

Electronic Supporting Information (ESI) for

Detection of parallel and antiparallel DNA triplex structures in living human cells using in-cell NMR

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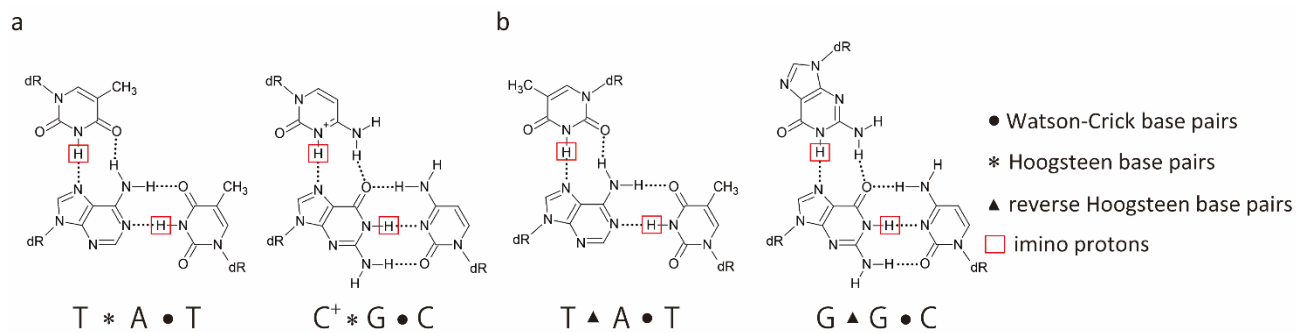


Fig. S1 The chemical structures of base triples in the parallel triplex ODN (T•A•T, and C⁺•G•C) (a) and antiparallel triplex ODN (T▲A•T and G▲G•C) (b).

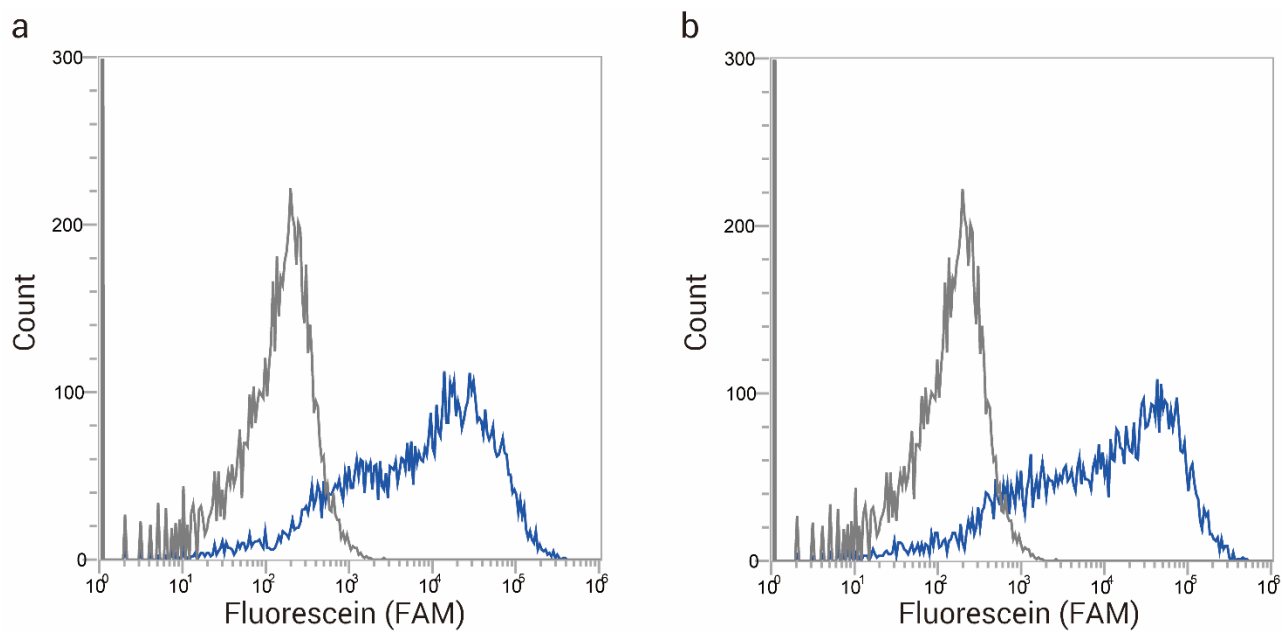


Fig. S2 FCM histogram for introduction of FAM-labelled PT-ODN (a) and APT-ODN (b) into cells. Cell counts with (blue) or without (grey) introduction of ODN are plotted against the intensity of FAM with gating of PI-positive cells.

