

RNA Localization in Bacteria

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ABSTRACT Diverse mechanisms and functions of posttranscriptional regulation by small regulatory RNAs and RNA-binding proteins have been described in bacteria. In contrast, little is known about the spatial organization of RNAs in bacterial cells. In eukaryotes, subcellular localization and transport of RNAs play important roles in diverse physiological processes, such as embryonic patterning, asymmetric cell division, epithelial polarity, and neuronal plasticity. It is now clear that bacterial RNAs also can accumulate at distinct sites in the cell. However, due to the small size of bacterial cells, RNA localization and localization-associated functions are more challenging to study in bacterial cells, and the underlying molecular mechanisms of transcript localization are less understood. Here, we review the emerging examples of RNAs localized to specific subcellular locations in bacteria, with indications that subcellular localization of transcripts might be important for gene expression and regulatory processes. Diverse mechanisms for bacterial RNA localization have been suggested, including close association to their genomic site of transcription, or to the localizations of their protein products in translation-dependent or -independent processes. We also provide an overview of the state of the art of technologies to visualize and track bacterial RNAs, ranging from hybridization-based approaches in fixed cells to *in vivo* imaging approaches using fluorescent protein reporters and/or RNA aptamers in single living bacterial cells. We conclude with a discussion of open questions in the field and ongoing technological developments regarding RNA imaging in eukaryotic systems that might likewise provide novel insights into RNA localization in bacteria.

INTRODUCTION

Spatial and temporal localization of macromolecules, including RNAs, reflects the compartmentalization of living cells and plays important roles in gene expression and regulation. In eukaryotic cells, physical separation between the transcription and translation machineries in the nucleus and cytoplasm, respectively, naturally results in the synthesis, processing, and translation

of mRNA to be spatially disconnected. Both mRNA localization and localized translation can be important regulatory mechanisms underlying embryonic patterning, asymmetric cell division, epithelial polarity, cell migration, and neuronal morphogenesis (1, 2). RNAs can be transported in the eukaryotic cell in several ways, such as (i) vectorial movement of mRNA by direct coupling to motor proteins, (ii) transport of mRNA by hitchhiking on another cargo, (iii) random transport of mRNA-motor complexes and local enrichment of mRNAs at target sites, or (iv) diffusion and motor-driven cytoplasmic flows with subsequent localized anchorage of the mRNA (3). Moreover, localized translation induction by phosphorylation and activation of translation initiation factors and their regulators in response to localized signals have been reported to impact gene regulation in eukaryotes (4).

In contrast, due to a lack of canonical membrane-bound organelles and a nuclear compartment, prokaryotic cells were long assumed to lack complex subcellular localization of macromolecules, and spatial localization has not been considered to play a significant role in expression and posttranscriptional regulation of bacterial

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mRNAs. This is also reflected by the classical picture of cotranscriptional translation of bacterial mRNAs, where transcription and protein synthesis are not spatially or temporally separated. Moreover, due to the much smaller size of bacterial cells compared to their eukaryotic counterparts, it has been more challenging to determine the subcellular organization of bacteria, to observe the subcellular distribution of biomolecules in bacterial cells, and to relate such organization and distribution to biological functions.

With the development of numerous labeling and imaging techniques as well as advanced microscopy approaches that can break the diffraction limit, it is now clear that the bacterial cells are also compartmentalized (5–7). Emerging evidence for differential localization of bacterial mRNAs indicates that the spatial organization in the cell can also impact gene expression and posttranscriptional regulation in prokaryotes (8–10). Commonly, localization patterns of mRNAs in bacteria include the nucleoid region, the cytoplasm, the cell poles, and the inner membrane. Frequently observed organizations of bacterial biomolecules include uniform distribution, distinct foci, and a putative helical structure, often in the vicinity of the cell envelope. Along with the visualization of transcript localization, it has also been shown that many enzymes and complexes involved in RNA metabolism, such as RNA polymerase (RNAP), ribosomes, and the degradosome, show distinct subcellular distributions, providing further support for the role of spatial organization in genetic information flow. Certain observations and conclusions in the study of bacterial RNA localization are still controversial, and the mechanisms underlying observed examples of subcellular localized transcripts remain to be further explored. However, it has nonetheless become clear that spatial control of RNA and related cellular machineries is likely important for gene expression and regulation in prokaryotes, just as it has been a well-established concept in higher organisms. These preliminary observations of distinct RNA localization patterns have brought attention to new phenomena and questions in bacterial posttranscriptional control, such as transcription-coupled versus transcription-uncoupled translation, translation-dependent and translation-independent mRNA localization, as well as localized degradation, stabilization, or regulation by small regulatory RNAs (sRNAs) and RNA-metabolizing complexes.

Posttranscriptional regulation by regulatory RNAs, RNA-binding proteins (RBPs), and RNases is a central layer of gene expression control in all kingdoms of life. Bacterial sRNAs (typically 50 to 300 nucleotides in

length) can control specific genes and/or coordinate expression of distinct regulons with clear physiological outcomes (11). While most sRNAs act as antisense RNAs by short and imperfect base-pairing, several can also directly bind to proteins and control their activity. sRNA-mediated regulation requires numerous and dynamic interplay with various cellular machineries, including RNAP, ribosomes, and degradosomes, and perturbs these machineries in the pathways of mRNA metabolism to broadly affect gene expression. The RNA chaperone Hfq serves as a key player in the sRNA regulatory pathways, where it functions in two main aspects: stabilization of sRNAs from degradation and promotion of the annealing between sRNAs and their target mRNAs (12). Base-pairing of sRNAs to their target mRNAs with the help of Hfq often leads to changes in translation and/or mRNA stability (positive or negative). Translation inhibition is often associated with RNase-mediated codegradation of the sRNA-mRNA pair. While posttranscriptional regulation in bacteria has mainly been studied at the population level in batch cultures, little is known about sRNA-mediated regulation at the single-cell level and even less about the extent and impact of subcellular localization of RNAs on regulatory processes in these organisms. Due to their important biological function, the subcellular localization of sRNAs and their interactions with target mRNAs, Hfq, and RNase E have become an intriguing research topic.

In this review, we describe recent advances in methods that allow for the investigation of RNA localization in bacterial systems, as well as findings regarding mRNA and sRNA localizations in these organisms. We discuss the models and mechanisms revealed by these examples of spatial control of RNA. In addition, we introduce new labeling and imaging methods recently developed in eukaryotic cells, most of which have not yet been applied to bacteria but have the potential to reveal new insights about prokaryotic transcript localization. Finally, we conclude by laying out open questions and future challenges in the field.

APPROACHES TO STUDY RNA LOCALIZATION

Biochemically, cell fractionation methods have been routinely used to study protein localization to the outer membrane, periplasm, inner membrane, and cytoplasm (13, 14). Similar approaches have also been used to investigate RNA localization. Particularly, when cell fractionation methods are combined with high-throughput RNA sequencing, the relative distribution of

transcripts between the membrane and the cytoplasm can be estimated at the whole-transcriptome level (15). Compared to fractionation-based approaches, visualization of RNAs by light microscopy techniques provides the most direct information on subcellular localization of individual RNA species. It is worth mentioning that three-dimensional cryo-electron tomography provides another remarkable imaging category with enhanced spatial resolution compared to conventional light microscopy, and has been applied to imaging subcellular organization of bacteria (reviewed in reference 16). However, compared to light microscopy, as in general no specific tags are introduced to the specific biomolecules of interest, cryo-electron tomography usually cannot provide selectivity of the specific biomolecules of interest during imaging.

Since many studies on bacterial RNA localization are fluorescence imaging based, here, we discuss labeling and imaging methods used in bacterial systems (see also several recent reviews on RNA imaging methods [2, 17, 18]).

