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Bioorthogonal chemistry-based RNA labeling technologies: evolution and current state

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Abstract

To understand the structure and ensuing function of RNA in various cellular processes, researchers greatly rely on traditional as well as contemporary labeling technologies to devise efficient biochemical and biophysical platforms. In this context, bioorthogonal chemistry based on chemoselective reactions that work under biologically benign conditions has emerged as a state-of-the-art labeling technology for functionalizing biopolymers. Implementation of this technology on sugar, protein, lipid and DNA is fairly well established. However, its use in labeling RNA has posed challenges due to the fragile nature of RNA. In this feature article, we provide an account of bioorthogonal chemistry-based RNA labeling techniques developed in our lab along with a detailed discussion on other technologies put forward recently. In particular, we focus on the development and applications of covalent methods to label RNA by transcription and posttranscription chemo-enzymatic approaches. It is expected that existing as well as new bioorthogonal functionalization methods will immensely advance our understanding of RNA and support the development of RNA-based diagnostic and therapeutic tools.

1 Introduction

In the hall of fame of biopolymers, RNA is uniquely recognized for its ability to participate in a myriad of cellular processes including coding, transfer and regulation of gene expression in prokaryotes and eukaryotes.[1,2] Apart from the major types (tRNA, rRNA and mRNA), the world of RNA encompasses several noncoding RNA (ncRNA), small nuclear RNA (snRNA), small nucleolar RNA (snoRNA), micro RNA (miRNA), enhancer RNA (eRNA), and so on, each performing an intricate function.[3–5] Like any music note, the functional syncing of several such RNA species orchestrates the biological symphony, and this is essentially achieved by the dynamic range of RNA structures, which enable them to bind to various metabolites, proteins and nucleic acids.[5–7] In order to unravel the functional intricacies associated with these biomolecules, it is pivotal to functionalize RNA with synthetic tags. The wide use of various biophysical tools, namely fluorescence, X-ray, EPR and NMR, to study RNA structure and dynamics led to the evolution of several strategies to label RNA with compatible probes.[8–13] Of these, the most routine methods use either

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enzymatic or solid-phase synthesis. These methods offer an easy means to conjugate a variety of functional tags and biophysical probes of interest with relatively minimal impact on the structure or function of RNA oligonucleotides (ONs).[14,15] Functionalized RNAs thus synthesized have not only tremendously advanced our understanding of the complexity of various RNA structures and their function but also have paved ways to develop RNA-based diagnostic and therapeutic tools.[14,16–18]

RNA labeling employing conventional solid-phase ON synthesis uses an automatized synthesizer. Here, the RNA ON of interest is grown, one nucleotide at a time on a solid support with modified nucleoside phosphoramidites using iterative rounds of deprotection, coupling, oxidation and capping reactions, before a global deprotection is performed to afford the functionalized ON. Even though this method is advantageous for introducing site-specific labels on RNA ONs, in many instances, modified phosphoramidite substrates do not withstand the relatively harsh chemical conditions of solid phase synthesis.[19] In addition, the length of RNA ONs synthesized is restricted (<50 mer) due to a gradual decrease in the efficiency of coupling with each iteration of nucleotide addition (due to the presence of additional 2′-O bulky protecting groups), which apparently is not encountered in the synthesis of DNA ONs. Alternatively, enzymatic synthesis exploits bacteriophage DNA dependent RNA polymerases like T7, SP6 and T3 to introduce modifications on RNA.[20–22] Although indiscriminate body labeling of RNA transcripts is a major drawback of this approach (unless unnatural base pairs are used), the method is not limited by the length of RNA and produces good amounts of the multiply labeled RNA.[23,24] Several groups including ours have employed such an approach to introduce modifications on RNA ONs.[25–27] Even though many chemical functionalities can be introduced on RNA using this approach, a drastic decrease in the efficiency of incorporation is observed when synthons contain bulky functional tags and more often the enzymes poorly process modified nucleotide substrates. Herein, incorporating a minimally perturbing chemical handle into RNA can open up post-synthetic routes to functionalize RNA. This is commonly performed using acid–amine coupling and thiol-maleimide Michael addition reactions.[28] Yet, the lack of selectivity and poor kinetics of these reactions have demanded robust conjugation strategies to tether synthetic tags.

With chemists becoming increasingly interested in developing tools to interrogate biological processes, a notable number of chemoselective transformations were elegantly standardized to be performed under biologically benign conditions.[29,30] Rigorous efforts in this direction led to the advent of bioorthogonal chemistry, which now is extensively used in investigating glycans,[31,32] proteins,[33,34] lipids,[35,36] DNA,[37,38] and more recently RNA.[39,40] Here, in this feature article, we provide an elaborate discussion on the contributions of various labs including ours in devising innovative approaches to covalently label RNA in cell-free and native cellular environments by using bioorthogonal chemistry. Alongside, we discuss how these approaches have been put to use in studying RNA and also in devising RNA-based diagnostic tools.

2 Covalent strategies to label RNA and their applications

As opposed to non-covalent methods, where binding is primarily dependent on the dissociation constant of the molecules involved, covalent strategies to label RNA promise a stable bond between the functional tag and the RNA molecule. Due to certain limitations of solid-phase chemistry, other techniques to label RNA initially focused on post-synthetic chemical tagging. Reactions like acid–amine, thiol–maleimide Michael addition, and periodate reaction gained particular attention towards labeling ONs.[41] However, limited reactivity, biocompatibility and specificity of the cognate counterparts used in these approaches reduced their frequent use and necessitated better labeling chemistries. Meantime, several traditional chemoselective reactions were tuned to functionalize biomolecules *in vitro* and subsequently optimized to work under cellular conditions. Such reactions were initially established on sugars, peptides and proteins and then on nucleic acids. However, their use in labeling RNA remained a major challenge largely due to the inherent instability of RNA. In the below sections, we discuss the evolution, applications and current state of biortho-gonal chemistry-based RNA labeling technologies.

2.1 Advent of biorthogonal chemistry for labeling nucleic acids

Cycloaddition reactions have been popular among organic chemists for several decades. Initial work by Michael and further elaborate work of Huisgen gave rise to the widely used 1,3-dipolar cycloaddition reaction between an azide and alkyne.[42,43] Although the use of high temperature limited its utility in many applications, independent work of Sharpless and coworkers as well as Meldal and coworkers in 2002 demonstrated the use of Cu catalyst in performing the reactions at room temperature.[44,45] Also, the use of Cu facilitated the formation of a specific regioisomer. A major leap in bioconjugation strategy was put forth by Bertozzi and coworkers, who explored Staudinger ligation and azide–alkyne cycloaddition reactions in a cellular setting and coined the term, bioorthogonal click reactions.[46,47] Here, the reactive moieties are orthogonal, biologically inert, non-toxic and largely preserve the function of labeled biomacromolecules.[48] The Staudinger ligation reaction used an azide and a phosphine substrate to tether various functional tags using a stable amide bond.[48] Although this chemistry provided a promising start to the development of a new class of bioorthogonal reactions, the decreased stability of phosphine counterparts and relatively slow kinetics impeded its extensive application. On the other hand, copper-assisted azide–alkyne cycloaddition (CuAAC) reaction outperformed Staudinger ligation as a fast and efficient reaction to tag various biomacromolecules *(e.g.* glycans, proteins, lipids and nucleic acids).[37,47,49] Even though the cytotoxicity of copper posed concerns for its use in biological systems, it was significantly overcome by the use of water-soluble Cu(I)-stabilizing ligands such as tris(3-hydroxypropyltriazolylmethyl)amine (THPTA) and tris-(benzyl-triazolylmethyl)amine (TBTA).[50–52]

