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Real-time Imaging of RNA Expression in Living Cells Using Bispyrene-modified RNA Probes

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We successfully monitored time-dependent RNA expression profiles using bispyrene-modified RNA probe in living cervical carcinoma cells stimulated with EGF. Fluorescence microscopy images clearly showed the RNA expression profile, and our time-dependent profiles were in good agreement with those obtained by RT-PCR. This suggests that our novel method can analyze cellular responses to specific stimuli in a time-dependent manner at a single-cell level.

The FANTOM Consortium revealed interesting features of RNA.[1] To determine RNA functions, molecular biological methods such as Northern blotting, RT-PCR, and DNA microarray analysis are commonly used. However, real-time RNA analysis cannot be performed since these methods require cell or tissue lysates. To elucidate expression profiles and their localization, methods to temporally analyze RNA expression at the single-cell level are needed. Several methods for detecting RNA in living cells using fluorescent probes have been reported.[2] Fluorescence-labeled linear oligonucleotides probes,[2a] molecular beacons,[2b] FRET probes,[2c] quenched autoligation probes,[2d] and fluorescent protein-based probes[2e] can sensitively detect target RNAs in living cells. However, these probes cannot distinguish RNA from DNA, and they require certain delivery systems. Thus, the time-dependent analysis of RNA in living cells has not been well demonstrated. In a previous report, we showed that bispyrene-modified 2′-*O*-methyl RNA probes (OMUpy2)[3] specifically detected target RNAs in cell-free media,[3] fixed cells,[4] and living cells.[4] OMUpy2 can distinguish RNA from DNA and can be delivered into cells without any drug delivery systems. Therefore, OMUpy2 could be employed for real-time RNA detection.

In this report, we describe a novel method for RNA expression profiling in a time-dependent manner in living cells in response to stimuli. We chose c-*fos* mRNA as a target RNA, which is a typical transcript known to be an immediately early response gene.[5] We demonstrated the time-dependent monitoring of c-*fos* mRNA transcription in living cells using OMUpy2. Thus, we successfully achieved RNA expression profiling.

We chose HeLa and C4II cells (cervical carcinoma cells) to establish the feasibility of our RNA expression profiling method. To study the c-*fos* mRNA expression profiles in these cell lines, RT-PCR analysis was used. As shown in Figure 1A (HeLa), at 0 min, c-*fos* mRNA was only minimally expressed. The expression level reached its maximum at 20–30 min after EGF[6] stimulation and then gradually decreased. For C4II cells (Figure 1B), at 0 min, c-*fos* mRNA was also minimally expressed. The expression level reached a maximum at 30–40 min after stimulation. The c-*fos* mRNA expression profiles were a little different between these two cell lines.

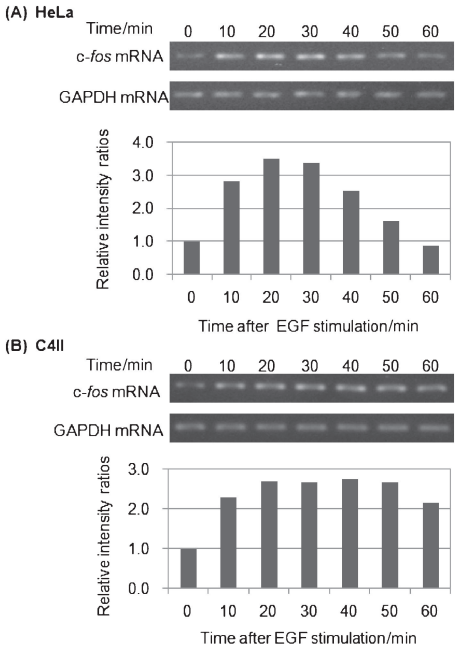

Figure 1. RT-PCR analysis of c-*fos* mRNA in HeLa and C4II cells after EGF stimulation. RT-PCR analysis of total RNA extracted from EGF treated HeLa cells (A) and C4II cells (B). (EGF: 10 ng mL^{-1}) PCR products were analyzed by 2% agarose gel electrophoresis and visualized with ethidium bromide. The c-*fos* mRNA expression levels were normalized to those of GAPDH mRNA. The results are relative intensity ratios to the expression level before EGF stimulation.

