

Nonlithographic Fabrication of Microfluidic Devices

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Abstract: A facile nonlithographic method for expedient fabrication of microfluidic devices of poly(dimethylsiloxane) is described. Positive-relief masters for the molds are directly printed on smooth substrates. For the formation of connecting channels and chambers inside the polymer components of the microfluidic devices, cavity-forming elements are adhered to the surfaces of the masters. Using this nonlithographic approach, we fabricated microfluidic devices for detection of bacterial spores on the basis of enhancement of the emission of terbium (III) ions.

Introduction

This publication describes an expedient and facile procedure for fabrication of microfluidic devices. Without using lithography, we prepared continuous-flow devices for light-emission detection of bacterial spores.

The efficiencies in reagent consumption, power usage, operation rate, and fabrication cost represent some of the advantages of micro- and nanofluidic devices.¹ The broadening of the applications of microfluidic devices as biosensors,^{2,3} massively parallel analyzers,⁴ microreactors^{5,6} and photonic components^{7,8} places a demand for efficient and facile prototyping and fabrication of such devices.

Poly(dimethylsiloxane) (PDMS) is a broadly used polymer material for fabrication of microfluidic devices.^{9,10} The masters for casting the PDMS components of such devices are usually prepared photolithographically using specialized equipment in a clean-room environment.¹¹ Here, we describe a nonlithographic approach, in which the masters are directly printed on smooth substrates, using regular office equipment, e.g., LaserJet printers.

Simple procedures for expedient detection of bacterial spores are important for areas such as health care, biotechnology, and defense against bioterrorism.^{12–14} Undertaking a nonlithographic approach, we fabricated a continuous-flow microfluidic devices for detection of bacterial spores. The spore detection is based on enhancing the emission of terbium (III) ions when ligated with 2,6-dipicolinic acid (DPA),^{15,16} which is a natural product comprising ~1–15% of the weight of bacterial spores.^{17–19} We investigated the performance of these devices.

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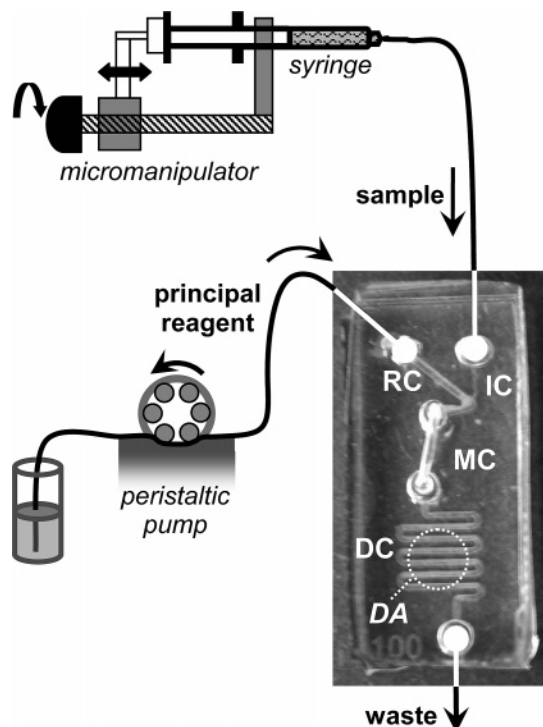
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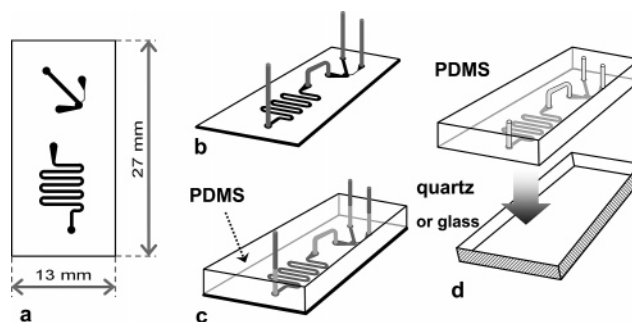
Scheme 1. Setup of a Continuous-Flow Microfluidic Device^a

^a IC = injection channel; RC = principal reagent channel; MC = mixing chamber; DC = detection channel; and DA = detection area.

Results and Discussion

Continuous-Flow Microfluidic Device. For detection of bacterial spores, we designed a continuous-flow device that comprises a principal-reagent channel, RC, an injection channel, IC, a mixing chamber, MC, and a wavy detection channel, DC, which carries the flow through the detection area, DA (Scheme 1). The principal reagent, which is expected to undergo emission enhancement, is continuously made to flow through the reagent inlet. Minute volumes of sample are introduced through the sample-injection inlet and are brought into contact with the principal reagent through the thin injection channel. The principal-reagent flow carries the sample into the mixing chamber. Upon mixing, species with enhanced emission are generated from components of the sample and the principal reagent. The solution exiting the mixing chamber flows into a wavy detection channel that covers an area large enough to be targeted with an excitation light beam with a cross-section diameter of $\sim 1\text{--}5$ mm. The small external dimensions of the microfluidic devices allowed us to place each of them in a 1-cm cuvette holder of a spectrofluorometer. Hence, we were able to readily monitor the changes in the emission properties of the solutions that flowed through the detection channel.

Nonlithographic Fabrication. The computer-aided-design (CAD) images of the PDMS components of the microfluidic devices were directly printed on polyester transparency films to produce the masters (Scheme 2a). For the formation of mixing

Scheme 2. Nonlithographic Fabrication of a Continuous-Flow Microfluidic Device

chambers and inlet/outlet connecting channels inside the PDMS components of the devices, polyethylene arcs and posts were adhered to the reservoir areas of the master printouts (Scheme 2b). The PDMS pre-polymer mixture was poured over the masters and allowed to cure (Scheme 2c). Because of the elasticity of PDMS, the cavity-forming polyethylene elements were readily removed from the bulk of the cured polymer, leaving hollow spaces with the desired shapes and sizes (Scheme 2d). This approach for generation of cavities in the device components allows for achieving: (1) complex connectivity within a single-device component, eliminating the need for fine alignment during the device assembly; (2) direct formation of channels for connecting the device with external tubing, eliminating the need for drilling through the cured polymer (such drilling through PDMS may cause a formation of cracks and damage the whole component); and (3) superior seal with external tubing due to the smoothness of the walls of the channels formed from posts adhered to the master.

The final assembly of the devices consists of irreversible adhesion of the PDMS components to the smooth surfaces of substrates such as glass, quartz, PDMS, or silicon (Scheme 2d and Figure 1).^{9–11}

For testing purposes, we also prepared devices with three or five parallel microfluidic channels and characterized the cross-section shapes and dimensions of their channels (Figure 2a,b). The relatively small sizes of the device allowed us to place each of them in a 1-cm cuvette holder of a spectrofluorometer and successfully detect the signals from solutions of fluorescent dyes that we flowed through the microchannels (Figure 2c).

Characteristics of the Printed Masters. In LaserJet printing, toner particles, comprising binder polymers, colorants, and charge-controlling agents,^{20,21} are deposited on a substrate, such as paper or transparency, and thermally treated. The elevated temperature causes melting of the binder polymer on the surface of the toner particles. Upon cooling and solidification, the polymer glues the particles together and adheres the printed feature to the surface of the substrate. The composition of the toner binder polymers varies among the different manufacturers. Usually the toner polymer contains polyesters, polystyrene, and epoxy resins.^{21–23}

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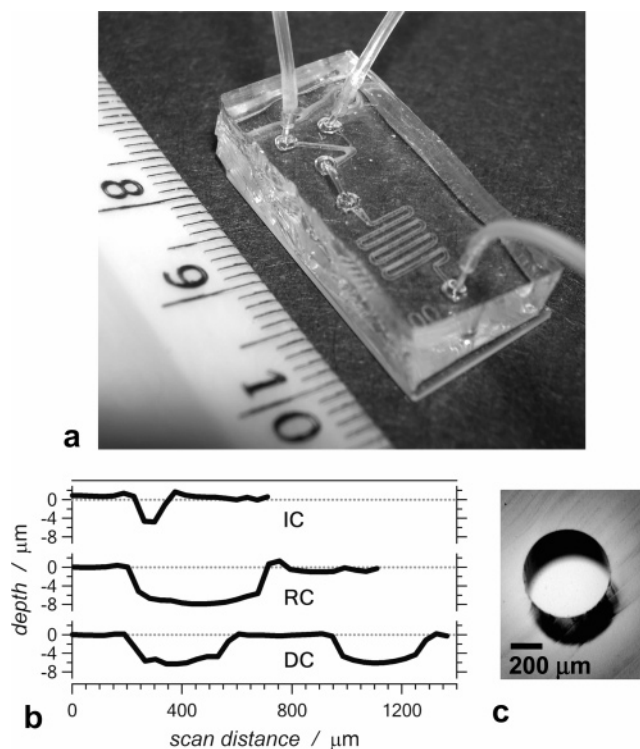


Figure 1. Continuous-flow microfluidic device: (a) a photograph of a device next to a metric ruler (the numbers represent the distance in centimeters); (b) profilographs from the surface of the PDMS component of a device scanned perpendicularly to various channels: injection channel (IC), principal-reagent channel (RC), and two neighboring detection channels (DC) (Scheme 1); and (c) a transmission microscope image of a PDMS slice cut across MC.

