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Abstract. Precise identification of eosinophils, basophils, and specific subpopulations of blood cells (B lymphocytes) in an unconventional automatic hematology analyzer is demonstrated. Our specific apparatus mixes two excitation radiations by means of an acousto-optics tunable filter to properly control fluorescence emission of phycoerythrin cyanin 5 (PC5) conjugated to antibodies (anti-CD20 or anti-CRTH2) and Thiazole Orange. This way our analyzer combining techniques of hematology analysis and flow cytometry based on multiple fluorescence detection, drastically improves the signal to noise ratio and decreases the spectral overlaps impact coming from multiple fluorescence emissions. © 2011 Society of Photo-Optical Instrumentation Engineers (SPIE). [DOI: 10.1117/1.3582162]

Keywords: flow cytometry; hematology; biological cells; multiple wavelength excitations; Thiazole Orange; fluorochromes; decrease compensations; multiple fluorescences.

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1 Introduction

A hematology analyzer allows the counting and characterization of blood cell populations by sampling a defined quantity of whole blood with electrical and optical measurements.¹⁻⁵ In particular, analyzers are able to determine cell concentrations and accurately discriminate up to eight populations of leucocytes [i.e., monocytes, neutrophils, basophils, eosinophils, lymphocytes, immature granulocytes, atypical lymphocytes (LYA), and high RNA content (HRC)] (i.e., Horiba ABX Pentra 120).⁶⁻¹¹ By comparison, a flow cytometer allows the separation of stained cells by using multiple fluorescence measurements and the precise discrimination of a lot of populations or subpopulations. Unfortunately, no precision on the sample volume is obtained, which prevents precise determination of absolute cell count (i.e., FACScan cytometer from Becton Dickinson).

In the great majority of routine diagnostics in hematology, automatic counting of the most common cells and their characterizations are sufficient to give enough information to the practitioner to assess the presence of a cell count imbalance or a cytological disorder. However, abnormal cell count results, compared with those that are normally expected, could be obtained during these conventional cytological analyses. These results may justify further investigations in order to refine the diagnostic. Furthermore, such analyses do not allow the accurate quantification of specific cells within a population or a family due to the failure of a hematology analyzer to measure cells bonded with fluorochrome conjugated to antibody. This is a major drawback particularly for detecting some pathologies and for monitoring their evolution.

In order to improve count results of a selected subpopulation of cells, complementary analyses are independently developed of those carried out using a conventional apparatus. For

example, discrimination of pathologies (e.g., immune reactions or leukemia) may be correlated with an abnormal level of particular leukocyte-type cell population. One of these complementary analyses may consist in preparing a blood smear followed by coloration and observation under a microscope. Cell identification and counting are, in this case, used in addition for those obtained using the automated equipment. The major drawbacks of this additional analysis include bad reliability, they are time consuming, and require experienced technicians. Another complementary analysis may consist in specifically labeling expected cells using selected antibodies coupled with fluorochromes or any other dyes (i.e., phycoerythrin cyanine 5).¹²⁻¹⁵ For example, characterization of all leukocyte cells can be realized with a flow cytometer and by using fluorometry.

Nowadays, one of the major drawbacks of cell diagnosis is the use of two different apparatus i.e., a conventional hematology analyzer and a flow cytometer. This way, mathematical cross-correlation between results obtained separately with these two systems is difficult and generates errors that drastically reduce analysis accuracy. Additionally, few laboratories are equipped with both a hematology analyzer and a flow cytometer and this imposes blood sample transportation toward specific centers. Last, a standard flow cytometer is complex and requires a higher skilled technician compared to a hematology analyzer, which is a semi-automatic apparatus.

In this paper, we present results of conventional and complementary analyses realized with a hematological analyzer and a flow cytometer, respectively, that can be coupled in the same apparatus.¹⁶ A dual laser excitation mixing blue and yellow CW laser sources is used to characterize up to eight populations of leucocytes more accurately than a conventional analyzer. Our experiment focuses on eosinophils and basophils because in normal blood samples these subpopulations are weakly represented, therefore, their detection and counting are difficult in

