Regional Cell Specific RNA Expression Profiling of FACS Isolated *Drosophila* Intestinal Cell Populations

Devanjali Dutta,[1] Nicolas Buchon,[2] Jinyi Xiang,[1] and Bruce A. Edgar[1]

[1]DKFZ-ZMBH Alliance, University of Heidelberg, Heidelberg, Germany
[2]Department of Entomology, Cornell University, Ithaca, New York

The adult *Drosophila* midgut is built of five distinct cell types, including stem cells, enteroblasts, enterocytes, enteroendocrine cells, and visceral muscles, and is divided into five major regions (R1 to R5), which are morphologically and functionally distinct from each other. This unit describes a protocol for the isolation of *Drosophila* intestinal cell populations for the purpose of cell type–specific transcriptome profiling from the five different regions. A method to select a cell type of interest labeled with green or yellow fluorescent protein (GFP, YFP) by making use of the GAL4-UAS bipartite system and fluorescent-activated cell sorting (FACS) is presented. Total RNA is isolated from the sorted cells of each region, and linear RNA amplification is used to obtain sufficient amounts of high-quality RNA for analysis by microarray, RT-PCR, or RNA sequencing. This method will be useful for quantitative transcriptome comparison across intestinal cell types in the different regions under normal and various experimental conditions.

Keywords: *Drosophila* intestinal stem cells • fluorescent-activated cell sorting (FACS) • cell type–specific RNA isolation • transcriptome profiling/RNASeq • enterocyte

How to cite this article:
Dutta, D., Buchon, N., Xiang, J., and Edgar, B.A. 2015. Regional cell specific RNA expression profiling of FACS isolated *Drosophila* intestinal cell populations. *Curr. Protoc. Stem Cell Biol.* 34:2F.2.1-2F.2.14.
doi: 10.1002/9780470151808.sc02f02s34

INTRODUCTION

The epithelial cell lining of the gastrointestinal tract in *Drosophila melanogaster* (*Drosophila*/fruitfly) is maintained by a continuous supply of cells, which arise by the differentiation of multipotent progenitors that originate from intestinal stem cells (Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2007). Intestinal stem cells (ISCs) proliferate throughout the life of the adults, replacing themselves and generating transient cells called enteroblasts (EBs), which differentiate into enterocytes (ECs) or enteroendocrine cells (EEs). Transcription factor *Escargot* (*esg*) is a common marker for both ISCs and EBs. In addition to these cell types, the midgut region of the intestine is ensheathed by two layers of visceral muscle (VM) cells and supplied with oxygen by the trachea. The proper regulation of intestinal stem cell maintenance, proliferation, and differentiation is critical for maintaining gut homeostasis (Buchon et al., 2009; Jiang et al., 2009, 2011; Osman et al., 2012). As the understanding of stem cell development and function in vivo becomes more sophisticated, it has become important to profile the various intestinal cell types at the transcriptome level. Recent studies have fine tuned our understanding of the midgut epithelium and demonstrated that the midgut is not a

Current Protocols in Stem Cell Biology 2F.2.1-2F.2.14, August 2015
Published online August 2015 in Wiley Online Library (wileyonlinelibrary.com).
doi: 10.1002/9780470151808.sc02f02s34

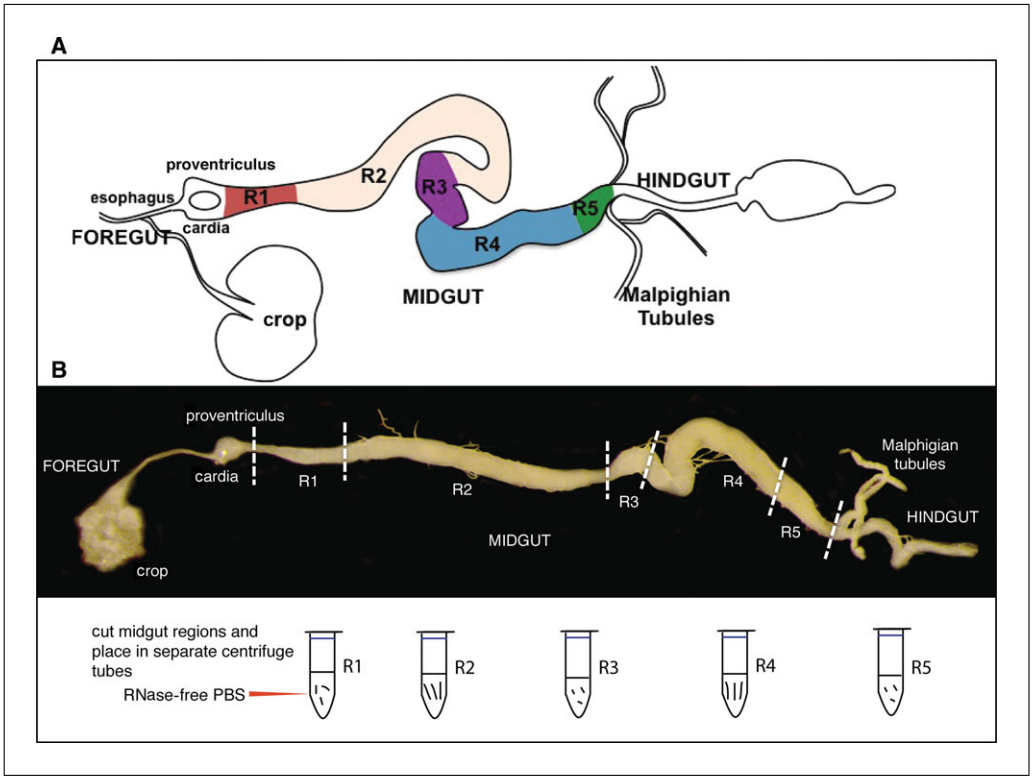

Figure 2F.2.1 The *Drosophila* midgut consists of five morphologically and functionally distinct regions or compartments (**A**) Schematic diagram showing the *Drosophila* midgut and the five regions. The white dotted lines show the start and end of each region. Incisions should be made at these positions to separate the regions. (**B**) Picture showing midgut regions. R1 starts from theproventricular boundary to the first hinge, where the crop physically interacts with the midgut. R2 starts from the end of R1 and ends just before the retrograde folding of the gut from where the copper cell region starts. R3 covers the copper cell region. R4 starts at the end of R3 and ends in the posterior midgut wheremidgut starts to narrow. R5 starts at the end of R4 and ends at the midgut hindgut junction, where the Malphigian tubules physically interact with the gut. Each region is cut and placed in separate microcentrifuge tubes for further processing.

