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Coping with artifact in the analysis of flow cytometric data

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ABSTRACT

This article highlights several sources of artifact that interfere with optimal analysis of flow cytometric data. Such problems are compounded when flow cytometry is performed on mechanically and enzymatically disaggregated solid tissues or on cultured cells, where subcellular debris, apoptotic or necrotic cells, and highly autofluorescent cells may comprise a substantial proportion of acquired events. We provide real-world examples of tissues that pose specific analytical challenges (bone marrow, breast cancer, lung cancer and adipose tissue) and suggest approaches to improve data analysis. These include the use of a sequential or hierarchical gating process, which envisions analysis as consisting of three parts: (1) removal of artifact; (2) defining classifying populations; and (3) measuring outcomes on the classifying populations. Tools for removal of artifact include use of the time parameter to detect and remove fluidic perturbations, use of doublet discrimination to avoid analysis of cell clusters, measurement of DNA content to remove subcellular debris and late apoptotic cells, Boolean gating to recognize and remove autofluorescent events, and the use of a dump gate (markers known to be negative on the population of interest, but expressed on interfering cells). Implementation of these strategies, as appropriate, extends the usefulness of flow cytometry to a wider range of applications.

1. Introduction

The field of flow cytometry has evolved to encompass cells and tissues from multiple sources, the simultaneous use of multiple fluorochromes, and the detection of rare cells among a very large denominator of interrogated events. Even in the best prepared samples, these applications bring with them multiple sources of artifact that can compromise analytical results unless recognized and addressed appropriately during data analysis. This article will provide three examples of difficult samples (bone marrow, solid epithelial tumors and adipose tissue), in order to demonstrate the problems and offer analytical solutions.

2. Materials and methods

2.1. Human samples

The bone marrow CD34 determination was performed by the UPMC Hematopoietic Stem Cell Laboratory as a diagnostic assay. The data were anonymized by an honest broker and are used under University of Pittsburgh IRB exemption (PRO14030569). The breast cancer and lung cancer samples were collected under IRB approved protocols (UPCI04-162 and UPCI99-053, respectively).

2.2. CD34 staining

Flow cytometry was performed using a commercially available FDA approved diagnostic assay (Stem-Kit, Beckman-Coulter Immunotech, Marseille, France, Cat. No. IM3630). All regents were included in the kit. Anti-CD45 FITC/CD34 PE (20 μL) was added to 12 × 75 polystyrene tubes in duplicate. A negative control cocktail (Isoclonic control), consisting of anti-CD45 FITC/CD34 PE plus a

Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; DNase, deoxyribonuclease; ECD, energy coupled dye; FAD, flavin adenine dinucleotide; FDA, food and drug administration; FITC, fluorescein isothiocyanate; FMO, fluorescence minus one; FMO*plus*, Fluorescence Minus Outcomes; HBSS, Hank's balanced salt solution; HIV, human immune-deficiency virus; IRB, internal review board; ISHAGE, international society for hematotherapy and graft engineering; KU, Kunitz units; NADH, reduced nicotinamide adenine dinucleotide; PBS, phosphate buffered saline; PE, phycoerythrin; PMT, photomultiplier tube; SVF, stromal vascular fraction; UPCI, University of Pittsburgh Cancer Institute; UPMC, University of Pittsburgh Medical Center; WBC, white blood cells.

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large excess of unlabeled anti-CD34, was added to a third tube. Cell suspension (100 μL of sample) was added quantitatively using a positive displacement pipettor (model 22 26 000-6, Eppendorf Brinkmann, Westbury, NY, USA). The tube was capped to prevent evaporation and incubated at room temperature in the dark for 20 min. Ammonium chloride-based lysing solution (2 mL) and 7AAD (20 μL) were then added. After a 10 min incubation at room temperature in the dark, 100 μL of Stem-Count calibration beads were quantitatively added at a known concentration of 1002 beads/μL and the tubes were held on ice. Samples were acquired on a 5-color Beckman Coulter FC500 cytometer, calibrated daily with FlowCheck and FlowSet beads (Beckman-Coulter). A wash tube was run between the two experimental tubes and the control tube to eliminate sample carryover. Three levels of process controls (CD-Chex CD34, level 1, level 2, level 3, Cat. Nos. 213336, 213346 and 213382, Streck Laboratories, Omaha, NE, USA) were run with each sample as positive controls of known low intermediate and high CD34 content.

2.3. Tumor disaggregation

Single cell suspensions were prepared from freshly excised tumor tissue as previously described. Step-by-step protocols are available in [1,2]. Briefly, tumors and lung tissue were minced with paired scalpels and digested with type I collagenase (0.4% in RPMI 1640 medium, Cat. No. C-0130, Sigma Chemicals, St. Louis, MO) and DNase (350 KU/mL, Sigma Chemicals, St. Louis, MO, Cat. No. D-5025) and disaggregated through 100 mesh stainless steel screens. Undigested tissue clumps were subjected to repeated rounds of digestion. Viable cells were separated from erythrocytes and debris on a Ficoll–Hypaque gradient (Histopaque 1077, Sigma Chemicals). Erythrocytes were lysed using an NH_4Cl-based lysing solution without fixative (Beckman-Coulter, Cat No. IM3630d).

