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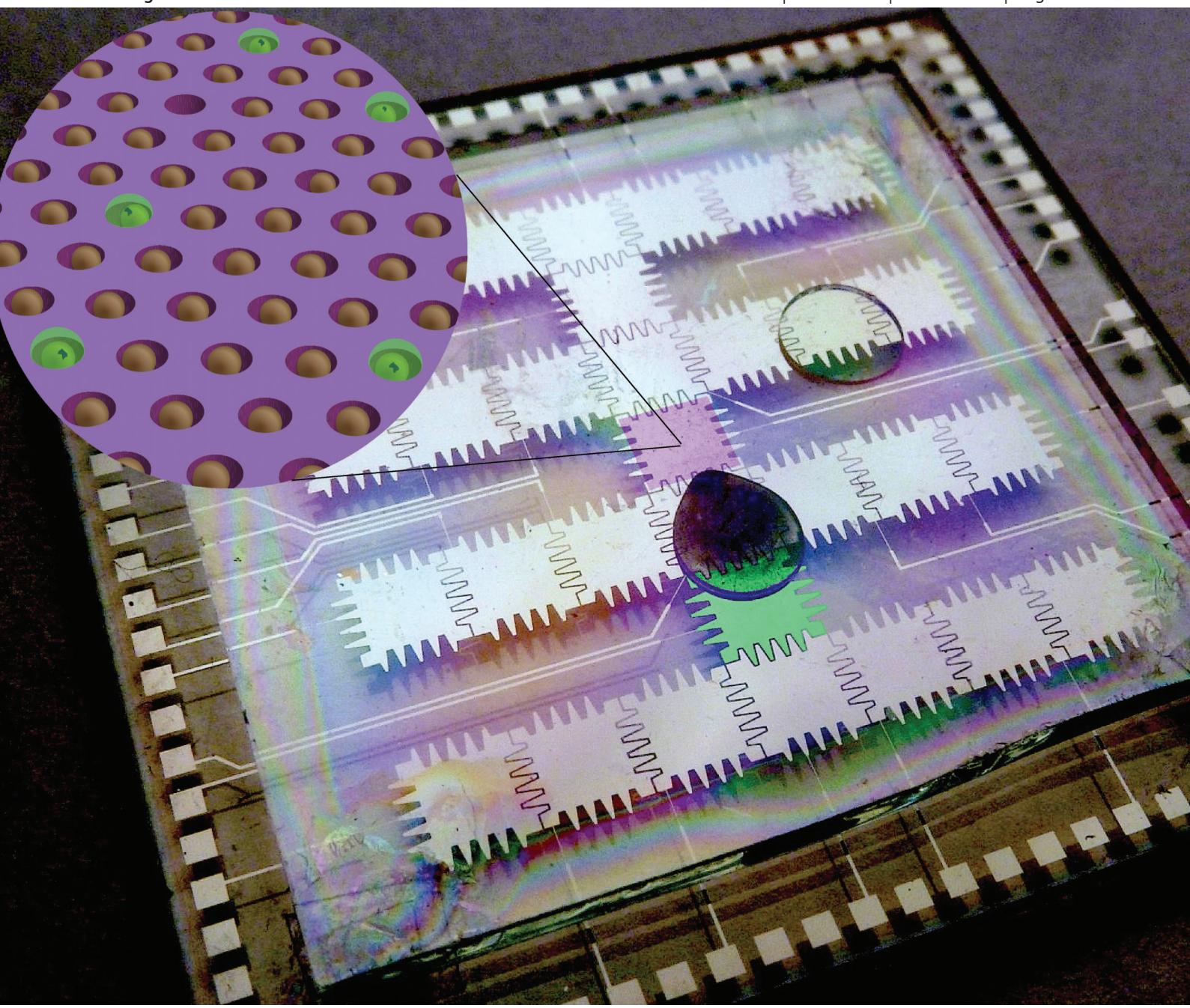

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Digital microfluidics-enabled single-molecule detection by printing and sealing single magnetic beads in femtoliter droplets

Daan Witters,[a] Karel Knez,[a] Frederik Ceyssens,[b] Robert Puers[b] and Jeroen Lammertyn*[a]

Digital microfluidics is introduced as a novel platform with unique advantages for performing single-molecule detection. We demonstrate how superparamagnetic beads, used for capturing single protein molecules, can be printed with unprecedentedly high loading efficiency and single bead resolution on an electrowetting-on-dielectric-based digital microfluidic chip by micropatterning the Teflon-AF surface of the device. By transporting droplets containing suspended superparamagnetic beads over a hydrophilic-in-hydrophobic micropatterned Teflon-AF surface, single beads are trapped inside the hydrophilic microwells due to their selective wettability and tailored dimensions. Digital microfluidics presents the following advantages for printing and sealing magnetic beads for single-molecule detection: (i) droplets containing suspended beads can be transported back and forth over the array of hydrophilic microwells to obtain high loading efficiencies of microwells with single beads, (ii) the use of hydrophilic-in-hydrophobic patterns permits the use of a magnet to speed up the bead transfer process to the wells, while the receding droplet meniscus removes excess beads off the chip surface and thereby shortens the bead patterning time, and (iii) reagents can be transported over the printed beads multiple times, while capillary forces and a magnet hold the printed beads in place. High loading efficiencies (98% with a CV of 0.9%) of single beads in microwells were obtained by transporting droplets of suspended beads over the array 10 times in less than 1 min, which is much higher than previously reported methods (40–60%), while the total surface area needed for performing single-molecule detection can be decreased. The performance of the device was demonstrated by fluorescent detection of the presence of the biotinylated enzyme β-galactosidase on streptavidin-coated beads with a linear dynamic range of 4 orders of magnitude ranging from 10 aM to 90 fM.

