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Stick, Flick, Click: DNA-guided Fluorescent Labeling of Long RNA for Single-molecule FRET

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Abstract: Exploring the spatiotemporal dynamics of biomolecules on a single-molecule level requires innovative ways to make them spectroscopically visible. Fluorescence resonance energy transfer (FRET) uses a pair of organic dyes as reporters to measure distances along a predefined biomolecular reaction coordinate. For this nanoscopic ruler to work, the fluorescent labels need to be coupled onto the molecule of interest in a bioorthogonal and site-selective manner. Tagging large non-coding RNAs with single-nucleotide precision is an open challenge. Here we summarize current strategies in labeling riboswitches and ribozymes for fluorescence spectroscopy and FRET in particular. A special focus lies on our recently developed, DNA-guided approach that inserts two fluorophores through a stepwise process of templated functionality transfer and click chemistry.

Keywords: Bioorthogonal · Fluorescence · Nucleic acids · Riboswitch · Spectroscopy

Fabio D. Steffen graduated from the University of Zurich in 2015 with a Master's degree in Biochemistry, working with Roland Sigel on RNA and their interactions with metal ions. He is currently pursuing his PhD studies in the same lab where he investigates the structure and function of different non-coding RNAs using a combination of single-molecule FRET and molecular modeling.

1. The Ideal Labeling Strategy for RNA

Tags and labels are ubiquitous in nature. Post-transcriptional and post-translational modifications (PTM) provide the cell with a rich chemical repertoire that generates functional diversity within the transcriptome and proteome. These decorations are critically involved in signaling (phosphorylation by kinases), epigenetics (*N*-acetylation of lysine), they can change expression patterns (methylation of adenine, m^6A), alter the cellular address of proteins and mark them for degradation (ubiquitylation), or report on their current folding status (glycosylation).[1] Like natural PTMs, extrinsic labels such as organic dyes, spin-labels or affinity tags can be incorporated into biomolecules both *in vitro* and *in vivo* to study their function or localize them in the cell.[2] For FRET and EPR applications, the challenge consists in making the modification specific to a unique site.[3] Compared to proteins, the alphabet of nucleic acid building blocks is more restricted and thus achieving site-selectivity becomes a major issue. The ideal RNA labeling method for fluorescence spectroscopy (1) can target any nucleotide within a biomolecule of any size; (2) it is site-specific down to a single nucleotide; (3) it is efficient, meaning the reaction is fast and gives a high yield; (4) it is bioorthogonal and proceeds without dye cross-reactivity, *i.e.* the reaction uses abiotic chemistry that is specific for each individual label; and (5) the method perturbs neither the RNA structure nor its function, thus the label should be small and not interfere with base-pairing.

A generally applicable method that would fulfill all the above criteria is lacking to date. Current labeling strategies for RNA suffer from the incompatibility between having full control over the labeling position and integrity of the RNA on the one hand, and the size of the biomolecule on the other (Fig 1). Purely chemical approaches such as solid-phase oligonucleotide synthesis (SPOS) use modified phosphoramidites and are the method of choice for short sequences with <60 nucleotides.[4] As such, SPOS gives the highest amount of flexibility in choosing the right dye position. To increase the size range of the target molecule, SPOS is often combined with enzymatic ligation where short chemically synthesized or *in vitro* transcribed fragments are combined into a single construct.[5] Using this approach, it has been possible to label riboswitches of up to about 200 nucleotides in length.[6] Still, the efficiency of the reaction depends on whether the secondary structure around the ligation site is tolerated by the enzyme. As an alternative to T4 DNA and RNA ligase, Silverman and coworkers have identified deoxyribozymes through *in vitro* selection that ligate a fluorescently labeled (oligo)nucleotide *via* a 2',5'-phosphodiester bond to an RNA of interest.[7]

A different approach learns from nature by imitating the functionality transfer reactions of natural PTM catalyzing enzymes.[8] A particularly attractive class are methyltransferases (MTase), which can be repurposed to deliver an *S*-adenosylmethionine (SAM) analog to the target RNA. Some of these promiscuous MTases are directed towards the 3'-terminus[9] or the 5'-cap[10] whereas others are programmable by a guide-DNA.[11] For live-cell imaging, chemo-enzymatic labeling emerges as a valuable alternative to genetically encoded RNA-fluorophore complexes like the spinach aptamer.[12]

On the other end of the spectrum are hybridization methods where short dye labeled DNA or peptide nucleic acids (PNA) oligonucleotides are attached to complementary regions on the RNA.[13] Usage of such fluorescent probes makes these strategies RNA size independent, at the expense of having a non-covalent and thus non-permanent linkage between the label and the target. Moreover, non-native loops often need to be inserted or elongated

