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Microfluidic-aided genotyping of zebrafish in the first 48 h with 100 % viability

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Abstract This paper introduces an innovative method for genotyping 1–2 days old zebrafish embryos, without sacrificing the life/health of the embryos. The method utilizes microfluidic technology to extract and collect a small amount of genetic material from the chorionic fluid or fin tissue of the embryo. Then, using conventional DNA extraction, PCR amplification, and high resolution melt analysis with fluorescent DNA detection techniques, the embryo is genotyped. The chorionic fluid approach was successful 78 % of the time while the fin clipping method was successful 100 % of the time. Chorionic fluid was shown to only contain DNA from the embryo and not from the mother. These results suggest a novel method to genotype zebrafish embryos that can facilitate high-throughput screening, while maintaining 100 % viability of the embryo.

Keywords Zebrafish · Genotyping · Microfluidics

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1 Introduction

Zebrafish (Danio rerio) are extensively used by biologists as a model organism for developmental research and preclinical drug discovery (Choudhury et al. 2012; Wielhouwer et al. 2011). Strengths of zebrafish include that their biological development and genetic mechanisms share significant homology with humans (Choudhury et al. 2012; Howe et al. 2013; Lieschke and Currie 2007), the overall drug toxicity in zebrafish embryos is comparable to mammals (Choudhury et al. 2012), and that zebrafish embryos have a shorter development time and are less expensive to maintain and study in research labs (Choudhury et al. 2012; Wielhouwer et al. 2011). Furthermore, zebrafish have high fecundity, and their embryos are conveniently sized for easy handling, and they are optically transparent, which aids visualization of phenotypes (Choudhury et al. 2012; Wielhouwer et al. 2011).

Most current methods and research tools for genotyping zebrafish mutants are tedious and require trained personnel, increasing time and costs for experiments. Currently for genotyping, embryos must be sacrificed; or fish must be raised to adulthood (age 2 to 3 months) in order to have live fin

amputations performed (Parant et al. 2009; Westerfield 2007). These limitations restrict the use of animals of known genotype under the age of 2–3 months, thus prohibiting drug or phenotype screens for mutants that are homozygous lethal or infertile. New technological advances including knock-out and knock-in approaches using TALENs and CRISPR/Cas9 also would benefit from identification of desired genotypes at an embryonic stage. Mutant genotyping, genetic screens, and drugs/therapeutics trials cannot be performed on animals of known genotype until an older age. Finally, characterization of pathology and development in embryos of known genotype is important for understanding disease processes. Thus, current approaches are inefficient and time consuming and can be considered a "genotyping bottleneck." Rapid, automated methods for genotyping early stage zebrafish embryos would represent a significant advance of wide utility.

Zebrafish embryos that are 1–2 days old range from 600 μm (without the chorion) to 1200 μm (with the chorion) in diameter (Wang et al. 2007). A chorion is a protective non-cellular membrane that naturally lasts until the fish is 2 days old and encapsulates the embryo in a liquid chorionic fluid. Despite the embryo's considerable size relative to most microfluidics devices, microfluidic research tools involving zebrafish embryos have been made for (1) continuous monitoring of zebrafish embryo development under different drug gradients, (Choudhury et al. 2012; Yang et al. 2011) (2) observing normal development of zebrafish embryos, (Wielhouwer et al. 2011) (3) droplet-based manipulation of live zebrafish embryos in digital microfluidics, (Son and Garrell 2009) (4) patterned delivery and expression of gene constructs in zebrafish embryos through electroporation to form desired mutants (Bansal et al. 2009). However, no work has been done to address the genotyping bottleneck for zebrafish research, a place where microfluidic technology could be expected to play a significant role. With the ability to precisely control the movement of small volumes of fluid and living organisms contained in the fluid, microfluidics can help collect genetic material from zebrafish embryos while they reside in an environment (small fluid volumes) very similar to their natural environment. This capability increases the survivability of the fish and also provides more process control to the researcher.

