Isolation and *in vitro* culture of primary cardiomyocytes from adult zebrafish hearts

Veronika Sander[1], Guillermo Suñe[1], Chris Jopling[2], Cristina Morera[1] & Juan Carlos Izpisua Belmonte[1,3]

[1]Center of Regenerative Medicine in Barcelona, Barcelona, Spain. [2]Centre National de la Recherche Scientifique, Unités Mixtes de Recherche (UMR)-5203, Institut de Génomique Fonctionnelle, Molecular Mechanisms of Regeneration, LabEx Ion Channel Science and Therapeutics (ICST), Département de Physiologie, Institut National de la Santé et de la Recherche Médicale (INSERM), U661, Universités de Montpellier 1 and 2, Montpellier, France. [3]Gene Expression Laboratory, Salk Institute for Biological Studies, La Jolla, California, USA. Correspondence should be addressed to J.C.I.B. (belmonte@salk.edu).

Published online 28 March 2013; doi:10.1038/nprot.2013.041

This protocol describes how to isolate primary cardiomyocytes from adult zebrafish hearts and culture them for up to 4 weeks, thereby using them as an alternative to *in vivo* experiments. After collagenase digestion of the ventricle, cells are exposed to increasing calcium concentrations in order to obtain high-purity cardiomyocytes. The whole isolation process can be accomplished in 4–5 h. The culture conditions we established allow the cells to preserve their mature sarcomeric integrity and contractile properties. Furthermore, adult zebrafish cardiomyocytes in culture, similarly to zebrafish *in vivo* heart regeneration, undergo partial dedifferentiation and, in contrast to their mammalian counterparts, are able to proliferate. Our protocol enables the study of structural and functional properties in close-to-native cardiomyocytes and allows the application of *in vitro* techniques and assays that are not feasible to perform in living animals.

INTRODUCTION

To investigate the best form of treatment for ischemic heart disease, a major cause of mortality in the Western world, the processes of wound healing and regeneration of the heart ventricle after myocardial infarction are being studied intensively. Adult mammalian cardiomyocytes proliferate insufficiently to regenerate injured myocardium; the damaged heart muscle is instead replaced by fibrotic scar tissue and the remaining cardiomyocytes undergo hypertrophic growth to compensate for loss of contractility. Suboptimal cardiac performance renders the heart more susceptible to failure and to future myocardial infarction events[1,2]. Commonly, primary cardiomyocytes isolated from adult hearts of mammalian model animals are studied for their ability to switch from their normal quiescent state to reactivation of proliferation. Despite the promising results of these studies, the proliferation capacity of adult cardiomyocytes is not yet sufficient to heal the diseased heart and restore its function[3,4].

The teleost species zebrafish (*Danio rerio*) has proven to be an excellent model organism for research on regeneration because of its ability to scarlessly heal the adult heart muscle after injury. In recent years, *in vivo* studies have shown that this astounding regeneration is accomplished by switching the cardiomyocytes from a mature to a partially dedifferentiated state, thereby enabling the reactivation of proliferation[5,6].

Despite the benefits of *in vivo* regeneration experiments, there are disadvantages to working with adult zebrafish. Surgical procedures on the heart are not easy to reproduce; they may lead to variations in the phenotypes obtained and require the examination of large numbers of animals. In addition, mortality can be high depending on the type of heart injury[7]. Technical difficulties include administration of chemical reagents to the fish (via aquarium water or injection).

Primary cardiomyocytes isolated from adult zebrafish hearts have been used mainly for immediate measurements on freshly isolated cells to compare the physiological parameters of fish and human cells[8,9]. The availability of this protocol for healthy long-term cultures of primary cardiomyocytes should provide a useful alternative to *in vivo* studies of the adult zebrafish heart.

Development of the protocol

Numerous isolation protocols optimized for different species are available, and culture conditions of primary cardiomyocytes are well established. However, it is still challenging to isolate high-quality adult cardiomyocyte populations and to keep cultures alive and healthy for more than 2 weeks[10].

Isolation procedures usually start by perfusing the heart with solutions containing zero or low Ca^{2+}, or by mincing the ventricle. Enzymatic digestion ensures complete disintegration of the tissue. It is followed by final steps to increase the purity of the cardiomyocyte population and restore physiological conditions.

There are two main approaches to *in vitro* culture of heart muscle cells. For the re-differentiation method, cardiac myocytes are cultured in serum-containing medium and in suspension. While in suspension, the cells acquire a rounded shape and partially lose the appearance and function of the *in vivo* heart muscle. At later stages of culture, re-differentiation occurs as the cells attach and regain some of the structures of mature cardiomyocytes, such as t-tubules and spontaneous contraction. In contrast, the rapid attachment technique uses serum-free conditions and coating of the culture dish. Consequently, the cells attach soon after plating and maintain near–*in vivo* characteristics, including spindle shape and sarcomeric striation. Without serum, the myocytes neither contract nor proliferate, and survive only up to 14 d (refs. 10,11).

Only a few reports describe cardiomyocyte isolation from fish hearts[8,9,12–14]. As these studies frequently aim to characterize the electrophysiological constitution of fish cardiomyocytes in order to assess their applicability for the human heart, little emphasis has been placed on developing conditions that promote their long-term survival in culture.

Technically, our protocol follows the general steps for cell isolation and is derived from the rapid attachment approach to culture[11].

To achieve high-quality cardiomyocytes in terms of purity, viability and preservation of *in vivo* properties, we modified the following parameters[15,16]. First, for tissue digestion, we use enzyme concentrations up to ten times higher and we incubate at 32 °C for 2 h instead of for 20–60 min as in most other protocols. Both modifications result from the suboptimal working temperature of the enzyme at 32 °C, as the fish cells cannot be stressed by a heat shock of 37 °C. We believe that cooler and slower digestion prevents cell damage and instead contributes to the preservation of cell integrity (**Supplementary Table 1**).

Second, we extended the normally low number of washes to a total of seven. The gentle, gradual reintroduction of Ca^{2+} from 0 mM (during digestion) to the physiological level of 1 mM at 4 °C, protects cardiomyocytes from the 'Ca^{2+} paradox', a phenomenon of severe damage of the sarcolemma caused by massive Ca^{2+} influx during restoration of Ca^{2+}-deprived cells to physiological ion levels[17,18]. As nonmyocardial cells have a low tolerance for Ca^{2+}, they die during the washes and get washed away, whereas live cardiomyocytes sediment during the mild centrifugation steps. In this way, we acquire high purity and viability without having to perform pre-plating or Percoll gradient centrifugation steps, as in other protocols, in order to separate cardiomyocytes from unwanted cell types such as fibroblasts (**Supplementary Table 2**).

Third, contrary to the original fast attachment approach, we do not observe slower or weaker cell attachment when using medium enriched with 5% (vol/vol) FBS. We therefore recommend growing cardiomyocytes in a serum-containing medium, as it seems advantageous for long-term survival (**Supplementary Table 2**).

The adult zebrafish is a particularly useful model because it can regenerate the heart via cardiomyocyte proliferation, whereas adult mammals cannot. The present protocol for isolation and *in vitro* culture of zebrafish cardiomyocytes will help researchers to understand how the fish cells manage to reactivate proliferation upon injury and why adult mammalian cardiomyocytes are unable to do so.

As most cardiac research focuses on understanding human heart disease, an adequate *in vitro* system should resemble the native situation of the heart muscle. Neonatal mammalian cardiomyocytes present a practical option because they are easier to isolate and maintain than adult cells. However, as neonatal cells still proliferate, they feature a less complex morphology, and their genetic setup of the contractile machinery and the ion channels is different from those of terminally differentiated adult cells[12,19,20]. We therefore consider adult cardiomyocytes as more relevant and applicable to modeling the *in vivo* heart muscle.