FISH

Single-molecule fluorescence *in situ* hybridization (smFISH) is one of the most widely used RNA-labeling strategies in both eukaryotic and bacterial systems (19–21). By fixing and permeabilizing the cells, DNA oligonucleotides covalently linked with fluorophores can access the interior and hybridize to the RNAs of interest, thereby labeling them (Fig. 1A). To enhance the signal-to-noise and target specificity, a few to tens of labeled oligos are used for each RNA, tiling along the nucleotide sequence. Enhanced fluorescent signals from multiple oligos on the same RNA appear as a single spot under the diffraction-limited fluorescence microscope, providing single-molecule sensitivity. Despite the limitation of FISH to “dead” cells, many important details can be gleaned from this approach, including the expression levels and localization of RNAs, which can be further used to derive the kinetic mechanisms of transcription or degradation (20, 22–24). FISH has been applied to both mRNAs and sRNAs in bacteria (for references, see the sections on mRNA and sRNA localization below). However, due to the relatively short length of sRNAs, the application of FISH to sRNAs may be case dependent, and be more applicable to sRNAs existing in high copy number.

FISH does not require genetic manipulation of the RNAs of interest, and normally does not perturb their function or certain features, such as lifetime. However, for fixed-cell imaging, fixation and permeabilization

conditions can potentially affect the native localization of biomolecules and/or the accessibility of the labeling reagents (25). While the accessibility issue is less of a concern for short FISH probes (usually ~20 nucleotides) compared to sizable antibodies in immunostaining protocol, it is still recommended that imaging results are validated by using multiple fixation and permeabilization methods. In addition, negative controls (such as a knockout strain of the RNA of interest) should always be used to examine the level of nonspecific binding of the FISH probes.

Fluorescent Protein Reporters

Live-cell imaging of RNAs allows for a direct observation of transcript motion, as well as the kinetics of RNA-associated activities. While such approaches are more challenging and require genetic manipulation, several methodologies have been developed for live-cell RNA imaging. One category of approaches relies on orthogonal protein-RNA interactions, consisting of an RNA-binding motif engineered into the transcript of interest, together with the cognate RBP fused to a fluorescent protein (FP) (2). The FP-fused RBP recognizes and binds to the RNA motif, thereby labeling the RNA. Repetitive RNA motifs of the same kind are often inserted into the RNA of interest to recruit multiple FP-fused RBPs, thereby enhancing the signal, even to single-transcript sensitivity (Fig. 1B). Commonly used RNA-RBP pairs include the MS2 and PP7 phage coat proteins with their respective RNA motifs, as well as the λ phage N-protein-*boxB* hairpin pair (26–28). There are several derivatives of this approach. To increase the signal brightness and photostability, FPs can be replaced by other visualizable tags on the RBPs, such as the SNAP-tag or an *Escherichia coli* dihydrofolate reductase tag (eDHFR-tag) (29–32). In these approaches, the tags can be labeled by the addition of organic dyes introduced into the cell. Such methods rely on the ability to introduce the dyes into cells by either their natural membrane permeability or manual delivery via transfection or microinjection. Since FP-fused RBPs are constitutively expressed and fluorescent in the cell, even if not bound to the target RNAs, they can result in significant background signals. To lower the background, another derivative of the FP live-cell approach employs complementation of the fluorescent reporter upon RNA binding. In this method, FPs are expressed as two independent halves, fused to either two RBPs or halves of the same RBP, respectively. When the two FP halves are brought into close proximity through binding to the same RNA molecule, a complete FP is reconstituted. For example, the RNA-

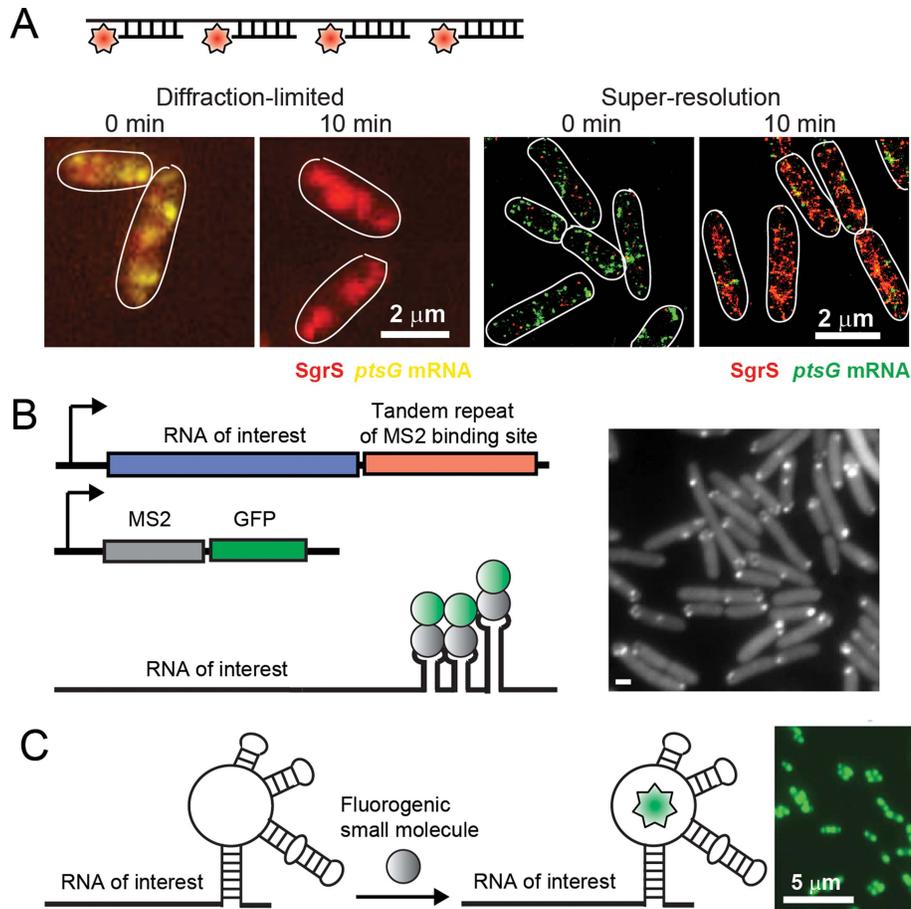


FIGURE 1 Methods to visualize bacterial RNAs. (A) smFISH and its application to imaging *SgrS* sRNA and its target mRNA *ptsG*. Images from diffraction-limited and super-resolution microscopes are shown for comparison. Adapted from reference 105. (B) Illustration of the FP reporter approaches with a FP-RBP and a corresponding RNA motif, using the MS2 system as an example, and its application to track mRNAs at the single-molecule level in live *E. coli* cells. Image adapted from reference 67. The scale bar in the image represents 1 μm . (C) The Spinach aptamer and its application to image mRNAs in live *E. coli* cells, in which a Spinach aptamer is fused to the RNA and with fluorescence detection upon addition of the organic ligand 3,5-difluoro-4-hydroxybenzylidene imidazolinone (DFHBI). Image adapted from reference 58 (licensed under a Creative Commons Attribution 4.0 International License [<http://creativecommons.org/licenses/by/4.0/>]).

binding eukaryotic translation initiation factor eIF4A can be expressed as two independent halves, each fused to a half of a split enhanced green fluorescent protein (EGFP) (33, 34). Upon binding of an eIF4A target inserted into a transcript of interest, fluorescent EGFP is reconstituted. It should be noted that the split eIF4A reporter system can only be applied to bacterial cells, as they natively lack eIF4A. Similarly, split EGFP has been fused to two different Pumilio homology domains (PUM-HD) of human PUMILIO1 (35), and the FP Venus has been split into two domains, fused to either the PP7 or MS2 coat proteins, and has been used to detect transcripts expressing adjacent PP7 and MS2 binding motifs (36).

