Microsyst Technol (2008) 14:673–681
DOI 10.1007/s00542-007-0515-x

TECHNICAL PAPER

RNA amplification chip with parallel microchannels and droplet positioning using capillary valves

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Received: 10 June 2007 / Accepted: 13 December 2007 / Published online: 2 February 2008

Abstract We present results from the MicroActive project which develops an instrument for molecular diagnostics. The instrument is first tested for patient screening for a group of viruses causing cervical cancer. Two disposable polymer chips with reagents stored on-chip are developed and will be inserted into the instrument for each patient sample analysis. The first chip will perform nucleic acid extraction from patient epithelial cervical cells, while mRNA amplification and fluorescent detection takes place in the second chip. This paper reports results on the amplification chip. Purified sample is inserted into the chip and split into ten smaller droplets for simultaneous amplification and detection of ten viruses. The droplets move in parallel channels, each with two chamber extensions containing dried reagents. Experimental results on parallel droplet movement using one external pump combined with hydrophobic restrictions show that the parallel droplet positions can be controlled. There are four valves with increasing burst pressures between 800 and 4,500 Pa in each parallel channel, positioning the droplets in metering zones and reaction chambers. The re-hydration times for the dried reagents in micro chambers have been monitored. After sample insertion, uniform concentration of the reagents in the droplet was reached after respectively 60 s and 10 min. These times are acceptable for successful amplification. Finally we show positive amplification of HPV type 16 viruses in a micro chamber.

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1 Introduction

The aim of the MicroActive project (MicroActive 2006) is to develop an instrument for molecular diagnostics intended for use in the doctors' office. The instrument will first be used for patient screening for a group of viruses causing cervical cancer, but the technology platform will have a wide applicability. Microfluidics and biotechnology form the basis for the development. Fully automated diagnosis systems based on microfluidics will widen the availability of advanced diagnostics for all citizens due to both higher availability and lower cost (Yager et al. 2006). Also, reducing the time from patient testing to diagnosis avoids anxiety and enables earlier treatment.

Human papillomavirus (HPV) is a group of sexually transmitted viruses that is related to the development of cervical cancer (Jenkins 2001; Walboomers et al. 1999). Cervical cancer is the second most common cancer type among women worldwide. Oncogenic proteins related to the five HPV-types 16, 18, 31, 33, and 45 are related to more than 97% of all cases of HPV-induced cervical cancer in Europe and these HPV viruses are the target of the NorChip PreTect HPV-Proofer©. The task of MicroActive is to miniaturize and automate the protocol of the PreTect

HPV-Proofer©, namely mRNA amplification and detection. Compared to commonly used approaches (e.g. DNA PCR amplification and immunoassay methods), mRNA detection largely avoids false positive results and has a high sensitivity (Lie et al. 2005; Kraus et al. 2004; Norchip 2007). It can currently be used to detect pre-cancer, cancer, STD and a range of respiratory diseases, to mention a few. New bio-markers are continuously being developed.

Within the MicroActive project the partners are working on:

1. Development of one disposable microfluidic chip for sample preparation including reservoirs containing all liquid reagents necessary to perform sample preparation consisting of cell concentration, lysis and nucleic acid purification.
2. Development of a second disposable microfluidic chip for multiple target amplification and fluorescent detection with dried spotted reagents stored in micro-channels.
3. Development of manufacturing methods for spotting and drying of reagents, surface coating and patterning, and polymer chip lamination that prevents inhibition of the bio-molecular processes.
4. Performing multi target detection from a single sample. This is possible due to simultaneous amplification and detection in separate parallel detection channels. Each parallel channel contains dried reagents for amplification of a disease (here: HPV type) specific marker.
5. Performing isothermal amplification of mRNA by nucleic acid sequence-based amplification (NASBA) (Compton 1991).
6. Performing tests on clinical samples, using test-chips for separate functions.
7. Providing the repeatable and stable fluid control required by a commercial system through use of simple pumps in combination with surface modification.
8. Developing an instrument without manual protocols.
9. Testing the instrument on clinical specimens and compare to gold standards.
10. Addressing factors such as reliability, usability and cost of the total instrument—factors which are crucial to acceptance by health care professionals.

In this paper we will focus on reporting experimental results on separate tests of the fluidic and biological functions on the second chip, the NASBA amplification and fluorescent detection chip, related to the points 2–7 above. The advantage of this NASBA amplification chip compared to previous microfluidic solutions for nucleic acid amplification is firstly that 11 mRNA markers can be detected simultaneously. Many symptoms may be caused by a range of viruses that now can be tested in one run. By splitting the sample droplet into e.g. 11 smaller droplets, each droplet can be mixed with dried primers identifying one marker and the 11 amplifications are performed in parallel channels before the positive/negative signal is read out separately for each channel. Cepheid (2007) performs PCR simultaneous amplification of four genes in one chamber, but this test is restricted to four genes only, due to detection of fluorescence at four separable wavelengths. The alternative to NASBA amplification of mRNA is reverse-transcriptase PCR (RT-PCR) which has not been an active field within microfluidics (Lien et al. 2007). NASBA has another advantage over RT-PCR, being a constant temperature reaction; it requires a simpler temperature control.

