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## FACS purification of *Drosophila* larval Neuroblasts for next generation sequencing

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### Abstract

Elegant tools are available for the genetic analysis of neural stem cell lineages in *Drosophila*, but a methodology for purifying stem cells and their differentiated progeny for transcriptome analysis is currently missing. Previous attempts to overcome this problem either involved using RNA isolated from whole larval brain tissue or co-transcriptional *in vivo* mRNA tagging. As both methods have limited cell type specificity, we developed a protocol for the isolation of *Drosophila* neural stem cells (neuroblasts, NBs) and their differentiated sibling cells by FACS. We dissected larval brains from fly strains expressing GFP under the control of a NB lineage-specific GAL4 line. Upon dissociation, we made use of differences in GFP intensity and cell size to separate NBs and neurons. The resulting cell populations are over 98% pure and can readily be used for live imaging or gene expression analysis. Our method is optimized for neural stem cells, but it can also be applied to other *Drosophila* cell types. Primary cell suspensions and sorted cell populations can be obtained within 1 d; material for deep-sequencing library preparation can be obtained within 4 d.

### Keywords

Neural stem cells; FACS isolation; mRNA-sequencing; *Drosophila*

## INTRODUCTION

Stem cells are the basis for the development of every multicellular organism and are involved in the maintenance of virtually all adult tissues. To carry out their function, stem cells have to remain in an undifferentiated state and maintain their ‘stemness’ properties over a series of cell divisions, while simultaneously generating developmentally restricted progeny. *Drosophila* larval neural progenitor cells (NBs) have proven to be an ideal model system<sup>1-3</sup> to investigate how the balance between stem cell identity and differentiation is sustained, and how failures in restricting the proliferation potential of stem cells can lead to the formation of tumors.

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NBs undergo repeated rounds of asymmetric cell division, the products of which are a larger cell that maintains stem cell identity and a smaller cell that will undergo neuronal differentiation. To date two different populations of NBs in the larval central brain and ventral nerve cord can be distinguished based on lineage and gene expression pattern<sup>4-6</sup>: ~400<sup>7,8</sup> asymmetrically dividing type I NBs produce a self-renewing NB and a ganglion mother cell (GMC) that divides once more to generate two postmitotic neurons or glial cells. The 16 type II NBs per brain also divide asymmetrically, but give rise to an intermediate neuronal progenitor (INP) instead of a GMC. INPs undergo a series of maturation steps and then divide asymmetrically for a limited number of times generating GMCs and ultimately neurons or glial cells<sup>4-6</sup>.

*Drosophila* genetics allows for easy manipulation and investigation of larval NB lineages, but the complete set of factors controlling NB self-renewal and lineage progression is not known. Identification of genes specifically expressed in larval NBs and their various lineage intermediates would be a first step towards discovering these factors. Hence, various methodologies were employed to generate NB specific transcriptome data. For example, gene expression in wild-type brains was compared to brains from mutants affecting asymmetric cell division, which causes the formation of brain tumors highly enriched for NBs<sup>9</sup>. Indeed, this method identified a number of NB specific genes. However, the approach is problematic, as the mutant NBs have lost the ability to undergo differentiation and they divide in an uncontrolled manner; therefore, they resemble wild-type NBs only to some extent. In addition, both mutant and wild-type larval brains contain a heterogeneous mixture of cell types (NBs, embryonic and larval neurons and glial cells), masking the true expression pattern of genes expressed in NBs and neurons. A second technique, thio-uracil (TU)-tagging, utilizes specific labeling of newly synthesized mRNAs with 4-thio-uracil (4TU) by cell type specific expression of uracil phosphoribosyltransferase (UPRT), an enzyme needed in order to incorporate 4TU into mRNA<sup>10</sup>. TU-labeling of mRNAs allows their subsequent purification and analysis by deep sequencing. The drawback of this method is the equal inheritance of UPRT by both NB daughters, resulting in labeling of mRNAs also in the differentiating population.

Clearly, the simplest way to identify NB transcriptomes would be to isolate large numbers of these cells and their differentiating progeny, followed by analysis of gene expression levels. Different cell populations can be separated either by magnetic bead sorting<sup>11</sup>, or by FACS, see *e.g.*<sup>12-15</sup>. Typically, these approaches use the expression of a transgene, usually mCD8::GFP, under the control of a cell type specific promoter. Cells expressing the transgene are captured by magnetic beads or are FACS sorted on the basis of their fluorescent signal. NBs and neurons, however, can not be purified this way, because the fluorescent marker protein will be inherited by both NB daughter cells and therefore mark all cells within the NB lineage.

To circumvent these problems, we developed a protocol for FACS isolation of larval NBs and neurons that uses both differences in cell size and in levels of GFP expression to separate both cell types from the same larval brain<sup>16</sup>. Our protocol relies on a sufficient, yet gentle dissociation of larval brains into single cells. NBs in this cell suspension are alive and maintain lineage characteristics of wild-type larval NBs<sup>16</sup> indicating that our method preserves the endogenous cell type specific transcription pattern well. We applied this method to determine the first complete expression profiles of NBs and neurons using 76-base paired-end *Illumina Solexa* sequencing<sup>16</sup>.

## Overview

This protocol is designed to obtain pure populations of cells from *Drosophila melanogaster* for downstream applications such as live imaging, expression analysis or western blotting.

The protocol is easy and straightforward (Fig. 1a), and allows the purification of pure cell populations within half a day. The first step is the dissection of larvae (Fig. 1b). The timing of this step depends on the dissection skills of the experimenter and should not exceed 60 min, because of risk of tissue degeneration. The brains are then enzymatically and mechanically disrupted, which takes another 60 minutes. After this 2-h procedure, the cells are subjected to FACS, which, depending on the number of dissected larval brains, takes up to 2 h (for 200 brains). We reproducibly sort around ~120 – 160 NBs and 18,000 – 25,000 neurons per larval brain. To obtain high quality RNA sequencing data without the need for excessive amplification cycles, the experimenter needs to purify 100,000 – 150,000 NBs and ~20 – 25 million neurons. NBs are directly sorted into TRIzol LS to preserve RNA. Neurons are sorted in FACS flow in a 1.5 mL tube, and spun down immediately after the tube is filled. After removal of the FACS flow, TRIzol LS is added and up to five tubes of sorted neurons are combined. Several sorts (NBs and neurons) are collected and stored at –80 °C until further processing. Depending on the application and the cell numbers required, this may take several days. After TRIzol LS purification, RNA quality is measured on an Agilent Bioanalyzer (Fig. 1c). After enrichment for poly(A)<sup>+</sup>-RNA, the mRNA is fragmented and subjected to first-strand cDNA synthesis. During second-strand synthesis, dUTP is used to specifically label second-strand cDNA. Second-strand cDNA is digested during library preparation for *Illumina Solexa* sequencing to preserve strand-specific transcript information. Preparation of double stranded cDNA takes 4 d. Libraries from NBs and neurons are sequenced by 76-base paired-end sequencing on a HiSeq2000 machine, adding another 11 d to the protocol. In total, at least 4 weeks have to be allocated to obtain transcriptome information for the cell type of interest, including setting up fly crosses for FACS.

### Other applications of the protocol and future uses

A vast array of cell type-specific driver lines are available for *Drosophila*, and therefore many cell types can be unambiguously defined using additional parameters such as cell size or granularity. Steps that are most likely to require tissue and cell type-specific optimization include cell type-specific labeling and the dissociation protocol, although the latter can easily be optimized by modifying the amounts of enzyme and incubation times.

