Application Note

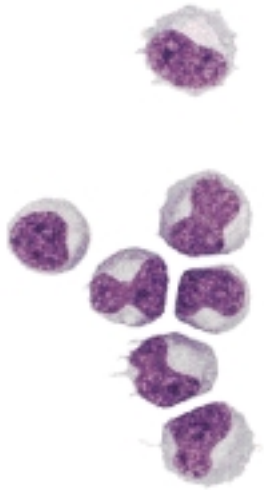
Peripheral Blood Dendritic Cells Revealed by Flow Cytometry

Identification of CD123+ (anti-interleukin 3 receptor α chain) and CD11c+ dendritic cell subsets

Scope

Dendritic cells (DCs) are specialized for antigen uptake, processing, and presentation to T cells.[1] This ability is shared by other *professional* antigen-presenting cells, such as B cells and monocytes. DCs are, however, distinguished by their ability to induce primary immune responses, ie, activation of immunologically naive T cells.[1] DCs are present in peripheral lymphoid tissues,[1] and a variety of populations with DC characteristics has also been found in blood and non-lymphoid organs and are considered to be precursors of the cells in lymphoid tissues[2–4] (reviewed in 1, 5, and 6).

Studies of DCs have been greatly hampered by their low frequencies in blood and tissues and by the lack of specific DC markers. Thus, knowledge about DCs has been obtained mainly from studies of DCs enriched by density centrifugation, culture, and/or negative selection.[7–11] These procedures utilize the density and adhesive properties of DCs, their selective growth in certain cytokine combinations, or their lack of expression of lineage markers for lymphocytes, monocytes and granulocytes (lin). These methods give, however, low yield and purity of DCs and are time consuming. In addition, the manipulation can alter the cells functionally and does not allow for quantitation of DC frequencies.



Freshly isolated CD123$^+$ DCs from PBMCs.

Recent reports identify CD83 and CMRF-44 as cell surface markers that are expressed at high levels on activated or cultured DCs from blood and lymphoid tissue.[12–15] Although excellent as DC activation antigens, these markers do not permit the characterization of unmanipulated cells, as they are absent or expressed at low levels in vivo. The search for antibodies that bind to freshly isolated DCs recently resulted in the identification of anti-interleukin 3 receptor α chain (Anti–IL-3Rα) as the first specific marker of a subclass of DCs in lymphoid organs.[16] These cells constituted the majority of DCs in mononuclear preparations of human peripheral lymphoid tissue. Antibodies to IL-3Rα (CD123) were furthermore sufficiently specific to allow a 200-fold enrichment of the CD123$^+$ DCs in mononuclear preparations of peripheral lymphoid tissue with a single positive immuno-magnetic selection.[16] The antibody was also used to identify the cells in sections stained by immunohistochemistry, and selectively recognized the CD123$^+$ DCs in extrafollicular, T cell rich areas.[16] This cell type is identical to the tonsillar DC described by Hart, et al,[17] and to the *plasmacytoid T-cell* DC described by Grouard, et al.[18] The CD123$^+$ DCs were also identified in peripheral blood[16] and are identical to the previously characterized CD11c$^-$ DC[19] and CD33^{dim}CD14$^-$CD16$^-$ DC.[20]

Because of the lack of DC specific markers, one of the main questions still unanswered is whether DCs constitute a separate lineage of cells. The confusion is related to results showing that DCs can be generated from mature peripheral blood monocytes as well as from unseparated CD34$^+$ progenitor cells when cultured in the presence of GM-CSF and TNF-α.[7,10,13,21,22] Using the CD123 antibody, a CD34$^+$ DC–committed progenitor population was identified.[16] These cells were distinct from the CD34$^+$ progenitors generating DCs of the Langerhans cell type.[16] Thus, a separate lineage of human DCs can be identified by high levels of CD123 staining, establishing a connection between DCs in different tissues.

