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Research Article

Transgenic expression in zebrafish embryos with an intact chorion by electroporation and microinjection

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ABSTRACT

Electroporation is regularly used to deliver agents into cells, including transgenic materials, but it is not used for mutating zebrafish embryos due to the lack of suitable systems, information on appropriate operating parameters, and the challenges posed by the protective chorion. Here, a novel method for gene delivery in zebrafish embryos was developed by combining microinjection into the space between the chorion and the embryo followed by electroporation. This method eliminates the need for chorion removal and injecting into the space between the chorion and embryo eliminates the need for finding and identifying key cell locations before performing an injection, making the process much simpler and more automatable. We also developed a microfluidic electroporation system and optimized electric pulse parameters for transgenesis of embryos. The study provided a novel method for gene delivery in zebrafish embryos that can be potentially implemented in a high throughput transgenesis or mutagenesis system.

1. Introduction

In drug discovery, vaccine development, human disease modeling, and gene delivery, a wide variety of animal models are deployed, each having its own set of limitations concerning cost and availability [1]. Among these models, the zebrafish (*Danio rerio*) emerged as an ideal organism to determine genetic and biochemical pathways, understand basic biological mechanisms, and for preclinical drug discovery. For any model system, the ability to modify the genome of the model (transgenesis) is a key requirement and the process can vary depending on cell types and models. In the case of zebrafish, transfection is best achieved at the embryo stage, preferably within the first hour of post-fertilization to ensure consistent gene expression across all cells. Microinjection is the prevailing and widely accepted method of zebrafish transfection. This method provides high gene expression efficiency (> 80 %) and high survival rate (> 70 %) including applications in genetics [2], virology [3], toxicology [4], and immunology [5]. However, manual injection is labor intensive, time-consuming, prone to error, requires high-resolution microscopy and trained technicians, limiting high throughput transgenesis.

Several research groups [6,7–9] have attempted to automate zebrafish embryo injections employing engineering tools that requires precision imaging and control systems to locate cells inside the embryo [10]. Additionally, orientation of the cell prior injection is achieved manually or using an automated system [11], including a high-resolution microscopy, detection control, and three-dimensional rotation capability of the embryo making the system expensive and complicated [12]. Therefore, a method for rapid and large-scale transgenesis of zebrafish embryos would be of great value to the research community if it could provide performance similar to microinjection in less time and at lower cost.

Electroporation is a potential alternative transfection method for zebrafish embryos that can alleviate the issues associated with manual microinjection. In electroporation, applied electric field to cells placed between two electrodes creates tiny yet temporary pores to the cell membrane enabling trans-membrane movement of various compounds like DNA, drugs, or other components [13]. Electroporation has been successfully implemented for: cancer research [14], virus transfection [15], drug and [14], DNA vaccine delivery [16,17]. Moreover, this method has been implemented for successful transgenic treatment of

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mice [18], sea urchins [19], cattle [20], birds [21] and fish [22,23] using different electrical conditions.

Previously, two groups demonstrated zebrafish embryo electroporation [24,25]. In the work by Huang [24], the setup processed multiple embryos simultaneously and claiming success for single-stage embryos but lacked description of metrics or methods for transgenesis with GFP-containing plasmid. Only an image with fluorescence in the embryos' yolk, which is a notoriously auto-fluorescent component, was provided. In [25], electroporation on embryos 3 and 24 h post fertilization (hpf) was performed after dechorionation. However, the set-up could accommodate only one embryo, and the survival rate of dechorionated embryos were poor at such early stage, limiting the efficacy. In [26], they attempted electroporation on 4–8 cell stage embryos and found it impossible with the chorion but were successful after removal with a commercially available, expensive Nepa Electroporator. Some research groups used electroporation on older embryos(> 24 hpf) [27] and even adult fish [22]. However, for non-mosaic expression of a gene, the embryos need to be electroporated at the earliest stage (0–1 hpf), making the process delicate and requiring precision.

The literature shows that the existing commercial electroporation systems for cells are unsuitable for zebrafish embryos. They require manual or chemical removal of chorion for success which is skill-intensive and chemical process, adversely affects the survival of the embryos. Moreover, these systems are not suitable for large-scale or high throughput transgenesis, which is required in the zebrafish workflow. Therefore, the demand for an affordable, high throughput electroporation system without the necessity of removing the chorion of embryos has yet to be fulfilled.

Here, we describe the development of a proof-of-concept electroporation approach for zebrafish embryos at 0 hpf without requiring chorion removal and that is demonstrated using a GFP-expressing plasmid. We tried multiple approaches and built corresponding prototypes to evaluate their efficacy before finding an approach that worked effectively. Thus, in this work we describe a novel concept incorporating a simple microinjection followed by electroporation that could lead to a relatively simple high throughput transgenesis system for zebrafish embryos.

2. Materials and methods

2.1. Ethics statement

Experiments with zebrafish embryos were performed according to the guidelines of the University of Utah Institutional Animal Care and Use Committee (IACUC), regulated under federal law (The Animal Welfare Act and Public Health Services Regulation Act) by the U.S Department of Agriculture and the Office of Laboratory Animal welfare at NIH.

2.2. Zebrafish stock and embryo raising

Adult fish were bred and maintained by standard protocols [28]. Briefly, embryos were grown at 28 °C in E3 media (5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, 0.33 mM $MgSO_4$ and 5−10 % Methylene Blue). After performing the electroporation and microinjection, the embryos were kept at 28 °C in an E3 medium for further observation up to 72 hpf. Every 24 hpf, dead or unfertilized embryos were removed, and fresh medium was provided.

2.3. Dechorionation process

Around twenty zebrafish embryos (0 hpf) were placed in a 1 mL tube with E3 media (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4 and 10−5 % Methylene Blue). A volume of 30 μl of 30 mg/mL pronase (Streptomyces griseus) was placed in the 1 mL tube containing the embryo tube, and the volume was adjusted to 1 mL. The tube was inverted gently to mix the reagents. Then, the tube was incubated at 37 °C for 4 min. Afterward, the embryos were gently pushed up and down ("triturate") with a transfer pipette until the chorions shed off. The embryos were washed three times with E3 media and transferred to a Petri dish.

