Chapter 16

Flow-Cytometric Analysis of Endocytic Compartments

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I. Introduction

The use of flow cytometry for the characterization of the endocytic pathway is the subject of this chapter. In particular, methods for measuring the pH of endocytic compartments in living cells, and for analyzing individual organelles, are presented here. It is our intent to provide sufficient detail about many of these techniques to allow their use without reference to previous publications. Information on topics not

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covered here, particularly flow-cytometric analysis of ligand binding, internalization, and degradation, may be found in several reviews (Sklar, 1987; Murphy et al., 1988; Murphy, 1988, 1989).

II.   Methods for Analysis of Living Cells

The most widely used applications of flow cytometry are the analysis of DNA content (cell cycle analysis) and the measurement of cell surface markers. These applications make use of a number of the features of flow cytometry, which include accurate quantitation of measured variables, lack of significant photobleaching (due to the short time for which each cell is analyzed), ability to acquire information on large numbers of cells (typically 10,000–100,000), correlated measurement of more than one variable per cell, and the potential for sorting individual cells for further analysis. Both of these applications involve *static* analysis of variables that do not change over the time course of typical experiments, and, because the measurements do not require living cells, samples are often fixed before analysis. Recently, the excellent temporal resolution of flow cytometry (the time at which individual cells are measured can be recorded with an accuracy <1 second) has been exploited for the analysis of the *kinetics* of physiological processes in living cells. Examples include continuous measurements of ligand binding and cellular responses (McNeil et al., 1985), changes in intravesicular pH (Sipe and Murphy, 1987; Cain and Murphy, 1988), and degradation of endocytosed substrates (Jongkind et al., 1986; Roederer et al., 1987). The following section focuses on methods for measuring intravesicular pH in living cells.

A.   General Methods

1.   SYNTHESIS OF FLUORESCENT CONJUGATES

For the examples described here, the following ligands and labeling procedures were used. Human transferrin (Tf) was labeled with fluorescein isothiocyanate (FITC) or lissamine rhodamine sulfonyl chloride (LRSC) as previously described (Sipe and Murphy, 1987). Dye–protein ratios were determined spectrophotometrically to be 4.3 (FITC–Tf) or 5.9 (LRSC–Tf). These ratios were optimal for both specificity of binding (>90%) and signal–autofluorescence ratio on cells (>3). Dextran, 70,000 D (Sigma) was labeled with FITC or substituted rhodamine isothiocyanate (XRITC) using the dibutyltin-dilaurate method (De Belder and

Granath, 1973). The labeled dextrans were purified using five cycles of ethanol precipitation to eliminate labeling artifacts due to low molecular weight contaminants (Preston et al., 1987). It is especially important to purify commercial preparations of labeled dextrans. FITC and LRSC were purchased from Molecular Probes and XRITC was purchased from Research Organics.

2.   FLOW CYTOMETRY

A modified FACS 440 (Becton Dickinson, Mountain View, CA) was used for all analyses reported here. For most experiments, an argon laser at 488 nm (400 mW) and a krypton laser at 568 nm (100 mW) were used for excitation. Forward scatter was collected by a photodiode. Side scatter was collected through a 488-nm bandpass filter (10-nm bandwidth) with a photomultiplier. Fluorescence from FITC was collected through a 530-nm bandpass filter (15-nm bandwidth) with a photomultiplier whereas XRITC or LRSC fluorescence was collected through a 625-nm bandpass filter (35-nm bandwidth). Because of the optical configuration of the FACS 440 dual-laser system, spillover between the two fluorescences was negligible. Data were collected in list mode using a VAXstation II/GPX computer (Digital Equipment Corp., Maynard, MA) and the Consort/VAX software package (Becton Dickinson Immunocytometry Systems, Mountain View, CA). In addition to the standard programs in this package, other utility programs were used in data analysis. These programs are available from the FACS Computer Users Group Library (c/o R.F.M.).

The methods described next should be applicable to any flow cytometer, with some restrictions. For accurate acidification kinetics, a dual-laser (either argon–krypton or argon–argon) cytometer is required. A dual-laser system is *not* required for acridine orange (AO) measurements or for many analyses of individual organelles. The cytometer should have the ability to acquire data in a time-resolved fashion, either by using an analog or digital clock and recording time as a parameter (Martin and Swartzendruber, 1980), or by recording the number of events that occur in each time interval (McNeil et al., 1985).

B.   Measurement of Acidification of Endocytosed Probes

Currently, there are at least three fluorescence methods used to measure ligand acidification kinetics *in vivo*. These include the dual-excitation ratio method (Ohkuma and Poole, 1978), the amine ratio

method (Murphy *et al.*, 1982a,b), and the dual-fluorescence ratio method (Murphy *et al.*, 1984). These methods are based upon the decreased quantum yield of fluorescein fluorescence at acidic pH (Martin and Lindquist, 1975). To determine the pH to which the ligand is exposed, measurements of the amount of ligand present and the relative quantum yield of the fluorescein label are required. The three methods differ in how these two measurements are made. A brief description of each of the methods is given here.

1. DUAL-EXCITATION RATIO METHOD

Ohkuma and Poole (1981) were the first to measure directly the pH of endocytic compartments using fluorescently labeled probes. The method is based upon the observation that the ratio of fluorescein emissions when excited at 450 and 490 nm is a monotonic function of pH. Using this ratio technique, they observed a pH of 4.5–4.8 in lysosomes of mouse peritoneal macrophages labeled with FITC–dextran for 24 hours. This technique has been widely used to assay intravesicular pH (e.g., Tycko and Maxfield, 1982; van Renswoude *et al.*, 1982). A major disadvantage of this technique is that it involves calculating the ratio of two pH-dependent emissions. Fluorescein fluorescence is highly quenched at low pH, thereby decreasing the signal–noise ratios for ligands with small numbers of receptors to levels comparable to cellular autofluorescence.

An illustration of the use of the dual-excitation method in flow cytometry is given in Section II,C on the use of membrane-permeant probes. The same general approach is applicable to fluorescein-conjugated ligands.

2. AMINE RATIO METHOD

An alternative to the dual-excitation method is the measurement of fluorescein fluorescence (with excitation at 488 nm) before and after neutralization of acidic compartments with an equilibrating agent, such as a weak base or ionophore (Murphy *et al.*, 1982a,b). The neutralization results in the unquenching of fluorescein fluorescence and thereby allows the measurement of total cell-associated fluorophore. The intravesicular pH is then calculated from the ratio of the fluorescence emissions before and after neutralization (by comparison with a calibration curve generated *in vitro* or *in situ*). This technique has been used to demonstrate that the acidification of FITC-conjugated epidermal growth factor is biphasic, with initial rapid acidification to pH 6.3 within 3–5 minutes followed by a

slower acidification to pH 5.3 over 40 minutes (Roederer and Murphy, 1986). When using this approach, care must be taken that the methods used to neutralize intravesicular pH result in reproducible equilibration to a known pH. This method is well suited for use with single-laser flow cytometers. The major disadvantage of the technique is that an average pH value is determined for a population of cells, and therefore, only discontinuous kinetics measurements can be made (i.e., by taking samples at defined time points and measuring them in the presence and absence of an equilibrating agent).

