Review

HORIBA Medical in Europe: Evolution of Technologies for White Blood Cell Differential at HORIBA Medical

Philippe NERIN

This article retraces the evolution of technologies for leucocyte differential, which is a key feature of hematology analyzers. It is emphasized here the way HORIBA Medical started using simple and robust technologies, based on CIS (Cell Impedance Signal) for 3 part DIFF, and then evolved toward more sophisticated bio-photonics designs including cut edge technologies, considered as pioneering works in the field of flow cytometry. This article was presented by myself at ISLH 2012, in Nice, France, at the luncheon session attended by about 200 worldwide customers.

Introduction

It is worth pointing out that the first attempts in automatic measurements of blood cells go back to the mid twentieth century, when two techniques were developed in parallel: impedance measurement (the so-called electronic gate) and the first optical scattering detector. Improved year after year along with specific reagents, the electrical and optical methods allowed considerable projections in the field of hematology since, in most cases, the main cellular populations (examined early back by a photonic microscope) are differentiated and taken into account with acceptable accuracy.

Nowadays, a hematology analyzer is a complex system involving various disciplines and technologies such as biology, biochemistry, chemistry, fluidics, optics, electronics, data acquisition and statistical data processing.[1] Basically, a hematology analyzer is a fully automated device able to sample the blood through a hermetic stopper, dilute the sample, add a specific reagent for single or multiple cell parameters detection, carry the cells within a flow stream toward one or several sensors, acquire and store the information resulting from several physical mechanisms, and finally process the raw data by using a classification algorithm. What definitely differentiates this new analyzer from a classic flow cytometer is it capacity to accurately manage whole blood volumes (traditionally per mm^3 or per µl) added to its high level of automation. Although all HORIBA ABX analyzers provide platelet, red blood cell and leukocyte measurement, this article will focus on leukocyte differential, pointing out the evolution of technologies from the beginning of HORIBA ABX products toward the last generation of apparatuses. Prior to this, it is necessary to point out that leukocyte differentiation and counting are strongly related to hematopoiesis. Any cell in circulating blood arises from a single cell, the stem cell, originating from

bone marrow.

The bone marrow is one of the most complex tissues in the body. Distributed as red marrow all over the skeleton, but mainly situated in the pelvis area, the sternum and the vertebrae, an adult weighing 70 kg has roughly 1.5 kg hematopoietic tissue. This comes in addition to 5-6 liters of blood, of which more than 2 kg consist of red blood cells.

The main process for blood production is:

(1) A few stem cells produce a large number of progeny cells. This goes on continuously, and a single stem cell may, in a week or two, have up to 10^6 descendants. The stem cells also maintain their own numbers by additional cell division, as showed in Figure 1.
(2) Immature cells become mature blood cells which are able to perform specialized functions in the body.
(3) A limited number of cell divisions occur in parallel with maturation, with a final stage where mature cells lose their ability to divide (erythrocytes)
(4) Mature cells are stored for a while in the bone marrow until they enter the bloodstream to perform their specialized functions in blood or tissues
(5) The multipotent stem cells have the ability to direct their development into all subclasses of hematopoietic cell types, including erythrocytes, granulocytes (neutrophils, eosinophils and basophils), mast cells, monocytes and macrophages, megakaryocytes and platelets, as well as the different types of lymphocytes, including B, T, and NK cells.

A few images of such cells are shown on Figure 1. The main objective of leukocyte differentiation is automatic classifying and counting of the lineages according to their morphology, size or more advanced criteria such

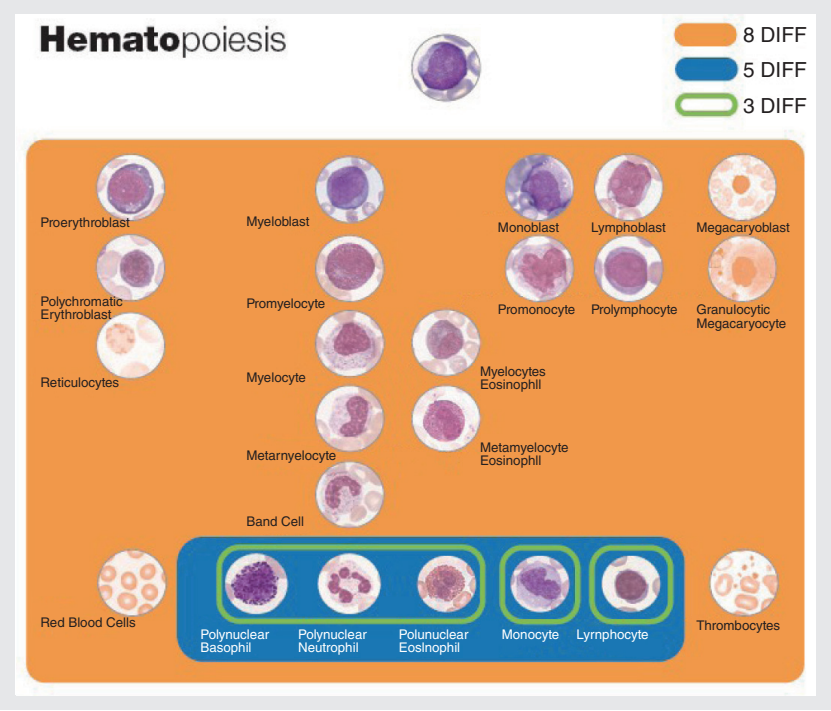

Figure 1 Leukocyte subsets according 3 DIFF, 5 DIFF and 8 DIFF definitions.

as the expression of some molecules like trans-membrane proteins or RNA/DNA content. In the area of automatic diagnosis, leukocyte classification has been organized according the following definitions:

3 DIFF: classification of three lineages of leukocytes (Lymphocytes, Monocytes and Granulocytes), so–called LMG classification.

5 DIFF: classification of five lineages of leukocytes (Lymphocytes, Monocytes, Neutrophils, Eosinophils, Basophils).

Extended-5 DIFF: classification of five lineages of leukocytes (Lymphocytes, Monocytes, Neutrophils, Eosinophils, Basophils) including the count of Large Immature Cells, the LIC parameter with a specific differential including IMM (Immature Monocytes), IML (Immature Lymphocytes) and IMG (Immature Granulocytes). The LIC parameter being used to flag samples containing abnormal leucocytes.

8 DIFF: classification of eight lineages of blood cells (Lymphocytes, Monocytes, Neutrophils, Eosinophils, Basophils, Blasts, Immature Granulocytes, Erythroblasts)

Today, manufacturers of *in vitro* diagnostic instruments are facing a dilemma: developing systems which enable ever more complex analyses for a more accurate and affordable diagnosis, providing robust and reliable methods to flag abnormal samples, while ensuring easily interpreted results for increased laboratory productivity, with a minimal false positive results thus reducing useless slides or flow cytometry reviews. The progress from 3 DIFF to 8 DIFF illustrates partly how technologies have been evolving during the last 10 years, pointing out how challenges have been taken up in terms of accuracy, throughput and reagent consumption, and how these advances have improved efficiency in the laboratory.

