Chapter 6
Optical Monitoring of Single Molecule Dynamics of RNA in Living Cells

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Abstract Live-cell optical imaging is a powerful approach to monitor the intracellular localization and dynamics of the molecules of interest for elucidating the mechanism of physiological function. Single-molecule imaging is a unique technique that provides information on the dynamics and association/dissociation of each molecule inside living cells. Despite having monitored a variety of biomolecules in living cells, live-cell imaging of RNA, which is an important component of living systems, has not been performed intensively. This can be attributed to the lack of techniques available for the optical labeling of RNA inside living cells. This review introduces labeling methods and microscopic techniques for monitoring RNA in living cells, focuses on an example of single-molecule imaging of a long non-coding RNA TERRA (telomeric repeat-containing RNA) using mPUM-technology, an RNA labeling method, along with the investigation of a single-molecule RNA imaging system in living cells.

Abbreviations

EGFP	Enhanced green fluorescent protein
FRET	Förster resonance energy transfer
PAMmer	PAM-presenting DNA oligonucleotide (PUM: protospacer adjacent motif)
PUM-HD	Pumilio homology domain. TERRA: telomeric repeat-containing RNA
TIRF	Total internal reflection fluorescence

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6.1   Introduction

Living cells consist of a myriad of biomolecule such as proteins, nucleic acids, lipids, and sugars. As a simple mixture of all the molecules involved in the living cells would not reconstitute such a cell, appropriate assembly, localization, and dynamics of these molecules are required for the generation and maintenance of the cellular lives. To understand the mechanism of living cells with respect to molecules, optical imaging is the most direct approach for observation such molecules in living cells. Optical microscopy, especially fluorescence microscopy, has been frequently used to visualize molecules in living samples owing to its non-invasiveness. Another advantage of fluorescence microscopy, apart from non-invasiveness, is selectivity to monitor fluorescence-tagged target molecules. A variety of fluorescent probes have been developed in recent decades for labeling and monitoring target molecules in living cells [1, 2]. Certain dyes that emit fluorescence upon binding to double-stranded nucleic acids are used to visualize DNA in living cells. Ions such as Ca^{2+} are visualized by probes obtained via organic synthesis that generate fluorescence during chelation of target ions [3] and protein-based probes that result in conformational change upon binding to Ca^{2+} and release FRET (Förster resonance energy transfer) signal [4]. Proteins in living cells can be conjugated with fluorescent proteins using genetic engineering techniques.

RNA is also an important element in living cells along with DNA, ions, and proteins. As described by the biological central dogma, RNA that functions as messenger RNA (mRNA) is produced via the transcription of genes in chromosomal DNA and transports genetic information for protein production. Previously mRNA was characterized only as a messenger of the genetic information, and attention was not paid to the intracellular localization and dynamics of mRNA. However, certain examples have been reported, in which the intracellular localization of mRNA plays important roles in physiological events [5, 6]. For instance, β-actin mRNA concentrates in leading edges involving lamellipodia, and filopodia of cells that are expanding and migrating upon stimulation with external growth and/or chemotactic signals [7–9]. This localization of β-actin mRNA supports rapid pseudopodia formation and cell expansion for quick response to external signals. Apart from mRNA, non-coding RNAs have also been implicated in various physiological processes. Among various species of non-coding RNAs, long non-coding RNAs (lncRNAs) exhibit crucial roles in cellular functions, such as gene regulation and genome stability [10].

Despite the importance of intracellular RNA localization and dynamics, techniques for visualizing RNAs in living cells are still developing, and hence the investigation of RNA based on its intracellular dynamics has been delayed. Under such circumstances, certain technologies have been developed recently for labeling and monitoring the RNA of interest. In this chapter, optical techniques that contribute to the visualization of RNA dynamics in living cells, such as microscopic methods and optical probe technologies, are introduced. Examples of single-molecule imaging of

RNA in living cells based on one of the technologies, named mPUM technology, are explained.

