Purification of hearts from zebrafish embryos

C. Geoffrey Burns and Calum A. MacRae

Massachusetts General Hospital, Charlestown and
Harvard Medical School, Boston, MA, USA

BioTechniques 40:274-282 (March 2006)
doi 10.2144/000112135

Quantitative assessments of gene expression in early embryonic organs are difficult to obtain from whole embryos. Although *in situ* hybridization can elucidate spatiotemporal patterns of messenger RNA (mRNA), it cannot enumerate fold differences in transcript abundance. Furthermore, *bona fide* quantitative techniques, such as quantitative PCR or microarray hybridization, when performed on whole embryo preparations, can ascertain transcript levels only in the aggregate. Ultimately, high-resolution measurements of gene expression in individual organs require the material to be purified away from surrounding tissues. Moreover, methods for isolating pure organs can facilitate culturing and transplantation of their constituents for *in vitro* studies and the *in vivo* evaluation of stem or progenitor cells, respectively.

Because mammalian embryos develop *in utero*, embryos and their internal organs are not readily accessible without laborious microsurgies (1). The limited number of offspring also restricts tissue availability. In contrast, zebrafish embryos develop externally and are available in significantly larger quantities. Therefore, we devised a method, void of microdissection, for isolating abundant embryonic organs from zebrafish focusing specifically on the developing heart.

Like all zebrafish organs, the heart develops rapidly. By 2 days postfertilization (dpf), it comprises two layers and two chambers, has established flow, and has undergone looping (2). Thereafter to day 4, the ventricular wall begins to thicken via addition of myocardial cells (3,4), and cardiac valves will soon arise from developing endocardial cushions (5).

To aid in tracking hearts, we used embryos that expressed green fluorescent protein (GFP) in the myocardium under control of the *cardiac myosin light chain 2 (cmlc2)* promoter (6). Embryos at the desired stages were resistant to enzymatic treatment with trypsin and collagenase. Serendipitously, we discovered that the fluid forces in media repeatedly drawn into and expelled from a needle and syringe ruptured embryonic yolk membranes and extricated, among other tissues, intact hearts (Figure 1). Subsequent size fractionation of the disrupted embryos and manual retrieval of GFP+ hearts afforded cardiac tissue with purity that approached 100%.

Embryos hemizygous for *Tg(cmlc2:GFP)* between 2 and 4 dpf were generated and handled as described (6). Prior to disruption, approximately 500 embryos were anesthetized, transferred to a 1.5-mL microfuge tube, washed three times with embryo disruption medium [EDM; Gibco Leibovitz's L-15 Medium (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (Hyclone, Logan, UT, USA), ice-cold at every step] and resuspended in 1.25 mL EDM. The microfuge tube, a 19-gauge needle (Precision Glide, 1 1/2 inches long, regular bevel; BD Medical, Franklin Lakes, NJ, USA) and a 6-mL syringe (Monoject syringe with a regular luer tip; Kendall Company, Mansfield, MA, USA) were secured to a ring stand (Figure 1A). The needle was centered in the x- and z-axes of the microfuge tube and aligned in the y-axis with the 0.1 mL volume mark.

Approximately 1 mL EDM containing embryos was drawn into the needle and syringe and immediately expelled back into the microfuge tube 30 times at a rate of 1 s per syringe motion. Fragmented embryos were

applied to 105 μ M nylon mesh (Small Parts, Miami Lakes, FL, USA), and the flow-through was collected in a 30-mm polystyrene Petri dish. The microfuge tube, needle, syringe, and mesh were washed with additional media that was added to the flow-through. The flow-through was subsequently applied to 40 μ M nylon mesh. The Petri dish that collected the 105 μ M flow-through was rinsed with additional media that was applied to the 40 μ M mesh. The 40 μ M mesh was inverted, and the retained material was washed off with EDM into a 30-mm Petri dish.

