Electronic Supplementary Information for

Site-specific one-pot triple click labeling for DNA and RNA

Marie-Luise Winz, Eva Christina Linder, Juliane Becker, Andres Jäschke

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Materials & Methods

Chemicals and further materials

Unless mentioned otherwise, all chemicals and lab-ware were purchased from either Sigma-Aldrich, Fluka, Acros or from the Zentrallager of Heidelberg University in the highest available purity. All oligonucleotides used in this study are given in Tables S3,S4.

Chemical synthesis of modified DNA and RNA

Synthesis and purification of single-modified (containing an octynyl modification) or double-modified DNA (containing TCO or BCnonene and octynyl modification) employed here (Table S3) was previously described[1] and was performed in an identical way for the additional double-modified DNA containing norbornene and octynyl modification. RNA (Table S4) synthesis was performed at 1 µmol scale on CPG-support using 2'-TBDMS-protected β-cyanoethylphosphoramidites with base labile *tert*-butylphenoxyacetyl (TAC) protection groups. Standard reagents (Sigma Aldrich Proligo) and standard protocols for RNA were used and synthesis performed on an ExpediteTM 8909 automated synthesizer. For introducing the norbornene modification, a norbornene-phosphoramidite[2] was coupled at the 5'-end. An alkyne (octynyl) moiety was introduced by internal coupling of C8-alkyne-dU phosphoramidite (baseclick). All phosphoramidites were used as 0.1 M solutions in acetonitrile (water content ≤ 10 ppm). After synthesis, the oligonucleotide was cleaved from the solid support and TAC-deprotected by incubation for 2 hours at room temperature with 1 ml aqueous ammonia (28-30%). After washing of the solid support with water, the combined aqueous fractions were extracted three times with 1 ml chloroform followed by lyophilisation of the aqueous phase. The lyophilized product was TBDMS-deprotected by incubation with TBAF (0.5 ml, 1 M in THF) for 24 h at room temperature, followed by addition of 900 µl H_2O, 150 µl 5 M ammonium acetate (0.5 M final concentration) and extraction (three times) with chloroform. Then, RNA was precipitated by addition of 2 volumes of isopropanol.[1] RNA was purified twice by 12% denaturing PAGE (12%, 1mm thick, run at 17W). Bands were visualized by UV-shadowing on a fluorescently coated thin layer chromatography plate (Polygram Sil G/UV254 plates) under UV light (254 nm), excised and eluted in 0.3 M sodium acetate overnight at 25°C, 550 rpm, and precipitated by addition of 2.2 volumes of 100% ethanol and centrifugation at maximum speed in a cooled

microcentrifuge. Pellets were washed twice with 70% ethanol and redissolved in MilliQ grade water.

Tailing of modified DNA with azido-modified nucleotides

Tailing of DNA was performed in varying batch sizes. Concentrations of reagents were generally the same while total amounts varied: 5 µM DNA and 1 U/µl TdT (Thermo Scientific) were incubated with 10 µM 3'-ddGTP (tebu-bio) in the commercial 1x TdT buffer with $CoCl_2$ for 12 h at 37°C, or with 100 µM N_6-*H*N_3-3'-dATP (Jena Bioscience; 10 µM were used in early experiments) for 2 h (12 h for early experiments with 10 µM N_6-*H*N_3-3'-dATP), at 37°C, followed by precipitation with isopropanol, in presence of 40 µg RNA-grade glycogen (Thermo Scientific). Isopropanol precipitation was performed as described above for ethanol precipitation. As DNA measurements at low concentrations are not very precise, DNA was not generally quantified at this step, and as near-quantitative recovery is generally observed in presence of glycogen, quantitative recovery was assumed for further calculations. Near-quantitative recovery was further corroborated by PAGE analyses, e.g., as shown in Fig. 2C, where equivalent amounts of tailed and non-tailed DNA were loaded on lanes 1 and 2 and led to comparable SYBR gold signals. Total amounts of reactants and total volumes used in all DNA tailing experiments shown in this work are detailed in Table S5.

Initial click optimization and general conditions

Click reagents used in this work are listed in Table S6. All reactions were generally performed in 50 mM phosphate buffer, pH 7, unless mentioned otherwise. DARinv optimization reactions were performed with 4 µM DNA with or without prior TdT tailing. For SPAAC optimization, DNA concentration (after TdT reaction) was kept at 2 µM, while DIBAC-Fluor 488 concentrations, reaction times and temperatures were varied as indicated. SPAAC reactions were stopped after the time indicated by addition of 1/10 reaction volume of 50 mM 3-azidopropyl acetate. CuAAC optimization was performed with 1 or 5 µM DNA without TdT tailing, and either 100 µM $CuSO_4$, 500 µM Tris (3-hydroxypropyltriazolyl-methyl)amine (THPTA) and 1 mM sodium ascorbate, or 500 µM $CuSO_4$, 2.5 mM THPTA and 5 mM sodium ascorbate. All reaction mixes were isopropanol precipitated and analysed by 18% denaturing PAGE.

Total amounts of reactants and total volumes for each test reaction shown in this work are shown in Table S7.

One-pot sequential triple click labeling of DNA

Triple-functionalized oligonucleotides were first subjected to DARinv (4 µM DNA, 80 µM tetrazine, 1 h, 25°C), then to SPAAC (2 µM DNA, 10 µM DIBAC Fluor 488, 20 min at 65°C), and then to CuAAC (1 µM DNA, 500 µM azide, 500 µM $CuSO_4$, 2.5 mM THPTA, 5 mM sodium ascorbate, 2 h at 50°C). All reaction steps were performed in 50 mM phosphate buffer, pH 7 - additional buffer was added alongside the other components in order to keep the concentration constant. To monitor the success of each reaction step, aliquots containing equivalent amounts of DNA were taken after each reaction step, frozen or stopped by addition of 1/10 volume of 50 mM 3-azidopropyl acetate (SPAAC only) and then purified alongside the final reaction products. All click reaction products were purified by precipitation with isopropanol in presence of glycogen and analyzed by denaturing PAGE. Conditions for triple labeling experiments with TCO- and BCnonene-modified DNAs are given in Supplementary Text. Total amounts of DNA and click reactants, as well as total volumes in each step for the different triple labeling experiments shown in this work are detailed in Table S8.