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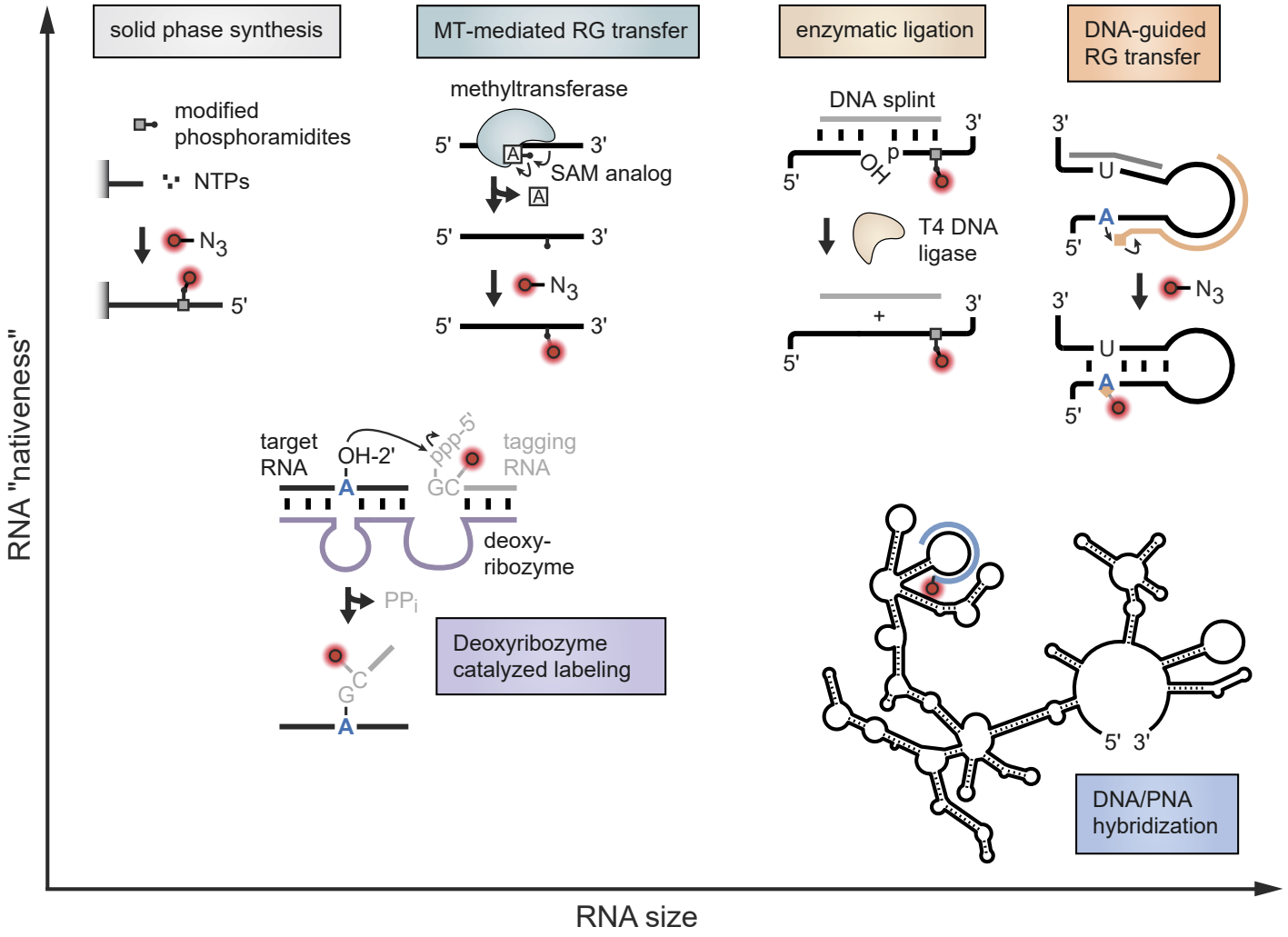

