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Assaying multiple restriction endonucleases functionalities and inhibitions on DNA microarray with multifunctional gold nanoparticle probes

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ABSTRACT

Herein, a double-stranded (ds) DNA microarray-based resonance light scattering (RLS) assay with multifunctional gold nanoparticle (GNP) probes has been developed for studying restriction endonuclease functionality and inhibition. Because of decreasing significantly melting temperature, the enzyme-cleaved dsDNAs easily unwind to form single-stranded (ss) DNAs. The ssDNAs are hybridized with multiplex complementary ssDNAs functionalized GNP probes followed by silver enhancement and RLS detection. Three restriction endonucleases (EcoRI, BamHI and EcoRV) and three potential inhibitors (doxorubicin hydrochloride (DOX), ethidium bromide (EB) and an EcoRI-derived helical peptide ($\alpha 4$)) were selected to demonstrate capability of the assay. Enzyme activities of restriction endonucleases are detected simultaneously with high specificity down to the limits of 2.0×10^{-2} U/mL for EcoRI, 1.1×10^{-2} U/mL for BamHI and 1.6×10^{-2} U/mL for EcoRV, respectively. More importantly, the inhibitory potencies of three inhibitors are showed quantitatively, indicating that our approach has great promise for high-throughput screening of restriction endonuclease inhibitors.

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1. Introduction

Endonucleases, a family of nuclease, are known as “molecular scalpel” with highly specific activity in cleaving the phosphodiester bond within DNA at defined positions (Galburt and Stoddard, 2002; Redondo et al., 2008). These enzymes have widely usages in PCR assay, gene mapping, medicinal chemistry, enzymatic amplification technique and nanostructures/nanodevices fabrication (Gao et al., 2010; Huang et al., 1986; Kanaras et al., 2007; Liu et al., 1999; Mitsuya et al., 1990; Qin and Yung, 2005; Schulze et al., 2012; Zhen et al., 2012). Endonucleases in prokaryotic organisms play the role of defensive system with the principal function of protecting host genome against foreign DNA (Galburt and Stoddard, 2002; Pingoud and Jeltsch, 2001). Accordingly, they have been deemed to be important targets in the discoveries of antimicrobial and antiviral drugs (Baughman et al., 2012; De Clercq, 2006; Tomassini et al., 1996). Therefore, sensitively and quantitatively assaying endonuclease functionality and inhibition is critical and useful in drug-development process.

A range of methods/techniques have been used for assaying endonucleases, including high-performance liquid chromatography (HPLC), enzyme-linked immunosorbent assay (ELISA), gel

electrophoresis, fluorescence resonance energy transfer (FRET), fluorescence polarization and gold nanoparticle (GNP)-based colorimetric methods (Alves et al., 1989; Feng et al., 2007; Huang et al., 2011a; Jeltsch et al., 1993; Li et al., 2007; Song et al., 2009; VanderVeen et al., 2005; Xu et al., 2007). Nevertheless, most of these methods/techniques can only detect one particular enzyme per assay, which cannot satisfy the requirement of modern molecular biology. Recently, gold nanobeacon- and quantum dot-based multiplex methods have been designed for simultaneously assaying restriction endonucleases activities and screening inhibitors (Huang et al., 2011b, 2011c). In these approaches, nanoprobe are functionalized with dsDNA substrates which are labeled by different fluorescent dyes or quenchers. Although the assays are effective in determining the activities of multiple restriction endonucleases (e.g., EcoRI and BamHI) simultaneously with high sensitivity and specificity, the photostability and spectral overlap of fluorescent labels limit their utilities in high throughput screening. In addition, different fluorescent labels need different excitation wavelengths which increase the cost of detection.

Due to their unique optical properties (surface plasma resonance (SPR) and resonance light scattering (RLS)), GNPs have been extensively applied as probes for developing homogeneous and/or heterogeneous sensing assays (Li et al., 2010; Matsui et al., 2005; Nam et al., 2003; Rosi and Mirkin, 2005; Taton et al., 2000; Wang et al., 2012, 2010). Specific recognition of proteins/DNAs by antibody/DNA functionalized GNPs followed by an enhancement step

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based on the electroless deposition of silver onto the GNPs has been employed for specific detection of proteins/DNAs on microarrays (Li et al., 2010; Nam et al., 2003; Taton et al., 2000). The microarray-based RLS assay represents a great step forward, towards higher sensitivity, with the eventual goal of detecting single biomolecular binding events. In addition, the microarray-based RLS assay can be visualized by a flatbed scanner with white light source, thereby providing significant cost savings on instrumentation.

