

# Detection of siRNA administered to cells and animals by using a fluorescence intensity distribution analysis polarization system

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## ABSTRACT

Small interfering RNA (siRNA) has excellent pharmacological features and is expected to be used for therapeutic drug development. To this end, however, new RNA technology needs to be established so that extremely small amounts (less than 1 pmol) of siRNA can be detected in organs of experimental animals and in human blood to facilitate pharmacokinetics studies. An important feature is that this new technology is not dependent on radioisotopes and can detect siRNA molecules identical to those used for drug development in preclinical tests with experimental animals or in clinical tests with humans. We report a convenient method that can detect small amounts of siRNA. The method uses high-power confocal microscopic analysis of fluorescence polarization in DNA probes that are bound to one of the strands of siRNA and directly quantitates the copy number of siRNA molecule after extraction from specimens. A pharmacokinetic study to examine the blood retention time of siRNA/cationic liposomes in mice showed that this straightforward method is consistent with the other reverse transcriptase polymerase chain reaction amplification-based method. We believe that the entire process is simple and applicable for a high-throughput analysis, which provides excellent technical support for fundamental research on RNA interference and development of siRNA drugs.

## INTRODUCTION

RNA interference (RNAi) is induced by incorporating double-stranded small interfering RNA (siRNA) of 21–25 nucleotides into the RNA-induced silencing complex that directs the cleavage of the complementary target mRNA in the cytoplasm (1). RNAi is a highly

sequence-specific post-transcriptional gene silencing event that permits degradation of specific mRNAs and thus has been used broadly as an excellent procedure for cellular gene knockout experiments. This excellent biological activity of siRNA has also been tested for therapeutic drugs. siRNA as a drug promises several advantages over traditional drugs, offering new types of medicines that have a very high target selectivity and that are effective at a low dose (nanomolar or subnanomolar concentrations) with low toxicity due to metabolism to natural nucleotide components (2–4).

Development of siRNA therapeutics, however, has been hampered by several issues, including poor intracellular uptake due to the intrinsic negatively charged ~13K molecular weight structure and limited stability in circulating blood. Another key issue has been the absence of a convenient method to detect small amounts of siRNA in its natural form to monitor the pharmacokinetics in cells, circulating blood and organs. A small amount of siRNA present in the circulating blood is difficult to quantitate by conventional column chromatographic procedures due to limited ultraviolet absorption of the administered siRNA.

Previously, as a pioneering effort, Sato *et al.* (5,6) measured the retention time of siRNA stabilized with cationic polymers in the circulating blood of mice by using fluorescence-labeled siRNA and polyacrylamide gel electrophoresis that allowed simultaneous isolation and visualization of siRNA. However, this method has several drawbacks: (i) it is unable to measure non-fluorescent (or non-radioactive) siRNA, such as unmodified siRNA in its natural form, (ii) it is technically tedious to run the gel electrophoresis, (iii) it fails to obtain accurate estimates of the amount of siRNA and (iv) it cannot handle many samples. Futami *et al.* (7) invented a new method to amplify siRNA of 21-mers containing 3'-TdT to DNA after 3'-terminal dG-tailing by using terminal deoxytransferase and reverse transcriptase polymerase chain reaction (RT-PCR) with a primer containing oligo-dC, referred to as the TdT/RT-PCR method.

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However, it can quantitate only siRNA having a 3'-terminal deoxynucleotidyl TdT sequence.

In this article, we describe a new method to estimate small amounts of siRNA that does not use RT-PCR but depends on the theory of fluorescence correlation spectroscopy (FCS) described by Edman (8). A single-molecule fluorescence detection system (MF20, Olympus Corporation, Tokyo, Japan), equipped with a confocal microscope and fluorescence spectroscopy, detects small fluorescence signal changes coming from a DNA probe hybridized with target siRNA molecules in a very small confocal volume of a light path chamber (1.5 fL). Samples at several nanomolar concentrations contain a few molecules of siRNA in such a small volume, so that signals directly reflect the concentration and the diffusion time of fluorescence molecules. Usually, the fluorescence-labeled DNA probe that increases the apparent molecular weight by 5–8-fold after hybridizing with specific target nucleic acids shows a reduced Brownian motion, resulting in an increased diffusion time in the confocal volume and a flickering of fluorescence that are used to estimate the degree of hybridization of the DNA probe. The number of fluorescence signal changes is expressed by the 'count-rate' that indicates the number of photons per second detected in the light path chamber being measured (9). Accordingly, in this study, we tested fluorescence intensity distribution analysis polarization (FIDA-PO) developed by Palo *et al.* (10,11) that overcomes the technical limitation of FCS that measures mainly fluorescence intensity changes due to the altered molecular mass and diffusion time of the fluorescent ligand. FIDA-PO has been used before for protein kinase assays, diagnosis of single-nucleotide polymorphism (12,13) and for studying the stability of fluorescence-labeled siRNA bound to cationic polymers in blood (5).

