Microarray-based label-free detection of RNA using bispyrene-modified 2'-*O*-methyl oligoribonucleotide as capture and detection probe

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Abstract— A novel oligonucleotide microarray that can detect RNAs without fluorescent labeling of sample RNAs was developed. As a capture and detection probe, bispyrene-modified 2'-*O*-methyl oligoribonucleotide (OMUpy2), whose fluorescence was dramatically increased when hybridized with its complementary RNA, was adopted. Fluorescence of the OMUpy2 tethered on the glass surface was enhanced as much as 22-fold by the addition of complementary oligoribonucleotide.

In the last decade, important discoveries concerned with RNAs, for example, small interfering RNA and microRNA (miRNA), have been reported. In particular, miRNAs have received much attention because of their important functions in gene regulation, diverse cellular processes ranging from cellular differentiation, proliferation, apoptosis, and metabolism to cancer[1–5]. The development of a more sophisticated method for RNA detection is an important issue in increasing the understanding of the function of RNAs in living cells. Oligonucleotide microarray technology, which can detect thousands of target nucleic acids on a chip, is one of the most superior technologies used to analyze RNA expression in the cell. Recently, new types of oligonucleotide microarray using an adopted functionalized oligonucleotide as a capture probe have been developed. Castoldi et al. adopted locked nucleic acid as a capture probe for oligonucleotide microarray analysis of miRNA expression of murine heart and liver[6]. Wang et al. immobilized a molecular beacon (MB), whose fluorescence was enhanced upon hybridization with its complementary DNA or RNA, as a capture and detection probe of an oligonucleotide microarray[7]. The MB-immobilized microarray was able to detect the hybridization without target labeling and washing protocol after hybridization. These technologies, which allow for rapid and highly sensitive detection of target nucleic acid on the oligonucleotide microarray, may contribute to the study of gene expression and gene diagnostics.

Here, we present a novel RNA-detection chip. The chip enabled label-free detection of RNAs using bispyrene modified 2'-*O*-methyl oligoribonucleotide (OMUpy2) as a capture and detection probe. Previously, we reported that OMUpy2, whose fluorescence at 480 nm was greatly enhanced when it hybridized with its complementary RNA (Figure 1, top), was useful for RNA detection in a homogeneous physiological solution[8,9]. If OMUpy2 probes complementary to target RNAs are immobilized in designated positions on a glass substrate (OMUpy2 array), various

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target RNAs could be detected simultaneously without target labeling and washing protocol. This is because only the probes that hybridize with target RNA can emit fluorescence at 480 nm at a specific position on the surface of a glass substrate (Figure 1, bottom). We studied the fluorescence characteristics of OMUpy2 tethered to the glass substrate with various linker molecules. The fluorescence enhancement of the OMUpy2 array upon the addition of oligoribonucleotide and its sequence specificity were also investigated.

Oligonucleotide sequences are listed in Table 1. To immobilize OMUpy2 on the surface of a glass substrate, several linker molecules (i.e., $d(T)_0$, $d(T)_6$, $d(T)_{12}$, and PEG-$d(T)_{12}$) were adopted. In the case of d(T)-linkers, the glass substrate was modified with 3-mercaptopropyltrimethoxysilane, and then 5'-thiol modified OMUpy2-CF3 (tether: $d(T)_0$, $d(T)_6$ and $d(T)_{12}$) was spotted on the glass substrate. In the case of PEG-$d(T)_{12}$-linker, 5'-thiol modified OMUpy2-CF3 (tether: $d(T)_{12}$) was spotted on a PEG_{2000}-maleimide-grafted glass substrate that was prepared according to reported procedure[10]. Detailed procedures for the immobilization of OMUpy2 were described in References and notes section[11].