Next, real-time RNA detection was performed. We attempted to detect c-*fos* mRNA expression profiles in both cell lines using bispyrene-modified RNA probes that are complementary to c-*fos* mRNA. Prior to monitoring c-*fos* mRNA in living cells, the cellular uptake and subsequent distribution of the probes were studied. We used 5′-fluorescein-labeled OMUpy2 with phosphorothioate linkages (F-OMUpy2-S).[4] Phosphorothioate linkages were introduced within the underlined sequences shown below to facilitate cellular uptake. The sequences for F-OMUpy2-S were: 5′-fluorescein-GAUGUGUUpyUpyCUCCUC-3′ (F-OMUpy2-fosS; complementary to c-*fos* mRNA), 5′-fluorescein-CUUCAACGUpyUpyCAUUC-3′ (F-OMUpy2-scrS; scrambled sequence).[4]

F-OMUpy2-S was delivered into quiescent HeLa and C4II cells for 4 or 6 h at 0.5 or 5 μM, respectively. The cells were washed 3 times with HBSS[6] and then observed with a fluorescence microscope at 37 °C. Real-time imaging data are shown in Figure 2A (HeLa) and Figure 2B (C4II). Fluorescence intensity per cell was quantified using ImageJ software, and the

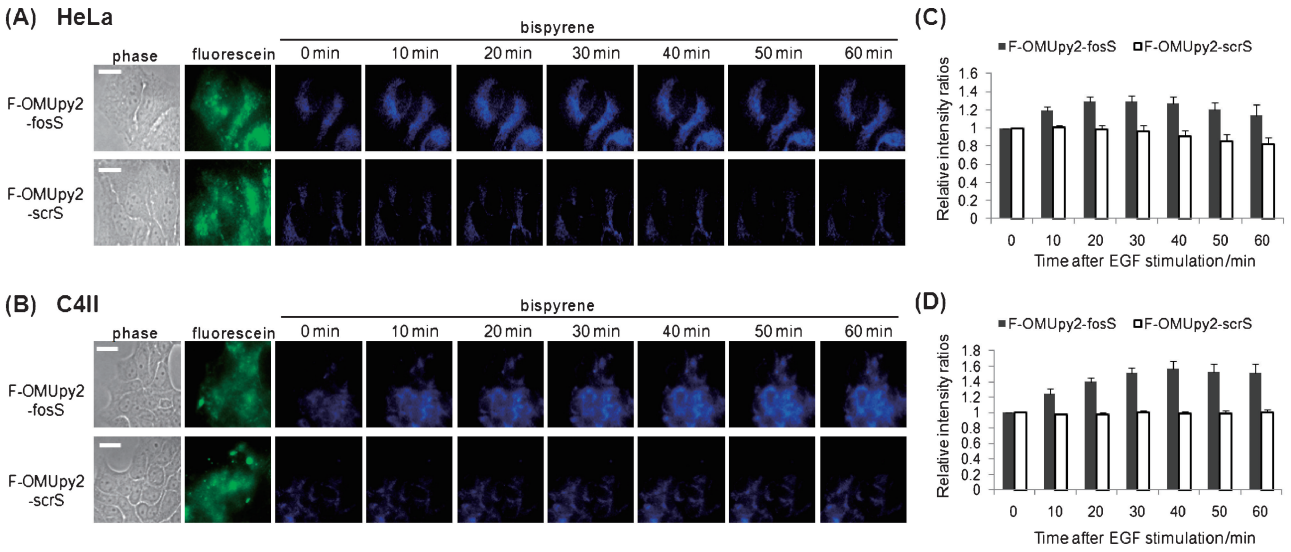

Figure 2. Real-time imaging of c-*fos* mRNA using F-OMUpy2-S. Real-time imaging data of c-*fos* mRNA using F-OMUpy2-S in living HeLa cells (A) and living C4II cells (B) after EGF stimulation. Fluorescence intensity was quantified using ImageJ and the relative intensity was calculated. Results are summarized for (C) HeLa and (D) C4II cells. Data were acquired using NIS-Element Ar software (Nikon). All fluorescent images were converted to pseudo-colors (fluorescein: green; bispyrene: blue). Fluorescence intensity per cell was quantified using ImageJ and background was subtracted from the total fluorescence intensity. Data at each time point were normalized to the intensity of EGF untreated cells and are given as relative intensity ratios. Scale: 20 µm. Filter sets: fluorescein (green), with Ex 450–490 nm, DM 505 nm, Em 520 nm; bispyrene (blue), with Ex 340/10 nm, DM 380 nm, Em 480/30 nm.

