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A Highly Sensitive Target-Primed Rolling Circle Amplification (TPRCA) Method for Fluorescent *in Situ* Hybridization Detection of MicroRNA in Tumor Cells

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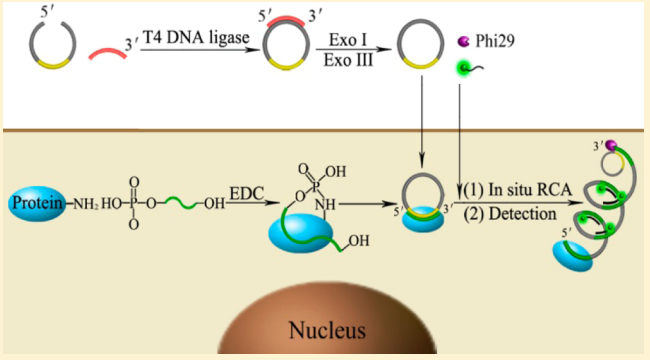
Supporting Information

ABSTRACT: The ability to detect spatial and temporal microRNA (miRNA) distribution at the single-cell level is essential for understanding the biological roles of miRNAs and miRNA-associated gene regulatory networks. We report for the first time the development of a target-primed RCA (TPRCA) strategy for highly sensitive and selective *in situ* visualization of miRNA expression patterns at the single-cell level. This strategy uses a circular DNA as the probe for *in situ* hybridization (ISH) with the target miRNA molecules, and the free 3′ terminus of miRNA then initiates an *in situ* RCA reaction to generate a long tandem repeated sequence with thousands of complementary segments. After hybridization with fluorescent detection probes, target miRNA molecules can be visualized with ultrahigh sensitivity. Because the RCA reaction can only be initiated by the free 3′ end of target miRNA, the developed strategy offers the advantage over existing ISH methods in eliminating the interference from precursor miRNA or mRNA. This strategy is demonstrated to show high sensitivity and selectivity for the detection of miR-222 expression levels in human hepatoma SMMC-7721 cells and hepatocyte L02 cells. Moreover, the developed TPRCA-based ISH strategy is successfully applied to multiplexed detection using two-color fluorescent probes for two miRNAs that are differentially expressed in the two cell lines. The results reveal that the developed strategy may have great potential for *in situ* miRNA expression analysis for basic research and clinical diagnostics.



MicroRNAs (miRNAs) are a class of short (approximately 22 nucleotides) endogenously expressed noncoding RNAs that act as important post-transcriptional regulators of gene expression by mediating mRNA cleavage or translational repression.[1] The miRNA precursors are transcribed from chromosomes and processed by an RNase III enzyme, Drosha, to yield 60−70-nt-long stem-loop structured pre-miRNAs. Pre-miRNAs are exported from the nucleus by Exportin-5 into the cytoplasm, where the pre-miRNAs are further processed by a Dicer-containing complex to form mature miRNAs. The mature miRNAs are loaded in the RNA-induced silencing complex (RISC), which can effect either transcriptional/translational repression or target breakdown through complementary sites with target mRNAs (mRNAs).[2−4] Recently, increasing evidence has revealed that miRNAs play crucial roles in many biological processes and, in particular, are recognized as potential diagnostic biomarkers as well as targets for drug discovery in cancers.[5] This has fueled the need to develop highly sensitive and selective detection methods for miRNAs. Currently, widely used miRNA analysis methods, including the real-time reverse transcription polymerase chain reaction,[6] Northern blotting,[7] and miRNA array technology,[8] could partly meet the detection requirement. Recent years have also witnessed an increasing interest in the developing of different isothermal amplification techniques for highly sensitive miRNA detection.[9−12] Despite the success of these methods for *in vitro* assays of miRNAs, there have been only a few methods that are capable of *in situ* detection of miRNAs.

Considering the complex stochastic nature of gene expression in mammalian cells, detection of the average miRNA expression in cell populations could lead to the loss of important information connecting miRNA expression with cell function.[13] Therefore, there is an increasing need for the methods that can achieve the expression of given miRNAs at single-cell resolution. Typically, *in situ* visualization of gene expression in cells has been performed using the fluorescence *in situ* hybridization (FISH) method. With the introduction of locked nucleic acid (LNA) oligonucleotides as hybridization probes, miRNA-FISH has become a powerful technique for imaging the spatial localization of miRNA at the tissue, cellular, and even subcellular level.[14−18] However, because of the absence of signal amplification, the FISH method usually shows

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Table 1. Sequences of the Oligonucleotides Used in This Work[a]

miR-222	5′-AGCUACAUCUGGCUACUGGGUCUC-3′
padlock probe **1**	5′ phosphate-**TGA CGT AGG CAA GAT** AGA GGA GAC CCA GTA GCC AGA TGT AGC TTC GTA GGA CTT AAA *GGT AGT TGG AGC TGT*-3′
ligation probe **2**	5′-**TCT TGC CTA CGT CAA** *CAG CTC CAA CTA CC*-3′
detection probe **3**	5′ FAM-GAG ACC CAG TAG CCA GAT GTA GCT-3′
padlock probe **4**	5′ phosphate-**TGA CGT AGG CAA GAT** AGA GTC GTG ATG GAC TCC GGT GAC TCG TAG GAC TTA AA*G GTA GTT GGA GCT GT*-3′
detection probe **5**	5′ FAM-ACT CGT AGG ACT TAA AGG-3′
scrambled padlock probe **6**	5′ phosphate-**TGA CGT AGG CAA GAT** AGA GAC AGG AGG TAG CTC GCA TAG CAT CTC GTA GGA CTT AAA *GGT AGT TGG AGC TGT*-3′
detection probe **7**	5′ FAM-ACA GGA GGT AGC TCG CAT AGC ATC-3′
miR-223	5′-UGUCAGUUUGUCAAAUACCCCA-3′
padlock probe **8**	5′ phosphate-**TGA CGT AGG CAA GAT** AGA GTG GGG TAT TTG ACA AAC TGA CAT CGT AGG ACT TAA A*GG TAG TTG GAG CTG T*-3′
detection probe **9**	5′ Cy3-TGG GGT ATT TGA CAA ACT GAC A-3′