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Introduction

Optical single-molecule detection methods have become highly valuable analytical techniques for a wide variety of bio-assays and have lead to new insights in various biochemical processes.[1,2] Moreover, they have enabled a very sensitive detection of relevant biomolecules down to the attomolar range, which is difficult if not impossible to achieve with other detection methods.[3] The ability to detect low concentrations of biomolecules, such as protein biomarkers, is crucial for the early-stage detection of many diseases, and is therefore indispensable for improving diagnostic tools for public health in the near future.[4]

Several methods have been introduced to isolate and optically detect single molecules. One of the most prominent and promising approaches for detecting single molecules consists of isolating protein biomarkers in ultra-small reaction wells, where they are detected by labelling them with enzyme reporters to form sandwich immunocomplexes.[5,6] Sensitive detection of proteins is then performed by confining these proteins to small reaction wells, in which an amplifiable fluorescent signal is generated, spatially confined and detected.[6–8] Recently, this concept has been further extended and optimized using superparamagnetic beads for capturing proteins in a sample more efficiently, and subsequently seeding these beads in femtoliter-sized wells.[5] Nevertheless, this promising approach relies on a few existing miniaturization technologies capable of patterning and sealing single superparamagnetic beads in femtoliter-sized reaction chambers. Ideally, such technologies should be capable of patterning single beads in high-density arrays of femtoliter-sized chambers with nearly 100% loading efficiency on a short time

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scale and in an automated way, while accurately sealing every reaction well for accumulating fluorescence signals and preventing evaporation.

Several reports have been published on the use of fiber-optic arrays for patterning and sealing superparamagnetic beads for single-molecule studies.[5,9–12] Here, single beads are loaded with centrifugal forces into an array of microwells fabricated at the tip of a bundle of optical fibers before being mechanically sealed with a silicone gasket in the presence of a droplet of fluorogenic substrate, thereby isolating the beads in femtoliter-sized wells. Fiber-optic microarrays allowed the first bead-based detection of single protein molecules by performing digital enzyme-linked immunosorbent assays (digital ELISA).[5] Nevertheless, this method strongly relies on high centrifugal forces and the manual sealing of microwells, which requires technically skilled staff for performing single-molecule experiments and offers little prospect for automation. Furthermore, due to the nature of the bead loading process, the distribution of single beads in the microwell array is random. Therefore, wells containing a bead first have to be identified (*e.g.*, by fluorescent labelling) for the precise determination of the ratio of ''on'' and ''off'' wells in a digital assay format.

Recently, Kan *et al.* (2011) reported the use of sequential fluid flows over microfabricated polymer arrays for isolating single superparamagnetic beads in femtoliter chambers.[13] Beads were loaded inside the microwells by gravity, after which oil was flowed over the array to remove excess beads and to seal the trapped beads in their suspension liquid that contained the fluorogenic substrate for single-molecule detection. This approach offers a higher degree of automation for performing single-molecule analysis compared to the fiber-optic arrays. Zhang *et al.* (2012) demonstrated the integration of a fiber-optic array in a microfluidic PDMS channel.[14] The cladding material of the fiber-optic array was selectively modified with a hydrophobic silane using a contact printing method, while the inner surface of the wells remained hydrophilic. This allowed selective filling of the hydrophilic wells with an aqueous solution and effective sealing with a subsequent stream of oil. This novel method avoided the need of complex mechanical sealing procedures usually needed for fiber-optic arrays and facilitated the use of fiber-optic arrays in an automated setting. Finally, Kim *et al.* (2012) fabricated a device that employs 1 million femtoliter wells at the bottom of a PDMS microfluidic channel.[15] The hydrophilic microwells were fabricated in a CYTOP fluoropolymer layer in order to generate hydrophilic-in-hydrophobic patterns. A suspension of beads was manually loaded into the device, after which the beads could settle by gravity before being sealed with fluorinated oil. Streptavidin-β-galactosidase could be detected with a limit of detection of 10 zeptomolar, which was 20-fold more sensitive than previous studies due to the interrogation of a larger number of beads.

In this study, we introduce digital microfluidics (DMF) as a promising alternative for printing and sealing single superparamagnetic beads in femtoliter-sized wells for performing single-molecule detection. DMF is an emerging technology capable of manipulating individual micro- to nanoliter droplets on arrays of actuation electrodes covered with a hydrophobic insulating layer by using the principle of electrowetting-on-dielectric (EWOD).[16–18] We recently demonstrated how DMF technology enables printing of femtoliter droplets in a high-throughput fashion by transporting microliter-sized droplets over a Teflon-AF surface that contains hydrophilic-in-hydrophobic micropatterns, thereby passively generating thousands of pico- to femtoliter-sized droplets per second by exploiting the selective wettability of the hydrophilic patches.[19] Here, we show how this approach can be modified and used as a tool for performing single bead patterning and single-molecule detection. We demonstrate how DMF allows suspended microbeads to be printed with single-bead resolution and unprecedentedly high loading efficiencies (98%) of single beads per microwell by shuttling droplets of suspended beads over tailored microwell arrays (Fig. 1). Its ability to perform single-molecule detection is further demonstrated by digitally quantifying the biotinylated protein β-galactosidase (BβG) down to attomolar levels. Furthermore, DMF offers the prospect of integrating all fluidic manipulations on-chip, which provides the possibility to perform fully automated single-molecule assays.