A second population of DCs with phenotypic characteristics distinct from those of $CD123^+$ DCs has been identified in peripheral lymphoid tissue, and can be defined as $CD11c^+HLA\text{-}DR^+lin^{dim/negative}$ cells. These DCs are localized in the germinal centers, as opposed to the $CD123^+$ cells.[23] The $CD11c^+HLA\text{-}DR^+lin^{dim/negative}$ DCs are also present in blood[19] and identical to the $CD33^{bright}CD14^{dim}CD16^-$ DCs.[20]

From these results it is clear that the DC system consists of multiple cell types with diVerent developmental pathways. Little is known, however, about the functional and potentially specialized roles of these cell types, and how their numbers are regulated under physiological and pathological conditions. Differences in immunophenotype and distinct localization in lymphoid organs might suggest disparate functional roles for the $CD123^+$ and $CD11c^+$ DCs.[16,19,23] The importance of such studies is emphasized by recent results on development of therapeutic strategies against tumors and infectious agents using manipulated DCs. Various reports now demonstrate that this approach holds promise in stopping tumor growth or even causing tumor regression.[24–26]

Utilizing the newly identified DC marker, the current system* for the first time allows for the simultaneous detection, quantitation, and isolation of two distinct DC subsets in freshly isolated peripheral blood. The procedure is based on three- or four-color flow cytometry, is fast, and permits use of small sample volumes. This assay should thus provide a tool that facilitates furthur studies elucidating the role of DCs in immune regulation[27] during physiological as well as pathological conditions.

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* *For research use only. Not for use in diagnostic or therapeutic procedures.*

Materials and Methods

Assay Strategy

The assay is designed to detect two different subsets of peripheral blood dendritic cells (DCs), $CD123^+$ (Anti–IL-3Rα^+) and $CD11c^+$. These populations are identified using a combination of multiple markers since CD11c and CD123 are not DC specific. Both DC populations express high levels of HLA-DR and low levels of lineage markers for monocytes, lymphocytes, and NK cells. Since identification of DCs depends on low expression of these lineage markers without discrimination between individual antibodies, the lineage markers are labeled with a single fluorochrome.[16,19] The lineage cocktail (lin 1) used in this assay contains CD3, CD14, CD16, CD19, CD20, and CD56.

In addition, the assay allows identification of basophilic granulocytes. Basophils are lineage negative and express similar levels of CD123 as the $CD123^+$ DC subset, but can be discriminated by their lack of HLA-DR expression.[16,28-32]

Cells

The assay is designed for whole blood (collected in EDTA, ACD, or sodium heparin) or peripheral blood mononuclear cells (PBMCs). For best results use blood within 24 hours of collection.

Reagents

1. BDIS fluorochrome-conjugated monoclonal antibodies for the detection of dendritic cells.

   4-color assay

   Lineage Cocktail 1 (lin 1) FITC (Catalog No. 340546). Contains CD3, CD14, CD16, CD19, CD20, and CD56.

   CD123 (Anti–IL-3Rα) PE* (Catalog No. 340545)

   Anti–HLA-DR PerCP† (Catalog No. 347364)

   CD11c APC* (Catalog No. 340544)

   Mouse IgG_1 PE (Catalog No. 349043)

   Mouse IgG_{2a} APC (Catalog No. 340473)

   3-color assay

   Lineage Cocktail 1 (lin 1) FITC (Catalog No. 340546). Contains CD3, CD14, CD16, CD19, CD20, and CD56.
   CD123 (Anti–IL-3Ra) PE (Catalog No. 340545)
   CD11c PE (Catalog No. 347637)
   Anti–HLA-DR PerCP (Catalog No. 347364)
   Mouse IgG_1 PE (Catalog No. 349043)
   Mouse IgG_{2a} PE (Catalog No. 349053)

2. FACS™ Lysing Solution‡ (10X) (Catalog No. 349202), dilute 1:10 in deionized water. Refer to the package insert for details.
3. Wash buffer: phosphate-buffered saline (PBS) (10X), Dulbecco's phosphate-buffered saline (GIBCO Catalog No. 14200-075), dilute 1:10 in deionized water.
4. 1% paraformaldehyde in 1X PBS.

