Electro-microinjection of fish eggs with an immobile capillary electrode

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Microinjection with ultra-fine glass capillaries is widely used to introduce cryoprotective agents and other foreign molecules into animal cells, oocytes, and embryos. The fragility of glass capillaries makes difficult the microinjection of fish eggs and embryos, which are usually protected by a hard outer shell, called the chorion. In this study, we introduce a new electromechanical approach, based on the electropiercing of fish eggs with a stationary needle electrode. The electropiercing setup consists of two asymmetric electrodes, including a μm -scaled nickel needle placed opposite to a mm-scaled planar counter-electrode. A fish egg is immersed in low-conductivity solution and positioned between the electrodes. Upon application of a short electric pulse of sufficient field strength, the chorion is electroporated and the egg is attracted to the needle electrode by positive dielectrophoresis. As a result, the hard chorion and the subjacent yolk membrane are impaled by the sharp electrode tip, thus providing direct access to the egg yolk plasma. Our experiments on early-stage medaka fish embryos showed the applicability of electro-microinjection to fish eggs measuring about 1 mm in diameter. We optimized the electropiercing of medaka eggs with respect to the field strength, pulse duration, and conductivity of bathing medium. We microscopically examined the injection of dye solution into egg yolk and the impact of electropiercing on embryos' viability and development. We also analyzed the mechanisms of electropiercing in comparison with the conventional mechanical microinjection. The new electropiercing method has a high potential for automation, e.g., via integration into microfluidic devices, which would allow a large-scale microinjection of fish eggs for a variety of applications in basic research and aquaculture. © 2015 AIP Publishing LLC.

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I. INTRODUCTION

Microinjection is a commonly used method for introducing exogenous material into oocytes and embryos of various species including teleost fishes.^{1–4} The technique has found many applications in basic research and fish aquaculture industry, including, most notably, production of transgenic fish and *in vitro* fertilization.^{5,6} In the last years, microinjection is increasingly being used in cryopreservation technology for loading fish eggs and embryos with cryoprotective agents (CPAs).^{7,8}

Eggs of teleost fishes have a complex multilayered structure with two distinct barriers with very different physical properties. Composed of cross-linked polypeptides, the hard outer egg-shell, called chorion, provides mechanical protection to the embryo during development. The second barrier is the vitelline membrane (lipid bilayer) that surrounds the yolk.⁹ Although some

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small CPAs, e.g., methanol, dimethyl sulfoxide (DMSO), etc., can slowly diffuse through the porous chorion, the even lower permeability of the vitelline membrane results in an insufficient loading of the large yolk and other egg's compartments with CPAs.^{10–13}

Recently, DMSO permeation into Japanese whiting (*Sillago japonica*) embryos was appreciably enhanced by electroporation with high-intensity short-duration electric field pulses, using large extracellular electrodes.¹⁴ However, the intracellular DMSO concentration (≤ 120 mM) attained with electroporation alone was not sufficient for successful cryopreservation of whiting embryos. Moreover, the use of excessive field strengths and pulse durations affected embryo viability,¹⁴ most likely, due to the irreversible permeabilization of the vitelline membrane via the electric breakdown mechanism.

In fact, both barriers, the chorion and the vitelline membrane, can be circumvented only by the direct injection of a CPA into the yolk.¹⁵ The very hard chorion, however, makes the application of conventional microinjection with an ultra-fine glass capillary needle difficult. Commonly, the injection needle is manually inserted into an immobilized egg under microscopic control using a precision micromanipulator. Manual microinjection is a laborious, time-consuming operation that relies largely on the operator's skills and experience. Because of its relatively low throughput, microinjection is rarely applied to large cell samples. The hard chorion also raises a problem of the fragility of thin glass capillaries, since broken tips may cause massive damage to embryonic cells and yolk membrane.

To simultaneously overcome the drawbacks of manual operation and glass fragility, we introduce here an electromechanical approach, based on the dielectrophoretic attraction and piercing of fish eggs with a stationary metallic needle microelectrode. In brief, the principle of electropiercing is as follows (Fig. 1). The space between the microelectrode and a large counter electrode is filled with a low-conductivity solution. A fish egg is first located at the tip of the microelectrode. Then, a short electric field pulse is applied, whose field strength is high enough to induce electroporation of the chorion. Due to the asymmetric electrode configuration and a large difference in the conductivities of the yolk plasma and external solution, the strongly inhomogeneous electric field exerts a transient positive dielectrophoretic force on the egg directed to the microelectrode.