Experimental

Reagents

BβG from *Escherichia coli*, the fluorogenic substrate fluorescein di(β-D-galactopyranoside) (FDG), bovine serum albumin (BSA), Tween-20, and $MgCl_2$ were purchased from Sigma-Aldrich (Bornem, Belgium). Lodestar superparamagnetic beads (2.7 μm diameter) functionalized with streptavidin were purchased from Agilent. Fluorinert FC-40 was obtained from 3M (St. Paul, MN, USA). Reagents for photolithography were obtained from Rohm and Haas (Marlborough, MN, USA) and Parylene-C dimer and Silane A174 were purchased from Plasma Parylene Coating Services (Rosenheim, Germany). Teflon-AF (6% w/w in FC-40) was obtained from Dupont (Wilmington, DE, USA). CR-7 chromium etchant was obtained from Cyantek (Fremont, CA, USA). The fluoroalkylsilane, Dynasylan F8263, was a kind gift from Evonik Degussa International AG (Essen, Germany). Vapor-Lock oil was obtained from Qiagen (Hilden, Germany).

DMF chip fabrication and operation: bottom plate

Glass wafers were rinsed in acetone, isopropyl alcohol and deionized water. A chromium layer (100 nm thickness) was then deposited *via* magnetron sputtering (Balzers BAE 370, Pfäffikon, Switzerland) and coated with S1818 positive photoresist before being exposed to UV-light through a chrome-on-glass photomask. Next, the photoresist was developed in 351 developing solution and the exposed chromium regions were etched with Cyantek CR-7 chromium etchant. Wafers were then cleaned in acetone and isopropyl alcohol to strip the photoresist, washed in deionized water and dried with a