In this work, we developed a dsDNA microarray-based RLS assay with ssDNA functionalized GNP probes for detecting restriction endonuclease functionality and inhibition. Taking advantage of the high-throughput feature of microarray and multiplex ssDNAs functionalized GNPs, our method is capable of multiplexing since, in principle, the detection of many different restriction endonucleases' functionalities and inhibitions can be carried out simultaneously on the same microarray.

2. Experimental section

2.1. Materials and reagents

Restriction endonucleases, NEBuffer EcoRI and BSA were obtained from New England Biolabs (NEB, UK). Synthetic oligonucleotides (as shown in Table S1) and doxorubicin hydrochloride (DOX) were purchased from Sangon Ltd. (Shanghai, China). Tetrachloroaurate (HAuCl_4), silver enhancer solution and methoxypolyethylene glycol amine (PEG-NH₂, MW=750) were acquired from Sigma-Aldrich Chemical Co. (USA). Ethidium bromide (EB) was purchased from Dingguo Ltd. (Beijing, China). Aldehyde 3-D glass slides were obtained from CapitalBio Ltd. (Beijing, China). Alpha4 peptide ($\alpha 4$, Ac-AIERSHKNISEIANFM-NH₂) was synthesized by ChinaPeptides Co., Ltd. (Shanghai, China). Sodium dodecyl sulfonate (SDS) was purchased from Aladdin Industrial Co. (Shanghai, China). Other chemicals were analytical grade. Milli-Q water (18.2 M Ω cm) was used in all experiments. The inactive EcoRI was obtained by heating 500 U/mL EcoRI solution at 65 °C for 20 min (1 U is defined as the amount of enzyme required to digest 1 μg of λ DNA in a total reaction volume of 50 μL at 37 °C for 1 h.). The stock solution of $\alpha 4$ was prepared by dissolving 1.57 mg $\alpha 4$ in 50 μL DMSO.

2.2. Preparation of DNA-modified GNP probe

The citrate stabilized 13 nm GNPs were synthesized by classical Turkevich–Frens method (Frens, 1973; Turkevich et al., 1951). DNA-modified GNPs (DNA-GNPs) were prepared by a previously reported procedure (Kanas et al., 2003). Generally, the GNPs solution (8 nM, 300 μL) was incubated with 15 μL mixture of alkanethiol-modified oligonucleotides EI-S, BI-S and EV-S (The molar ratios of GNP and total ssDNA are 1:150, 1:300, and 1:600; and the proportions of EI-S:BI-S:EV-S in the ssDNA mixtures are 1:1:1, 1:1.5:1, and 1:1.5:0.7, respectively.) in aqueous solution overnight, then diluted with equal volume PBS buffer (10 mM PB, 0.2 M NaCl, pH 7.5). After further incubation for 10 h, the solution was evaporated to 100 μL by vacuum centrifugation. Excess oligonucleotides were removed by repeated centrifugation (9000 rpm, 3 times). Finally, the DNA-GNPs were dispersed in probe reaction buffer (0.67 \times SSC, 0.1% (w/v) SDS) and stored at 4 °C for further use.

2.3. Fabrication of dsDNA microarray

The alkylamine-modified oligonucleotides (EI-P, BI-P and EV-P) were dissolved in spotting buffer (3 \times SSC, 1.5 M betaine, 0.005% (w/v) SDS) with desired concentrations and spotted onto the aldehyde 3-D glass slide by a SmartArrayer 96 system (CapitalBio

Ltd., Beijing, China). After an overnight incubation under 75% humidity at 37 °C, the slide was rinsed with washing buffer (1 \times SSC, 0.01% (w/v) SDS) and water, dried by centrifugation (480g for 1 min), then separated into 12 independent subarrays by PTFE grid. Subsequently, the slide was blocked with PEG-NH₂ (in PBS buffer (50 mM PB, 0.15 M NaCl, pH 7.5)) for 1 h at 30 °C to inactivate remaining aldehyde groups.