[a]Complementary sequences are given in the same font style. The sequence of detection probe **3** was the same as the underlined section in padlock probe **1**; both of them were complementary with the miR-222. The underlined section of padlock probe **4** was complementary with the mRNA of *β*-actin.

limited sensitivity and is not applicable to situations where miRNA is expressed in very low amounts. Rolling-circle amplification (RCA) is a site-anchored isothermal nucleic-acid signal amplification reaction based on the rolling-circle replication mechanism.[19−23] The FISH method combining with RCA can improve significantly the detection sensitivity. It has been reported that *in situ* detection of mitochondrial DNA molecules can be accomplished using a RCA reaction initiated by endonuclease-cleaved DNA targets.[24] Recently, a RCA-based method has been introduced for *in situ* sensitive detection of mRNA expression, by converting mRNA into cDNA as templates for the ligation of padlock probes and target-primed RCA.[25] Although the RCA reaction has been successfully used to detect miRNAs *in vitro*,[26,27] the development of a highly sensitive and selective technique for *in situ* visualization of miRNA expression patterns based on RCA has been largely unexplored.

We have previously demonstrated the utility of RCA in highly sensitive immunoassay and single-nucleotide polymorphism genotyping.[28−30] In this work, we introduced a modified approach for fixation of miRNAs in cells, in which the phosphate group at the 5′ terminus of miRNA was used for a covalent fixation of miRNAs.[31] By exploiting the free 3′ terminus of miRNA as the primer for RCA-based polymerase elongation, we developed for the first time a TPRCA strategy for highly sensitive and selective *in situ* detection of miRNA in cells. This strategy used a preliminarily synthesized circular DNA as the probe for *in situ* hybridization with miRNA, and the miRNA target then primed a RCA reaction to generate a long product with tandem repeated sequence localized at the miRNA target. The RCA product could be hybridized with thousands of fluorescent detection probes, thus allowing highly sensitive *in situ* visualization of the miRNA target. This strategy was demonstrated using miR-222 and miR-223 as the model system, whose expression level was shown to be closely correlated with advanced stage hepatocellular carcinoma tumors.[32] The results revealed that the developed strategy allows *in situ* multiplexed detection of miRNA targets in the cells with high sensitivity and selectivity, implying that it provided a promising platform for miRNA expression analysis at single-cell resolution.

EXPERIMENTAL SECTION

Chemicals and Materials. T4 DNA ligase, phi29 DNA polymerase, exonuclease I (*E. coli*), exonuclease III (*E. coli*), DNA polymerase I Klenow fragement, RNase I_f, and the mixture of deoxyribonucleotides (dNTPs) were purchased from New England Biolabs (Ipswich, MA, USA). 4′,6-Diamidino-2-phenylindole (DAPI) and l-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) were from Sigma Aldrich Chemical Coporation. Cy5-labeled ddCTP was obtained from GE Healthcare Company (Buckinghamshire, UK). Sodium chloride/sodium citrate (SSC, 20×) buffer was from Shanghai Sangon Biological Coporation (Shanghai, China). All other chemicals were of analytical grade and obtained from Sinopharm Chemical Reagent Co. Ltd. All solutions were prepared using ultrapure water, which was obtained through a Millipore Milli-Q water purification system (Billerica, MA, USA) and had an electric resistance >18.3 MΩ. Cell medium RPMI 1640 and DMEM were obtained from Clontech (Mountain View, CA). The oligonucleotides used in this work were synthesized by Takara Biotechnology Co. Ltd. (Dalian, China). The sequences of the synthesized oligonucleotides are listed in Table 1.

Preparation of Circular DNA Probe. In an aliquot of 36 μL hybridization buffer containing 30 mM Tris-HCl (pH 8.0), 100 mM KCl, 4 mM $MgCl_2$, 1 μL of padlock probe **1** (30 μM), and 3 μL of ligation probe **2** (30 μM) were added and incubated at 95 °C for 10 min, then at 55 °C for 2 h. After the mixture cooled to room temperature, 5 μL of T4 DNA ligase (60 U μL^{-1}) together with 7 μL of 10× ligase reaction buffer (500 mM Tris-HCl, pH 7.5, 100 mM $MgCl_2$, 100 mM DTT, and 10 mM ATP) and 48 μL of ultrapure water were added and incubated at 16 °C for 2 h. The mixture was heated at 65 °C for 20 min to inactivate the T4 DNA ligase. After adding 2 μL of exonuclease I (20 U μL^{-1}) and 2 μL of exonuclease III (100 U μL^{-1}), the mixture was incubated overnight at 37 °C to degrade the free and hybridized ligation probe **2**. The mixture was heated at 80 °C for 20 min to inactivate the exonuclease I and exonuclease III. The resulting solution of circular DNA probe was stored at 4 °C until use.