The materials that we used for the preparation of the masters (i.e., polyester transparencies, toner, and polyethylene posts and arcs) do not adhere strongly to PDMS. Therefore, we did not need to pretreat the surfaces of the masters before pouring the PDMS pre-polymer over them.

When the cured elastomer was pulled off the master, however, the polyethylene posts and arcs remained in the PDMS blocks. Therefore, we reattached the polyethylene elements to the surface of the master before using it again. We could use the same printed master up to about five times (Figure 3).

Although the reuse of the masters did not significantly compromise the dimensions of the channels, the roughness of the walls of the channels increased considerably when the same master was used multiple times. We observed a 2-fold increase in the root-mean-square (*rms*) of the profile traces of the bottoms of the channels obtained from masters that were reused six times (Figure 3). Microscope images of cured PDMS revealed black spots throughout the wall surfaces of the formed channels. Therefore, we believe that the observed increase in the roughness of the PDMS channel walls with reuse of the masters resulted from pulling off some of the toner particles from the printed relief features during the removal of the cured polymer. This loss of toner particles was the principal source of deterioration of the masters upon multiple uses. Therefore, the thinner sections of the channels and the areas of attachment of the polyethylene elements have to be examined for defects before reusing the masters.

The surface topography of the printed features on the masters, as well as the topography of the PDMS walls of the micro-

channels, reflects the LaserJet printing process. To form printed features, the toner particles are piled over one another and held together by the binder polymer. The topography of the surfaces of the PDMS walls of the channels clearly follows the external curvatures of the packed toner particles (Figure 4). The difference between the lowest and the highest points throughout the PDMS surfaces of the channels is about 1 μm (Figure 4c). The average *rms* value of the topography of the PDMS bottoms of the channels calculated from the AFM data is 0.20 ± 0.08 μm, which is slightly lower than the *rms* calculated from profilometry data (Figure 3).

To test the influence of the topography roughness of the PDMS surfaces on the microfluidic dynamics, we passed parallel flows of various aqueous solutions through microchannels that were about 1 cm long. The solution currents remained laminar, and we did not observe mixing along the lengths of the channels. Therefore, the roughness of the topography of the PDMS surfaces in the channels fabricated with LaserJet-printed masters does not appear to disturb the laminar nature of parallel microcurrents.

Dimensions and Shapes of the Relief Features. The lateral dimensions of the relief features on a master (and consequently, the features imprinted on the surface of a polymer block) were readily manipulated using the CAD software. The height of the relief features, however, showed dependence not only on the printing mode but also on the width of the printed channels. Profilometry measurements showed that LaserJet-printed masters produced features with inclined vertical walls resulting in channels with trapezoidal cross sections (Figures 1b, 2b, 3, and 5a).

The resolution of the printer sets the possible minimum of the lateral sizes that can be reproduced on the master. Although theoretically 1200 dpi resolution sets an absolute minimum of the lateral dimensions at about 21 μm, we could not reproducibly achieve the fabrication of microfluidic channels thinner than about 70 μm. The size of the toner particles and the spatial dispersion during their deposition on the printed surfaces set a constraint on the width of the printed channels. For printed features that are as thin as 20 μm, the toner particles are quite scattered over the printed area and do not form continuous lines (Figure 4a). The observed dispersion of toner particles makes the fabrication of channels thinner than ~50 μm quite susceptible to discontinuity defects formed during the printing process.

The height of the relief features and, hence, the depth of the formed channels depend on the amount of toner deposited on the surface of the master. We observed that the depths of the imprinted channels tend to decrease as they become narrower than about 200 μm (Figure 5), indicating that for increased resolution, when printing small features, the LaserJet printers decrease the amount of toner particles deposited per unit area. The observed trend of channel depth–width dependence was not identical for the different LaserJet printers we used. For example, upon altering the widths, the depths of the channels produced from masters printed with an HP 4200 LaserJet printer varied significantly less than the depths of the channels from masters printed with an HP 400TN printer (Figure 5). The masters for the spore-detection devices were printed with an HP 4200 LaserJet printer, and the heights of the different channels ranged between about 5 and 9 μm (Figure 1b).

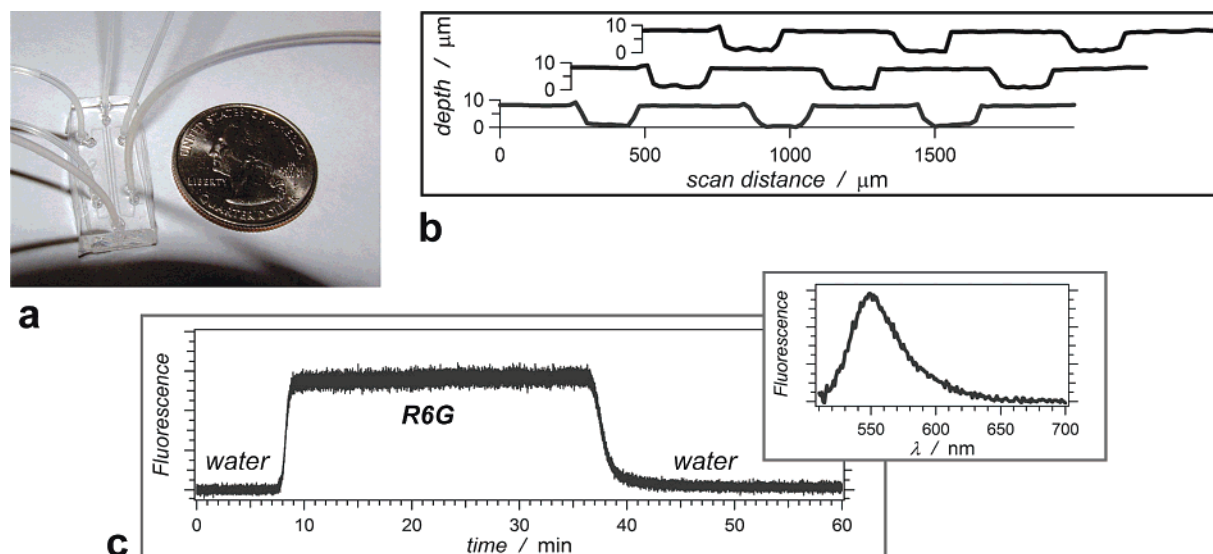


Figure 2. Flowing liquids through nonlithographically fabricated microchannels: (a) a photograph of a nonlithographically fabricated microfluidic flow-cell device next to a U.S. quarter. (b) Profilometry traces, perpendicular to three parallel channels, recorded at the surface of a PDMS component. The width of the three parallel channels is $200\ \mu\text{m}$. (c) A fluorescence signal was recorded as water and an aqueous solution of rhodamine 6G (R6G), $10\ \mu\text{M}$, are alternatively made to flow through the middle channel of the device. For this measurement, the microfluidic device was placed in a 1-cm cuvette holder of a spectrofluorometer ($\lambda_{\text{ex}} = 500\ \text{nm}$, $\lambda_{\text{em}} = 550\ \text{nm}$). (Inset) Fluorescence spectrum recorded as a R6G solution was made to flow through the middle channel of the device.