Signal to background ratio (SBR) is important for the accurate detection of target RNA in our microarray system. To evaluate the SBR of the OMUpy2 array, fluorescence microscopic analysis[12] of the OMUpy2 arrays whose surface was grafted with OMUpy2-CF3 *via* various linker molecules, was performed 30 min after the addition of the buffer solution (5 × SSC) of oligoribonucleotide complementary to OMUpy2-CF3 (cORN-CF3)[13]. Background fluorescence, fluorescence signal, and SBR of the OMUpy2 arrays are listed in Table 2. In all cases, fluorescence intensities of OMUpy2-CF3 on the glass surface were increased by the addition of cORN-CF3 (SBR > 1), although the SBRs were increased with the elongation of the linker molecule between OMUpy2 and the glass surface. The difference in the SBR is probably due to a difference in the background fluorescence and/or to the hybridization efficiency of OMUpy2 on the glass surface. Indeed, background fluorescence decreased with the elongation of the linker molecule, indicating that the interaction between the glass surface and pyrene in the OMUpy2 strand could be one of the reasons for the high background fluorescence observed in the case of $d(T)_0$ and $d(T)_6$-linker. On the other hand, in the case of PEG-$d(T)_{12}$, the SBR was 4-fold larger than that in the case of $d(T)_{12}$-linker, although the background fluorescence was similar in both cases. These results indicate that the hybridization efficiency of OMUpy2 on the glass surface was increased by the addition of PEG as a linker. The flexibility of the PEG linker largely might contribute to the improvement of the hybridization yield. Similar results were reported by Shlapak et al.[10] who found that the hybridization yield of the oligonucleotide tethered to PEG-grafted glass surface was 4-fold higher than that of the oligonucleotide tethered to a linkerless surface. They also concluded that the increase in the hybridization efficiency was caused by the flexibility of the PEG between the oligonucleotide capture probe and glass surface. Our results indicate that the OMUpy2 array can successfully detect complementary RNA without target labeling and washing protocol after the hybridization, and that the PEG-$d(T)_{12}$-linker is the most suitable linker among the four linkers examined.

Previously, we reported a unique character of OMUpy2 whereby the fluorescence intensity of OMUpy2 was enhanced by the addition of complementary oligoribonucleotide (cORN) but not by the addition of complementary oligodeoxyribonucleotide (cODN) in a homogeneous physiological solution[8]. Such a unique characteristic might be useful for RNA detection in crude biological samples, such as cell extract, tissue extract, and the reaction mixture of T7 RNA polymerase-based RNA amplification. To confirm whether this unique character is maintained on the PEG-grafted glass surface, fluorescence microscopic analysis of the OMUpy2 array was performed after the addition of

cORN or cODN solution whose sequences were complementary to OMUpy2-CF3 (cORN-CF3, cODN-CF3). The fluorescence intensity of OMUpy2-CF3 on the glass surface was significantly enhanced by the addition of cORN-CF3 solution but not by the addition of cODN-CF3 solution (Table 2). This result indicates that the unique RNA recognition-character of OMUpy2 in the homogeneous physiological solution was maintained by OMUpy2 immobilized on the PEG-grafted glass surface.

To evaluate the sequence specificity of the OMUpy2 array-based label-free detection of RNAs, three different OMUpy2 probes (OMUpy2-CF3, OMUpy2-JN2, and OMUpy2-CM3) were immobilized on a similar glass substrate *via* the PEG-d$(T)_{12}$ linker, and fluorescence microscopic analysis was performed after the addition of the buffer solution of cORN-CF3 or ORN-Ctl. Fluorescence intensity of the spot of OMUpy2-CF3 was increased 22-fold by the addition of cORN-CF3 but the fluorescence intensity of the other spots did not change (Figure 2, a–d). Moreover, the addition of ORN-Ctl, whose sequence was not complementary to any immobilized OMUpy2 probes, did not affect the fluorescence intensity of the spots (Figure 2, f–i). These results indicate that the OMUpy2 probe immobilized on the glass surface *via* the PEG-d$(T)_{12}$ linker can detect complementary RNA specifically from the enhancement of the fluorescence emission.

In conclusion, we developed a novel RNA detection array whose surface was grafted with OMUpy2. The OMUpy2 array can successfully detect its complementary RNA without target labeling and the washing protocol. Among the four linkers chosen, PEG-d$(T)_{12}$ was the most suitable for RNA detection. As the OMUpy2 array can detect target RNA by simply adding a sample solution to the array, it is expected that the OMUpy2 array is a useful tool for RNA expression analysis. The OMUpy2 array developed here may contribute to progress in the study of RNA expression, gene diagnostics, and the clinical usage of gene diagnosis. Detection of cellular RNAs based on the OMUpy2 array is now in progress.

