Lab-on-a-chip: Emerging analytical platforms for immune-mediated diseases.

Lab-on-a-chip: emerging analytical platforms for immune-mediated diseases

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Abstract

Miniaturization of analytical procedures has a significant impact on diagnostic testing since it provides several advantages such as: reduced sample and reagent consumption, shorter analysis time and less sample handling. Lab-on-a-chip (LoC), usually silicon, glass, or silicon-glass. Or polymer disposable cartridges, which are produced using techniques inherited from the microelectronics industry, could perform and integrate the operations needed to carry out biochemical analysis through the mechanical realization of a dedicated instrument.

Analytical devices based on miniaturized platforms like LoC may provide an important contribution to the diagnosis of high prevalence and rare diseases. In this paper we review some of the uses of Lab-on-a-chip in the clinical diagnostics of immune-mediated diseases and we provide an overview of how specific applications of these technologies could improve and simplify several complex diagnostic procedures.

1. INTRODUCTION

Miniaturization and automation of analytical procedures surely have a significant impact on diagnostic testing since they provide several advantages such as: reduced sample and reagent consumption, shorter analysis time and less sample handling. Analytical devices based on miniaturized platforms like Lab-on-a-chip (LoC) could provide an important contribution to the diagnosis of both common and rare diseases. Lab-on-a-chip is a device that integrates one or several laboratory functions on a single chip which may range from only millimeters to a few square centimeters in size. LoCs deal with the handling of extremely small fluid volumes, down to less than picoliters. Lab-on-a-chip devices are a subset of MEMS (Micro Electro-Mechanical Systems) devices and are often called "Micro Total Analysis Systems" (μTAS) as well. However, "Lab-on-a-Chip" indicates the scaling of single or multiple lab processes down to chip-format, whereas "TAS" are are dedicated to integrating the total sequence of lab processes [1].

The main advantage of the LoC analysis system is the possibility to create complete analytical microsystems by integrating various functional modules, i.e., mechanical, electrical and electronic, into a single device. The first LoC analysis system was a gas chromatograph that was developed in 1975 (S.C. Terry - Stanford University). However, only at the beginning of the 1990's did LoC research begin to grow as several research groups developed micropumps and flowsensors and started to elaborate the theoretical bases for integrated fluid treatments for analysis systems. These concepts demonstrated that integration of pre-treatment steps, which are usually done on a lab-scale, could extend the simple sensor functionality towards complete laboratory analysis, including for example, additional separation and sample pre-treatment steps.

The progress that has been made in microfluidics-based technologies is attributable to advances in material science, microfabrication processes and tools for the manipulation of fluids at small scales. It has led to progress in fields as disparate as molecular analysis, laboratory diagnostics, biodefense and consumer electronics. Microfluidics deals with systems involving fluid movement with geometries that have dimensions in the order of tens to hundreds of microns. Over the last 20 years there has been increasing interest in many bio-medical fields, ranging from basic research to commercial applications. LoC technologies have proven to be an interesting tool not only for genomics applications, such as capillary electrophoresis and DNA microarrays but also in a broad variety of areas including separation science [2], chemical synthesis [3,4], immunoassays [5,6], DNA amplification [7] and sequencing [8], protein

separations [9], single-cell analysis [10], tissue engineering [11] and in situ analysis of small multicellular organisms [12] (Fig1).

In this paper we review some of the uses of Lab-on-a-chip in the clinical diagnostics of immune-mediated diseases and we provide an overview of how specific applications of these technologies could improve and simplify several complex diagnostic procedures.

2. Methodology

2.1 Materials and manufacturing

Progress in materials science have given an additional push to LoC research and have opened up broader possibilities of application for these devices. Silicon and glass have been the most commonly used materials for the production of LoCs [13] due to the well known properties and fabrication techniques for this material. Nevertheless, the materials and the fabrication processes are quite expensive. However, during the last decade there has been increased focus on polymer-based chips, including polydimethylsiloxane (PDMS) [14], polycarbonate (PC) [15], polymethylmethacrylate (PMMA) [16], polyimide [17], polyethylene terephthalate (PET) [18], SU-8 [19], poly(cyclic olefin) [20], epoxy [21], and gene frame [22]. These materials are cheaper than silicon and more suitable for the production of disposable devices.

