump for the entire recovery period. The water should remain aerated until the fish is fully awakened.

# Sponge Preparation

* 1. Use regular kitchen sponge and make a longitudinal cut in the sponge to hold fish into during the injection and electroporation procedure (see3).

# Ultrasound Gel

7.1 Place small amount of highly conductive multi-purpose ultrasound gel next to the injection and electroporation setup. Ultrasound gel is essential to facilitate a tight contact between the zebrafish skin and electrodes—this will ensure adequate electrical conductivity, and consequently, distribution of electroporated cells throughout the entire telencephalon.

1. **Plasmid Solution Injection**
	1. Fill the prepared glass capillary with plasmid solution using microloader tips. Avoid the formation of any air bubbles inside of the capillary.
	2. Press “Menu/Change Capillary” on the injection device. Insert and secure the needle into the needle holder.
	3. Under a stereomicroscope with a magnification of 3.2x or 4x, cut only the tip of the capillary using fine-end forceps. Switch electroporation device from “Change Capillary” mode into “Inject” mode, then apply pressure with foot pedal to ensure that the plasmid solution is running easily out of the needle and without hindrance.
	4. Transfer the fish from the husbandry tank to the container (plastic box) with anesthetic solution. Wait a few minutes until the movement of the gills subsides.
	5. Place fish into the pre-wetted sponge with the dorsal side facing up. All following injection steps should done under the stereomicroscope to ensure the accuracy of procedure.
	6. Using a dissecting micro-knife from stainless steel with 40mm cutting edge and 0.5mm thickness, create a small hole in the fish skull at the posterior side of the telencephalon, just next to the border with optic tectum.

NOTE: This step should be performed carefully since the hole should be very small and superficial, penetrating solely the skull, to avoid brain damage.

* 1. Tilt the fish as necessary and orient the tip of the glass capillary towards the skull in the correct angle to facilitate penetration of the capillary tip through the hole.
	2. Insert the tip of the capillary through the hole in the skull carefully until it reaches the telencephalic ventricle. This will require penetration through the dorsal ependymal cell layer.

NOTE: This is a very delicate step. Be especially careful not to insert the capillary too deeply such as to come in contact with the brain tissue. Keep the capillary precisely in between the hemispheres, remaining inside the ventricle just after piercing the dorsal ependymal layer. The accuracy of procedure is improved by using pigmentation mutant lines such as Brassy (REF). allowing better visualization of glass capillary position during injection.

* 1. With the capillary tip inside of the ventricle, inject the plasmid solution by applying pressure with the foot pedal for about 10 s, which corresponds to approximately 1 µl of plasmid solution.

NOTE: If changing the needle puller, capillaries or injector, the system should be calibrated in order to always deliver 1 µl of plasmid solution.

* 1. Confirm success of previous step by observing the spread of liquid throughout the ventricle.
1. **Electroporation**
	1. Remove the fish from the injection setup while still holding it in the sponge.
	2. Immerse the inner side of the tip of the electrodes in the ultrasound gel.
	3. Cover the fish telencephalon with a small amount of ultrasound gel.
	4. Position the fish head between the electrodes, placing the positive electrode at the ventral side of the fish’s head and the negative electrode on the dorsal side, while still holding the fish’s body in the sponge. This sets the direction of the flow of the current necessary to electroporate ependymoglia positioned at subventricular zone.
	5. Press the electrodes gently and precisely against the telencephalon. Administer the current with the foot pedal. Hold the electrodes in place until all 5 pulses are finished.
2. **Fish recovery**
	1. Let the fish recover in the previously prepared, continuously aerated tank until it wakes up. Lidocaine gel could be applied on the skull in order to relieve any possibly developed pain.

**REPRESENTATIVE RESULTS:**

Described electroporation method allows delivery of plasmid DNA into ependymoglial cells, which are located superficially in the zebrafish telencephalon, just under the dorsal ependymal cell layer (**Figure 1A**).

If the result of electroporation is positive, labeled single ependymoglial cells (red cells in **Figure 2A, 2B**) can be observed among other ependymoglial cells (white in **Figure 2A, 2B**). Depending on the efficiency of the electroporation process, a higher (**Figure 2A**) or a lower (**Figure 2B**) number of ependymoglial cells may be labeled. Nevertheless, this protocol yields higher number of labeled cells than previously published(17, which is apparent in **Figure 3A** and **Video 1**. It is worth mentioning that the highest density of labeled cells tends to emerge mostly at the inner, ventricular side of both hemispheres (**Figure 3A**), due to the way in which the injected plasmid liquid distributes in between the hemispheres. In **Video 1**, one hemisphere of the zebrafish telencephalon is presented in 3D and the ependymoglial cells with radial processes can be seen from aside. The cells are co-electroporated and labelled with two plasmids, TdTomatomem and H2B-YFP plasmid (labelling nuclei). Notice the cell division of two nuclei surrounded by yellow circles.

Unsuccessful electroporation results in very low number or no labelled ependymoglial cells. This outcome can be generally explained by inaccurate injection where the tip of the capillary does not penetrate the dorsal ependymal cell layer. In this case plasmid solution spreads above the dorsal ependymal cell layer instead of filling telencephalic ventricle. This leads to ependymal cells solely being labeled (**Figure 3B**). Dorsal ependymal cells (blue arrows in **Figure 3B**) differ morphologically from ependymoglial cells (yellow arrow in **Figure 3A**). Their soma is larger, cuboid, and they do not possess radial, elongated processes. This is evident from comparing a side view of the ependymoglial cell layer (**Figure 4A, 4B**). TdTomatomem labeled cells are most likely ependymal cells, which are located above the layer of ependymoglia (**Figure 4B**). In contrast, in **Figure 4A**, TdTomatomem expressing plasmid is introduced to individual ependymoglial cells. Thus they TdTomatomem in addition to their initial labeling (in this case, transgenic *gfap:GFP* fish line, here seen in white).

