Technical note: Viability and motility of vitrified/thawed primordial germ cell isolated from common carp (*Cyprinus carpio*) somite embryos

Y. Kawakami, T. Saito, T. Fujimoto, R. Goto-Kazeto, E. Takahashi, S. Adachi, K. Arai and E. Yamaha

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Running head: Motility of vitrified primordial germ cell

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Y. Kawakami,*[,2] T. Saito,* T. Fujimoto,*[,3] R. Goto-Kazeto,*[,4] E. Takahashi,* S. Adachi,† K. Arai,[†] and E. Yamaha[*]

*Nanae Fresh Water Laboratory, Field Science Center of Northern Biosphere, Hokkaido University, Nanae, Hokkaido 041-1105, Japan

†Faculty and Graduate School of Fisheries Sciences, Hokkaido University, Hakodate 041-8611, Japan

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[2]Corresponding author. Tel: +81 138 65 2344; Fax: +81 138 65 2239

E-mail address: yutakakjp@hokudai-nanae.jp (Y. Kawakami)

[3]Current address: Center for Aquaculture and Environmental Research, Fisheries and Oceans Canada, West Vancouver, BC V7V 1N6, Canada

[4]Current address: Support Office for Female Researchers, Administration Center, Hokkaido University, Sapporo 060-0808, Japan

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ABSTRACT

The feasibility of cryopreserving common carp (*Cyprinus carpio*) primordial germ cells (**PGC**) by vitrification of whole embryos at the 22 to 28 somite stage was investigated. Green fluorescent protein (**GFP**)-labeled PGC were cooled rapidly using liquid nitrogen after exposure to a pretreatment solution containing 1.5 *M* cryoprotectant (ethylene glycol or dimethyl sulfoxide, 30 or 50 min) and a vitrification solution containing 3 *M* cryoprotectant and 0.5 *M* sucrose (5, 10, 20, or 30 min). Embryonic cells that were pretreated for 30 min and vitrified for 20 min with ethylene glycol had the highest rate of survival of embryonic cells (68.6%; $P < 0.01$), an optimal highest percentage of viable PGC (73.8% to 74.9%; $P < 0.05$), and no evidence of ice formation after thawing. The vitrified/thawed PGC were transplanted into blastula-stage embryos from goldfish (*Carassius auratus*). The PGC maintained their motility and moved to the gonadal ridge of the host embryo. Thus, the combination of vitrification and transplantation to produce germ-line chimeras is a powerful tool for the artificial production of next-generation offspring.

Key Words: common carp, germ-line chimera, goldfish, primordial germ cell, transplantation, vitrification

INTRODUCTION

Cryopreservation of gametes and embryos is a useful technique for conserving the genetic resources of a species. In teleosts, sperm has been successfully cryopreserved (Ohta et al., 2001; Kusuda et al., 2005; Cabrita et al., 2009). However, the development of cryopreservation techniques for teleost embryos has received less attention (Cabrita et al., 2009; Li et al., 2009).

Germ-line cells, including primordial germ cells (**PGC**), are the only cells that have the potential to transmit genetic information to the next generation. Thus, they have considerable use for gene banking and cryopreservation, particularly for the production of donor gametes via germ-line chimeras. In our recent studies, inter- and intra-species germ-line chimeras that ensured complete germ cell replacement using single PGC transplantation (**SPT**) methods have successfully produced offspring (Saito et al., 2008; Kawakami et al. 2010). Moreover, we developed a vitrification method for zebrafish (*Danio rerio*) embryos and viable offspring have been obtained from germ-line chimeras using PGC that were isolated from vitrified/thawed embryos (Higaki et al, 2010a; Kawakami et al., 2010). This suggests that the use of germ-line chimeras has considerable potential for the production of viable offspring. However, our method for vitrification of PGC has not yet been developed for other teleosts.

The common carp (*Cyprinus carpio*) is one of the most important commercial species in Japan due to their high market value for food and ornamental use (fancy carp: *Cyprinus carpio*). In this study we developed a method for the preservation of PGC from common carp embryos by vitrification. In addition, the viability of PGC that was derived from embryos after a vitrification/thawing process and the migratory activity of PGC after transplantation into a host (goldfish, *Carassius auratus*) was evaluated.