Fig. 1. Strategies for site-specific RNA labeling. Most current labeling methods are limited by the RNA size or its 'nativeness' (*i.e.* integrity of sequence and structure). Solid-phase oligonucleotide synthesis (SPOS): the RNA is chemically synthesized using phosphoramidite building blocks and labeled post-synthetically (*e.g.* by click chemistry).[4] Methyltransferase (MT) mediated reactive group (RG) transfer: the enzyme is promiscuous towards *S*-adenosyl methionine (SAM) analogs and can transfer a functional group (*e.g.* an alkyne) instead of the canonical methyl group.[8] Deoxyribozyme catalyzed labeling (DECAL): an *in vitro* selected deoxyribozyme couples a fluorescently labeled tagging RNA onto the target RNA.[7a] The tagging RNA can be as short as a single guanine.[7b] Enzymatic ligation: synthesized or transcribed oligonucleotides are enzymatically ligated with a DNA splint.[5] DNA-guided RG transfer: a bioorthogonal handle is guided towards a pre-selected adenine and subsequently clicked with a dye.[15] DNA/PNA hybridization: fluorescently labeled DNA or PNA oligonucleotides anneal non-covalently to engineered loops platforms.[13]

to accommodate the DNA or PNA oligo. Care must be taken not to disrupt key long-range tertiary contacts that would abrogate RNA function.

Recently, attempts have been made to combine the advantages of functionality transfer with the guiding capabilities of DNA-templated chemistry.[14] To label particularly long non-coding RNAs like riboswitches and ribozymes, we developed a novel site-directed labeling approach, which installs two fluorophores in a post-transcriptional and bioorthogonal manner.[15] In the following, we will review the conceptual basis of the labeling workflow with a special focus on the chemistry of the transfer and coupling reactions.

2. Guide, Transfer, Couple – RNA Labeling in Three Steps

Egloff *et al.* recently described an alkylation strategy to site-specifically incorporate etheno adducts on the Watson-Crick face of adenines and cytosines in single-stranded DNA oligonucleotides.[14a] If the alkylating agent is decorated with a bioorthogonal functional group, this moiety can be subsequently conjugated with a fluorophore or any other reactive probe.[16]

The applicability of this two-step approach has been demonstrated on a surface exposed single-stranded loop of a several hundred nucleotide long group II intron ribozyme.[16] To extend the scope of labeling positions, we introduced additional helper strands[17] which anneal upstream and/or downstream of the modification site and temporarily disrupt the local secondary structure pattern, making the selected nucleotide better accessible for the chemical modification (Fig. 2a).[15] The labeling sites are thus no longer limited to single-stranded regions but also base-paired nucleotides can be targeted. The functionalized guide-DNA then brings a vicinal diol into proximity of the exocyclic amine group of an adenine or cytosine. The diol is cleaved by periodate leaving an aldehyde to react with the nucleophilic primary amine. In a cyclization reaction, followed by elimination of water, a 1,N^6-ethenoadenine (εA) or 3,N^4-ethenocytosine (εC) is generated (Fig. 2b). The propargyl handle on position C8 is subsequently coupled to an azide derivatized fluorophore using Cu(I) catalyzed click chemistry (CuAAC). As a useful side-reaction of the *in situ* activation of the diol, the 3'-terminal ribose is oxidized and can be targeted in an orthogonal fashion using a second dye.[18]

3. Site Selection – Where to put the Label?

Single-molecule detection combined with Förster resonance energy transfer (FRET) has evolved into a versatile tool to monitor biomolecular interactions and dynamics on a broad range of

