GLASS-CAPILLARY-ACCESSIBLE DYNAMIC MICROARRAY FOR MICROINJECTION OF ZEBRAFISH EMBRYOS
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ABSTRACT
This paper describes a glass-capillary-accessible dynamic microarray for an efficient microinjection of zebrafish embryos. The microfluidic channel has an array of holes at the trapping sites and enabled a rapid alignment of eggs, direct injection, observation, and selective retrieval of embryos. Fertilized zebrafish eggs can be loaded without pumping, and genes of interest or antisense oligos are readily injectable using glass capillaries. We believe our chip will help high-throughput analysis of gene functions and the screening of zebrafish lines.

KEYWORDS: zebrafish, egg, microarray, microinjection

INTRODUCTION
Zebrafish, Danio rerio, is a widely used vertebral model organism to study developmental processes and genetics. In the last decade, it has also emerged as a human disease model because of the ease of transgenesis, loss of function analysis and a high genetic similarity to human [1]. A number of human disease genes and its functions are highly conserved in zebrafish [2]. It also gives numerous advantages for biological researchers: zebrafish embryos are optically transparent and develop outside the body, which makes it easy to investigate the “living” tissue and organ morphogenesis including angiogenesis, neurogenesis, and skeletal tissue formation. Moreover, all major organs form by 24 hours, and it becomes sexually mature within 3 to 4 month and is capable of laying 100 to 200 eggs per week. Thousands of zebrafish lines can be maintained at reasonable cost. These advantages make zebrafish an ideal model organism for a high-throughput analysis of genes involved in human disorders.

Recently, several groups have reported the microfluidic systems for zebrafish analysis [3–5]. For example, Martin et al have suggested a high-throughput vertebrate screening platform in which they can manipulate and orient hatched fish embryos for cellular resolution imaging. Choudhury et al developed a multi-channel microfluidic perfusion system that enables live tissue or organ imaging of zebrafish embryos under the influence of drugs. However, due to their inaccessibility, it was hard to directly manipulate the embryos incubated in the tanks.

Generally, gain and loss of function analysis are inevitable to know gene functions in living animals. In zebrafish, overexpression of gene of interest is easily achieved by injecting its mRNA synthesized in vitro. Injection of antisence oligos, morpholinos in general, leads to the knockdown of gene function by interfering with the protein translation. These methods transiently modulate gene expression level, especially at the early stages of embryonic development, but zebrafish lines that are stably expressing particular gene can be obtained by utilizing tol2 transposon technique [6]. To raise stable zebrafish lines, plasmid and tol2 transposon mRNA should be injected to one-cell stage embryo in order to promote genomic integration of the exogenous gene. Since one-cell stage lasts for only half an hour, researchers are required to align fertilized eggs in an injection plate as much as possible within a limited time. Dynamic microarray system is a multi-functional platform to transport, immobilize, observe, and retrieve selected particles [7]. Taking the advantages of this system, here we propose a glass capillary accessible dynamic microarray system which enables a rapid alignment of the fertilized zebrafish eggs for the effective microinjection and the subsequent analysis including embryo incubation, observation in a microfluidic channel (Fig. 1).
EXPERIMENT

Fabrication  The fabrication process is shown in Figure 2. Briefly, microfluidic channels were designed based on the equation reported previously [7] and fabricated in poly(dimethylsiloxane) (PDMS, Sylgard 184 Silicone Elastomer, Dow Corning). First, we designed a 1.3 mm-thick mold with 3D modeling software (Rhinoceros, AppliCraft) and used a commercial stereolithography modeling machine (Perfactory, Envision Tec, Germany) to fabricate the channel structure with photoreactive acrylates resin (R11). The exposed light is projected plane-by-plane by a digital micromirror device projector into a reservoir of R11. Light exposure made the R11 cross-linked and cured, realizing 3D channel structures by prototyping layer by layer while the base plate is gradually lifted up. The channel pattern was then used as a mold and PDMS with a 10 : 1 (w/w) ratio of crosslinking agent was poured into the R11 mold. After molding PDMS on patterns, holes were formed as inlets, outlets and injection holes. The molded PDMS was bonded to a glass substrate by treating with O₂ plasma, and then the device was baked on a hotplate for 1 h at 70°C to strengthen the bonding.

