Bioorganic & Medicinal Chemistry 20 (2012) 6034–6039

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

RNA-based diagnosis in a multicellular specimen by whole mount in situ hybridization using an RNA-specific probe

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ARTICLE INFO

Article history:
Received 13 July 2012
Revised 20 August 2012
Accepted 22 August 2012
Available online 28 August 2012

Keywords:
RNA diagnosis
RNA probe
In situ hybridization
Drosophila embryo

ABSTRACT

Recent RNA research has revealed the close involvement of various RNAs in cellular functions. RNAs are becoming the inevitable target molecules for research into details of gene expression. RNA and its related complexes are also promising targets for disease diagnosis. Multi cellular specimens such as organ tissues, histopathological specimens, and embryos are among the possible targets of RNA-based diagnostic techniques. In this report, we focused on a method that would provide such spatial and temporal information. We demonstrated that an RNA-specific probe (OMUpy2) was not only applicable to the detection of a specific mRNA in *Drosophila* embryos in a temporal and spatial manner but was also relatively quick and easy to use. The probe, OMUpy2, could be applied to other multi cellular systems for RNA-based diagnosis and research. The promising results of this manuscript show the great potential of RNA-based detection for both biological research and diagnostic medicine.

1. Introduction

Recent RNA research has revealed the close involvement of various RNAs in cellular functions. Not only RNA itself, but also RNA-protein complexes such as siRISC and miRISC are key players in cells.[1] RNAs are becoming the inevitable target molecules for research into details of gene expression. In order to clarify the roles of RNA-related molecules, the conventional protocols include Northern blotting,[2] RT-PCR,[3] and DNA microarray.[4] However, those protocols cannot practically study the differences in individual cells, tissues and embryos. In tissues, the individual cells may present different gene expressions. In embryos, the cells may be in different stages of differentiation. Therefore, new systems to meet the requirements are needed.

RNA and its related complexes are also promising targets for disease diagnosis.[5,6] Multi-cellular specimens such as organ tissues, histopathological specimens, and embryos are among the possible targets of RNA-based diagnostic techniques. Proof of the presence of certain RNAs in a specimen could provide information about diseases such as SARS[7] and newly-emerging virus infections.[8] Rapid and possibly quantitative analysis of a specific RNA could easily lead to rapid and precise diagnosis of diseases.

Taking this background into consideration, both in research into fundamental biology and in clinical disease diagnosis, selective and rapid detection of specific RNA in multi cellular specimens is essential. The conventional analytical method of RNA is in situ hybridization accompanied with an immune-staining protocol, which requires cDNA having a certain antigen such as digoxigenin and biotin.[9] Though this protocol has shown reliability to date, it requires skills and time-consuming procedures. Recently, elegant in situ hybridization protocols to detect RNA and its related substances have been reported. That is, RNA-imaging protocols have been intensively developed using fluorescent nucleic acid probes, and the rapid development of imaging technology has accelerated this study, allowing the visualization of RNA in cells, tissues and embryos. Several reports related to this new protocol include molecular beacons,[10,11] FRET-probes,[11,12] quenched autoligation probes [13] and fluorescent-protein based probes.[14] Though the probes used in those detection protocols have presented promising results for gene imaging studies, they were not RNA-specific. For further development of RNA-detection systems for the diagnosis of diseases, detection systems that can clearly detect RNA in specimens are urgently required.

We have developed a unique fluorescent RNA-specific probe having two consecutive pyrene-conjugated pyrimidine nucleosides. A pyrene was introduced at the 2′-position of a pyrimidine nucleoside denoted as Upy. Our probe, 2′-*O*-methyloligoribonucleotides (2′OMe ORN) having two consecutive modified nucleosides, denoted as OMUpy2, possesses two important characteristics. First, it is highly RNA-specific.[15,16] That is, the hybrids between OMUpy2 and the complementary RNA are highly fluorescent, whereas those between OMUpy2 and the complementary DNA are scarcely

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http://dx.doi.org/10.1016/j.bmc.2012.08.028

fluorescent. Even in hybrids between OMUpy2 and cRNA, a single mismatch causes significant quenching of the fluorescence. The spectroscopic studies revealed that the fluorescent behavior of the pyrene attached to the sugar was strongly affected by the surrounding environment of the pyrene.[17] And conformational studies using molecular dynamic simulations and ^{1}H NMR measurements revealed that the pyrene attached to the sugar in an RNA duplex was located outside of the helix, whereas that in a DNA duplex was lacated inside of the helix.[18] Therefore, the pyrene-modified RNA provides a useful tool for RNA detection. Secondly, OMUpy2 itself is hardly fluorescent. Therefore, a washing procedure to remove unbound probes from the detection system is not necessary, which facilitates the application of the probes to in situ RNA detection in living cells.

