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Fluorescent Probes for Imaging Endogenous β-Actin mRNA in Living Cells Using Fluorescent Protein-Tagged Pumilio

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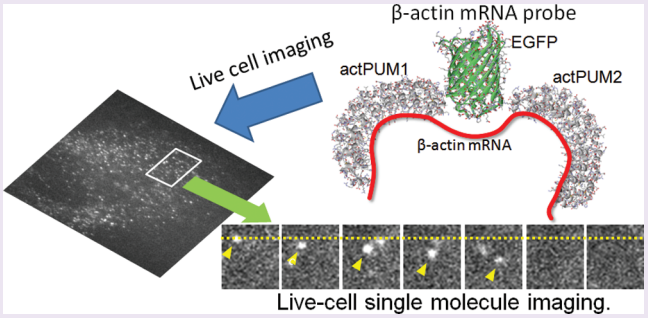
Supporting Information

ABSTRACT: Subcellular localization and dynamics of mRNAs control various physiological functions in living cells. A novel technique for visualizing endogenous mRNAs in living cells is necessary for investigation of the spatiotemporal movement of mRNAs. A pumilio homology domain of human pumilio 1 (PUM-HD) is a useful RNA binding protein as a tool for mRNA recognition because the domain can be modified to bind a specific 8-base sequence of target mRNA. In this study, we designed PUM-HD to match the sequence of β-actin mRNA and developed an mRNA probe consisting of two PUM-HD mutants flanking full-length enhanced green fluorescent protein (EGFP). Fluorescence microscopy with the probe in living cells revealed that the probe was labeled precisely with the β-actin mRNA in cytosol. Fluorescent spots from the probe were colocalized with microtubules and moved directionally in living cells. The PUM-HD mutants conjugated with full-length EGFP can enable visualization of β-actin mRNA localization and dynamics in living cells.



Various cellular functions are executed mainly by proteins that localize in specific cell regions. Protein localization is achieved not only by transportation of the proteins themselves but also through localization of the corresponding mRNAs.[1,2] Some mRNAs are recently known to localize in specific cell regions and play a part in the rapid protein localization. One of the example of such mRNAs is β-actin mRNA, which is concentrated in pseudopodia of fibroblasts[3] and growth cones and spines of neurons.[4] These regions require abundant β-actin proteins for actin network remodeling immediately after induction of external stimulation. In addition to the β-actin mRNA, various mRNAs reportedly localize in specific cellular regions; these behaviors of mRNAs are related to control of a variety of cell functions.[5,6] Although much information has been accumulated on this topic, more complete understanding of mRNA localization and dynamics demands novel techniques to visualize target mRNAs in living cells.

Intensive efforts have been undertaken to develop mRNA probes for live cell imaging.[7−12] A conventional strategy, such as a molecular beacon method,[9−12] is to use a probe of an oligonucleotide labeled with synthetic fluorophores. Because the oligonucleotide probes can be designed to have complementary sequences to the target mRNAs, the probes have high specificity to the target mRNA. This method, however, necessitates a procedure to inject the probe to individual cells each time. In contrast, genetically encoded probes make it easier to prepare the cells for imaging because the expression vector can be introduced to many cultured cells by transfection at a time. However, genetically encoded probes generally require additional sequences to the target mRNA; the additional sequences act as the recognition site of the probe. An example of the use of genetically encoded mRNA probes is in a method called the MS2 system.[13,14] In this method, the target mRNA is labeled with MS2 repeat sequences of mRNA, and the probe consists of a fluorescent protein and an MS2 coat protein that specifically binds to an MS2 region. The probes are accumulated on the MS2 repeat on the target mRNA and can be visualized as a fluorescent spot under a fluorescence microscope. This system has made it feasible to visualize a variety of mRNAs in living cells and animals. However, the mRNA visualized in this method is not endogenous but exogenous, and RNA engineering might engender misunderstanding of the localization and dynamics in living cells.

The ideal methodology of mRNA visualization is based on a genetically encoded probe that is designed to selectively recognize an endogenous target mRNA without additional tags. An RNA binding protein of pumilio can provide a useful component to develop such a probe. The pumilio-homology domain (PUM-HD) of human pumilio 1 comprises an array of eight elements (repeats 1−8) that recognizes the RNA sequence of UGUAYAUA (Y is C or U); each element specifically recognizes a single base of the RNA.[15] A

