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TITLE:

Database-guided Flow-cytometry for Evaluation of Bone Marrow Myeloid Cell Maturation

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Multiparameter flow cytometry, MFC, myelodysplastic syndromes, MDS, myeloid dysplasia, maturation database, bone marrow myeloid cells, database-guided analysis in MFC

SHORT ABSTRACT

The MDS diagnosis is difficult in the absence of morphological criteria or non-informative cytogenetics. MFC could help refine the MDS diagnostic process. To become useful for clinical practice, the MFC analysis must be based on parameters with sufficient specificity and sensitivity, and data should be reproducible between different operators.

LONG ABSTRACT

A working group initiated within the French Cytometry Association (AFC) was developed in order to harmonize the application of multiparameter flow cytometry (MFC) for myeloid disease diagnosis in France. The protocol presented here was agreed-upon and applied between September 2013 and November 2015 in six French diagnostic laboratories (University Hospitals of Saint-Etienne, Grenoble, Clermont-Ferrand, Nice, and Lille and Institut Paoli-Calmettes in Marseille) and allowed the standardization of bone marrow sample preparation and data acquisition. Three maturation databases were developed for neutrophil, monocytic, and erythroid lineages with bone marrow from “healthy” donor individuals (individuals without any evidence of a hematopoietic disease). A robust method of analysis for each myeloid lineage should be applicable for routine diagnostic use. New cases can be analyzed in the same manner and compared against the usual databases. Thus, quantitative and qualitative phenotypic abnormalities can be identified and those above 2SD compared with data of normal bone marrow samples should be considered indicative of pathology. The major limitation is the higher variability between the data achieved using the monoclonal antibodies obtained with the methods based on hybridoma technologies and currently used in clinical diagnosis. Setting criteria for technical validation of the data acquired may help improve the utility of MFC for MDS diagnostics. The establishment of these criteria requires analysis against a database. The reduction of investigator subjectivity in data analysis is an important advantage of this method.

INTRODUCTION:

In the absence of phenotypic markers specific to the dysplastic changes occurring in myeloid cells during MDS initiation and progression, a new approach has been proposed in recent years based on the evaluation of the maturation pathways (altered expression of myeloid antigens during the production of mature myeloid cells) or of the abnormal distribution of different cell types within bone marrow (BM) cell compartments[1,2].

This article presents a new method for standardized application of MFC in order to detect dysplastic changes in BM myeloid cell compartments related to myelodysplastic syndromes (MDS) or other myeloid hematological diseases. This study also shows the utility of using maturation databases for MFC data analysis.

Standardization of the sample preparation procedure, data acquisition, and analysis using the databases would allow the identification of the most relevant phenotypic abnormalities related to dysplastic changes in BM myeloid cells. Therefore, statistically selected subsets based on well-labeled and well-recognized formats (Automatic Population Separator (APS) diagrams, histograms, and dot plots) are required for developing an analysis strategy that can be used in subsequent analysis rounds. The discovery of robust phenotypic abnormalities in MDS would ease the diagnosis in cases with or without minimal morphological dysplasia and without cytogenetic aberrancies. Identification of discriminatory parameters allowing for the reduction of immunophenotypic panels may simplify the current scores[2], permitting their applicability in normal laboratories.

This method limits the subjective interpretations of cytometry data, as have been signaled by MDS diagnosis[3]. This step is a prerequisite for the development of automated tools for processing and analyzing flow data[4].

MDS comprises a heterogeneous group of clonal hematopoietic stem cell (HSC) disorders in which the spliceosome mutations cooperate with specific epigenetic modifiers to yield the MDS phenotype. It is now known that, along with HSC mutations, other mechanisms are involved in MDS pathophysiology, such as aberrant immune-mediated inflammation and interactions between malignant HSCs and the stromal microenvironment of the BM. However, these mechanisms remain poorly understood. The wide clinical and biological heterogeneity of MDS makes the diagnosis and selection of the optimal therapy a challenge. In the last decade, multiple studies have shown that MFC is often more sensitive in detecting dysplasia[2] than morphology, but technical and economic constraints make this technique difficult to standardize, with results often depending on the experience of the interpreter[3]. In addition, it is unclear how MFC can tip the balance toward MDS in cases with or without minimal morphological dysplasia and in the absence of cytogenetic anomalies, or in borderline cases such as hypocellular MDS, with a low blast count, from other non-clonal BM disorders such as bone marrow failure (*i.e.*, aplastic anemia). It also remains difficult to differentiate borderline cases of MDS with an excess of blasts from acute myeloid leukemia (AML). For all these reasons, the clinical guidelines do not integrate MFC testing into the MDS final diagnosis. In

2011, the US National Comprehensive Cancer Network (NCCN) recommended MFC for the estimation of the percentage of CD34+ cells, detection of paroxysmal nocturnal hemoglobinuria clones, and presence of cytotoxic T-cell clones in hypocellular MDS[5]. These two latter situations also involve a therapeutic goal because clinical data have shown a good response of these patients to immunosuppressive therapy[6]. The 2017 NCCN guidelines, citing the International Working Group (IWG) recommendations, listed aberrant immunophenotyping detection by MFC among the co-criteria for MDS diagnosis, but without making any specifications[6]. In addition, the recently published WHO classification stipulates that MFC findings alone are not sufficient to establish a primary diagnosis of MDS in the absence of conclusive morphological and/or cytogenetic data[7]. However, MFC can be used as an additional test showing the dysregulation of myeloid cell maturation patterns and quantifying the "distance from normal" for a patient at a specific time in the disease course.

This method is applicable at clinical laboratories interested in the evaluation of dysplasia in BM myeloid cells using MFC immunophenotyping, in order to refine the diagnosis in MDS or other myeloid disorders with dysplastic abnormalities.

PROTOCOL

The protocol listed below has been approved by the "Comité de Protection des Personnes" (Independant Ethics Committee) Sud-Est 1 from University Hospital of Saint-Etienne, France.

1. Cytometer Settings

Note: The cytometer settings were performed according to France Flow recommendations, in accordance with EuroFlow Procedure "EuroFlow Standard Operating Protocol (SOP) for Instrument Setup and Compensation (https://www.euroflow.org/usr/pub/protocols.php).

1.1 Monthly instrument setup

1.1.1 Turn on the cytometer. Ensure that all fluid levels are appropriate and open Diva 6.1.3. Perform fluidics startup: in the menu bar, select **Cytometer | Fluidics Startup**. Click **OK** when prompted. Allow the cytometer to warm up for at least 30 min.

1.1.2 Performance check - CST beads

Note: For this step, prepare 12 × 75 mm polystyrene tube, CST beads and sheath fluid (see **Table of Materials**).

1.1.2.1 Label a 12 × 75 mm polystyrene tube **CST.** Mix the provided bead vial by gentle inversion or very gentle vortexing. Add to the labeled tube: 0.35 mL of Sheath Fluid and 1 drop of CST beads. Vortex the tube gently and proceed to acquisition. Store the tube for up to 8 h at 2-25 °C in the dark if not acquiring immediately.

1.1.2.2 Perform the performance check: in the menu bar, select **Cytometer | CST**.

1.1.2.3 In the **Setup** tab of the CST module: confirm the Canto II as in the default 4-2H-2V configuration and also confirm that a baseline created using the current lot of CST beads exists for this configuration. If a baseline does not exist, refer to the CST Beads IFU. Confirm that this baseline has not expired: under **Setup Control**, select **Check Performance** from the drop-down menu.

1.1.2.4 Check **Load tube manually** and click **Run**. Confirm the lot number displayed. Gently vortex the diluted beads prepared above and when prompted, load the diluted beads and click **OK**.

1.1.2.5 When the performance check is complete, verify that Cytometer Performance passed. Click **View Report**. Re-run the performance check if the results did not pass. Save the Report in PDF format with the Performance Tracking Report Date.

1.1.3 Adjust **Fluorescent PMT voltages** with Rainbow Beads (**Table of Materials**).

Note: The target values are stipulated in the Euroflow Standard Operating Procedure (SOP) entitled "20180302_7th_Peak_Target_Values_Rainbow_Beads". This SOP is available on Euroflow site (www.euroflow.org; in the public area; Protocols tab).

1.1.3.1 Create a new experiment via **Monthly Instrument Setup Date | Specimen**.

1.1.3.2 In **Cytometer Settings**, choose the optical parameters and fluorochromes corresponding to the tubes concerned (FITC, PE, PerCPCy5.5, PE-Cy7, APC, APC-H7, V450, V500), and check the desired acquisition parameters (Log, A, H and / or W). Apply current CST settings (right click on **Cytometer Settings** of the Experiment). Set the threshold for FSC parameter at 10,000.

1.1.3.3 On the cytometer, turn the compensation off while setting fluorescence PMT voltages for Target MFI setting; for this purpose, go in the **Inspector**, navigate to the **Compensation** tab. Disable compensation by unchecking **Enable Compensation** option.

1.1.3.4 Create a worksheet **Target MFI** with all necessary dot plots (n = 2; FSC versus SSC, FITC versus PE), histograms (n = 8; one histogram for each fluorescence detector) and statistics showing the reference peak values (MFI and CV) for each fluorescence channel.

1.1.3.5 Dilute 1 drop of 8-peak Rainbow beads calibration particles in 1 mL of distilled water and vortex before use. Acquire without recording the 8-peak Rainbow beads solution at **LOW** flow rate. Store the tube for up to 8 h at 2-25 ˚C in the dark if not acquiring immediately.

1.1.3.6 Gate singlet beads **Population P1** in the FSC versus SSC bivariate dot plot and the 8th or 7th peak in the FITC versus PE bivariate dot plot (the brightest peak or the next one downwards, as it's stipulated in the Euroflow document entitled

"20180302_7th_Peak_Target_Values_Rainbow_Beads") and name this gate Population P2.

1.1.3.7 Continue the acquisition of the 8-peaks Rainbow bead suspension and adjust PMT voltages in all fluorescence channels to reach target MFI values according the Euroflow document "20180302_7th_Peak_Target_Values_Rainbow_Beads".