Applications of the method

Until recently, the phenomenon of how adult, quiescent zebrafish cardiomyocytes manage to reactivate the cell cycle during regeneration had not been elucidated. Now that the mechanism has been identified[5,6], research is directed toward finding factors and processes that initiate dedifferentiation of adult cardiomyocytes to a more immature state that allows cell division. Recent work by Jopling *et al.*[15] focused on hypoxia as a consequence of myocardial injury. Cultured cardiomyocytes were exposed to defined hypoxic, normoxic and hyperoxic conditions, and dedifferentiation and proliferation were assessed by immunocytochemistry. A significant increase in the number of dedifferentiated dividing cells was found under hypoxic conditions, whereas the opposite effect was seen in hyperoxia, indicating a stimulating effect of hypoxia on cardiomyocyte regeneration[15].

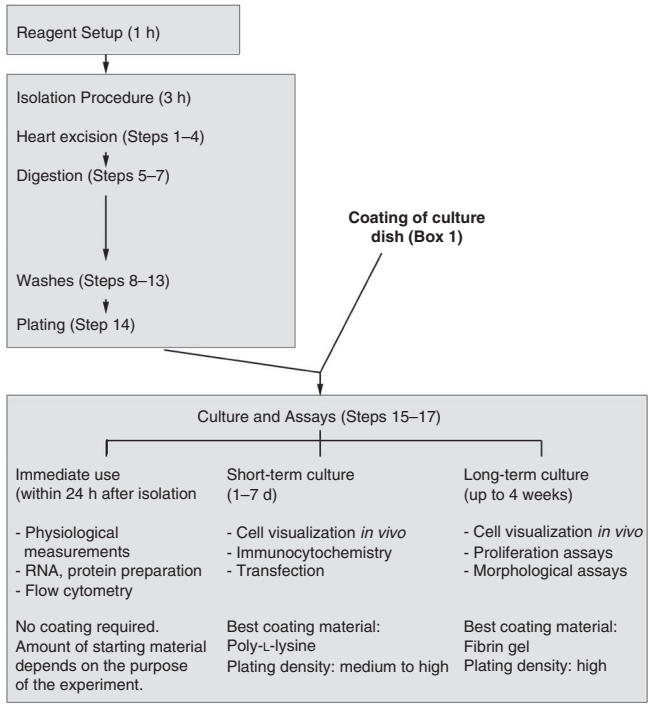

Figure 1 | Flowchart of the experimental procedures.

Cross talk between cardiomyocytes and other cardiac cell types, such as endothelial and epicardial cells, was shown to be involved in the regulation of regeneration of the heart muscle[21–23]. Isolation and culture of epicardial cells from adult zebrafish hearts was described recently[24], and coculture of myocytes with endothelial cells is commonly performed in mammalian cultures[25]. With our protocol on primary cardiomyocyte isolation and culture, it now becomes feasible to coculture defined cardiac cell populations from the zebrafish and investigate reciprocal effects.

Experimental design

The purpose of the experiment will determine the number of hearts required as starting material (**Fig. 1**). One can expect to obtain a sufficient number of cardiomyocytes from as few as three hearts. We do not recommend fewer than three hearts per analysis, as this will lead to very small cell pellets and increase the likelihood of high cell loss during the washes. When the cells are intended for *in vitro* studies on proliferation or morphology over a longer period of time, it is advisable to start with more than three hearts and medium-to-high plating density of the cells. We observed that medium and high cell densities promote the formation of cell-cell connections within the first 2 d of culture, rendering the culture's appearance to a network-like monolayer of rod-shaped cardiomyocytes (**Figs. 2** and **3**). The existence of a direct proportional relation between the amount of cell-cell connections, cell survival over an extended period of time and early onset of beating can be assumed.

Untreated cardiomyocytes begin to proliferate ~5 d after plating (**Supplementary Fig. 1a**). To render the cells conductive to cell division, the sarcomeric contractile apparatus, which occupies large parts of mature cardiomyocytes, disassembles[26]. This becomes morphologically apparent as the rod-shaped mature muscle cells attain

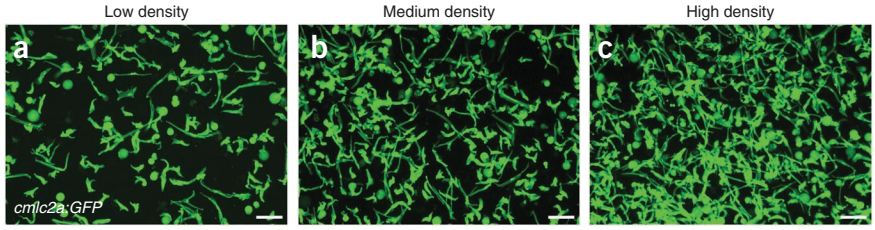

Figure 2 | Plating densities of isolated cardiomyocytes. (**a**) Cardiomyocytes isolated from hearts of *cmlc2a:GFP* zebrafish plated at low density. (**b**) Medium plating density. (**c**) High plating density. Scale bars, 100 µm.

a flattened appearance without striation, which enables them to firmly attach to the culture dish and divide (**Fig. 3b**). Extended cultures will contain both types of cell appearances. Notably, mature contractile cells stay connected to partially dedifferentiated cardiomyocytes (**Fig. 3c–f** and **Supplementary Videos 1** and **2**).

The initial lack of proliferation can be circumvented by isolating cardiomyocytes from regenerating hearts. These hearts will have undergone cell loss due to the injury; however, the cardiomyocytes of the surviving muscle will have started to regenerate all over the organ, and proliferating cells of flattened morphology can be seen from day 2 in culture[5,27].

The highest number of pure, viable cardiomyocytes is obtained from fish of 3–12 months of age. With increasing age, the hearts become harder to digest, leaving pieces of undigested tissue that negatively affect the yield and purity of the cardiomyocyte population. A change in tissue integrity is also observed in regenerating hearts, as these replace damaged or lost muscle tissue by a clot of extracellular matrix (ECM; scar tissue, composed mainly of fibrin and collagen), which should be removed during cleaning of the heart (Step 3).

Choice of culture dish and coating

As a rule of thumb, we use cardiomyocytes obtained from three hearts for cultures of low density when plated in one 24-well plate, whereas three hearts plated in one 48-well result in medium density, and four hearts plated in one 48-well result in high density. Examples of these three plating densities are shown in **Figure 2**. For immunocytological stainings, we found four-well slide dishes to be most practical, as they provide a sufficiently large surface for representative cell numbers even after some cell loss during the procedure.

Figure 1 gives an outline of the isolation procedure, including possible applications of isolated cardiomyocytes and guidelines for the choice of plating density and coating material. When plated on uncoated culture dishes, cardiomyocytes attach weakly to the surface, preferentially toward the edges of the dish, whereas floating cell clusters aggregate in the center of the dish. Within the first 7 d of culture, the cells can be completely removed by pipetting with a P1000 tip. When they are well attached, proliferating cells need to be processed, e.g. for RNA or protein preparation; trypsinization under mild conditions (0.1% (wt/vol) trypsin) for 5 min at 37 °C is possible.

