1 Introduction

1.1 Blood: The Most Information-Rich Biological Fluid

In addition to its essential role in transporting oxygen to the various organs of the body, blood is an extremely accessible and incredibly informative diagnostic fluid. Because all tissues and organs are vascularized, blood is infused with biomolecular clues that can potentially report on the physiological or pathological state of cells throughout the body. However, because blood is a complex fluid consisting of a large and varied cellular component and immense biochemical diversity, it presents key analytical challenges that must be met in order to successfully obtain pertinent information. As summarized in **Figure 1**, cells, including erythrocytes (red blood cells), leukocytes (white blood cells), and thrombocytes (platelets), constitute approximately 45% of the total blood volume, and the liquid component, or plasma, constitutes the remainder. Most diagnostic analyses on blood are typically carried out on the cell-free plasma component because the cells would otherwise introduce sample instability and matrix effects that would interfere with accurate analyte quantitation. Plasma is in itself an extraordinarily diverse medium that contains well over 100,000 different proteins spanning 12 orders of magnitude in concentration (10^{11}-10^{0} pg/mL).[1] As a result, for some analytical methods, high abundance plasma proteins, such as albumin and immunoglobulins, must be removed in order to detect low abundance proteins such as cytokines. In addition to these commonly discussed components, additional diagnostic indicators in blood include circulating tumor cells (CTCs),[2-5] DNA and even RNA,

which was long thought to be too unstable, especially in the ribonuclease-rich environment of the blood, to have any real diagnostic value.