homogenous organ, but consists of five morphologically and functionally distinct regions (R1 to R5), composed of specialized cells (Buchon et al., 2013; Marianes and Spradling, 2013). Thus the *Drosophila* midgut is a highly heterogenous tissue, rendering the understanding of individual cell type behavior complex to untangle. In order to understand tissue complexity, and characterize what makes the cells different and unique in each region, an in-depth profiling of cells in each region is required. Previously we had developed a method that could be useful for quantitative comparisons of gene expression across the different intestinal cell populations of the whole *Drosophila* midgut under physiological or any experimental condition of overexpression or knockdown (see the previous version of this unit at *http://www.currentprotocols.com/protocol/sc02F02*). We further refined our method in order to profile each cell type from each of the midgut regions. This unit begins with a description of the five regions and a method to dissociate and purify various intestinal cell types from those regions by enzymatic dissociation and FACS (see Basic Protocol 1), followed by a protocol to isolate and amplify RNA linearly (see Basic Protocol 2), and then by a protocol to stain the sorted cells to observe their morphology (see Basic Protocol 3). The amplified RNA can be used subsequently for gene expression profiling experiments. Figure 2F.2.1 shows the different positions where the midguts were cut to separate regions. A schematic overview of the major steps in the procedure is shown in Figure 2F.2.2.

NOTE: The following protocols are performed under Class II biohazard conditions.

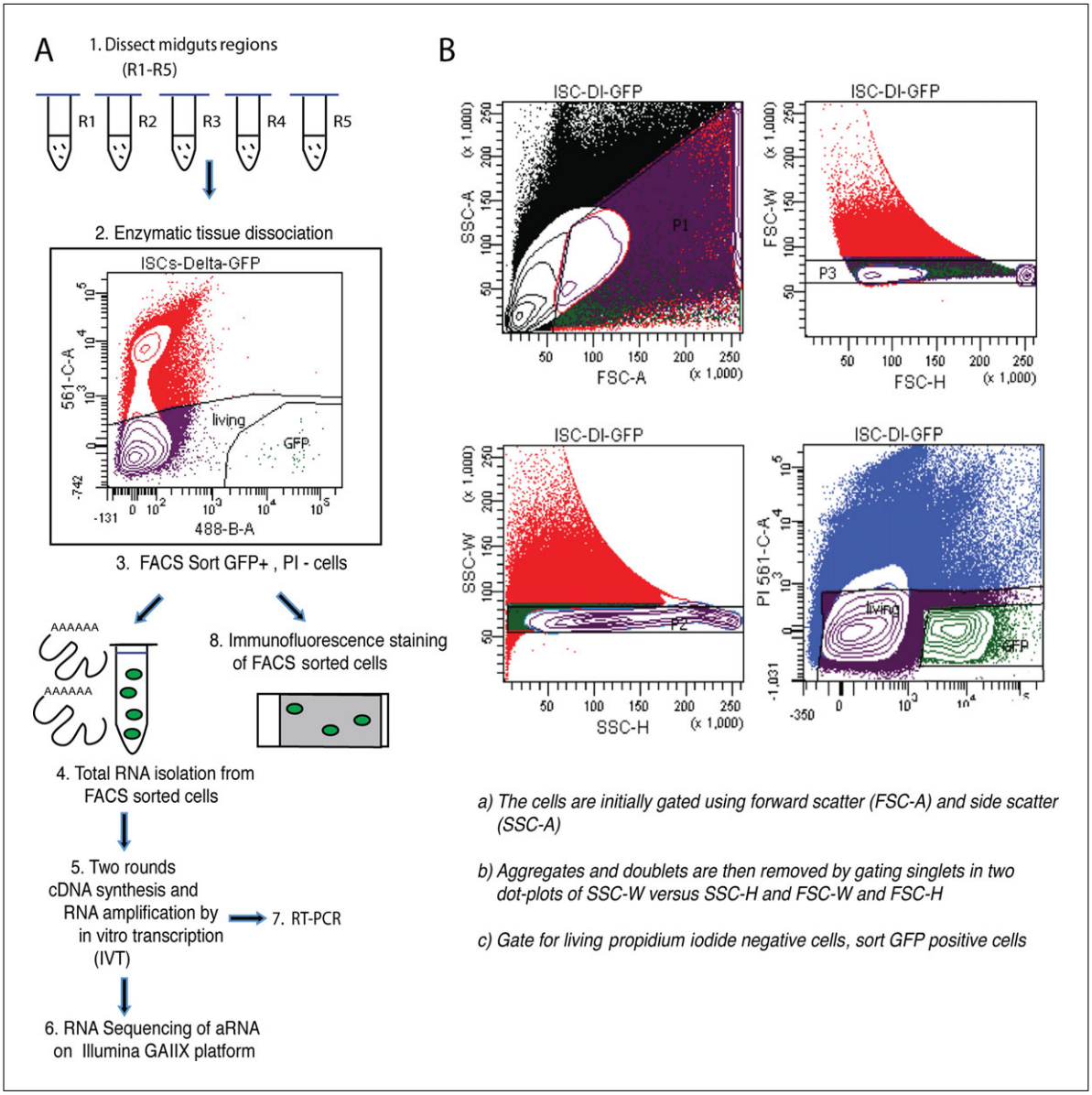

Figure 2F.2.2 FACS sorting of *Drosophila* intestinal cell populations for obtaining pure cell populations. (**A**) After dissection of midgut regions, tissue was disrupted by enzymatic treatment and the cell suspension was FACS sorted. Cells were gated based on GFP intensity and cell size. RNA was isolated from the FACS-sorted cells followed by two rounds of cDNA synthesis and in vitro transcription for RNA amplification. Amplified RNA was subjected to qRT-PCR analysis or Illumina GAIIX 72-bp mRNA sequencing. Immunofluorescence staining of sorted cells was performed to check for morphological changes. Genotypes used were esg-Gal4ts and UAS-GFP. (**B**) Detailed methodology of FACS sorting of cell populations. Cells were first gated for forward scatter (FSC-A) and side scatter (SSC-A); aggregates and doublets are then removed by selecting singlets using dot-plots of SSC-W versus SSC-H and FSC-W versus FSC-H. Finally, propidium iodide–negative (living) and GFP-positive cells were sorted out.

NOTE: All reagents and equipment should be sterile and RNase-free conditions should be maintained.