Earlier, positive NASBA reactions with wet, premixed reagents have been demonstrated on chip (Gulliksen et al. 2004, 2005). The new aspect of the present work is to dry the reagents in the micro chambers and to perform the amplifications in metered, separate nanoliter sized droplets. Droplet based analysis in a lab-on-a-chip was described by Burns (1998). We have reported splitting of the sample containing nucleic acids into 11 metered droplets in another paper (Mielnik et al. 2007). Metered sample droplets will be pushed into different channels where they mix with different dried regents for multiple analyses. Work on multiple analyses using different types of stored reagents have been performed by Weigl et al. (2006), for air drying and reactivation of PCR mixes in conventional macro scale volumes using 96-well plates. PCR in micro-chips have been performed by numerous groups, focusing e.g. on temperature cycling speed, miniaturization and chamber architecture and post-amplification detection (Zhang et al. 2006). For self-contained systems with pre-stored reagents, both liquid reagents and dried reagents have been introduced as plausible solutions for long-term storage of reagents for immunoassays on-chip (Linder et al. 2005).

Here we report experiments on parallel droplet movement control using one external pump only, combined with valves based on hydrophobic restrictions (Oh and Ahn 2006). We show seven droplets moving in seven parallel channels, each stopping controllable in three reaction chambers along the channel. Due to heating requirements for the reactions and localized heaters, it is important that all droplets are in the same reaction chamber at the same time. We also monitored the re-hydration times for the two necessary dried reagents using fluorescent markers and a confocal microscope. After sample droplet insertion, concentration of the reagents in the liquid droplet was not changing significantly in time after respectively 60 s and 10 min. These times are acceptable for successful amplification. Finally we have shown positive amplification of

HPV type 16 using dried enzymes stored in micro chambers. The re-activation of dried reagents is very sensitive to the environment, spotting and drying procedure, and positive amplifications are considered to be a breakthrough for the project.

2 NASBA amplification chip

In the NASBA amplification chip, purified RNA is mixed with dried reagents, heated and amplified. The amplified RNAs are detected by fluorescence. The input to the NASBA amplification chip is purified nucleic acids in elution buffer. The aim is that the sample preparation eventually will be performed in the sample preparation chip. However, the NASBA amplification chip can also be combined with laboratory routines for nucleic acid extraction. In the amplification experiments with droplets and dried enzymes reported in the end of this paper, HPV 16 oligos were amplified.

The input droplet of the NASBA amplification chip will be split into smaller reaction droplets. Each volume will be mixed with different reagents, so that a different mRNA strand is amplified in each reaction volume. In this way, the sample can be simultaneously analyzed for several HPV viruses, each giving either high risk of cervical cancer. In the amplification processes, a fluorescent beacon is attached to the amplicons, and the fluorescent signal of each reaction chamber is monitored, giving a positive or a negative HPV status for each HPV type.

Biochemical experiments have initially been performed in order to decide upon the design of the amplification chip. In particular, it was found that the reagents necessary for each of the NASBA amplifications must be dried in two separate chambers. It was also found that the chamber storing the second reagent mix also could be used for fluorescent detection, because the reagents dissolved so quickly that the optical signal was not disturbed. Thus, two reaction chambers are needed for each parallel HPV analysis. In order to provide a sufficiently strong fluorescent signal, the sample volume for each amplification was chosen to be 500 nl.

A prototype amplification chip has been designed and manufactured. The design (see Fig. 1) is based on numerous tests on sub-functions. Here we report on the sub-function tests performed on different test chips. Experiments on parallel motion control of the droplets, the re-hydration of dried reagents, and the RNA amplification, using dried enzymes are reported. A sketch showing the functions of the chip is seen in Fig. 1. Sample with extracted nucleic acids in buffer will be transferred from the sample preparation chip to the amplification chip. The output volume of the sample preparation chip is