To demonstrate the broad applicability of our method, we used our protocol to purify *Drosophila* adult intestinal stem cells (ISCs; R.C., unpublished data). We used the ISC-specific *escargot*-Gal4 driver line to label these cells with GFP<sup>17,18</sup>. Initial experiments revealed that the concentration of enzymes had to be increased in order to dissociate midgut tissue. We were able to separate a specific population of cells with high GFP signal and could show that these are probably ISCs. Nevertheless, further optimization of the method for this application and quality control experiments are indispensable.

In addition, our protocol can be combined with other *Drosophila* methodologies and thereby applied to the study of a variety of different biological questions. For example, it can be used to determine NB transcriptomes at various developmental stages (e.g., embryonic or pupal NBs) or under specific conditions (e.g., starvation, hormones and drugs). In addition, FACS can be combined with RNA interference (RNAi) to determine transcriptional changes upon loss of specific genes. Furthermore, the TARGET system<sup>19</sup> can be used to initiate GFP expression at specific time points to analyze transcriptional changes in a time-resolved manner. In combination with NB cell culture, for example, this might allow one to follow transcriptional changes in GMCs at various time points after mitosis. As the TARGET system can easily be combined with RNAi, the transcriptional changes occurring in NBs or their differentiating siblings upon inactivation of specific genes could be analyzed in a time-specific manner.

## Limitations and problems of the method

FACS relies on single cells passing a detection unit and their subsequent sorting according to, in our case, GFP expression and cell size. The biggest limitation of our approach is the histology of the larval brain, as it contains only very few NBs that give rise to many neurons and glia cells. Therefore, even if we were able to bulk-isolate many larval brains (as is possible for imaginal discs<sup>20,21</sup>), FACS sorting of NBs would remain the rate-limiting step in our protocol. During FACS, the cells need to be cooled to 4 °C to keep NBs from dividing, which decreases their survival and probably affects gene expression profiles.

Sorting according to cell size and GFP expression levels will be problematic if specific labeling of the cell type of interest and its progeny is not possible. This can be either because the transgene is also expressed in an unwanted subset of cells with similar characteristics, e.g., granularity and size, or because the stem cell and its progeny do not differ in any of the aforementioned features.

Complete dissociation of tissue is a prerequisite condition for FACS. Sorting of pure cell populations will not be possible if the tissue of interest cannot be properly dissociated. In general, enzymatic digestion, mechanical stress, different temperatures and removing the cells from their cellular context could potentially affect gene expression, signaling (e.g., Notch signaling) and cell survival. However, in our case, we could show that NBs maintain their lineage characteristics after dissociation and FACS<sup>16</sup>.

## Experimental design

Our protocol relies on sorting of GFP-labeled cells that can be separated on the basis of their size and strength of GFP expression. Exclusive labeling of stem cells using transgenic reporters, such as Gal4-driven GFP, is often not possible owing to the combination of a lack of driver lines that are exclusively expressed in the stem cells, and the perdurance of GFP (and thus its inheritance into the more differentiated daughter cells).

**Labeling of a cell population**—The larval brain is mostly made up of small neurons, medium-sized GMCs, INPs and NBs in the optic lobes, and of large NBs and glia cells. In addition to differences in cell size, subtypes of cells can be labeled with a fluorescent protein, for example, GFP. For this, either the UAS/Gal4 system in combination with a cell type-specific driver line or a line expressing GFP under the control of a cell type-specific promoter can be used. We decided to use the *asense (ase)*-Gal4 (ref. 22) line that is only expressed in type I but not in type II NB lineages or glia cells. We nevertheless checked for the presence of glia cells, but could not detect any Repo-positive cells after FACS<sup>16</sup>. We were also able to sort type II NBs, of which there are only 16 per brain, using the type II-specific driver lines *worniu*-Gal4, *ase*-Gal80 (ref. 23) or *Pointed*-Gal4 (ref. 24; data not shown). Therefore, in principle, more cell type-restricted Gal4 lines could be used to allow comparisons between different NB subpopulations. For GFP labeling of cells, we chose a strong nuclear GFP, called Stinger::GFP (stable insulated nuclear eGFP vectors<sup>25</sup>). This results in stronger resolution of the GFP signal, which gets weaker in differentiating daughter cells owing to reduced driver line expression and GFP degradation. We have also tested membrane-bound GFP (mCD8::GFP) to label cells. Compared with Stinger::GFP, this results in a much weaker GFP signal and less well-defined cell populations in the FACS plot. Even though the mCD8::GFP marker can be used to isolate highly enriched NB and neuron populations, we recommend Stinger::GFP for the initial establishment of the protocol. When Stinger::GFP is used, our method is sensitive enough to even distinguish homozygous from heterozygous GAL4/GFP-positive NBs, as shown in the FACS plot of Figure 2a.

**Material collection**—Once transgenic lines are established, and the specificity of fluorescent protein expression in the cell types of choice is confirmed, biological material can be collected. For our protocol, we collect crawling third-instar larvae, 5 d after a 24-h period of egg laying, from two or three bottles. Larvae are washed in PBS and 70% (vol/vol) ethanol immediately after collection to remove residual food. Larval brains are dissected (Fig. 1b) for a maximum of 1 h and kept in Rinaldini's solution on ice. Dissecting for longer times can decrease the number of viable NBs in the culture.

**Dissociation**—It is crucial to the success of the FACS sorting that tissues are sufficiently dissociated into single cells, but that the cells are not harmed by overly harsh or long treatment. Dissociation of larval brains with a combination of papain and collagenase I for 1 h at 30 °C has proven to be a good balance between sufficient dissociation, yield and survival of cells during FACS. For other applications, such as live imaging, a shorter incubation time of only 30 min might be sufficient, as the remaining neuron clusters are not problematic for imaging dividing NBs. Therefore, for different applications and tissues, incubation times and combinations of enzymes may need to be optimized.

**Amount of material**—The required amount of material to be collected depends on the downstream application of choice. For cell staining or live imaging, ~7,000 NBs will suffice, and this number can be obtained from 50 larval brains. In any case, we strongly recommend that live imaging and staining of sorted cells be used to evaluate the identity and viability of cells. To obtain enough cells for RNA extraction and subsequent mRNA sequencing, we routinely dissect ~150 larvae (in 1 h) per FACS round and conduct two consecutive sorts per day. We combine up to six FACS experiments to provide 300–500 ng of total RNA, which yields ~10 ng of double-stranded cDNA, from which libraries are prepared for deep sequencing. In such a large-scale preparation, the time devoted to the experiment and access time to a FACS machine can become the limiting parameters. For each sorting, 1 h of dissection time, 1 h of dissociation time and 2 h of FACS-sorting time need to be allocated. Therefore, sorting 36,000–48,000 type I NBs per day (from two experiments with ~300 larval brains) is conveniently possible using our methodology. Neurons, however, are highly abundant and can be sorted in essentially unlimited quantities (tens of millions).

## MATERIALS

### REAGENTS

*D. melanogaster* larvae, 5 d old, reared at 25 °C, e.g., *asense*-Gal4 (ref. 22)

PBS buffer (see REAGENT SETUP)

Ethanol (Roth, cat. no. P076)

Rinaldini's solution (see REAGENT SETUP)

NaCl (Sigma-Aldrich, cat. no. S3014)

KCl (Sigma-Aldrich, cat. no. P9541)

NaH<sub>2</sub>PO<sub>4</sub> (Sigma-Aldrich, cat. no. S9638)

Na<sub>2</sub>HPO<sub>4</sub> (Sigma-Aldrich, cat. no. S3264)

NaHCO<sub>3</sub> (Sigma-Aldrich, cat. no. S5761)

Glucose (Sigma-Aldrich, cat. no. G0350500)

Schneider's culture medium (Gibco, cat. no. 21720024)

Penicillin-Streptomycin solution (Sigma-Aldrich, cat. no. P4458)

Insulin solution from bovine pancreas (Sigma-Aldrich, cat. no. I0516)

L-Glutamine (Sigma-Aldrich, cat. no. G7513)

L-Glutathione (Sigma-Aldrich, cat. no. G6013)

FBS, heat inactivated (Sigma-Aldrich, cat. no. F4135)

Collagenase Type I (Sigma-Aldrich, cat. no. C2674)

Papain (Sigma-Aldrich, cat. no. P4762)

FACSFlow sheath fluid (BD Bioscience, cat. no. 342003) Poly-D-lysine-hydrobromide (Sigma-Aldrich, cat. no. P6282)

TRIzol LS reagent (Life technologies, cat. no. 10296-028)

!CAUTION TRIzol is highly toxic and should only be used in the fume hood. Gloves and lab coat should be worn when working with TRIzol.