The FP reporter systems use indirect labeling methods, in which significant modifications have to be introduced to the RNAs of interest. Therefore, it is important to know the potential pitfalls of these methods. First, FPs have a propensity to oligomerize (37, 38), and wild-type MS2 coat proteins, but not the V75EA81G double mutant, also have such propensity (39). Therefore, a careful choice of the FP-fused RBP is necessary to avoid the formation of artificial, RNA-independent foci. An independent labeling method, such as FISH, is recommended to be used to verify whether the fluorescence protein foci indeed also contain the RNA of interest. Second, the FP reporter systems have potential

risks of changing the mRNA processing, lifetime, and localization, due to protection by the bound proteins or due to the tandem array of the inserted RNA motif itself (40–44). Therefore, while this approach is very useful in revealing transcriptional activity, results have to be carefully interpreted when applied to studies of RNA degradation and localization. Recently, a reengineered MS2 system has been developed that has minimal effect on mRNA half-life (45). In addition, an mRNA degradation assay using rifampin can provide a good test of potential effects of the labeling method on mRNA turnover. Hereby, the signal of the fluorescent reporter on the mRNA should decay with the same kinetics as the native mRNA upon rifampin treatment, e.g., to rule out accumulation of a reconstituted or aggregated reporter with the mRNA part already being degraded. A comparatively long-lived fluorescent signal is therefore unlikely to reflect the correct localization of the mRNA. A strong overexpression, e.g., from an artificial promoter, might also impact on the transcript's properties, such as stability, function, or localization. Therefore, preference should be given to expression from a native promoter.

Fluorescent RNA Aptamers

RNA-protein interaction-based methods often raise the concern of changes of molecular and functional properties of the tagged RNA, due to the binding of multiple bulky FPs to the inserted repetitive and often structured RNA motifs. This approach may be especially problematic for short or structured transcripts such as sRNAs. The development of RNA aptamer-based imaging methods provides another possibility of live-cell RNA imaging. This approach utilizes RNA aptamer sequences added to the transcript of interest and fluorogenic small molecules that can freely diffuse into the cell and become fluorescent upon binding to the RNA aptamer (for recent reviews, see references 17 and 46–48). While not quite suitable for live-cell imaging, earlier developments of malachite green, thiazole orange, and dimethyl indole red aptamers, etc., provided proof of concept and illustrated the feasibility of such approaches (49–51). The Spinach system (Fig. 1C) is an example of the first generation of RNA aptamers for live-cell imaging (52). Since then, new aptamer systems have been developed, including Spinach 2 (53), Mango (54), Broccoli (55, 56), and Corn (57). Repetitive Spinach aptamers have been shown to increase the brightness by a maximum of 17-fold (with 64 repeats) compared to a single Spinach monomer at a cost of a significant lengthening of the aptamer sequence (58). Alternatively,

the fluorogenic small molecules have been designed into a covalently linked fluorophore-quencher pair, and the aptamers are selected to bind either the quencher or the fluorophore. In this design, the fluorophore is quenched by the linked quencher in the absence of the aptamer. Once either the quencher or the fluorophore binds to the aptamer, they are more physically separated and the fluorophore is dequenched (59, 60).

Despite their relatively broad applications in the area of metabolite sensing through engineering to various riboswitches (for reviews, see references 17 and 46–48), the use of RNA aptamers in single-molecule RNA imaging is still considered to be limited. This limited application of the aptamer-based method for single-molecule RNA imaging might be related to, e.g., the limited brightness of the tag and the requirement for correct folding of the aptamer *in vivo*. Further engineering of smaller and more stable aptamers, brighter fluorophores, and tandem arrays of the aptamer could collectively help to achieve single-RNA sensitivity. Similar to the FP reporter systems, the fusion of an aptamer sequence to a transcript of interest might also affect its properties, such as stability or function. Therefore, certain functional validation of the tagged RNA is required.

Super-Resolution Microscopy

With the various labeling methods described above, RNA can be directly visualized under the fluorescence microscope. However, due to the diffraction limit of visible light, conventional optical microscopy has a limited resolution of ~200 to 300 nm in the lateral direction and 500 to 700 nm in the axial direction.

While FISH can provide single-molecule sensitivity on RNA imaging, because of the small size of a bacterium (a few hundred nanometers to a few microns), only individual RNAs with very low abundance can be resolved in bacteria. Several super-resolution techniques that can break the diffraction limit have been developed. Among these techniques, single-molecule localization microscopies are most frequently applied in bacterial systems, in which photoactivatable FPs and photo-switchable dyes are used to label biomolecules, and can push the spatial resolution into the range of 10 to 20 nm (61–63). Therefore, the utilization of such super-resolution microscopy techniques can provide finer details on the RNA localization.

LOCALIZATION OF mRNAs

Besides distinct subcellular localizations of proteins and the nucleoid, diverse subcellular localizations of

mRNAs have also been reported in various bacteria. Commonly observed patterns of distribution of bacterial mRNAs include uniform expression throughout the cytoplasm, localization into distinct foci close to the nucleoid, formation of helical patterns along the cell axis, enrichment at the inner membrane, or concentration at the cell poles or the septum during cell division (Fig. 2). For example, using the split eIF4A approach in live cells, Broude and coworkers observed that *lacZ* mRNA was evenly distributed along the *E. coli* cell (Fig. 2A) (33). In contrast, another study observed limited dispersion of *lacZ* mRNA in *E. coli* as well as of several mRNAs in *Caulobacter crescentus* from their site of transcription using FISH in fixed cells (Fig. 2B) (20, 64). The *cat* mRNA, encoding a cytoplasmic chloramphenicol acetyltransferase, demonstrates a helix-like pattern in the cytoplasm in *E. coli* (Fig. 2C) (65). The *lacY* mRNA, encoding a membrane-bound lactose permease, localizes at or near the inner membrane (Fig. 2D) (65). In the Gram-positive bacterium *Bacillus subtilis*, MS2-GFP tagging revealed that *comE* mRNA, encoding the late competence operon, localizes to the cell poles (Fig. 2E) and the nascent septum that separates the daughter cells (Fig. 2F) (66). The sometimes disparate results of observed subcellular localizations of the same transcript might be due to the different RNA visualization technologies and expression systems used.

mRNA Accumulation Near the Site of Transcription

The observed nucleoid-associated transcript foci led to the proposal that bacterial mRNAs may remain localized close to their genomic site of transcription (64). Using a combination of MS2 tagging and validation by FISH, one study reported that the mRNAs of *groESL*, *creS*, *divJ*, *ompA*, and *fljK* of *C. crescentus* and the *lacZ* transcript in *E. coli* show limited dispersion from their site of transcription and are visible as distinct foci in the vicinity of the genomic DNA locus from where they are transcribed (64). Similarly, visualization of a transcript containing repeated MS2-binding motif sequences revealed that most transcripts moved randomly in a restricted spot near the midpoint or quarter-point of the cells, which corresponds to the localization of the F-plasmid, whereas a minority diffused throughout the cell or moved as chains (67). In this study, the observed distinct spots were suggested to be mRNAs that are still tethered to DNA by RNAP, while freely diffusing transcripts are completed transcripts.

mRNA Accumulation at the Sites of Protein Requirement

The observations that mRNAs accumulate at various other subcellular places in addition to close association with their genomic site of transcription (Fig. 2) indicate

