The MicroActive project: automatic detection of disease-related molecular cell activity

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ABSTRACT

The aim of the MicroActive project is to develop an instrument for molecular diagnostics. The instrument will first be tested for patient screening for a group of viruses causing cervical cancer. Two disposable polymer chips with reagents stored on-chip will be inserted into the instrument for each patient sample. The first chip performs sample preparation of the epithelial cervical cells while mRNA amplification and fluorescent detection takes place in the second chip. More than 10 different virus markers will be analysed in one chip. We report results on sub-functions of the amplification chip. The sample is split into smaller droplets, and the droplets move in parallel channels containing different dried reagents for the different analyses. We report experimental results on parallel droplet movement control using one external pump only, combined with hydrophobic valves. Valve burst pressures are controlled by geometry. We show droplet control using valves with burst pressures between 800 and 4500 Pa. We also monitored the re-hydration times for two necessary dried reagents. 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 have shown positive amplification of HPV type 16 using dried enzymes stored in micro chambers.

Keywords: Biomedical, sensor, molecular diagnostics, microfluidics, lab-on-a-chip, dried reagents, spotting, valve

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1. INTRODUCTION

The aim of the MicroActive project [1] 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 [2]. 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. Cervical cancer is the second most common cancer type among women worldwide. More than 80% of all adults will have at least one infection with HPV during their lifetime. However, nearly all these infections may be considered as the "cervical flu", and have nothing to do with cervical cancer risk. The development of cervical cancer is related to oncogenic proteins produced by transformed cells in the women's cervix. These cells are influenced by certain genetic sequences from a limited group of HPV-viruses and may develop into cervical cancer over a period of 10-25 years. 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. The NorChip Pre Tect HPV proofer technology [3-6] detects the oncogenic expression, and is therefore an accurate and cost-effective test for cervical cancer prevention. The task of MicroActive is to miniaturize and automate the protocol of this macroscopic 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. 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 will:

1) Develop 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) Develop a second disposable microfluidic chip for multiple target amplification and fluorescent detection with dried spotted reagents stored in micro-channels.
3) Develop manufacturing methods for spotting and drying of reagents, surface coating and patterning, and polymer chip lamination that do not inhibit the bio-molecular processes.
4) Perform 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) Perform isothermal amplification of mRNA by nucleic acid sequence-based amplification (NASBA) [7].
6) At early stages of the project, perform tests on clinical samples, using test-chips for separate functions.
7) Develop an instrument without manual protocols.
8) Test the instrument on clinical specimens and compare to gold standards.
9) Address factors such as reliability, usability and cost of the total instrument – factors which are crucial to acceptance by health care professionals.
10) Provide the repeatable and stable fluid control required by a commercial system through use of simple pumps in combination with surface modification.

In this paper we will focus on reporting the first experimental results on the fluidic and biological functions on the second chip, the NASBA amplification and fluorescent detection chip. Earlier, positive NASBA reactions with wet, premixed reagents have been demonstrated on chip [8, 9]. The new aspect of this work is to dry the reagents in the micro chambers and to perform the amplifications in metered, separate nanoliter sized droplets. The NASBA amplification chip is thus based on sample metering and droplet based microfluidics as described by Burns et al [10]. In our case the separate metered sample droplets are 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 and co-workers [11] for air drying and reactivation of PCR mixes in conventional macro scale volumes using 96-well plates. For self-contained systems, both liquid reagents and dried reagents have been introduced as plausible solutions for long-term storage of reagents on-chip [12].

Here we report experiments on parallel droplet movement control using one external pump only, combined with hydrophobic valves. We show 7 droplets moving in parallel channels, each stopping controllable in three reaction chambers along the channel. Due to heating requirements for the reactions, it is important that all droplets are in the same reaction chamber at the same time. We also monitored the re-hydration times for two necessary dried reagents using fluorescent markers and a confocal microscope. After sample droplet 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 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.