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Figure legends

Figure 1. Structure of OMUpy2 (a), fluorescence emission spectra of OMUpy2 in a homogeneous solution (b), and schematic illustration of microarray-based label-free detection of RNAs using OMUpy2 as a capture and detection probe (c).

Figure 2. Fluorescence microscopic images of the OMUpy2 array in the presence of oligoribonucleotide solution (a–d, f–i), and histograms of the fluorescence intensity of the microscopic images (e, j). Sequence of immobilized OMUpy2 probe is indicated on the right top of each image. a–e; In the presence of cORN-CF3, f–j; In the presence of ORN-Ctl. The colored line on the fluorescence images indicates the position used for the histogram analyses. [cORN-CF3 (or

ORN-Ctl)] = 1 μM in 5 × SSC buffer. Scale bar: 200 μm. Fluorescence intensity was corrected by the immobilized amount of the each probe quantified by fluorescence intensity when it hybridized with 6-FAM labeled complementary oligoDNA.

Abbreviations

cODN, complementary oligodeoxyribonucleotide; cORN, complementary oligoribonucleotide; miRNA, microRNA; MB, molecular beacon; OMUpy2, bispyrene-modified 2'-O-methyl oligoribonucleotide; SBR, signal to background ratio; SSC, saline sodiumcitrate

References and notes

1. Hutvágner, G.; McLachlan, J.; Pasquinelli, A. E.; Bálint, É.; Tuschl, T.; Zamore, P.D. *Science* **2001**, 293, 834.
2. Chen, J. F.; Mandel, E. M.; Thomson, J. M.; Wu, Q.; Callis, T. E.; Hammond, S. M.; Conlon, F. L.; Wang, D. Z. *Nat. Genet.* **2006**, 38, 228.
3. Brennecke, J.; Hipfner, D. R.; Stark, A.; Russell, R. B.; Cohen, S. M. *Cell* **2003**, 113, 25.
4. Calin, G. A.; Sevignani, C.; Dumitru, C. D.; Hyslop, T.; Noch, E.; Yendamuri, S.; Shimizu, M.; Rattan, S.; Bullrich, F.; Negrini, M.; Croce, C. M. *Proc. Natl. Acad. Sci. USA.* **2004**, 101, 2999.
5. Chan, J. A.; Krichevsky, A.M.; Kosik, K. S. *Cancer Res.* **2005**, 65, 6029.
6. Castoldi, M.; Schmidt, S.; Benes, V.; Noerholm, M.; Kulozik, A. E.; Hentze, M. W.; Muckenthaler, M. U. *RNA* **2006**, 12, 913.
7. Wang, H.; Li, J.; Liu, H.; Liu, Q.; Mei, Q.; Wang, Y.; Zhu, J.; He, N.; Lu, Z. *Nucleic Acids Res.* **2002**, 30, e61.
8. Mahara, A.; Iwase, R.; Sakamoto, T.; Yamana, K.; Yamaoka, T.; Murakami, A. *Angew. Chem. Int. Ed.* **2002**, 41, 3648.
9. Shimada, N.; Mahara, A.; Sakamoto, T.; Yamaoka, T.; Murakami, A. *Nucleic Acid Res. Symp. Ser.* **2004**, 48, 301.
10. Shlapack, R.; Pammer, P.; Armitage, D.; Zhu, R.; Hinterdorfer, P.; Vaupel, M.; Fruhwirth, T.; Howorka, S. *Langmuire* **2006**, 22, 277.
11. Immobilization of OMUpy2 on glass surface. Glass substrates (Micro Cover Glass; 22 × 24 mm, thickness 0.12–0.17 mm; Matsunami Glass Ind. (Osaka, Japan)) were soaked in 10% NaOH aq. soln. and incubated at room temperature for 12 h. After washing with water, glass substrates were soaked in 1% HCl aq. soln. and washed with water and then dried under nitrogen stream. After that, glass substrates were soaked in 1% 3-mercaptopropyltrimethoxysilane (MPTMS) or 3-glycidoxypropyltrimethoxysilane (GPTMS)/toluene and incubated at room temperature for 10 h and washed with acetone and then dried under nitrogen stream. GPTMS modified glass substrates were further modified with PEG-diamine and sulfosuccinimidyl 4-(p-maleimidophenyl)butyrate (SMPB) according to reported procedure[10]. Briefly, the epoxy group of GPTMS on the glass surface was hydrated with HCl and then $NaIO_4$ oxidation was performed to obtain an aldehyde-modified glass surface. A 17 mM PEG-diamine/$CHCl_3$ solution was poured onto the aldehyde-modified glass surface to graft PEG, and the glass substrate was incubated at 74°C for 40 h. The free terminal amino group of the grafted PEG was modified with SMPB. The solution of 5 μM 5'-thiol modified OMUpy2 in 20 mM PBS was spotted onto a MPTMS or PEG-SMPB modified glass surface by a microarray spotter (Stampman, NLE Lab., Nagoya, Japan) with split stainless-steel pin (ϕ = 0.15 mm). After spotting, the glass substrate was incubated at room temperature for 24 h in a humidified chamber. In the case of the PEG-SMPB modified glass surface, the maleimido group on the glass surface was blocked with 3-mercaptopropionic acid. Finally, glass substrates were washed with water and dried under

