Fly 5:3, 261-265; July/August/September 2011; © 2011 Landes Bioscience

Tissue-specific cell sorting from Drosophila embryos

Application to gene expression analysis

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Key words: cell purification, flow cytometry, heart development, RNA amplification, tissue specific, cell sorting, cell dissociation, mRNA expression

Abbreviations: GFP, green fluorescent protein; CRM, cis regulatory module; CM, cardiomyocyte; PC, pericardiac cells; FSC, forward scatter channel; SSC, side scatter channel; FACS, fluorescent activated cell sorter; WT, wild-type; aRNA, amplified RNA; qPCR, quantitative polymerase chain reaction; HL, hemolymph like

Comprehensive understanding of tissues and organs development requires a detailed description of tissues specific developmental programs. In particular, Gene Regulatory Networks need to be analyzed at the tissue level, requiring organ specific transcriptional landscapes to be established. Here, we describe an efficient and stringent strategy for cell purification of differentiating cells from Drosophila embryos by flow cytometry. This, combined to mRNA amplification, can be used for transcriptomic analysis of small, tissue-specific cell populations. We present an application to the Drosophila cardiac system, whose cell population represents 0.5 to 1% of total cells within the whole embryo. Based on widely available fluorescent reporter transgenes, this method should be applicable to a number of tissues and organs.

Introduction

The fly genome sequence has prompted most biological research and has enable researchers to carry out a number of functional genomic studies not previously feasible. Nevertheless, understanding how the information encoded can produce specific cell types and organs remains elusive. This is mainly due to the lack of tissue specific information's about how the genomic information is processed—in terms of transcript expression for instance—thus outlining the need to set methods to allow for organ specific transcriptome profiles.

Different approaches, like RNA tagging by TU incorporation[1] or laser microdissection,[2] are being developed to analyze gene expression from specific cell populations. These methods either require the construction of specific tools—for example in the case of tissue specific expression of TU—or are not suitable for isolating inner cell populations, which are not accessible to laser mediated micro-dissection.

Years of functional genetics and genomics in flies have lead to a variety of fly lines permitting tissue specific expression of fluorescent markers (mainly GFP), either directly under the control of tissue specific CRM,[3] or thanks to the constitution of libraries of Gal4 expressing lines (ref. 4 and http://kyotofly.kit.jp/stocks/GETDB/main.html), to name just a few. To manage tissue specific cell purifications, we decided to turn to cell sorting by flow cytometry. Compared to alternative methods, Fluorescent Activated Cell Sorting has a number of advantages. In particular, it allows purifying any cell type, irrespective of their accessibility, and it potentially allows isolating any cell component -being therefore not limited to transcripts analysis. As a matter of facts, some investigations in the fly already made use of flow cytometry. However, the methods were set for abundant cell types[5] or concerned circulating cells that do not require prior tissue dissociation.[6]

Here we describe a detailed and robust protocol for purifying small populations of fluorescently labeled cells from dissociated embryos at different developmental stages. This allows specific cell population to be sorted from whole embryos, even at late differentiated stages, when tissues are more resistant to cell dissociation. Linear amplification of mRNA further allows gene expression to be monitored from limited amount of purified cell.

This approach was applied to the differentiating cardiac system, which occurs late during embryogenesis, between stages 13 and 16. The cardiac system has a tube-like organization consisting essentially of two major cell types: the contractile cardiomyoblasts, which constitutes the lumen of the heart, and the associated non-myogenic pericardial cells (**Fig. 1A and B**). It forms from two rows of cells that are specified within the dorsal most mesoderm and subsequently join during dorsal closure to eventually

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Submitted: 03/24/11; Revised: 05/16/11; Accepted: 05/16/11
DOI:10.4161/fly.5.3.16509