6.2   RNA Labeling Methods for Live-Cell Optical Imaging

Several techniques for RNA labeling in living cells have been developed to monitor RNA localization, dynamics, and functions (Fig. 6.1) [11, 12]. The most popular method for RNA visualization in cell samples under an optical microscope is the fluorescence *in situ* hybridization method, in which the target RNA is hybridized with a fluorescence-labeled oligonucleotide probe. However, this method requires washing of excess probes and is limited for use in chemically fixed and permeabilized cell samples. To overcome these limitations, a technique was developed using an oligonucleotide-based tool known as molecular beacon [13–15]. The molecular beacon has a stem-loop hairpin structure and its 5′ and 3′ ends are conjugated with a fluorescent dye and a quencher, respectively. The fluorescent dye is located close to the quencher. Hence, the fluorescence is quenched in the absence of target RNA. In the presence of the target RNA, the stem-loop hairpin region dissociates and attaches to the target RNA sequence, leading to the separation of the fluorescence dye from the quencher and subsequent recovery of its fluorescence. Fluorogenic RNA aptamer is another nucleotide-based RNA probe that has recently been developed as a tool for RNA detection in living cells. The aptamer is an RNA molecule that specifically captures a nonfluorescent dye and converts it into the fluorescent form. As a typical example, an RNA aptamer "Spinach" captures an enhanced green fluorescent protein (EGFP) fluorophore-resembling dye 24-2-DFHBI and emits fluorescence [16–18].

In addition to oligonucleotide-based RNA probes, certain protein-based RNA probes have also been developed [19, 20]. The MS2 system is the most widely used
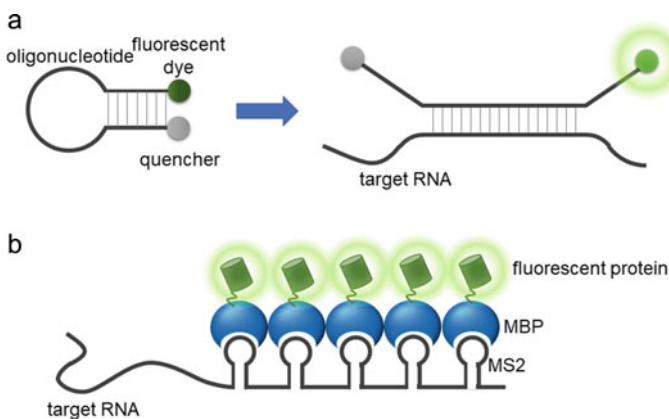

Fig. 6.1   Schematics of **a** molecular beacon and **b** MS2 system

method for labeling the RNA of interest using a protein-based probe. In the MS2 system, a repeat of a bacteriophage-derived RNA tag called MS2 is fused to the 3′ terminus of the target RNA. The MS2 region is specifically recognized and captured by MS2-binding proteins (MBP). In the MS2 system employed for monitoring the target RNA, MBP is fused to a fluorescent protein (FP) and introduced into the sample cells. In addition, the target RNA fused with an MS2 repeat sequence is expressed in the cells. The MBP-FP accumulates in the MS2 repeat region, and can be visualized as a bright fluorescent spot under a fluorescence microscope. This system originally existed only in bacteriophages. Hence, it has been used to label the RNA of interest in mammalian cells without off-target interactions with other RNAs. Another potential candidate for providing a platform for protein-based RNA probes is the Cas protein. Clustered regularly interspaced short palindromic repeats (CRISPR)/Cas systems have been typically used to target double-stranded DNA; however, several modifications have been obtained to alter the systems for targeting RNAs. Nelles et al. developed a fusion protein with GFP and dCas9 that incorporated a single guide RNA and PAMmer (PAM-presenting DNA oligonucleotide) that hybridizes to a target RNA [21]. The localization of β-actin mRNA in fixed cells was visualized using this method.

6.3 Design of the PUM-HD-Based RNA Probes

PUM-HD is an RNA-binding protein domain derived from human PUMILIO1 that binds to an 8-base RNA sequence of 5′-UGUAU(orC)AUA-3′ (Fig. 6.2) [22, 23]. Examinations performed with crystallography demonstrated that PUM-HD has eight repeated motifs, each capturing an RNA base via the interactions of hydrogen bonds
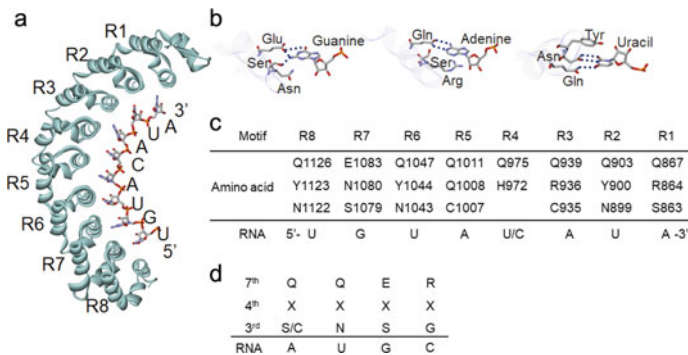