This work introduces two types of microfluidic devices for collecting zebrafish genetic material that are made in polydimethylsiloxane (PDMS) by replica molding (Duffy et al. 1998). Both devices help to resolve the genotyping bottleneck in zebrafish genetics-based research associated with the time-consuming process of DNA collection from zebrafish embryos that are 1–2 days old, while also preserving the life/health of the embryo. One device is used to collect the chorionic fluid surrounding the embryo and is referred to as "chorionic-fluid chip". The second device collects fin tissue as the genetic material and is referred to as the "fin-clip chip". The underlying functional methods by which these two devices work are referred as the "chorionic-fluid method" and the "fin-clip method" respectively.

2 Methods and materials

2.1 Chorionic-fluid method and chip design

There are three steps for the chorionic fluid method. First, zebrafish embryos at 24 hpf (hours post-fertilization) are exposed to pronase solution for 5–6 min at room temperature (22 °C). Pronase is a proteolytic enzyme that softens the chorion surrounding the embryo (Kim et al. 2004). Pronase solution is prepared by mixing 30 mg/ml of commercial Pronase (Part #. P-5147, Sigma-Aldrich, MO) in 1 ml of E3 buffer (for raising embryos; (Westerfield 2007)). Secondly, the embryos are washed with E3, as pronase over-exposure is harmful for embryos. This is done by exchanging the pronase solution containing the embryos with fresh E3 buffer three times. A temperature of 22 °C (instead of the typical 28.5 °C for zebrafish) was chosen because the lower temperature slows development and permits the experimenter a longer time-window. This was necessary because ages beyond 36 hpf the fish were observed to be stronger in resisting the fluid flow, making the fin clipping procedure difficult since the fish had to be appropriately positioned before fin-clipping. Similarly, in the chorionic-fluid method, which is also dependent on embryo age, the 22 °C provided a sufficient time-window to process enough embryos with pronase and through the chorionic-fluid chip.

In the final step the embryos are collected individually by suction in tubing ((~5 cm long) Part #06422-02, Cole Parmer Instrument Company, IL) connected to a disposable 3 ml syringe; only one embryo is collected in each tube. Caution is taken that during suction the embryo (along with the E3 buffer around it) does not enter or touch the syringe-tip. Then the free end of the tubing is connected to the inlet of a microchannel in the chorionic-fluid chip. The microchannel is designed so that its width tapers from 2 mm at the inlet to 0.75 mm at the outlet; the height remains constant at 1.7 mm (further details in Fig. 1). A typical size of a zebrafish embryo is approximately

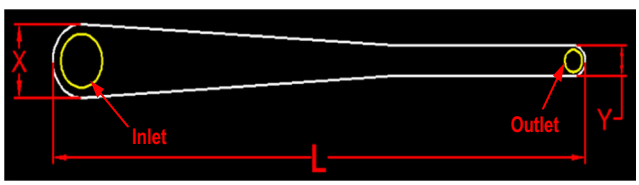

Fig. 1 Microchannel design for chorionic-fluid method. This is the top view of the channel. Channel outline is in white, with inlet/outlet ports depicted in yellow and labels in red. The embryo enters from left to right. Approximate channel dimensions for L, X, Y are 20 mm, 2 mm, 0.75 mm respectively with channel height of 1.7 mm

600 μm (without chorion) or 1200 μm (with chorion) (Wang et al. 2007). Hence as the embryos moves through the channel the channel's design allows shear and compressive forces to act on the chorion, but not on the embryo. This phenomenon tears the chorion while keeping the embryos safe from harm.

Once tubing is connected to the microchannel inlet, pulsating pressure is applied on the syringe's plunger by gently tapping the end of the syringe's plunger with a finger. This generates a pulsating flow inside the microchannel making the embryo travel across the microchannel in a series of small movements. The pulsating flow provides time for the embryo to adjust its body with varying microchannel width and avoid any harm to the embryo. Eventually the chorion tears and releases the chorionic fluid. When the embryo surfaces out of the microchannel at the outlet along with a small amount of fluid, the fluid (~60 μl) around the live embryo is collected manually using a pipette. The live embryo is separately collected by another pipette.

The tubing is then discarded and new tubing is used for the next embryo which undergoes the same process described earlier, but in a new/unused microchannel (each chorionic-fluid chip contains 10 identical microchannels to process 10 different embryos). This reduces the chance of DNA contamination between different embryos. The collected fluids (a mixture of chorionic fluid and E3) from processed embryos are then analyzed by standard genotyping techniques (polyermerase chain reaction (PCR) and high resolution melting analysis (HRMA)) and associated commercial hardware to genotype embryos of interest.