Chloroform/Trichloromethane (Roth, cat. no. 3313.4)

Isopropanol (Roth, cat. no. CP41)

RNase free water (Life technologies, cat. no. AM9932)

Diethylpyrocarbonate (DEPC) treated water (Life technologies, cat. no. 750023) Glycoblue (Life technologies, cat. no. AM9515)

Agilent RNA 6000 Nano Kit (Agilent Technologies, cat. no. 5067-1511)

Agilent RNA 6000 Pico Kit (Agilent Technologies, cat. no. 5067-1513)

Dynabeads mRNA purification Kit (Life technologies, cat. no. 61006)

Fragmentation buffer 5× (see REAGENT SETUP)

Tris base, adjusted to pH 8.2 (Sigma-Aldrich, cat. no. T1503)

Potassium acetate (Sigma-Aldrich, cat. no. P1190)

Magnesium acetate (Sigma-Aldrich, cat. no. M5661)

Sodium Acetate 3 M, pH 5.5 (Life technologies, cat. no. AM9740)

Random hexamer primers 3 µg/µL (Life technologies, cat. no. 48190-011)

dNTP Mix, 10 mM (Life technologies, cat. no. 18427-013)

SuperScript® II Reverse Transcriptase (Life technologies, cat. no. 18064-014)



SuperScript® III Reverse Transcriptase, in a set containing 5× first-strand buffer and 0.1 M DTT (Life technologies, cat. no. 18080-044)

iQ SYBR Green Supermix (Bio-Rad, cat. no. 170-8882)

RNaseOUT™ (Life technologies, cat. no. 10777-019)

Second-Strand Buffer (Life technologies, cat. no. 10812-014)

dUTP (Fermentas, cat. no. R0133)

dATP (Fermentas, cat. no. R0141)

dCTP (Fermentas, cat. no. R0151)

dGTP (Fermentas, cat. no. R0161)

DNA Polymerase I (*Escherichia coli*) (Life technologies, cat. no. 18010-017)

Ribonuclease H (Life technologies, cat. no. 18021-014)

*E. coli* DNA Ligase (Life technologies, cat. no. 18052-019)

MinElute Reaction Cleanup Kit (QIAGEN, cat. no. 28204)

Quant-iT Picogreen dsDNA Assay Kit (Life technologies, cat. no. P7589)

NEBNext DNA sample Prep Reagent kits (New England Biolabs, cat. no. E6040)

## EQUIPMENT

Foreceps Dumont Nr.5 (Fine Science Tools, cat. no. 11252-23)

Stereomicroscope SteREO Discovery.V8 (Zeiss)

Glass-bottom dishes, 35mm (MatTek Corporation, cat. no. P35G-0-14-C)

Steriflip (Millipore, cat. no. SCGP00525)

Thermo mixer compact (Eppendorf)

Cell strainer, 35 µm (BD Bioscience Falcon, cat. no. 352235)

FACSAriaIII, cell sorter (BD Bioscience)

Low-binding reagent tubes, 1.5 mL (Life technologies, cat. no. AM12450)

Phase-lock heavy gel tubes, 2 mL (5 PRIME, cat. no. 2302830)

Agilent 2100 Bioanalyzer (Agilent Technologies)

Magnetic rack (for example DynaMag™-2, Life technologies, cat. no. 12321D)

NanoDrop 3300 fluorospectrophotometer (Thermo Scientific)

Genome Analyzer IIX (GAIIIX; Illumina)

HiSeq 2000 sequence analyzer (Illumina)

## REAGENT SETUP

**PBS (1×)** To prepare 1× PBS dilute a 20× stock solution in distilled H<sub>2</sub>O and adjust the pH to 7.4. For the 20× stock solution, mix 151.94 g NaCl, 19.88g Na<sub>2</sub>HPO<sub>4</sub> and 8.28g NaH<sub>2</sub>PO<sub>4</sub> in 1 liter distilled H<sub>2</sub>O.

**Rinaldini's solution** To prepare 1× Rinaldini's solution mix 800 mg NaCl, 20 mg KCl, 5 mg NaH<sub>2</sub>PO<sub>4</sub>, 100 mg NaHCO<sub>3</sub>, 100 mg Glucose in 100 ml distilled H<sub>2</sub>O and filter sterilize using the Millipore Steriflip. Rinaldini's solution can be prepared as a 10× solution and stored at 4 °C for at least 3 months.

**Complete Schneider's culture medium** To prepare the culture medium mix 5 mL heat inactivated fetal bovine serum, 0.1 mL Insulin, 1 mL PenStrep, 5 mL L-Glutamine, 0.4 mL L-Glutathione and 37.85 mL Schneider's medium. Filter-sterilize the medium using the Millipore Steriflip. This solution can be stored at 4 °C for 1 week for FACS, and for 3 d for live imaging.

**CRITICAL** We realized that individual lots of Schneider's Medium differ in quality. Some batches can cause problems for the cells and batches need to be tested individually.

**Enzyme solutions** To prepare collagenase I and papain solutions, dissolve enzymes at a final concentration of 20 mg/mL in distilled H<sub>2</sub>O. Prepare single-use aliquots and store them at -20 °C for at least 2 months.

**CRITICAL** Avoid repeated freeze/thaw cycles, allow a maximum of one freeze/thaw cycle.

**Dissociation solution** For this buffer combine 450 µL of complete Schneider's culture medium with 25 µL of collagenase I and 25 µL of papain.

**CRITICAL** This solution can not be stored and always has to be freshly prepared.

**Fragmentation buffer (5×)** Combine Tris (pH 8.2) at a final concentration of 200 mM, KOAc at 500 mM and MgOAc at 150 mM in DEPC treated water, and filter-sterilize the buffer using the Millipore Steriflip. Fragmentation buffer can be stored at room temperature for at least 1 year.

## EQUIPMENT SETUP

**BD AriaIII cell sorter**—We used an AriaIII cell sorter equipped with a Coherent Sapphire Solid State 13mW laser, but in principle any sorter equipped with a 488 nm laser can be used. Sample station and collection module should be cooled to 4 °C during the entire duration of the sort. To ensure survival of cells, a 100 µm nozzle tip at a sheath pressure of 20 p.s.i. is recommended.

**Coating of glass-bottom dishes**—Glass-bottom dishes are washed once with freshly prepared 70% (vol/vol) ethanol and left to dry in a laminar flow hood. Poly-D-lysine at a final concentration of 0.01 % is added to the dishes, and then the dishes are left in the laminar flow hood for 30 min. After removal of the poly-D-lysine, the dishes are washed twice with distilled water and dried under UV-light.