2.4. Electroporation

The electroporation (EP) platform consists of a few electronic components, an EP circuit made with off-the-shelf components and a biochip. The EP biochip has a chamber for holding media and embryos and contacts with the electrodes for receiving pulses. As an electroporation circuit, two designs were used- one for handling high voltage pulses (50–500 V) and another for handling low voltage pulses (5–25 V). Three different EP biochip designs were tested based on the electrode and chip configuration; (1) a parallel plate set up, (2) a plate-needle electrode set up, and (3) a parallel plate set up with an EP chamber.

2.4.1. Electroporation circuit

A DC source (Keithley DC Power Supplies) provided voltage differentials ranging from 5 to 25 V across platinum electrodes. However, a controller was designed and employed to provide the voltages in pulses. The schematic of the circuit can be seen in Supplementary Fig. 1. The circuit contains an Arduino Uno board and a driver circuit (L298N). The driver circuit includes a MOSFET that acts as a switch, transferring pulses from the DC source to the electrodes during the on phase and ceasing when off. Moreover, negative pulses can also be provided by the circuit. The Arduino Uno controls the on/off phase.

The circuit diagram was changed for applying high voltages (50–500 V), wherein a power MOSFET was incorporated. The circuit diagram for the high voltages can be seen in Supplementary Fig. 2. A Piezo Driver/Power amplifier (Trek 2210) also was used as a power source in combination with a few other standard electronic components. The circuit diagram shows that the EP biochip receives zero voltage when the MOSFET is turned on, and the current goes to the ground. When the MOSFET is turned off, the device receives voltage from the DC source from 0 to 500 V. The on/off phase of the MOSFET was controlled by an Arduino Uno. By controlling the on/off phase, the device can produce various pulses with different durations.

2.4.2. Electroporation biochip setup

2.4.2.1. Parallel plate set up. The initial prototype developed in this work followed the system presented in [24], where two graphite electrodes were used at the top and bottom of the setup instead of platinum electrodes. The holder was 3D printed (PLA), and the chip was fabricated from laser-cut acrylic. The chip was attached with the bottom electrode using double sided tape. Each chip has ten wells and can contain 40 embryos at a time. Around 42 μL of media is required for each well to be filled. The gap between the electrodes was 3 mm, and the wells with the middle region were filled with media. The dimensions of the EP chip can be seen in Supplementary Table 1. Fig. 1(a) presents the cross-sectional schematic of the EP biochip, and Fig. 1(b) and (c) show the actual EP chip and holder, respectively. The EP system was used for the electroporation of zebrafish embryos at 0 hpf with chorion for high voltage application with different pulses and pulse durations. (100–500 V, number of pulses: 2–8, pulse duration: 15–50 ms, pulse gap 1 s) using the circuit presented in Supplementary Fig. 1. The system was tested with both Trypan blue as media and GFP-plasmid at a concentration of 1800 ng/μL.

2.4.2.2. Plate-Needle electrode system. The initial design presented in Section 2.4.2.1 was modified by making the well size smaller to accommodate one embryo and requiring less volume for electroporation. The EP chip well layer is divided into two parts. The well radius in

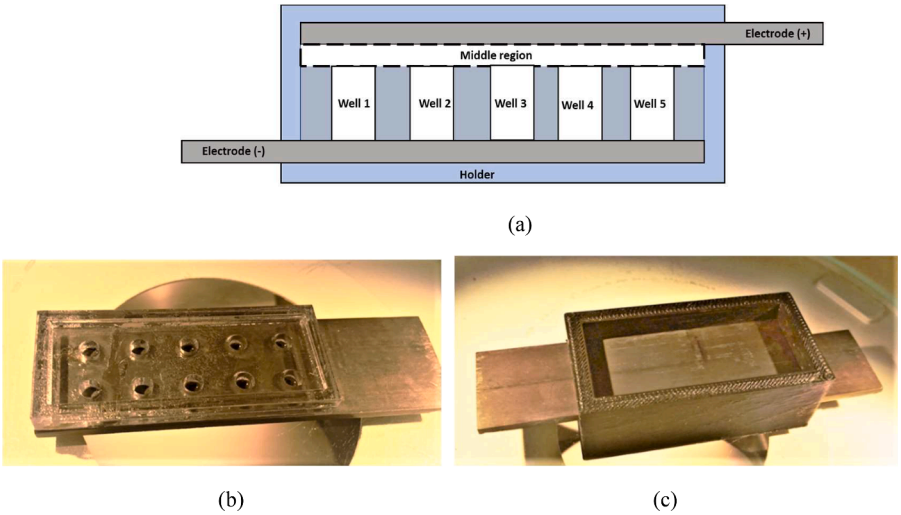


Fig. 1. Electroporation biochip parallel plate set up (a) schematic cross-sectional view (b) the prototype of the chip (c) The prototype of the holder.