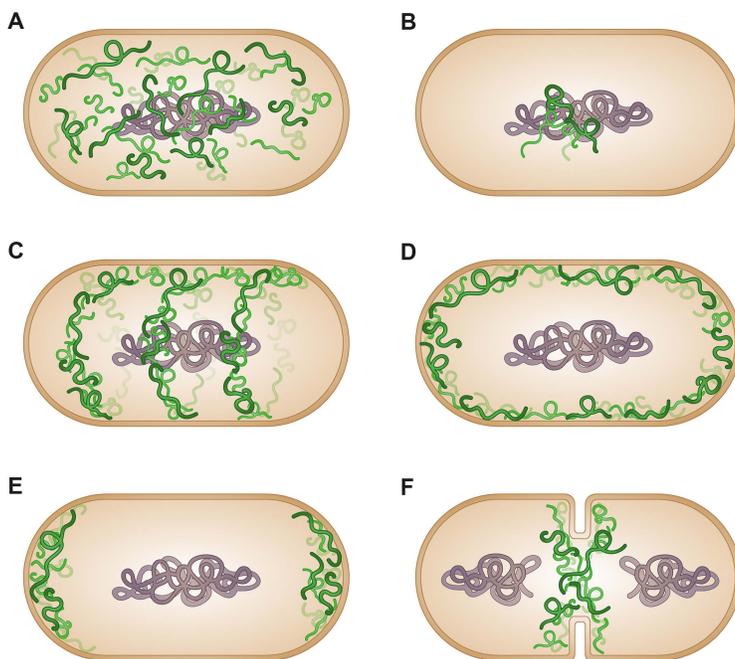


FIGURE 2 Diverse patterns of subcellular mRNA localization in bacteria. Schematic drawings of diverse mRNA localization patterns commonly reported in different bacteria. RNA molecules are shown in green, and the nucleoid in gray. (A) Distribution throughout the cytoplasm. (B) Localization at the site of transcription in the nucleoid. (C) Helical localization. (D) Enrichment at the inner membrane. (E) Localization at the cell poles and (F) septum.

that other cellular processes can affect the localization of the mRNA. For example, using a split aptamer approach for RNA labeling in live *E. coli* cells combined with independent validation by FISH, distinct spots of the endogenous *ptsC* mRNA, encoding an integral transmembrane transporter, could be detected, which do not colocalize with bulk DNA (68). Similarly, FISH analysis revealed distinct, uneven patterns of localization and specific foci at one or both poles for the dinitrogenase reductase-encoding *nifH* transcripts of *Klebsiella oxytoca* and *Azotobacter vinelandii* (69). One intriguing hypothesis is that mRNAs can be targeted to the subcellular domains where their encoded protein products are required (e.g., the cytoplasm, poles, or inner membrane) (65, 70, 71). For example, while the *cat* mRNA, encoding the cytoplasmic chloramphenicol acetyltransferase, was observed in a helix-like pattern in the cytoplasm of *E. coli*, the mRNA of *lacY*, encoding the membrane-bound lactose permease, was preferentially detected near the cytoplasmic membrane (65). In line with these imaging observations, upon fractionation of *E. coli*, the authors found that the *cat* and *lacY* mRNAs are enriched in cytosolic and membrane samples, respectively (65). Moreover, the same study reported the polycistronic *bglGFB* mRNA, which encodes both membrane-bound (BglF sugar permease) and soluble (BglG transcription factor and BglB phospho- β -glucosidase) components, was enriched at the cell membrane, indicating that envelope-targeting signals may be dominant. Furthermore, inhibition of translation with kasugamycin or chloramphenicol revealed that this membrane localization occurs in a translation-independent manner (65). This observation was further supported by introducing mutations that abolish *bglF* translation, which did not affect localization, indicating that *cis*-acting signals in the RNA itself dictate membrane localization (65). The *bglF* membrane-targeting signal, which is present in the sequence encoding the first two transmembrane helices of BglF, was found to be dominant over other operon components, because *bglG* mRNA expressed alone localizes to cell poles and *bglB* mRNA expressed alone is cytoplasmic (65). It has also been reported that *cis*-encoded RNA elements in the early coding region of mRNAs encoding Yop effector proteins (e.g., YopE and YopN) are required for the secretion of these effector proteins by type III secretion systems in *Yersinia* (72, 73). However, it is unknown if these transcripts are localized to the membrane or in proximity to the secretion apparatus. Similarly, N-terminal domains are required for secretion of flagellar proteins in diverse bacteria, and both

mRNA and peptide signals are recognized by the secretion apparatus and contribute to secretion efficiency (74). For example, secretion of heterologous proteins was facilitated by fusing the 173-bp 5' untranslated region (5' UTR) of the *fliC* gene (encoding flagellin) as well as a *fliC* transcriptional terminator (75).

While most studies have mainly looked at the localization of only a single or small set of mRNAs, recently Zhuang and coworkers investigated the spatial organization of mRNAs in *E. coli* on the transcriptome scale (70). They designed complex FISH probe sets to visualize defined sets of mRNAs, categorized by the subcellular localizations of their encoded proteins, allowing examination of ~27% of all *E. coli* mRNAs. This revealed that mRNAs encoding inner membrane proteins tended to be preferentially located at the inner membrane, whereas transcripts encoding cytoplasmic, periplasmic, and outer membrane proteins were generally detected more uniformly throughout the cytoplasm. Moreover, labeling of specific subgroups of mRNAs as a function of genomic localization of their genes revealed that spatial genome organization does not play a major role in the shaping of the cellular distribution of the transcriptome. Because inhibition of translation initiation using kasugamycin treatment disrupted envelope localization of membrane protein-encoding transcripts, Moffitt et al. concluded that membrane localization of these transcripts is translation dependent (70). Most inner membrane proteins are secreted via the signal recognition particle (SRP) pathway, which cotranslationally inserts proteins into the membrane, while outer membrane proteins are posttranslationally secreted via the SecB pathway (76). Examination of localization by FISH of fluorescent reporter fusions to SRP or SecB signal peptides confirmed that mRNAs with SRP signal sequences were found enriched at membranes, suggesting that these transcripts are recruited to the envelope during cotranslational secretion of the ribosome-bound nascent signal peptide (70). Furthermore, introduction of stop codon mutations into the *bglF* mRNA abolished its localization at the membrane, indicating that translation of its SRP signal sequence is required for membrane localization (70). Whether the protein signal sequence or solely the act of translation of its mRNA is required for directing the transcripts to the membrane is so far unknown. Another study also reported, based on cell fractionation, RNA-sequencing, and quantitative PCR, that mRNAs of inner membrane proteins are enriched at the membrane and depletion of the SRP receptor FtsY reduced the amounts of all mRNAs at the membrane (15). However, mRNAs encoding inner

membrane proteins were also found in the soluble ribosome-free fraction, which might represent a stage prior to their translation and targeting to the membrane. Mathematical modeling has suggested that translation initiation rates, the availability of secretory apparatuses, and the composition of the coding region define mRNA abundance and residence time near the membrane (77). In addition, modeling suggested that formation of membrane protein clusters might be facilitated by bursts of proteins translated from a single mRNA anchored to the membrane, and therefore spatiotemporal dynamics of mRNAs might strongly influence the organization of membrane protein complexes.

Recently, the mRNA encoding the major flagellin FlaA of the foodborne pathogen *Campylobacter jejuni* was also observed, using FISH, to localize to the cell poles in a translation-dependent manner (71). *C. jejuni* has two polar flagella, and polar *flaA* mRNA localization was primarily detected in short cells, which likely correspond to cells that have just divided and are building a new flagellum at the nascent cell pole. While polar localization was abolished for a translation-incompetent *flaA* mRNA with stop codon mutations before the N-terminal peptide, translation of the first 100 codons partially restored polar localization, suggesting that an N-terminal amino acid signal, or its translation, is sufficient for mRNA localization. Similarly, translation of the N-terminal peptide of type III secretion effector protein mRNAs has been shown to be required for secretion (72, 78).