## PROCEDURE

**Dissociation of larval brains** Timing 2 h 15 min



**1** Collect larvae of appropriate developmental time and genotype into a glass dish in 1 mL of PBS.

**2** Wash the larvae once in 1 mL of PBS.

**3** Wash the larvae in 1 mL 70% (vol/vol) of ethanol for 1 minute to remove all remaining food.

**4** Remove ethanol and add Schneider's culture medium.

**5** Fill a fresh 1.5 ml low binding reagent tube with cold Rinaldini's solution.

**6** Dissect larval brains with forceps under the stereomicroscope and directly transfer each brain into the tube with Rinaldini's solution (Fig. 1b).

**CRITICAL** Do not dissect for more than 1 h total to ensure tissue integrity of the dissected brains.

**7** Prepare the dissociation solution.

**8** After all the brains have sunken to the bottom of the tube, carefully remove the Rinaldini's solution and wash the brains once by adding 500  $\mu$ L of Rinaldini's solution. For washing, take up 200  $\mu$ L of liquid and expel it with force, causing the brains to stir up.

**CRITICAL** When you are washing and mixing, avoid sucking up brains into the pipette tip as they will stick to the plastic, thereby causing loss of material.

**CRITICAL** Visually check whether all the brains have sunken to the bottom. Do not let the brains dry out while removing the solution - leave a small amount to cover them.

**9** Remove the Rinaldini's solution and all material that was transferred into the tube during dissection and does not sink to the bottom of the tube (for example the remaining salivary glands).

**10** Add the dissociation solution to the brains and stir the brains up by pipetting in order to allow for complete mixing, again avoiding taking up brains into the pipette tip. Leave the tube for 1 h at 30 °C in a Thermomixer. Mix the content twice gently during incubation by taking up 200  $\mu$ L of dissociation buffer and expelling it with force.

**11** Remove the dissociation solution slowly without disrupting the brains at the bottom of the tube.

**12** Wash the brain samples carefully twice with 500  $\mu$ L Rinaldini's solution.

**CRITICAL** In all washing steps, add and remove the solution very slowly, so that no brains are stirred up. If brains were stirred up, let them settle down at the bottom of the tube on ice before continuing the washing step.

**13** Wash the samples carefully twice with 500  $\mu$ L Schneider's culture medium.

**14** After removing the medium from step 13, add 200  $\mu$ L of fresh Schneider's culture medium.

**15** Disrupt the tissue by using a 200  $\mu$ L pipette tip and pipette the brains and solution up and down, if possible without foaming, 10-20 times or until the solution appears homogenous.

## ? TROUBLESHOOTING

**16** Filter the cell suspension through a 30  $\mu\text{m}$  mesh 5 mL FACS tube.

**17** Depending on the number of dissociated brains, dilute the cell suspension with more Schneider's medium. We typically add Schneider's medium until the final amount adds up to  $\sim 10 \mu\text{L}$  of medium per dissected larval brain.

**18** Different downstream applications can be chosen at this point. For immunohistochemistry, place primary (i.e., unsorted) cell suspension onto a coated (poly-D-lysine) dish and proceed with standard staining protocols<sup>26</sup>. A typical primary cell suspension stained for the NB marker aPKC and the neuronal marker ELAV is shown in Fig. 2b-d.

**Sorting of NBs and neurons by FACS** Timing maximum up to 2 h

**CRITICAL** When you are proceeding with FACS, place the cell solution on ice and start sorting within the next 5-10 min.

**CRITICAL** The amount of medium in which the cells are suspended determines how fast the cells can be sorted. A solution that is too diluted can be sorted with maximum speed (flow rate of 5 on AriaIII), but the event rate might be low, thereby prolonging the sorting time and decreasing the survival rate of the cells. If the solution is too concentrated, the event rate can be high even at low sorting speeds, thus decreasing the sorting efficiency and leading to a lower yield of cells.

**19** Before sorting, resuspend the cells by flicking the tube several times before placing it into the refrigerated sample station.

**CRITICAL** Turn on sample agitation at 200-300 r.p.m. to ensure a homogeneous suspension of cells and to avoid sinking of cells to the bottom of the FACS tube.

**20** Run the cell sample in a FACS machine. Record at least 100,000 events to gain initial information on all cell populations present.

**CRITICAL** Depending on your tissue composition and cell size, axis scaling and the number of sorted events have to be adjusted. NBs are large cells and very rare compared with other neural cell types; therefore, we use logarithmic scaling and record a large number of events to be able to see a defined NB population (Fig. 3).

**21** Plot side scatter (SSC, log) and forward scatter (FSC, log). Two populations of the same size with different SSC signals should appear. The population with the higher SSC signal probably contains damaged or dying cells; therefore, the sorting gate is defined as the population with the lower SSC signal (Fig. 3a).

**22** Plotting the signal from the excitation of cells with the 488-nm laser [GFP FITC-A (area), log] and the phycoerythrin laser (PE-A, log) should separate auto-fluorescent (low GFP FITC-A signal) from GFP (high GFP FITC-A signal) expressing cells (Fig. 3b).

**23** A separate population of cells should be visible by plotting FSC-A versus GFP FITC-A. NBs are large and strongly GFP positive cells (Fig. 3c). Neurons are small and dimmer, and because of the much larger numbers of neurons per brain this population is easier to determine when less events are plotted. Thus, for a better resolution of the neuron population we recommend to plot only 10 % of the total recorded events.

**24** Cell clusters are excluded by plotting the area of the FSC signal versus its width (FSC-W, log). Events with a stronger FSC-W signal are excluded. Owing to the large size of NBs, the linearity of the scale is not maintained and the population appears bent (Fig. 3d).

**25** Sort the cells with an event rate of a maximum of 6,(000 events per second, which ensures good sort efficiency (>80 %).

**26** (Optional) Place a subset of the sorted NBs and neurons onto coated glass-bottom dishes to assess the identity and purity of the sorted cells by immunofluorescence. Typical cell cultures with sorted NBs and sorted neurons stained with the NB marker aPKC and the neuronal marker ELAV are shown in Fig. 2b-d. Owing to the high reproducibility between sortings and the low numbers of NBs obtained, this step was not performed in every experiment. Initially the populations of interest were gated and quality control experiments like expression analysis of known specific markers by quantitative PCR (qPCR) and immunohistochemistry were performed, to ensure that the sorted populations are reproducibly pure<sup>16</sup>.

## ? TROUBLESHOOTING

**27** Sort NBs into cooled 1.5 mL RNase-free tubes containing 750  $\mu$ L of TRIzol LS. Sort the neurons into empty 1.5 mL tubes, with 400,000 events per tube.

**CRITICAL** At low-pressure setting, the volume of one drop containing one event is 3 nL. Therefore, a maximum number of 83,000 cells can be added to 750  $\mu$ L of TRIzol, and a maximum of 500,000 events can be sorted in an empty 1.5 mL tube. We recommend, however, not filling up the tube to its full capacity to ensure easy handling in the following steps.

**28** Centrifuge the tubes containing neurons in FACS flow at 0.9 $\times$ g for 15 min at 4  $^{\circ}$ C directly after each tube is filled. Remove all but  $\sim$ 50  $\mu$ L of the FACS flow and add TRIzol LS. Up to five tubes filled with neurons can be combined in 750  $\mu$ L TRIzol LS.

**29** Fill up the tube containing NBs in TRIzol LS up to 1 mL with DEPC treated water.

**PAUSE POINT** Cells in TRIzol LS can be stored at  $-80^{\circ}$  C for several months or until enough material is collected for the desired downstream application.

### Generation of double-stranded cDNA for Solexa sequencing library preparation

Timing 4d

**30** Isolate total RNA from TRIzol according to the manufacturer's instructions, adjusted for a small amount of RNA. Use 2-ml phase-lock heavy gel tubes to ensure less genomic DNA carryover when removing the aqueous phase. Dissolve the RNA in 5  $\mu$ l of DEPC-treated water in an RNase-free low-binding tube.