CIS for 3 Part Differential

This measurement principle relies on an electronic gate designed to detect and generate an electronic pulse: the CIS (Cell Impedance Signal) related to the overall particle volume. According to Figure 2, an electric field is generated inside a micro-aperture placed inside a chamber, in which the particle, a blood cell, is pulled through by aspiration. Two electrodes are placed on either side of the aperture producing a continuous electric current through the aperture. A blood cell, when passing through the aperture, creates an obstacle to the electrical current and, therefore, increases the micro-aperture impedance. It can be experimentally shown that pulse current amplitude varies in accordance with cell volume, as long as the biological cell can be considered as slightly insulating. From the electronic point of view, a constant current source converts such current variations into pulsed voltage signals, measurable across the gate. A constant voltage supplies a basic network composed of a polarization resistor Rs, a shunt resistor R and feedback resistors R1 & R2. All of these resistors are

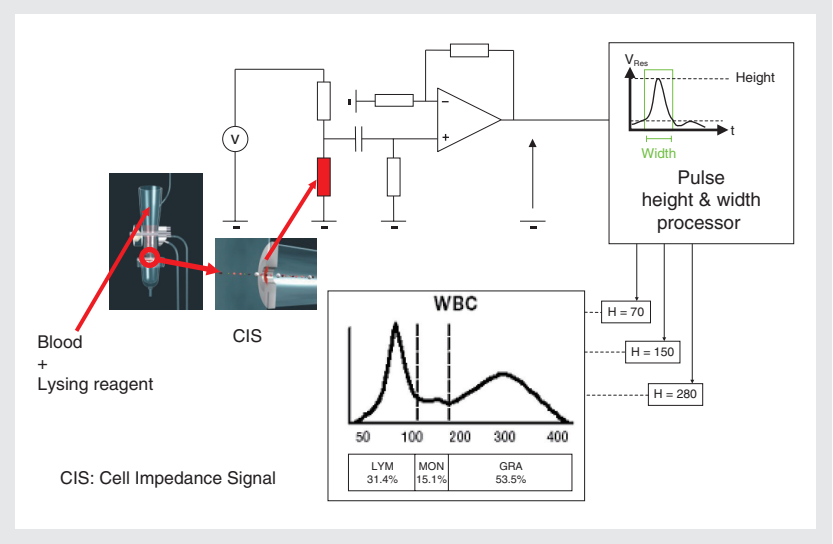

Figure 2 Principle of CIS for 3 Part Differential

optimized to simultaneously gain stable operation against temperature changes of the gate resistance Ra and they are set to optimize the electronic signal to noise ratio. The output signal is measured by using a specific electronic circuit designed to determine gate pulse parameters, namely their heights and widths.

It is worth noting that, whereas pulse heights inform about cell volume, widths of the pulses are used to filter the signal, rejecting undesirable pulses corresponding, for example, to simultaneous crossing of the gate by two cells.
The MICROS analyzer uses CIS technology. For 3 part DIFF, a specific reagent lyses red blood cells, but it preserves and prepares WBC membrane to react differentially according to cell features:

- When the lyse reacts with lymphocyte cytoplasmic membranes, it allows release of water-soluble cytoplasm and shrinks the cell membrane around the nucleus.
- When the lyse reacts with monocyte cytoplasmic membranes, it has an intermediate reaction, maintaining its large size in comparison to lymphocytes.
- When the lyse reacts with granulocyte cytoplasmic membranes, it has a limited reaction due to a molecule in their cytoplasmic structure which protects them from the shrinking action of the lyse. This limited reaction makes granulocytes the largest of the sub-populations in cell differentiation.

After the differential lysing action, the machine analyses the height of each pulse as cells pass through the electronic gate. These pulses are then digitized, grouped according to their size (30 fL to 450 fL), and mathematically processed to create the WBC distribution curve, which is also known as the WBC histogram.

The 3 sub-populations of WBCs are charted and classified according to the

following classes:
Lymphocytes (30 fL to 100 fL)
Monocytes (100 fL to 150 fL)
Granulocytes (150 fL to 450 fL)

This classification is also known as LMG. Results are printed according to a histogram as reported on Figure 2, specifying the percentage of each sub-population.

FROM 3 to 5 Part DIFF

The LMG differential lyse provides an efficient technique to separate Lymphocytes, Monocytes and Granulocytes. An improved diagnostic can be formulated if granulocytes are discriminated between themselves. For example, eosinophilia occurs in a wide range of conditions. In modern countries, its commonest causes are allergic diseases such as asthma and hay fever, whereas worldwide, the main cause is parasitic infection. It can also occur in relation to common skin diseases and drug reactions. Other rarer causes include: lung diseases, eg Loeffler's syndrome, vasculitis (inflammation of blood vessels), eg Churg-Strauss syndrome, some tumors, eg lymphoma, liver cirrhosis, some antibody deficiencies (not typically AIDS), other rare skin diseases, eg dermatitis herpetiformis and other unknown causes, labeled hypereosinophilic syndrome.

From a technological point of view, a new step was accomplished through simultaneous measurement of electronic volume and optical signature of cells travelling through an optical gate placed just above the electronic gate, Figure 3.
Each blood cell experiences a travel from the CIS sensor toward an optical gate where it interacts with the light beam, causing an optical power loss (the so-called optical extinction) measured by a photoreceptor. Different situations are depicted on Figure 2. When no cell crosses the optical gate, the light beam is totally transmitted, producing a stationary electrical signal called "the baseline". The light source (not represented on the Figure) is a thermal or semiconductor lamp. Usually, a non-coherent optical source is tailored by a beam shaper into a rectangular window with uniform illumination (the so-called optical gate) projected inside the flow cell.

HORIBA Medical instruments use a specific flow cytometer based on hydrofocusing to combine Cell Impedance Signal (CIS) and Optical Extinction. In this apparatus, cells are described by two parameters (volume and optical extinction) and displayed in a matrix where several sub-populations are identified by a specific algorithm. Low optical extinction corresponds to lymphocytes and monocytes which are poorly scattering particles whereas neutrophils and eosinophils produce high optical extinction corresponding to highly scattering particles. In this method, whole blood is mixed with a specific lytic reagent containing Chlorazol black as a stain reinforcing optical extinction, even if we have to recognize that the main contribution to optical extinction is scattering.

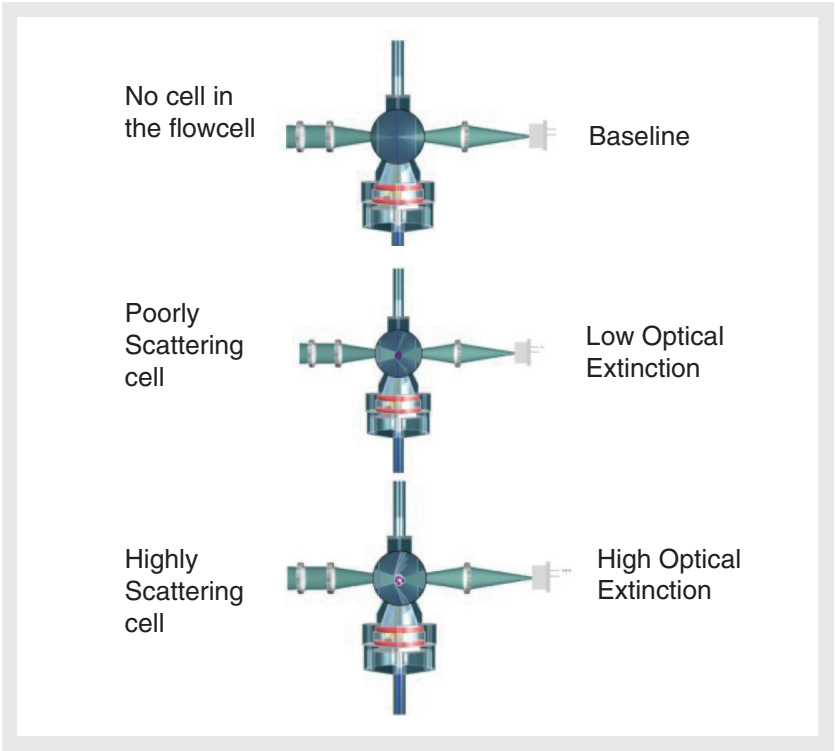

Figure 3 Schematic representation of the optical response to a travelling cell through the optical gate to work out the 5 part DIFF.

The Optical Extinction Phenomenon

While a cell crosses the optical gate, the optical detector is designed to provide a sensitive response to light losses including scattering and absorption.