We found that FIDA-PO can be used to estimate the amounts of fluorescence-labeled DNA probes in DNA/RNA heteroduplexes formed by hybridization with one of the strands of siRNA, even though the apparent molecular size of DNA probe increases by only 2-fold after annealing to siRNA. Here, we report that the FIDA-PO system combined with single-molecule fluorescence detection is convenient for quantitative and high-throughput analysis of many siRNA samples extracted from cells and blood of administered animals. We believe that this FIDA-PO method facilitates pharmacokinetics studies on siRNA and drug-delivery studies, expediting the development of siRNA medicines.

## MATERIALS AND METHODS

### siRNAs and fluorescence-labeled DNA probes

All siRNAs consisted of 19-bp RNA duplexes and two overhanging 3'-dTdT at the 3' termini. GL3-siRNA represents the sequence of part of the firefly luciferase gene (GL3, Promega) that is not present in human cells and was used in this study. It contains a duplex of strands containing sequences 5'-CUUACGCUGAGUACUUCG AdTdT-3' (sense strand) and 5'-UCGAAGUACUCAGC GUAAGdTdT-3' (antisense strand). Other siRNA

sequences used in this study were NS (nonspecific)-siRNA that contained sequences 5'-UUCUCCGAACG UGUCACGUdTdT-3' and 5'-ACGUGACACGUUCG GAGAAAdTdT-3' that were confirmed not to interact with any known mRNA sequence by homology search by using the Smith and Waterman method, and RecQL1-siRNA that contained sequences 5'-GUUCAG ACCACUUCAGCUUdTdT-3' (sense strand corresponding to the position 273–291 in RecQL1 mRNA) and 5'-AA GCUGAAGUGGUCUGAACdTdT-3' (antisense strand) (14). The  $T_m$  values for these siRNAs were 53.9°C for GL3-siRNA (GC content 47.4%), 56.0°C for NS-siRNA (GC content 52.6%) and 53.9°C for RecQL1 (GC content 47.3%). DNA probes that detect GL3-siRNA, NS-siRNA or RecQL1-siRNA consisted of 21-mer oligonucleotides that were chemically synthesized and were purified to over 95% homogeneity by using reverse phase column chromatography, and they were labeled at the 5' end by TAMRA (tetra methyl-rhodamine) (Sigma-Aldrich Japan, Tokyo, Japan) or Alexa Fluor 647 (Japan Bio Services Co., Ltd, Saitama, Japan).

### Cells and siRNA transfection

A549 (human lung carcinoma) cells obtained from ATCC (Manassas, VA, USA) were cultured at 37°C in a humidified chamber supplemented with 5% CO<sub>2</sub>. The culture medium and method were as described in the supplier's instructions. For transfection with siRNA, the cells were plated, proliferated by culturing for 24 h and then were incubated for 8 h with 40 nM siRNA duplex using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). The cells were then cultured in fresh medium under the conditions described before (14).

### Systemic administration of siRNA/liposome complex to mice and measurement of siRNA retained in blood

Animal procedures were approved by the committee for the institutional care and use of animals at GeneCare Research Institute Co. Ltd in accordance with the guidelines for animal experimentation prepared by the Japanese Association for Laboratory Animal Science. RecQL1-siRNA/LIC-101 complex (50 µg siRNA/mouse) was injected intravenously into ICR mice ( $n = 3$ ) purchased from CLEA Japan (Tokyo, Japan). The LIC-101 liposome consists of 2-*O*-(2-diethylaminoethyl)-carbonyl-1, 3-*O*-dioleoylglycerol and egg phosphatidylcholine. Mouse blood (30 µl) was taken from the tail vein at 0.5, 1, 3, 6, 12 and 24 h after administration of the siRNA/liposome complex. Half of the blood (15 µl) was diluted 10-fold by mixing with 135 µl of phosphate-buffered saline (PBS) containing 50 mM ethylenediamine tetraacetate (EDTA), and the siRNA was extracted as described in the 'Extraction of siRNA' section below. The formulation process of the RecQL1-siRNA/LIC101 complex was done in the laboratory of Nippon Shinyaku Co., Ltd (Kyoto, Japan) as reported by Yano *et al.* (15).

### Extraction of siRNA from cultured cells, mouse blood and organs

The siRNAs transfected *in vitro* to cells were extracted from cells after 6, 12, 24, 48, 72 and 96 h as we had described earlier (14). siRNAs administered *in vivo* to mice by intravenous injection were extracted from blood after 30 min and at 1, 3, 6, 12 and 24 h as we described earlier (7). siRNAs delivered to mouse organs were extracted, in a form of total RNA, from excised and ground organs 30 min after the intravenous injection of RecQL1-siRNA/LIC101 complex. Extraction was done by using phenol and chloroform, and the extracted total RNA was purified by using the miRNeasy Mini Kit (Qiagen, Hilden, Germany) following the instructions of the manufacturer. As the internal control, GL3-siRNA (50 pmol) was added to the grounded organ to standardize the recovery of RNA during the extraction process.