**PAUSE POINT** Extracted RNA can be stored at  $-80^{\circ}$  C for several weeks.

**31** Combine up to five sortings, or add DEPC treated water to a total of 25  $\mu$ L.

**32** Assess the quality and quantity of the extracted RNA on an Agilent Bioanalyzer using the Agilent RNA 6000 Nano Kit. Note that the machine cannot assign an RNA integrity number, as *Drosophila* 28S ribosomal RNA breaks into two similar-sized fragments that migrate close to the 18S rRNA band<sup>27</sup>. Visually ensure that only these two main rRNA bands are visible with early no background bands from degraded RNA. (Fig. 1c)

## ? TROUBLESHOOTING

**33** Heat ~300-500 ng of total RNA from step 31 to 65 °C to disrupt secondary structures; place the tubes on ice.

**34** To enrich for mRNA, aliquot 50 µL of well-suspended Dynabeads oligo(dT)<sub>25</sub> into a fresh RNase-free, low-binding tube.

**35** Wash beads twice with 50 µL binding buffer. After each washing, place the tube containing the magnetic beads onto the appropriate magnetic rack until all beads have migrated to the wall of the tube and the supernatant can be removed.

**36** Resuspend the beads in 25 µL of binding buffer, add the 25 µL of total RNA from step 33 and rotate the tube for 5 min at room temperature. All of the mRNA will be bound to the oligo(dT)-coated beads.

**CRITICAL** Owing to the small volume and high surface tension, flick the tube with your finger once every minute to make sure that the solution is mixed well.

**37** Remove the supernatant and wash the beads twice with 50 µL washing buffer B (included in the Dynabeads purification kit).

**38** Remove the washing buffer B and elute the mRNA by adding 10 µL 10 mM Tris-HCl and heating to 80 °C for 2 min.

**39** Separate the beads with the magnetic rack and transfer the supernatant containing the poly(A)<sup>+</sup>-enriched RNA into a fresh RNase-free, low-binding tube containing 40 µL of binding buffer; heat to 65 °C for 5 min, and then place it on ice. Keep the first tubes with the used beads for a second round of purification.

**40** Wash the used beads twice with 50 µL of washing buffer B.

**41** Add the poly(A)<sup>+</sup>-enriched RNA solution from step 39 (50 µl) to the beads from step 40 and incubate the mixture again at room temperature for 5 min while rotating the tube.

**42** Remove the supernatant and wash the beads twice with 50 µL of washing buffer B.

**43** Remove the washing buffer B and elute the mRNA from the beads by adding 10 µL 10 mM Tris-HCl and incubating it at 80 °C for 2 min.

**44** Separate the beads with the magnetic rack and transfer the supernatant into a fresh RNase-free, low-binding tube.

**CRITICAL** Check the depletion of rRNA from the poly(A)<sup>+</sup>-enriched RNA by qPCR with rRNA-specific primer pairs.

## ? TROUBLESHOOTING

### BOX 1

#### VERIFICATION OF rRNA DEPLETION BY qPCR

Timing 5h

1. Take a 1/10 volume of the poly(A)<sup>+</sup>-enriched RNA, as well as an untreated total RNA sample, and generate first-strand cDNA using SuperScript III with random hexamer primers according to the manufacturer's instructions.

2. Dilute the cDNA at least tenfold so as not to inhibit the subsequent qPCR reaction.
3. Set up the qPCR reaction using SYBR green according to the manufacturer's instructions with the following *Drosophila* rRNA primers and actin 5C primers as a housekeeping gene control.

rRNA	Forward primer	Reverse primer
18S rRNA	TAGACCGAGAGGTCCGGGTA	CAAAGGCAGGGACGTAATCAA
5.8S rRNA	CGATGAAGAACGCAGCAAACCTG	CATGGACTGCGATATGCGTTCA
28S rRNA left	CCTGATGGAAGACCGAAACAGT	CTGAGGGAAACTTCGGAAGGAA
28S rRNA right	GAGTTTGACTGGGGCGGTAC	GCTTTTGCTGTCCCTGTGTGTA
5S rRNA	ACGACCATAACCAGCTGAATAC	GCGGTCCCCATCTAAGTAC
Actin 5C	CCCCATTGAGCACGGTATCG	CACGCAGCTCATTGTAGAAGGT

4. We routinely use the following one-step PCR protocol:

1 × 95 °C 3 min

40 × 95 °C 10 s

62 °C 1 min (plate read)

At the end of this PCR protocol, we add the following steps to generate a melting curve, which allows us to evaluate the quality of the PCR products, as primer dimers and other PCR artifacts have a lower melting temperature:

1 × 95 °C 10 s

62 °C 1 min

62 °C, inclines of 0.5 °C up to 95 °C 5 s (plate read after each incline)

5. Normalize the data using actin 5C expression and calculate the fold change of rRNA depletion by comparing it with the rRNA expression in the untreated sample.

6. Expect at least an 80% reduction of rRNA concentration in the mRNA-enriched samples. With high sequencing depth, low levels of remaining rRNA will still allow for sufficient sequencing of large numbers of cDNA reads.

**45** For mRNA fragmentation, add 10 µL of fragmentation buffer and 30 µL of DEPC-treated water to the purified mRNA from step 44. After incubation at 94 °C for exactly 2.5 min, precipitate RNA with 5 µL (1/10 volume) of 3 M sodium acetate, 125 µL (2.5 volume) of 100 % (vol/vol) ethanol and 1 µL of glycogen overnight at -20 °C.

**46** Pellet the fragmented mRNA by centrifugation at maximum speed for 1 h at 4 °C and wash it once with 500 µL of ice-cold 80% (vol/vol) ethanol and once with 100% (vol/vol) ethanol. Air-dry the pellet for 10 min and dissolve it in 10 µL of DEPC-treated water. Analyze the fragment sizes on an Agilent Bioanalyzer using the Agilent RNA 6000 pico kit. The peak of fragment sizes should range from 200 to 300 nt..

**CRITICAL** Do not let the RNA air-dry for too long, because solubility decreases with increased drying time.

## ? TROUBLESHOOTING

**47** For first-strand cDNA synthesis, add 1 µL of random primers, 1.5 µL of dNTPs and 3.5 µL of H<sub>2</sub>O; heat up the mixture to 65 °C for 5 min and cool it on ice for at least 1 min.

**48** Add 6  $\mu\text{L}$  of 5 $\times$  first-strand buffer, 3  $\mu\text{L}$  of 1M DTT, 1.5  $\mu\text{L}$  of RNaseOUT, 1.5  $\mu\text{L}$  of Superscript II enzyme and 4  $\mu\text{L}$  of  $\text{H}_2\text{O}$ ; incubate the mixture for 5 min at 25  $^\circ\text{C}$  and for 50 min 42  $^\circ\text{C}$ , and then inactivate the enzyme at 70  $^\circ\text{C}$  for 15 min.

**49** Precipitate the first-strand cDNA with 3  $\mu\text{L}$  of NaCl (4 M) and 90  $\mu\text{L}$  of 96 % (vol/vol) ethanol at  $-20^\circ\text{C}$  for at least 2 h or overnight. After centrifugation at maximum speed at 4  $^\circ\text{C}$  for 25 min, remove the ethanol and wash once with 80 % (vol/vol) ethanol. Air-dry the pellet and dissolve it in 54  $\mu\text{L}$  of  $\text{H}_2\text{O}$ .

