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Quantitative Analysis in Nanoliter Wells by Prefilling of Wells Using Electrospray Deposition Followed by Sample Introduction with a Coverslip Method

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In contrast to performing assays on a substrate using immobilization techniques, wet analysis in nanoliter-sized wells allows quantitative monitoring of enzyme-based reactions by measuring luminescence with time. However, a suitable dispensing method is required to accurately deposit stabilized enzyme solutions into nanoliter wells in such a manner that the enzyme activities are preserved prior to and during measurements. Furthermore, an efficient method is required to add sample liquid to these wells in such a manner that evaporation of assay liquid is completely prevented during sample introduction and monitoring. A powerful methodology is presented in this paper allowing quantitative analysis of enzyme-based reactions in identical nanoliter volumes on-chip. In a first step, picoliter amounts of protein solutions are deposited as uniform dry dots into wells using our reported electrospraying technique (Moerman, R.; Frank, J.; Marijnissen, J. C. M.; Schalkhammer, T. G. M.; van Dedem, G. W. K. *Anal. Chem.* 2001, *73*, 2183−2189.). The silicon chips are then stored at temperatures as low as −80 °C. At the time of analysis, a sample solution is slid into the wells using a coverslip. With the edge of the coverslip, sample solution is pushed across the wells at a speed of 1.5−2.5 cm/s to prevent carryover of reagents to neighboring wells. Evaporation of assay liquid from the wells is prevented because the coverslip seals the wells and "bonds" to the chip by adhesion forces. Electrospraying appears to be an excellent method to deposit enzyme solutions containing up to 20% (w/v) of trehalose without being hampered by clogging of the capillary or splashing of droplets. After being sprayed on-chip (silicon nitride), the enzymes pyruvate kinase and lactate dehydrogenase remained stable for a period of 1.5−2 months at a storage temperature of −20 °C. The coverslip method completely prevented evaporation for minutes up to hours allowing monitoring of enzyme-based reactions in arrays of nanoliter wells.

Downscaling of assay volumes to the nanoliter and picoliter level reduces the costs associated with expensive and rare (bio)-chemicals and allows high-throughput screening of high-density arrays using very small amounts of sample liquid. So, blood samples, for example, can be screened for more and different compounds and more frequently than is currently done in clinical diagnostics. In addition, cell analysis, monitoring cell growth, and monitoring crystallization of expensive proteins are preferably performed in arrays of easily accessible and identical nanoliter or picoliter wells made in silicon[1,2] or plastic.[3] However, this requires a platform consisting of effective and cost-saving techniques allowing customers to fully benefit from the advantages of downscaling. First, a liquid deposition technique should allow for accurate dotting of picoliter amounts of protein solutions containing high concentrations of protectants to preserve the activities of antibodies and enzymes on-chip. As a consequence, the accuracy of deposition should be independent of liquid composition, and clogging of capillaries or channels due to high concentrations of additives should be prevented. Deposition techniques such as piezodispensing and contact-printing have restrictions regarding liquid composition, dot volume, and dot size. Furthermore, piezoheads and print heads are relatively expensive and vulnerable. In contrast, miniaturized electrospray dispensing[4−6] is based on liquid being slowly forced through a capillary toward the capillary tip where it forms a liquid cone that releases a continuous spray of nanometer-sized droplets by just applying an electric field. So, there is no shooting (piezoelectric) or contact-printing mechanism required, shear stress on biocompounds is

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negligible, and dispensing is not dependent on liquid composition because the released amount of liquid depends on the liquid feed. Capillaries are low cost, disposable, and available in various materials, diameters, and lengths. Furthermore, the electric field induces convection in the liquid cone, which prevents the compounds from crystallizing at the capillary tip. Finally, a sprayed liquid amount of 200 pL (room temperature) evaporates within 1 s upon landing onto substrate resulting in a nearly dry reagent dot, which is favorable to preserve protein stability.[7]

Second, the final analysis of a small amount of sample solution preferably is performed using a simple and automated setup instead of using complex dispensing tools such as a piezoelectric dispenser, which requires precise alignment for each chip combined with a method to prevent rapid evaporation of the nanoliters of assay liquid. We have reported on a coverslip method[8,9] ("chimney") to fill arrays of wells with a sample solution using a coverslip containing holes to degas wells. The chimney method is suitable for applications where relatively soft polymer substrates or fragile coated substrates are used because, after filling of the wells, the coverslip is just pushed downward onto the substrate to seal the wells. However, the chimney method has a few disadvantages: (1) it is difficult to handle manually because the coverslip has to be moved across the chip forward and backward in a very precise and fast manner to fill the wells adequately and at the same time prevent carryover; (2) the chimney is restricted to well depths of 40 ± 5 μm because deeper wells are filled with more difficulty while shallow wells may result in severe carryover of dot compounds; (3) the chimney is restricted to certain liquids that are pulled between the coverslip and the chip by capillary forces; (4) the chimney is not suitable for handling cell suspensions and cell-free extracts because the micrometer-sized cells and cell debris remain between the coverslip and the chip after sealing resulting in poor sealing of the wells; (5) arrays of micrometer-sized holes have to be drilled or etched into coverslips of quartz, glass, or plastic, which is relatively expensive.