### Detection of siRNA by FIDA-PO system

Fluorescence changes in the labeled DNA probe were monitored by using FIDA-PO before and after hybridization with siRNA, similar to its use to detect other biological materials (5,9,12,13). A standard curve was made for each labeled DNA probe by using a 2 nM DNA probe at varying concentrations (0–200 nM) of siRNA in 30  $\mu$ l of hybridization reaction mixture consisting of 20 mM Tris-HCL (pH 7.5), 1 mM EDTA and 100 mM NaCl. After hybridization under the conditions described below, the fluorescence changes in the DNA probe were measured and plotted. The non-linear standard curve obtained from the measurements of increasing concentrations of siRNA was smoothed by fitting equation  $y = \text{bottom} + (\text{top} - \text{bottom}) \times \{A_0 + S + K_d - [(A_0 + S + K_d)^2 - 4 \times A_0 \times S]^{1/2}\} / (2 \times A_0)$  (where bottom represents the value obtained with 0 nM siRNA, top represents the value with 200 nM siRNA,  $A_0$  is the concentration of the DNA probe,  $S$  is the concentration of siRNA and  $K_d$  is the dissociation constant between the DNA probe and siRNA antisense chain). The  $K_d$  value measured in the hybridization reaction mixture containing 100 mM NaCl was  $5.27 \pm 1.48$  nM, and it was used for the standard curve for FIDA-PO analysis.

RNA fractions, extracted from cultured cells or blood, were purified by using the column in the miRNeasy Mini Kit. The RNA solution (5–6  $\mu$ l) was diluted to a total of 30  $\mu$ l by adding 20 mM Tris-HCl (pH 7.5) buffer containing 1 mM EDTA and 100 mM NaCl. The single-stranded DNA probe was mixed in this solution at concentration 2 nM, and hybridization was done in Eppendorf tubes by incubation at the following temperatures: the annealing condition changed at interval times of 50 s from 95°C for 5 min to 90°C for 5 min, 80°C for 10 min, 70°C for 10 min, 60°C for 10 min, 50°C for 10 min, 40°C for 10 min, 30°C for 10 min and finally to room temperature (20°C). After hybridization of the fluorescent DNA probe with the complimentary strand of siRNA, each sample (30  $\mu$ l) was monitored five times by FIDA-PO analysis by using an MF20 apparatus (Olympus Corporation, Japan) equipped with a confocal microscope and a laser. Every measurement of the polarized fluorescent light

yielded from the 100  $\mu$ W of 543 nm laser light irradiation was done automatically for 15 s at each monitoring (a total of 75 s per sample). The increased diffusion time (microseconds: represented as K1) of the annealed labeled DNA probe/siRNA complex, as well as the change in the intensity of fluorescence polarization (FP: represented as mP), were measured and computed automatically by using softwares installed in MF20. All measurements were done at room temperature around 20°C.

### Quantitative analysis of siRNA by the TdT/RT-PCR method

The overhanging 3' terminal dTdT of siRNA can be efficiently 3'-oligo-dG-tailed by incubation with terminal deoxynucleotidyl transferase (EC2.7.7.31) and dGTP. The oligo-dG-tailed siRNA sequences are first converted to cDNA by oligo-dC-containing primer and reverse transcriptase, and then the resulting oligo-dC-containing cDNAs are amplified by PCR. Quantitative PCR can be done with these cDNA templates. This method works well for 3'-dTdT-containing siRNA. The method was invented by Futami and Furuichi (16) and was used previously to determine the retention time of RecQL1-siRNA in the circulating blood of mice that were intravenously injected with the RecQL1-siRNA/LIC-101 complex (7).

## RESULTS

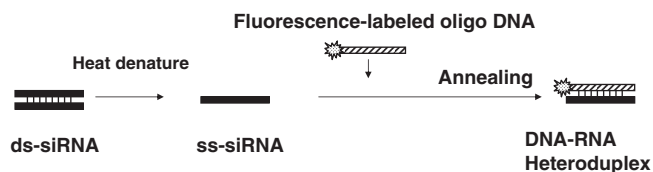
### Detection of the siRNA/DNA probe heteroduplex by FP

Various concentrations of GL3-siRNA were heat-denatured, were hybridized with a fluorescent TAMRA-labeled DNA probe, and the resulting siRNA/DNA probe heteroduplex underwent single-molecule fluorescence detection by using an MF20 apparatus to test if fluorescence emission from the labeled DNA probe may be affected after heteroduplex formation with siRNA (Figure 1). The MF20 apparatus has multiple functions, such as FCS, FIDA (fluorescence intensity distribution analysis) and FIDA-PO, which enable monitoring of the activity of the fluorescence-labeled molecules from various aspects. We found that FIDA-PO can monitor FP in accordance with molecular change in a DNA probe from single-stranded DNA to a base-paired DNA/RNA heteroduplex.

The labeled DNA probe at concentrations 0.5 nM, 2 nM and 10 nM was tested to detect GL3-siRNA of varying concentrations from 0.001 to 100 nM (Figure 2A). To our surprise, the FP of the DNA probe increased markedly after binding to siRNA (21-mers) even when the molecular weight shift of the DNA probe (21-mers) before and after heteroduplex formation was assumed to be only 2-fold. Figure 2A shows that the relative FP change (represented as mP) of the DNA probe increased dose-dependently as the siRNA concentration increased. The degree of FP change did not differ markedly between the three concentrations of the DNA probe, sharing a similar sigmoidal profile of FP change against increasing siRNA concentrations.

