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A novel method for microinjection into *Xenopus* eggs and embryos supported in methylcellulose solution

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ABSTRACT We have developed a novel method for microinjection into *Xenopus* eggs and embryos. Microinjection was performed into eggs or embryos that were placed in wells (ca. 2.5 mm x 2.5 mm x 0.8 mm for each well) at the bottom of a commercially available hybridoma dish, which was filled with 1.5% methylcellulose solution. Eggs or embryos, rotated to a desired orientation in the viscous methylcellulose solution with a hair loop, could remain in the orientation for more than twenty minutes. Accordingly, samples such as mRNAs, DNAs, proteins and antisense morpholino oligonucleotides could be easily and efficiently microinjected into any part (animal, vegetal, dorsal, lateral or ventral) of more than five hundred eggs and embryos in one day. In addition, methylcellulose did not interfere with the development of the injected eggs and embryos.

KEY WORDS: *microinjection, morpholino oligonucleotide, blastomere, methylcellulose 4000*

Introduction

Xenopus laevis is widely used as a model organism in developmental biology research. One of the experimental advantages of this animal is that it is suitable for functional assays by injecting plasmid DNA (Mohun *et al.*, 1986), mRNA (Brachet *et al.*, 1973) and anti-sense morpholino oligonucleotides (Heasman *et al.*, 2000) into eggs and embryos where endogenous proteins are expressed or in ectopic regions (Amaya *et al.*, 1991). On injecting into the cells of the vegetal pole region, embryos should be rotated 180° to the animal–vegetal axis because the animal hemisphere always faces upwards due to gravity. To date, we have usually used–nylon mesh or glass ladders to hold the inverted embryos in a 3–5% Ficoll 400 solution (Sive *et al.*, 2000). However, in either case, skill is required to perform microinjection into vegetal or lateral blastomeres of a large number of embryos in the limited time period.

Accordingly, we have developed a simple and efficient injection method by adopting a hybridoma dish with 700 wells filled with 1.5% methylcellulose solution, which is much higher in viscosity compared to 5% Ficoll 400. Eggs and embryos are placed in wells along a row, rotated to the desired orientation with a hair loop, and thereby they can keep their orientation for more than twenty minutes. Therefore, as samples can be efficiently microinjected into the desired part of the eggs and embryos, we can perform injection of up to ten different mRNA samples into a vegetal blastomere of more than 500 embryos, up to 700 embryos, in one day. In addition, methylcellulose has no negative effect on the development of embryos and is less expensive than Ficoll 400. This method may be especially useful to researchers who perform microinjections into various blastomeres in *Xenopus* embryos.

Experimental Protocols

An injection chamber

A commercially available dish with 700 wells (hybridoma dish, Greiner bio-one co., ltd. Ca. 633160) was used for the injection chamber. Lines were made with different colored felt-tipped pens on the underside of the dish to distinguish the position of injected embryos from uninjected ones. Methylcellulose 4000 (Wako chemicals, Ca. 136-02155) was dissolved by vigorous shaking in cold 1/3 Marc's Modified Ringers solution (1 x MMR: 0.1 M NaCl, 20 mM KCl, 1 mM $MgSO_4$, 2 mM $CaCl_2$, and 5 mM HEPES; pH 7.4) to the final concentration of 1.5 % (w/v), autoclaved, and then stored at 4°C. On the day before the injection experiment, about 20 ml and 5 ml of the methylcellulose solution was poured, with care not to produce

Abbreviations used in this paper: FDL, fluorescein dextran lysine, GPBC, germ plasm-bearing cell; MMR, Marc's modified Ringer's solution.

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air bubbles, into the hybridoma dishes and 5 cm culture dishes, respectively. They were kept at room temperature until use.

Embryos

Embryos were prepared essentially by the method of Sive *et al*. (2000). Briefly, *Xenopus* eggs, stripped from gonadotropin-injected females, were fertilized *in vitro* with sperm of surgically isolated testes from an adult male. Forty minutes after fertilization, eggs were dejellied with 2% cysteine-HCl in 1 x MMR (pH 8.0), rinsed with, and placed in 0.1 x MMR. With a Pasteur pipette, eggs were transferred into the 5 cm culture dish filled with methylcellulose solution with minimal carry-over of the 0.1 x MMR. They were then transferred into the injection chamber with minimal carry-over and aligned, with a hair loop, in wells (one embryo/well) along the colored line drawn on the underside of the chamber. Sixty embryos to be injected of one mRNA sample were transferred into one dish.

