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Whole Animal Cell Sorting of *Drosophila* Embryos

MARK A. KRASNOW,* SUSAN CUMBERLEDGE, GERARD MANNING,
LEONARD A. HERZENBERG, AND GARRY P. NOLAN†

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Use of primary culture cells has been limited by the inability to purify most types of cells, particularly cells from early developmental stages. In whole animal cell sorting (WACS), live cells derived from animals harboring a *lacZ* transgene are purified according to their level of β-galactosidase expression with a fluorogenic β-galactosidase substrate and fluorescence-activated cell sorting. With WACS, incipient posterior compartment cells that express the *engrailed* gene were purified from early *Drosophila* embryos. Neuronal precursor cells were also purified, and they differentiated into neurons with high efficiency in culture. Because there are many *lacZ* strains, it may be possible to purify most types of *Drosophila* cells. The same approach is also applicable to other organisms for which germ-line transformation is possible.

IN MANY ORGANISMS DIFFERENT DEvelopmental pathways originate because of differential allocation of egg cytoplasm and regulatory gene products to individual cells; subsequent development of these cells and their descendants is commonly modulated by signals between cells (*1*). Characterization of the biochemical differences among developing cells and a molecular understanding of the interactions between cells might be possible if the different cells of a developing animal could be isolated for analysis and for study in vitro. This has been an important experimental approach in studies of early amphibian development (*2*) and development of the mam-

malian nervous system (*3*) where, because of specialized cell and tissue morphologies, it is possible to identify and excise certain types of developing cells and tissues. Also, the ability to isolate cells by their surface markers has revolutionized the study of the immune system (*4*). Here we present a general method for purifying cells from developing animals based solely on differences in cellular patterns of gene expression. Whole animal cell sorting (WACS) combines the resolution and generality of current cell-marking techniques that use introduced transgenes with the purification and analytical power of fluorescence-activated cell sorting (FACS).

For WACS, embryos of a strain harboring an *Escherichia coli lacZ* transgene expressed in a particular cell type are grown to the desired developmental stage. The cells of the developing animals are dissociated and stained with a fluorogenic β-galactosidase substrate, and the fluorescent cells are purified by FACS. The purified cells can be analyzed directly or allowed to continue development in culture, alone or in the presence of other cells or developmental signaling molecules. There are several requirements for this strategy to be successful. First, strains that express β-galactosidase in the cells of interest at the appropriate developmental stage are required (thousands of *D. melanogaster* strains with *lacZ* under the control of various cell- and tissue-specific promoters and regulatory elements have been generated by germ-line transformation) (*5*). Second, the animals' cells must be dissociated and live cells in the preparations must be identified. Third, the β-galactosidase–expressing cells must be fluorescently stained and purified. Finally, the purified cells must be able to continue their development in vitro.

When the cells of *D. melanogaster* embryos are dissociated and grown as primary cultures, many morphologically distinct cells arise, including neurons, myocytes, and chitin-secreting cells (*6*). We prepared cells from ~10^3 4- to 6-hour-old embryos by dounce homogenization and stained them with propidium iodide (PI) and either fluorescein diacetate (FDA) or calcein blue acetoxymethyl ester (CBAM) to distinguish live cells from dead cells and debris by FACS analysis (Fig. 1, left). Live cells (stippled box) were considered as those that excluded PI (low PI fluorescence) and metabolized

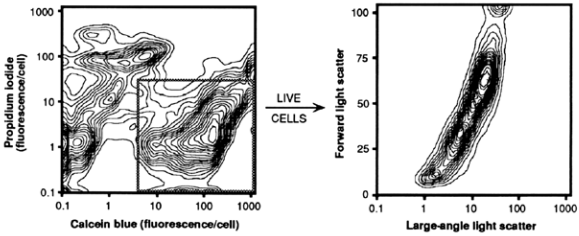

Fig. 1. Flow cytometric analysis of *D. melanogaster* embryo cells. We disrupted 4- to 6-hour-old embryos by dounce homogenization, stained the cells with PI and CBAM (*26*) and analyzed them by FACS (*27*). (**Left**) Contour plot of cellular fluorescence measurements. The live cell population is indicated by the stippled box. (**Right**) Light scatter measurements of the live cells.