Fig. 6.2 RNA recognition by PUM-HD [27]. **a** The structure of a PUM-HD and a recognized 8-base RNA. **b** RNA recognition by three amino acids in a repeat motif of PUM-HD. **c** The three amino acid combination in each repeat motif of wild type PUM-HD and the recognized RNA base. **d** Relationship between recognized RNA bases and the combination of three amino acids in the repeat motif

and van der Waals forces [24, 25]. Based on crystallographic studies, three amino acids at the third, fourth, and seventh positions in one helix of the motif were identified to be involved in these interactions with the RNA base, and hence in determining the specificity of the RNA base. Therefore, site-directed mutageneses of these three amino acids, especially on the third and seventh positions, alters the RNA base for recognition by the motif, and custom-designed PUM-HD mutants that recognize a specific 8-base RNA sequence can be prepared [24, 26–28]. Although no motifs recognize a cytosine base in the wild-type PUM-HD, several investigations with random mutations on the three amino acids have discovered a motif that recognizes a cytosine base [29]. The dissociation constant between a PUM-HD mutant (mPUM) and the eight-base sequence RNA identified is typically in the order of nM when the RNA initiates 5′-UGU-3′ at the 5′ terminus, and ~ 100 nM when the 5′ terminus is not 5′-UGU-3′ [24].

As a single mPUM can be considered to recognize an 8-base RNA sequence, the specificity of the target RNA sequence is 4^8, which might not fulfill required specificity for labeling to a target RNA in mammalian cells by using only a single mPUM species. One of the approaches employed in solving the potential off-target problem of mPUM is the use of a fluorescent protein reconstitution method [8, 9, 30]. In this method, the two split fragments of a fluorescent protein are fused to a pair of proteins of interest. When the pair of proteins approach each other, the fluorescent protein-fragments are brought into proximity, leading to the reconstitution of the full-length fluorescent protein and restoration of its fluorescence. Using this method, mPUM-based probes can be designed to recognize target RNA specifically. Two mPUM molecules that recognize different but adjacent regions in a target RNA are prepared and subsequently conjugated with N- and C-terminal fluorescent protein fragments. After both mPUMs bind to the target regions in the RNA of interest, the fragments of fluorescent protein come in proximity and reconstitute to form the fluorescent protein. In this design, the probe does not emit fluorescence without binding to the target RNA. Moreover, a 16-base sequence in the target RNA is required to be recognized by the probe system for emitting fluorescence signals, leading to sufficient selectivity for observing a specific RNA molecule in mammalian cells.

6.4   Microscope Setup for Single-Molecule Imaging of RNA in Living Cells

Fluorescent probes based on the reconstitution of split fluorescent protein are applied not only for conventional and confocal fluorescence microscope imaging but also for single-molecule fluorescence imaging to monitor the dynamics of the target molecules in real-time. The PUM-HD-based RNA probe also has the potential to label the RNA of interest for monitoring its single-molecule dynamics [8, 9, 31]. However, the RNA is typically localize in the cytosol and/or the nucleus, whereas the targets for single-molecule imaging comprise primarily the membrane molecules as

the total internal reflection illumination typically used for single-molecule imaging can irradiate the regions located in the vicinity of the plasma membrane. Hence, special techniques are required for the illumination of cells with a low background fluorescence signal in single RNA molecule imaging. Our microscopy setup for single-molecule imaging inside a cell sample is explained below.

Microscope systems for single-molecule RNA detection inside a cell are typically designed based on the total internal reflection fluorescence (TIRF) microscope (Fig. 6.3). We constructed a home-build TIRF microscope system that employs excitation laser optics to illuminate the sample, and detection optics for the separation of emitted fluorescence light based on the wavelength followed by detection with respective sCMOS cameras. Using this system, simultaneous dual- and triple-color fluorescence imaging of the RNA of interest and an associated protein labeled with another fluorescent dye can be conducted [27, 31].

