
CHAPTER 8

Preparation and Analysis of Pure Cell Populations from *Drosophila*

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I. Introduction

As the genetic analysis of development and cell function in *Drosophila melanogaster* has burgeoned over the last 15 years, so has our ability to distinguish various cell types in developing tissues, using molecular cell markers that have become available mostly through gene cloning. As our understanding of development and cell function *in vivo* becomes more sophisticated, it is increasingly important to isolate the various cell types so that they can be more fully analyzed and manipulated in various ways. This allows one to test the emerging models of the underlying cellular and molecular processes and to characterize these processes biochemically and discover new components.

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What has been needed is a convenient, reliable way to purify large quantities of different cell types from *Drosophila*.

A wealth of knowledge has emerged from studies of purified cells and continuous cell lines from vertebrates, with the mammalian immune system perhaps the most dramatic example (Parks *et al.*, 1989). In contrast, there have been only a few serious attempts to isolate and study pure populations of *Drosophila* cells. Mahowald and his colleagues have shown that highly enriched populations of pole cells (germ-line precursors) and neuroblasts can be obtained in reasonable quantity from embryos (Allis *et al.*, 1977; Furst and Mahowald, 1985), and other groups (Bernstein *et al.*, 1978; Storti *et al.*, 1978) have described procedures for the isolation of myoblasts (see Mahowald (Chapter 7) and Ashburner (1989a) for reviews). This pioneering work demonstrated the feasibility of cell purification from *Drosophila* embryos, and it showed that purified cells can retain the ability to differentiate appropriately into morphologically distinct cell types. The fractionation schemes relied primarily on differences in general physical characteristics of the cells, such as their size, shape, density, or adhesive properties. For example, pole cells, because they tend to have a low lipid content and are larger than most embryonic cells, can be purified by equilibrium density centrifugation followed by sedimentation velocity centrifugation (Allis *et al.*, 1977). Neuroblasts also tend to be large and can be selectively enriched by centrifugal elutriation and adherence to glass (Furst and Mahowald, 1985). However, most *Drosophila* embryonic cells, at least during early embryogenesis, are rather unexceptional in morphology and hence may not be amenable to purification by methods based solely on such physical characteristics. Methods for purifying these cells must rely on other properties of the cells, such as expression of cell type-specific molecular markers.

Surface markers have been widely used in mammalian systems to isolate specific cell types, particularly cells of the immune system (Parks *et al.*, 1989). Antibodies that recognize specific cell surface antigens are commonly employed in the purification by using the antibodies to fluorescently label the cells followed by flow cytometry/fluorescence-activated cell sorting (FACS) or by coupling the antibodies to a solid phase and selectively resorbing the cells of interest ("panning") (Wysocki and Sato, 1978). These techniques have not been applied to *Drosophila*, at least in part because few antibodies to cell type-specific surface antigens have been available until recently. However, in *Drosophila*, many intracellular markers are known, perhaps the most important of which is the *Escherichia coli lacZ* (β -galactosidase) gene, which is not normally present but is easily introduced by P-element-mediated transformation. Thousands of different strains expressing *lacZ* under control of various cell- and tissue-specific promoters and regulatory elements have been constructed, many by random insertion of a *lacZ* transposon such that *lacZ* expression comes under the control of an endogenous enhancer or regulatory element ("enhancer trap") (O'Kane and Gehring, 1987; Bier *et al.*, 1989; Bellen *et al.*, 1989). We have established a method, called whole animal cell sorting (WACS), for purifying the β -galactosidase expressing cells from such transgenic strains by FACS

(Krasnow *et al.*, 1991). The key technical innovation that opened the way to this approach was the development of a viable, fluorogenic β -galactosidase substrate (fluorescein di- β -D-galactopyranoside) that was shown to be effective in the analysis and purification of cultured mammalian cells engineered to express β -galactosidase (Nolan *et al.*, 1988; Fiering *et al.*, 1991).

The general scheme for WACS is as follows (Fig. 1). (1) Embryos carrying a *lacZ* transgene expressed in a specific cell type are grown to the desired developmental stage. (2) Cells of the developing embryos are dissociated and stained with FDG and then stained with a viable cell stain and a dead cell stain.

