Expanding the chemical scope of RNA: methyltransferases to site-specific alkynylation of RNA for click labeling

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ABSTRACT

This work identifies the combination of enzymatic transfer and click labeling as an efficient method for the site-specific tagging of RNA molecules for biophysical studies. A double-activated analog of the ubiquitous co-substrate S-adenosyl-L-methionine was employed to enzymatically transfer a five carbon chain containing a terminal alkynyl moiety onto RNA. The tRNA:methyltransferase Trm1 transferred the extended alkynyl moiety to its natural target, the N2 of guanosine 26 in tRNA Phe. LC/MS and LC/MS/MS techniques were used to detect and characterize the modified nucleoside as well as its cycloaddition product with a fluorescent azide. The latter resulted from a labeling reaction via Cu(I)-catalyzed azide-alkyne 1,3-cycloaddition click chemistry, producing site-specifically labeled RNA whose suitability for single molecule fluorescence experiments was verified in fluorescence correlation spectroscopy experiments.

INTRODUCTION

The covalent attachment of a chemical moiety to a target macromolecule is of general interest e.g. for labeling, crosslinking, immobilization or isolation of nucleic acids. Enzymatic group transfer reactions have an intrinsic potential for such ‘tagging’ of biomacromolecules. A simple form of tagging involves the use of cofactors (or cosubstrates) carrying a radioactively labeled transferable group. More sophisticated approaches use cosubstrate analogs to transfer chemically altered derivatives of the natural transfer group, which may carry e.g. dyes for fluorescent labeling, or biotin for capture experiments. Recently, tagging with bioorthogonal groups such as terminal alkynes, which can be further modified in a selective second reaction, has become popular. S-adenosyl-L-methionine (AdoMet or SAM, Figure 1A) dependent methyltransferases (MTases) are an attractive class of enzymes for tagging purposes. MTases, which cluster into at least five distinct classes (1–3) are a structurally highly diverse group of enzymes. This functional convergence of catalytic pockets with remarkably distinct structures (1), suggest that at least some MTases should be able to accommodate and turn over non-natural AdoMet analogs in their active site. Many AdoMet analogs have been extensively studied in the past to elucidate features of basic biochemistry (4,5), or as potential targets for pharmaceutical intervention (6), and for a limited number, transfer experiments with side chains other than methyl groups have been conducted. S-adenosyl-L-ethionine and S-adenosyl-L-n-propionine exhibited elevated binding constants in comparison to AdoMet and group transfer kinetics decreased drastically by going from methyl to ethyl and even more to n-propyl (7), making such AdoMet analogs with saturated side chains impractical for the introduction of reporter groups more sophisticated than a radioactive methyl group. Recently, the field has been reinvigorated by the introduction of conceptually new analogs of AdoMet (8). Aziridine-based analogs have been used to tag plasmid DNA with fluorescent groups or biotin (9–12). In
further developments, conceptually new analogs have recently been introduced, in which the transition state of the transfer reaction is stabilized by conjugation to an adjacent unsaturated bond within the side chain (13–17). Here we report the application of such ‘double-activated’ AdoMet analogs to the tagging of tRNA using RNA:MTases. The tagging reaction, during which a carbon chain ending in a terminal alkyne is transferred, opens the way for a number of alternative secondary functionalizations of the tagged tRNA via Cu(I)-catalyzed azide-alkyne 1,3-cycloaddition (CuAAC) (18,19). In this work, we demonstrate the usefulness of this type of enzymatic tagging and CuAAC click chemistry by introducing a fluorophore that allows single molecule detection of labeled RNA in fluorescence correlation spectroscopy (FCS).

MATERIALS AND METHODS

Saccharomyces cerevisiae tRNA\textsubscript{Phe} transcript was obtained by conventional run-off transcription using T7 RNA polymerase and pYF0 plasmid kindly provided by O. Uhlenbeck (20). All proteins used for methylation assays were His\textsubscript{6}-tagged and expressed in Escherichia coli BL21 (DE3), followed by purification by Ni-NTA affinity chromatography. tRNA:m\textsuperscript{2}G\textsubscript{36}-methyltransferase Trm1 from Pyrococcus furiosus (21), yeast S. cerevisiae tRNA:m\textsuperscript{3}C\textsubscript{34,40,48,49}-methyltransferase Trm4 (22), and P. abyssi tRNA:m\textsuperscript{A}\textsubscript{58}-methyltransferase TrmI (23) were described previously. Purified recombinant P. abyssi tRNA:m\textsuperscript{2}G\textsubscript{10}-methyltransferase Trm11 (24) was kindly provided by J. Urbonavicius and H. Grosjean. The AdoMet analog AdeEnYn\textsubscript{2} (17) and THPTA ligand (25) were synthesized as described previously. All other common chemicals were from Sigma-Aldrich.

