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Microfluidics-Mediated Isothermal Detection of Enzyme Activity at the Single Molecule Level

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Abstract — **Conventional analysis of enzymatic activity, often carried out on pools of cells, is blind to heterogeneity in the population. Here, we combine microfluidics with a previously developed isothermal rolling circle amplification-based assay to investigate multiple enzymatic activities in down to single cells. This microfluidics-meditated assay performs at very high sensitivity in picoliter incubators with small quantities of biological materials. Furthermore, we demonstrate the assay's capability of multiplexed detection of at least three enzyme activities at the single molecule level.**

I. Introduction

Classical methods for studying enzymatic activity in cells rely on measuring the average effect in hundreds of thousands or even millions of cells. However, even genetically identical cells in an *in vitro* population are not homogenous entities. Several studies have indicated that enzyme activity, gene expression, or response to signaling may vary considerably among cells [1]. Such cell-to-cell heterogeneity is usually masked in ensemble average measurements in conventional bulk studies. Therefore, assays capable of detailed analysis of single or even batches of a few cells would prove valuable for a number of scientific and clinical purposes.

We recently developed a multiplexed, single molecule level, Rolling circle Enhanced Enzyme Activity Detection (REEAD) system for the scientifically and clinically important endogenous human enzyme, topoisomerase I (TopI) and the related enzymes, Flp and Cre recombinases [2], [3]. TopI exerts important cellular functions by introducing transient single-stranded DNA breaks in the human genome allowing the relaxation of superhelical tension arising as a consequence of DNA tracking processes [4]. Clinically, the enzyme has attracted substantial interest, being the sole cellular target for the anticancer chemotherapeutic, camptothecin (CPT). The cytotoxic effect of CPT has been demonstrated to correlate directly with the intracellular TopI activity level [5]-[8]. However, because of drug resistance, approximately half of the patients subjected to CPT treatment do not respond. A few cells retaining low TopI activity compared to the average activity of the cell population may be the cause of CPT resistance. This hypothesis has not yet been investigated, primarily because of the lack of analytical methods sensitive enough to measure the cellular TopI activity level in few cells.

Specific and multiplexed detection of Flp and Cre activities together with TopI serve as a proof-of-principle for the feasibility of REEAD for multiplexed enzyme detection in crude biological samples, and is also likely to find immediate practical use. Flp and Cre both use the basic TopI cleavage-ligation mechanism [9], [10] to mediate highly controllable and conservative recombination between two specific sites (Flp Recombination Target, FRT sites for Flp [11] and LoxP sites for Cre [12]). Depending on the composition of the recombined DNA, Flp and Cre activity may lead to deletions, insertions, or inversions. Since Flp and Cre are not naturally expressed in mammalian cells, these reaction schemes have been successfully utilized to control gene expression in mammalian test systems by inserting the relevant recombination sites and expressing the partner recombinase in a controlled manner [13], [14]. Moreover, during recent years a massive effort has been put into the development of Flp- or Cre-based gene integration systems for therapeutic purposes [15]-[16].

In the present study we propose to detect DNA modifying enzyme activities in cells encapsulated in picoliter droplets [17], [18]. Combining microfluidics-generated picoliter droplets with REEAD has several advantages: (1) high sensitivity: cell lysate with density of 10,000 cells/mL is required in a typical bulk assay. Given identical reaction conditions (Rolling Circle Amplification, RCA), a few cells would suffice in providing signals within a reduced volume (~100 pL); (2) confined reaction: single cell lysate confined in a picoliter droplet would result in less sample consumption and faster reaction kinetics [19] and (3) screening for enzymatic activity: scanning of a droplet array allows rapid analysis of enzyme activity. Here, we demonstrate simultaneous detection of multiple enzymatic activities (TopI, Flp and Cre) from 1-5 human cells, enabled by a unique combination of microfluidics and REEAD.

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II. Material and Methods

Cell Preparations and Expression Plasmids

Human embryonic kidney HEK293 cells were cultured in GIBCO's Minimal Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin and 100 mg/mL streptomycin and incubated in a humidified incubator (5% CO_2/95% air atmosphere at 37 °C).

