

Chapter 12

Rare Event Detection and Analysis in Flow Cytometry: Bone Marrow Mesenchymal Stem Cells, Breast Cancer Stem/Progenitor Cells in Malignant Effusions, and Pericytes in Disaggregated Adipose Tissue

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Abstract

One of the major strengths of Flow Cytometry is its ability to perform multiple measurements on single cells within a heterogeneous mixture. When the populations of interest are relatively rare, analytical methodology that is adequate for more prevalent populations is often overcome by sources of artifacts that become apparent only when large numbers of cells are acquired. This chapter presents three practical examples of rare event problems and gives detailed instructions for preparation of single cell suspensions from bone marrow, malignant effusions, and solid tissue. These examples include detection of mesenchymal stem cells in bone marrow, characterization of cycling/aneuploid cells in a breast cancer pleural effusion, and detection and subset analysis on adipose-derived pericytes. Standardization of the flow cytometer to decrease measurement variability and the use of integrally stained and immunoglobulin capture beads as spectral compensation standards are detailed. The chapter frames rare event detection as a signal-to-noise problem and provides practical methods to determine the lower limit of detection and the appropriate number of cells to acquire. Detailed staining protocols for implementation of the examples on a three-laser cytometer are provided, including methods for intracellular staining and the use of DAPI to quantify DNA content and identify events with $\geq 2N$ DNA. Finally, detailed data analysis is performed for all three examples with emphasis on a three step procedure: (1) Removal of sources of interference; (2) Identification of populations of interest using hierarchical *classifier* parameters; and (3) Measurement of *outcomes* on classifier populations.

Key words: Rare-event analysis, Flow cytometry, Breast cancer, Cancer stem cells, Adult stem cells, Adipose pericytes, Bone marrow-derived mesenchymal stem cells

1. Introduction

This chapter will detail the flow cytometric identification and quantification of three rare populations: one in liquid tissue (mesenchymal stem cells in a bone marrow aspirate), one that

exists as aggregates in suspension (breast cancer stem/progenitor cells in pleural effusion), and one in solid tissue (pericytes in disaggregated adipose). Detailed protocols will be given for preparation of single cell suspensions from these sources. This will be followed by instructions for efficient staining with multiple antibodies, sample acquisition, and data analysis.

2. Materials

2.1. Supplies and Equipment

1. Polypropylene conical tubes: 15 mL, 50 mL, and 225 mL Falcon tubes.
2. 70 μ m nylon and 26 mm diameter Falcon cell strainers.
3. 425 and 180 μ m sieves (W.S. Tyler, Mentor, OH).
4. 1,000 mL Nalgene jars.
5. 1.5 mL Eppendorf microcentrifuge tubes.
6. Tubes for flow cytometry: 12 $\times$ 75 mm Falcon tubes with or without 35 μ m filter cap.
7. Hemacytometer; Drummond Pipet-Aid pipetting device; 10 mL pipettes.
8. Shaking water bath (such as Bellydancer; Stovall Life Science, Inc., Greensboro, NC).
9. Flow cytometer: Beckman Coulter CyAn, Beckman Coulter Gallios (Beckman Coulter, Inc., Fullerton CA), BD LSR II (BD Biosciences, San Jose, CA), or other flow cytometer equipped with 405 nm or UV laser, blue laser and red laser.
10. Data analysis software such as VenturiOne (Applied Cytometry, Sheffield, UK) or Kaluza (Beckman Coulter).

2.2. Tissues and Cells: Minimum Tissue Requirement

1. Bone Marrow: ten million cells (0.5 mL at 20×10^6 /mL) (see Note 1).
2. Solid epithelial tissue: 1 g.
3. Adipose tissue: 20 g.
4. Pleural effusion: ten million cells.

2.3. Reagents and Solutions

1. Ficoll/Hypaque (Histopaque-1077; Sigma-Aldrich, St. Louis, MO).
2. Human Serum Albumin (Albuminar-25, NDC 0053-7680-33).
3. Mouse serum and newborn calf serum.
4. Phosphate Buffered Saline without calcium or magnesium (PBS-A).
5. Staining buffer: PBS-A, 4% newborn calf serum, 0.1% (w/v) sodium azide.

6. Phosphate Buffered Saline with CaCl_2 and MgCl_2 (PBS).
7. Collagenase type I/DNase solution in PBS: 0.4% Collagenase type I (Sigma-Aldrich), 350 KU/mL DNase (Sigma-Aldrich), and 1% albumin.
8. Collagenase type II solution in PBS: 2.5 g/L Collagenase type II (Worthington Biochemical, Lakewood, NJ), and 1% albumin.
9. Ammonium chloride (NH_4Cl) lysing solution (Beckman Coulter) diluted to $1\times$ (see Note 2).
10. EDTA buffer: PBS-A, 1 mM EDTA, and 0.1% albumin.
11. Permeabilization solution: PBS, 0.1% (w/v) saponin, and 0.5% albumin.
12. DAPI (4',6-diamidino-2-phenylindole, dihydrochloride) stock solution: 200 $\mu\text{g}/\text{mL}$ in PBS.
13. 4% Formaldehyde in hypertonic PBS-A (100 mL): 14 mL $10\times$ PBS-A, 40 mL 10% methanol-free formaldehyde (Polysciences, Warrington, PA), and 46 mL dH_2O .
14. Solutions for cell counting: Tuerk's solution and Trypan Blue.
15. Beads for monitoring flow cytometer performance: SpectraAlign beads (DAKO, Glostrup, Denmark); 8-peak Rainbow Calibration Particles (Spherotech, Lake Forest, IL).
16. Beads for compensation: Calibrite beads (unlabeled, FITC, and PE), Calibrite beads (APC); CompBeads Anti-mouse Ig, κ (all from BD Biosciences).

2.4. Antibodies

1. Anti-human antibodies for surface staining of:
 - (a) *Bone marrow mesenchymal cells*: CD105-FITC (61R-CD105-DHUFT; Fitzgerald Industries International, Acton, MA), CD73-PE (550257; BD Biosciences), CD34-ECD (IM2709U; Beckman Coulter), CD90-PC5 (IM3703; Beckman Coulter), CD117-PC7 (IM3698; Beckman Coulter), CD31-APC (FAB3567A; R&D Systems, Minneapolis, MN) or CD133-APC (130-090-854; Miltenyi Biotech, Gladbach, Germany), CD45-APC-Cy7 (348805; BD Biosciences).
 - (b) *Pleural fluid*: CD44-PE (MCA89PE; Serotec, Raleigh, NC), CD90-biotin (555594; BD Biosciences), Streptavidin-ECD (IM3326; Beckman Coulter), CD14-PC5 (IM2640U; Beckman Coulter), CD33-PC5 (IM2647U; Beckman Coulter), Glycophorin A-PE-Cy5 (559944; BD Biosciences), CD133-APC (Miltenyi Biotech), CD117-PC7 (IM3698; Beckman Coulter), and CD45-APC-Cy7 (BD Biosciences).
 - (c) *Adipose pericytes*: CD3-FITC (IM1281U; Beckman Coulter), CD146-PE (A07483; Beckman Coulter),

CD34-ECD (IM2709U; Beckman Coulter), CD90-PC5 (Beckman Coulter), CD117-PC7 (Beckman Coulter), CD31-APC (R&D Systems), and CD45-APC-Cy7 (BD Biosciences).

2. Anti-human antibody for intracellular staining of pleural effusion cells: pan cytokeratin-FITC (IM2356; Beckman Coulter).

3. Methods

3.1. Preparation of Single Cell Suspensions

3.1.1. Bone Marrow

Bone marrow is usually obtained by aspiration from the posterior iliac crests (1). It can also be obtained from discarded surgical specimens (femoral head in hip replacements (1, 2), rib section in lung lobectomy (3), or from cadaveric vertebrae (4)). Bone marrow should be aspirated into heparinized syringes (sodium heparin, 10 U/mL) and held at ambient (not refrigerated) temperature.

