Knockdown of GFP-Expression—A Comparison of Fusion and Lipofection

This Application Note describes a new membrane fusion-dependent technique for siRNA transfer into mammalian cells, which is used to knockdown specific target genes. The protocol provides instructions on the handling of Fuse-It-siRNA, as well as a comparison of various siRNA transfection alternatives.

Keywords:
Fuse-It-siRNA, siRNA transfection, siRNA transfer, RNA interference, RNAi knockdown, gene silencing, small non-coding RNAs Lipofectamine RNAiMAX, METAFECTENE SI+

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1. Background

RNA interference (RNAi) represents an important experimental tool for knocking down specific target genes. It is a biological process, in which small non-coding RNA molecules prevent the translation of specific mRNAs. One central type of small non-coding RNA is small interfering RNA (siRNA). After complementary base pairing, siRNAs lead to target mRNA degradation. RNAi can be induced experimentally by the exogenous introduction of siRNA molecules. The main advantages of this gene-editing tool are high efficiency and specificity. However, delivering siRNAs in a mild fashion continues to be a challenge. [1]

There are three main techniques for siRNA transfer that differ in efficiency and toxicity: lipofection, electroporation, and viral-mediated methods. Lipofection is a widely used lipoplex-based delivery method. However, it is not compatible with all cell types and has a low *in vivo* efficiency. Another popular method is electroporation. It uses electrical pulses to deliver siRNA into cells. For cells that are difficult to transfect by lipofection, electroporation might be an option, but at the cost of higher cytotoxicity. Therefore, for stable siRNA expression and long-term gene knockdown, viral-mediated delivery is often used. Some synthetic viral vectors can integrate into the genome of their host cell. However, viral delivery methods can trigger antiviral responses and the use of a Class II biological safety cabinet is mandatory. [2]

ibidi's reagent Fuse-It-siRNA transfers siRNA with high efficiency and extremely low cytotoxicity into the target cell. It was created for siRNA transfer into the cytoplasm of mammalian cells by membrane fusion. As shown in Figure 1, the Fuse-It liposomal carrier, which includes the siRNA, simply fuses with the cell membrane and subsequently releases the siRNA directly into the cytoplasm. Unlike classical lipoplex-based delivery methods, cells do not internalize the siRNA by endocytosis and there

is no need for endosomal release agents. The RNAi pathway starts immediately, without the interfering processes of endocytosis and lysosomal degradation (Figure 1). [3]

Fuse-It-siRNA transfers siRNA within 5–20 minutes. In contrast, classic lipofection reagents need several hours to transfer sufficient amounts of functional siRNA by endocytosis.

