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Nanoarrays: A Method for Performing Enzymatic Assays

Heidelinde R. C. Dietrich,*,† Johann Knoll,‡ Lennert R. van den Doel,† Gijs W. K. van Dedem,‡ Pascale A. S. Daran-Lapujade,‡ Lucas J. van Vliet,† Rob Moerman,‡ Jack T. Pronk,‡ and Ian T. Young†

Department of Imaging Science & Technology, Faculty of Applied Science, Delft University of Technology, Lorentzweg 1, 2628 CJ Delft, The Netherlands, and Department of Biotechnology, Faculty of Applied Science, Delft University of Technology, Julianalaan 67, 2628 BC Delft, The Netherlands

Conventional enzymatic assays for alcohol dehydrogenase, pyruvate kinase, and enolase performed in 96-well microtiter plates were compared with assays monitored in 25-well nanoarrays. All miniaturized reactions could be performed in maximum volumes of 6.3−8 nL and were read out with a conventional fluorescence microscope system equipped with a scientific grade CCD camera. Substrate and cofactor were already present inside the wells after having been presprayed, or they were applied in solution to the wells of the nanoarray shortly before the assays started. For all of the assays, commercially available enzymes and enzymes present in cell-free extracts were used. Assays carried out in premixed nanoarrays gave results comparable to those performed in presprayed nanoarrays. Enzyme activities determined in nanoarrays by using two different methods were in good agreement with assays performed in microtiter plates. Also, good correspondence was found between expected and observed enzyme levels. In short, enzymatic assays performed in premixed and in particular in presprayed nanoarrays are a promising low-volume and low-reagent- and sample-consuming alternative to current methodology and could find applications in many different areas of analytical chemistry.

In recent years, the miniaturization[1,2] of bioanalytical assays and the development of new intelligent analysis systems have become a new scientific discipline in areas such as biotechnology, combinatorial chemistry, functional genomics, drug discovery, and medical diagnostics. This trend nicely fits the move toward automated high-throughput screening (HTS) and ultra-high-throughput screening due to the need for reducing development and operating costs.[3] In drug discovery screening technology, groups are already moving from 96-well microtiter plate formats to 384-well plates and higher density plate formats with even smaller sample volumes. We have observed two approaches toward further assay miniaturization: first, the reduction of sample volume and the increase of well densities, and second, the fabrication of devices suitable for continuous-flow assays. Miniaturization means a reduction in the amounts of biological and chemical reagents and samples and in a shortened throughput time due to massive parallelization. Miniaturization requires not only tuning or redesigning of already existing assays but also the development of new special techniques for an easier handling of downscaled systems. The concept of automation, miniaturization, and high-throughput screening requires the development of (among other things) very sensitive homogeneous assay formats. Therefore, the application of fluorescence-based detection techniques is becoming more widely used. The advantage of fluorescence techniques lies in the fact that the measurement sensitivity can be improved by increasing the illumination intensity.[4] In this paper, fluorescence intensity is used as a quantitation tool. The aim of our research program has been to develop a miniaturized intelligent molecular diagnostic system capable of measuring different metabolic compounds simultaneously, in a very short time, using minimal amounts of reagents and sample. Such a laboratory-on-a-chip system can serve as an aid in decision-making processes in professional and technical environments. Our first focus lay in the field of bioprocess technology. Understanding, controlling, and improving fermentation processes requires rapid and sensitive analysis of intracellular compounds, such as enzymes. As a result of these analyses, fermentation processes can be further optimized and strains improved by metabolic engineering. The method, described in this article, allows the performance of dynamic measurements of enzymatic reactions in arrays of wells whose individual capacity is on the order of 1 nL. We believe that it can serve as a useful method in metabolomics and in the search for new enzymes. The chip enzymatic assays, presented in this paper, belong to the class of assay protocols in which a nondetectable reagent is converted into a detectable one.[5] We have previously[6,7] reported a new technique for the deposition of pico- and nanoliter volumes of reagent solutions by means of an electrospray dispensing system. This technique allows for the

* Corresponding author. Phone: +31-15 27 83727. Fax: +31-15 27 86740. E-mail: Heidi@ph.tn.tudelft.nl.

† Department of Imaging Science & Technology.

‡ Department of Biotechnology.