1. Bone marrow mononuclear cells, depleted of mature erythrocytes and mature granulocytes, are prepared by Ficoll/Hypaque gradient centrifugation (5).
2. Dilute aspirated bone marrow or bone marrow flushed from bones to 30 mL with PBS–0.1% albumin (see Note 3). The total number of cells per tube should not exceed 50×10^6 .
3. Pipette 15 mL of Ficoll/Hypaque into a 50 mL polypropylene conical tube.
4. Using a 10 mL pipette, carefully layer 30 mL of diluted bone marrow over the Ficoll/Hypaque (see Note 4). Set centrifuge to 25°C, and spin at $400 \times g$ for 45 min (brake off).
5. After centrifugation, aspirate and discard the upper layer (diluent) with a 10 mL pipette (see Note 5).
6. Using a 10-mL pipette, carefully collect the Buffy Coat and pipette into a new 50-mL conical tube (see Note 6).
7. Wash the Buffy Coat twice with 50 mL of PBS–0.1% albumin ($400 \times g$, 7 min, 25°C).
8. Resuspend in 2 mL of PBS–0.1% albumin and hold on ice.
9. Count cells and record cell concentration and volume (see Note 7).

3.1.2. Malignant Pleural Effusions

Pleural effusions are collected into plastic containers by suction. Ideally they should be heparinized (sodium heparin, 10 U/mL) because they often contain serous fluid capable of clotting. They contain cells in suspension, but tumor is usually in clumps that range from microscopic to visible. The cell count and volume vary considerably from sample to sample (see Note 8).

1. Record the volume and cell count of the unmanipulated sample (see Note 9).
2. Concentrate the cells and cell clumps by centrifugation ($400\times g$, 7 min, 4°C).
3. Discard supernatant and resuspend the pellet in 10 mL Collagenase type I/DNase solution (see Note 10).
4. Place sample in a shaking waterbath (e.g. Bellydancer) at 37°C for 30 min, maximum agitation setting.
5. Place a $70\text{ }\mu\text{m}$ cell strainer in the mouth of a new labeled 50 mL conical tube.
6. Using a 10 mL pipette, transfer material from the collagenase digestion tube into the cell strainer.
7. Add 10 mL of PBS–0.1% albumin to the digestion tube and transfer any remaining material to the cell strainer. Discard strainer.
8. Bring volume of strained cells to 50 mL with PBS–0.1% albumin, centrifuge at $400\times g$ for 7 min, 4°C and discard supernatant.
9. Add 45 mL of NH_4Cl lysing solution and mix (see Note 11).
10. Centrifuge at $400\times g$, 4°C for 10 min.
11. Pour off the supernatant and loosen cell pellet.
12. Resuspend in 2 mL of PBS–0.1% albumin and hold on ice.
13. Count cells on a hemacytometer (Tuerk's solution to eliminate red blood cells (RBC) and Trypan blue for viability) (6).

3.1.3. Whole Adipose Tissue

Adipose tissue is a byproduct of aesthetic surgery. It may be removed in the form of solid tissue or lipoaspirate. This protocol assumes solid adipose tissue. The expected yield of stromal/vascular cells is approximately 1×10^6 cells/g of tissue.

1. Record the weight of adipose tissue.
2. Cut the tissue in large pieces using the sterile scissors and distribute into 50 mL conicals (approximately 10 g of fat per tube).
3. Thoroughly mince tissue in the 50 mL conical tubes using scissors.
4. Add 30 mL of Collagenase type II solution per tube.
5. Vortex the conical tube and incubate at 37°C in a shaking water bath for 15 min, maximal agitation.
6. Examine the tubes to estimate efficient digestion. The presence of excessive clumps indicates under-digestion. The appearance of a clear yellow lipid layer indicates over-digestion. Reincubate in the water bath if full digestion is not achieved. Repeat every 5 min for a maximum of 30 min total digestion time.

7. Add 10 mL of EDTA buffer to neutralize ongoing collagenase activity.
8. Centrifuge at $400\times g$ for 10 min at 25°C .
9. Collect all semi-solid top fat layers from all tubes into 50 mL polypropylene conical tubes. Add 30–40 mL of PBS–0.1% albumin and shake thoroughly to homogenize (see Note 12).
10. Vortex the remaining contents of the digestion conical tubes and pass the contents through the $425\text{ }\mu\text{m}$ and $180\text{ }\mu\text{m}$ large sterile sieves into the 1,000 mL Nalgene jar. Use a glass pestle if necessary.
11. Wash the sieves with up to 100 mL of PBS–0.1% albumin.
12. Collect the sieved sample from the basin to 50 mL or 200 mL conical tubes.
13. Add the diluted fat from the top layer of the digest to the sieves and pass through using the glass pestle.
14. Centrifuge the sieved cells at $400\times g$ for 7 min at 4°C .
15. Discard supernatant and combine all cell pellets in one to two 50-mL conical tubes.
16. Wash the cells with PBS–0.1% albumin (50 mL per tube).
17. Centrifuge at $400\times g$ for 7 min at 25°C .
18. Discard supernatant and loosen the cell pellet.
19. Add 45 mL of NH_4Cl lysing solution per tube and mix.
20. Centrifuge at $400\times g$, 4°C for 10 min.
21. Pour off the supernatant and loosen cell pellet.
22. Resuspend in 2 mL of PBS–0.1% albumin and hold on ice.
23. Count cells on a hemacytometer (Tuerk's solution to eliminate RBC and Trypan blue for viability) (6).

3.2. Surface Staining

1. Pellet cells at $400\times g$ for 10 min at 4°C . Discard supernatant (see Note 13).
2. Resuspend cell pellet in 5 μL of neat decomplexed (56°C , 30 min) mouse serum (see Note 14).
3. Pellet cells ($400\times g$, 10 min, 25°C) and aspirate the supernatant as thoroughly as possible without disturbing the pellet (see Note 15).
4. Stain the dry pellet for surface markers by the addition of 2 μL of each monoclonal antibody (see Note 16).
5. Add antibodies in the following order (see Note 17):
 - (a) Bone marrow mesenchymal cells
 - CD105-FITC
 - CD73-PE

- CD34-ECD
- CD90-PC5
- CD117-PC7
- CD31-APC or CD133-APC
- CD45-APC-Cy7

(b) Pleural fluid

- CD44-PE
- CD90-biotin-streptavidin-ECD
- Lineage cocktail: CD14-PC5, CD33-PC5, and Glycophorin A-PE-Cy5.
- CD133-APC
- CD117-PC7
- CD45-APC-Cy7

(c) Adipose pericytes

- CD3-FITC
- CD146-PE
- CD34-ECD
- CD90-PC5
- CD117-PC7
- CD31-APC
- CD45-APC-Cy7

6. Incubate for 30 min on ice in the dark.

7. Dilute surface stained cell pellets in 1 mL of staining buffer.

8. Centrifuge at $400\times g$ for 7 min at 25°C.

9. Discard supernatant and loosen the cell pellet.

3.3. Intracellular Staining

Staining for intracellular antigens requires fixation and permeabilization, and is usually performed after surface staining.

1. Following surface staining, pellet cells at $400\times g$ for 10 min at 4°C. Discard supernatant with care to create a *dry pellet*.
2. Fix stained cells for 20 min at ambient temperature with 200 μ L of PBS and 200 μ L of 4% methanol-free formaldehyde in hypertonic PBS-A (see Note 18).
3. Centrifuge at $400\times g$ for 7 min at 25°C.
4. Discard supernatant with care to create a *dry pellet* and flick to loosen.
5. Permeabilize fixed cells by addition of 200 μ L of permeabilization solution containing saponin (10 min at ambient temperature).

