Light-up Probes

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Contact-Mediated Quenching for RNA Imaging in Bacteria with a Fluorophore-Binding Aptamer**

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The absence of inherently fluorescent RNA has hampered the detection and imaging of RNA in living cells for a long time. Considering the essential roles and rich dynamics of RNA in cells, there are only a handful of methods to image RNA in a cellular environment. Among the more conventional methods are molecular beacons (MBs), hairpin-shaped small oligonucleotides with a fluorophore on one end and a quencher on the other,[1] and green fluorescent protein (GFP) fused to sequence-specific RNA binding proteins.[2,3] Both approaches have their merits, but the former suffers from delivery, stability, and cellular distribution of MBs, whereas the latter might alter the function of the target RNA due to the size of the tag.[4]

Recently, small-molecule-based RNA labeling methods have been of great interest owing to their good cell permeability and structural as well as spectral flexibility. Small RNA motifs (aptamers) that can stably bind to conditionally fluorescent dyes, which are non-fluorescent in solution but highly fluorescent when bound to a specific RNA aptamer, have already been identified using the in vitro selection system SELEX,[5] and they can potentially be used as tags for RNA labeling. Another approach to design fluorogenic probes to label RNA was the use of fluorophore-quencher conjugates in combination with quencher-binding aptamers where the quenching occurs via Förster resonance energy transfer (FRET) or photoinduced electron transfer (PeT).[6] Upon binding of aptamers to the dual-labeled probe, fluorescence increases, but the reported turn-on ratios were relatively low and the dissociation constants were high. Lately, a "Spinach" aptamer that binds 3,5-difluoro-4-hydroxybenzylidine imidazolinone (DFHBI), resembling the fluorophore in GFP, has been developed and utilized for in vivo RNA imaging, which was the first example of RNA imaging with small molecules in living cells.[7]

Herein we present an alternative approach based on contact quenching of fluorophores and fluorophore-binding aptamers to image RNA with small molecules in living cells. The reported aptamers binding to fluorescein,[8] sulforhodamine B,[8] 5-carboxytetramethylrhodamine (TAMRA),[9] and rosamine[10] have not found many applications for studying biological processes, which is mostly due to high background fluorescence. Thus, we converted fluorophores into fluorescent turn-on probes, which are non-fluorescent in solutions but become highly fluorescent upon binding to specific RNA aptamers (Figure 1 a). In principle, fluorophores were conjugated to small molecules that can efficiently quench the fluorescence via contact quenching. The direct (contact) interaction between fluorophore and quencher should induce the formation of a non-fluorescent intramolecular complex (OFF state).[11] However, in the presence of a fluorophore-binding aptamer, the fluorophore would prefer to interact with the aptamer rather than with the quencher, which would enhance the fluorescence (ON state). To test this hypothesis, we used the sulforhodamine B fluorophore (SR) and its known binding partner, the SRB-2 aptamer (Figure 1 b,c).[8] The developed method was then utilized for monitoring in vitro RNA transcriptions in real time as well as imaging RNA in live bacteria.

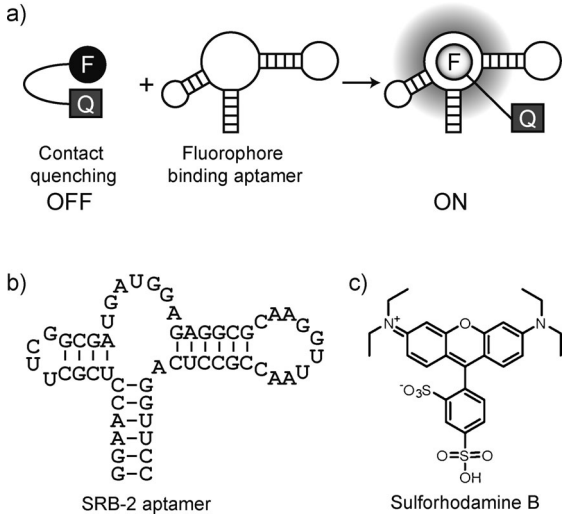

Figure 1. a) Detection principle of the fluorophore-binding RNAs by contact-quenched probes. The fluorophore-quencher (F-Q) conjugate is not fluorescent owing to contact quenching, and it becomes fluorescent upon binding to the RNA aptamer. b) Secondary structure of minimal SRB-2 aptamer. c) Structure of sulforhodamine B.

