Published in final edited form as:

Methods. 2012 January ; 56(1): 44–49. doi:10.1016/j.ymeth.2011.11.004.

Purification of cardiac cells from *Drosophila* embryos

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Abstract

Recent genome-level innovations have enabled the identification of the entire cadre of genes that are expressed in specific tissues at particular developmental times. However, to be informative as to how individual cell types develop, this process relies upon the successful and efficient purification of cells for a particular tissue. Here, we describe a method to isolate cardiac cells from *Drosophila* embryos. We generated transgenic embryos in which a cardiac-specific enhancer of the *Sulphonylurea receptor* (*Sur*) gene drove expression of the Green Fluorescent Protein (GFP) gene. Homogenized embryos were subjected to fluorescence activated cell sorting (FACS), resulting in approximately 50,000 cardiac cells purified. The prevalence of cardiac cells in the purified population was high, based upon a significant enrichment for cardiac-specific marker genes, including *Sur* and *Toll*. This enrichment also enabled the identification of cardiac-specific alternatively spliced isoforms of the *Zasp66* gene. In the future, this approach can be used to describe the cardiac transcriptome of *Drosophila* at distinct stages of embryonic development.

Keywords

Drosophila; heart; cell purification; cell sorting; *Sulphonylurea receptor*; method; FACS

1. Introduction

Unraveling the pathways that lead to the development of complex tissues and organs, such as the heart, requires an understanding of the cadre of genes that are expressed during the formation of that tissue, at sequential developmental stages. To a certain degree, this goal can be achieved for cardiac genes by studying gene expression in tissue culture cells that have been induced to develop as cardiac cells (see for example (1, 2)). However cells cultured *in vitro* fail to recapitulate all of the developmental steps that occur *in vivo*.

This obstacle can be overcome by directed dissection or purification of candidate tissues and organs. For the adult heart, several studies have analyzed transcriptional changes in the diseased heart of rodent models (see for example (3)), and such studies are facilitated by the ability to isolate readily the cardiac tissue by dissection. However, analysis of developmental processes requires the isolation of embryonic cardiac tissue, and for many of these cases cardiogenic cells must be carefully isolated from neighboring cell types. Latif et al (4) collected mouse embryos carrying an *Nkx2-5-eYFP* transgene, and then used fluorescence activated cell sorting (FACS) of homogenates to purify cardiogenic cells. This

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approach identified a broad range of cardiac-expressed genes, and also identified transcripts that were specific to different stages of the cardiogenic process.

The combination of this approach with a more genetically-amenable model system should enable not only the identification of cardiogenic factors, but also their rapid functional characterization. For *Drosophila*, this model system has already made significant contributions to our understanding of cardiac specification and development across the animal kingdom (5, 6). Chief among the cardiac regulatory factors in *Drosophila* is the NK homeobox gene *tinman* (*tin*), whose activity is essential to cardiac specification (7, 8), and which lies at the top of a complex transcriptional network for cardiac, skeletal, and visceral muscle development (9). Tin is also a transcriptional activator of the cardiac muscle differentiation program, where a number of direct transcriptional targets for Tin have been characterized. These targets include genes encoding the membrane antigens *Sulfonylurea receptor* (*Sur*) and *Toll* (*Tl*) receptor (10–12), among others.

On the morphological level, the open circulatory system in *Drosophila* consists of a pulsatory tube called the dorsal vessel, which becomes specified and differentiates within the one-day period of embryonic development. The posterior of the dorsal vessel, termed the heart proper, has a slightly wider diameter, and is the chamber into which hemolymph flows; hemolymph then is pumped more anteriorly through the region of the vessel termed the aorta, where the blood is deposited near the brain, before percolating back through the body cavity to re-enter the heart. Interestingly, the flow direction in the dorsal vessel may alternate.

Despite the relatively simple morphology, the cellular and molecular composition of the dorsal vessel is complex, comprising several cell types, that each possesses distinct morphological and molecular properties (reviewed in (13)). Firstly, muscular cardiac cells form the interior lining of the vessel, conferring contractile activity to the whole organ. Secondly, specialized cells form inflow tracts (termed ostia) in the heart proper. Thirdly, a diverse population of pericardial cells surrounds the muscular part of the dorsal vessel. The pericardial cells do not seem to participate in contractile function, but are important for hemolymph filtration and some aspects of rhythmicity (13, 14).

