On-chip Labeling via Surface Initiated Enzymatic Polymerization (SIEP) for Nucleic Acids Hybridization Detection

by

Vinalia Tjong

Department of Biomedical Engineering
Duke University

Date:_______________________

Approved:

Ashutosh Chilkoti, Supervisor

Stefan Zauscher

William Reichert

Gabriel Lopez

Aimee Zaas

Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Biomedical Engineering in the Graduate School of Duke University

2013
ABSTRACT

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Abstract

Current techniques for nucleic acid analysis often involve extensive sample preparation that requires skilled personnel and multiple purification steps. In this dissertation, we introduce an on-chip, isothermal, post-hybridization labeling and signal amplification technique that can directly interrogate unmodified DNA and RNA samples on a microarray format, eliminating the need for microarray sample preprocessing.

We name this technique Surface Initiated Enzymatic Polymerization (SIEP), where we exploit the ability of a template independent DNA polymerase called Terminal Deoxynucleotidyl Transferase (TdT) to catalyze the formation of long single-stranded DNA (ssDNA) chain from the 3’-end of a short DNA primer, which is tethered on the surface, and TdT’s ability to incorporate unnatural reporter nucleotides, such as fluorescent nucleotides. We hypothesize that polymerization of a long ssDNA chain while incorporating multiple fluorescent nucleotides on target DNA or RNA hybridized to probe printed on a surface will provide a simple and powerful, isothermal method for on-chip labeling and signal amplification.

We developed the SIEP methodology by first characterizing TdT biochemical reaction to polymerize long homopolymer ssDNA (> 1000 bases) starting from the 3’-OH of ten bases oligonucleotides. We found that the preferred monomers (deoxynucleotide, dNTP) are dATP and dTTP, and that the length of the ssDNA extension is determined
by the ratio of input monomer (dNTP) to initiator (short oligonucleotides). We also investigated TdT’s ability to incorporate fluorescent dNTPs into a ssDNA chain by examining the effect of the molar ratios of fluorescent dNTP to natural dNTP on the initiation efficiency, the degree of fluorophore incorporation, the length and the polydispersity of the polymerized DNA strand. These experiments allowed us to incorporate up to ~50 fluorescent Cy3-labeled dNTPs per kilobase into a ssDNA chain.

With the goal of using SIEP as an on-chip labeling method, we also quantified TdT mediated signal amplification on the surface by immobilizing ssDNA oligonucleotide initiators on a glass surface followed by SIEP of DNA. The incorporation of multiple fluorophores into the extended DNA chain by SIEP translated to a up to ~45 fold increase in signal amplification compared to the incorporation of a single fluorophore.

SIEP was then employed to detect hybridization of DNA (25 bases), short miRNA (21 bases) and long mRNA (1400 bases) by the post-hybridization, on-chip polymerization of fluorescently labeled ssDNA that was grown from the 3’-OH of hybridized target strands. A dose-response curve for detection of DNA hybridization by SIEP was generated, with a ~1 pM limit of detection (LOD) and a 2-log linear dynamic range while the detection of short miRNA and fragmented mRNA targets resulted in ~2 pM and ~10 pM LOD, respectively with a 3-log linear dynamic range.

We further developed SIEP for colorimetric detection by exploiting the presence of negatively charged phosphate backbone on the surface as target DNA or RNA
hybridizes on the immobilized probe. The net negative charge on the surface is further increased by TdT catalyzed polymerization of long ssDNA. We then used positively charged gold nanoparticles as reporters, which can be further amplified through electroless metallization, creating DNA spots that are visible by eye. We observed an increase of 100 fold in LOD due to SIEP amplification.

Overall, we demonstrated the use of SIEP methodology to label unmodified target DNA and RNA on chip, which can be detected through fluorescence signal or colorimetric signal of metallized DNA spots. This methodology is straightforward and versatile, is compatible with current microarray technology, and can be implemented using commercially available reagents.
To Mama, Kasey, and Aina
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1. Motivation and Background

1.1 Nucleic acid analysis: molecular diagnostic promises and challenges

Nucleic acids as genetic materials encode instructions that govern the development and functions in all living organism. Therefore they are an ideal biomarker and potentially more accurate, robust, reliable, and applicable across organism compared to traditional biotyping such as phenotyping of microorganisms or the use of protein and fatty acid analysis. The use of nucleic acids in the diagnosis of diseases, pathogens identification, and genetic studies has resulted in a term called molecular diagnostics. In recent years, advances in nucleic acids technologies have transformed molecular diagnostics, in particular, the diagnosis and management of infectious diseases; gene expression and regulations studies; and single nucleotide polymorphism (SNP) analysis. In general, nucleic acid testing is developed mainly for two purposes; to identify specific nucleic acids sequences and to quantify the amount of those particular sequences. These sequences are of interest because they are associated with certain types of pathogens, diseases etiology, or body responses to treatment or drugs. Some noteworthy examples of nucleic acid technologies that are widely used currently are polymerase chain reaction (PCR), terminal restriction fragment length polymorphism (T-RFLP), microarray technology, fluorescence in situ hybridization (FISH) assay, and the sequencing technologies.
Although these technologies in general have allowed more rapid and accurate diagnosis by using nucleic acid as biomarkers, there are still challenges that limit the application of these technologies only in hospital or reference labs where high-end instrumentations and skilled personnel are available. The main reason for this limitation is the amount of nucleic acid samples to start the analysis with. In most tests, DNA or RNA samples extracted from bodily fluids, stools, cells or tissue are limited or even insufficient for analysis and therefore require an amplification step.

In general, there are two strategies to overcome limited amount of samples (Figure 1-1); target amplification and signal amplification. Target amplification refers to procedures to amplify the amount of nucleic acid target, particularly the sequence of interest; while signal amplification refers to methods to apply multiple labels on the sequence of interest or procedures that amplify the presence of target nucleic acids. These amplification steps are often quite complex, depending on the type of amplification techniques, numbers of sequence being amplified at the same time (multiplexing), and number of purification steps. In addition, there are some intrinsic limitations that are specific to each amplification techniques. Due to the complexity of the samples processing (either target or signal amplification), the use of nucleic acid test in general is still restricted to hospitals, reference labs, and academic research labs, which have the resources for highly trained personnel and expensive instrumentations.
Next, we discuss some of the capabilities and limitations of these amplification techniques, which motivate the topic of this dissertation and the development of SIEP.

**Figure 1-1: Target amplification vs signal amplification**

Amplification techniques in nucleic acid testing comprise of target and signal amplification, which aim to amplify the amount of nucleic acid target and to apply multiple labels on the nucleic acid targets, respectively.

### 1.2 Nucleic acid target amplification

In this section, we will highlight some of the nucleic acid amplification techniques. This section is not meant to provide comprehensive review but rather to point out to readers how these techniques are useful in the analysis of nucleic acids as well as their limitations.

#### 1.2.1 Polymerase chain reaction (PCR)

PCR is the most popular technique to amplify a selected region of DNA molecules in vitro. The process requires a thermostable DNA polymerase, a template strand (the target), nucleotides, and one set of forward and reverse primer as the
initiation sites for polymerase to start replicating the template. Through multiple cycles of template denaturation, primer hybridization to the template (annealing step), and primer extension; the target DNA is amplified exponentially relatively quick. A more detailed discussion on the basic principle of PCR can be found in the published literature.\textsuperscript{11, 12}

The discussion we want to highlight here is the power and limitation of PCR as a technique for nucleic acids analysis, particularly in the application of PCR as diagnostics tools. As mentioned earlier, PCR allows exponential amplification of desired DNA targets, which means PCR is extremely sensitive. Theoretically, PCR has the potential to generate billions of copies of target DNA from a single copy in less than 1 h.\textsuperscript{13} Therefore, PCR enables the analysis of very small amounts of nucleic acid, placing PCR as one of the most sensitive techniques in nucleic acids testing. However, the extreme sensitivity comes with several pitfalls.\textsuperscript{13-15} First, nucleic acid samples preparation (template strand) for PCR is very critical because any slight contamination or errors in sample preparation will also be amplified during PCR, thus any errors could be detrimental to the accuracy of PCR amplification. Second, due to the sensitivity of sample preparation to errors, this also means that any variability, e.g. operators, reagents, in the PCR samples processing could also be amplified, resulting in the reliability issue of PCR as a test. Third, in the use of PCR as quantification method as well as amplification method during reverse transcription PCR (RT-PCR), accuracy of quantification could be impacted by the
variation in the amplification efficiency of different sequences, which potentially introduces errors and systematic bias in the original concentration. Finally, PCR techniques requires temperature cycling to denature, hybridize, and extend the DNA strands, which translates to an expensive piece of instrumentation that is only available in advanced laboratories.

There are some strategies in the development of PCR protocols that minimize the pitfalls discussed previously, such as the use of automation to set up PCR reactions, strict validation on the quantity and quality of template strands, samples normalization, the development of standard samples for competitive and non-competitive quantitative RT-PCR, and the development of digital PCR.\textsuperscript{14-16} However, these efforts further add complexity and cost to PCR as a test. Nevertheless, PCR is still one of the most popular techniques for nucleic-acid-based testing and specific PCR-based assays for different pathogens have been approved by FDA.\textsuperscript{13}

\subsection*{1.2.2 Isothermal amplification techniques}

One strategy to overcome the requirement of the PCR reaction for temperature cycling is the development of isothermal nucleic acid amplification techniques, where amplification is carried out at one particular temperature and therefore requires less complex and less expensive instrumentation. The significance on the development of isothermal techniques is their potential application in low-resource settings, compared to PCR which demands more advanced instrumentations.
There are many isothermal amplification techniques that have been developed. In general, they can be grouped based on their reaction principles (Table 1-1); techniques that exploit in vitro RNA transcription, DNA replications based on enzymatic duplex melting or primer annealing, and strand displacement phenomenon. In addition, comparing the reaction scheme of these techniques reveals several similarities, such as the use of enzymes (polymerases, helicase, ligase, recombinase, or endonucleases), the use of primers for reaction initiation, isothermal generation of ssDNA, exponential amplification, and the need to generate intermediate target for amplification. The differences lie in the temperature and time required for the reaction. The reaction temperatures, it can range between 30°C and 65°C, while the reaction time can be as short as 10 min to 2 h. Details on various isothermal amplification techniques have been reviewed elsewhere.

Although isothermal amplification only requires a simple instrumentation such as water baths or resistive heaters, some of the techniques can be quite complex involving three enzymes (NASBA) or a set of four to six primers (LAMP). In addition, due to the exponential nature of the amplification, there is still an issue of non-specific background amplification. Therefore, slower and more controlled progression is better suited for target quantification. Furthermore, although some techniques have shown some level of capabilities for multiplexing (at least two different targets), no data is available for most of the techniques on their multiplexing capability. Nevertheless, some
of these techniques have been commercialized and developed into nucleic acid tests that are approved by FDA such as TMA, NASBA, and SDA.

Table 1-1: Isothermal amplification techniques

<table>
<thead>
<tr>
<th>Reaction Principle</th>
<th>Enzymes</th>
</tr>
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<tbody>
<tr>
<td>In vitro RNA transcription</td>
<td>Transcription-mediated amplification (TMA)²⁰</td>
</tr>
<tr>
<td></td>
<td>Nucleic acid sequence-based amplification (NASBA)²¹</td>
</tr>
<tr>
<td></td>
<td>Signal-mediated amplification of RNA technology (SMART)²³</td>
</tr>
<tr>
<td>Enzymatic duplex melting</td>
<td>Helicase-dependent amplification (HDA)²³</td>
</tr>
<tr>
<td>Enzymatic primer annealing</td>
<td>Recombinase polymerase amplification (RPA)²⁴</td>
</tr>
<tr>
<td>Strand displacement</td>
<td>Loop-mediated amplification (LAMP)²⁵</td>
</tr>
<tr>
<td></td>
<td>Cross-priming amplification (CPA)²⁶,²⁷</td>
</tr>
<tr>
<td></td>
<td>Smart-amplification (Smart-AMP)²⁸</td>
</tr>
<tr>
<td></td>
<td>Rolling circle amplification (RCA)²⁹</td>
</tr>
<tr>
<td>Strand displacement and nicking</td>
<td>Strand displacement amplification (SDA)³⁰</td>
</tr>
<tr>
<td></td>
<td>Nicking enzyme amplification reaction (NEAR)³¹</td>
</tr>
<tr>
<td></td>
<td>Isothermal chain amplification (ICA)³²</td>
</tr>
</tbody>
</table>

1.3 Signal amplification: nucleic acid labeling and probes

Signal amplification strategies comprise of strategies to introduce multiple labeling on the target nucleic acid or the development of detection probes that can amplify the presence of target interest. In general, while target amplification aims to obtain as high a quantity of target materials as possible, signal amplification aims to increase the signal per unit target presence so that a smaller quantity of target can be measured. This section is not meant to provide a comprehensive review but to highlight some signal amplification strategies that have been used widely. Although we try to classify these strategies to different modes, they often overlap due to combinatory nature of the methods.
1.3.1 Fluorescence-based probes and signal amplification

The simplest form of signal amplification can be achieved through labeling of the target nucleic acids or target probes with fluorescence reporters. There are two sites for target nucleic acid labeling: end labeling at the 3’-end or the 5’-end and internal labeling that is distributed along the target (also termed as body labeling). Typically, 3’-end labeling attaching a single or a few fluorophores is limitedly accomplished by only a few template independent polymerases such as T4 RNA ligase,33 yeast poly A polymerase,34 and terminal transferase.35 On the other hand, internal labeling of multiple fluorescent nucleotides is typically attained using a template dependent DNA polymerase such as Taq polymerase during PCR reaction.36 Due to the polymerase inefficiency in incorporating bulky fluorescent nucleotides, multiple end or internal labeling can be obtained through indirect route by incorporating amino allyl modified nucleotide (amino allyl-dUTP),37 which can be further reacted to NHS-ester fluorescent dye. With the indirect labeling route, greater signal amplification is attained.

1.3.2 Affinity-based signal amplification

Another indirect route of target nucleic acid or probe labeling is through the incorporation of ligand or hapten such as biotin and digoxigenin by polymerases, which can bind to their corresponding affinity tag, streptavidin and digoxigenin antibody respectively.38, 39 To achieve signal amplification, streptavidin and digoxigenin antibody are conjugated to reporter molecules such as fluorescent dyes, photopolymerizable
chemicals for colorimetric detection,\textsuperscript{40} and horseradish peroxidase (HRP) enzyme which activate tyramide conjugated reporter.\textsuperscript{41, 42} Due to high affinity between biotin and streptavidin (Kd= $10^{-15}$), and the efficient incorporation of biotinylated nucleotides by most polymerases, this indirect strategy is preferred rather than incorporating fluorescent nucleotides directly.

Another strategy for affinity-based signal amplification includes the use of specific antibodies that recognizes duplex DNA\textsuperscript{43} or DNA-RNA hybrid.\textsuperscript{44, 45} Antibody to duplex DNA has been used to detect PCR product through subsequent reaction with horseradish peroxidase detection antibody while antibody to DNA-RNA hybrid is subsequently detected using fluorescently conjugated detection antibody in microarray assay.

1.3.3 Highly branched DNA signal amplification

There are two signal amplification probe technologies that are based on highly branched DNA structure; dendritic nucleic acid probe\textsuperscript{46} and branched-DNA assay (b-DNA).\textsuperscript{47} In dendritic nucleic acid probe, highly branched DNA structure is constructed from DNA monomer that is composed of two DNA strands with a complementary region in the middle such that when they hybridize, they form “X”-like structure. These monomers with the four dangling arms are then hybridizes with each other forming a branched structure that serve as the first layer. As the layers grow, it is crosslinked chemically to prevent dissociation. Fluorescent dyes are attached to the DNA dendrimer
through complementary hybridization between the one of the dangling arms and fluorescently tagged oligonucleotides while the probe sequence is attached to the DNA dendrimer through another complementary hybridization of the probe sequence to another dangling arm. With the dendrimer probe, at least 200 labels are attached which results in 200-fold enhancement on signal intensity and translates to 50-fold less total RNA required for analysis. Dendritic nucleic acid probe is commercialized by Genosphere, Inc as 3DNA dendrimer probes.

The branched DNA (b-DNA) assay was developed as an alternative molecular diagnostic to overcome the limitation of reverse transcriptase-polymerase chain reaction (RT-PCR) assay, where nucleic acid target amplification tends to skew the actual quantity of the target in samples. There are three key design strategies in b-DNA assay: (1) the cooperative hybridization of a set of probe (capture probe and extender) for a particular target that allows efficient and stable capture of messenger RNA (mRNA) target directly from cell lysate and tissue homogenate, (2) the sandwich approach for signal detection via label extender and preamplifier allows direct detection of unlabelled nucleic acid target and (3) the binding of multiple b-DNA amplifiers to alkaline-phosphatase labelled oligonucleotides, amplifies the hybridization signal. Although complex probe and amplifier oligonucleotides designs are needed to generate multiple layers of DNA hybridization for high selectivity and sensitivity, b-DNA has been successfully implemented in clinic to monitor patients on antiviral therapy for human
immunodeficiency virus (HIV), hepatitis C (HCV) and hepatitis B (HBV) and to stratify patients for therapy.47

1.3.4 Nanoparticle-based probes and signal amplification

Recent development in nanotechnology has resulted in the application of nanoparticles across many disciplines; one of it is in the development of sensitive nucleic acid analysis. Some of these nanoparticles are gold nanoparticles,51-53 quantum dots,54, 55 and organic or silica nanoparticles doped with fluorescent molecules.56-58 These nanoparticles are typically conjugated to the oligonucleotide detection probes to label DNA targets, allowing discrimination of single nucleotide mismatches with higher selectivity than fluorescence labeled target and two orders of magnitude higher in sensitivity than that of fluorescence labeled target by gold nanoparticle silver enhancement signal amplification.52

Although the development of various techniques for target and signal amplification has allowed highly sensitive nucleic acid analysis, to achieve robust detection of ultra-low target concentration, a combination of both target and signal amplification in one assay may be necessary. In addition, challenges remain in the sample processing to extract nucleic acids from cells or tissue, especially for RNA sample where the quality of extracted RNA can impact the outcome of analysis significantly.
1.4 Microarray chip technology

Microarray platform (Figure 1-1) has gained wider utility as a powerful tool for nucleic acids analysis due to its ability to perform high throughput parallel analysis of thousands of genes. Microarrays are now routinely used for analysis of gene expression,2, 3, 59, 60 to detect pathogens and their genotyping,61, 62 and are also being developed for the detection of microRNA.63-65 Microarray chip technology consists of several key components: (i) short DNA oligomers (19-60 mers) with a known sequence to be immobilized on a glass substrate as a capture probe, (ii) a nucleic acid sample to be analyzed as a target, (iii) signals produced by specific hybridization of the labeled target to the immobilized probe and (iv) powerful bioinformatics software for data analysis.

![Figure 1-2: Schematic of microarray technology](image)

Microarray technology comprises of immobilized DNA probes on a substrate which are exposed to fluorescently tagged targets. Immobilization of hundreds of thousands probes on different spots on the substrate allows unprecedented multiplexing and parallel analysis of different targets as demonstrated by the fluorescence scan image.
In recent years, the development of microarray platforms has overcome various technical obstacles, especially in the manufacturing of capture probes in a high throughput, reliable manner\textsuperscript{66-68} and the availability of powerful algorithms for data analysis.\textsuperscript{69,70} However, similar to any nucleic acid test, the major challenges remain in the detection of ultra-low amounts of target nucleic acid. As mentioned previously, the current methods to improve the limit of detection have focused primarily on two strategies:\textsuperscript{9,10} target amplification, mainly consisting of attempts to increase the amount of the initial target itself; and signal amplification which focus on various labeling schemes to increase the sensitivity of signal transduction. Although these target amplification and/or signal amplification methods have circumvented the limitation of detecting low amounts of target, most of these strategies introduce layers of complexity to the microarray processes and hence limit the applications of microarray analysis to the research laboratory setting.

1.4.1 Nucleic acid target amplification and labeling in microarray

Microarrays are less sensitive than PCR in detecting ultra-low concentrations of nucleic acids; hence microarray samples require pre-processing procedures that involve sample amplification (PCR or RT-PCR) and labeling steps. In microarray analysis, the target nucleic acid samples must be tagged with labels to enable imaging and detection when hybridization occurs. Therefore, a series of steps are often necessary to amplify and label the target molecule (Figure 1-1).
Microarray samples preparation includes extraction and purification of DNA or RNA sample from cells/tissues, amplification of genomic DNA or total RNA and labeling, hybridization of amplified and labeled target, and fluorescence signal acquisition. The proposed strategy for on-chip labeling allows direct interrogation of genomic DNA or total RNA by in situ labeling post hybridization.

Microarray samples normally consist of DNA or RNA targets, and are amplified by PCR, RT-PCR, or in vitro transcription, with labels introduced as the target is being amplified. The two main label incorporation strategies are the end labeling achieved using fluorescent-tagged oligonucleotides as primers during PCR or in vitro transcription and the body labeling through the random incorporation of nucleotides with reporter molecules in the amplicons. Of the two strategies, the body labeling method is usually preferred because it provides a much higher fluorescent signal for the same target due to the higher labeling density. The incorporation of reporter molecules in body labeling is achieved through the direct incorporation of modified nucleotides.
with fluorescent groups and indirect labeling is accomplished through the incorporation of modified nucleotides with amino allyl groups, biotin or digoxigenin (DIG). In direct incorporation, the labeling density in the amplicons is limited by the inefficient incorporation of bulky fluorescent molecules into the growing nucleotide chain. This limitation can be circumvented through indirect labeling using less bulky nucleotides with a specific functionality or ligand, which can be subsequently bound to fluorescent molecules either in the solution phase prior to hybridization or on the microarray after hybridization.

Our proposed strategy has a similar objective of incorporating multiple labels into the target molecules. However, instead of labeling during the amplification step, our technique aims to perform the labeling in situ, on-chip after hybridization. We hypothesize that labeling on-chip will avoid issues of inefficient hybridization due to the incorporation of unnatural nucleotides into the target and the bleaching of fluorescent molecules during long hybridization periods. In addition, we also envision that we can achieve a higher labeling density than the current methods through the formation of a long DNA chain during TdT extension; this could potentially increase the sensitivity of the assay and eliminate the target amplification steps (Figure 1-1).

1.4.2 On-chip signal amplification strategies

Another issue in the analysis of nucleic acids using DNA microarray technology is the ability to detect a limited copy number of certain sequences in the presence of
other more abundant sequences. As discussed above, solution phase target amplification approaches are routinely employed. Even after target amplification, however, the hybridization signals are still not sensitive enough to provide reliable information.\textsuperscript{10} Therefore, strategies to increase sensitivity have focused on increasing the hybridization signal of the probe-target duplex at the hybridization sites. The advantage of the on-chip amplification method is that it has the potential to provide the necessary sensitivity and specificity while retaining spatial multiplexing. Several techniques worth noting are dendritic nucleic acid probes,\textsuperscript{46} branched DNA (b-DNA),\textsuperscript{47} tyramide signal amplification (TSA),\textsuperscript{42} rolling circle amplification (RCA),\textsuperscript{72} and nanoparticle-based signal amplification using gold nanoparticles,\textsuperscript{51,52} quantum dots,\textsuperscript{54,55} and organic or silica nanoparticles doped with fluorescent molecules.\textsuperscript{56-58} Each of these techniques offers unique advantages and limitations related to the assay format. Therefore, extensive optimization has to be conducted to exploit the full potential of these approaches. One similarity of these techniques is that the signal amplification requires a new probe moiety that has to be pre-designed and pre-fabricated for the target of interest. For example, for dendritic nucleic acid probes, a pre-fabricated dendrimer molecule has to be designed to contain a complementary sequence to the captured target and must be made with the highest possible purity. b-DNA has a similar limitation in that proprietary software is needed to design the capture extender and the label extender, which have to be tailored against the target of interest. In addition, the synthesis of the branched oligonucleotide, which acts
as the key signal amplifier, is tedious and difficult. The assay design is less intensive for the RCA, TSA, and nanoparticle-based signal amplification methods, but pre-fabricated amplifier probes are still required. All of these techniques are reasonably sensitive and have been commercialized for research and diagnostic purposes. Our proposed technique offers a much simpler approach in which no prior design or pre-fabricated probe amplifier is needed. We use the target molecules with the 3'-end exposed as the amplifier and initiator for SIEP, generating a long DNA chain that contains multiple reporter molecules. This represents a significant simplification of the assay without compromising the signal amplification.
2. Surface Initiated Enzymatic Polymerization (SIEP)

2.1 DNA as a polymeric material

Polymer as a molecule is defined by its structure, which comprises of a repeating structural unit called monomer, forming a chain that is connected by covalent bonds and held by a backbone that spans across the repeating unit. As a macromolecule, polymers in their simplest form are typically made of hydrocarbon molecules with molecular weight determined by the number of repeating units, which can go up to hundreds of thousands of units. Examples of the chemical structure of the commonly used polymer are illustrated in Figure 2-1.

![Figure 2-1: Polymer structure and examples](image)
A DNA molecule is a linear chain polymer composed of repeating monomer units of deoxynucleotides (dNTP) (Figure 2-2), thus similar to traditional polymeric materials, the molecular weight of a DNA molecule is determined by the number of repeating monomer units (bases) per strand. As a macromolecule, DNA has four major components: the 5′-phosphate terminus, the 3′-OH terminus, the phosphate-sugar backbone and the nucleotides (bases) as side chains (Figure 2-2). The first two components are unique to DNA imparting its directionality and thus functionality and programmability while the last two are essentially the basic characteristic of DNA as polymeric materials. Although DNA main structure in general mimics a polymer chain, DNA as a polymer possesses additional characteristics that are unique to DNA.

These characteristic are:

1. Two DNA molecules can interact through π-π stacking and hydrogen bonds of the bases, forming a stable helix structure, as long as the two DNA strands are complimentary to each other. Complementarity is satisfied when adenine (A) base from one strand forms hydrogen bonds with thymine (T) base from another strand and cytosine (C) base complexes with guanine (G) base (Figure 2-2). This process is called hybridization and its precision, where each base binds to its pair, and coordination, where multiple base pairs form a tight bond.

2. DNA is a polyanion due to the presence of a phosphate group in every base, which forms a highly negative charged backbone (Figure 2-2), endowing DNA
with the characteristic feature of a polyelectrolyte. Due to its polymeric nature, the overall negative charge on DNA molecules is determined by the molecular weight of DNA and this highly charged backbone renders DNA with several unique physical characteristics, which can be exploited.

(3) DNA can be manipulated by nucleic acid modifying enzymes with molecular precision. These modifying enzymes can polymerize, digest, cut, ligate, and modify DNA strands by adding or breaking the phosphodiester bond, serving as an exceptional molecular tool kit (see some selected examples of nucleic acid modifying enzyme in Table 2-1).

(4) DNA can form secondary structures. The presence of bases on DNA as side chain moieties allows intra chain base to base interaction through weak non-covalent bonds (π-π stacking, hydrogen bonds, hydrophobic interaction and van der walls forces), which resulted in the formation of secondary DNA structures. Some examples of these secondary structures are aptamers, i-motif, and G-quadruplex.
Figure 2-2: DNA structure and hybridization
Double stranded DNA structure showing the phosphate backbone and the bases interaction, forming hydrogen bonds that correspond to complementary bases A-T and C-G.