MATERIALS AND METHODS

Fish

This study was carried out in accordance with the Guide for the Care and Use of Laboratory Animals in Hokkaido University. Fertilized eggs were obtained from common carp and normal and albino-type goldfish according to the methods described in Kawakami et al. (2011).

Microinjection of mRNA

Common carp embryos at the 1 to 4 cell stage were injected with 80 pg of green fluorescent protein (**GFP**)-cc(common carp)*nanos*-3'UTR strand-capped mRNA. The GFP-labeled mRNA was synthesized by SP6 transcription from a *Xba*I-linearized plasmid, which has a *Xba*I site downstream of cc*nanos*-3'UTR, using the mMESSAGE mMACHINE system (Ambion, Austin, TX), as described in Kawakami et al. (2011). Common carp PGC were visualized by the mRNA synthesized from GFP-cc*nanos*-3'UTR. Following injection, the embryos were cultured at 18°C in 96-well plates.

Vitrification of Donor PGC

Vitrification was performed according to the methods described in Kawakami et al. (2010).

In Experiment, 1 the effectiveness of the cryoprotectants ethylene glycol (**EG**) and dimethyl sulfoxide (**DMSO**) was compared by analyzing the ice formation and the number of live PGC in vitrified embryos, as follows. The embryos that were injected with GFP-cc*nanos*-3'UTR mRNA were cryopreserved by vitrification. Embryos at the 22 to 28 somite stage with the yolk sac removed were transferred into pretreatment solution (**PS**: 1.5 *M* EG [Wako, Tokyo, Japan] or 1.5 *M* DMSO [Wako]) in embryo medium (**EM**: 13.7 m*M* NaCl, 0.54 m*M* KCl, 0.025 m*M* Na_2HPO_4, 0.044 m*M* KH_2PO_4, 1.3 m*M* $CaCl_2$, 1.0 m*M* $MgSO_4$, 4.2 m*M* $NaHCO_3$ at pH 7.2, and 0.35% BSA [Wako]) at room temperature (20 to 23°C) for 30 min. The pretreated embryos were subsequently transferred to the vitrification solution (**VS**: EM containing 0.7% BSA, 0.5 *M* sucrose [Wako],

and 3 *M* EG or 3 *M* DMSO). The embryos were then incubated for 5, 10, or 20 min. These embryos were loaded into the mesh of the vitrification device and immersed in liquid nitrogen. The vitrified embryos were stored in liquid nitrogen for 1 d, after which they were warmed by immersing the vitrification device directly in thawing solution (EM supplemented with 0.7% BSA and 0.5 *M* sucrose) at room temperature for 1 min. The samples were then transferred to fresh thawing solution for 1 min before transfer to Ringer's solution.

To examine the ice formation in the embryos during cooling and survival of embryonic cells recovered from vitrified embryos, non-GFP treated embryos with the yolk sac removed were cooled rapidly after serial exposure to PS and VS. During warming of the embryos, ice formation was checked by visual inspection for the presence of a milky appearance, as described by Sheffen et al. (1986). Three embryos were used for analysis.

To examine the survival of PGC in vitrified/thawed embryos, GFP-labeled embryos with the yolk sac removed were photographed using a fluorescence stereomicroscope (MZ16F: Leica Microsystems, Inc., Bannockburn, IL) and cooled rapidly after serial exposure to PS and VS. Embryos were photographed 10 min after thawing and GFP-positive PGC, which are considered to be live PGC, were counted. Three embryos were used per replicate.

In Experiment 2, the effects of different exposure times to PS and VS containing EG on ice formation, embryonic cell viability, and the number of live PGC in vitrified embryos were compared as follows. The GFP-labeled and non-GFP treated embryos at the 22- to 28-somite stage with the yolk sac removed were transferred into PS (containing 1.5 *M* EG) for 30 or 50 min. The pretreated embryos were subsequently transferred to the VS (containing 3 *M* EG) for 10, 20, or 30 min. Ice formation and the number of live PGC in vitrified/thawed embryos were analyzed as in Experiment 1.

To assess the embryonic cell viability of vitrified embryos, vitrified/thawed non-GFP-labeled embryos at the 22- to 28-somite stage with the yolk sac removed were digested by pipetting in 30 μL of 0.25% sodium citrate in Ringer's solution. The homogenate was stained with 0.5% Trypan Blue (Nacalai Tesque, Kyoto, Japan). The number of Trypan Blue-positive and negative cells was counted using a hematocytometer. The

experiment was performed 3 times using 3 embryos per replicate.

