Amplified Fluorescence Detection of DNA and RNA Hybridization by Surface Initiated Enzymatic Polymerization (SIEP)[1]

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ABSTRACT

Detection of nucleic acid on-chip has been of great interest due to its potential as research tools and for diagnostic applications. We present a new on-chip, isothermal signal amplification technique for the direct detection of unmodified DNA and RNA on microarrays using *terminal deoxynucleotidyl transferase* (TdT), a template-independent DNA polymerase that catalyzes the sequential addition of deoxynucleotides (dNTPs) at the 3'-OH group of a DNA primer and *yeast poly(A) polymerase* (PaP), an RNA polymerase that catalyzes polyadenylation at the 3'-OH an RNA primer. We utilized their ability to catalyze the formation of long polynucleotide and TdT's ability to incorporate unnatural nucleotide substrates such as fluorescent nucleotides (F-dNTP) into a long polymer chain of single stranded DNA (ssDNA) from the 3'-OH of a target DNA or RNA that is tethered on the surface through hybridization. We obtained dose reponse curves of hybridized DNA and RNA with LOD in ~1-10 pM.

Keywords: DNA microarray, miRNA, hybridization, terminal transferase, fluorescence.

1 INTRODUCTION

Microarray technology has become a powerful analytical tool in biology and medical research because of their massively parallel analytical power and high throughput. It is a powerful method for DNA or RNA quantification and it has been used to investigate differential gene expression, detect pathogens, and more recently for the quantification of microRNA (miRNA) levels. Typically, for analysis of nucleic acids by fluorescence based arrays, front-end processing of samples prior to microarray analysis involves: (1) reverse transcription of mRNA to cDNA; (2) amplification of genomic DNA or cDNA; and (3) labeling the target DNA with fluorophores or other detection molecules prior to hybridization on the array.

This approach has several potential limitations. First, reverse transcription and PCR-based target amplification have variable efficiency depending on the sequence of the target, which potentially introduces errors and systematic bias in the original mRNA concentration. Second, it requires multiple steps of purification that are time consuming, labor intensive, and involve extensive sample manipulation, which has the potential to introduce errors in the original mRNA concentration due to loss of material. Third, the direct incorporation of labels into cDNA molecules can reduce their hybridization efficiency, further confounding analysis. Finally, because samples are labeled indiscriminately, even samples that do not hybridize can generate a false positive or background signal because of non-specific binding of unhybridized target molecules to the surface.

Because of these limitations, we believe that there is a strong rationale for the development of a microarray-based DNA and RNA assay that allows direct interrogation of DNA and RNA with the following attributes: (1) it can be carried out *in situ*, *i.e.*, is on-chip; (2) it ensures that only the analytes of interest (targets) are labeled by introducing a detection label on the target after hybridization, so as to minimize false positive signals and background signal due to non-specific adsorption; (3) it provides signal amplification by incorporating multiple chromophores, or other labels per binding event, so that the output can be read by low-cost optical scanners or cell phone cameras; and (4) it can be carried out under isothermal conditions to minimize its technological complexity and make it field portable for point-of-care analysis.

2 ON CHIP FLUORESCENCE LABELING OF DNA HYBRIDIZATION BY SIEP[2]

Herein, we report a new isothermal, on-chip, post-hybridization labeling and amplification scheme for DNA microarrays. This assay uses TdT, a template-independent DNA polymerase that catalyzes the sequential addition of deoxynucleotides (dNTPs) at the 3'-OH group of an oligonucleotide primer, and incorporates multiple fluorescent dNTPs into the ssDNA chain that is grown by TdT to provide post-hybridization, on-chip fluorescence detection of the hybridized DNA. We have named this methodology surface initiated enzymatic polymerization (SIEP) of DNA.

As shown in **Figure 1**, SIEP utilizes the target strand as the "in situ" signal amplifier, without prior need for a pre-synthesized detection probe or signal amplifier, which greatly simplifies the assay. We show that SIEP using TdT enables the facile incorporation of fluorescently labeled dNTPs directly into a growing ssDNA chain on the surface. By printing probes with their 3'-ends attached to the surface and an exposed 5'-end (Figure 1), we ensured the on-chip

detection and amplification by *in situ* growth of a DNA strand that incorporates a fluorescent dNTP only when a target DNA binds to the probe, as the only initiation sites for *in situ* polymerization of DNA on the surface by TdT are the exposed 3'-OH groups presented by target DNA bound to probes.