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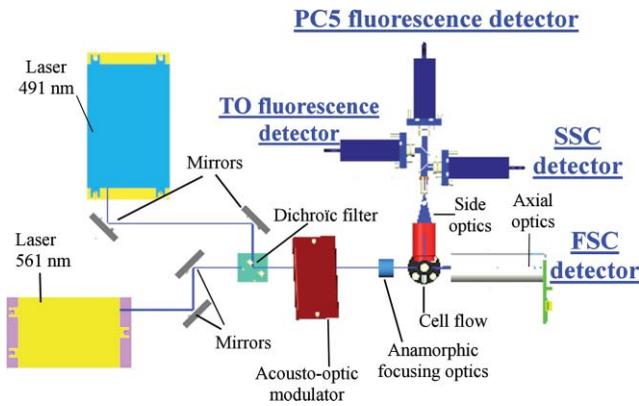


Fig. 1 Experimental set-up of the hematology analyzer. The particle flow circulates in perpendicular plane of this drawing.

routine hematology analysis. This multiparametric system is based on impedance, forward scattering, side scattering, and fluorescence analyses on a cell by cell approach. A comparison between a standard hematological analyzer using a single CW laser source and our new multiparametric system is shown to validate our new approach.

2 Material and Method

2.1 Hematology Analyzer

This automatic hematology analyzer has been developed by using flow cytometry principles based on hydrodynamic focusing.¹⁷ The cell flow is coupled to a specific optical layout as described in Fig. 1. The cell flow circulates in the perpendicular plane of the drawing. The optical system is composed of two solid state Cobolt laser sources (Calypso and Jive) emitting 25 mw of average power at 491 and 561 nm. An optical system composed of four highly reflecting mirrors and a dichroic

plate permits spatial combination of the light beams of the two lasers along the same optical axis. An acousto-optics tunable filter (AOTF nC.TN, company AA Opto Electronic) selects the wavelength and/or independently adjusts the transmitted intensity provided by the two optical sources. Beams are then focused in the flow cell through a rectangular window (150 $\mu\text{m}/50 \mu\text{m}$) and by means of two spherical and cylindrical lenses, respectively. Blood cells circulate in a microcapillary with a velocity close to 10 m/s.

Before passing in front of the laser beam, biological cells propagate through a microhole that is immersed in a liquid with a known conductivity. Impedance modification between electrodes placed on each side of the microaperture reveals the presence of a cell and gives information on its volume and count of cells. After electrical gating and cell illumination, light beams are scattered in two different directions. The forward scattering (FSC) is recorded by means of a photodiode in 1 to 3° annular pupil and it is more sensitive to the cell size than to other cellular characteristics.¹⁸ The side scattering (SSC) is picked up at an orthogonal direction in a numerical aperture of 0.8. This light is collected by means of a photomultiplier and it is more sensitive to cell structure or cellular granularity than to other cellular characteristics.¹⁸ A Semrock dichroic plate FF506-Di02, a Semrock filter FF506-Ex4, a focusing lens, and a photomultiplier (Hamamatsu) are used to select the scattered energy at the pump wavelength. Fluorescence measurement of Thiazole Orange (TO) allows identification of nucleic acid content of cells (RNA and DNA), whereas measurement of phycoerythrin cyanine 5 (PC5) allows identification of proteins bounded with fluorochrome conjugated antibody. The TO fluorescence is selected by means of two Semrock filters FF01-530/43 and FF650-Di01, a focusing lens, and a photomultiplier tube (Hamamatsu). The PC5 fluorescence is detected through a Semrock filter FF670/30, a focal lens, and a photomultiplier (Hamamatsu). Absorption and emission spectra of TO and PC5 conjugated to anti-CD20 or anti-CRTH2 are shown in Fig. 2. Let us note that fluorescence