Table 2-1: Selected nucleic acid modifying enzymes

<table>
<thead>
<tr>
<th>Main Function</th>
<th>Enzymes</th>
<th>Specific Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elongating and modifying DNA/RNA: Ligases Polymerases</td>
<td>T4 DNA ligase</td>
<td>Join DNA, RNA strand</td>
</tr>
<tr>
<td></td>
<td>T4 RNA ligase</td>
<td>Join and label RNA strand</td>
</tr>
<tr>
<td></td>
<td>Taq polymerase</td>
<td>Polymerize DNA from template</td>
</tr>
<tr>
<td></td>
<td>Klenow fragment</td>
<td>Polymerize DNA from template</td>
</tr>
<tr>
<td></td>
<td>Phi29 DNA polymerase</td>
<td>Polymerize DNA from template</td>
</tr>
<tr>
<td></td>
<td>Terminal transferase</td>
<td>Polymerize DNA, no template</td>
</tr>
<tr>
<td></td>
<td>Poly(A) polymerase</td>
<td>Polymerize ATP, no template</td>
</tr>
<tr>
<td>Digesting or cut DNA/RNA</td>
<td>Exonuclease I</td>
<td>Cut ssDNA from 3'-end to 5'-end</td>
</tr>
<tr>
<td></td>
<td>Exonuclease III</td>
<td>Cut dsDNA from 3'-end to 5'-end</td>
</tr>
<tr>
<td></td>
<td>DNAse I</td>
<td>Degrades DNA</td>
</tr>
<tr>
<td></td>
<td>Restriction enzymes</td>
<td>Cut DNA at specific sequence</td>
</tr>
<tr>
<td>Other functions: Unwinding dsDNA</td>
<td>Helicase</td>
<td>Unwind dsDNA to ssDNA</td>
</tr>
<tr>
<td>Convert RNA to DNA</td>
<td>Reverse transcriptase</td>
<td>Polymerize DNA from RNA template</td>
</tr>
<tr>
<td>Convert DNA to RNA</td>
<td>T7 RNA polymerase</td>
<td>Polymerize RNA from DNA template</td>
</tr>
</tbody>
</table>
2.2 Polymerization: chemical synthesis vs DNA polymerization

Synthetic polymers are made through a process called polymerization, where a mixture of initiators, monomers, and some form of catalysts are required for the chemical reaction. In general, the reaction links monomers to form a long chain of polymer. The specific reactions involved in polymerization are diverse depending on types of initiators, monomers, and catalysts (temperature, ultra-violet, radiation, organometallics, etc), however kinetically, polymerization reaction is characterized into two types of growth reactions; step-growth polymerization and chain-growth polymerization.

In step-growth polymerization, polymers are formed by the stepwise reaction of the functional groups on the monomers, resulting in high molecular weight when most monomers are converted into the polymer. In this reaction, a mixture of monomer in the presence of a catalyst is sufficient to drive the reaction without a need for initiator. In some step-growth reactions, polymerization of monomers produces condensates or small molecules as by-products, and thus often called condensation polymerization.

On the other hand, in chain-growth polymerization, initiator that carries active functional groups is required to jump start the polymerization reaction and monomers are then added into the initiator or growing polymer chain one at a time. The addition of a monomer one at a time for the growth of the polymer chain resulted in a term called addition polymerization. In addition polymerization, there are three major steps in the
polymer growth; initiation, propagation, and termination. A special case of addition polymerization called controlled living polymerization when termination reaction is removed and polymerization continues as long as monomers are available to be reacted to the growing chain. With the controlled living polymerization, polymers with precise molecular weight and tailored architecture (branched polymer such as star, comb, brush polymers) can be created.

A more elaborate explanation on the polymerization of synthetic polymers can be found in any polymer textbook. The objective of the paragraphs on polymerization is to draw readers’ attention to the similarity and differences between synthetic polymerization and polymerization occurring in nature, especially DNA polymerization.

In nature, DNA polymerization follows a similar process with the synthetic route, though with several distinct features. Similar to chemical synthesis, DNA polymerization requires (1) an initiator, which is a short oligonucleotide molecules (10-25 bases, often termed as primer in molecular biology); (2) monomers, which is a mixture of four bases or just individual bases; (3) an enzyme or polymerase, which functions as a catalyst for the phosphodiester bond formation; and (4) metal ions, which function as cofactors to the enzymatic reaction and omission of them will terminate the polymerization completely. Similar to addition polymerization, DNA monomer in the form of a base is added one by one into the DNA primer during the process and the
polymerization progresses like living polymerization whereby reaction ceases only when monomers have been consumed completely or when the catalyst is made to be inactive. Although DNA polymerization follows the kinetic of addition polymerization, it produces by-product of pyrophosphate that is proportional to the amount of bases incorporated into the growing DNA chain, a characteristic that is similar to condensation polymerization.

There are three unique characteristics of DNA polymerization; one is the ability of the enzyme to polymerize a DNA chain independent of or according to a template DNA chain. With the template dependent reaction, a template DNA chain is required to produce a DNA chain that is complementary to the template. This characteristic therefore allows a production of DNA chain with desired composition with ease and with high fidelity. On the other hand, in template independent reaction, DNA chain is polymerized randomly and therefore the composition is controlled by the input monomer or bases added into the reaction. The second unique characteristic is the directional property of DNA polymerization. The polymerases (the enzyme) only recognizes the 3’-OH of a DNA chain as the initiation sites for the catalysis of phosphodiester bond, thus extension of a DNA chain only start from the 5’-end to the 3’-end and not the other way around.

One last unique characteristic of DNA enzymatic polymerization is a phenomenon called enzyme processivity. Enzyme processivity is a measure of the
average number of nucleotides (dNTPs, the monomer) added by DNA polymerase as the enzyme associates/dissociates from the DNA template. By definition, only template dependent polymerization will have this property while template independent polymerization is classified as a non-processive reaction, carried out by non-processive enzymes. The implication of this phenomenon is in the speed of polymerization. A highly processive enzyme is able to polymerize long DNA chain at a significantly shorter time than a non-processive enzyme.

2.3 Surface initiated polymerization (SIP)

Surface initiated enzymatic polymerization (SIEP) as a method was inspired by surface initiated polymerization (SIP), which is a living polymerization method of forming well-defined thin polymer layer (typically termed as polymer brush) on a substrate with high density and architectural precision. In SIP (Figure 2-3a), an initiator is immobilized on a substrate on which polymer brush will be formed. The initiator is then exposed to solutions containing catalyst and monomer to generate polymer chains grown from the immobilized initiator. As a polymerization strategy, SIP can achieve exquisite control on the density, thickness, and functionality of the polymer chain by controlling the initiator density on the surface, polymerization time, and the type of monomer, respectively.
The similarity of (a) surface initiated polymerization (SIP) and (b) surface initiated enzymatic polymerization (SIEP) is illustrated above. The presence of initiator and the polymerization of monomer resulted in controlled formation of polymer chain grown from immobilized initiator. Some examples of controlled living polymerization method used in SIP are LRP (living radical polymerization), LROP (living ring opening polymerization), ATRP (atomic transfer radical polymerization), RAFT (reversible addition-fragmentation chain transfer polymerization), and ROMP (ring opening metathesis polymerization). Figures are adapted with permission.\textsuperscript{77, 78}

Similarly in SIEP of DNA, an initiator is immobilized on a surface followed by polymerization of the monomer with an enzyme as a catalyst (Figure 2-3b). In this scheme, the initiator is a short DNA molecule (primer) that is immobilized at the 5’-end, leaving the 3’-OH exposed to initiate the polymerization process. Utilizing DNA polymerase that recognizes the exposed 3’-OH of immobilized DNA, a long DNA chain can be grown from the immobilized DNA initiator by polymerizing deoxynucleotide (dNTP) as catalyzed by enzyme.\textsuperscript{79, 80} In short, SIEP is the biologically inspired version of SIP, where instead of using chemical synthesis methodology, biopolymers such as DNA
is synthesized through biological route, exploiting synthesis by nature into new applications, such as nanopatterning shown in Figure 2-3.

### 2.4 SIEP for nucleic acid analysis

The overall goal of this dissertation is to extend the application of SIEP of DNA, a technology that is developed previously to create single stranded (ss) DNA nanostructures grown from anchored short DNA chains, into an on-chip labeling technique for amplified sensing of nucleic acid. We exploit a unique template independent DNA polymerase –terminal deoxynucleotidyl transferase (TdT)– to polymerize long ssDNA chains from a surface and incorporate a diverse range of unnatural –chemically or optically functional nucleotides– into the growing DNA chain.

As shown in Figure 2-4, hybridization of target nucleic acid onto immobilized “receptor” probe allows for multiplexed and parallel assessment of different nucleic acid targets. This configuration is compatible with the commercial microarray platform and therefore is appropriate for the demonstration of SIEP on-chip labeling. SIEP assay utilizes the specificity of the immobilized probe and its orientation to hybridize target nucleic acid strand, exposing the unique 3’-OH of the target strand to provide an “initiation” site for the in-situ enzyme-catalyzed polymerization of long ssDNA chains that incorporate reporter nucleotides.
SIEP for the detection of DNA or RNA in a microarray format. An immobilized ssDNA (probe) captures a target DNA/RNA by hybridization, providing a short overhang with a free 3’-OH group that serves as the initiator for SIEP of a long strand of DNA that incorporates multiple unnatural nucleotides (fluorescent or chemically reactive). This technique allows on-chip labeling with the potential for a highly amplified signal by the incorporation of multiple reporters along the extended DNA chain.

There are several advantages of this assay; (1) it can be carried out \textit{in situ}, \textit{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 signals 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; (4) it can be carried out under isothermal conditions to minimize its technological complexity and make it field portable for point-of-care analysis; and (5) no
customized detection probes or other signal amplifiers that must be tailored for the target of interest is required, hence further simplify the assay.

In addition to exploiting the ability of TdT to incorporate functional or reporter nucleotides, the formation of long ssDNA can also be further utilized through the formation of highly negatively charged phosphate-ribose backbone of DNA strands. The polymerization of ssDNA initiated from the hybridized target increases the net negative charge on the spot where target nucleic acids bound. This increase in negative charges can act as signal amplifiers by interacting with positively charged reporter dyes or moieties.

As highlighted in Chapter 1, this research is mainly motivated by the need for a sensitive, amplified detection method that can both label and amplify a binding event and that is applicable to both DNA and RNA detection. To fulfill the goal of the proposed research, a systematic, mechanistic investigation and optimization of DNA polymerization by TdT will be carried out with a set of natural and unnatural nucleotides that embed optically and chemically reactive nucleotides into the polymerized DNA chains, which serve as a mean to attach reporter molecules. We hypothesize that the ability to polymerize long ssDNA chains while incorporating a large number of fluorescent or chemically reactive moieties into the DNA chain that is polymerized only from a bound analyte captured by an immobilized “receptor” probe
will provide a simple and powerful, isothermal method for on-chip labeling and signal amplification for nucleic acid biosensors and diagnostics.
3. Characterization of Terminal Deoxynucleotidyl Transferase (TdT)

3.1 Biochemical properties of TdT

The key component in SIEP technology is the enzyme, terminal deoxynucleotidyl transferase (TdT), a DNA polymerase that catalyzes the stepwise addition of deoxynucleotide (dNTP) to the 3′-OH termini of single-stranded DNA (ssDNA) chains. TdT was discovered in 1960 during the search for DNA polymerase when it was isolated from the calf thymus gland as a unique deoxynucleotide-polymerizing enzyme. In vivo, TdT is found to be responsible for the generation of random sequences that encode for the variation in vertebrate adaptive immune system. Specifically, TdT adds random nucleotides in the portion of antibody gene (V, D, and J exons), which variations during their recombination generate diversity in the gene that encodes for different antibodies and create a repertoire of antibodies for different pathogens.

In the early study of TdT, calf thymus gland is the source of TdT. In fact, TdT was first purified from calf thymus cell lysate. Initially, the isolated protein was reported to contain two polypeptides, α and β subunit. However, further investigation found that TdT is a monomeric protein with molecular weight ca. 58000 Da/520 amino acids. Although calf thymus is a cheap raw material, it is a significant challenge to obtain a homogenous TdT due to proteolytic degradation. In order to obtain TdT from a more reliable source, there were several efforts to produce TdT using recombinant technology.
by expressing TdT in *E. coli* \(^{87-89}\) and baculovirus.\(^90\) For this dissertation, we purchased TdT from Promega that produces TdT using *E. coli*.

There had been extensive studies on the biochemical property of TdT.\(^91\) Bollum *et al.* have investigated the nature of the calf thymus TdT reaction and kinetics by looking at initiators and substrate properties, buffer conditions, the effect of divalent metal ions, pH, and inhibitors on DNA polymerization by TdT.\(^92\) Some of those important findings are summarized in the following section.

### 3.1.1 Reaction stoichiometry of DNA polymerization catalyzed by TdT

DNA polymerization catalyzed by TdT is a template independent reaction which follows the reaction stoichiometry as shown in Figure 3-1a. In a template independent reaction, random polymerization of nucleotides is initiated from the 3’-OH of a primer, without a need for a template DNA strand (Figure 3-1b). In this enzymatic reaction, the monomers [dYTP] react with the 3’-OH of the primer [d(pX)], extending the primer at the 3’-end by forming phosphodiester bonds and releasing inorganic pyrophosphate as a by-product. According to this stoichiometry, polymerization of DNA catalyzed by TdT is distributive and irreversible so that when all primers and all monomers are being extended and polymerized, respectively, the length of the polymerization product should be proportional to the amount of monomer to primer ratio.
3.1.2 Initiator: primer requirement

Based on the study by Bollum et al., to catalyze template-independent polymerization, TdT requires 3'-OH of a primer initiator with a minimum of three phosphate groups and three deoxy residues.\textsuperscript{91} However, there is a study that shows TdT is capable of de-novo synthesis of short DNA fragment (up to 21 bases).\textsuperscript{93} In addition, characterization of TdT for the application of homopolymer tailing (<50 bases) revealed that TdT acts more efficiently on protruding ends compared to recessive or blunt ends.\textsuperscript{94}

![TdT reaction stoichiometry: template independent reaction](image)

**Figure 3-1: TdT reaction stoichiometry: template independent reaction**

TdT reaction stoichiometry, where the length of the extended chain is determined by the ratio of monomer to primer concentration. In this reaction, the dNTPs monomer react with the 3'-OH of the oligonucleotide initiator (primer), elongating the chain at the 3'-end and releasing inorganic pyrophosphate as by-product. The TdT reaction is a linear addition, condensation, and controlled living polymerization reaction.

3.1.3 Monomer: natural and unnatural dNTP

Although physiologically TdT incorporates dNTP randomly onto the 3'-end of a ssDNA, in vitro it can incorporate a wide range of unnatural dNTPs such as p-
nitrophenylethyl triphosphate, p-nitrophenyl triphosphate, cordycepin 5'-triphosphate, 2',3' dideoxynucleotides (ddNTPs), and dITP, however with preference of natural dNTP over unnatural dNTP. The broad range of substrates specificity suggests that the active site of the TdT enzyme interacts more strongly with the triphosphate moieties of the dNTP, whereas the sugar and base may have lesser importance. The promiscuity of TdT towards a wide variety of substrate has been exploited for end-tailing application in molecular biology and will be explored further in this dissertation.

3.1.4 Metal ion as cofactor

TdT, similar to other DNA polymerases, also requires divalent metal ions as the cofactor for catalysis. However, unlike other polymerases, TdT uses a variety of divalent cations such as Co²⁺, Mn²⁺, Zn²⁺, and Mg²⁺. Although TdT uses these cations, Mg²⁺ facilitates preferential incorporation of purine dNTP (dATP and dGTP), while Co²⁺ increases the polymerization efficiencies of pyrimidine dNTP (dTTP and dCTP). It was also found that addition of micromolar Zn²⁺ increases the efficiency of polymerization of all dNTPs.

3.2 TdT applications in molecular biology

TdT’s unique properties of catalyzing template independent DNA polymerization and its ability to incorporate unnatural nucleotides bearing reporter molecules or specific reactive groups, has transformed TdT to become a tool in molecular biology to manipulate the 3'-end of DNA. As shown in Figure 3-2, TdT has
been used as a reagent for end labeling\textsuperscript{96}, TA cloning, homopolymeric tailing in rapid amplification of cDNA ends (RACE)\textsuperscript{97}, and the detection of apoptosis (TUNEL assay)\textsuperscript{98}.

For the optimization of these applications, characterization of DNA polymerization using TdT has been concentrated on the polymerization of a short (15-30 bases) homopolymeric DNA tail\textsuperscript{94,99} and on several nucleotides or mononucleotides addition (<10 bases) for end labeling\textsuperscript{37-39,100}. For amplified labeling, we are interested in generating long ssDNA chains as a method to load multiple reporter molecules during polymerization. The absence of reports which characterize TdT-mediated polymerization of long DNA chains (≥1 Kb) has prompted us to carry out experiments and optimization to attain long DNA chains using TdT. In addition, despite extensive characterization of TdT isolated from calf thymus gland in the early 1960s, it is important to revisit the characteristics of recombinant TdT that is commercially available now. The experiments will be designed to focus on the two most important components in TdT catalyzed DNA polymerization, the primer initiator (I) and the nucleotide monomer (M). The optimum conditions for the synthesis of DNA chains by TdT will depend on the composition of the DNA polymer as well as the desired chain length.\textsuperscript{101}
Figure 3-2: Examples of TdT application in molecular biology

TdT ability to generate homopolymers specifically at the 3’-end of a DNA strand is used in RACE for the synthesis of cDNA and TA cloning to create “sticky” ends for the insertion of a DNA fragment into a vector. In addition, TdT ability to incorporate unnatural nucleotide is used in TUNEL assay for apoptosis as a way to label and quantify DNA fragments.

3.3 Characterization of TdT reaction for SIEP technology: formation of long homopolymer (≥1Kb) catalyzed 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 use this terminology to place the mechanism of TdT catalyzed DNA growth in the context of polymer chemistry. 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 varied the M/I ratio during TdT reactions. Using the optimized compositions and a reaction condition with M/I=1000, the kinetic of DNA polymerization catalyzed by TdT was also investigated by terminating the reaction at different time points and the length of extended primer was evaluated using gel electrophoresis.

### 3.3.1 Effects of initiator-monomer composition

First, we investigated the effects of the composition of the oligonucleotide primer ("initiator") and mononucleotide ("monomer") on the degree of polymerization in order to select the best combination of monomer and initiator for efficient polymerization of DNA catalyzed by TdT. Early work by Bollum et al. \(^9\) on the properties of TdT showed that initiation efficiency is affected by the type of oligonucleotide initiator and that the degree of polymerization is determined by the type of mononucleotide. Our previous work on TdT catalyzed DNA polymerization initiated from the surface also showed that the ssDNA oligonucleotide used as the initiator and the monomer composition affects polymerization efficiency.\(^{103,79}\)
Figure 3-3: The effects of monomer-initiator composition.
Agarose gel electrophoresis shows the effect of the composition of the oligonucleotide initiator and monomer at a constant M/I of 1000 on the polymerization product catalyzed by TdT for a 2 h reaction. Compositions that resulted in >80% initiation efficiency and relatively narrow dispersity of the product are enclosed within a box: I1+dATP [Cy5-dA\textsubscript{10}-(dATP)\textsubscript{n}], I2+dATP [Cy5-dT\textsubscript{10}-(dATP)\textsubscript{n}], and I2+dTTP [Cy5-dT\textsubscript{10}-(dTTP)\textsubscript{n}]. The band labeled (L) is a RNA MW ladder.

DNA polymerization was carried out to further explore this dependency, and the resulting ssDNA polymers were analyzed by gel electrophoresis (Figure 3-3). 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, which is consistent with TdT mediated polymerization studied previously on the surface as measured by ellipsometry.\textsuperscript{79} Polymerization of dGTP was not attempted in solution as synthesis of
poly(G) with a length greater than 25 bases is difficult due to the formation of secondary structure and aggregation of oligoGTP,\textsuperscript{104, 105} which results in decreased accessibility of the 3’-OH end of the growing chain to TdT.

In terms of oligonucleotide initiators, Figure 3-3 shows that Cy5-dT\textsubscript{10} was preferred by TdT over the other homooligomers tested, as shown by the absence of any residual initiator, indicating that the TdT catalyzed extension went to completion. In contrast, Cy5-dC\textsubscript{10} resulted in the least efficient primer initiation with initiation efficiency ~70\%, as estimated from the residual primer. Estimation of the length of the extension product showed that the combination of Cy5-dA\textsubscript{10} as the initiator and dATP as the monomer resulted in a DNA product whose average size of ~990 bases was closest to the expected chain length (1 Kb) as determined by M/I of 1000 and with the narrowest distribution of chain length as compared to all other combinations of primer and monomers that were tested. These results are a clear indication that this combination of initiator and monomer had the highest initiation efficiency and provided a near-monodisperse product. On the other hand, the combination of Cy5-dA\textsubscript{10} as the initiator and dTTP as the monomer resulted in the largest size distribution of extension product compared to other combinations tested. The longer than expected product from some initiator-monomer compositions such as Cy5-dA\textsubscript{10}-(dTTP)\textsubscript{n} and Cy5-dC\textsubscript{10}-(dTTP)\textsubscript{n} could be beneficial because the longer extension may allow additional incorporation of unnatural nucleotides. However, for these two combinations, initiation of TdT-catalyzed
DNA extension was relatively inefficient, as seen by the lower intensity of the extended product and the extension products also had a greater polydispersity. Due to the inefficient initiation step, the effective M/I changed during the reaction as more monomers are available for the polymerization reaction and hence resulted in a longer than expected extension product but at the expense of the molar yield of the product. Overall, considering high initiation efficiency and low polydispersity as the two metrics of interest, the three optimal combinations of initiator and monomer were the following, in decreasing order of preference: (1) dA10 (initiator) and dATP (monomer); (2) dT10 and dATP; and (3) dT10 and dTTP.

We further investigated the effect of initiator and monomer on the DNA polymerization catalyzed by TdT by reacting oligonucleotide primers (not homopolymers) that were terminated at the 3’-end with A, T, C or G, to determine the effect of the terminal nucleotide on the TdT catalyzed extension of DNA. As shown in **Figure 3-4**, using dATP or dTTP as monomers, we did not observe a significant effect of the primer sequence on TdT polymerization as all primers were initiated and extended to a similar extent. This result verifies that the type of monomer is the major factor that controls TdT catalyzed DNA polymerization.
Figure 3-4: The effects of initiator sequence on TdT-catalyzed DNA polymerization
Agarose gel electrophoresis shows that random primer sequences terminated at the 3’-end with A, T, C, and G has no effect on the polymerization of preferred monomer (dATP and dTTP) as all primers are extended with similar efficiency and the extended products have similar length.

3.3.2 Effects of monomer to initiator ratio

TdT polymerizes mononucleotides in a distributive manner; hence the chain length in TdT-catalyzed DNA polymerization is equal to the input monomer and initiator ratio (M/I) with a Poisson distribution in molecular weight, assuming that all primer served as an initiator and was extended and that the reaction has gone to completion. To demonstrate the control on the degree of polymerization, three M/I ratios (100, 1000, and 10000) were examined. Homopolymer composition of primer and monomer was chosen for simplicity and the preferred substrates (dATP and dTTP) were chosen as monomers. As shown in Figure 3-5a, long polymer chains up to ~8 Kb can be polymerized after a 24 hr reaction, while ~ 0.1 Kb and ~1 Kb can be readily obtained after 2 hr reaction. For both initiators –dA10 and dT10, using dATP and dTTP as
the monomer respectively, the polymerization efficiency approached a remarkable 100%, as no band that corresponds to the residual primer was observed. A second observation of interest is that the length of the DNA chain grown from the 3’-OH end of the primer was proportional to the M/I ratio (Figure 3-5b), 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. This experiment demonstrates that it is possible to generate long DNA chains (hundreds to thousands of bases long).

**Figure 3-5: The effects of monomer to initiator ratio.**

(a) ssDNA homopolymers (polydATP) and (polydTTP) with different lengths were generated at M/I (monomer: initiator) ratio of 100, 1000, and 10,000 using TdT to catalyze ssDNA growth from DNA initiators I1 (Cy5-dA10) and I2 (Cy5-dT10). As shown by agarose gel electrophoresis visualized by the Cy-5 fluorescence of the 5’-terminal fluorophore in the initiators, the length of the extended products (I1+dATP and I2+dTTP) correlate positively with the input M/I. (b) The exquisite control of the DNA polymerization catalyzed by TdT is further verified by reacting various M/I (100, 200, 500, 1000, 2000, and 3000) with Cy5-dA10 as initiator and dATP as monomer. The band labeled (L) is a RNA MW ladder.
3.3.3 Non-processivity of TdT: implication of stepwise polymerization of homopolymer ssDNA chain

In addition to the M/I ratio, the length of DNA chains catalyzed by TdT can also be controlled by the length of reaction due to the time dependent growth of DNA chains. This is a result of non-processive properties of TdT, which adds a single nucleotide in a stepwise fashion during polymerization (Figure 3-6). Although the stepwise polymerization allows a better control on the polymerization of DNA, the stepwise reaction can be detrimental when time is a limitation.

In order to utilize DNA polymerization as a means of labeling and signal amplification, we need to evaluate the speed of TdT catalyzed reaction in polymerizing DNA. Herein, we estimate the speed of DNA polymerizations by determining the length of polymerization product using gel electrophoresis. As shown in Figure 3-6, using M/I of 1000, ~100 bases product can be generated within 5 min of reaction and ~1000 bases ssDNA polymer can be obtained within an hour of TdT reaction. We selected the three best compositions; dA\textsubscript{10}-[dATP]\textsubscript{n}, dT\textsubscript{10}-[dATP]\textsubscript{n}, and dT\textsubscript{10}-[dTTP]\textsubscript{n} to compare their speed of reaction and found that the dT\textsubscript{10}-[dTTP]\textsubscript{n} composition is approximately two times faster compared to the other two compositions (within 10 min reaction) as shown in Figure 3-6. However, in order to generate long ssDNA chains (hundreds bases), a minimum of 30 min reaction is required for all compositions. In contrast, processive polymerase such as phi29 DNA polymerase can polymerize long ssDNA chains in tens
of thousands of bases within 1 h reaction. The result of this experiment provides an estimate on the time required to obtain long ssDNA chains (≥ 1000 bases) which is necessary for the incorporation of multiple reporter molecules for signal amplification.