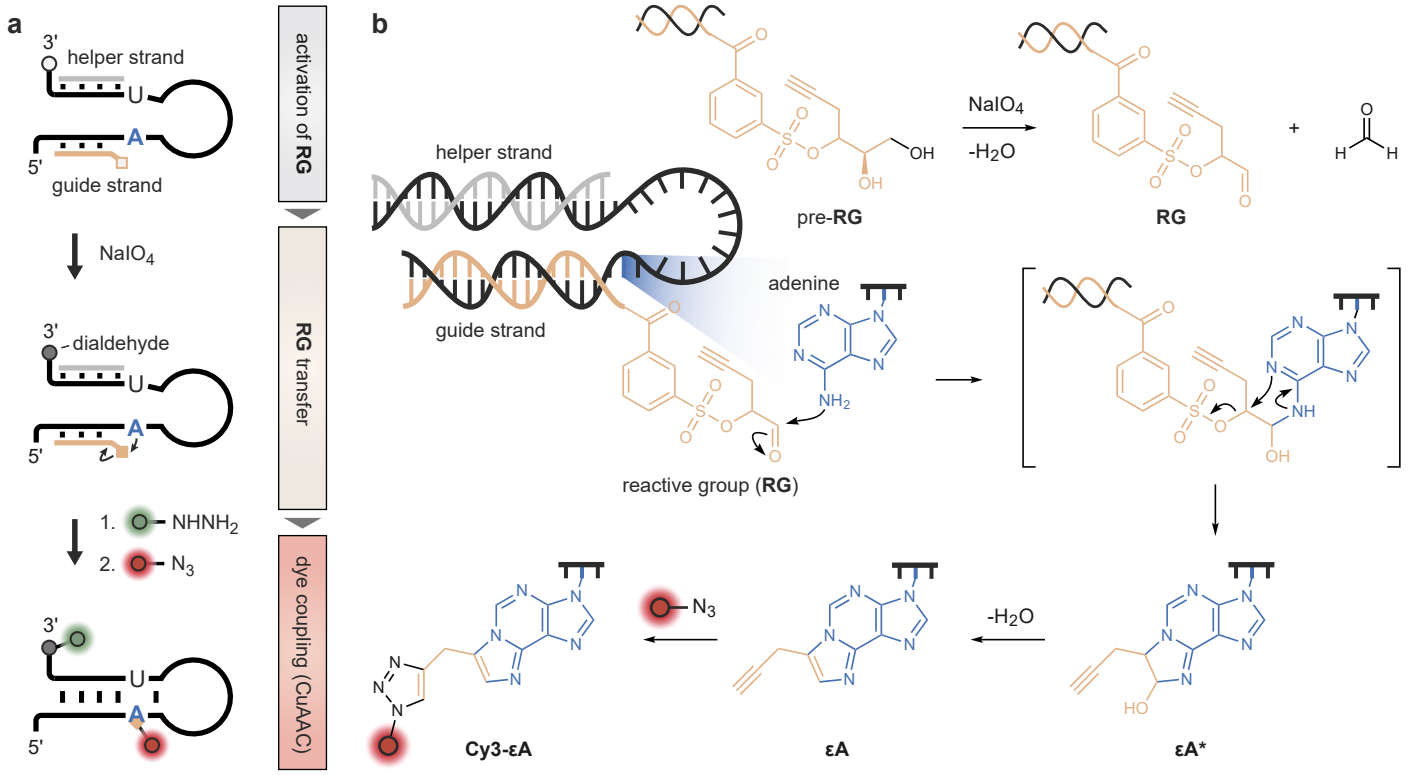

Fig. 2. DNA-guided fluorescence labeling of RNA in three steps. (a) Schematic overview of reactive group activation, transfer and bioorthogonal dye coupling. (b) Helper (gray) and guide (yellow) strands hybridize to the target RNA to bring a reactive group (**RG**) into the reach of a pre-selected adenine (blue). $NaIO_4$ activates the **RG** by oxidative cleavage. Concomitantly, the 3'-terminal ribose is converted to a dialdehyde. Etheno adduct formation proceeds through a hydroxyethano intermediate (εA*). In the last step, an azide functionalized dye is conjugated to the alkyne in a Cu(I) catalyzed cycloaddition reaction (CuAAC). A second, hydrazide derivatized fluorophore reacts with the 3'-terminal dialdehyde.

timescales from nanoseconds to minutes or even hours.[19] The selection of appropriate dye positions is thereby a prerequisite for informative FRET trajectories. To learn about RNA dynamics (*e.g.* a conformational rearrangement initiated upon binding of a metabolite or a protein), the dye pair should probe a functionally relevant reaction coordinate. In a two-state folding setting this can be two residues which are far apart in the unfolded state (low FRET) but come close together upon RNA collapse (high FRET). To use FRET as a spectroscopic ruler with maximum sensitivity,[20] the expected interdye distance r should match the Förster radius R_0 of the FRET pair (Eqn. (1)). Furthermore, reliable transfer efficiencies E require the dyes to rotate isotropically, which relates to the well-known 'κ^2-problem'.[21] Most importantly, the fluorophore positions have to be compatible with the RNA structure and function.

$$E = \frac{1}{1 + \left(\frac{r}{R_0}\right)^6} \qquad (1)$$

Riboswitches are regulatory RNAs that undergo a conformational change in response to binding of small-molecule metabolites such as S-adenosylmethionine (SAM),[22] flavin mononucleotide (FMN)[23] or cobalamins (*e.g.* coenzyme B_{12}).[24] Hence, riboswitches represent a prime example of biomolecular dynamics that can be probed by single-molecule FRET.[25] To showcase the applicability of our labeling protocol, we use a coenzyme B_{12} responsive riboswitch that controls the expression of an outer-membrane protein responsible for the transport of coenzyme B_{12} in *Escherichia coli*.[26]

To guide our search for appropriate labeling positions, we based our selection on a consensus secondary structure from a multisequence alignment (MSA) of cobalamin riboswitches[27] as well as a crystal structure of one representative.[28] We selected an adenine close to a kissing loop contact[27] (A_{35}) and another that is part of the ribosomal binding site (RBS) hairpin[29] (A_{213}). Both residues are thus near key functional elements but not themselves involved in long-range tertiary contacts. The two internal sites are complemented by the 3'-terminus (G_{240}) which gets modified concomitantly. In this way, FRET monitors the formation of the kissing loop and the RBS hairpin by probing a reaction coordinate from the donor labeled G_{240} to the acceptor labeled A_{35} or A_{213}, respectively.

