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RNA imaging in live cells

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Abstract

Traditional molecular biology tools have elucidated the identities and functions of RNA molecules, which are essential to the understanding of gene transcription and protein translation. Deepening this research field would further require the direct visualization of RNA dynamics such as the DNA-RNA interactions and RNA-protein interactions. Towards this goal, the rise of RNA imaging tools over the past 15 years has reformed how we looked at these processes. In this emerging topic, we first highlighted recent advances on three main RNA imaging tools based on the species of interacting molecules: RNA-RNA pairing, RNA-protein binding, and small molecule-RNA complex. We introduced the advantages of these tools from a technical viewpoint, including binding affinity, fluorescent turn-on ratio, stability, and impacts on targeted RNA. Next, we discussed new rising opportunities and future directions, echoing the state-of-the-art imaging tools in the fields of fluorescent proteins and small fluorescent molecules. Together, we believe this emerging field will bring new insights on how we study RNA biology in living systems.

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RNA imaging in live cells

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Keywords

fluorescent imaging| RNA imaging | Fluorogenic RNA

Abstract

Figure 1. RNA imaging in live cells with three main approaches: 1. hybridization between targeted sequence and fluorescen

Background:

RNA plays a central role in regulating the gene expression and protein translation. Among all different kinds of biomolecules, RNA is perhaps the most difficult one to be understood. It serves as the bridge between DNA, which stores our genetic information, and the proteins, which perform the functionalities. Besides the well-known mRNA, tRNA, and rRNA, other non-coding RNA such as miRNA and circRNA are retaining more and more attentions in recent years, due to its uniqueness in regulating translation processes. However, comparing to DNA and proteins, the developments of RNA imaging has lagged behind for almost a decade, due to several reasons: RNA has a smaller copy number and a lower stability, and its structures are highly dynamic in living systems. These make it a challenged task to be directly visualized in live cells.

Owning to the advances in the fluorescent imaging of DNA and proteins in live cells, varies of labeling and

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imaging tools have been developed for RNA in the past decade (Fig. 1). To visualize RNA in live cells, specificity is the major issue that needs to be addressed. Fluorescence in situ hybridization (FISH) technique, initially developed for labeling DNA on chromosomes, was directly adapted for RNA imaging; Motivated by the interactions between a specific RNA sequence and RNA-binding protein, RNA can be labeled via fluorescent proteins through expressing the RNA-binding protein together with fluorescent proteins in a tandem form; Inspired by the structures of Green Fluorescent Proteins (GFP), several RNA aptamer-small fluorescent molecules systems were recently developed for RNA labeling, in which the RNA aptamer serves as theβ -barrel, and the small fluorescent molecules serve as the core. In this context, we highlighted the recent advances during the developments of RNA imaging techniques in the past two decades, with a focus on the technical aspect for the three main approaches.

Recent Advances

I. FISH (fluorescent in situ hybridization)

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The Watson & Crick base pairing rule offers a natural way to assure specificity in labeling. Guided by this rule, DNA-FISH was initially developed to image DNA in fixed cells. To image mRNA with a length of several kilo nucleotides in cells, multiple synthetic RNA probes with fluorophores are usually required because of the limited length for synthetic RNA (<100 nucleotides) and the needs to exclude non-specific interactions with such short strands. As one of the few initial steps towards RNA-FISH, Tyagi et al. reported the imaging of individual mRNA molecules via multiple singly labeled RNA probes[1]. They demonstrated simultaneously imaging of three different mRNAs in mammalian cells via 48 fluorescent RNA probes, while each mRNA species corresponds to one specific fluorophore (Fig 2a). However, due to the large number of probes, this strategy cannot target transcripts shorter than about 800 nucleotides. Nevertheless, RNA-FISH technique still represents the first effort towards the imaging of RNA in fixed cells.

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The 1st generation RNA-FISH technique usually requires a high cost of synthetic fluorescent RNA probes. Moreover, their low stability and signal/noise (S/N) ratio also make it hard to be implemented into living systems. To address these issues, a variety of modified RNA-FISH techniques were reported. Among them, CRISPR Live FISH is the first FISH-based imaging technique for visualizing both DNA and RNA in live cells[2]. The compatibility with live cell imaging arises from the fact that the fluorescent gRNA (the guided RNA) cannot be degraded once dCas13d binds with the targeted RNA sequences. The working principle of Cas proteins family ensures a high specificity between RNA probes and targeted RNA sequences, thus eliminating the needs for multiple RNA probes. Through this technique, Qi et al. performed the simultaneous imaging of DNA and RNA, and visualize the transcript process in live cells (Fig 2b). Nowadays the Cas protein family is still commonly used to enhance the S/N ratio in nucleic acids imaging, due to their unique ability to assemble paired gRNA and targeted DNA/RNA sequence.