3. DUAL-FLUORESCENCE RATIO METHOD

The limitation of the amine ratio method can be overcome by using two different fluorescent conjugates of the same ligand, with one of the conjugates being pH-dependent (fluorescein) and the other being pH-independent (rhodamine) (Murphy *et al.*, 1984). Using this strategy, simultaneous measurements of total uptake (reflected mainly by rhodamine emission) and acidification (reflected mainly by fluorescein emission) can be made. This method has been used to make measurements of lysosomal pH (Cain and Murphy, 1986), to determine the kinetics of transferrin acidification (Sipe and Murphy, 1987), and to demonstrate the role of the Na^+,K^+-ATPase in regulation of endosomal acidification *in vivo* (Cain *et al.*, 1989).

The following protocols were used to determine the kinetics of acidification of transferrin (Sipe and Murphy, 1987), and can be used for other ligands with only minor modification. Three sets of information are required to calculate a pH value: measurements of rhodamine and fluorescein fluorescence as a function of time after internalization, measurements of the amount of ligand remaining on the cell surface as a function of time after warmup (in that surface-bound transferrin contributes to the fluorescence emission from the cell and would shift the measured pH toward neutrality), and a standard curve relating the fluorescein–rhodamine fluorescence ratio to pH.

a. Acidification Measurements. The same protocol is used for both adherent and nonadherent cells (nonadherent cells are washed by centrifugation at 800 g for 5 minutes, at 4°C). Chill ~1 × 10⁶ cells in phosphate-buffered saline (PBS; 0.14 M NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$) at 4°C for 15 minutes, wash three times with PBS, and label the cells at 4°C for 20–30 minutes in 2 ml of PBS containing 10 µg/ml FITC–Tf and 5 µg/ml LRSC–Tf. After incubation, wash the cells four times with PBS and scrape (adherent cells) or resuspend (nonadherent cells) into 1 ml of PBS
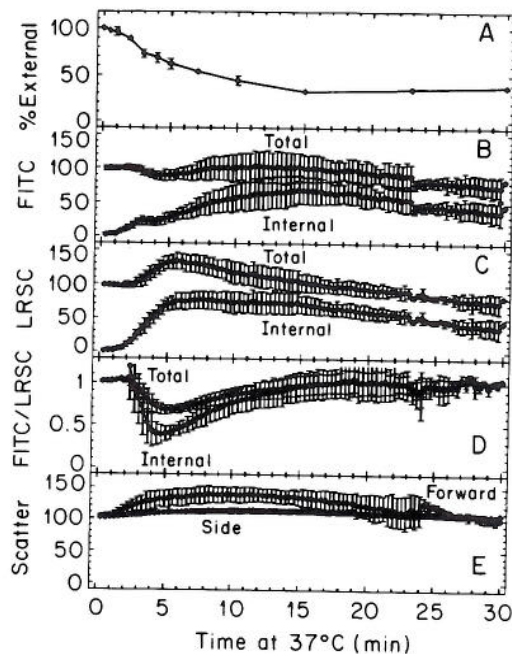
FIG. 1. Continuous measurement of transferrin acidification kinetics by flow cytometry. BALB/c 3T3 cells were allowed to bind labeled transferrin at 4°C, washed, and warmed to 37°C. Total FITC–Tf fluorescence (B) and LRSC–Tf fluorescence (C) were measured for individual cells as a function of time. The average values for six experiments, normalized to percentage of initial values, are shown. The percentages of transferrin on the surface at various times (A) were determined as described in the text and used to calculate the *internal* fluorescence (B,C). The ratio of these internal FITC–Tf and LRSC–Tf fluorescence values was calculated, and the results are shown in (D). Forward and side scatter are shown in (E). Error bars indicate 1 SD. From Sipe and Murphy (1987).

at 4°C. The amounts of FITC–Tf and LRSC–Tf can be adjusted to provide approximately equal signal–noise ratios for both probes. If an energy source is desired, 2 g/liter glucose can be added to PBS (incubation and warmup to 37°C in PBS containing 2 g/liter glucose yields results identical to those obtained in the absence of glucose).

Analyze the cell suspension by flow cytometry at 4°C for ~4–6 minutes to establish initial values for green and red fluorescence at the external pH. Warm the cells to the desired temperature and record forward and side light scatter, green and red fluorescence values, and time of analysis in list mode at ~200–400 cells/second. (This may be done using the kinetics option of the ACQ8 program in the Consort/VAX software.) Of practical importance is the temporal resolution with which data are collected. Because the computer word (or byte) used to store the list mode data has finite length, the total acquisition time for an experiment is inversely proportional to the temporal accuracy of the measurements. We generally use an accuracy of 1 second, allowing a maximum acquisition time of ~5 hours.

Background values for green and red fluorescence should also be recorded using unlabeled cells. These autofluorescence values are subtracted from those obtained with labeled cells before further analysis. Calculate average values for all parameters over 10- or 20-second intervals (the KINPRO program may be used for this purpose). An example of this type of analysis is shown in Fig. 1B,C,E.

b. Determination of Surface-Bound Transferrin. For receptor-bound probes, either the percentage of ligand remaining on the surface during the warmup must be determined or such ligand must be completely removed before analysis (e.g., using an acid wash or protease treatment), because surface ligand will artificially increase the estimate of the pH of ligand-containing compartments. Because removal of surface ligand makes continuous acidification measurements impossible, the former method is preferred.

