

CELL-FREE PROTEIN SYNTHESIS IN FEMTOLITER MICROCHAMBERS FOR ARRAYING ULTRA-HIGH DENSITY PROTEIN SPOTS

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ABSTRACT

Current methodologies for arraying proteins using cell-free protein synthesis on a chip have spatial limitations that prevent reaching ultra-high density necessary for high throughput analysis. To circumvent this, we developed an on-chip method based on microcompartmentalization of protein synthesis. Proteins are synthesized in arrayed micrometer scale chambers from confined DNA template molecules. Dimensions of the compartments are compatible with the best nanospotters for delivery of DNA templates and CFPS solutions in each microreactor. On-chip protein expression is highly efficient and the method can be used with the minimal amount of template i.e. single DNA molecules to perform digitalized cell-free protein synthesis (d-CFPS). A functionalized surface at the floor of the tightly sealed microchambers enables direct capture of expressed proteins. A density of 10^4 protein spots per mm^2 was achieved, which represents a gain by more than 3 orders of magnitude over conventional methods. Such a level of density would correspond to several proteomes on a single chip for high-throughput analysis with direct comparative studies.

KEYWORDS

Microchamber array, Protein array, Cell-free protein synthesis, Single DNA molecule.

INTRODUCTION

Protein microarray technology has developed into one of the most active areas in biotechnology today for drug screening, biomarker discovery and analysis of protein-protein interactions. In the present technology, proteins are separately expressed, purified and spotted to fabricate protein arrays, a method that costs time and effort. The challenge of arraying protein spots without these multiple steps has recently been benefited from on-chip cell-free protein synthesis (CFPS) [1]. However, for current on-chip CFPS, the large inter-spot spacing is necessary in order to prevent cross-contamination between protein spots since on-chip CFPS is up-to-now performed in a single large compartment. Here, we present an on-chip method based on microcompartmentalization of individual reactions for generating protein arrays with ultra-high density. We call synthesis from single or a few DNAs digitalized CFPS (d-CFPS), where arrayed PDMS microchambers are used to confine individual DNA molecules with a cell-free lysate capable of coupled transcription and translation (Fig. 1). A capture slide provides a functionalized surface in each microchamber, which serves to immobilize the newly synthesized proteins. The array of protein spots on a solid surface is obtained, with the exact pattern defined by the microchamber array, by peeling off the PDMS chip.

EXPERIMENTAL

PDMS chips and mold master were fabricated with conventional replica molding and photolithography using a negative-type photoresist. The patterned structure had a cylindrical shape, $7\ \mu\text{m}$ in diameter and $5\ \mu\text{m}$ in height. For easy release of the PDMS replica, the mold master was exposed to CHF₃ plasma and coated with a fluorocarbon layer using a Reactive Ion Etching machine (RIE-10NR, Samco Co.). Prepolymer of PDMS was mixed with curing reagent 10:1 mass ratio, poured over the mold master and the mixture cured at $110\ ^\circ\text{C}$ for 30 min. The fabricated PDMS chips were coated with a 2-methacryloyloxyethyl phosphorylcholine (MPC) polymer to prevent protein adsorption and ensure that the internal walls of the chamber are hydrophilic for easy injection of reagent. MPC

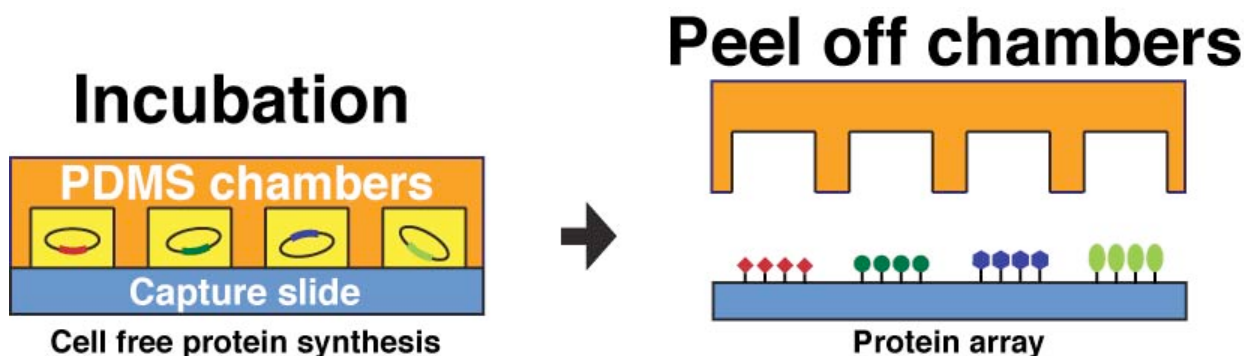


Figure 1. Experimental concept of CFPS. Proteins are synthesized from DNA molecules trapped in the presence of the cell-free protein synthesis system, and bound to a capture slide. Protein array is achieved after peeling off the PDMS microchamber array.