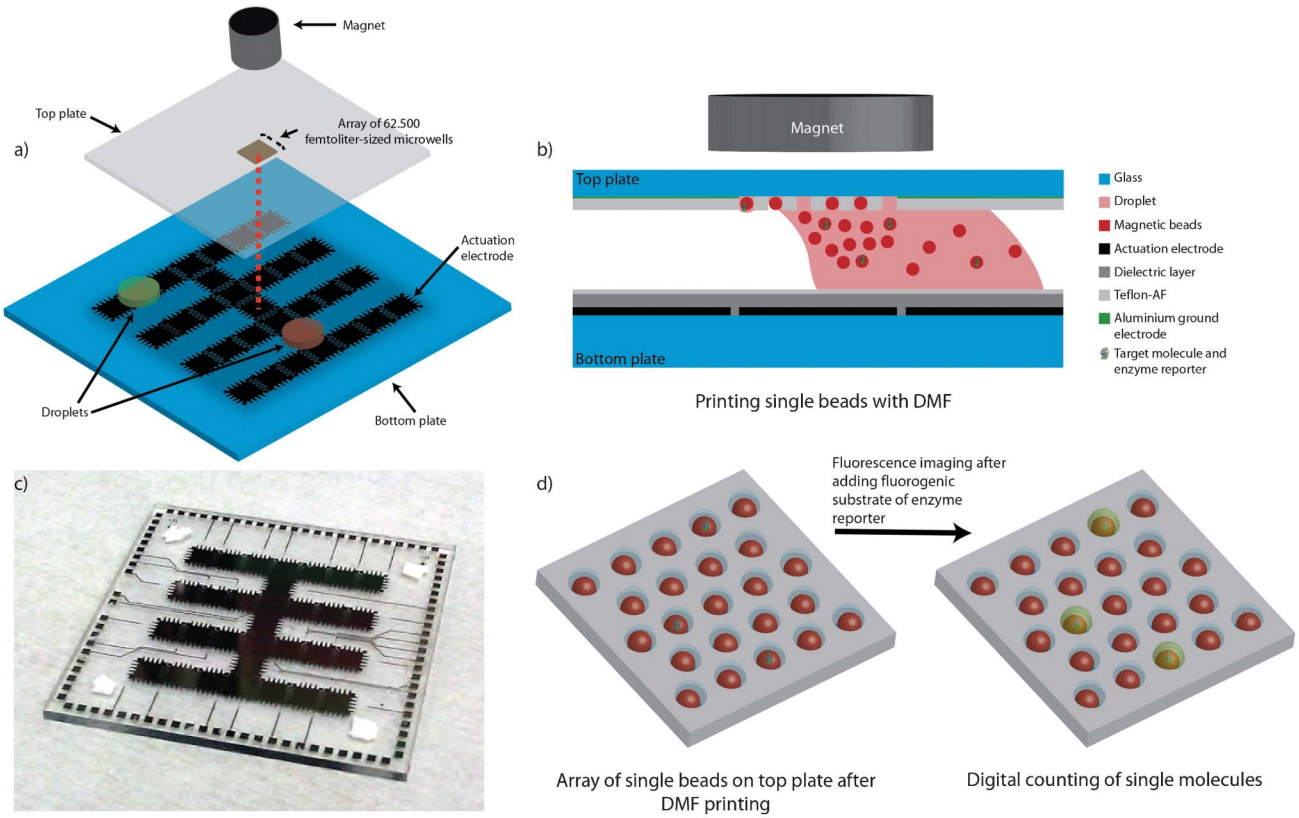

Fig. 1 Digital microfluidic (DMF) printing of single superparamagnetic beads for performing automated single-molecule detection. (a) An exploded view of a double-plate DMF device consisting of a bottom plate containing actuation electrodes, a dielectric layer and a Teflon-AF layer for electrostatic droplet actuation, and a top plate containing an array of 62.500 microwells designed for trapping and sealing single beads, centred above 1 actuation electrode. (b) The side view of a schematic representation of the DMF device demonstrating how suspended beads in a droplet are attracted towards the array of microwells, in which they are trapped, while the receding droplet meniscus removes excess beads off the surface. (c) A photograph of the bottom plate of the DMF chip used in this study. (d) A schematic representation of part of the array of printed single beads on the top plate. When single beads are isolated with the fluorogenic substrate of the enzyme reporter, only wells containing an enzyme reporter attached to a bead will fluoresce, allowing digital quantification of single molecules.

nitrogen gun. Subsequently, wafers were cleaned in O_2-plasma (120 s, 100 W), vacuum primed with silane A174 and coated with a ~3.5 μm layer of Parylene-C using chemical vapour deposition (AL 200, Plasma Parylene Coating Services, Rosenheim, Germany). Finally, a ~50 nm thin layer of Teflon-AF (3% w/w in Fluorinert FC-40) was spincoated (3000 rpm, 60 s) on top and baked for 5 min at 110 °C and 5 min at 200 °C. Every actuation electrode was connected to a contact pad at the edge of the chip. As illustrated in Fig. 1, DMF chips contained 31 actuation electrodes of 2.8 × 2.8 mm, each with inter-electrode gaps of 40 μm. Driving potentials of ~150 V_{DC} were applied to the actuation electrodes and controlled using homemade Matlab (MathWorks Inc., Natick, MA, USA) and Labview (National Instruments Corp., Austin, TX, USA) programs. Two pieces of double-sided tape (~300 μm thickness) were placed on the bottom plate as a spacer before both plates were manually aligned and placed on top of each other.

Fabrication of the top plate containing femtoliter-sized microwells

For capturing single beads, a thick layer of Teflon-AF was used on the top plate in order to trap the beads, preventing them from escaping from the microwells. We first coated clean cover glasses (0.7 mm thickness, Menzel-Gläser, Braunschweig, Germany) with a thin layer of aluminium *via* thermal evaporation, leaving a small window of 2.5 × 2.5 mm^2 free of aluminium in the centre of the top plate. The thin aluminium layer serves as a ground electrode for EWOD actuation and is still transparent enough to allow droplet visualization from above through a stereomicroscope. Teflon-AF exhibits a good adhesion to aluminium, but to improve the adhesion of Teflon-AF to the 2.5 × 2.5 mm^2 glass window in the centre of the top plate, it was first functionalized with a fluoroalkylsilane. Dynasylan F8263 was spincoated at 3000 rpm, rinsed in pure isopropanol and baked for 10 min at 100 °C. The wafers were then coated with a ~3 μm thick layer of Teflon-AF (6% w/w in fluorinert FC-40), which was spincoated at 500 rpm for 60 s and baked for 5 min at 110 °C and 10 min at 250 °C. For patterning of the Teflon-AF layer, a dry lift-off method was used.[20] Briefly, a Parylene-C mask (~500 nm thickness) was applied *via* chemical vapour deposition on top of the Teflon-AF layer. The deposited Parylene-C mask was then treated with a short O_2-plasma treatment (10 s, 42 sccm O_2, 100 mtorr, 60 W) to improve the adhesion of a metal hard mask needed for subsequent dry etching of the microwells. An

aluminium hard mask (100 nm thickness) was coated *via* thermal evaporation. This aluminium mask was patterned using standard photolithography using AZ1505 positive photoresist and wet chemical etching using Transene type A aluminium etchant. After patterning the aluminium hard mask, the exposed underlying Parylene-C and Teflon-AF layers were etched with O_2-plasma reactive ion etching (100 W, 150 mtorr, 42 sccm O_2) to reveal the array of hydrophilic patches on the glass surface. Finally, the Parylene-C mask was peeled off the top plate together with the aluminium hard mask using tweezers. By using this dry lift-off method, the hydrophobicity of the Teflon-AF layer remained intact, while standard photolithographic processes could be used to transfer the pattern to the Teflon-AF layer. This fabrication process resulted in 62.500 femtoliter-sized microwells of 4.5 μm diameter and 3 μm depth with an 8 μm centre-to-centre spacing, arranged in a hexagonal pattern in a square patch of 2 × 2 mm^2, which was centred in the transparent window of the aluminium ground electrode. The volume of each femtoliter well was calculated to be 38 fL.