**PAUSE POINT** First-strand cDNA samples can be stored at  $-80^\circ\text{C}$  for at least 1 year. **50** For second-strand cDNA synthesis, add 20  $\mu\text{L}$  of 5 $\times$  second-strand buffer, 1  $\mu\text{L}$  of random primers, 0.2  $\mu\text{L}$  of dATP, 0.2  $\mu\text{L}$  of dCTP, 0.2  $\mu\text{L}$  of dGTP, 0.2  $\mu\text{L}$  of dUTP, 2.66  $\mu\text{L}$  of DNA polymerase I, 0.66  $\mu\text{L}$  *E. coli* DNA ligase and 0.66  $\mu\text{L}$  RNase H to the first-strand cDNA; incubate the mixture at 16  $^\circ\text{C}$  for 2 h.

**CRITICAL** To be able to obtain strand specificity, second-strand cDNA is labeled with dUTP instead of dTTP.

**51** Purify the double-stranded cDNA using the MinElute reaction cleanup kit, and then quantify it using the Quant-iT Picogreen dsDNA assay in a NanoDrop 3300 fluorospectrophotometer.

#### Library preparation Timing 11 d

**52** Prepare the library for deep sequencing using a protocol from Illumina and the NEBNext DNA sample prep reagent kit. Digest dUTP-containing second-strand cDNA after size selection of adapter-ligated libraries but before PCR amplification of the adapter-modified fragments.

**53** Perform 76-base paired-end sequencing GAIIIX or HiSeq2000 machines.

## ? TROUBLESHOOTING

Troubleshooting advice can be found in Table 1.

**TIMING**—Steps 1-18, dissociation of larval brains: 2h 15 min

Steps 19-29, sorting of NBs and neurons by FACS: ~2 h

Steps 30-51, generation of cell type-specific double-stranded cDNA for Solexa-sequencing library preparation: 4 d (day 1: RNA isolation, overnight; day 2: mRNA enrichment and fragmentation of mRNA; day 3: first-strand cDNA synthesis; day 4: second-strand cDNA synthesis and purification of double-stranded cDNA)

Steps 52 and 53, library preparation: 11 d (day 1: end repair and adapter ligation; day 2: size selection of libraries, UDGase treatment, PCR enrichment of fragments and validation of libraries on a Bioanalyzer; days 3-11: sequencing)

Box 1, verification of rRNA depletion by qPCR: 5 h

## ANTICIPATED RESULTS

### Dissociation of larval brains (Steps 1-18)

The successful generation of a primary cell suspension is crucial for the procedure. It depends on a finely tuned interplay of the dissection procedure, time of enzymatic treatment



and mechanical disruption of the tissue. Larval dissection needs to be practiced, because a shorter dissection time increases the cell survival rate and helps preserve *in vivo* gene expression patterns. Depending on the specificity of the fluorescent labeling of the cells of interest, the precision of dissection may vary. In our case, *ase-Gal4* is also expressed in imaginal discs, and therefore we usually remove all attached discs and residual parts of the gut. We routinely monitor the quality of preparations by cell type-specific antibody staining and live imaging on a spinning disc microscope, monitoring several rounds of asymmetric divisions of both NBs and their direct progeny, the GMC<sup>16</sup>.

### Sorting of NBs and neurons by FACS (Steps 19-29)

Depending on the downstream application, we suggest adapting the duration of FACS sorting. If cells are used for live imaging or immunohistochemistry staining, we sort ~7,000 NBs, which should not take longer than 20-30 min. We directly sort the cells into complete Schneider's medium on poly-d-lysine-coated cell culture dishes and let them settle for 1-1.5 h. For transcriptome or qPCR analysis, we sort NBs directly into reaction tubes containing TRIzol LS reagent.

### Quality assessment of cell type-specific deep-sequencing data (Step 53)

After obtaining cell type-specific deep-sequencing data, the reads should be checked with FastQC (Babraham Bioinformatics, <http://www.bioinformatics.babraham.ac.uk/>), which is an important tool for quickly assessing the quality of the raw sequencing data. FastQC checks for general biases, for example, in sequence quality or sequence content per base. To estimate the probability of incorrect base calling, the PHRED score can be used. This score should be above 30, which would indicate a probability of 1 in 1,000 that a base is assigned incorrectly.

It is possible that the quality of the sequence reads is good, but that the sequencing depth is insufficient, and therefore the information content is inadequate. One reason could be incorrect fragment size distribution, which is assessed by aligning the fragments uniquely to the transcriptome. If fragments are too short, it is nucleotides from the adapters that are sequenced. In case of paired-end sequencing, too short fragments result in sequencing the same part of the fragment twice.

Another reason for reduced mRNA-sequencing depth could be contamination with, and thus sequencing of, RNA species other than poly(A) + RNA. This can also affect downstream analyses, as computing of large data sets is time- and resource-consuming. To assess rRNA contamination, the sequencing reads are aligned to all known rRNA species (using RefSeq), with the fraction of rRNA reads being a good indicator for the quality of the mRNA purification. Thus, bioinformatics analyses should preferably be done on reads that align only to mRNA sequences, with all other non-poly(A)<sup>+</sup>-containing RNA species being masked.

Finally, we also recommend manual inspection of the data in a genome browser. This helps visualize alternatively spliced genes, expression levels of transcripts and their different isoforms, genomic or intronic contamination or lack of strandedness.