the 1st layer is large compared to the 2nd layer to make the loading/unloading of embryos easier. There were 27 wells made in 9 columns, each with three wells. The lower and upper layer was made by laser-cut acrylic and attached using double-sided tape. The schematic of the well layers can be seen in Fig. 2(a), and the dimensions are provided in Supplementary Table 2. The lower or 2nd layer well radius can accommodate only one embryo per well. Here, in our Plate-Needle electrode setup, the top electrode is a needle electrode (30G-steel) which can be extended later for an array of electrodes. The bottom plate electrode made with graphite was attached to the EP chip's bottom using double-sided tape. The set up can be seen in Fig. 2(b). Conceptually, the motivation for using a needle electrode is to implement the lance array method presented in [29], where lance electrodes were used for transferring DNA inside the cell. In this method, the lance arrays (electrode shaped like lance) were exposed to DNA with a positive voltage (1.5 V). The DNAs because of its negative polarity was attracted to the positively charged lance arrays and attached itself to the electrode. After that, the arrays with attached DNA were inserted into the cell, and negative voltage was applied to them, releasing the DNA inside the cell. Here, our target was to insert the DNA into the chorion by using the Lance Array method. For these experiments, the wells were filled with GFP plasmid after placing the embryos inside the chamber. After that a positive voltage was applied to the needle electrodes and inserted to the wells 1st layer to attract the negatively charged DNA. At this point the needle was only touching the media and not the embryos. When the inserted needle reaches down to the 2nd layer of the well, it reaches to the chorion, and a negative voltage was used to release the plasmid inside the chorion. Due to the electric field, if pores are then created in the cell membrane, the negative voltage will drive the DNA/plasmid inside the cell through the pores created by electroporation. A precision micromanipulator was used for managing the movement of the needle electrode precisely to different layers of wells after testing multiple times. The schematic of the concept is presented in Supplementary Fig. 3(a) and (b). The EP circuit was modified to provide positive and negative pulses. The circuit shown in Supplementary Figure 1 was used for providing pulses.

An optical system is usually required to perform micro-needle insertion in most cell types. Such high-resolution optical systems can be costly. To avoid the high-cost optical system, an alternative process was utilized. This system was comprised of a micromanipulator where the positions of the wells on the X and Y-axes, as well as the needle travel distance to reach the chorion (Z-axis) were achieved with $a \pm 5$ μm of accuracy. The measured values were used as a standard value for needle insertion without a microscope. The lower layer well radius ensured that the embryos filled the well, and the needle was always positioned in the middle of each well for insertion. Multiple measurements were taken to determine the exact position of the needle to insert into the chorion of the embryos. The system and the well with an embryo can be seen in Figs. 2(b) and Supplementary Fig. 4, respectively.

This needle-based system was tested with Trypan blue dye and GFP-plasmid. In testing with Trypan blue, the parameters used were 10 V, 100 ms, six pulses (three positive pulses, three negative pulses), and a pulse gap of 1 s. Afterward, the embryos were removed from the wells with a transfer pipette and washed three times. After that the embryos were dechorionated using pronase (0.45 mg/mL) to check the condition of the cell. The age of the embryos was 0–2 hpf.

For testing with the plasmid, 0–2 hpf embryos were loaded in the chip wells, and the wells were filled with GFP plasmid (mnx:eGFP) of concentration 200 ng/ μL. As the well size was reduced compared to the

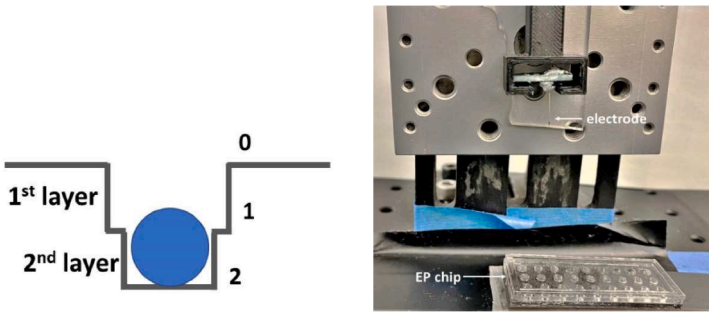


Fig. 2. The plate-needle EP system (a) schematic of the EP chip wells with two layers (b) Photograph of the plate needle set up with needle electrode and EP chip.

chip presented in Section 2.4.2.1, each well requires only 8 μL of plasmid volume. For 27 embryos, a total of 216 μL volume of media is required to test with each chip. The application voltage was 10 V, ten pulses (five positive and five negative), 1 s pulse gap with a pulse duration of 50 ms −200 ms

2.4.2.3. Parallel plate set up with EP chamber. The third and final EP system contains an electroporation chip made starting from a glass Petri dish. PDMS (Sylgard 184) was deposited and cured on the Petri dish. A 10 x 3 x 10 mm chamber was created by removing the cured PDMS using a sharp razor. Two platinum electrodes were inserted on the two opposite sides of the chambers. The gap between the two electrodes was 3 mm. The chamber can hold around 30 embryos with 200 μL of media volume. The loading and unloading of zebrafish embryos in the chamber can be easily done with a transfer pipette. The dimensions of the platinum electrodes are 10 x 10 x 0.1 mm (Newvision1981). The electrodes have a gold-plated copper handle that was coated with PTFE (polytetrafluoroethylene). A part of the gold-plated copper handle was exposed to act as a terminal for connection. The setup of the electroporation chip and a schematic of the system can be seen in Fig. 3. Fig. 3 (a) shows a schematic of the EP chamber with electrodes and Fig. 3(b) and (c) shows the physical set up and the platinum electrode respectively. For this setup, 5–25 V four positive pulses with six alternating positive and negative pulses were applied. The pulse gap was maintained at 50 ms and pulse width was ranged from 25–50 ms. The first four positive pulses were applied to create pores in the cell membrane and the trailing positive and negative pulses were applied for electrophoretic movement of plasmid into the cells. The applied pulse train is shown in Fig. 3(d) and the oscilloscope image can be seen in Supplementary Fig. 5. The circuit used for pulse application is presented in Supplementary Fig. 1.

The set up has been tested for GFP plasmid with chorionated and dechorionated embryos. The concentration of the plasmid was 200 ng/μL. The embryos were dechorionated by pronase treatment, loaded to the chamber of the EP chip and then pulses were applied to demonstrate the proof of concept of electroporation using this approach. The plasmid prepared for this experiment with dechorionated embryos was mnx:eGFP, and expression of this gene provides green fluorescence in motor neurons of zebrafish embryos starting from 24 hpf and continuing through 72 hpf. The dechorionated 2and 24 hpf embryos were placed inside the chamber with plasmid and PBS as media. After pulse application the embryos are carefully removed from the chamber and placed in a Agarose-coated Petri dish for further monitoring.

