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A FRET-based DNA nano-tweezers technique for the imaging analysis of specific mRNA

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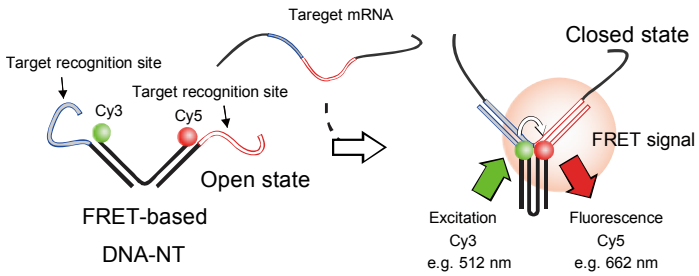

In this report, the feasibility of imaging analysis of target mRNA utilizing a novel FRET-based DNA nano-tweezers (DNA-NT) technique has been discussed.

Abstract

A DNA nano-tweezer structure (DNA-NT)-based target mRNA detection probe, which uses fluorescence resonance energy transfer (FRET) as a detection signal and works as a single molecule, has been developed. This FRET-paired fluorescent dye-modified DNA-NT, self-assembled from three single-stranded DNAs, alters its structure from open to closed states and produces a FRET signal in response to *in vitro* transcripts of Hes-1 mRNA. Our results showed that the FRET-based DNA-NT detected both GLUT1 mRNA as a pre-fixed target mRNA model and Hes-1 mRNA as a model expressed inside a living cell. These results confirm the feasibility of using the FRET-based DNA-NT for imaging analysis of target mRNA.

Text

The ability to monitor the gene expression status in a living cell holds great promise for analyzing molecular mechanisms of cellular responses and for evaluating the cellular quality of cell-based materials. Messenger RNA (mRNA) has been selected as the target molecule to be monitored in our study because it can act as a representative marker reflecting cellular status. Currently, there are few methods available for detecting a specific mRNA inside a living cell.

Molecular Beacon (MB)[1] is one of the most powerful tools for monitoring specific mRNA expression status without requiring a gene recombination process or destruction of the cells[2-9]. MB consists of single-stranded DNA that forms a stem-loop structure. The stem part has a fluorescent dye and a quencher, and the loop part has a complementary sequence of a partial target mRNA sequence. Usually the fluorescent dye and the quencher are held in close proximity at the stem part such that the fluorescent signal is off. Once the loop part recognizes the target and hybridizes to it, the stem portion is pulled apart and the distance between the dye and the quencher increases, relieving the effect of the quencher and resulting in the fluorescent signal turning on. Although MB has great potential for detecting specific target mRNA *in vitro*, MB is often digested by nucleases *in vivo*, which pulls the

stem portion apart, thus producing a false positive signal. To avoid generating this type of false positive signal, a dual fluorescence resonance energy transfer (FRET)-based MB system was developed[10,11]. This system uses two MBs whose fluorescent dyes are a FRET pair. These MBs recognize consecutive regions on the mRNA target, and the FRET signal is produced only when the two MBs hybridize to the same target. In this system, even when MBs are digested by a nuclease, the FRET signal is not generated and thus the amount of false positive signal is dramatically lower than in the original MB system. However, in this dual FRET-based MB system, both of the MBs need hybridize on the same mRNA to generate the FRET signal. This requirement leads to concerns that the signal responding speed is slow because all three molecules, two MBs and the target mRNA, have to form a triple molecule complex in a highly condensed intercellular environment. To overcome these problems, we have employed a DNA nano-tweezer structure (DNA-NT)[12] to act as a target mRNA detection probe. This DNA-NT also uses FRET as a detection signal, but it works as a single molecule (Fig. 1a). This molecule is expected to generate the FRET signal only when it recognizes a target mRNA as a result of a bimolecular reaction. Here, we discuss the feasibility of using our FRET-based DNA-NT for the imaging analysis of a specific mRNA in living cells.

The FRET-based DNA-NT was created by the annealing of three synthesized DNA oligonucleotides (the procedure is detailed in the supporting information, Fig. S1 and Table S1). Two of the oligonucleotides have a target recognition site whose sequence is complementary to the target mRNA. The third oligonucleotide is connected to FRET-paired fluorescent dyes, with Cy3 and Cy5 on its 5′ and 3′ ends, respectively.