Plasmids pCAGGA FLPe and pPGK Cre pA for expression of Flpe and Cre in human cells were kindly provided by Francis Stewart (Technische Universitaet Dresden, Dresden, Germany) and Klaus Rajewsky (Division of Pathology, Department of Medicine, Harvard Medical School, Boston, Massachusetts, USA), respectively. Transient transfection of plasmids into HEK293 cells was performed using lipofectamine2000 (Invitrogen) and 8 μg DNA and was carried out in GIBCO's Reduced Serum Medium (OPTI-MEM) according to the manufacturer's instructions. Transfection efficiencies as measured by the expression of a green fluorescent protein (GFP) control construct were 70%. Functional assays were conducted 24 h after transfection, and cells were harvested with 0.25% Trypsin-EDTA (GIBCO). The cells were resuspended in Phosphate-buffered Saline (1xPBS, Cellgro), 1% Pluronic F-68 (SIGMA), 0.1% BSA (Invitrogen) to a density of 1-5 million cells/mL by cell counting after harvest.

Synthetic DNA Substrates, Probes, and Primers

Oligonucleotides for construction of the S(TopI), S(Flp), S(Cre) substrates, the RCA primer (*p*), and the identification probes i(TopI), i(Flp), i(Cre) and were purchased from DNA Technology A/S, Aarhus, Denmark and synthesized on a model 394 DNA synthesizer from Applied Biosystems. The sequences of the oligonucleotides are identical to [3].

Rolling Circle Amplification

The previously presented single molecule TopI, Flp and Cre activity assay is based on enzyme-mediated circularization of synthetic DNA substrates (Figure 1A-C), followed by isothermal signal enhancement *via* Phi29 DNA polymerase-driven RCA (Figure 1D) (MBI Fermentas) on a solid support (CodeLink Activated Slides from SurModics). The DNA substrates comprise a single oligonucleotide, which is converted to a closed DNA circle by a single enzyme cleavage-ligation event. For each circularized substrate subsequent RCA results in the creation of one rolling circle product (RCP) consisting of multiple (up to 10^3) tandem copies of the circularized substrate [3], [20], [21]. Each RCP is optically detected at the single molecule level by hybridization to fluorescently labeled probes followed by microscopic analysis.

Microfluidics Device Fabrication and Operation

The microfluidic setup consists of two devices: a flow-focusing droplet generator (Figure 2A) and a droptrap (Figure 2B). Both devices were fabricated by conventional soft lithography techniques [7], casting and curing the PDMS prepolymer on a SU-8 3025 (MicroChem) master of a channel height at around 25 μm. PDMS prepolymer