Label cells as before using 15 μg/ml LRSC–Tf and no FITC–Tf. After washing, scrape or resuspend the cells into 2.5 ml of PBS at 4°C. To determine the total amount of Tf bound, remove 200 μl of the cell suspension and add it to 3 ml of ice-cold PBS. Warm the remaining cell suspension to the desired temperature, and at various periods of time during the warmup remove 200-μl aliquots and add them to 3 ml of ice-cold PBS. It is important that the PBS be ice cold to ensure that internalization of the LRSC–Tf is stopped as quickly as possible. After all samples have been collected, pellet the cells by centrifugation at 4°C. Resuspend the cell pellet into 250 μl of PBS containing 15 μg/ml FITC-conjugated goat anti-human Tf antibody (Tago, Burlingame, CA),

and incubate at 4°C for 20 minutes. After incubation, add 3 ml ice-cold PBS, mix, and pellet the cells by centrifugation. Resuspend the pellet in 3 ml of PBS, mix, and pellet by centrifugation. Wash three times with 3 ml of PBS, and resuspend the final cell pellet in 500 μl of PBS. Analyze ~10,000 cells at 4°C by flow cytometry. Determine the background fluorescence using unlabeled cells incubated with the FITC-conjugated antitransferrin antibody only, and subtract this nonspecific fluorescence to determine the specific transferrin fluorescence. Calculate the amount of transferrin remaining on the cell surface as a percentage of fluorescence intensity of the control sample held at 4°C. An example of this analysis is shown in Fig. 1A.

c. pH Calibration. Surface-label cells with FITC–Tf and LRSC–Tf,

and wash as described before. Scrape or resuspend the cells into 1 ml of various pH buffers at 4°C. The buffers are as follows: 0.05 M 3-[N-tris(hydroxymethyl)methylamino]-2-hydroxypropanesulfonic acid (HEPES), pH 7.6, 7.0, 6.6; 0.05 M 2-(N-morpholino)ethanesulfonic acid (MES), pH 6.0 and 5.6; 0.05 M NaOAc, pH 5.1 and 4.6; all containing 0.15 M NaCl. Analyze by flow cytometry at 4°C as described before. Calculate mean fluorescence values and subtract autofluorescence. An example of the resulting set of calibration curves is shown in Fig. 2.

To determine pH calibration curves for fluid-phase markers or ligands that dissociate from their receptors in response to low pH, it is necessary to generate an internal standard curve (Cain and Murphy, 1986). After internalization of the probe, incubate the cell suspension at 37°C in 200 mM 2-deoxyglucose and 40 mM sodium azide for 10 minutes to deplete
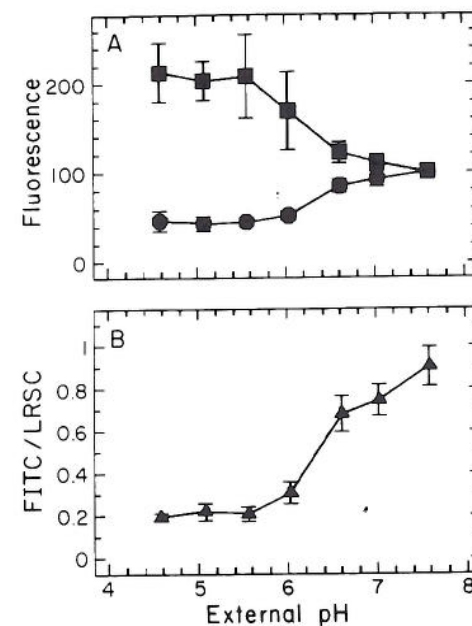
FIG. 2. pH calibration for FITC–Tf and LRSC–Tf. Cells were labeled, washed, and scraped into the appropriate buffer as described in the text. The pH dependence of fluorescence emission for surface-bound FITC–Tf (●) and LRSC–Tf (■) are shown in (A). The increase in LRSC fluorescence presumably corresponds to the conformational changes associated with the loss of iron from diferric transferrin. (B) The ratio of FITC and LRSC emissions was calculated from the data shown in (A). Note that while the fluorescence of both FITC–Tf and LRSC–Tf is pH-dependent, the fluorescence ratio is a monotonic function of pH. From Sipe and Murphy (1987).

cellular ATP pools. After incubation, dilute the suspension 1:1 into the pH calibration buffers used before (use at 0.1 M; final 0.05 M) and add nigericin (protonophore) to 75 μM for 10 minutes to equilibrate internal compartments to the external pH. Analyze by flow cytometry. Without the addition of the metabolic inhibitors, the cells are still able to maintain some pH gradients even in the presence of 75 μM nigericin.

d. Calculation of Internal pH. To allow comparisons between experiments, mean values for green (FITC–Tf) and red (LRSC–Tf) fluorescence for each experiment are divided by the mean initial fluorescence and averaged for all experiments. Internalization data are also averaged and interpolated over the same intervals, thereby allowing estimation of the amount of FITC–Tf and LRSC–Tf remaining on the cell surface at any time point during the experiment. After all measurements have been converted to percentages (of initial values), the percentage of external transferrin is subtracted from the mean fluorescence values for each interval. The ratio of the resulting *internal* green and red fluorescence values for each interval is then interpolated onto the normalized and averaged pH calibration curve, thereby allowing the calculation of an average pH for all compartments containing internalized transferrin (for each time interval). The calculations may be performed using the GENpH program (available through the FACS Computer Users Group Library, c/o R.F.M.). Typical results are shown in Fig. 3. For ligands that recycle, such as transferrin, acidification to approximately pH 6 is followed by alkalinization during recycling.

C. Acidification Measurements with Membrane-Permeant Probes

1. Yeast Vacuole pH Measurements with 6-Carboxyfluorescein Diacetate

As an illustration of the dual-excitation method, preliminary results on the analysis of yeast vacuole pH (Preston *et al.*, 1989) are described here. The vacuole in *Saccharomyces cerevisiae* resembles mammalian lysosomes in being an acidic organelle that contains hydrolytic enzymes. Yeast is an attractive experimental organism in that it allows facile application of classical and molecular genetic methodologies. However, it should be borne in mind that *Saccharomyces* cultures consist of free-living single cells with cell walls; this and other differences differentiate it from mammalian cells. The yeast vacuole differs from mammalian lysosomes both structurally and functionally, as does the plant vacuole. Of practical concern, *Saccharomyces* responds to drugs that are useful in

FIG. 3. Acidification kinetics of transferrin in BALB/c 3T3 cells. pH values were calculated over 20-second intervals as described in the text from the data shown in Figs. 1 and 2. Results are shown for cells warmed to 37°C (▲), 24°C (◆), and 17°C (no symbol). Note that alkalinization does not occur at the low temperatures. From Sipe and Murphy (1987).

studies of (mammalian) lysosome physiology in an idiosyncratic manner, if at all. For example, the minimal medium most widely used for yeast cultures contains nearly 0.1 M ammonium ion. How (and in fact whether) yeasts maintain any acidic compartment in the presence of this weak base remains to be shown. Another practical consideration involves the difficulty of labeling yeast vacuoles with macromolecular tracers, such as FITC–dextran, that have been widely used in studies of the endocytic pathway in mammalian cells (Preston et al., 1987). Recent results demonstrate the feasibility of using a low molecular weight fluorescent probe to assay vacuolar pH by the dual-excitation ratio method either by fluorescence microscopy (Pringle et al., Chap. 19, this volume) or by flow cytometry (Preston et al., 1989).

