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Automated screening for mutants affecting dopaminergic-neuron specification in *C. elegans*

Maria Doitsidou, Nuria Flames, Albert C Lee, Alexander Boyanov & Oliver Hobert

We describe an automated method to isolate mutant *Caenorhabditis elegans* that do not appropriately execute cellular differentiation programs. We used a fluorescence-activated sorting mechanism implemented in the COPAS Biosort machine to isolate mutants with subtle alterations in the cellular specificity of GFP expression. This methodology is considerably more efficient than comparable manual screens and enabled us to isolate mutants in which dopamine neurons do not differentiate appropriately.

Forward genetic screens for mutant organisms provide a powerful tool for understanding the molecular basis of many biological processes. The advantages of the model organism *Caenorhabditis elegans,* such as self-fertilization, short generation time, transparency and ease of handling, have prompted its extensive use in forward genetic screens that have yielded valuable insights into animal development and physiology[1,2]. Depending on the phenotype screened for, the actual isolation of mutants can be a major limitation in a forward genetic analysis. For example, screening for mutants in which individual cellular fates, visualized by *gfp*-based reporter gene technology[3], are not appropriately executed, can be a tedious undertaking. A technology that holds promise to automate phenotypic assessment in cell-fate screens is the COPAS Biosort system (Union Biometrica; 'worm sorter'). The worm sorter is a flow cytometry instrument adjusted to analyze and sort small living organisms the size of *C. elegans* on the basis of their optical density, size and fluorescence[4]. The worm sorter has not been previously tested for isolating mutants that do not execute specific cellular fates visualized by *gfp* reporters. Such screens can pose a challenge to worm-sorter technology as they often require the detection of worms in which only a small number of cells is labeled with a cell type–specific fluorescent marker. Moreover, desirable mutants may only lose marker-gene expression in a subset of cells, and the overall changes in fluorescence intensity may therefore be subtle. Testing the performance of the COPAS Biosort system in such specific conditions is necessary for assessing its applicability in screens for cell-fate mutants.

We describe here the use of the COPAS Biosort machine in a forward genetic screen for mutants defective in executing the dopaminergic cell fate. Dopaminergic neurons fulfill important functions across phylogeny, yet the genetic pathways that control dopaminergic cell fate are poorly understood to date[5]. A *gfp* reporter fusion to the promoter of the dopamine neuron–specific dopamine re-uptake transporter *dat-1* exclusively labels all dopaminergic neurons in *vtIs1 [dat-1::gfp]* transgenic worms[6,7] (**Fig. 1a**). As there are only eight dopaminergic neurons in the ~1,000

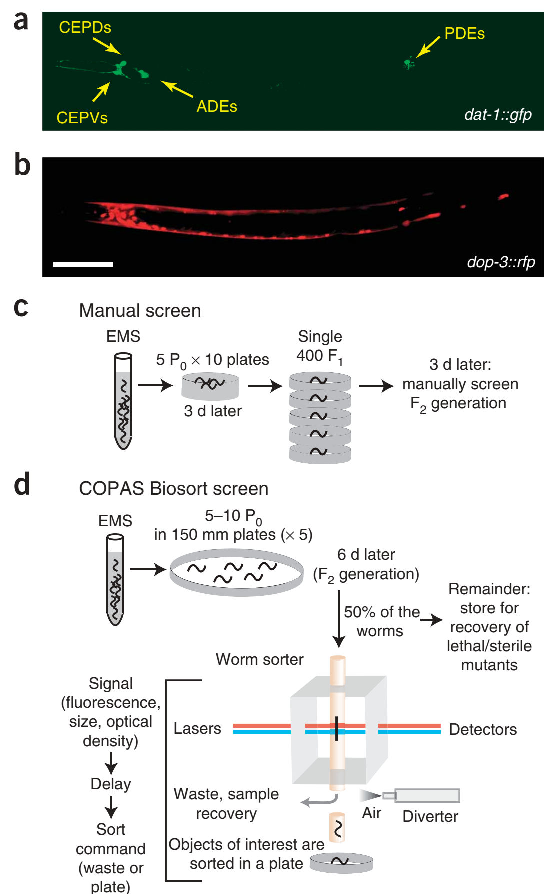

Figure 1 | Screening for dopaminergic cell-fate mutants. (**a**,**b**) Green (**a**) and red (**b**) fluorescence micrographs of a transgenic worm containing the *dat-1::gfp* and *dop-3::rfp* transgenes used for the worm sorter screen. Scale bar, 100 μm. (**c**,**d**) Outlines of the experimental procedures of the manual (**c**) and the worm-sorter (**d**) screens.

