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Visualization of *NRAS* RNA G-Quadruplex Structures in Cells with an Engineered Fluorogenic Hybridization Probe

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Supporting Information

ABSTRACT: The RNA G-quadruplex is an important secondary structure formed by guanine-rich RNA sequences. However, its folding studies have mainly been studied *in vitro*. Accurate identification of RNA G-quadruplex formation within a sequence of interest remains difficult in cells. Herein, and based on the guanine-rich sequence in the 5′-UTR of *NRAS* mRNA, we designed and synthesized the first G-quadruplex-triggered fluorogenic hybridization (**GTFH**) probe, **ISCH-nras1**, for the unique visualization of the G-quadruplexes that form in this region. **ISCH-nras1** is made up of two parts: The first is a fluorescent light-up moiety specific to G-quadruplex structures, and the second is a DNA molecule that can hybridize with a sequence that is adjacent to the guanine-rich sequence in the *NRAS* mRNA 5′-UTR. Further evaluation studies indicated that **ISCH-nras1** could directly and precisely detect the targeted *NRAS* RNA G-quadruplex structures, both *in vitro* and in cells. Thus, this **GTFH** probe was a useful tool for directly investigating the folding of G-quadruplex structures within an RNA of interest and represents a new direction for the design of smart RNA G-quadruplex probes.

Since RNA can fold onto itself and form fully paired regions or noncanonically paired regions,[1] such RNA secondary structures are crucial for gene regulation and function.[2] In the past years, RNA folding processes have been investigated in detail.[3] However, most experiments were performed *in vitro*. Only a few methods can be directly applied to investigate RNA structures in the cell, and even fewer for noncanonical RNA structures.[4]

RNA G-quadruplexes (G4s) are noncanonical secondary structures that are formed by guanine-rich (G-rich) RNA sequences via stacking of hydrogen-bonded G-quartets.[5] Computational searches for potential RNA G-quadruplexes have identified an overrepresentation of these structures in the untranslated regions (UTRs) of mRNAs and in long noncoding RNAs.[6] Accumulating evidence suggests that RNA G-quadruplexes play important functional roles in the control of a variety of cellular processes.[7] Thus, studying the structure–function relationships of RNA G-quadruplexes has attracted considerable attention.

Similar to the problems encountered while studying RNA secondary structures, approaches for identifying RNA G-quadruplexes have been largely limited to some *in vitro* biophysical techniques and computational prediction methods.[8] The current *in vitro* studies are not sufficient to understand how these RNAs fold and function in their native environment in the cell, which is densely crowded.[9] Notably, several fluorescent probes and antibodies have been developed to investigate RNA G-quadruplexes.[10] However, these molecules are not able to distinguish between different RNA G-quadruplexes, as they label all of the potential RNA G-quadruplexes in cells. Accordingly, there are no molecular probes that can selectively determine a particular G-quadruplex structure formed by specific G-rich RNAs of interest. Such technical difficulties greatly restrict specific investigations on the structure–function relationships of various RNA G-quadruplexes.

We recently developed an isaindigotone-based fluorescent light-up probe specific for G-quadruplex structures, but its selectivity for different G-quadruplexes was not satisfactory.[11] Therefore, we are interested in modifying this probe to develop a much smarter molecule that can individually visualize G-quadruplex structures formed by G-rich sequences of interest. Currently, one of the most common methods for detecting and localizing specific RNAs in cells is fluorescence *in situ* hybridization (FISH) techniques that use fluorescent probes that bind to parts of particular sequences with a high degree of sequence complementarity.[12] Enlightened by the idea of the FISH technique, we determined that attaching an oligonucleotide (anti-tail sequence) that could hybridize with a sequence that is adjacent (tail sequence) to the G-rich sequence of interest in **ISCH-1** would be a designable approach to improve our probe's selectivity. As shown in Figure 1A, we expect that the oligonucleotide will force the fluorophore located beside the defined G-rich sequence to trigger its fluorescent signal upon binding to the G-quadruplex structure. Thus, we called this engineered molecule a G-quadruplex-triggered fluorogenic hybridization (**GTFH**) probe.