4. Quality Checks – Site-specificity, Dye Integrity and RNA Switching

To test the precision of our DNA-guided functionality transfer reaction, we designed a reverse transcriptase assay, where a short ^{32}P-carrying primer is annealed downstream of the labeling site (here A_{35}) and is then stepwise extended by a polymerase (Fig. 3a). On a denaturing PAGE, bands appear for every nucleotide that is attached to the growing primer, except for the one where we expect the fluorophore to be conjugated to. The absence of this band suggests that the reverse transcriptase does not recognize the labeled nucleotide, as its Watson-Crick face is blocked, and therefore does not pause and release its cDNA product. This shows that out of two consecutive adenines (A_{35} and A_{36}) primarily A_{35} is labeled.

Fluorescence is ideally suited to interrogate dynamic processes in biomolecular settings because it is non-invasive and can cover a wide range of timescale from dye photophysics and diffusion (picoseconds to microseconds) to folding and binding kinetics (microseconds to minutes).[19] The fluorophores should thus be bright (*i.e.* have a high quantum efficiency), they should be photostable, water soluble and offer biocompatible coupling chemistries.[30] One popular class of dyes for single-molecule FRET are carbocyanines. Their photophysics and interaction profiles with nucleic

acids and proteins have been extensively characterized.[31] Long flexible linkers between the attachment point and the dye scaffold, as well as negatively charged sulfo groups help to minimize contacts with the biomolecule and enhance the free rotation of the dye.[32] Nevertheless, interactions particularly at the termini are not uncommon.[33] Consistent with previous fluorescence lifetime and time-resolved anisotropy measurements,[31c] the typical long fluorescence decay curves indicate that the dyes are still intact after conjugation to the RNA even if their flexibility is restrained by the biomolecule (Fig. 3b).

To evaluate the integrity and function of the riboswitch, we immobilize the RNA at the 5'-terminus *via* a streptavidin-biotin linker onto coated quartz slides and probe the RBS hairpin by single-molecule FRET.[34] In order to distinguish zero FRET molecules (interdye distance >10 nm) from donor only or acceptor bleached molecules, we additionally check the presence of the acceptor dye with a red laser in an alternating laser excitation (ALEX) scheme.[35] In this way, we can safely select only those molecules that are double labeled.

The single-molecule time traces and histograms suggest that the RBS exists in at least two major conformations, an open (low FRET) and a closed (high FRET) state (Fig. 3c). The open conformation possibly encompasses an ensemble of structures where the RBS is accessible for the ribosome to bind and translate the downstream gene. In the closed form, the RBS is sequestered and protein expression is downregulated.[29] Binding of the natural cofactor coenzyme B_{12} (CoB_{12}) to the aptamer region shifts the equilibrium towards the closed state, consistent with a negative feedback mechanism.[24a] By observing an increasing population of the high FRET state upon metabolite binding, we are confident that introduction of the bulky fluorophores at the designated positions does not impair the function of the riboswitch.

5. Multi-colored RNA Labeling – Limits and Prospects

We review here a recently established two-color labeling method which is applicable to RNAs of any size while preserving its native structure and sequence as good as currently possible. The labeling is precise and adaptable to different spectroscopic techniques such as FRET or EPR. With respect to the ideal labeling strategy there are still a few shortcomings: (i) Because ethenoadduct formation involves nitrogen atoms N1 and N6 of adenine or N4 and N3 of cytosine, the Watson-Crick base pairing of the modified residue is disrupted. Thermal melting experiments show that effects on the stability of longer RNAs are small, suggesting that only the tagged residue is affected. Special care is advised when targeting a functionally relevant, short and AU rich duplex. (ii) Double labeling yields are around 5% (15–35% for a single dye), which is usually enough for single-molecule measurements but may be a constraint for ensemble experiments. Optimization of the dye coupling stoichiometry and overall RNA recovery during purification might alleviate this issue. (iii) Separation of labeled from unreacted RNA is challenging. Size differences are small (usually <1 kDa) and the RNA is not significantly more hydrophobic due to the presence of the dyes alone, since common fluorophores are often sulfonated to minimize interactions. Unlabeled or mono-labeled riboswitches will compete for metabolites, but those species can be sorted out by dual-color excitation in single-molecule imaging.