After blocking process, the ssDNA microarrays were hybridized with the mixture of target oligonucleotides (EI-T, BI-T and EV-T were mixed at an equal molar ratio, 200 nM each) in hybridization buffer (4 \times SSC, 0.1% (w/v) SDS) for 2 h at 55 °C. Afterward, the slide was subjected to a series of rinses: (I) 55 °C hybridization buffer for 5 min (3 times); (II) 55 °C washing buffer for 5 min (2 times); (III) room-temperature washing buffer for 5 min (1 time); (IV) Milli-Q water for 3 min (3 times), respectively. Finally, the slide was dried by centrifugation.

2.4. Enzyme cleavage

The restriction endonuclease cleavage experiment was performed in 1 \times NEBuffer EcoRI (100 mM Tris-HCl, 50 mM NaCl, 10 mM MgCl₂, 0.025% Triton X-100, pH 7.5) containing 100 $\mu\text{g}/\text{mL}$ BSA (NEB recommend) on dsDNA microarray. 25 μL solutions containing various amounts of restriction endonucleases were applied to each subarray, respectively. For multiple restriction endonucleases cleavage, a mixed solution of EcoRI, BamHI and EcoRV was used. Corresponding control experiment was carried out by incubating one subarray with blank reaction buffer on the same slide. Following a period of incubation at 37 °C, the slide was washed with hybridization buffer at 50 °C for 5 min (3 times), washing buffer at 50 °C for 5 min (2 times), washing buffer at room-temperature for 5 min (1 times) and water for 3 min (3 times), respectively. Then, the slide was dried by centrifugation.

2.5. Labeling with DNA-GNP probes

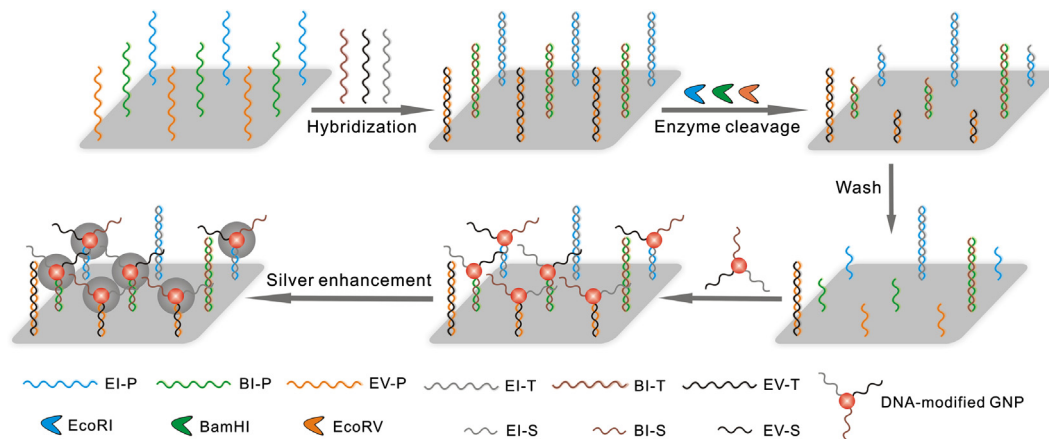
After enzyme cleavage, microarrays were incubated with 5 nM DNA-GNP probes (in 500 μL probe reaction buffer) at 37 °C for 1 h and then washed with hybridization buffer, washing buffer and water as previously described. After that, 1 mL silver enhancer solution (solution A (AgNO₃) and solution B (hydroquinone) were mixed with the volume ratio of 1:1) were applied to each slide for 8 min followed by washing with water (3 times) and drying with centrifugation.

