Anal. Chem. **2002,** *74,* 5071−5075

Reusable Platforms for High-Throughput On-Chip Temperature Gradient Assays

Hanbin Mao, Matthew A. Holden, Min You, and Paul S. Cremer*

Department of Chemistry, Texas A&M University, College Station, Texas 77843

This paper describes a reusable platform that can apply a linear temperature gradient to a lab-on-a-chip device. When a planar microfluidic device with a series of microchannels is placed on top of the platform with the channels perpendicular to the gradient, each channel is held at a discrete temperature. This allows temperature-dependent data for chemical or biochemical species flowed into the device to be obtained in a concurrent fashion. As a demonstration, a melting curve for dsDNA is performed by collecting all the data simultaneously. The gradient is stable enough to easily distinguish between 30-mers where the complement strand contains a single C−A mismatch or a single T−G mismatch or is a perfect match. On the other hand, a temperature gradient can be formed parallel to the direction of flow of the microchannels. This allows the temperature in each channel to vary continuously as the liquid flows downstream. If each microchannel in the array contains a distinct pH value, ionic strength, species concentration, or chemical composition, then a high-throughput two-variable experiment can be performed. We demonstrate this mode of data collection by measuring the fluorescence yield of fluorescein dye molecules in aqueous solution simultaneously as a function of concentration and temperature.

Over the last three decades, combinatorial strategies have become increasingly important in many fields of chemistry and biology.[1,2] Examples range from protein crystallization[3] to materials discovery[4] and catalysis.[5,6] The value of this approach stems from the ability to generate large data sets in a relatively short period of time. For example, split-pool methods have enabled vast libraries of small organic molecules to be created that can be quickly screened for their efficacy in ligand−receptor binding.[7] On the other hand, protein crystallization experiments require the probing of many subtly different solution conditions (e.g., ionic strength, concentration, pH, etc.). Setting up such an experiment can be accomplished rapidly in standard 96- or 384-well plate formats by placing the desired components in each robotically.

Despite the growing number of chemical and physical variables that can be probed in a combinatorial fashion, temperature is not usually considered one of them. This is because it would be impractical to hold each well in a 96-well plate format at a different temperature. This is unfortunate because high-throughput temperature methodologies in any of the above-mentioned fields would be invaluable. Recently, we introduced a technique that permits the generation of a linear temperature gradient inside a lab-on-a-chip device.[8] The principle of operation exploits the fact that heat flow in two dimensions between a heat source and cold sink leads to a linear gradient between the two when they are placed in parallel.[9] Shortly thereafter, Ross and Locascio showed that a temperature gradient in combination with an applied voltage can be exploited for on-chip analyte focusing.[10]

In the work presented here, we incorporate heating and cooling elements directly onto a planar glass substrate. The substrate is then employed as a reusable platform onto which planar microfluidic devices can be placed and removed (Figure 1). The linearity of the temperature gradient is confirmed directly in a first assay using thermocouple measurements. Next, demonstrations of DNA melting point curves are shown. Finally, two variable measurements of fluorescence quantum yield are made as a function of concentration and temperature. This final assay demonstrates the potential of this approach to massively parallel data acquisition in which only minute quantities of sample are required.

