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Using a Specific RNA–Protein Interaction To Quench the Fluorescent RNA Spinach

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Supporting Information

ABSTRACT: RNAs are involved in interaction networks with other biomolecules and are crucial for proper cell function. Yet their biochemical analysis remains challenging. For Förster Resonance Energy Transfer (FRET), a common tool to study such interaction networks, two interacting molecules have to be fluorescently labeled. “Spinach” is a genetically encodable RNA aptamer that starts to fluoresce upon binding of an organic molecule. Therefore, it is a biological fluorophore tag for RNAs. However, spinach has never been used in a FRET assembly before. Here, we describe how spinach is quenched when close to acceptors. We used RNA–DNA hybridization to bring quenchers or red organic dyes in close proximity to spinach. Furthermore, we investigate RNA–protein interactions quantitatively on the example of Pseudomonas aeruginosa phage coat protein 7 (PP7) and its interacting pp7-RNA. We utilize spinach quenching as a fully genetically encodable system even under lysate conditions. Therefore, this work represents a direct method to analyze RNA–protein interactions by quenching the spinach aptamer.

RNAs fulfill crucial functions in cellular processes such as gene regulation, nuclear organization, or RNA metabolism. They play regulatory roles in diverse signaling pathways.1,2 Also, they bring in essential proteins and prepare DNA for transcription.3 Moreover, RNA can function as decoy and drag proteins away from their original localization or bring proteins together in large complexes as scaffolds.4,5 Dysregulation or malfunction of their interaction networks with other biomolecules is involved in the onset and progression of neurodegenerative and cardiovascular diseases, as well as cancer.6–8 For detailed understanding of those processes and their connected diseases, homogeneous methods for the investigations of RNA interaction networks are essential but remain challenging. Genetically encodable tags such as fluorescent proteins (e.g., the green fluorescent protein, GFP) have revolutionized the field of protein biochemistry.9–11 As every fluorophore, those biological ones can undergo Förster Resonance Energy Transfer (FRET), once in close proximity to a suitable second fluorescent partner due to dipole–dipole interactions. For optimal FRET efficiencies, donor and acceptor must be oriented in parallel, whereas FRET cannot occur if both fluorophores are perpendicular to each other. The readout is either an acceptor intensity increase or donor quenching. FRET is an essential tool for the investigation of protein–protein interaction networks.12,13 For the analysis of RNA–protein interaction networks direct labeling of the RNA, encodable on genomic level, remains challenging. To label RNAs, there are either indirect methods via fluorescent hybridization probes available or direct linkage of an organic dye to the RNA during chemical synthesis.14 Disadvantages of both procedures include difficulty in chemical synthesis, as well as nonphysiological concentrations. Genetically encodable fluorescent labels for RNAs are only available via auxiliary RNA binding proteins that carry a GFP fusion15 or via RNA aptamer sequences that bind small molecules as chromophores.16–18 One of those aptamer–chromophore pairs is the spinach RNA, which can bind the small molecule (Z)-5-(3,5-difluoro-4-hydroxybenzylidene)-2,3-dimethyl-3,5-dihydro-4H-imidazol-4-one (DFHBI). DFHBI is a cell-permeable, non-cytotoxic chromophore, in which fluorescence increases excessively within the appropriate environment provided by the spinach RNA.19,20 This spinach–DFHBI complex (hereinafter “spinach”) as a fluorescent, genetically encodable RNA label has been used in diverse in cellulo applications like tracking mRNA or sensing small cellular metabolites.21,22 Up to now, spinach has not been shown to undergo quenching, which is an essential step toward its utilization in biochemical studies of RNA networks. Here, we show how binding to various fluorescently labeled biomolecules such as nucleic acids and proteins allows efficient quenching of spinach fluorescence. Additionally, we utilize a biological fluorescent protein label as an acceptor, allowing detailed and fully genetically encodable analysis of RNA–protein interactions.

