FACULTY OF TECHNOLOGY AND MARITIME SCIENCES

Application-oriented microfluidic LOC devices for the detection of microorganisms, toxic chemicals and serological biomarkers

Thesis submitted for the degree of Philosophiae Doctor

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Horten, 2015

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Preface

The thesis is submitted by a candidacy for the degree of Philosophiae doctor from the Department of Micro and Nano Systems Technology (IMST), Faculty of Technology and Maritime Sciences (TekMar), Buskerud and Vestfold University College (HBV).

The doctoral study was conducted from June 2011 to February 2015. Associate Professor PhD Tao Dong is the primary supervisor.

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Finally, my deepest thanks are presented to my family members for their understanding and selfless support during my PhD candidacy period.

Abstract

Lab-on-a-chip (LOC) technology has advanced over the past several decades. As miniaturized multiphase multistep reactors, LOCs are suitable for the implementation of complex liquid phase reactions in the field of biomedical detection. This doctoral thesis focused on the development of new LOC devices and relevant functionalities for various application cases, including microbe detection, water-safety testing, while also presenting preliminary studies on the measurement of signal molecules in blood.

The application-oriented R&D (research and development) strategy was employed in the studies on a series of biomedical LOC devices. Technological challenges, e.g. optimization of on-chip quantification NASBA (nucleic acid sequence-based amplification) protocols, system design etc. were resolved in individual cases. Based on a low-cost R&D strategy, most LOCs in this doctoral work were developed for bench-top equipment as disposable components. It is worth noting that a methodological concept is also developed and summarized in this thesis, i.e., the selection of decision support mechanisms (DSMs) for biomedical LOC devices.

The **DSM** is the supporting mechanism generating measurable signals in the LOC, and translating them into a meaningful conclusion, which can help users to make decisions in the real application field.

Namely, DSMs can enable LOC-based testing and are thus a distinguished feature of application-oriented LOC devices. The author categorized the research achievements presented in this thesis into three distinctive classes, as follows:

1) Conventional DSMs commonly employed in current biomedical experimental approaches.

Articles I, II and III focus on this category of DSMs. The series of LOCs in these reports were modelled on standard microtiter plates, and thus these chips are completely compatible with the microplate readers commonly utilized in biological laboratories.

Article I reported the design, fabrication and validation of a disposable 43-chamber LOC device for quantitative detection of waterborne pathogens. Its principle was similar to conventional ELISA(enzyme-linked immunosorbent assay) tests for microbes, entitled

immuno-NASBA assay. A synthetic peptide and two common waterborne pathogens (*Escherichia coli* and *rotavirus*) in artificial samples were used to validate the LOC functions, which indicated that the LOC device has the potential to quantify traces of waterborne pathogens with high specificity.

Article II described the development of a LOC platform for environmental investigations into aquatic microorganisms, on the basis of quantitative NASBA (Q-NASBA). The LOC system was composed of a membrane-based sampling module, a sample preparation cassette, and a 24-channel Q-NASBA chip. The DSM of the LOC was derived from the polyurethane-foam-unit (PFU) method, which has been widely used to evaluate environmental pollution in fresh water. The multifunctional system could simplify and standardize the complicated processes of microbial detection.

Article III addressed the implementation of a 384-chamber microfluidic simulator with the incorporated functions of pathogen identification and antimicrobial susceptibility testing (AST) for personalizing the antibiotic treatment of urinary tract infections (UTIs). Its DSM adopted the diagnosis principle of conventional ATP bioluminescence assay (ATP-BLA) for living microbial cells.

2) Unconventional DSMs for contrived LOC systems.

The second class of biomedical LOCs employs tailored DSMs, which are still derived from known working principles. However, the concrete mechanisms and diagnostic criteria are arbitrary in contrived functional LOCs.

Article IV investigated a bioluminescent-cell-based continuous-flow device, termed a ‘living-cell chip’, with a self-defining DSM to implement real-time broad-spectrum online monitoring of water quality. The prototype integrated a T-junction droplet generator, counter-flow micro-mixers, and time-delay channels. The LOC device can mix the water sample and *Vibrio fischeri* cell sensors into a droplet flow, and incubate the droplets in the time-delay channels before optical detection. Its DSM relies upon the relationship between the toxicant concentration in the water sample and the relative luminescence units of the bioluminescent cells in the running droplets, which is obviously different from the conventional intermittent method of ISO11384. The proposed LOC system shows great promise for an early warning system against potential toxicant chemicals in drinking water.

3) Developing prognostic/diagnosis DSMs for biomedical-database-dependent LOC systems with the aid of computational modelling.

The third category relies on computational modelling within a large-scale medical/healthcare database, which is currently emerging and is not yet completely developed.

Article V reported a pilot study on the design and fabrication of the LOC device for signal molecule profiling in blood. Articles VI proposed the potential roadmap and preliminary experimental approach for the construction of a human signal-molecule-profiling database (HSMPD) by the use of the former LOC device, leading to prognostic/diagnosis DSMs in the future.

List of papers

This thesis is based on the following articles:

I. **Compatible Immuno-NASBA LOC Device for Quantitative Detection of Waterborne Pathogens: Design and Validation,** Xinyan Zhao, T. Dong*, Z. Yang, N. Pires and N. Høivik, *Lab on a chip*, 12(3), 2012, Pages:602-12. DOI: 10.1039/c1lc20836e.
URL: http://pubs.rsc.org/en/content/articlehtml/2012/lc/c1lc20836e

II. **Multifunctional Sample Preparation Kit and On-Chip Quantitative Nucleic Acid Sequence-Based Amplification Tests for Microbial Detection,** Xinyan Zhao and T. Dong*, *Analytical Chemistry*, 84(20), 2012, Pages:8541-8. DOI: 10.1021/ac3020609. (**Nivå 2 journal**)
URL: http://pubs.acs.org/doi/abs/10.1021/ac3020609

III. **Rapid Identification and Susceptibility Testing of Uropathogenic Microbes via Immunosorbent ATP-bioluminescence Assay on a Microfluidic Simulator for Antibiotic Therapy,** T. Dong* and Xinyan Zhao, *Analytical Chemistry*, 87(4), 2015, Pages:2410-8. DOI: 10.1021/ac504428t. **(Nivå 2 journal)**
URL: http://pubs.acs.org/doi/abs/10.1021/ac504428t

IV. **A Microfluidic Device for Continuous Sensing of Systemic Acute Toxicants in Drinking Water,** Xinyan Zhao and T. Dong*, *International Journal of Environmental Research and Public Health*, 10(12), 2013, Pages: 6748-63. DOI:10.3390/ijerph10126748.
URL: http://doi.org/10.3390/ijerph10126748

V. **Design and Fabrication of Low-cost 1536-chamber Microfluidic Microarrays for Mood-disorders-related Serological Studies,** Xinyan Zhao and T. Dong*, *Sensors*, 13, 2013, Pages: 14570-82. DOI:10.3390/s131114570.
URL: http://doi.org/10.3390/s131114570

VI. **Design and Experimental Approach on the Construction of Human Signal-Molecule-Profiling Database,** Xinyan Zhao and T. Dong*, *International Journal of Environmental Research and Public Health*, 10(12), 2013, Pages: 6887-908. DOI:10.3390/ijerph10126887.
URL: http://doi.org/10.3390/ijerph10126887

Additional articles are partially encompassed in this thesis:

VII. **A 48-well microfluidic platform based on immuno-NASBA assay to quantify traces of soluble biomarkers in water environment,** Xinyan Zhao, T. Dong*, Z. Yang, F. Karlsen, E.B. Egeland and N. Pires, *Proceedings of 2011 International Symposium on Water Resource and Environmental Protection (IEEE ISWREP)*, Xi'an, China, Volume 2, 2011, Pages:1586-9. DOI: 10.1109/ISWREP.2011.5893334.
URL：http://ieeexplore.ieee.org/xpl/articleDetails.jsp?arnumber=5893334

VIII. **1196-chamber Compatible LOC Device with Bubble Valves for Signaling Molecular Profiling in Blood,** Xinyan Zhao and T. Dong*, *Proceedings of the 5th International Conference on BioMedical Engineering and Informatics (IEEE BMEI 2012)*, Chongqing, China, Volume 1, 2012, Pages:684-7. DOI: 10.1109/BMEI.2012.6512949.
URL: http://ieeexplore.ieee.org/xpls/abs_all.jsp?arnumber=6512949

IX. **A Household LOC Device for Online Monitoring Bacterial Pathogens in Drinking Water with Green Design Concept,** Xinyan Zhao and T. Dong*, *Proceedings of 35th Annual International Conference of the Engineering in Medicine and Biology Society* (*IEEE EMBC'13*), Osaka, Japan, Volume 1, 2013, Pages:1708-11. DOI: 10.1109/EMBC.2013.6609848.
URL: http://ieeexplore.ieee.org/stamp/stamp.jsp?arnumber=6609848

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Abbreviations

ACTH: adrenocorticotropic hormone;

AST: antimicrobial susceptibility testing;

ATP: adenosine triphosphate;

ATP-BLA: ATP bioluminescence assay;

BOD: biochemical oxygen demand;

CFU: Colony-forming unit;

CLIA: chemiluminescent immunoassay;

CMV: culture medium veins;

CPC: cetylpyridinium chloride;

Ct: the threshold time;

Dabcyl: 4-[4-(Dimethylamino)phenylazo]benzoic acid N-succinimidyl ester;

DEP: dielectrophoretic;

DEPC: diethyl pyrocarbonate;

DNA: deoxyribonucleic acid;

DSM: decision support mechanism;

> **Notes**: The DSM is the supporting mechanism generating measurable signals in the LOC, and translating them into a meaningful conclusion, which can help users to make decisions in the real application field.

EGF: epidermal growth factor;

ELISA: enzyme-linked immunosorbent assay;

EPO: erythropoietin;

E. coli: Escherichia coli;

$FMNH_2$: reduced flavin mononucleotide;

FSH: follicle-stimulating hormone;

GnRH: gonadotropin-releasing hormone;

HCG: human chorionic gonadotropin;

HPV: human papillomavirus;

HSMPD: human signal-molecule-profiling database;

IATP-BLA: immunosorbent ATP-bioluminescence assay;

IFNγ: interferon-gamma;

IGF-1: insulin-like growth factor 1;

LH: luteinizing hormone;

IgY: chicken egg yolk antibodies;

IL: interleukin;

IMRAMP: immune real time amplification;

LOC: lab on a chip;

LOD: limit of detection;

I/O: input/output;

MB: molecular beacons;

MEWQ: monitoring equipment for water quality;

mRNA: messenger RNA;

μTAS: micro total analysis systems;

NASBA: nucleic acid sequence-based amplification;

ORP: oxidation-reduction potential;

PBS: phosphate buffered saline;

PBST: phosphate buffered saline with 0.05% Tween-20;

PC: personal computer;

PCR: polymease chain reaction;

PDMS: polydimethylsiloxane;

PFU: polyurethane-foam-unit;

PMMA: polymethyl methacrylate;

PMT: photomultiplier tube;

POC: point of care;

PS: polystyrene;

Q-NASBA: quantitative nucleic acid sequence-based amplification;

RCHO: long-chain aliphatic aldehyde;

RNA: ribonucleic acid;

RNase: Ribonuclease;

R&D: research and development;

R.F.U.: relative fluorescence unit

R.L.U.: relative luminescence unit;

RT-PCR: Real-time polymerase chain reaction

SMP: signal molecular profiling;

S. aureus: staphylococcus aureus;

S. cerevisiae: saccharomyces cerevisiae;

TBI: translational bioinformatics;

TNF-α: tumor necrosis factor-alpha;

TOC: total organic carbon;

TSH: thyroid-stimulating hormone;

UTI: urinary tract infection;

VEGF: vascular endothelial growth factor;

V. fischeri: vibrio fischeri;

WHO: world health organization;

1 Introduction

Lab on a chip (LOC) refers to a precision instrument-related microchip of only a few square millimetre or centimetre sizes. A LOC is able to perform one or more functional laboratorial tests. It can handle very small volumes of biological fluids with complex biochemical reactions, and can finally output useful information or perform special functions.

The ability to perform laboratory operations on a small scale using miniaturized LOC devices is very appealing to end users.[1] Small volumes will reduce the time taken to synthesize and analyse a product; the unique behaviour of liquids at the micro scale allows greater control of molecular concentrations and interactions, and reagent costs and the amount of chemical waste can be much reduced.[1] In particular, LOC technology in the relevant fields of life science has broad application prospects. Biomedical LOC devices involve interdisciplinary knowledge of microsystems, microfluidics, mechanical engineering, surface physics, biochemistry, medical science *etc*. Although LOC technology has been studied for several decades, the majority of relevant research achievements currently stay in the laboratory stage, instead of entering the application stage. This doctoral thesis focuses on the challenges with regard to the application-oriented LOC technology in the biomedical field.

1.1 Background of application-oriented LOC systems

Probably owning to the interest tendency of LOC researchers, a large number of LOC-related projects have focused onto the academic value, instead of the applications and social impacts of the research achievements. However, the majority of relevant research achievements are currently on hold in the laboratory stage, instead of entering the commercial stage, implying that the research and development (R&D) strategy of LOC technology requires optimization. From the initial stages of R&D, the ultimate goals are to solve some of the existing problems in practice and to ensure that the innovative results

can be pragmatic. In fact, LOC technology is an innate applied science with great potential in the fields of biology and medicine, and has broad commercial prospects. The superiority of LOC systems is attributed to not only small sizes, but also other features derived from their spatial traits. When the volume of a biochemical reaction system is dramatically compressed, LOC exhibits some advantages which are the imperfection of conventional bench-top devices (Figure 1). The advantages of biomedical LOCs are summarized as follows:

- LOCs are particularly suitable for multiphase-involved multifield-coupling multistep biochemical assays. Besides, it is easy to implement large-scale parallel detection in one on-chip test, i.e., high-throughput testing;
- LOCs are often small and portable, which can reduce material costs in the mass production afterwards;
- Generally, LOCs are highly integrated; consequently, automation and standardization of LOC devices are easily realized;
- Compared with conventional laboratory tests, the consumption of energy, reagents and tested samples in a LOC assay is rather low, but the useful information obtained from the tests could be the same;
- Due to short diffusion distances, large specific surface areas, small heat capacity and other physical factors in the microscale, biochemical reactions probably achieve higher efficiency in LOCs;

Currently, LOC technology is still developing. The possible application fields of LOCs are shown in Figure 2. For example, this study involved in two relevant application fields. The first field is medical detection and diagnosis, referring to varied biological samples and chemical solutions employed in multistep biological reactions, and the second field is water quality monitoring, particularly the detection of waterborne pathogens, which involves multistep biological testing of aquatic samples.

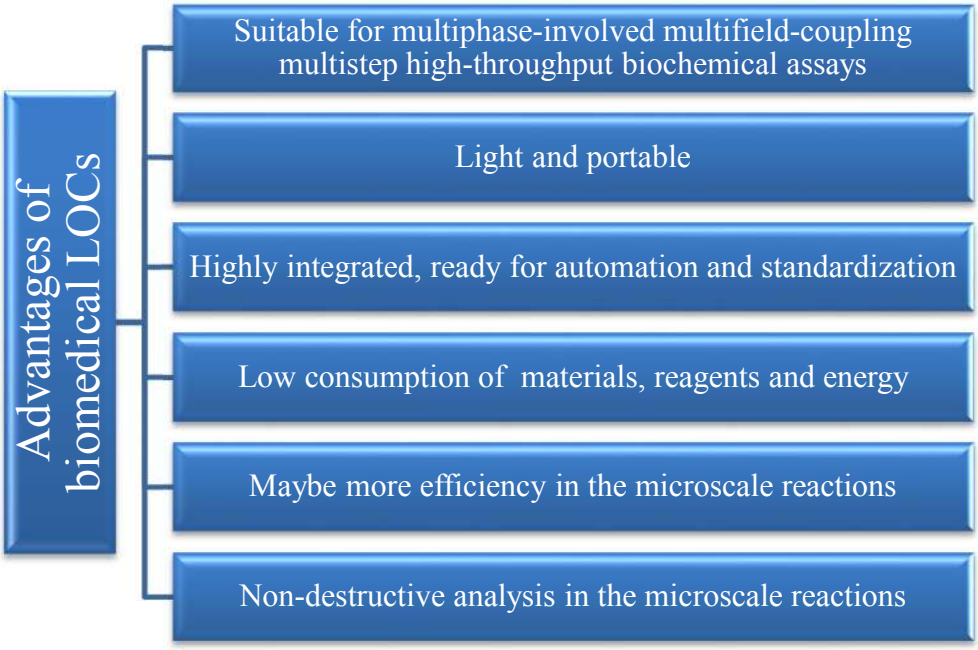

Figure 1. Technical superiority of biomedical LOCs.

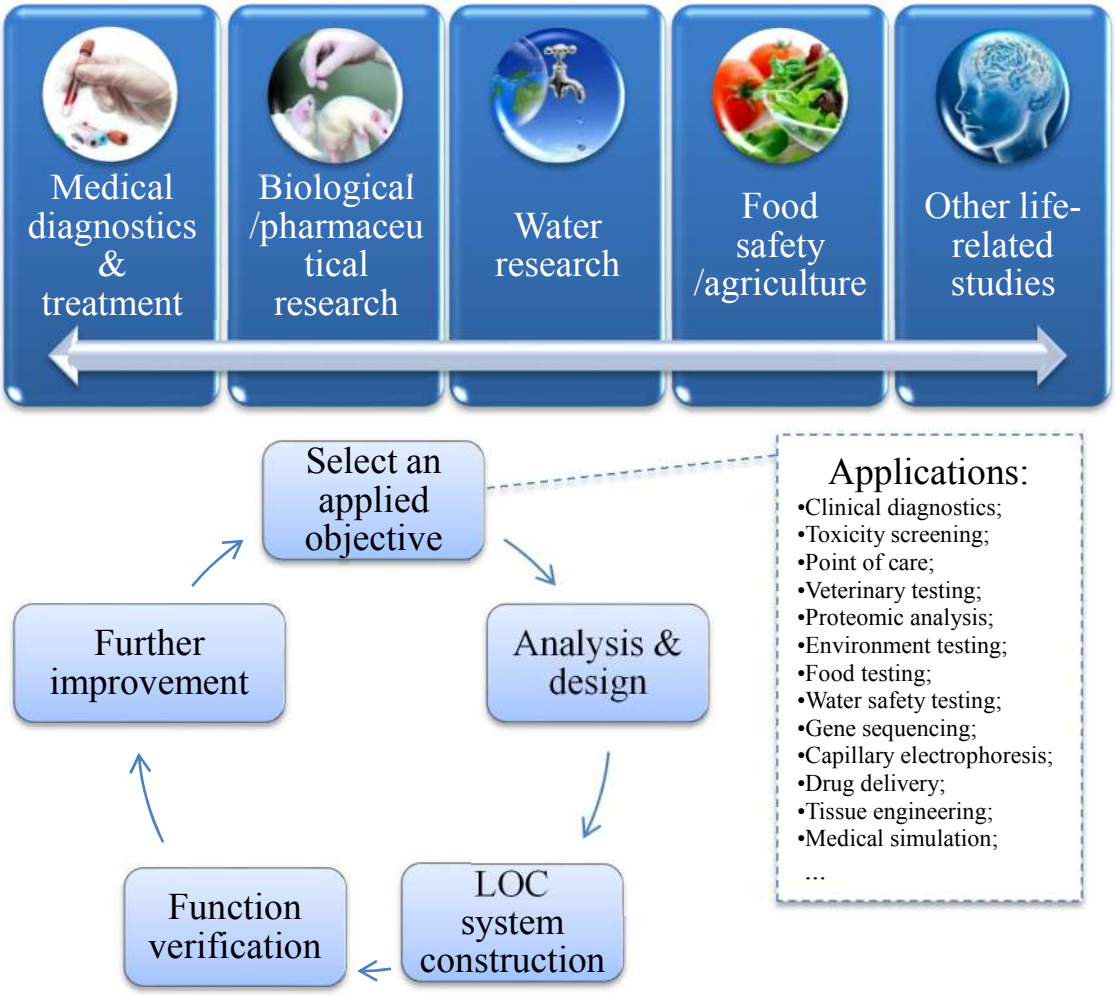

Figure 2. The concept of application-oriented biomedical LOC devices.

The application-oriented R&D strategy aims at a practical problem as the objective. The entire development cycle includes analysis, design, fabrication, validation and product optimization, focusing on the unique goal of resolving the targeted objective. Therefore, this R&D strategy has a fundamental guarantee that the research output will serve for practical demands. Moreover, actual conditions for the targeted objective will naturally exclude improper tracks within the technology roadmap for the system design, accordingly helping researchers reduce unnecessary efforts of R&D. The application-oriented R & D strategy is selected in this study, shown in Figure 2, lower part. Typical fields for application-oriented LOCs are enumerated as follows:

- Medical diagnosis and treatment

  Medical detection is relevant to the pre-treatment of heterogeneous biological mixtures, e.g. blood, urine, faeces etc. Biomedical testing often involves multiphase, multistep reactions, and makes extensive use of electric, magnetic fields and temperature control *etc*. Besides, more and more medical examinations require parallel detecting of multiple items in one sample simultaneously for the convenience of users. Obviously, LOC technology will perfectly match those requirements. Some commercial cases are demonstrated in Figure 3.

  In addition, if LOC tools dramatically increase the efficiency of medical detections, medical treatment programmes would be improved as well. Advanced tools in clinical medicine always play a key role in patient survival. For example, a patient in an emergency room is suspected to have some heart diseases. If a rapid diagnostic tool can return pivotal results on the spot, doctors are likely to give a reasoned treatment on the patient, rather than an empirical one.

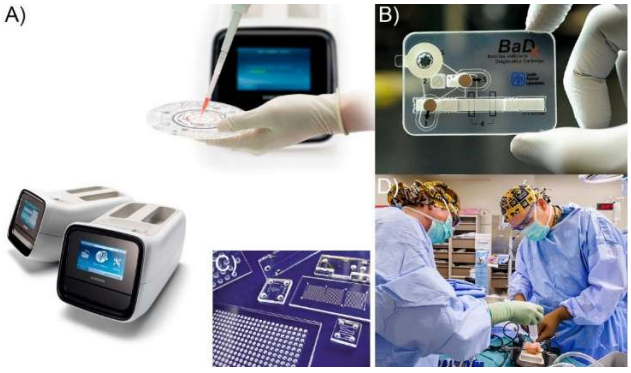

Figure 3. Examples of commercial LOC devices.[2-5] (a) Samsung™ LABGEO IB10 is a portable automatic immunoassay analyzer with centrifugal LOCs for blood testing;[2] (b) BaDx™ Portable Anthrax Detector;[3] (c) LOC devices made from glass by Micronit™ Microfluidics BV;[4] (d) MOPS (Missouri Osteochondral Allograft Preservation System): an example of tissue-on-a-chip.[5]

- Biological and pharmaceutical research

  Similarly, scientific research in biology research and drug development processes also involve the experiments concerning the sample treatment and identification of animal cells, tissues *etc*. High-throughput biomedical tests are always essential, see Figure 4. LOC technology shows great capabilities in this trajectory.

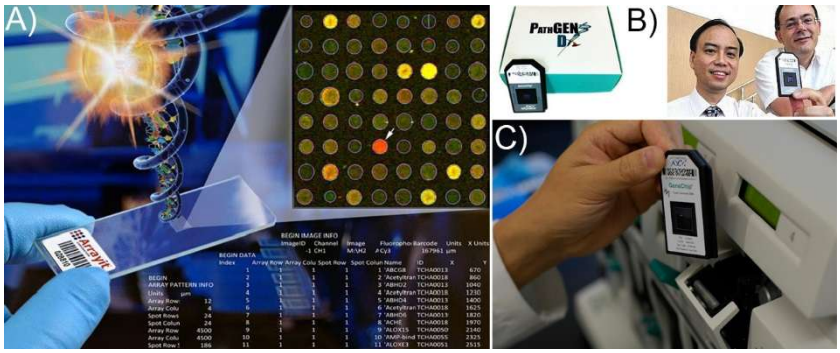

Figure 4. Commercial high-throughput biochips.[6-10] (a) Arrayit™ DNA microarrays-Pathways™ Focused Human Genome Microarrays enable targeted gene expression analysis of every major cellular pathway in the human body, which contains a complete set of 25,509 fully annotated human genes;[10] (b) PathGEN® PathChip can detect the presence of >70,000 viruses and bacteria from human samples within 2 days.[6,7] The PathChip runs on the Affymetrix GeneChip® System;[8] (c) An Affymetrix GeneChip® instrument at the University of British Columbia (Canada).[9]

- Water safety and environmental studies

  Water is the most important sustainable resource for urban populations. The enormous demand for water safety has led to the development of early warning technology for aquatic environment or municipal water consumption. Currently, the conventional monitoring technology for aquatic environments and distribution network of municipal water still rely upon the routine monitoring of some conventional parameters of water quality, such as pH, conductivity, free chlorine *etc*.[11,12] In fact, current detection methods can provide blameless protection with respect to water safety, but LOC technology here could potentially improve the degree of security.[11] Water quality monitoring, especially for the detection of waterborne pathogens, involves multiphase, multistep biochemical reactions, which is the suitable field for LOC technology[13-15].