On-chip femtoliter droplet printing and sealing

A solution of fluorescein in phosphate buffer was used for studying the principle of on-chip printing and sealing of femtoliter droplets in oil. A droplet of fluorescein (2.5 μL) was pipetted on the bottom plate of the chip before closing the device with the top plate. The droplet was then transported on-chip to the electrode, on top of which the array of microwells was aligned and incubated for 1 min to ensure all microwells were filled (Fig. 2). The filling of the microwells was observed with bright field microscopy as the contrast of a filled microwell and an unfilled microwell is clearly distinguishable (data not shown). After the microwells were filled, 90 μL of Qiagen Vapor-Lock oil was pipetted between both plates of the chip and held in place with capillary forces. Subsequently, the droplet of fluorescein was transported away from the array, thereby generating femtoliter-sized droplets of fluorescein inside the microwells because of their selective wettability. For visualization on an inverted fluorescence microscope, the chip was flipped upside down on the microscope stage after the droplet manipulations, which allowed easy visualization of the microwell array through the transparent window in the top plate.

On-chip printing of single superparamagnetic beads and single-molecule detection of biotinylated β-galactosidase

30 μL of Lodestar 2.7 streptavidin superparamagnetic beads (2.7 μm diameter) were washed in 470 μL washing buffer (PBS with 0.1% BSA and 0.1% Tween-20) 3 times. Next, 100 μL of this bead solution was added to 400 μL of blocking buffer (1% BSA, 0.05% Tween-20) containing various concentrations of BβG, and this mixture was placed on a rotational shaker for 4 h at room temperature. Next, beads were washed 3 times with washing buffer, 3 times with PBS containing 0.05% Pluronic F-68 and finally resuspended in 30 μL of PBS with 0.05% Pluronic F-68 before being manipulated on the DMF chip. Droplets of 2.5 μL were pipetted on an actuation electrode before the top plate sandwiched the droplet between both plates. Next, the droplet containing suspended beads was

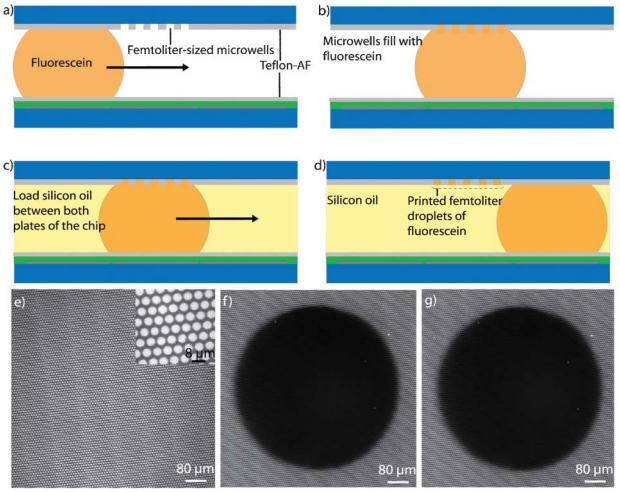

Fig. 2 DMF printing and sealing of femtoliter droplets. (a, b) A droplet of fluorescein is transported towards the array of microwells using EWOD actuation. The fluorescein wets the glass bottom surface of the wells in the top plate. (c) Vapor-Lock oil is pipetted between both plates of the chip, fills the space around the droplets and is held in place by capillary forces. (d) The droplet of fluorescein is transported away, thereby generating femtoliter droplets inside the microwells because of the hydrophilic nature of the wells. (e) A fluorescent image of the microwells immediately after the printing of fluorescein (inset represents a zoomed-in fraction of the array). (f) A fluorescent image of printed fluorescein droplets after the middle portion of the array was photobleached with high-intensity light. (g) A fluorescent image taken 30 min after photobleaching. The integrity of the bleached region indicates that no diffusion of fluorescein took place and the sealing was successful.

transported over the array of microwells multiple times, while a magnet (NdFeB, 6 mm diameter, 12.7 N, Supermagnete, Gottmadingen, Germany) was placed on top of the microwells (Fig. 1). While the magnet was maintained on top of the microwells, a 2.5 μL droplet of FDG in PBS buffer (100 μM) was transported to the microwells. Subsequently, Vapor-Lock oil was pipetted between both plates of the chip, which was kept into place due to capillary forces. Finally, the droplet of FDG was transported away from the microwells, thereby dispensing FDG over every single printed bead and sealing every bead with a femtoliter droplet of the fluorogenic substrate inside the oil. Immediately after sealing the fluorogenic substrate with the beads, an image was acquired which served as a background image. Beads were incubated with FDG for 30 min, after which another image was acquired with a 20× objective. All images were acquired with an exposure time of 1 s. The percentage of fluorescent wells ('active wells') was calculated as follows: $N_{FW}/N_B \times 100\%$, with N_{FW} being the number of fluorescent wells and N_B the total number of captured beads on the array. The number of beads could be obtained by exposing the beads to high-intensity light (excitation 530–550 nm, emission 575–625 nm) and subsequently visualizing their autofluorescence. Afterwards, beads could easily be counted using ImageJ software (US National Institutes of Health, http://rsb.info.nih.gov/ij/).