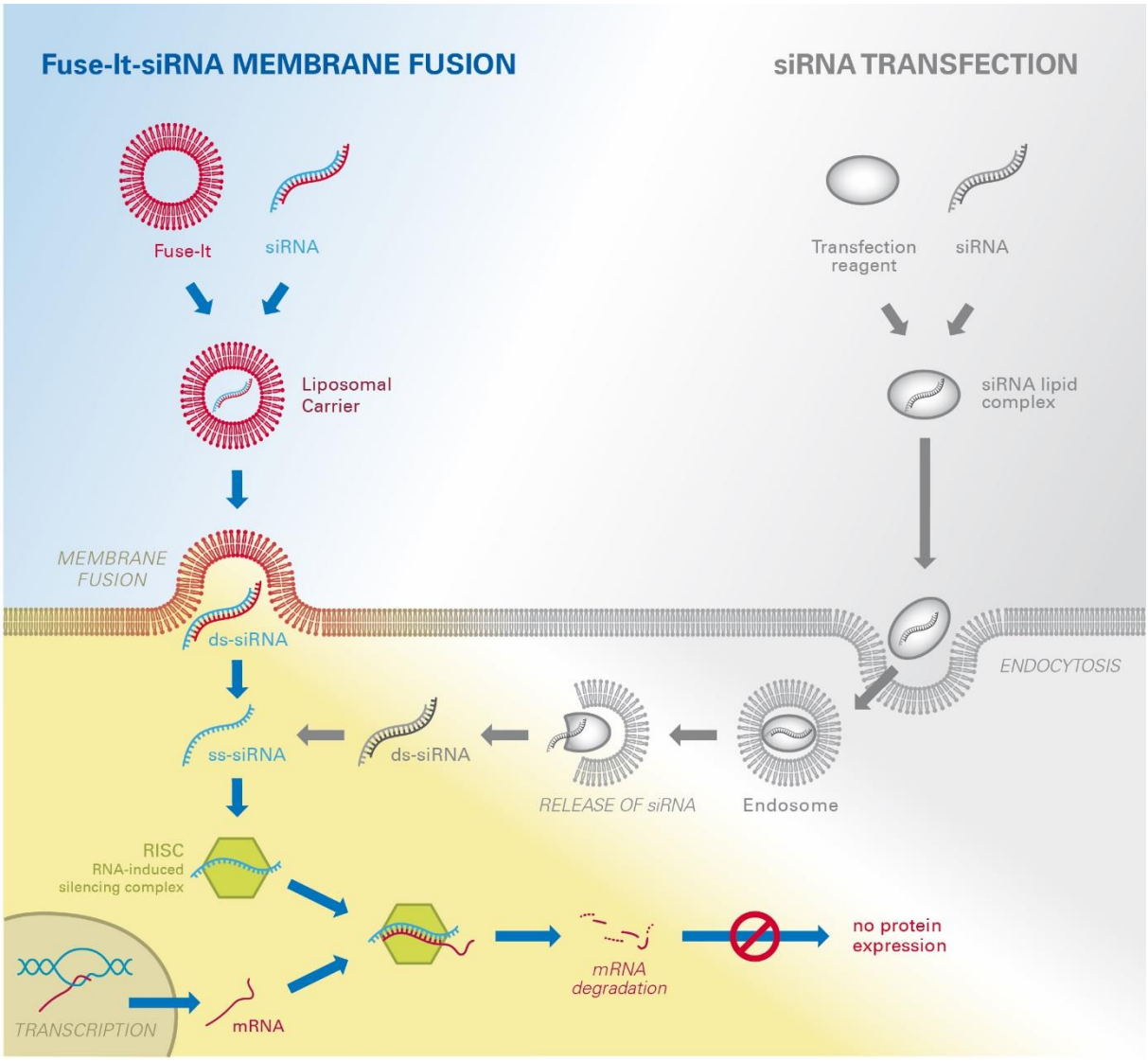

Figure 1: Simplified illustration of the membrane fusion mechanism of Fuse-It-siRNA vs. classical lipofection and the subsequent pathway of gene silencing by RNAi.

The double-stranded siRNA is separated, the first strand (passenger) is degraded and the second strand (guide) is loaded into the RNA-induced silencing complex (RISC). Both steps involve ribonuclease enzymes. The RISC recognizes its target mRNA by complementary base-pairing of the single-stranded siRNA with the endogenous mRNA. This leads to target mRNA degradation by the RISC, which results in translational inhibition and gene silencing.

2. Material and Equipment Required

For this protocol, the following materials are needed:

Table 1: Material and reagents needed for the knockdown of GFP in CHO-K1 cells.

Name	Company	Order No.
Fuse-It-siRNA	ibidi GmbH	60510
µ-Dish 35 mm, high, ibiTreat	ibidi GmbH	81156
CHO-K1 cells	DSMZ	ACC-110
DMEM-F12 Supplemented with 10% FCS	Sigma-Aldrich Chemie GmbH Sigma-Aldrich Chemie GmbH	D8437 F0804
Fibronectin	Corning	354008
Silencer® GFP (eGFP) siRNA	Thermo Fisher Scientific Inc.	AM4626
GFP-Plasmid, e.g. pEGFP-C1	Various, e.g. Clontech Laboratories, Inc.	Various
Lipofectamine™ 2000	Thermo Fisher Scientific Inc.	11668027
METAFECTENE SI$^+$ Kit	Biontex Laboratories GmbH	T100-1.0
Lipofectamine® RNAiMAX	Thermo Fisher Scientific Inc.	13778100
Opti-MEM® I Reduced Serum Medium	Thermo Fisher Scientific Inc.	31985070
1x PBS	Sigma-Aldrich Chemie GmbH	D8537
RNase-free water	Various	Various