Materials and Methods

Preparation of oligodeoxynucleotides (ODNs)

5'-AGAGAGAACCCCTTCTCTCTTTTCTCTT-3' and 5'-TCCTCCTTTTATAGGAGGATTTTGGTGGT-3' triplex ODNs, which were named parallel triplex ODN (PT-ODN) and antiparallel triplex ODN (APT-ODN), respectively, and their 5'-fluorescein (FAM)-labeled forms were synthesized, purified and de-salted by FASMAC Co., Ltd. (Kanagawa, Japan). ODNs corresponding to the duplex portions of PT-ODN (5'-AGAGAGAACCCCTTCTCTCT-3') (D^{PT}-ODN) and APT-ODN (5'-TCCTCCTTTTATAGGAGGA-3') (D^{APT}-ODN) were also synthesized, purified and de-salted by FASMAC Co., Ltd. (Kanagawa, Japan).

Introduction of ODNs into HeLa cells by streptolysin O (SLO) treatment

The procedure for introduction of ODNs into HeLa cells was described previously.¹ 4×10^7 HeLa cells were harvested in ice-cold phosphate-buffered saline (PBS). To the ice-cold cell suspension, SLO (Bioacademia) was added to a final concentration of 0.06 $\mu\text{g}/\text{mL}$. The cells were then incubated at 4°C for 10 min with gentle shaking. After washing three times with 10 mL of ice-cold PBS, 2.5 mL of transport buffer (TB: 25 mM HEPES-KOH (pH 7.0), 115 mM CH_3COOK , and 2.5 mM MgCl_2) was added and the cells were incubated for 5 min at 32°C with gentle shaking. 5 mL of TB was added to the cell suspension and the supernatant was removed by centrifugation. The cells were incubated with TB that contained cytosol² prepared from mouse liver cells, an ATP regenerating system (1 mM ATP, 50 ng/mL creatine kinase, and 2.62 mg/mL creatine phosphate), 1 mg/mL glucose, 1 mM GTP, and the ODN of interest at 32°C for 30 min with gentle shaking. At this time, 150 μL of the incubation solution was used per 1×10^7 cells. The DNA concentrations and volumes of the incubation solutions are shown in Table S1. Then, to reseal the pores formed by SLO, CaCl_2 was added to a final concentration of 1 mM, followed by incubation at 32°C for 5 minutes with gentle shaking. The cells were then washed three times with 10 mL of Leibovitz's L-15 medium containing 1 mM CaCl_2 .

Table S1 Concentrations of DNAs and final volumes of the incubation solutions for SLO treatment.

	Non-labeled DNA	FAM-labeled DNA	Total volume
In-cell NMR	1000 μM	-	600 μL
FCM and confocal microscopy	-	20 μM	50 μL
Gel electrophoresis	980 μM	20 μM	50 μL

Flow cytometry (FCM) analysis

The SLO-treated cells were suspended in PBS containing 5 $\mu\text{g}/\text{mL}$ propidium iodide (PI) and then subjected to FCM using an Attune NxT Flow Cytometer (Thermo Fisher Scientific).

Confocal fluorescence microscopy analysis

The SLO-treated cells were suspended in PBS containing 8 μM Hoechst 33342 and then incubated at room temperature for 20 minutes. Microscopy images were acquired with an Olympus FV1000 confocal scanning laser microscope equipped with a 60 $\times$ UPlanSApo objective.