**Figure 3-6: Non-processivity of TdT**
(a) Gel electrophoresis of TdT reaction products which reactions are terminated with EDTA at different time points. Three compositions were selected for this experiment; A\textsubscript{10-}[dATP]\textsubscript{n}, T\textsubscript{10-}[dATP]\textsubscript{n}, and T\textsubscript{10-}[dTTP]\textsubscript{n} with M/I of 1000. (b) The length of extended product is estimated using the reference ladder bands and plotted against the reaction time. Due the size distribution of the product, the length estimated on the graph is the average length of the product. The graph clearly distinguish T\textsubscript{10-}[dTTP]\textsubscript{n} from the other two compositions with faster polymerization kinetics and longer extension product.
3.4 Incorporation of unnatural nucleotides

Another property of TdT that will be exploited in SIEP assay is TdT’s ability to incorporate a wide range of dNTPs. We are particularly interested in the incorporation of nucleotides containing reporter molecules or functional groups that can be further reacted to reporter molecules. There were earlier studies on TdT reactivity towards several unnatural nucleotides, such as fluorescently labeled ribonucleotides\textsuperscript{107} or dideoxynucleotides (ddNTPs),\textsuperscript{108} amino allyl dNTPs,\textsuperscript{37} biotin labeled dNTPs,\textsuperscript{38} digoxigenin-dUTP,\textsuperscript{39} and modified triphosphate dNTPs,\textsuperscript{109} however, these studies are typically designed for end-tailing with a single or several (<10 bases) nucleotides incorporation. For SIEP, we are interested in quantifying the multiple incorporations (>10 bases) of unnatural nucleotides into a long DNA strand as they are being polymerized by TdT. In addition, to the best of our knowledge, the ability of TdT to directly incorporate fluorescent dNTPs and aldehyde dNTPs has not been explored.

In this section, we show the characterizations of three types of unnatural nucleotide (Figure 3-7): (1) fluorescent dNTPs, particularly Cy3-dATP and Cy3-dUTP; (2) amino allyl dUTP; and (3) aldehyde dNTPs (CHO-dNTPs), particularly CHO-dATP and CHO-dUTP.
3.4.1 Incorporation of fluorescent dNTPs

We selected an M/I of 1000, 2 h reaction, and three initiator-monomer combinations (Figure S-1c) that resulted in efficient initiation and a low polydispersity of the DNA product – Cy5-dA10/dATP, Cy5-dT10/dATP, and Cy5-dT10/dTTP to assess the incorporation of fluorescent dNTPs. Using these compositions and solution
polymerization reactions (see Methods), we determined whether and to what extent, fluorescent dNTPs – specifically Cy3-dATP and Cy3-dUTP – can be incorporated into growing ssDNA chains. Our goal is to develop a set of optimized conditions for TdT-catalyzed polymerization of fluorescent dNTPs. 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. Furthermore, we studied the effect of fluorescent dNTPs incorporation on (i) the number of chains that can be extended and (ii) the chain length. The molecular weight and its distribution were assessed by gel electrophoresis. 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 3-8a, 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. When we compared the incorporation of Cy3-labeled dNTP using TdT with the incorporation of Cy3-labeled
dUTPs obtained using PCR (Tli or Taq DNA polymerase), TdT can incorporate 4-50 dye/kb with relative efficiency of primer extension decreasing from 95% to 50% while PCR is only able to incorporate 2-20 dye/kb with lower yields (80%-15%). Taking the same reaction efficiency as a comparative measure (e.g. 80%), TdT can incorporate up to ~15 fold more dye/kb than PCR. Figure 3-8a also shows that the initiator-monomer combination that was most successful at incorporation of Cy3 labeled dNTP showed the following trend: Cy5-dT_{10}/dATP > Cy5-dA_{10}/dATP > Cy5-dT_{10}/dTTP. 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 3-8b). 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. Figure 3-8b shows that polymerization initiation in the presence of fluorescent nucleotide was >80% efficient for the primer-initiator combination of Cy5-dA_{10}-(dATP)_n, while a significant drop in polymerization initiation to ~50% was observed for Cy5-dT_{10}-(dATP)_n.

In addition, we studied the effect of fluorescent dNTP on the DNA chain length, by visualizing the length of the DNA product by gel electrophoresis. We found the DNA product was shorter when the ratio of Cy3-dNTP to dNTP was ≥0.1 (Figure 3-8c). This result showed the effect of the inclusion of fluorescent dNTP on the TdT reaction kinetics. As the fraction of fluorescent dNTP increased in the reaction mixture, a shorter
and more polydisperse product was generated. The greatest effect of fluorescent dNTP on TdT reaction kinetics was shown by the initiator/monomer combination of Cy5-dT\textsubscript{10}/dTTP/Cy3-dUTP, which suggests that dUTP is not a preferred substrate for TdT, as compared to dATP and dTTP.\textsuperscript{91}

Overall, our results show that the presence of fluorescent dNTP in the reaction affects the initiation step and the kinetics of TdT reaction, so that as the molar ratio of fluorescent dNTP to natural dNTP is increased, the fraction of DNA primer that is initiated during polymerization decreases and the length of extended product also decreases. We determined that up to ~50 Cy3-dATP per kb can be incorporated into extended ssDNA chain within a 2 h reaction time under experimental conditions that are optimized for the incorporation of fluorescent nucleotides.

### 3.4.2 Incorporation of amino allyl-dUTP

Amino allyl-dUTP (aa-dUTP) is an unnatural nucleotide with amino allyl modification at the fifth carbon position of a uridine base (Figure 3-7c). The exposed amine group reacts with other chemically reactive group such as N-hydroxysuccinimide ester (NHS-ester) in aqueous buffer, allowing attachment of reporter dyes. Aa-dUTP is currently being used as a reagent for indirect labeling of nucleic acids through their incorporation into polymerized DNA strand by polymerases. It is found that in contrast to fluorescent dNTP, aa-dUTP can be incorporated with the same efficiency as the natural dNTPs during enzymatic reaction.
Figure 3-8. Incorporation of fluorescent nucleotides by TdT

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-A_10 and Cy5-T_10] decreases. The data points in (a) and (b) were obtained from the average of three independent TdT reactions. (c) Cy5 scan of the agarose gel shows the length of extended product after fluorescent dNTP incorporation, where retardation in the dNTP polymerization is more evident as the amount of fluorescent dNTP increases. The band labeled (L) is a RNA MW ladder markers: 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8, 1.0, 1.5, and 2.0 Kb.
We are interested in re-evaluating aa-dUTP due to the unique strategy of SIEP. We hypothesize that the less bulky amine will be incorporated more favorably and subsequent reaction with NHS ester fluorescent dyes will result in higher labeling density compared to the direct incorporation of fluorescent dNTP, hence producing greater amplified signals.

We took a similar approach of examining aa-dUTP with fluorescent dNTPs. For simplicity, we chose to polymerize homopolymer, starting with Cy5-dT10 as initiator and dTTP/aa-dUTP as monomers. We selected M/I of 1000, 2 hr reaction, and various molar ratios of aa-dUTP to the natural dTTP to assess the ability of TdT to incorporate aa-dUTP into the polymerized DNA chain in solution. TdT reaction products were then analyzed with gel electrophoresis for its chain length and size distribution. Our studies also determined (i) the number of chains that can be extended and (ii) the number of labeling molecules that can be conjugated subsequently. The reactivity of the amine group in the extended chain was verified by subsequent conjugation with NHS-ester Cy3 dyes after inactivation of TdT reaction and removal of unincorporated aa-dUTP. The amount of extended chains and the relative amounts of Cy3 dyes in a chain is determined by measuring the amount Cy5 fluorescence signals and taking the relative amount of Cy3 to Cy5 fluorescence signals, respectively. Unmodified DNA (exclusively polymerized with dTTP) of the same sequence and length polymerized under the same conditions was used as the control for this experiment.
As shown in the initiator scan at the Cy5 emission wavelength (Figure 3-9a), the presence of aa-dUTP monomer alone (*lane*) in the reaction resulted in inefficient initiation with only a small fraction being extended. However, it is possible to obtain efficient ssDNA extension when the reaction contains a small amount of natural dTTP, e.g. the presence of 10 fold aa-dUTP in excess of dTTP allows a relatively efficient initiation and extension of ssDNA (Figure 3-9a, lane 10). This experiment verified our hypothesis that the less bulky allyl-dUTP (aa-dUTP) can be incorporated more favorably than the fluorescently labeled nucleotides, which could be exploited to generate higher labeling density.

We conjugated the amine group of the incorporated aa-dUTP to the Cy3 dye that has been modified with NHS-ester. After removal of unreacted aa-dUTP, the extended product is incubated with Cy3 NHS-ester at room temperature for 1 hr in the dark. As shown in the label Cy3 scan in Figure 3-9a, Cy3 dyes can be conjugated successfully and the relative amount of conjugated dyes is determined by the amount of aa-dUTP in the extended ssDNA chain as indicated by the increase in Cy3 intensity with increasing aa-dUTP molar ratio. Using the Cy3 signal intensity from the gel, we estimated an optimum ratio of aa-dUTP to dTTP is in the range of 0.5-2. To verify the optimum ratio that generates high labeling density while maintaining primer extension efficiency, we quantified the number of Cy3 dyes per chain (see Methods). Figure 3-9b and c shows the estimated number of Cy3 dyes per chain and the corresponding extension efficiency,
respectively. Although 5 fold more aa-dUTP than dTTP in the reaction mixtures resulted in the highest number of Cy3 dye per chain, the extension efficiency is relatively lower than other reaction conditions, especially when compared with aa-dUTP to dTTP ratio < 2. Correlating the gel electrophoresis and the quantification measurement, the optimum condition that resulted in high labeling density and high extension efficiency are when aa-dUTP is in equal molar or twice of dTTP, where ~200 Cy3 dyes/chain is being incorporated with >65% efficiency. The labeling density achieved with aa-dUTP incorporation is significantly higher compared to the direct incorporation of Cy3-dATP, where only a maximum of ~50 Cy3 dyes/chain is incorporated with ~50% extension efficiency.

It is to be determined if an additional labeling step is needed to achieve higher labeling density and a more efficient primer extension by incorporating aa-dUTP as a means of indirect labeling will translate into higher signal amplification in SIEP assay.
Figure 3-9. Incorporation of aminoallyl-dUTP by TdT

In (a), gel electrophoresis of Cy5-T10 primer after being extended in the presence of dTTP (C), aa-dUTP (*) and various ratio of aa-dUTP to dTTP (0.1, 0.2, 0.5, 1, 2, 5 and 10). The Cy5 scan shows the relative amount of primer being extended and the Cy3 scan shows the relative degree of aa-dUTP incorporation after conjugation with Cy3 dyes through NHS-ester coupling with the amine group. The labeling density is quantified in (b) by calculating the number of Cy3 dyes per DNA chain from the total Cy3 signal intensity with respect to the total amount of DNA chain as reflected in (c). The results clearly demonstrate that there is an optimum amount of aa-dUTP feed (*) in the TdT reaction mixture that produces high labeling density without compromising the reaction efficiency. The band labeled (L) is a RNA MW ladder with 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8, 1.0, 1.5, and 2.0 Kb markers.
3.4.3 Incorporation of Aldehyde dNTPs

In addition to amine reactive groups, we explored aldehyde modified nucleotide (CHO-dNTP), which has a modification that is bulkier than amino modified nucleotide, where a thiophene group links the nucleobase to the aldehyde group (Figure 3-7d and e). In particular, the thiophene group is used to generate an aromatic aldehyde modification on the nucleotide which is less reactive compared to aliphatic aldehyde, thus rendering the aldehyde group to react in more specific manner. The aldehyde functional group is selected due to several considerations. First, the aldehyde reactive group can be further reacted in aqueous environments with relatively mild reaction conditions, and they react rapidly, which are important attributes for efficient in-situ labeling. Second, aldehyde-functional groups are compatible with a wide range of commercially available linker and bioconjugation technologies (e.g., Solulink). In addition, recent work by Raindlova et al.\textsuperscript{110} showed that aldehyde–functionalized nucleotides were readily incorporated into DNA by several other polymerases. This promiscuity thus promises high incorporation by TdT. Finally, an important motivation for incorporating aldehyde-functional nucleotides into ssDNA during SIEP is that they can be selectively labeled in-situ through metallization (e.g., Ag).\textsuperscript{111, 112} We are particularly interested in using the CHO-dNTP to introduce metal particle labeling for SIEP colorimetric detection.
We used the same approach of evaluating CHO-dNTP with fluorescent dNTP and aa-dUTP. We selected homopolymer composition for simplicity by using Cy5-dT₁₀, Cy5-dA₁₀ as initiators and dTTP/CHO-dUTP, dATP/CHO-dATP as monomers, respectively. While using M/I of 1000 and 2 hr reaction, we varied the amount of CHO-dNTP with respect to its natural counterpart and examined the reaction products with gel electrophoresis, visualizing them through the Cy5 dye tag. To validate the presence of the aldehyde group, CHO-dUTP can be directly visualized by the fluorescent signal produced by the thiophene group embedded within the CHO-dUTP structure, while CHO-dATP has to be further reacted with Cy3-hydrazone dyes. Aligned with the goal to use CHO-dNTP as the seed for Ag metallization, the reactivity of the aldehyde group was further verified by exposing the extended DNA chain to a metallization solution (Tollens’ reagent), which selectively oxidizes aldehyde and reduces the Ag ions to metallic Ag, which can be quantified by Ag nanoparticles (NPs) absorbance at 400-450 nm.

We hypothesized that the less bulky aldehyde modification compared to fluorescent dye on the nucleobase will result in high incorporation efficiency, therefore we attempted to use high ratio of CHO-dNTP to its natural dNTP. However, we found that TdT has less preference towards CHO-dNTP as substrates when we observed no incorporation when using CHO-dUTP alone as the monomer and an extremely large product distribution when more CHO-dUTP was present than the natural dTTP in the
reaction, which indicates inefficient incorporation (Figure 3-10a). Therefore, we examined CHO-dNTP feed ratios that were similar with the incorporation of fluorescent dNTP.

We first studied the incorporation of CHO-dUTP due to the ability to assess its incorporation directly through thiophene fluorescence. As shown in Figure 3-10b, CHO-dUTP is incorporated into the extending ssDNA chain in a similar fashion as Cy3-dUTP, where increasing ratio of CHO-dUTP to dTTP resulted in larger product distribution and slower kinetic of polymerization. We quantified the amount of primer being extended and the number of CHO-dUTP incorporated (see Methods) by tagging the primer with Cy5 and exploiting the fluorescence properties of the thiophene group (excited at ~250 nm UV wavelength and emit at 433 nm). As shown in Figure 3-11, the maximum number of CHO-dUTP incorporated is ~17 bases per chain with ~60% extension efficiency. This number is surprisingly low considering the less bulky modification on the CHO-dUTP as compared to Cy3-dUTP.
Figure 3-10: Incorporation of aldehyde-dUTP by TdT
The reaction product incorporating CHO-dUTP into the extending ssDNA chain is analyzed using gel electrophoresis by exciting the gel with Cy5 laser and UV lamp. The initiator Cy5 scan in (a) shows the relative amount of initiator (I) being extended in the presence of only CHO-dUTP (*) and high ratio of CHO-dUTP to dTTP (10, 5, 2, 1). In (b), the initiator scan for Cy5 dye and UV scan for thiophene group in CHO-dUTP show the relative amount of initiator (I) being extended and the incorporated CHO-dUTP, respectively in the presence of only CHO-dUTP (*) and various ratio of CHO-dUTP and dTTP (0, 0.01, 0.02, 0.05, 0.1 and 0.2). L is RNA ladder and M is monomer CHO-dUTP.

Figure 3-11: Quantification of aldehyde-dUTP
Quantitative measurement of the extended product and the number of CHO-dUTP per chain was estimated using Nanodrop fluorospectrometer by taking the ratio of the total amount of CHO-dUTP to the amount of initiator/primer being extended.
We then investigated the incorporation of CHO-dATP by carrying out similar TdT reactions. We designed a longer linker between the nucleobase to the active aldehyde group for CHO-dATP (Figure 3-7e) because it has been shown previously that unnatural nucleotides with a longer linker is tolerated better by the polymerases.\textsuperscript{113} We compared the incorporation of CHO-dUTP and CHO-dATP and found that CHO-dATP is incorporated better by TdT, however with marginal preference. Figure 3-12a shows that increasing amount of CHO-dNTP in the reaction produces shorter TdT-catalyzed homopolymer. The impact of the presence of unnatural dNTPs on the kinetics of TdT-catalyzed reaction is more significant when CHO-dUTP is present than CHO-dATP, as shown by the shorter and larger product distribution of dT\textsubscript{25}-[dTTP/CHO-dUTP]\textsubscript{n} on gel electrophoresis (Figure 3-12a). We further verified the extent of CHO-dUTP and CHO-dATP incorporation by conjugating Cy3 hydrazide dyes to the aldehyde groups. As shown in Figure 3-12b, at CHO-dNTP to dNTP ratio of 0.2, CHO-dATP is incorporated better than CHO-dUTP, as indicated by the less polydisperse product in the initiator scan and higher intensity in the label scan. Although CHO-dATP appears to be incorporated more favorably than CHO-dUTP, the extent of incorporation is marginally better. We can deduce that from comparing the intensity of Cy3 signal in Figure 3-12b that the number of CHO-dATP incorporated into a polymerized strand is in the close range of ~17 bases per chain, as quantified earlier for CHO-dUTP.
The lower incorporation of CHO-dNTP compared to Cy3-dNTP and amino allyl-dUTP is unexpected based on their less bulky modification on the nucleobase. We speculate two possible reasons for the unfavorable incorporation; first, the thiophene group that links the nucleobase to the aldehyde group has a significant effect on TdT interaction with CHO-dNTP and second, the aliphatic linker that separates the nucleobase from the active groups is too short.

We attempted to improve the incorporation of CHO-dNTP by adding small amount of Zn$^{2+}$ divalent metal cofactor (25 µM) that is known to increase the polymerization of dNTPs by TdT. However, we did not observe any significant difference when we compared the reaction product with and without Zn$^{2+}$.

Despite the lower incorporation of CHO-dNTP compared to Cy3-dNTP and amino allyl-dUTP, we are interested to find out if the aldehyde groups can reduce Ag ions into metallic Ag as a seed for further metallization of the DNA strand. Our approach to investigate this was to incubate TdT polymerized DNA strand containing CHO-dNTP in a Tollens’ reagent. As shown in the reaction stoichiometry in Figure 3-13, one aldehyde group reduces two Ag ions into metallic Ag cluster. This specific Ag nucleation can be examined by monitoring the development of Ag NPs plasmon absorbance peak at 400-450 nm using UV/Vis spectroscopy. The total absorbance intensity of various reaction products generated from the different feed ratio of CHO-
dNTP to dNTP was compared and the minimum number of CHO-dNTP per chain to initiate significant Ag absorbance peak was determined.

![Figure 3-12: Comparison of CHO-dUTP and CHO-dATP incorporation](image)

**Figure 3-12: Comparison of CHO-dUTP and CHO-dATP incorporation**
The effect of CHO-dNTP on TdT reaction efficiency and kinetics of polymerization is shown in (a) where the presence of CHO-dUTP in the TdT reaction mixture results in shorter and more polydisperse product than CHO-dATP. In (b), the reactivity of incorporated aldehyde group is verified through the successful conjugation of Cy3-hydrazide dyes, as shown by the positive label scan when CHO-dNTP is added into the TdT reaction. The band labeled (L) is a RNA MW ladder.

$$2\text{Ag(NH}_3\text{)}_2^+ + \text{RCHO} + 3\text{OH}^- \rightarrow 2\text{Ag} + \text{RCOO}^- + 4\text{NH}_3 + 2\text{H}_2\text{O}$$

**Figure 3-13: Tollens’ reaction stoichiometry**
Reduction of two silver ions by one aldehyde group produces two Ag metal clusters.
We reconstituted polymerized DNA in Tollens’ reagent after removal of unreacted monomers and measure the absorbance after 1 hr incubation. We observed three distinct peaks; at 273 nm, 350 nm, and 430 nm, which correspond to DNA absorbance peak (poly-dT), thiophene group on CHO-dUTP, and Ag NPs, respectively (Figure 3-14). We utilized the DNA absorbance peak and the Ag NPs peak to quantify the polymerization efficiency and the incorporation of CHO-dUTP, respectively. Using the ratio of these two peaks, we identified the effective aldehyde groups density in a chain and their reducing capacities, as it is being incorporated into the DNA strand. In addition, we measured the development of Ag clusters over time as it is exposed to Tollens’ reagent by measuring the absorbance profile over time.

We hypothesized that as the number of CHO-dUTP per chain increases, the Ag labeling density increases, and this will be reflected by the total absorbance at 430 nm. As shown in Figure 3-15 and 3-16, after normalizing the Ag NPs absorbance intensity with the DNA absorbance intensity, we observe increasing Ag NPs formation as the density of the aldehyde groups in the DNA chain increases, which is attained by simply adding more CHO-dNTP in the TdT reaction (indicated by the higher CHO-dNTP/dNTP). In addition, the longer the aldehyde modified DNA strand is incubated in Tollens’ reagent, the stronger the Ag NP absorbance until the absorbance reaches a plateau when all the aldehyde groups have been exhausted (Figure 3-16).
Figure 3-14: Absorbance profile of TdT polymerized DNA strand with CHO-dUTP incorporation after 1 hr incubation in Tollens’ reagent.

Three distinct peaks are observed from the absorbance profile of \([dTTP/CHO-dUTP]\) polymer after incubation in Tollens’ regent; DNA peak at 273 nm, thiophene peak at 350 nm (CHO-dUTP), and Ag NP peak at 430 nm. Varying amount of CHO-dUTP added into TdT reaction resulted in different intensity of the absorbance peak. The ability of incorporated aldehyde group to reduce Ag ions is clearly demonstrated by the higher intensity at 430 nm as CHO-dUTP is added into TdT reaction.

Interestingly, although the presence of aldehyde groups increases the formation of Ag NPs, we found that the long homopolymer (poly-dT and poly-dA, ≥1 Kb) alone can cause reduction of Ag ions in Tollen’s reagent (Figure 3-15 and 3-16, dTTP and dATP data). We did not observe similar behavior when monomers (dTTP and dATP) were exposed to Tollens’ reagent. In addition, when we compared poly-dT and poly-dA (~1 Kb) on their ability to form Ag NPs (Figure 3-15 and 3-16, sample 1) after incubation in Tollens’ reagent, poly-dT showed better conversion of Ag ions into Ag NPs.
The ability of poly-dT and poly-dA to reduce Ag ions to Ag metal is unexpected, however after further literature investigation, we found that DNA bases can form DNA-metal complexes by coordination with metal ions, in particular Ag⁺, Hg⁺, and Pt²⁺.\textsuperscript{115, 116} We believe the coordination between Ag ions with the bases is further facilitated by the long homopolymer formation, creating clusters of Ag metal.

The non-specificity of Ag metal seed formation along DNA chains could be beneficial or detrimental to SIEP assay. It is beneficial because the formation of metal seeds for further metallization can be initiated along the DNA chain despite low incorporation of CHO-dNTP. However it can also be detrimental if the reaction becomes non-specific and leads to non-specific signal. Further discussion on the outcome of the strategy of using Tollens’ reagent will be elaborated in Chapter 6.
Figure 3-15: Effective reduction of Ag ions by [dTTP-CHO-dUTP], to form Ag NPs
The reduction of Ag ions to Ag NPs by CHO-dUTP in TdT polymerized DNA strand is measured by taking the ratio of Ag NP absorbance intensity to DNA absorbance intensity during Tollens’ reaction. As the amount of CHO-dUTP increases, the more Ag NPs form.

Figure 3-16: Effective reduction of Ag ions by [dATP-CHO-dATP], to form Ag NPs
3.5 Conclusions

We described the characterization of a template independent DNA polymerase, TdT, by its ability to polymerize long ssDNA homopolymer (>1 Kb). We studied the effect of initiator-monomer composition, the effect of monomer to initiator ratio, the kinetics of stepwise homopolymerization of DNA, and the incorporation of unnatural dNTP (fluorescent dNTP, amino allyl dUTP, aldehyde-dNTP) on the polymerization of DNA by TdT.

We found that long (>1 Kb and up to 8 Kb) ssDNA homopolymer of preferred monomers (dATP and dTTP) can be grown by TdT, and that the length of the ssDNA product was determined by the monomer to oligonucleotide initiator ratio. We also investigated TdT’s ability to incorporate unnatural dNTPs into a ssDNA chain by examining the effect of the molar ratios of unnatural dNTP to natural dNTP on the efficiency of primer initiation, degree of unnatural dNTP incorporation, polydispersity, and the length of the polymerized DNA strands. We observed efficient initiation (≥50%), narrow polydispersity of the extended product, and up to ~45 fluorescent-dNTP/chain when fluorescently labeled nucleotides were incorporated. On the other hand, incorporation of amino allyl dUTP allowed up to ~200 Cy3 dyes to be coupled to the incorporated amine group with >65% initiator being extended. Incorporation of another functional dNTP, aldehyde modified dNTP, was also investigated and we found the
incorporation to be the least favorable compared to fluorescent dNTP and amino allyl
dUTP.

The ability of TdT to polymerize long ssDNA chains and incorporating multiple
reporter or functional nucleotides has reaffirmed our hypothesis on the possibility of
using TdT in SIEP assay. It is to be determined if the reaction in solution can be
replicated on the surface.

3.6 Materials and methods summary

3.6.1 Materials

All oligonucleotide primers, probe, and target used in this study were
synthesized by Integrated DNA Technologies, Inc. TdT enzyme, TdT buffer, and natural
dNTP monomers (dATP, dTTP, and dCTP) were purchased from Promega. Fluorescent
nucleotides, Cy3-dATP and Cy3-dUTP were purchased from PerkinElmer, Inc.
Aldehyde-dUTP (CHO-dUTP) and aldehyde-dATP (CHO-dATP) were synthesized by
the Duke’s Small Molecule Synthesis Facility. Amino-allyl-dUTP, yeast
pyrophosphatase, Top Vision LE GQ agarose were purchased from Fermentas. Cy3 NHS
ester and Cy3 hydrazide were purchased from GE Healthcare Life Sciences. Silver
nitrate, ammonium acetate, and aniline were purchased from Sigma-Aldrich while
sodium hydroxide and ammonium hydroxide were purchased from EMD Chemicals,
Inc.
3.6.2 Instrumentation

The incorporation of fluorescent nucleotides and amino allyl-dUTP was determined by a Victor\textsuperscript{TM} microplate reader (Perkin Elmer Life Sciences). For Cy5 fluorescence measurement, an excitation band filter of 650 ± 4 nm and an emission band filter of 680 ± 5 nm was used, while for Cy3 fluorescence measurement, a 560 ± 4 nm excitation band filter and a 590 ± 10 nm emission band filter was used. The incorporation of CHO-dUTP was determined by Nanodrop Fluorospectrometer (Thermo Scientific) using the appropriate Cy5 (source: white light, emit: 665 nm) and thiophene (source: UV light, emit: 433 nm) excitation and emission filters. The absorbance of Ag NPs and DNA were measured using Nanodrop Spectrophotometers (Thermo Scientific).

3.6.3 Methods

Polymerization of long DNA chains catalyzed by TdT. The reaction mixture consisted of 1 μM Cy5-labeled oligonucleotide primer initiator [5’-Cy5-dT\textsubscript{10}, 5’-Cy5-dA\textsubscript{10}, 5’-Cy5-dC\textsubscript{10}, 5’-Cy5-TCA TTG TAC GCA T-3’, 5’-Cy5-TCA TTG TAC GCA A-3’, 5’-Cy5-TCA TTG TAC CAC-3’, or 5’-Cy5-TCA TTG TAC AG-3’], 100 μM, 1 mM, or 10 mM dNTP monomers (dTTP, dTTP, or dCTP), and 10 U of TdT in 10 μL of TdT buffer (1x, 100mM potassium cacodylate, 1 mM CoCl\textsubscript{2}, and 0.2 mM DTT, pH 7.2). For high molecular weight polymerization of DNA (>1 Kb), 10 mM MgCl\textsubscript{2} and 0.1 U of yeast pyrophosphatase was added to the dATP polymerization mixture, while 0.1 M glycine was added to dTTP polymerization reaction mixture.\textsuperscript{117} The reaction mixture was
incubated at 37 °C for 2 h or 24 h (for 10 mM dNTP) and stopped by heating at 70 °C for 10 min, according to the protocol supplied by the enzyme supplier.

