Development, History, and Future of Automated Cell Counters

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KEYWORDS

• Automated cell counter • Hematology • Blood • Hemoglobin
• Electrical impedance counting • Flow cytometry • Point of Care • Light scattering

KEY POINTS

- The invention of the microscope allowed the differentiation and counting of blood cells.
- Automated blood cell counters have greatly improved the speed and accuracy of cellular blood analysis, using optical light scattering or changes in electrical current induced by blood cells flowing through an electrically charged small opening.
- Novel methods under development include the addition of new parameters to the complete blood count and white cell differential and the development of smaller, portable instrumentation and of devices that allow in-vivo analysis of blood cells.

"…for the blood is the life"
—Deuteronomy 12:23

INTRODUCTION

Even in antiquity, blood was recognized as a singular bodily fluid that was the essence of life, possessing mysterious properties that provided sustenance for human survival. The unraveling of those mysterious properties only became possible once blood could first be characterized according to the appearance and number of its particulate components. Those critical steps comprised, in the first instance, the development of microscopy, enabling the visualization of component blood cells, and subsequently advances made possible through the techniques to measure physical properties of the formed elements of the blood as well as the electronic means of capturing this information.

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Abbreviations

CBC	Complete blood count
FOV	Field of view
RBC	Red blood cell
SRS	Stimulated Raman scattering
WBC	White blood cell

The development of the optical microscope made it possible to visualize individual human and plant cells, as well as bacteria. Even the simplest microscope developed by van Leeuwenhoek more than 300 years ago allowed the observation that blood is composed of small red globules, and their size was eventually determined. Progressive developments in optical microscopy followed during the next 2 centuries, such as the addition of an eyepiece to form a compound microscope and improved optics, including objective lenses comprising several lenses to correct for distortions. The application of dyes by Paul Ehrlich in the late 1870s allowed, for the first time, differentiation between different white cell types.[1,2] By then, it was evident that the number of blood cells changes in many diseases and it therefore became clear that it was important to more accurately quantify cell number by doing a blood count. Laborious manual measurements were introduced in which measured volumes of blood were placed on a calibrated slide chamber and cells were counted one by one, the total number being calculated from the known volume and geometry of the chamber. Although still in practice today because of its simplicity, manual counting of cells is prone to large errors and is both time consuming and labor intensive. Automated methods for counting blood cells became a necessity, and engineers began to work together with hematologists to find solutions to this problem. Over the ensuing decades, flow-based cytometers using light, impedance measurements, or both, were developed. These devices provided a large number of parameters related to enumerating and identifying blood elements, including erythrocytes (red blood cells [RBCs]), leukocytes (white blood cells [WBCs]), and thrombocytes (platelets), and differentiating the various leukocyte subtypes. In addition, with the recognition that qualitative differences in the appearance of red cells relating to their size, shape, and degree of hemoglobinization connoted certain types of anemia, the ability to simultaneously and independently measure hemoglobin concentration and hematocrit (or packed cell volume), together with the red cell count, provided Maxwell Wintrobe[3–5] with the tools to promulgate a set of red cell indices that included the mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration. This development allowed the morphologic classification of anemias into subtypes according to whether microcytic, normocytic, or macrocytic; and hypochromic, normochromic, or even hyperchromic according to degree of hemoglobinization. Later, it also became possible to quantify the size distribution of red cell populations, expressed as the red blood cell distribution width, thus enabling a further distinction into homogeneous and heterogeneous categories within each morphologic subtype.[6]

UTILITY OF A BLOOD COUNT

Of all the tissues in the body, the blood in circulation is the easiest and least invasive to sample. As such, a blood count represents a biopsy obtained through a simple venipuncture. A complete blood count (CBC) is therefore one of the most commonly used clinical laboratory tests. It provides a rapid and cost-effective assessment of various modalities of a patient's state of health as well as important clues to the