To confirm the target recognition ability *in vitro*, the *in vitro* transcript of hairy and enhancer of split-1 gene (Hes-1) mRNA[13] was used as a model target RNA (the procedure is detailed in the supporting information, Fig. S2). Hes-1 is known to be a key transcriptional factor for determining cell fate in embryonic stem cells (ES cells)[14]. The exact sequences of the Hes-1 mRNA targeting DNA-NT (Hes-1 DNA-NT) and the DNA-NT expected to fail to detect any mRNAs in living murine cells (control DNA-NT) are depicted in Fig. 1b and Fig. 1c.

First, the target recognition ability of DNA-NT was confirmed by electrophoresis (Fig. 2a). When Hes-1 DNA-NT was mixed with Hes-1 mRNA, the green band corresponding to the Cy3 of DNA-NT was observed at a slightly higher position (lane 5) than the band of the target mRNA observed only in the Gel Star-stained image (lane 4). In contrast, when control DNA-NT was mixed with the target mRNA, the DNA-NT retained its position (lane

3) and no green band was detected at the target mRNA position (lane 6). These findings indicate that DNA-NT has the ability to recognize a target mRNA *in vitro*. Further FRET analysis confirmed the target detecting ability of DNA-NT (Fig. 2b). With the addition of the target mRNA, the relative fluorescence intensity observed at 565 nm decreased, whereas the intensity at 662 nm increased. This indicates that DNA-NT recognizes the target mRNA and changes its structure from an open to a closed state, inducing the distal change of modified Cy3 and Cy5 and leading to a FRET signal. The FRET efficiency reflects the concentration of the target mRNA (Fig. 2c). As the concentration of the target mRNA is increased, the FRET efficiency also increases, up to a point. The lower detection limit was 0.035 (vs. DNA-NT), which is corresponding to 17.6 nM of Hes-1 mRNA in this experimental condition (Fig. S3). Past that threshold, the FRET efficiency is actually decreased. This decrease seen with high concentrations of target mRNA occurs because the DNA-NT has two target recognition sites, each of which has the ability to bind a target. The excess target induces the DNA-NT into an open state that is able to hold two targets in its two recognition sites independently, resulting in low FRET efficiency. Nevertheless, these results indicate that FRET-based DNA-NT can act as a specific mRNA-detecting fluorescent probe.

The distance between the recognition sites of our DNA-NT was then optimized using model DNA targets that have a different number of adenines between their targeted sites (termed as Hes-1 DNA(An) listed in Table S1). The FRET efficiency was calculated as fluorescence intensity at 662 nm/fluorescence intensity at 565 nm. The target recognition sites form a double-stranded structure with the target, presumably a helical structure. It is also known that the FRET occurs from a donor chromophore to an acceptor chromophore through non-radiative dipole – dipole coupling, and thus the FRET efficiency is affected not only by the donor – acceptor distance but also by the dipole relation between two fluorescent dyes. Therefore it was assumed that the FRET efficiency between the two fluorescent dyes on the DNA-NT in the target-recognizing form is not simply proportional to the number of adenines inserted between the targeted sites. In fact, as shown in Fig. 3, a gap of four bases (Hes-1 DNA(A4)) gave the strongest FRET signal. It is reported that the cyanine fluorophores such as Cy3 and Cy5 are predominantly stacked on the end of double-stranded DNA[15,16]. The molecular dynamics of Cy3 and Cy5 may therefore be restricted in some unfavorable dipole relations in case of A0 and A2, leading to the lower FRET efficiency. Also these short gap distances may result in steric interference or other interactions between Cy3 and Cy5 such as ground-state quenching, which also cause the

lower FRET efficiency as discussed in case of dual FRET molecular beacons[17]. Nevertheless, the target sites were spaced with gaps of four bases between them for all further experiments.