In this study, we therefore applied an alternative coverslip method,[10] referred to as the "Zamboney" method, allowing one to perform enzyme-based reactions in nanoliter wells by just manually sliding a coverslip with test solution across an array of wells. We show here progress curves (calibration curves) for enolase, lactate dehydrogenase (LDH), and pyruvate kinase (PK) by measuring the decrease in NADH fluorescence with time in wells.

In addition to spraying on flat substrates and in aluminum microliter wells as reported previously,[5] we show here spraying conditions to prefill 400-μm-wide and 50-μm-deep wells with relatively complex reagent cocktails containing up to 20% w/v trehalose. We used trehalose in this study because it is a well-known preservative that is successfully used in stabilizing LDH following freeze-drying[11] and freeze−thawing[12] and in stabilizing the labile phosphofructokinase (PFK) during air-drying[13] and during freeze-drying.[14]

We report here on novel results concerning preserving activities of LDH and PK in wells on a silicon nitride surface as a function of spraying conditions, enzyme and trehalose concentrations, storage time, and storage temperature.

EXPERIMENTAL SECTION

Chemicals. Brij 35, D(+)-trehalose, 2-phosphoglycerate (2-PG), phosphoenolpyruvate (PEP), and pyruvate were from Sigma (St. Louis, MO). NADH, adinosine 5′ diphosphate (ADP), enolase, LDH (from beef heart, 1250 units/mL), and PK (from rabbit muscle, 2000 units/mL) were from Roche. The enzyme suspensions contained 3.2 M $(NH_4)_2SO_4$.

Fabrication of Coverslips, Chips, and Storage Boxes. *Coverslips.* We used Pyrex coverslips allowing adequate light transmission for excitation of NADH molecules as reported previously.[15] Coverslips of 2 cm × 1 cm were cut from 1-mm-thick Pyrex plates. Prior to use and after use, the coverslips were cleaned by brushing them for 20 s with a 5% w/v solution of Brij 35 followed by rinsing with purified water for 1 min.

Substrates. At the Delft Institute of Microelectronics and Submicron Technology (DIMES), arrays of 25 (5 × 5) round and square wells were made in silicon substrates of 2 × 1 cm (500 μm thick) by means of plasma etching. The wells are 400 μm wide, 400 μm apart (edge to edge), and 50 μm deep. The chip and well surfaces were coated with a 50-nm-thick silicon nitride layer by means of chemical vapor deposition.

Aluminum Storage Boxes. Prefilled chips were stored in machined aluminum boxes having a storage volume of 10.5 × 20.5 × 0.7 mm.

Methodology of Performing and Measuring Enzyme-Based Reactions in Nanoliter Wells. Enolase and PK based assays were performed according to reactions 1 + 2 + 3, reactions 2 + 3 to show that the methodology can be used for complex systems that require spraying of complex cocktails of reagents. Calibration curves of enolase and PK were obtained by measuring the decrease in NADH concentration with time using fluorescence detection.

$$2\text{-PG} \overset{\text{enolase}}{\leftrightarrow} \text{PEP} \tag{1}$$

$$\text{PEP} + \text{ADP} \overset{\text{PK}}{\leftrightarrow} \text{pyruvate} + \text{AMP} \tag{2}$$

$$\text{pyruvate} + \text{NADH} \overset{\text{LDH}}{\leftrightarrow} \text{lactate} + \text{NAD} \tag{3}$$

The complete methodology to perform and measure reactions in nanoliter wells is shown in Figure 1. We sprayed reagent solutions into wells of 400 μm wide and 50 μm deep (Figure 1A). A prefilled

