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Analysis and Isolation of Adipocytes by Flow Cytometry

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Abstract

Analysis and isolation of adipocytes via flow cytometry is particularly useful to study their biology. However, the adoption of this technology has often been hampered by the presence of stromal/vascular cells in adipocyte fractions prepared from collagenase-digested adipose tissue. Here, we describe a multistep staining method and gating strategy that effectively excludes stromal contaminants. Initially, we set a gate optimized to the size and internal complexity of adipocytes. Exclusion of cell aggregates is then performed based on fluorescence of a nuclear stain followed by positive selection to collect only those cell events containing lipid droplets. Lastly, negative selection of cells expressing stromal or vascular lineage markers removes any remaining stromal contaminants. These procedures are applicable to simple analysis of adipocytes and their subcellular constituents by flow cytometry as well as isolation of adipocytes by flow sorting.

1. Introduction

Flow cytometry is a powerful technique that provides the ability to rapidly measure multiple cellular parameters across single cell suspensions and large cell populations with tremendous precision. During cytometry, analysis of light scatter can distinguish different cells based on their size, shape, and internal complexity. The presence and amount of specific intracellular and cell surface molecules can be measured with antibodies or ligands conjugated with fluorescent probes. Likewise, fluorescent indicators are available to measure the transport of ions across cellular membranes, as well as assess mitochondrial activity and other metabolic parameters. Specific cell subtypes can be isolated from mixed populations of single cell suspensions on specialized cytometers called “sorters.” In spite of the tremendous analytical power and advantages afforded by flow cytometry, this technology has been underutilized in the study of adipocyte biology.

A general assumption hampering the broad scale adoption of flow cytometry to the study of adipocytes is that they are too large (50–200 μm) and fragile to effectively analyze and sort using modern benchtop flow cytometers. However, the internal diameter of the fluidics of modern flow cytometers ranges from 150 to 250 μm —large enough to accommodate all but the largest fat cells. Moreover, typical flow pressures range from only 5 to 10 psi and the laminar flow within the cytometer exerts minimal shear stress on cells. In addition, adipocytes are fairly deformable. Their shape is largely defined by large cytoplasmic lipid droplets, which contain liquid triglyceride at room or higher temperature, rather than cytoskeletal elements. In collaboration with Amnis Corporation (Seattle, WA) we have acquired brightfield images of intact unilocular and multilocular adipocytes (and cell debris) in the fluidics stream of an ImageStream $\times$ Imaging Flow Cytometer (Fig. 15.1).

Selecting adipocytes and collecting them for study via flow sorting is a bit more complex than analysis alone. Sorting exposes cells to higher flow pressures, smaller fluidic diameters, and therefore, increased shear stress. Within the sorter, cells are encapsulated within droplets of sheath fluid. The trajectory of the droplets is controlled by imparting them with an electric charge. The path of the charged droplets is deflected toward an oppositely charged plate so that individual droplets may be captured in separate tubes. However, the high flow pressures and relatively small “tip” diameters (70–100 μm) required to produce the droplets may result in shear-induced adipocyte lysis. In spite of these conditions we find that a small percentage of fat cells survive sorting as determined by microscopy of the sorted cells (Fig. 15.2). Moreover, lysis of adipocytes during sorting can actually facilitate recovery of intact nuclei (Majka et al., 2010), nucleic acids, and other cellular constituents for further analysis (Majka et al., 2010; Schaedlich, Knelangen, Santos, Fischer, & Santos, 2010).

Applying these concepts, we and other laboratories have begun to exploit flow cytometry to study aspects of adipocyte biology with considerable success. For example, Shi and Kandror (2008) and Bruzzone et al. (2012) developed flow cytometry techniques to measure the degree of translocation of the insulin-stimulated glucose transporter, Glut4, to the membrane of 3T3-L1 adipocytes. Zhu et al. (2012) measured mitochondrial membrane potential and production of reactive oxygen species in Lym1-depleted 3T3-L1 adipocytes, while Festy et al. (2005) used flow cytometry to assess surface protein expression between human primary adipocytes and stromal cells. Other laboratories have employed flow cytometry to measure lipid accumulation in adipocytes (Lee, Chen, Wiesner, & Huang, 2004), assess production of Toll-like receptor 2 and tumor necrosis factor α in primary adipocytes (Murakami, Bujo, Unoki, & Saito, 2007), and track the differentiation of rat primary preadipocytes in response to anandamide (Karaliota, Sifakaki-Kapadai, Gontinou, Psarra, & Mavri-Vavayanni, 2009). We exploited flow cytometry and fluorescence deconvolution microscopy to demonstrate the production of adipocytes from bone marrow-derived progenitor cells (Crossno, Majka, Grazia, Gill, & Klemm, 2006; Majka et al., 2010, 2012). Additionally, we found that flow sorting of primary mouse adipocytes was a highly efficient means to isolate intact nuclei for cytogenetic analysis (Majka et al., 2010) and RNA for global gene expression analysis. Schaedlich et al. (2010) used similar methods to recover RNA from embryonic stem cell-derived adipocytes for RT-PCR analysis.

