Published in final edited form as:
*Lab Chip*. 2013 March 21; 13(6): 1011–1026. doi:10.1039/c2lc41104k.

# Microfluidic Sample Preparation for Diagnostic Cytopathology

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## Abstract

The cellular components of body fluids are routinely analyzed to identify disease and treatment approaches. While significant focus has been placed on developing cell analysis technologies, tools to automate the preparation of cellular specimens have been more limited, especially for body fluids beyond blood. Preparation steps include separating, concentrating, and exposing cells to reagents. Sample preparation continues to be routinely performed off-chip by technicians, preventing cell-based point-of-care diagnostics, increasing the cost of tests, and reducing the consistency of the final analysis following multiple manually-performed steps. Here, we review the assortment of biofluids for which suspended cells are analyzed, along with their characteristics and diagnostic value. We present an overview of the conventional sample preparation processes for cytological diagnosis. We finally discuss the challenges and opportunities in developing microfluidic devices for the purpose of automating or miniaturizing these processes, with particular emphases on preparing large or small volume samples, working with samples of high cellularity, automating multi-step processes, and obtaining high purity subpopulations of cells. We hope to convey the importance of and help identify new research directions addressing the vast biological and clinical applications in preparing and analyzing the array of available biological fluids. Successfully addressing the challenges described in this review can lead to inexpensive systems to improve diagnostic accuracy while simultaneously reducing overall systemic healthcare costs.

### Keywords

biofluids; rare cell analysis; cytology

## 1. Introduction

Analysis of cell samples is widely used for medical diagnostics. Some areas of use include (i) quantifying cellular components of blood like complete blood counts (CBC), (ii) reviewing tissue sections and liquid-based cellular solutions by pathology, and(iii) analysis and sorting of target cell populations with flow cytometry. Miniaturization of flow cytometry and differential blood cell counts have been a strong focus of the microfluidics community[1–4]. However, there has been less focus on analysis of histologic sections and cytology-based diagnostics, which often require a larger amount of sample preparation.

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While there has been significant progress made in developing cell detection and analysis technologies, the development of miniaturized and automated tools to prepare cellular specimens for sample analysis quietly lags behind. Sample preparation continues to be performed off-chip with macroscale instruments, like the bench top centrifuge, which limits the use of cell-based diagnostics in point-of-care settings, increases the cost, and reduces the consistency of tests performed through manually-performed steps[5]. Additionally, beyond the analysis of cells present in blood, processing and analyzing cells from other body fluids has diagnostic importantance. The preparation of other body fluid samples for the analysis of cells has unique challenges that impede efforts to move towards miniaturization and automation.

Here we review the assortment of biofluids clinically analyzed, along with their characteristics and diagnostic value. We investigate the conventional methods for cytological diagnosis and the challenges and opportunities in developing microfluidic devices for the purpose of the sample preparation of biofluids for cytodiagnostics, with a particular emphasis on preparing large volume samples. We then identify efforts in the microfluidics community to prepare and analyze various biofluids with a focus outside of traditionally analyzed cells in blood[6]. We hope to convey the importance of and help identify new research directions addressing the vast biological and clinical applications in preparing and analyzing the array of clinically-available biological fluids.

## 2. Standard Procedure for Liquid-Based Cytology

Examination of the free cellular content of body fluids, cytology, is routinely performed for disease detection and diagnosis. The CDC estimates that 6.8 billion laboratory tests are preformed yearly, where anatomic pathology and cytology account for 23% of testing. Samples for cytological examination are first collected in a clinical setting, by a physician, physician assistant, nurse, or dedicated technician. These samples are transported to the cytopathology lab which is often present within major medical centers, but may be off site for smaller community hospitals or clinics. In the cytology laboratory various sample preparation steps are performed prior to analysis including centrifugation to concentrate cells in dilute samples, followed by preparation and staining of slides, and examination under a light microscope (Figure 1).

### 2.1 Sample Collection

Sample collection is the first step in preparing a sample for analysis, in which the collection technique can often impact downstream cytodiagnostic assays that can be performed. Samples are obtained through collection techniques that vary in their level of invasiveness and risk to the patient. Sample collection techniques include phlebotomy, fluid aspiration via needle or syringe, or saline washing of a mucosal surface with catheter assistance. First impressions like color, odor, and volume are noted to determine whether further testing is required. For example, red-colored urine may indicate the presence of red blood cells in the urine sample due to underlying pathology, including infection or cancer. To further elucidate the cause of illness and make a proper diagnosis, additional tests may be ordered. Depending on the procedure that is required, sample collection is carried out different locations, and can be performed in a patient procedure room, at the hospital bedside of the patient, at an

outpatient office, or in the clinical laboratory. Once the sample is obtained, it is sent to a cytopathology lab and stored at 4°C. Generally, samples can be refrigerated for several days while maintaining sample integrity[7]. In some cases, samples are treated with anti-clotting agents to preserve cell morphology. For example, blood is collected into a collection tube containing an anti-coagulant to prevent cell clumping, and fixing agents are added to urine to avoid loss of cell viability due to fast degradation times of cells in urine.

### 2.2 Sample Preparation

Most laboratories use two or more methods of preparing liquid-based samples for further evaluation and analysis. From a sample, cell smears or cell blocks may be prepared for microscopic review by a cytopathologist using conventional or immunocytochemical stains. Cell solutions can also be further analyzed with flow cytometry or undergo cytogenetic analysis. To make a cell smear or cell block, an aliquot of the sample, typically 50 mL in pleural and peritoneal fluid specimens, is centrifuged with a bench top centrifuge. The supernatant is aspirated and the sediment is used for preparing direct cell smears or cell blocks. A direct smear is the conventional method whereby sedimented cells are manually transferred onto a glass slide for examiner review under the microscope. Alternatively, an automated instrument called the cytocentrifuge takes the cell sediment and evenly distributes the cells onto a designated circle on a glass slide in an automatic and reproducible fashion. Ultimately, a cell smear can take up to two centrifugation steps and multiple pipetting procedures, requiring as much as 10 minutes of preparation time per sample. Additionally, the presence of blood cells can prevent the formation of a uniform cell layer, which may make the cell smear difficult to interpret under microscopy examination. Cell blocks are formed from cell sediments that are then embedded in paraffin and cut into histological sections. Following cell preparation, the slides are stained with colored dyes to help differentiate cells by color-specific features of cellular morphology described in the sample analysis section. The slides are alcohol-fixed or air-dried to remove water content; this is then followed by a series of washing and staining with stains like Papanicolaou (Pap) or Romanowsky to highlight nuclear and cytoplasmic features. In some cases, biofluid samples are prepared for flow cytometry where the cells are labeled with compatible fluorescent dyes in liquid solution rather than on a glass slide. The remaining fluid specimens are stored in the refrigerator for further and repeated testing if needed. Slide preparations for cytology review can take up to 45 minutes in total for a single sample[8]. Manual sample preparation of slides and blocks can lead to increased costs and variation in quality from lab to lab.