Tritium incorporation assay and inhibition by AdoEnYn

Methylation activity of recombinant His\textsubscript{6}-tagged Trm1 was measured using 2\textsubscript{m}M tRNA\textsubscript{Phe} transcript as a substrate and 8\textsubscript{m}M [methyl-\textsuperscript{3H}]AdoMet as cosubstrate, essentially as described in (26). Incubation with Trm1 (0.5\textsubscript{m}M) was performed in buffer PUS (100 mM Tris–HCl, pH 8.0, 10 mM MgCl\textsubscript{2}, 100 mM NH\textsubscript{4}OAc, 0.1 mM EDTA and 0.5 mM DTT) at 50\textdegree C for 20 min. The reaction was initiated by addition of enzyme and stopped by pipetting onto Whatman 3MM paper pre-impregnated with 5% TCA, followed by an immediate wash with cold 5% TCA. After two successive washes with EtOH and drying, the radioactivity attached to the filter papers was measured by scintillation counting. The AdoMet analog AdeEnYn\textsubscript{2} was added to reaction mixtures at final concentrations ranging from 0 to 250\textsubscript{m}M.

EnYn-labeling of sctRNA\textsubscript{Phe} transcript

tRNA\textsubscript{Phe} transcript (45 \mu g, 6\textsubscript{m}M final concentration) was incubated at 50\textdegree C for 2 h in buffer PUS (see above) in the presence of 30\textsubscript{m}M AdeEnYn\textsubscript{2} and 6\textsubscript{m}M Trm1. After incubation, the modified transcript was recovered by phenol/CHCl\textsubscript{3} extraction, EtOH precipitation and dissolved in water. Kinetic studies were performed in PUS buffer at 50\textdegree C for 2 h using 2\textsubscript{m}g of tRNA\textsubscript{Phe} transcript (8\textsubscript{m}M final concentration) with 4\textsubscript{m}M of recombinant Trm1.

CuAAC click labeling with Alexa azide

EnYn-modified tRNA\textsubscript{Phe} (see above) was subjected to coupling with AlexaFluor 594 azide (Invitrogen) by CuAAC click chemistry. In brief, EnYn-modified tRNA\textsubscript{Phe} transcript (18\textsubscript{m}g, 14.4\textsubscript{m}M) was incubated at room temperature for 2 h in PUS buffer containing...
were prepared by complete cleavage of modified Samples for HPLC, LC–MS and LC–MS/MS analysis. Analysis by HPLC, LC–MS and LC–MS/MS was performed at 1.5 kV multiplier voltage in the MS/MS mode. The [M-H]+ ions of the two nucleosides Cu–THPTA complex were removed by gel-filtration spin column (Illumina G25, GE Healthcare). The sample was used for reverse transcription (RT) assay or enzymatic digestion for LC–MS/MS without further treatment.

Analysis by HPLC, LC–MS and LC–MS/MS

Samples for HPLC, LC–MS and LC–MS/MS analysis were prepared by complete cleavage of modified tRNA\textsuperscript{Phe} with nuclease P1, followed by phosphodiesterase treatment and de-phosphorylation with shrimp alkaline phosphatase (SAP). Modified tRNA\textsuperscript{Phe} sample (4 μg) was dissolved in 10 mM NH\textsubscript{4}OAc pH 5.3 and incubated overnight at 37°C in the presence of 3 U nuclease P1 (Roche) per 100 μg RNA. Snake venom phosphodiesterase ( Worthington, Lakewood, USA) was then added to a concentration of 0.06 U per 100 μg RNA, and the mixture was incubated at 37°C for another 2 h. Finally, 1/10 volumes of 10× SAP buffer (Fermentas, Lithuania) was added, followed by 3/20 volumes of H\textsubscript{2}O and 1/4 volumes of shrimp alkaline phosphatase (SAP stock at 1 U/μl; from Fermentas, Lithuania) to convert the resulting mixture of mononucleotides to free nucleosides. The mixture was incubated at 37°C for 1 h. The resulting nucleoside mix was directly applied onto a Synergy Fusion RP column (4 μm particle size, 80 Å pore size, 250 mm length, 2 mm inner diameter) pre-equilibrated with 5 mM NH\textsubscript{4}OAc pH 5.3 (buffer A) and eluted with CH\textsubscript{3}OH (0% for 3 min and gradients to 10% in 7 min, to 60% in 10 min and to 70% in 5 min). For analysis of the UV and fluorescence properties, the resulting reaction mixtures were analyzed on a Agilent 1100 series ChemStation equipped with a Diode Array Detector (DAD) (254 and 590 nm) and a Fluorescence Detector (FLD) (excitation 590 nm, emission 617 nm).