Excitation optics is the most important aspect of the microscopic system for single-molecule RNA imaging as the incidence angle of the excitation laser light on the sample is required to be modulated. The samples were illuminated by evanescent light that reaches the sample with a depth of approximately 150 nm from the coverslip in the TIRF microscopic visualizations. Under such excitation conditions, most of the intracellular molecules, including RNAs are not illuminated. Hence, they are
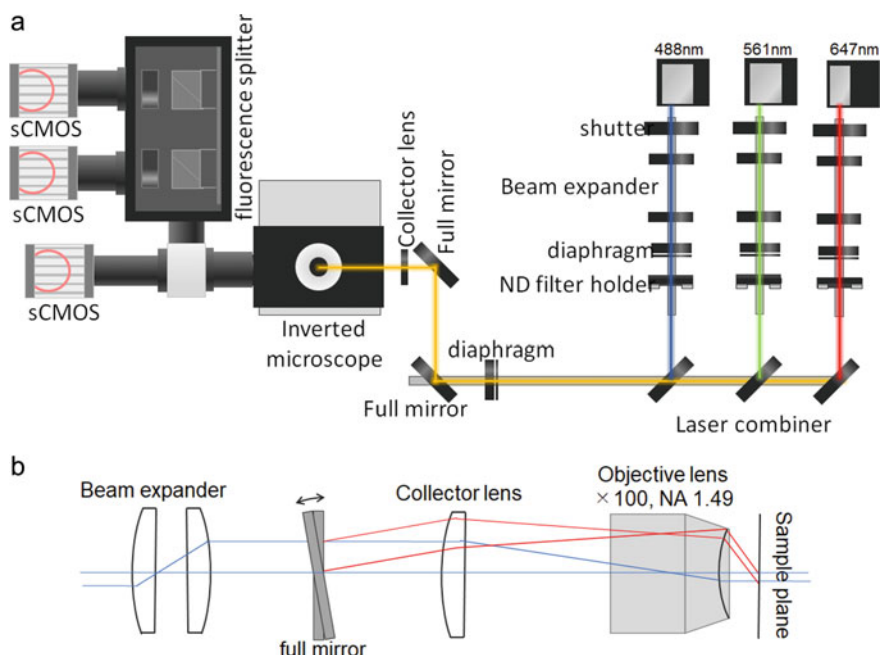

Fig. 6.3 Microscope setup for single molecule RNA observation [27]. **a** Overall picture of the microscope system used in RNA single molecule imaging. **b** Light path adjustment for TIRF and oblique illumination

invisible with the excitation during complete TIRF illumination. Highly inclined illumination or oblique illumination, in which the laser beam demonstrates a large incidence angle but smaller than the total internal reflection, deepen the illumination area and enables the monitoring of intracellular molecules with low background fluorescence [32].

The optical apparatus for excitation in the TIRF microscope system developed by us was composed of diode lasers, mechanical shutters, beam expanders consisting of a pair of convex lenses, neutral density (ND) filters, laser combiners, full mirrors, and a collector lens [27]. These components were placed on a vibration removal table along with a microscope and a detection system. The mechanical shutters were placed in front of the lasers. The two convex lenses in a beam expander exhibited a focus distance of 10 mm and 200 mm, respectively, which increases the diameter of the laser beam increased 20 times. The intensity of the laser lights was modulated via ND filters and combined using a dichroic mirror that act as a laser combiner. The laser light was introduced to the collector lens that focuses the laser light on the back focal plane of the objective lens. Subsequently, the laser beam is emitted from the objective lens as parallel light. The full mirror was placed 250 mm away from the collector lens with focus distance of 250 nm. This means that the mirror and collector lens are situated in a conjugate position. Alteration of the mirror angle slides the laser beam position after passing through the collector lens despite being kept parallel to the optical axis, making the incident angle of the laser beam on the sample to be oblique illumination that penetrates into the sample cell.

6.5 Application of PUM-HD for Monitoring RNA in Living Cells

The mPUM-based probe has been recently used for monitoring telomeric repeat-containing RNA (TERRA), which is a non-coding RNA, to investigate its mechanism [31]. TERRA is the transcription product obtained from the terminal region of the chromosomal DNA called telomeres [33–36]. TERRA consists of a subtelomeric region along with the telomeric-repeat region, and is implicated in telomere maintenance. Two different models have been proposed for explaining the mechanism of TERRA in the maintenance and regulation of telomeres: a scaffold model, which proposes that TERRA included in the ribonucleoprotein (RNP) complexes on a telomere aiding in their stabilization [37]; and a decoy model, which suggests that the protein components in the RNP complexes are transported or removed by TERRA [38]. Single-molecule monitoring of TERRA dynamics around a telomere in living cells would provide critical information for evaluating the true mechanistic model of the TERRA function.

