Chapter 18

Detection and Characterization of Rare Circulating Endothelial Cells by Imaging Flow Cytometry

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Abstract

Circulating endothelial cells (CECs) are angiogenic cells that appear in increased numbers in the peripheral circulation either as a result of vascular injury or in response to angiogenic stimuli. Elevated levels of CECs have been correlated with various disease states, indicating the use of CECs as a biomarker of disease. Flow cytometry is a widely accepted method for detecting and quantitating CECs. Flow cytometry provides statistical information on large numbers of cells but no information on morphological characteristics. Imaging flow cytometry combines traditional flow cytometry and microscopy, providing a streamlined, multiparameter approach to characterize the biological properties and morphology of large numbers of cells, and is particularly amenable for rare event analysis such as CECs. This approach for identifying and characterizing CECs allows the morphological characterization of large numbers of live, nucleated, single CECs, and alleviates the need for prior enrichment.

Key words Circulating endothelial cells, CECs, Imaging flow cytometry, Rare event flow cytometry, CD146

1 Introduction

The endothelium is a layer of cells providing the inner lining of blood vessels, forming a barrier between the blood and the outer portion of vessels. Direct assessment of endothelial damage or repair is difficult to accomplish with unobtrusive techniques, thus circulating endothelial cells (CECs) have been proposed as surrogate biomarkers of endothelial health in various diseases. CECs have been reported to be an indicator of vascular injury in many conditions such as sickle cell disease, SLE, pulmonary hypertension, vasculitis, rickettsial infection, cardiovascular disease and percutaneous coronary intervention, renal transplantation, and others. Levels of CECs have frequently been reported to be elevated in many types of cancers such as breast, lymphoma, prostate, pancreatic, renal, lung, and rectal cancer, among others. The extreme rarity of these cells necessitates the need for accurate resolution of

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one CEC among a background of millions of leukocytic and other non-endothelial cells and particles in the peripheral blood.

Several different approaches have been developed for detection and enumeration of CECs using combinations of surface markers. A problem common to many of the markers used to identify CECs is the lack of complete specificity for endothelial cells. CD34, for example, is well known to be expressed on endothelial cells [1, 2] but in the hematology and cytometry communities is more regarded as a marker of hematopoietic stem cells [3, 4]. CD31, also called PECAM-1, can mark endothelial cells but is also found on a wide variety of leukocytes [5, 6]. CD146, also known as MCAM, had been described as an endothelial-specific marker [7–10] but is now known to be expressed on a small percentage of circulating T lymphocytes whose expression can be upregulated by activation [11, 12].

Flow cytometric immunophenotyping is ideally suited for detection and quantification of CECs, as it can rapidly analyze tens of thousands of cells per second using multiple markers to identify cells of interest. Flow cytometry can accurately quantify the number of positive events, and, if desired, can physically separate these cells for further studies. Imaging flow cytometry offers many of the same benefits for identification of CECs as does regular flow cytometry (except for cell sorting), and in addition offers the ability to visualize these cells. Visualization not only offers morphologic information regarding these cells, but can also provide data concerning antigen co-localization, antigen capping, cell health, and so forth.

Millions or tens of millions of events from peripheral blood need to be analyzed in order to acquire a sufficient number of the rare CECs to assure, or at least suggest, statistical significance. Several special steps need to be taken in flow cytometric detection of rare events. Of great importance is the need for the cytometer to be as clean as possible, thus lessening the amount of debris or background material ending up in the data files collected. It is recommended to clean the cytometer after running the single color compensation controls and before running the $N-1$ control, because the latter control is the most critical for assessing background events. Crucial components of this assay in addition to thorough cleaning of the cytometer include: collection of a large number of events, use of a blocking serum to help minimize nonspecific binding, exclusion of dead cells, inclusion of mononuclear cells, exclusion of doublets and aggregates, use of a nuclear stain to ensure that cells rather than clumps of particles are examined, time-resolved gating to exclude system variation, CD45 and CD3 to exclude leukocytes, use of an $N-1$ control to help identify background events, ensure accurate compensation, and set gates, use of the brightest fluorochrome for the rare event marker, and the use of CD146 to identify endothelial cells.