### 2.3 Sample Analysis

Once samples are prepared, they are given to the cytopathologist for diagnosis. Cell smears and blocks are examined under light microscopy and described with particular attention given to important parameters including cell size, morphology, nuclear to cytoplasmic ratio, and the presence of multi-nucleation of cell aggregates. In the case of malignant cells, chromatin is generally more unfolded and will often appear darker[9,10]. The laboratory diagnosis may be reported with a heading such as `positive for malignant cells', `suspicious', or `negative for malignant cells'. Oftentimes, further testing is requested by the cytopathologist, especially in `suspicious' and `positive' cases. Specimens can also be analyzed with flow cytometry, cytogenetic testing using fluorescence in-situ hybridization

(FISH),or immunocytochemical analysis, which provides a more sensitive and specific approach to determine whether malignancy is present by using specific labels. Use of flow cytometry and cytogenetic analysis have aided inaccurately predicting certain disease states in a reproducible fashion. These studies have therefore become increasingly more important in defining disease characteristics, which is well illustrated by the routine use in the diagnosis and treatment of hematologic malignancies[11]. Regarding immunocytochemistry, for example, a sample from a patient with a history of lung cancer would require staining for biomarkers specific to the lung like EGFR, KRAS or TTF-1[12]. Frequently, immunolabeling of existing colorimetric slides are performed by washing out previous colorimetric labels and applying new labels with more specificity. Additionally, cytopathologists are interested in extracting nucleic acid sequence information from malignant cells within samples to identify mutations that are potential therapeutic targets, but this is still not routine clinic practice. One method of collecting pure malignant cells is to use laser capture microdissection[13,14], which uses a laser to cut out malignant cells from cell smears for molecular analysis[15].

## 3. Background and Applications of Biofluids

Biofluids are liquid-based cellular solutions that originate from the human body. Examples include urine, blood, pleural fluid, peritoneal fluid, cerebrospinal fluid, and wound exudates (Figure 2, Table 1). Sample preparation and analysis of biofluids is emerging as an application area that many microfluidic technologies may be able to address. Microfluidic research has in particular focused on the preparation and analysis of blood[16]. With already over 1,100 citations, much of the work thus far has related to the development of devices for cell and molecular analysis of blood samples [16–18][17]. While significant diagnostic information about the patient can be obtained from blood analysis, other biofluids may be as rich in terms of diagnostic information. These other biofluids present unique sample preparation and analysis challenges that microfluidic technologies are poised to address. Note that biofluids other than blood can contain elevated amounts of blood cells due to underlying pathology. Given that under these circumstances, the amount of blood cells present generally would not be equal to the amount present in whole blood, these biofluids can be considered to be analogous to diluted blood for sample preparation purposes and, thus, have similar challenges. It is noteworthy that the cellular profile within a biofluid that results from a non-malignant versus a malignant process can be significantly different. Additionally, fine needle aspirations and core biopsies, while not included in this review due to their high cellular content, can be considered biofluids when placed in liquid solution. Here, we highlight clinically relevant biofluids with a brief background, identify the diagnostic value and clinical applications for microfluidics, and discuss critical challenges with developing sample preparation tools for each biofluid. We categorize each biofluid with regard to fluid volume (mL), cellularity (cells/mL), fluid viscosity, and associated diseases. For some of these biofluids, researchers can gain access to remnant samples after all diagnostic tests are performed with expedited IRB review, which can speed investigation into simple approaches to prepare and analyze these diagnostically important fluids beyond blood[18].

### 3.1 Blood

**Background—**Blood harbors vast information about the physiological and pathological conditions of the human body. The sampling and analysis of blood plays a significant role in medical diagnostics. One example of blood testing includes surveying the cellular constituents of blood with a complete blood count (CBC) that quantifies the number of red blood cells (erythrocytes), white blood cells (leukocytes), and platelets present in the sample analyzed. Full blood counts have largely been automated by hematology analyzers and flow cytometers, becoming the gold standard for CBC determination. Blood will continue to be the biofluid that is the dominant focus for engineering specimen handling and analysis technologies. It is easily accessible from patients among researchers and has proven to be a valid proof-of-concept biofluid to be used in evaluating prototypes being developed for use with various other biofluids.

**Diagnostic Value—**A complete blood count (CBC) is routinely used as a laboratory test to determine health status. Typically a microliter of blood contains 5 million erythrocytes, 5,000–10,000 leukocytes, and 400,000 platelets[16]. Leukocytes are further classified into subpopulations of cells that have different physiological functions, including neutrophils, lymphocytes, monocytes, basophils, and eosinophils. An abnormal CBC is often indicative of underlying pathology, including infection or cancer. Noting the presence of abnormal numbers of specific populations of leukocytes[1,19,20] assists in diagnosing particular hematologic conditions, monitoring response to therapy, or determining the presence of disease progression. For example, the absolute number of peripheral CD4+ T cells (helper T-cells) is used to monitor HIV progression[21]. In another example, in patients with chronic myeloid leukemia, the BCR-ABL gene translocation results in the presence of a constitutively active tyrosine kinase that can be detected and is now a target for therapeutic intervention[22]. Preparation of a peripheral blood smear for review would be needed for confirmation purposes if cell counts appear to be low. Review of a peripheral blood smear is also in order if there is concern for certain infectious diseases, such as malaria or babesiosis. In the case of malaria, one can directly visualize the presence of the parasite in infected erythrocytes. The level of parasitemia (volume of blood occupied by the malaria parasite) can be estimated from review of the peripheral blood smear, which is important in terms of patient prognosis[23]. A current additional method for pathogen detection includes conventional blood culture, where an aliquot of blood is placed into blood culture media. If a microorganism is detected, further analysis is required for specific microorganism identification. This process usually takes 2–3 days, and can be suboptimal in terms of pathogen isolation[24]. Also, isolating bacteria is necessary to study the methods and consequences of biofilm formation and has significant implications in development and understanding catheter associated blood stream infections with staphylococcal organisms.

Finally, although not clinically used as of yet, there has been much work in isolating and identifying rare cells from blood. For example, the fraction of circulating tumor cells (CTC) present in blood could be as minute as one circulating tumor cell per billion blood cells; the capture and analysis of circulating tumor cells could provide information about cancer relapse or mutational state[25]. Similarly, fetal cells are present in maternal peripheral blood in rare amounts, and can help determine health of the developing fetus (see Amniotic Fluid)[26].

Notably, a recent shift has emerged for developing sample preparation tools to process large mL volumes of blood as a method to gain statistically significant access to rare cell populations in blood samples[27,28]. In these instances, the importance of processing large sample volumes brings about a critical challenge for rapid processing.

**Sample Preparation Challenges—**Many sample preparation challenges in preparing blood have been highlighted in Toner and Irimia's review[16]. Briefly, challenges include the high cellularity of samples and the propensity of cellular components to aggregate. In this case, chemicals that prevent platelet activation can be used, e.g. EDTA. While dilution may seem a possible remedy, this translates to greater processing times for a given blood volume. Another alternative is to lyse red blood cells which can remove >99% of the cellular content leaving a population of platelets and leukocytes. A distinct advantage of reducing cellularity enables researchers to exploit physical phenomena such as mechanical, electromagnetic, and fluidic forces that can encourage interaction of cells with surfaces for specific affinity capture, concentrate cell subpopulations by size, or stain, wash, and focus cells for analysis. Under circumstances where large (milliliter) volumes of blood are to be processed, additional technological advancements can allow for processing of whole blood in a continuous and rapid fashion. Using parallel devices, some have demonstrated that processing at sample flow rates of 1 mL/min[29] to 1 mL/hr[30,31] is achievable. An example where large volume concentration would be needed in the clinic is in developing a rapid blood culture test. Specific separation and concentration of a larger amount of bacterial components from a sample of blood would allow for rapid growth and analysis, perhaps shortening time from 2–3 days to 2–3 hours and significantly aiding clinical decision making.