PGC Transplantation

For transplantation, GFP-labeled embryos at the 22- to 28-somite stage with the yolk sac removed were treated with PS for 30 min and with VS for 20 min, using EG as the cryoprotectant. Ten to 15 embryos were loaded into the mesh of the vitrification device and immersed in liquid nitrogen. The vitrified embryos were stored in liquid nitrogen for 1, 15, 43, or 44 d. The donor (common carp) embryos containing visible PGC after thawing were dissociated into single cells using 0.25% sodium citrate in Ringer's solution after thawing. The PGC from the dissociated cells were transplanted at room temperature into host (normal or albino-type goldfish) blastulae following an SPT method that ensured complete germ cell replacement, which involved knockdown of endogenous PGC in the host embryo and subsequent transplantation of a visualized PGC from a donor (Saito et al., 2008; Kawakami et al., 2010). The host blastulae were initially incubated at 20°C but underwent a period of delayed development (incubation for approximately 1 d at 10°C) before transplantation. The transplantation procedure took between 2 and 5 h, during which 1 isolated PGC was picked up with a glass micro-needle under a stereomicroscope and transplanted into the marginal region of the blastodisc of a goldfish blastula. The normal developmental rate of albino-type goldfish embryos is slower than that of normal goldfish, as melanophore is low. This has the advantage that it is easier to observe the transplanted PGC in the albino-type host. Chimeric embryos were examined and photographed using a fluorescence stereomicroscope.

Statistical Analysis

Cell survival rates were subjected to angular transformation (arcsin $\sqrt{\%}$). The effects of the cryoprotectant used (EG or DMSO) and of the exposure time to VS on PGC survival rates and of the exposure time to VS and PS under EG on PGC survival rates and cell survival rates were analyzed by 2-way analysis of

variance (ANOVA). A computer program (JUSE-Stat Works Ver4.86 for Windows) was used for the statistical analyses. A $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

In previous studies of teleosts, PGC isolated from cryopreserved blastomeres and gametes produced germ-line chimeras (Kusuda et al., 2002; Kobayashi et al., 2007). In recent years, we have reported effective procedures in zebrafish. We succeeded in production of germ-line chimeras using PGC isolated from vitrified/thawed yolk-intact somite embryos or vitrified/thawed cultured embryoids with SPT transplantation methods (Higaki et al., 2010a; Kawakami et al. 2010). In these studies, mixed EG and DMSO were used as cryoprotectants. In previous studies, EG and DMSO have been the cryoprotectants of choice for preserving the embryos of a range of species (Kusuda et al., 2002, 2004; Kobayashi et al., 2007; Yasui et al., 2011). However, the toxicity of these vehicles is species-specific in teleosts (Cabrita et al., 2006). Higaki et al. (2010b) compared EG, DMSO, glycerol, methanol, propylene glycol, and 1,3-butylene glycol for use in vitrified zebrafish somite embryos. It was apparent that EG was a better cryoprotectant than DMSO. In the present study in common carp, the number of visible (live) PGC was greater in the embryos treated with EG than in those treated with DMSO ($P < 0.05$; Table 1, Exp. 1). Ethylene glycol should be first choice of cryoprotectant in cyprinid fish.

Ice crystal formation is assumed to be the most common factor in the failure of teleost embryo cryopreservation. Intracellular ice formation plays a central role in cell injury during cooling (Li et al., 2009). A number of techniques have been tested for the cryopreservation of fish embryos, including the use of different cryoprotectants, immersion times, and devices for immersing the embryos in liquid nitrogen**.** However, none have resulted in viable embryos or hatching after vitrification/thawing (Cabrita et al., 2006, Edashige et al., 2006, Li et al., 2009). The visible damage on the surface of the embryo (Figure 1A) suggests that ice crystals formed during cooling (Higaki et al., 2010a). In contrast, the somite structure can clearly be seen in the

absence of ice crystal formation (Figure 1B). There were differences in viability and survival among the groups that were exposed to different combinations of PS and VS exposure times ($P < 0.01$; Table 1). Optimal cell viability and numbers of live PGC were achieved after 30 min of pretreatment and 20 min of vitrification using EG. Moreover, little surface damage was observed in embryos that were vitrified with this protocol, and the protocol was adopted for vitrified embryos (Table 1, Exp. 2).