As alluded to above, setting up this assay for CECs requires the use of multiple fluorochrome-conjugated antibodies for staining the cells. A generally accepted principle in designing polychromatic flow cytometry assays is to use the fluorochrome with the greatest quantum yield (i.e., the "brightest") with the marker that is the least dense (i.e., the "dimmest") in order to achieve acceptably bright staining of the marker in question [13]. In rare event analysis not only does this apply, but also does the desire to have the most rare marker stain as brightly as possible in order to best separate stained cells from background. Markers such as CD146 are generally found as a continuum of intensities in peripheral blood rather than as a discrete population of events. Thus the use a bright fluorochrome with CD146 is highly desirable. It should be emphasized that the goal of imaging flow cytometry of CECs is the visualization and characterization of these cells, not their quantification. Imaging flow cytometry has much slower acquisition rates and creates much larger file sizes, making this technique difficult to replace routine flow cytometry as the method of choice for enumeration. However, imaging flow cytometry is clearly a preferred method for visualization of CECs as it is much quicker than manual analysis, is capable of using multiple fluorochromes, and yields data which can be gated in a Boolean manner and statically analyzed.

2 Materials

1. 8.5 ml Vacutainer tubes containing ACD solution A (BD Biosciences, San Jose, CA).
2. 50 and 15 ml conical polypropylene tubes.
3. ACK Lysing Buffer (Quality Biologicals, Gaithersburg, MD): A commercial product containing ammonium chloride and potassium designed to lyse red blood cells but not leukocytes; is utilized to lyse red blood cells in whole blood, leaving white blood cells intact.
4. FACS buffer: PBS pH 7.2, 0.5 mM EDTA, 0.2 % BSA. 0.5 mM EDTA, and 0.2 % BSA were added to the PBS to help prevent cells from aggregating, while pH 7.2 better enabled Hoechst labeling. Store FACS buffer at 4 °C.
5. Normal mouse serum.
6. Antibodies (*see* **Note 1**):

   CD146 PE (clone P1H12).

   CD3 AlexaFluor 647 (clone UCHT1).

   CD45 APC-Cy7 (clone 2D1).
7. Hoechst 33342.
8. 7-AAD.

9. 70 μ nylon mesh strainers.
10. ImageStream X or MKII imaging flow cytometer equipped with 405, 488, 561 (optional), 642 (or 658), and 785 nm lasers and two cameras/12 channels; INSPIRE acquisition software and IDEAS analysis software (Amnis, Seattle, WA).

3 Methods

3.1 Preparation of Sample for Immunophenotyping

1. Prior to beginning this procedure, all antibodies should be titrated according to McLaughlin, et al. [14] (*see* **Note 2**).
2. Collect whole blood (*see* **Note 3**) by venipuncture in vacutainer tubes containing ACD.
3. Centrifuge blood in the original vacutainer tubes for 10 min at 400 × *g* at room temperature.
4. Remove the top plasma layer by vacuum aspiration (or similar method) and discard. For more complete lysis of blood, remove as much of plasma layer as possible.
5. Transfer remaining blood cell pack (~3–4 ml) from vacutainer tube to a 50 ml polypropylene conical tube. Use polypropylene tubes for all subsequent steps to minimize cell loss. Use one 50 ml conical tube for each vacutainer used.
6. Dilute the blood cell pack 1:10 with ACK Lysing Buffer.
7. Lyse blood for 10 min at room temperature with gentle rocking.
8. Centrifuge the lysed blood for 10 min at 400 × *g* at room temperature.
9. Carefully remove the supernatant by vacuum aspiration (or similar method) and discard.
10. Resuspend cell pellet in 5 ml ACK Lysing Buffer. Cells can be combined into one 50 ml conical tube at this step.
11. Lyse cells a second time for 10 min at room temperature with gentle rocking.
12. Centrifuge the lysed blood for 10 min at 400 × *g* at room temperature.
13. Carefully remove the supernatant by vacuum aspiration (or similar method) and discard.
14. Resuspend the cell pellet in 10 ml FACS buffer to wash the cells.
15. Centrifuge for 10 min at 400 × *g* at room temperature.
16. Carefully remove the supernatant by vacuum aspiration (or similar method) and discard.