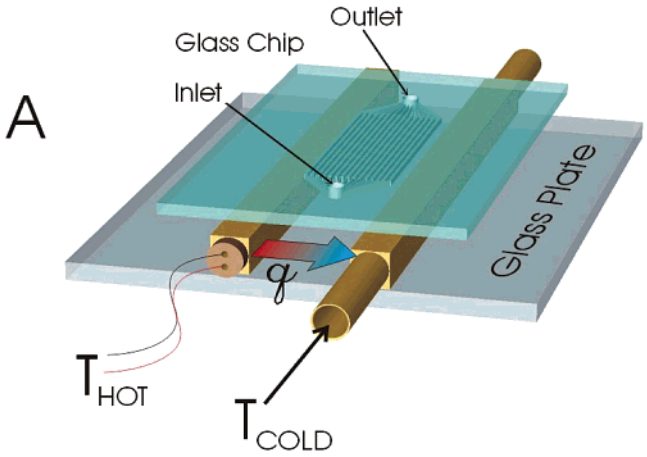
EXPERIMENTAL SECTION

Temperature Gradient Platform Fabrication. In a standard design, two square brass tubes (4 × 4 mm) hollowed out with circular holes (0.125-in. i.d.) were spaced 3.4 mm apart on top of a glass plate (50 × 75 × 1 mm, Corning) and fixed in place with epoxy glue (Figure 1). The cooling tube was regulated via a water circulator (Fisher Scientific, Pittsburgh, PA), while the heating tube was controlled with a cartridge heater (25 W, 0.125 in. × 2 in. (o.d. × length), Omega Engineering, Inc., Stanford, CT) regulated by a Variac (VWR Scientific Products Co.). By using this design, the temperature gradient easily spanned the range from 16 to 101 °C in the experiments presented here.

* To whom correspondence should be addressed. Tel: 979-862-1200. Fax: 979-845-7561. E-mail: cremer@mail.chem.tamu.edu.

(1) Tramontano, A.; Janda, K. D.; Lerner, R. A. *Science* **1986**, *234*, 1566−1570.
(2) Pollack, S. J.; Jacobs, J. W.; Schultz, P. G. *Science* **1986**, *234*, 1570−1573.
(3) Wiencek, J. M. *Annu. Rev. Biomed. Eng.* **1999**, *1*, 505−534.
(4) Xiang, X. D.; Sun, X. D.; Briceno, G.; Lou, Y. L.; Wang, K. A.; Chang, H. Y.; Wallacefreedman, W. G.; Chen, S. W.; Schultz, P. G. *Science* **1995**, *268*, 1738−1740.
(5) Janda, K. D.; Lo, L. C.; Lo, C. H. L.; Sim, M. M.; Wang, R.; Wong, C. H.; Lerner, R. A. *Science* **1997**, *275*, 945−948.
(6) Reddington, E.; Sapienza, A.; Gurau, B.; Viswanathan, R.; Sarangapani, S.; Smotkin, E. S.; Mallouk, T. E. *Science* **1998**, *280*, 1735−1737.
(7) Houghten, R. A.; Pinilla, C.; Blondelle, S. E.; Appel, J. R.; Dooley, C. T.; Cuervo, J. H. *Nature* **1991**, *354*, 84−86.
(8) Mao, H.; Yang, T.; Cremer, P. S. *J. Am. Chem. Soc.* **2002**, *124*, 4432−4435.
(9) Incropera, F. P.; DeWitt, D. P. *Fundamentals of Heat and Mass Transfer*, 3rd ed.; John Wiley & Sons: New York, 1990.
(10) Ross, D.; Locascio, L. E. *Anal. Chem.* **2002**, *74*, 2556−2564.

10.1021/ac025851z CCC: $22.00
Published on Web 08/27/2002



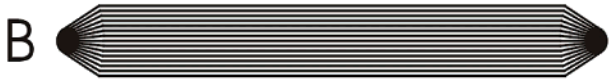


Figure 1. (a) Schematic diagram of a temperature gradient microfluidic device. The temperature gradient platform is formed by fixing two brass tubes onto a glass substrate with epoxy. The left tube houses a cartridge heater that serves as the heat source. The right tube allows liquid to flow through it and serves as the cooling element. A detachable chip containing microchannels is placed directly onto the platform, with the microchannels perpendicular to the temperature gradient. The arrow (q) represents the direction of heat flow. (b) Geometry of the glass channels in the microfluidic device.