The criteria for the selection of substrate material must not only take into consideration the cost of material, the machinability and the reusability, but the end use of the device as well. Nevertheless, the key characteristics of the materials that are used include :

- optical transparency for signal detection (fluorescent, visible, UV),

- surface chemistry and reactivity,

- the ability to functionalize materials with chemical moieties for the modulation of macromolecules/cell-surface interactions,

- the degree of mechanical rigidity or flexibility.

The polymer materials listed above fulfill the key requirements, therefore they can be used for different purposes. A variety of manufacturing methods have been developed to allow efficient micromachining of polymers with different physical and chemical properties. Micromachining techniques can use two kinds of approaches:

- replication; this involves the use of a precision template or master from which many identical polymer microstructures can be made;

- direct fabrication; this includes methods such as laser ablation, optical lithography and X-ray lithography, where individual polymer surfaces are fabricated separately [23]. Replication or direct techniques could be selected depending on the number of devices required and whether the design is likely to change.

3. LoCs for genomic analysis

LoCs for genomic analysis have progressed rapidly since the completion of the human genome project and thanks to the availability of public databases that are constantly enriched by useful tools for genome data mining.

DNA analysis involves several processes which may be complex and are often time consuming. A microfluidic system for genomic analysis should integrate several activities; first of all, purification of nucleic acids from biological samples that are highly complex matrices, then DNA amplification, and finally sequence variation detection.

Several microfluidic platforms have been developed for cellular lysis and DNA purification using chemical [24, 25] or mechanical methods [26, 27, 28] and electroporation [29].

Solid phase extraction of DNA, using silica support such as micropillar [30] silica beads or immobilized particles [31, 32, 33] were the first protocols for DNA purification that researchers attempted to transfer onto microfluidic platforms, but the protocol for DNA purification in all of the systems did not change as compared to the macro system. Of note is a device in silicon / PDMS that is able to carry out the extraction of DNA from whole blood using the electrostatic interactions between the DNA and the amino groups displaced on the surface of the microchip [34]. Thanks to its high efficiency, this type of microfluidic device could find potential applications in genomics studies on samples consisting of small amounts of material [35, 36].

3.1 PCR reaction

Almost all DNA and mRNA assays involve polymerase chain reaction (PCR) for genotyping (i.e., real time PCR), for the enrichment of DNA sequences before hybridization procedures (i.e., ASO APEX), or for gene expression studies.

The existing methods for performing PCR on microchips can be divided into two categories [37]:

1. Time domain PCR where the reaction mixture is stationary inside a reaction chamber and the temperature changes in order to carry out the thermal cycles.

2. Space domain PCR where the reaction mixture is moved within a continuous channel through the three zones at a fixed temperature. This method, which can be conducted at a relatively high speed, requires larger samples and a larger microfluidic platform.

The choice of material is very important for DNA amplification. PCR is a multi-component reaction that includes reagents with different chemical properties. Moreover, the surface to volume ratio in a microfluidic device is very high therefore the probability of one or more components binding to the inner surface of the chip should not be underestimated [7].

The first devices were fabricated in silicon [38, 39], glass [40, 41, 42] or as hybrid glass / silicon [43, 44, 45, 46, 47, 48] due to the excellent thermal conductivity of silicon, and the optical properties of glass that allow for visual inspection of the reaction chamber and enable real-time monitoring of the reaction using. fluorescence detection based systems. The microchips for PCR were also constructed using plastic

materials such as SU-8 [49], polyimide [50], PMMA [51], PDMS [52, 53]. More conventional plastics, such as polycarbonate, PC [54, 55, 56], Cyclic olefins co-polymer (COC) [57] were finally used because they could be manufactured on a large scale and at a low cost.