Work related to the sample preparation chip is not reported here, but a biobank has been established that stores patient cervical cell samples. Biobank samples are currently used for macroscopic comparisons between the HPV mRNA diagnostics method which the project is miniaturizing, and laboratory gold standards for cervical cancer diagnostics. Patient samples have also been processed in microfluidic test chips performing cell capture and lysis. The chosen procedure for cell capture and lysis has resulted in mRNA of sufficient concentration and quality for amplification, further optimization is under way. The first chips for nucleic acid purification have also been tested, in this case the quality of the extracted mRNA was too low and a new approach is currently explored. A prototype including all fluidic functions of the sample preparation chip has been manufactured and tested with stored liquid reagents, pumps and heating. The chip functioned roughly as expected; improvements will be made in the next generation prototype.

2. NASBA AMPLIFICATION CHIP

In the NASBA amplification chip, the purified sample output of the sample preparation chip is split into separate, smaller reaction volumes. Each volume is 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- or medium 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 the NASBA amplification 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 is based on numerous tests on sub-functions, below we report on the parallel motion control of the droplets, the re-hydration of dried reagents, and the amplification, using dried enzymes. A sketch showing the functions of the chip is seen in figure 1. Sample with extracted nucleic acids in buffer will be transferred from the sample preparation chip to the amplification chip via a disposable connection. The output volume of the sample preparation chip is approximately 20µl, the current design of the amplification chip allows for input volumes between 14µl 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 10 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, 10 equally sized sample droplets are snapped off and reside in the 10 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.

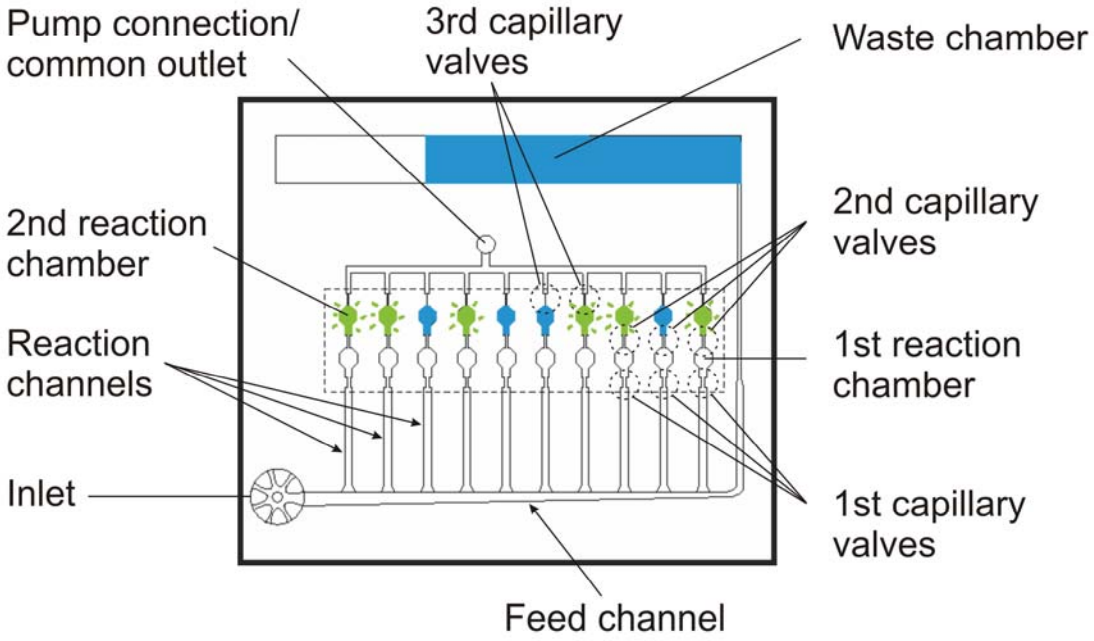

Figure 1. Sketch of the NASBA amplification chip. The star-shaped inlet is in the lower left corner. The horizontal feed channel leads to the 10 parallel reaction channels and to the large waste chamber on the top of the chip. The hydrophobic valves are spotted at the three restrictions in each parallel 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.

3. TEST CHIP MANUFACTURING

Test chips both for fluidic and biological experiments on sub functions of the amplification chip have been manufactured. All chips 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 O2 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 approx. 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 [xx]). The contact angle of DI water on Teflon surface was measured to be

approximately 100º. 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 combined with channel restrictions and hydrophobic valves [13]. The chips contained 7 parallel channels (800 μm × 200 μm, width × depth), each with four lateral restrictions of 380 μm, 150 μm, 75 μm and 33 μm (see figure 2). A total of 5 chips were tested.