possible presence of disease. A CBC provides information about the cellular components of the blood: RBCs, WBCs, and platelets. CBC data include not only information about the numbers of the 3 basic cell types but also provide information about the size, shape, and degree of hemoglobinization of the RBCs as well as the morphologically identifiable types of WBCs; the so-called WBC differential. The CBC provides clinicians with important information relevant to a patient's state of health and possible type of underlying disease; these data, along with clinical and other laboratory data, are often critical in constructing a differential diagnosis for a patient as well as for monitoring the progression of a disease and its responsiveness to treatment. At its most rudimentary level, the CBC gives information about low RBC levels (anemia), high RBC levels (erythrocytosis), low WBC levels (leukopenia), high WBC levels (leukocytosis), low platelet levels (thrombocytopenia), and high platelet levels (thrombocytosis). In addition, data on low and high absolute numbers of the different leukocyte types provide a wealth of information regarding the likely type and cause of underlying disease, whether infectious, inflammatory, neoplastic, or other. The blood cell count can therefore be an important first indicator of disease for many illnesses and is therefore a pivotal starting point in forming a clinical diagnosis, screening for changes in patient health, and for monitoring of disease progression or treatment. For example, in leukocyte disorders, the number of leukocytes reported and leukocyte differential can assist in assessing infections as well as in evaluating for hematologic malignancies (leukemias). Given this wealth of information that comes from what is essentially a single laboratory test, the move toward automation of the CBC was inevitable once the technical capabilities related to measurement, recording, reporting, and rapidity of throughput became available. Moreover, continued improvements in the various components of these integral steps of automated blood counting have led to progressive advances in both the quality and quantity of information that is obtained from a CBC. Modern blood counting instruments are able to closely simulate the qualitative information obtained through conventional microscopy with quantitative measurements of the numbers, dimensions, and properties of the cellular components of the blood, providing a composite multiparameter assessment of the state of the tissue that is the circulating blood.

HISTORY AND CURRENT METHODS

Counting of blood cells was one of the first quantitative methods for blood testing, and for some time has been the most widely used of tests in clinical settings. Initial measurements were based on careful wet sample preparation on a slide chamber, visualization, and manual counting with the aid of an optical microscope. Later techniques involved flow methods coupled with either impedance measurements or light scattering/fluorescence techniques to automatically enumerate cells one by one. There has been a more recent resurgence of microscopy-based techniques as a result of groundbreaking advances in imaging and image processing tools for automated cell counting (**Table 1**).

Manual Counting Methods

The first blood count is credited to Karl Vierordt[7,8] at the University of Tübingen, who published his research on the topic in a series of articles in 1852. His method involved drawing blood into a capillary tube and spreading a known volume onto a slide, followed by microscopic analysis. During the following years, incremental improvements of this method followed, including better specimen preparation and refinements in the counting chamber (including the use of elliptically shaped capillary tubes by Malassez[9]

Table 1
Brief history of early milestones in blood counting methods leading to automation

Discoverer, Year	Methodology
Vierordt,[7,8] 1852	Microscope
Oliver,[11] 1896	Light scattering and absorption measured by eye
Marcandier et al,[12] 1928	Light scattering and absorption measured with a photodetector
Coulter,[17] 1953	Impedance measurement
Fulwyler,[13] 1965	Impedance measurement and electrostatic cell sorting
Dittrich & Göehde,[14] 1968	Fluorescence-based flow cytometry
Julius et al,[15] 1972	Fluorescence-activated cell sorting
George & Groner,[16] 1973	Light scattering in flow cytometry

Data from Refs.[7,8,11–17]

as well as the addition of etched perpendicular grids in the chamber for easier enumeration of cells). These improvements resulted in counts becoming more accurate but still tedious and slow to perform. A description of subsequent modifications was reviewed and published by Gray.[10] Because of its simplicity, this method is still used in low-resource laboratories around the world.

Automated Counting Methods

Toward the end of the nineteenth century, the prototypes of automated blood counters were first developed, with rapid advances made throughout the twentieth century. These instruments performed measurements using either the light scattered and absorbed by blood cells, or changes in the electrical current induced by blood cells flowing through a small, electrically charged opening.