It is worth to analyse, from a physics point of view, the process of optical extinction as a unique feature in HORIBA Medical blood analyzers. Optical extinction is the capability of any particle to remove light from an original beam. This light removal produces power variation in the transmitted light beam. Two independant mechanisms, absorption and scattering, are responsible for optical extinction. Absorption is mostly a non radiative process reinforced by cytochemistry, most often converting light to thermal energy. Whereas scattering is a very complex phenomenon, involving specific features of the particle itself. For blood cells, optical thickness is so

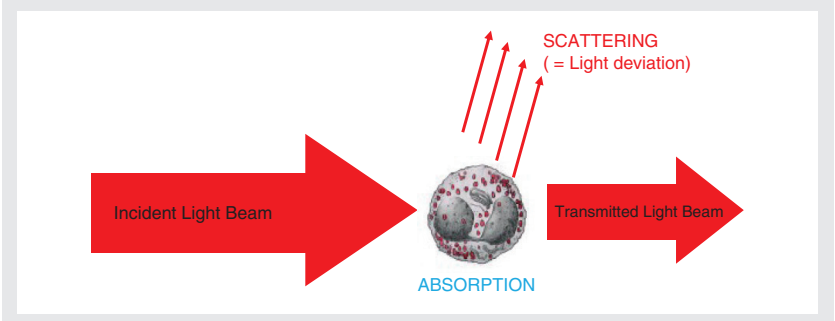

Figure 4 Optical Extinction Definition

small that absorption is often negligeable in comparison to scattering. Scattering by a perfect sphere has been definitely solved by Gustave Mie in 1907. Since then, a lot of progresses have been done using extensive computer calculations. A biological cell is a pseudo random particle with different compartments, each having a specific refractive index.

These refractive indexes have been measured by several research teams for cytoplasms, mitochondria and others biological species as reported in the left side of Figure 5. On the right side , top and bottom graphs represent computer calculations of light scattering versus scatter angle for a semi realistic cell , wherein nucleus, cytoplasm and granule refractive indexes have been taken into account.[2] These curves show that light scattering is dependant on cell granule or organelle concentration: higher concentration produces significant effect mainly at high scatter angle, more than 20 degrees, whereas forward scattering is not dependant on this organelle content. On the other hand, side scattering is dependant on nucleus size whereas forward scattering is not. Design tradeoffs involving parameters, such as optical numerical aperture of illumination/detection and light wavelength, are very important since they balance absorption and scattering strength responsible for the performance of the analyzer. Each cell being probed optically and electronically is identified by its two components X: impedance measurement, Y: optical extinction measurement.

According to Figure 6, the blue cluster represents Lymphocytes which are very small round shaped cells with condensed cytoplasm and large nucleus. These cells are normally positioned in the lower part of the optical Y-axis as well as the lower part of the volume X-axis because of their small volume. The far left side of the lymphocyte zone (LL) should normally be empty. Any detection of cells in the (LL) zone indicates small lymphocytes, Platelet aggregates, NRBCs (Nucleated Red Blood Cells), or improperly adjusted flow cell alignment. Background noise may also be detected in this zone if

Cell Component	Index	Reference
Cytoplasm, rat liver cells	1.38	(Beuthan et al., 1996)
Mitochondria, rat liver cells	1.40	(Beuthan et al., 1996)
Lipid	1.48	(Beuthan et al., 1996)
Cytoplasm	1.35	(Kohl and Cope, 1994)
Protein	1.50	(Kohl and Cope, 1994)
Cytoplasm, hamster ovary cells	1.37	(Brunsting and Mullaney, 1974)
Mitochondria, rat liver	1.42	(Liu et al., 1996)
Melanin	1.7	(Vitkin et al., 1994)
Cytoplasm	1.358-1.374	(Lanni et al., 1985)[2]
Cortical cytoplasm	1.353-1.368	(Bereiter -Han et al., 1979)
Dried protein	1.58	(Barer and Joseph, 1954)

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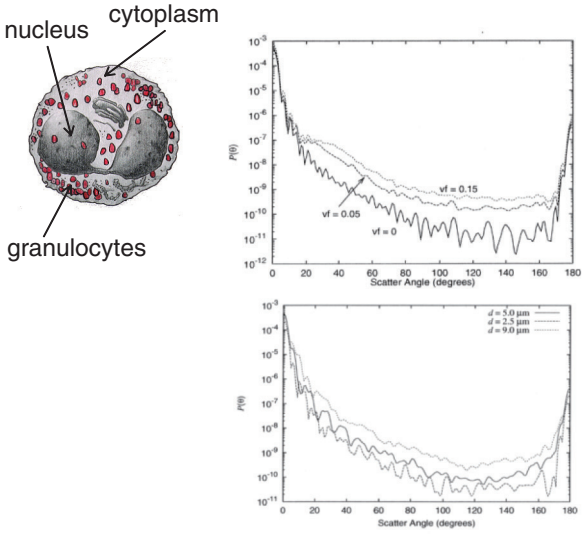


Figure 5 Refractive indexes of biological coumponds (left hand side) , a pseudo realistic model of cell (center), and numerical results of scattering diagrams for a simplified cell model taking into account inner granule concentration (top) and nucleus size (bottom).

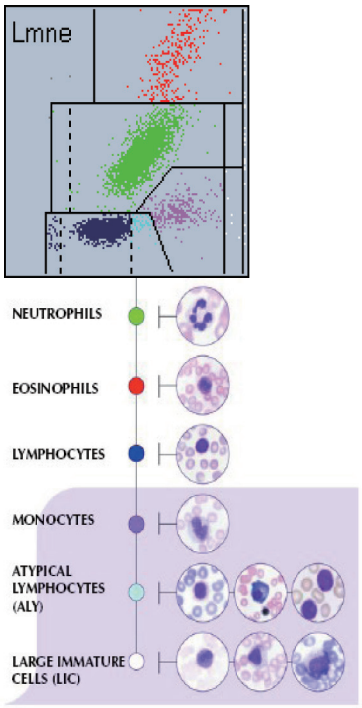


Figure 6 LMNE matrix representation according to CIS and optical extinction measurement.

biological interference takes place in a significant way.

As represented in Figure 6, optical extinction (Y-axis) is plotted against volumes (X-axis) to form an image with several "clusters" identified as follows:

The blue cluster represents Lymphocytes which are very small round shaped cells with condensed cytoplasm and large nucleus. These cells are normally positioned in the lower part of the optical Y-axis as well as the lower part of the volume X-axis because of their small volume. The far left side of the lymphocyte zone (LL) should normally be empty. Any detection of cells in the (LL) zone indicates small lymphocytes, Platelet aggregates, NRBCs (Nucleated Red Blood Cells), or improperly adjusted flow cell alignment. Background noise may also be detected in this zone if biological interference takes place in a significant way.

The purple cluster represents Monocytes which are very large irregular shaped cells with large convoluted nuclei. The nucleus contains folds and sometimes vacuoles. The cytoplasm is also large with non-granular intra-cellular material. These cells will not scatter or absorb a large amount of light when passing through the optical gate. They will therefore be positioned in the lower part of the optical Y-axis. Because monocytes are large cells, they will be placed on the right side of the volume X-axis.

The green cluster represents Neutrophils which are larger in size than lymphocytes. Neutrophils contain granular material in their cytoplasm along

with a segmented nucleus. Due to these cellular features, more light will be scattered as they pass through the optical gate, spreading up the cloud to higher values of the Y-axis depending on their granule concentration, and then spreading up along the X-axis according to their maturity. Hyper segmentation and increased granule content will place these cells higher on the optical Y-axis.

The red cluster represents Eosinophils which are somehow like neutrophils. They contain granular material and segmented nuclei within the cytoplasm. Because they scatter more light than other cells, they are placed at the top of the image. Hyper-segmentation of the nucleus and increased granule/ organelle content will spread this population across the right side of the matrix.

Additional parameters, ALY (Atypical Lymphocytes) and LIC (Large Immature Cells), are other cells identified in pathologic blood, they complete the matrix spectrum of leukocytes.

Basophils are rare cells, they are embedded in the LMNE matrix. To detect this rare population, a specific reagent is used (ABX BASOLYSE II) in order to lyse erythrocytes and shrink leukocytes except basophils which cytoplasm remains shaped. Based on cytochemistry of basophils, this process allows a basophil measurement based on CIS reading for volumetric analysis of the sample. An improvement was made in OCTRA technology where CIS and Optical Extinction are combined for basophil detection.