In this report, we present the concept of 'RNA-based diagnosis', conducted by an in situ RNA detection protocol using our RNA-specific probe in multi-cellular systems. This system is suitable for detecting RNA in its spatial and temporal aspects. In addition, the protocol employed in this study makes whole mount in situ hybridization (WISH), which is commonly used for embryo studies, easier and faster than ever, thanks to the elimination of washing steps. We chose *Drosophila* embryos and focused on *Krüppel* mRNA (Kr-mRNA). The *Krüppel* gene, a gap gene, is one of the first zygotic segmentation genes activated in early *Drosophila* embryos.[19,20] It is well known that the Kr-mRNA is abundantly expressed in the early stages of differentiation, and its temporal and spatial expression patterns have been well characterized.[21–23] Transcription of Kr-mRNA starts at the syncytial blastoderm stage (stage-3) and lasts up to 8 h after fertilization.[24] At the onset of transcription, the Kr-mRNA is detected mostly in the center domain of *Drosophila* embryos during stage-3-5[9,22–23]. Therefore, the OMUpy2 probe for Kr-mRNA is expected to emit signals in the center region of *Drosophila* embryos if the probe specifically hybridizes with the Kr-mRNA in the embryos.

2. Materials & methods

2.1. Probe preparation

The probes were synthesized according to previous reports using conventional phosphoramidite chemistry and purified by reversed-phase HPLC.[25] The sequences used in this study are listed in Table 1. The sequence of Kr-OMUpy2 is a 2′-OMe ORN complementary to the 304–318 nucleotide position of the Kr-mRNA (GenBank ID: 22024037). Kr-F is a 5′-fluorescein labeled 2′-OMe ORN which has the same nucleotide sequence as Kr-OMUpy2. Kr-OMUnonpy is a non-pyrene-labeled 2′-OMe ORN which has the same sequence as Kr-OMUpy2. Control-OMUpy2 consists of the same base composition as Kr-OMUpy2 with a scrambled sequence.

Table 1
RNA sequences used in this study

Kr-ORN	5′-AGC ACA AAA UUA GGA-3′
Kr-OMUpy2	5′-*UCC UAA* UpyUpy*U UGU GCU*-3′
Kr-F	5′-F *UCC UAA UUU UGU GCU*-3′
Kr-OMUnonpy	5′-*UCC UAA UUU UGU GCU*-3′
Control-OMUpy2	5′-*AGU CAC* UpyUpy*U UUC GUU*-3′
28S-ORN	5′-CGC UCA UCA GAC CCC AGA AAA G-3′
miR24-ORN	5′-UGG CUC AGU UCA GCA GGA ACA G-3′
miR21-ORN	5′-UAG CUU AUC AGA CUG AUG UUG A-3′

F, fluorescein; Italic letters, 2′-OMe RNA backbone; Upy, 2′-*O*-pyrenylmethyluridine. Kr-ORN: target sequence complementary to nucleotide positions (304–318) in Kr-mRNA. Kr-OMUpy2: antisense sequence for Kr-mRNA. Kr-F: 5′-fluorescein-labeled probe with same sequence as Kr-OMUpy2. Kr-OMUnonpy: non-pyrene-labeled probe with the same sequence as Kr-OMUpy2. Control-OMUpy2: scrambled sequence with the same base composition as Kr-OMUpy2. 28S-ORN: the sequence complementaery to nucleotide positions (1903–1924) in Human 28S ribosomal RNA gene. miR24-ORN: the sequence complementaery to nucleotide positions (50–71) in hsa-miR-24. miR21-ORN: the sequence complementaery to nucleotide positions (8–29) in hsa-miR-21.