Several on-chip signal amplification methods such as tyramide signal amplification,[3-4] immunoPCR,[5] rolling circle amplification (RCA),[6-7] and branched DNA technology[8] have been previously used for quantification of DNA and RNA. Although these signal amplification methods are sensitive, they require customized probes and signal amplifiers that have to be tailored for the target of interest, which introduces further complexity into the assay.

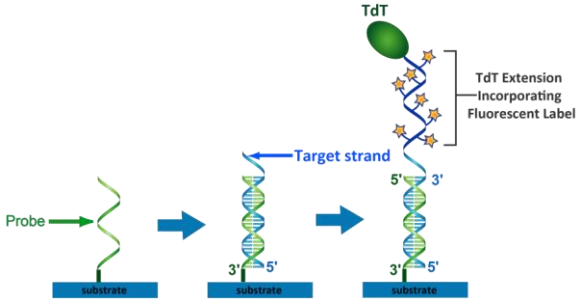

Figure 1. Schematic of SIEP for DNA detection that allows single step, isothermal, on-chip, post-hybridization fluorescence detection of DNA microarrays.

We show that on-chip fluorescence amplification by SIEP has a low pM limit of detection (LOD) and 2 to 3-orders of magnitude dynamic range, so that it is competitive with other techniques for DNA detection on surfaces.[9-10] This technology is compatible with commercial microarrays, because the orientation of the probes is the same as in many commercial microarrays[11] and is attractive, as it does not require prelabeling of all cDNA strands prior to labeling, is highly selective as it only labels positive "hits" on the surface, and is isothermal, so that it can be carried out directly on a DNA microarray with no need for specialized equipment.

2.1 Homopolymerization of DNA and Incorporation of Fluorescent dNTP by TdT

We focused on two parameters to characterize TdT catalyzed DNA polymerization in solution; they were the DNA primer that acts as an "initiator (**I**)" for the enzymatic polymerization of DNA and the nucleotide "monomer (**M**)." We selected short homo-oligomers (dA_{10}, dT_{10}, and dC_{10}) as primer initiator for simplicity and tagged with Cy5 dye at the 5'-end for the ease of visualization after gel electrophoresis. These primers were reacted with mononucleotide dNTPs; dATP, dTTP and dCTP and the products of polymerization were identified by the Cy5 dye. To study the effect of initiator-monomer composition on the degree of polymerization, each primer was reacted with various mononucleotides and the initiation efficiency as well as the length and the polydispersity of the extended product were examined using gel electrophoresis. To investigate the control on the degree of polymerization, we selected the type of monomers that are preferred by TdT and vary the M/I ratio during TdT reaction.

We found that the polymerization of dATP resulted in the narrowest size distribution of the extension product followed by the polymerization of dTTP. On the other hand, the polymerization of dCTP was not as favorable as that of dATP and dTTP, shown by the relatively shorter and significantly larger polydispersity of the extension products. Hence, the preferred order of monomer is dATP > dTTP >> dCTP.

Assuming that all primer served as an initiator and was extended and that the reaction has gone to completion, TdT-catalyzed DNA polymerization is equal to the input monomer and initiator ratio (M/I) with a Poisson distribution in molecular weight,[12] Using homopolymer composition of primer and monomer, we found that the length of the DNA chain grown from the 3'-OH end of the primer was proportional to the M/I ratio, so that the product length can be controlled simply using a higher M/I ratio and a longer reaction time to allow the reaction to go to completion.

We selected an M/I of 1000, 2 h reaction, and two types of fluorescent dNTP – Cy3-dATP and Cy3-dUTP to assess the incorporation of fluorescent dNTPs. Using these monomers and solution polymerization reactions, we determined whether and to what extent, fluorescent dNTPs can be incorporated into growing ssDNA chains. We found that TdT did not catalyze DNA polymerization efficiently when a fluorescent dNTP was used as the sole monomer in the reaction. Therefore, we examined the effect of varying the ratio of fluorescent dNTPs [Cy3-dATP and Cy3-dUTP] to their corresponding natural dNTPs [dATP and dTTP] on the incorporation of the fluorescent dNTP in the polymerized DNA. The amount of extended chains and the relative amounts of nucleotides incorporated were determined by tagging the primer initiator with Cy5 and incorporating Cy3-dNTPs which fluorescent intensities were measured using a microplate reader, after inactivating the reaction mixture and removing unreacted fluorescent dNTPs.