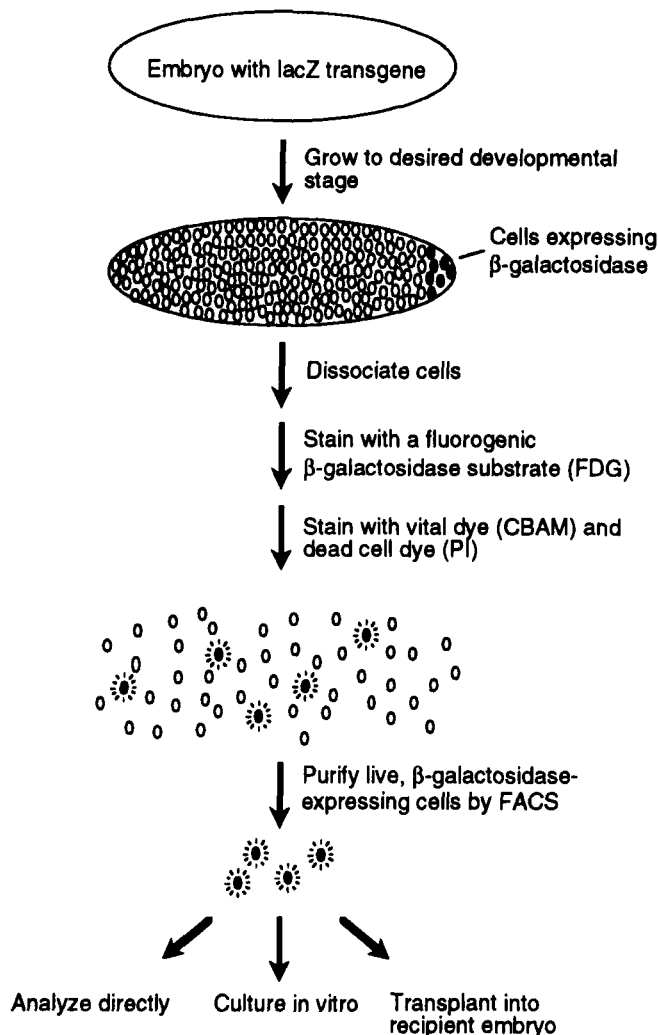


Fig. 1 Scheme for whole-animal cell sorting.

(3) Live cells expressing β -galactosidase are purified away from dead cells and nonexpressing cells by FACS. (4) The purified cells can then be analyzed directly, cultured *in vitro*, or reintroduced into a recipient embryo. The scheme requires only basic embryo handling and cell culture techniques, except for cell sorting, which is usually carried out in conjunction with a trained FACS technician. Once there is a large, healthy fly population with the appropriate *lacZ* expression pattern, the cells of interest are obtained in 2–3 hr from the time of embryo harvesting and cell dissociation.

This scheme has been used to obtain reasonable quantities ($\sim 10^5$ cells) of various types of *Drosophila* embryonic cells and to study their development *in vitro*. The same approach should be applicable to any cell type provided an appropriate *lacZ* strain exists and the cells can be efficiently dissociated and stained without disrupting their viability and development. Neuronal precursor cells, and cells expressing the segmentation genes *fushi tarazu*, *engrailed*, and *wingless* have all been purified by this approach (Krasnow *et al.*, 1991; Cumberledge and Krasnow, 1993). The cell populations obtained are completely viable and highly pure (typically 90% or greater), and they can continue their development *in vitro*. For example, the purified neuronal precursor cells were shown to divide and differentiate into neurons at high efficiency in culture (Krasnow *et al.*, 1991). We have also used the purified cells to study the dynamics of *engrailed* expression in isolated cells (Cumberledge and Krasnow, 1993), to reconstruct and analyze developmental signaling events between *engrailed*-expressing cells and the neighboring *wingless*-expressing cells (Cumberledge and Krasnow, 1993), and to investigate the homophilic and heterophilic adhesion properties of *engrailed*-expressing cells and *wingless*-expressing cells (S. Cumberledge and M. Krasnow, unpublished results). There are many potential applications of the purified cells that have not yet been explored, such as transplantation of the cells back into living animals, biochemical studies of cellular processes and purification of cell constituents, and the generation of cell type-specific cDNA libraries and monoclonal antibodies. While sufficient numbers of cells can be obtained by the current procedures for most cell-based analyses, a major challenge in the future will be to find ways to increase purification speed and yields so that preparative quantities of purified cells can be easily obtained at reasonable cost (see Section IV).

II. Purifying Embryonic Cells by Fluorescence-Activated Cell Sorting

A. Equipment and Reagents

1. Flow Cytometer/FACS Instrument

We have used a modified Becton Dickinson FACStar Plus flow cytometer, equipped with two argon-ion lasers. Dual laser flow cytometry, data collection, and multiparameter analysis are performed essentially as described by Parks

et al. (1986,1989). One argon-ion laser (488 nm, 400 mW output) is used to generate four signals: forward light scatter, large angle light scatter, fluorescein (detected through a 530/30-nm bandpass filter), and propidium iodide (detected through a 575/26-nm bandpass filter). A second argon-ion laser was used as an ultraviolet light source (351–363 nm, 50 mW) to excite calcein blue, whose emission was detected through a 405/20-nm filter. Data collection and multi-parameter analysis are carried out on a Digital VAX computer system using the FACS/DESK software (Moore and Kautz, 1986). For applications in which the highest degree of cell purity and viability are not required, calcein blue staining can be omitted and a single laser flow cytometer (488 nm excitation) used for cell isolation.