1.1.3.8 Once Target MFI values for the 8th or 7th Peak are reached, record the Target MFI achieved and the corresponding final PMT values. Acquire 5,000 events and record the data.

Note: That PMT values must be used below in step 1.2.3 (Performance Check – Confirmation of PMT Values with Rainbow Beads).

1.1.3.9 Record these values making a print screen with Worksheet **Target MFI** and instrument settings and save as .jpg picture.

Note: When a "New" tube is created, sometimes the PMT values vary unexpectedly. For this, a double checking of PMT is essential. Compare each time the PMT values to your notes, double check that **Target MFI** values and PMT values are correct!

1.1.3.10 Save the **Application Settings**. In the Browser, right-click on **Cytometer Settings**. From the drop-down menu, select **Application Settings**, and save. Click **OK**. If prompted, click **Yes** to maintain the threshold values.

Note: Save the Application Settings using the default name. Do NOT rename the settings.

1.1.4 Adjust the **FCS** and **SSC voltages** with lysed washed blood (LWB).

Note: For this step, 50 µL of a peripheral blood (PB) sample from a healthy volunteer, lysing solution (**Table of Materials**) and washing buffer are needed.

1.1.4.1 Pipets 50 µL of PB into a tube. Add 2 mL of freshly diluted lysing solution. Mix gently and incubate for 10 min at RT.

1.1.4.2 Centrifuge for 5 min at 540 x g.

1.1.4.3 Aspirate the supernatant without disturbing the cell pellet, leaving approximately 50 µL residual volume in the tube. Mix gently and add 2 mL of filtered wash solution.

1.1.4.4 Centrifuge for 5 min at 540 x g.

1.1.4.5 Repeat one more time the steps 1.1.4.3-1.1.4.4.

1.1.4.6 Add 250 µL of filtered washing buffer and mix gently.

1.1.4.7 In the Experiment created for Rainbow Beads acquisition, create a new **Specimen | New Worksheet**: draw a bi-parametric SSC-A / FSC-A graph.

1.1.4.8 Acquire the cells, gate the lymphocytes in a FSC versus SSC bivariate dot plot and adjust FSC and SSC voltages to reach the following mean target values for the gated lymphocyte population: FSC: 55,000 (range 50,000 – 60,000) and SSC: 13,000 (range 11,000 – 15,000).

1.1.4.9 Acquire and record the data with about 10,000 events. Verify the mean FSC and SSC target values for gated lymphocytes. Readjust FSC and SSC voltage if necessary.

1.1.4.10 Print screen and store the print of the target channel values that are obtained.

1.1.5 Fluorescence compensation settings.

Note: The single-stained compensation controls must be set after the Target MFI settings and FSC/SSC settings have been established. For this step, a PB from a healthy volunteer, Compensation Particles (**Table of Materials**), lysing solution and washing buffer are needed. The list of fluorochrome-conjugated antibody reagents used to setup the fluorescence compensation matrices and their reference populations are listed in the **Table 1**.

1.1.5.1 Label one tube per reagent to be used in setting up fluorescence compensation (FITC, PE, PerCPCy5.5, APC, V450, V500, PECy7 – CD117, APC-H7 – CD10, APC-H7 – CD14 and APC-H7 – CD71) and a “blank/unstained” tube.

1.1.5.2 Pipet 50 µL of PB into each tube or 1 drop of “negative” Compensation Particles + 1 drop of “positive” Compensation Particles in the compensation control tubes indicated above in **Table 1**.

1.1.5.3 Add appropriate amount of the antibody reagent to the tube. Add filtered washing buffer to reach a final volume of 100 µL per tube and mix gently. Incubate for 15 min at RT, protected from light.

1.1.5.4 Add 2 mL of freshly diluted lysing solution only in the tubes with the cells and mix gently. Incubate for 10 min at RT, protected from light.

1.1.5.5 Centrifuge for 5 min at 540 x g.

1.1.5.6 Aspirate the supernatant without disturbing the cell pellet leaving approximately 50 µL residual volume in each tube. Mix gently. Add 2 mL of filtered washing buffer.

1.1.5.7 Centrifuge 5 min at 540 x g.

1.1.5.8 Aspirate the supernatant without disturbing the cell pellet leaving approximately 50 µL residual volume in each tube. Add filtered washing buffer to reach a final volume of 250 µL per

tube and mix gently.

1.1.5.9 Create Compensation Controls.

1.1.5.9.1 From the menu bar, select Experiment created for Rainbow Beads acquisition. Create a new **Specimen | Compensation Setup | Create Compensation Controls**.

1.1.5.9.2 In the resulting dialog box, select the **Include separate unstained control tube/well** checkbox. Create generic (not label-specific) compensation controls for FITC, PE, PerCPCy5.5, APC, HV450, HV500. Create label-specific compensation controls for PE-Cy7– CD117, APC-H7 – CD10, APC-H7 – CD14 and APC-H7 – CD71. Click **OK**.

1.1.5.9.3 In a new worksheet, create a bi-parametric SSC-A / FSC-A graph and draw a gate on lymphocytes (P1) and the histogram corresponding to the fluorochrome that will be detected in each tube and draw a P2 gate for the positive peak. Display the hierarchy (right click on a graph and select **Show Population Hierarchy**) to visualize the number of events in P2, except for the Unstained Control tube.

1.1.5.9.4 In the Browser, expand the Compensation Controls specimen.

1.1.5.9.5 Vortex the unstained cells, prepared above, for 3-5 s. Install the prepared unstained cells on the cytometer. Adjust the flow rate to **Medium** and click **Acquire Data**. In the FSC-A vs SSC-A dot plot, adjust the P1 gate to fully encompass the lymphocyte population. Right-click on the P1 gate. Select **Apply to all compensation control**.

1.1.5.9.6 From the **Acquisition** Dashboard, click **Record Data** to acquire 5,000 events. For all the single-color stained control cells, verify that the P2 interval gate encompasses the positive population.

1.1.5.9.7 For the PE-Cy7 and APCH7 tubes, add a P3 interval gate to the histogram and ensure that it encompasses the negative population, and that the P2 encompasses the positive population.

1.1.5.9.8 Calculate Compensation. From the menu bar, select **Experiment | Compensation Setup | Calculate Compensation**. Name the compensation matrix: **Compensations date**. Select **Link and Save**.

1.1.5.9.9 Save the compensation matrix in the Catalog Application Settings: click on **Cytometer Settings | Application settings | Save**, name compensation matrix **Compensation date** and click **OK**.

1.1.5.9.10 In the Browser, click on **Cytometer Settings**. In the Inspector, navigate to the **Compensation** tab. Click **Print** in the lower right corner.

Note: This information can also be retrieved from the catalog.

1.1.5.9.11 Control of the compensation matrix.

1.1.5.9.11.1 Mix in one tube all the single stained tube (APCH7 of choice).

1.1.5.9.11.2 Create a new Experiment named **Compensation verification date**, add new Specimen, click right on the **Cytometer Settings** of this experiment and choose **Link | Unlink | Application Setting** saved in the step 1.1.3.10. Acquire 50,000 events from this tube with the new settings.

1.1.5.9.11.3 Apply a new Global Worksheet. Create 1 dot plot FSC-A/SSC-A and draw a gate to visualize the lymphocytes and n x (n-1)/ 2 other plots focused on the lymphocytes gate to visualize two-by-two parameters.

1.2 Daily Instrument setup

1.2.1 Turn on the cytometer. Ensure that all fluid levels are appropriate and open Diva 6.1.3. Perform fluidics startup: in the menu bar, select **Cytometer | Fluidics Startup**. Click **OK** when prompted. Allow the cytometer to warm up for at least 30 min.

1.2.2 Performance Check - CST Beads: repeat the steps 1.1.2.1 – 1.1.2.5.

1.2.3 Performance Check - Confirmation of PMT Values with Rainbow Beads.

1.2.3.1 Label a polystyrene tube as **Rainbow Beads** and check that the lot number is the one in use. Thoroughly mix the Rainbow Bead vial. Prepare the Rainbow Beads, add 1 drop of Rainbow Beads to 1 mL of deionized or distilled water. Protect from light.

***Note*:** Proceed to acquisition or store the tube at 2-8 ˚C until acquisition.

1.2.3.2 Create a new Experiment: **Rainbow Beads Date**.

1.2.3.3 Link the compensations: right click on **Cytometer Settings**, select **Link Setup**, select the appropriate compensation matrix created in step 1.1.5.9.9 and select **Overwrite**.

1.2.3.4 Unlink compensation: right click on **Cytometer Settings**, select **Unlink from the previously linked setup** and click **OK**.

1.2.3.5 Apply **Application Settings**: right click on **Cytometer Settings**, select **Application Settings**, apply the setting created in step 1.1.3.10 during the Monthly Setup and select **Keep the compensation value**.

1.2.3.6 Deselect **Enable compensation**.

1.2.3.7 Create a new Specimen in the Experiment with worksheet template for Rainbow Beads. Acquire the tube in **LOW** acquisition.

1.2.3.8 During the beads acquisition, adjust the P1 gate to include only the singlet bead population. Adjust the P2 gate on the FITC-A / PE-A dot plot to include only the singlet bead population. Record 10,000 events.

1.2.3.9 Check that the MFI and the CV values obtained for P2 population are in the pre-defined targets of the protocol. Otherwise, wash the cytometer and start the operation again. Save the report as PDF format.

2. BM Sample Preparation

Note: Perform the cell washing protocol just before the staining procedure.

2.1. Pipette 600 µL of primary sample into a 15 mL centrifuge tube.

2.2. Add 10 mL of washing buffer (PBS + 0.5% BSA [>98% pure BSA] + 0.09% NaN_3 filtered solution, pH 7.4). Mix the cell suspension well using a pipette.

2.3. Centrifuge for 5 min at 540 x g (wash 1). Discard the supernatant without disturbing the cell pellet.

2.4. Repeat steps 2.2–2.3 (wash 2).

2.5. Suspend the cell pellet in 400 µL of washing buffer.

2.6. Staining of backbone markers. Transfer the entire volume of the backbone antibodies to a polypropylene tube for FACS analysis, identified with the patient data and the "backbone". Add 350 µL of washed sample (the volume of the washed sample required to fill all the tubes on the panel). Mix well using a pipette.