We did not notice an improvement in cell attachment on gelatin- or collagen-coated dishes. However, cardiomyocytes attach markedly better to dishes coated with poly-L-lysine, and they are distributed more evenly than with all other coating materials we tested. We suggest the use of poly-L-lysine as the coating material when the visualization of single cells is desirable and when cell loss needs to be minimized, e.g. for immunocytochemistry, transfection, treatments that require changing of the culture medium, and measurement of exact, comparable cell numbers. For these applications,

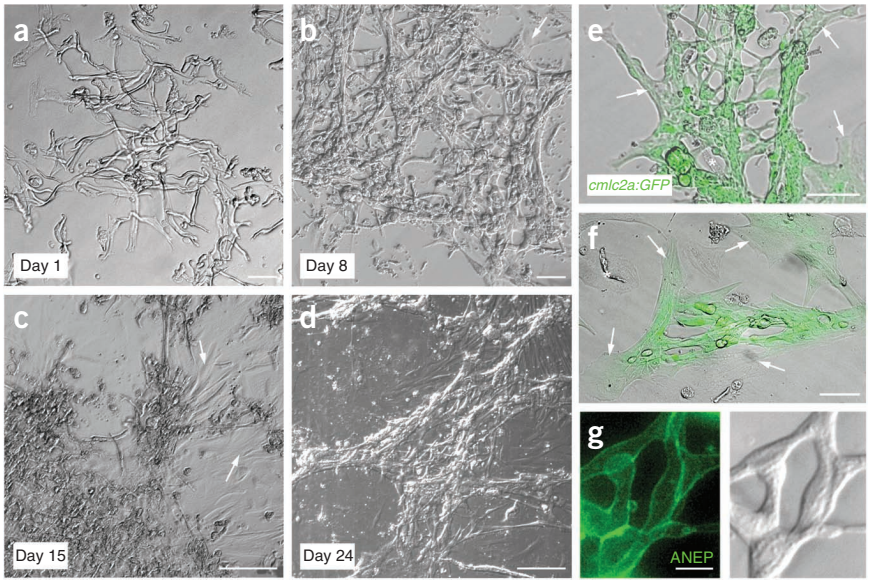

Figure 3 | Morphological characterization of cardiomyocyte cultures over 4 weeks. (**a**) Cardiomyocytes on day 1 after plating are located randomly, appear spindle shaped and of ~100 µm length, and do not contract at this early time point, even though the sarcomeric striations are visible under a light microscope. (**b**) One week after plating (day 8), the cells have formed connections, allowing them to beat in a synchronous manner. In addition, cells undergoing partial dedifferentiation can be seen (arrow) (**Supplementary Video 4**). (**c**) During the second week of culture (day 15), large areas are covered by dedifferentiated, proliferating cardiomyocytes that are still connected to mature cells (arrows) (**Supplementary Video 7**). (**d**) A 3- to 4-week culture (day 24) of cardiomyocytes appears as a tight network that shows strong, uniform contractions (**Supplementary Video 1**). (**e**,**f**) Cardiomyocytes isolated from the *tg(cmlc2a:GFP)* line strongly express GFP. Partial dedifferentiation of the cells after 2 (**e**) and 3 (**f**) weeks of culture is accompanied by a decrease in the expression of sarcomeric genes such as *cmlc2a* (arrows). Asterisks indicate dead cells. The mature cells remain connected to the ones undergoing dedifferentiation and division, and no signs of cell death are visible (**Supplementary Video 2**)[33]. Lineage tracing confirmed the myocardial origin of the dedifferentiated cells (**Supplementary Fig. 5a–c** and **Supplementary Video 8**). (**g**) High magnification of cell-cell connections in a 10-d culture, visualized by the voltage-sensitive dye Aminonaphthylethenyl pyridinium (ANEP) (**Supplementary Fig. 3**). A UV-filtered fluorescence image is shown on the left, and a bright-field image is shown on the right. Scale bars, 10 µm (**g**), 50 µm (**a**,**b**,**e**,**f**) and 100 µm (**c**,**d**).

24–48-well plates or 4- or 8-chamber slides are suitable, as they allow parallel examination of multiple samples under different treatments or conditions while keeping the required number of fish relatively low. For short-term experiments, the plating density can be low or medium. Notably, low-density cultures of more than 1 week on poly-L-lysine will start to degenerate because of insufficient cell-cell contacts. In that case, medium plating density will be a better choice (**Supplementary Table 2**).

Fibrin is produced immediately after heart injury as part of the blood clot that seals the wound, and it stimulates healing and cell migration; as described by Kim *et al.*[24], it can be used as a coating material for epicardial cell culture. While testing the behavior of isolated cardiomyocytes on fibrin, we observed that the cells formed aggregates and started strong, synchronous beating earlier than on other coating surfaces (**Supplementary Table 2** and **Supplementary Video 3**). This observation indicates that cardiomyocytes in culture behave similarly to cardiomyocytes *in vivo* when cultured on a fibrin matrix. The viscosity of the gel causes differences in the thickness of the coating when used in smaller dish formats (24 wells and smaller), making it less practical for imaging[24]. Larger dishes can be used to diminish this problem, but a higher number of cardiomyocytes will be required to produce medium-to-high-density cultures.

Advantages and limitations of the method

The major advantage of working with primary cardiomyocytes in culture is the possibility of performing assays under defined conditions (e.g., temperature, oxygen pressure); treating cells with exact concentrations of growth factors, chemicals or small molecule inhibitors; and examining transgenic zebrafish lines in parallel. All these approaches can be carried out in a highly reproducible manner while keeping the number of animals killed low compared with *in vivo* experiments. The effects on the cells upon treatment can be monitored easily, and even subtle changes in morphology or proliferation can be determined more precisely than in living fish[16]. Furthermore, the large number of *in vitro* tools and techniques, including transfection, imaging and fluorescence-activated cell sorting (FACS), can be adapted and applied to zebrafish cardiomyocytes. Specific gene knockdown using RNAi techniques is conceivable; however, one should keep in mind that manipulation by RNAi in cardiomyocytes in general is a challenging undertaking and of low efficiency.

Primary cell cultures represent physiologically more native conditions of a tissue than immortalized cell lines; thus, observations are likely to reflect the *in vivo* state of the myocardium and can be regarded as valuable information for predicting processes in the living animal. Working with primary zebrafish cells is also feasible for laboratories without fish facilities, by simply keeping the required number of fish in a temporary aquarium (as sold in pet shops) until cardiomyocyte isolation and culture.

However, cells in culture cannot replace *in vivo* experiments. Even though cardiomyocytes isolated with this protocol maintain their native intracellular structures and contractile properties over several weeks, the artificial settings of the culture system have to be considered, and cell death must be expected. In our hands, trypsinizing the cells led to high mortality and morphological change; therefore, passaging or freezing primary zebrafish cardiomyocytes is not yet feasible. Further practical limitations include the relatively small amount of starting material, the low proliferation rate and the weak attachment to most surfaces. As with all primary cultures, contaminations by other cell types cannot be completely avoided.