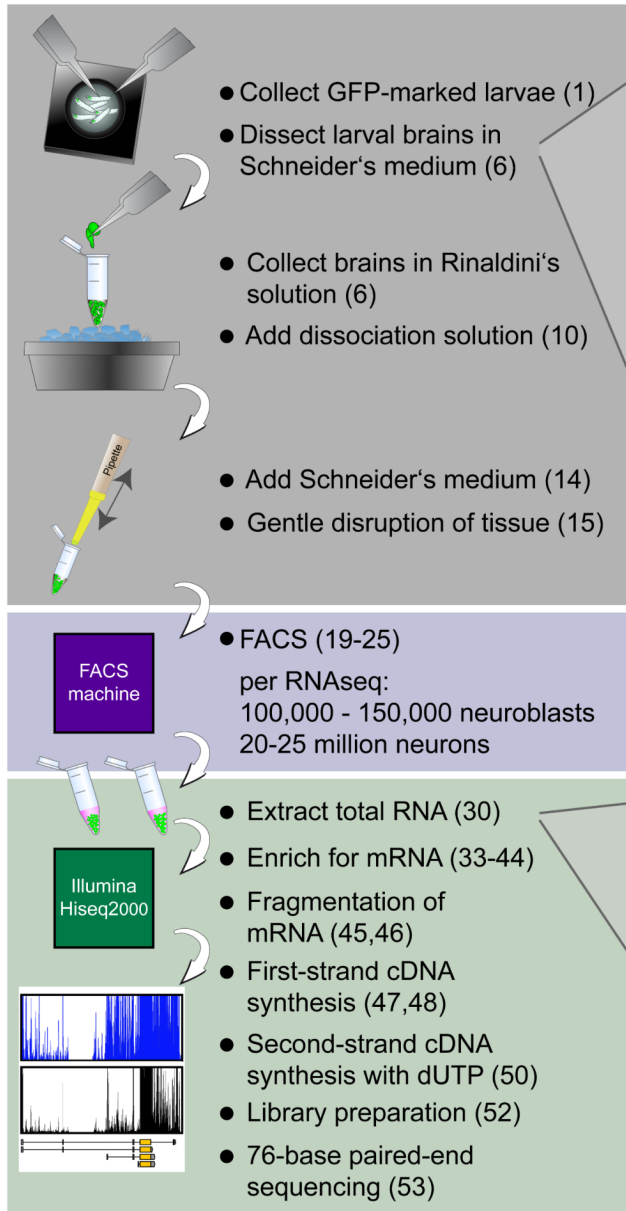
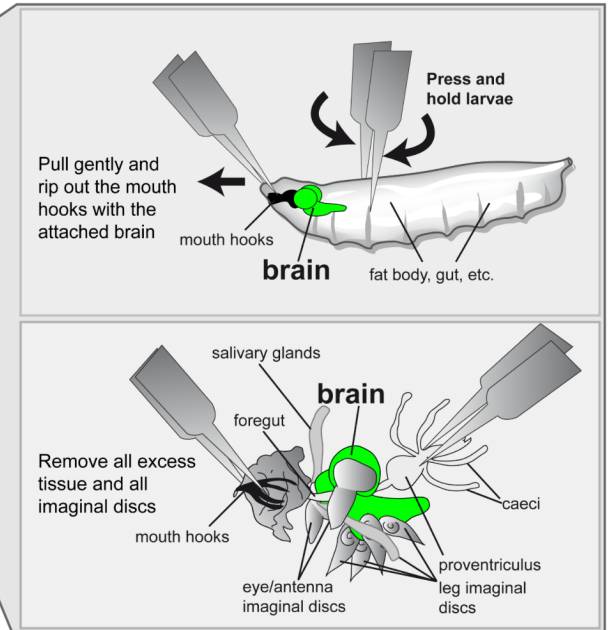
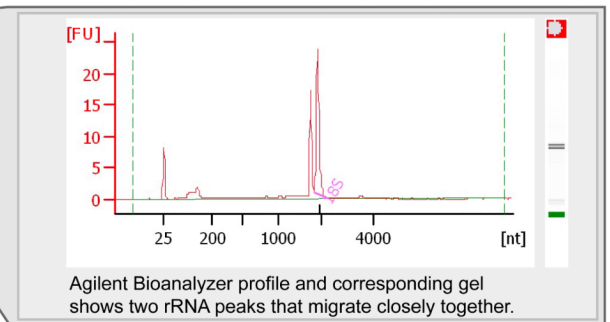
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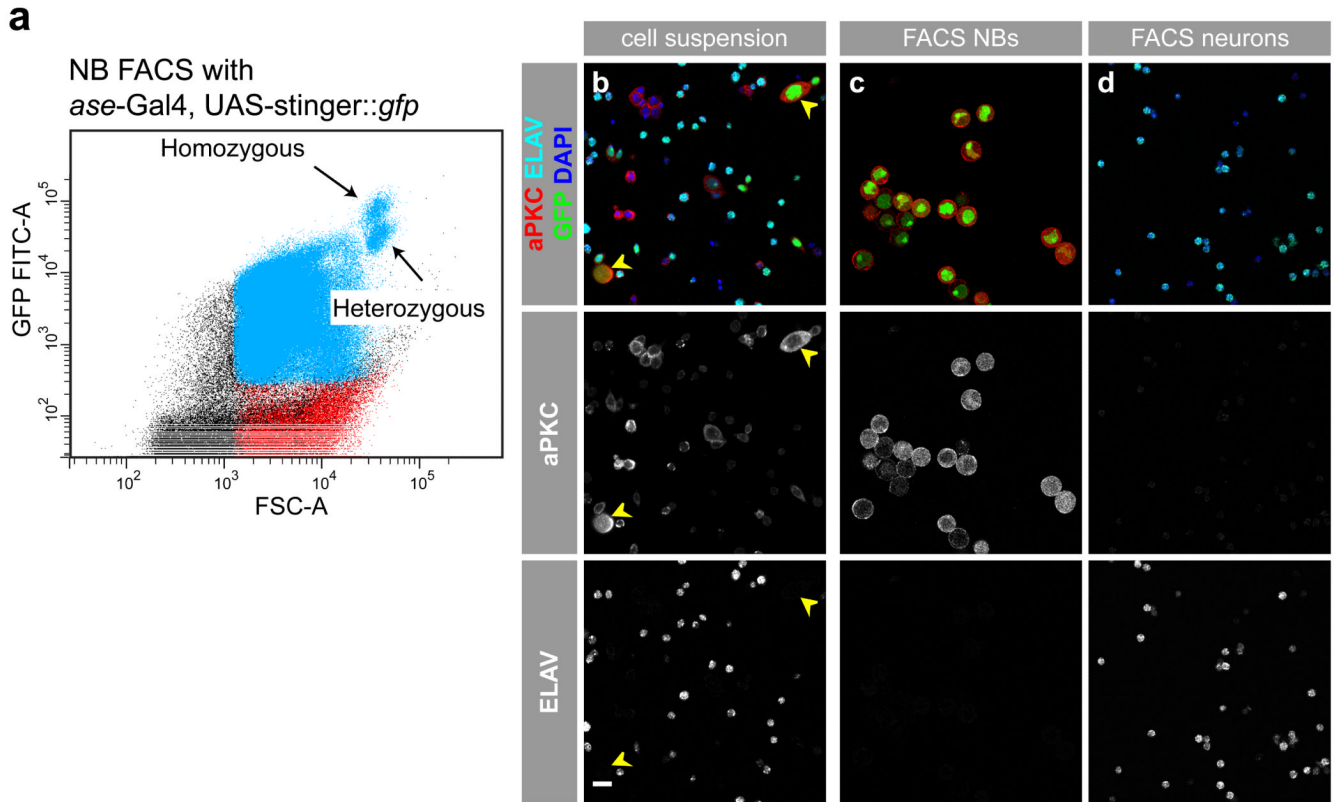
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**a. Overview of the FACS protocol****b. Schematic of brain dissection****c. Drosophila RNA quality****Figure 1.**

Schematic overview of the FACS purification protocol for transcriptome analysis of larval neuroblasts (NBs) and neurons, and dissection of larval brains. (a–c) Larvae of the appropriate developmental stage expressing GFP under the control of a neuroblast-specific Gal4 line are collected. Larval brains are dissected in Schneider's medium (a,b), and collected in ice-cold Rinaldini's solution. The tissue is enzymatically digested and gently disrupted by pipetting. The resulting cell suspension is subjected to FACS to separate NBs and neurons. The sorted cells are kept in TRIzol LS reagent to preserve the RNA. After RNA extraction, RNA quality is measured on an Agilent Bioanalyzer (c), the samples undergo enrichment for poly(A) + RNA, are fragmented, and then first- and second-strand cDNA syntheses are performed. Finally, after library preparation, samples are subjected to transcriptome analysis by paired-end mRNA sequencing. Corresponding PROCEDURE