Note: Calculate the total volume of backbone antibodies for surface membrane staining (as shown in **Table 2**).

2.7. Pipette equal amounts of the sample-backbone mix into 3 polypropylene tubes for FACS analysis, identified with the patient data and “tube number 1” to “tube number 3”. If necessary, use washing buffer to reach a final volume of 200 µL per tube.

CAUTION: Be careful not to leave any trace of the sample on the walls of the tubes; otherwise, these cells will not be stained. If necessary, vortex the cells and centrifuge the tube.

2.8. In each tube, add the appropriate volume of antibodies directed against cell surface

markers (except for the backbone markers), as specified in **Table 2**. Mix well using a pipette. Incubate for 30 min at RT protected from light.

2.9. Add 2 mL of lysing solution. Mix well using a pipette. Incubate for 10 min at RT protected from light.

2.10. Centrifuge for 5 min at 540 x g. Discard the supernatant without disturbing the cell pellet, leaving approximately 50 µL residual volume in each tube. Mix well using a pipette.

2.11. Add 2 mL of washing buffer to the cell pellet. Mix well using a pipette.

2.12. Repeat steps 2.10-2.11 (wash 2).

2.13. Centrifuge for 5 min at 540 x g. Discard the supernatant without disturbing the cell pellet and re-suspend the cell pellet in 200 µL of PBS. Mix well using a pipette.

2.14. Acquire the cells, preferably, immediately after staining or store at 4 °C, protected from light, for no more than 1 h until measured in the flow cytometer.

3. Data Acquisition

3.1. Open a New Experiment in Diva software and rename it according to the name, type of sample and date.

3.2. Create a new Specimen containing 3 tubes. Specify in the Experiment Layout the antibodies used in each tube.

3.3. Click right on the **Cytometer Settings**, choose **Application Settings** and apply the values obtained in Monthly Setup (step 1.1.3.10).

3.4. Open a new Global Worksheet and create the dot plots: SSC-A/ FSC-A, SSC-A/ CD45-HV500-A, SSC-A/ FITC-A, SSC-A/ PE-A, SSC-A/ CD34-PerCP-Cy5.5, SSC-A/ CD117-PECy7-A, SSC-A/ APC-A, SSC-A/ APCH7-A, SSC-A/ HLA-DR-HV450-A. In the SSC-A/FSC-A dot plot, create a gate to select singlet cells. In the SSC-A/CD45-BV500-A dot plot, create gates to select 4 populations: granulocytes, monocytes, blasts, and lymphocytes. Project these populations in the dot plots created previously.

3.5. Create a new Global Worksheet for compensation control as described in step 1.1.5.9.11.3.

3.6. Acquire the tube in **MEDIUM** acquisition and record 500,000 events/tube. After technical validation (evaluation of compensation and the proper staining), export the data as FCS3.0 files.

4. Data Analysis

Note: To construct the normal BM, databases were used files from healthy donors and from individuals without any evidence for a hematopoietic disease as follows 11 from 18 files for the Neutrophils_NM database, 10 from 18 files for Monocytes_NM database and 14 from 18 files for NRC_NM database. The files discarded showed various technical problems, as presented in the Representative Results section. The files were individually analyzed using the Infinicyt software (**Table of Materials**), conforming to the various strategies depicted in **Figures 1A(1-3)** for neutrophil lineage (Profile Neutrophils_Maturation.inp), **Figure 2A** for monocyte lineage (Profile Monocytes_Maturation.inp), and **Figure 3A(1-2)** for erythroid cell lineage (Profile NRC_Maturation.inp).

4.1 Strategy of analysis for neutrophils

4.1.1 Identify CD34+ neutrophil-committed blasts using an intersection of seven gates, allowing the selection of CD34+ CD117+ HLADR+low CD10- CD13+ CD11b- events (**Figure 1A.1**). Assign these events to the **Neutrophil** tab in the Population Hierarchy Tree and thereafter uncheck this tab in order to remove these cells (depicted in blue) from the display of the remaining events (gray).

4.1.2 Isolate the CD117+ CD34- CD13+ CD11b- HLADR+low neutrophil precursors using an intersection of six gates as depicted in **Figure 1 A**(2) and assign them to the **Neutrophil** tab.

4.1.3 Identify more mature neutrophils using an intersection of four gates, allowing for the discrimination of CD45dim SSCint-hi CD117- HLADR-cells and their assignment to the **Neutrophil** tab.

4.1.4 Uncheck the remaining events, keeping only the neutrophils visible, then export this population by clicking **File | Export** and verifying that all the required parameters are checked and save the data as FCS files.

4.1.5 In a merged file consisting of all exported FCS files, perform a quality check by evaluating the intensity of expression of markers for each subpopulation. Using APS plots with medians for each file and SD curves for each subpopulation shown, remove the cases outside the 2SD curves (see details in the Representative Results section) (**Figure 1B**).

4.1.6 In the resulting composed file with “Neutrophils” visible, draw the Maturation Pathway on an APS diagram (**Figure 1C** left) and save as a .cyt file.

Note: A comparison Maturation Diagram allows the visualization of all parameters from all files included in the Neutrophils_NM database represented against the normalized database. The diagram presented in **Figure 2C**, right side, shows that all files included in the Neutrophils_NM database (n=11) fit in 2 SD compared with median of the group.

4.2 Strategy of analysis for monocytes

4.2.1 Identify the monocytic lineage cells (CD117+/- CD64+hi HLADR+hi) using an intersection of four gates (**Figure 2A**). Assign these events to the **Monocytic** tab in the Population Hierarchy Tree.

4.2.2 Uncheck the remaining events, keeping only the monocytic cells visible, then export this population by clicking **File | Export** and verifying that all the required parameters are checked and save the data as FCS files.

4.2.3 In a merged file consisting of all exported FCS files, perform a quality check by evaluating the intensity of expression of markers for each subpopulation, then remove the cases outside the 2SD curves (details in Representative Results section) (**Figure 2B**).

4.2.4 In the resulting file with "Monocytic" cells visible, draw the Maturation Pathway on the APS diagram (**Figure 2C** left) and save this as a .cyt file.

Note: A comparison Maturation Diagram allows the visualization of all parameters from all files included in the Monocytes_NM database represented against the normalized database. The diagram presented in **Figure 2C**, right side, shows that all files included in the Monocytes_NM database (n=10) fit in 2 SD compared with the median of the group.

4.3 Strategy of analysis for nucleated red cells (NRCs)

4.3.1 Identify CD34+ erythroid committed blasts using an intersection of seven gates that allow the selection of CD34+ CD117+ HLADR+low CD105+ CD33- CD36+ CD71+ events (**Figure 3A**(1)). Assign these events to the "NRC" tab in the Population Hierarchy Tree and then uncheck this tab in order to remove these cells (depicted in red) from the display of the remaining events (gray).

4.3.2 Identify more mature NRCs using an intersection of four gates that allow the discrimination of CD45-/+dim SSClow CD36+hi CD71+hi CD105+/-cells. Assign these events to the "NRC" tab (**Figure 3A**(2)). The platelets (CD36+hi SSClow cells) must be removed from the NRC population (**Figure 3A**(2)).

4.3.3 Uncheck the remaining events, keeping only the NRC cells visible, then export this population by clicking **File| Export** and verifying that all the required parameters are checked and save the data as FCS files.

4.3.4 In a merged file consisting of all exported FCS files, perform a quality check by evaluating the intensity of expression of markers for each subpopulation, followed by the removal of the cases outside the 2SD curves (see details in Representative Results section) (**Figure 3B**).

4.3.5 In the resulting file with "NRC" cells visible, draw the Maturation Pathway on the APS

diagram (**Figure 3C**, left) and save this as a .cyt file.

Note: A comparison Maturation Diagram allows the visualization of all parameters from all files included in the NRC_NM database represented against the normalized database. The diagram presented in **Figure 3C**, right side, shows that all files included in the NRC_NM database (n = 14) fit in 2 SD compared with median of the group.

4.4 Evaluation of maturation in BM myeloid compartments using the Maturation Databases

4.4.1 Open the .cyt file corresponding to the lineage of interest *(i.e.*, Neutrophils_NM_GMFF.cyt for neutrophil lineage, Monocytes_NM_GMFF.cyt for monocytic lineage, and NRCs_NM_GMFF.cyt for erythroid lineage).

4.4.2 Right-click on **Maturation** tab below the tab corresponding to the lineage of interest (**Neutrophils | Monocytic| NRC**) and save the Maturation to Maturation Database.

4.4.3 Open a new FCS file and perform analysis as explained previously (step 4.1.1–4.1.3 for Neutrophils, step 4.2.1 for Monocytic cells, and step 4.3.1–4.3.2 for NRC).

4.4.4 Draw the maturation pathway for the population of interest.

4.4.5 Open the corresponding database in the **Tools** tab (Database Analysis) and compare the population to be analyzed with the corresponding Maturation Database. Check the data for compatibility with the available database: complete compatibility (green triangle); partial compatibility, in most cases discrepancies in the name of the parameters (yellow triangle); and incompatibility (red triangle).

4.4.6 If the data are compatibles or partial compatibles, the software creates the **Normalized Maturation Differences** diagram. To visualize the **Parameter Band Maturation Differences**, open a new diagram in **Diagram** tab, click **Maturation** and choose how many parameters to be displayed, click **OK** and the diagram appear. With right click in the diagram; changes can be made in Data Visualization, Database Visualization and Maturation Diagram Visualization.

4.4.7 To visualize the significance of the differences between the new file and data included in the database, configure a zoom (right-click on **Normalized Maturation Differences** and apply **Zoom**).