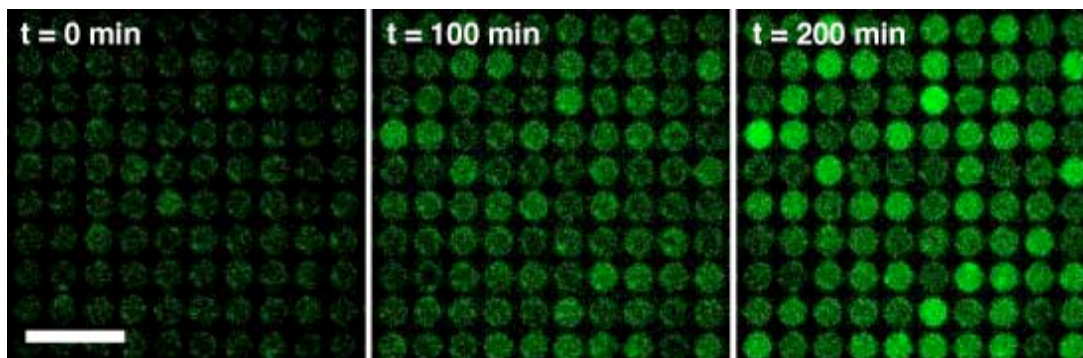


Figure 2. Time-lapse images of CFPS during incubation. Fluorescence intensities inside the chambers were gradually increased with time due to the accumulation of protein molecules. Scale bar is 30 μm .

coated PDMS sheets were soaked into water over night until use. The gene of Emerald GFP (EmGFP: S65T, S72A, N149K, M153T, I167T) in the plasmid pRSET/EmGFP (Invitrogen), was substituted by the one coding for the red fluorescent protein E2-Crimson. Commercialized CFPS reagents were mixed according to the manufacturer's instructions. A droplet of the cell-free synthesis system was sandwiched between the PDMS chip and the microscope coverslide. Microchambers were tightly sealed by pressing the PDMS chip against the microscope coverslide. Reactions were carried out at 30 $^{\circ}\text{C}$ on the microscope stage. Fluorescence was monitored with the appropriate filters for EmGFP and E2-Crimson. Calibration of the EmGFP and E2-Crimson concentrations in the microchambers was performed with a fusion protein E2-Crimson-EmGFP. For experiments, the illumination system was controlled by Andor iQ (Andor Technology Plc.). Image acquisition was obtained using iXonEM +885 EM-CCD Camera (Andor Technology Plc.).

RESULTS AND DISCUSSION

In order to test d-CFPS feasibility, DNA molecules were digitalized on the microchamber array by using a dilute solution of DNA so that the distribution of the number of DNA molecules sealed within the chambers follows a Poisson distribution. This simple configuration allows no need of special equipment for spotting a few or single DNA molecules. On-chip protein synthesis was monitored in real time by following the fluorescent signals for Emerald GFP (Fig. 2). Fluorescence signals from different chambers increased with time. This rate, corresponding to a single DNA molecule in a microchamber is almost identical to the bulk rate of 14 molecules per minute. The protein spots were obtained by peeling off the PDMS sheet after 6 to 7 hours of reaction. His-tagged proteins (E2-Crimson) were bound on Ni-NTA coated slide with the exact pattern defined by the PDMS microchamber array (Fig. 3), where the concentration was 0.6 DNA molecules per chamber. An analysis of the distribution of the fluorescent spots is well fitted by a Poisson distribution with the expected occupancy (Fig. 4). The Poisson distribution of DNA occupancy indicates that 20% of protein spots are synthesized from single DNA molecules. For E2-Crimson, the Ni-NTA slide captured close to 90% of the amount of protein synthesized during d-CFPS. With higher DNA concentration (24 DNA molecules per chamber), all the microchambers are statistically filled with several copies of the DNA template. The protein spots on the capture slide are 7 μm in diameter, a value identical to the diameter of the microchambers (Fig. 5). The result is consistent with the absence of any detectable "cross-talk" between neighboring chambers. The efficiency of the his-tagged protein binding to the Ni-NTA coated slides was assessed by comparing the fluorescence intensity from each chamber before and after peeling off the PDMS sheet.

