Point-of-Care Testing

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A technology platform for digital nucleic acid diagnostics at the point of care

Eine Technologieplattform für die digitale Nukleinsäurediagnostik vor Ort

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Abstract: The combination of digital amplification and centrifugal microfluidics can enable quantitative and fast diagnostics at the point of care (PoC). The new unit operation of centrifugal step emulsification allows high throughput droplet generation. Different methods for digital nucleic acid analysis, including PCR, recombinase polymerase amplification (RPA) and loop mediated isothermal amplification (LAMP), have already been demonstrated. Our novel approach of integrated sample-to-answer analysis is introduced, and examples for the detection of HIV and single cell analysis of antibiotic resistant bacteria are presented. Next to these LabDisk based systems, a microfluidic cartridge termed DropChip allows for digital amplification using only commercially available laboratory devices.

Keywords: digital nucleic acid diagnostics; lab-on-a-chip; point-of-care testing (POCT).

Zusammenfassung: Die Kombination von digitalen Amplifikationsmethoden mit zentrifugaler Mikrofluidik ermöglicht eine quantitative und schnelle Diagnostik vor Ort. Mit Hilfe der neuen Grundoperation der zentrifugalen Stufenemulsifikation können Tropfen im Hochdurchsatz erzeugt werden. Unter Anwendung dieser Grundoperation wurde die digitale Nukleinsäureanalytik mit verschiedenen Amplifikationsmethoden wie PCR, recombinase polymerase amplification (RPA) und loop-mediated isothermal amplification (LAMP) gezeigt. Wir stellen neue Ansätze zur integrierten und automatisierten Analyse von Probenmaterial am Beispiel der Detektion von HIV sowie der Untersuchung einzelner Zellen auf Antibiotikaresistenzen vor. Neben diesen LabDisk-basierten Systemen erlaubt der DropChip, eine weitere mikrofluidische Kartusche, die digitale Amplifikation unter Verwendung kommerziell verfügbarer Laborgeräte.

Schlüsselwörter: digitale Nukleinsäurediagnostik; Lab-on-a-Chip; Point-of-Care Testing (POCT).

Introduction

With the aid of centrifugal microfluidics complex laboratory procedures can be automated and integrated on lab-on-a-chip cartridges. The crucial interface between user and device can be simplified, providing a robust system. As previously reported [1–3] we developed a platform for digital nucleic acid analysis on rotating microfluidic cartridges.

For digital analysis of nucleic acids, the sample is partitioned in pL-nL volumes and the amplification of the target molecule occurs in each small compartment. Digital amplification offers many advantages over the reaction in bulk, such as absolute quantification without the need for running standard curves and much higher accuracy and sensitivity [4].

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We combined this methodology with centrifugal microfluidics, to overcome the disadvantages that current commercially available systems involve. This includes large dead volume of sample and reagents, high technical demand on the processing device as well as many handling steps with multiple pieces of equipment and disposables.

Centrifugal step emulsification for digital analysis of nucleic acids

The novel technique of centrifugal step emulsification can generate droplets dead volume free with negligible dispersity (CV of 2–4% in diameter) over a wide range of centrifugal pressures by simple means of rotation [1]. The system works without any pumps or tubing. An inlet chamber is connected to a droplet collection chamber by a channel. At the junction between the channel and the chamber a nozzle for droplet generation is implemented. After this nozzle, the structure first widens in one direction forming a terrace and then leads to a backward facing step, where additionally the depth increases (see Figure 1). The structure is loaded with fluorinated oil (continuous phase) and in the following aqueous liquid (disperse phase) is forced through the nozzle by centrifugation. At the backward facing step, capillary effects lead to the formation of droplets. Buoyancy then removes the latter from the nozzle, as

the density of the surrounding oil is higher. For a broad range of operation the dimensions of nozzle and terrace together with the interfacial tension of the liquids exclusively determine the size of the droplets. Therefore, neither pressure nor flow rate needs to be tightly controlled. To increase the throughput the number of nozzles can be parallelized [6]. Currently, up to 10^4 droplets can be produced per second with pre-selectable diameters from 30–170 μm resulting in droplets in the pL-nL range.