Glass Microfluidic Chip Fabrication. Standard 50 × 75 mm soda lime glass slides (Corning) were cleaned by boiling in 7X detergent (ICN), rinsing with copious amounts of DI water, and drying under a nitrogen stream. Photoresist (Shipley, S1813) was spun onto one side to a thickness of ~5 μm and soft-baked for 1 h at 90 °C in a convection oven. Photomasks were prepared by reducing a pattern printed with a 1200 dpi printer onto Kodak technical pan film. Samples were exposed using a Quintel 6000 mask aligner and developed in a 1:1 solution of Microposit developer concentrate (Microchem) and DI water. Slides were etched and bonded using a process adapted from Lin and co-workers.[11] This involved gently waving photopatterned slides in a buffer oxide etchant (BOE) solution (1:6 ratio of 48% HF/200 g of NH_4F in 300 mL of DI water) for 2.5 min, washing in a 1 M HCl solution for 30 s, and then placing them back into BOE for 2.5 min. This cycle of etching and washing could be repeated up to 6 times before the photoresist would degrade and peel away. The patterned lines were between 30 and 40 μm deep as determined by profilometry measurements. Chips were produced with 15 parallel microchannels that were 19 mm long, 120 μm wide, and spaced by 90 μm (Figure 1b). At each terminus, the microchannels converged to a common 1-mm-diameter outlet drilled into the glass using a diamond-coated drill bit (Wale Apparatus). A 25.0 × 37.5 mm soda lime glass slide section was used as a cover for the microchannels. Covers and etched chips were cleaned by boiling in 7X detergent and then placed in a warm 6:1:1 DI $H_2O/HCl/H_2O_2$ solution for 5 min, a warm 5:1:1 DI $H_2O/NH_4OH/H_2O_2$ solution for 5 min, rinsed with copious DI water, and finally dried with N_2. Each device was bonded by stacking the plates between weights in the following order: a 0.5-in.-thick solid brass substrate (which served as a base), a polished alumina flat, the etched chip (channels up), a soda lime glass cover, a second smaller polished alumina flat, and finally a 40-g brass weight. During thermal bonding, the weight would press on the alumina flat, causing the two glass surfaces to fuse. After the initial firing, the weight and the flat would be moved to an unbonded section and the firing schedule rerun. This process was repeated until all vital areas were bonded.[12] The firing sequence was as follows: From room temperature, a 280 °C/h ramp was applied until 400 °C and held at that temperature for 4 h. Next, a 280 °C/h ramp was applied up to 588 °C and held for 6 h. Finally, the kiln was shut off and allowed to cool to room temperature.

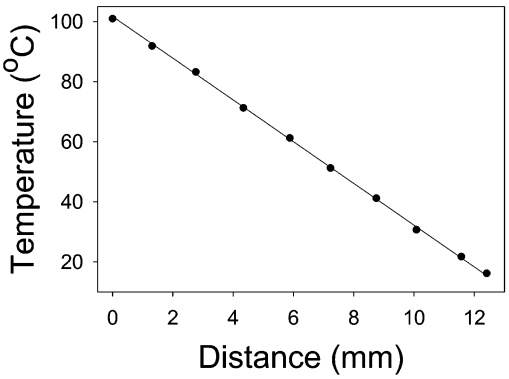


Figure 2. Temperature vs position inside a microfluidic device placed on the temperature gradient platform. Error bars for each point fit within the circles used to plot the data.

Temperature Gradient Apparatus. Initial temperature gradient characterization was done with a larger platform than described above, and the bass tubes were separated by 12.6 mm. This device consisted of a poly(dimethylsiloxane) (PDMS) mold bound to a planar glass surface. The PDMS (Dow Corning Sylgard Silicone Elastomer-184, Krayden, Inc.) architecture was fabricated as described previously.[13] Briefly, channels were formed in PDMS by replica molding on a photoresist patterned surface. The PDMS surface was then rendered hydrophilic by oxygen plasma treatment and bonded to a glass slide. Ten holes were punched into the device using a syringe needle (300-μm i.d.) through the top of the PDMS mold down to the glass substrate. The device was placed on top of the brass tubes with the holes oriented along the temperature gradient such that the two holes on either end were directly above the metal tubes. A thermocouple was inserted into the holes to probe the temperature at each location, from which a plot of temperature versus position was made for a range of 16−101 °C (Figure 2). It should be noted that a small amount of vacuum grease was applied to the surface of each brass tube to ensure uniform contact to the microfluidic device. Identical experiments were performed with the standard 3.4-mm gradient platforms described in the rest of this paper. These results were the same as the ones shown here, but only five holes were bored in parallel in a device because of the narrowness of the gradient.