Tailing of RNA with 2'-N_3-GTP

To obtain RNA for triple labeling, 1 nmol of preQ1 5'-RNA (20 µM concentration, in 50 µl) was incubated with 25 nmol 2'-N_3-GTP (tebu-bio, 500 µM concentration) and 1,200 U yeast PAP (Thermo Scientific, 24 U/µl) in 1x yeast PAP buffer RNA for 2 h at 37°C, followed by 10 min at 65°C for heat inactivation.

To obtain the full-length riboswitch construct for triple labeling, the preQ1 3'-RNA fragment was first radiolabeled and then ligated to the tailed preQ1 5'-RNA fragment in a splinted ligation, whereupon the DNA splint was digested. These procedures are described in detail in the following paragraph.

Radiolabeling of preQ1-3'-RNA and ligation to tailed RNA

40 pmol preQ1-3'-RNA (4 µM) was first dephosphorylated with 1 U shrimp alkaline phosphatase (SAP Thermo Scientific, 0.1 U/µl) in 1x SAP buffer for 1 h at 37°C in a total volume of 10 µl. The reaction was stopped at 70°C for 10 min. Re-phosphorylation was performed by mixing SAP reaction mixture (2.5 µl – 10 pmol RNA), with 13.33 pmol of γ-^{32}P-ATP (Hartmann Analytic, 3000 Ci/mmol), 10 U T4 polynucleotide kinase (Thermo Scientific), and 1 µl 10x PNK buffer, in a total volume of 12.5 µl. Radiolabeled RNA was purified by denaturing PAGE (12%, 1mm), radioactive bands were visualized using a storage phosphor screen scanned on a Typhoon 9400 scanner, and excised. Radiolabeled RNA was then eluted and ethanol precipitated as described above and quantified by Cherenkov counting in a LS6500 Multiple-Purpose Scintillation Counter (Beckman Coulter) prior to elution and after precipitation and re-dissolving in 5 µl H_2O. Radiolabeled RNA was set to ~1 µM volume by addition of water, according to Cherenkov counts.

1 nmol of non-labeled preQ$_1$-3'-RNA (5 µM in the final reaction volume), ~1 pmol of radiolabelled preQ$_1$-3'-RNA (~ 5 nM), 900 pmol of preQ-1-splint DNA (4.5 µM final) and 10 µl PAP reaction mixture, containing 200 pmol tailed preQ1-5'-RNA (1 µM final) were mixed (29 µl total volume) and heated to 90°C for 30 s. The mixture was then cooled at room temperature for 5 min. To the annealed mixture was added ligation buffer (50 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 500 µM DTT, 50 µM ATP final concentrations) and 4.22 µg RNA ligase 2 (RNL2, in-house preparation, 21.1 ng/µl final concentration) and water to 200 µl final volume. The reaction was allowed to proceed for 1 h at 37°C, followed by 10 min at 80°C to inhibit RNL2. 100 U DNase I (0.5 U/µl) were then added to the ligation mixture and incubated at 37°C for 15 min, to digest the splint DNA. The reactions were stopped by phenol extraction: H_2O and sodium acetate (0.3 M final) were added to obtain a final volume of 200 µl, and extraction was performed twice with 200 µl phenol, then 3x with 600 µl diethyl ether. RNA was then precipitated by addition of 450 µl 100% ethanol and further purified as described above and re-dissolved in 20 µl H_2O. Purification yields were near-quantitative according to radioactivity measurements.

One-pot sequential triple click labeling of RNA

Triple-functionalized, ligated preQ1 riboswitch RNA was first subjected to DARinv (2.2 µM RNA, 12.5 µM tetrazine; 1 h, 25°C), then to SPAAC (2 µM RNA, 20 µM DIBAC Fluor 488, 2 h at 65°C – excess was twice as high and incubation was longer for RNA than for DNA as

longer incubation and higher excess are needed to convert 2'-linked azide, compared to azide linked via a hexyl linker[3]), and then to CuAAC (1 µM RNA, 500 µM azide, 100 µM $CuSO_4$, 500 µM THPTA, 1 mM sodium ascorbate, 2 h at 37°C. The temperature was lower compared to DNA, in order to protect RNA from degradation, which is facilitated by copper ions). All reaction steps were performed in 50 mM phosphate buffer, pH 7 - additional buffer was added alongside the other components in order to keep the concentration constant. To monitor the success of each reaction step, aliquots containing equivalent amounts of RNA were taken after each reaction step, frozen or and then purified alongside the final reaction products. As an additional control reaction, SPAAC was performed with tailed, ligated $preQ_1$ riboswitch RNA, and purified alongside the other intermediate and final products. All RNA click reaction products were purified by precipitation with ethanol in presence of glycogen and analyzed by denaturing PAGE.

Total amounts of RNA and click reaction partners in each step are detailed in Table S9.

PAGE analysis of tailing and click reactions

Reaction products were analyzed on high-percentage, denaturing (7M Urea) polyacrylamide (PAA) gels (typically 18% and 1 mm thickness for DNA, 15% and 0.4 mm thickness for radiolabeled RNA), as indicated. Samples were diluted in 1 volume of loading buffer (1x TBE, 90% formamide) prior to gel loading. 1 mm gels were generally run at 17 W, 0.4 mm gels at 40 W, with 1x TBE as running buffer. Fluorescent labels were directly visualized on a Typhoon 9400 scanner (Table S10). To visualize oligonucleotides independently from labels, 1 mm PAA gels were stained with SYBR gold (Life Technologies) in a 0.5x - 1x dilution of SYBR gold in 1x TBE for ~10 min, orbital shaking at ~100 rpm and scanned on a Typhoon 9400 scanner (Table S10). Radioactivity was visualized by exposing a storage phosphor screen and scanning it in storage phosphor mode on a Typhoon 9400 scanner.

Image treatment

Overlays were generated and bands quantified as previously described[3, 4].