2.2. Extraction of total RNA from *Drosophila* embryos and purification of mRNA

All embryos used in this study were from wild-type *Drosophila* flies (Canton S). Embryos were collected at 2 h intervals and aged until the desired developmental stage on yeast juice agar plates at 25 °C. The 0–2-hr embryos and 3–5-hr embryos were dechorionated with 5% sodium hypochlorite in water for 1.5 min and then frozen in liquid nitrogen. After 500 μl of TRIzol® Reagent (Invitrogen) was added to the frozen embryos on ice, the embryos were homogenized quickly, and 500 μl TRIzol® Reagent and 200 μl chloroform/isoamyl alcohol (49/1, v/v) were added, followed by vigorous shaking. After incubation on ice for 5 min, the suspension was centrifuged at 12,000 rpm at 4 °C for 15 min. RNA in the aqueous phase was transferred to a sterilized tube. After addition of 500 μl of isopropanol to the tube, the solution was stored on ice for 5 min followed by centrifugation at 12,000 rpm at 4 °C for 10 min. Isopropanol was exchanged with 75% aq ethanol and the ethanol solution was centrifuged at 7500 rpm at 4 °C for 3 min three times. The precipitate, RNA, was suspended in 75% aq ethanol and stored at −20 °C for more than 12 h. The RNA suspension was collected by centrifugation and dissolved in PBS buffer, followed by treatment with an oligo (dT)-cellulose column (GE Healthcare).

2.3. Fluorescence measurements

Fluorescence spectra were measured with a spectrofluorophotometer (RF-5300PC, Shimadzu). The fluorescence spectra of Kr-OMUpy2 in the presence of the extracted mRNA were measured with a micro volume cell (Capillary Adaptor Cell; HELIX Biomedical Accessories; cell volume, 3 μl). The concentration of extracted mRNA solution used for measurements was 65 mg/ml and those of Kr-OMUpy2 and Control-OMUpy2 were each 30 μM.

2.4. Whole mount in situ hybridization

The collections of *Drosophila m.* Canton S wild-type embryos were dechorionated in a solution of 5% sodium hypochlorite in water for about 1.5 min in a small basket. Embryos were thoroughly washed with 0.07% NaCl/0.1% TritonX-100. The embryos were then fixed with 4% paraformaldehyde in 10 mM phosphate buffer containing 0.1 M NaCl and heptane (1/1: v/v) with gentle shaking for 20 min at 25 °C. After fixation, the aqueous phase was completely removed and embryos were vigorously shaken in a 1:1 mixture of heptane and methanol so that they were devitellinized. Embryos were then washed with methanol twice and stored in 100% methanol at −85 °C.[26] Prior to WISH using fixed embryos, the supernatant, methanol, was exchanged with PBT solution (10 mM phosphate buffer, pH 7.0/0.1 M NaCl/0.1% Tween 20), and embryos were stored at 25 °C for 15 min. After exchanging PBT solution, the probe solution (30 μM OMUpy2 in PBT) was then added to the fixed embryos, followed by incubation at 40 °C for 30 min, then at 4 °C for 2 h. The embryos treated with probes were then washed with PBT once. Embryos were mounted on a glass slide in 70% glycerol in PBS and observed by fluorescent microscopy using a 20 × objective lens.

2.5. Microscopic measurement and photography

Microscopic measurements and imaging of embryos treated with Kr-OMUpy2 and Control-OMUpy2 were performed on a Nikon ECLIPSE TE300 (Nikon) microscope equipped with a xenon

lamp. Fluorescent images were obtained under the following conditions: Filter sets: for Kr-OMUpy2 and Control-OMUpy2, EX 340/15 nm DM 380 nm EM 480/30 nm; For Kr-F, EX 470/20 nm DM 505 nm EM 535/25 nm. The objective lens was a 20×/0.45 Plan Fluor (Nikon) for differential interference contrast (DIC) images. The merge images were converted to pseudo-color (Fig. 5I, yellow; Fig. 5J, blue). Microscopic fluorescence spectra were measured with a Photonic Multi-channel Analyzer (PN-100, Hamamatsu Photonics, Hamamatsu) under the following conditions: EX 340/15 nm DM 380 nm. The exposure time for obtaining images was 1 s and that for obtaining spectra was 5 s.

