

SEED HAEMATOLOGY



Challenges in monocyte counting

Introduction

Monocytes are a type of leukocytes (white blood cells). They perform an important part of the immune defence of the organism. One of their functions is to destroy bacteria by phagocytosis and that is the reason why vacuoles are often seen in the cytoplasm of these cells.

Monocytes are produced in the bone marrow from precursor cells called monoblasts, which themselves are derived from haematopoietic stem cells. Monocytes circulate in the bloodstream for about one to three days and then typically enter tissue throughout the body. In tissue, monocytes mature into different types of macrophages in different anatomical locations. Macrophages are responsible for protecting tissue from foreign substances (microbes, cancer cells, cellular debris) in a process called phagocytosis.

Morphologically, monocytes are the largest leukocytes, with sizes varying between 10 and 20 μm . They usually have a large nucleus and a moderate amount of cytoplasm, which is grey-blue after May-Grünwald Giemsa staining and may contain fine, evenly distributed granules and sometimes vacuoles (Figs. 1 and 2). The reference ranges for monocytes are 5.2–15.2% or $0.29 - 0.95 \times 10^9/\text{L}$ for men and 4.2–11.8% or $0.25 - 0.84 \times 10^9/\text{L}$ for women [1]. Values outside this range do not necessarily indicate disease. It is recommended to always examine reference ranges for suitability in a given patient population according to the method recommended by the International Federation of Clinical Chemistry and Laboratory Medicine [2].