For testing with chorionated embryos, plasmid was injected inside the chorion before application of the voltage pulses. A beta-actin GFP plasmid was made with various concentrations (400–1200 ng/μL, and multiple volumes (30–150 nl) were injected into the chorion of the embryos. Expression of this gene provides green fluorescence in every tissue. 1 mm glass pipettes were used as a needle, and one side of the pulled needle tip was cut to around 50 μm. The plasmids were loaded into the needle and injected into the chorion one embryo at a time. The injection into the chorion leaves a hole in the chorion that does not repair. Five embryos were placed inside the EP chamber after injection and electroporated at one time. The chamber was filled with PBS media. The survival of the embryos was calculated at 24 hpf, and the expression percentage was calculated based on the number of surviving embryos.

2.5. Microinjection

Microinjection was performed on zebrafish embryos of 0–1 hpf using a stereo microscope, a microinjector from Harvard Apparatus (EC1–65–0001 PLI-100) and a needle holder or a 3-axis micromanipulator. A needle puller (Model P-97, Sutter Instrument CO.) was used to pull a 1 mm outer diameter glass pipette to make the needle. The tip of the needle was cut with forceps according to the diameter of the location to be injected. With the microinjector, injection time and pressure to the needle can be controlled, which controls the volume injected. The injection was performed at the cell and the chorionic space of the zebrafish embryos. The pipette tip size and volume to be injected were decided based on the location. A stereo microscope was used to visualize cutting the tip according to the volume and during injection [30].

2.6. Preparation of plasmid

A Qiagen maxiprep kit was used for the plasmid, which was prepared according to the manufacturer's directions. We used pDEST Tol2 beta-actin:eGFP polyA [31] and mnx:GFP [32] for injection.

2.7. Transgene analysis and imaging

We used a fluorescent microscope with a light source to observe whether the GFP plasmid had been expressed or not. For taking fluorescent images at 24 hpf, a Zeiss AxioImager M2 was used.

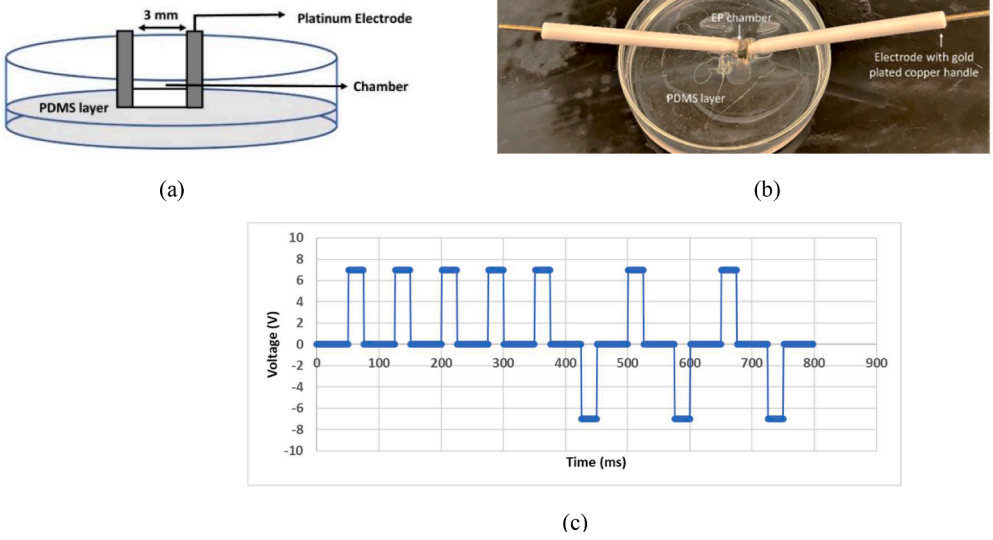


Fig. 3. The final EP chip design. (a) The schematic of the EP chamber with electrodes; (b) photograph of the physical setup; (c) a platinum electrode; (d) the applied pulse train for a 7 V excitation.

3. Results and discussion

The prototype setups presented in Section 2.4.2 were tested with Trypan blue dye and GFP plasmid. Testing with Trypan blue provided some visual information on the limitations of the system and method, which helped identify needed changes to the system's design and parameters, leading to eventual success in testing with plasmid.

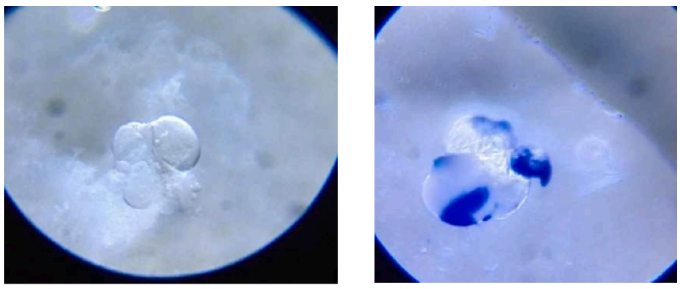
3.1. Testing with Trypan blue dye

3.1.1. Testing the parallel plate setup

The system presented in Section 2.4.2.1 was tested with Trypan blue dye and zebrafish embryos (0 hpf) without removing the chorion in an attempt to replicate the results presented by Huang et al. [24]. After the application of voltage to the electrodes (100–500 V, number of pulses: 2–8, pulse duration: 15–50 ms, pulse gap 1 s), it was observed that Trypan blue dye enters into the chorion and fills the perivitelline space (the space between the cell membrane and chorion membrane). Comparing with the negative control (no voltage), Fig. 4 shows that the intensity of Trypan blue inside the chorion is higher after voltage application, indicating more dye crosses the chorion. Fig. 4(a) shows the negative control embryos where no voltage was applied, and the embryos that were not exposed to Trypan blue. Fig. 4(b) shows the negative control embryos exposed to the Trypan blue only but not to the voltage. Fig. 4(c) shows the effect of 100 V application with two pulses of 15 ms and 1 s gap in the presence of Trypan blue using the setup presented in Section 2.4.2.1. The difference between the intensity of Trypan blue dye inside the chorion due to voltage and the intensity with no voltage application is apparent from the images. However, the testing material must enter the cell for successful transfection. It is not possible from the images presented in Fig. 4 to determine if the dye has entered the cell or not without removing the chorion. Moreover, the application of high voltage severely affected the embryos' survival (<60%). Therefore, testing with Trypan blue in this setup did not fully demonstrate successful electroporation, and the setup needed to be tested with GFP plasmid to determine if the electroporation process actually delivers molecules to the cells, and not just to the perivitelline space.