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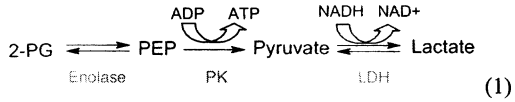
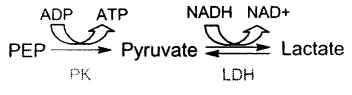
deposition of various reagents in well-defined dry spots onto a solid support. It is also possible to spray different substances in different wells on one and the same chip for parallel analysis. We were interested in depositing components required for enzymatic reactions in wells of nanoarray chips. Thus, it becomes possible to develop chips suitable for rapid analysis of certain analytes, used for instance in a medical laboratory, for examining various body fluids. Therefore, substrates, coenzymes, activator metals, and other essential reagents have to be sprayed into the wells of the nanoarray and immediately frozen to guarantee the stability of the various ingredients.

A single, small sample can be distributed across the wells, and the reactions corresponding to the presprayed reagents in each well will start. To reach this goal, different enzymatic assays were carried out in three formats: First, we miniaturized existing assays performed in cuvettes to the microliter level for use in microtiter plates, and second, we further adapted these assays for application in nanoarrays. The third step was the deposition of components necessary for certain enzymatic reactions into the wells of the nanoarray using the electrospraying method described in the Experimental Section. These chips were then filled with an enzyme solution for the determination of the enzyme activity. In this paper, we report the tuning of a number of enzymatic assays to the nanoliter scale and subsequent comparison with measurements done in microliter volumes using commercially available enzymes and enzymes derived from cell-free yeast extracts. Moreover, the influence of different solution compositions on enolase and pyruvate kinase assays was tested.

Methods for the assay of enolase, pyruvate kinase (PK), and alcohol dehydrogenase (ADH), which are involved in anaerobic metabolism, were tested. The determination of the enzyme activity was done by monitoring the production or consumption of NAD(P)H. Since this nucleotide is only at one stage directly involved in glycolysis, enolase and pyruvate kinase were measured by coupling them with at least one other enzyme, like lactate dehydrogenase (LDH) according to the following scheme:

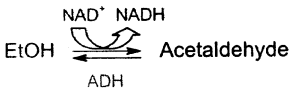


(1)



(2)

The assay for alcohol dehydrogenase was performed according to the following equation:



(3)

Enolase, pyruvate kinase, and alcohol dehydrogenase were acquired either from commercial sources or in the form of a cell-free extract[8] (CFE) that we derived from *Saccharomyces cerevisiae*. In this paper, we describe the monitoring of the activity of selected enzymes in nanoliter volumes by means of nanoarrays. Results, obtained by using two different methods, are compared with those obtained in the more conventional 96-well microtiter plate assays.

EXPERIMENTAL SECTION

Reagents and Materials. Magnesium sulfate, sodium hydroxide, hydrochloric acid (36−38%), and ethanol (absolute) were obtained from J. T.Baker (Deventer, The Netherlands). Calcium chloride, sulfuric acid, and glycine were purchased from Merck (VWR International B.V., Amsterdam, The Netherlands). Triethanolamine hydrochloride, D-(+)-trehalose dihydrate, Brij 35 solution (30% w/v), and hydrogen peroxide were bought from Sigma-Aldrich Chemie (Zwijndrecht, The Netherlands). Alcohol dehydrogenase (from yeast, EC 1.1.1.1), pyruvate kinase (from rabbit muscle, EC 2.7.1.40), L-lactate dehydrogenase (from beef heart, EC 1.1.1.27), bovine serum albumin (BSA, fraction V), and phosphoenol pyruvate (PEP) were obtained from Roche Diagnostics Nederland B.V. (Almere, The Netherlands). β-NADH, fructose 1,6-biphosphate, 2-phosphoglycerate (2-PG), ADP, and enolase (from baker's yeast, EC 4.2.1.11) were purchased from Sigma-Aldrich Chemie (Zwijndrecht, The Netherlands). β-NAD^+ was obtained from Fluka (Zwijndrecht, The Netherlands). P.A.L.D.-L. kindly provided CFE (Department of Biotechnology). MilliQ-purified water was used in all cases.