2.2 Fin-clip method and chip design

Figure 2a shows the design of the microfluidic chip used for the fin-clip method. The chip has two parts: a flow channel (highlighted in white and magenta) and a pneumatic channel (highlighted in cyan). The flow channel is ~700 μm wide and has two heights: ~550 μm (highlighted in white in Fig. 2a) and ~220 μm (highlighted in magenta in Fig. 2a which is the clipping chamber, where the fin is clipped). The pneumatic channel is ~110 μm high with two parts of different widths (~350 μm and ~200 μm). The wider part (~350 μm) interfaces with the flow channel and is used to pneumatically actuate a membrane in the clipping chamber. The 350 μm width is selected based on the stiffness of the membrane (quantified in another study (Samuel et al. 2014)) to provide a balance of membrane-deflection and membrane-strength to avoid membrane failure and perform fin clipping of embryos.

The chip is operated manually by two syringes and an external pneumatic line connected to the pneumatic channel at port X in Fig. 2. The other two syringes are used to control the position of the embryo in the flow channel and are therefore connected at ports Y and Z in (Fig. 2a). Port A is always open to the environment to provide access to air, extra E3 buffer, collection of fin tissue, and the fin-clipped embryo.

Embryos were manually dechorionated or dechorionated using pronase treatment and pipetting (Westerfield 2007). An embryo is collected in tubing (Part #06422-02, Cole Parmer Instrument Company, Vernon Hills, IL, USA) connected to syringe-1 by suction. Then the outlet of the tubing is connected to port Y. The outlet of syringe-2 is attached to identical tubing, and the other end of the tubing is connected at port Z. The external pneumatic line is connected at port X.

For the fin-clip device to work, it is required that the embryo enters the clipping chamber with its tail facing the chamber. However, there are two possible positions in which an embryo can enter at port Y: head-first or tail-first. If the embryo enters tail-first then it is transferred straight to the clipping chamber by simultaneous application of positive pressure from syringe-1 and negative pressure from syringe-2. If the embryo enters head-first it is transferred to the positioning chamber first (shown in Fig. 2) by only the supply of positive pressure from syringe-1, and then by only application of

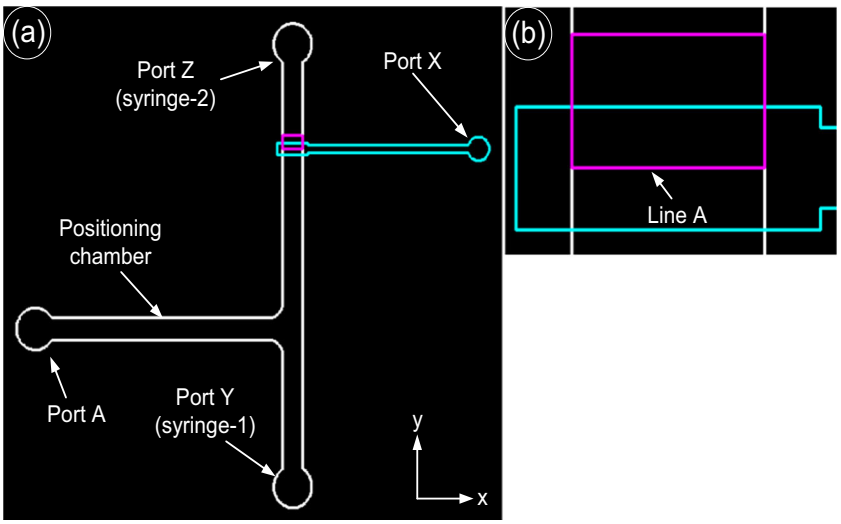

Fig. 2 **a** Fin-clip chip layout. **b** Expanded view of a part in the flow channel, including clipping chamber and depicting Line A

negative pressure from syringe-2 the embryo is transferred to the clipping chamber. The dimensions of the flow channel are such that the embryo cannot flip in any direction of its own will, yet the embryo can still move comfortably in the channel.