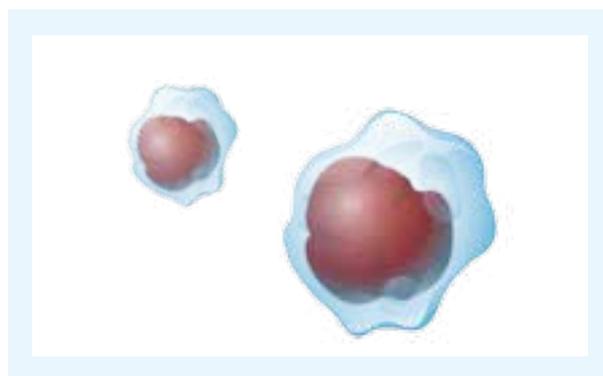


Fig. 1 Illustrations of monocytes

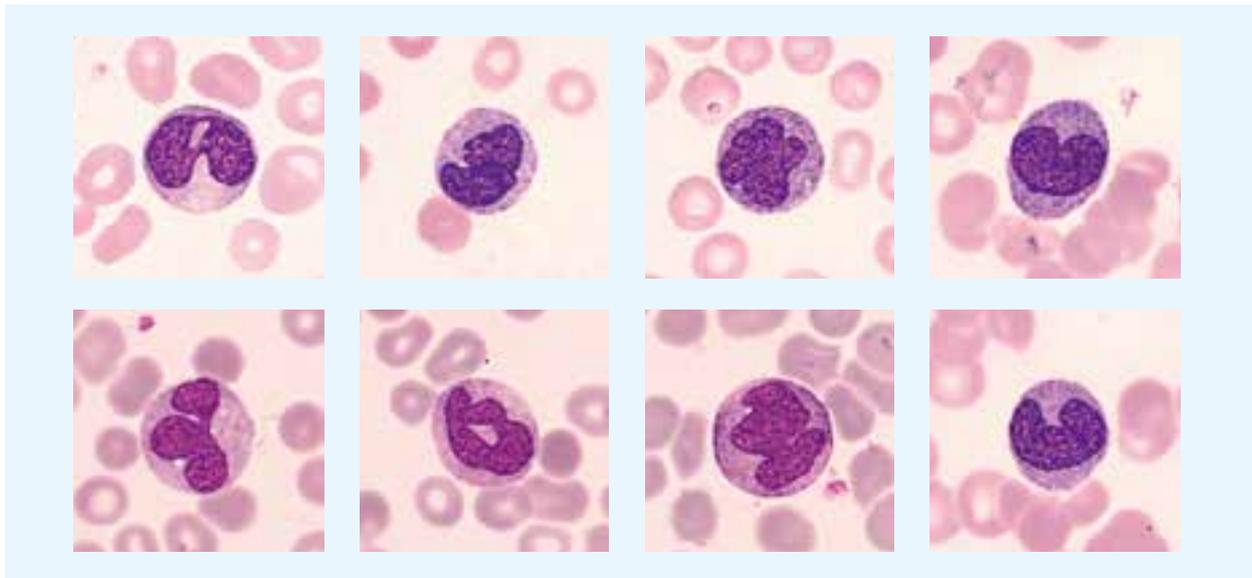


Fig. 2 Microscopy images of monocytes in a stained peripheral blood film

Function of the monocytes

Monocytes, including the macrophage and dendritic cell types that they differentiate into, have three main functions in the immune system.

a) Phagocytosis

Microbes and foreign particles are taken up by the cell, digested and that way destroyed.

b) Antigen presentation

Microbial protein fragments that remain after digestion can serve as antigens. They are placed on the monocyte's surface, which activates T lymphocytes that produce a specific immune response against the presented antigen.

c) Cytokine production

Many factors produced by other cells can regulate the chemotaxis and other functions of monocytes. For instance, some microbes can directly stimulate monocytes to produce pro-inflammatory and anti-inflammatory cytokines and help during the immune response.

The monocyte count

The monocyte count forms part of a white blood cell (WBC) differential count and is expressed either as a percentage of monocytes relative to the total WBC count, or as an absolute count. Both values are useful for the diagnosis of the patient. Two abnormal situations can occur when looking at the number of monocytes: monocytosis and monocytopenia.

1) Monocytosis

An increase in the number of monocytes circulating in the blood. A wide range of different diseases may produce this state. Some of these are different infectious diseases (e.g. tuberculosis, leprosy and salmonellosis), blood-based and immune system causes (chronic neutropenia and myeloproliferative disorders), autoimmune diseases and vasculitis, leukaemias (i.e. chronic myelomonocytic leukaemia (CMML)), recovery phase of neutropenia or an acute infection.

2) Monocytopenia

A decrease in the number of monocytes circulating in the blood. Monocytopenia is rare as an isolated finding. The causes include acute infections, stress, treatment with glucocorticoids, aplastic anaemia, hairy cell leukaemia, acute myeloid leukaemia, treatment with myelotoxic drugs and genetic syndromes.

The challenge of counting monocytes

When comparing manual and automated WBC differential counts, the following questions keep cropping up with respect to monocyte numbers:

- Why are monocyte counts obtained from manual differentiation often lower than those from automated counts?
- Why are there different reference values for monocyte counts obtained from manual and automated methods?

The reasons for the different monocyte counts can be found in the methods used, in the statistical principle and also in the capability of monocytes to adhere to microscope slides.

Methods

Manual WBC differentiation

Manual WBC differentiation is done by means of a peripheral blood smear: a thin layer of blood is smeared on a microscope slide and then stained in such a way that it allows the various blood cells to be examined microscopically. The aim is to find a region called monolayer, where the cells are spaced far enough apart to be counted and differentiated. The monolayer is found in the 'feathered edge' created by the spreader slide as it spreads the blood onto the slide (Fig. 3). After staining, the monolayer is viewed under a microscope, the individual cells are examined and their morphology is characterised and recorded.

The quality of smears (stained according to the May-Grünwald-Giemsa or Wright methods) is affected by many known factors, such as grease-free carrier slides, the quality of the spreader slide, the smearing angle and the staining protocol. Moreover, monocytes are particularly adherent cells. The glass surface of the carrier slide and the edge of the spreader slide provide excellent adhesion surfaces for this WBC subpopulation. As a result, the monocytes tend to accumulate more around the edge of the smear [3]. This is why it is difficult to detect them in the monolayer that is usually examined, which increases the difficulty of obtaining a reliable count.