Recently, the technology of microdrops has been introduced: the PCR mixture is contained in droplets moving through the channels of the microchip [8, 24, 58, 59]. Although the development of PCR microdrops technology is still on-going, there is considerable evidence showing the flexibility and great potential of this technique, especially for high-throughput PCR analysis of single molecules or on single cells.

3.2 Integrated systems for DNA analysis

Integration of all the processes needed to analyze DNA on a single miniaturized system, all the while providing reliable results and showing good sensitivity and specificity, is a goal that has not yet been fully achieved.

A first device of this type was built in 1996 and allowed for the integration of PCR and capillary electrophoresis [60]. This approach was further refined and improved over the years, allowing researchers to carry out multiple reactions in parallel and thus increasing the throughput [61,62].

A second example of integration was obtained between PCR reaction and DNA hybridization on glass/silicon [55] and polycarbonate [63] devices.

The situation became more complicated when an attempt was made to integrate the processes involving sample preparation and its subsequent amplification, since rather complex, multiple architecture, microfluidic reactions are required. The first attempt was made in 2001 [64], and since then similar platforms have been described [33, 65, 66], but the inability to perform parallel analyses has limited clinical use in standard laboratories.

In order to be useful in clinical laboratories, microfluidic devices must be able to integrate all the analytical processes into one chip faster than traditional systems using small volumes and handling multiple samples in parallel. These characteristics require the presence of multiple interconnected chambers for both mixing and reaction, thus further complicating the architecture of microfluidics. (Fig.2).

4. LoCs for genomic analysis: applications

A great deal of the excitement over microfluidics lies in its potential for producing revolutionary but practical devices. LoC-based assays for nucleic acid analysis have an immense spectrum of applications, mainly in molecular diagnostics and in the identification and characterization of pathogens (i.e., virus, bacteria, yeast).

Since one of the advantages of microarray analysis is the possibility to screen thousands of genes simultaneously, it could provide us with a better understanding of the mechanisms of current drug therapies, it may reveal new potential targets for therapeutic interventions and it could be used both to

evaluate transcription biomarkers for diagnostics and to predict the clinical outcome of several autoimmune diseases (67, 68, 69)

Despite the progress that has been made in understanding the genetic causes of genetic diseases, we are still at the dawn of molecular diagnosis for rare genetic disorders since it is slow, expensive, sometimes unreliable and insufficient. This results in a large gap between the goals of current genomic research and the possibility for patients and families affected with rare genetic diseases to receive a simple and rapid genetic diagnosis. Several factors make such testing difficult, including the vast number of diseases, the low number of patients per disease which thus results in a small number of requests per disease per laboratory, the nature of the disease mutation which is a private mutation, the advanced technology needed to detect mutations, and the high cost of testing.

Miniaturization and automation of analytical procedures surely have a significant impact on diagnostic testing since they provide several advantages such as: reduced sample and reagent consumption, shorter analysis time, and less sample handling. Analytical devices based on miniaturized platforms like Lab-on-a-chip could provide an important contribution to the diagnosis of these diseases.

Several LoCs for mutation detection have been developed to perform only a single step of the genotyping process, such as PCR or detection by probe hybridization [70, 71, 72, 73, 74, 75, 76].

Recently, some successful attempts have been made at integrating more than one step, but only one of the developed devices has been used in testing for the detection of the IVSI-110 G>A mutation in the human beta-globin gene allowing for correct genotyping [77].

A different scenario can be described for microfluidic devices that allow for the detection of pathogens in body fluids. We have seen several microfluidic-based platforms on the market, including Handylab (BD), that developed disposable cartridges with on-board dry reagents and a benchtop instrument. The device can potentially be used for near-patient diagnosis, and the manufacturer has released multiple tests for common hospital-acquired infections. Another example is Cepheid, which has developed an integrated benchtop analyzer ("GeneXpert") for the detection of Clostridium difficile, influenza and tuberculosis and for methicillin-resistant Staphylococcus aureus [78].