As shown in **Figure 2a**, the extent of incorporation of Cy3-dNTP in the extended chain increased as a function of the ratio of the fluorescent dNTP to natural dNTP. For a Cy3-dATP/dATP ratio of 0.2, up to 50 fluorophores were incorporated into the ~1 kb long ssDNA chains that was grown by TdT. On the other hand, as the ratio of fluorescent dNTP to natural dNTP increased in the reaction, the amount of primer (initiator) that was extended decreased (**Figure 2b**). This trend is consistent with other studies on fluorescent dNTP incorporation using Taq and other DNA polymerases, in which the yield of the PCR product was inversely related to the ratio of fluorescent dNTP to unmodified dNTP in the reaction.[13]

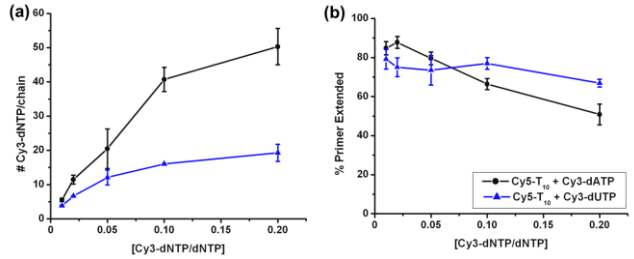

Figure 2. The presence of fluorescent dNTPs affects the polymerization of natural dNTPs by TdT as shown by the polymerization products of different molar ratios of fluorescent dNTPs to natural dNTPs (0.01, 0.02, 0.05, 0.1, and 0.2). (a) As the amount of Cy3-dATP increases in the TdT reaction mixture, the number of Cy3-dATP being incorporated increases while (b) the amount of extended oligonucleotide primer [Cy5-T_{10}] decreases.

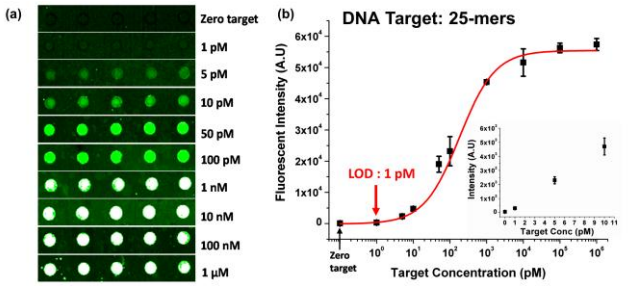

Figure 3. Dose response curve of DNA hybridization. (a) Fluorescence image of target DNA after hybridization with printed probes on glass, and in situ, TdT catalyzed SIEP of fluorescent DNA from hybridized target. (b) Fluorescence signal intensity as a function of target DNA concentration generates a dose response curve with a LOD of ~1 pM and a 2-log dynamic range. The inset shows the signal intensity at target concentration's ≤ 10 pM.

2.2 On-chip Detection and Signal Amplification of DNA Hybridization by SIEP

We demonstrated that the ability of TdT to incorporate fluorescent dNTPs could be used to carry out on-chip labeling of target DNA on a surface, as shown in **Figure 1**. A 25 base long target DNA was captured by an immobilized probe and selectively extended by TdT using a ratio of 0.005 (Cy3- dATP to dATP) in the TdT catalyzed extension of DNA. Multiple Cy3-dATP were incorporated into the extended target and the presence of the target was detected by quantification of the fluorescence intensity from each spot by a fluorescence scanner.