REPRESENTATIVE RESULTS

The 54 BM samples harvested in K-EDTA anticoagulant were included in the study. The MFC data were analyzed in the absence of any information about the patients. Retrospective study showed that the BM samples were from 7 healthy donors (5 males and 2 females with a median age of 47.4 [35-48], 11 individuals with no evidence of a hematopoietic disease (8 males and 3 females with a median age of 57.9 [35-72]) and 36 cases with various pathological conditions: 1 case with anemia and low creatinine level, 3 cases with anemia and high creatinine level, 8

cases with anemia of inflammation, 1 case with anemia caused by vitamin B_{12} deficiency, 4 cases with anemia ± other cytopenias caused by liver damage, 3 cases with autoimmune hemolytic anemia, 5 cases with idiopathic thrombocytopenic purpura, 1 case with macrophage activation syndrome, 3 cases with complete remission after lymphoma treatment (minimal residual disease < 0.01%) and 7 cases carrying hematological disorders (4 MDS, 1 AML, 1 chronic myelomonocytic leukemia and 1 myeloproliferative syndrome JAK2 positive). This last category included 18 males and 18 females with a mean of age 60.3 [17-100]. All samples were from Caucasian individuals. The building databases allowed identifying and fixing several issues related to sample preparation and acquisition. In our case, the final databases included 11/18 files for neutrophil maturation, 10/18 files for monocyte maturation and 14/18 files for NRC maturation. The files that were not included in databases posed various technical problems. The most common staining issues were observed for CD11b and CD13 in neutrophils, CD300e and HLADR in monocytes, and CD71 and HLADR in NRCs. Exporting data can be a source of errors; the most frequent in our dataset was the absence of FSC-H in exported files and the inversions of FSC-W with FSC-H that sometimes occur in exporting DIVA files[8]. The evaluation of new cases of cytopenias suspected of being MDS against the Myeloid Normal Maturation Databases allow for the identification of abnormal expression of maturation antigens of neutrophils lineage (**Figure 4**), monocytes (**Figure 5**), and NRC (**Figure 6**) even in cases without cytological or cytogenetic abnormalities (**Figure 7**). Otherwise, using routine acquisition software, such as Diva, these would be difficult or impossible to realize.

Moreover, the interpretation of the significance of differences between antigen expression on pathological settings and normal counterparts allows for "quantification" of these abnormalities, with the identification of those that are discriminant for a group of cases. This may also make it possible to rank them based on importance for the purposes of a follow-up. In this study, all 7 cases of hematological disorders with myeloid dysplasia features (4 MDS, 1 AML, 1 chronic myelomonocytic leukemia and 1 myeloproliferative syndrome JAK2 positive) were classified as abnormal when compared with the NBM databases. In addition, the evaluation against the Neutrophils_NM database eliminated the suspicion of myeloid dysplasia in 7 cases with toxic, inflammatory, autoimmune hemolytic anemia and anemia from chronic kidney disease. The evaluation against the Monocytes_NM database eliminated the suspicion of myeloid dysplasia in 4 cases with idiopathic thrombocytopenic purpura, while the evaluation against NRC_NM allowed for differentiation in 3 cases, 2 of inflammatory anemia and 1 of idiopathic thrombocytopenic purpura. The BM aspirates from the patients in complete remission after lymphoma or solid tumor treatments were within 2 SD from the median of the normal databases for all three lineages, and thus these samples may be alternatives to healthy donor BM samples.

FIGURES AND TABLES LEGENDS

Figure 1. Analysis strategies for neutrophil cell lineage. Selection of CD34+ CD117+ HLADR+low CD10- CD13+ CD11b- neutrophil progenitors was realized by intersection of seven gates as depicted in A1. The next step of maturation, CD117+ CD34- CD13+ CD11b- HLADR+low neutrophil precursors was chosen using an intersection of six gates as depicted in A2. The more mature neutrophils are finally identified using an intersection of four gates that allow

discrimination of CD45dim SSCint-hi CD117- HLADR- cells (A3). The most discriminant markers for neutrophil lineage were CD34, CD117, CD11b, and CD16. Along with CD13, these parameters allow identification of five subpopulations: CD34+ progenitors (CD34+ CD117+ CD13+^{low} CD11b- CD16-) (dark blue); CD34- CD117+ CD13+^{hi} CD11b- CD16- neutrophil precursors (blue); CD34- CD117- CD13+^{low} CD11b- CD16- neutrophils (3rd step of maturation; light blue); CD34- CD117- CD13+^{low} CD11b+ CD16+^{low} neutrophils (4th step of maturation; pink); mature neutrophils (CD34- CD117- CD13+^{hi} CD11b+^{hi} CD16+^{hi}; violet). Each colored circle represents the median of a subpopulation for the marker of interest from one sample (B). C displays the homogeneous distribution of the tested parameters during the neutrophil cell maturation when compared with 2 SD of the normal maturation database (n=11).

Figure 2. Analysis strategy for monocytes. Identification of monocytic lineage cells (CD117+/- CD64+hi HLADR+hi) is performed using an intersection of four gates (A). The most discriminant markers for monocytic lineage were CD14, CD300e (IREM2), CD35, and HLA-DR. Along with CD117, these parameters allow identification of three subpopulations: CD34- CD117+^{low}/- HLADR+^{hi} CD35- CD14- CD300e- (red); CD34- CD117- HLADR+^{med} CD35+^{med} CD14+^{med} CD300e- (orange); mature monocytes CD34- CD117- HLADR+^{med} CD35+^{hi} CD14+^{hi} CD300e+ (green) (B). C displays the homogeneous distribution of the tested parameters during the monocytic cell maturation when compared with 2 SD of the normal maturation database (n=10).

Figure 3. Analysis strategies for NRCs. The identification of CD34+ erythroid committed blasts was realized using an intersection of seven gates that allow selection of CD34+ CD117+ erythroid progenitors (A1). The more mature NRCs are identified using an intersection of five gates (A2). Exclusion of the platelets (CD36+hi SSClow cells) from NRC population is required (A2). The most discriminant markers for erythroid cell lineage were CD34, CD117, CD71, and CD105. Along with CD33, these parameters allow identification of three subpopulations: CD45+^{low} CD34+ CD117+ HLADR+^{med} CD71+^{med} CD36+^{med} CD105+^{hi} (red); CD34- CD117+ HLADR+^{low} CD71+^{hi} CD36+^{hi} CD105+^{hi} NRCs (2nd step of maturation; pink); CD34- CD117- HLADR-/+^{low} CD71+^{hi} CD36+^{med} CD105+^{low}/- NRCs (more mature NRCs; pink salmon) (B). C displays the homogeneous distribution of the tested parameters during the NRC maturation when compared with 2 SD of the normal maturation database (n=14).

Figure 4. Representative flow cytometry analysis of neutrophils in a del(7) MDS case. (A). In the Normalized Maturation Differences diagram, the gray area corresponds to the normal expression of antigens resulting from the analysis of the 11 normal files included in the neutrophil database (median± 2SD). The abnormal expression of several antigens was observed when compared the del(7) MDS case against Neutrophils_NM database (n=1 versus n=11 normal controls samples). (B). Parameter Band Maturation Diagrams allow the comparisons between the median intensity of expression of each marker at different stages of maturation (continuous full lines) and 2SD curves calculated for the 11 normal BM cases included in the database (continuous dashed lines). Phenotypic abnormalities of neutrophils observed in a del(7) MDS case as compared with the Neutrophil Normal Maturation Database are as follows: overall increased expression of CD34, slight increase of CD11b expression on immature neutrophils (stages 2), diminished expression of CD13 on immature neutrophils (stages 1-3) and

slight increase of CD16 expression on the first two stages of neutrophil maturation followed by moderate increases of CD16 expression on the mature neutrophils (stages 4-5). The panel C shows the corresponding dot plots as visualized in DIVA software. Image evaluation using the DIVA software allows for identification of a small number of phenotypic abnormalities in this MDS case: an increased percentage of CD34+ CD13+ precursors and the downregulation of CD13 expression. The abnormal expression of CD16 is difficult to observe in this type of representation.

Figure 5. Representative flow cytometry analysis of monocytes in a del(7) MDS case. (A). The Normalized Maturation Differences diagram offers an overall view of antigen expression at various stages of monocytic cell lineage maturation. The gray area corresponds to the normal expression of antigens resulting from the analysis of 10 normal files included in the monocytic database (median ± 2SD). The abnormal expression of several antigens exceeds 2SD, but for some markers, such as CD35 and HLADR, it exceeds 4SD. (B). In the Parameter Band Maturation Diagrams, the phenotypic abnormalities of monocytic cells observed in a SMD del(7) case when compared with Monocyte Normal Maturation Database (n=1 versus n=10 normal controls samples) are as follows: diminished expression of CD45 during stages 1–4 of monocytic cells, of CD117 at stages 1-2 of monocytic precursors, and of HLA-DR overall. The increased expression of CD35 in the first 3 stages of maturation of the monocytic cells was the most significant abnormality for this lineage. (C). The DIVA dot plots that allow evaluation of monocytic cells in a single normal BM. (D). The DIVA dot plots that allow evaluation of monocytic cells in del(7) MDS case. Image evaluation using the DIVA software allows for identification of two phenotypic abnormalities in this MDS case: the diminished expression of HLA-DR and the increased expression of CD35 in a part of monocytic precursors (red population).

Figure 6. Representative flow cytometry analysis of nucleated red cells in a del(7) MDS case. (A). The Normalized Maturation Differences diagram offers an overall view of antigen expression at different stages of NRC lineage maturation. The gray area corresponds to the normal expression of antigens resulting from the analysis of the 14 normal files included in the NRC Normal Maturation Database (median ± 2SD). The abnormal expression of several antigens exceeds 2SD, but for some markers, such as CD34, CD117, CD71, and CD105, it exceeds 4SD. (B). In the Parameter Band Maturation Diagrams, the phenotypic abnormalities observed in a SMD del(7) case when compared with the NRC Normal Maturation Database (n=1 versus n=14 normal controls samples) are as follows: diminished expression of CD34 in the first two stages of maturation, of CD117, CD105, CD71 and HLA-DR on stage 1 of erythroid lineage precursors. Image evaluation using the DIVA software allows for identification of CD71 abnormal expression on erythroid cells in this MDS case, but other modifications are not detectable.

