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COMMUNICATION

L-DNA-tagged fluorescence *in situ* hybridization for highly sensitive imaging of RNAs in single cells

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We report an effective fluorescence *in situ* hybridization strategy, named L-DNA tagged FISH (LT-FISH), for highly sensitive RNA detection in fixed cultured cells. LT-FISH includes two-step hybridization processes with a L-D chimera oligonucleotide probe and a fluorescence-labeled PCR product tethering a L-DNA tag. The degree of fluorescence enhancement, depending on the length of PCR products, was up to 14-fold when the 606-bp product was used. Endogenous mRNA and miRNA in cancer cells were visualized by utilizing this L-DNA-mediated signal amplification technique.

RNA fluorescence *in situ* hybridization (FISH) is an indispensable technique to visualize transcripts of interest in fixed cells or tissues using complementary nucleic acid probes to target RNA sequences. In most cases, a single-stranded oligonucleotide probe labeled with one or a few fluorescent dyes is used. Fluorescence-labeled oligonucleotide probes recognizing only a single site in the target RNA often offer a too-low sensitivity to enable visualization of intracellular mRNAs; thus, sets of multiple oligonucleotides that bind to several different regions in the target mRNA have been applied.[1,2] However, this approach is not applicable to short RNAs, such as miRNA, which are promising markers to identify cell types, because hybridizable regions in short RNAs are extremely limited. Therefore, to realize sensitive imaging of small RNAs, multiplexing of the hybridization sites or enhancement of fluorescence intensity per probe may be plausible solutions (Fig. 1). Several FISH methods, including fluorescence signal amplification, such as rolling circle amplification (RCA),[3,4] enzyme-labeled fluorescence (ELF),[5,6] and tyramide signal amplification (TSA),[7,8] have been developed for miRNA imaging. However, all these methods involve *in situ* enzymatic reactions to enhance the signal, where the degree of signal amplification could be strongly affected by the sample preparation conditions and/or cell-specific molecular environment.

Herein, we report a new FISH methodology, named L-DNA-tagged FISH (LT-FISH), which employs fluorescence-labeled PCR products bearing a single-stranded part of L-DNA (an enantiomer of a natural DNA molecule (D-DNA)) (Fig. 1). LT-FISH makes possible the sensitive and specific detection of endogenous RNA, without any conventional *in situ* signal amplification process; thus, it offers more stable and reliable signals.

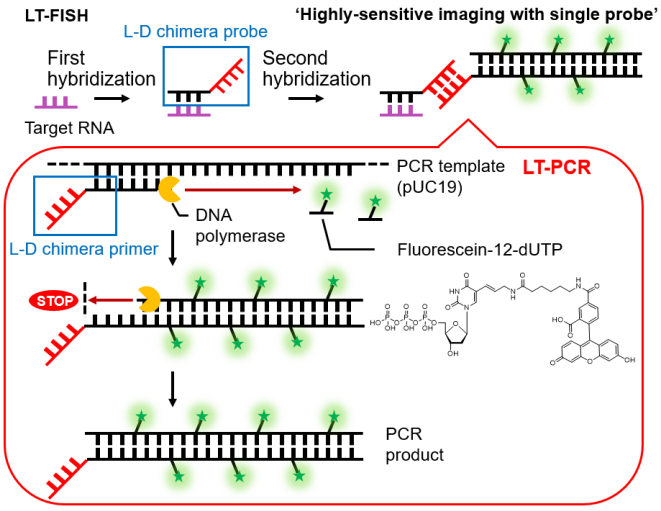

Fig. 1 Illustration of L-DNA tagged fluorescence *in situ* hybridization (LT-FISH). Fluorescence-labeled PCR product bearing L-DNA tag prepared from L-DNA tagged PCR (LT-PCR) using fluorophore-labeled dNTP and L-D chimera primer. The L-DNA, D-DNA, and target RNA are red, black, and purple, respectively.