Equipment

1. Sodium heparin (BD VACUTAINER® Catalog No. 367673), ACD (BD VACUTAINER Catalog No. 364816) or EDTA (BD VACUTAINER Catalog No. 367661) VACUTAINER tubes.
2. Disposable 12x75-mm Falcon™ polystyrene test tubes (BD Labware Catalog No. 2058).
3. Vortex mixer.
4. FACS® brand flow cytometer.
5. Centrifuge.
6. Vacuum cell aspirator.
7. Micropipettor with tips (Pipetman®, Rainin Instrument Co, or equivalent).

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* US Patent No. 4,520,110; European Patent No. 76,695; Canadian Patent No. 1,179,942.
† US Patent No. 4,876,190.
‡ US Patent Nos. 4,654,312; 4,902,613; and 5,098,849.

	FITC	PE		PerCP	APC
4-Color Assay					
Tube 1	lin 1 20 µL	CD123	5 µL for whole blood 10 µL for PBMCs	Anti–HLA-DR 10 µL	CD11c 5 µL
Tube 2	lin 1 20 µL	Mouse IgG_1		Anti–HLA-DR 10 µL	Mouse IgG_{2a}
3-Color Assay					
Tube 1	lin 1 20 µL	CD123	5 µL for whole blood 10 µL for PBMCs	Anti–HLA-DR 10 µL	
Tube 2	lin 1 20 µL	Mouse IgG_1		Anti–HLA-DR 10 µL	
Tube 3	lin 1 20 µL	CD11c 5 µL		Anti–HLA-DR 10 µL	
Tube 4	lin 1 20 µL	Mouse IgG_{2a}		Anti–HLA-DR 10 µL	

Table 1 Staining strategy for 3- and 4-color peripheral blood dendritic cell assay.

Direct Immunofluorescence Staining of Whole Blood

1. Add monoclonal antibodies to the appropriate tubes. See Table 1.

   NOTE: We recommend using 5 µL CD11c PE or APC, 10 µL Anti–HLA-DR PerCP, and 5 µL CD123 PE per test.

2. Add 100 µL of whole blood to each tube. Vortex and incubate 15 minutes at room temperature in the dark.
3. Add 2 mL of FACS Lysing Solution. Vortex and incubate 10 minutes at room temperature in the dark.
4. Centrifuge at 300 x *g* for 5 minutes. Aspirate the supernatant.
5. Vortex gently and add 1 mL of wash buffer.
6. Centrifuge at 300 x *g* for 5 minutes.
7. Aspirate the supernatant. Vortex gently and resuspend in 300 µL of 1% paraformaldehyde.
8. Analyze on a FACS brand flow cytometer. Samples can be stored at 2° to 8°C in the dark for up to 24 hours prior to analysis.

Direct Immunofluorescence Staining of PBMCs

1. Add monoclonal antibodies to the appropriate tubes. See Table 1.

   NOTE: We recommend using 5 µL CD11c PE or APC, 10 µL Anti–HLA-DR PerCP and 10 µL CD123 PE per test.

2. Add 50 µL (1 x 10^6 cells) PBMCs to each tube. Vortex and incubate 25 minutes on ice in the dark.
3. Vortex gently and add 1 mL of wash buffer.
4. Centrifuge at 300 x *g* for 5 minutes.
5. Aspirate the supernatant. Vortex gently and resuspend in 300 µL of 1% paraformaldehyde.
6. Analyze on a FACS brand flow cytometer. Samples can be stored at 2° to 8°C in the dark for up to 24 hours prior to analysis.

Data Acquisition and Analysis

1. Use CaliBRITE™ beads and FACSComp™ software to adjust the photomultiplier tube (PMT) voltages and fluorescence compensation, and to check the sensitivity of the instrument.
2. Use FACSComp, version 2.0 or later, for the 3-color assay. The 4-color assay requires a flow cytometer with dual laser and FACSComp, version 4.0. The forward scatter (FSC) amplifier can be adjusted manually.