The introduced sample is partitioned together with the required reagents for the particular application. The distribution of target molecules occurs randomly. If at least one target molecule is present in a droplet, the amplification reaction can take place, thereby generating a fluorescent signal (denoted a positive droplet). Due to chamber geometry, droplets form a stable monolayer during incubation and subsequent readout. If not stated otherwise, a microarray scanner was used for fluorescence imaging. An ImageJ routine [7] allows semi-automated image recognition [2]. Absolute quantification can be achieved by comparing the number of positive droplets to the total number of droplets analyzed. Dependent on the scanner, filter sets of up to four different wavelengths for excitation and emission were available, enabling the development of multiplexed reactions.

This technology provides a universal platform for several amplification methods. Digital droplet PCR (ddPCR) [3], as well as isothermal reactions, such as digital droplet

Figure 1: Concept of droplet generation in step emulsification (adapted from [5]).

Lines of figures represent the top view and cross sections of the nozzle over time. Continuous phase is shown in yellow. The dispersed phase (blue) is pushed into the nozzle by centrifugal forces and moves forward onto the terrace and over the backward facing step. As the size of the forming droplet in the following chamber increases, a constriction (neck) forms. The thread connecting the forming droplet and the liquid in the channel becomes unstable, ruptures and a droplet is formed, which is removed from the nozzle by buoyancy.

RPA (ddRPA) [1] and digital droplet LAMP (ddLAMP) [2], have already been demonstrated. To provide robust and reproducible testing at the PoC an even higher level of integration is necessary. Including sample preparation on a disposable microfluidic cartridge can enable a sample-to-answer bioanalysis [8–10] and further reduces the overall process time. For several clinical applications we are aiming to develop solutions for a highly integrated digital analysis.

Detection of antibiotic resistant pathogens

Infections with antibiotic-resistant bacteria are already widespread across the globe [11]. Methicillin-resistant *Staphylococcus aureus* (MRSA) is one of the most pathogenic bacteria in hospital and community-associated settings [12]. Bacteria culture is the current standard for MRSA detection in the clinics. However, this diagnostic method is laborious and usually takes several hours to days in a specialized laboratory before the results come out. Therefore, developing a rapid and automated diagnostic for MRSA detection is in urgent demand to facilitate MRSA spreading control and better treatment management of the patients.

A novel LabDisk, a disk-shaped microfluidic cartridge, operating on a portable device, can provide this diagnostic system for MRSA detection towards PoC. Simultaneous detection of bacterial species and antibiotic resistance at single cell level is targeted for goal oriented therapy. Analysis will directly be performed with a nasal swab sample from the patient. All processing steps, including sample preparation, emulsification, amplification and detection, run automatically.

Bacterial cells and their genetic material are separated and encapsulated in droplets. The approach of analyzing single cells allows the assignment of antibiotic resistance to the infecting agent, improving the positive predictive value of MRSA testing. The number of colony forming units (CFUs) per droplet follows Poisson distribution (see Figure 2). The frequency of a double allocation of *S. aureus* and *S. epidermidis* cells in one droplet after emulsification of a mixed culture also comply with Poisson distribution. It can therefore be concluded that the encapsulation of CFUs occurs independently and the assignment of antibiotic resistance to the bacterial species is possible.

Bacteria are lysed within the droplets and several genetic markers can be identified applying a multiplexed RPA reaction, generating different fluorescent signals. Combining in-droplet lysis with direct isothermal amplification facilitates the integration of sample preparation

Figure 2: Encapsulation of *Staphylococcus aureus* cells in droplets. (A) Distribution of colony forming units (CFUs) across droplets follows Poisson statistics. μ : Expected number of CFUs/droplet. n : Total number of analyzed droplets. (B) Microscopic image of bacterial cells encapsulated in droplets. Directly before encapsulation cells were stained with the green fluorescent dye SYTO® 9.

on the LabDisk and improves the overall process time to 30–60 min. A hyperspectral camera optical system for the detection of several fluorophores will be integrated in the processing device to be developed. Very few handling steps (placing the nasal swab into the LabDisk and starting the run) should enable hospital staff to perform the diagnostic analysis themselves. Verification of the system with clinical samples is planned for 2018.