First, we sought to find an optimal contact quencher that has an absorbance spectrum that does not significantly overlap with the emission spectrum of SR to avoid quenching by FRET. Known quenchers *p*-nitrobenzylamine (MN), dinitroaniline (DN), trinitroaniline (TN), carbazole (CZ), and 7-hydroxycoumarin-3-carboxylic acid (CM)[12] were attached to the fluorophore (Figure 2 a) and their fluorescence

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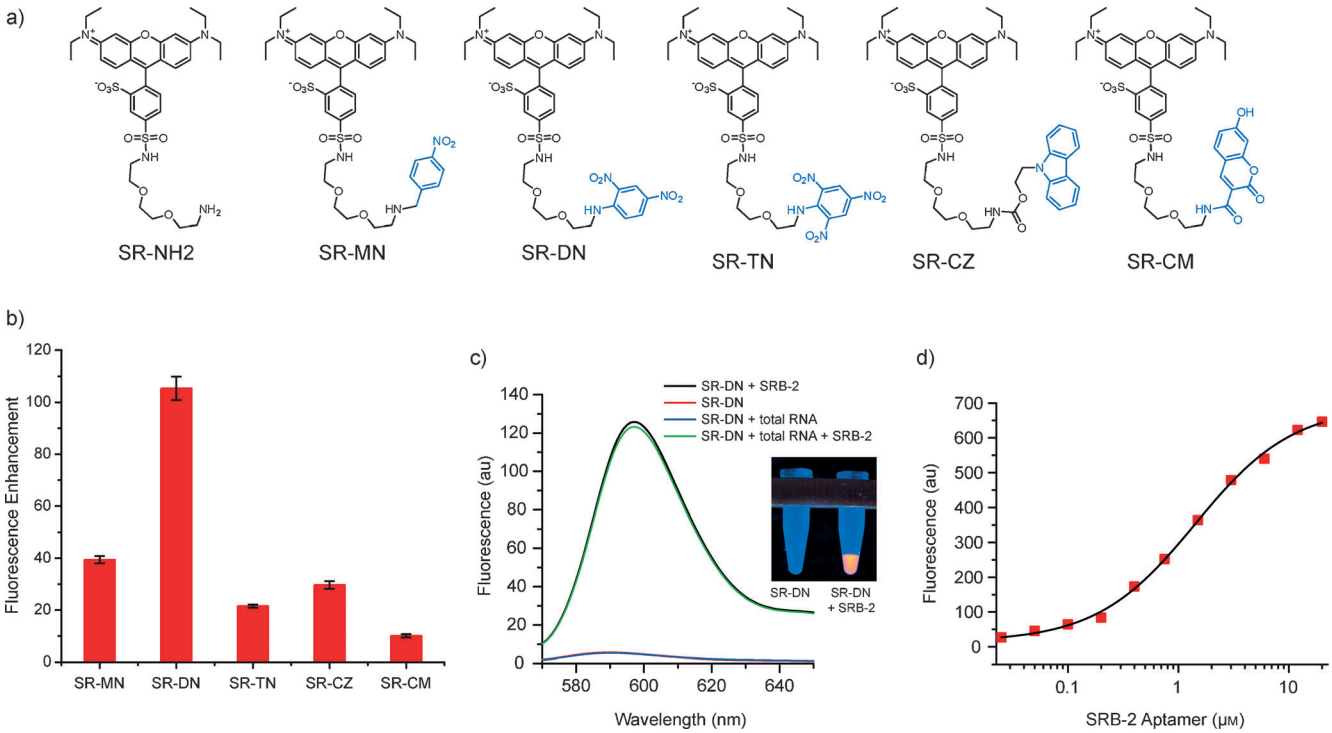