   NOTE: Proper instrument setup with the correct version of FACSComp is important to obtain appropriate results.

3. Acquire prepared samples on a flow cytometer. For data acquisition, use CellQuest™ software with a threshold on FSC to exclude debris.

   NOTE: Dendritic cells occur with low frequency in peripheral blood. Acquire a minimum of 50,000 events.

4. Use CellQuest, Attractors™ , or PAINT-A-GATE$^{PRO™}$ software for data analysis. Gating strategies for CellQuest and Attractors are demonstrated.

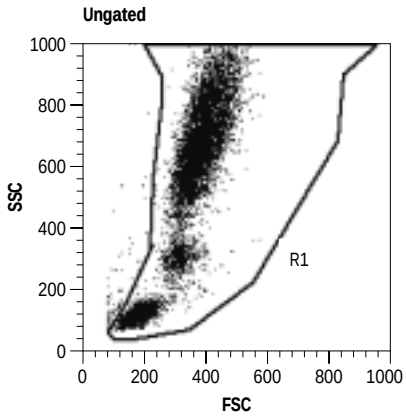
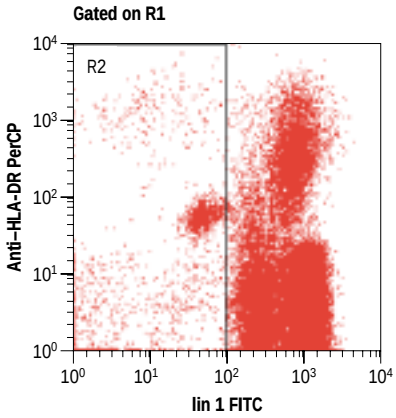


Figure 1 Whole blood, 4-color assay. Ungated SSC/FSC dot plot. 20% of total events are displayed.



Gate: Events excl. debris (R1)
Total Events: 50000

Gate	Events	% Gated	% Total
Events excl. debris (R1)	49659	100.00	99.32
lin 1 dim/-events (R2)	1962	3.95	3.92
G3	1962	3.95	3.92
Basophils	515	1.04	1.03
CD123$^+$ DCs	69	0.14	0.14
CD11c$^+$ DCs	102	0.21	0.20
G7	686	1.38	1.37

Figure 2 Whole blood, 4-color assay. Region R2 includes lin 1 dim and negative events, such as DC populations, basophils, and eosinophils. All R1 events are displayed (Figure 1).

CellQuest Analysis (3- and 4-color)

1. Discriminate cells from debris.

   Create a side scatter/forward scatter (SSC/FSC) dot plot (ungated). Draw region R1 to exclude debris and dead cells. See Figure 1.

2. Identify lin 1 dim and negative events.

   Create an Anti–HLA-DR/lin 1 dot plot gated on region R1. Draw region R2 to include lin 1 dim and negative cells. See Figure 2.

3. Identify peripheral blood DC subsets and basophils.

   NOTE: In a 4-color assay format, Tube 1 stains DC subsets and basophils and Tube 2 is the isotype control. In a 3-color assay format, Tube 1 and Tube 3 stain DC subsets and basophils and Tube 2 and Tube 4 are isotype controls. Use the isotype control tubes to identify non-specific staining.

   Go to the Gate List and define G3 as R1 and R2. Create an Anti–HLA-DR/CD123 dot plot and an Anti–HLA-DR/CD11c dot plot, each gated on G3. See Figure 3a and Figure 3b. These plots only display cells that are lin 1$^{dim/negative}$. Draw region R3 to define basophils (Anti–HLA-DR$^-$/CD123$^+$), region R4 to define CD123$^+$ DCs (Anti–HLA-DR$^+$/CD123$^+$), and region R5 to define CD11c$^+$ DCs (Anti–HLA-DR$^+$/CD11c$^+$).