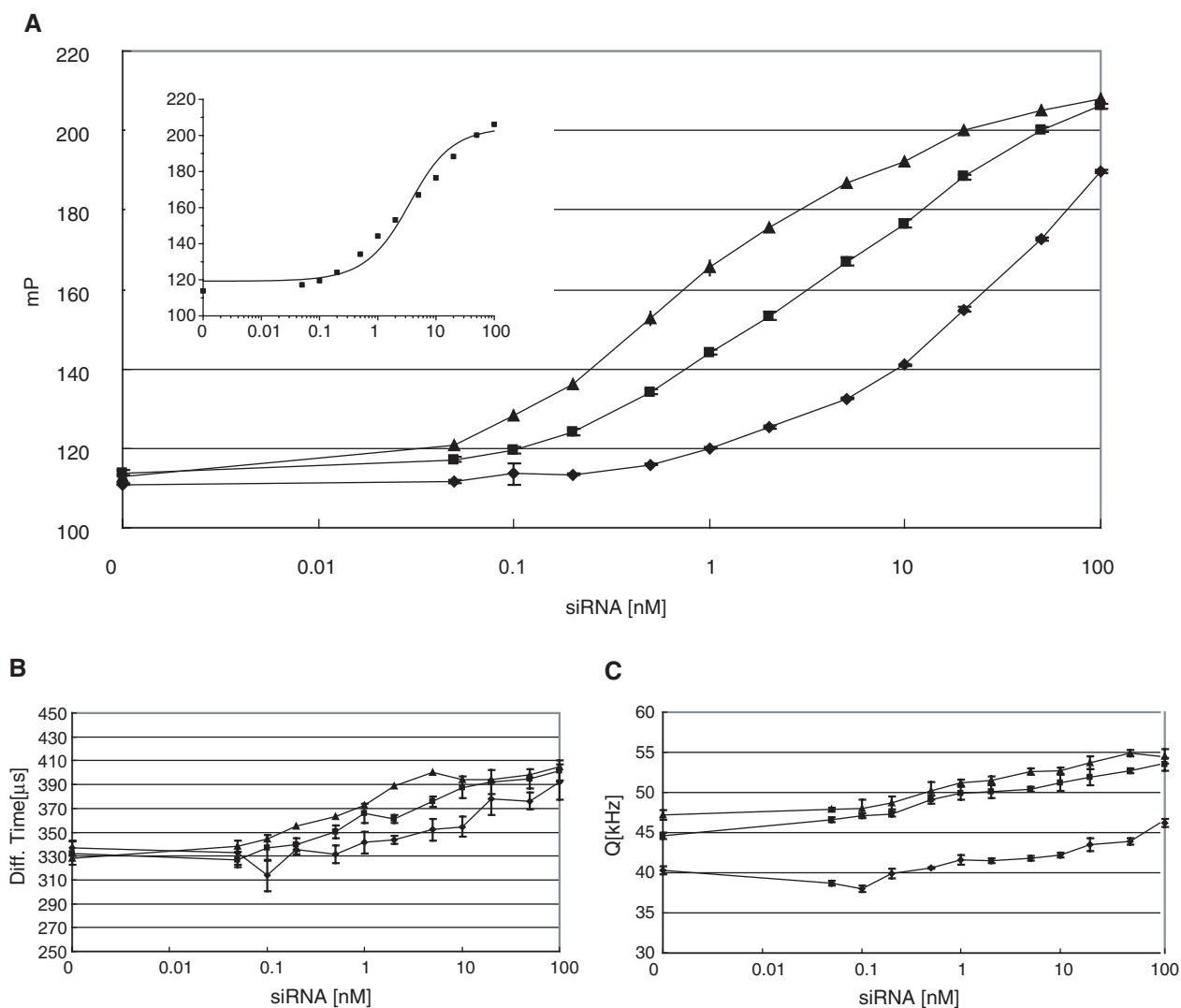
These results suggested that the FP change of labeled DNA probe in a heteroduplex can be used to detect