Side Scattering (SSC) versus Optical Extinction (EXT)

SSC versus EXT was investigated at HORIBA Medical using a customized flow cytometer measuring simultaneously Side Scattering and Optical

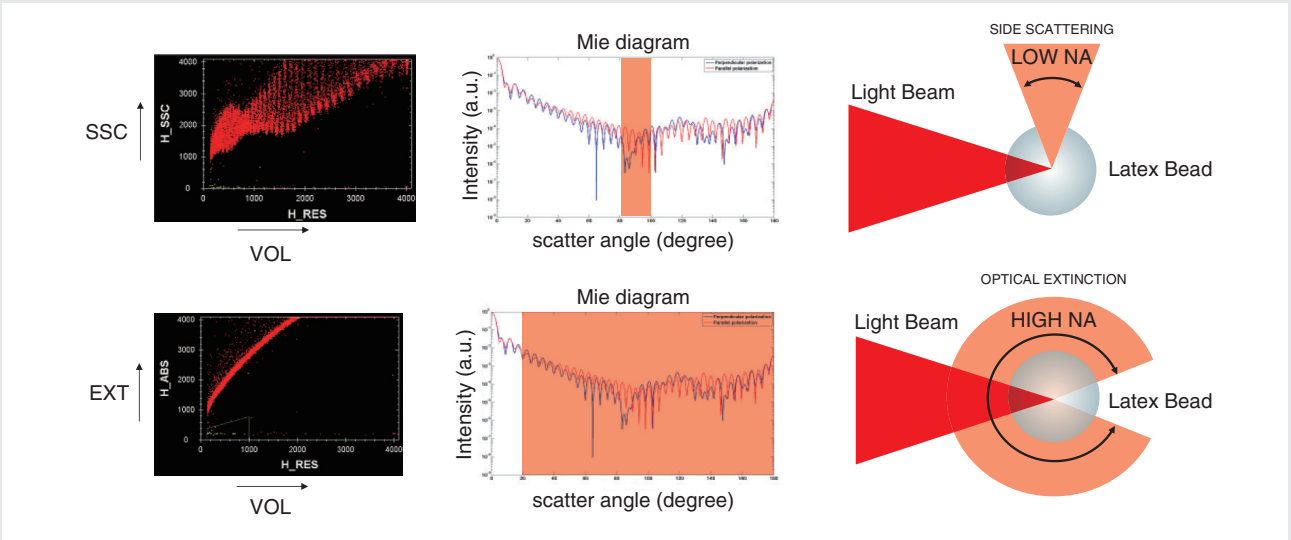


Figure 7 Comparison of Side Scattering Detector (SSC) placed at right angle of the light beam (top image) producing a relatively low numerical aperture (LOW NA) in comparison with optical Extinction (EXT) which provides High Numerical aperture (High NA) filtering therefore the Scattering profile derived from Mie Theory for both TE and TM polarizations.

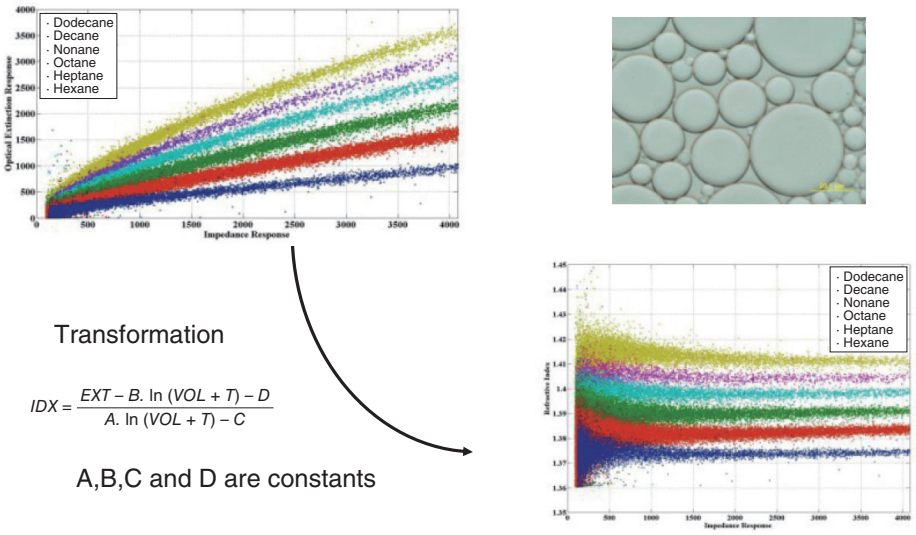


Figure 8 Calibration of bi-parametric representation CIS x EXT to determine a new mapping involving VOL x INDEX. The calibration process is based on emulsions of organic solvents: hexane (marine blue), heptane (red), octane (green), nonane (blue), decane (purple), dodecane (yellow).

Extinction. Figure 7 is an amazing demonstration of side scattering and extinction behaviors for polydisperse polystyrene beads, diameters ranging from 1 to 20 µm.

The horizontal axis represents particle volume and the vertical axis optical response. Side Scattering detector (SSC) produces a very strange behavior with strong oscillations whereas Optical extinction (EXT) has a smooth like response. Explanations of these results come from Mie theory where it is derived that scattering of a perfect sphere is an oscillating function of size parameter or scatter angle. In SSC, these oscillations are weakly filtered by the low numerical aperture of side optics (pink color). On the other hand, optical extinction takes place over a large numerical aperture, causing an

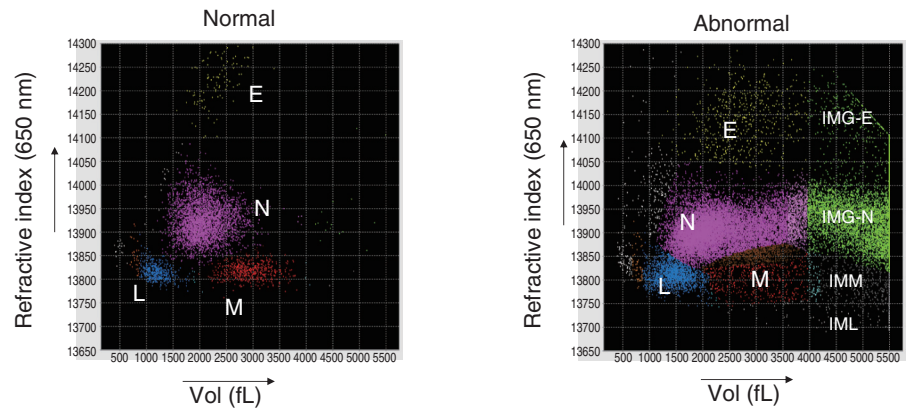


Figure 9 Normal (left) and Abnormal samples (right) according to orthogonal representation volume x refractive index.
This classification process was compared to microscopic examination of blood smears, and algorithms were optimized to match manual counts as much as possible. More specifically, we defined thresholds to identify immature granulocytes (green color), monoblasts (grey color) and lymphoblasts (blue color). These fundamental results were the starting point for flags on Pentra DX analyzer. Accordingly, ten years ago, HORIBA Medical introduced the extended 5 part diff comprising Immature Granulocyte, Monoblast and Lymphoblast counts based on volume and optical extinction measurements. The sum of IML, IMM and IMG is called Large Immature Cells parameter or LIC parameter.

efficient filtering process of optical oscillations, producing therefore a smooth response in comparison to a standard side scattering detector.

These considerations have been applied to calibrate VOL x EXT using specific emulsions made from organic solvants (hexane, heptane, etc..). In Figure 8, each emulsion have a specific refractive index, increasing from the bottom (blue) to the top (green). As you can see on this picture, each fluidic particle is a perfect sphere with a specific volume and refractive index represented in the matrix as a colored dot. Each refractive index isoline can be transformed into another one where volume dependence is removed to produce an orthogonal mapping of volumes and refractive indexes.
In this new orthogonal representation, normal and abnormal blood can be displayed and each cell can be identified by its volume and its average refractive index.