All concentration specifications mentioned in this paper are final concentrations and not initial concentrations. Micro titer plate assays were performed in 96-well microtiter plates with an HTS 7000 Plus Bio Assay Reader (Perkin-Elmer, Norwalk CT). Assays carried out on nanoarrays were monitored with a modified conventional microscope system (Zeiss Axioskop with Fluar 5×/0.75 objective) equipped with a back-illuminated CCD camera (Princeton Versarray 512B).[9,10] An electrospray device developed in our research program for dispensing reagents into wells in volumes ranging from 60 pL to 500 nL has been described in previous publications.[6,7,11]

All assays were performed at 25 °C. The HTS 7000 Plus Bio Assay Reader is equipped with an integrated cooling and heating system. The temperature for nanoarray experiments was controlled by a PE120 Peltier heating and freezing stage (Linkam). The catalytic activity of all enzymes was determined by the increase or decrease of NADH, by taking the initial slope of the progress curve. An excitation wavelength of 360 nm and an emission wavelength of 465 nm apply for all measurements. For each set of reactions, NADH calibration curves were made to allow quantification of enzymatic activities.

Microtiter Plate Assays. *Pyruvate Kinase.* The reaction mixture contained 0.05 M TEA-HCL buffer (pH 7.5), 2 mM PEP, 0.5 mM β-NADH, D-(+)-trehalose dihydrate (1 w/v %), Brij 35 (0.25 w/v %), 11 mM $MgSO_4$, 44 mM KCl, 0.5 mM fructose 1,6-biphosphate, 2 mM ADP, and 12.5 units/mL LDH. The reaction was started with 50 μL of PK (end concentrations: 0.14, 0.1, 0.06 unit/mL) or with 5, 10, and 15 μL of CFE.

Enolase (ENO). The assay solution contained 0.05 M TEA-HCl buffer (pH 7.5), 1 mM 2-PG, 0.5 mM β-NADH, D-(+)-trehalose dihydrate (1 w/v %), Brij 35 (0.25 w/v %), 5 units/mL LDH, 5 units/mL PK, 1 mM ADP, and 2 mM $MgSO_4$/KCl. The reaction

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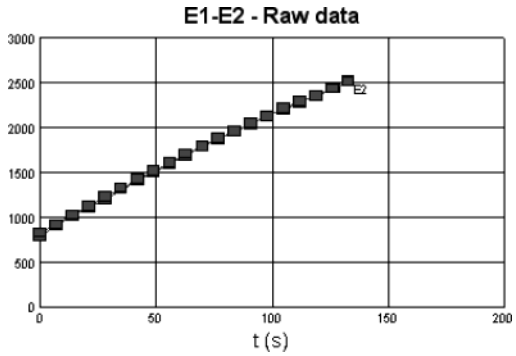

Figure 1. Typical progress curve of ADH (0.14 unit/mL) performed in a 96-well microtiter plate.

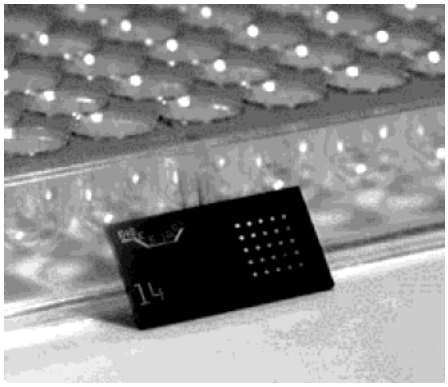

Figure 2. A 2 × 1 cm^2 nanoarray with two nanoarrays of 5 × 5 wells lying against a commercially available 12.78 × 8.55 cm^2 microtiter plate with 96 wells.

was activated with 50 μL of ENO (end concentrations: 0.14, 0.1, 0.06 unit/mL) or with 5, 10, and 15 μL of CFE.

Alcohol Dehydrogenase. The reaction solution contained 0.05 M glycine−KOH (pH 9.0), 0.5 mM β-NAD$^+$, BSA (1 w/v %), Brij 35 (0.25 w/v %), and 0.8 mM ethanol (absolute). The assay was initialized by adding 50 μL of ADH (final concentration: 0.14, 0.1, 0.06, or 0.075 unit/mL) or 5, 10, and 15 μL of CFE.

Figure 1 depicts a typical progress curve of an enzyme reaction using a 96-well microtiter plate.