Fig. 3 Feathered edge of a Wright-Giemsa-stained peripheral blood smear

Automated cell count

With flow cytometry, cells are labelled fluorescently and then examined with a semiconductor laser. A flow cytometer counts and classifies cells by irradiating them with a monochromatic laser beam and analysing their forward scatter (FSC), side scatter (SSC) and side fluorescence (SFL) signals. The intensities of the two types of scattered light (FSC and SSC) reflect cell surface structure, particle shape and size, nucleus form, refractive index and reflectivity of the cells. In general, the FSC signal increases with growing cell size, and the SSC signal becomes stronger as the intra-

cellular structures become more complex. The intensity of the side fluorescence light mainly reflects the type and amount of nucleic acids and cell organelles inside the cell. The differentiation and counting of the WBC subpopulations is done by using this technique, and it also allows the detection of abnormal cells and immature cells (Fig. 4).

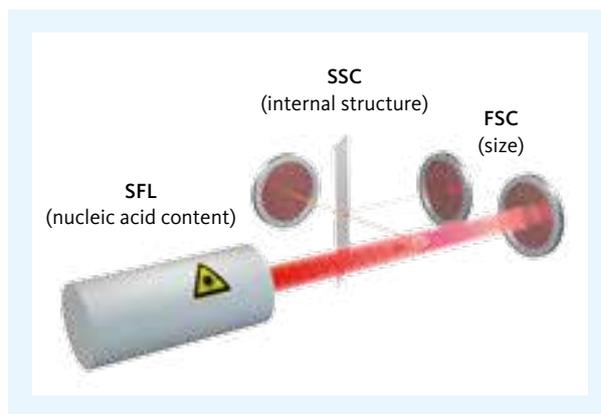


Fig. 4 Measurement principle of fluorescence flow cytometry

Technology of the XN analysers

The XN portfolio of haematology analysers consists of the XN-Series and XN-L Series. Four WBC populations (neutrophils, eosinophils, lymphocytes, monocytes) are examined by the WDF channel of the XN-Series (Fig. 5a) and five (the previous four plus basophils) in the XN-L Series (Fig. 5b).

In both series, the measurement principle is based on a unique reagent system consisting of a combination of a lysis reagent and a fluorescence marker, which are added to the blood sample. During this process, the first component, Lysercell WDF, causes the haemolysis and dissolution of red blood cells and platelets and perforates the cell membranes of white blood cells. What makes this reagent special is that the white cells remain largely intact during this process. The impact on cell morphology, its potential change and the permeabilization of the membrane depends on the individual characteristics of each type of white blood cell. These differences are distinguished by using side scattered light. These changes in the surface can be observed under the scanning electron microscope (SEM) (Fig. 6) and by means of other techniques [4]. Next, the fluorescence marker, Fluorocell WDF, penetrates the cells and labels the nucleic acids and cell organelles.