nitrogen stream.

12. All fluorescence images were obtained by a fluorescence microscope (ECLIPSE TE300, Nikon, Tokyo, Japan) equipped with a high-pressure Xe lamp, a digital CCD camera (ORCA-ER, Hamamatsu Photonics, Shizuoka, Japan), and an image intensifier unit (C8600, Hamamatsu Photonics, Shizuoka Japan). A 20-fold objective lens (SuperFluor 20×, Nikon, Tokyo, Japan) and a filter block consisting of band pass filters (340/15 and 480/30 nm) and a dichroic mirror (380 nm) were used for microarray observation and acquisition of fluorescence images. Fluorescence intensity was quantified from the fluorescence image by image processing software (Photoshop® 6.0, Adobe Systems Inc., CA, US)
13. Hybridization of oligonucleotides on OMUpy2 array. OMUpy2 array was pre-moisturized in water at room temperature for overnight. After rapid removal of water on the OMUpy2 array, the oligonucleotide solution (10 μl, 1 or 2 μM in 5 × SSC) was poured onto the surface of the OMUpy2 array. Thirty minutes after the addition of the oligonucleotide solution, fluorescence images were acquired by a fluorescence microscopic imaging system.

Table(s)

Table 1. Oligonucleotide sequence of OMUpy2 probe, ORN and ODN

Name	Sequence
OMUpy2-CF3	5'-thiol-C_6-d$(T)_{0,6,12}$-GAUGUGUUpyUpyCUCCUC-3'
OMUpy2-JN2	5'-thiol- C_6-d$(T)_{12}$-GAGGGCUpyUpyCGUGCGC-3'
OMUpy2-CM3	5'-thiol- C_6-d$(T)_{12}$-UUUpyUpyCAUUGUUUUCC-3'
cORN-CF3	5'-r(AGGAGAAACACAUC)-3'
ORN-Ctl	5'- r(GAAUGAACGUUGAAG)-3'
cODN-CF3	5'-d(AGGAGAAACACATC)-3'