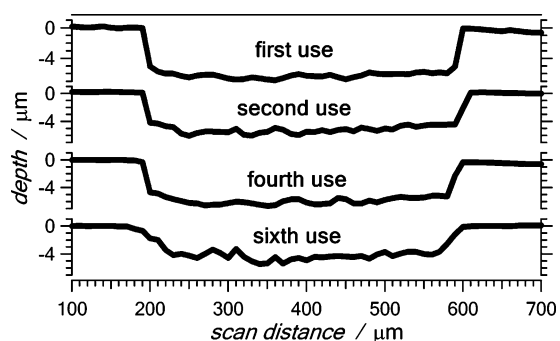


Figure 3. Profilometry traces across $400\text{-}\mu\text{m}$ channels on PDMS blocks prepared by reusing the same master (printed with HP 4050N LaserJet printer). After the cured polymer was removed from the master, fresh PDMS prepolymer was poured over the same master and allowed to cure. This process of reusing the master was repeated six times. The dimensions of the formed channels, average height, $\langle h \rangle$, and average width, $\langle w \rangle$, as well as the root-mean-square, rms , of the traces at the bottom of the channels are: for the first use: $\langle h \rangle = 4.4\ \mu\text{m}$, $\langle w \rangle = 398\ \mu\text{m}$, $rms = 0.31\ \mu\text{m}$; for the second use: $\langle h \rangle = 4.2\ \mu\text{m}$, $\langle w \rangle = 395\ \mu\text{m}$, $rms = 0.48\ \mu\text{m}$; for the fourth use: $\langle h \rangle = 4.2\ \mu\text{m}$, $\langle w \rangle = 399\ \mu\text{m}$, $rms = 0.46\ \mu\text{m}$; and for the sixth use: $\langle h \rangle = 4.2\ \mu\text{m}$, $\langle w \rangle = 395\ \mu\text{m}$, $rms = 0.59\ \mu\text{m}$.

To controllably increase the heights of the relief features, we repeatedly printed the CAD patterns on the same masters (Figure 6). The significant drawback of this approach is the requirement for exact alignment of the transparency films on the printer tray prior to each process of reprinting. The smallest observed deviations in the positions of consecutively printed patterns were in the order of a tenth of a millimeter. For example, two consecutive prints of: (1) $50\text{-}\mu\text{m}$ wide channels resulted in a wider channel with the same depth and a double-dip cross section (Figure 6, second trace); (2) $100\text{-}\mu\text{m}$ channels resulted in a twice wider channel with the same depth and a trapezoidal cross section (Figure 6, second trace); and (3) $200\text{-}\mu\text{m}$ wide channels resulted in a 50% increase in both the channel depth and the channel width (Figure 6, second trace). Three and four consecutive prints of the CAD pattern on the same master increased not only the depths but also the widths of the channels.

Furthermore, multiple-print masters produced channels with round-bottom cross sections (Figure 6). These findings suggest that the multiple-print approach for increase in the channel depths is not feasible for channels thinner than about $200\ \mu\text{m}$.

Unlike the rest of the microfluidic channels, the mixing chamber and the connecting channels, formed from the auxiliary 3D elements, have circular cross sections with smooth walls and diameters of $\sim 0.58\ \text{mm}$ (Figure 1c).

Sample Injection, Mixing, and Dilution. For testing the functionality of the devices and, in particular, the passive mixing of the sample with the principal reagent,²⁴ we recorded a series of injections of an opaque sample. Small volumes ($2\text{--}20\ \mu\text{L}$) of an aqueous solution of crystal violet were introduced through the injection channel, while neat water was constantly flowing as a principal reagent (Figure 7).

For each injection, we decreased the volume of the syringe with a micromanipulator, creating pressure in the injection reservoir higher than the pressure in the reagent channel. Due to the pressure difference, the sample solution flowed into the reagent channel until the pressure was again equilibrated. The rate, at which the volume of the syringe was decreased during the injection, did not interfere with the measured amount of injected sample. In most cases we observed that, except at the initial time of each injection, the injection channel was filled predominantly with the principal reagent (Figure 7). Due to the small volume of the injection channel, i.e., $\sim 600\ \text{pL}$, the uncertainty resultant from the diffusion of the principal reagent into the injection channel can be neglected for injections $\geq \sim 1\ \mu\text{L}$.

The clear separation between the laminar flows of the dye solution and the water, observed before the mixing chamber, was not evident in the detection channel. This observation demonstrates the efficiency of the passive mixing we incorpo-

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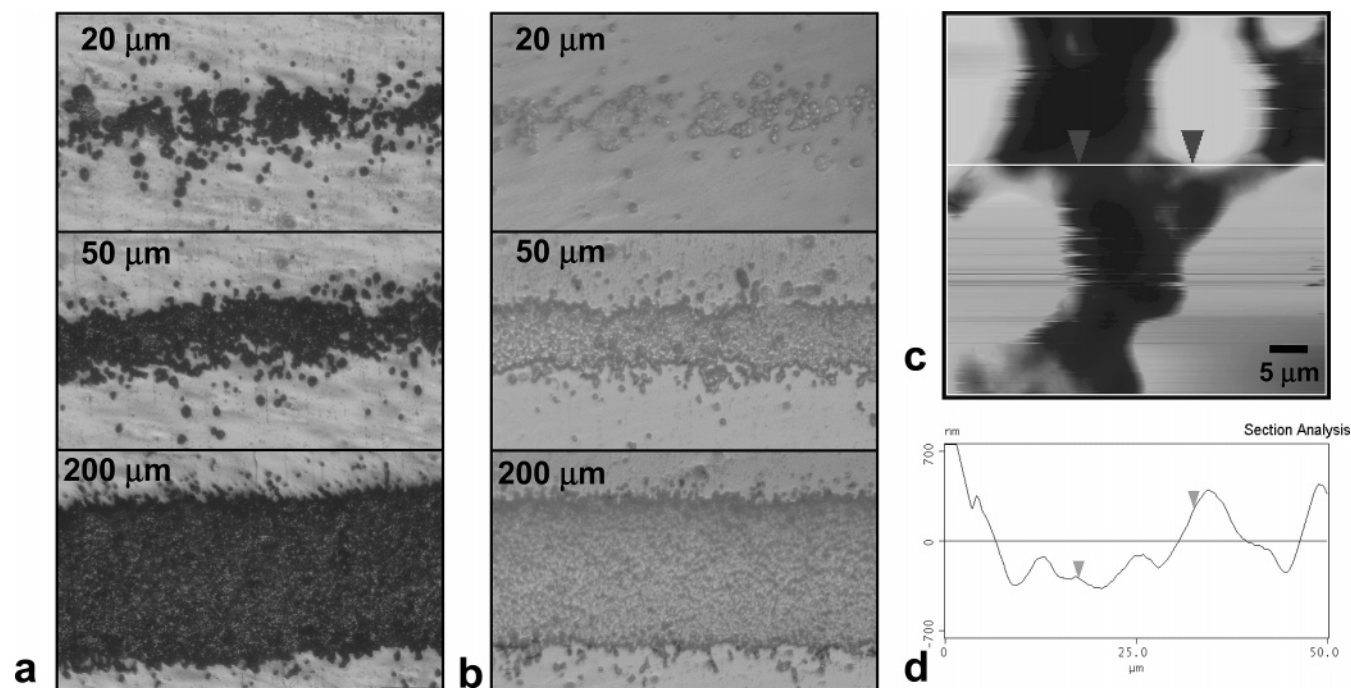


Figure 4. Topography of features printed with a LaserJet HP 1320 printer: (a) reflection microscope images of lines with various widths (20, 50, and 200 μm) printed on a transparency film, (b) reflection microscope images of negative-relief lines with various widths on the surface of a PDMS block cured over features printed with a LaserJet printer (the microscope was focused on the bottom of the negative-relief features), (c) atomic force microscopy (AFM) image (tapping mode) of the PDMS bottom of a channel formed from features printed with a LaserJet printer, and (d) section-analysis trace as indicated on the AFM image.

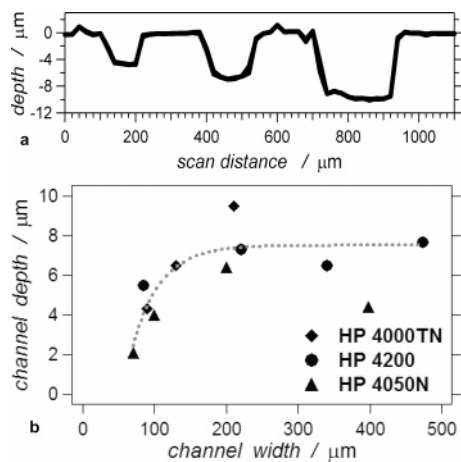


Figure 5. Correlation between the depth and the width of channels prepared from LaserJet-printed masters. (a) Profilometry traces, perpendicular to three parallel channels with different sizes, recorded at the surface of PDMS blocks. The master was printed with an HP 4000TN LaserJet printer. (b) Depth vs width relation for channels prepared with masters printed with three different LaserJet printers.

rated in the described devices. To quantify our observations, we performed a temporal and special analysis of the grayscale distribution at selected regions of the images collected with the video camera during the injections. We observed significant broadening of the signal from the opaque sample after passing through the mixing chamber (Figure 8b). In addition, the distribution of the opaque sample across the channel was changed and became more uniform (i.e., with the maximum intensity closer to the center of the channel) after passing through the mixing chamber (Figure 8c). These findings suggest there is achievement of efficient mixing and significant dilution of the sample with the principal reagent before entering the detection area of the device.