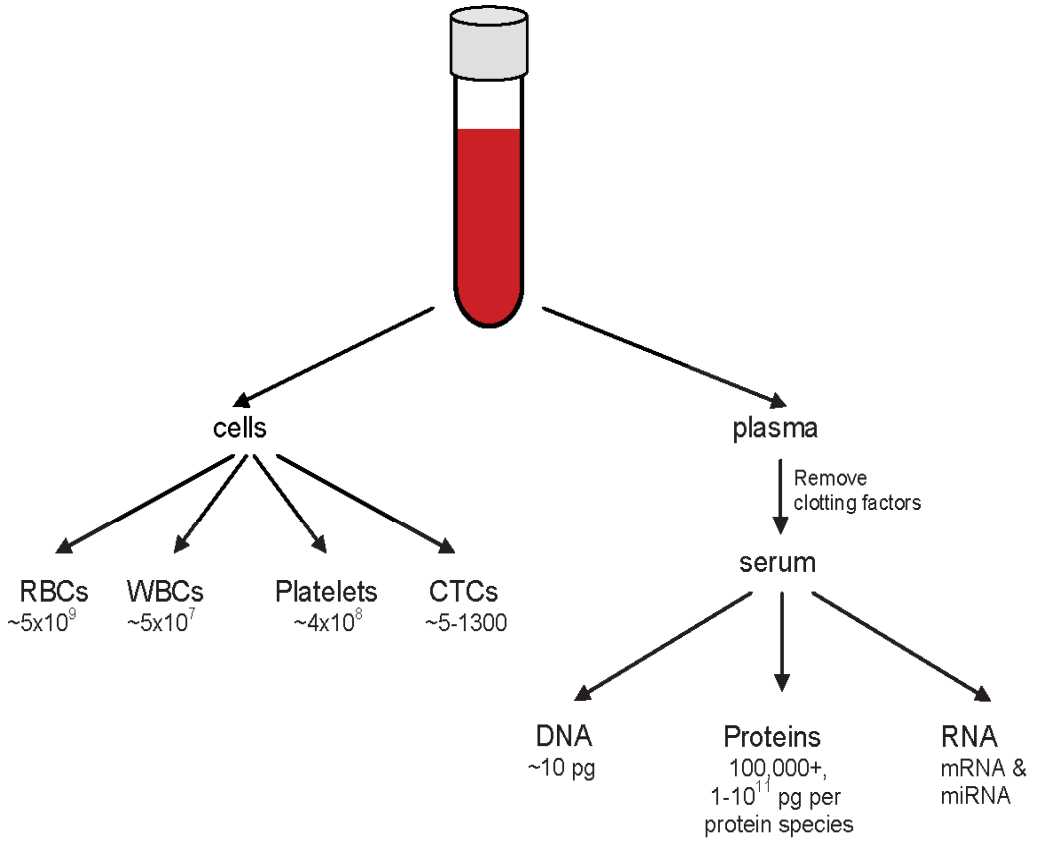

Figure 1 Composition of 1 milliliter of whole blood. (RBC=Red Blood Cell; WBC=White Blood Cell; CTC=Circulating Tumor Cell).

1.2 Proteomic Technologies

Because the plasma proteome is incredibly rich in organ- and disease-specific biomolecular detail, there has been a tremendous effort over the last decade to characterize multi-protein signatures for disease diagnosis rather than relying on single biomarkers.[6] Many studies have shown that multi-protein signatures can diagnose cancer and other diseases with greater specificity and sensitivity than single biomarkers.[1] However, these efforts have also

revealed a number of inadequacies in current proteomics technologies that have hindered their routine use for clinical diagnostic purposes. For example, 2-D poly(acrylamide) gel electrophoresis is a valuable research tool that was among the first technologies used for separation and detection of plasma proteins. However, the approach is too sample-intensive, low throughput, insensitive, and laborious for proteomic characterization of sample-limited clinical assays.[7]

Several versions of mass spectrometry have demonstrated the utility of multiplexed analyses, though they have not yet achieved broad clinical application. Surface-Enhanced Laser Desorption/Ionization Time of Flight Mass Spectrometry, SELDI-TOF/MS (substrates are commercially marketed by Bio-Rad under the name ProteinChip), are chromatographic surfaces that can be treated to have diverse affinities[5] to capture a subset of proteins based on their hydrophobicity or charge, amongst other chemical/physical properties.[6] Retained proteins are then identified by mass spectrometric analysis. Increased specificity can be realized by coating the SELDI surfaces with antibodies to proteins of interest.[7] Several reports in the literature have validated SELDI-TOF mass-spectrometric signatures for the plasma detection of prostate[8] and breast[9,10] cancer. Furthermore, the technique has shown promise for evaluating therapeutic responsiveness to chemotherapies.[11] The advantages of SELDI are its small sample volume requirements (~20 μL),[12] high sensitivity,[13] and speed. In addition, because patient classification is based on a differential spectral signature, it is not essential to be able to identify the actual proteins that are differentially expressed. However, the technology is limited in clinical utility by a lack of reproducibility,[14] sample processing time, and instrument expense.

A plasma proteomic technique that has shown great promise in recent years from the perspectives of multiplexing capability, throughput, and sensitivity is the protein microarray.[15]

Potentially, thousands of antibodies and/or proteins can be arrayed on a single protein microarray slide. In addition, the technology can be coupled with existing amplification techniques, such as gold or silver amplification or with rolling circle amplification (RCA), to greatly enhance sensitivity. Protein and antibody-based microarrays have been utilized to identify biomarkers for early diagnosis of epithelial ovarian cancers,[16,17] as well as for classification of patients with autoimmune disease and cancers of the prostate, bladder, pancreas, and stomach. In the vast majority of cases, a panel of differentially expressed plasma markers demonstrated significantly improved diagnostic accuracy as compared to each component protein. This technology is expected to continue to grow as the antibody repertoire becomes more comprehensive and as antibodies to different isoforms and post-translational modifications of proteins become available.

1.3 On-Chip Plasma Separation and Detection

The goals of separating plasma from whole blood on-chip are to be able to scale down sample volumes, increase sample processing speed, decrease the time from sample collection to detection, and avoid the variability associated with typical sample handling procedures. A number of strategies for separating plasma from whole blood on-chip have been reported in the last decade, including filtration, on-chip centrifugation, lattice sorting, and plasma skimming.