Nanoarray Assays. The wells of the nanoarray were either empty or already contained the assay reagents in dry form. This means that two different procedures ("modes") were applied. The premixed mode implied the mixing of commercially available enzymes or enzymes from cell-free extracts with all compounds necessary for the assay and the subsequent measurement of the reaction. In the second mode, called the presprayed mode, substrates and cofactor substances were sprayed into the wells of the nanoarray. The enzyme to be determined and any other essential assay components were then mixed together, the nanoarrays were filled with this solution, and reading was initialized immediately. The nanoarrays (Figure 2) were etched in silicon and consisted of a matrix of 5 × 5 wells with volumes of 6.3 (circular wells) and 8 nL (square wells). The chips were coated with silicon nitride. Each well had a size of 400 × 400 μm^2 (square wells) or a diameter of 400 μm (round wells), respectively, and a depth of 50 μm. The composition of the solutions used is already described under Microtiter Plate Assays. In the first method ("premixed mode"), 6 μL of the reaction solution was taken and applied onto the nanoarray. The wells were closed with a 2-mm-thick Pyrex coverslip to prevent evaporation. The closing of the wells was performed in a sliding motion, the coverslip was tightly pressed onto the nanoarray, and the redundant liquid was blown dry by a stream of air. In this way, the coverslip was held in place by van der Waals forces.[12,13] Readout of the nanoarray started ~60 s after initialization of the reaction. One single scan included five wells and took 6−7 s. The exposure and integration time per well was 0.1 s.

The presprayed mode used "presprayed" nanoarrays. The appropriate reagents, such as PEP, 2-PG, β-NADH, and β-NAD$^+$, were sprayed into the wells at a flow rate of 0.7 μL/h and a potential of 1.10 kV using our electrospray microdispensing method described earlier. The presprayed nanoarrays were immediately frozen at −24 °C. The reaction was initialized by depositing 6 μL of enzyme solution, including any other assay components, onto the chip surface and filling the wells. The wells were closed, and the readout was performed as mentioned above.

For comparison of the kinetics of enolase and pyruvate kinase for different solution compositions, only presprayed nanoarrays were used. The same measurement procedure was applied as described under nanoarray assays.

Figure 3 shows a set of characteristic progression curves of an enzymatic reaction monitored in a 25-well nanoarray.

Enolase. The first solution was made as follows: 0.05 M TEA-HCl buffer (pH 7.5), 1 mM 2-PG, 0.5 mM β-NADH, D-(+)-trehalose dihydrate (1 w/v %), and Brij 35 (0.25 w/v %) were sprayed into the wells. For the activation solution, 5 units/mL LDH, 5 units/mL PK, 1 mM ADP, 2 mM $MgSO_4$/KCl ,and enolase (0.14, 0.1, 0.06 unit/mL) were mixed together. The reaction was activated by filling the nanoarray with 6 μL of this mixture. The second solution additionally contained 5 units/mL LDH and 5 units/mL PK. The activation solution now contained 1 mM ADP, 2 mM $MgSO_4$/KCl, enolase (0.14, 0.1, 0.06 unit/mL), and 0.05 M TRIS-HCl buffer (pH 7.4). Again the reaction was activated by adding 6 μL of this solution. The third solution contained all ingredients except for enolase (0.14, 0.1, 0.06 unit/mL) and 0.05 M TRIS-HCl buffer (pH 7.4).

Pyruvate Kinase. The first solution contained 0.05 M TEA-HCl buffer (pH 7.5), 2 mM PEP, 0.5 mM β-NADH, D-(+)-trehalose dihydrate (1 w/v %), and Brij 35 (0.25 w/v %), which were sprayed into the wells. Then 1 mM $MgSO_4$, 44 mM KCl, 0.5 mM fructose 1,6-biphosphate, 2 mM ADP, and 12.5 units/mL LDH, and PK (0.14, 0.1, 0.06 unit/mL) were mixed together. The reaction was started by adding 6 μL of activation solution. In the second solution, 12.5 units/mL LDH was additionally sprayed. The activation solution contained 1 mM $MgSO_4$, 44 mM KCl, 0.5 mM fructose 1,6-biphosphate, 2 mM ADP, 0.05 M TRIS-HCl buffer (pH 7.4), and PK (0.14, 0.1, 0.06 unit/mL). For the third solution, all components necessary for the reaction were sprayed into the wells of the nanoarray except for pyruvate kinase. A 6-μL sample of the solution composed of 0.05 M TRIS-HCl buffer (pH 7.4) and PK (0.14, 0.1, 0.06 unit/mL) was taken to start the reaction.