ISOLATION AND PURIFICATION OF *DROSOPHILA* INTESTINAL CELLS BY FLUORESCENT-ACTIVATED CELL SORTING (FACS)

BASIC PROTOCOL 1

This protocol describes tissue preparation and isolation of the intestinal cell types from R1 to R5 by FACS. The following adult fly *Gal4* lines driving *UAS-GFP* or *UAS-YFP* expression—*esg*-Gal4 (ISCs +EBs), *Dl*-Gal4 (ISCs), *Su(H)*-Gal4 (EBs), *Myo1A*-Gal4

(ECs), *How*-Gal4 (visceral muscle), and *Rab3*-YFP or *Pros-v1-Gal4* (EEs)—should be used to select the different cell populations. All of the dissection tools and dishes should be wiped with RNaseZap from Ambion prior to use. Keep dishes placed on ice throughout. For each region, prepare a separate PBS microcentrifuge tube, and mark tubes as R1, R2, R3, R4 and R5 (Fig. 2F.2.1).

Materials

Transgenic adult fly *Gal4* lines driving GFP or YFP expression: *esg-Gal4* (ISCs +EBs), *Dl*-Gal4 (ISCs), *Su(H)*-Gal4 (EBs), *Myo1A*-Gal4 (ECs), *How*-Gal4 (visceral muscle) and *Rab3*-YFP or *Pros-v1*-Gal4 (EEs) and UAS-mcd8-GFP/UAS-nls-GFP
10 × PBS-DEPC (see recipe)
RNaseZap (Ambion) *or* 70% ethanol
Elastase (see recipe) *or* collagenase type IV (see recipe)
1 mg/ml propidium iodide
10% bleach
DEPC-treated water (see recipe)
Wild-type control w^{III8} flies

Dissection dishes
Dissecting tools: scissors, fine forceps
Dissecting microscope
1.5-ml microcentrifuge tubes
27°C heating block with shaking
Refrigerated microcentrifuge
25- and 70-μm filter units (BD Biosciences)
FACSAria II cell sorter (Becton Dickinson)
FACS tubes with filter tops (BD Biosciences)
BD FACSDiva v6.1.1 or similar software

1. Use CO_2-anesthetized adult female flies, as it is preferable to use either female or male midguts; mixing both sexes could skew the transcriptome profiles due to gender-specific variations.

   Use of female flies is recommended for obtaining more cells of each cell population due to the ease of dissection and larger size.

2. Using a scalpel, remove the head, wings, and legs of the fly or dissect the intact fly. Transfer fly into a well of a dissection dish containing 2 ml of 1 × PBS-DEPC to start dissections. Use a pair of fine forceps to gently hold the fly between the thorax and abdomen and gently squeeze the abdomen to push the midgut out from the anterior end. With another pair of forceps, pull out the gut slowly but steadily. Pull carefully to obtain the undamaged gut, cut off the crop, and then cut off the midgut/hindgut junction (cut where the Malphigian tubules branch onto the gut). Using forceps, cut the midgut at the main regions as indicated in Figure 2F.2.1. Boundaries are described with precision on Flygut (Buchon et al., 2013). If Malphigian tubules are pulled out as well, dissect them away from the gut.

   Removing the head makes the crop easily visible, and removing wings and legs helps in a cleaner dissection; however, this can be bypassed and the intact fly can be dissected as well. Dissecting 10 to 15 flies at a time is recommended to avoid exposing the midgut tissue to room temperature for long periods of time.

3. Use forceps to immediately transfer the gut regions into their respective 1.5-ml microcentrifuge tubes containing PBS-DEPC (~400 µl), placed on ice. Keep total dissection time <2 hr.

   To avoid variations in gene expression patterns as a function of temperature, the dissected midguts should be maintained at 4°C until the dissociation step.

4. For isolation of cells from whole midguts, collect ~150 midguts (for ISCs, EBs, EEs) and 100 midguts (for esg^+ cells, ECs, and VM) in a 1.5-ml microcentrifuge tube and digest with 100 µl elastase solution (1 mg/ml final concentration). For isolation of cells from regions, cut ~500 midguts (for ISCs, EBs, EEs) and 300 to 400 midguts (for esg^+ cells, ECs, and VM), and distribute all five dissected regions into their respective microcentrifuge tubes. Digest each tube with 75 to 100 µl elastase solution.

   More midguts are dissected for less abundant cell types like ISCs, EEs, and EBs in order to obtain enough cells (>500 cells) for regional profiling of the particular cell type after sorting. Collagenase type IV can also be used for isolating the esg^+ cells, depending on availability; however, the efficiency of elastase was found to be higher and more living cells could be obtained, particularly for flies expressing membrane GFP (mGFP). The efficacy of collagenase for ECs and the VM was not determined. Dissect the samples within 2 hr. The efficacy of elastase may vary with batch and storage, and thus needs to be checked regularly. ISC and EB numbers are very low in R1 and R3; EE numbers are low in R3; ECs are low in R1; and VM cells are low in R3 and R5.

5. Incubate tubes for 1 hr at 27°C in a heating block with shaking at 34 rpm. Agitate every 15 min by pipetting up and down at least 30 to 40 times to fully dissociate the tissue.

6. Microcentrifuge dissociated cells for 20 min at 300 × g, 4°C, and re-suspend pellet in 500 µl fresh PBS-DEPC for sorting. Add 1 µl of 1 mg/ml propidium iodide.

7. Filter dissociated cells using 25-µm (for ISCs, EBs, esg^+ cells, and EEs) and 70-µm (for ECs and VM) filters to remove clumps, which might otherwise block the nozzle of the FACS sorter. Maintain cells throughout at 4°C.

   The time and agitation for dissociation to obtain ECs and visceral muscle should be increased in case clumps are still seen even after 1 hr.

8. Pre-treat BD FACSAria II cell sorter with 10% bleach for 180 sec followed by washing three times, 180 sec each, with DEPC water. Sort samples at 4°C and 23 psi pressure using the 85-µm nozzle for the ISCs, EBs, ESG^+ cells, and EEs. Use100-µm sized nozzle for sorting ECs and visceral muscle cells.

   Use BD FACSDiva v6.1.1 or similar software for collection, storage, and analysis of the digital data.

9. To account for auto fluorescence, set gates using midguts from w^{III8} flies. GFP-positive cells from each region were sorted based on two criteria—fluorescence intensity and size of cells. Gate cells initially using forward scatter (FSC-A) and side scatter (SSC-A). Remove clumps and doublets by gating singlets in two linear scale dot-plots of SSC-W versus SSC-H and FSC-W and FSC-H (Fig. 2F.2.1 B).