Pentra DX and Nexus: the LIC Parameter

Pictures, in Figure 10, illustrate some Leucograms of Pentra DX and Nexus blood analyzers. Acute and chronic leukemias, displayed respectively on the left and on the right hand sides, show the LIC parameter including a differential count of IML, IMM, IMG. Many other blood diseases have been investigated by biologists in European laboratories and the LIC (Large Immature Cells) parameter was compared to cytology counts showing acceptable correlations between automatic and manual counts. The LIC parameter was therefore definitively used for flag purpose.
We recently investigated the capability of these analyzers to detect abnormal samples at Bordeaux Hospital. The experiment was based upon a total of 218 patients including 152 abnormal samples. These samples were selected to provide a large spectra of abnormal cells containing different etiologies for immature granulocytes and blasts. Within the scope of this study, sensitivity and specificity of the LIC parameter were investigated versus IG + BLAST

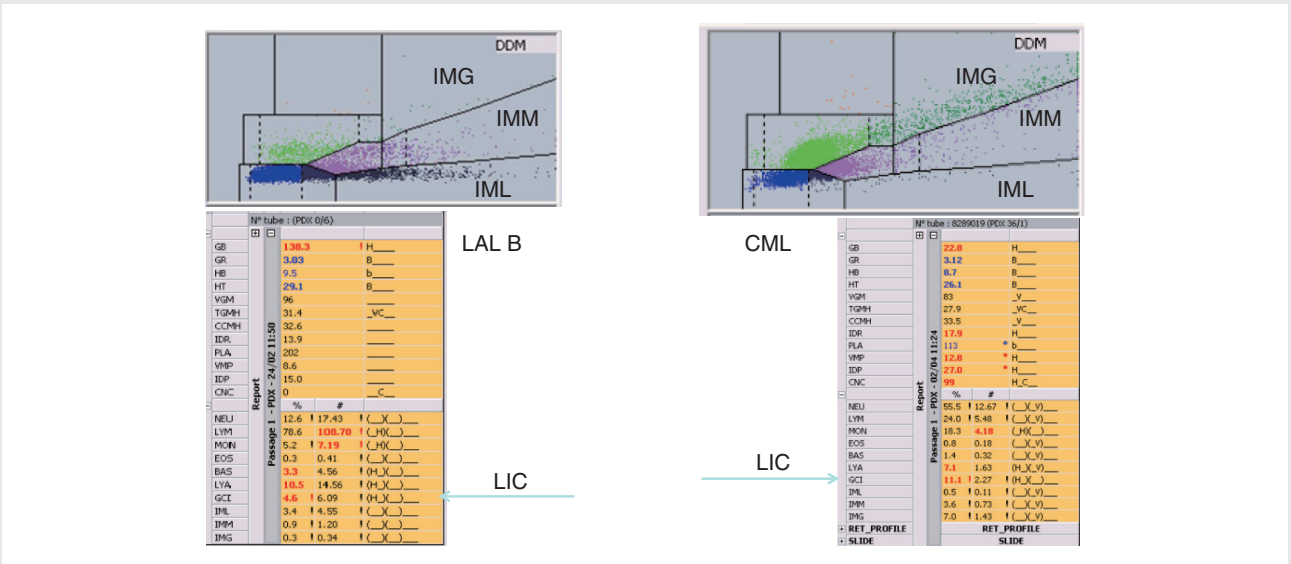
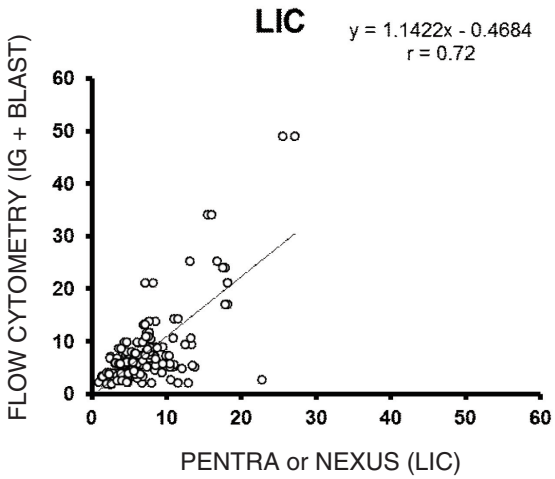


Figure 10 Example of two abnormal samples from leukemia patients: LAL B (left) and CML (right). LIC parameter is derived as the summation of IML, IMM and IMG events falling in corresponding boxes.



Sensitivity	0,94
Specificity	0,83
PV-	0,89
PV+	0,88

Figure 11 IG + BLAST derived by flow cytometry versus LIC derived by PENTRA and NEXUS.

derived from flow cytometry based on CD16, CHTR2, CD45 and CD11b (standard protocol used at Bordeaux Hospital). We reported that Pentra DX and NEXUS analyzers had a sensitivity of 94 % and a specificity of 83% to flag samples, with a negative predictive value of 89% and a positive predictive value of 88%.

For routine purpose, these results are highly satisfactory since we reported that the rate of false negative is lower than 10 %. In addition, it is worth pointing out that the absolute count of Immature cells can be derived by mulytiplying the LIC % parameter by the total count of white blood cells; nevertheless we have to recognize that this parameter is mainly designed to detect and flag abnormal cells in the sample. From a biophysics point of view, it is worth noting that despite the fact that measuring principles are far from each other, analysis being based on volume x refractive index measurements, Pentra DX and Nexus instruments deliver highly correlated results in comparison to flow cytometry immunomarking. (Figure 11)

FROM 5 to 8 Part DIFF: OCTRA Technology.

To reduce some discrepancies between routine and CD marker analyses, mainly for absolute count of immature cells and its interference with other leucocyte sub-populations, we recently developped a unique optical device, depicted in Figure 12. In addition to previous physical parameters (CIS, SSC and EXT), the cell signature is enriched by fluorescence measurement of RNA / DNA content. Based on this strategy, called OCTRA technology, we were looking for an improved method of detecting and counting immature cells including immature granulocytes, blasts and erythroblasts.

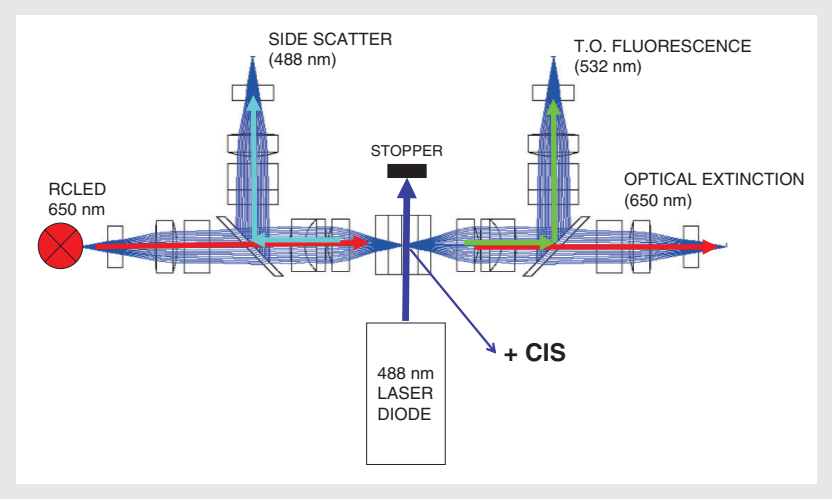


Figure 12 Schematic representation of the optical principle used in OCTRA Technology.

OCTRA Technology combines two optical beams in the flow cell. The red light beam passing through the flow chamber is used for optical extinction measurement. The Blue Laser diode is used to induce both scattering and fluorescence optical signals of cells. These three optical measurements are combined to Cell Impedance Signal (CIS) measurement. Optical Extinction, Fluorescence, Scattering and Cell Impedance measurements form a fourth dimensional space analysis where each blood cell signature is digitized by an electronic processor.

Analytical techniques based on fluorescence detection are very popular because of their high sensitivity and selectivity, together with advantages of spatial and temporal resolution. The principle of fluorescence relies on absorption and re-emission of light by a molecule, see Figure 13. Once the molecule is excited by absorption of a photon at 488 nm, it returns to the ground state with emission of a new photon at 530 nm. In OCTRA technology, the Thiazol Orange dye[3] is used and replaces the Chlorazol Black used in the Pentra Analyzer. Free in solution, the two aromatic cycles rotate about the Carbon-Carbon bound and absorbed photons are converted to mechanical motion: there is no significant fluorescence. On the other hand, this molecular compound fluoresces strongly when rotation is blocked by hybridization to DNA / RNA strands.
Early stages of blood cell maturation, normally occurring in bone marrow only, are denoted by their nucleic content. Nucleic acids (RNA and DNA) are therefore found in larger amounts in these immature cells. Mature leukocytes also exhibit different levels of nucleic acids depending on their nature and activity. The level of nucleic acid concentration can be measured through the use of Thiazole Orange (TO), as exemplified in the next section.