Cell Culture and Fixation. SMMC-7721 cells (human hepatoma cell line) and L02 cells (human hepatocyte cell line) were obtained from Cell Bank of the Committee on Type Culture Collection of the Chinese Academy of Sciences

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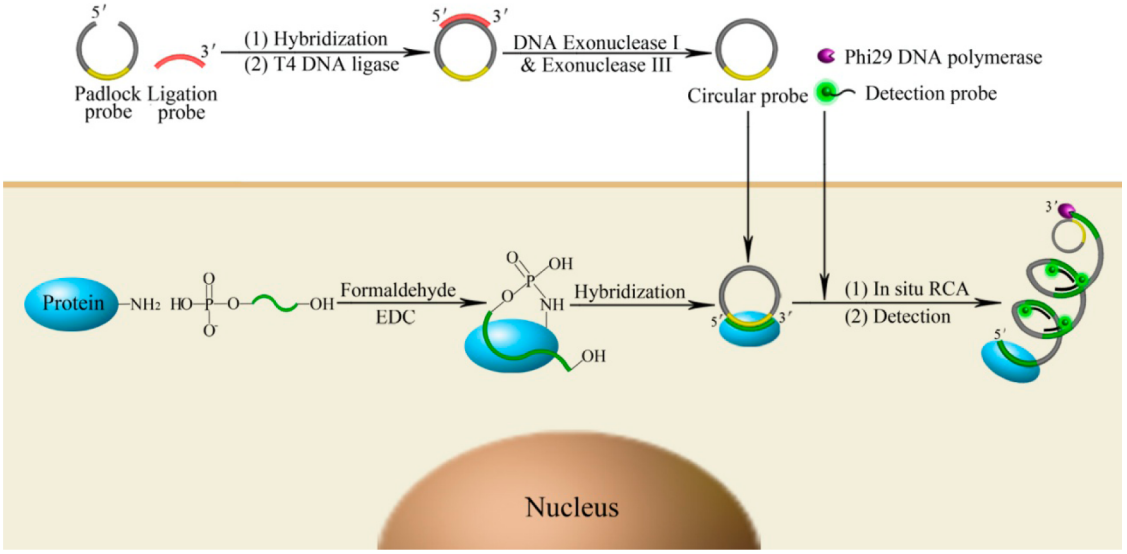
Scheme 1. Schematic Illustration of Fluorescence *in Situ* Hybridization (FISH) Process Based on TPRCA for Highly Sensitive and Selective Visualization of miRNA in Cells[a]



[a]After the cell was fixed using formaldehyde and EDC, the circular DNA probe diffused into the cell and was hybridized with the target miRNA, and the free 3′ terminus of miRNA then initiated an *in situ* RCA reaction in the presence of phi29 DNA polymerase and dNTPs, generating a long tandem repeated sequence. The hybridization of the RCA product with the fluorescent detection probe can provide information about the localization of target miRNA molecules at the single-cell level.

(Beijing, China). The cells were cultured in an RPMI 1640 medium supplemented with 15% fetal calf serum, 100 μg mL^{-1} of streptomycin, and 100 units mL^{-1} of penicillin. Cells were all cultured at 37 °C in a humidified incubator containing 5% CO_2.

SMMC-7721 and L02 cells were seeded on six-well culture slides and cultured in the culture medium for 24 h. The cells were first washed twice with phosphate buffered saline (PBS, pH 7.4, calcium and magnesium free) and then fixed on the slides with PBS containing 4% paraformaldehyde (PFA) for 15 min at room temperature followed by two PBS washes. Before the subsequent EDC fixation, to remove residual phosphate from the PBS washes, slides were first incubated twice for 10 min in 1 mL of a freshly prepared solution containing 0.13 M 1-methylimidazole and 300 mM NaCl (the pH of the solution was adjusted to 8.0 using 12 M HCl). The EDC fixation solution was prepared by adding 88 μL of freshly prepared EDC aqueous solution (~9.2 M) into 5 mL of 1-methylimidazole and 300 mM NaCl (pH 8.0) solution, followed by readjusting the pH to 8.0 using 12 M HCl (the final concentration of EDC was about 0.16 M). The EDC fixation was performed by adding 500 μL of EDC fixation solution on each slide in a humidified chamber and incubating for 1 h at 25 °C. After fixation, the slides were washed twice with PBS to remove excessive EDC.

***In Situ* Visualization of miRNA via TPRCA ISH.** The slides with fixed cells were first incubated in a humidified 37 °C incubator (Thermo Scientific Forma Series II Water Jacketed CO_2 Incubators, Waltham, MA, USA) for 1 h with 200 μL of probe hybridization solution containing 120 μL of circular DNA probe, 40 μL of formamide, and 40 μL of 10× SSC. After being washed twice with 2× SSC, the slides were dehydrated through a series of 70%, 80%, and 99% ice-cold ethanol for 3 min each and air-dried. Then, the TPRCA reaction was performed in the humidified 37 °C incubator for 1.5 h with 200 μL of reaction solution containing 20 μL of 10× phi29 DNA polymerase reaction buffer (500 mM Tris-HCl, 100 mM $MgCl_2$, 100 mM $(NH_4)_2SO_4$, and 40 mM DTT, pH 7.5), 20 μL of dNTP mixture (each 2.5 mM), 10 μL of phi29 DNA polymerase (10 U μL^{-1}), and 150 μL of ultrapure water. After three washes with PBS and two washes with 2× SSC, the slides were immediately dehydrated through a series of 70%, 80%, and 99% ice-cold ethanol for 3 min each and air-dried. A hybridization solution containing 100 nM FAM-labeled detection probe **3**, 2× SSC, and 20% formamide (200 μL) was added to the slides and incubated in a humidified 37 °C incubator for 30 min. The slides were washed with PBS twice for 3 min each. Before imaging, the slides were stained with 1 μg mL^{-1} DAPI solution for 10 min and then washed twice with PBS.

