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Making the Message Clear: Concepts for mRNA Imaging

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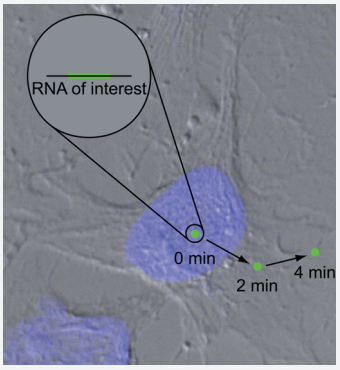
ABSTRACT: The transcriptome of each individual cell contains numerous RNA species, each of which can be controlled by multiple mechanisms during their lifetime. The standard transcriptome analysis focuses on the expression levels of the genes of interest. To gain additional insights into spatiotemporal RNA distribution and the underlying trafficking processes, RNA labeling and imaging are necessary—ideally in living cells. This perspective will summarize state-of-the-art RNA imaging methods including their strengths and weaknesses.



INTRODUCTION

The cell is the basic biological unit of living organisms and comprises many different types of RNA dedicated to particular functions. Several RNA types are well-known to regulate gene expression by interacting with mRNA *in trans*,[1−3] and additional mechanisms such as poly(A)-tail lengthening and shortening,[4] modifications,[5] or interactions with RNA-binding proteins can also act on mRNA and alter the expression level.[6] Currently, RNA modifications are increasingly being recognized as another mode of controlling mRNA metabolism and trafficking; e.g., N^6-methyladenosine was shown to affect nuclear export of mRNAs.[7,8] Furthermore, active transport and subcellular localization of mRNAs enable local translation into proteins.[9] The asymmetric localization of mRNA is one of the most remarkable mechanisms for regulating gene expression in large polarized cells. It is essential for normal *Drosophila* embryogenesis[10−12] and neuronal development,[13−15] and impairments in mRNA distribution have been associated with mental retardation (e.g., fragile X syndrome) and cancer metastasis.[16,17] In order to get a comprehensive picture of the cellular functions of a certain RNA, it is important to obtain spatial information in addition to its expression level in a cell or organism. Microdissection and subsequent analysis of axonal growth cones revealed a variety of mRNAs including diverse functions,[18] but the spatial resolution of this technique is limited. Hence, techniques offering better resolution and ideally providing information about the dynamics of mRNA trafficking and subcellular localization are required.

In this review we aim to highlight available and emerging methods to study mRNA localization in cells. We have grouped the available methods into four categories, namely, (i) aptamers, (ii) covalent modifications, (iii) RNA-binding proteins, and (iv) simultaneous RNA imaging and sequencing. The well-established MS2-based system that has been successfully used to study RNA localization in living cells from transcription to decay and has even been implemented in mice deserves a review on its own[19,20] and will not be covered here.

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APTAMERS

The most intuitive way for labeling a particular RNA for in-cell tracking is fusion of a reporter tag, similar to GFP-fusion proteins that have revolutionized protein labeling. Although fluorescent RNA-based reporters have not yet been identified in nature, *in vitro* selection by SELEX (Systematic Evolution of Ligands by EXponential enrichment) yielded an aptamer termed Spinach, which mimics the green fluorescent protein (GFP). Spinach binds to the cell-permeable small molecule 3,5-difluoro-4-hydroxybenzylidene imidazolinone (DFHBI, **1**) and turns on its fluorescence (Figure 1A).[21] The aptamer sequence can be appended to label the RNA of interest (ROI); however the affinity is not great ($K_d \approx 300-500$ nM),[22] and it likely misfolds, making a tRNA scaffold necessary to ensure the aptamer's folding *in vivo*.[21−24] A number of alternative aptamer−fluorophore complexes with improved binding affinities and/or fluorescence properties including different fluorescence wavelength have been reported since the original publication, e.g., Spinach2−DFHBI,[23] Broccoli−DFHBI[24] and—most notably—Mango-thiazole orange (**2**),[22] providing a toolbox for RNA labeling. Mango has recently been crystallized, showing a three tiered G-quadruplex,