10. Sort out propidium iodide–negative (living), GFP-positive cells from regions R1-R5 into five different microcentrifuge tubes. Perform the procedure three times to obtain biological triplicates. Enrich the cell type fractions using FACS Aria cell sorter (see

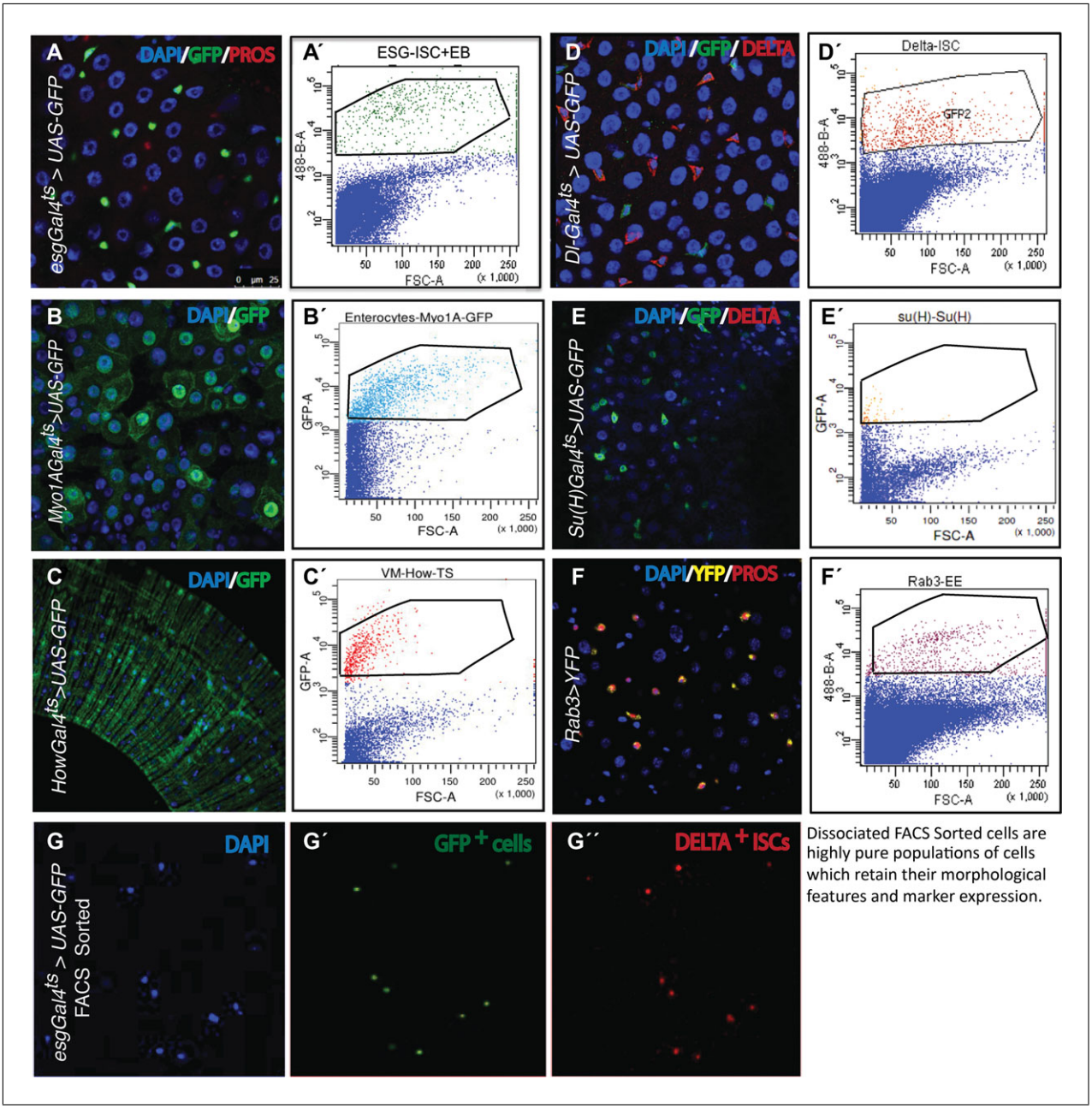

Figure 2F.2.3 Cell type–specific GAL4 drivers and FACS profiles of *Drosophila* intestinal cell populations. Various genotypes were used to isolate different cell populations. FACS profiles of various cell types are shown. (**A**, **A′**) *esg*-Gal4^{ts}, UAS-GFP; (**B**, **B′**) *Myo1A*-Gal4^{ts}, UAS-GFP; (**C**, **C′**) *How*-Gal4^{ts}, UAS-GFP; (**D**, **D′**) *Dl*-Gal4^{ts}, UAS-GFP; (**E**, **E′**) *Su(H)*-Gal4^{ts}, UAS-GFP; (**F**, **F′**) *Rab3*-YFP; (**G**, **G′**, **G′′**). Immunofluorescence staining for the intestinal stem cells showed no effect of FACS on cell morphology with cells expressing marker Delta and GFP respectively. Genotypes used were *esg*-Gal4^{ts}, UAS-GFP. Dissociated FACS-sorted cells are highly pure populations of cells that retain their morphological features and marker expression.

equipment setup) and cell type–specific marker drivers expressing green and yellow fluorescent protein (GFP/YFP) (Fig. 2F.2.3 A-F, A′-F′).

After cell sorting, immediately proceed to RNA isolation. Storage of sorted cells in extraction buffer even at –80°C decreases RNA yield.

For the purpose of culturing or immunostaining of sorted cells, sorting of cells directly into Schneider's medium is recommended.

RNA ISOLATION AND AMPLIFICATION

BASIC PROTOCOL 2

For the purpose of RNA isolation, one genotype should be dissected at a time and at least three biological replicates should be prepared for each cell type. When isolating cells from whole midguts, it is recommended that the same numbers of cells are sorted out and the same amount of RNA is amplified for each cell type or for comparing experimental conditions with controls. As fewer cells are recovered from single regions, isolate as many cells as possible from each region. Amplification of RNA is necessary for smaller cells like ISCs, EBs, and EEs due to their low yield of RNA, whereas RNA from esg^+ cells and ECs can be used directly for gene expression studies. However, as linear amplification introduces bias towards the longer transcripts and might skew analysis, it is thus not advisable to compare amplified and unamplified samples. Amplifying RNA from all the cell types being studied or compared is recommended.

NOTE: RNaseZap (Ambion) is used throughout to keep RNase-free conditions.