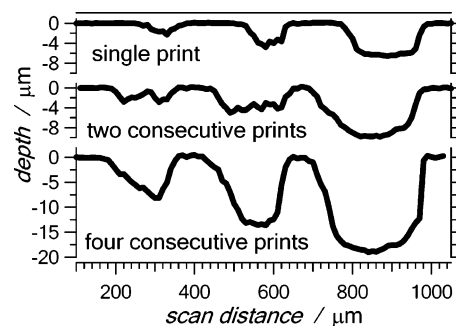


Figure 6. Profilometry traces showing the increase in the channel depths and widths as the CAD pattern is printed with an HP 4050N LaserJet printer on the same master two and four consecutive times. The traces were recorded perpendicularly to three parallel channels with initial widths of 50, 100, and 200 μm .

The thickness of the detection channel is about 50 times smaller than the thickness of the mixing chamber (Figure 1b,c) resulting in the difference in the grayscales of the image of the same solution when it is at the end of the mixing chamber and in the detection channel (Figures 7, 8a, and 9a). The angle of viewing the circular channel at the end of the mixing chamber additionally increases the observed difference in the grayscales: i.e. the diluted dye solution is significantly darker at the exit of the mixing chamber than in the detection channel (Figure 7).

Note, however, that in Figure 9c, while we have manually offset the individual curves laterally (i.e., along the x -axis) for their better distinction, the vertical steps that successively raise the baseline optical density have been reproduced exactly as measured. This overall darkening with time (injections at each volume were repeated four times; only one example of each is shown) was due to a gradual deposition of dye in the detection channel (resulting in the dark spots in Figures 8a and 9a). This

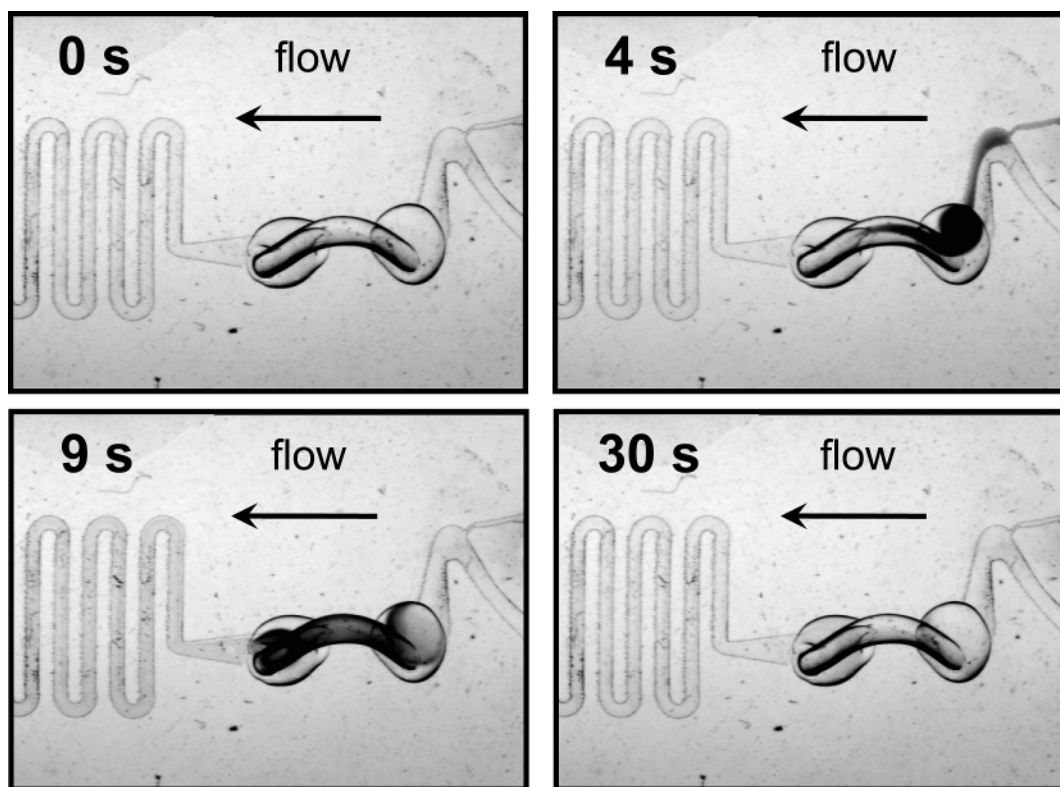


Figure 7. Transmission optical microscope images of a continuous-flow microfluidic device (top view) recorded during an injection of $6 \mu\text{L}$ of an aqueous solution of crystal violet (1 mM). Water flowed as a principal reagent at $0.96 \mu\text{L/s}$.

effect, along with the underexposure of a number of video pixels at higher injection volumes, allows us to draw only semiquantitative conclusions from the shown data. Nevertheless, the curves in Figure 9c truthfully reflect the increasing volumes of injected dye, and in particular Figure 9b provides an intriguing demonstration of the feasibility of this optical approach. As expected, in Figure 9b the time course of the “detection area” is paralleled by the mean density curve of region 2 (located inside the detection area shown in Figure 9a). Clearly, more detail is resolved by the latter curve. The curves of region 1 in Figures 8b and 9b, on the other hand, reveal the time interval and time course of the injection, reproducing even the oscillating action of the peristaltic pump used here to inflow water through the principal reagent channel. Comparison of the curves of regions 1 and 2 in Figures 8b and 9b shows a marked delay of the appearance of the maxima of the densities (about 5 s: see inset of Figure 8b), along with a lowering of the peak heights due to the dilution of the injected dye in the mixing chamber.

The cross section of the mixing chamber is about 75 times larger than the cross section of the principal-reagent channel (Figure 1b,c), causing significant decrease in the linear flow velocity of the solutions when entering the mixing chamber. This velocity decrease, together with the turns in the flow direction, could be the reason for the observed mixing.

Injection of DPA-Containing Samples. We tested the sensitivity of our devices with a series of injections of various volumes of DPA solutions with different concentrations. The principal reagent was a buffered solution of TbCl_3 . The excitation wavelength was set at 280 nm. During the injections of the DPA solutions, we recorded the changes in the emission intensity ($\lambda_{\text{em}} = 542 \text{ nm}$) with time. These injection signals appeared as temporal emission peaks (Figures 10a and 11a).

The shapes of the time-resolved curves recorded for the injections of the solution of crystal violet (Figures 8b, 9b,c) are identical with the shapes of the emission signals recorded for the DPA and spore samples (Figures 10a, and 11a). Furthermore, the temporal emission signals (Figures 10a and 11a) appeared about 6–10 s after the sample injections: it is similar to the delay we observed in the experiments with the opaque dye (Figure 8b, inset). Therefore, we believe that the analysis involving an opaque dye, described in the previous section, represents the fluidic processes occurring during the continuous-flow emission measurements.

We observed an excellent linear correlation between the areas of the temporal signal peaks and the amount of injected DPA (Figures 10d and 11b). The heights of the signal peaks, however, showed dependence not only on the amount of DPA injected but also on its concentration: i.e. for the same amount of DPA, the injection of small volumes of concentrated samples resulted in taller peaks than the injection of large volume of less concentrated samples. Therefore, the peak area is the quantity that we used for the data analysis.

Using glass (instead of quartz) for the fabrication of the microfluidic devices (Scheme 2d), offers the advantage of low cost. Despite the limited transparency of the glass material in the UV region, we were able to use PDMS/glass devices for conducting DPA-detection experiments based on emission enhancement of terbium (III) ions (Figure 10). The PDMS/glass microfluidic devices allowed us to detect nanomolar amounts of DPA: e.g. $C_{\text{DPA}} = 100 \mu\text{M}$, $V_{\text{inj}} = 10 \mu\text{L}$ with $S/N \approx 1 \text{ dB}$ (Table 1), which set the sensitivity limit of this approach with PDMS/glass devices.

