Lab on a Chip

Cite this: *Lab Chip*, 2012, **12**, 464

www.rsc.org/loc

PAPER

Highly-integrated lab-on-chip system for point-of-care multiparameter analysis†‡

Soeren Schumacher,*[a] Jörg Nestler,[b] Thomas Otto,[b] Michael Wegener,[c] Eva Ehrentreich-Förster,[a] Dirk Michel,[a] Kai Wunderlich,[a] Silke Palzer,[d] Kai Sohn,[d] Achim Weber,[d] Matthias Burgard,[e] Andrzej Grzesiak,[e] Andreas Teichert,[e] Albrecht Brandenburg,[f] Birgit Koger,[f] Jörg Albers,[g] Eric Nebling[g] and Frank F. Bier[a]

Received 27th July 2011, Accepted 30th September 2011
DOI: 10.1039/c1lc20693a

A novel innovative approach towards a marketable lab-on-chip system for point-of-care *in vitro* diagnostics is reported. In a consortium of seven Fraunhofer Institutes a lab-on-chip system called "Fraunhofer ivD-platform" has been established which opens up the possibility for an on-site analysis at low costs. The system features a high degree of modularity and integration. Modularity allows the adaption of common and established assay types of various formats. Integration lets the system move from the laboratory to the point-of-need. By making use of the microarray format the lab-on-chip system also addresses new trends in biomedicine. Research topics such as personalized medicine or companion diagnostics show that multiparameter analyses are an added value for diagnostics, therapy as well as therapy control. These goals are addressed with a low-cost and self-contained cartridge, since reagents, microfluidic actuators and various sensors are integrated within the cartridge. In combination with a fully automated instrumentation (read-out and processing unit) a diagnostic assay can be performed in about 15 min. *Via* a user-friendly interface the read-out unit itself performs the assay protocol, data acquisition and data analysis. So far, example assays for nucleic acids (detection of different pathogens) and protein markers (such as CRP and PSA) have been established using an electrochemical read-out based on redoxcycling or an optical read-out based on total internal reflectance fluorescence (TIRF). It could be shown that the assay performance within the cartridge is similar to that found for the same assay in a microtiter plate. Furthermore, recent developments are the integration of sample preparation and polymerase chain reaction (PCR) on-chip. Hence, the instrument is capable of providing heating-and-cooling cycles necessary for DNA-amplification. In addition to scientific aspects also the production of such a lab-on-chip system was part of the development since this heavily affects the success of a later market launch. In summary, the Fraunhofer ivD-platform covers the whole value chain ranging from microfluidics, material and polymer sciences, assay and sensor development to the production and assembly design. In this consortium the gap between diagnostic needs and available technologies can be closed.

1. Introduction

It is beyond question that lab-on-chip systems will be one of the mainstream technologies in the next decades.[1–4] Especially in the context of *in vitro* diagnostics the role and significance of lab-on-chip systems increases heavily due to two trends.[5–8] Firstly, diagnostics has to become more cost-effective since the pressure on health-care systems increases.[9,10] Reasons for that, among

[a]*Fraunhofer Institute for Biomedical Engineering IBMT, Am Mühlenberg 13, D-14476 Potsdam, Germany. E-mail: soeren.schumacher@ibmt.fraunhofer.de; Web: www.ivd-plattform.fraunhofer.de; Fax: (+49) 331/58187-119; Tel: (+49) 331/58187-314*
[b]*Fraunhofer Institute for Electronic Nano Systems ENAS, Technologie-Campus 3, D-09126 Chemnitz, Germany*
[c]*Fraunhofer Institute for Applied Polymer Research IAP, Geiselbergstr. 69, D-14476 Potsdam, Germany*
[d]*Fraunhofer Institute for Interfacial Engineering and Biotechnology IGB, Nobelstr. 12, D-70569 Stuttgart, Germany*
[e]*Fraunhofer Institute for Manufacturing Engineering and Automation IPA, Nobelstr. 12, D-70569 Stuttgart, Germany*
[f]*Fraunhofer Institute for Physical Measurement Technique IPM, Heidenhofstr. 8, D-79110 Freiburg, Germany*
[g]*Fraunhofer Institute for Silicon Technology ISIT, Fraunhoferstr. 1, D-25524 Itzehoe, Germany*

† Published as part of a LOC themed issue dedicated to German Research: Guest Editors Andreas Manz and Holger Becker.

‡ Electronic supplementary information (ESI) available. See DOI: 10.1039/c1lc20693a

others are demographic changes, an increasing global population, globalization or climate change.[11,12] Secondly, new trends in biomedicine lead to a new way of diagnostics with a higher degree of complexity because more parameters are being measured. Especially, in the context of personalized medicine, diagnostics can further be used as a tool for screening and monitoring of patients, leading to a customized therapy and subsequently to therapy control.[13,14] This indicates the need for a rapid and more frequent diagnostic tool at the point-of-need. In addition, more information should be accessible from a single sample leading to the concept of multiplex assays. The most advanced examples of this kind are microarrays, where thousands of probes are arranged on top of a glass slide or on a piece of silicon wafer.[15–17] Microarrays are mainly used for nucleic acid analysis and up to now only very few have found their way to routine diagnostics. One example of this kind is the cytochrome c assay for genetic variations, an example for companion diagnostics.[18] However, the trend towards multiplexing will continue, especially regarding protein and peptide arrays for biomarker research or drug screenings.[19,20]

Lab-on-chip systems are able to address the requirement of providing a fast multiplex diagnostic at low costs and at the point-of-need.[21–23] *De facto*, many interesting scientific approaches have been developed which are on different levels of design complexity and adaptability. Nevertheless, at present just a few systems are on the market due to the fact that many hurdles have to be taken in order to launch such a system as a commercial product. There are right now some examples which are already on the market. Some of these such as the Triage system from Alere or the DXpress Reader from Life Sign LLC are based on lateral-flow like cartridges which are after incubation with a sample quantified in reader. These systems are already commercially available for different diagnostic purposes such as cardiac infarction or drug of abuse testing. These systems can be seen as a first step since they are dependent on already existing technology and hence copying their drawbacks. Beside these also other systems are available which provide a more innovative cartridge design. Among others, systems like Afinion from Axis-Shield, Magnotech from Philips, Biocartis detection cartridge or Qualigen Fast Pack IP from Qualigen show differently designed cartridges for different application fields in the detection DNA as well as proteins or small molecules. Due to the innovative cartridge design the adaption of common assays is in most cases difficult and hence their easy transfer is not possible.