6. Intracellular cytokeratin staining of pleural effusion cells:
 - (a) Centrifuge at $400\times g$ for 7 min at 25°C .
 - (b) Decant supernatant with care to create a *dry pellet* and flick to loosen.
 - (c) Add 5 μL of neat mouse serum to cell pellet, incubate at ambient temperature for 5 min, centrifuge, and decant to *dry pellet*.
 - (d) Loosen cell pellet and add 2 μL of anti-pan cytokeratin-FITC for 30 min.
 - (e) Dilute cytokeratin stained cell pellets in 500 μL of staining medium.
 - (f) Centrifuge at $400\times g$ for 7 min at 25°C and discard supernatant to a *dry pellet*.
 - (g) Loosen cell pellets and dilute to a cell concentration of ten million cells/400 μL ($25\times 10^6/\text{mL}$) of staining buffer.
 - (h) Transfer to 12×75 mm tubes with 35 μm filter caps for flow cytometry.
7. DNA content by DAPI staining for bone marrow mesenchymal cells, adipose pericytes, and pleural effusion cells:
 - (a) Disaggregate cell pellets and dilute to a cell concentration of ten million cells/400 μL of staining buffer.
 - (b) Add 16 μL of DAPI stock solution to a final concentration of 8 $\mu\text{g}/\text{mL}$ (7) (see Note 19).
 - (c) Transfer to 12×75 mm tubes with 35 μm filter caps for flow cytometry.

3.4. Instrument Setup and Standards

3.4.1. IgG Capture Bead Staining

1. Vortex CompBeads thoroughly before use (see Note 20).
2. Label a separate 1.5 mL Eppendorf tube for each mouse monoclonal antibody conjugated to a tandem dye (e.g. ECD, PE-Cy5, PE-Cy7, and APC-Cy7).
3. Add one full drop (approximately 60 μL) of anti-mouse Ig CompBeads to each Eppendorf tube.
4. Centrifuge for 10 min at $400\times g$. Carefully aspirate supernatant to ensure a “dry pellet” (see Note 21).
5. Sonicate each tube for 10 s in a water bath sonicator (see Note 22).
6. Add 2 μL of each antibody directly to beads (one antibody per tube) and gently reflux.
7. Incubate for 15 min at room temperature in the dark (see Note 16).
8. Add 1 μL of mouse serum and incubate for 5 min at room temperature (see Note 23).

9. Add 100 μ L of staining buffer and reflux.
10. Sonicate each tube for 10 s.
11. Add 1 mL of staining buffer. For manual compensation, add one drop of negative CompBeads to each test tube that contains antibody stained beads (see Note 24).
12. Centrifuge beads for 10 min at $400\times g$, decant and carefully blot to remove residual supernatant (see Note 25).
13. Resuspend washed beads in 0.5 mL of staining buffer.
14. Transfer to 12 $\times$ 75 mm snap cap tubes for flow cytometry.
15. Sonicate for 10 s prior to acquisition on the flow cytometer.

3.4.2. Instrument Setup and Sample Acquisition

These protocols have been validated on Beckman Coulter CyAn and Gallios cytometers. This protocol is applicable to all cytometers equipped with a UV or violet laser, a blue laser, and a red laser.

1. The cytometer is calibrated to predetermined photomultiplier target channels prior to each use using SpectraAlign beads and 8-peak Rainbow Calibration Particles (see Note 26).
2. The data from 8-peak beads can also be used to monitor instrument sensitivity (resolution of dim peaks) and instrument linearity (8).
3. All fluorescence parameters are collected in the logarithmic mode, with the exception of DAPI emission at 455 nm which is collected in the linear mode (see Note 27).
4. Acquire unstained cells or beads first, and then each single stained sample (bead or cells) from the shortest emission wavelength (FITC) to the longest (e.g. CD45-APC-Cy7).
5. Run a rinse tube. Then run analytical samples with a rinse tube in between if needed (see Note 28). Do not apply spectral compensation. This will be done offline with analytical software.

3.5. How Many Events to Acquire

Not everything that is detected by a flow cytometer is a cell. In fact, in messy samples like disaggregated solid tissues, cells are sometimes in the minority. Rare event problems sometimes necessitate the acquisition of millions of events in order to obtain the required number of cells. The correct number depends on three factors: (1) The proportion of cells to debris; (2) The signal-to-noise ratio of the population of interest compared to all other events; (3) The frequency of the population of interest.

Discriminating between genuine events and potential sources of artifact such as debris will be illustrated in each example. The inclusion of dead or dying cells, autofluorescent events, or sub-cellular debris distorts both the numerator (population of interest) and denominator, and makes for an unreliable analysis.

Much of the literature dealing with flow cytometry of cultured or disaggregated cells suffers to a greater or lesser extent from inclusion of such sources of artifact.

The signal-to-noise ratio encompasses both the distance (in fluorescence or scatter intensity) between positive and negative populations, and the variability (spread) of those populations in multiparameter space. Isotype controls or other definitive negative populations are often helpful to determine the boundaries defining a population of interest. Even when positive and negative populations appear to be well separated, acquisition of millions of events often reveals a scattering of false positive events within the multiparameter space of the population of interest. Creative gating, including elimination of sources of artifact and use of multiple parameters (both positive and negative) to define the population of interest, can often increase the signal-to-noise ratio. Ultimately, the frequency of false positive events determines the lower limit of detection and thus the maximum number of cells to be acquired. Figure 1 shows an example in which five false

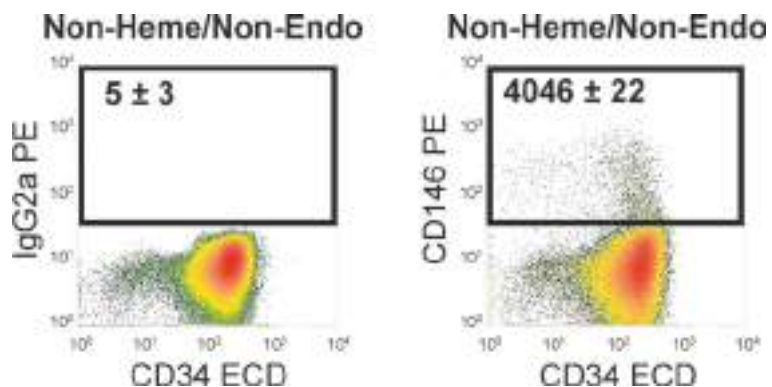


Fig. 1. Estimation of the lower limit of detection from an isotype control. The lower limit of detection is determined by the signal-to-noise ratio and thus is dependent on the mean fluorescence of negative and positive populations and their respective spreads. In this example we show an FMO (fluorescence minus one) control (*left panel*) for the detection of pericytes (*right panel*) in the stromal vascular fraction of adipose tissue. An equal number of events (910,000) are shown in each histogram and both have been gated on viable singlet events that are both non-hematopoietic (CD45[−]/CD3[−]) and non-endothelial (CD31[−]). The gates identifying CD146⁺ pericytes (*right*) and false positive events (*left*) are identical and, in this case have been placed to exclude all but 5 ± 3 (mean $\pm$ SD of triplicate determinations) events in the isotype control tube. The lower limit of detection is calculated as the log mean false positive rate plus $1.6445 \times \text{SD}$. This is an estimate of the 95th percentile of the false positive frequency. The antilog of this value, 0.0085% (1 event in 11,790) is considered the lower limit of detection and defines the lowest proportion of pericytes that can be reliably detected. Combining this information with the methods shown in Fig. 2, the optimal number of total events to acquire for this assay is $100 \times 11,790 = 1,179,000$. The frequency of CD146⁺ pericytes (*right histogram*) in this example is 2.5% of clean non-heme events or 0.278% of total events. In our example, acquiring 4046 CD146⁺ events would give a CV of 2% according to Poisson statistics.