For mass analysis, an LC/MS/MS system (Thermo Electron, Dreieich, Germany) consisting of a Surveyor HPLC (quaternary Surveyor LC pump plus, Surveyor autosampler plus with integrated column heater and cooled sample tray and double-wavelength Surveyor UV-Vis plus) and a triple stage quadrupole mass spectrometer (TSQ 7000 with API-2 ion source and performance kit) were used. UV-detection was performed at 254 nm. Note that shifts of retention times from LC/ DAD to LC/MS are due to different dead volumes of the systems. Subsequently, the eluent was introduced without splitting into the electrospray ion source (ESI) of the mass spectrometer. ESI interface parameters were as follows: middle position, spray voltage 4.5 kV, sheath gas (N\textsubscript{2}) 90 psi, aux gas (N\textsubscript{2}) 10 scales, capillary heater temperature 350°C. For identification of common nucleosides, C, U, G A, and EnYn-tRNA\textsuperscript{Phe} was loaded onto a 10% urea-polyacrylamide gel. After electrophoresis, the gel was scanned for AlexaFluor 594 fluorescence on a Typhoon 9410 (GE Healthcare) with excitation at 532 nm and emission at 610 nm (610 BP 30 filter). As loading control, the total tRNA\textsuperscript{Phe} amount per lane was quantified after subsequent staining with SYBRGold and a second fluorescence scan with Cy3 settings (532 excitation, 580 nm emission: 580 BP 30 filter). DNA size marker was run in the left gel lane.

Screening of different tRNA:MTases for EnYn/ AlexaFluor 594-labeling of yeast S. cerevisiae tRNA\textsuperscript{Phe} (sctRNA\textsuperscript{Phe}) was performed with recombinant Trm1, Trm4, Trm1 and Trm11. tRNA\textsuperscript{Phe} transcript was used at 6 μM final concentration, while the final concentrations of enzymes were 2 μM for Trm1, Trm4 and Trm1 and 9 μM for Trm1. After the reaction, modified transcripts were phenol/CH\textsubscript{3}Cl extracted and EtOH precipitated before CuAAC click reaction with AlexaFluor 594-azide. Products were separated by PAGE (10% urea gel) and scanned after staining as described above.

Fluorescence correlation spectroscopy

Fluorescence correlation spectroscopy (FCS) was carried out on an inverted optical microscope IX-70 (Olympus, Japan) equipped with a FV300 laser scanning unit (Olympus, Japan), a single-photon counting avalanche photodiode detector (Perkin-Elmer, USA) and a 60×/1.2 NA water immersion objective (Olympus, Japan). A TimeHarp 200 time-correlated single-photon counting card in combination with the software package
SymPhoTime (both PicoQuant, Germany) was used for data acquisition and analysis. Probes were excited with the 543-nm line of a Helium-Neon laser. Emission was collected after filtering through a LP565 long-pass filter. All samples were analyzed at click-labeled tRNA\textsuperscript{Phe} concentrations of ~10 nM in 20 mM Tris, 1 mM EDTA and 1 mM DTT at pH 7.4. Measurements were carried out in eight-well, polystyrene-chambered cover-glasses (Laboratory-Tek, Nalge Nunc International) at 22°C. Rhodamine 6G served as a reference dye of known diffusion coefficient to calibrate the confocal volume. Experimental AlexaFluor 594 autocorrelation curves were fitted with a single component model function accounting for triplet state kinetics. AlexaFluor 594-labeled tRNA autocorrelation curves were fitted with a model function accounting for two components plus triplet state kinetics. For these fits the diffusion time of one component was set to the previously determined diffusion time of free AlexaFluor 594. For fitting free AlexaFluor 594 and AlexaFluor 594-labeled tRNA\textsuperscript{Phe} autocorrelation curves, the structural parameter $S$ and the confocal volume $V$ of the setup were set to fixed values previously determined with a reference dye of known diffusion coefficient, i.e. Rhodamine 6G ($S \approx 6, V \approx 0.2$ fl). The diffusion coefficients of the samples were obtained from the fits and converted to their hydrodynamic radii through the Stokes–Einstein relation as described previously (28).