In more detail, lab-on-chip technologies have a broad range of design and complexity.[24] To serve the needs for systems which can be easily operated at the point-of-need, integration is one of the keywords to be addressed. Today, lab-on-chip systems usually do not include any active components. Pumps and valves have to be added from outside, typically by a read-out machine that also provides all liquids needed to run the biochemical assay. Thus a mechanical interface is unavoidable between microfluidics and the read-out device in order to connect channels and tubing for the transport of liquids. This interface always is a source of technical problems. Not only dead volumes increase with any connecting part, but also sources of leakage appear that will distort the outcome, spoil the performance or even the whole instrument. The opposite may also occur, that is any kind of dust or dirt may distort the outcome or even stop the liquids at all.

In a joint project of seven Fraunhofer Institutes a lab-on-chip system has been established which possesses a high degree of integration and modularity to serve the needs of the market as well as developments in biomedicine.[25] With this consortium it is possible to cover the whole value chain necessary to establish a lab-on-chip system that is ready for the market. The different sub-projects are described ranging from the microfluidic cartridge design, assay development and its transfer into the cartridge, the use of different sensor principles to the production of these cartridges to match the economies of scale.

2. Concept of the Fraunhofer ivD-platform

The aim of the Fraunhofer ivD-platform has been the establishment of a lab-on-chip system which meets the following three requirements.

2.1 Open platform for all kinds of common (bio-)medical assays

Common laboratory assays can be based on the detection of different substances such as small molecules (hormones, metabolites, drugs *etc.*), larger antigens (proteins, peptides, glycopattern *etc.*) or DNA. Hence, the Fraunhofer ivD-platform had to be designed for these different assay types. In addition, the Fraunhofer ivD-platform had to be open for different sample matrices such as urine, serum or whole blood. In this context, the main advantage provided by the Fraunhofer ivD-platform is to use common assay types which are then transferred to the cartridge design avoiding the transfer to other assay types such as bead-based ones. For the detection of small molecules or antigens there had to be the possibility to monitor a binding reaction between these analytes and the binding molecules. These binding molecules could be antibodies but also other binding molecules such as molecularly imprinted polymers, aptamers or specifically binding peptides. For DNA detection hybridization between probes and analyte DNA is carried out, usually following its purification and amplification. In addition, also different concentration ranges have to be measured. This is addressed by the possibility to choose either an optical or an electrochemical read-out.

2.2 High degree of integration

During the development stage it was one design rule to integrate as many steps as possible onto the lab-on-chip cartridge. In this regard, steps like pumping, reagent reservoirs, simple heating and sensors were integrated on the chip. This enables the production of a read-out and processing device to be quite small since pumps and reagent bottles are not necessary anymore. Hence, the base station has solely to provide the electronics necessary for the controlling and, in case of an optical read-out, the optical parts such as LED and camera or, in case of the electrochemical read-out, a potentiostat. This concept offers the possibility to make such base station unit as small as possible. Only for DNA amplification by a common PCR a more complex heating and cooling system has to be provided. Thus, depending on the application, the system can be miniaturized to serve the needs of the assay.

2.3 Possibility for serial production

In every step of the conception of the Fraunhofer ivD-platform the possibility of serial production was an important aspect. Hence, only materials which can be produced at low costs at high numbers have been used in the design. Furthermore, the complexity of the system itself was held to be as low as possible to circumvent demanding and cost-intensive assembly and processing steps. Since this concept was directly used to obtain the first cartridges a prototype status of the system was avoided, *i.e.* during the development phase materials and processes were used which are also used for a real mass production of such a system. Therefore, biomedical assays are directly adapted to a system which can be later launched to the market.

To further strengthen the possibility for a market launch, the whole process was structured in a work-flow (Fig. 1). In the first step a biomarker is chosen which is characteristic for a medical indication in a certain concentration. Consequently, an assay has to be developed which is able to detect and quantify the biomarker within the diagnostic window. This assay is then transferred into the lab-on-chip design by choosing the appropriate sensor principle (either electrochemical or optical). Further, the cartridge has to be adapted to this specific assay by surface treatments of some parts, different ways of spotting or the filling of the appropriate reagents. This workflow can also be understood as the different steps of production for a customized lab-on-chip system.

3. Cartridge-design

3.1 General aspects

The general cartridge concept is depicted in Fig. S1 (ESI). The layout is adapted from the layout of half a microtiter plate having outer dimensions of 60 × 80 mm^2 which is nearly the size of a credit card. The latest setup of the cartridge contains 8 reservoirs with volumes of 150 µL (reservoir 1–4) and 75 µL (reservoir 5–8), one sample reservoir (up to 45 µL), a sensor area and a waste reservoir. To avoid any fluidic interface to the read-out instrument, reagents can be stored directly in the reservoirs and are transported through the channel system by means of integrated, one-shot low-cost pumps.

The ivD-cartridge currently has a height of about 3.8 mm and consists of two main parts (Fig. 2).[26] The bottom part holds the micropumps and their electrical contacts. The injection molded top part made of cycloolefin copolymer (COC) holds the liquid reservoirs as well as the fluidic channel system. Filling of the reservoirs is achieved through filling holes on the top (automatic filling with a robot will be described later). The bottom part of the cartridge consists of a printed circuit board (PCB) which is used to contact the pumps and, if needed, valves. As already discussed, one of the main aims being addressed is a high degree of integration. Thus, the pumping, sensors and heating have been integrated onto the cartridge.