TERRA has a unique repetitive region with multiple 5′-UUAGGG-3′ sequences derived from a telomere [33]. Our TERRA probe was designed to address this region [31]. The TERRA probe consists of three domains: an EGFP N-terminal, an mPUM

designed to capture a 5′-UUAGGGUU-3′ sequence (mPUMt), and an EGFP C-terminal fragment. Three repeats of a nuclear localization signal sequence are fused to the N-terminus of the probe owing to the nuclear localization of TERRA. In the presence of TERRA, multiple probe molecules accumulate on the unique repetitive region. The EGFP N-terminal fragment in the probe and the C-terminal fragment of the next probe come in close proximity, leading to the reconstitution of EGFP and emission of fluorescence. Although many reconstituted fluorescent proteins are expected to be generated on a TERRA molecule because of the large number of telomeric repeats in TERRA, most of the observed fluorescent spots included a single fluorescent molecule, confirmed by single-step photobleaching and analysis of the distribution of fluorescence intensity of the spots. This suggested that mPUMt did not penetrate and attach to a G-quartet structure, which is formed by the telomeric repeats, but only captured the flexible terminal region of TERRA.

Using the developed mPUMt-based probe, endogenous TERRA was monitored with an oblique illumination microscope in the living U2OS cells. The U2OS cells express the mPUMt-based TERRA probe as well as a SNAP-fused telomere marker protein TRF1 that enables the simultaneous monitoring of TERRA and telomeres. Many TERRA was visualized in a diffuse motion in the nucleus, whereas telomeres were almost static in the resulting movie. We observed two modes of the TERRA motion, namely diffusive and stationary upon detailed analysis of the movie. In addition, TERRA demonstrated localization of a substantial part to a region about 1 μm apart from a telomere. However, the remaining portion was colocalized with a telomere. This suggested the presence of a scaffold for capturing TERRA in this region.

To investigate the function of the TERRA in the region around a telomere, triple-color single-molecule imaging of TERRA, telomere, and telomere-associated proteins was conducted with an oblique illumination microscope equipped with a triple-color fluorescence separating detection system. The telomere-related protein, HP1, which is included in the scaffold model, and hnRNPA1, which is a potential target in the decoy model, were observed. In addition, H2A that reportedly does not interact with TERRA, was adopted as a negative control. In this observation, TERRA and telomere-related proteins were observed in a diffusive motion, whereas the telomeres were almost static, in accordance with the results obtained with dual-color imaging. Colocalization with TERRA was detected in all three telomere-related proteins. Consequently, the colocalization duration time was analyzed. For H2A with which interaction with TERRA has not been reported, the colocalization with TERRA can be considered as an accidental overlapping, and the colocalization duration was less than 0.2 s. The colocalization duration for HP1 and TERRA was comparable to that of H2A and TERRA, whereas the duration of hnRNPA1-TERRA colocalization was significantly longer than that of H2A-TERRA. Thus, hnRNPA1 was identified as an interacting partner of TERRA. A radial distribution analysis demonstrated that the interaction between hnRNPA1 and TERRA was concentrated in the area approximately 1–1.5 μm apart from a telomere. On initiation of this colocalization, diffusive hnRNPA1 molecules arrived and interacted with a static TERRA in the area. Based on the results from single-molecule imaging, a potential model for the function of
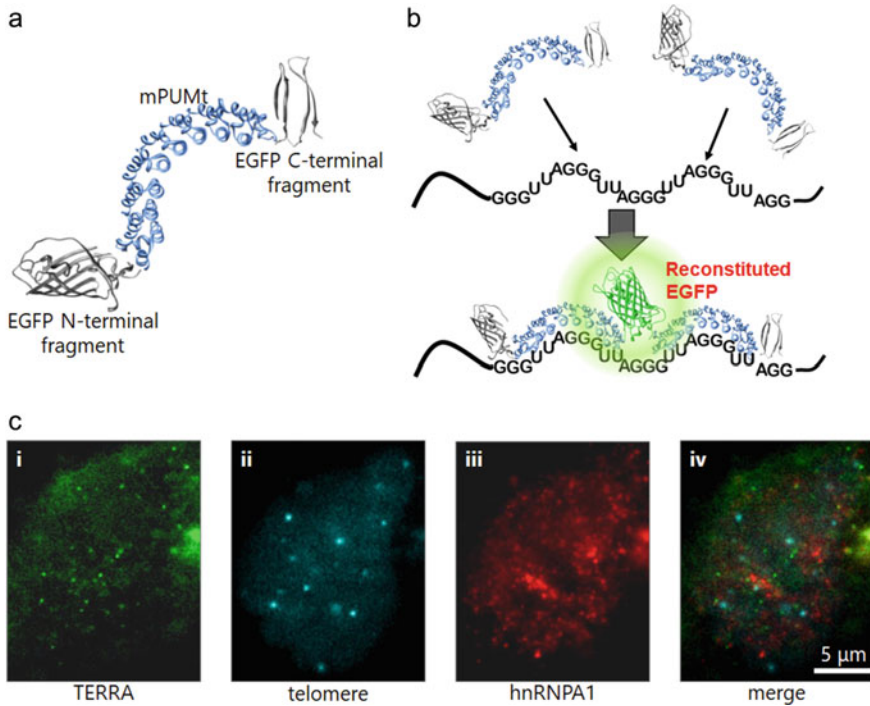
Fig. 6.4 Single molecule observation of TERRA [31]. **a** A schematic of mPUM-based TERRA probe. **b** Principle to detect TERRA by the mPUM-based probe. **c** Obtained single molecule fluorescence images of TERRA, telomere, and hnRNPA1