Nevertheless, the forming cardiac musculature in *Drosophila* is relatively small, and is not conducive to microdissection. Here, we describe a method that can overcome this limitation, developed from published assays aimed at isolating other cell types in *Drosophila* (15–17). We generated transgenic embryos in which the dorsal vessel expresses the green fluorescent protein (GFP). Following homogenization of staged embryos, we isolated cardiac cells via FACS. Molecular analyses of the collected cardiac samples confirmed their enrichment in known cardiac-specific molecular markers, and identified a new cardiac-specific splice-isoform of the *Zasp66* gene. This method can be further adopted for transcriptome and other high-throughput characterization of the dorsal vessel.

2. Materials and methods

2.1. Fly stocks

The *Sur-Gal4* plasmid was constructed by cloning the ~900-bp enhancer region of the *Sur* gene (11) into the pPTGAL vector (18) to control expression of *Gal4* in a tissue-specific manner. This plasmid was used to create transgenic animals by P-element mediated insertion of the *Sur-Gal4* construct, and a line was isolated for which the insertion mapped to the third chromosome. To create a stable fly line with fluorescent cardiac cells, a second chromosome carrying a *UAS-2XeGFP* insertion (19) was combined with the *Sur-Gal4* line, using standard

crossing techniques. Flies that were homozygous for both inserts were used directly for embryo production.

2.2. Embryo collection

This method requires large quantities of highly synchronized embryos. Dense fly cultures were kept in four plastic cages that were fitted with standard 100 mm Petri dishes containing grape juice agar and a smear of heat-killed yeast paste. For collection, plates were changed at 2-hr intervals, for a total of 10 h. The first collection was discarded since not all of the embryos from a first collection are synchronized; all the following collections were immediately placed at 4°C, where they were kept until the beginning of the staging phase. Cooling for a few hours was not harmful to the embryos, and it allowed synchronization between collection batches, since embryonic development was significantly slowed at 4°C, and resumed normally after embryos were placed at 25°C. In the staging phase of the protocol, embryos were allowed to develop for 10 hrs at 25°C (approximately corresponding to stage 13 of *Drosophila* development), when GFP fluorescence in developing cardioblasts became apparent.

2.3. Cell isolation

A schematic of the steps required for obtaining cell suspension from embryos is depicted in Fig. 1. Staged embryos were dechorionated with 50% bleach solution (sodium hypochlorite) for 3 min, extensively washed, and spread over a fresh grape juice agar plate. Using a low-magnification dissection microscope, any hatched larvae or old embryos were located and mechanically removed. At this step, the proper developmental stage of embryos can be monitored in a sample subjected to fluorescence microscopy, when mounted on a slide in a drop of 80% glycerol and covered with a coverslip. Next, batches of approximately $1.0\text{--}1.5 \times 10^3$ embryos were collected using a paintbrush, transferred into a Dounce homogenizer (Wheaton, cat. #432-1270) containing 1 ml of Insect Dissection Medium (Sigma, cat. #C1544), and disrupted with 7–8 strokes of the loose pestle. Resulting homogenates were combined in a 15-ml tube, pelleted at 500g for 5 min and, after resuspension in 1 ml of Schneider's medium (Gibco, cat. #11720), treated with 1 mg/ml elastase (Sigma cat. #E0258) at room temperature for 30 min, on a rotator. The enzymatic reaction was stopped with 4 ml Schneider's medium containing 10% FBS (Atlanta Biologicals, cat. #S10450), gravity-filtered through a 50 m syringe filter (BD Biosciences, cat. #340603), and centrifuged at 500g for 5 min. The resulting pellet was finally resuspended in 1 ml of Schneider's medium supplemented with: 10% FBS, 10 µg/ml Propidium Iodide (PI) (Invitrogen, cat. #P3566), 10 µg/ml Hoechst 33258 (Invitrogen, cat. #H3569), and 0.1% Pluronic F-68 (Sigma, cat. #P1300). After incubation for 20 min at room temperature, the cell suspension was kept on ice until sorting.

