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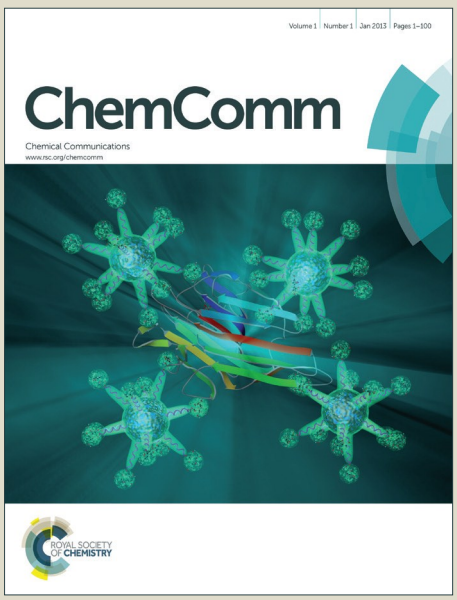

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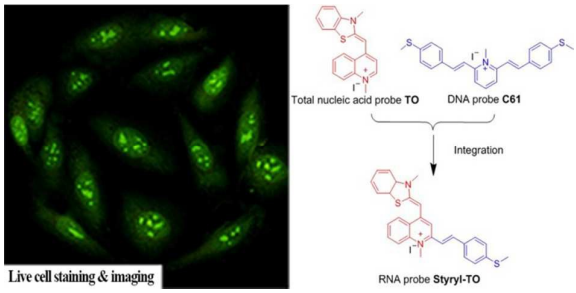
Graphical Abstract



Journal Name

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Molecular Fluorescent Dye for Specific Staining and Imaging of RNA in Live Cells: a Novel Ligand Integration from Classical Thiazole Orange and Styryl Compounds

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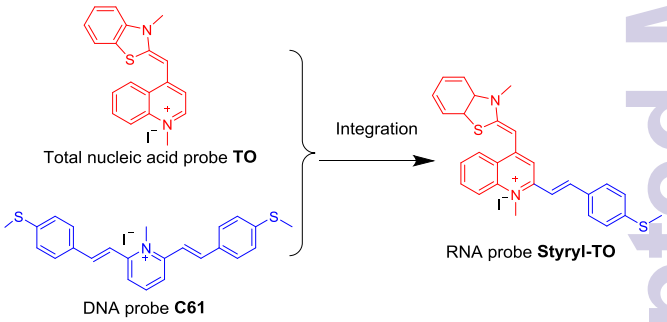
A new RNA-selective fluorescent dye integrated with a thiazole orange and a *p*-(methylthio)styryl moiety shows better nucleolus RNA staining and imaging performance in live cells than the commercial stains. It also exhibits excellent photostability, cell tolerance, and counterstain compatibility with 4',6-diamidino-2-phenylindole for specific RNA-DNA colocalization in bioassays.

Live cell imaging or staining by target-specific molecular fluorescent probes is a very important and useful technology for medical diagnosis and biomedical research because it allows the investigation of the distribution, migration and transcriptional dynamics of cell nucleus.[1] RNA molecules in living cells are known responsible for a wide variety of functions including physical transportation, interpretation of genetic information, regulation of gene expression, and some essential bio-catalytic roles.[2] However, the information on distribution dynamics and transcriptional activities of RNA in cell nucleus and the relationship with special secondary structures of DNAs, such as G-quadruplex and temporal/spatial processing of RNA, is still limited to-date.[3] It is probably due to the limiting RNA-selective technology has been developed during the past decades.