As obesity and metabolic diseases linked to adipose tissue continue to place health and financial burdens on society, scientists have begun to address complicated questions about adipocyte production and lineage, fat cell turnover, apoptosis and senescence. Flow cytometry is an important addition to the arsenal of sophisticated techniques that are now employed in these studies. The ability of flow cytometry to screen multiple adipocyte parameters over large cell populations is an advantage over microscopic methods. Moreover, even the best commercial laser confocal systems are limited to a nominal resolution of approximately 200 nm, making it difficult to discern the orientation and relationship between structures and markers in tissue sections and whole mounts. Flow cytometry overcomes this limitation by facilitating analysis of individual cells or events. Imaging flow cytometers, including the ImageStream $\times$ (Amnis, Seattle, WA) combine the large population/multiple parameter advantages of traditional flow cytometry, with the ability to acquire brightfield images of adipocytes in the flow stream. Finally, modern flow cytometers may exceed the ability of laser confocal microscopes to detect diffuse fluorescent signals (e.g., cytosolic EGFP), since fluorescence is integrated over the entire cell or event instead of individual pixels.

A final remaining challenge to using flow cytometry in the study of adipocytes is the presence of contaminating stromal/vascular cells. These additional cell types pose a complication in adipocyte fractions prepared by collagenase digestion. Repeated wash steps fail to remove all stromal cell contamination. We previously published a multistep flow cytometry procedure (Majka et al., 2012) that effectively excludes stromal/vascular cells from adipocyte preparations. The strategy involved gating or acquisition of cells whose size and internal complexity were greater than stromal/vascular cells, exclusion of cell aggregates, and exclusion of cells or events expressing stromal lineage markers. The procedure was validated by demonstrating distinct marker gene expression between adipocytes and stromal cells sorted by this strategy. More recently, we have made two important improvements to this strategy. First, we identify and collect single cells and exclude aggregates based on fluorescence of the nuclear stain, Vybrant DyeCycle Violet (Molecular Probes/Life Technologies, Eugene, OR). Second, we collect only events containing lipid droplets as determined by staining with HSC LipidTOX Neutral Lipid stain (also Molecular Probes).

In the following sections we provide detailed methods for preparation of free-floating adipocytes, staining of adipocytes with DyeCycle Violet, LipidTOX Deep Red, and fluorescent antibodies to stromal lineage markers. We further delineate our multistep gating regimen to capture and analyze adipocytes free of stromal contaminants.

2. Preparation of Adipocytes by Collagenase Digestion of Adipose Tissue

A single cell suspension of free-floating adipocytes is required for flow cytometry. Free-floating fat cells are isolated from adipose tissue by digesting the tissue in a buffered collagenase solution. Following digestion, undigested tissue fragments are removed by filtration, and the adipocytes are separated from stromal/vascular cells by centrifugation and/or flotation. There are numerous variations on this procedure in the literature; typically reflecting differences in digestion buffer composition and centrifugation/flotation

conditions. In general, most methods produce adipocyte suspensions of comparable quality. The procedure outlined below is routinely used in our laboratory however, it may be modified as appropriate based on specific assay parameters for successful flow cytometry of adipocytes.