Gel purification of triple-clicked DNA

190 pmol of DNA were triple modified with biotin-tetrazine, DIBAC-Fluor 488 and biotin azide (Table S7), were loaded and run on a denaturing PAA gel, visualized by scanning for

Fluor 488. The desired, triple labeled product was excised, eluted and precipitated as described above, in presence of 2 µg glycogen. DNA was then redissolved in H_2O and quantified on a NanoDrop ND-1000 Spectrophotometer (peqLab), which revealed a recovery of ~75 pmol, corresponding to ~40% isolated yield.

Supplementary Text

Attempts at SPAAC optimization using 3'-ddNTP-tailed DNA

Using 3'-N_3-ddG-tailed DNA and limited (≤10-fold) excess of the DIBAC-Fluor 488, incomplete turnovers of ≤40% were observed (Fig. S1). At a reaction temperature of 65°C, only small differences were observed between different reaction times (40 min or 2 h). At acidic pH, a non-fluorescent side-product was visible in addition to the desired, fluorescent product, which resulted from SPAAC between azide-DNA and the synthetic intermediate DIBAC-amine. We thus concluded that, for 3'-attached azide, higher SPAAC yields could only be achieved at higher DIBAC excess, or possibly at drastically increased reaction time or temperature, neither of which was desired in this context. We therefore opted against 3'-N_3-ddNTPs.

Attempts at tailing and triple click labeling or DARinv of DNA-TCO-alk or DNA-BCnonene-alk

5 µM of the respective DNA containing highly reactive dienophiles TCO or BCnonene and an alkyne moiety (Fig. S2A,B) were tailed with TdT in presence of 10 µM N^6-*H*N_3-3'-dATP for 12 h at 37°C. DNA was isopropanol precipitated and subjected to DARinv (4 µM DNA, 6 µM tetrazine-biotin, which is usually enough to convert the highly reactive dienophiles, 30 min at room temperature), then to SPAAC with DIBAC-Fluor 488 and CuAAC with biotin-azide, as described in Materials and Methods. After each reaction step, aliquots of reaction intermediates containing equivalent amounts were withdrawn and, in case of the SPAAC step, the reaction was quenched with 3-azidopropyl acetate. Additionally, to validate reactivity in DARinv, tailed and un-tailed DNA-TCO-alkyne and DNA-BCnonene-alkyne were subjected to DARinv alone (4 µM DNA, 20 µM tetrazine-biotin, 140 min at room temperature). All reactions were analyzed by PAGE. Triple-labeling gels were scanned for Fluor 488 and stained with SYBR gold. DARinv-only gels were stained with SYBR gold.

Side-reactions between azide and dienophile

TdT tailing reaction of DNA-TCO-alk or DNA-BCnonene-alk with N^6-*H*N_3-3'-dATP produced a range of (side-)products (Fig. S2C-F), of which one was running below the starting band. We interpreted the lower-running product as a cyclization product. Two major higher-running products were also observed. All (side-)products were still reactive in CuAAC (Fig. S2C,D), showing that the terminal alkyne was intact. There was hardly any reaction product detected after DARinv (Fig. S2C-F), whereas non-tailed DNA reacted efficiently in DARinv (Fig. S2E,F), indicating that the dienophile group was blocked after TdT tailing. Only the lower one of the two higher-running products disappeared after SPAAC, whereas the lowest- and highest-running product bands remained unchanged, indicating that the azide group was blocked in those (Fig. S2C,D). The most likely explanation for this is that the dienophiles had reacted with the azides, either intra-molecularly, to form a cyclized product (lower-running), or inter-molecularly, to form a product in which the 5'-norbornene is conjugated to an azide-bearing nucleotide (top band). This type of reaction proceeds very slowly under normal conditions, making DARinv and CuAAC[1] or DARinv and SPAAC[5] orthogonal. Prolonged tailing times (12h), and the presence of cobalt ions[6] may have allowed this type of reaction to proceed nonetheless. Should highly reactive dienophiles be required, shortening of tailing times and the use of alternative tailing buffers (without cobalt ions) may help circumvent this type of cross-reaction. In our case, however, we opted for the use of norbornene as the dienophile, which only showed minimal cross-reactivity under the final tailing conditions.

Reaction yields of click reactions

Reaction yields of click reactions were not generally quantified in an exact manner, for several reasons:

1) Relative yields

Relative yields can, in principle, be determined using gel analyses. However, this is only possible if signals are not influenced by the modifications attached. This is the case for radioactive signals, as that used to visualize the triple labeled RNA construct, for which a relative yield of ~40% desired, triple labeled construct was determined.

However, this is not the case for the SYBR gold signal used to visualize the DNA constructs after triple click reaction. Here, the addition of DIBAC Fluor 488 in SPAAC led to an increase

in signal, whereas the addition of Alexa Fluor 647 or Cy5 in DARinv or CuAAC led to quenching of the signal. Both phenomena make a reliable quantification impossible. We therefore do not report on relative yields for triple labeling, with the exception of triple clicked RNA.

2) Isolated yields

Several factors make the determination of isolated yields difficult. The first one is that quantification of the relatively low amounts of DNA employed in our click reactions are generally hard to quantify in a reliable manner. Measurements of absorbance at 260 nm as in a NanoDrop are only reliable at higher amounts, such as the ones used in the larger-scale approach for gel-eluted product (see Materials & Methods).

Measurements using dyes that bind oligonucleotides of different identity can be used to quantify lower amounts of material. However, these methods do not work when other fluorescent dyes are present in the mixture or on the oligonucleotide itself, as is the case for all our triple labeled constructs.

Furthermore, the presence of different species in a mixture would further complicate the exact determination of isolated yields.

We therefore only report an estimate for the isolated yield for the gel-purified, triple-labeled DNA (see Materials & Methods).

