From Manual Microscopy to Automated Cell Counters
for First Line Screening of Body Fluids
"But not without a special *body fluid* mode"

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Body Fluids

Cerebrospinal fluid and Synovial fluid

Body fluids (BFs) can be found in various cavities of the body under normal conditions (i.e., cerebrospinal fluid and synovial fluid), and others during pathological conditions (i.e., serous fluids). Cerebrospinal fluid (CSF) is mainly produced (80%) by the choroid plexuses, circulates through the ventricular system, subarachnoid space and down the spinal cord (1). In adults, CSF production rate is about 500 ml/day, with approximately 150 ml CSF in the central nervous system (CNS) at any given time (2). CSF is crystal clear and colorless, and serves a variety of purposes including protection of the brain from physical trauma, and the removal of waste products. Adult CSF contains no red blood cells (RBCs), and very few ($<5\times10^6$/L) white blood cells (WBCs). Roughly 70% of the WBCs are lymphocytes and 30% monocytes (3). The analysis of CSF is a key diagnostic element in the diagnosis of a variety of diseases including inflammatory conditions and infectious or non-infectious, amongst others, involving the CNS. Synovial fluid, also called joint fluid, is an ultra-filtrate of plasma combined with hyaluronic acid, found in synovial joints (i.e., knee and hip). Its main functions is to transport nutrients to the articular cartilage, and to lubricate the joints. Normal synovial fluid is highly viscous. It has a light yellow color, and it contains less than 200×10^6/L WBCs in a distribution of roughly 70% monocytes, <25% lymphocytes, and <25% neutrophils (4, 5). The number of RBCs varies, and an increase in their number may result from a traumatic tap or hemorrhage (1). The purpose of synovial fluid analysis is to detect arthritis and to place a fluid into one of several categories, in the diagnosis of joint diseases.

Serous fluids

Serous cavities (pleural, peritoneal) normally contain small amounts of fluid, formed by the filtration of

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plasma that acts as a lubricant to the parietal (cavity wall) and visceral (organ within the cavity) membrane surfaces. An accumulation of fluid in the serous cavities is called an effusion, and based on the underlying pathophysiology, they are classified into transudates or exudates. Transudates are usually the result of a systemic non-inflammatory disease such as congestive heart failure and hypertension, while exudates are associated with disorders such as inflammation, infection and malignancies, involving the organ. Peritoneal effusion (ascites) is defined as an abnormal accumulation of fluid in the peritoneal space. The main cause of ascites is due to liver cirrhosis (80%), followed by cancer (10%), congestive heart failure (3%), tuberculosis (2%) or other causes (6). Ascites fluids normally contain $<300\times10^6$ WBC/L with <25% neutrophils (7).

Pleural effusions, may result from congestive heart failure, bacterial pneumonia, neoplastic diseases or cirrhosis amongst others (8). The majority of cells found in normal pleural fluids are macrophages (75%). Other cells include lymphocytes (25%), while

neutrophils and eosinophils account for less than 2% each (9). Continuous ambulatory peritoneal dialysis (CAPD) fluid is not a biological body fluid, and is used as an alternative to hemodialysis to treat patients with end-stage renal disease. A catheter is inserted into the peritoneal cavity, where dialysate fluid fills the cavity and "dwells" for about 4-6 hours, while gradually removing waste products and extra fluid from the bloodstream. Fluid is then drained out, and replaced with fresh fluid. This procedure is repeated about 4 times a day. CAPD fluid normally contains $<50\times10^6$ WBC/L with a majority (80%) of cells being mononuclear cells (MN) (10).