Figure 2. a) Synthesized sulforhodamine B-contact quencher conjugates. b) Fluorescence enhancement factors, $F_{(RNA:Probe)}/F_{(Probe)}$, of the probes upon binding to the SRB-2 aptamer. Fluorescence spectra of the probes (1 μM) before and after binding to excess SRB-2 aptamer (20 μM) were recorded. Samples were excited at 580 nm, and the fluorescence emissions were recorded at 596 nm. c) Fluorescence emission spectra of SR-DN (1 μM); in the presence of SRB-2 aptamer (0.2 mg mL^{-1}, 10 μM, black), alone (red), in the presence of *E. coli* total RNA (0.5 mg mL^{-1}, blue) and in the presence of both total RNA and SRB-2 (green). An excitation wavelength of 560 nm was used. Inset: fluorescence emission upon excitation with a 366 nm UV hand-lamp. d) Determination of the dissociation constant between SR-DN and SRB-2 aptamer. SR-DN (1 μM) was titrated with increasing amounts of SRB-2 aptamer and the fluorescence increase was measured. $K_d = 1.4 \pm 0.1$ μM at 25 °C.

quenching was evaluated in an aptamer binding assay. The fluorescence value of the respective SR-quencher probe (1 μM) in the presence of excess SRB-2 aptamer (20 μM) was divided by its fluorescence in the absence of SRB-2 (Figure 2b). All of the screened molecules were able to significantly quench the SR fluorescence, and the fluorescence of SR was restored upon binding to the SRB-2 aptamer. The screened molecules MN, DN, TN, CZ, and CM showed 39-, 105-, 22-, 30-, and 10-fold fluorescence enhancement upon binding to SRB-2, respectively. These values compare very favorably to the previously reported FRET or PeT-based quencher-dye systems that yielded 5- to 6-fold fluorescence enhancements upon aptamer (50 μM) binding.[6] The major advantage of contact quenching is that it can be completely abolished by the disruption of the intramolecular heterodimer upon binding of the fluorophore to RNA, while FRET and PeT quenching may not be fully suppressed. The best contact quencher for SR was found to be dinitroaniline (Supporting Information, Figure S1) and was used for further experiments.

To demonstrate the specificity of the interaction between SR-DN probe and SRB-2 aptamer, SR-DN probe was incubated with *E. coli* total RNA and no fluorescence increase was observed (Figure 2c). The dissociation constant (K_d) between SRB-2 aptamer and SR-DN was calculated to be 1.4 ± 0.1 μM, a value that is about five times higher than the previously reported K_d between sulforhodamine B and SRB-2 aptamer[8] (Figure 2d), which is most likely due to the modification of the 5′-sulfonate group in SR, and the free energy required to disrupt the intramolecular heterodimer in SR-DN.

Next, we investigated the spectral properties of the SR-DN probe in detail (Table 1). A change in the absorbance spectrum of the fluorophore is a characteristic feature of contact quenching owing to the orbital interactions between the fluorophore and the quencher.[11,13] We observed that SR-NH2 has an absorbance maximum at 568 nm that is shifted to 574 nm for SR-DN, indicating a significant intramolecular ground-state heterodimer formation (Table 1). Furthermore, SR-NH2 and SR-DN conjugates have exactly the same fluorescence emission maxima at 590 nm, another indication for an intramolecular contact quenching (Supporting Information, Figure S2). The fluorescence quantum yield (QY) of SR-NH2 and SR-DN was determined as 0.34 and 0.026,

Table 1: Spectroscopic properties of free and SRB-2 aptamer-bound SR-DN.

	QY	Abs_{max} [nm]	Em_{max} [nm]
SR-NH2	0.34	568	590
SR-NH2/SRB-2	0.79	579	596
SR-DN	0.026	574	590
SR-DN/SRB-2	0.65	579	596

respectively, indicating a 13-fold decrease that is due to contact quenching. Complex formation between SRB-2 aptamer and SR-DN probe also results in changes in the absorbance and emission spectra: the absorbance maximum for SR-DN/SRB-2 complex is at 579 nm and the emission maximum at 596 nm. These results suggest that the fluorophore-quencher intramolecular complex is disrupted in the presence of SRB-2 aptamer, and that the fluorophore occupies a different microenvironment inside the RNA. This is also reflected in the high observed quantum yield of 0.65 when SR-DN is bound to the SRB-2 aptamer, which is 25 times higher than that of the SR-DN probe and about twice that of SR-NH2. Presumably, the hydrophobic environment and the limited rotational freedom inside the RNA binding pocket increase the quantum yield of the fluorophore.