Next, the ability of FRET-based DNA-NT to recognize a target mRNA inside a cell was confirmed by fluorescence *in situ* hybridization (FISH), in which the fixed target mRNA was detected by our FRET-based DNA-NT (The procedure is detailed in the supporting information). The mRNA of glucose transporter 1 gene[18] (GLUT1, Fig. S4) in mouse hepatocarcinoma Hepa 1-6 cells[19,20] was selected as our model, and a GLUT1-specific FRET-based DNA-NT was designed (GLUT1 DNA-NT, Fig. S5). As shown in Fig. 4, a clear FRET signal was observed in cells combined with GLUT1 DNA-NT, while no FRET signal was observed when control DNA-NT was used. In contrast to the FRET signal, the Cy3 and Cy5 signals were clearly observed both in cells treated with GLUT1 DNA-NT and in those treated with control DNA-NT. These results imply that the DNA-NTs attached to the cellular components non-specifically and were not removed completely with washing steps. In traditional methods with standard FISH probes, which simply bind to a target and generate a detection signal, this type of leftover probe causes a false positive signal. The FRET-based DNA-NT, in contrast, does not produce the FRET signal when it simply binds

non-specifically to a substance inside a cell. It produces the FRET signal only when it binds to the identical target mRNA with two target recognition sites, reducing the false positive signal dramatically.

Finally, the validity of detection of target mRNA inside a living cell with the FRET-based DNA-NT was tested. To assess the feasibility, we introduced the FRET-based DNA-NT into a living cell using Streptolysin O (SLO) that creates a pore on the cellular membrane[10]. Next, the cells were stained with cell tracker to be able to compare the cell location. The cells were then fixed to obtain the fluorescent images that reflect the status of DNA-NT recognizing the target mRNA in living cells (the procedure is detailed in the supporting information). The mRNA of Hes-1 in mouse ES cells (EB3 cells[21,22]) was targeted as a model mRNA for this experiment. When using Hes-1 DNA-NT with SLO treatment, clear confocal images of both the Cy5 signal and the FRET signal were obtained in the area in which the cell tracker was observed (Fig. 5, upper panels). On the other hand, in cells that had not undergone the SLO treatment, neither Cy5 signals nor FRET signals were observed (Fig. 5, middle panels). The comparison of these results confirmed the successful introduction of DNA-NT into a living cell. When control DNA-NT was used with SLO treatment, the Cy5 signal was observed but no FRET signal was obtained (Fig. 5, lower

panels). Therefore, we conclude that the FRET image obtained by the use of Hes-1 DNA-NT with SLO treatment reflects the status of Hes-1 mRNA expression. Although these images were obtained after the fixation of the cells, so the cells were no longer alive, the treatment was administered successfully to living cells and these results support the idea that the FRET-based DNA-NT can be used as a fluorescence probe for the imaging analysis of mRNA inside a living cell.

Conclusions

In this communication, we have developed a new DNA nano-tweezer structure (DNA-NT)-based target mRNA detection probe. This technique uses FRET as a detection signal and works as a single molecule. Our results suggest that this FRET-based DNA-NT can be used for an imaging analysis of a target mRNA inside a living cell. Although there are still several technical issues to overcome, such as the stability of the probe and the experimental settings for live cell imaging, the basic principle and the feasibility of using FRET-based DNA-NT for imaging analysis of target mRNA detection has been demonstrated.

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Figure legends

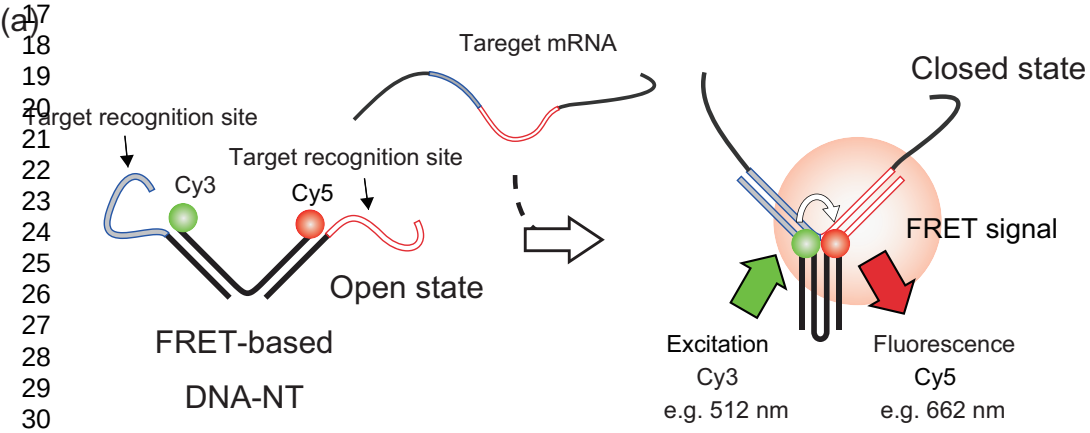
Fig. 1. Principle of target mRNA detection with FRET-based DNA-NT and the exact sequences of DNA-NTs. (a) When the FRET-based DNA-NT recognizes its target RNA, it alters its structure from an open to a closed state. Along with the structural change, the distance between the pre-modified FRET pair of fluorescent dyes, Cy3 and Cy5, changes, and this produces a FRET signal. (b) The sequence of Hes-1 DNA-NT. (c) The sequence of control DNA-NT.