3. Results

RNA-specific probes containing two consecutive pyrene-conjugated nucleotides (OMUpy2) were synthesized according to previous reports;[15] the structure of the core region of the probe is shown in Figure 1. The nucleotide position, nt304–nt318 in *Krüppel* mRNA (total nt, 2547), was chosen as the target site of OMUpy2 based on an estimation by the 2D-structure simulation software, RNA Structure. The sequence contains an ApA sequence, where UpyUpy in OMUpy2 can hybridize. Details of the RNA probes and ORNs are shown in Table 1. 2′-*O*-Methyloligoribonucleotides were employed because they represent high stability in physiological media and they form significantly stable hybrids with their complementary oligoribonucleotides (ORN). Fluorescence spectra of OMUpy2 complementary to Kr-mRNA (Kr-OMUpy2) in the presence and absence of the complementary ORN (Kr-ORN) are depicted in Figure 2. The equimolar mixture of Kr-OMUpy2 and Kr-ORN significantly fluoresced around 480 nm. Neither single-stranded Kr-OMUpy2 nor the equimolar mixture of Control-OMUpy2 and Kr-ORN emitted appreciable fluorescence.

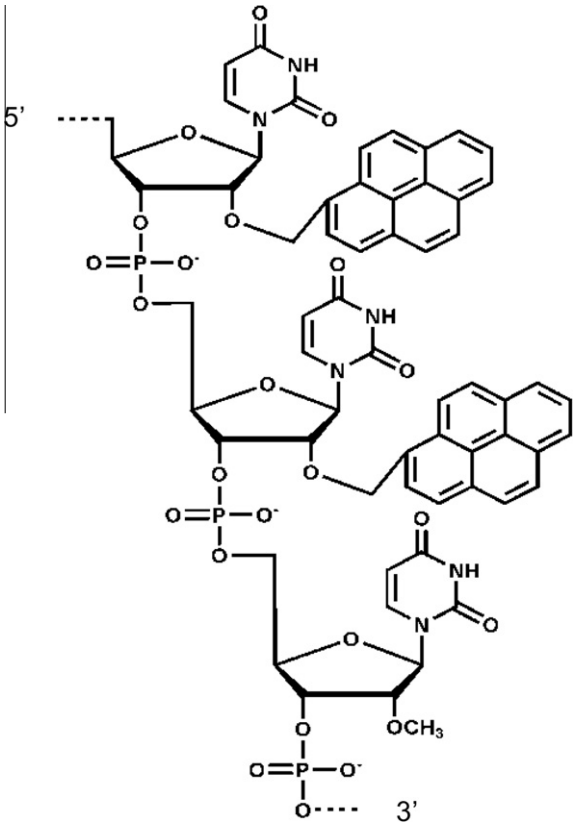

Figure 1. Structure of OMUpy2 consisting of 2′-OMe nucleotides and 2′-*O*-pyrenylmethyl uridines.

In addition, we observed a little emission from the equimolar solution of Kr-OMUpy2 probe and noncomplementary ORNs (28S-ORN, miR24-ORN, and miR21-ORN) (Fig. S1). Based on a previous report, we attributed the fluorescence around 480 nm to a pyrene excimer formed by a hybrid formation between OMUpy2 and its complementary RNA.[15] In order to evaluate the capability of OMUpy2 in terms of the quantitative analysis of RNA, the fluorescence intensity of OMUpy2 in the presence of the complementary ORN (cORN) was measured in the range of 10–1000 nM. The fluorescence intensity of OMUpy2 RNA hybrids versus the concentration of cORN had a linear relationship as shown in Figure 3, showing that the detection of target RNA by OMUpy2 was quantitative in the concentration range. These results clearly show that Kr-OMUpy2 can be a suitable RNA-specific fluorescence probe for RNA detection in a sequence specific manner.