MATERIALS

REAGENTS

- Zebrafish (*Danio rerio*), adult male and female, aged between 3 and 12 months. Lines used: wild type (AB), *tg*(*cmlc2a*:*GFP*), transgenic zebrafish expressing GFP under the control of the *cmlc2a* (*myl7*) promoter[28] to visualize cardiomyocytes *in vivo* ! **CAUTION** All animal experiments must be performed in accordance with relevant guidelines and regulations. ! **CAUTION** Cardiomyocytes obtained from fish older than 12 months show decreased viability (see the INTRODUCTION and **Table 1**).
- Fish water (aquarium water from the zebrafish facility)
- Tricaine (Sigma, cat. no. A5040, Reagent Setup)
- Minimum essential medium (MEM) cell culture medium (Gibco, cat. no. 10370)
- GlutaMAX (Gibco, cat. no. 35050-038)
- 2,3-Butanedione monoxime (BDM; Sigma, cat. no. B0753)
- Penicillin-streptomycin (Gibco, cat. no. 15140-122)
- Normocin (Invivogen, cat. no. ant-nr-2)
- FBS (HyClone, cat. no. CH30160.03)
- PBS, 1× (prepared in house or purchased from PAA Laboratories, cat. no. H15-002)
- Heparin sodium salt (Sigma, cat. no. H3393)
- HEPES (Sigma, cat. no. H3375)
- Taurine (Sigma, cat. no. T8691)
- Glucose (Sigma, cat. no. G7528)
- Calcium chloride (Sigma, cat. no. C4901)
- Collagenase type II (Gibco, cat. no. 17101-015)
- Collagenase type IV (Gibco, cat. no. 17104-019)
- Poly-L-lysine, 0.01% (wt/vol) (Sigma, cat. no. P-4832)
- Fibrinogen, bovine plasma (Fg; Calbiochem, cat. no. 341573-1GM, Reagent Setup)
- Thrombin, bovine (Calbiochem, cat. no. 605157)
- α-sarcomeric actin (α-SA) mouse monoclonal antibody, 1:400 (Sigma, cat. no. A2172)
- Tropomyosin mouse monoclonal antibody), 1:400 (Sigma, cat. no. T2780
- GFP chicken antibody, 1:250 (Aves, cat. no. GFP-1020)
- Myocyte enhancer factor 2c (Mef2c) rabbit polyclonal antibody, 1:25 (Abcam, cat. no. ab79436)
- Phosphohistone H3 (pSer28) rat monoclonal antibody, 1:500 (pHH3; Sigma, cat. no. H9908)
- BrdU rat monoclonal antibody, 1:25 (Santa Cruz Biotechnology, cat. no. sc-70441)

EQUIPMENT

- Screw-cap microcentrifuge tubes, 1.5 ml (Sarstedt)
- Falcon conical tubes, 14 ml and 50 ml (Sarstedt)
- Sterile micropipettes for Gilson pipettes, 2–1,000 µl, length of 1,000-µl tips: 79.0 mm (ART Molecular Bioproducts, cat. no. 2279)
- Plastic pipettes, 3 ml (Deltalab)
- Petri dishes, 90-mm and 55-mm diameter (Deltalab)
- Dissecting stereomicroscope (Olympus)
- Operation sponge (self-made; foam with incision of the size of a zebrafish to be placed and immobilized)
- Forceps (Dumont FST no. 5)
- Absorbent paper towels (Kimberley-Clark)
- Thermomixer (Eppendorf) or water bath set to 32 °C

Box 1 | Culture dish coating ● TIMING 10–60 min

Additional reagent setup required for fibrin coating:
• Aliquots (500 µl) of fibrinogen (Fg) stock solution (20 mg ml^{-1}); clottability determined as described in Kim *et al.*[24].
• Aliquots (25 µl) of thrombin stock solution (50 U ml^{-1})
1. As discussed in the INTRODUCTION, the format and coating of the culture dish depends on the type of experiment. If coating is required, it is best performed during heart digestion (Steps 5–7). For coating with poly-L-lysine, follow option A. Coating with fibrin gel is performed as described by Kim *et al.*[24] and described in option B.

(A) Poly-L-lysine
(i) Add a sufficient amount of 0.01% (wt/vol) poly-L-lysine solution to the culture dish to cover the surface completely.
(ii) Incubate the dish for 5 min at room temperature.
(iii) Aspirate the excess solution.
(iv) Thoroughly rinse with sterile water.
(v) Allow the dish to dry for at least 2 h.

(B) Fibrin gel
(i) Thaw one aliquot of Fg stock solution in a 37 °C water bath and keep it at room temperature.
(ii) Quickly defrost one aliquot of thrombin stock solution and keep it on ice.
(iii) Prepare the Fg working solution by diluting the stock aliquot 1:10 in prewarmed DMEM (final concentration: 2 mg ml^{-1}).
(iv) Add thrombin to the Fg working solution (final concentration of 0.2 U ml; mix quickly by inverting the tube and proceed immediately to the coating step.
(v) Quickly coat the culture dish of choice with the Fg-thrombin mixture using, e.g., 300 µl of solution per 24-well plate. Make sure the bottom of the well is completely covered.
(vi) Incubate in the culture hood for 30 min. After 20–30 min, check whether the fibrin gel has formed, and then proceed with plating of the cardiomyocytes.
For critical steps and troubleshooting during this procedure, please refer to the original protocol[24]. A 1-week cardiomyocyte culture plated on fibrin gel is shown in **Supplementary Video 3**.

• Cooled microcentrifuge (Eppendorf 5415 R)
• Cell culture hood with laminar flow and UV light (Telstar Bio-II-A)
• Cell culture incubator, 28 °C, 5% CO_2, humidified (Revco)
• Filcon sterile filter units, 100 µm, or cell strainers
• Multiwell plates (e.g., 48-well; Corning, cat. no. 3548)
• Four-chamber slides (Lab-Tek, cat. no. 177437 and 154917)

REAGENT SETUP

Tricaine solution Prepare a stock solution of 15.3 mM in H_2O. Aliquots can be stored at −20 °C for a few months. For euthanasia, freshly dilute 1,700 µl in 20 ml of fish water (end concentration: 1.3 mM). ! CAUTION All buffers used during the isolation and culture of cardiomyocytes must be sterile filtered.

Heparin buffer The buffer consists of 1× PBS plus 10 U ml^{-1} heparin and 100 U ml^{-1} penicillin-streptomycin and can be stored at 4 °C for up to 3 months. Fill a 5-cm Petri dish for heart collection (Step 3) and place it on ice.

Perfusion buffer Perfusion buffer consists of 1× PBS plus 10 mM HEPES, 30 mM taurine, 5.5 mM glucose and 10 mM BDM; it can be stored at 4 °C for up to 3 months.

Digestion buffer Digestion buffer consists of perfusion buffer plus 12.5 µM $CaCl_2$ plus collagenases II and IV (5 mg ml^{-1} each). Freshly prepare the required volume (number of hearts × 250 µl, plus some extra) and place it on a shaker at room temperature (20–25 °C) until use (Step 5).

Stopping buffers (SB) 1–7 Perfusion buffer plus: 10% (vol/vol) FBS and 12.5 µM $CaCl_2$ (12.5 µl of 1 mM $CaCl_2$ stock in 1 ml; SB1); 5% (vol/vol) FBS and 12.5 µM $CaCl_2$ (12.5 µl of 1 mM $CaCl_2$ stock in 1 ml; SB2); 5% (vol/vol) FBS and 62 µM $CaCl_2$ (6.2 µl of 10 mM stock $CaCl_2$ stock in 1 ml; SB3); 5% (vol/vol) FBS and 112 µM $CaCl_2$ (11.2 µl of 10 mM stock $CaCl_2$ stock in 1 ml; SB4); 5% (vol/vol) FBS and 212 µM $CaCl_2$ (21.2 µl of 10 mM stock $CaCl_2$ stock in 1 ml; SB5); 5% (vol/vol) FBS and 500 µM $CaCl_2$ (5 µl of 100 mM stock $CaCl_2$ stock in 1 ml; SB6); 5% (vol/vol) FBS and 1,000 µM $CaCl_2$ (10 µl of 100 mM stock $CaCl_2$ stock in 1 ml; SB7). Freshly prepare the required volumes and place them on ice until use (Steps 8–13).

$CaCl_2$ stocks Prepare $CaCl_2$ stock solutions of 100 mM, 10 mM and 1 mM concentrations and store them at 4 °C for a few months.

