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TECHNICAL NOTE

An efficient microinjection method for unfertilized eggs of Asian amphioxus *Branchiostoma belcheri*

Xin Liu · Guang Li · Jun Feng · Xi Yang · Yi-Quan Wang

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Abstract Amphioxus is a promising model animal for evolutionary and developmental studies. However, as an emerging model organism, amphioxus lacks most molecular techniques applied in other well-developed model animals. Microinjection is a powerful technique for gene manipulation, and thus it undoubtedly is one of useful approaches in the studies of gene function and embryonic development. Although the method has been exploited in Florida and European amphioxus, it still remains to be optimized and introduced into other amphioxus species. In order to introduce the technique into our lab, we followed and optimized the previous description and successfully performed microinjection on unfertilized eggs of Asian amphioxus *Branchiostoma belcheri*. We made six solutions for practice: 200 mM KCl, 100 ng/μl actin-*LacZ* or 100 ng/μl actin-*RFP* vectors, *LacZ* or *RFP* vectors without promoter and *RFP* capped mRNA. More than 99.2 % of eggs injected with KCl were able to be fertilized, 94.3 % of them could hatch normally and 55.9 % survived until 2-day larvae, all of which were nearly equivalent to those obtained from normally fertilized eggs. Embryos injected with two plasmid constructs also showed very high fertilizing and hatching ratios, but normally developing ratios were slightly lower than that of KCl injection. Of those injected embryos, 91.8 % expressed exogenous gene *LacZ* and 80.5 % exhibited foreign *RFP* expression, which were driven by a promoter from amphioxus *β-actin* gene. The data indicated a successful modified microinjection method for the unfertilized eggs of Asian amphioxus, and those modifications improved the feasibility and efficiency of microinjection on amphioxus.

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X. Liu · G. Li · J. Feng · X. Yang · Y.-Q. Wang (✉)
State Key Laboratory of the Cellular Stress Biology,
School of Life Sciences, Xiamen University,
Xiamen, Fujian 361005, People's Republic of China
e-mail: wangyq@xmu.edu.cn

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Introduction

Amphioxus (or lancelets) is a basal group of invertebrate chordates and represents the best available invertebrate proxy to vertebrates. Anatomically, they have a simple but similar body plan to that of vertebrates, and genetically, they retain a prototypical pre-duplicated chordate genome with respect to genome content and structure, and even to chromosomal organization. Therefore, those small marine animals have long been considered to be a good model for studying the basic patterning of chordate body plan and the evolutionary development of chordates.

Taxonomically, amphioxus belongs to the subphylum Cephalochordata including about 31 living species around the world (Wang and Fang 2005), but only four of them are commonly used for biological studies: the Floridian–Caribbean lancelet *Branchiostoma floridae*, the European lancelet *Branchiostoma lanceolatum*, and the Asian lancelets *Branchiostoma belcheri* and *Branchiostoma japonicum*, with the later two Asian ones are wrongly treated as the same species *B. belcheri* previously (Zhang et al. 2006). The experimental techniques most frequently used for amphioxus studies in the past two decades were in situ hybridization and immunohistochemical staining due to the limitation of living embryo supply. Researchers employed those methods to reveal the spatial expression of a given gene and deduced its potential function from the expression data. Techniques of using small molecules to inhibit or activate some signaling pathways were also introduced to the functional studies of amphioxus genes. As for macromolecules, Yu and Zhang (2005) tentatively performed sperm-mediated gene transfer in *B. japonicum*, but it was very

possibly a false result because endogenous green fluorescence was observed later in the species (Deheyn et al. 2007; Li et al. 2009) and no other papers using this method for amphioxus appeared after this report. In 2004, Holland and Yu first developed a reproducible method of unfertilized egg microinjection for manipulating gene expression in amphioxus (2004). The immediate applications of this method were microinjections of plasmid containing a reporter gene under the control of *cis*-regulator or a promoter sequence into the eggs of *B. floridae* for transient transgenics (Yu et al. 2004; Holland et al. 2008). Soon after, the method was employed to inject morpholino or mRNA to knock down/overexpress a given gene in amphioxus. And this technology was also performed in *B. lanceolatum*, however less frequently, to overexpress genes and obtain transient transgenic amphioxus. Undoubtedly, microinjection is a very powerful method in the functional studies of amphioxus genes. However, the method has not been performed successfully in Asian amphioxus *B. belcheri* or *B. japonicum* yet. Moreover, in contrast to chemical treatments, the manipulation of gene expression using microinjection techniques is less frequently and still remains to be optimized (Bertrand and Escriva 2011).

Actually, the current application of microinjection techniques is mainly hindered by acquirement of unfertilized eggs. After the first report of continuous culture of two amphioxus *B. belcheri* and *B. japonicum* in the laboratory (Zhang et al. 2007), we were perennially culturing those amphioxus in our lab and were endeavoring to make amphioxus spawning repetitiously. Taking advantage of our recent achievements in the control of amphioxus spawning behavior and the extension of their spawning duration (Li et al. 2012), we described here a feasible and more efficient microinjection method for unfertilized egg microinjection in amphioxus *B. belcheri* following the pioneering work (Holland and Yu 2004).