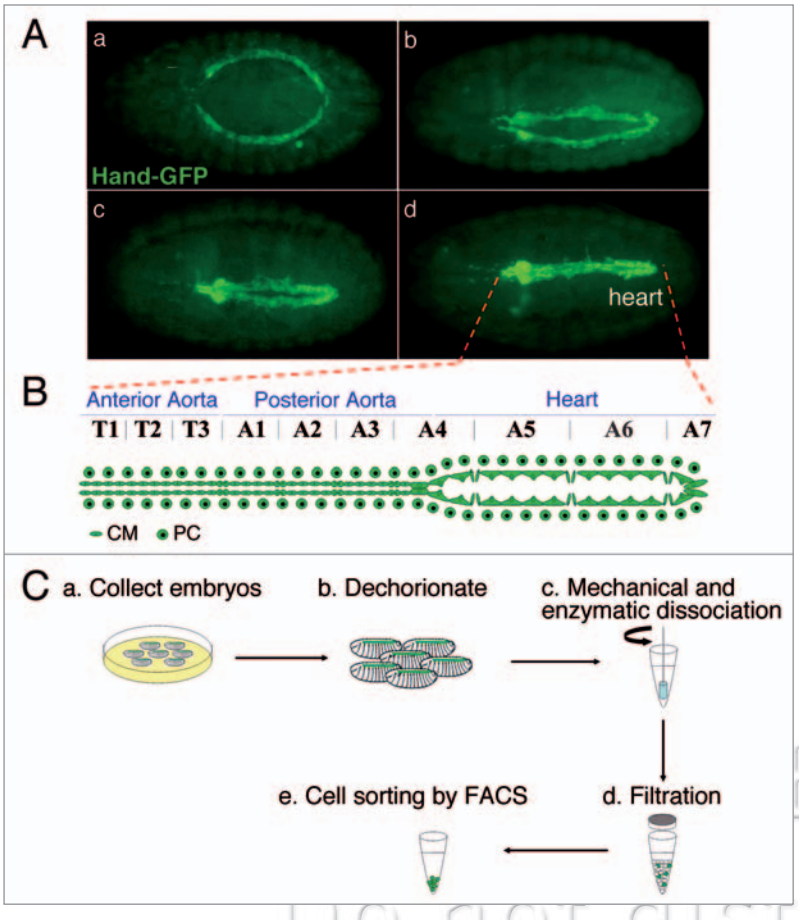

Figure 1. Hand-GFP reporter expression pattern used and overview of the dissociation and cell sorting protocol. (A) Anti-GFP labeling of differentiating Hand-GFP embryos (dorsal views). GFP expression starts at stage 13 (a), and continues during dorsal closure (b and c) up to the formation of the cardiac tube (d). (B) Schematic representation of the embryonic cardiac system. Cardiomyocytes (CM) form the tube proper from thoracic segment T1 to abdominal segment A7 and are associated to pericardiac cells (PC). (C) Schematic drawing of the GFP purification procedure. Timely collected embryos are dechorionated (a and b) and mechanically and enzymatically dissociated (c). Dissociated cells are recovered by differential centrifugation and filtration (d). GFP cells are selectively purified by flow cytometry (e).

make up the cardiac tube.[7] In this study, cardiac differentiating cells were purified from a GFP reporter line expressed specifically in the cardiac system.[8]

Results

Embryos dissociation. 4 hours egg collection followed by 10 to 14 hours of development at 25°C respectively allowed collecting stage 13–14 and stage 15–16 embryos (**Fig. 1A**). A flowchart of the dissociation procedure is given in **Figure 1C**. Briefly, staged, dechorionated embryos were dissociated by mechanical disruptions of tissues using a potter, followed by trypsin enzymatic digestion. In addition, DNAse treatment was used to remove genomic DNA released from damaged cells. Small speed centrifugation allowed to pellet the cells while removing debris and filtration on meshes removed most of non-dissociated cells. For late developmental stages (stages 15 and 16), extra mechanical disruption was required as well as a collagenase treatment, to ensure a better dissociation of differentiated tissues.

The quality of the dissociation was checked by visual inspection using Malassez cells and Tripan blue staining was used to monitor apoptotic cells. We routinely obtained above 95% of unstained (viable) dissociated cells (not shown). Overall, the dissociation procedure takes less than 1 hour.