2.4. Tumor staining

2.4.1. Prevention of nonspecific binding and surface staining

Non-specific binding of fluorochrome-conjugated antibodies was minimized by preincubating pelleted cells for 5 minutes with neat decomplemented (56 °C, 30 min) mouse serum (5 μL), centrifuged and decanted [2]. In the example shown in Figs. 2 and 3, prior to intracellular cytokeratin staining, cells were stained for surface markers (2 μL each added to the cell pellet, 15–30 min on ice; CD44-PE (Beckman-Coulter, Cat No. A32537), CD90-biotin (BD, Cat. No. 555594), Streptavidin-ECD (Beckman Coulter, Fullerton, CA, Cat. No. IM3326), CD14-PECy5 (Beckman-Coulter, Cat. No. IM2640U), CD33-PECy5 (Beckman-Coulter, Cat. No. IM2647U), Glycophorin A/CD235a-PECy5 (BD Biosciences, Cat. No. 559944), CD45-APCCy7 (BD, Cat. No. 348805)), and fixed with 2% methanol-free formaldehyde (Polysciences, Warrington, PA).

2.4.2. Intracellular staining for cytokeratin and DNA content

Gentle cell permeabilization after extracellular staining and fixation is necessary for intracellular staining. This permits the DNA intercalating dye DAPI to be used to measure DNA content, which we also used to exclude events with degraded DNA or no DNA. Surface stained, fixed cells were permeabilized with 0.1% saponin (Coulter) in phosphate buffered saline with 0.5% human serum albumin (10 min at room temperature), cell pellets were incubated with 5 μL of neat mouse serum for 5 min, centrifuged and decanted. The cell pellet was disrupted and incubated with 2 μL of anti-pan cytokeratin-FITC (Beckman Coulter, Cat. No. IM2356) for 30 min. Cells were diluted to a concentration of 10 million cells/400 μL of staining buffer and DAPI (Life Technologies, Grand Island, NY, Cat. No. D1306) was added 10 min before sample acquisition, to a final concentration of 7.7 μg/mL and 40 μL/10^6 cells. The intensity of DNA staining depends on both the cell and dye concentrations and can be quite variable. Lymphocytes in the tissue sample were identified (Fig. 2, pink region) during sample acquisition and used to adjust PMT gain, such that the brightness of the 2 N peak fell in a convenient fluorescence channel.

2.5. Adipose disaggregation

Single cell suspensions were prepared from whole fat tissue and lipoaspirates as previously described [3,4]. Briefly, fat tissue was thoroughly minced with scissors. The aqueous portion of lipoaspirate was removed by aspiration after centrifugation. The resulting fatty tissue was digested for 30 min in Hanks' Balanced Salt Solution (HBSS, Invitrogen) containing 3.5% bovine serum albumin (BSA, Millipore, Charlottesville, VA) and 1 mg/ml collagenase type II (Worthington, Lakewood, NJ) on a shaking water bath at 37 °C, and finally disaggregated through successive 425 and 180 μm sieves (W.S. Tyler, Mentor, OH). Mature adipocytes were eliminated by centrifugation (400 g, ambient temperature, 10 min) and cell pellets were resuspended in NH_4Cl-based erythrocyte lysis buffer (Beckman Coulter, Miami, FL, Cat. No. IM3630d), incubated for 10 min at ambient temperature, and washed in PBS. Some samples were depleted of debris on a Ficoll–Hypaque density gradient (Histopaque1-1077, Sigma). Freshly isolated cells from the stromal vascular fraction (SVF) were maintained on ice and stained for analytical flow cytometry as previously described [5].

2.6. Adipose staining

Cell suspensions were centrifuged (200 g, 7 min) and the cell pellet was preincubated with 5 μL neat mouse serum (Sigma), centrifuged and decanted, to minimize non-specific antibody binding. Cells were simultaneously stained with monoclonal mouse anti-human antibodies (CD105-FITC (Fitzgerald, Acton, MA, Cat. No. 61R-CD105-DHUFT), CD73-PE (BD Biosciences, Cat. No. 550257), CD146-biotin (Miltenyi Biotec, Auburn, CA, Cat. No. 130-092-852), CD14-PECy5 (Beckman Coulter, Cat. No. IM2640U), CD33-PECy5 (Beckman Coulter, Cat. No. IM2647U), Glycophorin-A/CD235a-PECy5 (BD Biosciences, Cat. No. 559944), CD31-PECy7 (Biolegend, San Diego, CA, Cat. No. 303117), CD90-APC (BD Biosciences, Cat. No. 559869), CD34 APC-Alexa 700 (Beckman Coulter, Cat. No. A86354), and CD45-APCCy7 (BD Biosciences, Cat. No. 348805), 2 μL each, on ice), washed and incubated with streptavidin-PE-Texas Red (ECD, Beckman Coulter, Cat. No. IM3326). Analytical samples were fixed with 2% methanol-free formaldehyde (Polysciences, Warrington, PA), permeabilized in PBS with 0.1% saponin (Coulter), 0.5% BSA for 10 min at ambient temperature and incubated with 7.7 μg/ml DAPI.

2.7. Sample acquisition and data analysis (tumor and adipose samples)

Multi-dimensional flow cytometric acquisition was performed using a 10-color Gallios cytometer (Beckman Coulter, Miami FL). The cytometer was calibrated to predetermined photomultiplier target channels prior to each use using 8-peak Rainbow Calibration Particles (Spherotech, Libertyville, IL, Cat. No. RCP-30-5A). Threshold was set on DAPI fluorescence to exclude subcellular debris and up to 1.8 million events were acquired per sample. The DAPI signal was acquired independently on two violet channels optimizing voltage settings of two individual photomultiplier tubes for removal of debris and cell cycle analysis. For compensation purposes, BD Calibrite™ beads (BD Biosciences) and single antibody-stained mouse IgG capture beads (BD Biosciences) were acquired for single fluorochromes (FITC, PE, APC) and tandem-dyes (ECD, PECy5, PECy7, and APCCy7), respectively.

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