4. Recreate the Gate List shown in Figure 4a.

5. Confirm the resolution of the populations.

   The resolution of individual cell clusters should be analyzed using all available parameters. Figure 4b shows the cluster resolution of CD123$^+$ DCs, CD11c$^+$ DCs and basophils in a 4-color, whole blood example. To display only the events of interest use the logical gates described in Table 2. Choose the Multicolor Gating option when formatting the plots in Figure 4b. For additional information on population resolution see the Remarks section.

6. Determine the cell frequency.

   Once the populations of interest are defined in the Gate List (Figure 4a), statistics can be obtained from dot plots gated on R1. See Figure 2.

   - CD123$^+$ DCs — R1 and R2 and R4
   - CD11c$^+$ DCs — R1 and R2 and R5
   - Basophils — R1 and R2 and R3

   NOTE: For 3-color analysis, appropriate statistics are obtained from two different data files. CD11c$^+$ DC statistics are obtained from the CD11c PE data file. CD123$^+$ DC and basophil statistics are obtained from the CD123 PE data file.

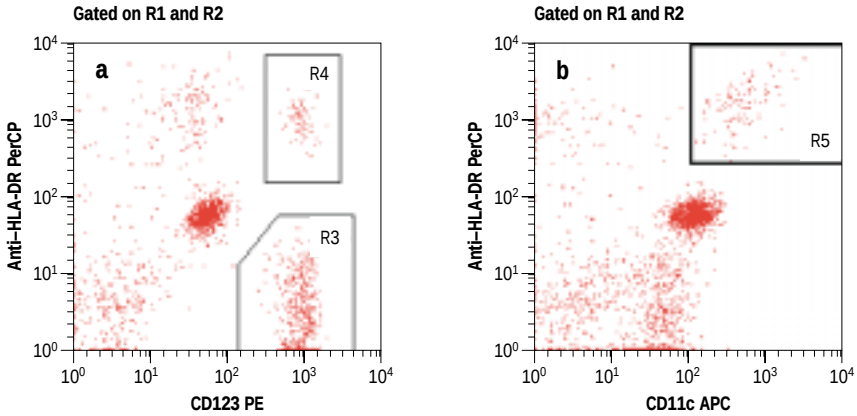


Figure 3 Whole blood, 4-color assay. Gated on events excluding debris and on lin 1 dim and negative events, defined in logical gate G3 (Figure 4a). (3a) Region R3 defines basophils and region R4 CD123⁺ DCs. (3b) Region R5 defines CD11c⁺ DCs. All G3 (R1 and R2) events are displayed.