Prior to establishing click labeling procedures on RNA, several groups successfully installed functional tags on DNA either *in vitro* or in cells using click chemistry.[37,38,53–58] Seela and Sirivolu incorporated various click-compatible nucleoside phosphoramidites bearing diyne groups into DNA using solid-phase synthesis.[59,60] Later, click compatible triphosphates of 5-ethynyl (EdUTP) and 5-diyne-substituted 2′-deoxyuridine were enzymatically

incorporated for the first time into DNA employing PCR.[54] These nucleotide analogs, which would later become highly popular for metabolic experiments, was used for deliberate high-density body labeling followed by click reaction. Subsequently, a seminal paper from Salic and Mitchison showed metabolic labeling of replicating DNA using EdU, which set the stage for the development of chemo-enzymatic RNA labeling methods discussed below.[57] Although labeling DNA using click chemistry was effectively used for a variety of applications, the inherent instability of RNA posed a major challenge for introducing bioorthogonal click functionalities. RNA labeling utilizing bioorthogonal functional tags requires the installation of clickable nucleotide analogs, which can be incorporated into RNA either (a) by a solid-phase ON synthesis protocol, (b) by employing transcription reaction or (c) post-transcriptionally using enzymes or ribozymes (Fig. 1). Several groups including ours have focused on developing techniques to generate clickable RNAs using RNA processing enzymes with the view of implementing such strategies in analysing RNA in cell-free and native cellular settings.[40,61]

2.2 Installing clickable labels on RNA employing transcription reaction

Click labeling of cellular RNA using alkyne-containing nucleosides—In the beginning, post-synthetic bioconjugation of RNA ONs by azide–alkyne cycloaddition reaction largely preferred the installation of alkyne functionality on ONs because stable alkyne-modified substrates suitable for solid-phase as well as enzymatic incorporation could be readily prepared (Fig. 2). Originally inspired by 5-bromouridine and 4-thiouridine,[62–65] alkyne probes were deployed for metabolic labeling of transcriptome on fixed cells and whole tissue mounts followed by staining with appropriate fluorophores.[66]

Salic and Jao reported a nucleoside analog, 5-ethynyl uridine (EU), which gets salvaged and incorporated into nascent RNA transcripts by RNA polymerases I, II and III.[39] Further, CuAAC reaction was performed on fixed and permeabilized cells, and also on tissue sections employing fluorescent azides. The ease of metabolic incorporation of EU has gained particular popularity for imaging and detecting nascent RNA levels in a wide range of systems.[67–71] Esteban and coworkers reported the capture and profiling of interactome of newly transcribed RNAs in stem cells by using EU.[72] The technology, namely capture of the newly transcribed RNA interactome using click chemistry (RICK), was used to enrich nascent RNA and its interacting proteins, which were crosslinked using UV irradiation (Fig. 3). The enriched nascent RNA transcripts were confirmed by sequencing and their interacting proteins were characterized by mass analysis. Very recently, van Oudenaarden and coworkers used EU for understanding the synthesis and degradation rates of mRNA in a single intestinal stem cell using the single-cell EU-labeled RNA sequencing technique (scEU-seq).[73] In this strategy, cells treated with EU were dissociated, fixed, permeabilized and subjected to click reaction with a biotinylated azide probe. Single cells were sorted and cDNA was generated by reverse transcription using poly-T primers having an additional unique DNA barcode. EU-labeled and unlabeled hybrids were separated by streptavidin coated magnetic beads before sequencing. This strategy marks an important step in deconvoluting the transcript dynamics of individual cells in heterogeneous cell populations. An important application of EU was demonstrated by de Haan and coworkers, where EU was used to visualize RNA synthesis in cells treated with coronavirus (MHV strain A59)

known to cause severe acute respiratory syndrome, SARS-CoV (Fig. 3).[74] Cells were infected with MHV followed by treatment with actinomycin D, an inhibitor of cellular DNA-dependent RNA synthesis, which does not affect viral replication. Cells were subsequently treated with EU and subjected to click reaction with azide-conjugated fluorophores. The staining pattern showed EU incorporation into perinuclear cytoplasmic foci. It might be also possible that such a strategy could be used for visualizing novel SARS-CoV-2 nascent RNA transcripts in cells with clickable metabolic probes.

Similar to EU, 5-ethynylcytidine (EC) was also used for nascent RNA click labeling.[75] Additionally, several independent groups incorporated various nucleoside analogs such as 7-ethynyl-8-aza-7-deazaadenosine (7-EAA),[76] N^6-propargyladenosine (N^6PA)[77] and 2-ethynyladenosine (2-EA)[78] for labeling nascent and polyadenylated RNAs in cells. These approaches are particularly useful for profiling mRNA production in response to stimuli.[78]

We reported an octadyne-modified uridine analog, which was used for functionalizing RNA ONs and cellular RNA transcripts (Fig. 4).[79] The modified triphosphate (ODUTP) is efficiently incorporated into RNA transcripts by bacterial and mammalian RNA polymerases. This allowed posttranscriptional functionalization of RNA with a variety of biophysical tags, namely fluorescent, amino acid, sugar and affinity tags, by clicking with the corresponding azide-containing counterparts. The specific incorporation of ODUTP by endogenous RNA polymerase also enabled the visualization of newly transcribing RNA in cells by click reaction with a fluorescent azide. The presence of an internal alkyne, which is inert to click reaction, makes it possible to detect its distinct Raman signatures at 2106 cm^{-1} and 2240 cm^{-1}, a region free from the cellular Raman signal.[80] This duality of the nucleoside analog can be potentially used in two-channel visualization of RNA in cells by click chemistry and Raman imaging. Further, ODU-labeled RNA is efficiently processed by reverse transcriptase and converted to cDNA, which is particularly useful in aptamer selection protocols.[81] Therefore, in principle this probe can be used for simultaneous nascent RNA detection using fluorescence imaging and stimulated Raman scattering, and can be used for aptamer selection.

Click labeling of cellular RNA using azide-containing nucleosides—A major drawback of CuAAC reaction, which limited its use in live cells, was the toxicity of the copper catalyst used. Alternatively, reagent-free approaches employing Staudinger ligation and strain-promoted azide–alkyne cycloaddition (SPAAC) reactions with an azide handle on the biomacromolecule have been developed for labeling protein and glycans in cells.[47,82] While the azide group serves as a common reactive partner in different bioorthogonal reactions, incorporating an azide group into ONs by chemical methods poses a major challenge as the azide group is not stable under solid-phase ON synthesis conditions.[83–86] Therefore, incorporating a minimally perturbing azide handle into RNA ONs can be considerably useful as it can provide a means to devise a versatile labeling technology, which would be modular and suitable for live cell analysis (Fig. 5). Gaur and coworkers incorporated an azide substrate namely 8-azidoadenosine triphosphate (8-N_3ATP) into RNA in *vitro* using T7 RNA polymerase.[87] Further, the labeled RNA was used for UV-induced photo-crosslinking reaction. However, during this time, click chemistry on nucleic acids was

still in its infancy, and therefore, its compatibility in cycloaddition and Staudinger reactions was not explored.

In order to establish a versatile RNA functionalization method, we relied on transcription reaction to introduce azide labels into transcripts and then we functionalized the transcripts in a modular fashion posttranscriptionally by performing Staudinger ligation, CuAAC and SPAAC reactions.[19,88] The nucleotide analog, 5-azidopropyl uridine triphosphate (APUTP), contains an azide group at the C5 position of uridine with a three-carbon linker (Fig. 5). The modified triphosphate was synthesized in a few steps (now commercially available from Jena Bioscience) and introduced into RNA transcripts utilizing T7 RNA polymerase. When the incorporation efficiency of APUTP was compared to natural UTP on a series of templates (modification installed at different positions on RNA ONs), we observed an efficiency on par with the natural UTP.

