Clickable PNA Probes for Imaging Human Telomeres and Poly(A) RNAs

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Supporting Information

ABSTRACT: The ability to bind strongly to complementary nucleic acid sequences, invade complex nucleic acid structures, and resist degradation by cellular enzymes has made peptide nucleic acid (PNA) oligomers as very useful hybridization probes in molecular diagnosis. For such applications, the PNA oligomers have to be labeled with appropriate reporters as they lack intrinsic labels that can be used in biophysical assays. Although solid-phase synthesis is commonly used to attach reporters onto PNA, development of milder and modular labeling methods will provide access to PNA oligomers labeled with a wider range of biophysical tags. Here, we describe the establishment of a postsynthetic modification strategy based on bioorthogonal chemical reactions in functionalizing PNA oligomers in solution with a variety of tags. A toolbox composed of alkyne- and azide-modified monomers were site-specifically incorporated into PNA oligomers and postsynthetically click-functionalized with various tags, ranging from sugar, amino acid, biotin, to fluorophores, by using copper(I)-catalyzed azide−alkyne cycloaddition, strain-promoted azide−alkyne cycloaddition, and Staudinger ligation reactions. As a proof of utility of this method, fluorescent PNA hybridization probes were developed and used in imaging human telomeres in chromosomes and poly(A) RNAs in cells. Taken together, this simple approach of generating a wide range of functional PNA oligomers will expand the use of PNA in molecular diagnosis.

INTRODUCTION

The potential of peptide nucleic acid (PNA) in gene therapy has long been contemplated because of its resistance to cellular hydrolytic enzymes and ability to bind strongly to complementary DNA and RNA.1-6 In addition, the tendency to stabilize triple helix7,8 and invade complex secondary structures9-11 has rendered PNA oligomers as very useful hybridization probes in molecular diagnosis. Needless to say, such applications greatly rely on PNA oligomers labeled with an appropriate reporter like a fluorophore, affinity tag, or magnetic resonance imaging agent.12-16 In particular, PNAs conjugated to fluorescent reporters have been frequently utilized in various hybridization assays for the detection, quantification, and visualization of specific nucleic acid sequences in cell-free and native cellular conditions.17-25

Probe-labeled PNA oligomers are commonly synthesized by using a procedure analogous to the solid-phase peptide synthesis protocol. Typically, native nucleobases attached to N-(2-aminooethyl)glycine (aeg) or a similar backbone are sequentially added from the C terminus (3'-end) to N terminus (5'-end) on the solid support by using tert-butylxycarbonyl (Boc) or fluorenylmethyloxycarbonyl chloride (Fmoc) chemistry.1,2,6 The desired label is then introduced at the N terminus by performing an acid-amine coupling reaction on the solid support before the final cleavage cum global deprotection step.2,3,27 This approach has been commonly used in preparing fluorescently modified PNA probes for imaging specific nucleic acid sequences in cells. Alternatively, base-modified monomers are incorporated into PNA oligomers at the desired position during solid-phase synthesis.28-33 However, this on-column labeling method has certain shortcomings. The tendency of many activated esters to undergo hydrolysis reduces the coupling efficiency, and certain modifications do not survive strong acid conditions used in the cleavage step (e.g., trifluoroacetic acid (TFA)/trifluoromethanesulfonic acid). Similar problems have also been encountered in both solid-phase peptide and oligonucleotide (ON) labeling methods. To circumvent these drawbacks, postsynthetic modification using chemoselective reactions has emerged as a valuable route to label glycans, proteins, and nucleic acids.34-44

In this approach, a small reactive handle, which is compatible with solid-phase or enzymatic labeling methods, is introduced into the oligomer and the desired label is attached by performing a chemoselective reaction between the reactive handle on the oligomer and the label containing the cognate reactive partner. This so-called “click chemistry” can be performed on the solid support as well as on the free oligomer in solution.45

Received: September 27, 2018
Accepted: October 31, 2018
Published: November 12, 2018

Supporting Information
Several chemoselective reactions, including copper(I)-catalyzed azide−alkyne cycloaddition (CuAAC), strain-promoted azide−alkyne cycloaddition (SPAAC), thiol−ene coupling, azide−phosphine Staudinger ligation, inverse electron demand Diels−Alder, and palladium-catalyzed reactions have been described for labeling biomolecules. In particular, azide−alkyne cycloaddition reaction has gained prominence as it is fast, highly chemoselective, and bioorthogonal, which has enabled its use in cell-free as well as in cellular systems. Initially, this bioconjugation method was performed on a solid support to prepare labeled PNA oligomers. Subsequently, solution-phase click labeling was conceived, wherein PNAs conjugated to cell-penetrating peptides, mesoporous silica nanoparticles, cross-linking agents, and fluorophores were prepared. However, given the usefulness of PNA in molecular diagnosis, establishment of chemical labeling strategies, which will provide direct access to a repertoire of PNA probes in a modular fashion, is constantly needed.