- Food safety and agricultural research

  Food safety is also associated with public health. Since quality detections of food involve multiphase multistep biochemical reactions, LOC technology is also

applicable in this field. The detection principle of these LOCs is similar to that for biology research.

- Furthermore, other life sciences-related fields probably also refer to the applied research of LOC technology, as long as those fields involve multiphase multistep biochemical reactions, for instance, toxicology research, bioinformatics *etc*.[16-18]

Among the relevant papers in this thesis, Articles I and II described the research work on the detection of pathogens in water.[15,19,20] Article III focuses on microbial detection in the urine for UTI patients.[15] Article IV reports on the studies on the continuous monitoring of toxic chemicals in water.[21] Articles V and VI aim at the profiling test of signal molecules in human blood.[18,22] Although these biomedical LOC devices are different and specific in the different application projects, commonness inside could be found. In general, the complete hardware of a concept LOC system is illustrated in Figure 5.

At present, one of major challenges in LOC research is how to make it useful in varied applications. From an engineering point of view, most technical challenges of LOCs are surmountable. Generally, a core LOC component should have at least two functions, which are to take the role of a multiphase reactor for multistep biochemical reactions and to generate detection signals. Namely, most biomedical LOCs should be some kind of convertors that can extract useful information from the samples. To obtain useful information, a series of supporting modules are essential, because core LOC components often require supporting instrumentation to implement specific functions. Typical supporting modules in a LOC system are summarized as below:

- The **sampling module** is responsible for sample collection and treatment, such as lysis, concentration and extraction. The refined outputs from the sampling module should be transferred into the LOC by specific interfaces, so the sampling module connects the macroscopic world and microscale channels in the LOC. Since high quality samples are important for quantitative analysis, sampling modules are often integrated partially or completely into LOCs, especially disposable LOCs, to reduce contamination and operating error. Furthermore, the complexity of biomedical samples and their viscousness often lead to the difficulty of thoroughly rinsing used vessels. Consequently, sampling modules and all sample-contact components have to be disposable.

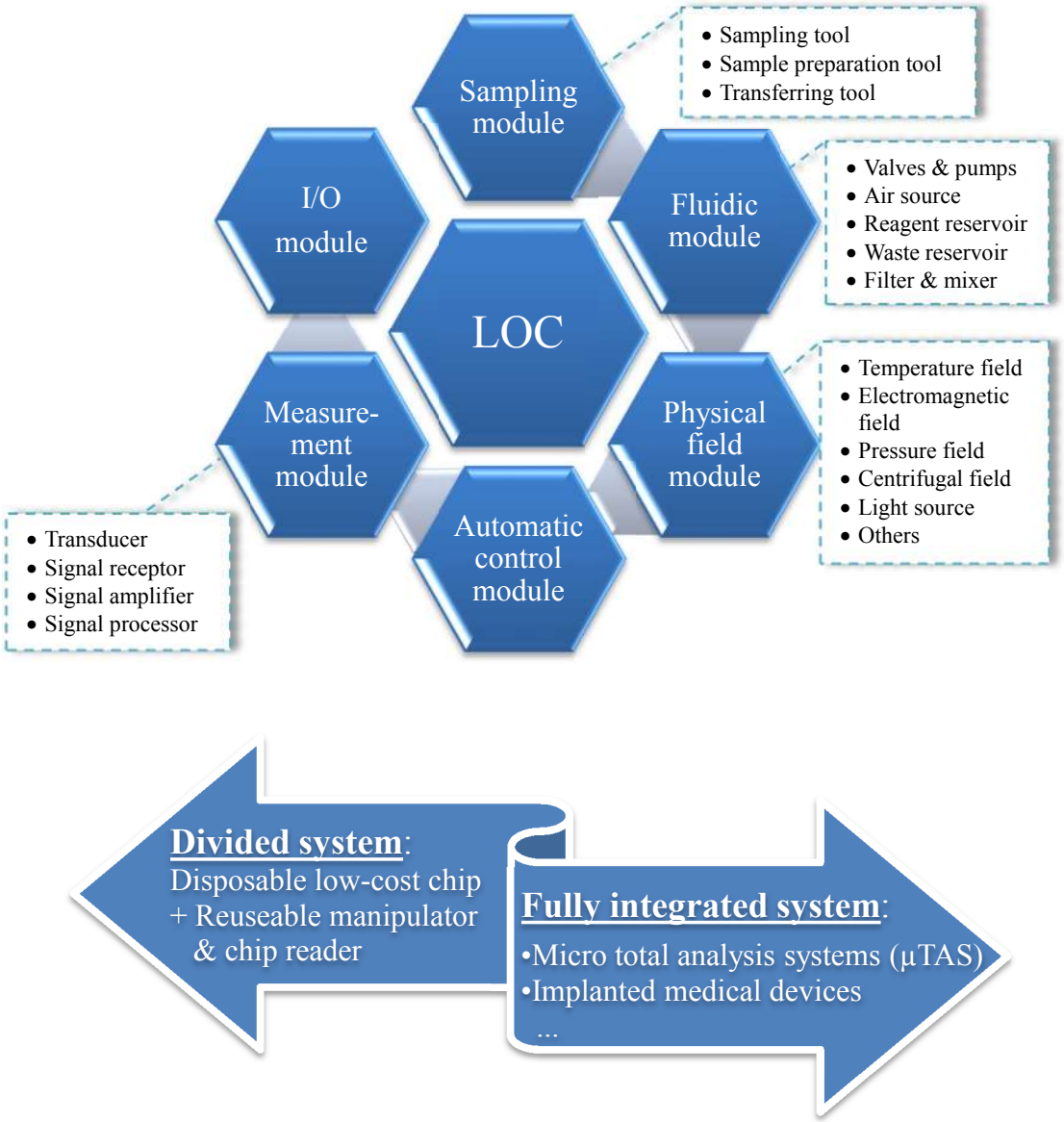

Figure 5. Typical hardware design of a complete LOC system.

- The **fluidic module** provides precise fluidic operation in multiphase on-chip reactions, comprising the valve and pump system, the high or low-pressure source, the filter, mixer, reagent and waste reservoirs, etc. Multistep reactions can be converted into instructions in the valve and pump system for a LOC system. Driven by pressure difference, various reagents sequentially or simultaneously flow through the reaction chambers, thus enabling complex biochemical reactions.
- The **physical field module** is responsible for providing physical field conditions on the LOC for specific biochemical reactions. For examples, lab-on-a-CD devices require the centrifugal field as the driving force.[23] PCR-based LOCs also require corresponding temperature fields, because PCR reactions for gene

detection usually require at least three different temperatures. [24,25] Magnetic micro beads are employed in many biomedical LOCs.[26,27] Inevitably, magnetic fields will be required in those LOC systems.[26,27] In fact, a multi-physics environment is the essential condition for a biomedical LOC.

- The function of **measurement module** is to read and amplify the signals on the LOCs, including denoising and other information processing. The core transducers are either integrated into the LOC core or arranged in the external reusable measurement module.
- The **input/output (I/O) module** is responsible for transmitting the information obtained by the measurement module to end users. The form of the I/O module maybe rely on the computers, but it is possible to build it on a new platform, e.g. a smart phone. Nowadays, more and more information is delivered to users through the Internet and smart phones. Mobile phones can help biomedical LOC devices to provide a friendly user interface.
- The **automatic control module** is the control centre of a LOC system. A complete LOC device should eventually develop into an easy-to-use automated system. Otherwise, LOCs will be nearly pointless to common users. Perhaps it is one of the reasons that many LOC prototypes have to be shelved in the laboratory. Owing to the rapid development of robotics, no significant challenges exist in the R&D of automatic control modules at present.

Obviously, a complete LOC system is always a complex instrument. Under present technical conditions, it is almost impossible to make the overall cost of such a complex system be cheap. There are two R&D strategies for application-oriented LOC systems (Figure 5, Bottom part):

- To develop a fully integrated system.

  All modules are miniaturized and highly integrated together to form a compact portable tool, such as micro total analysis systems (μTAS), point-of-care (POC) devices, etc. The integrated LOC system is also employed among some implanted medical devices. The application environment for the fully integrated system has strict limitations. For instance, implanted drug delivery devices will be used inside the human body for a long period to intelligently release specific drugs. Therefore, these implanted devices definitely should be much smaller than bench-top devices, and ought not to be heavy. Only a fully integrated LOC system can meet the

requirements in this case. Portable tools for rapid diagnosis on the spot are in demand in the emergency room, or a battlefield, or a disaster scene etc.

However, it is difficult to reduce the fabrication cost of a complex functional POC instrument, let alone a medical LOC appliance. Once a medical instrument was in contact with an individual clinical sample, it is not allowed to reuse it. The cost of fully integrated LOC systems became a major obstacle in the road leading to commercialization. Integrated LOC systems might not be amenable to the most common biomedical cases. Only in some special situations, e.g., emergency cardiac treatment, fully integrated LOC devices could meet real demands.

- To develop a divided LOC system

Another strategy is to select the framework of divided LOC systems, in which an entire system is split into two parts, i.e. an integrated reusable manipulator or 'chip reader', and disposable low-cost microfluidic chips. The former part could be a laboratory bench-top device. The manipulator should be reusable, and the cost of single measurement could be cost-efficient; it could be a large bench-top instrument, sacrificing mobility and cost-efficiency. On the other hand, disposable microfluidic chips are required to be low-cost, but sacrificing a part of LOC functions. With regard to a variety of multistep accurate fluidic operations, the simplified LOCs need function compensation and support from the reusable instrument. Thus, the strategy can reduce the cost of a single measurement using a LOC system.

Of course, compared with a fully integrated LOC device, the divided LOC system sacrifices efficiency and mobility. The microfluidic chips in such a divided LOC system turns into a low-cost component in the bench-top instrument to enhance its efficiency and convenience. Fortunately, divided LOC systems are able to fulfil the needs of users in common scenarios of biomedical fields, In particular, disposable LOCs could gain more acceptance because they can avoid the cleaning after testing medical or biological samples. In fact, sampling modules have been designed as disposable parts nowadays. Furthermore, the sampling modules are often integrated into the disposable LOCs directly. According to the strategy, disposable chips require cheap materials and cheap fabrication methods, such as injection moulding on polymers etc.[28-30] The hardware cost of a divided LOC system can be reduced within an acceptable range, which is the key to the commercialization of LOCs.[31,32]

On the other hand, a functional LOC system definitely does not only rely on hardware support. Direct signals from the chip are only some kind of physical quantity rather than meaningful conclusions. Without a translation mechanism, those signals are of no use in practical scenarios. The key translation mechanism for a LOC system is a 'decision support mechanism' (DSM), which can generate measurable signals in the LOC, and translate these signals into a meaningful conclusion in the real application field. A DSM is an information bridge, connecting the microscale LOC and the macroscopic world of applications. If a LOC system lacks of its DSM, the LOC has to be confined in the theoretical stage since its output is pointless. The DSM is essential to all application-oriented biomedical LOC systems. The roles of the modules in a LOC system are similar to those of the hardware in a PC, whereas the DSM is similar to the software system in a PC (Figure 6). The author of this PhD thesis describes the DSM concept as follows:

A) Reaction principles of LOC detection

   The detection principle and biochemical methods might be modified in the microscale world of LOCs. The form of the output signals can also vary considerably from the original, e.g. optical, electromagnetic, electrochemical etc. However, the reaction has to serve the purpose of the LOC, and the reaction principle is also determined by the purpose. For example, in the LOC system in Article III, living microbe pathogens in the urine samples of patients with UTIs were selected as the detection target; ATP-bioluminescence assay was employed as the basic principle of measurement, because the method is suitable for measuring living cells, and living microbes contain higher ATP concentrations. The feature is different from non-living particles. The initial step of developing an application-oriented biomedical LOC is to determine the detection purpose, and then select an appropriate reaction principle before any design of hardware components.

B) Criteria and scenarios for applications

   In the application cases, end users are often unconcerned about the specific reactions inside the microfluidic channels. Their concerns are the meaningful conclusions drawn by the LOC-based tests. DSM is critical here since it is responsible for interpreting the information of the LOC system.

   DSM will define the boundary conditions in a scenario for a specific purpose. For example, when a researcher starts to design a LOC to measure the concentration

of oestrogen in human blood, the physiological and pathological concentration ranges of oestrogen must be considered in the DSM, as well as other related factors e.g. the gender, age, etc. Otherwise, misleading results could be inferred, even if the on-chip tests were well performed. The LOC system requires developing a set of criteria to complete the interpretation regulations for the signals on LOCs. Despite the importance of DSMs, the existence of DSMs are often ignored in the publications concerning LOC systems. If the measuring window of a LOC device is far from the required range in its DSM, theoretically, the LOC system almost has no value in the target application.

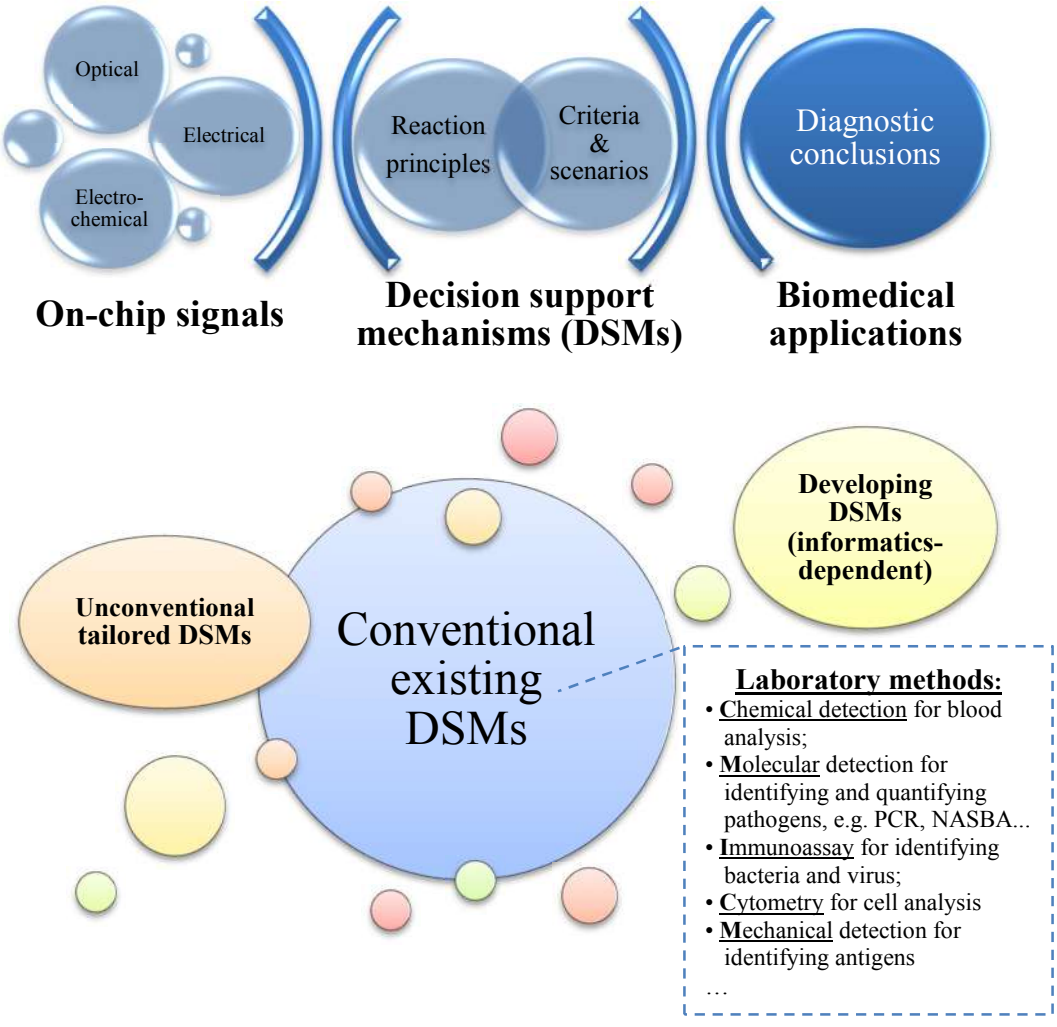

Figure 6. The roles and taxonomy of DSMs for a LOC system.

Based on the doctoral work, it is summarized that there are at least three different types of DSMs for LOC systems (Figure 6, lower part) as follows:

1. Conventional DSMs in common with current biomedical experimental approaches.

The overwhelming majority of biomedical LOC devices rely on existing diagnostic mechanisms in laboratories, because this strategy might reduce the difficulties in validating new LOC tools and avoid unnecessary interrogation of their working principles and diagnostic criteria. Thanks to the biological research of the last 70 years, biomedical experimental approaches have advanced significantly. In general, it is easy for LOC researchers to ascertain existing and confirmed biological methods for the detection of candidate targets, as well as the criteria and scenarios for specific applications. Developing a novel DSM without any specific purpose is risky. In this R&D model, LOC researchers only need to focus on how to miniaturize mature laboratory tests onto microfluidic chips. Of course, many technical challenges still exist. The most popular detection methods in LOCs are shown in Figure 6 (lower part).

However, the design for these LOC systems have to be limited by conventional DSMs. After all, there is a huge difference between the microstructure environment and the conditions in a laboratory. For instance, mixing of samples and reagents in a tube is easy in a conventional laboratory, but become challenges to LOCs. Considering the cost of LOCs and existing capacity of microfabrication, sometimes, conventional detection methods have to be modified slightly. When the change is too large, DSMs will be regarded as unconventional ones.

2. Unconventional tailored DSMs for contrived LOC systems on the basis of known principles.

The second class of biomedical LOCs employs some kind of tailored DSM, which is derived from the basic working principles of existing laboratory experiments, but concrete mechanisms and diagnostic criteria are arbitrary in terms of contrived functional LOCs. Compared with Class 1, the freedom in the system design has been extended for improved system performance. Actually, there are some advantages in the micro-reactors of LOCs. At first, the diffusion distances in LOCs are short, which is conducive to molecular collision in the on-chip reaction. Second, when the movement of particles is restricted by microstructures, those particles are easy to capture and separate. Third, the consumption of LOCs is rather small, leading to low maintenance costs. Therefore, it is

conducive for LOCs to sustain a long-term working style. According to these superior characteristics, conventional DSMs can be greatly modified for some LOCs to theoretically create outstanding detection systems.

Unfortunately, unconventional DSMs have to answer more questions or be subject to censure. In reality, verification of new methods and new tools is always required in large-scale and long-term experiments. If standard detection methods already exist, the challenges of these new methods increase, possibly only due to psychological factors. The large-scale validation experiments always need more financial support, which is an uncontrollable risk for most researchers. Therefore, most LOC developers prefer conventional DSMs, even though tailored DSMs probably lead to better performance.

3. Developing prognostic/diagnosis DSMs for biomedical-database-dependent LOC systems with the help of computational modelling.

With the rapid development of bioinformatics and so-called ‘big data’ technology, LOCs have also become attractive as biomedical information gathering tool by the bioinformatics branch.[33] The third category of DSMs is just emerging and is still not completely developed; it relies on computational modelling within a large-scale medical/healthcare database. Fascinatingly, this class of LOCs is developed prior to the birth of the DSMs. The LOC devices will be first employed to collect sufficient clinical data as input of a relevant biomedical database; after that, predictive computational models will be built by classification techniques on the basis of the relevant database; finally, various prognostic or diagnosis DSMs are created for the LOCs to detect health risks and identify dangerous health trends. At present, the research activities on these biomedical-database-dependent LOC systems remain in the stage of prospective study.

Undoubtedly, construction of relevant biomedical databases and the informatics research of data mining are arduous and time-consuming tasks to be undertaken before rendering these LOCs with prognostic/diagnosis functions. This category of LOCs generates both the tools to build their own supporting databases and to be the executive carriers of prognostic/diagnosis applications in the future. Compared with the previous two categories of DSM, this category has a longer development cycle, greater R&D investment and higher risks. Few researchers are willing to develop this kind of LOC, but

the author believes that the new-concept LOCs represent a promising development direction for the future.

1.2 Typical biomedical applications of LOC technology.

In considering the factors from both the technological and practical aspects, an application-oriented R&D strategy was chosen in this study and demonstrated in the following applications, leading to a series of relevant biomedical LOC devices with various DSMs.

1.2.1 LOC applications in water quality monitoring.

Inadequate access to clean water is hugely detrimental to both economic development and human health.[34] In the developing world, 2,200 children die daily from diseases transmitted through unsafe water and 780 million people still lack access to safe water.[35] Great effort has been invested in keeping water clean and safe. However, outbreaks of water pollution incidents still threaten people in both developed and developing countries in spite of the advancements in water treatment technology.[15] Industrial and environmental testing applications are always driven by the rollout of technology that can meet the increasing regulatory requirements and consumer demand for assuring food and water quality.[36] However, sending samples to the lab and inspecting growths in petri dishes under a microscope a week later remains the standard reference for accuracy; companies of checkout equipment are increasingly also using faster microfluidics tests in house for more immediate feedback, and for ongoing monitoring and optimization of their control and treatment processes.[36]

According to the different purposes, the methods and tools for monitoring water quality differ sharply. Close attention has been paid to two major application fields. One is the water safety of drinking water supply systems in modern cities, while the other concerns the environmental monitoring of natural water bodies. In addition, some special applications include water testing in swimming pools, water quality control of fisheries, the monitoring of wastewater, agricultural irrigation water, and the water supply to hospitals or biological laboratories etc. The different purposes of water quality testing determine the different standards, testing methods and tools.

The main purpose of environmental monitoring in natural water is to detect pollutions produced by human activities and to maintain ecological safety. Environmental monitoring has to be undertaken daily to deal with unpredictable events. As for general natural water, the ecosystem already exists there, and water quality testing mainly focuses on chemical contaminants, eutrophication, waterborne pathogens and ecological biomarkers etc. The research in this direction has a close relationship with hydrobiology and ecology. The degree of biological response ranges from a change in the molecular level of a single microorganism to the structure of a community and even to the function or structure of an ecosystem.[37] However, environment-oriented LOC research will usually study microorganisms in the water. The polyurethane-foam-unit (PFU) method was established and has been standardized and widely used in China to evaluate the toxic effects of pollutants, in which protozoans are selected as ecological indicators.[38,39] PFU blocks have to be exposed to an aquatic environment for several days before they recover and became subsequently enriched in living microorganisms.[15]

Furthermore, another group of microorganisms in water always attracts the interest of LOC researchers, i.e. waterborne pathogens. The data from all over the world provide evidence that waterborne pathogens are responsible for acute poisonings and various diseases in animals, wild and domestic, and in humans.[19] In Europe and North America, the direct-drinking water system is also vulnerable to waterborne pathogens (e.g. *Escherichia coli*, *Legionella*, *Acanthamoeba*, *adenoviruses*, rotavirus, etc.), which calls for a strong aspiration to develop high sensitive monitoring system against waterborne pathogens, so as to ensure the safety of the drinking water system.[19]

According to the website of World Health Organization (WHO), rotaviruses are estimated to be responsible for approximately 5 million deaths each year, with more than 85% of these deaths occurring in low-income countries in Africa and Asia, and over two million are hospitalized each year with pronounced dehydration.[19] Children under five years of age, especially those between six months and two years, are most vulnerable to the disease.[19,40,41]

Many LOC tools have been developed for monitoring these pathogens, which threaten public health not only in less developed villages, but also in modern cities. Tap water systems are always pivotal to public health in modern cities. Publicly available treated water has historically been associated with major increases in life expectancy and

improved public health, meanwhile water pollution accidents as well as some intentional contamination events in the municipal water system are also universal threats to urban residents.[42] Monitoring for the presence of possible contaminants in tap water has led to a common need for early warning devices that can sensitively detect toxicant chemicals and waterborne pathogens in tap water within the early stages of contamination events. Actually, real-time evaluation devices for water quality have been widely studied for a long time. Owing to the water industry being highly developed, rare pollution events can threaten the water safety of the water system in a city.[20]

However, dangerous factors still exist around the houses in the cities. In many cities, the sewage and water supply networks have been working for almost 100 years.[20] Exchanging the water supply network is often not frequent enough to ensure adequate water quality in all houses.[20] Besides, drinking water and sewage pipes often have imperceptible leaks.[20] The collapse of pipes causes interchange between drinking water and sewage, which can involve local pollutants in the drinking water supply.[20] Although high-frequency examinations will increase the costs for domestic consumers, periodic inspection is essential in reducing the impact of hidden accidents.[20] Moreover, the options of real-time analysis methods for a broad range of contaminants are still not flawless. The urban water supply systems require that the monitoring methods provide a rapid response; a real-time online testing system would be best, since every minute for an urban water system is of vital importance.

