High-throughput fluorescence-based isolation of live *C. elegans* larvae

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Published online 19 July 2012; doi:10.1038/nprot.2012.084

For the nematode *Caenorhabditis elegans*, automated selection of animals of specific genotypes from a mixed pool has become essential for genetic interaction or chemical screens. To date, such selection has been accomplished using specialized instruments. However, access to such dedicated equipment is not common. Here we describe live animal fluorescence-activated cell sorting (laFACS), a protocol for automatic selection of live first larval stage (L1) animals using a standard FACS system. We show that FACS can be used for the precise identification of GFP-expressing and non-GFP-expressing subpopulations and can accomplish high-speed sorting of live animals. We have routinely collected 100,000 or more homozygotes from a mixed starting population within 2 h, and with greater than 99% purity. The sorted animals continue to develop normally, making this protocol ideally suited for the isolation of terminal mutants for use in genetic interaction or chemical genetic screens.

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INTRODUCTION

C. elegans is an important model system for studying the fundamental genetic mechanisms underlying developmental and behavioral biology[1]. Among its many advantages as an animal model system is the fact that it can be easily grown to very large populations, and there is a vast infrastructure supporting this system that includes mutants affecting more than half of its genes (ref. 2 and D.G. Moerman and R. Waterston, personal communication), genome-scale RNAi reagents[3–5] and an extensive collection of GFP-marked strains[6]. These features make this model system well suited for large-scale studies[7–9].

It is estimated that 30% of the genes in *C. elegans* have a terminal phenotype such as embryonic lethality, maternal-effect lethality or sterility[10]. Although some terminal phenotypes (e.g., sterility) are viable, they do not produce viable offspring, and thus it is impossible to grow them as a pure population of homozygotes. Balancer chromosomes are key tools for preventing the loss of terminal alleles in a segregating population. In a balanced heterozygote, the wild-type allele is carried by a balancer chromosome, which bears chromosomal aberrations such as inversions or translocations that prevent recombination events. Some balancers carry recessive lethal alleles, causing homozygous balancer embryos to be inviable, thus preventing the proliferation of animals that have lost the recessive terminal allele. Many balancers contain GFP transgenes (**Table 1**), enabling the easy identification of heterozygous animals. As the balancer chromosome resists recombination, the GFP transgene is always associated with the wild-type allele, and thus homozygous mutant individuals can easily be identified by a lack of GFP expression (**Fig. 1**). However, manual isolation of homozygous individuals from a heterogeneous population is too labor intensive to be practicable for large-scale analyses.

The microscopic size of *C. elegans* facilitates the use of microfluidic and flow cytometric systems to analyze or sort and collect individual animals with specific optical properties. The COPAS (complex object parametric analysis and sorting) biosorter is an expert apparatus developed especially for the optical analysis and sorting of nematodes[11]. This specialized sorter is well suited to the fluorescence-based isolations of subpopulations in a heterogeneous batch[11] and is capable of much more, including stage-specific sorting and analysis of fluorescence within specific regions of the animals[11–13]. However, these dedicated machines are not widely available, and therefore accessibility to high-throughput techniques for the isolation of sterile or lethal mutants is restricted.

Recently, it was reported that a fluorescence-activated cell sorter, a more prevalent piece of equipment, can be used for automated sorting of live *C. elegans* larvae[14] and fixed embryos[15]. We have successfully used this technique, laFACS, to isolate over 100,000 larvae for RNAi genetic interaction screening of a maternal lethal mutant[14]. We used L1-stage larvae because their small size (about 250 μm long and 15 μm wide) allows them to run through the FACS fluidics system, and because starvation arrests growth at this stage and enables easy accumulation of developmentally synchronized animals. We used the GFP-negative balancer signature to rapidly identify homozygous mutant larvae in a mixed population and sort them to over 99% purity.

Such isolated larvae can be used for genetic interaction screens, chemical genetic screens or any other application in which large numbers of animals of a particular lethal or sterile genotype are required. There could conceivably be additional applications of laFACS. For example, it could be used to screen large populations for reporter gene activation. However, FACS machines and flow cytometers are designed to quantify fluorescence in relatively small particles, and they will not give a highly accurate quantitative readout of fluorescence in large objects such as *C. elegans* larvae. Unlike The COPAS biosorter, which is specifically designed to sort worms of all stages on the basis of size and/or degree of fluorescence, we have used FACS only to sort L1 larvae on the basis of the presence or absence of fluorescence. Case-by-case trials will have to be undertaken to investigate whether laFACS is suitable for alternative applications. Furthermore, because of size restrictions, the use of laFACS is limited to L1-stage larvae (and embryos). Nonetheless,

TABLE 1 | GFP-marked balancers in *C. elegans*.