The fluorophore and the quencher should be correctly oriented in space to interact efficiently with each other and to form a non-fluorescent intramolecular ground state dimer. Thus, the linker length between fluorophore and quencher is expected to play an important role. We synthesized four different SR-DN probes with a different number of ethylene glycol units ($n = 0, 1, 2, 3$), and measured their quantum yields and dissociation constants (Figure 3). The lowest quantum

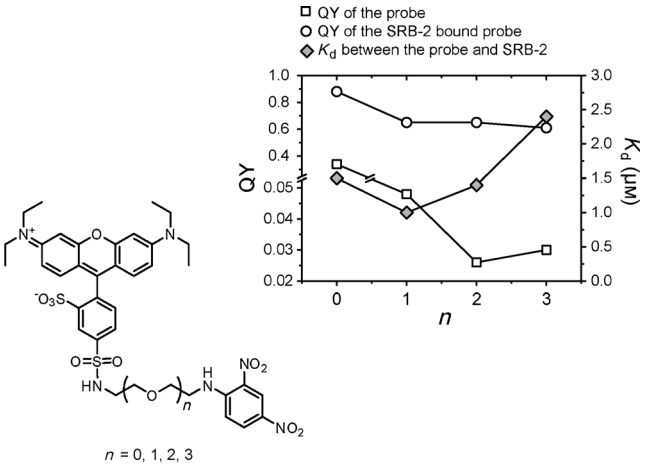

Figure 3. The structure of SR conjugated to DN through polyethylene glycol linkers with different length. Fluorescence quantum yields of free and SRB-2 bound probes as well as K_d of the complexes were shown. *n* denotes the number of ethylene glycol units in the linker.

yield of the free probes was observed for $n = 2$. Shorter linkers ($n = 0, 1$) may not allow fluorophore and quencher to interact properly in space. The probe with the longer linker ($n = 3$) showed only a slightly increased quantum yield. Aptamer-bound probes all showed similar quantum yields, and the dissociation constants varied in a range from 1 to 2.5 μM (Supporting Information, Figure S3).

We then applied the SR-DN probe to monitor the in vitro transcription of an arbitrarily chosen RNA of interest (ROI, 102 nucleotides) in real time. To prepare the template DNA, T7 promoter and SRB-2 sequences were fused to the 5′ and 3′ end of the ROI, respectively (Figure 4a). The time-dependent transcription of ROI-SRB-2 fusion as well as the ROI without

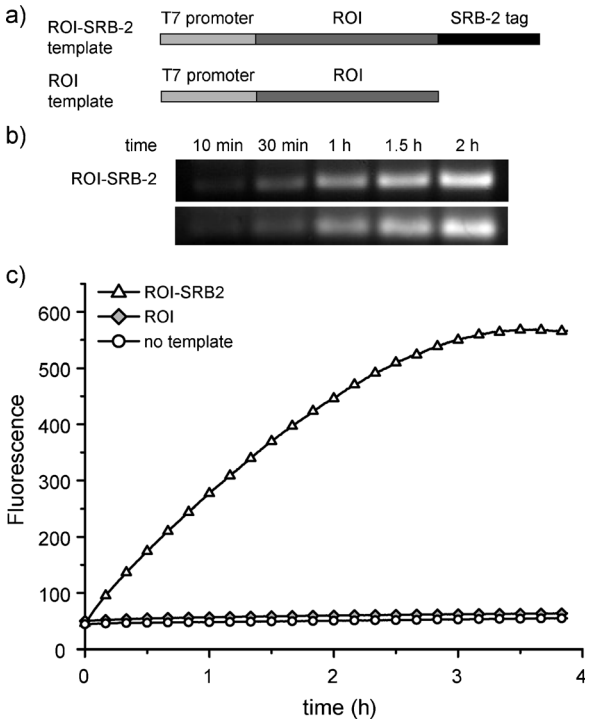

Figure 4. Monitoring in vitro transcription in real time. a) The SRB-2 sequence was fused to the 3′ end of an arbitrarily chosen RNA of interest (ROI), which has T7 promoter sequence at the 5′ end. The control template does not contain the sequence of SRB-2. b) Progress of the transcription was confirmed by agarose gel electrophoresis for both SRB-2 and control templates using ethidium bromide staining. c) Monitoring transcription of ROI-SRB-2 in real-time in the presence of SR-DN probe.