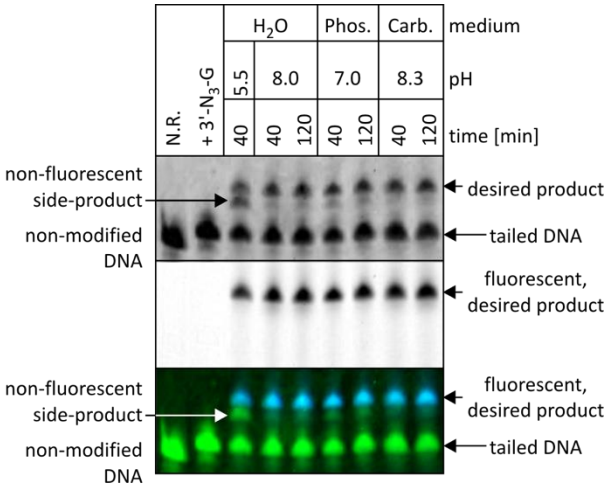

Fig. S1. Attempts at optimization of SPAAC conditions for dual or triple labeling using 3'-N_3-ddG-tailed DNA-alk (2 µM), DIBAC-Fluor 488 (20 µM) and 65°C reaction temperature. Buffer, pH, and incubation time were varied as indicated. Phos.: phosphate buffer pH 7.0, carb.: carbonate buffer pH 8.3. N.R.: no reaction control. PAGE analysis: gels were scanned for Fluor 488 (middle panel, blue in the overlay in lower panel), and then stained with SYBR gold (top panel, green in the overlay, presence of both results in a cyan-colored signal).

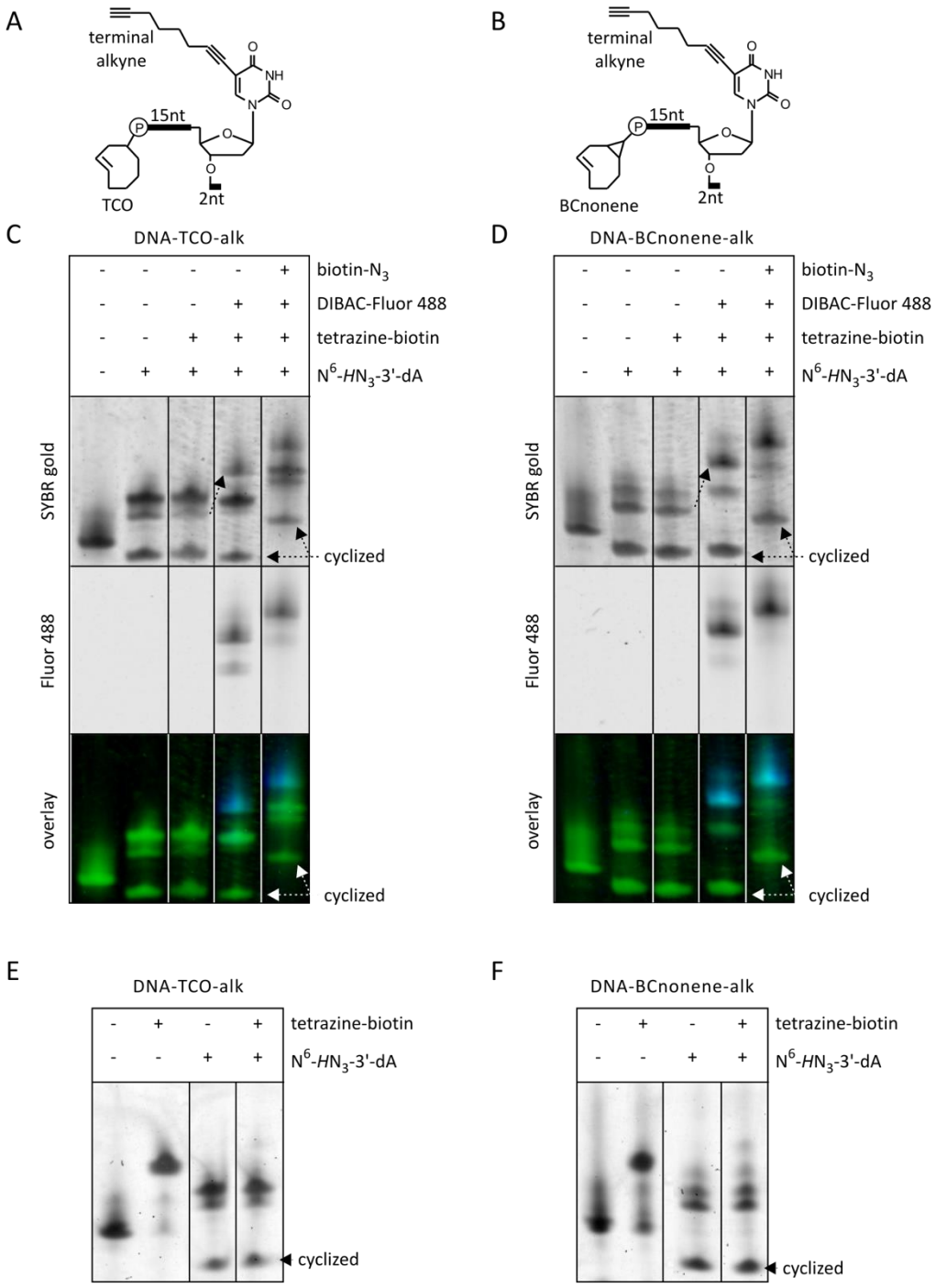

Fig. S2. Attempt of triple DNA labeling with two biotin moieties and one fluorescent dye, using TCO or BCnonene as the dienophile. (**A,B**) Schematic representation of DNA-TCO-alk (**A**) and DNA-BCnonene-alk (**B**). (**C,D**) Tailing with N^6-*H*N_3-3'-dA and subsequent triple labeling of DNA-TCO-alk (**C**) or DNA-BCnonene-alk (**D**). Separate scans and overlay of both (green: SYBR gold, blue: Fluor 488, presence of both: cyan) are shown. Arrows point to the cyclized DNA. Another pair of arrows indicate the single band shift observed upon SPAAC. (**E**,**F**) Validation of DARinv reactivity for non-tailed and N^6-*H*N_3-3'-dA-tailed DNA-TCO-alk (**E**) and DNA-BCnonene-alk (**F**). SYBR gold scans are shown. Lines in all four sub-figures indicate where different lanes of the same gels have been rearranged to facilitate comparison.