For sample preparation and PCR-application a similar layout is chosen. In this case the sensoric side of the cartridge is maintained. However, on the backside of the PCB there are additional electrodes for contacting additional pumps and valves as well as a second injection molded part. Here, reservoirs are placed to perform the sample preparation and PCR. To detect the amplified DNA, the PCR product is pumped to the sensoric side of the cartridge.

All parts used in the assembly are available at low costs to facilitate this system as a disposable. Nevertheless, the ivD-cartridge itself is in its size not yet adapted to a certain application. The goal of this design was to provide a basis for a variety of different types of biomedical assays. For a certain application the cartridge design will be appropriately adapted and only reservoirs really used for the assay are designed. Hence, the system can be even smaller than the here proposed credit card size, at lower costs with less waste.

3.2 Integrated pumping

As described elsewhere, pumping is achieved by the uni-directional deflection of a membrane due to electrolytic gas generation inside a hydrogel. Since external pumps are not necessary a high level of miniaturization is possible. By the electrolytically generated gas pressure, the membrane is displaced directly into the meandering reservoir channel, leading to a direct contact between the membrane and the liquid in the reservoir (Fig. S2, ESI). With this principle it is possible to displace liquids in a defined way into one direction by applying just an electrical

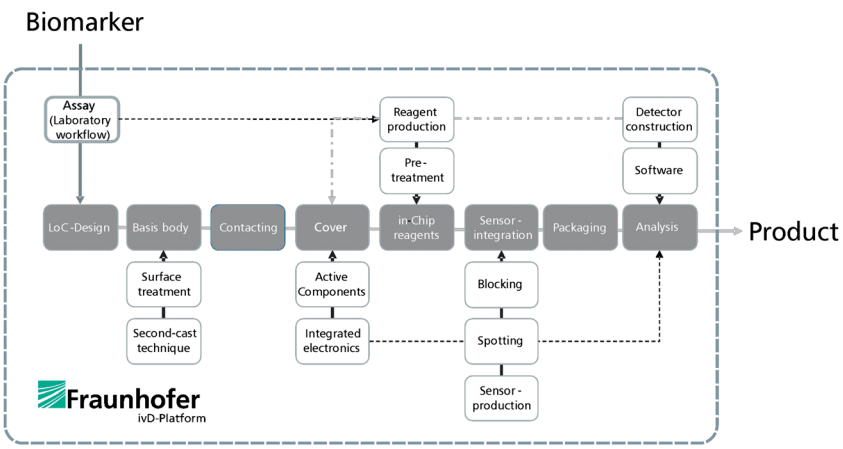

Fig. 1 Separable steps in development and production.

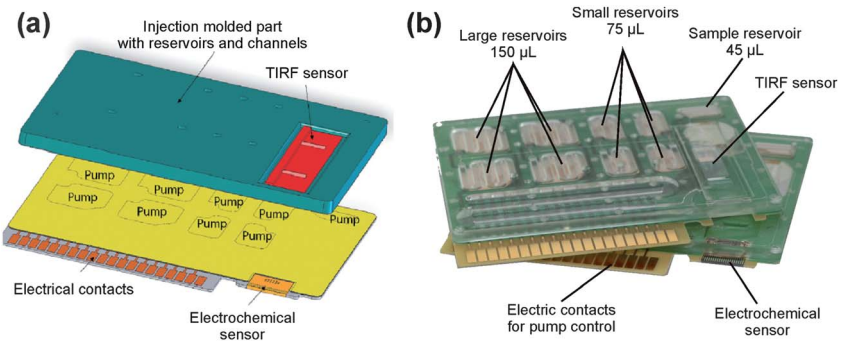

Fig. 2 Schematic (a) and real (b) pictures of the ivD-cartridges. The two cartridges (b) show different sensor configurations: the bottom cartridge is equipped with an electrochemical sensor, the top cartridge holds the optical TIRF sensor.

current.[27,28] In contrast to other proposed setups using electrolytic gas generation the use of a proper deformable polymer membrane improves both storage stability of the electrolyte and the controllability of the flow rates under different ambient conditions while it still allows the displacement of rather large liquid volumes.

As the membrane deflection is non-linear, using a constant current to drive the pumps would lead to flow rates which are more than 10 times larger at beginning of the pumping process than at the end. Therefore, the electric current and thus the deflection rates of the membrane are compensated by control electronics to achieve stable flow rates in a range between 0.1 to 1µL s^{-1} during the whole emptying process of each reservoir. As the overpressure underneath the membrane quickly reaches values larger than 1–2 bar, pressure drops in the channel system due to capillary effects or flow resistance can be neglected in most cases. A representative pumping sequence is shown in Fig S3 (ESI).

3.3 Integrated sensing

The cartridges can be configured to work either with a silicon-based electrochemical biosensor or an optical, fluorescence-based biosensor (Fig. 2). Since the system was designed to be as modular as possible the same injection molded parts can be used. Just *via* the assembling process it is possible to choose whether the sample and all reagents are directed to the optical slide or *via* another channel to the electrochemical sensor. Both sensors can be joined onto the cartridge in an easy way. By joining the sensors into the cartridge the flow cell is defined which matches the requirements of the sensor principle as well as that of the binding reaction.

3.3.1 Optical transducer. The measuring principle of the transducer is total internal reflectance fluorescence (TIRF).[29] Light is coupled in the foil through the prisms at an angle at which the in-coupled light is totally reflected at the boundary between the foil surface and the medium within the flow cell. The angle of incidence at the boundary between the polymer material and the surrounding medium is very close to the critical angle of total internal reflection to have an intensive evanescent field near the surface. This evanescent field is used for illumination of fluorescence-labeled biomolecules such as antibodies or DNA.[29,30] The fluorescence excited *via* the evanescence field is detected by a CCD camera, located after a filter for elimination of the excitation light. An advantage of this principle is that only fluorescent dyes located up to about 100 nm above the slide surface are illuminated. Accordingly, measurements can be accomplished in the presence of a fluorescent liquid or even in non-transparent liquids. Only the molecules which are located close to or directly bound to the surface are detected. By this principle, real-time measurements of binding reactions are possible. As an optical transducer a thin polymer foil slide is used, which at the same time works as a substrate for the immobilization of capture molecules such as antibodies as well as a wave-guiding element (Fig. 3a). Two prisms are located on the slide enabling in- and out-coupling of light. To ensure a cost-effective production the slide is produced by precision injection molding. The dimensions of the slide are 10 × 40 × 0.2 mm. An important property of the used polymer is its low auto-fluorescence. After various material tests a cyclic olefin polymer (COP) was chosen. For that material investigations showed that the auto-fluorescence is considerably lower than that of other polymers.[31]