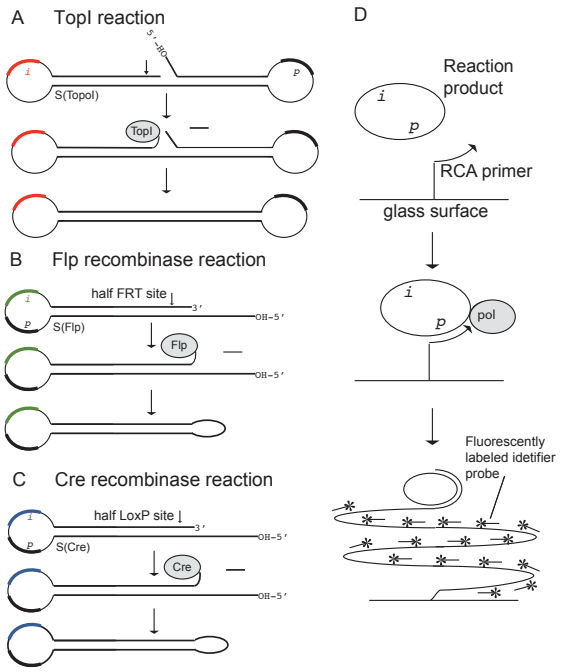

Figure 1: Schematic representation of REEAD. (A) S(TopI) folds into a dumbbell-shaped structure, with one loop containing an identifier element (marked *i*), the other containing the primer hybridization sequence (marked *p*), and the double-stranded stem region having a preferred TopI recognition sequence (TopI cleavage site marked with an arrow). Following TopI-mediated cleavage and ligation the dumbbell-shaped substrate is converted to a closed circle. (B) S(Flp) folds into a single-stranded loop structure containing an identifier element (*i*), a primer hybridization sequence (*p*), and a stem forming a half Flp Recombination Target (FRT) site, a minimal cleavage site for Flp. Flp cleavage and ligation converts this substrate to a closed DNA circle. (C) S(Cre) resembles S(Flp) except that the stem part of the substrate contains the sequence of half a LoxP site, which is the minimal cleavage substrate for Cre. The reaction of Cre is reminiscent of the Flp reaction and leads to a circular product. To minimize false positives, the RCA primers are designed to have the polymerase encounter the strand interruption before the identifier element. (D) RCA for visualization of generated circular DNA products. The *p* region of each of S(TopI), S(Flp), or S(Cre) is hybridized to a 5'-amine linked primer attached to a glass surface, which allows polymerase-assisted RCA of the circularized substrates (for unreacted open substrates the reaction terminates at the strand interruption). Visualization is performed by hybridization of fluorescently labeled probes to the RCP region corresponding to the *i* element of the dumbbell-substrate. Gray ellipses marked TopI, Flp, Cre, and Pol represents TopI, Flp, Cre, and Phi29 polymerase, respectively. The asterisks represent the fluorophores.

(Sylgard 184) was prepared in a 10 : 1 (base : curing agent) ratio and cured at 65°C for 1hr. Prior to the experiment, the channel was wet with oil/surfactant for at least 15 min. Two syringe pumps (Harvard Apparatus) were used to control the flow rates of oil/surfactant and reagents independently, forming monodisperse water-in-oil droplets at a frequency of 0.8-1.5 kHz. The droplet volume and generation frequency was controlled by the flow rate ratio, determined by the competition between continuous phase (carrier fluid: the oil/surfactant, flow rate 22.5 μL/min) and disperse phase (aqueous reagents: cells, lysis buffer and substrates, flow rate 2.5 μL/min). Carrier fluid (FC-40 fluorocarbon oil, 3M) was selected for its higher density than aqueous solutions, easing the collection of final products. Cells (see cell

preparation), lysis buffer (10 mM Tris-HCl pH 7.5, 0.5 mM EDTA, 1 mM DTT, 1 mM PMSF, 0.2% Tween 20), and substrates were prepared as stated above. The generated droplets were harvested by pipetting and placed on a primer printed glass slide. The PDMS droptrap was gently placed on top. The geometry of the droptrap was designed according to the size of generated droplets (Figure 2C). The droplets were left to dry for 12 hours. Wash, RCA, and hybridization of probes were performed similarly as bulk experiments described in [3].

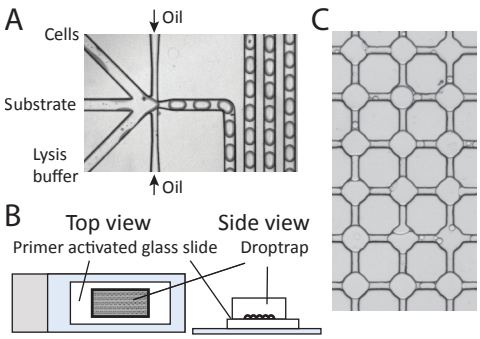

Figure 2: Unique combination of microfluidics and REEAD assay. (A) Cells-to-be-analyzed, DNA substrate and lysis buffer are confined in picoliter droplets in which the reaction took place. (B) After closed circular products are formed, the droplets are captured in a droptrap on a primer activated glass slide. Following drop exsiccation, enzymatic products are amplified by RCA, hybridized to fluorescently labeled probes and detected by microscopy. (C) Example of a section of the droptrap with confined droplets.

Microscopy

Epifluorescent and bright field images were captured with an inverted fluorescence microscope (Axio Observer, Zeiss). Monocolor emission from each fluorophore was collected and filtered through appropriate filters and dichroics. Image processing and analysis was performed with MetaMorph (v.7.6.5).