The dose-response curve show a sigmoidal response of the background corrected fluorescence signal intensity as a function of increasing DNA target concentrations with a linear range that spanned 2-orders of magnitude in fluorescence intensity (**Figure 3**). The dose-response curve also provided a 1 pM limit of detection (LOD), which is comparable to –and in some instances– better than other DNA target detection and amplification techniques, such as SPR-based detection with Au nanoparticle enhancement,[14] microsphere-based rolling circle amplification (RCA),[6] and fluorescent conjugated polymers.[15]

3 ON CHIP FLUORESCENCE LABELING OF RNA HYBRIDIZATION BY SIEP[16]

We have demonstrated the use of SIEP assay to detect DNA hybridization with one step, isothermal, on-chip, post hybridization fluorescence detection. Next, we are interested in developing an on-chip labeling assay by SIEP which can directly interrogate RNA. This assay builds on previous demonstration that the introduction of a unique 3'-OH moiety due to hybridization of a target DNA to an immobilized probe provides an "initiation" site for the *in situ* enzyme-catalyzed polymerization of long single stranded DNA (ssDNA) chains that incorporate fluorescently labeled nucleotides.[2]

Although TdT is very efficient at catalyzing the growth of DNA from a DNA initiator –the bound target in a target-probe duplex on the surface– and can incorporate multiple unnatural fluorescent nucleotides during SIEP into the growing DNA chain, it does not recognize a RNA initiator efficiently, as would be the case for hybridization of an RNA molecule to a probe on the surface. To solve this problem, herein we show that the combination of two enzymes: yeast poly(A) polymerase (PaP) and TdT, enable the *in situ* fluorescence detection of RNA molecules using SIEP. PaP is a template independent RNA polymerase that catalyzes polyadenylation at the 3'-OH group of RNA molecules. PaP however, does not accept fluorescently labeled ribonucleotides efficiently, so that *in situ* SIEP with PaP from an RNA initiator cannot be used for *in situ* post-hybridization fluorescence labeling and signal amplification of RNA. We have discovered, however, that PaP has the unusual attribute that it catalyzes the attachment of a few deoxyadenosine triphosphate (dATP) to the 3'–OH of an RNA initiator, creating a RNA-DNA hybrid. The 3'-end of this short DNA sequence can be recognized by TdT, and enables subsequent TdT catalyzed polymerization of a long ssDNA strand with multiple fluorophores, thereby enabling *in situ* fluorescence detection of hybridized RNA (**Figure 4**).

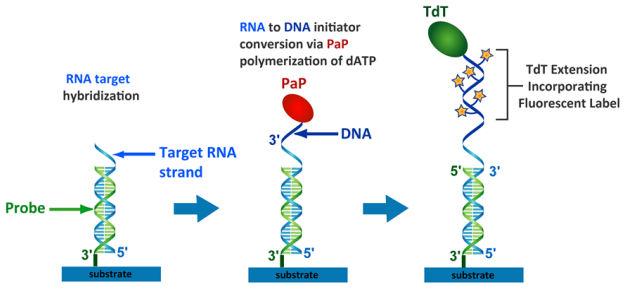

Figure 4. Direct RNA labeling via SIEP of DNA catalyzed by PaP and TdT. SIEP of DNA allows two steps direct, post-hybridization, isothermal, on-chip fluorescent labeling of RNA.

3.1 Detection of short and long RNA Target

After verifying that that the sequential reaction of PaP followed by TdT labeling is the preferred strategy for amplified direct labeling of RNA compared to one pot reaction,[16] we evaluated the sequential PaP and TdT reaction for amplified on-chip fluorescence detection of RNA hybridization to a surface-bound probe. We chose PNA capture probes for their high affinity for RNA target[17] and because they are not substrates for PaP or TdT, which eliminates the possibility of non-specific SIEP from the surface-bound probe. We printed the PNA probes on a "non-fouling" POEGMA slide because it prevents non-specific binding of biomolecules[18], and chose Cy5-dATP as the label by TdT catalyzed DNA polymerization because its fluorescence emission maximum is in the far-red region, which avoids any auto-fluorescence signals from the glass slide.

We obtained dose-response curves for short and long RNA targets as shown in **Figure 5**, respectively. For a short 21 base long synthetic RNA target that has the same length as most miRNA (**Figure 5a**), the LOD was ~2 pM. This LOD corresponds to 0.16 fmol target in an 80 µL hybridization buffer, which is comparable to previously reported LOD for unamplified samples on other array platforms for short RNA targets such as miRNA[19-22] and is similar to the LOD for DNA detection using TdT reported by us previously.[2] This result is the first demonstration of direct on-chip RNA fluorescence detection without the need for RNA manipulation or additional capture/ detection probe. The hybridized RNA target is directly quantified with sensitivity that is competitive to other more complicated techniques[21-24] by implementing a simple two-step, isothermal and on-chip reaction, using commercially available reagents.