17. Resuspend the cell pellet in 800 µl FACS buffer.
18. Add 200 µl normal mouse serum.
19. Incubate for 10 min at room temperature.
20. Perform a cell count and determine cell concentration.
21. Label 1.5 ml microcentrifuge tubes for the following (*see* **Note 4**):
    (a) Unstained control.
    (b) CD146 PE-only control.
    (c) CD3 AF647-only control.
    (d) CD45 APC-Cy7-only control.
    (e) Hoechst 33342-only control.
    (f) 7-AAD-only control.
    (g) $N-1$ control.
22. Transfer 1×10^6 cells to each control tube. Bring the volume in each control tube up to 100 µl with FACS buffer. Use remaining cells in 50 ml conical tube for CEC sample staining.
23. Add the appropriate amount of antibody, as determined in **step 1** of Subheading 3.1, to the tubes for controls and CEC sample. The $N-1$ control will contain CD3 AlexaFluor 647 and CD45 APC-Cy7, but should NOT contain CD146 PE. The CEC sample will contain CD146 PE, CD3 AlexaFluor 647, and CD45 APC-Cy7.
24. Incubate at room temperature for 30 min in the dark.

    (Prior to completion of sample preparation, the ImageStream should be started up, fluidics should be initialized, and all ASSIST tests and calibrations should be run. This can be done during the incubation steps.)
25. Add 1 ml FACS buffer to the control tubes and 10 ml FACS buffer to CEC sample tubes to wash the cells.
26. Centrifuge for 10 min at $400 \times g$ at room temperature.
27. Carefully remove the supernatant by vacuum aspiration (or similar method) and discard.
28. Resuspend the single-color controls and $N-1$ control in 50 µl FACS buffer, and the CEC cell pellet in FACS buffer to a concentration of ~ $3–5 \times 10^6$ cells in 50 µl.
29. Add Hoechst 33342 to the Hoechst only control, $N-1$ control, and CEC sample tubes to a final concentration of 0.5 µM per million cells and incubate at 37 °C for 30 min. For example, since the Hoechst-only control and $N-1$ control

have 1×10^6 cells in 50 μl, add 1.5 μl of 0.1 mM Hoechst 33342. For the CEC sample, multiply the number of millions of cells by 1.5 μl to determine how much 0.1 mM Hoechst 33342 to add. Do not wash cells after incubation.

30. Add 1.5 μl of a 50 mg/ml stock solution of 7-AAD per million cells to the 7-AAD-only control, $N-1$ control, and CEC sample tubes.

3.2 Imaging Flow Cytometry

3.2.1 Instrument Setup

Prior to completion of sample preparation, the ImageStream should be started up, fluidics should be initialized, and all ASSIST tests and calibrations should be run. This can be done during the incubation steps of the preparation of sample for immunephenotyping. All acquisition is performed in INSPIRE software.

This experiment was performed on a two camera ImageStream MKII equipped with 405, 488, 561, and 642 lasers. ImageStreams may have different lasers and varying powers. Modifications concerning which fluorochromes to use can be made based on instrument configuration. Instrument settings, laser powers, and acquisition criteria may be different on an ImageStream^X and on ImageStreams with varying laser powers.

1. For the panel described above, the following channels and lasers are used:
   (a) Bright-field imagery is collected in Channels 1 and 9 at an intensity of 800.
   (b) SSC imagery is collected in Channel 6 (745–800 nm filter) off of the 785 nm laser.
   (c) PE imagery is collected in Channel 3 (560–595 nm filter) off of the 488 and/or 561 nm laser.
   (d) 7-AAD imagery is collected in Channel 5 (660–745 nm filter) off of the 488 and/or 561 nm laser.
   (e) Hoechst imagery is collected in Channel 7 (430-505 nm filter) off of the 405 nm laser.
   (f) AlexaFluor 647 imagery is collected in Channel 11 (660–745 nm filter) off of the 642 or 658 nm laser.
   (g) APC-Cy7 imagery is collected in Channel 12 (745-800 nm filter) off of the 642 or 658 nm laser.
2. Set the desired magnification. In Samsel et al. [15], all data were collected at 60×. The collection speed will vary according to instrument model and magnification.
3. Laser powers for all lasers should be determined such that the events off a given laser are as bright as possible but are not saturating the cameras in those channels. This can be accomplished by running each single-color control sample, plotting the Raw Max Pixel (RMxP) feature (non-background-subtracted

pixel intensity) for that channel, and ensuring that events are not falling at or above a RMxP value of 4094. Increase or decrease laser power as needed. Return the sample, which will not be acquired until all laser powers have been determined. Repeat this process for each single-color control until all optimal laser powers have been established.