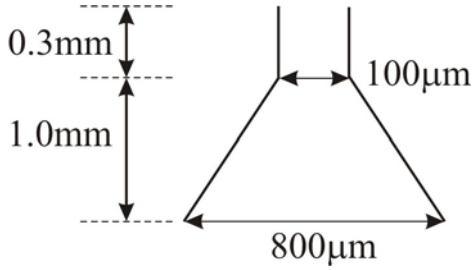

Figure 2. Details of capillary valve geometry (here, valve width is 100μm). The capillary valves contain a tapered part and a narrow restriction. The tapered part is included to aid spotting of the fluorpolymer for hydrophobization.

The chips were mounted in an aluminum frame and connected to a second chip containing a branch-like channel structure for distribution of the pressure to all parallel channels, see figure 3. A syringe pump (PHD2000 from Harvard Apparatus) was used to apply suction to the common chip outlet, withdrawing air from the 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 figure 4.

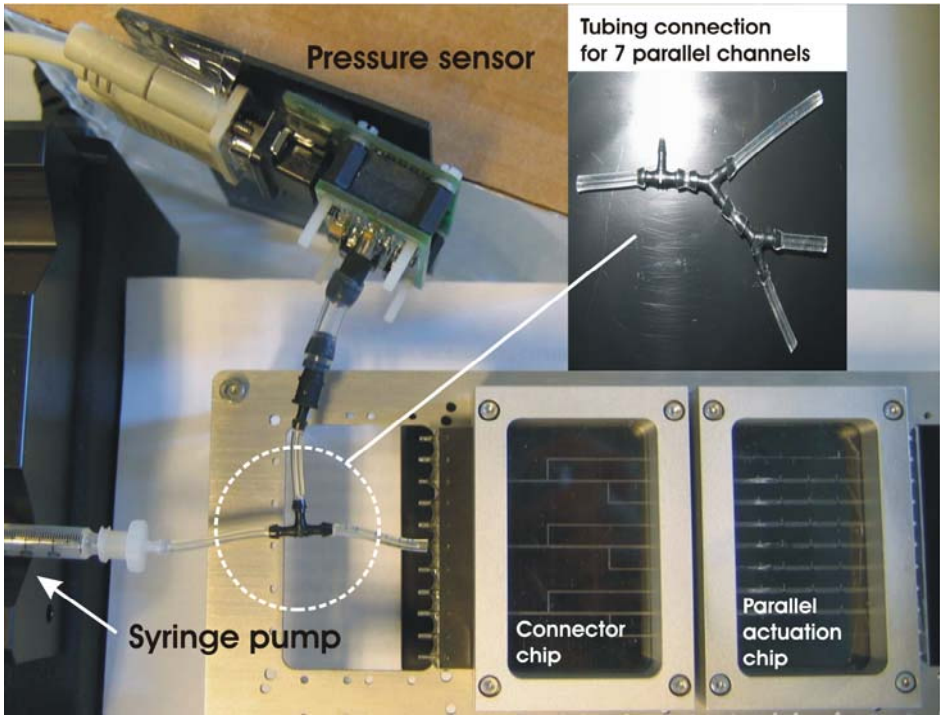

Figure 3. Test setup for monitoring of parallel droplet movement. The syringe connected to the pump is seen to the left, the MEMSCAP pressure sensor upper left. Tubing connects the pump and pressure sensor to the aluminum frame with the fluidic connectors, pressure distribution chip and the rightmost parallel droplet chip. The presented image shows the setup for actuation of three channels in parallel. For actuation of 7 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.