### 3.2 Pleural and Peritoneal Fluid

**Background—**Both the lungs and inner thoracic cavity are lined with visceral and parietal pleura respectively, which slide against one another when the lungs contract and expand. Between the two pleura there is a small amount of fluid called pleural fluid that acts as a lubricant, enhancing lung movement during inspiration and expiration under normal circumstances. However, under pathologic conditions, a pleural effusion may develop, where excess amount of pleural fluid accumulates in the pleural cavity. At times, volumes of 1–2 L of pleural fluid may accumulate. During a procedure called a thoracentesis, the operator will choose a location along the patient's back, insert a needle through the chest wall into the pleural space, and withdraw the pleural fluid present. Once collected, the fluid is described in terms of its gross, or clinical, appearance. Biochemical analyses are performed; using Light's criteria (criteria correlating the ratio of specific amounts of proteins within pleural fluid and serum to categories of disease states), results of these tests assist in determining if the pleural effusion has developed as the result of a transudative or exudative process[32]. Transudative pleural effusions result from an imbalance of hydrostatic pressure while exudative pleural effusions may result from an inflammatory process, examples including infection or cancer. Additional laboratory tests include total cell counts with differential cell count values, cultures for various microorganisms, and cytology. In the normal state, pleural fluid is largely acellular. However, depending on the process causing the pleural effusion to develop, a pleural fluid sample contains cells ranging from leukocytes

predominated by mononuclear or polymorphonuclear cells (1000–100,000 per μL), RBCs (1000–100,000 per μL), mesothelial cells that line the pleura cavity, cancer cells, and/or microorganisms such as bacteria. There has been considerable interest in extracting the malignant cell populations to study cancer invasiveness as well as the effusion microenvironment[33]. Peritoneal fluid is similar to pleural fluids but found in the peritoneal cavity surrounding the abdominal organs. Accumulation of this fluid, described as ascites, produces abdominal distension, which may be reflective of up to several liters in volume of ascitic, or peritoneal fluid. Peritoneal fluid is collected through a procedure called paracentesis, where an operator inserts a needle through the abdominal wall into the peritoneal space, and withdraws the fluid present.

**Diagnostic Value—**Possible cytolological diagnoses of these fluids include: positive for malignancy, suspicious for malignancy, and negative for malignancy. Patient samples determined to be negative for malignancy may have a cellular profile consistent with acute inflammation - associated with an increased neutrophil population, chronic inflammation - associated with a larger fraction of lymphocytes and histiocytes, reactive mesothelial changes, and lymphocytosis – associated with an increase of lymphocytes. The manifestation of malignant effusions in the pleural or peritoneal cavities typically indicates a poor prognosis. Malignant pleural effusions account for 10% of all pleural fluids[34]. Of those, lung and breast tumors account for 75% of pleural effusions[34], while intestinal and ovarian cancers make up a dominant portion of peritoneal effusions. Current cytopathology methods do not reveal a conclusive diagnosis in the analysis of a fair number of clinical samples analyzed; the accuracy of this cytopathology method relies on the experience of the technician as well as the cytopreparation method[35]. False negative findings may be a result of improper handling. Supplementary techniques have been used to improve sensitivity, like using immunocytochemistry. For example, biomarkers like carcinoembryonic antigen (CEA) are helpful for distinguishing malignant cells from reactive mesothelial cells. Additionally, nucleic acid analysis can give information about the malignant cells and whether specific drug-targeted gene mutations to EGFR or KRAS are present[36]. In cases with negative malignant effusions, specimens are analyzed for leukocyte prevalence.

**Sample Preparation Challenges—**These types of fluid samples may be cellular and full of proteinaceous debris in certain clinical situations. One difficulty, then, would lie in isolating particular cells from a background of more plentiful and less relevant cell populations, to aid nucleic acid analysis. Concentration of specific rare cell populations from a large sample volume can also be difficult. For example, during large volume paracentesis, as much as 9 L of fluid can be removed from the patient's abdomen – which is unable to be processed by traditional centrifugation. In this case, continuous processing and concentration of cells from a large volume of biofluid quickly can aid in nucleic acid or cytology analysis. Harvesting malignant cells from pleural and peritoneal fluid is challenging, especially when malignant cells can easily be confused with mesothelial cells during microscopic review, even with the trained eye. Both cell types are generally larger than the blood cell population of cells that may co-exist in such a sample, but they may be similar in size. Discriminating between such samples requires specific molecular or biophysical markers. Mechanical properties of mesothelial cells have been observed to be

different than malignant cells, for example[37]. Cell mechanophenotype can be used as a method of cell separation and may be a useful biomarker that can be linked with clinical outcomes[6,37,38]. Cell sorting using a label-free property such as cell stiffness can be a way to remove cellular background when imaging cell smears in order to aid in finding cells with specific gene mutations from within a now lower background of contaminating normal cells. Finally, automation of the current manual sample preparation approaches with chip-based concentrators, and solution exchange systems can aid in improving diagnostic accuracy independent of operator skill level.

### 3.3 Amniotic Fluid

**Background—**Amniotic fluid, the fluid in the amniotic sac that surrounds the developing fetus, contains a rich source of information for prenatal testing. It provides a safe and nourishing environment and a lubricated interface between the fetus and placenta. According to the National Center for Health Statistics, approximately 124,000 pregnant women per year undergo an invasive procedure called amniocentesis, where amniotic fluid is sampled. A typical test requires harvesting 20 mL of amniotic fluid for the screening of genetic diseases. Amniocentesis is considered a risky procedure as it can cause induced abortion or maternal injury. Amniotic fluid is composed mostly of water giving a fluid viscosity comparable to water with traces of cells sloughed off from the developing fetus.

**Diagnostic Value—**The isolation and genotyping of fetal cells (erythrocytes, leukocytes, and trophoblasts) is important for prenatal diagnosis. Cytogenetic analyses include karyotyping and FISH to detect aneuploidy of all chromosomes and structural chromosomal abnormalities that are present in clinical conditions such as Down syndrome. Harvested amniotic fluid also contains multiple cell types that differentiate along adipogenic, osteogenic, myogenic, endothelial, neurogenic and hepatic pathways[39,40]. Although not addressing a clinical problem, microfluidic technologies have been used for the culturing and differentiation of amniotic stem cells[41,42].

**Sample Preparation Challenges—**A major challenge with sample preparation of amniotic fluid is distinguishing between cells of maternal or fetal origin. This becomes critical when performing molecular analysis on the fetal cells is desired without result interference from maternal cells. Additionally, collecting sufficient numbers of fetal cells in high purity remains to be a challenge in order to initiate stem cell cultures and extract DNA for sequencing. Others have investigated maternal blood as a less-invasive alternative for isolating embryonic cells. Fetal cells migrate into the maternal peripheral blood and then can be isolated[43,44]. Recently, the detection of cell-free DNA in maternal blood has been demonstrated as a method for prenatal diagnosis[45]. Under these circumstances, a device that is able to process a large sample volume would be valuable.

### 3.4 Urine

**Background—**The genitourinary system includes the kidneys, ureters, urinary bladder and urethra. In the kidney, as blood is filtered and toxic metabolites are removed, urine is formed. Urine then flows along the genitourinary tract from the kidney, through the ureters, into the urinary bladder, where it collects until bladder emptying occurs. At that time, when

urine is voided, it exits the body from the bladder through the urethra, and may be collected either after spontaneous voiding, or by inserting a catheter into the bladder via the urethra. During cystoscopy the interior surface of the bladder is visualized with a fiber optic camera inserted through the urethra. To collect a bladder washing specimen, an operator may introduce a saline solution into the bladder, and then aspirate this fluid. The contents of this fluid are then further analyzed to determine if underlying pathology is present, such as malignancy. In general, the human body forms a total of 1–2L a day of urine. As a biofluid, in comparison to blood, under normal circumstances it is largely acellular, containing soluble metabolites and, at most, a small amount of protein[46]. However, particularly when underlying pathology is present, one may find cells, casts of cells, and/or crystals. Routine testing of urine includes urinalysis and urine microscopy. The term urinalysis describes a collection of tests done to determine whether particular metabolites and/or protein is present, and is performed usually as a group in order to screen for common diseases, whereas urine microscopy entails the microscopic examination of the sediment obtained from a centrifuged sample of urine.