Name of balancer[a]	Homozygous lethal?	Region balanced	Type of balancer	GFP marker
nT1[b] *[qIs51]*	Yes	Right end of chromosome IV through *unc-17*, left end of chromosome V through *unc-76*	Reciprocal translocation[c]	*myo2::GFP, pes-10::GFP, F2B7.9::GFP*
hT2[d,e] *[qIs48]*	Yes	Chromosome I from left end through *unc-101*, chromosome III from right end through *dpy-17*	Reciprocal translocation[c]	*myo2::GFP, pes-10::GFP, ges-1::GFP*
mIn1[f] *[mIs14]*	No	Chromosome II between *lin-31* and *rol-1*	Inversion	*myo2::GFP, pes-10::GFP, F2B7.9::GFP*
qC1 [qIs26][g]	Yes	Left portion chromosome III between *tra-1* and *dpy-1*	Unknown	*lag-2::GFP*
eT1[nIs267]	No	Chromosome V left end through *unc-23*, chromosome III right end through *unc-36*	Reciprocal translocation[c]	*myo-2::GFP*
mIs10[h]	No	Chromosome V between *unc-60* and *dpy-11*	Insertion	*myo2::GFP, pes-10::GFP, F2B7.9::GFP*
mIs11	No	Chromosome IV, unknown interval near *dpy-20*. This has been used successfully to balance deletions in *cyb-1*, *syn-4 tag-316*, *htp-1*, *mep-1* and *tag-137*	Insertion	*myo2::GFP, pes-10::GFP, F2B7.9::GFP*
mIs12	No	Chromosome II, unknown interval near *unc-4*	Insertion	*myo2::GFP, pes-10::GFP, F2B7.9::GFP*
mIs13	No	Chromosome I, unknown interval near *unc-54*. This has been used successfully to balance deletions in *tag-115*, *kin-1*, *npp-4*, *ero-1* and *bbs-1*	Insertion	*myo2::GFP, pes-10::GFP, F2B7.9::GFP*
okIs57	No	Chromosome X, unknown interval near *unc-3*	Insertion	*myo2::GFP, pes-10::GFP, F2B7.9::GFP*
okIs59	No	Chromosome I, unknown interval between *dpy-5* and *unc-13*. This has been used to balance deletions in *air-2*, *dao-5*, *npp-7*, *chn-1*, *tag-83* and *gly-2*	Insertion	*myo2::GFP, pes-10::GFP, F2B7.9::GFP*

[a]Information on balancers comes from WS228 (refs. 19 and 20) and author observations (M.L.E., unpublished observations). [b]*nT1[qIs51]* carries an uncharacterized lesion in *daf-15*. Some mutations lying within the balanced extents of *nT1* are unstable over *nT1[qIs51]*. [c]Reciprocal translocation balancers produce aneuploid inviable progeny[20], and this reduces the expected yield of viable homozygotes. [d]The *qIs48* element is sometimes lost by apparent rare recombination, leaving behind viable *hT2* homozygotes marked with the original *bli-4* mutation. [e]Some mutations lying within the balanced extents of *hT2* are unstable over *hT2[qIs48]*. [f]Some lethal alleles balanced by the original non-GFP *mIn1[dpy-10]* and lying near the left breakpoint are not balanced by *mIn1[mIs14 dpy-10]*. [g]*qIs26* also carries the dominant *rol-6* allele *su1006*. [h]Balancing activity of *mIs10* should be confirmed.

the ability to use a FACS instrument to sort live *C. elegans* greatly increases the accessibility and feasibility of high-throughput analyses in this model organism.

Experimental design

Strain construction. In the example presented here, we used laFACS to collect a pure population of maternal-effect lethal (*mel-28*, refs. 16,17) homozygotes that we then subjected to an RNAi-based genetic interaction screen[14]. First, we generated a GFP-balanced *mel-28* strain called PF405. In this strain, the *mel-28* allele and a *cis*-linked *unc-32* allele were carried on an otherwise wild-type chromosome, and were balanced by the *qC1* balancer containing the *qIs26* insertion (**Fig. 1**). The *qIs26* insertion includes both a *lag-2::GFP* reporter and the dominant *rol-6* allele *su1006* (ref. 18), and gives rise to recessive lethality. PF405 heterozygotes produce homozygous *qC1[qIs26]* animals that die as embryos, heterozygous *mel-28 unc-32/qC1[qIs26]* animals that have a Rol-6 phenotype and express GFP in the distal tip cells of the gonads and *mel-28 unc-32* homozygotes that are uncoordinated, do not express GFP and produce only dead eggs (**Figs. 1** and **2**).

Proper strain construction is crucial for the successful execution of laFACS. We used a balancer that carries, in addition to the

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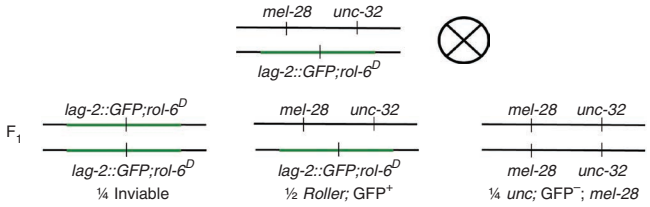

Figure 1 | Strain PF405 genetics. The *qC1*-balanced region of chromosome is shown in green. We used a version of *qC1* with the *qIs26* insertion that has the *lag-2::GFP* transgene and the dominant *rol-6*^{D} allele *su1006*. The *qIs26* insertion site created a recessive lethal allele such that *qC1[qIs26]* homozygotes are inviable. Heterozygotes have the Rol-6 phenotype and express GFP. Animals that have lost the balancer do not express GFP, are uncoordinated and are *mel-28* homozygotes (and thus produce only dead embryos).

GFP transgene, a dominant visible marker (*rol-6(su1006)*) and a recessive lethal lesion. The visible marker enables distinction of heterozygotes without the need for an epifluorescence microscope. If there are no appropriate balancers with this feature, it is recommended that the terminal allele of interest be kept in *cis* with a recessive visible marker. In our PF405 strain, the *mel-28* chromosome also carries an *unc-32* allele; thus, *mel-28* homozygotes from this strain are always uncoordinated (**Figs. 1** and **2f**).