In our LT-FISH method, L-DNA-tagged fluorescent DNA is a key molecule (Fig. 1). This DNA can be synthesized using fluorescence-labeled dNTP[9,10] in L-DNA-tagged PCR (LT-PCR),[11,12] in which a L-D chimera DNA containing L- and D-DNA at the 5′ and 3′ sides, respectively, is used as an LT-PCR primer. Because a L-DNA sequence is not recognized as a template by

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DNA polymerase, it remains as a single-stranded part after PCR. LT-FISH offers a simple two-step hybridization procedure; a L-D chimera DNA probe hybridizes to a complementary target RNA with its D-DNA part and, subsequently, a multiple fluorescence-labeled LT-PCR product hybridizes with the L-DNA part of the chimera probe on the target RNA. Of importance is that single-stranded L-DNA only forms a duplex with the complementary L-DNA strand, but not with a D-DNA strand, unlike many other artificial nucleic acid derivatives.[13,14] L-DNA has been shown to operate in an orthogonal fashion *in vitro* and in a complex cellular environment to avoid promiscuous hybridization against D-DNA and D-RNA.[15,16] The L-DNA part of the L-D chimera primer might be replaced with the D-DNA sequence linked to a primer part with a spacer.[17] However, the D-DNA tag sequence would need to be chosen to avoid complementarity to any relevant RNA sequence to avoid inhibition of amplification or false positives, whereas the L-DNA tag is inherently not complementary to the RNA. Therefore, the L-DNA part in the chimera probe would be an ideal molecular handle to recruit the fluorescence-labeled PCR products.

A 50-nt L-D chimera DNA primer L-FW (Fig. 2A and Table S1), of which the D-DNA part (20 nt) is complementary to pUC19 plasmid, was chemically synthesized (Fig. S1). Using L-FW, we carried out LT-PCR on pUC19 with KOD Dash DNA polymerase and fluorescein-labeled dUTP, in addition to a standard dNTP mix, to obtain a 131-bp duplex D-DNA with a 30-nt single-stranded L-DNA part. In this LT-PCR, fluorescein-labeled dUTP was selected as labeling reagent because fluorescein tends to be more efficiently incorporated into a PCR product with polymerase than other dyes.[18,19] In native PAGE analysis, the LT-PCR product was observed as a single specific band detected by fluorescence emission of both fluorescein and SYBR Gold (Fig. 2B), indicating the successful incorporation of fluorescein-labeled dUTP by KOD Dash DNA polymerase. The band was located between 131- and 161-bp PCR products, which were obtained by replacing L-FW with FW (20 nt; the primer without the L-DNA part) and D-FW (50 nt; the fully D-DNA-substituted primer), respectively (Table S1). Denaturing PAGE analysis revealed that the LT-PCR product consisted of two strands, with different lengths, each of which corresponds to 131- and 161-nt bands (Fig. 2C), indicating that the L-DNA part of the chimera primer remained as a single strand. We also synthesized longer LT-PCR products composed of 318- and 606-bp duplexes using L-FW and different reverse primers on a pUC19 template (Fig. S2). These products were expected to incorporate a greater number of fluorescein-labeled dU, leading to greater signal enhancement.

To determine the fluorescence intensity of LT-PCR products after binding to immobilized RNA strands, we set up an assay system using streptavidin-coated magnetic beads and biotin-labeled 22-nt RNA, biotin-let-7a (Fig. 3A). After immobilization of biotin-let-7a on the magnetic beads (as bait), different concentrations of fluorescence-labeled DNA were added. Then, the beads were washed and the fluorescence intensity was measured with plate readers. When a single fluorescein-labeled oligonucleotide probe (FAM-probe(let-7a)) was used as control, the observed fluorescence intensity linearly increased with an increase in concentration of the FAM-probe(let-7a) and plateaued at > 50 nM FAM-probe(let-7a) (Fig. 3B), indicating saturated hybridization at this concentration. This assay would be effective not only to optimize experimental conditions to avoid nonspecific binding, but also to define an appropriate detection range for the assay.

