Trends in Organic Chemistry
Vol. 17, 2014

Review

# Modified ECHO probes for highly specific RNA detection in living cells

**Akimitsu Okamoto***

Research Center for Advanced Science and Technology, The University of Tokyo, 4-6-1 Komaba, Meguro-ku, Tokyo 153-8904, Japan.

## ABSTRACT

The regulation of the mechanisms by which genomic information dictates cellular behavior is one of the most important and urgent research topics in the postgenomic era. Chemically modified nucleic acids would be key players in sensitive, quantitative, and real-time detection of specific transcripts. In this article, we review recent advances in nucleic acid detection using exciton-controlled hybridization-sensitive fluorescent oligonucleotide (ECHO) probe technologies. In ECHO probes, a hybridization-dependent fluorescent nucleotide regulated by the H-aggregation of thiazole orange dyes is incorporated into specific sequence contexts. ECHO probe serves as a fluorescent detection readout for target nucleic acids. In addition, auxiliary chemical functional modules such as pH sensitivity, photolabile ability, and enhanced hybridization property have been introduced into ECHO probes to accommodate a broad range of biological applications.

**KEYWORDS:** RNA, fluorescent nucleic acids, exciton, live-cell imaging, locked nucleic acid, caged nucleotide, desmethyl thiazole orange, phosphoramidite

## INTRODUCTION

Compared with DNA, which acts mainly as the storage of genetic information, RNA exhibits a wider range of functions, such as protein synthesis, gene regulation, and reaction catalysis [1-5]. Many transcriptional and post-transcriptional mechanisms, such as epigenetic regulation, splicing, RNA editing, and microRNA-mediated gene silencing, have been demonstrated to play critical roles in controlling the cellular behaviors, such as migration, division, differentiation, aging, and apoptosis. In addition, evidence is accumulating supporting the contention that the quantity, location, and timing of gene expression are highly regulated in, and critical for, cell functions. Spatiotemporal gene regulation has been observed in many cell types, particularly in cells with extraordinary geometry, such as neurons, in which an RNA molecule can be actively transported to specialized cellular compartments (e.g., synapses and growth cones) to perform its functions. The artificial nucleic acids based on photochemical ideas could be designed for observing both the spatial and temporal dimensions of the rich sequence information of a genome. In this article, we review recent advances in nucleic acid detection using exciton-controlled hybridization-sensitive fluorescent oligonucleotide (ECHO) probe technologies and further development of the probes by modification of their sugar backbone, nucleobase, and fluorescent dye for more specific RNA imaging.

## ECHO probes: principle, preparation, and imaging

Organic dyes are frequently conjugated to probes as a fluorescent readout for the detection of specific biomolecules, including nucleic acids, in highly heterologous environments. When excited by light in a specific range of wavelengths, such molecules emit fluorescence that illuminates specific

*okamoto@chembio.t.u-tokyo.ac.jp

targets in biological samples, usually via physical interactions between the probes and the target molecules. Background noise should be very low to allow sensitive and reliable detection of target biomolecules. As the photochemical properties of these dyes do not always differentiate their functional states (e.g., 'interactive' vs 'noninteractive' with a target), probes that are not interactive with targets have to be physically removed from the sample. If any residual fluorescent probes remain in the sample, they result in aberrant signals that interfere with detection of the true signal. In living cells, which rather resemble closed systems, complete removal of free or partially bound probes is difficult without causing damage to the cell; thus, the signal-to-noise ratio can be a serious problem that limits the effective detection of nucleic acids in living cells.

Photochemical fluorescence quenching technologies can play critical roles in designing smart probes that adapt to different photochemical properties for the recognition of target RNA. In these cases, changes in fluorescence properties can directly serve as readout for the detection of target RNA. Techniques based on photochemical quenching have been applied to monitor various types of RNA reactions using fluorescence. Some well-known nucleic acid imaging tools have been developed based on energy transfer processes, including molecular beacons [6], molecular break lights [7], Scorpion primers [8], and TaqMan probes [9]. When these approaches are used, efficient quenching is a prerequisite for reliable fluorescence observation. Although numerous photoquenching-based technologies have been developed that use different operating mechanisms and efficiencies, nucleic acid detection in a sensitive, quantitative, and instant fashion remains a challenge.

To develop a probe technology for nucleic acid imaging in which the fluorescent signals directly lead to target detection, we designed and synthesized a fluorescence-controlled nucleoside that is covalently conjugated to two thiazole orange (TO) moieties (Fig. 1a). TO is a fluorescent dye that binds to DNA via insertion into the DNA double-stranded structure. A substantial amount of research on linking TO dyes to artificial DNA strands or peptide nucleic acid strands has already been reported [10-19]. Indeed, the fluorescence intensities of these probes have been changed depending on hybridization with the complementary nucleic acids; however, the fluorescence in their single-strand states is often not sufficiently suppressed to be considered as negligible background fluorescence. Although such problems exist, there is no doubt that TO is a very effective dye for nucleic acid detection. A more precise design of fluorescent oligodeoxynucleotides (ODNs) based on characteristic photophysical properties of TO would give us ODN probes that are more sensitive.

The fluorescence of TO in aqueous solution is strongly suppressed when TO is involved in dimer formation [20, 21]. This suppression is due to an aggregated state of several fluorescent dye moieties arranged in parallel (H-aggregation). Under the exciton coupling effect, the excited state of the dye aggregates splits into two energy levels (E′ and E″) because of a resonance interaction (Fig. 1b). After excitation, the energy transition from the ground level to the lower energy level (E′) is blocked, whereas the transition to the upper energy level (E″) is allowed. This transition to the elevated energy state is followed by rapid conversions between the E′ and E″ levels, which effectively prevent radiative energy release from the excited dye aggregates, resulting in fluorescence quenching. Based on the excitonic coupling effect of TO dyes, we hypothesized that an ODN probe labeled with a TO dimer should exhibit suppressed fluorescence emission in the single-stranded state, but become highly fluorescent when forming double strands with target complementary nucleic acids. The fluorescence turn-on should follow the bis-intercalation of TO dyes into the probe-nucleic acid duplex after hybridization. This hypothesis underlies our rational design of a probe that exhibits a clear distinction in fluorescence intensity between the ON and OFF states, which can be used as an efficient fluorescent readout for nucleic acid imaging (Fig. 1c). In this probe technology with the exciton coupling effect, a single pyrimidine labeled with the homodimer of TO was designed (Fig. 1c). We termed this fluorescence-controlled nucleoside $D_{514}$, meaning doubly labeled nucleotide with an excitation maximum at 514 nm [22-25] (Fig. 1d).