Unlike the alkyne-labeled RNA, which can only be labeled by copper-based click reaction, the azide tag on RNA can additionally be functionalized by Staudinger ligation and SPAAC reactions. Notably, this meant that the azide on RNA could now push metabolic labeling to live cells without worrying about the toxicity of copper. Following the success we had with APUTP, AMUTP (containing a shorter one carbon linker) and ATUTP (containing a longer tetra ethylene glycol) were developed, which were found to be efficiently incorporated by T7 RNA polymerase.[89,90] The azide-labeled RNA transcripts were conveniently functionalized *in vitro* with various tags by CuAAC, SPAAC and Staudinger ligation reactions with very good isolable yields sufficient for downstream biophysical analysis. Having learned that our nucleotides can be incorporated into RNA by *in vitro* transcription reaction and further posttranscriptionally functionalized, we tested their use in labeling cellular RNA.

The azide-modified nucleosides, namely AMU, APU and ATU, did not get incorporated via the cellular salvage pathway. So we by-passed this step by transfecting the cells with respective triphosphates and rewardingly, AMUTP was incorporated successfully into nascent RNA transcripts, which were stained by click reaction using fluorescent alkynes (Fig. 6a).[89] Blocking RNA polymerase I and II using actinomycin D and ribonucleoside diphosphate reductase using hydroxyurea confirmed specific incorporation of AMUTP into RNA transcripts. In particular, SPAAC reaction was used for live-cell imaging of the newly synthesized transcripts using a fluorescent dibenzocyclooctyne reagent.

Further, dual-color imaging of RNA labeled with AMUTP and DNA labeled using EdU was also performed employing the orthogonality of azide and alkyne substrates. Notably, this was the first report on the specific incorporation of azide groups into RNA by endogenous RNA polymerases, which allowed the visualization of newly transcribing RNA by click reaction in fixed as well as in live cells.[89]

While C5 alkyne-modified uridine (EU) was incorporated into cellular transcripts by the salvage pathway, our experiments indicated that C5 azidomethyl uridine (AMU) is not compatible for incorporation by the salvage pathway.[89] The reasons could be that AMU is not internalized or it is not a good substrate for the phosphorylation process by cellular pyrimidine salvage enzymes. This issue was investigated by Kleiner's laboratory. Kleiner

and Zhang identified that uridine-cytidine kinase 2 (UCK2), an enzyme involved in monophosphorylating the nucleoside, restricted the incorporation of AMU into RNA.[91] The previously reported crystal structure revealed a tyrosine residue (Y65) at the active site of the enzyme, which was in close proximity to the C5 position of the incoming nucleotide (Fig. 6b).[92] It is likely that the tyrosine residue hindered the incorporation of relatively bulky C5 modified UTPs. Recombinantly expressed UCK2 favoured the efficient *in vitro* conversion of EU to its respective monophosphate but not AMU, which validated the observation from previous cell labeling experiments.[89] To circumvent this problem, Y65 in UCK2 was replaced with a glycine residue, which facilitated the monophosphorylation of AMU. Supporting the hypothesis, cells expressing this engineered UCK2 enabled the incorporation of AMU into cellular RNA without the need of transfection (Fig. 6a).[91] Additionally, the authors also observed that expression of engineered UCK2 in cells also increased the incorporation efficiency of EU into nascent RNA transcripts. Using the above approach, Spitale and coworkers subsequently showed cellspecific metabolic labeling wherein only cells expressing UCK2 were metabolically labeled with azide-containing uridine analogs.[93] Following our earlier reports on azide labeling, this group has also used various azide-modified purine nucleoside analogs for metabolic labeling of nascent RNA.[94] Recently, Luedtke and Tera introduced a cationic Sondheimer diyne derivative, namely DiMOC, wherein noncovalent interactions could facilitate the diyne core to intercalate in a co-facial fashion to sterically minimally accessible azide groups in DNA or RNA duplexes.[95] A second SPAAC reaction was performed on DiMOC to enable visualization of newly formed nucleic acids in cells.

The installation of site-specific labels on RNA as opposed to indiscriminate body-labeling is particularly desirable for applications which demand minimal impact on the RNA structure and its function. Site-specific incorporation of labels into RNA by transcription can be achieved by using unnatural base pairs, which are orthogonal to natural base pairing schemes (A–T and G–C pairs). Unnatural nucleobases developed by the groups of Hirao and Romesberg have set the platform for introducing various bioorthogonal click probes site-specifically on RNA including probes compatible for the inverse electrondemand Diels–Alder (IEDDA) reaction detailed in the section below.[23,96–99] Alternatively, posttranscriptional chemoenzymatic approaches using nucleic acid processing enzymes can also be used, which is discussed in later sections.

Functionalizing RNA employing IEDDA and Pd-mediated reactions—SPAAC chemistry offers a reagent-free approach to label RNA *in vitro* and in cells, but the decreased kinetics, bulkiness of commonly used cyclooctyne reagents and their relatively poor permeability demanded alternative reactions. IEDDA reaction between an electron-rich dienophile and an electron-poor diene, with high reaction speed, has gained particular attention for labeling biomacromolecules.[100,101] Luedtke and coworkers investigated the ability of a vinyl-labeled 2′-deoxyuridine to get metabolically incorporated into replicating DNA in cells and its subsequent functionalization employing IEDDA chemistry.[102] The feasibility of IEDDA reaction on RNA was first established by Jäschke and coworkers.[103] A strained dienophile, norbornene-modified guanosine initiator nucleotide, was incorporated into RNA ONs by *in vitro* transcription reaction, which was further functionalized using

cognate tetrazine counterparts conjugated to biotin or fluorescent tags. Rentmeister and coworkers incorporated a 4-vinylbenzyl group at the 5'-cap of RNA employing a variant of trimethylguanosine synthase 2 from *Giardia lamblia* (GlaTgs2-Var1).[104]

The vinyl label was functionalized using photoclick and IEDDA reactions. This work indeed provided a promising start to vinyltagging on RNA. However, it is to be noted that introducing a minimally invasive vinyl handle developed by Luedtke[102] could be better for internal labeling of RNA as such a modification would preserve the native function of RNA.

Conventional bioorthogonal click reactions result in the formation of residual chemical groups (e.g. triazole) upon reaction between cognate counterparts. Although residual functional groups are not a major concern for many bioconjugation strategies, these additional chemical moieties are not desirable if direct covalent installation of a biophysical probe is required. Here, Pd-based C–C bond forming reactions have a discernible upper hand and have been recently explored on biomacromolecules.[105] Despite Pd-based reactions gaining popularity for protein and DNA labeling, their utility in labeling RNA remained a challenge as reaction conditions typically use high temperatures and basic conditions. In this regard, we developed a two-in-one approach to functionalize RNA ONs having a vinyl handle employing Pd-mediated oxidative Heck and bioorthogonal IEDDA reactions (Fig. 7a).[106] The vinyl handle enabled the direct conjugation of various biophysical probes on RNA transcripts. Oxidative Heck reaction was performed with various heterocyclic aromatic boronic acid/ester substrates in the presence of Pd-EDTA catalyst and O_2, which resulted in fluorogenic RNA ON products showing up to 170-fold enhancement in fluorescence intensity as compared to the starting boronic acid/ester and the vinyl-labeled RNA ON substrates. IEDDA reaction on the RNA ON enabled the conjugation of tetrazine-modified biotin and Cy5 tags. Subsequently, two independent groups proved that VU and other vinyl-labeled nucleosides are good substrates for labeling cellular RNA transcripts by the salvage pathway and the newly transcribed vinyl tagged RNA could be further visualized by performing IEDDA reaction with fluorescent tetrazines (Fig. 7b).[107,108] Additionally, the approach was used for fluorescent detection of LS-180 tumour in live mice treated with VU employing a fluorogenic tetrazine.[108] Notably, these reports further push the utility of this probe for a variety of applications ranging from transcriptome analysis to visualizing viral infection in cells owing to its simple reagentless chemistry.

