

Detecting human circulating endothelial cells using the Attune® Acoustic Focusing Cytometer

Circulating endothelial cells (CECs) are mature cells shed from blood vessel walls during the natural process of endothelial cell turnover. Elevated levels of CECs have been reported in a host of pathological conditions including cardiovascular disorders, infectious diseases, immune disorders, post-transplantation analysis, and cancer [1]. In cancer research, CECs have been suggested as a noninvasive biomarker for angiogenic activity, providing insight into tumor regrowth, resistance to chemotherapy, early recurrence, and metastasis during or after chemotherapy [2]. In healthy individuals, CECs are reported to be present in very low numbers: 0.01%–0.0001% of all peripheral blood mononuclear cells [3].

Flow cytometry offers the necessary collection and analysis capabilities for detection of CECs, but is subject to numerous technical challenges. In comparison to traditional hydrodynamic focusing cytometers, the Attune® Acoustic Focusing Cytometer, with its fast acquisition times and increased precision, overcomes the technological hurdles involved in analyzing CECs.



Here we present a method for detecting human circulating endothelial cells using the Attune® Acoustic Focusing Cytometer. The method includes a number of conventional ways to improve rare-event detection: a blocking step, a viability stain (SYTOX® AADvanced™ Dead Cell Stain), and the use of a dump channel to eliminate unwanted cells and decrease background fluorescence. The challenge of collecting a large enough number of events in a reasonable amount of time is met by using a collection rate of 1,000 $\mu\text{L}/\text{min}$ with

the Attune® cytometer. This setting enables the collection of more than 4,000,000 live white blood cell (WBC) events in just 35 minutes; the acquisition time using a traditional hydrodynamic focusing cytometer would be 10–12 times longer, close to 6 hours. Furthermore, this method delivers additional time savings by eliminating wash steps to avoid sample loss and employing a simpler sample preparation method; details and specific panels designed for the blue/violet and blue/red Attune® cytometer configurations are outlined.

Basic protocol

Materials needed

- Attune® Acoustic Focusing Cytometer
- Attune® Performance Tracking Beads (Cat. No. 4449754)
- 12 x 75 mm tubes or other flow cytometry tubes
- 50 mL conical vials
- AbC™ Anti-Mouse Bead Kit (Cat. No. A10344)
- Phosphate-buffered saline (PBS) (Cat. No. 10010-023)
- Anti-coagulant coated collection tubes with EDTA (0.5 M), sodium heparin (140 USP units/mL), or sodium citrate (3.8% w/v)
- Human peripheral blood (use within 3 hr of collection)
- High-Yield Lyse fixative-free lysing solution (HYL) (Cat. No. HYL250)
- Normal Mouse IgG (Cat. No. 10400C)
- SYTOX® AADvanced™ Dead Cell Stain Kit (Cat. No. S10274)
- Directly fluorescent dye-labeled monoclonal mouse anti-human antibodies as described in Table 1

Titrate antibodies

Titrate each fluorescent dye-labeled monoclonal mouse anti-human antibody conjugate individually, and choose the dilution that provides the maximal separation of positive cells with minimal background of the negative population to determine the optimal staining concentrations.

NOTE: As with all immunophenotyping experiments, successful results depend on testing and titration of conjugated antibodies prior to preparing mixed antibody cocktails. We recommend using only directly labeled or Zenon® labeled antibodies; indirect antibody staining is not compatible with this no-wash staining protocol.

Obtain and block blood samples

1. Collect human peripheral blood following institutional and regional guidelines. Collect samples in anticoagulant-containing collection devices, or add 1/10 volume of anticoagulant (0.5 M EDTA, 3.8% w/v sodium citrate, or 140 USP units/mL sodium heparin) to samples just after collection. In either case, mix well immediately upon collection.
2. Block Fc-binding receptors by adding 50 µL normal mouse IgG per milliliter of whole blood. Incubate for a minimum of 20 min.

Table 1. Fluorescent dye-labeled monoclonal mouse anti-human antibodies for use in the procedure.