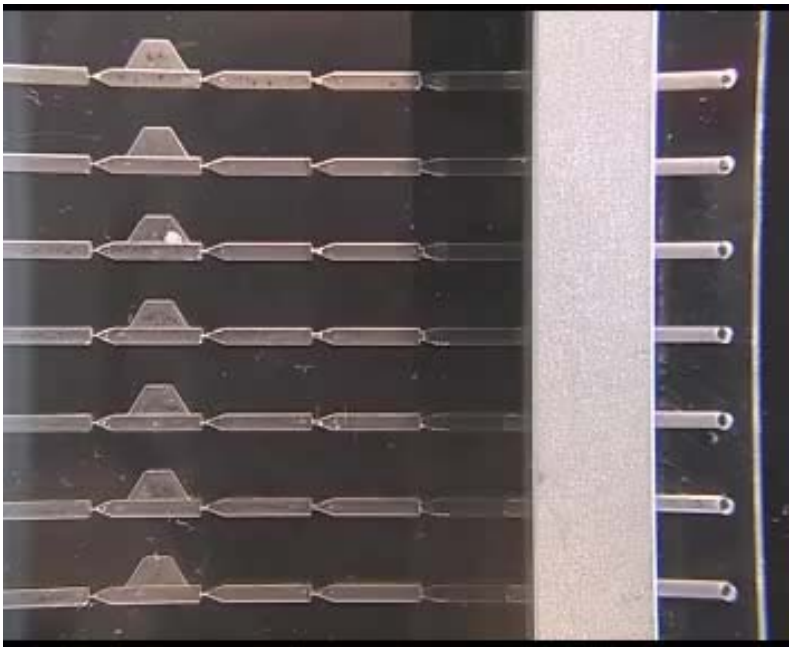

Figure 4. The seven parallel reaction and amplification test channels. Four restrictions with hydrophobic spots can be seen in each channel. Valve pressure strength increases from right to left. In the picture, 500 nl droplets are positioned before the rightmost, weakest valve.

The pressure as a function of time at the common pump connection for all channels was recorded during operation and is shown in figure 5. The pressure plot shows the absolute pressure. During pumping, the pressure in the system decreases until the pressure difference across the front water/air meniscus becomes higher than the burst pressure of the first hydrophobic valve. When the first droplet breaks through the valve, it enters the first chamber and the pressure in the common under pressure 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 pinning effects. No droplet passes to the second chamber 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 figure 5, where the pressure level of the first seven valves is clearly higher than that of the 2nd valves. Once all sample droplets reside in the first chamber, they proceed to cross their respective 2nd valves as the pumping continues. Passing of the 4th 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.

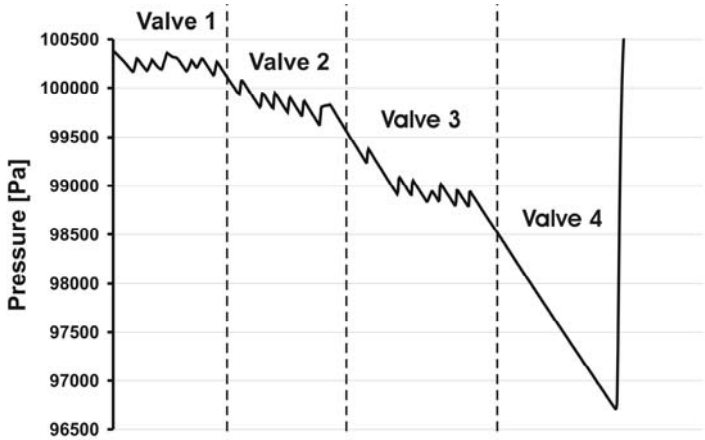

Figure 5. Common pulling pressure of parallel valves as a function of time. Parallel droplets are pulled at constant rate of 10 µl/min. Absolute pressure trace of seven parallel droplets moving through the parallel actuation chip. At the fourth valve, the first droplet to break the valve effectively ventilates the system, and hence the pressure in the system returns to atmospheric conditions.

The average burst pressures of the four valves are shown in figure 6. The data (open squares) represents average values from 5 chips with DI water as the working liquid. The error bars indicate the standard deviation of the differential pressure value. In addition to the use of DI water as working liquid, one experiment (open circles in figure 6) 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 approx. 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. After these experiments were finished, biochemical experiments showed that two reaction chambers in sequence are sufficient. Therefore, in the design of the final device, the number of valves can be reduced from 4 to 3, thus giving the possibility to separate the levels of burst pressure of the valves even further.