2. Fluorescent Dyes and β -Galactosidase Substrates

The structures of the compounds described below are shown in Fig 2. These structures, as well as some of the information provided below, are from a very useful catalog and handbook provided by Molecular Probes, Inc. (Haugland, 1992). Additional information about the compounds including literature references can be found in Haugland (1992) and in materials provided by the supplier with the reagents.

a. Fluorescein di- β -D-Galactopyranoside (FDG) (Fig. 2A)

Prepare a solution of 2 mM FDG (Molecular Probes, No. F-1179) in dH₂O. Mix the solution vigorously and heat it to 37°C because 2 mM is close to the solubility limit. (It is also possible to prepare 2 mM FDG by diluting a 200-mM FDG stock solution prepared in dH₂O/dimethylsulfoxide (1 : 1), but the effects of the residual dimethylsulfoxide on *Drosophila* cells are unknown.) Store small aliquots of 2 mM FDG at –20°C in the dark, where it is stable for months. After thawing an aliquot, heat it to 37°C for 10 min to ensure complete solubilization of the FDG. FDG is less stable in powder form than in solution, so prepare the stock solution as soon as the powder is received. The working solution should have a faint yellow color. FDG is not fluorescent, but it is converted into free fluorescein (via fluorescein mono- β -D-galactopyranoside) by β -galactosidase. Background fluorescence present in some FDG preparations, due to contaminants, can be eliminated by irradiating the working stock with the 488-nm argon laser beam of the flow cytometer for 1 min. Use protective eye wear when working near an exposed laser beam. For fluorescein, $\lambda_{\text{abs}} = \sim 490$ nm and $\lambda_{\text{em}} = \sim 514$ nm.

b. Phenylethyl β -D-Thiogalactoside (PETG) (Fig. 2B)

Prepare a stock solution of 50 mM PETG (Molecular Probes, No. P-1692) in dH₂O; store at 4°C, where it is stable for months. PETG is a reversible inhibitor of β -galactosidase, used to stop the β -galactosidase reaction. It readily enters cells, even at 4°C.

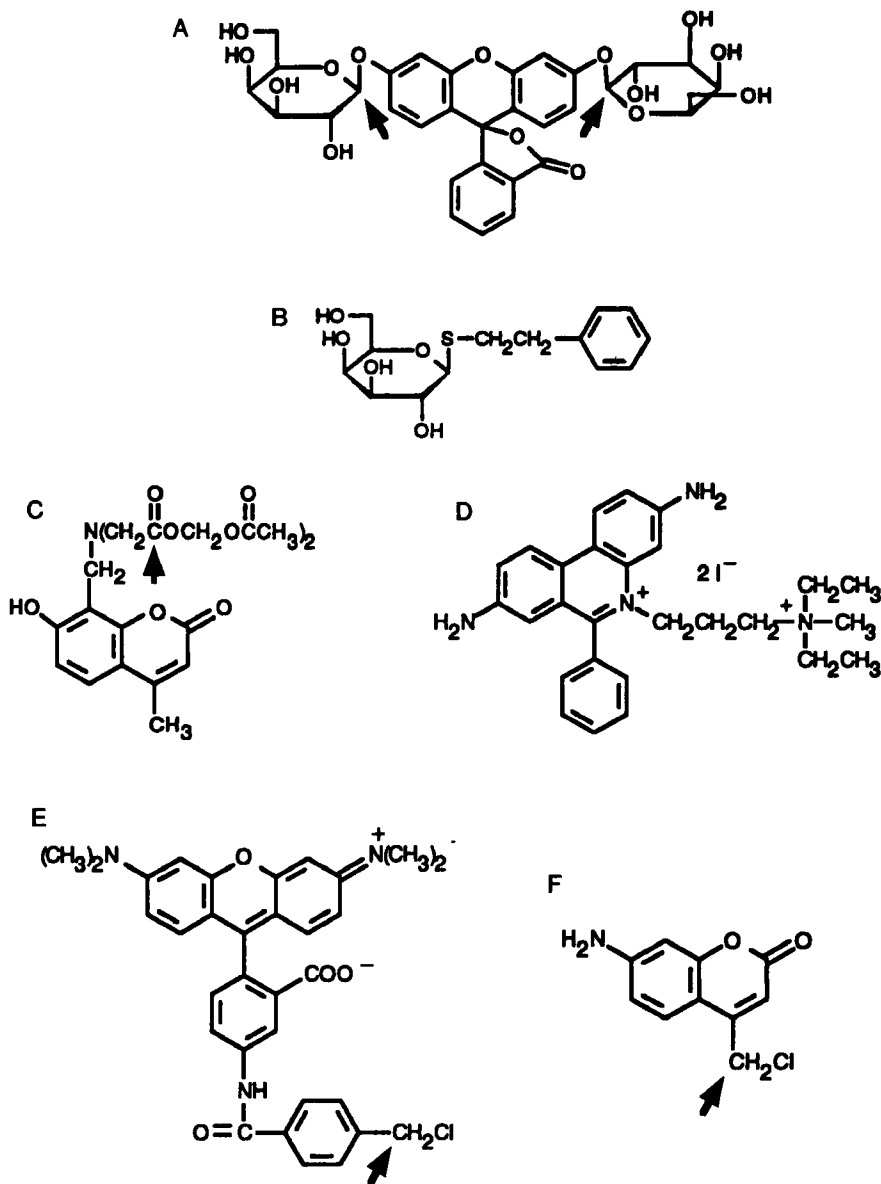


Fig. 2 Chemical structures of fluorescent dyes and β -galactosidase substrates. (A) Fluorescein di- β -D-galactopyranoside (FDG). The arrows show the positions of attack by H_2O in the hydrolysis reactions catalyzed by β -galactosidase, which liberates a free galactose group and generates a phenolic group on the dye. (B) Phenylethyl β -D-thiogalactoside (PETG). (C) Calcein blue acetoxy-methyl ester (CBAM). The arrow shows the position of attack by H_2O in the hydrolysis reaction catalyzed by cellular esterases, which generates a carboxylate group on the dye. (D) Propidium iodide (PI). (E) 5- (and- 6-)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine (CMTMR). The arrow shows the position of attack by intracellular thiol groups, which displaces the chloride group and forms a thioester linkage to the dye. (F) 7-Amino-4-chloromethylcoumarin (CMAC). The arrow shows the position of attack by intracellular thiol groups as in E.