Materials and methods

Collection of unfertilized eggs and sperm

The fertilization envelope of amphioxus eggs starts to rise up within several seconds after fertilizing, and the perivitelline space becomes very large in about 10 min. The fertilized eggs in fully elevated envelope were hard to be injected since they were not fastened and tended to escape from the microneedle. Besides, the fully elevated envelope is pliable and hard to be peeled off. So we also employed unfertilized eggs for microinjection in our practice. In general, we cultured amphioxus *B. belcheri* according to the previous descriptions (Zhang et al. 2007; Li et al. 2012) and adopted a heat-shock method (Fuentes et al. 2004) to induce amphioxus spawning with some modifications: First, we set artificial photoperiod cycle of the culture room at light from 23:00 to 13:30 (next day) and dark from 13:30 to 23:00 which made amphioxus spawning in the afternoon for the convenience of microinjection. Secondly, to obtain gametes as expected, we raised together 10 males and 10 females with well-developed gonads in a small plastic tank (20 cm in diameter and 30 cm in depth) sanded at the bottom and bubbled continuously, and then, kept them at approximately 22 °C for a minimal of 5 days before heat shock. Before the day when spawning was induced, we cleaned the amphioxus using fresh seawater and put them in 27 °C water bath. On the following day, we screened amphioxus out from the sand and put them individually into 250-ml plastic cups pre-filled with 10 ml filtered seawater, and then laid the cups in 27 °C water bath. After the light turned off at 13:30 we checked each cup every 30 min. Once amphioxus released the gametes, we immediately collected eggs/sperm in each cup. Those unfertilized eggs were subjected to microinjection immediately and the sperm was temporarily stored at 4 °C for fertilizing injected eggs later. Before microinjection, we detected each batch of eggs under a stereomicroscope, and those looked nicely round and without partially elevated envelope were qualified for microinjection (Holland and Yu 2004).

Preparation of microinjection solutions

KCl solution was usually employed as a control to evaluate the physical injury in the microinjection of zebrafish embryos. In order to evaluate the physical injury to amphioxus embryos, we injected 200 mM KCl into unfertilized eggs of *B. belcheri*, since this concentration had been proven nontoxic to *B. floridae* embryos (Yu et al. 2004). To establish microinjection technique in our lab, we applied two reporter gene vectors: actin-β-galactosidase (*LacZ*) reporter vector and actin-red fluorescent protein (*RFP*) reporter vector to judge the efficiency of the transgenic method. The original *LacZ* reporter vector was kindly given by Dr. J.K. Yu, and the *RFP* reporter vector (pmCherry-1 vector) was ordered from Clontech Co. In order to make the report gene driven by a stronger promoter, we cloned a promoter region from *β-actin* gene of *B. belcheri* via PCR method (Fig. 1) and respectively subcloned it into MCS of the two vectors. The sequences of PCR primers were Actin-F: 5′-CCCAAGCTTGCCCTAGATTGTTTCACATCTGG-3′ and Actin-R: 5′-TTTGCGGCCGCTGGAGCAGCAAAATGCAAGAG-3′. The recombined vectors were transfected into *Escherichia coli* DH5 α for propagation, and then they were extracted from the bacteria, dissolved in ddH_2O and stored at −20 °C for use. In addition, to compare with DNA injection, we synthesized capped mRNA of *RFP* gene in vitro. The coding sequence of *RFP* (*mCherry*) gene was subcloned into pXT7 vector in the sense orientation downstream of the T7 promoter. After verification by sequencing, the recombined plasmid *RFP*-pXT7 was

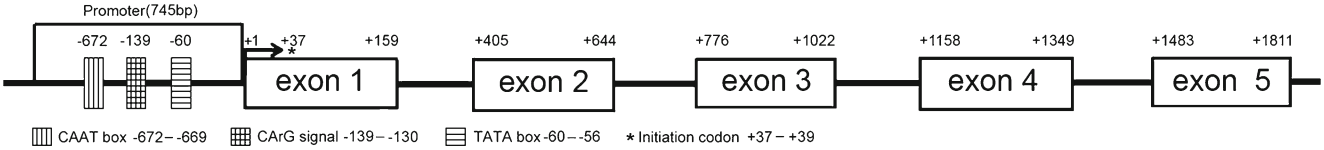


Fig. 1 Schematic diagram showing the genomic organization of *β-actin* gene. The transcription start site was denoted with *+1*, which was demonstrated using 5′ RACE. Exons were shown as *rectangles* and introns were shown as *solid lines*. The initiation codon (ATG) was indicated by a *star*. *Cis*-elements including CAAT box, CArG motif, and TATA box were shown in different boxes as indicated in the figure panel. These *cis*-elements essentially existed in the promoter of *β-actin* gene in other species. *Number above the exon and cis-element boxes* denoted the position of nucleotides in the gene

linearized with *BamH*I, and then was used as the template to synthesize the capped mRNA using mMESSAGE mMACHINE kit (Ambion Co.). The product was stored at −80 °C for subsequent microinjection.

Before microinjection, we separately confected six microinjection solutions: solution I containing 20 % glycerol (Sangon Co.) as a solution diffuser, 5 mg/ml Texas Red dextran (Invitrigen Co.) as an injection tracer, and 200 mM KCl; solution II containing 20 % glycerol, 5 mg/ml Texas Red dextran, and 100 ng/μl actin-*LacZ* vectors; solution III containing 20 % glycerol and 100 ng/μl actin-*RFP* vectors; solution IV containing 20 % glycerol, 5 mg/ml Texas Red dextran, and 100 ng/μl *LacZ* vector without promoter; solution V containing 20 % glycerol and 100 ng/μl *RFP* vector without promoter; and solution VI containing 20 % glycerol and 400 ng/μl *RFP* capped mRNA. We did not add Texas Red dextran into the solutions III, V, and VI, because the emission from this dye will probably disturb the observation on *RFP* gene expression under fluorescence stereomicroscope. All of above solutions were centrifuged for 5 min at 15,000×*g* before use to remove particulates that might clog the needle.