Cell sorting. GFP positives cells were purified using a Fluorescent Activated Cell Sorter (FACS). Cells were first discriminated from all events (chorion residuals, residual cell parts...) by analyzing FSC/SSC ratio. Then, single cells were selected using SSC and FSC parameters. On this population, the GFP positives cells were clearly discriminated in the scatter plot of GFP intensity (FITC filter, 525 nm) versus autofluorescence analyzed at 575 nm. Indeed, this cell population is absent in dissociated cells from WT embryos, which were systematically analyzed in parallel to set up the FACS parameters (yellow dots in **Fig. 2A**). Setting the gate based on these parameters, we isolated GFP labeled cardiac system cells. These parameters were sufficiently robust to permit for sorting with an efficacy greater than 95%, what allowed to purify an average of 30 x 10^3 GFP positives cells in 15 minutes, even thought the proportion of positives cells represent as few as 1% of all starting dissociated cells (**Fig. 2A** bottom).

Sorting efficiency was then assessed by re-analyzing sorted cells by FACS, what demonstrated that >99% of sorted cells were GFP positives (not shown). This was also confirmed by examination of cells under an epifluorescent microscope (**Fig. 2B**).

RNA preparation and gene expression analysis. We designed the protocol in order to reduce the delay between embryos collection and RNA extraction—thus limiting RNA degradation and potential changes in transcriptome landscape. Therefore, short cell sorting sessions (less than 20 minutes) were selected. In these conditions, around 30 x 10^3 cardiac system cells can be recovered using the Hand-GFP reporter line. Cells were directly sorted in RNA extraction buffer. On average, 50 ng total RNA was recovered from 30 x 10^3 cells following the method described by Baugh et al. Quality was tested with Agilent Bioanalyzer[10] (**Fig. 3A**). To allow for gene expression analysis, a step of mRNA linear amplification was added. Starting from 30 x 10^3 cells we routinely obtained around 4 µg of amplified RNA (aRNA, **Fig. 3B**). The cell sorting procedure and RNA preparation strategy was then validated by Q-PCR. We first tested GFP expression, which is dramatically enriched in purified cells compared to whole Hand-GFP embryos, *tinman* (*tin*) and *pericardin* (*prc*) expression, which are specifically expressed in subsets of cardiac system cells at the stage examined[11] were also analyzed. As expected, both are highly enriched in the sorted cell population. We also tested the expression level of *proctolin* (*proct*), which encodes a neuropeptide known to be

specifically expressed in neurons.[12] *proct* expression is significantly reduced in sorted cells compared to whole embryos (**Fig. 3C**). Amplified RNA can also be used for whole genome transcriptome analysis on microarrays (our unpublished results).

Materials and Methods

Fly stocks. The GFP reporter line used in this study places GFP under the control of the 3rd intron of the Hand gene deleted from visceral mesoderm enhancer (region 1,074–1,374, ref. 8). Transgenic flies carrying this construct retain Hand-like cardiac cells specific expression, including contractile cardioblasts and non-myogenic pericardial and lymph gland cells. Oregon R were used as wild-type control to set up FACS parameters.

Embryos collection and dissociation. Egg collection and embryo aging was performed at 25°C on apple juice agar plates. Embryos were dechorionated in 5° bleach for 2.5 min and extensively rinsed with H_2O. Then embryos were drained on absorbent paper, weighted and re-suspended in cold Schneider culture medium (GIBCO Invitrogen) at a 50 mg/ml concentration. About 1,000 one week old mated females were required to collect 50 mg embryos in 4 hours.

Unless otherwise stated, all subsequent steps were carried out at 4°C (in a cold room). 1 ml of embryos suspension was subjected to mechanical disruption using Dounce homogenizer. For stage 13/14 embryos, mechanical disruption was achieved after 3–5 strokes using a pestle mounted on a drill set at 50 rpm. For stage 15/16 embryos, the number of strokes was increased to 10 and 20 U of Collagenase A (Worthington) was added in the Schneider medium.