Here, we describe the synthesis and site-specific incorporation of a clickable toolbox composed of alkyne- and azide-modified uracil PNA base analogs and azidoacetic acid as an azide surrogate. The alkyne- and azide-modified PNA oligomers were efficiently clicked with a variety of tags, ranging from sugar, amino acid, biotin, to fluorophores, by CuAAC reaction. The ability of azide group to participate in different chemoselective reactions was further exploited in labeling PNA with biophysical probes in a modular fashion by using SPAAC and Staudinger ligation reactions. Using this labeling method, we designed fluorescent PNA hybridization probes to specifically visualize human telomeres in chromosomes and poly(A) tail-containing RNAs in cells. Further, the compatibility of clickable PNA oligomers to posthybridization labeling adds an advantage to this method as oligomers containing large reporters could potentially hamper the hybridization efficiency.

**RESULTS AND DISCUSSION**

**Synthesis of Alkyne- and Azide-Modified Uracil PNA Base Analogs 7 and 14.** The clickable uracil PNA base analogs containing the original aeg backbone were synthesized according to the steps illustrated in Schemes 1 and 2. In the monomer design, we have intentionally tethered the clickable component to the nucleobase via an alkyl or triethylene glycol linker so as to enhance the efficiency of the click reaction when incorporated into PNA oligomers. Further, the PNA analogs 7 and 14 contain a nonclickable internal alkyne, which can be used as a Raman-scattering label. The alkyne group displays a characteristic strong signal, which falls in the Raman-silent region of the cell. This unique bioorthogonal Raman label...
has recently been used in visualizing biomolecules, including nucleic acids and proteins, by the stimulated Raman-scattering imaging technique.\(^5\)\(^9\)\(^6\)\(^1\) Hence, these nucleobase analogs incorporated into PNA hybridization probes could potentially facilitate two-channel visualization of a specific nucleic acid sequence in cells by employing click chemistry (terminal alkyne or azide) and Raman spectroscopy (internal alkyne).

S-Iodouracil ethyl ester \(1^\text{62}\) was reacted with octa-1,7-diyne 2 under Sonogashira coupling reaction conditions to give octadiyne-conjugated uracil (ODU) ethyl ester 3 (Scheme 1). The ester group was hydrolyzed in the presence of LiOH and coupled with a commercially available Fmoc aeg−PNA backbone 5 to give compound 6. Further treatment with TFA gave the ODU-modified PNA monomer 7 required for the solid-phase PNA synthesis.

S-Iodouracil ethyl ester 1 was Boc-protected (8) at the N3 position.\(^5\)\(^2\) O-Tosylated tetraethylene glycol containing a terminal alkyne group (9) was prepared in two steps following a reported procedure.\(^5\)\(^3\) Compound 8 was coupled with 9 under Sonogashira cross-coupling reaction conditions, which was then converted into azide-modified uracil ester 11 by treating with NaN\(_3\) (Scheme 2). The ester was hydrolyzed and coupled with Fmoc aeg−PNA backbone 5, and then the tertiary butyl group was removed using TFA to afford the azide-modified uracil PNA building block 14.

**Post-synthetic Modification of Alkyne- and Azide-Labeled PNA Oligomers.** The suitability of modified PNA base analogs for post-synthetic chemical labeling was evaluated by using model PNA oligomers containing OD−uracil (16), azido−uracil (17), and azido−acetamide (18, Figure 1). These oligomers were prepared using lysine-loaded Rink amide resin and Fmoc-protected aeg−PNA monomers. PNA oligomers were synthesized with two C-terminal lysine residues to enhance the aqueous solubility. The oligomers were further purified by reversed phase high performance liquid chromatography (RP-HPLC) and characterized by mass analysis (Figure S1 and Table S1). The modified PNAs were subjected to CuAAC, SPAAC, and Staudinger ligation reactions in the presence of a variety of substrates containing cognate alkyne, azide, and phosphine reactive partners (a−i, Figure 2). The substrates included different fluorophores, sugar, amino acid, and affinity tags.