The use of a balancer that confers recessive lethality is preferable, but not required. To grow worms for this protocol, we chose heterozygous animals to start the initial stocks, and then expanded them for two generations without specifically picking heterozygotes. Thus the L1s we sorted were the F_2 of the heterozygotes initially picked. As our balancer was homozygous lethal and *mel-28* homozygotes do not produce larvae, all of these L1s arose from a heterozygous carrier, and thus one out of three are expected to be *mel-28* homozygotes (**Fig. 1**). When using homozygous viable balancers, with each generation there will be balancer homozygotes that have lost the allele of interest. This lowers the expected yield of desired animals homozygous for the recessive terminal allele. However, because this protocol allows for the sorting of hundreds of thousands of animals, it should still be possible to use this protocol to isolate substantial numbers of homozygotes using a balancer that is not homozygous lethal.

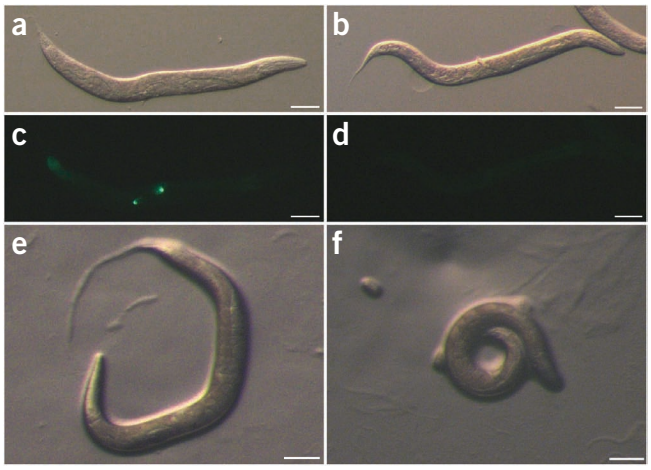

Figure 2 | Strain PF405 phenotypes. (**a**–**f**) Heterozygous and homozygous phenotypes are shown. (**a**,**c**,**e**) Heterozygous *mel-28 unc-32/qC1[qIs26]* adults (Rol-6 phenotype). (**b**,**d**,**f**) Homozygous *mel-28 unc-32* adults from the PF405 strain. Images were captured in bright-field mode (**a**,**b**,**c**,**f**), and with GFP filters (**c**,**d**). (**a**–**d**) Animals were anesthetized with levamisole and immobilized on an agar pad. (**e**,**f**) Characteristic behavior of Rol-6 (**e**) and Unc (**f**) adults on an NGM plate. Scale bars, 100 μm.

Sorting larvae. To prepare the larvae for sorting, we used sodium hypochlorite treatment to isolate embryos from PF405 adults and allowed these to hatch without food so that the animals were arrested at the L1 stage (**Fig. 3**). After filtering the hatched L1s to remove large debris, we used laFACS to isolate GFP-negative homozygous *mel-28* L1 animals from the population. To prime the FACS for larval sorting, we installed a 100-μm nozzle and set up a stream at a lower-than-usual drop-drive frequency (~16 kHz as opposed to ~38 kHz; see **Fig. 4a**) in order to optimize worm viability while passing through the apparatus.

To determine FACS parameters and establish a signature for worms that do not express GFP, we ran wild-type (N2) L1s. We set up a gate in a forward-scatter versus side-scatter plot in order to distinguish worm-sized events from debris. In effect, the larvae were so large that they were off scale (even at the lowest photomultiplier tube (PMT) voltage settings), and this 'worm gate' encompassed only the events with the highest forward-scatter values (**Fig. 4b**). This gate is especially useful when sorting strains that produce a lot of small debris (e.g., remnants of the inviable embryos present in the *mel-28* population (**Figs. 3c** and **4c**)). We evaluated only events that passed the worm gate for green fluorescent emission versus red fluorescent emission (emission around 530 and 610 nm, respectively; **Fig. 4b**,**c**). We adjusted voltage settings such that GFP-negative larvae showed an equal ratio of green to red (auto)fluorescence and could be distinguished from GFP-positive larvae, which showed relatively higher green than red fluorescence (**Fig. 4b**,**c**). We set gates to identify both GFP-positive and GFP-negative populations.

Controls. In going through the steps of this protocol, it is important to regularly inspect the samples microscopically. We looked for larval viability, genotypic purity (as determined by GFP fluorescence as well as Rol and Unc phenotypic characteristics), debris content, and clumping or coagulation. Synchronized and filtered wild-type larvae and a sample of pure GFP-positive larvae can be taken along as a negative and positive control, respectively, for the FACS run. These control samples are especially practical when first setting up the technique.

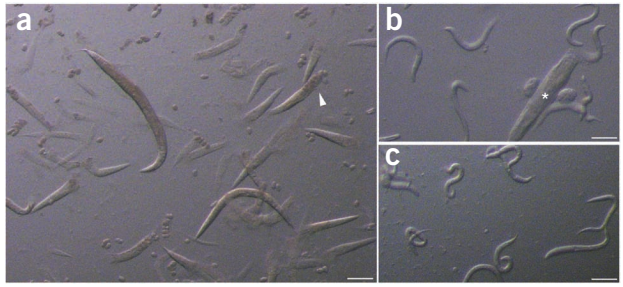

Figure 3 | Preparation of *C. elegans* for laFACS. (**a**) After an ~4 min treatment with sodium hypochlorite and agitation, most adults have burst and freed their embryos (arrowhead). (**b**) After overnight incubation in M9, many L1 larvae have hatched, but large segments of adult corpses (asterisk) remain. (**c**) After filtering, only small amounts of debris and L1 larvae remain. Scale bars, 200 μm (**a**) and 100 μm (**b**,**c**).