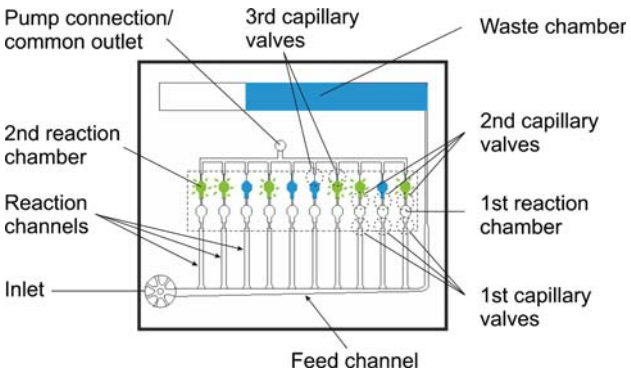

Fig. 1 Sketch of the NASBA amplification chip. The *star*-shaped inlet is in the *lower left* corner. The horizontal feed channel leads to the ten parallel reaction channels and to the large waste chamber on the *top* of the chip. The hydrophobic valves are spotted at the three restrictions in each parallel channel. The lower/first restrictions are wider than the upper/last ones. Dried master mix will be spotted in the first chamber and dried enzymes will be spotted in the second chamber. A peltier element is placed under the area of the *dashed line*, and the optical module will be scanned across the upper chambers. In the figure, the parallel reaction droplets have reached the second reaction chamber

approximately 20 µl, the current design of the amplification chip allows for input volumes between 14 and 35 µl. The sample enters the amplification chip via a star-shaped inlet and is pulled into a feed channel by capillary action. As the liquid moves through the feed channel, the ten vertical reaction channels are filled with sample up to the first capillary valve. The excess sample enters the rightmost waste channel and is pulled into the large, upper waste chamber. The waste chamber contains a filter which absorbs the surplus sample, so that the feed channel and the waste channel are drained. At this stage, ten equally sized sample droplets are snapped off and reside in the ten parallel reaction channels. One pump is connected to the system via one common outlet for all 10 parallel channels. The parallel droplets are pulled from the metering sections of the channels into the first reaction chamber, overcoming the resistance caused by the 1st capillary valves. When all droplets have crossed their respective capillary valves, the pumping stops. No sample droplets enter the second chamber yet, because the second set of capillary valves is designed to be stronger (having higher burst pressure) than the first. At this point, all sample droplets reside inside their 1st reaction chambers, and are allowed to mix with the dried reagents. After a prescribed time, the pumping is recommenced. When the pressure difference reaches a level corresponding to the strength of the second set of capillary valves, the droplets sequentially overcome the restrictions and travel to the second reaction chamber. When all droplets reside in the second chamber, the pumping is stopped and the chip is heated to 41°C. The sample droplets are left in this reaction chamber to mix

with dried enzymes, and the NASBA reaction is initiated. During the amplification process the reaction chambers are scanned sequentially by the optical module, and the fluorescence signal is recorded.

3 Test chip manufacturing

Test chips for both fluidic and biological experiments on sub functions of the amplification chip were manufactured in cyclic olefin copolymer (COC). The chips used for testing of the hydrophobic valves were milled and laser ablated, whereas test chips for re-hydration of dried reagents and for NASBA amplification experiments were hot embossed.

After manufacturing, the chips were cleaned in an ultrasonic bath, air dried, and O_2 plasma activated prior to coating with 0.5% polyethylene glycol (PEG) in methanol (Sigma Aldrich Norway AS, Norway). The PEG layer on the microchip surfaces after coating rendered the channel walls hydrophilic, with a contact angle to DI water measured to be approximately 30°.

The hydrophobic valves were created in bottlenecks of the microchannels by spotting 0,5% Teflon 1600 AF (DuPont) using the PipeJet spotting system (BioFluidix, Germany). The contact angle of DI water on Teflon surface was measured to be approximately 110°. Satisfactory coating of the valve structures was confirmed after spotting by microscope inspection. After coating and spotting, the chips were sealed with adhesive tape.

4 Sample droplets moving in parallel channels with hydrophobic valves

We have tested the feasibility of controlling the positioning of all sample droplets in the parallel channels by using one common pump only, combined with channel restrictions and hydrophobic patches separating the hydrophilic reaction chambers. The test chips contained seven parallel channels; the choice of seven channels was arbitrarily chosen for demonstrating the effect. The final prototype will as mentioned contain ten parallel channels. Each test channel had a width of 800 and a depth of 200 μm. All channels had four lateral restrictions with widths 380, 150, 75 and 33 μm, widths decreasing in the downstream direction (see Fig. 2). A total of five chips were tested.

The chips were mounted in an aluminum frame and connected to a second chip containing a branch-like channel structure for distribution of the pump under pressure to all parallel channels, see Fig. 3. A syringe pump (PHD2000, Harvard Apparatus) was used to apply suction to the common chip outlet, withdrawing air from the

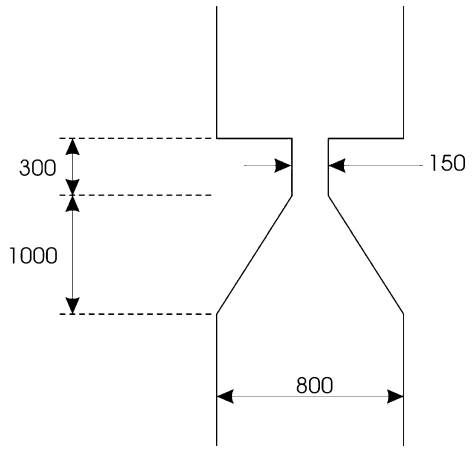

Fig. 2 Details of capillary valve geometry (here, valve width is 150 μm). All dimensions in μm. The capillary valves contain a tapered part and a narrow restriction. The tapered part is included to aid spotting of the fluorpolymer for hydrophobization