3.3.2 Electrochemical transducer. As an electrochemical transducer a silicon-based biochip is used (Fig. 3b).[32,33] This biochip is manufactured on 6- or 8-inch silicon wafers. For

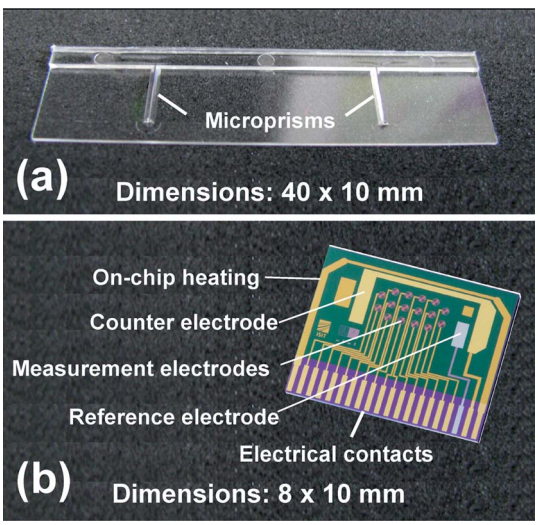

Fig. 3 (a) COP-slide with microprisms for optical transduction; (b) Electrical biochip for single electrode redox cyling.

production of the chips the following main process techniques are used: thermal oxidization of the base layer, vapor deposition for gold and iridium metallization, photolithography followed by a lift-off process for metal structuring, plasma enhanced chemical vapor deposition of silicon nitride as an insulating layer and photolithography followed by dry etching for structuring of the silicon nitride layer. The chip dimensions are 8 × 10 mm carrying 16 gold electrodes with a diameter of 350 μm each. Additionally, a gold counter electrode and an iridium/iridium-oxide reference electrode are integrated on this chip.

The transducing principle in this case is "single electrode redox cycling".[34,35] Instead of using a fluorescently labeled biomolecule for detection, the biomolecule is labeled with an enzyme. This enzyme is able to generate an electrochemically active substance which can be determined in parallel by 16 electrodes on the chip.

3.4 Integrated temperature control for hybridization

Considering the (bio-)medical assay especially for DNA analysis, additional heating steps for hybridization are required. Also for the binding of an antigen and an antibody a stable temperature could be beneficial for a reliable binding reaction. Consequently, the cartridge itself is able to provide such a kind of heating area below the sensor field of the optical waveguide. To obtain this heating, tiny resistor elements with a negative temperature coefficient (NTCs) are integrated onto the PCB *via* soldering. Since these parts can be produced at low cost, they are also suitable for disposable applications. Although these NTCs are commonly used as temperature sensors, the dissipated heat inside these resistors is used for heating. In this regard, the same control units as used for the pumps can be employed since the value of the current controls the temperature, thus simplifying the necessary control circuitry.

Four similar NTCs with a resistance of 4.7 kΩ are placed on the PCB in a small cavity below the sensing region to realize heating within the targeted temperature window up to 70 °C. To ensure a well-defined distribution of the temperature, a thermally conductive paste is filled into the cavity. By operating the NTCs with constant currents of 4.2 and 12 mA temperatures of 42 and 65 °C, respectively, could be stably realized. However, an additional control feedback has been established in the control electronics to allow for operation at different ambient conditions. Fig. S4 (ESI) shows the temperature distribution within the sensor region.

4. Instrumentation: processing and read-out instrument

4.1 Aspects for the design

Since the microfluidic cartridge exhibits a high degree of integration, the read-out and processing unit for the system has to combine the circuitry necessary for the cartridge control and the hardware which is required for the particular sensor. Furthermore, in case of the PCR-module devices for the realization of large heating and cooling rates have also to be provided.

The instrument was designed with regard to its later application in for example a physician's office. Hence, a user merely needs to place the cartridge into a compact reader (Fig. S5, ESI). In addition, the instrumentation holds a barcode scanner which enables a personalized data processing using the patients ID. Subsequently, a predefined process is performed automatically by the system: pumping of different reagents, waiting during incubation time, read-out and presenting results in an appropriate way.

4.2 Optical transducer and PCR-module

For a cartridge with an optical slide, the read-out unit has to hold mainly the optics necessary for the measurement. By using the TIRF principle, light has to be coupled into the polymer chip using microprisms integrated into the polymer element. The used excitation light source is a high power LED which enables a cheap production of the read-out unit. The used peak wavelength of the LED is at 627 nm with a spectral bandwidth of 26 nm. A typical value for the luminous flux is 465 lm. A collimator and a cylindrical lens were installed to shape the LED-beam to a bar which is slightly broader than the prism. This ensures the coupling of light into the waveguide even if slight geometrical changes of the slide due to production tolerances are present. The optical set-up is shown in Fig. S6 (ESI).

Then, a fluorescence image is taken using an uncooled CCD camera positioned behind an imaging optics and an interference filter for suppression of the excitation wavelength. For the detection of fluorescence, an interference filter with a bandpass from 663 nm to 737 nm is used. The filtered fluorescence light is spatially resolved to detect single dots in a microarray. The read-out area is 6 × 10 mm^2 in which more than 240 dots can be detected using a dot pitch of 0.5 mm. The camera can work at integration times between 62 μs and 67 s. For biochip applications, usually an integration time below 10 s is sufficient. The images are normalized to a specified integration time. The intensity of each dot is determined and the background of the area around each dot is subtracted from the intensity values of each dot separately. Using calibration curves, the concentration of each parameter is determined.