2.6. Enzyme inhibition

Briefly, the inhibitors were diluted to a series of concentrations with reaction buffer (1 \times NEBuffer EcoRI containing 100 $\mu\text{g}/\text{mL}$ BSA) and then mixed with the mixture of restriction endonucleases (final concentrations of restriction endonucleases were 250 U/mL of EcoRI, 250 U/mL of BamHI and 125 U/mL of EcoRV, respectively). Next, 25 μL of the cocktail solution was applied to each subarray. For $\alpha 4$, the mixture of restriction endonucleases and peptide were preincubated for 30 min at 37 °C before transferring onto microarray. As control experiment, one of the subarrays on the same slide was incubated with the same solution without inhibitor. After incubating at 37 °C in dark for 4 h, the slide was washed, dried, labeled by DNA-GNP probes and treated with silver enhancer solution as previously described.

2.7. Data acquisition and processing

RLS signal was acquired by ArrayIt SpotWare Colorimetric Microarray Scanner (Telechem. International Inc., USA) according to the manufacturer's preset parameters. The background originating



Scheme 1. The schematic representation of dsDNA microarray-based RLS assay with DNA-GNP probes for studying restriction endonuclease functionality. The illustration is not drawn to scale.

from the slide was recorded and subtracted prior to evaluation. The mean value and standard deviation of the RLS intensity were determined from 6 spot replicates per sample.

3. Results and discussion

3.1. Assay principle

Scheme 1 outlines the strategy of dsDNA microarray-based assay for studying restriction endonucleases functionalities. Briefly, single-stranded alkylamine-modified oligonucleotides (EI-P, BI-P and EV-P) are spotted and immobilized on commercial aldehyde-functionalized glass slide by a standard robotic procedure. The aldehyde groups on the glass surface react readily with the primary amines of oligonucleotides to form Schiff base linkages. Subsequently, the slide is treated with blocking solution to inactivate the remaining aldehyde groups. Afterward, dsDNA microarray is generated by hybridizing target oligonucleotides (EI-T, BI-T and EV-T) with immobilized oligonucleotides. The newly formed dsDNAs on the microarray contain palindromic recognition sequences which can be cleaved by corresponding restriction endonuclease. Because the melting temperature of enzymatically cleaved dsDNA is much lower than that of intact dsDNA, the enzymatically cleaved dsDNA unwinds to form ssDNAs at certain temperature (e.g., 50 °C), while the uncleaved dsDNA keeps its double helical structure. After unwinding the enzymatically cleaved dsDNA by thermal buffer solution, DNA-GNP probes were used to hybridize the ssDNAs on the microarray. Subsequently, a silver enhancement step was applied to the microarray for signal amplification since the RLS properties of gold nanoparticles by themselves are relatively poor, if the particles are smaller than ca. 40 nm (Yguerabide and Yguerabide, 2001). In the silver enhancement step, ionic silver is reduced to elemental silver by hydroquinone. Due to silver deposition on the gold nanoparticles, the average size of nanoparticles is increased from 13 to 100 nm (Taton et al., 2000; Wang et al., 2005). Gold and silver nanoparticles of this final size (ca. 100 nm) are very strong light scatterers and are readily detected even by a common flatbed scanner.

3.2. Optimization of experimental conditions

In order to enhance the sensitivity and selectivity of this method, the modifying conditions of DNA-GNP probes are first optimized. In this case, we investigate the effects of two factors (the molar ratio of GNP and total ssDNA probes in solution, and the proportions of three ssDNA probes (EI-S, BI-S and EV-S) in the DNA mixture) on the assay performance. Optimized results are obtained when molar ratio of total

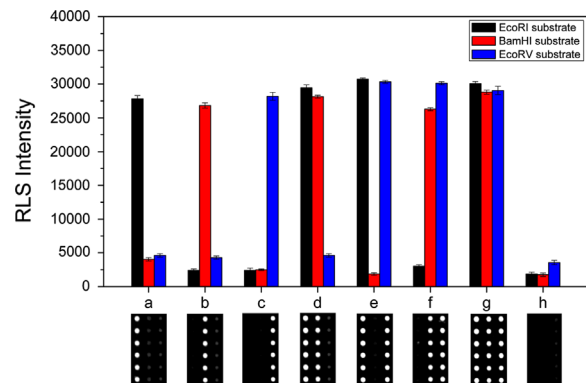


Fig. 1. RLS images (bottom) and corresponding RLS intensities of dsDNA substrates cleaved by different restriction endonucleases: (a) EcoRI, (b) BamHI, (c) EcoRV, (d) mixture of EcoRI and BamHI, (e) mixture of EcoRI and EcoRV, (f) mixture of BamHI and EcoRV, (g) mixture of EcoRI, BamHI and EcoRV, (h) heat inactivated EcoRI, respectively. The columns in RLS images contain (from left to right) EcoRI substrate, BamHI substrate, and EcoRV substrate, respectively. The concentration of restriction endonuclease is 500 U/mL each.