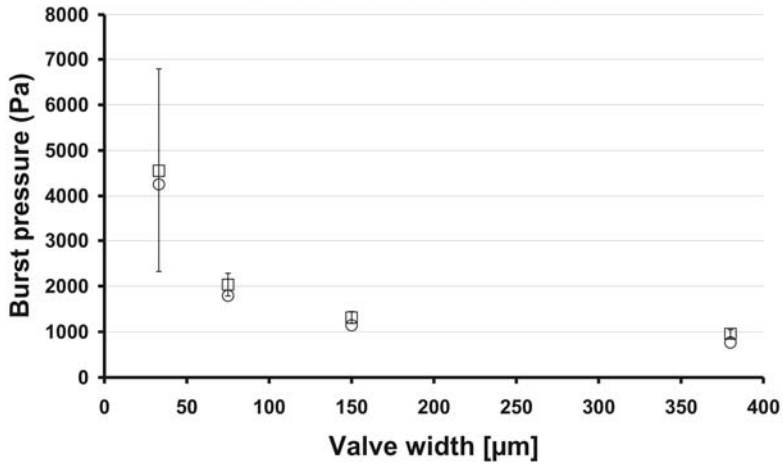

Figure 6. Burst pressures of the capillary valves as function of valve width. Symbols: □ DI water; ○ reagents; solid line represents the analytical values for water. Contact angle of DI water on Teflon was measured to be approx. 100º. The pressure data for each valve represents an average of 35 measurements (5 chips with 7 parallel channels each).

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 there will be two chambers with dried reagents, the first with 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.

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 approx. 30 seconds 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 490nm/520nm) 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 495nm/515nm) 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 (molecular weight AMV-RT, 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 limited, with optical slice thickness ~100μm. An Omnichrome Series 43 ArKr laser was used for sample illumination. The fluorescence filters were set for FITC detection, with excitation peak at 488nm and emission at >510nm.

The reaction chamber containing dried master mix was flooded [14] by injecting 1.5μl 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 12s time intervals (total chamber depth was 200μm). The re-hydration process of the dried master mix is shown in figure 7. As can be seen by inspection of the images, the re-hydration and diffusion of the fluorescent species is essentially completed after 60s. After that time, no significant changes in the fluorescent signal from the solution (neither with regard to intensity nor homogeneity) were observed. For the dried enzymes, the time before an equilibrium concentration of fluorescent IgG at 150 μm height was reached, was 10 minutes.