In the event of accidents or intentional contamination, municipal water is susceptible to two kinds of contaminants, i.e. chemical or biological pollutants. The detection methods of chemical pollutants are very different from those for biological ones. Moreover, unknown contaminants are continuously being discovered, while detection methods at a low-concentration level in drinking water have only recently become available.[43] The newly discovered pollutants will gradually turn into conventional ones over time. It is a fact that emerging contaminants are always present potentially evading the detection by the current real-time monitoring systems for municipal water, no matter how many test items are prepared in the real-time evaluating devices.[44]

Security events in an urban water supply system are typically processed in three stages:

1) Early warning stage: (to be conscious of an abnormality in the water.)

2) Hazard identification stage: (to confirm the problem of water quality by accredited methods.)

3) Crisis solving stage; (to solve the public crisis in the correct way)

The first two stages depend on varied methods of water quality detection, whereas Stage 3 is outside the scope of this Chapter. In Stage 2, current qualitative/quantitative detection methods are sophisticated for most pollutants. However, perfect technology for Stage 1 is still missing. Due to the lack of ideal wide-range online detectors, regular sampling and laboratory-based technique still dominates the work in Stage 1, but Stage 1 is the most important phase. The earlier contamination is found in water, the lower the loss caused by the pollution. At present, online monitoring equipment for water quality (MEWQ) is often expensive, and the measuring range or sensitivity has to be further improved.[45] MEWQ has been highly developed and commercialized, varying from bench-top precision equipment to mobile quantitative early warning monitors (Figure 7).

From the engineering point of view, the ideal solution is to merge the first two stages. That is, to design an ideal detector capable of continuously accurately measuring water quality parameters and to transmit real-time measurement results over long distances. Currently laboratory automation technology is sufficient for the tasks of detecting conventional parameters such as pH, redox potential, free available chlorine, conductivity, turbidity, etc.[46] At the same time, telecommunication technology is also advanced enough to support the function of remote real-time monitoring. However, the major barrier here is the high maintenance costs of online MEWQ in the waiting period of Stage 1. Typically, water quality has to be examined frequently to guarantee public water safety, since unpredictable water pollution accidents break out infrequently. However, the price for any kind of accurate measurement is always not cost-efficient, let alone a group of accurate measuring experiments used to exclude the presence of a variety of known pollutants. Thereupon, the maintenance cost will be considerably high. The expensive online quantitative methods and tools are only suitable for the pivotal of water supply network in a city. Furthermore, the measuring frequency will be actually reduced to control the maintenance cost in the waiting period of Stage 1, resulting in the risk of some rapid contaminant events during the measuring intervals. Another problem is that those

expensive online detectors can only cover the central part of the water supply network, instead of sub-pipelines and end branches.

Another strategy for water quality monitoring is to totally separate the purposes and methods between Stage 1 and Stage 2. That is, to use a low-cost long-term broad-spectrum online MEWQ for Stage 1, and to involve those expensive precision instruments and accurate methods for Stage 2 after triggering the alarms in Stage 1. This strategy could satisfy both respects from the security requirement on drinking water and the maintaining costs of the surveillance system. In fact, the major task for the online MEWQ in Stage 1 is the timely and correct discovery of the abnormality in the water, instead of accurate qualitative measurement.

The automation equipment can simplify or replace manual operations. Unfortunately, most of them are designed for Stage 2, whereas tools for Stage 1 are relatively insufficient, though many detectors are claimed to be real-time, online, and sufficiently low-cost. As implied by the product descriptions, these online water quality monitoring systems cannot monitor all kinds of specific contaminants directly, because monitoring of many different specific contaminants is currently not feasible.[45] Instead, research shows that many contaminants affect one or more water quality parameters such as chlorine residual, TOC, pH, conductivity, etc.[45,47] Therefore, current online MEWQ with the detecting capability of limited water quality parameters also has a potential to detect contaminants.[45] Although those devices have been quite advanced already, no suitable online MEWQ could be a perfect solution in meeting the high demand for safety and efficiency at a relative low expense.

LOC technology is probably a low-cost solution for MEWQ, because it is possible to reduce the reagent and energy consumption by miniaturization. With reference to numerous multiphase separation and multistep reactions, LOC technology is well suited for the implementation of complex reactions for biomedical detection.

Figure 7. Commercialized automatic instruments for water quality monitoring.[45,48-57] (a) YSI™ 5100 BOD laboratory setup (laboratory analysis for Biochemical Oxygen Demand/BOD, dissolved oxygen, pH, etc.).[48] (b) Hach™ eclox rapid response water test kit (parameters: chemiluminescence toxicity screening, arsenic, pesticide/nerve agents, chlorine, colour, total dissolved solids, pH).[49] (c) YSI™ 556 MPS (Multiprobe System) combines the versatility of an easy-to-use, easy-to-read handheld unit with all the functionality of a multiparameter system (dissolved oxygen, pH, conductivity, temperature, *etc*.).[50] (d) An online water quality monitoring system installed at a military facility.[45] (e) s::can™ Messtechnik GmbH, Submersible UV-VIS-spectrometer probe installed in a spring chamber. (Parameters: turbidity, SAC254, Nitrate, Total Organic Carbon/TOC, temperature and electric conductivity).[51] (f) Colifast ALARM™ (At-line automated remote monitor) detects total coliforms, thermotolerant coliforms or *E.coli* in drinking water. The fully automated system consists of an analysing instrument and a bacterial growth medium system. In addition, the Colifast ALARM can measure the turbidity level of the water.[52] (g) ADASA™ mobile stations for water quality monitoring.[53] (h) GE™ Sievers InnovOx® on-Line TOC analyser provides industry-leading sample handling robustness and instrument uptime for process, environmental, and wastewater TOC Analysis.[54] Typically, it can run 30 days without replacing reagents.[54] (i) A multiparameter water quality system for water quality monitoring

provides one of the most efficient solutions for the continuous on-line measurement of pH, Oxidation-Reduction Potential/ORP, conductivity, temperature, free chlorine, total chlorine, monochloramine, and oxygen.[55] (j) Q45WQ water quality panel (Analytical Technology™, Inc., USA) provides constant, on-line monitoring of any remaining disinfectant and up to 6 other parameters in water distribution systems and moveable water treatment facilities.[56] (k) The AppliTOX® on-line broadband toxicity analyzer is a fully automated batch bioassay based on the well-established ISO 11348 laboratory procedure. The original test protocol has been used since the 1980s and is accepted as a standard method in a large number of countries and applications.[57]

1.2.1.1 LOC technology for microbial detection.

Evaluation of microbes in water can protect people from illness due to consumption of water containing pathogens, thus prevent the outbreak of water related diseases.[13] Most current procedures for microbial analysis in water monitoring are based on periodic sampling and culture methods, which are slow, requiring 24–48 h. When the first results reach the decision-takers and trigger an alarm, significant time has already passed and the population may have been exposed to a health hazard.[58] There is a need for rapid, reliable detection of microbial contaminants.[58]

As a primary laboratorial method for microbial analysis, culture methods have a variety of drawbacks, though this method is probably the most authoritative. To monitor waterborne pathogens with high efficiency, immunoassay methods are widely used.[19] Besides, relevant researchers have increasingly moved toward molecular technologies to meet the need for rapid, multiplexed, species-level detection, ranging from PCR, NASBA to microarrays.[58,59] Based on the principle of PCR amplification, some isothermal DNA/RNA amplification assays have been developed, including loop-mediated isothermal amplification,[19] T7 based linear amplification[60], NASBA, etc. NASBA has been directly realized in waterborne pathogen detection,[61] which requires a procedure of extracting the mRNA from the sample.[19,62] However, the immunoassay methods, such as ELISA (enzyme-linked immunosorbent assay), can avoid the extraction of DNA or mRNA, making the original signal of microbes easier to be quantified.[19] Among the most common detection methods, immuno-PCR assay with femtomolar sensitivity and high specificity is the most sensitive, combining the advantages of both immunoassay and PCR.[19,63-67] Combined immunoassay and NASBA, IMRAMP (immuno-real-time-amplification) is also highly sensitive, with sensitivity at least 10^3 to 10^5 fold higher than the ELISA technology[19,68,69], shown in Figure 8.

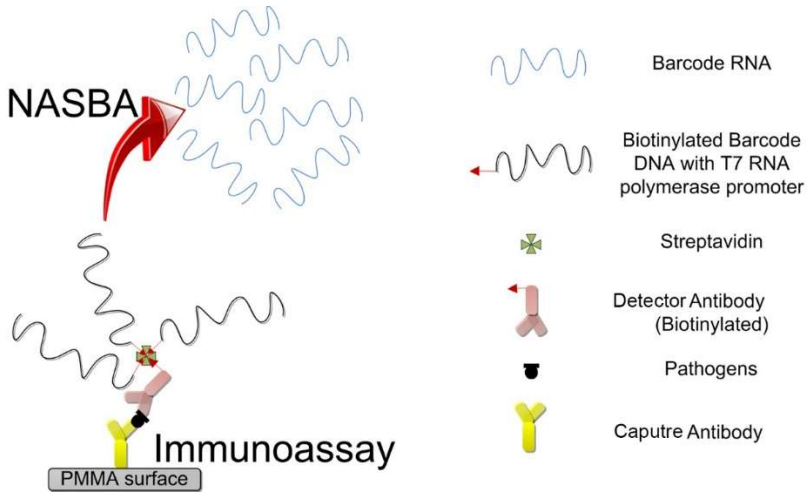

Figure 8. Amplification mechanism of IMRAMP (immuno-NASBA) assay.[19] Reproduced from Ref. 19 with permission from the Royal Society of Chemistry. The principle can be simply understood as immunoassay followed by NASBA amplification. The immunoassay is like a sandwich ELISA, but its report group is a barcode DNA with a T7 RNA polymerase promoter, not an enzyme. The barcode DNA plays a role as a bridge to connect immunoassay and NASBA amplification. After the NASBA amplification, the weak immune signals are enlarged by a million times and above. Eventually, fluorescent signals are released with the help of beacon probes.[19]

Furthermore, various methods for biological detection have been developed. For example, the ATP bioluminescence assay (ATP-BLA) is a multistep process involving the luciferase enzyme, luciferin substrate, oxygen, magnesium cation and adenosine triphosphate (ATP), which will induce the efficient emission of photons (550-570 nm).[70-72]

In fact, most biological methods rely on the optical properties of analytes, including light-scattering detection,[73] enzymatic fluorescence detection,[74] chemiluminescence methods,[74] electrochemiluminescence detection,[75] Raman spectroscopy,[76] dye-loaded microsphere detection,[77] etc.[58,78] These existing methods enable the quantification of very low concentrations of microbes with high specificity.[58] Generally, limit of detection (LOD) of the methods without molecular amplification could only reach the level of 1×10^3 cells/mL of analytes in the water samples.[58] Unusually, the LOD of ATP-BLA could be up to the level of 200 cells/mL.[58] When the methods contain a molecular amplification stage, the LOD could reach 1 cell/mL or even a lower level.[79]

Detection of microorganisms at very low concentrations is still a challenge, especially aiming for online monitoring.[58] Accordingly, current microbial detection mainly relies upon intermittent biological methods, while continuous broad-spectrum methods and tools for monitoring microbes are still insufficient. Of course, increasing the frequency of

intermittent experiments, and implement broad-spectrum detection by using high-throughput biochips will be a solution for online monitoring of waterborne pathogens, but testing costs would increase dramatically. Moreover, high-throughput biochips still cannot solve the detection problem of unknown pathogens in water.

In fact, various LOC devices for monitoring of pathogens have been widely studied.[20,80,81] Taguchi *et al*. investigated an immune-capture LOC device to trap protozoa for subsequent fluorescence tests.[82] By using this concentration tool, 10mL of a sample mixture of 10^7 oocysts/mL could be simply captured on the chip within 1 h.[82] Dielectrophoretic (DEP) LOC is another method of concentrating pathogen samples, and DEP LOCs for environmental microorganisms have been reported.[83] Some concentration factors in these applications could achieve 10^4-10^5.[34] Subramanian *et al.* reported an immunological LOC based on the principle of surface plasmon resonance for detection of *E. coli* O157:H7 with a LOD of 10^4-10^6 cells/mL,[84] inferior to those of ELISA LOCs.[85] It is worth noting that molecular-biology-based microarray technology has become a standard high-throughput method for the screening of pathogens. Wong C. *et al.* have developed a DNA chip that can identify 70,000 different viruses and bacteria in one test.[6,86]

Cell-based LOC is another important branch for microbial detection.[87] The usage of LOCs could result in better environmental control over cells or bacteria during cell culturing. At present, the invention of 'organ-on-a-chip' could lead to improvement in traditional culture-based pathogen-monitoring approaches.[34,88] LOCs might also allow the design of micro-environment to promote the culture of microorganisms, which are not yet achieved in a standard biological lab.

At last, one of key factors to commercialization for applied-oriented LOCs is faster and easier fabrication.[20] Sometimes, the cost of a LOC is more attractive than its precision of measurement.[20] Moreover, LOCs in this field are often disposable chips, therefore expensive and time-consuming fabrication methods are naturally excluded.[20]

1.2.1.2 LOC technology for monitoring chemical contaminations.

A huge market for water quality monitoring always exists. The global market for drinking water reached EUR 250 billion in 2008, with investment of over EUR 33 billion per annum.[89] For decades, researchers have placed great importance on the detection of

chemical pollutants; hundreds of accurate MEWQs have been commercialized for chemical analysis. Therefore, developing LOCs to measure chemicals in water accurately seems not very demanding, but LOCs for early warning of chemical contaminations in water are still promising.

Most commercial online MEWQs are relatively expensive (Figure 7). In addition, these MEWQs (e.g. Figure 7, insets D, G, I and J) are strongly dependent on the model and optimization software to deduce some typical contamination events, however many atypical events are often neglected. Existing systems employing living cells (e.g. Figure 7, inset F) are often impractical in size or involves sample preparation. Miniaturization of the above-mentioned system could probably result in the reduction of system costs. LOC technology has provided the possibility to miniaturize the instruments. Based on the LOC structural frame, the ideal early warning devices for water quality should fulfil the following conditions:

1) Have a low maintenance cost;
2) Be able to work continuously online;
3) Be able to monitor a broad range of harmful chemicals over a threshold of sensitivity and accuracy for the guarantee of timely and correct alarms;

The main purpose of an early warning device for water quality is not to quantify the accuracy parameters in water. Instead, qualitative diagnosis is sufficient. The most critical challenge is how to make an early warning system with a broad-spectrum detection range able to work continuously at a low cost. Notably, the early warning devices cannot replace the existing MEWQs for accurate analysis. They are compatible with the existing system.

Electrochemical sensors are the most popular tools for online detection of specific chemicals in water, because they can real-time measure specific chemicals continuously at a low cost. However, the sensitivity of electrochemical sensors could be a challenge, particularly in highly purified drinking water, since water quality standards for drinking water in most developed countries are often close to the LOD for ordinary electrochemical sensors.[46] Moreover, the electrochemical principle is for specific chemicals, instead of a naturally broad-spectrum method. Most MEWQs in Figure 7 are composed of electrochemical sensors, each of which is only able to measure a specific parameter.

Another frequent online warning method for detecting chemicals is the spectrometric method, which has also been commercialized, for example, in Figure 7, inset E. That tool can continuously work in theory, because the maintenance cost of spectrometric measurement mainly lies in the consumption of light sources and lens smearing. At present, these tools are mainly employed in the monitoring of sewage discharging into the aquatic environment, instead of quality tests for drinking water. Unlike electrochemical sensors, spectrometric ones could be natural broad-spectrum for organic compounds. However, a precise optical instrument often requires a high maintenance cost to keep working in the field.

In fact, biological sensors have obvious advantages in the early warning of chemicals. When using a living organism as the core sensor to judge the presence of chemical contaminants, the living organism is the natural authority and has an innate broad range of detection. Besides, the maintenance cost for the survival of the living organisms is not very high. Living organisms can evaluate the total toxicity of aqueous samples, instead of concentrations of specific chemical constituents.[13,21] For example, the Toxprotect™ fish monitor (GmbH, Schwentinental, Germany) was developed to detect toxins in water by analysing the swimming activity of fishes.[90] If the fish in a river shows anomalies, it is an indication for toxins in the water.[21]

However, animal models have a high resistance to varied factors and are the subject of some ethical issues. Cell-based toxicity tests provide an alternative to animal experiments.[21] These systems can monitor a broad range of chemicals, including some unknown chemicals.[21,91] Model mammalian or human cells display similar physiological responses to the cells in the human body; however, it is difficult to maintain the viability of mammalian cells.[21,92] Bioluminescent bacteria have a higher growth rate, and are less costly to propagate, and easy to detect.[21,93] Thus, bioluminescent bacteria represent promising sensors for use in continuous-sensing devices against toxicants.[21] Specially, the light-emission ability of the natural marine bacterium *Vibrio fischeri* was widely employed in sensitive and rapid luminescence-based tests, described in ISO 11384.[21,94-96]

The living-organism system can be easily disturbed by various factors, so the standardization of living organisms should be treated as a serious requirement. For example, the experimental method of ISO11384 employs a periodic standardized procedure to solve the challenge. *V. fischeri* luminesces through the production of

luciferase, which catalyses the oxidation of a long-chain aliphatic aldehyde (RCHO) and a reduced flavin mononucleotide ($FMNH_2$).[21,94] The free energy in this reaction is released in the form of light at a wavelength of 490 nm:[21]

$$FMNH_2 + RCHO + O_2 \rightarrow FMN + RCOOH + H_2O + h\gamma\ (490\ nm)$$

The presence of acute toxicants that affect this reaction can suppress or extinguish the light emission.[21] The method has been widely used in the rapid qualitative assessment of water quality.

Furthermore, it was also used in a few automatic systems, storing standard copies of lyophilized *V. fischeri* cells. Periodically, the automatic system will use one copy in each individual test for water quality. The DSM of such kind of automatic systems is a typical conventional one. The design is not very suitable for continuous early warning of water quality, because there are long intervals between the adjacent intermittent tests. Given sufficient conditions, such an instrument could be miniaturized into a LOC, but it may not be necessary. Actually, promising alternative solutions have emerged.

A new branch of LOC, 'integrative biology' technology, casts a hopeful ray on the cell-based early warning device of water safety. This solution integrates the living cells[21,97,98] or small tissues[99-101] or organoids[102-104] into a LOC device, which turns into a chimeric long-term working device with 'life', including cell-based LOCs in the early years, microfluidic cell and culture systems or body-on-chip or tissue-on-a-chip, or organ-on-a-chip in recent years. In theory, this type of LOC is promising in solving the above-mentioned challenge in the field of early warning of water quality, because not only the living 'component' on the chip retains a broad detection band, but also the LOC structure limits the independent variables. The integrative LOC could probably generate an explicit output signal, compared with the present devices. However, the DSMs of new integrative LOCs have to be determined by the design of LOC systems, which belongs to typical unconventional DSMs.

1.2.2 LOC applications in medical diagnosis and treatment

Medical diagnosis is another typical field for LOC applications. The LOCs, called 'sample-to-answer' systems, are able to perform fast and low-cost tests on small volumes of biological samples and to seek for diagnostic conclusions,.[23] Over the past few decades,

research has been conducted in this field to detect patterns of biomarkers and provide information on their concentration in biological samples for robust diagnosis. In fact, the working principles and basic structure of the LOCs in the medical field are quite similar to those LOCs designed for water quality monitoring. Especially in pathogen detection, the methods of quantifying microbes in human urine or blood samples are almost the same as those of waterborne pathogens, demonstrated in Chapter 1.2.1.

However, due to their different purpose and sample properties, two cohorts of LOCs are not exactly the same. For medical detection, the biological samples e.g. urine, blood etc. are usually more complex than water samples. Consequently, the challenges in the sample treatment of medical LOCs are much larger than LOCs in the cases of water quality detection. On the other hand, medical LOCs are clearly biased towards the function of rapid in-situ detection, whereas the LOCs for water quality detection aim at continuous online working over a long period. The most common types of commercial LOC in the medical field are lateral flow devices, i.e. dipsticks or strips.(Figure 9) Lateral flow devices embody all the characteristics of medical LOCs. They are small, portable, easy-to-use, cheap and disposable. In addition, the turnaround time of strips has been shortened into several minutes. Their unique drawbacks lie in the measurement precision. Usually, lateral flow devices are used as widespread qualitative tools for medical screening.

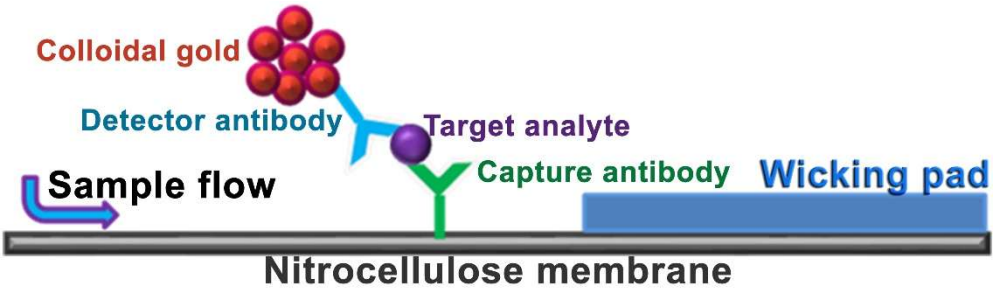

Figure 9. Schematic mechanism of a lateral flow device.[105] The LOC is based on immuno- or antibody-based test. It is often comprised of a nitrocellulose membrane covered by a sample pad on one end and a wicking pad on the other end. The sample pad will be submersed in a sample solution, e.g. urine, blood, etc. The solution wicks up the nitrocellulose membrane on the strip, causing the fluid to pass over an area containing an excess of gold-labelled detector antibody specific to the target protein. After that, the antibody-protein complex will continue moving up the strip with the flow of fluid. When the gold-labelled antibodies with bound proteins pass over the test line, the antibody-protein complex forms a sandwich with the immobilized capture antibody. This results in the formation of a visible line on the strip indicates that the target protein is present in the sample.[105]

The field of medical LOCs has been well-reviewed, though it is still a research hotspot for researchers and companies, shown in Figures 3 and 4. Recent reviews are good reference materials for a general overview of medical LOCs.[106-111]

It is not necessary to duplicate the similar work in this PhD thesis. Here, we only discuss several frequent but critical questions about the study of medical LOCs.

1) Why do current medical systems need LOCs?

Excitingly, the modern medical system has established a complete laboratorial methodology for medical diagnosis, which is able to handle almost all kinds of medical cases. Except for emergent cases, sending the sample to the lab and inspecting the growth in a petri dish under a microscope within a week remains the acceptable standard reference in the medical field. However, LOCs could reduce the turnaround time of medical tests in house or in the screening stage for more immediate feedback, which will be beneficial to patients in avoiding unwanted medical examination and optimizing the treatment processes. Medical LOCs are able to undertake these diagnostic and screening tests out of the lab and direct at the point of care. However, advanced LOCs are still significantly expensive, so the established system of laboratorial tests still maintains its entrenched status. From the point of view of DSMs, the risk-adverse medical system still trusts in the accuracy of the traditional laboratory tests, more than the faster LOCs with new DSMs. LOCs have started to make inroads in emergency rooms, particularly for diagnosing cardiac events and identifying the bacteria causing severe infections, where saving time saves lives.[36]

2) Which application fields are medical LOCs well suited for?

Demand for LOC devices for research in pharmaceuticals, life sciences and medical sciences is increasing strongly. As the research moves to more complex biological analysis, traditional laboratorial tests have switched to high-throughput automatic processing. LOC is a perfect platform to implement high throughput biomedical tests. In particular, medical LOCs are suited for handling experiments into living cells or biological samples, since researchers always intend to understand the underlying processes of the body response on the cellular level, which needs more complex high-density chips with thousands of reaction chambers and high precision structures.[36]

3) What are the most commonly used biological samples for medical LOCs?

Since medical LOCs are good at performing multiphase multistep biochemical reactions, the most commonly used biological samples are various body fluids, including blood, urine, saliva etc. In the daily work of a hospital, urinalysis and blood tests may be the most common options for biochemical examinations. LOC researchers are also interested in these demanding fields.