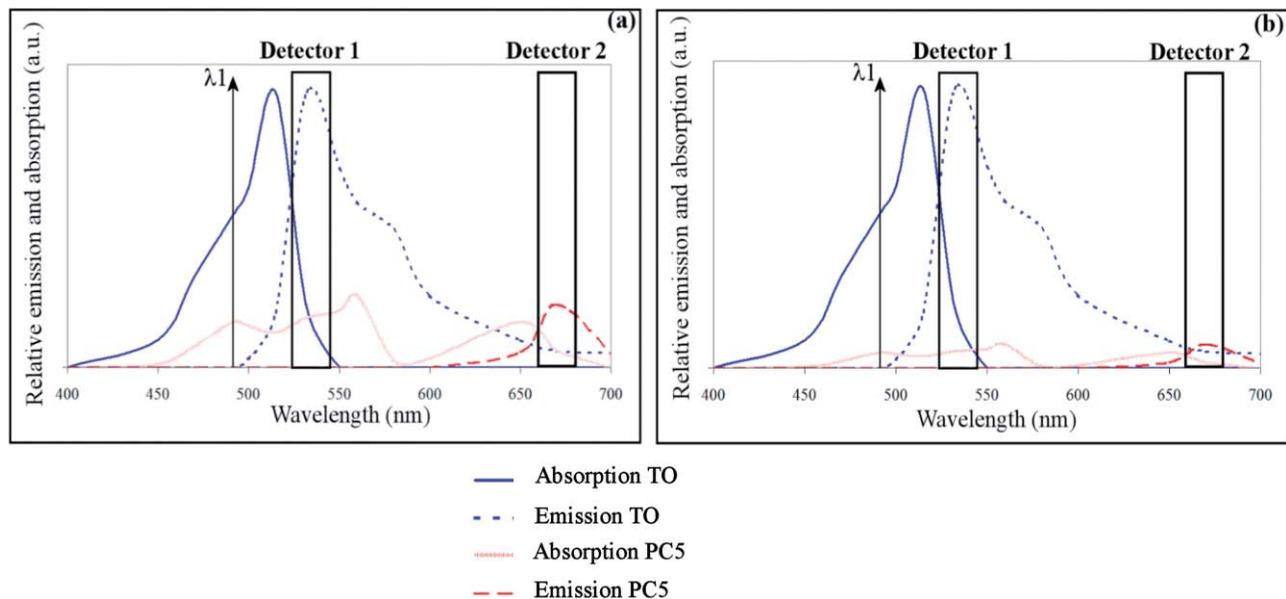


Fig. 2 Absorption and emission spectra of TO and PC5 conjugated to (a) anti-CD20 or (b) anti-CRTH2 antibodies. Arrow: laser excitation at 491 nm; rectangles: band-pass filters on detectors 1 and 2 (508,5–551,5 and 655–685, respectively).

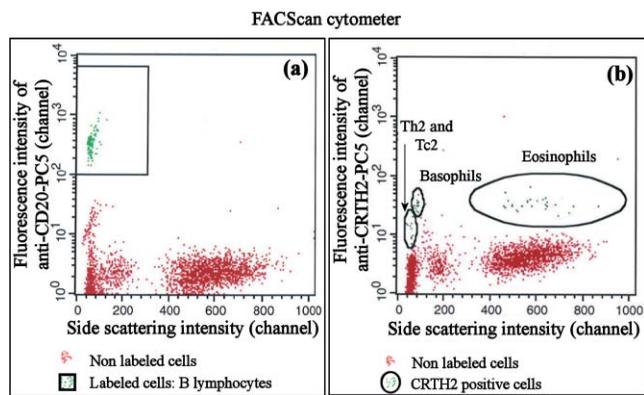


Fig. 3 FACS cytometer. Cytograms of PC5 fluorescence and side scattering discrimination of (a) B lymphocytes and (b) eosinophils, basophils, and subpopulations of T cells (Th2 and Tc2).

efficiency of TO is more important than fluorescence efficiency of PC5 conjugated with anti-CD20 or anti-CRTH2 because of their staining surface (Fig. 2).

Emission spectrum of the population labeled with PC5, is spectrally overlapped by TO fluorescence. Therefore, a specific mathematical computation is applied to data coming from detector 2 in order to extract the PC5 signal only. This method called “compensation”¹⁹ allows one to specifically eliminate the fluorescence signal of nonspecific microscopic elements by subtracting energy with an amount proportionally equal to the spectral overlap between TO and PC5 spectral emissions. The problem induced by compensations is that their calculation factors are based on average values. For a same fluorochrome family, compensation factors depend on important variations of physical properties, in particular depending on the supplier, causing a critical problem for calibration of instruments. In the field of diagnosis, an uncontrolled supply origin could lead to erroneous measurements and thus potentially dangerous results. For example, PC5 (phycoerythrin-cyanine 5) quantum yield is not steady enough to accept final software compensations, in particular because it is a tandem, which is a particularly unstable chemical structure.