Useful microenvironment-sensitive fluorescent nucleoside analogs have been generated by conjugating heterocycles onto purine and pyrimidine bases.[8,9] Typically, such nucleoside analogs are synthesized by Pd-catalyzed cross-coupling reactions and then converted to respective phosphoramidite and triphosphate substrates before incorporation by either chemical or enzymatic methods.[105] However, microenvironmentsensitive nucleoside analog-labeled ONs can be constructed by directly performing C–C bond formation reaction on ONs, which obviates the synthesis of phosphoramidites and triphosphates. This was possible due to the development of water-soluble Pd-ligand catalytic systems, which enabled post-synthetic functionalization of proteins and DNA ONs.[109–111] Suzuki–Miyaura coupling was established on DNA ONs containing halogen-modified nucleosides by the labs of Manderville, Jäschke and Davis.[112–114] However, its feasibility for functionalizing RNA was not reported until recently. Okamoto and coworkers in their study to recognize fat mass and

obesity associated (FTO) demethylase, which recognizes N^6-methyladenosine (m^6A), first incorporated the diazirine group on RNA using the Suzuki–Miyaura reaction utilizing diazirine-containing boronic ester and 5-iodouridine-labeled RNA.[115]

We investigated the scope of coupling several probe-containing boronic acid and ester substrates onto RNA ONs enzymatically.[116] 5-Iodouridine triphosphate (IUTP) was synthesized and incorporated into RNA transcripts by *in vitro* transcription. IUTP was incorporated into transcripts almost as efficiently as natural UTP. Further, the iodo-functional group on RNA was coupled to various boronic acid and ester substrates using a catalytic system consisting of 2-aminopyrimidine-4,6-diol or dimethylamino-substituted ADHP ligand and $Pd(OAc)_2$ (Fig. 8). The incorporation of benzofuran and benzofuran vinyluridine into RNA ONs *via* this coupling reaction enabled the attachment of microenvironment-responsive fluorescent analogs in a ligand-controlled stereo-selective fashion. Although the toxicity and slow reaction kinetics have precluded the use of Pd in cellular systems, a report on the successful use of Pd-nanoparticles in effecting cross-coupling reactions in cells is encouraging.[117]

2.3 Site-specific chemo-enzymatic labeling of RNA and its applications

Several groups have independently devised strategies to site-specifically label RNA employing posttranscriptional RNA labeling enzymes and ribozymes. A comprehensive description of these approaches is presented in an earlier review from our group in 2017,[40] followed by other reviews.[61,118,119] Yeast and *E. coli* poly(A) polymerase (PAP) and terminal deoxynucleotidyl transferase (TdT) have been shown to be promiscuous to dNTPs and NTPs labeled with azide handles at various positions (C-2′, C-3′ and C-8 of adenosine).[120–122] This enabled site-specific RNA labeling at the 3′-end with various functional tags. Also, Jäschke and coworkers have established a single-pot click reaction on RNA incorporated with orthogonal reactive handles.[122]

Rentmeister and coworkers incorporated multiple azide-labeled residues at the 3′-end of RNA, which can be click functionalized using SPAAC reaction.[123] Yeast poly(A) polymerase was used for incorporating azide handles onto ARCA-capped mRNA coding for eGFP and luciferase enzyme. The azide handles were click reacted with fluorescent reporter molecules. This allowed simultaneous visualization of the mRNA and the product eGFP protein after translation (Fig. 9). Subsequently, the same group reported that 3′-poly(A) RNA labeled with bioorthogonal handles can be used for subcellular localization and tracking of mRNA in developing zebrafish embryos.[124] The labeled mRNA was also efficiently translated and visualized.

3′-RNA labeling has also been shown with DmHen1 and human HsHEN1 2′-O-methyltransferases, which transfer chemical functional groups on the Adomet cofactor to the terminus of RNA.[125] Fluorophores and affinity tags were either directly tagged on RNA or tagged with a bioorthogonal handle for subsequent functionalization. 3′-RNA labeling employing animal Hen1 enabled the introduction of FRET probes on RNA. Although these approaches are particularly advantageous as modifications can be accepted irrespective of the nucleotide at the 3′ end, the feasibility of an internal modification using this chemistry

needs to be further investigated. Additionally, adding modified residues at the sugar could alter the native structure of RNA and thus its biological functioning.

The discovery of an adaptive immune system, namely clustered regularly interspaced short palindromic repeats (CRISPR), gave rise to a new revolution in the field of programmed gene editing. In this technology, an RNA sequence called a guide RNA can be programmed to direct the Cas9 protein to a specific genomic region of interest for inducing double stranded DNA breaks which can result in indels (insertion or deletion).[126,127] The CRISPR system has been used for a wide range of applications such as site-specific display of functional proteins for fluorescent labeling, base-editing, transcription activation/ repression and chromatin remodelling.[128,129] Site-specific synthetic tagging of target genes employing chemo-enzymatic RNA labeling approaches and gene targeting tools can lead to novel gene engineering technologies. Brown and coworkers used click chemistry for the facile synthesis of guide RNAs, wherein 3′ alkyne-labeled crRNA and 5′ azide-labeled tracrRNA were ligated to form the full-length sgRNA.[130] Thus obtained sgRNA was functional, cleaving target DNA *in vitro* and in cells without any unexpected off-target effects.

Recently, our group reported an ingenious technology, namely sgRNA-Click (sgR-CLK), wherein synthetic molecules were installed site-specifically on a target gene locus using the CRISPR-dCas9 gene targeting tool and bioorthogonal click chemistry.[131] In order to site-specifically label the CRISPR system, we used the promiscuity of a terminal uridylyl transferase (TUTase) to repurpose the sgRNA with multiple azide handles using AMUTP. The azide-handles on sgRNA were then displayed on the target gene of interest using dCas9 and further functionalized using CuAAC and SPAAC reactions (Fig. 9a). sgR-CLK allowed us to enrich a specific region of chromatin from mouse embryonic stem cells and can be further explored for displaying small molecule probes, drug conjugates and diagnostic tools on a specific gene locus *in vivo*. In addition to this technology, the strategy we devised for incorporating C5 modified UTP analogs site-specifically on RNA using TUTase can have vast implications ranging from constructing biophysical probes for single molecule studies to the installation of fluorescent tags for RNA FISH.

Recently, Frischmuth and coworkers employed a dual RNA labeling approach for incorporating azide- and alkyne-modified nucleotide analogs into mRNA indiscriminately using T7 RNA polymerase and site-specifically at the terminus employing a poly(A) polymerase.[132] The whole eGFP mRNA was labeled with EU, whereas the poly(A) tails were labeled with 7-ethynyl-7-deazaadenosine 5′-triphosphate (EATP) and 3′-end labeled with 3′-azido-2',3′-dideoxyadenosine (AzddATP) using a poly(A) polymerase. The click-labeled mRNA was visualized upon functionalization with appropriate azide/alkyne fluorophores. The orthogonal click functional tags at different regions in mRNA assisted in labeling a single mRNA with different modifications.