First, urine is an abundant bio-fluid that can be readily obtained by convenient means.[112] As the wastes of the bloodstream extracted by the kidneys, urine contains high concentrations of urea, inorganic salts, creatinine, ammonia, organic acids, various water-soluble toxins and pigmented products of haemoglobin breakdown.[113] Nowadays, urinalysis methods are mainly used in labs and hospitals, mostly including physical urinalysis tests, microscopic examinations and chemical assays.[114] The advantages of urinalysis mainly lie in its non-invasiveness and high sensitivity. The results of urinalysis could be employed to detect personal health changes at an early stage.[115] Compared with blood sampling processes, the sampling of urine is much easier and can be repeatable in a single day. Medical studies have been carried out to prove the correlation between urinary components and respective human diseases, such as diabetes, kidney diseases, UTIs etc.[114] Besides, urinary metabolomics or urinary proteome is a perspective method for diagnosis, especially appropriate for kidney cancers and chronic inflammatory diseases.[116,117] The results of urine tests can also reflect some toxicological or metabolic responses of the body, because metabolites and proteins in the urine mainly come from plasma proteins.[118] In the current study of proteome, urine has shown some unique advantages so it is considered to be a suitable source for both physiological research and disease biomarker discovery. Some changes in the plasma proteome may be distinctively reflected in urine, the waste of the human body, whereas those changes may be concealed by the steady state inside the body.[119] Many studies have been undertaken in the field of urinary metabolomics and proteomics.[113,119] More than 2600 metabolite species and at least 1500 proteins have been found in human urine, among which considerable associations to human diseases were discovered, such as UTI, bladder cancer, diabetes, etc.[113,119,120]

The study and analysis of human urine was the starting point for laboratory medicine in the history. At present, urinalysis is generally adopted by hospitals, and actually, it is also

suitable for monitoring daily health conditions at home. Urinalysis devices are widely available for chemical examinations in hospitals, such as chemical auto-analysers etc. The microscopic examination is another class of traditional urinalysis methods. Becoming more intelligent and automatic, current urinalysis tests rely more and more on automatic instruments. The emergence of automatic urinalysis instruments can not only offer the opportunity to standardize the microscopic and chemical examinations of urine samples, but also reduce the operating error, the labour intensity and the operation time of urinalysis.[121] The urinalysis system for home users has aroused general interest among researchers, since urine tests are suitable for the household daily monitoring of personal health conditions. Obviously, urinalysis LOCs are logical solutions for this purpose, but inadequate importance has been placed on urinalysis LOCs, due to the lack of developed DSMs. The knowledge of urinary diagnostics is accumulating in line with the development of urinalysis tools, including LOCs.

Second, blood testing is another common technique used in hospitals, with detection in a single test typically ranging from several items to tens of biomarkers. Since blood belongs to the internal environment of the human body, its importance in medical diagnosis is beyond any doubt. Compared with urine, blood samples have high stability in a normal person. When the composition of blood changes dramatically, great changes may occur inside the human body. Owing to decades of medical research, doctors and scientists have discovered profuse knowledge of blood diagnostics, which becomes conventional DSMs for LOCs of blood tests.

However, blood contains unknown knowledge which needs to be discovered, accordingly more developing DSMs for blood test LOCs will emerge in theory.[122] At present, around four hundred types of signal molecules in the blood stream are known, whereas the most commonly referenced in a hospital is less than fifty.[22] Although the concentrations of signal molecules are extremely low, signal molecules are important to maintain routine functions of human bodies. Signal molecular profiling (SMP) can provide holographic data of the entire human body.[18] SMP is related not only to human gene mapping, but also to the interactions between the human body and the surroundinenvironment.[18,123] In most cases, dramatic changes of SMP indicate the advent of disease (e.g., cardiovascular disease, cancer and invasion of pathogens).[18,124] Although much effort has been made to discover the diagnostic relationships between serological biomarkers and their respective diseases, the progress is hampered by the absence of a high-capacity clinical database on

human serological information.[18] Only a small proportion of such diagnostic knowledge has been uncovered.[125] In fact, the network of signal molecules has a well-ordered structure.[18,126-128] Translational relationships between signal molecules and specific diseases are more complex than those in physiology or pathology.[18]

To build such a medical database, high-throughput serological tests are required to use high throughput tools, e.g. microarrays. SMP records will be good resources for data mining in the field of translational bioinformatics (TBI). The hidden information behind the SMP data will be discovered for better understanding of physical conditions of individual patients in the future.[18]

Current protein microarrays are too expensive as the practical tools for SMP data acquisition.[22] They are often built on a flat glass slide or matrix-based material. The reaction interface on the slide might be easily smeared when manipulated carelessly; due to the slow speed of molecular diffusion, complete bio-hybridization assays usually require several hours, or even a couple of days. The demand of the SMP study calls for specific low-cost high-throughput LOCs, which DSMs are typically within the category of developing ones. In Articles V and VI, the study on SMP and the related LOCs were reported.

1.3 Aims and tasks.

In this thesis, the researcher aims to solve three specific practical problems in various cases by using LOC technology, including water quality detection, UTI diagnosis and serological study. A methodological study of LOC systems is also demanded during the R&D of various LOCs. The main objectives were shown as follows:

- Aim 1: To evaluate water quality by using LOC technology at a low cost, especially in the drinking water system.

Task 1: To design and realize a disposable sensitive LOC for qualitative or semi-quantitative detection of multiple microorganisms in water at the same time. In addition, the LOC should be compatible with a cost-efficient or common laboratory instrument to reduce the system cost.
Task 2: To implement a high-throughput LOC for precise quantification of microorganisms in water, and design the sampling module with seamless connection to the LOC. Similarly, the LOC should also be a compatible component to the cost-efficient or common laboratory instrument in Task 1.
Task 3: To develop a cost-efficient continuous LOC system for long-term monitoring of a broad-spectrum of toxic chemicals in water.

- Aim 2: To evaluate physical conditions or prognosticate diseases through a simple serological test by using LOC technology.

Task 4: To realize high-throughput quantification of signal molecules in serum by using a simple low-cost LOC, and design a roadmap for building a relevant biomedical database to support its diagnosis or prognosis functions of the LOC.

- Aim 3: To efficiently identify pathogen types and their antibiotic resistance properties in the diagnosis and treatment of UTIs.

Task 5: To design and validate a disposable high-throughput LOC for detection of microbial pathogens in a bacteriuria sample and further investigation of antibiotic resistance properties of the microbes. Similarly, the LOC should be compatible with the cost-efficient or common laboratory instrument in Task 1.

1.4 Contributions of the thesis.

The research tasks have been fulfilled in association with the primary supervisor, who provided the primitive ideas in the study. The chip design and system integration was mainly undertaken by the candidate under the guidance of the primary supervisor, but a part of chip fabrication was processed with the help of SINFEF™ MiNaLab, Xiamen University(XMU, China) and Nanjing University of Science and Technology (NUST, China). The experimental installation and validation was also performed by the candidate with the laboratorial resources in the partner institutes, including Institute of Hydrobiology at Chinese Academy of Sciences (IHB-CAS, China), XMU, NUST, Chongqing Technology and Business University (CTBU, China), Ziyang Maternal and Child Health Hospital (ZMCHH, China) and Chongqing Xiji Hospital (CXH, China). The candidate conducted the entire work of data acquisition and analysis. Thanks to the comprehensive contacts of the primary supervisor, my doctoral work could obtain so many interdisciplinary resources from the above-mentioned partners.

The main scientific contributions of this doctoral work are as follows:

1. The invention of a series of disposable LOCs to be compatible with standard microplate readers in common biological laboratories, serving as a low-cost model for other LOC systems;
2. The development of *V. fischeri* cell-based LOCs for continuous early warning of chemical toxicants in water; especially, the invention of the cell-based LOC with a coral-reef bioreactor. (unpublished)
3. The realization of sensitive and complicated immuno-NASBA and Q-NASBA assays on microfluidic LOCs, with the development of a novel analytical algorithmic method for NASBA-based quantification tests.
4. The development of a microfluidic LOC of Immunosorbent ATP-bioluminescence Assay (IATP-BLA) for rapid pathogen identification and antimicrobial susceptibility testing (AST) in the diagnosis of UTIs, as a good model of microfluidic simulators for medical diagnosis and treatment.
5. The development of low-cost LOCs, named ‘SMP chips’, for simultaneous quantification of hundreds of signal molecules in blood. Based on the SMP chips, the construction roadmap of the human signal-molecule-profiling database (HSMPD) is also studied.

6. The concept of 'decision support mechanism' (DSM) is proposed for the R&D of application-oriented LOC devices. Moreover, three categories of DSM for application-oriented LOCs are demonstrated for the first time, including conventional DSMs, unconventional DSMs and developing prognostic DSMs.

Most research achievements have been reported in international scientific publications. Furthermore, a summary of these scientific discoveries is presented in Chapter 2. The candidate prepared all manuscripts with the revision by the co-authors.

2 Summary of the articles

This chapter presents six articles capturing the research contributions of this doctoral thesis. This doctoral work involved with two primary fields of application: 1) water research, reported in Articles I, II and IV; and 2) medical diagnosis, reported in Articles III, V and VI. The research in each article is application-oriented, with the result that each portion of the work was focused on a different application with a different background and different objectives. Although each article is relatively independent, together they have generated a common body of knowledge of LOC. In particular, the DSMs for these application-oriented LOCs can be categorized as shown in Table 1. The collected articles were organized according to their DSM categories, rather than their publication dates.

Characteristics of LOCs	Decision support mechanism (DSM)		
	1) Conventional DSM	2) Unconventional DSM	3) Developing prognostic DSM
	Articles I, II and III	Article IV	Articles V and VI
High-density chip	Yes	×	Yes
Disposable chip	Yes	×	Yes
Able to work continuously	×	Yes	×
Able to perform complicated tests	Yes	Yes	Yes
Application field /objectives	Microbial detection	Monitoring of toxic chemicals in drinking water	Medical diagnosis, e.g., from blood

Table 1: The categories of DSMs and LOCs in this doctoral work.

2.1 Functional LOC devices with conventional DSMs.

The overwhelming majority of biomedical LOC devices rely on existing diagnostic mechanisms in laboratories, which may reduce the validation difficulty of new LOC tools. This strategy avoids unnecessary investigations of the working principles and diagnostic criteria. However, the degrees of freedom in the system design will be limited by conventional laboratory methods.

2.1.1 Compatible immuno-NASBA LOC device for quantitative detection of waterborne pathogens.

- **Background**

See also Chapter 1.2.1.1

- **Objectives:** (Article I)

To develop a disposable sensitive LOC with a friendly reusable supporting instrument for the parallel detection of multiple microbial pathogens in water.

- **Main relevant projects:**
  - FoU-forprosjekt i VRI Fund (Immuno-NASBA kretser, Project No.: 38043)
  - Oslofjord Fund (Real-time quantitative IMRAMP microfluidic system, Project No.: 202444);
- **Challenges:**
  - Is it possible to use existing equipment in a common biomedical laboratory as a low-cost reusable supporting platform for the disposable LOCs?
  - How can the LOD of the low-cost LOC system be improved?
  - How can parallel detection of multiple targets be implemented on a low-cost LOC?
- **Design of the LOC system and its DSM:**

Common microplate readers present in standard biological laboratories were selected as the low-cost reusable supporting platform for the disposable LOC, entitled ‘immuno-NASBA LOC’. It is modelled on a 96-well ELISA microplate (Figure 10).[19] The prototype LOC has 43 chambers available to perform independent assays to

simultaneously monitor up to six targets in one water sample. Compatibility between new LOCs and mature detection devices could significantly reduce the R&D challenge. The most remarkable feature of this immuno-NASBA LOC is its compatibility with microplate readers, allowing users to avoid purchasing any additional equipment or analytical software for the new LOCs.[19]

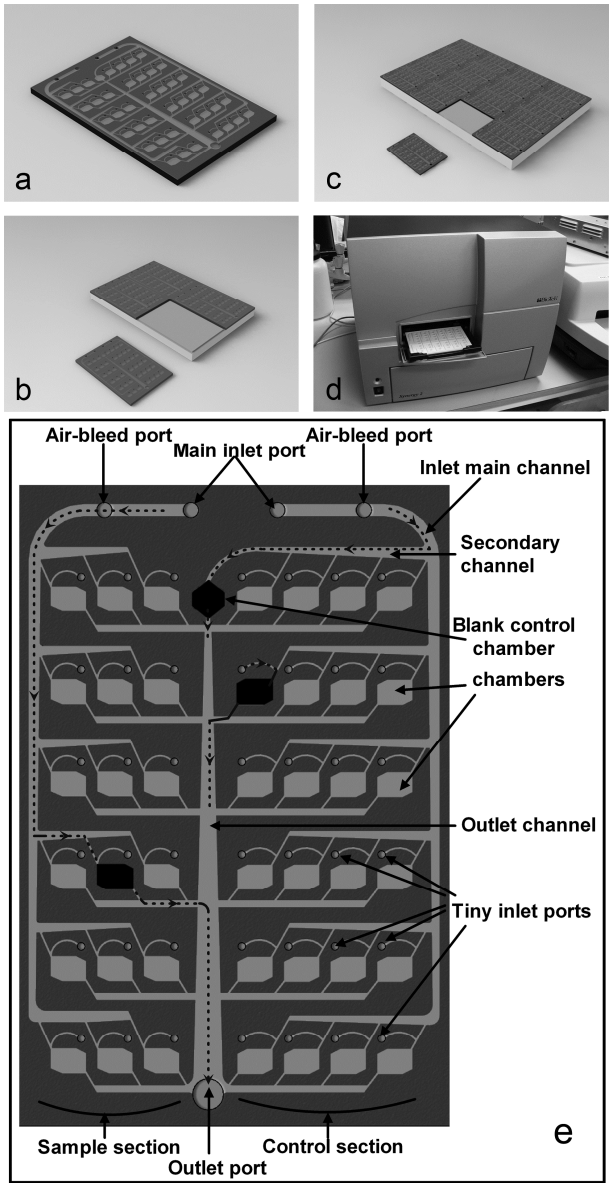

Figure 10. Architecture of the compatible immuno-NASBA chips.[19] Reproduced from Ref. 19 with permission from the Royal Society of Chemistry. This prototype 6-channel immuno-NASBA chip was designed and fabricated according to the dimensions of standard 96-well microtiter plates. It is composed of a polymethyl methacrylate (PMMA) plate and a transparent cover, see also Article I.[19]

The DSM of the immuno-NASBA LOC is based on IMRAMP as described in Figure 8. The principle of this technique is close to that of the traditional ELISA assay. At first,

different capture antibodies are used to recognize different biological targets in each chamber. Then, the signals are amplified by follow-up NASBA assays prior to direct detection on a microplate reader as the LOC has the same dimensions as a 96-well microtiter plate. The external valve system is shown in Figure 11. It is used to carry out parallel reactions in the LOC.[19]

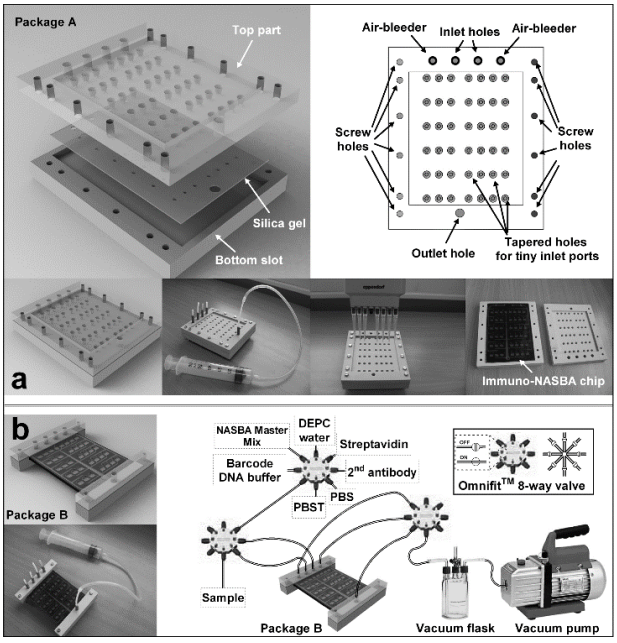

Figure 11. Reusable valve and pump system for immuno-NASBA LOC.[19] Reproduced from Ref. 19 with permission from the Royal Society of Chemistry. Inset (a) depicts Package A for chip pre-treatment. Antibody reagents can be loaded into the tapered ports by using a pipette. Inset (b) shows Package B for the detection assay, see also Article I.[19]

The IMRAMP assay has been almost shelved for many years. An important reason is its complicated protocol, whereas this LOC device can semi-automatically perform multiple protocols of the IMRAMP assay.[19] The LOCs were made of PMMA, a common inexpensive material, so it is feasible for efficient production of the LOCs by multiple methods such as laser ablation, hot embossing or injection moulding technology.

- **Experiments:**

A series of antigen-antibody reactions were used to test the performance of the LOC prototype. The two common waterborne pathogens, *Escherichia coli* (*E.coli*) and *rotavirus* were used as the testing targets, while a peptide, adrenocorticotropic hormone (ACTH) was selected as a reference target. ACTH, *E.coli* cells and inactivated rotavirus were serially diluted in PBS buffer. Moreover, the 5×10^{-13}mol.L^{-1} ACTH solution was

used as the 'ACTH sample' in the experiment. The 1.2×10^{6} cells/mL (equivalent to 2×10^{-15} mol.L^{-1}) *E.coli* solution was prepared as the '*E.coli* sample'.[19] The 1×10^{-2} μg/ml rotavirus solution was employed as the 'rotavirus sample'.[19] Three types of multiplex samples were also prepared.[19] The schematic protocol is depicted in Figure 12.[19] (See also Article I)

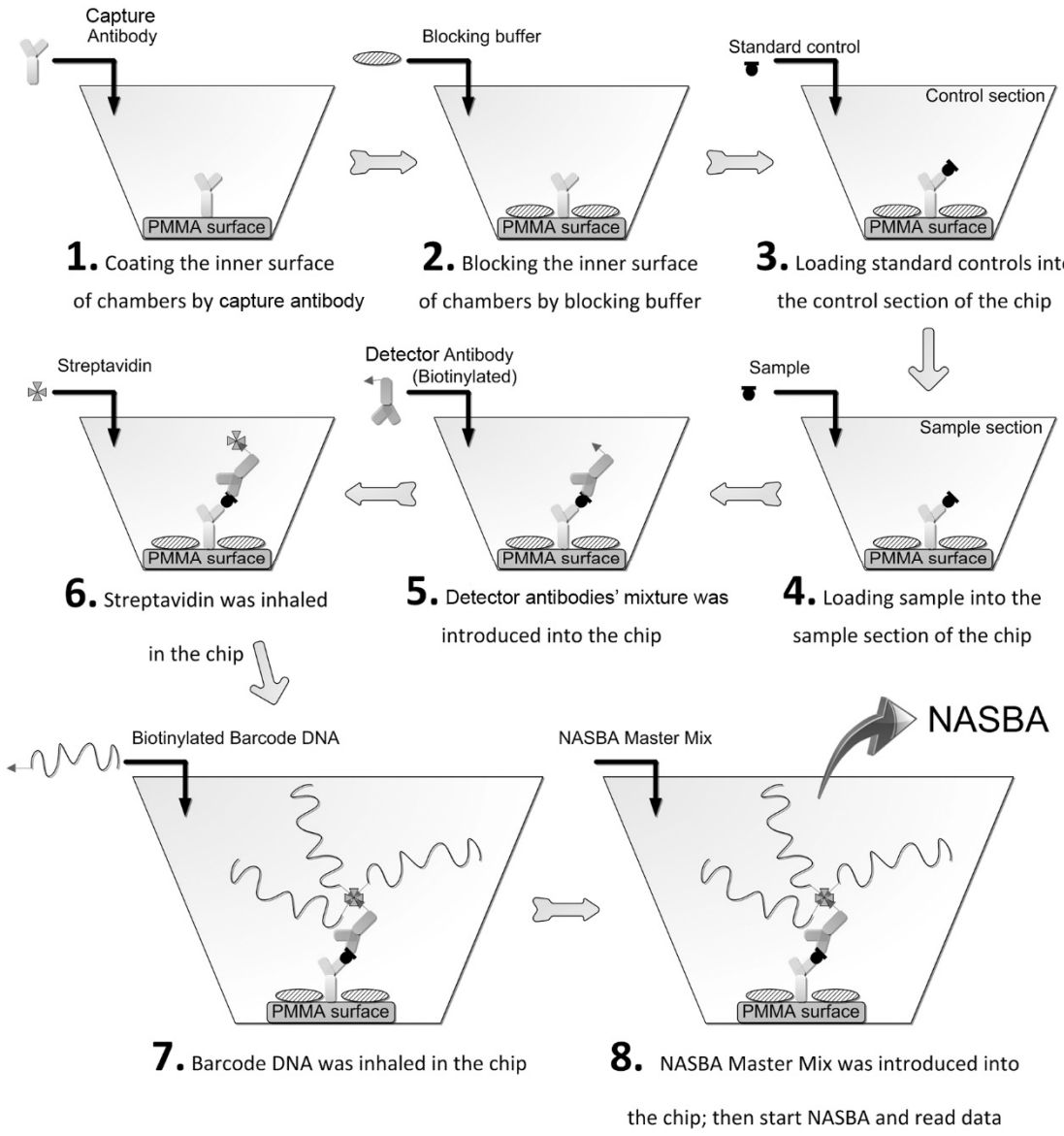

Figure 12. The schematic protocol of the on-chip IMRAMP assay. Reproduced from Ref. 19 with permission from the Royal Society of Chemistry. (See also Article I for the manipulation details)[19]

The tests used polyclonal antibody sera against *E.coli* and rotavirus; a pair of monoclonal antibodies with high affinity to ACTH were chosen for the detection of ACTH. The on-chip IMRAMP assay is initialized on the surface of the PMMA inside the reaction

chamber. All reagents are prepared from DEPC-treated water. Diethyl pyrocarbonate (DEPC) was obtained from Sigma©.[19] Barcode DNA was purified and biotinylated from the PreTect™ HPV-Proofer kit (Norchip©). The positive amplification product for HPV subtype 16 contained a large number of DNA tags. Since the barcode DNA was obtained from the kit, the same kit was suitable for the subsequent NASBA assay.[19,129]

- **Results and discussion:**

The data of on-chip IMRAMP tests were recorded by a microplate reader and showed in Figure 13, see also Article I. The concept of 'threshold time' was defined artificially for the comparison of amplification signals, according to the concept of the cycle threshold (C_t) value used in RT-PCR (Real-time polymerase chain reaction) assays. The C_t represents the time required for the fluorescent signal to exceed background noise level. Higher target concentrations will result in shorter threshold times in the amplification assay.[19]

The relationship between sample concentration and threshold time is a decreasing function. The LOD of IMRAMP assay is about 10^5 CFU/mL, similar to other antibody-based methods. This result indicated that the LOD of the immuno-NASBA may be predominantly determined by the antibody affinity rather than by the follow-up amplification step. However, the amplification of biological signals can reduce the requirement of detection instrumentation, thus reducing the system cost. An immuno-NASBA LOC allows for the detection of up to six different targets. ACTH, *E. coli* and rotavirus in multiplex samples were also tested, which results indicated that the interference within multiplex IMRAMP assays might occur, see also Article I.

Contamination of RNase is a significant problem during the IMRAMP assay for protection of RNA molecules in the test. The RNase present in the sample cannot be avoided, and likely enters the system upon sample loading. To reduce the potential for RNase contamination, the chambers are frequently rinsed by using buffers. DNA rather than RNA was chosen here as the barcode tag for detector antibodies because of its resistance to RNase. However, high concentration of barcode DNAs in the reagent might become other contaminants, leading to false-positive results.

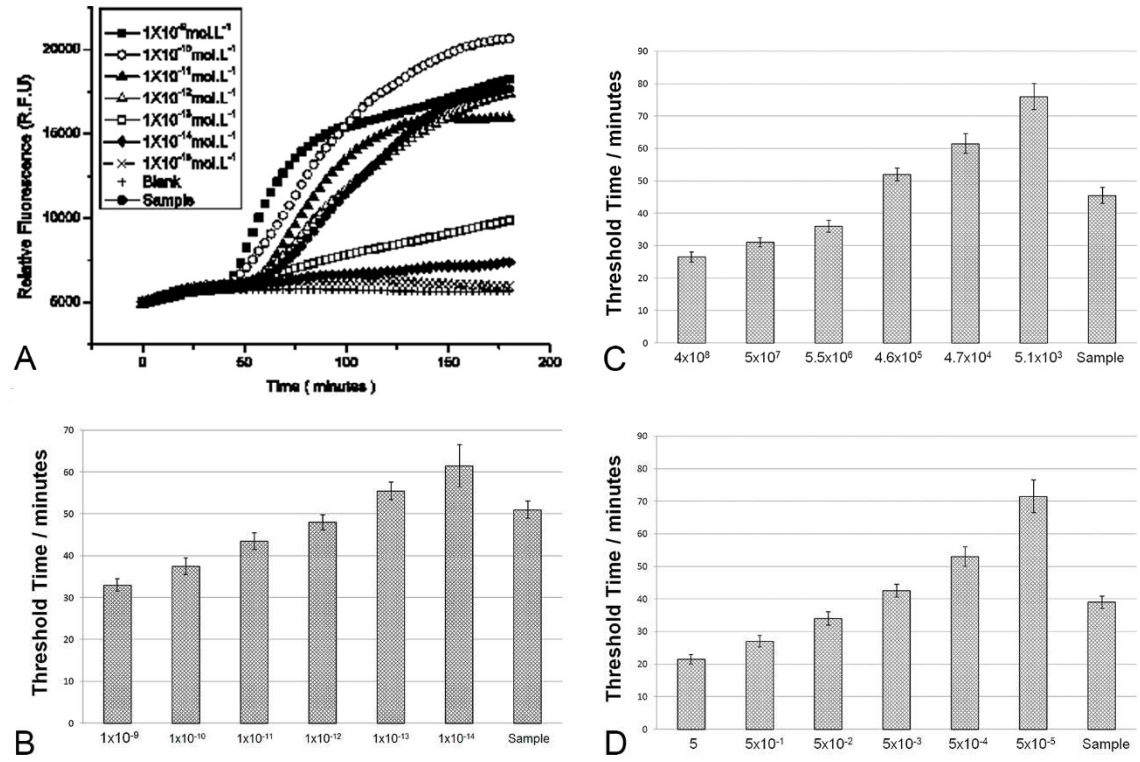

Figure 13. Quantitative detection assays on immuno-NASBA chips.[19] Reproduced from Ref. 19 with permission from the Royal Society of Chemistry. (a) The IMRAMP assays produces standard amplification curves at ACTH concentrations between 1×10^{-9} mol.L^{-1} and 1×10^{-12} mol.L^{-1}. (b) The plot of the threshold times for the ACTH standard solutions in the IMRAMP assays. The 'sample' in inset A or inset B refers to an ACTH solution of 5×10^{-13} mol.L^{-1}. (c) The threshold time of *E.coli* sample (at 1.2×10^{6} cells/mL) is between those of the 5.5×10^{6} cells/mL and 4.6×10^{5} cells/mL standards. (d) The threshold times for diluted rotavirus solutions were similar as inset C. The 'sample' in inset D refers to the rotavirus solution of 1×10^{-2} μg/ml.[19]

- **Summary of the study:**
  - The divided LOC system is easy to implement, especially given that the low-cost LOC is compatible with ubiquitous bench-top instrumentation.
  - There are four main limitations of low-cost LOCs: the limited machining precision, the limited accuracy of fluid operations, limited measurement equipment, and the intrinsic characteristics of on-chip biochemical tests. Since the cost remains a limiting factor, the biochemical tests carried out on LOCs are likely general opportunities for further improvement of LOCs.
  - This LOC employed a DSM similar to traditional ELISA testing to meet the needs of multi-channel detection of biological targets. It remains an obstacle: only a semi-quantitative test could be achieved in the study.
  - A series of higher-density LOC devices could be derived from this LOC, which is designed to be compatible with standard 96-well microtiter

plates. The 384 or 1536 well microtiter plates can also be read in the common microplate readers. If the chamber density of the LOC increases accordingly, the divided LOC framework will remain functional.