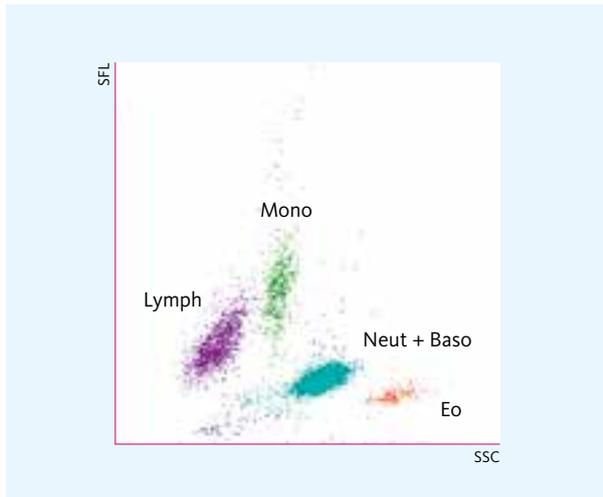


Fig. 5a WDF scattergram, XN-Series

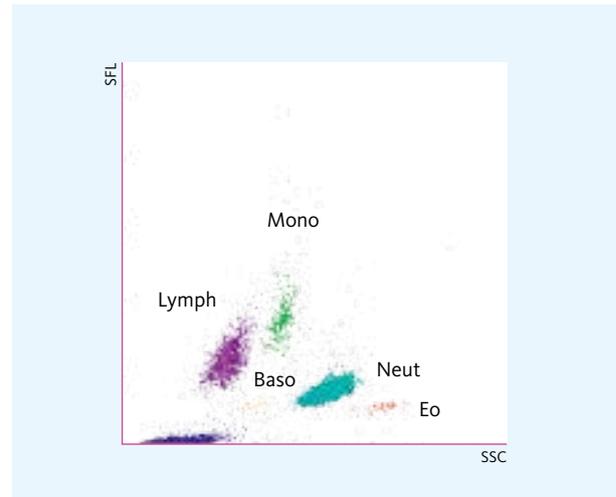


Fig. 5b WDF scattergram, XN-L Series

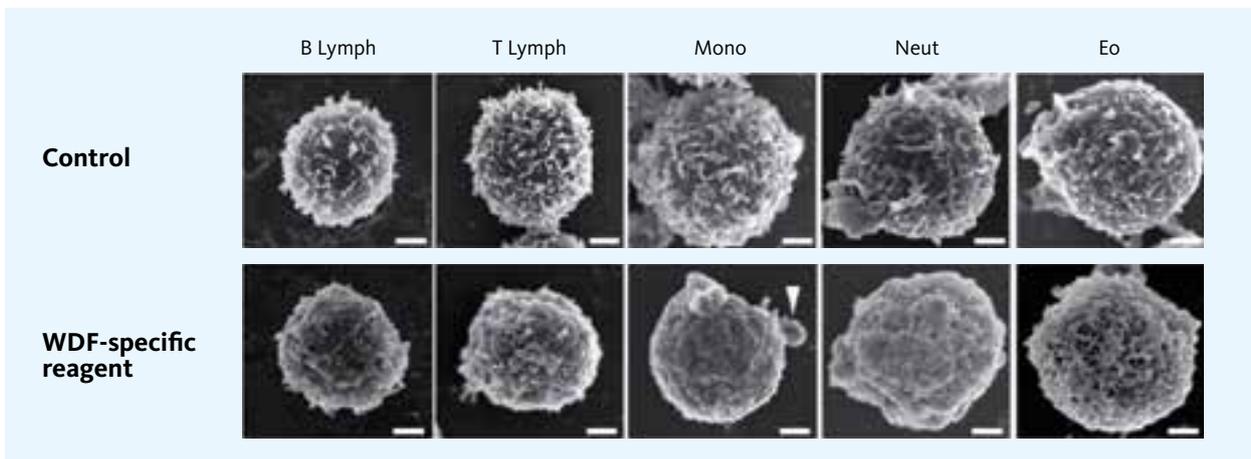


Fig. 6 SEM images of isolated subtypes of leukocytes before and after being treated with Lysercell WDF. Bar width = 1 µm.

Following the incubation period, the sample is analysed by means of flow cytometry, using the semiconductor laser and measuring the SFL, FSC and SSC signals. For additional cell analysis, these measured signals relating to each individual cell are recorded simultaneously and represented in scattergrams (Figs. 5a and 5b). The measurement provides excellent counting accuracy and flagging sensitivity due to special shape recognition analysis of each subpopulation and flexible gating. The adherence of monocytes is not important anymore, since we are not using glass slides in the counting procedure.

How to get the most accurate counts?