2.4. Fluorescence-Activated Cell Sorting (FACS)

The cell suspension was sorted using a high-speed Legacy MoFlo cell sorter (Beckman Coulter), equipped with UV and argon lasers. Machine settings were adjusted to the single cell sorting mode. As a special requirement for *Drosophila* cell viability, the sheath fluid was replaced with Seecof saline, containing: 6 mM Na_2HPO_4 , 3.67 mM KH_2PO_4 , 106 mM NaCl, 26.8 mM KCl, 6.4 mM MgCl_2 , 2.25 mM CaCl_2 , pH 6.8, and additionally supplemented with 0.05% Pluronic F-68 (Sigma, cat. #P1300). The latter is a surfactant reagent that greatly enhances cell survival through the sorting procedure. Cells were sorted by gating for GFP-positive and Hoechst-positive events, and collected directly into RLT lysis buffer provided by the RNeasy Mini kit (Qiagen cat. #74104). Special attention was paid to not exceed dilution of the lysis buffer by more than two-fold. Samples obtained on different days were kept frozen in the lysis buffer until further use.

For quality control, some cell samples were sorted in 10 ml of Schneider's medium. Sorted cells were pelleted at 500g for 10 min, restored in 50 μ l of the medium with 10 μ g/ml of PI and examined under a fluorescence microscope. GFP, PI, and Hoechst fluorescence was used to assess the purity and viability of the sorted cells.

2.5. RNA extraction and PCR analysis

Multiple samples in RLT lysis buffer were combined, and total RNA was isolated according to the RNeasy kit manufacturer's protocol. A 15-min, on-column DNase I treatment was included, following the protocol's options (Qiagen, cat. #79254). Purified RNA was re-suspended in 30 μ l of water. cDNA synthesis was performed with 12 μ l of isolated RNA and random primer hexamers (Roche, cat. #11034731001), using the SuperScript II Reverse Transcriptase kit (Invitrogen, cat. #18064) in accordance with the manufacturer's protocol. The cDNA mixture that was obtained was diluted ten-fold and kept frozen. PCRs were assembled in 15 μ l total volume, using 1 μ l of the diluted cDNA, 0.5 μ l of specific primers (concentration 25 mM each), and 0.1 μ l Pfx Platinum polymerase (Invitrogen, cat. #11708). For splicing analysis, putative alternative splice sites were selected based on annotated transcript data obtained from FlyBase.org, and their sequences are listed in Table 1. Reactions were run for 35 cycles, using primer annealing temperatures 50–55°C.

3. Results

In order to genetically tag cardiac cells in the *Drosophila* embryo, we cloned the enhancer region corresponding to the *Sur* gene (11) upstream of the *Gal4* gene, using the pPTGAL vector (18), and generated transgenic fly lines by P-element mediated germline transformation. Utilizing the well-characterized binary Gal4/UAS expression system (20), the cardiac-specific activity of this construct was confirmed by crossing the flies with *UAS-2xeGFP* (Fig. 2 A,B). The GFP expression was first detected at embryonic stage 13 in cardiac progenitor cells that were migrating as two lateral rows towards the dorsal midline to form the dorsal vessel (Fig 2A). At stage 16, when the dorsal vessel was formed, bright GFP fluorescence was predominant in the cardiac cells (Fig. 2B). Some cells within the cardiac tube were slightly brighter, although we have not defined to which cardiac sub-population these cells correspond. Some residual signal was also detected in a subpopulation of pericardial cells, and a few cells in the central nervous system. Since similar non-cardiac expression was observed with the cloned *Sur* enhancer in a different transgenic construct and different genetic background (11), we conclude that it is a *bona fide* property of this enhancer. To reduce the collection of non-cardiac cells in our experiments, we used embryos developed up to stage 13, when the *Sur* expression was only observed in cardiac progenitors.