2.1.2 Multifunctional sample preparation kit and on-chip quantitative NASBA tests for microbial detection.

- **Background**

See also Chapter 1.2.1.

- **Objectives:** (Article II)

To develop a set of convenient low-cost tools for the quantitative investigation of microorganisms present in aquatic environments.

- **Main relevant projects:**
  - FoU-forprosjekt i VRI Fund (Intelligent vannprøvetakeutstyr, Project No.: 38042)
  - Oslofjord Fund (Real-time quantitative IMRAMP microfluidic system, Project No.: 202444);
  - The Norwegian Micro-Nanofabrication Facility, Norfab (197411/V30)
- **Challenges:**
  - Is it possible to readjust the microplate reader-compatible immuno-NASBA LOC for the investigation of environmental microorganisms?
  - How can a seamless connection between field sampling and in-house detection be achieved?
  - How can the parallel quantitative capability of multiplex molecular detection be improved?
  - How can the method of quantification for NASBA tests be improved?
- **Design of the LOC system and its DSM:**

The LOC in this work was named the ‘Q-NASBA chip’, and the DSM was derived from the existing PFU method to evaluate environmental pollution in fresh water. In general, the PFU method is based on the statistics of the microorganisms living in an aquatic environment, as described in the Chapter 1.2.1. The implementation of the traditional PFU method is manual and time-consuming, severely limited in two stages: the sampling

process and the detection process. If the laboratory is located far from the sampling point, the composition of samples may change during transport. As for the detection process, operators with sufficient knowledge of microbiological taxonomy have to manually identify and count different types of living microorganisms under a microscope. In this work, a sampling kit was developed for the collection of microbes, including a specific sampling tool with optional built-in membrane filters, and a coordinated sample preparation cassette for the optimisation of the sampling process (Figure 14, see also Article II).[15]

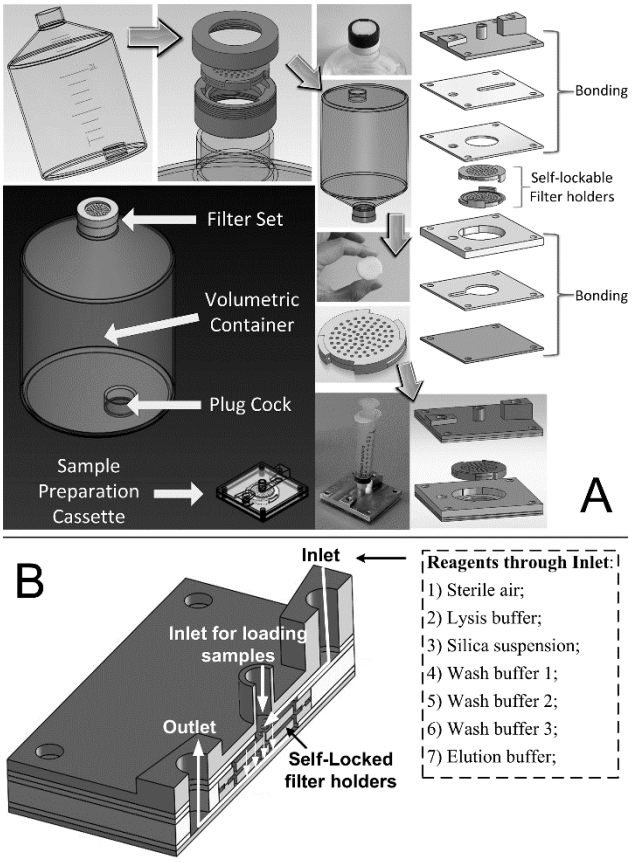

Figure 14. Components of the multifunctional sample preparation kit.[15] Reprinted with permission from Ref. 15, Copyright 2012, American Chemical Society. (see also Article II)

This low-cost sampling kit is seamlessly connected with the subsequent sample preparation. The microbes collected in the filter set can be immediately stored in a specific preservation buffer to protect DNA/RNA molecules, which provides enough time to transport samples to a core laboratory.[15] We altered the reported six-channel immuno-NASBA LOC (Figure 10) to obtain a 24-channel chip for Q-NASBA assays (Figure 15).[15] The on-chip Q-NASBA assay can be regarded as a sandwich hybridisation-based NASBA

assay (Figure 16),[15,130] similar to a multiplex NASBA assay. Different RNA templates are enriched in different chambers to avoid any interference between samples in the system. As a result, different molecular beacons (MBs) in the multiplex NASBA system can share the same fluorescence group, which reduces the requirement for detectors.[15] Furthermore, a novel analytical algorithmic method for better quantification of on-chip NASBA was also developed.

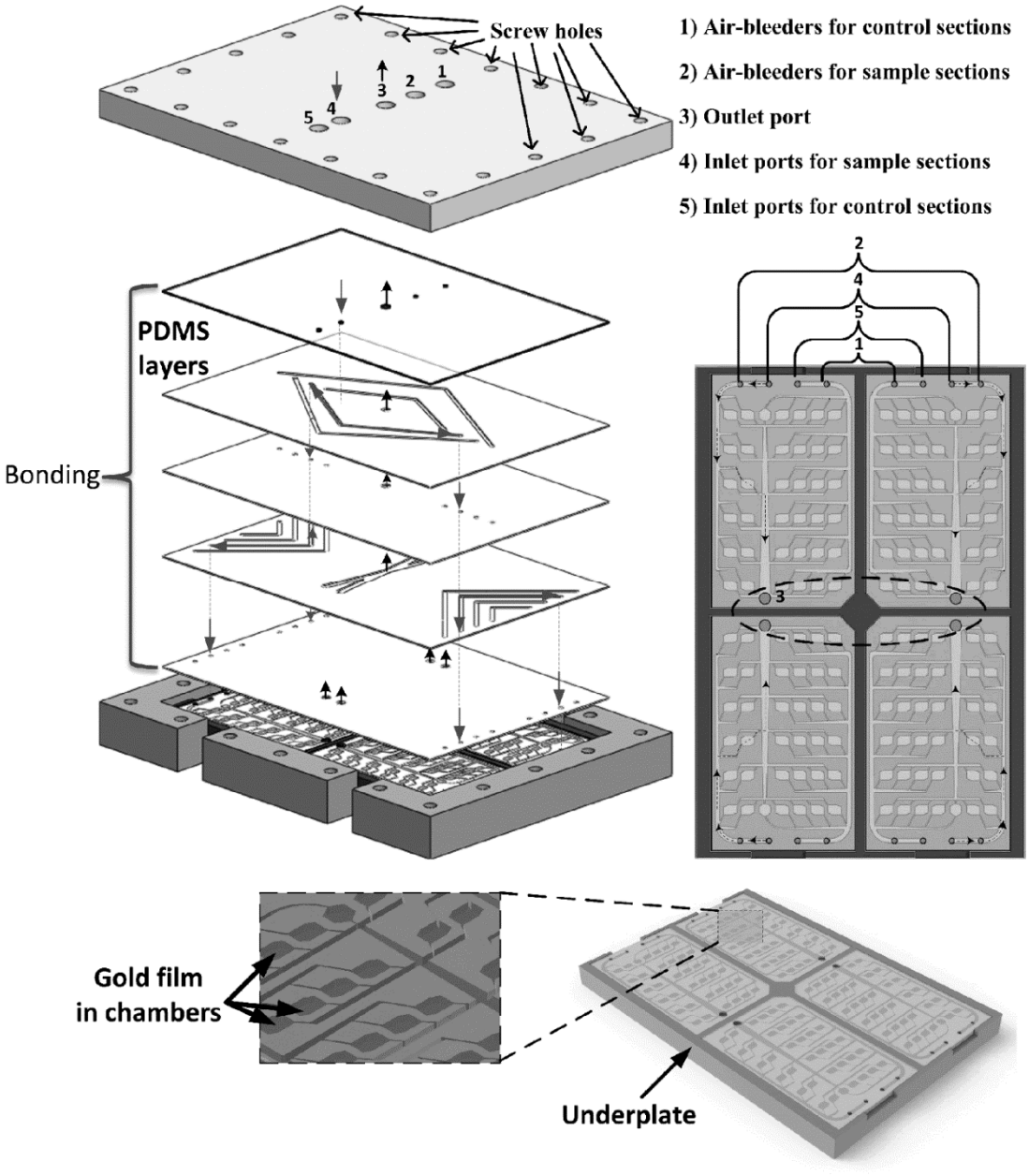

Figure 15. Architecture of the Q-NASBA chip.[15] Adapted with permission from Ref. 15, Copyright 2012, American Chemical Society. The six-channel LOC with micro-channels and chambers was made using both polymer and silicon. A gold film was deposited on the surface of each chamber. A common microplate reader can be used to read the LOC as a standard 384-well microtiter plate.[15] (See also Article II)

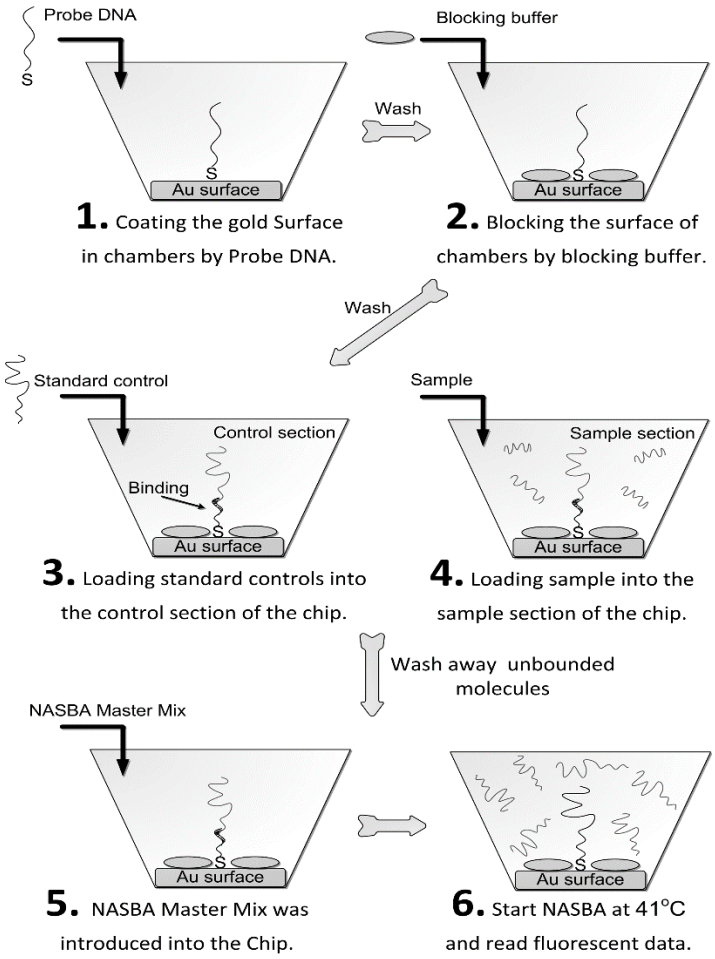

Figure 16. Mechanism of amplification for the on-chip Q-NASBA assay.[15] Adapted with permission from Ref. 15, Copyright 2012, American Chemical Society. 'Capture probes' are specific single-stranded DNA molecules that can anchor onto the gold surface layer to capture corresponding RNA templates for NASBA assays. The 5'-end of each probe was furnished with a 15-mer thymine linker and modified by a mercapto group that can chemically couple with gold atoms.[15,131,132] The 3'-end of each probe contained the identical sequence as the 'reverse primers' in the NASBA assays.[15]

- **Experiments:**

The sampling and transport processes are used to condense microorganisms from an up to 2L water sample onto the membrane filter in a specific filter set. The sample preparation cassette was designed to lyse the microbial cells retained on the filter set and to extract their DNA/RNA contents to minimize contamination during the procedure (Figure 14). The Q-NASBA chips and relevant components were prepared using both polymers (PDMS and PMMA) and silicon. The inner surfaces of reaction chambers were manually treated using pipettes, as previously described.[15,131] In this work, *Saccharomyces cerevisiae*, *Staphylococcus aureus* and *E. coli* were selected as the testing targets to demonstrate the system function. (See also Article II)

Three groups of probes and primers were used in Q-NASBA assays, including 'pan-fungal probe and primers' for *S. cerevisiae*, 'pan-gram-positive probe and primers' for *S. aureus*, and 'pan-gram-negative probe and primers' for *E. coli*. Zhao *et al.* reported the following primers and probes in 2009:[15,133]

1. Pan-fungal probe and primers: (Target: 28S rRNA)[133]

   Forward primer: 5' *CGGCTCTTCCTATCATACCG* 3'

   Reverse primer: 5' *AATTCTAATACGACTCACTATAGGGCTAAACCC AGCTCACGTTCC* 3'

   Molecular beacon: 5'FAM-*CGCGATATTCGGTAAGCGTTGGATTGAT CGCG* –Dabcyl 3'

   Capture probe: 5'SH-$(T)_{15}$ –Reverse primer-3'

2. Pan-gram-positive probe and primers: (Target: 16S rRNA)[133]

   Forward primer: 5' *TACGGGAGGCAGCAGT* 3'

   Reverse primer: 5' *AATTCTAATACGACTCACTATAGGGGCTGCTGG CACGTAGTTAGCCGTGGCTTTC* 3'

   Molecular beacon: 5'FAM-*CGAGCTAGCAACGCCGCGTGAGTGAA GCTCG*–Dabcyl 3'

   Capture probe: 5'SH-$(T)_{15}$ –Reverse primer-3'

3. Pan-gram-negative probe and primers: (Target: 16S rRNA)[133]

   Forward primer: 5' *CCTGATGCAGCCATGCCGCGTG* 3'

   Reverse primer: 5' *AATTCTAATACGACTCACTATAGGGCACGGAGTTA GCCGGTGCTT* 3'

   Molecular beacon: 5'FAM-*CGAGCTTGAAGAAGGCCTTCGGGTTGTA AAGAGCTCG* –Dabcyl 3'

   Capture probe: 5'SH-$(T)_{15}$–Reverse primer-3'

The chambers in the LOC were coated with different probes with different detection specificies.[15] Then, the LOC was operated through an external pump and valve system, see also Article II.

- **Results and discussion:**

To verify the accuracy of Q-NASBA assays performed using a microplate reader, the initial tests were conducted manually on PCR plates. In the present study, the total RNA *of S. cerevisiae* was purified and used as standard samples for Q-NASBA tests. The results are presented in Figure 17, inset A. At total RNA concentrations between 1 ng·L^{-1} and 1 µg·L^{-1}, the Q-NASBA assays exhibit standard amplification curves.

The threshold time (C_t) is redefined artificially as the time required for the fluorescent signal to exceed the background noise. However, as the noise levels of NASBA assays can vary, the artificial threshold for C_t values lacks a solid standard. To address this concern, a moving threshold was defined as 105% of the average fluorescence value of 5 min before every measurement. The amplification time when the measured value exceeded its moving average threshold for the first time was defined as C_t. Subtracting each measured value from its moving threshold yields the value of difference value, as demonstrated in Figure 17, Inset B.

$$Threshold\ (n) = 105\% \times \frac{\sum_{n-5}^{n-1} Fluoresenc\ e \cdot Value(i)}{5}$$

However, the measured values may occasionally exceed their moving thresholds within the first 20 min because of the initial temperature fluctuations inside the microplate reader，but significant C_t values are always found to occur after 25 min; therefore, the first 20 min can be ignored if abnormal data are also observed for the negative control. This method yields a good linear curve between C_t and the logarithm of template concentration (Figure 17, Inset C). *S. cerevisiae* was used to demonstrate the function of Q-NASBA chips, whereas *S. aureus* and *E. coli* were employed in the subsequent recovery tests on Q-NASBA chips. The testing results of *S. cerevisiae* are presented in Figure 17, insets D, E and F. The preliminary tests confirmed that the on-chip Q-NASBA is a sensitive method for the quantification of *S. cerevisiae* in water. The LOD of the Q-NASBA platform for microbes in water samples is above 10^2 cells·L^{-1}. Known amounts of *S. cerevisiae*, *S. aureus*, and *E. coli* were added to the natural water samples acquired from a reservoir, and analysed in the LOC devices. Excellent recoveries were obtained for microbe cells in the samples ranging from 90% to 120%, see also Article II.

The hybridisation of target nucleic acids to immobilized probes is a critical challenge to on-chip Q-NASBA tests. It is subject to probe length and density effects, as well as to surface charge and chemistry.[15,130,134,135] The tolerable LOD of this LOC may also be also attributed to the low efficiency of RNA extraction and hybridisation. One improving method could involve amplifying the templates by multiplex PCR/NASBA and extending the hybridisation time to enhance output signals.[15,136-138]

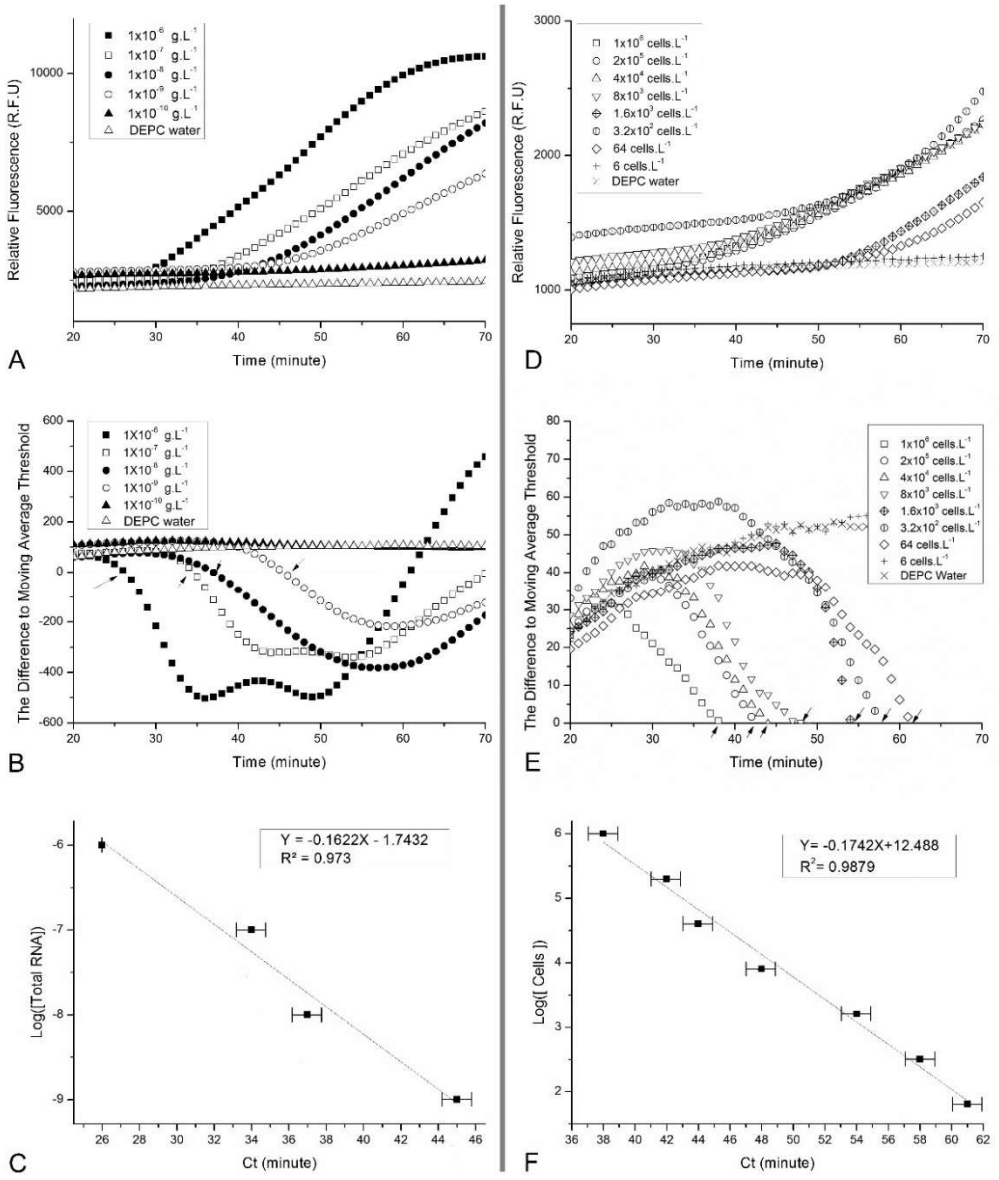

Figure 17. Typical results of Q- NASBA assays.[15] Adapted with permission from Ref. 15, Copyright 2012, American Chemical Society. Insets A, B and C present the testing results from PCR plates, whereas insets D, E and F present the results obtained with the Q-NASBA chip. The arrows in Insets B and E show the locations of each 'Ct' point, where the abscissa presents the relative Ct values. (See also Article II)[15]

- **Summary of the study:**

- The results of this study indicated that Q-NASBA assays can be performed successfully in a common microplate reader. Moreover, the method of quantification for NASBA tests was also improved. In addition, we found that C_t values always occur before 1 h; therefore, the total reaction time of Q-NASBA assays can be shortened to 70 min.
- This system design reflects a simple principle that sample preparation is critical for error propagation throughout the whole procedure. Without accurate sample preparation, any result in the detection cannot be completely trusted.[15] The seamless connection between the sampling module and the detection module helps operators decrease the possibility of contamination and sample loss, the most destructive factors for such quantitative tests.
- The multifunctional LOC system successfully simplified and standardized the complicated processes of multiple Q-NASBA assays, but the system design still contains some redundancies. More in-depth studies are required before this LOC is utilized in practical applications.
- This series of microplate-compatible LOCs could likely be further developed to produce a low-cost microarray.

2.1.3 Rapid identification and susceptibility testing of uropathogenic microbes via an immunosorbent ATP-bioluminescence assay on a microfluidic simulator for antibiotic therapy.

- **Background**

UTIs are among the most common bacterial infections and mainly caused by bacteria.[70,121,139,140] The most common UTI-causing organism is *E. coli*, with 80%–85% of cases originating from these bacteria. *Staphylococcus saprophyticus* are responsible for 5%–10% of UTI cases, and UTIs also can be caused by viral or fungal infections.[121] Other groups of bacteria can also cause UTIs.[121] UTIs are often treated with antibiotics.[70,141] The standard diagnosis of UTIs in hospitals relies on culture-based identification and antimicrobial susceptibility testing (AST), which have typical delays of a couple of days.[70,142]

In the absence of a definitive microbiological diagnosis, doctors frequently initiate an imprecise empirical broad-spectrum antibiotic treatment to meet the urgent needs of

patients.[139] A growing problem of worldwide concern is the increasing resistance of pathogens to conventional antibiotics[10].[143] Although the diagnostic technology for UTIs has advanced, the existing methods or tools remain too slow to achieve timely and judicious antibiotic treatment in practice.[70]

- **Objectives:** (Article III)

To achieve rapid diagnosis of UTI pathogens using high-throughput LOC technology and provide a low-cost efficient medical simulation platform for AST, enabling the selection of appropriate antibiotics.