White Blood Cells (WBCs)

Activation of the immune system is a coordinated effort, and may be detected by an increased number of markers including WBCs. WBCs are an important part of the body's defense against microbial invaders. They attack and engulf infected or dead cells, and they play an immediate and delayed role with their response. Neutrophils (10 to 15 μm) are primarily involved in the first line defense against infection/inflammation, particularly bacterial infection; eosinophils (10 to 14 μm) are mostly encountered in anti-parasitic and allergic reactions; lymphocytes (8 to 10 μm) are involved in recognizing foreign particles such as viruses and antigens, and monocytes (15 to 20 μm) play multiple roles in immune function including their transformation into highly phagocytic macrophages (Figure 1). Neutrophils together with eosinophils and basophils form the polymorphonuclear (PMNs) cells, whereas the combination of lymphocytes and monocytes are called MNs.

Apart from WBCs, other cells can be seen in a variety of BFs. These include lining cells (mesothelial, leptomeningeal, synoviocyte), phagocytic cells (histiocytes/macrophages, erythrophages, siderophages, lipophages), malignant cells (blasts, lymphoma cells and non-hematopoietic malignant cells), but also, bacteria, fungi and yeast cells (11). Mesothelial cells form the lining of serous cavities. These cells are large (12 - 30 μm), have a large round nucleus, and can appear in single or clustered forms. Non-hematopoietic malignant cells (i.e., adenocarcinoma, breast carcinoma, primary brain tumors) are also large, with large nucleoli, and are known to form tight clusters. Differentiating between mesothelial cells and malignant cells can be difficult because reactive mesothelial cells can be large, and may cluster together to resemble malignant cells (1). The (unexpected) finding of malignant cells in BFs, especially in patients with unknown malignant diseases, is of utmost interest, and should always be reported.

Relevance of cell counts

Changes in the cellular components of BFs are a reflection of disease pathogenesis and disease stage. For example, *bacterial meningitis*, a life threatening infection of the CNS, is characterized by an elevated WBC ($>1000\times10^6$/L) and PMN predominance (>50%) in CSF. The less severe *viral meningitis* is characterized by CSF WBCs between 10 and 1000×10^6/L with a lymphocytic pleocytosis (14) (Table 1). The number of RBCs in CSF can aid in distinguishing between a traumatic tap and a subarachnoid hemorrhage (18). Historically, pleural effusions have been dichotomized into *transudates* and *exudates* based on the following

Table 1. WBC counts in normal state and in various disease states (inflammatory and infectious) in a variety of BF. CSF= Cerebrospinal fluid, PMN=polymorphonuclear, MN=mononuclear

Normal state				Disease state				
	WBC (×10⁶/L)	PMN %	MN %		WBC (×10⁶/L)	PMN %	MN %	Refs
CSF	≤5	<2	>90	Bacterial Meningitis	>1000	>50		(1, 12-14)
				Viral Meningitis	10 - 1000		>80	
				Fungal Meningitis	20 - 2000		>50	
				Eosinophilic Meningitis		>10%EO or >10 EO/μL		
CAPD fluid	<50	12	>80	Peritonitis	>100	>50		(10, 15, 16)
				Eosinophilic peritonitis		>10%EO or >40×10⁶EO/L		
Pleural fluid	1716	<10	25	Transudate	<1000			(8, 9)
				Exudate	>1000			
Peritoneal fluid	281	27	-	Spontaneous Bacterial Peritonitis	≥250×10⁶/L			(7, 17)
Synovial fluid	<200	<25	<80	Non-inflammatory	0 - 5000	<30		(1, 5)
				Inflammatory	2000 - 200,000	>50		
				Infectious	50,000 - 200,000	>90		
				Hemorrhagic	50 - 10,000	<50		