For direct filtration, size-exclusion barriers are lithographed within microfluidic channels such that cells, which are larger than openings in the barrier, are blocked from entering, while plasma passes through freely. The major disadvantage of direct filtration methods is clogging of the on-chip filter within a relatively short period of time.[18] By comparison, cross-flow filtration,

in which the fluid is filtered transverse to its direction of flow, is more resistant to clogging and can extend device longevity. [19-21]

Using the principle of deterministic lateral displacement, Davis *et al.* created an array of posts with specifically defined post-to-post and row-to-row distances, in order to "bump" cells that are above a critical hydrodynamic diameter along the angle of the array, while particles below this critical diameter flowed straight through the device without being displaced laterally.[22] In this manner, cells of different sizes could be separated from each other, and cell-free plasma could be obtained with almost no dilution of plasma and at a volume flow rate of 1μL/minute. In the lab-on-a-disk method for plasma separation, metering chambers, siphon-based hydrophilic extraction channels, plasma collection and detection chambers were positioned at different radial distances on a CD-sized disk and fluid flow between the chambers could be controlled by adjusting the spin speed. [23,24]

An alternative method for plasma separation involves plasma skimming, wherein differential flow rates at the bifurcation of a channel are utilized to preferentially guide cells into one daughter channel while cell-free plasma enters the other. Cells travel preferentially into the higher flow-rate channel due to the pressure gradient and shear forces created by the flow-rate differential. Yang and co-workers have described several devices that are able to obtain plasma purities approaching 100%.[25,26]

1.4 Thesis Overview

All of the techniques described to this point are accompanied by the limitations stemming from the inherent instability of proteins. Any degree of sample manipulation or purification therefore introduces error into the analysis.[6] Direct methods of analysis, while difficult to

implement, have distinct advantages. One such direct analytical platform, the Integrated Blood Barcode Chip (IBBC) described in **Chapter 2**, integrates plasma separation and target detection analysis onto a single chip for rapid and unadulterated plasma proteomic analysis. [27] Plasma skimming is utilized to achieve on-chip plasma purification, while multiplexed protein analysis is achieved via the DNA-Encoded Antibody Library (DEAL) technology.[28] Eight different biomarkers, corresponding to liver, prostate, and immune function, can be assayed in each lane within only ten minutes, using as little as a fingerprick of blood. In a separate experiment on pre-centrifuged plasma from breast- and prostate-cancer patients, patterns of biomarker up- and down-regulation could be discerned that distinguished the two cancers as well as subgroups of patients having the same cancer.

In **Chapter 3**, the assay panel is expanded to 35 oncologically-relevant proteins in order to define a plasma biomolecular signature that can distinguish patients with glioblastoma multiforme, the most aggressively malignant of brain tumors, from healthy controls. The panel is also used to identify a signature that can stratify GBM patients based on their responsiveness to chemotherapy (i.e. the anti-VEGF monoclonal antibody, Avastin). In both cases, the differential expression of a number of proteins yielded excellent clustering of patients into separate experimental and control groups, allowing for highly accurate classification and diagnosis of test samples. Although these studies were conducted within ELISA-like wells rather than on-chip, detection of the validated biomarker set could easily be accomplished within an IBBC. The ability to quickly run this many protein assays in parallel from a single drop of blood would be expected to significantly reduce assay costs by minimizing reagent requirements and labor. In the future, it could also facilitate dynamic biomarker monitoring by allowing assays to be performed on a minute-by-minute or hourly basis, with minimal blood loss or discomfort to the patient.

In **Chapter 4**, the computational and analytical tools that were developed in-house to quickly process and analyze large data sets are introduced and described in detail. The software takes as its input the intensity values acquired from the fluorescent scans of the assayed slides. The output files consist of statistical analyses and graphs of the entire data set, as well as files that interface in automated fashion with Excel statistics software, *Cluster 3.0*, and Java *TreeView* in order to create cluster maps for patient classification and diagnostic testing. The software also affords relatively quick and straightforward analysis of differential protein expression between experimental and control groups, thereby facilitating data-mining for disease biomarkers.

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