III. Results and Discussion

High Sensitivity Enabled By Confining the Signal

We used human embryonic kidney cells (HEK293 cells) transfected with plasmids allowing the expression of a recombinant thermally stabile mutant of Flp (Flpe) and Cre. Cells were harvested and resuspended to a density of 1-5 million cells/mL before they were introduced into the microfluidic channel. Estimated from the Poisson distribution, around 9-30% of droplets contained one cell in a 100 pL droplet [27]. The two other channels were fed with a divalent cation-free lysis buffer and substrates, respectively. Subsequent to generation of droplets, a design of serpentine channel was included to ensure complete mixing among cells, lysis buffer, and DNA substrates. After lysis of the cells, the enzymes were released and able to interact with the substrates, thus creating closed DNA circles (Figure 1A). The droplets were subsequently captured in the droptrap, exsiccated, and the closed DNA circles hybridized to the printed primer, before subjection to RCA. The resulting RCPs were visualized by hybridization to the appropriate fluorescent probes followed by microscopic analyses. Shown in Figure 3, the REEAD and microfluidics-mediated approach enabled higher detection sensitivity as compared to conventional bulk assay when using the same amount of cells. Based on the microscopic analysis, few cells would be sufficient in providing detectable signal within a 100 pL.

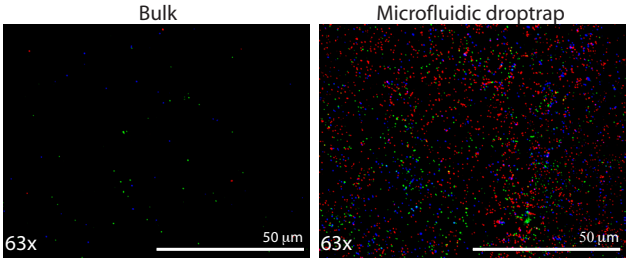

Figure 3. Comparison between bulk and microfluidics-mediated assay: A mixture of DNA substrates (300 nM of S(TopI) and S(Flp), and 100 nM pre-ligated control circles) was fed into one channel of the microfluidic chip, while 5 million cells/mL HEK293 cells (transfected with the Flpe expression vector) and lysis buffer were introduced into the other two channels. The generated droplets were captured in the droptrap and exsiccated. The ligated substrates then hybridized to the pre-printed primers, followed by RCA. Enzymatic activities were captured by fluorescently labeled probes: FAM-labeled i(Flp), TAMRA-labeled i(TopI), and Cy5-labeled i(control), respectively. Cell lysate from around 1600 cells were analyzed. The microfluidics assay showed significantly higher detectable responses when compared to its bulk counterpart. Note that the droptrap has 3200 cavities for trapping drops, of which 50% contain a cell based on the Poisson distribution when using the abovementioned cell density.

Multiplexed Detection of Enzyme Activities on Droptrap

As demonstrated in Figure 4, cells expressing TopI and recombinant Flpe and Cre gave rise to blue, green, and red fluorescent spots, respectively. This result demonstrates the suitability of the presented microfluidic REEAD assay for multiplexed detection in few, if not, single cells. The specific detection of TopI activity in one or few human cells may be of substantial cancer prognostic value, as TopI is the sole target for several anticancer therapeutics. The single molecule detection of RCPs, allowing the visualization of multiple, such as Flp, Cre, or TopI cleavage-ligation events, opens up the possibility of very sensitive measurement down to the single cell or, in principle, even the single enzyme level. A similar sensitivity may be obtained using a PCR-based readout format. However, the thermal cycling principle of PCR would compromise detection of individual cleavage-ligation events and likely the accuracy of the assay, owing to the exponential accumulation of inaccuracies inherent to PCR. We believe that the presented single molecule microfluidic REEAD assay presents unique advantages for both basic and applied science allowing subtle variations between individual cells in a large population to be recognized. The successful multiplexed enzyme detection presented here, holds promise for the possibilities of developing even more extended setups allowing the simultaneous detection of enzyme collections relevant for different industrial or clinical applications.