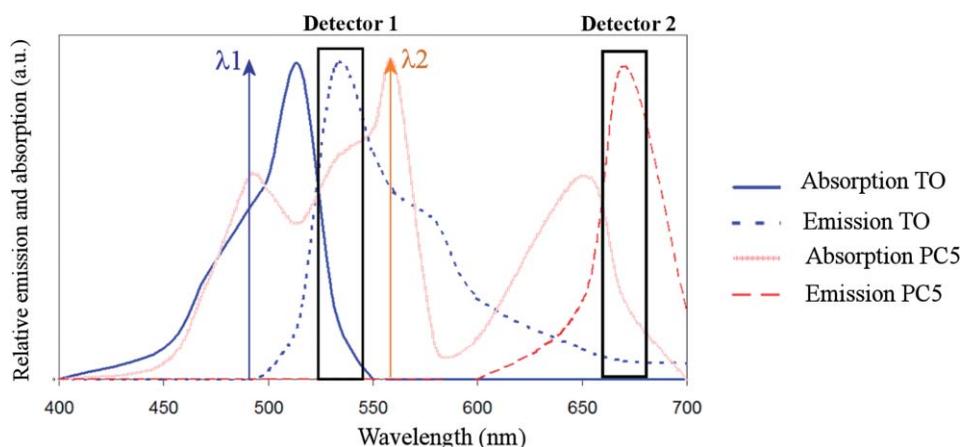


Fig. 5 Absorption and emission spectra of TO and PC5; laser excitation at 491 nm (λ_1) and at 561 nm (λ_2); rectangles: band-pass filters on detectors 1 and 2 (508,5–551,5 and 655–685 nm, respectively).

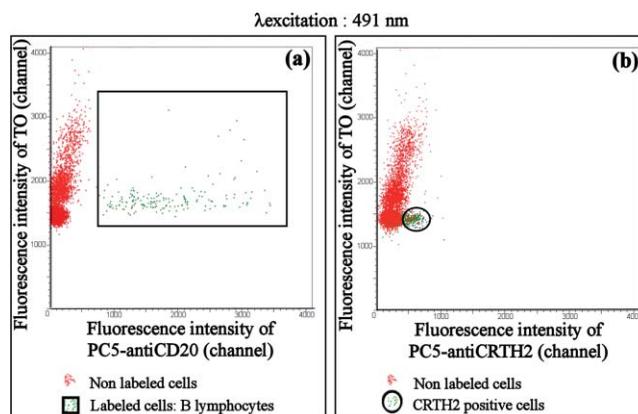


Fig. 4 Hematology analyzer (excitation wavelength: 491 nm). Cytograms of TO and PC5 fluorescences. TO versus PC5 fluorescence conjugated to (a) anti-CD20 antibody and (b) anti-CRTH2 antibody. One dot one cell.

A standard microfluidic device is used to sample, mix, and incubate the blood sample with a reagent²⁰ in order to lyse the red blood cells and to label the nucleic acids (RNA and DNA) with TO. Dilutions are adapted to the analyses and are different for each operation. For a conventional analysis, 2 mL of reagent are mixed with 30 μ L of blood and 88 μ L of this sample (blood and reagent) are analyzed, which corresponds to 15 s of diagnosis.

For a complementary analysis, cells are stained before a cytology experiment. The blood sample is incubated with a solution containing antibodies (i.e., anti-CD20 or anti-CRTH2) coupled with PC5 (phycoerythrin cyanine 5) fluorochrome. PC5 conjugated with anti-CD20 (Beckman Coulter) characterizes B lymphocytes representing 3.5% of human standard blood cells. PC5 conjugated with anti-CRTH2 (Beckman Coulter) characterizes eosinophils and basophils ($\approx 2\%$ and $\approx 1\%$ in human standard blood cells, respectively) and subpopulations of T lymphocytes [Th2 and Tc2 ($\approx 0,5\%$ in human blood cells)].^{21,22} A total of 60 μ L of blood and 10 μ L of antibodies coupled with PC5 are mixed and incubate during 30 min in a dark chamber at room temperature. After incubation, 2 ml of reagent with 35 μ L of blood are mixed and 88 μ L of the sample (stained blood cells and reagent) are analyzed during 15 s.

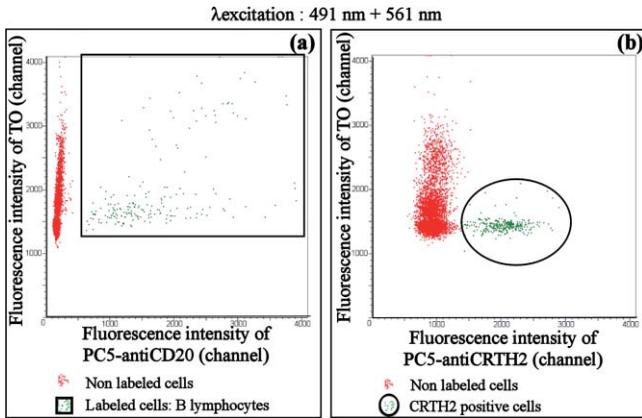


Fig. 6 Hematology analyzer (excitation wavelengths: 491 + 561 nm). Cytograms of TO and PC5 fluorescence. TO versus PC5 fluorescence conjugated to (a) anti-CD20 antibody and (b) anti-CRTH2 antibody. One dot one cell.