Received: June 14, 2017
Published: July 6, 2017

DOI: 10.1021/acscentsci.7b00251
ACS Cent. Sci. 2017, 3, 701−707

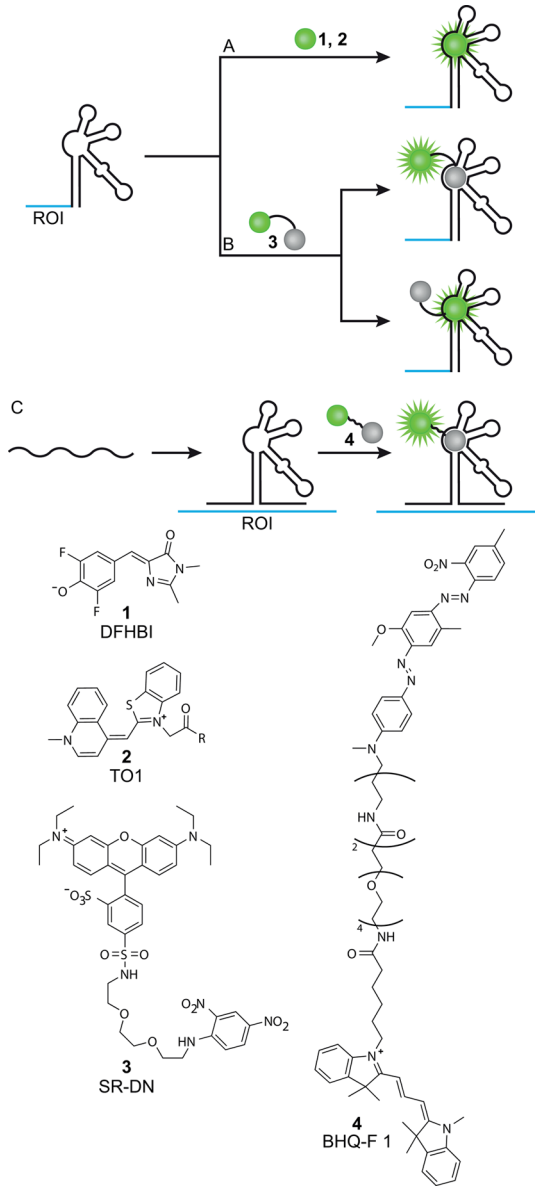

Figure 1. Aptamer-based RNA imaging strategies. (A) The ROI (blue) is extended by an aptamer that binds and activates the fluorophore (green dot to green star) DFHBI (**1**)[21,23,24] or thiazole orange (TO1, **2**, R = biotin-PEG3-amine).[22] (B) SR-DN (**3**) is one possible fluorophore-quencher complex. The aptamer binds either to the quencher or to the fluorophore moiety perturbing the complex and resulting in fluorescence (green star).[31,32] (C) The RNA-targeting aptamer binds to the ROI *in trans*. Correct hybridization induces folding and enables binding and activation of BHQ-F 1 (**4**) (green star). The upper part of **4** is the quencher, followed by the linker and the fluorophore Cy3 (lower part).[29]

which curiously binds the complete thiazole orange molecule comprising the linker and biotin.[25] Mango was further improved by a microfluidic-based selection to yield Mango variants (II–IV) with high turn-on ratios and high affinity.[26,27] Although reports on mRNA live cell labeling are still scarce,[28−30] this technology will likely become more prominent in the near future and might outcompete the established MS2-MCP-based system, which requires a big tag.