To obtain an ODN probe containing $D_{514}$, a synthetic nucleoside with two amino linkers was first prepared from a uridine derivative **1** with an acrylate group at position C5 [26, 27] (Scheme 1). Two primary

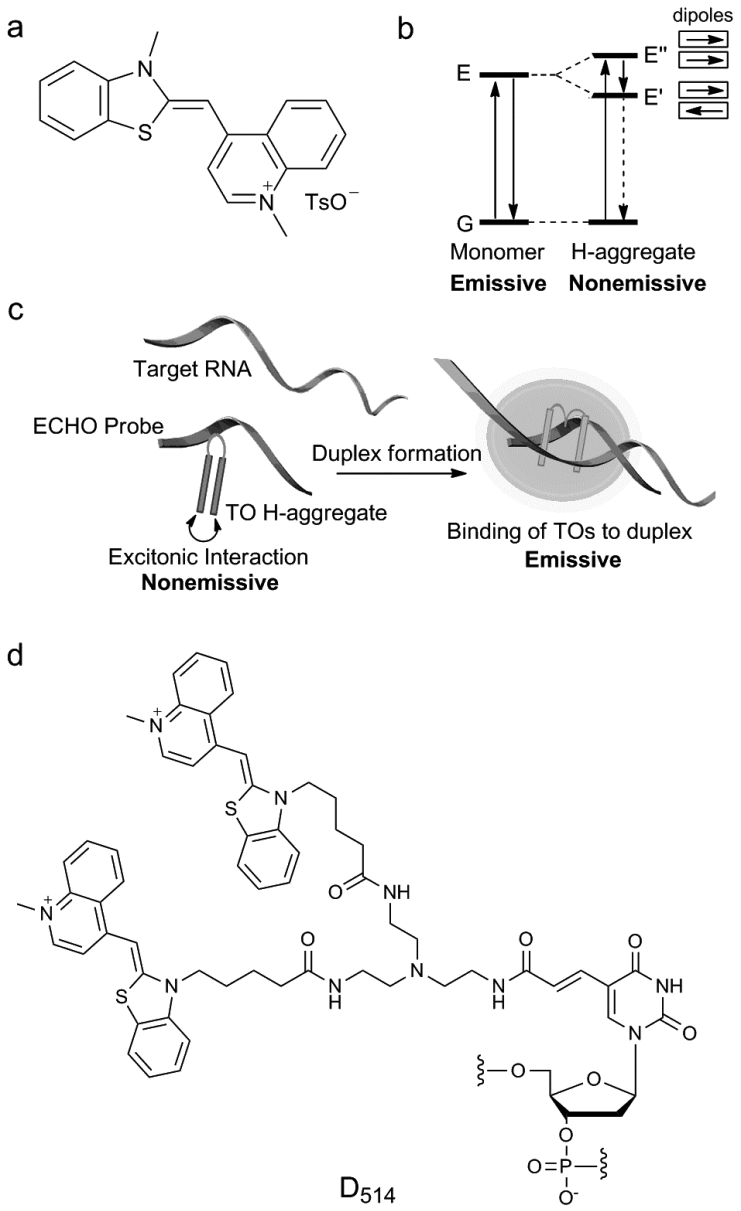
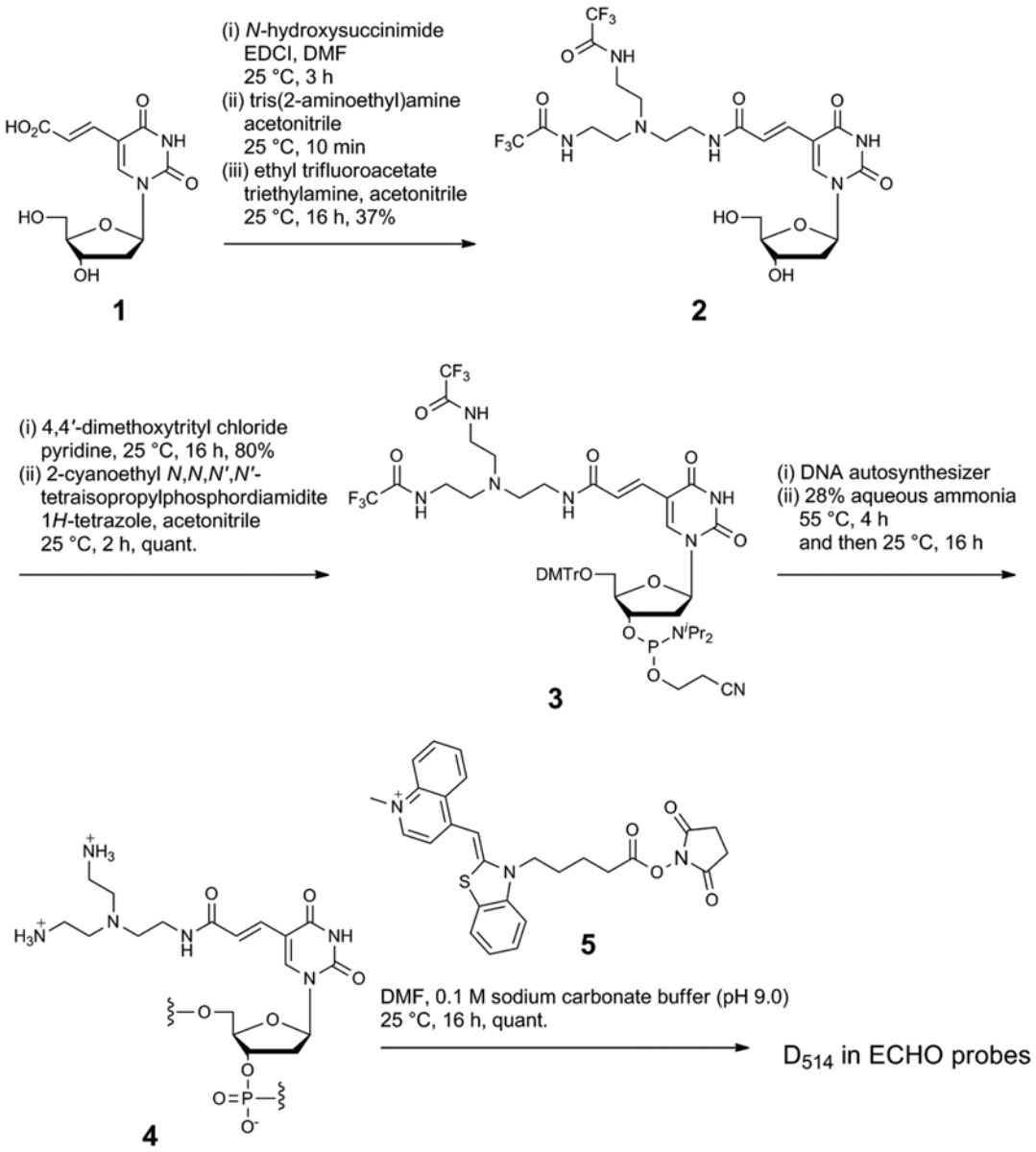