Ultimately, the key advantage over other existing approaches is the independence of RNA length, while establishing a covalent and thus permanent linkage between the RNA and the dye. As such, the method combines the main benefit of DNA/PNA hybridization with direct dye coupling as in solid-phase oligonucleotide synthesis. The current design of the reactive group has been tested to tag adenines and cytosines but should be extendable to guanines as well.[36] Furthermore, alternative transfer chemistries at different positions on the base or sugar ring are conceivable. PNA instead of DNA-guided delivery of the RG would allow to shorten the guide strand and possibly increase labeling yields at highly buried residues. Lastly, the reactive group chemistry could be reversed (*i.e.* conjugate a RG-azide with a dye-alkyne) to enable orthogonal dye couplings at two or more internal sites. To avoid the cellular toxicity of Cu(I), other bioorthogonal reactions (*e.g.* copper-free click chemistry with strained cyclooctynes,[37] Staudinger ligation[38] or inverse-electron demand Diels-Alder reactions[39]) could be exploited in the future. All these developments build upon the modular nature of the method (DNA-guided delivery – stick, RG transfer – flick, and dye coupling – click) and further expand the scope of possible labeling sites within biologically relevant RNAs.

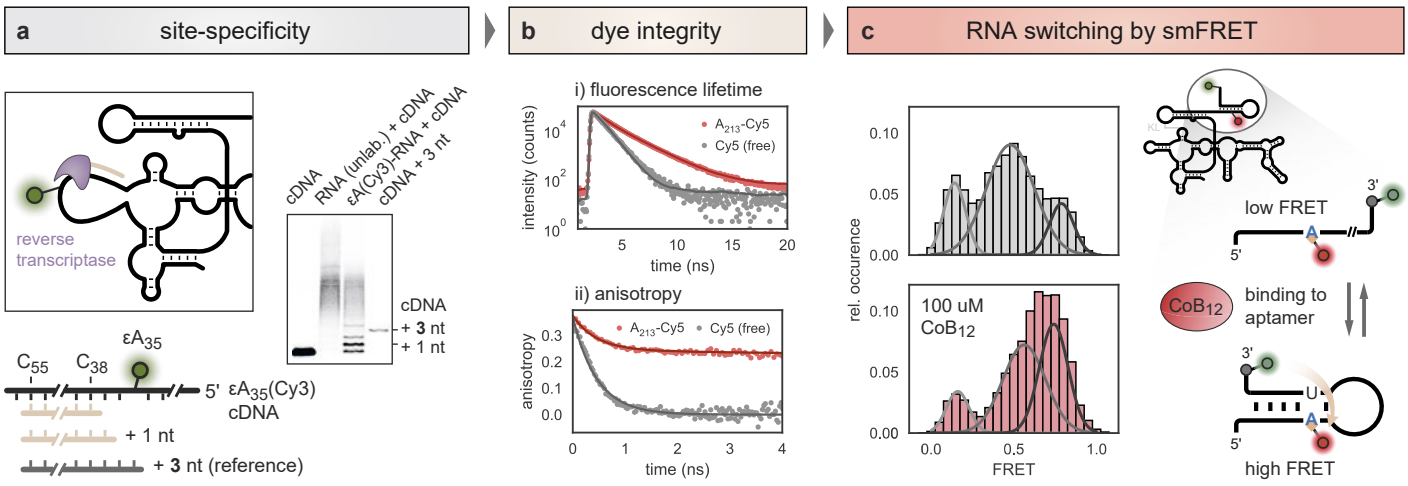

Fig. 3. Quality checks to assess the site-specificity, fluorophore integrity and RNA switching. (a) Reverse transcriptase assay probing the internal labeling of a B_{12} responsive riboswitch with Cy3 on A_{35}. The denaturing PAGE gel shows a ^{32}P-labeled cDNA primer which gets extended by a reverse transcriptase. The band corresponding to the cDNA primer + 3 nucleotides, which is opposite to the labeling site, is missing because the reverse transcriptase does not recognize εA_{35}-Cy3 with its Watson-Crick face being blocked. The enzyme therefore does not pause at his position and no cDNA product is released. Gel reproduced from ref. [15] with permission from Oxford University Press. (b) Prolonged fluorescence lifetime and time-resolved anisotropy decays indicate that the fluorophores are conjugated to the RNA and are still intact. Decays adapted from ref. [15]. (c) Riboswitch dynamics interrogated by single-molecule FRET. Binding of coenzyme B_{12} (CoB_{12}) to the RNA aptamer region triggers a switch on the expression platform, pushing the ribosomal binding site (RBS) hairpin into a closed form (high FRET), which in turn downregulates gene expression. Histograms adapted from ref. [15].