Plating medium Plating medium consists of MEM plus 2 mM GlutaMAX, 5 mM BDM, 5% (vol/vol) FBS, 100 U ml^{-1} penicillin-streptomycin and Normocin (1/500 dilution) and can be stored at 4 °C for 2 weeks.

Fibrin gel Prepare as explained in Kim *et al.*[24] and as described in **Box 1**.

PROCEDURE

Heart excision ● TIMING 5–8 min per heart

1| Place each fish in 1.3 mM Tricaine solution until no signs of life are visible (3–5 min).
▲ CRITICAL STEP Steps 1–11 can be performed on a clean lab bench using sterile instruments and filter pipette tips.

2| Transfer each fish onto a wet operation sponge and place it under a dissecting microscope (**Fig. 4a**).

3| Use forceps to remove the scales and tear an opening of about 4 mm in the ventral muscle longitudinally. Take care not to damage the underlying heart. Remove the pericardium (silver). Grab the heart, ideally on the outflow tract (white), and pull out the whole organ. Quickly place the excised heart in heparin buffer, in a 55-mm Petri dish on ice. Completely remove

non-ventricle tissues (outflow tract, atrium, pericardium) and gently tear open ventricles to wash out the blood (**Fig. 4b–e**).
▲ **CRITICAL STEP** Proceed quickly through Steps 3–5, as extended time (>15 min) in heparin buffer decreases cell viability.

4| Transfer hearts into screw-cap tubes prefilled with 500 µl of heparin buffer on ice, with a maximum of 3 hearts per tube.
▲ **CRITICAL STEP** If a large number of hearts are processed, transfer them to tubes that have been prefilled with perfusion buffer instead of heparin buffer to avoid cell damage.

Digestion ● TIMING 2 h

5| Replace heparin or perfusion buffer with 250 µl of digestion buffer per heart (maximum 750 µl per tube) (**Fig. 4f**). Digest the hearts for 2 h at 32 °C in a thermomixer at 800 r.p.m. Alternatively, the tubes can be placed in a water bath set to 32 °C and gently flicked every 10 min.

6| When using a thermomixer, flick the tubes approximately every 30 min to help with the disaggregation of the tissue. Make sure no tissue gets stuck to the cap of the tube.
▲ **CRITICAL STEP** Digestion time can vary between 1.5 and 2 h depending on the activity of the collagenase batch and on the integrity of the tissue (Experimental design). Digestion for longer than 2 h or at higher temperatures is not advisable, as the cells will be damaged by overdigestion. Indigestible material is best removed by filtration using sterile filters of 100–150 µm size.
? TROUBLESHOOTING

7| During digestion, coat the cell culture dish with the coating material of choice (**Box 1**).

Washes ● TIMING 30–60 min

8| After digestion, flick the tubes to completely disaggregate the tissue.
▲ **CRITICAL STEP** None or only a few small remaining pieces of tissue should be visible at this point.

9| Add an equivalent volume of SB1 (maximum 750 µl per tube); flick again or gently pipette up and down using P1000 tips (**Fig. 4g,h**) and place on ice. Avoid bubble formation during pipetting. After pipetting, all remaining tissue pieces should be dissolved, leaving a homogenous cell suspension (**Fig. 4h**).
▲ **CRITICAL STEP** Use only pipette tips with relatively large openings to minimize tearing of the cells.
? TROUBLESHOOTING

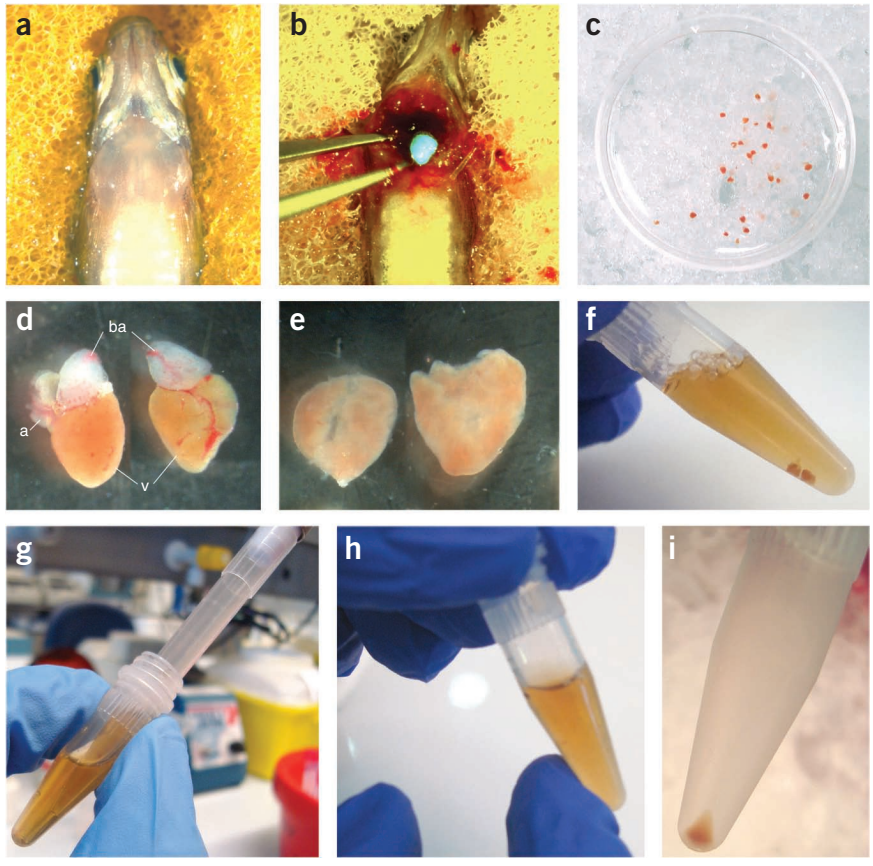

Figure 4 | Critical steps during cardiomyocyte isolation from zebrafish hearts. (**a**) Euthanized zebrafish placed ventral side up on an operation sponge. (**b**) *Tg(Cmlc2a:GFP)* zebrafish after careful opening of the chest and removal of the pericardium with sterilized forceps. Under UV light as used here, the ventricle appears in green because of the constitutive GFP expression in the cardiomyocytes of this transgenic fish line. (**c**) Hearts are placed in a Petri dish filled with heparin buffer on ice immediately after excision. To prevent cell death, incubation in heparin buffer should not exceed 15 min. (**d**) Close-up of hearts before cleaning, with atrium and outflow tract (bulbus arteriosus) and coagulated blood still attached to the ventricle. v, ventricle; a, atrium; ba, bulbus arteriosus. (**e**) Ventricles after removal of nonventricular tissues. By using forceps, the heart muscle is gently opened up to rinse out residual blood from the inside of the ventricle. (**f**) Two or three hearts are placed in one screw-cap tube for digestion with digestion buffer. (**g,h**) After digestion and gentle flicking and pipetting, the cell suspension should become homogenous without visible residual undigested pieces of tissue. (**i**) Appearance of the pure cardiomyocyte pellet after seven rounds of washes.

10| Centrifuge at 250*g* (or slower, see **Table 1**) for 5 min at 4 °C (**Fig. 4i**).
? TROUBLESHOOTING

11| After washing with SB1, carefully take off the supernatant and resuspend the pellet in 1 ml of the next sequential SB (Reagent Setup) by gentle pipetting.

12| Repeat Steps 10 and 11 until the cells are pelleted in SB7.

13| After the last wash, resuspend the pellet in plating medium for culture. Alternatively, if the cells are used for RNA or protein isolation or FACS, continue according to specific protocols.