For this protocol, the following equipment and instruments are needed:

- Ultrasonic bath (power 50 W, frequency 35 kHz)
- Fluorescence microscope equipped with an appropriate filter set for GFP (fluorescence properties: Ex_{max} 483 nm / Em_{max} 509 nm), and a stage top incubator (37°C, 5% CO_2)

3. Experimental Procedure

In this Application Note, the knockdown of GFP by siRNA transfer into transient GFP-expressing CHO-K1 cells is described. For the delivery of siRNA, three different reagents were compared: Fuse-It-siRNA, Lipofectamine RNAiMAX, and METAFECTENE SI$^+$. Additionally, a control experiment without the delivery of siRNA was carried out. Here, two technical replicates of each condition were performed, therefore eight µ-Dishes were required.

1.1. Coating the µ-Dishes with Fibronectin

1. For a final volume of 3200 µl, mix 48 µl of the fibronectin stock solution (1 mg/ml) and 3152 µl of 1x PBS. The final concentration of fibronectin is 15 µg/ml.
2. Add 400 µl of fibronectin dilution to each µ-Dish.
3. Incubate 30–40 minutes at 37°C.
4. Aspirate the fibronectin solution from each µ-Dish.
5. Wash each µ-Dish once with 400 µl of 1x PBS.

1.2. Seeding Cells

Seed 60,000–80,000 CHO-K1 cells in each µ-Dish (growth area 3.5 cm²). Incubate the cells for 24 hours at 37°C using standard cell culture conditions. The optical cell density at the time of transfection should be ~80%.

1.3. Transfection with GFP-Plasmid

Important Note: GFP-plasmids from various companies can be used for the transfection. However, the compatibility of the siRNA with the respective GFP-plasmid must be ensured.

1. Add 8 µl of Lipofectamine2000 to 1592 µl of OptiMEM, mix by pipetting, and incubate for 5 minutes at room temperature.
2. Add 8 µg (= 8 µl) of GFP-Plasmid to 1592 µl of OptiMEM, mix by pipetting, and incubate for 5 minutes at room temperature.
3. Mix both dilutions and incubate for 15 minutes at room temperature.
4. Aspirate the cell culture medium.
5. Wash the cells once with 1x PBS.
6. Add 400 µl of transfection mix to each of the eight µ-Dishes.
7. Incubate the cells for 1.5-2 hours at 37°C using standard cell culture conditions.

Important Note: Due to the long lifetime of GFP-protein, do not incubate the cells longer than 2 hours with the GFP-plasmid transfection mix. Otherwise, the expressed protein will superimpose the knockdown of GFP-mRNA.

1.4. siRNA Preparation

Dissolve the siRNA in the appropriate RNase-free buffer at a concentration of 50 pmol/µl (= 50 µM) for the stock solution. Prepare 5 µl aliquots and store them at -80°C. Add 45 µl of RNase-free water to one aliquot of the siRNA stock (1:10 dilution) to get a final concentration of 5 pmol/µl.

1.5. siRNA Delivery

Two technical replicates are performed for each siRNA delivery method.

1.5.1 Fusion Protocol with Fuse-It-siRNA

The standard protocol given in the “Fuse-It-siRNA Instructions” should be followed for the first trial of Fuse-It-siRNA with CHO-K1 cells.

1. Cool down the ultrasonic bath with an ice pack.
2. Mix 20 pmol of siRNA (= 4 µl of the 5 pmol/µl solution) and 4 µl of Neutralization Buffer (NB) thoroughly. Incubate for 10 minutes at room temperature.
3. Vortex the Fusogenic Solution (FS) until the solution is homogeneous.
4. Transfer 2.5 µl of the FS in an RNase-free micro reaction tube and sonicate it in the ultrasonic bath for 5 minutes at room temperature or lower.
5. Add the neutralized siRNA (siRNA + NB) and mix thoroughly.
6. Sonicate the mixture in the ultrasonic bath for 5 minutes.
7. Dilute the fusogenic mixture in 1x PBS to make a final volume of 500 µl.
8. Sonicate the dilution in the ultrasonic bath for 5 minutes.
9. Remove the transfection mix from the cells and wash once with 1x PBS.