criteria: WBC $<1000\times10^6$/L (transudates) and WBC $>1000\times10^6$/L (exudates) (19, 20). However, because of the significant overlap when this criteria was used, classification of effusions is currently based on biochemical tests (Light's criteria) (21) and less so on cytological parameters. Although the total WBC count is of limited diagnostic value in distinguishing transudates from exudates, the WBC differential count can narrow the diagnostic possibilities. For example, the finding of neutrophilia heightens suspicion for parapneumonic effusion; whereas, a lymphocytosis (>50%) profile is indicative of tuberculous effusions and malignancies. Some even suggest that hemorrhagic pleural fluid (RBC$>10,000\times10^6$/L) can predict malignancy (22). Cirrhotic patients with ascites are highly susceptible to *spontaneous bacterial peritonitis* (SBP). According to guidelines, SBP is present when $>250\times10^6$ PMNs/L are counted in ascites (17). The frequent occurrence of *peritonitis* is a major complication of CAPD in patients undergoing dialysis. If the following criteria's are present: cloudy effluent containing more than 100×10^6 WBC/L with ≥50% PMNs, and symptoms or signs of peritoneal inflammation, peritonitis is deemed the probable cause (15). In synovial fluids, the WBC and PMN counts are important diagnostic markers in enabling classification between *non-inflammatory* (WBC $<5000\times10^6$/L, PMN<30%), *inflammatory* (WBC $2000-200,000\times10^6$/L, PMN >50%), *septic* (WBC 50,000 - $200,000\times10^6$/L , PMN >90%) and *hemorrhagic disorders* (WBC: 50 - $10,000\times10^6$/L, PMN <50%) (23).

It is important to know that no diagnosis is made solely on the WBC/WBC differential results. However, their role as an important diagnostic parameter has undoubtedly been recognized, and is currently included in many clinical guidelines to help point the way to more specific testing, and possibly empiric treatment when necessary. Inaccurate results may lead to inappropriate diagnosis and therapy; therefore, accurate, precise and rapid laboratory results are of major clinical relevance.

Measuring cells in BFs

Traditional manual microscopy
The hemocytometer, also referred to as "counting chamber", is traditionally used to determine the concentration of total WBCs and RBCs in BFs. For WBC differentiation into MNs and PMNs, a stained cytocentrifuged slide is prepared. Cytocentrifugation is a cell preparation system that uses centrifugal forces to deposit cells onto a slide. The slide is stained, and followed by a 100 - 200 cell count differentiation (depending on laboratories protocol). The operating parameters (speed, timing, sample volume) generally vary from lab to lab, and this can influence the quality of the slide, and even result in quantitative variations in evaluation studies. The combined techniques are considered the gold standard; however, they have their limitations (24, 25). The hemocytometer is subjective, it is labor intensive, and has a high inter and intra-assay imprecision. Major concerns of the differential method correspond to the preparation of the cytospin slide. Because of the centrifugation, vulnerable cells are lost or obtain aberrant morphology, and macrophages or mesothelial cell clusters can be mistaken for malignancy. Collectively, results obtained from traditional microscopic methods should be viewed skeptically because their potential sources of errors can contribute to misleading results. The above described components emphasize the deficiencies of manual microscopy. However, it has remained the gold standard for BF analysis although, part of this method (hemocytometer) has been abolished decades ago as a reference method for blood samples (26). Because of the significant limitations of the manual method, along with the continuous increase of workload and shortage of skilled personnel (24/7), many laboratories opted for alternative methods by adopting the use of automated hematology analyzers, and more recent, urine analyzers to perform BF cell counts.

Automated analyzers
Up until the mid-1950s, cells in blood samples were routinely counted manually. The first automated cell counter was invented by Wallace Coulter based on electrical impedance principle (27). The Coulter principle revolutionized blood counting, and substantially reduced the time-consuming manual blood cell counts. His principle also laid the groundwork for subsequent development of modern day cell counters which employ similar techniques, but slightly different approaches. The two basic principles applied are "flow cytometry" and "electric impedance" technology. The basic principle of flow cytometry is the passage of fluorescently labelled cells in single file (hydrodynamic focusing) through a flow cell that is being intersected by a laser light. Photodetectors collect and measure the light in different wavelength ranges and scatter wavelengths by the use of specific optical filters. To this classic method, fluorescent dyes has been added to newer devices (28). Cells are then categorized based on the combination of sideward scattered light (cell granularity), forward scattered light (size of the cell) and fluorescent light (RNA/DNA content). In impedance technique, cells are sized and counted by detecting and measuring changes in electrical resistance when a particle passes through a small aperture. The change in voltage generates a pulse and the number of pulses is proportional to the number of cells counted. The size of the voltage pulse is also directly proportional to the volume or size of the cell. Most instruments generate two types of data graphic display for laboratory review: scattergrams and histograms. Histogram depicts the relative number of cells plotted against cell size, and scattergrams provide information on the WBC differential cells.