In order to detect and measure fluorescence signals over a wide dynamic range that is common in multiparameter analyses multiple images can be taken per microarray, each image with increasing exposure time. The detected images are analyzed fully automatically. A rectangular grid is fitted to the microarray image and each dot is characterized. As a background correction the grey values of the background in the vicinity of a dot are subtracted from the value of the whole grey value found for each dot. By integration of the pixels and calculating their median a value for each dot is created. These values are subsequently normalized with regards to their integration time.

With special emphasis on the DNA detection, the read-out unit has to provide heating and cooling cycles for a polymerase chain reaction in which a target DNA can be amplified. In the ivD-System a peltier element with a maximum heating power of 9.5W is used for heating and cooling. On one side of the peltier element a block of copper, a heat sink and a fan are installed for cooling. On the other side of the peltier element a heat spreader of aluminium is fixed. In this heat spreader the temperature control sensor is placed. This setup allows a fast, accurate and reliable temperature profiling which is a prerequisite for a PCR-reaction to be specific.

4.3 Electrochemical transducer

For the electrochemical transducer a simpler read-out system can be designed. For this purpose microprocessor controlled systems have been developed which are able to control the nine pumps on the cartridge for the assay sequences and the heater for temperature control at hybridization steps. Beside the circuitry for pump controlling a potentiostat has been inserted which enables the measurement of the redox-active species. The measurement of the redox-active species is realized by switching the potentials of the electrodes. The integrated potentiostat carries out chronoamperometric measurements. Due to the constant production of the electrochemically active substance, the current rises over time and the signal can be quantified. Since just electric components are needed, the electrochemical transducer can further be miniaturized.

5. Example assays

5.1 Microarray technology

In the context of novel emerging diagnostics like personalized medicine or companion diagnostics but also due to new finding in biomedicine, the influence and significance of multiparameter analyses are growing enormously.[22] Many attempts have been reported to develop a multiparameter analysis and in many cases bead-based approaches are being used. Especially for lab-on-chip systems the use of magnetic beads is reported by many research groups and companies since the easy displacement and control of these beads makes this effort promising. Nevertheless, an additional development step for transferring common and established assay formats to this bead-based approach is required. Hence, the Fraunhofer ivD-platform targets an easier and more generic approach. Starting from conventional immunoassays the transfer to a multiparameter analysis *via* the design of a microarray was chosen to be the most adaptable method due to the fact that microarrays are reported for a great variety of different binding molecules such as peptide, DNA or antibodies just to name the most common ones. Their application can either depend on immobilized binding molecules such as antibodies or on immobilized antigens to provide detection of allergens or infectious diseases. That is why different parameters have to be considered as type of interaction, kind of surface, the chemistry for immobilization and the appropriate blocking reagents. In the following sections four different example assays are described: The simultaneous detection of the C-reactive protein (CRP) and prostate-specific antigen (PSA) based on the optical transducer (5.2.1) or the electrochemical chip (5.3), the detection of DNA on the optical waveguide externally (5.2.2) and on-chip (5.2.3) making use of the integrated temperature control.

5.2 Optical transducer

5.2.1 Protein assay: simultaneous detection of CRP and PSA.

As first example a sandwich assay for the simultaneous detection of CRP and PSA has been established. Both parameters were chosen because of their different clinically relevant detection limits. CRP as an acute phase protein is a biomarker which displays an infection. Its threshold value is about 10 mg L^{-1}. In contrast, PSA as a marker for prostate cancer has to be detected in the lower μg L^{-1} range showing a cut-off value of 2.5 μL g^{-1}. By choosing these parameters, the dynamic range of the system could be evaluated while showing that both medical ranges can be covered.

For the chosen sandwich assay an antibody towards a certain antigen, *e.g.* CRP or PSA is immobilized on the COP-slide. After incubation with the sample the sensor field is washed with a washing buffer to remove all unspecifically bound molecules. After the washing step a fluorescently labeled antibody is rinsed over the sensor field. This antibody can bind from the opposite site of the antigen while the concentration of the bound CRP can be quantified *via* the fluorescence label.

Before spotting, the COP-slide was pretreated in a comparatively short procedure containing different washing steps. After desiccation, a spotting layout of 10 x10 spots was chosen (Fig. S7, ESI) and prepared using a non-contact piezo spotter (Sciflex arrayer). Each spot contains about 1.5 nL of 1mg mL^{-1} polyclonal antibody in bicarbonate buffer solution (100 mM, pH 9.6). Besides the specific antibodies for CRP and PSA two non-specific antibodies were spotted as negative control. Furthermore, guiding spots and carbonate buffer as a spotting control were deposited.

To run an assay within the cartridge, reservoirs were filled with required reagents, *i.e.* different washing buffers enriched with detergent, water and labeled detection antibodies (AB) specific for PSA or CRP. Afterwards the sample was applied *via* the sample reservoir.

The assay was performed by setting-up a pumping sequence to realize the allocation of the reagents such as PBS buffer, detector antibodies *etc.* in a certain sequence (Table 1). This was done *via* a user-friendly interface of the read-out unit. The used flow rates were 0.1μL s^{-1} for sample and antibodies and 1μL s^{-1} for the cleaning buffer. After the pumping protocol, including washing and incubation sequences, the microarray was evaluated using the optical read-out unit.

Since CRP as well as PSA were chosen to be detected simultaneously, it was important to validate the degree of cross-reactivity. Hence, the assay was first performed with one analyte. Then the values for the specific antibody-antigen interaction were compared with values obtained for the reaction of the

Table 1 Pumping protocol for simultaneous detection of CRP and PSA within the ivD-cartridge

Step	Time	Process
1	120 s	Pumping of PBS, pH 7.0 solution (pump 1)
2	200 s	Pumping of sample (pump 9 - slow pumping)
3	120 s min	Flushing with PBS (pump 2)
4	160 s	Pumping of detection antibodies – 1μg mL^{-1} (pump 5 - slow pumping)
5	70s	Flushing with PBS (pump 3)
6	160 s	Labeled Cy5 antibody – 1μg mL^{-1} (pump 6 - slow pumping)
7	70 s min	Flushing with PBS (pumps 4 + 7 + 8)

Total assay time: approximately 15 min

antibody with the other analyte. It was found for both antibodies that CRP and PSA can cross react but the degree can be neglected since there is always a quantifiable difference in their signal (Fig. S8, ESI).