The PGC migrate from their emergent site to the gonadal ridge during embryonic development (Mölyneaux and Wylie, 2004). In a previous study, both EG and DMSO were used for the vitrification of zebrafish embryos that contained GFP-labeled PGC. The authors then successfully obtained functional gametes from vitrified/thawed GFP-labeled PGC using germ-line chimeras. Thus, vitrified/thawed PGC can be kept alive, have motility, and maintain the ability to differentiate (Higaki et al., 2010a; Kawakami et al., 2010). Successful migration of donor PGC to the host gonadal ridge is used to determine PGC survival after vitrification. In our study, the migration ability of PGC isolated from vitrified/thawed embryos was analyzed by transplantation of single PGC into gold fish blastulae. The PGC isolated from embryos kept in liquid nitrogen for more than a month had migration ability (11.3% and 14.4% of manipulated embryos). After transplantation, GFP-positive PGC were visible in the host gonadal ridge (Figure 3) and the donor GFP-labeled PGC were visible in the chimeras even at 24 d post fertilization. Thus, our procedure may be used for vitrification of PGC in common carp.

In summary, we developed a method for the vitrification of common carp PGC that preserves their motility upon thawing. We used a related host species (goldfish) and we did not check the growth and differentiation of donor PGC. Common carp spawn in early summer, thus it is difficult obtain a constant supply of embryos. However, using our techniques for vitrification, we have the ability to produce common carp PGC for transplantation at any time. We hope to develop inter- and intra-species germ-line chimeras using a range of host species. This technique would provide a useful alternative to sperm cryopreservation for the production of viable offspring.

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Legends for Figures

Figure 1. Gross morphology of common carp embryos (yolk-removed) at the 22 to 28 somite stage after cooling and warming. (A) An embryo that experienced ice formation during cooling and has a severely degraded embryonic body. The picture was taken 10 min after thawing. (B) An embryo that did not experience ice formation during cooling and has a leathery surface embryonic body. The picture was taken 10 min after thawing. (C) A fresh, control embryo (yolk-removed). The embryo with green fluorescent protein (GFP)-labeled primordial germ cells (PGC) were injected with GFP-cc*nanos*-3'UTR mRNA. Bar = 500 µm.

Figure 2. Time-lapse images of a live primordial germ cell (PGC) from a vitrified/thawed embryo that was preserved for 43 d using an ethylene glycol based cryoprotectant and a pretreatment-vitrification cycle of 30/20 min. The pictures were taken using light (A1, B1, C1, D1) and fluorescence microscopy (A2, B2, C2, D2). (A) 10 min after thawing, (B) 90 min after thawing, (C) 270 min, and (D) 450 min. Bar = 200 µm.

Figure 3. Migration of donor primordial germ cel (PGC) in a vitrified/thawed embryo. Arrows indicate the location of the fluorescent protein-labeled PGC. (A) Host at 3 d post fertilization (dpf), (B) 6 dpf, and (C) 24 dpf. Bars = 500 µm.

Table 1. Percentages of live cell and the number of live primordial germ cell (PGC) recovered from 22 to 28 somite embryos that were cooled rapidly after serial exposure to a pretreatment solution (PS) and a vitrification solution (VS)

Experimental group of cryoprotectant	Exposure time (min)		Ice formation[1]	Cell viability (%)	Live PGC (%)[2]	No. of visible PGC[3]
	PS	VS				
None (control)						3 to 16
Exp. 1						
Ethylene glycol	30	5	+		$71.1 \pm 7.7^{E, c}$	
	30	10	±		$76.7 \pm 25.1^{F, c}$	
	30	20	–		$74.9 \pm 11.7^{F, c}$	
Dimethyl sulfoxide	30	5	±		$8.6 \pm 9.1^{G, d}$	
	30	10	±		$44.4 \pm 9.6^{H, d}$	
	30	20	±		$54.0 \pm 11.2^{H, d}$	
Exp.2						
Ethylene glycol	30	10	–	$53.9 \pm 3.6^{A, a}$	$78.3 \pm 20.2^{I, e}$	
	30	20	–	$68.6 \pm 9.2^{B, a}$	$73.8 \pm 15.1^{I, e}$	
	30	30	±	$50.2 \pm 3.7^{A, a}$	$48.8 \pm 38.1^{J, e}$	
	50	10	±	$45.8 \pm 12.7^{C, b}$	$47.8 \pm 25.5^{K, f}$	
	50	20	±	$60.7 \pm 5.8^{D, b}$	$38.3 \pm 18.9^{K, f}$	
	50	30	+	$33.1 \pm 13.0^{C, b}$	$0.0 \pm 0.0^{L, f}$	

Values are mean ± SD.