In vitro NMR measurements

The ODN of interest was dissolved in TB (pH 7.0) containing 10% D_2O and 10 μM 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) to a final concentration of 150 μM . The solution was heated at 95°C for 5 min and then annealed at a rate of -1°C/min for 60 min. ^1H NMR spectra were recorded at 10°C by the band-selective optimized-flip-angle short-transient (SOFAS³) technique with PC9 and rSNOB pulses for water suppression. The *in vitro* NMR spectra were accumulated for ca. 30 min. The numbers of scans were 2048. All NMR measurements were performed using a Bruker BioSpin AVANCE III HD 600 spectrometer equipped with a cryogenic probe.

In-cell NMR measurements using a bioreactor system

In-cell NMR measurements were performed with a bioreactor system.^{4,5} A 80% slurry of the SLO-treated cells was mixed with the same volume of 0.9 $\times$ Leibovitz's L-15 medium containing 3% of low-melting temperature agarose (Lonza), 10% D_2O , and 10 μM DSS to the final concentrations of 40% cells and 1.5% agarose. The mixture was transferred by means of a syringe to a Teflon tube with an inner diameter of 0.5 mm and then cooled on ice for 10 min. The thread-like agarose gel containing cells in the Teflon tube was extruded into a 5 mm NMR tube with 140 μL of 3% agarose gel (lower gel) containing 0.9 $\times$ L-15 medium, 10% D_2O , and 20 μM DSS at its bottom. Onto the crumpled thread-like gel containing cells, another 140 μL of 1.5% agarose-gel-thread (upper gel) without cells was extruded, which was solidified in a silicon tube with an inner diameter of 1 mm. The NMR tube was connected to the bioreactor system shown in Fig. S2. In Fig. S2, Teflon tubes that connected to individual components are indicated by black lines. The 0.9 $\times$ Leibovitz's L-15 medium containing 10% D_2O and 10 μM DSS was supplied from a syringe pump at a flow rate of 25 $\mu\text{L}/\text{min}$. The L-15 medium was used to keep a pH value constant without CO_2 incubation. During in-cell NMR experiments, the medium was delivered into the NMR sample tubes, the overflowed medium being drained to the waste bottle. Due to prolongation of cell viability by the bioreactor system, it was possible to obtain in-cell NMR signals over 20 hr, although it is generally impossible to obtain in-cell NMR signals over 2-4 hr without the bioreactor system. Either inhomogeneous broadening or noise caused by the bioreactor system turns out to be moderate, if any.

^1H NMR spectra were recorded in the same way as *in vitro* spectra at 10°C by the SOFAST technique. The in-cell NMR signals were accumulated from 1.5 hr to 17.5 hr for PT-ODN and from 1.5 hr to 20.5 hr for APT-ODN after the introduction of triplex ODNs into HeLa cells to obtain one one-dimensional spectrum, respectively. The numbers of scans were 66560 and 79872, respectively. For reference, HeLa cells without the introduction of nucleic acids gave no signal in the imino proton region.^{1,6}

For quantification of the populations of the triplex and duplex, we used a well-separated peak or a well-separated group of peaks, as specified in a main text. Therefore, integration of a peak (or a group of peaks) was performed without applying deconvolution of signals.