Results and discussion

We report a novel method for printing single magnetic beads in femtoliter droplets and show how to seal these droplets in an oil environment. The use of the DMF chip for detecting single molecules in solution is demonstrated, as well as the detection of single enzyme molecules captured on paramagnetic beads. All of these operations can be executed on a single DMF platform, which offers advantages for user-friendly, high-throughput single-molecule analysis.

DMF printing and sealing of femtoliter droplets in oil

First, the on-chip printing and sealing of femtoliter droplets is evaluated. Therefore, a droplet of fluorescein was transported towards the array of microwells in an air environment to ensure proper wetting of the microwells with fluorescein solution (Fig. 2). The filling of the microwells can be observed in bright field with an inverted microscope, as the contrast of the wells changes upon filling and occurs almost instantaneously. Then, Vapor-Lock oil was loaded onto the chip, after which the droplet of fluorescein was transported away from the microwells using EWOD actuation. Due to the selective wettability of the hydrophilic bottom of the microwells, femtoliter-sized droplets were retained in the microwells as expected (Fig. 2e). The uniform fluorescence intensity resulting from the microwells indicates that the deposited femtoliter droplets are monodisperse, which is an important feature for performing single molecule experiments. For evaluating the effectiveness of the sealing method, we photobleached the centre of the array of fluorescein droplets by exposing it to high-intensity light for 5 min. This resulted in a well-defined dark circle surrounded by fluorescent microwells. After 30 min, there was no increase in fluorescence intensity in the dark region, indicating that there is no fluorescein leaking from the fluorescent wells to the photobleached wells. These results show that the low-viscosity Vapor-Lock oil is an effective strategy for sealing femtoliter droplets on a double-plate DMF chip and prevents their evaporation, which would potentially inhibit the study of single molecules in solution. Using this strategy, 62.500 femtoliter droplets could be printed and sealed in oil by 1 actuation step on the DMF chip. The ability to measure solution-based enzyme concentrations in a digital assay format on the DMF chip was evaluated by loading different concentrations of BβG in the presence of FDG in the femtoliter wells and subsequently sealing them in oil. Concentrations ranging from 540 fM to 10.7 pM of BβG were loaded on-chip in 62.500 microwells in the presence of FDG. The fluorogenic substrate FDG is hydrolysed to fluorescein in microwells that contain a BβG molecule, which accumulates inside the microwell and results in a local high concentration of a fluorescent product that can be detected with a standard fluorescence microscope. The number of 'active' wells with respect to the number of active and inactive wells is then a measure for the enzyme concentration in the sample.[5] The relationship between enzyme concentration and the fraction of active wells was found to be linear within the tested concentration range (Fig. 3).

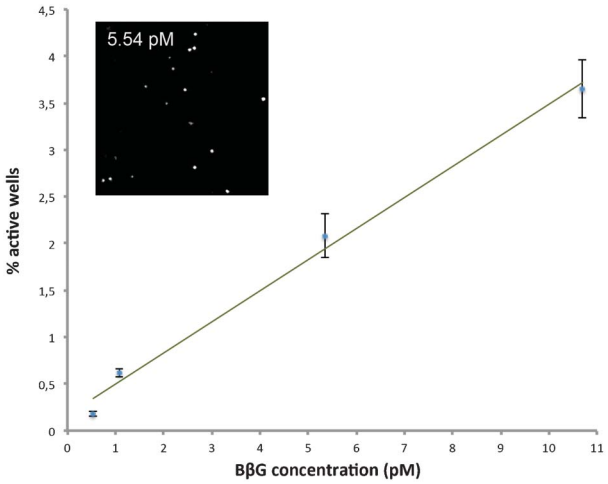

Fig. 3 Digital quantification of solution-based BβG molecules on a DMF chip. The ratio of active wells is linearly dependent on the concentration of BβG molecules (R^2 = 0.99). Error bars represent the standard deviations of 3 repetitions.

High-throughput printing of single superparamagnetic beads on a DMF chip

Fig. 1b shows a schematic representation of the bead printing process. A droplet containing suspended superparamagnetic beads is transported towards the microwell array. The magnet on top of the array attracts the beads towards the array, trapping part of the beads inside the microwells, while the receding droplet meniscus removes beads that are on top of the Teflon-AF surface in between the microwells. The hydrophilic nature of the bottom surface of a microwell, the geometrical trapping of the bead inside the well, and the magnetic force exerted on the superparamagnetic beads cause the beads to remain inside the microwells, while the droplet passes over the array. Because a magnet can be used to speed up the transfer of beads towards the array, there is no need for letting the beads settle by gravity, which shortens the total time needed for the patterning process. Moreover, because of the reconfigurable nature of EWOD actuation on DMF chips, the bead suspension can be dragged over the microwell array many times by simply programming the droplet to shuttle over the array. We hypothesized that this can result in a near 100% loading efficiency of single beads per microwell. As demonstrated in Fig. 4a, the fraction of microwells containing a single bead increases as the droplet is transported over the array more times. Without the use of a magnet for attracting the beads inside the microwells, a loading efficiency of max ~70% can be obtained after 10 seeding cycles (Fig. 4b). For these experiments, the beads were allowed to settle by gravity on the top plate of the DMF chip by flipping the chip upside down and letting the beads sink down on the array for 2 min before starting to shuttle the droplet back and forth. In contrast, when a magnet was placed on top of the array for attracting the beads, a loading efficiency of 98% was obtained (Fig. 4a). 10 seeding cycles proved to be satisfactory for obtaining these high loading efficiencies and resulted in a CV