Once the crossreactivity had been established a concentration dependent measurement of both parameters was performed. Therefore increasing concentrations of PSA and CRP were added into the sample reservoir and the assay was performed within 15 min according to the pumping sequencing. After completion, the values could be quantified giving high dynamic measurement ranges of 5–1060 ng mL^{-1} for PSA and 16–2000 ng mL^{-1} for CRP which are suitable for medical application (Fig. 4).

5.2.2 DNA assay: multiparameter detection of human pathogens in a microarray format on the polymeric waveguide (off-chip). As an example for point-of care nucleic acid detection, several human pathogens involved in sepsis were selected, comprising prokaryotic pathogens as well as the human pathogenic fungus *Candida albicans*. The main issue in sepsis diagnosis is the time-span from collecting a patient's blood sample to indentification of the causative agent. By utilization of microarray technologies this time period can be minimized dramatically. Further improvement results especially from transferring the assay to the microfluidic system.

Short pathogen-specific oligonucleotides for four sepsis causing microbes (*Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida albicans*) were used as probes for the hybridization of Cy-labeled PCR products from 16*S*- or 18*S*- ribosomal DNA (rDNA), respectively. With this approach, a specific detection of pathogen DNA could be accomplished on such a COP slide.

The workflow of this nucleic acid assay consists of several distinct steps, which so far were carried out with external devices. The first step in the pathogen detection assay is the isolation of nucleic acids from a biological sample, which is meant to be implemented in the sample preparation unit. From here, the purified microbial nucleic acids will be pumped into the PCR compartment, where the following step, a multiplex PCR, takes place. Two degenerated primer sets are used for the amplification and simultaneous fluorescence labeling of species-specific sequences of prokaryotic and eukaryotic ribosomal DNA sections. This on-chip PCR will be realized by the implemented peltier element capable of heating and cooling the mixture directly inside the PCR compartment. After fragmenting the PCR products by DNase treatment, they are pumped over the prespotted COP slide in a fourth step. Heating of the hybridization chamber ensures specific binding of the target DNA to the probes. After the washing steps, the hybridized array gets directly evaluated by the prisms of the TIRF waveguide. Due to its microfluidic nature, the total assay time is reduced significantly.

For the functionalization of the COP surface, different surface modifications and surface coatings were applied. The surfaces were treated with barrier discharging, poly lysine coating, silicium oxide coating, epoxy silanization and a combination of these procedures. The most consistent results in application, reproducibility and comparability to commercially available coated glass slides were found in the combination of barrier discharging and epoxy silanization which therefore were selected for further proceeding. This leads to the additional benefit that conventional protocols and reagents for glass slides can simply be transferred to hybridization on COP slides.

Spotting of the COP slides, which in comparison to glass slides are considerably smaller and more flexible, could be accomplished by utilization of custom-manufactured carriers with a conventional ring-pin spotter. Thus, handling of COP slides is as easy accomplished as handling of glass slides.

For the detection of pathogenic DNA in a sample, a species-specific sequence from the respective ribosomal DNA is amplified with two universal, degenerated primer pairs in a multiplex PCR. Simultaneously, PCR products are labeled by incorporation of Cy-labeled nucleotides. Before hybridization PCR-products are fragmented by DNase digestion to ensure specific binding to the short pathogen-specific oligonucleotides.

One example of a hybridized COP slide array is shown in Fig 13. The target sequences were amplified in a multiplex PCR with template DNA from *S. aureus* and *P. aeruginosa*, respectively. Amplification was carried out in 30 cycles with Phusion High Fidelity Polymerase in the provided GC buffer and in presence of dNTPs, Cy5-dCTP and the specific primer pairs (10μM each). Different controls were integrated into the assay to guarantee specificity. First of all, a spotting control was used by spotting Cy-labeled oligonucleotides from sequences of *S. aureus*. Secondly, Cy3-labeled antisense oligonucleotides for *C. albicans* probes were spiked into the hybridization mixture as a measure for hybridization accuracy and efficiency. The specifically

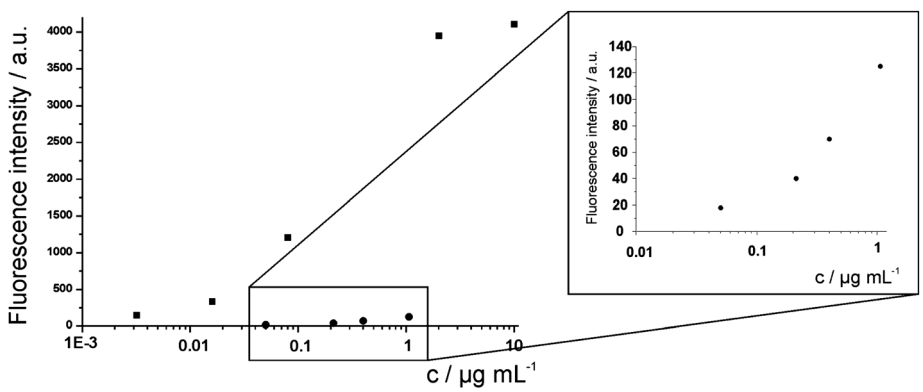

Fig. 4 Concentration dependence on CRP (■) and PSA (●) obtained within the ivD-cartridge.

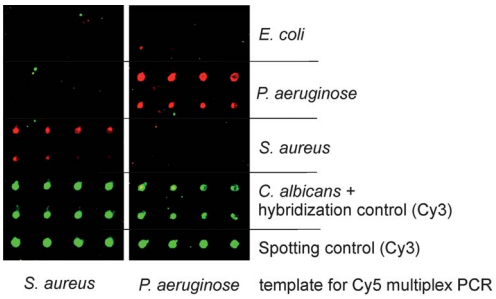

Fig. 5 False-color fluorescence image of spotted and hybridized COP slides. On the right side the applied species-specific oligonucleotide probes are indicated, the last lane of was used as a spotting control. On both slides, Cy3-labeled *C. albicans* antisense oligonucleotides were spiked into the hybridization mixture as a control for successful hybridization. The red spots are specifically hybridized PCR products from the multiplex PCR with the respective template DNA, indicated at the bottom of the image.