Figure 7. Representative flow cytometry analysis of neutrophils, monocytes and NRCs in an early stage MDS case without morphological dysplasia and without cytogenetic aberrancies. (A). The distribution of immature and mature cells within different maturation stages for the three myeloid lineages: neutrophils, monocytes and NRCs. An increased number of mature monocytes (stage 5) and the slightly diminution of monocytic immature cells (stage 2 of maturation) was observed. (B). The Normalized Maturation Differences diagram shows that the

phenotypic abnormalities exceed 2SD, but for CD34, CD117, CD11b, CD16 and HLADR, they are higher at 4SD. Phenotypic abnormalities observed for neutrophils are as follows: slightly increased SSC values in neutrophil immature precursors (stage 1), diminution of expression of CD34 (stage 1), of CD117 (stage 1), of CD16 (stage 1-2) and of HLADR (stages 1-3 of maturation). An increased expression of CD11b was observed on neutrophil immature precursors (stage 1). (C). The Normalized Maturation Differences diagram shows that the most important phenotypic abnormalities observed for the monocytic lineage (exceeding 10SD) are increased expression of CD35 and early acquisition of CD300e on the immature monocytic precursors. Other phenotypic abnormalities observed on the monocytic lineage are as follows: diminution of FSC and SSC on more mature monocytes (stages 3–5) and slight diminution of expression of CD14 on stages 2-3 of monocytic cells. (D)

. The Normalized Maturation Differences diagram and the Parameter Band Maturation Diagrams shows that the most important phenotypic abnormalities observed for the NRC are: increased expression of CD117 (exceeding 10SD) on stages 2-3 of NRC maturation, increased expression of CD34 (exceeding 5SD) during the second stage of maturation of NRC and decreased expression of CD36 on the early erythroid precursors (stages 1–3). In addition, the SSC on immature NRC is lower than on the normal counterparts.

Table 1. List of fluorochrome-conjugated antibody reagents used to setup the fluorescence compensation matrices with their reference populations

Table 2. Combination and quantities of antibodies used for evaluation of maturation of neutrophil, monocytes and erythroid cells in BM

DISCUSSION

The quality of BM aspirate could impact on the final results. The hemodilution of the BM aspirate could distort the distribution of cells in different stages of maturation due to the absence of progenitors or precursors cells. Probably employing a bulk lysing method may help in normalization of BM aspirates for hemodilution in flow cytometric analyses. In addition, the critical steps for the evaluation of BM myeloid dysplasia by flow-cytometry are the sample processing and staining, data acquisition, and interpretation[2,3]. The sample processing and staining should be performed up to 72 h after BM harvesting. The data should be acquired, preferably, immediately after staining or store the samples at 4 °C, protected from light, for no more than 1 h until measuring in the flow cytometer. The database-guided interpretation avoids the subjective evaluation of BM myeloid cell maturation.

The antibody combinations and the sample preparation were broadly conformed to the EuroFlow procedures[9,10]. The difference compared with the EuroFlow panel was that all antibodies except CD300e were provided by BD Bioscience. The flow cytometry standardization made it possible to obtain data with high levels of reproducibility[11], but this entails joining a group working on the standardization of the panels in question. In our group, the standardization of SSC remains an area to be improved. The major issue regarding MFC databases building is the staining procedure. The initial distribution of the non-backbone antibodies in FACS tubes before adding the equal amounts of the sample-backbone mix avoids

repeating the backbone staining in case of eventual errors in the distribution of other non-backbone antibodies. In addition, to overcome the staining problems there are two possibilities: either increase the incubation time of antibodies from 15 to 30 minutes, or use the recombinant antibodies, which provide greater reproducibility, according to the manufacturer's specifications[12]. The analysis was performed in conformity to recently published data[13-15]. Concerning monocytes analysis, we frequently observed the absence of CD34+ CD117- monocytic precursors, as has been previously reported by Shen et al.[16]. For this reason, we eliminated from the analysis strategy a supplementary gate for isolation of this particular population. The intersection of gates allowing isolation of CD64+F HLADR+F/int and CD117+/- includes the immature precursors (CD117+ CD34+low/-) and the more mature monocytes.

The evaluation of new FCS files compared to a database contributes to a more objective, standardized analysis and data interpretation and avoids misinterpretations of the so-called patterns recognition, which is difficult to standardize[3]. The quantification of the amplitude of phenotypic abnormalities by comparison with normal databases makes it possible to rank these abnormalities, which may help improve MFC scores for MDS diagnosis. Moreover, this method allows precise identification of the phenotypic abnormalities on immature CD34+ and/or CD117+ precursors and in more mature cells, which are not evident when using the current analysis strategies in acquisition software. This method is relevant in the diagnosis of MDS cases that do or do not have evident morphologic abnormalities, or that do or do not carry cytogenetic recurrent aberrancies.

Improvement of antibody staining is needed, and the addition of new normal BM data in databases is required in order to increase the robustness, reliability, and sensitivity of the analysis. This method could be applied, with further investigation, to cytopenias from other causes, or to clonal hematopoiesis of indeterminate potential (CHIP), in order to identify the phenotypic changes reliably related to dysplastic processes.

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DISCLOSURES

The authors declare that they have no competing financial interests. The Flow-Cytometry Laboratory at the University Hospital of Saint-Etienne is a member of EuroFlow Consortium.

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Figure_1 Click here to download Figure Figure 1 neutrophils analyse.tif

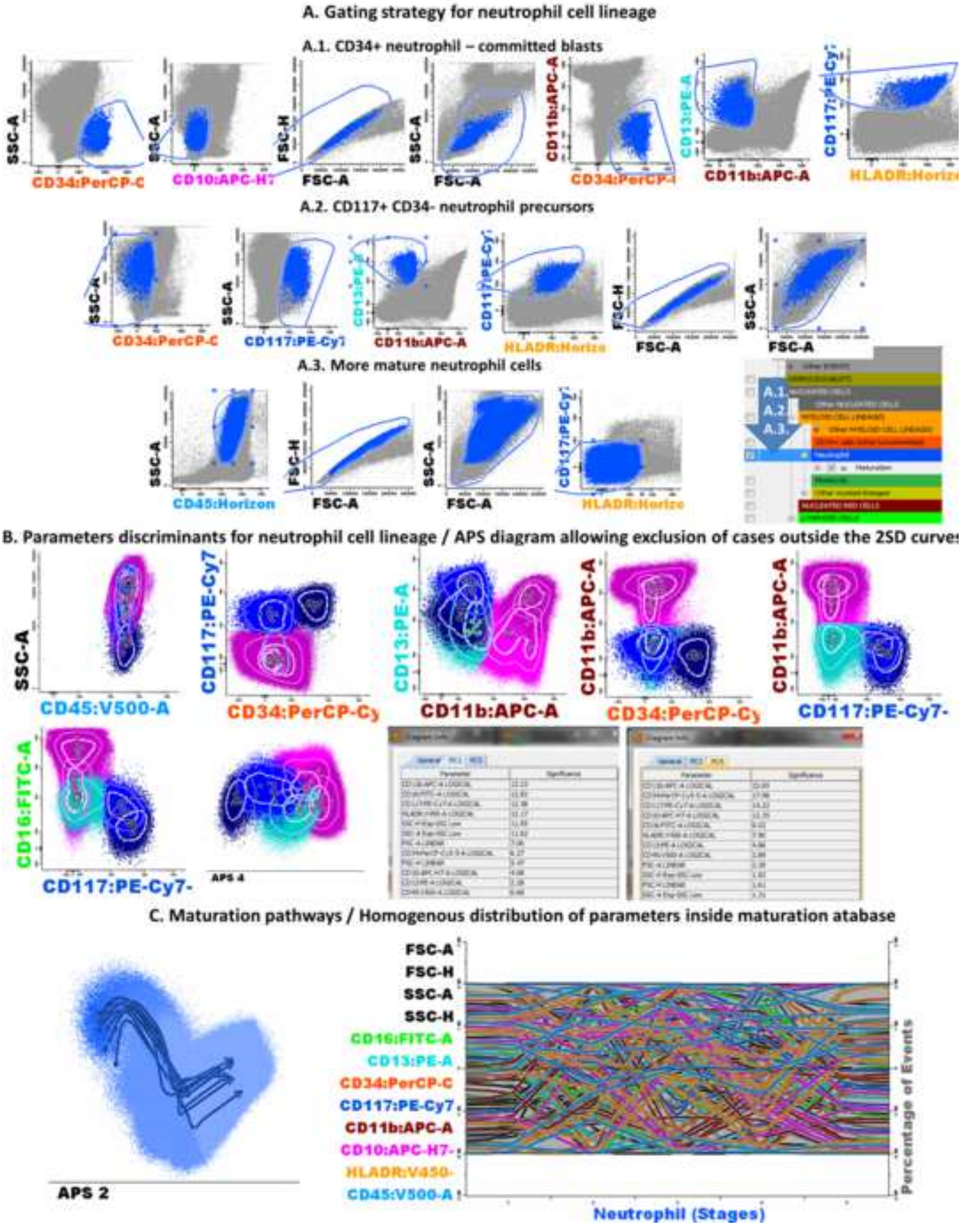
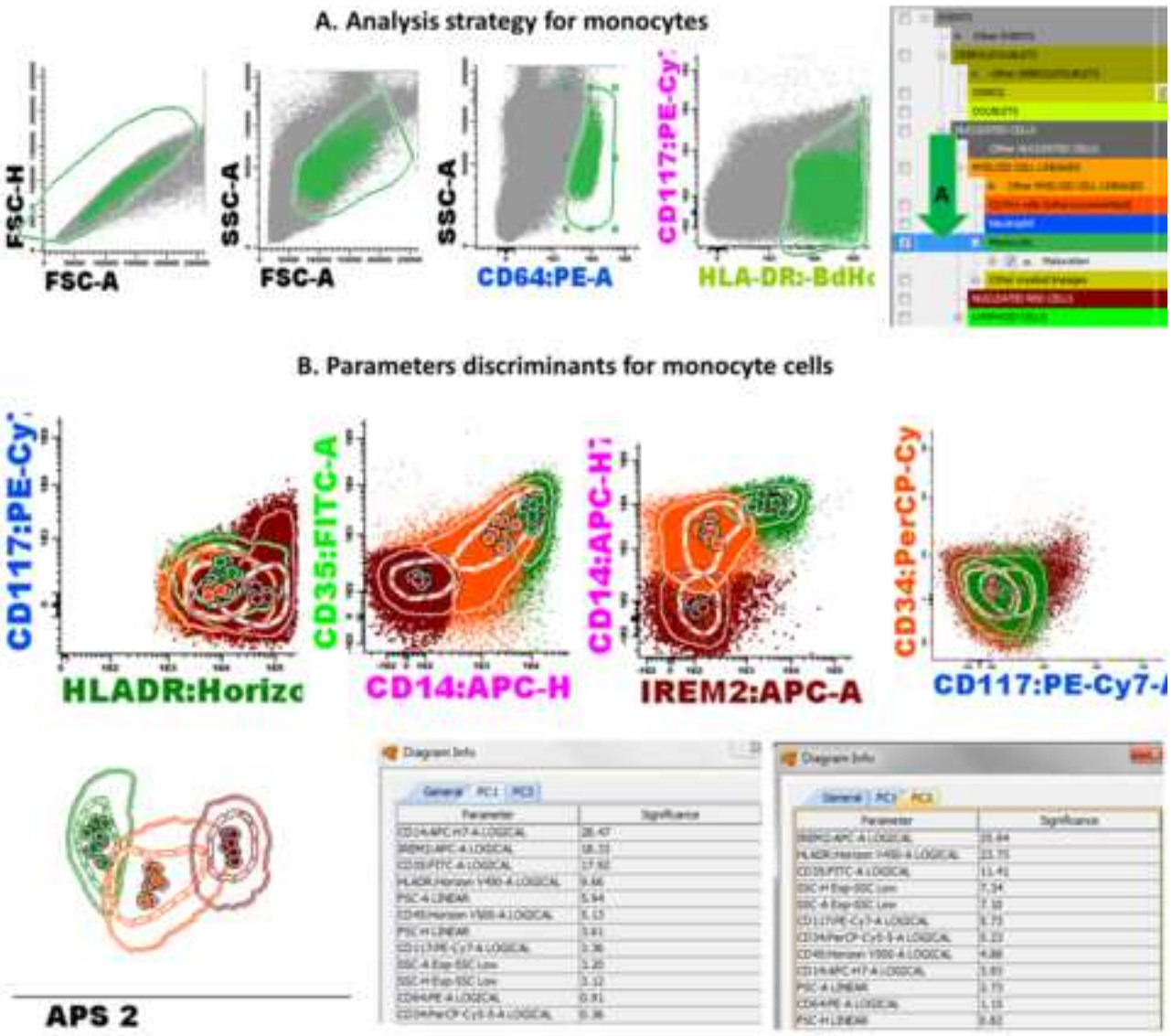