ssDNA probes and GNPs is 300:1 and the proportion of EI-S:BI-S:EV-S is 1:1.5:0.7 in the ssDNA mixture (as shown in Fig. S1).

The components of probe reaction buffer is second optimized because the ionic strength can influence the efficiency and specificity of DNA hybridization (Gong and Levicky, 2008). Using EcoRI as typical example, the effect of SSC amount on RLS intensity is examined. Relative high specificity of EcoRI detection and RLS intensity are obtained under the probe reaction buffer containing $0.67 \times$ SSC and 0.1% (w/v) SDS (as shown in Fig. S2).

In order to achieve higher cleavage efficiency, we also optimize the concentration of alkylamine-modified ssDNA in spotting buffer and the concentration of PEG-NH₂ in blocking solution since the crowding and intertwining of DNAs can cause steric hindrance on the interactions of dsDNAs with enzymes (Nakano et al., 2011). Using EcoRI as typical example, maximum cleavage efficiency is achieved when the concentration of EI-P is above 30 μ M in spotting buffer (as shown in Fig. S3) and the concentration of PEG-NH₂ is above 4 mg/mL in blocking solution (as shown in Fig. S4). In addition, we find that the cleavage efficiency is also dependent on the incubation time. It is increased by elongating incubation time and begins to saturate after 4 h incubation (as shown in Fig. S5).

3.3. Specificity of the multiplex method

A key problem in multiplex detection is the nonspecific/crossing-reactions of the components in the reaction mixture. In this