Protocol. Yeast cultures are grown, handled, and labeled with 5 μM 6-carboxyfluorescein diacetate (6-CFDA) as described in detail in this volume (Pringle et al., 19). After labeling, the cells are washed with YEPD (1% yeast extract, 2% Bacto-peptone, 2% glucose) at 0°C and kept on ice at 1×10^8 cells/ml in YEPD. The label appears to be vacuole-specific (by fluorescence microscopy) and stable for at least 2 hours under these conditions. Analysis by flow cytometry is performed as described earlier, using dual-argon lasers at 458 nm and 488 nm, and with minimal

laser power consistent with useful signal–noise ratios (we use ≤40 mW at both wavelengths). For the analysis, the cells are diluted 1 : 100 into appropriate buffers or test media at ambient temperature (24°C). At 24°C, the rate of loss of 6-carboxyfluorescein (6-CF) is minimal (~5–10%) over this period. The pH–fluorescence ratio standard curves (Fig. 4) are constructed by analyzing cells after equilibration for 10 minutes at 24°C in analysis buffer (50 mM KCl, 50 mM NaCl, 50 mM HEPES, 50 mM MES), at various pH values, in the presence or absence of a mixture of clamping agents [0.2 M NH$_4$Ac, 10 mM NaN$_3$, 10 mM 2-deoxyglucose, 25 μM carbonyl cyanide chlorophenylhydrazone (CCCP)]. The clamping agents do not affect 6-CF localization over the time of the analysis. Glucose, 110 mM, was added to the unclamped cells to prevent coalescence of the vacuole (Pringle et al., Chap. 19, this volume).

An important criterion for the validity of pH measurements using fluorescence methods is the demonstration of agreement between *in vitro* and *in situ* standard curves (Cain and Murphy, 1986). These curves are
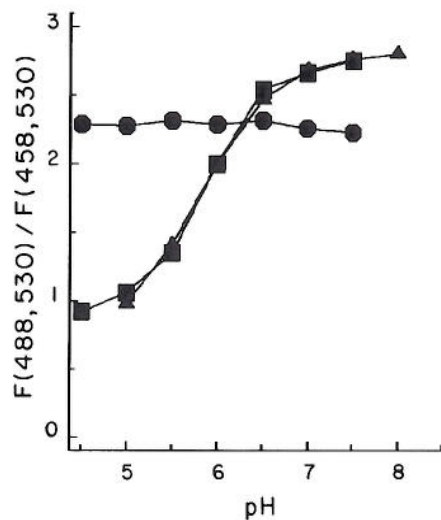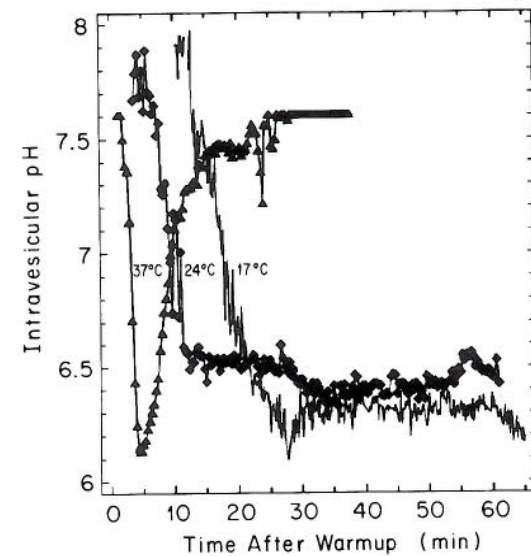

FIG. 4. Dual-emission ratio measurement of yeast vacuolar pH. Cells labeled using the protocol described in the text were diluted into analysis buffer at 24°C at the indicated pH values, in the absence (●) and presence (■) of protonophores. The fluorescence ratio was determined 10 minutes after dilutions were made. The curve obtained in the presence of protonophores constitutes the *in situ* standard curve (see text). An *in vitro* standard curve (▲) was obtained by fluorometry of solutions of hydrolyzed 6-CFDA (see text), and normalized such that the value at pH 6.0 was equal to that of the clamped curve. From Preston et al. (1989).

μg/ml unlabeled 7.5-μm polystyrene beads (Flow Cytometry Standards Corporation, Research Triangle Park, NC). The presence of the beads allows for the determination of background fluorescence from the AO in solution by the analysis of the apparent fluorescence of the unlabeled beads. Measure baseline autofluorescence at 37°C by analyzing the sample on the flow cytometer. Logarithmic amplifiers should be used for all fluorescence parameters in order to record accurately the large changes in fluorescence typically observed (convert data to linear scale prior to further data analysis; this is done by the KINPRO program automatically). Add AO (Kodak Laboratory and Specialty Chemicals, Eastman Kodak Co., Rochester, NY) to a concentration of 200 ng/ml (0.663 μM), and monitor AO uptake for 15 minutes. Add NH$_4$Cl to 100 mM, and continue analysis for 15 minutes to monitor loss of red fluorescence and recovery of green fluorescence as discussed later. Maintain sample temperature at 37°C and a flow rate of ~150 events per second. Upper and lower forward-scatter gates are used in the analysis of the data in order to analyze separately the fluorescence from live cells and beads.

It is important to note that the initial rate of AO accumulation in acidic compartments is dominated by existing proton gradients, and does not indicate the rate of acidification. For example, the chloroquine-resistant mutant CHL60-64, which has been shown to have a defect in the rate but not extent of lysosomal acidification, shows no difference in the initial amount of AO accumulated when compared to parental cells (Cain and Murphy, 1988). Upon addition of amines, red fluorescence is rapidly lost, indicating the loss of pH gradients within the cell. This loss of red fluorescence is stable for at least 15 minutes. Green fluorescence is also rapidly lost; however, this loss is only transient. Green fluorescence shows an exponential recovery to a value ~80% higher than the initial value. The recovery of green fluorescence represents the accumulation of AO within compartments due to continued proton pumping. The lack of recovery of red fluorescence indicates that the concentration of AO does not reach a sufficient concentration to allow multimer formation. This would occur because, in the presence of amines, pH gradients are not regenerated despite continued pumping; the compartments vacuolate as a result of osmotic pressure, thereby reducing the effective AO concentration within the compartment (de Duve *et al.*, 1974). Therefore, the recovery of green fluorescence is a measure of the total cellular rate of acidification, and, as shown in Fig. 5, the rate of recovery was almost three times higher in parental cells than in CHL60-64.

expected to be similar because the fluorescence of fluorescein derivatives is relatively insensitive to nonspecific ionic effects and molecular self-association. To construct an *in vitro* calibration curve, 6-CFDA was hydrolyzed at pH 10 and diluted to 1 μM in the buffers of different pH values described before. Fluorescence-emission curves were acquired with excitation at both 458 and 488 nm using a Gilford Fluoro IV spectrofluorometer, and the region from 515 to 545 nm was integrated. These conditions were chosen to approximate closely the detection conditions of the flow cytometer. The resulting calibration curve is in excellent agreement with the clamped *in situ* curve (Fig. 4). Comparison of the unclamped curve with the clamped calibration curve yields a value of ~6.2 for the vacuolar pH of log-phase cells, regardless of the external pH (between 5.0 and 8.0).