A fluorescent signal upon binding can also be obtained by creating fluorophore/quencher pairs, whose quenching properties are abrogated in response to binding. One example is the use of the contact-quencher dinitroaniline, which is coupled with a triethylene glycol linker to a fluorophore (sulforhodamine-dinitroaniline, SR-DN, **3**) and quenches it efficiently. Dinitroaniline can quench several fluorophores, and the quencher-fluorophore dyes are cell-permeable.[31] Through aptamer binding of either the quencher[32] or the fluorophore,[31] the fluorophore-quencher complex is destroyed turning on fluorescence (Figure 1B). A ROI extended by the respective aptamer was successfully imaged in living bacteria.[31,32] Even two different RNAs—one extended with the quencher-binding aptamer and the other one with the fluorophore-binding aptamer—were imaged simultaneously using two different fluorophore-quencher complexes.[32] Another method uses tandem repeats of a tobramycin-binding aptamer, so-called IMAGE tags (Intracellular MultiAptamer Genetic tags) that can theoretically be appended to a ROI. With the use of Cy3- and Cy5-tobramycin a FRET signal is observed, which has been used for imaging transcriptional activity in live yeast cells.[33]

Finally, aptamers can be engineered to target an ROI *in trans*. Herein, hybridization of the RNA-targeting aptamer leads to formation of a black hole quencher (BHQ)[34] recognition loop. Therefore, only after correct hybridization the BHQ-part of the BHQ-fluorophore conjugate 1 (BHQ-F 1, **4**) can be bound and fluorescence occurs (Figure 1C). With this method mRNA imaging was performed in living HeLa cells.[29] Recently, a similar RNA-targeting aptamer approach was realized in live *Escherichia coli* cells using a truncated form of the Spinach aptamer and DFHBI.[28] It can be anticipated that the development of improved fluorogenic aptamers (e.g., the Mango aptamer described above) will propel this approach in the near future.[27]

COVALENT MODIFICATIONS

Compared to aptamers, covalent modification of the ROI is a more direct and stable labeling approach, because the modification is permanently linked to the ROI. A small functional group can often be incorporated into the ROI by metabolic labeling, if suitably modified precursors are provided and accepted. Click chemistry can then be used to attach a fluorophore and visualize the RNA (Figure 2).[35] While visualizing nascent transcripts is important for certain cell questions (e.g., visualization of RNA production as a stress response), the major challenge of this approach is transcript specificity—i.e., avoiding that the functional group is incorporated into all cellular RNAs. To achieve specificity for certain RNA types or even transcripts, post-transcriptional modification based on cosubstrate promiscuous RNA-modifying enzymes has proven successful.[36−41] One specific example is the use of the archaeal $tRNA^{Ile2}$-agmatidine synthetase (Tias), which conjugates azide/alkyne-bearing agmatine analogues, like 2-propynylamine (**5**), and is absent in mammalian cells. When a $tRNA^{Ile2}$-5S fusion RNA and Tias were expressed in the presence of 2-propynylamine in cells, subsequent labeling via Cu(I)-catalyzed azide alkyne cycloaddition was possible in fixed cells using Sulfo-Cy5-azide (Figure 2A).[42] For future live-cell imaging based on this approach, cell-permeable fluorogenic dyes will be required.

Covalent modifications can also be installed by RNA itself. One type of ribozymes catalyzes self-alkylation with a fluorophore, like fluorescein iodoacetamide (FIA, **7**), which bears an electrophilic reactive group (Figure 2C).[43] Another noteworthy self-alkylating ribozyme reacts with a 2,3-disubstituted epoxide at *N*7 of a specific guanosine. Using an azide-epoxide probe (2-(3-(3-methyloxiran-2-yl)propoxy)ethyl 4-