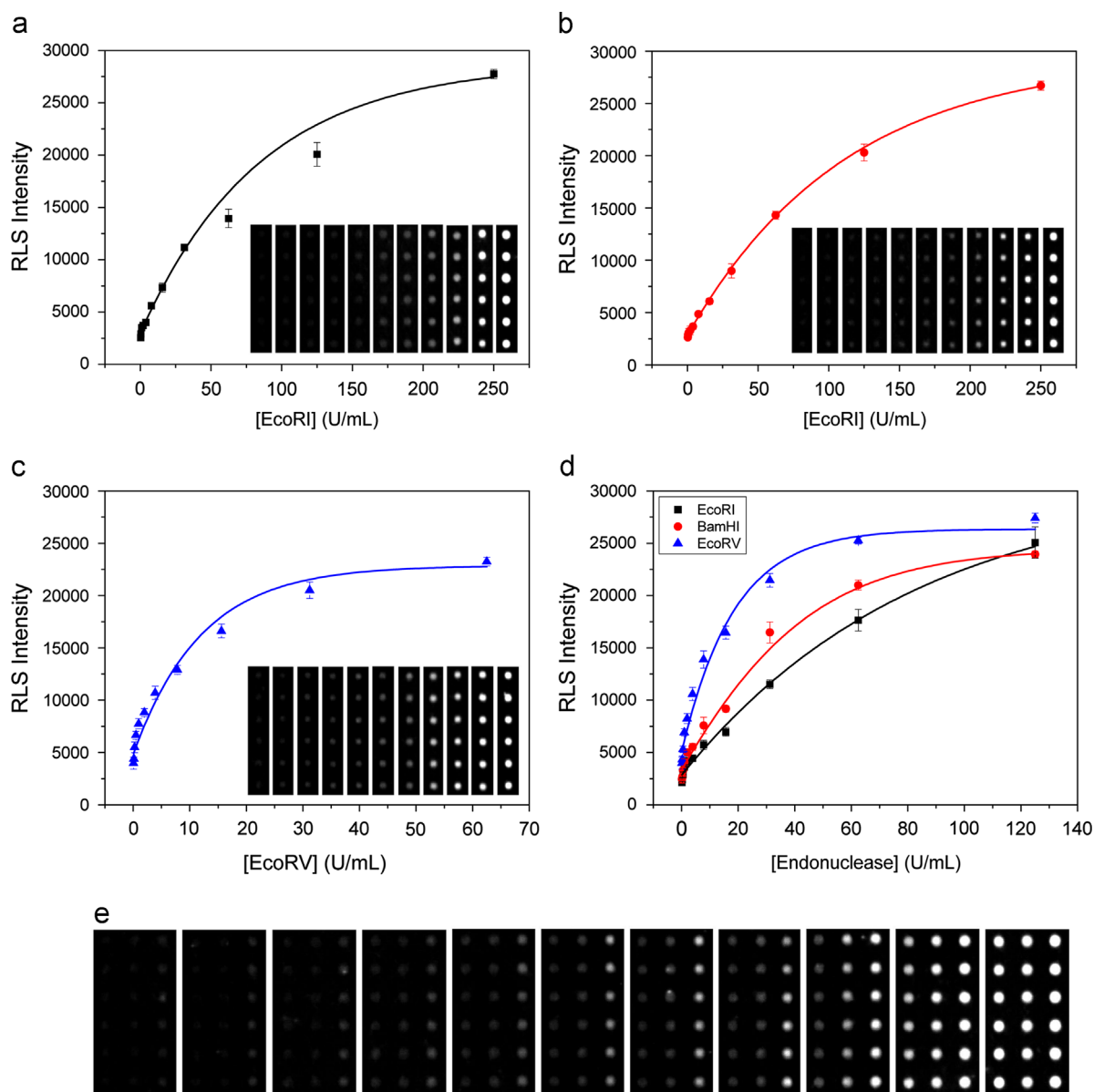


Fig. 2. RLS images (insets and e) and corresponding curves of RLS intensities responding to various concentrations of restriction endonucleases: (a) single EcoRI, (b) single BamHI, (c) single EcoRV, (d) mixture of EcoRI, BamHI and EcoRV (three restriction endonucleases are mixed at equal concentrations), respectively. The columns in RLS images contain (from left to right) EcoRI substrate, BamHI substrate, and EcoRV substrate, respectively.

case, three restriction endonucleases EcoRI, BamHI and EcoRV are selected as model enzymes to address the multiplexed detection ability of this assay. To evaluate the specificity of this method, assays of three individual restriction endonucleases, mixture of two and three restriction endonucleases, and inactivated EcoRI with three substrates are carried out, respectively. The results of this dsDNA microarray-based restriction endonuclease assay are shown in Fig. 1. As anticipated, only the spots reacted with specific restriction endonucleases give strong positive signals, i.e., RLS intensity of specific substrate-restriction endonuclease is more than 6 times stronger than that of non-substrate-restriction endonuclease. The RLS intensity of mixed restriction endonucleases assay is slightly stronger than that of single restriction endonuclease assay, which is probably caused by star activity or slightly nonspecific cleavage of restriction endonuclease (Conlan et al., 1999; Wei et al., 2008). In addition, the RLS intensities of inactive EcoRI-substrates are almost equal to that of control sample because inactive EcoRI can not cleave any of dsDNA substrates on microarray. The results demonstrate that the dsDNA

microarray-based assay has excellent specificity and can be used to discriminate multiple restriction endonucleases simultaneously.

3.4. Sensitivity of the multiplex method

In order to detect the sensitivity of the assay, a series of standard restriction endonuclease solution with various concentrations are prepared and applied to different subarrays. As shown in Fig. 2, the RLS intensity is increased proportionally with increasing concentration of restriction endonuclease, and the signal trends to saturate above 250 U/mL of EcoRI, 250 U/mL of BamHI and 62.5 U/mL of EcoRV, respectively. The detection limits (estimated as 3 times of the standard deviation of RLS signals of control samples) are 2.0×10^{-2} U/mL for EcoRI, 1.1×10^{-2} U/mL for BamHI and 1.6×10^{-2} U/mL for EcoRV, respectively. These are much lower than those of the previously reported methods (e.g., gel electrophoresis, cationic conjugated polymer-based assays, gold nanorod-based method, graphene oxide-based method and dsDNA microarray-based fluorescence assay) (Deng