Single Nucleotide Extension-Based ISH and Fluorescence ISH Analysis. The slides with fixed cells were first incubated in a humidified 37 °C incubator for 1 h with 200 μL of probe hybridization solution containing 120 μL of circular DNA probe, 40 μL of formamide, and 40 μL of 10× SSC. After being washed twice with 2× SSC, the slides were dehydrated through a series of 70%, 80%, and 99% ice-cold ethanol for 3 min each and air-dried. A single nucleotide extension reaction was carried out in a humidified 37 °C incubator for 2 h with 200 μL of reaction solution containing 10 mM Tris-HCl (pH 7.9), 50 mM NaCl, 10 mM $MgCl_2$, 100 nM Cy5-labeled ddCTP, and 2 U DNA polymerase I Klenow fragment. The slides were washed with PBS twice for 3 min each. Fluorescence ISH was performed by directly incubating the fixed cells with 200 μL of hybridization solution containing 100 nM FAM-labeled detection probe **3**, 2× SSC, and 20% formamide at 37 °C for 1 h. The slides were washed twice with PBS. Before imaging, all slides were stained with 1 μg mL^{-1} DAPI solution for 10 min and then washed twice with PBS.

Confocal Fluorescence Imaging System. Fluorescence imaging was performed using a confocal laser scanning fluorescence microscope setup consisting of an Olympus IX-

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70 inverted microscope with an Olympus FluoView 500 confocal scanning system. The cellular images were acquired using a 40× objective. An Ar^+ laser (488 nm) was used as an excitation source for a FAM-labeled detection probe, and a 515 nm (±10 nm) bandpass filter was used for fluorescence detection. A red He Ne laser (633 nm) was used as an excitation source for the Cy5-labeled probe, and a 660 nm long-pass filter was used for fluorescence detection. The DAPI dye was excited with a 405 nm laser line and detected with a 460 nm (±10 nm) bandpass filter. The fluorescence images were presented after processing by Maestro version 2.2 software and Image J version 1.38x software.

RESULTS AND DISCUSSION

Design of Fluorescence ISH Based on TPRCA for miRNA Visualization. The design of the fluorescence ISH based on TPRCA for highly sensitive miRNA detection is schematically illustrated in Scheme 1. A circular DNA probe containing a sequence complementary to the target miRNA was first prepared using a padlock probe and a ligation probe via DNA ligase reaction. Initially, the cells were fixed using formaldehyde and EDC. During the fixation process, the miRNA 5′ phosphate end was cross-linked with amino groups in the protein matrix by EDC, and the 3′ terminus of the target miRNA molecule was still free to serve as a primer for RCA-based polymerase elongation. So, after the circular DNA probe diffused into the cells and was hybridized with the target miRNA, the free 3′ terminus of miRNA could initiate an *in situ* RCA reaction in the presence of phi29 DNA polymerase and dNTPs, generating a long tandem repeated sequence. The hybridization of the RCA product with the fluorescent detection probe provided the information about the localization and distributions of target miRNA molecules at the single-cell level.

As the first demonstration of incorporating TPRCA in fluorescence ISH for highly sensitive miRNA visualization detection, there are indeed several novel advantages. First, a RCA reaction can generate a long tandem repeated sequence with thousands of complementary segments, which, after hybridization with the fluorescent detection probe, can be observed as a superbright spot under a conventional fluorescence microscope and easily distinguished from the background in a cell. This high signal intensity can greatly enhance the detection sensitivity and improve the cellular resolution and makes this technique suitable especially for examining less-abundantly expressed miRNAs and determining small changes in the expression levels of miRNA, which usually have functional importance. Second, other RNA transcripts such as mRNA or pre-miRNA, even though containing a high homologue in their sequence, do not have a free 3′ terminus in the homologous region and will not initiate the RCA reaction, so there will be no positive signal for other RNA transcripts. In this sense, the self-priming design in the presented technique can offer high selectivity and allow highly specific detection of mature miRNA in the presence of pre-miRNA and other RNA transcripts, which is distinctly advantageous over the current FISH methods.

Demonstration of the miRNA-Primed RCA Reaction. We first tested whether a miRNA could serve as a primer to initiate the RCA reaction. A circular DNA probe containing a sequence complementary with miR-222 was prepared via T4 DNA ligase reaction, and RCA was then performed in the presence of miR-222, dNTPs, and phi29 DNA polymerase. The RCA product was analyzed by agarose gel electrophoresis (Figure S1 in Supporting Information). A bright band for the large-molecule-weight product could be observed in this case (as shown in lane 1). In contrast, the RCA product was not obtained in the absence of miR-222 (lane 2) or in the absence of T4 DNA ligase reaction (lane 3). In addition, no products were seen when dNTPs (lane 4) or phi29 DNA polymerase (lane 5) was not added during RCA reactions. Moreover, no RCA products were obtained when using a noncomplementary scrambled padlock probe (lane 6) or replacing the target miR-222 with a nonspecific miR-223 as the primer (lane 7). These results indicated that it was feasible to use a miRNA target to initiate RCA reactions, allowing the development of an *in situ* detection technique for miRNAs.