4. Set lasers at the optimal powers determined.

3.2.2 Sample Acquisition and Enrichment by Gating

Setting acquisition gates or cell classifiers in INSPIRE acquisition software (Amnis Corporation, Seattle, WA) designates which events will be acquired into the data file, and which events will be eliminated. This provides a powerful tool for rare event cytometry, in allowing for enrichment of the cell of interest at the time of acquisition. Due to the extremely rare nature of CECs and the large number of cells that must be imaged in order to find CECs, a single raw image file (.rif) data file from the ImageStream containing sufficient events for detection and analysis of CECs would need to contain several million events and the file size would be hundreds of gigabytes. The analyses of files of such size are not practical using standard desktop computer systems; therefore it is necessary to acquire ImageStream data differently than is usually done for flow cytometry. To minimize file size, data should be collected using an acquisition gate or cell classifier (live acquisition gate) so that the majority of CD146 PE negative events are not collected, thus enriching for $CD146^+$ events in the acquired data file. All CECs are $CD146^+$, but only a small percentage (2–3 %) of other mononuclear cells express this marker. Using this approach, a .rif data file containing 10,000 events could be collected, having a file size of roughly 1–4 GB. Even using this enrichment by gating approach, it is necessary to collect multiple 10,000 event .rif files in order to analyze the entire aliquot of any given sample. Each .rif is then analyzed individually.

This gating by enrichment or cell classifying can be accomplished several ways. One way would be to use the Intensity feature of the CD146 PE channel (Channel 3) to identify CD146 negative and positive events. However, to minimize the number of different acquisition plots used, we used the Raw Max Pixel_CD146 PE (Channel 3) feature, which we also used to eliminate saturating events. Whichever features you choose, however, extreme caution should be taken and these gates should be set generously so that cells of interest are not inadvertently omitted.

Acquisition gates for an ImageStream MKII were set as follows (Fig. 1) (*see* **Note 5** for setting acquisition gates for an ImageStream^X).

1. Load the unstained sample.
   (a) Create a two-parameter dot plot of the Area_Channel 1 feature versus the Aspect Ratio_Channel 1 feature and

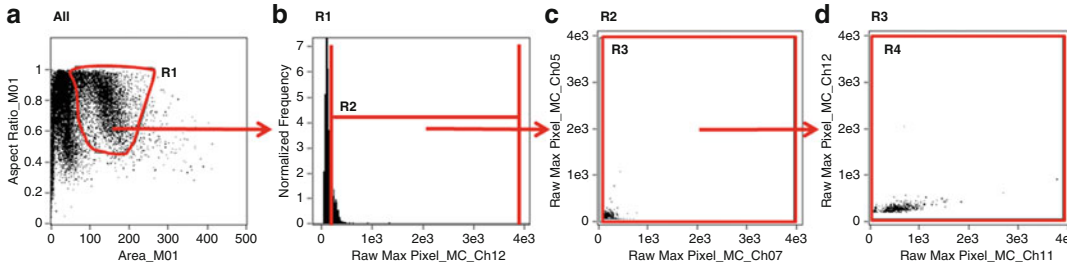

Fig. 1 Gating strategy for enrichment of CECs during acquisition on an ImageStream MKII. Debris and large aggregates were eliminated with the R1 gate (**a**). The majority of CD146 PE-negative events were eliminated with the R2 gate to enrich for CD146-positive cells. Great care must be taken to ensure all PE-positive events are included in the R2 gate. The left edge of the R2 gate should be set generously. Events which may have saturated the camera in the PE channel were also eliminated in the R2 gate (**b**). 7-AAD or Hoechst fluorescence which may have saturated the camera was gated out (**c**), as was any saturating CD3 AlexaFluor 647 or CD45 APC-Cy7 fluorescence (**d**)

draw a generous amorphous gate ("R1") around the single-cell population to eliminate the acquisition of debris and aggregates into the acquired data file (Fig. 1a).

(b) Create a histogram, gated on "R1," of the Raw Max Pixel_Channel 3 feature. Create a region ("R2") whose left edge excludes the majority of the unstained cells and whose right edge is set at a RMxP_Ch 3 value of 4090 to exclude events which were saturating the camera (Fig. 1b). Great care must be taken to ensure that dim CD146 PE positive events are included in this gate. The gate should be set generously. Stricter gating can be done in subsequent analysis after compensation has been performed. Exact values for the lower RMxP_Ch 3 setting are not given here, as the actual lower RMxP_Ch 3 setting may vary from instrument to instrument, with laser powers, and with staining conditions.