mRNA Localization by Specific *trans*-Acting Factors

Emerging examples have also indicated that there are specific *trans*-acting factors that posttranscriptionally regulate mRNA localization. For example, overexpression of the cold shock protein CspE increased the fraction of membrane protein encoding mRNAs in the ribosome-free fraction and their amount on the membrane and positively affected their translation, indicating potential regulation of subcellular RNA localization (15). Moreover, the 98-amino-acid protein ComN, a posttranscriptional regulator of competence gene expression, localizes to the division site and cell poles via direct interaction with DivIVA, a key protein involved in cell pole differentiation in *B. subtilis* (66). ComN-DivIVA interaction promotes accumulation of *comE* mRNA to septal and polar sites, indicating that localized regulators can also impact mRNA localization in bacteria. Furthermore, localized mRNA translation of ComE proteins might be required for efficient compe-

tence development. During a global analysis of the direct RNA regulon of the translational regulator CsrA by RNA immunoprecipitation sequencing (RIP-seq), *flaA* mRNA was revealed as the major translationally repressed target of CsrA in *C. jejuni* and the abovementioned polar *flaA* mRNA localization was detected to be posttranscriptionally regulated by the CsrA-FliW regulatory system (71). Deletion of the CsrA protein antagonist FliW releases CsrA and in turn allows translational repression of *flaA* mRNA, abrogating its polar localization. This observation suggests both that the *flaA* mRNA localization is translation dependent and that spatial control of bacterial transcripts can be regulated posttranscriptionally. It remains to be seen how many other flagellar mRNAs localize to the flagella apparatus and whether polar localization of the *flaA* mRNA or membrane localization of inner membrane protein mRNAs has any effects on the efficiency of secretion and/or assembly of larger complexes, or is just a by-product of cotranslational secretion.

LOCALIZED mRNA TRANSLATION AND DEGRADATION

While transcription and translation occur at distinct places in the nucleus and cytoplasm in eukaryotes, translation can already initiate cotranscriptionally in the cytoplasm of bacteria and translation can continue after transcription completion and release of the mRNA. Furthermore, the abovementioned examples show that mRNAs can migrate outside the nucleoid and translation can take place at distinct locations in the cell, such as at the membrane during cotranslational secretion of inner membrane proteins. Moreover, it has been reported that the RNAP transcription machinery and ribosomes occupy partially different subcellular regions within different bacterial cells (79–81). For example, in *B. subtilis*, RNAP has been primarily detected inside and ribosomes outside the nucleoid, respectively (82), indicating that transcription and translation are spatially separated. Cryo-electron tomography indicated that 70S ribosomes of *Spiroplasma melliferum* are distributed throughout the cytoplasm, with 15% in close proximity to the membrane (83). Similar electron cryotomography analysis of the cellular ultrastructure of logarithmically growing cultures of *C. jejuni* revealed ribosome exclusion zones at cell poles (80). Also consistent with extranucleoid distribution of ribosomes, 5S rRNA was detected as an array of fluorescent particles distributed along the cell or the cell poles in *E. coli* (33), indicating specific localization of ribosomes outside the nucleoid in

E. coli, which is in agreement with enrichment of ribosomal proteins at the cell periphery and cell poles in both *E. coli* (84) and *B. subtilis* (82, 85). While several ribosomal proteins (L1, S2, and L7/L12) are enriched at either of the cell poles and the translation factor EF-Tu colocalizes with the bacterial cytoskeleton protein MreB, it remains unclear how many of these localized translation factors are incorporated into actively translated ribosomes (reviewed in reference 86). It should be noted that the cellular localization can differ for unbound subunits versus actively translating ribosomes or transcribing RNAP. For example, it has been reported that mRNA-free ribosome subunits are not fully excluded from the nucleoid, thereby allowing for translation initiation on nascent mRNAs throughout the nucleoid and cotranscriptional translation (81). Moreover, a large fraction of RNAPs, presumably the transcribing population, has been reported to be primarily located at the periphery of the nucleoid and thus is close to the pool of ribosomes excluded from the nucleoid (87). In *C. crescentus* and another alphaproteobacterium, *Sinorhizobium meliloti*, ribosomes are detected throughout the cell, including in the nucleoid region (64, 90), which is different from the ribosome/nucleoid segregation observed in *E. coli* and *B. subtilis* (82, 84). Hereby, it was suggested that mRNA-bound ribosomal subunits show limited mobility in *C. crescentus* due to the observed limited dispersion of their mRNA targets from their site of transcription (64, 90, 91).

The specific organization of ribosomes has also been reported to change during different growth conditions in some bacteria (88). The distinct subcellular localization of ribosomes, mRNAs, and RNAP indicates that transcription and translation are not necessarily coupled in bacteria and localized translation by specific ribosomes at subcellular locations might also play a role in bacterial gene expression. For example, inner membrane-bound ribosomes in *E. coli* that are actively engaged in translation (89) might play a role in specific translation of inner membrane proteins.

Similar to the specific and dynamic localization of ribosomes and transcripts in bacterial cells, distinct subcellular distribution of RNA-degrading proteins and complexes has also been reported in prokaryotes (92–94). The *E. coli* degradosome initiates most of the RNA decay in bacteria and contains RNase E, the RNA helicase RhlB, polynucleotide phosphorylase (PNPase), and enolase (95). The *B. subtilis* degradosome consists of PNPase; RNases J1, J2, and Y; as well as the DEAD-box RNA helicase CshA, enolase, and phosphofructokinase (96). Components of the *E. coli* degradosome

have been reported to associate with the membrane or to assemble into helical filaments (97–100). Using super-resolution imaging of 24 FP fusions to RNA degradation and processing enzymes in *E. coli*, Moffitt et al. detected that only the four proteins (RNase E, PNPase, RhlB, and the polyadenylation enzyme PAPI) were enriched at the membrane, whereas the other tested fusions were mainly uniformly distributed throughout the cytoplasm (70). The membrane localization of the degradosome is mediated by membrane anchoring of segment A of RNase E (98). In line with a colocalization of inner membrane protein-coding mRNAs and degradosome components at the cell envelope, it has been observed that these transcripts had shorter half-lives than the mRNAs of cytoplasmic, periplasmic, or outer membrane proteins (70). Moreover, artificial targeting of mRNAs to the membrane via fusion to SRP signal sequences destabilizes these mRNAs. Thus, cocolocalization of certain mRNAs with RNA degradation components can lead to their specific degradation. Interestingly, the degradosome localization can be impacted by growth conditions: a redistribution of RNase E/enolase from membrane-associated patterns under aerobic to diffuse patterns under anaerobic conditions results in stabilization of DicF sRNA and filamentation of the bacteria (101).

In *C. crescentus*, RNase E shows a patchy localization pattern and the clustered localization of this enzyme is determined by the location of DNA, independent of its mRNA substrates (64, 90). Hereby, the localization of RNase E clusters was found to correlate with two subcellular chromosomal positions that encode the highly expressed rRNA genes, indicating that RNase E-mediated rRNA processing occurs at the site of rRNA synthesis (90). Although mediated by apparently different mechanisms compared to *E. coli*, the association of RNase E with DNA in *C. crescentus* also indicates that there is spatially organized mRNA decay in this organism. Such a subcellular organization of the RNA degradation machinery likely also applies to other prokaryotes. For example, the membrane-bound RNase Y of Gram-positive bacteria (e.g., *B. subtilis* and *Staphylococcus aureus*) appears to be assembled at similar cellular locations as other RNA degradation enzymes (93, 102). Recently, it has been reported that RNase Y is recruited to lipid rafts via flotillin in *S. aureus* and that flotillin increases RNase Y function (103), indicating localized RNA degradation in bacterial membrane microdomains. How many and which mRNAs are mainly targeted by such localized degradation remains to be seen.

