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

Fabio D. Steffen*, Richard Börner, Eva Freisinger, and Roland K. O. Sigel*

*SCS-Metrohm Award for best oral presentation in Medicinal Chemistry & Chemical Biology

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 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 fluoresceinously 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

*Correspondence: F. D. Steffen, Prof. R. K. O. Sigel
E-mail: fabio.steffen@chem.uzh.ch, roland.sigel@chem.uzh.ch
Department of Chemistry, University of Zurich, Winterthurerstrasse 190, CH-8057 Zurich
To extend the scope of labeling positions, we introduced additional helper strands 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).

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\textsubscript{6}-ethenoadenine (ε\textsubscript{A}) or 3,N\textsubscript{4}-ethenocytosine (ε\textsubscript{C}) 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.

### 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 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.\footnote{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. 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.} 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. 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. 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.

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.\footnote{To extend the scope of labeling positions, we introduced additional helper strands 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). 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\textsubscript{6}-ethenoadenine (ε\textsubscript{A}) or 3,N\textsubscript{4}-ethenocytosine (ε\textsubscript{C}) 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.}
The fluorophores should thus be bright as well as a crystal structure of one representative. We selected an adenine close to a kissing loop contact and another that is part of the ribosomal binding site (RBS) hairpin 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 G240 to the acceptor labeled A35 or A213, 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 35P-carrying primer is annealed downstream of the labeling site 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. 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 primarily A35 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 timescales from nanoseconds to minutes or even hours. 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, the expected interdye distance r should match the Förster radius R mín of the FRET pair (Eqn. (1)). Furthermore, reliable transfer efficiencies E require the dyes to rotate isotropically, which relates to the well-known ‘κ’-problem. Most importantly, the fluorophore positions have to be compatible with the RNA structure and function.

\[
E = \frac{1}{1 + \left(\frac{r}{R_{\text{m}}}\right)^6}
\]  

Riboswitches are regulatory RNAs that undergo a conformational change in response to binding of small-molecule metabolites such as S-adenosylmethionine (SAM), flavin mononucleotide (FMN) or cobalamins (e.g. coenzyme B12). Hence, riboswitches represent a prime example of biomolecular dynamics that can be probed by single-molecule FRET. To showcase the applicability of our labeling protocol, we use a coenzyme B12-responsive riboswitch that controls the expression of an outer-membrane protein responsible for the transport of coenzyme B12 in Escherichia coli.

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 as well as a crystal structure of one representative. We selected an adenine close to a kissing loop contact and another that is part of the ribosomal binding site (RBS) hairpin 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 G240 to the acceptor labeled A35 or A213, respectively.

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Fluorescence is ideally suited to interrogate dynamic processes in biomolecular settings because it is non-invasive and can cover a wide range of timescales from dye photophysics and diffusion (picoseconds to microseconds) to folding and binding kinetics (microseconds to minutes). The fluorophores should thus be bright (i.e. have a high quantum efficiency), they should be photostable, water soluble and offer biocompatible coupling chemistries. 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,[34] 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.[35] In order to distinguish zero FRET molecules (interdyne 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.[36] 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.[37] Binding of the natural cofactor coenzyme B12 (CoB12) to the aptamer region shifts the closed state towards the closed state, consistent with a negative feedback mechanism.[38] 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:

- 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 monolabeled 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.[39] 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-azole with a dye-alkyne) to enable orthogonal dye couplings at two or more internal sites. To avoid the cellular toxicity of Cu(t), 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.
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