The **GTFH** probe was designed to contain the following two parts: (1) the fluorescent light-up moiety derived from **ISCH-1** for identifying the G-quadruplex and (2) an oligonucleotide for directing hybridization. Accordingly, an alkyne side chain was first introduced onto **ISCH-1** to create the new molecule **ISCH-oa1** (Figure 1B, Scheme S1 and Figures S1–S4). This modification enabled a convenient and accessible conjugation

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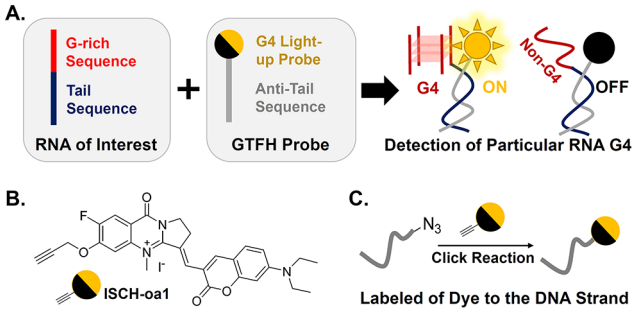

Figure 1. Illustration of **GTFH** probe. (A) **GTFH** probe design principle. (B) Chemical structure of **ISCH-oa1**. (C) **GTFH** probe preparation.

process with commercially purchased azido-modified oligonucleotides via a click reaction (Figure 1C).

To test the application of the **GTFH** probe, the most studied G-rich sequence, **G4T25**, which is located in the 5′-UTR of the *NRAS* mRNA, was chosen as the target (Figure 2).[13] The first

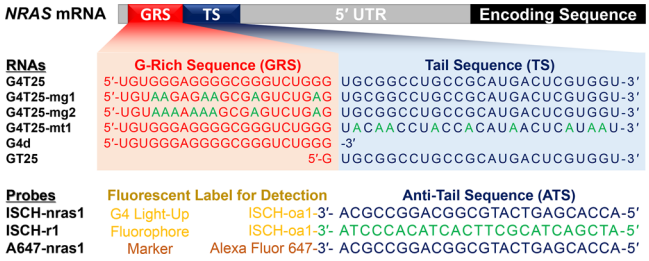

Figure 2. Illustration of G-rich RNA sequences and the related **GTFH** probes used in this study.

GTFH probe, **ISCH-nras1**, which included an **ISCH-oa1** labeled on the 3′-end of a 25-mer DNA sequence complementary to the nearby downstream tail sequence of the G-rich sequence on **G4T25**, was then designed and synthesized through a click reaction. A negative reference called **ISCH-r1**, whose anti-tail sequence was mutated to prevent hybridization, was also prepared (Scheme S2, Table S1, and Figures S5−S6). In addition to **G4T25**, RNAs with mutations (**G4T25-mg1** and **G4T25-mg2**) and deletion (**GT25**) of the G-rich sequence, as well as a mutation (**G4T25-mt1**) and deletion (**G4d**) of the tail sequence, were also used as controls. CD, TDS, and EMSA studies demonstrated that only **G4T25**, **G4T25-mt1**, and **G4d** could form G-quadruplex structures, and the probe **ISCH-nras1** could hybridize with **G4T25**, **G4T25-mg1**, **G4T25-mg2** as well as **GT25** (Figures S7−10).

The fluorescence properties of **ISCH-nras1** with different RNAs were initially investigated by fluorescence spectroscopy *in vitro* (Figure S11). As shown in Figure 3, **ISCH-nras1** alone in buffer displayed weak emission. An emission peak at ∼650 nm appeared and was significantly enhanced upon gradual addition of the RNA G-quadruplex formed by the **G4T25**. Concentration-dependent experiments indicated that the detection limit of **ISCH-nras1** for **G4T25** in solution was 0.024 μM (Figure S12). In contrast, we observed a much weaker fluorescent enhancement or even a decrease in signal when the **G4T25-mg1**, **G4T25-mg2**, **GT25**, **G4T25-mt1**, **G4d** RNAs and other G-quadruplexes were used (Figure S13). Furthermore, in the control experiments, negligible fluorescent enhancement was observed upon addition of **G4T25** into the negative reference **ISCH-r1** (Figure S14A). In contrast, the

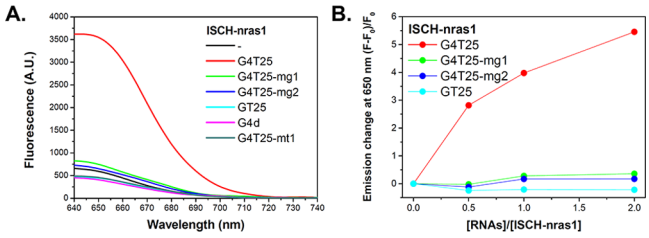

Figure 3. Fluorescence spectrum of **ISCH-nras1** with RNAs. (A) Fluorescence spectrum of 1 μM **ISCH-nras1** with or without 2 μM RNAs. (B) Fluorescence emission change of 1 μM **ISCH-nras1** at 650 nm against the ratio of [RNAs]/[**ISCH-nras1**] at λ_{ex} = 630 nm.

fluorescence of the precursor fluorophore **ISCH-oa1** was greatly enhanced by the **G4T25**, **G4T25-mt1** RNAs and other G-quadruplex structures (Figures S15−S16). Accordingly, these results confirmed that attaching an oligonucleotide to the G-quadruplex probe that could hybridize with a sequence adjacent to the G-rich sequence of interest would improve its selectivity. To further confirm that the **ISCH-nras1** emission originated from the G-quadruplex structure, we incubated **ISCH-nras1** with **G4T25** in several G-quadruplex unfolding conditions.[14] The **ISCH-nras1** emissions in these conditions were obviously weak (Figures S14B, S17−S18). Taken together, the results show that the **ISCH-nras1** probe has promise for the selective detection of *NRAS* RNA G-quadruplex.