DNA Melting Curve Measurements. Oligonucleotides (Integrated DNA Technologies, Coralville, IA) were prepared at 113

(11) Lin, C. H.; Lee, G. B.; Lin, Y. H.; Chang, G. L. *J. Micromech. Microeng.* **2001**, *11*, 726−732.

(12) Fan, Z. H.; Harrison, D. J. *Anal. Chem.* **1994**, *66*, 177−184.

(13) Yang, T.; Jung, S. Y.; Mao, H.; Cremer, P. S. *Anal. Chem.* **2001**, *73*, 165−169.

nM concentration in Tris (Sigma) buffer solution at pH 8.0. After mixing 15-μL aliquots of complementary strands at equimolar ratio, the resulting mixture was heated to 94 °C for 5 min and allowed to cool to room temperature. A 15-μL aliquot of of SYBR Green I (Molecular Probes, Inc., Eugene, OR, 1:5000 dilution) in Tris buffer (pH 8.0) was added to the DNA solutions, resulting in a final concentration of 56 nM for the dsDNA. The solution was incubated in the dark for 20 min before injection at room temperature into the all-glass microchannel device. An all-glass device was employed for this experiment because PDMS−glass hybrid devices seemed to imbibe SYBR Green I dye and DNA into the polymer. After sample injection, the microfluidic device was brought into contact with the temperature gradient platform. The hot end of the stage was maintained at 77 °C and the cold end at 36 °C as verified by thermocouple measurements prior to every experiment. The fluorescence intensity was directly proportional to the signal intensity measured by our CCD camera, and it was therefore possible to relate the flourescence signal to the degree of DNA melting. The fluorescence signal from the SYBR Green I was detected under a standard fluorescence microscope (E800, Nikon). The influence of temperature on the fluorescence of SYBR Green I was corrected by 2%/°C in accordance with standard literature procedures.[14]

RESULTS AND DISCUSSION

Single-Nucleotide Polymorphism (SNP) Analysis. Monitoring the thermal transition between dsDNA and ssDNA is the principle diagnostic tool used in many DNA-based assays.[15,16] For example, during PCR amplification, the melting curve of dsDNA is used to follow reaction progress and product purity.[17] Although measuring DNA melting curves is essential for these techniques, current methods are hindered by the need to ramp the temperature sequentially.[18−20] In PCR, this is often done with a special thermal cycler.[17] For example, Ririe and co-workers demonstrated simultaneous PCR and melting curve analysis by integrating a microvolume fluorometer into a thermal cycler.[21] Mirkin and colleagues used similar temperature ramping methods to measure DNA melting curves on chip.[22]

Below we describe the combination of intercalation dye chemistry with an on-chip temperature gradient to produce a one-shot melting curve for dsDNA. The intercalation dye, SYBR Green I, was mixed with DNA samples and injected into a microchannel array while fluorescence microscopy was performed. SYBR Green I is known to fluoresce when incorporated between stacked base pairs of dsDNA but lose fluorescence in aqueous solution.[23]

The synthetic oligonucleotides employed in this study are shown in Table 1. DNA fragments 1 and 2 were complementary,

Table 1. DNA Sequences of Four Oligonucleotides[a]

1	3′ TAA TTA CGA TAC GTC TTT TAG AAT CTC ACA 5′
2	5′ ATT AAT GCT ATG CAG AAA ATC TTA GAG TGT 3′
3	5′ ATT AAT GCT ATG C**G**G AAA ATC TTA GAG TGT 3′
4	5′ ATT AAT GCT ATG CA**A** AAA ATC TTA GAG TGT 3′

[a]Letters in boldface type and underlined represent the positions of mismatched bases.