Microinjection

Before the microinjection, we pulled the 1B100F-4 borosilicate glass capillary (WPI Co.) to a very long and fine tip microneedle using a P97 horizontal puller (Sutter Co.) with the parameters (pressure=300, heat=545, pull=50, velocity =50, time=250), and then filled the solution into the microneedle from unpulled end by capillary action. Just before the injection, we broke the needle tip to a diameter of about 2 μm using a fine forceps under a stereomicroscope and then fixed the microneedle in the capillary holder. In order to carry the eggs for microinjection, we coated two 24 mm×32 mm coverslips with 2 μl of 0.25 mg/ml polylysine (mol. wt 150,000–300,000, Sangon Co.) solution. After drying the coverslips in air for about 1–2 min, we put one of the coverslips in a 6-cm Petri dish and gently covered it with about 1 ml filtered seawater (salinity is 26 ‰), and then carefully laid two rows of unfertilized eggs (about 100 eggs of each row) on the coverslip using a fine tip pipette. Microinjection was performed under an IX71 inverted microscope (Olympus Co.) using FemtoJet microinjector and TransferMan NK2 micromanipulator (Eppendorf Co.). Usually, the injection pressure was set at 800 Pa with a pulse length of 200 ms and compensation pressure at 200 Pa. For injecting solutions without Texas Red dextran, we set the injection pressure at about 100 Pa with a pulse length of 200 ms and compensation pressure at 20 Pa. The volume of each injection was adjusted by injecting drops into a dish containing vegetable oil. The radius of the drops was about 6–7 μm, which is about 1 pl in volume measured by the CellSense software. After setting down the facilities, we needled the egg from upper side at a 45° angle and completed the injection of each dish in less than 20 min to avoid evaporation.

Egg fertilizing and culturing

After microinjection, we immediately fertilized the eggs with 10 μl of fresh sperm suspension. The sperm would still be usable in 2 days if it was kept at 4 °C, but more sperm suspension (about 200 μl) was added. Meanwhile, a wild type control (WT, noninjected eggs) from the same batch of eggs and sperm was set to evaluate the efficiency of fertilization and gamete quality. During the elevation of fertilization envelope, we added 10 ml freshly filtered seawater into the Petri dish and gently rotated the dish to detach the eggs from the slip. When the envelopes were fully elevated, we poured the eggs together with seawater into a new Petri dish and replaced a portion of the seawater with fresh one to remove the excess sperm and polylysine dissolved from the coverslip. After that, we checked the eggs under a SZX2-FOF fluorescence stereomicroscope with a rhodamine filter (Olympus Co.) and removed those eggs without red fluorescence emission if the injected solution contained Texas Red dextran. Since all of those manipulations were performed at room temperature, the eggs started to cleavage in 40–45 min after fertilization. The embryos were raised in an incubator setting the temperature at 25 °C and the humidity at 85 % until desired stages. After hatching, dead eggs/embryos and the discarded fertilization envelopes should be removed instantly to keep the seawater clean and one third of the seawater should be replaced with fresh filtered seawater every day. Once the larvae mouth opened,

they should be fed with unicellular algae (*Dicrateria zhangjiangensis*) once a day.

Detection of LacZ and RFP signals

The procedures of LacZ detection were essentially after the description of Yu et al. (2004). To detect the expression of *LacZ* gene driven by *β-actin* promoter, injected embryos at different developmental stages were fixed in 1 % glutaraldehyde confected in seawater at room temperature for 30 min and subsequently washed four times (10 min each) with PBST (0.1 % Tween-20 in PBS). Then, the embryos were transferred into fresh staining solution (4 mM $K_4Fe(CN)_6$, 4 mM $K_3Fe(CN)_6$, 50 mM $MgCl_2$, 1 mg/ml X-gal, and 0.1 % Tween-20 in 1× PBS) and incubated at 37 °C in the dark for 1 h or overnight. When the reaction was completed, the embryos were washed three times (10 min each) with PBST and postfixed in 4 % paraformaldehyde confected with 1× PBST for 50 min at room temperature. Finally, the embryos were mounted on glass slides in 80 % glycerol solution and photographed under inverted microscope. Some of the embryos and larvae were cut into