1. Adipose tissue is weighed, rinsed with phosphate-buffered saline, and minced with scissors or razor blades to produce fragments of approximately 1 mm.
2. The tissue fragments are transferred to a 50-ml screw cap conical polypropylene centrifuge tube and digestion buffer (Krebs–Ringers – HEPES + 2.5 mM glucose + 2% fetal bovine serum + 200 μ M adenosine + 1 mg/ml collagenase (Sigma, C2139), pH 7.4) is added at roughly 1 ml/0.25 g tissue. Krebs–Ringers–HEPES contains 120 mM NaCl, 4.7 mM KCl, 2.2 mM CaCl_2 , 10 mM HEPES, 1.2 mM KH_2PO_4 , and 1.2 mM MgSO_4 .
3. The suspension is incubated for 1 h at 37 °C at 100 rpm on an orbital shaker.
4. Following tissue digestion the suspension is passed through a 150- μ m mesh Celltrics filter (Partec GmbH) followed by an equal volume of wash buffer (Hanks balanced salt solution [Cellgro 21-022-CV], 2% fetal bovine serum (FBS), 200 μ M adenosine, pH 7.4).
5. The filtered suspension is centrifuged at $150 \times g$ for 8 min. During centrifugation, the adipocytes will form a layer at the top of the liquid.
6. Transfer adipocytes to a clean 15-ml conical tube and add 3–4 volumes of wash buffer. Gently resuspend the adipocytes by rocking the sealed tube.
7. Centrifuge the suspension at $150 \times g$ for 8 min and transfer the adipocyte layer to a clean 12 $\times$ 75-mm polypropylene tube.

Comments and troubleshooting

1. Following collagenase digestion, filtration of the cell suspension through the Celltrics filter can be facilitated by placing a double layer of gauze above the filter mesh to retain large tissue fragments that may impede filtration.
2. Some protocols suggest centrifugation of adipose cell suspensions at $300\text{--}500 \times g$. We find this results in considerable adipocyte lysis evident by the presence of large, clear triacylglycerol droplets in the adipocyte layer following centrifugation. Centrifugation at $150 \times g$ prevents mechanical damage to the fat cells.
3. We generally transfer the floating adipocyte layer between tubes using a 1-ml PipetMan pipettor. We snip 1–2 mm off the end of the plastic pipette tip to enlarge the bore of the tip. This facilitates transfer of the viscous adipocyte layer.
4. Polypropylene plasticware is preferred over polystyrene and other materials.
5. Rocking rather than vortexing is preferred for resuspending adipocytes in wash buffer as it minimizes cell damage.

3. Staining Of Single Cell Suspensions From Adipose Tissue For Flow Cytometry Analysis

To isolate and analyze adipocytes free from contamination by stromal/vascular cells we have devised a flow cytometry strategy based on the (1) the significant size difference between large adipocytes and considerably smaller stromal cells, (2) exclusion of cell aggregates, (3) presence of large lipid droplets in adipocytes, and (4) exclusion of cells bearing stromal lineage markers. Initial “separation” of adipocytes and stromal cells by their size is based on differences in forward scatter (FSC) and side scatter (SSC) distributions between the two populations (described in Section 4). Single cells containing nuclei are distinguished from cell aggregates by DyeCycle Violet fluorescence, and events containing lipid droplets are identified by LipidTOX Deep Red fluorescence. Finally, any remaining stromal contaminants are excluded based on their staining with phycoerythrin (PE)-conjugated antibodies to stromal cell lineage markers. Figure 15.3A shows an example of this gating scheme. The following sections describe the procedures we routinely use to stain free-floating adipocytes with these reagents, which generally follow the recommendations of the reagent suppliers.

3.1. LipidTOX Deep Red neutral lipid staining of lipid droplets

Lipid droplets are often stained with reagents like Oil Red O or Nile Red. Oil Red-stained lipid droplets are easily identified by their intense red coloration under visible light or strong red fluorescence (Reusch & Klemm, 2002). However, staining with Oil Red O requires fixation of cells and staining in the presence of organic solvents (e.g., 60% isopropanol) and is, therefore, limited to the analysis of dead cells. Nile Red, while soluble in DMSO and other organic solvents, can be diluted into aqueous buffers for the staining of live or dead adipocytes. However, its emission maximum at 528 nm may overlap with green (FITC, GFP) and yellow–orange (PE) fluorophores in flow cytometry. For our flow cytometry applications, we have found LipidTOX stains useful as they can be applied in aqueous solutions to either live or fixed/dead cells. LipidTOX dyes are available with green (emission maximum 505 nm) or red (609 nm) fluorescence for applications where other green- or red-emitting fluorophores are not an issue, or in far-red emission (655 nm) to prevent overlap with green, yellow, or orange fluorophores. The following procedure details the steps we use to stain free-floating adipocytes with LipidTOX Deep Red.