**RESULTS**

tRNA:methyltransferase activity is inhibited by a double activated AdoMet analog

As a potential synthetic cosubstrate for enzymatic transfer of a terminal alkynyl group, the AdoMet analog AdoEnYn \textbf{2} (Figure 1A) was tested. AdoEnYn \textbf{2} contains a pent-2-en-4-ynyl (EnYn) side chain instead of the methyl group (17). The double bond in $\beta$-position to the sulfonium center counteracts unfavorable steric interactions within the $S_{2,2}$-like transition by conjugative stabilization (13,14,29) and the terminal alkyn serves as reactive bio-orthogonal functionality. Trm1 is an enzyme known to transfer the methyl group from AdoMet \textbf{1} to N2 of guanosine 26 in tRNAs, including an unmodified transcript of yeast tRNA\textsuperscript{Phe} (21,30). In our hands, preparations of recombinant Trm1 from \textit{Pyrococcus furiosus} exhibited robust \textit{in vitro} activity in a tritium incorporation assay with various tRNA substrates (26) and therefore was chosen as test system. We found the tRNA\textsuperscript{Phe} methylation reaction to be inhibited by micromolar concentrations of AdoEnYn (Figure 1B). A more detailed characterization at various concentrations was performed and analyzed in an Eadie-Hofstee plot (Figure 1C). The analysis indicated a mixed type of inhibition and an IC$_{50}$ around 100 nM. This value is comparable to other AdoMet analogs tested on a tRNA-m\textsuperscript{G}-methyltransferase (4) and about two orders of magnitude above typical $K_m$ values for AdoMet (31), which is not unexpected given the bulkiness of the side chain.

Direct transfer of EnYn reactive group to tRNA substrate

To test for transfer of the EnYn side chain by Trm1, tRNA\textsuperscript{Phe} was reisolated from a reaction mixture containing Trm1 and AdoEnYn \textbf{2}, but no AdoMet. After enzymatic digestion by nuclease P1, snake venom phosphodiesterase, and alkaline phosphatase, the resulting nucleosides were separated on an RP-18 HPLC column and analyzed by LC/MS (Figure 2). In addition to UV detection of the major nucleotides, MS-based analysis revealed an additional signal at 17.4 min, whose $m/z$ value of 347.7 corresponds to the expected alkynylated guanosine \textbf{3} (calc. 348.1 for [M+H$^+$]). Fragmentation of the glycosidic bond by LC/MS/MS produced a signal at $m/z = 216.1$, matching exactly that of the modified nucleobase \textbf{4} (calc. 216.1 for [M+H$^+$]). We conclude that the EnYn moiety has been enzymatically transferred to a guanosine residue of the tRNA.

Click-labeling of EnYn-modified tRNA\textsuperscript{Phe} with fluorescent Alexa dye

To evaluate the usefulness of enzymatic EnYn incorporation for fluorescent labeling via CuAAC click chemistry (Figure 3A), tRNA modified as described above was reacted with a fluorescent dye carrying an azide group. Reaction mixtures included CuSO$_4$, ascorbate as a reducing agent to generate Cu(I) \textit{in situ}, as well as the Cu(I)-ligand THPTA (25), and AlexaFluor 594 azide (Figure 3A). Under conditions which had been pre-optimized with a synthetic oligodeoxyxynucleotide containing a terminal alkyn (not shown), neither DNA nor RNA (loading controls in Figure 3B) showed any signs of degradation. After reacting EnYn-modified tRNA\textsuperscript{Phe} with AlexaFluor 594 azide, small molecules were removed by microscale gel-filtration and the RNA was analyzed by denaturing urea PAGE (Figure 3B). A scan of AlexaFluor 594 fluorescence revealed a fluorescent band that superimposes to the loading control obtained by RNA staining with SYBRGold. In control reactions, no fluorescence signal was obtained upon omission of either Cu(I), AdoEnYn \textbf{2}, or enzyme. This demonstrates that the fluorescence labeling is indeed the result of a CuAAC reaction, since this reaction is contingent upon the presence of each of the above compounds.