**Polymerization of DNA chains with various M/I.** The reaction mixture consisted of 1 μM Cy5-labeled oligonucleotide primer initiator [5’-Cy5-dA10], 100 μM, 200 μM, 500 μM, 1 mM, 2 mM, or 3mM dATP monomers, and 10 U of TdT in 10 μL of TdT buffer was incubated at 37 °C for 10 h and the reaction was then stopped by heating at 70 °C for 10 min, according to the protocol supplied by the enzyme supplier.

**Gel Electrophoresis.** Gel electrophoresis was carried out on a horizontal electrophoresis system (C.B.S Scientific Company, Inc) at 120 V for 40-60 min depending on the expected length of the extension product. Half of the extended product from the 10 μL TdT reaction mixture was loaded into a 2% agarose gel. The gels were then imaged using a Typhoon 9410 variable scanner (GE Healthcare Life Sciences) using 633 nm (Cy5), 532 nm (Cy3), and 488 nm (SYBR Green II) laser excitation or bench top UV excitation gel imager for CHO-dUTP bands. The size of the extended product was estimated using a 0.1-2 Kb or 0.5-10 Kb RNA ladder (Invitrogen, CA).

**Incorporation of fluorescent nucleotides by TdT.** The reaction mixture consisted of 1 μM Cy5-labeled oligonucleotide primer initiator [5’-Cy5-dT10 or 5’-Cy5-dA10], 1 mM
dNTP monomers (dATP or dTTP), and 10 U of TdT in 10 μL of TdT buffer (1x, 100mM potassium cacodylate, 1 mM CoCl₂, and 0.2 mM DTT, pH 7.2). Different amounts of fluorescent dNTPs (Cy3-dATP and Cy3-dUTP) were added to the TdT reaction mixture. The ratios of fluorescent to natural dNTPs were 1:5, 1:10, 1:20, 1:50, and 1:100 at 1mM total dNTP concentration. The reactions were carried out in a total volume of 10 μL, which was incubated at 37 °C for 2 h, and terminated by heating the solution at 70 °C for 10 min.

**Determination of number of fluorescent nucleotides incorporated per DNA chain by TdT.** The TdT reaction product was subjected to a purification step to remove the non-extended primer initiator (<20 bases) and unreacted monomers, especially the unreacted fluorescent dNTPs, by adding 40 μL filtered water to the mixture and passing it through a Probe Quant™ G50 spin column (GE Healthcare). To determine the mole fraction of primer that was extended and the degree of fluorescent dNTPs that were incorporated to the polymerized DNA, the purified product was then diluted with 100 μL deionized water, and its fluorescence was measured on a Victor™ microplate reader (Perkin Elmer Life Sciences) using two sets of excitation and emission filters for Cy3 and Cy5 fluorescence measurement. The amount of primer and fluorescent dNTPs was determined by establishing a linear standard curve. The number of fluorescent dNTPs per DNA chain can be calculated by taking the molar ratio of the fluorophores with the
primer. The initiation efficiency of the primer (% primer extended) is determined relative to the control reaction with only natural dNTP presence in the reaction.

**Incorporation of aminoallyl-dUTP:** The TdT reaction mixture consisted of 1 μM Cy5-labeled oligonucleotide primer [5’-Cy5-dT10], 1 mM dTTP and 10 U of TdT in 10 μL of TdT buffer (100 mM potassium cacodylate, 1 mM CoCl₂, and 0.2 mM DTT, pH 7.2). To study incorporation efficiency, different amounts of aa-dUTP (Fermentas) were added to the TdT reaction mixture. The ratio of aa-dUTP to natural dTTP were 10:1, 5:1, 2:1, 1:1, 1:2, 1:5 and 1:10 at 1 mM total dTTP concentration. The reaction was carried out in a total volume of 10 μL, which was then incubated at 37 °C for 2 hr and terminated by the addition of 20 mM EDTA.

**Indirect incorporation of fluorescent dye into the extended ssDNA by coupling to amino allyl-dUTP:** The extended product was purified using Microcon YM-50 spin column (Millipore, Bedford, MA) to remove the unincorporated aa-dUTP. The purified product was reacted with 0.1 mM Cy3-NHS ester (GE Healthcare) in borate buffer (pH 9.0) for 1 hr at room temperature in the dark. The product was further purified with Probe Quant™ G50 spin column (GE Healthcare, Piscataway, NJ) to remove the unreacted dyes and analyzed with gel electrophoresis. The number of Cy3 dyes coupled into the extended chain was quantified as described earlier, using a microplate reader.
**Incorporation of aldehyde-dNTP:** The typical TdT reaction mixture consists of 1 μM Cy5-labeled oligonucleotide primers [5’-Cy5-TCA TTG TAC GCA A-3’ or 5’-Cy5-TCA TTG TAC GCA T-3’], 1 mM dATP or dTTP and 10 U of TdT in 10 μL of TdT buffer (100mM potassium cacodylate, 1 mM CoCl₂, and 0.2 mM DTT, pH 7.2). To study incorporation efficiency, different amounts of CHO-dNTPs (CHO-dATP and CHO-dUTP, Duke SMSF) were added to the TdT reaction mixture. The ratios of CHO-dNTPs to natural dNTPs were 10:1, 5:1, 2:1, 1:1, 1:5, 1:10, 1:20, 1:50, and 1:100 at 1 mM total dNTP concentration. The reaction was carried out in a total volume of 10 μL, which was then incubated at 37 °C for 2 hr and terminated by the addition of 20 mM EDTA.

**Determination of the number of aldehyde-dUTP incorporation into the extended DNA chain:** The number of CHO-dUTP in the extended ssDNA was quantified by using Nanodrop Fluorospectrometer (Thermo Scientific) by measuring the fluorescence emission of the thiophene group linker. Using UV excitation ~250 nm and emission at 433 nm, the amount of CHO-dUTP incorporated was determined with the established standard curve. The number of fluorescent dNTPs incorporated per DNA chain was obtained from the ratio of the total amount of fluorophores and the total amount of primer (obtained from Cy5 fluorescence signal).
Conjugation of Cy3-hydrazide to aldehyde groups: The extended product was purified using Microcon YM-50 spin column (Millipore, Bedford, MA) to remove the unincorporated CHO-dNTP. The purified product was reacted with 40 µM Cy3-hydrazide (GE Healthcare) in 0.1M ammonium acetate buffer and 10 mM aniline as catalyst for 1 hr at room temperature in the dark. The product was further purified with Probe Quant™ G50 spin column (GE Healthcare, Piscataway, NJ) to remove the unreacted dyes and analyzed with gel electrophoresis.

Aldehyde-functionalized nucleotide directed DNA metallization in solution: The extended product containing CHO-dNTP was purified using Probe Quant™ G50 spin column to remove unreacted CHO-dNTP. Purified products (~50 µl) were mixed with 5 µl centrifuged Tollens’ reagent. The Tollens’ reagent was prepared by mixing aq. AgNO₃ (0.5 M, 625 µl) and NaOH solution (3 M, 125 µl) followed by subsequent addition of NH₄OH solution (14%, 140 µl) until the black precipitate is completely dissolved. The formation of Ag nucleation was monitored by UV/Vis spectroscopy (Nanodrop Spectrophotometer, Thermo Scientific) and the development of 430 nm for Ag NPs and 270 nm for DNA absorbance peak was measured at different time intervals (e.g 5 min, 15 min, 30 min, 1 hr, and 2 hr).
4. Amplified On-Chip Fluorescence Detection of DNA Hybridization by SIEP

4.1 Overview

Having shown that TdT is able to facilely polymerized long DNA strands (>1 Kb) from a short oligonucleotide initiator and to incorporate fluorescent dNTP or amino allyl-dUTP in Chapter 3; we now aim to apply TdT to SIEP technology for the detection of DNA hybridization. We selected the microarray platform to demonstrate TdT-catalyzed SIEP assay.

DNA microarrays have become a powerful and near ubiquitous analytical tool in biology and medical research because of their massively parallel analytical power and high throughput. Microarrays are now routinely used for analysis of gene expression, and are also being developed for the detection of microRNA. Fluorescence-based DNA microarrays remain the most widely adopted format because of their high sensitivity and the easy availability of fluorescence scanners. 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. The disadvantage of pre-hybridization labeling and amplification is that it adds significant up-front complexity to the assay. Furthermore, because labeling is carried out prior to hybridization, only a few
fluorophores can be incorporated into the DNA target along the DNA chain without disrupting hybridization,\textsuperscript{119} and all DNA from a complex clinical sample is labeled indiscriminately, potentially introducing noise in the assay due to non-specific binding of the target to the surface.

Several on-chip signal amplification methods such as tyramide signal amplification,\textsuperscript{120, 121} immunoPCR,\textsuperscript{122} rolling circle amplification (RCA),\textsuperscript{123, 124} and branched DNA technology\textsuperscript{47} 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.

Using SIEP methodology, we devise a new isothermal, on-chip, post-hybridization labeling and amplification scheme for DNA microarrays. SIEP uses terminal deoxynucleotidyl transferase (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.

As shown in Figure 4-1, 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 4-1), we ensured the on-chip detection and amplification by \textit{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 \textit{in situ} polymerization of DNA on the surface by TdT are the exposed 3’-OH groups presented by target DNA bound to the probes. This technology is compatible with commercial microarrays, because the orientation of the probes is the same as in many commercial microarrays. This method 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.

\textbf{Figure 4-1: Schematic of SIEP for DNA detection}

SIEP of DNA allows single step, isothermal, on-chip, post-hybridization fluorescence detection of DNA microarrays.
4.2 Signal Amplification by TdT-catalyzed reaction

We studied the ability of TdT to utilize DNA strands on the surface as the “in situ” signal amplifier by immobilizing ssDNA on a surface, as shown in Figure 4-2. After optimizing the polymerization conditions for TdT catalyzed DNA growth in solution (Chapter 3), we selected two optimal combinations from these solution studies for subsequent SIEP: dA25 and dT25 as the initiators and a mixture of dATP and Cy3-dATP as the monomers. Although previous work has shown that SIEP using TdT can lead to a 50 nm thick poly(A) homopolymer on the surface, those studies did not attempt to incorporate a fluorescent nucleotide into the DNA chain grown by SIEP. We immobilized an oligonucleotide initiator on a glass substrate followed by polymerization of nucleotides using a mixture of dATP and Cy3-dATP. All initiators were modified at their 5′-end for covalent immobilization on the surface and labeled internally with Cy5 in order to visualize the microspots of the printed primers on the surface and to provide an independent estimate of the spotting density of the probe (Figure 4-2). The Cy5 dye on the immobilized initiator was placed 15 bases away from the 3′-end to prevent any energy transfer from Cy3 to Cy5. We selected ratios of Cy3-dATP to dATP of 0.005, 0.01, 0.02 and 0.05 for SIEP. Two types of control experiments were also carried out; (1) SIEP in the absence of TdT, to examine the effect of nonspecific binding of fluorophores to the surface; (2) SIEP with Cy3-dideoxynucleotides (Cy3-ddATP) instead of Cy3-dATP. As ddATP is a chain terminator, incorporation of
Cy3-ddATP only allows a single fluorophore to be incorporated into the DNA strand grown by TdT (Figure 4-2). Comparison of the Cy3 fluorescence from spots amplified with Cy-dATP and with Cy3-ddATP provided a metric to compare the level of fluorophore incorporation by SIEP using Cy3-dATP, and thereby a measure of the degree of fluorescence signal amplification possible with SIEP.

Figure 4-2: Schematic of SIEP incorporating single terminal Cy3-ddATP and multiple Cy3-dATP

As shown in Figure 4-3, spots of immobilized Cy5-labeled DNA initiator [5′-NH₂-(CH₂)₁₂-dT₁₀-Cy5-dT₁₅] were successfully extended by SIEP and incorporated Cy3-dATP into the DNA chain, as seen by the high level of Cy3 fluorescence intensity from the spots after SIEP. Furthermore, the spots exposed to Cy3-ddATP (left panel) showed some Cy3 fluorescent intensity, albeit with significantly lower intensity than spots that were exposed to the reaction mixture containing Cy3-dATP (right panel). The inclusion of Cy3-ddATP into the reaction mixture ensured that a maximum of only one fluorophore could be incorporated into the DNA chain polymerized by SIEP, as incorporation of a single Cy3-ddATP causes chain termination. Comparison of the
Cy3/Cy5 fluorescence intensity’s in the left panel with the right panel (Figure 4) also clearly indicates the incorporation of multiple fluorophores when Cy3-dATP is used, and shows the potential signal amplification that can be achieved by SIEP. In addition to the greater Cy3 fluorescence intensity from spots that were exposed to Cy3-dATP, we also observed an increase in the diameter of the spots compared to the Cy-5 signals that emanates solely from the immobilized primer, which could be due to the dispersion of a large fluorescence signal or an increase in the footprint of the DNA spots due to the polymerization of long DNA chains that spillover the margin of the printed spots of the covalently immobilized oligonucleotide primer.

The control spots that were exposed to the TdT reaction mixture without any enzyme in the reaction mixture did not show any detectable Cy3 fluorescence signal, which demonstrates that non-specific adsorption of free fluorescent dNTPs was below the detection limit and that the Cy3 fluorescent signal is generated exclusively from fluorescent dNTPs that are incorporated into the DNA chains whose polymerization is catalyzed by TdT.
The direct incorporation of fluorescent dNTPs is initiated on the surface using immobilized oligonucleotide initiators [5'-NH2-(CH2)12-T10-Cy5-T15]. The signal amplification is clearly demonstrated by comparing the Cy3 spots with single fluorophore (Cy3-ddATP) and multiple fluorophores (Cy3-dATP) incorporated into the ssDNA chain after 1 hr TdT-mediated SIEP at 37°C. The negative control (reaction without TdT) shows no detectable spots with Cy3 fluorescent signal, which means the non-specific adsorption of free fluorescent dNTPs was below the detection limit and the Cy3 fluorescent signal is generated exclusively from the polymerized fluorescent dNTPs.

Mean ratio of Cy3 and Cy5 fluorescence intensity of spots with single and multiple fluorophores incorporation is plotted against the ratio of fluorescent dNTP (Cy3-ddATP or Cy3-dATP) with unmodified dNTP (dATP). As shown in (a) and (b), incorporation of a single Cy3 dye in TdT reaction with Cy3-ddATP resulted in a similar Cy3 intensity across the different reaction conditions, while incorporation of multiple Cy3 dye in TdT reaction with Cy3-dATP resulted in increasing Cy3 intensity as more fluorescent dNTP was added in the reaction. The incorporation of multiple Cy3-dATP into the polymerized DNA is clearly illustrated by the significant increase in mean Cy3/Cy5 intensity.
4.3 Effects of immobilized DNA concentration and composition on signal amplification

In order to quantify the ability of TdT to add multiple fluorophores into a DNA chain that is polymerized from a short DNA initiator and that is immobilized on a surface, DNA spots with a single fluorophore Cy3-ddATP per chain were used as the reference to determine the Cy3 signal amplification. We inferred the average number of Cy3-ddATP incorporated per ssDNA on the surface by comparing the fluorescence intensity ratio of Cy3-ddATP to Cy5-tagged oligonucleotide initiator from DNA spots that incorporated multiple Cy3-ddATP with the ratio of Cy3-ddATP to Cy5-tagged initiator from control spots that incorporated only a single Cy3-tagged “dye-terminator” nucleotide into the extended chain (Figure 4-4). We also compared two surface initiators, dT25 and dA25, to study the effect of initiator-monomer composition on signal amplification. In addition, we spotted 2µM and 10µM DNA initiator onto a glass substrate to examine the effect of surface initiator concentration on the DNA polymerization and overall signal amplification after multiple fluorophores incorporation (Figure 4-4). We were only able to test a limited range of immobilized initiator concentrations due to the limited ability to control the surface density of the initiator. When we spotted < 2µM DNA solution, DNA spots with an inconsistent morphology and fluorescence intensity were observed, while spotting DNA solution > 10µM resulted in saturated fluorescence intensity after TdT reaction with Cy3-ddATP. As
shown in Figure 4-5, there was no significant change in the signal amplification as the DNA spotting solution concentration increased, which indicates that the DNA polymerization is independent of the initiator concentration on the surface in the range that we tested. However, we observed differences in the level of signal amplification, as a function of the initiator and monomer combination. Figure 4-5 shows that the immobilized dT25 initiator resulted in higher signal amplification compared to immobilized dA25 as an initiator. The higher signal amplification observed when using dT25 as initiator is contributed mainly by the lower Cy3/Cy5 value of the reference spots. This observation is consistent with the solution experiment where a greater number of Cy3-were incorporated when oligodT was used as the initiator.

The difference in the signal amplification when dA25 and dT25 were used as the initiator may imply that different initiators will result in a different labeling efficiency. However, we believe the difference in the signal amplification is caused by the higher degree of Cy5 quenching by the multiple adenosine moieties of the dA25 initiator,125 which resulted in a higher Cy3/Cy5 value of the reference spots and thus lower signal amplification when dA25 was used as the initiator. In addition, we have shown in Chapter 3 that the product of TdT extension is identical for primers with different sequences, especially when the preferred monomers –dTTP or dATP– were added as monomers to the reaction mixture (Figure 3-4). As most initiators in a DNA microarray
are unlikely to consist of long stretches of a single nucleotide, these results suggest that the effect of the initiator sequence on DNA extension by TdT is likely to be minimal.

We also observed an increase in signal amplification as the concentration of fluorescent dNTP increased in the reaction mixture, which again corresponds well with results obtained in solution shown in Chapter 3 (Figure 3-8). The highest signal amplification achieved in this experiment corresponded to approximately a 45-fold increase in fluorescent signal (equivalent to ~45 Cy3-dATP/chain) compared to reference spots with a single fluorophore per DNA chain. When we compared the fluorescence signal from DNA spots with multiple fluorophores to reference DNA spots with a single fluorophore along the extended DNA chain, the increase in fluorescence signal clearly demonstrates the capacity of TdT to incorporate multiple fluorophores and the possibility of using TdT mediated DNA polymerization as a method for on-chip labeling and signal amplification.

Although the large number of incorporated dyes has the potential to lead to self-quenching and if self-quenching between Cy3 dye did occur, its effect was negligible at the reaction conditions we tested, as we observed significant enhancement in the fluorescence signal upon TdT amplification with fluorescent nucleotides as compared to the control sample, which incorporated only a single fluorescent nucleotide. Furthermore, self-quenching—should it become a problem—can be ameliorated by controlling the average spacing between the fluorescent nucleotides by controlling the
length of extended product and by controlling the ratio of fluorescent dNTP and unmodified dNTP in the reaction mixture.

**Figure 4-5: Effects of immobilized DNA concentration and composition on signal amplification**

The effect of composition of oligonucleotide initiator (Cy5-dA25 or Cy5-dT25), its concentration, and the mole fraction of fluorescent Cy3-dATP (when mixed with dATP) on the number of Cy3-dATP moieties incorporated into the DNA chain grown by SIEP. The data for the level of signal amplification (mean ± s.t.d.) for each sample was averaged from five replicate spots from three independent SIEP reactions. *P< 0.05 (Student’s t-test), comparing the effect of the type of oligonucleotide initiator and the spotting concentration to the signal amplification generated from different TdT reaction conditions.

**4.4 Direct incorporation of fluorescent label by SIEP for DNA sensing**

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 4-1**.

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 the quantification of the fluorescence intensity from each spot by a fluorescence scanner. The dose-response curve shows 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 4-6). 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, microsphere-based rolling circle amplification (RCA), and fluorescent conjugated polymers.

We also examined the specificity of TdT reaction to the target strand by exposing the probe to the TdT reaction mixture containing fluorescent dNTP in the absence of the bound target. We observed undetectable fluorescence signal from the surface, indicating that the TdT catalyzed extension of DNA solely occurs from the bound target. However, as the ratio of fluorescent dNTP to its corresponding natural dNTP increased, we found that the non-specific signal from immobilized probes also increased, for reasons that we do not yet understand.
Figure 4-6: Dose response curve of DNA hybridization

Dose-response of SIEP mediated on-chip fluorescence detection of DNA hybridization in a DNA microarray. (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.

4.5 Indirect incorporation of fluorescent label by SIEP of amino allyl-dUTP

We investigated the signal amplification of indirect fluorescent labeling through SIEP of amino allyl-dUTP and subsequent Cy3-NHS ester conjugation due to the favorable results in solution studies (Chapter 3.4.2). We carried out similar experiments to SIEP of fluorescent dNTP (Figure 4-2) in order to compare the signal amplification of direct and indirect labeling. We used DNA spots with a single fluorophore Cy3-ddATP per chain as the reference to quantify the multiple Cy3 dye incorporation or conjugation. We inferred the average number of Cy3 dyes incorporated per ssDNA on the surface by
comparing the fluorescence intensity ratio of Cy3 to Cy5-tagged oligonucleotide initiator from DNA spots that incorporated multiple Cy3 dyes (directly or indirectly) with the ratio of Cy3-ddATP to Cy5-tagged initiator from control spots that incorporated only a single Cy3-tagged “dye-terminator” nucleotide into the extended chain. Two types of oligonucleotide initiator (dA_{25} and dT_{25}) were immobilized on a glass substrate to determine the effect of initiator composition on signal amplification. In this experiment, in order to compare the efficiency of direct and indirect labeling, we used the same amount of Cy3 dyes in the reaction. We selected 0.005 and 0.02 Cy3-dATP to dATP ratio for direct incorporation of Cy3-dATP which amount to 0.5 µM and 2 µM of Cy3-NHS ester dyes for indirect labeling. In addition, to compare the labeling density of direct and indirect labeling via SIEP, we grew the same length of DNA chains by adding the same amount of monomer (dATP for direct and dTTP for indirect) in the SIEP reaction.

As shown in **Figure 4-7**, indirect labeling through the incorporation of aa-dUTP by SIEP resulted in signal amplifications that are significantly lower than the direct incorporation of Cy3-dATP. This result contradicts the quantification of indirect labeling in solution when incorporation of aa-dUTP and subsequent Cy3 dye coupling resulted in up to 4 fold higher number of Cy3 dyes per chain. The reason for such discrepancy could be due to several reasons. First, the efficiency of NHS-ester conjugation to primary amine is influenced by the rate of NHS-ester hydrolysis, resulting in a less efficient crosslinking, especially for reaction on surfaces. Second, the inefficiency of NHS-ester
conjugation often requires high molar excess of the active NHS-ester moieties to the primary amine. This requirement is not limiting the reaction in solution because the unreacted dyes can be removed through a purification step. However, for a reaction that occurs on a surface, high molar ratio of NHS-ester dye produces high surrounding background. Our attempt to increase the Cy3-NHS ester dyes amount during on-chip coupling has resulted in extremely high background that masked the actual sample spots.

Figure 4-7: Comparison of direct and indirect on-chip labeling by SIEP
Quantification of signal amplification obtained from direct incorporation of Cy3-dATP and indirect labeling through aa-dUTP incorporation, followed Cy3 NHS-ester dyes coupling. The superiority of direct Cy3-dATP is consistent across different amount of dye in the reaction and different type of immobilized initiators.
The comparison between direct and indirect on-chip SIEP labeling has shown that the additional step involved for indirect labeling is not beneficial despite the TdT ability to incorporate more aa-dUTP. The limiting step for indirect labeling is in the conjugation of the dye to the amine group on the aa-dUTP, which is an inefficient process and a non-specific reaction. Taking this observation into consideration, we decided to abandon the indirect labeling strategy for hybridization detection via SIEP.

4.6 Conclusions

We presented a new methodology for signal amplification on a surface by direct incorporation of multiple nucleotides bearing fluorophores by TdT catalyzed DNA polymerization. Our results clearly showed multiple fluorescent nucleotides can be incorporated into the extended ssDNA chain on a surface, which translated to a ~45 fold signal amplification compared to the incorporation of a single fluorophore. We then exploited this finding to develop a post-hybridization, on-chip detection and amplification of DNA hybridization in a microarray format. We generated a dose-response curve for detection of DNA hybridization by SIEP, with a ~1 pM limit of detection and a 2-log linear dynamic range.

SIEP as a method has the attractive attributes that it is both isothermal and on-chip—as the fluorophores are covalently incorporated into a ssDNA chain that is grown from a tethered DNA strand at 37 °C. Compared with other amplification techniques, SIEP offers an alternative signal amplification methodology that is straightforward and
can be achieved by a one-step isothermal reaction under conditions that are compatible with commercial microarrays.

4.7 Materials and method summary

4.7.1 Materials

All oligonucleotide primers, probe, and target used in this study were synthesized by Integrated DNA Technologies, Inc. TdT enzyme, TdT buffer, and natural dNTP monomers (dATP and dTTP) were purchased from Promega. Fluorescent nucleotides, Cy3-dATP and Cy3-dideoxynucleotides (Cy3-ddATP) were purchased from PerkinElmer, Inc. Amino-allyl-dUTP was purchased from Fermentas while Cy3 NHS-ester was purchased from GE Healthcare Life Sciences. Tween 20 were purchased from Sigma-Aldrich (St Louis, MO). Succinimidyl 4-formylbenzoate (SFB) linker was purchased from Pierce (Rockford, IL). Sodium acetate and sodium citrate were purchased from Chemicals, Inc. Hydrazinonicotinamide modified glass slide (Hylink Glass slides) was purchased from Solulink (San Diego, CA).

