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Hybridization-Sensitive Fluorescent Oligonucleotide Probe Conjugated with a Bulky Module for Compartment-Specific mRNA Monitoring in a Living Cell

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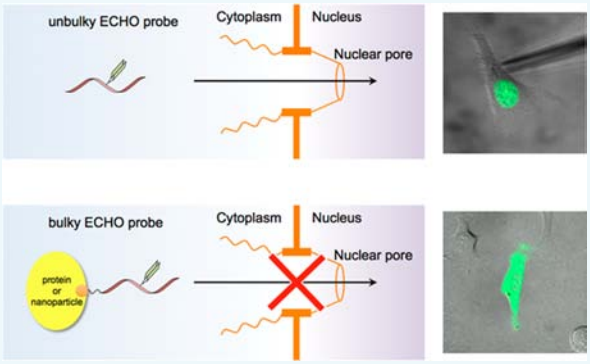
S *Supporting Information*

ABSTRACT: Live-cell RNA imaging at specific intracellular locations is technically limited because of the diffusive nature of small oligonucleotide probes. The bulky fluorescent light-up probes that possess streptavidin or gold nanoparticles at the end of oligonucleotides were designed and synthesized. The bulky probes allowed nucleus- and cytoplasm-selective monitoring of endogenous mRNAs through nuclear and cytoplasmic microinjection, respectively. Simultaneous use of bulky and unbulky probes conjugated with different fluorescent dyes enabled dual color imaging of mRNAs present in nucleus and cytoplasm. Furthermore, we observed that the fluorescence near the cell edge in a living HeLa cell traveled over time in coordination with the dynamic formation and deformation of the pseudopodial protrusions after lipofection of the bulky probes.



INTRODUCTION

Many of the mRNA species control cell fate and function by localizing into subcellular compartment such as organelles, cellular poles, and pseudopodial projections through *cis*-acting localization elements and/or specific binding proteins.[1,2] Visualization of mRNA molecules in living cells may provide key information for answering biological questions, understanding disease mechanism, and designing new therapeutic strategies. One of the most popular techniques for live-cell RNA imaging is the use of nucleic acid probes, which have a complementary sequence against the target RNA and a fluorescent molecule.[3,4] Such nucleic acid probes have in principle few restrictions to detect any endogenous RNAs, as it is not necessary to attach any engineered sequences for tagging RNAs of interest.

In the live-cell RNA imaging, where probes cannot be washed out to reduce the background signal such as done in in situ hybridization (ISH) experiments, several fluorescent turn-on probes have been developed to increase the signal-to-noise ratio.[5−7] A molecular beacon, that is, a hairpin-shaped single-stranded nucleic acid probe with an internally quenched fluorophore whose fluorescence is recovered upon binding to target RNA species, is the most prevalent technique.[8−10] Our group has developed another type of turn-on probe, called an ECHO (Exciton-Controlled Hybridization-sensitive fluorescent Oligonucleotide) probe.[11,12] The background signal of the ECHO probe in the unbound state is suppressed low owing to the excitonic interaction provided by H-aggregation of the bis-thiazole orange moiety. On the other hand, stronger emission is shown after hybridization with target RNAs through disruption of H-aggregation and intercalation of thiazole orange dye into the duplex structure. Over 10 kinds of thiazole-orange derivatives with different fluorescence properties have been synthesized and incorporated to give ECHO probes for multicolor imaging.[13,14] Although these turn-on nucleic acid probes have succeeded in real-time monitoring of target RNA species in living cells, practical methods for cellular compartment-specific imaging are still under development due to the diffusive property of the probes. Notably, it has been reported that oligonucleotides introduced into living cells through microinjection or endocytosis are localized in the nucleus for unknown reasons.[15,16] We have also observed that microinjected or transfected ECHO probes, which consist of polyT sequences that were designed to detect polyA tail of mRNA, tended to be located at the cell nucleus.[11,13]

To overcome the problem of nuclear accumulation of oligonucleotide probes, there have been several efforts,

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including attachment of signal molecules such as nuclear export peptides or tRNA to exploit endogenous active transport pathway[17] and large proteins that cannot traverse the nuclear pore complex.[18,19] In this work, we have established bulky ECHO probes (Figure 1), which have streptavidin tethered at the 5′- and 3′-ends of the oligonucleotides, and examined cytoplasm-selective mRNA imaging. A gold nanoparticle that is even larger than streptavidin was also attached and tested for intracellular localization. Simultaneous use of both bulky and unbulky ECHO probes enabled two-color imaging, in which mRNAs localized in the nucleus and cytoplasm were distinguished in different fluorescence emission wavelengths. Furthermore, we observed the restricted fluorescence of bulky ECHO probe near the edge of a HeLa cell in conjunction with the dynamic formation and deformation of pseudopodia after lipofection of the bulky probe.