**Fig. 1.** The synthetic nucleotide $D_{514}$ with fluorescence emission control: (a) chemical structure of a thiazole orange (TO) dye; (b) Jablonski energy diagram illustrating the exciton coupling effect of an H-aggregate; (c) design of a fluorescence emission controllable hybridization-sensitive nucleotide that 'lights up' after recognition of the complementary target; (d) chemical structure of $D_{514}$ in the ECHO probe.

amino groups obtained by the coupling of **1** with tris(2-aminoethyl)amine were protected by trifluoroacetyl groups to give nucleotide **2**. Protection of the 5′-hydroxy group and the following phosphoramidation of the 3′-hydroxy group gave **3**, which was then incorporated into a single ODN strand using the conventional phosphoramidite method on a DNA autosynthesizer to give **4**. The TO derivative **5** was synthesized from a benzothiazole derivative. The diamino-modified ODN strand was then mixed with **5** and the reaction product was purified using reverse-phase HPLC coupled to UV-vis monitoring. In support of our hypothesis, ODN probes carrying a single $D_{514}$




**Scheme 1.** Synthesis route of ECHO probes using a DNA autosynthesizer.

showed the characteristic photophysical behavior of the exciton coupling effect. The absorption spectra obtained from two $D_{514}$-containing ODN probes had two major bands (at 480 and 510 nm) with different signal intensities. In the absence of complementary nucleic acid, the 480 nm band showed higher signal intensity, which indicates the dimer state of TO under the influence of the exciton coupling effect; however, the band at 510 nm became predominant after the $D_{514}$-containing ODN was hybridized to the complementary nucleic acid, which is consistent with a conformational change of the TO dimer to a monomer (Fig. 2). Concomitantly, the fluorescence emission of the ODN probes was robustly turned on, indicating the release of the excitonic interaction and the intercalation of TO dyes into the duplex [28]. Remarkably, the fluorescence emission of a polypyrimidine sequence $T_6D_{514}T_6$ was almost completely quenched in the single-stranded state, and addition of polyadenine to the probe solution resulted in >160-fold increase in fluorescence intensity at 530 nm. The excitation spectra displayed a single

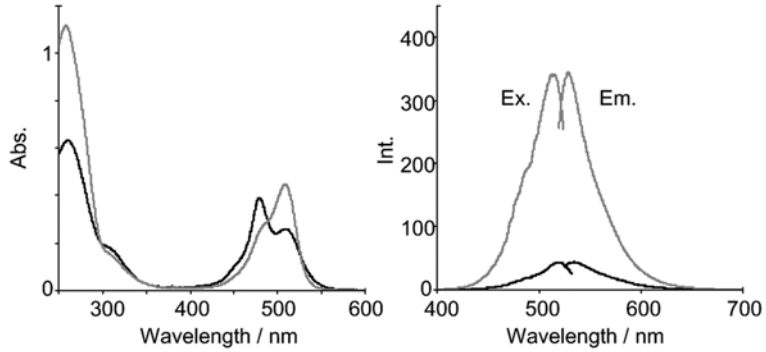



**Fig. 2.** Absorption (left), excitation, and emission spectra (right) of CGCAATD$_{514}$TAACGC in the absence (black) or presence (gray) of GCGTTAAATTGCG.

broad peak at about 510 nm, regardless of the single- or double-stranded state of the probes. This wavelength is in good agreement with the predominant absorption band observed at 510 nm when the probe is in the duplex state, indicating that only absorption at 510 nm effectively participates in the fluorescence emission. Exciton-controlled fluorescent nucleotides represent a novel molecular design for emission-controlled nucleic acid imaging, which we have now termed exciton-controlled hybridization-sensitive fluorescent oligodeoxyribonucleotide (ECHO) probe technology.

Fluorescence *in situ* hybridization (FISH) is a powerful imaging method used in karyotyping, cytogenotyping, cancer diagnosis, species specification, and gene expression analysis [29-34]. Although widely used in both the academic and clinical sectors, conventional FISH methods are still cumbersome and time-consuming. Taking advantage of the target-dependent fluorescent turn-on mechanism of ECHO probes, we developed a high-resolution FISH protocol by adapting the ECHO probes as a fluorescent readout (ECHO-FISH) [35]. The new protocol includes no stringent wash steps, resulting in a 25-min procedure from fixation to mounting. Because of the superior signal-to-noise ratio of ECHO probes compared with conventional ODN probes, clear fluorescence detection of poly(A)$^+$ RNA in HeLa cells was achieved (Fig. 3). Poly(A)$^+$ RNAs and gene-specific transcripts were successfully detected using the stringency to single mismatches. Because of the simplicity of the protocol, ECHO-FISH is highly reproducible, stringent, and compatible with other fluorescent cellular labeling techniques,

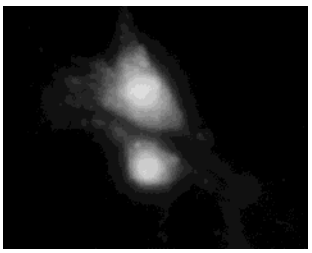


**Fig. 3.** ECHO-FISH of dissociated rat hippocampal primary cultures stained with $T_6D_{514}T_6$.

allowing analysis of gene expression in specific cell types in a highly heterologous cell population. Poly(A)$^+$ RNAs in HeLa cells and dissociated hippocampal primary cultures were identified in intranuclear speckles, and mRNAs located in the distal dendrites of hippocampal neurons were identified using ECHO probes. Therefore, the fine subcellular localization patterns of RNA expression can be studied using ECHO-FISH. In addition to RNA detection, telomeric and centromeric DNA on metaphasic mouse chromosomes were also detected using specific probes that hybridize to the repeated sequences.

## ECHO-LNA chimeric probes: backbone modification for sequence-specific and tight hybridization

RNA is highly diverse in terms of size, structure, mass, function, location, and expression timing, as well as in terms of sequences [1-5]. In particular, the structural and sequential diversity of RNA, such as higher-ordered structure formation and one-base

mutations, plays an important role in characterizing the function of RNA. However, the functions of fluorescent nucleic acid probes, even ECHO probes, are sometimes insufficient for effective detection of a variety of RNA molecules, because accessing the RNA sequence that forms a higher-ordered structure and distinguishing the target RNA sequence from the one-base mutants are often thermodynamically difficult for fluorescent nucleic acid probes [36].