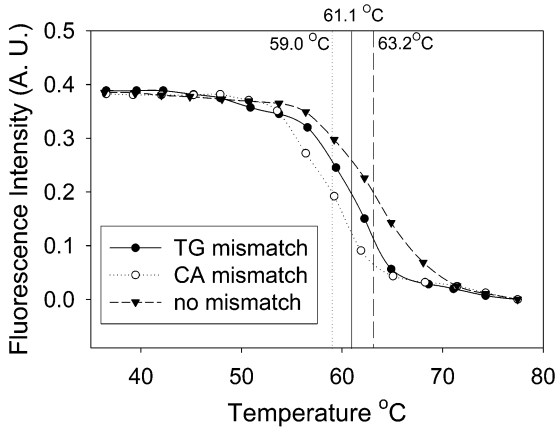


Figure 3. Fluorescence intensity of SYBR Green I dye vs temperature in the presence of complementary DNA strands (▼), DNA strands with a single T−G mismatch (●), and DNA strands with a single C−A mismatch (○).

while 1 and 3 contained a T−G mismatch and 1 and 4 had a C−A mismatch. Since a single base pair mismatch reduces the amount of hydrogen-bonding interactions, the T_m of complementary dsDNA will be higher than the T_m of dsDNA with a mismatch. This effect was observed in Figure 3, where the melting curves of 1 and 2, 1 and 3, and 1 and 4 were 63.2, 61.1, and 59.0 °C, respectively. These results matched expectations since T−G base pair interactions are known to be more stable than C−A base pair interactions.[24] All curves were repeated several times with different devices and yielded essentially identical results each time.

This method has several advantages compared to conventional DNA melting curve measurements. While standard techniques usually require at least hundreds of microliters and tens of minutes for a single curve, combining a microfluidic chip with a reusable temperature gradient platform allows us to perform the same measurement with hundreds of nanoliters in just one shot (i.e., a few seconds). Since the fluorescence at all temperatures is detected simultaneously, the signal-to-noise ratio of the overall process is improved with respect to sequential analysis. This is because any variations in the light source intensity or detector yield as a function of time are avoided. Furthermore, the SYBR Green I dye is subjected to far less photodamage and thermal damage due to the reduction in time of exposure to the excitation source and to temperature extremes. Finally, the current geometry of this method could be easily adapted to acquire multiple DNA melting curves simultaneously. This could be accomplished by injecting different DNA strands into each channel and employing the strategy described below for multidimensional on-chip analysis.

(14) Jobs, M.; Fredriksson, S.; Brookes, A. J.; Landegren, U. *Anal. Chem.* **2002**, *74*, 199−202.
(15) Brown, P. O.; Botstein, D. *Nat. Genet.* **1999**, *21*, 33−37 Suppl.
(16) Pirrung, M. C. *Angew. Chem., Int. Ed.* **2002**, *41*, 1277−1289.
(17) Wittwer, C. T.; Ririe, K. M.; Andrew, R. V.; David, D. A.; Gundry, R. A.; Balis, U. J. *BioTechniques* **1997**, *22*, 176−181.
(18) Kopp, M. U.; de Mello, A. J.; Manz, A. *Science* **1998**, *280*, 1046−1048.
(19) Lagally, E. T.; Medintz, I.; Mathies, R. A. *Anal. Chem.* **2001**, *73*, 565−570.
(20) Khandurina, J.; McKnight, T. E.; Jacobson, S. C.; Waters, L. C.; Foote, R. S.; Ramsey, J. M. *Anal. Chem.* **2000**, *72*, 2995−3000.
(21) Ririe, R. K.; Rasmussen, R. P.; Wittwer, C. T. *Anal. Biochem.* **1997**, *245*, 154−160.
(22) Taton, T. A.; Mirkin, C. A.; Letsinger, R. L. *Science* **2000**, *289*, 1517−1560.
(23) Wittwer, C. T.; Herrmann, M. G.; Moss, A. A.; Rasmussen, R. P. *BioTechniques* **1997**, *22*, 130−139.
(24) Frutos, A. G.; Pal, S.; Quesada, M.; Lahiri, J. *J. Am. Chem. Soc.* **2002**, *124*, 2396−2397.