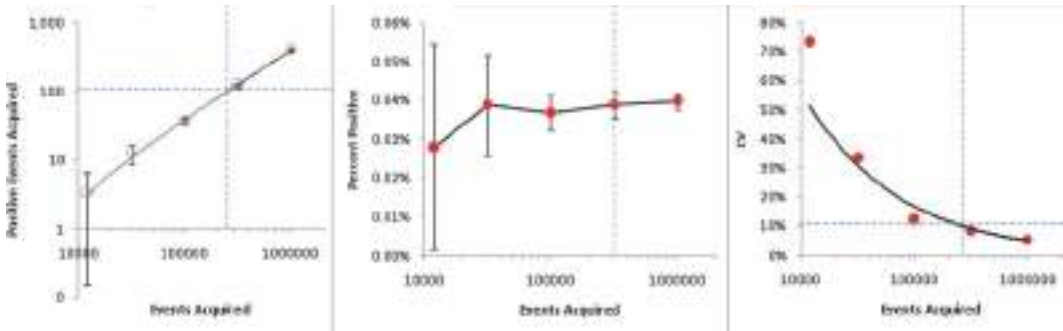


Fig. 2. How many events to acquire? Here we are quantifying CD45⁺/CD34⁺/CD105⁺/CD73⁺ mesenchymal stem cells in freshly isolated human bone marrow. A single file was acquired for a total of 4,074,618 events. We divided the file into three equal segments of varying number to simulate the acquisition of different numbers of cells in triplicate (13). The *graph on the left* shows the number of positive events (mesenchymal stem cells) as a function of total events acquired (mean of triplicate determinations, bars = 1 SD). The *dashed lines* show the number of total events (X-axis) required to detect 100 positive events (Y-axis). The *center graph* shows the calculated mean percent of mesenchymal stem cells (bars = 1 SD) as a function of the number of events acquired. The dashed line intersects with the estimated percent positive when 100 positive events are acquired. The *right graph* shows the calculated coefficient of variation (SD/mean) of the percent positive (red circles) as a function of the total number of cells acquired. The *solid black line* is a predicted CV based on Poisson counting statistics in which the CV% is equal to the $100/\sqrt{\text{positive events acquired}}$ (14). The dashed lines demonstrate that a CV of 10% is obtained when 100 positive events are detected. In this example, this requires a total of 250,000 events.

positive events were detected in an isotype control sample in which 910,000 gated events were analyzed. By performing replicate determinations, we were able to calculate the 95th percentile of the false positive event frequency is 0.0085% or 1 event in 11,790. Since acquiring 100 events of a population of interest is sufficient to give a CV of 10% (see Fig. 2), acquiring 1,179,000 events will take the assay to its limit of sensitivity, and acquiring more cells will do nothing to improve the result.

Figure 2 shows the effect of acquiring triplicate samples of increasing numbers of cells. As expected, the number of positive events increases linearly with the number of events acquired and the variability between triplicate frequency estimates (Percent positive) decreases. Importantly, the measured coefficient of variation (CV, calculated as the standard deviation/mean) of the triplicate determinations decreases markedly. This empirical demonstration also shows the adequacy of the CV determined by a simpler alternative approach, Poisson statistics. Poisson statistics deal with the probability distribution of rare events. The Poisson CV of the counting error, defined as $100/\sqrt{\text{positive events counted}}$, is 10% when 100 events are counted. In this example, the frequency of positive events (0.04%) requires that 250,000 events be acquired to achieve a CV of 10%.

There is one more aspect of signal-to-noise that counting statistics do not take into account, and that is the distribution of the population of interest in multiparameter space. Figure 3 shows

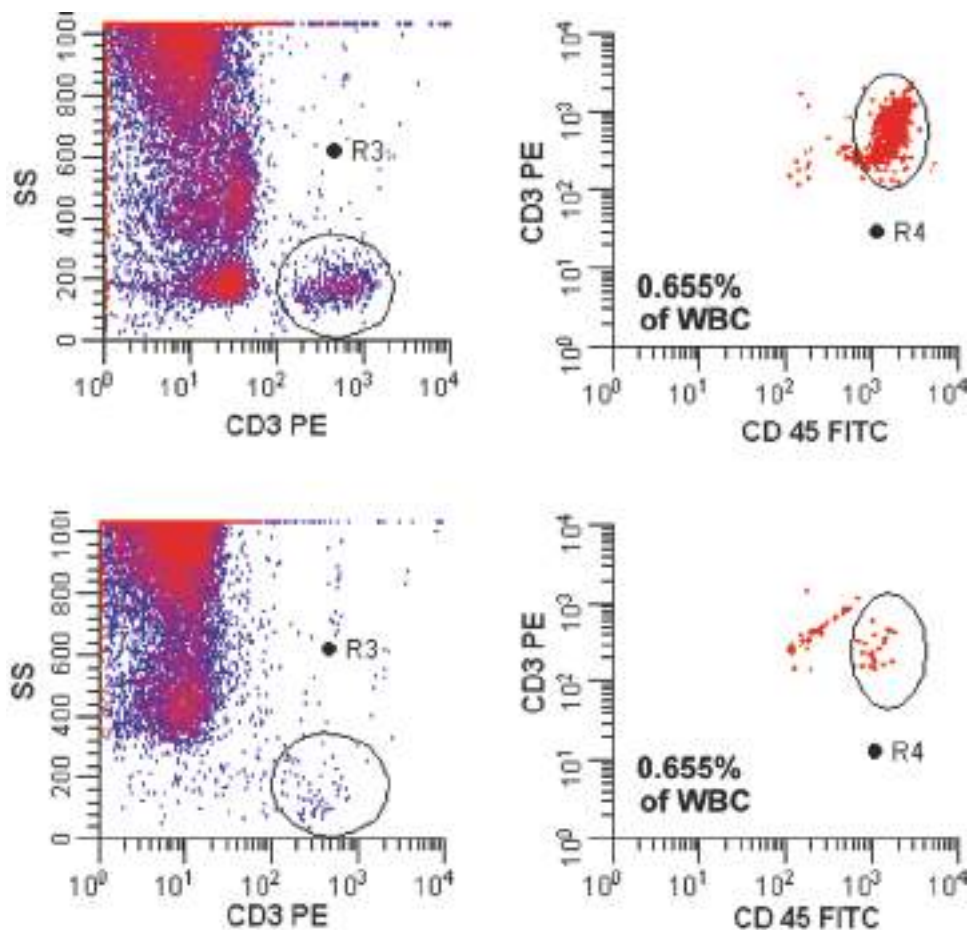


Fig. 3. Knowledge of the spatial distribution of a population of interest can be used to increase the signal-to-noise ratio. T-cell determinations were performed at two time intervals on a patient undergoing immunoablative therapy with cytoxan, fludarabine, and anti-thymocyte globulin. In the upper histograms a plot of CD3 versus side scatter reveals a clear population of CD3+ cells. Further gating on CD45 versus CD3 demonstrates that T-cells have relatively little variability in the expression of either marker. This permits the elimination of several outlying events that in all probability do not represent viable T cells. The bottom row shows the same analysis on a sample that contains far fewer T-cells. Knowledge of the location of *bona fide* T cells in multiparameter space, and careful standardization of the instrument permit the same analytical gates to be applied, even though there is not a clearly discernable population in the plot of CD3 versus side scatter. Projection of these events onto a plot of CD45 versus CD3 reveals a small population of T cells (23 events) and a more numerous diagonal streak of artifactual events. Although the Poisson CV of the T-cell population is an unreliable 20.9%, its distribution in space lends credibility to the analysis. The actual assays were performed in triplicate, using 7-AAD exclusion as a criterion of viability (not shown) to help substantiate the conclusion that the T cells were genuine and viable, even though they represented only 0.028% of CD45+ events.

peripheral T-cell determinations performed on patients undergoing immunoablative therapy (9). Given the knowledge that T-cells are very constrained with respect to the range of CD3 and CD45 expression, very low numbers of positive events (in this case 23 CD3+ events, Poisson CV=20.9%) can be reliably detected.