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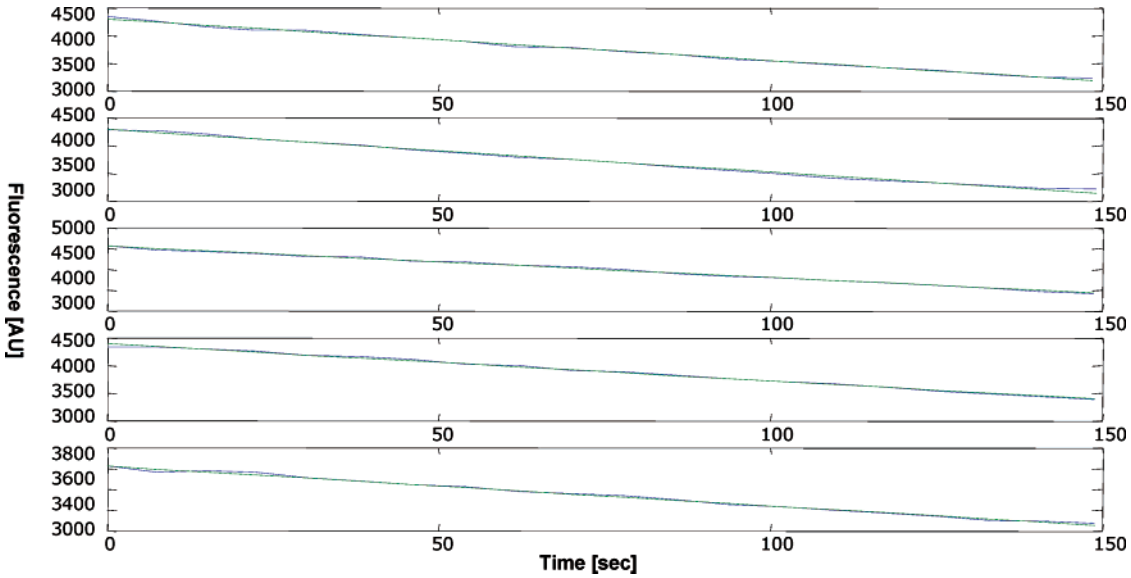

Figure 3. Typical progress curve of PK (0.14 unit/mL) performed in a column of a 25-well nanoarray. The blue line represents the actual measurement line. The green line shows the model line of pseudo-first-order kinetics.

RESULTS AND DISCUSSION

The screening and detection of intermediates and products in biotechnological processes require fast, high density, and easy to handle assays using minute sample volumes. We have achieved this goal by the design and production of nanoarray chips with 6.3- and 8-nL volumes. For this work, conversion rates for enolase, alcohol dehydrogenase, and pyruvate kinase obtained by using presprayed and premixed nanoarrays were compared with each other but also with rates obtained using microtiter plates. Enzyme activities were determined using commercially available enzymes and CFE. The evaluation of enzyme activities in CFE was done using standard spectrophotometric methods[8,14] using cuvettes. These data gave additional insight into the quality of the enzymatic assays performed in microtiter plates and presprayed and premixed nanoarrays. In this paper, the values referred to as nominal values are the activities indicated on the label of the commercial enzyme preparations. Assays performed in microtiter plates were carried out in duplicate. The obtained NADH fluorescence signals were recalculated using eqs 1 and 2, and the NADH concentrations were displayed as a function of time. The slope obtained was used to calculate the enzyme activity (expressed in μM/(min·mL)), which was compared with activities acquired from premixed and presprayed nanoarrays. Measurements using nanoarrays were performed in quintuplicate and apparent outliers were omitted. The slopes were taken to calculate the conversion rate of each enzyme and, subsequently, the calculation of the mean value and the standard deviation, to determine the confidence interval.