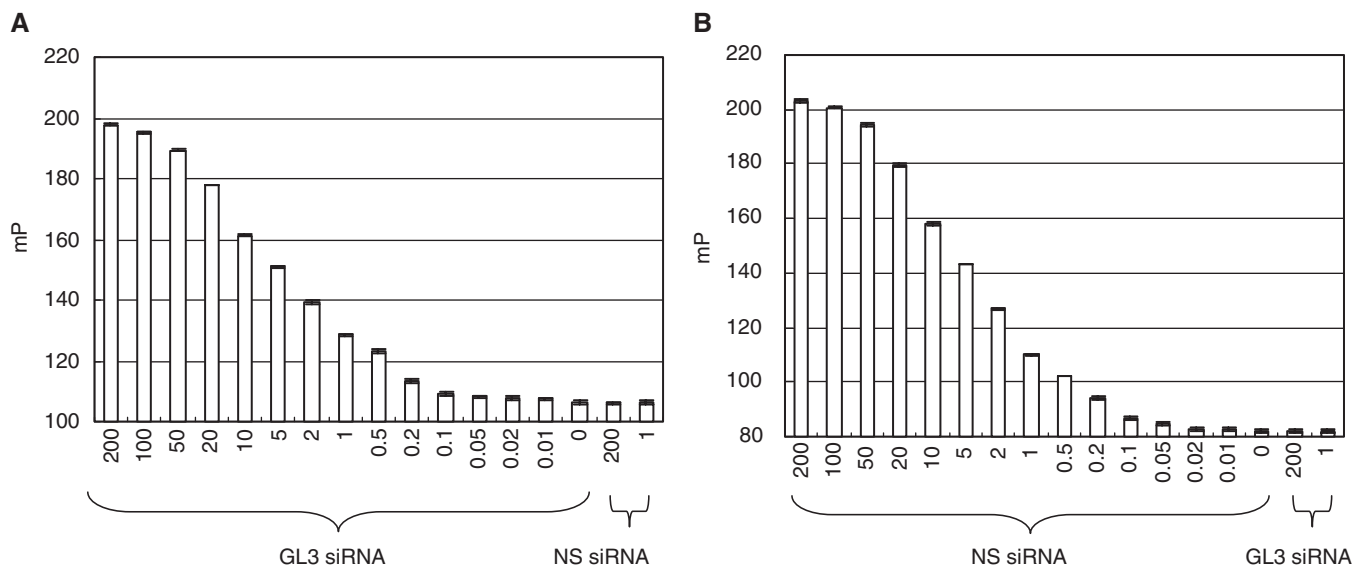


**Figure 1.** Schematic representation of siRNA detection by DNA probe/siRNA heteroduplex formation and fluorescence polarization. A DNA probe labeled at the 5' or 3' terminus with a fluorescent ligand, which is illustrated by a bar having diagonal lines and a jagged blob, binds by annealing to the complimentary single-stranded siRNA. The fluorescence polarization changes generated by the DNA-RNA heteroduplex formation were detected by using a confocal microscope without removal of the free DNA probe.

siRNA from its lowest limit of 0.5 nM to over 50 nM under the appropriate DNA probe concentration. The labeled DNA probe of 2 nM, however, seemed to be most appropriate, because it showed a wider coverage in detection of siRNA (approximately from 0.5 to 50 nM) with a linear dose dependency. The data obtained with the 2 nM DNA probe was smoothed to serve as the standard curve by fitting a program (Figure 2A, inset), and the later experiments were done with this 2 nM DNA probe condition. When these experimental data were analyzed from the aspect of diffusion time of annealed DNA probe by using FCS, the diffusion time increased as if the Brownian movement of the



**Figure 2.** Quantitation of siRNA by FIDA-PO of the DNA probe/siRNA heteroduplex. Various concentrations of GL3-siRNA (0.001–100 nM) were hybridized with 0.5, 2 and 10 nM DNA probe (21-mer) that consisted of a 21-mer GL3-siRNA-specific sequence and a 5' TAMRA-labeled terminus. Hybridization was done as described in the 'Materials and Methods' section. The reaction mixtures were placed in an automated MF20 sample feeder and underwent analysis with FCS, FIDA and FIDA-PO. Each sample was analyzed five times for 15 s, a total of 75 s and the averaged values were recorded. Fluorescent light (or polarization) emitted by irradiation of 100 μW laser light (543 nm) was measured and translated as diffusion time (K1) or as polarized fluorescent intensity (mP). (A) FIDA-PO analysis, (B) FCS analysis and (C) FIDA analysis. Closed triangle: 0.5 nM, closed square: 2 nM, closed diamond: 10 nM. Error bars show standard deviation (SD).



**Figure 3.** Sequence-specific quantitation of siRNA by FIDA-PO. (A) GL3-siRNAs of increasing concentrations from 0 to 200 nM underwent FIDA-PO analysis with a 2 nM GL3-DNA probe. NS-siRNAs (1 nM, 200 nM) were tested as negative controls. (B) NS-siRNAs of increasing concentrations from 0.01 nM to 200 nM underwent FIDA-PO analysis with a 2 nM NS-DNA probe. GL3-siRNAs (1 nM, 200 nM) were tested as negative controls. Error bars show the SD.

heteroduplex of the DNA/siRNA was reduced, i.e., the apparent molecular weight of the complex increased as the concentration of siRNA increased (Figure 2B). The results with FCS showed that the diffusion time of the GL3-DNA probe increased dose dependently, but only slightly from 330  $\mu$ s (microseconds) to about 400  $\mu$ s (Figure 2B). We noted that these diffusion time changes were distinct, but they were not large enough to be used to quantitate siRNA.