Figure 2. RNA-FISH imaging. (a) Imaging of three mRNAs (FAM105A, PTGS2 and FKBP5) in A549 cells via 48 fluorescent RNA probes. Adapted from Ref. [1]. Scale bars: 5 μm. (b) Tracking the real-time dynamics of RNA transcription in live cells via Dual CRISPR DNA/RNA LiveFISH. Adapted from Ref. [2]. Scale bars:10 μm.

II. RNA-binding proteins

RNA-protein interactions exist in many vital processes such as DNA transcription, RNA processing, and RNA translation. The vast pool of fluorescent proteins inspires scientists to question whether they can be

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tagged onto targeted RNA sequences via RNA-protein interactions. Such conception, if can be achieved, will greatly facilitate the imaging of RNA in live cells. The first system, bacteriophage MS2 was pioneered by the Singer Lab in the 1990s, and now still remains to be the gold standard for RNA imaging in live cells. MS2 coat proteins (MCP) are 129-amino acid proteins that specifically bind with MS2 RNA sequence. In the original design, Singer et al. attached six MS2 RNA sequence with the targeted mRNA. Meanwhile, GFP was fused with MCP so that one targeted mRNA can recruit multiple GFPs to enhance the S/N ratio. As an example of utilizing the MS2 system, Singer et al. reported single-molecule imaging of nascent peptides (SINAPS) to assess translation in live cells (Fig. 3a). The MCP and Sun-tag were fused together with the targeted RNA sequence so the mRNA and the synthesized protein can be visualized simultaneously [3].

Although RNA-binding proteins systems such as MS2 bridge the gap between fluorescent proteins and RNA imaging, they still suffer from high background in live cell RNA imaging, as all fluorescent proteins are constitutively fluorescent. In 2019, Jaffrey lab, who pioneered the developments of fluorogenic RNA aptamers, flipped this question and devised a fluorescent "turn-on" system for imaging RNA[4]. They designed a bifunctional peptide "tDeg", which contains a degron sequence and a Tat peptide. When fluorescent proteins were tagged with "tDeg", they will be rapidly degraded in live cells because of the degron sequence (Fig. 3b). However, when Tat peptide bind with an RNA hairpin TAR (*trans* -activation response element) variant-2, named Pepper RNA, it will shield the degron and thus prevent the protein degradation, giving a "turn-on" fluorescence. Such mechanism accounts for its low background fluorescence as all unbound fluorescent proteins are degraded.

Figure 3. RNA imaging via RNA-proteins interactions. (a) Single-molecule imaging of nascent peptides (SINAPS) technique enables the simultaneously visualization of proteins and mRNA during the translation processes. The proteins are tagged with GFP while the mRNA is tagged with MCP-RFP. Adapted from Ref. [3]. Scale bar: 5 μm. (b) The Pepper RNA-regulated protein destabilization domain system (tDeg) with a "turn-on" strategy for imaging RNA. Fluorescent proteins will be degraded unless Pepper RNA binds with tDeg, thus preventing its degradation. Peptide tDeg confers Pepper RNA-dependent regulation on mNeonGreen, mCherry, and the luciferase NanoLuc in HEK293T cells. Adapted from Ref. [4]. Scale bar: 40 μm.

III. RNA aptamer and small molecules

The charming bioluminescent jellyfish inspires the discovery of Green Fluorescent Proteins (GFPs). Nowadays although thousands of fluorescent proteins have been developed, naturally-existing RNAs are all not fluorescent. Despite of this, chemical biologist created artificial "fluorescent RNA" by integrating RNA aptamer and synthesized fluorescent molecules, which resemble the β -barrel and fluorophore of GFP respectively. The new approach, named fluorogenic RNA aptamer, usually refers to short RNA sequences with a few tens of nucleotides that could specifically bind with small fluorophore. The strategy offers an exceptional to label RNA in the absence of proteins, thus giving minimal impacts on the biological functions of targeted RNA.