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[1] a) J.-Y. Roignant, M. Soller, *Trends Genet.* **2017**, *33*, 380; b) C. T. Walsh, S. Garneau-Tsodikova, G. J. Gatto, *Angew. Chem. Int. Ed.* **2005**, *44*, 7342.
[2] a) J. A. Prescher, C. R. Bertozzi, *Nat. Chem. Biol.* **2005**, *1*, 13; b) D. Schulz, A. Rentmeister, *ChemBioChem* **2014**, *15*, 2342.
[3] a) S. Saha, A. P. Jagtap, S. T. Sigurdsson, in 'Methods in Enzymology', Eds. P. Z. Qin, K. Warncke, Elsevier, Amsterdam, **2015,** p. 397; b) S. Solomatin, D. Herschlag, in 'Methods in Enzymology', Ed. D. Herschlag, Elsevier, Amsterdam, **2009**, p. 47; c) M. Sustarsic, A. N. Kapanidis, *Curr. Opin. Struct. Biol.* **2015**, *34*, 52.
[4] a) S. Seidu-Larry, B. Krieg, M. Hirsch, M. Helm, O. Domingo, *Chem. Commun.* **2012**, *48*, 11014; b) S. H. Weisbrod, A. Marx, *Chem. Commun.* **2008**, 5675.
[5] a) M. Moore, P. Sharp, *Science* **1992**, *256*, 992; b) N. Vušurović, R. B. Altman, D. S. Terry, R. Micura, S. C. Blanchard, *J. Am. Chem. Soc.* **2017**, *139*, 8186.
[6] a) J. M. Esquiaqui, E. M. Sherman, J.-D. Ye, G. E. Fanucci, in 'Methods in Enzymology', Ed. D. H. Burke, Elsevier, Amsterdam, **2014**, p. 287; b) K. Lang, R. Micura, *Nat. Protoc.* **2008**, *3*, 1457.
[7] a) D. A. Baum, S. K. Silverman, *Angew. Chem. Int. Ed.* **2007**, *46*, 3502; b) L. Büttner, F. Javadi-Zarnaghi, C. Höbartner, *J. Am. Chem. Soc.* **2014**, *136*, 8131.
[8] L. Anhäuser, A. Rentmeister, *Curr. Opin. Biotechnol.* **2017**, *48*, 69.
[9] A. Plotnikova, A. Osipenko, V. Masevičius, G. Vilkaitis, S. Klimašauskas, *J. Am. Chem. Soc.* **2014**, *136*, 13550.
[10] J. M. Holstein, L. Anhäuser, A. Rentmeister, *Angew. Chem. Int. Ed.* **2016**, *55*, 10899.
[11] M. Tomkuviene, B. Clouet-d'Orval, I. Cerniauskas, E. Weinhold, S. Klimasauskas, *Nucleic Acids Res.* **2012**, *40*, 6765.
[12] J. S. Paige, K. Y. Wu, S. R. Jaffrey, *Science* **2011**, *333*, 642.
[13] a) A. G. Schmitz, S. Zelger-Paulus, G. Gasser, R. K. O. Sigel, *ChemBioChem* **2015**, *16*, 1302; b) G. J. Smith, T. R. Sosnick, N. F. Scherer, T. Pan, *RNA* **2005**, *11*, 234.
[14] a) D. Egloff, I. A. Oleinich, E. Freisinger, *ACS Chem. Biol.* **2015**, *10*, 547; b) K. Jahn, E. M. Olsen, M. M. Nielsen, T. Tørring, R. M. Zadegan, E. S. Andersen, K. V. Gothelf, J. Kjems, *Bioconjugate Chem.* **2011**, *22*, 95; c) K. Onizuka, Y. Taniguchi, S. Sasaki, *Bioconjugate Chem.* **2009**, *20*, 799.
[15] M. Zhao, F. D. Steffen, R. Börner, M. F. Schaffer, R. K. O. Sigel, E. Freisinger, *Nucleic Acids Res.* **2018**, *46*, e13.
[16] D. Egloff, I. A. Oleinich, M. Zhao, S. L. B. König, R. K. O. Sigel, E. Freisinger, *ACS Chem. Biol.* **2016**, *11*, 2558.
[17] B. M. Fuchs, F. O. Glockner, J. Wulf, R. Amann, *Appl. Environ. Microbiol.* **2000**, *66*, 3603.