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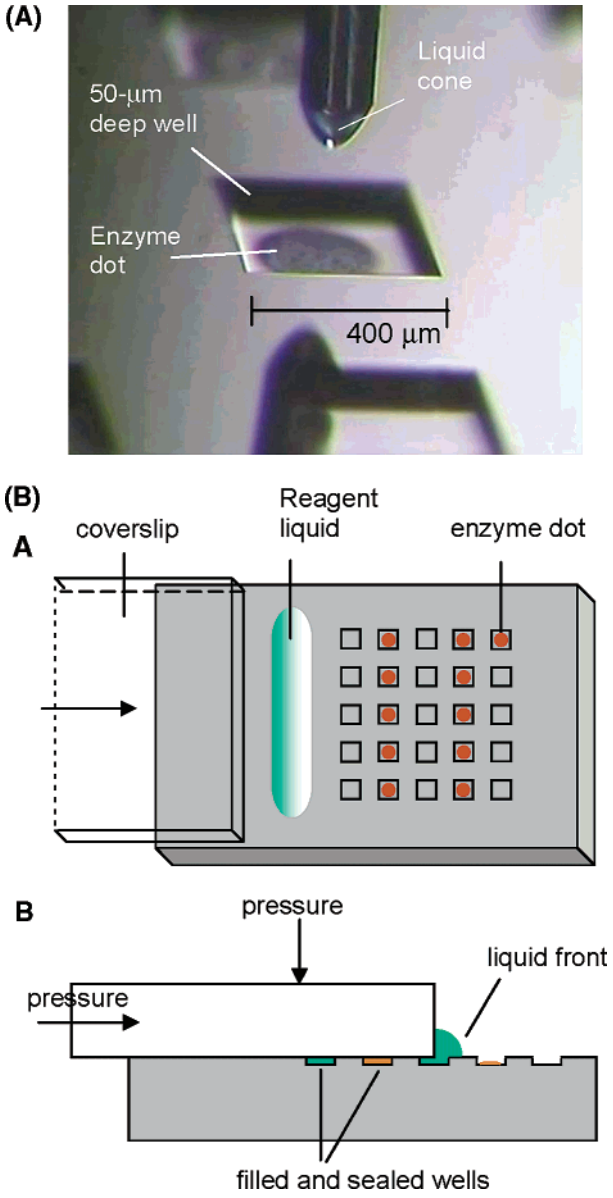

Figure 1. Methodology to perform quantitative reactions in nanoliter volumes. (A) Prefilling of nanoliter wells: image (180× magnified) of the spraying in a 400-μm-wide and 50-μm-deep well. (B) Schematic of the principle of filling of an array of wells with "sample" solution using the coverslip method. In panel A (topview) of (B), reagent liquid is pipetted onto a chip and a coverslip is pressed onto the chip and moved toward the liquid line. In panel B (sideview) of (B), the edge of the coverslip pushes the liquid across the wells and seals the filled wells allowing to monitor the well contents for hours up to days.

chip was put in an aluminum box that was stored at low temperature to preserve the activities of the sprayed reagents. Before measurement, an aluminum box containing a chip was allowed to accommodate to room temperature and then the chip was placed in a holder. Liquid was pipetted onto the chip, and the array of wells was filled using the coverslip (Figure 1B). This chip was placed onto a computer-controlled $x-y-z$ table to monitor the reactions with time in a sequential manner using a specially adapted microscope−CCD−camera setup. From the initial slopes of the progress curves, we calculated the initial reaction rates in picomoles of converted substrate per minute per well.

Compositions of the Spraying and Calibration Solutions. All substrate solutions that were electrosprayed contained 1% w/v Brij 35 and 1% w/v trehalose in 0.05 M triethanolamine buffer (TEA). To determine the PK calibration curve, a solution containing 2 mM NADH and 8 mM PEP was sprayed into five wells of a single column. In each well, 2 nL of substrate solution was deposited. Just before measurements, arrays of wells were filled with solutions containing 0.05, 0.1, and 0.2 unit/mL PK, 12.5 units/mL LDH, 44 mM KCl, 11 mM $MgSO_4$, and 2 mM ADP in 0.1 M TEA buffer. So, at the start of the reaction the filled square wells having a volume of 8 nL contained $^2/_8$(2 mM NADH and 8 mM PEP) = 0.5 mM NADH and 2 mM PEP, assuming homogeneous mixing of the compounds.

To determine the enolase calibration curve, a solution containing 2 mM NADH and 4 mM 2-PG was sprayed into five wells of a single column. In each well, 2 nL of substrate solution was deposited. Prior to measurements, arrays of wells were filled with solutions containing 0.1 and 0.5 unit/mL enolase, 5 units/mL LDH, 5 units/mL PK, 2 mM ADP, 44 mM KCl, and 11 mM $MgSO_4$ in 0.1 M TEA buffer. So, at the start of the reaction, the filled square wells (8 nL) contained 0.5 mM NADH and 1 mM 2-PG.

Preservation of Enzyme Activities after Spraying and Storage. Solutions of 5 units/mL LDH and 10 units/mL PK were sprayed in a solution of a 0.05 M TEA containing 0.5−2% w/v Brij 35 and 0.5−20% w/v trehalose.

Just before measurements, solutions containing 2 mM pyruvate and 0.5 mM NADH in 0.1 M TEA buffer were added to arrays of LDH-containing wells using the coverslip method. To arrays of PK-containing wells, solutions were added containing 2 mM PEP, 0.5 mM NADH, 2 mM ADP, 44 mM KCl, 11 mM $MgSO_4$, and 12.5 units/mL LDH in 0.1 M TEA buffer.