In recent years, a considerable amount of effort has been done for the development of small-molecule-based fluorescent dyes for RNA imaging in live cells.[4] Some fluorescent molecular ligands like crescent-shape and V-shape probes[5a,b], styryl and **E36** dyes,[5c,d] and a near-infrared probe[5e] were examined for RNA detection. In comparison with DNA dyes, a common difficulty encountered is the low specificity of small molecular dyes for RNA, especially the nucleus RNA dyes for live cell imaging, due to small molecules usually have better affinity to DNA. The poor nuclear membrane permeability of the RNA dyes is also a big problem to be solved.[6] SYTO RNASelect is the only commercially available dye for RNA imaging in live cells, but its molecular structure is not known for further modification in order to fit for different purpose. Molecular design of new target-specific fluorescent compound is the key to address the problems. Styryl-based molecular dye have been recently reported and proved to be selective binding with RNA[5c] in nucleoli and cytoplasm of live cells[5d]. The findings indicate that the molecular structure of styryl is crucial for RNA-specific binding.[7a-c] In addition, thiazole orange (**TO**, Scheme 1) is a well-known and widely used nucleic acid fluorescent probe due to its high fluorescence quantum yield. **TO** conjugates have recently been reported as a G-quadruplex-selective fluorescent probe and demonstrated as a good structural platform for designing novel fluorescent probes targeting on different types of nucleic acids.[8] However, **TO** has never been explored as a RNA dye because of its poor selectivity.



Scheme1. Molecular design of **Styryl-TO** through ligand integration of thiazole orange **TO** and DNA dye **C61**.

1-Methyl-2,6-bis(4-(methylthio)-styryl)-pyridinium iodide **C61** is an example of styryl compounds, which is also known as a promising green fluorescent DNA probe.[9a] The most attractive property of **C61** is that it provides a broader color option for biological imaging[9b] and *p*-(methylthio)styryl

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moiety has an unique functionality for discrimination of dsDNA and RNA[9c]. We therefore attempt to design a novel RNA-specific fluorescent dye that possesses both of the advantages of **TO** and **C61** by integrating their special scaffolds into a single molecule. The concept of ligand integration to merge with two or more unique characteristics and functionality of small organic molecules could be a promising approach for RNA-specific dyes development. Herein, we report a new RNA-specific switch-on fluorescent dye (**Styryl-TO**), which is designed by the integration of a **TO** structure and a *p*-(methylthio)styryl moiety of **C61** and demonstrate its performance for nuclear RNA staining and imaging in live cells.

Table 1. Fluorescence characteristics of **Styryl-TO** and its binding constants with different nucleic acids.

Nucleic acid	B shift (nm)[a]	Concentration of nucleic acid[b] (μM)	K_d ($x10^5 M^{-1}$)[c]
RNA	5	15	12.32
ds26	12	12	4.55
htg21	10	16	3.22
st-DNA	11	8	1.78
da21	15	8	2.17

Concentration of **Styryl-TO**: 5μM. ds26: self-complementary duplex DNA; htg21: telomere G-quadruplex; st-DNA: salmon testes DNA; da21: Single-stranded purine.
[a] Hypsochromic shifts; [b] Concentration of nucleic acids used for the fluorescence intensity reaching a plateau; [c] Equilibrium binding constants.

Styryl-TO was prepared by integrating a **TO** and a *p*-(methylthio)styryl moiety of **C61** through multi-step synthesis (Scheme S1, ESI†). To prove the concept, the functionality, binding specificity, and fluorescent property of the new dye interacting with RNA and various DNAs were investigated by fluorescence titration. The fluorescence intensity increases approximately 152±23 folds upon **Styryl-TO** binding with RNA as shown in Figure 1a. Nevertheless, under similar conditions, the treatment with a wide variety of DNA substrates including duplex DNA (salmon testes stDNA, ds26: self-complementary duplex DNA), single-stranded DNA (da21), and G-quadruplex DNA (htg21), it induced much smaller fluorescence signal enhancement (Figure 1b). This new molecular scaffold surprisingly changes the binding preference of **TO** moiety to RNA rather than DNA; it also retains the merit fluorescence-signalling property of **TO** dyes. **Styryl-TO** only shows very weak background fluorescent signal in blank solutions without RNA. In order to further explain the selectivity of the dye towards RNA, the equilibrium binding constants (K_d) of the compound and different nucleic acids were investigated and calculated (Table 1). Obviously, the RNA shows much bigger binding affinity (from 3 to 7 folds in terms of K_d values) than other DNA substrates, which indicated that **Styryl-TO** has higher specificity towards RNA. The good specificity is attributed to the new integrated *p*-(methylthio)styryl moiety which is proved