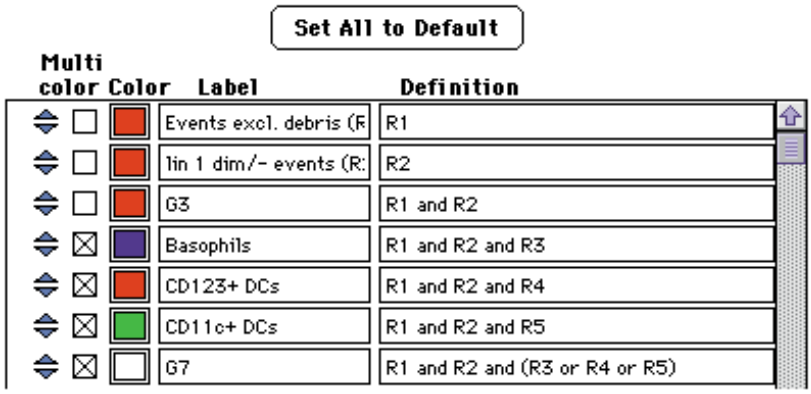


***Figure 4a** CellQuest Gate List.*

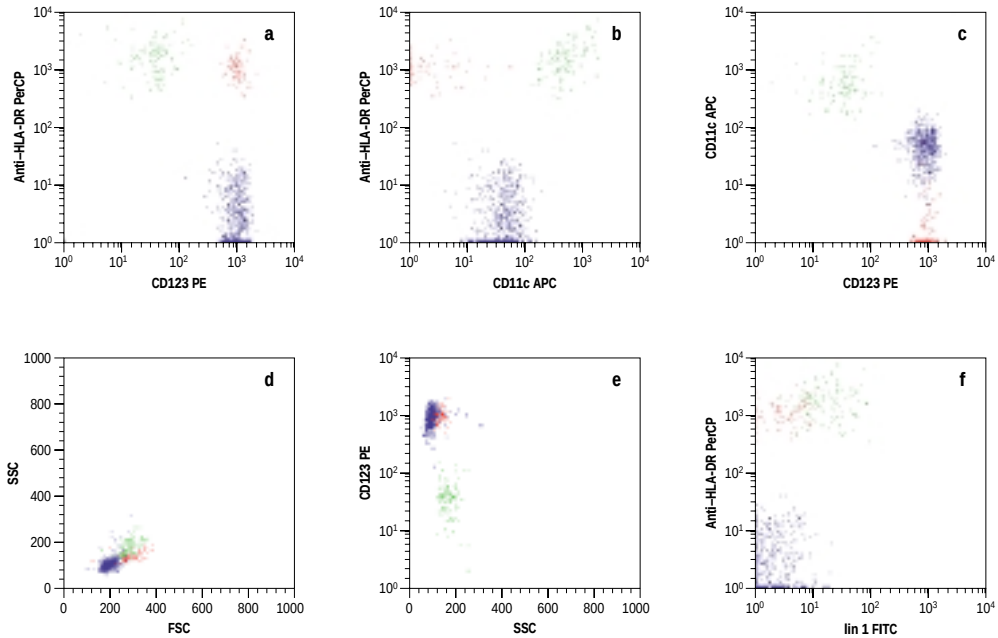


Figure 4b Whole blood, 4-color assay. Gated on G7, defined as [R1 and R2 and (R3 or R4 or R5)], see Figure 4a. This logical gate displays only the populations of interest. Red events = CD123⁺ DCs, green events = CD11c⁺ DCs, blue events = basophils.

Assay	Population of Interest	Logical Gate
4-color	Basophils, CD123$^+$ DCs, CD11c$^+$ DCs	R1 and R2 and (R3 or R4 or R5)
3-color	Basophils, CD123$^+$ DCs	R1 and R2 and (R3 or R4)
3-color	CD11c$^+$ DCs	R1 and R2 and R5

Table 2 Logical gating for 3- and 4-color detection of peripheral blood DCs and basophils.

Attractors Analysis (3- and 4-color)*

Good working knowledge of Attractors software is recommended for this application. Refer to the *Attractors Software User's Guide* for introductory instructions and details.

Step 1 through step 4 describe how to create a 4-color attractor template to identify CD123$^+$ and CD11c$^+$ peripheral blood DCs and basophils. This template uses the same principles described in the CellQuest analysis section.

For a 3-color assay, you need to design two attractor analysis documents. To identify the CD11c$^+$ DC population, create a first document following all the steps except step 3c and step 3d. To define CD123$^+$ DCs and basophils, design a second document, following all steps except step 3e.

1. Discriminate cells from debris.

   a. Create an SSC/FSC dot plot and place a series of fixed black hole attractors to exclude debris and dead cells from further analysis. See Figure 5a, Debris 1 and Debris 2 attractors.

   b. Create an Anti–HLA-DR/lin 1 dot plot and cover all events with a fixed attractor. Adjust the attractor color to gray. See Figure 5b, All events attractor.

   NOTE: Ensure that the entire plot is covered with the attractor.

2. Identify lin 1 dim and negative cells.

   a. Select the All events attractor in the Anti–HLA-DR/lin 1 dot plot. Then place a fixed subattractor over lin 1 dim and negative events. Adjust the attractor color to light gray. See Figure 5c, lin 1dim/negative events attractor.