Control Experiments. The defined spraying and defined sample solutions that were prepared for on-chip measurements were first assayed on a photospectrometer in volumes of 200 μL at a temperature of 25 ± 1 °C. The solutions that gave correct progress curves (appropriate nominal ratios) were also used on the same day to prefill wells by means of spraying and to fill wells using the coverslip method. To differentiate between loss of enzyme activity caused by spraying or caused by contact with the silicon nitride or Pyrex surfaces, progress curves were obtained from nanoliter wells that were filled with premixed assay using the coverslip method. These assays were made by pipetting 50 μL of a defined spraying solution and 50 μL of a defined enzyme solution in a vial followed by mixing on a vortex mixer for 5 s.

Prefilling of Nanoliter Wells by Means of Electrospraying. A syringe was filled with a fresh enzyme solution that had been stored for no longer than 1 h at 4 °C. The syringe was refilled every 15 min. After alignment, electrospraying was started at the topside of a chip to obtain a stable Taylor cone followed by spraying into the third column of wells or into the second and fourth columns of wells. Reagent dots were deposited by spraying for 1 up to 10 s into each well, depending on the reagent concentrations required. Rows of dots were sprayed in a computer-controlled manner by moving the table 150 μm downward (spraying stops), 800 μm sideways, and 150 μm upward (spraying resumes) in a total time period of 0.25 s. We sprayed at flow rates of 160 and 200 pL·s^{-1} at spray distances ranging from 250 to 400 μm.

Coverslip Method. The principle of filling (sub)nanoliter wells (containing presprayed reagent dots) with a "sample" liquid while

avoiding carryover is to fill and seal the wells that rapidly at a speed of 400 μm/0.03−0.02 s that all dissolved reagent material is trapped in the wells. As is shown schematically in Figure 1B, this is achieved by using a cleaned (brushed with Brij 35) and smooth coverslip allowing a rapid horizontal movement over the array of wells, thereby pushing the pipetted liquid forward across the wells, resulting in wetting of the wells and subsequent removal of the air from the wells with the liquid flow. The filled wells are immediately sliced off and sealed off by the coverslip, thus avoiding carryover and evaporation for a long period of time. To prevent liquid being pulled between coverslip and chip by means of capillary action, pressure is applied onto the coverslip prior to and during movement of the coverslip. The ultrathin layer of liquid that remains between coverslip and chip after sealing allows long-term "bonding" of chip and cover by powerful adhesive forces.

Prior to pipetting, a prefilled chip was fixed in a laboratory-made PMMA holder that was placed in a fixed position underneath a zoom microscope to inspect the wells and the chip surface and to verify appropriate filling of the wells prior to detection. The 0.5−1.0 μL of aqueous solution was deposited ∼1 mm in front of the first column of wells using a flexible pipet tip (OD of 0.68 mm) from Bio-Rad (Catalog No. 223-9915). A Pyrex coverslip was placed on top of the chip, ∼1 mm in front of the liquid line. A pressure of 1−3 kg/cm^2 (measured by placing the PMMA holder onto a mass balance) was applied by pressing the index finger (pressure area ∼1 cm^2) onto the front half of the coverslip while the coverslip was pushed with the thumb in a horizontal direction toward the liquid line and across the wells at a speed of 1−3 cm/s, which we verified by recording the sliding of the coverslip with a Sony digital video camera followed by playback of each frame of 0.04 s. Immediately after filling, the vertical pressure was increased to 4−7 kg/cm^2 while excess liquid was removed using pressurized air (4−5 bar). After 5−20 s, the pressure was released resulting in a "bonded" chip and cover.

Carryover. Carryover of sprayed LDH and PK to neighboring wells that were not prefilled with enzyme solution ("empty") was determined by measuring NADH fluorescence with time in both the prefilled and the wells containing only the test solution. If carryover of sprayed enzyme to neighboring wells occurs, NADH is also converted into NAD^+ in these wells, resulting in false positive progress curves. The slope of this progress curve is a measure for the amount of enzyme that has been carried over.

Detection. *Fluorescence Measurements.* The PMMA holder containing a filled chip was fixed at a defined position onto an *x*−*y*−*z* stage allowing precise and repeatable alignment underneath the objective of a Zeiss Axioscope microscope that was coupled to a Princeton Versarray 512B back-illuminated CCD camera with a Tektronic 512 × 512 DB chip, as reported previously.[15,16] Prior to monitoring of the reactions, the system was calibrated by measuring NADH solutions in the range of 0−0.5 mM. Enzyme-based progress curves were obtained by measuring NADH fluorescence ($\lambda_{exc.}$ = 340 nm, $\lambda_{em.}$ = 465 nm) from 25 wells in a sequential manner (cycle time of 30 s) for a total period of 240−500 s. With each fluorescence measurement, a fluorescent image was recorded from a single well to verify the presence of small air bubbles.