Locked nucleic acid (LNA) is a modified nucleotide that contains a methylene bridge connecting the 2′ oxygen and the 4′ carbon of the ribose [37] (Fig. 4). ODNs containing LNAs have enhanced hybridization properties and thermostability with complementary DNA or RNA molecules; therefore, they exhibit properties that are desirable to increase detection sensitivity and specificity in nucleic acid imaging. In particular, LNA trinucleotides surrounding a mismatched nucleotide maximize discrimination efficiency and their strand invasion property has been used to promote probe accessibility to RNA regions with higher structures. Based on these properties of LNA nucleotides, we constructed ECHO-LNA conjugates in which some residues of the ECHO probe were modified to LNA [38].

Insertion of LNA nucleotides into the hybridization-sensitive fluorescent probes facilitated analysis of higher-ordered structures [39]. HIV-1 TAR RNA, which is the transactivation responsive element located at the 5′-end of HIV-1 mRNA [40], is a small RNA hairpin consisting of a stem–loop structure with a three-nucleotide bulge [41, 42]. The 16-mer ECHO-LNA chimeric probe was prepared for fluorescent detection of the TAR RNA stem-loop structure (Fig. 5). Four LNA nucleotides were incorporated into the probe to achieve high binding affinity to TAR RNA. The ECHO-LNA chimeric probe before hybridization showed low fluorescence intensity, which was at the same level as that of a DNA-backbone probe. In contrast, the ECHO-LNA chimeric probe showed more effective on-off switching of fluorescence emission, and the fluorescence intensity was higher after hybridization with TAR RNA than that of a DNA-backbone probe (1.7 times at 60 °C). The higher fluorescence intensity of the hybrid formed using a well-designed LNA-incorporating probe suggested that use of LNA nucleotides and temperature control was effective for clearer on-off fluorescence observation of an RNA strand with a higher-ordered structure.

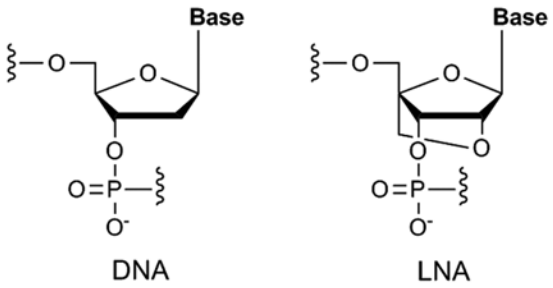



**Fig. 4.** Chemical structure of LNA-modified nucleotide.

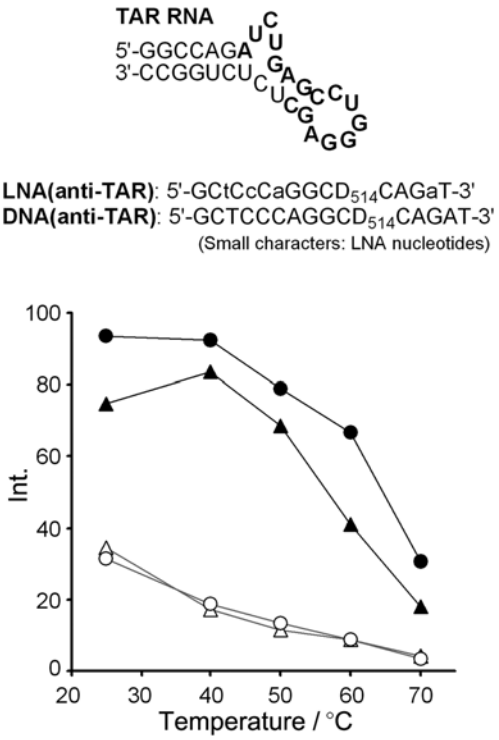



**Fig. 5.** ECHO probes binding to TAR RNA. The fluorescence intensities at 531 nm at different temperatures are plotted with open circles for ECHO-LNA chimeric probe **LNA(anti-TAR)**, closed circles for its hybrid with TAR RNA, open triangles for **DNA(anti-TAR)**, and closed triangles for its hybrid with TAR RNA.

LNA trinucleotides have been reported to be useful for recognition of one-base alteration [43]. The ECHO probe for fluorescent detection of a single-nucleotide polymorphism (SNP) has also been designed. SNP is among the very important research

targets for progress in gene diagnosis and personalized medicine. The target SNP was placenta-specific 4 (PLAC4) rs130833, which is an SNP located in the transcribed regions of placental-expressed mRNA in maternal plasma [44, 45]. We prepared ECHO-LNA conjugates, in which the LNA trinucleotide recognizes the target SNP site of PLAC4 RNA, and compared the SNP selectivity of fluorescence emission with DNA probes. ECHO-LNA chimeric probes for C-allele and T-allele showed high $T_m$ values for hybridization with each of the matched PLAC4 RNA sequences, but much lower $T_m$ values with mismatched sequences (11-27 °C decrease). In contrast, the gap in $T_m$ values of DNA probes for hybridization with matched and mismatched RNA was less than 3 °C, although the matched hybrid was only a little more thermostable. The large gap between $T_m$ values of matched and mismatched hybrids observed for LNA-incorporating probes meant that appropriate temperature control was effective for clear fluorescence detection of SNP. Actually, LNA-containing probes distinguished one-nucleotide difference of the target RNA more sensitively compared with DNA probes and showed high fluorescence emission only when the sequence was matched. Combination of a hybridization-sensitive fluorescent probe and an SNP-recognizing LNA trinucleotide was effective for mix-and-read fluorescent SNP typing.

## Caged ECHO probes: nucleobase modification for pinpoint photoactivation

Design of a fluorophore that can be area-specifically activated by remote control, such as photoirradiation, is also desirable. The molecular caging technology has been used in many biological applications to activate effective substances in spatiotemporal manners [46-51]. To obtain a caged ECHO probe, an α-methyl-2-nitro-4,5-dimethoxybenzyl alcohol was tethered to the thymine base N3 of $^{NB}D_{514}$ [52] (Fig. 6). The photolabile nitrobenzyl (NB) unit effectively suppressed the function of the probe by decreasing the ability of probes to form duplexes with target DNAs or RNAs. For example, the $T_m$ of the hybridization between $T_6{}^{NB}D_{514}T_6$ and $A_{13}$ RNA oligonucleotide was decreased from 54 °C to 22 °C. As a result, little fluorescence emission of $T_6{}^{NB}D_{514}T_6$ was observed in the presence of $A_{13}$ at room temperature.

To uncage protected ECHO probes, a brief irradiation using either a 360 nm UV laser or a 405 nm solid-state LED laser is efficient for removing the NB group from $D_{514}$. The caged ECHO probe, $^{NB}D_{514}$-containing ODNs, only emitted weak fluorescence in the presence of the complementary RNA strand. The emission spectrum excited at 513 nm showed negligible fluorescence, and the fluorescence intensity after addition of the complementary RNA was still low.