3.1.2. Testing with plate-needle electrode system

The system presented in Section 2.4.2.2 was tested with Trypan blue first. After loading the embryos in the chip, the wells were filled with Trypan blue dye. Initially, the voltage was applied for a range of 10–100 V with five pulses of 50 ms duration and a 50 ms pulse gap to check the survival of the embryos. As the needle moved closer to the embryo, the distance between the two electrodes (needle and plate) became small, and the electric field intensity became higher. The survival data (Supplementary Table 3) for various voltages (10–100 V) shows that above 20 V, viability was reduced to less than 50 %. Based on this data, the voltage range was kept between 5–25 V for further testing. Fig. 5 shows the results of testing with Trypan blue dye. As shown in Fig. 5, when voltage was applied, Trypan blue stained the yolk, but it could not be determined whether Trypan blue was inserted into the cell or not. Here,



(a) (b)

Fig. 5. (a) Control dechorionated embryo- No electric voltage, only exposed to Trypan blue (b) 10 V application of 5 pulses with 50 ms pulse duration and 50 ms pulse gap.

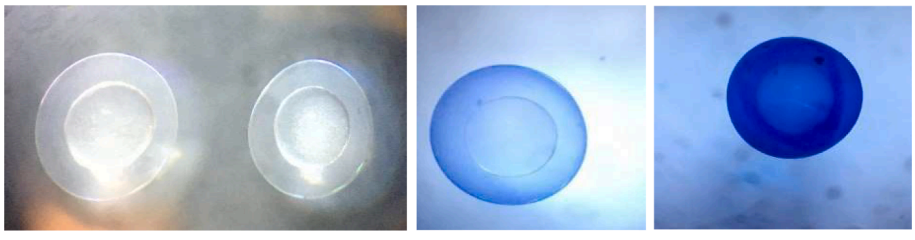
Fig. 5(a) shows a dechorionated control embryo that was only exposed to Trypan blue dye but not to any electric field. Fig. 5(b) shows the dechorionated embryo after exposure to the electric field and Trypan blue dye. Similar results were observed for five repetitions on two consecutive days. Furthermore, survival was 0 % following the pronase and dechorionation step, showing a real need to avoid this process.

3.2. Testing with plasmid

Testing with Trypan blue dye did not provide a definitive answer regarding the success of electroporation. Testing with the two systems presented in Sections 2.4.2.1 and 2.4.2.2 did not confirm the dye entry inside the cell. Therefore, a method that could confirm delivery into the cell was needed to evaluate the success of electroporation and so we turned to plasmids.

3.2.1. Testing with initial parallel plate setup

Testing with a plasmid (mnx:eGFP) using the parallel plate setup presented in Fig. 1 showed no gene expression. For testing with the plasmid, voltages from 50 to 100 V were applied with two pulses of 15 ms pulse duration and 1 s pulse gap. Ten embryos of 0–2 hpf were loaded in the wells, filling the chip (the wells and middle region) with a plasmid (mnx:eGFP). However, no gene expression was found in the embryos. The plasmid test was performed only once as the well, and the middle region required a high volume of plasmid (around 42 μL for each well and 1 mL for the middle region), which is not practical compared to the microinjection process in terms of plasmid volume. Additionally, the application of high voltage directly affects the survival of the embryos. The chip design needed to be improved, considering the required plasmid volume. Testing with this set up provided us insight on the practical design of the chip in terms of reducing the plasmid volume requirement. Furthermore, the results indicated that the Trypan blue experiments were most likely only able to deliver the dye to the perivitelline space and not all the way into the cells of the embryo, as dye was clearly present around the cells, but in these experiments, no genes



(a) (b) (c)

Fig. 4. (a) Negative control embryos- no voltage or Trypan blue dye(b) Negative control with exposure to Trypan blue but no voltage (c) Embryo after application of 100 V with two 15 ms pulses, 1 s pulse gap in the presence of Trypan blue.

from the plasmid were expressed.

3.2.2. Testing with plate-needle electrode system

Due to the absence of polarity in dye particles, the Lance-Array method could not be realized using Trypan blue testing with the Plate-Needle setup. For testing with plasmid, application of 10 V with alternate positive negative pulses did not provide any gene expression. Moreover, the same parameters used in [29] were also tested but no gene expression was found. Supplementary Table 4 shows the various parameters used for the testing. At this point, there was no proof of electroporation or pore formation due to the application of an electric field. Testing with dechorionated embryos with GFP-plasmid would likely have provided evidence of electroporation [26]. However, The EP chip was unsuitable for testing with dechorionated embryos as the wells are small and the embryos become easily damaged during the loading-unloading stage. Moreover, the conductivity of the steel needle is relatively low, which could contribute to the low gene delivery and no gene expression. In addition, this set up did not provide a significant improvement in terms of the plasmid volume required. A new approach was needed that would be easier for loading and unloading the embryos and suitable for testing with dechorionated embryos and used less plasmid volume in addition to reusing the plasmid.

3.2.3. Testing with parallel plate set up with EP chamber

As there had been no evidence of gene incorporation based on EP so far, we moved to testing with dechorionated embryos with plasmid to determine if the electroporation was working at all. Therefore, a new setup was developed where the loading and unloading of dechorionated embryos was simple to perform without adverse effects on the survival of these delicate embryos. The parallel plate setup with the EP chamber presented in Section 2.4.2.3 proved to be a solution to the loading and unloading issue and could accommodate 30 embryos simultaneously. Moreover, the chamber was wide enough to recollect part of the plasmid using a transfer pipette after testing for reuse.