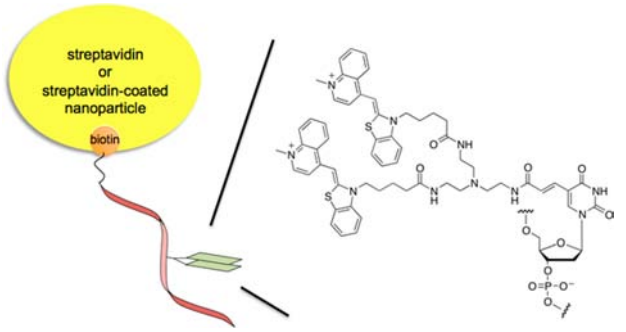

Figure 1. Illustration of bulky ECHO probe and chemical structure of D_{514}.

RESULTS AND DISCUSSION

Design, Synthesis, and Characterization of Bulky ECHO Probe. Nuclear pore complexes (NPCs), which constitute large aqueous transport channels embedded in the nuclear membrane, are the sole gateway to mediate the bidirectional exchange of small molecules, nucleic acids, and proteins between nucleus and cytoplasm.[20,21] The molecules possessing molecular mass >40 kDa are not capable of passive diffusion through NPCs. Nucleic acid probes (normally <20 kDa) conjugated with large proteins or particles would allow nucleus- and cytoplasm-selective RNA monitoring. Bulky ECHO probes bearing streptavidin or gold-nanoparticles were designed to prevent the probes from passing through the nuclear pores. We synthesized 5′ and 3′ biotin-linked ECHO probes that consist of a poly(dT) sequence for targeting polyA tails of mRNA and doubly fluorescent dye-labeled nucleotides, D_{514} or D_{600}, which emit green or red fluorescence, respectively.[13]

The bulky ECHO probes retain "light-up" characteristics upon hybridization through the same mechanism as conventional ECHO probes. Photochemical properties of the bulky ECHO probes conjugated with streptavidin were investigated in vitro by fluorescence and UV−visible absorption spectroscopy. In the presence of the complementary polyA sequence, both D_{514} and D_{600} probes gave strong fluorescence emission, whereas only slight signals were observed in the single-stranded state of the probes (Figure 2A and B). Notably, the differences of fluorescence intensity between hybridized and unhybridized states of D_{514} and D_{600} probes were more than 184-fold at 533 nm and 33-fold at 640 nm, respectively. The absorption spectra

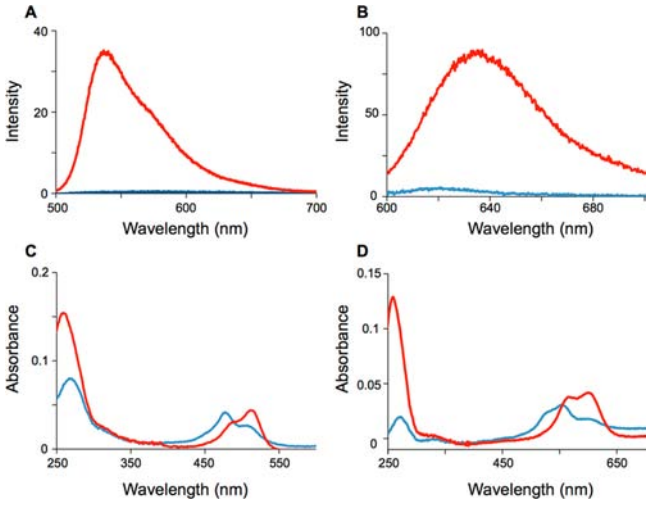

Figure 2. Photochemical properties of bulky ECHO probes. (A) Fluorescence spectra of streptavidin-tethered D_{514} probe (5′-TTTT-TTD_{514}TTTTTT-biotin-streptavidin-3′) excited at 488 nm. (B) Fluorescence spectra of streptavidin-tethered D_{600} probe (5′-streptavidin-biotin-TTTTTTTD_{600}TTTTTT-3′) excited at 514 nm. (C) Absorption spectra of D_{514} probe. (D) UV−vis absorption spectra of D_{600} probe. Hybridized state and unhybridized state were shown in red and blue, respectively. In each experiment, a 13mer polyA oligonucleotide was used as the complementary strand.

of the dye moieties on both ECHO probes showed red shift upon hybridization to the complementary strands (Figure 2C and D), indicating that H-aggregation of the two adjacent chromophores was released to form a fluorescently active state.