Leukocyte Classification in OCTRA Technology.

In OCTRA technology, whole blood is mixed with a specific reagent performing simultaneously the following operations:
- Removal of erythrocyte by lytic action

- Chemical poration of nucleated cells for fast staining purpose, while maintaining membrane voltage to accelerate penetration of Thiazole Orange (electropositive ion).
- Staining of intra cellular nucleic acids by TO, which is an intercalative dye, highly specific to nucleic acids.

Once incubation completed (a few seconds), the cell suspension is pulled through a flow chamber equipped with the electro-optical device depicted in Figure 12.

In OCTRA technology, multiple cells crossing through CIS or EXT gates are detected by pulse width measurements: undesirable events are filtered based on pulse/height ratio information. The four space analysis is used to design algorithms in a self-learning approach, after intensive testing based on a large spectrum of blood samples including normal and abnormal. In addition to the standard matrix CIS x EXT available in the Pentra instrument, OCTRA technology provides a new matrix where ERB, BASOPHILS, IG and HRC cells are identified.

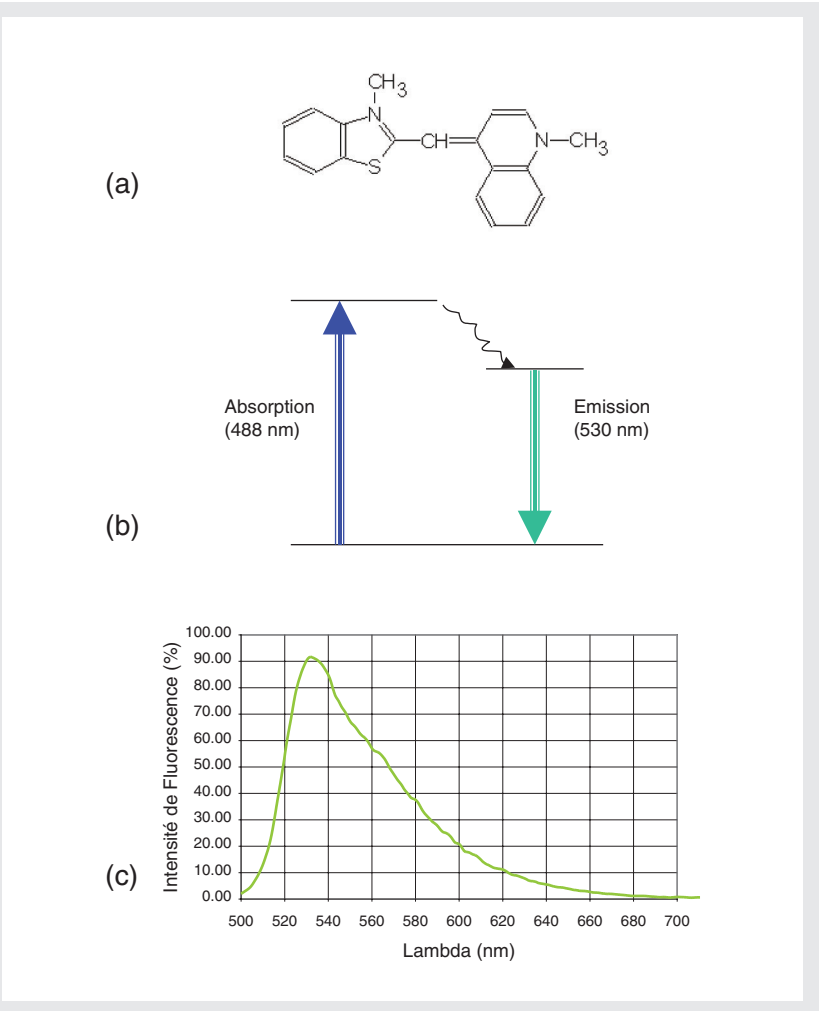


Figure 13 Simplified Jablonsky diagram of Thiazole Orange dye
(a) and associated fluorescence spectra (c) for λ_{ex} = 488 nm.

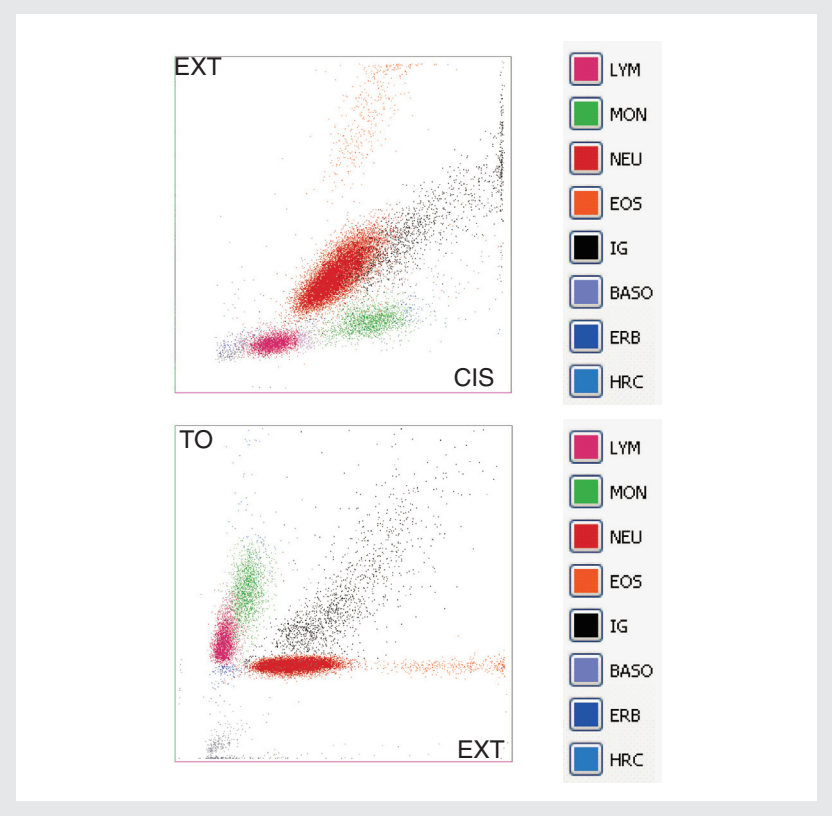


Figure 14 Standard bi-parametric representation in OCTRA technology involving CIS x EXT and EXT x TO images.

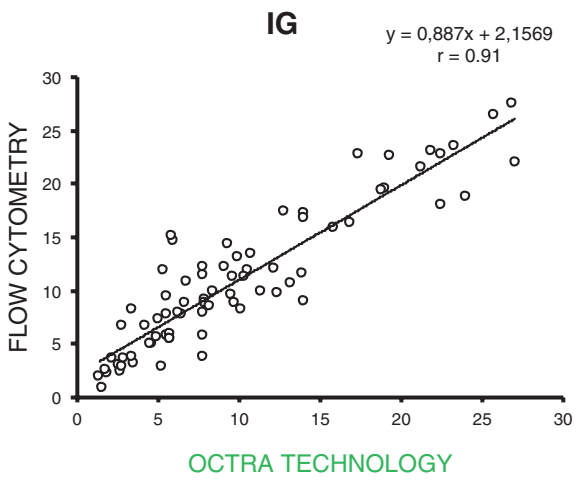
Figure 14 displays a representation using standard parameters CIS x EXT and EXT x TO.

Stromas, platelet aggregates and other fragments (grey color), having no genetic material, are easily separated by their poor level of TO fluorescence. Platelet aggregates are themselves denoted by their higher SSC response.

Being lysed in the same way as erythrocytes, the remaining part of Nucleated Red Blood Cells (marine blue), which have small nucleuses; produce weak level of signals in the four measurement spaces.

Lymphocytes (purple color) are larger in size than NRBC. Being mono-nucleated, and having small cytoplasm, they produce a bigger response for EXT and SSC. Depending on their maturation and their activation state, they produce a weak or high level of CIS and TO fluorescence.