The following steps were only used to eliminate saturating events. These are not strictly necessary, but are highly valuable in gating out events which are saturating the camera, and thus could have incorrectly calculated feature values.

(c) Create a two-parameter dot plot, gated on the CD146 positive gate "R2", of the Raw Max Pixel_Channel 7 feature (Hoechst) versus Raw Max Pixel_Channel 5 feature (7-AAD). Create a large rectangular gate ("R3"), which includes all events except those containing saturating fluorescence (Fig. 1c). Set the upper left and lower right vertices to a raw max pixel value of 4090.

(d) Create a two parameter dot plot, gated on the "R3" gate, of the Raw Max Pixel_Channel 11 feature (CD3-AF647)

versus the Raw Max Pixel_Channel 12 feature (CD45 APC-Cy7). Create a large rectangular gate ("R4") (Fig. 1d) which includes all events except those containing saturating fluorescence. Set the upper left and lower right vertices to a raw max pixel value of 4090. Setting the gate in this manner allowed the study of Th17 phenotype identified by CD146 expression on $CD3^+$ cells [16].

2. Set the destination folder, enter the file name, and number of events to collect.
3. Designate the R1 acquisition gate in the File Acquisition area in INSPIRE. It is necessary to use the R1 gate at this step, as the R2 gate is for acquiring PE-positive events, which should not occur in the unstained sample. Acquire 10,000 events of the unstained sample.
4. Load and acquire 10,000–20,000 events of the $N-1$ sample using the R1 acquisition gate. Similarly as for the unstained sample, it is necessary to use the R1 gate at this step, since the R2 gate is set for acquiring PE-positive events, which should not occur in the $N-1$ sample. Any PE positive events that do occur in the acquired file will have arisen as a result of nonspecific binding or dead cells, etc., and will help with eliminating false PE positive events from the final CEC gate.
5. Load the CEC sample and ensure that the acquisition gate acquisition gate in the File Acquisition area in INSPIRE is set ("R4"). The cells which are being currently viewed (top left pull-down menu in INSPIRE) can also be set to R4 to visualize only the events of interest as they are being acquired.
6. Acquire multiple 10,000–20,000 event files.

3.2.3 Acquiring Single-Color Compensation Controls

It is absolutely essential that compensation controls be acquired properly and that the compensation matrix is accurate (see Subheading 3.3.1 for creating the compensation matrix). Single-color controls for compensation must be run with bright field and the 785 nm laser turned off, and with all other lasers set to the power used while collecting the CEC samples. The acquisition gates will be different for the single-color controls than they were for acquiring the unstained, $N-1$, or CEC sample because turning off bright field invalidates the R1 acquisition gate (as no events will fall in the R1 bright-field gate). In fact, the acquisition gates will be different for each single color control, corresponding with which fluorescence channel is currently being used. Further, extremely bright events which saturate the camera should be eliminated from the acquisition file using the Raw Max Pixel feature, to ensure proper compensation. Since the CEC sample was acquired uncompensated, the single-color controls can be acquired after the samples.

Acquisition gates for the single-color controls were set as follows:

(a) Turn off both bright field and the 785 laser. Files acquired with bright field turned off will be annotated with "_NoBF.rif". These files should be used when creating the compensation matrix in IDEAS following acquisition.

(b) Create a histogram gated on All events using the Raw Max Pixel_Channel 3 (CD146 PE) feature and draw a region so that events above 4090 are eliminated from the acquired file. Use this gate while acquiring the CD146 single-color control.

(c) Acquire 10,000 events.

(d) Repeat **steps** (**b**) and (**c**) above for each fluorescence channel used, changing the Raw Max Pixel feature channel as appropriate for each single-color control. A new acquisition gate, which eliminates the saturating fluorescence for the channel currently being used, will need to be created for each channel used, and set as the acquisition gate.

3.3 Data Analysis

All data analysis is performed in IDEAS analysis software.