Blue/violet panel					
Target/label	Supplier	Cat. No.	Fluorescence channel	Laser	Filter
CD45/FITC	Life Technologies	MHCD4501	BL1	488 nm	530/30
CD3/FITC	Life Technologies	MHCD0301	BL1	488 nm	530/30
CD19/FITC	Life Technologies	MHCD1901	BL1	488 nm	530/30
CD34/PE	BD Biosciences	348057	BL2	488 nm	574/26
SYTOX® AADvanced™ stain	Life Technologies	S10274	BL3	488 nm	640 longpass
CD31/Pacific Blue™ dye	BioLegend	303114	VL1	405 nm	450/40
Blue/red panel					
Target/label	Supplier	Cat. No.	Fluorescence channel	Laser	Filter
CD45/FITC	Life Technologies	MHCD4501	BL1	488 nm	530/30
CD3/FITC	Life Technologies	MHCD0301	BL1	488 nm	530/30
CD19/FITC	Life Technologies	MHCD1901	BL1	488 nm	530/30
CD34/PE	BD Biosciences	348057	BL2	488 nm	574/26
SYTOX® AADvanced™ stain	Life Technologies	S10274	BL3	488 nm	690/50
CD31/Alexa Fluor® 647 dye	BioLegend	303112	RL1	638 nm	660/20

Prepare a solution of 0.2 µM SYTOX® AADvanced™ Dead Cell Stain in High-Yield Lyse fixative-free lysing solution (HYL)

Add 2 µL of 1 mM SYTOX® AADvanced™ Dead Cell Stain stock solution in DMSO for every 10 mL of HYL.

Compensation controls

Prepare single-color compensation controls for each labeled antibody according to Table 2, with AbC™ capture beads from the AbC™ Anti-Mouse Bead Kit [4,5]. Run the SYTOX® AADvanced™ Dead Cell Stain compensation control sample using prepared blood (step 7 below).

Using antibody capture beads for the compensation controls, as recommended in this protocol, not only saves

valuable sample but is also critical for properly compensating markers with low event rates.

1. Prepare an unstained control by adding 1 drop of AbC™ anti-mouse control beads (component B) to tube 1, then adding 1 mL PBS.
2. Add one drop of AbC™ capture beads (component A) to a labeled sample tube for each antibody conjugate included in the panel.
3. Add each mouse antibody conjugate at the manufacturer's recommended staining concentration, or at the optimal titration concentration, to the appropriate sample tubes as outlined in Table 2.
4. Incubate for 15 min at room temperature, protected from light.

5. Add 3 mL PBS and centrifuge for 5 min at 200 x g.
6. Carefully remove and discard the supernatant, and resuspend the bead pellets by adding 1 mL PBS.
7. Prepare the blood plus dead cell stain control by adding 100 µL of prepared blood to tube 4. Then add 2 mL of 0.2 µM SYTOX® AADvanced™ stain and incubate, protected from light, for 20 min at room temperature.

Table 2. Staining overview for the Attune® cytometer.

	CD45 FITC	CD3 FITC	CD19 FITC	CD34 PE	Dead cell stain in HYL	CD31 conjugate*
Compensation controls						
1	Beads unstained	-	-	-	-	-
2	Beads plus CD45 FITC	Add	-	-	-	-
3	Beads plus CD34 PE	-	-	Add	-	-
4	Blood plus dead cell stain in HYL	-	-	-	Add	-
5	Beads plus CD31 conjugate*	-	-	-	-	Add
Gating controls						
6	Unstained cells	-	-	-	HYL only	-
7	FMO minus FITC	-	-	Add	Add	Add
8	FMO minus CD34 PE	Add	Add	Add	-	Add
9	FMO minus dead cell stain	Add	Add	Add	Add	Add
10	FMO minus CD31 conjugate*	Add	Add	Add	Add	-
Test samples						
11	All markers	Add	Add	Add	Add	Add

* For the blue/violet configuration, the conjugate is to Pacific Blue™ dye. For the blue/red configuration, the conjugate is to Alexa Fluor® 647 dye.

Staining protocol—fluorescence minus one (FMO) gating controls and test samples [6,7]

1. Pipet all antibodies into each of seven labeled 50 mL conical vials as outlined in Table 2, depending on your Attune® cytometer configuration.
2. Add 1 mL prepared blood to each vial and mix well.
3. Incubate for 20 min at room temperature (or as recommended by the manufacturer), protected from light.
4. Add 20 mL HYL (without dead cell stain) to tube 6, the unstained cell control.
5. Add 20 mL of 0.2 μ M SYTOX® AADvanced™ Stain in HYL to each remaining tube and incubate all tubes for 20 min at room temperature, protected from light.
6. Place samples on ice.

Data collection

Get started

1. Verify instrument performance with the Attune® Performance Tracking Beads.
2. Create a new experiment using the instrument settings optimized for HYL staining.