**FIGURE AND TABLE LEGENDS:**

**Figure 1: Schematic representation of coronal section of the everted zebrafish telencephalon**

Scheme of a coronal section of zebrafish telencephalon, highlighting the position of ependymoglial cells, which are lining ventricular surface and building the ventral ventricular wall. Dorsal ependymal layer is bridging the two hemispheres and covering the ventricle (V), located in between two cell layers – ependymoglial and ependymal. Black arrow and the representation of the eye indicate view shown in Figures 2 and 3.

**Figure 2: Micrographsshowing effective electroporation outcomes**

(**A, B**) 3D representation of a larger (**A**) or smaller (**B**) number of electroporated ependymoglial cells in the adult zebrafish telencephalon, as viewed from above. The electroporations were done in Tg(*gfap:GFP*) fish line expressing GFP fluorescent protein (depicted in white) in all ependymoglial cells. Individual electroporated cells are labeled with pCS2-TdTomatomem plasmid 3. Scale bars: 50 µm in both A and B.

**Figure 3: Confocal micrographs depicting the difference between successful and unsuccessful electroporation**

(**A**) 3D confocal image of BABB-cleared zebrafish telencephalon (REF) with a large number of pCS-TdTomatomem electroporated ependymoglial cells. Note the morphology of the ependymoglial cells with long, elongated processes (yellow arrows). Both telencephalic hemispheres, highlighted with yellow dashed lines, can be observed.

(**B**) Confocal image of unsuccessful electroporation of pCS2-TdTomatomem plasmid. Mostly dorsal ependymal cells are labelled and only a few ependymoglial cells express TdTomatomem plasmid. Note the clear difference in the morphology of ependymal cells (blue arrows). Scale bars: 50 µm in both A and B.

**Figure 4: 3D lateral views of electroporated and non-electroporated ependymoglial cells**

(A) 3D lateral representation electroporated ependymoglial cells, positive for both Tg(*gfap:GFP*) and pSC2-TdTomatomem (yellow arrow).

(B) 3D lateral representation of unsuccessful electroporation. Note that the location of pCS2-TdTomatomem positive cells is above the Tg(*gfap:GFP*) ependymoglia layer. Most likely dorsal ependymal layer cells were electroporated (blue arrow). Scale bars: 30 µm in both A and B.

**DISCUSSION:**

This electroporation protocol is a reliable *in vivo* method of labeling individual ependymoglial cells. Apart from labeling, reported electroporation protocol can be used as a very fast and straightforward platform of gene editing with the use of Cre recombinase or CRISPR Cas9 techniques 19. However, this protocol has several critical steps. First, during the injection step, one needs to be especially careful that the injected plasmid amount is equal for each individual fish, such that the number of labelled cells remains comparably similar. This can be achieved by controlling the size of the glass capillary opening, meaning that the cut of each tip should be constant among different capillaries or calibration should be performed for each individual capillary. Additionally, the duration of injection, regulated by foot pedal, should be the same for each individual injection. Second, the proper position of a hole in the skull made with the micro-knife is crucial for the proper dispersion of the plasmid liquid throughout the telencephalon. It is equally important to penetrate the dorsal ependymal cell layer with the capillary tip, as stated in the protocol. Moreover, it is also essential that the hole created is not too big, as to prevent the plasmid mixture and the cerebro-spinal fluid from leaking out of the telencephalon. Another critical step is the strength of the applied electrical current. It is important to make sure that the electroporation device is operating as precisely as possible, such that the strength of the applied current does not deviate much from the voltage appearing on the screen, which is not always accurate. If these values are not consistent, it is necessary to adjust the strength of the current on the electroporation device, since an administered current higher than the recommended 54-57V may compromise fish survival.

Compared to the other methods for a plasmid delivery and cell labelling commonly used in the field, electroporation has obvious advantages. In contrast to lipofection for example, during electroporation, cationic liposomes (e.g. lipofectamine) are not used and thus toxicity connected with its usage is completely avoided20. It was previously reported that lipofection and electroporation have equal efficiency rates (from 20 to 50 cells per telencephalon21). However, optimized protocol reported here generally yields from 100 to 200 cells per telencephalon. In comparison with viral vectors, biosafety is not an issue with electroporation. In addition, commonly used AAVs or lentiviruses fail to produce detectable expression of transgenes in zebrafish brain14,22. Finally, although the Cre-lox system is nowadays commonly used in zebrafish, plasmid electroporation is faster since it does not require long waiting times necessary for fish breeding and growing and allows individual cell labeling and tracing.

 Presented electroporation protocol is optimized to be a fast, highly-efficient method of electroporating a large number of ependymoglial cells with all the necessary precautions to obtain optimal results. Electroporation of the adult zebrafish telencephalon is crucial in order to visualize individual ependymoglial cells and study their role in neurogenesis and in the regeneration process. Recently, success has been achieved in the simultaneous disruption of multiple genes of the adult zebrafish telencephalon through gene editing via the electroporation and StagR-Cas9 techniques19. This opens a wide field of possibilities and future applications of electroporation for a broad range of genetic manipulations.

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**DISCLOSURES:**

Authors have nothing to disclose.

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