Principles of optical measurement used for the determination of the leukocyte differential part on Pentra® series

P. Nerin, Research Director HORIBA Medical
E. Tournier, Hematology Product Manager HORIBA Medical

Introduction

One of the main objectives of the automation of the leukocyte differential part is to analyse the cells in suspension, identical to their native state in blood circulation or in the tube. The systematic slide review is abandoned and is only kept for special cases.

The cell and its interaction with light

Generally, the different cellular compartments of the cells have various spectroscopic properties and refractive index values which are wavelength dependent. These compartments compose the internal structure of the cell and are for example the chromatin, the cytoplasmic organelles: mitochondria, cytochrome C, Golgi apparatus, vacuoles, lysosome, endoplasmic reticulum...

The leukocytes are optically anisotropic, which means they are not necessarily perfectly spherical on the one hand, and they have structure heterogeneities linked to more or less random distribution of material of the cells compartments on the other hand.

From an optical point of view, absorption and refractive index vary in each point of the cell at the light wavelength. It is the result of the interaction of the cell with light which gives information on the optical properties of the cell.

The characteristic of the light (spatial and spectral characteristic of the light beam) have an impact on the performance of the optical measurement. An optical measure sensitive to absorption effects and cell refractive index is an extinction measurement (term generally used in optical physics).

![Fig.1: example of a cell in transverse section highlighting the complexity of its internal structure (pseudo-random spatial distribution of the heterogeneities of optically active material).]
Measurement using low coherence optical source

1. Properties of the cytochemical treatment

The cell treatment with cytochemical reagents, Eosinofix(1) or Leucodiff(2), allows to maintain practically the cells in their native form. Two important elements of the cellular identification are preserved: the volumetry and the nucleo-cytoplasmic ratio of each cell. This morphology being preserved, the identification is more accurate.

(1) Eosinofix is the reagent used on ABX Pentra 60/80/120 ranges
(2) Leucodiff is the reagent used on ABX Pentra DX/DF ranges

2. Measurement

In the HORIBA Medical analysers, the extinction measurement is made using low coherence optical source (extended source). The low coherence optical source is characterised by the different spectral and spatial components of the light beam. This measurement reveals the morphology of the different cells compartments on the one hand, and intra-cytoplasmic spectroscopic characteristics on the other hand.

The high numeric aperture of the light beam allows the observation of cells from different angles. This measurement is less sensitive to cell anisotropy, and its position or orientation in the beam (Fig.2), and gives information depending on the intrinsic characteristics of the cell. We can schematically talk about stereoscopic analysis (vision in perspective).

Extended source
In polychromatic light using a beam of low spatial coherence (divergent beam), the measurement is less sensitive to the position or orientation of the cell in the beam.

Laser source
In monochromatic light using high spatial coherence (parallel beam), the measurement is more sensitive to the position or orientation of the cell in the beam.

Fig.2: the measurement is slightly affected by the orientation or by the cell position.

Fig.3: the measurement result is affected by the orientation or the cell position.
Contrary to the HORIBA Medical technology, the laser beam (Light Amplification by Stimulated Emission of Radiation) is a monochromatic light generating a perfectly parallel beam and consequently allows a vision of the cell from one angle. The cell position or orientation within the light beam will give different results (Fig.3). The example of erythrocytes, which are perfectly anisotropic, show that the results would be completely dissimilar if they were not spherised.

a) Accuracy of the light beam

The principle used in the HORIBA Medical measurement analysers is based on the Köhler or Newton lighting principle. In these systems, the light beam is perfectly homogeneous at the measurement point (Fig.4).

The accuracy of the optical analysis is much more accurate than the one obtained by Gaussian light distribution provided by a laser. This system is also more robust and less subject to misalignment.

The laser optical source generates a light beam distribution having a Gaussian shape and consequently this process is not homogeneous at the measuring point. This involves important technological constraints, requires hydrofocalisation, and many adjustments. Using a laser optical source, the analysis is potentially more inaccurate (Fig.5).

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**Extended source**

![Extended source diagram](image1)

**Laser source**

![Laser source diagram](image2)

Fig.4: The light is homogeneous at measurement point.

Fig.5: The beam intensity is not homogeneous at measurement point.
b) Influence of the flow cell on accuracy

The cell flow is never perfectly linear. As a result the cells never cross the light beam at the same position. Extended source is more suitable for accurate optical measurement in comparison to a laser source as depicted in the following figure.

Extended source

Laser source

Whatever the crossing position at the measurement point, the signal is always characteristic of the cell.

If the cell is not centred on the measurement axis, the light response might be distorted.