c. Calcein Blue Acetoxymethyl Ester (CBAM) (Fig. 2C)

Prepare a 10-mM stock solution of CBAM (Molecular Probes, No. C-1429) in anhydrous dimethylsulfoxide; store small aliquots in the dark at -20°C . CBAM is a fluorescent coumarin derivative used to label live cells. It readily enters cells, and once inside it is converted by cellular esterases into calcein blue, a fluorescent molecule that is retained within live cells with intact membranes but lost from dead cells. For calcein blue, the longest-wavelength absorption maximum (λ_{abs}) is ~ 355 nm, and the wavelength of maximal fluorescence emission (λ_{em}) is ~ 440 nm.

d. Propidium Iodide (PI) (Fig. 2D)

Prepare a stock solution of PI at 0.5 mg/ml (Molecular Probes, No. P-1304) in dH_2O ; store at 4°C , where it is stable for months. PI is excluded from live cells, but it readily enters dead cells (and cells with compromised membranes), where it binds DNA and fluorescently stains the nucleus. PI is a mutagen, so use caution when handling. For PI, $\lambda_{\text{abs}} = \sim 536$ nm and $\lambda_{\text{em}} = \sim 617$ nm.

e. 5- (and 6-)-(((4-Chloromethyl)benzoyl)amino)tetramethylrhodamine (CMTMR) (Fig. 2E)

Prepare a stock solution of 80 μM CMTMR (Cell Tracker Orange, Molecular Probes, No. C-2927) in dH_2O . Store in the dark at -20°C in small, single-use aliquots. CMTMR and CMAC (see below) are used for stable marking of the purified cells (Section IIIC). They are membrane-permeant fluorescent dyes with a chloromethyl reactive group. They readily enter cells and are stably retained, probably by conjugation of the chloromethyl moiety to intracellular thiols (Zhang *et al.*, 1992). For CMTMR, $\lambda_{\text{abs}} = \sim 541$ nm and $\lambda_{\text{em}} = \sim 565$ nm.

f. 7-Amino-4-chloromethylcoumarin (CMAC) (Fig. 2F)

Prepare a stock solution of 80 μM CMAC (Cell Tracker Blue, Molecular Probes, No. A2110) in dH_2O ; store at -20°C in small, single-use aliquots. For CMAC, $\lambda_{\text{abs}} = \sim 354$ nm and $\lambda_{\text{em}} = \sim 469$ nm.

3. Media and Buffers

a. S2 Medium

Schneider's *Drosophila* medium (Gibco, No. 350-1720AJ; Ashburner, 1989b) supplemented with 11% fetal bovine serum (heat treated at 56°C for 30 min), penicillin at 50 units/ml, and streptomycin sulfate at 50 $\mu\text{g}/\text{ml}$.

b. SPC Medium

Schneider's *Drosophila* medium + 10 $\mu\text{g}/\text{ml}$ PI + 10 μM CBAM, both added just before use.

c. SPP Medium

Schneider's *Drosophila* medium + 10 $\mu\text{g/ml}$ PI + 1 mM PETG, both added just before use.

d. Phosphate-buffered saline (PBS)

137 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4 , 1.8 mM KH_2PO_4 ; pH 7.4.

e. PBSB

PBS + 0.5% bovine serum albumin.

B. Methods

1. Embryo Preparation

The embryo preparation and cell dissociation protocol is a modification of the method of Furst and Mahowald (Furst and Mahowald, 1985; Ashburner, 1989b). Refer to Ashburner (1989a,b) for related protocols and for general information on embryo preparation and cell culture. Most of our studies have used germ band elongation stage embryos (3–5 hr after egg lay at 25°C), as described below. Except for pole cells, it may not be possible to obtain intact, viable cells from embryos much younger than 3 hr because cellularization is not complete. We have also used somewhat older embryos (up to 7 hr after egg lay) successfully, but we have not attempted purification from embryos older than this or from animals at later stages of development, which may require additional methods, such as treating the cells with proteases or calcium-chelating agents such as EGTA to facilitate their dissociation.

Transgenic flies of the appropriate *lacZ* strain are maintained at 25°C in a large population cage (~50 gm, or $\sim 5 \times 10^4$, adult flies in an ~12-liter cage) and fed daily with trays of standard yeast/glucose/agar medium seeded with live yeast paste (see Elgin and Miller (1978) or Roberts (1986) for basic techniques for maintaining large fly populations). These conditions are usually adequate for obtaining $\sim 10^3$ or more embryos in a 2-hr collection; the size of the population should be adjusted according to the egg laying rate of the strain and the number of embryos that are needed for the purification.

On the day of the collection, prefeed the flies for 1 hr or more with a fresh yeast tray. This helps reduce the number of “held” (overly mature) eggs obtained in the collection.