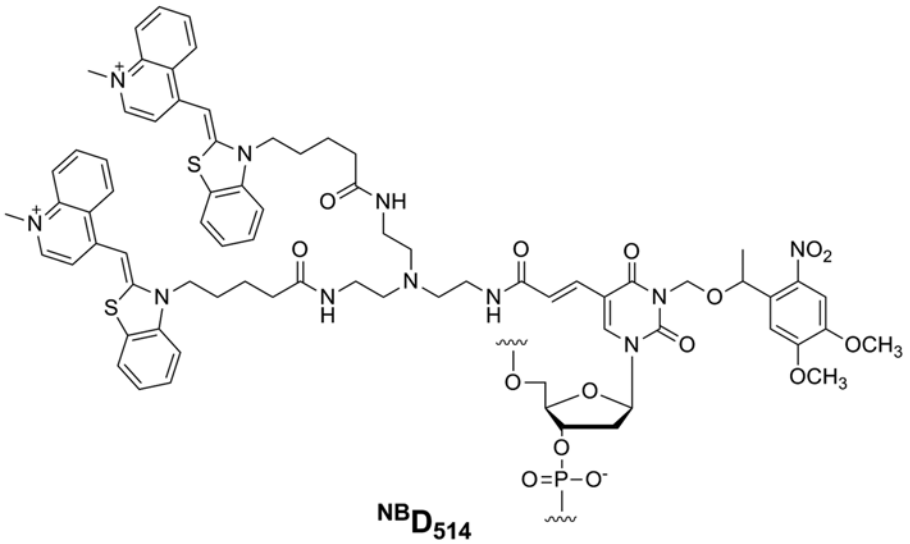



**Fig. 6.** Chemical structure of the caged fluorescence nucleotide $^{NB}D_{514}$.

However, although the probe solution irradiated with 360 nm light exhibited negligible fluorescence on excitation at 513 nm, the emission spectrum of the solution showed a strong fluorescence band at 532 nm when the complementary RNA was added. The behavior of the ODN after 360 nm irradiation was the same as that of the original uncaged probe.

The caged ECHO probes were incorporated into a living HeLa cell and photoirradiation was carried out. We injected $T_6{}^{NB}D_{514}T_6$, which will bind with poly(A)$^+$ RNA represented by mRNA poly(A) tail, into the cell nuclei using a pneumatic injector. The probe emitted weak fluorescence in the cell. We irradiated a blue light onto the cell nuclei using a 405 nm solid-state LED laser. After irradiation for 106 ms, the green fluorescence in the nuclei increased dramatically (Fig. 7). The fluorescence intensity increased up to 2–3-fold that of the caged state.

Therefore, pinpoint uncaging makes monitoring of RNA diffusion in a nucleus possible. Effective monitoring of the expression and behavior of mRNA in a cell nucleus is significant for cell biologists [53-57]. To detect subnuclear mRNA diffusion using a fluorescence signal, development of fluorescent probes binding with the poly(A) sequence of mRNA may be one of the most effective approaches. A $T_6{}^{NB}D_{514}T_6$-injected cell initially emitted weak fluorescence. After irradiation of the small defined area in a cell nucleus, uncaging immediately occurred in the irradiated area and showed a large increase

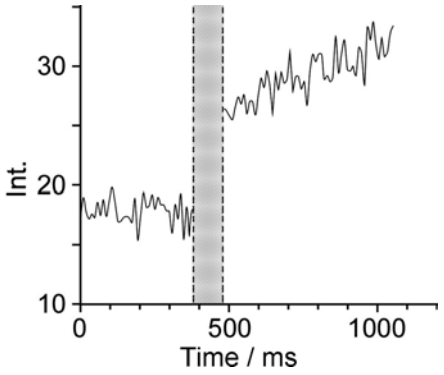



**Fig. 7.** Uncaging in a HeLa cell and increase in fluorescence. The gray column in the figure shows the period of irradiation with a 405 nm laser (376 − 482 ms). Bar, 10 μm.

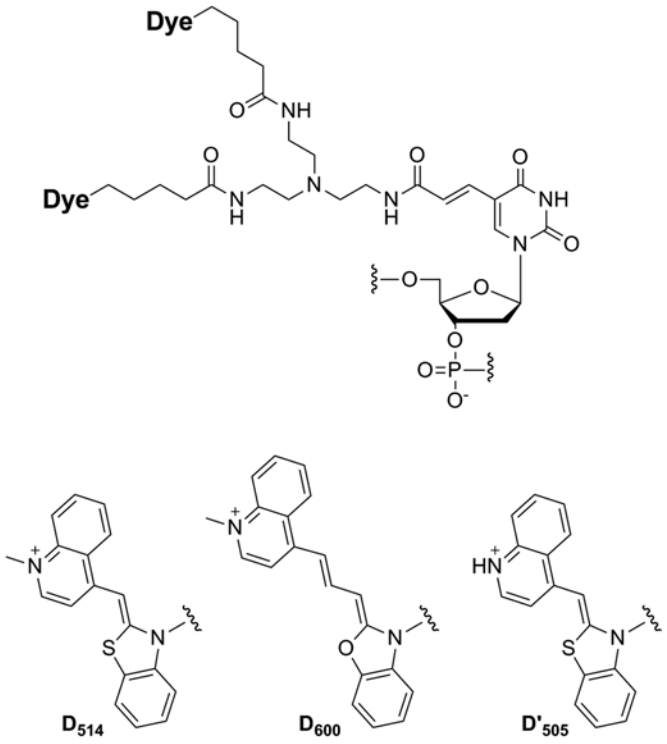



**Fig. 8.** ECHO probes containing nucleotides $D_{514}$, $D_{600}$, and $D'_{505}$.

in fluorescence intensity. In the initial 10 s after finishing photoirradiation, the fluorescence started spreading out toward the surrounding area. The fluorescence of the irradiation area was initially strong but gradually reduced with time, whereas the fluorescence intensity of the surrounding area gradually increased for 1 min after irradiation and then the fluorescence started to decrease.