Twelve different blood samples from healthy patients are analyzed with different proportions of B lymphocytes and basophils and/or eosinophils.

2.2 FACSscan Cytometer Reference

All measurements realized with our new apparatus are directly compared with data obtained with a standard cytometer used as a reference. This cytometer is the FACSscan from Becton Dickinson. It is equipped with an air-cooled 15 mW argon ion laser emitting at 488 nm and permits one to realize an axial diffraction measurement (FSC), orthogonal diffusion measurement (SSC), and three orthogonal fluorescence measurements. Only measurements of FSC, SSC, and a single fluorescence with PC5 (670 LP filter) are used in our experiments. Bi-parametric representations allow one, in a first case, to discriminate B cells [Fig. 3(a)] and, in a second experiment, eosinophils, basophils, and subpopulations of T cells (Th2, Tc2) [Fig. 3(b)]. CRTH2 is highly expressed on peripheral blood basophils and eosinophils and is also expressed on human subpopulation T cells.^{23,24}

It is also important to note that a hematology analyzer counts cells within a given amount of a blood sample, whereas a FACSscan cytometer counts a sufficient number of stained cells in an undetermined volume of a blood sample. In each case, the percentage of labeled cells are obtained assuming that blood samples used in each experiment come from the same blood.

3 Experiments

The aim of the experiment is to demonstrate that a dual laser excitation mixing (blue and yellow) CW laser sources can drastically improve cell characterization.

3.1 Experiment with a Single Laser Source

The first experiment consists in the selection of blood cells by using a single excitation wavelength at 491 nm. In the first configuration, the laser radiation excites TO and PC5 conjugated to anti-CD20 [Fig. 4(a)] for the identification of B lymphocytes, and, in the second configuration, it excites TO and

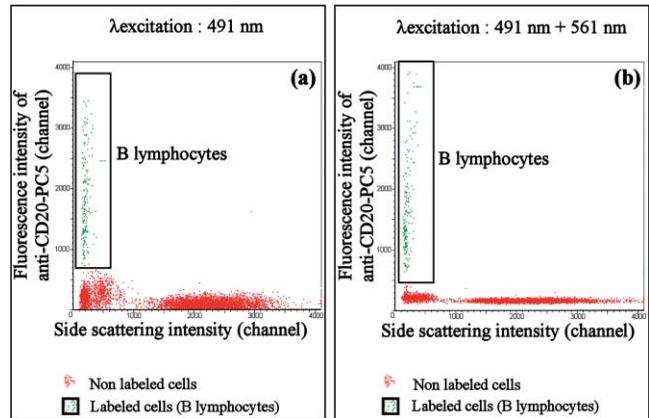


Fig. 7 Hematology analyzer [(a) excitation with a single wavelength (491 nm); (b) excitation with two wavelengths (491 + 561 nm)]. Bi-parametric representations obtained for labeled B lymphocytes by using PC5 fluorescence conjugated to anti-CD20 and SSC.

PC5 conjugated to anti-CRTH2 [Fig. 4(b)] for the identification of eosinophils, basophils, and subpopulations of lymphocytes T (Th2 and Tc2 cells). Due to a relatively strong antigenic expression, each B lymphocyte sufficiently fluoresces to produce a strong signal far from the floor noise induced by TO fluorescence. The compensation factor introduced in the data processing permits clear discrimination of B cells with respect to other nonlabeled elements [Fig. 4(a)]. On the contrary, and because