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RESULTS

Prefilling of Nanoliter Wells by Means of Electrospraying. *Spraying Conditions.* To preserve enzyme activities during spraying and storage, we sprayed enzyme solutions containing 0.5−20% w/v trehalose at room temperature at flow rates of 160 and 200 pL·s^{-1}. During spraying of solutions containing 20% w/v trehalose, clogging of the capillary, and crystallization of trehalose on the outside of the capillary were completely prevented because the electric field induced a severe convection in the liquid cone. As shown in Figure 1A, we achieved excellent spraying of the reagent cocktails in the center of the wells without any indications of splashing. By spraying for 1−3 s into a single well at spraying distances of 250 and 400 μm, uniform dots were obtained of 250 and 350 μm in diameter, respectively. Spraying for 1 s or less into each well is preferred because rapid evaporation results in "dry" dots already within 1 s after electrospraying, which is favorable because it increases the enzyme stability. Moreover, the total time required to prefill an array of 25 wells is reduced to 25 s. By applying the correct voltage difference between capillary tip and wells relative to the spray distance, the liquid cone is formed instantly resulting in reproducible spraying with a deviation of 2−3%.

Coverslip Method. Determining quantitative biochemical reactions requires the following: (i) complete filling of an array of identical wells to obtain identical liquid volumes of 8 (square wells) or 6.3 nL (round wells); (ii) prevention of carryover of sprayed reagents to neighboring wells during filling and after sealing, especially for applications where wells are prefilled with different reagents at various concentrations. Therefore, optimal filling conditions were evaluated using Pyrex coverslips.

Optimal Filling Conditions. Condensation onto the surface and into the wells of a prefilled chip was prevented in most cases by storing the prefilled chips in aluminum boxes. In addition, the chips can be wrapped in Parafilm to eliminate condensation.

Arrays of wells were successfully filled by pipetting an amount of 0.7−0.8 μL of liquid in the form of a line approximately 0.6 cm long and 1.3−1.6 mm wide. Pipetting of 0.5−0.6 μL of liquid resulted in entrapment of small air bubbles whereas pipetting of 1.0 μL resulted in a thicker line, meaning that the time between liquid entering the well and sealing by the coverslip increased, which resulted in an increase of carryover by ∼5%.

By applying a pressure of 3 kg/cm^2 onto the coverslip, capillary action was prevented adequately. The vertical pressure should not exceed 3 kg/cm^2 because then the cover and the chip stick together too much, resulting in an uncontrolled sliding movement that results in loss of contact between chip and cover. This should be avoided at all times because from the moment contact is lost, carryover occurs and most of the wells are filled with air bubbles because the liquid is merely pulled over the wells (discontinuous dewetting[3]).

After filling of an array, a powerful bonding of cover and chip was achieved by applying a vertical pressure of 7−8 kg/cm^2 onto the cover for 10 s resulting in an array of "microreactors" containing identical amounts of liquid that remained filled for days and some for weeks by using cleaned coverslips and cleaned chips. By applying these optimal filling and sealing conditions, we achieved complete filling of 25 wells while reducing carryover to less than 3%.

Enzyme Calibration Curves. When measuring enolase and PK activities from a sample solution in nanoliter wells, the reaction rates of enolase and PK depend on the concentrations of *active* enzymes, cofactor (NADH), and substrates (PEP and 2-PG) in nanoliter wells. The activities of these reagents may be influenced by the spraying procedure, coverslip method, and silicon nitride and Pyrex materials. Therefore, calibration curves of enolase and PK were made. An array of 25 wells, of which only the wells of the middle column were prefilled with reagents, was filled with a defined solution containing the enzymes followed by 20 s of pressing and 15−20-s transfer time before the PK- and enolase-based reactions were monitored by measuring NADH fluorescence eight times in each well (8 cycles) over a total period of 240 s. Figure 2A represents 25 fluorescence images recorded from 25 wells of an array at the start of the reactions during the first cycle. Only in the wells of the third column are fluorescence signals obtained because only in these wells do the enzyme reactions occur. In the middle column of Figure 2B, three progress curves are shown obtained from wells of the third column of a chip representing the conversion of 0.5 mM NADH into NAD^+ with time that corresponds to 0.5 mM 2-PG converted by 0.5 unit/mL enolase, according to reaction schemes 1−3. The graphs presented in columns 1 and 3 of Figure 2B (columns 2 and 4 of a chip) represent background signals (3000 ± 200 AU) caused by silicon nitride and Pyrex (1100 AU) and are caused by the defined sample solution (1900 AU). In contrast to quenching of NADH occurring in wells on 96-well microtiter plates, we obtained straight initial slopes from 50-μm-deep wells indicating that there was no quenching of NADH molecules in these shallow wells. Moreover, this indicated that the enzyme molecules were very well mixed with the dissolved reagents, which is another advantage of shallow wells.