Replace the prefeeding tray with a fresh yeast tray to begin egg collection. Use heat-killed yeast (e.g., treated for 60 min at 80°C) to decrease the likelihood of yeast contamination if the cells are to be cultured after purification. Remove the plate after 2 hr and allow the embryos to age for 3 hr at 25°C. The embryos obtained are 3 to 5 hr old; to obtain embryos at other stages, adjust the aging period accordingly.

Rinse the embryos from the food tray with a solution of 0.7% NaCl in a squirt bottle and a soft-hair paintbrush. Filter the embryos through a $\sim 440\text{-}\mu\text{m}$ mesh Nitex sieve to remove any adult flies and large debris, and collect the embryos on a $\sim 110\text{-}\mu\text{m}$ mesh Nitex sieve.

Wash embryos extensively with 0.7% NaCl to remove the remaining yeast. If cells are to be cultured after FACS, all subsequent steps are performed under sterile conditions and in a tissue culture hood whenever possible.

Transfer embryos from the sieve to a small wire mesh basket using a spatula or paintbrush or by rinsing them in with a squirt bottle. The basket can be constructed by gluing or melting a 1-inch section of plastic tube (cut from a 15-ml polypropylene tube) onto a wire mesh screen (Thomas Scientific No. 3435-B95). Immerse the embryos in a solution of 2.5% sodium hypochlorite for 2 min, swirling gently, to dechorionate the embryos.

Wash the embryos four times with 0.7% NaCl and then two times with Schneider's *Drosophila* medium, to remove all traces of sodium hypochlorite. Drain most of the liquid and then transfer the embryos to a 15-ml (17×120 mm) polypropylene screw-cap tube with printed graduations (Sarstedt No. 62.554.002). Bring the total volume of embryos and medium to ~ 10 ml.

2. Embryo Disruption and Cell Dissociation

All subsequent steps (except where indicated) are carried out at 4°C to prevent further development of the embryos.

Allow the embryos to settle in the tube, then aspirate off the supernatant. Resuspend the embryos in ~ 20 times their settled volume of S2 medium (e.g., 10 ml of S2 medium for 0.5 ml of settled embryos), using a Pasteur pipet. The final concentration of embryos can affect the efficiency of cell dissociation and the amount of cell lysis that occurs during douncing; for other developmental stages the optimal concentration may have to be determined empirically.

Transfer $\sim 5\text{-ml}$ aliquots of the resuspended embryos to a 7-ml glass dounce tissue homogenizer. Disrupt the embryos and dissociate the cells using seven slow strokes of a type A (loose) pestle. Cell yields are usually about one-third the estimated number of embryo cells; the rest are probably destroyed during douncing. Transfer the cell suspension to a 15-ml polypropylene tube.

Wash the cells by pelleting them in a tabletop centrifuge (5 min, 500 g). Carefully decant the supernatant and gently resuspend the cells in the residual medium. Cells isolated from early embryos can be fragile, so cells should be resuspended gently, but thoroughly, by briskly tapping or flicking the tube. Add 20 vol of S2 medium to the resuspended cells (e.g., 10 ml of medium for 0.5 ml of settled embryos). Repeat the wash and resuspend the cells in 10 vol of Schneider's *Drosophila* medium (e.g., 5 ml of medium for 0.5 ml of settled

embryos). Schneider's *Drosophila* medium is used here and in the next step because S2 medium may have higher background fluorescence, which could interfere with the FACS analysis.

Pellet the cells and resuspend them in Schneider's *Drosophila* medium. Use a volume of medium equal to the volume of embryos used (e.g., 0.5 ml of medium for 0.5 ml of settled embryos). The final cell concentration should be $\sim 10^7$ cells/ml. Pass the sample through a 50- μ m mesh Nitex filter to remove large cell clumps and large debris.

3. Staining Cells with Fluorescent Dyes

The FDG staining protocol is a modification of the procedure developed by Nolan *et al.* (1988) for staining mammalian cells in culture. Because it is large and hydrophilic, FDG does not readily enter cells. To allow efficient loading of the substrate, the cells are briefly treated with high concentrations of FDG under hypotonic conditions at 25°C. β -Galactosidase converts FDG to free fluorescein, which readily passes through the plasma membrane at 25°C but not at 4°C. Thus, immediately after FDG loading, the cells are chilled to 4°C to prevent leakage of fluorescein from the cells. Refer to Nolan *et al.* (1988), Fiering *et al.* (1991), Roederer *et al.* (1991), and information provided with the reagent by Molecular Probes for additional information about FDG staining.

Staining reactions are carried out with 200- μ l aliquots of cells in 6-ml (12 \times 75 mm) polystyrene round-bottom tubes (Falcon No. 2058). Add 10 μ l of 50 mM PETG to one of the aliquots; this inhibits β -galactosidase activity and serves as a negative control (see Step 4).

Equilibrate the cells at 25°C for 5 min. Add 200 μ l of 2 mM FDG and mix gently but thoroughly. This dilutes the Schneider's medium to approximately half its normal concentration.

After 2 min at 25°C, add 4.5 ml cold (4°C) SPC medium and mix well to restore isotonicity and chill the cells. SPC contains the dyes PI and CBAM, which are used to distinguish the live cells during cell sorting (Step 4).