Approximating the fish egg as a transient electric dipole $\mu(t)$ induced by a time-varying (e.g., pulsed) electric field $E(t)$, the expression for the time-dependent dielectrophoretic force $F_{DEP}(t)$ acting on the egg can be written as¹⁶

$$F_{DEP}(t) = (\mu(t) \cdot \nabla)E(t), \quad (1)$$

where ∇ is the nabla operator. The induced dipole moment of a spherical particle is defined as

$$\mu(t) = 4\pi\epsilon_e R_1^3 U(t)E(t), \quad (2)$$

where R_1 is the egg radius, $\epsilon_e \approx 80 \epsilon_0$ is the external permittivity, and $U(t)$ is the Clausius-Mossotti-factor which represents the effective polarizability of the egg relative to suspending medium. An expression for U of medaka fish eggs has been derived previously on the basis of a three-shell dielectric model.¹⁷ Driven by the positive dielectrophoresis (DEP), the fish egg is attracted to the microelectrode. While hitting the sharp electrode tip, both the chorion and the vitelline membrane are penetrated, thereby providing direct access to the yolk plasma. The localized electroporation of the chorion caused by the very high electric field at the electrode tip also seems to play a key role in electropiercing. After egg impalement, the microelectrode serves as the injection needle. Therefore, our technique can be viewed as a combination of microinjection and electroporation process with asymmetric electrodes.

Introduction to cell manipulation by DEP and related AC electrokinetic techniques is given in detail elsewhere.^{18–25} The effects of high-intensity electric pulses (e.g., electroporation of cells, dielectric breakdown of aqueous solutions, etc.) have been widely investigated in the last two decades.^{26–28} In previous studies, tiny sharp microelectrodes mounted on a micromanipulator and driven with a high-frequency electric field in the MHz range have been used as

FIG. 1. Principle of electropiercing. A fish egg immersed in a low-conductivity medium is located between the tip of a needle electrode (1) and a large planar counter-electrode (2). A high-voltage DC pulse applied to the asymmetric electrodes generates a strongly inhomogeneous electric field (dashed lines in (a)). The field induces a transient dipole μ within the egg, mainly due to the very fast polarization (ns-range) of the highly conductive yolk compartment. The interaction between field and dipole gives rise to a DEP force (F_{DEP}) directed towards the needle electrode. Simultaneously, the very high local electric field of strength around 0.5 MV/cm at the electrode tip induces electroporation (electric breakdown) of the chorion in the contact area. As a result, the chorion and the yolk membrane are impaled by the needle electrode, thus providing direct access to the egg yolk plasma.

dielectrophoretic tweezers for trapping, holding, and precise positioning of nano- and micrometer-sized particles, including yeast cells with a diameter of $\sim 5 \mu\text{m}$.^{29–31} Recently, the dielectrophoretically accessed, intracellular membrane-potential measurement method has been developed for electrophysiological studies of human cells with a diameter of $\sim 15 \mu\text{m}$.³² DEP has also been successfully used for sorting bovine oocytes ($\sim 100 \mu\text{m}$ in diameter) according to their developmental competence.³³

In this communication, we show a proof of principle for the applicability of the DEP-based electro-microinjection technique to extra-large biological particles, such as fertilized eggs and early stage embryos of Japanese medaka fish, measuring 1.2 mm in diameter. We have optimized the electropiercing parameters for medaka eggs with respect to the field strength and the pulse duration, as well as the conductivity of the bathing medium. We have also microscopically examined the injection of a dye solution into pierced eggs and analyzed the impact of electropiercing on embryos' viability and development.

II. MATERIALS AND METHODS

A. Medaka (*Oryzias latipes*) fish eggs and bathing solutions

Breeding populations of medaka were maintained in freshwater at 24–26 °C under an artificial 12-h light/dark cycle to stimulate continuous egg deposition. Naturally fertilized eggs were collected after natural spawning. Embryo stages were categorized according to Iwamatsu.⁹ The long attaching filaments were removed to facilitate electromanipulation. The eggs were thoroughly washed in deionized water ($\sigma \approx 1 \mu\text{S/m}$) obtained from a Milli-Q water purification

system (Millipore, Tokyo, Japan). Figure 2 illustrates the structure of an early-stage medaka fish egg. The small embryoblast and the very large yolk, covered with the vitelline membrane, are enclosed in the hard eggshell chorion.

Medaka eggs and early stage embryos are nearly perfect spheres measuring uniformly 1.2 mm in diameter.⁹ With a diameter of ~ 1.1 mm, the yolk sphere forms the largest compartment ($\sim 80\%$ in volume) of medaka eggs.

Prior to electropiercing, the eggs were transferred into a diluted phosphate buffer saline (PBS, pH 7.4) containing NaCl as the major solute. PBS solutions were prepared by diluting the isotonic 300 mOsm stock PBS with deionized water. The osmolality of PBS used in electropiercing experiments ranged between 4 and 20 mosmol/kg (denoted hereafter as mOsm). The osmolality and electrical conductivity of PBS solutions were measured using an osmometer (Type 13/13DR-Autocal, Roebing, Germany) and conductometer (B-173, Horiba, Japan), respectively.

