DIGITAL MICROFLUIDIC FEMTOLITER DROPLET PRINTING: A VERSATILE TOOL FOR SINGLE-MOLECULE DETECTION OF NUCLEIC ACIDS AND PROTEINS

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ABSTRACT

We demonstrate how single protein and nucleic acid molecules can be detected on a digital microfluidic (DMF) chip. Superparamagnetic particles are first used to capture single target DNA molecules or proteins and are subsequently labeled with an enzyme reporter. Microliter-sized droplets containing these suspended beads are then transported over a hydrophilic-in-hydrophobic array situated in the top plate of a double-plate DMF chip and sealed in an oil matrix in the presence of a fluorogenic substrate. The fluorophores generated due to the presence of a single target molecule accumulate in the femtoliter chambers, allowing easy visualization of single DNA or protein molecules with standard fluorescence microscopy.

KEYWORDS: Digital microfluidics, femtoliter droplet printing, single-molecule detection

INTRODUCTION

Single-molecule detection methods allow detection of ultralow concentrations of biomolecules [1] and have lead to new insights in various biochemical processes. One of the most promising approaches to detect single molecules is to isolate them in small reaction chambers and to use enzyme labels that allow the generation of fluorophores that accumulate inside those reaction chambers. Due to the confinement of fluorophores generating from these enzyme labels, local high concentrations of fluorescent molecules enable detection of single biomolecules with standard fluorescence microscopy. Nevertheless, such single molecule detection methods rely on technologies that are capable of generating and sealing femtoliter droplets and can preferentially print and seal magnetic microbeads at single bead resolution, as these beads are useful tools to capture and concentrate the target molecules. Most research efforts have been focused on the use of fiber-optic arrays to perform this kind of single-molecule detection [2]. Here, we show how digital microfluidics is a promising alternative for high-throughput printing and sealing of single magnetic beads and to use them for detecting single protein and DNA molecules.

CHIP FABRICATION AND PRINTING OF SINGLE SUPERPARAMAGNETIC BEADS

Digital microfluidic chips are fabricated with standard microfabrication techniques as described previously [3,4] and are mounted in a custom-made holder that houses electrical connections for DMF droplet actuation (Figure 1).

The top plates of our double-plate DMF device consisted of a glass wafer on top of which a thin aluminum layer was deposited as a ground electrode while a transparent window of 2.5 × 2.5 mm² was left free of aluminum in the center. A layer of Teflon-AF (~3 µm thickness) was then spincoated on top. Subsequently, a thin layer of Parylene-C (500 nm) was deposited on top of the Teflon-AF layer. Next, an aluminum hard mask was coated on top of this polymer stack and patterned with standard photolithography and wet chemical etching to obtain an array of 62500 circles of ~4.5 µm diameter centered in the transparent window of the aluminum ground electrode. This latter aluminum layer served as a hard mask for reactive ion etching of the underlying Parylene-C and Teflon-AF layers to reveal an array of microwells with a hydrophilic glass surface on the bottom of the wells. Finally, the Parylene-C mask could be mechanically peeled off the top plate together with the aluminum hard mask, resulting in an array of 62500 microwells with ~4.5 µm diameter.
and 3 µm depth arranged in a hexagonal array. This dry lift-off method allowed standard photolithographic processes to be used without compromising the hydrophobicity of the Teflon-AF layer, a crucial feature for effective digital microfluidic droplet actuation. After device assembly, droplets containing suspended superparamagnetic beads are shuttled over the array while a magnet attracts beads with single bead resolution inside the array. This results in very high loading efficiencies (98%) of single beads in microwells (Figure 2), which is higher than earlier reported results (40-60%) [5]. Moreover, the use of hydrophilic-in-hydrophobic microwells allows the use of magnet to speed up the transport of magnetic microbeads towards the array of microwells while the receding droplet meniscus enables efficient removal of excess beads off the chip surface. This results in high resolution patterns of single beads in microwells as can be observed in Figure 2.

![Figure 2: SEM image of single beads in microwells after digital microfluidic bead printing.](image)

**RESULTS AND DISCUSSION**

The ability of detecting single protein molecules is demonstrated by detecting the biotinylated enzyme β-galactosidase (BβG) on streptavidin-coated beads. Hereto, different concentrations of BβG were incubated with a fixed concentration of streptavidin-coated microbeads. Subsequently, these beads were printed on a DMF chip by shuttling a droplet with suspended beads over an array of microwells while a magnet was placed on top of the microwell array. After printing, a droplet of the fluorogenic substrate fluorescein di(β-D-galactopyranoside) (FDG) is transported towards the bead array. Subsequently, oil is pipetted between both plates of the DMF device and the droplet of FDG is transported away from the array, thereby printing FDG on every bead and sealing every bead with the fluorogenic substrate inside the oil. After a 30 min incubation, fluorescence images were taken by flipping the chip upside down on an inverted fluorescence microscope. The count of fluorescent microwells was plotted against the corresponding BβG concentration and resulted in a linear digital detection range between 10 aM and 90 fM of protein (Figure 3).

![Figure 3: Digital quantification of biotinylated β-galactosidase (BβG) on a digital microfluidic chip. The percentage of fluorescent microwells is plotted against the corresponding concentration of biotinylated β-galactosidase. Inset: fluorescent image of the digital quantification of a 1.71 fM concentration of BβG.](image)
We also demonstrate how target DNA from *Pseudomonas aeruginosa* can be detected with single-molecule resolution. Therefore, we first captured target DNA on magnetic beads that are functionalized with capture DNA probes that specifically capture the target molecule of interest. Next, biotinylated detection probes are hybridized to the DNA target molecule followed by the binding of a streptavidin-conjugated labeling enzyme (streptavidin β-galactosidase) to the DNA complex. Beads are then printed and sealed in the presence of a fluorogenic substrate as was mentioned in the previous paragraph. Figure 4 shows that there is a clear increase in single-molecule count for increasing target DNA concentrations and detection down to femtomolar levels is demonstrated.

**Figure 4: Digital quantification of DNA in pure buffer conditions. For an increasing concentration of target DNA, an increase in fluorescent microwells is observed.**

**CONCLUSION**

DMF enables femtoliter droplet printing and single bead patterning on a highly automated way. The use of hydrophilic-in-hydrophobic microwells allows the use of a magnet to speed up the bead transfer process to the microwells, while DMF’s reconfigurable droplet actuation allows droplets with suspended microbeads to be shuttled back and forth over the array of microwells until virtually all microwells contain a single bead. We demonstrated how this technique facilitates single-molecule detection of proteins and nucleic acids with femtomolar sensitivity.

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