Incubate the cells at 4°C to allow the β -galactosidase reaction to proceed. Typically, we allow the reaction to proceed for ~ 30 min, but this can vary from a few minutes to an hour or more, depending on the levels of β -galactosidase activity in the cells, and the optimal time may have to be determined for each *lacZ* strain. (Also during this incubation, cellular esterases convert the CBAM to calcein blue.) Cells must be kept at 4°C at all times from this point on to prevent leakage of fluorescein from the cells.

Pellet the cells (5 min, 500 g) and resuspend the cell pellet in 1 ml cold SPP medium. SPP medium contains PETG to stop the β -galactosidase reaction, which is sometimes necessary to keep the background fluorescence from increasing in the cells that don't express *lacZ*. This step also serves to remove the free CBAM in the medium.

4. Flow Cytometry and Cell Sorting

For what follows, the reader may wish to consult a general introduction to flow cytometry (e.g., Parks *et al.*, 1989; Radbruch, 1993). Carry out the standard calibration of the FACStar instrument (Parks *et al.*, 1986,1989), set the forward light scatter amplifier to $8\times$, and set the forward light scatter gates to help exclude small debris and cell aggregates from the analysis. Typically, gates from 200 to 820 (scale of 0 to 1000) are used on this flow cytometer. *Drosophila* embryonic cells are heterogenous in size (and many are smaller than mammalian cells that are commonly analyzed by flow cytometry), and their large-angle light scatter values are generally well correlated with their forward light scatter values (Fig. 3, right). Analyze the fluorescence of a small number of cells in the forward light scatter range noted above. After correcting for cellular autofluorescence and for fluorescein spectral overlap into the PI channel by standard methods (Parks *et al.*, 1989), there should be at least two distinct subpopulations differing in their calcein blue fluorescence and PI fluorescence (Fig. 3, left). Set the calcein blue fluorescence and PI fluorescence gates to include only the live cell population (high calcein blue, low PI fluorescence). To set the sorting gates for the β -galactosidase-expressing cells, analyze the fluorescein fluorescence of the live cells (forward light-scatter 200–820, high calcein blue fluorescence, low PI fluorescence). (Note: To prevent fluorescein leakage from the stained cells, they must be kept cold at all times from FDG staining until after cell sorting, even in the cell sorter, where they should be surrounded by ice or chilled water in the input reservoir.) The fluorescein profile

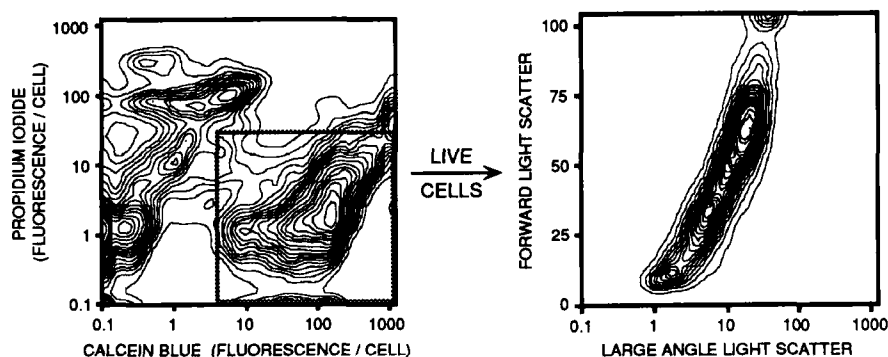


Fig. 3 Flow cytometric analysis of *D. melanogaster* embryo cells. Embryos 4 to 6 hr old were disrupted by dounce homogenization and the cells were stained with PI and CBAM and analyzed by flow cytometry. (Left) Contour plot of cellular fluorescence measurements. The live cell population (low PI fluorescence, high calcein blue fluorescence) is indicated by the stippled box. (Small cell debris and large cell clusters were excluded from the analysis by setting the forward light scatter gates as described in the text.) (Right) Light scatter measurements of the live cells. Reproduced from Krasnow *et al.* (1991).

is usually rather heterogenous, presumably due to heterogeneity in the levels of β -galactosidase in the individual cells and in the amount of FDG taken up by each, and there is overlap between the populations of β -galactosidase-expressing and nonexpressing cells (Fig. 4B; see also Krasnow *et al.*, 1991). In the best cases, there may be a distinguishable peak or "shoulder" of cells with high fluorescein fluorescence. More commonly, the overlap with the much larger population of cells that does not express β -galactosidase obscures the peak, and the β -galactosidase-expressing cells appear as a "tail" of highly fluorescent cells on the distribution of nonexpressing cells. It is therefore often useful to compare the fluorescein fluorescence profile of the experimental sam-