Figure_2 Click here to download Figure Figure 2 monocytes analyse.tif



C. Maturation pathways / Homogenous distribution of parameters inside maturation database

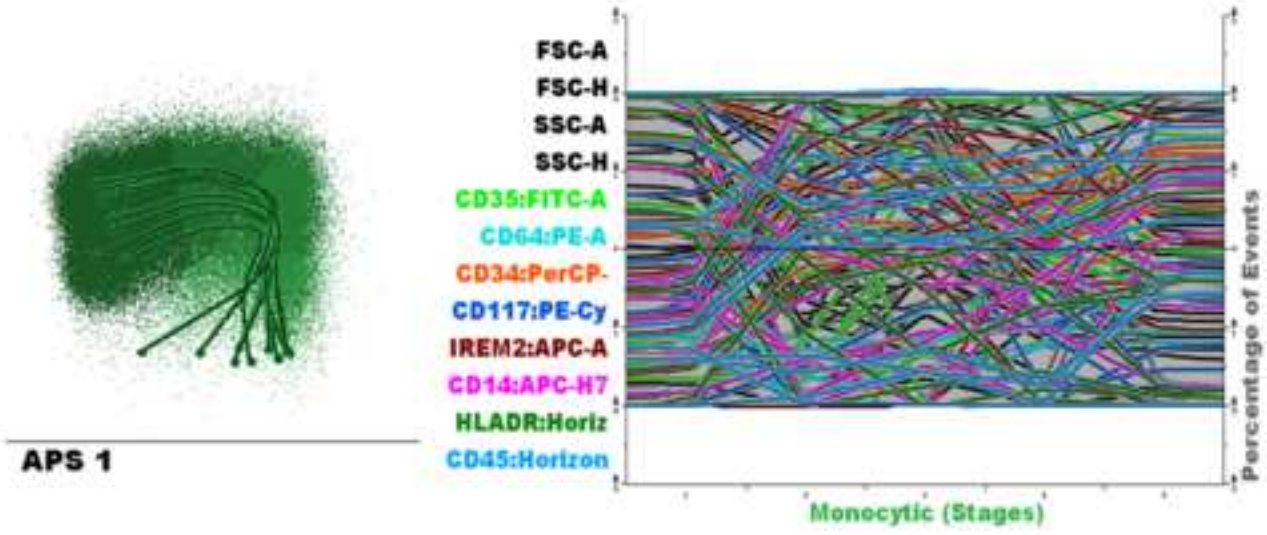


Figure_3

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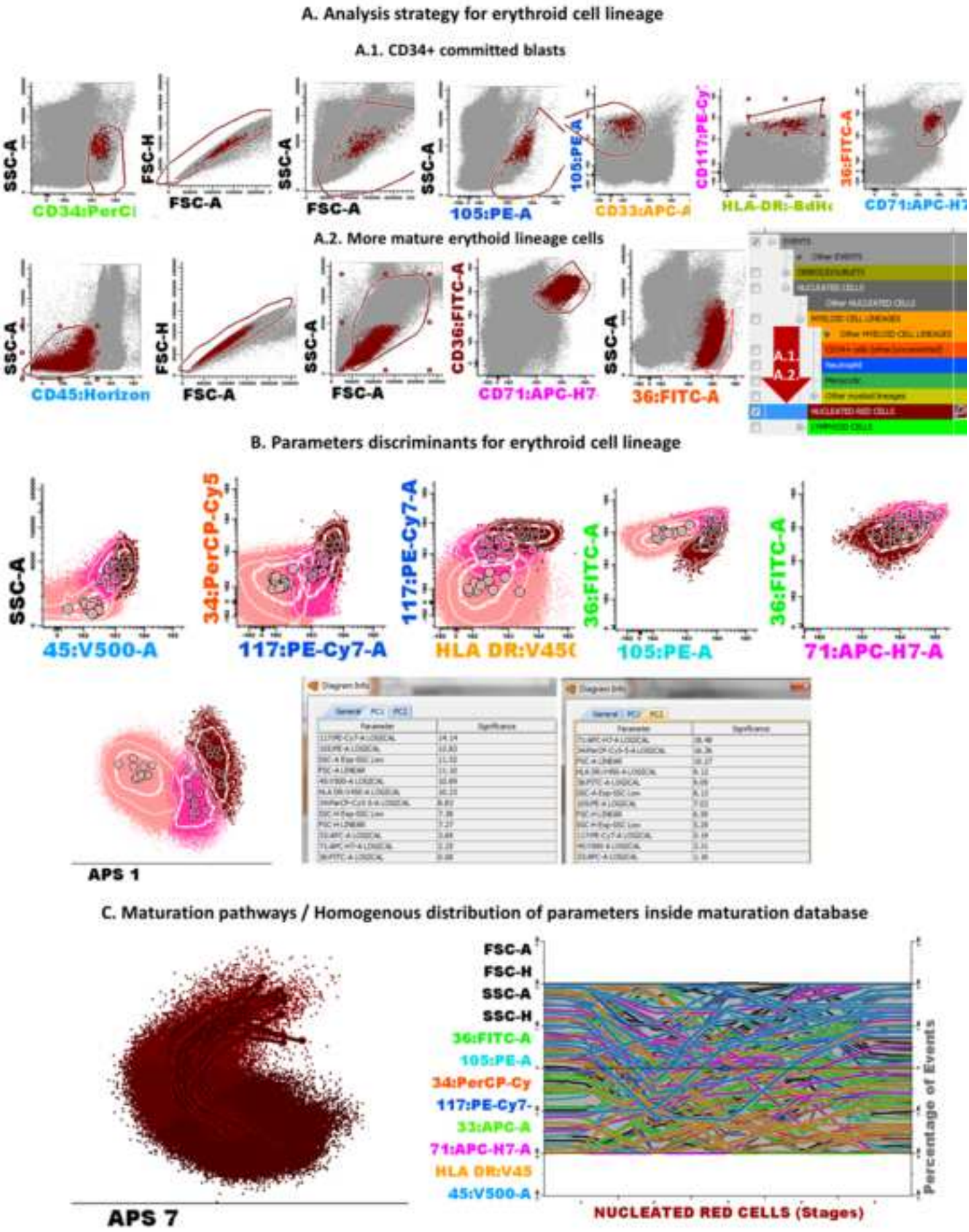