The eggs were allowed to equilibrate in PBS for at least 1 h. Within the osmolality range of PBS used here (4–20 mOsm), medaka eggs kept their original spherical shape without visible deformations or yolk shrinkage. For comparison, transient concave deformations (due to osmotic shrinkage) have been observed when medaka eggs were exposed to extremely hypertonic PBS solutions of 450–900 mOsm.¹⁷ In that study, the initial shrinkage of the egg (including the yolk) was apparently caused by the osmotically driven water efflux from the egg. After that, the concavity disappeared within the 5–10 min and the egg chorion and the yolk returned to their original spherical shapes.¹⁷ The observed restoration of egg shape and yolk volume in hypertonic PBS is indicative not only of the high chorion permeability for NaCl but also of the efficient osmoregulatory mechanisms in the yolk membrane, similar to those operating in mammalian cells exposed to anisotonic media.^{34,35}

B. Electro-microinjection system

The electropiercing setup was composed of two asymmetric electrodes, including a sharp needle electrode and a large-scaled counter-electrode. The microcapillary needle was made of electroformed-nickel (outer diameter 70 μm , inner diameter 50 μm , and apical angle 40°). Given this geometry, the electrode tip was approximated by a sphere with an effective radius of ~ 10 μm for estimating the local electric field in the following considerations. The

FIG. 2. Schematic drawing of a medaka egg at a stage 10–12 of development (a). The numbers denote oil drops (1), blastoderm containing embryonic cells (2), yolk plasma (3), perivitelline space (pvs, 4), chorion (5), and vitelline (yolk) membrane (6). The diameter of medaka eggs ranges between 1.15 and 1.24 mm. Part (b) illustrates a medaka egg placed between the needle electrode and the large plane counter-electrode. Encircled is the small concave deformation of the chorion surface caused by the needle electrode prior to electropulsing.

microelectrode was aligned perpendicularly to a planar counter-electrode (height 2 mm and width 5 mm), made of stainless steel. The capillary needle and the counter-electrode were connected, respectively, to the positive and negative (GND) terminals of a high-voltage DC pulse generator (Fig. 3(b)). The pulse generator supplies a single (or a train of) rectangular pulse of up to 7 kV with durations longer than $0.5\ \mu\text{s}$, as described in a previous publication.³⁶

Figure 3(a) provides a schematic diagram of the electro-microinjection system. To perform electropiercing, the egg was first placed between the electrodes as illustrated in Fig. 3(b). Using a micromanipulator (MMO-203, Narishige, Japan), the capillary needle was oriented along the egg radius, and the needle tip was pressed gently on the chorion surface. After the initial contact, the needle was moved slowly toward the egg by $\sim 50\ \mu\text{m}$, which produced a slight concave deformation of the chorion, as seen in the photograph in Fig. 2(b). With the egg held in this position, a high-voltage electric pulse was applied to the electrodes. During electropulsing, the egg was attracted to and pierced by the immobile needle electrode. Electropiercing was defined as having occurred if the tip of the needle electrode gained access to the yolk interior, as illustrated in Fig. 1(d). The entire electropiercing process was observed under a light microscope (CK-2, Olympus, Tokyo, Japan) and recorded using a hi-vision digital camera (HDR-XR500, Sony, Tokyo, Japan).

To prove whether exogenous substances can be introduced into electropierced eggs, we used trypan blue (Wako, Osaka, Japan) as an indicator molecule. A saturated trypan blue solution was prepared in PBS, at room temperature. The dye solution was gently delivered into the yolk using a micro syringe (MS-GLL025, Ito Co., Japan) connected via a silicone tube to the open end of the capillary needle, at a flow rate of $\sim 0.2\ \mu\text{l}/\text{min}$.

C. Impact of electropiercing on embryos' development

Fifty embryos were electropierced with an electric pulse of 300 V intensity and 2-ms duration in $390\text{-}\mu\text{S}/\text{cm}$ PBS solution (8 mOsm). The embryos were then incubated for 2 days in deionized water at room temperature. Embryos' development was inspected microscopically. The survival ratio was calculated as the number of eggs capable of development divided by the total number of eggs ($N = 50$).

III. RESULTS

To develop an efficient protocol for electro-microinjection of fish eggs, we conducted a series of experiments aimed at optimizing not only the field strength and the duration of the electric pulses but also the electric conductivity of the bathing solution.

FIG. 3. Schematic of the electro-microinjection setup (a) with a magnified outline of the electropiercing chamber (b). The latter consists of a very sharp nickel needle and stainless steel plate (GND), serving as a counter-electrode. During electropulsing, the egg is immersed in a diluted NaCl solution. The capillary needle is positioned by a micromanipulator. A high voltage electric pulse is applied between the needle and plate electrodes. The distance between the electrodes ($\sim 1.2\ \text{mm}$) corresponds to the diameter of medaka eggs.