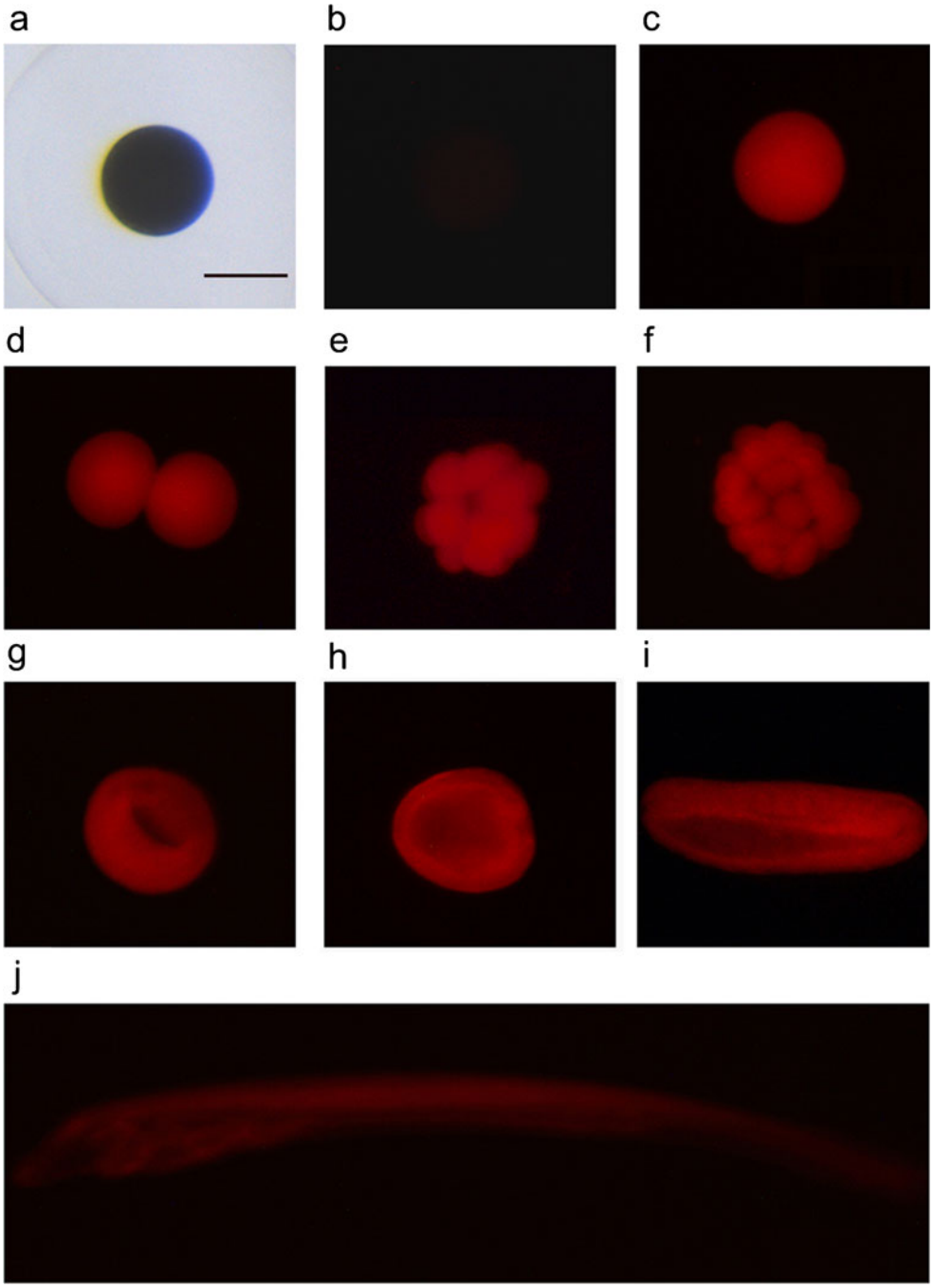


Fig. 2 Embryonic development of amphioxus microinjected with solution I. The embryos and larvae were photographed under a fluorescence stereoscope with rhodamine filter. The noninjected fertilized eggs did not present endogenous red fluorescence (**a, b**), and the injected embryos displayed visible red fluorescence emitting from the dye (**c**–**j**). **a** One-cell stage of noninjected embryo photographed under brightfield. **b** One-cell stage of noninjected embryo photographed with rhodamine filter. **c** One-cell stage after fertilization. **d** Two-cell stage. **e** 16-cell stage. **f** 32-cell stage. **g** Mid-gastrula. **h** Early neurula. **i** Mid-neurula. **j** 2-day larva. *Scale bar*=100 μm

5 μm serial histological sections after being double embedded in agar-paraffin, and then restained with eosin. To examine the expression of *RFP*, the living embryos were observed and photographed directly under a fluorescence stereomicroscope using rhodamine filter (Olympus Co.).

Whole-mount in situ hybridization

In order to detect the expression pattern of *β-actin* gene using whole-mount in situ hybridization, the 3′ untranslated region of the gene was amplified with a pair of primers (F: 5′-ACTGGAACGAAGTTAGGAC-3′ and R: 5′-GATCACAGCATTGCAGATGG-3′) and subcloned into pGEM-T easy vector for synthesizing antisense RNA probe in vitro. Digoxigenin-labeled antisense RNA probe was prepared according to the manufacturer's instruction (Promega Co.). The whole-mount in situ hybridization was carried out according the previous description (Holland 1999). The embryos were photographed under the inverted microscope.