Plating ● TIMING 10 min
14| Plate cells on your dish format and coating of choice (**Box 1**) and culture them at 28 °C in 5% CO_2.

Culture ● TIMING variable; days–weeks
15| Leave cells to attach for 24 h before treatment with chemical reagents (e.g., growth factors, BrdU).

16| Because of the presence of the contraction-inhibitor BDM in the plating medium, beating of the cardiomyocytes starts after 2–3 d in culture (**Supplementary Videos 3** and **4**). For faster recovery from BDM, the medium can be changed to plating medium without BDM. Change the medium by careful aspiration, leaving about 10% (1 mm) of the liquid on the cells to prevent detachment. Add fresh prewarmed medium dropwise. For long-term cultures, change the culture medium once a week.
▲ CRITICAL STEP Unnecessary medium changes should be avoided to preserve the number and network structure of the cells.
? TROUBLESHOOTING

17| Use standard protocols for immunocytological stainings of cultured cardiomyocytes. Details are described in Kim *et al.*[24].
▲ CRITICAL STEP Avoid aspiration of weakly attached cells during the fixation procedure. Remove the culture medium carefully with a Pasteur pipette. Leave about 1 mm of medium on the cells. Gently add a fixative (e.g., 4% (wt/vol) paraformaldehyde) onto the cells, with single drops against the side of the culture dish. For immunocytological stainings of cardiomyocytes cultured on fibrin gels, we recommend placing glass coverslips into multiwell dishes before coating.

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
6	Variable digestion times	Variability between batches of collagenase	When starting a new batch of collagenase, check the progress of digestion frequently (every 15 min) by flicking the tube. Note the time for complete tissue disintegration for each batch
6, 9	Indigestible pieces of tissue	Hearts containing a large amount of ECM, e.g., regenerating hearts that accumulate ECM as part of the wound-healing process, or older fish	Dissect clearly identifiable ECM parts off the ventricle If undigested chunks persist after the first wash in SB1, pass the cell suspension through a sterile 100–150-ìm filter Use younger fish (<12 months)
10, 16	Contamination with nonmyocardial cell types (low purity)	Incomplete removal of nonmyocardial tissues	Carefully remove all identifiable nonmyocardial cell types (connective tissue) after heart excision Use *cmlc2a* lineage–labeled zebrafish line to easily identify the myocardium during heart excision and cleaning of the tissue Digest longer, homogenize gently and ensure that no pieces of tissue are visible after the first wash with SB1 Centrifuge more slowly (150*g* instead of 250*g* for the last three out of seven washes)

(continued)

TABLE 1 | Troubleshooting table (continued).

Step	Problem	Possible reason	Solution
16	Low yield	Small amount of starting material (small hearts, or regenerating hearts with large portions of ECM	Use more hearts
		High cell loss during washes	Aspirate the supernatants carefully
		Incomplete digestion due to low active collagenase batch	Digest longer or change enzyme batch
		Old fish	Use younger fish (<12 months)
	Large number of dead cells (low viability)	Rough handling during isolation	Pipette gently, use pipettes with large openings to minimize mechanical stress
		Excessive digestion or a highly active collagenase batch	Shorten the digestion time or change the enzyme batch
	Irreproducible results	Cell density	Plate in higher density (see INTRODUCTION section on 'Choice of culture dish and coating')
	Lack of long-term survival	Contamination	Work as aseptically as possible. Sterile-filter all solutions and clean equipment thoroughly. Use filter tips. Change the medium once a week to refresh the antibiotic and antifungal formulations included in the plating medium. When culturing cells in slide dishes, place the slide inside a 10-cm Petri dish for better protection
		Low plating density	Plate in medium or high density, or use a smaller culture dish for the same number of isolated cardiomyocytes

● TIMING

Steps 1–4, heart excision: 5–8 min per heart
Steps 5–7, digestion: 2 h
Steps 8–13, washes: 30–60 min
Step 14, plating: 10 min
Steps 15–17, culture: variable; days–weeks
Box 1, culture dish coating: 10–60 min

ANTICIPATED RESULTS

The life expectancy of cultured zebrafish cardiomyocytes averages 4 weeks, during which time it is sufficient to change the culture medium once per week. We observed an accelerated decrease in viability after 4 weeks, when dead cells accumulated and the contractile activity of the culture slowed down (**Supplementary Fig. 2** and **Supplementary Video 5**). Dead cells are clearly distinguishable from partially dedifferentiated cardiomyocytes because they have a rough surface, are disconnected from the network of living cells and, most importantly, do not contract at all. Dedifferentiated cells, in contrast, frequently continue beating weakly because of their stable connections to mature cardiomyocytes. Examples of dead cells are highlighted in **Figure 3a**,**e**,**f** and in **Supplementary Video 6**. Viability depends on many factors, most prominently on the handling of the cells during isolation and the age of the fish (**Table 1**). Cardiomyocytes, because of their internal structure, are delicate cells to work with. Therefore, the excessive mechanical stress of pipetting should be avoided. Plating the cells on fibrin gels seems to promote the survival of long-term cultures.

Zebrafish cardiomyocytes show synchronous contraction without external stimulation. This behavior serves as a good measure of healthy cardiomyocytes. Further, the morphological appearance of the cells, including striation pattern, spindle shape and functional cell-cell connections, indicates their close-to-native integrity (**Fig. 3g** and **Supplementary Fig. 3**). We determined the proliferation rate and found 17±3% of cardiomyocytes were positively labeled for BrdU at day 7 of culture (**Supplementary Fig. 1**). This value corresponds to the BrdU incorporation index calculated for regenerating hearts at 7 d after amputation *in vivo*[29], and it strengthens the physiological relevance of the cardiomyocyte population obtained with our isolation method.

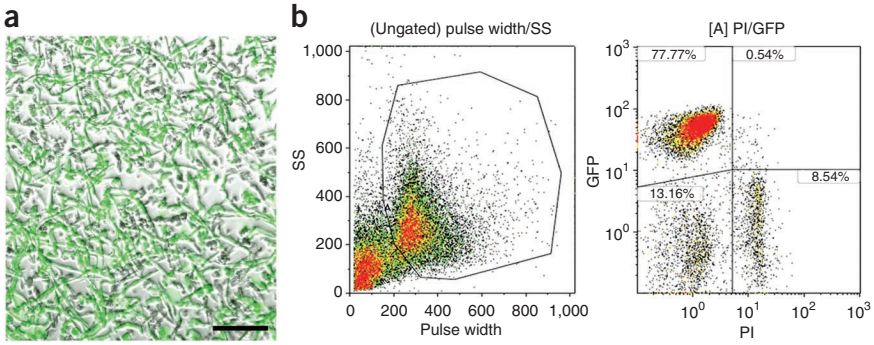

Figure 5 | The yield and purity of isolated cardiomyocytes. (**a**) Overlay of GFP and bright-field pictures showing ~80% GPF+ cardiomyocytes. (**b**) FACS analysis of purified cardiomyocytes. Cells isolated from three hearts of *tg(cmlc2a:GFP)* zebrafish were analyzed on a MoFlo cell sorter. The gated population of cells (excluding debris) was analyzed for propidium iodide (PI) and GFP. Scale bar, 100 µm.

By using GFP-expressing cardiomyocytes, we estimate a minimum of 80% purity (**Fig. 5** and **Supplementary Fig. 4**)[30]. We confirmed this percentage by flow cytometric analysis and counted an average yield of 5,000 viable cardiomyocytes per ventricle (**Fig. 5b** and **Supplementary Table 3**).