Labeling RNA at the 5′-end is also particularly important for a variety of applications. Rentmeister and coworkers developed several chemo-enzymatic 5′-RNA labeling approaches[104,133] Bioorthogonal reactive labels were introduced using *Giardia lamblia* trimethylguanosine synthase (GlaTgs2), which methylated the N^2 position of a dinucleotide RNA cap, m^7GpppA, employing AdoMet. A mutant GlaTgs-var1 incorporated various

clickable reactive functionalities at the 5′-end of RNA. Following this success, they also achieved metabolic labeling of RNA MTase target sites *in vivo* with propargyl groups.[134] The internally incorporated propargyl tags were 65% terminated during reverse transcription when undergoing conjugation with biotin employing CuAAC reaction and upon further incubation with streptavidin. The resultant fragments of cDNA were subjected to sequencing for mapping the methylation sites.

The research groups of Heemstra and Beal reported a click-based RNA labeling system to detect adenosine to inosine (A to I) performed by the ADAR family of enzymes. In this work, the authors optimized a wildtype and mutant (E1008Q) recombinantly expressed ADAR1 enzyme, which converted adenosine at the HER1 RNA substrate to inosine.[135] The inosine-converted RNA hairpin was selectively labeled at the N^1 position with a Michael acceptor, *N*-(4-ethynylphenyl)acrylamide (EPhAA). The alkyne-labeled RNA was further functionalized using CuAAC reaction with an azide-labeled cyanine5 fluorophore. The technique provides a promising strategy for detecting inosine to adenosine edits *in vitro* and in cells.[136,137]

Apart from these chemo-enzymatic approaches, methods have been developed to label nucleic acids using ribozymes.[40] Ribozyme-based strategies use a catalytic nucleic acid sequence evolved by systematic evolution of ligands by exponential enrichment (SELEX). Notable contributions from the labs of Höbartner, Heemstra and Muller have been particularly beneficial in functionalizing RNAs and characterizing their interaction partners.[138–141]

3 Conclusions and outlook

Several revolutionary technologies like high-throughput next generation sequencing, single-cell sequencing, nucleic acid origami, super-resolution imaging and CRISPR gene-editing/targeting tools have benefited from bioconjugation strategies.[128,142–146] With the rise of these advanced tools and techniques, there is a constant need to upgrade as well as develop newer nucleic acid labeling methods that support such technological developments. In this context, bioorthogonal chemistry has taken the pole position in advancing RNA labeling technologies that are compatible in cells and *in vivo* settings. The work in this direction started with incorporating chemo-selective reactive handles on RNA ONs by chemical means and desired biophysical tags/probes were installed post-synthetically by click reactions. Subsequently, transcriptionbased approaches and methodologies employing enzymes that catalyze posttranscriptional modifications enabled in-cell analysis and in certain cases site-specific tagging of RNA. This was possible only due to the development of substrate mimics, which seamlessly enter the cellular metabolic pathways and display minimally invasive reactive handles for further functionalization by using bioorthogonal reactions. Several of these methodologies have provided practical solutions to (i) probe RNA structure and dynamics, (ii) track and profile RNA and (iii) develop robust diagnostic tools. The next major step forward would be to develop techniques for site-specific covalent labeling *in vivo* that would allow real-time monitoring of the location, structure and dynamics of a specific endogenous RNA sequence. Repurposing chemical reactions that

have been recently developed for labeling protein, lipid and DNA would expand the repertoire of tools available for probing RNA.[147–150]

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Biographies

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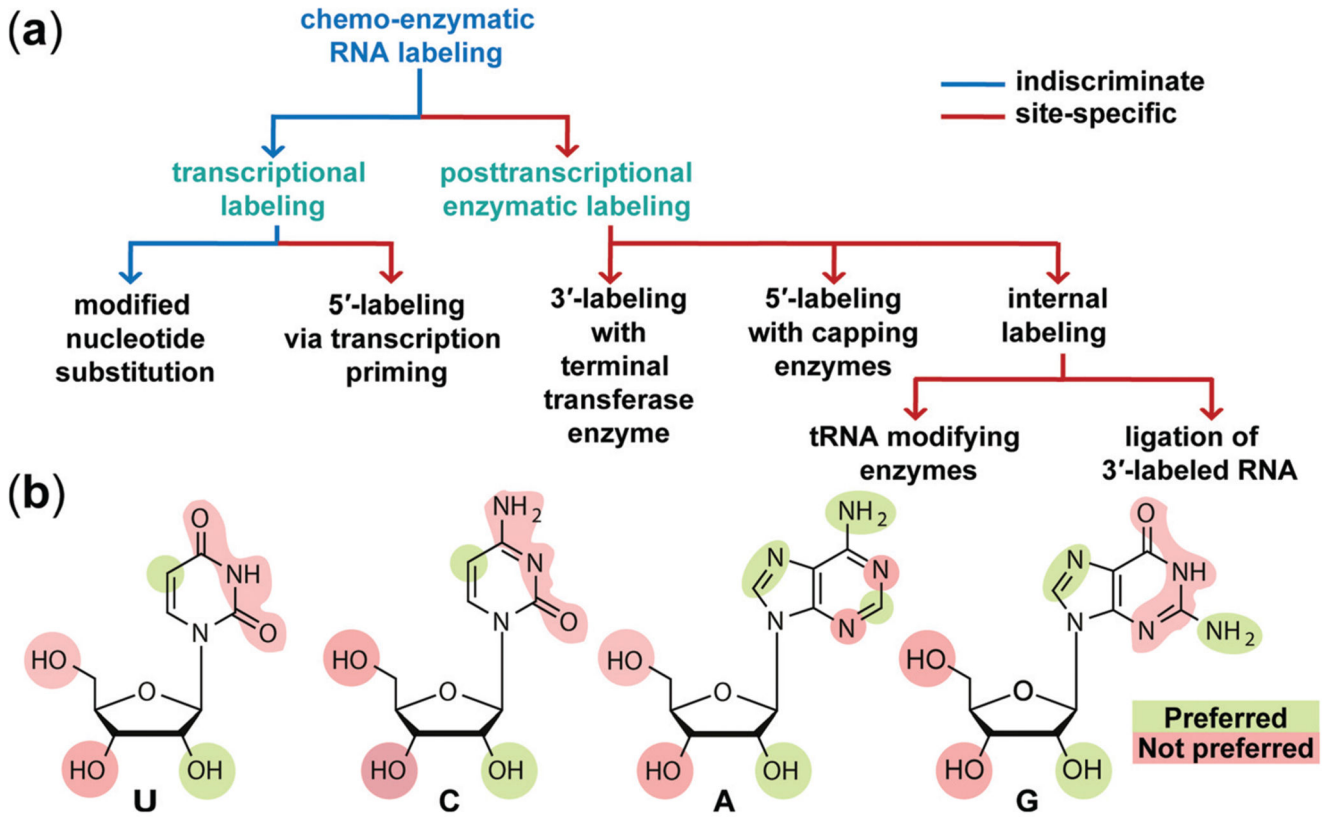

Fig. 1.

(a) Flow chart depicting transcriptional and posttranscriptional enzymatic strategies to generate clickable RNA using indiscriminate body labeling (blue line) or site-specific labeling (red line). (b) The preferred labeling sites on ribonucleosides for enzymatic incorporation of modified functional groups are shown.