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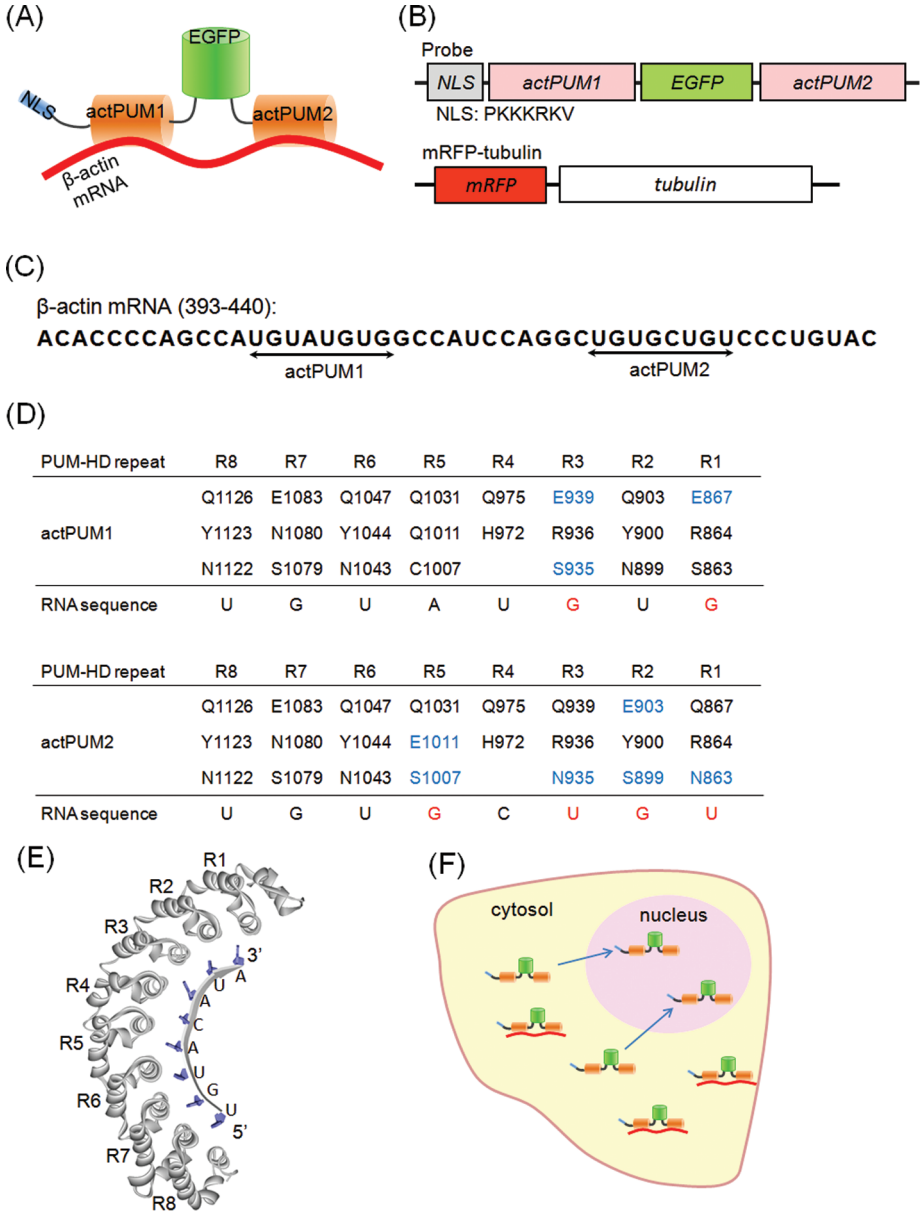

Figure 1. (A) Schematic of the probe recognizing β-actin mRNA. This probe has two PUM-HD mutants, actPUM1 and actPUM2, which are modified to recognize tandem sequences of β-actin mRNA (405−412 and 424−431, respectively) using site-directed mutagenesis. (B) Schematic structure of the cDNA of the probe and mRFP-tubulin. NLS is the nuclear localization signal. (C) The portion of β-actin mRNA sequences recognized by the PUM-HD mutants (arrows). (D) RNA sequences recognized by actPUM1 and actPUM2. The amino acids that interact with RNA bases are shown. Mutated amino acids and their recognition bases in PUM-HD repeats are shown, respectively, in blue and red. (E) The structure of 8-repeat motifs (R1−R8) of PUM-HD and the recognized RNA. The PDB ID code is 1M8Y. (F) The principle of visualization of β-actin mRNA in the cytosol using the mRNA probe. The probe that binds to β-actin mRNA stays in the cytosol. The excess probe that does not bind to the mRNA is transported into the nucleus.

