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# Cell-Selective Bioorthogonal Metabolic Labeling of RNA

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Supporting Information

**ABSTRACT:** Stringent chemical methods to profile RNA expression within discrete cellular populations remains a key challenge in biology. To address this issue, we developed a chemical−genetic strategy for metabolic labeling of RNA. Cell-specific labeling of RNA can be profiled and imaged using bioorthogonal chemistry. We anticipate that this platform will provide the community with a much-needed chemical toolset for cell-type specific profiling of cell-specific transcriptomes derived from complex biological systems.

RNA molecules have emerged as key players in nearly every facet of cellular regulation.[1,2] They are critical for cell survival and can be molecular drivers of disease.[3−5] RNA expression profiling is a critical first step toward discovering and characterizing the RNA content of a cell. Profiling RNA expression, and nascent RNA synthesis, from a single cell type within a complex structure or network is an extremely difficult task.

Nascent RNA expression programs can be studied through bioorthogonal metabolic labeling. For example, alkynyl-[6−8] and azido-containing[9] nucleoside metabolic intermediates can be introduced into cells, and nascent RNA can be tracked through bioorthogonal chemical reactions. Both Cu(I)-catalyzed azide−alkyne cycloaddition (CuAAC) and strain-promoted alkyne−azide cycloaddition (SPAAC) can be utilized for imaging or biotinylation and streptavidin enrichment. Although powerful, these approaches suffer from the key drawback that they are not cell-specific.

Cell-specific metabolic labeling has found widespread use for proteins[10−13] and more recently glycans,[14,15] and has proven invaluable for the description of macromolecular composition during development and within whole animals.[16−19] While these studies have advanced the profiling of proteins and sugars in a cell-type specific manner, there remains a paucity of analogous tools for RNA. Making bioorthogonal metabolic labeling of RNA cell-specific would open the door to a more detailed and mechanistic understanding of the complexity of cell-specific gene expression dynamics.

The modified nucleobase 4-thiouracil (4TU) can be incorporated into cellular RNA by expressing the bacterial enzyme uracil phosphorisyltranferase (UPRT). UPRT converts uracil and phosphoribosyl pyrophosphate (PRPP) to 5′-phosphoro-4-thiouridine, which is eventually incorporated into nascent RNA.[20] This approach has proven useful for studying RNA synthesis and decay.[20,21] However, recent reports have demonstrated that thiouridine enrichment suffers from vulnerabilities associated with the transient nature of disulfide bonds.[22] This could be overcome by instead installing bioorthogonal handles capable of forming stronger covalent products for enrichment. Furthermore, the reliance on disulfide cross-linking precludes imaging which has become critical for analysis of gene expression and RNA biology.

The aforementioned limitations have somewhat precluded the widespread usefulness of cell-specific metabolic labeling of RNA. We envision that extending the toolset for cell-specific RNA labeling will necessitate the design and implementation of additional and new biologically inert bioorthogonal nucleoside intermediates that can be recognized by specifically expressed enzymes to permit their incorporation into cellular RNA (outlined in Figure 1). To go further, stringent and parallel

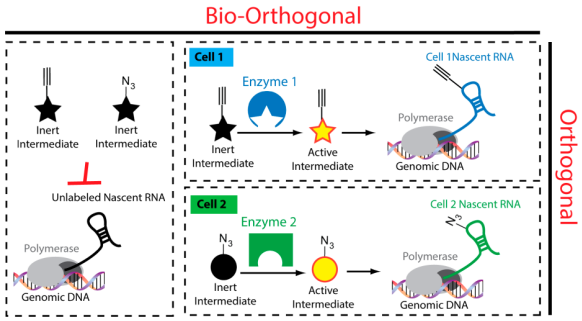


**Figure 1.** Outline of cell-type specific metabolic labeling of RNA.

metabolic labeling of RNA, with orthogonal handles, would set the stage for the examination of multicellular tissues, tumor-healthy cell interactions, host−pathogen interactions, and even different cell types along a single developmental trajectory.