For a particular experiment, the fin-clip chip is placed under a microscope so that the position of the embryo can be observed. The entire flow channel is initially filled with E3 buffer and the embryo enters from port Y. Once the embryo reaches the clipping chamber with the appropriate position, the membrane is actuated as soon as point B (see Fig. 3) on the embryo is at level with Line A in the clipping chamber. Point B is where the caudal blood artery connects with the caudal blood vein, which is about 300 μm from the tip of the tail at −36 hpf (all fin-clip experiments were carried out on embryos that were approximately 36 hpf in age). When the membrane comes down on the embryo's tail, its natural reaction is to escape in the negative y-direction (according to Fig. 2). This reaction results in clipping of a thin portion of its fin which is just below the caudal blood artery. Then the embryo and the clipped tissue are both transported to port A sequentially (using actuation from the two syringes) where both are collected by a pipette for further analysis. However, while collecting the clipped tissue the pipette inevitably collects ~10 μl of E3 buffer. The chip is thoroughly cleaned with ethanol and deionized water after fin-clipping of 5 embryos. Animated movies are available as electronic supplementary information to aid in more comprehensive understanding of the fin-clip method.

Fig. 3 Depiction of Point B on a zebrafish embryo at −36 hpf (Westerfield 2007)

2.3 Chip fabrication

The microfluidic chips were fabricated in polydimethylsiloxane by replica molding. The required micromolds were made by laser ablation of adhesive vinyl films and polymethylmethacrylate (PMMA) sheets. The entire fabrication of the chips and micromolds is articulated in the electronic supplementary information.

2.4 DNA extraction for Fin-clip method

The collected volume of 10 μl of E3 buffer (containing the clipped tissue) is placed in a 40 μl volume of DNA lysis buffer (Parant et al. 2009). 5 μl of Proteinase K (10 mg/mL) (Part # 03115879001, Roche Applied Science, IN, USA) are added, and the tube is placed in a water bath at 50 °C for 4 h to digest all proteins and cellular components. Next, the tube is placed in a boiling water bath for 15 min to denature the Proteinase K enzyme; and then stored at −20 °C until PCR.

2.5 DNA amplification and detection

A polymerase chain reaction (PCR) was carried out to amplify Gal4 or eif2B5, and then High Resolution Melting Analysis (HRMA) was used to detect the PCR product. Detection of the Gal4 amplicon was used to determine if the DNA was maternal of embryonic in origin (see below for further description). The *eif2B5* amplicon was used as a sensitivity test to determine in what percentage of samples PCR amplification could be successfully performed. PCR sensitivity tests were performed for both methods consisting of a sample pool of 18 embryos for the chorionic fluid method and 15 embryos for the fin-clip method.

Prior to PCR, the samples in tubes (only for the chorionic fluid method) were placed in a boiling water bath for 10 min to denature any Pronase enzyme. PCR reactions were performed in 96-well, hard-shell plates (Part#. HSP9665, Bio-Rad Inc., CA, USA) in 10 μl volume: 2 μl of master mix (Lightscanner 5× Master Mix, Biofire Diagnostics Inc., UT, USA), 1 μl of 5 μM forward primer, 1 μl of 5 μM reverse primer and 6 μl of sample. HRMA was carried out on a 96-well LightScanner instrument from Biofire Diagnostics, Inc. Supplementary

Table 1 lists the parameters used for PCR and HRMA and the primer sequences.

2.6 Tests for chorionic-fluid method and fin-clip method

Based on the described methods, experiments were performed to determine the following important aspects of the work:

1. The ability of both methods to collect the genetic material without damaging or killing the embryo;
2. The presence of sufficient DNA in chorionic fluid for genotyping embryos;
3. The source of the DNA from the chorionic fluid (maternal or embryonic).

For testing the chorionic-fluid method, we performed a cross of wild-type male fish to female fish heterozygous for a Gal4 transgene (Tg(*otpb*:*Gal4-VP16*)^{zc57}; Fujimoto et al. 2011). The Gal4 transgene is carried on Tol2-based transposon construct integrated into the genome. The construct includes a transgenesis marker consisting of GFP (Green Fluorescent Protein) expressed in the heart (Kwan et al. 2007). Embryos from this cross are either wild-type (GFP-negative), or Gal4+ (GFP-positive). After collecting chorionic fluid samples from these two groups of fish, PCR and HRMA was carried out on the samples.