noteworthy feature of PUM-HD is that PUM-HD can be modified to recognize 8-base RNA sequences different from the original one by substitution of specific amino acids in the elements.[16] We first demonstrated the usefulness of PUM-HD mutants for mRNA imaging in combination with a split GFP reconstitution technique. Two PUM-HD, fused to the N-terminal and C-terminal halves of split enhanced GFP (EGFP), were mutated to recognize two closely adjacent eight-nucleotide sequences in the mRNA of NADH dehydrogenase subunit 6.[17] Upon binding of the PUM-HD mutants to the mRNA, the EGFP fragments were brought close together and reconstituted. The reconstituted fragments recovered the fluorescence and visualized the localization of the mRNA in the mitochondrial matrix. This technology based on PUM-HD mutants and split EGFP reconstitution has been applied also for live cell imaging of the genomic RNA of tobacco mosaic virus (TMV) in plant cells[18] and β-actin mRNAs in the cytosol of living mammalian cells.[19]

Although the technique of the reconstitution of EGFP fragments reduces background fluorescence and improves the signal-to-background ratio in fluorescence images, this technique presents some limitations. A major limitation is that the reconstitution of EGFP fragments takes tens of minutes, which might hamper the analysis of mRNA dynamics before completing of the reconstitution. Another limitation is the requirement of two plasmids for expressing the probes in

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target cells. Balanced expression of the two probes is not controllable in each cell when general techniques of plasmid transfection are used. Different expression levels of the probes result in different fluorescence intensities even in the same cell line used for a study, which may lead to wrong results in a single cell analysis. A possible method to avoid such limitations is to use a full-length EGFP, because it produces fluorescence just after the maturation of the protein. In addition, only a single expression vector is sufficient to generate the probe in target cells.

In this study, we produced an mRNA probe consisting of two PUM-HD mutants and full-length EGFP for visualizing an endogenous mRNA in living cells. This probe is entirely genetically encoded, comprising a single polypeptide; its fluorophore is generated immediately after maturation of EGFP. To eliminate background fluorescence, a nuclear localization signal (NLS) peptide was connected with the N-terminus of the probe, which brought the probe into the nucleus. We also demonstrated single-molecule imaging of mRNA in a specific experimental condition. Using this probe, we showed that the probe can be used for the spatiotemporal analysis of endogenous β-actin mRNA in living cells.

RESULTS AND DISCUSSION

Probe Design. The probe for mRNA consisted of two PUM-HD mutants, a full-length EGFP, and a nuclear localization signal (NLS) sequence (Figure 1A and B). We used two 8-base sequences of β-actin mRNA, 405−412 (UGUAUGUG) and 424−431 (UGUGCUGU), as the recognition sequences of the PUM-HD mutants (Figure 1C) because they are mutually close and because the sequences resemble that recognized by wild type PUM-HD (5′-$U_1G_2U_3A_4C_5A_6U_7A_8$-3′). In particular, the consensus sequence beginning with 5′-UGU-3′ is quite important for the recognition of R8-R6 in the PUM-HD repeat. Although the R5 in the wild type PUM-HD recognizes purine, its recognition is not strict. The sequence 5′-$U_5A_6U_7A_8$-3′ is relatively variable.[16,20] On the basis of these general rules, we introduced three amino acid substitutions (Q867E, C935S, Q939E) into wild type PUM-HD to produce the mutant that binds to UGUAUGUG (Figure 1D and E). The other PUM-HD mutant that recognizes UGUGCUGU was generated by introducing six amino acid substitutions (S863N, N899S, Q903E, C935N, C1007S, Q1011E) to a wild type PUM-HD.

The principle of visualizing β-actin mRNA is based on a separation of the probe that binds to mRNA from the free one in living cells (Figure 1F). The probe for β-actin mRNA is generated in the cytoplasm and then binds to β-actin mRNAs. Successive expression of the probe produces an excess amount of the probe that cannot bind to β-actin mRNA. The excess probe is removed into the nucleus with the NLS function, eliminating the background fluorescence in the cytosol. Consequently, β-actin mRNA in the cytosol can be visualized with the probe under a low background fluorescence condition.

Immunoprecipitation and RT-PCR To Confirm the Binding of the Probe to β-Actin mRNA. We first examined binding abilities of the probe to β-actin mRNA by immunoprecipitation and subsequent reverse transcription-PCR (IPRT-PCR) experiments. The Cos7 cells expressing the probe were lysed and incubated with an anti-GFP antibody. The probe-antibody complex was then collected using protein G sepharose and was subjected to reverse transcription, by which the mRNAs binding to the probe were converted to cDNAs. The cDNAs were amplified by PCR. Electrophoresis of the PCR products showed a single band of the same size as that of β-actin cDNA (Figure 2, upper left). This result obviously