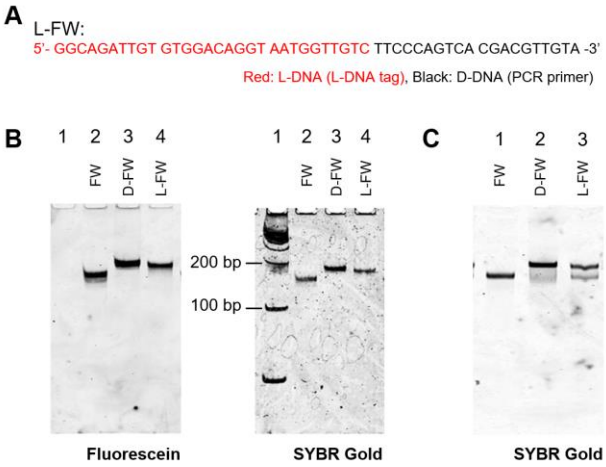

Fig. 2 Analysis of LT-PCR products. (A) Sequence of the L-D chimera DNA primer for LT-PCR on pUC19 plasmid DNA. (B) Native PAGE analysis of LT-PCR product. DNA ladder (lane 1), 131-bp PCR product (lane 2), 161-bp PCR product (lane 3), and LT-PCR product (lane 4) were loaded onto 10% polyacrylamide gel after purification. Band images were obtained through direct detection of fluorescein or after SYBR Gold staining. (C) Denaturing PAGE analysis of LT-PCR product. 131-bp PCR product (lane 1), 161-bp PCR product (lane 2), and LT-PCR product (lane 3) were loaded onto 15% polyacrylamide gel containing 7 M urea. Gel bands were stained with SYBR Gold.

Using this assay system, the fluorescence intensities of the multiple fluorescence-labeled probes synthesized by LT-PCR were investigated. L-probe(let-7a), a L-D chimera probe, was mixed with RNA-immobilized magnetic beads for the first hybridization. After excess probe was washed out, the multiple fluorescence-labeled probes synthesized by LT-PCR were mixed in different concentrations during the second hybridization step. Enhancement of the fluorescence signal here compared with FAM-probe(let-7a) was estimated from the slope of increased fluorescence upon an increase in concentration of each multiple fluorescence-labeled probe (Fig. 3C). The LT-PCR products with 131-, 318-, and 606-bp showed 1.8-, 5.2-, and 14.4-fold enhancement of fluorescence signals, respectively. The LT-PCR product with a longer sequence exhibited fluorescence enhancement, which was sufficient to observe intracellular RNA, although the enhancement appeared to not be very high, given that a number of thymine residues exist in the PCR products, because quenching of fluorescence signal occurs in highly labeled PCR product[18] and the incorporation efficiency of fluorescence labels is decreased by dye–dye or dye–polymerase interactions.[10,19]

Using the brightest 606-bp LT-PCR product for a multiple fluorescence-labeled probe, LT-FISH was conducted to visualize mRNA of the survivin gene, which is known to be highly expressed in many cancer cell lines.[20,21] A L-D chimera probe, L-

probe(survivin), synthesized for survivin mRNA (Table S1 and Fig. S3), was treated at 37 °C for 1.5 h with formaldehyde-fixed HepG2 cells, derived from human liver carcinoma. After removal of excess probe, the LT-PCR product was hybridized at 45 °C for 2 h. We observed a relatively strong fluorescence signal from the cytosol region based on confocal fluorescence microscopy analysis (Fig. 3A). The fluorescence signals in the nucleus were much weaker than those in the cytosol, which is well consistent with earlier observations.[22] On the other hand, negative control samples, in the absence of L-probe(survivin) or with a monotonal D-DNA probe (D-probe(survivin)) instead of L-probe(survivin), showed ignorable signals, indicating that nonspecific interaction between LT-PCR product and the fixed sample is negligible and that the observed fluorescence signals depend on L-DNA hybridization between L-DNA parts of L-probe(survivin) and LT-PCR product. A single fluorescein-labeled probe (10 nM), FAM-probe(survivin), was also treated for FISH, instead of using a set of L-probe(survivin) and a multiple fluorescence-labeled