1. LipidTox Deep Red is added to the adipocyte suspension at a 1:125 dilution and mixed gently by flicking the tube with a finger or by gentle inversion.
2. Incubate cells at 37 °C for 25 min with gentle inversion after 10–15 min.
3. Centrifuge cell suspension at $150\text{--}200 \times g$ for 2 min. Remove the supernatant from the layer of floating adipocytes.
4. Wash the cells by resuspending the adipocytes in an equal volume of wash buffer and centrifugation at $150\text{--}200 \times g$ for 2 min. Remove supernatant.
5. Resuspend cells to the previous volume with flow buffer (HBSS + 5% FBS + 200 μM adenosine).

Comment

At this stage we remove the supernatant rather than transferring the adipocyte layer (supernatant) between tubes to limit handling and lysis of the fragile fat cells.

3.2. Fluorescent antibody staining to exclude stromal/vascular cells from adipocytes

A crucial step in isolating or analyzing adipocytes by flow cytometry is exclusion of stromal/vascular cells from the adipocyte population. Our strategy to identify and exclude stromal cells is based on staining the free-floating adipocyte fraction with fluorescent antibodies to stromal lineage markers. For most experiments with mouse adipocyte preparations we use PE-conjugated antibodies to myeloid cells (CD11b, BD Pharmingen 557397), T lymphocytes (CD3, BioLegend 100206), B lymphocytes (CD45R, BioLegend 103208), red blood cells (TER-119, BioLegend 116208), granulocytes (Gr-1, BioLegend 108408), megakaryocytes and platelets (CD61, BioLegend 104308), endothelial cells (CD34, BD Pharmingen 551387), and neurons (NCAM, R&D Systems FAB5674P). In practice, it is possible to substitute a PE-conjugated antibody to the pan-leukocyte marker CD45 (BD Pharmingen 553081) for the cocktail of individual lineage-specific antibodies and exclude over 99% of stromal contaminants.

Antibody staining is done concurrent with LipidTOX Deep Red. Antibodies are used at a concentration of 0.25 μ M per 10^6 cells. Since accurate counts are often difficult to obtain with adipocytes, suspension volume is used as a proxy for cell number (i.e., if cells can be suspended easily in 0.5 ml it is assumed there are 10×10^6 or fewer cells. If a larger volume is needed the antibody amount is increased). Antibodies are added after LipidTOX Deep Red and the procedure above is followed. If not staining with LipidTOX Deep Red the antibody incubation is done on ice rather than at 37 °C, as adipocytes are somewhat more susceptible to lysis at warm temperatures needed for LipidTOX staining.

3.3. DyeCycle Violet staining to identify events with nuclei and distinguish singlets from aggregates

The study of adipocytes has often been thwarted by the inability to distinguish or separate adipocytes from stromal/vascular cells present in fat tissue. With its ability to analyze individual “events,” flow cytometry and sorting affords investigators a powerful tool to distinguish individual adipocytes from stromal contaminants based on optical and/or fluorescent properties. Single cells can be distinguished from cell aggregates by optical methods during flow cytometry. However, we have found that staining the adipocyte suspension with DNA stains allows us to distinguish single cells from aggregates, and unambiguously identify events that contain single nuclei. The following procedure describes our steps for staining free-floating adipocytes with the DNA stain, DyeCycle Violet.

1. After incubating adipocytes with LipidTOX and fluorescent antibodies and washing, the cells are resuspended to the previous volume and DyeCycle Violet is added to a dilution of 1:333 to the suspension and mixed gently.
2. Incubate at 37 °C for 30 min with one gentle mix after 15 min.

3. Cells are kept warm and transported for flow cytometric analysis with no further washing steps.

Comments

1. Hoechst 33342 can also be used to identify single cells as it stains nuclei in live or dead cells, and has an emission maximum close to that of DyeCycle Violet. When used with unfixed cells, DAPI and propidium iodide stain nuclei in dead cells or free-floating nuclei making them useful for distinguishing live versus dead cells. DyeCycle Violet is recommended for staining of nuclei in live cells by the manufacturer, but we find that it provides intense nuclear fluorescence in both live and dead cells, and can be used with unfixed or paraformaldehyde-fixed cells. We also find that DyeCycle Violet is generally more “forgiving” than DAPI, Hoechst, or propidium iodide, affording more flexibility with regard to concentration and incubation times.
2. The DyeCycle manufacturer recommends that cells be maintained at 37 °C following staining. We find this also prevents the adipocytes from congealing into a semisolid “clump” that is difficult for cytometer fluidics to aspirate. We transport cells between our laboratory and the flow cytometry core in an insulated container filled with glass beads preheated to 37 °C.