- **Main relevant projects:**
  - Oslofjord Fund (Touchsensor, Project No.: 234972);
  - The Norwegian Micro-Nanofabrication Facility, Norfab (197411/V30)
- **Challenges:**
  - Is it possible to readjust the previously developed disposable LOCs that are compatible with microplate readers for this new application of urine testing?
  - How can a low-cost LOC be developed that integrates the multiple functions of immunoassay, cell culture, rapid quantification of living cells and AST?
  - How can the multiple tests on the disposable LOC be accelerated?
  - How can the qualitative method of the ATP bioluminescence assay be converted to a semi-quantitative on-chip method with some specificity?
- **Design of the LOC system and its DSM:**

This work reports the design of a cell-based LOC system to accomplish the integrated functions of rapid microbe identification and AST, named ‘microfluidic simulator’ or ‘medical simulator’. Compared with other emerging methods, the culture-based method remains robust and reliable, with the results that the culture-based method is selected for the DSM of this LOC. Target microbes in the urine sample will be captured by specific antibodies in the LOC device and encapsulated by the hydrogel in situ. Given enough growth medium, the immobilized microbes can grow there prior to quantification and follow-up testing. Accordingly, the immunosorbent ATP-bioluminescence assay (IATP-BLA) was selected as a fundamental measurement method for the LOC to identify and

evaluate the properties of uropathogenic bacteria. The ATP bioluminescence assay (ATP-BLA) has been described in Chapter 1.2.1.1. The magnitude of the ATP bioluminescence signal is proportional to the amount of living microbes. Because the specificity of immunoassay and the high sensitivity of the ATP-BLA can be perfectly integrated into the 384-chamber LOC, this device can simultaneously perform rapid microbial tests on 13 types of microbe strains prior to AST for eight selected antibiotics. The entire processes of immunoassay, cell culture, ATP-BLA and AST were smoothly integrated in the LOC. Compared with traditional urine culture testing, the LOC accelerates the time to result from a few days down to hours.(Figure 18)[70] (See also Article III)

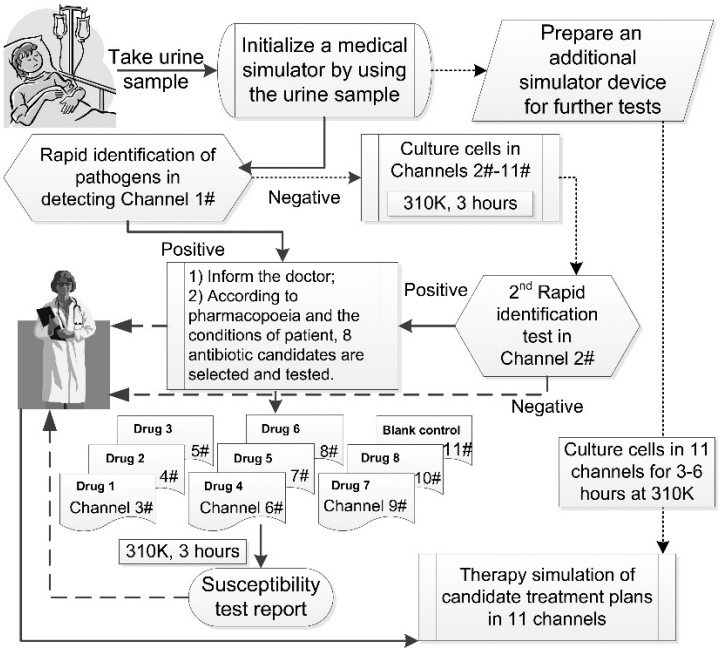

Figure 18. The typical process of the microfluidic simulator in a UTI case.[70] Reprinted with permission from Ref. 70, Copyright 2015, American Chemical Society. (See also Article III)

The LOC is designed with 384 vertical reaction chambers with dimensions corresponding to those of a standard 384-well microtiter plate as in Chapter 2.1.1 and 2.1.2, as shown in Figure 19. This design ensures that the signals from the microfluidic LOCs can be directly read using a common microplate reader. The schematic diagram of the on-chip IATP-BLA is shown in Figure 20. Due to the hydrophilicity of the glass fibres, the aqueous phase penetrates laterally towards the nearby reaction chambers with small particles such as bacteria, but large clots or mucilage are obstructed due to the limited size (<2.7 μm) of the apertures inside the fibreglass membrane. The antibodies immobilized on the glass

fibres capture target microbes. The captured microbes are encapsulated in a thin film of calcium alginate gel by the following gelation reaction:[144]

$$Ca^{2+} + HA\text{lg} + NaA\text{lg} = Ca(A\text{lg})_2 + H^+ + Na^+$$

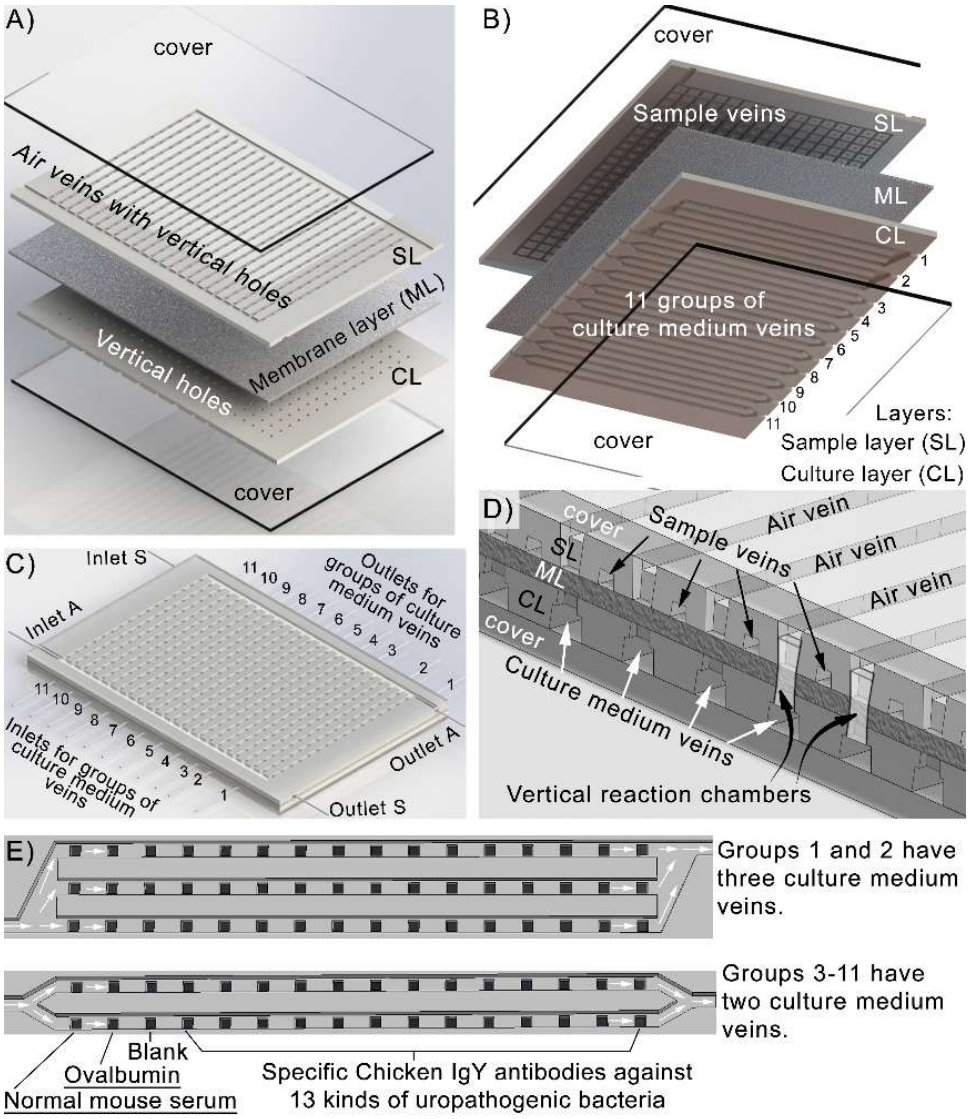

Figure 19. The architecture of the microfluidic LOC.[70] Reprinted with permission from Ref. 70, Copyright 2015, American Chemical Society. (a) Downward view of the chip layout. Three layer components: the Sample Layer (SL), the Membrane Layer (ML) and the Culture Layer (CL). (b) Upward view of the chip layout. 11 groups of culture medium veins (CMVs) are engraved on the lower face of the CL. (c) The inlets and outlets of the device. (d) Vertical reaction chambers in the cross-sectional view. (e) Groups of detection channels. Each group of CMVs is an independent detection channel. Every CMV covers 16 reaction chambers, including 13 IgY-coated detection units for uropathogens, an ovalbumin control, a normal mouse serum control, and a blank control. Groups 1-2 have three replicates, respectively; Groups 3-11 have only two replicates.[70] (See also Article III)

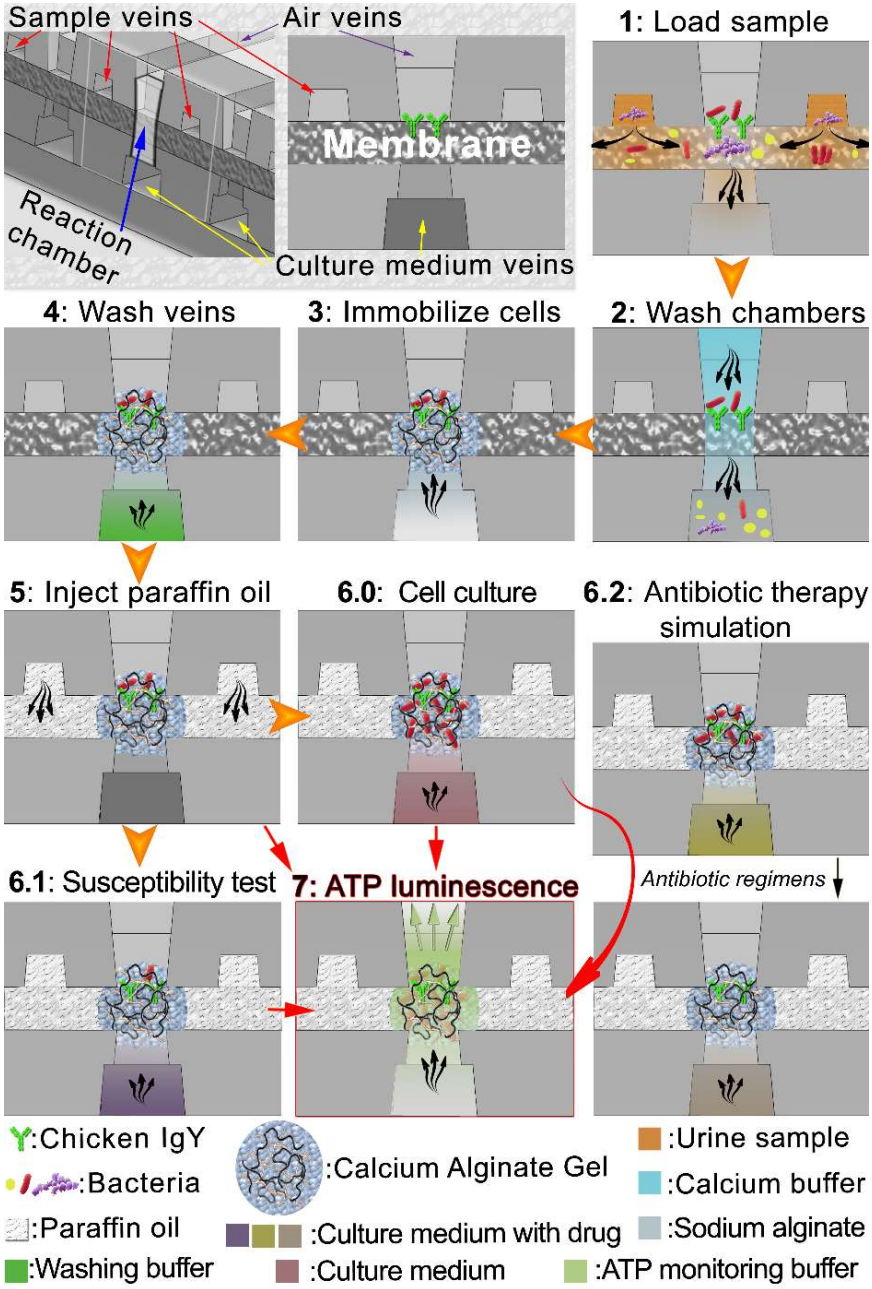

Figure 20. The typical protocol of the IATP-BLA test in the microfluidic LOC.[70] Reprinted with permission from Ref. 70, Copyright 2015, American Chemical Society. Specific IgY antibodies were immobilized on the fibreglass membrane during fabrication. The standard operating procedure is: (1) the urine sample is loaded into the sample veins, and the specific microbes are captured by IgY antibodies; (2) unbound microbes are removed; (3) the captured cells are encapsulated in situ by calcium alginate gel; (4) the sodium alginate solution is washed away; and (5) paraffin oil is injected to isolate each reaction chamber. Subsequently, different channels will be processed differently. (6.0) The captured microbes can be reproduced before ATP-BLA; (6.2) the captured microbes can be inhibited by a series of antibiotic regimens before ATP-BLA; or (6.1) the captured microbes can be inhibited by a single antibiotic in AST. Either way, (7) the cells will finally be quantified by ATP-BLA using a microplate reader.[70] (See also Article III)

- **Experiments:**

The microfluidic device is constructed of two white polystyrene (PS) sheets that were processed by laser ablation, between which a piece of Waterman™ grade GF-D fibreglass membrane filter was tightly clipped. Various reagents can be added to each reaction chamber through 24 CMVs. Each group of CMVs shares the same inlet and outlet pair. In the LOC, 16 vertical reaction chambers are designed for each CMV. The fibreglass membrane inside of the 13 chambers is immobilized with 13 types of specific chicken egg yolk antibodies (IgY) against their respective corresponding uropathogenic microbes, including *E. coli*, *Staphylococcus saprophyticus*, *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Klebsiella pneumonia*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Enterobacter cloacae*, *Enterococcus faecium*, *Enterococcus faecalis*, *Streptococcus viridans*, *Streptococcus pyogenes* and *Candida albicans*. The other three chambers in each CMV are for controls. (See also Article III)

Surface treatment of the fibreglass membrane was prepared in the same manner as poly-L-lysine-coated glass slides.[145] The protein solutions were transferred by pipetting into the corresponding chambers from the top of the air veins, with approximately 0.6 μL of solution added per chamber. The resulting components were incubated for 1 hour at 298K and then freeze-dried and sealed with transparent sealing tapes.[70] The LOCs were operated by a manual valve and pump system.[70] The 13 types of microbe clones were provided by cooperating hospitals and were the same strains as those used as the antigens for immunization. Flinn Scientific© artificial urine was spiked with the 13 types of microbes, and a series of microbe-containing artificial urine samples were made and tested. The relative luminescence unit (R.L.U.) data were collected by a microplate reader and analyzed by using Microsoft Excel® and OriginPro®. To validate the on-chip AST, an artificial urine sample containing 1000 cells/ml was loaded into a microfluidic LOC and tested by ampicillin, carbenicillin and erythromycin. The R.L.U. data of ATP-BLA were then collected.[70] (See also Article III)

- **Results and discussion:**

All operations from sample processing to ATP-BLA are smoothly integrated to reduce the turnaround time. For example, the sample processing employs lateral-flow across the fibreglass membrane, which not only allows for the immune capture of target microbes

but also acts to filter irrelevant particles from the sample. The tests showed that microfluidic LOCs can specifically recognize the microbes in the artificial urine samples containing multiple microbes (Figure 21).[70] A significant difference can be observed between microbes and ovalbumin-coated negative controls at concentrations above 1000 cells/ml, but it is also indicated that the on-chip ATP-BLA results cannot directly reflect the number of living microorganisms regardless of species. The method can be used to evaluate the numbers of microbes within the same species. We were not able to distinguish the difference at target microbe concentrations below 100 cells/ml.[70] Interestingly, the R.L.U. value of *E. coli* in a sample of 1×10^6 cells/ml generally appeared to be saturated, shown in Figure 21, inset B. This implies that the general measurement range of immediate IATP-BLA ranges from 1×10^3 cells/ml to 1×10^5 cells/ml, which is within the clinical cutoff of urine culture tests.[146] Moreover, cell cultivation prior to the 2nd round of ATP-BLA can improve the LOD, because in general, pathogenic microbes can grow exponentially in the growth medium. For example, if one *E.coli* cell is initially captured, hundreds of *E.coli* cells will proliferate in situ after 3 h of cultivation, which can be easily detected by the 2nd round of the ATP-BLA test.[70]

The signals of the blank controls are lower than those of negative ovalbumin controls (Figure 21, inset A), implying that the coated protein on the fibreglass potentially increase the nonspecific absorbance of microbes compared with that of raw fibreglass. Furthermore, the normal mouse serum controls were more similar to the detection items than to the negative controls (Figure 21, insets A and C). The specific absorbance of normal mouse serum should be related to Protein A and Protein G, which are derived from *S. aureus* and *Streptococcus* species. Both proteins have binding sites for the Fc portion of mammalian IgG, including the IgG in the mouse serum. For this reason, IgY antibodies from chicken were finally selected to replace the common IgG antibodies of mammalian.[70]

Given the proper cell culture conditions, hydrogel encapsulation is not an essential requirement for the growth of cells, but the hydrogel can restrict the movement of microbes. Aeration is another important factor for the on-chip AST. Parameter optimisation enabled good AST results using the LOC (Figure 21, inset D). The rate-limiting step of AST is the bacterial growth. According to our experience, cultivation for three hours could increase the limit of detection by at least one order of magnitude.

However, no firm conclusions can be drawn regarding this cultivation as different strains have different growth rates.(See also Article III)[70]

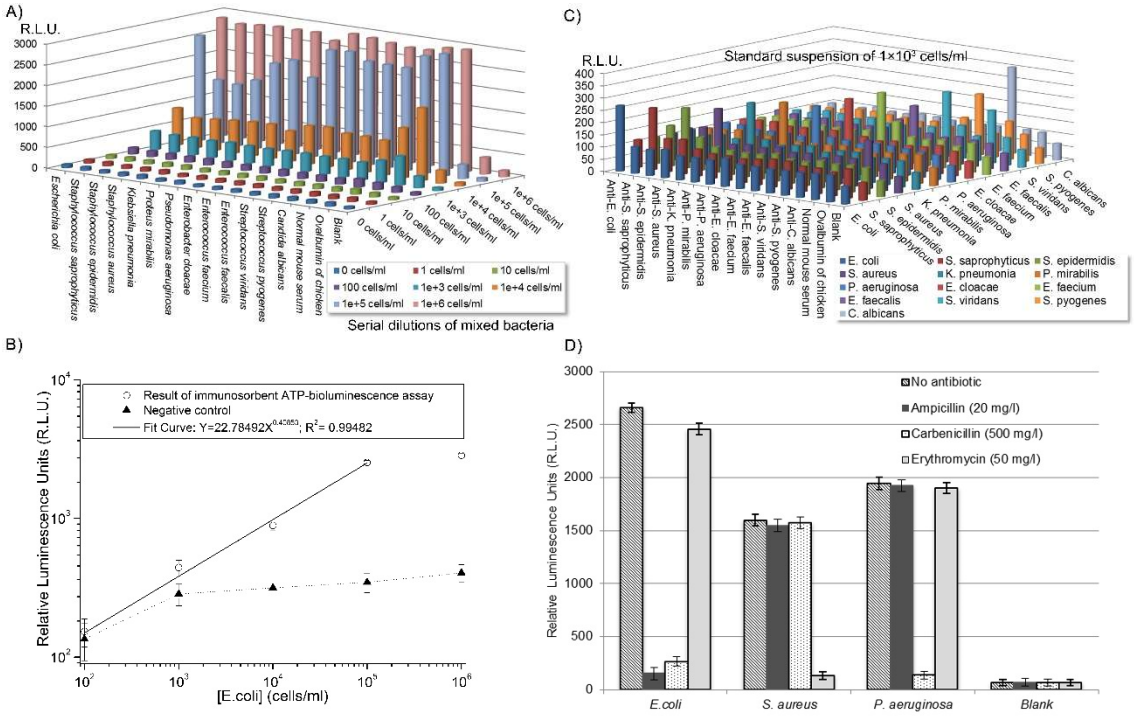

Figure 21. The rapid identification of 13 microbes and AST using the microfluidic LOCs.[70] Adapted with permission from Ref.70, Copyright 2015, American Chemical Society. (a) Serially diluted samples of artificial urine containing multiple microbes were tested. (b) The concentrations of *E. coli* in the serially diluted samples of artificial urine were measured, whereas the R.L.U. data of the negative controls are also shown as the background. A power regression analysis on R.L.U. values and relative cell concentrations (from 100 cells/ml to 1×10^5 cells/ml) produced a good fit curve (n=6, R^2=0.99482). (c) Thirteen standard samples with 1000 cells/ml of different single species were measured. (d) A typical on-chip AST was demonstrated. In the test, a sample of artificial urine containing 1×10^3 cells/ml was treated with 20 mg/L ampicillin, 500 mg/L carbenicillin and 50 mg/L erythromycin (n=4).[70] (See also Article III)

- **Summary of the study:**
  - This microfluidic simulator is a patient-specific modelling device to enable the personalized treatment of UTIs. The LOC operates on a similar principle as conventional urine culture and AST in petri dishes, but accelerates the testing. Although this approach provides a promising new option for the diagnosis and treatment of UTIs, large-scale clinical validation is required.
  - The integration of the specificity of immunoassays and the sensitivity of the ATP-bioluminescence assay enables this sensitive cytometric method

for the detection of specific living cells. Due to the high efficiency of on-chip IATP-BLA tests, the LOC device supports the rapid identification of the most common causative agents of UTIs and enables subsequent AST testing within 3-6 hours.

- Cell-based LOC technology could use cells, pathogen-containing tissues or tumour cells from individual patients to construct personal rapid simulation models for personalized clinical treatment, thereby reducing the risks of therapy. With increasing demand for personalized treatments, more and more medical simulators will be developed, especially for chronic incurable diseases such as chronic hepatitis, diabetes, cancer, and rheumatism.[70,147]
- A low-cost LOC system is a feasible way to implement such complex detection. Due to its compatibility with a microplate reader, the series of LOCs provides a good example of functional integration on a low-cost chip. The divided LOC system is suitable for the implementation of complex automation associated with high-throughput detection, particularly for tests of living cells.

2.2 LOC devices containing integrative biological systems with unconventional DSMs: a microfluidic device for continuous sensing of systemic acute toxins in drinking water

Compared with the LOCs based on conventional DSMs, the designs of these unconventional LOC systems allow for better performance. However, unconventional DSMs often require more evaluation; accordingly, large-scale long-term experiments are required for the validation of their designed functions.

- **Background**

See also Chapter 1.2.1.2.

- **Objectives:** (Article IV)

To develop a continuous, cost-efficient LOC system to evaluate the threat of acutely toxic chemicals.

- **Main relevant projects:**
    - Norwegian long term support from NorFab (living-cell-based LOC project);
    - Oslofjord Fund (Et cellebasert digitalt mikrofluidisk system, Project No.: 220635);
    - The Norwegian Micro-Nanofabrication Facility, Norfab (197411/V30);
- **Challenges:**
    - Is it possible to translate the traditional discrete-time protocol of the bioluminescence toxicity test using *V. fischeri* into a continuous test carried out on a chip?
    - How can a continuously functioning monitor be implemented at low cost?
    - How can the continuous steps of sample loading, mixing and detection be carried out in the LOC?
- **Design of the LOC system and its DSM:**

The principle of the luminescent bacteria test relies on the inhibition of light emission by *V. fischeri* in response to the effects of toxic chemicals.[21] Generally, the inhibition test in ISO11384 is accomplished by tests with specified volumes of a suspension of luminescent bacteria in a cuvette. The test criterion is the decrease in luminescence, measured after 15 min and again after 30 min. Parallel measurements of changes in the luminescence intensity of standard control samples can be used to determine the inhibition rate.[21] Here, the unconventional DSM of the *V. fischeri* cell-based LOC was developed according to traditional periodic tests, named a 'living-cell chip' (Figure 22).

In the LOC, water samples and a suspension of luminescent bacteria will be continuously added to the micro-mixers at a fixed flow rate. The mixture has to flow through a long spiral-shaped micro-channel before reaching the observation chamber, with the result that 20-30 min has passed from the time of mixing to the time of optical measurement. Theoretically, the basic principle of the on-chip test is similar to that of the periodic test. In addition, when the flow rates and other conditions of the LOC system are fixed, the flow will result in a steady state luminescent intensity in the observation chamber. The

inhibition rate based on the light emission can be evaluated by comparison with a parallel control system.