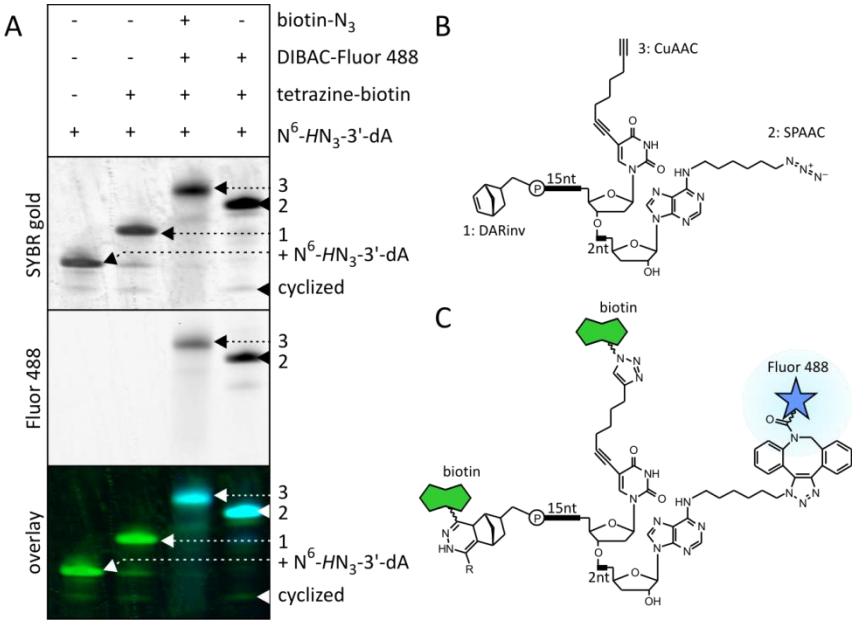

Fig. S3. Triple DNA labelling with two biotin moieties and one fluorescent dye. (**A**) PAGE analysis of reaction intermediates after different reaction steps and final products. Separate scans and overlay of both (green: SYBR gold, blue: Fluor 488, presence of both: cyan) are shown with original gel loading. (**B**,**C**) Pre- and post-click DNA with different reactive groups (**B**) and clicked labels (**C**). Related to Fig. 3.
Each reaction step leads to the appearance of a new, shifted band, and the (near-) complete disappearance of the previous main product.