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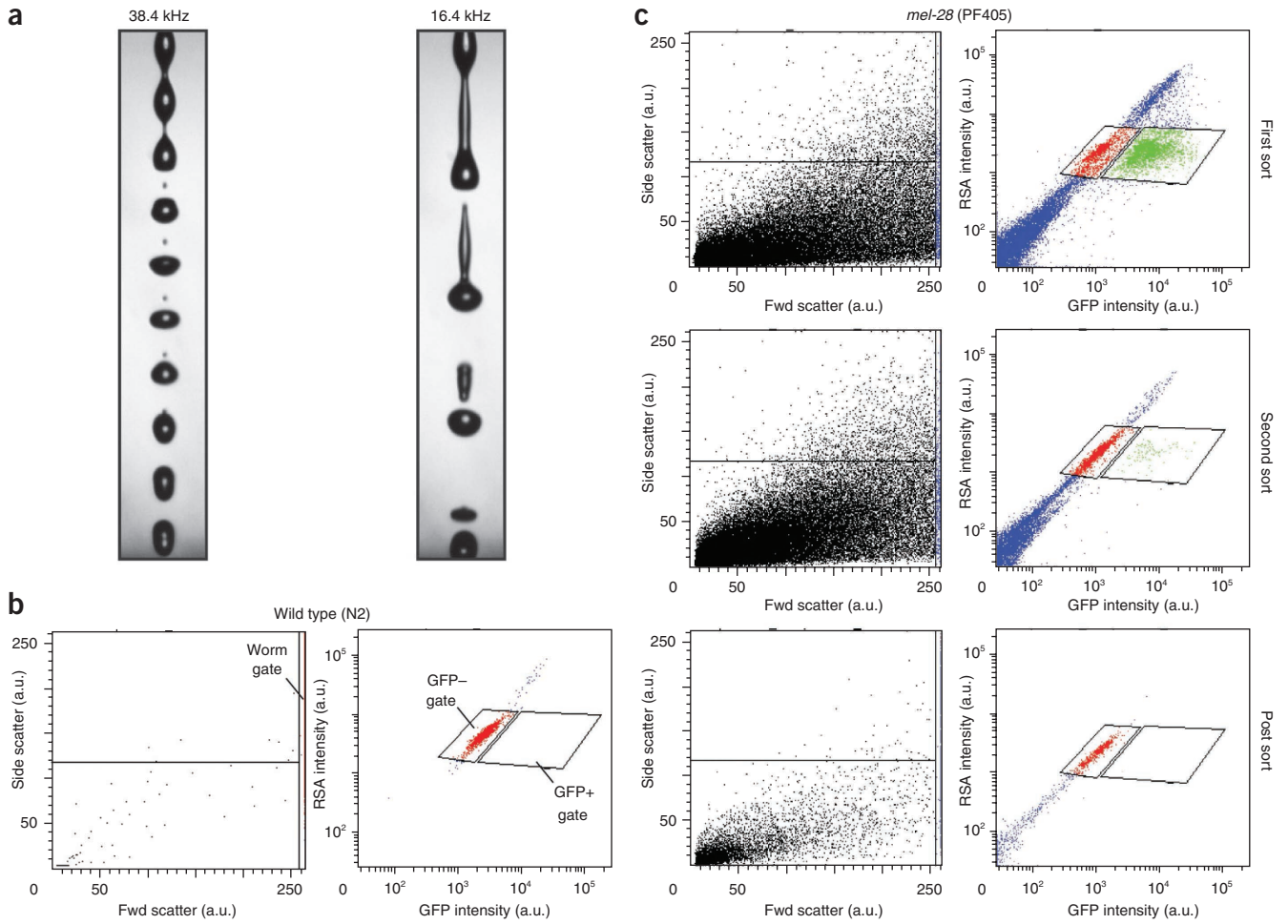

Figure 4 | Sorting *C. elegans* with laFACS. (**a**) Low-frequency drop-drive stream set up on the FACS facilitates larval survival. (**b**) We assessed wild-type (N2) L1 larvae in order to visualize purely GFP-negative larvae. We set a gate to distinguish larvae from debris in the plot of forward (fwd) scatter versus side scatter (worm gate; left), and two gates were set to identify GFP-negative and GFP-positive events in the GFP intensity (488-nm excitation, 530/30-nm emission) versus red spectrum autofluorescence (RSA; 488-nm excitation, 610/20-nm emission) plot (GFP-negative and GFP-positive gates, respectively; right). (**c**) Successive sorts showing the purification of GFP-negative larvae from a PF405 L1 population. Events that pass the worm gate are labeled in blue, events that subsequently pass the GFP-positive gate are labeled in green and the GFP-negative gates are labeled in red. a.u., arbitrary units.