The vitrification period for these embryos was 1 d.

[1]Ice formation during cooling: '+', '–', or '±' indicates that all of the embryos experienced ice formation, none of the embryos experienced ice formation, or some of the embryos experienced ice formation, respectively.

[2]Live PGC (%) indicates the ratio of vitrified/thawed green-fluorescent proetin (GFP)-labeled PGC per pre-vitrification GFP-labeled PGC in embryos that were injected with GFP-cc*nanos*-3'UTR mRNA.

[3]No. of PGC indicates the range in the number of GFP-labeled PGC per embryo that was injected with GFP-cc*nanos*-3'UTR mRNA.

[A,B], [C,D] Means of values with the same exposure period to PS without a common superscript differ ($P < 0.01$ and n = 4 embryos).

[a,b] Means of values with the same exposure period to VS without a common superscript differ ($P < 0.01$ and n = 4 embryos).

[E,F], [G,H], [I,J], [K,L] Means of values with the same exposure period to PS without a common superscript differ ($P < 0.05$ and n = 3 embryos).

[c,d] Means of values with the same exposure period to VS between ethylene glycol and dimethyl sulfoxide without a common superscript differ ($P < 0.05$ and n = 3 embryos).

[e,f] Means of values with the same exposure period to PS without a common superscript differ ($P < 0.05$ and n = 3 embryos).

Table 2. Localization of primordial germ cell (PGC) recovered from vitrified somitogenic-stage embryos 4 days post fertilization (dpf) and transplanted into blastula-stage goldfish embryos at 20°C

Trial No.	Characterization of donor	Stage (donor)	No. of cryopreservation days of donor	Host	No. of manipulated embryos	No. of fish at 4 dpf			
						Normal fish (%)	Localization of PGC		
							Total (%)	Gonadal ridge (%)	Ectopic (%)
1	Cryo-embryo	22 to 28 somite	1	GF-A	49	22 (44.9)	17 (34.7)	2 (4.1)	15 (30.6)
2	Cryo-embryo	22 to 28 somite	15	GF-A	43	13 (30.2)	8 (18.6)	2 (4.7)	6 (14.0)
3	Cryo-embryo	22 to 28 somite	15	GF	17	12 (70.6)	6 (35.3)	2 (11.8)	4 (23.5)
4	Cryo-embryo	22 to 28 somite	43	GF	30	24 (80.0)	16 (53.3)	4 (13.3)	12 (40.0)
5	Cryo-embryo	22 to 28 somite	44	GF	27	21 (77.8)	12 (44.4)	3 (11.1)	9 (33.3)

Cryo-embryo: Cryopreserved embryo with green fluorescent protein -labeled PGC injected with GFP-cc*nanos*-3'UTR mRNA, GF; goldfish, GF-A; albino-type goldfish.