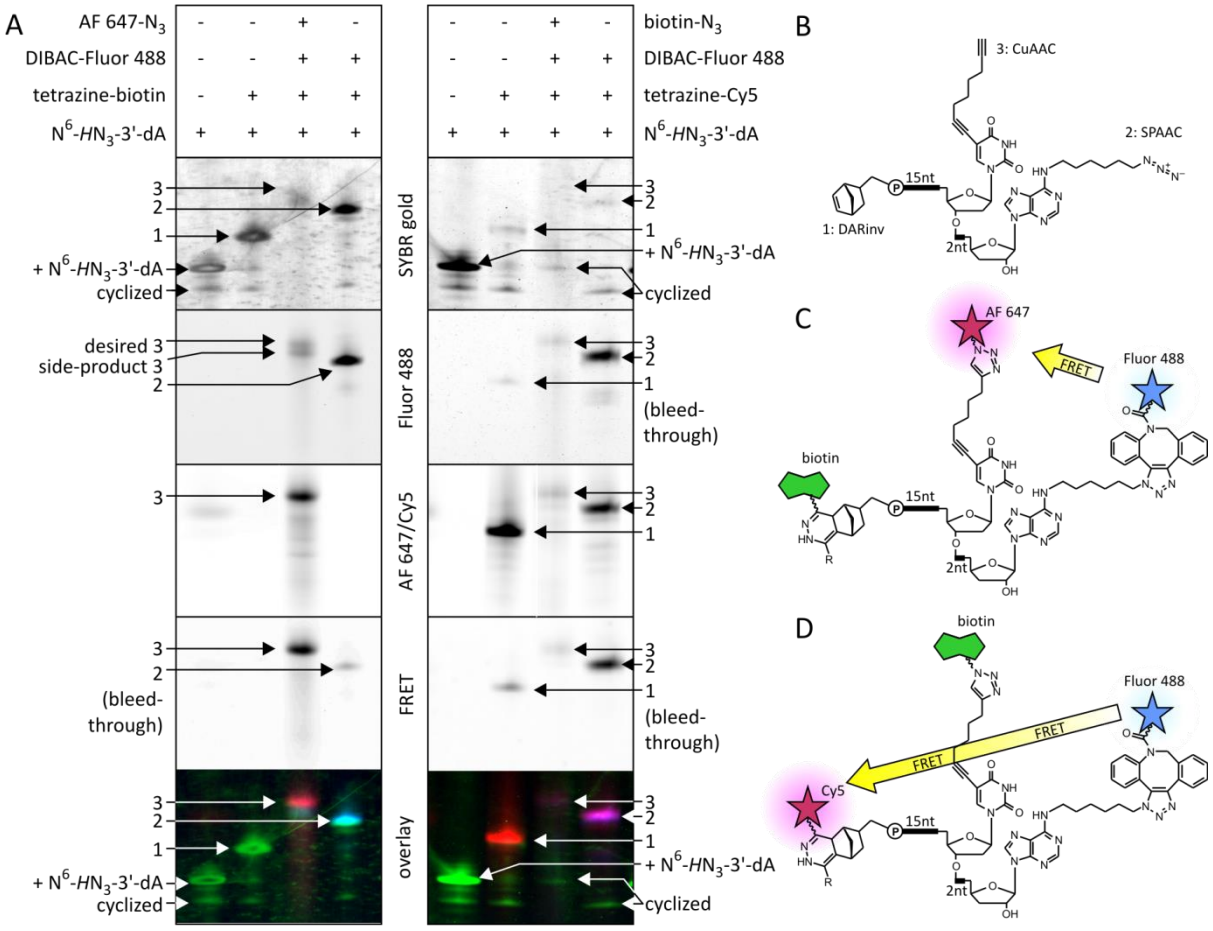

Fig. S4. Triple labeling of N^6-*H*N_3-3'-dA-tailed DNA-norb-alk with biotin-tetrazine and two dyes (DIBAC-Fluor 488 and Alexa Fluor 647-azide / DIBAC-Fluor 488 and Cy5-azide). (**A**) PAGE analysis of reaction intermediates after different reaction steps and final products. Panels show separate scans and overlay of different channels (green: SYBR gold, blue: Fluor 488, red: Alexa Fluor 647 / Cy5, green + blue: cyan, blue + red: magenta, presence of all: white or bright signal). "FRET" shows emission close to Alexa Fluor 647 / Cy5 emission maxima upon excitation at Fluor 488 excitation wavelength. Gels are shown with original gel loading. Note that we observed quenching of SYBR gold signal upon addition of Cy5 or Alexa Fluor 647 and of Fluor 488 upon addition of Cy5 or Alexa Fluor 647. As we were also able to record FRET signals, we concluded that FRET quenching occurred because the two dyes were relatively close to each other within the DNA sequence, even under denaturing conditions in the urea gel. (**B**-**D**) Schematic depiction of pre-click DNA (**B**) (tailed DNA-norb-alk), modified with norbornene, terminal alkyne and azide, and final triple-clicked DNA products (**C**,**D**) after reaction with (**C**) tetrazine-biotin, DIBAC-Fluor 488 and Alexa Fluor 647-azide [left panel of (**A**)] or after reaction with (**D**) tetrazine-Cy5, DIBAC-Fluor 488 and biotin-azide [right panel of (**A**)]. In (**C**) and (**D**) FRET between Fluor 488 and Alexa Fluor 647 or Cy5 is indicated by arrows. Related to Fig. 3. As observed in (**A**), each reaction step leads to (near-) complete disappearance of the previous major reaction product. In case of the attachment of Alexa Fluor 647 or Cy5, the resulting band appears fainter in the SYBR gold and Fluor 488 scans. This is, at least in part, due to quenching effects (mentioned above). Below the desired band, a significant cyan-colored band appeared, which we attribute to a side-reaction, most likely with another azide-containing moiety present in the reaction mixture, as this band is also shifted compared to the previous reaction product.

For the attachment of biotin after Fluor 488 and Cy5 (last step - CuAAC - in right panel in (**A**), see also scheme in (**D**)), the resulting band is very faint, probably due to poor recovery caused by high hydrophobicity of the triple-labelled product. However, disappearance of the intermediate from the previous reaction step indicates that the reaction proceeded (near-) quantitatively, also in this case.

Note that we cannot fully exclude partial degradation of DNA in the CuAAC step, although a 5-fold excess of ligand THPTA over copper ions in the reaction mixture should reduce this possibility to a minimum. Degradation might explain the appearance of some of the faint shorter products and smear observed in the left panel of (**A**) after CuAAC. Degradation could also, in part, account for the very faint product band after CuAAC in the right panel of (**A**). However, arguing against high levels of degradation, shorter products in the right panel do not appear stronger in intensity after CuAAC than after DARinv or SPAAC. Furthermore, other bands, such as the one corresponding to the cyclized product, do not decrease strongly in intensity either, indicating that significant degradation is not likely to have occurred.

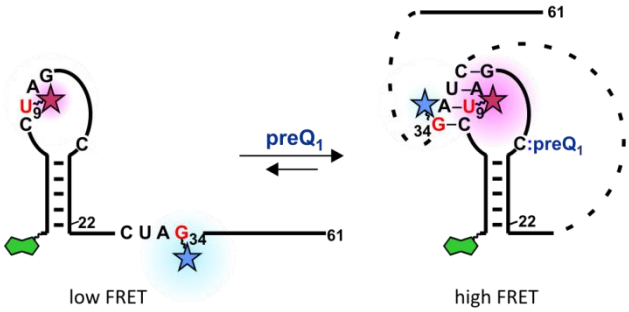

Fig. S5. Choice of attachment positions for triple labeling of the $preQ_1$ riboswitch RNA. Conformational changes that the $preQ_1$ riboswitch undergoes upon ligand binding[7] are shown. We chose U9 and G34 as attachment positions for dyes (stars) and the 5'-terminus as attachment position for biotin, which allows immobilization for smFRET studies. Upon $preQ_1$-binding both dyes would be brought into close proximity. This would lead to a change in FRET efficiency. Adapted from[7].