First, we were interested if spinach fluorescence could be utilized for Förster Resonance Energy Transfer (FRET). Therefore, three conditions have to be fulfilled: as FRET
occurs via dipole–dipole interactions the orientation of the fluorophores should not be perpendicular, the emission spectrum of the donor has to overlap with the absorption spectrum of the acceptor molecule, and the distance of both molecules must be within the range of $1 - 10$ nm.\textsuperscript{23} Accordingly, we used the very predictive and well characterized formation of an RNA–DNA hybrid to place a red fluorescent acceptor dye in close proximity to the spinach chromophore DFHBI. We modulated the P1 stem region of spinach, which is known to be robust against changes as fluorescence is not altered upon variation of this region.\textsuperscript{24,25} The 3′-end of spinach was shortened by 15 nt ("spinachΔ15"), which leads to a single stranded, nonhybridized, and accessible 5′-end (Figure 1a, Figure S1a). Additionally, we designed ssDNA oligonucleotides complementary to this region, which were labeled with various organic acceptor dyes on either their 5′- or their 3′-end. As the precise three-dimensional structure of the bound state is unknown, we added linker regions between nucleic acid probe
and fluorophore to allow for flexibility and thereby enrich the possibility of the correct orientation (Table S1). Binding of each DNA oligonucleotide to spinachΔ15 was verified by RNA Electrophoretic Mobility Shift Assay (REMSA). Under non-denaturing conditions, each sample showed a specific signal for spinachΔ15 fluorescence. Also, all acceptor-modified DNAs

Figure 2. Detection and analysis of RNA−protein interactions via spinach quenching. (a) Structural model of one possible spatial orientation of PP7−protein−pp7-spinach was generated by arranging crystal structures of PP7−pp7 (light gray and gray surface, PP7ΔFG dimer; black, pp7-RNA; pdb 2qux) and spinach-DFBHI (green, spinach-RNA; red, DFHBI; pdb, 4ts2) using nucleotides 36G and 43C (spinach) and 1G and 25C (pp7-RNA) for superimpositioning (Coot34 and Pymol35). Protein labeling with TF3 is shown (two red spheres). Distance to spinach chromophore DFHBI is measured (Pymol35) and indicated accordingly (red line). (b) Binding of spinach-pp7 and labeled PP7-protein dimer via REMSA. Spinach-pp7 (250 nM) was mixed with different concentrations of PP7-N93C-TF3 protein (0−12.5 μM, 1:1 dilution) and analyzed electrophoretically. Fluorescence was detected at 520 nm for spinach-pp7 fluorescence and at 620 nm for TF3-labeled proteins. (c) Upon addition of TF3-labeled cysteine mutants spinach-pp7 fluorescence is quenched significantly. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$. Data shown as mean ± SEM of triplicates. (d) PP7 protein variants are titrated against spinach-pp7. PP7-N93C-TF3 shows a concentration dependent quenching effect of spinach-pp7. Data shown as mean ± SEM of triplicates. (e) Reversibility of complex formation was shown by competition with unlabeled PP7ΔFG-protein. Data shown as mean ± SEM of triplicates.
(Figure 1b, Figure S1c–e) showed a fluorescent signal for the excess of free probe in the gel. Independently of the labeling position, all complementary oligonucleotides showed binding to the RNA complex as their fluorescent band partially shifted and thereby matched with the spinach channel. No spinachΔ15 association could be shown with any of the noncomplementary oligonucleotide probes as their fluorescent bands appeared as free probes only (Figure 1b). In principle, FRET allows acceptor fluorescence increase. Under homogeneous assay conditions, we did not observe any significant acceptor increase. This might be due to the solvent exposed DNA labels and accordingly other relaxations of the energy than fluorescence. Also, bleed through of the TMR dye in combination with its high extinction coefficient could hide an acceptor increase (Figure S2 and Table S2). However, radiationless energy transfer is also quantified via donor fluorescence decrease. Therefore, quantitative changes in spinach fluorescence upon binding of all labeled DNA probes were monitored (Figure 1c). The FRET efficiency can be calculated by the ratio of donor fluorescence (spinach) in the presence (Dₐ) and absence (D) of the acceptor (DNA probes). All of the labeled DNA probes fulfill the spectroscopic criteria for FRET (Figure S1). For 3' labeled probes, quenching efficiencies were in the range of 30–50%, whereas 5' labeled probes reached up to 85%. This is in the range of known fluorophore–acceptor pairs. To analyze a pure quencher molecule under our assay conditions, we used the IowaBlack F (IBF) quencher and observed similar quenching efficiencies as the previously tested red dyes (Figure 1c). The significant difference in quenching efficiencies between 3' and 5'-labeled probes is due to a 4 nm variation in distances from the two labeling positions to the spinach chromophore (Figure S1a). This strong distance dependency is common for resonance energy transfer and is therefore often used to analyze complex formations. In our system, as expected, the closer the fluorophores, the more efficiently spinach is quenched. To determine the spinachΔ15-RNA–DNA-probe interaction more closely, we titrated each DNA oligonucleotide to spinachΔ15 and monitored its change in donor fluorescence. As expected, titration of all DNA probes, which are identical in their sequence and differ only in the type of fluorescent acceptor dye, the more efficiently spinach is quenched. To show reversibility of the system, we designed an unlabeled DNA oligonucleotide, complementary to spinachΔ15 and monitored its change in donor fluorescence. As expected, titration of all DNA probes, which are identical in their sequence and differ only in the type of fluorescent label, showed similar affinities with a Kᵦ of about 70 nM (Figure 1d, Figure S1, Table S2). To show reversibility of the system, we designed an unlabeled DNA oligonucleotide, complementary to the fluorescent DNA probe, to compete with spinachΔ15 for binding. Fluorescence of spinachΔ15 was almost completely restored (~90%) in a concentration dependent manner indicating that the RNA aptamer is not disturbed by any effects throughout the whole procedure (Figure 1e). Our experiments showed efficient quenching of spinachΔ15 fluorescence presumably via FRET upon binding of DNA probes with various fluorescent acceptor dyes. All red chromophores quenched spinach with similar quenching efficiencies as the IBF quencher.