Then, we analyzed the conjugation between biotin-labeled ECHO probe and streptavidin by gel shift assay (Supporting Information Figure S1). When more than 2 equiv streptavidin was added against ECHO probe, the band of unconjugated ECHO probe completely disappeared. Because streptavidin monomer forms a stable homotetramer in the biological condition, the bulky ECHO probe mixture containing more than 4 equiv of streptavidin would predominantly exist in a one-to-one complex of streptavidin tetramer with a single ECHO probe.

Nucleus- and Cytoplasm-Specific mRNA Imaging. To evaluate the intracellular behavior of the bulky ECHO probes for mRNA visualization, the streptavidin-tethered probe containing D_{514} nucleotide was microinjected into living HeLa cells. When the probe was injected into the cytoplasm, the fluorescence signals were mainly observed from the outside of the nucleus (Figure 3A). On the other hand, nuclear injection of the same probe showed fluorescence signals from the nucleus (Figure 3B). However, conventional unbulky ECHO probes, which were microinjected into the cytoplasm or nucleus, showed signals only from the nucleus (Figure 3C and D). Attachment of a bulky moiety to the ECHO probes restricts the area of probe localization probably due to prevention of them passing through nuclear pores. Time-lapse imaging of the bulky ECHO probe showed that cytoplasm-selective fluorescence signals were retained for 10−20 min after cytoplasmic injection, whereas the fluorescence from the nucleus was also observed after 30 min (Figure 3E), suggesting that a streptavidin-linked ECHO probe might not be large enough to impair passive diffusion between the nucleus and cytoplasm.

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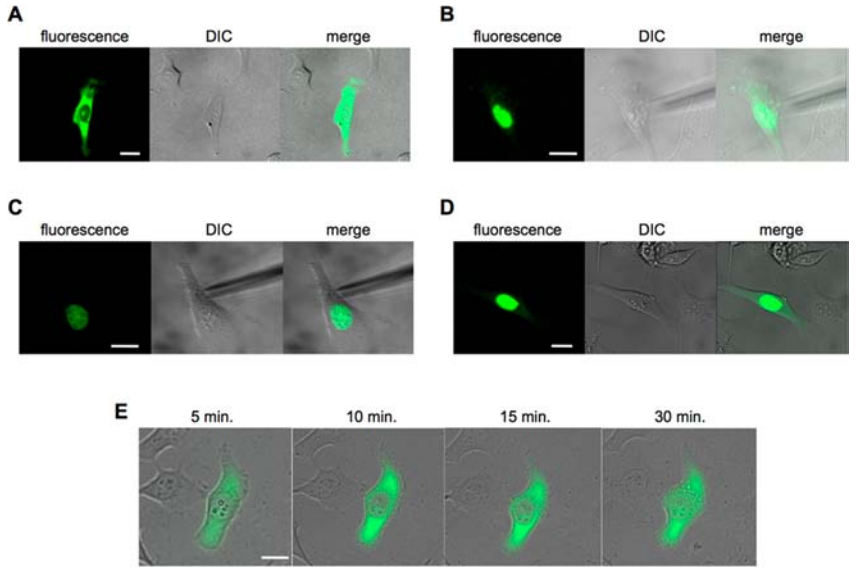

Figure 3. Fluorescence from the living HeLa cells after microinjection of bulky and unbulky ECHO probes. A streptavidin-tethered ECHO probe was injected into the cytoplasm (A) and nucleus (B). A conventional unbulky ECHO probe was also injected into the cytoplasm (C) and nucleus (D). (E) Time-lapse monitoring of a bulky probe microinjected into the cytoplasm was performed. All fluorescence images were obtained by excitation wavelength of 488 nm and emission filter of >505 nm. Scale bars show 20 μm.

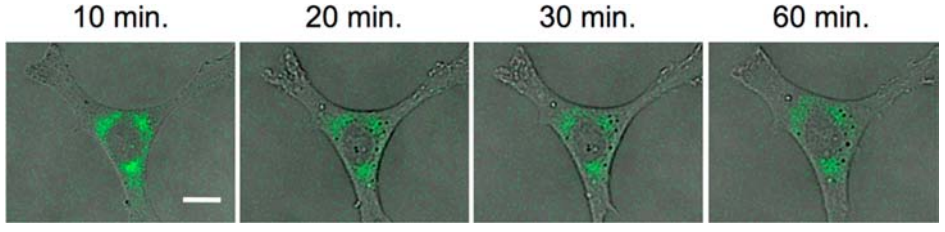

Figure 4. Time-lapse imaging of microinjected bulky ECHO probe conjugated with gold-nanoparticle. All fluorescence images were obtained by excitation wavelength of 488 nm and emission filter of >505 nm. Scale bar shows 20 μm.