Materials

Sorted cells (see Basic Protocol 1)
Arcturus PicoPure RNA isolation kit (Applied Biosystems)
70% RNase-free ethanol
Arcturus RiboAmp HS PLUS RNA amplification kit (Applied Biosystems)
SuperScript III reverse transcriptase (Invitrogen, cat. no. 18080-44)

1.5-ml microcentrifuge tubes
Parafilm
42°C water bath
Refrigerated microcentrifuge

Table 2F.2.1 RiboAmp HS PLUS Thermal Cycler Program Round One[a]

	Temperature (°C)	Time
First-strand synthesis	65	5 min
	4	Hold
	42	1 hr
	4	Hold
	37	30 min
	95	5 min
	4	Hold
Second-strand synthesis	95	2 min
	4	Hold
	25	10 min
	37	30 min
	70	5 min
	4	Hold
IVT	42	6 hr
	4	Hold (optional overnight hold)
	37	
	15	
	4	Hold

[a]From Applied Biosystems.

Table 2F.2.2 RiboAmp HS PLUS Thermal Cycler Program Round Two[a]

	Temperature (°C)	Time
First-strand synthesis	65	5 min
	4	Hold
	25	1 hr
	37	30 min
	4	5 min
Second-strand synthesis	95	2 min
	4	Hold
	37	30 min
	70	5 min
	4	Hold
IVT	42	6 hr
	4	Hold (optional overnight hold)
	37	
	15	
	4	Hold

[a]From Applied Biosystems. The program is not intended for automatic progression from one time and temperature set to another. The program lists a 4° to 8°C hold after each incubation or incubation cycle when it is necessary to remove the reactions from the thermal cycler to add reagents. After the addition of reagents, place the sample back into the thermal cycler and resume the program. Using a thermal cycler with a heated lid is important. The heated lid ensures proper temperature distribution within the reaction tube and prevents evaporative condensation that alters the reaction mixture concentrations. The 4° to 8°C steps in the thermal cycler program allow for buffer and reagent addition and mixing steps at certain points during the amplification process and are not intended for indefinite hold unless noted (Applied Biosystems).

Thermal cycler
Qubit 2.0 fluorometer (Invitrogen)

1. Use the Arcturus PicoPure RNA isolation kit for RNA isolation with the following modifications—sort as many cells as possible for regional cell type–specific profiling each into five different 1.5-ml microcentrifuge tubes containing 500 μl of RNA extraction buffer (place immediately on ice).

   A minimum of 2000 cells is highly recommended for each sorting.

   The ArrayPure Nano-scale RNA Purification Kit (Epicentre, cat. no. MPS04050) and MessageBOOSTERTM cDNA Synthesis Kit (Epicentre, cat. no. MB060124) can also be used for RNA isolation and RNA amplification.

2. Seal sorted cells in microcentrifuge tubes with Parafilm and incubate 1 hr in a 42°C water bath.

   Incubation times can vary between 45 min and 1 hr but not less.

3. After incubation, microcentrifuge cells 4 min at 3000 × *g*, 4°C. Transfer supernatant into a new microcentrifuge tube and add 500 μl of 70% RNase-free ethanol, and mix well. Follow the Arcturus PicoPure RNA isolation kit per the manufacturer's instructions, adding the extraction buffer–alcohol mixes in increments of 300 μl to the RNA extraction columns.

4. Elute the RNA with 12 μl of elution buffer and store at –80°C or proceed to amplification.

5. Program the thermal cycler as per Arcturus RiboAmp HS PLUS RNA amplification kit instructions (Tables 2F.2.1 and 2F.2.2).

   The amplification cycle steps should be saved as a single program with breaks at 4°C. The machine should not be switched off any time in between the procedure.

6. Quantify RNA using the Qubit 2.0 Fluorometer. Use 2 ng of RNA for amplification. For regional cell profiling <1 ng RNA might be obtained. In such cases, use the whole amount for amplification. Amplify the RNA using Arcturus RiboAmp HS PLUS RNA amplification kit. To normalize, use a constant amount of amplified RNA as input for downstream processes like RNA-Seq etc.

7. Use amplified RNA directly for RT-PCR, microarray, or RNA sequencing.

IMMUNOFLUORESCENCE STAINING OF SORTED STEM CELLS

BASIC PROTOCOL 3

The staining protocol for *Drosophila* intestinal stem cells is adapted from the protocol used for the staining of germline cells, as described in *UNIT 2E.3*. It has been concluded that the FACS sorting procedure does not cause any detectable modification of ISC properties (Fig. 2F.2.2 G-G″). Positively charged slides were used and the procedure was performed in a humidified chamber (wet tissues in petri plate) and without exposure to direct light. Midguts were stained with mouse monoclonal anti-Delta (1:100, rabbit polyclonal anti-GFP, 1:1000, Invitrogen) and DNA was stained with Hoechst 33258 (Invitrogen).

Materials

Sorted cells (see Basic Protocol 1)
Schneider's medium (Prom°Cell)
Fetal bovine serum (FBS)
16% w/v formaldehyde (Alfa Aesar)
0.15% PBST (see recipe)
5% NGS in PBST (see recipe)
Antibodies: mouse monoclonal anti-Delta (1:100), rabbit polyclonal anti-GFP (1:1000) (Invitrogen)
0.1% DAPI
Vectashield mounting medium (Vector)

1.5-ml microcentrifuge tubes
Refrigerated microcentrifuge
Positively charged slides
Petri plates
Coverslips
Microscope

1. Sort out 10,000 cells in a 1.5-ml microcentrifuge tube containing 500 µl of Schneider's medium with 10% FBS (S-FBS).

2. Microcentrifuge 5 min at 500 × *g*, 4°C and resuspend in 100 µl of S-FBS.

3. Pipet suspension onto a positively charged slide in a humidified chamber (wet tissues in a petri plate) and allow cells to settle for 1.5 hr.

   Avoid exposure to direct light during the staining procedure (cover chamber with a tissue or foil and place in dark).

4. Fix cells by adding 500 μl of 1:3 (v/v) formaldehyde/Schneider's medium onto the slide and allow cells to stand for 30 min in the humidified chamber (away from direct light) at room temperature.
5. Hold the slide at a 45° angle and wash at least five times with 1 ml of 0.15% PBST.
6. Block by adding 500 μl of 5% NGS in PBST onto the slide for 30 min. Wash off blocking solution by slowly pipetting 1 ml of 0.15% PBST onto the slide to drain off excess blocking solution. Wash at least three times.
7. Add 500 μl primary antibody in PBST with 5% NGS onto the slide and incubate for 1.5 hr at room temperature. Wash off excess primary antibody five to six times with 1 ml of 0.15% PBST.
8. Add 500 μl secondary antibody in PBST with 5% NGS and incubate for 1 hr. Rinse with PBST.
9. Add 0.1% DAPI (1:200 in 1× PBS) onto the slide and let stand for 5 min at room temperature. After incubation, hold slide at a 45° angle and wash five to six times with 1 ml PBST.
10. Add 25 μl mounting medium (Vectashield) and add coverslip onto the slide. Observe under a microscope.