Intact GFP+ ventricles were identified under fluorescent light and collected selectively with a p20 micropipet under transmitted light (Figure 1, D and E). Approximately 40 ventricles were collected each time the micropipet was filled. To minimize contamination by noncardiac tissues, the hearts were subsequently expelled into a 30-mm Petri dish containing fresh EDM and retrieved selectively a second time before being accumulated in a microfuge tube on ice. From 500 embryos, approximately 250 total hearts were retrieved in the span of 1 h. Hearts were pelleted, the media was decanted, and the preparations were frozen in a dry ice/ethanol bath prior to storage at -80°C.

At this time, visual inspection suggested that cardiac tissue purity approached homogeneity (Figure 1, F and G). The percentage of ventricles with attached complete atria was often between 10%–30% but as high as 50% in some preparations (Figure 1, F and G). The remainder of ventricles retained partial or no atria (Figure 1, F and G). We were unable to identify more than a few sole atria in each preparation. The atria that were unaccounted for were either retained in the embryo's bodies or assumed to be sufficiently fragmented so as to evade identification. In general, we found that using a smaller number of syringe motions increased the percentage of ventricles with atria but decreased the percentage of hearts recovered. Nonfluorescent outflow tracts (e.g., bulbus arteriosus) were attached to the majority of ventricles (Figures 1, F and G). Spontaneous rhythmic contractions were observed when the media

Figure 1. Purification of embryonic hearts from zebrafish. A needle and syringe (shown in A) were used to fragment 3-day-old myocardial green fluorescent protein (GFP)+ embryos as described. (B and C) Examples of embryos prior to (left) and after (right) disruption. Fluid forces created by the needle and syringe sheered off the yolk membranes and extricated fluorescent hearts. (D and E) Hearts were found intact in the >40 μ M, <105 μ M size fraction. A p20 micropipet (shown schematically) was used to further purify hearts (arrows) away from nonfluorescent noncardiac tissue (*). (F and G) Purified cardiac tissue approached homogeneity. Only GFP+ hearts with nonfluorescent bulbus arteriosus (ba) are apparent. Approximately 20% of ventricles retained full atria (a; heart on left), and the rest retained partial or no atria (heart on right). The fluorescence intensity of GFP in atria is lower than that in ventricles, and this is an inherent characteristic of the transgenic line. Embryos and tissues were illuminated with transmitted (B, D, and F) or fluorescent (C, E, and G) light.

was warmed to room temperature, confirming tissue viability (see Supplementary Video 1 available online at www.BioTechniques.com).

To objectively assess the content and purity of heart preparations, we measured the relative amounts of seven tissue-specific markers in three tissue fractions with quantitative PCR (see Table 1 and References 7–14). Total RNA was isolated with TRIzol[®] reagent (Invitrogen), with each heart yielding between 1.5 and 3 ng. Total RNA was random primed and used as templates in first-strand synthesis (SuperScript[™] First Strand Synthesis System for RT-PCR; Invitrogen). Quantitative PCR was performed with SYBR[®] green DNA dye and an ABI PRISM[®] 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. All signals were normalized to the ubiquitous β -actin mRNA (5'-GCTGTTTTCCCTCCATTGTT-3', 5'-TCCCATGCCAACCATCACT-3').

Heart preparations harbored 340-fold more *cmlc2* mRNA than whole embryos, indicating a substantial enrichment for myocardium (Table 1). They also contained 79-fold more *atrial myosin heavy chain* mRNA, confirming the presence of atria. Lastly, purified hearts retained endocardium, as evidenced by a 23-fold greater quantity of *endothelial-tie2* transcripts in the heart fraction. Negligible amounts of other tissue-specific mRNAs were present in the heart fraction (Table 1), thereby confirming tissue purity suggested by visual inspection (Figure 1, F and G). Enrichment of specific tissues in other size fractions suggests that manual or automated retrieval of these organs with a micropipet or fluorescence-activated cell sorting (FACS), respectively, could yield pure tissue preparations for other organs (Table 1).