Herein, we have extended the chemical toolkit for cell-type specific metabolic labeling of RNA. First, we have examined the substrate tolerance for UPRT to enable cell-type specific labeling with functionalities that permit cellular imaging and biotinylation. We have also developed a second, orthogonal small molecule−enzyme pair, using the enzyme penicillin G amidase (PGA). PGA can release a "caged" 2′-azidoadenosine analog to endow RNA with azido functionality, which is also amenable to cellular imaging and biotinylation. We demonstrate these two sets are orthogonal to each other. The

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described tools and approaches will be invaluable to researchers focused on understanding dynamic gene expression patterns within discrete cell populations.

We began by attempting to extend the substrate tolerance of UPRT. We reasoned this would be a good starting point, as UPRT is known to be functional in mammalian cells and can accept exogenous 4TU to subsequently label RNA. Investigation of the UPRT crystal structure with uracil bound suggested that the 5-position of uracil would be amenable to derivatization (Figure 2A; Supplementary Figure 1). We

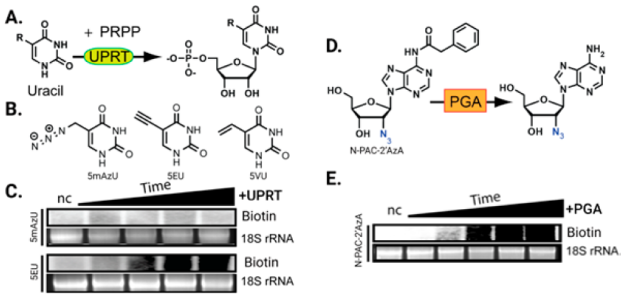


**Figure 2.** Establishing a modified uracil-UPRT pairs for RNA incorporation into living cells. (A) Pathway of UPRT to make 5-phosphouridine. (B) Analogs (5 mAzU, 5 EU, and 5VU) designed and tested in this study. (C) Time-course transfer blot analysis of analog incorporation into cellular RNA, dependent on UPRT. The blot demonstrates the presence of biotin on the RNA appended through the CuAAC reaction. Time points are, 30 min, 1 h, 3 h, 5 h. nc = 5 h no UPRT. (D) Pathway of uncaging 2′-azidoadenosine. (E) Time-course transfer blot analysis of analog incorporation into cellular RNA, dependent on PGA. Time points are 0 min, 30 min, 1 h, 3 h, 5 h. nc = 5 h no PGA. The blot demonstrates the presence of biotin on the RNA appended through the CuAAC reaction.

designed and synthesized three 5-position modified uracil analogs (Figure 2B, Supporting Information). We first demonstrated that both the 5-methylazidouracil (**5 mAzU**) and 5-ethynyluracil (**5 EU**) were amenable to functionalization by conventional CuAAC reactions (Supplementary Figure 2). Unfortunately, the 5-vinyluracil (**5VU**) was not high yielding enough to our several attempts of inverse electron demand Diels–Alder chemistry (IEDDA) with tetrazenes (Supplementary Figure 2). This is consistent with previous reports, which demonstrated that the large HOMO–LUMO energy gap of the 5-vinyluridine limits its utility with IEDDA.[23] We therefore focused on **5 mAzU** and **5 EU** for RNA incorporation screening.