    Round GFP-positive cells can be observed, which represent the progenitor cells. Delta antibody marks the intestinal stem cells specifically and co-localizes with GFP-positive cells.

REAGENTS AND SOLUTIONS

For culture recipes and steps, use sterile tissue culture–grade water. For other purposes, use deionized, distilled water or equivalent in recipes and protocol steps.

Collagenase solution, 1% (w/v)

Dissolve lyophilized collagenase type IV (Sigma, cat. no. C8051) in RNase-free PBS to a concentration of 50 mg/ml. Filter through a 0.22-μm filter. Prepare 1-ml aliquots and store up to 3 months at –20°C. Thaw stock solution from freezer immediately before use and dilute into 1× PBS-DEPC (see recipe) to a final concentration of 10 mg/ml.

DEPC-treated water and PBS-DEPC, 0.1% (v/v)

Add 1 ml of fresh diethyl pyrocarbonate (DEPC) to 1 liter of distilled water or 1 × PBS (10 × PBS, Ambion). Shake well to disperse the DEPC through the solution and leave overnight on a shaker at room temperature (room temperature, 22° to 25°C). Autoclave at 15 psi on liquid cycle for 20 min to inactivate the remaining DEPC. The solution remains stable for many months in RNase-free conditions at room temperature.

Elastase solution, 0.4% (w/v)

Reconstitute elastase (Sigma, cat. no. E0258) in cell dissociation buffer at a concentration of 4 mg/ml and store at –20°C. Avoid multiple freeze-thaws.

NGS in PBST, 5% (v/v)

Add 5 ml NGS (Sigma) to 100 ml PBST

PBST, 0.15% (v/v)

Mix 0.15 ml Triton X-100 (Merck) in 100 ml of 1 × PBS

COMMENTARY

Background Information

While isolation of frequently occurring cell types is relatively easy and enables transcriptome, proteome, and metabolome studies, profiling of rare cell types still remains a challenge. Several methods have been described for the isolation, purification, and profiling of rare cell types and adult stem cells from various mammalian and *Drosophila* tissues. These include (1) 4-thiouracil (TU) tagging, e.g., from *Drosophila* larvae, embryos, and adults and other mammalian tissues as described by Miller et al., 2009; (2) RNA co-immunoprecipitation or mRNA tagging, e.g., from muscles and sensory neuronal cells in *C. elegans* (Roy et al., 2002; Kunitomo et al., 2005); (3) isolation of nuclei tagged in specific cell types (INTACT), e.g., from *C. elegans* muscle cells, from the mesoderm of *Drosophila* embryos, and from neurons as described by Steiner et al. (2012); (4) fluorescent-activated cell sorting (FACS), e.g., from *Drosophila* neuronal stem cells and embryonal germline cells and mammalian tissues (Shigenobu et al., 2006; Berger et al., 2012; Pasut et al., 2012); and (5) microdissection or mechanical purification, e.g., for various plant tissues (Leonhardt et al., 2004; Schmid et al., 2012). While mechanical methods and microdissection require the cells to be detectable for isolation, TU-tagging is recommended for frequently occurring cells where modified 4-thiouracil can be incorporated into newly synthesized RNA in cells expressing uracil phosphoribosyltransferase (UPRT), thus enabling cell type–specific RNA isolation. The INTACT method involves affinity purification of nuclei from specifically tagged cells, and thus renders the isolation of whole cells unnecessary. It has the added advantage of allowing the study of chromatin features or epigenetic profiling of rare cell types. However, more optimization of the method is needed. mRNA tagging involves tagging mRNA from a specific tissue by expressing a FLAG-tagged poly(A) binding protein (PABP) and separation of the mRNA of the specific tissue by co-immunoprecipitation using an anti-FLAG antibody (Yang et al., 2005). FACS facilitates isolation of rare cell types fluorescently tagged with cell type–specific markers, from complex tissues. It is an extremely sensitive method and allows isolation of cells with very low false positives, which makes it a powerful and straightforward tool for cell type specific studies. Using various reporters and the bipartite *GAL4/UAS* system (Fischer et al., 1988; Brand and Perrimon, 1993) several *Drosophila* cell types have been purified. Recently, female undifferentiated germ line cells from the adult *Drosophila* ovaries were isolated using FACS (*UNIT 2E.3*; Lim et al., 2012).

The *Drosophila* midgut has well characterized markers, with cell type–specific *GAL4* drivers available for all the different cell populations. The objective was to develop a reproducible, fast, easy, and efficient method to profile the various cell types with high accuracy using the existing resources and without requiring unusual equipment or technical specialization. A combination of the GAL4/UAS system, FACS, RNA isolation, and linear RNA amplification (using commercially available kits) has been used to facilitate gene expression profiling of the *Drosophila* intestinal cell types. Described in this unit is a protocol that can be used to isolate the endodermally derived undifferentiated stem (ISCs), progenitor cells (EBs), the differentiated enterocytes and enteroendocrine cells (ECs and EEs), and the mesodermally derived visceral muscle (VM) from all five midgut regions with high precision. The robustness of the process can be demonstrated by the fact that even regional cell type profiling gives accurate transcriptome profiles, which comply with most previous studies on the gut. Using that method, we have started to unravel the transcriptional landscape of midgut cell types and their variation across midgut regions (see previous version of this unit at *http://www.currentprotocols.com/protocol/sc02F02*). With minor variations in the protocol for each cell type, it can be used to study the gene expression profile of any cell population in the *Drosophila* midgut under any experimental condition of knockdown or gene over expression (Korzelius et al., 2014; Loza-Coll et al., 2014; Zhou et al., 2015) or even for cell cycle analysis (Zielke et al., 2014). This makes it a powerful tool to study cell type–specific variations in the *Drosophila* intestinal populations.

In addition to FACS, TU-tagging, INTACT, microdissection, and magnetic bead sorting have been used to purify RNA from specific cell populations from tissues. TU-tagging, PABP CoIP, or the INTACT method could in principle be used for the fly gut instead of the FACS protocol, but optimization for use in the *Drosophila* intestine would be required. The yield after CoIP of RNA from stem cells might be problematic, making FACS a more

feasible method. While magnetic bead sorting is believed to be a milder method than FACS for cell purification that might cause less cell death (Iyer and Cox, 2010; Zou et al., 2011), with the FACS protocol described here, >90% propidium iodide–negative (living) cells have been consistently observed, indicating that the conditions in which cell isolation is performed are not harsh and do not cause enormous cell damage or death.