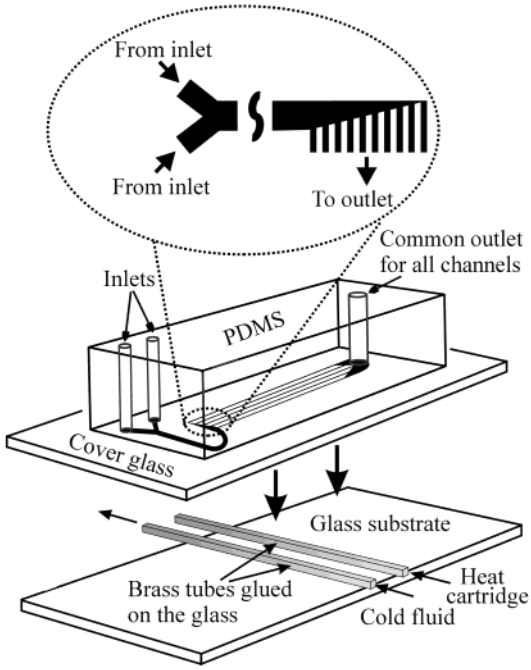


Figure 4. Schematic diagram of a microfluidic device for two-variable analysis. The brass tubes are situated orthogonally to the long axis of the microchannels.

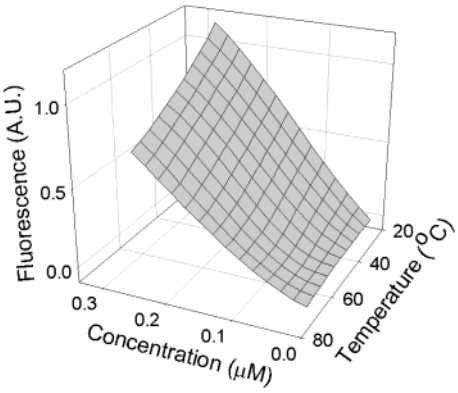


Figure 5. Three-dimensional plot of fluorescence intensity of carboxyfluorescein dye molecules in aqueous solution as a function of their concentration (0.00715−0.266 μM) and temperature (28−74 °C). The plot was mapped over 110 data points (excluded for clarity) gained from 11 temperature measurements across 10 microchannels. The grid intersections do not represent data points, but serve simply as a guide to the eye.

Multidimensional On-Chip Assays. Highly parallel and combinatorial assays are needed to satisfy the accelerating pace of bioinformatic data collection.[25] The capabilities of the temperature gradient platform can be combined with variables such as pH, ionic strength, or concentration to perform multivariable experiments. A first variable can be created with the present platform by orienting the temperature gradient parallel to the direction of flow inside the microchannel device (Figure 4). This causes the temperature of the analyte to change continuously as liquid flows downstream. To obtain a gradient of a second variable such as concentration, the microchannel array can contain a range of concentrations of a soluble molecule.

We[26] and others[27−29] have designed methods for concentration dilution inside an array of microchannels. In our approach, two streams of liquid are combined at a Y-junction and allowed to diffuse into each other as they flow downstream side by side. Only diffusional mixing occurs because the Reynolds number inside the channel is low enough to prevent turbulence. After a fixed distance (2 cm), the channel can be separated into a series of smaller tributaries that emanate from the main flow stream. (Figure 4, inset) In practice, substrate is infused into one inlet port while pure buffer is injected into the other. This method was employed to create a dilution series that ranged over a factor of 37 in concentration in 10 parallel channels when the flow rate was set to 2 μL/min at each inlet. The fluorescein concentration injected at the inlet was 0.266 μM. A linear temperature gradient from 28 to 74 °C was established along the length of the channels after separation of the dye into the microchannels. After separating, the concentrations of fluorescein in each channel could be calculated relative to one another, since the fluorescence intensities of fluorescein were linearly related to concentration under the low concentration conditions employed. Even though the dye was constantly flowing through the microchannels, the temperature at any given point along a microchannel was considered to be at equilibrium. This assumption was deemed valid because small volumes of aqueous solutions in microchannels have been shown to equilibrate in similar local environments as fast as 10^7 °C/s.[30] As can be seen from Figure 5, the highest intensity was observed at the highest concentration and lowest temperature; however, the intensities varied in a complex manner. The two-variable fluorescein assay demonstrates the potential of this technique to collect data in a massively parallel fashion. As with the single-variable assay described above, this assay uses only low analyte volumes and provides excellent S/N, while effectively squaring the amount of data that can be collected.