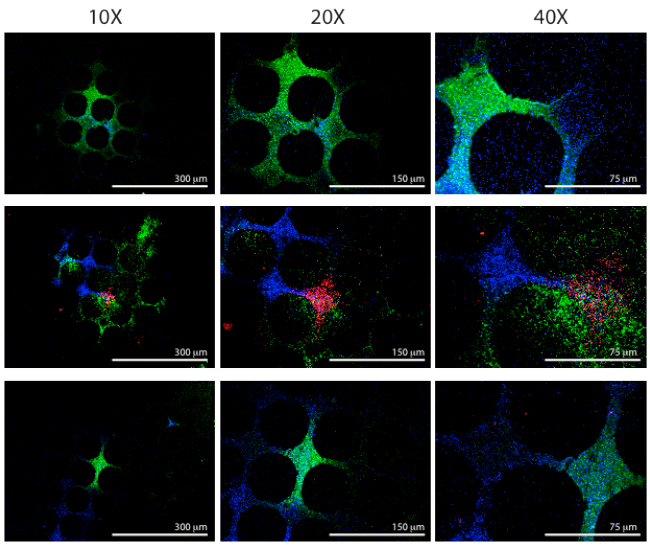

Figure 4: Multiplexed detection of TopI, Flp, and Cre. A mixture of 300 nM of each of S(TopI), S(Flp),and S(Cre) was fed in one channel of the microfluidic chip with 5 million cells/mL HEK293 cells transfected with both the Flpe and the Cre expression vectors and lysis buffer, respectively, in the other two channels. The generated droplets were captured in the droptrap, exsiccated, and the ligated substrates hybridized to the primers prior to RCA and hybridization to FAM-labeled i(Flp), TAMRA-labeled i(Cre), and Cy5-labeled i(TopI).

IV. Conclusion

The proposed microfluidics-mediated REEAD assay represents a unique approach to study enzymatic activities possibly down to the single cell level. Confining the reactions in picoliter droplets greatly enhances the detection sensitivity, compared to conventional bulk assay. Results described herein also show the capability of multiplexed enzyme activity detection. We expect this assay to be useful both practically for clinical diagnostics and fundamentally for deciphering biological heterogeneity.

References

[1] L. A. Doyle. Topoisomerase expression in cancer cell lines and clinical samples. *Cancer Chemother Pharmacol.* **34**, pp. 32-40, 1994

[2] M. Stougaard, J. Lohmann, A. Mancino, S. Celik, F. F. Andersen, J. Koch, B. R. Knudsen. *ACS Nano* **3**, pp. 223-33, 2009

[3] F. F. Andersen, M. Stougaard, H. L. Jørgensen, S. Bendsen, S. Juul, K. Hald, A. H. Andersen, J. Koch, and B. R. Knudsen. Multiplexed detection of site specific recombinase and DNA Topoisomerase activities at the single molecule level. *ACS Nano* **3**, pp. 4043–4054, 2009

[4] J. J. Champoux. Mechanism of catalysis by eukaryotic DNA Topoisomerase I. *Adv. Pharmacol., **29A***, pp. 71–82, 1994

[5] Y. Pommier. Topoisomerase I inhibitors: camptothecins and beyond. *Nat. Rev. Cancer* **6**, pp. 789–802, 2006

[6] Z. Liao, R. W. Robey, J. Guirouilh-Barbat, K. K. To, O. Polgar, S. E. Bates, Y. Pommier. Reduced expression of DNA Topoisomerase I in SF295 human glioblastoma cells selected for resistance to Homocamptothecin and Diflomotecan. *Mol. Pharmacol.*, **73**, pp. 490–497, 2008

[7] J. Soret, M. Gabut, C. Dupon, G. Kohlhagen, J. Stevenin, Y. Pommier, J. Tazi. Altered serine/arginine-rich protein phosphorylation and exonic enhancer-dependent splicing in mammalian cells lacking Topoisomerase I. *Cancer Res.* **63**, pp. 8203–8211, 2003

[8] K. Taniguchi, K. Kohno, K. Kawanami, M. Wada, T. Kanematsu, M. Kuwano. Drug-induced down-regulation of Topoisomerase I in human epidermoid cancer cells resistant to Saintopin and Camptothecins. *Cancer Res.*, **56**, pp. 2348–2354, 1996