A. Mechanical piercing of medaka eggs with a nickel needle

Prior to electropulsing experiments, we examined whether the fine nickel needle was suitable for mechanical piercing of the very hard chorion of medaka eggs. In these experiments, the tip of the needle was first oriented along the radius of the target egg and pressed gently onto the chorion surface using a micromanipulator. The initial contact position of the needle (z_0) was noticed. The needle was then moved at a constant velocity of $\sim 10 \mu\text{m/s}$ toward the egg center. The gradual movement of the needle resulted in increasing deformation and finally in the mechanical penetration (puncture) of the egg. The needle position at the moment of puncture z_p was also noticed. In our experiments, the mean egg deformation ($\Delta z_p = z_p - z_0$, or needle displacement) required for mechanical piercing of medaka eggs was $\Delta z_p \approx 330 \pm 45 \mu\text{m}$, which is 22 times larger than the chorion thickness $d_{\text{ch}} \approx 15 \mu\text{m}$.¹⁷ Given that the egg radius is $\sim 600 \mu\text{m}$, the relatively large Δz_p value means that the egg/chorion was strongly deformed before being mechanically punctured. The measured Δz_p values ranged between 260 and $420 \mu\text{m}$ ($N=10$ eggs, $SD=45 \mu\text{m}$), suggesting some variability in the chorion stiffness among individual medaka eggs. These results also point towards the need for a careful control of the needle movement for piercing each individual egg with a conventional (non-electrical) microinjector.

The mean Δz_p value of $330 \mu\text{m}$ obtained here with a nickel needle compares favorably with the egg deformation of $\sim 400 \mu\text{m}$ measured on medaka eggs microinjected with a glass capillary (see Figs. 13 and 15 in Ref. 32). According to Tan *et al.*,³⁷ a medaka egg experiences a critical puncture force of $F_p \approx 12.5 \text{ mN}$ at the moment of chorion breakage, i.e., at a deformation of $\sim 400 \mu\text{m}$. Judging from the puncture force data reported by Tan *et al.*,³⁷ the chorion of medaka embryos is much harder (~ 10 times) than that of zebrafish, whose critical puncture force is in the range of $0.8\text{--}1.1 \text{ mN}$. Therefore, mechanical microinjection of medaka eggs requires relatively thick glass capillaries with a tip radius of $12.5 \mu\text{m}$, as compared to the $7.5 \mu\text{m}$ used for piercing zebrafish eggs.³⁷ For comparison, the electrode tip used in our experiments can be approximated by a sphere with an effective radius of $\sim 10 \mu\text{m}$ (see Section II B). Tan *et al.*³⁷ also point out that egg deformation imposed by an injection needle gives rise to a significant non-linear increase of the internal pressure within the egg, which may be important for the integrity of the embryonic cell membranes.

B. Determination of the minimum field strength required for electropiercing

The electric conductivity of suspending medium σ_e is one of the major parameters determining the direction and magnitude of DEP force acting on a biological cells.^{18,21,22} Theoretically, only cells whose intracellular conductivity is larger than that of the suspending medium would exhibit a transient positive DEP in response to a DC pulse.³⁸ Therefore, in addition to the effects of field strength and pulse duration, we examined the impact of conductivity of bathing solution on the efficiency of electropiercing of medaka eggs. The conductivity of the yolk interior, σ_{yolk} , which forms the largest compartment of medaka eggs, is $\sim 4 \text{ mS/cm}$.¹⁷

We found that the needle electrode never penetrated medaka eggs, if electropulsing was performed at a relatively high external conductivity σ_e ranging between 2.6 and 10.2 mS/cm (see supplementary Fig. S1).⁴⁸ At conductivities 2.6 and 5.1 mS/cm , i.e., those comparable to the conductivity of the yolk interior σ_{yolk} ($\approx 4 \text{ mS/cm}$), the DEP force acting on the egg was apparently too weak for piercing the chorion (see supplementary Fig. S1, lines 1–2).⁴⁸ Upon electropulsing at the highest conductivity of 10.2 mS/cm , the fish egg was even repelled from the needle electrode (see supplementary Fig. S1, bottom line).⁴⁸ In this case, the egg experienced a repulsive (i.e., negative) DEP force because σ_e was significantly larger than σ_{yolk} .

In order to ensure a sufficient attraction of medaka eggs to the needle electrode by positive DEP, we performed the following electropiercing experiments at conductivities σ_e below σ_{yolk} , i.e., by varying σ_e between $1 \mu\text{S/cm}$ and 1.7 mS/cm . For each pulse duration, ranging between $10 \mu\text{s}$ and 10 ms , and each conductivity, we determined the minimum (or critical) voltage V_{min} of a single pulse required for electropiercing. Starting with a sub-critical voltage (i.e., not sufficient for chorion penetration), we increased the voltage in steps of 20 V until the egg was

penetrated by the needle electrode to provide access to the yolk interior. After each pulse, the egg was allowed to relax for at least 2 min. The minimum voltages $V_{\min}$ required for electropiercing at different pulse durations and conductivities were determined, and the $V_{\min}$ data were plotted *versus* the pulse duration as shown in Fig. 4. At all tested conductivities, the application of an electric pulse of sufficient field strength (i.e., $\geq V_{\min}$) and of relatively short duration (e.g., $<200 \mu\text{s}$) caused the eggs to move quickly by the positive DEP toward the needle electrode and to become impaled on the needle, as illustrated in Fig. 1(d).