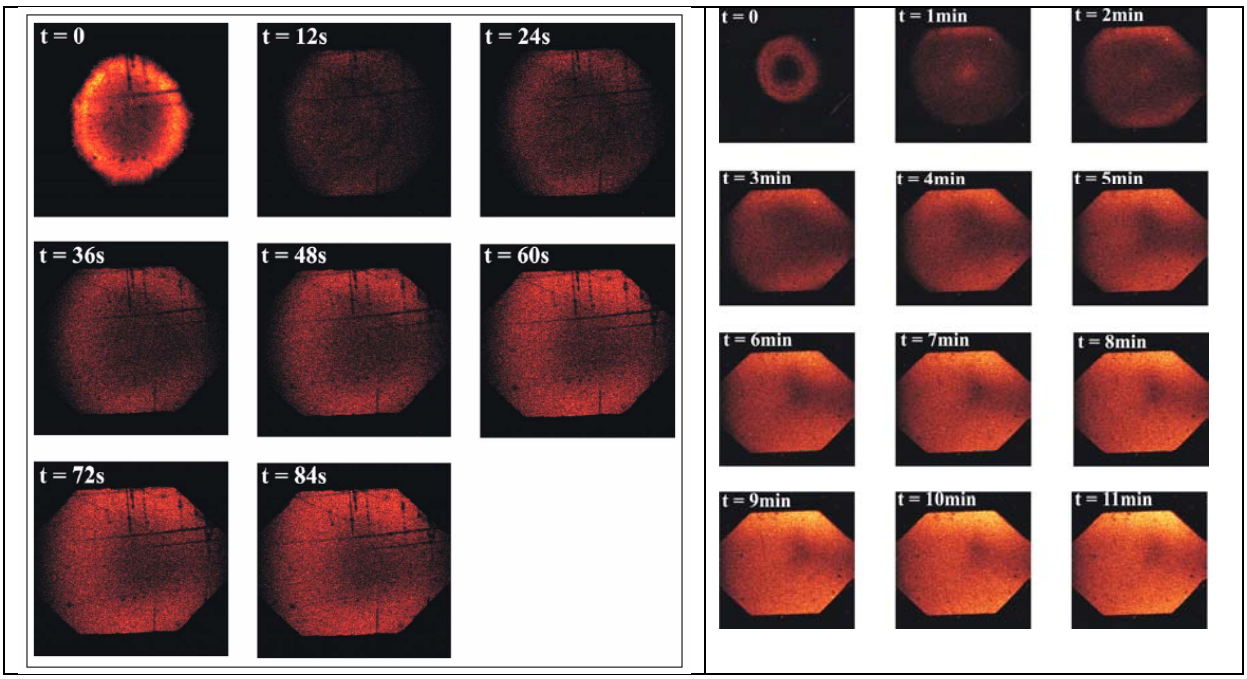

Figure 7. The left sequence of CLSM images shows the temporal evolution of fluorophore concentration, initially dried with the master mix at the bottom of the chamber. The focal plane is positioned 150 μm above the bottom of the chamber. Time between images is 12s. The image at t=0 is taken without the 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 image sequence on the right 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.

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:

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

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

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

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 minutes.

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 is observed without the aid of external actuation.

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. The hot embossed 500 nl reaction chambers in COC were also coated with PEG. Milled reaction chambers 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 NASBA reagents were provided in the PreTect HPV-Proofer kit (NorChip AS, Norway). A stabilizing agent, 0.5% PEG 8000 (Sigma Aldrich Norway AS), was added to the enzyme solution prior to spotting and drying [15]. The microchips were spotted with enzymes using the Nanoject II from Drummond Scientific Company, dried in room temperature for up to 1 day and sealed with adhesive tape.

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 first incubated at 65˚C for 3 minutes followed by 3 minutes at 41˚C. The microchip containing the dried enzymes was tempered to 41˚C before 1 μl reaction mixture was applied to each of six reaction chamber in parallel on the microchip. The sample droplets were positioned in the reaction chambers by applying hydrophobic spots in the narrow channels leading in and out of the chamber. A custom-made instrument recorded the fluorescent signal of the amplification reaction at 41˚C [3], scanning 6 reaction chambers on one chip. Figure 8 shows amplification curves of the 500 nl reaction chambers for reactivation of spotted and dried enzymes.

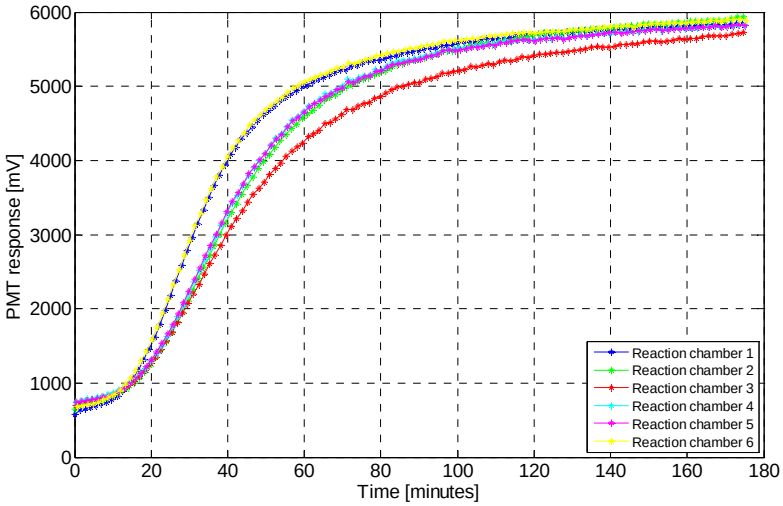

Figure 8. Amplification curves of the six 500 nl reaction chambers run simultaneously on one microchip employing a sample of positive control for HPV type 16 (0.1 µM). The dried enzymes including PEG 8000 were re-hydrated after 1 day at room temperature. No amplification was observed when PEG 8000 was excluded in the drying procedure.