**Diagnostic Value—**Upon collection, one notes the color and odor of the urine sample, which may indicate the presence of disease. For example, tea-color or frankly red urine may signify that red blood cells, or hematuria is present. Urine contains a variety of metabolites that are of diagnostic importance. For example, urine may be screened for whether glucose is present in excess, to determine if a patient has diabetes mellitus. Urine pregnancy tests evaluate urine for the presence of human chorionic gonadotrophin. Concern for infection would prompt a clinician to send urine samples for microbiologic evaluations, such as staining and culturing for microorganisms. Unlike epithelial cells, some microorganisms can survive the harsh degrading and high shear environments in urine, proliferate, and cause ascending urinary tract infection. In the case of acute kidney injury, examination of urine sediment is virtually required as part of the diagnostic evaluation. In this case, cell casts representing damaged renal epithelial cells, indicate that renal tubules have been damaged. Under other pathologic conditions, the presence of red or white blood cells in excess will assist in determining an appropriate diagnosis. Additional findings that may be present during microscopic evaluation of urine include the presence of red or white blood cell casts and/or crystals. Urine cytology assists in diagnosing malignancy, especially those that arise from cells originating from the genitourinary system, e.g. bladder cancer. Urine cytology may also be important in determining the etiology of acute kidney injury in renal transplant patients. For example, investigators were able to distinguish renal transplant graft rejection from cyclosporine toxicity in renal allograft recipients[47]. Cytodiagnostic urinalysis is also useful in diagnosing the cause of kidney allograft injury, where allograft dysfunction may be due to allograft rejection, the presence of polyoma virus, or calcineurin inhibitor toxicity[48].

**Sample Preparation Challenges—**Sample preparation of urine is difficult due to its acellular nature, even with bloody samples that indicate microorganism infection or malignancy. In the latter case, small quantities of malignant cells are shed from the kidney, prostate or bladder, and released into the urine. There is considerable interest in harvesting these cells but large milliliter volume assessment and repetitive testing are generally required. Analysis for the presence of microorganisms such as bacteria or fungi is currently

performed by inoculating culture media with an aliquot of urine. Specific detection of a species may take up to the 2–3 days, and is currently done by exploiting the metabolic differences present between species as they consume the nutrients present in culture media. Isolation and identification of a specific organism within a shorter time after urine sample collection (rather than 2–3 days later), would likely require concentrating all of the colony forming units from a large volume of urine to a small volume, a difficult task when considering the typical volumes of urine that may be collected. Similarly, the concentration of rare cells from urine may also be difficult. Urine may also contain debris such as red or white cell casts or crystals, further complicating the task of isolating specific cells for the purposes of cytology review. Additionally, the high urea concentration present in urine degrades cells rapidly; thus, samples should be prepared and processed quickly to increase the yield of cytologic review.

### 3.5 Bone Marrow Aspiration

**Background—**Bone marrow is located in the fatty core of cancellous bone (sternum, rib, and pelvis), and the long bones (femur, tibia, and humerus). Cells that give rise to the cellular components of blood are housed in bone marrow. Collecting a sample of bone marrow for further evaluation and testing would include either obtaining a bone marrow aspiration or a bone marrow biopsy. During a bone marrow aspiration, a needle is inserted into the bone marrow space; a sample is then withdrawn though the needle into a syringe. Bone marrow tissue is spongy semisolid tissue, and typical bone marrow specimens are highly cellular. Further analysis of a bone marrow specimen is usually indicated if there is concern that a pathologic hematologic process is present. An example of such a process would be a hematologic cancer or a deficiency in one or more hematologic cell lines.

**Diagnostic Value—**Cytology review of a bone marrow aspirate is in order in many clinical scenarios. Bone marrow aspiration is commonly performed in the evaluation of hematologic malignancies. Cytology review gives important information regarding the behavior of cells present in marrow. Particular abnormalities that can be appreciated during microscopic review of a prepared slide will allow for accurate diagnosis of a clinical condition. Specific cell surface markers that have been identified are known to correlate with the clinical behavior of particular malignancies. This makes the identification of cell surface markers important in the diagnosis, determination of appropriate therapies available to the patient, prediction of the clinical course, as well as in estimating the prognosis for the patient. Usual testing performed on bone marrow aspiration specimens include slide preparations, as well as immunophenotypic, cytogenetic, molecular genetic studies such as fluorescence in situ hybridization, and flow cytometry analysis[49]. Also, bone marrow aspirations may be sent for microbiologic studies, including microscopic review, culture for microorganisms, and molecular testing.

**Sample Preparation Challenges—**Bone marrow aspirates are highly cellular and have a spongy semisolid consistency. This makes separation of specific cell populations more difficult. Typically, a volume of less than 500 microliters is harvested for analysis; a portion is sent for glass slide preparation for microscopic review, and the remaining sample is sent for further testing. Separation of specific cell populations is important for diagnostic

reasons. Also, this is important in harvesting cells to be used for clinical treatment; for example, hematopoietic stem cells are used for bone marrow engrafting in patients undergoing bone marrow transplantation. Given that low volumes are typically available for testing, the efficiency of a microfluidic device to be used for the purposes of isolating specific cell populations from a small volume should be high. As well, where there is a clinical condition causing a paucity of cells present in the bone marrow of a patient, the ability to isolate rare cells would provide a distinct advantage.

### 3.6 Cerebrospinal Fluid

**Background—**Cerebrospinal fluid (CSF) is formed by the choroid plexus, which is located within the cerebral ventricular system, and surrounds the structures that comprise the central nervous system (CNS), which includes the cerebrum and the spinal cord[50]. It protects the CNS, providing shock absorbency, and also allows for the elimination of chemical waste. Approximately 150 mL of CSF surrounds the adult brain. A sample of CSF, when sampled, should have less than 2 leukocytes per microliter, and no red blood cells; an abundance of red cells may be present, however, if inadvertently red cells are introduced into the sample during the collection process. The most common procedure performed to collect a sample of CSF for analysis is lumbar puncture. A needle is introduced into the space surrounding the lumbar spinal cord, which is occupied by CSF (as is the case for the entire spinal cord). A sample is then taken and sent for biochemical, cytologic, and microbial studies. Under normal conditions, CSF is a clear, thin, watery fluid.

**Diagnostic Value—**Sampling of CSF is indicated in the evaluation of a patient with disease involving the central nervous system. Routine testing of CSF includes biochemical, microbiologic, and cytologic analysis. Additional tests that may be performed, if clinically indicated, would be molecular genetic, cytogenetic, or immunophenotypic analysis, as well as flow cytometry[51]. For example, consider a clinical scenario where there is concern that meningitis may be present. In addition to microscopic review of a glass slide for the presence of microorganisms, the number of cells present in the sample is quantified. Also, the specific population of cells present is determined; i.e., the number and type of white blood cells, and the number of red blood cells. Immunophenotypic and flow cytometry analysis is especially important where there is concern for central nervous system lymphoma.

**Sample Preparation Challenges—**In particular regarding CSF, the isolation of rare cells from a fluid sample is a dilemma. Where a sample contains a large amount of red blood cells because of trauma related to the procedure, removal of red blood cells before cytologic analysis is important. Also, given the number of tests that may be requested for a finite volume of collected sample, one can anticipate that only a small volume of fluid will be available for each test requested. A benefit, then, would be for any given device to have the ability of isolating cells in an efficient manner while working with a small volume of fluid.