3.2.3.1. Testing with dechorionated embryos using GFP-plasmid. To test whether dechorionated embryos could be electroporated, voltages of 5, 10, 15 and 20 V were applied with four positive pulses followed by six positive and negative pulses of duration 50 ms and a pulse gap of 50 ms. For 5 V, the gene did not express at all, and only 16.67 % of embryos survived. However, after applying 10 V, two fish expressed the gene in at least a few cells, as shown in Fig. 6(a). However, only 10 % of the fish survived. For 15 and 20 V, none of the embryos survived. This result shows that electroporation can transfect the zebrafish embryos when the chorion is removed. However, removing the chorion by forceps or chemical processes directly reduces the survival of the embryos.

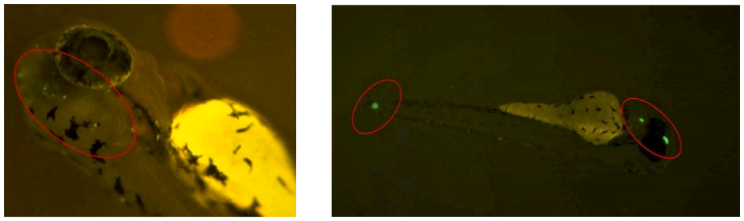
We also tested electroporation with dechorionated 24 hpf embryos as dechorionation does less harm at this age. Beta-actin: GFP plasmid was used for this test, which expresses in every tissue and is easy to characterize. However, the gene was expressed only in 1–2 cells (shown in Fig. 6b), which is not sufficient for most zebrafish researchers. Therefore, a process still needed to be developed by which zebrafish embryos could be consistently genetically modified through electroporation at the age of 0 hpf without removing the protective chorion layer, so as to improve survival while enabling significant genetic modification.

3.2.3.2. Testing with embryos using microinjection and electroporation. The results presented in previous sections (testing with dechorionated embryos shown in Section 3.2.3.1 and testing with chorionated embryos presented in Section 3.2.1) showed that the plasmid DNA cannot reach and/or cross the cell membrane and cannot be expressed when the chorion is present during electroporation. This could be due to the large plasmid size (10 kbps) being unable to penetrate the small holes in the chorion membrane [33]. Or the distance that needs to be traveled for the plasmid to reach the cell membrane is sufficiently long such that the provided electric field cannot create enough electrophoretic motion to move the plasmid across the space in time. If the first reasoning is correct, then the chorion pores could be a size below the plasmid size, restricting the movement of the plasmid. Moreover, we have seen in Section 3.2.1 those high voltages from 100–500 V were applied which creates an electric field of greater than 500 V/cm and still we did not get expression. Additionally, increasing the pulse duration and voltage was not feasible as it negatively affects the health of the embryos. However, testing with dechorionated embryos with much lower electric field provided some expression. This indicated that the electric field is sufficient for the plasmid to reach the cell for expression if the chorion is not present. To test the first potential explanation, plasmids were injected into the perivitelline space, and pulses were applied after placing the embryos in the EP chamber. This approach could validate the concept of the small pore size of chorion limiting the entry of plasmid as the primary reason for unsuccessful electroporation for embryos.

Fig. 7 shows the steps of the experiment where Fig. 7(a) shows the injection of the plasmid to chorion by a glass needle, and Fig. 7(b) illustrates the electroporation after injection.

Four controls were considered for the experiments. Embryos in control group 1 had no voltage applied, and no plasmid was injected into them. This was used to calculate the zebrafish clutch survival. In control group 2 embryos, the plasmid was injected (1 nL) inside the cell to demonstrate expected plasmid gene expression and it provided expression in > 80 % ($n = 50$) embryos indicating the plasmid is in good condition and served as a positive control. In control group 3, the plasmid was injected into the chorion ($n = 50$), but no voltage was applied. This was done to identify if gene expression could be obtained without voltage application. However, none of the embryos in control group 3 showed gene expression demonstrating that without the application of voltage, the plasmid does not express. For control 4, the embryos were exposed to the voltages but no plasmid to check whether they survived the voltage application or not. The voltages for which > 50 % survived were considered for further experiments. Fig. 7c) shows the beta-action; GFP expression of a embryo after 6 day post fertilization for voltage application of 7 V and injection of 1200 ng/ μL, 30 nL plasmid inside the chorion. Fig. 8 shows the gene expression and survival percentages of embryos for various voltage application after injection of 1200 ng/ μL, 30 nL plasmid inside the chorion.

Initially, a high concentration plasmid was prepared (1200 ng/μL), and a 30 nL volume was injected into the chorion of the embryos. After



(a) (b)

Fig. 6. (a) Expression of mnx: GFP at 72 hpf (b) Expression of beta-actin: GFP at 72 hpf.