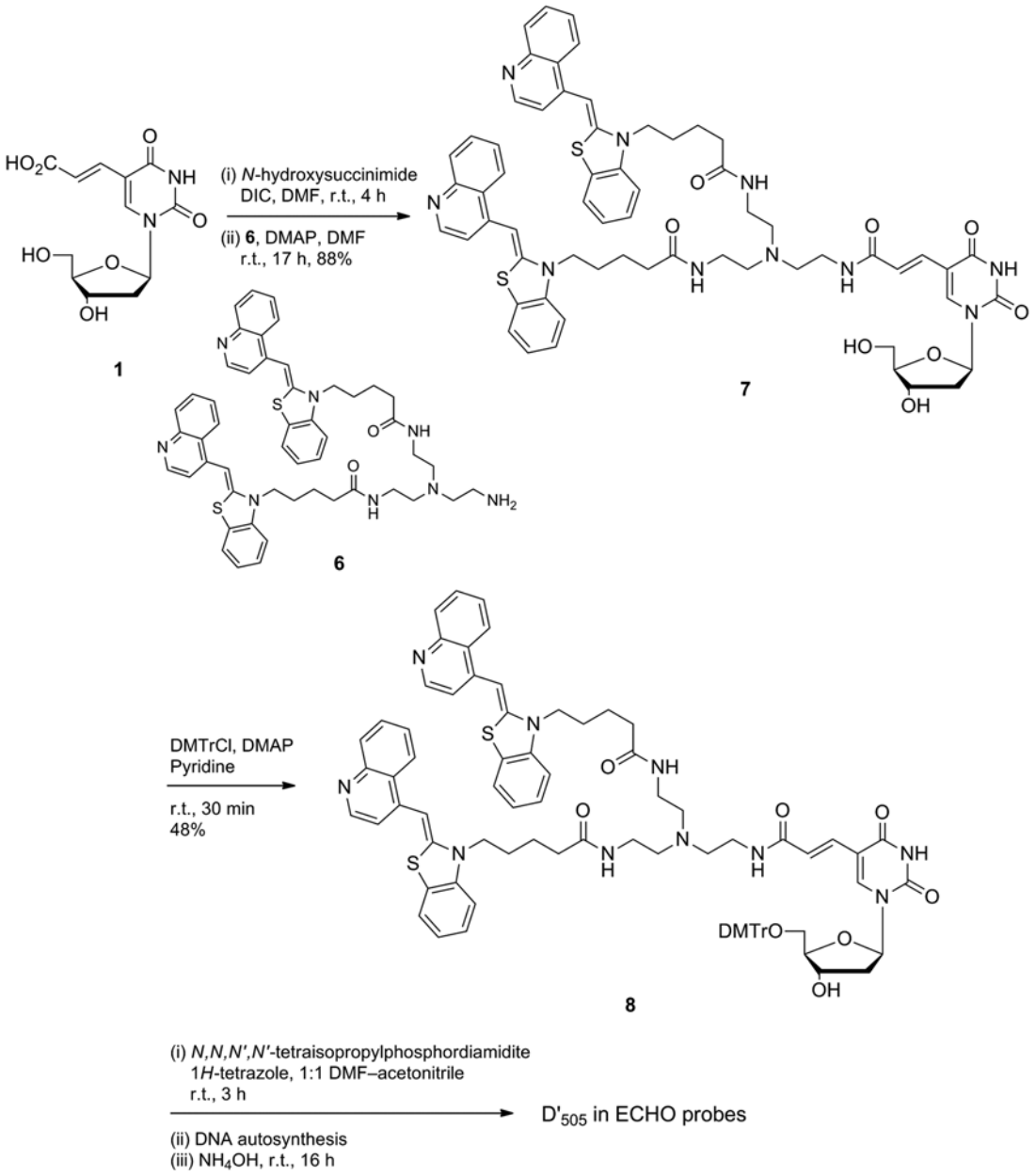
**pH-sensitive ECHO probes: fluorescent dye modification for easy probe preparation**

ECHO probes possess a thymidine derivative labeled with a positively charged dye, such as $D_{514}$ or $D_{600}$ (Fig. 8). Although several dye-labeled nucleotides for ECHO probes having different colors and functions have been developed [24, 27], their simultaneous incorporation into one probe molecule is impossible, because a post-synthetic modification procedure is used in which positively charged dyes are attached to the extra amino groups of the synthetic ODN. The difficulty in incorporating differently colored nucleotides with effective ECHO probe functions into one ODN strand prevents us from expanding the diversity of the functions and uses of ECHO probes. The key to solving this problem is to design a dye-tethering nucleoside that is soluble in several organic solvents. In this case, we can use DNA autosynthesis through the phosphoramidite of the dye-tethering nucleoside. Development of the dye-tethering nucleoside facilitates the dye introduction step and subsequent product purification step.

Desmethyl thiazole orange [58, 59] has been tethered with the nucleoside unit of DNA autosynthesis to produce a new type of ECHO probe ($D'_{505}$) [60]. $D'_{505}$ nucleoside, in which a derivative is substituted for the TO moiety of $D_{514}$, was prepared through several chemical synthesis steps (Scheme 2). $D'_{505}$ nucleoside is soluble in several organic solvents, e.g., chloroform and DMF, because it is uncharged. The solubility of $D'_{505}$ nucleoside in organic solvents made handling of the nucleoside unit in organic synthesis and preparation of the ODN with fluorescent dyes easier. The $D'_{505}$ nucleoside was protected with a dimethoxytrityl group to give a synthetic unit **8** for DNA autosynthesis. The doubly dye-labeled nucleoside **9** was incorporated into ODNs through DNA autosynthesis using acetonitrile and DMF after conversion into the phosphoramidite form (coupling yield, >99%). The ODN containing a fluorescent nucleotide $D'_{505}$ was obtained after deprotection with ammonia. The synthesized $D'_{505}$-containing oligonucleotides can be used for RNA imaging after purification without any of the further modifications required in conventional ECHO probes.

Fluorescence emission of a $D'_{505}$-containing ODN $T_6D'_{505}T_6$ at 522 nm was observed on excitation at 505 nm after addition of the $A_{13}$ RNA, whereas the emission was suppressed in the unhybridized state. $T_6D'_{505}T_6$ works as a good ECHO probe to emit fluorescence through recognition of the complementary RNA. However, desmethyl thiazole orange, which is used for dyes of $D'_{505}$, is weakly emissive [59]. The p$K_a$ of desmethyl thiazole orange has been reported to be approximately 7.6, and the protonation of $D'_{505}$ in the ECHO probe plays an important role in hybridization-sensitive fluorescence emission. The measurements at pH 5-7 showed on-off switching of fluorescence and shift of absorption depending on addition of the complementary RNA strand, which is similar to conventional ECHO probes. In contrast, the fluorescence from $T_6D'_{505}T_6$ at higher pH (pH 8-10) was weak even in the presence of the complementary RNA strand. The absorption spectra of $T_6D'_{505}T_6$ at higher pH were predominantly observed at 500 nm regardless of the presence of the complementary RNA. The small band shift suggests that the unprotonated desmethyl thiazole orange dyes of $D'_{505}$ do not form H-aggregates before hybridization. Although fluorescein quenching by H-aggregation is not expected, desmethyl thiazole orange is originally weakly emissive and, as a result, the fluorescence from the deprotonated form at higher pH is strongly suppressed. $T_6D'_{505}T_6$ lost the function of hybridization-sensitive fluorescence control at higher pH.