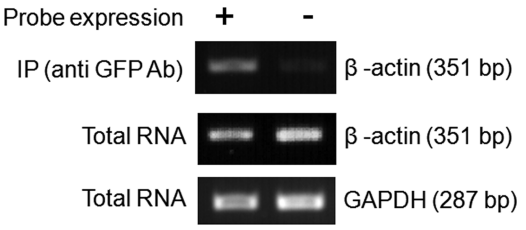

Figure 2. Immunoprecipitation and RT-PCR experiments used to verify the ability of the probe to bind β-actin mRNA. RNA extracted from the immunoprecipitated probe was subjected to RT-PCR using primers of β-actin mRNA (top, left panel). From cells expressing the probe, a specific band at the size of β-actin is visible (left lane).

indicates that the probe binds to β-actin mRNA in living cells. In contrast, a faint band of β-actin cDNA was also observed in the case of the cells without expressing the probe (Figure 2, upper right). This weak band may be an amplified product from the residual β-actin cDNA adsorbed on the sepharose beads or in the washing solution. Total RNAs of the cells were also subjected to the RT-PCR experiment to estimate the amount of β-actin mRNA. The result revealed that the amount of β-actin mRNAs was almost identical with the presence or absence of the probes. From these results, we concluded that the probe bound to β-actin mRNA in living cells.

Intracellular Distribution of the Probe Observed by Epifluorescence Microscopy. Next, the probe was subjected to live cell imaging of β-actin mRNAs. We investigated the localization of the probe in living Cos7 cells using epifluorescence microscopy. Although most of the probe was localized in the nucleus, a portion of the probe was observed in the cytosol (Figure 3A). The fluorescence in the nucleus indicates that the NLS of the probe brought it into the nucleus. In contrast, fluorescence in the cytoplasm was lower than that in the nucleus. These results suggest that the excess probes that did not bind to β-actin mRNAs were transported from the cytosol to the nucleus.

Previous reports demonstrated that β-actin mRNA forms the RNA−protein complex[21,22] and is transported along cytoskeletons by a motor protein.[3,19,23] In particular, Fusco *et al.* reported that β-actin mRNA are colocalized and transported on microtubules in Cos7 cells.[23] We then examined whether the present β-actin mRNA probe enables visualization of colocalization of β-actin mRNA with microtubules in living Cos7 cells. We prepared Cos7 cells expressing the probe and tubulin-RFP and observed the cells by epifluorescence microscopy. The fluorescence image of tubulin-RFP showed microtubule fibrous structures in the cytoplasm. The merged image of the probe with tubulin RFP revealed that the fluorescence from the probe was colocalized with that of the RFP specifically in the cell peripheral region (Figures 3B), demonstrating the colocalization of β-actin mRNA with microtubules.

Single-Molecule Observation of β-Actin mRNA by TIRF Microscopy. We performed single-molecule fluorescence imaging of β-actin mRNA in living cells. To remove the background fluorescence, total internal reflection fluorescence (TIRF) microscopy was used in the present experiment. Cos7 cells were observed only 4 h after the plasmid transfection. In

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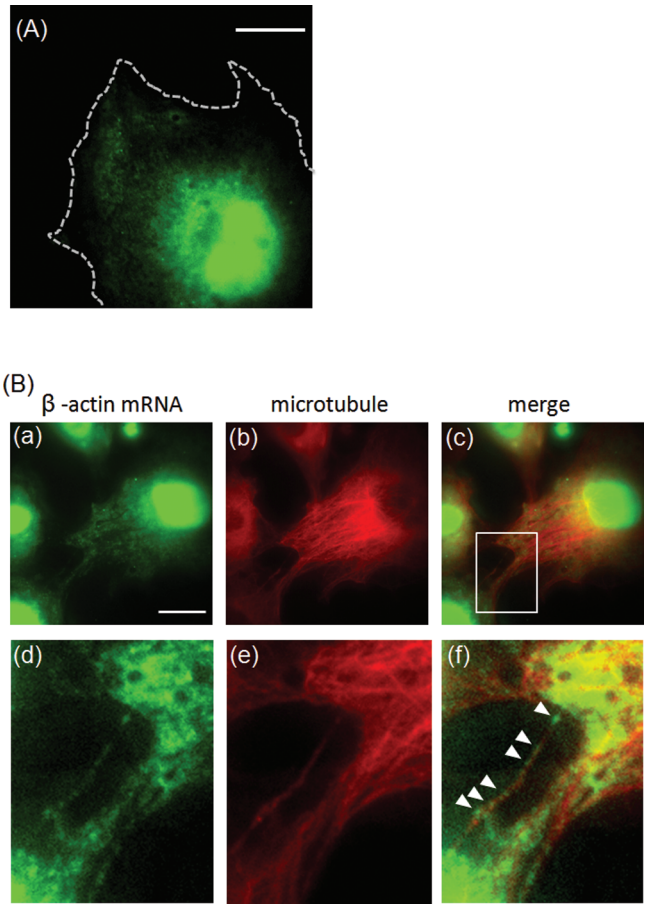

Figure 3. (A) Epifluorescence image of the Cos7 cell expressing the probe. The gray broken line represents the cell contour. Scale bar, 20 μm. (B) Epifluorescence images of the Cos7 cell expressing the probe (a), mRFP-α-tubulin (b), and the merged image (c). (d–f) Enlarged images in the white box shown in panel c. Overlapping of the probe on microtubules is indicated by arrowheads in panel f. Scale bar, 20 μm.