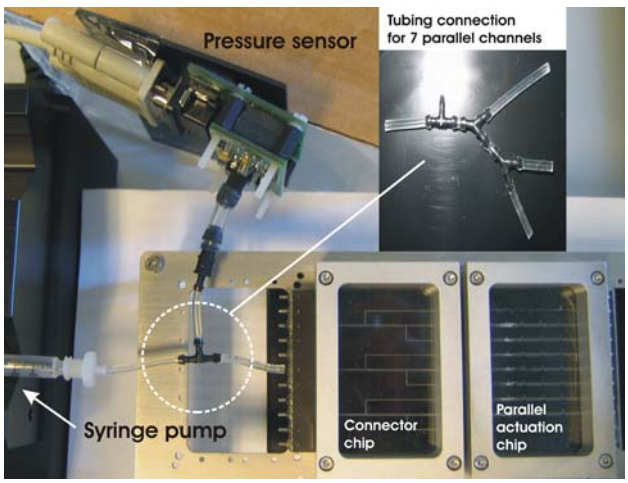

Fig. 3 Test setup for monitoring of parallel droplet movement. The syringe connected to the pump is seen to the *left*, the MEMSCAP pressure sensor *upper left*. Tubing connects the pump and pressure sensor to the aluminum frame with the fluidic connectors, the pressure distribution chip and the rightmost parallel droplet actuation chip. The presented image shows the setup for actuation of three channels in parallel. For actuation of seven channels simultaneously, the tubing connection between the syringe, the pressure sensor and the aluminum frame is exchanged with the connection shown in the *upper right* corner of the image

system at a rate of 10 μl/min. A pressure sensor (TP3100 001A 0P from MEMSCAP) was used for monitoring the pressure in the tube connection to the parallel channels. A close-up image of the parallel actuation chip with liquid droplets in front of the first restriction is shown in Fig. 4.

The pressure as a function of time at the common pump was recorded during operation and is shown in Fig. 5. The pressure plot shows the absolute pressure. During pumping, the pressure in the system decreases until the pressure

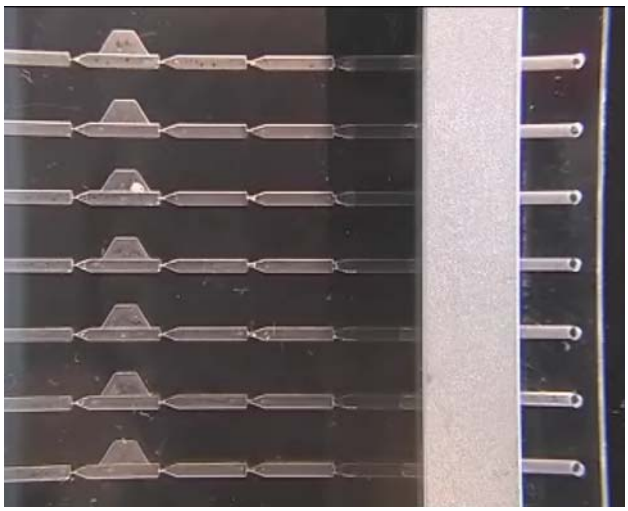

Fig. 4 The test chip for parallel droplet movement control. Seven parallel reaction and amplification test channels are seen, each with four restrictions with hydrophobic spots. Valve pressure strength increases from *right* to *left*. In the image, 500 nl droplets are positioned before the rightmost, weakest valve

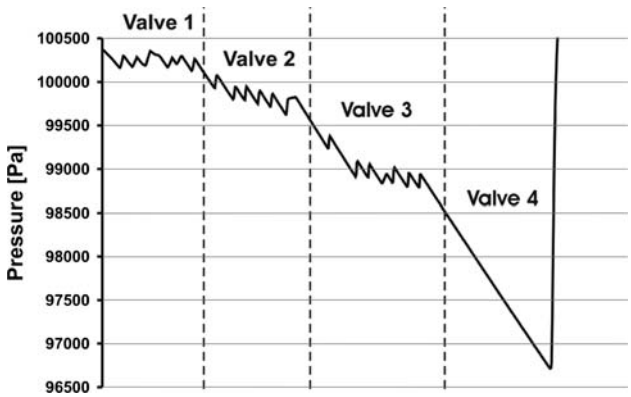

Fig. 5 Pressure characteristics (absolute pressure) of droplet movement through the parallel actuation chip containing seven parallel channels. A constant flow rate (suction) of 10 µl/min is applied at the common downstream end of the chip. At the fourth valve, the first droplet to break the valve effectively ventilates the system, and hence the pressure in the system returns to atmospheric conditions

difference across the front water/air meniscus becomes higher than the burst pressure of the first hydrophobic valve. When the first droplet breaks through its valve, it enters the first chamber and the pressure in the common downstream region is slightly relaxed due to the reduced volume between the liquid droplets and the syringe. The parallel liquid droplets sequentially pass their respective first valves; the order in which the droplets move is arbitrary, depending on slight variations in burst pressure of the seven valves due to manufacturing tolerances and surface roughness effects. No droplet passes to the second chamber