The greatest advantage of incorporation of a 'ready-made' fluorescent nucleotide with the function of hybridization-sensitive fluorescence switching into ODNs is to make it possible to incorporate a different fluorescence color by combination with the conventional ECHO probe procedure. The $D'_{505}/D_{600}$ system, ODN($D'_{505}$,$D_{600}$) TTTTTTD$'_{505}$ TTTTTTD$_{600}$TTTTTT, which contains two fluorescent colors in one strand, was prepared. Förster resonance energy transfer (FRET) between $D'_{505}$ and $D_{600}$ was observed when the donor




**Scheme 2.** Synthesis of $D'_{505}$-containing ODN.

nucleotide $D'_{505}$ was excited by irradiation at 505 nm. Emission from FRET acceptor $D_{600}$ (621 nm) as well as emission from $D'_{505}$ (524 nm) was observed. This FRET efficiently occurred only when ODN($D'_{505}$,$D_{600}$) was hybridized with the complementary RNA.

The FRET system of a multicolored ECHO probe is applicable to RNA imaging and provides a way to simplify the monitoring of specific cells and specifically locating RNA in a cell through acceptor bleaching assay [61-63]. A poly(A)$^+$ RNA-targeting $D'_{505}$/$D_{600}$ probe, ODN($D'_{505}$,$D_{600}$), was transfected into living HeLa cells. The cells (mainly nuclei) emitted strong fluorescence through a 600-735 nm filter when excited with a 488 nm laser (red in Fig. 9). The fluorescence observed through a 500-590 nm filter (green) was weak, suggesting that the FRET process of ODN($D'_{505}$,$D_{600}$) was active even in living cells. One of the strongly emitting

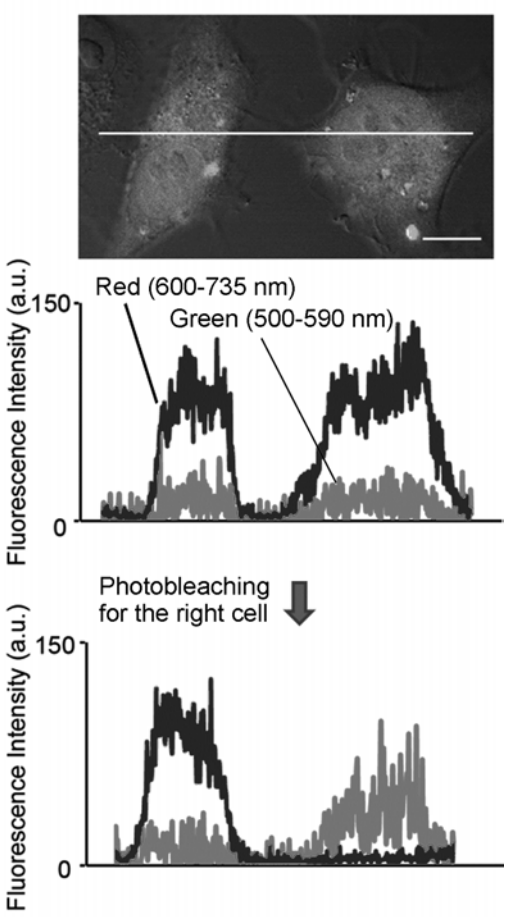



**Fig. 9.** Live-cell RNA imaging and acceptor bleaching. An image of ODN($D'_{505}$,$D_{600}$)-injected HeLa cells before bleaching (Top). Short scale bars, 10 μm. Only the nucleus of the right-hand cell was scanned repeatedly at 561 nm. Fluorescence intensities along the long white lines drawn in the images were plotted (middle and bottom).

cells was selected and then scanned repeatedly with a 561 nm laser. The fluorescence observed through a red filter was reduced to 16% only in the irradiated cell. The $D_{600}$ dyes in the probe were photobleached and were no longer able to work as FRET acceptors. The strong fluorescence of $D_{514}$, the FRET donor, was recovered through a green filter (Fig. 9).

## CONCLUSION

Based on the exciton coupling energy transfer principle, we created ECHO probes for hybridization-sensitive fluorescent nucleic acid detection and modified their sugar backbone, nucleobase, and fluorescent dye for extra functions, such as detection of a single nucleotide difference, higher binding ability to sequences with higher-order structure, intracellular pinpoint photoactivation, facile preparation of probes, multicoloring of probes, and addition of pH sensitivity. We hope that the discussion above has clearly demonstrated the feasibility and importance of ECHO probes in a suite of nucleic acid detection and imaging assays. With these technical advances, we are at the starting point of the development of practical assays for the quantitative detection of RNAs in living cells, tissues, and even intact organisms, with high resolution.

## CONFLICT OF INTEREST STATEMENT

None.

## REFERENCES

1. Bloomfield, V. A., Crothers, D. M. and Tinoco, I. Jr. 2000, Nucleic Acids: Structures, Properties, and Functions, University Science Books, Sausalito, CA.
2. Kiss, T. 2001, EMBO J., 20, 3617.
3. Limbach, P. A., Crain, P. F. and McCloskey, J. A. 1994, Nucleic Acids Res., 22, 2183.
4. Al-Hashimi, H. M. and Walter, N. G. 2008, Curr. Opin. Struct. Biol., 18, 321.
5. Sharp, P. A. 2009, Cell, 136, 577.
6. Tyagi, S. and Kramer, F. R. 1996, Nat. Biotechnol., 14, 303.
7. Biggins, J. B., Prudent, J. R., Marshall, D. J., Ruppen, M. and Thorson, J. S. 2000, Proc. Natl. Acad. Sci. USA, 97, 13537.
8. Whitcombe, D., Theaker, J., Guy, S. P., Brown, T. and Little, S. 1999, Nat. Biotechnol., 17, 804.
9. Holland, P. M., Abramson, R. D., Watson, R. and Gelfand, D. H. 1991, Proc. Natl. Acad. Sci. USA, 88, 7276.
10. Hanafi-Bagby, D., Piunno, P. A. E., Wust, C. C. and Krull, U. J. 2000, Anal. Chim. Acta, 411, 19.
11. Wang, X. and Krull, U. J. 2002, Anal. Chim. Acta, 470, 57.
12. Wang, X. and Krull, U. J. 2005, Bioorg. Med. Chem. Lett., 15, 1725.
13. Asseline, U., Chassignol, M., Aubert, Y. and Roig, V. 2006, Org. Biomol. Chem., 4, 1949.
14. Lartia, R. and Asseline, U. 2006, Chem. Eur. J., 12, 2270.