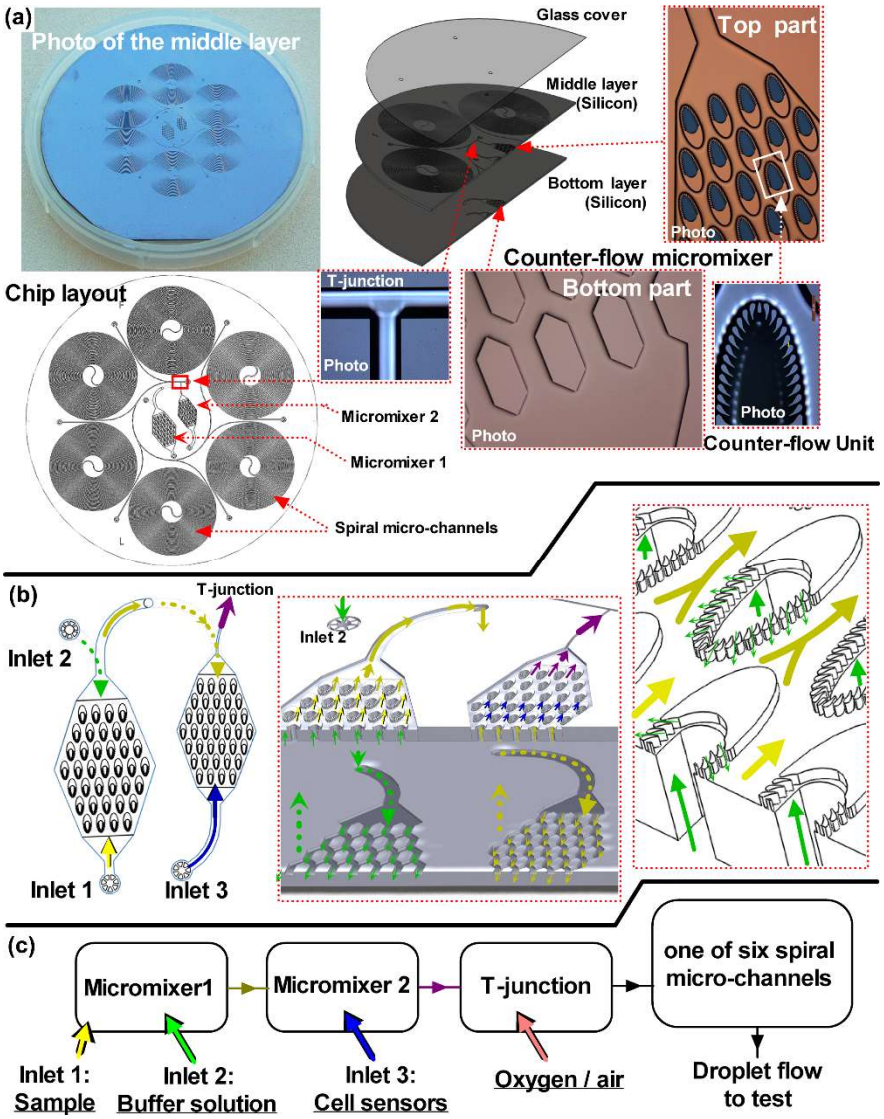

Figure 22. Construction of the cell-based LOC device.[21] The map of the chip domains is shown at the bottom. The sample, buffer solution and cell suspension are mixed to form droplets within the airflow (see also Article IV).[21]

The complete LOC system is composed of five major components, including a sampling module, peripheral actuating devices (i.e., valves and pumps, etc.), a cell-culture module for *V. fischeri*, a cell-based LOC device and a photomultiplier-tube-(PMT)-based photo-sensing module. The non-clogging micro-concentrator in the previous report was employed as the sampling module for drinking water.[148] Copper (Cu^{2+}), zinc (Zn^{2+}), potassium dichromate and 3, 5-dichlorophenol were selected as typical toxins to validate the LOC system.[21]

- **Experiments:**

The toxicity of the water sample was evaluated by measuring the intensity of emitted light measured by the PMT-based detection module, as shown in Figure 23.[21] The PMT was used as a sensitive detection module with low power consumption and low noise, and these signals were imported into a computer prior to analysis using Microsoft Excel® and OriginPro® software.[21] (See also Article IV)

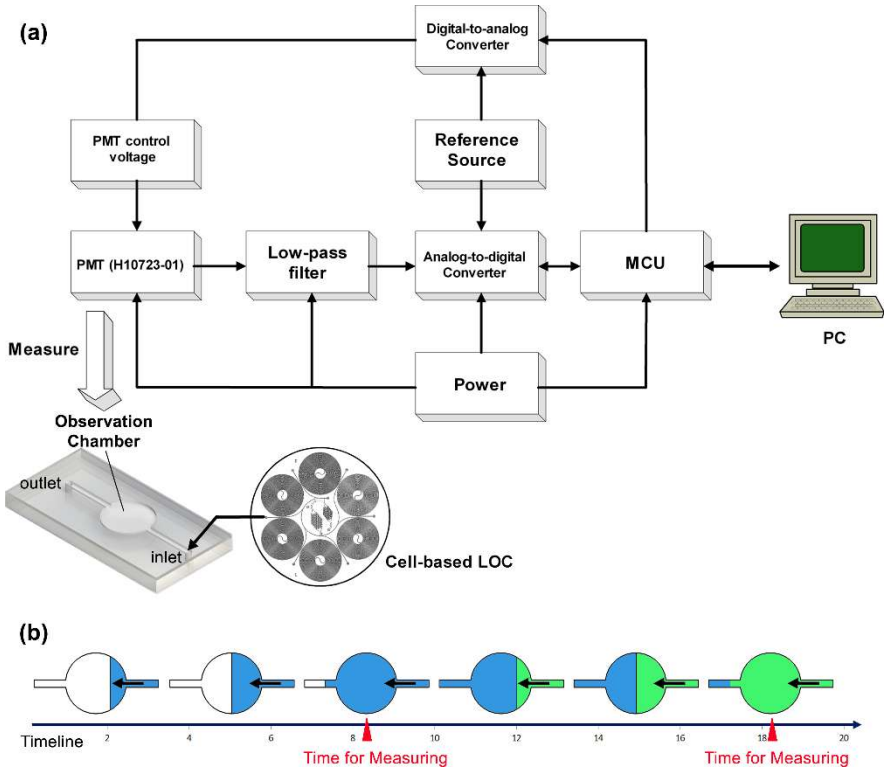

Figure 23. Schematic of the detection system.[21] The droplets continuously flow through the observation chamber. After a short period, the contents of the observation chamber are totally replaced. Then, the detection system performs periodic measurements of the observation chamber.[21] (See also Article IV)

The $CuSO_4$ and $ZnSO_4$ solutions were serially diluted in deionized water as model toxin samples, which concentrations ranged from safe levels below the guidelines in potable water to poisonous levels far above the legal limit for drinking water quality.[149,150] The solutions of 50 mg/L potassium dichromate and 4 mg/L 3, 5-dichlorophenol were prepared in deionized water and used as the artificial contaminated water samples. Deionized water and 2% NaCl were used as blank solutions. A solution of 60 mg/L cetylpyridinium chloride (Weifa®, Oslo, Norway) was employed as a positive control, since cetylpyridinium chloride (CPC) is a broad-spectrum antibacterial agent. *V. fischeri*

came from the BO1243-500 BioToX™ Kit (Aboatox Oy®, Masku, Finland). See also Article IV.

The volumetric flow rates for the test samples, buffer solutions, bacterial suspensions and air flows were at a constant ratio of 1:1:2:4. The 'droplet flow rate' is defined as the total volumetric flow rate of the three liquid solutions. The 'response time' of the sensing device is defined as the period from the moment the first droplet is formed to the moment that the observation chamber is filled by the droplets. After the response time and sampling interval of the LOC system were studied in initial tests using serial dilutions of $CuSO_4$, the 20 μL observation chamber was selected as the default chamber volume. Moreover, the droplet flow rate was fixed at 4×10^{-11} m^3/s for all other toxicity tests.[21] Because the system continuously monitors the water samples, the observation chamber is measured over a specific period, which is defined as the 'sampling interval'.[21] This period is set to coincide with the period over which the droplets in the observation chamber are expelled and replaced by fresh ones. (See also Article IV)

- **Results and discussion:**

Six Cu^{2+} solutions were tested in the cell-based sensing device at several droplet flow rates (Figure 24, inset A).[21] The LOC responses to approximately 0.05 mg/L Cu^{2+} or Zn^{2+} solutions are similar to the responses for deionized water, which is in line with the WHO standards for drinking water quality (< 2 mg/L).[150] The results for the group with 0.05 mg/L Cu^{2+} were very similar to the results for the deionized water group. However, the solutions of 0.5 mg/L Cu^{2+} and 2.5 mg/L Cu^{2+} exhibited some toxicological effects on the *V. fischeri* cells (Figure 24, inset A). The results for the positive control group (60 mg/L CPC) were in line with expectations (i.e., their bioluminescent signals were close to zero).[21] The Zn^{2+} solutions were also measured using the standard settings and analysed (Figure 24, inset B). A non-linear relationship between the Zn^{2+} concentration and the R.L.U. was observed, but this LOC system is useful as a broad-spectrum early warning tool rather than as a quantitative system.[21] The artificial samples of tap water were also tested in the cell-based LOC system, and the results indicated that the cell-based LOC can easily distinguished toxic contamination from safe drinking water(Figure 24, inset E).[21]

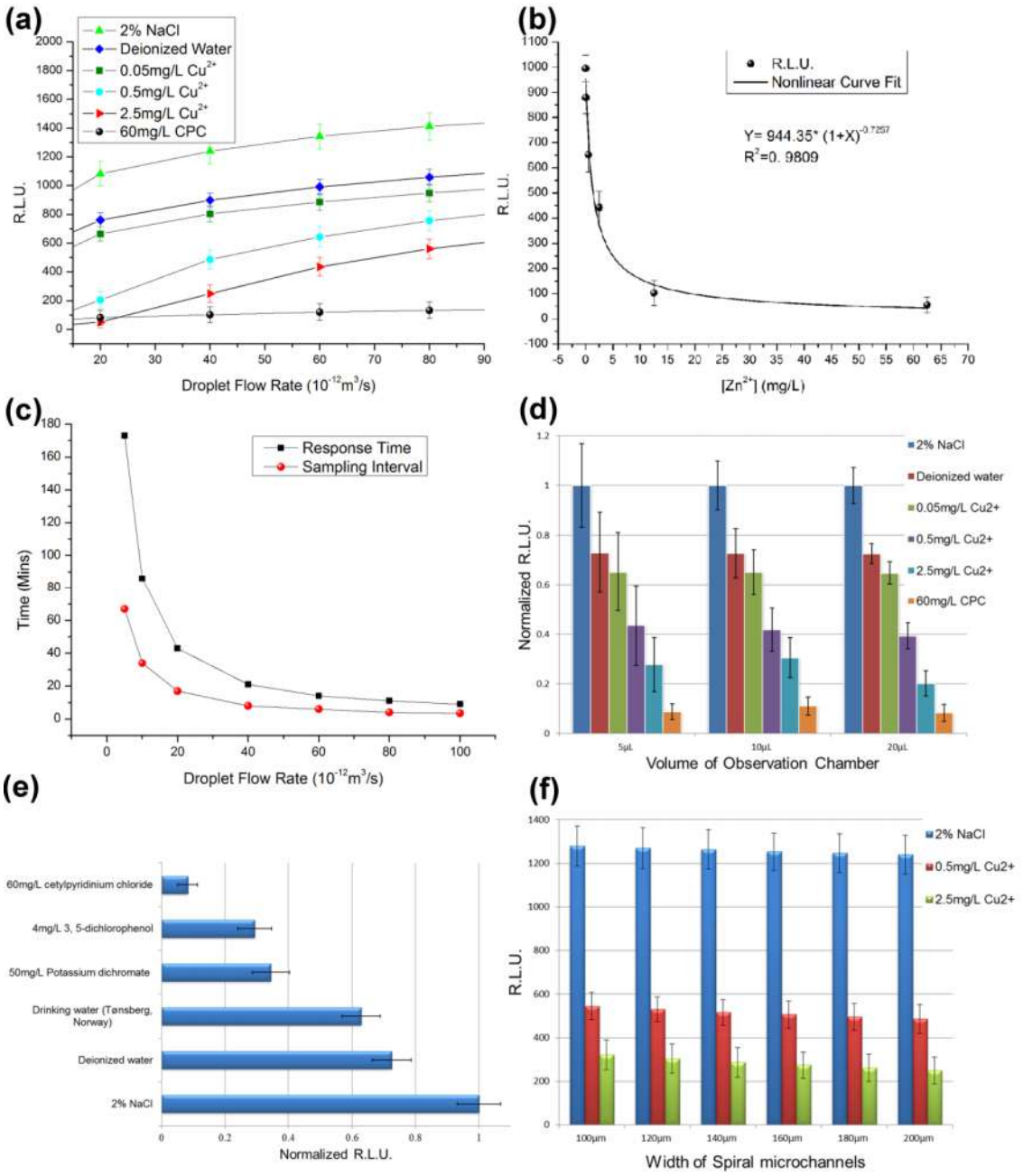

Figure 24. Toxicity test results for the *V. fischeri* cell-based LOC system.[21] (a) The six sample solutions (2% NaCl, deionized water, 0.05 mg/L Cu^{2+} solution, 0.5 mg/L Cu^{2+} solution, 2.5 mg/L Cu^{2+} solution and 60 mg/L CPC) were tested at different droplet flow rates. (b) Nonlinear regression between [Zn^{2+}] and the RLU data. (c) Response time and sampling interval of the sensing device. (d) Observation chambers with volumes of 5 μL, 10 μL and 20 μL were tested. (e) The drinking water sample and five artificial solutions were tested, including 2% NaCl, deionized water, a drinking water sample, 50 mg/L potassium dichromate, 4 mg/L 3, 5-dichlorophenol and 60 mg/L CPC. (f) Parallel tests for the six spiral microchannels.[21] (See also Article IV)

When the droplet flow rate decreased below 2×10^{-11} m^3/s, the response time increased to more than 30 min (Figure 24, inset C). The standard droplet flow rate was selected as 4×10^{-11} m^3/s to limit the response time to within 30 min. When different observation

chambers and spiral micro-channels were tested in parallel experiments, the results showed that using a smaller observation chamber led to larger relative errors (Figure 24, inset D), whereas switching among different spiral micro-channels had no significant impact on the results (Figure 24, inset F). The 20 µL observation chamber was preferred.[21]

In this study, the converted EC_{50} value for copper (II) was approximately 0.5 mg/L, and the equivalent exposure time was almost 30 min. The traditional toxicity assessment using *V. fischeri* cells as biosensors is based on the inhibition of luminescence. The toxicity is usually captured as the EC_{50}, which is the effective concentration of the tested chemical at which 50% of the luminescence is inhibited.[151] The reported EC_{50} values for copper (II) exposure of *V. fischeri* range from 0.15-0.58 mg/L at an exposure time of 30 min.[152]. Here, the continuous tests in the LOC device were in line with the traditional periodic tests, but the standard reference values for the on-chip tests have to be modified due to the different physical environment of the LOC. [21]

- **Summary of the study:**
    - This living-cell chip is an appropriate early warning device to assess possible threats from toxic chemicals in water. Note that this cell-based LOC device was developed to assess the toxicity of chemicals in water rather than their concentrations. This study demonstrated that a traditional DSM could be modified for a specific LOC to realize a special purpose. The unconventional tailored DSM is generated for the contrived LOC.
    - The chip design of this LOC employs the infrastructure of digital microfluidics, which is a good solution for sample mixing, oxygen supply, and continuous detection. However, the digital microfluidic structure might be not optimal for the continuous culture of cell sensors. This LOC system has to employ an external bioreactor as the long-term source of living cell sensors, increasing the total cost. Furthermore, the tiny droplet volume leads to extraordinarily high requirements for the optical detection module. The LOC system should be further improved to address these limitations.
    - Currently, the living-cell chip has to work in a stable environment, making it difficult to deploy for field-testing.
    - Integration of the bioreactor and observation chamber into a single LOC would enhance the system performance and greatly reduce the system cost.

- **Further study on a series of living-cell chips.**

Based on colorimetric reactions in a specific growth medium during the proliferation of living microbes, another tailored DSM was also investigated in the living-cell chip, the continuous monitoring of living bacteria in water.[20] However, the response time was far from the desirable rapid response. Then, the digital microfluidics infrastructure was totally removed from the new version of the living-cell chip. Inspired by the natural coral reef arrangement, the 2nd generation living-cell chip was developed with a new design to enable early warning of the presence of toxic chemicals in drinking water. These results remain unpublished.

The 2nd generation living-cell chip includes a coral-reef-like microfluidic bioreactor to accommodate bioluminescent cell sensors. A protected hydrostatic area is present behind each pillar unit, intended for the propagation of living organisms (Figure 25). The flow consisting of the water sample, nutritional substances and oxygen will continuously pass by pillar units as ocean currents pass through coral reefs. The semi-open structures take full advantage of the compactness of the microchip, resulting in the simple and efficient diffusion of oxygen, nutrients, and chemical analytes. The cell sensors can stay and multiply within the protected area; if the cell population is overcrowded, the excess cells will spread out of the protected area and be flushed away by the continuous flow of water.

Given fixed conditions that meet the needs of the cells, the populations of living cell sensors in a LOC will reach a spontaneous steady state. Until toxic chemicals are introduced in the water samples, all the cell colonies in the LOC will be affected synchronously. Consequently, the bioluminescent signals will be stronger than the previous living-cell chip by several orders of magnitude, although the LOC still relies on the simple and low-cost microstructure. The bioreactor and observation chamber, separate in the 1st version of the chip, are perfectly integrated in the new version of the chip design. Moreover, the LOC naturally works continuously rather than periodically, and the water sample flows in a continuous stream around the resident cell colonies of the LOC.

Compared with commercial online water quality monitoring equipment (see also Chapter 1.2.1, Figure 7), the miniaturised LOC system is able to achieve long-term continuous monitoring with low consumption of reagents and energy. Moreover, due to the nature of living cell sensors, the cell-based LOC system has the ability to simultaneously detect

thousands of harmful substances. This 2nd generation living-cell chip has the potential to become an excellent early warning system for water safety. This device has entered the commercialisation process, and an international patent is currently being processed.

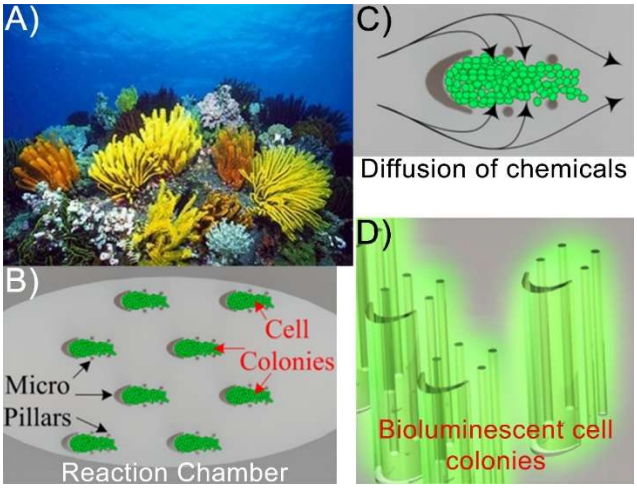

Figure 25. The principle of the coral-reef-like microfluidic bioreactor present in the 2nd generation of the living-cell chip.(unpublished) (a) A photo of a natural coral reef in the ocean current. (b) The bioluminescent cell sensors can form stable cell colonies in the reaction chamber with micro-pillar arrays. (c) The chemicals present in the water sample will continuously diffuse past the cell colonies. (d) Schematic sketch of bioluminescent cell colonies in the LOC.

Although the DSMs of living-cell chips are still derived from known principles of existing biological protocols, their specific criteria were greatly modified to match the various LOC hardware designs. Compared with conventional cell-based toxicity tests, the series of LOCs are able to operate continuously over a long period to monitor a broad spectrum of harmful agents in drinking water. However, many doubts have been raised about the unconventional DSMs, and a long-term large-scale verification is required to demonstrate the function of living-cell chips. A follow-up research project has been initiated to address these questions.

2.3 Database-oriented LOC devices to construct a prognostic DSM with computational modelling.

Fascinatingly, this category of LOCs are developed prior to the birth of their DSMs. The LOC devices are employed to collect sufficient clinical data from relevant biomedical databases; based on these databases, predictive computational models can start to be established by using classification techniques; finally, various prognostic or diagnosis

DSMs can be created for these LOCs to evaluate health risks and identify dangerous health trends. Such LOCs will be both tools for building their own supporting databases and executive carriers of prognostic/diagnosis applications afterward.

Blood testing is one of the main application field for prognostic LOCs due to the importance and frequency of these tests in medical diagnosis. The study of blood-related LOCs often extends beyond the scope of the microsystem due to its intrinsic interdisciplinary foundation. An acceptable LOC for blood testing will also involve knowledge from medical science, biology, biostatistics, materials science, ethics, and other fields. Any emerging biomedical LOC in this field requires a long period of research and validation.

Many automatic instruments have been commercialized and implemented in hospitals for routine blood tests. For this reason, it is not necessary to develop new LOC tools for routine blood tests with the same DSM and function. However, blood samples contain an enormous amount of health-related information beyond that obtained through routine blood tests. This unused information could be used to detect diseases at an early stage, see also Chapters 1.1 and 1.2.2. The concept of a biomedical-database-dependent LOC system has been proposed for the acquisition of holistic data from blood tests. These emerging LOCs rely on the development of prognostic DSMs related to computational modelling in those biomedical databases. At present, research on these biomedical-database-dependent LOC systems remains prospective. Undoubtedly, the construction of relevant biomedical databases and informatics research using data mining are arduous time-consuming tasks that must be carried out before these LOCs can achieve prognostic/diagnostic functions.

The following work is a preliminary attempt in this direction.

2.3.1 Design and fabrication of low-cost 1536-chamber microfluidic microarrays.

- **Objectives:** (Article V, and a part of Article VI)

To produce a low-cost LOC for the high-throughput quantification of signal molecules in blood, i.e., signal molecule profiling (SMP)

- **Challenges:**

Is it possible to readjust the previous disposable LOCs that are compatible with microplate readers into a low-cost SMP chip?

- **Design of the LOC system and preliminary validation:**

A 1536-chamber microfluidic microarray chip, called the 'SMP chip' was designed (Figure 26).[22] This chip can standardize the simultaneous detection of up to 384 types of signal molecules in a blood sample, with three parallel measurements of every target molecule. Similar to previous microplate-compatible chips, the SMP chip has the same dimensions as the 1536-well microtiter plate, thus making the SMP chip readable in a microplate reader. The low-cost material, PMMA was selected to construct the LOC. Moreover, PMMA is amenable to low-cost fabrication methods, *i.e.*, carbon dioxide laser ablation in the R&D stage and injection moulding in the next stage of mass production. As there are no more than 400 hormones and cytokines present in the blood, the LOC contains sufficient measurement capacity.