The "Verigene" is an innovative analyzer that allows for simple testing of nucleic acid and protein on a single platform. The instrumentation is benchtop and it consists of a reader and one or more processors. It combines automated nucleic acid extraction purification on one cartridge and amplification and hybridization on an microfluidic device. The technology is based on nanosphere particles which are functionalized, depending upon the application, with either a defined number of oligonucleotides or a defined number of antibodies that are specific to a particular protein of interest.

Microfluidic chip technology has also been the choice for the innovative, next generation sequencing analyzer, i.e., the Ion Personal Genome Machine (PGM™) (Life Technology). The PGM sequencer offers multiple Ion chip densities, thus allowing the sequencing from 10 Mb (Ion 314 chip) to more than 1 Gb of sequence (Ion 318 chip) in about 2 hours.

4.1 A possible application: Systemic auto-inflammatory syndromes

Systemic auto-inflammatory syndromes could be a model for the development of these technologies. These emerging morbid conditions are rather uncommon disorders and are a challenge for the clinician since they are poorly understood (the first proposals for nosological organization date back to about ten years ago), highly debilitating, and sometimes fatal if not diagnosed promptly. However, they are curable, albeit with expensive drugs (the so-called biotech drugs that target anti-inflammatory activity). Although these diseases are rare, coming to a clinical diagnosis is a mandatory step of any diagnostic algorithm for the management of fevers of unknown origin. Moreover, the opportunity to diagnose these conditions in real time thanks to emerging technologies would surely trigger the interest of the scientific community at large, and in particular, would likely lead to the request for advanced diagnostic aids by hospitals that are characterized by high case mix. Systemic auto-inflammatory syndromes are characterized by recurrent episodes of systemic inflammation involving several tissues and organs, as well as joints and skin. They are disorders of the innate immune system. Since the identification of these defects, the attention of the medical community and of clinical practice has focused on individual genes.

Hereditary periodic fever syndromes are monogenic diseases which present with recurrent inflammation and unexplained fevers, and are classified as auto-inflammatory in nature [79, 80] . The causative genes encode proteins involved in innate immunity, mainly by affecting proinflammatory cytokines and apoptosis pathways. While Familial Mediterranean Fever (FMF) is the most frequent auto-inflammatory syndrome in the Mediterranean basin and is considered the prototype of the auto-inflammatory syndrome, most hereditary periodic fever syndromes are rare diseases. FMF is a genetic disease with an autosomal recessive mode of transmission and is caused by mutations in the MEFV (marenostrin/pyrin) gene. The disease typically manifests in the pediatric population with recurrent short-lasting febrile attacks. Amyloidosis is a complication of FMF and it occurs many years after the first disease onset. Other rarer hereditary recurrent fevers are due to mevalonate kinase deficiency (MKD, gene MVK) and two dominantly inherited diseases: tumor necrosis factor receptor-associated periodic syndrome (TRAPS, gene TNFRSF1A) and cryopyrin-associated periodic syndrome (CAPS, gene NLRP3). Genetic testing for these syndromes is a logical and feasible way to corroborate clinical diagnosis.

Molecular analysis on mutational hot-spot regions (where variants that are clearly shown to be pathogenic and are frequently identified in patients are located) is a practice shared by geneticists and clinicians working in the field of hereditary periodic fever syndromes. [81].

Moreover, some mutations are present at high frequency only in specific populations [82]. For instance, the four clearly pathogenic MEFV variants are almost exclusively found in Mediterranean populations, while the p.Glu148Gln variant has a frequency of 20% in Asian countries [83].

The p.Phe479Leu is especially relevant in Greek and Iranian patients [83]. The p.Pro75Leu of TNFRSF1A gene is frequent in Arabic and African populations [84].