FDA to fluorescein (*7*) or CBAM to calcein blue (high calcein blue flourescence); they represented about 50% of the preparation and were well resolved from other populations, which probably include dead cells, nuclei, yolk, and debris. The live cell population had a broad distribution of forward light scatter values because of heterogeneity in cell size and the presence of some incompletely dissociated cells (Fig. 1, right). Unlike in many heterogeneous cell populations (*4*), however, in the live cell population there were no subpopulations with distinctive forward and large-angle light scatter profiles, possibly because there has been little cellular differentiation at this early stage in development. In the following experiments, we used PI staining and forward light scatter measurements to exclude from analysis dead cells, large cell aggregates, and most debris; in some experiments, we also used CBAM staining to exclude cell-sized debris and a rare population of autofluorescent particles.

Cultured mammalian cells that express β-galactosidase can be fluorescently stained with the β-galactosidase substrate fluorescein di-β-D-galactopyranoside (FDG) and remain viable (*8*). Nonfluorescent FDG is briefly introduced into cells at 37°C under hypotonic conditions, and the fluorescent reaction product fluorescein accumulates in cells expressing β-galactosidase. The β-galactosidase reaction is carried out under iso-

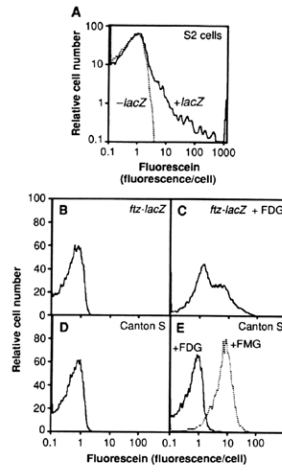

Fig. 2. Staining of live *D. melanogaster* culture cells and embryo cells with FDG. (**A**) S2 cells were transiently transfected by the calcium phosphate technique with 0.5 μg of pP_{ac}lacZ (solid line) or with pP_{ac} (dotted line), a control plasmid without the *lacZ* sequences (*29*). The cells were collected 45 hours after transfection, then resuspended in SM medium and stained with FDG and PI (*30*). The β-galactosidase reactions were for 1 hour and cellular fluorescence was determined by FACS. (B through E) Cells were prepared from 4.5- to 6.5-hour-old 43.04 embryos which harbor a *ftz-lacZ* transgene (*11,14*), or from wild-type (Canton S) embryos of the same age (*26*). Washed, filtered cells were mock-loaded in hypotonic SM or loaded with 1 mM FDG (*30*) or 1 mM FMG (*31*) for 1.5 min at 23°C. Isotonicity was restored, cells were stained with PI, and the β-galactosidase reactions were continued for 45 min and then stopped by addition of PETG. Fluorescence values of the live cells are shown. (**B**) *ftz-lacZ* cells, mock-stained. (**C**) *ftz-lacZ* cells, stained with FDG. The fluorescent population may trail into the nonfluorescent population because of heterogeneity in β-galactosidase expression among developing embryos of slightly different ages. (**D**) Canton S cells, mock-stained. (**E**) Canton S cells, stained with FDG (solid line) or FMG (dotted line). FMG was used to test the substate loading of the cells. Less than 0.5% of live cells had fluorescence values greater than four units after FDG staining.

M. A. Krasnow, S. Cumberledge, G. Manning, Department of Biochemistry, Stanford University, Stanford, CA 94305.
L. A. Herzenberg and G. P. Nolan, Department of Genetics, Stanford University, Stanford, CA 94305.

