Few and far between

Tools and strategies for rare-event detection using flow cytometry.

The ability to accurately detect and analyze rare cells in a cell population is critical, not only for the study of disease progression but also for our understanding of key pathways in normal development. Flow cytometry is the method of choice for detecting rare-cell populations—including stem cells, circulating endothelial cells, circulating tumor cells, and residual disease cells—in blood, bone marrow, and a wide variety of other samples. Thanks to technological advances in instrumentation combined with better detection reagents and more sophisticated analysis strategies, the identification of rare cells at frequencies as low as 0.0001% has been reported.

Flow cytometry offers several advantages for the identification, enumeration, and characterization of rare cells. Foremost among these is the ability to perform multiple quantitative measurements on each cell in a cell population, and to subsequently sort these cells for further downstream testing. The availability of flow cytometers with multiple lasers and detection channels, in conjunction with the development of new fluorophores and conjugates that span the visible to near-infrared spectrum, has allowed for improved panel design in multiplex assay protocols (see "Flow cytometry panel design: The basics" on page 20). These multiparameter measurements increase assay specificity while also providing a strategy for identifying and eliminating cells from further analysis (e.g., see "Gating strategies for maximizing assay specificity" on the next page), each of which is critical to the detection of rare cells.

Flow cytometry, however, also poses several technical limitations for rare-cell detection, including the time required to process large sample volumes (or, alternatively, to perform enrichment techniques prior to analysis), and the lack of visual confirmation of cell identity. Several excellent review articles discuss these challenges in detail [1,2]. Here we focus on a few major obstacles in rare-cell detection, specific strategies to address them, and examples of successful rare-cell analysis by flow cytometry both from recently published reports and from our own labs. We also demonstrate that acoustic focusing cytometry can dramatically increase sample acquisition rates compared with conventional flow cytometry, enabling a larger number of rare cells to be analyzed in a single experiment.

Watch the Science/AAAS webinar on rare-cell detection and analysis

For more information on how to maximize your rare-event studies, view the webinar "Overcoming challenges in cellular analysis: Multiparameter analysis of rare cells" by Andrea Cossarizza (University of Modena and Reggio Emilia School of Medicine, Italy) and David Cousins (University of Leicester, United Kingdom) at **webinar.sciencemag.org/webinar/archive/overcoming-challenges-cellular-analysis**. In this webinar, the speakers discuss advances in flow cytometry that have improved the detection of rare-cell events and provide examples of the isolation and analysis of innate lymphoid cells (ILCs), circulating antigen-specific lymphocytes, and innate-like cells such as natural killer T cells and circulating endothelial cells (CECs).

How many events must be acquired?

In flow cytometry, an "event" is defined as a single particle detected by the instrument. The term "rare" generally refers to a frequency of 0.01% and below. Accurate detection of rare-cell events using flow cytometry requires the ability to detect single cells with specific characteristics in a heterogeneous population of cells. This detection can be additionally complicated by the challenge of detecting the cells of interest in a limited sample or in the presence of cell debris or other artifacts of sample preparation. Minimal sample preparation is recommended, to avoid these artifacts and minimize cell loss. "No-lyse/no-wash" or "lyse/no-wash" procedures can help maintain the cells' native characteristics while maximizing recovery of the rare population (see "First, do no harm" on page 28). For example, the Invitrogen™ High-Yield Lyse Solution is a premixed, fixative-free erythrocyte-lysing solution for flow cytometry that eliminates red cells from whole blood without a subsequent wash step, minimizing loss of rare blood cell populations [3].

When investigating a rare population of cells, it may be necessary to acquire millions of events to obtain a sufficient number of cells for statistically significant detection. The number of events needed for analysis depends on three main factors: the ratio of cells to debris in the sample; the signal-to-noise ratio of the detected cells compared to background fluorescence; and the frequency of

Table 1. Number of cells required to achieve a given precision in rare-event analysis. Adapted from Allan AL, Keeney M (2010) *J Oncol* 426218.