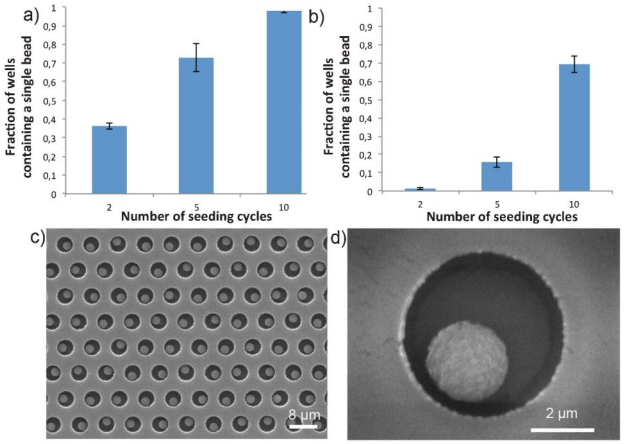

Fig. 4 The loading efficiency of patterning single superparamagnetic beads (2.7 µm diameter) in femtoliter-sized microwells on a DMF chip when (a) using a magnet for attracting beads inside the microwells and (b) without using a magnet. (c) Scanning electron microscope images of part of an array of single beads patterned after 10 seeding cycles and (d) close-up of a single bead in a microwell.

value of 0.9%, showing the high repeatability of the bead loading process when performing 10 seeding cycles with magnet attraction. Increasing the number of seeding cycles to more than 10 did not prove to increase the loading efficiency (data not shown). Nevertheless, this outperforms previously reported loading efficiencies that were reported to be around 40–60%.[5,13–15] The use of DMF technology presented here clearly proves its advantages for obtaining high-density single bead patterns. Considering the low CV of the process when using 10 seeding cycles, this strategy potentially avoids the need for counting wells that contain beads or fluorescently labelling beads for determining the number of wells containing a bead, which is needed in other reported technologies for knowing the exact ratio of 'active' *vs.* 'inactive' wells containing single beads. Moreover, due to the high density of single beads, the total surface area needed for performing single-molecule detection becomes smaller.

On-chip single-molecule detection of β-galactosidase using DMF

To test the ability of the DMF chip to detect single enzyme molecules captured on beads, BβG was detected by first capturing it with streptavidin-coated superparamagnetic beads. A fixed concentration of beads was incubated with different concentrations of BβG (1.7 aM to 170 fM) in a fixed volume, after which the beads were printed on the DMF chip using 10 seeding cycles and magnetic attraction of the beads towards the array. Subsequently, a droplet of FDG was transported to the beads before oil was inserted into the device. Femtoliter droplets of FDG were printed and sealed over the beads by transporting the droplet of FDG away from the array on the chip. The linear dynamic range of the device was then determined by its ability to differentiate between wells showing fluorescence and wells lacking fluorescence. A fluorescent image was taken right after the sealing process and subtracted from a fluorescent image taken 30 min after the