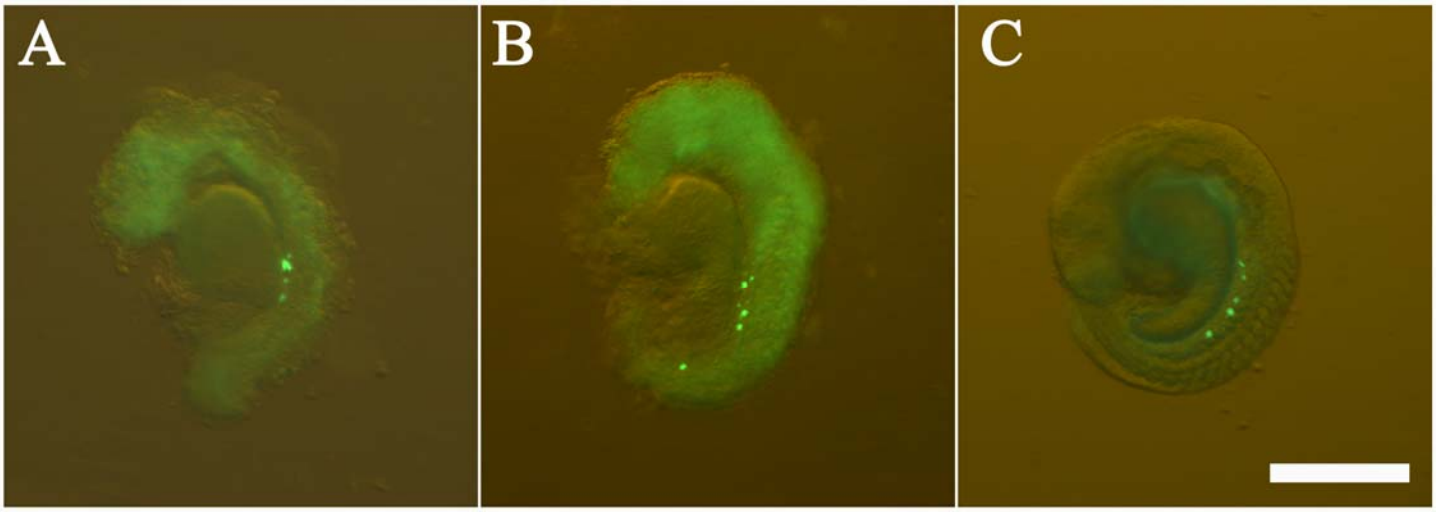

Fig.1

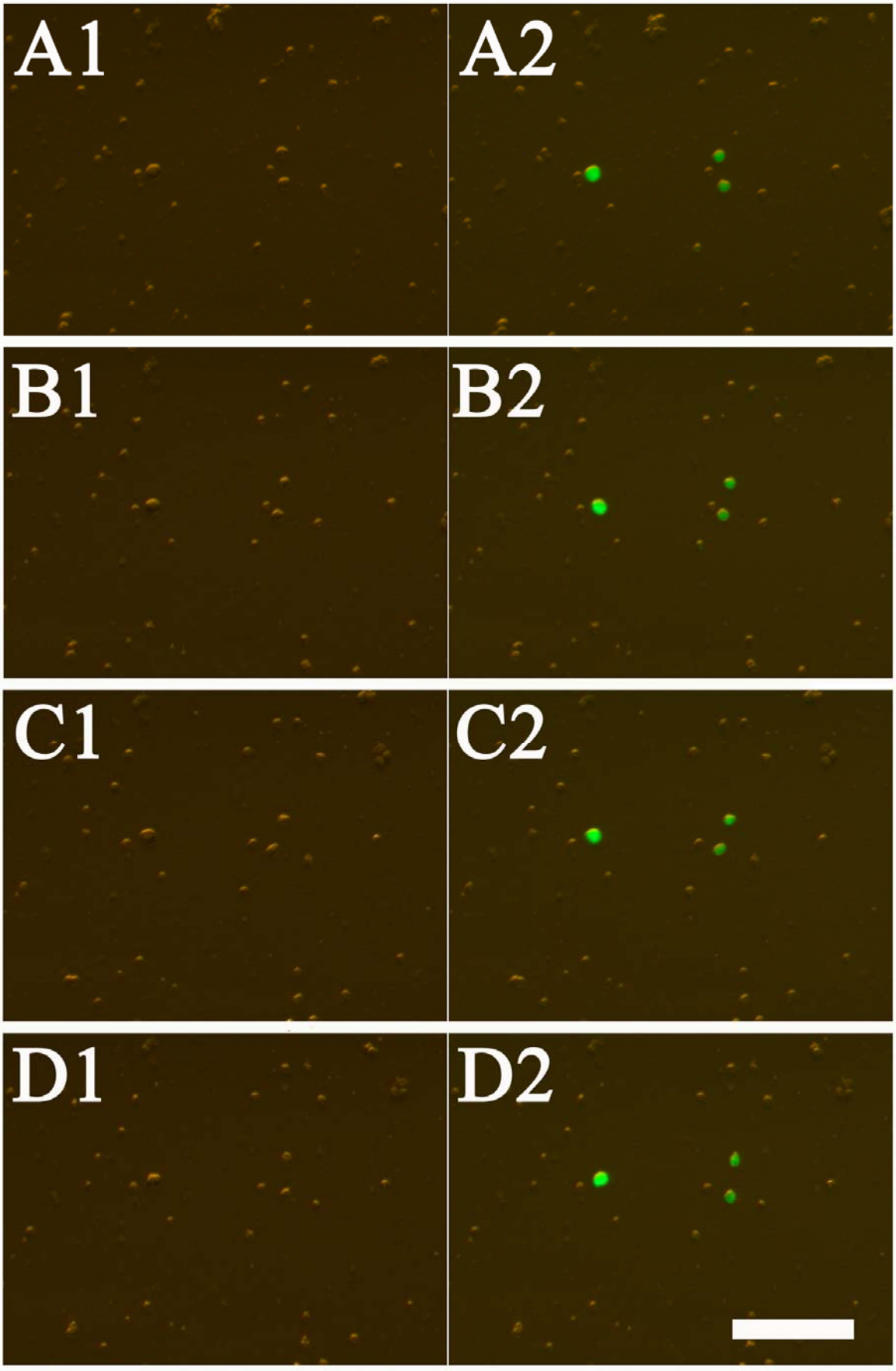