2. ACIDIFICATION MEASUREMENTS WITH ACRIDINE ORANGE

A rapid and fairly simple method to determine whole-cell acidification activity, which we have used to screen for potential acidification mutants, is the monitoring of the pH-induced accumulation of acridine orange (AO) (Cain and Murphy, 1988). Acridine orange is a weak base that accumulates in acidic compartments in the same manner as the vacuologenic amines (i.e., chloroquine and NH$_4$Cl). The unprotonated form is membrane-permeant, whereas the protonated form has reduced membrane permeability. In this manner, AO becomes trapped and accumulates within acidic compartments. When the concentration of AO becomes sufficiently high, dimers and higher order multimers form, resulting in spectral shifts in absorbance and fluorescence emission from green to red. Green fluorescence (excitation 488, emission 530 nm) may be used as a measure of total cell-associated AO, whereas red fluorescence (excitation 568, emission 625 nm) may be used as a measure of the extent of stacking of AO within acidic compartments. Both green and red fluorescence can be monitored on single-laser cytometers using 488-nm excitation and separate 530-nm and 625-nm emission filters. However, significant spillover from green to red fluorescence is observed, and thus the dual-laser method is preferred. In either case, the advantages of the AO method are that it allows for the rapid screening of a large number of mutants, and that it does not rely on the endocytosis and proper delivery of probes to acidic compartments.

Protocol. Harvest cells and dilute to 1 $\times$ 10^6 cells/ml in PBS. Add 20

characterize the endocytic pathway because biochemical analyses may be performed on isolated fractions. This technique is also limited in that the material separated for further analysis is not homogeneous, and as a result, colocalization studies are difficult to perform and interpret. Free-flow electrophoresis has now been used to identify multiple endocytic compartments and as a preparative tool for the identification of proteins present within endosomes (Schmid *et al.*, 1988). However, trypsinization of the vesicles was required before adequate separation was achieved, and the vesicle populations isolated were again not homogeneous.

It is therefore desirable to have a method to identify compartments based on their contents (temporally defined, if necessary), rather than by morphological or biophysical criteria. The feasibility of analyzing organelles by flow cytometry has been demonstrated previously (Murphy, 1985). We have coined the term single-organelle flow analysis (SOFA) to refer to the concept of measuring the properties of individual organelles in flow (Roederer *et al.*, 1989). In addition to our studies on endocytic organelles, a similar approach has been used to analyze plasma membrane vesicles (Gorvel *et al.*, 1984; Moktari *et al.*, 1986) and mitochondria (O'Connor *et al.*, 1988; M. Roederer, D. M. Sipe, J. Halow, A. Koretsky, and R. F. Murphy, unpublished results).

The SOFA technique has several advantages over techniques currently used to investigate endocytosis.

1. A major advantage of this technique is that it can be extremely rapid; lysates can be analyzed within 2 minutes of cell disruption. At this time, pH gradients are still present in endocytic vesicles (Murphy, 1985).
2. Because of the large number of events that can be analyzed (a typical analysis rate is 3000 particles per second), meaningful statistical analyses can be performed, and low-frequency events can be detected.
3. Vesicles can be analyzed for more than one parameter at a time, including forward and side scatter (related to size and optical density) and several fluorescence parameters. Thus, colocalization studies can be performed on an individual-vesicle basis.
4. The method can be used to localize enzyme activity to single-vesicle populations, using fluorogenic substrates (Murphy, 1985).
5. Flow sorting can be used to sort vesicles based upon the fluorescence of internalized ligands, thus allowing for the isolation of highly purified populations of endocytic vesicles (Murphy, 1985).
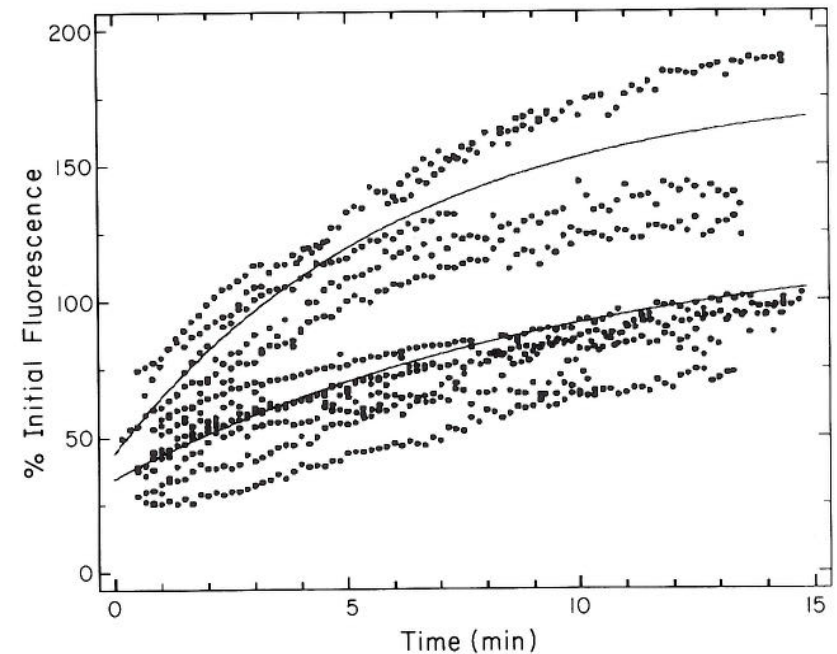
FIG. 5. Effect of ammonium chloride on AO accumulation *in vivo*. The recovery of AO green fluorescence after the addition of NH$_4$Cl was measured for parental 3T3 cells (upper trace) and CHL60-64 (lower trace) as described in the text. The data for multiple (five to seven) separate experiments are shown with fluorescence values normalized to percentage of initial fluorescence. The data have also been aligned so that the start of the recovery period is plotted at 0 minute. Each point represents the average of all events over a 10-second interval ~1500 cells) for a single experiment. The line is an exponential function drawn using the average values for extent of recovery, extent of loss, and the average rate. From Cain and Murphy (1988). Reproduced by copyright permission of the Rockefeller University Press.