The suspension was then filtered on a 100 μm mesh and centrifuged at 850 rpm for 1 min in a 1.5 ml eppendorf. Cells and aggregates were then washed in 500 μl of 'hemolymph like' (HL) buffer (KCl; 25 mM; NaCl: 90 mM; $NaHCO_3$: 4.8 mM; D-glc: 80 mM; Trehalose: 5 mM; L-Gln: 5 mM Hepes: 10 mM; pH = 6.9 final) without Ca^{2+} nor Mg^{2+} and centrifuged at 850 rpm for 1 min. Cells were then re-suspended in HL containing 0.25% trypsin (Invitrogen) and incubated for 10' at 37°C for enzymatic digestion. Trypsin reaction was stop by adding 400 μl of Schneider and 100 μl of goat serum (20% final serum). 2 U of RQ RNase-Free DNase (Sigma) was added and incubation was extended for 5' at 37°C. Cells were then pelleted by centrifugation (850 rpm, 1 minute) and resuspended in 500 μl of cold Schneider. Cells were filtered on a 30 μm mesh (Miltenyi Biotec) and 10 μl were used for visual examination and count on a Malassez cell, with Tripan blue staining. We routinely obtained 1 to 5 x 10^7 cells per ml among which less than 5% were positive for tripan blue staining.

Fluorescent activated cell sorting. For sorting, we used a Bekton-Dickinson high-speed cell sorter FACSAria system flow cytometer equipped with a 488 nm argon laser. Cardiac system cells were sorted by gating for GFP-positive and autofluorescence-negative events, as well as light-scattering parameters. For GFP and autofluorescence detection, we used 525 nm and 575 nm band-pass filters, respectively. Cells were sorted through a flow chamber with a 100 μm flow cell tip under 9 psi sheath fluid pressure. For microscopy analysis and antibody staining, cells were collected in PBS. For RNA preparation, the sorted cells were collected into 1.5 mL tubes containing 300 μl Trizol (Invitrogen).