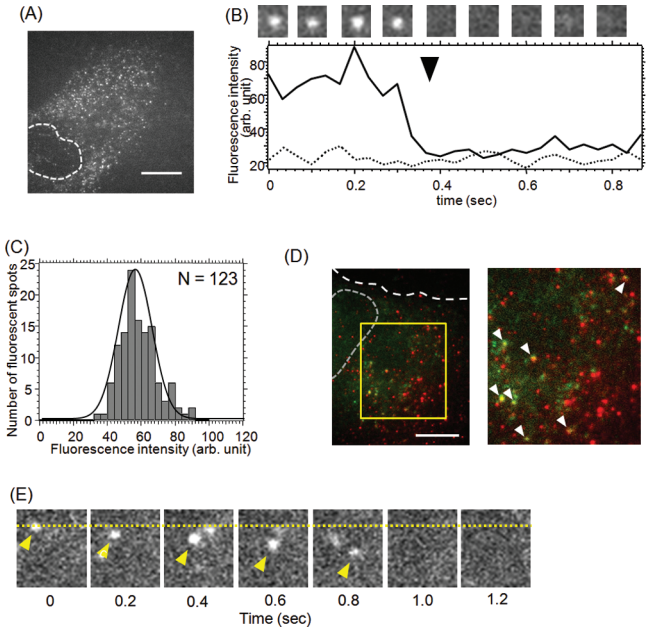

Figure 4. Single molecule imaging of the probe by TIRF microscopy. (A) Fluorescence image of a Cos7 cell expressing the probe. The broken line indicates the contour of the nucleus. Scale bar, 10 μm. (B) Time-course analysis of fluorescence intensities. The solid line indicates the intensity of a fluorescent spot shown above the graph. The broken line represents the background intensity. The black arrowhead indicates the time point of the single-step bleaching. (C) A histogram of fluorescence intensity of fluorescent spots. The solid line indicates a Gaussian fitting curve of the histogram. (D) Left: merged image of the present probe and Texas-Red-labeled oligonucleotide hybridized on β-actin mRNA. The white and gray broken lines represent the contours of the cell and the nucleus, respectively. Scale bar, 10 μm. Right: enlarged image in the yellow box. The white arrowheads indicate the point at which the fluorescence from GFP and Texas Red are colocalized. (E) Directional motion of a fluorescent spot. The yellow dotted line indicates the initial vertical position. The yellow arrowheads indicate the position of the spot.

this experimental condition, many fluorescent spots were obtained only in the cytoplasm (Figure 4A, Supporting Movie S1), suggesting that the excess amount of the probe is so few that most of the probes were not transported to the nucleus but bound to β-actin mRNA in the cytoplasm.

To examine whether the individual fluorescent spots indicate single probe molecules, we analyzed the time-dependent changes of the fluorescence intensity of a single spot. Small fluctuation of the fluorescence intensity was observed for 0.3 s, and the fluorescence was rapidly bleached thereafter (Figure 4B, Supporting Movie S2). This single-step bleaching is a typical phenomenon in single-molecule fluorescence. We also measured the fluorescence intensities of different spots and analyzed the intensity distribution. The histogram of the fluorescence intensities of the spots (N = 123) fitted with a single Gaussian distribution (Figure 4C), indicating that each fluorescent spot includes the same number of probes. These results demonstrate that the individual fluorescent spots originated from single probe molecules.

We next performed an *in situ* hybridization experiment to confirm that the fluorescent spots of the probes precisely indicate the localization of β-actin mRNAs. Cos7 cells were fixed by 3.7% formaldehyde 4 h after transfection with the plasmid. The cells were stained with a Texas-Red-labeled oligonucleotide probe for β-actin mRNA and then were observed by TIRF microscopy. We found that the fluorescent spots derived from the PUM-HD based probes were overlapped with the fluorescence of Texas Red (Figure 4D). This result indicates that the PUM-HD based probes precisely represent the localization of β-actin mRNAs.