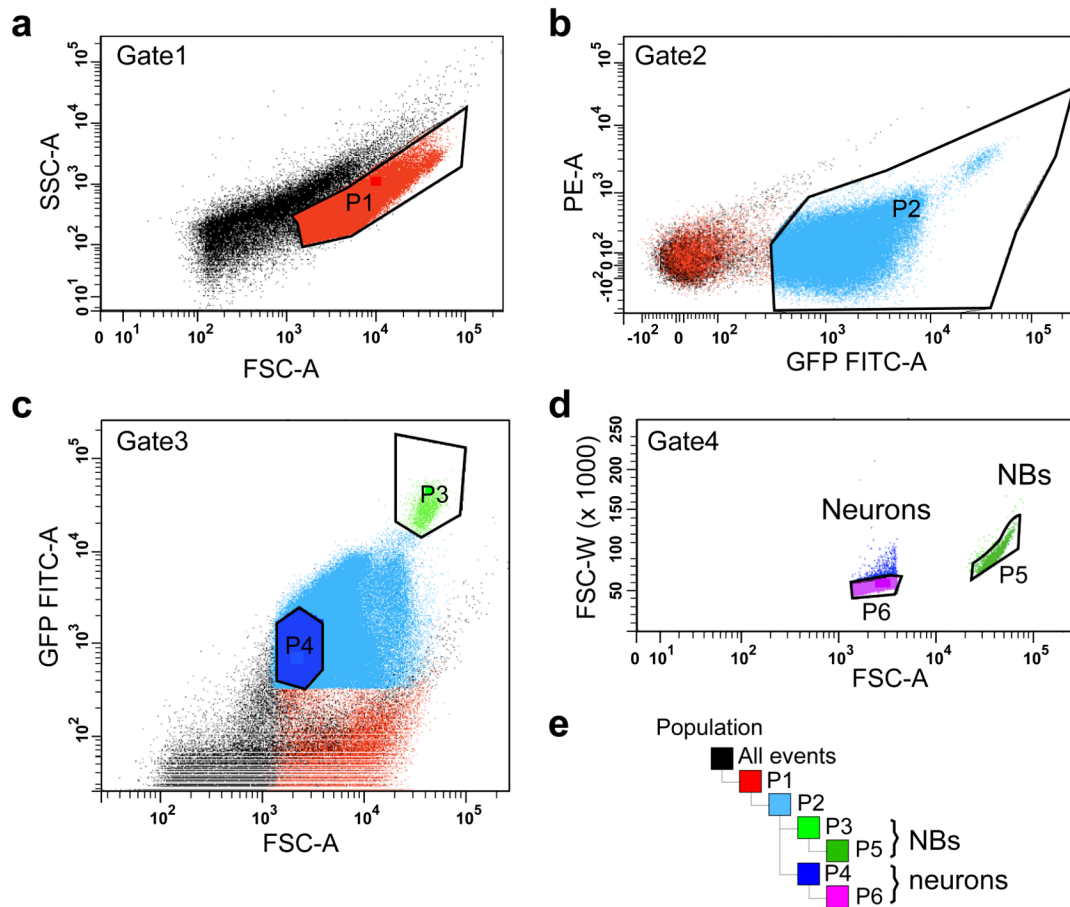
steps are indicated in parentheses. **(b)** For the dissection of larval brains, hold the larva with two forceps, one at the anterior end and one in the middle of the body (upper panel). Gently rip out the mouth hooks with the attached brain, imaginal discs, salivary glands and gut (lower panel). Remove all excess tissue and transfer the isolated brain with forceps into the tube with Rinaldini's solution on ice. **(c)** Assurance of quality and quantity of the extracted RNA using an Agilent Bioanalyzer with the Agilent RNA 6000 nano kit. A typical *Drosophila* RNA profile is shown with its corresponding gel. Note that 28S rRNA is not visible, as this rRNA breaks into two similar-sized fragments that migrate close to the 18S rRNA band<sup>27</sup>. It is important that only the two main rRNA bands are visible, with no background bands from degraded RNA.



**Figure 2.**

Pure populations of NBs and neurons can be separated by FACS. **(a)** By using a Gal4 fly strain harboring both heterozygous and homozygous Gal4 and UAS-Stinger::*gfp*, we were able to separate two NB populations when plotting the strength of the GFP signal (GFP FITC-A) to the size of the cells (FSC-A). Both populations are of equal cell size but are different in the strength of the GFP signal, as heterozygous insertions result in weaker GFP signals than homozygous insertions. **(b-d)** Confocal images of representative cell cultures before and after sorting by FACS. **(b)** Images of a primary (i.e., unsorted) cell suspension stained with the NBs marker aPKC in red, the neuronal marker ELAV in cyan, the DNA marker DAPI in blue and the Stinger::GFP driven by *asense-Gal4* in green. Large NBs in a primary cell culture are marked with yellow arrowheads and show aPKC expression **(b, middle)** but no ELAV **(b, bottom)** expression. **(c)** Sorted NBs are positive for aPKC **(c, middle)** but not for ELAV **(c, bottom)**. **(d)** Sorted neurons are negative for the NB marker aPKC **(d, middle)** but positive for the neuronal marker ELAV **(d, bottom)**. Scale bar, 10  $\mu\text{m}$ .



FACS gating strategy for sorting of NBs and neurons with *ase-Gal4, UAS-stinger::gfp***Figure 3.**

Representative flow cytometry data to illustrate the gating strategy for FACS purification of larval NBs and neurons. **(a)** To discriminate between healthy and damaged or dying cells, side scatter (SSC-A, log) and forward scatter (FSC-A, log) are plotted. Higher SSC-A signals indicate increased granularity and therefore likely dying cells. Thus, one should define the sorting gate as the population with the lower SSC-A signal (P1). **(b)** To gate for GFP-positive cells, plot the signal from the excitation of cells with the 488-nm laser (GFP FITC-A, log) against the signal from the PE laser (PE-A, log). The population with the higher GFP FITC-A signal is chosen for sorting (P2). **(c)** Plotting the GFP signal against FSC-A reveals a population of bright and large cells (NBs, P3), and smaller cells with a weaker signal (neurons, P4). **(d)** Exclude cell clusters by sorting only events with a low FSC width (FSC-W) versus area (FSC-A) signal (P5, NBs; P6, neurons). **(e)** Gating hierarchy.

**Table 1**  
**Troubleshooting table**

Step	Problem	Possible reason	Solution
15	Tissue pieces in cell suspension	Insufficient enzymatic digestion	Increase enzyme concentration or incubation time. Do not exceed 2 mg mL <sup>-1</sup> of final concentration of enzyme, or 1.5 h of incubation
			Prepare fresh enzyme stocks
			Do not freeze and thaw enzymes more than twice
			Try different enzyme combination (e.g., trypsin or elastase)
15	Cells die after dissociation	Tissue is damaged during dissection procedure	Reduce the period of dissection to maximum 45 min
		Enzymatic treatment is damaging tissue	Reduce time of enzymatic treatment (e.g. 30 min)
			Reduce the concentration of enzymes, or use either papain or collagenase I only
			Reduce incubation temperature (i.e. incubate at room temperature)
		Mechanical disruption is damaging tissue	Try to be as gentle as possible (avoid foaming); do not use a pipette tip smaller than 200 µL and do not pipette the tissue up and down more than 30 times
26	Fewer cells than expected during FACS (expected yield 50-70% of total NB numbers)	Insufficient enzymatic digestion/ cells die after dissociation	See troubleshooting guidance for step 15
		Incorrect FACS instrument setting	Reduce pressure to a low pressure, maximum of 20 p.s.i.
			Use 100 µm nozzle
32	Insufficient amounts of RNA isolated; at least 300-500 ng of total RNA is required per RNA-seq analysis	Not enough material was sorted during FACS	Increase number of dissected larva for each FACS round Increase number of FACS rounds to collect more cells to isolate RNA from.
32	Low RNA yield, denatured RNA, or DNA contamination in RNA Prep	Multiple steps during RNA isolation can be causal	We suggest following the advice given in the TRIzol LS manual
			Adapt the RNA isolation protocol to a small amount of material or change the method of RNA isolation. For example, use the RNeasy micro kit from Qiagen (cat. no. 74004), which includes an optional on column DNase digest step (RNase-free DNase set, Qiagen cat. no. 79254)

Step	Problem	Possible reason	Solution
44	Insufficient mRNA enrichment	mRNA isolation protocol does not work well	Do not exceed the manufacturer's recommended amount of total RNA for enrichment of mRNA with Dynabeads, but also do not use <100 ng of total RNA for mRNA purification
			Chose a different protocol to enrich for mRNA (for example the RiboZero Magnetic Kit from Epicentre (cat. no. MRZH116))
46	Wrong mRNA fragment sizes	Incubation with fragmentation buffer	Incubation with fragmentation buffer is either too long or too short. Set up a time-course experiment with different incubation times at 94 °C to optimize fragment size, and check the sizes on an Agilent Bioanalyzer with the Agilent RNA 6000 Pico Kit