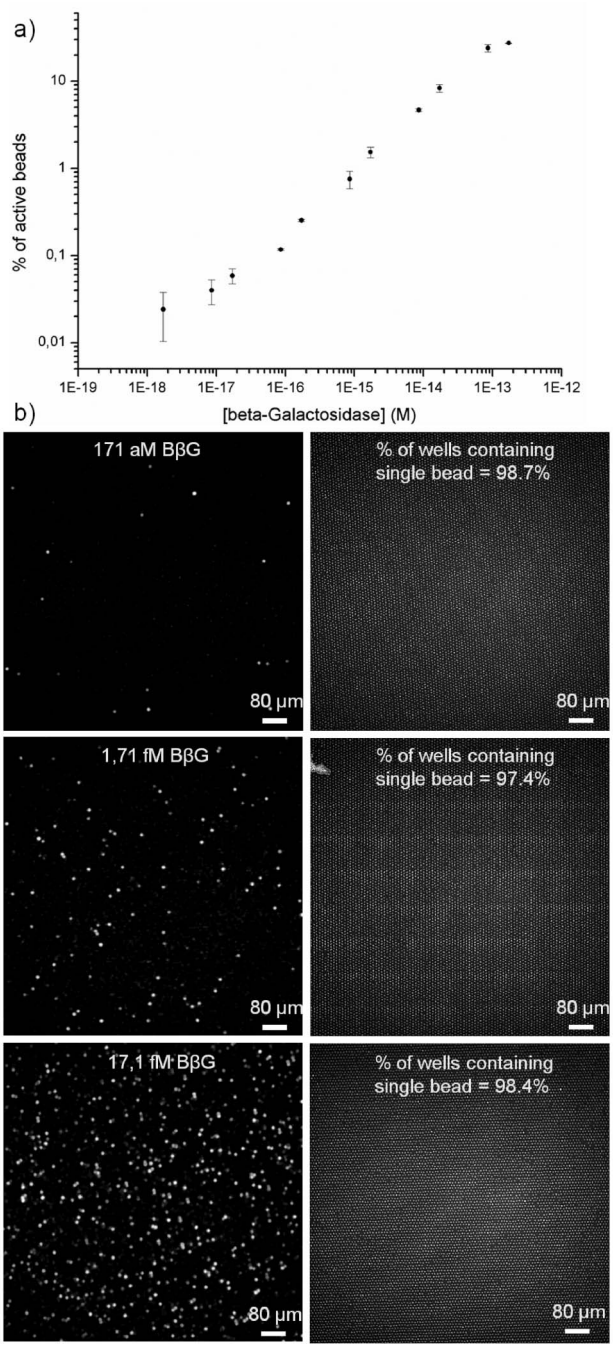

Fig. 5 Single-molecule detection of biotinylated β-galactosidase (BβG) on a DMF chip. (a) A log–log plot of % of fluorescent wells as a function of the concentration of BβG captured on streptavidin-coated superparamagnetic beads. Error bars represent the standard deviations of 3 repetitions. (b) Fluorescent images of the digital quantification of BβG on a DMF chip and their respective images of patterned single beads on the right. The displayed field of view corresponds to ~1/4 of the total array surface.

sealing. Fig. 5b shows fluorescence images of part of the array and illustrates how the number of wells showing fluorescence increases as the concentration of BβG increases. Fig. 5a shows a log–log plot of the concentration of BβG as a function of the percentage of fluorescent wells. A linear trend in digital

enzyme quantification is observed for BβG concentrations between 10 aM and 90 fM (part of the curve with R^2 = 0.99), covering a wide linear dynamic range of 4 orders of magnitude. The percentage of wells containing a single bead was visualized for every experiment by exposing the beads to high-intensity light and detecting their autofluorescence (excitation 530–550 nm, emission 575–625 nm). This percentage was always found to be around 98%, demonstrating the high and repeatable loading efficiencies that are obtained by printing single beads with DMF technology.

Due to an excess of streptavidin capture molecules immobilized on the superparamagnetic beads, the capturing of BβG molecules with beads is much more efficient from a kinetic perspective when compared to BβG detection without beads (Fig. 3), as the amount of target molecules can be concentrated and maximized before the beads are loaded inside the wells. Therefore, lower protein concentrations can be detected when capturing the target molecules on magnetic beads first as compared to directly detecting them as solution-based protein concentrations.

After performing a single-molecule experiment, the top plate of the DMF chip was easily regenerated in a simple way in order to prepare it for a new experiment. Although Teflon-AF has a very limited adhesion to substrates, the top plates of the DMF devices were fabricated with materials to which Teflon-AF exhibits good adhesion. This allows us to treat the top plates with sonication without removing the Teflon-AF from the substrate due to bad adhesion. The beads were trapped strongly inside the wells, necessitating a sonication treatment of 2 min in acetone and 2 min in isopropanol in order to remove all of the beads. After sonication, the top plates were rinsed in deionized water and could be reused many times without any remaining fluorescence or beads interfering in the following experiments.

The ratio of ''active'' and ''inactive'' wells in our experiments for very low BβG concentrations deviates from the theoretically expected ratio that one would expect if every single molecule would be captured with beads and a representative fraction of those beads would be seeded on the DMF chip. Presumably, some loss of single-molecules occurs due to the non-specific adsorption of BβG to tube walls during incubation and washing of the beads, and when transporting and trapping beads with captured BβG on the Teflon-AF surface of the DMF chip. The possible loss of BβG activity over time and the limited presence of unconjugated biotin and β-galactosidase might also contribute to a lower-than-expected count at low protein concentrations. The reported method will therefore be further optimized in the near future for detecting lower concentrations of BβG by optimizing the buffer conditions used for capturing BβG on beads, and trying different surfactants and blocking agents (*i.e.* different types of Pluronics and blocking proteins) to prevent non-specific adsorption of captured single molecules on the Teflon-AF surface of the chip and tube walls. Nevertheless, our study demonstrates DMF as a tool with unique advantages for performing detection of single protein molecules captured on magnetic beads.

Conclusions

In summary, we demonstrated how DMF can be used as an innovative tool for printing and sealing single superparamagnetic beads in femtoliter-sized microwells with unique advantages for performing single-molecule detection. Femtoliter droplets were successfully printed and sealed in oil on a DMF chip for the first time by transporting droplets of aqueous solution towards an array of microwells, subsequently filling the space between both plates of the chip with oil, and transporting the aqueous droplet away from the array. As such, thousands of femtoliter droplets could be printed and sealed per actuation step. The principle of printing single superparamagnetic beads was demonstrated by shuttling a droplet of suspended superparamagnetic beads over the array of microwells, while placing a magnet on top of the array. 10 seeding cycles resulted in a 98% loading efficiency of single beads in microwells in less than 1 min, outperforming the bead loading capacity of previously reported methods. Other microfluidic methods have been reported that pattern and seal single magnetic beads for performing single-molecule detection by using microchannel-based microfluidic devices and sequential fluid flows.[13–15] Here, we demonstrated that DMF technology enables higher loading efficiencies of single beads in microwells and faster bead patterning times due to its unique feature of reconfigurable and fast droplet actuation combined with magnetic attraction of beads in microwells. Moreover, we demonstrated that the bead loading principle is very repeatable, and thereby can avoid the need for counting the number of captured beads in the future. We demonstrated the ability to detect single molecules on this platform by detecting BβG captured on streptavidin-coated magnetic beads with a linear digital dynamic detection range between 10 aM and 90 fM.

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