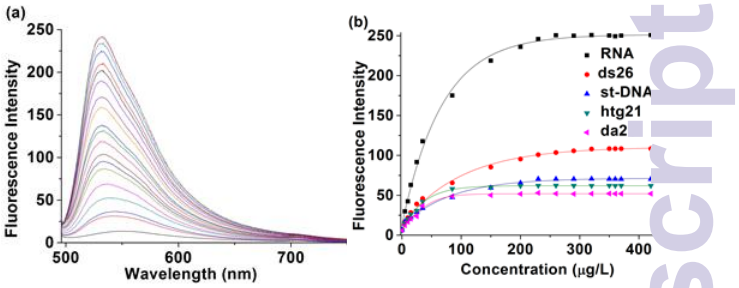

Figure 1. (a) Fluorescence titration of **Styryl-TO** with an increasing concentration of RNA (λ_{ex} = 476 nm); (b) The plots of fluorescence intensity (λ_{em} = 535nm) versus various nucleic acids at different concentrations.

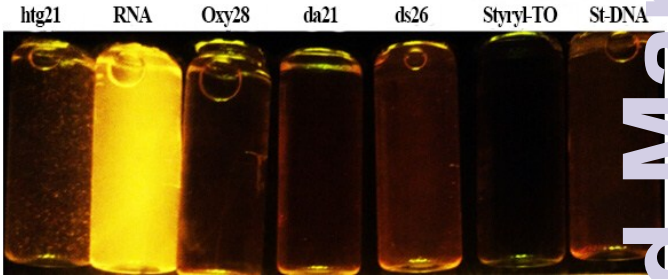

Figure 2. The fluorescence changes of **Styryl-TO** under UV-illumination (λ_{ex} = 302 nm) with the presence of various nucleic acids: G-quadruplex htg21, Oxy28, Single DNA da21, duplex DNA ds26 and St-DNA in Tris-HCl buffer.

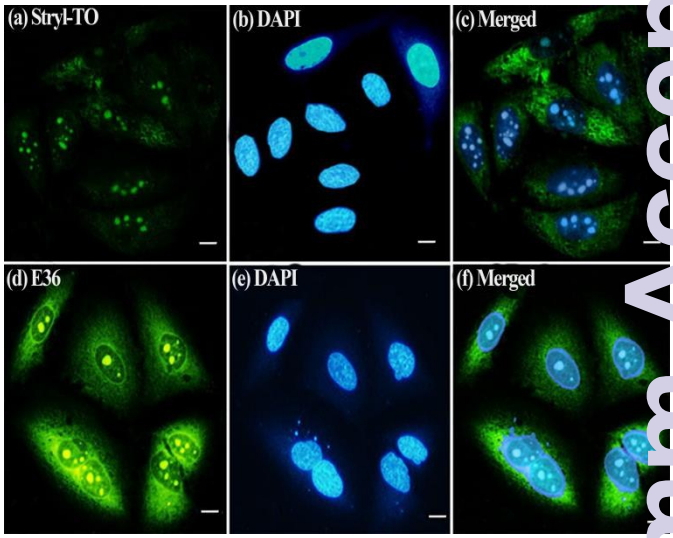

Figure 3. (a) - (c): PC3 cells staining and counterstaining with **Styryl-TO** and **DAPI**; (d) - (f):comparison study with **E36** and **DAPI**. The images were taken under FITC channel for **Styryl-TO** and **DAPI** channel for **DAPI**. 1000× magnification was utilized in the imaging. Scale bar is 10 μm.

binding RNA with high selectivity[5b-e,7a-c,9c]; **TO** and its analogues are known unable to differentiate RNA from DNA.[10]

Photophysical properties of **Styryl-TO** in both aqueous and organic media were studied and the results (Table S3 & Figure S7, ESI†) showed the fluorescence quantum yields (Φ_f) of the dye in aqueous is much lower (Φ_f = 1.6 $x10^{-3}$) than that in dichloromethane (Φ_f = 5.3 $x10^{-3}$). Interestingly, the dye upon bound with RNA in aqueous is significantly increased about 35

times (Φ_f = 5.6 x10^{-2}). The RNA induced fluorescence signal (orange) is strong enough for observing by naked eye under UV-illumination as shown in Figure 2.