Because of the ability of the present probe to visualize β-actin mRNAs on a single molecule level, we expected that dynamics of the mRNAs could be evaluated. In the TIRF microscopy imaging, we found that some fluorescent spots showed directional motion in the cytoplasm (Figure 4E, Supporting Movie S3). The velocity of this spot was calculated to be 1.63 μm/s. This value was comparable to that of previous reports in which β-actin mRNA is transported by motor proteins like dynein and kinesin.[24,25]

Discussion. In this study, we generated a new β-actin mRNA probe consisting of full-length EGFP and two PUM-HD mutants. Using epifluorescence microscopy, we visualized the colocalization of β-actin mRNA with microtubules. In addition, we succeeded in imaging β-actin mRNAs in cytoplasm on a single molecule level, in which directional motion of the mRNA was observed. Colocalization with microtubules and directional movement of β-actin mRNAs observed in this study are consistent with the reported mechanism of RNA transportation. For example, Fusco *et al.* demonstrated an

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observation of β-actin mRNA using the probes based on MS2-system. They showed that β-actin mRNAs were colocalized and moved along microtubules,[23] and the motion of the mRNAs was categorized into four modes: diffusive, corralled, static, and directed.[23] The fraction of directed and static modes was estimated at nearly 20%, and that of diffusive and corralled ones was nearly 30%. In the present study, we could clarify the four modes for some β-actin mRNAs in the obtained movie (Supporting Movie S1) at a single molecule level. However, it was difficult to quantify the fraction of the four modes because the fluorescence intensity of individual EGFP fluctuated. The instability of the fluorescence intensity hampered precise detection and tracking of some fluorescent spots of EGFP. In order to analyze the motion of each spot quantitatively, it will be necessary to develop a new system to track all fluorescent spots precisely. When the system is realized, the present probe would be a more useful tool for discriminating the motion of a target mRNA in living cells.

Theoretical consideration allows us to estimate the selectivity of the probe. The probe in this study has two PUM-HD mutants so that it can distinguish 4^{16} (about 4.3×10^9) transcripts. Accordingly, two PUM-HD mutants are sufficient to uniquely identify the target mRNA. In addition, the selectivity of PUM-HD and its mutants has been well investigated.[16,20] It has been reported that the dissociation constant between a particular PUM-HD mutant and its recognition sequence varies from 0.50 to 18 nM, which is larger than that of the wild type PUM-HD (0.48 nM).[16] In the present study, we inserted several mutations into the wild type PUM-HD to match the sequence of β-actin mRNA. The affinity of the PUM-HD mutants to their target sequences of mRNA is expected to be lower than the wild type PUM-HD. However, we used two PUM-HDs in the β-actin mRNA probe to increase the affinity. It was reported that PUM-HD can be engineered with 16 RNA-binding repeats by insertion of eight repeats of PUM-HD into another PUM-HD at the position between repeats 5 and 6. This engineered PUM-HD recognizes a specific 16-base RNA sequence with higher affinity and selectivity compared with the case of recognition of an 8-base RNA sequence.[26] Therefore, the present probe including the two PUM-HD mutants could also bind strongly to the target sequences of mRNA.

We tested full-length EGFP in this study for developing the probe. The present probe design differs from the PUM-HD based probes reported previously,[19] in which the probes consisted of split EGFP fragments connected with PUM-HD mutants. A salient advantage of the use of split EGFP is that the fluorophore is generated only after the probes bind to the target mRNA. Therefore, this technique makes it possible to eliminate the background fluorescence and to visualize the target mRNA with small steric hindrance. Such fluorescent protein reconstitution technique has been adopted in the previous PUM-HD based probes and some probes based on the MS2 system[17−19] and succeeded in single fluorescent molecule imaging of β-actin mRNAs.[19] However, it takes more than 30 min to recover the fluorescence,[27] which makes it difficult to observe events before the reconstitution is completed. To complement the limitation in the probe with the EGFP reconstitution technique, another design of PUM-HD based mRNA probe was necessary for imaging such events. Full-length EGFP emits fluorescence, irrespective of binding to the target mRNA. The probe described here will make it possible to visualize phenomena that occur within tens of minutes after the target mRNA is synthesized. Although we could not find any applications to observe dynamics of mRNAs in an early stage after generation of the mRNA, the mRNA probe based on full-length EGFP has a strong potential in the observation of phenomena like export of the target mRNA through the pores of the nuclear membrane if the nuclear membrane could be illuminated selectively.

The probe based on PUM-HD and full-length EGFP also has the advantage that the present system needs transfection of only a single plasmid. The method with EGFP reconstitution technique is performed with a pair of probes and, therefore, requires two plasmids transfection to the target cells. The requirement of multiple plasmids makes it difficult to control the expression of the probe molecules to be equal. On the other hand, an mRNA probe based on full-length EGFP consists of a single protein molecule and therefore requires induction of only a single plasmid into the cells. This simplicity will allow for application of two- or multiple-color fluorescent probes to visualize different mRNAs simultaneously. Accordingly, the probe with full-length EGFP will become a convenient tool for simultaneous observation of multiple mRNAs with different fluorescent proteins in single living cells.