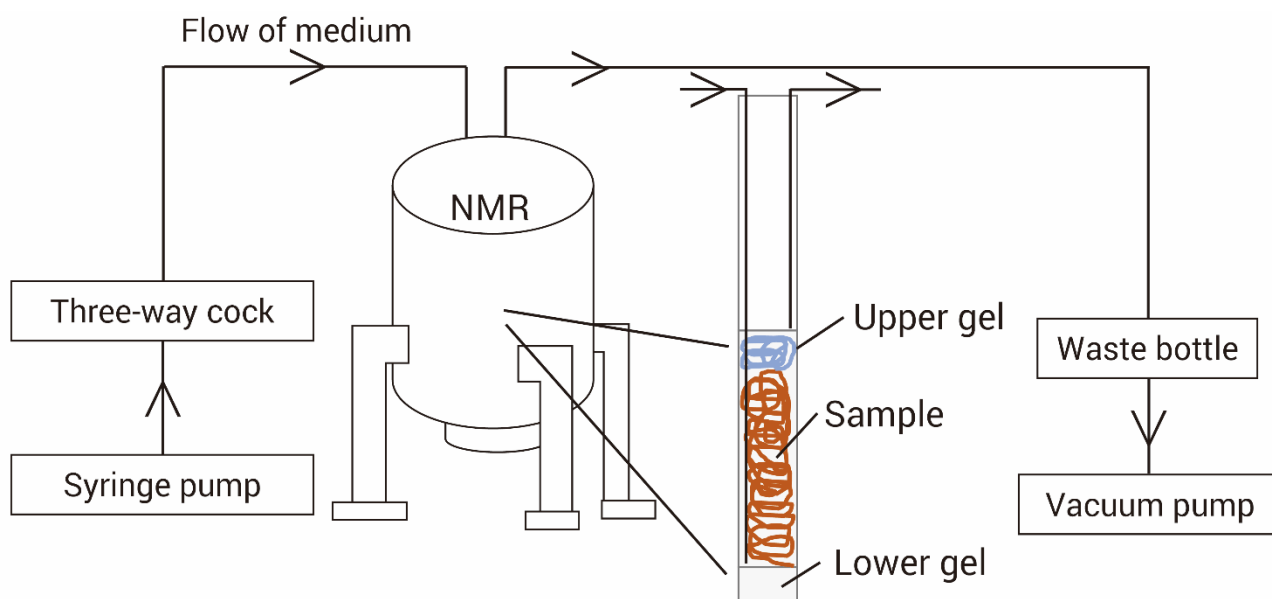


Fig. S3 Schematic diagram of bioreactor.

Analysis of introduced ODNs by electrophoresis

50 μL of a suspension of cells treated by SLO with FAM-labeled ODN was divided into five portions, which were incubated at 10°C for each time period. After incubation, the cell suspension was washed with 500 μL of L-15 medium and centrifuged at $300 \times g$ for 5 min. 54 μL of lysis buffer (10 M urea, 50 mM Tris-HCl, 48.5 mM boric acid, 2 mM EDTA) was added to each cell pellet and the cells were disrupted by freezing and thawing five times. After centrifugation of the disrupted cells at $300 \times g$ for 5 min, each 5 μL of the supernatant was analyzed by 20% denaturing polyacrylamide gel electrophoresis with 8 M urea. A flowchart of the experiment is shown in Fig. S4.

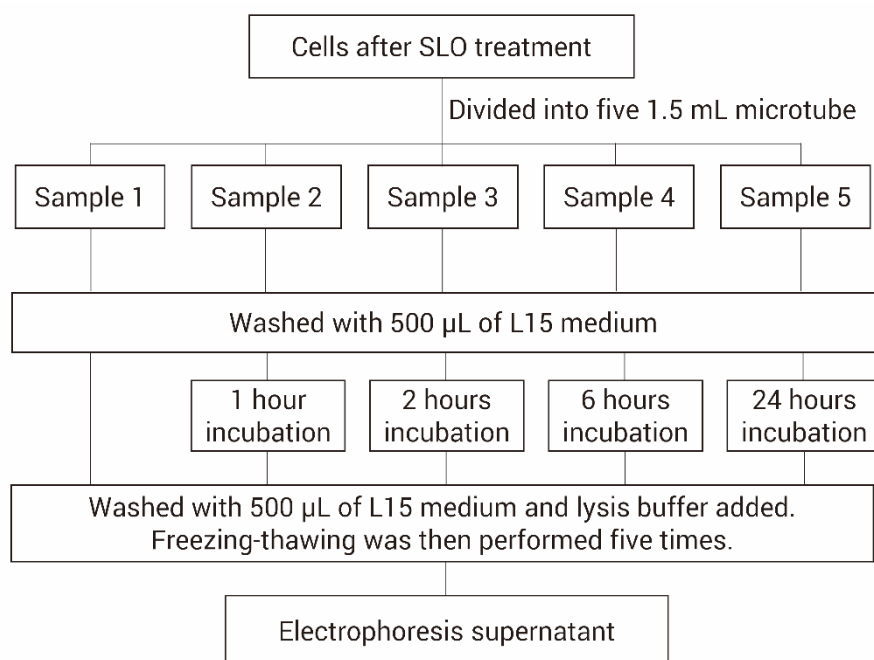


Fig. S4 Flowchart of gel electrophoresis analysis of intracellular ODNs.