Encouraged by the *in vitro* results, we prepared to transfect the **ISCH-nras1** into cells. In view of the extremely low concentration of a particular RNA in a single cell, targeted RNA secondary structure detection in cells can be achieved by either amplifying the fluorescent signal or transfecting the RNA of interest into cells.[12a,15] At this stage, we chose to transfect the RNAs that we used in the *in vitro* experiments into cells to further validate the performance of **ISCH-nras1** (Figure 4A). The conventional FISH probe **A647-nras1** (Figure 2) was used as a marker to study the transfection efficiency and hybridization activity in cells.

As shown in Figure 4B, upon transfection of the RNAs containing the native tail sequence, the cell samples stained by **A647-nras1** exhibited strong fluorescent spots in the cytoplasm. The approximate number of spots for **A647-nras1**

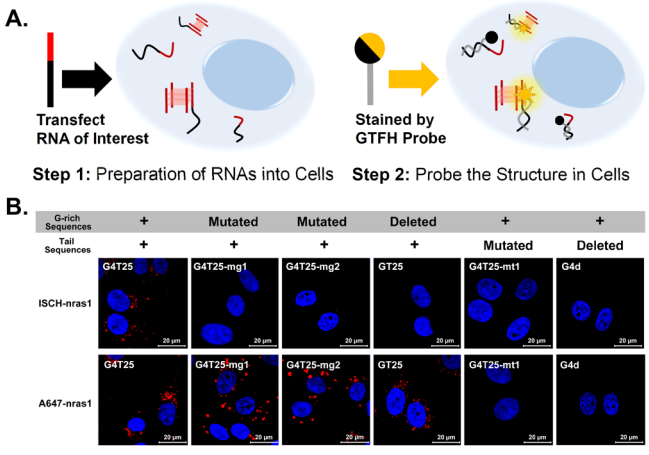

Figure 4. Detection of the targeted RNA G-quadruplexes inside cells. (A) Illustration of the transfection and tracking of the RNAs inside cells. (B) Confocal imaging of RNA-transfected cells stained with **ISCH-nras1** and **A647-nras1**.

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among the different cell samples revealed similar and stable transfection efficiency under our experimental conditions (Figure S19). Then, the cell samples were stained with **ISCH-nras1**. Remarkably, fluorescent spots were observed only in the cell samples containing **G4T25**, and not in the cells containing **G4T25-mg1**, **G4T25-mg2**, or **GT25**. This result was consistent with the *in vitro* fluorescence studies, showing that **ISCH-nras1** exhibits considerable selectivity for the G-quadruplex formed by **G4T25**. Notably, no fluorescent signal was observed in the cells transfected with **G4T25-mt1** or **G4d** and stained with **A647-nras1** and **ISCH-nras1**, suggesting the complementary sequence is crucial for recognition. To better understand their hybridization in cells, RNase A and H digest experiments were conducted in the cells transfected with **G4T25** (Figure S20).[16] The original enhanced fluorescence signals due to **A647-nras1** and **ISCH-nras1** in the cytoplasm clearly disappeared after RNase A and H treatment. The addition of the complementary strand **P25c** to the tail sequence of **G4T25** in the staining processes also resulted in the disappearance of the fluorescent signals (Figure S21). The **ISCH-r1** and **ISCH-oa1** probes that could not hybridize with the tail sequence of **G4T25** also showed negligible fluorescence in the experiments (Figure S22). Taken together, these results confirmed that hybridization occurred between **ISCH-nras1** and **G4T25** inside cells and that **ISCH-nras1** can be used to visualize the specific G-quadruplex structures formed by this G-rich sequence.

To further ascertain the **ISCH-nras1** emission originated from its fluorescent light-up moiety binding to G-quadruplex structure in cells (Figure S23A), the complementary strand **G4c** to the G-rich sequence and the ligand **IZCM-7**, which binds to the *NRAS* G-quadruplex (Figure S24), were used to unfold the G-quadruplex and displace the **ISCH-nras1** light-up moiety from the targeted G-quadruplex structure, respectively.[17] The original enhanced fluorescence due to **ISCH-nras1** in the cytoplasm gradually decreased after **G4c** or **IZCM-7** treatment (Figure S23B). Accordingly, these results confirmed the function of the G-quadruplex light-up moiety in the **GTFH** probe.