Yet, the principal disadvantage of the devices made of glass slides is their opacity in the far-UV region: i.e. at the excitation

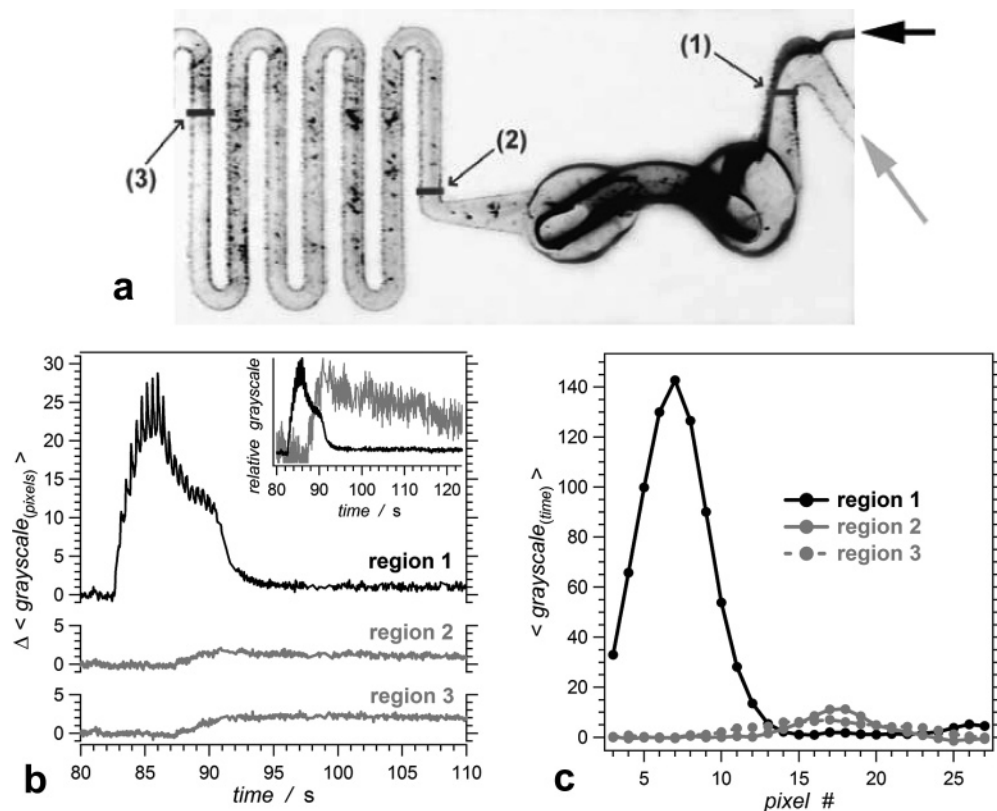


Figure 8. Injection of 2 μL of an aqueous solution of crystal violet in a device with water (0.96 $\mu\text{L/s}$) continuous flowing as a principal reagent. (a) Videomicrograph of the device during an injection with the cross sections of the channels, used for the image analysis, indicated with numbered black rectangles: (1), (2), and (3). The dark spots throughout the channels resulted from the dye staining the walls of the channels after multiple usage of the device. (b) Time-resolved changes in the grayscale averaged over the pixels covering the regions marked with black rectangles in Figure 8a. The intensity oscillations of the trace representing "region 1" result from the pulses in the flow of the principal reagent generated by the peristaltic pump. (Inset) Normalized curves representing the time-resolved grayscale changes in the regions 1 and 2. (c) Distribution of the grayscale intensity along the cross sections perpendicular to the channels recorded at the maxima of the time-resolved traces in b.

wavelength, 280 nm, the optical transmission of the glass slide that we used is only 6%. Using devices made of quartz and PDMS allowed us to significantly increase the sensitivity of this method of detection (Figure 11). While injection of 1 nmol DPA in PDMS/glass devices, for example, resulted in signals with $S/N \approx 1$ dB, the injections of 40 pmol DPA in PDMS/quartz devices (e.g., $C_{\text{DPA}} = 10 \mu\text{M}$, $V_{\text{inj}} = 4 \mu\text{L}$) resulted in signals with $S/N \approx 2.3$ dB (Table 2).

Injection of Spore-Containing Samples. Under identical conditions, we conducted tests with samples containing spores of *Bacillus subtilis* (variety "niger") also known as *B. atrophaeus* and *B. globigii* (Figure 12).²⁵ The three methods for extraction of DPA from bacterial spores are: (1) mechanical breaking of the spores, (2) causing the spores to germinate and release their DPA, and (3) chemical breaking of the spores at elevated temperature.¹⁷ We utilized the latter. The spore samples were thermally treated in the presence of dodecyl amine prior to the injections. The quantity of DPA released from the spores during treatment with microwave radiation was 1.3% ($\pm 0.5\%$) of their dry weight. Conventional thermal heating, at $\sim 95^\circ\text{C}$ for about 30 min, resulted in extraction of DPA amounting to 0.7% ($\pm 0.4\%$) of the dry-spore weight (Figure 13). The amounts of DPA extracted from the spore samples is lower than previously

reported.^{19,26} This discrepancy most probably is a result of the inefficiency of the extraction procedure due to unexpectedly high thermal resistance of the bacterial spores we used; microscope images revealed a significant number of intact spores in samples that were thermally treated with microwaves or conventional heating in the presence of dodecyl amine.

Injections of microwave-pretreated samples of bacterial spores in a continuous-flow device, through which terbium (III)-containing principal reagent was made to flow, yielded emission signals resultant from the DPA released from the spores (Figure 14). Because the targeted analyte is DPA (a natural product abundant solely in bacterial spores),²⁷ vegetative bacteria and other biological samples are unlikely to give false positives (Figure 14a). Inherently, a spore-detection method based on DPA sensing cannot provide discrimination between different types of spores: e.g. we cannot discern virulent from nonvirulent species by monitoring the changes in the emission of lanthanide ions.

Calibration of the devices with DPA samples with known concentrations (Figure 11b), allowed us to determine that injection of 6×10^5 , 1.2×10^6 , 1.8×10^6 , and 3×10^6 spores resulted in emission signals that correspond to 3.2 ± 0.7 ng, 5.6 ± 1.6 ng, 8.1 ± 0.8 ng, and 14 ± 5.4 ng DPA, respectively.

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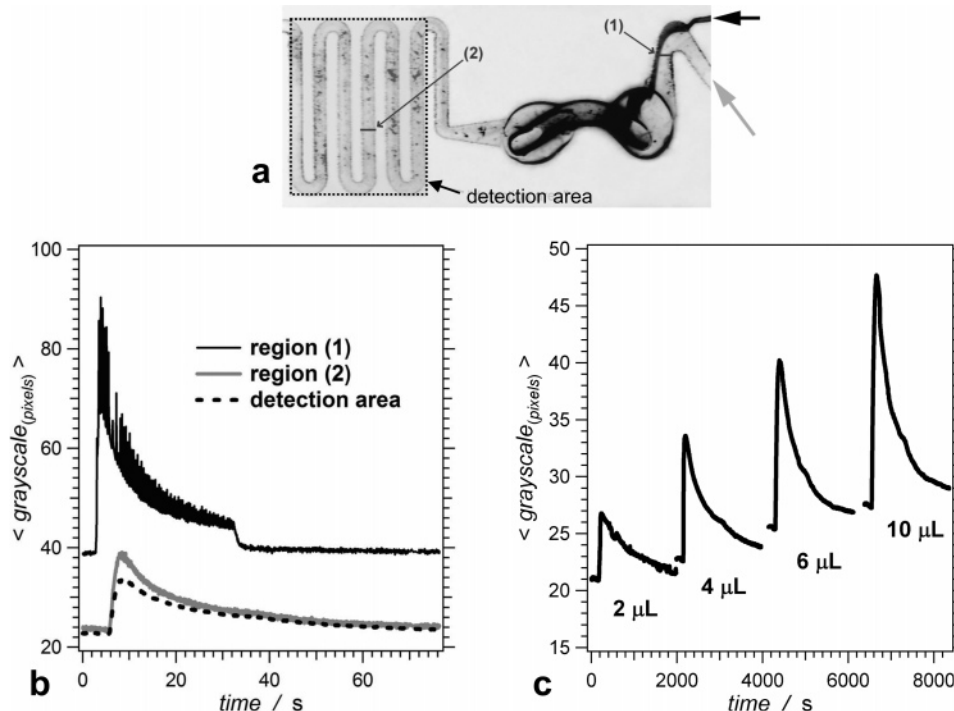


Figure 9. Injection of aqueous solution of crystal violet in a device with continuous flow of water ($0.96 \mu\text{L/s}$) as a principal reagent. (a) Videomicrograph of the device during an injection of the crystal violet solution with the cross sections of the channels indicated with black rectangles numbered with (1) and (2), and the detection area (Scheme 1), used for the image analysis. The dark spots throughout the channels resulted from the dye staining the walls of the channels after multiple usage of the device. (b) Time-resolved changes in the average optical densities of the cross-sectional regions and the detection area during the injection of $4 \mu\text{L}$ of crystal violet solution. (c) Time-resolved changes in the average optical densities of the detection area during consecutive injection of 2, 4, 6, and $10 \mu\text{L}$ of crystal violet solution. The individual curves are offset along the x -axis for better distinction.