Investigation of the miRNA Fixation Method. The miRNA fixation method was then investigated. In a conventional miRNA ISH assay, a formaldehyde fixation method was commonly used. However, it has been reported that this method has the problem of miRNA release and diffusion out of the cells during the subsequent incubation at high temperature. To prevent the loss of miRNA, an additional miRNA fixation step was added after the formaldehyde treatment, in which the water-soluble EDC was used as cross-linker to condense the miRNA 5′ phosphate with amino groups in the protein to form stable linkages.[31] Here, we investigated the effect of the two different fixation methods by conducting a TPRCA-based ISH assay using miR-222 as the model system. The recent study revealed that miR-222 was commonly overexpressed in hepatocellular carcinoma tissues and cell lines compared with normal liver controls.[32] In this work, SMMC-7721, a human hepatocarcinoma cell line, and L02, a normal human hepatocyte cell line, were selected as the target cells. The relative expression levels of miR-222 in these two cell lines were determined using quantitative PCR, and the results confirmed that the L02 cell line had a lower miR-222 expression level than the SMMC-7721 cell line (Figure S4 and Table S1 in Supporting Information). As shown in Figure 1, for human hepatoma SMMC-7721 cell with highly expression of miR-222, the abundant and bright cytoplasmic signals were observed in

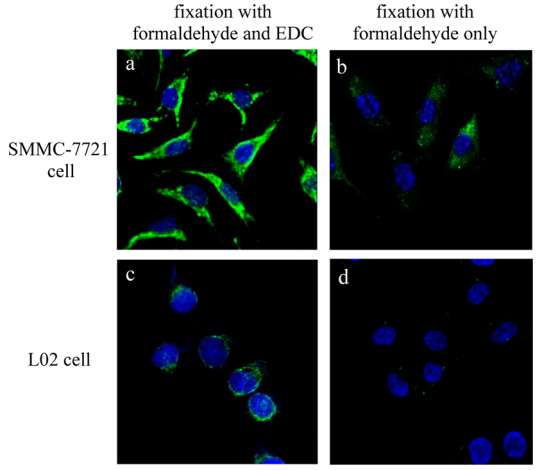

Figure 1. TPRCA-based fluorescence ISH detection of miR-222 in human hepatoma SMMC-7721 cells (a and b) and human hepatocyte L02 cells (c and d) after the fixation with formaldehyde and EDC (a and c) or with formaldehyde only (b and d). The green fluorescence represents FAM-labeled detection probe (PMT 600 V, gain 4%, and offset 2%), and cell nuclei are stained with DAPI (blue).

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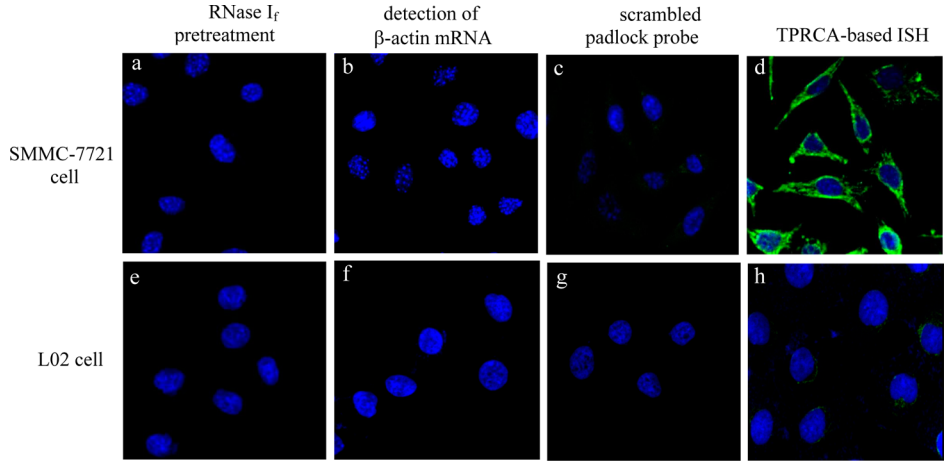

Figure 2. TPRCA-based fluorescence ISH detection in human hepatoma SMMC-7721 cells (a, b, c, and d) and human hepatocyte L02 cells (e, f, g, and h) subjected to RNase I$_f$ pretreatment before hybridization with a padlock probe (a and e), detection of β-actin mRNA by the TPRCA-based ISH method (b and f), hybridization with a scrambled padlock probe (c and g), and a normal TPRCA-based ISH (d and h), respectively. The green fluorescence represents a FAM-labeled detection probe (PMT 600 V, gain 4%, and offset 2%), and cell nuclei are stained with DAPI (blue).

the formaldehyde and EDC-fixed sample (Figure 1a), whereas a very dim signal was obtained in the formaldehyde-fixed sample (Figure 1b). On the other hand, for the human hepatocyte L02 cell that expressed miR-222 in a relatively low amount, the almost undetectable signals in the formaldehyde-fixed sample (Figure 1d) were also improved moderately by adding the EDC treatment (Figure 1c), which were mainly localized to the perinuclear region of cell. These results indicated that the leakage of miRNA can be eliminated sufficiently by introducing an irreversible EDC cross-link between the 5′ phosphate of the miRNA and amino groups in the protein. In addition, it can be observed that the fluorescent signals were clearly more abundant in SMMC-7721 cells than those obtained in L02 cells whether the formaldehyde and EDC-fixed samples or the formaldehyde-fixed samples were detected, indicating that the miR-222 expression level in human hepatoma cells was significantly higher than that in human normal hepatocyte cells, which was consistent with the results obtained by quantitative PCR.