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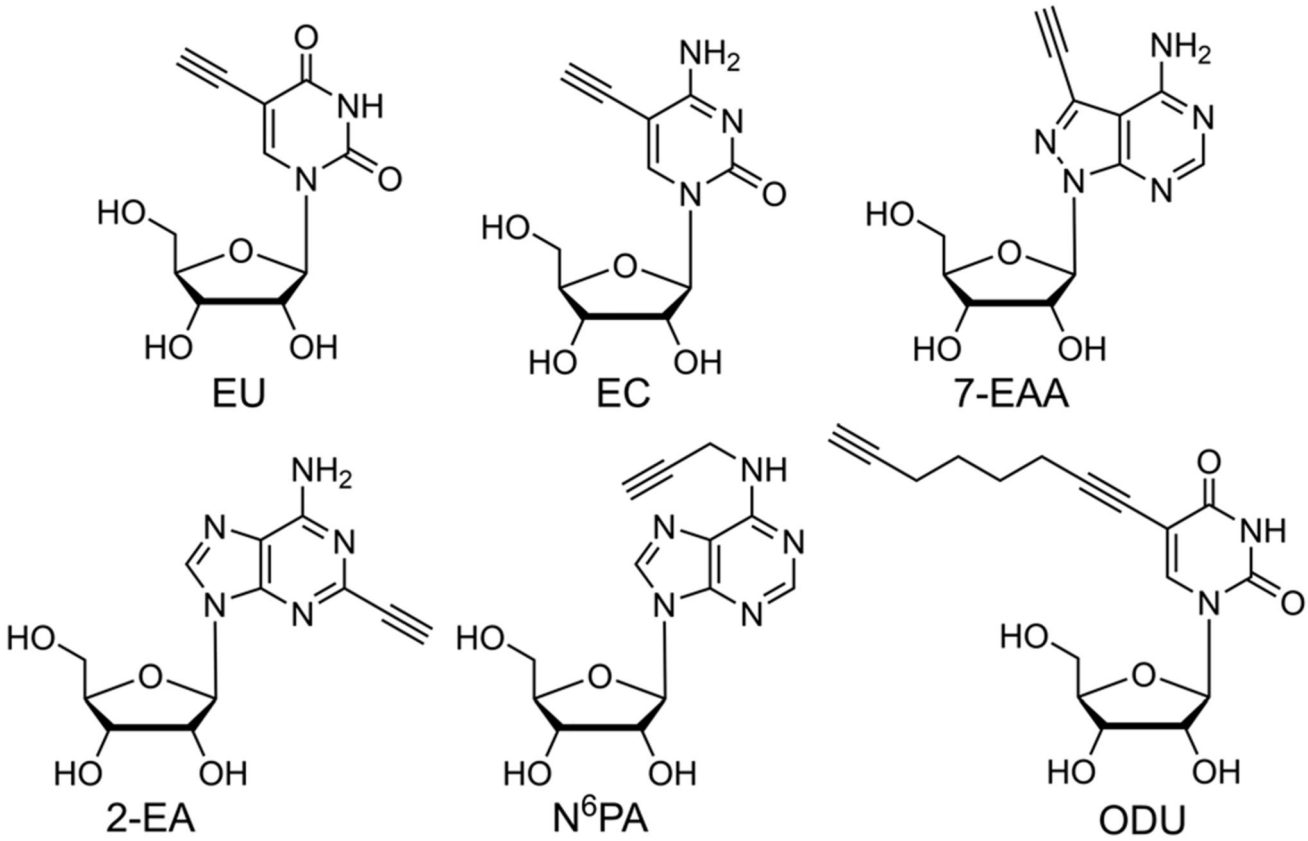

Fig. 2. Chemical structures of alkyne-modified nucleoside analogs.

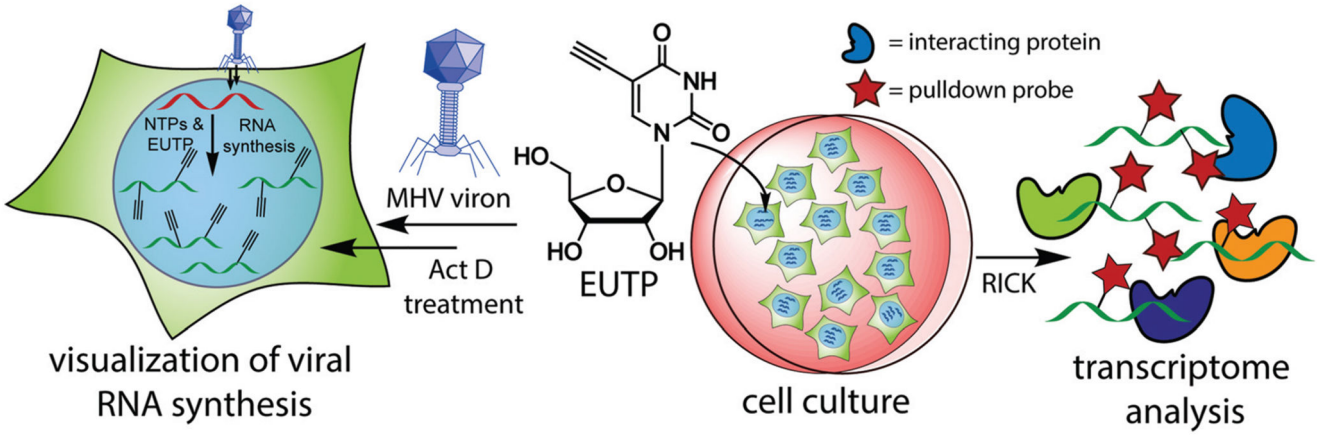

Fig. 3.

Metabolic labeling of nascent RNA transcripts can be used for visualizing viral RNA synthesis in cells infected with the MHV virion. Cells were treated with actinomycin D (Act D) to prevent incorporation of EU into host cell RNA.[74] EU labeling enables RICK technology used for enriching nascent RNA and its interacting proteins.[72]

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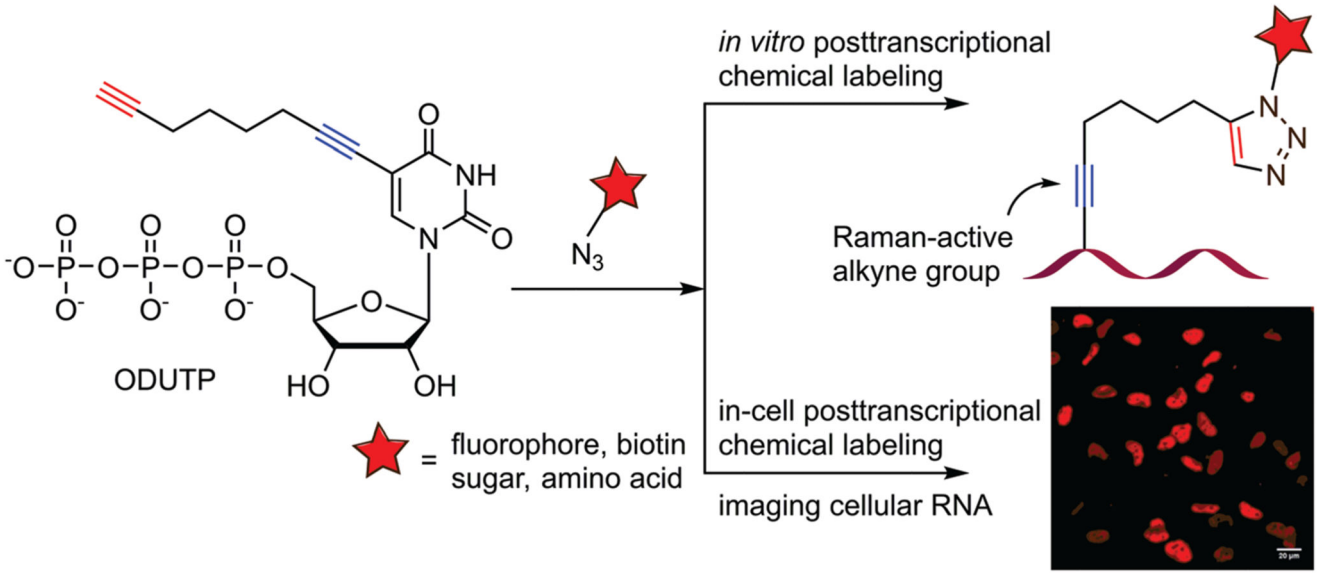

Fig. 4.