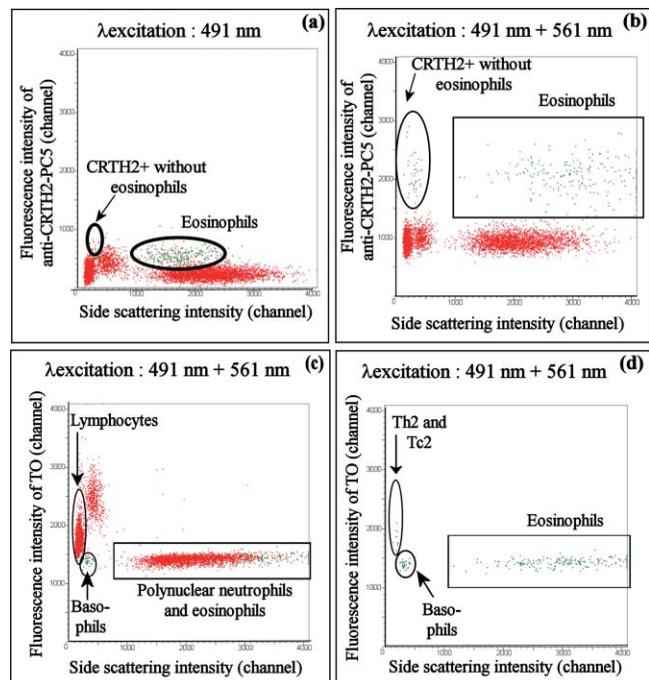


Fig. 8 Hematology analyzer; bi-parametric representations (a) and (b) allowing discrimination of eosinophils and the other CRTH2 positive cells through the use of PC5 fluorescence conjugated to anti-CRTH2 and SSC; (a) excitation with a single wavelength (491 nm); (b) excitation with two wavelengths (491 + 561 nm). For excitation with two wavelengths, bi-parametric representations (c) and (d) of fluorescence TO and side scattering allowing discrimination of basophils and subpopulations of T cells (Th2 and Tc2); (d) CRTH2 positive cells.

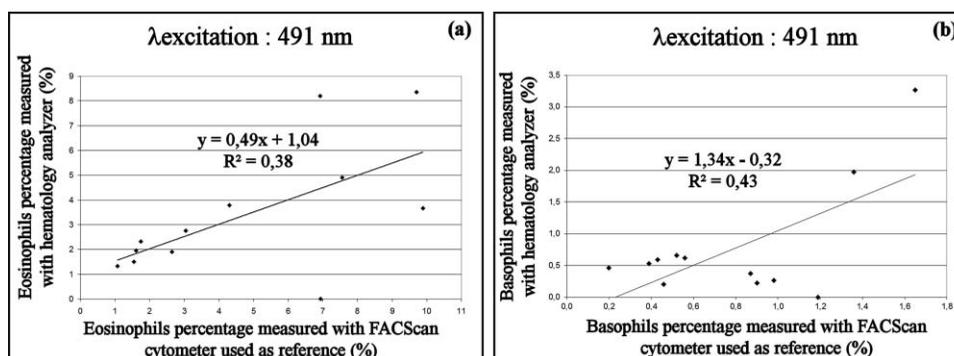


Fig. 9 Population percentages measured with a hematology analyzer including a single excitation wavelength and FACScan cytometer used as a reference for 12 blood samples for (a) eosinophils and (b) basophils. Line: linear regression model.

of their weak antigenic expression, CRTH2 positive cells do not sufficiently fluoresce to provide a strong signal to noise ratio. Thus, a bad discrimination between labeled and nonlabeled cells is obtained [see Fig. 2(b)]. Unfortunately, compensation factors introduced in the data processing are used but they do not permit improvement for better cell discrimination.

3.2 Experiment with Two Laser Sources

In order to improve cell discrimination, a second light source is added. So, the optical system is now composed of two lasers emitting in blue (491 nm) and yellow (561 nm) (see Fig. 5). An acousto-optics tunable filter allows independent adjustment of the two beam intensities. Radiation at 561 nm (Refs. 25 and 26) is judiciously chosen to selectively excite PC5 without inducing any excitation of TO. In this configuration, the first source at 491 nm excites both TO and PC5 without any possibility to independently control their absorption and fluorescence efficiency ratio and thus decrease or eliminate problems of multiparametric flow cytometry. The second laser beam at 561 nm permits a selective increase of the absorbed energy of PC5 only and to control its fluorescence emission level to obtain a better signal to noise ratio. It is then possible to obtain a clear fluorescence signature from stained B lymphocytes (PC5 conjugated to anti-CD20) and from stained CRTH2 positive cells (PC5 conjugated to anti-CRTH2) when PC5 and TO are coupled in the same apparatus (see Fig. 6).