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MATERIALS

REAGENTS

- *C. elegans* strain carrying the allele of interest balanced by a GFP-containing balancer chromosome (GFP balancer strains are available through the *Caenorhabditis* Genetics Center, http://www.cbs.umn.edu/CGC/)
- OP50 strain of *E. coli* (*Caenorhabditis* Genetics Center, http://www.cbs.umn.edu/CGC/)
- Nematode growth medium (NGM; US Biological, cat. no. N1000)
- Tryptone (Fisher Scientific, cat. no. BP1421)
- $CaCl_2$ dihydrate (Fisher Scientific, cat. no. BP510)
- NaCl (Fisher Scientific, cat. no. BP358)
- KCl (Fisher Scientific, cat. no. BP366)
- KH_2PO_4 (Fisher Scientific, cat. no. BP362)
- K_2HPO_4 (Fisher Scientific, cat. no. BP363)
- Na_2HPO_4 heptahydrate (Fisher Scientific, cat. no. BP331)
- $MgSO_4$ heptahydrate (Fisher Scientific, cat. no. BP213)
- NaOH (Fisher Scientific, cat. no. BP359)
- Sucrose (Fisher Scientific, cat. no. BP220)
- Sodium hypochlorite solution (4–6%, wt/vol; we used household bleach, such as Clorox) **! CAUTION** Use proper ventilation when working with sodium hypochlorite.
- Deionized water

EQUIPMENT

- Plates (100 mm; Fisher Scientific, cat. no. 08-757-12)
- Plates (150 mm; Fisher Scientific, cat. no. 08-757-14)
- Cell strainer (40 μm; BD Falcon, cat. no. 352340)
- Conical tubes (50 ml; Fisher Scientific, cat. no. 14-432-22)
- Collection tubes (15 ml; Fisher Scientific, cat. no. 05-527-90)
- FACS instrument equipped with a 488-nm excitation laser and 530/30-nm and 610/20-nm emission filters (BD FACSAria I)
- Epifluorescence dissecting microscope suitable for imaging GFP (such as the Leica MZ16FA)
- Accudrop beads (BD Biosciences, cat. no. 34529)

REAGENT SETUP

PBS, 1× To 800 ml of deionized water, add 8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4 and 0.24 g of KH_2PO_4 and stir until it is dissolved. Adjust the pH to 7.4 with 5 M NaOH or HCl. Bring the volume to 1 liter using deionized water, and then autoclave. The PBS solution (1×) may be stored indefinitely at room temperature (20–25 °C).

Sucrose, 60% (wt/vol) To 250 ml of deionized water, add 600 g of sucrose and stir until most of it is dissolved. Add another 250 ml of deionized water and stir until the sucrose is completely dissolved, and then bring the volume

to 1 liter using deionized water and autoclave. This solution may be stored indefinitely at room temperature, but we recommend storing it at 4 °C so that it is prechilled in preparation for Step 4 of the PROCEDURE.

$CaCl_2$, 1 M To 250 ml of deionized water, add 147.01 g of $CaCl_2$ and stir until most of it is dissolved. Add another 250 ml of deionized water and stir until the $CaCl_2$ is completely dissolved, and then bring the volume to 1 liter using deionized water and autoclave. Allow the solution to cool to room temperature. This solution may be stored indefinitely at room temperature.

$MgSO_4$, 1 M To 250 ml of deionized water, add 246.48 g of $MgSO_4$ and stir until most of it is dissolved. Add another 250 ml of deionized water and stir until the $MgSO_4$ is completely dissolved, and then bring the volume to 1 liter using deionized water and autoclave. Allow to cool to room temperature. This solution may be stored indefinitely at room temperature.

K_2HPO_4, 1 M (dibasic) To 250 ml of deionized water, add 174.18 g of K_2HPO_4 and stir until most of it is dissolved. Add another 250 ml of deionized water and stir until the K_2HPO_4 is completely dissolved, and then bring the volume to 1 liter using deionized water and autoclave. Allow the solution to cool to room temperature. This solution may be stored indefinitely at room temperature.

KH_2PO_4, 1 M (monobasic) To 250 ml of deionized water, add 136.09 g of KH_2PO_4 and stir until most of it is dissolved. Add another 250 ml of deionized water and stir until the KH_2PO_4 is completely dissolved, and then bring the volume to 1 liter using deionized water and autoclave. Allow it to cool to room temperature. This solution may be stored indefinitely at room temperature.

Potassium phosphate buffer, 1 M (pH 6.0) Prepare by mixing 132 ml of prepared 1 M K_2HPO_4 with 868 ml of prepared 1 M KH_2PO_4 in a sterile bottle. This solution may be stored indefinitely at room temperature.

Standard NGM plates, 100 mm For 1 liter of NGM, mix 23.005 g of nematode growth medium in 975 ml of deionized water. Autoclave with a stir bar in the solution. After removal from the autoclave, stir the molten solution on a stir plate until it has cooled to ~55 °C, and then add 24.7 ml of 1 M potassium phosphate solution, 1 ml of 1 M $CaCl_2$ and 1 ml of 1 M $MgSO_4$. After mixing, pour 28 ml into each 100-mm plate. If pouring by hand, aim to obtain approximately 30 plates per liter. Let the plates sit at room temperature for 1–3 d in order to allow the condensation to evaporate. Unseeded NGM plates may be stored tightly sealed in plastic sleeves and kept at 4 °C for months. To seed plates, grow a 50-ml culture of OP50 *E. coli* in LB without antibiotics, using standard sterile microbiological techniques[18]. This culture may be stored at 4 °C for 4 weeks. Add 200 µl of OP50 culture onto each poured NGM plate and let it sit at room temperature overnight. Seeded plates may be stored at 4 °C for 4 weeks.