The effect of the number of counted cells on the reliability of the results is explained below by an example with an assumed WBC concentration of 8,000/µL and a monocyte fraction of 5%.

- **Manual differentiation:** Usually, 100 WBC are evaluated in routine laboratory tests when manual differentiation is performed (n = 100 cells).
- **Automated differentiation:** For the analysis of WBC, using XN-Series analysers as an example, the sample is diluted 1: 61 with reagents. The analysed volume is 58.2 µL and leads to the following cell count:

$$\frac{8,000 \text{ cells} / \mu\text{L}}{61 \text{ (dilution)}} \times 58.2 \mu\text{L (analysed volume)} = 7,633 \text{ WBC}$$

Therefore, if for example a WBC count of 8,000/µL is displayed, in fact 7,633 cells have been analysed by the XN-Series (n = 7,633).

The Rümke Table [5] permits a statistical prediction about the accuracy of counting parameters (see Table 1).

Table 1 95% confidence limits for the actual number of cells per 100 WBC. A = observed cells per 100 WBC.

A	Actual number of cells per 100 WBC				
	n = 100	n = 200	n = 500	n = 1,000	n = 10,000
0	0–3.6	0–1.8	0–0.7	0–0.4	0–0.1
1	0.0–5.4	0.1–3.6	0.3–2.3	0.5–1.8	0.8–1.3
5	1.6–11.3	2.4–9.0	3.3–7.3	3.7–6.5	4.5–5.5
10	4.9–17.6	6.2–15.0	7.5–13.0	8.2–12.0	9.4–10.7
15	8.6–23.5	10.4–20.7	12.0–18.4	12.8–17.4	14.3–15.8
20	12.7–29.2	14.7–26.2	16.6–23.8	17.6–22.6	19.2–20.8
30	21.2–40.0	23.7–36.9	26.0–34.2	27.2–32.9	29.1–31.0
40	30.3–50.3	33.2–47.1	35.7–44.4	36.9–43.1	39.0–41.0
50	39.8–60.2	42.9–57.1	45.5–54.5	46.9–53.1	49.0–51.0
70	60.0–78.8	63.1–76.3	65.8–74.0	67.1–72.8	69.0–70.9
80	70.8–87.3	73.8–85.3	76.2–83.4	77.4–82.4	79.2–80.8
90	82.4–95.1	85.0–93.8	87.0–92.5	88.0–91.8	89.3–90.6
100	96.4–100	98.2–100	99.3–100	99.6–100	99.9–100

If 5% monocytes are observed in a smear using n = 100 cells, this means that the true value lies between 1.6 and 11.3. There is a very wide underlying confidence interval, because the number of differentiated cells is very low. In comparison, the XN analysers differentiate thousands of cells from the same sample (see the example calculation above). With this number of differentiated cells, the monocyte result would actually vary at most between 3.7 and 6.5 cells. In statistical terms, therefore, a larger number of evaluated cells leads to a more accurate result.

Publications

Studies that compare the various methods with each other (manual differentiation, fluorescence flow cytometry and/or automated differentiation using X-Class or XN-Class instruments), show excellent results regarding the accuracy in the count of the automatic haematology analysers.

- Seo JY *et al.* (2015): ‘Performance evaluation of the new hematology analyzer Sysmex XN-Series’ [6].

The following Table (Table 2) taken from this study shows the correlation coefficients derived from the comparison of the WBC differential from the ‘Low WBC’ mode of the XN-Series with the automated (X-Class) and manual differential.

Table 2 Comparison of the leukocyte differential from the ‘Low WBC’ mode of the XN-Series with automated and manual differential performed on original samples

	Automated differential R ²	Manual differential R ²
Neutrophil %	0.98	0.87
Lymphocyte %	0.97	0.86
Monocyte %	0.92	0.84
Eosinophil %	0.84	0.67
Basophil %	0.10	0.00

- Briggs C *et al.* (2012): ‘Performance evaluation of the Sysmex haematology XN modular system’ [7].