Next, we asked the question whether it is possible to quench spinach fluorescence via protein–RNA interactions. Therefore, we utilized the well characterized Pseudomonas aeruginosa...
bacteriophage coat protein 7 (PP7) and its interacting pp7-RNA wild-type hairpin. As described before, deletion of the residues 67−75 of the central FG loop (PP7ΔFG) leaves it without any further cysteines and also keeps it from forming higher oligomeric structures.27−29 PP7ΔFG is a 13.6 kDa protein that forms an intertwined homodimer with a large β-sheet surface that serves as a platform to interact tightly with a short 25 nt pp7-RNA hairpin (Figure 2a). We designed three cysteine mutants, A22C, A62C, and N93C for chemical labeling via thioether formation with the red acceptor fluorophore Tide Fluor 3 (TF3).30 Efficient FRET was ensured by flexible label orientation in nonrigid regions of the protein itself and additionally by the thioether linker between the cysteine side chain and the TF3 dye. As one pp7-RNA is bound by a PP7ΔFG homodimer, each RNA−protein complex carries two red fluorescent labels, one in each protomer. To label the pp7-RNA, we integrated its sequence at the tip of spinach stem P3 (“spinach-pp7”), which was also used in the past to generate spinach based biosensors and therefore made it our preferred entry point for construct design.31−33 Spinach-pp7 is able to bind DFHBI with comparable affinities and fluoresces similarly to the unmodified spinach aptamer (Figure S3, Table S3). Afterward, we checked if the new spinach-pp7 RNA construct is able to bind DFHBI with comparable affinities and fluoresces similarly to the unmodified spinach aptamer (Figure S3, Table S3). We purified and tested the fusion proteins for binding and quenching together with the spinach-pp7 complex (Figure 3). Linkers between both proteins derived from cloning overhangs (linker sequence ENLYFQG) and ensured a flexible orientation of mCherry to spinach. Complex formation was also verified via REMSAs in which each fusion protein was titrated against mCherry-PP7 and PP7-mCherry), which comprises spectral characteristics similar to the organic labels (Figure S5). We purified and tested the fusion proteins for binding and quenching together with the spinach-pp7 complex (Figure 3). Linkers between both proteins derived from cloning overhangs (linker sequence ENLYFQG) and ensured a flexible orientation of mCherry to spinach. Complex formation was also verified via REMSAs in which each fusion protein was titrated against spinach-pp7. Both mCherry tagged PP7 proteins bound in a concentration dependent manner similar to unlabeled PP7ΔFG, while mCherry alone did not show any binding (Figure 3a). Interestingly, only the N-terminally tagged fusion protein was able to quench spinach-pp7 (~20%), whereas the C-terminally fused PP7-mCherry did not quench significantly in comparison to controls (Figure 3b). Due to an overall low energy transfer from the donor and potential additional radiationless relaxations, an acceptor increase could not be observed (Figure S6). To analyze the RNA−protein interaction in a quantitative manner, we titrated mCherry-PP7 to spinach-pp7 RNA. Based on the fluorescence intensity, a KD of 140 nM (Figure 3c, Table S4) was determined. The lower affinity compared to a previously reported value of 1.6 nM obtained by REMSA may result from the addition of the mCherry label or be due to varied experimental conditions (e.g., buffer, ion strength).31 Then we tested the reversibility of the system by adding increasing amounts of untagged PP7ΔFG to the preformed complex, thereby competing with mCherry-PP7. Fluorescence could be restored up to 95% compared to the RNA-aptamer fluorescence alone (Figure 3d). Overall, the increased complex size dropped the spinach quenching