Results and discussion

Normally developing ratio of injected eggs and efficiency of microinjection

Although amphioxus has been considered as a good model in evolutionary and developmental studies since many years ago,

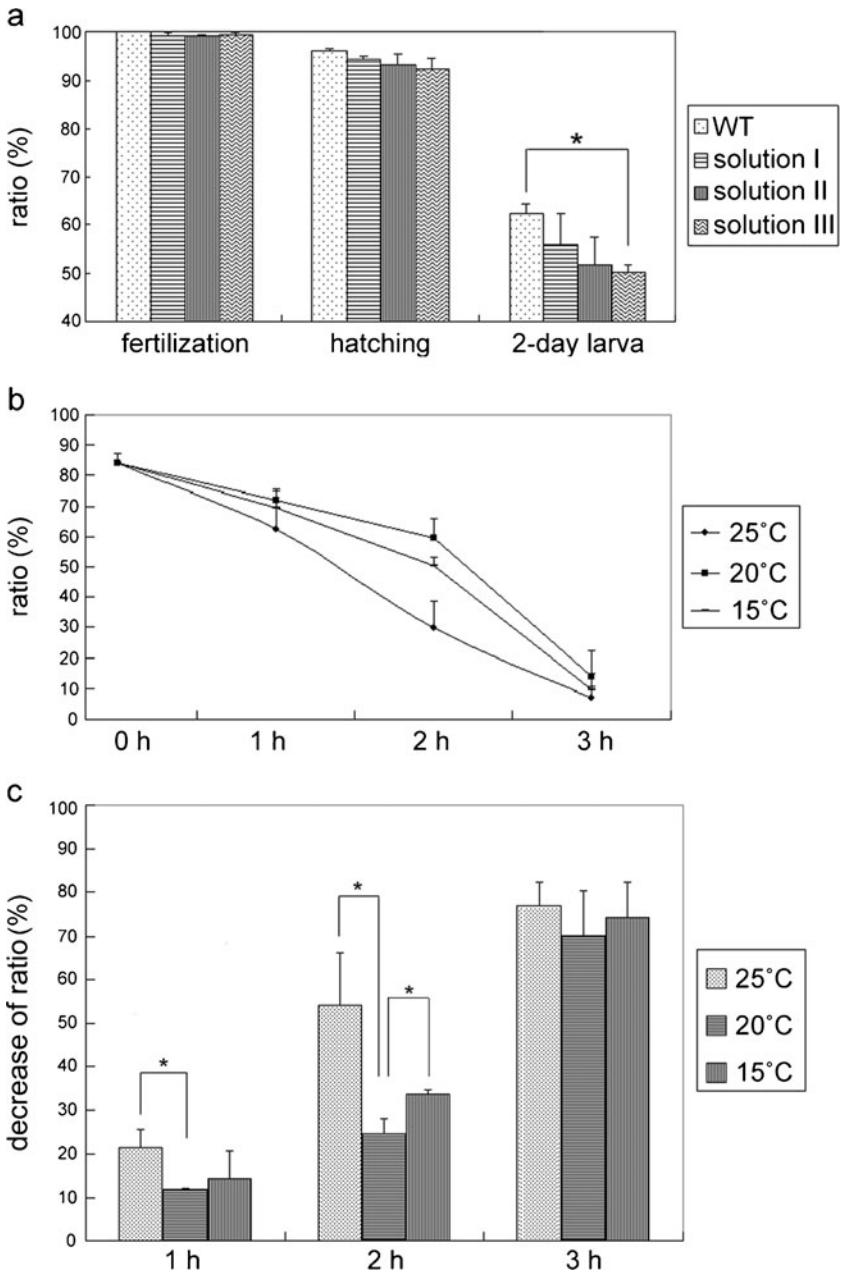


Fig. 3 Statistical results of embryonic development. **a** The ratios of fertilization, hatch, and normal development of 2-day larva. Statistical analyses were performed with SPSS version 13.0. Results were given as mean ± SD, and statistical analysis was carried out by *t* test. P values less than 0.05 were considered as statistically significant and those more than 0.05 were not shown. **b** The relationship between normally developing ratios of 2-day larvae and the temperature combining with the duration of postponed fertilization. A portion of the eggs were fertilized immediately after spawning (0 h). The rest of unfertilized eggs were preserved at 25, 20, and 15 °C for 1–3 h before fertilization. **c** The decrease of normally developing ratio after postponed fertilization (1, 2, and 3 h) vs that of 0 h. *P* values less than 0.05 were considered as statistically significant and those more than 0.05 were not shown

Table 1 The ratios of fertilization, hatch, and normal development larva

Injected solution	Unfertilized egg		Fertilization		Hatched neurula		2-Day larva	
	Batch No.	No.	No.	Ratio (%)	No.	Ratio (%)	No.	Ratio (%)
WT	1	304	304	100	289	95.07	198	65.13
	2	297	297	100	287	96.63	179	60.27
	3	358	358	100	344	96.09	220	61.45
	Total	959	959	100	920	95.90	597	62.30
Solution I	1	232	229	98.7	221	95.26	142	61.21
	2	225	224	99.6	212	94.22	106	47.11
	3	208	207	99.5	194	93.27	124	59.62
	Total	665	660	99.2	627	94.30	372	55.90
Solution II	1	262	260	99.2	246	93.89	139	53.05
	2	251	249	99.2	234	93.23	150	59.76
	3	295	294	99.7	267	90.51	146	49.49
	4	240	237	98.8	230	95.83	111	46.25
	Total	1,048	1,040	99.2	977	93.20	546	52.10
Solution III	1	239	239	100.0	221	92.47	121	50.63
	2	264	262	99.2	236	89.39	128	48.48
	3	215	213	99.1	204	94.88	112	52.09
	Total	718	714	99.4	661	92.10	361	50.30