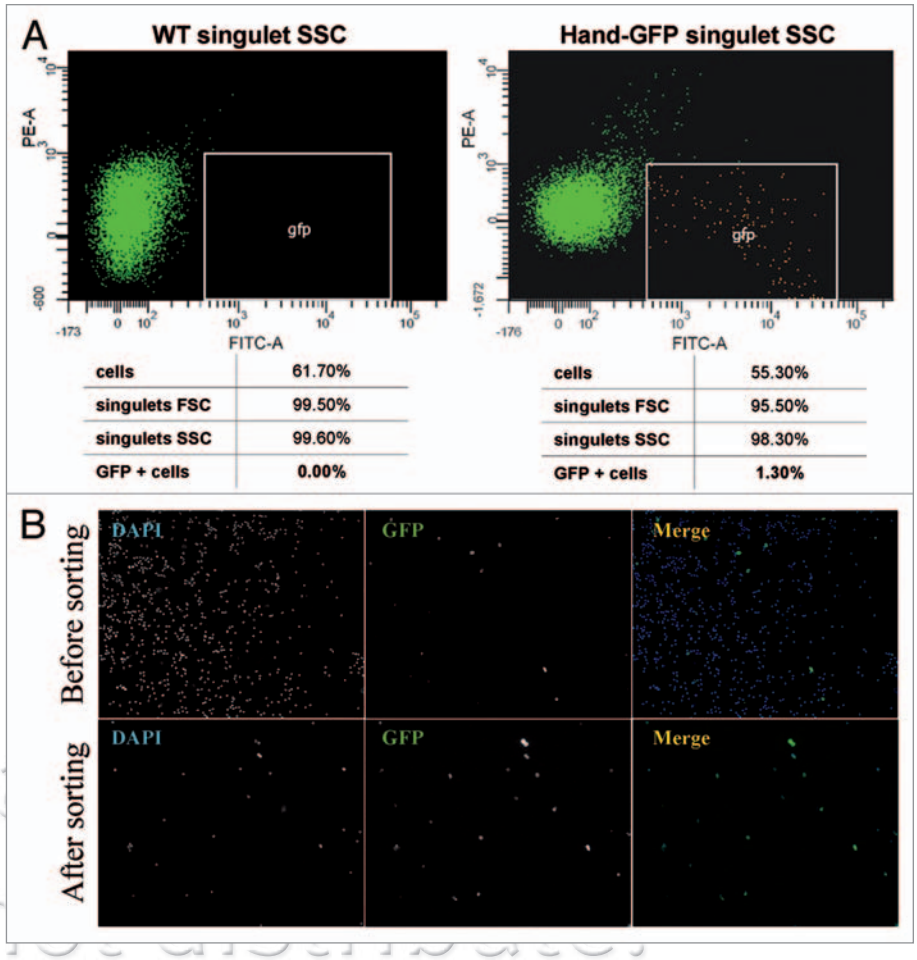

Figure 2. FACS analysis of cells from staged dissociated embryos. (A) Dissociated cells from WT (left) and Hand-GFP (right) 14–18 hours embryos were analyzed by FACS. GFP positive population is discriminated by fluorescence at 575 nm versus fluorescence at 525 nm. No GFP positives (GFP^+) cells are observed in WT ("GFP" window), whereas in this experiment GFP^+ cells represent 1.3% of total cells in Hand-GFP embryos (yellow dots, tables at the bottom). (B) GFP positives cells were sorted and checked for GFP expression by immunofluorescence compared to non sorted cells (input, top). DAPI, which stains DNA, allow labeling cell nuclei. Cell purification procedure allowed reaching >95% purity. Similar results were obtained when starting from 10 h–14 hours embryos (Data not shown).

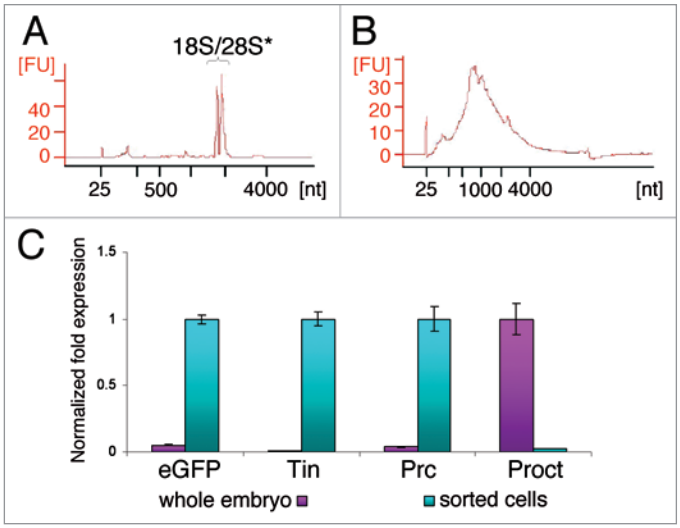

Figure 3. Analysis of total RNA and a RNA from sorted cells. (A and B) High quality migration profiles of Total RNA and amplified RNA (aRNA) obtained with Agilent Bioanalyser procedure are shown in (A and B), respectively. In (A), the typical two main peaks corresponding to 18S and 28S cleaved ribosomal RNA is indicated. (C) GFP, *tin*, *prc* and *proct* expression levels was monitored by Q-PCR in 14–18-hours old Hand-GFP embryos as well as in GFP$^+$ sorted cells from same embryos preparations. GFP, *tin* and *prc* are highly enriched in sorted cells. On the opposite, the neuropeptide encoding *proct* transcript is depleted in purified cardiac cells compared to Hand-GFP embryos.

Using these parameters, a minimum of 30 x 10^3 GFP positives cells were collected in 15 minutes.

Immunostaining. Immunostaining of embryos was performed as previously described in reference 14, using anti-GFP. Staining of dissociated cells was performed on Lab-Tek™ chamber slides. Chambers were coated with 200 µl of Poly-L-Lysine 0.01% (Sigma) during 1 hour at 37°C. A 50 µl drop of cell suspension was deposited at the middle of each chamber and left for 1 hour to allow for cells sedimentation. Cells were then fixed 10 minutes in 4% formaldehyde diluted in PBS. After permeabilisation and saturation (1 hour in PBS triton 0.3%, BSA 3%), cells were incubated 2 hours with primary antibody in PBS 1x 0.3% triton 1% BSA. Secondary antibody was diluted in PBS and incubated for 1 hour. Lab-Tek™ chamber slides were mounted in Vectashield containing DAPI (Vector Laboratories). Slides were observed on a Zeiss LSM 510 confocal microscope.