Assuming that a single bacterial spore weighs $0.5 \pm 0.2 \text{ pg}$, we estimated that the amount of DPA measured with the microfluidic continuous-flow devices corresponds to $0.95 \pm 0.23\%$ of the dry weight of the pretreated spores injected in the devices. This number is in an acceptable agreement with the amount of released DPA determined from steady-state emission measurements (Figure 13).

Signal-to-Noise Ratios and Sensitivity. Although the peak area depends solely on the amount of the injected sample (DPA or spores), the peak height and, hence, the peak appearance depend on the sample concentration as well. Increase in the sample concentration (and decrease in the injection volume) leads to increase in the peak height and decrease in the peak width, i.e., the peak area remains the same,

$$C_{\text{DPA}}^{(1)} V_{\text{inj}}^{(1)} = C_{\text{DPA}}^{(2)} V_{\text{inj}}^{(2)}$$

Because the signal-to-noise ratio, S/N , depends on the peak height, h_{peak} (i.e., $S/N [\text{dB}] = 20 \log(h_{\text{peak}}/r.m.s)$), it is expected that increase in sample concentration will increase S/N and hence, improve the sensitivity of the measurements. Table 1 shows the values for S/N of the emission signals resultant from the injections of 1–20 nmol of DPA in a PDMS/glass continuous-flow device. For the detection of relatively small amounts of sample, i.e., $\leq 5 \text{ nmol}$ that is close to the detection sensitivity, the increase in the DPA concentration indeed leads to significant increase in S/N . For example, the signal from the injection of 2 nmol DPA from $200 \mu\text{M}$ solution has close to 4-fold higher S/N than the signal from 2 nmol DPA injected from $100 \mu\text{M}$ solution (Table 1). Similarly, tripling the DPA concentration for introducing 3 nmol DPA, leads to doubling

the S/N of the measured peaks (Table 1). Similar trends were observed for injections in PDMS/quartz devices (Tables 2 and 3).

Conclusions

Simplicity, expedience, and low cost are principal advantages of the described nonlithographic fabrication procedure. These advantages present venues for fast prototyping of microfluidic devices. The described nonlithographic approach offers a research and development alternative for environments where specialized microfabrication facilities are not available. We believe that the outlined continuous-flow type of microfluidic devices can be readily adapted for analyses of a broad variety of chemical and biological samples.

Experimental Section

Materials. Precleaned microscope glass slides (Corning, $75 \text{ mm} \times 50 \text{ mm}$) and quartz slides ($25 \text{ mm} \times 50 \text{ mm}$) were purchased from VWR and SPI Supplies, respectively, and cut into $25 \text{ mm} \times 10 \text{ mm}$ pieces before use. Pre-polymer of PDMS (Sylgard 184 silicone elastomer base kit) was purchased from Dow Corning Corporation. Polyethylene tubing was purchased from VWR. Polyethylene line cords (South Bend Corporation) were purchased from a hardware distributor. Transparency films (3M visual system division) were obtained from office-supply distributors. Terbium chloride hexahydrate (99.9%) was purchased from Stanford Materials Corporation and used without further purification. Crystal violet (95%) and 2,6-dipicolinic acid (DPA) were purchased from Sigma-Aldrich and used as received. Solvents, spectroscopic grade, were obtained from VWR.

B. subtilis (variety “niger”) endospore culture was obtained from the U.S. Army Research Laboratory facility, as desiccated powder. Before measurements, dry spores were suspended in deionized double

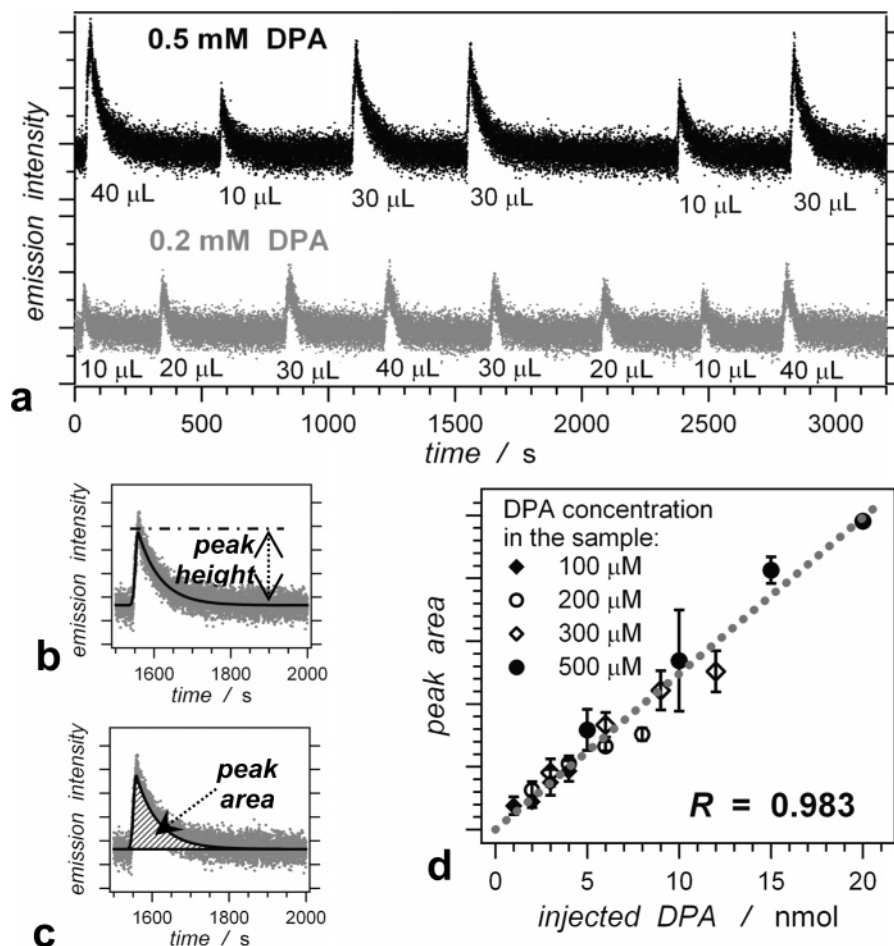


Figure 10. Detection of DPA with a PDMS/glass device using emission enhancement of Tb(III) ions. The reagent that flows through the microfluidic device is an aqueous solution of 1 mM TbCl₃ in 10 mM acetate buffer, pH = 6 ($\lambda_{\text{ex}} = 280$ nm, $\lambda_{\text{em}} = 542$ nm). (a) Emission signals recorded during injections of different volumes, V_{inj} , of aqueous solution of DPA with two different concentrations, C_{DPA} . For $C_{\text{DPA}} = 200$ μM , the recorded peaks are for $V_{\text{inj}} = 10, 20, 30, 40, 30, 20, 10,$ and 40 μL ; for $C_{\text{DPA}} = 500$ μM , the peaks correspond to $V_{\text{inj}} = 40, 10, 30, 30, 10,$ and 30 μL . (b) An emission injection peak resulting from the injection of 30 μL of 0.5 mM DPA with data fit of the change in the emission signal and definition of *peak height*; (c) definition of *peak area*. (d) Linear correlation between the peak area and the amount of DPA ($C_{\text{DPA}}V_{\text{inj}}$) injected, with the corresponding correlation coefficient, R .