In Table 1, the corresponding initial reaction rates (picomole of converted substrate per minute per well) for known amounts of PK and enolase (μunits/well) are listed. Each reaction rate is an average obtained from four to five progress curves measured from five wells (middle column) on a single chip. Standard deviations are ∼10% as a result of carryover (0−3%), pipetting errors, and spraying errors (2−3% deviation). In Table 1, the initial reaction rates (y), given in picomoles of converted substrate per minute per well, of PK and enolase are listed with the corresponding enzyme concentrations (x) given in microunits per well. In addition, nominal ratios are listed for on-chip measurements as well as for microtiter plate measurements. The nominal ratios for PK and enolase obtained from on-chip measurements are approximately 50 and 25% lower than the nominal ratios obtained from microtiter plate measurements. Premixing experiments on-chip (controls) also resulted in 50 and 25% lower nominal ratios. So, the lower reaction rates were caused by a reduced activity of the enzymes PK and enolase (adsorption of PK and enolase on the Pyrex glass or silicon nitride) instead of being caused by a reduced activity of the NADH, PEP, or 2-PG as a result of the spraying or freezing procedure. The 25% activity loss of enolase on-chip was probably also affected by the activity loss of PK on-chip since PK serves as an auxiliary enzyme (reaction 2). Nominal ratios of LDH obtained from premixing and prefilling experiments on-chip were identical to those obtained from microtiter plate

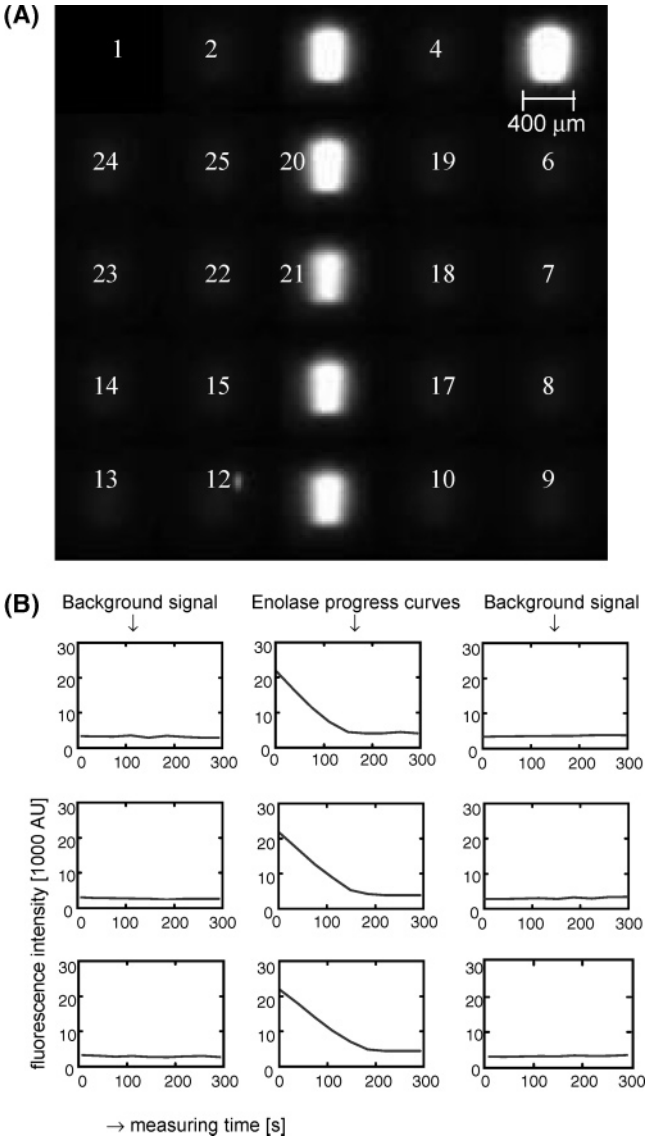

Figure 2. Enolase-based reactions performed in nanoliter wells on-chip. (A) The five wells of the middle column were prefilled with enolase-based reagents, and a defined solution containing NADH, LDH, PK, and enolase was added to all 25 wells using the Zamboney method. So, only in these wells isNADH fluorescence visible while in the other wells background fluorescence is negligible. These 25 fluorescence images were taken at the initial sequence of measurements, ∼20 s after filling. The order of numbering corresponds to the sequence of measurement. (B) Three characteristic enolase (0.5 unit/mL) progress curves obtained from the wells (prefilled with reagents) from the middle column by measuring the decrease in NADH fluorescence with time in a sequential manner. These three progress curves represent the conversion of 0.5 mM NADH into NAD^+ with time, which corresponds to 0.5 mM 2-PG converted by 0.5 unit/mL enolase, according to reaction schemes 1−3. The graphs from the left and right columns show background fluorescence signals (3000 ± 200 AU units) obtained from the wells that were not prefilled with reagents but only contain the defined enzyme solutions. The nine graphs shown here consist of eight fluorescence measurements each with 30 s of interval between two measurements.

control experiments ($y = 0.65x$, $R^2 = 0.99$), so, LDH activity was not inhibited by the Pyrex or silicon nitride.