Kr-OMUpy2 was then added to mRNA extracted from *Drosophila* embryos expressing Kr-mRNA and the fluorescence profile was examined. Mahara et al.[15] demonstrated that OMUpy2 could recognize RNAs with a higher-ordered structure by monitoring fluorescence from the probe using *E. coli* 5S-rRNA. The OMUpy2 (15-nt) emitted fluorescence only when it hybridized to the complementary RNA. That is, appreciable fluorescence observed from OMUpy2 solution indicated that the site is single-stranded. We therefore measured emission spectra of the Kr-OMUpy2 solution in the presence of mRNAs extracted and purified from *Drosophila* embryos. Total mRNA was extracted from embryos 3–5 h after fertilization, where Kr-mRNA was abundantly expressed (3–5-hr-mRNA). Kr-OMUpy2/3–5-hr-mRNA solution and Control-OMUpy2/3–5-hr-mRNA were concentrated into two microliters and their fluorescence characteristics were evaluated with a micro volume capillary adaptor quartz cell. As a result, Kr-OMUpy2/3–5-hr-mRNA solution showed significant emission at around 480 nm. In contrast, solutions of Control-OMUpy2/3–5-hr-mRNA, Kr-OMUpy2 alone, and Control-OMUpy2 alone scarcely emitted fluorescence signals, respectively (Fig. 4A).

We then used the extracted mRNA from 0–2 hr after fertilization of embryos (0–2-hr-mRNA), where Kr-mRNA was scarcely transcribed. A solution of Kr-OMUpy2/0–2-hr-mRNA emitted fluorescence at around 480 nm, and the intensity was 25% compared with that of Kr-OMUpy2/3–5-hr-mRNAs solution (Fig. 4A). Though it is practically difficult to estimate the amount of Kr-mRNA in the extraction solution, these results confirm that Kr-OMUpy2 is capable of selectively detecting Kr-mRNA.

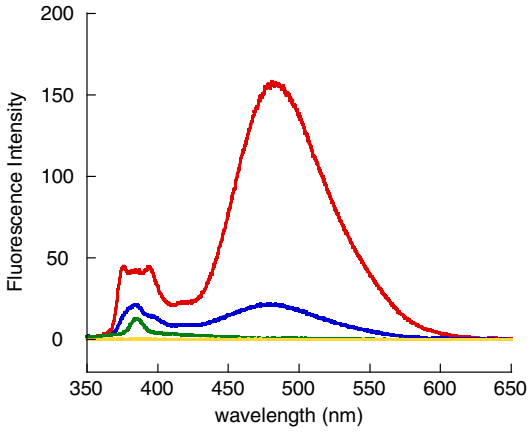

Figure 2. Fluorescence spectra of Kr-OMUpy2 and Control-OMUpy2 in the presence and absence of Kr-ORN. Red line, Kr-OMUpy2/Kr-ORN; blue line, Kr-OMUpy2; green line, Control-OMUpy2/Kr-ORN; yellow line, Control-OMUpy2; [Kr-OMUpy2] = [Control-OMUpy2] = [Kr-ORN] = 0.75 μM in 10 mM phosphate buffer (pH 7.0) containing 100 mM NaCl, 11 °C, λ_{ex} = 342 nm.

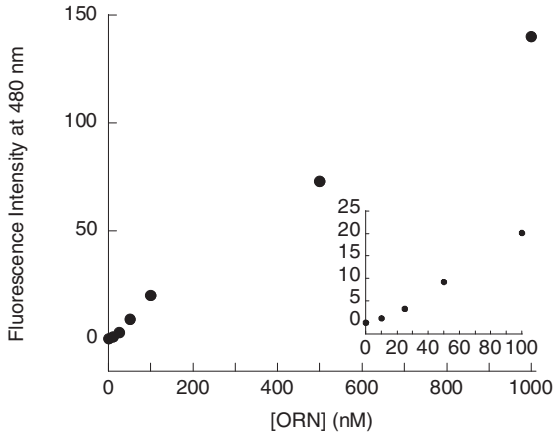

Figure 3. Fluorescence intensity at 480 nm of OMUpy2 in the presence of various concentration of the complementary oligo RNA. [Kr-OMUpy2] = 1 μM, [Kr-ORN] = 10–1000 nM Ex:340 nm, Em: 480 nm, 10 mM phosphate buffer (pH 7.0) containing 100 mM NaCl.