Superseeded 5× peptone plates, 150 mm For 1 liter of NGM, mix 23.005 g of nematode growth medium and 10 g of tryptone in 975 ml of deionized water. The tryptone will not go into solution completely until autoclaved. Autoclave with a stir bar in the solution. After removal from the autoclave, stir the molten solution on a stir plate until it has cooled to ~55 °C, and then add 24.7 ml of 1 M potassium phosphate solution, 1 ml of 1 M $CaCl_2$ and 1 ml of 1 M $MgSO_4$. Pour ~55 ml per 150 mm plate. If pouring by hand, aim to obtain 18 plates per liter. Let the plates sit at room temperature for 1–3 d in order to allow the condensation to evaporate. Unseeded plates may be stored tightly sealed in plastic sleeves and kept at 4 °C for months. To superseed the plates, prepare a culture of OP50 in LB using standard sterile microbiological techniques[18], and then pellet it and resuspend it in one-tenth the volume of M9 (e.g., a 200-ml solution of OP50 would be pelleted and resuspended into 20 ml of M9). Use a sterile bent glass rod to spread ~1 ml of the concentrated OP50 across each 150-mm 5× peptone plate, ensuring that the bacteria cover the surface area of the plate. Seeded plates may be stored at 4 °C for 4 weeks.

M9 Mix 3.0 g of KH_2PO_4, 6.0 g of Na_2HPO_4 and 5.0 g of NaCl in 1 liter of deionized H_2O. Autoclave the solution and then cool it to room temperature. By using sterile techniques, add 1 ml of sterile 1 M $MgSO_4$ and swirl to mix. Prepared M9 may be stored at room temperature indefinitely, but we recommend storing one aliquot of M9 at 4 °C so that it is prechilled in preparation for Step 4 of the PROCEDURE.

Sodium hypochlorite solution Mix 30 ml of household sodium hypochlorite solution (bleach) with 60 ml of deionized H_2O and 10 ml of 10 N NaOH. Store at room temperature in the dark for no more than 1 month. **! CAUTION** Hypochlorite solutions lose activity over time. Use proper ventilation when working with sodium hypochlorite.

EQUIPMENT SETUP

FACS preparation Clean and sterilize the FACS fluidics system, if required, and use PBS as sheath fluid. Install a 100-µm nozzle and set the sheath pressure at 20 p.s.i. Set up a stable stream with low drop-drive frequency. Standard operating parameters for the FACSAria run FACS drop-drive frequency in the vicinity of 38 kHz when using the 100-µm nozzle, thereby creating a stable droplet break-off point at the correct distance from the nozzle. However, at this frequency, we noted a low recovery rate (± 50%), indicating that a large proportion of the larvae did not survive the sort. A stable break-off point can be obtained at lower frequency (± 16 kHz; **Fig. 4a**). At this lower frequency, it is also possible to set an accurate drop delay and stable side streams (as determined by Accudrop beads). Sorting with this setup led to a greatly improved recovery rate (± 80%). Set the sample agitation function (if available) to ensure that larvae do not settle to the bottom of the sample tube during the sort.

Run a wild-type control consisting of purified, non-GFP L1 larvae (**Fig. 4b**). Adjust the flow rate so as to obtain approximately 100–300 events per second. Prepare a scatter plot analysis of forward scatter area (FSC-A) versus side scatter area (SSC-A; **Fig. 4b**). Set low power to the FSC PMT; intact L1 larvae will give a FSC-A signal that is off scale, and debris will give relatively weaker signals. Set SSC-A PMT power to visualize the widest possible range of events. This scatter plot will make it possible to monitor debris content in your sample (**Fig. 4b**,**c**). Set a gate to isolate events with the highest FSC-A signal; these contain the larvae (**Fig. 4b**). Prepare a scatter plot analysis of green fluorescence (GFP; 488-nm excitation and emission at 530/30 nm) and red fluorescence (red spectrum autofluorescence (RSA); 488-nm excitation and emission at 610/20 nm) and display only the population that passes the worm gate set in the FSC-A versus SSC-A plot (**Fig. 4b**). Adjust the GFP and RSA PMT settings to center the event population representing the GFP-negative, intact larvae (**Fig. 4b**). This scatter plot will make it possible to distinguish true GFP fluorescence from autofluorescence by determining the ratio of green to red fluorescence. Use compensation settings to adjust for spectral overlap between GFP emission and the RSA filter set (settings will depend on the equipment and reporter strain used). This will aid in the distinction between GFP-positive and GFP-negative populations.

Prepare a 15-ml collection tube in the sort block. The collected larvae will remain viable in the PBS that accumulates in the collection tube throughout the sort. Trial sorts of negative- and positive-control samples should be performed and inspected microscopically to assess recovery rate, viability and purity of the sorted animals.

PROCEDURE

Worm preparation ● TIMING 6–12 d

1| Place 12 GFP-balanced heterozygous hermaphrodite young adults on each of ten 100-mm standard NGM plates seeded with OP50 and allow them to grow at 20 °C until the plate is full of young adults, but the worms are not starved (i.e., some of the OP50 lawn remains on the plate).

2| Wash the worms from the plates using M9 (1–3 ml per plate) and collect the M9 worm suspension into a 50-ml conical tube.

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3| Centrifuge the collected worms at 700*g* for 5 min at room temperature and carefully decant the supernatant.

4| Add 25 ml of cold M9 and 25 ml of cold 60% (wt/vol) sucrose to the worm pellet and mix by inversion.
▲ **CRITICAL STEP** Store autoclaved solutions of M9 and 60% (wt/vol) sucrose at 4 °C for this purpose.

5| Centrifuge at 1,500*g* at 4 °C for 5 min.

6| Remove gravid worms from the top using a serological pipette and place them in a new 50-ml conical tube.

7| Wash the collected worms with M9 by filling the tube with M9, centrifuging at 700*g* for 5 min at 4 °C and then carefully decanting the supernatant.

8| Resuspend the worm pellet in ~25 ml of sodium hypochlorite solution.