Conclusion

Giving the complexity of the structure and morphology of the leukocytes, the identification and counting method combining cytochemistry and low coherent light measurement (extended source and low coherence illumination) proves to be more discriminating and more robust compared to laser methods.

The optical measurement done on Pentra analysers allow to provide more accurate information on the volume, nucleo-cytoplasmic ratio and internal structure of each cell, and this robustness is particularly useful for abnormalities identifications.
Definitions

**Mitochondrion**
In cell biology, a mitochondrion (plural mitochondria) is a membrane-enclosed organelle found in most eukaryotic cells. These organelles range from 0.5 to 10 micrometers (µm) in diameter. Mitochondria are sometimes described as "cellular power plants" because they generate most of the cell's supply of adenosine triphosphate (ATP), used as a source of the chemical energy.

http://en.wikipedia.org/wiki/Mitochondrion

**Cytochrome C**
Cytochrome c, or cyt c (horse heart:PDB 1HRC) is a small heme protein found loosely associated with the inner membrane of the mitochondrial. It belongs to thecytochrome C family of proteins. Cytochrome c is a highly soluble protein, unlike other cytochromes, with a solubility of about 100g/L and is an essential component of theelectron transport chain, where it carries one electron. It is capable of undergoing oxidation and reduction, but does not bind oxygen. It transfers electrons between Complexes III and IV. Cytochrome c is also an intermediate in apoptosis, a controlled form of cell death used to kill cells in the process of development or in response to infection or DNA damage.

http://en.wikipedia.org/wiki/Cytochrome_c

**Golgi apparatus**
The primary function of the Golgi apparatus is to process and package macromolecules, such as proteins and lipids, after their synthesis and before they make their way to their destination; it is particularly important in the processing of proteins for secretion. The Golgi apparatus forms a part of the cellular endomembrane system.


**Anisotropy**
Anisotropy is the property of being directionally dependent, as opposed to isotropy, which implies homogeneity in all directions. It can be defined as a difference, when measured along different axes, in a material's physical property (absorbance, refractive index, density, etc.) An example of anisotropy is the light coming through a polarizer.

http://en.wikipedia.org/wiki/Anisotropy

**Köhler illumination**
Köhler illumination is a method of specimen illumination used in transmitted- or reflected-light microscopy. It was designed by August Köhler in 1893, and overcame the limitations of previous techniques of sample illumination (ie: critical illumination). Prior to the advent of Köhler illumination, the filament of the bulb used to illuminate the sample could be visible in the sample plane. This created what is known as a filament image. Various techniques were used to remove the filament image, for example lowering the power of the light source, using an opal bulb, or placing an opal glass diffuser in front of the light source. However, all these techniques, although effective in reducing the filament image to a certain degree, had the effect of reducing the quality and uniformity of light reaching the sample. Reducing the power of the light source and introducing an opal bulb both caused a reduction in the spectrum of incident light. For transmitted-light microscopy wide spectrum white light is desirable in order to realize the maximum amount of contrast. Further, adding an opal glass diffuser will cause the light reaching the sample to be uneven. Uniformity of light is essential to avoid shadows, glare, and inadequate contrast when taking photomicrographs. Köhler illumination overcomes these limitations.

http://en.wikipedia.org/wiki/K%C3%B6hler_illumination
**Gaussian beam**

In optics, a Gaussian beam is a beam of electromagnetic radiation whose transverse electric field and intensity (irradiance) distributions are described by gaussian functions. Many lasers emit beams with a Gaussian profile, in which case the laser is said to be operating on the *fundamental transverse mode*, or “TEM$_{00}$ mode” of the laser’s optical resonator. When refracted by a lens, a Gaussian beam is transformed into another Gaussian beam (characterized by a different set of parameters), which explains why it is a convenient, widespread model in laser optics.

http://en.wikipedia.org/wiki/Gaussian_beam

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Barbara J.Bain Professor of Diagnostic Haematology St Mary’s Hospital Campus of Imperial College Faculty of Medicine, London and Honorary Consultant Haematologist, St Mary’s Hospital London.

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Alfons Hoestra University of Amsterdam, The Netherlands.
Valeri Mal’tsev Institute of Chemical Kinetics and Combustion, Novosibirsk, Russia.
Gorden Videen Army Research Laboratory, Adelphi, MD, USA, University of Amsterdam, The Netherlands.

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Riccardo Pratesi Dipartimento di Fisica, Università di Firenze, Sesto Fiorentino (Fi), Italia.


Philippe Nerin, Research Director Horiba Medical, PhD
Didier Lefèvre, R&D Department