*To whom correspondence should be addressed.
†Current address: Whitehead Institute for Biomedical Research, 9 Cambridge Center, Cambridge, MA 02139.

tonic conditions, and at 4°C so that fluorescein will not leak from the cells (*8*). It was possible to stain transiently transfected *D. melanogaster* Schneider's line 2 (S2) cells (*9*) expressing β-galactosidase with FDG under similar conditions, although FDG was loaded at 23°C instead of 37°C to prevent heat shock. A fraction of the transfected cell population (Fig. 2A) had high fluorescein fluorescence (solid line), and the fluorescent cells were absent when the cells were transfected with a control plasmid without *lacZ* sequences (dotted line) or when FDG was omitted (*7*). The heterogeneity in fluorescence probably reflects heterogeneity in β-galactosidase expression among the transfected S2 cells (*10*). Because most cells were unchanged in fluorescence after FDG treatment, there must be little transfer of the fluorescent products from cells that are expressing β-galactosidase to those that are not.

We next determined if FDG could be used for labeling cells derived from whole *D. melanogaster* embryos. In strains harboring a *fushi tarazu* (*ftz*)–*lacZ* transgene, β-galactosidase is expressed, as is the endogenous *ftz* gene, in seven regularly spaced rows of cells (stripes) along the anterior-posterior axis (*11*). Cells from 4.5- to 6.5-hour-old *ftz-lacZ* embryos were loaded with FDG under the conditions used for S2 cells, and β-galactosidase reactions were continued for 0, 1, or 45 min. A significant cell population with fluorescence values of four units or more appeared after 1 min (*7*), and by 45 min the fluorescent cells represented ~35% of the live cells (Fig. 2C). This is in good agreement with the fraction of β-galactosidase–expressing cells determined by histochemical staining of intact embryos (*11*). There were few fluorescent cells if FDG was omitted (Fig. 2B), if cells were from Canton S embryos that lack a *lacZ* transgene (Fig. 2, D and E), or if the *E. coli* β-galactosidase inhibitor phenylethyl-β-D-thiogalactoside (PETG) was added to 1 mM before FDG addition (*7*). Also, when Canton S cells were treated with fluorescein mono-β-D-galactopyranoside (FMG), a fluorescent compound similar in structure to FDG, the entire population became fluorescent (Fig. 2E); this suggests that all of the embryo cells take up the β-galactosidase substrate and nonfluorescent cells are not simply deficient in substrate loading. These experiments demonstrate that fluorescent staining with FDG is specific for *E. coli* β-galactosidase, and that one can use FDG to distinguish β-galactosidase–expressing cells from nonexpressing cells in heterogeneous cell populations derived from whole animals.

We carried out similar experiments with a strain harboring an *engrailed-lacZ* transgene to show that WACS can be used to purify cells solely on the basis of their pattern of gene expression. Early in embryogenesis there are no morphological differences between *en*-expressing cells and nonexpressing cells, although the expressing cells become the posterior part of each segment (*12*). Cells from 4- to 6-hour-old *en-lacZ* embryos were treated with FDG, the fluorescent cells were sorted, and β-galactosidase and *en* expression in the cells were determined by antibody staining (Fig. 3). Essentially all sorted cells expressed both proteins, whereas only rare cells in a nonfluorescent control population expressed either; we obtained a similar result using protein immunoblots of cell extracts probed with anti–β-galactosidase antibodies (*13*). Thus, the FACS assay accurately measures expression of the endogenous gene, and WACS can separate stripes of *en*-expressing cells, the incipient posterior compartment cells, from nonexpressing cells of the interstripes.