Adding the second excitation wavelength and an acousto-optics tunable filter allows a balance of the fluorescence emission from TO and PC5 conjugated to anti-CRTH2 by managing the intensities of the two laser beams. Thus, a better discrimination of eosinophils and basophils is obtained.

3.3 Bi-parametric Representation of Fluorescence and Diffraction Labeled Cells

Another representation is realized by using the fluorescence analysis of PC5 conjugated to anti-CD20 or anti-CRTH2 antibodies and side scattering. Comparison between the use of a single wavelength and two wavelengths for excitation is shown in Fig. 7 (PC5-anti-CD20) and Figs. 8(a) and 8(b) (PC5-anti-CRTH2).

With a single wavelength at 491 nm, and because of efficient fluorescence emission of PC5 conjugated to anti-CD20, B lymphocytes are well separated from nonlabeled cells. Adding a second excitation wavelength at 561 nm increases the separation of the two cell populations even more and slightly improves the signal to noise ratio. On the contrary, identification of eosinophils and basophils by using a single excitation wavelength is impossible. The fluorescence emission of TO hides the fluorescence emission of PC5 conjugated to anti-CRTH2. Then spatial superimposition of cell populations is observed (Fig. 8). In this case, the second excitation wavelength (561 nm) significantly increases the signal from PC5-anti-CRTH2, which permits proper

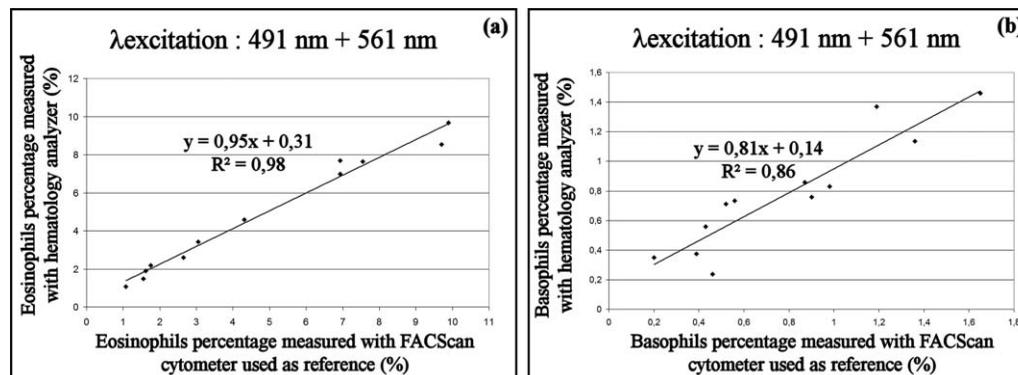


Fig. 10 Population percentages measured with hematology analyzer including two excitation wavelengths and FACScan cytometer used as reference for 12 blood samples for (a) eosinophils and for (b) basophils. Line: linear regression model

Table 1 Comparison between percentages of labeled cells obtained with hematology analyzer including a single and two laser sources and FACScan cytometer (12 samples are processed).

	Blood number	1	2	3	4	5	6	7	8	9	10	11	12
Eosinophils percentage (%)	Excitation wavelength 491 nm	3,7	–	1,9	8,2	3,8	1,3	2,8	4,9	1,5	8,3	2,3	2,0
	Excitation wavelengths 491 nm + 561 nm	9,7	7,0	2,6	7,7	4,6	1,1	3,4	7,6	1,5	8,5	2,2	1,9
	FACScan reference	9,9	6,9	2,6	6,9	4,3	1,1	3,0	7,6	1,6	9,7	1,8	1,6
Basophils percentage (%)	Excitation wavelength 491 nm	3,3	–	0,2	0,4	0,7	0,2	0,5	2,0	0,5	0,3	0,6	0,6
	Excitation wavelengths 491 nm + 561 nm	1,5	1,4	0,8	0,9	0,7	0,2	0,4	1,1	0,3	0,8	0,6	0,7
	FACScan reference	1,6	1,2	0,9	0,9	0,5	0,5	0,4	1,4	0,2	1,0	0,4	0,6

identification of basophils and eosinophils and others subpopulations of CRTH2 positive cells (Fig. 8). Bi-parametric representation SSC-PC5 [Fig. 8(b)] allows discrimination of eosinophils and other CRTH2 positive cells from other leucocytes. Results are obtained from multiparametric investigations and by determining inter-relationships between each recorded parameter (electric, FSC, SSC, and TO fluorescence).^{20,27–29} Then, classification of each of the cell subpopulations is obtained. Bi-parametric representation SSC-PC5 [Fig. 8(b)] allows discrimination of eosinophils and other CRTH2 positive cells from other leucocytes. Lymphocytes and basophils can be discriminated by using the SSC-TO bi-parametric representation [Fig. 8(c)]. T cellular subpopulations (Th2 and Tc2) are located in the lymphocyte area and can be identified by using intercorrelation between Figs. 8(b) and 8(c) [Fig. 8(d)].