To study the higher-order nuclear organization of RNA molecules, cell staining with a fluorescent RNA-specific dye is needed to counter labelled with **DAPI**, which is a DNA-selective dye.[11a] Figure 3a shows the fluorescence imaging for the treatment of PC3 cells(human prostate cancer cell line) with **Styryl-TO** that produces a strong and bright fluorescence response signal mainly confining in certain regions of the nucleus. Some green spots with weak brightness are also observed in the cytoplasm. A reported RNA probe[11b-c] **E36** was also employed for performance comparison (Figure 3d-f). **E36** stains the nucleus but most regions of the cell are also stained (Figure 3d). The comparison study indicates that **Styryl-TO** shows much better RNA specific staining performance in cells. The cellular localization of **Styryl-TO** in PC3 cells is similar to that of **E36** and other RNA fluorescent probes. In addition, the fluorescent intensity of **Styryl-TO** in the nucleoli where RNA undergoes transcription is found much higher than that of in the nucleus; however, no cell morphology or viability change was observed during the imaging experiments. Figure 3c further demonstrates the cell imaging performance of **Styryl-TO** by co-staining with the blue nucleus dye **DAPI** in the FITC or Cy3 channel. **Styryl-TO** exhibits a very low fluorescence response to DNA and good counter labelling properties with the DNA-selective dye **DAPI** in the living PC3 cells.[5e] The images clearly reveal different patterns of RNA-DNA co-localization in the live cells.[11d] This observation further supports that **Styryl-TO** has a very good counterstaining compatibility with the DNA-selective dye (**DAPI**). In addition, the utility of the dye was also demonstrated in other cell lines (HUVEC, NIH-3T3, L929) and it gave very good RNA specificity and imaging performance (Figure S10B, ESI†).

To confirm the specificity of **Styryl-TO** towards RNA in cells, deoxyribonuclease (DNase) and ribonuclease (RNase) digest tests were performed.[12] In the DNase digest test, only DNA substrates are hydrolysed in the cells while in the RNase digest test, only RNA substrates are hydrolyzed. The fixed-permeabilized PC3 cells were used in the experiment and **E36** and SYTO RNASelect were selected as the control. As expected, in the DNase digest test (Figure 4 DNase), no obvious diminishing of fluorescence in the nucleoli stained with **Styryl-TO** was found; in contrast, for RNase digest test, the originally intensive fluorescence signal of the nucleoli stained with **Styryl-TO** in cells was dramatically disappeared (Figure 4 RNase). Similar photo-behaviour was also observed in the control study with **E36** and SYTO RNASelect. These results evidently indicate that the enhanced fluorescence signal is originated from the interaction of **Styryl-TO** with RNA in the nucleoli of PC cells.[13]

Photostability is always a key factor to determine the usefulness of a dye for cell imaging. **Styryl-TO** is evaluated in live PC3 cells with an inverted fluorescence microscope. Figure 5 shows the photo-stability comparison of SYTO RNASelect and **Styryl-TO**. The fluorescence intensity of SYTO