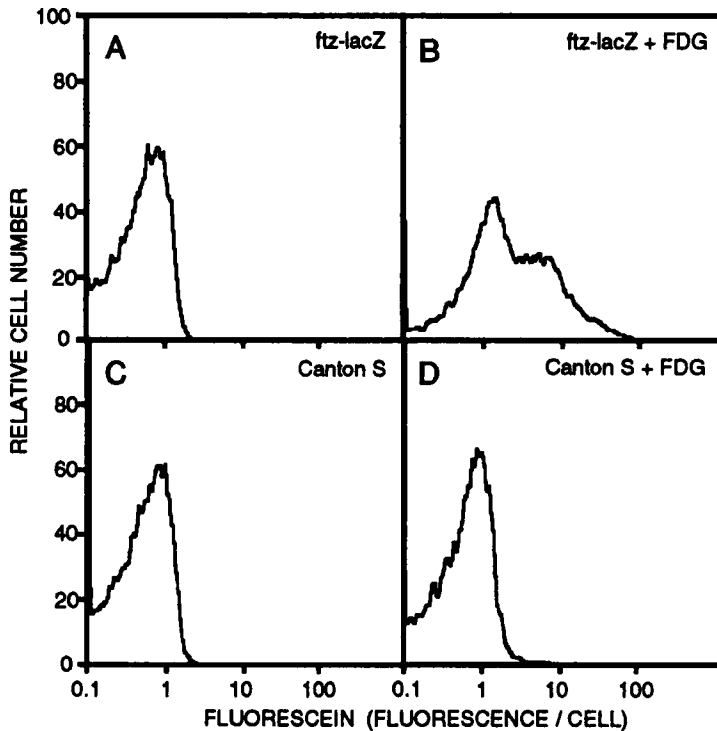


Fig. 4 Staining of *D. melanogaster* embryo cells with the fluorogenic β -galactosidase substrate FDG. Cells were prepared from 4.5- to 6.5-hr old embryos harboring a *ftz-lacZ* transgene (expressed in cells of the even-numbered parasegments) or from 4.5- to 6.5-hr old wild-type (Canton S) embryos. The washed, filtered cells were mock-treated with hypotonic Schneider's medium, or treated with 1 mM FDG in hypotonic Schneider's medium, 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 at 4°C and stopped by the addition of PETG to 1 mM. Fluorescence values of the live cells (see Fig. 3) are shown. (A) *ftz-lacZ* cells, mock-treated. (B) *ftz-lacZ* cells, treated with FDG. (C) Canton S cells, mock-treated. (D) Canton S cells, treated with FDG. Reproduced from Krasnow *et al.* (1991).

ples with that of negative control cells (cells in which PETG was added before FDG to inhibit the β -galactosidase reaction, see Section IIB3 above), or FDG-treated cells from a strain that does not express *lacZ* (Fig. 4D), and to set the fluorescence gate above that of the highest fluorescein fluorescence detected in this sample to maximize purity.

Up to a few thousand cells can be sorted per second, or $\sim 10^7$ cells per hour. If, for example, 1% of the cells express β -galactosidase, then theoretically $\sim 10^5$ cells can be isolated per hour. However, when we have determined the number of purified cells by microscopy immediately after sorting, the number is much less, typically only 10 to 40% of the number of sorting events registered by the cell sorter. (The reasons for the low yield are unclear, especially since many mammalian cell types give yields of 60% or greater on the FACS (Parks *et al.*, 1986). To determine the number of cells by microscopy, add an equal volume of a solution containing 3 $\mu\text{g}/\text{ml}$ acridine orange and 5 $\mu\text{g}/\text{ml}$ ethidium bromide to the cells and view under a fluorescence microscope; acridine orange stains live and dead cells and appears green, and ethidium bromide stains the nuclei of dead cells and appears orange/red. Alternatively, cells can be stained with an equal volume of 0.25% (w/v) solution of trypan blue and viewed under a light microscope. Dead cells stain dark blue, while viable cells exclude the dye and remain unstained; by this method, though, it is sometimes difficult to distinguish live cells from debris.

III. Culturing and Analysis of Purified Cells

A. Short-Term Culturing

Cells are sorted directly into a tissue culture chamber slide (Lab-Tek, 16-well, available from Nunc Inc., No. 178599) containing 75 μl of S2 medium, and the cells are allowed to settle on the slide. The medium is carefully replaced with fresh S2 medium (to remove the sheath fluid that accumulates during sorting), and the cells are cultured at 25°C in a humidified chamber. Optimal culture conditions should be determined empirically and will depend on the type of cells and the length of the culture period. We have cultured purified cells for a few days, but we have not yet attempted to establish long-term cultures from purified cells. It may help to coat the slide or culture vessel with extracellular matrix components before culturing; we often pretreat the slides by growing *D. melanogaster* Schneider line 2 (S2) cells to near confluence in the chamber slides for 16–20 hr and then removing the cells by washing the slides with S2 medium (Cumberledge and Krasnow, 1993). Conditioned S2 medium, prepared by culturing cells from dissociated *Drosophila* embryos for 1 day and then removing the cells by centrifugation and filtration through a 0.45- μm filter, can also aid in culturing (Krasnow *et al.*, 1991).

B. Fixation and Staining with Antibodies

If cells are to be stained with antibodies, they are collected directly onto polylysine-coated (Ashburner, 1989b) chamber slides or transferred to such slides after culturing. (Culturing on polylysine-coated slides is not generally recommended as it may adversely affect the cells.) Cells are gently pelleted onto the slide by low-speed centrifugation and then fixed in a solution of 4% paraformaldehyde at room temperature for 10 min. Even after fixation, attachment of the cells to the slide is usually weak and extreme care must be taken during all subsequent incubation and washing steps to keep cells from dislodging from the slide. When changing buffers, slowly aspirate off most of the old solution using a drawn-out capillary tube or long micropipet tip and add new solutions by slowly streaming down along the wall of the chamber well. For staining intracellular antigens, cells can be permeabilized by incubating in PBS + 0.1% Triton X-100 for 5 min. Wash the cells for 1 min with PBSB five times and then incubate twice in PBSB for 15 min each. Remove the PBSB, add the primary antibody diluted in PBSB, and incubate at room temperature for 1 hr. Wash the cells again with PBSB as above. The cells are now ready for staining with a secondary antibody and detection. For antigens present in low abundance, we have used a biotinylated secondary antibody and horseradish peroxidase immunocytochemistry (Vectastain ABC, Vector Laboratories, No. PK-4001).