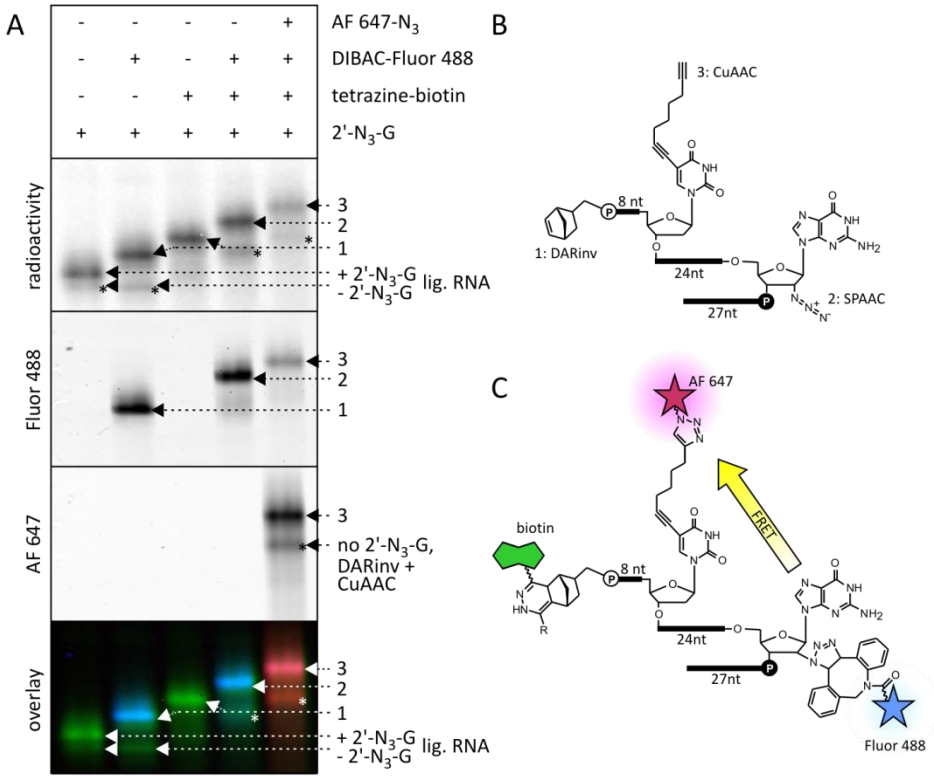

Fig. S6. Triple RNA labelling with biotin and two dyes (DIBAC-Fluor 488 and Alexa Fluor 647). (**A**) PAGE analysis of reaction intermediates after different reaction steps and final product. Different panels show separate scans and overlay of different channels (green: radioactive signal, blue: Fluor 488, red: Alexa Fluor 647 (AF 647) / Cy5, green + blue: cyan, green + red: yellow/orange, presence of all: white or brighter signal). Bands resulting from a fraction of RNA in which the preQ$_1$-5'-RNA was not successfully tailed with 2'-N$_3$-G, and which do not contain the azide to undergo SPAAC are indicated with a star (*). These side products are visible in the radioactivity and AF 647 channels, but not in the Fluor 488 channel. (**B**) Starting RNA (tailed preQ$_1$-5'-RNA, ligated to 5'-radiolabelled preQ$_1$-3'-RNA), modified with norbornene, terminal alkyne and azide. Radioactive label (^{32}P in the phosphate) is indicated by a white P on black. (**C**) Resulting triple-clicked RNA after reaction with tetrazine-biotin, DIBAC-Fluor 488 and Alexa Fluor 647-azide. FRET that should occur in a folded state is indicated by an arrow. Related to Fig. 4.

Supplementary Tables

Table S1. Reaction conditions that allowed (near-) quantitative turnover of functionalized DNA. Conditions that were later selected for DNA triple labeling are shown in bold.

Reaction	DNA concentration [µM]	Label concentration [fold-excess/µM]	Temperature [°C]	Duration [min]	Copper concentration [µM]
SPAAC	**2**	**5x/10**	**65**	**20**	-
		10x/20	65	5-10	
		10x/20	37	15	
CuAAC	5	100x/500	37	**120**	500
	1	500x/500	37		**500**
	1	**500x/500**	**50**		100
DARinv	**4**	**20x/80**	**R.T. (25°C)**	**≥60**	-

Table S2. Reaction conditions for RNA triple labeling.

Reaction	RNA concentration [µM]	Label concentration [fold-excess/µM]	Temperature [°C]	Duration [min]	Copper concentration [µM]
SPAAC	**2**	**10x/20***	**65**	**120***	-
CuAAC	**1**	**500x/500**	**37**	**120**	**100†**
DARinv	**2.2**	**5.6x/12.5‡**	**R.T. (25°C)**	**60**	-

*Higher DIBAC excess and longer reaction time used for RNA than for DNA, to allow for efficient conversion of 2'-attached azide, which is less reactive than azide linked via hexyl linker[3].

†Lower copper concentration and reaction temperature used for RNA than for DNA, to minimize RNA degradation.

‡Lower tetrazine concentration used for RNA than for DNA, due to extended reaction times in SPAAC, during which DARinv continues to proceed.

Table S3. Modified DNA for test reactions and the generation of triple-labeled DNA by TdT tailing, DARinv, SPAAC, and CuAAC.

ID	Sequence	Provenience
DNA-alk	5'-GGAGCTCAGCCTTCAC (5- (C8-alkyne)-dU)-GC-3'	in-house solid-phase oligonucleotide synthesis
DNA-norb-alk	5'-Norbornene-GGAGCTCAGCCTTCAC (5- (C8-alkyne)-dU)-GC-3'	
DNA-TCO-alk	5'-TCO-GGAGCTCAGCCTTCAC (5- (C8-alkyne)-dU)-GC-3'	
DNA-BCnonene-alk	5'-BCnonene-GGAGCTCAGCCTTCAC (5- (C8-alkyne)-dU)-GC-3'	

Table S4. Oligonucleotides used for synthesis of triple-modified RNA by PAP tailing, splinted ligation, DARinv, SPAAC, and CuAAC. *F. nucleatum*: *Fusobacterium nucleatum*.

ID	Sequence	Provenience	Comments
preQ1-5'-RNA	5'-Norbornene-AGAUGUGC- (5- (C8-alkyne)-dU)-AGCAAAACCAUCUUUAAAAAACUA-3'	in-house solid-phase oligonucleotide synthesis	*F. nucleatum* $preQ_1$-riboswitch[7] aptamer
preQ1-3'-RNA	5'-p-ACUUGGGGUGCAAGUCCCCUUUUUUAU-3'	Biomers	*F. nucleatum* $preQ_1$-riboswitch[7] expression platform
preQ1-splint	5'-ATAAAAAAGGGGACTTGCACCCCAAGTCTAGT TTTTTAAAGATGGTTTTGCTAGCACATCT-3'	Biomers	splint for ligation of tailed $preQ_1$-5'-RNA and $preQ_1$-3'-RNA

Table S5. Exact amounts of material used in tailing of DNA to obtain starting material for click reactions with DNA (related to Fig. 2,3,S1-S4).

Experiment	Amount of DNA	Amount of nucleotide	Total volume	Reaction conditions
Tailing DNA-alk with N_6-*H*N_3-3'-dATP to test SPAAC (Fig. 2A)	400 pmol	800 pmol	80 µl	12 h, 37°C, 10 min, 70°C
Tailing DNA-norb-alk with with N_6-*H*N_3-3'-dATP to test DARinv (Fig. 2C)	400 pmol	800 pmol	80 µl	12h, 37°C, no heat inactivation
Tailing DNA-norb-alk with with N_6-*H*N_3-3'-dATP for triple labeling with biotin tetrazine, DIBAC Fluor 488 and biotin azide (Fig. 3A;S3)	200 pmol	4 nmol	40 µl	2h, 37°C, no heat inactivation
Tailing DNA-norb-alk with with N_6-*H*N_3-3'-dATP for triple labeling with biotin and dyes (Fig. 3B,C;S4) and with biotin tetrazine, DIBAC Fluor 488 and biotin azide for gel purification	400 pmol	8 nmol	80 µl	2 h, 37°C, no heat inactivation
Tailing DNA-alk with 3'-N_3-ddGTP to test SPAAC (Fig. S1)	300 pmol	600 pmol	60 µl	12 h, 37°C, 10 min, 70°C
Tailing DNA-TCO-alk/DNA-BCN-alk with N_6-*H*N_3-3'-dATP to test DARinv and triple labeling (Fig. S2)	400 pmol	800 pmol	80 µl	12 h, 37°C, no heat inactivation

Table S6. Click reagents used in this work.