ODUTP represents a multifunctional nucleotide analog, which can be potentially used in RNA aptamer selection by the Click-SELEX protocol, and in two-channel detection of cellular RNA transcripts by click chemistry and Raman spectroscopy.[79] This figure is partially adapted from a figure reported in our article.[79]

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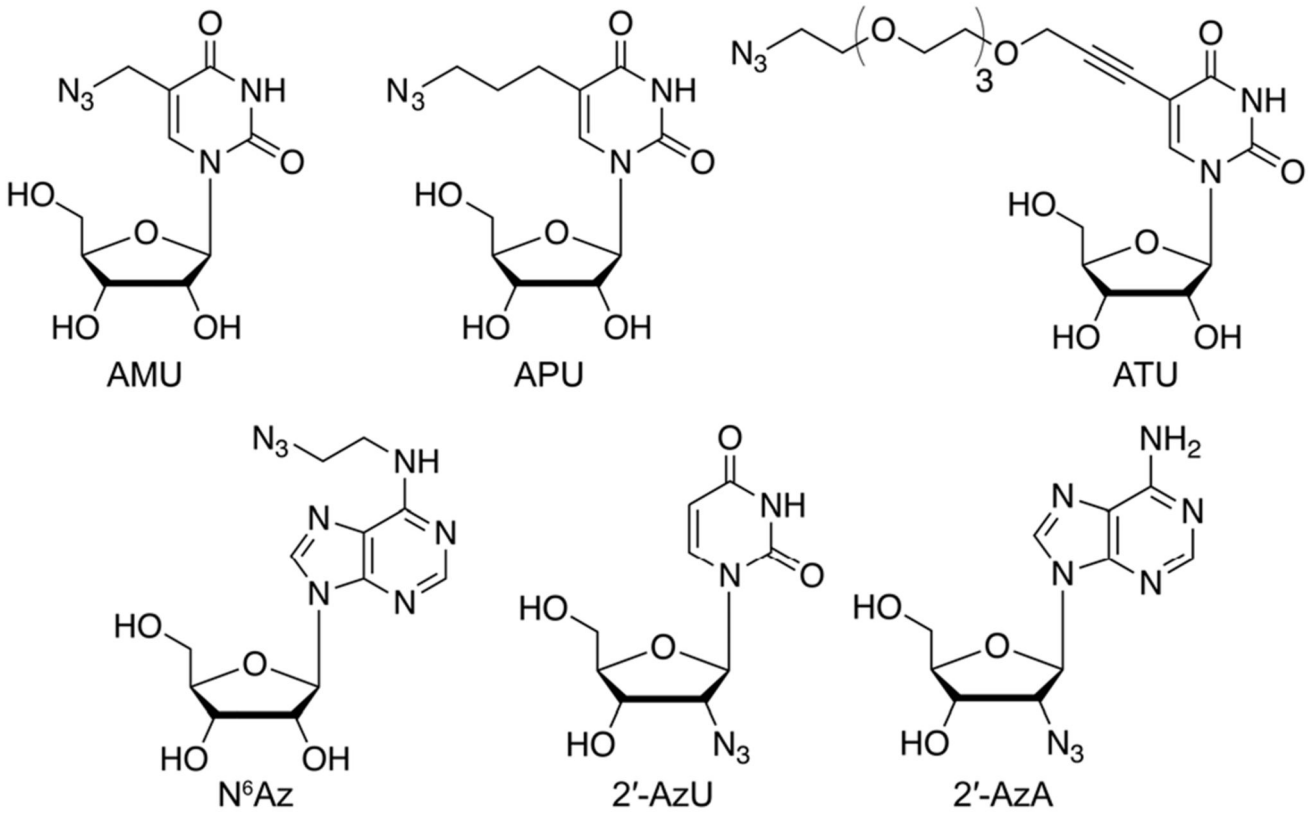

Fig. 5. Chemical structures of azide-modified nucleoside analogs.

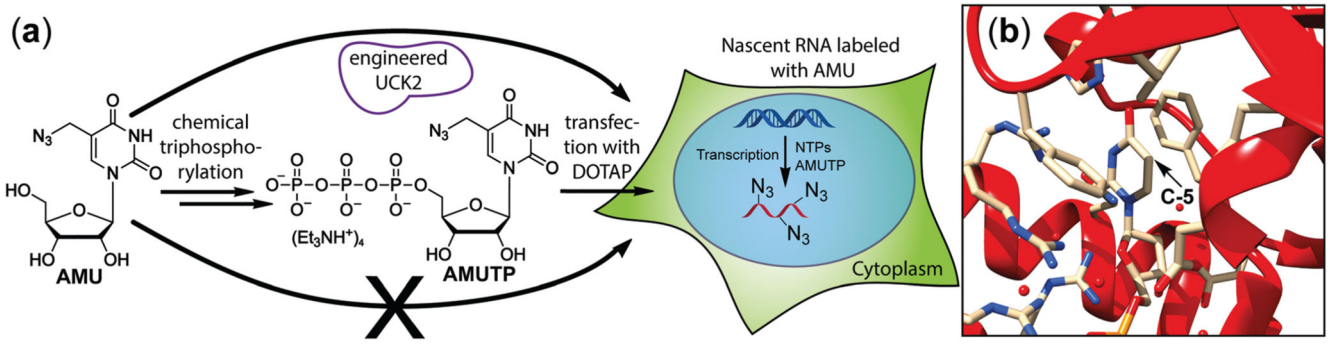

Fig. 6.

(a) Steps involved in the metabolic labeling of nascent RNA with AMU. Since AMU is not metabolically incorporated into RNA, the corresponding triphosphate (AMUTP) is transfected using 1,2-dioleoyl-3-trimethylammonium propane (DOTAP), which is then efficiently incorporated into nascent RNA by endogenous RNA polymerases.[89,90] Other transfection agents like Lipofectamine did not work well as compared to DOTAP. Alternatively, an engineered UCK2 enzyme involved in the pyrimidine salvage pathway readily assists in metabolic incorporation of AMU into RNA.[91] (b) Tyrosine residue (Y65) in the active site hinders the C5 position of the substrate analog of UTP (PDB: 1UEI).[92]

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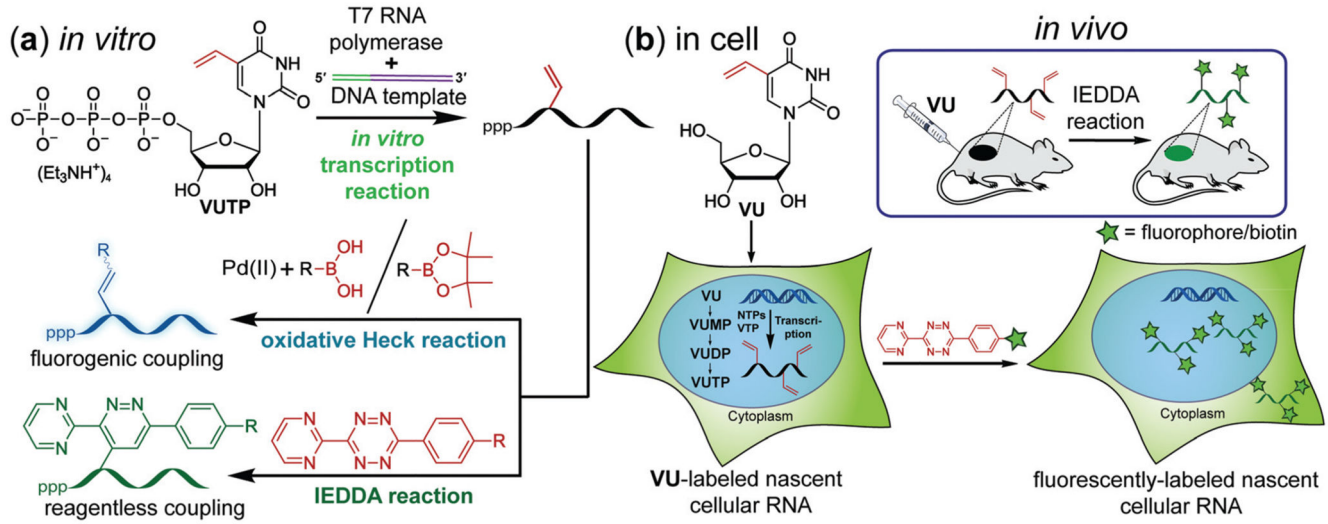

Fig. 7.