The first difference between measurements performed in microtiter plates and nanoarrays was the shape of the NADH calibration curve. The curve obtained using a microtiter plate showed the form of a shifted hyperbola at NADH concentrations above 150 μM. This phenomenon is probably caused by the longer optical pathway through the liquid in the microtiter plate wells that have a depth of 1067 μm compared to a nanoarray with a depth of 50 μm. The nonlinear response of the NADH fluorescence was fit to the empirical equation

$$\text{fluorescence} = A(1 - \exp(-B[\text{NADH}])) + C \quad (1)$$

Using the estimated parameters *A*, *B*, and *C*, the NADH concentration in the sample wells can be calculated using

$$[\text{NADH}] = -1/B \ln(1 - (\text{fluorescence} - C)/A) \quad (2)$$

These instantaneous NADH values thus calculated were plotted as a function of time, and from the calculated slopes of these (straight) lines, the conversion rates were obtained. The NADH calibration curves using nanoarrays were linear and therefore did not need to be modeled. The slopes of these calibration curves could immediately be used for the determination of each conversion rate.

Alcohol Dehydrogenase. The ADH assay performed in microtiter plates gave an ~1.5 times higher conversion rate compared to the premixed nanoarray (Table 1). Measurements using presprayed nanoarrays gave a 1.7 times lower conversion rate compared to the ones performed in microtiter plates (Table 1).

Table 1. Determination of ADH Activity Comparing Premixed and Presprayed Nanoarrays and Microtiter Plates Using Commercially Available Enzymes

ADH concn (μM/min·mL)	conversion rate (μM/min·mL)		conversion referring to the nominal value (%)	
nominal value	nanoarray	microtiter plate	nano-array	microtiter plate
premixed				
0.075	0.053 ± 0.003	0.075 ± 0.01	70.7	100.0
0.1	0.085 ± 0.003	0.110 ± 0.00	85.0	110.0
0.14	0.096 ± 0.004	0.146 ± 0.01	68.6	104.3
presprayed (nanoarray only)				
0.06	0.050 ± 0.005	0.084 ± 0.004	83.3	140.0
0.1	0.085 ± 0.004	0.141 ± 0.005	85.0	141.0
0.14	0.115 ± 0.005	0.190 ± 0.005	82.1	135.7

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Table 2. Determination of ADH in CFE by Comparing Premixed and Presprayed Nanoarrays and Microtiter Plates

ADH concn (μM/min·mL)	conversion rate (μM/min·mL)		conversion referring to the nominal value (%)	
spectrophotometer nominal stvalue	nanoarray	microtiter plate	nano-array	microtiter plate
premixed				
19.60 ± 0.2	13.40 ± 0.15	15.51 ± 0.84	68.3	79.1
presprayed (nanoarray only)				
8.77 ± 0.06	5.14 ± 0.25	6.93 ± 2.34	58.6	79.5

Table 3. Determination of PK Activity Comparing Premixed and Presprayed Nanoarrays and Microtiter Plates Using Commercially Available Enzymes

PK concn (μM/min·mL)	conversion rate (μM/min·mL)		conversion referring to the nominal value (%)	
nominal value	nanoarray	microtiter plate	nano-array	microtiter plate
premixed				
0.06	0.040 ± 0.006	0.042 ± 0.007	67.0	70.0
0.1	0.085 ± 0.006	0.069 ± 0.007	85.0	69.0
0.14	0.123 ± 0.004	0.115 ± 0.003	87.9	82.1
presprayed (nanoarray only)				
0.06	0.042 ± 0.004	0.066 ± 0.006	70.0	110.0
0.1	0.085 ± 0.010	0.103 ± 0.011	85.0	103.0
0.14	0.117 ± 0.008	0.155 ± 0.001	83.6	110.7

The fact that all essential reagents are sprayed and the enzyme solution is added to these dry substances may hamper the complete dissolution of the presprayed reagents and the desired mixing of presprayed reagent and enzyme solution. This might lead to a significant reduction in conversion. This may be a result of different spot morphologies. One can imagine that a spot which is evenly spread dissolves faster than one which shows a hemispherical morphology. Another negative influence on the result could reside in the possible vibration of the spraying needle as a result of an unstable, oscillating floor in the laboratory.