Previously, *Drosophila* intestinal cells have been analyzed by FACS to determine the cell cycle phases of cells under various experimental conditions (Amcheslavsky et al., 2011). However, the described dissociation protocol uses trypsin for dissociation, which is found to be damaging for the cells, as fewer living cells were obtained subsequent to treatment (<60%). Also, due to the time required for the described preparation, it would not be ideal for gene expression studies. Milder enzymes (collagenase, elastase) that are optimized for isolation of all cell types of the midgut, either membrane or nuclear GFP labeled, are used. Immunofluorescent staining of FACS-sorted ISCs showed that cells retained their morphological characteristics and marker expression after sorting, and thus the FACS procedure appears not to cause any major modification of the stem cell properties. This protocol might also be adapted for cell type–specific studies of other cellular constituents such as chromatin, proteins, lipids, or various metabolites, provided that the end-analysis methods are sensitive enough for the relatively small number of cells (2000) obtained. Since this FACS protocol isolates living cells, it could also conceivably be used to purify stem cells for in vitro culture studies.

Using this protocol, transcriptome profiles of each *Drosophila* intestinal cell population under physiological conditions and also after enteric infection by *Pseudomonas entomophila* (*P.e*) have been generated. Such a dataset can be used to study the complex changes occurring in the fly midgut upon infection in a cell type–specific manner. As the profiling has been done under homeostatic conditions, the dataset can be used as a reference for comparison with new transcriptome data prepared from these cell types under new experimental conditions, as long as the new data were generated using the same protocol. The resource can be used to find novel cell type–specific markers and for developing new cell type–specific Gal4 "driver" lines. Using the cell type–specific transcriptome datasets, several novel markers for *Drosophila* intestinal stem cells have been identified and verified (unpub. observ.).

Critical Parameters and Troubleshooting

An equal number of cells should be sorted out for each cell type. For regional profiling, isolate as many cells as possible from each region. Following amplification, use a constant amount of RNA for RNA-Seq to normalize the process. All steps from dissection of midguts up to RNA isolation should be performed on the same day, since cell storage at –80°C in extraction buffer was found to reduce the yield of RNA. To avoid delays during the procedure, all the settings for the FACS and PCR machines should be set beforehand. Pretreat the FACS machine as described with bleach and DEPC-treated water before each use. As ECs are polyploid and larger in size, and visceral muscle cells are elongated, use 70- to 100-μm filters and the largest nozzle size available (100 μm) for the flow cytometer when sorting ECs or visceral muscle. Also, a longer dissociation time (1.5 hr) is needed for complete dissociation of ECs and visceral muscle cells. The protocol has been verified under different experimental conditions and works well for the cell types, with esg^+ cell sorting being the most efficient. Use of collagenase or elastase is optional, depending on availability. The authors have found elastase to be more efficient. For flies expressing membrane-bound GFP (mGFP), collagenase was found to attenuate the GFP intensity; thus, use of elastase is recommended in such cases. The RNA isolation kit is used for microdissected samples, which require much lower buffer volumes; the columns thus will not take >300 μl at one time. The yield is better if one column is used to bind all the RNA instead of multiple columns before proceeding to the washing and elution step. To obtain maximum yield, keep all conditions RNase-free at all times and thoroughly clean the sorter with bleach and alcohol before starting the procedure. Clean sorter with DEPC-treated water between samples of different genotypes and regions. The RNA amplification procedure involves a two-step amplification of RNA and might be costly if experiments are done in replicates. The amplification step should be bypassed in cases where enough RNA is obtained after RNA isolation (e.g., for esg^+ cells or ECs) for downstream analysis. Amplify the replicates of one genotype in different batches to avoid amplification biases due to handling. For data analysis of the RNA-Seq data

under different experimental conditions, we suggest comparing the fold changes between samples and controls for each replicate, rather than RPKM values only. We noticed rare and mild variations in the raw RPKM values of biological replicates, which could be due to handling differences of samples at different times or variation of the amplification process. This could alter the statistical power of the analysis by increasing the noise of gene expression, especially for genes expressed at low levels. However the fold change between the samples and controls (for each experiment) remains constant between experimental replicates, which increase the ability to identify differentially regulated genes,

Anticipated Results

From whole midguts, this protocol generates ~200 to 300 ng of total RNA for the esg^+ cells, 300 to 400 ng for enterocytes, and <50 ng for the rest of the cell types—namely intestinal stem cells (ISCs), enteroendocrine cells (EEs), enteroblasts (EBs), and the visceral muscle (VM). When 2 ng RNA is amplified, yields ranging from 50 to 125 μg are obtained. For regional profiling, less than 1 ng might be obtained, which on amplification yields 30 to 35 μg RNA. (sufficient for microarray, RT-PCR, and RNA sequencing).

Time Considerations

The dissection of midguts should be accomplished within 2 to 3 hr, followed by cell dissociation by enzymatic treatment for 1 to 1.5 hr depending on cell type. The gates for FACS sorting should be pre-set to avoid any delay in the procedure. Dissociated cells are FACS sorted, and RNA should be isolated from the sorted cells immediately, which takes 4 to 5 hr. All procedures from dissection up to RNA isolation (see Basic Protocol 1) take in total ~10 hr and should be performed on the same day to obtain high yields of RNA. The timeline of this part of the protocol should be carefully planned beforehand. The isolated RNA can be stored at –80°C until amplification. Amplification of RNA by standard methods takes 3 days (two 6-hr incubations for in vitro transcription), including quantification by a Qubit fluorometer. The immunofluorescence staining protocol starting from dissection to staining takes 8 hr. Amplified RNA can be stored at –80°C until further gene expression profiling experiments are performed.

Acknowledgements

The authors thank Monika Laglotz from the ZMBH FACS facility for help with the cell sorting, and David Ibberson at the Cell Networks-Deep Sequencing Core Facility of Universität Heidelberg for help with RNA sequencing. The authors also thank Hanna Reuter for helping with the FACS dissociation protocol, as well as Jerome Korzelius for the Rab3-YFP picture and scientific discussions and Parthive Patel and Norman Zielke for generous help with flies and helpful insights. Funded by DFG SFB873, DKFZ A220, and ERC Advanced Grant 26515 to B.A.E.

Literature Cited

Amcheslavsky, A., Ito, N., Jiang, J., and Ip, Y.T. 2011. Tuberous sclerosis complex and Myc coordinate the growth and division of *Drosophila* intestinal stem cells. *J. Cell Biol.*193:695-710.