Microinjection of visible dye and fluorescein dextran lysine into a vegetal blastomere of 32-cell stage embryos

Before injection, embryos in wells were turned upside down with a hair loop. The inverted embryos were held in position in the 1.5% methylcellulose solution until the injection was complete. Eighteen nl of a mixture of 1% Xylenecyanol FF (Sigma, 335940) and Bromophenol blue (Sigma, 18030) in 10 mM Tris-HCl solution (pH 7.5) was injected, with a microinjector (Drummond, 3-00-203-XV), into one of the vegetal blastomeres of 32-cell stage embryos, and then a photograph was taken just after the injection. Separately, 4.5 nl of 100 mg/ml of fluorescein dextran lysine (FDL, Molecular probe, Ca. D1820) was injected as a lineage tracer into one of the germ plasm-bearing cells (GPBCs) of 32-cell stage embryos, which were then allowed to develop until stage 46 (Nishiumi *et al*., 2005). After microinjection, about 20 ml of 1/3 MMR was added to the hybridoma dish, which was slowly shaken several times to reduce the methylcellulose concentration. On the next day, after shaking several times the solution was replaced by 20 ml of fresh 1/3 MMR. The same procedure was repeated several times to reduce the concentration of methylcellulose.

Cells derived from the FDL-injected blastomere

Embryos injected with FDL were fixed at stage 46 with 4% paraformaldehyde in phosphate-buffered saline (PBS; 1.5 mM KH_2PO_4, 8.1 mMNa_2HPO_4, 2.7 mM KCl and 136 mM NaCl), dehydrated with an ethanol series, and embedded in paraffin. Serial sections were cut 8 μm thick. The FDL-labeled cells derived from the injected vegetal blastomere were identified with a fluorescence microscope (Olympus BH-2, Olympus Kogaku, Tokyo), as described previously (Nishiumi *et al*., 2005).

Results

Determination of appropriate concentration of methylcellulose

In order to determine the appropriate concentration of methylcellulose, we rotated embryos until the vegetal pole was upward in 1, 1.5 and 2% methylcellulose solutions, and then examined whether the initial position was restored after 10 min. Embryos in 1% solution turned around to the initial position, while those in the 1.5% and 2% solutions remained in the rotated position (data not shown). However, as 2% methylcellulose solution was difficult to pipette and handle because of its high viscosity, 1.5% methylcellulose solution was, therefore, chosen for the following procedure. To confirm the results, we observed the embryos in 1.5% methylcellulose solution at 10 and 20 min after rotation. As indicated in Fig. 1A, none of the embryos were restored to the initial position. Even after 30 minutes, they were fixed in the position (data not shown). Additionally, Fig. 1B shows a magnified image of the well in the upper left corner in Fig. 1A, which shows that the embryo in the well was held to the initial axis even after cleavage. These results indicate that microinjection into blastomeres of a large number of embryos can be performed efficiently within ca. 30 min, which roughly corresponds to the interphase between cleavages.

In addition, as the viscosity of methylcellulose was somewhat different from batch to batch, the appropriate concentration for new batches should be determined beforehand.

The effect of methylcellulose on the development of embryos

Dejellied embryos were cultured in either 1.5% methylcellulose in 1/3 MMR, or in methylcellulose-free 1/3 MMR for one day. Then, embryos of both groups were transferred to 1/3 MMR and allowed to develop until stage 46. Development of embryos in both groups was carefully examined and the number of normally-developed tad-