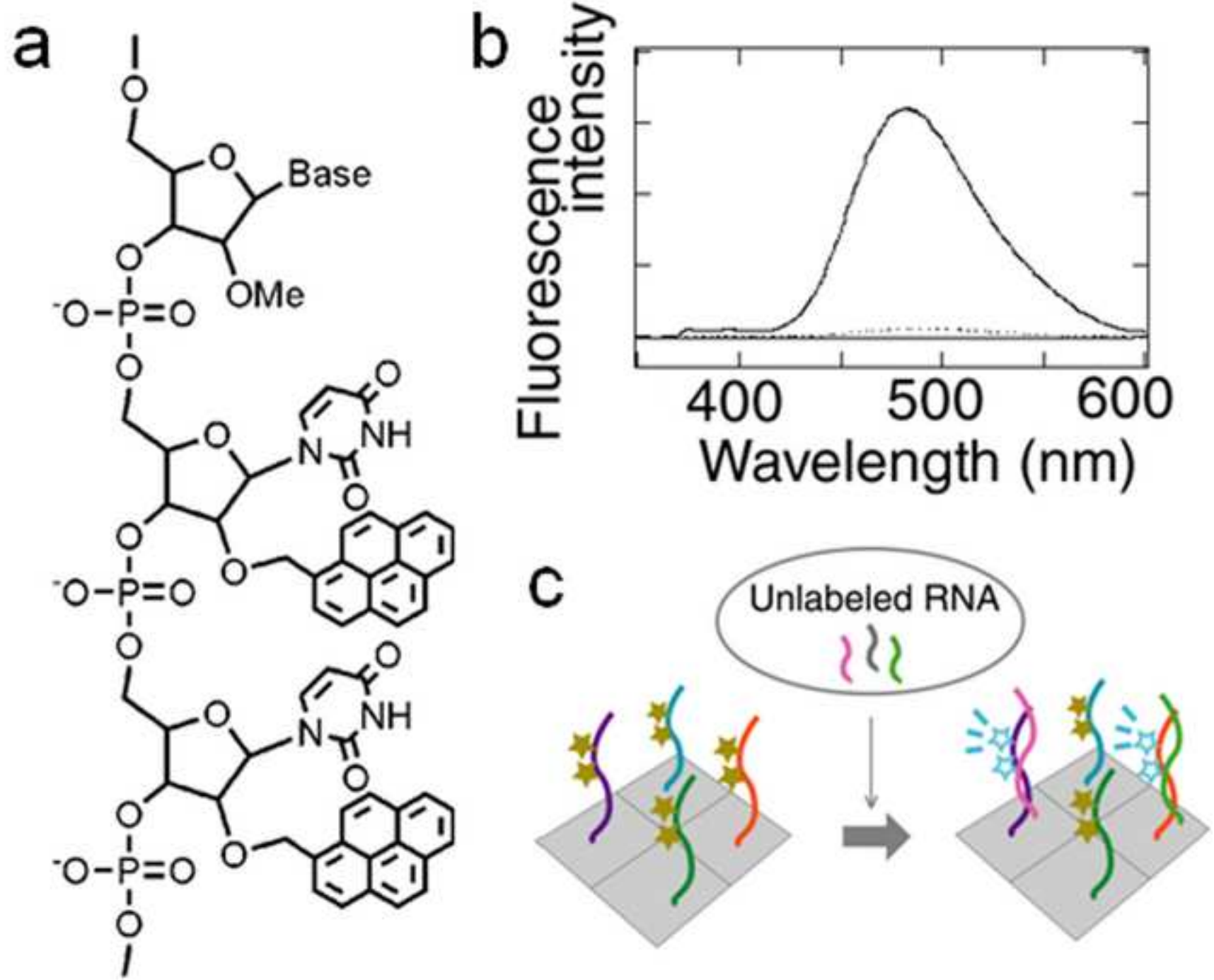
Underlined character indicates 2'-*O*-methylribonucleoside. Upy indicates 2'-*O*-pyrenylmethyluridine.

Table 2. Background fluorescence (BF), fluorescence signal (FS), and signal to background ratio (SBR) of the OMUpy2 array and OMUpy2 in homogeneous solution