As recently described for larval zebrafish cells[31], we also found adult cardiomyocytes to be suitable for FACS. Taking advantage of the increasing amount of transgenic zebrafish lines available, FACS allows collection of fluorescently tagged cardiomyocytes of highest purity. Furthermore, subpopulations, e.g. proliferating cardiomyocytes expressing EGFP under the control of the *cyclin B1* promoter, can be sorted[32]. Even small samples of sorted cells (ranging from 1,000 to 10,000 cells) provide sufficient material for modern technologies of RNA extraction, amplification and subsequent transcriptome analysis, such as microarrays and next-generation sequencing.

Primary cardiomyocyte cultures are well suited for *in vivo* imaging of lineage-specific fluorescent protein expression and immunocytological labeling on fixed cells. Treatments affecting cell morphology can be monitored by labeling components of the sarcomeric apparatus, including tropomyosin and α-SA (**Supplementary Fig. 5** and **Fig. 6**), or by visualization of *tg(cmlc2a:XFP)* lineage tracer lines (**Figs. 2** and **3e**,**f**; **Supplementary Videos 7** and **8**), whereas proliferation can be traced by BrdU labeling (**Figs. 6b** and **7**) or pHH3 antibody staining (**Fig. 6d**). Additional approved zebrafish antibodies can be found at https://wiki.zfin.org/display/AB/ZFIN+Antibody+Wiki.

Among the most common liposomal transfection reagents, the best result we obtained in terms of transfection efficiency was 10–15% using Lipofectamine

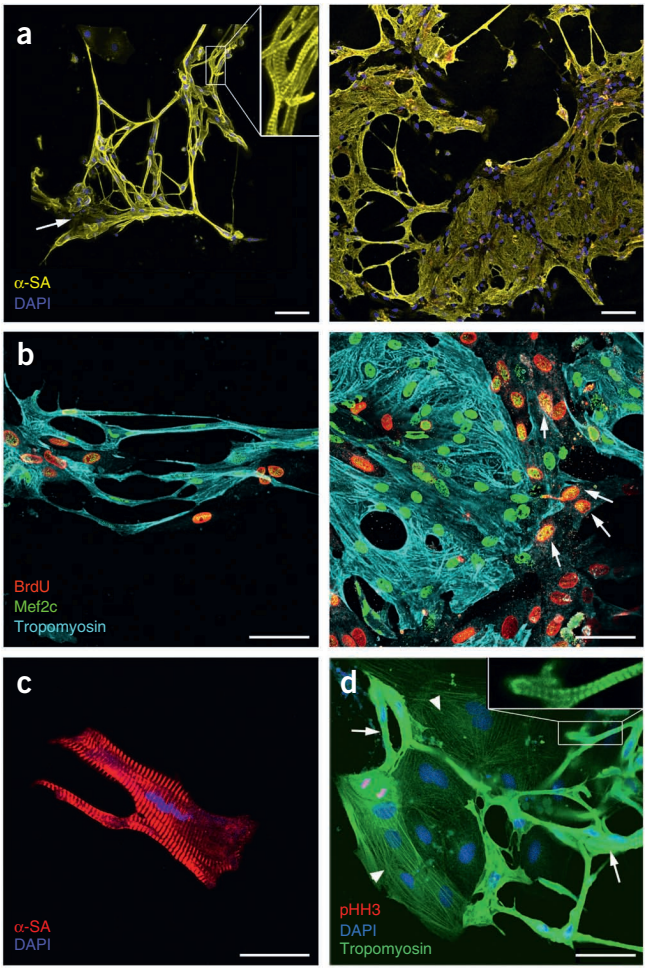

Figure 6 | Sarcomeric structure and proliferation of cultured cardiomyocytes. (**a**) One-week culture of cardiomyocytes isolated from normal (left) and regenerating hearts (right, after partial ventricle amputation 7 d before isolation), immunocytologically stained for α-SA (yellow) and DAPI (blue). Cells obtained from nonregenerating hearts appear mainly rod shaped with a clear striation pattern of the sarcomeres (inset). Beginning dedifferentiation can be identified by decreasing the α-SA signal owing to disassembly of the sarcomeres, and the flattened, nonstriated morphology (arrow in the left image). In contrast, cardiomyocyte cultures prepared from regenerating hearts cover large areas of the culture dish with dedifferentiated, proliferating cells that show less-intense α-SA staining. (**b**) The same cell population as in **a**, labeled with antibodies against tropomyosin (an actin-binding protein located in the sarcomeric apparatus; light blue), Mef2c (a transcription factor specifically expressed in adult cardiomyocytes[34]; green) and BrdU incorporation (red). The number of proliferating cells is increased in cardiomyocytes prepared from regenerating hearts (right). Note the overlap of partially dedifferentiated cells (identifiable by the weak label of Tropomyosin) and BrdU (arrows in the right image). Even with reduced expression of a sarcomeric marker such as Tropomyosin, Mef2c expression in every nucleus proves the purity of the culture (**Supplementary Fig. 5d–h**). (**c**) Example of a mature cardiomyocyte showing a sarcomeric structure labeled by α-SA (red; DAPI in blue). (**d**) Network of cardiomyocytes undergoing partial dedifferentiation and division. A nucleus in telophase is labeled with pHH3-specific antibody (red). The signal of tropomyosin staining (green) is shown overexposed in order to clearly demonstrate that all cells (nuclear staining with DAPI, blue) are cardiomyocytes expressing either high (arrow and inset) or low (arrowheads) levels of tropomyosin, depending on their grade of dedifferentiation. Scale bars, 100 µm (**a**,**b**,**d**) and 25 µm (**c**).

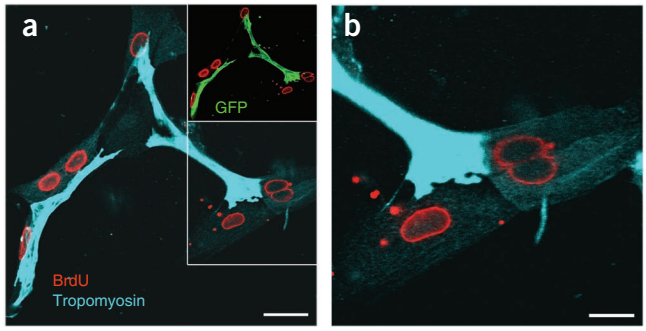

Figure 7 | Dividing cardiomyocytes. (**a**) Reduced labeling of tropomyosin (light blue) and *cmlc2a:GFP* (green; upper inset) indicates partial dedifferentiation in regions of active cell division (BrdU-positive nuclei; red). (**b**) High magnification of the cell in **a**. Scale bars, 100 μm (**a**) and 75 μm (**b**).

2000 (**Supplementary Fig. 6**). This finding proves that adult zebrafish cardiomyocytes are capable of transfection *per se*; however, optimization of the transfection protocol or method (e.g., infection with adenovirus as used in mammalian cardiomyocytes) will be necessary.

Our protocol for isolation and *in vitro* culture of primary zebrafish cardiomyocytes provides a novel and valuable system for investigating the properties of this cell type and may help in discovering the regulatory mechanisms involved in heart regeneration.

Note: Supplementary information is available in the online version of the paper.

ACKNOWLEDGMENTS We thank A. Raya for help on the protocol, I. Berniakovich and C. Newey for critical reading of the manuscript and C. Fabregat, D. Mulero and J. Vaquero for technical assistance. This work was supported by an INSERM ATIP-AVENIR grant (C.J.). Work in the laboratory of J.C.I.B. was supported by TERCEL-ISCIII-MINECO, Fundacion Cellex, CIBER, The Leona M. and Harry B. Helmsley Charitable Trust and the G. Harold and Leila Y. Mathers Charitable Foundation.