Perform compensation setup

1. In the experiment browser, right-click “Compensation” and select “Compensation Setup”.
2. In the Compensation Setup dialog box, select to Compensate on “Height” and select the parameters required for your panel: BL1, BL2, BL3, and VL1 for the blue/violet configuration; or BL1, BL2, BL3, and RL1 for the blue/red configuration. Deselect any unused parameters so that only the channels of interest are recorded.
—Pulse height is used for both scatter and fluorescence signals because it provides lower measurement standard deviations than pulse area.

NOTE: For this application, pulse height will provide better scatter and fluorescence precision than pulse area. Unused parameters should be deselected because the Attune® software limits acquisition to 500,000 events if more than 10 parameters are recorded. When 10 parameters or fewer are selected, the limit is 2 million events, although this can be increased to 20 million by appending files together.
—Match one single-color compensation control sample to each parameter selected.

3. Click “OK” to create the compensation control samples.

Optimize cytometer settings

1. Create the workspace for your specimen by double-clicking on the first sample for the specimen in the experiment browser. Alternatively, the workspace may be made by double-clicking on the “Workspace” option in the experiment explorer.
2. Create a bivariate plot of forward scatter (FSC) vs. side scatter (SSC).
3. Run the unstained cells without recording the data and optimize FSC vs. SSC voltages so that all the cells are on-scale and the typical populations of lymphocytes, monocytes, and granulocytes can be distinguished. Use the standard collection rate of 1,000 μ L/min. Alternatively, you may use a slower collection rate of 200 μ L/min for the initial phases of determining the optimal voltages because this will provide more time to observe changes while adjusting thresholds and PMT voltages. However, it is best to use a 1,000 μ L/min flow rate to fine-tune final adjustments and for acquisition of controls and test samples.
4. Place a polygon gate around the events in the FSC vs. SSC plot to eliminate the debris (Figure 1A).
5. Optimize the FSC threshold to include all events within the

lymphocyte and granulocyte populations.

6. Optimize remaining fluorescence PMT voltage levels by running each of the single-color bead control tubes without recording data. Adjust fluorescence PMT voltages so that cell autofluorescence is well above system noise by placing the autofluorescence around a median fluorescence of 10^3 . Next, verify that all stained beads for each of the labeled antibodies remain on-scale. Where events become off-scale, you should lower PMT voltages or reconsider what dyes are being used for your targets. In multicolor staining, use the brightest dyes (e.g., PE) for the lowest-expressing targets. Save the optimized settings and use these to create an experimental template to simplify instrument setup when running your study samples.

NOTE: It is best to optimize instrument PMT voltages and threshold values during antibody titration and panel development experiments. As you determine optimal instrument settings, save them in a “.gis” instrument settings file named for your panel. This settings file can then be loaded as your default instrument settings when creating new experiments, and should require, at most, minimal adjustment. You will save valuable time and sample when running your panel by simply loading your predefined instrument settings at the start of your experiment.

Acquire data from the compensation control samples

1. Select the first compensation control tube.
2. Since the beads used for compensation controls have scatter characteristics different from those of white blood cells in whole blood, the forward and side scatter voltages and threshold may need to be adjusted to place the bead singlets on scale. Do not, however, change fluorescence PMT voltages from those optimized for your panel. Compensation controls and the stained-cell panel must be run at the same fluorescence voltages to obtain a valid compensation matrix.
3. Set the recording conditions to collect 10,000 R1 events (R1 is the default gate for compensation samples), the acquisition volume to 500 μL , and the collection rate to 1,000 $\mu\text{L}/\text{min}$.
4. Place the unstained bead compensation control (containing component B) on the SIP and press "Run". Adjust the R1 gate to include only bead singlets and record the data.
5. Following acquisition, right-click the R1 gate and select "Apply gate shape to all compensation controls". Run each compensation control in order.
6. For acquisition of the SYTOX[®] AADvanced[™] Dead Cell Stain compensation control, adjust R1 to include the lymphocyte and granulocyte populations (Figure 1A), and the FSC and SSC PMT voltages to the values optimized for cells (in step 3 of *Optimize cytometer settings*).
7. After completing the compensation controls, return the FSC and SSC PMT voltages to values optimized for cells, if changed.

Run experimental samples

1. Select the first sample tube of stained cells.
2. Set the collection rate to 1,000 $\mu\text{L}/\text{min}$, and adjust the acquisition volume and recording criteria to obtain the desired number of events.
3. Proceed with data acquisition for test samples.