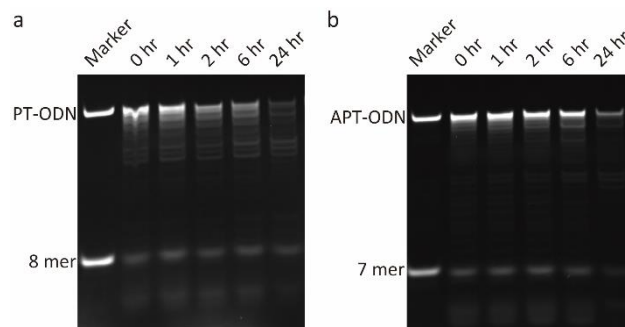


Fig. S5 Examination of the degradation of the triplex ODNs introduced in the cells over time. Denaturing gel electrophoreses of the DNA fractions collected from the cells for PT-ODN (a) and APT-ODN (b). The times indicate the durations after the SLO and resealing treatment. PT- and APT-ODNs were applied in the Marker lane of (a) and (b), respectively, as a reference.

The initial concentration of PT-ODN and APT-ODN in cells were estimated to be ca. 20 μ M on the basis of the intensity of the fluorescence. The relative amounts of the intact triplex ODN and degraded ODNs were determined by quantifying the band intensities in the same time period as that used for in-cell NMR measurements. The band intensities for the time points between the examined ones (1, 2, 6, and 24 hrs) were estimated assuming the liner change of the band intensity. The relative amount of the intact triplex ODN (T) was calculated using equation (1).

$$T = \frac{B_i}{B_i + B_f} \times 100 \quad (1)$$

where B_i is the integrated band intensity of the intact triplex ODN and B_f the integrated band intensity of the degraded ODNs (Fig. S6). The average of T was calculated for the time period used for in-cell NMR measurements, 1.5 hr to 17.5 hr for PT-ODN and 1.5 hr to 20.5 hr for APT-ODN, respectively.

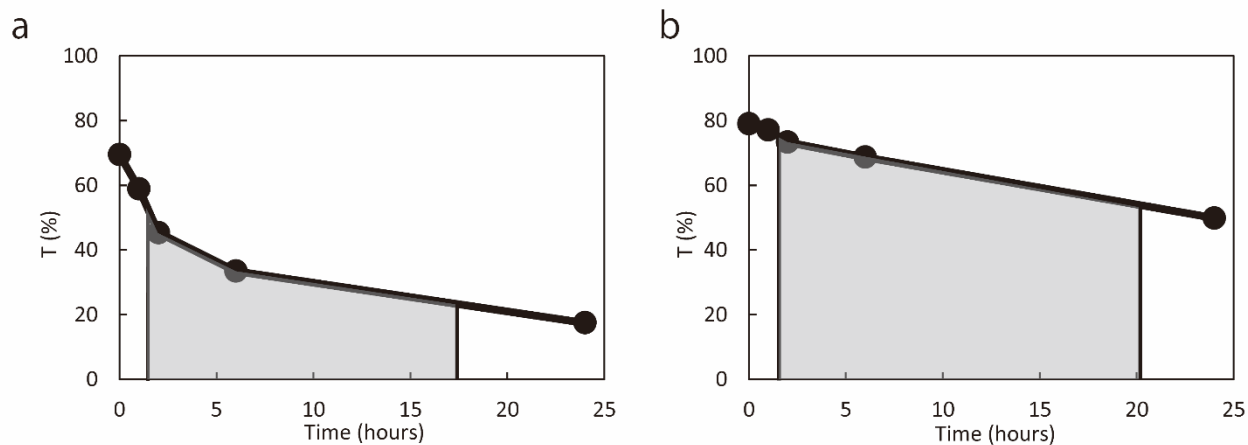


Fig. S6 The plot of the relative amount of the intact triplex ODN (T) calculated using equation (1) against various time points for PT-ODN (a) and APT-ODN (b). The area corresponding to the time period used for in-cell NMR measurement is shaded.

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