Howard Hughes Medical Institute, Department of Biochemistry and Molecular Biophysics, Columbia University Medical Center, 701 W. 168th Street, New York, New York 10032, USA. Correspondence should be addressed to M.D. (md2398@columbia.edu) or O.H. (or38@columbia.edu).

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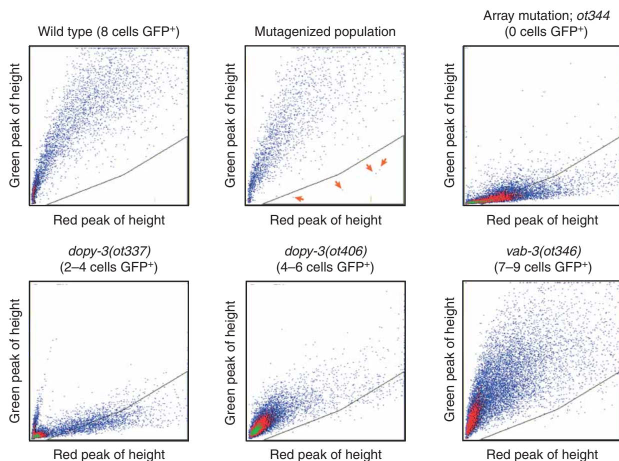

Figure 2 | Relative fluorescence intensity plots between red and green channels of sorted worms. Scale is defined by the sorting parameters (**Supplementary Methods**). Each dot corresponds to a single worm. Shown are plots for a non-mutagenized population of the sorting strain, for a mutagenized population of worms (red arrows indicate individual worms that fall into the manually set sorting window) and for homogenous populations of several retrieved mutants. The triangular area at the bottom right of each panel is an example of a sorting region chosen to demonstrate the profile differences between various populations. The actual sorting region used in the screening process was usually larger to minimize the possibility of losing mutants.

cell-containing *C. elegans* hermaphrodite, the use of *dat-1::gfp* provides a challenging test system for the sensitivity of the worm sorter. We first ran a sorting test in which the transgenic strain carrying *dat-1::gfp* was mixed with non-transgenic, that is, non–GFP-expressing worms. Non-transgenic worms could indeed be sorted out efficiently (**Supplementary Table 1** online). To control for the variability in fluorescent intensities among individual transgenic worms, we introduced a chromosomally integrated, broadly expressed *rfp* transgene (*vsIs33 [dop-3::rfp]*)[8] in the background of our screening strain as an internal reference of fluorescence intensity. This strain expresses *rfp* in some body-wall muscles and in various head neurons, ventral nerve cord neurons, tail neurons and two mechanosensory neurons, the PVDs, but not in dopaminergic neurons (**Fig. 1b**). We set the worm sorter to compare fluorescence between green and red channels to plot their ratio (**Fig. 2**) and to sort worms with reduced green to red ratio of fluorescence. This substantially increased the efficiency of sorting GFP-negative worms (**Supplementary Table 1**).

To test the practicality of the worm sorter–based genetic screens for dopaminergic-fate mutants, we conducted two different genetic screens for ethyl methanesulfonate (EMS)-induced mutations, a manual one and an automated worm sorter screen, and compared the outcomes. For the manual F_1 clonal screen we mutagenized worms with EMS, singled F_1 progeny onto individual plates and screened their progeny with the help of a stereo dissecting microscope with a fluorescent light source (**Fig. 1c**). We screened 11,000 EMS-mutagenized haploid genomes in the course of several months and identified 10 mutants that expressed *dat-1::gfp* in fewer cells than normal (**Table 1** and see **Supplementary Table 2** online for a more comprehensive version of this table).

In the automated screen we used the worm sorter to screen approximately 80,000 EMS-mutagenized haploid genomes (**Fig. 1d**), focusing on isolating mutants that show reduced *gfp* expression. We obtained fluorescence distribution plots of wild-type and mutagenized populations (**Fig. 2**): in the mutagenzied population some worms had reduced green to red fluorescence ratio compared to that of wild-type worms (**Fig. 2**). The worms falling into the specified sorting region were sorted by the COPAS instrument, and we then inspected them under a fluorescence stereoscope to confirm the presence of a phenotype.