To prepare samples for FACS sorting, we developed a protocol shown schematically in Fig. 1. To obtain a homogenous cell suspension, embryos were mechanically disrupted in an homogenizer and additionally treated with elastase. Although mechanical disruption is usually sufficient for isolating cells from early embryos (up to stage 6), additional enzyme treatment was required to enhance release of single cells from stage 13 embryos. Before sorting, the cell suspension was incubated with PI to mark damaged and dead cells, and with cell-permeable Hoechst 33258 to unambiguously label all cell nuclei. In the flow cytometric analysis, three populations of cells were clearly distinguishable on the plot of green (GFP) and red (PI) fluorescence (Fig. 2C, left panel): dead cells, that stained strongly with PI (gate "1"); unstained cells and debris, that were not fluorescent in either channel (predominantly residing within gate "3"); and GFP-positive cells (gate "2"). As expected, the proportion of GFP-positive cells was low, ranging from 0.05% to 0.1 % of all plotted events.

In a separate graph, plotted against green and blue (Hoechst) fluorescence, gated GFP-positive cells showed heterogeneity that we attribute to single cells with various DNA

content (gate “4”), as well as multi-cellular clusters (gate “5”) that must have escaped exclusion despite the stringent selection that we used. To ensure the highest purity, particles with especially high Hoechst fluorescence were excluded from sorting. Examination by fluorescence microscopy of collected test samples confirmed that our sorting strategy was successful in selecting GFP-positive cells (referred to as “Sur>GFP” sample), reliably achieving the presence of GFP in more than 90% of the sorted cells (not shown), and that the sorting process was not harmful to cell survival (Fig. 2D).

As a control, viable cells regardless of their GFP fluorescence were sorted from the same cell suspension used to generate the Sur>GFP sample (Fig. 2C, gate “3”). This control provided an average molecular profiling across all embryonic tissues, that was time-synchronized with the GFP-positive cells that were collected. From our preliminary analyses of different cell populations fluorescently tagged using general mesoderm-specific drivers (such as *Tw-Gal4* and *24B-Gal4*), we estimate the ratio of mesoderm-derived cells in such a general cell mix to be at approximately 20%.

In the course of this study we sorted approximately 5×10^5 cells for each sample, where the size-limiting sample was the Sur>GFP. This amount of sorted material allowed us to extract 75 ng/sample of total RNA. Using approximately half of the extracted RNA in a reverse transcriptase reaction, and subsequently diluting the synthesized cDNA ten-fold, we generated 200 μ l of cDNA per sample. This amount is estimated to be sufficient for 200 PCR amplifications, when using 1 μ l of cDNA per reaction.

The prepared cDNA was next used in PCR-based molecular validation of the Sur>GFP sample. From this analysis, the Sur>GFP sample was enriched in muscle-specific transcripts, such as muscle-specific actin (*Act57B*) and troponin I (*TnI*). Moreover, the Sur>GFP sample was significantly enriched in known cardiac-specific transcripts, such as the endogenous *Sulphonylurea receptor* gene (*Sur*), and the *Toll* gene (*Tl*) (Fig. 3A). These data indicate that we have successfully collected samples that are enriched in cardioblasts.

Next, we carried out a comparative analysis, looking for differences in alternative splicing patterns of muscle-specific transcripts between cDNA synthesized from Sur>GFP cells, versus cDNA synthesized from non-sorted cells, anticipating that this sample would predominantly contain non-cardiac muscle transcripts. In this assay, we used PCR primers that recognized sequences flanking putative alternative splice-sites, and that could generate products with predictably different sizes. Although a number of targets that were tested failed to identify any differences in their alternative splicing between Sur>GFP and non-sorted samples, we found that transcripts of the muscle structural gene *Zasp66* showed splice variants that were unique to the Sur>GFP sample (Fig. 3B).

Overall, our results indicate that the proposed sorting procedure of genetically tagged cardiac cells is effective in generating sufficient amounts of RNA for PCR-based molecular profiling, demonstrates enrichment in expression of general muscle genes as well as cardiac-specific genes, and, finally, is sensitive enough to detect novel molecular signatures of the cardiac muscle.

4. Discussion

In this work, we describe a method to collect enriched populations of cardioblasts from the *Drosophila* embryo. Our sorting method is based upon pioneering studies addressing FACS-based sample collection, for different types of *Drosophila* cells (15–17). However, we have developed some modifications to this method, in order to maximize the yield of surviving cardiac cells, and to enhance detection of cardiac-specific transcripts. Recently, a manuscript by Salmand et al (21) described a similar approach to isolation of GFP-labeled cells from

the *Drosophila* dorsal vessel. The authors of this parallel and independent study report some methodological differences to our approach, such as choice of protease for enzymatic treatment and the applied sorting logic. Interestingly, the authors do not mention the technical challenges of sorting *Drosophila* cells, which were met in the course of our study and which we address below in more detail.