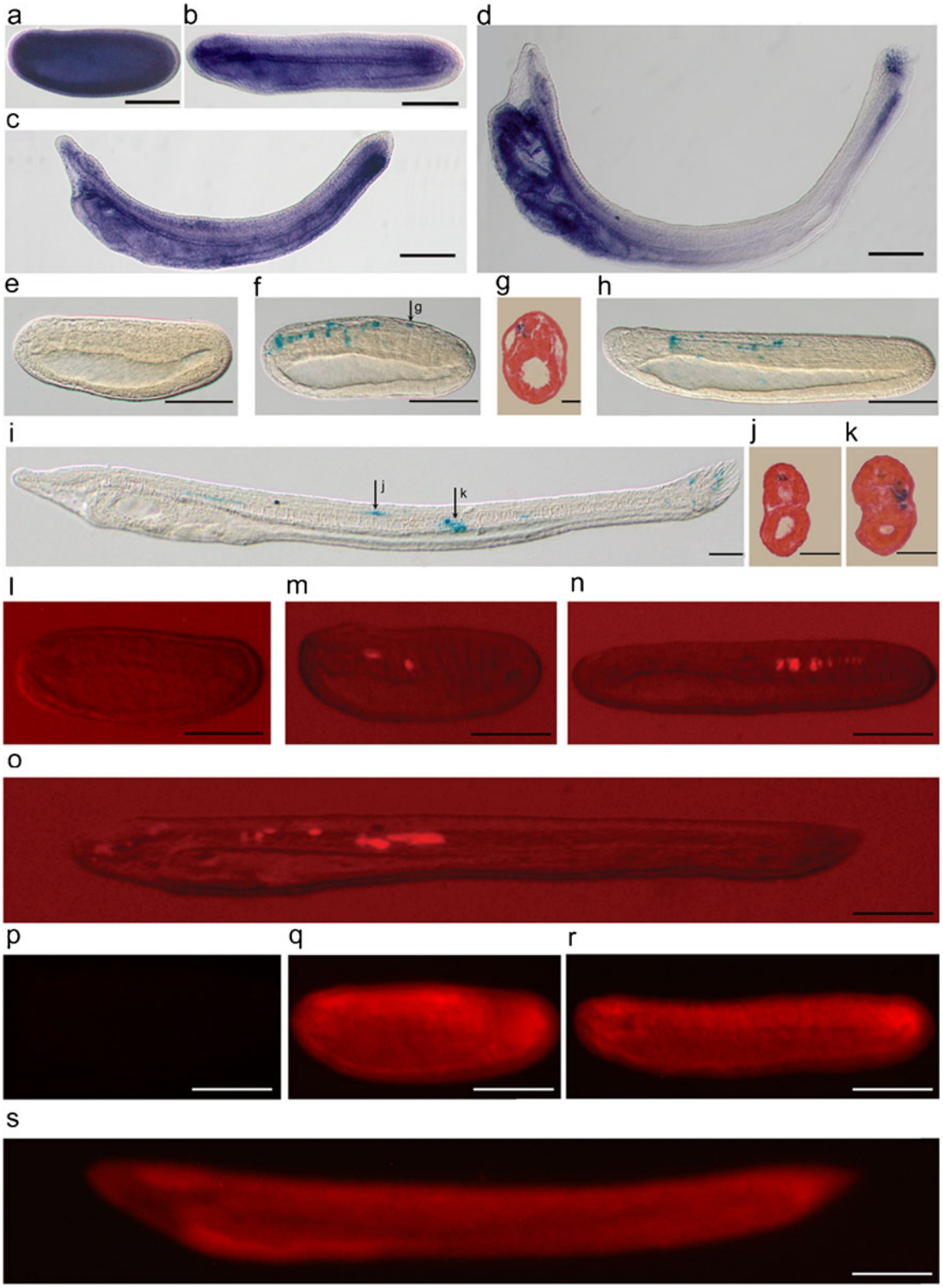
its application is hindered by the lack of an efficient embryonic manipulation method. The laboratory culture, prolonged breeding duration, as well as inducement of spawning of amphioxus provide us more opportunities to find ways to control their spawning behavior (Fuentes et al. 2004; Zhang et al. 2007). Fortunately in 2011 breeding season, we made some evident progress in the breeding control for amphioxus (Li et al. 2012). This allowed us to develop an efficient microinjection method on *B. belcheri* in our lab.

In our experiments, we applied six different injection solutions respectively containing KCl, actin-*LacZ* or actin-pmCherry-1 vectors, the vectors without promoter, and capped *RFP* mRNA (see "Materials and methods") for microinjection of the unfertilized eggs. Initially, we intended to evaluate the injuries to the embryos due to the injection of above solutions and thus performed following injecting experiments of each solution on more than three batches of eggs.

First, we injected more than 600 unfertilized eggs with solution I and compared their developing ratio with that of noninjected eggs (WT). All of those injected eggs emitted visible red fluorescence from the dye under a fluorescence stereomicroscope indicating successful injection (Fig. 2). The results displayed that both fertilizing and hatching ratio of injected eggs were almost equal to that of WT, but the normally developing ratios of injection seemed to be slightly lower than that of control (Fig. 3a), suggesting that solution I had no obvious physical injury of microinjection or chemical deleterious effect upon the embryos. Then, we respectively performed microinjections on three batches of unfertilized eggs using solutions II and III. The statistic results showed that there were also no significant difference between injected eggs and WT in fertilizing or hatching ratio, but normally developing ratios were above 10 % lower than that of WT and also slightly lower than that of solution I-injected embryos (Table 1), hinting that exogenous gene expression might affect embryonic development in some ways.