### 3.7 Bronchoalveolar Lavage Fluid

**Background—**Since the introduction of the fiber optic bronchoscope in the early 1970's, bronchoalveolar lavage has been used as an investigative tool in the study and evaluation of

acute and chronic pulmonary disorders[5253]. In the lungs, gas exchange between inspired air and blood occurs. Upon inhalation, air is carried through the respiratory tree, a structure that begins with the trachea and progressively branches into small airways until it reaches the blind ends of the respiratory passages, termed the alveolar sac[50]. The walls of alveolar sacs are lined with alveoli, the site where gas exchange occurs[50]. As the respiratory tree further branches and subdivides, the population and identity of cells that make up the mucosal lining changes, as there are regional distinctions[54]. Examples of the cells found along respiratory mucosa include pseudostratified, tall, columnar, ciliated epithelial cells and mucus secreting goblet cells. Alveolar macrophages and recruited neutrophils are among the cells that protect against inhaled debris and invading pathogens. During bronchoalveolar lavage, after a bronchoscope is introduced into respiratory tree, aliquots of normal saline are introduced into the respiratory airways; this fluid is then aspirated for analysis.

**Diagnostic Value—**Bronchoalveolar lavage (BAL) is indicated in patients with abnormalities of unclear etiology seen on chest imaging[55].Recovered aliquots of bronchial alveolar lavage fluid are analyzed for the presence of microorganisms, the profile of cells present, and the type of soluble biochemical factors present. Upon collection, one notes the volume and gross appearance of uncentrifuged fluid. A portion of fresh sample may be sent to the microbiology laboratory for quantitative bacterial culture. The supernatant and cell pellet in a portion of the sample that is centrifuged is further analyzed. The type and amount of soluble factors present in the supernatant can be determined with biochemical methods. The resuspended cell pellet may be sent for additional tests, including flow cytometry, nucleic acid analysis, glass slide preparation for microscopic review, and further microbiologic studies. A complete profile of cells present in the sample is determined, and accuracy is enhanced by analyzing 400–500 cells[56].There are reports available in the literature regarding the expected amounts and profiles of cells found in BAL fluid analysis[54]. Identification of the population of cells present, as well as the determination of cell phenotypes is used to assist in the evaluation of patients with lung pathology, including those with an acute respiratory infection, interstitial lung disease, or malignancy. In addition, regular long term monitoring of BAL fluid profiles with lung tissue sampling is performed in patients who have undergone lung transplantation to assess for acute and chronic long term graft and host interactions[54].

**Sample Preparation Challenges—**BAL fluid should be processed promptly if cells are collected in a nutrient poor media, such as normal saline[56]. In addition, note that mucus production increases the viscosity of respiratory secretions, and may interfere with BAL fluid specimen preparation; care should be taken as samples are collected to minimize this. Often, collected fluid is strained though material such as cotton gauze, to remove debris and allow for more adequate sample preparation. Isolation of specific cell populations is important for diagnostic purposes, so that the determination can been made whether the presence of these cells correlates with particular lung pathology. On average, a larger volume of 100 to 250 mL of collected fluid is available for analysis. The number of cells present may vary from a sparse amount to a highly cellular specimen. While the majority of cells present may be neutrophils, lymphocytes present in smaller numbers may be more difficult to isolate and further analyze.

### 3.8 Synovial Fluid

**Background—**Synovial fluid is the biofluid that lines the cavitated space in synovial joints. Examples of synovial joints include the elbow and knee. The synovial membrane is the boundary layer of this space, and consists of synoviocytes. Synoviocytes secrete hyaluronic acid and proteins, as well as synovial fluid, which functions as a lubricant, a shock absorbent, and provides nutrition for articular hyaline cartilage. Synovial fluid is a clear fluid that is a filtrate of plasma; it is viscous, containing hyaluronic acid, proteins, and few cells. Normal synovial fluid has fewer than 180 leukocytes per microliter. The abundance of these cells should be mononuclear cells. In particular disease states, this number of leukocytes can increase. The percentage profile of subpopulations of leukocytes present may change, for example, the predominating leukocyte population may be granulocytes (polymorphonuclear cells) instead of mononuclear cells. In addition, the presence of crystals may be noted. The characteristics and profile of synovial fluid may be determined by obtaining a sample of fluid during arthrocentesis. During arthrocentesis, a sample of synovial fluid is aspirated through a needle that is inserted into the joint space. This procedure may be performed by a physician in the hospital at the bedside or in the office[57].

**Diagnostic Value—**Under normal circumstances synovial fluid is clear; cloudy or bloody fluid may be present in conditions where an inflammatory, infectious, or traumatic process has affected the joint from which the fluid was aspirated. The appearance of the fluid is noted at the time of initial collection. With regard to conditions causing joint inflammation, or arthritis: in the case of osteoarthritis, aspirated synovial fluid may appear clear; however, in the case of gout, where monourate crystals within the joint space elicit an inflammatory response, aspirated synovial fluid may appear cloudy, but translucent. To assist in accurately diagnosing the cause of arthritis, a sample of synovial fluid is obtained with arthrocentesis. In general, a 1–2 mL aspirate of fluid is adequate for routine diagnostic testing. Metabolites that are routinely quantified include glucose, total protein, and lactate dehydrogenase levels[58]. Additionally, the total number of leukocytes and specific leukocyte subpopulations present is quantified. Further testing include performing a wet mount, or glass slide preparation, for review under normal light and compensated polarized light microscopy. Microscopy allows for the identification of crystals that may be responsible for the joint inflammation causing patient symptoms. If there is concern for infection, for example, a sample of fluid is sent for Gramstain and microbial culture, to determine if any microorganisms are present. Further cytology studies are also appropriate, as a malignancy may affect the synovium as a primary tumor or as the result of tumor metastasis[59]. It has also been reported that the proportion of synovial fluid mesenchymal stem cells present in freshly collected joint aspirates inversely correlates with disease severity in osteoarthritis[60].

**Sample Preparation Challenges—**Typically, arthrocentesis may yield 1–5 mL of fluid for analysis, leaving one with a small volume available for analysis. This collected volume may be highly cellular, or acellular, depending on what process is present in the joint that prompted the patient to seek medical attention. Also, depending on the underlying pathology present, if any, aspirated fluid may have high viscosity, requiring high pressure to allow for fluid flow through the intended device. As noted above, crystals may also be present, which

should be considered in the preparation of a sample where cells are to be further characterized and analyzed. The isolation of specific cell populations is important for diagnostic and prognostic purposes.

## 4. Critical Challenges in Microfluidic Sample Preparation

Retrieving target cell populations in solution and preparing them for analysis with minimal perturbation is a complex sample preparation task. Our survey of various diagnostically-important biofluids has revealed several classes of sample preparation challenges which microfluidic technologies are poised to address: 1) concentrating rare cells from large volumes of biofluids, 2) efficiently preparing small volume samples, 3) preparing samples with high cellularity, 4) automating multi-step sample preparation, and 5) obtaining high purity for molecular assays (Table 2).

Many microfluidic approaches to address sample preparation have aimed to scale down macroscale techniques. For example, cells can be isolated in a conical tube by incubating with immunomagnetic beads followed by placing a strong magnet in proximity to the tube and performing multiple rinse steps. Microscale technologies followed suit by integrating with on-chip and off-chip magnets for separating cancer cells, bacteria, and fungi from blood[61]. Other concepts were borrowed from analytical chemistry, like affinity and size-based chromatography, using instead “columns” sized for cells and affinity approaches specific to cell biomarkers like size or surface proteins. Approaches that make use of unique physics accessible in microfluidic systems are also poised to make an impact[62–65]. We refer the reader to several comprehensive reviews that discuss the physical operating mechanisms of various microfluidic cell separation and concentration approaches[66,67,27].