Accordingly, many laboratories have adopted a two-step strategy—that is, an initial search for the most common pathogenic variants, followed, if necessary, by an extended search spanning the complete coding sequence of the various genes. In this context, developing a cheap, easy-to-use, portable assay based on LoC technology, which is able to integrate nucleic acid purification, DNA amplification and signal detection in a monolithic shape, and that can quickly detect the most frequent mutations, even in a

peripheral clinical center, could relieve the workload of specialized central laboratories from first level analysis. This would result in more efficient management of hereditary periodic fever testing.

5. LoCs for immunoassays

A wide range of diseases, ranging from viral and bacterial infections, parasitic infestations, cancer, and immuno-mediated diseases are characterized by changes in protein concentrations in body fluids. High sensitivity and specificity immunoassays are routinely used to detect and quantitate clinically significant markers. Traditional immunoassay experiments can take up to several hours to complete, moreover, the use of large volumes of precious samples and antibody reagents continue to be a limit for these tests. Promising platforms for microfluidic immunoassays have been explored in recent years, but despite great efforts to develop them, LoC immunoassays are still limited in several aspects: low sensitivity, inability to quantify the target and inability to detect multiple targets at the same time. There are some examples of applications of microfluidic immunoassays in clinical diagnostics [85]. A plug-based microfluidic chip capable of performing agglutination assays for ABO and D (Rh) blood typing and blood group has been developed by Kline et al [86]. Becker and co-workers designed a microfluidic cartridge that carries out blood group determination in two minutes using an agglutination assay [87].

A point-of-care blood polymer chip that uses a sandwich immunoassay has been developed by Song et al. for the detection of cardiac troponin I [88].

The presence of autoantibodies directed to a variety of intra-cellular antigens are a diagnostic feature of autoimmune diseases [89, 90].

Although LoC and many other assay technologies have been developed over the past 50 years, indirect immunofluorescence (IIF) rem11ains the recommended autoantibody assay for screening the sera of many autoimmune conditions because it is a relatively inexpensive method and has acceptable diagnostic specificity. Another aspect of the IIF technique for autoantibody detection is that, as compared to immunometric assays, they lack the ability to precisely quantitate autoantibody titer and to distinguish autoantibodies directed to specific molecular targets [91].

On the other hand, one advantage of IIF over immunoassay is the ability to simultaneously detect multiple autoantibodies when used as the screening test of a single serum [92, 93]. Nevertheless, in the very near future this analytical approach may no longer suffice. The pathways through which autoantibodies develop and perpetuate are complex and not completely understood. There is some evidence that apoptosis plays a key role in the perpetuation of autoantibodies, and there is growing evidence that exosomes [94] and related subcellular particles released from living cells may be a source of autoantigens. Moreover, there has been substantial evidence linking human autoantibodies to major histocompatibility complex (MHC). Future autoimmune disease markers will probably include single nucleotide polymorphisms [95, 96], functional non-coding RNA molecules, novel RNA-binding proteins, novel autoantibodies, and level of circulating DNA [97, 98, 99, 100].

The spectrum of autoantibodies that is described in autoimmune diseases continues to expand, and the various autoantibody patterns observed in patients could lead to differential clinical interpretations if read in a complex diagnostic and therapeutic algorithm [101] that also takes into account genetic (i.e., MHC) information and other emerging biomarkers [97, 102, 103, 104, 105, 106]. This evolution in autoimmune

disease biomarkers needs to use multiplexed, miniaturized and ultra-sensitive technologies than can simultaneously identify multiple analytes in a routine clinical setting [107, 108, 109, 110, 111, 112, 113, 114]. LoCs could provide full integration and automation of multiple analyses on a single chip, therefore they would appear to be a promising technological platform for parallel analysis of multiple, clinically significant biomarkers.

Despite the high potential of LoC technology there are few examples of applications in autoimmune diseases [115, 116].