For further detailed characterization of CuAAC derivatization products, fluorescent tRNA was reisolated, enzymatically digested to nucleosides, and analyzed by HPLC as described before for EnYn-labeled tRNA\textsuperscript{Phe}. Prior to characterization by LC/MS and LC/MS/MS, a DAD and a FLD were employed to detect the AlexaFluor 594 chromophore (Figure 4A–C). Apart from the major nucleotides, an additional compound eluted after ~20–22 min, which showed absorption and fluorescence properties characteristic for AlexaFluor 594. Further thorough analysis by LC/MS and LC/MS/MS identified the fluorescent adduct of AlexaFluor 594 azide and the modified guanosine \textbf{3} (Figure 4F), as well as the corresponding fragmentation product resulting from breakage of the glycosidic bond (Figure 4G). Note that the introduction of the Alexa dye results in an additional...
Figure 2. LC/UV (254 nm) and LC/MS (m/z 348) analysis of modified tRNA\textsuperscript{Phe} transcript after enzymatic digestion shows the formation of alkynylated guanosine \textit{3}; an MS taken from the chromatogram at 17.4 min is shown in the lower left. LC/MS/MS shows the typical fragmentation of \textit{3} (m/z 348) to the alkynylated nucleobase \textit{4} (m/z 216; lower right).

Figure 3. Click derivatization of EnYn-modified RNA by a fluorescent azide. The reaction scheme is shown in (A). Reactions were analyzed by denaturing PAGE and subsequent scanning for AlexaFluor 594 fluorescence (Excitation 532 nm, Emission 610 nm; upper left). A loading control was obtained after RNA staining with SYBRGold (B).
Figure 4. Identification of the fluorescent adduct of AlexaFluor 594 azide and the modified guanosine 3. Panels on the left side show HPLC with DAD detection of nucleosides (A) and Alexa dye (B), and fluorescence detection (C). Panels on the right side show detection on an LC/MS/MS setup by UV absorption (D) and tracing of the [M+H]^{2+} mother ion (E). Panel (F) shows an MS of the peak eluting at 20.5 min (note that the given structural formulas contain a positive charge and single protonation will lead to an ion with m/z = 2). LC/MS/MS fragmentation of the mother ion yielded a strong signal resulting from loss of the ribose moiety (G). Note that, due to altered capillary lengths between the various detectors, the peaks show slightly different retention in the DAD/FLD setup (A–C) than in the LC/MS/MS setup (D–G).
positive charge in the positive mode (Figure 4F–G). Yet further confirmation was obtained by identifying the adduct and its fragmentation product in negative MS mode (not shown). No unreacted alkynylated guanosine was detected, suggesting that the click derivatization was quantitative.

Localization of modification position by RT

Determination of the position of modification inside the RNA chain was conducted by an RT arrest assay. When a complementary DNA primer was hybridized to the tRNA’s 3’-end, its enzymatic elongation stopped after incorporation of a dNTP complementary to C27, one nucleotide before the modified G26. This is clearly recognizable in an autoradiography of a denaturing PAGE on which the 32P-radiolabeled RT-mixture was separated alongside RT-based sequencing reactions (Figure 5). Thus, the Trm1 enzyme incorporates the EnYn moiety faithfully at the natural target position for methylation.

Optimal conditions for EnYn tRNA\textsuperscript{Phe} modification by Trm1

The EnYn transfer-click-PAGE sequence was further used as a readout for a more detailed characterization of the transfer reaction of EnYn onto RNA (Figure 6A). An optimal concentration of AdoEnYn 2 around 40 μM
(Figure 6B) is in agreement with its IC50 value of 100 µM. Variation of the enzyme concentration revealed a linear dependence of labeling efficiency and enzyme concentration, with maximal labeling at near stoichiometric concentrations of enzyme and tRNA substrate (not shown). A side-by-side comparison of labeling efficiency with a synthetic oligodeoxynucleotide bearing a terminal alkyne using the EnYn transfer-click-PAGE assay suggests a transfer of EnYn to 30% of the tRNA molecules present in the reaction (not shown).