DOI: 10.1021/acscentsci.7b00251
ACS Cent. Sci. 2017, 3, 701−707

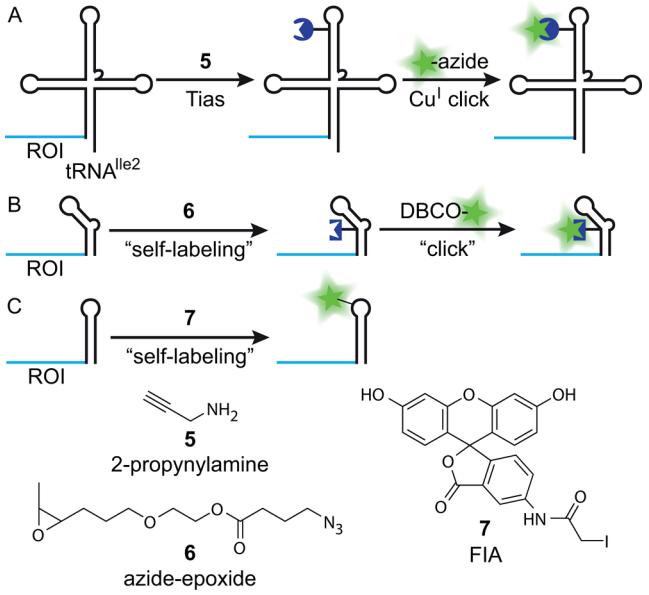

Figure 2. RNA labeling via covalent modification. (A) The ROI (blue) is extended by an archaeal tRNA, which is modified with an alkyne (**5**) by exogenous Tias. Subsequent copper-catalyzed click reaction with Cy5-azide leads to specific fluorescent labeling (green star).[42] (B) The ROI is extended by a self-alkylating 42 nt RNA reacting with an epoxide such as **6**. Copper-free click reaction can then be performed using DBCO-TAMRA (green star).[44] (C) A ribozyme catalyzing self-alkylation with the fluorophore FIA (**7**) can be used to extend and label an ROI.[43]

azidobutanoate, **6**) enables a subsequent click reaction, as shown for the fluorophore tetramethylrhodamine (TAMRA) (Figure 2B).[44] The approach is appealing; however, both self-alkylating appendages were only used in cell lysate until now. Similar to the aptamer approaches, a turn-on effect or the appendage of multiple fluorophores will be necessary to obtain the signal intensity and signal-to-noise ratio required for *in vivo* applications.

RNA-BINDING PROTEINS RCAS9 AND CAS13A

A completely different approach to recognize and label endogenous RNA sequence-specifically harnesses RNA-binding proteins or RNP-complexes fused to fluorescent proteins (FPs).[45] Among the known sequence-specific RNA-binding proteins, two families—namely, Pumilio[46,47] and PPR (pentatricopeptide repeat)[48,49] proteins—stand out, because they are built in a modular fashion and recognize the nucleobase rather than the sugar or phosphate backbone. Although the direct sequence-specific interaction is advantageous with respect to the simplicity of the fusion construct, and numerous advances in engineering these proteins have been made, obtaining protein variants with tailor-made sequence-specificity is still labor intense and not entirely predictable.[50−55]

Therefore, RNA−protein complexes relying on Watson−Crick interactions of a guide RNA for sequence-specificity hold promise to be a valuable alternative. The CRISPR/Cas9 system[56] can be adapted to recognize ssRNA instead of DNA.[57] Herein, the RNA-targeting nuclease-deficient Cas9 (RCas9) is fused to an FP. A single-guide RNA (sgRNA), bound by RCas9-FP, accounts for sequence-specific RNA targeting in the nucleus and export of the whole complex. For a strong interaction of the sgRNA/RCas9-FP complex with the ROI, a so-called PAMmer is essential, which binds upstream of the target RNA site and contains the mismatched PAM (protospacer adjacent motif, 5′-NGG-3′, Figure 3A).[58] This PAMmer consists of 2′-*O*-methylated RNA and ssDNA to be nuclease-resistant and not induce RNaseH degradation and therefore has to be transfected.[57] The other two components—RCas9-FP and sgRNA—can be produced by the cellular machinery.[58] The key advantage of this approach is flexibility regarding the target sequence. Even multiple RNA sequences can be targeted with the same RCas9-FP, if appropriate sgRNAs are provided. Besides, the RCas9 system prevents fluorescent background signals in the cytoplasm, because the key protein localizes to the nucleus. Consequently, only the bound protein is exported from the nucleus in complex with its target RNA.[58]