3. Identify peripheral blood DC populations and basophils.

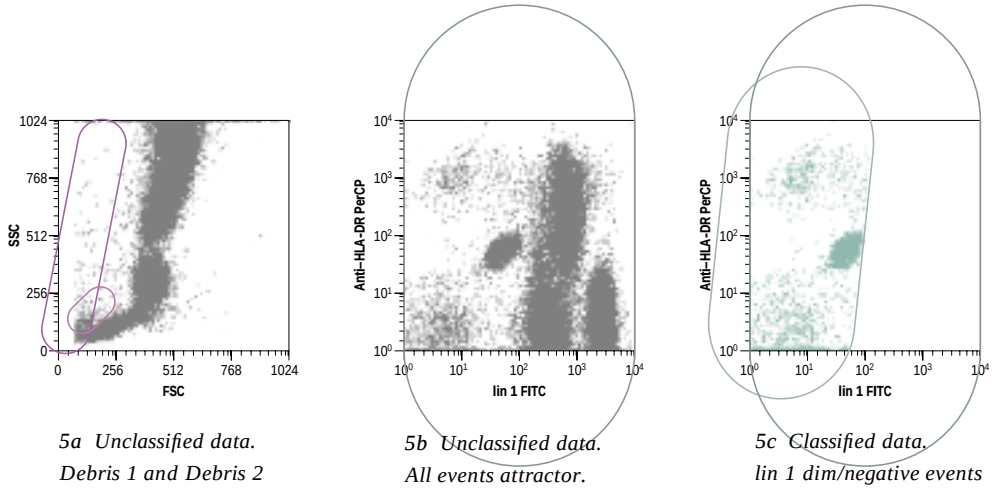
   a. Create an Anti–HLA-DR/CD123 and an Anti–HLA-DR/CD11c dot plot. See Figure 5d and Figure 5e.

   b. Select the lin 1 dim/negative events attractor in the Anti–HLA-DR/lin 1 dot plot. Choose Display from the Apple menu and then Selected Population Only and Classify Data. See Figure 5c, lin 1 dim/negative events attractor.

   c. On the Anti–HLA-DR/CD123 dot plot, define the CD123$^+$ DC population. Place a subattractor over the Anti–HLA-DR$^+$/CD123$^+$ high events. Adjust the subattractor color to red. See Figure 5d, CD123$^+$ DCs attractor.

   d. Select the lin 1 dim/negative events attractor in the Anti–HLA-DR/lin 1 dot plot. On the Anti–HLA-DR/CD123 dot plot, define the basophils. Place a subattractor over the Anti–HLA-DR$^-$/CD123$^+$ events. A skew vector is recommended for this population to offset the classification radius from the attraction boundary toward the x-axis. Adjust the subattractor color to blue. See Figure 5d, Basophils attractor.

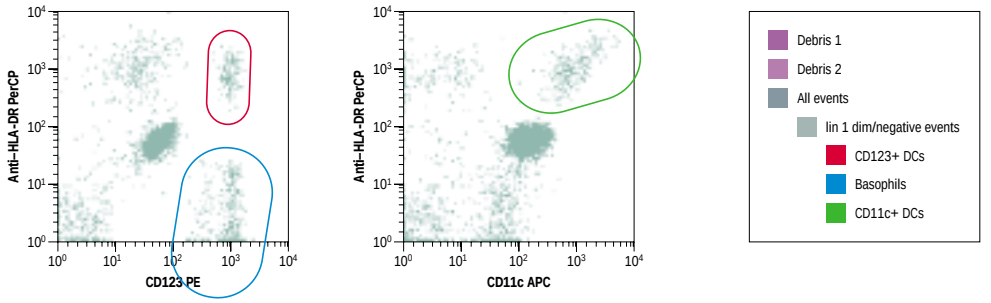
* US Patent No. 5,627,040; 5,739,000; and 5,795,727



5a Unclassified data. Debris 1 and Debris 2 attractors.

5b Unclassified data. All events attractor.

5c Classified data. lin 1 dim/negative events attractor.



5d Classified data. CD123⁺ DCs and Basophils attractors.

5e Classified data. CD11c⁺ DCs attractor.

5f Attractor hierarchy.