To employ the multidimensional assay without flowing the analyte, certain modifications will need to be made to prevent diffusional or convectional flow within each microchannel. One approach could be to create a series of hermetically sealed compartments at fixed distances along each channel after the sample has been introduced. In a PDMS device, pneumatic valves[31] could be used to seal separate compartments. On the other hand, walls could be erected inside glass devices through in situ polymerization.[32] Either way, the ultimate device would resemble a 96- or 384-well plate format with temperature varied along one direction while a second parameter is varied along the

(25) Collins, F. S.; Patrinos, A.; Jordan, E.; Chakravarti, A.; Gesteland, R.; Walters, L.; Fearon, E.; Hartwelt, L.; Langley, C. H.; Mathies, R. A.; Olson, M.; Pawson, A. J.; Pollard, T.; Williamson, A.; Wold, B.; Buetow, K.; Branscomb, E.; Capecchi, M.; Church, G.; Garner, H.; Gibbs, R. A.; Hawkins, T.; Hodgson, K.; Knotek, M.; Meisler, M.; Rubin, G. M.; Smith, L. M.; Smith, R. F.; Westerfield, M.; Clayton, E. W.; Fisher, N. L.; Lerman, C. E.; McInerney, J. D.; Nebo, W.; Press, N.; Valle, D. *Science* **1998**, *282*, 682−689.
(26) Mao, H.; Yang, T.; Cremer, P. S. *Anal. Chem.* **2002**, *74*, 379−385.
(27) Weigl, B. H.; Yager, P. *Science* **1999**, *283*, 346−347.
(28) Jacobson, S. C.; McKnight, T. E.; Ramsey, J. M. *Anal. Chem.* **1999**, *71*, 4455−4459.
(29) Dertinger, S. K.; Chiu, D. T.; Jeon, N. L.; Whitesides, G. M. *Anal. Chem.* **2001**, *73*, 1240−1246.
(30) Lowe, H.; Ehrfeld, W. *Electrochim. Acta* **1999**, *44*, 3679−3689.
(31) Unger, M. A.; Chou, H. P.; Thorsen, T.; Scherer, A.; Quake, S. R. *Science* **2000**, *288*, 113−116.
(32) Beebe, D. J.; Moore, J. S.; Bauer, J. M.; Yu, Q.; Liu, R. H.; Devadoss, C.; Jo, B. *Nature* **2000**, *404*, 588−590.

other. The advantage to such a technique would not only be in the use of temperature as one of the variables but also in the very small volumes that could be easily introduced in such an on-chip microtiter plate format. One application of such plates could be in the field of protein crystallization, which is very sensitive to chemical concentration, ionic strength, and pH as well as temperature.[33] Other assays for (a) the identification of optimal reaction conditions, (b) the screening of cell behavior under varying conditions, or (c) the formation of platforms for combinatorial materials discovery can also be envisioned.

(33) Juárez-Martinez, G.; Steinmann, P.; Roszak, A. W.; Isaacs, N. W.; Cooper, J. M. *Anal. Chem.* **2002**, *74*, 3505−3510.

ACKNOWLEDGMENT

This work was supported by ARO (DAAD19-01-1-0346), an ONR-YIP Award (NOOO14-00-1-0664), a Beckman Young Investigator Award, and a Nontenured Faculty Award from 3M Corp. We also acknowledge support from the Center for Integrated Microchemical Systems at Texas A&M University and the use of the TAMU/CIMS Materials Characterization Facility.

Received for review June 10, 2002. Accepted July 19, 2002.

AC025851Z