### 4.1 Concentrating Rare Cells from Large Volumes of Biofluids

Biofluid analysis may require large sampling volumes of tens to hundreds of milliliters, especially for rare cell applications. Relevant biofluids where one would envision large volume analysis include blood, urine, pleural, peritoneal, and BAL fluid samples. For example, 50 mL of pleural fluid is adequate to gather enough material for a cell smear in malignant pleural fluid analysis[68]. In another case, the presence of >5 cancer cells within 7.5 mL of blood was shown to be an independent predictor of overall patient survival in metastatic breast cancer[25]. Oftentimes, a sample with a low cellularity combined with inadequate sampling and the presence of rare cells leads to unsatisfactory results, prompting further diagnostic testing. In order to achieve large volume processing, potential devices should process samples at flow rates in the mL/min scale to satisfy the workload requirements of a clinic with a single machine or achieve rapid turnaround to aid in quicker clinical decisions. One challenge for a device using high flow rates is that cells can be exposed to high shear stress in the microchannels leading to cell damage. Scaling up of flow rates is possible by creating massive parallel arrays or increasing the channel dimensions of the device enabling more volume throughput while maintaining acceptable forces on cells.

In developing new microfluidic approaches for this challenge researchers should design systems that can be expanded in a parallel fashion and still retain the fundamental mechanics[62,69,70]. These mechanics typically take advantage of particle size, shape,

deformability and density using hydrodynamic forces induced by microchannel features. For example, Deterministic Lateral Displacement (DLD) and inertial microfluidics use various channel shapes and orientations to guide particles into specified outlets for collection. Since these channels can easily be arrayed, there is no limit to the amount of parallelization that can take place, offering macroscale regime flow rates in a compact microfluidic device. In another technique, acoustic waves maneuver cells to defined pressure nodes[71–73]. While able to process high flow rates, the technology requires an external piezo or patterned interdigitated transducer (IDT) to actuate the fluid, which may present a challenge for parallelization. Still, forces from these systems are large compared to dielectric and magnetic forces and therefore systems operate with appreciable flow rates (0.05 – 0.5 mL/min) with only a single channel. The ideal technology would be one where microchannels can be stacked indefinitely to obtain rapid processing times.

### 4.2 Efficiently Preparing Small Volume Samples for Multiple Assays

There are circumstances where the sample volume available for testing is limited. For example, typically 300 microliters of bone marrow is collected during a single bone marrow aspiration. Also, in diagnostically challenging cases, clinicians may require multiple tests from a single sample to aid in determining the final diagnosis. In both examples, it is very important that the volume of sample available for testing is efficiently prepared so that an optimal number of analyses can be completed. Factors that may reduce the sample available for further analysis include dead volume in a chip or external tubing, parallel assays, and loss of cells due to an inefficient on-chip sample preparation process. Innovative approaches to load and pump fluids without significant dead volume will lead to a reduction in the effective volume of sample needed for analysis, while sequential assays can make the most of a sample volume.

Traditionally, the microfluidic field has touted the small volume processing capabilities as an inherent advantage of the small scales of operation. Methods have been developed that take advantage of microchannel dimensions as well as the materials used to create the microchannels. In capillary-driven flow, fluid is actuated through surface tension, in which the flow can be driven by differences in surface energy upon wetting of a channel, network of channels, or porous structure such as paper[74–76]. In another technique using vacuum-driven flow, fluid is actuated by using the porous structure of normal Polydimethylsiloxane (PDMS) as a negative pressure source after the device is taken out of a vacuum chamber[77,78]. While both techniques offer a standalone system for working with small liquid volumes and concentrating cells with no moving parts and external components, these approaches potentially have long processing times, which may be prohibitive for certain point-of-care diagnostic applications. Another technique that requires additional external components makes use of centrifugation of a small volume of biofluid on a microfluidic chip to achieve pumping. As with traditional centrifugation – but with much smaller volumes – this approach allows for separation of plasma from whole blood and the subsequent mixing of the plasma with lyophilized reagents for detection[79]. A recent innovation that works most effectively with microscale volumes separates cells specifically bound to magnetic micro beads by a surface antigen by, instead of moving fluid, passing the bead-bound cells themselves through an immiscible phase into a second wash volume[80].

### 4.3 Preparing Samples with High Cellularity

Biofluids that have high cellularity, like blood or bone marrow aspirates, will contain diverse and large populations of cells. High cellularity samples can be challenging for many reasons, including that within these samples cells can interact within fluid flow to prevent accurate separations, cells have a higher likelihood of aggregating, and clusters of cells can clog microfluidic channels. While one may consider a dilution step to ameliorate the potential for cell aggregation and microchannel clogging, this has the effect of increasing sample volume, which may then require more sample processing time. One may also consider designing assays where cell separation occurs at the microchannel surface, rather than within the crowded fluid phase, such as affinity capture-based approaches[81,82]. Alternatively the crowded fluid phase may be used to aid in separation through the margination of particular cell populations to preferred locations in a flow[20,83]. Analysis may also be facilitated by segmenting a highly cellular solution into more easily analyzed single cells. It is important to isolate and analyze single cells with particular genetic mutations to move towards companion diagnostics for mutation-targeted drug therapies.

There has been much progress in dealing with samples that have high cellularity, especially for isolating specific cell populations from complex fluids like blood. One mechanism for cell separation is by size, for example DLD directs particles to move at an angle with respect to flow through an array of microposts. Larger cells undergo a bumping mode whereby cells are deflected from the normal trajectory while smaller particles follow streamlines in a zigzag mode, and the approach operates effectively even at very high cellularities. In another method, the microchannels are coated with antibodies that are specific to receptors of target cell populations so that cells are captured on-chip. These affinity based-platforms have been developed for isolating circulating tumor cells from blood[31,30,81,82] and tuning the shape of structures in the flow or creating mixing flows to allow surface contact even in highly cellular solutions is a challenge that is being addressed[31,81,84]. While these systems offer rapid throughput and high efficiency of cell capture, a successful technology should be able to balance these criteria in addition to high purity and ability to retrieve cells in solution (See Section 4.5). A possible solution is to segment highly cellular solutions into droplets containing single cells using droplet generators[85,86]. One main advantage is that single cells can be detected using fluorescent imaging and separated into different zones for further analysis.

### 4.4 Automating Multi-Step Sample Preparation

Automated handling of samples is needed for standardizing the sample preparation process. This includes centrifugation, pipetting, and cell staining, all operations usually manually performed in clinical labs. For example, when working with large volumes of biofluids, technicians must aliquot, centrifuge and manually pipette the mixed sample to make cell smears. However, mishandling and user error in processing such as neglecting a step to homogenize the sample before performing a decanting step may lead to misleading results. A standardized fluid plumbing system in which an entire volume is mixed and processed through a device would address this issue by removing user sampling bias; it can also allow for processing large liquid volumes. Automation of these steps to minimize sample handling

will preserve sample integrity and lead to less error overall, ultimately leading to cost reduction and increase in the diagnostic accuracy.

Some microfluidic technologies already offer multiple steps that are integrated onto a single platform. Centrifugal microfluidics can prepare blood samples through a series of pumping, valving, volume metering and mixing[87]. Abaxis currently sells this sample-to-answer technology in the Piccolo clinical blood analyzer system for both medical and veterinary diagnostics. Other technologies like the Centrifuge Chip, a method that recreates the functions of a benchtop centrifuge in a microfluidic format, combines cell concentration, separation, and staining, and is particularly useful for samples with low cellularity[70]. In another technique, droplet-based microfluidic approaches miniaturize reactions by segmenting in droplets containing femto- to micro-liter liquid volumes, aiding in biochemical screening, enzyme kinetic studies, and assays[88]. Since each compartmentalized droplet performs one reaction, multiple reactions can be performed in parallel, which offers superior throughput[85,86]. Methods to merge droplets can then be used to perform sequential sample preparation and staining steps on individual cells[89–91]. Digital microfluidics, a method for manipulating droplets using electrowetting, can be utilized to perform sequential operations on cell and tissue samples fixed to slides[92] and has advantages in working with small volumes in a non-continuous format as well. An ideal technology would automate cell staining techniques in a microfluidic platform to enable efficient uniform labeling using traditional and immunocytochemical stains as well as to enhance the performance of cytogenetic analysis e.g. FISH.