Control Experiments of Fluorescence ISH Based on TPRCA. To demonstrate that the bright fluorescence shown in Figure 1a was indeed caused by the target miRNA-primed *in situ* RCA reaction, we designed three control experiments. In the first one, RNase I$_f$, a single strand specific RNA endonuclease which will cleave at all RNA dinucleotide bonds was used to pretreat the formaldehyde and EDC-fixed samples at 37 °C for 2 h before hybridization with the padlock probe. We noticed no signals were detected in this case (Figure 2a), whereas the sample without the pretreatment of RNase I$_f$ showed highly intense fluorescent signals (Figure 2d), verifying that the signals were RNA dependent, rather than induced by nonspecific priming from DNA. To further preclude the interference from mRNA and rRNA, another control experiment was performed in which a housekeeping mRNA (β-actin) was detected using the TPRCA method. We designed a padlock probe containing a sequence specific to the β-actin mRNA and a fluorescence labeled detection probe complementary with RCA products (padlock probe **4** and detection probe **5** in Table 1). In this case, no distinct fluorescent signals were observed (Figure 2b), implying that the TPRCA method was able to eliminate the interference from mRNA in the detection of miRNA in our assay. This ability was attributed to the fact that mRNA does not have a free 3′ terminal complementary with the circle probe and cannot act as the primer to initiate RCA. Likewise, it can be inferred that rRNA would not generate bright fluorescence images because of its inability to act as a primer for RCA. To demonstrate that the signals resulted from specific hybridization of the padlock probe with target miRNA, we performed a further control experiment using a padlock probe with randomly scrambled sequence (padlock probe **6** in Table 1) and the corresponding detection probe **7** instead of the original complementary padlock probe and detection probe to conduct the TPRCA-based ISH. It could be seen that an extremely faint green fluorescence background in the SMMC-7721 cell was detected in Figure 2c, which might arise from the nonspecific adsorption of detection probe **7**. In contrast, when the complementary padlock probe was employed, an intense fluorescent signal was obtained (Figure 2d), indicating that the detection was highly dependent on the specific hybridization reaction between the padlock probe and the target miRNA, and only in the state of hybridization could a RCA reaction be initiated effectively by miRNA. Taken together, these results validated the ability of the TPRCA-based ISH method for specific *in situ* detection of miRNA at the level of single cells.

Sensitivity Enhancement of Fluorescence ISH Based on TPRCA. As mentioned above, because a miRNA target molecule can initiate an *in situ* RCA reaction, which produces a long tandem repeated sequence with thousands of complementary segments to a FAM-labeled detection probe, the TPRCA-based ISH method is expected to obtain a high sensitivity for miRNA *in situ* visualization. To demonstrate the method's high sensitivity of detection, we arranged a single nucleotide extension (SNE)-based ISH assay for miRNA *in situ* detection, in which a Cy5-labeled ddCTP could be added at the 3′ terminus of target miRNA through a DNA polymerase I Klenow fragment after hybridization with the padlock probe. Because only one fluorophore was added on a target miRNA molecule, this method did not involve the signal amplification and was used as a reference method to compare the sensitivity with the TPRCA-based ISH. As shown in Figure 3, for human hepatoma SMMC-7721 cells that highly expressed miR-222, the TPRCA-based ISH detection exhibited highly intense cytoplasmic fluorescent signals under a relatively low voltage of

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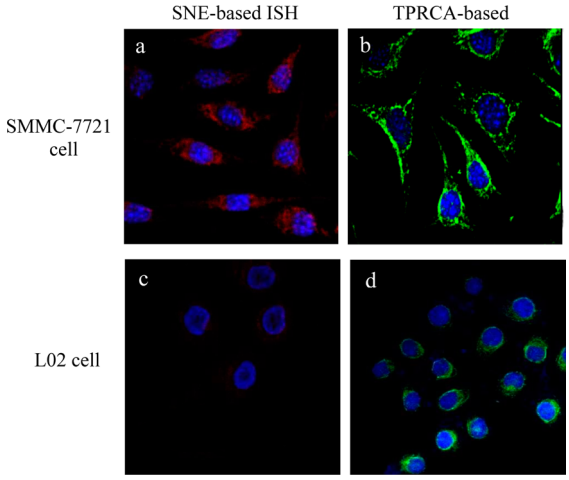

Figure 3. Visualization of miR-222 in human hepatoma SMMC-7721 cells (a and b) and human hepatocyte L02 cells (c and d) through single nucleotide extension (SNE)-based ISH (a and c) and TPRCA-based ISH (b and d), respectively. The green fluorescence represents a FAM-labeled detection probe (PMT 600 V, gain 4%, and offset 2%). The red fluorescence denotes Cy5-labeled ddCTP (PMT 800 V, gain 4%, and offset 2%), and cell nuclei are stained with DAPI (blue).

photoelectric multiplication tube (PMT) of 600 V (Figure 3b), whereas almost no detectable signals could be observed under the identical voltage of PMT when SNE-based ISH was detected (data not shown). When the voltage of PMT was further increased to 800 V, a moderately intense fluorescent signal was obtained (Figure 3a), indicating that the TPRCA-based ISH can indeed enhance the sensitivity of miRNA *in situ* visualization and provide a high imaging contrast. Additionally, for human hepatocyte L02 cells that expressed miR-222 in a relatively low amount, rare but bright fluorescent signals localized to the perinuclear region of the cell could be clearly observed by the TPRCA-based ISH method under a relatively low PMT voltage of 600 V (Figure 3d), whereas no signals could be detected in the SNE-based ISH assay even under a relatively high PMT voltage of 800 V (Figure 3c). These results clearly demonstrated that the TPRCA-based ISH method could greatly enhance the detection sensitivity and cellular resolution, which is significantly important for the detection of miRNAs expressed in low amounts as well as the determination of small changes in the expression levels of miRNA.