CuAAC reaction was performed by incubating alkyne-modified PNA 16 and azide-modified PNA 17 and 18 with azide substrates a−d and alkyne substrates e−g, respectively (Figures 2, 3, and 4). Reactions were carried out in the presence of a water-soluble Cu(I) stabilizing ligand, tris(3-hydroxypropyltriazolylmethyl)amine (THPTA), CuSO\(_4\) and a reducing agent, sodium ascorbate, at 37 °C for 2 h. The reaction mixture was resolved by RP-HPLC, and the clicked product was isolated and characterized by matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass analysis (Figures S2, S3, and Table S2). In a reaction with different azide substrates (a−d), the alkyne-modified PNA 16 was completely consumed within 2 h and respective clicked products were isolated in respectable yields (Table S2). Typically, a reaction performed at 15 nmol scale of PNA 16 gave 5.8−7.8 nmol of the product (16a−16d). CuAAC reaction between azide-modified PNA 17 and 18 with alkyne substrates e−g proceeded to completion, and the products were isolated in moderate yields (Figure 4 and Table S2). The emission spectrum of naphthalimide-labeled PNA products (16a, 17e and 18e) also confirmed the fluorescence labeling by click reaction (Figure S4). Next, PNAs 17 and 18 were subjected to SPAAC reaction with biotin−cyclodextrin (h). The reaction completed in 1 h, and the clicked products 17h and 18h were recovered in good yields (Figure 5 and Table S3). Although Staudinger ligation between azido−uracil-modified PNA 17 and biotinylated triarylphosphine (i) was poor, reaction with azido−acetamide-modified PNA 18 gave good yields of the ligated product 18i (Table S3). Collectively, these results underscore the potential of the click-labeling approach in synthesizing various functionalized PNA oligomers in amounts sufficient for subsequent biophysical analysis.

**Clicked-Labeled PNA Hybridization Probes Allow Visualization of Specific Nucleic Acid Sequences.**

**Imaging Human Telomeric DNA Repeat Sequence.** To illustrate the utility of this post-synthetic labeling method in visualizing nucleic acids, we first chose to target the human telomerases, which are present at the ends of chromosomes and protect them from end-to-end fusion and degradation.\(^6\)\(^4\)\(^6\)\(^5\) The human telomeres are composed of thousands of tandem hexamer nucleotide repeat (TTAGGG)\(_n\) which terminate into 100−200 nucleotide long 3’ single-stranded overhang.\(^6\)\(^6\) Abnormal shortening of telomeric repeats and maintenance of telomere length by the telomerase activity can lead to genomic instability and tumor progression.\(^6\)\(^7\)\(^−\)\(^6\)\(^9\) Telomeric DNA repeat overhang has been shown to form G-quadruplex structures in vitro and in cellular environment.\(^6\)\(^8\)\(^−\)\(^6\)\(^9\) Recently, we developed a graphene oxide-based platform to detect telomeric DNA repeat in vitro by using the ability of a complementary PNA probe to invade G-quadruplex structure and form a stable duplex.\(^6\) Prompted by this key observation, we sought to use the post-synthetic labeling approach to develop a fluorescently labeled PNA hybridization probe to visualize telomeres on the chromosome ends.

An 18-mer azide-labeled PNA probe 19 complementary to the human telomeric DNA repeat sequence (TTAGGG)\(_n\) was synthesized (Figure 1 and Table S1). When subjected to CuAAC, SPAAC, and Staudinger ligation reactions with fluorescent and biotin substrates, we could isolate the clicked products in reasonable amounts (Figure 2 and Table S4). Although biotin-labeled PNA probes (19h and 19i) could be used for immunofluorescence staining, Alexa594-labeled PNA probe (19j) can be directly visualized post hybridization with telomeric DNA.