In the FIDA assay that measured the fluorescence intensity (as shown by Q [kHz]) of individual DNA probes, the intensity of fluorescence from the DNA probe also increased dose dependently, but only marginally at about 5–10 Q [kHz] increments under all DNA probe concentrations tested (Figure 2C). The increments were not large enough to be used to estimate siRNA concentration. In the FIDA assay, the intensity of fluorescence of each DNA probe molecule tended to be influenced by the initial concentration of the DNA probe, i.e. the higher the concentration of DNA probe, the lower was the intensity of fluorescence emitted from each molecule of DNA probe (Figure 2C).

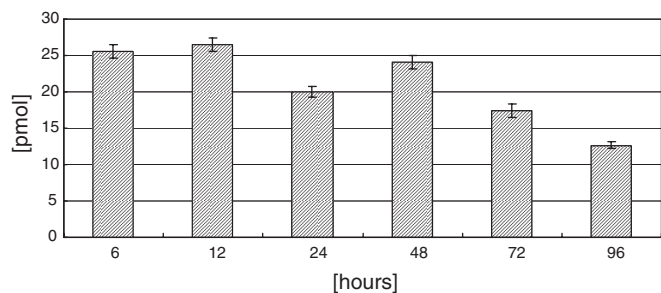
#### DNA probe sequence-specific detection of siRNA by FIDA-PO

To test if the FP change in DNA probe is sequence-specific and is also hybridization-dependent, we tested the effect of siRNA sequences other than GL3-siRNA. NS-siRNA containing an arbitrary sequence and a fluorescence-labeled DNA probe that can detect NS-siRNA was prepared, and it was used for cross-hybridization experiments with GL3-siRNA and its cognate DNA probe. The dose dependency was also examined for a wide range of siRNA concentrations from 0.01 to 200 nM with 2 nM of fluorescence-labeled DNA probes (Figure 3). The GL3-DNA

probe detected GL3-siRNAs at a successive range of concentrations from 0.1 to 100 nM, but it did not interact with non-matching NS-siRNA, even at high concentration 200 nM (Figure 3A). The FP of the the NS-DNA probe was unaffected *vice versa* by non-matching 1 nM and 200 nM GL3-siRNA (Figure 3B). These results indicate that FIDA-PO analysis with a 21-mer DNA probe can measure siRNA of concentrations from as low as less than 0.1–50 nM sequence-specifically.

#### Detection of siRNAs in cells

We tested FIDA-PO analysis to monitor the metabolism of siRNA transfected to cells under cultivation. We used RecQL1-siRNA that can silence endogenous RecQL1 helicase mRNAs existing abundantly in growing cancer cells to understand the fate of siRNA functioning in cells (17). RecQL1-siRNA in a complex with lipofectamine was transfected into human lung cancer A549 cells at concentration 40 nM (containing 40 pmol siRNA in 1 ml of medium) by incubation at 37°C for 8 h. A549 cells were then removed from the Petri dish, were washed with PBS and then the total cellular RNA was extracted at times 6, 12, 24, 48, 72 and 96 h and the amounts of RecQL1-siRNA were measured by FIDA-PO analysis by using the RecQL1-specific DNA probe as described in the ‘Materials and Methods’ section. The RecQL1-siRNA-specific DNA probe consisting of a sense sequence does not react with cellular nucleic acids such as tRNA, ribosomal RNA, endogenous microRNAs and RecQL1 mRNA. The A549 cell RNA extract was monitored separately by a DNA probe complimentary to non-specific siRNA as a negative control. As an internal control, NS-siRNA (20 pmol) was added to the cell lysate to monitor the recovery of siRNA during the extraction process. Figure 4 shows the time course of

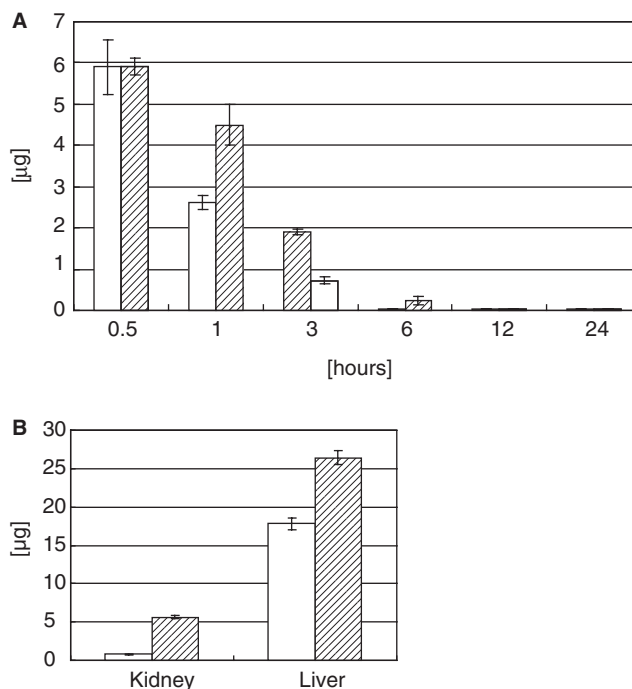


**Figure 4.** Detection of siRNA in cells by FIDA-PO analysis. RecQL1-siRNA was transfected to A549 human lung carcinoma cells ( $1.5 \times 10^4$ ) with Lipofectamine as described in the 'Materials and Methods' section. At 8 h after transfection, cells were washed and cultured in fresh medium. siRNA was extracted from cells at the indicated time after transfection. Extraction of RNA was done as described in the 'Materials and Methods' section, and the amount of RecQL1-siRNA present in the extracts was determined with a 2 nM RecQL1-DNA probe. Error bars show the SD.