The first fluorescent RNA aptamer that has been widely recognized was reported by Jaffrey lab in 2011, named Spinach[5]. The fluorescent core DMHBI (3,5-dimethoxyl-4-hydroxybenzyl imidazole porphyrone) has a similar chemical structure with the core of GFP (Fig. 4a). Through systematic evolution of ligands by exponential enrichment (SELEX), they selected RNA aptamer that specifically binds with DMHBI, leading to a ˜2000-fold enhancement in fluorescence intensity *in vitro* . However, Spinach suffers from low fluorescence in live cells. To address this issue, Spinach2 was developed with nearly identical photophysical properties but enhanced stability and better folding in live cells. By imaging the CGG repeating RNAs in live cell, they found Spinach2 retains 80% of its fluorescence.

The screening of a brand-new RNA aptamer for a fluorescent core usually requires a large amount of induced mutation experiments. To expedite the screening process, Jaffrey lab developed a platform, named SELEX-FACS by combining the SELEX and fluorescence-activated cell sorting (FACS) techniques[9]. Aptamers obtained through this approach are compatible with cellular expression and intracellular imaging, enabling

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rapid identification of folded aptamers highly optimized in the cellular context. The shorter aptamer sequence Broccoli, developed based on this platform, exhibits much stronger fluorescent intensity in cells. To extend the spectral range of the fluorophore, Jaffrey lab further developed the Corn-DFHO ((5-fluorinated 3,5-difluorine 4-hydroxybenzyl imidazolinone-2-oxime)) complex (Fig. 4b), where DFHO is on the basis of the naturally occurring fluorophore in RFP[6]. Comparing to Spinach and Broccoli, Corn has to be used in a quasi-symmetric homodimer form, to improve its stability and binding affinity with DFHO. Through this strategy the authors visualized the Pol III transcription in live cells[6].

In addition to the above small molecules designed based on the structures of fluorophore in fluorescent proteins, fluorogenic RNA aptamers were also developed base on nucleic acid fluorescent dyes. Thiazole orange (TO) is one of the most widely used non-specific nucleic acid fluorescent dyes. Unrau lab found that the acetylation of TO lead to the attenuations of non-specific interactions with RNA, but still retains high binding affinity with RNA Mango. Comparing to Spinach, Mango has a better thermal stability and a two-fold fluorescence efficiency. With a newer version, the Mango II, designed for intracellular environment, Unrau and collaborators demonstrated its feasibility in imaging of single RNA molecules in live mammalian cells[7].

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Although the Spinach and Mango families have been shown to be powerful for imaging mRNAs in mammalian cells, they still suffer from several disadvantages including instability in live cell, low brightness, and limited availability of dye/aptamer pair with a wide spectral range. A potential reason might be the presence of G-quadruplexes in those aptamer structures. The G-quadruplex has been found to be crucial for fluorophore rigidification in the aptamers *in vitro* , but will likely be degraded in mammalian cells. Furthermore, most of the Spinach and Mango families are multimeric structures, which may impede their application in imaging low abundance mRNAs in live cells. The developments of novel fluorogenic RNA may requires a re-design of fluorophore with tunable structures, higher brightness, and better binding affinity with a monomeric and stable RNA sequence.

Figure 4. RNA imaging in live cells via fluorogenic RNA aptamers. (a) The structure of HBI (green) in the context of GFP and GFP-like fluorophore DMHBI for RNA Spinach. Imaging of $5S$ RNA in live cells via DMHBI derivatives. Adapted from Ref.[5]. Scale bar: 10 μm. (b) The structure of Corn-DFHO, and imaging of RNA (green) and DNA (blue) in live cells. Adapted from Ref. [6]. Scale bar: 10 μm. (c) Two color channel fluorophores for RNA Mango and imaging of RNA via TO1 in live cells. Adapted from Ref. [7]. Scale bar: 10 μm. (d)The structure of HBC and RNA pepper, lighting up different HBC-like fluorophores with a wide range of emission spectrum. Adapted from Ref. [8].