C. Stable Fluorescent Marking of Purified Cells

For applications in which different populations of purified cells are intermixed, such as cell adhesion or signal transduction assays, it is sometimes useful to distinguish the different cell populations during culturing or to reisolate the different cell types after culturing to determine the effects of intermixing. We have used the dyes CMTMR and CMAC to stably label purified cells for these purposes, and they also may be useful for marking purified cells before introducing them into recipient embryos. These are membrane-permeant fluorescent dyes that contain a chloromethyl reactive group. They readily enter cells, where they are stably retained, probably by reaction of the chloromethyl group with intracellular glutathione (Zhang *et al.*, 1992). After sorting, the purified cells are incubated in S2 medium + 80 nM CMTMR, or S2 medium + 160 nM CMAC, for 5 min at 25°C. After the cells are washed twice with S2 medium to remove unbound dye, two differentially marked populations can be cultured together (or cultured with unmarked cells) and viewed by fluorescence microscopy, or they can be resorted according to their CMTMR or CMAC fluorescence to reisolate the two populations. Note that the residual fluorescent products from the CBAM staining may interfere with the detection of the CMAC fluorescence because of spectral overlap. This is not a problem if the cells are cultured for several hours before analysis, as the products of

the CBAM staining eventually dissipate, but if cells are to be analyzed soon after sorting CBAM staining may have to be omitted.

IV. Conclusions

WACS is a relatively new technique that has been successfully used to isolate and study a number of different early embryonic cell types *in vitro*. In its current form, it can be used to prepare analytical quantities of almost any early embryonic cell type, provided a *lacZ* strain that expresses β -galactosidase specifically in the cells of interest is available. So far, we have used WACS to purify cells from early stages of embryogenesis; applying WACS to later stages of development will probably require the use of other methods for cell dissociation besides the mechanical procedure (douncing) described here, as most cells become more adherent as the embryo matures. Although traditional methods such as treatment with trypsin or EGTA can be used, we can also envision the introduction of a transgene that can be conditionally activated to disrupt cell associations when needed. It will also be necessary to determine whether endogenous β -galactosidase activity is present at these later developmental stages, which could interfere with the procedure unless it can be specifically inhibited, for example by treatment with chloroquine (Fiering *et al.*, 1991).

Although FDG works well in this procedure, better substrates for detecting *lacZ* expression might improve both the yield and purity of cells obtained by WACS. FDG is not freely permeable across plasma membranes, requiring hypotonic conditions to introduce the substrate into cells and making it difficult to load high concentrations. Also, the fluorescent product of the β -galactosidase reaction is not efficiently retained at 25°C and the cells must be kept at 4°C until sorting is complete. New substrates with better uptake properties that give rise to products that are retained in the cells at 25°C would be useful, as would substrates that are converted into products of greater fluorescence, to allow clean separation of β -galactosidase-expressing cells from nonexpressing cells; new FDG analogs that have been described (Zhang *et al.*, 1991; Haugland, 1992) should be evaluated. Also, development of whole new ways of marking and purifying cells, such as using antibodies against cell surface antigens (see below), developing viable, fluorogenic or luminescent probes for other intracellular markers such as luciferase, or expression of *Aequorea victoria* green fluorescent protein, would also be useful adjuncts to the current method as they would allow combinations of markers to be used and thereby increase the variety of cell types that could be isolated.

Perhaps the major limitation of the current methodology, at least for some applications, is in the number of cells that can be obtained in a reasonable period of time and at reasonable cost. Adequate numbers of cells can be obtained for most cell-based analyses such as antibody staining of fixed cells, and for protein immunoblots, but for many applications it would be extremely useful

to have an essentially unlimited supply of purified cells, particularly for biochemical analysis and for preparative purposes such as protein isolation from the purified cells. The limiting factor is the speed of the cell sorter, which can analyze and sort cells at rates of up to $\sim 10^7$ cells per hour. At these rates, it would be impractical to purify adequate quantities of even the most abundant cell types in a preparation for most preparative purposes. We are considering two possible solutions. One is to use another method for isolating marked cells that does not rely on flow cytometry. For example, "panning" with cell-type-specific antibodies immobilized on a solid support would increase throughput, and it would also avoid the expense of cell sorting, which becomes prohibitive for large-scale purifications. However, this approach requires an antibody against a cell-type-specific surface marker, or the production of a transgenic strain that expresses an exogenous cell surface marker in the cells of interest, and it would not take advantage of the large battery of existing β -galactosidase-expressing strains. An alternative approach is to attempt to immortalize cells purified by the standard procedure, for example by transforming them with activated oncogenes, to establish cell lines of identified cell type.

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