Desired CV (%)		1	5	10	20	40
Number of events of interest* (r)		10,000	400	100	25	6
When occurring at a frequency of (%)	1 : n	Total number of events that must be collected †				
10	10	10^5	4×10^3	10^3	2.5×10^2	6.25×10^1
1	100	10^6	4×10^4	10^4	2.5×10^3	6.25×10^2
0.1	1,000	10^7	4×10^5	10^5	2.5×10^4	6.25×10^3
0.01	10,000	10^8	4×10^6	10^6	2.5×10^5	6.25×10^4
0.001	100,000	10^9	4×10^7	10^7	2.5×10^6	6.25×10^5
0.00001 ‡	10,000,000	10^{11}	4×10^9	10^9	2.5×10^8	6.25×10^7

*For cell-based assays such as flow cytometry, a simple calculation can be used to determine the size of the database or sample that will provide a given precision: $r = (100/CV)^2$, where r is the number of events meeting the required criterion, and CV is the coefficient of variation of a known positive control. † With a WBC count in the low-normal range (~5×10^9 cells/L), 10 mL of blood would contain ~5×10^7 events. ‡ Estimated frequency of CTCs in the peripheral blood of cancer patients.

the cell population of interest in the sample. Poisson statistics apply when counting randomly distributed populations, where precision increases as more events are acquired [1]. To determine the size of the sample (number of cells) that will provide a given precision, the equation $r = (100/CV)^2$ is used, where r is the number of cells meeting the defined criterion of the rare event, and CV is the coefficient of variation of a known positive control. Table 1 shows the number of events required to achieve various levels of precision in rare-cell analysis [4].

Strategies for optimizing instrument sensitivity

For extremely rare cell populations such as circulating endothelial cells—occurring at a frequency of 0.01–0.0001% in a background of peripheral mononuclear cells—1 million to 10 million cells must be analyzed. Acquiring this many cells can pose challenges, both in terms of the volume of sample (e.g., blood) and the instrument time required, as well as the size of the data files for analysis. As discussed below, acoustic focusing cytometry can mitigate some of these issues because its detection speeds (up to 35,000 events per second) and sample flow rates (up to 1,000 µL per minute) are dramatically higher than those of conventional hydrodynamic focusing cytometers, allowing more cells and a larger sample volume to be analyzed without compromising data (Table 2).

Table 2. Comparison of collection rates obtained using hydrodynamic focusing cytometry and acoustic focusing cytometry.

Instrument collection rate	Time to acquire 10^6 granulocyte events*	Relative rate
Hydrodynamic focusing at "High" flow rate	63 min 33 sec	—
Acoustic focusing at 200 µL/min	13 min 20 sec	4.8x faster
Acoustic focusing at 500 µL/min	5 min 47 sec	11.0x faster
Acoustic focusing at 1,000 µL/min	3 min 13 sec	19.7x faster

*A blood sample from an aplastic anemia individual with a PNH neutrophil population was analyzed on a conventional hydrodynamic focusing cytometer and on the Attune™ Acoustic Focusing Cytometer, each with a stop gate set on one million granulocyte events, and the time of acquisition was recorded.

Two other often-overlooked factors to consider when optimizing the sensitivity of a flow cytometry assay are the cleanliness of the instrument and the integrity of the sample. It is important to make sure the instrument and fluids used are clean and free of particles that could contribute falsely to the rare population. Additionally, during acquisition, it can be helpful to include time as a parameter, looking for any bursts or breaks of data during acquisition that may indicate a problem with the sample (such as clumping) or with the fluidics of the instrument.

Gating strategies for maximizing assay specificity

High assay specificity is a critical requirement for detecting a maximum number of true positive cells while at the same time minimizing false-positive and false-negative events. Phenotypic differences between the rare cells and the background cells can be exploited by using markers that are specific to each population. Increasing the number of markers that distinguish rare cells can lead to enhanced assay sensitivity and specificity; this multiparameter gating strategy is facilitated by a flow cytometer with multiple lasers and 10 or more fluorescence detectors, such as the Attune™ NxT Acoustic Focusing Cytometer (see page 19).

To maximize detection of the rare event, more than one fluorescence parameter should be used to positively identify the cell of interest (compound gating). Likewise, there should be at least one fluorescence parameter for which the rare event is negative (negative gating). This negative channel, which is used to exclude a →