DLD-1 cells in culture were treated with colcemid to arrest the cell cycle in the metaphase stage.\(^6\) The cell suspension was...
incubated in a hypotonic solution (75 mM KCl), and the pelleted cells were resuspended in a fixative solution. The cells were then dropped on a glass slide to prepare the metaphase chromosome spreads. Chromosomes were hybridized with freshly click-labeled Alexa PNA probe 19j and counterstained with 4′,6-diamidino-2-phenylindole (DAPI). Images of the spread clearly indicated four fluorescent spots (red) consistent with the binding of the PNA probe to the telomeres present at the ends of chromosomes (Figure 6A). A commercially available FITC-labeled PNA probe prepared by acid–amine coupling also showed similar telomere staining pattern (green, Figure 6B). Notably, both the probes stained all chromosomes in a given spread, highlighting the comparable efficiency of the staining process by click-labeled and commercial PNA probes.

Imaging Poly(A) RNAs.
With the success of imaging telomeric DNA, we expanded the utility of click PNA labeling by CuAAC, SPAAC, and Staudinger ligation reactions, respectively. See the Supporting Information for synthesis of substrates and commercial source.

Figure 2. Azide-, alkyne-, and triarylphosphine-modified substrates (a–l) used in the postsynthetic PNA labeling by CuAAC, SPAAC, and Staudinger ligation reactions, respectively. See the Supporting Information for synthesis of substrates and commercial source.

Figure 3. Postsynthetic chemical functionalization of alkyne-modified PNA oligomer 16 with azide substrates a–d by using the CuAAC reaction.
approach by developing a fluorescent poly(T) PNA probe to visualize cellular poly(A) RNAs. Poly(A) tailing is an important mRNA maturation process, which provides stability and promotes translation of mRNA. Poly(A) tail length shortening causes mRNA splicing and initiates degradation process. Fluorescent poly(T) ON probes are commonly used to track and visualize poly(A) RNAs inside cells, although the stability and hybridization efficiency of ON probes in a cellular environment is a concern. Since PNA oligomers are resistant to nucleases and proteases and they bind more...
strongly to RNA than the complementary DNA ON, we synthesized a 12-mer fluorescent poly(T) PNA probe by click-reacting alkyne-modified PNA with Alexa594 azide (Figure 2 and Table S4).

DLD-1 cells in culture were fixed, permeabilized, and hybridized with PNA in a hybridization buffer at 37 °C for 2.5 h. Cells were washed and then DAPI-stained and imaged using a confocal microscope. The images revealed a punctate nuclear staining and uniform cytoplasmic staining in the Alexa594 channel (red, Figure 7A). As a positive control, cells were incubated with Cy5-(dT)30, a commonly used DNA ON probe for poly(A) RNA imaging. The DNA probe produced a staining pattern similar to that of the PNA probe (Figure 7B). The binding of PNA–DNA probes to poly(A) RNAs was further confirmed by RNase A treatment. Cells treated with RNase A and then hybridized with the probes revealed no detectable fluorescence signal from the cells (Figure 7).

**Posthybridization Click Labeling of PNA–DNA ON Duplex.** Although PNA binds to its target ON strongly, certain bulky modifications could potentially affect its binding efficiency. Hence, we attempted to perform click reactions on PNA–DNA heteroduplexes. Azide-modified (19) and alkyne-modified (21) PNAs were hybridized with a complementary telomeric DNA ON repeat. Azide-labeled duplex 19·23 reacted quite efficiently with Alexa azide substrate k (lane 6). A control reaction between unmodified PNA–DNA duplex 22·23 and Alexa azide k did not produce any fluorescent click product, substantiating the high chemoselectivity of this labeling method. It is important to mention here that the observed mobility of click-labeled duplexes relative to the substrate...
duplexes is due to a combined effect of increased molecular weight and overall charge on the products.

**CONCLUSIONS**

We have established a postsynthetic chemical functionalization method to generate PNA probes by using bioorthogonal reactions like azide–alkyne cycloaddition and Staudinger ligation reactions. The clickable PNA oligomers, generated by incorporating azide- and alkyne-modified PNA base analogs, were used in installing a repertoire of biophysical tags in a modular fashion by click reactions. This labeling method was further utilized in synthesizing fluorescent PNA hybridization probes complementary to the human telomeric DNA ON repeat and poly(A) repeat, which enabled the specific visualization of human telomeres in chromosomes and poly(A) tail-containing RNAs in cells. Collectively, our results demonstrate that this PNA bioconjugation approach is modular and will complement existing methods by providing access to PNA probes for various applications.