10. Add 250 µl of the fusogenic mixture, drop-wise, onto the cells of each of the two µ-Dishes.
11. Incubate for 8 minutes at 37°C.
12. Replace the fusogenic mixture of each µ-Dish with 2 mL of fresh culture medium.
13. Incubate the cells for 24 hours at 37°C using standard cell culture conditions.

1.5.2 Transfection Protocol with Lipofectamine® RNAiMAX

The standard protocol for Lipofectamine® RNAiMAX was optimized for the siRNA transfection of CHO-K1 cells in a µ-Dish.

1. Add 7 µl of Lipofectamine RNAiMAX to 193 µl of OptiMEM, mix by pipetting, and incubate for 5 minutes at room temperature.
2. Add 20 pmol of siRNA (= 4 µl of the 5 pmol/µl solution) to 196 µl of OptiMEM, mix by pipetting, and incubate for 5 minutes at room temperature.
3. Mix both dilutions to get a final volume of 400 µl and incubate for 5 minutes at room temperature.
4. Remove the transfection mix from the cells and wash once with 1x PBS.
5. Add 200 µl of the siRNA transfection mixture, drop-wise, onto the cells of each of the two µ-Dishes.
6. Add 1.8 mL of fresh culture medium to the cells of each µ-Dish.
7. Incubate the cells for 24 hours at 37°C using standard cell culture conditions.

1.5.3 Transfection Protocol with METAFECTENE SI+

The standard protocol for METAFECTENE SI+ was optimized for the siRNA transfection of CHO-K1 cells in a µ-Dish.

1. Mix 192 µl of 1x SI+ buffer, 9.2 µl of METAFECTENE SI+ and 120 pmol of siRNA (= 24 µl of the 5 pmol/µl solution).
2. Incubate for 10 minutes at room temperature.
3. Remove the transfection mix from the cells and wash once with 1x PBS.
4. Add 100 µl of the siRNA transfection mixture, drop-wise, onto the cells of each of the two µ-Dishes.
5. Add 400 µl of Opti-MEM to the cells.
6. Add 1.5 mL of fresh culture medium to the cells of each µ-Dish.
7. Incubate the cells for 24 hours at 37°C using standard cell culture conditions.

Important Note: The most important difference between Fuse-It-siRNA and classical lipofection reagents is the short incubation time (minutes instead of hours). Additionally, liposomal complexes are prepared in low osmolarity buffers instead of media by use of ultrasonication steps.

1.6. Live Cell Imaging

Image the cells using phase contrast and fluorescence microscopy. Liposomal fusion efficiency is most easily detected by tracking the infrared control dye at 780 nm. In this Application Note, live cell images were taken on a Zeiss Axio Vert.A1 that was equipped with a AxioCam ICm1 camera.

4. Results and Discussion

The GFP-knockdown results are presented in Figure 2. ibidi's Fuse-It-siRNA showed the highest knockdown efficiency, with up to an 85% reduction of GFP positive cells when compared to a nontreated control. Even though the same or higher amounts of siRNA were used, Lipofectamine RNAiMAX and METAFECTENE SI$^+$ showed lower knockdown efficiencies when compared to Fuse-It-siRNA (Figure 2, top).