Hematology analyzers were originally intended for measuring cells in whole blood. However, WBCs in blood samples are at least 1000 times higher than that of BF samples, rendering these analyzers (using standard blood software) less suitable for BF analysis due to their high imprecision in the lower concentration range, and due to misclassification of tissue cells (i.e., mesothelial cells counted as WBCs) (29, 30). Currently there are a number of manufacturers on the market

with instruments (hematology and urine) suitable for BF cell counting (31). Each analyzer uses either impedance, flow cytometry, digital imaging flow cytometry or a combination of these technologies. Since their introduction, efficiency and productivity have significantly improved by reducing turn-around-time, increasing precision and accuracy, and eliminating inter-observer variability compared to traditional manual methods (32-38).

Evolution of Sysmex's automated cell counters for BF analysis

Sysmex Corporation (Kobe, Japan) is currently the market leader in Europe for BF analysis. In 1999, Sysmex launched its fully automated hematology analyzer, the XE-2100, initially developed for the analysis of whole blood samples. A few years later, this analyzer was FDA cleared for measuring a wide variety of BFs with the exception of CSF due to its high background limits (LoQ WBC: 50×10^6/L). In 2007 Sysmex launched the XE-5000, a fully automated hematology analyzer, which contains unique software tailored for BFs analysis (the body fluid mode). It allows instant quantification of CSF, serous fluids,