Table 2 Pumping and heating sequence of hybridization experiments within the ivD-cartridge

Step	Temperature	Process
1	42 °C	Preheating
2	42 °C	Prehybridization buffer (BSA-containing 4 × SSC)
3	25 °C	Water (deionized)
4	65 °C	Heating
5	65 °C	Sample flushing (DNA-strands in 4 × SSC buffer)
6-8	25 °C	Washing buffer I–III (2 × SSC; 0,1 × SSC; 0,01 × SSC)

hybridized Cy5-labeled PCR fragments clearly demonstrate the specificity of the assayed probes (Fig. 5). Also, the high signal to noise ratio combined with good signal intensities indicate that the COP slides can be considered as qualitatively comparable to commercial glass slides.

Our current efforts focus on implementation of the complete sample preparation process into the lab-on-chip device, including PCR amplification of target DNA.

5.2.3 DNA hybridization on-chip including integrated heating. To show the applicability of the integrated heating below the sensor field a hybridization experiment was conducted as a first application. Several nucleic acid probes (probe 1–3) were immobilized on a COP-slide and the spotting design was chosen to be a grid of 4 × 5 spots. In addition to these three probes also a spotting control and a negative hybridization control were deposited onto the slide. To perform the assay the reservoirs were filled with approximately 90 μL each prehybridization buffer (4 × SSC, 1% BSA), water and different washing buffers with changing ionic strength (2 × SSC; 0,1 × SSC; 0,01 × SSC). The sample, *i.e.* differently labeled DNA-strands in 4 × SSC buffer, was inserted into the sample reservoir (approximately 50 μL) and a certain pumping protocol was started (Table 2). Besides the pumping sequence also different temperatures were adjusted within the sensor field and maintained for a certain time span. For the assay, mainly two different temperatures were used: 42 °C as prehybridization temperature and 65 °C for hybridization.

As displayed in Fig. 6 it can be seen that the probes hybridized specifically at 65 °C. Furthermore, as a negative control the same pumping sequence was used without any heating. Hybridization did not take place at all. This clearly confirms that the heating was successful and a temperature at 65 °C is essential for the success of the hybridization.

5.3 Electrochemical transducer

Besides the optical read-out also an electrochemical detection based on "single-electrode redoxcycling" on microarrays has been performed. In case of the electrochemical chip the spatial resolution is defined by the electrode array where capture molecules such as antibodies are immobilized on the electrode positions. The electrochemical chip contains 16 electrodes with a diameter of 350 μm and so 16 different capture molecules could be deposited. For comparison to the optical read-out also CRP and PSA are transferred to this design.

The spatial resolution for deposition of the capture molecules is realized by a piezo-driven microdispensing device (nanospotter (Gesim)). On each position droplets of approximately 12 nL volume containing the different capture molecules in phosphate buffered saline (PBS) were spotted. The immobilization of these capture molecules was carried out by thiol-gold interaction and hydrophobic adsorption. The spotted chips were designed as disposable to detect only one sample each.

In the example system for detection of PSA and CRP the electrical biochips are spotted as shown in Fig. S9 (ESI). Each three positions contain the positive control, the anti-CRP antibody, the anti-PSA antibody or a negative control. As positive control a capture protein is spotted to allow direct binding of the enzyme label while bovine serum albumine (BSA) serves as a negative control as well as blocking agent. 100–500 μg mL^{-1}.

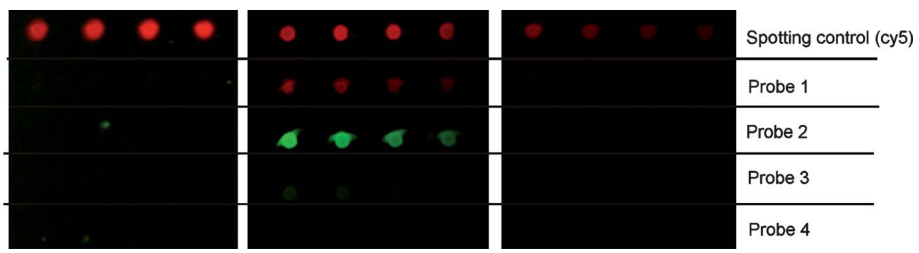

Fig. 6 False-color fluorescence image of a microarray with immobilized DNA probes, before their hybridization, after hybridization at 65 °C and pumping sequence shown in Table 2, and the experiment without any heating at room temperature.

Table 3 Pumping protocol for performing an electrochemically based assay within the ivD-cartridge in about 20 min

Step	Time	Process
1	40 s	Pumping of PBS (pump 5) – with integrated chip test
2	7 min	Sample incubation (pump 9) with switching between stop flow - slow
3	1 min	Flushing with PBS (pump 1)
4	4 min	Detector antibody 1μg mL^{-1} (pump 6) (stop flow – slow pumping)
5	1 min	Flushing with PBS (pump 2)
6	4 min	Enzyme-conjugate (pump 7) (stop flow – slow pumping)
7	2 min	Flushing with PBS (pumps 3 + 4)
8	20 s	Pumping of substrate solution (pump 8)
9	30 s	Stop flow for detection
Total assay time: 20–30 min		

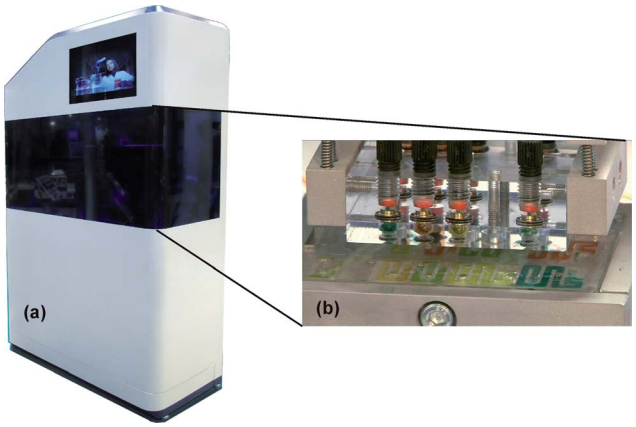

Fig. 8 Already realized filling module within a stand-alone (a) module. (b) Automated filling head for reagent and washing buffer charging into the ivD-cartridge.