Fig. 2. Characterization of a FRET-based DNA-NT with *in vitro* transcripts. (a) Native agarose gel electrophoresis was performed to assess the target-recognition ability of DNA-NT. Gel Star-stained image (left panel) and fluorescence (Cy3) image (right panel). Lane 1, RNA ladder; Lane 2, Hes-1 DNA-NT; Lane 3, control DNA-NT; Lane 4, Hes-1 mRNA, Lane 5, Hes-1 mRNA + Hes-1 DNA-NT; and Lane 6, Hes-1 mRNA + control FRET-based DNA-NT. (b) Fluorescence spectra of FRET-based DNA-NT with different concentrations of target mRNA. (c) Dose response of FRET-based DNA-NT (N = 3). Experimental details are described in Supporting Information.

Fig. 3. Optimization of the distance between targeted sites. Synthesized DNA oligonucleotides with a different number of adenines [termed as Hes-1 DNA(An)] were utilized as target models. The results of the figure are shown as means ± standard deviation of three replicates (N = 3).

Fig. 4. Representative fluorescent images of Hepa-1-6 cells that were stained with FRET-based DNA-NTs after fixation. Upper images, GLUT1 DNA-NT; lower images, control DNA-NT. Details of experimental settings to obtain these images and the calculation for FRET image analysis were described in Supporting Information.

Fig. 5. Representative confocal fluorescent images of DNA-NT introduced into EB3 cells. DNA-NTs were introduced into living cells by treatment with SLO, and the cells were stained with CellTracker™ Blue CMF2HC. The cells were then fixed, and fluorescent images were obtained. Details of experimental settings to obtain these images and the calculation for FRET image analysis were described in Supporting Information.



(b)