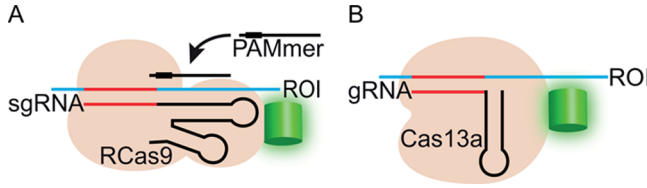

Figure 3. RNA-binding proteins RCas9 and Cas13a. (A) RCas9: A FP (green) is fused to RCas9 (brown), which binds directly to the ROI (blue). Its RNA recognition depends on sgRNA (red part hybridizes to red part of ROI) and a PAMmer that has to be transfected.[58] (B) Cas13a: The catalytically inactive Cas13a targets the ROI via hybridization of the guide RNA (gRNA, red parts).[59] The fusion of a FP (green) to Cas13a holds promise to become a novel tool for RNA imaging in the future.

Currently, another member of the CRISPR family termed Cas13a (previously known as C2c2) is emerging as a promising alternative for RNA targeting.[59−61] Cas13a is an RNA-guided RNase containing two HEPN (Higher Eukaryotes and Prokaryotes Nucleotide-binding) domains. If the catalytic HEPN residues are substituted, the resulting variants lose their cleavage activity, but retain their ability to bind RNA sequence-specifically via the gRNA (Figure 3B). The major advantage of Cas13a over RCas9 is that the additional oligonucleotide (the PAMmer) becomes unnecessary. Therefore, although no publication on Cas13a-based RNA-imaging has been reported to the best of our knowledge, Cas13a will likely be utilized for live-cell RNA imaging in the future.[59]

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SIMULTANEOUS RNA IMAGING AND SEQUENCING

A recent ground-breaking development termed fluorescent *in situ* sequencing (FISSEQ) uses fixed cells or tissues for transcriptome-wide RNA sequencing and at the same time obtains the spatial information via imaging.[62] FISSEQ is based on reverse transcription (RT) of RNA in fixed cells with short random primers. Incorporation of aminoallyl-dUTP allows for cross-linking with an amine-reactive linker to prevent diffusion (Figure 4A). The resulting cDNA is then subjected to rolling-