Fig.2

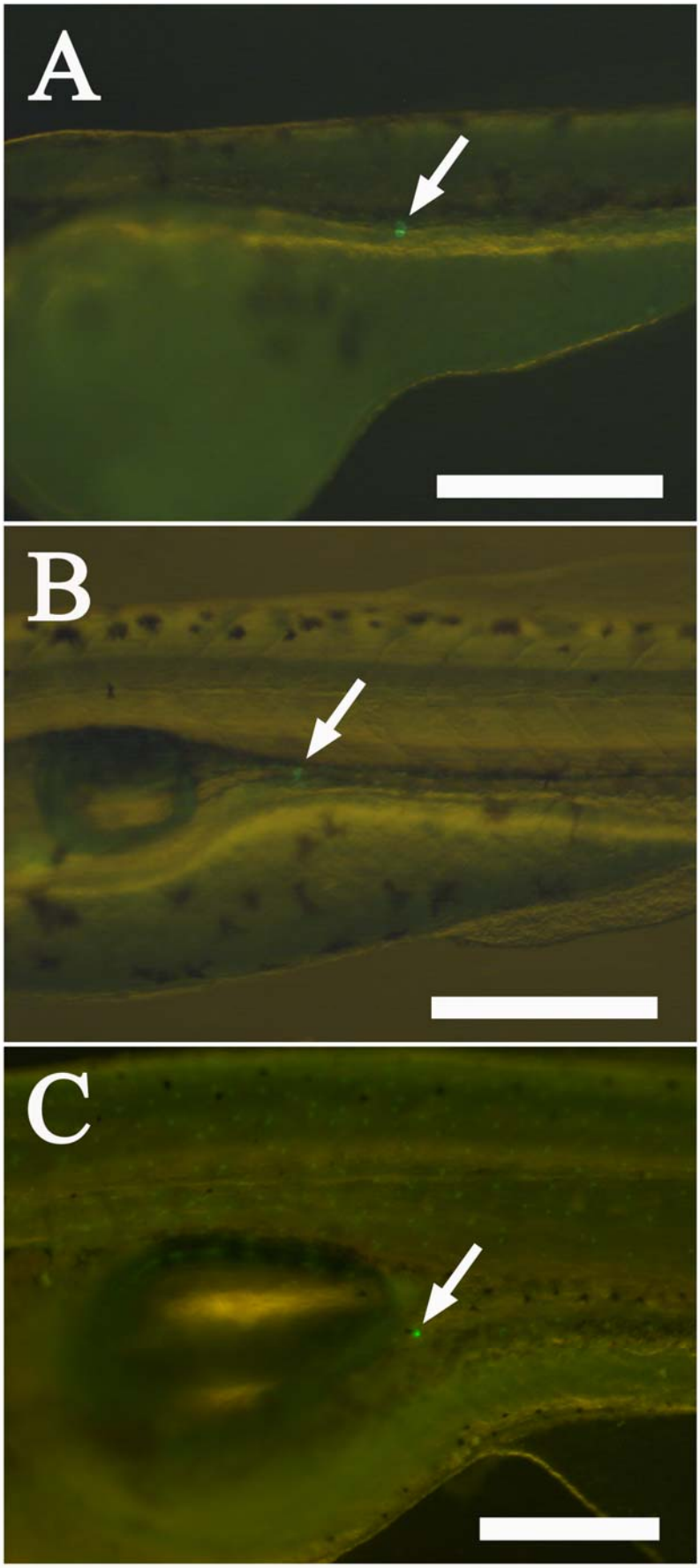

Fig.3