Long-Term Storage of Sprayed Enzymes. A future picoliter dosing platform should provide a way to prefill many chips with various enzyme and reagent solutions that remain stable on-chip during

Table 1. Reaction Rates and Nominal Ratios Obtained from Progress Curves of Enolase and Pyruvate Kinase in Nanoliter Wells[a]

PK (μunit/well)	reaction rate (pmol min^{-1} per well)	enolase (μunit/well)	reaction rate (pmol min^{-1} per well)
0.40	0.11 ± 0.01	0.80	0.17 ± 0.02
0.40	0.13 ± 0.02	0.80	0.19 ± 0.02
0.80	0.25 ± 0.02	3.15[b]	0.94 ± 0.08
1.60	0.53 ± 0.07	4.00	0.92 ± 0.10
$y = 0.30x$ ($R^2 = 0.99$)[c]		$y = 0.233x$ ($R^2 = 0.99$)[c]	
PK control (microtiter) $y = 0.60x$ ($R^2 = 0.99$)[c]		enolase control (microtiter) $y = 0.30x$ ($R^2 = 0.99$)[c]	

[a] Reaction rates (picomoles of converted substrate per minute per well) and corresponding enzyme concentrations (microunits per well). [b] Experiment performed in round wells, which contain 0.79 × 4.00 μunits = 3.15 μunits. [c] Nominal rates: y represents the reaction rate in picomoles of converted substrate per miute per well, and x represents the corresponding enzyme concentration in microunits per well.

Table 2. Relative Activities of LDH and PK as a Function of Trehalose Concentration, Spraying Conditions, and Storage Temperature[a]

storage temp, °C	trehalos, % w/v	spray distance, μm	voltage kV	enzyme activity, % relative[b]
LDH Stored for 2 Days				
−80	0.5[c]	400	1.27	0
−80	1	400	1.27	0
−80	4[d]	400	1.27	20
−80	10	400	1.26	95
−80	20	400	1.26	95
LDH Stored for 2 Months				
−80	10	400	1.26	90[e]
−80	20	400	1.26	95[e]
PK stored for 4 days				
+4	10	300	1.20	60[e]
−80	10	300	1.19	50[e]
−20	20	250	0.97	85
PK Stored for 1.5 Months				
−20	20	300	1.00	100
−20	20	250	1.04	40[e]
−20	20	250	1.12	0
−20	20	250	1.14	0

[a] The LDH and PK solutions contained 1% Brij 35 and 5 units of LDH/mL and 10 units of PK/mL, respectively. [b] Percentage of enzyme activity relative to the nominal ratios obtained from premixing experiments. [c] The solution contained 0.5% w/v Brij 35. [d] The solution contained 2% w/v Brij 35. [e] Duplicate measurements (2 chips).

long-term storage. Therefore, we assessed optimal solution compositions and optimal spraying and storage conditions to optimally preserve the activities of the model enzymes LDH and PK. Solutions containing 5 units/mL LDH and 10 units/mL PK were sprayed into wells of columns 2 and 4 resulting in enzyme concentrations of 0.25 (column 2) and 1 unit/mL (column 4) prior to monitoring. The spraying and storage conditions and related relative enzyme activities, given in percentages of the nominal ratios obtained from premixing experiments, are listed in Table 2. In our previous study,[5] we showed that the activities of LDH and PK were not reduced by the spraying procedure alone when enzyme solutions containing 0.5 and 1 wt % trehalose were sprayed into aluminum microliter wells. However, as is shown in Table 2, after spraying of LDH solutions containing 1 wt % trehalose onto silicon nitride followed by additional freezing for 2 days, LDH had lost its activity completely, probably due to freeze denaturation. Surprisingly, addition of 10 or 20% w/v trehalose to 5 units of LDH/mL resulted in >90% preservation of the activity of LDH after spraying at room temperature followed by storage at −80 °C for 2 months. This means that trehalose is a very powerful preservative that stabilizes LDH on silicon nitride during air-drying, freezing, and defrosting.