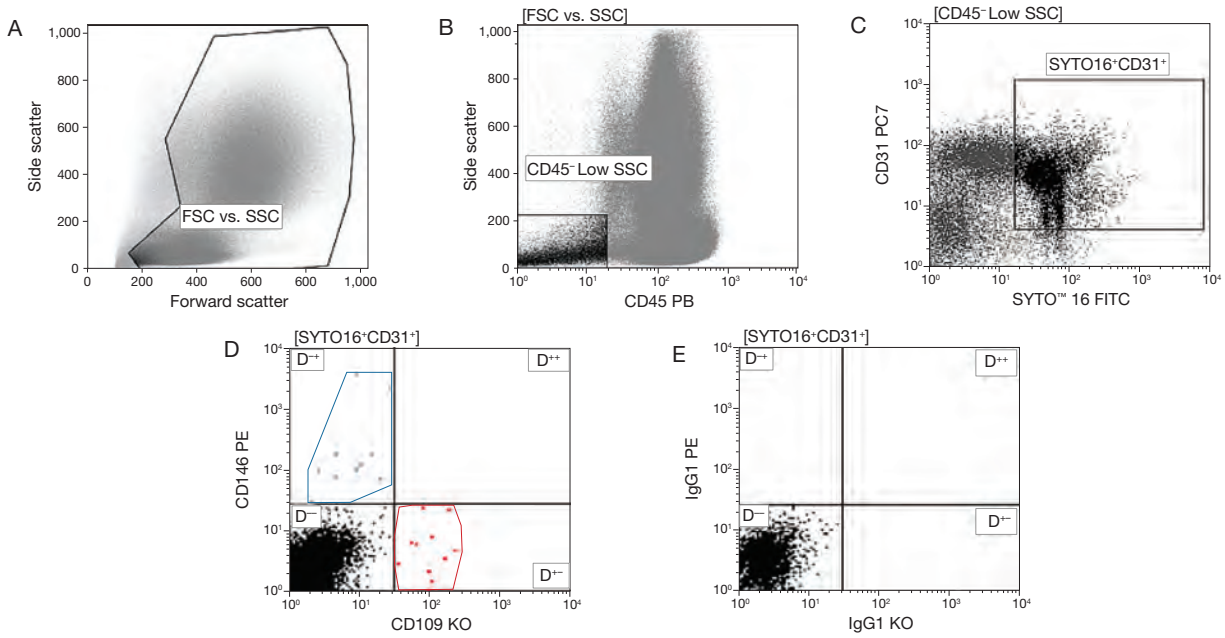

Figure 1. Gating strategy to detect circulating endothelial cells (CECs) expressing CD109 and CD146. The gating strategy includes exclusion of debris **(A)** and selection of CD45-negative **(B)**, nucleated (positive for SYTO™ 16 stain, Cat. No. S7578) and CD31-positive **(C)** cells, followed by the identification of CECs as positive for CD109 (red) or CD146 (blue) **(D)**. **(E)** Negative control. Reprinted with permission from Manusco P et al. (2014) *PLoS One* 9(12):e114713.

population of events from further analysis, is commonly referred to as a "dump channel". Events typically excluded from analysis are dead and dying cells, cell aggregates and debris, and cells with unwanted markers or characteristics. Additionally, fluorescent-minus-one (FMO) controls—in which every fluorescent marker except one is used to determine the fluorescence contribution of all other markers to the detection channel for the excluded marker—are useful in determining points of separation between positive and negative populations.

To illustrate such a gating approach, a strategy for the detection of circulating endothelial cells (CECs) developed by Manusco and coworkers [5] is shown in Figure 1. CECs are vascular cells that have been shed from the vascular wall into the bloodstream. CECs and their progenitor cells are extremely rare in normal blood (0.01–0.0001% of peripheral mononuclear cells) but have been found to be elevated in various disease states, including cardiovascular disease and several cancers [6]. To detect CECs expressing CD109 or CD146 (two subpopulations enriched in the blood of cancer samples), the researchers used the cell-permeant SYTO™ 16 nucleic acid stain to discriminate between DNA-containing cells and cell debris, as well as a panel of fluorescent monoclonal antibody conjugates that included anti-CD45 (to exclude hematopoietic cells), anti-CD31 and anti-CD34 (endothelial cell markers), and anti-CD109 or anti-CD146 (markers expressed in the CECs of interest) antibodies. Figure 1D shows the rare CD109⁺ (red) and CD146⁺ (blue) cell populations identified by combining compound gating with negative gating.

As a second example, Figure 2 shows two negative gating strategies designed to eliminate cell debris (using forward scatter vs. side scatter) and dead cells (using the SYTOX™ AADvanced™ Dead Cell Stain) during the detection of CECs with a panel of fluorescent anti–human CD antibodies. SYTOX™ AADvanced™ stain is a cell-impermeant nucleic acid dye for dead-cell detection that is compatible with excitation from the common 488 nm argon-ion laser. A detailed protocol for detecting human CECs using the Attune™ cytometer is provided at **thermofisher.com/attuneappnotesbp71**.