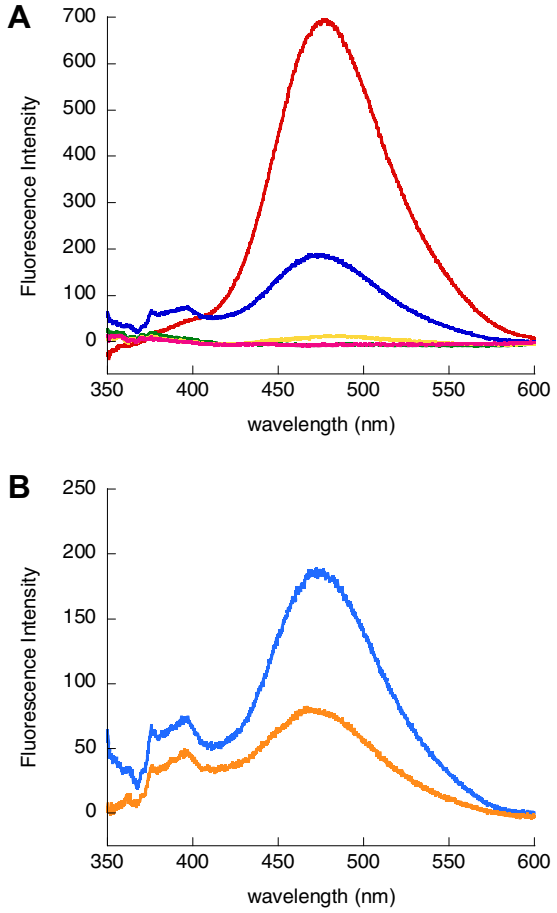

Figure 4. (A) Fluorescence spectra of Kr-OMUpy2 and Control-OMUpy2 in the presence or the absence of mRNAs extracted from *Drosophila* embryos expressing Kr-mRNA. Red line, Kr-OMUpy2/3–5-hr-mRNA; blue line, Kr-OMUpy2/0–2-hr-mRNA; green line, Control-OMUpy2/3-5-hr-mRNA; yellow line, Kr-OMUpy2; pink line, Control-OMUpy2; [Kr-OMUpy2] = [Control-OMUpy2] = 30 μM [mRNA] = 65 mg/ml 10 mM phosphate buffer (pH 7.0) containing 100 mM NaCl. (B) Competition assay with Kr-OMUnonpy. Blue line, Kr-OMUpy2/0-2-hr-mRNA; orange line, Kr-OMUnonpy/Kr-OMUpy2/0–2-hr-mRNA. [Kr-OMUpy2] = 30 μM [Kr-OMUnonpy] = 15 μM [mRNA] = 65 mg/ml 10 mM phosphate buffer (pH 7.0) containing 100 mM NaCl.

To verify whether the emission in Kr-OMUpy2/0–2-hr-mRNA solution was attributed to sequence specific hybridization of Kr-OMUpy2 with the targeted sequence in Kr-mRNA, we performed a competition assay using Kr-OMUnonpy, whose sequence is the same as that of Kr-OMUpy2 except the presence of two consecutive pyrenes. Addition of Kr-OMUnonpy to the solution of Kr-OMUpy2/0–2-hr-mRNA solution caused the large reduction of the fluorescence intensity at around 480 nm (Fig. 4B), suggesting that Kr-OMUpy2 recognized Kr-mRNA in a sequence-specific manner.

The detection of Kr-mRNA in the fixed embryos was carried out using both stage-1 and stage-5 embryos at the beginning of formation of the cellular blastoderm. Using a Kr-F (15-nt) probe whose 5′end was modified with fluorescein, we first examined the distribution of OMUpy2 in the fixed embryos at stage-5. Figure 5A shows DIC images of the embryos at the stage-5, and Figure 5E shows fluorescence images after careful washing of Kr-F with PBT once. The fluorescent signal of Kr-F was detected in the entire region of the embryo, indicating that Kr-F was almost evenly distributed in the embryo.

Kr-OMUpy2 was then employed to verify the presence of Kr-mRNA in the fixed *Drosophila* embryos with just one washing step using PBT. Figures 5B and C show the DIC images of the embryos at stage-5 after treatment with Kr-OMUpy2 and Control-OMUpy2, 'respectively'. Figure 5D shows the results with an early embryo (stage-1) prior to nuclei migration toward the cortex. In stage-5 embryos treated with Kr-OMUpy2, a fluorescence signal was apparently detected in the center region and around the posterior region of the embryo (Fig. 5F and Fig. S2). The localization of the signal in the center region of the embryo exactly corresponds to the reported Kr-mRNA expression pattern in early embryos[9,21–23]. The signal obtained in the posterior region of the embryo was similar to the observation reported by Hoch et al.[27]. In the case of stages-5 embryo treated with Control-OMUpy2 and stage-1 embryo treated with Kr-OMUpy2, no fluorescence was observed (Fig. 5G and H). To confirm whether the signal in the stage-5 embryo treated with the Kr-OMUpy2 (Fig. 5F) was derived from the bis-pyrene moiety, the emission spectra were measured with a microspectroscope (Fig. 6). The spectrum of the stage-5 embryo treated with Kr-OMUpy2 peaked at around 480 nm (Figs. 5F and 6), and it closely resembled that of Kr-OMUpy2/Kr-ORN (Fig. 2), whereas other spectra with small intensity at 480 nm were obtained from embryos shown in Figure 5G and H.