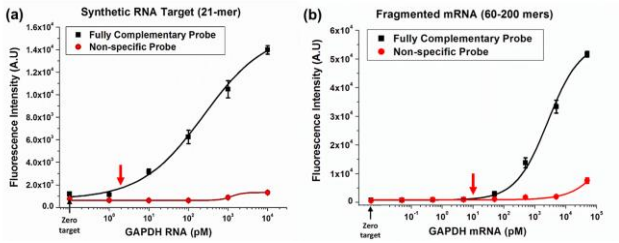

Figure 5. Dose response curves of RNA target, (a) short, 21-mer RNA target and (b) fragmented mRNA target. Red arrows indicate LOD.

We next investigated the direct detection of a more challenging target, a full-length mRNA target with a 17 base complementary sequence for a surface-bound PNA probe. Due to the limitation imposed by longer mRNA target, we include an RNA fragmentation step, a method that is known to increase the detection sensitivity of long targets in microarray.[25-26] The RNA fragments resulting from this cleavage primarily contain a 3'-phosphate group as opposed to the desired 3'-OH group necessary for polymerization by PaP or TdT. Therefor, we devised an additional end-repair step by phosphatase treatment following fragmentation to remove the 3'-phosphate and recover the 3'-OH. We used Antarctic Phosphatase for this purpose and carried out an on-chip repair step before SIEP reaction. We obtained a 10 pM LOD for fragmented and end-repaired full length mRNA input (**Figure 5b**), which is a 100 fold improvement from the 1 nM LOD for the non-fragmented full-length mRNA and similar to the LOD obtained for the short, synthetic RNA target (**Figure 5a**).

3.2 Multiplexed RNA Detection

We further investigated the potential of SIEP to detect multiple mRNA targets in a microarray format. We were also interested in examining whether this assay would be immune to sequence bias, which is critical for quantitative and robust multiplexed detection. To do so, we performed a microarray analysis of a mixture of mRNA target molecules consisting of full length GAPDH, PRSS21, and IFI44 mRNA transcripts that were fragmented, and then captured by probes, followed by fluorescence detection by sequential TdT and PaP catalyzed SIEP. As shown in **Figure 6a**, we can detect individual fragmented mRNA target in a mixture and maintain the sensitivity for the assay at ~10 pM for all targets, suggesting that the presence of three targets simultaneously does not degrade the sensitivity of the assay given the large number of short RNA strands generated by the fragmentation of the three full-length mRNA transcripts. Control experiments were also performed in which one or more of the targets were omitted from the hybridization mixture to confirm the specificity of the assay (**Figure 6b**). However, we would like to highlight that specificity of the target labeling is also determined by the stringency of the hybridization between the probe and the target RNA strand, thus like any hybridization-based RNA detection, our technique is limited by the specificity of the probe or the quality of the probe design.

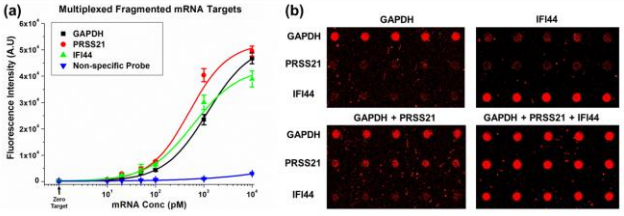

Figure 6. Multiplexed detection of RNA target by SIEP shows (a) dose response curves of simultaneous dtectection of three targets and (b) selectivity of SIEP.

4 CONCLUSION

We have demonstrated direct labeling of unmodified DNA and RNA using one step and two steps, respectively, post hybridization, isothermal, on-chip fluorescence labeling by SIEP. This method is shown to be sensitive, specific, versatile yet straightforward and is compatible with current microarray set-up by simply using commerically available reagents. SIEP as a method is currently being developed for amplified DNA and RNA colorimetric detection.

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