Basophils (blue color) have roughly the same size as that of lymphocytes, but being poly-nucleated, they produce higher SSC levels. On the other hand, the TO level remains weak like other granulocytes. Neutrophils (violet) and eosinophils (pink) also produce this TO level, but being bigger, they produce medium RES and EXT levels; being more complex, they exhibit a much higher SSC response. Due to their granule size, Eosinophils produce a higher EXT response than neutrophils with respect to their volume (CIS). We have to point out that mature granulocytes produce a TO baseline from which the



Sensitivity	0,95
Specificity	0,8
PV-	0,88
PV+	0,85

Figure 15 Immature Granulocyte parameter derived from OCTRA technology in comparison with four color cyometry for CD16, CHTR2, CD45 and CD11b.

immature cells are detected.

Immature granulocytes (black) show higher TO level than mature granulocytes, and high EXT. High CIS responses are produced because of their large volumes. Other large leukocytes are HRC (High ARN Content) (sky blue), which produce high CIS and TO responses. These cells have a high TO level due to their high RNA content.

Monocytes (green), being mono-nucleated and having no granules, produce a weak to medium SSC level and a medium to high EXT, TO and CIS levels.

In OCTRA Technology, the HRC box gathers lymphoblast, monoblast and plasma cells.

We investigated the capability of OCTRA technology to detect abnormal samples containing immature granulocytes. The experiment included 196 patients, selected to provide a large spectrum of abnormal cells containing different etiologies for immature granulocytes, blasts and atypical lymphocytes. Within the scope of this study, sensitivity and specificity of the IG parameter and its flag capability, were investigated versus IG derived from flow cytometry based on CD16, CHTR2, CD45 and CD11b (standard protocol used at Bordeaux Hospital). Based on a cut-off of IG (Octra) > 2% and IG (Flow cytometry) > 2%, we reported that OCTRA technology had a sensitivity of 95% and a specificity of 80% to recognize and flag samples containing immature granulocytes, with a negative predictive value of 88%

and a positive predictive value of 85%. These results are highly satisfactory for routine application, and it is worth noting that RNA / DNA expression measurements of leucocytes provide an exceptionally simple and affordable technique to meet flow cytometry standards both for detecting and counting IG cells as reported in Figure 15.

Biomolecule Detection for Leucocyte Recognition and Counting

Many membrane or cytoplasmic proteins of leukocytes have been studied at laboratory level.[4] Availability of monoclonal antibodies directed against

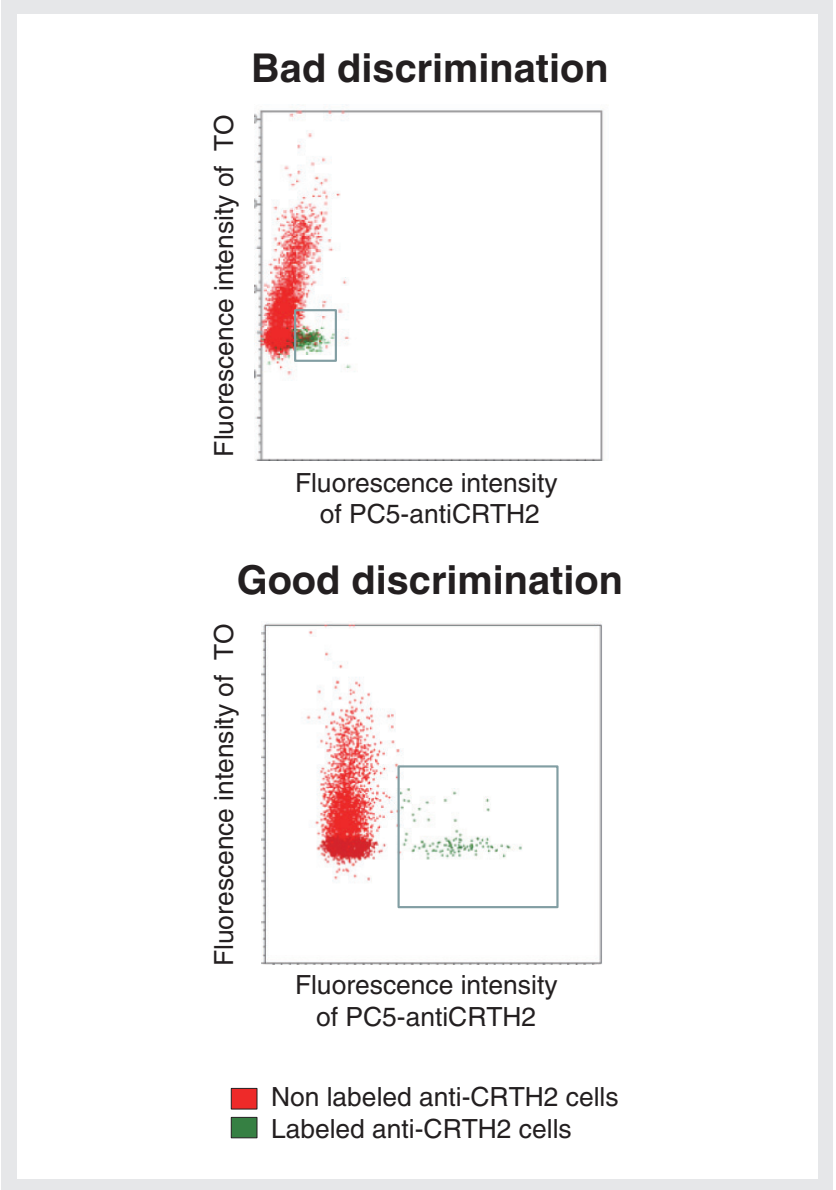


Figure 16 Top: demonstration of a single wavelength LIF experiment based on PC5 –CRTH2 and TO –RNA/DNA showing poor separation of labeled and non-labeled cells. Bottom: dual wavelength LIF experiment where PC-antiCRTH2 is correctly measured despite unbalanced biomolecule expression.

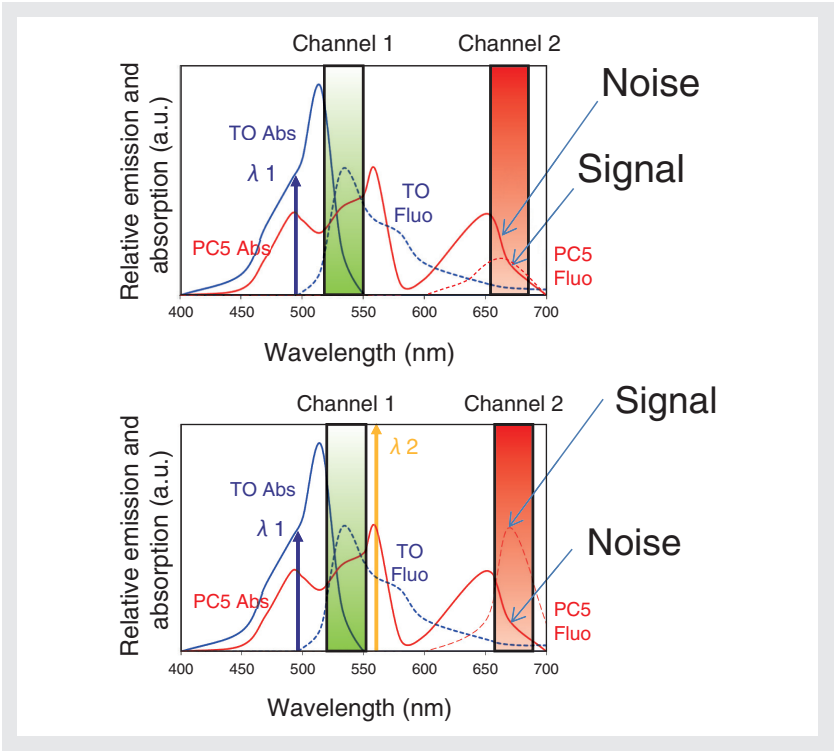


Figure 17 Illustration of spectral overlaps, single (top) and dual (bottom) LIF experiments.

these proteins allows flow cytometric analysis of leukocyte lineages, mainly based on fluorescence immuno-markers.[5] In this context, the standard diagram is replaced by a similar diagram, where cells are fully described by their antigenic expression. A specific cell is described by a set of CD markers, each having a specific expression. Obviously, this concerns the field of standard flow cytometry, except that at HORIBA Medical, we took into account limitations and drawbacks existing in marketed flow cytometers.[6] A first limitation comes from detection of unbalanced fluorescence signals measured in a multi-channel flow cytometer. To illustrate this pitfall, we carried out an experiment where a weak fluorescence (Phycoerythrin Cyanine 5 coupled to an antibody denoted PC5-antibody) is investigated in the presence of a strong fluorescence background (TO from DNA / RNA). In Figure 16, the situation of PC5-antiCRTH2 is reported here whereas several other combinations were investigated.[6]

Figure 16 (top) reports an experiment using a single wavelength operation showing that anti-CRTH2 is weakly detected in presence of TO fluorescence, while Figure 16 (bottom) reports a dual wavelength excitation exhibiting a larger fluorescence for labeled anti-CHRTH2 cells.