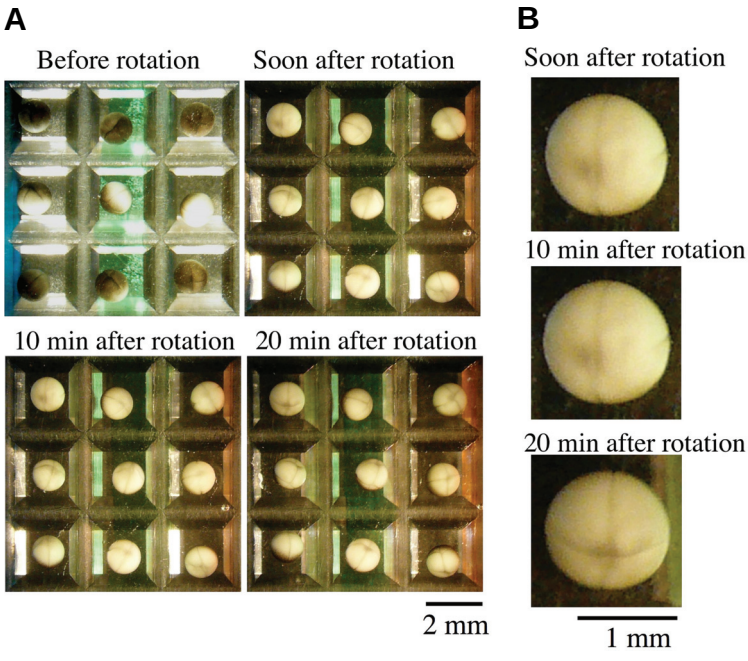

Fig. 1. Embryos were rotated upside down with a hair loop in 1.5% methylcellulose solution, and observed soon after rotation, and at 10 and 20 min after rotation. *As shown in Fig.* **(A)***, none of the inverted embryos were restored to the initial position or to the animal pole being upward. In addition, an embryo in the upper-most well of the left column at 20 min after rotation in (A) was held to the inverted position even after the second cleavage* **(B)***. Bars in (A,B) represent 2 and 1 mm, respectively.*

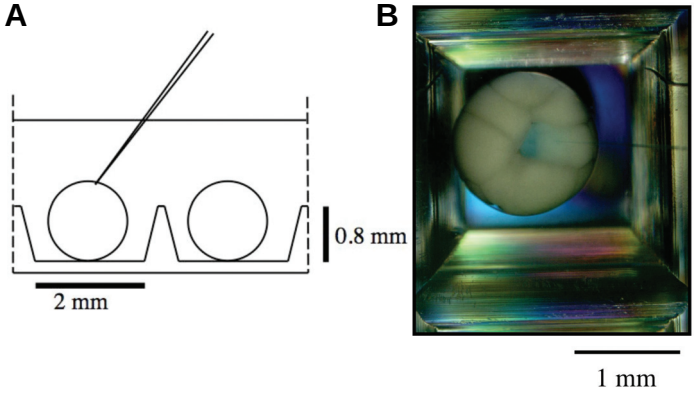

Fig. 2. Schematic diagram of microinjection procedure (A). *32-cell stage embryos were rotated upside down with a hair loop in a well at the bottom of the hybridoma dish filled with 1.5% methylcellulose solution. Visible dye was injected into one of the vegetal blastomeres with a microinjector. Just after injection the blastomere was stained blue* **(B)**. *The horizontal and vertical bars in (A) are 2 and 0.8 mm, respectively. The bar in (B) is 1 mm.*

poles was counted. In three independent experiments, there was no indication of developmental retardation in embryos of either group. The rate of normally-developed tadpoles on day 3 was 94% (114/121) in 1.5% methylcellulose-1/3 MMR and 92% (97/107) in methylcellulose-free 1/3 MMR. These findings indicated that the development and survival rate of the embryos exposed to methylcellulose solution were not significantly different from those of the control embryos.

Microinjection in 1.5% methylcellulose solution

To verify whether sample was really injected into blastomeres by the present injection method, visible dye, a mixture of 1% Xylenecyanol FF and Bromophenol blue solution, was injected into one of the vegetal blastomeres of 32-cell stage embryos (Fig. 2). As a vegetal blastomere of an embryo was stained blue just after the injection, it means that microinjection was successfully carried out. Then, FDL was injected into one of the germ plasm-bearing cells (GPBCs) of 32-cell stage embryos, which were allowed to develop to stage 46. FDL-labeled cells from the injected GPBC were examined in experimental tadpoles from the injected embryos. FDL-labeled cells were found in the intestine and in the genital ridges, the latter of which were thought to be primordial germ cells (Fig. 3). This is in good agreement with the FDL-labeled cells observed in experimental tadpoles of our previous study when FDL was injected into GPBCs of 32-cell stage embryos (Ikenishi *et al.*, 2006). In three independent experiments, the survival rates of injected embryos at stage 46 were 89% (53/64), 92% (45/49), and 96% (50/52), respectively. These rates were almost the same as those obtained in our preliminary experiments with Ficoll 400.