III. Methods for Single-Organelle Flow Analysis

Endocytic compartments are extremely heterogeneous both morphologically and biophysically, making analysis and separation by standard techniques very difficult. Electron microscopy, which has been used to obtain structural and spatial information about the endocytic pathway in great detail, is limited for several reasons: only small numbers of samples can be examined easily, the information obtained is inherently static, and it is difficult to quantitate enzymatic and biochemical properties. Density gradient centrifugation, on the other hand, has been widely used to
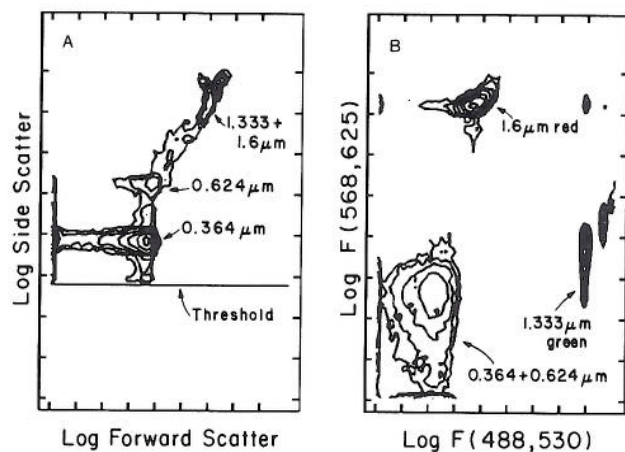
FIG. 6. Calibration standards and selection of light-scatter threshold for SOFA. A mixture of 1.6-μm red and 1.333-μm green beads (Polysciences), and 0.624-μm and 0.364-μm beads (Duke Scientific) were analyzed by flow cytometry and data for 30,000 events collected. Panels (A) and (B) show two-dimensional histograms of (A) scatter (log side scatter versus log forward scatter) and (B) fluorescence (488-nm excitation, 530-nm emission versus 568-nm excitation, 625-nm emission). The line in (A) indicates the position of the side-scatter threshold used for acquisition. Contour intervals are drawn at levels from 10 to 640 events per bin with a factor of 2 separating each contour, and tics are drawn at 0.5-log intervals.

A. General Methods

1. FLOW-CYTOMETRIC ANALYSIS

Because the small size of many organelles is at the limit of sensitivity and because of the heterogeneity in fluorescence intensity, logarithmic amplifiers, which provide 3–4 decades of intensity, should be used for all detectors. A fixed number of channels per decade should be used in all cases to facilitate conversion of data from log to linear and to allow accurate comparison between experiments.

Because the forward-scatter signal on most instruments is collected with a photodiode, which has less sensitivity than the PMT used for side scatter, a side-scatter threshold is used for all vesicle analyses. In order to set the side-scatter threshold consistently and for comparison between experiments, a mixture of 1.6-μm red and 1.333-μm green beads (Polysciences) and 0.624-μm and 0.364-μm beads (Duke Scientific) are analyzed at the beginning and end of each experiment. The threshold is set such that the 0.364-μm beads form a tight population several channels above the threshold. An example of the analysis of the bead mixture is presented in Fig. 6. At these settings, particulate matter in the normal sheath fluid gives a high background event rate; therefore, sheath fluid and all sample buffers should either be centrifuged at 27,500 g_{max} overnight or filtered through 0.1 μm Durapore membrane filters (Millipore) prior to use. In addition, light noise from extraneous sources should be reduced as much as possible by shielding the sample chamber and checking for light leaks into the optics housing. With filtered sheath fluid, there is still a "background" rate of ~40 events per second. This rate is from particulates in the sheath, PMT noise, scattering from the stream, and electronic noise. Sample flow rate or concentration should be adjusted such that event rates are at ~1000 per second to ensure a low percentage of background events. A minimum of 50,000 events should be collected for each sample.

different fluorescence signals in order to distinguish the probes. Therefore, it is often advantageous to calculate a derived parameter that represents the ratio of these two fluorescences. Because the data are collected in log mode, the log of the ratio may be obtained simply by taking the difference of the two parameters for each event (the CALC4 program may be used for this purpose). A constant offset is normally added to this log ratio to adjust the range of values obtained to within the bounds of the histogram.

B. Acridine Orange Uptake in Isolated Vesicles

As mentioned earlier, AO has been used to measure whole-cell acidification activity by flow cytometry (Cain and Murphy, 1988). In addition, AO accumulation has been used to determine the acidification activity in populations of vesicles from homogenized cells by fluorometry (e.g., Marnell *et al.*, 1984; Timchak *et al.*, 1986). We have demonstrated the feasibility of using the flow cytometer to analyze the

2. DATA ANALYSIS

Frequently, only a small percentage of the events recorded are from the population of interest (e.g., containing the fluorescent probe). These labeled events often appear as a tail from the distribution of unlabeled events. Therefore, a sample prepared from unlabeled cells should always be analyzed to obtain a "background" signal that can be used in the postacquisition windowing and analysis of the labeled samples.

Experiments that detect colocalization of probes involve the use of two
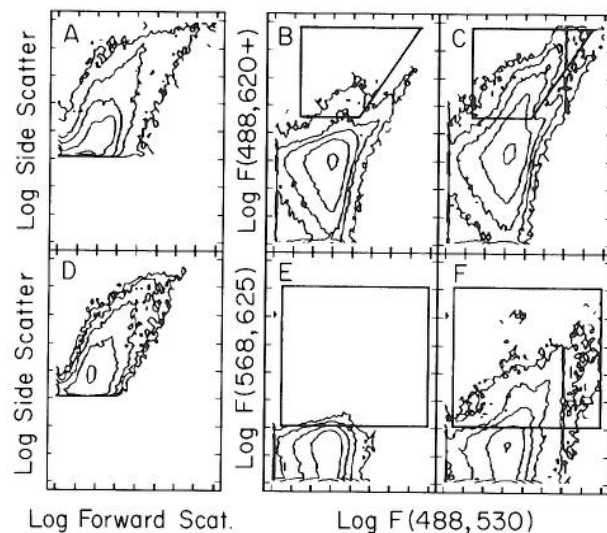
accumulation of AO within acidic compartments on a vesicle-by-vesicle basis using the flow cytometer. A protocol describing the technique is provided here.