AUTHOR CONTRIBUTIONS V.S., C.J. and J.C.I.B. designed the study; V.S., G.S., C.J. and C.M. carried out the experiments; and V.S. and J.C.I.B. wrote the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

1. Major, R.J. & Poss, K.D. Zebrafish heart regeneration as a model for cardiac tissue repair. *Drug Discov. Today Dis. Models* **4**, 219–225 (2007).
2. Kuhl, S.J. & Kuhl, M. Improving cardiac regeneration after injury: are we a step closer? *Bioessays* **33**, 669–673 (2011).
3. Bersell, K., Arab, S., Haring, B. & Kuhn, B. Neuregulin1/ErbB4 signaling induces cardiomyocyte proliferation and repair of heart injury. *Cell* **138**, 257–270 (2009).
4. Engel, F.B. *et al.* p38 MAP kinase inhibition enables proliferation of adult mammalian cardiomyocytes. *Genes Dev.* **19**, 1175–1187 (2005).
5. Jopling, C. *et al.* Zebrafish heart regeneration occurs by cardiomyocyte dedifferentiation and proliferation. *Nature* **464**, 606–609 (2010).
6. Kikuchi, K. *et al.* Primary contribution to zebrafish heart regeneration by *gata4*$^{+}$ cardiomyocytes. *Nature* **464**, 601–605 (2010).
7. Gonzalez-Rosa, J.M. & Mercader, N. Cryoinjury as a myocardial infarction model for the study of cardiac regeneration in the zebrafish. *Nat. Protoc.* **7**, 782–788 (2012).
8. Brette, F. *et al.* Characterization of isolated ventricular myocytes from adult zebrafish (*Danio rerio*). *Biochem. Biophys. Res. Commun.* **374**, 143–146 (2008).
9. Zhang, P.C., Llach, A., Sheng, X.Y., Hove-Madsen, L. & Tibbits, G.F. Calcium handling in zebrafish ventricular myocytes. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **300**, R56–R66 (2011).
10. Louch, W.E., Sheehan, K.A. & Wolska, B.M. Methods in cardiomyocyte isolation, culture, and gene transfer. *J. Mol. Cell Cardiol.* **51**, 288–298 (2011).
11. Mitcheson, J.S., Hancox, J.C. & Levi, A.J. Cultured adult cardiac myocytes: future applications, culture methods, morphological and electrophysiological properties. *Cardiovasc. Res.* **39**, 280–300 (1998).
12. Grunow, B. *et al.* *In vitro* developed spontaneously contracting cardiomyocytes from rainbow trout as a model system for human heart research. *Cell Physiol. Biochem.* **27**, 1–12 (2011).
13. Nurmi, A. & Vornanen, M. Electrophysiological properties of rainbow trout cardiac myocytes in serum-free primary culture. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **282**, R1200–R1209 (2002).
14. Warren, K.S., Baker, K. & Fishman, M.C. The *slow mo* mutation reduces pacemaker current and heart rate in adult zebrafish. *Am. J. Physiol. Heart Circ. Physiol.* **281**, H1711–H1719 (2001).
15. Jopling, C., Sune, G., Faucherre, A., Fabregat, C. & Izpisua Belmonte, J.C. Hypoxia induces myocardial regeneration in zebrafish. *Circulation* **126**, 3017–3027 (2012).
16. Jopling, C., Suñe, G., Morera, C. & Izpisua Belmonte, J.C. p38α MAPK regulates myocardial regeneration in zebrafish. *Cell Cycle* **11**, 1195–1201 (2012).
17. Frank, J.S., Rich, T.L., Beydler, S. & Kreman, M. Calcium depletion in rabbit myocardium. Ultrastructure of the sarcolemma and correlation with the calcium paradox. *Circ. Res.* **51**, 117–130 (1982).
18. Singal, P.K., Matsukubo, M.P. & Dhalla, N.S. Calcium-related changes in the ultrastructure of mammalian myocardium. *Br. J. Exp. Pathol.* **60**, 96–106 (1979).
19. Brand, N.J., Lara-Pezzi, E., Rosenthal, N. & Barton, P.J. Analysis of cardiac myocyte biology in transgenic mice: a protocol for preparation of neonatal mouse cardiac myocyte cultures. *Methods Mol. Biol.* **633**, 113–124 (2010).
20. Nakagawa, O. *et al.* Rapid transcriptional activation and early mRNA turnover of brain natriuretic peptide in cardiocyte hypertrophy. Evidence for brain natriuretic peptide as an "emergency" cardiac hormone against ventricular overload. *J. Clin. Invest.* **96**, 1280–1287 (1995).
21. Brutsaert, D.L. Cardiac endothelial-myocardial signaling: its role in cardiac growth, contractile performance, and rhythmicity. *Physiol. Rev.* **83**, 59–115 (2003).
22. Kikuchi, K. *et al.* Retinoic acid production by endocardium and epicardium is an injury response essential for zebrafish heart regeneration. *Dev. Cell* **20**, 397–404 (2011).
23. Vieira, J.M. & Riley, P.R. Epicardium-derived cells: a new source of regenerative capacity. *Heart* **97**, 15–19 (2011).
24. Kim, J., Rubin, N., Huang, Y., Tuan, T.L. & Lien, C.L. *In vitro* culture of epicardial cells from adult zebrafish heart on a fibrin matrix. *Nat. Protoc.* **7**, 247–255 (2012).
25. Hedhli, N. *et al.* Endothelial-derived neuregulin is an important mediator of ischaemia-induced angiogenesis and arteriogenesis. *Cardiovasc. Res.* **93**, 516–524 (2012).
26. Jopling, C., Boue, S. & Izpisua Belmonte, J.C. Dedifferentiation, transdifferentiation and reprogramming: three routes to regeneration. *Nat. Rev. Mol. Cell Biol.* **12**, 79–89 (2011).
27. Porrello, E.R. *et al.* Transient regenerative potential of the neonatal mouse heart. *Science* **331**, 1078–1080 (2011).
28. Huang, C.J., Tu, C.T., Hsiao, C.D., Hsieh, F.J. & Tsai, H.J. Germ-line transmission of a myocardium-specific GFP transgene reveals critical regulatory elements in the cardiac myosin light chain 2 promoter of zebrafish. *Dev. Dyn.* **228**, 30–40 (2003).
29. Poss, K.D., Wilson, L.G. & Keating, M.T. Heart regeneration in zebrafish. *Science* **298**, 2188–2190 (2002).
30. McCurley, A.T. & Callard, G.V. Characterization of housekeeping genes in zebrafish: male-female differences and effects of tissue type, developmental stage and chemical treatment. *BMC Mol. Biol.* **9**, 102 (2008).
31. Manoli, M. & Driever, W. Fluorescence-activated cell sorting (FACS) of fluorescently tagged cells from zebrafish larvae for RNA isolation. *Cold Spring Harb. Protoc.* http://dx.doi.org/10.1101/pdb.prot069633 (2012).
32. Kassen, S.C. *et al.* The *Tg(ccnb1:EGFP)* transgenic zebrafish line labels proliferating cells during retinal development and regeneration. *Mol. Vis.* **14**, 951–963 (2008).
33. Dispersyn, G.D. *et al.* Dissociation of cardiomyocyte apoptosis and dedifferentiation in infarct border zones. *Eur. Heart J.* **23**, 849–57 (2002).
34. Wang, J. *et al.* The regenerative capacity of zebrafish reverses cardiac failure caused by genetic cardiomyocyte depletion. *Development* **138**, 3421–3430 (2011).