3.3.1 Compensation Matrix

1. Prior to creating the compensation matrix, each single color control file can be separately analyzed to identify true positively stained cells, and a new "_NoBF" rif can be created which contains only true positively stained cells, eliminating debris, particles staining nonspecifically for the dye, or autofluorescence (*see* **Note 6**).
2. Follow the steps in the compensation wizard to create a compensation matrix utilizing the "_NoBF" single color control rif files.
3. View the created matrix and ensure that there are values for each channel used. It is absolutely imperative that all compensation values in the matrix be verified and modified if necessary. All single-color controls should be checked individually against every other single color control used in the panel with the calculated compensation matrix to ensure that appropriate values have been determined. If values are over- or under-compensated, the matrix should be edited until compensation is accurate.

3.3.2 Gating Strategy

A hierarchical gating strategy is created to identify CECs (Fig. 2).

1. Open a CEC rif file and apply the optimized compensation matrix.
2. Create a Brightfield_Area versus Intensity_Side Scatter plot to identify and create a gate on low side scatter mononuclear cells (Fig. 2a).

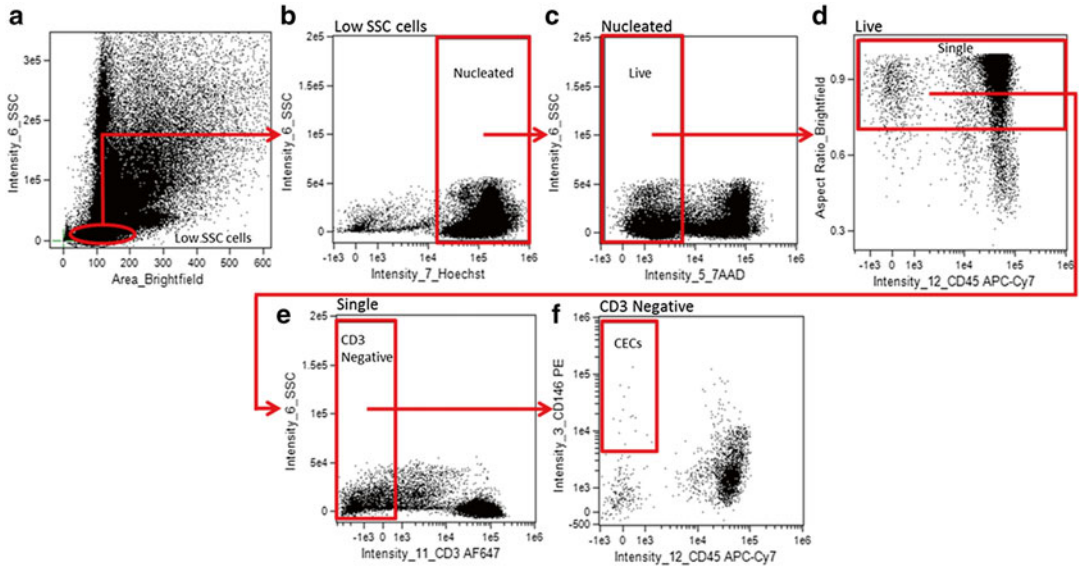

Fig. 2 Analysis gating strategy for identifying CECs from Samsel, et al. [15]. CECs were identified as low SSC (**a**), Hoechst positive (nucleated) (**b**), 7-AAD negative (live) (**c**), aspect ratio high (single cells) (**d**), CD3 negative (non-T-cells) (**e**), CD45 negative (non-leukocytic) CD146-positive cells (**f**)

3. Create an Intensity_Hoechst (Channel 7) versus Intensity_Side Scatter (Channel 6) plot, gated on the low side scatter mononuclear cells, to identify and gate on Hoechst positive (nucleated) cells (Fig. 2b).
4. Create an Intensity_7AAD (Channel 5) versus Intensity_Side Scatter (Channel 6) plot, gated on nucleated low side scatter cells, to identify and gate on Live (7AAD negative) cells (Fig. 2c).
5. Create an Intensity_CD45 APC-Cy7 (Channel 12) versus Aspect Ratio_Brightfield plot, gated on Live, Nucleated, low side scatter cells, to identify and gate on single cells (Fig. 2d). Single, round cells have a higher aspect ratio while elongated cells, aggregates, or more than one cell in a frame have a lower aspect ratio.
6. Create an Intensity_CD3 AlexaFluor 647 (Channel 11) versus Intensity_Side Scatter plot, gated on Live, nucleated, single cells with low side scatter, to identify and gate on CD3 negative cells (Fig. 2e).
7. Create an Intensity_CD45 APC-Cy7 (Channel 12) versus Intensity_CD146 PE (Channel 3) plot, gated on Live, nucleated, single, CD3-negative cells with low side scatter, to identify and gate on CD146+ CD45− cells (Fig. 2f). Save this file and use it as a template to verify the location of all gates in the $N-1$ control file.