LOCALIZATION OF sRNAs

Localization of bacterial sRNAs has been investigated predominantly with FISH. The housekeeping transcript transfer-messenger RNA (tmRNA, also known as SsrA), involved in stalled ribosome rescue, was one of the first sRNAs investigated regarding its subcellular location (104). tmRNA contains both tRNA-like and mRNA properties. It forms an RNP complex (tmRNP) with the small protein B (SmpB) to function in *trans*-translation. During *trans*-translation, the tmRNP binds to stalled ribosomes and adds a proteolysis signaling tag to the stalled peptide, thereby recycling the ribosome and facilitating the degradation of the aberrant protein product. By FISH labeling of tmRNA and immunostaining of several relevant proteins, including SmpB and RNase R, in *C. crescentus*, Russell et al. demonstrated a helix-like pattern of tmRNA, SmpB, and RNase R in the swarmer cells (G1 phase), whereas in S phase after initiation of DNA replication, tmRNA molecules are largely degraded by RNase R, with the remaining transcripts becoming homogeneously distributed in the cytoplasm (104). Both tmRNA and SmpB show a high degree of colocalization, consistent with the formation of a tmRNP, whereas the helical structures formed by the tmRNP and RNase R are mostly distinct from each other. The helical organization of tmRNA relies on SmpB; however, the underlying molecular basis of this distinct organization remains unclear. In addition, such helical organization is not disrupted upon translation inhibition, suggesting that it is not related to *trans*-translation activity. These results indicate that the most likely biological relevance of such a helical organization is to protect tmRNA from RNase R-mediated degradation during G1, as they are localized “out of phase,” whereas in the S phase, proteolysis of SmpB would release tmRNA from its helical location away from RNase R, allowing it to be degraded in order to regulate the abundance and function of tmRNA in a cell-cycle-dependent manner.

Compared to mRNAs, studies of several sRNAs suggest that the distribution of these transcripts is less compartmentalized. With smFISH and super-resolution imaging, Fei et al. showed that SgrS, an sRNA involved in the glucose-phosphate stress response, is roughly homogeneously distributed in the cytoplasm when its copy number is high, whereas when its expression is lower, it appears to be specifically absent from the central nucleoid region (105). A more comprehensive study of the localization of several sRNAs, including GlmZ, OxyS, RyhB, and SgrS, found equal preference for localization within the cytoplasm and nucleoid region and no pref-

erential localization at the membrane or cell poles, as often observed for mRNAs by quantifying the signal overlap between RNA FISH and 4',6-diamidino-2-phenylindole (DAPI) staining of DNA (106). Interestingly, the ability of a particular transcript to freely diffuse into the nucleoid region was correlated with the length and the translation activity of the RNA, because shortening and reducing translation of *gfp* mRNA led to the same localization as sRNAs and reduced nucleoid exclusion. Therefore, in general, longer RNAs, including coding RNAs, have a lower propensity toward nucleoid localization compared to shorter, often noncoding transcripts. So far, the available studies on a handful of examples of sRNAs suggest an unbiased cellular localization (105, 106). It should be noted that investigation of sRNA localizations is predominantly focused on noncoding regulatory RNAs. Even though SgrS is a dual-functional sRNA, which also encodes the small protein SgrT (107) under the conditions of previous investigations, SgrT was not actively translated. Therefore, SgrS is still considered a regulatory RNA only under the investigated conditions, and demonstrates the same localization behavior as other tested regulatory sRNAs (108).

The unbiased localization of regulatory sRNAs investigated so far seems to be consistent with the fact that typically each sRNA species can regulate multiple mRNA targets (11, 109), which themselves might each adopt different cellular localizations. Therefore, the availability of sRNAs throughout the cell will ensure that all targets can be regulated. On the other hand, the localization of the target mRNA might kinetically affect regulation by the local availability of its sRNA regulator or other protein factors. Computational simulations have shown that a biased localization of an sRNA (such as membrane versus cytoplasmic localization) can lead to a distinct regulation of different target mRNAs, providing an opportunity for regulatory hierarchy among different targets (110). For example, a membrane-localized sRNA would regulate a membrane-localized target mRNA more efficiently compared to a cytoplasmic counterpart. However, the observation that sRNAs tested so far all exhibit an unbiased localization suggests that mRNA targets, regardless of their localization, would be equally sampled by the same sRNA species. Therefore, any regulatory hierarchy would be attributed to the other factors, such as localization of Hfq and RNase E, and the strength of base-pairing interactions, etc. As an example, the SgrS-mediated degradation of the mRNA encoding a fusion of *ptsG* to *crp* has been shown to be significantly reduced upon elimination of

the transmembrane encoding domains of PtsG (111). This observation is reminiscent of the observed faster endogenous turnover of membrane-localized mRNAs compared to mRNAs with other cellular localizations (70) and of the membrane localization of RNase E.

Hfq is an important chaperone for sRNAs in bacteria and also facilitates sRNA-mediated gene regulation via base-pairing (12, 112). Therefore, understanding the localization of Hfq can provide insight into spatio-temporal themes of sRNA-based regulation. However, results from various studies using different labeling and imaging approaches to study Hfq localization are conflicting, and therefore its localization in the cell remains controversial. Hfq has been observed to adopt a diffuse cytoplasmic localization outside of the nucleoid region by immunofluorescence staining (79), as well as preferential membrane localization by electron microscopy (113), which was recently recapitulated in an *in vitro* system using artificial vesicles (113, 114). A helical organization of Hfq along the longitudinal direction of the cell has also been observed by immunofluorescence staining (99, 113, 115). In contrast with all the fixed-cell experiments, single-molecule tracking of FP-tagged Hfq in a live cell reveals that Hfq is essentially freely diffusing inside the cell, with the diffusion rate slowed down when Hfq binds to the newly transcribed RNA attached to the nucleoid (116). Future experiments are needed to resolve this discrepancy in observation of Hfq localization. Despite the absence of Hfq significantly affecting the stability and abundance of sRNAs (105), it has minimal effect on the localization of tested sRNAs (106). As mentioned above, localization of the *ptsG* mRNA to the inner membrane has been reported to strongly contribute to its efficient repression by the sRNA SgrS, together with Hfq, during phosphosugar stress (111). So far, it remains unclear if Hfq can actively localize SgrS to the membrane to facilitate sRNA-*ptsG* mRNA interactions or if, in contrast, SgrS-*ptsG* mRNA complexes might instead lead to Hfq localization. Moreover, membrane localization of Hfq might also be due to interactions with the degradosome.

EMERGING STRATEGIES AND APPROACHES

Recently, new developments in chemical biology and molecular engineering have expanded the toolkit for imaging RNA localization and measuring their activities *in vivo*. These tools have been developed mostly for RNA imaging in eukaryotic systems, but some of them might have the great potential to be applied to bacterial systems as well.

First, various new developments in FISH methods have allowed for signal amplification and high-throughput imaging. For example, in the *in situ* hybridization chain reaction (HCR) (117, 118), two fluorescently labeled oligonucleotides that alone exist as metastable hairpin structures are designed (Fig. 3A). A third initiator probe is then introduced to bind to the target RNA of interest. This initiator probe has an overhang region that can open one of the two fluorescently labeled hairpins by branch migration, making the first hairpin-containing oligo capable of hybridizing to the second hairpin oligo. The two fluorescent oligos bind to each other in an alternating fashion in the hybridization chain reaction, thereby linking multiple fluorescently labeled probes to the RNA of interest and amplifying the signal. *In situ* PCR has also been used for FISH signal amplification (119–122). In this method, padlock probes (long oligonucleotides whose ends are complementary to the target RNA, where hybridization results in circularization of the probe) are hybridized to cDNAs generated from endogenous RNAs of interest *in situ*, which generates nicked single-stranded DNAs that are further ligated to be circular DNAs (Fig. 3B). Rolling circle amplification from these circular DNA templates by DNA polymerase can generate products carrying repetitive sequences for the hybridization of the fluorophore-labeled secondary probes. Such FISH strategies with signal amplification can be adapted to bacterial cells, and could be beneficial for imaging sRNAs, to which it is difficult to attach multiple conventional FISH probes due to their short length. To increase the throughput of RNA imaging, various multiplexed FISH imaging methods have also been developed in eukaryotic cells. Multiplexed imaging can be achieved by fluorescent barcoding, in which different combinations of fluorophore-labeled oligos hybridize to separate RNA species to generate pseudocolors (123), or repetitive hybridization and imaging cycles (124–127). However, such multiplexed imaging methods rely on the ability to spatially resolve individual transcripts. With diffraction-limited microscopy, only RNAs with very low abundance can be resolved individually in bacterial cells due to their small size, making the application of the multiplexed imaging method practically difficult in bacterial systems.