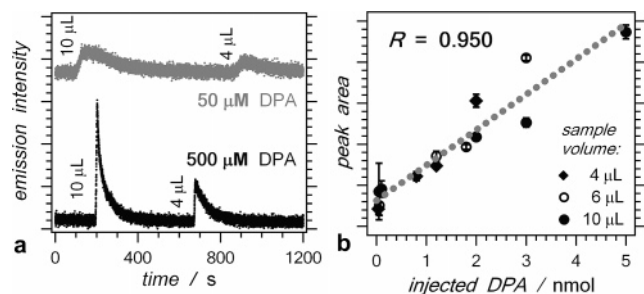


Figure 11. Detection of DPA with a PDMS/quartz device using emission enhancement of Tb(III) ions. The reagent that flows through the microfluidic device is an aqueous solution of 1 mM TbCl₃ in 10 mM acetate buffer, pH = 6 ($\lambda_{\text{ex}} = 280$ nm, $\lambda_{\text{em}} = 542$ nm). (a) Emission signals recorded during injections of 4 and 10 μL of 0.05 and 0.5 mM aqueous solution of DPA. (b) Linear correlation between the peak area and the amount of DPA ($C_{\text{DPA}}V_{\text{inj}}$) injected, with the corresponding correlation coefficient, R .

distilled water (DDW) and washed by centrifugation, with plenty of ice-cold DDW. In order to remove possible contaminants nonspecifically bound to spore coats, spores were additionally purified by density gradient centrifugation through sodium bromide (EMD Chemicals, ACS purity) 40% solution in DDW, with density 1.155.^{28,29} Bottom fractions

(28) Williams David, D.; Turnbough Charles, L., Jr. Surface layer protein EA1 is not a component of *Bacillus anthracis* spores but is a persistent contaminant in spore preparations. *J. Bacteriol.* **2004**, *186* (2), 566–569.

of thus purified cultures were washed with cold DDW and kept on ice as DDW suspensions. For the optical-microscopy imaging, 0.1% TWEEN-20 was added to the samples in order to suppress the formation of spore aggregates.

Vegetative bacteria were grown from the same spore cultures overnight (~18 h) in sterilized 2% Luria–Bertani medium (Sigma) at 30 °C, were shaken at 240 rpm in aerated tubes, until the density was $\sim 10^9$ cells/mL, and then washed with plenty of ice-cold DDW and kept as DDW suspensions on ice until use.³⁰ The bacteria were seen at 400 \times and 600 \times magnification as viable *Bacillus* colonies of average size of ~ 5 – 10 cells each. All cells were counted under a phase-contrast microscope at 400 \times using Petroff-Hausser chamber. Original concentrated spore stocks were diluted with DDW toward final count $\sim (7 \pm 3) \times 10^8$ per mL and stored at 2 °C in the dark for no longer than 48 h until use.

For the thermal treatments, dodecyl amine (DDA) was added to the vegetative bacteria or spore samples. The pH of the aqueous stock solution of DDA was adjusted to 6. The final concentration of DDA in the samples was 0.1 (w/w). The samples were heated either on a sand bath (thermostated at 95 °C) for about 30 min, or in a microwave

(29) Laflamme, C.; Ho, J.; Veillette, M.; Latremouille, M.-C.; Verreault, D.; Meriaux, A.; Duchaine, C. Flow cytometry analysis of germinating *Bacillus* spores, using membrane potential dye. *Arch. Microbiol.* **2005**, *183* (2), 107–112.

(30) Premasiri, W. R.; Moir, D. T.; Klempner, M. S.; Krieger, N.; Jones, G., II; Ziegler, L. D. Characterization of the Surface Enhanced Raman Scattering (SERS) of Bacteria. *J. Phys. Chem. B* **2005**, *109* (1), 312–320.

Table 1. Signal-to-Noise Ratios, S/N , of Measured Emission Signals from Injection of DPA Solutions in a PDMS/Glass Device^a

C_{DPA} (μM)	10 μL	20 μL	30 μL	40 μL
100	0.926 \pm 0.628 dB (1 nmol)	2.96 \pm 0.72 dB (2 nmol)	5.74 \pm 0.38 dB (3 nmol)	6.41 \pm 0.59 dB (4 nmol)
200	11.0 \pm 0.8 dB (2 nmol)	13.4 \pm 0.3 dB (4 nmol)	14.4 \pm 0.4 dB (6 nmol)	14.9 \pm 0.3 dB (8 nmol)
300	11.7 \pm 0.5 dB (3 nmol)	15.4 \pm 0.9 dB (6 nmol)	15.9 \pm 0.5 dB (9 nmol)	17.5 \pm 1.2 dB (12 nmol)
500	14.8 \pm 0.4 dB (5 nmol)	18.2 \pm 1.6 dB (10 nmol)	19.7 \pm 0.4 dB (15 nmol)	20.8 \pm 0.6 dB (20 nmol)

^a Results for four different injection volumes and four different concentrations, C_{DPA} , are shown. The values of S/N are reported in dB. The amounts of injected DPA are displayed in parentheses under the corresponding S/N values.

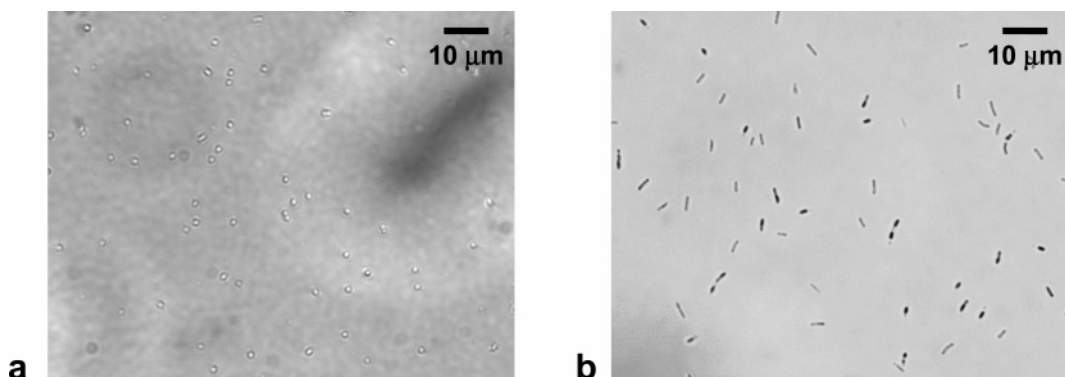


Figure 12. DIC microscope images of *B. subtilis* (variety “niger”): (a) dormant endospores from a suspension, purified by density-gradient centrifugation and stored at 2 °C (the samples were allowed to warm up room temperature for 2 h prior taking the image) and (b) vegetative bacteria from an aqueous solution stored at 2 °C for 24 h and allowed to warm up to room temperature for 2 h. The samples were “wet mounts”: 10 mM aqueous Tris buffer (pH = 7.6) and 150 mM saline. No changes in the viability of the culture were observed in an 8-h period. Storage at 2 °C for about 96 h resulted in less than 10% sporulation.

Table 2. Signal-to-Noise Ratios, S/N , of Measured Emission Signals from Injection of DPA Solutions in a PDMS/Quartz Device^a

C_{DPA} (μM)	4 μL	6 μL	10 μL
10	2.29 \pm 0.98 dB ^b (40 pmol) ^c	5.13 \pm 0.25 dB (60 pmol)	7.40 \pm 0.92 dB (100 pmol)
50	12.1 \pm 0.3 dB (200 pmol)	14.5 \pm 0.4 dB (300 pmol)	19.5 \pm 0.7 dB (500 pmol)
100	14.4 \pm 0.9 dB (400 pmol)	17.6 \pm 0.8 dB (600 pmol)	19.5 \pm 0.7 dB (1 nmol)
200	16.5 \pm 0.9 dB (800 pmol)	22.7 \pm 1.2 dB (1.2 nmol)	26.1 \pm 0.7 dB (2 nmol)
300	20.6 \pm 0.7 dB (1.2 nmol)	24.9 \pm 0.9 dB (1.8 nmol)	28.1 \pm 0.9 dB (3 nmol)
500	24.1 \pm 0.5 dB (2 nmol)	28.5 \pm 0.1 dB (3 nmol)	31.9 \pm 0.1 dB (5 nmol)

^a Results for three different injection volumes and six different concentrations, C_{DPA} , are shown. ^b The values of S/N are reported in dB. ^c The amounts of injected DPA are displayed in parentheses under the corresponding S/N values.

(Sanyo, EM-S5002W) at maximum power for about 20 s. To compensate for the evaporation during heating, DDW was added to the cooled treated samples up to their original volumes.