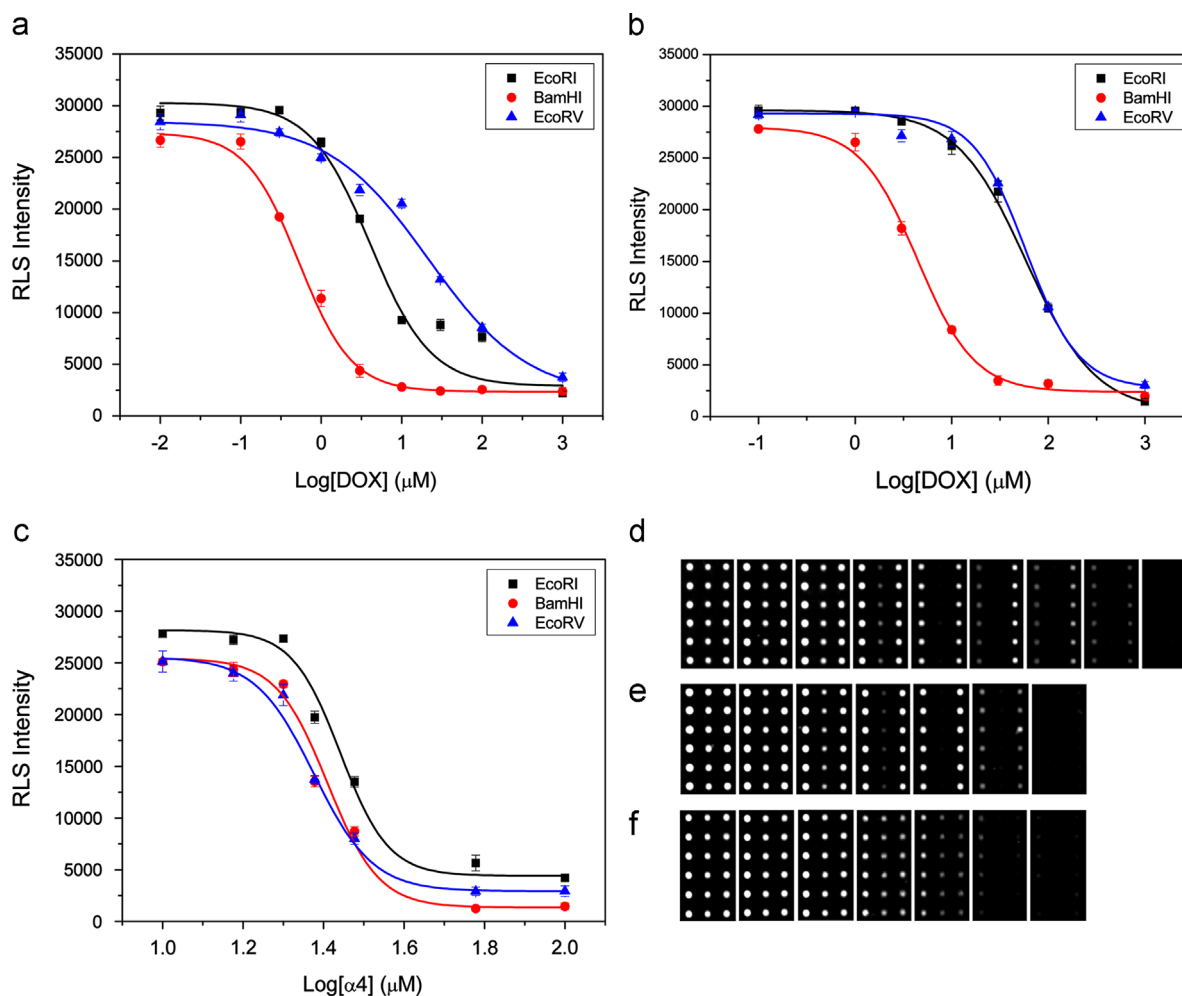


Fig. 3. Curves of RLS intensities responding to successive concentrations of inhibitors: (a) DOX, (b) EB, (c) $\alpha 4$, and corresponding RLS images ((d) DOX, (e) EB and (f) $\alpha 4$). The columns in RLS images contain (from left to right) EcoRI substrate, BamHI substrate, and EcoRV substrate, respectively. Microarrays are incubated with the mixture solution containing various concentrations of inhibitors and certain amount of restriction endonucleases (250 U/mL EcoRI, 250 U/mL BamHI and 125 U/mL EcoRV).

et al., 2012; Feng et al., 2007; Jeltsch et al., 1993; Lee et al., 2011; Ma et al., 2013; Pu et al., 2010).