8. Open the $N-1$ control file with the final compensation matrix and the CEC template created in **step 7**. Adjust the final CD146+ CD45− CEC gate location as necessary to eliminate background and non-specific events, and minimize those background events from the CD146+ CD45− CEC gate. Adjust other gates as needed. Ensure that all data are properly compensated and adjust matrix if necessary. Save this file and use as a template for analyzing all CEC files.
9. Open and analyze each 10,000–20,000 event file individually using the final compensation matrix and template adjusted with the $N-1$ control.
10. Because these are rare events, the number of CECs may vary greatly in each file. To visualize a larger number of CECs, it is possible to merge files containing CECs.

4 Notes

1. Panel design: There are many guidelines and considerations which should be followed for choosing antibodies and fluorochromes when designing a panel for rare event analysis.
   (a) Instrument configuration: Instrument configurations vary depending on the wavelengths and number of lasers and channels available. Panels should be designed to perform optimally with the configuration of the instrument being used.
   (b) Antibody and fluorochrome selection, minimizing spectral overlap: The brightest fluorochromes in a panel should be utilized for the antibody specific to the rare event marker, or marker with lowest antigen density [17]. For this reason, we used phycoerythrin (PE), which has a high staining index, for CD146, the marker used for detecting CECs. In addition, care should be taken to minimize the spectral overlap between the fluorochromes used in the panel. Spectral overlap and fluorochromes being excited by more than one laser can both lead to data spread which can negatively affect sensitivity [18]. For example, even though PE-Cy5 has a higher staining index than PE, we chose not to use tandems due to spillover optics. All lasers available in the instrument configuration should be used to spread fluorochromes out across channels and reduce spectral overlap.
   (c) Specificity and exclusion gating: Several markers have been described for identification of CECs, but none are specific only to endothelial cells, and are found on other cell types [7–10]. One such marker, CD146, also known

as MelCAM and MCAM, was originally thought to be an endothelial-specific marker. However, CD146 was subsequently found to be present on a subset of activated T-lymphocytes [11, 12, 16]. It is important to include additional markers in the panel which will exclude non-CEC events that also express the antigen of interest (in the case of CECs, CD146) and ensure specificity. Elimination of events not meeting your criteria is referred to as exclusion gating, or a "dump" channel. For example, the pan-leukocyte marker CD45 is expressed on all leukocytes in the peripheral circulation. As such, CD45-positive events are commonly excluded from CEC detection so that the vast majority of non-CEC events will be eliminated from the analysis. For this reason, we included CD3 and CD45 antibodies into our panel to exclude T-lymphocytes which also express CD146. By excluding or "dumping" CD3 positive and CD45 positive cells out of further analysis, only CD146-positive cells which are negative for expression of CD3 and CD45 could be considered as a CEC.

(d) Viability and nuclear staining: It is important to remove dead cells from the analysis as dead cells may bind antibodies nonspecifically, thus giving rise to background and false-positive events. Additionally, use of a nuclear dye can be used to ensure that events being detected as CECs are single cells, rather than clumps or particles. Because of the desire to spread fluorochromes across all lasers, the collection channels still available, and because the cells were not fixed, Hoechst 33342 was chosen as the nuclear dye and 7-AAD was chosen as a viability dye in this panel.

2. Antibody titration: In order to optimize the staining of cells with fluorochrome-conjugated antibodies, the antibodies were first titered to determine the concentration that maximally distinguishes cells staining positive for the respective antigen from the cells which do not bind to the antibody. This was accomplished by testing dilutions (in this case from 1:2 to 1:100) of each antibody against one million PBMCs from a healthy donor. The concentrations of the initial stock solutions of each antibody were determined by the vendors, thus the titers used will vary from vendor to vendor and even from lot to lot of antibody. 10 μl of the antibody dilutions was added to 100 μl of PBS containing the 10^6 PBMCs and incubated for 30 min at room temperature. The cells were then washed once, centrifuged, and analyzed immediately. The cells were analyzed on a Beckman Coulter MoFlo and the staining index (SI) calculated for each antibody. The SI is the mean channel number of the positive staining divided

by the mean channel number of the negative staining. The dilution yielding the highest SI was selected as the concentration to be used for all subsequent staining.