Methods based on optical measurements

In 1896 a new method for blood cell counting was proposed by George Oliver,[11] based on the measurement (by eye) of light loss caused by scattering and absorption in a test tube filled with diluted blood. This method could be considered the forerunner of eventually the automated blood count, and it provided an RBC count without the need for manual counting of individual cells. However, the inability to accurately quantify the light loss as well as problems related to variations in cell size, shape, or hemoglobin content prevented this method from becoming widely used. Nevertheless, during the 1920s, novel developments in photodetectors (made possible by the discovery of the photoelectric effect and the invention of the photodiode) revived interest in using light scattering and absorption for blood cell counting. Marcandier and colleagues[12] showed in 1928 that, by measuring the light transmitted through a solution of diluted blood, a blood count could be derived after properly calibrating the photometer. However, as in the previously described method by Oliver,[11] the count was not accurate if there were variations in cell size, shape, or hemoglobin content. To address these issues, a flow device that isolated cells in small liquid droplets was first developed by Fulwyler[13] in 1965 for the purpose of separating cells based on their size. In 1968, Dittrich and Göhde[14] coupled a laser beam to this flow device and successfully demonstrated fluorescence-based cytometry. Because of the high speed of the flow, in which thousands of cells pass through the laser beam per second, high-throughput cytometry became a reality. This development may be seen as a watershed in the transition from manual to automated blood counting. In the early 1970s, Julius and

colleagues[15] demonstrated fluorescence-based cell sorting, which he called fluorescence-activated cell sorting. The use of fluorescence labels enabled the identification, in addition to the separation, of many types of cells and therefore added a new dimension to the blood count. Up to 11 different fluorophores could be used simultaneously, and, through the use of deconvolution algorithms that allow separation of overlapping spectra, the number could be even larger. Light scattering was also implemented in flow cytometers by George and Groner[16] in 1973, allowing the discrimination of different types of WBCs based on their size/scattering properties. In addition, using light scattering measurements at 2 different angles, they were able to obtain measurements of red cell size (low-angle light scatter) and hemoglobin content (higher-angle scatter) after the cells were isovolumetrically sphered.[16]

Methods based on electrical measurements

During the late 1940s, Wallace Coulter was working on a method to assess particulates in paint. Spurred by his experiences in the Navy and witnessing the effects of the atomic bombs dropped at the end of the Second World War, he looked for ways to apply his technique to blood cell counting. In a discovery that came to be known as the Coulter effect, he started to develop a simplified blood cell analysis tool that could be used for rapid screening of blood from large numbers of people. The Coulter effect is based on the phenomena that cells are poor electrical conductors compared with a saline solution and individual particles passing thorough an orifice at the same time as an electric current produce a change (decrease) in the current caused by the particle-induced increase in electrical impedance. Furthermore, the change in impedance (and therefore in the measured current) is proportional to the volume of the particle, which is the foundation for size-based counting and separation. This simple but elegant concept laid the groundwork for subsequent development of modern automated blood cell counters. After Coulter's[17] first patent on this topic in 1953, many subsequent developments followed, including the popular flow cell format, in which the cells are directed through a flow channel or chamber rather than being passed through an aperture. A significant modification to the system was made in 1965 by Fulwyler,[13] who used a Coulter counter to measure cell volume and then partitioned the cells into droplets of the medium. Because the charge of the droplets is related to the cell volume, an electrostatic field applied to the droplets can deflect them into a collection vessel. Cells can thus be sorted and later reused. Like the fluorescence-based approach pioneered by Julius and colleagues,[15] this approach also played an important part in laying the foundation for automated cell-sorting techniques.

The addition of RNA-binding dyes such as acridine orange enabled automated discrimination and enumeration of reticulocytes as a component of the blood count and substantially improved the accuracy, speed, and precision of reticulocyte counting. Another milestone was the addition of precise size discriminators in electrical and light scattering instruments, which revolutionized platelet counting by enabling automation of this previously laborious measurement.