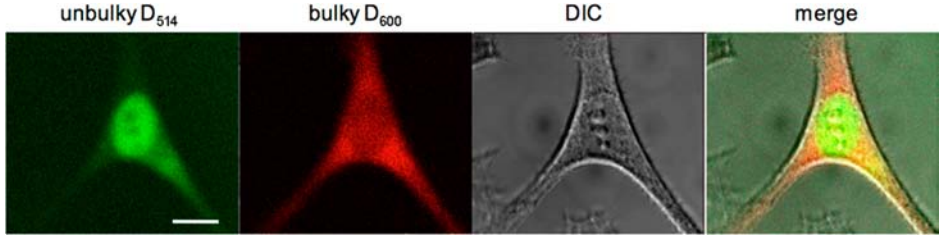

Figure 5. Dual color imaging by the combinatorial use of an unbulky ECHO probe containing D_{514} and a bulky ECHO probe containing D_{600}. The mixture of the probes was microinjected into the cytoplasm of living HeLa cells. Fluorescence images were obtained by an excitation wavelength of 488 nm and an emission filter of >505 nm for the D_{514} probe and an excitation wavelength of 543 nm and an emission filter of >615 nm for the D_{600} probe. Scale bar shows 20 μm.

To investigate the sequence dependence of this phenomenon, we synthesized 6 different biotin-labeled ECHO probes including 4 specific mRNA-binding sequences and 2 negative controls (Supporting Information Table S1). In vitro fluorescence measurements revealed that all of probes showed turn-on characteristics in the presence of complementary strands (Supporting Information Figure S2). After mixing with 4 equiv of streptavidin to form bulky probes, the probes were microinjected into the cytoplasm of HeLa cells. The fluorescence signals were observed from the cytosol and did not diffuse to the nucleus, whereas the probes without streptavidin were visualized mainly from the nucleus (Supporting Information Figure S3). It is notable that injection of the same amount of negative control probes did not show any fluorescence, suggesting that the observed fluorescence signals were derived from target specific hybridization. These results support the premise that cytosolic RNA visualization with bulky ECHO probes can be applied for various target sequences.

By controlling the bulkiness of the ECHO probe, it might be possible to image certain intracellular regions more precisely. A more bulky probe was designed which was modified with a streptavidin-coated gold nanoparticle. The diameter of streptavidin and the gold-nanoparticle is ~4 and ~15 nm, respectively. After microinjection of the nanoparticle-conjugated probe into the cytoplasm of a living HeLa cell, time-lapse monitoring of fluorescence signals was performed. No signal leakage into the nucleus was observed even after 60 min of injection, indicating the effect of the bulkiness (Figure 4).

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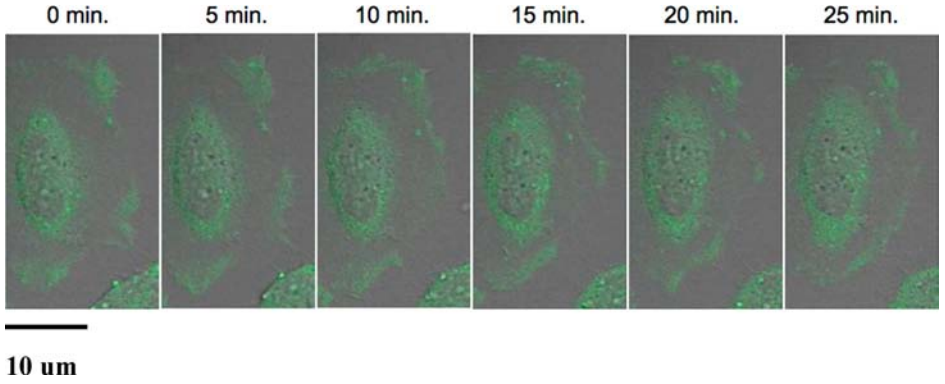

Figure 6. Time-lapse fluorescence imaging of a moving HeLa cell transfected with a streptavidin-linked ECHO probe by lipofection. The perinuclear region and cell edges were mainly stained. All fluorescence images were obtained by excitation wavelength of 488 nm and emission filter of >505 nm. Scale bar shows 10 μm.