4. Analysis and Sorting of Adipocytes by Flow Cytometry

The following flow cytometry steps and gating regimen represent our day-to-day strategy performed on a MoFlo XDP Flow Cytometer/Sorter (Beckman Coulter, Inc., Brea, CA). However, we have used these same parameters on a CyAn ADP Analyzer and a Gallios Flow Cytometer (both Beckman Coulter), and they should be applicable to most modern flow cytometer systems.

4.1. MoFlo XDP settings

Adipocytes are sorted using a Moflo XDP cell sorter with Summit 5.3 software (Beckman Coulter). A 100- μ m nozzle tip is used with a sheath pressure of 30 psi and a drop drive frequency of 46,700 Hz and amplitude of 15 V. The sheath fluid consists of Isoflow (Beckman Coulter, Fullerton, CA). The sample and collection tubes are maintained at 15 °C using an attached Haake recirculating water bath. To keep cells in suspension the Moflo is equipped with a SmartSampler sample station with the sample agitation set to maintain an agitation cycle of 4 s on and 5 s off. Sort mode is set to Purify 1 to prevent the capture of cells close to unwanted events (e.g., clusters, debris, events lacking a nucleus or lipid droplet, events positive for lineage markers) in the droplet queue. Appropriate signal compensation is set using single color control samples.

Samples are analyzed using the following excitation lasers and emission filters: PE—552-nm laser and 580/23 bandpass filter; LipidTOX Deep Red—641-nm laser and 670/28 filter; DyeCycle Violet—UV laser and 447/60 bandpass filter. We use UV as an alternate excitation wavelength for DyeCycle Violet in lieu of the standard 405 nm violet excitation, because the MoFlo XDP is equipped with a Co-Lase (Propel Labs, Ft. Collins, CO) colinear

red-violet laser system and spillover of the DyeCycle Violet signal into the LipidTOX Deep Red detector precludes the use of the 405-nm laser for excitation of the DyeCycle Violet.

4.2. Isolation of adipocytes from stromal cells based on cell size: FSC versus SSC gating

Adipocytes are substantially larger (50–200 μm) than stromal cell populations (typically <20 μm), and the presence of highly refractile lipid droplets in their cytoplasm endows them with tremendous light scattering character. These features make it possible to separate adipocytes from the majority of stromal cells and cell debris during initial FSC versus SSC gating. Light scattered in line with the incident laser beam, the FSC, is influenced by cell size, whereas light scattered to the side, the SSC parameter, by organelles and lipid droplets is influenced by internal complexity. Analysis of flow cytometry data generally begins by plotting these two parameters on a scatter diagram. Because of the large size and refractile nature of adipocytes, we plot the log value for each parameter rather than their linear values.

Figure 15.3B shows a typical FSC/SSC plot for stromal/vascular cells. When compared to FSC/SSC distribution for free-floating adipocytes in Fig. 15.3A, it is evident that there is some overlap in the distribution of the events in each population. However, there are clearly cells with very high FSC and SSC values present in the adipocyte fraction that are not observed in the stromal/vascular fraction. We begin our isolation of adipocytes from stromal cells by gating solely on these large, refractile cells as indicated in the figure. There are some small adipocytes that do not fall within this gate, and if it necessary to analyze the entire fat cell population, one may gate on the entire cell population for further analysis.

4.3. Separation of single cells (singlets) from cell aggregates

Aggregates of adipocytes and stromal cells are common in fat cell fractions following collagenase digestion. To characterize or sort single cells, cell aggregates must be excluded from the analysis or sort. During flow cytometry this may be accomplished optically, by comparing the fluorescence pulse peak height (either FSC or SSC) to the pulse peak width or area. The ratio between peak height and peak width or peak area is proportional for single cells, which form a vertical distribution on a scatter plot of peak height versus peak width (Fig. 15.3C) or a diagonal distribution when peak height is plotted against peak area. Cell aggregates exhibit increased peak width and area compared to peak height and, therefore, do not fall on the distinct vertical (width) or horizontal (area) distributions of singlet events. Setting a gate around the singlets effectively excludes cell aggregates from further analysis and sorting.

Because of the unique optical properties of adipocytes (single and multiple refractile cytoplasmic lipid droplets), we have found that a similar gating strategy based on DyeCycle Violet fluorescence parameters is a more reliable method of identifying single cells. Figure 15.3A, second panel from the left, shows a plot of DyeCycle Violet fluorescence height versus fluorescence area for free-floating adipocytes. The diagonal distribution of single cells is easily distinguished from aggregates, and is gated for further analysis. The use of DyeCycle Violet in this step also ensures that all gated events contain a nucleus and are not free-floating lipid droplets or other debris.