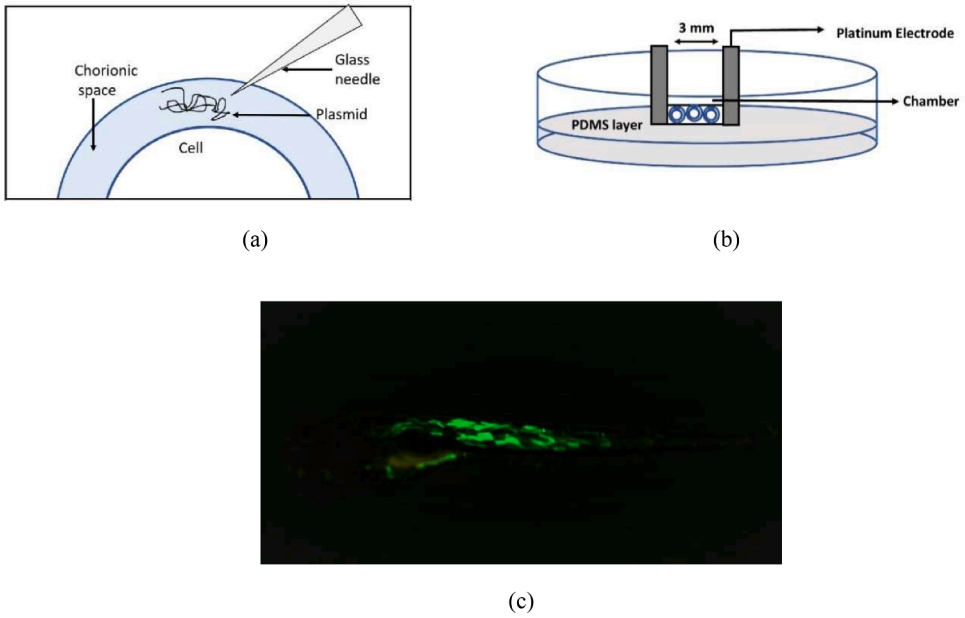


Fig. 7. Microinjection and Electroporation (a) Injection to chorion (b) Electroporation after injection (c) Expression of beta-actin: GFP at 6 dpf.

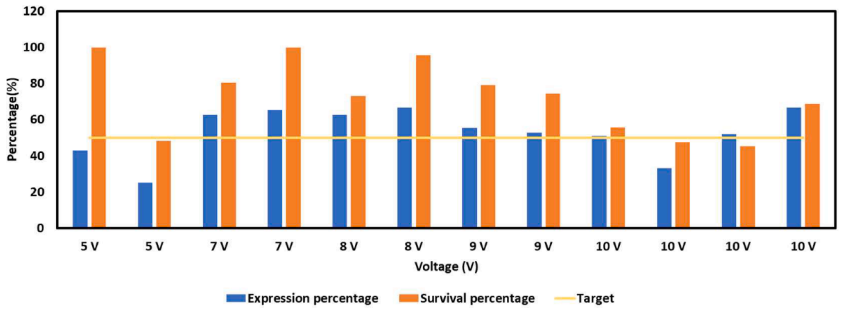


Fig. 8. Expression and Survival percentage of embryos for different voltage application after injection of 1200 ng/ μL, 30 nL plasmid inside chorion.

injection, the embryos were electroporated with 5–10 V, 25 ms pulse duration, four positive pulses followed by alternate six positive and negative pulses, and the pulse gap was 50 ms. For all voltages applied, we achieved gene expression. This outcome indicates that the chorion was inhibiting plasmid entry and the subsequent gene expression and electroporation success was achieved when the plasmid was injected inside the chorion. The results are presented in Fig. 8. The best performance in terms of gene expression was observed for 7 and 8 V, with more than 60 % expression. For 7 V, 25 out of 40 embryos survived and for 8 V, 16 out of 24 surviving embryos provided gene expression. The data for different voltages are presented in Supplementary Table 5. The voltages higher than 10 V severely affected survival. For 15 V, only 2 out of 18 embryos survived. Higher pulse duration (50 ms) and multiple pulse groups were also tested. For example, 10 V, 50 ms duration, 50 ms gap, and 10 pulses are one pulse group. The gap between the two pulse groups were 30 s. This experiment is to check whether multiple pulse groups increase the expression by allowing plasmid to first pass the chorion and then be present to be moved into the cells. However, that approach affected the survival of the embryos severely. For 50 ms pulse duration, 10 out of 20 embryos survived the 10 V application. Moreover, for the three-pulse group with 10 V, the survival rate reduced to 10 % with only 2 embryos surviving out of 20. The respective data can be seen in Supplementary Table 6.

To determine whether the concentration and volume of plasmid injected into the chorion affect the expression percentage, various concentrations and volumes were tested. The concentration was varied from 400–1200 ng/μL. The volumes varied from 30–150 nL. 400 ng/μL concentration with 30 nL injected did not show any expression after applying voltage, but 400 ng/μL with 150 and 90 nL provided expression. However, a high volume of plasmid (> 90 nL) clogged the needle often during the injection. Therefore, we needed to inject a lower volume of plasmid for this experiment. Previously, injecting 1200 ng/μL in 30 nL provided expression without the issue of needle clogging. Therefore, to avoid needle clogging during injection, the volume injected should be kept under 30 nL.

However, 1200 ng/μL for 30 nL volume for each embryo is a significant amount of plasmid compared to the microinjection to zebrafish cell where only 1 nl of 200 pg/μL is used. To achieve gene expression with the least amount of plasmid possible, different concentrations (400–1200 ng/μL) with a volume of 30 nL were tested for voltage 7 V. The data is presented in Fig. 9 and Supplementary Table 7. 600 ng/μL provided the best performance in terms of expression (66.67%) for n = 30 embryos. 400 ng/μL also provided expression (17 %, n = 46), but only ~1–2 cells expressed the transgene. A different volume of plasmid (5–15 nL) was also tested for 600 ng/μL, and the data is presented in Fig. 10 and Supplementary Table 8. 10 nL (8.3 %, n = 24) and 15 nL (31.57 %, n = 19) provided very low expression compared to 30 nL volume. So, the lowest concentration that can provide good performance in terms of expression is 600 ng/μL. Therefore, 600 ng/μL with 30 nL volume injection with 7 V, 25 ms pulse durations, four positive pulses, and six alternate positive, negative pulses provided the best result for our system with sufficiently high survival and expression percentages.

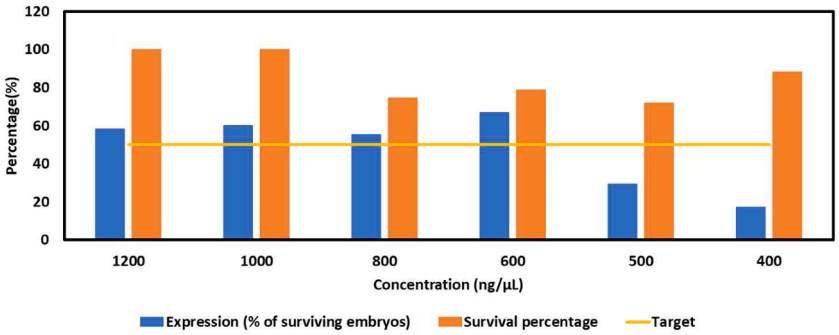


Fig. 9. Expression and Survival percentage of embryos for different concentration of plasmid injected inside chorion before voltage application. The voltage was 7 V, and the volume injected was 30 nL to the chorion.