Actually, amphioxus is an ecologically *r*-selected organism which owns special traits that include high fecundity, small body size, early maturity onset, but high mortality also. A ripe female amphioxus could release almost ten thousands of eggs in one night, and therefore larvae usually have a

Fig. 4 Gene expression in developing amphioxus embryos. The endogenous expression pattern of *β-actin* revealed by in situ hybridization (**a**–**d**). **a** At early neurula stage, expression was particularly intense at the mesendoderm. **b, c** At late neurula stage and the stage with opened mouth, expressions were especially intense at the pharyngeal endoderm, notochord, and the posterior region of the embryo. **d** At 2-day larva stage, expression also continued throughout the pharynx, the posterior region of gut, and tail bud. **e** Early neurula stage of embryo injected with solution IV. The control embryo did not show any *LacZ* expression. Exogenous *LacZ* gene expression in developing embryos injected with solution II (**f**–**k**). **f** Early neurula stage. **g** Cross section through level **g** in photo **f**. **h** Mid-neurula stage. **i** 2-day larva stage. **j, k** Cross sections through levels **j** and **k** in photo **i**. **l** Early neurula stage of embryo, injected with solution V. Those control embryos did not emit any exogenous red fluoresce. Exogenous *RFP* expression in developing embryos injected with solution III (**m**–**o**). **m** Early neurula stage. **n** Mid-neurula stage. **o** Larva with opened mouth. Translation of *RFP* mRNA in developing amphioxus embryos injected with solution VI (**p**–**s**). **p** Early neurula of noninjected embryo as a negative control. **q** Early neurula. **r** Mid-neurula. **s** Larva with opened mouth. Whole mounts (**a**–**s**) in lateral view with anterior at left. *Scale bar* in **g**, **j**, and **k** indicate 20 μm, and those in other panels indicated 100 μm



high mortality ratio. So the process of microinjection and egg incubation applied in amphioxus should be efficient enough to produce large number of injected eggs for subsequent statistical analysis and reduce the mortality during the embryo development. In Florida amphioxus, 500 or more eggs could be injected in one night of spawning and 50 % of those eggs survived till neurula stage (Holland and Yu 2004). In our experiments, one practiced person was able to inject more than 200 eggs on single coverslip within 15–20 min and could inject about 2,000 eggs in one spawning afternoon. The surviving

ratio of hatched neurula was above 92 % in general, much higher than that of previous report (Holland and Yu 2004).

This higher normally developing ratio might benefit from the following modifications in our experiment. To hold unfertilized eggs, Holland and Yu (2004) laid eggs on the bottom of a coated dish but we put eggs on a coated coverslip. The latter was smaller than the bottom of a Petri dish and thus less polylysine was adopted. In addition, we immediately diluted polylysine by adding some fresh seawater and transferring the eggs to a new dish after microinjection, which was not mentioned in their protocol. Moreover, we cultured the eggs in a high-humidity incubator to keep constant salinity, which is important for embryos developing well.

Expression of *LacZ* reporter construct in amphioxus embryos

Gene reporter systems played an important role in gene expression and regulation studies in many animal models. Being one of a widely used reporter, *LacZ* is a reporter gene sensitively to be detected in transgenic studies and useful in *cis*-regulator element and promoter analysis. So we employed this system to evaluate the efficiency of our microinjection on amphioxus unfertilized eggs.

To have a high expression level for our test, we adopted the promoter of amphioxus *β-actin* gene (Fig. 1) in our reporter constructs. Actually, endogenous expression of *β-actin* gene was throughout the body in the mesendoderm at early neurula stage (Fig. 4a) and especially intense at the pharyngeal endoderm, notochord, and the posterior region of the embryo at late neurula stage and the stage when the mouth opened (Fig. 4b, c). At 2-day larva stage, the expression continued throughout the pharynx, the posterior region of gut, and tail bud but disappeared or was hard to be detected in the middle part of the body (Fig. 4d). Using the actin-*LacZ* reporter construct, we totally injected about 1,000 unfertilized eggs and fixed the embryos/larvae at different developmental stages to detect *LacZ* expression. The results showed that 91.8 % (385/419) of neurula and 53.4 % (62/116) of larvae displayed blue stains, indicating the *LacZ* gene expression in the embryos. The expression ratio of *LacZ* gene in our microinjection was higher than that first reported by Yu et al. (2004). However, the embryo injected with *LacZ* vector without the promoter did not show any *LacZ* expression (Fig. 4e). We speculate that this result is mainly due to the stronger promoter of *β-actin* gene in our reporter system than that of *AmphiFoxD* in Yu's reporter system. Even though, the expression ratio gradually fell down along with the embryonic developing indicating a dilution of the plasmid over cell generations during development, and the gene expression was also mosaic like that in Florida amphioxus (Yu et al. 2004; Holland et al. 2008). Although most of the expression was located at the endogenous expression domains of *β-actin* gene, the *LacZ* signal was sometime detected in somites in the middle part of the body (Fig. 4k), suggesting that the signal might not indicate the *β-actin* expression but nonspecific. Thus our results just proved that the exogenous gene was successfully introduced into unfertilized amphioxus eggs and expressed in the developing embryos.