Berger, C., Harzer, H., Burkard, T.R., Steinmann, J., van der Horst, S., Laurenson, A.S., Novachkov, M., Reichert, H., and Knoblich, J.A. 2012. FACS purification and transcriptome analysis of *Drosophila* neural stem cells reveals a role for Klumpfuss in self-renewal. *Cell Rep.* 2:407-418.

Brand, A.H. and Perrimon, N. 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* 118:401-415.

Buchon, N., Broderick, N.A., Poidevin, M., Pradervand, S., and Lemaitre, B. 2009. Drosophila intestinal response to bacterial infection: Activation of host defense and stem cell proliferation. *Cell Host Microbe* 5:200-211.

Buchon, N., Osman, D., David, F.P.A., Fang, H.Y., Boquete, J.P., Deplancke, B., and Lemaitre, B. 2013. Morphological and molecular characterization of adult midgut compartmentalization in *Drosophila*. *Cell Rep.* 3:1725-1738.

Fischer, J.A., Giniger, E., Maniatis, T., and Ptashne, M. 1988. GAL4 activates transcription in *Drosophila*. *Nature* 332:853-856.

Iyer, E.P.R. and Cox, D.N. 2010. Laser capture microdissection of *Drosophila* peripheral neurons. *J. Vis. Exp.* 39:2016.

Jiang, H., Grenley, M.O., Bravo, M.J., Blumhagen, R.Z., and Edgar, B.A. 2011. EGFR/Ras/MAPK signaling mediates adult midgut epithelial homeostasis and regeneration in *Drosophila*. *Cell Stem Cell* 1:84-95.

Jiang, H., Patel, P.H., Kohlmaier, A., Grenley, M.O., McEwen, D.G., and Edgar, B.A. 2009. Cytokine/Jak/Stat signaling mediates regeneration and homeostasis in the *Drosophila* midgut. *Cell* 137:1343-1355.

Korzelius, J., Naumann, S.K., Loza-Coll, M.A., Chan, J.S., Dutta, D., Oberheim, J., Gläßer, C., Southall, T.D., Brand, A.H., Jones, D.L., and Edgar, B.A. 2014. Escargot maintains stemness and suppresses differentiation in *Drosophila* intestinal stem cells. *EMBO J.* 33:2967-2982.

Kunitomo, H., Uesugi, H., Kohara, Y., and Iino, Y. 2005. Identification of ciliated sensory

neuron-expressed genes in *Caenorhabditis elegans* using targeted pull-down of poly (A) tails. *Genome Biol.* 6:R17.

Leonhardt, N., Kwak, J.M., Robert, N., Waner, D., Leonhardt, G., and Schroeder, J.I. 2004. Microarray expression analyses of *Arabidopsis* guard cells and isolation of a recessive abscisic acid hypersensitive protein phosphatase 2C mutant. *Plant Cell* 16:596-615.

Lim, R.S.M., Osato, M. and Kai, T. 2012. Isolation of undifferentiated female germline cells from adult *Drosophila* ovaries. *Curr. Protoc. Stem Cell Biol.* 22:2E.3.1-2E.3.14.

Loza-Coll, M.A., Southall, T.D., Sandall, S.L., Brand, A.H., and Jones, L. 2014. Regulation of *Drosophila* intestinal stem cell maintenance and differentiation by the transcription factor Escargot. *EMBO J.* 33:2983-2996.

Marianes, A. and Spradling, A.C. 2013. Physiological and stem cell compartmentalization within the *Drosophila* midgut. *Elife* 2:e00886.

Micchelli, C.A. and Perrimon, N. 2006. Evidence that stem cells reside in the adult *Drosophila* midgut epithelium. *Nature* 439:475-479.

Miller, M.R., Robinson, K.J., Cleary, M.D., and Doe, C.Q. 2009. TU-tagging: Cell type-specific RNA isolation from intact complex tissues. *Nat. Methods* 6:439-444.

Ohlstein, B. and Spradling, A. 2007. Multipotent *Drosophila* intestinal stem cells specify daughter cell fates by differential notch signaling. *Science* 315:988-992.

Osman, D., Buchon, N., Chakrabarti, S., Huang, Y.T., Su, W., Poidevin, M., Tsai, Y., and Lemaitre, B. 2012. Autocrine and paracrine unpaired signaling regulate intestinal stem cell maintenance and division. *J. Cell Sci.* 125:5944-5949.

Pasut, A., Oleynik, P., and Rudnicki, M.A. 2012. Isolation of muscle stem cells by fluorescence activated cell sorting cytometry. *Methods Mol. Biol.* 798:53-64.

Roy, P.J., Stuart, J.M., Lund, J., and Kim, S.K. 2002. Chromosomal clustering of muscle-expressed genes in *Caenorhabditis elegans*. *Nature* 418:975-979.

Schmid, M.W., Schmidt, A., Klostermeier, U.C., Barann, M., Rosenstiel, P., and Grossniklaus, U. 2012. A powerful method for transcriptional profiling of specific cell types in eukaryotes: Laser-assisted microdissection and RNA sequencing. *PLoS One* 7:e29685.

Shigenobu, S., Arita, K., Kitadate, Y., Noda, C., and Kobayashi, S. 2006. Isolation of germline cells from *Drosophila* embryos by flow cytometry. *Dev. Growth Differ.* 48:49-57.

Steiner, F.A., Talbert, P.B., Kasinathan, S., Deal, R.B., and Henikof, S. 2012. Cell type-specific nuclei purification from whole animals for genome-wide expression and chromatin profiling. *Genome Res.* 22:766-777.

Yang, Z., Edenberg, H.J., and Davis, R.L. 2005. Isolation of mRNA from specific tissues of *Drosophila* by mRNA tagging. *Nucleic Acids Res.* 33:e148.

Zhou, J., Florescu, S., Boettcher, A.-L., Luo, L., Dutta, D., Kerr, G., Cai, Y., Edgar, B.A., and Boutros, M. 2015. Dpp/Gbb signaling is required for normal intestinal regeneration during infection. *Dev. Biol.* 399:189-203.

Zielke, N., Korzelius, J., van Straaten, M., Bender, K., Schuhknecht, G.F.P., Dutta, D., Xiang, J., and Edgar, B.A. 2014. Fly-FUCCI: A versatile tool for studying cell proliferation in complex tissues. *Cell Rep.* 7:588-598.

Zou, K., Hou, L., Sun, K., Xie, W., and Wu, J. 2011. Improved efficiency of female germline stem cell purification using fragilis-based magnetic bead sorting. *Stem Cells Dev.* 20:2197-2204.