4.7.2 Instrumentation

On-chip TdT labeling and DNA hybridization dose response was determined by scanning the glass slides for the Cy5 (635 nm) and Cy3 (532 nm) fluorescent signal using an Axon GenePix Pro 4200 scanner (Molecular Devices) at 10 µm resolution with optimized PMT and gain settings.
4.7.3 Methods

**Immobilization of oligonucleotide primer on glass.** Glass substrates functionalized with hydrazinonicotinamide (Hylink Glass slides) were used as the substrates for immobilization of Cy5-labeled oligonucleotide initiator. In order to immobilize the oligonucleotide initiators covalently on the glass substrate, 5'-amine modified oligonucleotides [5’-NH₂-(CH₂)$_{12}$-dA$_{10}$-Cy5-dA$_{15}$ and 5’-NH₂-(CH₂)$_{12}$-dT$_{10}$-Cy5-dT$_{15}$] were conjugated to SFB by reacting a 20-fold molar excess of SFB with the amine-modified oligonucleotides in the modification buffer (100 mM sodium acetate, 150 mM NaCl, pH 7.2) overnight in the dark. After removal of unreacted SFB using Microcon YM-3 spin columns (Millipore), the SFB modified oligonucleotides were reconstituted in conjugation buffer (100 mM sodium citrate, 150 mM NaCl, pH 6.0) at two different concentrations (10 μM and 2 μM) and spotted onto the Hylink glass slides using a non-contact piezoelectric printer (Piezzorray, Perkin Elmer, Inc). The spotted slides were then incubated overnight in a humidified chamber to allow covalent immobilization of the spotted oligonucleotides to the surface. To remove residual, unreacted oligonucleotides, the slides were rinsed in 1x SSC buffer (15 mM sodium citrate, 150 mM NaCl, pH 7.0) with 0.1% Tween 20 (Sigma, St. Louis, MO), then rinsed with filtered water and spin dry on microarray slide centrifuge for 2 min. The slides with spotted DNA were stored under vacuum until further use.
Direct Cy3-dATP Incorporation by SIEP. SIEP was performed on the glass slide spotted with Cy5-labeled oligonucleotide primers by incubating each well on the slide with 10 U of TdT, 100 μM of dATP monomer, and 0.5 μM, 1 μM, 2 μM or 5 μM of Cy3-dATP nucleotide or a dye-terminator, Cy3-dideoxynucleotides (Cy3-ddATP) in 100 μL 1x TdT buffer. Wells incubated with Cy3-ddATP served as a reference, as the extended oligonucleotides initiator incorporated only a single Cy3-ddATP “dye-terminator” per chain. SIEP was carried out for 1 h at 37 °C, after which the slides were washed three times with 1x SSC buffer containing 0.1 % Tween 20 to facilitate the removal of any non-specifically bound reactants.

Comparison of direct and indirect fluorescent labeling via SIEP. Direct fluorescent labeling by SIEP was performed on the glass slide spotted with Cy5-labeled oligonucleotide primers by incubating each well on the slide with 10 U of TdT, 100 μM of dATP monomer, 0.1% Tween 20, and 0.5 μM, or 2 μM of Cy3-dATP nucleotide or a dye-terminator, Cy3-dideoxynucleotides (Cy3-ddATP) in 100 μL 1x TdT buffer. Wells incubated with Cy3-ddATP served as a reference, as the extended oligonucleotides initiator incorporated only a single Cy3-ddATP “dye-terminator” per chain. For indirect labeling, Cy5-labeled oligonucleotide primers were exposed to 10 U of TdT, 50 μM of dTTP monomer, 50 μM of aa-dUTP monomer, and 0.1% Tween 20, followed by subsequent exposure to Cy3-NHS ester (0.5 μM or 2 μM) in borate buffer (pH 9.0) for 30
min at room temperature. SIEP was carried out for 1 h at 37 °C, after which the slides were washed three times with 1x SSC buffer containing 0.1 % Tween 20 to facilitate the removal of any non-specifically bound reactants.

**Analysis of fluorescence signal amplification from DNA spots.** Cy5 and Cy3 fluorescent signals were analyzed using GenePix 6.0 (Molecular Devices) by localized background subtraction; only signals that had a signal to noise ratio (SNR) > 3 were included in the analysis. Cy5 fluorescent signals from the immobilized oligonucleotide initiators were used to normalize the amount of immobilized primer across the different DNA spots. This step is necessary to exclude the increase in fluorescent signal due to the variations in the printing volume. The ratio of fluorescent signal intensity of Cy3 to Cy5 was then used as the measure of the fluorescent dNTP incorporation. By comparison of the ratio of Cy3/Cy5 signal intensities of the well incorporating multiple Cy3-dATP into each primer by SIEP with the reference wells that can only incorporate a maximum of a single Cy3-ddATP per DNA primer, we could determine the magnitude of signal amplification from the incorporation of multiple fluorophores in the DNA chain extended from the 3’-OH end of the primer by TdT.

**On-chip fluorescent labeling of DNA hybridization by TdT.** The 25-mer target sequence [5’-GAG CTT CTG TGG AGT TAC TCT CTT T-3’] was selected from the core
protein of Hepatitis B Virus. The fully complementary probe [5'-AAA GAG AGT AAC TCC ACA GAA- (CH₂)n- NH₂-3'] was spotted using a non-contact printer (Piezzorray, Perkin Elmer, Inc.) to covalently attach the probe to the glass surface. The slides spotted with the probes were then incubated overnight in a humidified chamber and rinsed in 1x SSC buffer with 0.1% Tween 20 followed by rinsing with filtered water and spun dry. A dose-response curve of hybridized target was generated by incubating the printed probes to a range of target DNA concentrations (1 pM-1 μM) for 4 h at 37 °C in 3x SSC buffer, followed by SIEP using TdT to incorporate Cy3-dATP (0.1 U/μl TdT, 100 μM dATP and 0.5 μM Cy3-dATP in 1x TdT buffer) for 1 h at 37 °C. The slides were then rinsed in 1x SSC buffer with 0.1% Tween 20 for 30 min and scanned immediately on a GenePix scanner. The average signal intensity of the spot was then plotted as a function of the target DNA concentration.

**LOD Determination.** LOD was determined by determining the concentration that corresponds to the signal at zero concentration plus three times the standard deviation from the sigmoidal curve fit of the target versus concentration (“dose-response”) curve.
5. Direct Fluorescence Detection of RNA on Microarrays by SIEP

5.1 Overview

We have shown in Chapter 4 on 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. Using a similar platform technology, i.e. microarray; we investigated the use of SIEP for direct on-chip RNA detection. Herein, direct detection implies that no or minimal modification of RNA target is required for its detection by the SIEP assay.

Microarray technology is a powerful method for RNA quantification that is capable of analyzing thousands of genes in parallel, and has been used to investigate differential gene expression, detect pathogens, and more recently has been used for the quantification of microRNA (miRNA) levels. Although they are sensitive, many RNA analysis techniques require converting the target RNA molecules to complementary DNA (cDNA), a molecule that is less susceptible to degradation, through a reverse transcription step, followed by an optional linear (in vitro transcription-based) or exponential (PCR-based) amplification of the original target and subsequent detection. Reverse transcription and target amplification are often accompanied by enzymatic or chemical labeling of cDNA molecules for detection.
This approach has several potential limitations. First, reverse transcription and PCR-based target amplification have variable efficiencies depending on the sequence of the target, which potentially introduces errors and systematic bias in the original mRNA concentration.\(^1\) 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 materials.\(^1\)\(^3\)\(^4\) Third, the direct incorporation of labels into cDNA molecules can reduce their hybridization efficiency, further confounding analysis.\(^1\)\(^9\) 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 RNA assay that allows direct interrogation of RNA with the following attributes: (1) it can be carried out \textit{in situ}, \textit{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.

To devise an RNA microarray assay with these attributes, herein we introduce a direct RNA detection scheme that involves the direct hybridization of unlabeled RNA molecules with a printed complementary probe on a non-fouling surface\textsuperscript{136} followed by in situ –on-chip– RNA target labeling and signal amplification using surface initiated enzymatic polymerization (SIEP).\textsuperscript{78, 137} To the best of our knowledge, this is the first demonstration of an on-chip fluorescence detection methodology that can directly interrogate RNA target (short (miRNA) and long (mRNA)) without reverse transcription or other pre-hybridization labeling.\textsuperscript{138}

This assay builds on previous demonstration (Chapter 4) that the introduction of a unique 3’-OH moiety due to hybridization of a target DNA to an immobilized probe DNA provides an “initiation” site for the \textit{in situ} enzyme-catalyzed polymerization of long single stranded DNA (ssDNA) chains that incorporate fluorescently labeled nucleotides. In our original implementation of SIEP shown in Chapter 4, we used terminal deoxynucleotidyl transferase (TdT), a template independent DNA polymerase that catalyzes the sequential addition of deoxynucleotides (dNTPs) at the 3’-OH group of DNA strands and during this process also incorporates multiple fluorescent dNTPs into the polymerized ssDNA.\textsuperscript{137}
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, and has been previously used by Corn and coworkers for SPR imaging-based detection of RNA hybridization to a surface bound probe. 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 5-1).
Figure 5-1: 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

5.2 RNA labeling by DNA polymerization using TdT

RNA samples isolated from cells or tissues are generally chemically or enzymatically labeled prior to detection. Chemical labeling of RNA samples is accomplished through reactive chemistries such as periodate, activated phosphate or activated succinimide esters, thiol, or “click” chemistry. These labeling strategies chemically attach labels internally or at the end of the RNA chain. Enzymatic labeling of RNA offers another option for site selective attachment of labels. Examples include: T7 RNA polymerase that incorporates various guanosine analogs into RNA transcripts during in vitro transcription; T4 polynucleotide kinase (PNK) that transfers a radioactive phosphate from ATP to the 5’-end of RNA; T4 RNA ligase that incorporates
radioactive dNTPs, fluorescent dinucleotides, and biotinylated short oligonucleotide at the 3'-end of RNA;\textsuperscript{33, 146, 147} *Klenow fragment of DNA polymerase I* that incorporates biotinylated dNTPs into RNA primer according to the template DNA strand;\textsuperscript{129, 132} *Yeast Poly(A) polymerase* (PaP) that incorporates radioactive cordycepin triphosphate,\textsuperscript{148} biotin-ATP, digoxigenin-UTP (DIG-UTP) and a limited number of fluorescein-ATP;\textsuperscript{34} and *terminal deoxynucleotidyl transferase* (TdT) that incorporates a limited number of biotin-\textsuperscript{dUTP} or DIG-\textsuperscript{dUTP} at the 3'-end of RNA.\textsuperscript{149}

We reported in Chapter 3.4.1 the direct incorporation of multiple fluorescent dNTPs into a ssDNA chain from a short oligonucleotide DNA initiator by TdT catalyzed DNA polymerization. We showed that long (>1 Kb) homopolymer ssDNA could be polymerized from short DNA initiators (~10 bases) and that up to ~50 fluorescent Cy-3 labeled dNTPs could be incorporated per kilobase of DNA.\textsuperscript{137} Although there is a report of RNA labeling by incorporating a few DIG- or biotin-\textsuperscript{dUTP} as at the 3'-end of RNA using TdT,\textsuperscript{149} investigation of the direct labeling of RNA with fluorescent dNTPs using TdT has not been previously reported. We hence examined TdT’s ability to directly incorporate multiple fluorescent dNTPs (Cy3-dNTPs) for amplified RNA labeling. Throughout the paper, we often refer to the RNA primer as “initiator” and the nucleotides as “monomers” to place the reaction of TdT catalyzed DNA growth in the context of surface initiated chemical polymerization.
As shown in Figure 5-2a, Cy5-tagged RNA primers (Cy5-rA15) are poor initiators for TdT catalyzed DNA polymerization, as seen by the presence of the majority of the RNA strands at the bottom of the gel, which corresponds to the original length of the RNA primer (lane P). This observation is expected because RNA is not the native substrate for TdT. Consequently, in a TdT reaction mixture that contains dATP and Cy3-dATP, the low reaction initiation efficiency results in a limited amount of Cy3-dATPs being incorporated into the extended chain, as indicated by the intense bands of unreacted Cy3-dATPs in Figure 5-2b. Consistent with this observation, a small fraction of the RNA primers serve as initiators for the polymerization of dATP as seen by presence of faint high MW, Cy5 labeled bands in the upper part of the gel in Figure 5-2a. In addition, the low level of Cy3-dATP incorporated in the product is visible in the gel scanned at the Cy3 emission wavelength in Figure 5-2b, which indicates that TdT can incorporate Cy3-dATPs into the extended polydA, from a short RNA primer, albeit with very low efficiency.
Figure 5-2: RNA labeling with TdT or PaP by incorporation of fluorescent nucleotides
Gel electrophoresis shows the extent of incorporation of Cy3-labeled nucleotides into the polymerized DNA and RNA product that is grown from the 3’-end of a short RNA primer by TdT and PaP. A Cy5-tagged RNA (Cy5- rA15) is used as the primer (initiator) with ATP and Cy3-ATP (for PaP catalyzed polymerization) or dATP and Cy3-dATP (for TdT catalyzed polymerization) as monomers. Extended products are visualized by the appearance of higher MW bands in gels imaged at the Cy5 fluorescence emission wavelength due to labeling of the primer with Cy5 fluorophore (initiator scan), while the degree of incorporation of Cy3-labeled nucleotides are visualized in gels that are imaged at the Cy3 fluorescence emission wavelength (label scan). (a) Extent of RNA polymerization for the TdT and PaP catalyzed reactions; (b) incorporation of Cy3 nucleotides into polymerized product by TdT and PaP catalyzed reactions. The ratio of Cy3 labeled nucleotides and the corresponding natural nucleotides as monomers used in each reaction is indicated by the numbers on the top of each lane. Lanes “L”, “P”, “C” correspond to ssDNA ladder, RNA primer (no polymerization), and a positive control for the TdT catalyzed reaction, where a DNA primer was extended by TdT, respectively.

5.3 RNA labeling by RNA polymerization using PaP

Due to the inefficiency of TdT in recognizing a short RNA primer as an initiator, we investigated the ability of yeast PaP, a RNA polymerase, to catalyze the polymerization of RNA from an RNA primer with ribonucleotides (NTPs) as the monomer. Previous studies have shown that yeast PaP can incorporate various
unnatural NTP analogs, such as the chain-terminating 3'-deoxy ATP, biotin-UTP, DIG-UTP, fluorescein-ATP, and aminoallyl-UTP. However, for detection of surface immobilized RNA, the incorporation of these analogs requires an additional signal amplification step through the binding of streptavidin and anti-fluorescein alkaline phosphatase or dye conjugation to amine groups for signal detection. We examined the ability of yeast PaP to incorporate Cy3-ATP, for direct fluorescent detection, from a reaction mixture that also contains the native substrate –ATP– of PaP, recognizing that PaP is unlikely to catalyze polymerization solely using Cy3-ATP. As shown in the last four lanes of Figure 5-2a, wherein the gel was scanned at the emission wavelength of Cy5 (that labels the primer), PaP catalyzed polymerization is efficient as seen by the appearance of new high MW bands. However, the corresponding lanes in Figure 5-2b show that these bands show virtually no Cy3 fluorescence, indicating that little Cy3-ATP is incorporated into the polyA that is polymerized by PaP. The lack of Cy3 labeling suggests that PaP has a strong preference for ATP as the substrate, so that Cy3-ATP is excluded from the polymerized chain. These results clearly indicate that using yeast PaP to incorporate fluorescent labels by polymerization of RNA is not likely to be a viable approach for the detection of RNA by SIEP.

5.4 RNA labeling by DNA polymerization using TdT and PaP

We found one report that yeast PaP could incorporate dATP at the 3’end of an RNA primer. We verified this finding by extending an RNA primer with PaP, using
dATP as the monomer in the polymerization mixture. We found that RNA primers were modified with up to two dATP moieties at the 3’-OH terminus after a 2 h reaction (Figure 5-3). Although this process is kinetically slow, it suggested an interesting solution to the conundrum of extending an RNA target efficiently while also incorporating fluorophores into the polymerized strand by using PAP to add a short oligo(dA) tail at the 3’-OH end of the RNA target, which could subsequently initiate DNA polymerization and incorporation of fluorescently labeled dNTPs by TdT, as shown in Figure 5-1.

**Figure 5-3: Limited polymerization of dATP by PaP**
Denaturing polyacrylamide gel electrophoresis verifies the limited polymerization of dATP into RNA primer. After 2 hr PaP reaction, only addition of 2 dATPs is observed. (Data is courtesy of Dr. Hua Yu).

We investigated this observation in more detail in two separate experiments where the reactions catalyzed by PaP and TdT were either carried out in “one-pot”, or sequentially (PaP first, then TdT) using a Cy5-tagged RNA primer (Cy5-rA15), and a
mixture of dATP and Cy3-dATP as the monomers. As shown in Figure 5-4, the sequential PaP and TdT reaction shows a higher primer extension efficiency and greater incorporation of Cy3-dATP in the polymerized ssDNA strand as compared to the one-pot reaction. More importantly, most of the input primer was extended in the sequential reaction of PaP followed by TdT. The extended product length was ~1-2 Kb, and is a function of the monomer (dATP) to initiator (RNA primer) ratios (M/I) in the reaction. We were also able to incorporate multiple fluorescent dNTPs in the extended product in the one-pot or sequential PaP and TdT reactions, which was not possible with PaP alone. We were able to obtain up to ~45 Cy3 fluorophores per extended RNA primer (Figure 5-5b). Furthermore, in comparison between the one-pot and sequential reactions of PaP and TdT, we obtained better overall efficiency in the sequential approach as seen by greater extension efficiency, longer and more uniformly sized products (Figure 5-4), and a higher degree of incorporation of Cy3-dATP (Figure 5-5b).

We next compared the TdT alone, the “one-pot”, and the sequential PaP and TdT catalyzed reactions by quantifying the efficiency of primer extension and the incorporation of Cy3-dATP in the polymerized product (Figure 5-5). We included the reaction with TdT alone to demonstrate the necessity of using PaP with TdT combination to efficiently label RNA and generate signal amplification. The quantification excludes any unextended RNA primer or any RNA chain that is < 20 bases in length, as well as unreacted monomers (dATP and Cy3-dATP). In terms of
primer initiation efficiency, TdT alone only extended ~3-6% of the RNA primer, while in
the “one-pot” PaP and TdT reaction, the percentage of primer extended was ~17-33%
depending on the [Cy-3dATP]/[dATP] ratio. The sequential PaP and TdT catalyzed
polymerization exhibited the highest efficiency, with polymerization initiated from
~50% of RNA primers at all [Cy-3dATP]/[dATP] ratios (Figure 5-5a). We also measured
the degree of labeling per polymerized ssDNA chain, by quantifying the number of Cy3-
dATPs that were incorporated into the extended chain (Figure 5-5b). Reaction with TdT
alone resulted in the highest degree of labeling per extended chain, whereas the two
enzyme methods exhibited fewer Cy3-dATP incorporated per chain, with the sequential
reaction incorporating more Cy3 labels compared to the “one-pot” reaction.

Taking both the primer extension efficiency and number of fluorophores
incorporated in each extended chain into account, and normalizing the total Cy3 signal
by the amount of total initiator (input RNA primer) in each reaction, Figure 5-5c shows
the overall “effectiveness” of labeling for a given amount of RNA initiator. Clearly the
sequential reaction is more efficient and generates a greater degree of labeling, as
indicated by the average of ~20 Cy3-dATP incorporated per input RNA primer. These
results clearly establish that the sequential reaction of PaP followed by TdT labeling is
the preferred strategy for amplified direct labeling of RNA.
**Figure 5-4: RNA labeling using TdT and PaP by incorporation of fluorescent dNTPs**

Gel electrophoresis shows the extent of incorporation of Cy3-labeled nucleotides into the polymerized DNA product that is grown from the 3’-end of a short RNA primer by a mixture of the two enzymes TdT and PaP in “one pot” or sequential reaction. A Cy5-tagged RNA (Cy5- rA₁₅) is used as the primer (initiator) dATP and Cy3-dATP as monomers. Extended products are visualized by the appearance of higher MW bands in gels imaged at the Cy5 fluorescence emission wavelength due to labeling of the primer with Cy5 fluorophore (initiator scan), while the degree of incorporation of Cy3-labeled nucleotides are visualized in gels that are imaged at the Cy3 fluorescence emission wavelength (label scan). (a) Extent of DNA polymerization for the by TdT and PaP in “one pot” and sequential PaP and TdT reactions; and (b) incorporation of Cy3-dATP in a “one pot” and sequential PaP, TdT reactions. The ratio of Cy3 labeled nucleotides and the corresponding natural nucleotides as monomers used in each reaction is indicated by the numbers on the top of each lane. Lanes “L”, “P”, “C” correspond to ssDNA ladder, RNA primer (no polymerization), and a positive control for the TdT catalyzed reaction, where a DNA primer was extended by TdT, respectively.
Figure 5-5: Quantification of fluorescent nucleotide per RNA chain
Quantification of incorporation of Cy3-labeled nucleotides into the polymerized RNA product that is grown from the 3’-end of a short RNA primer by TdT, and by a mixture of TdT and PaP. A Cy5-tagged RNA primer (Cy5-rA15) was subjected to TdT, “one pot”, or sequential PaP and TdT catalyzed reactions as a function of the molar ratio of Cy3-dATP to dATP ranging from 0.01 to 0.2. (a) The fraction of extended RNA primer, which represents the fraction of input primer that was extended to >20 base for the TdT, and TdT and PaP (“one-pot” and sequential) reactions as a function of the molar ratio of Cy3-dATP to dATP. (b) Number of Cy3 incorporated per extended chain as function of Cy3-dATP to dATP in polymerization mixture. (c) The average degree of labeling that accounts for the number of Cy3-dATP incorporated normalized to the amount of input primer in the reaction.
5.5 Direct on-chip detection of RNA target by SIEP amplification

Next, 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 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, 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.

5.5.1 Detection of short RNA target (miRNA)

We obtained dose-response curves for short and long RNA targets as shown in Figure 5-6 and 5-7, respectively. For a short 21 base long synthetic RNA target that has the same length as most miRNA (Figure 5-6), 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 and is similar to the LOD for DNA detection using TdT (Chapter 4.3) reported by us previously. 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\textsuperscript{45, 55, 65, 139} by implementing a simple two-step, isothermal and on-chip reaction, using commercially available reagents.

![Image of dose response curve and spot morphology](image)

**Figure 5-6: Dose response curve of 21-mer RNA target hybridization**

(a) Dose-response curves of RNA hybridization shows successful on-chip fluorescence detection by SIEP of a short synthetic 21 base long RNA target. The image of the probes with labeled bound RNA target is shown in (b). Arrows indicate the LOD (~2pM) as determined by the target concentration at signal from no target control plus 3× standard deviation.

### 5.5.2 Detection of long RNA target (mRNA)

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. The LOD of a long 1.4 Kb RNA target was 1 nM, which is 3 orders of magnitude larger as compared to the short RNA target (Figure 5-7). The source for the decline in performance is largely due to the long target mRNA molecule. This observation is
consistent with other studies reported in the literature, where large nucleic acid targets (cDNA or mRNA) reduce the sensitivity and specificity of the microarray analysis.\textsuperscript{151, 152}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure5-7.png}
\caption{Dose response curve of mRNA (GAPDH) target hybridization}
\begin{enumerate}
\item (a) Dose-response curves of RNA hybridization shows successful on-chip fluorescence detection by SIEP of a full length 1.4 Kb long mRNA target. The image of the probes with labeled bound RNA target is shown in (b). Arrows indicate the LOD (~1 nM) as determined by the target concentration at signal from no target control plus 3× standard deviation.
\end{enumerate}
\end{figure}

There are several possible reasons for this lower LOD. First, in all surface based assays, including microarrays printed on a surface, diffusion of the targets to the surface-immobilized probes could be a limiting step. For a typical microarray assay, hybridization of the mRNA target is carried out with mild fluid agitation/rotation in a reaction chamber. Thus at low target concentrations, hybridization occurs in the mass transport limited regime and the diffusive flux of the target molecules is the rate limiting step that governs the capture of the probe by the target.\textsuperscript{153} In the diffusion-limited regime, the steady-state accumulation of target molecules on the probe spot is
proportional to the diffusion coefficient (D) of the target. We calculated that D for the 21 base long RNA is $1.4 \times 10^{-10}$ m$^2$/s, while for a 1.4 Kb RNA, D is estimated to be $7.7 \times 10^{-12}$ m$^2$/s using a reported diffusion coefficient for a reference 7.5 Kb RNA and a scaling rule for DNA diffusion coefficients on a similar length scale. The lower D for 1.4 Kb RNA decreases the detection sensitivity of long target mRNA molecules. In addition, the target region in the 1.4 Kb RNA may be buried within self-complementary secondary structures, thus making it less accessible for hybridization. Furthermore, once hybridized to the probe, hydrodynamic drag on the free hanging strands may disrupt the hybrid that is only 17 bp long. Finally, after the target is hybridized to the probes, the secondary structure of a long RNA may hinder SIEP, due to the inaccessibility of the 3’-OH end that acts as the initiation site for SIEP.

We attempted to improve the sensitivity of mRNA detection by carrying out an RNA fragmentation step, a method that is known to increase the detection sensitivity of long targets in microarray. Although there are other solutions to relieve problems associated with the detection of long mRNA, such as targeting the mRNA to the probe by electrostatic or magnetic fields, by pumping the fluid sample past the sensor, by using a special fluid chamber geometry, and by employing 3D hierarchical microsensors with nanoscale features to enhance probe display, fragmentation of long targets is the most straightforward approach while maintaining the simplicity of our assay without major modifications to our processing steps. The benefits of RNA
fragmentation are: (1) by breaking a long target into small fragments, the rate of diffusion of the fragment that bears the target sequence is dramatically enhanced; (2) target sequence on small fragments is more accessible to surface-bound probes and more likely to stay attached to the surface during labeling by SIEP; (3) the 3’-OH in small fragments is more accessible for labeling by PaP and TdT as compared to a longer target.

We carried out standard Zn-mediated RNA hydrolysis using commercially available reagents and protocols (Ambion), which cleaves long RNA molecules into 60-200 nucleotide fragments in less than 20 min. 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. We hence 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 experiment in solution to verify the repair step. The gel electrophoresis of fragmented mRNA target, with and without phosphatase treatment showed that phosphatase repair greatly improves the PaP and TdT reaction initiation efficiency and hence incorporation of Cy3-dATP (Figure 5-8).
Figure 5-8: Labeling of fragmented RNA after end-repair step with PaP and TdT

Gel electrophoresis of fragmented mRNA, phosphatase treated (lane 4-6) and untreated (lane 1-3), undergoing PaP and TdT sequential labeling. SYBR Green II stain shows the unextended (lane 1 and 4) and extended products (lane 2-3 and 5-6) while Cy3 scan shows extended product with incorporated Cy3-dATP (lane 3 and 6). Lane L represents the RNA ladder.

After fragmenting the 1.4 Kb long GAPDH mRNA followed by isothermal, on-chip 3’-end-repair by phosphatase, we carried out a sequential PaP and TdT catalyzed polymerization using a mixture of dATP and Cy3-dATP as the monomers, as described previously (see Figure 5-9). We obtained a 10 pM LOD for fragmented and end-repaired full length mRNA input (Figure 5-10), which is a 100 fold improvement from the 1 nM LOD for the full-length mRNA (Figure 5-7) and similar to the LOD obtained for the short, synthetic RNA target (Figure 5-6). The additional steps of mRNA fragmentation and on-chip phosphatase repair are easy to perform using established protocols and
commercially available reagents, so that they do not significantly increase the complexity of the assay.

Figure 5-9: Fragmented mRNA labeling via SIEP with on-chip 3’-end-repair
SIEP for fragmented mRNA samples requires additional step of on-chip 3’-end-repair using alkaline phosphatase.

Figure 5-10: Dose response curve of fragmented mRNA (GAPDH) hybridization
(a) Dose-response curves of RNA hybridization shows successful on-chip fluorescence detection by SIEP of a fragmented mRNA target, where improved detection sensitivity for 1.4 Kb long RNA by fragmentation and on-chip phosphatase end-repair is demonstrated. The image of the probes with labeled bound RNA target is shown in (b). Arrows indicate the LOD (~10 pM) as determined by the target concentration at signal from no target control plus 3× standard deviation.
Figure 5.11: Dose response curve of fragmented mRNA (GAPDH) in the presence of 1 ng/µl yeast background.