Protocol. Scrape or resuspend 10^7 cells into 4 ml of homogenization buffer (HB; 0.25 M sucrose, 2 mM EDTA, 10 mM HEPES, pH 7.4) at 4°C. Homogenize the cell suspension using 10 strokes of a tight-fitting glass–Teflon homogenizer, and then prepare a postnuclear supernatant (PNS) by centrifugation at 1000 g for 10 minutes. Hold the PNS on ice and perform all subsequent incubations and analyses at room temperature. Dilute an aliquot of the PNS 1 : 5 into histidine buffer (30 mM histidine, 130 mM NaCl, 20 mM KCl, 2 mM MgCl$_2$, pH 7.0), and incubate at room temperature for 1 hour to allow for dissipation of existing proton gradients. Analyze the sample on the flow cytometer as described before to obtain background autofluorescence levels. Add AO to a concentration of 7.5 μM, incubate for 10 minutes, and analyze on the flow cytometer to determine the extent of acidification remaining after vesicle isolation. Add ATP to 2 mM, incubate for 10 minutes, and analyze to determine acidification activity. To dissipate pH gradients, add NH$_4$Cl to 100 mM, incubate, and analyze.

The results of the analysis of AO uptake are presented in Fig. 7. From these results, it is clear that this method can be used to determine qualitatively the acidification properties of isolated vesicles.

C. Colocalization of Endocytic Markers

One powerful aspect of SOFA is the demonstration of colocalization of endocytic markers on a vesicle-by-vesicle basis. We have used this technique to determine the kinetics with which newly endocytosed material is delivered to preexisting lysosomes and to follow the segregation of XRITC–dextran and FITC–Tf during endocytosis (Roederer *et al.*, 1989).

Labeling of Lysosomes and Endosomes. Label cells with 20 mg/ml XRITC–dextran in growth medium for 1 hour at 37°C, wash cells extensively (minimum of three changes) with PBS at 4°C, and chase in growth medium for 20 minutes. Wash the cells extensively at 4°C and add prewarmed (37°C) growth medium containing 20 mg/ml FITC–dextran for 10 minutes. Wash extensively at 4°C, prepare a PNS, and analyze the PNS by flow cytometry as described earlier. It is important that the cells be thoroughly washed, because even small amounts of free labeled dextrans can lead to a dramatic increase in the fluorescence background. If there is free dextran present, the entire population of vesicles (including
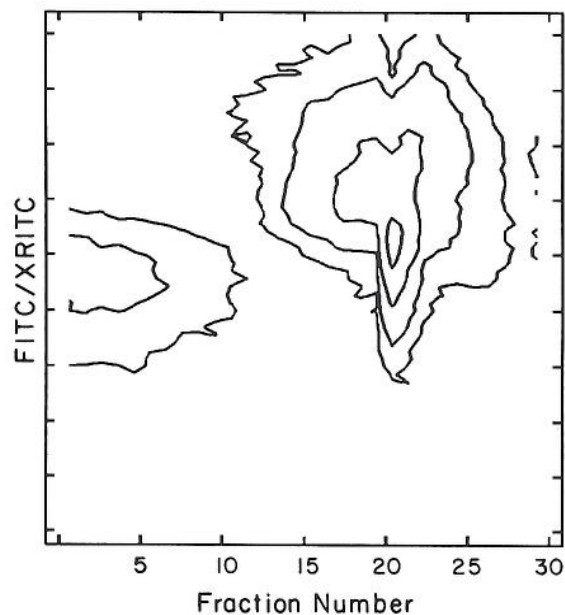
FIG. 7. Analysis of acidification of individual vesicles using AO. A PNS from Swiss 3T3 cells was prepared and assayed for AO uptake as described in the text. During analysis, 200,000 events were acquired. Contour lines in all panels are drawn to include at least 99%, 97%, 70%, and 10% of the total events. The regions drawn in the panels indicate AO-positive events. (A) A correlated histogram of side scatter versus forward scatter indicating the threshold used for acquisition. (B), (C) Two-dimensional histograms of fluorescence (488-nm excitation, ≥620-nm emission) before (B) and after (C) ATP addition. Before addition of ATP only 0.6% positive vesicles were detected compared to the unlabeled sample (data not shown). With the addition of ATP the AO-positive population increased to 11.3% of the total events recorded. This increase was due to acidification activity within the vesicles, because addition of 100 mM NH$_4$Cl decreased the frequency of positive events to 1.9% (data not shown). (D) A correlated histogram for forward scatter and side scatter for the AO-positive vesicles in (C). Because of spillover between the two emissions (see text) resulting from 488-nm excitation, the increase in AO concentration was monitored directly by exciting the higher order multimers at 568-nm light (E,F). Only 1.6% of the events were above background level before addition of ATP (E), whereas this amount increased to 10.2% after addition of ATP (F). From Roederer *et al.* (1989).

unlabeled vesicles) will be "shifted" in fluorescence as compared to an unlabeled sample.

The results of a typical analysis are shown in Fig. 8. Comparisons between an unlabeled sample (Fig. 8A) and the labeled sample (Fig. 8B) clearly demonstrate that endocytic compartments can be detected using fluorescently labeled fluid-phase markers and that endosomes (10-minute pulse) and lysosomes (1-hour pulse and 30-minute chase) can be kinetically defined with fluid-phase markers by SOFA. As discussed later, we
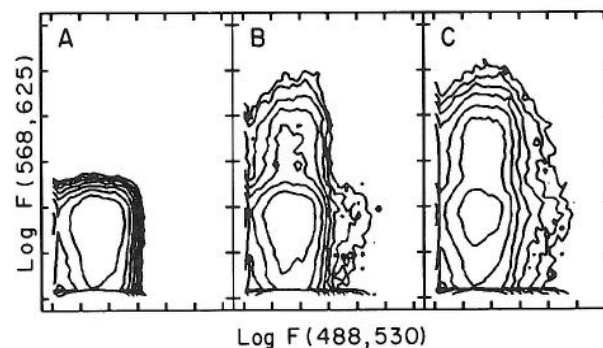
FIG. 8. Labeling of endosomes and lysosomes and the analysis of microsomal pellets. Human K562 cells were labeled and analyzed by flow cytometry for FITC– and XRITC–dextran fluorescence as described in the text. During analysis 250,000 events were recorded. Two-dimensional histograms of XRITC versus FITC fluorescences are shown for unlabeled (A) and labeled PNS (B), and a labeled microsomal pellet (C). Contour intervals are drawn at levels of 10–640 events with a factor of 2 separating each contour, and tic marks are drawn at 0.5-log intervals. Note the appearance of events positive for both red and green fluorescence after pelleting.

are currently investigating the feasibility of isolating these compartments by flow sorting for biochemical analysis. To eliminate contamination by cytoplasmic proteins present in the PNS, vesicles may be pelleted onto a sucrose cushion (see protocol in Section III,D). The effect of this purification is shown in Fig. 8C.

The SOFA technique can also be combined with density gradient centrifugation to permit correlation of fluorescence parameters with physical parameters. Fractions from density gradients (or other separation techniques) may be analyzed in sequence; if the data files from these fractions are combined in such a way that a new parameter corresponding to original fraction number is generated, the distribution of any flow-cytometer parameter versus density can be obtained (the COMBINE program from the Consort/VAX system may be used for this purpose). The analysis of a sequence of fractions is facilitated by automated sample-handling devices, such as the FACS Automate.