The following trends are evident from Fig. 4. At a given external conductivity, the minimum voltage required for electropiercing decreases with increasing field pulse duration. At pulse durations longer than 2 ms, $V_{\min}$ approaches asymptotically a value of $\sim 200 \text{ V}$, for all tested conductivities. At pulse durations shorter than $\sim 100 \mu\text{s}$, $V_{\min}$ decreases with increasing external conductivity. Since the positive DEP force acting on a living cell usually decreases with increasing conductivity,³⁸ the observed conductivity dependence of $V_{\min}$ was unexpected, suggesting that electropiercing relied not only on the dielectrophoretic attraction but can also involve other mechanisms (see Section IV).

In deionized water with conductivity of $\sim 1 \mu\text{S/cm}$, electropiercing could be performed by varying the pulse duration over a wide range between $10 \mu\text{s}$ and 20 ms (Fig. 4, circles). In contrast, electropulsing in NaCl solutions (PBS) gave rise to gas bubble formation by electrolysis mainly on the microelectrode, if pulse duration exceeded a critical value. The minimum pulse duration leading to gas bubble formation decreased with increasing solution conductivity. For a 200-V pulse, gas bubbles formed at a pulse duration of $\sim 50 \text{ ms}$ in deionized water ($\sim 1 \mu\text{S/cm}$). At the same voltage, the pulse duration of gas bubble formation decreased to $\sim 10 \text{ ms}$ as the conductivity was increased 200–390 $\mu\text{S/cm}$, and to 1 ms at 1.73 mS/cm. Electropiercing was not possible under the conditions of gas bubble formation.

From the data shown in Fig. 4, the optimum pulse duration for electropiercing was found to lie between $100 \mu\text{s}$ and 1 ms (dashed circle in Fig. 4). Within this range, moderate voltages between ~ 200 and 500 V were sufficient for electropiercing of medaka eggs over the entire conductivity range. Below the optimum range of pulse duration (i.e., $<100 \mu\text{s}$), electropiercing required very high voltages of up to 2 kV. At pulse durations longer than 2 ms, electropiercing was hindered by gas bubble formation at the needle electrode. The optimum pulse durations of 0.1–2 ms were used in the following experiments involving microinjection of trypan blue and viability tests.

C. Trypan blue injection into the yolk and viability tests

The series of micrographs in Fig. 5 illustrate the process of electropiercing followed by the injection of trypan blue solution into the yolk of a medaka egg. In many cases, penetration of

FIG. 4. Minimum pulse voltage $V_{\min}$ required for electropiercing of medaka eggs as a function of pulse duration. Experiments were conducted at the indicated conductivities σ_e of bathing solution. Each point is the mean value ($\pm$ range) of 3–4 independent trials. The RHS ordinate denotes the field strength at the tip of the needle electrode E_{tip} attached to the chorion. E_{tip} was calculated by applying Equation (8) from Ref. 39: $E_{\text{tip}} = V/R_{\text{el}}$, where V and R_{el} ($\approx 10 \mu\text{m}$) are the voltage and the radius of the electrode tip, respectively. Encircled are optimum conditions (i.e., pulse strength and duration) for electropiercing of medaka eggs. For further details, see text.

the vitelline membrane by the needle electrode triggered a rapid shrinkage of the yolk (Figs. 5(b)–5(d)). During shrinkage, the yolk plasma was extruded through the puncture hole into the perivitelline space (Fig. 5(c)). Within the first minute after electropiercing, the yolk volume decreased by 50%–60% with respect to its original size (Fig. 5(d) vs 5(a)). The observed yolk plasma extrusion through the pierced hole was apparently driven by the hydrostatic pressure in the yolk compartment caused, most likely, by the contraction of the actin cytoskeleton underlying the vitelline membrane. Cortically located actin microfilament network has been found in the oocytes of various teleost fishes.^{40,41}

The observed yolk contraction did not affect the access to the yolk interior via the needle electrode. Thus, upon injection of trypan blue solution through the needle, the yolk regained slowly its original size, as evident from Figs. 5(e) and 5(f). Unlike the yolk interior, the perivitelline space remains largely unstained during trypan blue injection (Fig. 5(e)), suggesting that no significant dye diffusion occurs through the yolk membrane. The latter implies that electropulsing did not affect the barrier function of the yolk membrane (e.g., by the irreversible