Microfabrication. The computer-aided-design (CAD) images of the microfluidic devices were printed on a polyester transparency film (3M Inc.) using Hewlett-Packard LaserJet 4200, 4000TN, 4050N, or 1320 printers. Up to 11 device patterns, shown in Figure 1a, were printed in a circular area of about 50 cm². The circular areas with the printed reliefs were cut off the transparencies and immobilized at the bottom of polystyrene Petri dishes, which we used for molds for the polymer components. Polyethylene posts and other three-dimensional (3D) cavity-forming elements were adhered to the top of designated areas (e.g., on the reservoirs at the termini of the channels) on the master printouts. PDMS pre-polymer was poured into the Petri dish with the master. After 2 h thermal curing (at 75 °C), the PDMS block with the

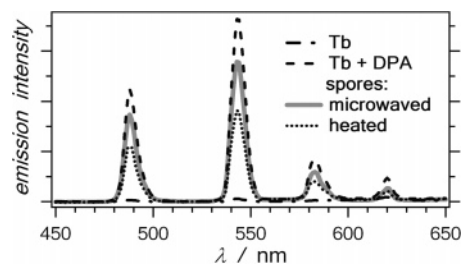


Figure 13. Emission spectra ($\lambda_{\text{ex}} = 280$ nm) of TbCl_3 (1 mM) by itself and in the presence of DPA (1.2 μM = 0.2 mg/L) and thermally treated spores of *B. subtilis* (8.3 mg/L). All samples were buffered with 10 mM acetate buffer, pH 6. The spores were thermally pretreated in an aqueous solution of dodecylamine (0.1% w/w, pH 6) by microwaving for 20 s or heating at ~ 95 °C for 30 min.

3D cavity-forming elements in it was removed from the Petri dish. The 3D elements were carefully removed from the bulk of the cured polymer, leaving cavities with the desired shapes and sizes. The PDMS components for the devices were cut from the polymer block. Precleaned glass or quartz slides (sequentially washed with hexane, ethanol, ethanol/ HCl_{conc} mixture, and water) and the PDMS components were treated with oxygen plasma (March Plasmod, March Instruments, Inc.), pressed against one another, and heated at 100–120 °C for ~ 12 h. The latter steps resulted in irreversible adhesion between the elastomer components and the slides, yielding the assembled devices. Polyethylene tubes (o.d. 1.09 mm, i.d. 0.38 mm, purchased from VWR) were inserted into the connecting channels of the device (Figures 1a and 2a).

Microscopy and Profilometry. The injection and mixing video images, as well as the cross-section images (e.g., Figures 1c, 7, 8a, and 9a), were recorded on a Zeiss SV11 stereoscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY) in a transmission mode. The images of the printed transparency films and the surface of the PDMS imprints (e.g., Figure 4a,b) were recorded on a Hirox KH-3000

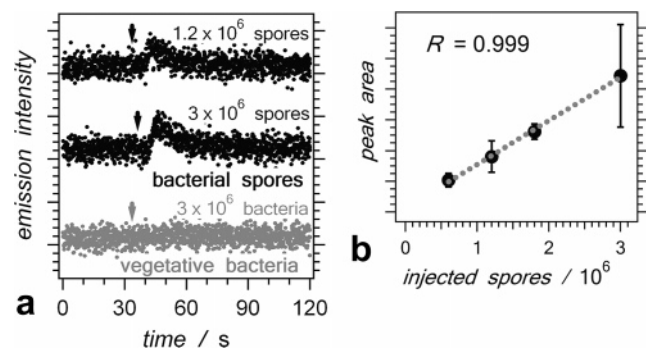


Figure 14. Detection of bacterial endospores with a PDMS/quartz device using emission enhancement of Tb(III) ions. The reagent that flows through the microfluidic device is an aqueous solution of 1 mM TbCl₃ in 10 mM acetate buffer, pH = 6 ($\lambda_{\text{ex}} = 280$ nm, $\lambda_{\text{em}} = 542$ nm). (a) Injection emission signals from microwave-pretreated samples of bacterial spores (*B. subtilis*, 3×10^8 spores/mL, $V_{\text{inj}} = 4$ and $10 \mu\text{L}$) and vegetative bacteria (*B. subtilis*, 3×10^8 cells/mL, $V_{\text{inj}} = 10 \mu\text{L}$). The amounts of injected spores and bacteria are shown above the data curves. The arrow heads indicate the times of injection of the samples. (b) Linear correlation between the area of the temporal injection peaks and the amount of spores injected, with the corresponding correlation coefficient, R .

Table 3. Signal-to-Noise Ratios, S/N , of Measured Emission Signals from Injection of Microwave-Pretreated Spore Suspensions in a PDMS/Quartz Device^a

C_{spore} (spores/mL)	V_{inj} (μL)	spores injected ($V_{\text{inj}} \times C_{\text{spore}}$)	S/N (dB)
3×10^8	2	6×10^5	4.03 ± 1.14
3×10^8	4	1.2×10^6	5.58 ± 1.47
3×10^8	6	1.8×10^6	8.10 ± 0.86
3×10^8	10	3×10^6	7.48 ± 2.71

microscope (Hirox-USA, Inc., River Edge, NJ) in a reflection mode. Slides of Bacillus spores and vegetative forms, mounted wet specimens (e.g., Figure 12), were observed with the Olympus BX-50 upright light microscope (Olympus, Inc., Melville, NY) using differential interference contrast (DIC) illumination, at magnifications $200\times$ (dry), $400\times$ (dry, water immersion), and $600\times$ (oil immersion).

Atomic force microscopy (AFM) images were collected with a commercial Nanoscope III (Digital Instruments, Santa Barbara, CA) using optical beam deflection to monitor the displacement of an aluminum-coated silicon nitride cantilever having a spring constant 40 N/m (Nanosensors). The AFM was performed in a tapping mode (150 kHz) under ambient laboratory conditions.

Profilometry data were collected on an Alpha-Step 500 profiler (KLA Tencor, San Jose, CA).

The roughness of the surface was determined by measuring the root-mean-square (rms) of the average of height (z) taken from the mean data planes.

Injection. A syringe filled with the sample solution was placed on a micromanipulator, connected to the sample inlet of the device (Scheme 1 and Figure 1a), and primed: i.e. the bubbles, if any, were removed, and the injection reservoir, as well as the injection channel, was filled with the sample solution. The peristaltic pump, which flowed the principal reagent, was turned on, and the device was allowed to equilibrate for a few minutes. For each injection, the volume of the syringe was decreased with the micromanipulator, creating pressure in the injection reservoir higher than the pressure in the reagent channel.

The grayscale image analysis was performed with Windows software developed by Volkmar Heinrich (vheinrich@ucdavis.edu). This software allows the definition of up to eight regions of interest in a video image and records features like grayscale intensity profiles or average intensities for each region.

Emission Measurements. The steady-state and microfluidic emission measurements were performed on FluoroLog-3 (HORIBA Jobin Yvon, Inc., Edison, NJ) and PTI/FeliX (Photon Technology International, Inc., Birmingham, NJ) spectrofluorometers.

A spore-detection device was placed in a 1-cm cuvette holder of a spectrofluorometer. The detection area of the prepared spore detection device (Scheme 1) was illuminated with the excitation beam. The fluorometer was used in a time-based mode at constant excitation and emission wavelengths. The emission slit widths were set to 2 nm. Data points were recorded every 100 ms.

In all measurements, involving terbium (III) ions, the excitation and emission wavelengths were set at 280 and 542 nm, respectively. To block the scattered light from the excitation beam that partially passes through the emission monochromator as a $2 \times \lambda$ signal, we placed a long-wavelength-pass 395-nm optical filter on the emission optical path immediately before the emission monochromator.

Analysis of the Temporal Emission Data. For each sample concentration, C_{sample} , and injection volume, V_{inj} , at least five measurements were performed. The change in the emission during the injections was recorded. Due to the background fluorescence, the changes appeared as relatively weak signals on a strong DC background. The DC offset was subtracted from the collected data prior to the analysis. For each set of measurements of spore samples with a microfluidic device, the device was calibrated with DPA samples with known concentrations. The emission intensity scales for plotting the signals were chosen to give the best representation of the presented data (i.e., zoom without stretching out of scale), and hence, they are not identical in the different graphs.

Each temporal emission peak was fit to a curve that follows the changes in the emission intensity. Linear combinations of Gaussian and exponential functions were applied for data fits. The height of the peak is defined as the difference between the baseline and the peak maximum (Figure 10b). The peak area represents the total integrated area under the peak (Figure 10c). Student t -distribution was used for the error analysis. The error bars (e.g., Figures 10d, 11b, and 14b) were calculated for a confidence interval of 95%. The signal-to-noise ratios (S/N) were calculated from the peak height (h_{peak}) and the rms of the residuals obtained from the data fit of the signal: S/N [dB] = $20 \lg(h_{\text{peak}}/rms)$. All data analysis was performed on MSWindows XP-professional workstations equipped with IgorPro software, version 4.0 (Wavemetrics, Inc.).

Acknowledgment. Financial support for this work was provided by the U.S. Army Research Laboratory and the UCR Academic Senate Research Funds. We extend our gratitude to Prof. Nosang Myung and Ms. Silvia Lee for assisting with the imaging work presented in Figure 4a and b.

Supporting Information Available: Video recordings of sample injections. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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