An illustration of this approach is shown in Fig. 9. Briefly, cells were labeled with XRITC–dextran for 1 hour, washed, chased in label-free medium for 120 minutes, and labeled with FITC–dextran for 15 minutes. Postnuclear supernatants were prepared and centrifuged on 27% Percoll gradients as described by Merion and Poretz (1981). Briefly, 2 ml of a PNS was mixed with 12 ml of a Percoll stock, to a final concentration of $1\times$ HB and 27% Percoll. These were centrifuged in an SA600 rotor at 25,000 g_{max} for 151 minutes in an RC5C Ultracentrifuge (DuPont Instru-

FIG. 9. Correlation of buoyant density and flow-cytometric parameters. Swiss 3T3 cells were labeled with 10 mg/ml XRITC–dextran for 60 minutes, washed at 4°C, and chased for 120 minutes. The cells were then labeled with 20 mg/ml FITC–dextran for 15 minutes. After labeling, the cells were washed at 4°C, and a PNS was prepared and fractionated on a 27% Percoll gradient as described in the text. Fractions of 0.4 ml were collected and analyzed for forward and side scatter, and FITC and XRITC fluorescence. A derived parameter, the log of the ratio of FITC–Tf and XRITC–dextran fluorescence values, is displayed versus fraction number. Because this analysis is several hours after isolation of the vesicles, pH gradients have dissipated and the fluorescein fluorescence is not quenched by low pH. Therefore, the derived parameter simply reflects the relative amounts of FITC– and XRITC–dextran in each vesicle. Contour intervals are drawn at levels of 100, 300, 1000, and 3000 events. Axis tics are drawn at 0.5-log intervals. From Roederer et al. (1989).

ments, Wilmington, DE). Fractions were collected from the bottom and analyzed on the flow cytometer for FITC and XRITC–dextran fluorescence. Note that the FITC–dextran positive events (high F/X ratio) are restricted to the low-density portion of the gradient, and that some intermediate-density events that are positive for FITC–dextran only are observed. We interpret the variation in density as resulting from maturation of endosomes (low density) into lysosomes (high density); the results indicate that the density change can occur without fusion with the dense XRITC–dextran-containing compartments (low F/X ratio).

D. Sorting of Endocytic Compartments

As discussed earlier, one of the advantages of SOFA is the possibility of obtaining organelle fractions of extreme purity. The procedures required to do this are no different from standard flow-sorting methods, with two exceptions. Only these exceptions will be discussed further. First, the nozzle head drive (which induces droplet formation) can induce significant noise in the scatter and fluorescence signals. This can be minimized by careful alignment of the optical system, with particular attention to the laser obscuration bars or beam stops. These bars act to prevent laser light scattered from the stream from entering the scatter and fluorescence detectors; when the droplet drive is on, scattering from the stream is increased and may be observed as an oscillation on the constant signal from the detectors and/or a dramatic increase in the event rate. Adjustment of the position of the obscuration bar to minimize this oscillation should significantly reduce this problem.

Second, because sorting of vesicles occurs in droplets, and these droplets contain volume from the original sample, contamination from cytoplasmic constituents is a potential problem. This is especially true when identification of proteins specific to endocytic compartments is desired. The solution is the removal of soluble cytoplasmic components by gel exclusion chromatography or pelleting onto a sucrose cushion.

Preparation and Analysis of Microsomal Pellets. The following protocol was adapted from Marsh *et al.* (1987). Cells (K562) were labeled as described earlier for labeling of endosomes and lysosomes. All of the following steps were performed at 0°C. Using a 3-ml syringe and 22.5-gauge needle, gently overlay 2.5 ml of HB onto 0.5 ml of cushion buffer (1 M sucrose in 10 mM HEPES, 2 mM EDTA pH 7.4; HB with 1 M sucrose as opposed to 0.25 M) in a 5-ml Beckman ultraclear centrifuge tube. Next, overlay the remaining PNS on top of the HB (this is possible because a small amount of PBS used in the wash steps remains during the preparation of the PNS, and therefore, the concentration of sucrose is slightly less than HB alone). Reserve 0.25 ml of the PNS for analysis. The layer of HB serves to reduce further the amount of contamination by cytoplasmic proteins. Spin at 4°C in an SW50.1 rotor at 100,000 g for 40 minutes. The microsomal pellet and the sucrose cushion are removed using a 3-ml syringe and 22.5-gauge needle and are added to 1.5 ml of HB without sucrose (final sucrose concentration is 0.25 M). Analyze both the PNS and the resuspended microsomal pellet. If the event rate is too high, dilute the samples with HB.

The results of the analysis are shown in Fig. 8C. As can be seen, there

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is an increase in the frequency of red and green positive events. This enrichment may be due to removal of cellular debris. In addition, a new population of vesicles containing both FITC and XRITC–dextran has appeared after preparation of the microsomal pellet. The appearance of this population could result from fusion and/or aggregation of the vesicles during the centrifugation or from the enrichment of a low-frequency population. We are currently investigating these possibilities.

Perhaps the most exciting prospect of this technique is the capability to specifically sort defined populations of vesicles for further biochemical characterization. The FACS is capable of sorting several thousand vesicles per second; preliminary vesicle-sorting experiments have been successful.

IV. Summary and Future Directions

A number of straightforward extensions of the methods described in this chapter may be envisaged. Recycling vesicles may be distinguished by coincubating with transferrin and dextran, and selecting high-transferring, low-dextran containing compartments. Endosomes may be defined as medium-transferrin, medium-dextran containing compartments. Lysosomes can be defined temporally, as late (high-density) dextran-containing (transferrin-free) compartments (Roederer *et al.,* 1989). Future experiments will utilize fluorescently tagged antibodies that are specific to the cytoplasmic tails of membrane proteins. We have obtained preliminary results on the use of antibodies to cytoplasmic determinants. Results for anticlathrin antibodies, either indirectly (D. M. Sipe and R. F. Murphy, unpublished results) or directly conjugated (F. Brodsky, unpublished results), have demonstrated that coated structures can be identified by SOFA. By the use of appropriate fluorescently labeled antibodies, in combination with endocytic markers, it will become easier to identify various compartments. Much as immunologically relevant cells have been classified on the basis of surface molecules, vesicles may be similarly identified ("vesiculotyping").

The technique of single-organelle flow analysis is the first isolation method that does not rely on a biophysical property; rather, it relies on biological properties. Analytically, it can distinguish the contents of individual vesicles at high speed to provide statistically significant data. Preparatively, it can provide the means by which highly purified populations of vesicles can be obtained.

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