NOVEL METHODS UNDER DEVELOPMENT

Despite the significant improvements in the technologies described earlier, there are still several limitations to current automated blood counters, such as complex and time-consuming sample preparation, limited number of parameters that can be detected, the need for a blood draw, and lack of capability for continuous monitoring. With rapid technological advancements in optics, electronics, and microfluidics, further improvements are now being directed toward (1) the development of novel

contrast agents and methods that will allow higher throughput and multiplexing capabilities, as well as adding new parameters to the measurements such as the analysis of subpopulations of blood cells or counting of smaller particles in blood; (2) the development of smaller, portable, automated blood counters; and (3) the development of in-vivo blood counting techniques.

New Contrast Agents and Methods

Current flow-based blood counters can detect up to approximately 13 parameters simultaneously (11 color and 2 scattered light [side and forward scatter])[18] in a high-speed laminar flow (up to 20 m/s), leading to the discrimination of phenotypic subpopulations of cells and a throughput of up to 100,000 cells per second. However, the heterogeneity and complexity of the immune system as reflected in lymphocyte subsets that have clinical diagnostic relevance may require even higher speed and multiplexing capabilities.

This improvement can be achieved by adding new contrast agents, such as quantum dots or rare earth metals that have narrower emission bands, or through the use of intrinsic markers that are easier to multiplex, such as those based on Raman scattering.[19] The main disadvantage of the Raman-based technique is the significantly reduced speed of measurement caused by the low Raman intensity compared with fluorescence or light scattering. To address this issue, molecular markers that can be used in combination with surface enhanced Raman spectroscopy and are capable of multiparameter blood analysis have been developed.[20] Other possibilities include the use of techniques that can provide additional contrast, such as those based on photothermal and photoacoustic measurements. However, increasing the multidimensionality of the information requires novel computational tools to help analyze these complex datasets and reveal new information, such as the identification of diverse populations of cells. Shekhar and colleagues[21] give an example of a tool that enables automated classification of nearly 40 different proteins to recover the large diversity of $CD8^+$ T cells. In addition, with the recent interest in cellular exosomes, the ability of flow cytometers to measure particles significantly smaller than 1 μm is currently being explored.

Because thousands of blood counts are performed each day in conventional large clinical laboratories, the development and improvement of machine-aided flagging algorithms is also a necessity, because they help reduce the number of false-positives and false-negatives by triggering morphologic review by an expert.

The Need for Portability

Current automated blood counting systems are large devices, the use of which is generally restricted to centralized laboratories. However, the need for delivery of improved but less expensive health care has posed newer challenges to scientists and engineers for the development of automated blood counters that are portable and can be used, for example, in doctor's offices, in disaster areas, in low-resource or remote areas, for monitoring astronauts in space, or even for home testing. The many developments over the past 20 years in microfluidics, optics, electronics, computers, and integration and miniaturization of devices based on such principles are bringing that goal ever closer. Sample preparation has been simplified by the use of microfluidic devices that require much smaller volumes of blood to perform a blood count. Although miniaturized optical components allow the development of smaller devices, faster electronic detectors help increase the throughput of such measurements. In addition, the analysis of recorded data is now aided by computers that

can help improve the accuracy of the counts by using better statistical analysis or pattern recognition algorithms.

Miniaturization of flow-based cytometers

Several research groups recently reported the development of miniature flow devices. Such examples include on-chip impedance spectroscopy for a WBC differential count that has a 95% correlation against a commercial flow-based optical/Coulter counter,[22] or fluorescence-based measurements within a sheathless microflow device for a 4-part leukocyte differential count.[23]