For testing the fin-clip method, fin tissue from 6 GFP-positive (and hence Gal4-positive) embryos was collected using the method described earlier. After collection, conventional DNA extraction, PCR, and HRMA are carried out on the samples.

For viability tests, wild-type embryos were processed through both methods. The embryos were observed for 5 days. Additionally, 3 wild-type embryos had their fins clipped by the fin-clip method and were monitored for 5 days for fin regrowth.

3 Results and discussion

3.1 Micromolds and fabricated chips

Figure 4 shows micromolds and fully fabricated zebrafish genotyping chips. These micromolds can only be used for rapid prototyping of PDMS microfluidic devices that contain microchannels at different heights. Therefore the fabrication process is designed to build micromolds for single use, as after multiple use the micro-features of the molds can detach from the petri dish surface. The strength of these molds can be increased and therefore their multiple-use can be made possible by using films with stronger adhesives. Also, since laser ablation was used to pattern the films, the molds do not have

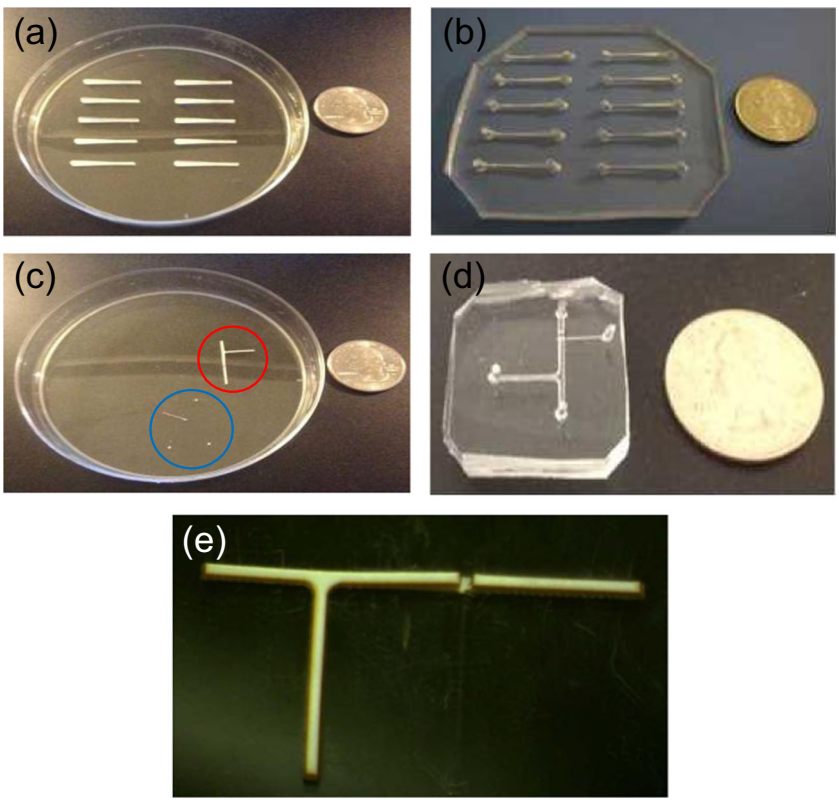

Fig. 4 Micromolds and fully fabricated chips alongside a US quarter coin; **a** micromold for chorionic-fluid chip; **b** fully fabricated chorionic-fluid chip with 10 separate chambers for chorionic fluid extraction; **c** micromold for fin-clip chip. The mold for the flow channel is highlighted by a red circle and the mold for the pneumatic channel is highlighted by a blue circle; **d** fully fabricated fin-clip chip; **e** a magnified picture of the mold for the flow channel of the fin-clip chip

smooth walls. The rough sidewalls could contribute to the ability of the chips to rupture the chorions.

The experimental setups for testing both types of chips are shown in Supplementary Fig. 1 and Supplementary Fig. 3.