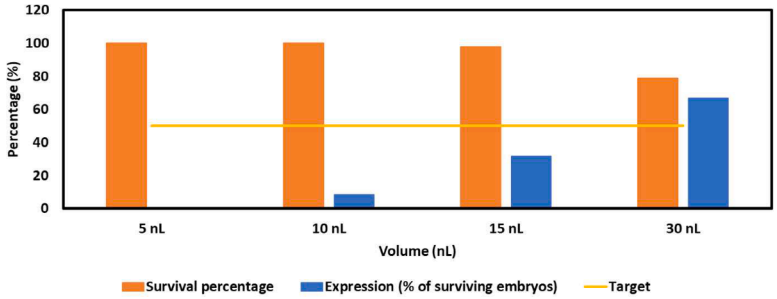


Fig. 10. Expression and survival percentage of embryos for different volume of plasmid injected inside chorion before voltage application.i. The voltage was 7 V, and the plasmid concentration was 600 ng/μL.

The best transfection efficiency with the current set up was found for injection of 30 nL, 600 ng/μL plasmid injection to chorion of each embryo with application of 7 V. The voltages depend on the distance between electrodes and might need to be modified for other geometries depending on the chip design. The plasmid volume required for the current set up is very high compared to the conventional microinjection which might not be cost effective, but the plasmid solution could be used repeatedly for many embryos. However, upon calculation it has been found that, considering $30 for a plasmid kit for making 160 μL of 1875 ng/μL concentration and labor cost of $15/ h, 1000 embryos can be transfected for <$6 with an added advantage to less execution time for the current set up. Considering the cost of the system, cost of the plasmid, transfection efficiency and survival percentage of the embryos, this method with an automated platform could replace the conventional microinjection process. In the future, this work could potentially be extended for CRISPR/Cas9 testing by changing the electrical parameters.

4. Conclusion

This work introduces a novel method of transgenesis of zebrafish embryos. In this method, genetic material is injected into the chorionic fluid space before the application of electrical pulses for electroporation. The experimental results presented in this paper show that the chorion inhibits electroporation of zebrafish embryos at 0 hpf by physically blocking entry of the plasmid into the cell. Moreover, removing the chorion significantly affects the survival rate of the embryos at this early stage, which is not desirable to most researchers. The novel method of injecting genetic material inside the chorion before electroporation presented in this work solves the issue with chorion removal and gives a standard transfection success of > 60 %. The microinjection process used in this paper did not require a high-resolution microscope as the injections were performed on chorionated embryos and only needed to penetrate the chorion (and not the cells). Chorionated embryos are visible to the naked eye, so a microscope was not needed, especially since the injections only needed to penetrate into the space between the chorion and the embryos and were not dependent on embryo orientation in the system. Moreover, these reduced requirements are advantageous for making a high-throughput transgenesis system without the requirement of costly microscopy or imaging systems. In this direction, we have developed an electroporation chip and an electroporation circuit with off-the-shelf components that can deliver the required energy for electroporation through pulses. We have also optimized the electroporation parameters, such as the amplitude of the voltage, pulse durations, and pulse number using a custom-made electroporator to achieve optimized results in terms of expression efficiency and survival of the embryos. We have also optimized the plasmid concentration and volume to be injected inside the chorion.

This presented method reduces the zebrafish embryos' survival concern by leaving the chorion intact. The results show significant improvement in embryo survival using this method compared to the electroporation of dechorionated embryos. To replace microinjection with electroporation as a transfection method for zebrafish embryos the success rate should be similar or better than the former. Although, the electroporation method presented could not reach the 80 % transfection rate of microinjection, it can significantly improve execution time and overall throughput. In the demonstrated method with the current set up, injecting into the chorion and providing pulses for 20 embryos takes only 5 min including the loading and unloading to the chambers. In one hour, around 200 embryos can be transfected which provides at least 5 times reduction in terms of experimental and operator time compared to conventional microinjection. This improvement can be further extended by automating the injection process, while the electroporation takes almost no time. By combining the concept of injection into the chorion and electroporation, a high throughput automated system could be developed which could transfect thousands of embryos in minutes rather than hours. Moreover, the system will be less expensive if the position of the embryo holders can be mechanically determined by repeated testing, eliminating the requirement for an imaging system. The presented electroporation system has been developed using off-the-shelf

components capable of electroporating zebrafish embryos. It was found that 7 V with a 50 ms pulse gap, 25 ms pulse duration, and four positive pulses followed by alternate six positive negative pulses provide 66.67 % expression efficiency with a plasmid volume and concentration of 30 nL and 600 ng/μL respectively. In the future, this work could be extended for CRISPR/Cas9 testing by changing the electrical parameters. The method and the system can be used directly and could be applied to an automated high-throughput rapid transgenesis system for zebrafish embryos.

CRediT authorship contribution statement

Nusrat Tazin: Conceptualization, Methodology, Validation, Investigation, Writing – original draft, Writing – review & editing. **Christopher Jordon Lambert:** Conceptualization, Methodology, Writing – review & editing. **Raheel Samuel:** Conceptualization. **Tamara J. Stevenson:** Conceptualization, Methodology, Validation, Investigation, Writing – review & editing. **Joshua L. Bonkowsky:** Conceptualization, Resources, Supervision, Writing – review & editing. **Bruce K. Gale:** Conceptualization, Resources, Software, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Bruce K. Gale reports financial support was provided by National Institutes of Health.

Data availability

The datas are shared in Supplementary material document and further can be provided upon request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.btre.2023.e00814.

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