DOI: 10.1021/acscentsci.7b00251
ACS Cent. Sci. 2017, 3, 701−707

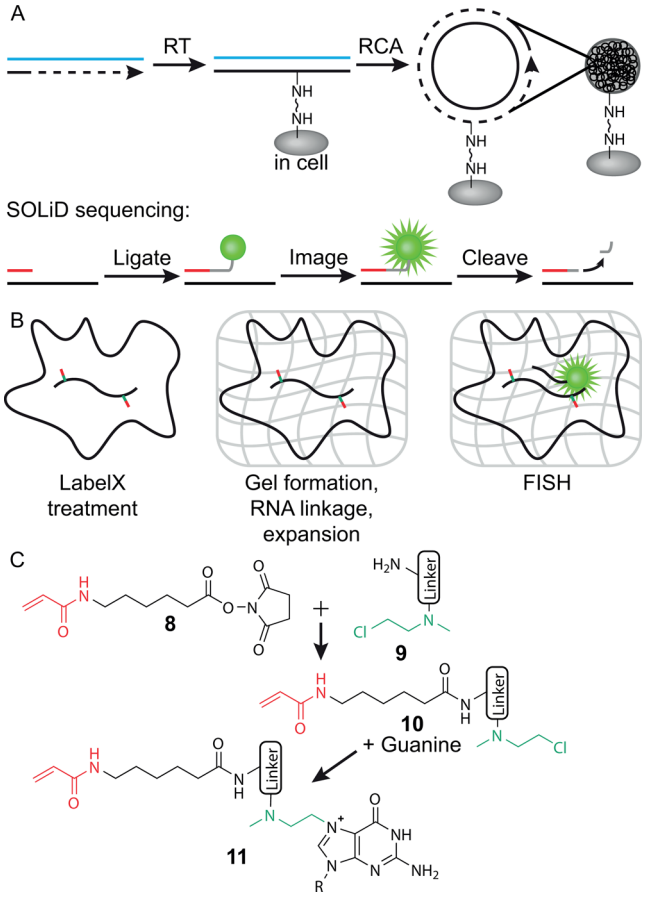

Figure 4. Scheme of simultaneous RNA imaging and sequencing. (A) FISSEQ: The ROI (blue) is reverse transcribed (RT) to cDNA, which is cross-linked inside the cell. After circularization and RCA a cross-linked, concentrated point of specific cDNA-copies is present in the cell. In the next step SOLiD sequencing is performed on each of the cDNA amplicons. After primer (red) hybridization a fluorescent probe is ligated, whose fluorescence (green star) is imaged in a confocal microscope. The end of the probe is cleaved again and the next round of sequencing is performed until the whole cDNA was sequenced.[62,63] (B) Expansion microscopy: The RNA in the cell is reacted with LabelX (**10**), which covalently attaches the RNA to the expansion microscopy gel after its formation. Subsequently, the gel expansion is performed, resulting in sub-nanometer resolution when imaging with FISH probes (green fluorescence).[64,65] (C) Chemistry underlying expansion microscopy: Cross-linking is based on LabelX (**10**), which is produced by NHS-ester chemistry using Acryloyl-X SE (**8**) and Label-IT amine (**9**). **10** reacts with RNA (e.g., with guanine, **11**) and cross-links the RNA to the expansion microscopy gel (attachments in red-green in B).[64]

circle amplification (RCA) to create clusters containing ~1000 copies of the cDNA sequence. SOLiD sequencing (Sequencing by Oligonucleotide Ligation and Detection) of these nanoball-shaped clusters is performed by ligation of fluorescently labeled nucleotides and imaging each step with a confocal microscope (Figure 4A).[63] In FISSEQ several aspects need to be considered to obtain useful images and distinguish the signal from the cellular noise. First, the signal density has to be reduced by focusing on just a subset of ROIs. This is achieved by lengthening the RT-primer by one or two nucleotides. Second, the sequence of colors at a single cluster is compared to known transcript sequences. Instead of using general intensity thresholds, only these known sequences are considered. With increasing sequencing length, the accuracy of true hits increases. Overall, FISSEQ provides information about RNA localization and its expression level even for single cells.[62,63] The technique is ground-breaking and opens a new era for transcriptome-wide studies of RNA localization, which are relevant for studying mutations at the single-cell level (e.g., in cancer) or allele-specific expression. What it cannot do, however, is to provide dynamic information aimed at studying mRNA trafficking, simply because fixation and amplification are required to allow for in-cell sequencing.

Expansion microscopy (ExM) was recently developed and can be combined with FISSEQ to enable imaging with sub-nanoscale resolution. In ExM, a compound termed LabelX (**10**) is used to attach RNA covalently to a polyelectrolyte gel. The resulting gel matrix is then expanded by osmotic swelling, and this physical magnification leads to a more defined imaging result (Figure 4B).[64] The compound LabelX (**10**) itself is synthesized from Acryloyl-X SE (6-((acryloyl)amino)hexanoic acid, succinimidyl ester, **8**) and Label-IT amine (**9**) using NHS-ester chemistry and reacts with RNA, preferentially with the *N*7 position of guanines (**11**, Figure 4C).[64] In conclusion, the RNA can be covalently anchored to the ExM gel, and the whole ExM protocol,[65] including gel formation and expansion, can be performed (Figure 4B).[64] Single-molecule imaging is then possible using hybridization of fluorescently labeled oligonucleotides, called FISH (Fluorescence *In Situ* Hybridization).[66,67] A combination of ExM and FISSEQ is a valuable tool that provides transcriptome and localization data at sub-nanometer resolution.

OUTLOOK

Thanks to recent developments and discoveries, a variety of approaches are now available for RNA labeling and imaging. However, there is no "generally ideal" method for localizing and tracking a large number of different transcripts in living cells. Instead, a method should be chosen depending on the application and the question to be addressed. For instance, methods involving simultaneous RNA imaging and sequencing can only be used in fixed cells or tissues, but provide data for (almost) the whole transcriptome. Besides, expansion microscopy in combination with FISH enables single-molecule RNA imaging. However, FISSEQ is very time-consuming—one experiment including evaluation takes 16 days.