Recently, CRISPR (clustered regularly interspaced short palindromic repeat)-Cas systems have been engineered to label both DNA (128–130) and RNA (131, 132) in live cells. An early example of RNA labeling with CRISPR-Cas systems originated from the finding that

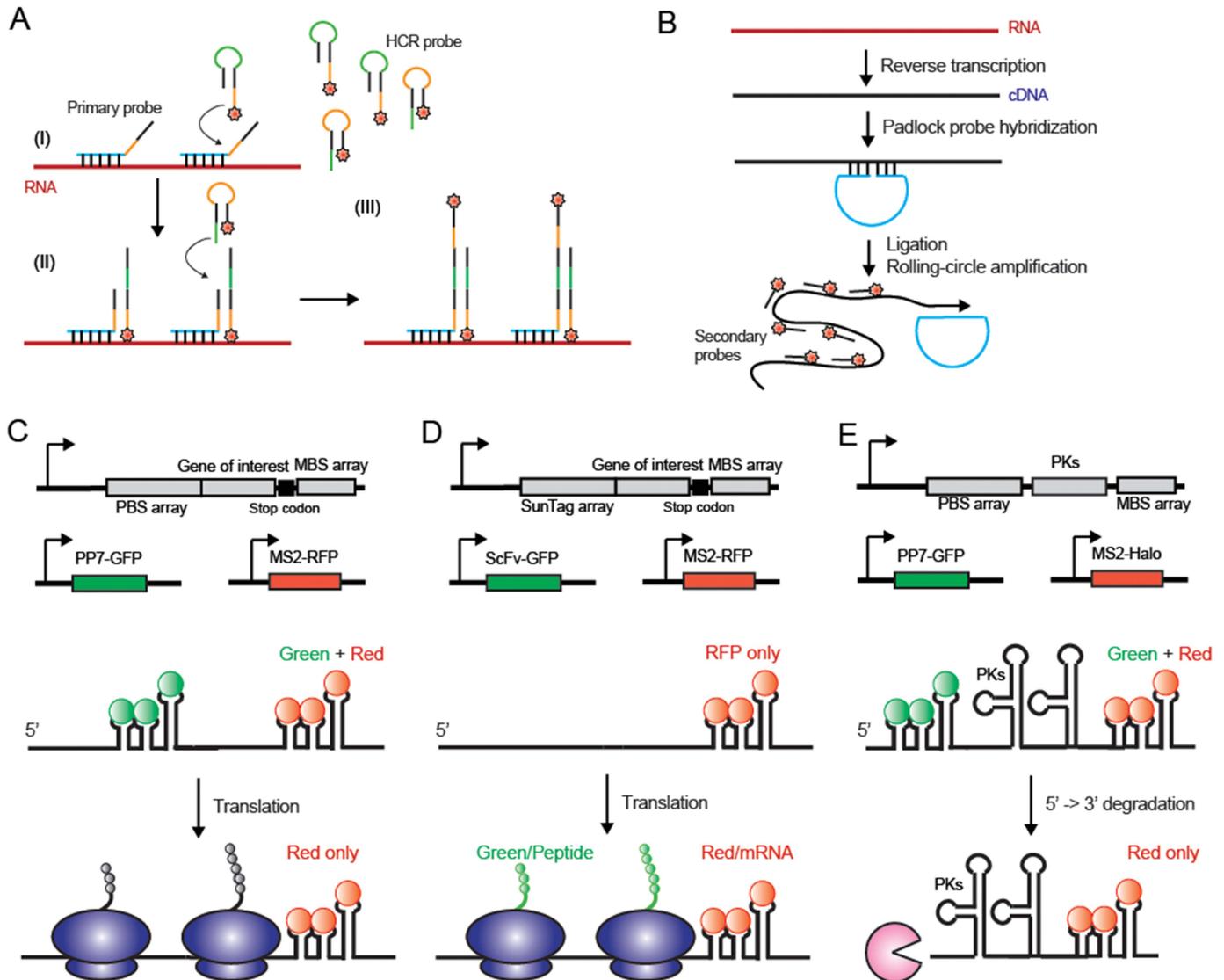


FIGURE 3 Emerging mRNA imaging methods in eukaryotic systems. (A) In the *in situ* HCR, binding of the primary probe initiates the alternating binding of two HCR probes, thereby amplifying the signal. (B) In the *in situ* PCR, a cDNA is first generated from the RNA of interest. Padlock probes are hybridized to the cDNA and ligated to be circular DNAs. Fluorophore-labeled secondary probes are then hybridized to the products generated from rolling circle amplification of these circular DNA templates. (C) Schematic representation of the TRICK reporter construct. (D) Schematic representation of the SunTag construct. (E) Schematic representation of the TREAT reporter construct.

Cas9, an RNA-guided DNase, can also target single-stranded RNA by providing the PAM (protospacer adjacent motif) as part of an oligonucleotide (PAMmer) (133). Thereby, a catalytically inactive Cas9 (dCas9), fused to an FP and in complex with the PAMmer and single guide RNA (sgRNA), can target and label the RNA of interest in a programmable fashion (131). Recently, Cas13a, an RNA-guided RNA-targeting CRISPR-

Cas effector, has been applied for RNA tracking in mammalian cells (132). Compared to the MS2/PP7 systems mentioned above, one advantage of the CRISPR-based imaging is that no additional tagging sequence must be fused to the RNA of interest, as the sgRNA can be flexibly programmed to target any RNA sequence of interest. Nevertheless, similar to other FP reporter systems, CRISPR-based labeling might affect mRNA pro-

cessing, degradation, or localization for a specific RNA of interest. Moreover, to distinguish target RNA-bound fusion proteins from those that are unbound, which could contribute to high level of background fluorescence, additional modifications are necessary. One such strategy could be to utilize multiple sgRNAs to tile along the RNA sequence of interest to enhance the signal on the RNA compared to background level, in a similar fashion as is employed in FISH labeling. Another strategy would be to reduce the unbound fraction of the fluorescent Cas proteins. For example, in a recent approach, a zinc-finger binding domain and the transcription repressor KRAB have been fused to Cas13a-FP, and the zinc-finger binding site was inserted into the promoter controlling the expression of the fusion protein (132, 134). In this way, a negative feedback loop is created, and the unbound Cas13a-FP therefore auto-represses its expression to control the background fluorescence.

Single-molecule RNA tracking has also been combined with additional reporters to correlate their translation or degradation activities in eukaryotic cells. A biosensor called TRICK (translating RNA imaging by coat protein knockoff) has been developed that reports the first round of translation (135). In this imaging system, cassettes of the PP7 binding site (PBS) and the MS2 binding sites (MBS) are integrated into the coding region and the 3' UTR, respectively (Fig. 3C). Newly synthesized mRNAs carry both GFP-fused PP7 proteins and red fluorescent protein (RFP)-fused MS2 proteins, generating colocalized green and red signals under the microscope. During the first round of translation after export of the mRNA to the cytoplasm, ribosomes displace the GFP-PP7 fusion from transcripts, leaving only RFP signals. The SunTag system allows for real-time tracking of multiple cycles of translation on individual mRNAs (136–140). In this technique, a tandem array of a sequence coding for a short peptide is inserted into the open reading frame of interest. Once translated, the resulting polypeptide, containing multiple such short peptide epitopes, is recognized and bound by a specific single-chain variable fragment (scFv) antibody fused with GFP coexpressed in the cell (Fig. 3D). In parallel, the mRNA of interest is also labeled by either the MS2 or PP7 system with a different FP (e.g., RFP) at the 3' UTR. Therefore, actively translating mRNAs generate two fluorescence signals, whereas untranslated mRNAs only generate an RFP signal. In addition to reporters of translation, a biosensor called TREAT (3'-RNA end accumulation during turnover) has been developed to track mRNA turnover in eukaryotes (126,