Antibodies used were Chicken Anti-GFP (Aves Labs, Inc., used at 1/1,000) and Alexa 488 coupled goat anti-chicken (Molecular Probe used at 1/500).

RNA extraction, amplification and Q-PCR analysis. Sorted cells were collected in 300 µl of Trizol and RNA extracted according to Baugh et al. Isolated total RNA (~50 ng) was analysed on Agilent 2100 Bioanalyser and RNA 6,000 Picochips following manufacturer instructions to monitor RNA quality. Then, mRNA were selectively amplified with the Amino Allyl MessageAmp™ II aRNA Amplification Kit (Ambion) based on the RNA amplification protocol developed by Eberwine.[15] The aRNA procedure begins with total RNA that is reverse transcribed using an oligo(dT) primer containing a T7 RNA polymerase promoter sequence. The reaction is treated with RNase H to cleave the mRNA into small fragments. These small RNA fragments serve as primers during the second-strand synthesis reaction producing a double-stranded cDNA template for T7 in vitro transcription (IVT). aRNA quality was verified on Agilent Bioanalyser.

For Q-PCR gene expression analysis, 500 ng of aRNA were reverse transcribed with 100 U of Reverse transcripase (SuperScript Invitrogen) in a 20 µl reaction volume. In all cases, three technical replicates were performed on three biological samples for mRNA quantification.

The relative abundance of GFP, *tinman*, *pericardin* and *proctolin* mRNAs was assessed using *rp49* to normalize data. Reactions in SYBR® GreenER qPCR SuperMix (Invitrogen) were performed using the CFX® Real-Time PCR Detection System (Bio-Rad Laboratories Inc.). For 25 µl PCR reaction, 10 ng of cDNA were used together with 200 nM of each primers. The sequences of the specific primers are:

-rp49 rev 5'-AAA CGC GGT TCT GCA TGA G-3'
for 5'-GAC GCT TCA AGG GAC AGT ATC-3'
-GFP rev 5'-CCA TGC CGA GAG TGA TC-3'
for 5'-GAA GCG CGA TCA CAT GG-3'
-tinman rev 5'-GAG ACC GCT AGC AAC TCG TC-3'
for 5'-AGT CGA AAG CGA CAC TCC AG-3'.
-pericardin rev 5'-TCA GCT CGA TCA GTA ACA GCA-3'
for 5'-GGA AGC AAA CAG GGA ACT CTT-3'
-proctolin rev 5'-CAC CTG TGT CCA CTT CCA CA-3'
for 5'-CTC CAT CGA AAA ACA CAA ACC-3'.

Conclusions

We present here a fast, accurate and amenable method for analyzing gene expression profiles of purified cell population, isolated from developing Drosophila embryos. Our method is flexible, and needs small-scale egg collection. Therefore, it can easily be applied to genetically modified individuals (either mutants or gain of function) without the need to collect large amount of virgin females. This method should also be useful for other applications, such as primary cell cultures or tissue specific proteomics.

In addition, most of currently available cell sorters allow purifying cells based on multiple fluorescent markers. Hence, combining Gal4 (or newly developed QF[13]) driven fluorescent markers with other tissue specific markers will allow extending and/or refining tissue specificity of the cell sorting.

Acknowledgments

We thank A. Paululat for Hand-GFP fly stock, S. Long for stocks maintenance, M. Barad for his expertise in cell sorting all lab members for fruitful discussions and L. Röder for careful reading of the manuscript. This work was supported by ANR, partner of the ERASysBio+ initiative supported under the EU ERA-NET Plus scheme in FP7, by an ANR "JCJC" grant and by funding from the AFM to Laurent Perrin. Pierre-Adrien Salmand. was supported by a doctoral fellowship from AFM.

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