synovial fluids and CAPD without any pretreatment, and counts three times more cells than the XE-2100, resulting in improved precision at the lower concentration range (LoQ: 10×10^6/L). Sysmex's latest hematology analyzer, the XN-Series, was launched in 2011. It also contains a BF mode, measures a variety of BFs and counts two times more cells than the XE-5000 to increase precision (LoQ: 5×10^6/L). These analyzers adapted the combination of fluorescent flow cytometry with a semiconductor laser and impedance technique to measure the following cellular parameters: size, volume, granularity, surface area, and fluorescent signal. This data is used to determine the total nucleated cell count (TNC), RBC, WBC, MN, PMN, and high fluorescent (HF-BF) cells such as macrophages, malignant and mesothelial cells. These HF-BF cells are found just above the MN cluster, and are not included in the WBC (differential) count. However, they are included in the TNC (Figure 1). In 2014, Sysmex released new BF software for its urine analyzer UF-1000*i*, initially developed for measuring urine samples. The UF-1000*i* BF mode identifies cells based on forward scatter, fluorescence and adaptive cluster analysis, and reports a TNC, WBC and RBC count.
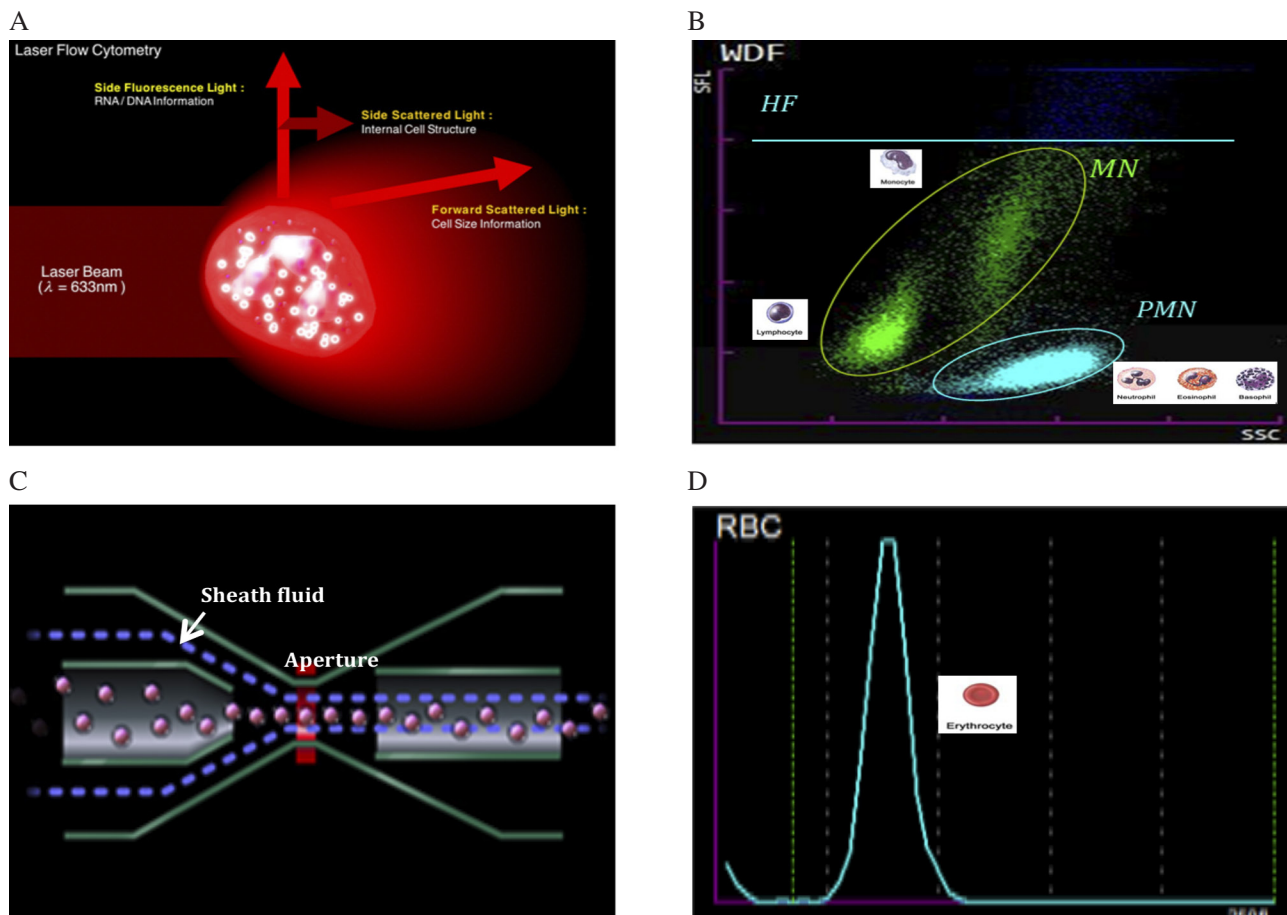
A

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Figure 1. Principles adapted by Sysmex hematology analyzers to determine WBCs and RBCs. (A) Fluorescent flow cytometry principle, used to count and differentiate WBCs. (B) WBC differential (WDF) scattergram, with the various WBC cell types indicated. Within the MN cluster, the clusters for lymphocytes (lower left) and monocytes (upper right) are clearly visible; within the PMN cluster eosinophils (not present in this example) will appear as a separate cluster on the right of the neutrophil cluster that is clearly visible in this picture. The HF cluster represents cells with high nucleic acid content and large size such as macrophages, mesothelial and tumour cells. (C) Hydrodynamic focussing impedance technique, used to count RBCs. (D) RBC histogram. In graph B, the x-axis (SSC) represents the side scattered light and the y-axis (SFL) represents the side fluorescent light signal. MN= monocytes + lymphocytes and PMN= neutrophils+ eosinophils+ basophils. HF= high fluorescent cells.