Discussion

We developed a novel microinjection method to facilitate performing microinjection into one of the vegetal blastomeres of 32-cell stage *Xenopus* embryos. This method, with the hybridoma dish and methylcellulose solution, has the following advantages; 1) The eggs and embryos can be easily rotated to a desired orientation with a hair loop, without affecting the orientation of other eggs and embryos because only one egg or embryo is in each well of the hybridoma dish. 2) The eggs and embryos, once rotated to the desired orientation, can remain in the orientation in the methylcellulose solution for more than 20 minutes. 3) The eggs and embryos can develop healthily because each egg or embryo was partitioned in a well of the hybridoma dish, which prevents contact between eggs or embryos and guarantees sufficient oxygen. 4) Methylcellulose is much less expensive compared to ficoll. In addition, the method does not require any specific skills, so that researchers who want to microinject into any part of the egg and embryo will be able to perform the injection easily and quickly.

Because methylcellulose is often used in cultures for colony formation of blood stem cells (Kubota and Preisler, 1982) and for cloning of hybridomas (Davis *et al.*, 1982), it is known to be non-toxic and, therefore, may be suitable as an embryo-supporting medium. In fact, methylcellulose did not give rise to any detrimental effect on the development of embryos up until hatching when the concentration of methylcellulose was gradually reduced by replacement with fresh MMR after 1 day of culture. On the other hand, when embryos were left in the methylcellulose solution they died, probably due to the asphyxia after hatching because they could not move in the methylcellulose solution. However, within a limited time period, methylcellulose solution may be useful to aid detailed observations of non-anesthetized tadpoles, such as quickly moving eyes, blood cells flowing in vessels and a beating heart, because even tadpoles could not move in the methylcellulose solution.

Using the method described here, we have routinely performed microinjection of mRNA and antisense morpholino oligo-

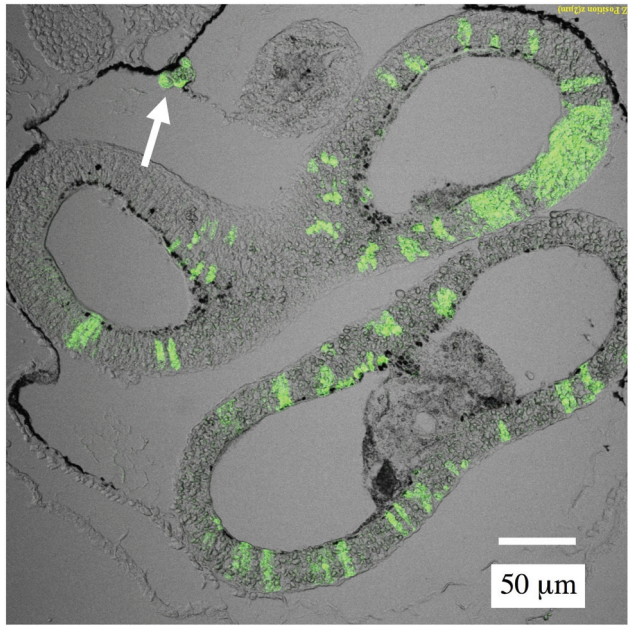

Fig. 3. A merged image of fluorescent and phase-contrast micrographs of a transverse section of a stage-46 tadpole derived from an FDL-injected embryo. *FDL was injected into a single GPBC of embryos at the 32-cell stage. FDL-positive cells are observed in digestive tracts and in the genital ridge. Arrow indicates primordial germ cells in the genital ridges. Upper left is dorsal and lower right ventral. Bar represents 50 μm.*

nucleotides into *Xenopus* eggs and embryos without any problems. We believe that this method may be especially useful to researchers who perform microinjections to various blastomeres of *Xenopus* embryos.

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