4.4. Identification of events containing lipid droplets

Adipocytes contain one or more large triglyceride droplets, which distinguish them from relatively lipid-deficient stromal/vascular cells. This characteristic provides another means of separating fat cells from stromal contaminants. Lipid droplets (both within intact adipocytes and free-floating) stain intensely with LipidTOX dyes and are easily distinguished from lipid-free events. For gating purposes we compare the fluorescence profile (histogram) of a small portion of unstained adipocytes to the fluorescence of LipidTOX-stained cells (Fig. 15.3A, second panel from the right). The lipid-positive cells are gated for further analysis or sorting.

4.5. Exclusion of stromal/vascular cells

The final gating step in our strategy removes any remaining stromal contaminants based on their staining with PE-conjugated antibodies to stromal cell lineage markers. This step is necessary to remove any stromal cells that may adhere to free lipid droplets and thus not be excluded based on DyeCycle and LipidTOX staining in the previous gating steps. As with LipidTOX staining and analysis, we compare the PE fluorescence profile of a small aliquot of unstained cells to that of adipocytes stained with a cocktail of PE-conjugated antibodies to lineage markers on a histogram of PE fluorescence (Fig. 15.3A, far right panel). The small percentage of PE-positive events is excluded from the gated PE-negative adipocytes.

4.6. Validation of adipocyte isolation strategy

QRT-PCR for various stromal-specific factors was performed on RNA purified from adipocytes before and after flow sorting with our multistep gating strategy. Figure 15.3D shows that stromal-specific RNAs are absent in flow-purified adipocytes, but are readily detectable in the preflow adipocyte fraction.

5. Summary

This chapter presents a method for the separation of free-floating adipocytes from stromal/vascular cell contaminants during flow cytometry or flow sorting. The technique is eminently suited to the rapid analysis of multiple parameters in a large number of adipocytes, and can be adapted to suit a range of assay requirements. We routinely use this method to follow the production of adipocytes from progenitors expressing green fluorescent protein (detected with a 488-nm laser and 529/28 bandpass filter), and to obtain purified adipocyte nuclei for cytogenetic analysis. Obviously, antibodies to lineage markers conjugated to a wide variety of fluorophores are available commercially, and LipidTOX stains are available in green, red, and deep red versions. Therefore, a variety of color combinations compatible with many experimental requirements and instrument laser configurations are available. Given its many advantages, we encourage investigators to adopt flow cytometry to their arsenal of techniques as they unravel the intricacies of adipocyte biology.

Acknowledgments

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Figure 15.1.

Brightfield images of unilocular and multilocular adipocytes, and cell debris acquired with an ImageStream X Imaging Flow Cytometer at Amnis Corporation. The images demonstrate the feasibility of using flow cytometry to study intact adipocytes.

Figure 15.2.

Brightfield images of an intact unilocular and an intact multilocular adipocyte after flow sorting. Free-floating adipocytes were sorted on a MoFlo XDP instrument equipped with a 70- μ m tip. Adipocytes were collected in a 12 $\times$ 75-mm polypropylene tube containing 150 μ l of flow buffer. Images were acquired with a conventional light microscope.

Figure 15.3.

Multistep flow cytometry/sorting strategy. (A) Gating strategy for adipocyte isolation is diagrammed from left to right. In the first step, adipocytes are identified by their large size and refractile properties in a plot of FSC versus SSC. A gate is placed around the population of cells that are larger and more refractile than those present in the stromal/vascular fractions (B). In the next step, DyeCycle Violet (DCV) fluorescence of the gated adipocytes is evaluated in a plot of peak height versus peak area (note the linear scale). The singlets that form a diagonal distribution are gated, while cell aggregates are ignored. Singlets can also be identified optically by comparing SSC (or FSC) peak height to peak width or peak area as shown in (C). In the third step, LipidTOX fluorescence of the singlets is evaluated on a histogram. We compare the fluorescence signal distribution of LipidTOX-stained cells to a small portion of unstained cells to positively identify events containing a lipid droplet. Finally, any remaining stromal contaminants are excluded based on their labeling with PE-conjugated antibodies to stromal markers. The purified adipocytes can undergo further analysis and/or sorting. (D) QRT-PCR was used to verify the staining and gating strategy. The results show the presence of several stromal cells markers in adipocyte fractions prior to sorting. These markers were virtually undetectable in flow-purified adipocytes.