In 2019, Yang lab and collaborators synthesized a series of GFP fluorophore-like dye with a more rigid electron acceptor and a stronger electron donor, named HBC, with a wide spectral range covering from cyan to red channel[8](Fig. 4d). The corresponding RNA sequence, Pepper (not to be confused with the aforementioned Pepper system based on fluorogenic proteins) induced more than 3000 times enhancement in fluorescent intensity with good photostability when binding with HBC. With only 43 nucleotides, Pepper has an extremely high binding affinity with HBC (Kd [?]3.5 nM), but minimal perturbation on the functionalities of targeted RNA sequence. Especially when comparing to red channel fluorogenic RNAs such as Broccoli and Corn, Pepper is truly monomeric, thermally stable, and highly fluorescent in live cell; thus, enables the two-color visualizations of RNA translation for live-cell RNA imaging. More importantly, Yang et al. demonstrated the feasibility of Pepper620 in structured illumination microscopy (SIM) imaging. This represents the first step towards super-resolution imaging of RNA in live cells with fluorogenic RNA aptamers.

Potential and Challenges

Over the past decade the toolbox of RNA imaging has expanded enormously, prompting massive scientific

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findings in RNA research from a new perspective. As fluorescent proteins have become a routine tool for life science research, we envision that the fluorogenic RNAs will be widely adopted by the community as well. Nevertheless, several challenges still exist on the technical aspect, and overcoming them may require cooperation from chemist, biologist, and even optical engineer.

Trade-off between stability and impact on biological functions of RNA . An ideal fluorescent label should be as small as possible with strong fluorescence and good stability. Unlike DNA, which normally exist in double-stranded form, RNA is single-stranded, and the formation of RNA aptamers relies on intra-molecular hydrogen bonding. Hence, labeling RNA with a good stability usually require multi-valent interaction, such as the MS2 and dimerization of fluorogenic RNA aptamer system, though there is always some concern on whether these bulk labelling would impact the biological functions of targeted mRNA. In this case, shorter fluorogenic RNA aptamer sequences (˜40 nt) is believed to be a better choice, despite of its weaker stability. It is yet another level of challenge to label small non-coding RNAs such as miRNA and circRNA because they are usually tens of nucleotides long. Recently the developments of synthetic fluorescent nucleobases emerged as a powerful tool to report the location of DNA molecules, and it is further tempting to speculate whether these non-canonical nucleobases could tagged with RNA sequence in living systems via epigenetic tools of RNA modifications. Potentially, only one single base is needed to give fluorescent signal without forming secondary structures. Such approach will bring the imaging of nucleic acids onto a new exciting level.

Photoswitchable fluorophore for super-resolution imaging . The discovery of GFP lightened the field of molecular biology by allowing the direct visualization of living systems. However, the spatial resolution of optical imaging is restrained by the diffraction limit of the light, about half of its wavelength (˜300 nm for visible light). Super-resolution imaging techniques, together with the engineering of photo-switchable fluorescent proteins and dyes, broke this diffraction limit and pushed the resolution down to about ten nanometers; thus, give new insights into the heterogeneity of intracellular environments at the nanoscale. Although Pepper620 has been demonstrated to be successful in SIM technique with ˜100 nm spatial resolution. It is still highly demanding to design new photo-switchable fluorogenic RNA aptamers that are compatible with single-molecule localization microscopy. That can not only push the spatial resolution down to ten nanometers level, but also visualize RNA dynamics from a single-molecule perspective. The RhoBAST (Rhodamine Binding Aptamer for Super-resolution Imaging Techniques) system reported by Jäschke and collaborators marks a milestone towards the single-molecule localization imaging of RNA in living systems[10]. RhoBAST is based on a linked fluorophore-quencher system, and the fast association and dissociation kinetics generate the intermittent fluorescence, meaning that the fluorescence can only be turned on when bound to the RNA aptamers. Nonetheless, there are plenty of room left to improve the fluorescence turn-on ratio and the binding affinity of these photo-switchable RNA aptamers. The integration of photo-switchable dyes and fluorogenic RNA aptamers may unlock new possibilities of highly stable and photo-switchable fluorogenic RNA aptamer for a precise localization of RNA in living systems at the single molecule level.

Conclusions

In sum, in this emerging topic we discussed several important RNA imaging tools in living systems, and compare their advantages on a technical aspect. These imaging tools have greatly empowered biologist to understand the role of RNA during transcription and translation. We look forward to the future development and application of this charming field, and we believe these tools will soon be widely adopted in labs of molecular biology all over the world, just like what GFP did decades ago.

Acknowledgements

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RNA imaging in live cells with three main approaches: 1. hybridization between targeted sequence and fluorescent RNA pr