The following Table (Table 3) shows the correlation between the complete blood count of XN-Series and the XE-2100; data are shown for WBC counts.

Table 3 Correlation statistics of complete blood count results of XN-Series compared to XE-2100

	R ² value	Slope	Intercept
WBC	0.99	1.07	- 0.76
Neutrophil	0.99	1.00	- 0.02
Lymphocyte	0.99	1.14	- 0.3
Monocyte	0.91	0.90	+ 0.09
Eosinophil	0.99	0.98	0.00
Basophil	0.76	1.15	+ 0.01
IG	0.94	1.14	+ 0.02

- Kawauchi S *et al.* (2014): ‘Comparison of the Leukocyte differentiation Scattergrams between the XN-Series and the XE-Series of Hematology Analyzers’ [3].

The paper explains the different effects of the WDF and DIFF reagents on leukocytes and why the WDF scattergram of the XN-Series shows a better separation of the different cell populations, especially between lymphocytes and monocytes.

In summary, the results of the cited publications demonstrate precise results of the XN’s automated differentiation in comparison with the X-Class and the manual method. This comparison between the methods, and also the Rümke table, permit the conclusion that automated differentiation should be the method of choice for determining cell numbers. However, in the case of a pathological result from automated differentiation with warning flags such as ‘Blasts/ Abn Lympho?’, assessment of cell morphology should be based on a blood smear. The analyser, although able to detect the presence of pathological cells and inform the laboratory, is not able to ‘see’ in detail the morphology of the cells in the same way as human eyes can, therefore a look at the cell images, e.g. through a microscope, is needed.

Nowadays, this microscopy can be facilitated considerably with the help of automated image analysis systems such as the Sysmex DI-60* (Fig. 7).

Automated microscopic differentiation

The Sysmex DI-60 is an automated cell-locating image analysis system, which can be fully integrated within the haematology workflow.

The device itself consists of motorised objectives, a high-quality digital camera and a computer system that collects and pre-classifies the cells from the stained blood smears. It automatically locates the cells on the slide and captures an image, after which it analyses and pre-classifies these cells using advanced image processing. The analyser is equipped with artificial neural network software, which is programmed to recognize blood cell characteristics. The software checks about 300 features of each cell and then compares them with the neural network database.

The number of white blood cells that are analysed is user-definable. After the DI-60 has acquired and pre-classified the cells, the operator verifies and, if needed, modifies the suggested classification of each cell. The operator may also introduce additional observations and comments, if needed. Tabé Y *et al.* [8] found a very good accuracy and agreement between results obtained from the DI-60 and manual microscopy. The overall analytical accuracy of the pre-classification of WBC by the DI-60, including pathological WBC populations, was 88.4%. In addition, blasts were correctly classified with 95% sensitivity and 99% specificity.