The determination of ADH in cell-free extract by means of premixed nanoarrays led to a 1.2-fold higher conversion rate compared to that in presprayed nanoarrays (Table 2). The implementation in microtiter plates gave again a higher conversion rate. There was no significant difference between assays using premixed and presprayed nanoarrays. Also, the ADH activity in CFE was comparable to the nominal values obtained by the standard spectrophotometric method using cuvettes.

Pyruvate Kinase. Conversion rates obtained using premixed nanoarrays were comparable to those obtained using microtiter plates (Table 3). Experiments using presprayed nanoarrays gave results similar to the ones performed in premixed nanoarrays (Table 3), whereas conversion rates from microtiter plates were more than 100% compared to the nominal values. The determinations of pyruvate kinase in cell-free extract using premixed and presprayed nanoarrays gave nearly the same conversion rates of 41% (Table 4). The activity of pyruvate kinase determined by a spectrophotometer was again higher and amounted to 7.71 units/mL.

Table 4. Determination of PK in CFE by Comparing Premixed and Presprayed Nanoarrays and Microtiter Plates

PK concn (μM/min·mL)	conversion rate (μM/min·mL)		conversion referring to the nominal value (%)	
spectrophotometer nominal value	nanoarray	microtiter plate	nano-array	microtiter plate
premixed				
7.71 ± 0.06	3.19 ±0.29	2.07 ± 0.25	41.4	26.8
presprayed (nanoarray only)				
7.71 ± 0.06	3.17 ± 0.92	2.65 ± 0.23	41.1	34.4

Table 5. Determination of ENO Activity Comparing Premixed and Presprayed nanoarrays and microtiter plates using commercially available enzymes

PK concn (μM/min·mL)	conversion rate (μM/min·mL)		conversion - referring to the nominal value (%)	
nominal value	nanoarray	microtiter plate	nanoarray	microtiter plate
premixed				
0.06	0.074 ± 0.006	0.057 ± 0.002	123.0	95.0
0.1	0.107 ± 0.006	0.104 ± 0.005	107.0	104.0
0.14	0.154 ± 0.013	0.157 ± 0.008	110.0	112.0
presprayed (nanoarray only)				
0.06	0.034 ± 0.004	0.048 ± 0.006	57.0	80.0
0.1	0.084 ± 0.012	0.103 ± 0.006	84.0	103.0
0.14	0.152 ± 0.027	0.135 ± 0.002	108.6	96.4

Table 6. Determination of ENO in CFE by Comparing Premixed and Presprayed Nanoarrays and Microtiter Plates

PK concn (μM/min·mL)	conversion rate (μM/min·mL)		conversion referring to the nominal value (%)	
spectrophotometer nominal value	nanoarray	microtiter plate	nanoarray	microtiter plate
premixed				
0.50 ± 0.06	0.31 ± 0.180	0.44 ± 0.03	62.0	88.0
presprayed (nanoarray only)				
2.52 ± 0.17	0.53 ± 0.44	1.15 ± 0.34	21.0	45.6

The differences in enzyme activities between measurements monitored in microtiter plates, cuvettes, and nanoarrays could be explained by temperature variations. The temperature in the microtiter plate reader can be tuned by changing the software-specified target temperature. The temperature achieved cannot be controlled directly in the screening environment of the reader. It has to be assumed that the target temperature is stable. However, there is no guarantee and the temperature could change over time, especially when the ambient temperature varies. This could be an explanation why measurements performed in microtiter plates sometimes gave a higher enzyme activity.

The temperature in nanoarrays, however, can be controlled in a more sophisticated manner. The nanoarray lies directly on a heating and freezing stage. The actual temperature on the nanoarray was measured by means of a thermocouple. Since the nanoarray stays in direct contact with this stage, the temperature can be considered as very stable, even if the ambient temperature