3.2 Chip design and genotyping

First we tested the sensitivity of our approach for detecting and amplifying DNA from chorionic fluid. When 18 embryos were processed using the chorionic-fluid method the sensitivity for amplification of embryonic DNA was 78 % (14 of 18 embryos had amplification of the control *eif2B5* gene).

We carried out tests using the chorionic-fluid chip to determine the source of chorionic DNA amplified by PCR. Our concern was whether maternal cells could be present in the chorionic fluid, and thus result in contamination of results from chorionic fluid. We found that GFP-negative, wild-type embryos did not show any PCR amplification of Gal4. The Gal4 transgene is stably integrated into the maternal genome (Kwan et al. 2007), so that absence of PCR amplification demonstrates that no maternal cells or DNA was contained in the chorionic fluid (Fig. 5). Conversely, all GFP-positive embryos carrying the Gal4 transgene successfully amplified. That is, every GFP-positive embryo was positive for the Gal4 PCR product; and every GFP-negative embryo did not have the Gal4 PCR product.

The reduced sensitivity in the chorionic-fluid method for PCR amplification was likely due to the low volume of chorionic fluid as a genetic material source for genotyping – corroborated by the large number PCR cycles required (60 cycles) for appropriate amplification of chorionic DNA (shown in Supplementary Table 1). The low volume of chorionic fluid places stringent demands on the collection of chorionic DNA; which in turn is reflected as an important design metric of the chorionic-fluid chip. This design metric requires that the collected chorionic-fluid should have minimum dilution by E3 buffer that is used to push the embryo across the channel by pulsating flow, since chorionic DNA is not abundant in quantity. However, the pulsating flow can negatively affect the sensitivity of the device by excessive dilution of DNA. Therefore channel dimensions in Fig. 1 should be such

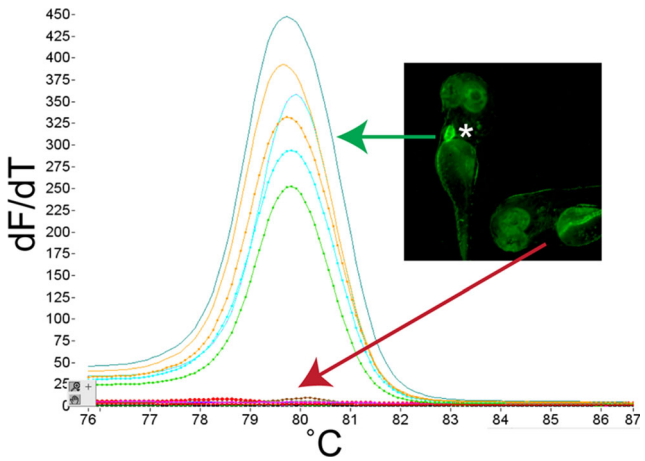

Fig. 5 HRMA melt-curve analysis of Gal4 PCR amplicon (differential of fluorescence change, y-axis vs. temperature in °C, x-axis) demonstrates that chorionic fluid carries embryonic cells and DNA can be amplified from chorionic fluid extracted with the microfluidics device. Inset: live view of whole embryos under a fluorescent microscope show lateral views of a Gal4-transgenic embryo, visualized because of cardiac GFP expression (asterisk), and a non-transgenic embryo. The method amplifies the Gal4 PCR product (*green arrow*), but the non-transgenic embryo, which has a transgenic mother, does not generate PCR product (*red arrow*)