Figure_4

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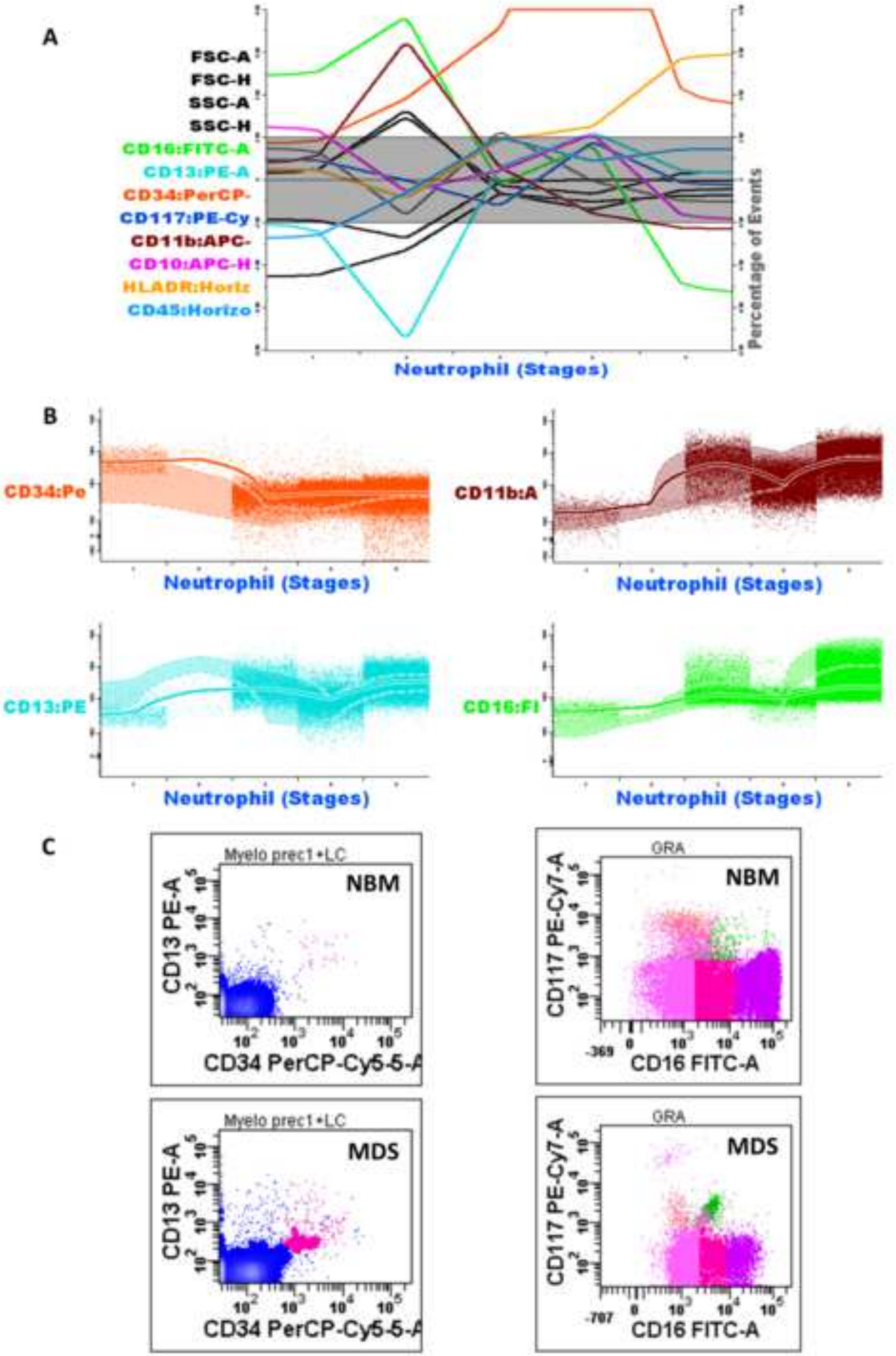

Figure_5

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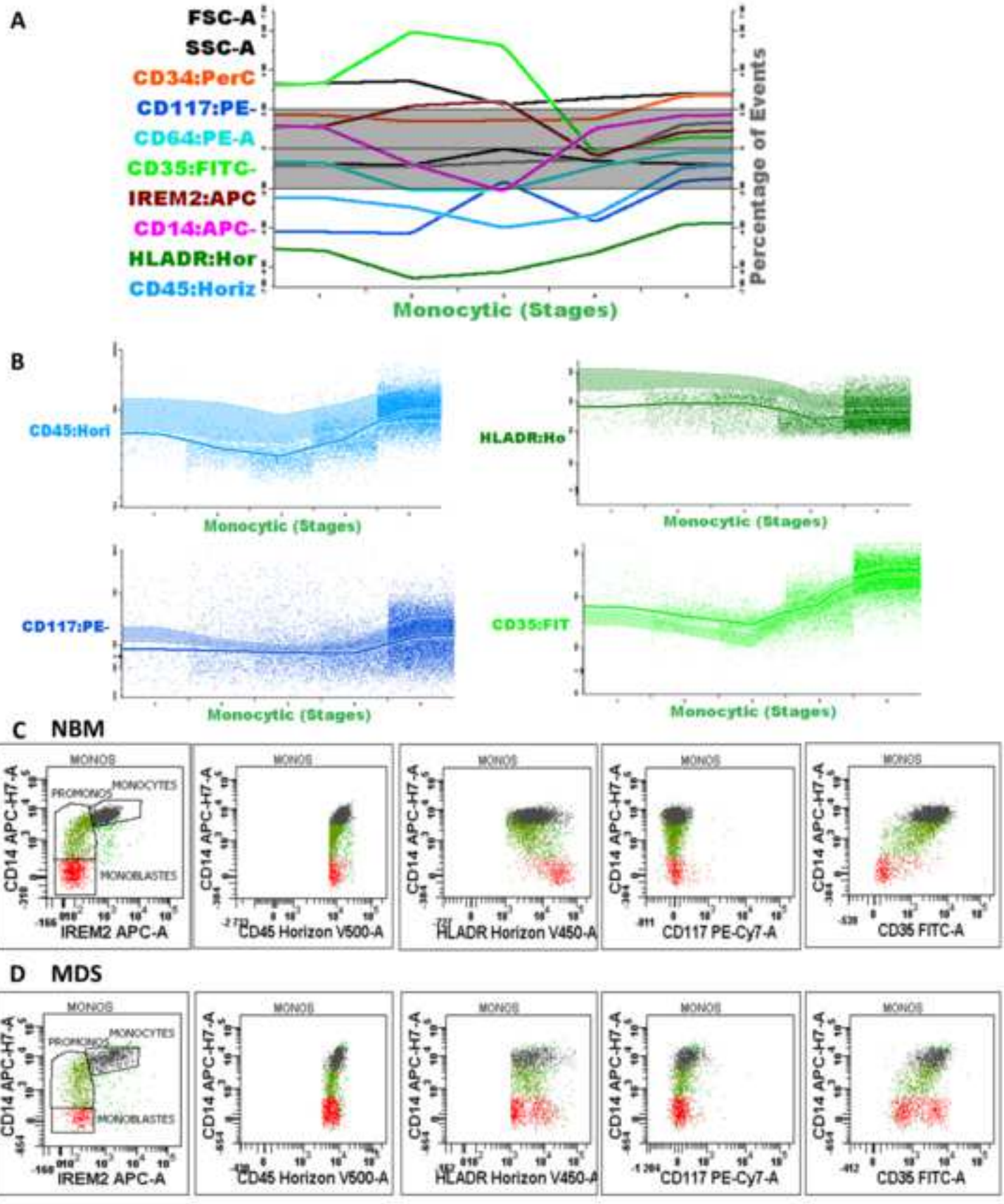

Figure_6

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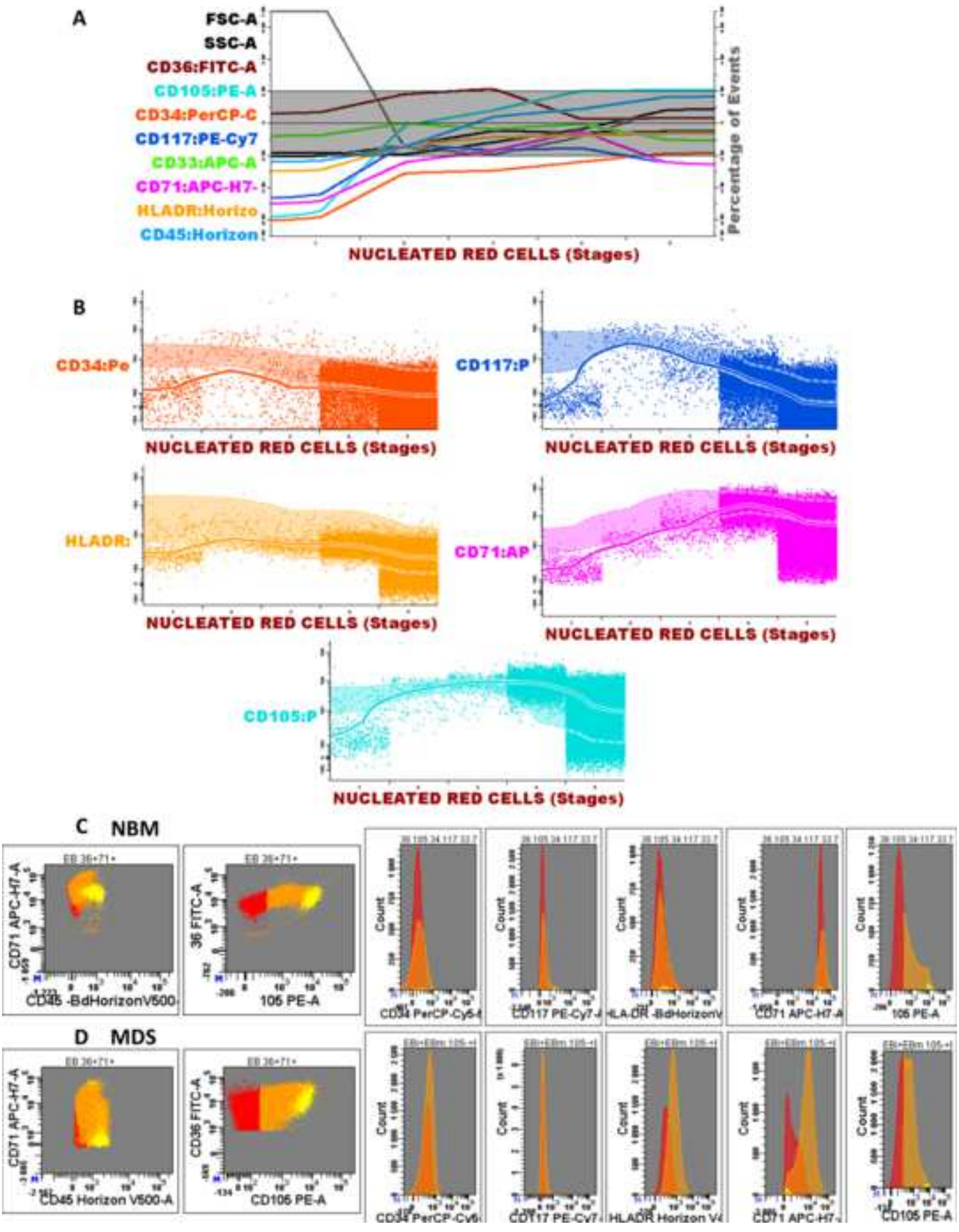