Table 7. Determination of ENO Activity in Presprayed Nanoarrays and Microtiter plates and different solution compositions using commercially available enzymes

solution composition	ENO concn (μM/min·mL) nominal value	conversion rate (μM/min·mL) presprayed nanoarray	conversion rate (μM/min·mL) microtiter plate	conversion referring to nominal value (%) presprayed nanoarray	conversion referring to nominal value (%) microtiter plate
2-PG/NADH	0.06	0.010 ± 0.004	0.035 ± 0.025	21.7	58.4
	0.1	0.022 ± 0.004	0.053 ± 0.012	37.0	53.2
	0.14	0.037 ± 0.004	0.061 ± 0.008	26.4	40.9
2-PG/NADH/LDH/PK	0.06	0.052 ± 0.003	0.01 ± 0.004	16.7	86.3
	0.1	0.072 ± 0.000	0.02 ± 0.003	20	71.5
	0.14	0.098 ± 0.001	0.03 ± 0.009	21.4	65.3
2-PG/NADH/LDH/PK/ADP/MgSO4/KCl	0.06	0.01 ± 0.002	0.068 ± 0.005	16.7	113.5
	0.1	0.04 ± 0.011	0.125 ± 0.000	40.0	124.8
	0.14	N/A	0.190 ± 0.008		126.5

varies. It can be assumed that reading performed in nanoarrays is more reliable than the ones performed in microtiter plates.

The comparison of different pyruvate kinase substrate solutions, in which NADH/PEP/LDH and NADH/PEP/LDH/ADP/$MgSO_4$/KCl/fructose 1,6-biphoshate were combined in one solution, could not be performed in presprayed nanoarrays. These nanoarrays were presprayed and immediately frozen for one night to be measured, and the following day no fluorescence signal could be detected. The same event occurred when ∼2 mL of this solution was frozen and measured the next day with a microtiter plate reader. This could be due to a small amount of pyruvic acid in the PEP bottle. It is likely that LDH began to initiate the conversion of PEP into lactate. This means that the available NADH had already been slowly converted to NAD^+, which is not fluorescent.

Enolase. The performance of this enzymatic assay in premixed nanoarrays was quite comparable to the assay performed in microtiter plates (Table 5). The conversion rates obtained using presprayed nanoarrays were again similar to those obtained from microtiter plates. It is striking though that with a presprayed nanoarray and an ENO concentration of 0.14 unit/mL the rates were higher than those obtained using a microtiter plate (Table 5). This is despite the fact that the concentrations of 0.06 and 0.1 unit/mL were lower than those using microtiter plates. This might be due to an unevenly sprayed nanoarray. By evaluating more and more data for this paper, it became apparent that the spraying of the substrate solutions sometimes was not constant. Usually the first two wells contained more substrate. The last well always gave the weakest signal compared to the rest of a column. This could be due to an unstable working voltage. Another influence could be the pumping system itself. It might be that the speed of the spraying procedure is too fast for the pump. Thus, a delay is built up. This means that at the first two wells the pump still can follow the movement of the stage, but from well three onward the stage moves too quickly so that the fluid cannot be transported in a regular manner.

Enolase activity in cell-free extracts using a premixed nanoarray was found to be 62% compared to 88% obtained by using microtiter plates (Table 6) relative to the activities obtained by the spectrophotometric method. The same measurement but with presprayed nanoarrays gave a conversion of only 21% (Table 6). But with the use of a microtiter plate, the conversion was only 45.6% compared to the activity obtained using the spectrophotometric method. Possibly, the spectrophotometric assay did not give the correct data. This can be deduced from the fact that all measurements on cell-free extracts performed in microtiter plates always gave a good approximation to conversion rates obtained by spectrophotometric methods.

The determination of enolase activity using different substrate solution compositions was rather interesting. In general, measurements performed on each presprayed nanoarray were very bad in the sense that only low conversion rates (less than 50%) could be achieved (Table 7). However, assays carried out by using microtiter plates resulted in a continuous increase of enolase activity by adding all necessary reagents (except for enolase) in one solution (Table 7).

CONCLUSIONS

We have shown that enzyme activity assays for alcohol dehydrogenase, pyruvate kinase, and enolase are possible in nanoarrays using premixed and presprayed reagents. We have also shown that our methods are applicable to commercially available enzymes as well as to enzymes present in cell-free extracts. It seems that there are no significant differences in conversion rates for ADH and PK using commercially available enzymes using presprayed and premixed nanoarrays. Only in the case of enolase in premixed nanoarrays, could a higher rate have been achieved compared to rates obtained using presprayed nanoarrays. For enzyme determinations in cell-free extracts, premixed and presprayed nanoarrays are equally good and reproducible.

The performance of enzymatic assays using nanoarrays is quick, cost-effective, and low in sample use. This makes it an excellent substitute for microtiter plate and spectrophotometric tests.

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