9| Swirl the solution every minute or so. After about 4 min, the solution should become cloudy, indicating that many adults have dissolved. Immediately fill the tube with M9 and centrifuge at 1,500*g* at room temperature for 2 min. Carefully decant the bleach solution without disturbing the worm embryo pellet.

10| Wash the embryo pellet. Fill the tube with M9 and vortex to resuspend the pellet; centrifuge at 1,500*g* for 2 min at room temperature and decant the M9.

11| Repeat Step 10 two more times.

12| Resuspend the washed embryos in 5–10 ml of M9.

13| Allow the collected embryos to hatch in M9 by rotating them at 25 °C for 14–20 h.

14| Estimate the number of hatched larvae by mixing the solution of larvae in M9, extracting 1 µl and dropping it on a slide, and counting the number of larvae in the extracted droplet. Extrapolate the total number of larvae using the total volume. (For example, if you counted 100 larvae in 1 µl and have 10 ml of larvae, then you have a total of 100 × 10,000 = 1,000,000 larvae.) Use a micropipetter to plate 25,000–50,000 synchronized larvae in M9 onto 150-mm 5× peptone plates superseeded with OP50. Incubate the plates at 20 °C until most plated worms are gravid adults (3–6 d, depending on the strain). There should be thousands of embryos present on the plate at the time of harvest.
? TROUBLESHOOTING

15| Although the population will be mostly synchronized at this step, purify it further by performing another sucrose float (as in Steps 2–7) to separate gravid adults from any remaining larvae. Use one 50-ml conical tube per five full plates of worms.

16| Treat the collected worms with sodium hypochlorite solution to isolate embryos (as in Steps 8–11).
▲ **CRITICAL STEP** The embryos from this step will hatch to form the L1s that will be sorted the following day, and thus it is important for these embryos to be as clean as possible in order to avoid adding extraparticulate matter through the FACS. Ideally, at this stage, the adult worm bodies are dissolved completely by the bleach, leaving only a pure solution of embryos. However, overbleaching at this step will kill embryos, lowering the L1 yield. Different bottles of bleach have different potencies, and thus the amount of time spent bleaching these worms must be determined empirically. After each minute in bleach solution, mix the worm suspension and remove 1 µl to examine under a stereoscopic microscope. When 75–90% of the gravid adults have burst open and released their embryos (**Fig. 3a**), proceed to the M9 washes.

17| Allow the collected embryos to hatch in at least 5–10 ml of M9 in a 50-ml conical tube while rotating at 25 degrees for 14–20 h (**Fig. 3b**).

18| Dilute the L1s by filling each conical tube to the top with M9 and filter each tube of L1s twice using a 40-µm nylon cell strainer (**Fig. 3c**).

19| Estimate the number of larvae as described in Step 14. Concentrate the larvae by centrifuging at 750*g* for 5 min at room temperature, and then resuspend them in M9 to a concentration of 200–300 L1s per µl. Transfer the larval solution to a tube suitable for loading onto the FACS instrument.

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FACS ● TIMING ~2 h

20| Inspect the suspension microscopically and examine it for debris content and viability. The solution should be largely composed of larvae and embryos (**Fig. 1c**), with very little large debris (**Fig. 1b**). Live larvae tend to writhe in solution.

21| Run 0.5–10 ml of the collected larvae from Step 20 through the FACS system and adjust the flow rate to approximately 100–300 events per second.
? TROUBLESHOOTING

22| Set gates encompassing the GFP-positive and GFP-negative populations (**Fig. 4b**,**c**). Larvae with GFP expression will form a population of events off the diagonal in the plot of GFP versus RSA (with a high GFP-to-RSA ratio) that is not seen in the wild-type control sample (**Fig. 4b**,**c**).
? TROUBLESHOOTING

23| Sort the GFP-negative larvae into a 15-ml collection tube.

24| Estimate the number of larvae as described in Step 14. It is likely that there will be both larvae and embryos in the recovered solution, and the yield of sorted larvae should be at least 80% of the expected number of GFP-negative animals. (With the balancer we used, we expected one-third of the L1s from Step 19 to be GFP negative.) Microscopic examination of the sorted sample should reveal that GFP-positive animals are rare (but not completely absent yet).
? TROUBLESHOOTING

25| Concentrate the sorted larvae by centrifuging at 750*g* for 5 min at room temperature, and then resuspend them in PBS to a concentration of 200–300 L1s per μl.

26| Sort the larval suspension a second time (repeat Steps 21–23; **Fig. 4c**).

27| To determine whether the twice-sorted larval population is sufficiently pure, run a small (~0.5 ml) sample through the FACS instrument again to record the number of GFP-positive events that are still present (**Fig. 4c**). Allow the FACS to sort about 1,000–10,000 events to verify purity. In our experience, after the second sort, fewer than 0.5% of the collected animals were GFP positive, and this was sufficient purity for our RNAi screen. If a purer sample is desired, repeat Steps 21–23. In addition, inspect the suspension microscopically; there should be no GFP-positive animals (or they should be exceedingly rare), and the vast majority of the collected animals should have the desired genotype.
? TROUBLESHOOTING

28| Concentrate the larvae by centrifuging at 750*g* for 5 min at room temperature and resuspend in a medium suitable for the final application. (We used the sorted homozygous mutants in an RNAi-based genetic interaction screen performed in S medium using 96-well plates[14].)

29| Clean and sterilize the FACS fluidics system.

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 2**.

TABLE 2 | Troubleshooting table.