RecQL1-siRNA metabolism in A549 cells. The amount of RecQL1-siRNA detected by the specific DNA probe decreased gradually as A549 cell culturing proceeded, giving rise to a half-life of about 96 h. Over 90% of RecQL1 mRNA was silenced in 24 h by the RNAi reaction, as we determined by using RT-PCR (7). In contrast, the non-specific DNA probe that was used in the negative control experiment showed no sign of FIDA-PO, justifying the specific interaction of the RecQL1-DNA probe with the transfected RecQL1-siRNA.

#### Quantitation of siRNAs in circulating blood

We used FIDA-PO analysis to monitor the retention of intravenously injected siRNA in circulating blood of mice. The RecQL1-siRNA/LIC-101 complex (50  $\mu$ g siRNA/mouse) was administered to mice, and their blood was taken from the tail vein at 0.5, 1, 3, 6, 12 and 24 h after administration. The blood was diluted 10-fold by extraction buffer, and RNA was extracted as described in the 'Materials and Methods' section. RNA was dissolved in a small volume of buffer (30  $\mu$ l), and aliquots were analyzed for the amount of siRNA by using the FIDA-PO system. Figure 5A shows the time course of the change in concentration of RecQL1-siRNA in the circulating mouse blood. The data show that RecQL1-siRNA in the complex with LIC-101 liposome disappeared from circulating blood quickly with an initial half retention time of <0.5 h. To find if the kinetics data obtained by this FIDA-PO system is consistent with the data obtained by other methods, we compared the data with data obtained by the PCR-mediated, amplification-based method previously described (7,16). The method of this study is referred to as the TdT/RT-PCR method that can estimate the amount of siRNA having a 3'-TdT sequence. The results obtained from these two independent methods were almost identical at 0.5 and 3 h. In this study, the RecQL1-siRNA/LIC101 complex was delivered preferentially and quickly to the liver after intravenous administration into the tail vein of mice because LIC101 is a liver-prone cationic liposome (7,15).



**Figure 5.** FIDA-PO analysis for the pharmacokinetics of intravenously injected siRNA in mouse blood and siRNA distribution in mouse organs. RecQL1-siRNA (50  $\mu$ g) was injected intravenously into mice ( $n = 3$ ) in the form of a siRNA/liposome complex or a RecQL1-siRNA/LIC101 complex in 10% maltose solution. (A) Pharmacokinetics in mouse blood; mouse blood (30  $\mu$ l) was extracted from the tail vein at 30 and 60 min and at 3, 6, 12 and 24 h after administration, and an RNA fraction was extracted as described in the 'Materials and Methods' section. The amount of RecQL1-siRNA was determined by using the FIDA-PO system and was represented as siRNA weight ( $\mu$ g) per whole blood (2 ml) of mouse. The results were compared with results using the TdT/RT-PCR method (7,16). White columns show data obtained by FIDA-PO analysis, and hatched columns show data by using the TdT/RT-PCR method. Error bars show the SD. (B) Determination of siRNA distribution in mouse organs; after 30 min, an RNA fraction was extracted from excised mouse organs as described in the 'Materials and Methods' section. The amount of siRNA was determined by using the FIDA-PO system and was represented as siRNA weight ( $\mu$ g) per whole tissue of mouse. White columns show data obtained by FIDA-PO analysis, and hatched columns show data by using the TdT/RT-PCR method. Error bars show the SD.

The pharmacokinetics of RecQL1-siRNA formulated with LIC-101 liposome are distinct, because most RecQL1-siRNA was delivered to the liver within 5 min after administration, whereas most of the other remained for about 3 h in circulating blood and then disappeared gradually within 6 h. This kinetic data associated with the liver-prone siRNA/liposome complex was confirmed by the FIDA-PO system and TdT/RT-PCR method. Thus, the pharmacokinetic study of siRNA drug at nanomolar levels can be achieved by FIDA-PO analysis without amplifying siRNA by RT-PCR or by preparing and handling cumbersome radioactive siRNA. This straightforward method that detects siRNA molecules by using a confocal microscope with a single-molecule fluorescence detection system can avoid bias of predicting the initial amount of siRNA after amplification by RT-PCR.