For the study of development in vitro, the purified cells must be capable of executing their normal developmental program in culture. To test for developmental competency, we purified neuronal precursor cells from a strain with *lacZ* expression in neuroblasts and cells derived from neuroblasts (*14*) (Fig. 4). After growth in culture for 1 day, 33 to 41% of the purified cells had long slender processes characteristic of neurons (Fig. 4) (*15*), and the cell bodies and processes of the differentiated cells stained with anti–horseradish peroxidase (HRP) antisera (*7*), which recognizes a neuronal surface antigen (*16*). Only a few percent (1.6 to 5%) of unpurified control cells had this morphology under the same culture conditions. Purified cells that did not acquire neuronal morphology may have arrested at an earlier developmental stage, possibly because culture conditions are suboptimal or because differentiation of these cells requires interactions with other cell types.

In the purified culture, neurons with one or two processes were most common, but there were also rare cells with more than two neurites. Many cells were present in clusters of two or more cells of similar morphology (Fig. 4); such clusters probably derive from individual precursor cells (*17*), suggesting that the purified cells can divide as well as differentiate. The purified cells also appeared to interact with each other, as neuronal processes commonly bridged neighboring cells. Most of the morphological changes took place after more than several hours in culture; after 3.5 hours only about 2% of cells had neuronal morphology, and of these most had only short processes. The time course of differentiation of the purified cells thus appears similar to the time course of neural differentiation in its normal context, where axonogenesis begins 8 to 10 hours

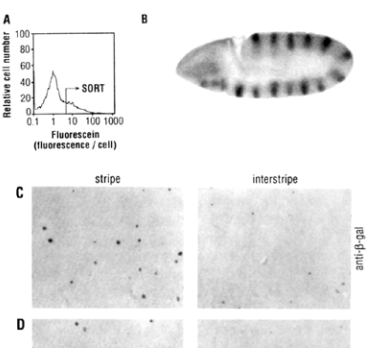

Fig. 3. Purification of the stripes of *en*-expressing cells by WACS. Cells from 4- to 6-hour-old 2.5EPLHT + 5.0R embryos, which harbor an *en-lacZ* transgene expressed in the posterior compartment of each segment (*32*), were prepared and stained with FDG, PI, and CBAM. β-Galactosidase reactions were for 20 min. Live cells with high fluorescein fluorescence (~7 to 990 units), or a low-fluorescence control population (~0.2 to 0.8 unit), were sorted onto cold, polylysine-coated slides and fixed with 4% paraformaldehyde. Fixed cells were stained with a rabbit polyclonal anti–β-galactosidase antiserum or a mouse monoclonal anti-*en* antibody (*33*) and then treated with a biotinylated secondary antibody and HRP immunochemistry. The stained cells were photographed at ×400 under interference-contrast optics; antigen-positive cells appear darker than the antigen-negative cells, which appear gray. (**A**) Fluorescein fluorescence of live cells from *en-lacZ* embryos. The sort gate for the high-fluorescence cells is indicated. (**B**) An intact *en-lacZ* embryo stained with anti–β-galactosidase antiserum as described (*25*). An anterior stripe of *lacZ*-expressing cells appears slightly later than those shown. (**C**) High-fluorescein fluorescence cells (*en* "stripe" cells; left panel) or low–fluorescein fluorescence cells ("interstripe" cells; right panel) stained with anti–β-galactosidase antiserum. (**D**) High–fluorescein fluorescence cells (left panel) or low–fluorescein fluorescence cells (right panel) stained with anti-*en* antibody.