Results of cell identifications obtained with our new hematology analyzer including two excitation wavelengths are quantitatively compared to the data obtained with a standard FACScan cytometer used as a reference. Twelve blood samples are analyzed by using one and two excitation wavelengths. In each sample, we determined the percentage of eosinophils and basophils and we plotted the results obtained with the hematology analyzer with respect to the one obtained with our reference (FACScan cytometer). Correlation curves resulting from those experiments are presented in Figs. 9 and 10. All of the data are also summarized in Table 1 (12 samples are processed).

By using a single excitation wavelength in the hematology analyzer, the determination coefficients R^2 , which measure adequacy between linear regression model and data, are close to 0.38 and 0.43 for eosinophils and basophils (Fig. 9). Determination coefficient R^2 is the square of the correlation coefficient r , which is the covariance (between x explanatory variable and y variable to be determined) divided by multiple standard deviations. This means that poor reliability of cell identification is obtained by using our hematology analyzer with one excitation wavelength (491 nm). In contrast, correlation coefficients significantly increase close to 0.98 and 0.86 for eosinophils and basophils, respectively, when cell identification is realized by using two excitation wavelengths (Fig. 10). These observations quantitatively confirm the blood cell identification improvement

by mixing two wavelengths to excite TO and PC5, which reduces multiparametric problems.

4 Discussion and Conclusion

Multiparametric analysis by using a single hematology analyzer offers a lot of advantages such as precision, reliability, and simplicity and allows production of full and extensive results without a need for successive multiple and separate analyses. Nevertheless, this technique is difficult to implement because of important spectral overlapping between fluorescence emissions of cell membranous and intracellular staining. A data processing called compensation method can partially eliminate a fluorescence signal of nonspecific microscopic elements but not for all types of stained cells. For example, it is difficult to simultaneously use TO and PC5 conjugated to anti-CRTH2 to identify nucleic acid, eosinophils, and basophils in just one analysis by using a single excitation wavelength centered at 491 nm close to the absorption peak of TO. Indeed, the fluorescence level of TO is more important than the fluorescence level of PC5 mainly. The use of a second laser source with an emission wavelength properly chosen to avoid TO excitation (but to excite PC5), can drastically improve analysis accuracy and enables elimination of most misinterpretations of the results.

We demonstrated that separation of eosinophils and basophils from other leucocytes [i.e., monocytes, neutrophils, lymphocytes, immature granulocytes, atypical lymphocytes, High RNA Content (HRC)] and separation of specific populations or subpopulations, such as B cells, can be achieved by mixing two excitation radiations by means of an acousto-optics tunable filter. The principle of this method is to separately control the energy of fluorescence emissions coming from different fluorochromes with unbalanced fluorescence efficiency. We show that this method is particularly efficient for identification of cell subpopulations labeled with antibody (conjugated with fluorochrome) having a weak or unbalanced antigenic expression even in the presence of TO. A two-excitation wavelength hematology analyzer provides a significant advantage for the separation of basophils and eosinophils. These preliminary experiments for discriminating Th2 and Tc2 cells show a great enhancement for accurate detection and rare cells counting.

Additionally, the analyzer provides the total amount of leukocytes and labeled cells for a given volume of blood, and then their precise absolute count.

This analysis concept can be expanded to other fluorochromes as phycoerythrin Texas Red (PE-TR), Texas Red (TR), Phycoerythrin Cyanin 7 (PC7), Alexa Fluor 568, etc. The system and method described in this paper would be advantageous for determining blood pathologies linked to leukocytes. The use of other laser excitation wavelengths, such as orange laser (Cobolt Mambo),³⁰ could decrease the spectral overlap between fluorochrome emissions. Some research is extensively carried out to extend this method using a supercontinuum optical source providing a set of excitation wavelengths (>2) and a set of fluorescence channels (>2). This new approach is expected to provide a correct measurement of cells exhibiting different antigenic expressions, fluorescence being well conditioned by selecting the best beam, and its intensities associated to each wavelength.

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