At the top of Figure 17, blue and red curves are respectively the absorption spectrum of TO and that of PC5. Dashed lines are respectively the fluorescence spectrum of TO and that of PC5. Using one wavelength illumination, overlap of TO spectrum in channel 2 induces a background optical signal producing quantum noise for PC5 fluorescence measurement. At the bottom of Figure 17, a second wave is added in the flow chamber at 565 nm reinforcing significantly the PC5 fluorescence strength independently

of the TO fluorescence, thus improving to some extent the signal to noise ratio for channel 2.

Wavelength Multiplexing: A Flow Cytometry Paradigm.

A generalisation of the previous concept is depicted in Figure 18. Here, multi-wavelength illumination is proposed to improve the signal to noise ratio for multi color flow cytometry measurements with spectral overlaps.[6]

Looking at Figure 18, let's consider a fifth channel flow cytometer designed to adress the following fluorochormes: Alexa Fluor 405 (AF405 blue) for channel 1 (CH1) , Cyanine 2 (marine blue) for channel 2 (CH2), Cyanine 3 (orange) for channel 3, Cyanine 5 (red) for channel 4 (CH4) and Cyanine 5.5(brown) for channel 5, each coupled to a monoclonal antibody. This general scheme can be used for unbalanced CD expression measurements involving, for example, CD16, CRTH2, CD45, CD11b and CD34 as a standard protocol for leucocyte differentiation purpose. In this set-up, the wave λ 1 excites AF405. The wave λ 2 excites CY3. The wave λ 3 excites CY4. The wave λ 4 excites CY5 and the wave λ 5 excites CY5.5. This scheme is a generalisation of our principle for which each antibody is associated to a specific wave and a specific channel.

Thus, this set-up requires multi-wavelength operation. Each CD marker being adressed by a specific wave of defined wavelength and amplitude. To provide flexibility on multi-wavelength operation and selection, HORIBA Medical promoted the development of a specific laser based on non linear optics inside crystal fibers.[7, 8]

Figure 18 shows the set-up investigated at research level and corresponding

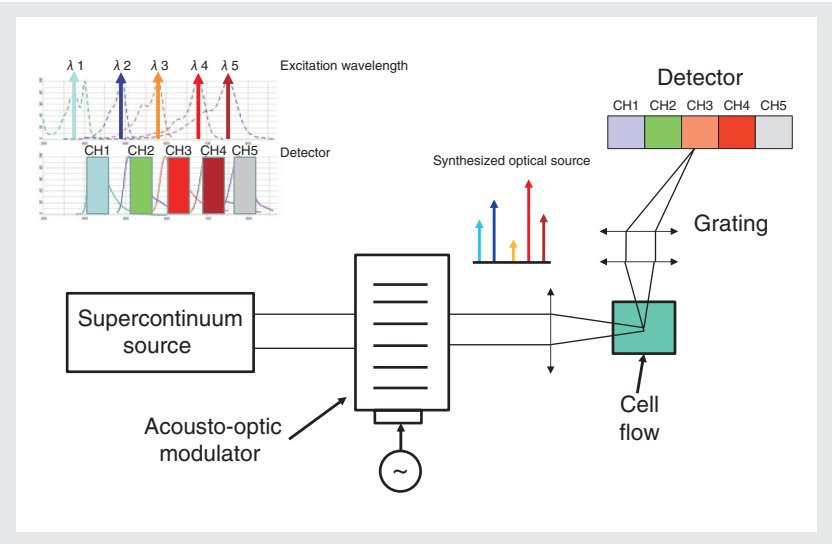


Figure 18 Design principle of a multiwavelength and multispectral detection architecture based on supercontinuum optical source.

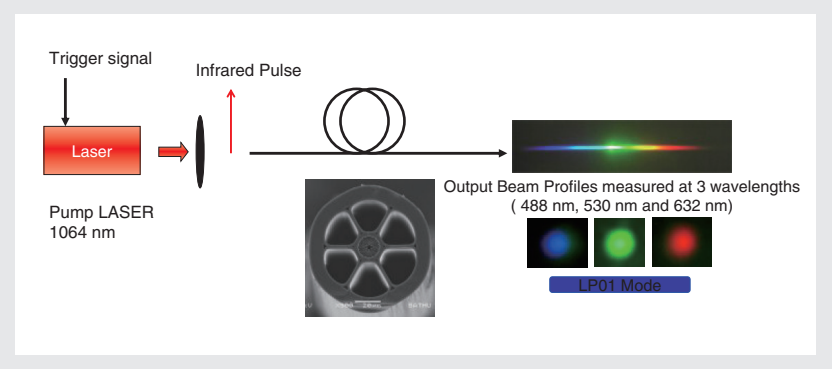


Figure 19 The supercontinuum optical source principle used at HORIBA Medical

to a compact solid-state flow cytometer with multiplexing-demultiplexing capabilities for CD expression measurements. Somewhere, it stands as a paradigm in the field of flow cytometry since the device is expected to be fully programmable thanks to a wavelength selector acting as an optical synthesizer tailoring both wavelength and wave amplitude for CD marker expression measurement. Preliminary results of this promising technology have been accepted for publication.[9]

In Figure 19, the optical source is depicted. It is based on a crystal fiber is composed of a small silica core surrounded by air galeries. Non linear effect takes place when an optical infrared pulse is sent into the tiny optical fiber core. When the pulse matches some technical conditions defined by the fiber itself, non linear optical processes convert the original pulse to a supercontinuum light source comprising all wavelengths, from the visible range to the short infrared. The beam emerging from this optical source looks like a white laser with a high spatial coherence. This rainbow-like image corresponds to dispersion of the beam by an optical grating. Thus, the white laser beam can be filtered at specific wavelengths providing LP01 modes identically to a conventional laser beam.

Conclusions

WBC differential technology covers a wide range of products at HORIBA Medical, depending on needs, habits and laboratory organization. Micros and Pentra analyzers provide flexible, accurate and reliable solutions for routine hematology, with satisfactory correlations with many references such as manual counts or cross correlated instruments based on physical measurements. We have to point out that incoming OCTRA technology will bring higher insight with improved correlations to flow cytometry «gold standards».

In addition to continuous improvement in biophysical measurement principles applied in routine analysis, HORIBA Medical explores the way cellular immunophenotypes can be carried out using optical wavelength multiplexing. This methodology is expected to fulfill requirements for multiple measurement of biomolecules, both improving cell identification and accuracy. To some extent, wavelength multiplexing alleviates software compensations applied in standard flow cytometers, which are the root cause

of inaccurate measurements in clinical applications, including diagnosis of hematology disorders or Minimal Residual Disease (MRD) monitoring. MRD is part of the concept of chronic disease follow-up requiring an accurate and sensitive methodology to monitore patients all along their lives. We are expecting that routine and innovative technologies will improve sensitivity and efficiency of clinical care and preventive medicine, which are the driving guidelines for the WHO and European Community in their fight against chronic disease while promoting active and healthy aging.

About the author: Philippe NERIN (MSc, PhD) is author of about 20 peer reviewed publications and inventor of about 25 patents. He has a 20 years experience in Applied research encompassing Measuring Science, Biophysics and Biophotonics. Since 2003, he is leading the Research and Innovation at HORIBA Medical, Montpellier.

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Philippe NERIN
Research Director.
HORIBA ABX SAS
PhD.