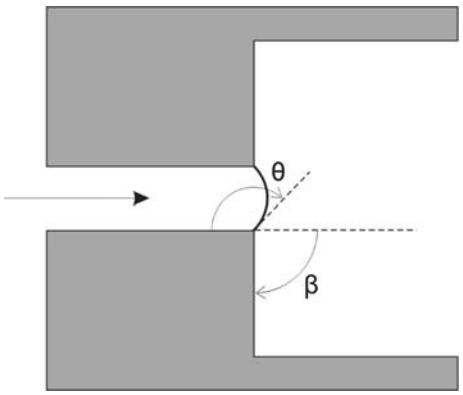

Fig. 6 Definition of wetting angle θ and diverging angle β

before all droplets reside in the first chamber, because of the higher strength (burst pressure) of the second set of valves. This is clearly visible in Fig. 5, where the pressure level of the first seven valves is higher than that of the second valves. Once all sample droplets reside in the first chamber, they proceed to cross their respective second valves as the pumping continues. Passing of the fourth valve results in one pressure rise only, because once a single sample droplet crosses this valve, the system is vented to atmospheric pressure and further pumping does not cause any more movement of any droplet.

The pressure necessary to overcome a hydrophobic restriction for a liquid with surface tension γ and wetting angle θ is given by the Young-Laplace equation, which in the case of a rectangular channel can be written as (Probstein 1994):

$$\Delta p = -2\gamma\cos(\theta)\left(\frac{1}{w}+\frac{1}{h}\right) \tag{1}$$

where w and h are the width and depth of the restriction, respectively. Here, identical wetting angle of all four walls is assumed. In the present case, the capillary valve consists of a hydrophobic restriction terminated by a suddenly diverging channel section (with diverging angle β), see Fig. 6.

In order to drive the liquid forward, the applied pressure difference across the meniscus must be sufficient not only to overcome the strength of the hydrophobic restriction, but also to force the meniscus to achieve wetting angle θ with the diverging wall. In such case, the pressure required to drive the liquid forward may be expressed as:

$$\Delta p = -2\gamma\left(\frac{\cos(\theta)_I}{w}+\frac{\cos(\theta)}{h}\right) \tag{2}$$

where $\theta_I = \min[(\theta+\beta), 180°]$ (see e.g. Cho et al. 2007). For a channel with hydrophobic walls, $\theta > 90°$ and in our case β is 90°. Then $\theta_I = 180°$ and thus the pressure required to overcome the valve becomes:

$$\Delta p = 2\gamma\left(\frac{1}{w} - \frac{\cos(\theta)}{h}\right) \quad (3)$$

the pressure necessary to overcome the capillary valves was estimated from Eq. (3) and compared to experimentally obtained pressure values. The average burst pressures of the four valves are plotted in Fig. 7. The data (open squares) represents average values from five chips with DI water as the working liquid. The error bars indicate the standard deviation of the differential pressure value. The solid line represents the theoretical values calculated from Eq. (3). As evidenced by the data in Fig. 7, good comparison between the experimental and theoretical values for the burst pressure is achieved.

In addition to the use of DI water as working liquid, one experiment (open circles in Fig. 7) using pre-mixed sample with NASBA reagents was conducted to verify the system functionality. The measured burst pressures are seen to be slightly below that found for DI water. This is attributed to the slightly lower wetting angle of the sample mixture, which was measured to be approximate 95°.

The parallel droplet experiments demonstrate that the burst pressures for the sequential valves do not overlap, and that the principle of parallel droplet positioning using one single pump and hydrophobic valves is feasible.

5 Re-hydration of dried master-mix and enzymes

The NASBA amplification chip will have the all necessary reagents stored on chip in a dry state. In each parallel channel the two chambers separated by hydrophobic valves will contain dried reagents. The first chamber will store the NASBA nucleotide ion-adjusted master mixture (master mix) that is specific for each HPV type mRNA amplification, and the second chamber containing the dried enzymes. One crucial question is whether the dried reagents will dissolve into the sample droplet in an acceptable time, or if further mixing by e.g. moving the droplet back and forth over the dried reagents is needed. Here we report on experiments monitoring the dissolution of the master mix and the enzymes.