results are shown in Figures 2C and 2D. Fluorescein emission (green) was observed in the cytoplasm in both cell lines, which indicated that probes were taken up by cells and then distributed into the cytoplasm (Figures 2A and 2B). There were no differences in distribution for the probe sequences or cell lines. For HeLa cells (Figures 2A and 2C), when F-OMUpy2-scrS was delivered into the cytoplasm, bispyrene emission (blue) was minimally observed in the cytoplasm and its intensity remained nearly unchanged throughout the observation period (0–60 min). In contrast, the bispyrene showed a characteristic emission pattern in F-OMUpy2-fosS treated HeLa cells (Figure 2A). At 0 min, the fluorescence intensity was essentially equal to F-OMUpy2-scrS treated HeLa cells, and gradually increased up to a maximum at 20 min. These results are in good agreement with the RT-PCR analysis shown in Figure 1A. The fluorescent spectrum from F-OMUpy2-fosS treated cells was confirmed to have a maximum at 480 nm, which indicated that the emission was due to c-*fos* mRNA–F-OMUpy2-fosS hybrids.[4] At 0 min, the fluorescence intensity of C4II cells (Figures 2B and 2D) was essentially equal to that of F-OMUpy2-scrS treated C4II cells, and gradually increased up to a maximum at 40 min. These results were in good agreement with those obtained by RT-PCR analysis shown in Figure 1B. We also performed a time-dependent analysis of c-*fos* mRNA expression using OMUpy2-S that was not conjugated with fluorescein. These data were in good agreement with the data using F-OMUpy2-S as shown in Figure 2 (data not shown). Based on these results, our method can detect a real difference in RNA expression profiles between these two cell lines. After reaching a maximum level, the fluorescence intensity did not decrease appreciably. This suggested that the binding of F-OMUpy2-fosS to c-*fos* mRNA could affect the rate of c-*fos* mRNA degradation.

In conclusion, we successfully monitored the time-dependent c-*fos* mRNA expression in living cells. This RNA live-cell profiling method is a novel means to analyze the responses time of cells to specific stimuli at the single-cell level. This method could be applied generally to analyze other RNA expression. Furthermore, this method could be an effective means to elucidate the functions of noncoding RNA and RNA-induced silencing complexes (RISC).

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References and Notes

1 The FANTOM Consortium, *Science* **2005**, *309*, 1559.
2 a) T. Kubota, S. Ikeda, H. Yanagisawa, M. Yuki, A. Okamoto, *Bioconjugate Chem.* **2009**, *20*, 1256. b) S. Tyagi, F. R. Kramer, *Nat. Biotechnol.* **1996**, *14*, 303; N. Nitin, P. J. Santangelo, G. Kim, S. Nie, G. Bao, *Nucleic Acids Res.* **2004**, *32*, e58. FRET, fluorescence resonance energy transfer: c) K. Okabe, Y. Harada, J. Zhang, H. Tadakuma, T. Tani, T. Funatsu, *Nucleic Acids Res.* **2011**, *39*, e20; P. J. Santangelo, B. Nix, A. Tsourkas, G. Bao, *Nucleic Acids Res.* **2004**, *32*, e57. d) H. Abe, E. T. Kool, *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 263. e) L. Haim, G. Zipor, S. Aronov, J. E. Gerst, *Nat. Methods* **2007**, *4*, 409.
3 A. Mahara, R. Iwase, T. Sakamoto, K. Yamana, T. Yamaoka, A. Murakami, *Angew. Chem., Int. Ed.* **2002**, *41*, 3648.
4 R. Waki, A. Yamayoshi, A. Kobori, A. Murakami, *Chem. Commun.* **2011**, *47*, 4204.
5 M. E. Greenberg, E. B. Ziff, *Nature* **1984**, *311*, 433.
6 EGF, epidermal growth factor; HBSS, Hanks balanced salt solution. HBSS contains 25 mM HEPES and 0.1 mM non-essential amino acids (NEAA).