Figure 5 Attractor template. 4-color whole blood assay. Detection of CD123⁺ and CD11c⁺ DC populations and basophils. (5a to 5e) 2-parameter dot plots. 5d and 5e show only events of the selected attractor lin 1 dim/negative events.

e. Select the lin 1 dim/negative events attractor in the Anti–HLA-DR/lin 1 dot plot. On the Anti–HLA-DR/CD11c dot plot, define the CD11c⁺ DC population. Place a subattractor over the Anti–HLA-DR⁺/CD11c⁺ events. Adjust the subattractor color to green. See Figure 5e, CD11c⁺ DCs attractor.

f. Turn off Selected Population Only and Classify Data.

4. Check for resolution of populations.

   Highlight the attractor of interest, select Display from the Apple menu, and then choose Selected Population Only.

Frequencies and cell counts of the peripheral blood DC populations and basophils are located in the results page of Attractors software (not shown).

PAINT-A-GATE^{PRO}

Generate dot plots as shown in Figure 4b. Paint and remove debris and dead cells in an SSC/FSC dot plot. Continue using the same analysis strategy as described for CellQuest.

Parameter	CD123+ DCs	CD11c+ DCs	Basophils	See Figure
lin 1	-	-/+	-	4b (f)
Anti–HLA-DR	++	+++	-	4b (a,b,f)
CD123	+++	+	+++	4b (a,c,e)
CD11c	-	+++	+	4b (b,c)

Table 3 Immunophenotype of CD11c+, CD123+ DCs, and basophils (fluorescence intensities).

Results of CellQuest Analysis

DCs are found in low frequencies in peripheral blood. Figure 2 contains statistics from a normal whole blood sample using the 4-color assay. These statistics demonstrate the importance of collecting 50,000 events.

The immunophenotype of the populations of interest is summarized in Table 3.

Remarks

This assay was optimized using fresh peripheral blood from healthy donors. Its utility has not been characterized in blood from abnormal samples. We recommend using fresh blood within 24 hours. Consider the following when performing this assay:

- Incomplete separation of CD11c+ DCs.

  Prolonged or stressed sample storage may cause cell activation or differentiation. Upon activation, lineage markers can be upregulated on the DC subsets, resulting in incomplete separation of the CD11c+ DCs from the CD11c+lin 1+ non–DC leucocytes.

  CD11c+ DCs express low levels of CD14 when freshly isolated from peripheral blood.[20]

- Cluster resolution of CD123+ DCs.

  We recommend using the lineage cocktail to descriminate CD123+ DCs. In an ungated Anti–HLA-DR/CD123 distribution, CD123+ DCs cannot always be separated from other CD123-expressing leucocytes. Using the lineage cocktail to gate on the lin$^{dim/negative}$ cells, the CD123+ DCs are clearly separated.

- SSC high events.

  CD123+ DCs, CD11c+ DCs, and basophils have scatter characteristics that place them between the lymphocytes and monocytes on an SSC/FSC dot plot (see Figure 4b [d]). If SSC high events are observed in the SSC/FSC dot plot when determining the resolution of populations (step 5, CellQuest analysis), we recommend applying an additional low-scatter gate to exclude these false-positive events.

- CD11c/CD123 double-positive events.

  Very low frequencies of CD11c+/CD123+ events have been observed. These events are not yet understood. In a 4-color assay, these double-positive events can be excluded from the analysis by gating on a CD11c APC/CD123 PE dot plot.

- Cell aggregation.

  Cell activation has been observed in aged (>24 hour), cultured, or stressed samples. Activated DCs express high levels of adhesion molecules and tend to stick to one another and to other cell types. Aggregation of DCs will have an impact on cell frequency measurements.

- Anti–HLA-DR isotype control.

  Mouse IgG_{2a} PerCP can be used as an isotype control for Anti–HLA-DR PerCP.

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BD Biosciences publishes this method as a service to investigators. Detailed support for non–flow cytometric aspects of this procedure may not be available from BD Biosciences.

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