To test for RNA incorporation, analogs **5 mAzU** and **5 EU** were introduced to wild-type cells and a transfected cell line expressing UPRT. 5-Ethynyluracil (**5 EU**), but not 5-methylazidouracil (**5 mAzU**), was incorporated into cellular RNA, only in the UPRT-expressing cells. A robust signal for the incorporation of **5 EU** into nascent RNA is observed in as little as 30 min, very similar to the positive control 4TU (Figure 2C; Supplementary Figures 3–5). No incorporation was observed in cells not expressing UPRT, even after 5 h. Notably, the nucleoside analog 5-ethynyluridine is widely used as a non-cell-specific tracer for metabolic labeling due to its ability to be enriched through CuAAC and imaging, in both cell culture and live animals;[7] as such, we anticipate this initial finding will be of great use to the community. Overall, these data suggest that compound **5 EU** is robustly incorporated in a cell-type specific manner and extends the chemical approach of UPRT-dependent metabolic labeling of cellular RNA.

Having identified **5 EU**–UPRT as our first pair, we shifted focus on attempts to introduce a handle that would permit orthogonal tagging to the alkyne and further extend cell-specific metabolic labeling of RNA. We reasoned the introduction of azide functionality could permit strain-promoted alkyne–azide cycloaddition (SPAAC), which has been demonstrated to be orthogonal to CuAAC for parallel labeling reactions and, as such, may enable simultaneous RNA profiling in multiple cell types with high stringency.[24] We also reasoned it may be more beneficial to utilize an analog that is preferential to mRNAs, as the expression of these RNAs is of high interest to the community.

Along these lines, we recently demonstrated that 2′-azidoadenosine is a robust substrate for polyA polymerase (mRNA polyA tail tagging); this distribution results in significant staining of the cytoplasmic pool of RNA.[9] We demonstrated that RNA labeling and imaging is suitable by SPAAC or CuAAC. In the same study we also noted that N6-derivatized adenosine analogs were not as robustly incorporated into RNA as 2′-azidoadenosine, which led us to hypothesize that protecting the exocyclic amine with a bulky group would limit RNA incorporation into cells. As such, caging of 2′-azidoadenosine could impart orthogonal metabolic labeling of RNA.

As a second enzyme to target for gating the incorporation of bioorthogonal nucleosides, we turned our attention to penicillin G-amidase (PGA). PGA is an enzyme not expressed in mammalian cells, and it has been demonstrated to work on a variety of substrates to uncage functional molecules.[10,25,26] With PGA in mind, we designed and synthesized N6-phenylacetyl-2′-azidoadenosine (Figure 2D; **N-PAC-2′AzA**; Supporting Information).

*In vitro* incubation of N6-protected 2′-azidoadenosine with recombinant PGA demonstrated successful uncaging of an N6-protected 2′-azidoadenosine (Supplementary Figure 6). Following this encouraging result **N-PAC-2′AzA** was introduced to wild-type cells and a transfected cell line expressing PGA (Supplementary Figure 7) to test for RNA incorporation. RNA was isolated and appended with a biotin handle through CuAAC. As shown in Figure 2E (Supplementary Figure 8), PGA expressing cells smoothly incorporated **N-PAC-2′AzA** into nascent RNA. Wild-type cells had no incorporation over background, even after 5 h of incubation. Signal for the incorporation of **N-PAC-2′AzA** is observed in as little as 30 min, suggesting that there is similar kinetics between the **5 EU**–UPRT and **N-PAC-2′AzA**–PGA pairs for paralleled metabolic incorporation. Overall, these results demonstrate that we have identified two new bio-orthogonal small molecule–enzyme pairs that could be used for bioorthogonal metabolic labeling of nascent RNA and could orthogonally endow RNA with unique chemical functionalities in parallel.

Having established small molecule–enzyme pairs, we next sought to more stringently test its specificity. Cell-specific incorporation of **5 EU** was imaged with Alexa-488 azide utilizing CuAAC (Figure 3A; Supplementary Figure 9). Cell-type specific incorporation of **N-PAC-2′AzA** was imaged with Cy5 alkyne through CuAAC (Figure 3B; Supplementary Figure 10). Staining of analog **5 EU** was mostly limited to the nucleolus, which is consistent with the possibility that the majority of the probe is incorporated into rRNA. This is in agreement with staining and RNA profiling observed with 5-