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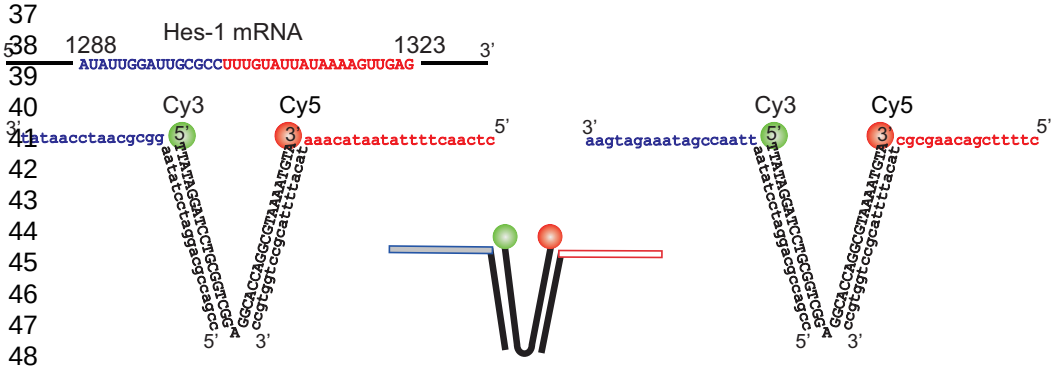
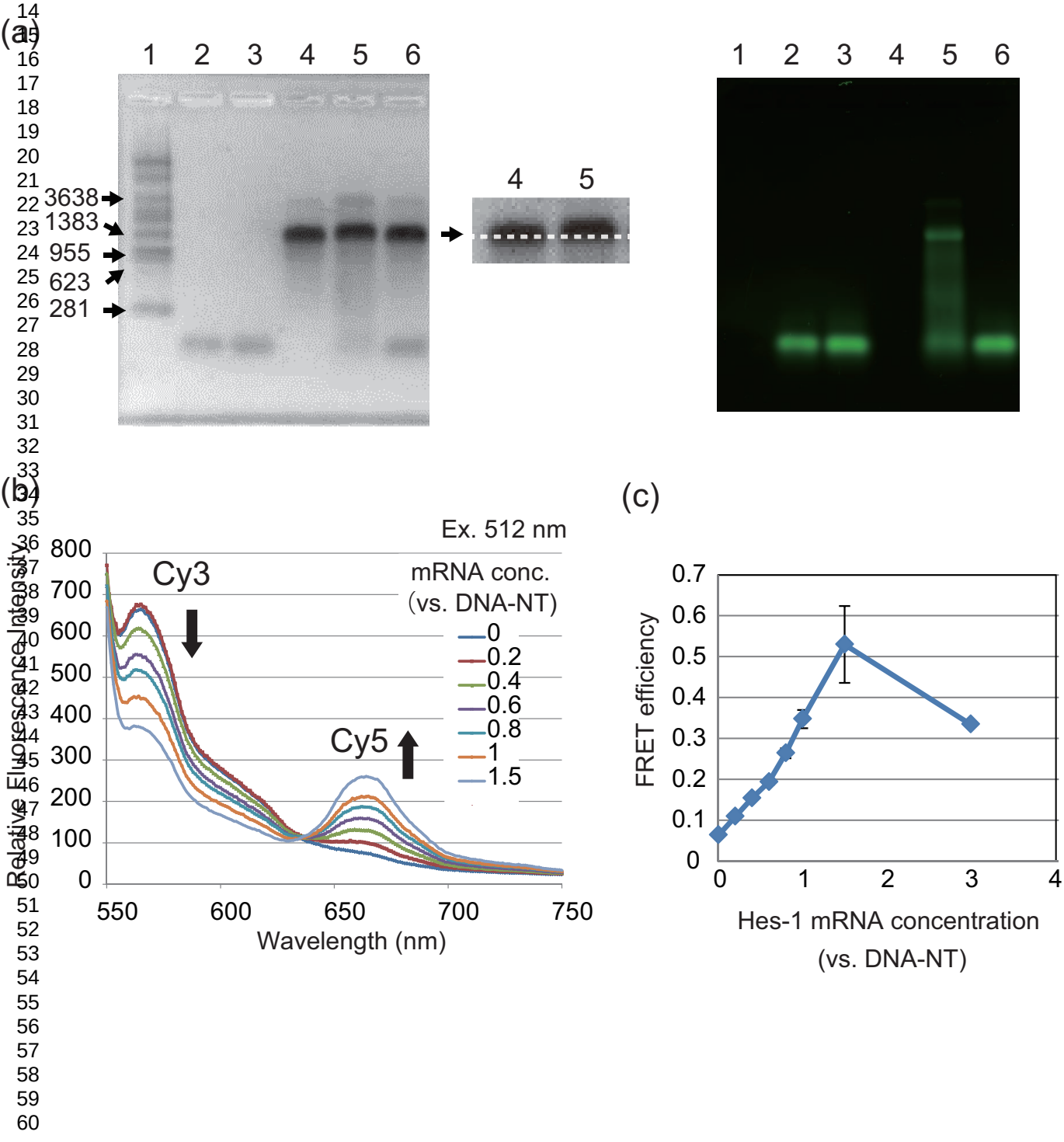


Figure 1



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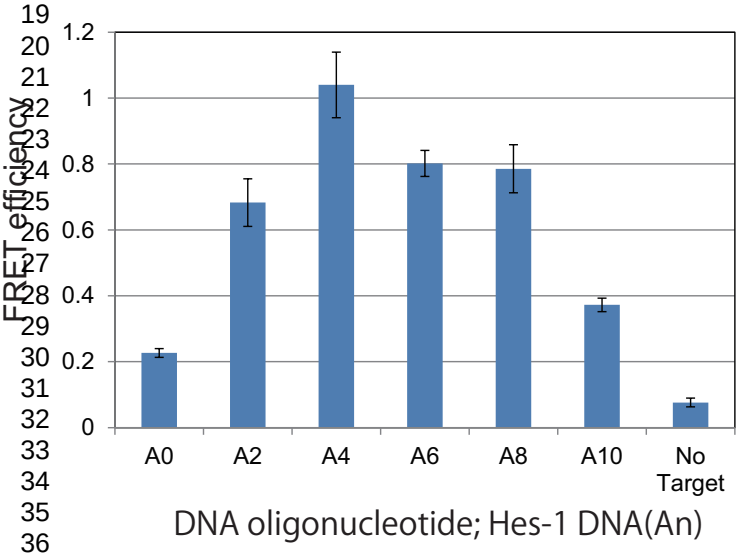