A limitation of this system is the observation area inside the cells. Dynamics of the fluorescent molecules that can be visualized is just those molecules that happen to migrate into the TIRF distance. In this study, the excitation light was set at an angle slightly larger than the critical angle so that the illumination depth was thicker than the perfect TIRF condition. In addition, we used Cos7 cells, which have a planar shape with thin cytoplasm. Consequently, we were able to observe dynamics of the β-actin mRNAs that moved in parallel with the surface of glass slides. However, when cytosol of a target cell is thick in comparison to the illumination depth, perpendicular motion of the fluorescent molecule has to be considered. In such cases, it may be difficult to evaluate accurate velocities of the mRNA movements inside the cells.

In conclusion, we developed a novel β-actin mRNA probe that was composed of two PUM-HD mutants and full-length EGFP. Using this probe, we observed cytosolic β-actin mRNA and visualized colocalization of the mRNA with microtubules in living cells under an epifluorescence microscope. Moreover, we succeeded in single-molecule imaging of β-actin mRNA in living cells using TIRF microscopy. Only one plasmid is necessary for induction of the probe to target cells. Therefore, it is more convenient than the probe using EGFP reconstitution technique. This simplicity allows for induction of two- or multiple-color fluorescent probes to visualize different mRNAs simultaneously. In addition, if the expression of the probe is controlled lower than the target mRNA, it is possible to track the mRNA on a single molecule level. This probe will therefore become a powerful tool for simultaneous observation of multiple single mRNAs with different fluorescent proteins in living cells.

METHODS

Construction of Plasmids. The cDNAs encoding the RNA binding domain of human PUMILIO1 (PUM-HD) (828−1176 amino acids) and EGFP were generated by PCR and then cloned to pBluescript vector (Stratagene, CA). One PUM-HD mutant (Q867E, C935S, Q939E), named actPUM1, and the other PUM-HD mutant (S863N, N899S, Q903E, C935N, C1007S, and Q1011E), named actPUM2, were generated using a mutagenic PCR technique. The cDNA fragments were subcloned into a mammalian expression vector, pcDNA3.1 (+) (Invitrogen Corp., CA). A cDNA encoding a nuclear

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localization signal (NLS) sequence (PKKKRKV) was inserted to the 5′-terminal end of the probe. The cDNA of mRFP-α-tubulin fusion was generated by replacing the cDNA of AcGFP in pAcGFP1-tubulin (Clontech, CA) with that of mRFP. The cDNA fragments were also subcloned into a mammalian expression vector, pcDNA3.1 (+) (Invitrogen Corp.).

Cell Culture and Transfection. Cos7 cells were cultured in Dulbecco's Modified Eagle Medium (D-MEM; Gibco, CA) supplemented with 10% fetal bovine serum (Gibco) at 37 °C in 5% CO_2. The cells were transfected with plasmids using Lipofectamine 2000 (Invitrogen Corp.) according to the manufacturer's protocol.

Immunoprecipitation. Cos7 cells grown on a 10 cm dish were transfected with pcDNA3.1 (+) vector including the probe cDNA. After 2 days' incubation, the cells were washed with phosphate buffered saline (PBS; 10 mM Na_2HPO_4, 2 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4) and collected by scraping. The cell suspension was centrifuged at 4,000 rpm for 1 min at 4 °C. The supernatants were removed, and then 400 μL of a lysis buffer (150 mM NaCl, 5 mM EDTA, 50 mM NaF, 0.5% NP-40, and 10 mM Tris−HCl, pH 7.4) was added to the precipitate. The sample was rotated for 1 h at 4 °C. The resultant lysate was centrifuged at 12,000 rpm for 5 min at 4 °C. Then the supernatant was transferred to a fresh tube. The supernatant was mixed with 1 μL of RNase inhibitor (Takara, Japan), and 100 μL of the sample was added to 300 μL of the lysis buffer. Anti-GFP antibody (Roche Diagnostic Systems Inc., Indianapolis, IN) was then added to the lysates and was rotated for overnight at 4 °C. To collect the antibody-probe complex, 30 μL of Protein G Sepharose (GE Healthcare, U.K.) was added to the lysate. The suspension was rotated for 1 h at 4 °C and centrifuged at 5,000 rpm for 1 min at 4 °C. The obtained precipitation was washed four times with 500 μL of the lysis buffer and was mixed with 200 μL of the lysis buffer, 200 μL of TRIzol (Invitrogen Corp.), and 40 μL of chloroform. The mixtures were incubated for 3 min at RT and then centrifuged at 12,000*g* for 15 min at 4 °C, and the water layer was transferred to fresh tubes. After RNA precipitation with chloroform, isopropyl alcohol, and then 70% ethanol, the solution was treated with DNase and subjected to RT-PCR experimentation.