**3.6. Data Analysis
of Three Examples:
Bone Marrow
Mesenchymal
Stem Cells,
Cytokeratin + Cells
in a Malignant Pleural
Effusion, and Pericytes
in Human Adipose
Tissue**

We perform spectral compensation and data analysis offline, using VenturiOne or Kaluza software both of which have been designed to accommodate very large datafiles. After creating compensation matrices using the data from our single-stained beads, we create a playlist of datafiles in which each datafile is associated with an analysis template and a compensation matrix. Arriving at an adequate analysis template is an iterative process that used to be quite painful. The use of playlists to organize datafiles and compensation files from different directories, and the ability to export results to spreadsheets greatly facilitates reanalysis and revisiting data sets with new questions. Our analysis strategy usually proceeds in three steps:

1. First we eliminate sources of interference with logical gates. These may include event bursts (from transient fluidic disturbances), cell-cell doublets and clusters, subcellular debris, dead cells, and autofluorescent events.
2. Next, we decide on our *classifier* parameters. These are used to define the major population(s) of interest.
3. Next *outcome* parameters to be measured on each classifier population are determined. The distinction between classifier and outcome parameters is sometimes clear, but sometimes it is quite fluid, especially when exploring a new combination of analytes. At this stage it is often helpful to color-event cells positive for the outcome parameters and examine their expression on plots of the classifier parameters (10).

**3.6.1. Elimination
of Sources of Interference**

Interference can come from many sources including fluidic disturbances, nonspecific binding of antibodies (dead and dying cells are adept at this), and cells with intrinsic autofluorescence (particularly cultured cells and some populations from fresh disaggregated tissues).

1. Fluidic disturbances alter laminar flow within the flow cell and results in increased variability in measurements, particularly light scatter. It is easy to spot transient fluidic disturbances by plotting a time parameter (or event count) versus log side scatter. Such a disturbance can be seen in the pleural effusion example (Fig. 4). These events can be examined in isolation and removed from the analysis with a logical gate, if they prove to have altered marker expression.
2. Cell doublets and clusters, resulting from physical aggregation or coincidence are also problematic when large numbers of events are acquired. For DNA analysis, a doublet appears as a single cell with 4N DNA (11). For phenotypic analysis a T-cell/B-cell conjugate looks like a single cell with coexpression of T- and B-cell markers. Clusters are easily removed by pulse analysis of the triggering parameter. In our examples,

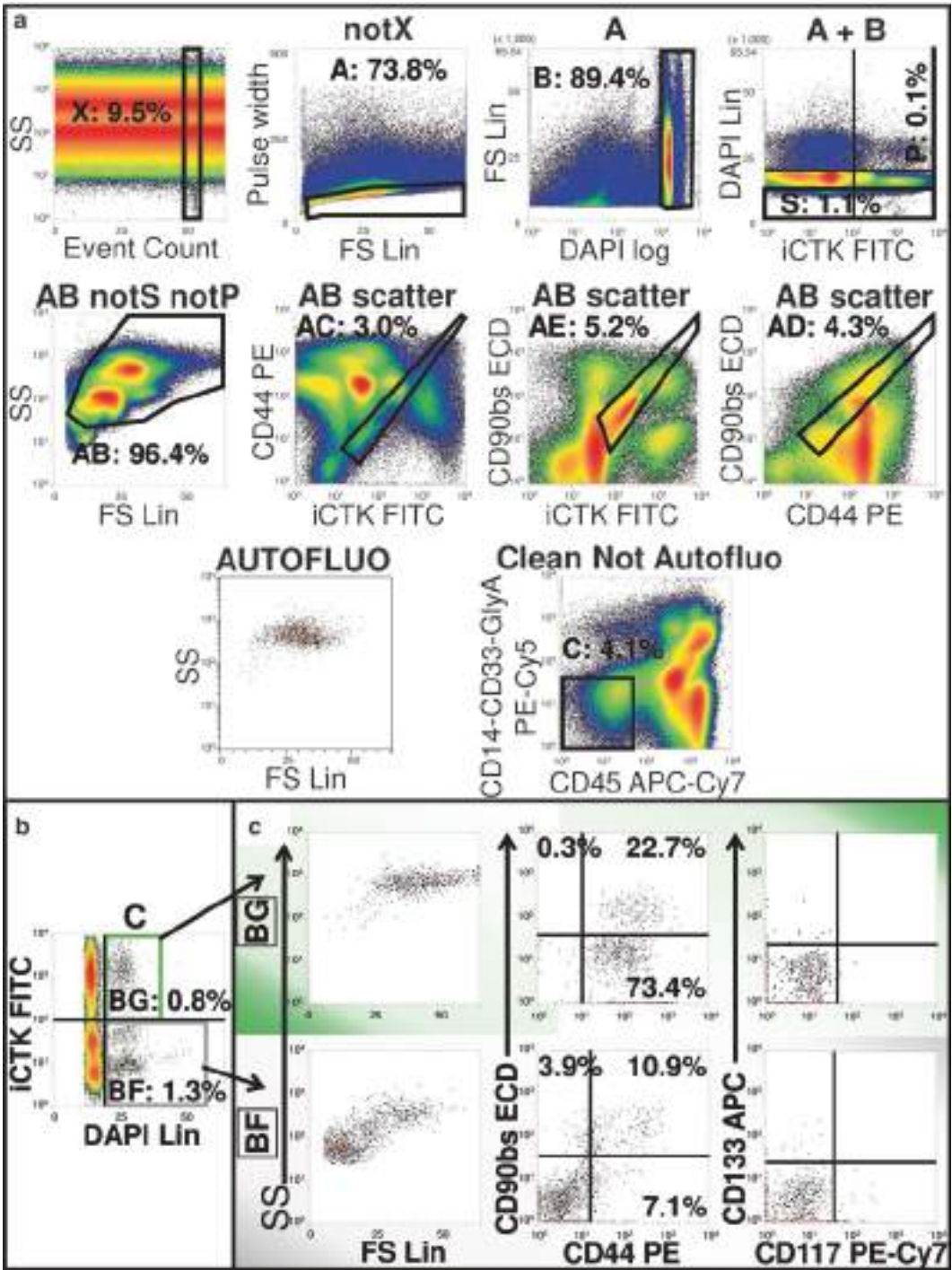


Fig. 4. Analysis of cycling/aneuploid cells in a breast cancer pleural effusion. (a) Elimination of sources of artifact prior to analysis. This data is from a cryopreserved breast cancer malignant pleural effusion. Pleural effusions are heterogeneous and often have more inflammatory cells than tumor cells. The goal was to detect aneuploid/cycling cells in subpopulations of cytokeratin+ (epithelial) cells. The first step in data analysis is to remove events which can interfere with detection of the events of interest. We always monitor light scatter as a function of time. Log side scatter is very sensitive to

we compare forward scatter pulse height (labeled FS lin) to forward scatter pulse width (labeled Pulse width) and eliminate event clusters that are too wide (i.e. have too long a time of flight) for their pulse height. In our examples the frequency of clusters ranges from 5% (Fig. 6, bone marrow) to 26% (Fig. 4, malignant pleural effusion).