[18] a) D. Proudnikov, A. Mirzabekov, *Nucleic Acids Res.* **1996**, *24*, 4535; b) P. Z. Qin, A. M. Pyle, *Methods* **1999**, *18*, 60.
[19] a) R. Börner, D. Kowerko, H. G. Miserachs, M. F. Schaffer, R. K.O. Sigel, *Coord. Chem. Rev.* **2016**, *327-328*, 123; b) B. Schuler, H. Hofmann, *Curr. Opin. Struct. Biol.* **2013**, *23*, 36.
[20] a) B. Schuler, E. A. Lipman, P. J. Steinbach, M. Kumke, W. A. Eaton, *Proc. Natl. Acad. Sci. U. S. A.* **2005**, *102*, 2754; b) L. Stryer, R. P. Haugland, *Proc. Natl. Acad. Sci. U. S. A.* **1967**, *58*, 719.
[21] R. E. Dale, J. Eisinger, W. E. Blumberg, *Biophys. J.* **1979**, *26*, 161.
[22] R. K. Montange, R. T. Batey, *Nature* **2006**, *441*, 1172.
[23] A. Serganov, L. Huang, D. J. Patel, *Nature* **2009**, *458*, 233.
[24] a) M. Mandal, R. R. Breaker, *Nat. Rev. Mol. Cell Biol.* **2004**, *5*, 451; b) A. Nahvi, N. Sudarsan, M. S. Ebert, X. Zou, K. L. Brown, R. R. Breaker, *Chem. Biol.* **2002**, *9*, 1043.
[25] a) E. D. Holmstrom, J. T. Polaski, R. T. Batey, D. J. Nesbitt, *J. Am. Chem. Soc.* **2014**, *136*, 16832; b) C. Manz, A. Y. Kobitski, A. Samanta, B. G. Keller, A. Jäschke, G. U. Nienhaus, *Nat. Chem. Biol.* **2017,** *13***,** 1172; c) K. C. Suddala, J. Wang, Q. Hou, N. G. Walter, *J. Am. Chem. Soc.* **2015**, *137*, 14075.
[26] M. D. Lundrigan, W. Koster, R. J. Kadner, W. Köster, *Proc. Natl. Acad. Sci. U. S. A.* **1991**, *88*, 1479.
[27] A. Nahvi, J. E. Barrick, R. R. Breaker, *Nucleic Acids Res.* **2004**, *32*, 143.
[28] J. E. Johnson, F. E. Reyes, J. T. Polaski, R. T. Batey, *Nature* **2012**, *492*, 133.
[29] X. Nou, R. J. Kadner, *Proc. Natl. Acad. Sci. U. S. A.* **2000**, *97*, 7190.
[30] T. Ha, *Methods* **2001**, *25*, 78.
[31] a) E. Lerner, E. Ploetz, J. Hohlbein, T. Cordes, S. Weiss, *J. Phys. Chem. B.* **2016**, *120*, 6401; b) M. Levitus, S. Ranjit, *Q. Rev. Biophys.* **2011**, *44*, 123; c) F. D. Steffen, R. K. O. Sigel, R. Börner, *Phys. Chem. Chem. Phys.* **2016**, *18*, 29045.
[32] S. Sindbert, S. Kalinin, H. Nguyen, A. Kienzler, L. Clima, W. Bannwarth, B. Appel, S. Müller, C. A. M. Seidel, *J. Am. Chem. Soc.* **2011**, *133*, 2463.
[33] a) A. Iqbal, S. Arslan, B. Okumus, T. J. Wilson, G. Giraud, D. G. Norman, T. Ha, Lilley, David M J, *Proc. Natl. Acad. Sci. U. S. A.* **2008**, *105*, 11176; b) M. E. Sanborn, B. K. Connolly, K. Gurunathan, M. Levitus, *J. Phys. Chem. B* **2007**, *111*, 11064.
[34] R. Roy, S. Hohng, T. Ha, *Nat. Methods* **2008**, *5*, 507.
[35] A. N. Kapanidis, N. K. Lee, T. A. Laurence, S. Doose, E. Margeat, S. Weiss, *Proc. Natl. Acad. Sci. U. S. A.* **2004**, *101*, 8936.
[36] L. Kronberg, R. Sjöholm, S. Karlsson, *Chem. Res. Toxicol.* **1992**, *5*, 852.
[37] N. J. Agard, J. A. Prescher, C. R. Bertozzi, *J. Am. Chem. Soc.* **2004**, *126*, 15046.
[38] E. Saxon, *Science* **2000**, *287*, 2007.
[39] J. Schoch, S. Ameta, A. Jäschke, *Chem. Commun.* **2011**, *47*, 12536.