Overall research questions

Since their introduction in clinical laboratories, the use of automated cell counters has led to major improvements in BF analysis, and gained increasing, though still limited acceptance as an alternative to manual microscopy. The current limitations of BF modes applies to automated analyzers: lack of precision in the low ($<20\times10^6$/L) counting range, inability to detect malignant cells, interferences by non-cellular particles (bacteria, lipids, crystals) leading to spurious WBC and/or RBC results, and their inability to flag abnormality (33, 35, 38, 39). Therefore, in this thesis we investigated whether the currently available automated analyzers (by Sysmex) containing a dedicated BF mode are, *I*) sufficiently precise to measure blood cells in non-malignant BFs in clinically relevant ranges, *II*) sufficiently accurate in the WBC and WBC differential count, *III*) able to discriminate normal from infectious/inflamed samples (diagnostic test accuracy), *IV*) capable of detecting and flagging interfering particles, and *V*) suitable for replacing manual microscopy.

Scope of thesis

To date, there is an ongoing discussion about the use of automated cell counters versus conventional microscopy for body fluid cell counts. In **Chapter 2**, a review of the literature is presented on the clinical relevance of blood cell counts in BFs and contemporary methods for measuring them in samples suspected of inflammatory diseases. Currently, there remains a discussion about which diagnostic parameter (WBC or PMN) best predicts peritonitis in dialysis patients. To this end, **Chapter 3** describes a retrospective study using the automated XE-5000 WBC count and differential results in effluent dialysate samples for distinguishing between infectious and non-infectious peritonitis.

In 2007, Sysmex launched the XE-5000 BF mode, and this analyzer was evaluated extensively in our laboratory and also by others. The two main limitations resulted from its evaluation include high imprecision in the low concentration range and overestimation of CSF PMNs. Consequently, Sysmex developed new software to solve these issues. With the new software, gating algorithms for separation of unknown particles such as cell debris or fragments in the WBC/PMN count has been improved. Therefore, in **Chapter 4** we evaluated this new software in comparison with the current software on the XE-5000 in CSF samples. Next, in 2011 Sysmex released the XN-Series which is their latest hematology analyzer. The XN-BF mode has several new features compared to the XE-5000 BF mode. It is able to count four times more WBCs than the XE-5000, aspirates less sample volume (88 µL) compared to the XE-5000 (130 µL), and offers open or closed tube sampling for BFs. In **Chapter 5**, we compared the XN-1000 BF mode with the hemocytometer for counting cells in CSF, CAPD and serous fluids. Because of the continued demand for low imprecision in the low concentration range and high accuracy in CSF samples, Sysmex developed a new high sensitive Analysis mode (hsA) on the

XN-Series, specifically for counting cells in CSF, which we evaluated in **Chapter 6**. The XN-hsA mode has several new features such as it provides a 4-part differential count, uses flow cytometry technique for counting RBCs, aspirates more sample volume (180 µL) than the XN-BF mode and it counts twice as many cells, which in turn increases its precision and finally has new gating algorithms to detect and flag abnormal cells in CSF samples. Optical urine sediment analyzers are also being used for BF analysis, although they are not designed and certified for this application. At present, the Sysmex UF-1000i analyzer contains a urine mode and a dedicated BF mode. The BF mode contains new gating and modified algorithms to enhance cell counting and decrease interference with cell fragments (observed when measuring BF in the urine mode). This brings us to **Chapter 7**, where we report on a validation study between the UF-1000i and reference methods for counting cells in serous fluids. Disadvantages of automated analyzers include potential interfering factors from non-cellular particles including liposomal particles. Patients with neoplastic meningitis are treated with the chemotherapeutic drug DepoCyt (cytarabine encapsulated in liposomal particles). The active drug is uniformly distributed throughout the CSF by its sustained release, and is detectable up to 14 days after administration. Interferences of DepoCyt particles are of concern because reporting falsely elevated WBC counts can have potential adverse clinical consequences in patients with neoplastic meningitis. In **Chapter 8**, the interference of liposomal particles on the XN-1000 BF mode is studied. Lastly, **Chapter 9** discusses the main findings of this thesis in a broader perspective, and gives suggestions for future research.

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