3. Dead and dying cells may also have altered marker expression, so it is always desirable to eliminate them from the analysis. This can be approached in several ways. In all of the examples given here, cells were permeabilized after staining and fixation in order to facilitate DAPI staining of cellular DNA. This has two benefits: (1) subcellular debris and hypodiploid (apoptotic) cells are easily identified and removed on a plot of DAPI log fluorescence intensity versus FS; (2) display of DAPI fluorescence on a linear scale provides a low-resolution cell-cycle analysis (see Notes 29 and 30). In our adipose example (Fig. 5), 14% of events, most of them subcellular debris resulting from tissue digestion, had $<2N$ DNA. Even after limiting the analysis to cells with DNA content $\geq 2N$, early apoptotic events with intact DNA may still be present. These can be identified and eliminated by their characteristic light scatter profile (generally low forward scatter with too much side scatter relative to forward scatter). In our adipose example the T-cell marker CD3 was used as a “Dump Gate” and CD3+ T cells were color evented and backgated on the light scatter plot (red colored events in the electronic version,

Fig. 4. (continued) fluidic perturbations which show up as a decrease in FS. Here a transient disturbance was eliminated with “NOT” gate (X). The next step is to use pulse analysis to remove doublets and cell clusters. Pleural effusions are susceptible to clumping, and cell clusters will have marker expression characteristic of the aggregate. In this case 73.8% of events were singlets. The cells in this analysis were permeabilized with saponin after surface staining and fixation. DAPI can be used to measure DNA content in permeabilized cells. DAPI log is useful for eliminating events with no or little DNA (about 10% of singlets). In the linear mode, DAPI fluorescence is used for cell-cycle determinations. Here it is used to remove hypodiploid (apoptotic) cells (1.1% of nucleated singlets). In the same histogram a small proportion of events (0.1%) are so brightly stained with cytokeratin-FITC that they have saturated the PMT and therefore appear in the last channel. These events cannot be spectrally compensated, because their true fluorescence intensity is unknown. Next a conservative gate eliminates events with too little light scatter to be viable cells. Together, the last three histograms in the second row are used to identify and eliminate cells with autofluorescence. Any events falling within the intersection of regions AC, AE and AD (AUTOFLUO) are candidates for elimination. The high light scatter of autofluorescent cells suggests that they are granulocytes. Finally, CD45 negative, hematopoietic lineage negative cells are identified (gate C) as tumor cell candidates. These cells represented only 2.54% of the 3.6 million events acquired and represent the denominator for subsequent rare event analysis. (b) After removing sources of artifact, and excluding cells expressing hematopoietic lineage markers, we defined our classifier populations (populations of interest) as cytokeratin+ (BG) and negative (BF) cells with $>2N$ DNA content, as determined by DAPI staining. We detected a total of 767 Cytokeratin+ cells with $>2N$ DNA content, representing 0.8% of heme-lineage negative cells and 0.02% of acquired events. (c) Outcomes measured on these two populations included expression of the adhesion molecule CD44 (96% of cycling cytokeratin+ cells) and the stem cell marker CD90 (23%). Credible subpopulations of CD117+ and CD133+ cells were not seen. This analysis was repeated on heme-lineage negative, cytokeratin negative aneuploid/cycling cells, which have much lower light scatter, and smaller proportions of CD44+ and CD90+ cells.

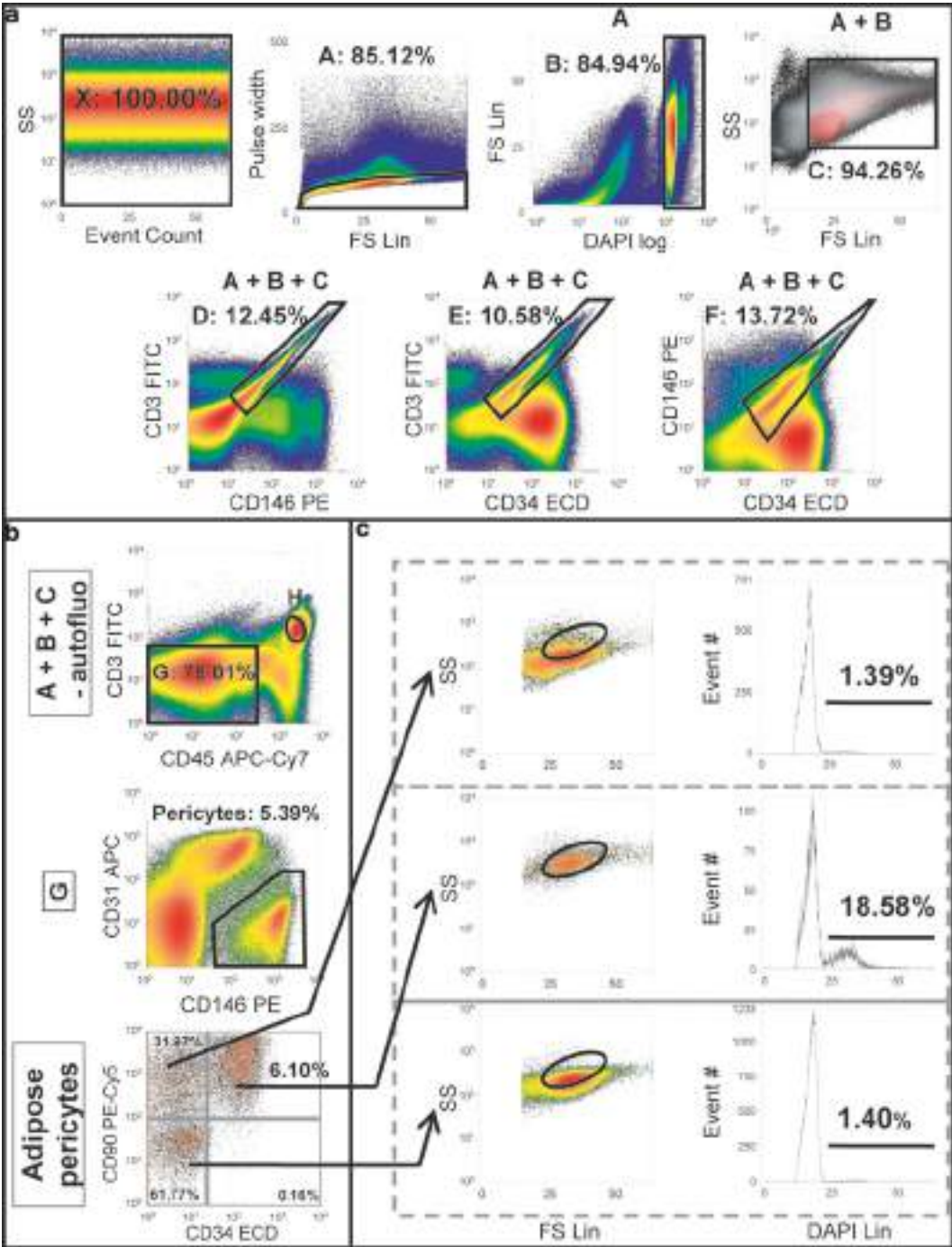


Fig. 5. Flow cytometric analysis of pericytes subsets in adipose tissue. (a) Elimination of irrelevant events by selection of cellular nucleated singlets. *Left to right*. We check for disturbances using time versus log side scatter. Cell clusters are eliminated using pulse analysis (*region A*). Nucleated cells were stained with the nuclear dye DAPI after gentle permeabilization (*region B*). Cell-events can be further defined using known internal cellular landmarks. In this example, resting CD3+ CD45+ T-lymphocytes (H) have been color-evented red (within *region C*). The knowledge of the light scatter localization of resting T cells serves as a landmark to exclude smaller subcellular events and preapoptotic cells. Selection of

Fig. 5). This gives us a point of reference to eliminate events with lower forward light scatter. This method assumes that the cells of interest have at least as high light scatter as small resting T-cells (see Note 31).