Use of labeled tRNA\textsuperscript{Phe} in FCS

Click-labeled tRNA\textsuperscript{Phe} molecules were successfully employed in FCS studies. Figure 7 shows normalized FCS autocorrelation curves and the corresponding fits of free AlexaFluor 594 dye and AlexaFluor 594-labeled tRNA\textsuperscript{Phe}. The fits were done using a single component model for AlexaFluor 594 and a dual component model for AlexaFluor 594-labeled tRNA\textsuperscript{Phe} with one component fixed to the diffusion time of free AlexaFluor 594. Both models accounted for triplet state kinetics. The diffusion coefficients of the samples were obtained from the fits and converted to their hydrodynamic radii through the Stokes–Einstein relation. The hydrodynamic radius of free AlexaFluor 594 was 1.2 nm, that of AlexaFluor 594-labeled tRNA\textsuperscript{Phe} 5.7 nm, similar to that obtained from labeled tRNAs obtained by splint ligation techniques (32,33) (correlation data not shown), illustrating that labeled tRNAs can be detected on the single-molecule level.

tRNA labeling using other tRNA:methyltransferases

In our initial screen, three other RNA:MTases with chemically distinct modification sites had been investigated (Figure 8). These enzymes were yeast \textit{S. cerevisiae} tRNA\textsuperscript{m\textsuperscript{1}C\textsubscript{34,40,48,49}}-methyltransferase Trm4 (22), \textit{P. abyssi} tRNA\textsuperscript{m\textsuperscript{1}A\textsubscript{58}}-methyltransferase TrmI (23) and \textit{P. abyssi} tRNA\textsuperscript{m\textsuperscript{2}G\textsubscript{10}}-methyltransferase Trm11 (24). Enzyme concentrations were adjusted to yield similar activity in the tritium incorporation assay under standard conditions, and fluorescent dye incorporation was monitored by EnYn transfer-click-PAGE assay. A robust fluorescence signal was obtained only with the RNA:MTase Trm11 from \textit{P. abyssi} in addition to Trm1. Interestingly, both active enzymes identified so far are specific for the the exocyclic amino group at position 2 of guanosine. Target nucleotides of all tested enzymes are involved in tertiary interactions and similarly hidden inside the tRNA structure. A cautious interpretation of these findings suggests that the chemical nature of the target nucleophile rather than the accessibility of the alkylnyl moiety to click reagents is a limiting factor for efficient RNA labeling. Clearly, further screens must be conducted to reveal if the scope of this tagging technique is determined by the type of chemistry, AdoMet analog binding to the enzyme active site, by the specific activity of enzyme preparation or even the sterical accessibility of the alkylnyl moiety.

DISCUSSION

Post-synthetic enzymatic modification of biopolymers is an attractive approach to introduce labels for biophysical studies. Many biophysical experiments, such as FCS, or single molecule fluorescence resonance energy transfer (smFRET) (33) demand complete site-specificity of labeling, yet require flexibility with respect to the type of label, e.g. the spectral properties of attached dyes. For proteins (17,34–36) and DNA (37–40), the demands for versatile labeling have been addressed by click chemistry such as CuAAC (18,19). CuAAC allows a biopolymer carrying a terminal alkyne (or azide) to be linked with azide (or terminal alkyne) derivatives of a variety of fluorescent dyes, but has so far only rarely been used for the labeling of RNA (41). Here we present an enzymatic posttranscriptional modification approach for RNA, which proved to be stable under the reaction conditions despite the presence of Cu ions, which are known to cleave RNA via redox chemistry (42,43). In our reaction sequence, RNA is efficiently and site-specifically modified.
by an RNA:MTase and an AdoMet analog with a terminal alkyne, which is then used to click on a fluorescent label. The sequence can be exploited for efficient labeling of RNA, which can then be investigated in biophysical studies. An obvious advantage over pre-synthetic labeling methods is that it may be applied to native RNA which is otherwise difficult to label with site specificity (44–45). As the site specificity resides in the RNA:MTases, so does the scope of this method. For example, one may envisage to screen the large variety of rRNA:MTases that is constantly increasing as a result of resistance mechanisms (46,47), in order to label ribosomes for single molecule studies (48). In-depth analysis of additional enzymes must determine how far this labeling approach can be adapted to further types of RNA. Given the huge number of RNA:MTases, and considering that DNA and protein MTases also accept the AdoMet donor, we foresee that this approach will turn out highly versatile.

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