Expression of *RFP* reporter construct in amphioxus embryos

Though it has been widely used in many model animals including amphioxus, *LacZ* reporter system could not be utilized to visualize reporter gene expression in vivo. Therefore, a fluorescent protein gene such as *GFP* or *RFP* is needed for monitoring gene expression in living organisms. This approach is particularly useful in amphioxus for its external embryonic development and transparent body throughout its whole life cycle. However, endogenous green fluorescent protein was discovered in amphioxus *B. floridae* (Deheyn et al. 2007), and more than 10 amphioxus *GFP* genes were characterized from three species of genus *Branchiostoma* (Li et al. 2009; Bomati et al. 2009) so far. They emitted green fluorescence from different tissues/organs during the embryonic development and disturbed the observation on report gene expression. So we reconstructed a *RFP* reporter system in pmCherry-1 vector instead of *GFP*.

In this study, we injected 628 unfertilized eggs with the actin-pmCherry-1 vector, then fertilized the eggs and cultured the embryos as above description. The results showed that about 80.5 % (211/262) of neurula and 47.5 % (58/122) larvae emitted red fluorescence. The *RFP* expression was also mosaic in the developmental embryos (Fig. 4m–o) and the signal mainly appeared in notochord and somites. A slightly lower statistical ratio might result from the missing injected embryos, which had not been picked out in this experiment because no trace dye was added in the solution III (see "Materials and methods"). Further, we injected pmCherry-1 vector-only (without promoter) into the unfertilized eggs as negative controls, and all of those injected embryos did not show any exogenous *RFP* expression during the development (Fig. 4l). Moreover, we also injected capped mRNA of *RFP* gene into amphioxus eggs, which diffused to all cells and synthesized red fluorescent protein in the whole embryo (Fig. 4q–s). Thus, the results suggested a highly efficient method and also demonstrated the usability of *RFP* reporter assay in the study of amphioxus embryos.

Nevertheless, the observation might be potentially disturbed by unicellular algae fed in larval gut while using *RFP* report system because the algae granules sometime looked like reddish under a fluorescence stereomicroscope with rhodamine filter (data not shown). For safety, we took count of the larvae emitting red fluorescence before their mouth

opened or excluding the fluorescence from gut of larvae with opened mouth (Fig. 4o).

Factors influencing on the efficiency of microinjection

In order to have a high efficiency of microinjection, several factors should be considered in the experiments. First, both eggs and sperm should be healthy and vigorous. Freshly collected eggs looked rounded and none of them appeared partially elevated fertilization envelope. The dead eggs and debris should be removed timely because they would not only clog the injection needle but also deteriorate the water quality. The vigor of eggs will decline after spawning if it is not fertilized instantly. In European amphioxus, eggs could be fertilized normally within about 1 h after spawning and the embryos developed well (Theodosiou et al. 2011). To test the vigorous duration of eggs, we put unfertilized eggs at different temperatures for a certain time before fertilization. The results indicated that the fertilizing ratio was not evidently affected when the eggs were kept at 15–25 °C for less than 3 h, but the ratio of normally developing larvae was decreased gradually (Fig. 3b). The morphological defects observed in abnormal larvae were mainly anterior truncation, curved, and shrivel body. The normally developing larvae respectively decreased by 21.52 % (1 h), 54.09 % (2 h), and 76.68 % (3 h) when the eggs were kept at 25 °C, by 11.92 % (1 h), 24.45 % (2 h), and 70.11 % (3 h) at 20 °C, or by 14.32 % (1 h), 33.64 % (2 h), and by 74.34 % (3 h) at 15 °C (Fig. 3c). The results indicated that 20 °C might be better than the other two temperatures for keeping the vigor of the eggs. This allows us to inject more unfertilized eggs from the same egg batch. As regards to sperms, they were still able to fertilize eggs after two or more days if preserving them at 4 °C, but more sperms were needed. Although stale sperm could fertilize eggs, the mortality or abnormal ratio of embryos might increase as the storage time extended longer.

In zebrafish, embryos are held in wedged-shaped troughs shaped in 1.5 % agarose for microinjection. But this method is not suitable for amphioxus eggs because they are too small to be arranged in wedged-shaped troughs. So the second key in this procedure is to immobilize amphioxus eggs for microinjection. In *B. floridae*, Yu et al. (2004) used polylysine-coated Petri dish to hold the eggs, and they noticed that too much polylysine was harmful because toxicity of polylysine and overadhering led a lower fertilization and higher malformation ratio. In our experiments, we employed coverslip to carry the eggs and tried different volumes (10, 5, 2, and 1 μl) of 0.25 mg/ml polylysine solution to coat two coverslips. As 1 μl of polylysine solution was not enough to immobilize eggs, we finally applied 2 μl of polylysine for coating the coverslips. This is very convenient to be performed just before using and easy to detach the eggs from the slip by gently rotating the dish after microinjection. Moreover, this method reduced the chemical injury towards the eggs because less polylysine was used.

Thirdly, the volume of each injection should be accurately controlled via adjusting injection pressure. In the experiment of zebrafish, the appropriate injection volume of plasmid DNA (100 ng/μl) is about 200 pl for each one-cell stage fertilized egg, which is about 0.18 % of egg's volume. For amphioxus unfertilized eggs, we strictly controlled the injection volume less than 2 pl for each egg, which is about 0.14 % of the volume of amphioxus egg. And a larger injection volume (>8 pl) tended to break the eggs immediately.

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