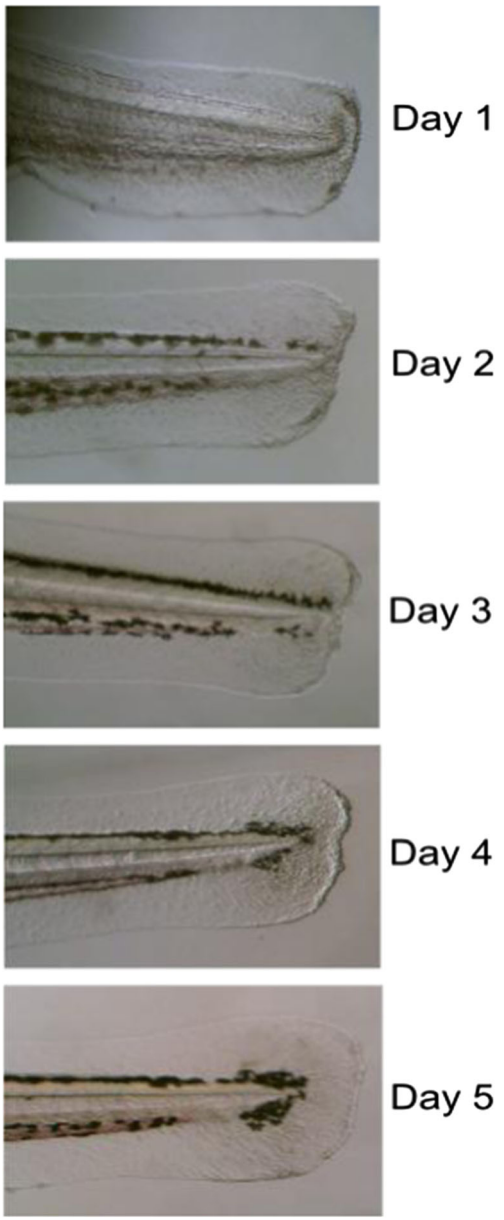

Fig. 6 Fin regeneration of an embryo over a period of 5 days, after being processed by the fin-clip method. Views show the tail of live embryo, rostral to the left, dorsal top

that they retain minimum volume as possible to inhibit excess dilution of chorionic DNA.

In contrast, the fin-clip method had a sensitivity for PCR amplification of 100 % (n=15 embryos). Though the fin-clip method has better sensitivity, the appropriate positioning of the embryo in the clipping chamber is crucial to clip the fin without damaging the embryo. Therefore this process requires continuous awareness of the position of the embryo inside the chip and associated use of syringe pumps, making the entire process more complicated. In addition, since the embryo is not anesthetized, it will try to move away from the chamber as it is drawn into it. We were not able to use anesthesia for the fin-clip method because the reflex motor response of the embryo is necessary for a successful fin-clip to occur.

Viability results show 100 % viability for both methods (see Supplementary Table 2) as the embryos showed sensitivity to touch with normal swimming behaviors, and had normal morphological development. Additionally, 3 wild-type fish had their fins clipped by the fin-clip method and were monitored for fin regrowth at room temperature of 22 °C for 5 days (Fig. 6).

4 Conclusions

In this work two types of microfluidic chips for collection of genetic material from zebrafish embryos (1–2 days old) have been developed: the chorionic-fluid method and the fin-clip method. Our results show that both methods provide an ability to preserve an embryo's life while determining its genotype at an early stage and can therefore increase the use of zebrafish embryos in biomedical research. The results also reveal that the source of chorionic DNA is embryonic; and that the chorionic-fluid method will require further optimization for higher sensitivity.

Our approach currently has three limitations. First, it is not automated, which would improve speed of throughput and thus increase the number of animals that could be screened. Second, we use pronase enzyme softening of the chorion prior to the chorionic-fluid method. Pronase enzyme can vary in strength; and pronase pre-treatment could cause small holes to develop in the chorion with resultant loss of fluid, leading to reduced sensitivity. Future improvements in our method can be focused on developing an entirely enzyme-free approach for the dechorionation. Finally, embryos are not anesthetized because the reflex motor response of the embryo is necessary to help with the fin-clip method; but this also makes correct positioning of the embryo in the channel slower. Further design improvements in the fin-clip chip to handle anesthetized embryos would be beneficial.

Currently, there are no similar methods for genotyping at an embryonic stage in zebrafish. While fluorophore labelling of transgenes offers a method for confirmation of transgenic line status (Kwan et al. 2007), actual genotyping of a mutant or variant is not possible without killing the animal. The ability to genotype and sort at embryonic stages, and maintain and grow the zebrafish embryos is important for small molecule screens for therapies in mutant backgrounds. In addition, with increased use of zebrafish to test genetic variants identified from next-generation sequencing approaches, ability to identify and sort embryos carrying a desired mutation is important.

Further research can help scale these methods for high-throughput screening and increase the utility and cost-effectiveness of using zebrafish models.

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Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval "All applicable international, national, and/or institutional guidelines for the care and use of animals were followed."

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