141). In this approach, the 3' UTR is engineered to include two viral pseudoknots (PKs) flanked by PP7 and MS2 binding sites (Fig. 3E). Since the PK structures block 5'-to-3' degradation of the transcript by the 5'-3' exoribonuclease Xrn1 (142), TREAT allows one to distinguish between intact and partially degraded mRNAs. The feasibility of applying such functional reporters to bacterial systems remains to be tested. For example, FP reporters in these systems are generally tagged with a nuclear localization signal sequence. This sequence can effectively reduce the amount of unbound FPs in the cytoplasm and thereby reduce the imaging background. Such a segregation of unbound fluorescent reporter from the mRNA-bound signal cannot be applied in bacteria because they lack a separation of nucleus versus cytoplasm. In addition, given that there are fundamental differences in mRNA metabolism between prokaryotes and eukaryotes, such as transcription-coupled translation in bacteria versus spatially separated transcription and translation in eukaryotes, and the much shorter lifetimes of mRNA in bacteria compared to the ones of eukaryotic mRNAs, these functional reporters may need to be significantly reengineered for their applications in bacterial cells.

In addition to imaging of mRNAs, a new method, FASTmiR, has been developed for the imaging of eukaryotic microRNAs (miRNAs) in live cells (143). FASTmiR was designed based on the Spinach system, in which a sensory domain that can base-pair with the miRNA of interest is fused to a modified Spinach aptamer. Binding of a miRNA to the FASTmiR helps the folding of the Spinach aptamer and forming the DHFBI binding pocket, thereby generating a fluorescence signal. The concept of FASTmiR may be further developed into a platform for imaging bacterial sRNAs. However, given that sRNAs are in general longer than miRNAs, and most contain secondary structures, the structural design of FASTmiR might need to be significantly modified to allow efficient sRNA detection in bacteria.

Finally, in addition to optical approaches that enhance the spatial resolution, expansion microscopy utilizes swellable polymer networks to physically expand the specimen. Such physical magnification leads to an enhanced effective spatial resolution (126, 141). In addition to immunofluorescence, expansion microscopy has recently been combined with FISH imaging of RNA (144), and iterative expansion microscopy further enhances resolution from ~70 nm to ~25 nm (145). Expansion microscopy has already been applied to bacteria (146), and should allow for studies of biomolecular location with finer resolution.

OPEN QUESTIONS AND FUTURE CHALLENGES

Despite the emerging evidence of subcellular RNA localization in bacteria, we are still only at the beginning of understanding the underlying mechanisms and potential functions of this process. One of the major questions is what are the deterministic factors or the driving forces for mRNA localization in bacteria. So far, several *cis*- and *trans*-acting factors have been suggested to affect transcript localization. For example, many mRNAs encoding inner membrane proteins are preferentially localized at the membrane, and this localization can be mediated in a translation-dependent manner during synthesis and secretion of N-terminal signal peptides. However, for certain mRNAs encoding inner membrane proteins or secreted effectors, it has been reported that signals within the mRNA itself are instead required for localization/secretion or that the mRNAs have multiple localization signals, including RNA sequence-based signals and those encoded in the N-terminal peptide amino acid sequence (10, 72, 74, 147). It remains to be seen how many bacterial mRNAs have a eukaryotic-like RNA zip code, and what the sequence and/or structural features of these elements are. Likewise, the features embedded in N-terminal signal sequences of the encoded proteins that might mediate localization of their cognate mRNA are unknown. While eukaryotic mRNAs can carry their zip codes at either the 3' UTR, 5' UTR, or coding region (1), the few examples of RNA elements directing localization of bacterial mRNAs have been reported to be located at 5' ends of mRNAs or within open reading frames (10). It has been suggested that an enrichment for uracils in mRNAs encoding integral membrane proteins might be a physiologically relevant signature of this group of mRNAs (148). Furthermore, it will also be interesting to see which (protein) factors bind to bacterial RNA zip codes and how they transport RNAs in the cell. In eukaryotes, many mRNAs are actively transported by RNA-motor complexes along polarized cytoskeleton structures and localize at local anchor signals such as the dynein-1 motor (3). Similar mechanisms involving binding to cytoskeleton proteins, or even cell division factors, might apply in bacteria. In addition, localized protection from RNases, as observed in eukaryotes, might mediate subcellular localization of bacterial transcripts.

It also remains unclear what the functions and physiological roles of localized RNAs and/or localized translation are in the cell, and how perturbation of such localization affects phenotypes related to the encoded protein. The organization and coexpression of function-

ally related genes in operons in bacteria might be in line with the observation that RNAs stay close to the site of transcription and are also translated close to the nucleoid so that protein complexes can be assembled. On the other hand, localized translation, e.g., at the membrane, might increase the efficiency of assembly of larger protein complexes at the future site of action, especially for those that might be hydrophobic. Such a localized translation at the membrane especially would make sense for secreted factors such as type III secretion system effectors or flagellins that might be translationally repressed until the secretion machinery is completed, and their translation might only be activated upon completion of the secretion machinery. Nevertheless, it is also possible that the membrane localization of mRNAs is just a by-product of secretion of the nascent N-terminal peptide of the translated protein. Moreover, it is still unclear whether the localization of certain mRNAs is regulated during changing growth or stress conditions or whether this is connected to cell division. In eukaryotes, alternative splicing and polyadenylation can control incorporation of localization signals into mature transcripts, and after nuclear export, diverse RBPs, adaptors, and cytoskeleton motors are recruited to localizing mRNAs (1, 2). It has been observed that posttranscriptional regulators, such as the competence regulator ComN in *B. subtilis* or the CsrA-FliW regulatory network of *C. jejuni*, can impact RNA localization in bacteria (66, 71). Although this can be an indirect effect via regulation of translation, these first examples show that posttranscriptional regulatory networks can impact RNA localization.

Compared to mRNA localization, even less is known about sRNA localization in bacteria. Several sRNAs also encode small proteins (for a review, see reference 149). It still remains to be studied whether these small-protein-encoding sRNAs might show a more defined cellular localization compared to solely noncoding RNAs. And if yes, it remains to be seen whether the final cellular locations of the encoded small-protein products affect the localization of their encoding dual-function sRNAs. Localization of sRNAs and their cognate mRNAs to certain sites in the cell might impact the efficiency and hierarchy of target gene regulation and in turn affect the functionality/outcome of genetic circuits. Therefore, considerations about subcellular localization might also be relevant for the design of synthetic gene regulatory circuits, since differential localization of the encoded protein and/or mRNA (membrane versus cytoplasmic) might impact efficiency of regulation if not all components are expressed in close vicinity.

The ongoing technological developments listed above, together with additional new approaches that emerge, will help in the further study of RNA localization in bacteria. So far, it is more challenging to apply existing mRNA imaging methods to sRNAs. For example, smFISH has a significantly compromised signal-to-noise ratio for sRNA imaging because of the much shorter length of sRNAs compared to mRNAs and resulting lower number of probes that can be applied. Similarly, live-cell imaging of sRNAs remains challenging because tagging with FPs or RNA aptamers could impact their regulatory properties and/or localization. Beyond simple detection of transcripts, ideally one would also simultaneously image RNA and translation/protein localization *in vivo* to decipher whether localization and localized translation impact translation, protein abundance, and physiology. Considering the sometimes controversial observations that have been reported so far regarding bacterial RNA localization, it is also recommended that findings are validated using different approaches, and that the experimental designs maintain transcript expression and characteristics as close to native as possible. Certainly, the continued technological advancements for studying RNA localization in these small organisms will reveal the previously unappreciated extent of bacterial compartmentalization and its contribution to physiology.

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