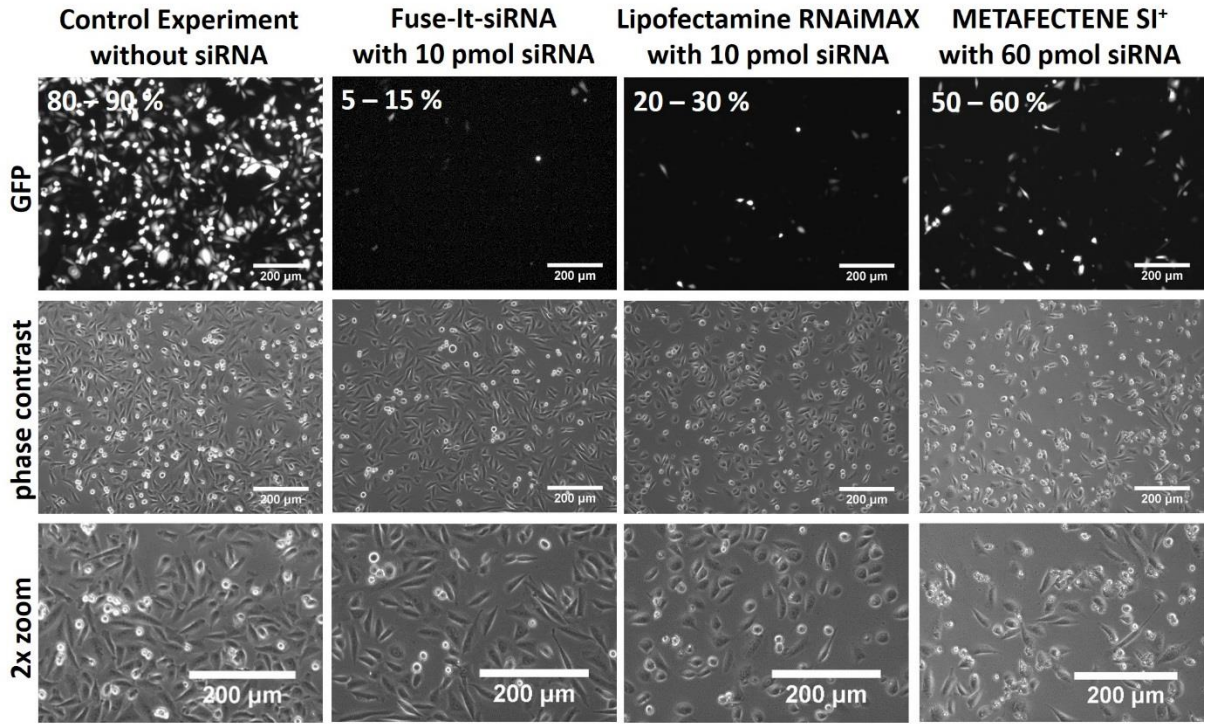

Figure 2: Documentation of GFP-transfected CHO-K1 cells 24 hours after the transfer of GFP-siRNA by use of indicated reagents. Percentages depict GFP-positive cells per field.

Further, cells treated with Fuse-It-siRNA showed a healthy morphology, which was similar to nontreated control cells. Instead, METAFECTENE SI$^+$- and Lipofectamine RNAiMAX-treated cells displayed a round morphology, indicating cell stress (Figure 2, middle and bottom).

Our results reveal that—in contrast to lipoplex-based methods—the siRNA transfer using Fuse-It-siRNA works in a very mild fashion with extremely low cytotoxicity. Only very short incubation times, as well as low amounts of liposomal carrier, are required, which results in a maximal siRNA transfer. These features protect even sensitive cells from the potential toxic effects of carrier reagents.

5. References

[1] R. W. Carthew and E. J. Sontheimer, “Origins and Mechanisms of miRNAs and siRNAs,” *Cell*, vol. 136, no. 4. pp. 642–655, 2009.

[2] T. Braun, C. Kleusch, E. Naumovska, R. Merkel, and A. Csiszár, “A bioanalytical assay to distinguish cellular uptake routes for liposomes,” *Cytom. Part A*, vol. 89, no. 3, pp. 301–308, 2016.

[3] K. Jensen, J. A. Anderson, and E. J. Glass, “Comparison of small interfering RNA (siRNA) delivery into bovine monocyte-derived macrophages by transfection and electroporation,” *Vet. Immunol. Immunopathol.*, vol. 158, no. 3–4, pp. 224–232, 2014.