Fig. 7 Digital imaging analysis system Sysmex DI-60*

*DI-60 is manufactured by CellaVision AB - www.cellavision.com

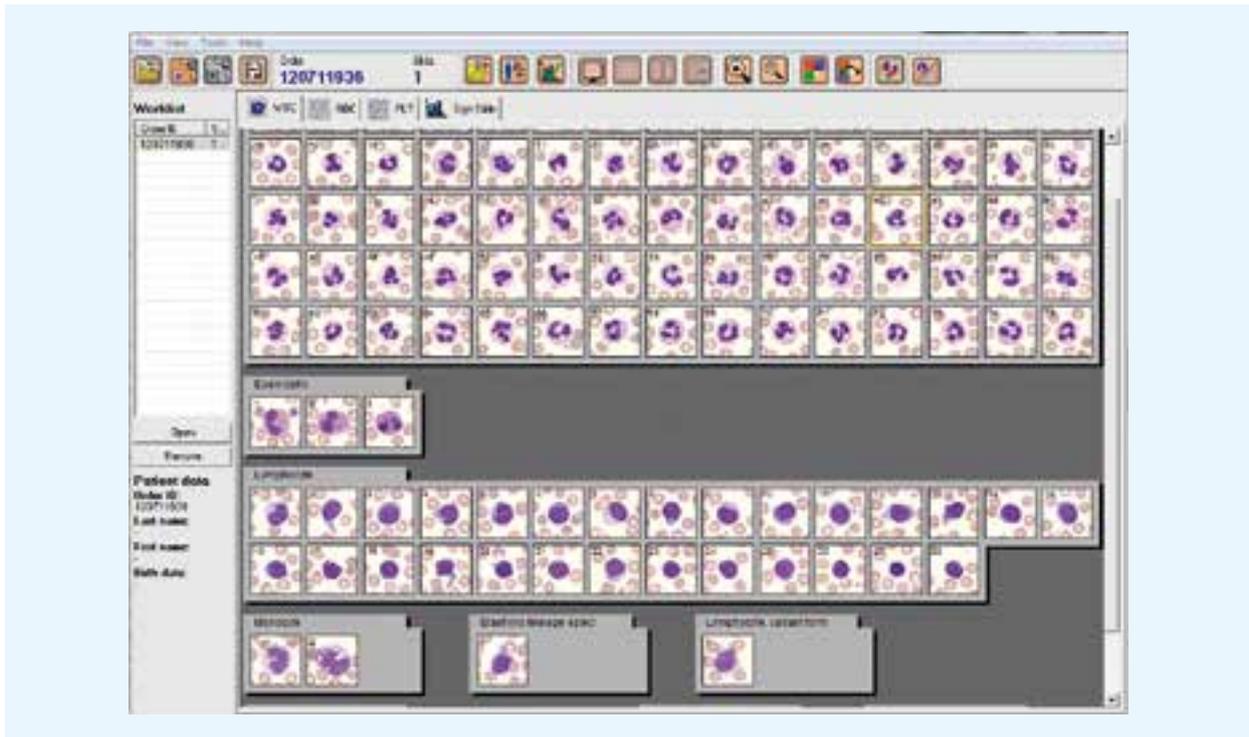


Fig. 8 Cell images produced by DI-60* presented in the result display

The DI-60* is able to pre-classify different cell types as follows:

- Pre-classification of WBC: segmented and band neutrophils, eosinophils, basophils, monocytes, lymphocytes, atypical lymphocytes, plasma cells, promyelocytes, myelocytes, metamyelocytes, blasts
- Pre-classification into non-WBC categories: smudge, artefacts, giant platelets, platelet clumps and nucleated red blood cells

The device also presents an overview that can be used to characterise the red blood cells' morphology and to estimate the platelet concentration.

The results and the cell images from this automated analysis of the smear are displayed on the screen (Fig. 8) and evaluated by the examiner and subsequently transferred to the laboratory information system (LIS).

Conclusions

Monocytes, despite being the largest of the WBC, can be difficult to detect in the manual smear due to their strong adhesiveness to microscope slides. Nevertheless, a reliable count is required in order to know how the situation of the patient is, since having for instance a monocytosis might be indicative of various diseases or the state of a disease.

The fluorescence flow cytometry technology allows the laboratory to obtain accurate monocyte counts. The automated haematology analysers that use this technology also count a higher number of cells, making the count statistically more precise. Moreover, if the morphology of the monocytes needs to be checked visually, an integrated, automated solution of digital imaging analysers allows their visualisation and even the pre-classification of the different cells, reducing the turnaround time in the laboratory and increasing the standardisation and accuracy of the results.

As outlined in this article, manual and automated differential cell counts are systematically different. For correct result interpretation, different reference ranges for manual and automated differentials, particularly for monocytes, should be in place in every laboratory.

*DI-60 is manufactured by CellaVision AB - www.cellavision.com

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