Aside from its sensing mechanism, the selectivity of **ISCH-nras1** was also confirmed by a competition assay, in which we tested the ability of **ISCH-nras1** to retain enhanced fluorescence emission by cotransfecting cells with **G4T25** and the abundant G-rich competitors (Figures S25–S26).[18] In the assay, **G4T25** was labeled with FAM for tracking in cells. In the presence of various amounts of competitor, the enhanced fluorescence of **ISCH-nras1** with the G-quadruplex formed by **G4T25** was only slightly affected. Overlap with the FAM-labeled **G4T25** was evident. These results demonstrate the promising potential of **ISCH-nras1** to serve as a highly selective probe for the specific G-quadruplex structure formed by the **G4T25**, even in harsh competitive environments.

The full-length *NRAS* 5′-UTR RNA contains 254 nucleotides, which is much longer than the **G4T25** sequence. We are curious to know whether **ISCH-nras1** could be applied to detect the G-quadruplex structure within the full-length RNA because previous nucleic acid probes labeled with FRET fluorophores or pyrene excimers could not achieve this goal.[19] To this end, full-length *NRAS* 5′-UTR RNAs (**UTR-full**) were prepared as validation models and transfected into cells. Aside from **UTR-full**, long RNAs with a mutation (**UTR-mutG**) or deletion (**UTR-delG**) of the G-rich sequence, as well as a mutation (**UTR-mT25**) of the tail sequence, were also used as controls. As shown in Figure 5, only cells transfected with **UTR-full** could be stained by **ISCH-nras1**. Mutation or

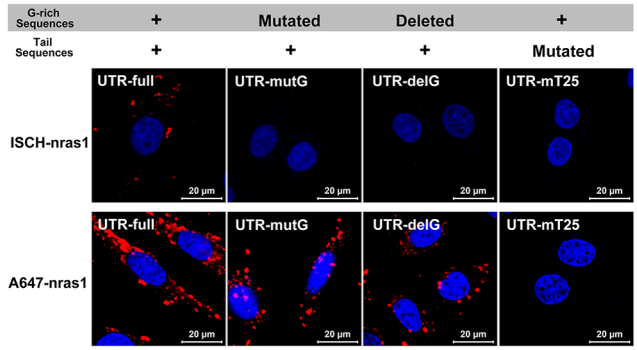

Figure 5. Confocal imaging of cells transfected with long RNAs and stained with **ISCH-nras1** and **A647-nras1**.

deletion of the G-rich sequence in full-length RNA inhibited the fluorescence signal. By contrast, the cell samples stained with **A647-nras1** exhibited strong fluorescence spots in the cytoplasm. Additionally, no fluorescent signal was observed when the cells containing **UTR-mT25** were treated with **ISCH-nras1** and **A647-nras1**. These findings are consistent with the results observed from the above staining assays, showing that **ISCH-nras1** displays practical and promising application prospects for the selective detection of the specific G-quadruplex formed by the G-rich sequence within the short and long forms of the *NRAS* mRNA 5′-UTR.

In summary, we have successfully developed the first **GTFH** probe, **ISCH-nras1**, for the unique visualization of the G-quadruplex structure formed by the G-rich sequence within the 5′-UTR of *NRAS* mRNA. Notably, the synthesis of this probe is convenient and accessible. Researchers can design and synthesize their own **GTFH** probes for investigating the folding of specific G-quadruplex structures within RNAs of interest in cells by following the idea and application examples given in this study. The smart **GTFH** probe represents a novel tool for deciphering the folding of individual RNA G-quadruplex structures and understanding how G-rich RNA structures evolved within cells. Concentration-dependent experiments demonstrated the detection limit of **ISCH-nras1** for *NRAS* RNA was about 0.1 fmol per cell (Figures S27–S28). Thus, in the present visualization approach, RNAs needed to be transfected into cells in view of the extremely low concentration of particular RNAs in a single cell. Notably, we constructed a 5′-UTR of the *NRAS* mRNA reporter and transfected the plasmid into cells to increase the endogenous RNA concentration. After treatment of **ISCH-nras1**, enhanced fluorescence could be found by quantification of cells using high-content imaging platform (Figures S29–S30), but the *in situ* detection of spots of particular RNA G-quadruplex in a single cell is still a challenge (Figure S31). The design of the **GTFH** probe and the collective results from this study may be the first steps to achieving this aim. Further improvements on the probe to enable the *in situ* visualization of particular G-quadruplex, including modification of the fluorescent light-up moiety and the nucleotides or length of anti-tail sequence are now underway.

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■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b04799.

Synthesis and characterization of the probes, experimental procedures, and supplemental spectra and graphs (PDF)

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Notes

The authors declare no competing financial interest.

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