FIG. 5. Micrographs of a medaka egg taken before electropulsing (a) and immediately after electropiercing (b), followed by yolk shrinkage and injection of trypan blue solution (c)–(f). Micrographs (c), (d), (e), and (f) were taken at the indicated time intervals after the start of dye injection (with a rate of $\sim 0.2 \mu\text{l}/\text{min}$). The arrow in (e) indicates the embryonic cells (blastoderm), which, unlike the yolk plasma, are not stained with trypan blue.

electroporation mechanism). Likewise, the small embryonic cells seemed to tolerate well the high-voltage pulse, as proved by their ability to exclude trypan blue (arrow in Fig. 5(e)). This experiment demonstrates the feasibility of using electro-microinjection for the complete replacement of the yolk plasma with an exogenous solution, without affecting the viability of embryonic cells.

Interestingly, if the needle electrode was removed from the egg shortly after electropiercing, the puncture hole in the yolk membrane sealed rapidly (within few min) and the yolk leakage stopped. The process of “self-healing” of the punctured yolk membrane is illustrated in Fig. 6.

To assess the impact of electropiercing on egg viability, we scored the developmental capacity of medaka embryos in electropierced and control samples. In these experiments, only embryos at blastula stage were used, such as shown in the upper line of Fig. 7 (stage ~ 10 , according to the Iwamatsu⁹ stages). Prior to electropiercing, the embryos were immersed in a PBS solution of conductivity $390 \mu\text{S}/\text{cm}$. At this conductivity, electropiercing could be performed with a nearly 100% efficiency by applying a moderate pulse voltage of 300 V at a pulse duration of 2 ms (2nd line in Fig. 7). Immediately after electropiercing, the needle electrode was withdrawn, which allowed the yolk membrane to seal the pierced hole (Fig. 6), thus preventing excessive leakage of the yolk plasma. Under these conditions, the yolk shrank to $\sim 50\%$ of its original volume (3rd line in Fig. 7). Control embryos were treated similarly but without field pulse application.

The micrographs in the 2 bottom lines of Fig. 7 illustrate the development of early-stage embryos after electropiercing. Despite partial yolk plasma release, the embryos progressed from blastula (stage ~ 10) to the early somite stage (stage ~ 20) within 1 day after electropiercing (3rd line in Fig. 7), and further to the late somite stage (stage 23–25) within the next 24 h (bottom line in Fig. 7).

In our experiments, 19 out of 50 medaka embryos survived *electropiercing* with a needle electrode of $70\text{-}\mu\text{m}$ diameter, which corresponds to a survival rate of 38%. For comparison, none of five tested embryos survived if they were *mechanically* pierced with the same $70\text{-}\mu\text{m}$ thick nickel needle. The survival of control embryos without piercing was 92%, i.e., 46 out of 50 eggs underwent development. Our survival data show that electropiercing procedure can be harmful for early stage medaka embryos, indicating the need for further optimization, e.g., via the use of thinner needle electrodes.

A poor survival after mechanical piercing seems to be a common feature of fish embryos. Thus, puncture with a glass capillary has significantly reduced the survival rate in zebrafish embryos from $\sim 80\%$ to $\sim 50\%$.¹³ Studies on early fertilized turbot eggs also show relatively low survival rates after microinjection or even simple puncture.⁴²

IV. DISCUSSION

The data presented above prove the feasibility of electro-microinjection of fish eggs by means of an immobile capillary electrode. Upon application of a short electric pulse of

FIG. 6. Micrographs of the pierced hole in the yolk membrane during the self-healing process. The egg bathed in water at a conductivity of $\sim 1 \mu\text{S}/\text{cm}$ was electropierced with a pulse of 450 V and 2 ms duration. The needle was removed shortly after electropiercing. The micrographs show the appearance of the pierced hole at the indicated time intervals after the removal of the needle. Spheroids are oil drops inside the yolk.

FIG. 7. Development of medaka embryos after electropiercing. Early-stage embryos (stage ~ 10) were immersed in 390- $\mu\text{S}/\text{cm}$ PBS (1st line) and electropierced with an electric pulse of 300 V and 2 ms duration (2nd line). The needle electrode was immediately withdrawn. The 3rd and bottom lines show the same embryos during development, respectively, 1 and 2 days after electropiercing.

sufficient voltage, a fish egg (immersed in low-conductivity medium) is strongly attracted by the positive DEP force (F_{DEP}) toward the needle electrode. As a result, the hard outer shell (chorion) and the subjacent soft yolk membrane are impaled by the sharp electrode tip, thereby providing direct access to the egg yolk plasma.