HIV diagnostics via digital droplet RT-LAMP

Companion diagnostics play an important role by testing biomarkers for personalized medicine. More than 35 million people worldwide are suffering from HIV- and/or the closely related HTLV (human T-lymphotropic virus) infections. An effective therapy requires concurrent analysis of the respective viral load. Within a current research project, the development of a specific LabDisk for digital amplification of HIV- and HTLV-RNA using reverse transcription LAMP (RT-LAMP) from a blood sample is intended. A fast and automated processing of the sample within the microfluidic cartridge could enable the diagnostic testing at the PoC, e.g. during a doctor's visit. This reduces the psychological stress of the patient and the results could directly initiate an effective therapy.

Applying isothermal amplification a time-to-result below 30 min can be reached. The combination of LAMP with the digital approach allows for quantification. Less than 250 RNA copies per mL blood (less than 15 RNA copies per sample analyzed) are aimed to be detected. To reach this high sensitivity a combination of LAMP with a novel real time detection technology is under development.

Digital nucleic acid analysis with standard laboratory devices

Complementary to the LabDisk, a microfluidic cartridge in the standardized format of a microscope slide, termed DropChip, was developed (Figure 3). It allows for digital amplification using only commercially available

Figure 3: Microfluidic cartridge for digital nucleic acid diagnostics. The DropChip was designed to have the standardized format of a microscope slide. This allows for digital amplification using only commercially available laboratory devices (Picture by Bernd Müller).

laboratory devices. Droplets are generated as described above by rotating the DropChip in a bench-top microcentrifuge. Amplification and fluorescence detection were performed on-chip in an in situ thermocycler and a microarray scanner, respectively.

ddLAMP has already been shown with *E. coli* DNA as target [2]. Absolute quantification of the DNA in the range of 15–1500 copies per μL was performed (Figure 4). Results were comparable to existing commercial ddPCR devices. Furthermore ddLAMP was robust to temperature changes and relatively insensitive to primer concentration variations.

In addition to isothermal amplification, the DropChip is suitable for ddPCR [3]. We have demonstrated the transfer of non-invasive prenatal diagnostic (NIPD) methods from a reaction tube to a lab-on-a-chip format. Cell-free fetal DNA (cffDNA) was extracted from the mother's blood [13] and used for the quantitative detection of the deletion p.Phe508del. In homozygous form this mutation leads to the autosomal recessive disease cystic fibrosis.

A further application of NIPD is the rhesus D (RhD) genotyping during pregnancy of RhD negative women. Simultaneous detection of *RHD* exon 5, *RHD* exon 7 and *RASSF1A* was achieved in a multiplexed ddPCR. Based on the real-time PCR protocol, ddPCR was established and target sequences were identified with different fluorescent labelled hydrolysis probes. The assay was useful for detecting cffDNA from plasma of RhD negative woman with RhD positive fetus. Fetal rhesus genotyping in RhD negative pregnant women is challenging due to low gene copy numbers. At the current stage, the sensitivity is lower compared to standard quantitative PCR. Optimization of this novel ddPCR approach can constitute a technology suitable for population screening purpose.

Figure 4: ddLAMP in DropChip (adapted from [2]).

(A) Comparison of the ddLAMP and ddPCR show good concordance for amplification of *E. coli* target DNA in the range of 15–1500 copies per μL . (B) Sections of false color fluorescence images of ddLAMP. Detection was performed with a microarray scanner.

Conclusions

For PoC diagnostics, there is the need to detect disease-related biomarkers in a reliable and rapid way, which is additionally easy to perform. The described applications illustrate the versatile exploitation possibilities of the LabDisk and the DropChip for nucleic acid analysis. The integration of sample preparation and digital analysis on a single disposable cartridge is of added value for the user and can enable sensitive and absolute quantification at the PoC.

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