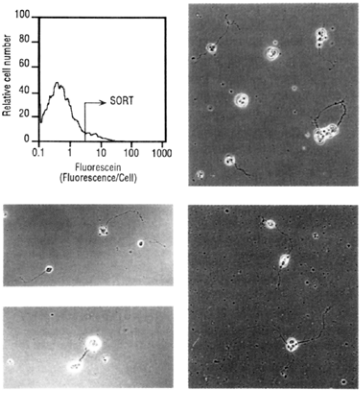

Fig. 4. Purification of neuronal precursor cells by WACS and development of these cells in vitro. Cells were prepared from 4- to 6-hour-old embryos of strain F249, which express β-galactosidase in neuroblasts and their progeny (*14*), and stained with FDG and PI. β-Galactosidase reactions were for 1 hour. Live cells ($\sim 6 \times 10^8$) with high fluorescein fluorescence (>4 units), or a mock-sorted control population (all fluorescence values), were sorted into 0.1 ml of M/CM medium (*34*) in the center of a 35-mm culture dish. Cells were allowed to attach for ~1 hour, 0.2 ml of M/CM was added, and the culture was placed in a humidified chamber at 25°C for 1 day. After culturing, there were $\sim 3 \times 10^3$ cell clusters in the experimental and the control cultures. Differentiated cells were photographed without fixation at ×400 with a water immersion objective and phase-contrast optics. The graph at the top left shows fluorescein fluorescence values of the live cells from F249 embryos. The sort gate for the high-fluorescence cells is indicated. The color panels are photomicrographs of the live, fluorescent cells after culturing. Growth cones can be seen at the ends of most neurites. Similar results were obtained in two independent experiments.

after fertilization and continues for several hours throughout embryogenesis (*18*).

Other purification methods, such as elutriation (*19*) and differential cell adhesion (*19, 20*), have been successfully applied to *D. melanogaster* embryo cells to enrich for certain cell types, but they lack the purification power of WACS, and they are based on intrinsic physical differences among cells and so are not inherently generalizable. In contrast, WACS is applicable to virtually any cell type at any stage of development, so long as the cells can be selectively marked with β-galactosidase and viably separated from neighboring cells. Given the large number of existing *lacZ* strains and the ease of generating new ones (*5*), it may be possible to mark and purify most types of *D. melanogaster* cells (*21*). If necessary, FDG staining of β-galactosidase–expressing cells can be used in combination with other cell markers, including other fluorescent markers that can be distinguished from fluorescein by means of multiparameter FACS.

The stage is now set to begin purification of the different cells of an animal from a particular developmental stage for molecular analysis and for reconstructing developmental interactions from purified cells in vitro. For instance, it should be possible to purify *wingless*-expressing cells, which neighbor the *en*-expressing cells in each segment, to test in vitro the proposal that *wingless*-expressing cells are required for maintenance of *en* expression during embryogenesis (*22*). WACS may be of special importance in the study of the nervous system, where many cell types interact to form a functional network. WACS should also be applicable to other animals for which germ-line transformation with *lacZ* transgenes is possible, such as the mouse and the nematode worm (*23*).

Because maximal sorting speeds of current FACS instruments approach $\sim 10^7$ cells (or $\sim 10^3$ embryos) per hour, 10^5 to 10^7 purified cells can be obtained in several hours by WACS if the cells of interest represent a reasonable proportion of total cells. This is enough for most cell-based analyses, and it should be enough for constructing cell type–specific cDNA libraries and for detecting proteins and DNA binding activities using sensitive techniques (*24*). Cell yields can be increased if other purification techniques are used to enrich for the cells of interest before WACS, or it may be possible to establish continuous cell lines from the purified cells.