Positive dielectrophoresis apparently provides the primary force for the attraction of the egg to the needle electrode. Both the time course and the magnitude of the transient DEP force exerted on a medaka egg upon application of DC pulse can be roughly estimated from the following considerations based on the experimental electrorotation (ROT) spectra of medaka eggs reported previously.¹⁷ The supplementary Fig. S2⁴⁸ illustrates the theoretical ROT and DEP spectra of medaka eggs, presented, respectively, as the imaginary and real parts of the complex polarizability factor U^* over the kHz–MHz frequency range. The spectra were calculated for four different external conductivities σ_e matching those used in our electropiercing experiments (Fig. 4). For these calculations, we used the dielectric data of early-stage medaka embryos derived from their electrorotation (ROT) spectra on the basis of a three-shell model (TSM).¹⁷ The main co-field peak in the ROT spectra (f_{cy} , supplementary Fig. S2(a))⁴⁸ results mainly from the ionic polarization of the yolk plasma.¹⁷ The latter also gives rise to the high-frequency dispersion in the DEP spectra at 10–100 MHz (supplementary Fig. S2(b)).⁴⁸ It is well known that a dispersion in the frequency spectrum implies a relaxation process in the time domain (see Appendix 4 in [Jones, 1995¹⁸]). Using the $f_{\text{cy}} \approx 50$ MHz at $\sigma_e = 0.2$ mS/cm (supplementary Fig. S2(a)),⁴⁸ the relaxation time of the positive DEP force in response to a DC electric pulse can be calculated as $\tau_{\text{yolk}} = (2\pi f_{\text{cy}})^{-1} \approx 3$ ns. This means that the F_{DEP} reaches its maximum value U_{max} very fast, i.e., within $5 \times \tau_{\text{yolk}} \approx 15$ ns after application of a DC pulse. It is also evident from supplementary Fig. S2(b),⁴⁸ that the U_{max} value decreases with increasing external conductivity σ_e , e.g., from $U_{\text{max}} \approx 0.8$ at 1 $\mu\text{S}/\text{cm}$ to $U_{\text{max}} \approx 0.5$ at 1.6 mS/cm. Consequently, increasing external conductivity will reduce the magnitude of DEP force acting on the medaka egg.

In accordance with the theoretically expected conductivity-dependence of the DEP force (supplementary Fig. S2(b))⁴⁸ discussed above, electropiercing failed at relatively high external conductivities, such as ranging between 2.6 and 5.1 mS/cm (see supplementary Fig. S1, lines 1–2).⁴⁸ This finding suggests that if σ_e is comparable to the yolk plasma conductivity $\sigma_{\text{yolk}} \approx 4$ mS/cm, the DEP force might have been too weak and insufficient for piercing the hard chorion. Moreover, the egg was even repelled from the needle electrode upon electropulsing at σ_e of 10.2 mS/cm (see supplementary Fig. S1, bottom line).⁴⁸ In this case, i.e., when σ_e was more than twice as high as σ_{yolk} , the egg might have experienced negative DEP force.

In sharp contrast to the high-conductivity conditions illustrated in supplementary Fig. S1,⁴⁸ electropiercing could reliably be achieved when medaka eggs were immersed in solutions of lower conductivities (Fig. 4). Particularly, at conductivities σ_e ranging between 0.2 and 1.7 mS/cm, electropiercing could be performed with a moderate voltage of ~ 300 V and a pulse duration of ~ 0.5 – 2 ms (Fig. 4).

Using the dipole-based approach,¹⁸ we can roughly estimate the magnitude of the DEP force required for electropiercing. The details of calculations, including equations, structural, and dielectric properties of medaka eggs, are given in Ref. 17. At a voltage of 300 V, the local field strength in the midpoint between the electrodes ($z = R_1 = 0.6$ mm; i.e., corresponding to the egg center) is $E = 33$ kV/m, and the field gradient along the z axis $\nabla_z E = 95$ MV/m². For these calculations, the electric field was approximated by a field between a point charge at the tip of the needle electrode and a charged plate electrode. Substituting these values into Eq. (2) yields $\mu = 4.3 \times 10^{-14}$ C m for the induced dipole moment within the egg. The corresponding DEP force acting on the egg at a pulsing voltage of 300 V is 4.1 μ N, as calculated using Eq. (1).

With a pulse duration of 0.5 ms, 300 V were sufficient for electropiercing at conductivities 0.2–1.7 mS/cm (Fig. 4). With the same pulse duration, electropiercing at the lowest conductivity of 1 μ S/cm required a much larger voltage of 500 V (Fig. 4, blue symbols). At this pulse voltage, the egg experiences a DEP force of ~ 11.5 μ N. It should be noted that the calculated range of 4–11.5 μ N for the DEP forces is only a rough approximation. This is because the theoretical DEP spectra in supplementary Fig. S2(b)⁴⁸ were derived assuming the field inhomogeneity to be small in comparison to the particle size. This assumption, however, does not hold for the setup used here, i.e., for a mm-sized fish egg attached to the microelectrode (Fig. 1(b)).⁴⁸