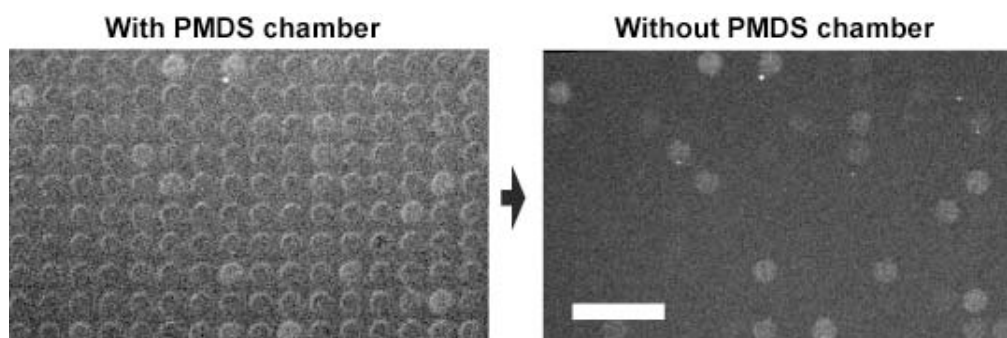


Figure 3. Fluorescence images before and after peeling off the PMDS sheet for his-tagged E2-Crimson. His-tagged proteins were bound on Ni-NTA coated slide with the exact pattern defined by the PDMS microchamber array. Scale bar is 30 μm .

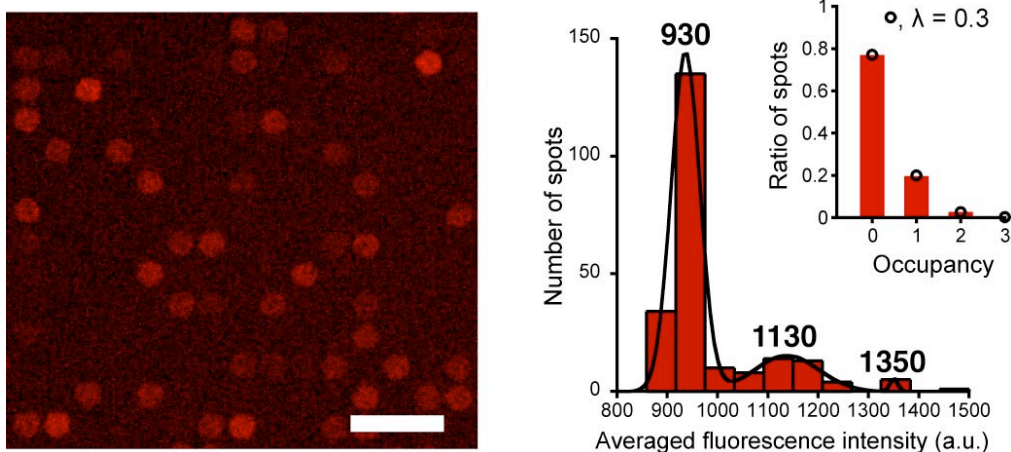


Figure 4. Spots of E2-Crimson captured on Ni-NTA coated slide (top). Scale bar is 30 μm . Statistical analysis of each spots (bottom).

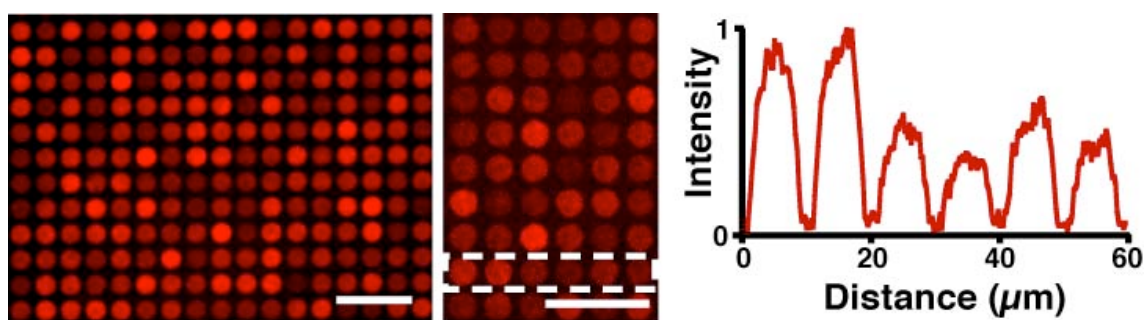


Figure 5. Ultra high-density protein spots for E2-Crimson arrayed on a Ni-NTA coated slide (top). Scale bars are 30 μm . Fluorescence intensity profiles for white dashed-rectangle on the magnified image (bottom).

CONCLUSION

We achieved a density of 10^4 protein spots per mm^2 , which represents a gain by more than 3 orders of magnitude over conventional methods. This is a further step towards miniaturization and the generation of protein arrays with extremely high densities in the micrometer scale. Such arrays would allow direct comparative analysis of several proteomes displayed on a single chip and improve the high-throughput analysis capacity.

REFERENCES

[1] "Printing protein arrays from DNA arrays," M. He *et al.*, Nat Methods, **5**, 175 (2008)

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