### 4.5 Achieving High Purity Cell Populations

Achieving high purity of specific cell populations from heterogeneous solutions presents a critical challenge in biofluid processing. Purity can be important in preparing cellular samples for nucleic acid analysis, for cell counting of specific selected sub-populations, and for reducing the presence of background cells and other non-cellular particulates that can mask optical observation of cells of interest. For example, bloody specimens containing leukocytes can contaminate molecular analysis results when attempting to detect gene mutations or perform nucleic acid sequencing in a target cell population[12]. Strategies directed toward the removal of contaminating cells can also aid in the development of platforms for cell counting and sorting of specific sub-populations. For example, in reviewing standard cytology slides, target cells are often more difficult to examine when the background density of less clinically relevant cells increases. In these circumstances, the removal of blood cellular components and the concentration of target cells into a small field of view may expedite and increase the accuracy of cytology examinations[2793,70].

A few microfluidic approaches address these issues and are capable of extracting cells at high purity[31,30], [94]. However, it is worth noting that some methods lack the ability to make cells readily available in solution after sample preparation, limiting the ability to integrate these techniques with downstream cell-based analysis tools like flow cytometry and some imaging-based techniques. Some technologies have been successful in recovering cells after on-chip processing[95,70]. In the Centrifuge Chip, target cancer cells are selected by size from a bloody sample, collected in a concentrated solution, and imaged in a small field of view[70].

In another technique, viable cells are recovered from an affinity-based cell isolation chip using a hydrogel coating layer which can be subsequently degraded to release cells for downstream molecular assays[95]. Both microfluidic methods provide significant promise in addressing this challenge.

## 5. Broader Perspectives on Preparing Clinical Samples

Sample preparation of biofluids has significant importance in the microfluidic and clinical communities. As we design new technologies to address current deficiencies, or apply current technologies to new biofluids of importance outlined here, we need to consider all stakeholders involved, including clinicians, clinical lab professionals, regulatory agencies, such as the FDA in the United States, and insurance companies. Most importantly, we must consider the very patients who stand to benefit from new advances in the field. It is crucial to involve stakeholders as early as possible in the development process. Making use of a multidisciplinary approach would yield fruitful innovative ideas rooted in a desire to address the real problems currently present. Also, it can also provide motivation for more vigorous investigation of cutting edge applications. Understanding what requirements a new medical diagnostic device should meet to be approved for use, as set by the regulatory agency present in a particular region of interest, should then prompt designers to incorporate these ideas during prototype development and testing. With regard to medical diagnostics, innovators should consider what their target patient population is, benchmarks that may already be presently used, and how to accurately detect differences among patients within the population of interest. Also, knowledge of reimbursement policies may guide the approach to be undertaken in terms of diagnostic development. Indeed, reduced sample preparation time and increased consistency should lead to more accurate clinical results received quicker, and eventually, improved patient outcomes. These outcomes justify reimbursement proposals through arguments of systemic savings. In addition, standardization of sample preparation should improve sample quality while also reducing cost to the clinical laboratory. Successfully addressing the challenges described in this paper is critically important in achieving the ultimate goal of translating microtechnologies from benchtop research to bedside medicine.

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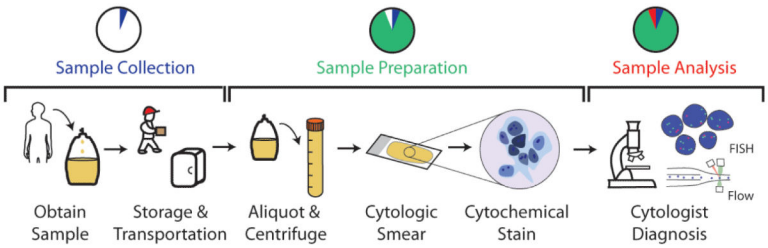


**Figure 1.**

Standard procedure in collecting, preparing and analyzing biofluid samples for cytodiagnostics. Biofluid specimens are collected from the patient and transported to a cytopathology laboratory. Freshly collected samples are prepared with multiple centrifugation and manual handling steps including cell fixing, washing, and cytochemical staining. These are necessary steps for preparing cell-based assays including cell smears, cell blocks and cell solutions. Prepared samples undergo microscopic examination, flow cytometry, and cytogenetic analysis. From the initial collection of biofluid samples until final analysis is complete, the time spent during the sample preparation portion has the longest duration, relative to sample collection and sample analysis.

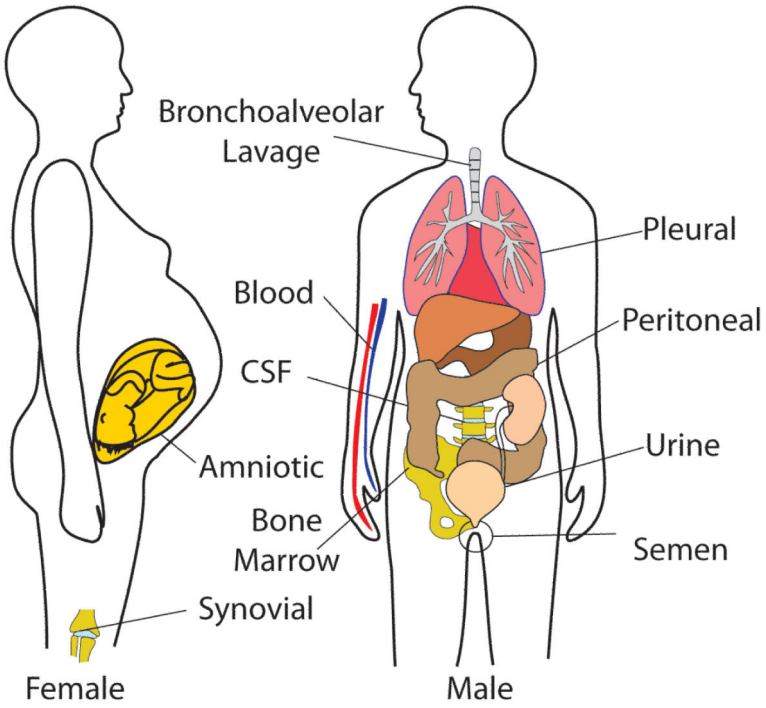


**Figure 2.**

The human system produces biofluids to complete bodily functions. The constant secretion, absorption, and circulation of fluid in the body and the rich source of cellular material make it a valuable medium for liquid-based cytology. Such liquid-based samples present vast opportunities in bridging medical and clinical applications with microfluidic technologies for sample preparation and analysis.

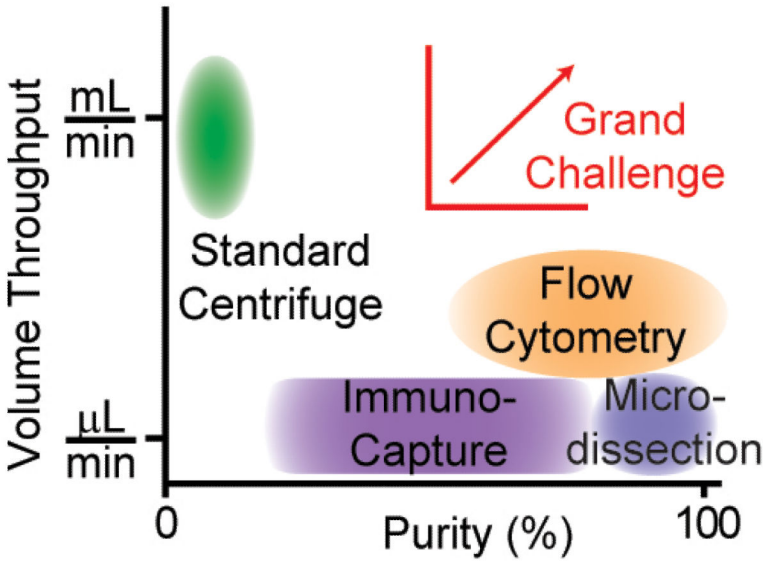


**Figure 3.**

Sample preparation of biofluids to improve throughput and purity. Macroscale instruments like the centrifuge specialize in throughput while microscale technologies emphasize on purity. Both high throughput and purity are useful alone, but for some samples it will be clinically useful to have technologies that can process fluids at high throughputs and with high purity (e.g. isolating malignant cells from pleural fluids for nucleic acid analysis).