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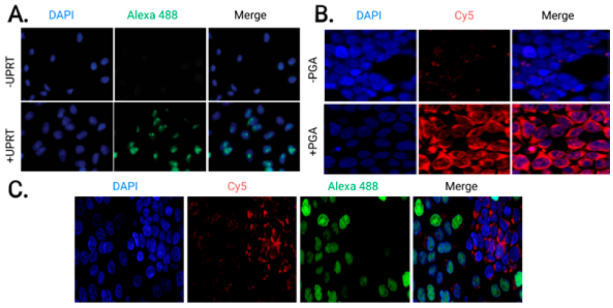


**Figure 3.** Imaging demonstrating stringent cell-type specific metabolic labeling of RNA. (A) Imaging of 5 EU RNA incorporation in the presence of UPRT. (B) Imaging of N-PAC-2′AzA RNA incorporation in the presence of PGA. (C) Demonstrated RNA incorporation and cell-type specificity within a coculture system. Alexa 488 was used to stain 5 EU incorporation and Cy5 was used to stain 2′-azidoadenosine incorporation.

ethynyluridine incorporation.[7] Uncaged **N-PAC-2′AzA** is also imaged in the nucleus (nucleolus), but the majority of signal is in the cytoplasm. This is in agreement with our previous results which showed that 2′-azidoadenosine is robustly incorporated into mRNAs through polyA polymerase.[9] RNase digestion of total RNA and cell cultures almost completely abrogated signal from the analogs, further demonstrating that they are incorporated into RNA (Supplementary Figure 11). These results demonstrate that our approach of RNA labeling is dependent on the presence of specific enzymes and that incorporation can be assayed by imaging.

We next imaged incorporation of each nucleoside in a coculture system. Coculturing of PGA and UPRT transfected cells were exposed to **5 EU** and **N-PAC-2′AzA** simultaneously. Incorporation of each analog was measured by the same means as Figure 3A and B. As shown in Figure 3C (Supplementary Figure 12), RNA labeling signals were mutually exclusive; the Alexa 488 signal did not overlap with that of Cy5. As such, the orthogonal pairs have cellular specificity within the detection limit of our imaging experiments.

Lastly, we performed a coculture experiment to test our approach in an affinity assay. Isolation of total RNA after cocultured PGA- and UPRT-expressing cells was subjected to RT-PCR for the PGA and UPRT mRNAs. PGA mRNA was only enriched when taken through an alkyne−biotin affinity purification. UPRT mRNA was only enriched when taken through an azide−biotin affinity (Supplementary Figure 13). Overall, these data demonstrate the feasibility of our approach and demonstrate the specificity of our metabolite−enzyme pairs for RNA labeling.

In this work, we have successfully extended the bioorthogonal toolkit for metabolic labeling of RNA. Further, we have developed analogues for cell-type specific metabolic RNA labeling that are highly specific and permit parallel multicellular RNA labeling within coculture conditions. To accomplish this, we have synthesized a new suite of nucleobase/nucleoside probes that contain bioorthogonal handles. Further, we have demonstrated their ability to react with enzymes to permit their incorporation into cellular RNA. Labeled RNA can also be biotinylated, which will permit stringent enrichment protocols for downstream analysis of gene expression. Such methods could next be extended to work in tissues comprised of high cellular complexity. It is well established that nucleoside analogs can be incorporated into many tissue types and complex environments within living animals,[7] even smoothly passing the blood−brain barrier.[27] As such, we anticipate our strategy will be extremely useful for investigating RNA expression changes and function within any cell type. Future efforts will be focused on extending these findings into complex cellular environments, *in vivo*.

## ■ ASSOCIATED CONTENT

### Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/jacs.6b11401.

Experimental methods, synthetic schemes, and spectra, for all compounds (PDF)

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⊥K.N. and M.F. contributed equally.

### Notes

The authors declare no competing financial interest.

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