6. Future prospectives

Over the next few years we expect to see an increasing number of commercially available, disposable microfluidic devices. Due to rapid progress in manufacturing techniques for polymer-based chips, production costs have reached a level at which single-use applications have become economically competitive. Nevertheless, integration of all the analytical protocols on the same micro-device, which is essential for analysis on complex matrices such as biological fluids that often require pretreatment, and the parallelization of multiple analyses on a single device, could be a major critical point for the development and the diffusion of microfluidic -based devices. One of the possible scenarios for the future could be, at least for some applications, the widespread diffusion, even in remote laboratories, of cheap and easy to use Lab-on-a Chip based platforms connected to complex diagnostic software for data analysis and interconnected to medical databases hosted in reference medical centers. Not only will this likely decrease the costs, but it could allow for efficient diagnosis, both in large medical centers and in remote laboratories and peripheral hospitals.

7. Take-home messages

1. Lab-on-a-chip (LoCs) are devices that integrate one or several laboratory functions on a single chip of only millimeters to a few square centimeters in size. LoCs provide an important contribution to the diagnosis of common and rare diseases.

2. The most important advantage of the LoC analysis system is the possibility to create complete analytical microsystems by integrating various functional, mechanical, electrical and electronic modules in a single device

3. LoC technology has proven to be an interesting tool for genomics applications, such as DNA microarrays, immunoassays, DNA amplification and sequencing, protein separations, single-cell analysis, tissue engineering.

4. The development of cheap, easy-to-use, and portable assays based on LoCs technology, which is able to integrate nucleic acid purification, DNA amplification and signal detection in a monolithic shape and that can quickly detect the most frequent mutations in a peripheral clinical center, could relieve the workload of specialized central laboratories from first level analysis, thus resulting in more efficient management of hereditary periodic fever testing.

5. The evolution in autoimmune disease biomarkers needs to utilize multiplexed, miniaturized and ultra-sensitive technologies than can simultaneously identify multiple analytes in a routine clinical setting.

6. LoCs technology allows for the integration of multiple analyses on a single chip, thus requiring a relatively small amount of reagents and materials. Moreover, this technology has the potential to be completely automated.

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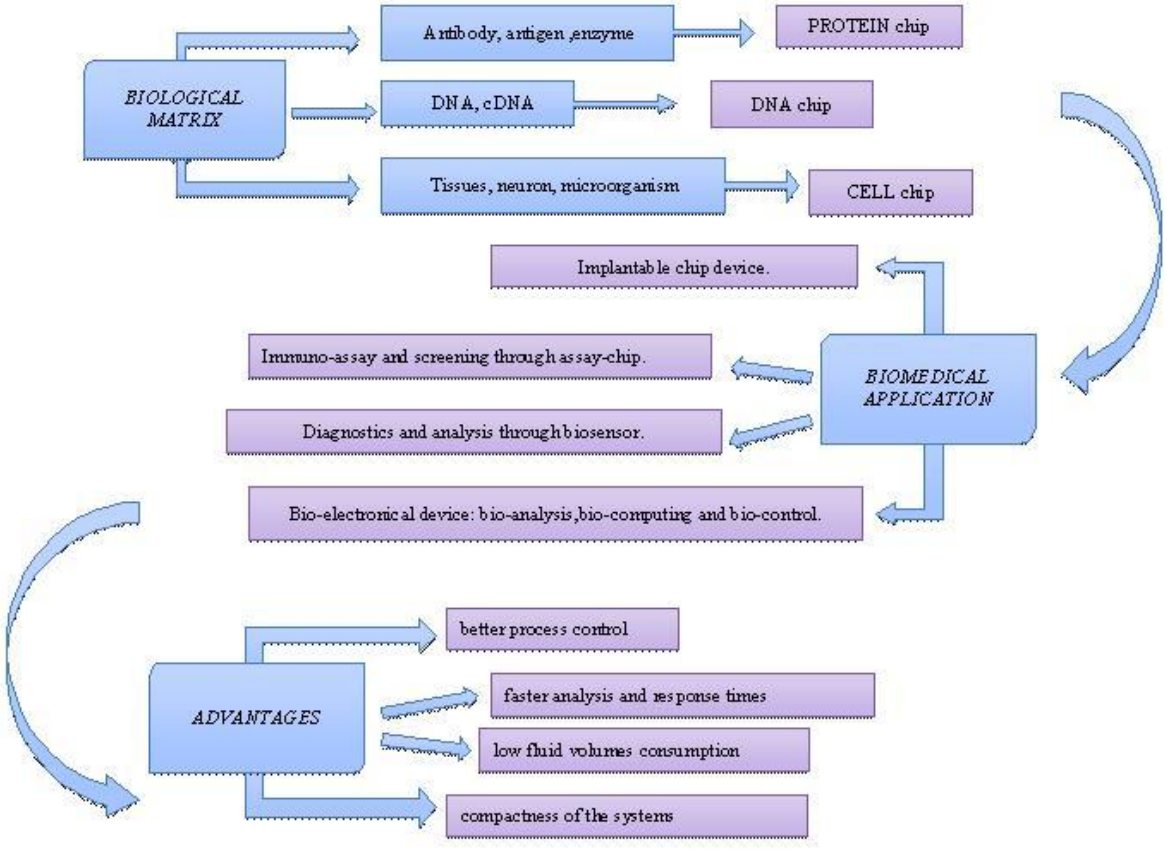