3. Quantity of whole blood needed: Because the number of CECs is extremely low in normal human peripheral blood and varies in different diseases and disease states, the volume of whole blood needed may vary [19]. We utilized two 8.5 ml vacutainer tubes for basic immunophenotyping and detection of CECs by imaging flow cytometry. A higher volume of blood may be desired to obtain a higher "*N*" of detected CECs, for further characterization of CECs with additional markers, for validation of immunophenotype, or if subject type is normal control blood. If a higher number of detected CECs are desired, or if the subject type is normal control, scale up the amount of whole blood used. Validation of this immunophenotypic imaging flow cytometry method for CEC detection was accomplished by sorting a portion of the sample which was used for imaging, and performing Q-RT PCR for endothelial genes [15]. Because enough CECs were needed for both sorting followed by Q-RT PCR *and* detection of CECs by imaging flow cytometry, and because CECs are a rare population, we started with three 8.5 ml vacutainer tubes when performing both sorting and imaging simultaneously. Further, if using an ImageStream^X, only approximately 50 % of the sample loaded can be imaged due to priming of the pumps, while using an ImageStream MKII, approximately 95 % of the sample loaded can be imaged. If an ImageStream^X is being used, starting with 2–3 tubes of whole blood can be advantageous.
4. Controls: Controls are needed for performing spectral compensation and proper setting of gates. To ensure proper correction of spectral overlap, single color controls are run to perform compensation. Each single color control should contain only one antibody/dye. The Hoechst single color control will need to be incubated at 37 °C for 20 min after addition of Hoechst. The $N-1$ control should contain CD3 AlexaFluor 647, CD45 APC-Cy7, Hoechst, and 7AAD, but should NOT contain CD146 PE. For the $N-1$ control, antibody labeling should be performed first per the protocol, cells should be washed with FACS buffer, the supernatant should be removed, cells should be resuspended in FACS buffer, and incubated with Hoechst at 37 °C for 20 min. After Hoechst incubation, the cells should not be washed. 7-AAD is added last.
5. Setting cell classifiers for acquisition on ISX: Images were acquired on a 2 camera, 12-channel ImageStream^X (Amnis Corporation, Seattle, WA) utilizing 405, 488, 658, and 785 nm lasers. Bright field was collected in Channels 1 and 9 at an intensity of 800, SSC was collected in Channel 6 at a 785 nm

power of ~2 mW, Hoechst was detected in Channel 7 (430-505 nm filter), PE and 7AAD were detected in Channels 3 (560–595 nm filter) and 5 (660–745 nm filter), respectively, and AlexaFluor 647 and APC-Cy7 were detected in channels 11 (660–745 nm filter) and 12 (745–800 nm filter), respectively. Cell classifiers were set for channel 1 area lower limit of 25 to eliminate collection of debris, channel 3 raw max pixel lower limit of 20 (non-background-subtracted pixel intensity of the PE channel) such that the majority of CD146 PE-negative events were not collected, and a raw max pixel upper limit of 4094 for all channels used so that events containing saturating fluorescence were not collected. Care must be taken to ensure that you are eliminating any PE-positive events.

6. Creating compensation matrix: Because accurate compensation is critical for this procedure, it can be helpful to analyze each single color control file before creating the compensation matrix. In doing this, new *.rif files can be created which contain only true single positive cells, and events which appear to be positive for the channel of fluorescence but may be debris, particles staining non-specifically for the dye, or autofluorescence can be eliminated. To do this, open each single color *.rif without applying any compensation. Plot of intensity value of the single-color channel currently being used against an adjacent channel. For example plot the Intensity of Channel 3 for PE against the Intensity of Channel 2. The true PE-positive cells should be identifiable by clustering together and can be verified by clicking on the events to visualize the imagery. A region can be drawn around the true positively stained cells, thus creating a new population. A new *.rif file can be created for this true positive staining population by clicking on "Tools," "Create new data file from population," and selecting the true positive staining population. This can be done for each single color control prior to calculating the compensation matrix, and will help with obtaining a more accurate compensation matrix.

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