[9] S. G. Hansen, R. F. Frøhlich, B. R. Knudsen. Type IB Topoisomerases and tyrosine recombinases: distinct functions within related structural frameworks. *Curr. Top. Biochem. Res.* **5**, pp. 149–159, 2003

[10] I. Grainge, M. Jayaram. The integrase family of recombinase: organization and function of the active site. *Mol. Microbiol.* **33**, pp. 449–456, 1999

[11] B. J. Andrews, G. A. Proteau, L. G. Beatty, P. D. Sadowski. The FLP recombinase of the 2 micron circle DNA of yeast: interaction with its target sequences. *Cell.* **40**, pp.795–803. 1985

[12] K. Abremski, R. Hoess, Bacteriophage P1 site-specific recombination. Purification and properties of the Cre recombinase protein. *J. Biol. Chem.* **259**, pp. 1509–1514, 1984

[13] A. L. Garcia-Otin, F. Guillou. Mammalian genome targeting using site-specific recombinases. *Front Biosci.* **11**, pp. 1108–1136, 2006

[14] C. S. Branda, S. M. Dymecki. Talking about a revolution: the impact of site-specific recombinases on genetic analyses in mice. *Dev. Cell* **6**, pp. 7–28, 2004

[15] S. Bolusani, C. H. Ma, A. Paek, J. H. Konieczka, M. Jayaram, Y. Voziyanov. Evolution of variants of yeast site-specific recombinase Flp that utilize native genomic sequences as recombination target sites. *Nucleic Acids Res.* **34**, pp. 5259–5269, 2006

[16] A. Akopian, M. W. Stark. Site-specific DNA recombinases as instruments for genomic surgery. *Adv Genet.* **55**, pp. 1–23, 2005

[17] D. Qin, Y. Xia, G. M. Whitesides. Soft lithography for micro- and nanoscale patterning. *Nature Protocols* **5**, pp. 491-502, 2010

[18] H. Song, D. L. Chen, R. F. Ismagilov. Reactions in droplets in microflulidic channels. *Angewandte Chemie* **45**, pp. 7336-56, 2006

[19] M. E. Vincent, W. Liu, E. B. Haney and R. F. Ismagilov, Microfluidic stochastic confinement enhances analysis of rare cells by isolating cells and creating high density environments for control of diffusible signals, *Chem. Soc. Rev.* **39**, pp.974-984, 2010

[20] A. Fire, S. Q. Xu. Rolling replication of short DNA circles. *Proc. Natl. Acad. Sci. U.S.A.* **92**, pp. 4641–4645, 1995

[21] L. Lam, R. Iino, K. V. Tabata, H. Noji. Highly sensitive restriction enzyme assay and analysis: a review. *Anal. Bioanal. Chem.* **391**, pp. 2423–2432, 2008

[22] K. Healy, Nanopore-based single-molecule DNA analysis. *Nanomedicine* **2**, pp. 459–481, 2007

[23] G. Charvin, T. R. Strick, D. Bensimon, V. Croquette. Tracking topoisomerase activity at the single-molecule level. *Annu. Rev. Biophys. Biomol. Struct.* **34**, pp. 201–219, 2005

[24] J. van Mameren, E. J. Peterman, G. J Wuite. See me, feel me: methods to concurrently visualize and manipulate single DNA molecules and associated proteins. *Nucleic Acids Res.* **36**, pp. 4381–4389, 2008

[25] K. M. Herbert, W. J. Greenleaf, S. M. Block. Single-molecule studies of RNA polymerase: motoring along. *Annu. Rev. Biochem.* **77**, pp. 149–176, 2008

[26] L. Wang, M. B. O'Donoghue, W. Tan. Nanoparticles for multiplex diagnostics and imaging. *Nanomedicine*, **1**, pp. 413–426, 2006

[27] J. F. Edd, D. Di Carlo, K. J. Humphry, S. Köster, D. Irimia, D. A. Weitz, M. Toner, Controlled encapsulation of single-cells into monodisperse picolitre drops. *Lab on a Chip* **8**, pp.1262-1264, 2008