SRB-2 tag was confirmed by agarose gel electrophoresis (Figure 4b). Then, the transcription of the ROI-SRB-2 fusion was monitored at 37 °C in the presence of SR-DN probe. As shown in Figure 4c, the fluorescence increases over time as SR-DN binds to the transcribed ROI-SRB-2 fusion. On the other hand, in control reactions, where either no template or template without the SRB-2 sequence was added into the reaction mixture, no fluorescence increase could be observed.

Finally, we investigated whether RNA can be imaged in live bacteria by using SRB-2 aptamer and the fluorogenic probe SR-DN. For stabilization against nucleolytic degradation, the SRB-2 sequence was placed within a tRNA scaffold structure (Supporting Information, Figure S4)[14] and cloned into pET28 bacterial expression plasmid (pET28-SRB-2). The tRNA scaffold did not affect the affinity of the SRB-2 aptamer to the probe and the quantum yield of the aptamer bound fluorophore (Figure S5). A control plasmid with only the tRNA scaffold (pET28-tRNA) was also prepared. *E. coli* was transformed with these plasmids and transcription was induced by the addition of IPTG. After brief incubation with SR-DN (1 μM) at 37 °C, a significant fluorescence signal was observed in the bacteria transformed with the SRB-2 carrying plasmid, whereas no fluorescence was detected in bacteria carrying the control plasmid (Figure 5). Quantitative real-time PCR data proved that both plasmids produce similar amounts of transcripts in bacteria (Supporting Information,

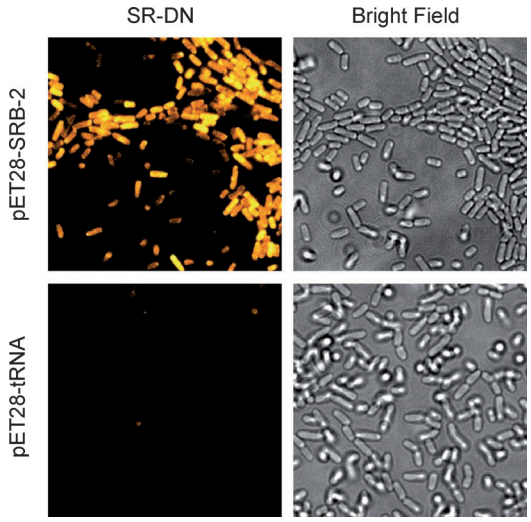

Figure 5. Imaging SRB-2 aptamer in live bacteria with SR-DN probe. Bacteria were transformed with either pET28-SRB-2 (top row) or pET28-tRNA (bottom row) plasmid. Transcription of aptamers was induced by the addition of IPTG (1 mM). Bacteria were briefly incubated with SR-DN probe (1 μM) and imaged at 37 °C without any washing step.

Figure S6), yet only the SRB-2 transcribing bacteria were selectively labeled. Furthermore, leaving out IPTG induction step completely removed the fluorescence signal from bacteria harboring the SRB-2 plasmid (Supporting Information, Figure S7). It should be noted that bacteria were not washed and the excess probe was not removed from the medium during imaging, highlighting the advantages of the turn-on probe. Moreover, we did not observe any toxic effects of SR-DN to bacteria (Supporting Information, Figure S8).

In summary, we identified dinitroaniline as an efficient contact quencher for sulforhodamine B. Fluorescence of the SR-DN conjugate is significantly reduced when free in solution, but increases more than 100-fold upon binding to the SRB-2 aptamer. Then, the SRB-2 aptamer was used to monitor an in vitro transcription in real time. Moreover, we could image the SRB-2 aptamer in live *E. coli* with the SR-DN probe, demonstrating the utility of SRB-2 as a genetically encoded tag for biological applications. The modular nature of this RNA labeling approach may permit the development of various turn-on probes with different colors and their respective aptamers with better affinities. In the future, this may allow the detection of low abundant RNA and imaging of multiple RNAs simultaneously.

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