Although DEP is the primary driving force, the DEP mechanism alone does not fully account for the results of our electropiercing experiments performed within the conductivity range from 1 μ S/cm to 1.7 mS/cm (Fig. 4). Thus, contrary to the conductivity-dependence of the DEP force (supplementary Fig. S2(b)),⁴⁸ the voltage required for electropiercing increased with decreasing conductivity (Fig. 4). This is particularly evident for the pulse duration of 0.5 ms, at which the minimum piercing voltage V_{min} grew from ~ 300 to 500 V (and the DEP force increased) as the conductivity σ_e was decreased from 0.2 mS/cm to 1 μ S/cm. Moreover, the estimated DEP force range of 4.1–11.5 μ N operating during electropiercing in this study is at least three orders of magnitude lower than the force of 12.5 mN (Ref. 37) required for mechanical puncture of medaka eggs by conventional microinjection.

The above findings suggest that, besides the DEP force, further mechanisms might be essential for electropiercing of fish eggs. We can conceive of at least two interrelated mechanisms which may affect the mechanical properties (e.g., elasticity) of the chorion. These are (1) the dielectric breakdown/electroporation of the chorion caused by the very high local electric field, and (2) the related high ionic current density (and energy dissipation) through the microscopic segment in the chorion attached to the tip of the needle electrode.

- (1) As illustrated in Fig. 1(a), the electric field lines converge on the tip of the needle electrode, thus producing a very high local field strength E_{tip} in front of the electrode tip. Using the approach outlined in Ref. 39, we found that at a pulse voltage of ~ 500 V the local electric field strength E_{tip} within the chorion can reach a very high value of ~ 0.5 MV/cm ($= 50$ MV/m; see the RHS ordinate in Fig. 4). Interestingly, the estimated E_{tip} operating in our experiments approaches the field strength of 60–80 MV/m which are sufficient to induce the dielectric

breakdown of aqueous solutions of different conductivities.²⁶ As a result, the strong electric field may reduce significantly the elastic modulus of the chorion (e.g., via the mechanism known as electroporation or dielectric breakdown), which would in turn enable the needle electrode penetration.

- (2) A further factor possibly responsible for the unexpected conductivity effect appears to be the Joule energy dissipation P_w [W/m³] in the microscopic segment in the chorion attached to the tip of the needle electrode. In dielectric materials with Ohmic losses, the volume-specific energy dissipation P_w is proportional to the square of the local electric field E times the local specific conductivity σ : $P_w = E^2 \cdot \sigma$ (i.e., P_w scales linearly with ionic conductivity σ). A detailed analysis of the electric energy absorption/dissipation in biological cells is given in Ref. 43. Due to its porous structure and high permeability to electrolytes, the chorion of medaka eggs has a relatively high ionic conductivity σ_{ch} , which is about half the external conductivity σ_e : $\sigma_{ch} \approx 0.5\sigma_e$.¹⁷ Accordingly, the local P_w in the chorion material at the electrode tip also increases linearly with increasing external conductivity. Based on the above considerations, we hypothesize that the conductivity-mediated facilitation of electropiercing (Fig. 4) may have resulted, at least in part, from a decrease in the mechanical strength (e.g., softening) of the chorion, which is composed of cross-linked glycoproteins, due to the local electrical energy dissipation at the electrode tip. In view of the rigorous discussion in the literature concerning “thermal” vs. “electronic” mechanisms for dielectric breakdown in liquids,²⁶ the two mechanisms possibly responsible for the alteration of the mechanical properties (dielectric breakdown vs energy dissipation) are apparently interrelated.

A further possible interpretation of the results shown in Fig. 4 would include pump effects of the medium in and around the gap between the needle electrode and the chorion. In such an interpretation, external medium with a polarizability higher or lower than the effective polarizability of the egg would be pumped either into or out of the gap. This would lead to the repulsion of the chorion from the electrode at high conductivities of the external medium, while a low conductive medium would be squeezed out by the higher polarizable medium of the egg, i.e., the egg would be attracted towards the electrode.^{44,45}

V. CONCLUSION

This study provides proof-of-principle for a new electro-microinjection technique for manipulation of fish eggs and early-stage embryos surrounded by a hard eggshell. The method, which is a combination of microinjection and electroporation, can be used for the rapid exchange of the egg-yolk plasma with a solution of desired composition, including dyes, drugs, and, most importantly, cryoprotective agents. Among other features, the immobile microinjection needle makes this method an attractive alternative to the conventional microinjection with movable glass capillaries. Although this work focuses on medaka eggs, the approach presented here can be generally applied to eggs and embryos of many teleost fishes and other animals, including those of biotechnological interest. The electro-microinjection presented here also has the potential for automation, e.g., via integration into microfluidic devices, such as reported for electroporation and mechanical microinjection systems,^{3,46,47} which would allow a large-scale microinjection of fish eggs for a variety of applications in basic research and aquaculture.

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