Figure_7

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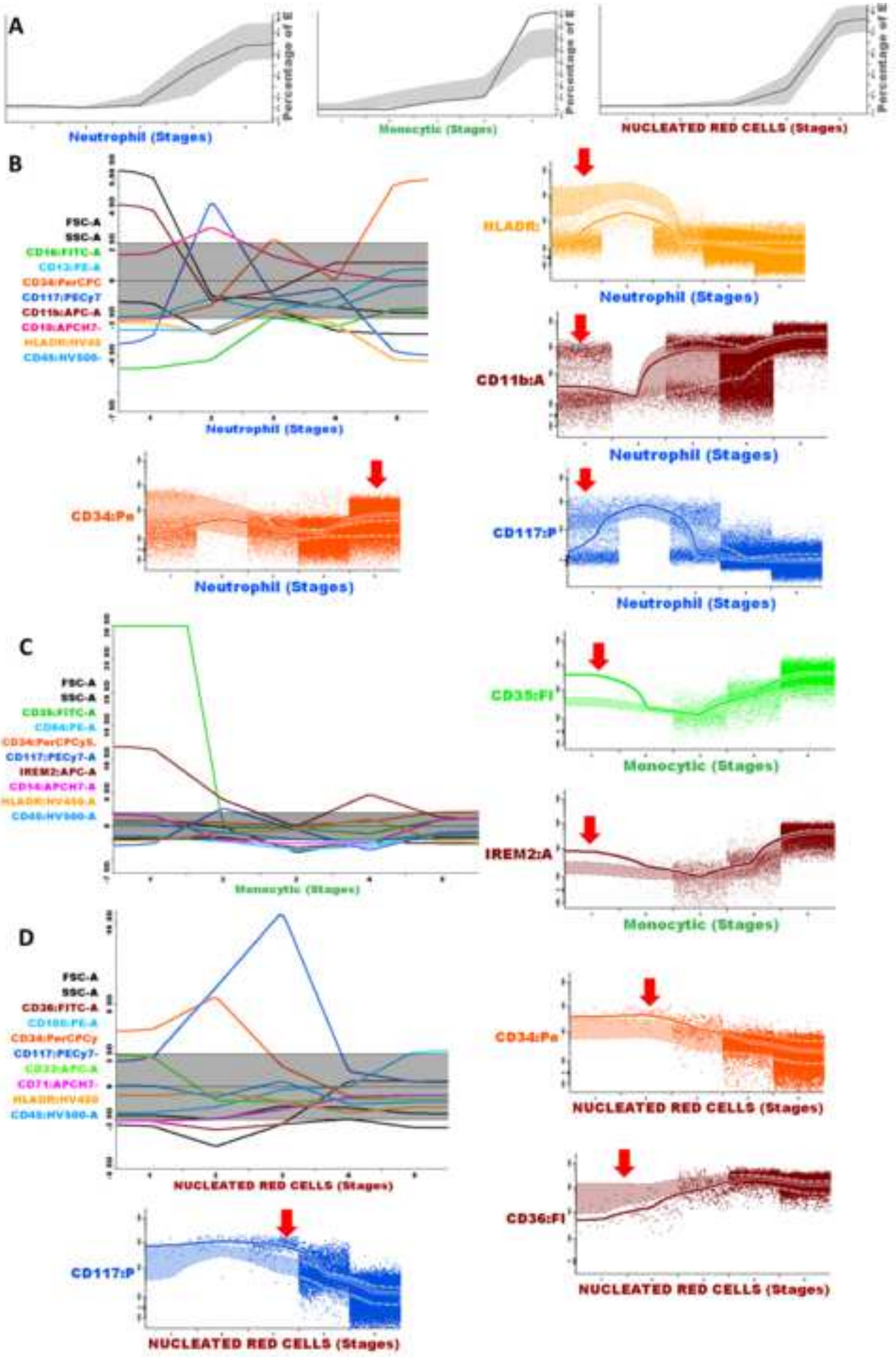

Table 1

Generic fluorochromes		
Generic targets	**Positive target**	**PECy7 target**
HLA-DR HV450 BD; clone L243; 5 µL/test	B-cells & HLA-DR+ T-cells	**CD117** BD; clone 104D2D 5 µL/test
CD45 HV500 BD; clone HI30; 5 µL/test	Lymphocytes	
CD8 FITC BD; clone SK1; 20 µl	CD8hi T-cells	
CD8 PE BD; clone SK1; 20 µL/test	CD8hi T-cells	
CD5 PerCPCy5.5 BD; clone L17F12; 15 µL/test	CD5+ T-cells	
CD8 APC BD; clone SK1; 5 µL/test	CD8hi T-cells	

Provide 1 additional tube: Unmarked blood cells

° Artificially CD14- monocytes created by "appending" 5000 ever

Negative CompBead used as negative reference population

Tandem fluorochromes		
Positive target	**APCH7 targets**	**Positive target**
CompBead	**CD10** BD; clone HI10A; 5 µL/test	CompBead
	CD14° BD; clone MøP9; 5 µL/test	Monocytes
	CD71# BD; clone M-A712; 5 µL/test	CompBead

ıts from the unstained tube to this tube during the acquisition

Table 2

FITC	PE	PerCPCy5.5*	PECy7*	APC	APCH7
CD16	CD13	CD34	CD117	CD11b	CD10
20 µL	7 µL	10 µL	5 µL	5 µL	5 µL
CD35	CD64	CD34	CD117	CD300e	CD14
5 µL	20 µL	10 µL	5 µL	5 µL	5 µL
CD36	CD105	CD34	CD117	CD33	CD71
5 µL	5 µL	10 µL	5 µL	10 µL	2 µL

*Backbone markers

V450*	V500*
HLADR	CD45
5 µL	1 µL
HLADR	CD45
5 µL	1 µL
HLADR	CD45
5 µL	1 µL

Materials Table

Click here to download Materials Table JoVE_Materials_R3.xls

Name of Material/ Equipment	Company
BD FACSCanto II flow-cytometer	BD Biosciences, CA, USA
Awel C48-R Centrifuge	AWEL Industries, FR
Pipetts of 10µl and 200µl	
Pasteur pipettes	
15 mL Falcon tubes	
polypropylene tube for FACS	
Mouse Anti-Human HLA-DR	BD Biosciences, CA, USA
Mouse Anti-Human CD45	BD Biosciences, CA, USA
Mouse Anti-Human CD16	BD Biosciences, CA, USA
Mouse Anti-Human CD13	BD Biosciences, CA, USA
Mouse Anti-Human CD34	BD Biosciences, CA, USA
Mouse Anti-Human CD117	BD Biosciences, CA, USA
Mouse Anti-Human CD11b	BD Biosciences, CA, USA
Mouse Anti-Human CD10	BD Biosciences, CA, USA
Mouse Anti-Human CD35	BD Biosciences, CA, USA
Mouse Anti-Human CD64	BD Biosciences, CA, USA
Mouse Anti-Human CD300e	Immunostep
Mouse Anti-Human CD14	BD Biosciences, CA, USA
Mouse Anti-Human CD36	BD Biosciences, CA, USA
Mouse Anti-Human CD105 Mouse Anti-Human CD33	BD Biosciences, CA, USA
Mouse Anti-Human CD71	BD Biosciences, CA, USA
Lysing Solution 10X Concentrate (IVD)	BD Biosciences, CA, USA

FACSFlow Sheath Fluid	BD Biosciences, CA, USA
FACSDiva CS&T IVD beads	BD Biosciences, CA, USA
RAINBOW CALIBRATION PARTICLES, 8 PEAKS	Cytognos, Salamanca, Spain
Compensation Particles Multicolor CompBeads (CE/I	BD Biosciences, CA, USA
Diva software versions 6.1.2 and 6.1.3	BD Biosciences, CA, USA
Phosphate buffered saline tablets	R&D Systems, Minneapolis, USA
Bovine serum albumin (BSA)	Sigma-Aldrich, France
Sodium azide 99%	Sigma-Aldrich, France
Infinicyt software version 1.8.0.e	Cytognos, Salamanca, Spain

Catalog Number	Comments/Description
SN: V33896301336	3-laser, 4-2-2 configuration
SN: 910120016; Model No: 320002001	low speed centrifuges; capacit
655874	clone L243 Mouse BALB/c IgG2a, κ
560777	clone HI30 Mouse IgG1, κ
656146	clone CLB/fcGran1 Mouse BALB/
347406	clone L138 Mouse BALB/c X C57BL/6 IgG1,
347222	clone 8G12 Mouse BALB/c IgG1, κ
339217	clone 104D2 Mouse BALB/c IgG1
333143	clone D12 Mouse BALB/c IgG2a, κ
646783	clone HI10A Mouse BALB/c IgG1, κ
555452	clone E11 Mouse IgG1, κ
644385	clone 10.1 Mouse BALB/c IgG1, κ
IREM2A-T100	clone UP-H2 Mouse BALB/c IgG1, k
641394	clone MoP9 Mouse BALB/c IgG2b, κ
656151	clone CLB-IVC7 Mouse IgG1, κ
560839	clone 266 Mouse BALB/c IgG1, κ
345800	clone P67.6 Mouse BALB/c IgG1, κ
655408	clone M-A712 Mouse BALB/c IgG2a, κ
349202	

342003
656046
SPH-RCP-30-5A lots EAB01, EAC01, EAD05, EA
ref. #51-90-9001229 + #51-90-9001291

5564
A9647
199931

Filters and mirrors details: https://www.bdbiosciences.com/documents/BD_FACSCanto_II_FilterG

:y 60 FACS tubes

Fluorochrome Horizon V450
(Ex max 404 nm/

(Ex max 415 nm/
Em max 500 nm)
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Fluorochrome FITC
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