To demonstrate the stability/reproducibility of the assay, EcoRI was used as typical example. Under optimized conditions, 96 identical reactions (125 U/mL EcoRI react with arrays and DNA-GNP probes from several batches) were analyzed. The signal-to-noise ratio (S/N) and quality Z' factor were calculated (as shown in Fig. S6). The relative high S/N and large Z' factor indicate that the assay has good reproducibility and stability.

3.5. Inhibition assay

Two commonly used DNA-binding molecules (EB and DOX) and an EcoRI-derived helical peptide ($\alpha 4$) are used to address the ability of the assay for screening restriction endonuclease inhibitor. The chemical structures of EB and DOX are shown in Chart S1. It is well known that DNA-binding molecules are potential inhibitors of restriction endonuclease which can disrupt the protein-DNA interactions. EB is a typical DNA intercalator (Luedtke et al., 2003). DOX is a widely used anticancer drug and shows preferentially GC binding affinity by intercalating the chromophore between bases with the daunosamine moiety projecting into the minor groove (Cutts et al., 2005). $\alpha 4$ is derived from the interfacial $\alpha 4$ helix peptide of EcoRI, which retains monomeric helical conformation in aqueous solution and can disrupt the formation of restriction endonuclease dimers by

Table 1
 IC_{50} values (μM) of the inhibitors.

| Inhibitors | EcoRI | BamHI | EcoRV |
|------------|-------|-------|-------|
| DOX | 6.0 | 0.7 | 37.2 |
| EB | 66.1 | 5.5 | 77.6 |
| $\alpha 4$ | 30.9 | 26.9 | 26.3 |

interacting with the interfacial region of the enzymes (Brickner and Chmielewski, 1998). $\alpha 4$ could bind with three restriction endonucleases because all of them are dimeric enzymes and possess α helix-containing dimerization interfacial region (Winkler et al., 1993; Newman et al., 1994; Venclovas et al., 1994; Vipond and Halford, 1993).

Fig. 3 shows the inhibitory curves of inhibitors. As expected, the RLS intensities are decreased with increasing the concentrations of inhibitors. The DNA-binding molecules follow the inhibition efficiency order of DOX > EB for the three restriction endonucleases. The result is consistent with the result of our previous work (Ma et al., 2013). In particular, $\alpha 4$ shows nearly same inhibition efficiency towards three restriction endonucleases. The half maximal inhibitory concentrations (IC_{50}) were calculated by the fitted equations of sigmoid curves (as shown in Table 1). The results suggest that our method not only has the potential to screen the abilities of inhibitors qualitatively but can also be used to yield

quantitative data on the inhibition efficiencies of different inhibitors, including substrate (dsDNA)-binding inhibitor and enzyme-binding inhibitor.

4. Conclusions

In summary, a dsDNA microarray-based multiplex assay has been developed for detecting restriction endonucleases with high selectivity and sensitivity and screening inhibitors of restriction endonucleases. The experimental results demonstrate that our approach not only have the potential to detect multiple restriction endonucleases simultaneously, but also can be used to evaluate the efficiency of restriction endonuclease inhibitor. Three restriction endonucleases and three inhibitors are chosen here to establish this new microarray format by proof of principle experiments. However, the present technique can be readily reconfigured for high-throughput analysis of restriction endonuclease functionality and inhibition by assembling dozens kinds of ssDNAs on one GNP surface and immobilizing thousands kinds of dsDNA substrates on microarray. In addition, the assay may also be employed to screen methylated DNA targets because DNA methylation inhibits DNA cleavage by the corresponding restriction endonuclease.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.bios.2013.08.045>.

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