We identified 22 mutants with reduced GFP expression. Among these, 3 mutants had merely reduced overall GFP expression in all 8 dopaminergic neurons (data not shown) and 2 mutants

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Table 1 | Mutants retrieved from genetic screens

Gene name	Molecular identity	Manual screen	Sorter screen	Allele names	Inheritance	Pleiotropies
dopy-2	Unknown	0 alleles	3 alleles	*ot340, ot345 ot406*	Recessive	–[c]
dopy-3	Unknown	0 alleles	1 allele	*ot337*	Dominant	–
dopy-4	Unknown	1 allele[a]	0 alleles	*ot260*	Recessive	–
dopy-5	Unknown	4 alleles[b]	0 alleles	*ot283, ot284, ot296, ot298*	Recessive	Sterile
dopy-6	Unknown	1 allele[a]	0 alleles	*ot263*	Recessive	–
dopy-7	Unknown	0 alleles	2 alleles	*ot399, ot347*	Recessive	Sick
lin-32	bHLH	2 alleles	4 alleles	*ot259, ot297, ot341, ot343, ot338, ot366*	Recessive	–
ham-1	No homologies	2 alleles	6 alleles	*ot253, ot257, ot342, ot361, ot367, ot339, ot371, ot364*	Recessive	–
vab-3	Paired and homeodomain	0 alleles	1 allele	*ot346*	Recessive	Notched head
Total number of alleles		10	17			
Genomes screened		11,000	80,000			
Allele frequency per genomes screened		1/1,100	1/4,700			
Time investment		100 d[d]	25 d[d]			
Allele frequency per time		1 allele per 10 d	1 allele per 1.5 d			

[a]As these two mutants only affect *gfp* expression in the two PDE neurons, it is possible that these two mutants were only retrieved by the manual screen because *gfp* expression in the PDEs is dimmer than in the other dopaminergic neurons and a loss of *gfp* expression only in these neurons is the most challenging phenotype to detect. In fact, one of the two missing PDE mutants was isolated in the manual screen only because of its concurrent 'extra PDEs' phenotype (see quantification of phenotypic data in **Supplementary Fig. 1**). [b]This sterile mutant was not isolated by the worm sorter screen because we did not pursue sorted mutants that could not easily be maintained as homozygotes. In contrast to semi-clonal manual screens, in which it is easy to maintain a sterile mutant strain by identifying and maintaining heterozygous adult worms from the parental plate, plates that were analyzed by the worm sorter contained significantly more complex population of mutants, which makes the re-isolation of the parents labor intensive, though still possible, in principle. [c]No obvious pleiotropies. [d]The 100 days dissecting scope work is full-time microscopy while 25 days of worm sorting involves mainly machine running and casual observation of functioning of sorter.

showed complete loss of GFP expression owing to mutations in the transgenic array (see **Supplementary Methods** online); we did not consider either of these mutant classes further. The remaining 17 mutants had a reduced number of cells expressing GFP in at least one class of dopaminergic neurons (**Table 1** and **Supplementary Table 2**). The mutants showed striking cellular specificity: individual mutants had distinct subsets of dopaminergic neurons affected; one mutant had been affected in as little as one subclass of the eight dopaminergic neurons (**Table 2**, **Fig. 3** and **Supplementary Fig. 1** online). The sorting profiles of homozygous populations of some of these mutants are presented in **Figure 2**.

We mapped the mutants retrieved from both screens using high-throughput single-nucleotide polymorphism mapping[9], performed complementation tests (data not shown) and sequenced candidate genes in the regions in which we mapped individual mutants. In total, we recovered 6 alleles of the Math/atonal-like bHLH gene *lin-32*, previously known to have a role in specifying one subtype of dopaminergic neurons[10], but not previously known to also affect the specification of other dopaminergic neurons (**Table 1**, **Supplementary Fig. 2** and **Supplementary Table 3** online). We recovered one allele of the Pax6/Eyeless-like gene *vab-3* (ref. 11), which had not previously been implicated in dopamine neuron development (**Table 1**, **Supplementary Fig. 2** and **Supplementary Table 4** online) and eight alleles of the *ham-1* gene, previously known to be involved in controlling asymmetric cell divisions[12] (**Table 1**, **Supplementary Fig. 2** and **Supplementary Table 5** online). In addition to these known genes, we isolated several additional, apparently new mutants that fall into six complementation groups, that we mapped to different chromosomal locations (**Table 1**, **Fig. 3** and **Supplementary Fig. 1**). We termed these genes ‘dopy’ for dopaminergic neuron atypical. *dopy* mutants appear to affect *dat-1::gfp* expression in a cell type–specific manner, suggesting that on some level, there is differential regulation of the dopaminergic fate among the various classes of dopaminergic neurons (**Fig. 3c**). Based on the allele recovery rate, we estimated that our worm-sorter screen reached at least 78% saturation (**Supplementary Table 6** online).