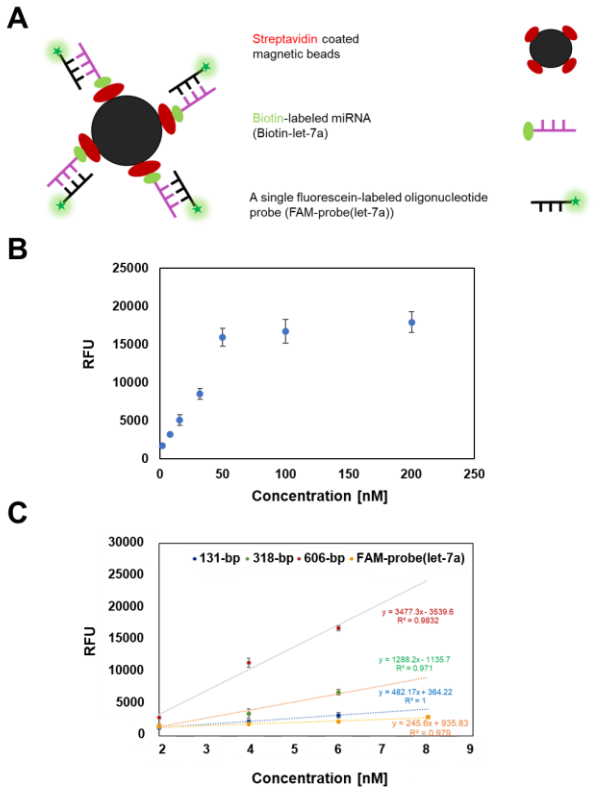

Fig. 3 Quantification of a single fluorescein-labeled probe and PCR products. (A) Schematic illustration of the hybridization of a single fluorescein-labeled probe FAM-probe(let-7a) with let-7a on the magnetic beads. (B) Fluorescence intensity of various concentration of FAM-probe(let-7a). (C) Fluorescence intensity of various concentration of FAM-probe(let-7a), 131-, 318-, and 606-bp PCR products.

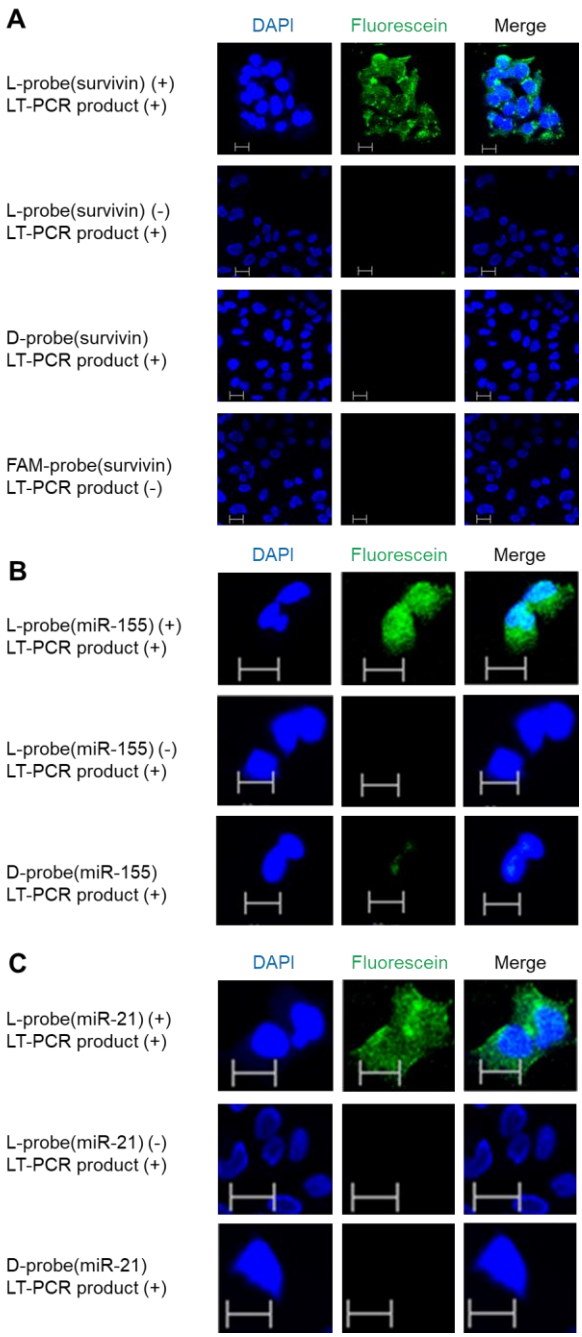

Fig. 4 LT-FISH for imaging of mRNA and miRNA in fixed cells. (A) Survivin mRNA in HepG2 cells. It was visualized by LT-FISH with L-probe(survivin) as L-D chimera probe and fluorescence-labeled LT-PCR product. Control samples are without L-probe(survivin), with D-probe(survivin) and with FAM-probe(survivin). (B) miR-155 in HepG2 cells. Control samples are without L-probe(miR-155) and with D-probe(miR-155). (C) LT-FISH was conducted to visualize miR-21 in A549 cells. Control samples are without L-probe(miR-21) and with D-probe(miR-21). Cell nuclei were stained with DAPI, shown in blue. Scale bars: 20 μm.