For live-cell applications and tracking, fluorogenic aptamers are currently emerging as the best and most readily implementable approach. They require genetic engineering to tag the ROI and in theory, such an extension could affect an RNA's fate. However, compared to the established MS2-MCP system the tag is very small. Once the aptamers and fluorophores have been sufficiently improved to allow for routine application in living cells, it will become clear, whether the rather stable aptamer structure and potentially required additional stabilizing elements will affect RNA turnover and/or localization. This includes clarifying what is actually being imaged. In the worst case scenario the ROI might already be degraded and just the remaining—more stable—aptamer might be visualized. Currently, many efforts are undertaken to further improve the affinity and the turn-on effect of aptamers, and it will be exciting to watch the "evolution" of Mango variants and the development of novel aptamer/ligand pairs in the near future.

An interesting variation of the aptamer approach is the development of RNA-targeting aptamers, which can be produced by the cell and hybridize specifically to the target RNA *in trans*—no tag is required at the ROI. Ideally, fluorescence should occur only after binding to the ROI, because this leads to formation of

DOI: 10.1021/acscentsci.7b00251
ACS Cent. Sci. 2017, 3, 701−707

the aptamer's stem−loop structure. It remains to be seen how specific this hybridization will be and what happens if off-targeting occurs or if binding to structured or protein-occupied sequences is inefficient, a challenge that hybridization-based methods face in general. Definitely the combination of turn-on aptamers and trans-binding can be expected in the near future, and it will be exciting to see how far the signal and signal-to-noise ratio of such an approach can be pushed.

Sensitivity problems are encountered when self-alkylating ribozymes are used as tags. An advantage compared to aptamers is the permanent linkage, which might turn out to be useful for long-term tracking. However, the approaches reported to date were only used in cell lysate and will most likely require a turn-on effect or a better signal-to-noise ratio, if live cell applications are envisaged in the future. Alternatively, covalent modifications can be installed by enzymes, but this approach faces similar challenges: the ROI needs to be extended (e.g., by a tRNA) to obtain specificity for a certain transcript. The advantage of this system is the use of an exogenous enzyme, with its inherent specificity and multiple turnover. Specificity for their respective tRNA was reported for the bacterial tRNA guanine transglycosylase[68] and the archaeal $tRNA^{Ile2}$-agmatidine synthetase[42] and used to label tRNA-extended RNA in living cells. If a tag on the ROI should be avoided, an RNA-modifying enzyme with substrate specificity and the ability to use an orthogonal cosubstrate is required. This has proven challenging so far, but a promising example is a methyltransferase, which specifically modifies the 5′ cap of mRNAs, but shows cosubstrate promiscuity and therefore allows site-specific installation of bioorthogonal groups. This approach was successful to modify mRNA *in vitro* and to perform the subsequent fluorescent labeling of the cap-modified mRNAs in living cells.[36−40] If the enzyme can be engineered to preferentially convert cosubstrate analogues, this approach bears potential for in-cell mRNA labeling in the future.

Finally, RNA-binding proteins appear to be very promising for applications in living cells, because they can be produced by the cell itself and bind RNA with high affinity. The most prominent example is RCas9, whose sequence-specificity is simply determined by the accompanying sgRNA. Therefore, different RNAs can be targeted with the same protein by the use of different sgRNAs. Background signal from unbound protein is mostly limited to the nucleus, because only bound protein is exported from the nucleus. However, RCas9 is a rather big protein and might cause aberrant localization of the ROI. Furthermore, the PAMmer needs to be synthesized and subsequently transfected, which is a significant problem for most biological questions. The alternative Cas13a protein raises high hopes, because it can be used as a sequence-specific RNA-guided RNA-binding protein (without the additional PAMmer) and might become a readily programmable RNA-labeling approach in the future.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

A.R. acknowledges financial support by the Deutsche Forschungsgemeinschaft within (RE 2796/2-1) and the Fonds der Chemischen Industrie (Dozentenstipendium). K.R. thanks the SFB858 for financial support.

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DOI: 10.1021/acscentsci.7b00251
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