Data analysis

1. Ensure that the R1 gate excludes debris in an FSC vs. SSC plot (Figure 1A).
2. Derive a BL3 vs. SSC daughter plot from the FSC vs. SSC R1 gate, and create a "Live" gate of cells negative for SYTOX[®] AADvanced[™] dead cell staining, to remove dead cells from subsequent analysis (Figure 1B).
3. Derive a BL1 vs. SSC daughter plot from the "Live" cell population. Gate on the negative population from this dump channel (Figure 2). CECs are negative for CD45 (pan-leukocyte marker), CD3 (T cell marker), and CD19 (B cell marker). All three antibody conjugates are run in the FITC channel to exclude from analysis cells of known immunophenotype that are not of interest.

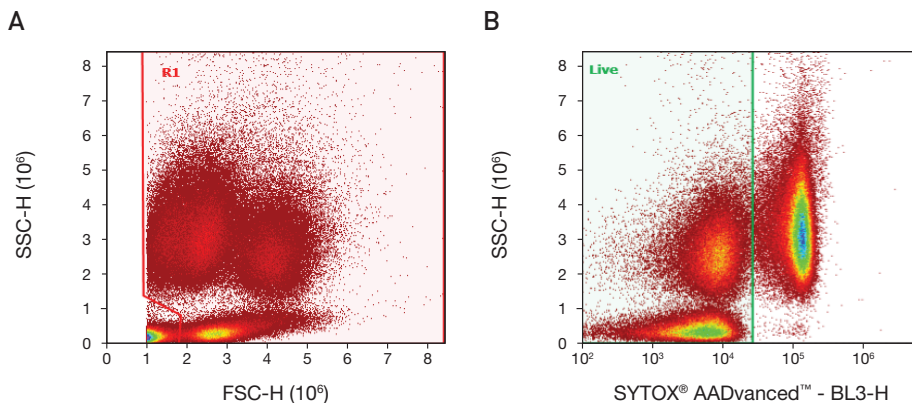


Figure 1. Gating to eliminate debris and dead cells. (A) Density plot of forward scatter (FSC-H) vs. side scatter (SSC-H) showing both the threshold level and the R1 gate to remove debris. **(B)** Gated on events in the R1 region from plot 1A. Density plot of SYTOX[®] AADvanced[™] stain fluorescence (BL3-H) vs. side scatter shows the live cell gate eliminating dead cells.

Example of analysis using the Attune® cytometer blue/violet configuration

CECs were quantified in human whole blood on an Attune® Acoustic Focusing Cytometer, following the instructions in this application note.

A daughter density plot was created from the CD45⁻/CD3⁻/CD19⁻ gate (Figure 2). FMO controls were used to place the quadrant marker. The final plot of CD34-PE (BL2-H) vs. CD31-Pacific Blue™ (VL1-H) shows CECs in the upper right-hand quadrant (Figure 3).

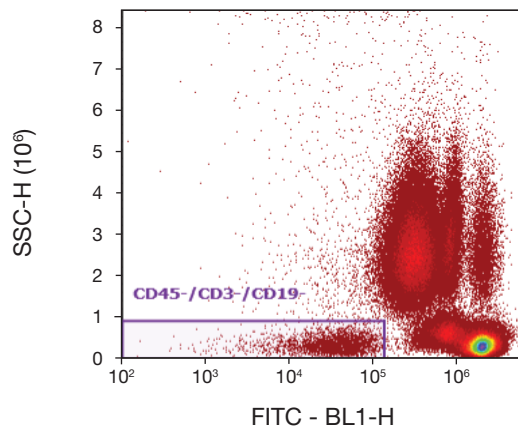
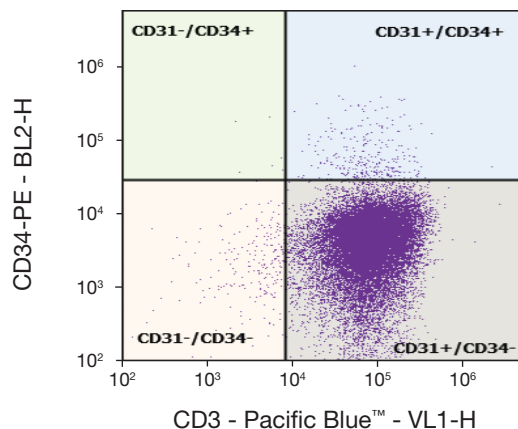


Figure 2. Gating the negative population. Gated on live cells. Density plot of the FITC dump channel (BL1-H) vs. side scatter (SSC-H), showing gate on CD45⁻/CD3⁻/CD19⁻ cells. Since CECs are negative for all three of these markers, all positive cells can be eliminated from further analysis using only one fluorescence channel.