(a) VU represents a two-in-one nucleoside analog that enables RNA modification using oxidative Heck and IEDDA reactions. While the former approach can be used for direct coupling of fluorogenic probes onto RNA ONs, the latter approach can be used for bioorthogonal labeling in cells.[106] This figure is partially adapted from a figure reported in our article.[106] (b) Metabolic incorporation of VU into nascent RNA in cells and in detecting tumour in live mice.[107,108]

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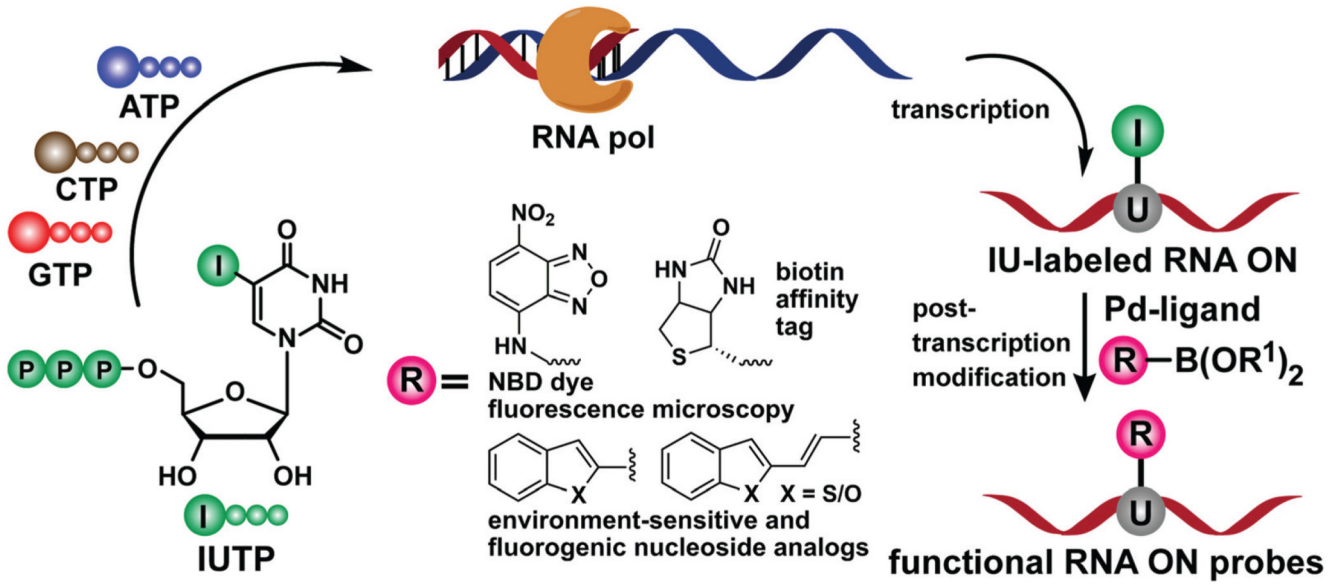

Fig. 8.

Posttranscriptional Suzuki–Miyaura cross-coupling reaction offers direct access to RNA ONs labeled with fluorogenic environment-sensitive probes for nucleic acid structure and recognition studies, fluorescent probes for microscopy and an affinity tag for immunoassays. This figure is reproduced by the permission of Nucleic Acids Research: Oxford Journals.[116]

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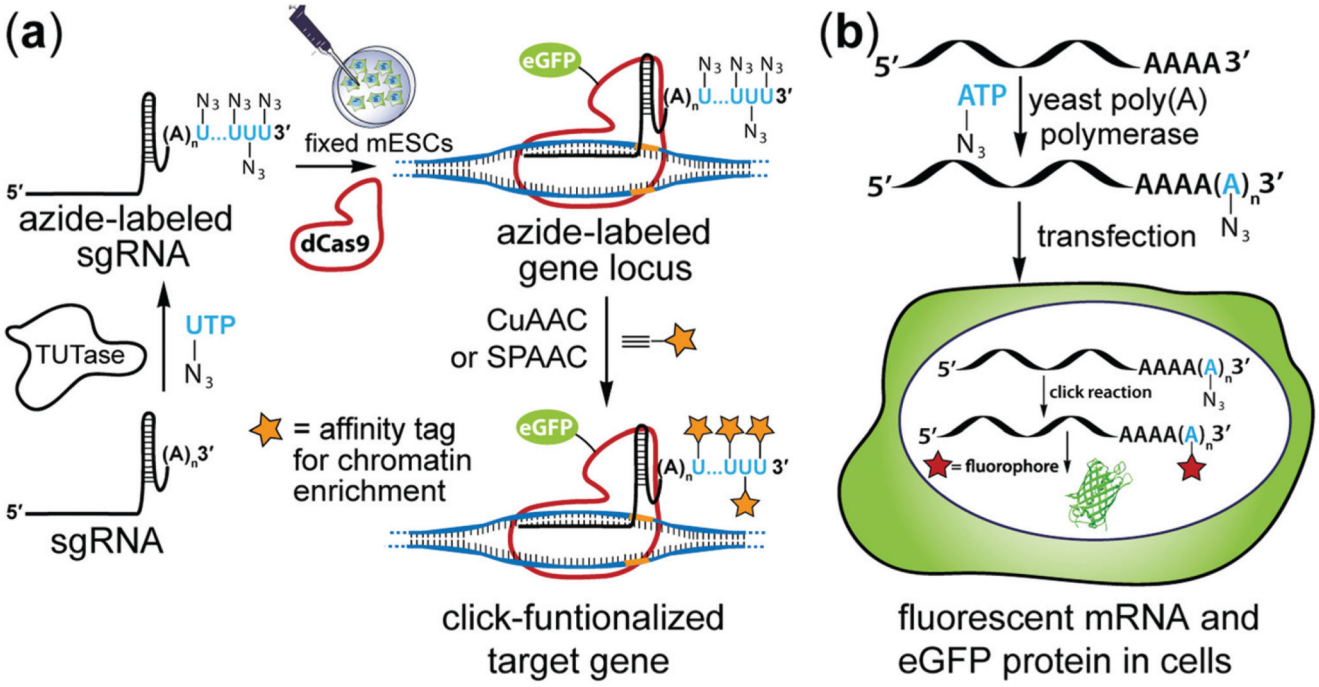

Fig. 9.

(a) Step-wise depiction of sgR-CLK technology developed in our lab for performing site-specific labeling of a target gene with synthetic molecules employing CRISPR-dCas9 and bioorthogonal click chemistry. The strategy can be used to display small-molecules on target genes.[131] This figure is partially adapted from a figure reported in our article.[131] (b) 3′-RNA labeling strategy developed by Rentmeister and coworkers for visualizing poly(A) tails of mRNA and its translated protein.[123]

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