Dose-response curves of fragmented GAPDH mRNA target in the presence of 1 ng/µl yeast RNA background (equivalent to ~ 1 nM mRNA transcript) show a drop in LOD to 100 pM (arrow) compared to 10 pM LOD without the presence of yeast RNA background.

We further tested the robustness of SIEP for RNA labeling on chip by carrying out a dose response study of GAPDH mRNA target in the presence of yeast total RNA as background (fragmented at 1 ng/µl, equivalent to ~ 1 nM mRNA transcript). Although the LOD decreased to 100 pM (Figure 5.10) compared to 10 pM LOD without the yeast RNA background, a dose-response curve was obtained, indicating that even with a high level of background, it is possible to carry out an RNA hybridization assay by SIEP.

5.5.3 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 that were spotted on POEGMA-coated glass slides, followed by fluorescence detection by sequential TdT and PaP catalyzed SIEP. As shown in Figure 5-11a, 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. A control probe that is non-complementary to any of the fragments generated from the mRNA transcripts was also used in the assay, and showed a near flat dose-response curve with low signal, demonstrating that non-specific SIEP in the absence of hybridization of the targets to its probe is minimal. 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 5-11b). 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 5-12: Multiplexed RNA detection via on-chip SIEP labeling

The sensitivity and specificity of the probes and fluorescence detection by SIEP are demonstrated by multiplexed detection of three mRNA transcripts. In (a), the dose-response curves of fragmented in-vitro transcribed GAPDH, PRSS21, and IFI44 genes, analyzed simultaneously in heterogeneous mixture samples has an LOD of 10 pM and in (b), specificity of the assay is demonstrated with 100 pM target when individual targets or a mixture of targets are exposed to the microarray.

5.6 Conclusions

We reported the first demonstration of surface initiated enzymatic polymerization (SIEP) for the direct detection of RNA in a fluorescence microarray format. This new method incorporated multiple fluorophores into a RNA strand using the two-step sequential and complementary reactions catalyzed by PaP to incorporate dATP at the 3'-OH of an RNA molecule, followed by TdT to catalyze the sequential addition of a mixture of natural and fluorescent dNTPs at the 3'-OH of RNA-DNA hybrid. We efficiently converted RNA into DNA (~50% conversion) by polymerization of dATP using yeast PaP, and the short DNA strands appended to the end of the RNA by PaP functioned as the initiator for the TdT catalyzed polymerization of longer DNA strands from a mixture of natural and fluorescently dNTPs that contained up to ~45 Cy3
fluorophores per 1 Kb DNA. We obtained a ~2 pM limit of detection (LOD) and a 3-log linear dynamic range for hybridization of a short 21 base long RNA target to an immobilized peptide nucleic acid probe, while fragmented mRNA targets from three different full length mRNA transcripts yielded a ~10 pM LOD with a similar dynamic range in a microarray format.

This methodology has the attractive attributes that: (1) it is both carried out isothermally on-chip, (2) it is able to directly detect short RNA (miRNA) and long mRNA targets from a heterogeneous mixture, and (3) the fluorophore labels are incorporated covalently only upon hybridization of the RNA target by a printed probe, thereby minimizing non-specific labeling. Compared to other on-chip enzymatic labeling techniques, SIEP offers an alternative signal amplification methodology that is straightforward and versatile in that, it can detect DNA, miRNA, and long mRNA transcripts using protocols that are compatible with current microarray technology. Furthermore, the concept of SIEP can be applied to other biosensing platforms beyond microarray such as electrochemical sensors, SPR, quartz crystal microbalance, ellipsometry or atomic force microscopy, through signal amplification via DNA polymerization or incorporating other types of reporter nucleotides.
5.7 Materials and method summary

5.7.1 Materials

Cy5-labeled RNA oligonucleotide primer [5’-Cy5-rA\textsubscript{10}], synthetic RNA target, and PNA probes were synthesized by Gene Link, Integrated DNA Technologies, Panagene (Daejeon, Korea), respectively. TdT enzyme, TdT buffer, and dATP were purchased from Promega while Cy3-dATP was purchased from Perkin Elmer. Yeast PaP and buffer were supplied by Affymetrix, while antarctic phosphatase was purchased from New England Biolabs. Plasmids containing the genes for GAPDH, PRSS21, and IFI44 were purchased from ATCC. The in vitro transcription kit (Ambion MEGAscript Kits, SP6 promoter) to express the full length mRNA from these plasmids was purchased from Ambion. Top Vision LE GQ agarose was supplied by Fermentas. Phosphate buffer and Tween 20 were purchased from Sigma-Aldrich. The non-fouling poly(oligo(ethylene glycol) methacrylate) (POEGMA) brush substrate for PNA probe immobilization were supplied by Mr. Angus Hucknall and made in-house following protocols previously described\textsuperscript{136} while streptavidin for anchoring the DNA/PNA was purchased from Sigma.

5.7.2 Instrumentation

The incorporation of fluorescent nucleotides in solution was determined by fluorescence measurements on a Victor\textsuperscript{3TM} microplate reader (Perkin Elmer Life Sciences). For Cy5 fluorescence measurement, an excitation band filter of 650 ± 4 nm and
an emission band filter of 680 ± 5 nm was used, while for Cy3 fluorescence measurement, a 560 ± 4 nm excitation band filter and a 590 ± 10 nm emission band filter was used. On-chip TdT labeling was determined by scanning the glass slides for the Cy5 (635 nm) and Cy3 (532 nm) fluorescent signal using an Axon GenePix Pro 4200 scanner (Molecular Devices) at 10 µm resolution with optimized PMT and gain settings.

5.7.3 Methods

RNA Extension and Labeling in Solution. In all RNA extension reactions, the concentration of RNA primer that acts as the initiator (I) for SIEP was 1000 fold less than that of the monomer nucleotides (M) to drive the polymerization of an RNA chain. The ratio of fluorescent dNTPs to natural dNTP were 1:5, 1:10, 1:20, 1:50, and 1:100 at ~1 mM total monomer concentration (M).

(1) Reaction with TdT: a reaction mixture consisting of 1 µM Cy5-labeled RNA primer [5’-Cy5-rA10], 1 mM dATP monomers, various amounts of Cy3-dATP, and 10 U of TdT in 20 µL of TdT buffer (1x, 100mM potassium cacodylate, 1 mM CoCl₂, and 0.2 mM DTT, pH 7.2) were incubated at 37 °C for 2 h.

(2) “One pot” reaction with PaP and TdT: a reaction mixture consisting of 1 µM RNA primer [5’-Cy5-rA10], 1 mM dATP monomers, various amounts of Cy3-dATP, 600 U of PaP in PaP buffer (1x, 20 mM Tris-HCl, 0.6 mM MnCl₂, 20 µM EDTA, 0.2 mM DTT, 100 µg/ml acetylated BSA, 10% glycerol, pH 7.0) and 10 U of TdT in TdT buffer (1x) in 20 µl total reaction volume were incubated at 37 °C for 2 h.
(3) Sequential PaP and TdT reactions were carried out in two steps: in the first step, a mixture of 1 μM RNA primer [5’-Cy5-rA10], 1 mM dATP monomers, and 600 U of PaP in 10 μL of PaP buffer (1x) was incubated at 37 °C for 1 h. Without an intervening purification step, in the second step, various amounts of Cy3-dATP and 10 U of TdT in TdT buffer (1x) were added to the reaction mixture to make up a total of 20 μL of reaction and further incubated at 37 °C for 2 h.

A control reaction was carried out similarly using Cy5-labeled DNA primer as the initiator [5’-Cy5-dA10] with 1 mM dATP as the monomer to compare the efficiency of RNA and DNA primer extension using TdT.

**Determination of the Number of Fluorescent Nucleotides Incorporated per RNA Chain.** For each type of extension reaction, the reaction product was subjected to a purification step to remove the non-extended primer (<20 bases) and unreacted monomers, by adding filtered water to make up a 50 μl mixture and passing it through a Probe Quant™ G50 spin column (GE Healthcare). To determine the mole fraction of initiator that was extended and the number of fluorescent dNTPs that were incorporated into the polymerized DNA, the purified product was then diluted with 100 μL deionized water, and its fluorescence was measured on a Victor™ microplate reader (Perkin Elmer Life Sciences) using two sets of excitation and emission filters for Cy5 and Cy3 fluorescence measurement. The amount of initiator and fluorescent dNTPs were
determined by establishing a linear standard curve. The average number of fluorescent dNTPs per DNA chain was determined by dividing the total amount of fluorophores by the amount of primer. The initiation efficiency of the extended primer (% primer extended) is determined relative to the input primer amount in the reaction.

**Fragmented RNA Extension and Labeling.** In vitro transcribed mRNA (~1 µg/ml) was incubated in the RNA fragmentation reagent from Ambion Inc. at 70 °C for 15 min and purified and concentrated using Amicon Ultra filter (Millipore) with 3000 molecular weight cut off. A fraction of fragmented RNA was then reacted with phosphatase (0.25 U/µl Antarctic phosphatase and 0.05% BSA in 1x phosphatase buffer) and further purified using Amicon Ultra (3K) filter. About 300 ng of fragmented RNA (as initiator) was then reacted following the sequential reaction protocol (3) using PaP and TdT, with dATP and Cy3-dATP as the monomers.

**On-chip Fluorescent labeling of RNA Hybridization by SIEP.** We selected a DNA sequence from the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene as a model system to test our assay. The fully complementary 5'-biotinylated 17-mer PNA probe [5’-Ac-GTC CAC CAC CCT GTT GC- lysine- biotin-3’, 1µM] and non-specific PNA probe derived from the hepatitis B virus (HBV) sequence [5’-Ac-ACC TTG TCA TGT ACC AT- lysine-biotin-3’, 1 µM] were individually mixed with streptavidin (2.5
µM) and then spotted using a non-contact printer (Piezzorray, Perkin Elmer) on a nonfouling poly(oligo(ethylene glycol) methacrylate) (POEGMA) brush grown on a glass substrate. The slides spotted with the probes were then incubated overnight in a vacuum chamber and rinsed with 1x SSC buffer containing 0.1% Tween 20 before use. A dose-response curve of hybridized target was generated by incubating the printed probes with a solution of the target RNA that covered a 1 pM-0.1 µM concentration range. Each target solution was heated at 95 °C for 10 min prior to hybridization, then quenched in ice and incubated with the printed probe. We carried out the assay using two targets: (1) a 21-mer synthetic RNA target [5’-rGrCrA rArCrA rGrGrG rUrGrG rUrGrG rArCrC rUrCrA-3’] that is complementary to the probe; and (2) full length in vitro transcribed GAPDH mRNA (~1.4 Kb, details found in SI). Each target was incubated overnight (~16 h) with mild shaking at 42 °C in the hybridization buffer (3x SSC, 0.1% Tween 20, and 4.95 M urea). The synthetic RNA was used as received while the in vitro transcribed GAPDH mRNA was either used as received or fragmented (details in SI) by incubation in the fragmentation reagent at 70 °C for 15 min. For fragmented RNA targets, after rinsing (3x) with wash buffer (1x SCC, 0.1% Tween-20), the bound fragmented RNA target was enzymatically dephosphorylated on-chip (0.0625 U/µl phosphatase in 0.125% BSA and 0.1% Tween 20) at 37 °C for 1 h to ensure the availability of the 3’-OH. The 3’-OH of the bound RNA target was then converted to DNA by SIIEP using PaP to incorporate a short oligo-dATP (6 U/µl PaP and 500 µM
dATP in 1x PaP buffer) at 37 °C for 2 h, followed by SIEP using TdT to incorporate Cy5-dATP (0.1 U/μl TdT, 100 μM dATP and 0.5 μM Cy3-dATP in 1x TdT buffer) at 37 °C for 1 h. The slides were then rinsed in 1x SSC buffer with 0.1% Tween 20 for 30 min and scanned immediately on a GenePix scanner. The average signal intensity of the spot (background subtracted) was then plotted as a function of the target RNA concentration.

**Multiplexed Detection of mRNA via SIEP.** We printed a PNA probe for GAPDH gene, serine protease 21 (PRSS21) gene [5’-Ac-CTT CGG TGA CTC AGG TG-lysine-biotin-3’], 1μM], and interferon-induced protein 44 (IFI44) gene [5’-Ac-CTG AGA CGA ATG CTA TG-lysine-biotin-3’, 1μM] as individual spots on a polymer brush substrate. These probes were then exposed to a mixture of fragmented mRNA targets (corresponding *in vitro* transcribed GAPDH, PRSS21, and IFI44 mRNA targets) spiked into the hybridization buffer. Following overnight hybridization step at 42 °C, the targets were treated for the on-chip dephosphorylation step and the on-chip labeling steps as described earlier.

**LOD Determination.** LOD was determined by determining the concentration that corresponds to the signal at zero concentration plus three times the standard deviation from the sigmoidal curve fit of the target versus concentration (“dose-response”) curve.
6. Amplified Colorimetric Detection of DNA and RNA by SIEP

6.1 Overview

Recent interest in the development of point of care technologies that provide inexpensive, unaided, visual readout for nucleic acid detection has led to the development of alternative reporters other than fluorescent molecules. The leading reporter that fulfills these qualities is metal nanoparticles. For example, to provide colorimetric visual readout, gold or silver nanoparticles are typically used as a seed for further metallization deposition\textsuperscript{52, 55, 161, 162} or the aggregation of gold nanoparticles (Au NPs) produces color change from red to blue under exposure to ambient light (known as localized surface plasmon resonance)\textsuperscript{163-165}.

We devise a strategy to utilize the ability of SIEP to polymerize long DNA chain and to incorporate functional nucleotides, i.e. aldehyde modified dNTP to develop an amplified colorimetric detection assay. The aldehyde group is used to site specifically reduce Ag ions in Tollens’ reagent to form Ag NP seeds that can be enhanced for more metal deposition\textsuperscript{111, 112}. A similar scheme to DNA or RNA hybridization detection with SIEP of fluorescent label (Chapter 4 and 5); the \textit{in situ} growth of DNA strand from bound targets with aldehyde groups incorporated into the chain is used to drive metallization only when targets are bound to the probe (\textbf{Figure 6-1}).
We investigated the possibility of aldehyde directed metallization, as elaborated in details in **Chapter 3.4.3**. We found increased amount of aldehyde in the DNA chain indeed caused more Ag NPs formation. However, we also found that homopolymers of polydT and polydA can cause Ag metal formation through coordination of DNA bases with Ag ions. Although the solution studies showed encouraging data that support our hypothesis on using SIEP incorporated aldehyde groups for metallization, our attempts to obtain similar results with reactions on the surface had failed. We observed significant non-specific silver deposition on the substrate after further development of Ag NPs seeds and little metallization on spots immobilized with DNA that were extended with SIEP.

![Scheme of SIEP colorimetric assay by incorporating aldehyde dNTPs](image)

**Figure 6-1:** Scheme of SIEP colorimetric assay by incorporating aldehyde dNTPs

Colorimetric detection by SIEP of aldehyde modified nucleotides, which reduces Ag ions to Ag NPs seed upon exposure to Tollens’ reagent. Further metallization on seed Ag NPs is expected to generate metal deposition spots that are visible by eyes.
The non-specific metal deposition led us to revise our strategy for SIEP based colorimetric assay. Instead of using aldehyde groups for metallization, we turned to the negatively charged phosphate backbone of a DNA chain. We reasoned that SIEP of DNA produces long DNA chain and thus generate more negatively charge phosphate groups, increasing the net negative charge on the surface. As shown schematically in Figure 6-2, SIEP of bound DNA or RNA target using TdT or PaP, respectively produces long DNA or RNA chains that are exclusively extended from the 3'-OH of the target. Using peptide nucleic acid (PNA) as a probe, which has peptide neutral backbone; the increase in negative charge solely comes from bound target and the in situ SIEP of the target. For colorimetric detection, we use positively charged gold nanoparticles (Au NPs) to bind to the negatively charged backbone as seeds for further metallization enhancement, creating spots of metal deposition that correspond to the amount of target bound and are visible by eye.

Similar strategies that exploit negatively charged DNA backbone for sensing have been demonstrated. These strategies rely on the electrostatic interaction of positively charged reporters to interact with the negative phosphate backbone. For example, cationic conjugated polythiophene derivatives have been used in rapid and sensitive detection of DNA, where polymers bound to single stranded DNA undergo a major conformational change compared to double stranded DNA, generating strong fluorescence emission. Other successful examples utilize PNA capture probes and
cationic reporters, which include conjugated polymers (CCPs), enzymes and gold or silver nanoparticles. PNA is used as capture probes due to its neutral peptide backbone while maintaining bases specificity, which upon hybridization with target DNA generates localized negative charge that corresponds to the amount of DNA target bound.

Figure 6-2: Scheme of SIEP colorimetric assay using the negatively charged phosphate backbone of long DNA chain
Colorimetric detection by SIEP of nucleotides, forming a long ssDNA chain extended from hybridized target which in turn increases the net negative charge on the surface. The negative charge attracts positively charged Au NPs that can be enhanced for further gold deposition, producing metallized spot that can be visually detected by eye.

The use of Au NPs for DNA detection has been largely in the form of a tag. Generally, the DNA target is labeled with Au NPs through affinity tag (biotin-streptavidin, antibody) or a detection probe that can recognize the targets and are labeled with Au NPs. Although this strategy has been successful, the need for
affinity tag labeling on the target, designing a probe, and exposure to the detection probes introduce additional complexity in the assay. To simplify the assay, several strategies that can interrogate DNA target directly have been developed, particularly by exploiting the polyelectrolyte property of a DNA molecule, the electrostatic interaction of Au NPs and a polyelectrolyte, or charge screening phenomenon.\textsuperscript{162-165} For example, Kim \textit{et al.}\textsuperscript{162} immobilized PNA probes to hybridize with DNA target which subsequently detected by positively charged Au NPs. Due to the electrostatic interactions, the Au NPs selectively attach to the hybridized, negatively charged DNA, which can be visualized by the naked eye or an optical flatbed scanner after the treatment with metal enhancement solution.

Our strategy is very similar in the way that we exploit the electrostatic interactions between the negatively charged DNA phosphate backbone and the positively charged Au NPs. However, in order to increase the net negative charge on the hybridized target, we will apply our unique SIEP technology as an on-chip signal amplification method. We expect SIEP to improve the sensitivity and the selectivity of the colorimetric assay by simply adding a one step, post-hybridization, and isothermal reaction. Overall, amplified colorimetric SIEP assay has all the positive attributes of SIEP assay and in addition the ability to assess hybridization of DNA or RNA target visually without the need for fluorescent microscope or scanner.
6.2 Positively charged gold nanoparticles (Au NPs)

6.2.1 Synthesis of positively charged Au NPs

Generally, Au NPs are synthesized by the reduction of gold salt, resulting in nucleation of metal clusters and followed by the growth of the nuclei into metallic nanoparticles. The most popular and straightforward protocol for Au NPs synthesis follows the steps described by Turkevich and Frens, where reduction of gold chloride (HAuCl₄) with sodium citrate is carried out in aqueous solution at boiling temperature.¹⁷³, ¹⁷⁴ In this protocol, sodium citrate acts as a reducing agent as well as a capping agent, decorating the Au NPs with citrate ions and prevents particle aggregation. Due to weak reducing and capping characteristic of citrate, this protocol reliably produces Au NPs with sizes between 16 nm to 150 nm in diameter. Another popular method to produce smaller Au NPs is described by Brust et al.,¹⁷⁵ where instead of sodium citrate, a stronger reducing agent, sodium borohydride (NaBH₄) and a stronger capping agent, dodecanethiol (C₁₂H₂₅SH) are used. With this method, nanoparticles with diameters in the range of 1-3 nm can be produced.

In order for Au NPs to function as reporter, there are two important characteristics that have to be controlled; the size and the surface functionalization. As described previously, size can be tailored by controlling the reducing agent, the capping agent, and the growth step of the Au NPs.¹⁷⁶ For surface functionalization, in particular to produce positively charged Au NPs, there are several methods reported in the
literature. The first method is to functionalize the Au NPs surface with amine terminated thiol by direct synthesis or ligand exchange reaction.\textsuperscript{177} For direct synthesis, the reduction of gold chloride is carried out in the presence of cysteamine hydrochloride,\textsuperscript{178} while ligand exchange reaction is typically carried out on citrate stabilized Au NPs, followed by exposure to thiocetic acid and then amine functionalized thiols, which displace the citrate ions.\textsuperscript{179} The second method is to expose citrate stabilized Au NPs to high concentration of cationic surfactant, in particular cetyltrimethylammonium bromide (CTAB).\textsuperscript{162} For this method, it is hypothesized that the CTAB imparts positive charges on the Au NPs by forming double layer\textsuperscript{181} on the surface of Au NPs through electrostatic interaction with the citrate ions or replacing the citrate ions with positively charged ammonium ions.\textsuperscript{162} The third method involves the use of positively charged polyelectrolytes, such as polyethyleneimine (PEI) as reducing or stabilizing agents by simply mixing gold chloride and PEI,\textsuperscript{182} with heating at 80 °C,\textsuperscript{183} or with addition of strong reducing agent such as sodium borohydride.\textsuperscript{184}

We utilized various sizes of Au NPs (~1.4 nm, 5 nm, 10 nm, 20 nm, and 50 nm in diameter) and particles produced with the three different methods of surface functionalization to impart positive charges. For amine thiol functionalized Au NPs, we purchased ~1.4 nm and ~ 5 nm (in diameter) positively charged Au NPs from Nanoprobes and Nanocs, respectively while we prepared < 10 nm in diameter Au NPs in-house with CTAB and PEI stabilization. For larger size Au NPs, we purchased citrate
stabilized stock from Ted Pella and surface functionalized them in-house with CTAB. Our objective is to investigate the effect of size and different surface functionalization of Au NPs on the particles interaction with the negatively charged DNA backbone.

### 6.2.2 Characterization of positively charged Au NPs

#### 6.2.2.1 Transmission Electron Microscopy (TEM)

We characterized and verified the morphology and the size of the nanoparticles we made by carrying out TEM microscopy on the positively charged Au NPs. In particular, we imaged the CTAB and PEI stabilized Au NPs (Figure 6-3). We found that the size of CTAB and PEI stabilized particles are ~ 5 nm and ~ 2 nm in diameter, respectively. As shown in Figure 6-3, we have made reasonably monodisperse nanoparticles with sizes <10 nm in diameter.

![TEM micrograph of CTAB (a) and PEI (b) stabilized Au NPs](image)

**Figure 6-3**: TEM micrograph of CTAB (a) and PEI (b) stabilized Au NPs

TEM image reveals the size of CTAB functionalized Au NPs as ~5 nm in diameter, shown in (a) while PEI stabilized Au NPs as ~2 nm in diameter, shown in (b).
6.2.2.2 Zeta potential measurement

The overall positive charges on the Au NPs we synthesized and functionalized with CTAB or PEI was verified by measuring their electrophoretic mobility across two electrodes, which is translated to a parameter called zeta potential. Using zeta potential we can qualitatively assess the change in the polarity of the Au NPs by comparing the zeta potential of citrate stabilized particles with CTAB and PEI stabilized particles. As shown in Table 6-1, the zeta potential of citrate stabilized Au NPs is more negative than CTAB modified Au NPs or PEI stabilized Au NPs, even in the presence of saline buffer (PBS) and a small amount of detergent (Tween 20). These results verify that we have prepared positively charged (or to be accurate, more positively charged) particles with CTAB functionalization and PEI stabilization.

Table 6-1: Zeta potential of citrate, CTAB and PEI stabilized Au NPs

<table>
<thead>
<tr>
<th>Gold Nanoparticles</th>
<th>Zeta Potential (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citrate stabilized</td>
<td>-33.03 ± 2.45</td>
</tr>
<tr>
<td>CTAB stabilized</td>
<td>48.87 ± 5.08</td>
</tr>
<tr>
<td>CTAB stabilized in PBS and 0.1% Tween 20</td>
<td>30.03 ± 0.11</td>
</tr>
<tr>
<td>PEI stabilized</td>
<td>12.6 ± 1.70</td>
</tr>
<tr>
<td>PEI stabilized in PBS and 0.1% Tween 20</td>
<td>7.73 ± 3.24</td>
</tr>
</tbody>
</table>

6.2.2.3 Electrostatic binding of Au NPs and DNA

We verified our hypothesis on the use of SIEP as a signal amplification method and the electrostatic binding between positively charged Au NPs and DNA backbone
for colorimetric assay by immobilizing PNA and DNA molecules with the 5’-end or the 3’-end exposed on a polymer brush substrate (POEGMA polymer brush, 136, 160 similar substrate used in Chapter 5) grown on a glass substrate. We selected PNA (17-mer) as a negative control due to its neutral peptide backbone so that to verify the selectivity of SIEP and Au NPs labeling towards DNA. We also included a negative DNA control for SIEP by immobilizing DNA molecules at their 3’-end, precluding any SIEP reactions on them and two positive DNA controls, 8 Kb DNA molecules (extended with TdT in solution) and DNA molecules immobilized at their 5’-end, exposing the 3’-OH end for SIEP reaction. In addition, we are interested to see if there is a difference between 5 nm and 10 nm (in diameter) Au NPs on the intensity of metallization spots they produced.

Figure 6-4a shows the flatbed scanned image of the glass slide on which DNA is immobilized after 30 min Au NPs exposure and a few minutes of the gold enhancement step. Without SIEP reaction, no metallization spots appear on the row with PNA spots (R1), which verifies the selectivity of positively charged Au NPs towards DNA. Figure 6-4a also shows that metallization is proportional to the length of DNA molecules as the highest intensity without SIEP reaction is generated on the row with 8 Kb polydA spots (R3). On the other hand, after 1 hr SIEP reaction, only faint metallized spots develop on the PNA row due to non-specific binding of Au NPs, while a drastic change in the intensity of dT25 spots appears (R4), which has the 3’-OH end exposed for SIEP extension. The intensity of the negative control DNA spots (R2) remains the same even
after SIEP reaction because the exposed 5’end is unreactive towards SIEP extension, indicating that only limited Au NPs bind to the immobilized short DNA strand (15-mer). As for the 8 Kb polydA spots (R3), after SIEP reaction, there is a slight increase in their intensity. This increase could be due to limited SIEP extension because the 3’-OH might be buried due to the extremely long DNA chain adsorption onto the substrate. As for the different nanoparticles size tested in this experiment, similar intensities are produced by spots exposed to 5 nm and 10 nm Au NPs.

A further investigation on the morphology of unextended PNA and extended DNA spots were carried out by imaging the substrate with an optical microscope. As shown in Figure 6-4b, marked differences in the metal deposition can be observed before and after the SIEP reaction, especially on the negatively charged DNA. This result validates that the SIEP reaction does extend DNA with 3’-OH exposed on the surface and that the Au NPs is positively charged, is able to bind electrostatically to the negatively charged DNA phosphate backbone, and the amount bound is proportional to the length of ssDNA chain.

6.3 Colorimetric assay optimization

We studied several important parameters in the development of SIEP colorimetric assay; they are the size of the nanoparticles; the type of the monomer used in SIEP extension and the monomer concentration in SIEP reaction; the surface functionalization to impart positive charges on the Au NPs; and the gold enhancement
solution that produce minimal non-specific metal deposition. Our objective is to study the nanoparticles interaction with surface immobilized long ssDNA and then establish a protocol that allows high sensitivity and selectivity for colorimetric detection, with SIEP amplification.