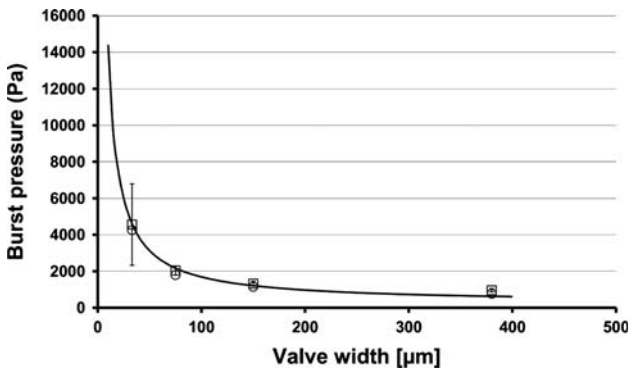

Fig. 7 Burst pressures of the capillary valves as function of valve width. Symbols: *open square* DI water; *open circle* reagents; *solid line* represents the analytical values for water. Contact angle of DI water on Teflon was measured to be approximate 110°. The pressure data for each valve represents an average of 35 measurements (5 chips with 7 parallel channels each)

The spotting of reagents and enzymes into the 500 nl hot embossed reaction chambers was performed using the spotter Nanoject II from Drummond Scientific Company. A volume corresponding to the required amount of reagents for a 500 nl sample size was spotted sequentially into the chambers, 30 nl at a time. The spotted liquid was allowed to dry for approximate 30 s between each deposited droplet. In this manner, a well-defined lump of reagents was deposited on the bottom surface of the reaction chambers. After spotting, the chips with dried reagents were stored at room temperature for at least three days prior to the re-hydration experiments.

In the actual master mix, molecular beacons which contain both a fluorophore and a quencher are present in the solution. The quenchers suppress the fluorescent signal of the fluorophore as long as the targeted mRNA is not present; upon detection, the fluorophore and the quencher are separated within the molecular beacon, releasing the fluorescent signal (in presence of external excitation). For the re-hydration experiments, molecular beacons (excitation/emission 490 nm/520 nm) without quenchers were added to the solution prior to spotting and drying. In this manner, the master mix was fluorescent without the need of actual amplification, and its re-hydration from solid state and diffusion into the sample could be monitored. In contrast to the master mix, the enzymes do not contain any fluorescent components. In order to permit fluorescent detection of the re-hydration and diffusion process of the dried enzymes, the antibody IgG (Southern Biotech, Birmingham, AL) fluorescently labelled with FITC (excitation/emission 495/515 nm) was added to the enzyme suspension prior to spotting and drying in the reaction chambers. The fluorescent IgG tracer (molecular weight 150 kDa) thereby modeled the largest enzyme AMV-RT (molecular weight 160 kDa).

The processes of re-hydration of the dried master mix and enzymes in the liquid samples were investigated experimentally by confocal laser scanning microscopy (CLSM). A Leica DM RXA epifluorescent microscope equipped with Leica TCS 4D confocal unit was used for the measurements. The imaging of the reaction chambers was performed via an HC PL Fluotar objective with 5-fold magnification and $NA = 0.15$. The low magnification was necessary in order to image the entire reaction chamber within the field of view of the microscope. As a consequence, the depth-wise resolution of the measurements was