Reagent (alternative name)	Provenience
Alexa Fluor 647-azide (AF 647-N_3)	Life Technologies
Biotin-azide (biotin-N_3)	in-house synthesis[8]
Biotin-tetrazine	in-house synthesis[2]
Cy5-tetrazine	kind gift from M. Wiessler (DKFZ)
DIBAC-Fluor 488 (sold as Dibenzylcyclooctyne-Fluor 488)	Jena Bioscience
THPTA	in-house synthesis[4]
3-azidopropyl acetate	in-house synthesis

Table S7. Exact amounts of material used in test click reactions of DNA.

Experiment	Amount of DNA (per condition)	Amount of click reaction partner	Total volume (per condition)	Reaction time
Test SPAAC with DNA-alk tailed with N_6-*H*N_3-3'-dATP (Fig. 2A)	20 pmol	40-200 pmol, as indicated by excess in Fig. 2A	10 µl	As indicated in Fig. 2A
Test CuAAC with DNA-alk (Fig. 2B)	20 pmol (1 µM) / 100 pmol (5 µM)	2 nmol (for 20 pmol DNA) / 10 nmol (for 100 pmol DNA)	20 µl	2h
Test DARinv with DNA-norb-alk tailed with N_6-*H*N_3-3'-dATP (Fig. 2C)	20 pmol	100-800 pmol, as indicated by excess in Fig. 2C	5 µl	As indicated in Fig. 2C
Test SPAAC with DNA-alk tailed with 3'-N_3-ddGTP to (Fig. S1)	20 pmol	200 pmol	10 µl	As indicated in Fig. S1
Test DARinv with DNA-TCO-alk/DNA-BCN-alk non-tailed or tailed with N_6-*H*N_3-3'-dATP (Fig. S2E,F)	20 pmol	100 pmol	5 µl	140 min

Table S8. Exact amounts of material used in triple labeling reactions of DNA, shown for each individual reaction step.

Experiment	Step	Amount of DNA	Amount of click reactant	Total volume	Aliquot removed	Reaction conditions
Triple labeling of DNA-norb-alk tailed with with N_6-*H*N_3-3'-dATP with biotin tetrazine, DIBAC Fluor 488 and biotin azide or Alexa Fluor 647 azide, as indicated in figures (Fig. 3A,B;S3,S4)	1: DARinv (biotin tetrazine)	60 pmol	1.2 nmol	15 µl	5 µl	1h, 25°C
	2: SPAAC (DIBAC Fluor 488)	40 pmol	200 pmol	20 µl	10 µl	20 min, 65°C
	3: CuAAC (as indicated)	20 pmol	10 nmol	20 µl	(all)	2h, 50°C
Triple labeling of DNA-norb-alk with with N_6-*H*N_3-3'-dATP with Cy5 tetrazine, DIBAC Fluor 488 and biotin azide (Fig. 3C; S4)	1: DARinv (Cy5 tetrazine)	80 pmol	1.6 nmol	20 µl	5 µl	1h, 25°C
	2: SPAAC (DIBAC Fluor 488)	60 pmol	300 pmol	30 µl	10 µl	20 min, 65°C
	3: CuAAC (biotin azide)	40 pmol	20 nmol	40 µl	(all)	2h, 50°C
Triple labeling of DNA-TCO-alk/DNA-BCN-alk tailed with N_6-*H*N_3-3'-dATP (Fig. S2C,D)	1: DARinv (biotin-tetrazine)	60 pmol	90 pmol	15 µl	5 µl (20 pmol DNA)	5 min, room temperature
	2: SPAAC (DIBAC Fluor 488)	40 pmol	200 pmol	20 µl	10 µl (20 pmol DNA)	20 min, 65°C
	3: CuAAC (biotin-azide)	20 pmol	10 nmol	20 µl	(all)	2h, 50°C
Triple labeling of DNA-norb-alk tailed with with N_6-*H*N_3-3'-dATP with biotin tetrazine, DIBAC Fluor 488 and biotin azide for gel purification	1: DARinv (biotin tetrazine)	190 pmol	3.8 nmol	47.5 µl	none	1h, 25°C
	2: SPAAC (DIBAC Fluor 488)	190 pmol	950 pmol	95 µl	none	20 min, 65°C
	3: CuAAC (biotin azide)	190 pmol	95 nmol	190 µl	(all)	2h, 50°C
			Isolated yield: ~75 pmol (~40%)			

Table S9. Exact amounts of material used in triple labeling reaction of RNA, shown for each individual reaction step.

Experiment	Step	Amount of DNA	Amount of click reactant	Total volume	Aliquot removed	Reaction conditions
Triple labeling of $preQ_1$ riboswitch RNA (Fig. 4;S6)	1: DARinv (biotin tetrazine)	50 pmol	280 pmol	22.5 µl	4.5 µl	1h, 25°C
	2: SPAAC (DIBAC Fluor 488)	40 pmol	400 pmol	20 µl	5 µl	2h, 65°C
	3: CuAAC (Alexa Fluor 647 azide)	30 pmol	15 nmol	30 µl	(all)	2h, 37°C
	Additiona reactionl: SPAAC alone (DIBAC Fluor 488)	10 pmol	100 pmol	5 µl	(all)	2h, 65°C

Table S10. List of scan settings for PAGE. (BP: band pass; SP: short pass). Unless mentioned otherwise, all scans were performed at a pixel size of 200 µm. PMT-values were adjusted between 300 and 800 V to assure optimal contrast, without causing overexposure.

Dye (s)	Excitation	Emission
Fluor 488	488 nm	520nm BP 40
Alexa Fluor 647/Cy5	633 nm	670 nm BP 30
FRET between Fluor 488 and AF 647/Cy5	488 nm	670 nm BP 30
SYBR gold	532 nm	526 nm SP

Supplementary References

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