Figure 6-4: Spots of immobilized PNA and DNA molecules with and without SIEP amplification after exposure to Au NPs and gold enhancement

The glass substrate immobilized with PNA and DNA molecules is scanned with a flatbed scanner after treatment with SIEP and exposure to positively charged Au NPs, followed by gold enhancement step, as shown in (a). Comparing images with and without SIEP, especially on DNA spots with 3’-OH exposed (R3 in (a) and R2 in (b)); SIEP treatment results in marked increase in the intensity of the spots. Morphology of the metallized PNA and DNA spots are investigated with an optical microscope and is shown in (b). The scale bar is 150 µm.
6.3.1 Effects of nanoparticle size

Our preliminary studies showed similar intensity of metallized spots after exposure to 5 nm and 10 nm Au NPs in diameter. Although it has been demonstrated before that smaller particles interact with DNA better and thus exhibit a stronger response,\textsuperscript{162, 185} we are interested to find out if long ssDNA polymerized by SIEP generates a similar response.

We immobilized dA\textsubscript{10} primers on a substrate at their 5’-end, exposing the 3’-OH for SIEP reaction by TdT enzyme. We then carried out TdT reaction for 1 hr at 37°C, polymerizing dATP at the 3’-OH of the immobilized primer, followed by exposure to positively charged Au NPs that are CTAB functionalized with 5 nm, 20 nm, or 50 nm diameters and gold enhancement step for further metal deposition. As shown in Figure 6-5, with or without SIEP, drop in intensities occur as the diameter of Au NPs increases. Although spots of DNA that undergo SIEP have higher intensities, the trend on the effects of nanoparticles size is similar with DNA spots that are not extended with SIEP. When 50 nm Au NPs is used as reporter, the effect of SIEP amplification is negated by the inefficient binding of the Au NPs to the DNA backbone. It is postulated that a large particle has lower accessibility to the DNA chain than a small particle\textsuperscript{185} and even it binds to the DNA chain, due to hydrodynamic drag, a large particle can fall off/rinse off easily. This result demonstrates that in order to achieve efficient labeling and to
maximize the metal deposition, Au NPs ≤ 10 nm should be used for SIEP colorimetric assay.

Figure 6-5: Effects of Au NPs size on the intensity of metallized DNA spots with and without SIEP amplification

Increase in the size of Au NPs cause inefficient labeling of DNA chain and thus produces lower intensities of metallized spots. The effect of large Au NPs size is detrimental on SIEP amplification when the diameter of Au NPs ≥ 20 nm. The images of metallized DNA spots (~150 µm) are taken using an optical microscope and intensities are averaged from five DNA spots.

6.3.2 Effects of monomer type and concentration

We have demonstrated in Chapter 3 that SIEP reaction catalyzed by TdT can polymerize dATP and dTTP efficiently. Therefore, we are interested to see if the type of monomer affects the binding of Au NPs to homopolymer DNA chains produced by SIEP reaction. We are also interested to see if within 1 hr SIEP reaction, the length of ssDNA chain polymerized by TdT is limited by the amount of input monomer in the reaction.

We immobilized dT25 primers on a substrate at their 5’-end, exposing the 3’-OH for SIEP reaction by TdT enzyme. We then carried out TdT reaction by adding different
amounts of dATP and dTTP for 1 hr at 37°C, followed by exposure to ~ 5nm positively charged Au NPs that are coated with CTAB and gold enhancement step for further metal deposition. **Figure 6-6** shows the signal intensities of the DNA spots after SIEP reaction polymerizing dATP and dTTP. Clearly, homopolyA generates higher signal intensity compared to homopolyT in all reaction conditions. This result can be explained by the intrinsic affinity of adenine nucleotides for gold, resulting in better binding of Au NPs to homopolydA DNA chains, which translates to a higher intensity due to the higher amount of Au NPs seeds for further metal deposition. **Figure 6-6** also shows that SIEP reaction in the presence of 0.1 mM monomer is sufficient to generate considerable signals and higher monomer concentration produce marginal or insignificant improvement.

![Figure 6-6: Effects of monomer type and concentration during SIEP reaction](image)

SIEP amplification by dATP polymerization produces higher signal intensity compared to dTTP polymerization. In addition, for 1 hr SIEP reaction, 0.1 mM monomer input is sufficient to produce signal amplification and increase in monomer input resulted in marginal signal improvement. The image of metallized DNA spot is taken using an optical microscope and intensities are averaged from five spots.
6.3.3 Effects of positively charged Au NPs surface functionalization

We investigated the effects of Au NPs surface functionalization on the particles interaction with the phosphate backbone of a DNA chain by immobilizing DNA primers (dA₁₀) on a substrate and subjecting them to SIEP colorimetric assay. We compared thiol amine modified, CTAB modified, and PEI stabilized nanoparticles interactions with homopolydA. These particles have sizes of 1.4 nm, 2 nm, and 5 nm in diameter.

As shown in Figure 6-7, Au NPs with different surface functionalization produce similar intensities and have little impact on the overall signal intensity with or without SIEP reaction. This means that once the particle surface is positively charged, the difference in the charge density and polarity of the positive charge on Au NPs have little influence on their interaction with negatively charged DNA backbones.

The only difference is the higher signal produced by 1.4 nm thiol-amine modified Au NPs. However this higher signal also resulted in higher background signal around the substrate. This observation is consistent with the result reported previously by Kim et al.,¹⁶² where 1.4 nm exhibited highest intensity with a strong background signal due to non-specific binding of the small size Au NPs. In addition, the effect of SIEP is less significant when we use 1.4 nm Au NPs as shown by the lowest difference between signal without and with SIEP.

In addition to examining the background signal generated on the substrate, we also assessed the non-specificity of Au NPs binding to the PNA molecules, which was
immobilized at the 3’-end, simulating the orientation of the immobilized probes in SIEP colorimetric assay. We find the CTAB modified Au NPs produce the lowest signal from the PNA spots, giving the highest specificity of labeling DNA against PNA molecules. Considering the signal amplification, the background signal, and the specificity of Au NPs towards DNA, we find that the CTAB modified particles is the best candidate for SIEP colorimetric assay.

Figure 6-7: Intensity of metallized DNA spots labeled with three types of functionalization on positively charged Au NPs
Comparison of the signal intensities from immobilized DNA molecules, with and without SIEP, produced after exposure to various Au NPs that are subjected to different method of surface functionalization shows similar intensity across different Au NPs, thus little impact of surface functionalization on the signal amplification.

6.3.4 Gold enhancement step

In order to generate metal spots that can be identified visually, an additional step after Au NPs exposure is required. The gold or silver enhancement step is to grow the positively charged Au NPs seeds.\textsuperscript{52, 162} It is critical to control the enhancement step for
generating metallized spots with intensity that are proportional to the amount of DNA target bound. We selected gold enhancement instead of silver enhancement for their lower background and less volatile reaction, allowing a more controlled development of the Au NPs growth. Kim et al. uses gold enhancement solution sold by Nanoprobes in their assay, allowing the reaction to last for 1 min. We attempted this commercial gold enhancement solution, but we observed significant background and development of non-specific metallization on PNA spots.

We found another recipe for gold enhancement solution developed by the Braun group for DNA nanowire fabrication, which utilized a mixture of potassium thiocyanate (KSCN), gold chloride (HAuCl₄), and hydroquinone. We used this recipe in our initial experiments; however we find the development is still too fast to generate repeatable results. Our further research into gold enhancement solution resulted in several strategies to slow down the development of gold. The strategies that were effective are: (1) increasing the ionic strength of the gold enhancement solution by adding 0.1-0.5 M NaCl, (2) adding viscosity modifier such as polyethylene glycol or glycerol (10%), and (3) adding a small amount of detergent (0.1%) such as Tween-20. After several trials, we settled on adding 0.1 M NaCl to our gold enhancement solution which lengthens the exposure time to 4-7 min, allowing better handling and rinsing step after gold enhancement step.
6.4 Colorimetric detection of DNA and RNA hybridization by SIEP

We have established that SIEP colorimetric assay is feasible using positively charged Au NPs followed by the gold enhancement step. Using optimized protocol that comprised of dATP for SIEP polymerization, ~5 nm CTAB modified Au NPs as seeds for metallization, and in-house developed gold enhancement solution, we generated dose response curves for DNA and RNA target, using PNA as the capture probe.

6.4.1 Dose response curve of DNA target hybridization

We assessed SIEP colorimetric assay for the analysis of DNA targets by hybridizing 22-mer DNA targets onto immobilized PNA probes on a non-fouling substrate. The hybridized targets were then exposed to TdT reaction containing 0.1 mM dATP for 1 hr at 37 °C, selectively polymerizing dATP from the 3'-OH of bound target. The SIEP amplified target was then exposed to positively charged Au NPs (~ 5nm in diameter, CTAB modified) for 30 min. After rigorous rinsing, gold enhancement step was carried out for 5 min. The substrate with metallized DNA spots was then spun dry and imaged with a flatbed scanner for intensity analysis and an optical microscope for detailed morphology.

As shown in Figure 6-8, hybridized targets that undergo SIEP reaction can be distinguished visually from non-specific spots at 1 nM concentration compared to 100 nM for target without SIEP amplification. However, when we plot the intensity of spots...
(background corrected mean intensity) generated from hybridized target with and without SIEP amplification, the LOD for both assay is the same (LOD is determined from identifying the concentration with signal intensity of zero targets plus three times the standard deviation). Although technically SIEP does not improve the LOD of DNA hybridization assay, visually SIEP improve the assay sensitivity by 100 fold. In addition, from our experience generating dose response curves, SIEP amplification produce a more consistent increase in signal intensities than without SIEP amplification as the target concentration increases.

We compared our result with the result published by Kim et al.,\textsuperscript{162} where they hybridized 18-mer target to a PNA probe and carry out Au NPs labeling and gold enhancement. They showed data with LOD of 10 pM and claimed that their assay is as sensitive as fluorescent labeled targets. In our hand, this result is not repeatable. The discrepancy between our data and their finding is probably due to variations in sample processing and assessment of the data (their data is 8-bit grayscale while our data is 16-bit grayscale, see methods). Another key step probably lies in the gold enhancement step as we often observed significant signal produced by PNA probe alone without target exposure, which is likely caused by the binding of the Au NP to the bases in the PNA\textsuperscript{188} and result in high level of “background” signal within the spot itself (not from the substrate) as shown in optical microscopy image. This high level background within a spot makes it difficult to assess target hybridization at low concentrations (< 1 nM).
Figure 6-8: Visual assessment and dose response curve of hybridized 22-mer DNA target on PNA probe, with and without SIEP amplification

Optical microscope image of metallized spots with fully complementary (left column) and non-specific (right column) PNA probes after exposure to a range of 22-mer DNA target concentration is shown. We determine visually a sensitivity of 100 nM (highlighted) without SIEP amplification, while with SIEP, the sensitivity is 100 fold higher at 1 nM (highlighted). However, when the LOD is determined from the background corrected signal intensities (see dose response curve) measured using a flatbed scanner, SIEP amplification results in 1 nM LOD that is similar to the LOD obtained without SIEP.

6.4.2 Dose response of RNA target hybridization

We demonstrate the use of SIEP colorimetric assay for the detection of hybridized RNA target by using PaP enzyme to replace the role of TdT enzyme. As shown in Chapter 5, TdT is not efficient in initiating polymerization at the 3’-OH of an RNA, therefore a template independent RNA polymerase enzyme such as PaP is an ideal alternative. We have demonstrated that PaP can polymerize ATP from the 3’-OH of an RNA initiator and generate a long homopolyA (Figure 5-2a). When this
characteristic is used to grow long homopolyA from the RNA target bound to the immobilized probe, signal amplification can be attained through the increase in the net negative charge from the bound RNA target.

We assessed the SIEP amplification on the RNA target by hybridizing 28-mer RNA target onto the immobilized PNA probe. The hybridized target was then exposed to PaP reaction containing 0.1 mM ATP for 1 hr at 37 °C, followed by incubation with positively charged Au NPs (~ 5nm in diameter, CTAB modified) for 30 min. After rigorous rinsing, gold enhancement step was carried out for 5 min. The substrate with metallized DNA spots was then spun dry and imaged with a flatbed scanner for intensity analysis and an optical microscope for detailed morphology.

We obtain 100 pM sensitivity and selectivity after SIEP amplification compared to 10 nM without SIEP, as shown by the optical microscope image in Figure 6-9. This level of sensitivity is 10 fold more sensitive than the DNA detection. We believe the improvement in the sensitivity of RNA hybridization detection is due to higher affinity of PNA towards RNA than DNA.\textsuperscript{150} When we plot the background corrected intensities of spots generated from RNA hybridization, we obtain ~100 pM of LOD for SIEP amplified spots and ~ 1 nM LOD for hybridized RNA without amplification. The discrepancy in the sensitivity level is probably due to the technical definition of LOD. Nevertheless, visual assessment and intensity plot consistently show that SIEP amplification improves the detection sensitivity of RNA hybridization.
Figure 6-9: Visual assessment and dose response curve of hybridized 28-mer RNA target on PNA probe, with and without SIEP amplification

Optical microscope image of metallized spots with fully complementary (left column) and non-specific (right column) PNA probes after exposure to a range of 28-mer RNA target concentration is shown. We determine visually a sensitivity of 10 nM (highlighted) without SIEP amplification, while with SIEP, the sensitivity is 100 fold higher at 10 nM (highlighted). However, when the LOD is determined from the background corrected signal intensities (see dose response curve) measured using a flatbed scanner, the LOD without SIEP amplification improves to 1 nM while the LOD with SIEP amplification is maintained.

Overall, SIEP amplification on hybridized DNA and RNA target has improved the sensitivity of colorimetric detection by 100 fold, especially by comparing the metallization spot of specific probe with non-specific probe visually. In other words, SIEP amplification allows a more sensitive detection and a more robust assessment of positive hybridization events.
6.5 Conclusions

We presented a scheme for SIEP-based assay with colorimetric detection. In this scheme we utilized the ability of TdT and PaP to polymerize long DNA/RNA chains from the 3’-OH of a DNA/RNA initiator and the electrostatic interaction of positively charged Au NPs with the negatively charged DNA backbone. We demonstrated that in situ polymerization of DNA/RNA by SIEP, initiated from the hybridized DNA or RNA target produced long ssDNA/RNA chains that increases the net negative charge on the surface. This increase in negative charge attracted positively charged Au NPs, which then served as seeds for further metal deposition, allowing visual readout of target hybridization. We observed long homopolyadenine and Au NPs with 5 nm diameter and CTAB functionalization produced high metal deposition. Using SIEP amplification, visual readouts on hybridized DNA and RNA targets were obtained with sensitivities that were 100 fold higher than unamplified assay. Finally, this work has demonstrated the versatility of SIEP amplified assay for nucleic acid hybridization detection, extending signal detection from fluorescent to visual readout.

6.6 Materials and methods summary

6.6.1 Materials

All oligonucleotide primers and targets used in this study were synthesized by Integrated DNA Technologies, Inc, while the PNA probes were synthesized by Panagene. TdT enzyme, TdT buffer, and dNTP monomers (dATP and dTTP) were
purchased from Promega while ATP monomer, yeast PaP, and buffer were supplied by Fermentas and Affymetrix. Chemicals for nanoparticles synthesis and gold enhancement such as chloroauric acid (HAuCl₄), cetyltrimethylammonium bromide (CTAB), sodium borohydride (NaBH₄), phosphate buffer (PBS), Tween 20, polyethyleneimine (PEI, branched, Mw: 25,000), potassium thiocyanate (KSCN), postassium gold (III) chloride (KAuCl₄), and hydroquinone were purchased from Sigma-Aldrich (St Louis, MO) while sodium chloride and citric acid were purchased from EMD Chemicals, Inc. Gold nanoparticles, 1.4 nm positively charge particles, 5 nm thiol-PEG-amine modified, and 10 nm, 20 nm, 50 nm citrate stabilized particles were purchased from Nanoprobes, Inc, Nanocs, and Ted Pella, respectively. The nonfouling poly(oligo(ethylene glycol) methacrylate) (POEGMA) brush substrate for DNA primer and PNA probe immobilization were supplied by Mr. Angus Hucknall and made in-house following protocols previously described¹³⁶ while streptavidin for anchoring the DNA/PNA was purchased from Sigma.

**6.6.2 Instrumentation**

The nanoparticles size characterization were imaged using transmission electron microscopy (TEM) FEI Tecnai G² Twin and the zeta potential were measured using Zetasizer Nano ZS (Malvern Instrument). The developed glass slides were scanned using Spotware™ colorimetric microarray scanner from ArrayIt and imaged with Nikon Eclipse TE2000-U microscope.
6.6.3 Methods

Synthesis and characterization of CTAB stabilized gold nanoparticles. We followed the protocol described by Kim et al. Briefly, 1.97 mg of HAuCl4 was dissolved in 20 ml citric acid solution (0.38 mM), followed by the addition of 0.6 ml NaBH₄ (0.1 M) at room temperature. This protocol resulted in ~5 nm diameter Au NP. To impart positive charges on the nanoparticles surface, equal volume of CTAB (0.2 M, dissolved by heating at 60-80 °C) and nanoparticles solution (made in-house or purchased from Ted Pella) were mixed. After ~ 5 min, in order to simulate hybridization buffer, gold nanoparticles solution with CTAB were mixed with phosphate buffered saline (1x PBS) with 0.1 % Tween (PBST buffer) of equal volume. The solution of CTAB stabilized nanoparticles in PBST buffer is stable for months at room temperature. The particles morphology and size were determined with TEM microscopy operated at 160 kV while the confirmation of positive charge was measured with zeta potential and obtained from averaging three independent measurements.

Synthesis and characterization of PEI stabilized gold nanoparticles. We followed the protocol described by Sun et al. with modification. Briefly, 80 µl of HAuCl₄ (30 mM) was added into 10 ml Milli-Q water (18 MΩ) and stirred for 1 min. After which 20 µl PEI (18.3%, requires some HCl for dissolution) was added into the gold chloride solution and stirred for 1 min. In order to reduce the gold chloride, 0.3 ml NaBH₄ (0.1 M, kept in
ice) was added into the mixture drop by drop during vigorous mixing and the mixture was further stirred for 5 min. The nanoparticles solution was then stored at 4 °C and used as is for colorimetric assay. The particle morphology and size were determined with TEM microscopy operated at 200 kV while the confirmation of positive charge was measured with zeta potential and obtained from averaging three independent measurements.

**Gold enhancement solution.** We followed the protocol described by Keren et al.\textsuperscript{187} with modification. Briefly, a solution of KSCN (50µl ml, 60 mg/ml) was mixed with a solution of KAuCl4 (50µl, 23 mg/ml). The mixture was then centrifuged at 2000 rpm for 1 min and the supernatant was removed from the orange precipitate. After which, Sorensen’s buffer (800 µl, 0.5 M KH\textsubscript{2}PO\textsubscript{4}, pH 5.0) containing 0.1 M NaCl was added to dissolve the precipitate. A solution of hydroquinone (40 µl, 5.5 mg/ml) was prepared fresh or kept in ice and added into the mixture right before the gold enhancement step.

**Feasibility study and optimization of SIEP colorimetric assay.** The DNA primers [1 µM of 5’- CGG GCA ACA TAC CTT-biotin-3’, 5’-biotin-dA\textsubscript{10}-3’, and 5’-biotin-dT\textsubscript{25}-3’] and PNA primers [5’-Ac- CGG GCA ACA TAC CTT - lysine-biotin-3’] were individually mixed with streptavidin (2 µM) and then spotted using a non-contact printer (Piezzorray, Perkin Elmer) on a nonfouling poly(ethylene glycol) methacrylate)
(POEGMA) brush\textsuperscript{136, 160} grown on a glass substrate. The slides spotted with the primers were then incubated overnight in a vacuum chamber and rinsed with 1x SSC buffer containing 0.1% Tween 20 before exposure to SIEP reaction at 37 °C for 1 h. SIEP reaction comprised of 10 U of TdT and dATP or dTTP monomers (0.1 mM, 0.5 mM, or 1 mM) in 100 μL of TdT buffer (1x, 100mM potassium cacodylate, 1 mM CoCl\textsubscript{2}, and 0.2 mM DTT, 0.1% Tween 20, pH 7.2). The slides were then rinsed three times with 1x SSC buffer with 0.1% Tween 20. Following SIEP reaction, the slide were exposed to various sizes of positively charged gold nanoparticles (1.4 nm, 5 nm, 10 nm, 20 nm, and 50 nm) for 30 min and then rinsed three times with PBST buffer (1x PBS + 0.1% Tween 20) before exposure to the gold enhancement solution for 4-7 min.

**Metallization spot analysis.** The 16-bit image of metallized DNA spots were scanned using ArrayIt colorimetric flatbed scanner in the inverted mode (Figure 6-10b) with the appropriate gain setting (typically 0.8 to 1.0) using their proprietary software (SpotWare\textsuperscript{TM}). The individual spot intensity was then determined using GenePix 6.0 (Molecular Devices) by localized background subtraction (Figure 6-10c) and the average intensities of 5 spots were plotted for comparison.
Figure 6-10: Image of metallized DNA spots scanned with a flatbed scanner and localized background corrected image after analysis

Metallized DNA spots hybridized on fully complementary probes (left column) and non-specific probe (right column) is scanned with a flatbed scanner is shown in (a). The inverted image of (a) is shown in (b) while the inverted, localized background corrected intensities is shown in (c).

Colorimetric SIEP assay for hybridization of DNA target. The fully complementary PNA probe [1µM, 5’-Ac-CAA TGC CAG CCC CAG CG- lysine- biotin-3’] and non-specific PNA probe [1µM, 5’-Ac-GTT GCT GGT AGT TTA TG- lysine- biotin-3’] were individually mixed with streptavidin (2 µM) and then spotted using a non-contact printer (Piezzorray, Perkin Elmer) on a glass slide coated with a non-fouling polymer brush. The slide spotted with the primers was then incubated overnight in a vacuum chamber and rinsed with 1x SSC buffer containing 0.1% Tween 20 before exposure to the DNA target. A dose-response curve of the hybridized target was generated by incubating the printed probes to a range of target DNA concentrations [1 pM-1 µM, 5’-CGC TGG GGC TGG ACT TGC CCT C-3’] for 4 h at 42 °C in 3x SSC buffer. After rinsing
three times (1xSSC + 0.1% Tween 20), the slide was exposed to SIEP reaction using TdT to grow polydA (0.1 U/µl TdT, 0.1 mM dATP in 1x TdT buffer) for 1 h at 37 °C. The slides was then rinsed three times in 1x SSC buffer with 0.1% Tween 20 followed by another set of rinse in PBST. For gold nanoparticles labeling, the slides was exposed to 5 nm CTAB stabilized particles for 30 min. After three times rinsing in PBST, the slide was exposed to gold enhancement solution for 5 min.

**Colorimetric SIEP assay for hybridization of RNA target.** The fully complementary PNA probe [1µM, 5’-Ac- CAA TGC CAG CCC CAG CG - lysine- biotin-3’] and non-specific PNA probe [1µM, 5’-Ac- GTT GCT GGT AGT TTA TG- lysine- biotin-3’] were individually mixed with streptavidin (2 µM) and then spotted using a non-contact printer (Piezzorray, Perkin Elmer) on a glass slide coated with non-fouling polymer brush. The slide spotted with the primers was then incubated overnight in a vacuum chamber and rinsed with 1x SSC buffer containing 0.1% Tween 20 before exposure to DNA target. A dose-response curve of hybridized target was generated by incubating the printed probes to a range of target RNA concentrations [1 pM-1 µM, 5’- UUU GAC GCU GGG GCU GGC AUU GCC CUC-3’] for 4 h at 42 °C in 3x SSC and 4.95 M urea buffer. After rinsing three times (1xSSC + 0.1% Tween 20), the slide was exposed to SIEP reaction using yeast PaP to grow polyA (6 U/µl PaP, 0.1 mM ATP in 1x PaP buffer) for 1 h at 37 °C. The slides was then rinsed three times in 1x SSC buffer with 0.1% Tween 20.
followed by another set of rinse in PBST. For gold nanoparticles labeling, the slides was exposed to 5 nm CTAB stabilized particles for 30 min. After rinsing three times in PBST, the slide was exposed to gold enhancement solution for 5 min.
7. Performance of on-chip labeling via SIEP

In the development of any new analytical/clinical assays or sensing technologies, it is important to assess their performance, advantages, and limitations. We have demonstrated the SIEP capabilities and highlighted advantages of SIEP-based assay in previous chapters. In this chapter, we discuss the limitations of SIEP, its analytical performance against other techniques and if SIEP can be used to analyze clinically relevant samples without target amplification.

There are several performance parameters that are generally used to benchmark different assays or instruments for analytical purposes. They are sensitivity, limit of detection (LOD), and dynamic range. These parameters can be limited to the instruments’ performance but also assay specific, depending on the platforms (surface vs solution based reaction), signal transduction (e.g. fluorescent or colorimetric), and signal amplification. In this chapter, in order to evaluate the performance of SIEP and to eliminate other extrinsic factors, we study the use of SIEP for fluorescence detection of DNA hybridization (25-mer target) on the microarray platform. We used target DNA that is chemically tagged with a single Cy3 dye at the 3’-end as an ideal target for its comparison with SIEP on-chip labeling.
7.1 SIEP signal amplification and sensitivity

We compared on-chip SIEP labeling of DNA target using TdT against an identical target sequence that is labeled via chemical synthesis with a single Cy3 fluorophore at the 3’-end on microarray platform. For on-chip SIEP-TdT reactions where the ratio of Cy3-dATP to dATP is 0.005, we observed a ~2 fold signal amplification when the target concentration is 1 nM as compared to synthetically made Cy3-tagged target (Figure 7-1). The modest signal amplification is due to the low ratio of fluorescent dNTP to the natural dNTP used in the reaction (one fluorescent nucleotide for every 200 natural nucleotides). As shown in the experiment quantifying SIEP mediated signal amplification using TdT on the surface (Chapter 4.2), reaction condition with Cy3-dATP to dATP ratio of 0.005 resulted in ~6 fold signal amplification. Taking into account that we are now labeling a hybridized strand instead of covalently immobilized ssDNA, the drop in the signal amplification is to be expected due to low surface concentration of the target initiator strand. This experimental result also suggests that the efficiency of TdT SIEP labeling is lower than 100% as compared to ~95% labeling efficiency of chemically labeled targets and thus may also contribute to the lower than expected signal amplification. Although we cannot achieve massive signal amplification, our hypothesis that TdT labeling is able to incorporate multiple fluorescent reporters is verified by the result of this experiment where the overall signal intensity of TdT labeling is higher than
the target tagged with a single fluorophore, despite lower labeling efficiency, especially when the target concentration ≤ 10 pM.