**METHODS**

**Postsynthetic Modification of Azide- and Alkyne-Modified PNA Oligomers by CuAAC Reaction.** Catalyst mix was prepared by mixing a solution of THPTA (4.2 μL, 90 mM), CuSO₄ (4.2 μL, 45 mM), and sodium ascorbate (4.2 μL, 90 mM) in water. This mixture was added to an aqueous solution of alkyne- or azide-modified PNA oligomer (25 μL, 0.6 mM). Stock solutions (7.5 mM) of azide (a–d) and alkyne (e–g) substrates were prepared in dimethyl sulfoxide (DMSO). Azide or alkyne substrate (10 μL, 7.5 mM) was added to the reaction mixture, and the volume was adjusted to 50 μL by adding water. The final concentration of reaction components was the following: THPTA (7.50 mM), CuSO₄ (3.75 mM), sodium ascorbate (7.50 mM), PNA oligomer (0.30 mM, 15 nmol), azide or alkyne substrate (1.5 mM), and DMSO (20%). The reaction mixture was incubated at 37 °C for 2 h on a thermomixer (500 rpm). The clicked product was purified by RP-HPLC (Figure S2). The peak corresponding to the clicked product was isolated and characterized by MALDI-TOF mass analysis. For the structure of clicked products, see Figures 3 and 4, and for yield and mass data, see Table S2.

**Postsynthetic Modification of Azide-Modified PNA Oligomers by SPAAC Reaction with Biotin—Cyclooctyne.** A solution of azide-modified PNA oligomer 17/18 in water (12.5 μL, 0.6 mM) was mixed with cyclooctyne substrate h (2.25 μL, 10 mM) dissolved in DMSO. DMSO (2.27 μL) was added to the above reaction mixture, and the total volume was adjusted to 25 μL by adding water. The final concentration of PNA oligomers was 0.3 mM (7.5 nmol), cyclooctyne substrate h was 0.9 mM, and DMSO was 20%. The reaction mixture was incubated at 37 °C for 1 h, and the clicked product was purified by RP-HPLC. The peak corresponding to the product was isolated and characterized by MALDI-TOF mass analysis. For the structure of clicked products, see Figure S5, and for yield and mass data, see Table S3.

**Postsynthetic Modification of Azide-Modified PNA Oligomers by Staudinger Ligation Reaction with Biotinylated Triarylphosphine Substrate i.** A solution of azide-modified PNA oligomer 17/18 in water (6.0 μL, 1.0 mM) was mixed with 50 mM phosphate-buffered saline (PBS) buffer (10 μL, pH 8.0). Biotinylated phosphine substrate i (1.2 μL, 50 mM) in DMSO was added to the above solution and mixed well. DMSO (8.8 μL) was added to the above reaction mixture, and the volume was adjusted to 50 μL by adding water. The final concentration of reaction components was the following: PNA (0.12 mM, 6 nmol), i (1.2 mM), and DMSO (20%). The reaction mixture was incubated at 37 °C for 12 h, and the ligated product was purified by RP-HPLC. The ligated product was isolated and characterized by MALDI-TOF mass analysis. For the structure of the ligated products, see Figure S5, and for yield and mass data, see Table S3.

**Imaging Telomeric DNA Repeats.** Metaphase Chromosome Spreads. DLD-1 (Human colon cancer cells ATCC CCL-221) cells were cultured in RPMI1640 medium (Gibco by Life Technologies, 61870-036) supplemented with 10% fetal bovine serum (Gibco by Life Technologies, 10437028) and penicillin–streptomycin (Gibco by Life Technologies, 15070-063) under humidified atmosphere at 37 °C with 5% CO₂. Cells were seeded in a 100 mm culture dish and allowed to grow till 70% confluency, and then cells were further treated with Colcemid (Roche 10295892001) 0.1 μg/mL for 90 min in an incubator. Subsequently, cells were trypsinized to form a single-cell suspension and incubated in a hypotonic solution (75 mM KCl). Cells were then pelleted by centrifugation and resuspended in fixative solution (methanol/acetic acid = 3:1). Metaphase spreads were prepared by dropping the cell suspension (10 μL) in fixative on a glass slide under moist condition. Glass slides with spreads were then air-dried and stored at room temperature until use.