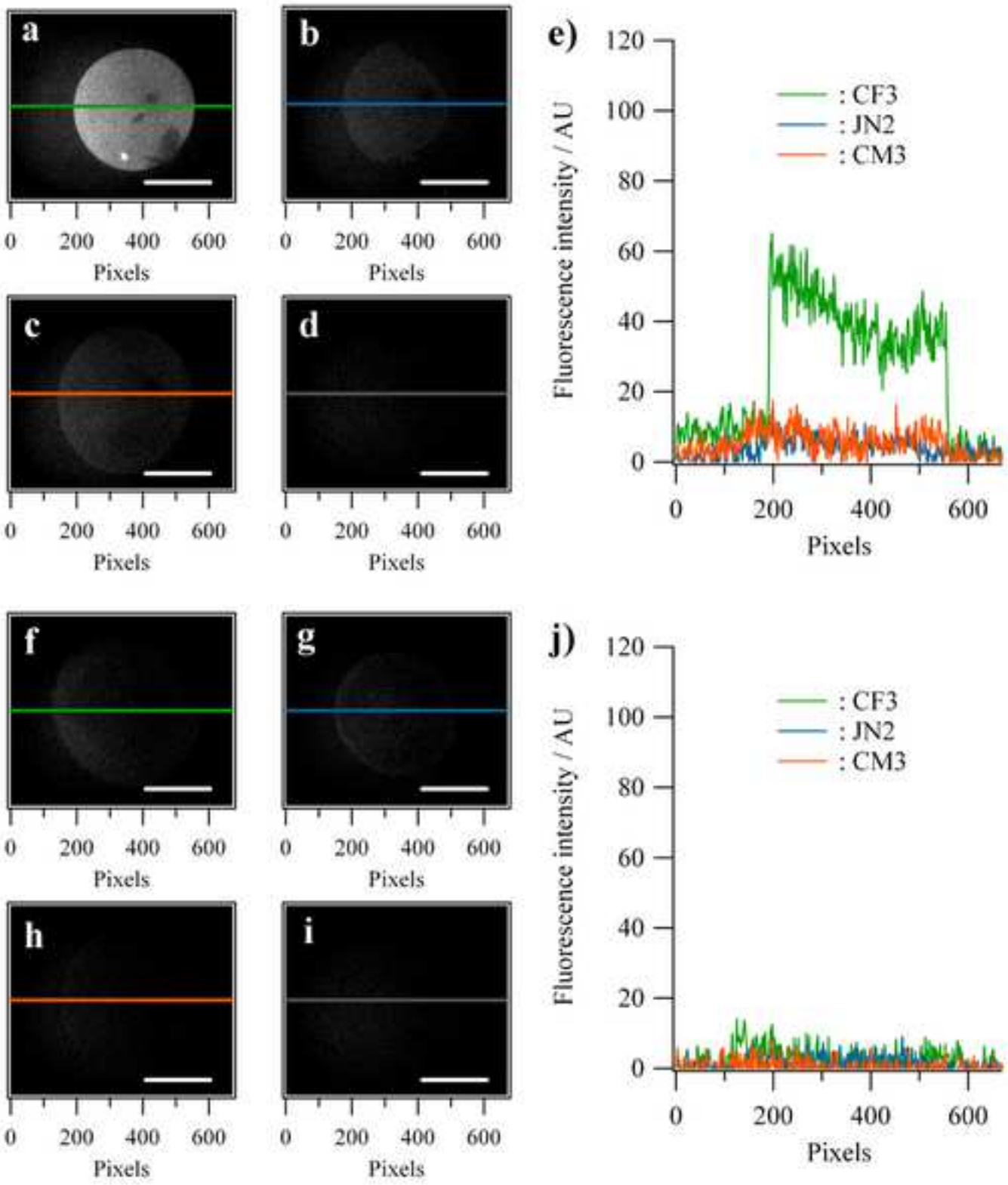
	Oligonucleotide added	BF	FS	SBR
OMUpy2 array[a]				
d$(T)_0$	cORN-CF3	73	117	1.6
d$(T)_6$	cORN-CF3	21	61	2.9
d$(T)_{12}$	cORN-CF3	2	11	5.4
PEG-d$(T)_{12}$	cORN-CF3	2	44	22
PEG-d$(T)_{12}$	ORN-Ctl	2	6	2.9
PEG-d$(T)_{12}$	cODN-CF3	4	10	2.5
In homogeneous solution[b]				
	cORN-CF3	2	176	88
	cODN-CF3	2	5	2.5

[a][cORN (or cODN)] = 2 μM in 5 × SSC.
[b][OMUpy2-CF3 (d$(T)_0$)] = 1 μM, [cORN (or cODN)] = 1 μM in 5 × SSC. Fluorescence intensity at 480 nm was measured by a spectrofluorophotometer with excitation at 342 nm at room temperature.

Figure(s)


a
Base
OMe
NH
NH
b
Fluorescence intensity
400
500
600
Wavelength (nm)
c
Unlabeled RNA

Figure(s)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40
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: CF3
: JN2
: CM3