We increased the Cy3-dATP to dATP ratio to 0.02 (4 fold higher) in an attempt to produce higher signal amplification. Although we achieved higher signal amplification (~8 fold amplification at 1 nM concentration), we observed significant non-specific labeling when the probe was exposed to SIEP reaction without any prior target exposure as highlighted in Figure 7-2. In addition, due to the higher amount of fluorescent dNTP in the reaction, the surrounding background has significantly higher fluorescence signals as well, reducing the signal to noise ratio of the DNA spots, especially for concentration < 10 pM. We have several hypotheses for the source of non-specific signal; (i) the non-specific adsorption of TdT that contains fluorescent dNTP in the active binding pockets, (ii) the non-specific adsorption of fluorescent dNTP, and (iii) the non-specific activity of TdT, such as de novo DNA synthesis ability.93

We have attempted several solutions to eliminate or reduce the non-specific signal generated by the SIEP reaction. First, we treated the surface with Proteinase K (a protease) to remove the non-specifically adsorbed TdT. However the result of this treatment did not show any significant removal of the non-specific signal. Second, we tried to manage the non-specific activity of TdT by the inclusion of “ghost” primer to prevent any non-specific activity on the surface; yet this attempt likewise did not result in any significant removal of the non-specific signal as well. Despite our efforts to
remove these non-specific signals, we were not able to eliminate it. Our conclusion is that the non-specific signal from the probe can be kept at a manageable level by maintaining a low ratio of fluorescent-dNTP to dNTP for example at 0.005.

Sensitivity of detection is defined as the ability of a method to discriminate small differences in analyte concentration. This parameter is affected by the noise of the measurement and the slope of the calibration curve, which is assessed by the relationship of the signal intensity measured and the target concentration. The noise of the measurement can be detrimental to sensitivity especially at the low target concentration (< 10 pM). As shown in Figure 7-1 and 7-2, the sensitivity of SIEP labeling is higher than the hybridization of fluorescently tagged DNA target, judging from the slope of the signal intensity with respect to the target concentration, when concentration is greater than 10 pM. In addition, the difference in the slope of dose response curve at this concentration range (>10 pM) increases as the ratio of Cy3-dATP to dATP increases, indicating that increasing number of fluorophore in the target DNA extended with SIEP improves sensitivity of DNA hybridization assay. However, when the target concentration is low (< 10 pM), the dose response curve slope is more tapered and there is little difference between SIEP labeled and chemically labeled target.

From the assessment of signal amplification and sensitivity of SIEP labeling in comparison with chemically tagged target, it is consistently shown that the effectiveness and advantages of SIEP on-chip labeling occurs when the target concentration is >10 pM.
This observation could be explained by following reasons. First, at low target concentration (<10 pM), the amount of target bound is limited. At such a low amounts of target bound, the efficiency of target labeling is critical and it is possible that the multiple labels incorporated by TdT are compensating for the less than 100% labeling inefficiency, which results in lower than expected signal amplification. Second, at low target concentrations, the presence of non-specific signal could be detrimental to the ability to differentiate specific signal from noise. In SIEP labeling, due to the presence of fluorescently tag nucleotides in the reaction mixture, the probability of having high background signal is higher, thus resulting in lower signal to noise ratio and masking the effectiveness of SIEP signal amplification.

Overall, on-chip labeling and signal amplification by SIEP improve sensitivity but at low target concentration, sensitivity is limited by SIEP labeling efficiency and the non-specific signal generated by physically adsorbed fluorescent nucleotides.
Figure 7-1: Comparison of on-chip SIEP labeling (Cy3-dATP/dATP: 0.005) with chemically synthesized Cy3-labeled DNA target
The dose response curve of SIEP labeling (Cy3-dATP/dATP: 0.005) against chemically synthesized Cy3-tagged target shows ~2 folds signal amplification at 1 nM. Signal amplification and increase in sensitivity is observed only at concentration > 10 pM.

Figure 7-2: Comparison of on-chip SIEP labeling (Cy3-dATP/dATP: 0.02) with chemically synthesized Cy3-labeled DNA target
The dose response curve of SIEP labeling (Cy3-dATP/dATP: 0.02) against chemically synthesized Cy3-tagged target shows ~8 folds signal amplification at 1 nM. The increase in signal amplification and sensitivity due to increase in the amount of fluorescent nucleotides in the SIEP reaction is observed only at concentration > 10 pM. The box at zero target concentration highlights the non-specific signal generated by SIEP.
7.2 Limit of detection (LOD) and limit of quantitation (LOQ)

Limit of detection (LOD) and limit of quantitation (LOQ) are often associated with the sensitivity of an assay, although by definition they are not the same. By definition, LOD is used to describe the lowest concentration that generates a signal which can be reliably distinguished from the blank signal while LOQ is used to describe the lowest concentration that generates a signal which can not only be reliably detected but also satisfy some predefined precision and threshold above bias.

The threshold of the signal generated at LOD is normally determined based on the noise of measurement (in terms of standard deviation, \( \sigma \)) and the signal generated using a blank sample. A guideline to determine LOD is published by the Clinical and Laboratory Standards Institute (Publication EP17) and has been summarized in essence by previous publications.\(^{190, 191}\) For this dissertation, we use the general accepted value for LOD, which is typically determined as the concentration at which the measured signal is equal to the blank signal plus 3\( \sigma \) of the blank sample.\(^ {189} \)

We determined that the LOD of SIEP based assay for the detection of nucleic acid hybridization is in the low pM range (1 pM-10 pM), depending on the size of the target (Chapter 5). Although the method of LOD determination could vary,\(^ {191} \) we find that the LOD we obtained from SIEP labeling is typical of surface-based assay and it is comparable –and in some instances– better than other DNA or RNA target labeling and signal amplification techniques.\(^ {45, 55, 63-65, 123, 126, 127} \)
Based on the LOD definition, the LOD of a sensing technique is limited by the non-specific signal when there is no targets are present. Therefore for SIEP on-chip labeling, although we expect that incorporation of multiple fluorophores can improve detection sensitivity, the LOD, in practice is limited by the signal generated at zero target concentration and the noise ($\sigma$), which largely arises from non-specific signal generated by surface exposure to fluorescent nucleotides during SIEP reaction. The lower the signal at zero target concentration (blank), the lower the LOD, which in turn limits the amount of fluorescent nucleotides in the SIEP reaction.

As shown in Figure 7-1 and 7-2, the second factor that fundamentally influences the LOD of DNA detection is the signal generated at low target concentration, which has to be significantly higher than the blank signal. This low target concentration signal is affected by multiple factors such as the hybridization affinity of the DNA target and the probe, the SIEP labeling efficiency, and the signal amplification. We have discussed the limitation on labeling efficiency which translates to the limited signal amplification. Next, we discuss the limitation imposed by the hybridization affinity of the immobilized probes and the hybridized targets. The hybridization affinity is often characterized by constant called dissociation equilibrium constant ($K_D$), which for a simple hybridization between two species, a probe immobilized on a surface and a free single-stranded target in solution, can be modeled as a Langmuir adsorption isotherm reaction (see Appendix A). Using a dose response we established for 25-mer DNA targets, we determined that
the $K_D$ for the hybridization of the target to probe is $\sim 1.8 \times 10^{-10}$ M, which is typical of surface-based reaction (Appendix A). Unfortunately, SIEP on-chip labeling is not able to generate high enough signal amplification at low target concentrations ($< 10$ pM or 10 fold below $K_D$) and therefore producing LOD that is comparable with a chemically synthesized target that is tagged with a single fluorophore. Ultimately, a lower LOD can be attained by reducing the non-specific signal at blank and increasing the specific signal at low target concentrations. Specific signals at low target concentrations can be improved by having higher hybridization affinity and increasing the number of target bound by introducing mixing or nanostructures for probe immobilization.

There is no generally accepted method to determine LOQ, therefore it is typically determined at some predefined level above bias and certain precision. The only guideline in the LOQ determination is that LOQ may be equivalent to LOD or it could be at a much higher concentration, however it cannot be lower than LOD. From the results we obtain by hybridizing DNA or RNA on microarray platform, either using SIEP on-chip labeling or chemically synthesized target; we determined the LOQ is at 10 pM (10 fold below the Kd), where we consistently see higher signal than blank and above the noise level of the assay for all target types (DNA, short RNA, long RNA). LOQ here is determined as the concentration at which the measured signal is equal to the blank signal plus 10σ of the blank sample.
7.3 Dynamic range

Dynamic range is defined as the range of analyte concentration that spans from the lower quantitation limit to the upper quantitation limit. Typically, dynamic range is a region within the calibration curve where the analyte concentration versus the response signal remains linear with correlation coefficient close to 1. We observe similar dynamic range for SIEP-TdT labeling and chemically tagged target as shown in Figure 7-1, where a linear dynamic range spans across 2-orders of magnitude (1 pM- 100 pM in the log-log plot) for DNA target hybridization. This 2-orders of magnitude is typical of fluorescence-based detection technique with a standard DNA microarray scanner. We believe that this dynamic range is influenced by the transduction method (fluorescence) and the instrumentation to measure fluorescence, therefore can be further extended by optimizing the scanner gain settings or using a scanner that can combine signals scanned at two different gain settings for different regimes of target concentration.

Interestingly, we observed 3-order of magnitude (1 pM-1 nM in the log-normal plot) dynamic range for 21-mer RNA target dose response curve, hybridized to immobilized PNA probe (Chapter 5). Although the signal transduction for both DNA and RNA hybridization is based on fluorescence detection, the increase in dynamic range for RNA hybridization could be attributed by the lower overall signal generated in using PNA probe in RNA hybridization SIEP assay than in using DNA probe in DNA
hybridization assay and the higher affinity for RNA-PNA interaction than DNA-DNA interaction.\textsuperscript{150}

### 7.4 Can SIEP detect clinically relevant samples?

We evaluate the ability of on-chip SIEP labeling to detect RNA target in clinically relevant sample by finding out the level of mRNA and miRNA in blood. We identified several mRNA targets, i.e. GAPDH, RSAD2, IFI44, PRSS21, which are relevant to the diagnosis of influenza and respiratory viral infections\textsuperscript{196} and several miRNA targets i.e. \textit{miR-15b}, \textit{miR-16}, \textit{miR-24} in blood\textsuperscript{197} as comparison to assess if SIEP on-chip labeling can be used to detect clinically relevant samples. We determined in-house the concentration of GAPDH, RSAD2, IFI44, and PRSS21 mRNAs in total human RNA from blood samples using quantitative PCR (qPCR) by establishing calibration curve using corresponding plasmid genes purchased from ATCC (data is courtesy of Mr. Marshall Nichols). The concentration of GAPDH, RSAD2, IFI44, and PRSS21 mRNAs in total human RNA (1 µg total RNA) averaged from 10 blood samples are ~10 pM, ~100 fM-10 pM, ~100 fM-1 pM, and ~10 fM, respectively. As for the presence of miRNA in blood, a study quantifying circulating miRNA in blood reveals that the amount of \textit{miR-15b}, \textit{miR-16}, and \textit{miR-24} are in the range of ~15 fM-0.2 pM.\textsuperscript{197}

Based on the assessment of SIEP LOD and LOQ, SIEP on-chip labeling at its best can be used to detect GAPDH, RSAD2, and IFI44 mRNAs without the amplification step but not PRSS21. However, as shown from the low level of circulating miRNA, SIEP on-
chip labeling is not sensitive enough to directly interrogate miRNA in blood and target amplification is required in the process.

Overall, by knowing the advantages and the limitations of on-chip labeling by SIEP, one can design and apply this assay without the additional target amplification step when the target nucleic acid is within the LOD and LOQ of SIEP. In addition, we believe the performance of SIEP is strongly related to the type of platform on which SIEP is performed. As briefly covered in Chapter 5, at low target concentration, most surface-based reaction is mass-transport limited and limited by the amount of probes immobilized on the surface. In order to overcome this physical limitation and to improve LOQ, active mixing during hybridization process or 3D hierarchical microsensors to enhance probe display can be employed.

7.5 Materials and method summary

7.5.1 Materials

All the oligonucleotide probes and targets used in this study were synthesized by Integrated DNA Technologies, Inc. TdT enzyme, TdT buffer, and dATP were purchased from Promega. Fluorescent nucleotides, Cy3-dATP was purchased from PerkinElmer, Inc. Tween 20 were purchased from Sigma-Aldrich (St Louis, MO). Succinimidyl 4-formylbenzoate (SFB) linker was purchased from Pierce (Rockford, IL). Sodium acetate and sodium citrate were purchased from EMD Chemicals, Inc.
Hydrazinonicotinamide modified glass slide (Hylink Glass slides) was purchased from Solulink (San Diego, CA).

7.5.2 Instrumentation

On-chip TdT labeling and DNA hybridization dose response was determined by scanning the glass slides for the Cy5 (635 nm) and Cy3 (532 nm) fluorescent signal using an Axon GenePix Pro 4200 scanner (Molecular Devices) at 10 µm resolution with optimized PMT and gain settings.

7.5.3 Methods

Comparison of on-chip labeling by TdT with Cy3-tagged DNA target hybridization.

The 25-mer target sequence [5’-GAG CTT CTG TGG AGT TAC TCT CTT T-3’ or 5’-GAG CTT CTG TGG AGT TAC TCT CTT T-Cy3-3’] was selected from the core protein of Hepatitis B Virus. The fully complementary probe [5’-AAA GAG AGT AAC TCC ACA GGA- (CH$_2$)$_6$- NH$_2$-3’] was spotted using a non-contact printer (Piezzorray, Perkin Elmer, Inc.) after modification with SFB to covalently attach the probe to the glass substrate functionalized with hydrazinonicotinamide (Hylink Glass slides). The slides spotted with the probes were then incubated overnight in a humidified chamber and rinsed in 1x SSC buffer with 0.1% Tween 20 followed by rinsing with filtered water and spun dry. A dose-response curve of hybridized target was generated by incubating the printed probes to a range of target DNA concentrations (1 pM-1 µM) for 4 h at 37 °C in 3x SSC buffer. For SIEP on-chip labeling, TdT was used to incorporate Cy3-dATP (0.1
U/μl TdT, 100 μM dATP and 0.5 μM Cy3-dATP in 1x TdT buffer) for 1 h at 37 °C. The slides were then rinsed in 1x SSC buffer with 0.1% Tween 20 for 30 min and scanned immediately on a GenePix scanner. The average signal intensity of the spot was then plotted as a function of the target DNA concentration.
8. Conclusions and Future Directions

8.1 Conclusions

We have developed a new on-chip labeling and signal amplification technique for the detection of nucleic acids hybridization on a microarray platform by exploiting a template independent DNA polymerase (TdT and PaP) ability to polymerize long DNA or RNA chains (thousands of bases) and incorporating unnatural reporter nucleotides such as fluorescent nucleotides. We term this methodology Surface Initiated Enzymatic Polymerization (SIEP) for the labeling and polymerization of DNA that is initiated from the surface bound nucleic acid targets.

We first characterized the ability of the TdT enzyme to polymerize long ssDNA chains and to incorporate fluorescent nucleotides in solution. We verified the multiple incorporation of fluorescent nucleotide by quantifying the number of fluorophores per extended chain. We found fluorescent incorporation up to ~50 fluorophores per chain and that increasing fluorescent nucleotides input in the enzymatic reaction resulted in increasing number of fluorophores being incorporated while at the same time decreasing the reaction efficiency.

After we verified the multiple fluorophores incorporation on the surface using SIEP and quantified the signal amplification (up to ~45 fold), we generated a dose response curve for the hybridization of DNA, miRNA, and mRNA target, which yielded LOD of ~1-10 pM depending on the size of the target nucleic acids. In addition, we
further demonstrated the versatility of SIEP assay by developing a colorimetric assay using SIEP, exploiting the electrostatic interaction between the negatively charged DNA backbones and positively charged gold nanoparticles.

SIEP as a method has several attractive attributes: (1) it is carried out on-chip isothermally; (2) it is able to directly detect DNA, miRNA, and mRNA from heterogenous mixture without the need for labeling step or additional detection probe; (3) multiple fluorescent labels are incorporated into surface initiated polymerization of DNA, resulting in signal amplification; and (4) it is versatile as the SIEP concept can be tailored for fluorescence and colorimetric amplification.

Although SIEP performance is comparable and in some instances, better than other DNA or RNA target labeling and signal amplification techniques, the LOD and signal amplification of SIEP are limited by labeling efficiency, especially at low target concentrations and the non-specific signals generated due to surface exposure to fluorescent nucleotides. Nevertheless, SIEP offers an alternative signal amplification that is straightforward and versatile in that, it can detect DNA, miRNA, and long mRNA transcripts through fluorescence or colorimetric detection using commercially available reagents and protocols that are compatible with current microarray technology.
8.2 Future Directions

There are several strategies, specifically in the use of SIEP for sensing and improvement in SIEP performance that has not been explored in this dissertation. The strategies suggested here are designed to further exploit TdT reaction, to explore other platforms on which SIEP is performed, and to further exploit the increase in the net negative charge of SIEP amplified DNA or RNA spots.

8.2.1 Explore TdT promiscuity

We have shown in detailed characterization studies that TdT is a promiscuous DNA polymerase with the ability to polymerize several unnatural nucleotides to a relatively significant extent. Therefore it would be interesting to utilize TdT in polymerizing other functional nucleotides that are available in the repertoire of unnatural nucleotides. Some examples of these unnatural nucleotides are ‘click’ nucleotides such as alkyne- or azide-dNTP, amine modified nucleotides such as N6-(6-amino)hexyl-dATP or 7-deaza-7-propagylamino-dATP, cyclooctyne modified dUTP (5-Dibenzylcyclooctyne-2’-deoxyuridine 5’-triphosphate) for copper-free click chemistry, and nucleotides labeled with redox dyes for electrochemical transduction. These unnatural nucleotides are commercially available from Jena Bioscience and by utilizing SIEP methodology of polymerizing long DNA chain while incorporating multiple of these unnatural nucleotides, further signal amplification studies using several suggested nucleotides should be pursued. In addition to pursuing the incorporation of unnatural
nucleotides for sensing applications, the use of TdT to build hybrid macromolecules through DNA polymerization would be interesting using these functional unnatural nucleotides.

In line with the theme to explore TdT promiscuity is to carry out site-directed mutation on TdT active center to generate mutant TdT that is tailored and screened for particular unnatural nucleotides. Using available literature on the active site of TdT enzyme and the sequence for recombinant TdT, as well as the screening method developed in Chapter 3, one can envision the development of mutant TdT that is specific for certain applications.

8.2.2 Exploit the formation of pyrophosphate for signal amplification

Another product of TdT reaction that has not been explored is the formation of pyrophosphate (PPi) by product as TdT forms long DNA chains. From the reaction stoichiometry (Figure 3-13), PPi is produced in the amount that is equivalent to the number of monomer (dNTP) being polymerized. Using a principle that is used in pyrosequencing which converts PPi to a bioluminescence signal through biochemical reaction of adenosine 5’ phosphosulfate (APS), ATP sulphurylase, luciferase, and luciferin, PPi by product from TdT reaction can be exploited for signal amplification. Briefly, ATP sulphurylase converts PPi to ATP in the presence of APS, which is then followed by luciferin conversion to oxyluciferin by luciferase as fueled by ATP. The formation of oxyluciferin generates visible light that is proportional to the amount of
ATP. A similar strategy of exploiting PPI as reporters has been pursued previously using the polymerizing capability of T7 DNA Polymerase on a signal amplification cassette to generate vast amounts of PPI. Nevertheless, this strategy is perfect for SIEP, particularly in the form of bead-based assay, where target bound to the beads can be used as the initiation site for TdT catalyzed polymerization, generating vast amounts of PPI that is proportional to the amount of hybridized target in a single step and thus bioluminescence signals for quantification. One caveat in this strategy is to avoid the use of dATP as the monomer for TdT catalyzed reaction because dATP also fuels luciferase catalysis and thus interferes with the assay specificity.

8.2.3 Applying SIEP to other platforms

Bead-based assay

The application of SIEP using microarray platform has demonstrated several limitations that are platform related. For example, the mass transport limited reaction of DNA hybridization on a two-dimensional surface, the low efficiency of SIEP reaction, and the non-specific adsorption of fluorescent-dNTP has kept the LOD of on chip SIEP labeling at a low pM range. Although the microarray platform allows high multiplexing and high throughput analysis, applying SIEP on other platforms that provide a better mass transport condition could possibly push the LOD lower and sensitivity higher than a two-dimensional microarray platform. Bead-based assay is an alternative platform that combines the specificity of a surface-based reaction and yet the three-dimensional nature
of the bead suspension system provides convenient and reliable way of mixing, which results in faster hybridization kinetics and possibly improves the mass transport of analyte to the immobilized probe as well as the efficiency of the SIEP reaction. In addition, bead-based assay utilizes hundreds to thousands of beads containing the probes that will capture the nucleic acid target, allowing higher probability of capturing rare target molecules in a low concentration mixture.

One example of a suitable bead assay platform was developed by Luminex Corporation, where low multiplexing (up to 500 different analyte) is achieved by using polymer beads that are doped with different amount of dyes (red and infrared fluorophores) as a tagging mechanism on the beads. In addition to multiplexing tag, to facilitate rinsing and removal of non-specific adsorption of fluorescent-dNTP, Luminex beads are also magnetic. Using magnetic bead, rinsing can be conveniently carried out using magnetic separator and can be accomplished within minutes. Furthermore, to achieve high sensitivity of fluorescence detection, a bead analyzer that functions like a flow cytometer, equipped with multiple lasers, photodiodes and photomultiplier, is used to detect the fluorescence intensity generated by the binding of target to individual beads, allowing accumulation of signals from multiple beads. Although it is arguable which platform (microarray or bead-based assay) is more sensitive for the target analyte detection (nucleic acid, protein) and there are challenges that are specific to the use of
bead based assay, development of SIEP on bead will expand the applicability of SIEP beyond the microarray platform.

**Electrochemical transduction**

The discussion on the non-specific fluorescence signal in Chapter 7 has demonstrated the disadvantages of using fluorescent-dNTP as a labeling scheme, particularly due to non-specific adsorption that generates non-specific signals. Therefore it would be interesting to explore other reporter and transduction mechanism such as electrochemical detection, which has been shown to be an effective transducer for DNA hybridization assay.\(^{203}\) The application of SIEP with the electrochemical transduction is appropriate because electrochemical transduction is a surface sensitive phenomenon. By using redox active nucleotides as discussed in an earlier section or cationic redox markers, SIEP will generate electrochemical signal amplification on target hybridized to immobilized probe on a gold electrode. However, using electrochemical transduction does not change the sensing platform, whereby the capture probe is immobilized on a flat gold electrode and thus detection of target nucleic acid at low concentrations is still limited by diffusion of the target to the probe.

**8.2.4 Explore other positively charged reporter to improve sensitivity**

Chapter 6 in this dissertation has demonstrated the increase in the net negative charge on the surface of SIEP amplified DNA spots. The generality in the electrostatic interaction between the negatively phosphate backbone and any positively charged
reporters should be explored further. Although this strategy requires an additional step of exposing SIEP amplified spots to positively charged reporters, we can increase the efficiency of SIEP labeling on the surface by simply polymerizing TdT natural substrate, dATP or dTTP. Some candidates for positively charged reporters are the positively charged fluorescent dyes (SYTO dyes, e.g. SYTO 61),\textsuperscript{204} cationic redox marker,\textsuperscript{205} and other optically active nanoparticles such as positively charged quantum dots. One consideration that is critical for the success of nanoparticle labeling on SIEP extended ssDNA is that the size of the nanoparticles should be ≤ 10 nm.
Appendix A

*Binding constant determination for nucleic acid hybridization on the microarray platform using Langmuir adsorption isotherm model*

We determine the equilibrium binding constant (expressed as the dissociation constant, $K_D$) of DNA target hybridization to the immobilized probes using the Langmuir adsorption isotherm model. This model is valid for our experimental set-up for the following reasons:\textsuperscript{192} (1) we are measuring the hybridization reaction at equilibrium for different target concentrations; (2) the spot of immobilized probes only carry one type of probe and it is monodisperse in size and sequence; (3) the probes are exposed to a single, unique target where hybridization solutions contain only one type of sequence; (4) the target comprises of non-complementary ssDNA and no self-hybridized, hairpins, or loops; (5) each target hybridizes with a single probe so that the target length and sequence as well as the separations between probes prevent any possibility of a single target hybridizing with two separate probes; and (6) there is a minimum interaction between the probes.

We model the hybridization process following the hybridization stoichiometry shown below:

$$[P] + [T] \leftrightarrow [P.T]$$  \hspace{1cm} (1)

where $[P]$ represents the probe, $[T]$ represents the target, and $[P.T]$ represents the hybrid of the probe and the target.
According to the stoichiometry shown in equation (1), the binding/hybridization constant is expressed as:

$$K_A = \frac{k_{on}}{k_{off}} = \frac{[P.T]}{[P].[T]}$$  \hspace{1cm} (2)

When the amount of probe $[P]$ at a given time is expressed as $[P]_0-[P.T]$ and the equilibrium fraction of probes that forms hybrid as $X$, where $X = \frac{[P.T]}{[P]_0}$, equation 2 can be expressed as:

$$[T]K_A = \frac{[P.T]}{[P]_0-[P.T]} = \frac{X}{1-X}$$  \hspace{1cm} (3)

In the binding event where $K_A$ is independent of $X$ and the hybridization on the small spot of probe on the surface does not affect $[T]$, $X$ should be proportional to the intensity of the spot ($I$), and is expressed as:

$$X = \frac{I}{I_{max}}$$  \hspace{1cm} (4)

Rearranging equation (3) and (4), the relationship between intensity ($I$) and target concentration $[T]$ can be obtained as:

$$I = \frac{I_{max}[T]K_A}{1+[T]K_A}$$  \hspace{1cm} (5)

Using equation (5) and the sigmoidal fit of the dose response curve we established using fluorescence SIEP assay, $K_D$ can be determined when $I/I_{max}$ is equal to $\frac{1}{2}$ and $[T]$ is equal to $1/K_A$ or $K_D$, as shown in Figure A1.
Figure A1: Equilibrium dose response curve for DNA hybridization measured by fluorescence SIEP assay

Using the Langmuir adsorption isotherm model, K₀ is the target concentration at the half of the maximum intensity of the dose response curve as established by the on-chip labeling using fluorescence SIEP assay. The dose response curve is fitted using the growth/sigmoidal dose response fit with Origin 9.0 and K₀ is determined as the calculated EC50 from the curve fit.
References


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Biography

Vinalia Tjong was born on December 26, 1979 in Lubuk Linggau, a small town in South Sumatra, Indonesia. There she spent her childhood and teenage year until she was 15 at which time she left home for Bandung in neighboring Java, where she spent her high school years studying science at St. Angela High. She then left Indonesia for Singapore in 1998 to pursue an undergraduate study in Materials Engineering at the Nanyang Technological University with a tuition grant from the Singapore government. After completing her undergraduate study with honors in 2002, she was given a fellowship from the Singapore-MIT Alliance to pursue a master in molecular and chemical engineering at the National University of Singapore. The sponsorship from Singapore government for her undergraduate studies obliged her to remain in Singapore, where she worked for the Institute of Materials Research and Engineering, a member of Agency of Science, Technology and Research (A-Star) from 2003 to 2007. After nine years, she left Singapore for the United States to pursue her doctorate degree in Biomedical Engineering at Duke University. At Duke, her research proposal has helped to secure a 3-year NSF grant (CBET 1033621) and she has participated in a DARPA funded project. To date, she has published a total of 8 peer-reviewed papers and one book chapter.