Reverse Transcription and Polymerase Chain Reaction (RT-PCR). The RNA solutions obtained in the experiment described in the immunoprecipitation section were suspended in 100 μL of RNase-free water. The portion of the suspension containing 0.5 μg of RNA was used for the following reverse transcription. Single-stranded cDNAs were prepared using oligo-p(dT) primers and the SuperScript 3 First-Strand Synthesis System (Invitrogen Corp.) according to the protocol. The cDNAs were subjected to PCR using either a pair of β-actin primers (5′-CCAACCGCGAGAAGATGACCC-3′ and 5′-TCTCCAGGGAGGAGCTAGAAG-3′). For the control experiment, total RNA of Cos7 cells is extracted using the following method. Cos7 cells were washed and collected in the same way as described in the immunoprecipitation section. Total RNA was extracted from the collected cells using TRIzol (Invitrogen Corp.) according to the manufacturer's protocol. The total RNA solution was subjected to RT-PCR experiments in the same method as used for immunoprecipitated samples. The PCR primer pair for amplification of GAPDH cDNA was 5′-GGAGTCAACGGATTTGG-3′ and 5′-AAGACGCCAGTG-GACTC-3′. The amplified PCR products were separated using agarose gel electrophoresis. The existence of β-actin and GAPDH was examined through detection of the corresponding bands in the electrophoresis images.

Epifluorescence Microscopy. Fluorescence imaging was performed using an inverted microscope (IX-71; Olympus Optical Co. Ltd.) equipped with the PlanApo 100× oil immersion objective (numerical aperture, 1.40). The light from a 100 W mercury arc lamp was collected on the back focal plane of the objective to illuminate the fluorophores in samples. Emission from the fluorophore was introduced to a cooled EM-CCD camera (iXon; Andor Technology, Northern Ireland) through the objective. Metamorph software (Molecular Devices Corp., CA) was used to control the microscope and to capture the fluorescence images.

Fluorescence *in Situ* Hybridization (FISH). FISH experiment was carried out in almost the same method as previously described.[19] Cos7 cells expressing the probe on coverslips were washed once in PBS and fixed for 30 min at RT with 3.7% formaldehyde in PBS. After the cells were washed twice with PBS, the cells were permeabilized by treatment with 70% ethanol overnight at 4 °C. The cells were hydrated for 5 min at RT in 2× SSC (300 mM NaCl, 30 mM sodium citrate, pH 7.0) containing 50% formamide. β-Actin mRNAs were hybridized with 5′ Texas-Red-labeled antisense oligonucleotide probe (5′-TGTAAAACTTTGGGGGATGCTCGCTCCAACCGACTGCTGT-CACCTTCACCGTTCCAGT-3′) overnight at 37 °C using 40 μL of a mixture containing 10% dextran sulfate, 2 mM vanadyl-ribonucleoside complex, 0.02% RNase-free BSA, 40 μg *E. coli* tRNA, 2× SSC, 50% formamide, and 30 ng of the oligonucleotide probe. The cells were washed twice for 30 min in 2× SSC supplemented with 50% formamide at 37 °C. Then, the cells were soaked in PBS and observed by using a fluorescence microscope (Olympus IX81) equipped with a EM-CCD camera (ImagEM, Hamamatsu photonics) and a home-build TIRF illumination system.

Total Internal Reflection Fluorescence (TIRF) Microscopy and Fluorescent Spots Tracking. Single molecule imaging was performed using an inverted microscope (IX-81; Olympus Optical Co. Ltd.), equipped with a home-build TIRF apparatus, a 488 nm laser and PlanApo 100× oil immersion objective with a numerical aperture of 1.49. For excitation illumination, the laser beams placed off the optical axis (but parallel to the optical axis) so that they passed near the edge of the objective lens to make a total internal reflection, which illuminates just an evanescent field of the sample. Emission from the fluorophore was collected by the objective and transmitted to a cooled EM-CCD camera (ImagEM; Hamamatsu photonics, Japan). Aquacosmos and HC-Image softwares (Hamamatsu photonics) were used to control the microscope system and to capture the fluorescence images, respectively. The fluorescent spots were tracked, and their velocity was calculated using the Image J software.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Movie of a Cos7 cell expressing the probe observed by TIRF microscopy (Movie S1). Single step bleaching of a fluorescence spot observed by TIRF microscopy (Movie S2). Directional motion of a fluorescent spot observed by TIRF microscopy (Movie S3). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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