Interestingly, the localized area of the fluorescence signals was restricted only at the perinuclear regions. This nanoparticle-conjugated ECHO probe might be too bulky to diffuse into some dense spaces such as cell edges where cytoskeletal fibers are continuously synthesized. These bulky ECHO probes consistently failed to pass through the nuclear pores and, thus, enabled cytoplasm-selective mRNA visualization.

Dual Color RNA Imaging for Nucleus and Cytoplasm. Multicolor imaging in living cells offers an advantage in investigation of the relationship between two or more target molecules or spaces at the same time. We distinguished cytoplasmic and nuclear mRNA by simultaneous use of unbulky and bulky ECHO probes containing two different dye-labeled nucleotides, D_{514} and D_{600}, respectively. The mixture of unbulky ECHO probe with D_{514} and streptavidin-tethered ECHO probe with D_{600} was microinjected into a living HeLa cell and monitored in two different fluorescence channels (520−550 nm for D_{514} and >615 nm for D_{600}). While the fluorescence signals derived from D_{514} were observed mainly from the cell nucleus, D_{600} signals were detected from the cytoplasm (Figure 5). The compartment-specific fluorescence emission suggests that the dual color imaging with the combination of unbulky and bulky ECHO probes could offer a new approach to investigating behavioral differences of nuclear and cytoplasmic mRNAs.

Monitoring of mRNA Movement at Cell Edge. Chemical-based transfection such as lipofection is also widely used for nucleic acid transfection against millions of cells. Lipofection has often been used for cell uptake of ECHO probes.[13] To observe the intracellular localization of a bulky ECHO probe after lipofection, streptavidin-tethered $U_{11}D_{514}U_{11}$, which has nuclease-resistant 2′-OMe uridines instead of T,[22] was transfected into living HeLa cells. In a freely moving cell, fluorescence was restricted to the cytoplasm especially in the perinuclear region and at mobile edges. The fluorescence near the edge of cells "traveled" over time in coordination with the dynamic formation and deformation of the pseudopodial protrusions (Figure 6 and Supporting Information movie). The fluorescence localization was reminiscent of the previously reported β-actin mRNA that localizes to the proximity of cell edges where actin filament assembly is actively promoting forward protrusions.[2] A large proportion of the $U_{11}D_{514}U_{11}$-biotin-streptavidin labeled polyadenylated RNA in the subcytoplasmic regions might have represented mRNAs coding cytoskeletal proteins such as actin. The fluorescent imaging with the streptavidin-tethered ECHO probe hints that specific subcytoplasmic regions may contain an "anchor" complex to restrict the movement of specific mRNAs.

We have developed a new type of ECHO probe that possesses a "bulky" moiety for cytoplasm-selective mRNA visualization. Bulky moieties such as streptavidin and gold nanoparticles restricted cytoplasmic localization of the probes in a cell due to inhibiting passive diffusion between the cytoplasm and nucleus through nuclear pores and gave us simultaneous visualization of cytoplasmic and nuclear mRNAs in two different fluorophores. The functionalized ECHO probe made it possible to monitor mRNA movement at the cell edge. Since techniques aiming at intracellular space-selective mRNA monitoring are not well-established yet, these ECHO probes will facilitate visualization and understanding of behaviors of compartmentalized endogenous RNAs.

EXPERIMENTAL SECTION

Synthesis of Bulky ECHO Probe. ECHO probes used in this study were prepared as previously described.[11,13] Briefly, oligonucleotide syntheses were performed by a conventional phosphoramidite method using an H6 DNA/RNA synthesizer (Nihon Techno Service Co., Ushiku, Japan). Besides standard phosphoramidites of dA, dG, dC, and dT, 5′-Biotin Phosphoramidite and 3′-BiotinTEG CPG were purchased from Glen Research Inc. and used to label biotin at the 5′- and 3′-ends, respectively. The diamino nucleotide phosphoramidite unit chemically synthesized was introduced into oligonucleotides as previously reported,[11] and then thiazole-orange derivatives were coupled to obtain ECHO probes. The coupling time of modified nucleotides on the DNA synthesizer was extended to 9 min. The synthesized oligonucleotides were cleaved from the support with 28% aqueous ammonia and deprotected at 25 °C for 10 h. The products were purified by reversed-phase HPLC on a CHEMCOBOND 5-ODS-H column with elution solution containing 0.1 M triethylamine acetate (TEAA, pH 7.0) and acetonitrile. MALDI-TOF mass spectrometry (microflex, Bruker Daltonics) was used for identification of synthesized oligonucleotides with 2′,3′,4′-trihydroxyacetophenone as a matrix. A 10 mg/mL solution of the 2,3,4-trihydroxyacetophenone (2,3,4-THAP) in 50% acetonitrile and a 10 mg/mL solution of 2,4,6-trihydroxyacetophenone (2,4,6-THAP) and 1 mg/mL diammonium citrate (DAC) in 50% acetonitrile were mixed at a ratio of 2:1 (v/v).