4. Elimination of events with saturating fluorescence. It is highly desirable to adjust PMT gains such that all positive events are on scale. In rare cases this is not possible without unbalancing PMT gain or obscuring dim positive events. In Fig. 4, cytokeratin expression occupies such a large dynamic range that 0.1% of all events fall within the last channel (i.e. are saturated with respect to green fluorescence). Although this is a relatively small proportion of events, they must be removed from the analysis. Because their fluorescence is unknown, they cannot be spectrally compensated and will appear positive in the adjacent PE channel.
5. Cellular autofluorescence results from expression of naturally fluorescent biomolecules such as flavinoids (12). Autofluorescence can be distinguished from fluorescence specific to most of the dyes used in cytometry by its broad emission spectrum. Autofluorescent biomolecules are often excited better with short wavelength light than with long wavelengths. If the cell population of interest is autofluorescent, one is generally limited to long excitation wavelengths (e.g. red diode laser). In our examples, mesenchymal stem cells, adipose pericytes, and cytokeratin+breast cancer cells, the cells of interest are not autofluorescent, so we can use compound logical gates to eliminate events that excite with 488 nm light and emit in the ranges detected by FL1, FL2, and FL3 channels (e.g. using 530/30, 584/42, and 675/20 bandpass filters, respectively). In the pleural effusion example (Fig. 4), relatively few events fall within all three diagonal gates, and these are uniformly cells with high light scatter.
6. Nonspecific fluorescence (autofluorescent or caused by nonspecific antibody binding) can be eliminated using a *dump gate*, that is, a marker which the population of interest is known not to express. If the dump gate uses a dye such as FITC,

Fig. 5. (continued) autofluorescent events in the three first fluorescent channels (FITC, PE, and ECD) was performed as in Fig. 4. (b) Selection of adipose pericytes by use of classifiers (CD3, CD45, CD31, and CD146). Remaining autofluorescent events are eliminated using the CD3-FITC signal as a dump gate. Adipose pericytes are non-hematopoietic (region G), non endothelial (CD31– cells), and express the cell adhesion molecule CD146 (Region Pericytes). In this example, pericytes account for 5.39% of non hematopoietic events, 4.25% of the adipose stromal vascular fraction, and 2.62% of total events. CD90 and CD34 serve as secondary classifiers, as pericytes can be divided into three distinct subpopulations based on CD90 and CD34 expression. The majority of pericytes do not express CD34 (93.1% of pericytes). One third express CD90 exclusively, and a minor population coexpress CD34 and CD90 (0.16% of total events). (c) Outcome: The rarest population, CD34+ CD90+ pericytes, is larger (higher light scatter compared to CD34 negative pericytes) and shows a higher proliferation level (DAPI lin >2N, 18.58% of CD34+ CD90+ pericytes). CD34+ CD90+ pericytes may represent a transit amplifying population of cells in transition from pericytes to supra-adventitial adipose stromal cells (15).

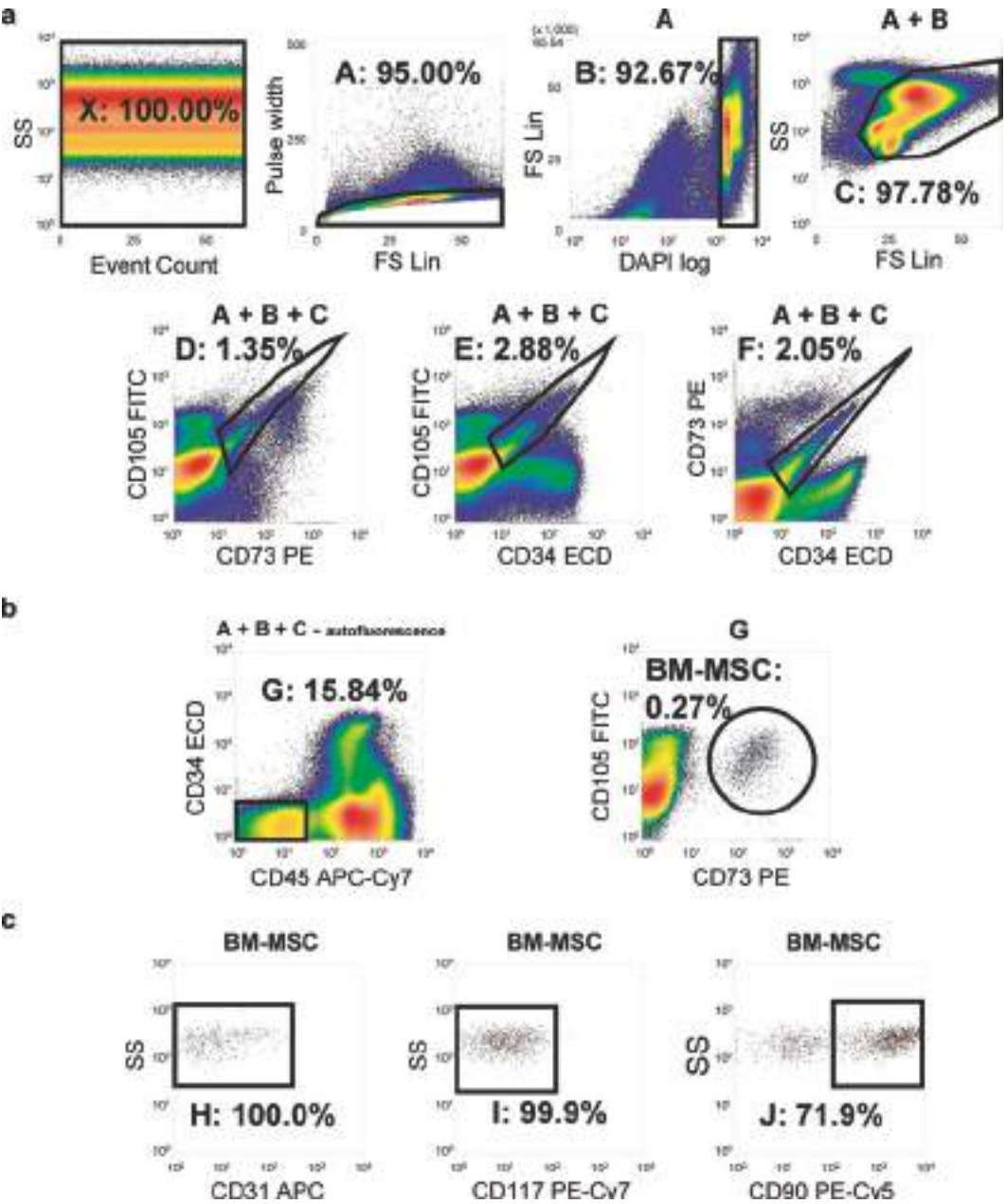


Fig. 6. Detection of bone marrow mesenchymal stem cells in freshly isolated bone marrow. A total of four million events were acquired. (a) Elimination of sources of interference. Fluidic disturbances, elimination of cell clusters, selection of nucleated events, and exclusion of debris, subcellular particles and early apoptotic cells were done as in Figs. 4 and 5. The lower row shows the selection of autofluorescent events in the three first fluorescence channels. All events present concomitantly in *regions D, E, and F* were eliminated. (b) Identification of a classifier population. Bone marrow mesenchymal stem cells are detected as CD34[−]/CD45[−]/CD73⁺/CD105⁺ cells. (c) Measurement of outcomes on the classifier population. CD31 (an endothelial marker) and CD117 (c-Kit) are all negative on BM-MSC. The majority of BM-MSC are positive for the stem cell marker CD90.

which is within the range of autofluorescent emission, it eliminates cells binding the antibody specifically and nonspecifically, and autofluorescent events as well. This can be seen in Fig. 5, where non-hematopoietic cells are identified as CD3-FITC negative, as well as CD45 negative, prior to the identification of adipose pericytes. Nonspecific antibody binding is also minimized by a preincubation/blocking step with normal mouse serum prior to staining.

3.6.2. Identification of Classifier Parameters

Classifier parameters are those used to identify populations of interest. Thinking of parameters as either primary or secondary markers, or outcomes creates a hierarchical model, focuses the analysis, and eliminates the “all possible combinations” problem encountered in multi-parameter analysis.