It is noteworthy that because of its high sensitivity, the RCA technique can in principle generate bright fluorescent spots that allow the counting of target molecules. However, in our study, the bright spots can be observed only for miRNAs with low expression levels, such as miR-222 in an L02 cell, when the fluorescent images were presented at a sufficiently large magnification (for example, see Figure S5 in the Supporting Information). In order to observe the cell-to-cell expression variations of miRNA and the average expression level of miRNA in the cell population, the fluorescent images were presented at a relatively low magnification. Moreover, for a high miRNA expression level, such as miR-222 in the SMMC-7721 cell, the density of the bright spots was so high that it was difficult to obtain single miRNA fluorescent spots. Hence, we can only observe continuous but heterogeneous bright islands that may include many undistinguished spots in our fluorescent images.

Selectivity Improvement of Fluorescence ISH Based on TPRCA. In conventional fluorescence ISH methods, a fluorophore-labeled detection probe complementary to target miRNA was commonly used. Such an oligonucleotide probe, however, can also anneal nonspecifically to precursor miRNA (pre-miRNA), causing false positive readings for expression levels of mature miRNA, because pre-miRNA levels are not indicative of their corresponding mature miRNA levels. So, a major challenge for miRNA *in situ* detection is discrimination between pre-miRNA and mature miRNA. The TPRCA-based ISH detection relies on the fact that the free 3′ terminus of target miRNA can serve as a primer to trigger an *in situ* RCA reaction. Because pre-miRNA has a 60−70-nt-long stem-loop structure, in which the mature microRNA is located in the stem with the 5′ terminus far from the loop and the 3′ terminus adjacent to the loop,[33] it does not have a free 3′ terminus in the region complementary to the circular DNA probe and cannot initiate the RCA reaction. In this sense, the developed TPRCA-based ISH strategy should in principle allow the selective detection of mature miRNA even in the presence of pre-miRNA. To test the high selectivity of TPRCA-based ISH detection, a direct fluorescence ISH assay for miRNA visualization was performed by using a FAM-labeled detection probe to hybridize with the target miRNA. As shown in Figure 4, for human hepatoma SMMC-7721 cells that highly expressed

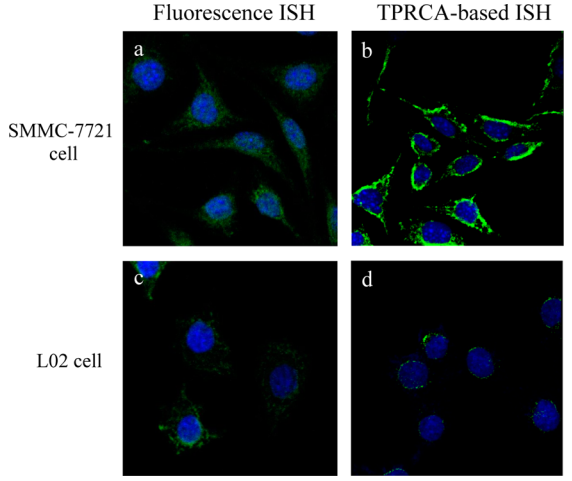

Figure 4. Fluorescent images of human hepatoma SMMC-7721 cells (a and b) and human hepatocyte L02 cells (c and d) obtained by fluorescence ISH (a and c) and TPRCA-based ISH (b and d) with FAM-labeled detection probe, respectively. The green fluorescence represents the FAM-labeled detection probe (PMT 600 V, gain 4%, and offset 2%), and cell nuclei are stained with DAPI (blue).

miR-222, the TPRCA-based ISH experiment revealed bright fluorescent signals predominantly localized to the cytoplasm of cells, not to the nucleus (Figure 4b). However, besides cytoplasmic signals, abundant signals inside nuclei could also be clearly observed in the direct fluorescence ISH assay (Figure 4a). It is known that the precursor miRNA present in the nucleus is first exported to the cytoplasm and then cleaved by Dicer enzyme to generate mature miRNA.[2−4] So, it can be inferred that the mature miRNA is very much confined to the cytoplasm, and the abundant signals inside nuclei obtained in direct fluorescence ISH detection should be induced by nonspecific hybridization of the FAM-labeled detection probe with pre-miRNA present in the nuclei of cells. Similar results were obtained when human hepatocyte L02 cells with a relatively low amount of miR-222 were detected. Moreover,