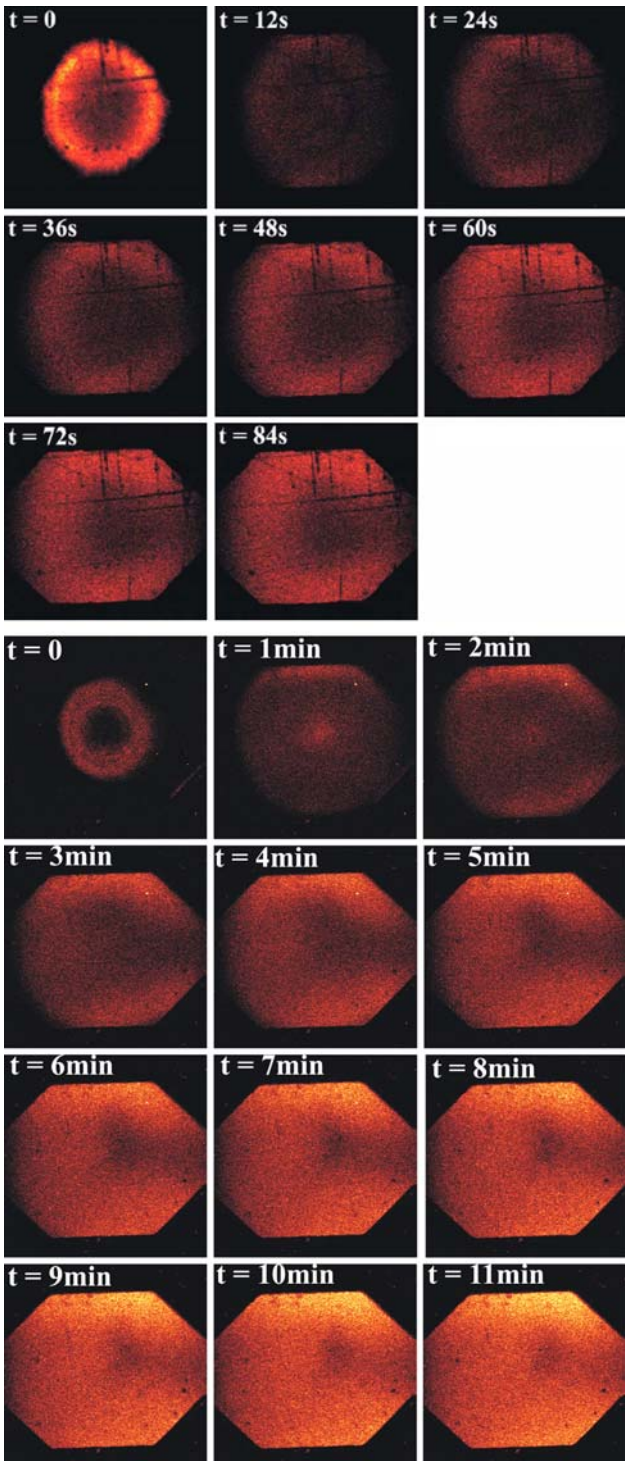

Fig. 8 The upper sequence of CLSM images shows the temporal evolution of fluorophore concentration, initially dried with the master mix at the bottom of the chamber. The focal plane is positioned 150 μm above the bottom of the chamber. Time between images is 12 s. The image at $t = 0$ is taken without the aqueous solution present in the chamber, with the focal plane at the bottom wall of the chamber; hence the high fluorescence intensity in the image. The lower image sequence shows the concentration time evolution of fluorescent IgG at the same height. The fluorescent IgG was initially dried at the bottom of the chamber with the NASBA enzymes. Time between images is here 60 s

limited, with optical slice thickness ~ 100 μm. An Omnichrome Series 43 ArKr laser was used for sample illumination. The fluorescence filters were set for FITC detection, with excitation peak at 488 nm and emission at >510 nm.

The reaction chamber containing dried master mix was flooded (Goldschmidtboeing 2006) by injecting 1.5 μl of the relevant solution containing 15% DMSO and 375 mM Sorbitol in water into the microchip via the chip supply channel. Sequences of images at a plane 150 μm above the bottom wall of the chamber were acquired in 12 s time intervals (total chamber depth was 200 μm). The re-hydration process of the dried master mix is shown in Fig. 8. As can be seen by inspection of the images, the fluorescence intensity is essentially unchanged beyond 60 s, although some weak local fluorescence intensity variations can still be discerned in the image sequence beyond that time. Thus, the re-hydration and diffusion of the fluorescent species is sufficiently completed after 60 s. After emptying the reaction chamber, no fluorescent reactant residues were detected at the chamber bottom, thus confirming the successful re-hydration process.

For the dried enzymes, the time before an equilibrium concentration of fluorescent IgG at 150 μm height was reached, was approximately 10 min.

In order to investigate whether the dissolution time of the dried reagents is dominated by the re-hydration or by the diffusion process, we compare these results with the theoretical estimate for the diffusion time of similarly sized molecules from the bottom of the chamber to the measurement plane, i.e. a distance of 150 μm. The average distance of diffusion L of a particle with a diffusion coefficient D is proportional to the square root of time t:

$$L = \sqrt{2Dt}. \tag{4}$$

The Stokes–Einstein diffusion coefficient for a spherical particle suspended in an aqueous solution is given by:

$$D = \frac{kT}{3\mu\pi d_p} \tag{5}$$

where k is the Boltzmann constant, T is the absolute temperature, μ is the dynamic viscosity and d_p is the particle diameter. We have estimated the diameter of the fluorescent molecular beacons in the master mix to be $d_{MB} \approx 2.8$ nm, while the diameter of the fluorescently labelled IgG is estimated to be $d_{MB} \approx 7$ nm. Using these sizes, we estimate an average diffusion time of 70 s for the fluorescent beacons to reach the 150 μm plane. The larger IgG proteins will have an average diffusion time of approximately 3 min.

Although the estimates given above are crude, they indicate that the diffusion time for the molecular beacons roughly corresponds to the experimentally observed time needed to obtain a homogeneous suspension of master mix in DMSO/sorbitol/water solution. This, in turn, implies that the dissolution process is diffusion-limited. Re-hydration itself is rapid, occurring nearly instantaneously as compared to the time scale of diffusion.

For the enzymes, we observe that the time required to achieve a homogeneous concentration of fluorescent IgG is significantly larger than the estimated diffusion time. This implies that the re-hydration of the enzymes is slow and dominates the process of dissolution. Nevertheless, complete dissolution was observed.