*Lab Chip*. Author manuscript; available in PMC 2014 June 02.

**Table 1**

Body Fluids used for Cytodiagnostics

| Body Fluid | Location | Procedure Required (Level of Risk 0–3) | Volume Obtained per Test | Viscosity (mPa·s) | Diagnostics, Diseases or Applications | Incidence (Cases/year in U.S.) | Cell Type and Number (cells/mL) | Microfluidic Technologies |
|---|---|---|---|---|---|---|---|---|
| Blood | Veins | Venipuncture[1] | 1–7.5 mL[96] | 1.7–3.2 [97] | CBC | 12 M[98] | RBCs(5 B)<br>WBCs (5–10M)<br>Platelets (400 M) | 16 |
| | | | | | HIV, Hematologic Malignancies | 48 K[99] | Lymphocyte subsets CD4+ (1.5K) | 19,1 |
| | | | | | Sepsis, Biofilm Investigation | 0.75 K[100] | Bacteria(>30 cfu)[101] Fungi | 61 |
| | | | | | Metastastic Cancer | * | CTCs (5)[25] | 28 |
| | | | | | Prenatal Diagnosis | * | Fetal Cells (NA) | 4443 |
| Urine, Bladder Washings | Urethra | Voided[0] | 8–50 mL[102] | 1.2[103] | Urothelial Carcinoma | 9 M[98] | Epithelial Cells RBCs (>13 K)[104] WBCs | NA |
| | | | | | Infection | 8.2 M[105] | Bacteria (>$10^5$ cfu) | NA |
| | Bladder | Catheterization[2] | 100 mL[106] | 1 | | | RBCs and Nucleated Cells (6–9×$10^4$)[107] | NA |
| Semen | Seminal vesicles | PESA[3] | >2 mL | | In-Vitro Fertilizations | 13 M[108] | Spermatozoa (20M) | 109 |
| Pleural | Pleura | Thoracentesis[3] | 50 mL | 1.25–1.68[110] | Mesothelioma Malignancy Leukemia Infection Inflammation | 1.5 M[111] | Mesothelial cells<br>RBCs<br>WBCs<br>Cancer cells | 6,37 |
| Peritoneal | Peritoneum | Paracentesis[3] | 50 mL | 1.425[112] | | | | NA |
| Pericardial | Pericardium | Pericardiocentesis[3] | 250 mL | | | | | NA |
| Cerebrospinal Fluid | Subarachnoid space | Lumbar puncture[3] | 15 mL | 1.26–1.39[113] | Meningitis CNS Lymphoma Multiple Sclerosis | 0.4 M[114] | WBCs (>5) | NA |
| Nasorespiratory Fluid | Respiratory Tree | Bronchoalveolar Lavage – bronchoscopy[3] | 100–300 mL[115] | | Infection Malignancy Inflammation | 0.5 M[116] | WBCs[54]<br>RBCs<br>Dendritic cells Eosinophils | 117 |
| Amniotic Fluid | Amniotic Sac | Amniocentesis[3] | 20 mL | 1.006–1.008 | Prenatal Diagnosis | 124,000 (NCHS) | Differentiated Cells, Trophoblasts[118] | 41 |
| Synovial Fluid | Joints | Arthorcentesis[2] | 1–2 mL | 6[119] | Joint pain Inflammation | | Inflammatory Cells | NA |
| Bone Marrow Aspiration | Pelvis Bone | Bone marrow aspiration[3] | 300 μL | 37.5[120] | Leukemia, Multiple Myeloma, Lymphoma, Anemia, | | RBCs<br>WBCs<br>CTCs<br>Progenitor Cells | NA |

Footnote:

NCHS = National Center for Health Statistics

Risk Level (rated 0–3): 0 indicates least invasive or procedural difficulty; 3 indicates very invasive and an elevated potential risk of harm to patient.

NA = Data not available

* = currently under investigation

cfu = colony forming units

**Table 2**

Critical Challenges with Microfluidic Sample Preparation

| Challenges | Requirements | Methods | Throughput | Max Volume | Operational Cellularity | Integration Ability | Average Purity | References |
|---|---|---|---|---|---|---|---|---|
| **Concentrating Rare Cells from Large Volumes** | 1) High volumetric flow rate (mL/min) 2) Ability to parallelize channels 3) Continuous flow | DLD | 0.35 mL/h, ~$10^7$cells/min | None | Diluted blood (50%) | Detection | 99% | 69 |
| | | Inertial | ~$10^6$ cells/min | None | Diluted blood (~2%) | Detection | 80–99.71% | 62,70,121,122 |
| | | Acoustics | 80 μL/min, ~$10^8$ cells/min | None | Whole blood with sheath | Detection | 100% | 71,72,73 |
| **Preparing Small Volume Samples for Multiple Assays** | 1) Reduced dead volume 2) Sequential assays | Capillary | 0.8–20 nL/s | 300 nL | NA | Assays, Detection | NA | 74 |
| | | Vacuum | 0.5–2 nL/s | 5 μL | Whole blood | Assays, Detection | 99% | 77,78 |
| | | Centrifugal | 5 nL/s–0.1 mL/s | 2 mL | Whole blood | Assays, Detection | >99% | 79 |
| | | Mechanical Filters | 0.75 mL/min, ~$10^9$ cells/min | None | Whole blood | Assays, Detection | 90% | 29 |
| | | Immiscible Phase | 1 cm/s | 20 μL | While blood | Assays, Detection | >80% | 80 |
| **Preparing Samples with High Cellularity** | 1) Cell separation at channel surface 2) Segment highly cellular populations into single cells | Affinity-Based | 1 mL/hr | None | Whole blood | Assays, Detection | 9.2–68% | 31,30,81,82 |
| | | Cell Margination | NA | 15–70 μL | Whole blood | Assays | NA | 20,83 |
| | | Droplet | 8 μL/hr | NA | Diluted blood (10%) | Assays, Detection | 99% | 85,86 |
| | | DLD | 0.35 mL/h, ~$10^7$ cells/min | None | Diluted blood (50%) | Detection | 99% | 69 |
| **Automating Multi-Step Sample Preparation** | 1) Standardize fluid plumbing system 2) Minimize sample handling 3) Ability to stain, concentrate, and lyse cells | Inertial Solution Exchange | NA | None | Diluted blood (10%) | Assays | NA | 123 |
| | | Centrifugal | 5 nL/s–0.1 mL/s | 2 mL | Whole blood | Assays, Detection | >99% | 79 |
| | | Droplet | 8 μL/hr | NA | Diluted blood (10%) | Assays, Detection | 99% | 85,86 |
| | | Digital | NA | >1 μL | NA | Assays, Detection | NA | 92 |
| **Achieving High Purity Cell Populations** | 1) Reduce background of cells 2) Concentrate cells into small field of view 3) Make cells readily available in solution | Biopolymer System | 2 μL/min | NA | Whole blood | Assays, Detection | NA | 95 |
| | | Centrifuge Chip | 0.1 mL/h | None | Diluted blood (5%) | Assays, Detection | ~40% | 70 |