Another obstacle in any immunophenotyping experiment is the background fluorescence that arises from nonspecific antibody binding. Unlabeled normal mouse IgG antibody is commonly used to block Fc-binding receptors in samples prior to any staining protocols in order to reduce this nonspecific binding, decrease background fluorescence, and increase signal-to-noise ratios.

Detecting rare tumor cells in cancer studies

In cancer research, flow cytometry has been used to detect tumor cells by the presence or absence of specific cell-surface markers, typically in blood, bone marrow, and other fluids. The tumor cells of interest are often "buried" within a background of normal cells,

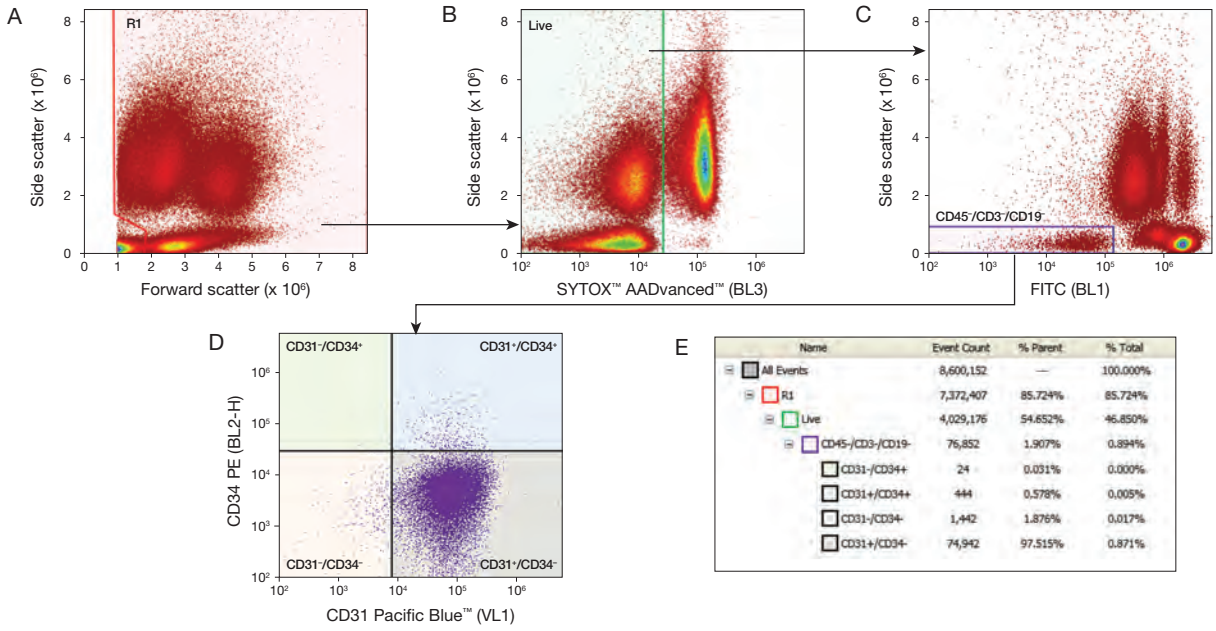

Figure 2. Gating to eliminate debris and dead cells for detection of circulating endothelial cells (CECs). (A) A density plot of forward scatter (FSC) vs. side scatter (SSC) shows both the threshold level and the R1 gate to remove debris. **(B)** Gated on R1 events, this density plot of SYTOX™ AADvanced™ stain (Cat. No. S10274) fluorescence (BL3) vs. side scatter shows the live cell gate, which eliminated dead cells. **(C)** Gated on live cells, this density plot of the FITC dump channel (BL1) vs. side scatter shows the gate on CD45⁻ CD3⁻ CD19⁻ cells. Since CECs are negative for all three of these markers, all positive cells can be eliminated from further analysis using only one fluorescence channel. **(D)** Gated on live CD45⁻ CD3⁻ CD19⁻ cells, CECs are identified as CD31⁺ CD34⁺ in the upper right-hand quadrant. **(E)** CECs are 0.578% of the parent CD45⁻ CD3⁻ CD19⁻ cells and 0.011% of the total live WBCs.