**Figure 4.** Quenching of spinach-pp7 from overexpressing *E. coli* BL21(DE3) cell lysates. (a) Emission spectra of lysates from spinach-pp7 overexpressing *E. coli* BL21(DE3) cell supplemented with 1.3 μM DFHBI after addition of either mCherry-PP7 or mCherry (66 μM). λEm = 425 nm. (b) Quantification of donor quenching effect of samples shown in panel a at spinach-pp7 peak emission wavelength (dashed line, λEm = 505). Data shown as mean ± SEM of triplicates. *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001. n.s. = not significant.
efficiency as expected, due to larger distances of both fluorophores. Nevertheless, we successfully utilized mCherry as a biological fluorophore to determine an RNA–protein interaction quantitatively.

Finally, we were interested if spinach quenching is possible in a more complex environment (cellular lysate). For that purpose, the spinach-pp7 RNA was expressed in BL21(DE3) cells, and lysates were supplemented with DFHBI (final concentration 1.3 μM) as well as mCherry or mCherry-PP7 (66 μM). Knowing that fluorescence intensity and quenching efficiency highly depend on the spectral setting, influences of the excitation wavelength as well as bandwidths was investigated (Figure S7). To ensure minimal acceptor (quencher) fluorescence bleed through, we chose the following settings: λ<sub>ex</sub> = 425 nm, BW<sub>ex</sub> and BW<sub>em</sub> = 5 nm. Using this setup, emission spectra (465–700 nm) were recorded (Figure 4a). The lysate (gray) shows only spinach-pp7 fluorescence. Upon addition of mCherry-PP7 protein, we observe a significant decrease of donor fluorescence (QE = 20%) (Figure 4b), which is in line with the behavior of the purified components (Figure 3c). Importantly, addition of the mCherry protein only (lacking the PP7 fusion element) does not result in a reduction of donor fluorescence (Figure 4b). In both mCherry containing samples, due to residual bleed through, some mCherry fluorescence is observed (maximum at 615 nm). In line with the results above, we do not observe FRET-based increase of acceptor fluorescence (Figures 4a and S8). In summary, our data show the usage of the spinach aptamer in RNA–protein interaction measurements for the first time, even under lysate conditions.

Investigations of RNA interactions are of crucial interest, but simple homogeneous methods such as FRET are rare. For FRET, the RNA of interest needs a fluorescent tag. The spinach aptamer is a genetically encodable RNA label, binding to the DFHBI chromophore and thereby activating its fluorescence. Here we describe how spinach is quenched upon interaction with biomolecules such as nucleic acids or proteins. Binding induces proximity based radiationless energy transfer to acceptor dyes like quenchers and red fluorophores. We could monitor and quantify the specific hybridization to complementary DNA probes. Also, direct RNA–protein interactions can be observed and analyzed for their binding affinity. Additionally, we successfully substitute chemical dyes for genetically encodable fluorescent proteins such as mCherry and could show that donor quenching can be detected in lysates. This setup can possibly be used under in cellulo conditions, if better fluorescent RNA tags as well as more suitable FRET pairing fluorescent proteins are developed. Optimizing the overall signal strength and FRET efficiency, these systems might increase acceptor fluorescence, which is preferred in some cases over donor quenching.

### METHODS

Experimental procedures and methods are described in detail in the Supporting Information.

### ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.7b00332.

Supplemental methods detailing RNA hybridizing oligonucleotides, cloning of RNA and protein constructs, RNA in vitro transcription, protein expression, purification and labeling, REMSAs, quenching and competition measurements, and fluorescence analysis of E. coli expressed spinach-pp7 lysates, supplemental figures showing spinachΔ15 and labeled DNA probes, emission spectra spinachΔ15 and 5′-TMR binding probe, spinach-pp7 binding to PP7-protein mutants, emission spectra analysis of PP7-N93C w/o spinach-pp7, spectral properties of spinach-DFHBI and mCherry protein, emission spectra analysis of mCherry tagged PP7 protein without spinach-pp7, optimization of plate reader settings, spectroscopic analysis of spinach-pp7 expressing E. coli lysates, and supplemental tables showing oligonucleotide details used in hybridization assay, spectroscopic properties of the used fluorophores, spectroscopic and biochemical properties of the used spinach constructs, and spectroscopic and biochemical properties of the used labeled PP7 proteins (PDF)

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**Notes**

The authors declare no competing financial interest.

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### REFERENCES