Comparing the manual and automated screening approach, we found that the worm-sorter screen is more efficient in several regards. First, as less worm picking is required, the preparation of mutagenized worms for screening with the sorter requires much less operating time than that in the manual screen (**Fig. 1c**,**d**). Moreover, in the actual screening process, it takes about 1 h of intense effort at the stereomicroscope to screen 60 mutagenized genomes (for which ~1,000 individual worms need to be screened in our design) whereas the worm sorter can screen up to 2,000 mutagenized genomes (~50,000 individual worms) in the same amount of time. The workload was on average 10 laborious days of manual screen at the stereomicroscope per isolated mutant as opposed to 1.5 part-time days that involve mostly casual supervision of the proper function of the worm sorter (**Table 1** and **Supplementary Methods**). This high-throughput screening rate of the worm sorter–based method is unprecedented as the only other technology available for automated mutant isolation, the use of microfluidics[13], offers a screening speed that is orders of magnitude lower (few hundred worms per hour as opposed to several thousands).

Table 2 | Phenotypes retrieved using the worm sorter

Number of dopaminergic neurons expressing GFP	Number of alleles	Allele name
0 out of 8	2	*ot344, ot373* (array mutations)
2 out of 8	3	*ot399, ot347, ot366*
3 out of 8	1	*ot337*
4 out of 8	5	*ot340, ot406, ot341, ot343, ot338*
6 out of 8	7	*ot342, ot361, ot367, ot339, ot371, ot363, ot346*
7 out of 8	1	*ot345*

For mutants displaying multiple phenotypes, we considered here only the phenotype on the basis of which the mutant was isolated.

Comparison of the spectrum of mutants retrieved by manual and automated screening illustrates the effectiveness and sensitivity of the worm-sorter approach. Although we retrieved alleles of two loci (*lin-32, ham-1*) by both the manual and automated screen (with more alleles retrieved by the latter), we retrieved alleles of four loci (*dopy-2, dopy-3, dopy-7, vab-3*) exclusively in the automated screen. These mutant alleles would have likely surfaced in manual screens as well, but it would have taken much more time to isolate them. The sensitivity of the worm sorter is also illustrated by its ability to recognize mutants with as little as one cell having lost GFP expression (**Table 2**).

The worm sorter can be used to automate the phenotypic selection step for various cell-fate markers; for example, we also used the worm sorter to isolate mutants with aberrant expression of neuronal-fate markers normally only expressed in two (AIYL and AIYR) or as little as one (ASEL) neuronal subtypes (V. Bertrand, F. Zhang, M.D. and O.H., unpublished data). Use of the worm sorter dramatically sped up the screening process and, in the case described here in detail, allowed identification of a variety of mutants that displayed abnormal dopaminergic neuron differentiation. An implicit feature of automation is scalability. Given the minimal effort involved in growing and sorting worms, one can interrogate the genome to as saturating a degree as any mutagenesis approach may allow. In combination with the recently developed strategies that allow for easy identification of the molecular lesion, such as transposon tagging[14] or whole-genome sequencing[15],

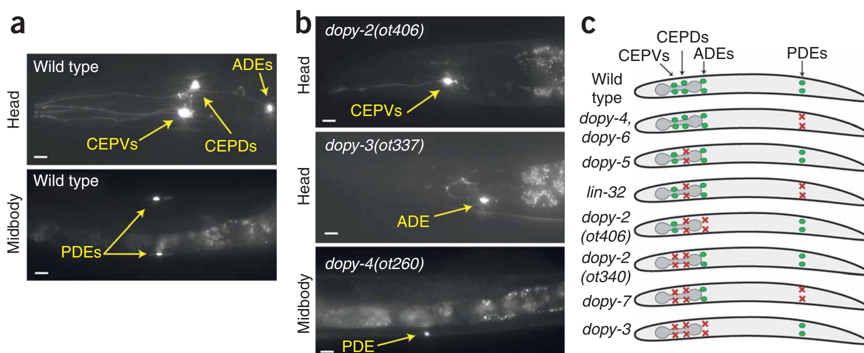

Figure 3 | Phenotypes of isolated *dopy* mutants. (**a**,**b**) Micrographs of representative wild-type (**a**) and mutant worms (**b**) expressing *dat-1::gfp*. Scale bars, 10 μm. (**c**) Schematic summary of cell-type specificity of dopaminergic mutant phenotypes.

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worm sorter–based screens will enable an exhaustive analysis of genetic pathways involved in development.

Note: Supplementary information is available on the Nature Methods website.

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AUTHOR CONTRIBUTIONS
M.D. conducted the genetic screens (manual and sorter), implemented the sorter strategy, mapped and characterized mutants, and co-wrote the paper; N.F. implemented the sorter strategy and conducted sorter screens; A.C.L. conducted manual screens; A.B. assisted in mapping mutants; and O.H. initiated and supervised the project, and co-wrote the paper.

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