Dissolution of the enzymes will be the final step on the NASBA chip prior to detection of the fluorescent signal. Microscope inspection of the reaction chamber after re-hydration revealed that no residue of the dried material is left on the chamber wall. Therefore, the bottom of the reaction chamber does not disturb the optical detection of the amplification process, and it may be performed in the same chamber as the dissolution of the enzymes. The amplification is time-consuming, requiring approximately 90 min before unambiguous decision about presence/absence of the targeted species can be reached.

Based on these experiments we conclude that no active mixing mechanisms are necessary neither for the dissolution of the master mix, nor the enzymes. In both cases, the mixing time is most probably sufficiently fast for the functionality of the NASBA chip.

6 NASBA amplification in nanoliter chambers with dried enzymes

The critical test of the NASBA amplification chip is whether it is possible to obtain and detect successful NASBA amplification of oligos or HPV mRNA using separate sample volumes of 500 nl and dried reagents. Tests have been performed on re-hydration and re-activation of dried enzymes. The enzymes needed for the NASBA reaction are AMV-RT, RNaseH and T7 RNA polymerase (PreTect HPV-Proofer kit, NorChip). COC hot embossed 500 nl reaction chambers with feeding and ventilation channels were coated with PEG. In contrast, COC milled chips with the same geometries had no positive amplifications; this may be due to insufficient PEG coating of rough surfaces and thereby the adsorption of enzymes on the walls, which inhibits the reaction.

The enzyme solution was spotted in the micro-chambers, on top of the PEG. The microchips were spotted with enzymes using the Nanoject II from Drummond Scientific Company, dried in room temperature for up to 1 day and then sealed with adhesive tape.

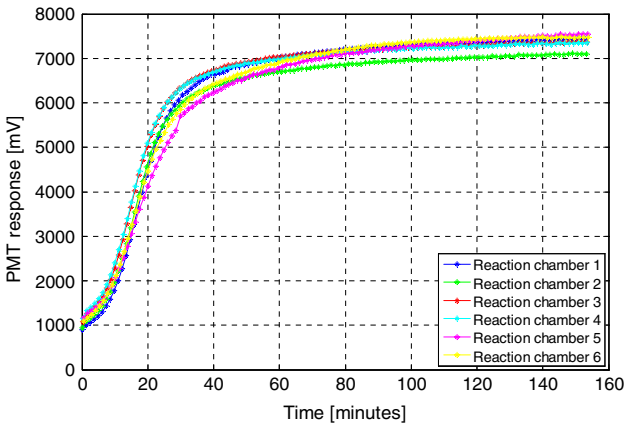

Fig. 9 Amplification curves of the six 500 nl reaction chambers run simultaneously on one microchip employing a sample of positive control for HPV type 16 (0.1 μM)

The amplification reaction was performed by first manually mixing the NASBA reagents, except the enzymes, and a positive control sample of human papillomavirus (HPV) type 16. The mixture was incubated at 65°C for 3 min followed by 3 min at 41°C. The sample droplets were pulled into the microchip by an under-pressure and positioned in the reaction chambers with dried reagents with the help of hydrophobic spots applied in the narrow channels leading in and out of the chamber. A custom-made instrument (Gulliksen et al. 2005) recorded the fluorescent signal of the amplification reaction at 41°C, scanning 6 reaction chambers on one chip (Gulliksen et al. 2007). Figure 9 shows positive amplification curves of the 500 nl reaction chambers.

The present work demonstrates on-chip storage of dried enzymes which are reactivated upon re-hydration of sample. These results are promising with regard to the development of disposable self-contained microchips for NASBA.

7 Conclusions

We have presented experimental results on test chips, examining sub-functions of a prototype microchip for simultaneous detection of multiple mRNA targets. It was shown that successive passive valves based on hydrophobic channel restrictions could have increasing burst pressures well separated and above pinning pressures. This enabled the simultaneous droplet positioning in parallel channels to be controlled by one external pump only. The two types of dried reagents (master-mix and enzymes) necessary for NASBA amplification were spotted, dried and re-hydrated in micro-chambers. Re-hydration with fluorescent markers

showed that the dried reagents were sufficiently re-hydrated on time scales acceptable for on-chip NASBA reactions. The most important result in the project so far is that spotted and dried enzymes in a nanoliter-sized reaction chamber can be re-hydrated and re-activated and may result in positive NASBA amplifications. To our knowledge, this is the first time the NASBA enzymes have been successfully dried and re-activated on-chip.

The next step in the project is to use the prototype NASBA amplification chip with all functions included, in order to verify that the fluidic and biochemical sub functions work when they are combined.

Acknowledgements The MicroActive project is funded by the European Commission, contract IST-NMP-CT-2005-0173319.

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