### Quantitation of siRNAs delivered to organs

We tested the FIDA-PO procedure to determine the distribution of siRNA in mouse organs after systemic administration by intravenous injection. The RecQL1-siRNA/LIC-101 complex (50 µg siRNA/mouse) was used for this experiment, and the RNA fractions were extracted from the liver and kidney at 0.5 h after administrations. Three mice were used in this experiment, and one sample from each mouse underwent FIDA-PO analysis independently after appropriate dilutions. The data indicate that a substantial portion of the administered RecQL1-siRNA was delivered to the liver, consistent with the liver-prone property of the LIC-101 carrier (Figure 5B). Only a small amount of RecQL1-siRNA was found in the kidney. These data are also consistent with previous conclusions obtained by using the TdT/RT-PCR procedure. Noteworthy is that measurements of siRNA by FIDA-PO analysis are consistent and reproducible.

### DISCUSSION

RNAi technology is now indispensable for basic research, and siRNA-mediated screening is quite popular in pharmaceutical industries to find target genes to discover drugs that manage disorders in various disease fields. Efforts to use the siRNA molecule itself for therapeutic drugs have also been increasing in various fields of human diseases. The development of siRNA drugs has been realized after establishing the basis of the siRNA function in silencing specific mRNA, and after solving several problems associated with the therapeutic use of siRNA, such as an off-target effect caused by less strict sequence matching (18), an interferon-inducing effect caused by a high dose of siRNA (19), TOL3 receptor interacting RNA molecules (20) and setting up large-scale chemical synthesis of pure siRNA (21).

Many examples exist in research that show anti-cancer activities of siRNA in various animal models. For example, siRNAs that can silence the expression of vasoactive epidermal growth factor required for angiogenesis of malignant cancers prohibit the growth of prostate cancer cells, resulting in regression of cancers (22). In mouse models of metastatic lung cancer, siRNAs that can suppress the expression of Bcl-2 that represses cancer cell apoptosis are efficacious (15). Acute depression of PLK1 (Polo-like kinase 1) that participates in cell-cycle progression by siRNA results in marked inhibition of growth of bladder cancers (23). However, these findings have not so far been substantiated by accurate pharmacokinetic data of siRNA concentrations present in cancer cells or blood. Previously, we showed that RecQL1-siRNA, which silences the expression of RecQL1 DNA helicase participating in DNA repair during cell-cycle progression, selectively kills a wide spectrum of cancer cells without affecting normal cells (14). During our study, we noted that siRNA drug development needs reliable methods that can monitor small amounts of siRNA distributed at low concentrations in cells, blood and targeted organs.

Imperative is that such methods can monitor the same siRNA species to be used for a therapeutic purpose and do

not monitor the labeled siRNA prepared specially for a tracking purpose. Using highly radioactive siRNA for human use is not practical. Chemically modified siRNA, such as siRNA labeled with a multi-ring fluorescein molecule, does not necessarily represent the true pharmacological nature of siRNA. Rhodamin dye tends to be incorporated preferentially into cancer cells instead of into normal cells by binding to specific surface proteins or through the active channel function of cancer cells (24). In this context, the FIDA-PO system provides convincing measurements of siRNA based on straightforward methods, including only hybridization with a DNA probe and confocal single-molecule fluorescence detection, and thus it can directly quantitate the same siRNA preparations administered in preclinical or clinical studies without having complicated processes of amplification that are laborious and are often associated with error and inaccuracy.

From the sensitivity viewpoint, the FIDA-PO system can easily detect 0.5–50 nanomolar concentrations of siRNA existing in 30 µl of reaction mixture (Figures 2 and 5). Because confocal volumes needed for measurement are only 1 fL, and the hybridization volume can be lowered to a few microliters, siRNA of less than 100 fmol (about 1 ng of siRNA of molecular weight 13 kDa) can be quantitated. From the viewpoint of reaction time, FIDA-PO permits a rapid determination of about less than 1 min for each sample by using the fully automated MF20 instrument. The total time, including of the hybridization reaction, is about 90 min and the whole process is simple; and no washing-off or removal of free DNA probe is necessary after annealing to the complimentary strand of siRNA. The method is reproducible and is resistant to contamination of high molecular weight RNAs, such as transfer RNAs and ribosomal RNAs.

In this study, we initially used a TAMRA-labeled DNA probe that is activated by He-Ne laser (543 nm) and emits fluorescence light. To investigate if the fluorescence ligand influences the quantitation of siRNA, we tested fluorescence ligand molecules other than TAMRA and found that quantitative detection of siRNA was possible with other kinds of fluorescence markers, such as Alexa Fluor 647 that is excited by laser light of wavelength 633 nm (data not shown). These results indicated that FIDA-PO analysis can achieve simultaneous detection of multiple siRNA species independently by using DNA probes containing different emission wavelengths.

When the RecQL1-siRNA/LIC-101 complex was intravenously injected into mice, the results of analyzing its retention in circulating blood and distribution in organs by using the FIDA-PO method fall within the similar ranges of siRNA concentration obtained by using the independent TdT/RT-PCR method (Figures 4 and 5). This comparative study indicated that both methods are compatible and perhaps can complement each other to assure the conclusion of pharmacokinetic experiments. Although the precise physico-chemical mechanism(s) underlying FIDA-PO changes in the DNA probe after annealing to siRNA remains to be studied, we believe that the FIDA-PO system will contribute to quantitation



of siRNA and microRNA, and to the clinical development of siRNA medicines.

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