15. Svanvik, N., Nygren, J., Westman, G. and Kubista, M. 2001, J. Am. Chem. Soc., 123, 803.
16. Köhler, O., Jarikote, D. V. and Seitz, O. 2004, Chem. Commun., 2674.
17. Jarikote, D. V., Köhler, O., Socher, E. and Seitz, O. 2005, Eur. J. Org. Chem., 3187.
18. Marin, V. L. and Armitage, B. A. 2006, Biochemistry, 45, 1745.
19. Serndl, S. and Wagenknecht, H.-A. 2009, Angew. Chem. Int. Ed., 48, 2418.
20. Kasha, M. 1963, Radiat. Res., 20, 55.
21. McRae, E. G. and Kasha, M. 1958, J. Chem. Phys., 28, 721.
22. Okamoto, A. 2010, Chem. Rec., 10, 188.
23. Okamoto, A. 2011, Chem. Soc. Rev., 40, 5815.
24. Wang, D. O. and Okamoto, A. 2012, J. Photochem. Photobiol. C-Photochem. Rev., 13, 112.
25. Hayashi, G. and Okamoto, A. 2013, Chem. Rec., 13, 209.
26. Ikeda, S. and Okamoto, A. 2008, Chem. Asian J., 3, 958.
27. Ikeda, S., Kubota, T., Yuki, M. and Okamoto, A. 2009, Angew. Chem. Int. Ed., 48, 6480.
28. Kubota, T., Ikeda, S. and Okamoto, A. 2009, Bull. Chem. Soc. Jpn., 82, 110.
29. Langer, P. R., Waldrop, A. A. and Ward, D. C. 1981, Proc. Natl. Acad. Sci. USA, 78, 6633.
30. Nielsen, P. E., Egholm, M., Berg, R. H. and Buchardt, O. 1991, Science, 254, 1497.
31. Nilsson, M., Malmgren, H., Samiotaki, M., Kwiatkowski, M., Chowdhary, B. P. and Landegren, U. 1994, Science, 265, 2085.
32. Femino, A. M., Fay, F. S., Fogarty, K. and Singer, R. H. 1998, Science, 280, 585.
33. Silahtaroglu, A., Pfundheller, H., Koshkin, A., Tommerup, N. and Kauppinen, S. 2004, Cytogenet. Genome Res., 107, 32.
34. Larsson, C., Grundberg, I., Söderberg, O. and Nilsson, M. 2010, Nat. Methods, 7, 395.
35. Wang, D. O., Matsuno, H., Ikeda, S., Nakamura, A., Yanagisawa, H., Hayashi, Y. and Okamoto, A. 2012, RNA, 18, 166.
36. Kubota, T., Ikeda, S., Yanagisawa, H., Yuki, M. and Okamoto, A. 2010, PLoS ONE, 5, e13003.
37. Petersen, M. and Wengel, J. 2003, Trends Biotechnol., 21, 74.
38. Sugizaki, K. and Okamoto, A. 2010, Bioconjug. Chem., 21, 2276.
39. Sannes-Lowery, K. A., Hu, P., Mack, D. P., Mei, H.-Y. and Loo, J. A. 1997, Anal. Chem., 69, 5130.
40. Dingwall, C., Ernberg, I., Gait, M. J., Green, S. M., Heaphy, S., Karn, J., Lowe, A. D., Singh, M. and Skinner, M. A. 1990, EMBO J., 9, 4145.
41. Roy, S., Delling, U., Chen, C.-H., Rosen, C. A. and Sonenberg, N. 1990, Genes Dev., 4, 1365.
42. Cordingley, M. G., LaFemina, R. L., Callahan, P. L., Condra, J. H., Gotlib, L., Schlabach, A. J. and Colonno, R. J. 1990, Proc. Natl. Acad. Sci. USA, 87, 8985.
43. You, Y., Moreira, B. G., Behlke, M. A. and Owczarzy, R. 2006, Nucleic Acids Res., 34, e60.
44. Lo, Y. M. D., Tsui, N. B. Y., Chiu, R. W. K., Lau, T. K., Leung, T. N., Heung, M. M. S., Gerovassili, A., Jin, Y., Nicolaides, K. H., Cantor, C. R. and Ding, C. 2007, Nature Med., 13, 218.
45. Go, A. T. J. I., Visser, A., Mulders, M. A. M., Blankenstein, M. A., van Vugt, J. M. G. and Oudejans, C. B. M. 2007, Clin. Chem., 53, 2223.
46. Berger, C. L., Svensson, E. C. and Thomas, D. D. 1989, Proc. Natl. Acad. Sci. USA, 86, 8753.
47. Walker, J. W., Reid, G. P., McCray, J. A. and Trentham, D. R. 1988, J. Am. Chem. Soc., 110, 7170.
48. Dantzig, J. A., Higuchi, H. and Goldman, Y. E. 1998, Methods Enzymol., 291, 307.
49. Adams, S. R. and Tsien, R. Y. 1993, Annu. Rev. Physiol., 55, 755.
50. McCray, J. A. and Trentham, D. R. 1989, Annu. Rev. Biophys. Biophys. Chem., 18, 239.
51. Mitchison, T. J., Sawin, K. E., Theriot, J. A., Gee, K. and Mallavarapu, A. 1998, Methods Enzymol., 291, 63.
52. Ikeda, S., Kubota, T., Wang, D. O., Yanagisawa, H., Umemoto, T. and Okamoto, A. 2011, ChemBioChem, 12, 2871.
53. Molenaar, C., Abdulle, A., Gena, A., Tanke, H. J. and Dirks, R. W. 2004, J. Cell Biol., 165, 191.
54. Tokunaga, K., Shibuya, T., Ishihama, Y., Tadakuma, H., Ide, M., Yoshida, M., Funatsu, T., Ohshima, Y. and Tani, T. 2006, Genes Cells, 11, 305.

55. Enninga, J., Levy, D. E., Blobel, G. and Fontoura, B. M. 2002, Science, 295, 1523.
56. Politz, J. C., Tuft, R. A., Pederson, T. and Singer, R. H. 1999, Curr. Biol., 9, 285.
57. Taneja, K. L., Lifshitz, L. M., Fay, F. S. and Singer, R. H. 1992, J. Cell Biol., 119, 1245.
58. Yue, S. T. 1997, US Patent, 5656449.
59. Ikeda, S. and Okamoto, A. 2007, Photochem. Photobiol. Sci., 6, 1197.
60. Okamoto, A., Sugizaki, K., Yuki, M., .
Yanagisawa, H., Ikeda, S., Sueoka, T., Hayashi, G. and Wang, D. O. 2013, Org. Biomol. Chem., 11, 362.
61. Miyawaki, A. 2003, Dev. Cell, 4, 295.
62. Valentin, G., Verheggen, C., Piolot, T., Neel, H., Coppey-Moisan, M. and Bertrand, E. 2005, Nat. Methods, 2, 801.
63. Ikeda, S., Kubota, T., Wang, D. O., Yanagisawa, H., Yuki, M. and Okamoto, A. 2011, Org. Biomol. Chem., 9, 6598.