One of the challenges in our study was that the sample has to be collected from late embryos. By stage 13 the embryonic tissues become strongly adherent, and cannot be broken into single-cell suspension by merely mechanical forces, as can be achieved with early stage embryos. Thus, enzymatic treatment with elastase was a necessary element in our protocol. In addition to elastase, we also found that trypsin produced suitable results in separating cells.

A second challenge that we faced was the poor sorting and survival of *Drosophila* cells. In fact, our pilot FACS runs did not succeed in detectable collection of viable cells. Since FACS is primarily developed for sorting mammalian cells, common FACS reagents are not amenable to the *Drosophila* system, especially considering the different osmolarity of *Drosophila* hemolymph compared to mammalian buffers (22). To this end, using Seecof saline as a sheath fluid improved the amount of sorted material in our samples. In addition, we report here that addition of the surfactant reagent Pluronic F68 to the cells before sorting, as well as to the sheath fluid, substantially increased survival of the sorted cells. In one study, we found that whereas normal mammalian sorting conditions resulted in 100% death of sorted *Drosophila* S2 cultured cells, addition of Pluronic tremendously minimized cell death down to only 5% (not shown). The property of Pluronic as a mechanical damage-reducing agent is recognized in biotechnology methods dealing with large-scale cultures grown in bioreactors (23), and seems suitable for a similar role here. Overall, our modifications of the sorting protocol have resulted in successful sampling of *Drosophila* cardioblasts.

The flow-sorting technique that we have presented strongly depends upon the specificity of tagging of the desired tissue with fluorescent probes. Therefore, the extensive analysis of genetic markers intended to be used in cell sorting is a crucial prior step of the method presented. In many instances, transgenic constructs with tissue-specific activities will expand their pattern of expression as embryonic development proceeds, causing a reduction in the specificity of the sorted samples. We obviated the complication caused by late expression of *Sur>GFP* in embryonic neuronal cells, by tightly controlling the developmental stage at which sampling occurred, which, however, introduced an undesired stringency to the isolation protocol. In the parallel study of Salmand et al (21), sorting of cells from the dorsal vessel was mediated by a fluorescent reporter constructed on the basis of the *Hand* enhancer. This alternative reporter also shows expression outside of the cardiac cells, including pericardial and lymph gland cells. Hence, finding an accurate cardiac-specific reporter might be a cumbersome task. We propose that the specificity of cell purification could be tremendously improved by expressing two-color fluorescent proteins, from distinct enhancers that show the required tissue-specificity. Cells could then be purified by setting up the gating in order that both of the fluorescent tags are selected during sorting. For instance, one could use the *Sur* enhancer to express *GFP* and the *Mef2* cardiac enhancer (24) to express mCherry. In this case, simultaneous selection for both green and red signals (while keeping negative selection against multi-cellular aggregates with the Hoechst dye) should result in collection of pure cardiac cell sample at broader developmental stages, even though each enhancer shows a small amount of activity in other, but not overlapping tissues.

The genetic material that we collected from sorted *Sur>GFP* cells was small, but sufficient for PCR-based molecular analysis. However, much broader perspectives can be achieved

with samples of larger size: microarray or next-generation sequencing technology is poised to provide vast, comprehensive information about the cardiac transcriptome, whereas cDNA library cloning may be useful in functional studies of cardiac transcription factors. In this regard, we anticipate that the size of cardiac samples produced by FACS can be scaled up, as there is no foreseeable technical limitation restraining the size of collected samples. In this report we show that collections can be made and stored independently, to be combined later for RNA extraction. Additionally, sample amplification methods can be utilized, as currently used for single-cell transcriptome studies (25, 26). RNA amplification was applied to the sorted cardiac sample from *Drosophila* dorsal vessel in the recent parallel study by Salmand et al (21). It is expected that the modern transcriptome applications, such as next-generation sequencing approaches, will provide a wide range of novel data about molecular signatures and features of the sorted cardioblasts, thereby broadening our understanding of cardiogenesis, and providing new cardiac-expressed factors to subject to genetic analysis.