A



B

Name	Event Count	% Parent	% Total
All Events	8,600,152	---	100.000%
R1	7,372,407	85.724%	85.724%
Live	4,029,176	54.652%	46.850%
CD45-/CD3-/CD19-	76,852	1.907%	0.894%
CD31-/CD34+	24	0.031%	0.000%
CD31+/CD34+	444	0.578%	0.005%
CD31-/CD34-	1,442	1.876%	0.017%
CD31+/CD34-	74,942	97.515%	0.871%

Figure 3. Dot plot analysis of human CECs using the Attune® cytometer blue/violet configuration. (A) Final plot is gated to remove debris, then gated to only include live CD45⁻/CD3⁻/CD19⁻ cells. Marker placement was determined using the FMO control samples with CECs in the upper right-hand quadrant. (B) CECs are 0.578% of the parent CD45⁻/CD3⁻/CD19⁻ cells and 0.011% of the total live WBCs (444/4,029,176).

Example of analysis using the Attune® cytometer blue/red configuration

CECs were quantified in human whole blood on an Attune® Acoustic Focusing Cytometer, following the instructions in this application note.

A daughter density plot was created from the CD45⁻/CD3⁻/CD19⁻ gate (Figure 4). FMO controls were used to place the quadrant marker. The final plot of CD34-PE (BL2-H) vs. CD31-Alexa Fluor® 647 (RL1-H) shows CECs in the upper right-hand quadrant (Figure 5).

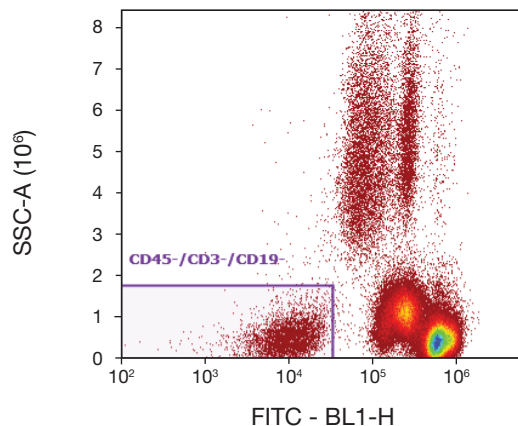
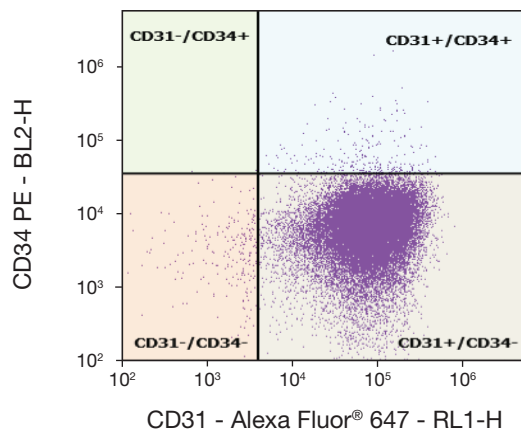


Figure 4. Gating the negative population. Gated on live cells. Density plot of the FITC dump channel (BL1-H) vs. side scatter (SSC-H), showing gate on CD45⁻/CD3⁻/CD19⁻ cells. Since CECs are negative for all three of these markers, all positive cells can be eliminated from further analysis using only one fluorescence channel.

A



B

Name	Event Count	% Parent	% Total
All Events	3,928,759	—	100.000%
R1	3,376,695	85.948%	85.948%
Live	3,019,852	89.432%	76.865%
CD45-/CD3-/CD19-	241,058	7.982%	6.136%
CD31-/CD34+	14	0.006%	0.000%
CD31+/CD34+	178	0.074%	0.005%
CD31-/CD34-	2,546	1.056%	0.065%
CD31+/CD34-	238,320	98.864%	6.066%

Figure 5. Dot plot analysis of human CECs using the Attune® cytometer blue/red configuration. (A) Final plot is gated to remove debris, then gated to only include live CD45⁻/CD3⁻/CD19⁻ cells. Marker placement was determined using the FMO control samples with CECs in the upper right-hand quadrant. (B) CECs are 0.074% of the parent CD45⁻/CD3⁻/CD19⁻ cells and 0.0059% of the total live WBCs (178/3,019,852).

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