or may be extremely dilute (e.g., after detaching from a solid tumor and entering the bloodstream). One of the early applications of rare-event detection was the analysis of minimal residual disease (MRD) in leukemia patients. MRD refers to the small number of residual cancer cells remaining in a subject during or after treatment, which may be undetectable by morphologic analysis. Using multicolor flow cytometry, however, researchers have detected MRD in leukemia considered to be in remission by morphologic criteria. Figure 3 demonstrates the detection of a leukemia-associated immunophenotype (CD34⁺ CD13⁺ CD45^{WEAK} CD25⁺) in an acute myeloid leukemia (AML) sample after induction chemotherapy. In this study, the MRD represents only 0.003% of all nucleated peripheral blood cells [7]. →

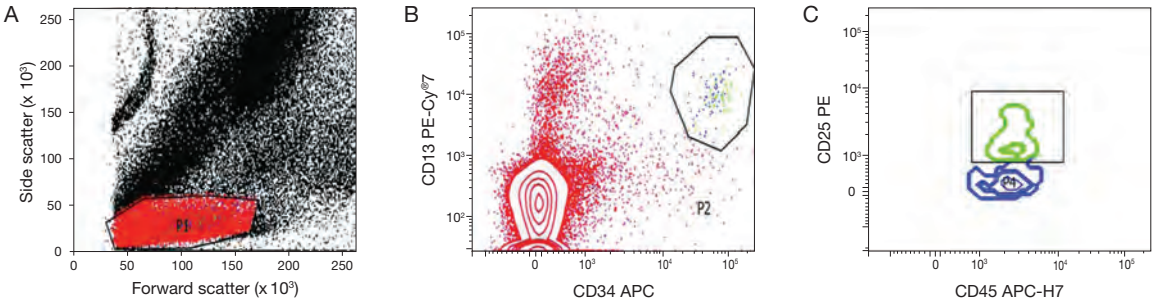

Figure 3. Flow cytometric detection of MRD after induction chemotherapy in the peripheral blood of an AML patient. In the scattergram **(A)**, a gate (P1) is set around cells with low forward scatter (FSC) and side scatter (SSC), reflecting small to intermediate size and low granularity, respectively. In the blast gate **(B)**, cells within P1 with high CD34 and high CD13 expression are selected away (P2) from CD34⁻ CD13⁻ cells (red dots), representing predominantly lymphocytes. Some CD34⁻ CD13⁺ cells are monocytes caught in P1. In the blast cell contour plot **(C)**, MRD is detected within the CD34⁺ CD13⁺ gate based on the expression of CD25 and intermediate CD45 staining (green cluster). These cells with the patient's LAIP features (CD34⁺ CD13⁺ CD45^{WEAK} CD25⁺) account for 0.003% of all nucleated cells, a common denominator for MRD definition. The blue CD25⁻ cluster represents normal myeloid precursor cells (CD34⁺ CD13⁺ CD45^{WEAK} CD25⁻) caught in P2. Reprinted with permission from American Society of Hematology: Paietta E (2012) *Hematology Am Soc Hematol Educ Program* 2012:35–42.