4. Discussion

The development of RNA research has inaugurated a new era of life science, and the ripple effects extend to many different fields of life science. This study focuses on RNA-based diagnosis, which could be a key to research in the future life sciences including medical science. From the information obtained by DNA diagnosis, we can understand the presence of genetic alteration in specimens such as cells, tissue, and organs. The finding of a single base alteration in DNA, for example, suggests the possibility that the cells may develop into cancerous cells. However, this alteration does not always mean the development of the cancer. In medical diagnosis, it is quite important to find the signs of transformation in cells prior to their development into malignancy. RNA could be a suitable target for detecting signs of cancerous change in cells.[5,6] To date, many methods of detecting and analyzing RNAs in cells have been developed.[2–4] One such area of research is the development of probes that can detect a specified RNA from the viewpoint of temporal and spatial localization in living cells. We have developed such an RNA-specific probe, OMUpy2.

In this study, Kr-OMUpy2 complementary to Kr-mRNA emitted a fluorescence signal in the presence of extracted mRNA from embryos expressing Kr-mRNA (Fig. 4A), suggesting that Kr-OMUpy2

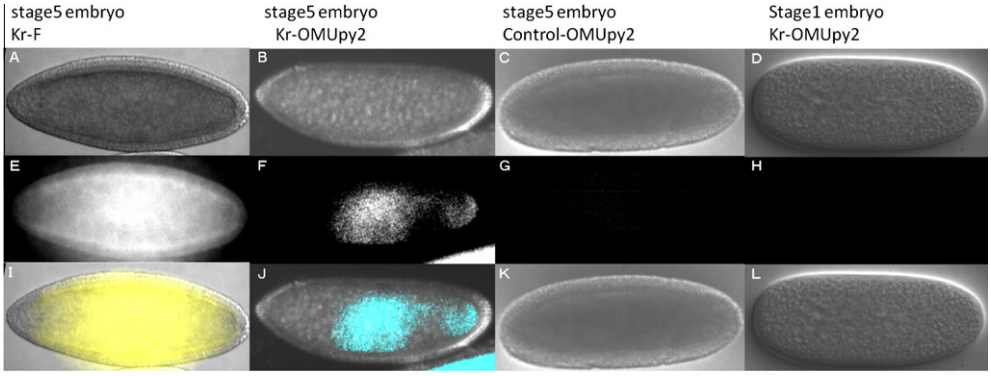

Figure 5. DIC and fluorescent images of fixed *Drosophila* embryos. Anterior is to the left. (A–D) DIC (differential interference contrast) images. (E–H) Fluorescence images. (I–L) merge images. [Kr-OMUpy2] = [Kr-F] = [Control-OMUpy2] = 30 μM, Kr-OMUpy2, Control-OMUpy2: (Ex, 340/15 nm; Em, 480/30 nm), Kr-F: (Ex, 470/20 nm; Em, 520 nm (BA)) Objective: Plan Fluor 20X, NA 0.45. The merge images were converted to pseudo-color (I, yellow; J, blue).

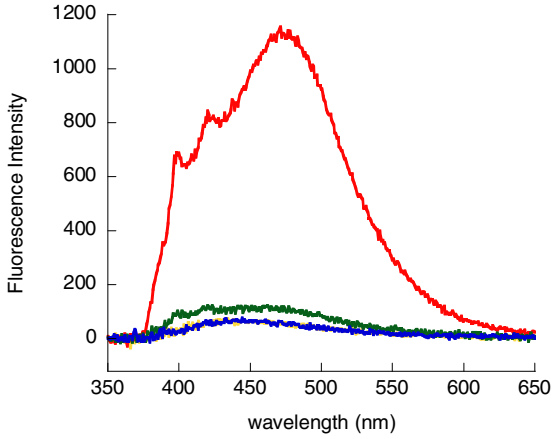

Figure 6. Microscopic fluorescence spectra of the embryos after whole-mount in situ hybridization. Red line, stage-5 embryo treated with Kr-OMUpy2; green line, stage-5 embryo treated with Control-OMUpy2; blue line, stage-5 embryo treated without probes; yellow line, stage-1 embryo treated with Kr-OMUpy2.