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15. β-Galactosidase–expressing cells from strain F249 or a mock-sorted control population were purified by WACS and cultured for 1 day (Fig. 4). The total number of cells and the fraction with neuronal morphology were determined. Cells with one or more thin processes (neurites) one cell diameter in length or longer were scored as neurons. Mature cultures contained isolated cells and small clusters of two to approximately ten cells; clusters were treated as single cells in the analysis.
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21. For certain transitory cell types, careful staging of embryos, or the use of altered *lacZ* transgenes with shortened β-galactosidase half-lives, may be necessary so that contamination with cells more mature than those desired is avoided.
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26. Embryo cells were prepared by a modification of the procedure of Furst and Mahowald (*19, 25*). Eggs were collected for 2 hours from cages containing 20 $\times 10^3$ to 40 $\times 10^3$ healthy flies fed on heat-killed yeast; the first collection of the day was not used. Eggs ($\sim 10^3$) were aged at 25°C, washed with 0.7% NaCl, and dechorionated for 2 min in 2.5% sodium hypochlorite. Subsequent steps were at 4°C. Embryos were washed with Schneider's medium (SM) (Gibco #350-1720AJ), resuspended in approximately five times their settled volume of SM, and disrupted by dounce homogenization (seven strokes, type A pestle). The cell suspension was centrifuged (5 min, 400*g*), and the cell pellet was resuspended in SM. This washing procedure was repeated three times, and the cell suspension was filtered through Nitex (~50 by 50 μm pore size) to remove large debris and cell aggregates. PI was added to 10 μg/ml and CBAM (Molecular Probes) to 10 μM. The cells were incubated at 4°C for 10 min or more to allow hydrolysis of CBAM. Just before FACS, we pelleted cells and resuspended them in fresh SM containing PI (10 μg/ml) to reduce background fluorescence from free CBAM. CBAM treatment at 1 to 10 μM had no significant effects on S2 cell doubling time or on the efficiency of differentiation of unpurified embryo cells into neurons and myocytes.
27. Dual laser FACS, data collection, and multiparameter analysis were with a modified Becton Dickinson FACStar Plus and data analysis regimes of FACS/DESK (*28*). One argon laser (488 nm, 400 mW output) drove light scatter, and fluorescein (530/30 nm bandpass) and PI (575/26 nm bandpass) detectors; a second argon laser (380 nm, 50 mW) drove the CBAM (460 nm beam splitter, 405/20 nm bandpass) detector. The forward scatter amplifier

was set at ×8. Standard procedures were used to correct for fluorescein spectral overlap into the PI channel (*28*) and for compensation for cellular autofluorescence [S. Alberti, D. R. Parks, L. A. Herzenberg, *Cytometry* **8**, 114 (1987)]. We excluded small debris and large clusters of cells from analysis using forward scatter gates set from 100 to 920 (scale 0 to 1000); in experiments where PI but not CBAM was used to identify live cells, more restrictive gates (~200 to 920) were used to help exclude debris. Data was collected on 3×10^4 cells per sample.

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30. FDG staining was as described (*8*) with the following modifications. Cells (10^6 to 10^7 per milliliter) were loaded with FDG by placement in hypotonic SM (SM diluted 1:1 with distilled H_2O) containing 1 mM FDG for 1.5 or 2 min at 23°C. We restored isotonicity by adding a tenfold excess of cold (4°C) SM containing PI (10 μg/ml). The β-galactosidase reactions continued at 4°C for 1 to 60 min. In most experiments, reactions were stopped by addition of PETG to 1 mM. CBAM, when used, was added just after the cold SM and the cells were further processed as described (*26*).
31. We centrifuged cells treated with FMG (5 min, 400*g*) and resuspended them in SM containing PI before FACS to reduce background fluorescence from free FMG.
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34. M/CM is a 1:1 mixture of M [SM supplemented with 18% (v/v) heat-inactivated fetal bovine serum and penicillin-streptomycin] and CM (conditioned medium, prepared by culturing cells from 4- to 6-hour-old embryos in M for 1 day and then removing cells by centrifugation and filtration through a 0.45-μm filter).
35. We thank D. Parks, S. Fiering, L. Herzenberg, and Y. Hiromi for valuable discussions; Y. Hiromi, M. Koelle, C.-N. Chen, and T. Kornberg for unpublished strains and plasmids; and E. Martin-Blanco, T. Kornberg, and A. Boulet for antibodies. M.A.K. is a Lucille P. Markey Scholar in Biomedical Science, S.C. was supported by National Research Service Awards training grant CA09151, and G.M. is a Howard Hughes Medical Institute Predoctoral Fellow. Supported by grants from the Lucille P. Markey Charitable Trust and the National Institutes of Health to M.A.K.

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