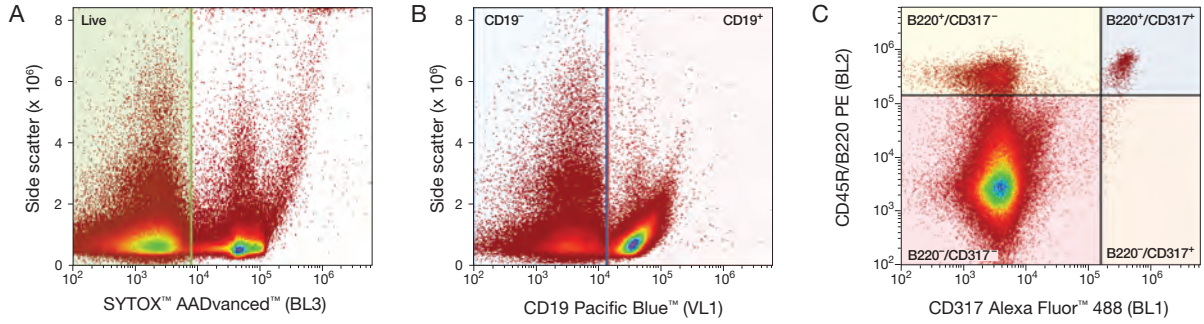

Figure 4. Mouse plasmacytoid dendritic cell (pDC) gating and analysis. (A) A gate was made on live cells using SYTOX™ AADvanced™ Dead Cell Stain (Cat. No. S10274; channel BL3, 640 nm longpass filter). **(B)** Live cells were then gated on CD19⁻ cells (channel VL1, 450/40 nm bandpass (BP) filter). **(C)** A 2-parameter plot of CD45R/B220 vs. CD317 was used to detect pDCs (channel BL1, 530/30 nm BP filter; and channel BL2, 574/26 nm BP filter); pDCs were identified as dual B220⁺/CD317⁺ (upper right quadrant) and comprise 0.851% of live CD19⁻ cells, which is 0.194% of total splenocytes. A collection rate of 500 µL/min was used to acquire 1.3 million total cells; total acquisition time was 23 min, which is 3x faster than the same sample run on a traditional hydrodynamic focusing cytometer.

Circulating tumor cells (CTCs) are another active area of cancer research. CTCs have been detected in the blood of subjects with solid tumors, and their presence can be predictive of disease state and clinical outcome [8]. Flow cytometry has been used to analyze tumor metastasis to bone marrow and lymph node in preclinical models [9] and to detect rare tumor cells in human blood samples [10]. Today, flow cytometry is an essential tool for the detection of rare tumor cells in cancer research [1].

Acoustic cytometry for improved rare-event detection

The large number of cells required for rare-event detection can translate into very long acquisition times on a traditional hydrodynamic focusing flow cytometer. By comparison, acoustic focusing cytometry aligns cells using acoustic forces rather than hydrodynamic forces, delivering much higher throughput compared to traditional flow cytometry and enabling more cells to be analyzed in a shorter period of time (Table 2). Because there is minimal data variation regardless of sample-throughput rate, acoustic focusing cytometry is ideal for detecting rare-cell events as well as for analyzing dilute samples such as cerebrospinal fluid, in which low cell numbers necessitate large sample volumes.

The recently introduced Attune™ NxT Acoustic Focusing Cytometer achieves sample-throughput rates over 10 times faster than those of other cytometers (up to 1,000 µL/min) and data acquisition speeds of 35,000 events/sec, enabling rapid detection of rare events with precision and accuracy and without aborting data. The Attune™ NxT cytometer retains all the acoustic focusing benefits of the first-generation Attune™ cytometer, while also providing additional lasers (up to 4 spatially separated lasers), more detection channels (up to 14 emission channels plus forward and side scatter channels), and improved software that can manage the large data sets (up to 20 million events per run) generated during multiparameter analyses. Figure 4 shows a successful gating strategy for identifying mouse plasmacytoid dendritic cells (pDCs)—which are 0.194% of total splenocytes—using the Attune™ NxT cytometer.

Bring rare-event detection to your lab

Rare-event detection has become an essential tool for a host of applications, such as identification of antigen-specific cell populations, monitoring of hematological cancers, and detection of circulating tumor cells and endothelial cells. The success of rare-event cell detection is affected by many parameters, including sample quality, frequency of the cells of interest, sample preparation, specificity and expression levels of the chosen markers, and robustness and reproducibility of the assay. The next-generation Attune™ NxT Acoustic Focusing Cytometer provides a benchtop instrument with the performance and reliability that rare-cell detection requires. To request an in-lab demonstration, visit **thermofisher.com/attunebp71**. ■

References

1. Hedley BD, Keeney M (2013) *Int Jnl Lab Hem* 35:344–350.
2. Zimmerlin L, Donnenberg VS, Donnenberg AD (2011) Methods Mol Biol 699:251–273.
3. Gratama JW, Menéndez P, Kraan J et al. (2000) *J Immunol Methods* 239:13–23.
4. Allan AL, Keeney M (2010) *J Oncol* 426218.
5. Manusco P, Calleri A, Gregato G et al. (2014) *PLoS ONE* 9:e114713.
6. Boos CJ, Lip GY, Blann AD (2006) *J Am Coll Cardiol* 48:1538–1547.
7. Paietta E (2012) *Hematology Am Soc Hematol Educ Program* 2012:35–42.
8. Yu M, Stott S, Toner M et al. (2011) *J Cell Biol* 192:373–382.
9. Goodale D, Phay C, Postenka CO et al. (2009) *Cytometry A* 75:344–355.
10. Watanabe M, Uehara Y, Yamashita N et al. (2014) *Cytometry A* 85:206–213.