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Absorption and Fluorescence Measurement. UV−vis absorption and fluorescence spectra of single-stranded (unhybridized) and double-stranded (hybridized) probes (2.0 μM) were measured using spectrophotometers UV2550 (Shimadzu) and RF-5300PC (Shimadzu), respectively, in a quartz cuvette (1 cm path length) with buffer solution (100 mM sodium chloride, 50 mM phosphate, pH 7.0). The probes containing D_{514} and D_{600} were excited at 488 and 594 nm, respectively. Oligonucleotides for complementary strands were purchased from Eurofins Genomics.

Cell Culture. HeLa cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing heat-inactivated fetal bovine serum (10%), penicillin (50 mg/mL), and streptomycin (50 mg/mL) under a humidified atmosphere (5% CO_2) at 37 °C. Reagents for culturing were purchased from Sigma. For the following imaging experiments, cells were cultured in glass-based dishes (Matsunami). Prior to microscopic observation, the culture medium was washed and exchanged to a phenol red-free DMEM (OPTI-MEM, GIBCO).

Microinjection and Transfection. Microinjection of probes was performed using a pneumatic injector (FemtoJet express, Eppendorf) with glass needles (FemtoTip, Eppendorf) and 3-D manipulators (Narishige). Injection of each probe (50 μM) was performed at 100 hPa for 1.0 s in each cell (100 fL volume). For two-color imaging, the mixture of bulky probe containing D_{600} (20 μM) and a normal probe containing D_{514} (20 μM) was microinjected into HeLa cells cultured in phenol-red-free DMEM. Approximately 30% of the cells showed an abnormal change in cell shape as a result of the injection; those cells were not used in the experiments.

$U_{11}D_{514}U_{11}$-biotin (0.5 μM final concentration) was mixed with streptavidin at the molar ratio 2:1 in OPTI-MEM and incubated at r.t. for 15 min before lipofection into the HeLa cell. The probe was introduced using a transfection reagent (Lipofectamine 2000, Invitrogen) following the manufacturer's protocol. After 1 h incubation at 37 °C with the Lipofectamine 2000 and a probe, the cells were washed three times with PBS and S9 observed in phenol-red-free DMEM. The cells were maintained under culture conditions using an incubation system during fluorescence imaging.

Imaging. Cells were maintained in the culturing condition (37 °C, 5% CO_2) by an incubation system (INU; Tokai Hit) during the observation. Images were acquired with a motorized, inverted Axio Observer Z1 microscope (Carl Zeiss, Jena, Germany) equipped with a 636 objective (PlanApochromat NA 1.4, oil immersion) and an EM-CCD camera (evolve, Roper) and LSM510 META confocal unit (Zeiss). Each probe was excited with a He−Ne laser or an Ar laser. Differential interference contrast fluorescent images were acquired with a confocal unit LSM 510 META (Zeiss) equipped with Ar and He−Ne lasers and diode lasers. Appropriate excitation laser and fluorescent filters were used for each color probe (D_{514}: Ex 488, Em 505LP; D_{600}: Ex 543, Em 615LP). Excitation power (percentage of the laser output, 0.5%) and the gain of the detector (854) were fixed throughout these experiments. The acquired images were analyzed and processed with operation software (Zen2008, Zeiss).

Streptavidin Gel Mobility Shift Assay. ECHO probe (5 μM) and streptavidin (promega; 0, 2.5 μM, 5.0 μM, 10 μM, 20 μM, or 40 μM) mixture were incubated at 37 °C for 15 min and then combined with gel electrophoresis loading buffer. The sample was electrophoresed on a 12% PAGE denaturing gel containing 7 M urea (300 V, 15 min) and stained by incubation with SYBR Green II (Life Technologies) for 15 min.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Tables and figures as described. MALDI-TOF MS data. Dynamic formation and deformation of the pseudopodial protrusions video. 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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