TERRA was proposed. The hnRNPA1 was originally located in an RNP complex on a telomere. When TERRAs appear around the telomere and form a scaffold, hnRNPA1 is captured with the TERRA scaffold upon release from telomeres. Subsequently, the component of the RNP is altered leading to a change in state of the telomere and the on/off regulation of the function of telomere maintenance. While further analyses of the dynamics and biochemical properties of TERRA and the associated molecules are required to obtain proof of this hypothesis, these results are achieved by development of the TERRA probe based on mPUM and split EGFP reconstitution techniques and unique of a single-molecule imaging approach (Fig. 6.4).

6.6 Perspective

The single-molecule RNA imaging study using a probe based on mPUM and split fluorescent protein techniques was used to obtain unique information different from previous biochemical experiments. Single-molecule imaging provides information that other methods cannot discern. However, certain limitations can be attributed on

the single-molecule RNA imaging using the probe introduced here. The observation time is limited to just a few second owing to photobleaching and the lack of quantitativity due to the irreversibility of fluorescent protein reconstitution. In order to apply the mPUM technology to different situation of RNA imaging analysis from short-term single-molecule imaging, some technical variations are expected. An approach for introducing such modifications is to swap the split fluorescent protein portion with other protein tools. For instance, quantitative RNA probes could be designed using a split luciferase reconstitution technique [30] or a fluorescent protein FAST [39], whose reconstitution reactions are reversible. When higher specificity of the target RNA is required, a modified mPUM consisting of 14 or 16 repeated motifs can be applied to the design of mPUM-based RNA probes [29]. Thus, the mPUM-based probe has a potential to modify the design to fit with the requirements of the investigations.

The evolution of microscopic methods will improve RNA imaging in living cells. The study introduced above used an oblique illumination technique. Hence, the resulting data are two-dimensional (2D) images, whereas RNA demonstrates three-dimensional (3D) diffusion in the cells. Recently, several techniques have been developed to obtain 3D fluorescence images in living cells. One such method for developing 3D images is based on the use of 2D diffraction grating to separate fluorescence light to multiple beams and to induce different areas on an image sensor [40]. The respective area has an image of the sample at obtained different depths. By the combination of such images, a 3D image is constructed. Another method to create a 3D image is based on the use of a light sheet as the excitation source. The use of lattice light sheet is a technique for obtaining high-resolution 3D images [41, 42]. Such techniques involving modified detection and excitation optical systems will support the 3D observation of the motility of target molecules in living cells.

In conclusion, the properties of mPUM, such as design flexibility and specificity for the target RNA sequence have been used for protein-based tools for live-cell optical imaging of RNAs. Even the single-molecule dynamics of an RNA of interest can be visualized in living cells combining the mPUM technology and optical microscope techniques. With further development of these technologies in the near future, the molecular mechanisms of RNA function in living cells can be elucidated.

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