Here, the SMP chips operate by a chemiluminescent immunoassay (CLIA). CLIA is a sensitive detection method characterized by femtomolar sensitivity and high specificity. In addition, another type of SMP chip was also developed (Figure 27)[18] operating according to the same quantitative method of IMRAMP (see also Chapter 1.2.1.1, Figure 8 and Chapter 2.1.1). The SMP chip can be considered a low-throughput protein microarray with a 3D structure, which reaction interface is protected by a stereo structure. Current instruments available for handling ordinary microtiter plates could also be converted into instruments for the surface treatment of SMP chips.[22]

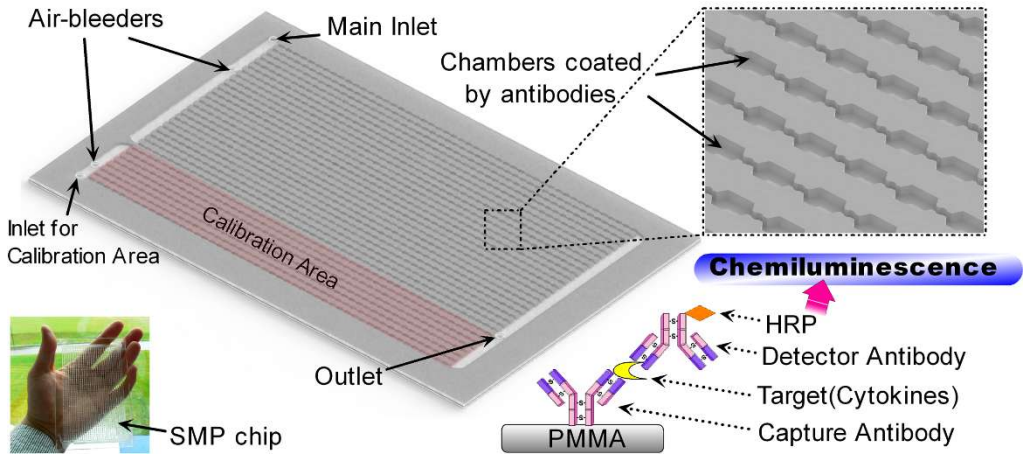

Figure 26. The layout of 1536-chamber microfluidic LOC.[22] (See also Article V)

The chip was modified by 16 types of different capture antibodies to measure relevant signal molecule targets, including thyroid-stimulating hormone (TSH), tumor necrosis factor-alpha (TNF-α), cortisol, follicle-stimulating hormone (FSH), luteinizing hormone (LH), human chorionic gonadotropin (HCG), erythropoietin (EPO), gonadotropin-releasing hormone (GnRH), interferon-gamma (IFNγ), insulin-like growth factor 1 (IGF-1), epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), interleukin (IL)-1 beta, IL-2, IL-6 and IL-8.[22] (See also Article V and Article VI)

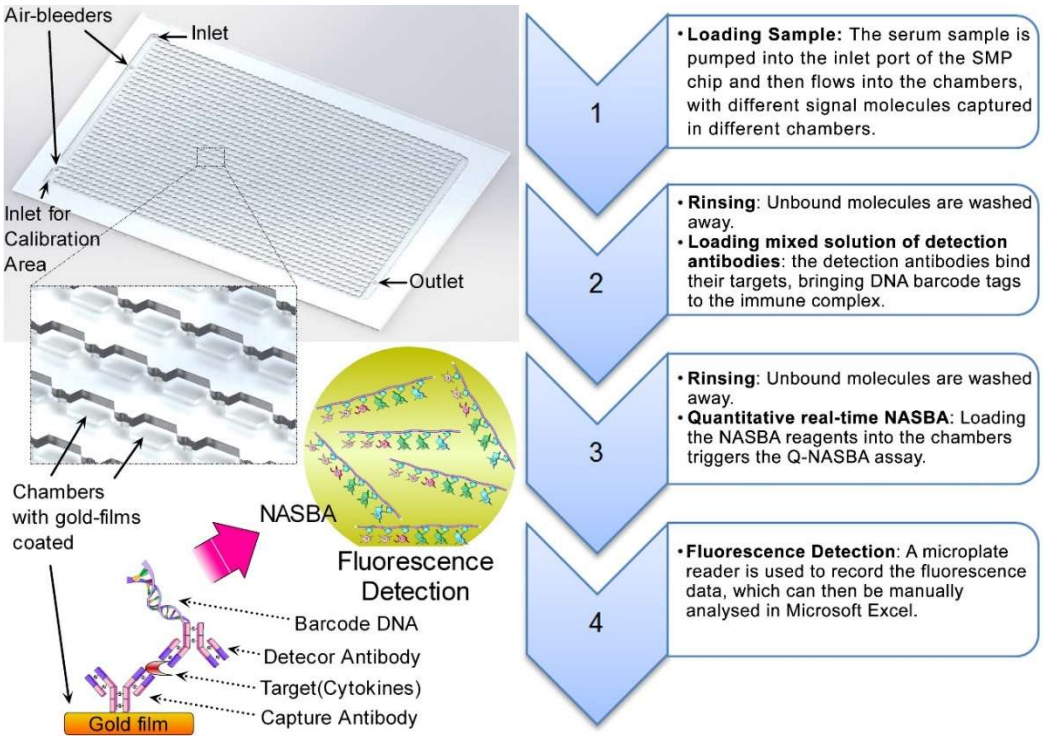

Figure 27. The operating process of the SMP chip (Type B).[18] (See also Article VI)

- **Results and discussion:**

Normalisation of measurements between the two types of SMP chips provides data comparable to traditional ELISA methods. For example, the testing results of the IMRAMP-based SMP chips are presented in Figure 28.

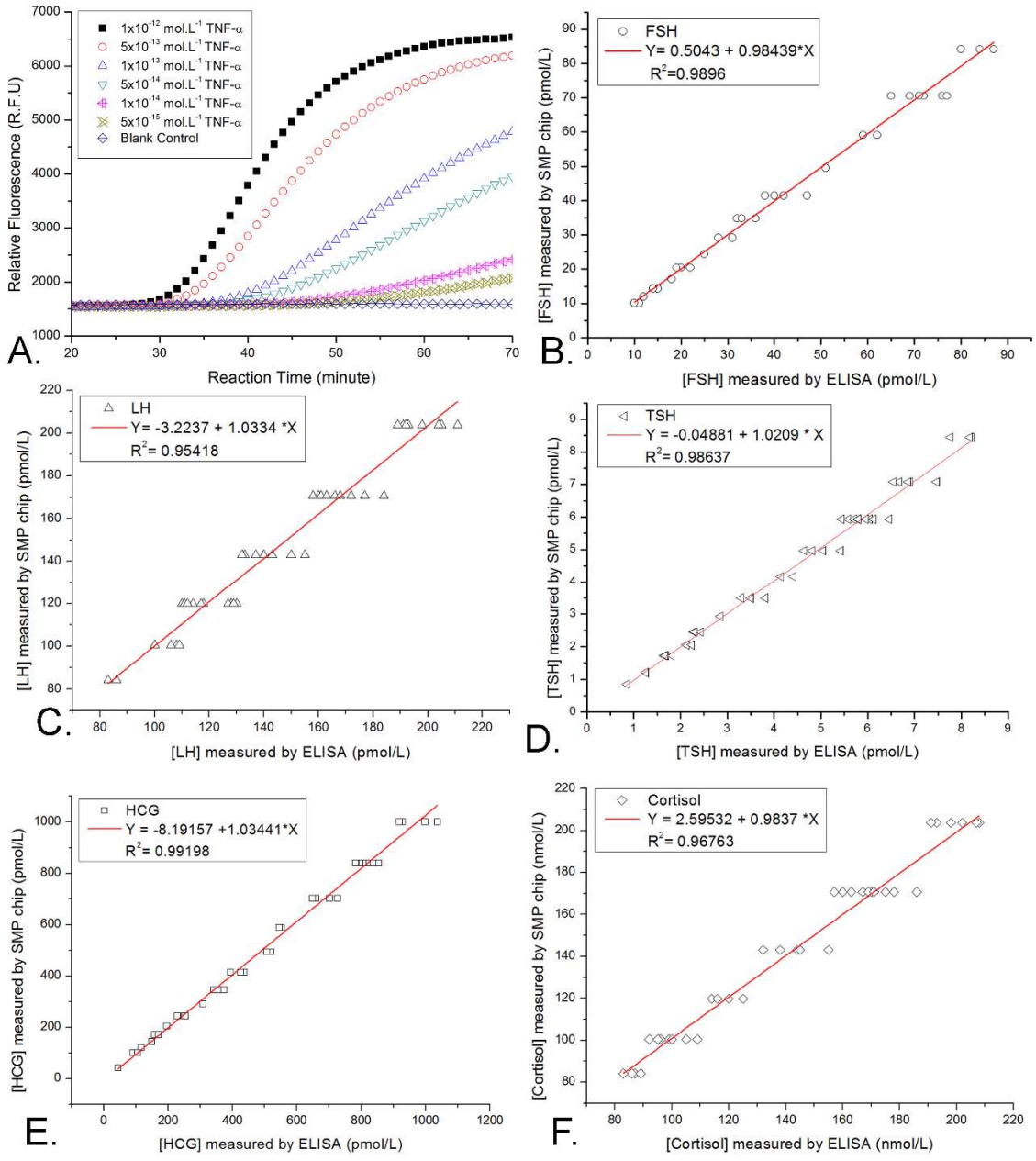

Figure 28. Comparison plots for results obtained using the traditional ELISA method vs. IMRAMP tests on the SMP chips.[18] (a) IMRAMP results for detection of the TNF-α standard samples. (b) Plots comparing the test results for FSH. (c) Plots comparing the test results for LH. (d) Plots comparing the test results for TSH. (e) Plots comparing the test results for HCG. (f) Plots comparing the test results for cortisol.[18]

The LODs of both SMP chips are approximately 1×10^{-14} mol·L^{-1}, in the typical range of detection of common ELISA tests. Both tests can be regarded as extended blood examinations that aim to measure hundreds of the most important biomarkers in the blood, *i.e.*, cytokines and hormones, which can fulfil the common requirements of medical diagnosis and the demands of data acquisition to construct a biomedical database.[18]

2.3.2 Experimental approach to the construction of a human signal-molecule-profiling database.

- **Objectives:** (Article VI)

To study the developing prognostic DSM of the SMP chip and design a low-cost roadmap for the construction of its corresponding database, the human signal-molecule-profiling database (HSMPD).

- **Challenges:**
    - Is it feasible to develop a prognostic DSM for the SMP chip?
    - How can the HSMPD database for prognostic DSMs be practically constructed?
    - Is it possible for the SMP chip to be applied in hospitals in the absence of a mature database?
    - How can the medical system accept the SMP chip prior to the development of the first prognostic DSM?
- **System design and its developing prognostic DSM:**

The HSMPD will be an invaluable source of information for translational bioinformatics (TBI) research. It has been proposed to develop an HSMPD without creating an enormous scientific project. The success of the HSMPD project requires the cooperation of IT, instrumentation engineering, the traditional healthcare system and the TBI community (Figure 29).[18]

The cost of data acquisition for developing a medical database is an impassable challenge, but it could be partially supported by the medical system as patients pay for their physical examinations. After all, SMP chips are more expensive than traditional diagnostic

reagents. The shortfall should be covered by public funding or else shifted to the database users in the TBI community.

The data prioritisation scheme was designed based on the comments of potential users. HSMPD will adopt the framework of a relational database. Each record in HSMPD is divided into three interrelated sections with different information sources (Figure 30).[18] The typical process of data acquisition in the HSMPD system is performed by hospitals, operating according to the procedures in Figure 31.[18]

The SMP chips were provided as new tools for ordinary blood testing to clinical laboratories in the cooperating hospital. The test samples for the SMP chips were diluted sera, so a small volume of blood sample is sufficient for a single SMP chip. Namely, the remaining sample left from an ordinary blood test is usually sufficient to perform an additional SMP test.[18] (See also Article VI.)

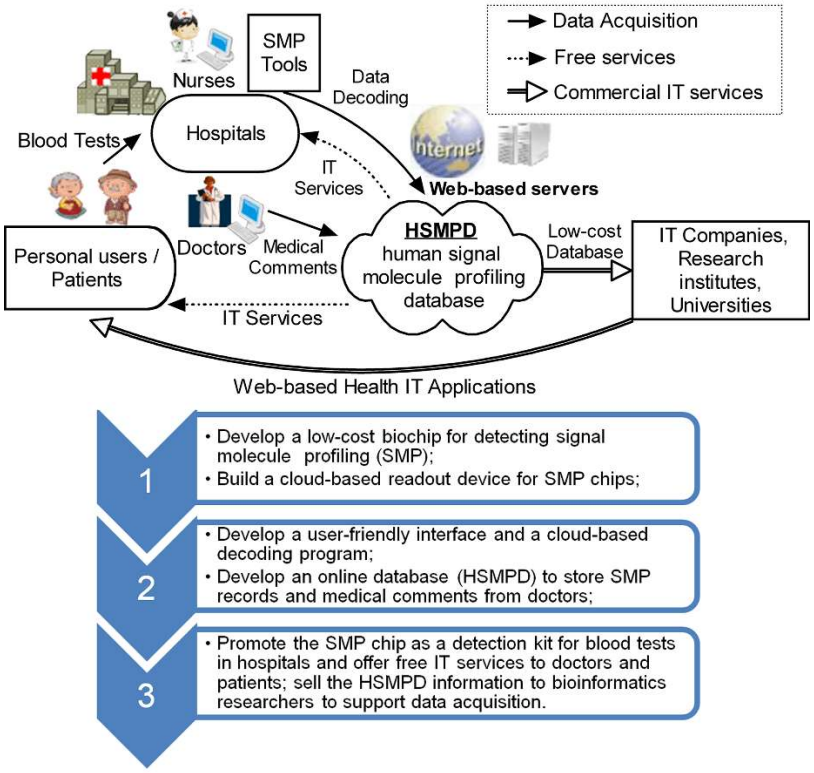

Figure 29. Technology roadmap for the construction of the HSMPD.[18] The HSMPD is a bridge to connect traditional health care systems with the TBI community. The HSMPD services to the TBI community can provide a stable stream of income to support the process of data acquisition for the HSMPD, resulting in a self-motivating mechanism.[18] (See also Article VI)

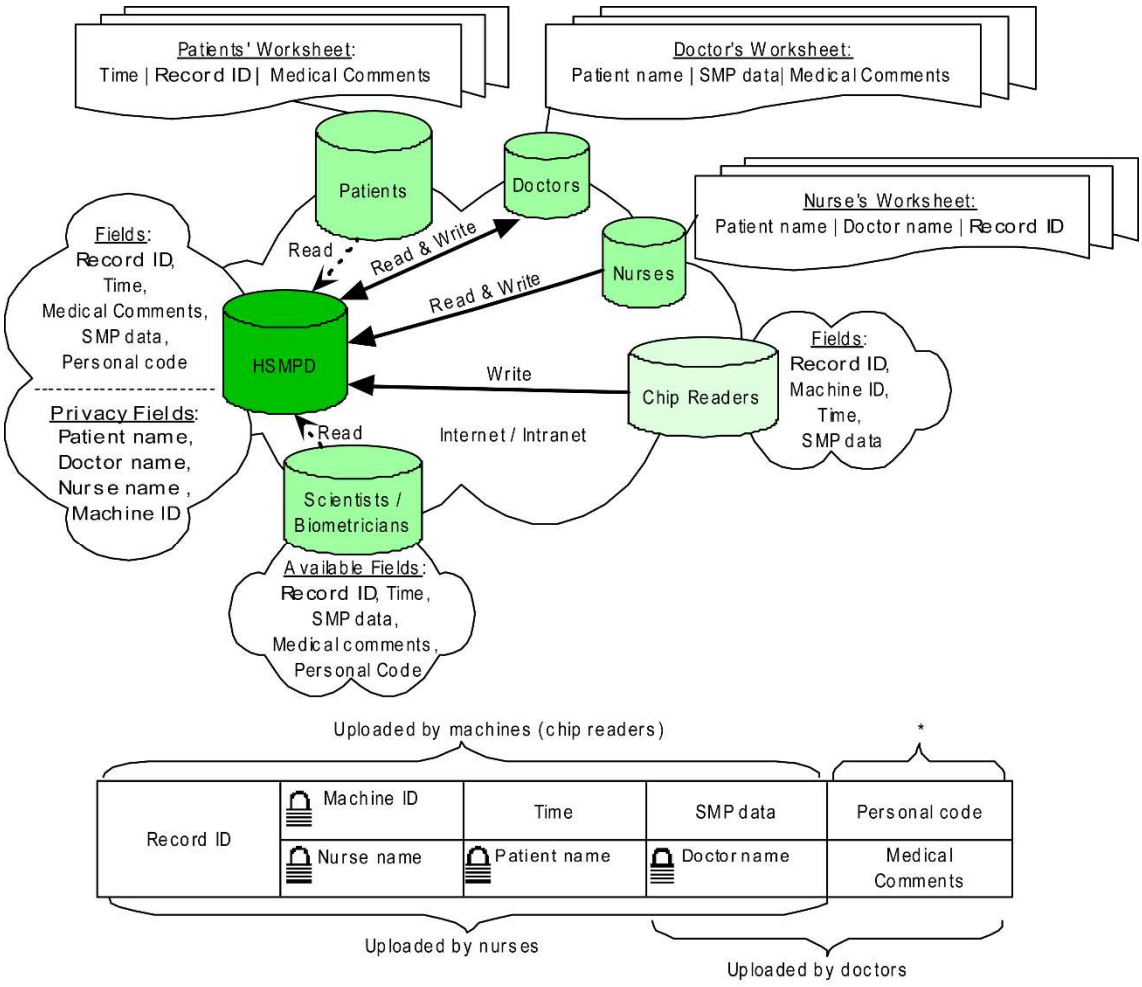

Figure 30. Data structure of the HSMPD.[18] Doctors, nurses and chip readers will input the clinical records into the HSMPD. The information in every record is categorized into two parts. The medical SMP data are open to all researchers and patients without unnecessary ethical review, whereas the private patient information is protected behind a firewall and can be accessed only by a trusted intermediary.[18] (See also Article VI)

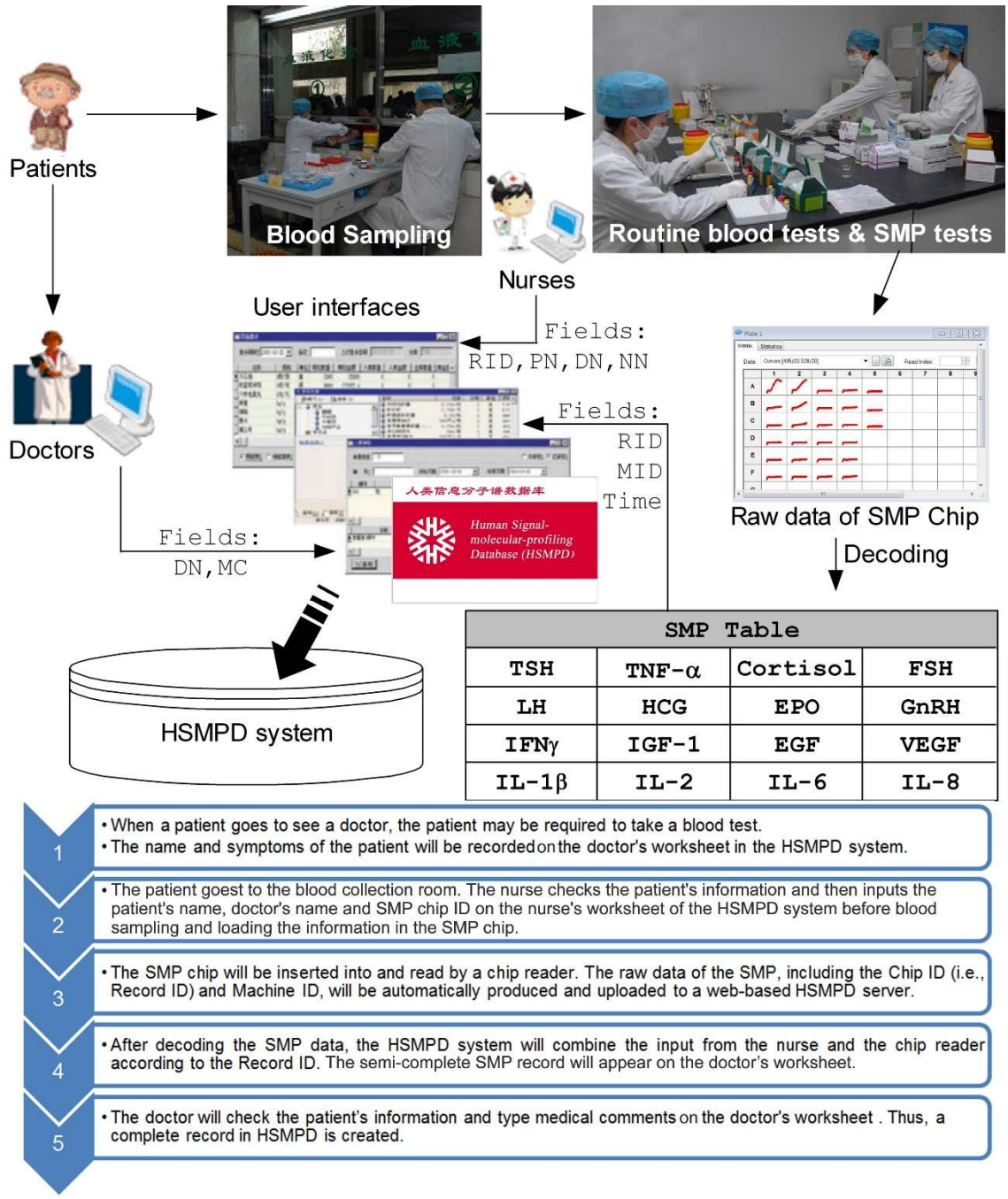

Figure 31. Typical process of data acquisition in HSMPD system.[18] (See also Article VI)

- **Discussion and Outlook:**

This pilot study for an ambitious project concerning human disease diagnosis and prognostication tools enabled the development of a roadmap and specific LOC tools for data acquisition. The feasibility and methodology of data acquisition for the HSMPD was tested through the survey study and simulation tests. From the technical point of view, the LOC tools and relevant information technology plan have no serious problem. However,

due to the ethical risks and the work habits of doctors and nurses, it is difficult for a new tool or method to enter the existing medical system. It is even more difficult for the SMP chips that do not have a mature DSM or a biomedical database to enable their diagnostic and prognostication functions.[18] Therefore, in the initial stage, the SMP chip has to be compatible with standard blood testing in the traditional diagnostic system until the relevant database and the first DSM come into being. Furthermore, the SMP chip has to be easy-to-use and low-cost to ensure participation by doctors and patients. Only wide adoption of such prognostic LOCs will enable the establishment of the relevant databases that will make these LOCs useful in medical practice. (See also Article VI)

3 Main conclusions and perspectives

This doctoral thesis described an application-oriented strategy for the R&D of LOC systems, which was demonstrated in R & D activities in three aspects for detecting microorganisms, toxic chemicals and serological biomarkers. Various DSMs of these biomedical LOC devices were also analysed. The primary conclusions and perspectives of this thesis are summarized as follows:

- A divided LOC system is easy to implement, especially given that low-cost LOCs are compatible with ubiquitous bench-top instrumentation. A series of LOCs has been developed to be compatible with standard 96/384/1536-well microtiter plates and is thus, compatible with microplate readers commonly utilized in standard biological laboratories. As the measurement stability of these microplate-compatible LOCs has improved, microplate-compatible microarrays have been developed for multi-channel synchronous detection of biological targets, including microorganisms and serological biomarkers. Due to its compatibility with microplate readers, this series of LOCs provides a good example of functional integration on a low-cost chip. The divided LOC system is suitable for the implementation of complex automation associated with high-throughput detection, particularly for tests involving living cells. However, four main limitations still exist for low-cost LOCs: limited machining precision, limited accuracy in fluid operations, limited measurement equipment, and the intrinsic characteristics of on-chip biochemical tests. For cases in which costs are limited, it is feasible to enhance the performance of low-cost LOCs by improving on-chip biochemical tests.
- These LOCs are capable of simplifying and standardizing the complicated processes involved in complex biological tests: 1) The IMRAMP assay is similar to traditional ELISA testing in a biomedical laboratory, and the signals in the test

can be amplified at least a million fold by follow-up NASBA assays. 2) Q-NASBA assays have also been demonstrated as suitable for microplate-compatible LOCs, in which the total reaction time can be shortened to 70 min. Both methods are characterized by a femtomolar sensitivity and high specificity. Moreover, a novel analytical algorithmic method has been generated for NASBA-based quantification tests, which improve the measurement ability of these LOCs. 3) The IATP-BLA test integrates the specificity of immunoassays and the sensitivity of ATP-BLA for living pathogens, which can be perfectly implemented on a 384-chamber LOC, followed by a variety of in-situ operations, including urine culture, antimicrobial susceptibility testing and antibiotic therapy simulation. Due to the system design, this LOC supports rapid identification of the most common causative agents of UTIs and enables rapid AST testing within 6 hours.

- For chemical contamination in water, most existing instruments for water quality detection are designed for a definite quantitative determination of specific chemicals, rather than a continuous early warning of broad-spectrum toxic chemicals. The LOC known as a 'living-cell chip' is an appropriate early warning device for assessing possible threats from toxic chemicals in water. This LOC device can continuously mix a water sample and *Vibrio fischeri* cell sensors into droplets, and this mixture is incubated for a long time before optical detection. The system represents a low-cost broad-spectrum monitor against potential toxins, such as heavy metal ions, phenol, etc. Moreover, the 2nd generation of this 'living-cell chip' has been developed, which carries a coral-reef-like micro-bioreactor with the aim of improved performance.(unpublished)
- A pilot study for an ambitious project concerning human disease diagnosis and prognostication tools has been carried out. The relevant LOC tools, called SMP chips, were designed, fabricated and tested. The candidate also studied the feasibility and process of data acquisition for a human signal-molecule-profiling database (HSMPD). Due to the work habits of doctors and nurses, it is difficult for SMP chips to be included in existing medical systems. In the initial stage, the SMP chip must be compatible with standard blood testing in the traditional diagnostic system until the relevant database and the first DSM are established.

This doctoral study shows that an application-oriented strategy is feasible in the field of biomedical LOC research. More importance should be attached to the application

potential of research activities from the initial stages. With the further development of LOCs, the first category of LOC systems with conventional DSMs will gradually become disposable components employed on reusable laboratory instruments, except for POC devices. In fact, relevant companies in biomedical instruments have been moving in this direction. The second category of LOCs with unconventional DSMs is required in particular application scenarios, for example, in long-term biological experiments with complicated cell culturing. Traditional DSMs will be modified for specific contrived LOCs to realize a given purpose. The third category of LOCs is dependent on developing prognostic DSMs, which have not yet been established. Only the wide adoption of such prognostic LOCs will enable the establishment of relevant biomedical databases, which can then enable their diagnostic and prognostication functions. As the first category of LOCs is gradually commercialized, the second and third categories of LOCs will become mainstream in LOC research field.

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