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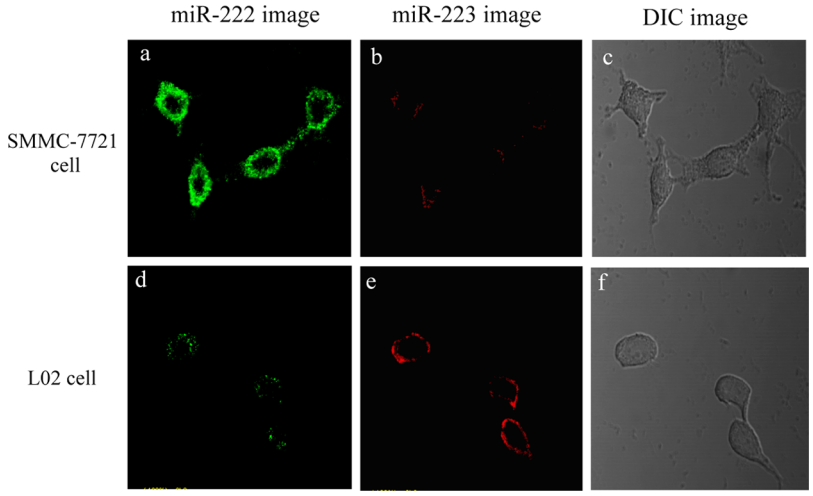

Figure 5. Dual-color fluorescence images of miR-222 and miR-223 in human hepatoma SMMC-7721 cells (a and b) and human hepatocyte L02 cells (d and e) using the TPRCA-based fluorescence ISH detection. The green fluorescence represents the FAM-labeled detection probe (PMT 600 V, gain 4%, and offset 2%), and the red fluorescence denotes the Cy3-labeled detection probe (PMT 600 V, gain 4%, and offset 2%). Parts c and f represent differential interference contrast (DIC) images of SMMC-7721 cells and L02 cells, respectively.

when using the direct fluorescence ISH assay, no distinct differences in fluorescence intensity between the SMMC-7721 cell and L02 cell were observed (Figure 4a and c), indicating that the total expression levels of pre-miRNA and mature miRNA in these two types of cells were comparable. In contrast, when using TPRCA-based ISH detection, an obvious difference in fluorescence intensity between the SMMC-7721 cell and L02 cell was obtained (Figure 4b and d). These results verified the ability of the proposed TPRCA-based ISH method to perform highly selective *in situ* detection of mature miRNA. Furthermore, we noticed that the fluorescent signals obtained with the TPRCA-based ISH method were obviously more intense than those provided by direct fluorescence ISH assay, which also demonstrated the high sensitivity of the TPRCA-based ISH strategy.

Dual-Color *in Situ* Detection of miRNAs. To test the ability of the TPRCA method for multiplex detection of miRNAs' expressions, we designed padlock probe **8** and the corresponding detection probe **9** (Table 1) for another miRNA, miR-223. With the probes for miR-222 and miR-223, we performed dual-color detection of miR-222 and miR-223, in L02 and SMMC-7721 cell lines. Contrary to miR-222, miR-223 is reported to be commonly repressed in hepatocellular carcinoma.[34] The result of quantitative PCR also confirmed that the L02 cell line had a higher miR-223 expression level than the SMMC-7721 cell line (Figure S6 and Table S2 in the Supporting Information). The fluorescence images obtained in the dual-color assay were shown in Figure 5. For the detection of miR-222, abundant and bright cytoplasmic signals were observed in human hepatoma SMMC-7721 cells (Figure 5a), whereas dim fluorescence signals were obtained in human hepatocyte L02 cells (Figure 5d). In contrast, L02 cells gave more intensive fluorescent signals for miR-223 than SMMC-7721 cells (Figure 5b and e). These results indicated that the fluorescence intensities obtained with the TPRCA-based ISH method were correlated to the expression levels of miR-222 and miR-223 in these two cell lines, which were consistent with the results of quantitative PCR.

CONCLUSIONS

We develop a TPRCA strategy for highly sensitive and selective *in situ* visualization of miRNA expression patterns at the single-cell level. This strategy uses a circular DNA as the probe for *in situ* hybridization with the target miRNA molecules, and the free 3′ terminus of miRNA then initiates an *in situ* RCA reaction to generate a long tandem repeated sequence with thousands of complementary segments. After hybridization with the FAM-labeled detection probe, target miRNA molecules can be visualized under a conventional fluorescence microscope with ultrahigh sensitivity and spatial resolution, which enables the examination of less-abundantly expressed miRNAs and the determination of small changes in the expression levels of miRNA. The introduction of an irreversible EDC cross-link between the 5′ phosphate of the miRNA and amino groups in the protein can notably decrease miRNA diffusion and improve miRNA retention in cells. In addition, the TPRCA strategy for miRNA *in situ* visualization can eliminate effectively the interference from precursor miRNA through a RCA reaction initiated by the free 3′ end of target miRNA molecules, which is distinctly advantageous over the current fluorescence ISH methods. Furthermore, it is noteworthy that the miRNA-primed TPRCA-based ISH protocol described here is both fast and efficient and can be completed within approximately 6 h starting with fixation of the cells. This could become a decisive factor when choosing an appropriate ISH method for detecting disease-associated miRNAs in cancer cells. This strategy has been successfully applied to the detection of miR-222 expression levels in human hepatoma SMMC-7721 cells and hepatocyte L02 cells, and the results are consistent with previous studies of miR-222. Hence, we believe that the TPRCA-based ISH method has great potential for reliable disease association studies and miRNA expression analysis as a powerful tool for basic research and clinical diagnosis.

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■ ASSOCIATED CONTENT

Supporting Information

Experimental details and additional figures as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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