Comparing the stability results obtained from PK as listed in Table 2, it appears that the activity of PK reduces drastically at relatively high electric fields, which is in agreement with our previous results.[5] So, spraying of labile enzymes should occur at normal electric fields to impose not too much stress onto these enzymes. In addition, a higher concentration of Brij 35, e.g., 2% w/v, may reduce this stress. To fully stabilize PK at −20 °C, 20% w/v trehalose should be added to a 10 units/mL solution of PK. Moreover, PK preferably is stored at +4 or −20 °C instead of at −80 °C, which resulted in an extra 10% loss of activity, most likely caused by freeze denaturation. These results suggest that by storing sprayed LDH at +4 or −20 °C instead of at −80 °C, the LDH activity is most likely 100% preserved.

DISCUSSION

Our complete methodology allows quantitative analysis of biochemical reactions in identical nanoliter wells without the need to immobilize enzymes or other reagents. Already upon filling of the prefilled wells with a test solution, the sprayed reagents dissolve and mix rapidly with the test compounds by means of diffusion, resulting in homogeneous reaction mixtures that are similar to reaction mixtures obtained on microtiter plates.

In contrast to other techniques such as piezoelectric dispensing that suffer from clogging, our spraying technique allows prefilling of nanoliter wells with solutions containing up to 20% w/v trehalose, which is required to stabilize relatively labile enzymes such as LDH and PK on-chip for months. As a result, the chips do not need to be used for quantitative measurements directly but can be shipped to other analytical laboratories where they can be stored for a few months and still be used for quantitative biochemical analysis in a reproducible manner. For high-throughput applications, dotting preferably is performed by placing capillaries in rows or arrays at distances of 600−800 μm.[17] In addition, the speed of spraying can be increased by using high-speed actuators and by decreasing the spraytime to 0.5 s or less.

It is most likely that enzymes other than LDH and PK might be successfully stored on-chip in solid state at a temperature of −20 °C, since stability studies have shown that PFK,[14] glucose-6-phosphate dehydrogenase,[18] L-asparaginase,[19] and catalase[20] remained stable during freeze-drying by using additives such as trehalose, sucrose, maltose, lactose in combination with borate, Zn^{2+}, Co^{2+}, Cu^{2+}, or Ni^{2+}. In addition, effective protectants such as hydroxypropyl-β-cyclodextrin, 3-[(3-cholamidopropyl)dimethyl-

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ammonio]-1-propanesulfonate (CHAPS), and sucrose fatty acid monoester proved to stabilize LDH upon freeze-drying and freeze-thawing.[12]

Future automation of the Zamboney method just requires a powerful actuator to move the coverslip in horizontal direction across a chip while a second actuator presses the coverslip onto the chip. In contrast to the chimney method, the Zamboney method is also suitable to fill arrays of wells with cell suspensions and cell-free extracts because the cells are pushed forward across the wells and chip surface instead of being trapped between the coverslip and chip surface. In addition, the Zamboney method is suitable for filling wells with viscous liquids such as glycerol or volatile liquids such as ethanol. A coverslip preferably is thicker than 0.2 mm and can be a few centimeters wide to fill several arrays at a time by pipetting several lines of one or more sample liquids. In addition to glass coverslips, PMMA coverslips can be used as long as the surface is reasonably hydrophilic, rigid enough to withstand the vertical pressure, and as smooth as possible for long-term sealing. The silicon chips, which are coated with silicon nitride, can be reused up to 30−40 times before the silicon nitride layer gets damaged. In addition to silicon nitride-coated chips, polymer-based chip material can be used as long as it is hydrophilic and rigid enough to achieve successful filling of wells followed by adequate bonding of coverslip and chip. A properly filled and covered chip can be monitored with commercially available microscopes or array scanners. In addition, (fast) kinetics can be monitored by integrating a photodiode array into a coverslip or chip.

Preliminary results have shown that yeast cells grow and multiply in these silicon nitride wells after the cell suspensions are "pushed" into the wells with a Pyrex coverslip. In addition, preliminary results have shown that peroxide-based reactions (using an Amplex Red kit[6]) can be successfully monitored in these wells.

CONCLUSIONS

A straightforward platform is presented here for the performance of quantitative analyses of (bio)chemical reactions in nanoliter wells based on two independent methods: (i) reproducible spraying of (sub)nanoliter amounts of (bio)chemicals into arrays of 50-μm-deep nanoliter wells on-chip followed by storage; (ii) rapid filling of arrays of wells using a coverslip followed by powerful well sealing to completely prevent evaporation. Promising stability results were obtained after spraying of solutions containing LDH and PK, nonionic surfactant in 10−20% w/v trehalose creating the possibility to preserve activities of a wide variety of proteins in nanoliter wells for at least 1.5−2 months.

Arrays of prefilled wells can be very easily filled by using our coverslip method with a wide variety of solutions, and the wells can be sealed for minutes up to days which opens up interesting possibilities in the fields of combinatorial chemistry, small-scale PCR, and metabolomics (single cell) among others.

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