To perform an assay, the reagents are filled into the ivD-cartridge and a specific pumping sequence including washing and labeling is applied. The whole assay can be performed in about 20 min (Table 3). In contrast to the optical read-out an electrochemically active substance has to be present for signal generation after rinsing the electrodes with the detection antibody. Thus, the last step performs pumping of a substrate which can be converted by the enzyme to yield p-aminophenol (p-AP, 1 mg mL^{-1}), a redox-active substance. To obtain a spatial resolution of the generated p-AP additional valves are closed to ensure a static reaction volume on the chip.

As an analytical signal the production of p-AP is then monitored at each of the 16 electrodes in parallel. Hence, spatial resolved reduction and oxidation behavior of p-AP can be monitored. This is achieved by switching between +200 mV and −350 mV with a frequency of 1 Hz and measuring the resulting electrical current. The mostly linear current slope within the first few seconds of stop flow is proportional to the concentration of PSA or CRP in the sample. It could be shown that the assay is sensitive to CRP and PSA (Fig. 7). Furthermore, the capability for storage of all compounds within the system was evaluated. It has been demonstrated that the capture molecules on-chip as well as the required assay reagents are stable for more than 1 year at 4 °C.

6. Assembly and production line

There have been several attempts to develop automated manufacturing processes for the entire lab-on-chip system. We decided to go for a hybrid approach using automated and manual process steps in order to minimize the costs for the implementation phase. A consequent response to increasing costs, time and innovation pressures in a systematic analysis of the requirements and a specific design concept.

The work piece carrier is designed in a special manner, so that it can be used throughout the production line and for all component parts. The proposed approach and developed planning system involves optimal selection of the processes with proper manufacturing steps. We decided to use a very modular manufacturing line in order to meet all the requirements: variability of the order volumes, investment costs of the manufacturing equipment and performance of the quality tools. We propose a manufacturing line with several modules that represents the individual functionalization (coating, spotting *etc.*), assembly and quality steps (Fig. S10, ESI) The system then allows the realization of the required packaging and production techniques in a biomedical environment.

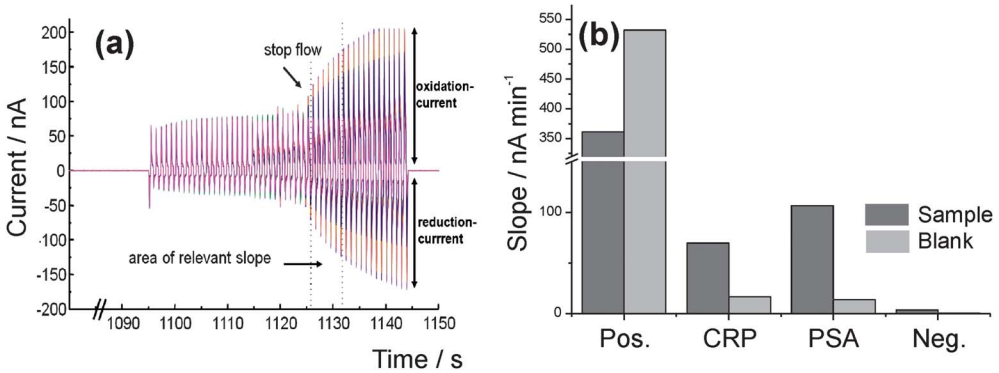

Fig. 7 (a) Raw data obtained from redox cycling at a frequency potential switching at 1 Hz; (b) measurements for a sample (dark bars) containing 10 ng mL^{-1} CRP as well as PSA in comparison to a blank (light bars).

A modular system design enables a very flexible reaction for the particular requirement in the application areas of molecular diagnostics. Therefore, the assembly line provides a decent grade of autonomy in order to guarantee a high quality production whether for low or high number of pieces. Up to now, two modules have already been realized. More modules are designed and are in the process of realization.

Beside of the development and consulting during the implementation of biochemical methods and planning of the interlinked processes, concepts for some new technologies have also been developed. Especially coating and dispensing technologies have been proved and adapted for the requirements of the LoC manufacturing systems. The aim was an automation of wet processes in order to deliver high manufacturing quality. The filling of the cartridge with analysis liquid is a key process to ensure a proper and reproducible result. An automated process for precise dispensing of eight different reagents with an adjustable volume has been realized (Fig. 8b).

Each single module can be used as a stand-alone equipment (Fig. 8a), but it can also be integrated in an overall manufacturing control to build up a production line. Regarding the increasing complexity of technical devices the ergonomic issues have been considered in order to guarantee a highly user-friendly operation.

7. Conclusion and further perspectives

The Fraunhofer ivD-platform has been developed and established to provide a technological platform for various kinds of (bio-) chemical assays. Hence, the technological basis for transferring all necessary steps, like washing or labeling as well as heating, could be realized in this system. Even intense cooling and heating cycles as needed for PCR could be obtained and installed within the read-out unit. Due to the high degree of integration the system can be miniaturized as, for example, external pumps are not required anymore. In addition, the system can be produced in mass production processes matching the economies of scales. First test assays could already be realized on this system. In this regard, more assays will be adapted to the platform. This will be done aiming at two different targets. One target will be the establishment of different assay types to show the technological applicability of the cartridge. The other target will be to define multiparameter assays for a certain application which will act as a lead application with a known market share and potential for a market launch.

Acknowledgements

The work of the Fraunhofer ivD-platform was supported by the FhG internal programs under grant no. WISA 819 638. The consortium would like to gratefully acknowledge the support from the Fraunhofer Society and from the advisory board over the last five years.

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