606-bp probe, but there was very weak fluorescence signal from HepG2 cells. Thus, LT-FISH has much higher sensitivity for endogenous mRNA imaging compared with the conventional FISH.

We also looked at two different miRNA species, miR-155 and miR-21, which are expressed in HepG2 and A549 cells,

respectively.[23,24] We used chemically synthesized specific L-D chimera probes, L-probe(miR-155) and L-probe(miR-21) (Fig. S3). It has been reported that miR-155 abnormally expresses in hepatocellular carcinoma and it is expected to serve as a potential therapeutic target in the early stages of tumorigenesis.[25] LT-FISH, using a set of L-probe(miR-155) and a multiple fluorescence-labeled 606-bp probe, was conducted to visualize miR-155 in HepG2 cells, which were human liver cancer cell lines. We observed strong fluorescence both in the cytosol and nucleus. The fluorescence in the cytosol may be partially due to the pri-miRNA and pre-miRNA, which are precursors of mature miRNA. On the other hand, negative control samples, in the absence of L-probe(miR-155) showed negligible signals, and with a monotonal D-DNA probe (D-probe(miR-155)) instead of L-probe(miR-155) showed very weak fluorescence in the nucleus. This method is useful for the imaging of another miRNA in another cell. miR-21 is known to be highly expressed in the cytoplasm of tumor cells and represents a pivotal prognostic marker for lung cancers.[26] Strong fluorescence was observed grater signal in the cytosol than in the nucleus in LT-FISH for miR-21 in A549 cells (Fig. 4B). This observation was also in good agreement with findings reported earlier for miR-21 localization in lung cancer cells. On the other hand, negative control samples, in the absence of L-probe(miR-21) or with a monotonal D-DNA probe (D-probe(miR-21)) instead of L-probe(miR-21), showed negligible signals.

In summary, by employing combinatorial use of a L-D chimera probe and a multiple fluorescence-labeled LT-PCR product, we developed a novel concept of fluorescence signal enhancement for FISH. LT-FISH does not require any *in situ* enzymatic amplification. It is only necessary to design each L-D chimera probe for a specific target endogenous RNA. The multiple fluorescence-labeled LT-PCR product can be used for sensitive RNA imaging regardless of the target RNA sequence. We believe that this LT-FISH technique could be expanded by exploiting a LT-PCR product containing different fluorescent dyes and orthogonal L-DNA tag sequences, to offer a more sensitive detection of intracellular RNAs.

Conflicts of interest

There are no conflicts of interest to declare.

Acknowledgments

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TOC One sentence text:

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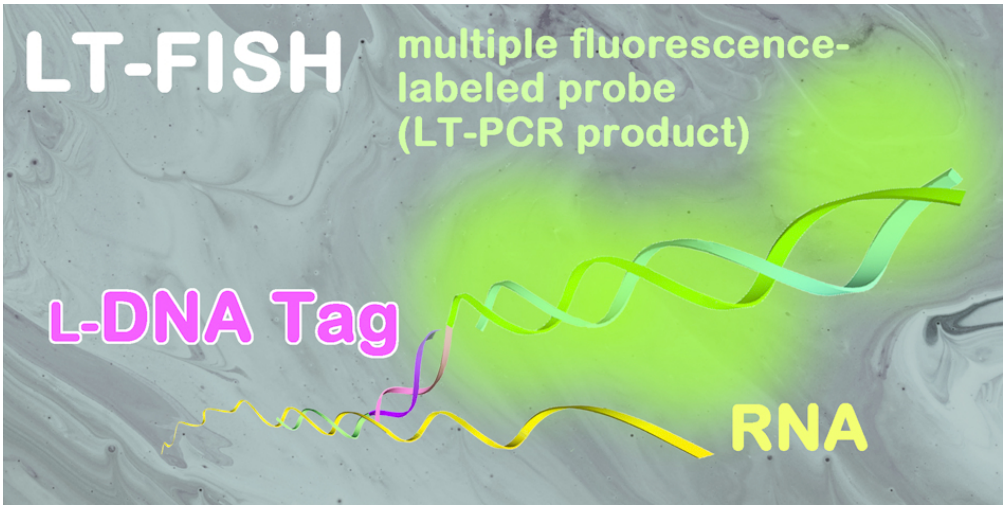

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