Step	Problem	Possible reason	Solution
14	Many eggs do not hatch	Did not allow enough time to hatch	Allow longer time for eggs to hatch, some strains might require a longer incubation
		Overbleaching	Bleach for a maximum of 4 min
		Not enough M9 was added or tube was not agitated	Use at least 5 ml of M9, have worms in a 50-ml conical tube, and be sure they are rotating during the entire incubation

(continued)

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TABLE 2 | Troubleshooting table (Continued).

Step	Problem	Possible reason	Solution
14		Balancer itself gives rise to embryonic lethality	Homozygous-lethal balancers and reciprocal translocation balancers produce some inviable embryos. Some proportion of embryos will not hatch, and this should not disrupt further applications, provided there are enough live L1s left for sorting
21	Recurrent clogged nozzle on the FACS	Stock is not bleached well enough, leading to excess of debris	At Step 16, bleach for a longer amount of time to reduce debris from adult worm bodies
		Filtering is insufficient	Dilute the worm solution more before filtering or filter an additional time
		Worm suspension is too concentrated	Dilute the worm suspension
22	No clearly distinct GFP-negative population is visible	Samples with high debris content will have more debris that passes through the FSC-versus-SSC worm gate (**Fig. 4c**). Such samples will display events in the GFP-versus-RSA plot with a wide range of autofluorescent intensity, visible as a diagonal smear obscuring the GFP-negative population (**Fig. 4c**)	Use the upper and lower limits of RSA intensity of the GFP-positive gate to guide the placement of the GFP-negative gate (**Fig. 4c**)
24	Some inviable larvae are recovered	Reciprocal translocation balancer is used	In GFP-marked reciprocal translocation balancers, the GFP insertion is present on just one of the translocated chromosome arms. Depending on the location of the terminal allele, there could be GFP–half-translocation (aneuploid) animals that are not homozygous for the terminal allele. If the recovered animals are to be used for a chemical or genetic screen, then the presence of some GFP-inviable larvae of the wrong genotype should not disrupt the screen, provided there are enough viable larvae collected. To avoid aneuploidy, use a balancer that is not a reciprocal translocation
27	Collected GFP-negative larvae do not have the desired genotype	The balancer is unstable	Check GFP animals from balanced stock to determine whether they have the desired genotype. If not, the balancer may have broken down and permitted recombination. Thaw a frozen stock of the strain to recover the original balanced line. If this is a recurrent problem, then try a different balancer

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● TIMING

The total timing of the protocol varies depending on the strain used, and it can from 6 to 12 d. After a sufficient population of the strain has been grown and the L1s isolated, FACS setup takes 30 min and the actual sort takes about 2 h.

Step 1: 3–6 d
Steps 2–12: ~2 h
Step 13: 14–20 h
Step 14: 3–6 d
Steps 15 and 16: ~2 h
Step 17: 14–20 h
Steps 18 and 19: ~1 h
Steps 20–29: ~2 h

ANTICIPATED RESULTS

We generally achieved an 80% or greater recovery rate (larvae recovered/events sorted). The unrecovered fraction of larvae disintegrates during the sort and is seen in subsequent re-sorts as debris (**Fig. 4c**). Sorted larvae were viable and showed no reduced growth or survival in subsequent growth assays (compared with an unsorted control; data not shown). The purity of the sorted larvae (GFP-negative larvae/total larvae) was generally 90% or higher after the initial FACS run in our example sort of *mel-28* larvae (this will depend on the initial ratio of GFP-positive to GFP-negative larvae and debris content). A subsequent re-sort of the collected larvae typically gave a final purity of >99% GFP-negative larvae (**Fig. 4c**).

To ensure a yield of at least 100,000 pure, homozygous *mel-28* larvae, we started out with ~660,000 larvae. With the PF405 strain, one in three of the prepared L1 larvae are homozygous mutants and therefore GFP negative (~220,000 larvae). The expected proportion of homozygous larvae will vary depending on the balancer used. With an 80% recovery rate (~176,000 successfully sorted larvae) and 90% purity (~158,000 GFP-negative larvae), this first sort was re-sorted to obtain ~127,000 GFP-negative larvae with >99% purity. This isolation required 1 week of growth and larval preparation time and 3 h of reserved FACS time. We used a FACSAria I instrument in a core facility shared by many other users, and none of the subsequent applications were affected by sorting *C. elegans* larvae.

ACKNOWLEDGMENTS We thank E. Andersen, D.G. Moerman and R. Waterston for sharing unpublished data; P.-L. Ip, J. Lucas and K. Erikson for technical assistance; and S.D. Weatherbee for critically reviewing the manuscript. Funding sources included the Eunice Kennedy Shriver National Institute of Child Health and Human Development (NICHD; R01HD046236) and the National Human Genome Research Institute (NHGRI; U01 HG004276) to F.P., the US National Institutes of Health (NIH; R01GM078279-01) to K.D.B. and the National Science Foundation (0827858) and Fairfield University start-up funds to A.G.F. Nematode strains were provided by the *Caenorhabditis* Genetics Center, which is funded by the NIH National Center for Research Resources (NCRR).

AUTHOR CONTRIBUTIONS F.P. and A.G.F. initially developed the idea; B.O.R.B. and A.G.F. developed the FACS conditions; and F.P., A.G.F., B.O.R.B., E.K.M., M.L.E. and K.D.B. contributed to the realization of the protocol.

COMPETING FINANCIAL INTERESTS The authors declare no competing financial interests.

Published online at http://www.nature.com/doifinder/10.1038/nprot.2012.084. Reprints and permissions information is available online at http://www.nature.com/reprints/index.html.

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