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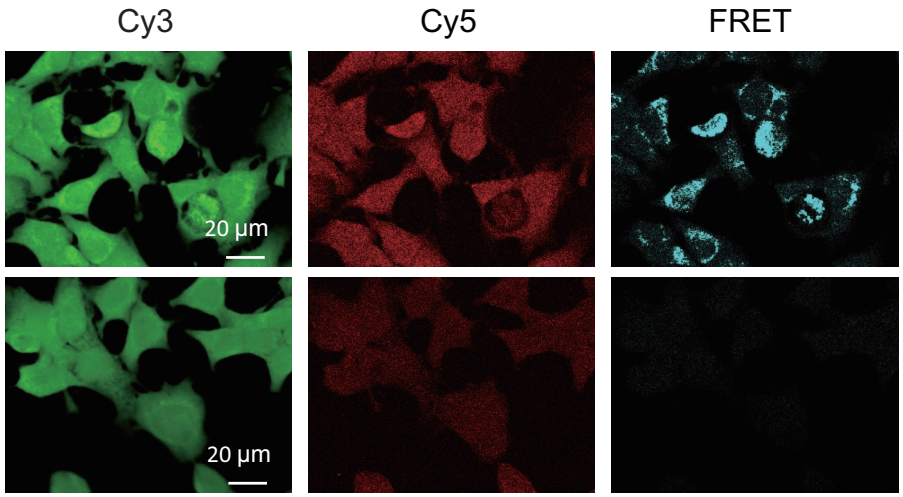

Figure 4

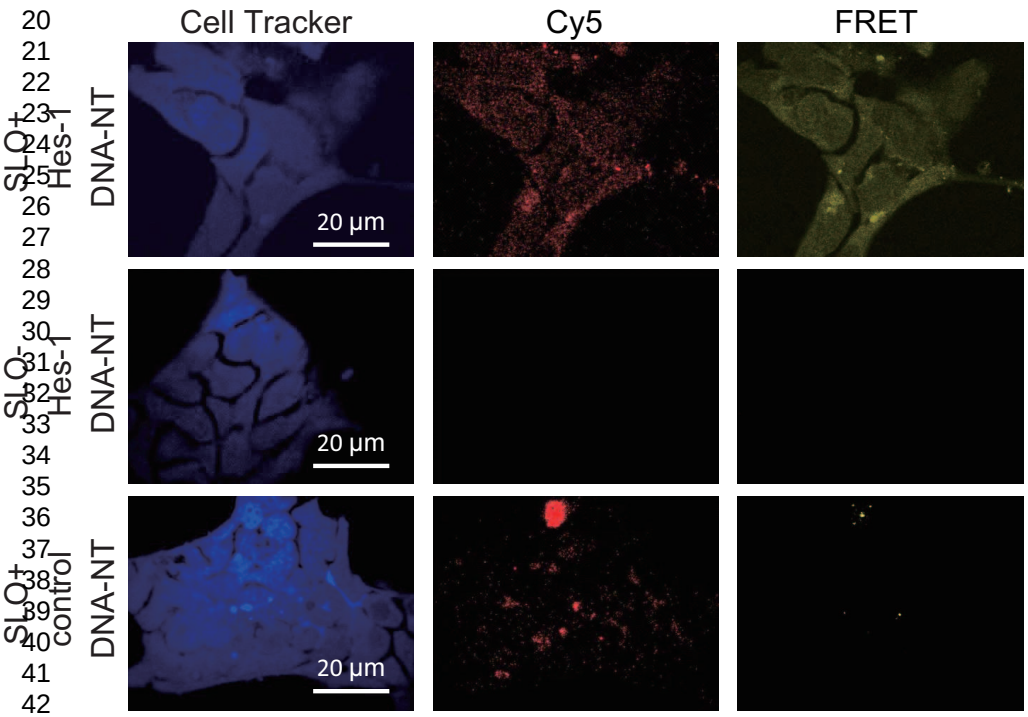

Figure 5