The resurgence of image-based blood counters

Before the development of flow-based techniques, blood counting was performed by hand, with technicians examining cells under a microscope using a hemocytometer. The procedure was extremely laborious and it often required hours to generate a blood count. Despite this, image-based counting has some advantages, such as easier sample preparation and handling, and the ability to provide morphologic information that can be useful for the classification of immature and abnormal cells. In addition, imaging systems can be significantly smaller, more portable, and more robust than flow systems. For that reason, there has been a revival of image-based counters, particularly spurred by the development of high-quality, inexpensive camera sensors and of complex, robust image analysis algorithms. Ceelie and colleagues[24] recently examined the performance of 2 state-of-the-art automated image-based instruments and concluded that their accuracy in providing morphologic classification of RBCs and WBCs depends on the type of pathologic changes in the blood sample. One main drawback of image-based blood counters is the limited field of view (FOV), which depends on the magnification and is usually in the range of hundreds of micrometers. For accurate and fast measurements of WBCs and rare cells, the examination of larger areas is needed. Although scanning and tiling together multiple images is possible, a significant advance has been made by the development of a lens-free, in-line holographic imaging technique that uses partially coherent light to record the shadow of cells on an imaging sensor. Cell information can be extracted through the recovery of the phase information of each cell for very large FOVs, limited only by the size of the imaging chip. RBC, WBC (with granulocyte, monocyte, and lymphocyte differential), and hemoglobin concentrations could be measured in this way.[25] More recently, an automated image-based blood counting method that includes RBCs, WBCs with 3-part differential, and platelets has been reported. This method uses a simple sample preparation (that includes WBC and platelet staining) and automated image analysis for performing a blood count on very small volumes of blood and can be adapted to portable devices.[26]

In-vivo Automated Blood Count

Despite the advantages of automated ex-vivo blood counting, the ability to perform an automated in-vivo blood count is appealing because of the potential for noninvasive and near-continuous measurements. Other advantages include that no sample preparation is needed and sampling of larger volumes of blood is possible such that the detection of rare abnormal cells might become feasible.

The blood cells naturally flow through blood vessels, and therefore an adaptation of flow devices first comes to mind. However, there are many challenges that prevent a simple adaptation of the ex-vivo technology to in-vivo measurements. First, the flow is significantly slower ($\sim$0.002 m/s in microvessels and $\sim$0.2 m/s in large vessels,

compared with ~10 m/s in flow cytometers) in humans, which would make the measurements significantly slower. Second, poor in-vivo optical conditions require the development of novel contrast mechanisms. Third, the speed of cells is constantly changing, which makes quantitative measurements difficult. An excellent review of in-vivo blood measurements has been published by Tuchin and colleagues[27] and it describes recent efforts to apply optical (absorption, fluorescence, elastic scattering, inelastic scattering [Raman]) as well as ultrasonographic, photothermal, and photoacoustic methods for this purpose. Other potential contrast methods are those that allow deeper penetration of light in the tissue. Examples include optical coherence tomography, nonlinear microscopies such as second harmonic generation, multiphoton fluorescence excitation microscopy, coherent anti-Stokes Raman scattering, and stimulated Raman scattering (SRS). As an example, SRS has been used for in-vivo label-free visualization of RBCs flowing through a capillary.[28]

FUTURE

The difficulty in predicting what the future holds for blood cell counting is best exemplified by considering how difficult it would have been to predict the current situation 50 years ago. However, there will be the dichotomy of building better blood counters for improved diagnostics versus smaller, more portable, or even wearable and implantable in-vivo devices that can provide certain parameters that may be useful as early indicators for changes in disease status and more elaborate tests.

Better, more accurate, and more comprehensive instruments will include more parameters in the blood count, such as the detection of rare-event blood cells, microvesicles, and exosomes, or subpopulations of cells with particular phenotypes that may be of significant clinical value. Measurement of dynamic functional changes in circulating cells is another possibility. Being able to provide multiplexed chemical information will also be important additions to future blood counting devices. Higher throughputs will also likely be the focus of future instruments, and could be achieved by developing new multimodal methodologies based on existing technologies or through the application of newer contrast technologies.

Smaller, portable devices that could do a blood count at the point of care and devices designed for in-vivo measurements will benefit from the use of high-resolution cameras and high-speed transmittance digital microscopy. In this way, cells can be examined under native conditions within the circulation through the use of photoacoustic detection, opening up new possibilities for assessment of cell-cell interactions, detection of circulating tumor cells, in-vivo cell deformability in diseases like sickle cell anemia and other intrinsic cell membrane disorders, and visualization of platelets during thrombus formation.[27]

Ten or more years into the future, automated blood counting instruments are likely to be significantly different from what they are today. Whether any of the directions predicted in this article will come into being is unknown at this time. The opportunity to contemplate this question is one that scientists are free to engage in. It is from such exercises that past developments stemmed and are likely to arise in the future.

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