We present a simple method for isolating intact embryonic hearts from zebrafish. This technique will permit quantitative comparisons of gene expression in wild-type and mutant hearts. Single transcripts can be assessed using quantitative PCR.

Table 1. Relative Amounts of Tissue-Specific mRNAs Present in Whole Embryos and Three Tissue Fractions Measured by Quantitative PCR

Tissue-Specific mRNA	Tissue(s)	Tissue Fraction and Fold Differences Relative to Whole Embryos				Primers	References
		Whole Embryos	<40 μM	>40 μM, <105 mM			
				Heart ^a	Nonheart		
<i>cmhc2</i>	Myocardium ^b	1.0	0.4	340.2	3.5	F: 5'-GGAGAGAAGCTCAATGGCACA-3' R: 5'-GTCATTAGCAGCCTCTTGAAGTCA-3'	7
<i>amhc</i>	Atrial myocardium	1.0	1.1	78.8	1.0	F: 5'-TGGCGATGCTGACGTTTCTT-3' R: 5'-TCACGGTGACACAAAACAGCC-3'	8
<i>tie2</i>	Endothelium	1.0	17.0	22.8	2.1	F: 5'-TGGAGGAGCGCAAGACATATG-3' R: 5'-CATTATTGCTGCAGTGCATCAG-3'	9
<i>βE1 globin</i>	Red blood cells	1.0	16.9	1.2	0.3	F: 5'-TGGTTGTGTGGACAGACTTCGA-3' R: 5'-CGATAAGACACCTTGCCAGAGC-3'	10
<i>lfabp</i>	Liver	1.0	2.8	0.1	3.2	F: 5'-GAGCCATCTCTCTGCCAGAAGA-3' R: 5'-GGAGTTTTGGAGGTGATGGTGA-3'	11
<i>insulin</i>	Endocrine pancreas	1.0	24.8	0.8	3.2	F: 5'-GTGGATCTCATCTGGTCGATGC-3' R: 5'-AGGAGGAAGGAAACCCAGAAGG-3'	12
<i>ifabp</i>	Intestine	1.0	0.5	0.0	2.3	F: 5'-AGTCAGCACTTTCCGCACACT-3' R: 5'-TCCCTCTATGACCCAGGATCCT-3'	13
<i>six3b</i>	Brain/eyes	1.0	0.1	0.0	0.0	F: 5'-CTTACCCCAATCCGAGCAAGA-3' R: 5'-GCTGAAGCCTGTTTTTGGCA-3'	14

Three tissue fractions were prepared from *Tg(cmhc2:GFP)* embryos on day 3 postfertilization as described and analyzed by quantitative PCR. Signals in all samples were normalized to the ubiquitous messenger RNA (mRNA), *β-actin*, and reported as fold-differences relative to whole embryos. Bolded numbers identify the fraction(s) enriched with each transcript. *six3b* mRNAs were used as a control for tissues not predicted to detach from the embryos. F, forward primer; R, reverse primer.

^aFold differences in the heart sample represent the averages of two experiments.

^b*cmhc2* is expressed in both atrial and ventricular myocardium.

Alternatively, expression profiling of myriad genes can be accomplished with microarrays or serial analysis of gene expression (SAGE). This technique will be particularly useful for identifying downstream effectors of proteins that, when mutated, cause defects in cardiomyocyte proliferation or endocardial cushion maturation.

ACKNOWLEDGMENTS

We thank Caroline Erter Burns, David Milan, and Shannon Fishman for critical reading of the manuscript. C.G.B. was funded by National Institutes on Aging/National Institutes of Health (NIH) grant no. 1K01AG023562.

COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

REFERENCES

1. Nagy, A., M. Gertsenstein, K. Vintersten, and R. Behringer. 2003. Manipulating the Mouse Embryo: A Laboratory Manual. CSH Laboratory Press, Cold Spring Harbor, NY.