Highlights

- A cardiac-specific GFP-expressing line was generated.
- Homogenized embryos were subjected to cell sorting to enrich for cardiac cells.
- Up to 50,000 cardiac cells could be isolated from whole-embryo homogenates.
- The purified cells showed strong enrichment for cardiac markers.

Abbreviations

GFP	green fluorescent protein
FACS	fluorescence activated cell sorting
PI	propidium iodide

Acknowledgments

This work was supported by HL080545 from the NIH/NHLBI, and a March of Dimes Birth Defects Foundation grant, awarded to RMC. Sample collection for this study was conducted at the Flow Cytometry Shared Resource Center, supported by the University of New Mexico Health Sciences Center and the University of New Mexico Cancer Center.

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Figure 1. Schematic of cardioblast isolation from *Drosophila* embryos

Figure 2. Activity of the *Sur>GFP* reporter in *Drosophila* embryos, and purification of cardioblasts

The *UAS-2XeGFP; Sur-Gal4* line generated embryos in which the cardiac precursors and the dorsal vessel were clearly visible by immunostaining against GFP. A: At stage 13, cardiac progenitors could be observed as they migrate dorsally to form the cardiac tube (arrowhead); B: at stage 16, the mature dorsal vessel (arrow) was labeled in its entirety. At this stage, a small number of neurons also have enhancer activity (asterisk). Embryos are shown from the dorsal side in A and oblique laterally in (B); anterior to the left. C, left panel: FACS profiles of cells obtained from stage 13 *Sur>GFP* embryos. In the plot of red and green fluorescence channels, GFP-positive cells (gate “2”) are clearly discriminated

from dead, PI-positive cells (gate “1”). Gate “3” signifies the control, all living cells. Right panel: the GFP gated cells, when plotted in a graph of green versus blue channels, show further separation into fractions of single-cell (gate “4”) and multi-cellular (gate “5”) clusters. D: Cells selected by gate “2” and gate “4” in (C), visualized by fluorescence microscopy.

Figure 3. Characterization of gene expression and alternative transcript utilization in purified cardioblasts

A: Results of PCR analysis of the expression of muscle differentiation markers (*Act57B* and *TnI*), and cardiac identity markers (*Tl* and the endogenous *Sur* gene), between control embryonic cells, collected regardless of their GFP fluorescence (non-sorted), and Sur>GFP cells. Note that the Sur>GFP sample is enriched for muscle markers, and shows strong enrichment for *Sur* and *Tl*. There is residual *Tl* expression in the non-fluorescent cell population, since *Tl* is expressed in other embryonic tissues at this stage (27). Detection of the housekeeping actin gene *Act5C* is shown as a loading control. B: Differences in alternative splicing of *Zasp66* between Sur>GFP cells and non-sorted cells. Cardiac transcripts, not present in the non-sorted sample, are indicated with arrowheads. Splicing schemes representing the detected transcripts are shown at the left. A non-spliced amplicon of *TnI* serves as a loading control. Asterisk marks non-specific product.

Table 1

Primers used in this study.

TARGET	Forward Primer (5'->)	Reverse Primer (5'->)
<i>Act57B</i>	TGCAGTTGCCTAGCACCA	GACCCCATCCGCTCTATC
<i>Act5C</i>	AAGGATCGCTTGTCTGGG	GTATATCATATCATATCTCATGTGG
<i>Sur</i>	CCTTGCTATCGGCGCTTCT	GGTGTCAATTCAAGAGCCAAGGT
<i>Tm1</i>	AGACCCAGGAAGCCCTGAC	TCAGAGCGCTCCAAGTCTTC
<i>Zaps66</i>	TCCACAAGCAATTCAACTCG	GATACTGGCGCTGATACTGG
<i>Tn1</i>	GAAGGTCTCCAAATACGAAA	GCTGTTGTTGTTTATTGACTTC
<i>Tl</i>	GTGGACGCCAGGATACAAGGA	TCGGAAGCGGCCTTTAGTC