Fig. 1 Integrated microfluidic Chip

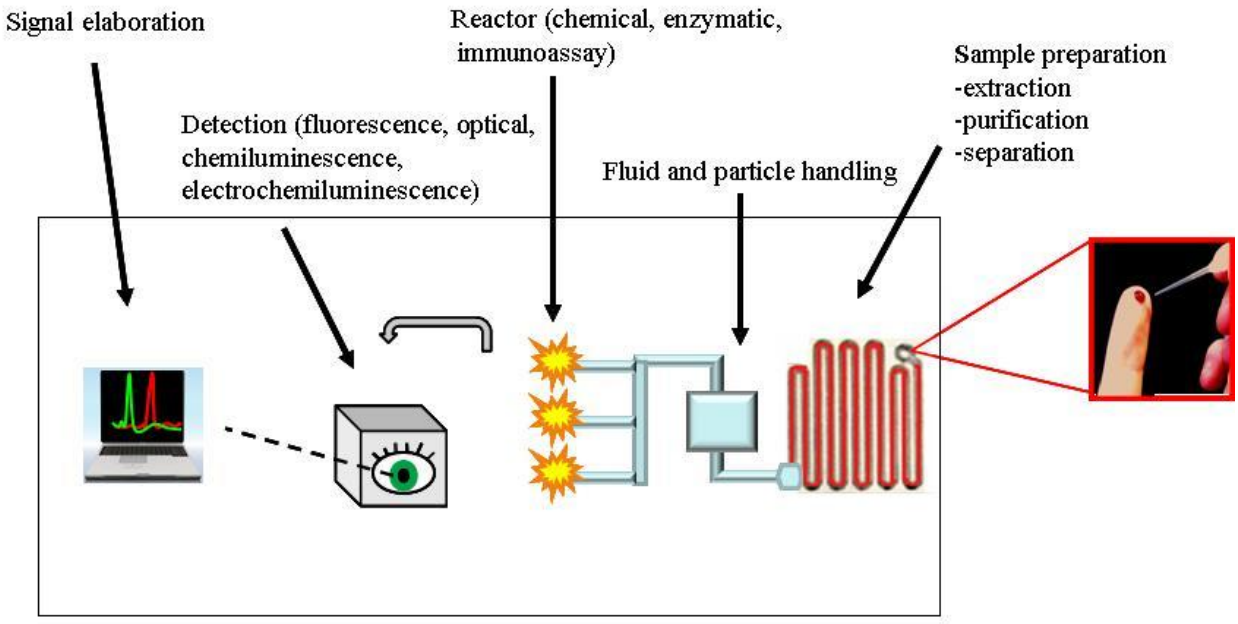

Fig. 2 Units and technologies that need to be integrated into Lab-on a-chip