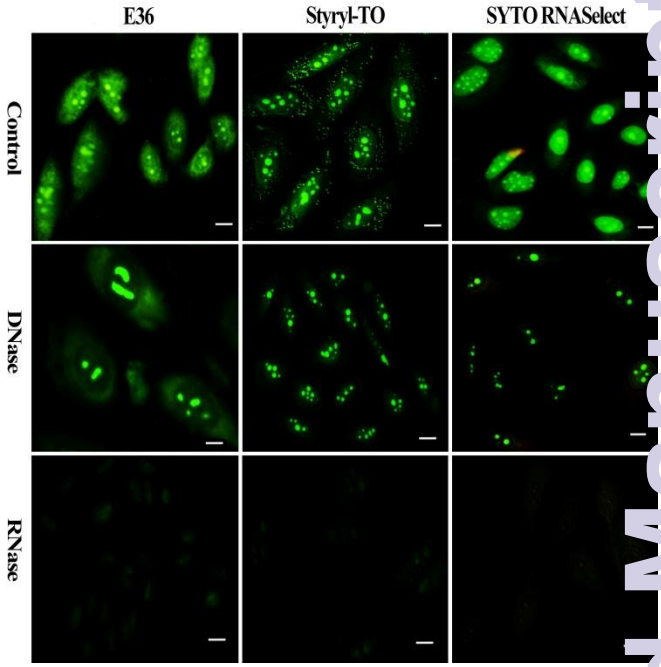

Figure 4. DNase and RNase digest test images of **E36**, **Styryl-TO**, and SYTO RNASelect. Equal exposure was used for the same dye imaging. **E36**, **Styryl-TO**, and SYTO RNASelect were tested in the concentration of 5 µM. **E36**, **Styryl-TO** and SYTO RNASelect (green: FITC channel) are shown. 1000× magnification was utilized in the imaging. Scale bar is 10 µm.

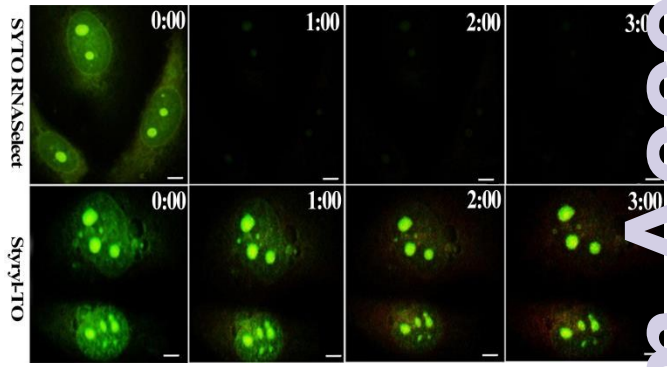

Figure 5. Time-lapse imaging of nucleolus RNA in PC3 cells with SYTO RNASelect (top) and **Styryl-TO** (bottom) in the range of 0-3 h. 1000× magnification was utilized in the imaging. Scale bar is 10 µm.

RNASelect was found decreased more than 80% after 1 h irradiation while the intensity of **Styryl-TO** was almost no changes. After 3 h, the fluorescence signal of SYTO RNASelect was completely disappeared; nonetheless, the fluorescence signal of **Styryl-TO** in the nucleoli of the PC cells was still retained obviously (approximately 90% of the intensity in beginning). Moreover, the photo-stability of **Styryl-TO** in solution conditions was examined and it also exhibited better stability than SYTO RNASelect (Figure S11, ESI†). The experiments conducted in a buffered RNA solution, under continuous irradiation for 100 min and 600 scans, the enhanced fluorescence signal of **Styryl-TO** shows no observable

intensity changes; while in the case of SYTO RNASelect, only about 25 % of its original intensity was retained. The results demonstrate that **Styryl-TO** is a robust RNA-specific dye for staining and imaging of nucleolar RNA in live cells and it is particularly attractive for the experiments require long time irradiation.[14]

In conclusion, a new and robust RNA-specific switch-on fluorescent dye was developed from ligand integration of a classical nucleic acid fluorescent probe and a DNA dye moieties. The newly developed molecular scaffold holds both the merit properties of their parent compounds. The study demonstrated the concept of ligand integration and proved that the dye is able to offer excellent RNA specificity, photo-stability, and cell tolerance. The dye shows very good counterstain compatibility with **DAPI** for specific RNA-DNA colocalization investigations in live cells. Also, the dye was successfully utilized in staining of nucleolus RNA for cell imaging and its performance is found much better than the commercial SYTO RNASelect dye.

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Graphical Abstract