specifically detected the Kr-mRNA in the mRNA pool. This was further confirmed by competition experiments using Kr-OMUnonpy, which had no pyrene moieties. The fluorescent signal was found to be faint in the solution of Kr-OMUpy2/0–2-hr-mRNA from 0- to 2-hr embryos, in which Kr-mRNA would have scarcely been transcribed. These results suggest that Kr-OMUpy2 is specifically hybridized with Kr-mRNA without purification (Fig. 5F and Fig. S2).

In the commonly used in situ RNA hybridization methods with *Drosophila* embryos, the steps from the embryo fixation to observation take at least 3 days [26,28]. In that protocol, cDNA probes are prepared by reverse transcription reaction on mRNA, and their lengths are usually more than 100-nt. Using these cDNA-probes, it can take a long time, sometimes one day, to attain optimum hybridization with the target mRNA in the fixed embryos, and repeated washing steps are inevitably necessary. Furthermore, visualization procedures such as immunostaining are required to specify the target RNA. In contrast, using the Kr-OMUpy2 probe we developed, several washing steps could be omitted and the hybridization time was shortened to a few hours. This was attributed to the unique characteristics of Kr-OMUpy2, which consists of a short oligonucleotide (15-nt), and emits signals only when it is excited in the presence of its complementary RNA. Thus, unbound and non-fluorescent Kr-OMUpy2 can be ignored in imaging protocols.

For RNA-based diagnosis using OMUpy2 in multi cellular specimens, the results of probing should be quantitative. As shown in Figure 3, the quantitative range in detecting RNAs by the OMUpy2 system is between 10–1000 nM. Though the exact copy number of expressed mRNA in individual cells cannot be specified, OMUpy2 could be applicable to detecting mRNA in fixed embryos and other multicellular specimens.

As shown in Figure 5F, clear fluorescence was observed in the center region of fixed *Drosophila* embryos at the stage-5, and its fluorescence spectrum showed a similar spectrum of the hybrid of Kr-OMUpy2 and Kr-ORN. Previous studies using conventional WISH protocols have reported that Kr-mRNA was observed in the center region of the embryo as a broad band. We concluded that the signal in the fluorescence image (Fig. 5F and Fig. S2) was derived from the Kr-OMUpy2 hybridized with Kr-mRNA in the embryo. Namely, Kr-OMUpy2 clearly achieved the visualization of the localization of Kr-mRNA. Using the protocol, it is practically possible to specify target RNAs from the viewpoint of spatial and temporal localization in multicellular specimens. These results suggest that the study of gene expression in multicellular specimen has wide-ranging potential in RNA-based diagnosis and is broad enough to cover academic studies in which the probe would be used.

5. Conclusions

Molecular biological studies often investigate the features of cells irrespective of the timing of their individual functions, resulting in a static, oversimplified picture of the dynamic whole. Given the advances of research on signaling, we think that future RNA-based research methods will have to be able to detect when RNAs transcribed, when RNAs migrate, where RNAs are localized, and when or under what circumstances RNAs are degraded. In this report, we focused on a method that would provide such spatial and temporal information. We demonstrated that an RNA-specific probe (OMUpy2) was not only applicable to the detection of a specific mRNA in *Drosophila* embryos in a temporal and spatial manner but was also relatively quick and easy to use. The probe, OMUpy2, could be applied to other multi cellular systems for RNA-based diagnosis and research. The promising results of this manuscript show the great potential of RNA-based detection for both biological research and diagnostic medicine.

Acknowledgements

This work was supported by a Grant-in-aid from the Japan Society for the Promotion of Science (JSPS) (AM, AY, 21350093; AK, 22750154; AY, 22750153) and by a Grant-in aid from Japan Science and Technology Agency (JST) (AM, 231Z02809).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmc.2012.08.028.

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