Zebrafish egg array  Zebrafishes were kept on a 14-hour light-10-hour dark cycle using appropriate timers and maintained in a well-conditioned water at 26 – 28 °C with the biological filtering system. They were fed with dry food flakes and living Artemia twice a day to keep them in good breeding condition. For setting up pair matings and collecting eggs, one female and one male each were caught using small nets and are carefully transferred to the mating box at the previous night to the embryo collection. To collect fertilized eggs, the water from the mating container was poured through a plastic tea strainer. Collected eggs were then rinsed with 0.03% salt water containing methylene blue, and kept at 20 °C to slow the embryonic development.

Microinjection  For microinjection, glass capillaries (arishige Scientific Instrument Lab, Tokyo, Japan, 1 x 90 mm) were pulled on a needle puller (arishige) to give a stubby and narrow quickly to a tip. The end is then cut using fine forceps to give an opening of between 0.05 mm and 0.15 mm diameter. A microinjection set-up consists of a stereomicroscope M80 (Leica Microsystems, Wetzlar, Germany) connected to a digital camera IC80 HD (Leica) and a micromanipulator system (WPI, UK) attached to a microinjector, FemtoJet (Eppendorf, Hamburg, Germany) (Fig. 3A). FITC labeled dextran (FITC-dextran, Mw = 4000, Sigma, St Louis, MO) was dissolved in 1x Danieau’s solution (58 mM NaCl, 0.7 mM KCl, 0.4 mM MgSO₄, 0.6 mM Ca(NO₃)₂, and 5 mM HEPES, pH7.6) at final concentration of 10 mM.
and briefly centrifuged at 12,000 \( \times g \) to remove undissolved particles before loading to the injection needle.

**RESULTS AND DISCUSSION**

To make an array of one-cell stage zebrafish embryos, fertilized eggs should be aligned as much as possible within 30 min. We first sealed the injection holes of a chip with thin PDMS membrane and placed the eggs in an inlet chamber. The eggs were easily loaded by removing the salt water from the outlet chamber and returning it again to the inlet chamber (Fig. 3B). Almost all of the trapping sites were occupied with eggs by repeating the flushing cycle two or three times. It took only a few minutes to fulfill the trapping sites (eighteen eggs). PDMS membrane covering the microarray was then gently removed before microinjection. To demonstrate the microinjection of the arrayed eggs, FITC-labeled dextran (FITC-dextran) was loaded into the glass capillary and injected in the one-cell stage embryos through the injection holes (\( \phi = 1 \text{ mm} \)) (Fig. 3C). Each embryos were successfully immobilized within the trapping sites even after the injection. After finishing the injection, holes for injection was gently sealed again with the PDMS membrane for further incubation of embryos at 28 °C. Fluorescence microscopic observation revealed that injected FITC-dextran was expanded and kept in all cells divided during the early embryonic development. Almost all of the trapped embryos were viable for at least four days in a microchannel, and no apparent abnormalities were observed in the grown embryos (Fig. 4). These results show that our system enabled rapid alignment of one-cell stage embryos, and the subsequent observation and biological analysis were possible within the dynamic microarray system. For example, founder transgenic embryos can be observed at each trapping site, and embryos with a high transgenesis rate can be retrieved through the injection holes by flushing the salt water with the selected injection holes opened.

**CONCLUSION**

The presented dynamic microarray system is expected to be applied in the microinjection of DNA, mRNA, or morpholino antisense oligo to investigate the gene functions during the zebrafish development. The readily injectable microarray will improve the efficiency of microinjection as well as the embryo handling to observe and retrieve the embryo with interesting phenotype.

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**REFERENCES**


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