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 sub-functions of a microchip for simultaneous detection of multiple mRNA targets, specifically HPV viruses that predispose for cervical cancer. It was shown that by manufacturing passive valves consisting of channel restrictions with hydrophobic spotting, the simultaneous movement of droplets in parallel channels could be controlled by one external pump, only. Tests were also performed on the re-hydration of the reagents needed for NASBA amplifications. Pre-tests had shown that master mix and enzymes had to be dried in two separate chambers. Spotting and drying of reagents in chambers, and later re-hydration with fluorescent markers, showed that the dried reagents were re-hydrated on time scales acceptable for on-chip NASBA reactions. The most important result in the project so far is that re-activation of dried enzymes in a nanoliter-sized reaction chamber is possible 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 sub functions work when they are combined. Also, the re-activation of dried master mix will be developed.

ACKNOWLEDGEMENTS

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REFERENCES

1. MicroActive FP6 STREP, contract IST-NMP-CT-2005-0173319 http://www.sintef.no/microactive
2. P. Yager, T. Edwards, E. Fu, K. Helton, K. Nelson, M. R. Tam and B. H. Weigl, "Microfluidic diagnostic technologies for global public health", *Nature* 442, 412-418 (2006)
3. A. K. Lie et al., " DNA versus RNA based methods for human papilloma virus detection in cervical neoplasia", *Gynecologic Oncology* 97, 908-915 (2005)
4. Walboomers et al. "Human papilloma virus is a necessary cause of invasive cervical cancer worldwide", *J. Pathol.* 189(1), 12-19 (1999)
5. I. Kraus, T. Molden, L. E. Ernø, H. Skomedal, F. Karlsen and B. Hagmar, "Human papillomavirus oncogenic expression in the dysplasticportio; an investigation of biopsies from 190 cervical cones", *British Journal of Cancer* 90, 1407-1411 (2004)
6. http://www.norchip.com/
7. J. Compton, "Nucleic acid sequence-based amplification," *Nature* 350, 91 (1991).
8. A. Gulliksen, L. Solli, F. Karlsen, H. Rogne, E. Hovig, T. Nordstrøm and R. Sirevåg, "Real-time Nucleic Acid Sequence-Based Amplification in Nanoliter Volumes", *Anal. Chemistry* 76, 9-12 (2004)
9. A. Gulliksen, L. Solli, K. S. Drese, O. Sörensen, F. Karlsen, H. Rogne, E. Hovig and R. Sirevåg, "Parallel nanoliter detection of cancer markers using polymer microchips", *Lab Chip* 5, 416-419 (2005)
10. M. A. Burns, B. N. Johnson, S. N. Brahmasandra, K. Handique, J. R. Webster, M. Krishnan, T. S. Sammarco, P. M. Man, D. Jones, D. Heldsinger, C. Mastrangelo, and D. T. Burke, "An integrated nanoliter DNA analysis device", *Science*, 282, 484-487 (1998)
11. B. H. Weigl, J. Gerdes, P. Tarr, P. Yager, L. Dillman, R. Peck, S. Ramachandran, M. Lemba, M. Kokoris, M. Nabavi, F. Battrell, D. Hoekstra, E. J. Klein and D. M. Denno, "Fully integrated multiplexed lab-on-a-card assay for enteric pathogens,", *Proc. of SPIE* 6112, 1-11 (2006)
12. V. Linder, S. K. Sia and G. M. Whitesides, "Reagent-loaded cartridges for valveless and automated fluid delivery in microfluidic devices", *Anal Chem*, 77, 64 (2005)
13. K. W. Oh, C. H. J. Ahn, "A review of Microvalves", *Micromech. Microeng* 16, R13-R39 (2006)
14. F. Goldschmidtboeing, M. Rabold, and P Woias, "Strategies for void-free liquid filling of micro cavities", *Journal of Micromech. and Microeng*., 16 (7), 1321 - 1330 (2006)
15. E. Garcia, J. R. Kirkham, A. V. Hatch, K. R. Hawkins and P. Yager, "Controlled microfluidic reconstitution of functional protein from an anhydrous storage depot", *Lab Chip* 4, 78 (2004)