1. Primary classifiers can be strung together as a Boolean AND gate. In Fig. 6, the primary classifiers identifying mesenchymal stem cells are absence of CD45 and CD34 expression AND presence of CD73 and CD105.
2. Secondary classifiers branch into multiple populations of interest. In Fig. 5 adipose pericytes, identified by primary classifiers (CD3-/CD45-/CD31-/CD146+) are subsetted into three subpopulations by the secondary classifier markers CD34 and CD90. In Fig. 4, the secondary classifier markers cytokeratin and DAPI identify populations of cycling/aneuploid cells for further analysis.

3.6.3. Measurement of Outcome Parameters on Populations Identified by Classifiers

The division between classifiers and outcomes is not rigid and often depends on how one frames a biologic question. For example, do we wish to start with cells that secrete interferon gamma (classifier) and examine lymphocyte subsets as outcomes, or wish to define lymphocyte subsets (classifiers) and determine (as outcomes) the proportion of cells secreting interferon gamma in each? As such, outcomes are final branch points in our hierarchical mode. In Fig. 4, the outcomes are light scatter, CD44, CD90, CD117, and CD133 expression on cycling/aneuploid cytokeratin+ and negative non-hematopoietic cells. In Fig. 5, they are light scatter and DNA content in adipose-derived pericytes. In Fig. 6, the outcome parameters are CD90, CD117, and CD31 expression on mesenchymal stem cells.

4. Notes

1. Bone marrow aspirates are naturally diluted in peripheral blood. When correctly performed, a bone marrow aspirate should contain approximately 20×10^6 mononuclear cells/mL. Lower concentrations indicate excessive hemodilution.

2. If you use a different product make certain that it does not also contain a fixative.
3. Bovine serum albumin or human serum albumin may be used. The recommended concentration (0.1%) is equivalent to 2% serum. Two percent newborn calf serum is more economical, equally effective, and can be used for many applications. Serum albumin protects cells from shear stress encountered during centrifugation.
4. Watch your favorite bartender prepare a "Black and Tan." With the tube slightly tilted, slide the pipette down the wall of the tube until it is close to the Ficoll/Hypaque. Using a pipetting device (Drummond Pipet-Aid), dispense the cell suspension slowly and evenly, carefully sliding the pipette tip up the wall as you go. A successful gradient shows a sharp interface between the bone marrow suspension and the Ficoll/Hypaque layer. Some investigators prefer to add the cell suspension to the tube first and then carefully underlayer the Ficoll/Hypaque using a pipette or a syringe and a bone marrow biopsy needle.
5. The bone marrow mononuclear cells will appear as a whitish-tan layer at the Ficoll/Hypaque interface. This is often referred to as the Buffy Coat. Take care not to disturb the Buffy Coat when removing and discarding the diluent.
6. Prolonged exposure to Ficoll/Hypaque is toxic to the cells. Take care to include as little as possible when recovering the Buffy Coat.
7. We routinely use a Beckman-Coulter Act10 hematology analyzer for cell counting. This instrument detects cells by a change in conductivity that occurs when cells displace electrolyte. It can be fooled by cell-sized debris and fat globules. Train your eyes to estimate cell number from the size of the cell pellet. Hemacytometers are inexpensive and yield reliable counts after a little practice.
8. Tumor cells are usually in the minority in malignant effusions, the majority population being acute or chronic inflammatory cells. Mesothelial cells are also often seen.
9. If the sample collected is more than 200 mL, use 225 mL polypropylene conical tubes.
10. DNase and collagenase require divalent cations and will not work in calcium/magnesium free PBS. The albumin serves as a buffer against cell digestion by nonspecific proteases.
11. Treatment with ammonium chloride lyses red blood cells. This step may be omitted if sample is not visually bloody.
12. This fatty layer often contains trapped cells.

13. The number of cells to be stained in a single pellet can range from 0.5×10^6 to 10×10^6 or more without changing the amount of antibody added. The key issue in antibody staining is the final concentration of the antibody in the reaction mixture and not the number of cells. Best practice is to determine the optimal antibody concentration by titration. Alternatively, read the manufacturer's instructions paying attention to the recommended dilution of stock antibody, but not the number of cells. For example, if the instructions call for suspending the cells in 0.1 mL of buffer and then adding 10 μ L of antibody, this means that the antibody can withstand a ten-fold dilution. By minimizing the sample volume you will minimize the amount of antibody required per test.
14. Addition of mouse serum, which contains mouse immunoglobulins, blocks nonspecific binding of murine monoclonal antibodies.
15. We refer to this as a *dry pellet* elsewhere in the protocol. Cells pelleted in a 15 mL conical tube and aspirated dry actually contain anywhere from 10 to 50 μ L of residual liquid.
16. Tandem dyes are easily degraded by exposure to light. Reagents, stained cells, and stained beads must be carefully protected from ambient light. We perform staining in an unilluminated biological safety cabinet, and cover stained cells and beads with aluminum foil to minimize light exposure.
17. The choice of antibodies and fluorochromes is specific to the question being addressed and the available instrumentation, and can be modified at will. The order of antibody addition may influence staining as binding occurs very rapidly and sequential addition of many antibodies progressively reduces the concentration of the individual antibodies in the mixture.
18. The choice of formaldehyde is critical. We always use EM grade methanol-free material purchased at a low concentration stock solution (10%). It is not necessary or advisable to make up fresh paraformaldehyde solution from powder.
19. Unlike antibody staining, the concentration of cells and the concentration of dye are critical for consistent results. This protocol is specific to cells at 25×10^6 /mL.
20. Single stained compensation standards are essential for correct spectral compensation. We find it best to use internally stained Calibrite beads for FITC, PE and APC, and Ig capture beads (CompBeads) for antibodies conjugated to tandem dyes. DAPI does not require spectral compensation against these dyes so no single stain preparation is required.

21. Beads do not pack as tightly as cells so care must be taken not to aspirate the beads.
22. Sonication is not essential, but is very useful because it disaggregates bead clumps better than vortexing or refluxing.
23. Addition of mouse serum prevents clumping of beads due to antibody crosslinking.
24. Negative beads are not required for some automated compensation algorithms, such as those implemented on Beckman-Coulter instruments or in VenturiOne software.
25. Decanting and blotting must be done in one smooth motion to prevent loss of beads.
26. Determining balanced PMT settings is an art in itself and has been addressed by us elsewhere (9). Once these settings have been established, and bead target channels have been determined, they can be reproduced from experiment to experiment by adjusting PMT voltage to place the brightest peak of the Rainbow particles in the predetermined target channel. The intensity of DAPI staining will vary somewhat from specimen to specimen, because it is measured on a linear scale and is highly dependent on the total amount of DNA in the sample. PMT voltage for the DAPI channel is adjusted for each sample to place the median fluorescence of the 2N population at about channel 64 of 1,023.
27. Newer three-laser cytometers make use of high resolution digital pulse analysis. All native data is linear. Logarithmic transformations are performed mathematically. Referring to data collected in the “logarithmic mode” is a throwback to the days when analog pulse measurements were directed to either a log or linear amplifier.
28. Sample carryover can be a killer in rare event analysis. Instruments vary widely in the degree of sample carryover. You can tell if you have a carryover problem if you see excessive events when running a rinse (filtered water or PBS) tube after your sample tube.
29. Cell-cycle analysis can be optimized by using fixation and permeabilization methods less favorable for surface marker staining.
30. If permeabilization is not possible (as is the case when sorting viable cells), uptake of DAPI or another DNA-binding dye such as propidium iodide or 7-aminoactinomycin-D, can be used to identify and exclude dead cells, but this method does not exclude subcellular debris or early apoptotic cells.
31. Some cells (e.g. embryonic stem cells) are very small and have lower light scatter.

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