2. Stainier, D.Y., B. Fouquet, J.N. Chen, K.S. Warren, B.M. Weinstein, S.E. Meiler, M.A. Mohideen, S.C. Neuhauss, L. et al. 1996. Mutations affecting the formation and function of the cardiovascular system in the zebrafish embryo. *Development* 123:285-292.

3. Antkiewicz, D.S., C.G. Burns, S.A. Carney, R.E. Peterson, and W. Heideman. 2005. Heart malformation is an early response to TCDD in embryonic zebrafish. *Toxicol. Sci.* 84:368-377.

4. Mably, J.D., M.A. Mohideen, C.G. Burns, J.N. Chen, and M.C. Fishman. 2003. *heart of glass* regulates the concentric growth of the heart in zebrafish. *Curr. Biol.* 13:2138-2147.

5. Beis, D., T. Bartman, S.W. Jin, I.C. Scott, L.A. D'Amico, E.A. Ober, H. Verkade, J. Frantsve, et al. 2005. Genetic and cellular analyses of zebrafish atrioventricular cushion and valve development. *Development* 132:4193-4204.

6. Burns, C.G., D.J. Milan, E.J. Grand, C.A. MacRae, and M.C. Fishman. 2005. High-throughput assay for small molecules that modulate zebrafish embryonic heart rate. *Nat. Chem. Biol.* 1:263-264.

7. Yelon, D., S.A. Horne, and D.Y. Stainier. 1999. Restricted expression of cardiac myosin genes reveals regulated aspects of heart tube assembly in zebrafish. *Dev. Biol.* 214:23-37.

8. Berdough, E., H. Coleman, D.H. Lee, D.Y. Stainier, and D. Yelon. 2003. Mutation of *weak atrium/atrial myosin heavy chain* disrupts atrial function and influences ventricular morphogenesis in zebrafish. *Development* 130:6121-6129.

9. Motoike, T., S. Loughna, E. Perens, B.L. Roman, W. Liao, T.C. Chau, C.D.

-
- Richardson, T. Kawate, et al.** 2000. Universal GFP reporter for the study of vascular development. *Development* 128:75-81.
10. **Brownlie, A., C. Hersey, A.C. Oates, B.H. Paw, A.M. Falick, H.E. Witkowska, J. Flint, D. Higgs, et al.** 2003. Characterization of embryonic globin genes of the zebrafish. *Dev. Biol.* 255:48-61.
11. **Denovan-Wright, E.M., M. Pierce, M.K. Sharma, and J.M. Wright.** 2000. cDNA sequence and tissue-specific expression of a basic liver-type fatty acid binding protein in adult zebrafish (*Danio rerio*). *Biochim. Biophys. Acta* 1492:227-232.
12. **Argenton, F., E. Zecchin, and M. Bortolussi.** 1999. Early appearance of pancreatic hormone-expressing cells in the zebrafish embryo. *Mech. Dev.* 87:217-221.
13. **Andre, M., S. Ando, C. Ballagny, M. Durlat, G. Poupard, C. Briancon, and P.J. Babin.** 2000. Intestinal fatty acid binding protein gene expression reveals the cephalo-caudal patterning during zebrafish gut morphogenesis. *Int. J. Dev. Biol.* 44:249-252.
14. **Seo, H.C., Drivenes, S. Ellingsen and A. Fjose.** 1998. Expression of two zebrafish homologues of the murine *Six3* gene demarcates the initial eye primordia. *Mech. Dev.* 73:45-57.

Received 16 November 2005; accepted 13 January 2006.

Address correspondence to C. Geoffrey Burns, Developmental Biology Laboratory, Cardiovascular Research Center, Massachusetts General Hospital, 149 13th Street, Charlestown, MA 02129, USA. e-mail: gburns@cvrc.mgh.harvard.edu

To purchase reprints
of this article, contact
Reprints@BioTechniques.com