Hybridization and Imaging. Slides containing metaphase spreads were heated at 65 °C for 10 min and immersed in 1× PBS for 15 min. Subsequently, metaphase spreads were fixed using 4% formaldehyde in 1× PBS (50 mM MgCl₂) for 15 min. Slides were then washed with 1× PBS for 3 min twice and treated with 3 μg/μL RNase A for 3 h in a moist condition at 37 °C. After RNase treatment, slides were washed with 2× saline-sodium citrate (SSC) buffer, autoclave water, and then immersed in 0.005% pepsin solution prepared in 10 mM HCl (pH 2.0) for 5 min at 37 °C. Next, the slides were washed with 1× PBS and dipped in fixative (formaldehyde) solution for 10 min. Slides were then washed with 1× PBS 5 min twice and allowed to dehydrate in the presence of chilled ethanol series (70, 90, and 100%) for 5 min. Above slides were air-dried and subjected to hybridization with Alexa594-labeled PNA probe 19 or FITC-labeled Pangene PNA probe (300 nM) in the hybridization buffer [70% formamide, 20 mM Tris (pH 7.4), 20 mM Na₂HPO₄ (pH 7.4), and 0.1 μg/mL salmon sperm DNA in 2× SSC buffer] at 85 °C for 6 min. The above slides were then incubated for 12 h at 37 °C in a moist condition.

After hybridization, slides were washed with wash buffer I (70% formamide and 10 mM Tris pH 7.2) twice for 15 min and then with wash buffer II (70% formamide, 50 mM Tris pH 7.4, 150 mM NaCl, and 0.05% Tween20) twice for 10 min. Slides were then dehydrated with ethanol series as mentioned earlier and air-dried. Metaphase spreads were counterstained with DAPI in 2× SSC for 3 min and washed with 0.1× SSC. Further, 7 μL of antifade mounting media was added to the spreads on the slide, covered with coverslips, and sealed with nail polish. Cells were imaged using Zeiss Axio Imager Z1 with oil immersion using 100x lens.

**Imaging Cellular Poly(A) RNAs.** DLD-1 cells were cultured in RPMI1640 medium (Gibco by Life Technologies, 61870-036) supplemented with 10% fetal bovine serum.
Hybridization buffer (150 mM KH2PO4, 2.7 mM KCl, 137 mM NaCl, 500 μM vanadylribonucleoside complex (VRC), pH 7.4) prepared in 2 μM and fixed in 500 μM of 4% paraformaldehyde containing 500 μM VRC for 15 min. Subsequently, cells were permeabilized with 95% chilled methanol for 5 min. Cells were then washed with 500 μL of 2X SSC buffer (0.3 M sodium citrate, 0.03 M sodium chloride, 500 μM VRC, pH 7.4). The coverslip was placed upside down on a Parafilm strip containing 50 μL of hybridization buffer (10% (w/v) dextran sulfate, 40% (v/v) formalde, 30 ng/μL salmon sperm DNA, and 500 μM VRC prepared in 2X SSC) and incubated for 30 min at 37 °C. Cells were washed with 500 μL of 2X SSC buffer and hybridized with the fluorescent PNA probe 20k (1.0 μM) in hybridization buffer for 2.5 h at 37 °C. In a positive control experiment, cells were hybridized with 0.5 μM Cy5-(dT)30 under similar conditions. Cells were then washed with 500 μL of 2X SSC and 500 μL of 0.1X SSC buffer. Cells were counterstained with 500 μL of DAPI (55 nM in 2X SSC) for 3 min and washed with 500 μL of 0.1X SSC. Coverslips were then placed on a microscope slide with 7 μL of antifade mounting media and sealed with nail polish. Cells were imaged using a confocal laser scanning microscope with oil immersion using 40X lens. Images were acquired by using the following excitation and emission settings. DAPI (λex = 405 nm and λem = 420-480 nm, blue channel); Alexa594-modified PNA probe (λex = 561 nm and λem = 570-680 nm, red channel); and Cy5-(dT)30 probe (λex = 633 nm and λem = 640-760 nm, red channel). ImageJ software was used to process the images.

**RNase A Treatment.** Cells were incubated in 500 μL of RNase A (0.5 μg/mL in 1X PBS) solution for 1 h at 37 °C. Cells were washed with 500 μL of 2X SSC and then hybridized with 0.5 μM of Cy5-(dT)30 or 1.0 μM of labeled PNA probe 20k, as mentioned above.

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